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# Complexation and Phase Transfer of Nucleotides by Gramicidin S†

Eric M. Krauss‡ and Sunney I. Chan*

ABSTRACT: Gramicidin S (GrS), an amphiphilic cyclosymmetric decapeptide produced by *Bacillus brevis* G-B and Nagano, binds nucleotides in water to yield a complex which partitions into organic solvents. The observed phase-transfer efficiencies at a given pH increase in the order AMP < ADP < ATP. The lipophilic complexes have well-defined stoichiometries, which were determined to be 1:1 for ADP–GrS at pH 7 and ATP–GrS at pH 3 and 1:2 for ATP–GrS at pH 7. The interaction is primarily ionic, involving coordination of the ornithine $N^{\delta}H_3^+$ groups of the peptide and the phosphoryl groups of the nucleotide, with little contribution from the nucleoside moiety. Exchange of organic and inorganic phosphates was also found to be mediated by GrS. The nucleotide complexes are sparingly soluble in water and self-associate extensively in $CHCl_3$, most likely by cross-$\beta$-aggregation, to yield large, ribbonlike aggregates which give rise to broad NMR resonances. Structures for the 1:1 and 1:2 complexes are proposed. In the latter, two GrS molecules envelop the nucleotide, orienting their apolar faces externally in opposite directions, while the lateral faces retain considerable polar character and direct aggregation in organic media. The 1:1 complex possesses a single apolar face and is less lipophilic. Binding constants were estimated by simulation of the extraction data. For the 1:1 complexes, $K_{1:1} \simeq 4 \times 10^4$ $M^{-1}$ for either ADP or ATP. Phase transfer of the ATP complex at pH 7 could be modeled either by stochastically independent binding to two noninteracting sites on the nucleotide with $K_1 \sim K_2 \sim K_{1:1}$ or by a sequential process with $K_1 \sim K_{1:1}$ and $K_2/K_1 < 100$. It is concluded that the apparent selectivity of GrS for ATP over ADP is a consequence of the greater lipophilicity and tendency to aggregate of the 1:2 complex, rather than an intrinsically higher binding affinity for triphosphates. GrS is, to our knowledge, the first peptide known to possess phase-transfer activity toward nucleotides; this is, in addition, the first molecular recognition process in which GrS is demonstrated to participate in vitro at physiologically active concentrations.

Considerable attention has been given macromolecules which form stable coordination complexes with ionic substrates in solution. Through judicious placement of charged and polar groups, it has been possible to design complexing agents, or complexones, which bind substrates strongly and with considerable selectivity (Lehn, 1978). Complexones serve as models for the study of biomolecular recognition and transport processes (Lehn, 1978; Tabushi et al., 1978, 1980, 1981) and have important applications in chemical catalysis (Montanari et al., 1982). Much of the recent research on anion complexones of potential biological significance (Dietrich et al., 1979, 1981; Graf & Lehn, 1976; Kimura et al., 1981, 1982) has centered on natural and synthetic polyamines which interact with nucleotides (Dietrich et al., 1981; Kimura et al., 1982; Nakai & Glinsmann, 1977; Tabushi et al., 1980, 1981). Of the latter class of compounds, the simplest examples are the linear amines putrescine, spermidine, and spermine, which form nucleotide complexes that may serve a regulatory function in cell growth (Nakai & Glinsmann, 1977). Macromonocyclic salts containing repeating units of ethylene-, propylene-, and butylenediamine bind nucleotides more tightly than the acyclic ligands (Dietrich et al., 1981; Kimura et al., 1982), presumably because the cyclic compounds are less conformationally mobile and possess a higher charge density. A highly constrained, lipophilic ethylenediamine congener, *N,N'*-distearyl-1,4-diazabicyclo[2.2.2]octane (DS-Dabco),[1] has been shown to bind nucleotides and transfer them efficiently to $CHCl_3$ (Tabushi et al., 1980, 1981). All of these complexes involve ion pairing between the phosphate moieties of the nucleotide and the positively charged amino groups of the ligand and are stabilized by electrostatic and, where possible, hydrogen-bonding interactions.

Gramicidin S [GrS, *cyclo*-(Val[1]-Orn[2]-Leu[3]-D-Phe[4]-Pro[5])$_2$; Figure 1] is a cyclosymmetric decapeptide antibiotic produced by mature cultures of *Bacillus brevis* G-B and Nagano which has been the subject of many physicochemical studies [see Chapter 5 of Izumiya et al. (1979)] but whose biological

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[1] Abbreviations: GrS, gramicidin S; $Me_6$GrS, [2,2',$N^{\delta}$-trimethylornithyl]gramicidin S; ChaGrS, 4,4'-bis(D-cyclohexylalanyl)gramicidin S; DS-Dabco, *N,N'*-distearyl-1,4-diazabicyclo[2.2.2]octane; AMP, adenosine 5'-monophosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; GTP, guanosine 5'-triphosphate; CTP, cytidine 5'-triphosphate; UTP, uridine 5'-triphosphate; TTP, thymidine 5'-triphosphate; 2'-dTTP, 2'-thymidine 5'-triphosphate; 2',3'-dideoxy-TTP, 2',3'-dideoxythymidine 5'-triphosphate; $CHCl_3$, chloroform; $CDCl_3$, deuteriochloroform; $CCl_4$, carbon tetrachloride; EtOH, ethanol; $Me_2CO$, acetone; MeOH, methanol; $Me_2SO$-$d_6$, dimethyl-$d_6$ sulfoxide; $PP_i$, inorganic pyrophosphate; $P_i$, inorganic phosphate; Orn, ornithine; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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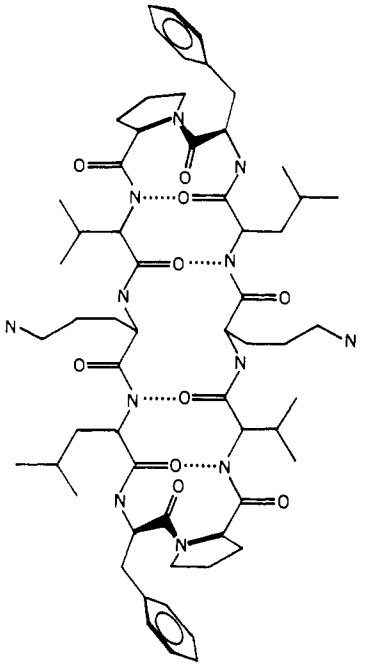

FIGURE 1: Diagrammatic representation of the decapeptide antibiotic GrS, illustrating antiparallel $\beta$-pleated backbone structure.

functions remain unclear. The solution structure is well characterized (Jardetzky & Roberts, 1981). On inspection, GrS appears to be a likely candidate for an anion complexone: it is a divalent cation, and the positive charges, because of the rigidity of the peptide backbone (Stern et al., 1968; Allerhand & Komoroski, 1973; Krauss & Chan, 1982a) and the relative lack of motional freedom of the Orn side chains (Komoroski et al., 1975; Krauss & Chan, 1982b), are constrained to occupy positions on one face of the ring, an average of less than 8 Å apart (Krauss & Chan, 1982b). The side chains of the remaining hydrophobic residues are arrayed toward the opposite face of the ring (Stern et al., 1968; Few, 1956), suggesting that if GrS were indeed an anion complexone it might possess phase-transfer activity as well. The anion binding properties of GrS have not been investigated previously in vitro. In vivo, GrS produces remarkable effects on phosphate metabolism in microorganisms; strains of *B. brevis* actively synthesizing the peptide exhibit a nearly complete loss of extractable nucleoside di- and triphosphates (Silaeva et al., 1965; Glazer et al., 1966) accompanied by a rapid loss of $^{31}P$ NMR signals from intracellular ATP (Vostroknutova et al., 1981). These effects were attributed to inhibition of membrane-bound respiratory enzymes by adsorption of GrS to acidic phospholipids (Ostrovskii et al., 1976) rather than to nucleotide binding. Direct evidence of such an interaction with bacterial membranes has not been obtained, however (Kaprel'yants et al., 1977).

In this study, it is reported that GrS efficiently binds nucleotides in water to yield a complex which partitions into organic solvents such as $CHCl_3$, establishing this molecule as the first phase-transfer reagent for nucleotides which is a natural product and the first nucleotide complexone which is not an aliphatic polyamine. GrS exhibits a pattern of selectivity similar to those of the synthetic complexones (Tabushi et al., 1981; Dietrich et al., 1981), with the extraction efficiencies generally increasing in the order AMP < ADP < ATP. The interaction is primarily ionic, with little or no contribution from the nucleoside moiety, and is subject to competition by charged species. In the organic phase, the complexes associate extensively, most likely by cross-$\beta$-aggregation. In contrast to the situation with the synthetic ligands, where the stoichiometry is uncertain or unknown (Tabushi et al., 1981) or is assumed a priori to be 1:1 (Dietrich et al., 1981; Kimura et al., 1982) even though other possibilities exist, the stoichiometries of the extracted complexes formed by GrS are obtained by NMR and confirmed by simulation of the extraction profiles. Knowledge of the stoichiometries permits estimation of binding constants through systematic study of the extraction and thereby affords an understanding of the molecular basis for the observed selectivity among the nucleotides. Our observations strongly indicate that a direct nucleotide–GrS interaction underlies the anomalously low energy charge and vigorous nucleotide efflux observed in late cultures of *B. brevis* actively synthesizing the peptide and, possibly, the lethal effects exerted by GrS on susceptible microorganisms. The implications of GrS-induced phase transfer of nucleotides for selective nucleotide transport through biological membranes are considered.

