Lipophilic Diammonium Cation Having a Rigid Structure Complementary to Pyrophosphate Dianions of Nucleotides. Selective Extraction and Transport of Nucleotides

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Abstract: 1,4-Diazabicyclo[2.2.2]octane was attached symmetrically by two stearyl chains on nitrogens to afford two cationic centers 2.4 Å apart from each other. Various nucleotide anions were transferred from an aqueous solution to a chloroform solution via salt formation with the ammonium cation. The number of phosphate units in nucleotides was thus recognized to give the relative order tri- > di- > monophosphate. The selectivity ratios were extremely high, e.g., 45 for ADP/AMP and 7500 for ATP/AMP at pH 3. The pH dependence of the degree of extraction revealed the mode of salt formation so that the geminate or vicinal dianions interacted most favorably with the diammonium cations. The highly efficient extraction of nucleotides made it possible to use the diammonium salt as a carrier of nucleotide transport through a chloroform liquid membrane. Salt and pH gradients across the membrane successfully drove active as well as passive transport of nucleotides. The transport rate decreased in the order tri- > di- > monophosphate to afford high overall selectivity ratios, e.g., 60 for ATP/AMP and 51 for ADP/AMP.

Nucleoside phosphates are energy mediators in a large number of biological reactions. The free energy derived principally from a series of biological oxidations is largely converted into chemical energies through the formation of pyrophosphate linkage of nucleotides, e.g., ADP → ATP. In return, the hydrolysis of pyrophosphate linkage, e.g., ATP → ADP, generates free energies available to drive endoergonic reaction sequences forward.[1] A large number of essential biological reactions are dependent on such nucleotide transformations, e.g., muscle contraction, photosynthesis, biosynthesis of proteins, nucleic acids, carbohydrates, or lipids among many others. In these nucleotide-dependent reactions, there certainly operates an ingenious device that discriminates among subtle structural differences of the nucleotides.[2] The recognition of mono-, di-, and triphosphates is of primary importance for maintaining the vectorial (one-way) reaction of bioorganisms.

Intracellular and certainly intramitochondrial environments where most biological reactions occur are not simple aqueous solutions. Indeed, the binding sites of enzymes or biological membranes are best understood as nonaqueous and in certain instances aprotic as well.[3] In such environments, the phosphate anions should be desolvated to some extent and stabilized through ionic interactions with the ion(s) or dipole(s) of the binding site. α-Amino terminus of polypeptides and ϵ-amino or guanidium grouping of lysine or arginine, respectively, are suggested[4] to be responsible for such interactions, but the detailed mechanism of recognition has never been clarified on the basis of molecular biology.

Our approach modeling these fundamentally important biological recognitions is based on the use of intramolecular diammonium cations, which were fixed into a rigid bicyclic structure so that the distance (2.4 Å) might be complementary to those of vicinal pyrophosphate, $^{-}O-P-O-P-O^{-}$, or geminate phosphate, $^{-}O-P-O^{-}$, dianions.[5] The ammonium salt was soluble in not only organic media (and practically insoluble in water) but also media liphophilic enough to extract very hydrophilic nucleotides from an aqueous to an organic phase. Thus di- and triphosphate anions of purine as well as pyrimidine nucleosides were selectively extracted into organic phase by the ion-pair formation at pH 3 where the monophosphate remained in an aqueous phase.

The lipophilic diammonium salt was also used as a carrier to facilitate the selective transport of nucleotides through a chloroform liquid membrane. pH and salt gradients across the membrane could successfully drive the active as well as passive transport of nucleotides with high selectivities. This is the first successful example of the selective transport of nucleotides with use of an artificial carrier molecule.[6]

Experimental Section

Commercially available chemicals were used directly unless otherwise noted. Infrared spectra were obtained on a Hitachi Model 215 spectrophotometer. ^1H NMR spectra were recorded with Varian EM-360 and HA-100D instruments. Electronic spectra were measured with a Hitachi 340S spectrophotometer. pH measurement was performed on a Toa electronics Model HM-5B instrument. The separation and quantitative analysis of nucleotide mixture were carried out by a Shimadzu-Du Pont high-pressure liquid chromatograph, Model 830 (column, Permaphase AAX, 1.5 m; eluant, 0.1 M NaH_2PO_4).

Preparation of the Diammonium salt. 1,4-Diazabicyclo[2.2.2]octane, DABCO (1.0 g, 8.9 mmol), was heated with stearyl iodide (20 g, 53 mmol) in 50 mL of dimethylformamide (DMF) at 70 °C for 2 days. The DMF solution was added dropwise to 200 mL of benzene and the mixture filtered to remove excess stearyl iodide. The solid was then washed with 100 mL of water at 60 °C for 1 h and the solution filtered to remove a monoammonium salt. The solid was recrystallized from DMF to give colorless prisms, **1** (6.5 g, 83%): mp 234-237 °C; IR (KBr) 2900, 2850, 1460, 1350, 1110, 1050, 870, 840, and 710 cm^{-1}; NMR (Me_2SO-d_6) δ (from HMDS) 0.80 (br, 6 H, CH_3), 1.18 (br, 60 H, CCH_2), 1.64 (br, 4 H, $NCCH_2$), 3.48 (br, 4 H, NCH_2), 3.82 (br, 12 H, NCH_2CH_2N). Anal. Calcd for $C_{42}H_{86}N_2I_2$: C, 57.79; H, 9.93; N, 3.21; I, 29.11. Found: C, 57.72; H, 10.43; N, 2.91; I, 28.88.

