*J. Biochem.* **92**, 1413–1424 (1982)

# Mild and Efficient Conjugation of Rabbit Fab′ and Horseradish Peroxidase Using a Maleimide Compound and Its Use for Enzyme Immunoassay

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Received for publication, April 15, 1982

A mild and efficient procedure for conjugating rabbit Fab′ and horseradish peroxidase using a maleimide compound was developed. The enzyme was treated with *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl) maleimide to introduce maleimide groups. Then, the maleimide-enzyme was allowed to react with thiol groups of Fab′, and the conjugate formed was separated from unreacted components by gel filtration with Ultrogel AcA 44. In the peak fraction of the separated conjugate, 98% of peroxidase was associated with Fab′ and 90% of antibodies was associated with peroxidase. The recoveries in the conjugate of peroxidase and Fab′ incubated for conjugation were 65–74%. The conjugate formed appeared to be largely monomeric. Both the enzyme activity and antigen-binding activity of Fab′ were fairly well preserved in the conjugate. The cross-link formed was stable at 4°C at least 6 months. Use of the conjugates obtained by this method gave greater sensitivity in sandwich enzyme immunoassay for human ferritin and human thyroid-stimulating hormone than conjugates prepared by the periodate method. The conjugation using *N*-hydroxysuccinimide ester of *m*-maleimidobenzoic acid provided a similar monomeric preparation but was less efficient.

We first used *N,N′-o*-phenylenedimaleimide for conjugating IgG (*1*) and Fab′ (*2*) with β-D-galactosidase [β-D-galactoside galactohydrolase EC 3.2.1.23] from *Escherichia coli*, and the use of Fab′-β-D-galactosidase conjugates thus obtained allowed us to perform sandwich enzyme immunoassays for macromolecular antigens with attomole ($1 \times 10^{-18}$ mol) sensitivities (*3*, *4*). Later, *N*-hydroxysuccinimide ester of *m*-maleimidobenzoic acid was used to conjugate IgG with β-D-galactosidase (*5*). However, we noticed that these maleimides were not stable at neutral pH and we synthesized a more stable maleimide, *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl) maleimide (*6*), to conjugate IgG and Fab′ with glucose oxidase [β-D-glucose: oxygen 1-oxidoreductase EC 1.1.3.4] from *Aspergillus niger* (*7*). No attempt has been reported to conjugate antibodies

with horseradish peroxidase [donor: hydrogen-peroxide oxidoreductase EC 1.11.1.7] by using maleimides.

This paper describes a mild and efficient conjugation of rabbit Fab′ and horseradish peroxidase with minimal polymerization by using *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl) maleimide and compares the conjugates obtained with those prepared by using periodate, which have been very widely used in both immunohistochemistry and enzyme immunoassay for the past few years (*8*). A preliminary report of this study has been published elsewhere (*9*).

## MATERIALS AND METHODS

*Materials*—Horseradish peroxidase was obtained from Boehringer Mannheim, Mannheim (grade I), Sigma Chemical Co., St. Louis and Toyobo Ltd., Tokyo (grade I.C.). *N*-Hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl) maleimide was obtained from Zieben Chemicals Co., Tokyo. *N*-Hydroxysuccinimide ester of *m*-maleimidobenzoic acid was obtained from Sigma. Sephadex G-25 and G-150, Sephacryl S-200, CNBr-activated Sepharose 4B and concanavalin A-Sepharose 4B were obtained from Pharmacia Fine Chemicals AB, Uppsala. Ultrogel AcA 44 was a product of LKB. Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co., Chicago. Alkaline phosphatase [orthophosphoric-monoester phosphohydrolase alkaline optimum EC 3.1.3.1] from calf intestine, human luteinizing hormone (LH) and human follicle-stimulating hormone (FSH) were obtained from Boehringer Mannheim, Mannheim. Human thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (CG) were obtained from Calbiochem-Behring Corp., La Jolla. Rabbit anti-human IgG serum, goat anti-rabbit IgG serum and fluorescein-labeled rabbit anti-human IgG IgG were obtained from Miles Laboratories, Inc., Kankakee. Polystyrene balls (3.2 mm in diameter) were obtained from Precision Plastic Ball Co., Chicago. Other chemicals were obtained from Nakarai Chemicals, Ltd., Kyoto.

*Preparation of Human Ferritin and Anti-Human Ferritin*—Ferritin was prepared from human liver and anti-ferritin sera were raised in rabbits as described previously (*10*). The preparation of ferritin used was electrophoretically pure. Anti-ferritin sera obtained showed no cross-reactivity with other serum proteins or with cellular components on examination by a double-immunodiffusion technique and immunoelectrophoresis.

*Preparation of IgG, $F(ab')_2$ and Fab′*—IgG was prepared by fractionation of sera with $Na_2SO_4$ followed by passage through a diethylaminoethyl cellulose column, and $F(ab')_2$ was prepared by digestion of IgG with pepsin (*11*). In order to prepare fluorescein-labeled Fab′-peroxidase conjugate, $F(ab')_2$ was mixed with one-fortieth weight of fluorescein-labeled non-specific $F(ab')_2$, which was prepared by passing fluorescein-labeled anti-human IgG $F(ab')_2$ through a human IgG-Sepharose 4B column.

