Stereochemical Aspects of the Biosynthesis of Spectinomycin

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Abstract: The biosynthesis of spectinomycin (**1**) has been studied with specifically and stereospecifically labeled glucose as precursors. The results further define the mode of conversion of glucose into the actinamine (**2**) moiety of **1** and show that the formation of the cyclitol portion by *myo*-inositol-1-phosphate synthase involves stereospecific loss of the *pro-R* hydrogen from C-6 of glucose 6-phosphate. The TDP-glucose oxidoreductase reaction is implicated in the formation of the 4,6-dideoxyhexose moiety of **1** by the demonstration of an intramolecular hydrogen transfer from C-4 to C-6 of the hexose, which occurs with the same stereochemistry, i.e., replacement of OH at C-6 by H-4 in an inversion mode, that has been demonstrated for the enzyme from *E. coli* and from another streptomycete.

Spectinomycin (**1**) (Figure 1) is an unusual aminoglycoside antibiotic produced by several species of *Streptomyces*.[1,2] It inhibits protein synthesis at the ribosomal level, by selectively inhibiting initiating ribosomes,[3] and is used clinically for the treatment of gonorrhea as well as for veterinary purposes. Its structure has been elucidated by Wiley et al.[4] and confirmed by a single-crystal X-ray analysis,[5] which also established the absolute configuration, as well as by recent syntheses.[6,7]

Biosynthetic studies by Mitscher et al.[8] have shown that **1** is derived from two building blocks. The two *N*-methyl groups are derived from methionine, and glucose is the ultimate precursor of the aminocyclitol moiety constituting ring A and of the six-carbon unit which makes up ring C and the attached *C*-methyl group. The latter represents a 4,6-dideoxyhexose, and [6-^{3}H]-glucose specifically labels the *C*-methyl group in addition to contributing radioactivity to the actinamine (**2**) moiety. [2-^{14}C]*myo*-Inositol is an efficient precursor of the actinamine portion of **1**, indicating that the incorporation of glucose into ring A of **1** may proceed via this cyclitol. [2-^{14}C]Actinamine was incorporated seven times less efficiently than *myo*-inositol, a finding interpreted to indicate that **2** is not an intermediate in the formation of **1**, but can be converted into a pathway intermediate, and that *N*-methylation takes place after the assembly of the ABC ring system. This interpretation was, however, refuted in later mutant experiments[9] which showed that **2** is an obligatory intermediate in the biosynthesis of **1**. A more recent ^{13}C NMR study from Rinehart's laboratory[10] has further defined the mode of incorporation of D-[6-^{13}C]glucose into **1** by showing that the primary sites of labeling are C-6 and C-6′ (2.34 and 2.64 atom % excess ^{13}C), with the *N*-methyl groups being the only other labeled positions (0.74 and 0.73 atom % excess ^{13}C).

In the present paper we report the results of further studies on the biosynthesis of spectinomycin which deal with stereochemical aspects of the conversion of glucose into this antibiotic.

Results and Discussion

While the results of Stroshane et al.[10] define which carbon of the actinamine moiety of **1** is derived from C-6 of glucose, they still leave an ambiguity between two possible modes of incorporation of *myo*-inositol into this unit. These are outlined in Figure 2. Pathway *a* corresponds to the mode of formation of streptidine from glucose;[11,12] it is the one favored by the authors[10] and requires inversion of configuration at one chiral center of *myo*-inositol, the carbon derived from C-4 of glucose. The alternative pathway b requires configurational inversion at two centers, the carbons derived from C-4 and C-6 of glucose. These two possibilities can be distinguished by following the fate of specific hydrogens of glucose in the conversion to **2**. As shown in Figure 2, both routes will result in loss of the original hydrogens from C-3 and C-4 of glucose and retention of H-2.[13] Route a will in addition result in retention of H-1 and one of the hydrogens from C-6 of glucose, whereas route b will instead lead to retention of the hydrogen derived from the 5 position of glucose. The finding by Mitscher et al.[8] that [6-^{3}H]glucose labels both C-6′ and the actinamine

