Urokinase Conjugated with Water-Soluble Dextran

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Urokinase, a plasminogen activator, was conjugated with dextran by the cyanogen bromide activation-coupling method. The resulting water-soluble conjugate was purified by gel permeation chromatography on Sephadex G-200. The maximal activity was obtained when the ratio of urokinase/dextran was 1/20 for the coupling. The final preparation showed 5 CTA units/mg conjugate, 300 CTA units/mg protein, 8.4% activity retention, and 47% protein retention. The urokinase-dextran conjugate had good thermal, pH and storage stabilities. In addition, it showed greater resistance to the inhibitory effect of human plasma than native urokinase. Also *in vitro* biological half-life of urokinase increased 40 times by this conjugation. In view of activity, excellent stability and increased half-life, the conjugate can be a potential fibrinolytic agent in an injectable form.

Introduction

Plasminogen activators are serine proteases with trypsin-like specificity which convert the inactive zymogen, plasminogen to enzymatically active plasmin and thereby initiate fibrinolysis[1,2]. Among them, a human urinary type plasminogen activator, known as urokinase has been used clinically for treatment of thrombosis and/or vascular obstruction.

For the purpose of thrombolytic therapy, immobilization of urokinase was attempted to increase *in vivo* half-life, or to obtain local fibrinolytic activity for thromboembolic disorders, or to develop antithrombogenic materials[3-8]. Previous results suggested the potential clinical application of immobilized urokinase[4-8]. Water-insoluble matrices, such as Sepharose, nylon, polyester and others have been used for the immobilization of urokinase. In the immobilization of urokinase, steric considerations appeared to be important and we showed previously[9] that the maximal activity for an agarose-NH-$(CH_2)_n$-CO-NH-$(CH_2)_2$-CO-NH-urokinase was obtained when spacer length (n) was 7.

In this work, we coupled urokinase to a water-soluble dextran which has a potential clinical application used as a blood volume expander[10]. The resulting urokinase conjugate can be an intravenous injectable fibrinolytic agent with enhanced biological half-life.

Experimental

Materials. A partially purified urokinase (2×10^{-5} CTA[a] units/vial) from human urine, human thrombin (100 NIH units/vial), fibrinogen (1 g clottable protein/vial), and human plasminogen were kind gifts[b]. The plasminogen was purified as described previously[9]. Dextran (average MW: 7×10^4) from *Leuconostoc mesenteroides,* dextranase (288 units/mg) from *Penicillium* species and cyanogen bromide were used as received[c]. All other reagents were commercially available reagent grades.

Urokinase Assay. The activity of urokinase was determined by the fibrin plate method[11]. One fibrin plate contained 2.1 m*l* of 0.2% human fibrinogen dissolved in veronal-HCl buffer, pH 7.8 and 100 μl of human thrombin (2 units). After incubating the plate with 5 μl of urokinase solution at 37°C for 8-16 hr, diameter of lytic zone was measured with a caliper. If required, caseinolytic assay for the urokinase activitiy was also carried out for a comparative purpose to secure the obtained data[9].

Determination of Contents of Protein and Carbohydrate. The contents of protein and carbohydrate were determined by the methods of Lowry *et al.*[12] and Dubois *et al.*[13] by using bovine serum albumin and free dextran (MW: 7×10^4) as authentic materials, respectively.

Conjugation of Urokinase with Water-Soluble Dextran. Coupling reaction was carried out by the method of Marshall and Rabinowtiz with a slight modification[14]. One gram of dextran was dissolved completely in 100 m*l* of distilled water and then was adjusted to pH 10.5 with 0.5 *N* NaOH solution. Two portions of CNBr (0.25 g each) were added at 30 min time interval with vigorous stirring. The reaction mixture was maintained at pH 10.5 during this activation process by addition of 0.5 *N* NaOH solution with Dosimat pH stat[d]. Thirty min after the second addition, of CNBr, pH of the solution was brought to 9.0 with 0.1*M* HCl. The activated dextran was dialyzed at 4°C for 2 hr against 4 *l* of distilled water, pH 9.0 (adjusted with sodium carbonate solution) and 0.05 g of urokinase (1×10^5 CTA units) was added. The pH was readjusted to 9.0 and the coupling was allowed to proceed for 20 h at 4°C with a slow stirring. The unreacted imidocarbonate group on dextran was blocked by using 0.5 g of glycine for further 12 h

