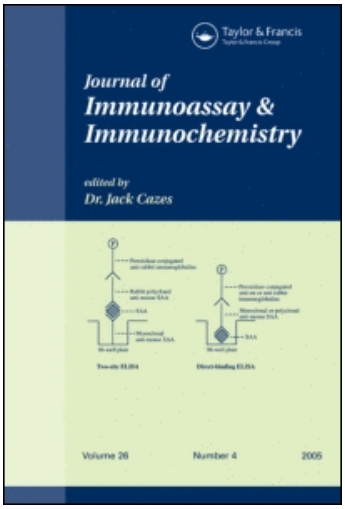

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Enzyme-Labeling of Antibodies and Their Fragments for Enzyme Immunoassay and Immunohistochemical Staining

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ENZYME-LABELING OF ANTIBODIES AND THEIR FRAGMENTS FOR ENZYME IMMUNOASSAY AND IMMUNOHISTOCHEMICAL STAINING

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I. INTRODUCTION

The use of an enzyme as a label has a number of advantages over the use of other labels in both immunohistochemistry and immunoassay. Immunofluorescence techniques are not suitable for ultrastructural research on cells, and ferritin-labeled antibodies allow only electronmicroscopic studies. By contrast, enzyme-labeled antibodies permit localization of cellular antigens in relation to tissue structures under light microscope and also demonstration of cellular antigens at an ultrastructural level by electronmicroscopy. Antibody or antibody fragments labeled with enzymes of small molecular weight can more readily permeate cells of tissue sections than ferritin-labeled antibodies. The color of tissue sections prepared by immunoenzymatic techniques is stable for years, while immunofluorescence of tissue sections decreases rapidly when exposed to light. Radioisotope-labeled reagents decay with time; there are health hazards due to radio-

isotopes; and disposal of radioactive waste is becoming increasingly difficult. By contrast, enzyme-labeled antigens and antibodies are stable for months or even years and there are no problems either of health hazards or of waste disposal with appropriate choice of enzymes and substrates. Under favourable conditions, enzyme immunoassays may be even more sensitive than radioimmunoassays (1). Enzyme-labeled antigens and antibodies have found increasing use during the past decade, reinforced by improvements in enzyme-labeling methods.

The existing reviews of enzyme-labeling methods (2-5) do not include recent developments. The present work reviews available methods for enzyme-labeling in which enzymes are cross-linked with antibodies or antibody fragments through covalent bonds and describes more recent and useful methods in detail.

II. DEVELOPMENT OF LABELING METHODS

Before development of the glutaraldehyde method

Before the glutaraldehyde method was developed, several cross-linking reagents were tested for their ability to conjugate enzymes with antibodies. These included cyanuric chloride, 4,4'-difluoro-3,3'-dinitro-diphenylsulfone, toluene-2,2'-diisocyanate, N,N'-dicyclohexylcarbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, and bisdiazotized o-dianisidine (3). They all had various disadvantages, and none is currently in use.

One-step glutaraldehyde method

Proteins can be readily cross-linked by reaction of glutaraldehyde under mild conditions mainly with amino groups of lysine

residues. Conjugation of enzymes with IgG by using glutaraldehyde was first reported in 1969 (6). Glutaraldehyde was added to a mixture of enzyme and human, rabbit or sheep IgG, and the reaction was allowed to proceed at pH 6.8 at room temperature for 2 h. The enzymes tested were horseradish peroxidase, glucose oxidase from Aspergillus niger, tyrosinase from mushroom and alkaline phosphatase from Escherichia coli and chicken intestine. The conjugates produced were highly polymerized and heterogeneous.

The characteristics of this method were revealed more clearly by later studies in which horseradish peroxidase was conjugated with rabbit Fab (7) and IgG from different species including sheep (7), goat (8) and rabbit (9). The IgG was largely polymerized and only a very small proportion of the available enzyme was conjugated with IgG. Although enzyme activity was fairly well retained in the conjugate, a serious loss of antibody activity was found by hemagglutination, agarose block precipitation and radial immunodiffusion.

Similar characteristics were also observed in the conjugation of IgG with alkaline phosphatase from calf intestine. Rabbit IgG and the enzyme were converted to highly polymerized conjugates, the yield in terms of antigenic activity of rabbit IgG being about 1 % (10), although 60-70 % of the enzyme activity was retained (11). When goat (anti-human IgG) IgG was conjugated with alkaline phosphatase, increasing concentrations of glutaraldehyde resulted in increasing losses of enzyme and antibody activities with low efficiency of enzyme labeling and extensive

polymerization of IgG (12). Similarly, when protein A was conjugated with alkaline phosphatase by the one-step method, its ability to bind to IgG was greatly reduced and labeling efficiency was very low (13). It was suggested that each protein was polymerized to a homopolymer because of different reactivities to glutaraldehyde and that the binding sites of IgG or protein A were buried in large aggregates with consequent loss of binding reactivity to antigens or IgG (12, 13). No improvement resulted from varying either glutaraldehyde concentration or conjugation reaction time (12, 13).

In contrast to alkaline phosphatase, lactoperoxidase was more efficiently (49 %) conjugated with goat IgG at a low concentration of glutaraldehyde without losing its enzyme activity and loss of antibody activity was only 21 % (12). This may be because of similar reactivities to glutaraldehyde of the two proteins, unlike alkaline phosphatase which is much less reactive. The goat IgG-lactoperoxidase conjugate obtained was, however, highly polymerized and heterogeneous.

IgG and ß-D-galactosidase were also mostly converted to highly polymerized conjugates in the one-step glutaraldehyde method (14). Activities both of the enzyme and of IgG were significantly impaired, and the nonspecific binding of the polymerized conjugates to solid phase was greater than with conjugates prepared by the maleimide or pyridyl disulfide methods described below.

Glucose oxidase also has been conjugated by the one-step glutaraldehyde method with rabbit IgG (15), sheep IgG (16) and

heat aggregated human and rabbit IgG (17), and ß-D-galactosidase has been conjugated with sheep (18) and rabbit (19) IgG, but the conjugates obtained were not characterized.

Two-step glutaraldehyde method

The two-step glutaraldehyde method was first reported in an incomplete form in 1971 (11). Alkaline phosphatase was allowed to react with glutaraldehyde for 1 h and then rabbit IgG was added. The antigenic sites of rabbit IgG thus conjugated retained more activity than that found with the one-step method. In a later report, alkaline phosphatase was treated with glutaraldehyde, the excess of glutaraldehyde was removed, and the glutaraldehyde-alkaline phosphatase complex was allowed to react with protein A (13). The activities of both the enzyme and protein A in the conjugate were well retained, although the enzyme was polymerized. It was suggested that protein A became conjugated to the outer surface of the enzyme polymers, thereby protecting the activity of protein A from steric hindrance. The enzyme activity which requires access of only low molecular weight substrate to the active site is likely to be much less influenced by steric hindrance.

An even more successful conjugation by the two-step glutaraldehyde method was described with horseradish peroxidase (7). The enzyme alone was not insolubilized by treatment even with excess of glutaraldehyde probably because the majority of amino groups in the enzyme molecules were blocked by allylisothiocyanate which was present in the horseradish extracts (20), and a monomeric antibody-peroxidase conjugate resulted from the two-step

method (7, 21, 22). A high proportion of peroxidase in the conjugates could be achieved by increasing the amount of peroxidase (7). The enzyme was, however, partly dimerized or even polymerized by treatment with glutaraldehyde (7, 21), the extent varying with the enzyme preparation used (7), and the molecular weight of the conjugate and its enzyme/antibody molar ratio also varied (7, 21). The peroxidase activity was reduced by 30-50 % (9, 21, 23). A considerable decrease of rabbit anti-human IgG antibody activity was also observed after conjugation with the enzyme, although less than in the one-step method (8, 21, 23, 24). The recovery of the enzyme in conjugates prepared by the two-step proceduce was as low as in the one-step method (7, 8, 21, 24).

Periodate method

The periodate method was reported in 1974 (25). The first step of this method was to completely block amino groups remaining reactive in horseradish peroxidase molecules by treatment with 1-fluoro-2,4-dinitrobenzene to minimize self-conjugation. The second step was to oxidize carbohydrate moieties of the glycoprotein enzyme with $NaIO_4$ to generate aldehyde groups. In the third step, the aldehyde groups generated were allowed to react at pH 9.5 with amino groups of IgG antibodies to form a Schiff's base. The final step was to reduce the Schiff's base using $NaBH_4$ to stabilize the cross-linkages. The reduction with $NaBH_4$ caused 18 % loss of the enzyme activity. The recovery of the enzyme in the conjugates increased with increasing degree of the oxidation and was 68 % when oxidized with 0.08 % $NaIO_4$ for 30

min. However, increase in the recovery was accompanied by increase in both the molecular weight of the conjugates formed and loss of antibody activity.

Later reports further defined the peroxidase-IgG conjugates prepared by the periodate method (21, 23). The enzyme activity in the conjugate was 30-55 % of the original activity, and a considerable loss of rabbit anti-human IgG antibody activity by conjugation was noted by hemagglutination, the loss being more than with the two-step glutaraldehyde method. The molecular weight of the conjugate was more than 400,000. In another report, 14-18 % of peroxidase activity and 30-40 % of rabbit anti-α-fetoprotein activity were obtained in the conjugate, much of the loss of antibody activity resulting from reduction with $NaBH_4$ (26). In some reports, the reduction step with $NaBH_4$ was even omitted, because it caused a considerable loss of enzyme activity (27) or antibody activity (28).

An attempt was made to minimize self-conjugation of peroxidase by lowering the pH after oxidation of the enzyme with periodate (29, 30). Only 5 % of oxidized peroxidase was self-conjugated at pH 4.4, while 35 % was self-conjugated at pH 9.5 even with prior blocking of amino groups by 1-fluoro-2,4-dinitrobenzene. The maximum amount of the enzyme that could be incorporated into 90,000 molecular weight Fab'-enzyme conjugate was 34 %. When the enzyme/antibody molar ratio was less than or more than one, conjugates showed a reduced antibody avidity. In another report, more than 95 % of rabbit IgG and about 60 % of

Fab' were conjugated with more than 95 % and 60 % of peroxidase, respectively, although conjugates obtained were largely polymeric, and the antibody and enzyme activities in the conjugates were reduced (22).

Maleimide method

Maleimide groups react under mild conditions fairly rapidly with thiol groups and very slowly with other functional groups of proteins, and various maleimide compounds have been synthesized for cross-linking proteins (31). We used one of them, N,N'-o-phenylenedimaleimide for enzyme-labeling of IgG in 1975 (32). IgG was reduced with 2-mercaptoethylamine to generate thiol groups in the hinge and treated with excess of the dimaleimide to introduce maleimide groups. Then, the maleimide-IgG was allowed to react with thiol groups in the native form of ß-D-galactosidase. Fab', which contained approximately one thiol group per molecule, was also conjugated with ß-D-galactosidase in the same way (33, 34).

This was the only method at that time in which the antibody conjugation site was predictable and was not associated with antigen-binding sites, and the conjugates obtained were characterized more precisely than other conjugates (35, 36). The dimaleimide method can be performed at pH 6.0-6.5. There was no loss of ß-D-galactosidase activity during the conjugation reaction, and the antibody activity was well retained in the conjugates. There was little formation of homopolymers of IgG or of Fab'. The recovery of ß-D-galactosidase in the conju-

gate was more than 99 % and the conjugate fractions obtained by gel filtration contained little free enzyme. The recoveries of IgG and Fab' in the conjugates were 26 and 43 %, respectively. The cross-link was stable at 4°C at pH 6-7 for at least a year.

The dimaleimide method is a general method that is applicable to the conjugation of almost every kind of peptide and protein, since thiol groups can be readily introduced into most peptides and proteins at neutral pH. It is highly reproducible, if the content of thiol and maleimide groups in the protein to be conjugated is confirmed before conjugation. However, some caution is required. Maleimide groups are not stable at neutral or higher pH, and thiol groups are not stable in the absence of EDTA (35, 36). Alkaline phosphatase loses 20-40 % of its enzyme activity after introduction of thiol groups (37) and is inactivated by EDTA, which is added to prevent the oxidation of thiol groups. α-D-Glucosidase from yeast loses 30 % of activity by incubation with the dimaleimide (37, 38) and maleimide groups have instead to be introduced into the antibody or antibody fragment to be labeled. The reduction of antibodies at higher pH than 6 increases the production of polymerized conjugates in which more than one β-D-galactosidase molecule is associated with an individual conjugate molecules.

Donkey (anti-sheep IgG) IgG conjugation with β-D-galactosidase by a modified dimaleimide method (39, 40) was performed as follows. Thiol groups were introduced into donkey (anti-sheep IgG) IgG adsorbed on sheep IgG-Sepharose by treatment with methyl

4-mercaptobutyrimidate, and the (anti-sheep IgG) IgG with thiol groups was eluted at pH 2.5, treated with excess N,N'-o-phenylenedimaleimide and conjugated with ß-D-galactosidase. Up to 80 % of the enzyme was conjugated to immunologically active antibody with 90 % retention of the enzyme activity.

Another dimaleimide, N,N'-oxydimethylenedimaleimide, was tested for the conjugation of IgG with enzymes such as peroxidase, glucose oxidase, ß-D-galactosidase and penicillinase (41). Thiol groups were introduced into both peroxidase and IgG using S-acetylmercaptosuccinic anhydride, and mercaptosuccinylated enzyme or IgG was treated with the dimaleimide to introduce maleimide groups and then allowed to react with mercaptosuccinylated IgG or enzyme. Both peroxidase and antibody activities were well retained in conjugates, in contrast to significant decreases in both activities when the periodate method was used. The recovery of peroxidase in conjugates was 44-77 %. The use of N,N'-oxydimethylenedimaleimide was claimed to be advantageous over that of N,N'-o-phenylenedimaleimide, because the former was more soluble in acetone and resulted in conjugates with lower non-specific binding. However, these findings were not confirmed in our laboratory. N,N'-o-phenylenedimaleimide was sufficiently soluble in N,N-dimethylformamide for introduction of maleimide groups. These two dimaleimides had similar stability in aqueous solutions, and the quality of conjugates obtained using them were similar. Furthermore, N,N'-o-phenylenedimaleimide is commercially available, while N,N'-oxydimethylenedimaleimide is not.

Unfortunately, maleimides are less stable at higher pH. The observation that N-ethylmaleimide is much more stable at pH 7 than N,N'-o-phenylenedimaleimide in which the maleimide group is directly attached to a benzene ring stimulated us to synthesize a more stable maleimide, N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate which is now commercially available (Zieben Chemical Co., Ltd., Tokyo and Pierce Chemical Company, Rockford, Illinois) (37, 42). Maleimide groups of this compound decompose at pH 7 at 30°C only 4 % within 2 h.

N-Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate was used to prepare IgG- and Fab'-glucose oxidase conjugates (42). Glucose oxidase was treated with the reagent to introduce maleimide groups, and the maleimide-enzyme was allowed to react with thiol groups of reduced IgG or Fab'. The recoveries of antibody and enzyme in the conjugates were more than 40 %. Anti-human IgG antibody activity was almost fully preserved, while glucose oxidase activity was reduced by 15 and 26 % during the conjugation reaction with Fab' and IgG, respectively. Although there was no self-conjugation of IgG, Fab' or glucose oxidase, the conjugates obtained were heterogeneous with a molar ratio (IgG or Fab'/glucose oxidase) of 1-3. In the same way, Fab' was conjugated with horseradish peroxidase (43-45). The conjugate obtained was largely monomeric, probably because the number of amino groups in the enzyme was very small. The yield was 65-75 %. There was no loss of the enzyme activity, and the antigen-binding activity of Fab' was better retained in the conjugate than in the glutaraldehyde and periodate methods. The use

of Fab'-peroxidase conjugate resulted in more sensitive enzyme immunoassays and more efficient immunohistochemical stainings with lower background and higher specific binding than that of conjugates prepared by the glutaraldehyde and periodate methods. Alkaline phosphatase could also be conjugated with IgG or Fab' in the same way, although the enzyme activity decreased 20-40 %.

N-Succinimidyl m-maleimidobenzoate was used to introduce maleimide groups into donkey (anti-sheep IgG) IgG, and the maleimide groups introduced were allowed to react with thiol groups of ß-D-galactosidase (39). Eighty per cent of the enzyme used was conjugated to immunologically active antibody with approximately 90 % retention of enzyme activity, and 50 % of antibody was conjugated with ß-D-galactosidase. A later paper also confirmed that both activities of ß-D-galactosidase and IgG were better retained in this method than in the one-step glutaraldehyde method (14). However, the conjugation was not definded in more detail, for example, by estimating the number of maleimide groups introduced and IgG molecules conjugated.

A drawback in the use of N-succinimidyl m-maleimidobenzoate was demonstrated when it was used for the preparation of Fab'-horseradish peroxidase conjugate in place of N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (45). When maleimide groups were introduced into the enzyme at pH 7.0, the average number of maleimide groups introduced per enzyme molecule was about 0.6 with the former and 1.0-1.2 with the latter, and the recovery of the enzyme in the conjugate less than 50 % and 65-75 %

respectively. Maleimide groups introduced using the former are much more labile at pH 7 than those introduced using the latter (37, 43) and might have been decomposed in part. The introduction of maleimide groups at pH 6.5 was even less efficient with both reagents. This is apparently because N-hydroxysuccinimide ester is less reactive with amino groups at lower pH.

Pyridyl disulfide method

Pyridyl disulfide derivatives were utilized for enzyme-labeling by two groups in 1978. One group used N-succinimidyl 3-(2-pyridyldithio)propionate as a cross-linking reagent (46). 2-Pyridyl disulfide groups were introduced into both peroxidase and rabbit anti-human transferrin IgG by treatment with this reagent. The 2-pyridyl disulfide groups introduced into IgG were reduced by dithiothreitol to generate thiol groups and the two proteins were conjugated by a disulfide interchange under mild conditions. The peroxidase activity was well retained with 45 % recovery in the conjugate.