Table I. Incorporation of Specifically Tritiated Glucoses into Spectinomycin

	[6-^{14}C,6-^{3}H]glucose	[6-^{14}C,4-^{3}H]glucose
glucose fed	1.37×10^7 dpm ^{14}C, ^{3}H/^{14}C 2.34	9.6×10^6 dpm ^{14}C, ^{3}H/^{14}C 2.34
spectinomycin obtained	^{14}C incorporation 0.3%, ^{3}H/^{14}C 1.78 = 76% ^{3}H-retention	^{14}C incorporation 0.1%, ^{3}H/^{14}C 0.57 = 24% ^{3}H-retention
actinamine	^{3}H/^{14}C 1.20 = 49% ^{3}H-retention	^{3}H/^{14}C 0.02 = 1% ^{3}H-retention
acetic acid from C-5′ + C-6′	^{3}H/^{14}C 2.25 = 96% ^{3}H-retention	^{3}H/^{14}C 1.34 = 57% ^{3}H-retention

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(13) Loss of a hydrogen isotope label in the conversion of *myo*-inositol to **1** can be expected from the carbons which have undergone inversion of configuration[14] and definitely from those carrying nitrogen, because introduction of the nitrogen must involve transamination to a keto intermediate.
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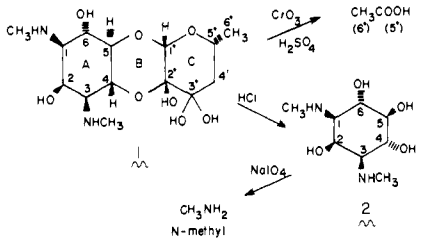

Figure 1. Degradation of spectinomycin.

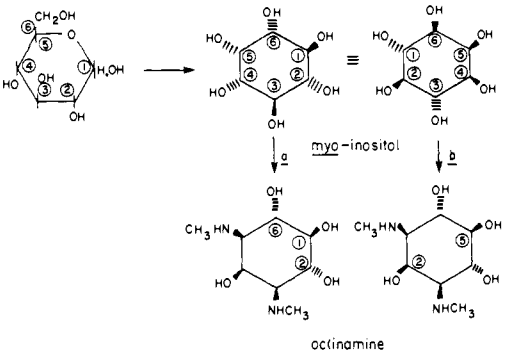

Figure 2. Two possible modes of incorporation of glucose and *myo*-inositol into the actinamine moiety of **1**. (The encircled numbers refer to the positions of the glucose molecule which contribute a carbon-bound hydrogen isotope.)

moiety of **1** can be interpreted to support pathway a. However, in view of the results of Stroshane et al.[10] the possibility exists that the tritium in the actinamine moiety was located in the *N*-methyl groups rather than at C-6.

To further probe this question and to lay the ground for the experiments described below, we fed to spectinomycin-producing cultures of *Streptomyces flavopersicus* several specifically tritiated glucoses, each mixed with [6-^{14}C]glucose, and determined the $^{3}H/^{14}C$ ratios of the precursor, the derived **1** and its degradation products. The results are summarized in Table I. As predicted for both pathways, tritium from C-4 of glucose is not incorporated into the actinamine moiety of **1**, obtained by acid hydrolysis of the antibiotic. Glucose tritiated in the 5 position was not fed because a label in this position is not a good indicator; C-5 of glucose 6-phosphate is transiently dehydrogenated to a ketone in the *myo*-inositol-1-phosphate synthase reaction and isotope effects in this reaction[15] may distort the $^{3}H/^{14}C$ ratios. Actinamine retains almost exactly half of the tritium from C-6 of glucose, in agreement with loss of one of the two methylene hydrogens in the *myo*-inositol-1-phosphate synthase reaction and retention of the other one in the conversion of *myo*-inositol into **2** by pathway a. To check if the incorporation is specifically into C-6 rather than the *N*-methyl groups, a sample of **2** derived from [6-^{14}C]glucose was degraded by periodate oxidation. The methylamine from the *N*-methyl groups contained no more than 25% of the total radioactivity of **2**. Thus, under our experimental conditions, feeding of only small amounts of labeled precursor, the majority of the tritium from C-6 of glucose is incorporated into the ring of **2** where, presumably, it residues at C-6.[16]

The conclusion that tritium from C-6 of glucose is incorporated specifically at C-6 of **1** is further supported by the finding (see below) that this incorporation is stereospecific. Conversion of C-6 of glucose via 3-phosphoglycerate and serine into the methyl group of methionine and then into the *N*-methyl groups of **1** should show no discrimination between the two heterotopic hydrogens. The operation of pathway a rather than b in the conversion of *myo*-inositol into **2** is also supported by the result of an experiment (not shown) which indicates incorporation of tritium from C-1 of glucose into **2**. These results are in accord with the previous

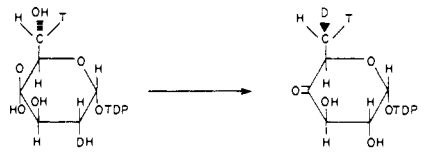

Figure 3. Steric course of the TDP-glucose oxidoreductase reaction.

suggestion[10] that the mode of formation of the actinamine moiety of **1** parallels that of streptidine from *myo*-inositol.