[a]As difined by the Committee on Thrombolytic Agents. [b]Korea Green Cross Corp., Seoul, Korea. [c]Sigma Chemical Co., St. Louis, MO 63178. [d]Titator E526 and Dosimat E535, Metrohm Herisau, Switzerland. [e]Microfraction Collector FC-80K, Gilson Medical Electronics, Inc., Middleton, WI 53562.

reaction. The urokinase-dextran conjugate was lyophilized and followed gel filtration on a Sephadex G-200 column (90 × 1.5 cm) to remove uncoupled free enzyme. The lyophilized conjugate dissolved in 5 m*l* of 50 mM sodium phosphate buffer (pH 7.4) and 3 m*l* fraction was collected at a flow rate of 7.2 m*l*/h by using the same buffer[e]. The conjugate fractions which showed urokinase activity and positive carbohydrate reaction[13] as well were pooled and lyophilized.

The Properties of Urokinase-Dextran Conjugate. Heat stability was studied using solutions containing urokinase-dextran conjugate (50 μg protein/m*l*) in 0.05M phosphate buffer, pH 7.4. The solutions were incubated at 60 and 70°C. As a control, urokinase solution was heated at the same conditions as described above in the presence of free dextran at the concentration of 10 mg/m*l*. Five μl aliquots were taken at appropriate time intervals from the reaction mixtures for the determination of remaining activities. Electrophoresis of the conjugate on 5% polyacrylamide gel was carried out according to the method of Weber *et al.*[15] Inhibitory effects of blood plasma on the fibrinolytic activity of the conjugated urokinase were examined. About 3 mg of the conjugate (15-20 CTA units) were dissolved in 100 μl of 0.05 *M* phosphate buffer (pH 7.4) and 5 μl of the solution was mixed with various aliquots of plasma immediately followed by the assay on the fibrin plate as described. The human blood was obtained from healthy volunteers by a venipuncture technique. The blood was treated by adding 3.8% sodium citrate (1:9 parts) and centrifuged at 3,000 rpm for 20 min. Rat plasma was also prepared by the same method. The effect of dextranase treatment on the activity of the conjugate was determined by incubation of 30-40 CTA units of the conjugate and 29 units of the dextranase in 1.0 m*l* of the reaction mixture of 0.05 M phosphate buffer, pH 7.4 at 37°C during the period of 3 h. 5-10 μl of aliquots were taken for the assay according to the time course. Free urokinase was also treated under the same conditions and remaining activities were determined. The *in vitro* stability of the native and conjugated urokinases was studied with 0.1 m*l* aliquot of the human plasma which was added to each of the Eppendorf tubes containing the appropriate amounts (about 200 CTA units each) of native and conjugated urokinase (total volume 1.0 m*l*). After incubation at 37°C, 5 μl aliquots were taken and assayed on the fibrin plate.

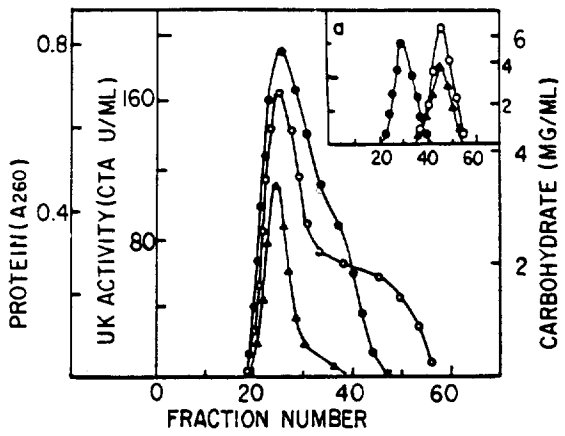

Figure 1. Sepahadex G-200 column chromatography of the urokinase-dextran conjugate. O: absorbance at 260 nm for protein. ▲: urokinase activity. ●: carbohydrate content. (Inset:a) the mixture of authentic urokinase and dextran.

TABLE 1: Comparative Results of Conjugate Formation by Different Ratio of Urokinase/Dextran for Coupling

UK/Dextran Ratio	Activity Retention (%)[a]	Extent of Conjugation (%)[b]
1 : 10	5.2	35
1 : 20	8.4	47
1 : 50	4.8	54
1 : 100	4.6	65
1 : 400	4.0	80

[a] Amount of the enzyme used for conjugation was expressed as 100%.
[b] Means % protein bound to dextran compared the amount of protein used initially for conjugation (100%).