The iodide salt exhibited a UV absorption (λ_{max} = 218 nm) that overlapped with those of nucleotide bases and made it difficult to determine the amount of nucleotide in the extraction experiment. Thus the counterion was converted to the chloride via the hydroxide. Silver oxide

(1) The oxidative phosphorylation was first documented by: Kalckar, H. *Enzymologia* **1937**, *2*, 47-52; *Biochem. J.* **1939**, *33*, 631-641; Belitser, V. A.; Tzibokawa, E. T. *Biokhimiya* **1939**, *4*, 516-535. The other phosphorylation comes from photophosphorylation. Numerous references are now available for these biologically important processes.

(2) Boyer, P. D., Ed. "The Enzymes", 3rd ed., Academic Press: New York, 1973; Vol VIII.

(3) (a) Penefsky, H. S. In "The Enzymes", 3rd ed., Boyer P. D., Ed.; Academic Press: New York, 1974; Vol. X, pp 375-384. (b) Abrams, A.; Smith, J. B. *Ibid.* pp 395-429. (c) Hasselbach, W. *Ibid.* pp. 431-467.

(4) (a) Roustan, C.; Pradel, L. A.; Kassab, R.; Fattoum, A.; Thoai, N. V. *Biochim. Biophys. Acta* **1970**, *233*, 369-379. (b) Noerby, J. G.; Jensen, J. *Ibid.* **1971**, *233*, 104-116, 395-403. (c) Hegyvary, C.; Post, R. L. *J. Biol. Chem.* **1971**, *246*, 5234-5240.

(5) (a) Tabushi, I.; Imuta, J.; Seko, N.; Kobuke, Y. *J. Am. Chem. Soc.* **1978**, *100*, 6287-6288. (b) Tabushi, I.; Kobuke, Y.; Imuta, J. *Ibid.* **1980**, *102*, 1744-1745.

(6) The transport of anionic species is reported only by: (a) Behr, J.-P.; Lehn, J.-M. *J. Am. Chem. Soc.* **1973**, *95*, 6108-6110. Uptake of anionic species into organic media is observed by: (b) Graf, E.; Lehn, J.-M. *Ibid.* **1978**, *98*, 6403-6405. (c) Lehn, J.-M.; Sonveaux, E.; Willard, A. K. *Ibid.* **1978**, *100*, 4914-4916. (d) Dietrich, B.; Fyles, T. M.; Lehn, J.-M.; Pease, L. G. *J. Chem. Soc., Chem. Commun.* **1978**, 934-936.

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Table I. Extraction of Adenosine Mono-, Di-, and Triphosphates by Diammonium Salt 3 and Monoammonium Salt

ammonium salt	10^{-4} concn, M 3 or 4 nucleotide		pH	$AXP_{extracted}$, % AMP	ADP	ATP	K_{AXP}[a] AMP	ADP	ATP
3	2.5	1.0	3.0	1.3 ± 0.4	37 ± 2	99 ± 0.5	(1)	45	7500
3	2.5	1.0	5.0	29 ± 6	89 ± 0.8	>99.6[b]	31	610	32000[c]
3	2.5	1.0	8.0	82 ± 10	97 ± 0.3	>99.6[b]	350	2500	111000[c]
4	5.0	1.0	3.0	0.7 ± 0.4	6 ± 3	6[d]	(1)	9	9
4	5.0	1.0	5.0	1.1 ± 0.5	9 ± 3.5	7[d]	1.6	14	11
4	5.0	1.0	8.0	0 ± 0.6	6 ± 0.8	9[d]	0	9	14
3	0.5	0.2	3.0	1.0 ± 0	40 ± 0	65 ± 1	(1)	66	180
3	0.5	0.2	5.0	41 ± 0	84 ± 1	92 ± 1	69	520	1100
3	0.5	0.2	8.0	69 ± 6	94 ± 1	95 ± 0	220	1600	1900

[a] $K_{AXP} = ([AXP]_{CHCl_3}/[AXP]_{aq})_{pH\ i}/([AMP]_{CHCl_3}/[AMP]_{aq})_{pH\ 3}$; $i = 3$, 5, or 8. [b] No trace of ATP was detectable in the aqueous phase. [c] These values were estimated from competitive binding experiments. See ref 9. [d] Values obtained by a single extraction experiment.

was prepared just before use from 8.2 g of silver nitrate and aqueous NaOH (2.65 M, 20 mL) and washed thoroughly with water. This was added to the solution of the iodide salt **1** (5.0 g, 3.0 mmol) in 1 L of methanol containing 10 mL of water. The reaction temperature was kept at 40 °C for 40 min, when the elimination of I^- was complete as ascertained by the complete disappearance of the UV absorption of I^-. The solution was separated from silver salt by filtration, and into the solution was added 2.3 mL of concentrated HCl, where the solution became acidic. Methanol was evaporated in vacuo to give 3.81 g (96%) of a colorless solid. The solid was recrystallized successively from DMF and methanol to give 2.1 g of pure **3**,[7] mp 92–93 °C. Anal. Calcd for $C_{42}H_{86}N_2Cl_2 \cdot H_2O$: C, 69.48; H, 12.49; N, 3.86; Cl, 9.77. Found: C, 69.41; H, 12.56; N, 3.99; Cl, 9.71. In earlier experiments, the diammonium salt **3** partly decomposed during storage. In this report, all of experiments were undertaken with use of freshly prepared material. Some experimental values of the extraction and the transport reported in the previous communications[5] were corrected in this paper.