Aliquots of the samples of **1** from [6-^{14}C,6-^{3}H]- and [6-^{14}C,4-^{3}H]glucose were also degraded by Kuhn-Roth oxidation to give acetic acid from C-5′ and C-6′ (Figure 1). As shown in Table I, tritium from the 6 position of glucose is incorporated into C-6′, confirming Mitscher's results,[8] and there is essentially no loss of tritium relative to the ^{14}C label. More interestingly, tritium from C-4 of glucose is also incorporated into C-6′ of **1**, indicating a transfer of a hydrogen from C-4 to C-6 in the biosynthesis of the 4,6-dideoxyhexose moiety of **1** from glucose. The less than complete retention of tritium at C-6′ may be due to an isotope effect or may reflect competing metabolic reactions of the substrate resulting in loss of tritium from C-4 but not ^{14}C from C-6. A 4 → 6 hydrogen transfer is characteristic of the TDP-glucose oxidoreductase reaction,[17,18] a reaction widely involved in the biosynthesis of deoxyhexoses. Earlier work in our laboratory had established the steric course of this hydride transfer both for the purified enzyme from *E. coli*[19] and for the *in vivo* reaction leading to the formation of the 2,6-dideoxyhexose moiety of the antibiotic granaticin in *Streptomyces violaceoruber*.[20] In both instances, the transfer of a hydride from C-4 to C-6 of the hexose was found to be strictly intramolecular and to involve replacement of the OH group at C-6 by H-4 in an inversion mode (Figure 3).

To attain further evidence relative to the involvement of the TDP-glucose oxidoreductase reaction in the formation of the 4,6-dideoxyhexose moiety of **1** and the steric course of this reaction in another streptomycete, we carried out feeding experiments with (6*R*)- and (6*S*)-[6-^{14}C,4-^{2}H,6-^{3}H]glucose. This precursor was available from our earlier work[19,20] and was labeled in such a way that every tritiated molecule also carried deuterium; the ^{14}C label, on the other hand, was strictly in separate molecules. If the replacement of the OH group at C-6 by the migrating deuterium is stereospecific, the resulting methyl group will contain ^{1}H, ^{2}H, and ^{3}H in a chiral arrangement of either *R* or *S* configuration. However, in view of the large dilution of the added labeled precursor by unlabeled glucose from the medium and from endogenous synthesis, this will be the case only if the migration of the deuterium is strictly intramolecular, because a methyl group can only be chiral if ^{1}H, ^{2}H, and ^{3}H are present in the same molecule. An aliquot of the spectinomycin from each of these feeding experiments was subjected to Kuhn-Roth oxidation and the acetic acid from C-5′ and C-6′ was analyzed for its chirality by the method of Cornforth et al.[21] and Lüthy et al.[22] This analysis procedure involves conversion of the acetate into acetyl-coenzyme A and condensation with glyoxylate, catalyzed by malate synthase, to give malate. An isotope effect in the malate synthase reaction gives rise to an unsymmetrical tritium distribution between the two diastereotopic hydrogens of malate, if the acetate is chiral, which can be determined by incubation with fumarase. Thus an *F* value (percentage tritium retention in the fumarase reaction) of 79 indicates that the sample analyzed is chirally pure acetate of *R* configuration; an *F* value of 21 indicates

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Table II. Incorporation of Stereospecifically Labeled Glucoses into Spectinomycin