## Materials and Methods

*Materials.* Gramicidin S dihydrochloride was obtained from Sigma Chemical Co. and lyophilized prior to use. It migrated as a single spot in several solvent systems on TLC. Adenosine 5′-triphosphate, disodium salt (Sigma), adenosine 5′-diphosphate, disodium salt (Sigma), adenosine 5′-monophosphate, monosodium salt (Sigma), uridine 5′-triphosphate, trisodium salt (Sigma), thymidine 5′-triphosphate, trisodium salt (Sigma), 2′-thymidine 5′-triphosphate, tetrasodium salt (P-L Biochemicals), 2′,3′-dideoxythymidine 5′-triphosphate, tetrasodium salt (P-L Biochemicals), guanosine 5′-triphosphate, trilithium salt (Calbiochem), and cytidine 5′-triphosphate, disodium salt (Calbiochem), were used without further purification. Other chemicals were of reagent grade.

*Spectroscopy.* UV spectra were measured on a Beckman Instruments ACTA CIII or a Cary 219 spectrophotometer by using 1-cm path-length cells at 25 °C. Concentrations of standard solutions of the nucleotides were determined from published molar extinction coefficients (Dawson et al., 1969). Nucleotide concentration in the aqueous phase after extraction was determined by measurement of the absorbance at 270 nm, where the absorbance of the peptide is negligible.

NMR spectra were obtained on a Bruker WM-500 multinuclear spectrometer at the Southern California Regional NMR Facility. The operating frequencies were 500.13 MHz for $^1H$ and 202.49 MHz for $^{31}P$.

*Extraction Procedure.* $CHCl_3$ constituted the organic phase for most of the extractions. The volumes of the aqueous and organic phases were equal. All operations were performed at room temperature. Aqueous solutions containing GrS, nucleotide, and (where desired) additional salts were combined in a stoppered 12-mL conical tube and adjusted to a specified pH with dilute NaOH or HCl. The organic solvent was added and the tube shaken vigorously by hand (four strokes per second) for 2 min. Phase separation was hastened by brief centrifugation and the pH of the aqueous layer readjusted to the starting value, where necessary. The decrease in pH during the initial 2-min extraction was generally <1 unit at pH 7 and nil at pH 3. The tube was shaken for an additional 2 min and centrifuged, and the absorbance ($A$) of the aqueous phase was determined. The extraction efficiency ($E$) was defined (Tabushi et al., 1981) by eq 1 where $A_{270,i}$ and $A_{270}$ refer to the

$$E = 1 - \frac{A_{270}}{A_{270,i}} \quad (1)$$

initial and final absorbances at 270 nm, respectively. The use

Table I: Extraction Efficiencies at pH 7[a]

| nucleotide | [nucleotide][b] | [GrS][b] | other | $E$ |
|---|---|---|---|---|
| ATP | 20 | 40 | | 0.88 |
| | 40 | 40 | | 0.48 |
| | 10 | 20 | 0.02 M NaCl | 0.75 |
| | 10 | 20 | 0.02 M $P_i$ | 0.45 |
| | 10 | 20 | 0.02 M $PP_i$ | ~0 |
| | 10 | 20 | 0.01 M $MgCl_2$ | 0.19 |
| | 10 | 20 | 1-octanol[c] | 0.61 |
| | 100 | 200[d] | | ~0 |
| ADP | 20 | 40 | | 0.78 |
| | 50 | 50 | | 0.51 |
| AMP | 20 | 40 | | 0.72 |

[a] $H_2O/CHCl_3$ system. [b] In $10^{-6}$ M. [c] Substituted for $CHCl_3$. [d] 1,5-Pentanediamine.

of longer extraction times afforded no increase in $E$.

Whereas neutral salts were added to maintain constant ionic strength in titrimetric studies of nucleotide complexation in water (Dietrich et al., 1979, 1981), this was not done in the present study in order to avoid competitive effects. Neutral salts were not used either in the extractions employing DS-Dabco (Tabushi et al., 1978, 1980, 1981) or in the quantitative study of the binding and extraction of alkali metal salts by macrotetralide antibiotics by Eisenman et al. (1969). The concentrations of $Na^+$ and $Cl^-$ present as counterions and for the purpose of pH adjustment were invariably too low ($\leq 10^{-3}$ M) to exert competitive effects in the extraction runs employing GrS.

*Data Reduction.* As described under Results, the variation of $E$ with the initial nucleotide concentration, $[a]_i$, is, for the 1:$n$ interaction with $\zeta = 1$, $E/[1 - (1 + K_p^{-1})E]^{n+1} = (n\text{-}[a]_i/1.2)^n KK_p$ neglecting aggregation. Attempts to fit the extraction profiles to this equation by iterative minimization of $\sum_j (E_j^{obsd} - E_j^{calcd})^2$ were unsuccessful, since the design matrix contained explicit $[a]_i^{-n}$ dependence which spuriously weighted each datum by this factor. Successful simulation made use of the equivalent relation $Y = \ln KK_p([a]_i n/1.2)^n + (n + 1) \ln (1 - q/K_p)$, where $Y \equiv \ln [E/(1 - E)^{n+1}]$, $q \equiv E/(1 - E)$, and the algorithm minimized $\sum_j (Y_j^{obsd} - Y_j^{calcd})^2$ with respect to $K$ and $K_p$. All calculations, including those outlined in the Appendix, were performed on a VAX 11/780 computer administered by the Division of Chemistry and Chemical Engineering at the California Institute of Technology.

## Results

GrS promotes phase transfer into $CHCl_3$ of adenine nucleotides (Table I). As shown in Table I, the extraction efficiency ($E$) decreases significantly for ADP and AMP at pH 3, but ATP exhibits a smaller pH dependence for the extraction. Table II also compares extraction data for GrS to those obtained for DS-Dabco under similar conditions (Tabushi et al., 1981). At neutral pH, GrS is the more efficient phase-transfer agent, while at pH 3, the reverse holds. The abilities of these complexones to discriminate between the adenine nucleotides at either pH is qualitatively the same.

The extraction of ATP into $CHCl_3$ by GrS at pH 7 is efficient, as illustrated by the value of 0.60 measured for $E$ when [ATP] = $2 \times 10^{-6}$ M and [GrS] = $4 \times 10^{-6}$ M. Purine nucleotides are extracted slightly more efficiently than pyrimidines. At $10^{-5}$ nucleotide, $2 \times 10^{-5}$ M GrS, and pH 7, the extraction efficiencies ($E$) are 0.83, 0.86, 0.70, and 0.80 for ATP, GTP, CTP, and UTP, respectively.

*Nucleotide Binding in Water.* Combining aqueous solutions of GrS and ATP at concentrations exceeding ~$10^{-5}$ M at pH 7 results in precipitation, demonstrating that binding occurs without the necessity of a second process involving phase transfer. Unfortunately, the very low aqueous solubility of the triphosphate–GrS complexes precluded titrimetric, UV, or NMR studies. It was, on the other hand, possible to observe by NMR the interaction between ADP and GrS when [GrS] < $10^{-4}$ M. $^1H$ NMR spectra at 500.13 MHz revealed a progressive downfield shift in the central unresolved component of the Orn $C^\delta H_2$ multiplet, an ABXX′ system in $D_2O$, with increasing [ADP] (Figure 2). There were no shifts in the aromatic resonances which might indicate stacking of the adenine and phenylalanine rings.

In order to obtain $^{31}P$ spectra, it was necessary, because of the low solubility of the GrS complexes, to utilize the alkylated GrS derivative [2,2′,$N^\delta$-trimethylornithyl]-GrS ($Me_6$GrS) (Krauss & Chan, 1982b) which binds nucleotides considerably more weakly than the native peptide. Addition of $1.5 \times 10^{-3}$ M $Me_6$GrS to $7.5 \times 10^{-4}$ M ATP at pH 7 resulted in downfield shifts of 0.5 ppm of $P^\gamma$, 0.4 ppm of $P^\beta$, and 0.1 ppm of $P^\alpha$, with sufficient line broadening to obscure the multiplet structure of the $P^\beta$ resonance (Figure 3). These chemical-shift changes

Table II: pH Effect and Comparison with DS-Dabco[a]

| | pH 7 | | pH 3 | |
|---|---|---|---|---|
| | GrS | DS-Dabco[b] | GrS | DS-Dabco |
| ATP | 0.99 | 0.95 | 0.90 | 0.99 |
| ADP | 0.96 | 0.94 | 0.25 | 0.37 |
| AMP | 0.72 | 0.69 | 0.02 | 0.01 |

[a] Extraction efficiencies for the $H_2O/CHCl_3$ system. Data for DS-Dabco from Tabushi et al. (1981). Concentrations: $2 \times 10^{-5}$ M nucleotide, $5 \times 10^{-5}$ M complexone (pH 7); $10^{-4}$ M nucleotide, $2.5 \times 10^{-4}$ M complexone (pH 3). [b] pH 8.

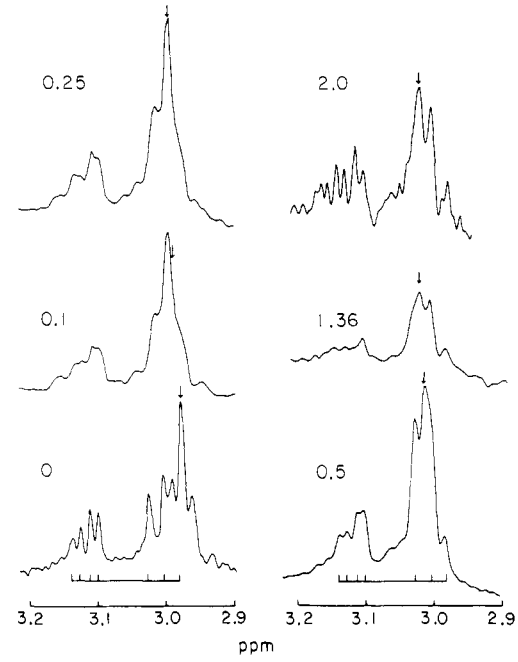


FIGURE 2: $^1H$ NMR spectra at 500.13 MHz of GrS in $^2H_2O$ under progressive addition of ADP. The Orn $C^\delta H_2$ and D-Phe $C^\beta H_2$ resonances lie in this region. For each spectrum, $[GrS]_i = 10^{-4}$ M and $[ADP]_i/[GrS]_i \equiv 1/\zeta$ is indicated above the trace; $[GrS]_i = 7.5 \times 10^{-5}$ M for $1/\zeta = 2$. The arrows indicate the position of the central unresolved component of the Orn $C^\delta H_2$ resonance. The grids above the chemical-shift scales show the positions of the invariant D-Phe $C^\beta H_2$ peaks. The apparently narrower line width of the $1/\zeta = 2$ spectrum is the result of Gaussian multiplication of the free induction decay.