Extraction of Nucleotide. A chloroform solution (8 mL) of the ammonium salt was shaken at 25.0 °C with an aqueous solution of a given nucleotide (1.0×10^{-4} M, 8 mL) at specified pH values. The ammonium salts employed were diammonium salt **3** (2.5×10^{-4} M), trioctylmethylammonium chloride (**4**) (5.0×10^{-4} M), or stearyltrimethylammonium chloride (**5**) (5.0×10^{-4} M). The pH in aqueous phase was adjusted to 3.0, 5.0, and 8.0 by the addition of 0.1 N sodium hydroxide or 0.1 N hydrochloric acid. The flask was shaken by hand for a few minutes. Extraction of the nucleotide from aqueous to organic phase shifted pH to lower values. Thus the pH was frequently readjusted to the original setting value. This procedure was repeated a couple of times. Then the shaking was conducted by an incubator thermostated at 25.0 ± 0.1 °C for 30 min, after which the pH was measured and adjusted, if necessary. The latter procedure was repeated at least three times. When no more change in pH values was observable for successive three measurements, the concentration of the nucleotides that remained in the aqueous phase was determined by UV spectrometry at each absorption maximum. When the extraction was too poor (less than 1%) to determine precisely the amount that decreased in the aqueous phase, the nucleotide concentration in chloroform was measured to determine the extraction percentage. On the other hand, adenosine triphosphate showed almost quantitative (more than 99%) extractions at pHs 5 and 8. In these cases, the competitive extraction in the presence of a 5–20 M excess amount of ADP was used to estimate the relative apparent extraction coefficient from which the equilibrium constants were calculated for ATP. All experimental values for the extraction are averages of at least three independent runs.

The ADP concentration in chloroform was determined as follows. An aqueous solution of ADP (1.0×10^{-4} M, mL, 8.0) was shaken with a chloroform solution containing the diammonium salt (2.5×10^{-4} M, 8 mL) to leave 0.208×10^{-4} M solution of ADP in the aqueous phase. The lower chloroform layer (6 mL) was sampled out with care not to be contaminated by the chloroform–water boundary layer. The chloroform solution was then shaken with an aqueous solution of sodium perchlorate (1.0×10^{-2} M, 6 mL, pH 3.0). The ADP in chloroform was almost quantitatively (99.2%) extracted back into the aqueous solution to give 0.786×10^{-4} M solution of ADP. The original chloroform solution gave the UV absorbance ($\log I_0/I$) of 1.10 at a slightly longer wavelength (262 nm) than that in an aqueous solution. Thus the molar extinction coefficient of ADP in chloroform was calculated to be 1.40×10^4 which was used as the ϵ_{max} for chloroform throughout the experiments.

Transport. The transport experiment was conducted by the use of Pressman cell.[8] Four cells were placed in a thermostated (25.0 ± 0.1 °C) bath, below which four magnetic rotors were connected to a central rotor to get a same rotation rate. This central rotor was driven by a synchronous motor in order to achieve the exactly same rotation rate throughout the transport run. Several rotation rates were obtained by changing the diameter of the central rotor relative to that of the motor. A typical transport condition is as follows. In a cell, the inner aqueous part I (3.43 cm^2) containing a nucleotide (1.0×10^{-2} M, 5 mL) and the outer aqueous part II (3.65 cm^2) containing 5 mL of water (no nucleotide) were introduced. Aqueous parts I and II were separated by a central glass tube. The organic layer (10 mL of chloroform containing 2.5×10^{-4} M of diammonium salt **3**) lay below two aqueous phases to bridge them. The chloroform layer was stirred with a small magnetic bar, which was rotated at a constant speed (204 rpm) with a magnetic rotor described above. The concentration of nucleotide appearing in aqueous part II was determined by the UV absorption at specified time intervals. The pHs of aqueous parts I and II were monitored by the glass electrodes inserted into both aqueous phases and readjusted very frequently by the addition of a tiny amount of NaOH (1.0 N) or HCl (1.0 N) to keep the pH deviation within ±0.1 pH unit at the most. For a check of the rotational rate dependence, a slower rotation rate (164 rpm) was adopted in some runs of ADP transport. However the identical transport rates were obtained. Some trials were also undertaken at a higher rotation rate (250 rpm), but the swirling of the aqueous phase into the chloroform phase made the results less reliable. Thus the rotation rate was fixed at 204 rpm in all runs described in the text.

For a transport by the application of salt gradients, sodium bromide was added to aqueous part II (1.0×10^{-2} M), while the pH values were kept at 5.0 ± 0.1 for both aqueous phases as described above.

Salt Exchange. A chloroform solution of **3** (2.5×10^{-4} M, 50 mL) was shaken with an aqueous solution of ADP (1.0×10^{-4} M, 100 mL, pH 5.0). This chloroform solution contained 0.79×10^{-4} M of the ion pair ADP–**3**, and a 10-mL portion of this solution was vigorously shaken by use of an incubator at 25.0 °C with an aqueous solution (10 mL, pH 5) of various inorganic salts, including $NaClO_4$, NaSCN, NaBr, $H_4P_2O_7$, Na_2SO_4, NaCl, NaF, CH_3COONa, NaH_2PO_4, and H_3BO_3. The pH of the aqueous solution generally deviated from the initial setting to slightly higher values and was readjusted every hour by the addition of 0.1 N HCl. The amount of ADP liberated into the aqueous phase was monitored by UV spectrometry.

Results and Discussion

Recognition of Nucleotides. Adenosine mono-, di-, and triphosphates were selectively extracted from aqueous to organic phases by ion-pair exchange with the lipophilic diammonium salt **3**. The results were compared in Table I with those obtained by using monoammonium salt **4**. The number of phosphate units in adenine nucleotides was successfully recognized by **3** so that the degree of the extraction decreased in the order ATP > ADP > AMP. The ratios of "apparent" partition coefficients calculated were listed in the last three columns of the table and exhibited significantly large values: e.g., ATP and ADP were recognized more favorably than AMP by factors 7500 and 45, respectively, at pH 3. The same trend holds in the extraction at pH 5. From the data of pK_a values,[10] adenine nucleotides are dissociated into

(7) The pure chloride was reasonably stable for a couple of weeks but slowly decomposing. Especially a trace amount of OH^- seriously accelerated the decomposition.