For the conjugation by the maleimide methods, Fab′ was prepared by reducing $F(ab')_2$ with 10 mM 2-mercaptoethylamine at pH 6 at 37°C for 1.5 h followed by gel filtration with Sephadex G-25 (*12*). For the conjugation by the periodate method, Fab′ was prepared in two different ways. 1) Fab′ was prepared as described above, and thiol groups generated (0.97–1.1 per Fab′ molecule) were blocked by incubation with 24 mM *N*-ethylmaleimide in 0.1 M sodium phosphate buffer, pH 6, at room temperature for 30 min followed by gel filtration with Ultrogel AcA 44 using 0.15 M NaCl to remove $F(ab')_2$, if any. 2) $F(ab')_2$ was reduced by 10 mM 2-mercaptoethanol at pH 8.2 at room temperature for 1 h, and thiol groups generated (1.6 per Fab′ molecule) were blocked by incubation with 12 mM sodium monoiodoacetate at 4°C for 16 h (*13*).

*Preparation of IgG- and CG-Sepharose 4B*—IgG (10 mg) or CG (30,000 U) was coupled to CNBr-activated Sepharose 4B (1 g) following the instructions of Pharmacia.

*Affinity-Purification of Anti-Human IgG $F(ab')_2$*—Anti-human IgG $F(ab')_2$ (20.9 mg) was adsorbed on a human IgG-Sepharose 4B column (5 × 14 mm) and eluted with 0.05 M glycine-HCl buffer, pH 2.9. The eluate was immediately neutralized with 0.5 M Tris-HCl buffer pH 8.0. The amount of purified anti-human IgG $F(ab')_2$ was 2.5 mg.

*Preparation and Affinity-Purification of Anti-TSH*—Anti-TSH sera were raised in rabbits as described previously (*14*). Anti-TSH IgG was

affinity-purified by elution at pH 2.3–2.5 from a TSH-Sepharose column and used for coating polystyrene balls (*14*). Anti-TSH $F(ab')_2$ was affinity-purified by elution at pH 2.3–2.5 from a TSH-Sepharose column in the same way as anti-TSH IgG and used for conjugation with peroxidase.

*Determination of Thiol and Maleimide Groups*—Thiol groups were determined using 4,4′-dithiodipyridine, and maleimide groups were determined by incubation with a known amount of 2-mercaptoethylamine and then measuring remaining thiol groups using 4,4′-dithiodipyridine (*11*).

*Measurement of Fluorescence Intensity*—Fluorescence intensity was measured using a Shimadzu fluorophotometer (RF-510, Shimadzu Seisakusho Ltd., Kyoto).

*Assay of Peroxidase Activity*—Peroxidase activity was fluorimetrically determined using *p*-hydroxyphenylacetic acid (*15*). Ten μl samples or polystyrene balls were incubated with 0.25 ml of 0.5% *p*-hydroxyphenylacetic acid in 0.05 M sodium acetate buffer, pH 5.0, containing 0.1% bovine serum albumin at 30°C for 5 min. Then, the reaction was started by adding 0.05 ml of 0.01% hydrogen peroxide, followed by incubation at 30°C for 20 min, unless otherwise indicated. The reaction was stopped by adding 2.5 ml of 0.1 M glycine-NaOH buffer, pH 10.3 and fluorescence intensity was measured by adjusting that of 1 μg/ml of quinine dissolved in 0.1 N $H_2SO_4$ to a scale of 100. The wavelengths used for excitation and emission analysis were 320 and 405 nm, respectively.

*Assay of Alkaline Phosphatase*—Alkaline phosphatase in 0.1 ml of 0.1 M glycine-NaOH buffer, pH 10.3, containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.05% $NaN_3$, and 0.025% egg albumin was incubated with 0.05 ml of $3 \times 10^{-4}$ M 4-methylumbelliferyl phosphate at 30°C for 10 min. The reaction was stopped by adding 2.5 ml of 0.5 M $K_2HPO_4$-KOH buffer, pH 10.4, containing 10 mM EDTA (*16*), and fluorescence intensity was determined using $1 \times 10^{-7}$ M 4-methylumbelliferone in the same buffer as a standard. The wavelengths for excitation and emission analysis were 360 and 450 nm, respectively.

*Conjugation by the Maleimide Method (I)—Introduction of maleimide groups:* Horseradish peroxidase (2 mg) was dissolved in 0.3 ml of 0.1 M sodium phosphate buffer, pH 7.0, and incubated with *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl) maleimide (1.6 mg, 100-fold molar excess) in 20 μl of *N*,*N*-dimethylformamide at 30°C for 1 h with continuous stirring. Precipitates formed were removed by centrifugation and the supernatant was subjected to gel filtration on a column (1.0 × 45 cm) of Sephadex G-25 using 0.1 M sodium phosphate buffer, pH 6.0. Fractions containing protein were pooled and concentrated using a collodion bag on ice.

*Conjugation of maleimide-peroxidase and Fab′:* The maleimide-peroxidase obtained (about 1.8 mg) in 0.1 M sodium phosphate buffer, pH 6.0 was incubated with rabbit Fab′ (about 2 mg) in 0.1 M sodium phosphate buffer, pH 6.0 containing 5 mM ethylenediaminetetraacetate at 4°C for 20 h. The concentration of both the maleimide-peroxidase and rabbit Fab′ in the reaction mixture was 0.05–0.15 mM. After incubation, the reaction mixture was subjected to gel filtration with a column (1.5 × 45 cm) of Ultrogel AcA 44 using 0.1 M sodium phosphate buffer, pH 6.5. Absorbance at 280 and 403 nm and peroxidase activity of each fraction were measured. For peroxidase assay, an aliquot of each fraction was diluted 2,500-fold with 0.01 M sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl and 0.1% bovine serum albumin and 10 μl of the diluted sample was used. Fractions containing the conjugate were stored at 4°C in the presence of 0.1% bovine serum albumin and 0.002% of thimerosal.

