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Immunoassay Using a Metal-complex Compound as a Chemiluminescent Catalyst. I. Iron(III)phthalocyanine as a Labeling Reagent

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A synthesized metal-complex compound with catalytic activity for a chemiluminescence reaction has been used for the first time as a labeling reagent for immunoassay. Several complex compounds of iron(III) and cobalt(II) were synthesized, and their catalytic activities for the chemiluminescence reaction between 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and hydrogen peroxide (H_2O_2) were examined. Iron(III) 4,11,18,25-tetracarboxyphthalocyanine (TCP–Fe(III)) showed the highest catalytic activity. The immunoassay of free albumin was carried out by a competitive method using albumin labeled with TCP–Fe(III) by means of the carbodiimide method; free albumin in the range of 2 to 1000 μg/tube could be determined with a coefficient of variation of from 6 to 16% in 5 analyses.

In order to determine a trace amount of component in a biological sample, various immunoassays, including radioimmunoassay (RIA), enzyme immunoassay (EIA), fluoroimmunoassay, spin immunoassay, and metalloimmunoassay, have been reported. EIA is an excellent method for the determination of a trace amount of a component in a biological sample, but it has the following defects: 1) an inhomogeneous antigen-antibody reaction caused by the steric hindrance of a large enzyme molecule as a labeling reagent, 2) the additional time necessary for the enzyme reaction, and 3) the instability of the enzyme.

Chemiluminescence immunoassay (CLIA) has also become a method of interest since the defects in EIA can be more or less overcome. Moreover, CLIA has the following characteristics: 1) high sensitivity, 2) rapid analysis after the separation of bound and free antigens, and 3) a simple and inexpensive apparatus. Various natural substances,[1-3] such as hemin, cytochrome c, vitamin B_{12}, ferritin, catalase, hemoglobin, and peroxidase, have been used as a labeling agent for CLIA. These labeling agents are limited by the kind and number of functional groups available for labeling, and by their instability and purity.

The use of a synthesized metal-complex compound instead of the above-mentioned natural substances as a catalyst in CLIA is supposed to make CLIA sensitive, inexpensive, and reproducible, and also to make labeling easy. No immunoassay has, however, been reported in which a synthesized metal-complex catalyst is used as a labeling reagent. In this connection, several metal-complex catalysts were synthesized by the authors, and their catalytic activities were examined, TCP–Fe(III) being found to be most promising. In the present study, albumin was selected as a model compound for the analysis of a protein. It was determined by CLIA using a metal-complex catalyst as follows: the free albumin as an analyte and labeled albumin with TCP–Fe(III) as a competitor were made to react competitively with an antibody immobilized on glass beads, and the TCP–Fe(III) on the bound fraction after the separation of the bound and free fractions was determined by measuring the chemiluminescence intensity between luminol and H_2O_2.

Experimental

Preparation of Labeled Albumin. TCP–Fe(III) as a labeling reagent was synthesized by the method of Shirai *et al.*[4,5] The human serum albumin (HSA) with TCP–Fe(III) was labeled by the carbodiimide method as follows. Five milligrams of TCP–Fe(III) was dissolved in 10 cm^3 of a potassium dihydrogenphosphate–disodium hydrogenphosphate buffer solution (pH 7.0)(Buffer Soln (A)); 20 mg of *N*-cyclohexyl-*N'*-(2-morpholinoethyl)carbodiimide - methyl *p*-toluenesulfonate (Fluka) was then added to the solution, and it was mixed for 20 min. Thirty milligrams of HSA (Sigma, Fraction V) was added to the above mixture, and the resulting mixture was incubated for 48 h at 4 °C. After the reaction, the mixture was centrifuged at 15000 *g*, and the precipitate was removed. To the supernatants we added 10 cm^3 of a saturated ammonium sulfate solution, after which the mixed solution was kept at 4 °C overnight. The supernatants was removed by centrifugation, and the precipitate thus obtained was dissolved in 2 cm^3 of Buffer Soln (A), after which the solution was dialyzed against Buffer Soln (A) to remove the ammonium sulfate. After dialysis, the solution was submitted to gel chromatography on a TOYOPEARL HW-50 (TOYO SODA) column (150 cm^3) by the use of Buffer Soln (A) as an eluent; a 30-cm^3 portion of an albumin fraction containing albumin labeled with TCP–Fe(III) was thus obtained.