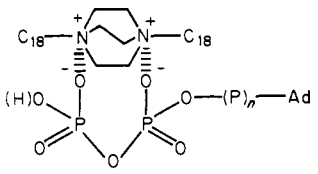
(8) Pressman, B.; Harris, E. J.; Jagger, W. S.; Johnson, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 1949–1956.

Table II. Extraction of Nucleotides of Guanine and Uracil by Diammonium Salt 3[a]

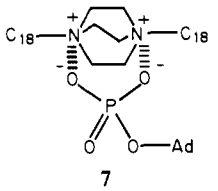
nucleotide	pH	nucleoside phosphate extracted, %			K_{NXP}[b]		
		mono	di	tri	mono	di	tri
guanine	3.0	1.2 ± 0	77 ± 1.4	93 ± 0.6	(1)	280	1100
	5.0	35 ± 4	89 ± 0.6	99 ± 0	44	670	8200
	8.0	95 ± 0.6	99 ± 0.6	99 ± 0.6	1600	8200	8200
uracil	3.0	1.0 ± 0.2	67 ± 0.8	93 ± 0.5	(1)	200	1300
	5.0	24 ± 1.1	94 ± 0.5	97 ± 0	31	1600	3200
	8.0	72 ± 4	98 ± 0.5	99 ± 0.5	250	4900	9800

[a] The concentrations of diammonium salt 3 and the nucleotide were 2.5×10^{-4} M and 1.0×10^{-4} M, respectively. [b] K_{NXP} follows a similar notation for K_{AXP}, where N is G or U. See note a under Table I.

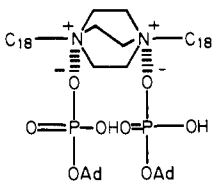
AMP^-, ADP^{2-}, and ATP^{3-} with ring protonation at pH 3, AMP^-, ADP^{2-}, and ATP^{3-} at pH 5, and AMP^{2-}, ADP^{3-}, and ATP^{4-} at pH 8. Comparison of the extraction data with the dissociated species of these nucleotides clearly indicates the actual mode of binding as



6, $n = 0$ or 1



7



8

Vicinal dianions (ADP and ATP at all pH values investigated) most favorably interacted with diammonium cations as shown in **6**. The rigid bicyclic skeleton is considered to provide an appropriate site for such duplicated ionic interactions. Contrastingly, the extraction of AMP showed a rather drastic change on the variation of pH values. AMP was extracted significantly into the chloroform phase only at pH 8, where AMP was dissociated to a geminate dianion and could interact with the diammonium cations favorably as shown in **7**. Similar dissociation is also possible at this pH range for the terminal phosphates of ADP or ATP, but it is difficult to determine the relative contribution of these interactions (types **6** and **7**), although it is clear that **6** makes a more important contribution on the basis of the weaker binding of AMP than ADP or ATP even at pH 8. The data listed in Table I also indicate that the monoanion of AMP (at pHs 3 and 5) is difficult to be extracted into chloroform. This may be explained by the picture that the salt formation of two nucleotide anions with one diammonium cation, **8**, will result in an unfavorable entropy effect.

The extremely efficient extraction of ATP should require additional comments. The apparent discrepancy between the number of ammonium cations and that of phosphate anions (three at pH 3 or pH 5, while four at pH 8) may need some sort of aggregation between certain numbers of diammonium and phosphate ions. The same situation occurs in the extraction of ADP at pH 8. In order to test this possibility, we examined the concentration dependence in the extraction, the results of which were included in the last three runs of Table I. When the concentrations of a nucleotide and diammonium salt **3** were lowered, the extraction percentages decreased in all the cases cited above.[11] This decrease in the degree of the extraction is compatible with the mechanism of an aggregate formation, because the aggregation should depend on the higher orders with respect to both concentrations of a nucleotide and the ammonium salt.

Another possible mechanism to account for the efficient extraction of ATP is derived from the nature of ATP molecule. Although the detailed structure of the ion pair ATP–**3** is difficult to be elucidated at present, it is highly probable that a remaining anionic site(s) of the triphosphate (two are ion paired with **3**) may be stabilized by a further ion pairing with a certain cationic species. This countercation association mechanism is supported by the observed dependence of the ATP transport rate on the nature and the concentration of the countercation. Where the countercation concentration is relatively low (Na^+, 2.0×10^{-2} M), the transport rates showed a complex profile probably due to the formation of a higher assembly where the countercation is N^+ of **3**. Thus 1.0×10^{-2} M NaCl was added to both aqueous phases in the transport setup. The transport rate of ATP increased from 2.2 to 3.3 $\mu M/cm^2/h$ (runs 1 and 4), while the corresponding value for ADP decreased slightly to 3.3 from 3.6 $\mu M/cm^2/h$ (runs 2 and 5). The presence of metal cations clearly assisted the ATP transport, and further details will be discussed in the later section. Here it should be emphasized that ATP is most probably extracted into chloroform accompanied by countercations.

Contrary to the highly selective and efficient extraction of nucleotides by use of diammonium salt **3**, monoammonium salt **4** was found very poor in nucleotide selectivity and in the degree of extraction. The poor extraction by **4** was most distinguished in cases where vicinal or geminate phosphate anions were involved. In the extraction of vicinal or geminate dianions of nucleotides, two ammonium cations are required to be gathered together. This process is assumed to be entropically unfavorable, if not unfavorable in enthalpy.