*Conjugation by the Maleimide Method (II)*—The conjugation by the maleimide method (II) was performed using *N*-hydroxysuccinimide ester of *m*-maleimidobenzoic acid essentially in the same way as the maleimide method (I) except for the following conditions. Peroxidase was treated with the ester at 25°C instead of 30°C, and the maleimide-peroxidase was subjected to gel filtration with a Sephadex G-25 column using 0.05 M sodium acetate buffer, pH 5.0, since maleimide groups of *m*-maleimidobenzoic acid were not stable at higher pH (*6*, *9*).

*Conjugation by the Periodate Method*—The conjugation by the periodate method was performed as described by Wilson and Nakane (*13*) except for the following conditions. Peroxidase was oxidized by sodium metaperiodate for 10 min instead of 20 min. The oxidation was stopped by

adding ethylene glycol followed by gel filtration with Sephadex G-25 as described by Boorsma and Streefkerk (*17*) instead of dialysis. Thiol groups of Fab′ used were blocked with *N*-ethylmaleimide or monoiodoacetate as described above. The concentration of both Fab′ and oxidized peroxidase incubated for conjugation was 0.02–0.05 mM. Gel filtration of the reaction mixture for conjugation and further processes were performed in the same way as in the maleimide method (I).

*Test for the Purity of Conjugates*—To test the presence of peroxidase (probably dimer) not associated with Fab′ in the conjugate preparations, the preparation of normal rabbit Fab′-peroxidase conjugate (210 ng) in 0.1 ml of 0.01 M sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl and 0.1% bovine serum albumin was passed through a column (3.5 × 27 mm) of goat (anti-rabbit IgG) IgG-Sepharose 4B at a flow rate of 1 ml/h using the same buffer, and peroxidase activity in the effluent was compared with the activity applied. As a control, a normal goat IgG-Sepharose 4B column was used in the same way.

To test the presence of antibodies (probably $F(ab')_2$) not associated with peroxidase in the conjugate preparations, the preparation of fluorescein-labeled Fab′-peroxidase conjugate (45 μg) in 0.1 ml of 0.1 M sodium acetate buffer, pH 6.0, containing 1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.2% bovine serum albumin, was passed through a concanavalin A-Sepharose 4B column (3 × 40 mm) at a flow rate of 1 ml/h using the same buffer, and the fluorescence intensity (490 nm for excitation and 510 nm for emission analysis) in the effluent was compared with that applied. As a control, fluorescein-labeled $F(ab')_2$ (23 μg) was passed through the same column.

*Assessment of the Molecular Weight of Fab′-Peroxidase Conjugate Prepared by the Maleimide Method (I)*—One ml of 0.1 M sodium phosphate buffer, pH 6.5 containing 1 mg of peroxidase, 2 mg of fluorescein-labeled Fab′, 0.25 mg of Fab′-peroxidase conjugate, 7 mg of bovine serum albumin, 2 mg of fluorescein-labeled $F(ab')_2$ and 1 mg of alkaline phosphatase was subjected to gel filtration using a column (1.5 × 45 cm) of Ultrogel AcA 44 equilibrated with the same buffer. The volume of each fraction was 0.86 ml. Absorbance at 280 and 403 nm, fluorescence intensity (490 nm for excitation and 510 nm for emission analysis), peroxidase activity and alkaline phosphatase activity were measured. Fab′ used was obtained by reducing $F(ab')_2$ at pH 6 and treating it with *N*-ethylmaleimide as described above.

*Test for the Antigen-Binding Ability of Conjugates*—To test the antigen-binding ability of conjugates, rabbit anti-human IgG Fab′-peroxidase conjugates (200 ng) in 0.1 ml of 0.01 M sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl and 0.1% bovine serum albumin were passed through a column (3.5 × 27 mm) of human IgG-Sepharose 4B at a flow rate of 1 ml/h using the same buffer, and peroxidase activity in the effluent was compared with the activity applied. As a control, a normal rabbit IgG-Sepharose 4B column was used in the same way.

*Test for the Stability of the Cross-Link*—Fluorescein-labeled Fab′-peroxidase conjugate (250 μg in 0.5 ml) prepared by the maleimide method (I) and stored under appropriate conditions was subjected to gel filtration with an Ultrogel AcA 44 column (1.0 × 45 cm) using 0.1 M sodium phosphate buffer, pH 6.5. Fluorescence intensity (490 nm for excitation and 510 nm for emission analysis) of each fraction was measured to detect release of Fab′ from the conjugate.

*Preparation of Anti-Ferritin and Anti-TSH IgG-Coated Polystyrene Balls*—Polystyrene balls were coated with IgG by physical adsorption (*3*). Anti-ferritin IgG used was not affinity-purified. Anti-TSH IgG used was affinity-purified, passed through a CG-Sepharose 4B column to remove antibodies which cross-reacted with LH, FSH, and CG and diluted 10-fold with normal IgG (*14*). The concentration of IgG used for coating was 0.1 mg/ml.