Preparation of Glass Beads Immobilized Antibodies. One cubic centimeter of a saturated ammonium sulfate solution was added to 1 cm^3 of rabbit anti-HSA serum (Medical and Biological Laboratories), after which the mixture was kept at room temperature for 1 h. The precipitate was collected by centrifuging it at 10000 *g* for 10 min and was then dissolved in 2 cm^3 of a 0.005 mol dm^{-3} phosphate buffer solution (Buffer Soln (B), pH 7.2) consisting of sodium dihydrogenphosphate and disodium hydrogenphosphate, the resulting solution being dialysed overnight against Buffer Soln (B). The precipitate thus produced was centrifuged, and an immunoglobulin G fraction was obtained by purifying the supernatants on a DEAE-cellulose column equilibrated with a Buffer Soln (B).

On the other hand, the glass-bead surface was activated by holding 100 g of glass beads (4 mm diam.) at 100 °C for 6 h in 100 cm^3 of a 50 wt% sodium hydroxide solution. The glass beads were washed and dried; alkylamino-bonded glass beads were then prepared by holding the glass beads for 6 h under refluxing in 300 cm^3 of a 2% (3-aminopropyl)triethoxysilane toluene solution. The immobilization of antibodies onto alkylamino-bonded glass beads was done by the glu-

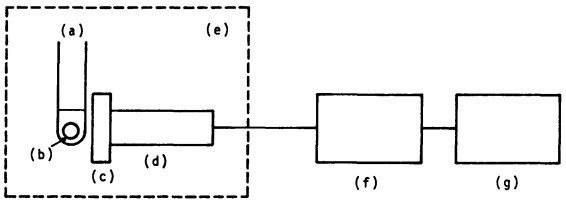

Fig. 1. Apparatus for CLIA.
a: Test tube, b: glass bead, c: shutter, d: photomultiplier, e: dark box, f: photon counter, and g: recorder.

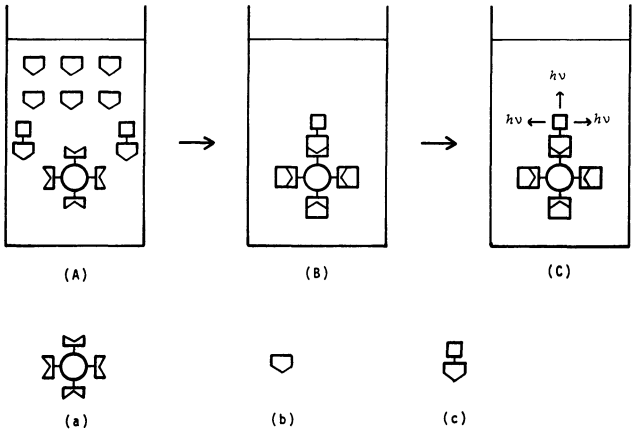

Fig. 2. Procedure for immunoassay.
A: Antigen-antibody reaction, B: separation of bound and free fractions, and C: measurement of chemiluminescence intensity. a; Glass bead immobilized with antibody, b; HSA, and c; labeled albumin.

taraldehyde method as follows. Thirty grams of the glass beads were dipped in 50 cm^3 of 5% glutaraldehyde/Buffer Soln (B) (0.01 mol dm^{-3}), and the mixture was made to react for 24 h at room temperature. After washing with Buffer Soln (B), the activated glass beads were dipped in 50 cm^3 of Buffer Soln (B) containing one-third of the antibodies which were taken out of 1 cm^3 of the antiserum; the mixture was then held at 4 °C for 48 h. The glass beads thus immobilized with antibodies were preserved at 4 °C in a Buffer Soln (B) containing 0.1% sodium azide and 0.1% bovine serum albumin.