	(6*R*)-[6-^{14}C,4-^{2}H,-6-^{3}H] glucose	(6*S*)-[6-^{14}C,4-^{2}H,-6-^{3}H] glucose
glucose fed	9.1 × 10^6 dpm ^{14}C, ^{3}H/^{14}C 2.87	8.2 × 10^6 dpm ^{14}C, ^{3}H/^{14}C 2.67
spectinomycin obtained	^{14}C incorporation 0.4%, ^{3}H/^{14}C 1.59 = 55% ^{3}H-retention	^{14}C incorporation 0.2%, ^{3}H/^{14}C 2.77 = 104% ^{3}H-retention
actinamine	^{3}H/^{14}C 0.47 = 16% ^{3}H-retention	^{3}H/^{14}C 2.03 = 76% ^{3}H-retention
acetic acid	^{3}H/^{14}C 2.48 = 86% ^{3}H-retention; *F* value 31	^{3}H/^{14}C 1.64 = 61% ^{3}H-retention; *F* value 70
configuration of Me group at C-6′	*S*	*R*

chirally pure *S*-acetate.[23] As shown in Table II , the feeding experiment with glucose of 6*R* configuration gave spectinomycin in which the *C*-methyl group had *S* configuration and the 6*S* isomer of the precursor gave **1** with a *C*-methyl group of *R* configuration. Thus the 4 → 6 hydrogen migration does occur stereospecifically and intramolecularly and with the stereochemistry shown in Figure 3; i.e., the OH group at C-6 of the hexose is replaced by H-4 in an inversion mode. These results completely parallel the earlier findings,[19,20] further implicating the TDP-glucose oxidoreductase reaction in the biosynthesis of the 4,6-dideoxyhexose moiety of **1** and indicating that the reaction in *S. flavopersicus* proceeds with the same stereochemistry and involves the same mechanism as deduced earlier for the enzymes from *E. coli*[19] and *S. violaceoruber*.[20]

In the cyclization of glucose 6-phosphate to *myo*-inositol 1-phosphate, one of the heterotopic methylene hydrogens from C-6 of the hexose is eliminated. Kirkwood and co-workers[24] reported earlier that the *myo*-inositol-1-phosphate synthase from rat testis and rat mammary gland catalyzes cyclization of glucose 6-phosphate with stereospecific removal of the *pro*-6*S* hydrogen. Degradation of the samples of **1** from the above experiments with stereospecifically tritiated glucose to give **2** enabled us to examine the steric course of the *myo*-inositol-1-phosphate synthase reaction in *S. flavopersicus*. As shown in Table II, glucose of 6*R* configuration gives **2** with predominant loss of tritium, whereas the 6*S* isomer of glucose is converted into **2** with predominant retention of tritium. Hence the reaction proceeds with stereospecific loss of H_R from C-6 of the hexose. This result is obviously at variance with the earlier findings on the mammalian enzyme, and this discrepancy has caused us to reexamine the steric course of this reaction in other systems. In collaboration with Drs. Mary W. and Frank A. Loewus[25] of Washington State University, we determined that the purified enzymes from beef testis and from pollen of *Lilium longiflorum* catalyzed the synthesis of *myo*-inositol 1-phosphate with stereospecific loss of the *pro*-*R* hydrogen from C-6 of glucose 6-phosphate. This result thus conforms to the present findings in *S. flavopersicus*.

Retention of H_S and loss of H_R from C-6 of glucose 6-phosphate in the *myo*-inositol-1-phosphate synthase reaction, i.e., cyclization in a retention mode, is in line with expectations based on biochemical precedence. The mechanism for this reaction, as proposed by Loewus and Kelly[26] and supported by ample evidence,[27]

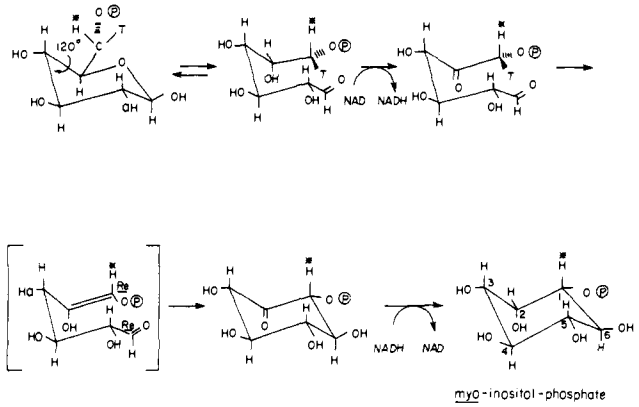

Figure 4. Stereochemistry and mechanism of the *myo*-inositol phoshate synthase reaction.