It is worth mentioning here that any adenine nucleotide decreased from aqueous phase was quantitatively found in chloroform solution as an ion pair with diammonium salt **3**. Stearyltrimethylammonium chloride **5**, however, exhibited a contrasting situation. ADP in an aqueous phase decreased from 1.0×10^{-4} M to 0.26×10^{-4} M by shaking at pH 5 with a chloroform solution of **5** (5.0×10^{-4} M), but no ADP was detected in the chloroform solution. ADP seems to be assembled at the water–chloroform interphase as a thick, opaque third layer which was observed in the extraction. From these results, it is concluded that diammonium salt **3** is a powerful phase-transfer reagent for nucleotides and that stearyltrimethylammonium salt **5** acts as a typical micellar reagent. The diammonium salt **3** is different from conventional micellar reagents in a sense that the cationic centers

(9) A mixture of ADP (0.6×10^{-3} M) and ATP (1.2×10^{-4} M) was shaken at pHs 5 and 8 with a chloroform solution of **3** (2.5×10^{-4} M). Then the chloroform solution was shaken with a 1.0×10^{-2} M solution (pH 5) of sodium perchlorate, and all of nucleotides were quantitatively extracted back into an aqueous solution. The total nucleotide concentration was measured by UV spectrometry (1.3×10^{-4} M for both pHs), and the ratios ATP/ADP were determined by HPLC to give 2.1 and 3.1 at pHs 5 and 8, respectively, from which the ratios of equilibrium constant K_{ATP}/K_{ADP} were calculated to be 39 and 44, respectively. Similar competitive extractions were undertaken by use of 1.2×10^{-4} M ATP and 1.1×10^{-3} M or 2.2×10^{-3} M ADP. The apparent equilibrium constants listed in Table I are mean values obtained from these three competitive experiments.

(10) (a) Alberty, R. A.; Smith, R. M.; Bock, R. M. *J. Biol. Chem.* **1951**, *193*, 425–434. (b) Izatt, . M.; Christensen, J. J. *J. Phys. Chem.* **1962**, *66*, 359–360. (c) Bock, R. M.; Ling, N. S. *Arch. Biochem. Biophys.* **1956**, *62*, 253–264.

(11) The values reported in the previous communication were too poor. The effect of decomposition of diammonium salt **3**, which was described in the Experimental Section, seems to be significant especially for the extraction in low concentrations of **3**.

Table III. Carrier-Mediated Transport of Nucleotide across a Chloroform Liquid Membrane

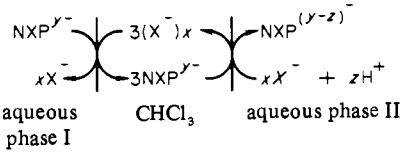
	aqueous phase I					$CHCl_3$	aqueous phase II			transport rate	
run	nucleotide	concn, M	pH	salt, M	surface area, cm^2	3, M	pH	salt, M	surface area, cm^2	$\mu M/h$	$\mu M/h/cm^2$ [a]
1	ATP	1.0×10^{-2}	5.0		3.43	2.5×10^{-4}	0.5		3.65		2.2
2	ADP	1.0×10^{-2}	5.0		3.43	2.5×10^{-4}	0.5		3.65	12.5	3.6 ± 0.0
3	AMP	1.0×10^{-2}	5.0		3.43	2.5×10^{-4}	0.5		3.65		0.07 ± 0.02
4	ATP	1.0×10^{-2}	5.0	NaCl (1.0×10^{-2})	3.43	2.5×10^{-4}	0.5	NaCl (1.0×10^{-2})	3.65		3.3 ± 0.4
5	ADP	1.0×10^{-2}	5.0	NaCl (1.0×10^{-2})	3.43	2.5×10^{-4}	0.5	NaCl (1.0×10^{-2})	3.65		3.3 ± 0.1
6	ATP	1.0×10^{-2}	5.0	KCl (1.0×10^{-2})	3.43	2.5×10^{-4}	0.5	KCl (1.0×10^{-2})	3.65		4.2 ± 0.7
7	ATP	1.0×10^{-2}	5.0	NaCl (1.0×10^{-1})	3.43	2.5×10^{-4}	0.5	NaCl (1.0×10^{-1})	3.65		0.36
8	ADP	1.0×10^{-2}	5.0	NaCl (1.0×10^{-1})	3.43	2.5×10^{-4}	0.5	NaCl (1.0×10^{-1})	3.65		0.07
9	ATP	1.0×10^{-2}	5.0	NaBr (1.0×10^{-2})	3.43	2.5×10^{-4}	0.5	NaBr (1.0×10^{-2})	3.65		0.1
10	ADP	1.0×10^{-2}	5.0	NaBr (1.0×10^{-2})	3.43	2.5×10^{-4}	0.5	NaBr (1.0×10^{-2})	3.65		0.04
11	ADP	1.0×10^{-2}	5.0		3.43	1.0×10^{-4}	0.5		3.65		3.1 ± 0.1
12	ADP	1.0×10^{-2}	5.0		3.43	0.5×10^{-4}	0.5		3.65		2.5 ± 0.07
13	ADP	1.0×10^{-2}	5.0		3.43	0.25×10^{-4}	0.5		3.65		1.3 ± 0.03
14	ADP	1.0×10^{-2}	5.0		4.75	2.5×10^{-4}	0.5		2.01	18.1	
15	ADP	1.0×10^{-2}	5.0		3.65	2.5×10^{-4}	0.5		3.43	12.6	
16	ADP	1.0×10^{-2}	5.0		2.01	2.5×10^{-4}	0.5		4.75	9.25	
17	ADP	1.0×10^{-2}	8.0		3.43	2.5×10^{-4}	0.5		3.65		3.7 ± 0.1
18	AMP	1.0×10^{-2}	8.0		3.43	2.5×10^{-4}	0.5		3.65		0.4 ± 0.07
19	ADP	5.0×10^{-3}	5.0		3.65	2.5×10^{-4}	5.0	NaBr (1.0×10^{-2})	3.43		3.3
20	ADP	5.0×10^{-4}	5.0		3.65	2.5×10^{-4}	5.0	NaBr (1.0×10^{-2}) ADP (5.0×10^{-4})	3.43		2.6 ± 0.3
21	UDP	1.0×10^{-2}	5.0		3.43	2.5×10^{-4}	0.5		3.65		0.55
22	UMP	1.0×10^{-2}	5.0		3.43	2.5×10^{-4}	0.5		3.65		0.019
23	CDP	1.0×10^{-2}	5.0		3.43	2.5×10^{-4}	0.5		3.65		0.078
24	CMP	1.0×10^{-2}	5.0		3.43	2.5×10^{-4}	0.5		3.65		0.013
25	Phe	5.0×10^{-2}	(0.1 N KOH)			1.0×10^{-2} [b]	(0.1 N HCl)				5.0[sa]
26	Tyr	5.0×10^{-2}	(0.1 N HCl)			1.0×10^{-2} [c]	(0.1 N KOH)				3.7[sa]
27	K^+	1.0×10^{-2}	100			1.5×10^{-3} [d]	10	KPic (1.0×10^{-5})			1.8[15]