Antigen-antibody Reaction. Twenty-five cubic millimeters of labeled albumin (containing about 0.6 μg of conjugated TCP–Fe(III)) were added to 1 cm^3 of Buffer Soln (B) containing a definite amount of albumin as an analyte, while one glass bead immobilized with an antibody was soaked in its solution. Thus the labeled albumin and the free albumin as an analyte were made to react competitively at 4 °C overnight with an antibody immobilized on the glass bead (competitive immunoassay).

Measurement of Chemiluminescence Intensity. The apparatus used for the measurement of the chemiluminescence intensity is shown in Fig. 1. One cubic centimeter of a 1.0×10^{-3} mol dm^{-3} luminol solution (pH 10.2, boric acid–potassium hydroxide buffer) and 1 cm^3 of a 2.5×10^{-3} mol dm^{-3} H_2O_2 solution were mixed in a 90×10 mm diam. test tube. The test tube was placed in front of the photomultiplier (Hamamaust TV, R464) equipped with a shutter (Copal No. 3), after which it was allowed to stand for 30 min before measurement in order to stabilize the chemiluminescence background.

After the antigen-antibody reaction, the glass beads immobilized with antibodies were washed twice with Buffer Soln (B) and then put into the above mixed solution. The shutter of the photomultiplier was immediately opened, and the chemiluminescence intensity was measured by means of a photon counter (Hamamatsu TV, C1230), the output being recorded by a Matsushita Communication Industrial apparatus (VP-6521A). Since the chemiluminescence intensity showed a maximal value about 3 min after the glass beads were put in, the maximal value was taken as the chemiluminescence intensity of a sample.

The procedure for immunoassay is shown schematically in Fig. 2.

Results and Discussion

Selection of a Labeling Reagent. The requirements for the catalyst as a labeling reagent in CLIA may be summarized as follows: 1) the catalytic activity for a chemiluminescence reaction is large enough for the sensitive detection of an analyte; 2) it has a functional group available for labeling; 3) a labeling reaction is feasible under mild conditions, 4) it is a stable compound, and 5) it can easily be prepared. Though metal ions such as cobalt(II), iron(III), copper(II), and osmium(VIII) show high catalytic activity for chemiluminescence reactions between luminol and H_2O_2, these metal ions can not satisfy all the requirements.

On the other hand, it is well known that chelate compounds, such as peroxidase, microperoxidase, hemin, hematin, and (*N,N'*-disalicylideneethylenediaminato)-cobalt(II),[6] strongly catalyze the chemiluminescence reaction between luminol and H_2O_2. Therefore, some kinds of chelate compounds may be supposed to be useful as labeling reagents. The common structure in the above-mentioned chelate compounds has the following characteristics: 1) the center of a molecule is occupied by iron(III) or cobalt(II), whose coordination number is six, 2) four coordination sites in a planar square are strongly linked with four donor atoms in a quadridentate ligand, and 3) two axial sites are weakly linked with donor atoms.