The other group used methyl 3-(4-dithiopyridyl)propionimidate or a combination of imidates and 4,4'-dithiodipyridine (47, 48). Pyridyl disulfide groups were introduced into the first protein to be conjugated by amidination with methyl 3-(4-dithiopyridyl)propionimidate or with either methyl 3-mercaptopropionimidate, methyl 4-mercaptobutyrimidate or 2-iminothiolane in the presence of 4,4'-dithiodipyridine. The first protein with pyridyl disulfide groups was then allowed to react with the second protein which had been thiolated. The specific activity of

peroxidase in sheep (anti-human IgE) IgG-peroxidase conjugate was 74 % of the original activity, and antibody activity was also well retained.

Other methods

The conjugation of sheep IgG with various enzymes such as peroxidase, alkaline phosphatase, glucose oxidase and ß-D-galactosidase by using p-benzoquinone was reported in 1976 (49, 50). p-Benzoquinone reacts with functional groups of proteins such as amino and thiol groups at pH 6, and p-benzoquinone-treated protein becomes reactive with functional groups of proteins at pH 8, although not reactive at pH 6. Therefore, a two-step conjugation is possible using this reagent. Activities of enzymes and antibodies show little or no modification by treatment with p-benzoquinone, but 20-50 % of enzyme activities and about 40 % of antibody activities are lost after conjugation. Peroxidase and antibody are recovered 15 and 60 %, respectively, in the conjugate. Although no enzyme polymers are present after treatment with p-benzoquinone, conjugates obtained are heterogenous. However, adequate conditions yield a monomeric antibody-peroxidase conjugate.

Iodoacetic acid or iodoacetamide is well known to react readily with thiol groups and form a thioether, and the use of N-succinimidyl iodoacetate was reported for the conjugation of ovalbumin and IgG from dog and mouse in 1978 (51). This method may also be useful if thiol groups in the hinge of Fab' are used for the conjugation.

Comparison of IgG and its fragments for enzyme-labeling

Nonspecific binding of enzyme-labeled IgG to solid materials is higher than that of enzyme-labeled Fab', although specific bindings of both conjugates are similar (35, 52, 53). Monomeric Fab' conjugates more readily penetrate into cells of tissue sections (5, 30). Therefore, Fab' conjugates are more useful in performing both highly sensitive sandwich enzyme immunoassays for macromolecular antigens and immunohistochemical staining of tissue sections.

Summary

The glutaraldehyde method has some advantages. Glutaraldehyde is inexpensive, and the procedures used are very simple and reproducible. The two-step method with glutaraldehyde provides a monomeric conjugate of horseradish peroxidase and antibodies or antibody fragments. The cross-link formed is stable, although its exact structure is unknown. However, these advantages appear to be outweighed by the following factors. Enzymes and antibodies or antibody fragments are polymerized, and antibody activity is significantly impaired by the conjugation. Polymerization is a serious problem, since it increases nonspecific binding of enzyme-labeled conjugates both in immunohistochemical staining and in quantitative enzyme immunoassay, and limits penetration of conjugates into cells of tissue sections. In addition, only a small proportion of the horseradish peroxidase used is converted to conjugates.

The periodate method provides antibody-horseradish peroxidase conjugates in high yield, a remarkable advance compared with the

glutaraldehyde method. The cross-link formed is very stable, since the Schiff's base formed between the oxidized peroxidase and antibodies or antibody fragments is reduced. This method is, however, applicable only when the enzymes contain carbohydrate moieties which are not involved in enzyme activity, and the enhanced yield of antibody-horseradish peroxidase conjugate is accompanied by appreciable polymerization.

The maleimide method has several advantages. The procedures used are mild and reproducible. Both enzymes (horseradish peroxidase and β-D-galactosidase from Escherichia coli) and antibodies or antibody fragments lose little activity by conjugation. The yield of conjugates is satisfacotrily high. There is no self-conjugation of enzymes, antibodies or antibody fragments. Fab' has approximately one thiol group per molecule in the hinge, remote from the antigen-binding site. It can be conjugated with enzymes through the hinge by the maleimide method. Monomeric Fab'-horseradish peroxidase conjugate thus offers more efficient staining of tissue sections and higher sensitivity of enzyme immunoassay than that of the corresponding conjugates prepared using amino groups of Fab' in the glutaraldehyde and periodate methods, suggesting that the use of thiol groups in the hinge of Fab' may also be advantageous in conjugation with other enzymes. The use of Fab'-β-D-galactosidase (1, 54-56) and Fab'-peroxidase (57) conjugates resulted in attomole level sandwich enzyme immunoassays for clinically important antigens such as thyroglobulin (54), IgE (55), ferritin (1), thyroid-stimulating

hormone (56) and growth hormone (57). Of these assays, the human ferritin assay was demonstrated to be more sensitive than the corresponding radioimmunoassay (1). A disadvantage of the maleimide method is that it is not applicable to conjugation with enzymes in which thiol groups are essential for the enzyme activity.

The pyridyl disulfide method has almost the same advantages as the maleimide method, and in addition the cross-link formed can easily be split by reduction. However, the yield of conjugates is lower in the pyridyl disulfide than in the maleimide method.

III. PREPARATION OF IgG AND ITS FRAGMENTS

A. Preparation of IgG (58, 59)

1. Add slowly 0.18 g of Na_2SO_4 to 1 ml of serum with stirring, and continue stirring at 22-25°C for 30 min after a complete dissolution.
2. Centrifuge the mixture at 10,000 rpm for 10 min at 22-25°C.
3. Dissolve the precipitate with 1 ml of sodium phosphate buffer, pH 6.3, 17.5 mmol/L and dialyse the solution against the same buffer.
4. Apply the supernatant to a DEAE cellulose column equilibrated with sodium phosphate buffer, pH 6.3, 17.5 mmol/L. The wet volume of DEAE cellulose for processing 10 mg of protein in the supernatant is 1 ml.
5. Calculate the amount of IgG from its absorbance at 280 nm by taking the extinction coefficient at 280 nm and molecular

weight of IgG to be 1.5 $g^{-1}.L.cm^{-1}$ (60) and 150,000 (61), respectively.

B. Preparation of Reduced IgG

IgG can be reduced in the same way as $F(ab')_2$ (III-D). The average number of thiol groups in the reduced IgG is 2.0-3.2 per molecule. Thiol groups generated in the hinge of IgG molecules are fairly stable in the presence of EDTA, although much less stable in the absence of EDTA than those of Fab' (53).

C. Preparation of $F(ab')_2$ (62)

1. Dialyse 10-20 mg of IgG in 1 ml against sodium acetate buffer, pH 4.5, 0.1 mol/L, at 5°C.
2. Add 0.05 ml (1/20 volume) of 2 mol/L NaCl to the dialysed IgG solution.
3. Dissolve pepsin from porcine gastric mucosa (0.2 mg/10 mg of IgG) in the dialysed IgG solution.
4. Incubate the mixture at 37°C for 15-24 h. The duration of incubation depends upon the preparation of pepsin and the species of animal from which the IgG was obtained.
5. Adjust the pH of the digested IgG solution to 8 using 1 mol/L NaOH.
6. Apply digested rabbit IgG solution at pH 8.0 to a Sephadex G-150 column (1.5 x 45 cm for 1.0-1.5 ml and 2.0 x 45 cm for 2.0-2.5 ml) using sodium borate buffer, pH 8.0, 0.1 mol/L. Ultrogel AcA 44 provides a sharper separation of $F(ab')_2$ than

does Sephadex G-150. The $F(ab')_2$ fragment from goat, sheep, guinea pig and probably other animals except for rabbit should be subjected to gel filtration with Ultrogel AcA 44 at pH 6-7 rather than pH 8.0, since its elution is retarded at pH 8. Washing of columns with a buffer containing bovine serum albumin before gel filtration helps to increase the recovery of $F(ab')_2$, when it is available only in small quantity.

7. Calculate the amount of $F(ab')_2$ from its absorbance at 280 nm by taking its extinction coefficient at 280 nm and molecular weight to be 1.48 $g^{-1}.L.cm^{-1}$ (63) and 92,000 (64, 65), respectively.

D. Preparation of Fab' (62)

1. Prepare 0.1-3 mg of $F(ab')_2$ in 0.45 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L.
2. Add to the $F(ab')_2$ solution above 0.05 ml (1/9 volume) of 0.1 mol/L 2-mercaptoethylamine in sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 5 mmol/L EDTA freshly prepared.
3. Incubate the mixture at 37°C for 1.5 h.
4. Apply the reaction mixture to a Sephadex G-25 column (1 x 30 cm) using sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 5 mmol/L EDTA.
5. Calculate the amount of Fab' from absorbance at 280 nm by taking the extinction coefficient at 280 nm and molecular weight of Fab' to be 1.48 $g^{-1}.L.cm^{-1}$ (63) and 46,000 (64, 65), respectively.
6. Measure the content of thiol groups in Fab'.

Measurement of thiol groups in Fab' (35, 36, 66)

1. Adjust the concentration of Fab' in a total volume of 0.5 ml sodium phosphate buffer, pH 6.0, 0.1 mol/L to give an absorbance at 280 nm of 0.2-1.0 (0.13-0.67 g/L, 2.9-14 μmol/L).
2. Add 0.02 ml of 5 mmol/L 4,4'-dithiodipyridine to this 0.5 ml Fab' solution.
3. Incubate the mixture at room temperature for 10-20 min.
4. Read absorbance at 324 nm.
5. Calculate the average number of thiol groups per Fab' molecule using the molar extinction coefficient at 324 nm of pyridine-4-thione which is 19,800 $M^{-1}.cm^{-1}$ (66), the extinction coefficient at 280 nm of Fab' which is 1.48 $g^{-1}.L.cm^{-1}$ (63) and the molecular weight of Fab' which is 46,000 (64, 65). The average number of thiol groups per Fab' molecule is 1.0 with a range of 0.97-1.1 (n=5) (36). The content of thiol groups does not significantly decrease in the presence of EDTA at 23°C for at least 4 h, while it decreases slightly in the absence of EDTA (36). However, caution is required, since thiol groups at lower concentrations are oxidized more rapidly.

A complete split of disulfide bond in the hinge of $F(ab')_2$ should be confirmed by subjecting Fab' to gel filtration with Ultrogel AcA 44 after blocking thiol groups (III-E). In rabbit $F(ab')_2$ thus prepared, there is little protein that can not be split. $F(ab')_2$ prepared from goat and guinea pig usually contains proteins (5-10 %) resistant to the reductive split.

$F(ab')_2$ from rat and mouse contains more and should be subjected to gel filtration with Ultrogel AcA 44 after reduction to separate Fab' from other proteins.

E. Preparation of SH-Blocked Fab'.

1. Reduce $F(ab')_2$ by incubation with 2-mercaptoethylamine at 37°C for 1.5 h as described above (III-D).
2. Add 0.1 ml of 0.1 mol/L N-ethylmaleimide, and incubate the mixture at 30°C for 20 min. (Or add 0.09 ml of 70 mmol/L sodium monoiodoacetate in Tris-HCl buffer, pH 8.2, 2 mol/L, and incubate at 4-6°C for 16 h (30).)
3. Apply the reaction mixture to a Ultrogel AcA 44 column (1.0 x 45 cm) using 0.15 mol/L NaCl.

IV. LABELING WITH HORSERADISH PEROXIDASE

A. Assay of Peroxidase

For the assay of peroxidase activity, there are three major methods: colorimetric, fluorimetric and luminescent. The fluorimetric assay with p-hydroxyphenylpropionic acid (67) has several advantages over the colorimetric assay with o-phenylenediamine or 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid-6) (ABTS). The fluorimetric assay is more sensitive than the colorimetric assay. Both background (reagent blank) and fluorescence generated by the enzyme reaction are stable at room temperature for at least 4 h. In the colorimetric assay, background increases with incubation time for the assay, although

color developed by the enzyme reaction is stabilized by the presence of reducing reagents such as Na_2SO_3. The substrate solution in the fluorimetric assay can be stored at -20°C for a long period of time, but it has to be freshly prepared in the colorimetric assay. Luminescent assay with luminol is less sensitive than the fluorimetric assay, and Pholas dactylus luciferin which can provide highly sensitive femtogram level assay (68) is not commercially available. In our laboratory, therefore, the fluorimetric assay with p-hydroxyphenylpropionic acid is used in preference to colorimetric and luminescent assays.

Fluorimetric assay with p-hydroxyphenylpropionic acid

Horseradish peroxidase in solution has a higher activity in the presence of bovine serum albumin, but the background is increased. Therefore, albumin concentration should not exceed 10 mg/L in the reaction mixture. Fab'-peroxidase conjugates bound to solid phase show the same activity in the presence and absence of bovine serum albumin.

Commercially available preparations of p-hydroxyphenylpropionic acid give rather high backgrounds and those preparations have to be purified for sensitive assays. An adequate purification can lower the background to a relative fluorescence intensity of 1-2 when fluorescence intensity of 1 mg/L of quinine in 0.1 N H_2SO_4 is adjusted to a scale of 100. The sensitivities in 10 and 100 min assays with such a low background are 0.2 and 0.02 pg (5 and 0.5 amol) of peroxidase, respectively. The assay with p-hydroxyphenylacetic acid is slightly less sensitive.

1. Dissolve 0.25 g of p-hydroxyphenylpropionic acid in 50 ml of 0.1 mol/L sodium phosphate buffer, pH 8.0 to give a concentration of 5 g/L. The pH of the solution becomes 7.0 after dissolving the substrate.
2. Add 0.01 ml of the enzyme in phosphate buffer, pH 7.0, 10 mmol/L containing 250 mg/L bovine serum albumin and 100 mmol/L NaCl, or an enzyme-bound polystyrene ball washed with the same buffer, to 0.25 ml of the substrate solution.
3. Incubate the mixture at 30°C for 5 min.
4. Start the enzyme reaction by adding 0.05 ml of 0.03 % H_2O_2 and continue the incubation at 30°C for 10-60 min in most experiments. (Dilute 30 % H_2O_2 with deionized water before use and find the concentration which gives the maximal activity. Store 30 % H_2O_2 in the cold.)
5. Stop the enzyme reaction by adding 2.5 ml of glycine-NaOH buffer, pH 10.3, 0.1 mol/L.
6. Measure fluorescence intensity using 320 nm for excitation and 405 nm for emission. Use 1 mg/L of quinine in 0.1 N H_2SO_4 as a standard.

B. Maleimide Method (I) for Labeling Fab' with Peroxidase (43-45)

The maleimide method (I) uses a stable maleimide compound, N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Zieben Chemical Co., Ltd., Tokyo or Pierce Chemical Company, Rockford, Ill.) (Fig. IV-1). In the first step, horseradish peroxidase is treated with the maleimide compound to introduce

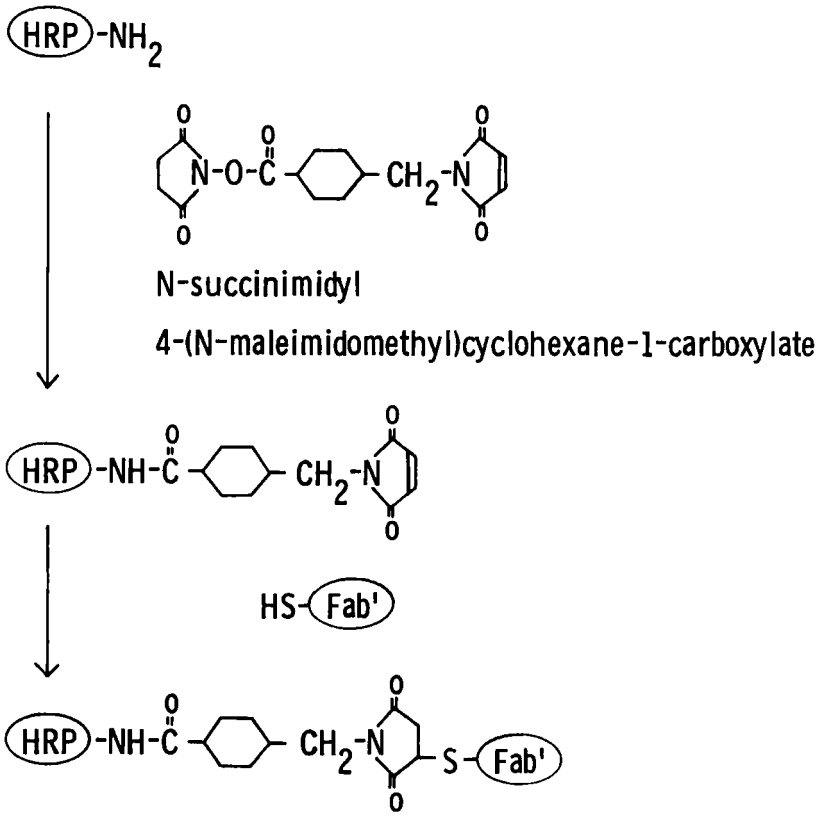


FIGURE IV-1. Preparation of Fab'peroxidase conjugate by the maleimide method (I). HRP = horseradish peroxidase.

maleimide groups. In the second step, the maleimide-peroxidase is allowed to react with thiol groups in the hinge of Fab' followed by gel filtration with Ultrogel AcA 44 to separate the conjugate formed from unconjugated peroxidase and Fab'.