[a] The value divided by the surface area at uptake site. [b] Tricaprylmethylammonium chloride in toluene. [c] Dinonylnaphthalenesulfonate in toluene. [e] Cryptand [2.2.C_8].

are attached symmetrically by two long alkyl chains. This structural characteristic apparently prohibits an appropriate alignment of the reagent at a water–chloroform interphase and is favorable for acting as a phase-transfer reagent of an unusually strong binding.

The extraction by use of diammonium salt **3** was also tried for other purine and pyrimidine nucleotides at the same pH range. Results are listed in Table II. There was observed no significant change in the extractability of different nucleotide bases.[12] A general trend found in the extraction of adenine nucleotides could be observed also in guanine and uracil nucleotides. Triphosphates were extracted almost quantitatively at all pHs employed and monophosphates extracted significantly only at pH 8. Diphosphates were between these two extremes. These results are compatible with the mechanism that the recognition is based primarily on the regiospecific ionic interaction between the diammonium cation and the vicinal, $^-O–P–O–P–O^-$, or geminate, $^-O–P–O^-$, dianions of phosphate. It is concluded that diammonium salt **3** is a powerful phase-transfer reagent to recognize nucleoside phosphates in the order triphosphate > diphosphate > monophosphate.

Scheme I



The high efficiency of the diammonium salt to extract nucleotides from aqueous to organic phases makes it possible to use this reagent as a carrier of the nucleotide transport which is another interesting aspect of bioenergetics. The nucleoside phosphates are suggested to be encapsulated in intrinsic carrier molecules such as ionophoric proteins in mitochondria[13] to facilitate the entry of otherwise highly hydrophilic nucleotides into a lipophilic biological membranes. Thus we investigated the selective transport through a chloroform liquid membrane by applying potential differences of a proton or an inorganic salt concentration across the membrane (Scheme I for a proton gradient)

Adenine nucleotides were dissolved in an aqueous solution (aqueous phase I, 1.0×10^{-2} M, pH 5) and transported through

(12) The amount of guanine or uracil nucleotide found in the chloroform phase was less then that decreased from the aqueous phase. This observation shows a contrast to the case of adenine nucleotides, where the nucleotide decreased from an aqueous phase was found quantitatively in a chloroform phase. GDP in the chloroform solution was 67 and 55% of the amount decreased from the aqueous phase at pHs 5 and 8, respectively. The corresponding values for UDP were 96 and 74%. The residual part may locate in the boundary region of water–chloroform or precipitated.

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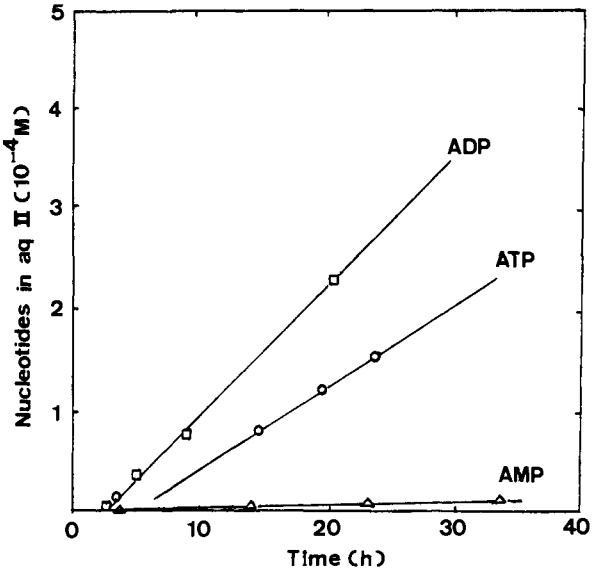

Figure 1. Nucleotide appearing in aqueous phase II as a function of time in a three-phase transport system: aqueous phase I, 1.0×10^{-2} M nucleotide, pH 5, aqueous phase II, pH 0.5, and $CHCl_3$, 2.5×10^{-4} M **3**, for ATP (○), ADP (□), and AMP (△).