Cobalt(II) and iron(III) complex compounds were

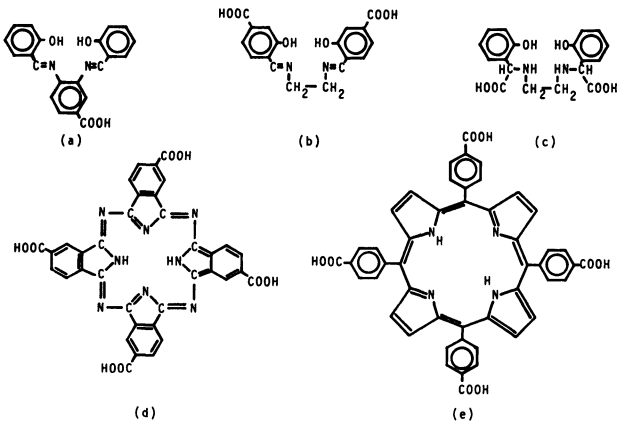

Fig. 3. Structure of ligands.
a: 3,4-Bis(salicylideneamino)benzoic acid, b: *N,N'*-bis-(4-carboxysalicylidene)ethylenediamine, c: *N,N'*-bis[2-(*o*-hydroxyphenylacetic acid)]ethylenediamine, d: 4,11, 18,25-tetracarboxyphthalocyanine, and e: 5,10,15,20-tetrakis(4-carboxyphenyl)porphine.

TABLE 1. ACTIVITY OF VARIOUS CATALYSTS IMMOBILIZED[a] ON A GLASS DISK

Catalyst		Chemiluminescence intensity/Hz	Relative activity
Ligand	Metal		
Blank		840	1.0
N,N'-Bis[2-(*o*-hydroxyphenylacetic acid)]ethylenediamine	Iron(III)	1900	2.3
	Cobalt(II)	1700	2.0
5,10,15,20-Tetrakis(4-carboxyphenyl)porphine	Cobalt(II)	1400	1.7
4,11,18,25-Tetracarboxyphthalocyanine	Iron(III)	350000	420
	Cobalt(II)	5200	6.2
N,N'-Bis(4-carboxysalicylidene)ethylenediamine	Cobalt(II)	35000	42
3,4-Bis(salicylideneamino)benzoic acid	Iron(III)	82000	98
	Cobalt(II)	7600	9.0
Hematin[b]		300000	360

a) An alkylamino-bonded glass disk was soaked in 20 cm^3 of a 1.0×10^{-3} mol dm^{-3} aqueous complex catalyst solution, and to it a 84.7-mg portion of *N*-cyclohexyl-*N'*-(2-morpholinoethyl)carbodiimide-methyl *p*-toluenesulfonate was added, the mixture then being allowed to react at room temperature for 24 h. b) Hematin was made to react with dicyclohexylcarbodiimide.

prepared by the use of the following quadridentate ligands, each of which has four donor atoms besides the carboxyl group used for the labeling reaction: 5,10,15,20-tetrakis(4-carboxyphenyl)porphine,[7,8] 4,11,18,25-tetracarboxyphthalocyanine, 3,4-bis(salicylideneamino)benzoic acid, *N,N'*-bis[2-(*o*-hydroxyphenylacetic acid)]ethylenediamine (Dojin Laboratories, EDDHA), and *N,N'*-bis(4-carboxysalicylidene)ethylenediamine[9] (Fig. 3). Their catalytic activities for the chemiluminescence reaction between luminol and H_2O_2 were measured by means of the flow-injection analysis system[10,11] and compared with each other. The flow diagram of the flow-injection analysis system and its experimental conditions are shown in Fig. 4, while the results obtained are shown in Fig. 5.

To examine the catalytic activity of a labeled complex compound, an experiment was carried out as follows: the surface of a 12-mm-diam. alkylamino-bonded glass disk was immobilized with a complex catalyst by means of the carboidimide method, and then the glass disk was placed in a stream of a mixed solution (pH 10.2, flow rate 1.96 cm^3 min^{-1}) of luminol (5.0×10^{-4} mol dm^{-3}) and H_2O_2 (1.25×10^{-3} mol dm^{-3}); after the chemiluminescence intensity was then measured with the apparatus[12] shown in Fig. 6. The results are shown in Table 1.