Preparations of peroxidase from Boehringer, Sigma and Toyobo were all suitable for this method. The maleimide compound used in this method is not readily soluble in buffers and has to be

dissolved first in organic solvents such as N,N-dimethylformamide followed by addition to the peroxidase solution. N-Hydroxysuccinimide ester reacts with amino groups of proteins more readily at higher pH and temperature (Table IV-1), but maleimide groups are stable at lower pH and temperature (37, 52, 53). Therefore, the pH of the reaction mixture should not be higher than 7 during the introduction of maleimide groups and should be lowered to 6 following the reaction. The average number of maleimide groups introduced into peroxidase at pH 7.0 at 30°C reaches a maximum of 1.6-1.7 per molecule within 1 h, while at pH 6.5 at 30°C it increases gradually up to 0.92 per molecule within 2 h (Table IV-1). The reaction between thiol and maleimide groups is little affected by pH between 5 and 7. It is helpful to monitor labeling efficiency by adding a small amount of fluorescein-labeled $F(ab')_2$ as a tracer to the $F(ab')_2$ to be labeled, although the same result can be achieved in alternative ways as described below.

Introduction of maleimide groups into peroxidase

1. Dissolve 2 mg (50 nmol) of horseradish peroxidase in 0.3 ml of sodium phosphate buffer, pH 7.0, 0.1 mol/L. Calculate the amount of the enzyme from the absorbance at 403 nm by taking the extinction coefficient at 403 nm and the molecular weight of the enzyme to be 2.275 $g^{-1}.L.cm^{-1}$ and 40,000, respectively (69).
2. Dissolve 0.8-1.6 mg (2400-4800 nmol) of N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (m.w. 334.33) (Zieben

TABLE IV-1

Number of Maleimide Groups Introduced into Horseradish Peroxidase under Different Conditions

Treatment with ester		Maleimide groups per peroxidase molecule			
pH	Time (h)	[a] CHM at 30°C	[a] MBA at 25°C	[a] MBA at 30°C	[b] MAA 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	1.2-1.4	0.60, 0.62	0.47, 0.16	—
7.0	1	1.6-1.7	0.53-0.65	0.33, 0.24	—
7.0	2	—	0.54, 0.40	0.25, 0.10	0.86

[a] 100-fold molar excess of N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (CHM) or N-succinimidyl m-maleimidobenzoate (MBA).
[b] 50-fold molar excess of N-succinimidyl maleimidoacetate (MAA).

Chemical Co., Tokyo or Pierce Chemical Co., Rockford, Ill.) in 0.03 ml of N,N-dimethylformamide. Warm the solution at 30°C for 1-2 min before addition to the peroxidase solution. This prevents the precipitation of the reagent when added to the peroxidase solution.

3. Add the reagent solution to the peroxidase solution and incubate the mixture at 30°C for 0.5-1 h with continuous stirring.
4. Centrifuge the reaction mixture briefly to remove excess precipitated reagent and apply the clear supernatant to a Sephadex G-25 column (1.0 x 45 cm) using sodium phosphate buffer, pH 6.0, 0.1 mol/L. Adjust the flow rate to 0.5-0.7 ml/min and the fraction volume to 0.6-0.8 ml.
5. Pool fractions showing absorbance at 403 nm and concentrate the pooled solution in a collodion bag in the cold. Do not use NaN_3 as a preservative, since it inactivates peroxidase and accelerates the decomposition of maleimide groups.

Measurement of maleimide groups in treated peroxidase

Samples are incubated with a known amount of 2-mercaptoethylamine, and the remaining thiol groups are measured (35, 52, 66).

1. Prepare the treated peroxidase in 0.45 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L with an absorbance at 403 nm of 0.7-1.0 (0.31-0.44 g/L, 7.7-11 μmol/L). Use 0.45 ml of the same buffer as a control.
2. Mix 0.01 ml of freshly prepared 0.1 mol/L (11.36 mg/ml) 2-mercaptoethylamine-HCl (m.w. 113.6) and 2 ml of 0.05 mol/L EDTA, the pH of which is adjusted to 6.0 using 1 mol/L NaOH. The final concentration of 2-mercaptoethylamine is 0.5 mmol/L.

3. Add 0.05 ml of the 2-mercaptoethylamine-EDTA mixture to the 0.45 ml treated peroxidase and incubate the reaction mixture at 30°C for 20 min.
4. Add 0.02 ml of 5 mmol/L (1.10 mg/ml) 4,4'-dithiodipyridine (m.w. 220.32), and incubate the mixture at 30°C for 10 min.
5. Read the absorbance at 324 nm. Calculate the average number of maleimide groups introduced per peroxidase molecule from the absorbance at 324 nm using the molar extinction coefficient at 324 nm of pyridine-4-thione which is 19,800 $M^{-1}.cm^{-1}$ (66), the extinction coefficient at 403 nm of peroxidase which is 2.275 $g^{-1}.L.cm^{-1}$ (69) and the molecular weight of peroxidase which is 40,000 (69).

Conjugation

1. Prepare about 1.8 mg (45 nmol) of the maleimide-peroxidase in 0.2-0.4 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L.
2. Prepare about 2.0 mg (43 nmol) of Fab' plus 0.05 mg of fluorescein-labeled Fab' in 0.2-0.4 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L containing 5 mmol/L EDTA (III-D).
3. Mix the two solutions above and incubate the mixture at 4°C for 20 h or at 30°C for 1 h. The final concentrations of the maleimide-peroxidase and Fab' in the reaction mixture for conjugation should be 50-100 μmol/L. It may be better to block remaining thiol groups with N-ethylmaleimide, since they may react with thimerosal added as a preservative.
4. Apply the reaction mixture to a Ultrogel AcA 44 column (1.5 x 45 cm) using sodium phosphate buffer, pH 6.5, 0.1 mol/L.

Adjust the flow rate to 0.3-0.5 ml/min and the fraction volume to about 1.0 ml.

5. 1) Read the absorbance of each fraction at 280 and 403 nm. 2) Measure fluorescence intensity (490 nm excitation, 510 nm emission) of each fraction using 1-10 nmol/L fluorescein as standard, when fluorescein-labeled Fab' is used. 3) Determine the peroxidase activity of each fraction (IV-A).
6. Store the Fab'-peroxidase conjugate at 4°C after adding thimerosal to give a final concentration of 20-50 mg/L and bovine serum albumin to give a final concentration of 1 g/L. Do not use NaN_3 as a preservative.

C. Characterization of Rabbit Fab'-Peroxidase Conjugate Prepared by the Maleimide Method (I) (43-45)

a. Purity of the Conjugate

The conjugate was almost completely separated from free monomer peroxidase and Fab' by gel filtration. Since the molecular weights of Fab' and peroxidase are similar (46,000 and 40,000) (Fig. IV-2), dimer and/or polymers of peroxidase, if any, can not be separated from the conjugate by gel filtration. To test for their presence in the conjugate, rabbit Fab'-peroxidase conjugate in the peak fraction (Fig. IV-2) was passed through a goat anti-(rabbit IgG) IgG-Sepharose 4B column, and only 1-2 % of peroxidase activity applied was found in the effluent (Table IV-2). When passed through a normal goat IgG-Sepharose 4B column, 95 % was recovered in the effluent. These results indicated that 98-99

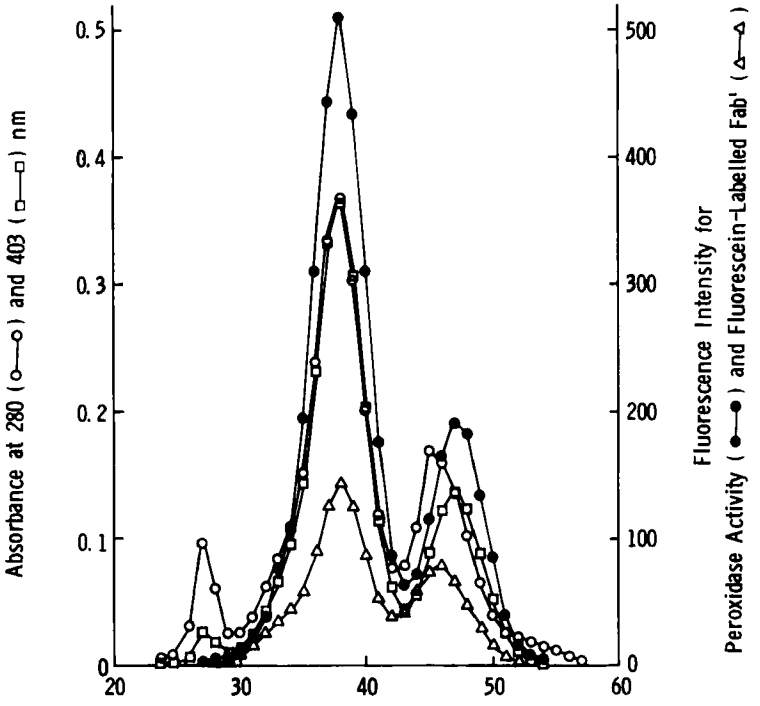


FIGURE IV-2. Elution profile from a Ultrogel AcA 44 column of rabbit Fab'-peroxidase conjugate prepared by the maleimide method (I). The amounts of peroxidase, Fab' and fluorescein-labeled normal Fab' used were 1.3, 1.5 and 0.04 mg, respectively. The concentrations of the maleimide-peroxidase and Fab' incubated for conjugation were 0.05 mmol/L. The column size used was 1.5 x 45 cm, and the fraction volume was 1.0 ml. Fluorescence intensity of fluorescein-labeled Fab' was measured by adjusting that of 10 nmol/L fluorescein to a scale of 100. Peroxidase activity was determined by 10 min assay using p-hydroxyphenyl-propionic acid. The recovery in the conjugate of peroxidase incubated for the conjugation was calculated from absorbance at 403 nm to be 73 %, and the recovery in the conjugate of Fab' incubated for the conjugation was calculated from fluorescence intensity of fluorescein-labeled Fab' to be 67 %.

TABLE IV-2

Purity of Rabbit Fluorescein-Labeled Fab'-Horseradish Peroxidase Conjugate Prepared by the Maleimide Method (I)

Sepharose 4B column	Peroxidase activity found in the effluent	Fluorescence intensity found in the effluent	
		conjugate	$F(ab')_2$
	%	%	%
Goat anti-rabbit IgG IgG	1-2	—	—
Normal goat IgG	95	—	—
Concanavalin A	0	5-10	96

The conjugates were applied to various columns, and peroxidase activity and fluorescence intensity in the effluent were expressed in percentages of those applied.

% of peroxidase in the peak fraction was associated with rabbit Fab' and that there was no significant formation of unconjugated dimers or polymers of peroxidase.

Test for the presence of unconjugated peroxidase

1. Apply about 200 ng of rabbit Fab'-peroxidase conjugate in 0.1 ml of sodium phosphate buffer, pH 7.0, 0.01 mol/L, containing 0.1 mol/L NaCl and 1 g/L bovine serum albumin to a goat anti-(rabbit IgG) IgG Sepharose 4B column (3.5 x 27 mm) at a flow rate of 1 ml/h using the same buffer, and collect the first 3 ml of the effluent. (10 mg of goat anti-(rabbit IgG) IgG was coupled to 1 g of CNBr-activated Sepharose 4B.)

2. Compare peroxidase activity in the effluent with the activity applied.

IgG and/or $F(ab')_2$ may be present in the conjugate fractions from a Ultrogel AcA 44 column, when the digestion and/or separation of IgG is incomplete or the reduction with 2-mercaptoethylamine is insufficient (III-C and III-D). Fab' may be partly reoxidized to $F(ab')_2$ during the conjugation reaction. To demonstrate this, fluorescein-labeled Fab'-peroxidase conjugate in the peak fraction from a Ultrogel AcA 44 column was passed through a concanavalin A-Sepharose 4B column, and 5-10 % of fluorescence intensity applied was found in the effluent (Table IV-2). When $F(ab')_2$ was passed through the same column, 96 % was recovered in the effluent. These results indicated that pepsin digestion was incomplete and/or that Fab' was partly reoxidized to $F(ab')_2$.

Test for the presence of unconjugated $F(ab')_2$

1. Apply about 0.05 mg of fluorescein-labeled Fab'-peroxidase conjugate in 0.1 ml of sodium acetate buffer, pH 6.0, 0.1 mol/L containing 1 mol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 1 mmol/L $MnCl_2$ and 2 g/L bovine serum albumin to a column (3 x 40 mm) of concanavalin A-Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala) at a flow rate of 1 ml/h using the same buffer, and collect the first 3 ml of the effluent.
2. Measure fluorescence intensity of the effluent using 490 nm for excitation and 510 nm for emission, and compare it with that applied.

b. Molar Ratio of Peroxidase and Fab' in the Conjugate

The molar ratio of peroxidase to Fab' in the conjugate can be calculated from the absorbances at 403 and 280 nm of the conjugate using the extinction coefficients of peroxidase (2.275 $g^{-1}.L.cm^{-1}$ at 403 nm and 0.73 $g^{-1}.L.cm^{-1}$ at 280 nm) (69) and Fab' (1.48 $g^{-1}.L.cm^{-1}$ at 280 nm) (63) and the molecular weights of peroxidase (40,000) (69) and Fab' (46,000) (64, 65). The extinction of peroxidase at 280 nm was about one third of that at 403 nm but varied to some extent with the preparation.

The molar ratio of peroxidase to Fab' in the conjugate was 1.00-1.09 both before and after purification by affinity chromatography using concanavalin A-Sepharose 4B to remove unconjugated $F(ab')_2$ (IV-D).

c. Molecular Size of the Conjugate

The molecular weight of rabbit Fab'-peroxidase conjugate was assessed to be 80,000-90,000 by gel filtration with Ultrogel AcA 44 using marker proteins such as horseradish peroxidase, fluorescein-labeled SH-blocked rabbit Fab' (III-E), bovine serum albumin, fluorescein-labeled rabbit $F(ab')_2$ and alkaline phosphatase from calf intestine (45). This indicates that one molecule each of Fab' and peroxidase were cross-linked to form a monomeric conjugate.

d. Recovery of Peroxidase and Fab' in the Conjugate

Loss of peroxidase and Fab' by gel filtration and concentration after the introduction of maleimide groups or after the reduction of $F(ab')_2$ before the conjugation was less than 10 %.

The recovery in the conjugate of the initial peroxidase can be calculated from the absorbance at 403 nm or from peroxidase activity in the elution profile obtained by gel filtration. The recovery in the conjugate of the initial Fab' can be calculated either from the fluorescence intensity in the elution profile obtained using fluorescein-labeled Fab' or from the absorbance at 280 nm of Fab'. The absorbance of Fab' at 280 nm can be calculated from the total absorbance at 280 nm, corrected for peroxidase absorbance by measuring absorbance at 403 nm and using the ratio of absorbances at 403 and 280 nm for the peroxidase preparation used. The recovery of Fab' can also be calculated from the recovery of peroxidase, the molar ratio of (Fab' to peroxidase) in the conjugate and the initial amounts of peroxidase and Fab' used. These calculations agree fairly well, implying that fluorescein-labeled Fab' reacts with the maleimide-peroxidase essentially in the same way as non-labeled Fab'.

The recovery in the conjugate of the initial peroxidase and Fab' reached 65-75 % after 15-20 h incubation at 4°C or 1 h incubation at 30°C, when the initial concentrations of the maleimide-peroxidase and Fab' were 50 μmol/L.

e. Antigen-Binding Activity of the Conjugate

Antigen-binding activity of Fab'-peroxidase conjugate may be examined by comparing the proportions of $F(ab')_2$ and Fab'-peroxidase conjugate adsorbed to antigen-Sepharose 4B. The proportion of anti-human IgG Fab'-peroxidase conjugate adsorbed to a human IgG-Sepharose 4B column was only slightly smaller than

that of anti-human IgG $F(ab')_2$ (Table IV-3). Normal rabbit Fab'-peroxidase conjugate was almost completely recovered from a human IgG-Sepharose 4B column. These results indicated that antigen-binding activity of Fab' was well retained in the maleimide method (I).

Test for antigen-binding activity of the conjugate

Apply about 200 ng of rabbit anti-human IgG Fab'-peroxidase conjugate in 0.1 ml of sodium phosphate buffer, pH 7, 10 mmol/L containing 100 mmol/L NaCl and 1 g/L bovine serum albumin to a human IgG-Sepharose 4B column (3.5 x 27 mm) at a flow rate of 1 ml/h using the same buffer, and collect the first 3 ml effluent. (10 mg of human IgG was coupled to 1 g of CNBr-activated Sepharose 4B.) Compare peroxidase activity in the effluent with that applied.

f. Stability of the Cross-Link in the Conjugate

Rabbit fluorescein-labeled Fab'-peroxidase conjugate was stored in sodium phosphate buffer, pH 6.5, 0.1 mol/L containing 100 mmol/L NaCl, 1 g/L bovine serum albumin and 20 mg/L thimerosal at 4°C and was subjected to gel filtration with an Ultrogel AcA 44 column in the same way as in the preparation of the conjugate. Little release of fluorescein-labeled Fab' was observed for at least 9 months.

g. Stability of peroxidase activity in the conjugate

No significant change in peroxidase activity was observed for at least a year, when rabbit Fab'-peroxidase conjugate was stored

TABLE IV-3

Adsorption to a Human IgG-Sepharose 4B Column of Rabbit Anti-Human IgG Fab'-Peroxidase Conjugate Prepared by the Maleimide Method (I)

Affinity-purification of anti-human IgG	Anti-human IgG used	Number of exp.	Adsorption to		
			human IgG-column	rabbit IgG-column	goat anti-rabbit IgG-column
				%	
No	$F(ab')_2$	3	10.8 (9.1-12.1)	2	—
No	Fab'-peroxidase conjugate	4	9.6 (8.6-10.7)	3	98, 99
Yes	$F(ab')_2$	2	90, 96	2	—
Yes before conjugation	Fab'-peroxidase conjugate	2	86, 87	4	99, 99
Yes after conjugation	Fab'-peroxidase conjugate	6	90 (88-91)	—	100

in sodium phosphate buffer, pH 6.5, 0.1 mol/L containing 100 mmol/L NaCl, 1 g/L bovine serum albumin and 20 mg/L thimerosal at 4°C.

h. Characteristics of Conjugates with Fab' from Various Animals Prepared by the Maleimide Method (I)

When peroxidase was conjugated with Fab' from goat, guinea pig, sheep and rat, elution profiles of conjugates from an Ultrogel AcA 44 column were similar to that of rabbit Fab'-peroxidase conjugate, indicating that the conjugates formed were mostly monomeric. However, conjugates containing more than one molecule of peroxidase and/or Fab' were formed in a limited amount, when the conjugation was performed using 100 μmol/L of Fab' from goat and rat.