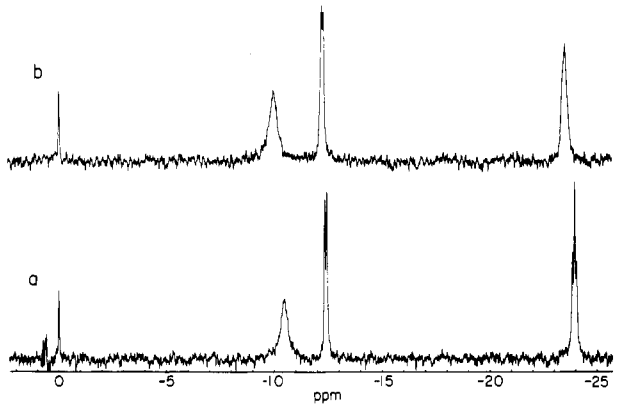


FIGURE 3: $^{31}P$ NMR spectra at 200.49 MHz of $7.5 \times 10^{-4}$ M ATP in water containing 10% $^2H_2O$ at pH 7.0 in the absence (a) and presence (b) of $1.5 \times 10^{-3}$ M [2,2′,$N^6$-trimethylornithyl]-GrS. Chemical shifts are referenced to internal $5 \times 10^{-4}$ M $P_i$ (=0.0 ppm).

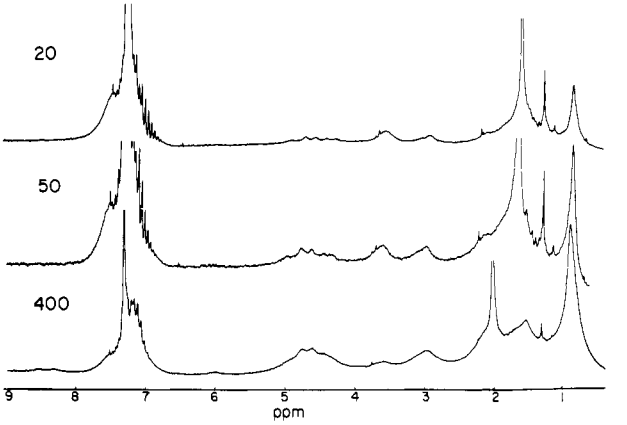


FIGURE 4: $^1H$ NMR spectra of ATP–GrS complexes in $CDCl_3$. The numbers to the left of the traces are the initial nucleotide concentrations in water (in $10^{-6}$ M) prior to extraction.

demonstrate that an interaction with the phosphate groups has occurred; the data are unfortunately insufficient to identify preferred sites of binding on the nucleotide.

Except for minor light scattering effects, the absorption spectrum of ADP above 245 nm did not change upon addition of GrS. At shorter wavelengths, absorption of the peptide group predominates.

*Phase Transfer.* Extraction of nearly stoichiometric solutions of GrS and nucleotides with $CHCl_3$ invariably yielded a clear aqueous phase and shape boundary. Nucleotide removed from the aqueous phase was quantitatively transferred to the organic layer, as verified by UV analysis. The partition coefficient of GrS into $CHCl_3$ ($K_G$) was determined independently by UV analysis to be 0.2 in the absence of salts. Because of the low solubility of the free antibiotic in the organic phase and the high apparent affinity of GrS for the nucleotides, the concentration of free GrS in $CHCl_3$ should be relatively low after extraction. As long as GrS is not initially present to large molar excess, the organic phase should after extraction contain almost exclusively nucleotide–GrS complexes.

Use of deuterated solvents permits measurement of $^1H$ NMR spectra of the extracted ATP–GrS complexes in $CDCl_3$ (Figure 4). While characteristic resonances of both components are discernible, the line widths are too broad (>50 Hz) to allow detailed study of conformation. Some dependence of the line width on concentration between $2 \times 10^{-5}$ and $40 \times 10^{-5}$ M nucleotide (initial concentration in water) is apparent, suggesting that it is a concentration-dependent aggregation of complexes, rather than an intrinsically high aggregation number for the individual complex, which accounts for the broad lines. Similar spectra were obtained for the ADP complex.

Extraction with $CHCl_3$ was facile, and the solubility of the ATP–GrS complex in this solvent exceeded $10^{-2}$ M. Attempts to use other organic solvents in the extraction were less successful. With 1-octanol, a slightly lower $E$ was measured (Table I). The use of hydrocarbons or $CCl_4$ resulted in interfacial precipitation. However, it was possible to obtain solutions of the ATP–GrS complex in either $CCl_4$ or toluene by repeated addition of the second organic solvent to a concentrated $CHCl_3$ solution and evaporation to near dryness. The complex is soluble in $CHCl_3$–hexane mixtures but is practically insoluble in the pure hydrocarbon.

Crystals were obtained by slow evaporation of a solution of the ATP–GrS complex in EtOH–$Me_2CO$ (1:1) (colorless needles, typically 2 × 0.02 mm).

*Nature of the Nucleotide–Peptide Interaction.* The NMR spectra in water demonstrate coordination of the ammonium and phosphoryl groups in the complex. Two other interactions are possible: (i) ring stacking of the nucleoside base and the side chain of D-Phe and (ii) formation of additional hydrogen bonds between the nucleoside and the peptide backbone. The lack of NMR chemical-shift changes indicative of ring stacking in the spectra of the ADP complex in water argues against (i), but no determination could be made in $CHCl_3$ because of the poor resolution in this solvent. The hydrogenated derivative 4,4′-bis(D-cyclohexylalanyl)-GrS (ChaGrS) was therefore prepared according to the method of Ruttenberg et al. (1966), and its interactions were compared with those of GrS. No difference in extraction efficiencies was found, and the minimum lethal concentration of ChaGrS against *Staphylococcus aureus* in liquid culture was $1.6 \times 10^{-6}$ M, vs. $1.5 \times 10^{-6}$ M for GrS. From these observations, it is concluded that base stacking contributes negligibly to the stabilization of the complex and that the aromaticity of residue 4 is unnecessary for full antibiotic potency.

For further investigation of (ii), the extraction of the thymine nucleotides TTP, 2′-dTTP, and 2′,3′-dideoxy-TTP by GrS was investigated. Again no difference was found between the analogues (data not shown), suggesting that hydrogen bonds between the sugar moiety and the peptide do not form. Other hydrogen bonds between the nucleoside base and the peptide are still possible, however, and may account for the observed small differences in $E$ between the various triphosphates.

In view of the importance of ion pairing in complex formation, it is not surprising that competitive effects are observed in the presence of other charged species. Among the anions, inorganic pyrophosphate ($PP_i$) at pH 7 competes most effectively, blocking 50% of the transfer of $10^{-5}$ M ATP at a concentration of $7 \times 10^{-5}$ M. The relative abilities of anions to block phase transfer of ATP at pH 7 decrease in the order $PP_i > P_i > (CH_2COO^-)_2$, $Br^-$, $SCN^-$, $ClO_4^- > Cl^-$, $CH_3COO^- > H_2BO_3^-$. Among the cations, $Mg^{2+}$, 1,4-butanediamine (putrescine), and 1,5-pentanediamine (cadaverine), which form soluble nucleotide complexes, compete about as effectively toward nucleotides as $P_i$ toward GrS, when added as the chlorides.

Equilibrium exchange of anions across a $CHCl_3/H_2O$ interface was mediated by GrS. In one experiment, a solution of ATP ($1.25 \times 10^{-5}$ M) and GrS ($2.5 \times 10^{-5}$ M) was extracted with $CHCl_3$ in the usual fashion. The organic phase was withdrawn and shaken with 0.02 M phosphate buffer, pH 7. After the second extraction, ~50% of the nucleotide was

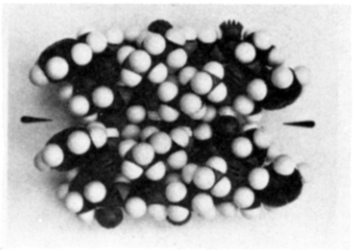

FIGURE 5: Cross-$\beta$-association of two molecules of GrS (nucleotides omitted), viewing the polar faces (side chains of Orn, D-Phe, and Pro visible). Pointers indicate line of contact of the ring edges, where intermolecular pleating occurs.

present in the aqueous phase. In contrast, distilled water or saline afforded <5% back-extraction. In a second experiment, a solution of $2.5 \times 10^{-5}$ M GrS and a variable concentration of $P_i$ were extracted with $CHCl_3$; the organic layer was removed and shaken with a second aqueous layer containing $1.25 \times 10^{-5}$ M ATP. The $CHCl_3$ phase, presumably containing a lipophilic phosphate complex of the peptide, induced phase transfer of ATP with a limiting $E$ at high $[P_i]$ comparable to that expected for a single extraction of ATP by GrS at the same initial concentrations.

*Complex Aggregation.* Once formed, the nucleotide–GrS complexes appear to interact strongly with one another, as shown by the formation of precipitates in water prior to extraction and the marked NMR line broadening in $CHCl_3$. In water, self-association is most likely directed by hydrophobic interactions, since the two charged side chains of the peptide, which confer its modest degree of aqueous solubility, are blocked. At low ($\leq 10^{-4}$ M, for ADP) concentrations, relatively small aggregates remain in solution and give rise to NMR spectra with moderately broadened lines, while bulk precipitation occurs at higher concentrations.