Figure 5 and Table 1 confirm that iron complexes generally show a higher catalytic activity and that their activities remain even after the bond formation of a carboxyl group. Especially, the catalytic activity of TCP–Fe(III) was higher than that of hematin,[13] which had been reported to be one of the most sensitive catalysts for chemiluminescence reactions. Hemin[2] has to be labeled in dimethyl sulfoxide, while TCP–Fe(III) can be labeled easily in water. Judging from these results, TCP–Fe(III) is superior to hemin.

Labeling of Albumin with TCP–Fe(III). The labeling in the immunoassay has to be carried out under mild conditions, since the analyte to be labeled is

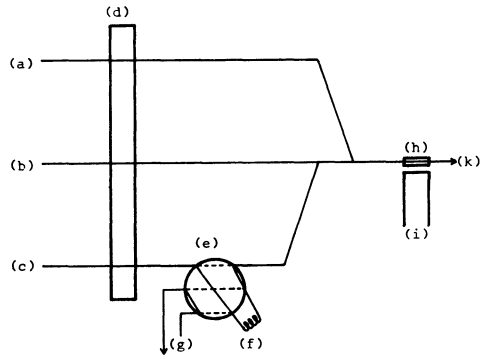

Fig. 4. Flow diagram of the flow-injection system.
a: Luminol (1.0×10^{-3} mol dm^{-3}, pH 10.2, flow rate 2.5 cm^3 min^{-1}), b: H_2O_2 (2.5×10^{-3} mol dm^{-3}, flow rate 2.5 cm^3 min^{-1}), c: buffer solution (flow rate 2.5 cm^3 min^{-1}) d: pump, e: sample inlet, h: flow-cell, i: photomultiplier, and k: waste.

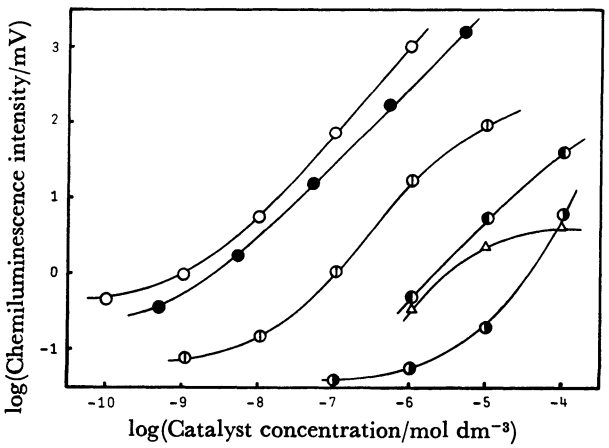

Fig. 5. Relationship between catalyst concentration and chemiluminescence intensity.
○: TCP–Fe(III), ●: hematin, ◫: Co(II) *N,N'*-bis(4-carboxysalicylidene)ethylenediamine, ◐: TCP–Co(II), △: Co(II) 5,10,15,20-tetrakis(4-carboxyphenyl)porphine, and ◑: Fe(III) 3,4-bis(salicylideneamino)benzoic acid.

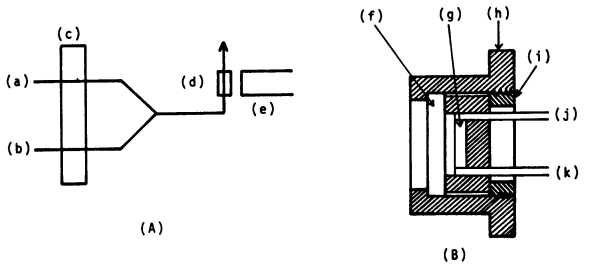

Fig. 6. Apparatus for measurement of chemiluminescence intensity based on the catalyst on the glass disk.
A: Flow diagram, a; luminol, b; H_2O_2, c; pump, d; flow-cell, and e; photomultiplier. B: Flow-cell. f; Window-glass, g; glass disk, h; cell holder, i; pressure screw, j; outlet, and k; inlet.