D. Further Purification of Fab'-Peroxidase Conjugate Prepared by the Maleimide Method (I)

a. Removal of $F(ab')_2$

Unconjugated $F(ab')_2$ can be removed using concanavalin A-Sepharose 4B, since peroxidase, but not $F(ab')_2$, is a glycoprotein and is adsorbed to concanavalin A-Sepharose 4B. Fab'-peroxidase conjugate adsorbed can be eluted by 0.1-0.2 mol/L α-methyl-D-mannoside. The column size of concanavalin A-Sepharose 4B (Pharmacia) required for 1 mg of the conjugate is 1 x 6 cm. The eluted peroxidase activity (73 % of the applied) was almost completely (98 %) bound to an anti-rabbit IgG IgG-Sepharose

4B column. It is possible in this way to separate the conjugate from any unconjugated $F(ab')_2$.

b. Preparation of Specific Fab'-Peroxidase Conjugate by Affinity Chromatography

The Fab'-peroxidase conjugate prepared by the maleimide method (I) can be purified by affinity chromatography. Rabbit anti-human IgG Fab'-peroxidase conjugate (0.04 mg) in 1.0 ml of sodium phosphate buffer, pH 6.5, 0.1 mol/L containing 1 g/L bovine serum albumin was applied to a human IgG-Sepharose 4B column (0.3 x 1.0 cm), and the specific anti-human IgG Fab'-peroxidase conjugate adsorbed on the column was eluted using glycine-HCl buffer, pH 2.8-2.9, 50 mmol/L. The eluate was neutralized with Tris-HCl buffer, pH 8.0, 0.5 mol/L. The peroxidase activity of the specific anti-human IgG Fab'-peroxidase conjugate was almost completely adsorbed to both a human IgG-Sepharose 4B column (88-91 %) and a goat anti-rabbit IgG IgG-Sepharose 4B column (100 %), indicating that the cross-link is stable at pH 2.9 (Table IV-3). Goat anti-human α-fetoprotein Fab'-peroxidase conjugate was also affinity-purified in the same way and tested in the sandwich enzyme immunoassay for human α-fetoprotein (Fig. IV-3). The sensitivity for human α-fetoprotein was enhanced from 100 amol/tube to 10 amol/tube by affinity-purification. Horseradish peroxidase is stable in glycine-HCl buffer, pH 2.7-2.9, 50 mmol/L for at least 20 min but loses its activity at pH 2.9 in the presence of 100 mmol/L NaCl or at pH 2.5 in the absence of

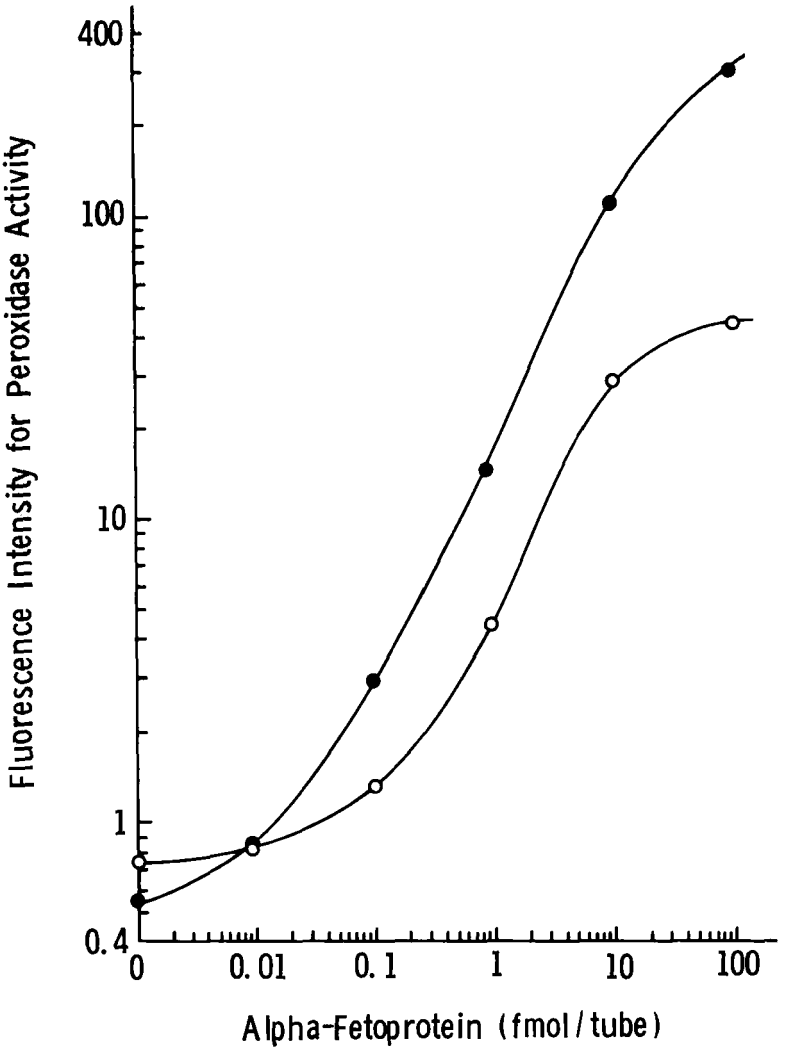


FIGURE IV-3. Sandwich enzyme immunoassay of human α-fetoprotein with goat anti-human α-fetoprotein Fab' which was conjugated with horseradish peroxidase and purified by affinity chromatography using a human α-fetoprotein-Sepharose 4B column. A goat anti-human α-fetoprotein IgG-coated polystyrene ball was incubated successively with human α-fetoprotein at 37°C for 4 h and with goat anti-human α-fetoprotein Fab'-peroxidase conjugates (5 ng/tube) at 37°C for 4 h. Peroxidase activity bound was determined by 40 min assay using p-hydroxyphenylacetic acid. Open and closed circles indicate the standard curves before and after affinity-purification.

NaCl. Elution at pH 2.5 enhances the non-specific binding of the conjugate to polystyrene balls.

E. Other Methods for Labeling Fab' with Peroxidase

a. Maleimide Method (II) for Labeling Fab' with Peroxidase

The maleimide method (II) uses N-succinimidyl m-maleimidobenzoate in place of N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate used in the maleimide method (I). Other conditions are the same as in the maleimide method (I) except for pH and temperature used.

This method provided monomeric Fab'-peroxidase conjugate similar to that obtained by the maleimide method (I). However, the yield of conjugate was less (35-49 %) than that in the maleimide method (I). The reason for this appeared to be that the introduction of maleimide groups by this method was not so efficient as in the maleimide method (I) (Table IV-1). The average number of maleimide groups introduced by treatment at pH 7.0 at 25°C for 1 h was 0.53-0.65 per peroxidase molecule, while it was 1.6-1.7 in the maleimide method (I). Treatment for a longer time than 1 h resulted in a decrease of maleimide groups introduced. Treatment at pH 6.5 at 25°C or at pH 7 at 30°C was less effective. These results appeared to be due to the fact that m-maleimidobenzoate was less stable than 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Fig. IV-4). This was supported by the fact that N-succinimidyl-maleimidoacetate was more stable and more effective in the introduction of

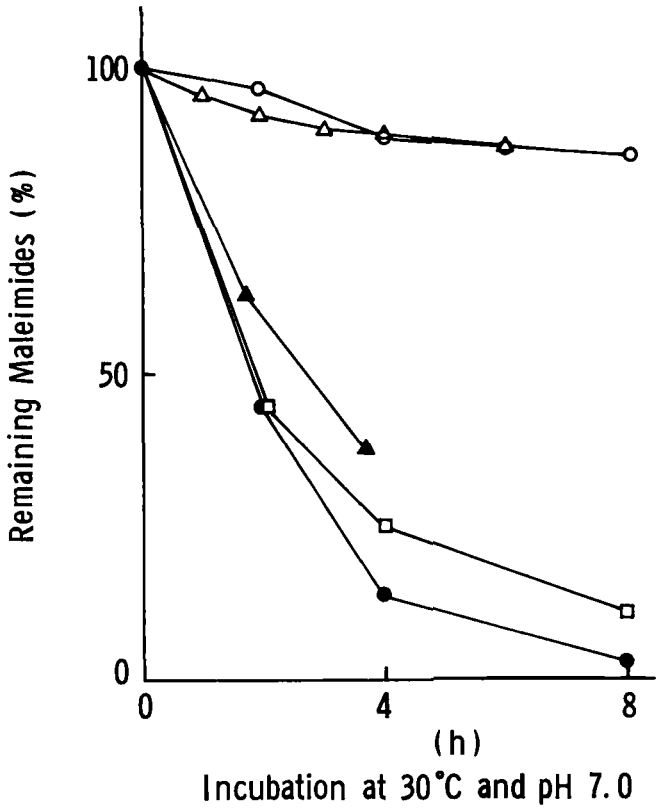


FIGURE IV-4. Stability of maleimide groups. Various maleimide compounds were incubated in sodium phosphate buffer, pH 7.0, 0.1 mol/L, at 30°C, and remaining maleimide groups were determined. Open circles, triangles, and squares indicate N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, N-succinimidyl maleimidoacetate, N-succinimidyl m-maleimidobenzoate, respectively. Closed circles, and triangles indicate N,N'-o-phenylenedimaleimide, N,N'-oxydimethylenedimaleimide, respectively.

maleimide groups into peroxidase than N-succinimidyl m-maleimidobenzoate (Table IV-1).

Introduction of maleimide groups into peroxidase

1. Dissolve 2 mg (50 nmol) of horseradish peroxidase in 0.3 ml of sodium phosphate buffer, pH 7.0, 0.1 mol/L.

2. Dissolve 1.57 mg (5,000 nmol) of N-succinimidyl m-maleimidobenzoate (m.w. 314.2) in 0.02 ml of N,N-dimethylformamide.
3. Add the reagent solution to the peroxidase solution and incubate the mixture at 25°C for 1 h.
4. Centrifuge the reaction mixture briefly to remove excess of precipitated reagent and apply the clear supernatant to a Sephadex G-25 column (1.0 x 45 cm) using sodium acetate buffer, pH 5.0, 50 mmol/L.

b. Maleimide Method (III) for Labeling Fab' with Peroxidase

The maleimide method (III) uses N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate to introduce maleimide groups into peroxidase as in the maleimide method (I) but does not use thiol groups in the hinge of Fab' for conjugation. Thiol groups of Fab' generated in the hinge by reduction with 2-mercaptoethylamine are blocked by monoiodoacetate or N-ethylmaleimide (III-E), and new thiol groups are introduced by treatment with S-acetylmercatosuccinic anhydride. The maleimide-peroxidase is then allowed to react with the mercaptosuccinylated SH-blocked Fab' (Fig. IV-5). The yield of conjugate increased with increasing numbers of thiol groups introduced per SH-blocked Fab' molecule. However, the increased yield was accompanied by an increased formation of polymerized conjugates and by an apparent heterogeneity of conjugates. Antigen-binding activity was less than with the conjugate prepared by the maleimide method (I). This method has, therefore, no apparent advantage over

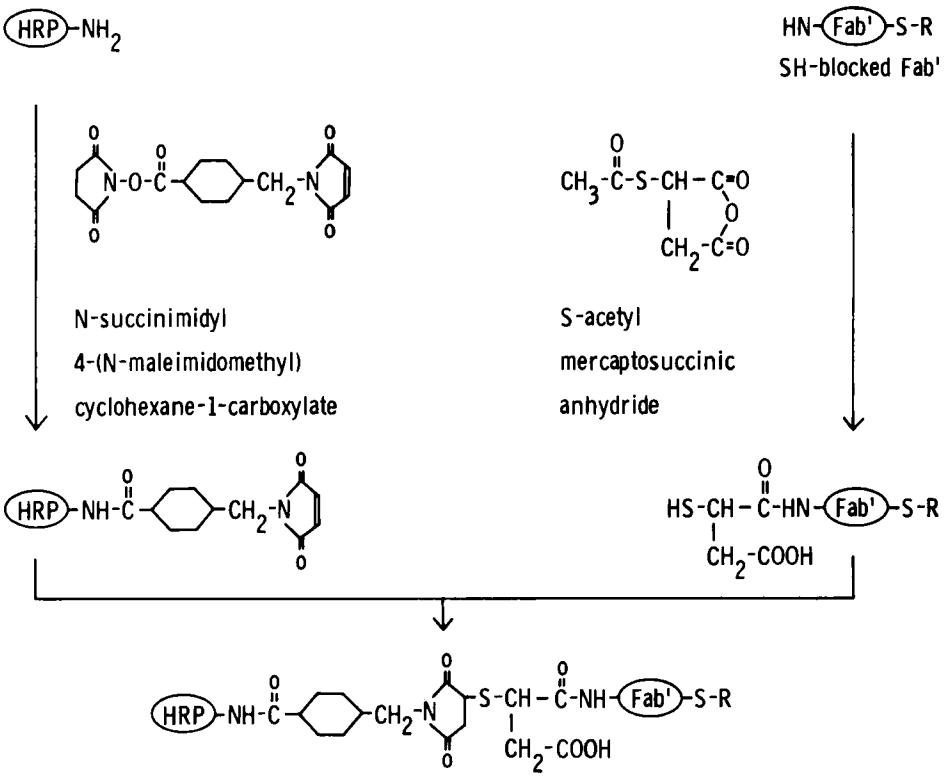


FIGURE IV-5. Preparation of Fab'-peroxidase conjugate by the maleimide method (III). HRP = horseradish peroxidase. R = $-CH_2COOH$

the maleimide method (I) and was used only to demonstrate that the antigen-binding activity was better preserved in Fab'-peroxidase conjugate prepared by using thiol groups in the hinge of Fab' than in the conjugate prepared using amino groups of Fab'.

Introduction of thiol groups into the SH-blocked Fab'

1. Prepare 2.2 mg (48 nmol) of the SH-blocked Fab' (III-E) in 0.44 ml of sodium phosphate buffer, pH 6.5, 0.1 mol/L.

2. Dissolve 0.21-0.84 mg (1,200-4,800 nmol) of S-acetylmercaptosuccinic anhydride (m.w. 174.18) in 0.01 ml of N,N-dimethylformamide.
3. Add the anhydride solution to the SH-blocked Fab' solution, and incubate the mixture at 25°C for 30 min.
4. Add 0.1 ml of Tris-HCl buffer, pH 7, 0.1 mol/L, 0.01 ml of 0.1 mol/L EDTA, pH 7 and 0.1 ml of 1 mol/L hydroxylamine, pH 7, and incubate the mixture at 30°C for 5 min.
5. Apply the reaction mixture to a Sephadex G-25 column (1.0 x 45 cm) equilibrated with sodium phosphate buffer, pH 6.0, 0.1 mol/L containing 5 mmol/L EDTA. The average number of thiol groups introduced was 0.99-1.8 per molecule (III-D for measuring thiol groups).

c. Pyridyl Disulfide Method (I) for Labeling Fab' with Peroxidase

The pyridyl disulfide method (I) uses N-succinimidyl 3-(2-pyridyldithio)propionate (Pharmacia Fine Chemicals AB, Uppsala and Pierce Chemical Co., Rockford, Ill.) in place of N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate used in the maleimide method (I). In the first step, horseradish peroxidase is treated with the reagent to introduce pyridyl disulfide groups. In the second step, the pyridyl disulfide groups introduced are allowed to react with thiol groups in the hinge of Fab' (Fig. IV-6). This method provides monomeric Fab'-peroxidase conjugate with little loss of activity of Fab' and peroxidase as in the maleimide method (I). However, the yield of conju-

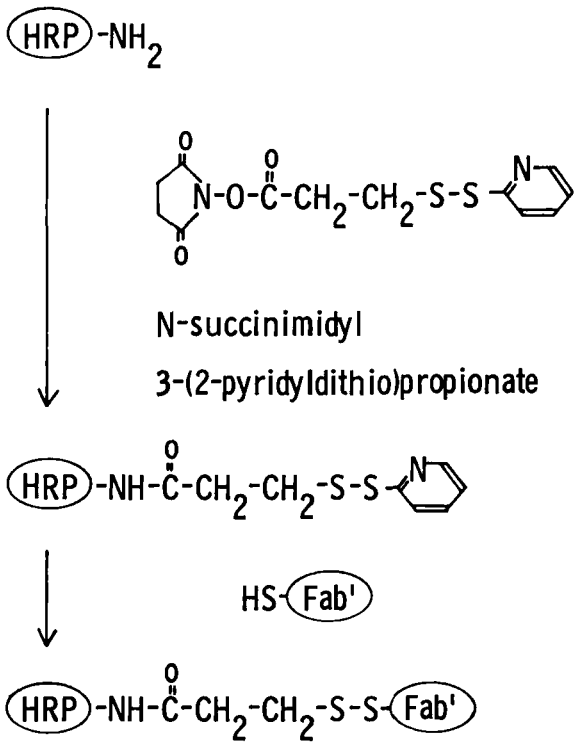


FIGURE IV-6. Preparation of Fab'-peroxidase conjugate by the pyridyl disulfide method (I). HRP = horseradish peroxidase.

gate is lower (54-59 %) than in the maleimide method (I) (65-75 %) (Table IV-4).