Results

Synthesis of Urokinase-Dextran Conjugate. The preparation of urokinase: water-soluble dextran conjugate by CNBr activation coupling method as described by Marshall and Rabinowitz gave good yields. As shown in Figure 1, the chromatography of the conjugate mixture on Sephadex G-200 showed that the majority of the conjugated form was eluted at void volume of the column as an associated form with dextran, although some of the free dextran remained in the conjugate. As shown in Figure 1a (inset), a mixture of urokinase and free dextran on the same column revealed that the two components are separable. Fractions from 21 to 28 were pooled and lyophilized to obtain the final products. Analyses showed that its specific activity was 300 CTA units per mg of protein, compared with 2,000 units of native enzyme. The activity was 5 CTA units/mg conjugate. The ratio of dextran to urokinase affected significantly the conjugation of the enzyme as expected. As shown in Table 1, the best specific activity was obtained when the ratio of urokinase/dextran was 1/20, although more protein was bound as more dextran was added.

Another urokinase-dextran conjugate which was prepared by using oxirane (1,4-butanediol diglycidyl ether) for the coupling[16] showed a good conjugate as well (data not shown). However, the study was terminated due to its weak stability to the variation of temperature and pH.

On 5% polyacrylamide gel electrophoresis, the conjugated protein barely penetrated to the gel presenting a diffused band, while free urokinase and dextranase showed sharp bands. Digestion of the conjugate with dextranase restored the urokinase band on the gel indicating that enzyme was covalently bound to dextran (Figure 2).

Properties for Urokinase-Dextran Conjugate. Figure 3 shows heat stability of the conjugate enzyme compared with native urokinase. At 60°C, a half of the enzyme activity of the conjugate was maintained, while over 80% activity of the native was lost for about 60 min. At 70°C, the activity loss of the native enzyme was more severe, indicating that over 90% activity disappeared within 20 min. No stabilization effect by free dextran appeared at all when native urokinas was treated in the presence of dextran as shown in Figure 3.

In addition, storage of the lyophilized conjugate in 0.05 *M*

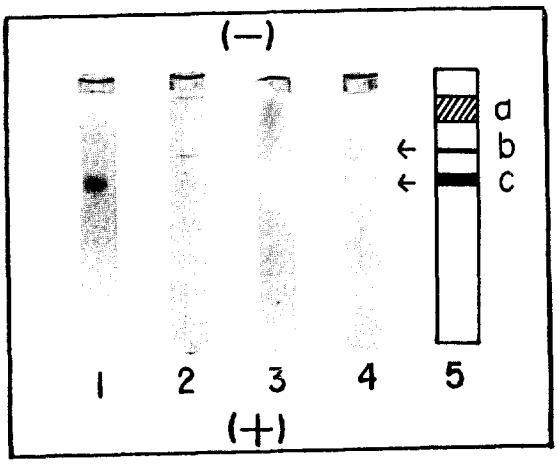

Figure 2. 5% Polyacrylamide gel electrophoresis of various urokinases. (1) authentic free urokinase; (2) authentic free dextranase; (3) urokinase-dextran conjugate; (4) urokinase-dextran conjugate treated with dextranase. Schematic representation: (a) diffused urokinase-dextran conjugate; (b) dextranase; (c) urokinase.

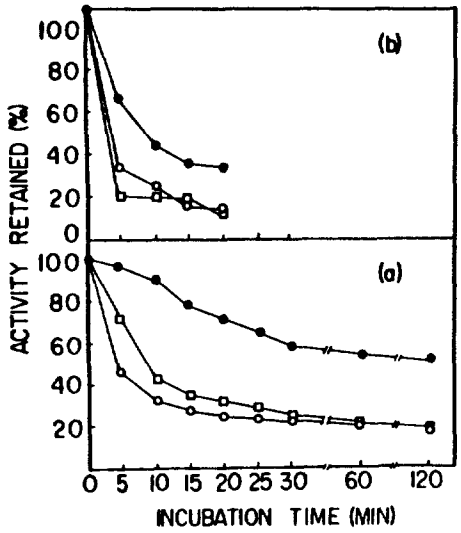

Figure 3. Loss of urokinase activity on heat treatment at 60°C (a) and 70°C (b). O: native urokinase. □: mixture of urokinase and dextran. ●: urokinase-dextran conjugate.

sodium phosphate buffer, pH 7.4 at 4°C for 6 months gave no influence on the fibrinolytic activity. Figure 4 shows retention of the activity of the conjugate for 20 days. The conjugate enzyme was stable without loss of activity, while the native one retained only 75% activity under these storage conditions.