chemically unstable. Antigen as an analyte can be easily labeled with TCP–Fe(III) under mild conditions by the use of a water-soluble carbodiimide reagent, because water-soluble TCP–Fe(III) has carboxyl groups which are used for the formation of an amide linkage between TCP–Fe(III) and an analyte. The absorption spectrum of free TCP–Fe(III) in Buffer Soln(A) gave two peaks at 637 and 325 nm, while conjugated TCP–Fe(III) showed a peak at 630 nm and two shoulders at 576 and 338 nm. The retention volume of albumin labeled with TCP–Fe(III) in gel chromatography was slightly smaller than that of albumin alone. Judging from these results, it was concluded that albumin can be certainly labeled with TCP–Fe(III).

In addition, the precipitin arc of labeled HSA with TCP–Fe(III) in an immunodiffusion technique using rabbit anti-HSA serum was the same as that of HSA. This means that the activity of albumin for antigen-antibody reactions is not lost by its labeling.

The catalytic activities of conjugated TCP–Fe(III) and free TCP–Fe(III) for chemiluminescence reactions were also examined (Fig. 7). The concentration of conjugated TCP–Fe(III) in a labeled albumin solution was estimated from the absorption of the solution at 325 nm. As can be seen from Fig. 7, there was no marked difference between the catalytic activities of conjugated TCP–Fe(III) and free TCP–Fe(III). Thus, TCP–Fe(III) was shown to be useful as a labeling reagent for immunoassay.

Immunoassay. The relationship between the time consumed for an antigen-antibody reaction and the chemiluminescence intensity is shown in Fig. 8. The chemiluminescence intensity of a bound fraction for a certain time consumed for an antigen-antibody reaction is shown in Fig. 8 on the basis of the chemiluminescence intensity of the bound fraction which was made to react with albumin labeled with TCP–Fe(III) for 42 h. As can be seen from Fig. 8, the chemiluminescence intensity of a bound fraction was almost constant after 8 h. In all the following experiments, the antigen-antibody reaction was done overnight at 4 °C.

Figure 9 shows a calibration curve obtained by the competitive method. According to this calibration curve, albumin in the range of from 2 to 1000 μg/tube can be determined with a coefficient of variation of from 6 to 16% for 5 analyses. This calibration curve was linear, but not sigmoidal as is seen in a usual immunoas-

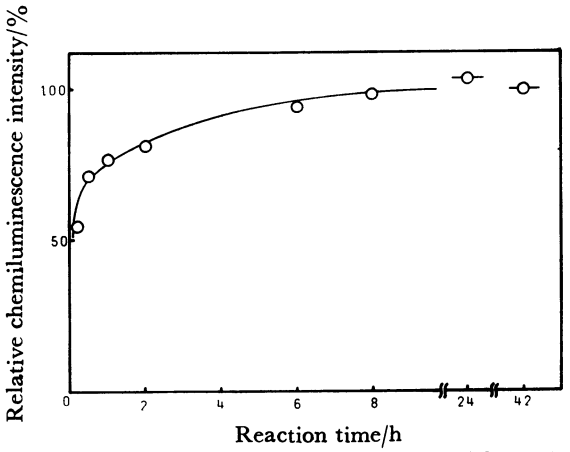

Fig. 8. Relationship between time consumed for antigen-antibody reaction and relative chemiluminescence intensity.

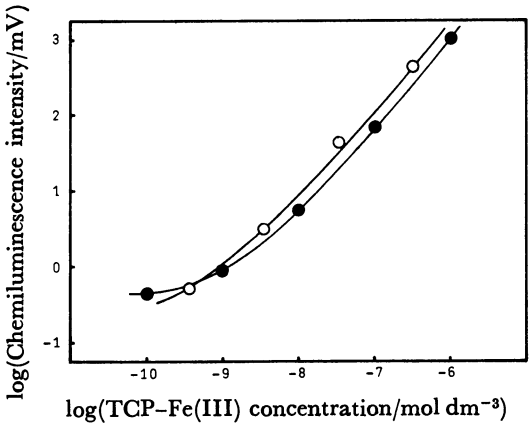

Fig. 7. Relationship between concentration of conjugated TCP–Fe(III) and chemiluminescence intensity.
○: Conjugated TCP–Fe(III) and ●: free TCP–Fe(III).

