1, 83 (1980).
(11) L. J. Filar and M. G. Wirick, p. 169 of Ref. 1c.
(12) P.J. Cheng and H.K. Schachman, *J. Polymer Sci.*, **16**, (1955).
(13) M. L. Huggins, *J. Amer. Chem. Soc.*, **64**, 2716(1942).
(14) D. T. F. Pals and J. J. Hermans, *Rec. Trav. Chim.*, **71**, 433 (1952); *ibid*, **71**, 458 (1952).
(15) P. J. Flory and T. G. Fox, Jr., *J. Amer. Chem. Soc.*, **73**, 1904 (1951).
(16) R. L. Cleland, *Biopolymers*, **6**, 1519 (1968).
(18) K. Nagasawa, Y. Tohira, T. Inoue and N. Tanoura, *Carbohydr. Res.*, **18**, 95 (1971).
(18) B. Chakrabarti and J. W. Park, *CRC Rev. Biochem.*, **8**, 225 (1980).

Enzymatic Synthesis of Cephaloglycin

Doo Hyun Nam, Heon Soo Sohn and Dewey D. Y. Ryu†
Department of chemistry, Korea Advanced Institute of Science and Technology, P. O. Box 150, Chongyangni, Seoul 131, Korea (*Received October 4, 1982*)

Cephaloglycin was synthesized directly from D-α-phenylglycine methyl ester and 7-aminocephalosporanic acid using whole cell enzyme of *Xanthomonas citri* (IFO 3835). Some optimal conditions for cephaloglycin synthesis were investigated, and yield improvements for its production by several methods were attempted. Using the whole cell enzyme system, the reaction kinetic model for cephaloglycin synthesis is proposed, and the kinetic constants for D-α-phenylglycine methyl ester hydrolysis, cephaloglycin synthesis, and cephaloglycin hydrolysis were determined. The K_m values of D-α-phenylglycine methyl ester, 7-aminocephalosporanic acid, and cephaloglycin were 11 mM, 24 mM, and 167 mM, and K_i value of D-α-phenylglycine was 15 mM, respectively. The pattern of product inhibition was found to be competitive one.

Introduction

New antibiotics discovered each year continue to increase linearly but those that were put into commercial production declined rapidly after 1950's,[1] because it became increasingly difficult to isolate new antibiotics sufficiently superior to existing products to warrant its introduction into clinical practice. A significant effort was focused on the modification of side groups that are capable of increasing the antibiotic potency or spectrum. A variety of semisynthetic cephalosporins have also been created by the substitution of appropriate side chains[2-4]. A large number of microorganisms have been found to have the synthetic activity of cephalosporin derivatives from 7-amino-3-cephem nucleus and several side chain compounds[5-16]. This biological synthesis of cephalosporins is relatively simple and convenient due to its mild condition, one step reaction, and high conversion, whilst the chemical process requires fairly large number of reaction steps for protection of reactive functional groups. Considering the merits of enzymatic synthesis, studies on the properties of cephalosporin synthesizing enzyme and the methods of increasing the conversion for production of cephaloglycin were undertaken.

Materials and Methods

Materials. The D-α-phenylglycine methyl ester (PGM) was prepared from D-α-phenylglycine (PG) (Aldrich Chemical Co., WI) and methanol using thionyl chloride. The 7-aminocephalosporanic acid (7-ACA) was kindly supplied from Wyeth Laboratory Inc., PA and the authentic cephaloglycin (CEG) was obtained from Sigma Chemical Co., MO. All other chemicals used were analytical reagents grade.