Introduction of pyridyl disulfide groups into peroxidase

1. Dissolve 2 mg (50 nmol) of horseradish peroxidase in 0.3 ml of sodium phosphate buffer, pH 7.5, 0.1 mol/L.
2. Dissolve 0.78 mg (2,500 nmol) of N-succinimidyl 3-(2-pyridyldithio) propionate (m.w. 312.5) in 0.06 ml of ethanol.
3. Add the reagent solution to the enzyme solution and incubate the mixture at 25°C for 30 min.

TABLE IV-4

Recovery of conjugated peroxidase in the maleimide method (I) and pyridyl disulfide method (I) under various conditions of incubation

Concentration of Fab' and enzyme (mmol/L)	pH	Temperature (°C)	Time (h)	Recovery of peroxidase (%)
Maleimide method (I)				
0.05	6	4	5	60
0.05	6	4	20	65-75
0.05	6	30	1	67
Pyridyl disulfide method (I)				
0.05	6	4	20	37
0.10	6	4	2.5	19
0.05	6	30	2.5	45, 46
0.10	6	30	2.5	54
0.10	6	30	20	59
0.10	7.5	30	2.5	57
0.10	7.5	30	20	57

4. Apply the reaction mixture to a Sephadex G-25 column (1.0 x 45 cm) equilibrated with sodium phosphate buffer, pH 6.0, 0.1 mol/L.

Measurement of pyridyl disulfide groups

1. Prepare a sample in 0.5 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L, with an absorbance at 403 nm of 0.3-0.6.

2. Add 0.02 ml of 0.1 mol/L (15.4 mg/ml) dithiothreitol (m.w. 154.25).
3. Incubate the mixture at 30°C for 20 min.
4. Read absorbance at 343 nm. Calculate the average number of pyridyl disulfide groups introduced per peroxidase molecule from absorbance at 343 nm using the molar extinction coefficient of pyridine-2-thione which is 8,080 $M^{-1}.\ cm^{-1}$ (70), the extinction coefficient at 403 nm of peroxidase which is 2.275 $g^{-1}.L.cm^{-1}$ (69) and the molecular weight of peroxidase which is 40,000 (69). The average number of pyridyl disulfide groups introduced under the above condition was 2.5-2.7 per peroxidase molecule.

Conjugation

1. Prepare about 1.8 mg (45 nmol) of the pyridyl disulfide-peroxidase in 0.22 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L.
2. Prepare about 2 mg (43 nmol) of Fab' in 0.22 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 5 mmol/L EDTA.
3. Mix the two solutions above and incubate the mixture at 30°C for 2.5 h. The final concentrations of the pyridyl disulfide-peroxidase and Fab' should be 100 μmol/L or higher.
4. Gel filtration and further processes are the same as in maleimide method (I).

d. Pyridyl Disulfide Method (II) for Labeling Fab' with Peroxidase

The pyridyl disulfide method (II) uses S-acetylmercaptosuccinic anhydride to introduce thiol groups into peroxidase and

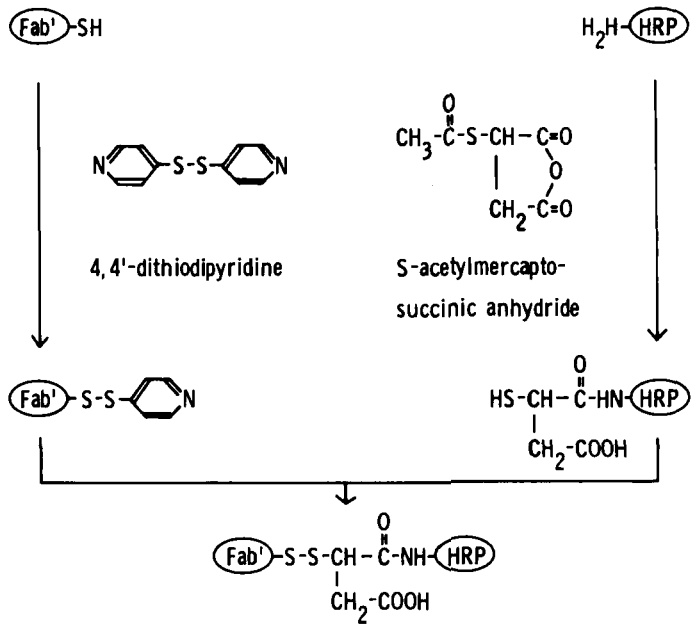


FIGURE IV-7. Preparation of Fab'-peroxidase conjugate by the pyridyl disulfide method (II). HRP = horseradish peroxidase.

4,4'-dithiodipyridine to introduce pyridyl disulfide groups into Fab' using thiol groups in the hinge. The mercaptosuccinylated peroxidase is allowed to react with the pyridyl disulfide-Fab' (Fig. IV-7).

This method uses only inexpensive reagents, 4,4'-dithiodipyridine and S-acetylmercaptosuccinic anhydride, providing monomeric Fab'-peroxidase conjugate with little loss of activity of Fab' and peroxidase as in the maleimide method (I) and pyridyl disulfide method (I). However, the recovery of peroxidase and Fab' in the conjugate is rather low (40-49 %).

Introduction of thiol groups into peroxidase

1. Dissolve 2 mg (50 nmol) of horseradish peroxidase in 0.25 ml of sodium phosphate buffer, pH 7.5, 0.1 mol/L.
2. Dissolve 1.74 mg (10,000 nmol) of S-acetylmercaptosuccinic anhydride (m.w. 174.18) in 0.01 ml of N,N-dimethylformamide.
3. Add the anhydride solution to the peroxidase solution, and incubate the mixture at 30°C for 30 min with continuous stirring.
4. Add 0.05 ml of Tris-HCl buffer, pH 7, 0.1 mol/L; 0.005 ml of EDTA, pH 7, 0.1 mol/L, and 0.05 ml of hydroxylamine, pH 7, 1 mol/L.
5. Incubate the mixture at 30°C for 5 min.
6. Apply the reaction mixture to a Sephadex G-25 column (1.0 x 45 cm) equilibrated with sodium phosphate buffer, pH 6, 0.1 mol/L, containing 5 mmol/L EDTA. The average number of thiol groups introduced under the above condition was 2.2 per peroxidase molecule (III-D for measuring thiol groups).

Introduction of pyridyl disulfide groups into Fab'

1. Prepare 2.2 mg (24 nmol) of $F(ab')_2$ in 0.18 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L containing 1 mmol/L EDTA.
2. Add 0.02 ml of 0.1 mol/L 2-mercaptoethylamine-HCl (m.w. 113.6), pH 6 containing 5 mmol/L EDTA to the $F(ab')_2$ solution.
3. Incubate the mixture at 37°C for 1.5 h.
4. Add 0.25-0.7 ml of sodium phosphate buffer, pH 6, 0.1 mol/L and 0.05-0.10 ml of 0.5 mol/L 4,4'-dithiodipyridine (m.w. 220.32) in N,N-dimethylformamide.

5. Incubate the mixture at 30°C for 10 min.
6. Apply the reaction mixture to a Sephadex G-25 column (1.0 x 30-45 cm) equilibrated with sodium phosphate buffer, pH 6, 0.1 mol/L. Pyridyl disulfide groups are measured in the same way as in the pyridyl disulfide method (I) except that calculation is made from absorbance at 324 nm using an extinction coefficient of 19,800 $M^{-1}.cm^{-1}$ (66). The average number of pyridyl disulfide groups introduced under the above condition was 0.70-0.84 per Fab' molecule.

Conjugation

The conjugation and further processes are the same as in the pyridyl disulfide method (I) except that the mixture for conjugation was treated with 0.01 ml of 100 mmol/L N-ethylmaleimide at 30°C for 10 min before gel filtration.

e. Pyridyl Disulfide Method (III) for Labeling Fab' with Peroxidase

The pyridyl disulfide method (III) uses N-succinimidyl 3-(2-pyridyldithio) propionate in place of N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate used in the maleimide method (III) (Fig. IV-8). Pyridyl disulfide groups are introduced into peroxidase as in the pyridyl disulfide method (I) and allowed to react with the mercaptosuccinylated SH-blocked Fab' prepared as in the maleimide method (III). The characteristics of the conjugate obtained were similar to those prepared by the maleimide method (III).

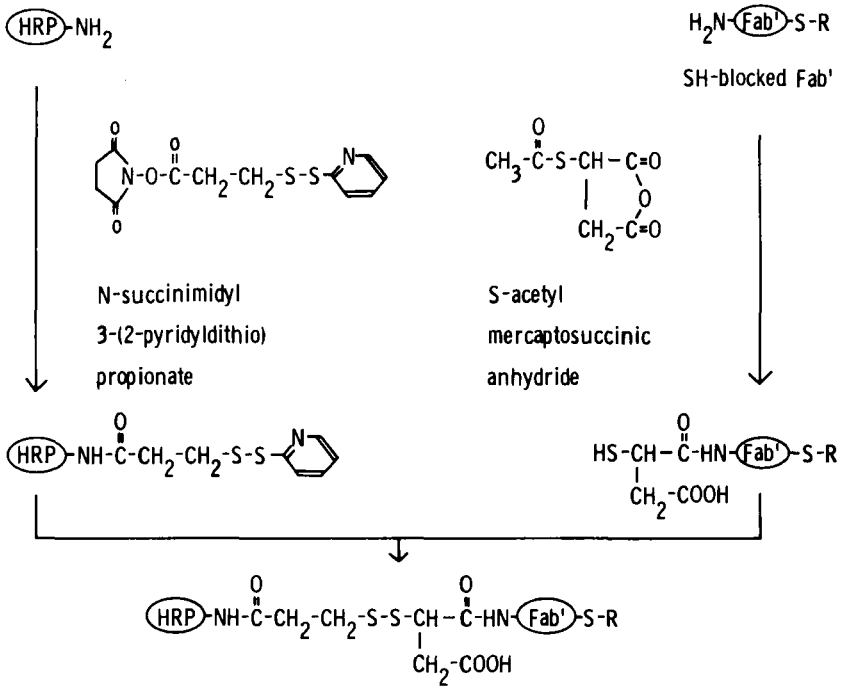


FIGURE IV-8. Preparation of Fab'-peroxidase conjugate by the pyridyl disulfide method (III). HRP = horseradish peroxidase.

f. Two-Step Glutaraldehyde Method for Labeling Fab' with Peroxidase (7, 22)

In the first step, horseradish peroxidase is treated with excess of glutaraldehyde to become reactive with amino groups. In the second step, the glutaraldehyde-treated peroxidase after removing dimer and polymers of the enzyme by gel filtration is allowed to react with amino groups of the SH-blocked Fab' (22).

Treatment of peroxidase with glutaraldehyde

1. Dissolve 10 mg of horseradish peroxidase in 0.19 ml of sodium phosphate buffer, pH 6.8, 0.1 mol/L.

2. Add 0.01 ml of 25 % glutaraldehyde to a final concentration of 12.5 g/L, and incubate the reaction mixture at room temperature for 18 h.
3. Apply the reaction mixture to a Ultrogel AcA 44 column (1 x 45 cm) using 150 mmol/L NaCl to remove excess of glutaraldehyde and dimer and/or polymers of peroxidase, if any.

Conjugation

1. Prepare 1.8 mg (39 nmol) of the SH-blocked Fab' in 0.73 ml of 150 mmol/L NaCl (III-E).
2. Prepare 8.0 mg (200 nmol) of the glutaraldehyde-treated peroxidase in 0.73 ml of 150 mmol/L NaCl.
3. Add 0.1 ml of sodium carbonate buffer, pH 9.5, 1 mol/L, to the mixture of the SH-blocked Fab' and the glutaraldehyde-treated peroxidase. The final concentrations of the SH-blocked Fab' and glutaraldehyde-treated peroxidase are 25 and 120 μmol/L, respectively.
4. Incubate the reaction mixture at 4°C for 24 h.
5. Add 0.05 ml of 0.2 mol/L L-lysine-HCl in sodium phosphate buffer, pH 8, 0.25 mol/L, to the reaction mixture, and incubate the mixture at 4°C for 2 h.
6. Gel filtration and subsequent steps are the same as in the maleimide method (I).

g. Periodate Method for Labeling Fab' with Peroxidase (22, 30)

Horseradish peroxidase is oxidized by $NaIO_4$ followed by addition of ethylene glycol to stop the oxidation (22). It is

then subjected to gel filtration using sodium acetate buffer, pH 4.4, 1 mmol/L. Aldehyde groups generated in the enzyme are allowed to react with amino groups of the SH-blocked Fab', and the Schiff's bases formed are reduced with sodium borohydride. The yield of conjugates increases with increasing oxidation of peroxidase and with increasing concentrations of Fab' and oxidized peroxidase. Highly polymerized conjugates were, however, inevitably produced as yields of conjugates increased, and loss of peroxidase activity also became more severe.

Oxidation of peroxidase

1. Dissolve 2 mg of horseradish peroxidase in 0.5 ml of deionized water.
2. Add 0.1 ml of 0.1 mol/L $NaIO_4$ to the peroxidase solution.
3. Incubate the mixture at room temperature for 10 min.
4. Add 0.05 ml of ethylene glycol to the reaction mixture, and incubate the mixture at room temperature for 5 min.
5. Apply the reaction mixture to a Sephadex G-25 column (1 x 45 cm) using sodium acetate buffer, pH 4.4, 1 mmol/L.

Conjugation

1. Prepare 1.8 mg (39 nmol) of the SH-blocked Fab' in 0.38 ml of 150 mmol/L NaCl (III-E).
2. Prepare 1.6 mg (40 nmol) of the oxidized peroxidase in 0.38 ml of sodium acetate buffer, pH 4.4, 1 mmol/L.
3. Add 0.02 ml of sodium carbonate buffer, pH 9.5, 1 mol/L, to the mixture of the SH-blocked Fab' and oxidized peroxidase. The final concentrations of the SH-blocked Fab' and oxidized peroxidase are about 50 µmol/L.

4. Incubate the reaction mixture at 25°C for 2 h.
5. Add 0.04 ml of 4 g/L of sodium borohydride.
6. Incubate the reaction mixture at 4°C for 2 h.
7. Gel filtration and further steps are the same as in the maleimide method (I).

F. Comparison of Fab'-Peroxidase Conjugates Prepared by Various Methods

a. Purity of Conjugates

The proportions of unconjugated peroxidase in the rabbit Fab'-peroxidase conjugate fractions eluted from a Ultrogel AcA 44 column were estimated by passing through a goat (anti-rabbit IgG) IgG-Sepharose 4B column (IV-C). As summarized in Table IV-5, unconjugated peroxidase was present only in small proportions (2-5 %) and more than 95 % of peroxidase was associated with Fab' in the maleimide (I-III) and pyridyl disulfide (I-III) methods. By contrast, unconjugated peroxidase in the conjugate fractions prepared by the glutaraldehyde and periodate methods amounted to 19-40 % and 6-16 %, respectively. This may have been partly due to the dimerization of peroxidase, although dimer and polymers were removed by gel filtration before conjugation in the glutaraldehyde method (IV-E). It may also have been due to an incomplete separation of the conjugate from unconjugated peroxidase in the glutaraldehyde method, since more than 90 % of peroxidase used remained unconjugated (Table IV-5 and Fig. IV-9G).

More than 90 % of Fab' in the conjugate fractions prepared by the maleimide method (I) was associated with peroxidase, and the

TABLE IV-5

Purity of rabbit Fab'-peroxidase conjugate and recovery of conjugated peroxidase in various conjugation methods

Conjugation method	Proportion of unconjugated peroxidase[a]	Recovery of peroxidase
	%	%
Maleimide method (I)	2	65-74
(II)	2	35-49
(III)	3	52-74
Pyridyl disulfide method (I)	2	54-58
(II)	3	40-49
(III)	5	58
Glutaraldehyde method	19-40	4-9
Periodate method		58-66
monomeric	6-16	-
polymeric	1	-

[a]Rabbit Fab'-peroxidase conjugate was applied to a goat (anti-rabbit IgG) IgG-Sepharose 4B column, and the enzyme activity in the effluent was taken as unconjugated enzyme and expressed as a percentage of that applied.

unconjugated $F(ab')_2$ present was less than 10 % as described above. No information on other methods is available .

b. Recovery of Peroxidase and Fab' in the Conjugates

The recoveries in the conjugates of peroxidase incubated for the conjugation were calculated from absorbance at 403 nm in the elution profiles obtained by gel filtration with Ultrogel AcA 44

(Fig. IV-9 A-H) and are summarized in Table IV-5. In general, the recovery increases with increasing concentration of peroxidase and of Fab' used for conjugation. The recovery was: maleimide (I) > pyridyl disulfide (I) > maleimide (II) = pyridyl disulfide (II) > glutaraldehyde. The recoveries in the maleimide (III) and pyridyl disulfide (III) methods increased with increasing number of thiol groups introduced into the SH-blocked Fab'. The recovery in the periodate method increased after prolonged oxidation of peroxidase with periodate.