In $CHCl_3$, precipitation does not occur, but there is a greater degree of NMR line broadening than in aqueous solutions of similar concentration. The most plausible explanation for this is lateral polymerization of the complexes in the organic solvent to form linear cross-$\beta$-aggregates (Figure 5). Cross-$\beta$-aggregation of GrS in oriented poly(ethoxyethylene) has been shown to occur by IR dichroism (Ingwall et al., 1975). A maximum of four interamide hydrogen bonds can form between each pair of GrS molecules, each of which can interact similarly with the backbone of a GrS molecule in a second complex. The aggregation numbers in $CHCl_3$ are likely to be large, because interamide hydrogen bonding itself is favorable in media of low polarity (Franzen & Stephens, 1963), particularly when several bonds can form in concert between the interacting molecules (Susi & Ard, 1966). The surface properties of these large, ribbonlike aggregates ensure high solubility in organic media, while slow rotational diffusion accounts for the broad NMR line widths. Interaction between aggregates should be weak, which may account for the large axial ratio of the crystals.

*Stoichiometry and Structure of the Complexes.* The molecularity of phase transfer was established directly by $^1H$ NMR. Following evaporation of $CHCl_3$, the extracted complexes were dissolved in MeOH-$d_4$–$Me_2SO$-$d_6$ (1:1), and the resonances of the nucleotide and peptide components, were integrated. It was determined that ATP and GrS form an extractable 1:2 complex at pH 7 and a 1:1 complex at pH 3. ADP and GrS form a 1:1 complex at pH 7. The measured stoichiometries did not depend on the initial concentration ratio of the components in the aqueous phase and represent an irreducible minimum exclusive of higher degrees of aggregation.

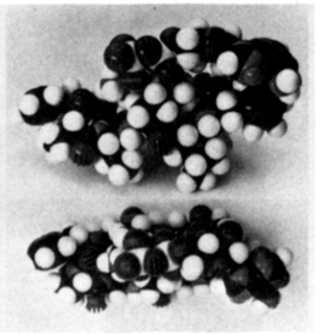

FIGURE 6: CPK model of the 1:1 ADP–GrS complex viewed along (top) and perpendicular to (bottom) the symmetry axis of the peptide. The location of the adenine moiety relative to the peptide is arbitrary.

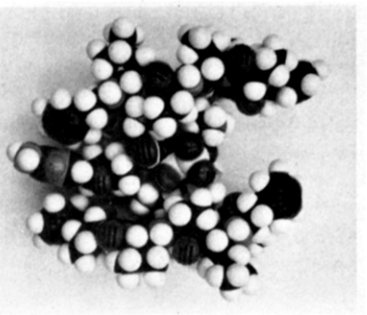

FIGURE 7: Lateral view of a CPK model of the 1:2 ATP–GrS complex. The nucleotide is sandwiched between the peptide molecules, which are proposed to lie with ring planes and major ring axes parallel. The exposed hydrophobic surfaces are parallel to the ring planes and perpendicular to the plane of the figure.

The dependence of stoichiometry on pH for ATP is the result of the change in ionization state of the nucleotide. At pH 7, ATP is predominantly tetravalent (Dawson et al., 1969) and binds the charged Orn termini of two peptide molecules. At pH 3, the nucleotide carries an overall charge of 2– because of adenine $N^1$ protonation and forms a neutral 1:1 complex. The relatively low extraction efficiency measured for ADP at pH 3 (Table II) is presumably a manifestation of the lower stability of complexes containing 2 equiv of nucleotide per peptide (Tabushi et al., 1981). AMP should itself be predominantly uncharged at pH 3 and is barely extracted by GrS.

From what has been determined concerning nucleotide–GrS binding and the stoichiometries and colligative properties of the complexes, it is possible to propose structures for the complexes in solution. In the 1:1 complex (ATP, pH 3; ADP, pH 7), the nucleotide is apposed to the polar face of the peptide (Figure 6) with the anionic moieties in intimate contact with the $NH_3^+$ groups of Orn. Since polar groups remain solvent exposed over this face, the 1:1 complex clearly possesses polar and nonpolar surfaces. Four externally directed amide groups of the peptide backbone are available for hydrogen bonding in $CHCl_3$ along each ring edge.

In the 1:2 complex (ATP, pH 7; Figure 7), two GrS molecules envelop the nucleotide. The charged groups of both components are solvent shielded, and the externally directed surfaces of the complex which are parallel to the ring planes are nonpolar. Cross-$\beta$-aggregation in organic media should occur readily if the ring planes and major ring axes are parallel (Figure 7). While this complex is expected to be more hydrophobic than the 1:1 complex by virtue of the larger number of exposed nonpolar side chains, the lateral aspect of the 1:2 complex, shown in the figure, retains considerable polar character; aggregation in water most likely proceeds via interaction of the hydrophobic surfaces, leaving the polar lateral

Table III: Estimated Binding and Partition Constants for GrS[a]

| nucleotide | pH | stoichiometry | method | log $K_1$ | log $K_2$ | log $K_p$ | log $K_w$ | log $K_p^2K_o$ |
|---|---|---|---|---|---|---|---|---|
| ADP | 7 | 1:1 | eq 4 | 4.6 ± 0.2 | | 0.4 ± 0.1 | | |
| | | | eq 5 | 4.5 ± 0.5 | | | ~6 | ~6 |
| ATP | 3 | 1:1 | eq 4 | 4.5 ± 0.4 | | 0.4 ± 0.2 | | |
| ATP | 7 | 1:2 | eq 6 | 10.8 ± 0.3[b] | | 1.3 ± 0.1 | | |
| | | | eq 7 (case I) | 4.5 ± 0.5 | 4.5 ± 0.5 | | ~8.5 | ~9.5 |
| | | | eq 7 (case II) | 4.5 ± 0.5 | 5 ± 1[c] | | *d* | *d* |

[a] In log $M^{-1}$, except log $K_p$. [b] log $K$, in log $M^{-2}$. [c] $K_2/K_1 < 100$ (see text). [d] Varies with $K_2$ (see Figure 12).

faces, which direct aggregation in solvents such as $CHCl_3$, relatively exposed.

Whereas species such as $ATP^{3-}$ or $ADP^{3-}$ were generally present in solution under the conditions employed in the extractions, 2:3 stoichiometries were not observed. This is not surprising in view of the large energetic cost incurred in forming such complexes from, e.g., the interaction of a free GrS molecule with a dimer of negatively charged 1:1 complexes in which the uncomplexed residual negative charges are closely apposed. Furthermore, the GrS ring axes in 2:3 complexes cannot be aligned parallel to one another so that self-association of the complexes in organic media is greatly hindered. Since the phase-transfer stoichiometries do not reflect the equilibrium mixture of ionization states of the nucleotide, either proton shifts must occur or the extracted complexes are not uniformly neutral. That the pH decreases significantly during the initial period of extraction of ATP at pH 7 suggests that transfer of the uncharged complex is predominant.

It should be emphasized that what is transferred to $CHCl_3$ are specific nucleotide complexes or aggregates of specific complexes, and not micellar aggregates. The possibility of micelle formation must be considered in light of the large apparent size of the aggregates, inferred from the NMR line widths in $CHCl_3$, and the known amphiphilicity of the peptide. While GrS may form a monolayer at the phase boundary (Few, 1956; Melnick et al., 1978), there is no evidence that it is capable of forming or entering a micelle in the absence of other amphiphiles. The observation of distinct and well-defined stoichiometries of phase transfer for different nucleotides eliminates the possibility of micellar entrapment.

*Analysis of Binding and Phase Transfer: 1:1 Complexes.* The 1:1 equilibrium extraction of monovalent cations into organic solvents by macrotetralide antibiotics has been treated previously by Eisenman et al. (1969). Whereas the analysis of the extraction of nucleotides by GrS parallels this to a certain extent, there are several important differences, namely, (a) the high efficiency of the extraction, which raises the possibility that the partition coefficient of the complex, rather than the binding constants, can limit $E$, (b) multistep binding in the 1:2 complexes, and (c) aggregation of the complexes in the aqueous and organic phases.

The binding constant for the 1:1 interaction in water is defined by

$$a + g \rightleftarrows ag \qquad K \equiv \frac{[ag]}{[a][g]} \tag{2}$$

and the macroscopic partition coefficient is defined by eq 3, where lower- and upper-case letters denote species in the aqueous and organic phases, respectively. An additional

$$ag \rightleftarrows AG \qquad K_p \equiv \frac{[AG]}{[ag]} \tag{3}$$

parameter ($\zeta$) specifies the initial ratio of GrS to nucleotide

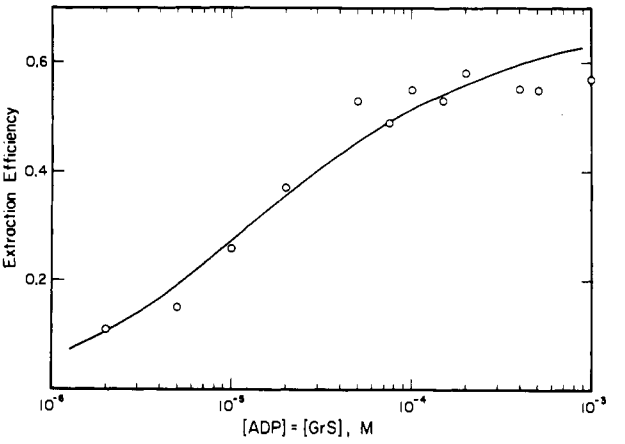


FIGURE 8: Extraction of ADP by GrS at pH 7 with $\zeta = 1$. The curve was plotted according to eq 4 by using the fitted parameters $K$ and $K_p$ listed in Table III.

concentration and is defined for a 1:$n$ interaction by $\zeta = [g]_i/(n[a]_i)$.