Experimental. A mutant of *Xanthomonas* citri (IFO 3835) was used throughout this work. The culture medium and fermentation conditions used, and the method of whole cell enzyme preparation were the same as those described previously[9]. To 1 m*l* of whole cell suspension in 0.1 *M* sodium phosphate buffer (pH 6.4) 2 m*l* of substrate in the same buffer solution was added and the reaction mixture was incubated at 37 °C for 5 min. The products were then assayed. Since both hydrolysis and synthesis of CEG occured simultaneously in this reaction system, the optimal conditions had to be determined by measuring the total accumulated concentration of CEG. The effect of enzyme loading on the reaction profile was also evaluated. The whole cell enzyme was concentrated and the conversion resulted from using the concentrated enzyme was compared with those using the original enzyme strength. Acetone treated whole cell enzyme was also evaluated for its possible improvements in the reaction rate and permeability of substrate and product. In order to suppress hydrolysis of PGM to PG by water, some organic solvents were selected based on their properties like Lewis basicity, relative ability of proton donor and degree of dipole moment[19], and added 10 % (v/v) to the reaction systems. The cumulative yield of CEG was measured and the conversion was compared with that achieved in aqueous reaction system. For kinetic study, the products in reaction mixture were analysed using high performance

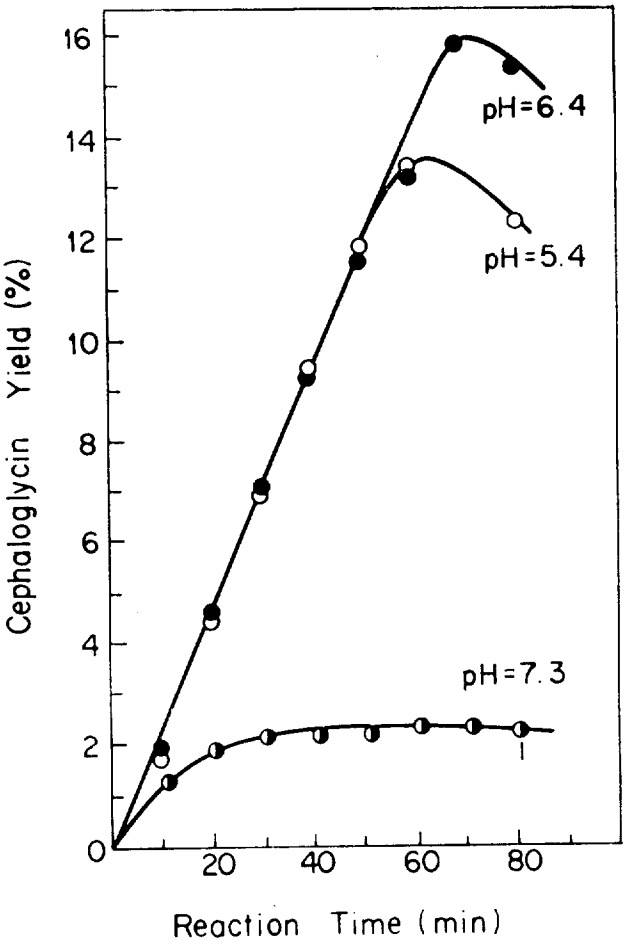

Figure 1. Effect of pH on CEG Synthesis. Reaction condition; 36°C, 60 m*M* of PGM, 20 m*M* of 7-ACA: 0.2 unit of enzyme was loaded.