The recovery of Fab' in conjugates was similar to that of peroxidase, since the molar ratio of Fab' to peroxidase was approximately one in both the reaction mixture for conjugation and in the conjugates formed in all the methods tested except for the glutaraldehyde method.

c. Molecular Size of Conjugates

As described above (IV-C), rabbit Fab'-peroxidase conjugate prepared by the maleimide method (I) was largely monomeric. Elution profiles of conjugates prepared by the maleimide (II), pyridyl disulfide (I) and (II) and two-step glutaraldehyde methods indicated that these conjugates were also largely monomeric (Fig. IV-9 A,B,D,E and G). However, the conjugates obtained by the maleimide (III), pyridyl disulfide (III) and periodate methods were heterogeneous (Fig. IV-9 C,F and H). Heterogeneity of the conjugates prepared by the maleimide (III) and pyridyl disulfide (III) methods increased with increasing number of thiol groups

introduced into the SH-blocked Fab'. In the periodate method, the formation of highly polymerized conjugates increased after prolonged oxidation of peroxidase with periodate.

d. Antigen-Binding Activity of Conjugates

The proportion of rabbit anti-human IgG Fab'-peroxidase conjugate adsorbed to a human IgG-Sepharose 4B column was larger in the maleimide method (I) than in the periodate method (Table IV-6). This suggested that the antigen binding activity of Fab' was fairly well preserved when Fab' was conjugated through thiol groups in the hinge, but was significantly impaired when Fab' was conjugated through its amino groups.

Conjugation of Fab' with an increased number of peroxidase molecules may impair antigen-binding activity in the maleimide (III), pyridyl disulfide (III) and periodate methods.

e. Peroxidase Activity of Conjugates

There was little loss of peroxidase activity in the maleimide (I, II and III) and pyridyl disulfide (I, II and III) methods. In contrast, we observed significant losses of peroxidase in the glutaraldehyde and periodate methods. When peroxidase was oxidized by periodate for 20 min, 29-64 % of peroxidase activity was in the monomeric conjugate and 62-76 % in the highly polymerized conjugates. However, peroxidase activity was found to be well preserved, when oxidation lasted only for 10 min and was followed by ethylene glycol.

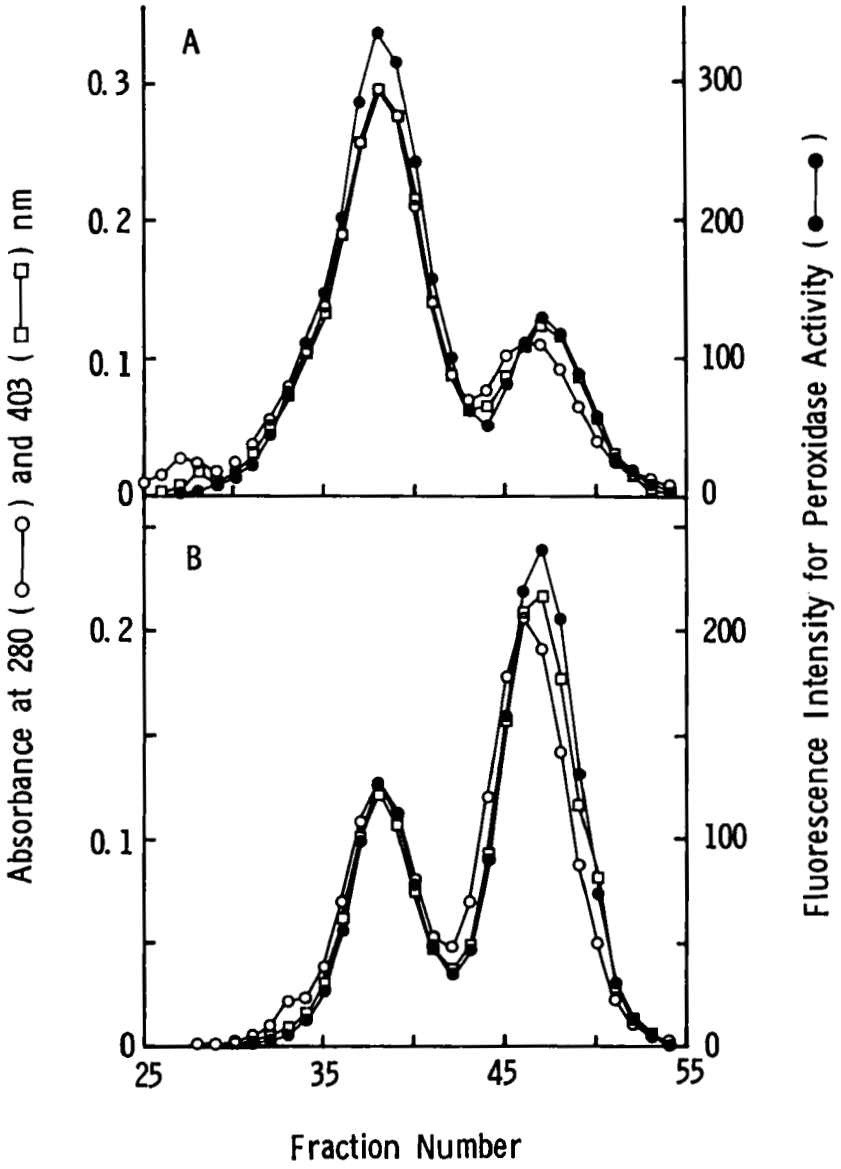


FIGURE IV-9A-H. Comparison of elution profiles from a Ultrogel AcA 44 column of rabbit Fab'-peroxidase conjugates prepared by the maleimide methods (I) (A), (II) (B) and (III) (C), pyridyl disulfide methods (I) (D), (II) (E) and (III) (F), glutaraldehyde (G) and periodate (H) methods. Peroxidase activity was determined by 10 min assay using p-hydroxyphenylacetic acid.

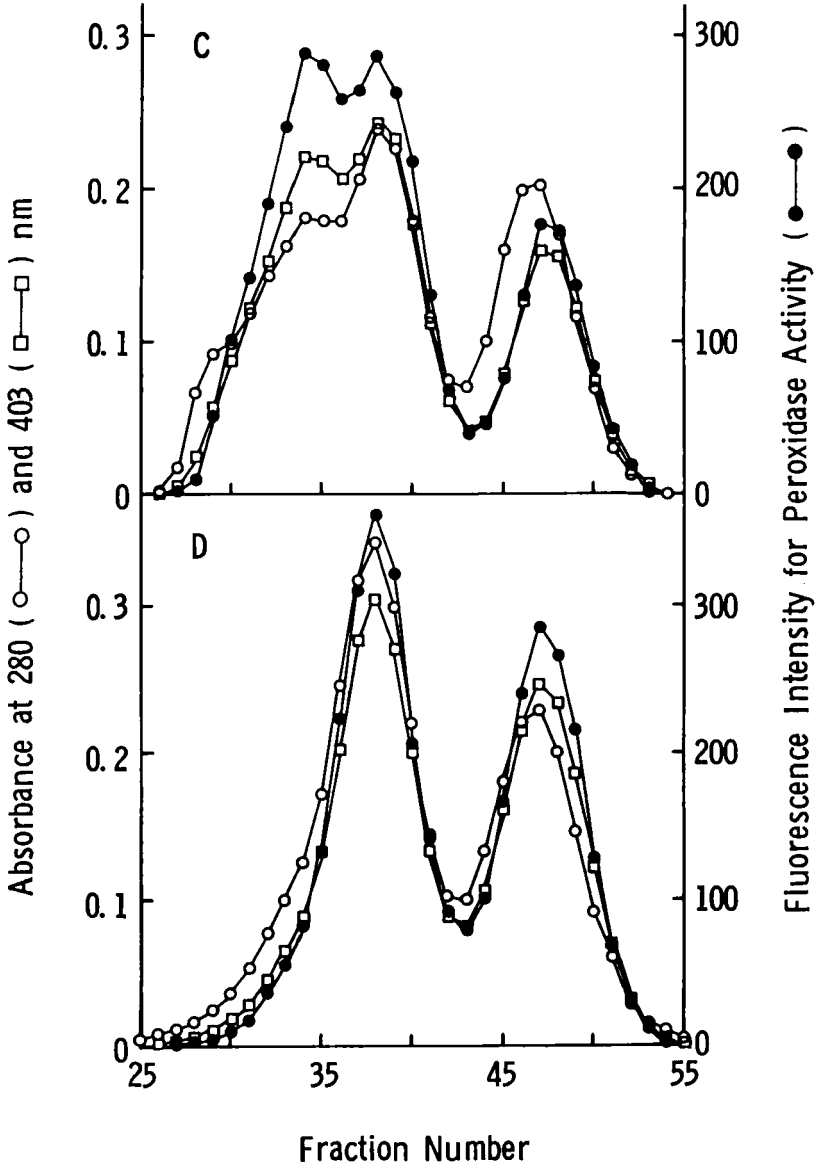


FIG. IV-9 (continued)

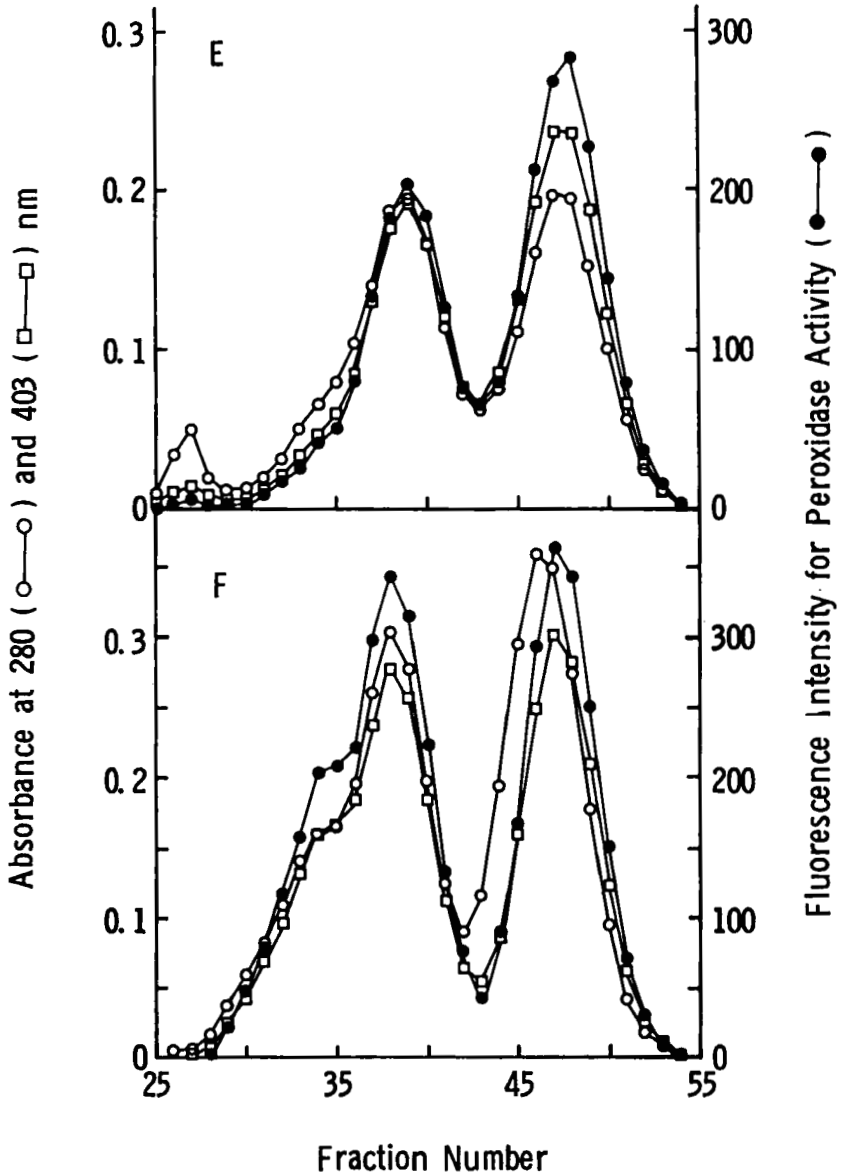


FIG. IV-9 (continued)

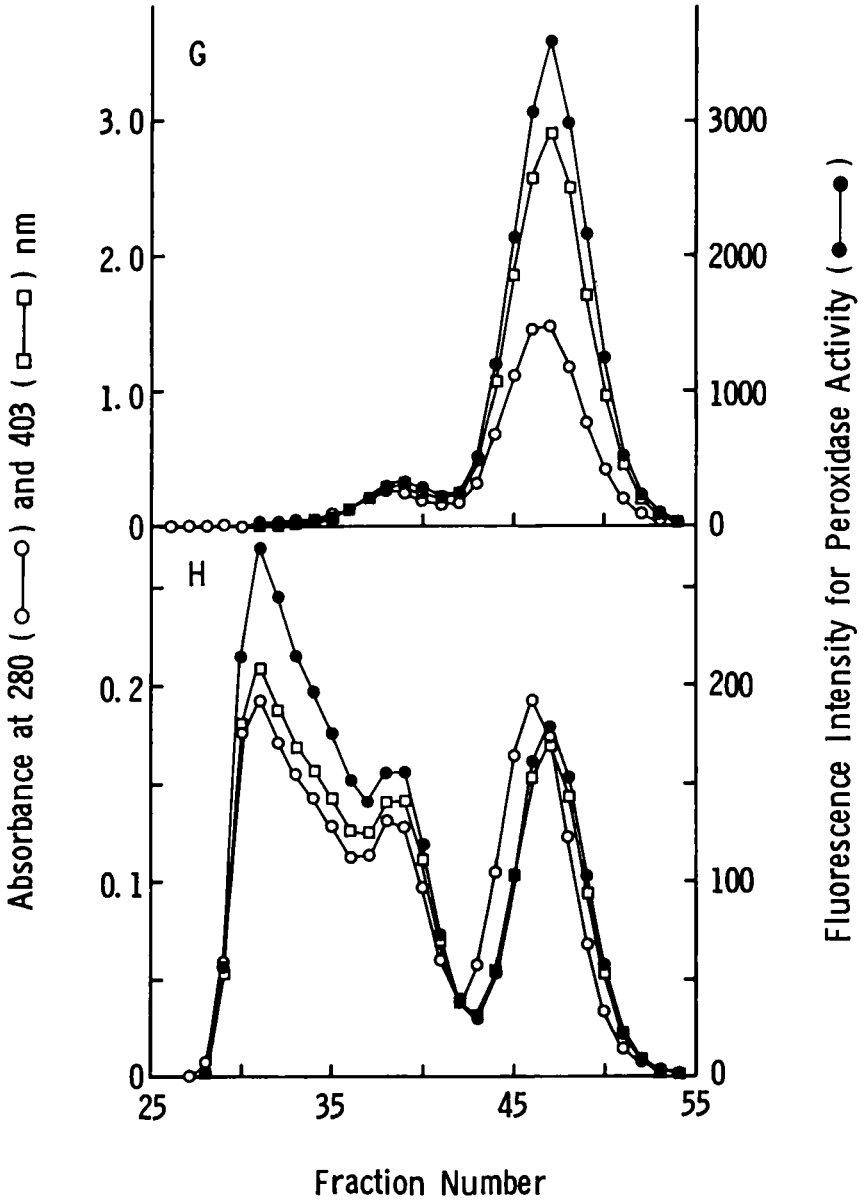


FIG. IV-9 (continued)

TABLE IV-6

Adsorption to a human IgG-Sepharose 4B column of affinity-purified rabbit anti-human IgG Fab'-peroxidase conjugates prepared by the maleimide method (I) and periodate method

Method for conjugation	Adsorption to		
	human IgG-column	rabbit IgG-column	goat anti-rabbit IgG-column
		%	
Maleimide method (I)	86, 87	4	99
Periodate method			
monomeric	61	4	84-94
highly polymeric	78	4	99

f. Usefulness of Conjugates in Sandwich Enzyme Immunoassay

Rabbit anti-human chorionic gonadotropin (hCG) Fab'-peroxidase conjugates prepared by various methods were tested in a sandwich enzyme immunoassay for hCG (Table IV-7). Nonspecific binding of the conjugates prepared by the maleimide methods (I) and (II) and pyridyl disulfide methods (I) and (II) were the lowest (0.0040-0.0046 % of peroxidase activity added) and specific binding, the highest. Nonspecific binding of other conjugates was 4-16 fold higher, and specific binding of other conjugates, 33-74 % lower.

TABLE IV-7

Sandwich enzyme immunoassay of hCG with rabbit anti-hCG Fab'-peroxidase conjugates prepared by various methods

Conjugation method	Fluorescence intensity for peroxidase activity bound			
	hCG added (mU/tube)			
	0	0.1	1	10
Maleimide method				
(I)	6	15	66	156
(II)	6	15	53	141
(III)	33	38	65	87
Pyridyl disulfide method (I)	6	14	57	152
(II)	6	14	52	145
(III)	24	28	57	94
Glutaraldehyde method	49	50	64	88
Periodate method				
monomeric	44	47	77	110
polymeric	98	98	132	188

A rabbit anti-hCG IgG-coated polystyrene ball was incubated successively with hCG at 37°C for 4 h and with anti-hCG Fab'-peroxidase conjugates (200 ng/tube) at 37°C for 6 h. Peroxidase activity bound was determined by 20 min assay using p-hydroxyphenylacetic acid.

Other antibodies tested included rabbit Fab' against human IgG, human ferritin (44, 45) and human thyroid-stimulating hormone (45), sheep Fab' against rabbit fibrinogen and goat Fab' against human α-fetoprotein. These Fab'-peroxidase conjugates prepared

by the maleimide method (I) gave lower nonspecific binding and higher specific binding than the periodate conjugates. Nonspecific binding of these conjugates was lower in the incubation at 20°C, although data shown (Table IV-7) were obtained at 37°C. Nonspecific binding of conjugates prepared using thiol groups in the hinge were even lower than those using amino groups.

These results indicate that the sensitivity of sandwich enzyme immunoassay can be improved by incubation at 20°C using conjugates prepared with thiol groups in the hinge. In fact, the use of Fab'-peroxidase conjugates prepared by the maleimide method (I) enabled us to measure 3 amol of human growth hormone (57) and 10 amol of human α-fetoprotein (Fig. IV-3) by the sandwich technique.

g. Usefulness of Conjugates in Immunohistochemical Staining

Fab'-peroxidase conjugates prepared using thiol groups in the hinge were also superior in immunohistochemical staining to those prepared using amino groups of Fab' (71).