If complex aggregation is, for the moment, neglected, and the volume ratio of the phases is assumed to be unity, eq 2 and 3 may be solved to yield a simple expression for $E$ which satisfactorily describes the observed extraction profiles (the variation in $E$ with $[a]_i$) for fixed $\zeta$:

$$\frac{E}{(1 - PE)(\zeta - PE)} = [a]_i \frac{KK_p}{1 + K_G} \tag{4}$$

Here, $P \equiv 1 + K_p^{-1}$, and $K_G = 0.2$ is the partition coefficient of free GrS into $CHCl_3$. Two types of saturation phenomena are clearly predicted by eq 4. The first, phase saturation, is encountered at high $[a]_i$ for $\zeta \geq 1$, where $(1 - PE) \rightarrow 0$ or $E \rightarrow P^{-1}$, a function of $K_p$ only. The second, stoichiometric saturation, limits $E$ when $\zeta < 1$, since GrS can extract no more of the nucleotide than it can bind stoichiometrically. In this case, $E \rightarrow \zeta P^{-1}$ with increasing $[a]_i$. The two forms of saturation are discussed further in connection with the phase transfer of ATP. It should be mentioned concerning eq 4 that, for $K_p$ sufficiently large and $[a]_i$ sufficiently small, the coefficients in the denominator on the left side may be taken as unity, with the resulting expression essentially equivalent to that derived for the phase transfer of cations by macrotetralides (Eisenman et al., 1969).

The extraction data obtained for the 1:1 interactions between ADP and GrS at pH 7 (Figure 8) and between ATP and GrS at pH 3 (Figure 9) are displayed with curves fitted to the data according to eq 4 by utilizing the procedure outlined under Materials and Methods. The fitted values of $K$ and $K_p$ are given in Table III. There is no significant difference in these parameters between the two types of complex.

Here, $K$ for ADP represents the equilibrium constant for the concerted formation of two ion pairs between the Orn $N^{\delta}H_3^+$ groups of GrS and the vicinal phosphoryl groups of the

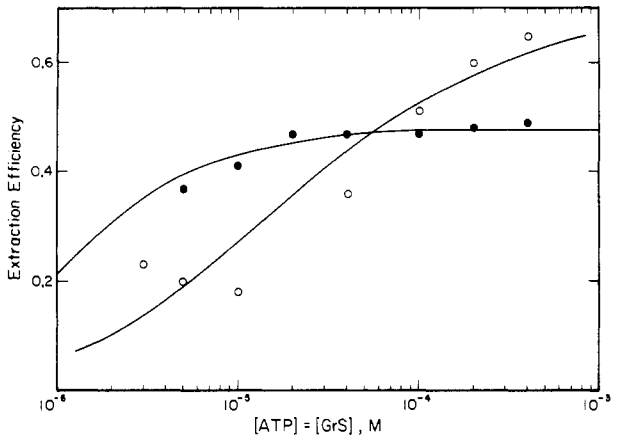


FIGURE 9: Extraction of ATP by GrS at pH 3 (open circles, 1:1 stoichiometry) and pH 7 (closed circles, 1:2 stoichiometry) with [GrS]/[ATP] = 1, with curves plotted according to eq 4 and 6, respectively. The parameters fitted to the $\zeta = 1$ profile at pH 7 by eq 6 (Figure 11) were utilized for the pH 7 profile here.

nucleotide. If the ion pairs are equivalent, a $\Delta G°$ of −3.1 kcal $mol^{-1}$ is estimated for each, to which both electrostatic attraction and hydrogen bonding contribute. In the case of ATP, GrS can interact either with vicinal or ($\alpha$,$\gamma$) phosphoryl groups. However, if adenine $N^1$ forms an intramolecular salt bridge in $CHCl_3$, as seems likely, it should most easily do so with the terminal phosphoryl; thus, in its final configuration, the groups which interact with the peptide in the 1:1 ATP complex are vicinal, as in the ADP complex, and the binding constant is nearly the same. The similarity in the estimated values of $K_p$ is also not unexpected, as the complexes possess similar surface properties.

The question of complex aggregation emerges when the extraction profiles for $\zeta > 1$ are considered. When $\zeta = 2$, for example, $E$ plateaus at significantly higher values than expected for the partition coefficients estimated from eq 4 (>0.95 for ADP, >0.99 for ATP at pH 7). Since a significant change in stoichiometry with $\zeta$ appears ruled out by NMR, it is necessary to consider explicitly the colligative properties of the complexes. This cannot presently be done rigorously, because we lack the means for measuring the aggregation state of the complex in solution. However, by generalizing the scheme outlined above to include the noncooperative association of complexes, it is possible to reproduce the extraction profiles with reasonable accuracy while removing the artificial restriction on activity coefficients implied in eq 3. In the Appendix, a binding equation is derived which takes into account the effects of aggregation (eq 5) where $r = KK_w[a][g]$ and

$$(1 - r)^2([a] - [a]_i) + \frac{r}{K_w}(1 + K_p) + \left(\frac{r}{K_w}\right)^2 K_p(K_pK_o - K_w) = 0 \quad (5)$$

from which $E$ may be computed directly. The concentrations of all species are sufficiently low ($< 5 \times 10^{-4}$ M) that activity coefficients are assumed close to unity. This derivation is based on the following provisions concerning the aggregation process: (i) Self-association occurs noncooperatively in both phases, characterized by second-order association constants $K_w$ (aqueous) and $K_o$ (organic) with $K_w < K_o$, and (ii) the apparent partition coefficient of an aggregate, because of structural rearrangements occurring on phase transfer which sequester solvophobic groups, is a slowly varying function of the number of contained monomeric complexes. It is further proposed that excess GrS, which is largely confined to the aqueous phase, interacts weakly with the aggregates as an amphiphile and causes a decrease in the average aggregation number in water (defined in the Appendix). According to this scheme, GrS in superstoichiometric amounts brings about a decrease in the observed $K_w$ and, consequently, an increase in the total concentration of aggregates in water, which promotes greater transfer across the phase boundary into $CHCl_3$.

As indicated in Figure 10, simultaneous simulation of both the $\zeta = 1$ and $\zeta = 2$ extraction profiles for ADP at pH 7 is possible only for values of the binding constant falling within a limited range. Good agreement with the experimental results is obtained when $10^4\ M^{-1} \leq K \leq 10^5\ M^{-1}$, in which case modest (2–10) aqueous aggregation numbers are estimated. When $K$ is too small, there is excessive curvature in both computed profiles, resulting in particularly large discrepancies between the calculated and observed values of $E$ at low $[a]_i$. For $K \geq 10^5\ M^{-1}$, the aggregation numbers for the aqueous phase ($\zeta = 1$) approach unity, and the $\zeta = 2$ simulation consequently fails. Thus, despite the difficulties inherent in the inclusion of complex association in the quantitative treatment of phase transfer, it is still possible to obtain a reasonably precise estimate of the 1:1 binding constant ($K_{1:1}$). This will prove useful in the analysis of the 1:2 binding problem, since $K_{1:1}$ should constitute a lower limit to the association constants for the

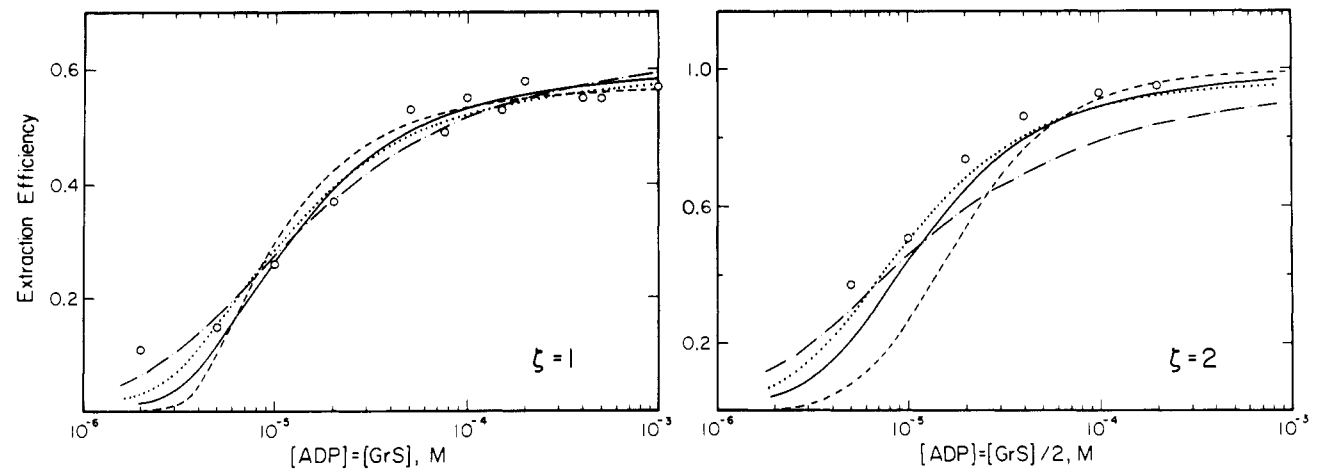


FIGURE 10: Computed extraction profiles (eq 5) for the extraction of ADP by GrS with $\zeta = 1$ (left panel) or $\zeta = 2$ (right panel). The method of calculation is described in the Appendix. $\zeta = 1$: (---) $K = 3 \times 10^3\ M^{-1}$, $K_w = 1.5 \times 10^7\ M^{-1}$, $K_p^2K_o = 2 \times 10^7\ M^{-1}$; (—) $K = 3 \times 10^4\ M^{-1}$, $K_w = 1 \times 10^6\ M^{-1}$, $K_p^2K_o = 1.5 \times 10^6\ M^{-1}$; (···) $K = 6 \times 10^4\ M^{-1}$, $K_w = 7 \times 10^5\ M^{-1}$, $K_p^2K_o = 1 \times 10^6\ M^{-1}$; (-·-) $K = 3 \times 10^5\ M^{-1}$, $K_w = 1.5 \times 10^5\ M^{-1}$, $K_p^2K_o = 2.5 \times 10^5\ M^{-1}$. $\zeta = 2$: $K$ and $K_p^2K_o$ as above; $K_w = 1 \times 10^4\ M^{-1}$.