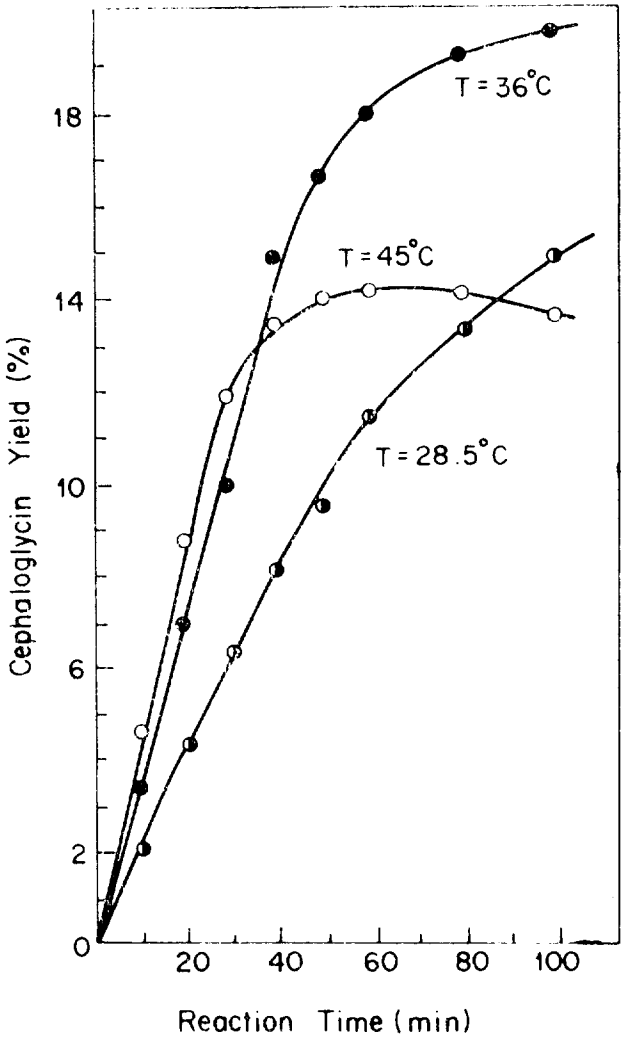

Figure 2. Effect of Reaction Temperature on CEG Production. Reaction condition; pH 6.4, 60 m*M* of PG*M*, 20 m*M* of 7-ACA: 0.2 unit of enzyme was loaded.

liquid chromatography (HPLC) (Model 440 LC, Waters Associates Inc., MA) as well as Fujii's spectrophotometric method[8]. The kinetic parameters involved in PGM hydrolysis were determined while varying PGM and PG concentrations and the kinetic parameters involved in CEG synthesis were determined under the saturated condition of PGM (45 m*M*). Waters Associates μ-Bondapak C_{18} column was employed using 0.35 *M* sodium acetate buffer (pH 4.0) as an eluent at 0.7 ml/min of flow rate and ultraviolet absorption detector (254 nm) in HPLC.

Results and Discussion

Cephaloglycin Production by Whole Cell Enzyme. The production of CEG by *X. citri* was confirmed by chromatographic methods. The CEG in reaction mixture had R_f value of 0.77 which corresponded to authentic CEG control in paper chromatographic assay (solvent system; 1-propanol: water =7 : 3 by volumetric ratio, Whatman No. 1 paper), and it also had the same retention time as the authentic one, 11 min, in HPLC assay. The optimal pH for CEG synthesis by whole cell enzyme was near 6.4, and the optimal temperature 36°C a shown in Figures 1 and 2. These reaction conditions are similar to those in the case of cephalexin (CEX) synthesis from 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) and PGM by *X. citri*[11]. It is desirable to determine the optimal conditions based on cumulative amount of CEG instead of initial reaction rate. The effect of enzyme loading on conversion of 7-ACA to CEG is shown in Figure 3. The maximum yield increased somewhat with the amount of enzyme. Its increment was, however, not so significant in spite of a proportional increase in initial reaction rate of CEG synthesis and CEG hydrolysis with the increase of enzyme loading. The difference of conversion profile between CEG synthesis and CEX synthesis, especially maximal conversion achieved, may be attributed to the different affinity of enzyme with the substrates, 7-ACA and 7-ADCA[9,11]. The overall conversion of 7-ACA to CEG reached only 20 % on a molar base. Kato *et al.*[17] could increase the overall conversion in synthetic reaction by reducing the ionic strength of reaction mixture so as to suppress the dissociation of enzyme molecule. An attempt to increase overall yield by lowering ionic strength failed when reaction was carried out in absence of phosphate. Acetone treatment of whole cell enzyme did not cause any significant change in CEG production.