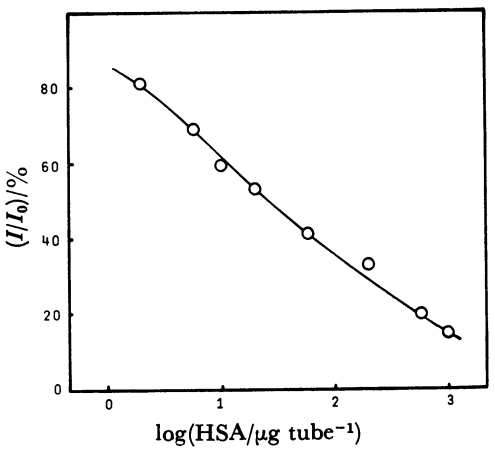

Fig. 9. Calibration curve.
I, I_0: Chemiluminescence intensity of the sample in the presence or absence of HSA.

say. This may be supposed to be due to the competitive reaction under unfavorable conditions,[14] *e.g.*, the ues of heterogeneous labeled albumin.

The present method is superior to the conventional methods of immunoassay, such as RIA and EIA, in the following points: 1) no counterplan is necessary for hazards of radioactivity in RIA; 2) the time necessary for measurement is short in comparison with EIA because the measurement is based on the chemiluminescence reaction, and 3) an analytical value is not so much influenced by contaminants[3] because the bound and free fractions are easily separated by the use of glass beads immobilized with antibodies.

It is concluded from the above-mentioned results that a synthesized metal-complex compound which works as a catalyst for a chemiluminescence reaction is useful and promising for immunoassay. Though the sensitivity and precision in the present work were not always satisfactory, they are now being improved by investigating the conditions for antigen-antibody reactions, chemiluminescence reactions, and instrumental measurements. Additional results will be reported in the near future.

References

1) Corning Glass Works, Jpn. Kokai Tokkyo Koho, 2790133 (1979); U. S. Appl. 815091 (1977); *Chem. Abstr.*, **90**, 182799f (1979).
2) Y. Ikariyama, S. Suzuki, and M. Aizawa, *Anal. Chem.*, **54**, 1126 (1982).
3) H. Arakawa, M. Maeda, and A. Tsuji, *Anal. Biochem.*, **97**, 248 (1979).
4) H. Shirai, S. Yagi, A. Suzuki, and N. Hojo, *Makuromol. Chem.*, **178**, 1889 (1977).
5) H. Shirai, A. Maruyama, K. Kobayashi, and N. Hojo, *Makuromol. Chem.*, **181**, 575 (1980).
6) I. Diehl and C. C. Hach, *Inorg. Synth.*, Vol. III, 196 (1950).
7) A. D. Alder, *J. Org. Chem.*, **32**, 476 (1967).
8) L. D. Rollmann, *J. Am. Chem. Soc.*, **97**, 2132 (1975).
9) R. H. Bailes and M. Calvin, *J. Am. Chem. Soc.*, **69**, 1886 (1947).
10) K. Tsukagoshi, M. Toriyama, and T. Hara, *Sci. Eng. Rev. Doshisha Univ.*, **23**, 119 (1982).
11) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **56**, 1382 (1983).
12) T. Hara, M. Toriyama, and M. Imaki, *Bull. Chem. Soc. Jpn.*, **55**, 1854 (1982).
13) H. A. Neufeld, C. J. Conklin, and R. D. Towner, *Anal. Biochem.*, **12**, 303 (1965).
14) C. J. Halfman, *Anal. Chem.*, **51**, 2306 (1979).