The effect of storage time on the stability of the conjugated enzyme in various pHs was also compared with that of the native. Results indicated that pH 7.4 was the best for the stabilities of both enzymes (Figure 5). In any pH condition, however, stability of the native enzyme was poorer than that of the conjugate. The optimum pH of the conjugate urokinase was between 7.5 and 8.0, which was the same for free and Sepharose immobilized urokinase[9].

Inhibition of Plasmas to Urokinase-Dextran Conjugate. Inhibition effects of human and rat plasma on the fibrinolytic activities of the conjugate and native urokinases were investigated. Figure 6-a and b illustrate the results. The conjugates enzyme showed more resistance to the inhibitory effect of

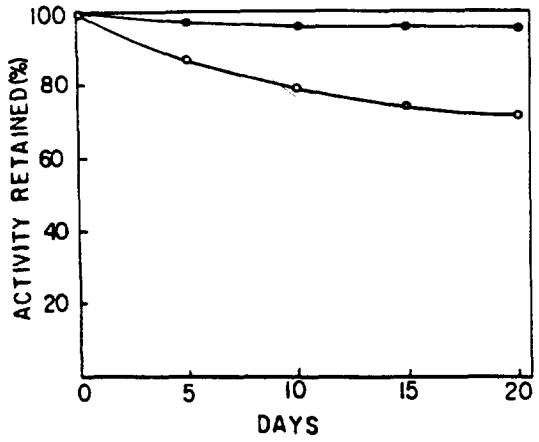

Figure 4. Storage stability of the urokinase activity in 0.05M phosphate buffer, pH 7.4 at 4°C. O: native urokinase. ●: urokinase-dextran conjugate.

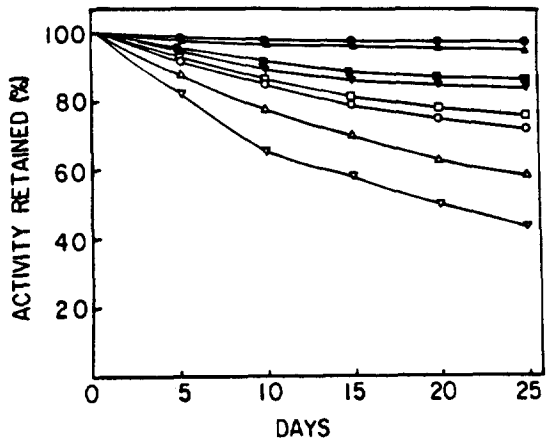

Figure 5. Storage stability of the urokinase activity under various pH conditions. ∇: and ▼: 0.05 *M* acetate buffer, pH 5.6. Δ: and ▲: 0.05M phosphate buffer, pH 6.6. O and ●: 0.05 *M* phosphate buffer, pH 7.4. □ and ■: 0.05 *M* phosphate buffer, pH 8.2. (Open mark) native urokinase. (Closed mark) urokinase-dextran conjugate.

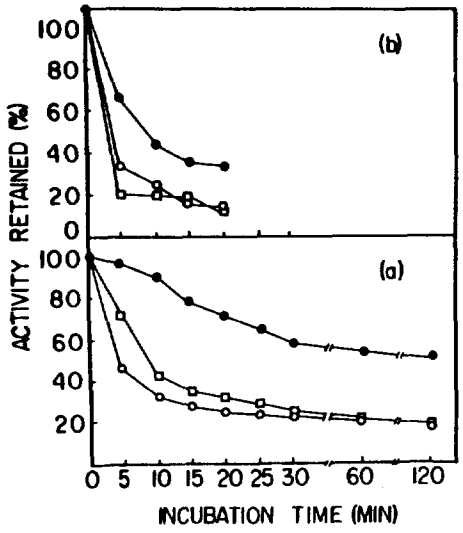

Figure 6. Inhibition of the urokinase activity by human plasma (a) and rat plasma (b). O: native urokinase. ●: urokinase-dextran conjugate.

plasma than that of the native enzyme. Especially, rat plasma revealed stronger inhibition to the urokinase activity. As shown in Figure 7, inhibition by plasma increased significantly when