chloroform containing 2.5×10^{-4} M diammonium salt **3**. The concentration of a nucleotide appearing in the other aqueous phase, II (pH 0.5), was plotted against time in Figure 1. The transport rate followed zeroth-order kinetics and was only dependent on the surface area (S) to give $(d[AXP]/dt)_{aq\,II} = kSt$, for all adenine nucleotides investigated after a short induction period (within 4 h). The rates measured from Figure I were listed in Table III (runs 1, 2, and 3). As was discussed above, the transport rate of ATP was much affected by the presence of metal cations. When the concentration of a countercation was relatively low (2.0×10^{-2} M), the transport rate of ATP was smaller than that of ADP. However, the ATP transport was accelerated in the presence of a higher concentration (1.0×10^{-2} M more) of the Na^+ or K^+ cation (runs 4 and 6). The use of a too high concentration (1×10^{-1} M) of a metal salt (runs 7 and 8) decreased the transport rate, probably due to the competitive anion (Cl^-) binding to the carrier molecule. This inhibition was most clearly demonstrated in runs 9 and 10, where sodium bromide significantly blocked the nucleotide transport. Although the detailed mechanism is not clear at the present stage of investigation, the effect of metal cations should be correlated with the association between the residual anion of ATP and the countercation as discussed above. For further insights into this transport mechanism to be gained, precise membrane dynamics should be investigated.[14] The transport selectivity ratios obtained are extremely high, calculated to be 60 for ATP/AMP (runs 6 and 3) and 51 for ADP/AMP (runs 2 and 3). These results illustrate the first successful example for the selective transport of nucleotides.

Overall transport rates, however, should depend on many factors such as carrier concentration and chemical potentials applied across the membrane. In the first place the rate-determining step was determined from possible elemental processes, uptake, diffusion, and release processes. Thus, the carrier concentration was varied ranging from 0.25×10^{-4} to 2.5×10^{-4} M for the ADP transport (runs 11–13 and 2). The rate increased with first-order with respect to the carrier concentration but was apparently saturated near 1.0×10^{-4} M. This strongly indicates that the rate-determining step should be changed during this concentration range. Then the rate-determining step was tried to be elucidated by changing surface areas of the water–chloroform interface at uptake and release sites (runs 14–16 and 2). The apparent rates (μM/h) were observed to decrease linearly with decreasing surface areas at the uptake site. Thus the transport rate is determined primarily by the rate of the nucleotide uptake, and the rate per unit surface area at the uptake site is calculated to be practically constant, 3.7 ± 0.3 μM/h/cm^2, for these four runs listed.

The other factor affecting the transport rate is chemical potential differences applied across the membrane. In runs 17 and 18, the pH in aqueous phase I was raised from 5.0 to 8.0 without changing other conditions. The rate enhancement was observed in the AMP transport by a factor of 6, probably due to the further ionization to the geminate dianion which was more strongly recognized by **3**.

These transport rates should be compared with those reported for other material transported through a liquid membrane. In runs 25 and 26 are listed the transport rates of phenylalanine with use of tricaprylmethylammonium chloride or tyrosine with dinonylnaphthalene sulfonate, the fastest combinations for the amino acid transport.[5a] Run 27 shows the potassium transport mediated by a cryptand [2.2.C_8], again the fastest combination for the metal cation transport by use of cryptands.[15] A direct comparison of these data with ours is certainly difficult, because many factors influencing the transport rate are not thoroughly elucidated. If the rate is allowed to be compared per mole of substrate and per mole of carrier concentration for a trivial measure, runs 2 (ours), 25, 26 and 27 are 1.4×10^6, 1.0×10^4, 7.4×10^3, and 1.2×10^5 μM/h/cm^2/mol of carrier/mol of substrate, respectively. The transport rate of nucleotides is quite large by factors 10–190 than those reported as the fastest for amino acids or alkali-metal cations. Note, however, that chemical potentials could not be standardized in spite of large differences from runs to runs, and we should wait to give more detailed discussions until a much deeper understanding of membrane dynamics is available in the future.

Other pyrimidine nucleotides were also transported successfully as shown in runs 21–24. It is now safely approved as a general rule that the diammonium salt **3** is a carrier that is better for the transport of diphosphates than monophosphates. It is noteworthy however that big differences were observed for the transport rates of ncleotides having different bases, rate ratios being 1:1/6.5:1/46 for ADP:UDP:CDP. In the extraction experiment described in the earlier section which is an equilibrium study, there could not be observed any significant differences between adenine and other nucleotides. It is an interesting possibility that these nucleotides behave differently in the membrane dynamic but not in the thermodynamic events.

In the above cases where the pH gradient was applied, ADP and ATP were partially hydrolyzed to AMP in aqueous phase II. The HPLC analysis showed that ADP in aqueous phase II was composed of 86% AMP and 14% ADP after 68 h in run 2, while ADP in aqueous phase I (pH 5.0) remained unchanged. The hydrolytic cleavage of nucleotides is known to have a sharp pH dependence.[16] In order to suppress the concomitant (undesired) hydrolysis, the pH of aqueous phase II should be kept at at least 5.0 and the transport should still be accelerated. Then we examined the release of the bound nucleotide driven by the application of the concentration gradient of various inorganic anions. The aqueous solution of an organic salt (1.0×10^{-2} M) was stirred with a chloroform solution of the ion pair ADP–**3** (7.9×10^{-5} M). The amount of ADP appearing in the aqueous phase was shown in Figure 2. The relative exchangeability of inorganic anions follows in the order[17] $SCN^- > ClO_4^- > HSO_4^- > Br^- > PP_i > Cl^- > H_2PO_4^- > CH_3COO^- > F^- > H_2BO_3^-$. The polarizable anion such as perchlorate or thiocyanate possesses a high rank. Similar order of the salt effect was observed also in the inhibition

(14) The rates of uptake and release were determined for ADP and ATP by using a two-phase system where one of the aqueous phases was excluded from the transport setup. However, the rate constants obtained were not enough to describe the overall transport rates of ADP and ATP or their relative order. One or more unknown slow rate processes should thus be involved when vectorial membrane potentials were applied as in the transport system.