When polystyrene balls were coated with anti-TSH IgG which was affinity-purified and passed through a CG-Sepharose 4B column, CG (up to 0.2 U/tube), LH (up to 1.3 mU/tube), and FSH (up to 1.2 mU/tube) were not detectable by the sandwich enzyme immunoassay described below.

*Test for the Nonspecific Binding*—An anti-ferritin IgG- or normal rabbit IgG-coated polystyrene ball was incubated with various forms of peroxidase in 0.15 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.1% bovine serum albumin and 0.002% thimerosal at

37°C for 6 h with continuous shaking and washed twice with 1 ml of the same buffer. Peroxidase activity bound was determined as described above. Each form of peroxidase was used at 3 different levels within a range of 20–60 ng/tube of free forms (intact and oxidized-reduced) or 19–88 ng/tube of conjugates. Nonspecific binding increased proportionately with increasing amounts of various forms of peroxidase used.

*Enzyme Immunoassay*—An anti-ferritin or anti-TSH IgG-coated polystyrene ball was incubated with ferritin or TSH in 0.15 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.1% bovine serum albumin and 0.002% thimerosal at 37°C for 6 h with continuous shaking. The polystyrene ball was washed twice with 1 ml of the same buffer and incubated with 19.2 ng of anti-ferritin or anti-TSH Fab′-peroxidase conjugate in 0.15 ml of the same buffer at room temperature overnight without shaking and at 37°C for 6 h with continuous shaking. The polystyrene ball was washed twice with 1 ml of the same buffer, and peroxidase activity bound to the polystyrene ball was determined as described above.

*Conjugates Used for Various Tests*—Conjugates used were taken from fractions 37–39 prepared by the maleimide method (I), from fractions 37–39 prepared by the periodate method as monomeric conjugates and from fractions 29–31 prepared by the periodate method as highly polymerized conjugates (Fig. 1).

*Calculations*—1) The amount and concentration of Fab′ were calculated from the absorbance at 280 nm by taking the molecular weight and extinction coefficient at 280 nm to be 46,000 (*18*, *19*) and 1.48 $cm^2$/mg (*20*), respectively. The amount and concentration of peroxidase were calculated from absorbance at 403 nm by taking the molecular weight and extinction coefficient at 403 nm to be 40,000 and 2.275 $cm^2$/mg, respectively (*21*).

2) The average number of maleimide groups introduced per peroxidase molecule was calculated by using the molecular weight and extinction coefficient at 403 nm of peroxidase as described above.

3) The specific binding of conjugates was calculated by subtracting peroxidase activity nonspecifically bound in the absence of antigens from that bound in the presence of antigens.

## RESULTS

*Conditions for Introduction of Maleimide Groups—Maleimide method (I):* Various conditions were tested for the introduction of maleimide groups into peroxidase by treatment with *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl) maleimide. Treatment at pH 7.0 was more effective than at pH 6.5, and introduction of maleimide groups reached a plateau after 1 h treatment at pH 7.0. The average number of maleimide groups introduced per peroxidase molecule by treatment at pH 7.0 for 1 h was 1.0–1.2 (Table I), which was highly reproducible with peroxidase preparations not only from Boehringer but also from Sigma and Toyobo.

*Maleimide method (II):* Treatment with the *N*-hydroxysuccinimide ester of *m*-maleimidobenzoic acid was also more effective at pH 7.0 than at pH 6.5, but treatment at pH 7.0 for more than 1 h resulted in a decrease of the average number of maleimide groups introduced per peroxidase molecule. The maximal average number of maleimide groups introduced per peroxidase molecule was 0.63 and was less than that in the maleimide method (I) (Table I). Treatment at 30°C was less effective than at 25°C.

TABLE I. Number of maleimide groups introduced under various conditions.

| Conditions for treatment with ester | | Number of maleimide groups introduced per peroxidase molecule | |
|---|---|---|---|
| pH | Time (h) | CHM[a] at 30°C | MBA[b] at 25°C |
| 6.5 | 0.5 | 0.45, 0.26 | 0.12, 0.13 |
| 6.5 | 1 | 0.58, 0.59 | 0.35, 0.31 |
| 6.5 | 2 | 0.92, 0.92 | 0.44, 0.43 |
| 7.0 | 0.5 | 0.82, 0.78 | 0.60, 0.62 |
| 7.0 | 1 | 1.0–1.2(6)[c] | 0.63, 0.56 |
| 7.0 | 2 | 1.1, 1.0 | 0.54, 0.40 |

[a] *N*-Hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl) maleimide. [b] *N*-Hydroxysuccinimide ester of *m*-maleimidobenzoic acid. [c] The number of experiments is indicated in parenthesis. Other conditions were the same as described in " MATERIALS AND METHODS."