G. Maleimide Methods for Labeling IgG, $F(ab')_2$ and Fab with Peroxidase

IgG, $F(ab')_2$ and Fab can be labeled with peroxidase in the same way as in the maleimide method (III) for labeling Fab'. Maleimide groups are introduced into peroxidase using N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate as in the maleimide method (I) and thiol groups are introduced into IgG,

$F(ab')_2$ or Fab using S-acetylmercaptosuccinic anhydride as in the maleimide method (III). The maleimide-peroxidase is then allowed to react with the mercaptosuccinylated IgG, $F(ab')_2$ or Fab. In order to completely conjugate the mercaptosuccinylated IgG and $F(ab')_2$, the average number of thiol groups introduced per IgG molecule should be more than 4, and an excess of the maleimide-peroxidase should be added, since it is difficult to separate IgG- or $F(ab')_2$-peroxidase conjugates from unconjugated IgG or $F(ab')_2$ by gel filtration. Maleimide-peroxidase can also be conjugated with reduced IgG (the maleimide method (IV)). It is, however, difficult to completely convert reduced IgG to conjugates, since the number of thiol groups of reduced IgG is only 2 per molecule.

Maleimide method (III) for labeling IgG with Peroxidase

Introduction of maleimide groups into peroxidase

The introduction of maleimide groups into peroxidase is performed in the same way as in the maleimide method (I) for labeling Fab' (IV-B).

Introduction of thiol groups into IgG

1. Prepare 5 mg (33 nmol) of IgG in 0.5 ml of sodium phosphate buffer, pH 6.5, 0.1 mol/L.
2. Dissolve 0.6 mg (3,400 nmol) of S-acetylmercaptosuccinic anhydride (m.w. 174,18) in 0.01 ml of N,N-dimethylformamide.
3. Add the anhydride solution to the IgG solution, and incubate the mixture at room temperature for 30 min with continuous stirring.

4. Add 0.02 ml of 100 mol/L EDTA, 0.1 ml of Tris-HCl buffer, pH 7.0, 0.1 mol/L, and 0.1 ml of hydroxylamine-HCl, pH 7.0, 1 mol/L.
5. Incubate the reaction mixture at 30°C for 4 min.
6. Apply the reaction mixture to a Sephadex G-25 column (1.0 x 45 cm) using sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 5 mmol/L EDTA. Adjust the flow rate to 0.6 ml/min and the fraction volume to 0.6 ml.
7. Pool and concentrate fractions containing the mercaptosuccinylated IgG. When 50 and 100 molar excess of S-acetylmercaptosuccinic anhydride were used, the average numbers of thiol groups introduced were 2.3 and 4.3-7.3 per IgG molecule, respectively (III-D for measuring thiol groups).

Conjugation

1. Prepare 3.0 mg (75 nmol) of the maleimide-peroxidase in 0.25 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L.
2. Prepare 2.3 mg (15 nmol) of the mercaptosuccinylated IgG in 0.25 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 5 mmol/L EDTA.
3. Mix the maleimide-peroxidase and mercaptosuccinylated IgG solutions above, and incubate the reaction mixture at 4°C for 20 h. The final concentrations of IgG and peroxidase are 30 and 150 μmol/L, respectively.
4. Apply the reaction mixture to a column (1.5 x 45 cm) of Ultrogel AcA 34 using sodium phosphate buffer, pH 6.5, 0.1 mol/L.

H. Characteristics of IgG-, $F(ab')_2$- and Fab-Peroxidase Conjugates

a. Purity of Conjugates

IgG- and $F(ab')_2$-peroxidase conjugates are clearly separated from unconjugated peroxidase but are not completely separated from unconjugated IgG (Figs. IV-10 and 11) and $F(ab')_2$ by gel filtration, since the molecular weights of these conjugates are not sufficiently different from IgG and $F(ab')_2$. The amount of unconjugated IgG or $F(ab')_2$ can be minimized by increasing the number of thiol groups introduced per IgG or $F(ab')_2$ molecule and increasing the molar ratio of the maleimide-peroxidase to the mercaptosuccinylated IgG and $F(ab')_2$ in the reaction mixture for conjugation. However, this is accompanied by an increase in the number of peroxidase molecules conjugated per IgG molecule, which may cause loss of antigen-binding activity.

Fab-peroxidase conjugate can be almost completely separated from unconjugated Fab and peroxidase with the Fab'-peroxidase conjugate prepared by the maleimide method (III).

b. Molecular Size of Conjugates

The numbers of peroxidase molecules conjugated per IgG, $F(ab')_2$ or Fab molecule increase with increasing number of thiol groups introduced per IgG, $F(ab')_2$ or Fab molecule, and these conjugates are heterogeneous to a various extent as with the Fab'-peroxidase conjugate prepared by the maleimide method (III) (Figs. IV-9C, IV-10 and 11). When 33 μmol/L of the mercapto-

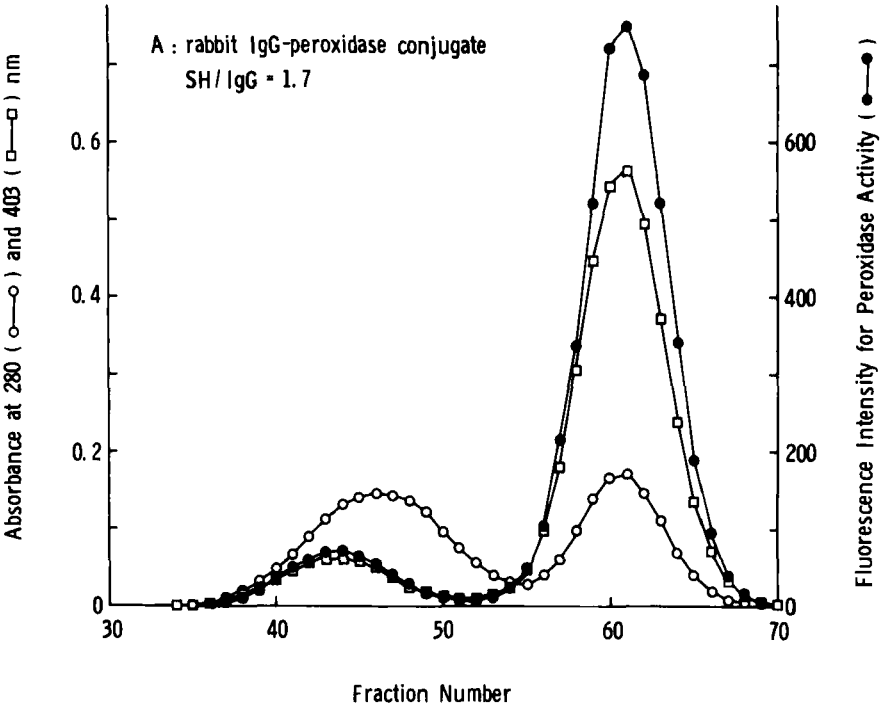
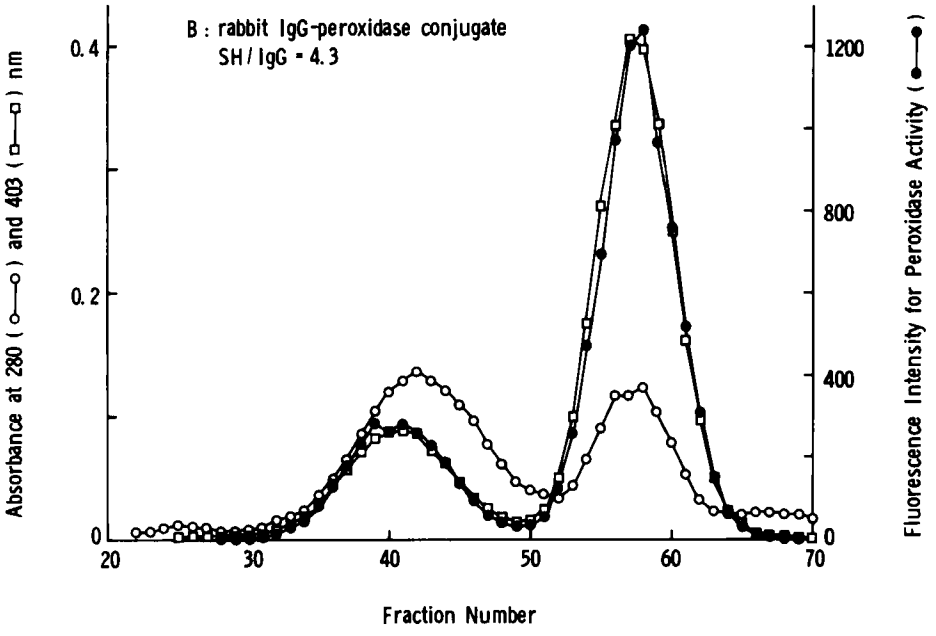


FIGURE IV-10. Elution profile from a column of Ultrogel AcA 34 of rabbit IgG-peroxidase conjugate prepared by the maleimide method (III). The average numbers of maleimide groups introduced into peroxidase molecule were 1.4 (A), 0.91 (B), 1.1 (C). The average numbers of thiol groups introduced per IgG molecule were 1.7 (A), 4.3 (B) and 7.3 (C). The concentrations of peroxidase incubated for the conjugation were 0.100 (A), 0.165 (B), 0.174 (C) mmol/L. The concentrations of the mercaptosuccinylated IgG for the conjugation were 0.020 (A), 0.033 (B) and 0.035 (C) mmol/L. The incubation for conjugation was performed at 4°C for 20 h. The column size used was 1.5 x 45 cm, and the fraction volume was 0.98-1.0 ml. The recovery of peroxidase in the conjugate was calculated from absorbance at 403 nm to be 12.7 % (A), 24.0 % (B) and 62 % (C). The average numbers of peroxidase molecules conjugated per IgG molecule was calculated to be 1.2 (B) and 3.1 (C).



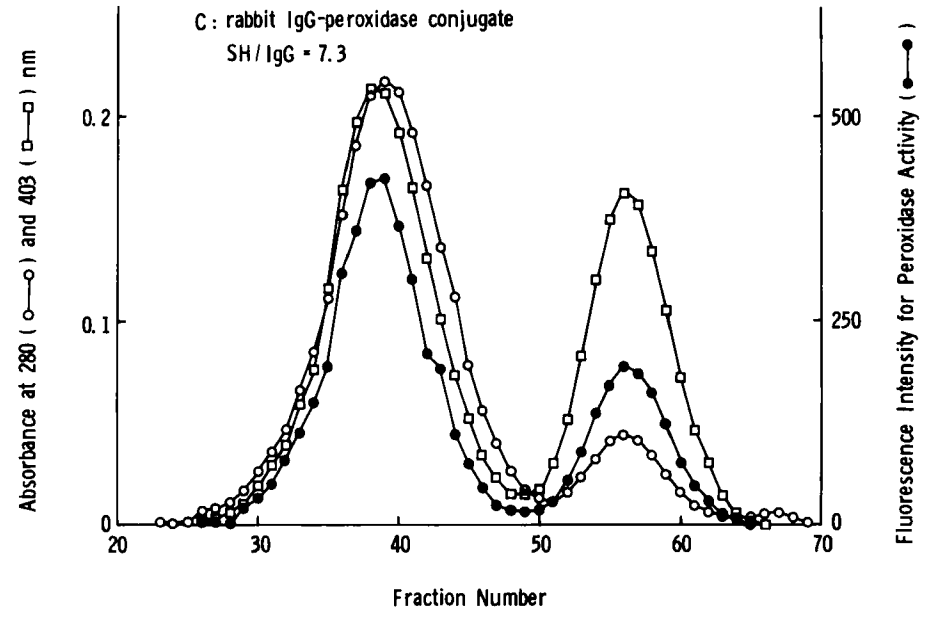


FIG. IV-10 (continued)

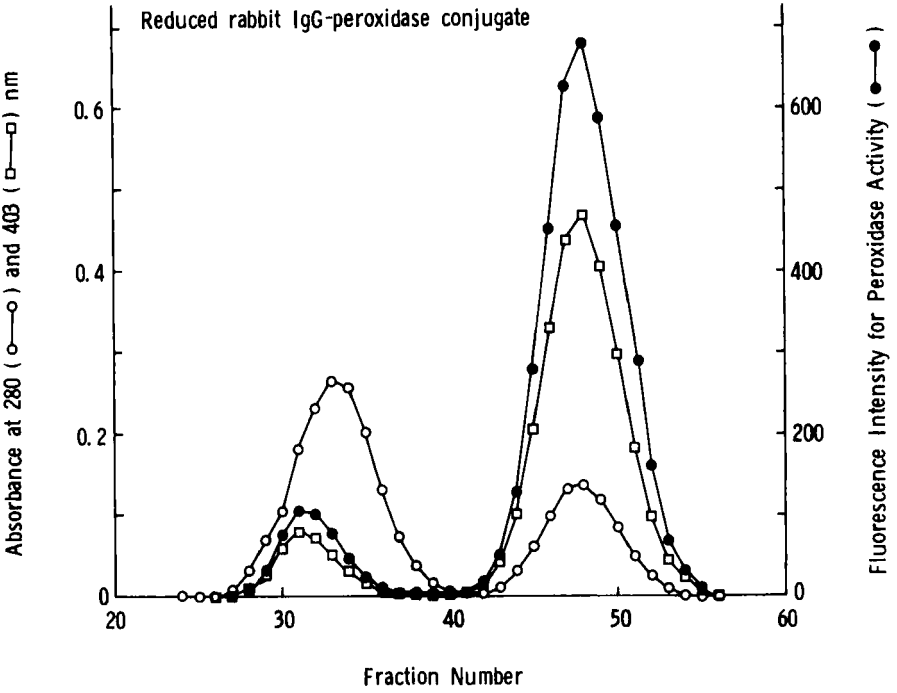


FIGURE IV-11. Elution profile from a column of Ultrogel AcA 44 of rabbit IgG-peroxidase conjugate prepared by the maleimide method (IV). The average numbers of maleimide groups introduced into peroxidase molecule and thiol groups generated per IgG molecule were both 1.3. The maleimide-peroxidase (0.174 mmol/L) and the mercaptosuccinylated IgG (0.035 mmol/L) were incubated at 4°C for 20 h. The column size used was 1.5 x 45 cm, and the fraction volume was 1.0 ml. The recovery of peroxidase in the conjugate was calculated from absorbance at 403 nm to be 11.7 %.

succinylated IgG with an average number of thiol groups of 4.3 per molecule was incubated with 165 μmol/L of maleimide-peroxidase, the number of peroxidase molecules conjugated per IgG molecule ranged from 1 to 4 as calculated from the ratio of absorbances at 403 and 280 nm (Fig. IV-10B). When 35 μmol/L of the mercaptosuccinylated IgG with an average number of thiol groups of 7.3 was

incubated with 174 μmol/L of maleimide-peroxidase, the number of peroxidase molecules conjugated per IgG molecule ranged from 2 to 5 (Fig. IV-10C). (When 1, 2, 3 and 4 molecules of peroxidase are conjugated with single molecules of IgG, the ratios of absorbance at 403 nm to that at 280 nm are 0.36, 0.64, 0.87 and 1.06, respectively.)

c. Yield of Conjugates

The yields of IgG-, $F(ab')_2$- and Fab-peroxidase conjugates increase with the number of thiol groups introduced. When 33-35 μmol/L of the mercaptosuccinylated IgG with an average number of thiol groups of 4.3 and 7.3 was incubated with 165-175 μmol/L of maleimide-peroxidase, the recovery of peroxidase in the conjugate was 24 and 62 %, respectively and the mercaptosuccinylated IgG appeared to be all converted to conjugates (Fig. IV-10B and C).

d. Nonspecific and Specific Bindings of Conjugates

Nonspecific binding of IgG- and $F(ab')_2$-peroxidase conjugates were higher than those of Fab'-peroxidase conjugates with thiol groups in the hinge, although specific binding was similar (Table IV-8). Sensitivity of sandwich enzyme immunoassay can, thererfore, be improved more easily using Fab'-peroxidase conjugates.

e. Application

As described above, IgG-, $F(ab')_2$- and Fab-peroxidase conjugates are heterogeneous and have higher nonspecific binding in

TABLE IV-8

Nonspecific and Specific Bindings of Anti-Ferritin-Peroxidase Conjugates

Conjugate	Molar ratio peroxidase/ antibody	Binding of anti-ferritin-peroxidase conjugate (mean ± SD, n = 4)		Ratio B/A
		Nonspecific (A) (%)	Specific (B) (%)	
Maleimide Conjugate				
Fab' (I)[a]	1.1	0.013 ± 0.002	1.55 ± 0.07	119
$F(ab')_2$ (III)[a]	1.6	0.045 ± 0.007	0.99 ± 0.04	22
IgG (III)[a]	1.8	0.061 ± 0.005	1.47 ± 0.10	24
Periodate conjugate				
IgG, polymeric	1.9	0.57 ± 0.005	3.84 ± 0.23	6.7
monomeric	1.9	0.20 ± 0.02	1.62 ± 0.04	8.1

Peroxidase activity nonspecifically and specifically bound was examined by sandwich enzyme immunoassay in the absence and presence of 0.45 ng (1 fmol) of ferritin per tube and expressed in percentage of the activity added per tube.
The amounts of peroxidase in conjugates used per tube were 9.4 ng on the basis of absorbance at 403 nm throughout. [a](I) and (III) indicate the maleimide method (I) and (III), respectively (IV-B and G).

sandwich enzyme immunoassays for macromolecular antigens than Fab'-peroxidase conjugates with thiol groups in the hinge. IgG- and probably $F(ab')_2$-peroxidase conjugates penetrate with difficulty into tissue sections, and these conjugates may give higher background staining. There is, therefore, no reason to

use IgG conjugates, unless sensitivity is not at issue or the antigen-binding activity of IgG is lost by pepsin digestion when preparing $F(ab')_2$.