Kinetics of Cephaloglycin Synthesing Enzyme. In the reports of recent studies, two different reaction kinetic

TABLE 1: Chromatographic Analysis of Reaction Pattern in CEG Synthesizing Reaction by *X. citri* Enzyme

Substrate	Detected compound	Expected reaction*
D-α-phenylglycine methyl ester (PGM)	PGM, PG	PGM ⟶ PG
D-α-phenylglycine (PG)	PG	Unreacted
7-aminocephalosporanic acid (7-ACA)	7-ACA	Unreacted
Cephaloglycin (CEG)	7-ACA, PG, CEG	CEG ⟶ 7-ACA+PG
PGM, 7-ACA	PGM, 7-ACA, PG, CEG	PGM+7-ACA ⟶ CEG
		PGM ⟶ PG
PG, Methanol	PG	PG ⟶×⟶ PGM
CEG, Methanol	7-ACA, PG, CEG	CEG ⟶ 7-ACA+PG
		CEG ⟶×⟶ 7-ACA+PGM
PG, 7-ACA	PG, 7-ACA	PG+7-ACA ⟶×⟶ CEG

*× represents that the reaction does not proceed.

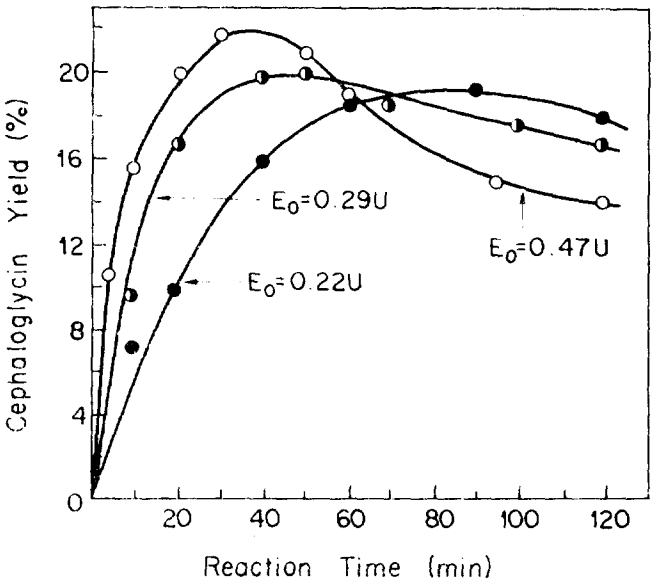

Figure 3. Effect of Enzyme Loading on CEG Synthesis. Reaction condition; 36°C, pH 6.4, 60m*M* of PGM, 20 m*M* of 7-ACA.

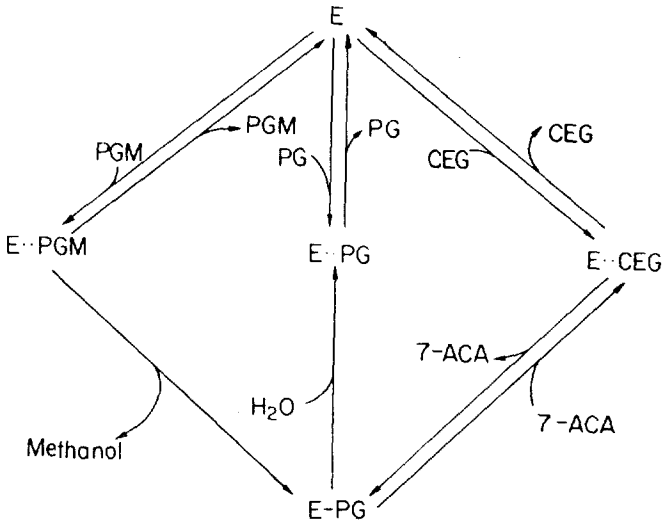

Figure 4. Proposed Reaction Model for Enzymatic Synthesis of CEG. E···PGM, E···PG, E···CEX; Enzyme substrate binding comlex; E—PG; Acyl enzyme intermediate.