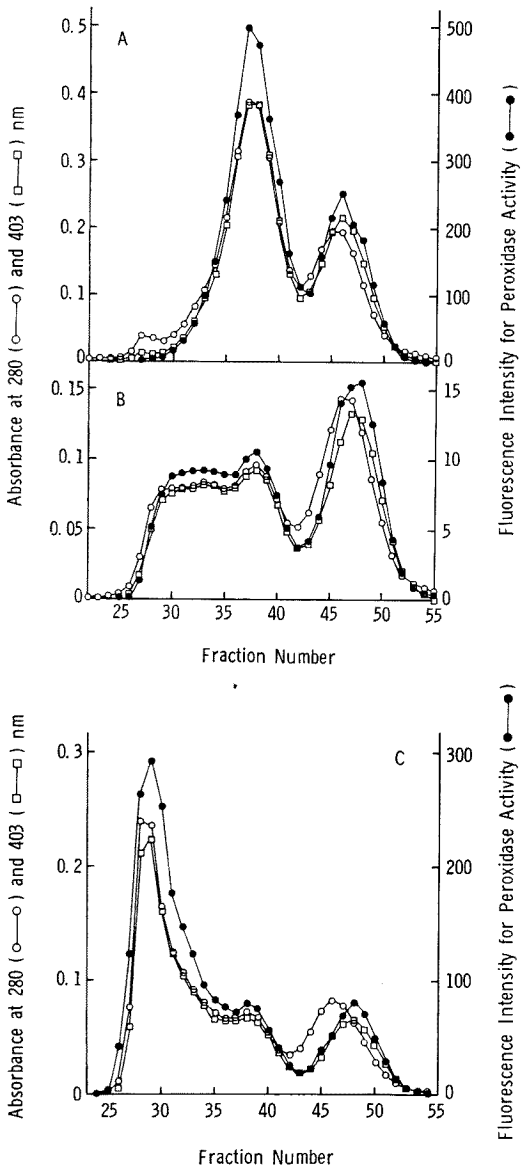


Fig. 1. Elution profiles from an Ultrogel AcA 44 column of Fab′-peroxidase conjugates obtained by the maleimide method (I) (A) and the periodate method (B and C). The concentrations of Fab′ and peroxidase incubated for conjugation were 0.1 mM in the maleimide method (I) and 0.02 mM (B) and 0.05 mM (C) in the periodate method. Peroxidase was oxidized by periodate for 10 min and subjected to gel filtration.

These differences between the two maleimide esters may be largely due to the fact that the former maleimide was more stable at neutral pH at 30°C than the latter one (*6*, *9*), and a high yield of conjugate could be expected only by using the former maleimide ester, which was therefore used in the subsequent experiments, unless otherwise specified.

*Separation of Conjugates from Unreacted Fab′ and Peroxidase*—Conjugates formed were efficiently separated from unreacted Fab′ and peroxidase by gel filtration with Ultrogel AcA 44 (Fig. 1), while separation with Sephacryl S-200 (*9*) or Sephadex G-150 (unpublished) was less efficient.

*Purity of Conjugates*—The presence of peroxidase (probably dimer) not associated with Fab′ and antibodies (probably $F(ab')_2$) not associated with peroxidase in the conjugate preparation was examined.

When normal rabbit Fab′-peroxidase conjugate prepared by the maleimide method (I) was passed through normal goat IgG- and goat (anti-rabbit IgG) IgG-Sepharose 4B columns, 96 and 1.4%, respectively, of peroxidase activity applied were detected in the effluent, indicating that 98% of peroxidase in the conjugate preparation was associated with rabbit Fab′. By contrast, when monomeric and highly polymerized conjugates prepared by the periodate method were applied, 10–16 and 0.5–0.7%, respectively, were found in the effluent (Table II).

When normal rabbit fluorescein-labeled Fab′-peroxidase conjugate prepared by the maleimide method (I) was passed through a concanavalin A-Sepharose 4B column, 9.5% of the fluorescence intensity applied was found in the effluent (Table II), suggesting that Fab′ was partly reoxidized to $F(ab')_2$ during the conjugation reaction. Peroxidase activity bound to the column could be eluted by 0.2 M α-methylmannoside and the eluted activity (73% of the applied) was almost completely (98%) bound to an anti-rabbit IgG IgG-Sepharose 4B column. Thus, it was possible to separate the conjugate from unconjugated antibodies.

*Recovery of Fab′ and Peroxidase*—The overall recovery of peroxidase after the introduction of maleimide groups, gel filtration and concentration was about 89–91%, and that of Fab′ after the

TABLE II. Binding of rabbit Fab′-peroxidase conjugate to goat anti-rabbit IgG IgG- and concanavalin A-Sepharose 4B columns.

| Conjugate | Peroxidase activity[a] in the effluent from | | Fluorescence intensity in the effluent[b] from concanavalin A column | |
|---|---|---|---|---|
| | Anti-rabbit IgG-column | Normal goat IgG-column | Fluorescein-labeled Fab′-peroxidase conjugate | Fluorescein-labeled $F(ab')_2$ |
| | % | | % | |
| Maleimide conjugate (I) | 1.4 | 96 | 9.5 | 95 |
| Periodate conjugate | | | | |
| Monomeric | 10, 16 | 99 | — | — |
| Highly polymerized | 0.5, 0.7 | 96 | — | — |

[a] Peroxidase activity of normal rabbit Fab′-peroxidase conjugate in the effluent was expressed as a percentage of the activity applied. [b] Fluorescence intensity in the effluent was expressed as a percentage of the fluorescence intensity of fluorescein-labeled Fab′-peroxidase conjugate or $F(ab')_2$ applied.

reduction, gel filtration and concentration was about 93–99%.

The recovery in the conjugate of peroxidase incubated for conjugation was calculated from the absorbance at 403 nm in the elution profile (Fig. 1), and a typical time course of the conjugation reaction is shown in Fig. 2. The recovery of peroxidase in the maleimide method (I) increased rapidly up to about 60% during the first 5 h incubation, and the subsequent 5–15 h incubation resulted in a recovery of 65–74% ($n=5$). The recovery of Fab′ in the conjugate must have been similar, since the molar ratio of peroxidase to Fab′ was one in the reaction mixture for the conjugation and was also approximately one in the conjugate as described above. In the maleimide method (II), the recoveries were lower than 50%.