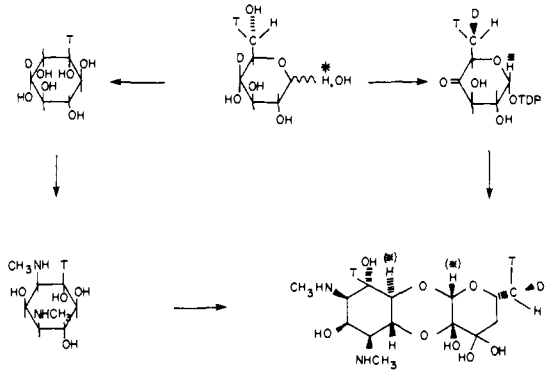

Figure 5. Stereochemistry of spectinomycin biosynthesis from glucose.

involves dehydrogenation of C-5 of the hexose phosphate followed by an aldol type ring closure between C-6 and C-1 and reduction of C-5 back to the alcohol (Figure 4). Seven of eight aldolases examined so far were found to catalyze replacement of H by C in the carbon–carbon bond formation step in a retention mode, possibly because the base that catalyzes proton abstraction also acts as a conjugate acid in the condensation, donating a proton to the carbonyl oxygen at the other reaction center.[28] For the eighth enzyme, deoxyribose-5-phosphate aldolase, the steric course could not be deduced because of a competing exchange reaction.[29] Our results on the steric course of the *myo*-inositol-1-phosphate synthase reaction thus fit in with the mechanistic classification of this enzyme as an aldolase and with the general stereochemical pattern of enzymatic aldol condensations.

As shown in Table II, the percentages of tritium retention in **2** from H_{6R} and H_{6S} of the substrate differ significantly from the theoretical values of 0 and 100%, respectively. This finding can be rationalized by the composite effect of two factors. In the studies with beef testis synthase, we observed tritium retentions of 21–31% for H_R and 95–96% for H_S of C-6 of glucose 6-phosphate.[25] This suggests that in this system nonstereospecific proton abstraction may compete with the stereospecific process, the former involving an intramolecular isotope effect, but that once the proton is abstracted, stereospecific ring closure takes place. If a similar situation exists with the *S. flavopersicus* enzyme, the *myo*-inositol serving as precursor of **2** would show tritium retentions of 20–30 and 95%, respectively. It should be remembered that because of the nature of the labeled precursor, all the tritiated *myo*-inositol molecules, but not the ^{14}C-labeled ones, will also carry deuterium at C-3 and that the C-H bond at C-3 has to be cleaved in a later epimerization step. An isotope effect in this epimerization reaction would retard the tritiated

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(28) K. R. Hanson and I. A. Rose, *Acc. Chem. Res.*, **8**, 1 (1975); I. A. Rose and K. R. Hanson in "Applications of Biochemical Systems in Organic Chemistry", J. B. Jones, C. J. Sih, and D. Perlman, Eds., Part II, Wiley, New York, 1976, p 507–553.
(29) D. L. Corina and D. C. Wilton, *Biochem. J.*, **157**, 573 (1976).

relative to the ^{14}C-labeled molecules and could thus decrease the $^{3}H/^{14}C$ ratio of **2** relative to the precursor *myo*-inositol.

In conclusion, this study has elucidated several stereochemical aspects of the biosynthesis of the antibiotic spectinomycin, as summarized in Figure 5.

Experimental Section

General Methods and Materials. (6*R*)- and (6*S*)-[4-^{2}H,6-^{3}H]glucose were obtained by alkaline phosphatase cleavage[20] of the corresponding glucose 6-phosphates available from earlier work.[19] [1-^{3}H]-, [4-^{3}H]-, [6-^{3}H]-, and [6-^{14}C]glucose were purchased from Amersham/Searle and bacterial (*E. coli*.) alkaline phosphatase was from Sigma. Materials for culture media were from Difco Laboratories, except for phytone which was obtained from B.B.L. Purification of (6*R*)- and (6*S*)-[4-^{2}H, 6-^{3}H]glucose was carried out by preparative TLC on 1-mm cellulose plates, 20 cm × 20 cm (Brinkmann, precoated), and on 0.25-mm cellulose plates, 20 cm × 20 cm (Eastman, precoated), using as the solvent system BuOH:AcOH:H_2O = 12:3:5.

Radioactivity was measured in a Beckman LS-7000 scintillation counter in 15 mL of Bray's solution, except for actinamine, which was counted in 10 mL of Aquasol. [^{3}H]- and [^{14}C]toluene were used as internal standards to determine counting efficiencies.