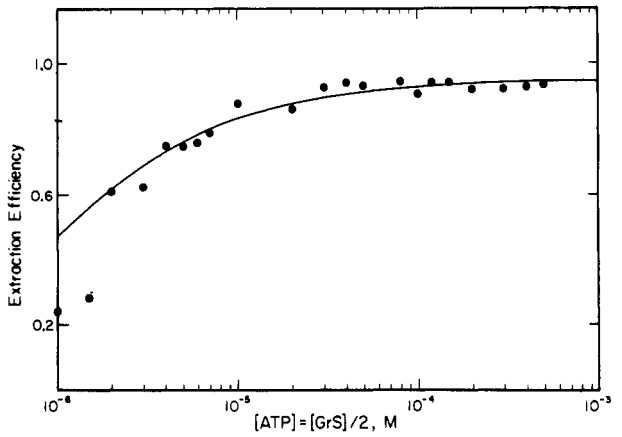


FIGURE 11: Extraction of ATP by GrS at pH 7 with $\zeta = 1$. The curve was plotted according to eq 6 by using the fitted parameters $K$ and $K_p$ listed in Table III.

interaction of GrS with ATP at pH 7. It should be noted that the concordance between the values of $E$ estimated for the 1:1 interaction by eq 4 and 5 is largely fortuitous, expected only if the aqueous aggregation numbers are sufficiently low. The parameter estimates obtained for the extended treatment are given in Table III.

*Extraction of 1:2 Complexes.* The change in stoichiometry of the ATP extraction with pH is clearly reflected in the extraction profiles in Figure 9. In each case, $[g]_i/[a]_i = 1$, but the curvature and limiting efficiencies differ, and the profiles intersect when $[a]_i \approx 5 \times 10^{-5}$ M. At pH 3, $\zeta = 1$ and phase saturation is encountered at high $[a]_i$, as described above, while at pH 7, $\zeta = 0.5$ and stoichiometric saturation is observed. When $[g]_i$ is not limiting, the 1:2 ATP extraction is even more efficient than the superstoichiometric ($\zeta = 2$) ADP extraction (compare Figure 10 and Figure 11).

As a starting point in the analysis, the ATP data were fitted to eq 6, the analogue of eq 4 for the 1:2 interaction, where $K$

$$\frac{E}{(1 - PE)(\zeta - PE)^2} = \left(\frac{2[a]_i}{1 + K_G}\right)^2 KK_p \qquad (6)$$

$\equiv [ag_2]/([a][g]^2)$; i.e., sequential binding with full cooperativity is assumed (Table III). Not surprisingly, the estimated $K_p$ is significantly larger than that obtained for the 1:1 complexes through application of eq 4. In addition, it is found that $K > K_{1:1}^2$; however, this estimate of $K$ must be regarded as suspect, since eq 6 appears consistently to overestimate $E$ for low $[a]_i$ (Figure 12), where the slope and sensitivity to $K$ of the extraction profile are greatest.

A systematic treatment of the 1:2 extraction requires inclusion of the aggregation constants $K_w$ and $K_o$ and, in addition, definition of the separate binding constants $K_1$ and $K_2$ for the two binding steps. The question of two-step binding is approached by considering GrS to be a bidentate ligand which complexes one of two available pairs of charges on the triphosphate. Two limiting cases are then defined. In the first, the charge pairs constitute two independent, noninteracting sites for peptide binding, and the microscopic binding constants formally are $K_1 \equiv [ag]/([a][g]) = [ag_2]/([ag'][g])$ and $K_2 \equiv [ag']/([a][g]) = [ag_2]/([ag][g])$ (case I). It is possible, although somewhat misleading, to regard each site as corresponding to a specific pair of phosphoryl groups. In case II, binding is obligatorily sequential. After the first binding step, a second binding site becomes available, conceivably through facilitated deprotonation of trianionic nucleotide species present in ag complexes. Cooperative effects may arise if, after the

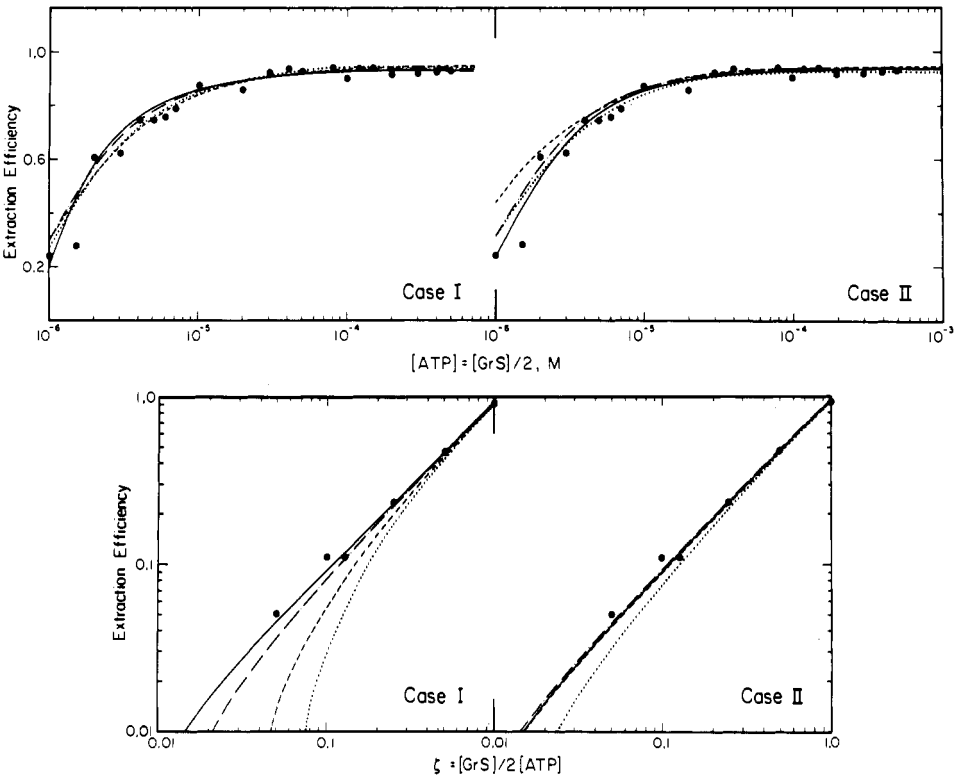


FIGURE 12: Computed extraction profiles (eq 7) for the extraction of ATP by GrS with $\zeta = 1$ (top panel) and for the pseudotitration (bottom panel). The two cases are defined in the text, and the method of calculation is described in the Appendix. Case I: (—) $K_1 = 3 \times 10^4$ M$^{-1}$, $K_2 = 3 \times 10^4$ M$^{-1}$, $K_w = 1 \times 10^9$ M$^{-1}$, $K_pK_o^2 = 1.5 \times 10^{10}$ M$^{-1}$; (-·-) $K_1 = 1 \times 10^5$ M$^{-1}$, $K_2 = 1 \times 10^5$ M$^{-1}$, $K_w = 7 \times 10^7$ M$^{-1}$, $K_pK_o^2 = 1 \times 10^9$ M$^{-1}$; (- - -) $K_1 = 1 \times 10^5$ M$^{-1}$, $K_2 = 1 \times 10^6$ M$^{-1}$, $K_w = 1 \times 10^7$ M$^{-1}$, $K_pK_o^2 = 1.8 \times 10^8$ M$^{-1}$; (···) $K_1 = 3 \times 10^4$ M$^{-1}$, $K_2 = 3 \times 10^6$ M$^{-1}$, $K_w = 3 \times 10^7$ M$^{-1}$, $K_p^2K_o = 5.4 \times 10^8$ M$^{-1}$. Case II ($K \equiv K_1K_2$, $\rho = K_2/K_1$): (—) $K = 3 \times 10^9$ M$^{-2}$, $\rho = 1$, $K_w = 2 \times 10^8$ M$^{-1}$, $K_p^2K_o = 3 \times 10^9$ M$^{-1}$; (-·-) $K = 3 \times 10^{10}$ M$^{-2}$, $\rho = 10$, $K_w = 2 \times 10^7$ M$^{-1}$, $K_p^2K_o = 3 \times 10^8$ M$^{-1}$; (- - -) $K = 3 \times 10^{11}$ M$^{-2}$, $\rho = 100$, $K_w = 3 \times 10^6$ M$^{-1}$, $K_p^2K_o = 4.5 \times 10^7$ M$^{-1}$; (···) $K = 1 \times 10^{11}$ M$^{-2}$, $\rho = 1$, $K_w = 7 \times 10^6$ M$^{-1}$, $K_p^2K_o = 9 \times 10^7$ M$^{-1}$.

second GrS molecule is bound, rotameric isomerization within the triphosphate improves the topochemical fit with the cationic moieties of both peptides. Here, $K_1 \equiv [ag]/([a][g])$ and $K_2 \equiv [ag_2]/([ag][g])$. In either case, it is assumed that only $ag_2$ species undergo phase transfer and that $K_1, K_2 \geq \log^{-1} 4.5$ $M^{-1}$. The extended equation for binding, including complex aggregation, is (see the Appendix)

$$(1 - r)^2([a] + u(K_1,K_2)[a][g] - [a]_i) + \frac{r}{K_w}(1 + K_p) + \left(\frac{r}{K_w}\right)^2 K_p(K_pK_o - K_w) = 0 \quad (7)$$

where $r = K_wK_1K_2[a][g]^2$ and $u(K_1,K_2) = K_1 + K_2$ (case I) or $K_1$ (case II). The binding equilibria are summarized below:

$$\text{case I: } a + g \overset{K_1}{\rightleftharpoons} ag \qquad a + g \overset{K_2}{\rightleftharpoons} ag'$$

$$\text{case II: } a + g \overset{K_1}{\rightleftharpoons} ag \qquad ag + g \overset{K_2}{\rightleftharpoons} ag_2$$

The calculated profiles are compared with the simple extraction profile for $\zeta = 1$ and a pseudotitration in which $E$ is measured as a function of $\zeta$ ($\zeta < 1$) with $[a]_i = 10^{-4}$ M (Figure 12). Use of the two sets of data makes it possible to define acceptable ranges for the binding constants in each case, much as the $\zeta = 2$ profile had provided helpful constraints on the likely values of $K_{1:1}$. (A $\zeta = 2$ profile could not accurately be measured here, since $E \sim 1$ over most of the concentration range.) The pseudotitration is noteworthy in that $E \sim \zeta$ for values of $\zeta$ as low as 0.05, which would, in the absence of colligative effects, indicate highly cooperative binding.