In the periodate method, 57–76% ($n=4$) of Fab′ and 58–77% ($n=4$) of peroxidase used were recovered in conjugates, and their recoveries in monomeric conjugate ranged from 22 to 29% ($n=4$). When Fab′ and oxidized peroxidase were incubated at higher concentrations for conjugation, their recoveries in conjugates were significantly enhanced but the formation of monomeric conjugate was decreased, that is, the formation of highly polymerized conjugates was increased (Fig. 1C), and the same tendency was observed on prolonged oxidation of peroxidase.

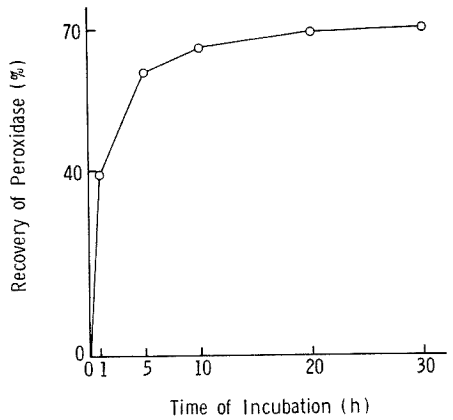


Fig. 2. Time course of the conjugation reaction in the maleimide method (I). The recovery of peroxidase in the conjugate was calculated from the absorbance at 403 nm in the elution perofile from an Ultrogel AcA 44 column.

*Size of Conjugate Molecules*—Molecular weights of conjugates were assessed by gel filtration with Ultrogel AcA 44 using five different proteins including peroxidase, fluorescein-labeled Fab′ bovine serum albumin, fluorescein-labeled $F(ab')_2$ and alkaline phosphatase (Fig. 3). (The molecular weights of these proteins were taken to be 40,000 (*21*), 46,000 (*18*, *19*), 66,200 (*22*), 92,000 and 100,000 (*23*), respectively.) The molecular

weight of Fab′-peroxidase conjugate in peak fraction 38 prepared by the maleimide method (I) (Fig. 1A) was assessed to be between 80,000 and 90,000. This indicated that the conjugate was largely monomeric, being formed mainly by combining one molecule each of Fab′ and peroxidase. This was consistent with the facts that the average numbers of thiol groups in Fab′ (see "MATERIALS AND METHODS") and maleimide groups introduced into peroxidase (see "RESULTS") were approximately one per molecule and that the molar ratio of peroxidase to Fab′ in the conjugate in the peak fraction prepared by the maleimide method (I) was calculated to be approximately one as

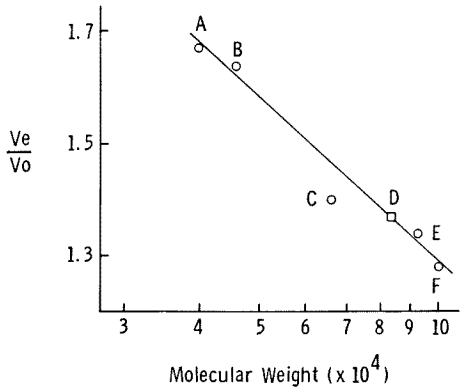


Fig. 3. Assessment of the molecular weight of Fab′-peroxidase conjugate prepared by the maleimide method (I). Peroxidase (A), fluorescein-labeled Fab′ (B), bovine serum albumin (C), rabbit Fab′-peroxidase conjugate (fraction 38) (D), fluorescein-labeled $F(ab')_2$ (E), and alkaline phosphatase (F) were subjected to gel filtration using Ultrogel AcA 44. The volume of each fraction was 0.86 ml. $V_e$: elution volume. $V_o$: void volume.

described previously (*9*).

By contrast, in the periodate method, conjugates were polymerized to various extents and their molecular weights were widely distributed (Fig. 1, B and C). When peroxidase was oxidized by periodate for 20 min and/or conjugated at a concentration of 50 mM, part of the conjugates formed was eluted in the same fraction as blue dextran (Fig. 1C).

*Peroxidase Activity in Conjugates*—The specific activity of peroxidase in the conjugates obtained by the maleimide method (I) was 99–100% of the original level on the basis of absorbance at 403 nm. By contrast, peroxidase activity tended to decrease slightly (89–97%) in the periodate method. When peroxidase was oxidized for 20 min and dialyzed without addition of ethylene glycol (*13*), peroxidase activity was lower: 29–64% in less polymerized conjugates and 68–76% in highly polymerized conjugates.

*Antigen-Binding Activity of Fab′ in Conjugates*—Antigen-binding activity of Fab′ in conjugates was examined using affinity-purified anti-human IgG Fab′-peroxidase conjugate, since only 10–11% of anti-human IgG Fab′-conjugate before affinity-purification was adsorbed on a human IgG column (Table III). Affinity-purified anti-human IgG Fab′-peroxidase conjugate prepared by the maleimide method (I) was adsorbed on a human IgG column to the extent of 89–90%, while monomeric and polymeric conjugates prepared by the periodate method were 61 and 78% adsorbed, respectively (Table III). This indicated that the antigen-binding activity was better maintained in the maleimide method (I) than in the periodate method.