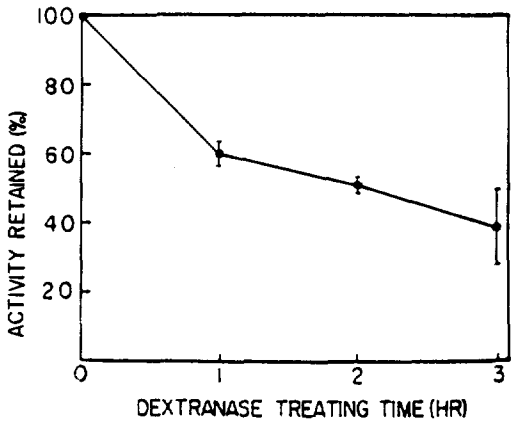

Figure 7. The effect of dextranase treatment on the susceptibility of the urokinase-dextran conjugate to the inhibitory effect of human plasma.

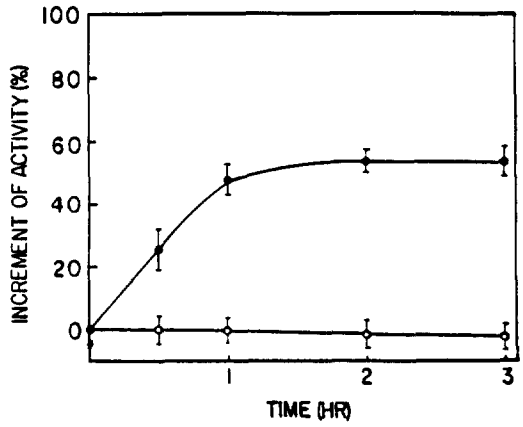

Figure 8. Effect of dextrnase treatment on the urokinase activity. O: native urokinase. ●: urokinase-dextran conjugate.

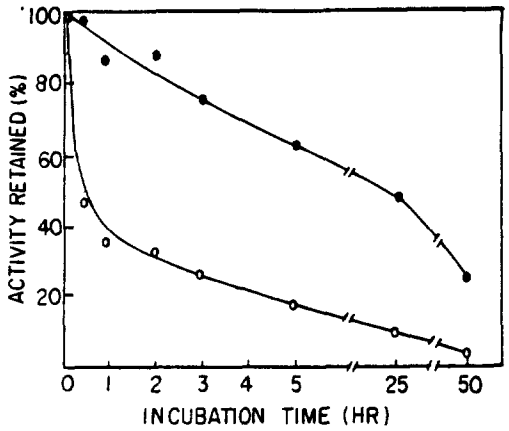

Figure 9. *In vivo* stability of the urokinase activity in the presence of human plasma. O: native urokinase. ●: urokinase-dextran conjugate.

the urokinase conjugate was treated with dextranase, resulting in free urokinase from a conjugated form. This explained well the fact that the conjugate form is more resistant to the inhibition as seen in Figure 6. This is due to the significant increase of the fibrinolytic activity (about 55%) as the conjugate was digested with dextranase in the absence of plasma (Figure 8).

Figure 9 illustrates *in vitro* biological half-life of the fibrinolytic activities of the two different forms of urokinase with the presence of human plasma. Half-life of urokinase was greatly improved by this conjugation from 15-45 min. of native urokinase to 20-24 hr of the conjugate enzyme. The conjugate had about 40 times longer biological half-life compared to the native form.

Discussion

Urokianse has been immobilized onto various matrices, and the results suggest a potential clinical use for thrombolytic therapy[3-9]. Nevertheless, water-insoluble matrices have been used and hence they are inadequate for the use of injectable fibrinolytic therapy. We, therefore, preapred urokinase-water soluble dextran conjugate as an injectable form. Since the dextran is being used as a blood volume expander and is a nonimmunogenic macromolecule[10], the injectable form of urokinase-dextran conjugate may have dual function potential as a proper blood volume expander and a fibrinolytic agent as well, showing enhanced biological half-life.

We prepared the urokinase-dextran conjugate by 2 different methods. The CNBr activation coupling method[14], forms covalent linkage between imidocarbonate groups of polysaccharide and ε-amino groups of lysine enzyme and the oxirane coupling method[16], gives $-CH_2-O-CH_2-CHOH-O-(CH_2)_4-O-CH_2-CHOH-CH_2-$ as a spacer between enzyme and dextran. The latter products, however, prepared by oxirane coupling showed very poor stabilities on pH and temperature.