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(17) The kinetic and equilibrium values for the liberation of nucleotide exhibited some differences. Thus the equilibrium value was adopted as a measure of their relative order.

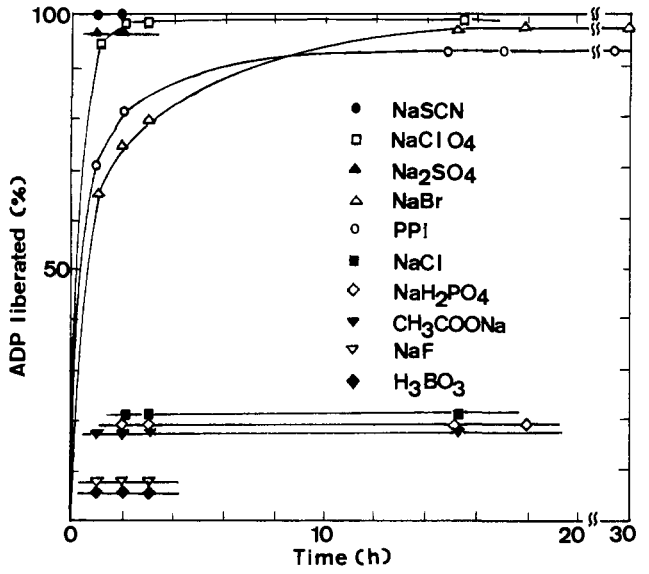

Figure 2. ADP liberated from the chloroform phase by an exchange with inorganic salts in aqueous phase. Initial conditions: 0.79×10^{-4} M ADP–3 complex in $CHCl_3$; 1.0×10^{-2} M inorganic salt in aqueous phase, pH 3.

of acetacetate decarboxylase,[18] affinity for Dowex 2 (tetraalkylammonium salt).[19] The anions having large polarizabilities are easily desolvated, being accessible to form ion pairs in a hydrophobic environment. Perchlorate and thiocyanate are thus highly efficient to extract out all of bound nucleotide and capable of forming stable ion pairs with the diammonium salt. These anions, however, inhibited a further transport when used as an anion-exchange reagent in aqueous phase II due to the poor dissociation of the ion pair to regenerate the active diammonium salt in the membrane phase. Bromide anion was moderately effective in the nucleotide liberation and still moderately dissociative and appropriately employed as an excellent exchange reagent in aqueous phase II. Thus NaBr was dissolved in aqueous phase II (1.0×10^{-2} M) to make a concentration gradient while the pHs were maintained at 5.0 for both aqueous phases. ADP was successfully transported (run 19) without suffering hydrolysis to an observable extent.

An active transport system was built up by using this salt gradient technique. ADP was dissolved in both aqueous phases (5.0×10^{-4} M) and NaBr was dissolved only in aqueous phase II. The ADP concentration in aqueous phase II increased with a rate, 2.6 ± 0.3 $\mu M/cm^2$, comparable to that obtained in the corresponding passive transport (run 12).

Conclusion

The lipophilic diammonium cation **3** is a very efficient and selective phase-transfer reagent of nucleotide anions. Two cationic centers are separated by 2.4 Å which is complementary to vicinal or geminate dianions of nucleotide phosphates. Nucleotide binding decreased in the order triphosphate > diphosphate > monophosphate, and their selectivity was high as exemplified by 45 for ADP/AMP and 7500 for ATP/AMP. Compared to this diammonium salt, trioctylmethylammonium chloride, a typical phase-transfer reagent, was much less effective for the phase transfer of nucleotides. Stearyltrimethylammonium chloride, a typical micelle-forming reagent, bound nucleotides at the boundary region of water–chloroform. Diammonium salt **3** facilitated effectively the transport of nucleotide across a chloroform liquid membrane. Active as well as passive transport was driven by pH or salt gradient across the membrane. The selectivity ratios of the transport rate of ATP or ADP to that of AMP were estimated to be as high as 60 or 51, respectively.

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α-Amino Acids as Chiral Educts for Asymmetric Products. Amino Acylation with *N*-Acylamino Acids

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Abstract: α-*N*-Acylamino acids have been developed as useful reagents for the preparation of optically pure α-aminoalkyl aryl ketones. Protection of the amino group as either the ethoxycarbonyl or benzenesulfonyl derivative allows alanine to serve as an effective educt for the chirally specific synthesis of a variety of structures containing the phenylethylamine backbone. Benzene undergoes Friedel–Crafts acylation with the *N*-acylalanine acid chloride. Catalyst complexation with oxygenated aromatics, however, prohibits acylation of aryl ethers. An arylmetallo reaction scheme overcomes this problem and also affords regiospecificity not attainable in conventional acylations. As examples, optically pure ephedrines and amphetamines were directly synthesized without recourse to resolution since the chirality of the amino acid educt was entirely conserved throughout the process.

The synthesis of optically pure α-aminoalkyl aryl ketones is of general interest in that it provides a direct route to a large variety of biologically significant compounds containing the phenylethylamine moiety in their active, asymmetric configurations. We have explored their preparation from the various acyl derivatives of readily available optically pure α-amino acids as educts. This has been done from two perspectives: (a) Friedel–Crafts acylation and (b) arylmetallo reactions. Our work demonstrates that these two methods are effective and uniquely complementary in several arylation applications with complete retention of optical activity during the synthetic process.

Friedel–Crafts Applications

The Friedel–Crafts acylation of aromatic compounds has been widely exploited since its initial appearance in the literature.[1]

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