models of CEX synthesizing enzyme from *X. citri* were suggested; one was carboxylic acid ester hydrolase, a similar pattern with acyl enzyme intermediate[14], and the other ordered bi-uni reaction considered as penicillin amidohydrolase[10]. Konecny[15] also reported that acyl enzyme intermediate of penicillin acylase from *Bacillus megaterium* may be responsible for the acylation of 7-ADCA with PGM to yield CEX. Between the two, bi-uni reaction model could not fully explain the reaction mechanism since three reactions simultaneously occurs by single enzyme; PGM hydrolysis to PG, transfer 7-ACA to PGM to form an amide bond of CEX, and hydrolysis of CEX[12,14,16]. In CEG synthesizing system, the reaction steps were analyzed and determined by HPLC (Table 1), and the hydrolytic steps of PGM and CEG were confirmed to be irreversible. A kinetic model having acyl enzyme intermediate was proposed as drawn in Figure 4. This reaction model differs from Kato's in that CEX hydrolytic step is irreversible[14]. Konecny proposed that all reaction steps were reversible[15]. Hydrolysis of PGM was found to be competitively inhibited by PG produced as shown in Figure 5, and this product, PG, also acted as a noncompetitive inhibitor in transfer reaction of 7-ACA to PGM to yield CEG (Figure 7). In CEX syn-

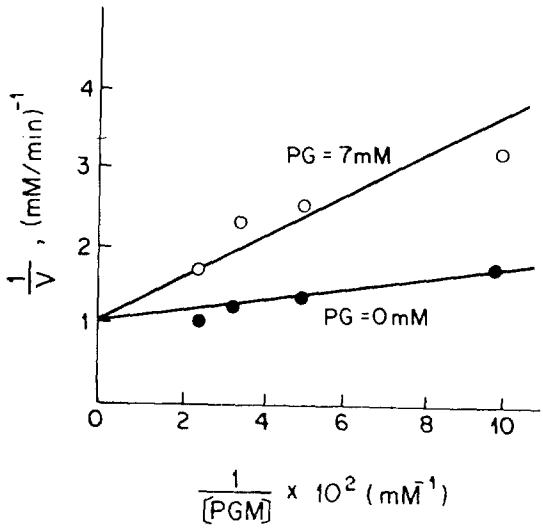

Figure 5. Lineweaver–Burk Plot of PGM Hydrolysis. Reaction condition; 37°C, pH 7.0 without PG (●) or 7 m*M* of PG (○); 4.5 mg of whole cell enzyme was employed.

thesizing reaction, excess PGM was reported to inhibit hy- and 0.23 m*M*/min/mg of cell (Figure 6). From the K_i value of PG obtained, the K_m value of 7-ACA (K_{7-ACA}) and drolysis of PGM[16] and 7-ADCA transfer to PGM[9]. This

TABLE 2: The Effect of Organic Solvents on CEG Synthesis

Solvent	Dipole Moment	Proton Donor[19]	Relative CEG Productivity (%)
Water	0.26	0.40	100
Triethylamine	0.32	0.61	13.9
Ethyl Ether	0.34	0.55	90
2-Methyl-1-propanol	0.22	0.55	15.8
2-Propanol	0.00	0.54	51.8
1-Hexane	0.00	0.00	110.1
Cyclohexane	0.00	0.00	100
2, 2, 4-Trimethylpentane	0.00	0.00	95.7