For case I (Figure 12, left curves), it is found that simulation of the $\zeta = 1$ extraction profile is possible for a wide range of values of $K_1$ and $K_2$. This is evidently not true of the pseudotitration, however, where the calculated curves fall well short of the data unless $K_1 \sim K_2 \sim K_{1:1}$. For binding constants satisfying the latter criteria, the apparent cooperativity in the pseudotitration arises from efficient complex aggregation in both phases (Table III). Case II suggests a range of $K$'s which does not greatly differ from that deduced for case I (Figure 12, right curves). The $\zeta = 1$ profile is well approximated if $K_2/K_1 \leq 10$ when $K_1K_2 > 10^{11}$ $M^{-2}$. For high values of the product of the binding constants with strong cooperativity, the computed and observed profiles diverge at low $[a]_i$ in a manner similar to that seen in Figure 11. Simulation of the pseudotitration, on the other hand, requires that when $K_1K_2 > 10^{11}$ $M^{-2}$, $K_2/K_1 > 10$. The data are thus consistent with case II for the ranges of binding constants listed in Table III, corresponding to $K_1 \sim K_{1:1}$ with weak cooperativity ($1 \leq K_2/K_1 < 100$).

Differentiation between the two cases is not possible on the basis of the phase-transfer data. Of the two cases, case I appears inherently simpler, involving only stochastically independent binding to a polyanion possessing two equivalent binding sites with microscopic affinity essentially equal to that estimated for the 1:1 interaction. However, since both cases ultimately provide estimates of $K_1$ and $K_2$ which are not appreciably larger than $K_{1:1}$, the extended analysis clearly shows that complex aggregation is a major determinant of the apparent selectivity of GrS for the triphosphates in phase transfer.

*Origin of Nucleotide Selectivity.* Analysis of the phase-transfer data has, in summary, provided similar estimates of the binding constants for the interaction between ADP and GrS at pH 7, ATP and GrS at pH 3, and the first step of the ATP–GrS interaction at pH 7, with $\Delta G_1^\circ = -RT \ln K_{1:1} \simeq 6.2$ kcal $mol^{-1}$. The binding constant for the second step of the ATP–GrS interaction is likely to be the same as or only slightly greater than this, and $\Delta G_1^\circ - \Delta G_2^\circ < 2$ kcal $mol^{-1}$. The selectivity of GrS for ATP over ADP in water at pH 7 is thus critically contingent upon the stoichiometry change and is a consequence of differences in colligative behavior and an enhanced partition coefficient into the organic phase. It is not a manifestation of an intrinsically higher affinity of GrS for the triphosphate in the initial encounter complex.

## Discussion

GrS is, to our knowledge, the first peptide known to possess phase-transfer activity toward nucleotides. This property was suggested initially by physicochemical studies in this laboratory and elsewhere (Krauss & Chan, 1982a,b) which showed GrS to be a conformationally rigid, amphiphilic molecule containing two positively charged residues whose motions are limited by the formation of intramolecular hydrogen bonds to the backbone. In the present work, a combination of chemical and spectroscopic methods has enabled us to characterize the structure, stoichiometry, and binding constants of several of the nucleotide coordination complexes of GrS which undergo transfer to organic media.

Although its solution structure is relatively well elucidated, the physiological role of GrS in the producer strain of *B. brevis* and the mechanism of antibiotic action against susceptible microorganisms are unclear. Concerning the latter, much consideration has been given the possibility of a direct interaction with membranes, either by insertion into the bilayer (Susi et al., 1979; Nakagaki et al., 1981; Wu et al., 1978) or by electrostatic interaction with phospholipid head groups (Ovchinnikov & Ivanov, 1975). Toxic effects would then result from disruption of permeability barriers [Hunter & Schwartz, 1967; see Chapter 6 of Izumiya et al. (1979)] or inhibition of elements of the respiratory chain (Ostrovskii et al., 1976; Dergunov et al., 1981). GrS is a surfactant (Few, 1956; Melnick et al., 1978), and experimental evidence of its interaction with synthetic (Finer et al., 1969; Pache et al., 1972; Susi et al., 1979; Nakagaki et al., 1981; Wu et al., 1978) or natural (Eremin et al., 1979) membranes has been reported. However, the low sensitivity of the methods used to monitor GrS–membrane interactions invariably necessitates use of concentrations ~1000-fold greater than the minimum lethal concentration in vivo. Such methods have in a rigorous study by Wu et al. (1978) failed to reveal any difference in membrane-binding affinity between GrS and a derivative containing a single positive charge, in contrast to the results of numerous bioassays [see Chapter 4 of Izumiya et al. (1979)]. On the other hand, nucleotide binding and transport clearly occur in vitro at physiologically active concentrations of the antibiotic. The present study indicates that GrS should enter cells with little difficulty, either unassisted (possibly in monoprotonated form) or assisted by extracellular $P_i$ as the lipophilic complex. The formation of nucleotide–GrS complexes within the cell should produce a variety of toxic effects related to the depletion of high-energy phosphate metabolites; in addition, the ATP complex could partition into the membrane, conceivably as the cross-$\beta$-aggregate, causing functional or structural impairment.

It has been amply demonstrated in previous investigations that GrS is associated with derangement of phosphate metabolism in microorganisms. Rapid efflux of nucleotides from a GrS-sensitive *Micrococcus* strain was observed to occur at antibiotic concentrations exceeding $10^{-6}$ M, which was the minimum lethal concentration for this organism (Miki, 1960). Whereas this might be attributable to a nonspecific lytic effect, rather than any direct interaction with the nucleotides, copious

nucleotide secretion has been observed as well in mature cultures of *B. brevis* synthesizing GrS (Glazer et al., 1966). Here, the effect is very likely mediated directly by GrS in its capacity as a phase-transfer agent, since nucleotide efflux proceeds without evidence of destruction of the vegetative culture. Interestingly, the profile of depleted nucleotides in the producer strain (Silaeva et al., 1965) consists essentially of those for which GrS possesses the highest apparent selectivity. The relation of changes in the nucleotide pool to sporulation is unclear since *B. brevis* strains which are incapable of GrS synthesis and do not undergo nucleotide depletion nevertheless sporulate (Nandi & Seddon, 1978; Seddon & Nandi, 1978).

Study of the nucleotide–GrS interaction provides a further insight into biological transport phenomena not previously suggested by investigations of synthetic anion complexones. Control of binding selectivity has generally been achieved through the synthesis of complexing agents which topochemically match their substrates (Lehn, 1978). Presumably, the optimal model for a receptor site is the complexone which forms the most stable substrate complex with the highest degree of selectivity. All of the macrocyclic polyaza compounds reported (Dietrich et al., 1981; Kimura et al., 1982) show a greater or lesser degree of preference for ATP over ADP, however, most likely because of the critical importance of charge density in binding to these agents, and no complexone selective for ADP is presently known. The cytoplasmic terminus of the bongkrekate-sensitive ADP/ATP antiport of the inner mitochondrial membrane (Klingenberg, 1976) is an example of a naturally occurring carrier site which is highly selective for ADP.

From the results of the present investigation, it is proposed that a relatively short solvent-exposed region of a membrane protein which is conformationally rigid, amphiphilic, and dicationic can function as the proximate receptor in a nucleoside diphosphate selective carrier. Such a region is capable of binding other nucleotides, but under physiological conditions, only a diphosphate will afford a neutral complex in which it is possible, by a conformational change in the membrane protein, for the nucleotide to be delivered through a hydrophobic zone to a second (cationic) acceptor or channel. Two such binding regions, closely apposed, would constitute a triphosphate-selective site; an antiport can be constructed from di- and triphosphate-selective sites on opposite sides of the membrane which function alternately as primary and secondary acceptors. The GrS–nucleotide phase-transfer paradigm suggests that selectivity in polyanion transport can be conferred, or even modulated, by dimeric association of structurally basic binding elements in membrane systems.

Finally, the induction of phase transfer of nucleotides by GrS raises the intriguing possibility that phase transfer of nucleic acids might occur as well. Current investigations have shown this to be the case, as will be reported in detail in a future publication.

## Appendix

*1:1 Association.* When the notation introduced in the text is used, the conservation of mass relations for nucleotide and peptide species is

$$[a]_i = [a] + \sum_j j[ag^j] + \sum_j j[AG^j] \quad (A1)$$

$$\zeta[a]_i = (1 + K_G)[g] + \sum_j j[ag^j] + \sum_j j[AG^j] \quad (A2)$$

where the superscripts indicate the number of monomeric complexes in the aggregate. Combining eq A1 and A2 provides a simple relationship between [g] and [a]:

$$[g] = ([a]_i(\zeta - 1) + [a])/(1 + K_G) \quad (A3)$$

The binding constant $K$ was defined by eq 2.