TABLE III. Binding of rabbit anti-human IgG Fab′-peroxidase conjugate to human IgG-Sepharose 4B.

| Affinity-purification of anti-human IgG Fab′ | Conjugate | Peroxidase activity in the effluent from | |
|---|---|---|---|
| | | Human IgG-column | Rabbit IgG-column |
| | | % | |
| No | Maleimide conjugate (I) | 89, 90 | 97 |
| Yes | Maleimide conjugate (I) | 10, 11 | 96 |
| Yes | Periodate conjugate | | |
| | Monomeric | 39 | 96 |
| | Highly polymerized | 22 | 96 |

*Stability of Cross-Link*—No release of Fab′ from Fab′-peroxidase conjugate prepared by the maleimide method (I) was detected when the conjugate was stored in 0.1 M sodium phosphate buffer, pH 6.5, containing 0.1% bovine serum albumin and 0.002% thimerosal at 4°C for 6 months.

*Nonspecific Binding of Various Forms of Peroxidase*—Nonspecific bindings of conjugates to antibody-coated solid phases limits the sensitivity of sandwich enzyme immunoassay for macromolecular antigens. Therefore, nonspecific bindings of anti-ferritin Fab′-peroxidase conjugates to anti-ferritin IgG-coated polystyrene balls were examined (Table IV). Nonspecific bindings of conjugates were very much higher (0.010–0.165%) than those of free peroxidase (intact or oxidized-reduced) (0.001%), and the maleimide conjugate (I) showed much lower nonspecific binding than the periodate conjugates. No difference in nonspecific binding of the periodate conjugates was observed whether thiol groups of Fab′ were blocked by *N*-ethylmaleimide or monoiodoacetate. Nonspecific bindings of normal rabbit Fab′-peroxidase conjugates to normal rabbit IgG-coated polystyrene balls were similar, indicating that these results with anti-ferritin conjugates were not inherent to anti-ferritin but were generally valid.

*Usefulness of Conjugates in Enzyme Immunoassay*—The usefulness of the conjugates was tested in sandwich enzyme immunoassay for human ferritin (Fig. 4A) and human TSH (Fig. 4B).

TABLE IV. Nonspecific binding of various forms of peroxidase to anti-ferritin IgG-coated polystyrene balls.

| Form of peroxidase added | Peroxidase activity bound (Percentage of the activity added per tube) |
|---|---|
| Free | |
| Intact | 0.000–0.001 |
| Oxidized-reduced[a] | 0.000–0.001 |
| Maleimide conjugate (I)[b] | 0.010–0.012 |
| Periodate conjugate[b] | |
| Monomeric | 0.058–0.092 |
| Highly polymerized | 0.135–0.165 |

[a] Peroxidase was oxidized and reduced in the same way as in the periodate method but not conjugated with Fab′.
[b] The conjugates used were anti-ferritin Fab′-peroxidase.

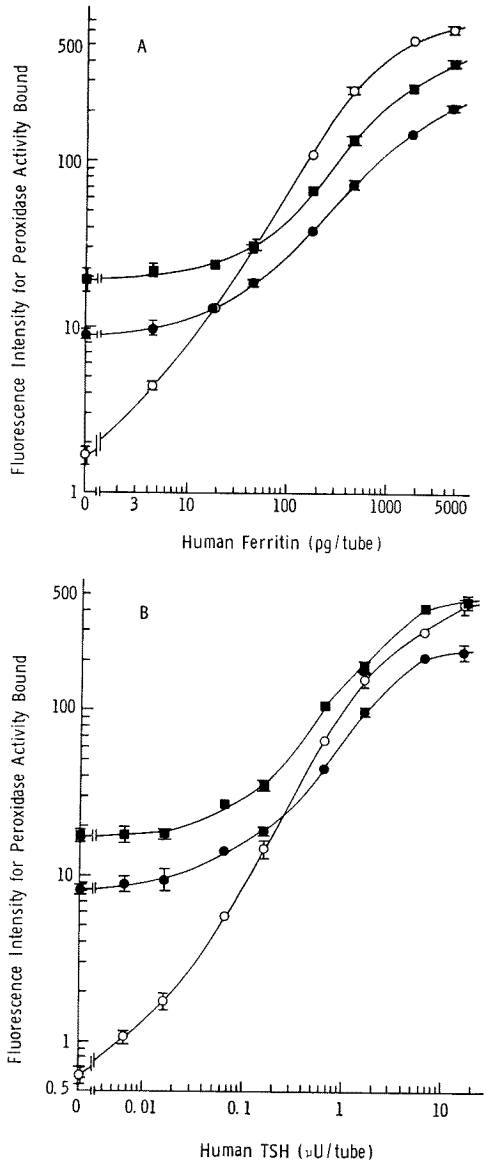


Fig. 4. Sandwich enzyme immunoassay of human ferritin (A) and human TSH (B). Peroxidase activity bound in the absence and presence of 6 and 16 nU of TSH was determined by assay for 60 min. Open circles indicate assays with the conjugates prepared by the maleimide method (I). Closed circles and squares indicate assays with monomeric and highly polymerized conjugates, respectively, prepared by the periodate method. Vertical bars indicate standard deviations ($n=5$ for each point).