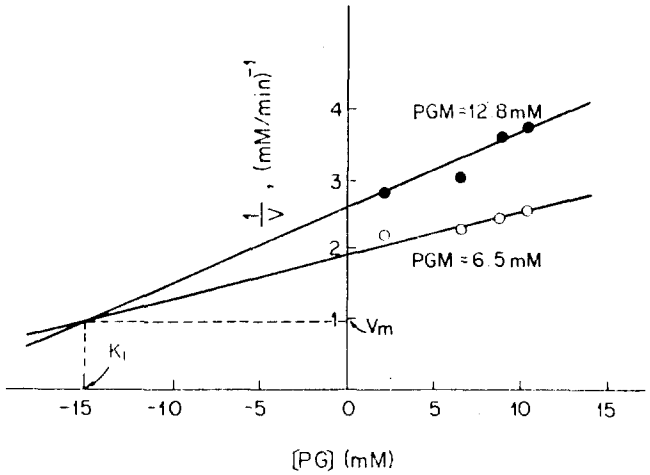

Figure 6. Dixon Plot of PGM Hydrolysis. The concentration of PGM were; (●) 12.84 m*M*; (○) 6.45 m*M*: Reaction condition; 37 °C, pH 7.0: 4.5 mg of whole cell enzyme was loaded.

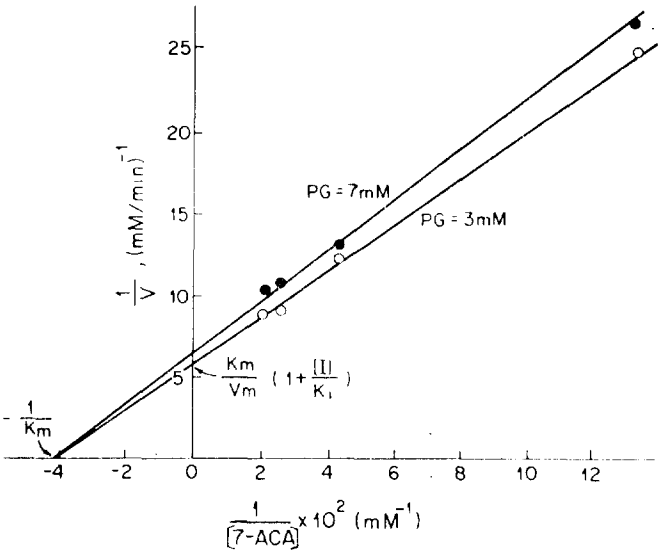

Figure 7. Lineweaver-Burk Plot of 7-ACA Transfer ot PGM to yield CEG. Reaction condition; 37 °C, pH 7.0, 3 m*M* (○) or 7 m*M* (●) of PG, saturated concentration of PGM: 4.5 mg of whole cell enzyme was loaded.

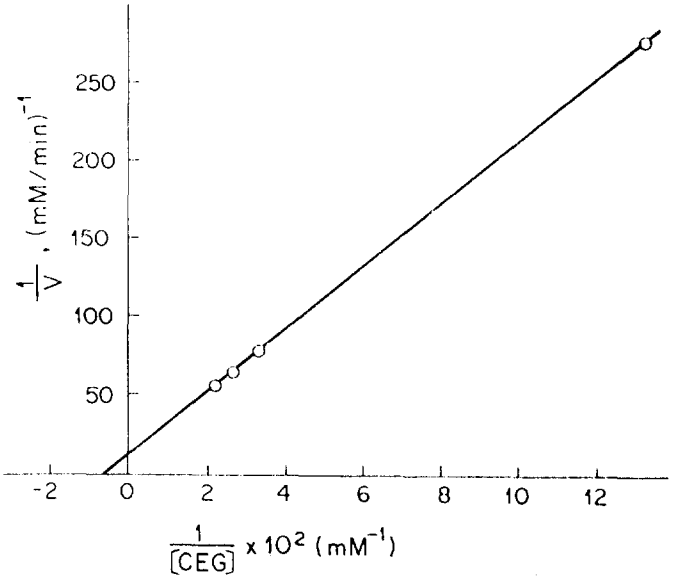

Figure 8. Lineweaver-Burk Plot of CEG Hydrolysis. Reaction condition; 37° C, pH 7.0, 4.5 mg of whole cell enzyme was employed.