It is assumed that aggregation occurs with negligible cooperativity. Concerning the organic phase, it has been shown for the polymerization of *N*-methylacetamide in $CCl_4$ (Løwenstein et al., 1970) that a single association constant is sufficient for all higher ($n > 2$) aggregates and that the magnitude of this constant is ≤30 times that of the dimerization constant. Even this small degree of cooperativity is unlikely for GrS if, as discussed in the text, aggregation in $CHCl_3$ occurs by cross-$\beta$-association through interamide hydrogen bonding, given the symmetry and rigidity of the peptide backbone. In water, where aggregation involves interaction of the apolar faces of the complexes, the relatively small size indicated for the soluble aggregates by the observation of high-resolution NMR spectral features and the total absence of precipitation after phase equilibration are noted. Second-order association constants for water ($K_w$) and the organic phase ($K_o$) are thus defined for all $j$, $k$ = 1, 2, 3...:

$$ag^j + ag^k \rightleftarrows ag^{j+k} \qquad K_w \equiv [ag^{j+k}]/([ag^j][ag^k])$$

$$AG^j + AG^k \rightleftarrows AG^{j+k} \qquad K_o \equiv [AG^{j+k}]/([AG^j][AG^k])$$

From these, it is possible to express the sums of species in closed form:

$$\sum_j [ag^j] = r/[K_w(1 - r)] \quad (A4)$$

$$\sum_j j[ag^j] = r/[K_w(1 - r)^2] \quad (A5)$$

$$\sum_j [AG^j] = R/[K_o(1 - R)] \quad (A6)$$

$$\sum_j j[AG^j] = R/[K_o(1 - R)^2] \quad (A7)$$

where $r \equiv KK_w[a][g]$ and $R \equiv K_o[AG^1]$. In addition, it is recalled from the definition of the extraction efficiency that

$$\sum_j j[AG^j] = [a]_i E \quad (A8)$$

These relations enable us to write eq A1 analytically:

$$[a]_i = [a] + r/[K_w(1 - r)^2] + [a]_i E \quad (A9)$$

An additional expression is necessary which relates the concentration of species across the phase boundary. It is convenient to define the aggregation number in the organic phase:

$$\langle N \rangle_o \equiv \sum_j j[AG^j]/\sum_j [AG^j] = 1/(1 - R) \quad (A10)$$

$\langle N \rangle_w$ may be defined analogously:

$$\langle N \rangle_w \equiv \sum_j j[ag^j]/\sum_j [ag^j] = 1/(1 - r) \quad (A11)$$

In defining the partition coefficient, it is recalled that the forces directing aggregation, and consequently the surface characteristics of the aggregates, differ between the phases. It is useful to consider initially two limiting situations which differ in important respects from the phase-transfer process at hand, in order to obtain a suitable expression for $K_p$. (i) If aggregation proceeds in an identical fashion in both aqueous and organic media, the macroscopically observed partition coefficient should simply be the ratio of total monomer concentrations, the energy of transfer of an aggregate scaling as the number of subunits, so that $K_p = \sum_j j[AG^j]/\sum_j j[ag^j] = (R/r)(K_w/K_o)(\langle N \rangle_o/\langle N \rangle_w)^2$; $E = K_p r/[K_w[a]_i(1 - r)^2]$ is then

seen to be independent of $K_o$. (ii) If the differences between the aggregates in the two phases are so great that structural rearrangement on phase transfer is energetically equivalent to complete disaggregation in one phase and reaggregation in the second, $K_p = [AG]/[ag] = (R/r)(K_w/K_o)$. Here, it is found that $[AG^j] = K_o^{-1}(K_oK_pr/K_w)^j$ is unbounded with increasing $j$, unless $K_oK_p/K_w < r^{-1}$, but for $\langle N\rangle_w > 1$, $r$ is O (1), and since $K_p \geq 1$ for the monomer, this requires that $K_o/K_w \leq 1$, which is contrary to our observations.

Between the two extremes, neither of which appears satisfactory, are cases where aggregates undergo direct phase transfer but with structural rearrangements which limit solvophobic contacts. Such cases correspond to $K_p = (R/r)(K_w/K_o)(\langle N\rangle_o/\langle N\rangle_w)^t$ $(0 < t < 2)$, where the free energy of phase transfer of $ag^j$ is between $-\infty$ and $j$ times that of monomeric ag. Aggregates of the nucleotide–GrS complex, by virtue of the amphiphilic character and compact structure of the monomeric unit, should undergo relatively facile internal reorganization on traversal of the phase boundary in order to sequester effectively the solvophobic groups in either phase (alkyl groups in water; amide and uncharged phosphoryl groups in $CHCl_3$). The apparent partition coefficient of an aggregate should then vary slowly with the number of contained monomeric complexes, suggesting that the most appropriate expression for the macroscopic partition coefficient, corresponding to $t = 1$, is just the quotient of aggregate concentrations:

$$K_p = \frac{R}{r}\frac{K_w}{K_o}\frac{\langle N\rangle_o}{\langle N\rangle_w} = \sum_j [AG^j]/\sum_j [ag^j] \qquad \text{(A12)}$$

If eq A6 and A10 are combined, $\sum_j [AG^j] = (\langle N\rangle_o - 1)/K_o$, while from eq A7, A8, and A10, $K_o[a]_iE = \langle N\rangle_o^2 - \langle N\rangle_o$; thus, solving for the aggregate concentration in the organic phase

$$\sum_j [AG^j] = [(4K_o[a]_iE + 1)^{1/2} - 1]/(2K_o) \qquad \text{(A13)}$$

Combining eq A4, A12, and A13

$$E = \frac{K_pr}{K_w[a]_i(1-r)}\left[1 + \frac{K_pK_or}{K_w(1-r)}\right] \qquad \text{(A14)}$$

which may be combined with eq A9 to yield

$$(1-r)^2([a] - [a]_i) + \frac{r}{K_w}(1 + K_p) + \left(\frac{r}{K_w}\right)^2 K_p(K_pK_o - K_w) = 0 \qquad \text{(A15)}$$

Equations A13 and A15 are solved for $r$, and $E$ was subsequently obtained from eq A14 as a function of $K$, $K_p$, $K_w$, $K_o$, $[a]_i$, and $\zeta$. The extraction profiles computed from these equations were optimized for a given $K$ by variation of the remaining parameters. There was a high degree of correlation between $K_p$ and $K_o$, so that this treatment actually involves adjustment of three parameters, $K$, $K_w$, and $K_p^2K_o$ (subject to $K_w < K_o$).

*1:2 Association.* Equations A9 and A3 are replaced by A16 and A17:

$$[a]_i = [a] + [ag] + r/[K_w(1-r)^2] + [a]_iE \qquad \text{(A16)}$$

$$[a] = [a]_i(1-\zeta) + ((1 + K_G)[g] - [ag])/2 \qquad \text{(A17)}$$

where $r \equiv K_1K_2K_w[a][g]^2$. Equations A14, A16, and A17 are solved for $E$ as a function of $K_1$, $K_2$, $K_p$, $K_w$, $K_o$, $[a]_i$, and $\zeta$, where either $[ag] = (K_1 + K_2)[a][g]$ (case I) or $[ag] = K_1[a][g]$ (case II).

**Registry No.** GrS, 113-73-5; ChaGrS, 23619-04-7; ADP, 58-64-0; AMP, 61-19-8; ATP, 56-65-5; TTP, 23198-01-8; 2′-dTTP, 365-08-2; 2′,3′-dideoxy-TTP, 611-60-9.

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# Amino Acid Sequence of the Heavy Chain Variable Region from the A/J Mouse Anti-Arsonate Monoclonal Antibody 36-60 Bearing a Minor Idiotype†

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ABSTRACT: In addition to the predominant idiotype family ($Id^{CR}$) associated with the murine A/J anti-azophenylarsonate response, a second idiotype family ($Id^{36-60}$) was defined on the basis of serologic cross-reactivity and amino acid sequence homology among monoclonal antibodies [Marshak-Rothstein, A., Margolies, M. N., Benedetto, J. D., & Gefter, M. L. (1981) *Eur. J. Immunol. 11*, 565–572]. The complete variable region amino acid sequence of the A/J IgG2a hybridoma protein 36-60 heavy chain was obtained by automated Edman degradation of the intact chain and fragments generated by cleavage with CNBr and by tryptic digestion of a succinylated CNBr peptide. A comparison of the $Id^{36-60}$ heavy chain sequence to that of the $Id^{CR}$ heavy chain variable region reveals only 45% homology between them. The structural data indicate that different genes encode the $V_H$, D, and $J_H$ gene-encoded sequences in the two idiotypes.

Idiotypes are serologically defined markers on immunoglobulin variable regions that often involve the antigen combining site. Idiotypes in inbred strains of mice have been instrumental in the understanding of antibody diversity and immune regulation. Certain predominant idiotypes may be present on the majority of antibodies synthesized in selected immune responses. The major cross-reacting idiotype (here designated $Id^{CR}$)[1] in A/J mice immunized with *p*-azophenylarsonate (Ars) linked to protein carriers is an example of such a response (Kuettner et al., 1972). The origin of diversity in this Ars-associated idiotype has been addressed through structural studies both at the DNA level (Siekevitz et al., 1982, 1983; Sims et al., 1982) and of the protein products (Estess et al., 1979, 1980; Marshak-Rothstein et al., 1980; Alkan et al., 1980; Margolies et al., 1981, 1983; Siegelman & Capra, 1981). The somatic cell fusion technique has made it possible to structurally characterize monoclonal antibodies of the desired idiotype. In the course of an examination of several randomly selected hybridoma proteins lacking the predominant $Id^{CR}$, a second idiotype family ($Id^{36-60}$) was defined, which also consists of a set of closely related molecules. Hybridoma proteins bearing $Id^{36-60}$ were regulated independently of $Id^{CR}$ in suppression experiments (Marshak-Rothstein et al., 1981). The frequent appearance of both $Id^{CR}$ and $Id^{36-60}$ among anti-Ars antibodies suggested that they each arise directly from germ-line genes. As it is not known why $Id^{CR}$ predominates in A/J mice, it is important to establish the degree of phenotypic diversity in these two families of monoclonal antibodies and to relate this diversity to the number and sequence of the relevant germ-line genes.

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[1] Abbreviations: $Id^{CR}$, a predominant cross-reacting idiotype in the A/J strain of mouse defined by rabbit antisera; Ars, *p*-azophenylarsonate; $Id^{36-60}$, a second idiotype expressed by a subpopulation of anti-Ars antibodies in A/J mice; HPLC, high-pressure liquid chromatography; Pth, phenylthiohydantoin; KLH, keyhole limpet hemocyanin; CDR, complementarity-determining region; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate.

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