Background, that is, peroxidase activity of Fab′-peroxidase conjugates nonspecifically bound in the absence of antigens was 5.6 (ferritin) or 13 (TSH) fold higher with monomeric conjugates and 12 (ferritin) or 28 (TSH) fold higher with highly polymerized conjugates prepared by the periodate method than with conjugates prepared by the maleimide method (I). Peroxidase activity specifically bound in the presence of antigens was higher with conjugates prepared by the maleimide method, except that it was slightly higher with polymerized anti-TSH conjugate prepared by the periodate method, but lower with monomeric conjugates prepared by the periodate method. As a result, more sensitive sandwich enzyme immunoassays were possible with conjugates prepared by the maleimide method (I).

Conjugates prepared by the periodate method using monoiodoacetate-blocked Fab′ gave essentially the same results with higher backgrounds, although specific binding was slightly decreased.

*Conjugation with Fab′ from Guinea Pig, Sheep, and Goat*—Similar monomeric conjugates were also obtained with Fab′ from guinea pig, sheep, and goat, although their characteristics were not examined in detail.

## DISCUSSION

For the past decade, antibody-horseradish peroxidase conjugates have been very widely used as an analytical tool in both immunohistochemistry and enzyme immunoassay, and there are two frequently used methods available to prepare those conjugates (*8*). One uses glutaraldehyde. A one-step method with glutaraldehyde is simple but gives polymerized conjugates by random conjugation (*24*, *25*). A two-step method with glutaraldehyde can provide monomeric conjugates but suffers from a very low recovery (less than 10%) of peroxidase in the conjugate (*24*, *26*, *27*). The other method that has been widely used for the past few years involves the oxidation of peroxidase with periodate, providing conjugates in high yields, but polymeric conjugates are inevitably formed with increasing recoveries (*13*, *26*, *27*). Therefore, no method has been available for preparing monomeric conjugates in high yields, and the maleimide method (I) is the first and only method that can offer monomeric conjugates of Fab′ and peroxidase in high yields.

There are at least two reasons why a highly efficient and reproducible method to provide monomeric conjugates of Fab′ and peroxidase could be developed using the maleimide compound. One is the fact that the number of maleimide groups introduced per peroxidase molecule by treatment even with an excess of the reagent was approximately one. This was highly reproducible and could be due to the characteristic of horseradish peroxidase that there seems to be approximately one amino group per individual peroxidase molecule which is reactive with the *N*-hydroxysuccinimide ester and allows the one maleimide group introduced to react with thiol groups of Fab′. This amino group may not be useful in the glutaraldehyde method, since the very low coupling efficiency in the glutaraldehyde method is well documented as described above. The second reason is the use of one thiol group in individual Fab′ molecules which can be readily generated in the hinge of $F(ab')_2$ by reduction with 2-mercaptoethylamine. In general, protein conjugates which are formed by introducing maleimide and thiol groups using amino groups present in proteins are more widely distributed on gel filtration than Fab′-peroxidase conjugate prepared by the maleimide method, even when the average number of maleimide and thiol groups introduced per protein molecule is one. In our experience, the average number of maleimide and thiol groups introduced per protein molecule should be more than one to conjugate them in high yields, though this is accompanied by heavier polymerization and a wider distribution of conjugates. Therefore, one thiol group of Fab′ also appeared unique in its reactivity with the maleimide-peroxidase to give the monomeric conjugate in a high yield.

The maleimide method (I) has the following advantages over the periodate method or/and glutaraldehyde method. 1) Peroxidase activity was almost fully maintained in the conjugate prepared by the maleimide method, while it was lowered to various extents in the periodate method (see "RESULTS"). It was also reported to be lowered more or less in both the periodate and glutaraldehyde methods (*25*, *27–29*). 2) Nonspecific bindings to the solid phase of the conjugates prepared by the maleimide method (I) were lower than those of monomeric conjugates prepared by the perio-

date method (Fig. 4, A and B, and Table IV) and were slightly lower than those of monomeric conjugates prepared by the glutaraldehyde method (to be published elsewhere). The periodate method inevitably produced polymerized conjugates, and their nonspecific bindings were higher than that of monomeric conjugates (Fig. 4, A and B, and Table IV). This is consistent with the previous reports that polymeric peroxidase conjugates give a high background in tissue staining (*13*) and that the background in sandwich enzyme immunoassay is higher with polymeric β-D-galactosidase conjugates (*11*). 3) Specific bindings of the maleimide conjugates were higher than those of monomeric conjugates prepared by the periodate method (Fig. 4, A and B) and much higher than those of monomeric conjugates prepared by the glutaraldehyde method (to be published elsewhere). This indicates that antibody activity was more or less impaired by the periodate and glutaraldehyde methods, and is consistent with the previous reports that antibody activity is impaired to various extents in both the glutaraldehyde and periodate methods (*25, 27–32*). In the maleimide method (1), thiol groups of Fab′, that is, thiol groups in the hinge of IgG molecules are used for conjugation, and the hinge is remote from the antigen-binding site of IgG molecules. Therefore, antibody activity may be fully retained in the conjugate prepared by the maleimide method. In this connection, it should be noted that antibody activity of Fab′ conjugated with β-D-galactosidase (*12*) and glucose oxidase (*7*) using its thiol groups is well retained in the conjugate.

The sandwich enzyme immunoassay for ferritin with the maleimide conjugate described above is 10–100 fold more sensitive than most of the previously reported sandwich enzyme immunoassays (*10, 33–37*) and as sensitive as the best of them (*38, 39*). The sensitivity for TSH with the maleimide conjugate described above is 5–333 fold greater than those previously reported (*40–44*).

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