Culture Conditions. *Streptomyces flavopersicus* NRRL 2820 was maintained on sterilized soil and seed cultures were prepared by inoculating 100 mL of medium, containing 0.5% phytone, 0.5% yeast extract, 1% glucose, 0.1% Bacto-Casetone, and 0.5% NaCl, with spores on soil particles. The cultures, contained in 500-mL Erlenmeyer flasks, were incubated at 32 °C on a New Brunswick rotary shaker at 160 rpm for 72 h. Production cultures were inoculated with 2 mL of vegetative inoculum from seed cultures. Based on time-course studies, addition of labeled precursors 24 h after inoculation and harvest of the cultures 24 h later was chosen as the standard condition for the feeding experiments. The precursors were added to the cultures as Millipore-sterilized aqueous solutions.

Isolation and Purification of Spectinomycin. The cultures were filtered, the filtrate was acidified to pH 6.0 with 0.1 N HCl, and spectinomycin was adsorbed onto a column of ion-exchange resin IRC-50 (Na^+) (20 mL). The resin was washed with 50 mL of water, then eluted with 0.5 N HCl (75 mL). The eluent was adjusted to pH 6.0 with 0.1 N NaOH and evaporated to dryness. The residue was taken up in 5 mL of cold absolute MeOH and filtered from salts; the spectinomycin was recrystallized with carrier (40 mg) from water/acetone until the specific radioactivity and $^{3}H/^{14}C$ ratio were constant.

Degradations of Spectinomycin. The Kuhn-Roth oxidations were carried out as described.[30] The chirality analysis of sodium acetate was carried out by the method of Cornforth et al.[21] and Arigoni and coworkers,[22] using essentially Eggerer's procedure.[23]

Hydrolysis of **1** to **2**, isolated as the dihydrochloride, was carried out as described by Wiley et al.,[4] as was the periodate oxidation of **2** to give methylamine, which was trapped and analyzed as the hydrochloride.

Acknowledgment. We thank Dr. T. G. Pridham, USDA Northern Regional Research Laboratory, Peoria, Ill., for the strain of *S. flavopersicus*; Dr. Paul F. Wiley of the Upjohn Company, for a gift of spectinomycin; and Mrs. Janet Weaver of this department for carrying out the chirality analyses of the acetate samples. This work was supported by the National Institutes of Health through NIH Research Grants AI 11172 (to H.G.F.) and CA 17047 (to L.H.H.) and through Fogarty International Fellowship TW 02567 (to O.A.M.).

(30) H. Simon and H. G. Floss, "Bestimmung der Isotopenverteilung in markierten Verbindungen", Springer-Verlag, Berlin, 1967, p 26.

The Relationship between Complex Stability Constants and Rates of Cation Transport through Liquid Membranes by Macrocyclic Carriers[1]

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Abstract: The relationship between the rate of carrier-facilitated transport of metal cations through chloroform membranes containing macrocyclic ligand carriers and the stability constant of the cation–carrier complex in methanol solution was investigated. Several macrocyclic ligand carriers were used in transporting Na^+, K^+, Rb^+, Cs^+, Ca^{2+}, Sr^{2+}, and Ba^{2+}. For maximum cation transport, an optimum range in value of the cation–carrier complex stability constant was shown to exist. The rate of cation transport decreased rapidly at stability constant values higher or lower than this range. The maximum observed transport occurred for carriers having log K_{MeOH} values from 5.5 to 6.0 for K^+ and Rb^+ and 6.5 to 7.0 for Ba^{2+} and Sr^{2+}. For all cations, little or no transport occurred with carriers having log K_{MeOH} less than 3.5–4.0. An equation was derived which correctly predicts the observed variation of cation transport rate with log K_{MeOH} over a wide range of log K_{MeOH} values. This equation makes possible the estimation of either log K or cation-transport rate in certain cases if the other of the two values is known.

Introduction

Macrocyclic ligands such as crown ethers and their derivatives (Figure 1, **1–7**, **9–11**) and cryptands (Figure 1, **12–13**) have been used as cation-transport carriers in hydrophobic liquid membranes.[2-9] A liquid membrane system based on the Schulman bridge has provided a simple means to study transport of this type.[2,3,5-7,9] In this system, the macrocyclic carrier, dissolved in the membrane, facilitates the transport of salt from the source phase through the membrane to the receiving phase by solubilizing the cation into the membrane solvent. In a continuing program to quantitatively describe the factors which influence cation

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