Urokinase was bound covalently to dextran which is evidenced by the results of gel permeation chromatography on Sephadex G-200, polyacrylamide gel electrophoresis, and dextranase treatments. Marshall *et al.*[14] reported that the conjugation of enzyme with polysaccharide produces high molecular weight aggregates consisting of enzyme and polysaccharide molecules which are covalently linked both intramolecularly and intermolecularly. From the result of the polyacrylamide gel electrophoresis, our finding is similar to the Marshall's result.

The conjugated urokinase after purification on Sephadex G-200 showed 5 CTA units/mg conjugate, 8.4% activity retention, and 47% protein retention when the ratio of urokinase/dextran was 1/20 for the coupling. The activity per unit weight of product can be increased if the purified or concentrated enzyme is used initially for coupling. Specific activity of the conjugate was 300 CTA units/mg protein bound, compared to 2,000 CTA units of native urokinase. It is likely that loss of the activity is due to both the coupling process and unfavorable steric hindrance between conjugate and macromolecular substrate plasminogen. Kim *et al.*[9] suggested that activity of immobilized urokinase can be significantly affected by spacer length. This phenomenum was also observed in the urokinase-dextran conjugate as expected. Dextranase treatment of the conjugate increased the fibrinolytic activity by 1.6 (Figure 8).

Heat treatment of the conjugate at 60°C showed that it is much more stable compared to its native part. When heated at 60°C, half-life of the native and conjugate urokinase was 5 min and 50 min, respectively. The enhanced stability may result from multisite binding of polypeptide chains with a support, partial dehydration of protein molecule surfaces and/or crosslinking of protein with support.[17] Also dextran may serve to maintain tertiary structure of the enzyme by intermolecular

crosslinking to prevent unfolding.

In terms of the potential application of the urokinase conjugate as a therapeutic agent, we examined the inhibitory effect of blood plasma on the fibrinolytic activity of the conjugate and *in vitro* biological stability using human plasma. We found that *in vitro* biological half-life increased about 40 times longer. Also considering the conjugate as a potential fibrinolytic agent, the resistance to plasma inhibition is important.[18,19] At the concentration of 5 μl of plasma in the reaction mixture which corresponds to the concentration of plasma in blood, 30% activity of the conjugate was inhibited, while 90% activity of the native urokinase was inhibited.

In conclusion, urokinase-dextran conjugate showed high fibrinolytic activity, good stability on pH, temperature and storage, and excellent *in vitro* biological half-life with human plasma. Specially, it can provide injectable form. Therefore, it shows good potential as a fibrinolytic agent as well as a blood volume expander.

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Platinum(II) Complexes of 2,2′-Diaminobinaphthyl

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Platinum(II) complexes of R-2,2′-diaminobinaphthyl (R-dabn), $[Pt(R\text{-}dabn)(H_2O)_2]Cl_2$, $[Pt(R\text{-}dabn)(R\text{-}Pn)]Cl_2$, $[Pt(R\text{-}dabn)(R\text{-}bn)]Cl_2$, and platinum(II) complexes of S-2,2′-diaminobinaphthyl (S-dabn), $[Pt(S\text{-}dabn)(H_2O)_2]Cl_2$, $[Pt(S\text{-}dabn)(S\text{-}Pn)]Cl_2$, and $[(Pt(S\text{-}dabn)(S\text{-}bn)]Cl_2$ have been prepared. (R-Pn and S-Pn are, respectively R- and S isomer of 2,3-diaminobutane). R-Pn and S-bn are, respectively R and S isomer of 2,3-diaminopropane). In the vicinity of the B-absorption band region of dabn, the circular dichroism spectra of platinum(II) complexes of R-dabn series show a positive B-band followed by a negative higher energy A-band, which is generally understood as the splitting pattern for a λ conformation, while the circular dichroism spectra of platinum(II) complexes of S-dabn series show a negative B-band followed by a positive higher energy A-band in the long-axis polarized absorption region as expected for a δ conformation.

Introduction

The various methods have been applied to the determination of absolute configurations of metal complexes. Among the methods being used frequently are as following[1,2];

(a) Empirical and semiempirical regional rules including octant sign method, ring-pairing method, and sextent rule,

(b) Chiroptical spectroscopic methods

(i) Empirical methods based on the d-d transition utilizing circular dichroism (CD) and optical rotatory dispersion (ORD) spectroscopic data

(ii) Nonempirical method based on exiton theory (exiton splitting theory),