substrate inhibition may be explained alternatively by competitive or noncompetitive inhibition of PG produced from high concentration of PGM. By using Dixon plot, the K_m value of PGM (K_{PGM}), K_i value of PG (K_{PG}), and V_m value of PGM hydrolysis (V_{PGM}) were determined as 11 m*M*, 15m*M*, and 0.23 m*M*/min/mg of cell (Figure 6). From the K_i value of PG obtained, theK_m value of 7-ACA (K_{7-ACA}) and V_m value of CEG synthesis (V_{7-ACA}) were estimated from Lineweaver-Burk plot (Figure 7) as 24 m*M* and 0.049 m*M*/min/mg of cell, and the K_m value of CEG (K_{CEG}) and V_m value of CEG hydrolysis (V_{CEG}) were also estimated as 167m*M* and 0.023m*M*/min/mg of cell (Figure 8). The ratio of maximum velocity of PGM hydrolysis, 7-ACA transfer to PGM, and CEG hydrolysis (V_{PGM}: V_{7-ACA}: V_{CEG}) were 100: 21: 10. This ratio determined was somewhat different from that reported by other 100: 49: 20 [13,16], where CEX was treated as a final product. The estimated K_{PGM} was similar to that obtained from CEX synthesizing system (K_{PGM}=14.5 m*M*)[9], but the calculated K_{7-ACA} was significantly different from K_{7-ADCA} in case of CEX synthesis (K_{7-ADCA}=3.7 m*M*)[9]. This difference in kinetic parameters may be due to different conversion profile of CEG synthesis and CEX synthesis. From the proposed scheme of reaction model (Figure 5), an enhancement of overall conversion of 7-ACA to CEG could be achieved by preventing PGM from its hydrolysis. This was in part verified by Kato *et al.* in amoxicillin synthesizing reaction [17]. The overall conversion of 6-aminopenicillanic acid (6-APA) to amoxicillin reached as high as 90 % by an addition of 10 % (v/v) 2-butanol. Recently, Lilly and his coworkers[18] also reported that reduction of water activity in reaction mixture by using 45 % (w/v) of polyethylene glycol (PEG) caused enhancement of enzyme stability and overall conversion in the acylation system of 6-APA by *Escheri-*

chia coli penicillin amidohydrolase. In CEG synthesizing system, the replacement of water with other organic solvents was attempted for the enhancement of overall conversion (Table 2). Most solvents (10 v/v %) tested, however, did not result in any significant improvement on CEG production. Only 1-hexane showed a slight improvement of CEG yield. It was also observed that solvents having higher ability of proton donor than water was unfavorable to stimulating CEG production.

References

(1) Y. Aharonowitz and G. Cohen, *Scient. Amer.*, **245**, 140 (1981).
(2) J. L. Spencer, E. H. Flynn, R. W. Roeske, F. Y. Siu and R. R. Chauvette, *J. Med. Chem.*, **9**, 746 (1966).
(3) C. W. Ryan, R. L. Sinnon and E. M. van Heyningen, *J. Med. Chem.*, **12**, 310 (1969).
(4) M. Neuman, *Drugs Exptl. Clin. Res.*, **6**, 491 (1980).
(5) T. Takahashi, Y. Yamazaki, K. Kato and M. Isono, *J. Amer. Chem. Soc.*, **94**, 4035(1972)
(6) W. Marconi, F. Bartoli, F. Cecere, G. Galli and F. Morisi, *Agric. Biol. Chem.*, **39**, 277 (1975).
(7) M. Shimizu, T. Masuike, H. Fujita, K. Kimura, R. Okachi and T. Nara, *Agric. Biol. Chem.*, **39**, 1225(1975)
(8) T. Fujii, K. Matsumoto and T. Watanabe, *Process Biochem.*, (Oct), 21 (1976).
(9) D. K. Rhee, S. B. Lee, J. S. Rhee, D. D. Y. Ryu and J. Hospodka, *Biotechnol. Bioeng.*, **22**, 1237 (1980).
(10) W. G. Choi, S. B. Lee and D. D. Y. Ryu, *Biotechnol. Bioeng.*, **23**, 361 (1981).
(11) I. H. Kim, D. H. Nam and D. D. Y. Ryu, *Appl. Biochem. Biotechnol.*, **8** in press (1983).
(12) T. Takahashi, K. Kato, Y. Yamazaki and Isono, *Japan. J. Antibiot.*, **30**, S-230 (1977).
(13) K. Kato, K. Kawahara, T. Takahashi and A. Kakinuma, *Agric. Biol. Chem.*, **44**, 1075 (1980).
(14) K. Kato, *Agric. Biol. Chem.*, **44**, 1083 (1980).
(15) J., Konecny, *Biotechnol. Letter*, **3**, 107 (1981).
(16) T. Takahashi, Y. Yamazaki and K. Kato, *Biochem. J.*, 137, 497 (1972).
(17) K. Kato, K. Kawahara, T. Takahashi and S. Igarasi, *Agric. Biol. Chem.*, **44**, 821 (1980).
(18) B. McDougall, P. Dunnill and M. D. Lilly, *Enzyme Microb. Technol.*, **4**, 114 (1982).
(19) L. R. Snyder, *J. Chromatogr.*, **92**, 223 (1974).

Studies on the Cation Exchange Elution Behaviors of Metal Complexes

Yong Soon Chung†

Department of Chemistry, Gyeongsang National University, Jinju, Gyeongnam 620, Korea

Byung Kiu Lee

Department of Science Education, Gyeongsang National University, Jinju, Gyeongnam 620, Korea

Chang Eon Oh

Department of chemistry, Yeungnam University, Gyongsan 632, Korea

(*Received October 12, 1982*)

The elution behaviors of a series of metal complexes, such as $Co(gly)_3$, $[Ni(en)_3]^{2+}$, $[Ni(phen)_3]^{2+}$, $[Fe(phen)_3]^{2+}$, $[Co(phen)_3]^{3+}$, $[Co(tn)_3]^{3+}$, $[Co(en)_3]^{3+}$ and $[Co(NH_3)_6]^{3+}$ (where gly; glycine, phen; phenanthroline, tn; trimethylenediamine, en; ethylenediamine), were studied in aqueous solution by measuring the retention volumes ($\bar{v}$ values) on SP-Sephadex C-25, cation exchange resin. It was found that the elution behaviors of metal complexes were apparently affected by salt concentrations, kinds of cations in eluent and kinds of anions in eluent, and according to the degrees of their effects coulombic forces, ion exchange capacities, the 'solvent effect' of resin backbone, hydrophobicity and hydrophilicity were applied to explain the elution mechanism.

Introduction

The variety of chromatographic techniques has been used to separate metal complexes.[1-5] Also, the widespread use of chromatographic methods has advanced considerably to the study of adsorption mechanism of metal complexes on ion exchange resin. In elution behaviors, in particular, the choice of elution methods and conditions seems to be largely empirical.[6] A brief review about the elutions of metal complexes by ion exchange resin is as follows: the adsorption behaviors of various Co(III) complexes were studied by paper chromatography and TLC[7-9]; the differences in $\bar{v}$ values of metal complexes due to the difference either in their electric charges or in their polarities[10,11]; ion association between complex ions and eluent anions was the main factor in the difference of $\bar{v}$ values[12,13]; electrostatic interaction between the complex ions and the functional groups in the stationary phase and/or between the complex ions and the anions in moving phase was used to interpret the adsorption mechanism.[13-15] Also, elution behaviors of metal complexes were explained by using water-structure maker and water-structure breaker.[16-19] These experiments were carried out by SP-Sephadex C-25