V. LABELING WITH β-D-GALACTOSIDASE

A. Assay of β-D-Galactosidase

The usual substrates for fluorimetric and colorimetric assays for β-D-galactosidase activity are 4-methylumbelliferyl β-D-galactoside and o-nitrophenyl β-D-galactoside, respectively. The sensitivities of 10 and 100 min assays with 4-methylumbelliferyl β-D-galactoside under the condition described below are 100 and 10 fg (0.2 and 0.02 amol) of the enzyme, respectively. The sensitivities of 10 and 100 min assays with o-nitrophenyl β-D-galactoside are 530 and 53 pg (1 and 0.1 fmol), respectively.

Fluorimetric Assay

1. Prepare enzyme samples in a total volume of 0.1 ml of sodium phosphate buffer, pH 7.0, 10 mmol/L, containing 100 mmol/L NaCl, 1 mmol/L $MgCl_2$, 50-1000 mg/L bovine serum albumin and 1 g/L NaN_3 (buffer A, pH 7.0). In this buffer, 4-methylumbelliferyl β-D-galctoside is slowly hydrolysed, increasing the background of the assay. The concentration of bovine serum albumin should be reduced to 50-100 mg/L to reduce hydrolysis, when the assay is performed with long incubation periods.
2. Dissolve 10 mg (0.03 mmol) of 4-methylumbelliferyl β-D-galactoside (m.w. 338.3) in 2.0 ml of N,N-dimethylformamide by shaking

at 37°C for 4-5 h and add 98 ml of deionized water to give 300 μmol/L.

3. Incubate the sample solution at 30°C for 5 min, and then start the enzyme reaction by adding 0.05 ml of the substrate solution.
4. Continue the incubation at 30°C for 10-60 min in most assays.
5. Stop the enzyme reaction by adding 2.5 ml of glycine-NaOH buffer, pH 10.3, 100 mmol/L.
6. Measure fluorescence intensity using 10-1000 nmol/L 4-methyl-umbelliferone (m.w. 176.2) in glycine-NaOH buffer, pH 10.3, 0.1 mol/L as standard. Use 360 nm for excitation and 450 nm for emission analysis.

B. Dimaleimide Methods for Labeling Fab' with β-D-Galactosidase

Two methods are described. In the dimaleimide method (I), Fab', which contains approximately one thiol group in the hinge, is treated with excess of N,N'-o-phenylenedimaleimide to introduce maleimide groups, and the maleimide-Fab' obtained is allowed to react with thiol groups in the native form of β-D-galactosidase from Escherichia coli (Fig. V-1). In dimaleimide method (II), β-D-galactosidase is treated with excess of N,N'-o-phenylene dimaleimide to introduce maleimide groups, and the maleimide-enzyme obtained is allowed to react with thiol groups in the hinge of Fab' (Fig. V-2).

The dimaleimide method (II) is more useful especially for efficient labeling of Fab' in a case where 0.1 mg or less of

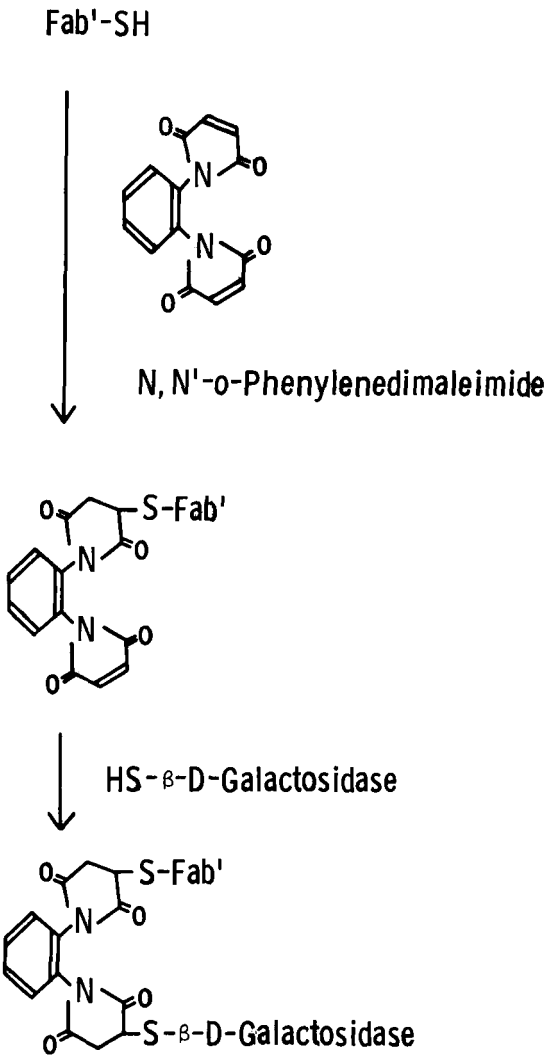


FIGURE V-1. Preparation of Fab'-ß-D-galactosidase conjugate by the dimaleimide method (I).

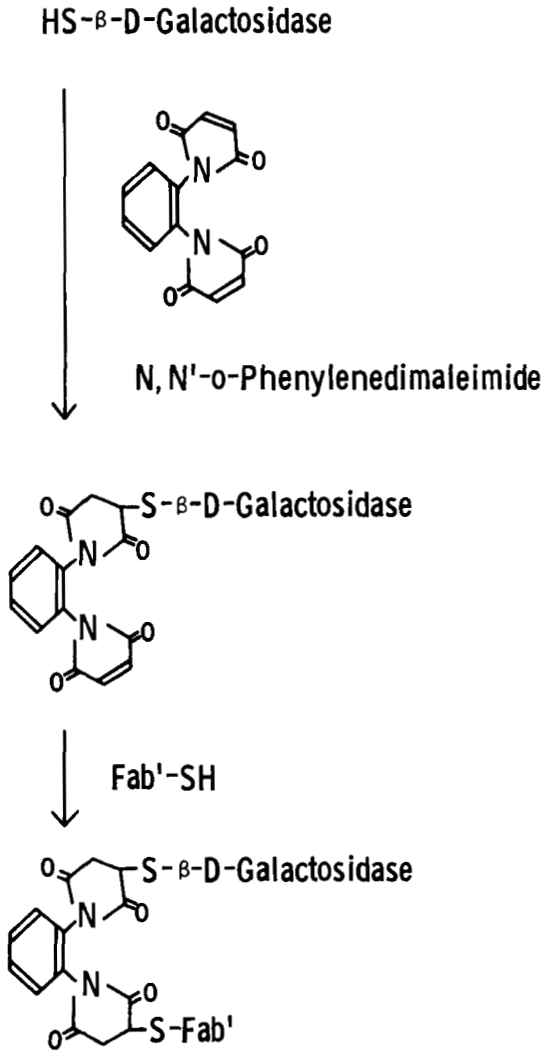


FIGURE V-2. Preparation of Fab'-β-D-galactosidase conjugate by the dimaleimide method (II).

affinity-purified $F(ab')_2$ is available. In the previously reported method, $F(ab')_2$ is subjected twice to gel filtration after reduction with 2-mercaptoethylamine and treatment with the dimaleimide (35, 36, 52). In the dimaleimide method (I) described here, gel filtration is required only once but $F(ab')_2$ has to be concentrated to 0.1 mg/0.18 ml to minimize the amounts of 2-mercaptoethylamine and N,N'-o-phenylenedimaleimide used, since the solubility of the dimaleimide in buffers is limited. Concentration to such a small volume is accompanied by a large loss of $F(ab')_2$. The difficulties are minimized in the dimaleimide method (II).

A method in which maleimide groups are introduced into Fab' or Fab using crosslinking reagents such as N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate and N-succinimidyl m-maleimidobenzoate is not recommended. There are three reasons for this. First, it is not easy to control the number of maleimide groups introduced into Fab' or Fab using these reagents, and the conjugates are formed in low yields with few maleimide groups and with polymerization. Secondly, the antigen-binding activity of Fab' or Fab is not retained when conjugation involves amino groups of these fragments, as shown in the maleimide methods for labeling Fab' with horseradish peroxidase (IV-F). Thirdly, it is not possible to monitor conjugation efficiency using fluorescein-labeled fragments when amino groups are used for conjugation.

These dimaleimide methods are reproducible with due caution. The content of thiol groups in ß-D-galactosidase should be defined

before use, since it is not sufficient in some commercially available preparations (35, 36, 52). The maleimide groups introduced into Fab' and ß-D-galactosidase should also be estimated. Maleimide-Fab' and ß-D-galactosidase should be kept at low pH and temperature to ensure stability (35-37, 57). Fab'-ß-D-galactosidase conjugate cannot be separated from free enzyme by gel filtration, since the molecular weight of ß-D-galactosidase is much larger (540,000) than that of Fab' (46,000). Therefore, excess of maleimide-Fab' or Fab' should be used to completely convert the enzyme to the conjugate. It is helpful to monitor conjugation efficiency by adding to the $F(ab')_2$ a small amount of fluorescein-labeled $F(ab')_2$.

a. Dimaleimide Method (I) for Labeling Fab' with ß-D-Galactosidase

Introduction of maleimide groups into Fab'

1. Prepare 0.1-1.4 mg (1.1-15 nmol) of $F(ab')_2$ plus 2.5-35 µg of fluorescein-labeled normal $F(ab')_2$ in 0.18 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L (III-C).
2. Add to the $F(ab')_2$ solution 0.02 ml of 0.1 mol/L 2-mercaptoethylamine in sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 5 mmol/L EDTA, which should be freshly prepared before use.
3. Incubate the mixture at 37°C for 90 min.
4. Add to the reaction mixture 0.25-0.7 ml of sodium acetate buffer, pH 5.0, 0.1 mol/L, and then 50-100 µl of 0.4 mol/L (107 g/L) N,N'-o-phenylenedimaleimide (m.w. 268) in N,N-dimethylformamide.

5. Incubate the mixture at 30°C for 20 min, and then centrifuge at 3,000 rpm for 10 min to remove excess of precipitated reagent.
6. Apply the reaction mixture to a Sephadex G-25 column (1.0 x 30-45 cm) using sodium acetate buffer, pH 5.0, 20 mmol/L.
7. For measuring maleimide groups introduced, prepare the maleimide-Fab' in 0.45 ml of sodium acetate buffer, pH 5.0, 20 mmol/L, with an absorbance at 280 nm of 0.7-1.0, and perform further steps in the same way as in labeling with peroxidase (III-D and IV-B). The average number of maleimide groups introduced per Fab' molecule under the above condition was 0.83-0.87. Do not use NaN_3 as a preservative, since maleimide groups decompose very rapidly in its presence.

Preparation of ß-D-galactosidase

Prepare 0.11-1.6 mg (0.2-3.0 nmol) of ß-D-galactosidase from Escherichia coli by centrifugation when commercial enzyme suspensions in 3.2 mol/L $(NH_4)_2SO_4$ are used. The enzyme preparation should be shown to contain more than 10 thiol groups per enzyme molecule (III-D for measuring thiol groups). ß-D-Galactosidase suspensions from Boehringer usually contains 11-16 thiol groups per enzyme molecule, although they may sometimes contain less than 10 (35, 36, 52). Lyophilized preparations of ß-D-galactosidase from Boehringer usually contain 20-24 thiol groups per enzyme molecule. Calculate the amount of ß-D-galactosidase from its absorbance at 280 nm by taking the extinction coefficient at 280 nm and the molecular weight of the enzyme to be 2.09 $g^{-1}.L.cm^{-1}$ and 540,000, respectively (72). The amount of enzyme in

commercially available suspensions should be measured from absorbance at 280 nm after removing bovine serum albumin which remains in the supernatant (35, 36, 52).

Conjugation

1. Mix the maleimide-Fab' and ß-D-galactosidase in sodium phosphate buffer, pH 6.0-6.5, 20-100 mmol/L. The maleimide-Fab' solution should be concentrated either before or after mixing with ß-D-galactosidase, and the pH of the maleimide-Fab' solution should be adjusted to 6.0-6.5 before mixing with ß-D-galactosidase. The final concentration of ß-D-galactosidase in the reaction mixture should be 1-10 µmol/L, and the molar ratio (Fab'/enzyme) in the reaction mixture for conjugation should be 3-10.
2. Incubate the mixture at 4°C for 20 h.
3. Apply the reaction mixture to a Sepharose 6B column (1.5 x 45 cm) using sodium phosphate buffer, pH 6.5, 10 mmol/L, containing 0.1 mol/L NaCl, 1 mmol/L $MgCl_2$, 1 g/L NaN_3 and 1 g/L bovine serum albumin (buffer A, pH 6.5).
4. 1) Assay ß-D-galactosidase activity in each fraction. 2) Determine fluorescence intensity of fluorescein-labeled Fab' in each fraction using 1 nmol/L fluorescein as a standard to monitor labeling efficiency, using 490 nm for excitation and 510 nm for emission. 3) Store the conjugate in buffer A at 4°C, since ß-D-galactosidase activity is lost at 4°C in the absence of proteins such as serum albumin and also by freezing-thawing even in the presence of bovine serum albumin. How-

ever, it was reported that the conjugate could be lyophilized in the presence of 2.5 g/L of bovine serum albumin, and that the lyophilized conjugate was stable at -20 and 4°C for at least 2 months (39).

b. Dimaleimide Method (II) for Labeling Fab' with ß-D-Galactosidase

Introduction of maleimide groups into ß-D-galactosidase

1. Prepare 0.11-1.6 mg (0.2-3.0 nmol) of ß-D-galactosidase in 0.2 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L (V-B-a).
2. Add 1-6 µl (200-1,200 nmol) of 0.2 mol/L (53.6 g/L) N,N'-o-phenylenedimaleimide in N,N-dimethylformamide.
3. Incubate the mixture at 30°C for 20 min.
4. Apply the reaction mixture to a Sephadex G-25 column (1.0 x 30-45 cm) using the same buffer containing 0.1-1 g/L SH-blocked bovine serum albumin, which is prepared by incubation with 0.1 mol/L N-ethylmaleimide at 30°C for 60 min followed by gel filtration (35, 36, 52). The average number of maleimide groups introduced into ß-D-galactosidase is 12 per molecule.

Preparation of Fab'

Prepare Fab' from 0.1-1.4 mg (1.1-15 nmol) of $F(ab')_2$ plus 2.5-35 µg of fluorescein-labeled normal $F(ab')_2$ as described in the section of III-D.

Conjugation

1. Incubate the maleimide-ß-D-galactosidase and Fab' in sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 1 mmol/L EDTA

in the same way as in the dimaleimide method (I) described above.

2. Add 1/100 volume of 100 g/L NaN_3 to the reaction mixture to destroy excess of maleimide groups. Blocking of maleimide groups by incubation with 1 mmol/L 2-mercaptoethanol, 2-mercaptoethylamine or N-acetyl L-cysteine gives conjugates which are equally useful in sandwich enzyme immunoassay.
3. Perform further steps in the same way as in the dimaleimide method (I) described above.

C. Pyridyl Disulfide Method (II) for Labeling Fab' with ß-D-Galactosidase

The principle of this method is the same as that of the pyridyl disulfide method (II) for labeling Fab' with horseradish peroxidase (IV-E-d). Pyridyl disulfide groups are introduced into the hinge of Fab' using 4,4'-dithiodipyridine and then allowed to react with thiol groups in the native form of ß-D-galactosidse. The use of 2,2'-dithiodipyridine results in a lower conjugate yield.

Introduction of pyridyl disulfide groups into Fab'

Prepare the pyridyl disulfide-Fab' from 0.5-1.4 mg (5.4-15 nmol) of $F(ab')_2$ plus 12.5-25 µg of fluorescein-labeled normal $F(ab')_2$ in the same way as in the pyridyl disulfide method (II) for labeling Fab' with horseradish peroxidase (IV-E-d).

Conjugation

Incubate the pyridyl disulfide-Fab' (0.45-1.2 mg, 10-26 nmol) and ß-D-galactosidase (0.5-2.1 mg, 1-4 nmol) in sodium phosphate

buffer, pH 6.5-7.5, 0.1 mol/L, at 30°C for 2 h or at 4°C for 20 h. Add 0.1 M N-ethylmaleimide to a final concentration of 4 mmol/L to block remaining thiol groups. Concentrate the pyridyl disulfide-Fab' solution either before or after mixing with ß-D-galactosidase. The final concentrations of the pyridyl disulfide-Fab' and ß-D-galactosidase for conjugation should be 5-10 and 50 μmol/L, respectively.

D. Characterization of Fab'-ß-D-Galactosidase Conjugate

a. Purity of the Conjugates

Fab'-ß-D-galactosidase conjugates could be completely separated from unconjugated Fab' by gel filtration, since the molecular weights of the enzyme and Fab' are 540,000 (72) and 46,000 (64, 65), respectively (Fig. V-3). There was no significant formation of dimer or polymer of Fab' during the conjugation reaction.

In the dimaleimide methods described above, excess of Fab' is incubated with ß-D-galactosidase to completely convert the enzyme to Fab'-enzyme conjugate, since this cannot be separated from unconjugated enzyme by gel filtration. In order to test for the presence of free enzyme in the preparation of rabbit Fab'-ß-D-galactosidase conjugate obtained by the dimaleimide method (I), 10 milliunits of the conjugate in 0.1 ml of buffer A, pH 7.0 (V-A) was applied to a goat (anti-rabbit IgG) IgG-Sepharose 4B column (3 x 30 mm) using buffer A, pH 7.0. More than 99 % of ß-D-galactosidase activity was adsorbed to the column, and the conjugate was recovered 93-99 % from a normal goat IgG-Sepharose 4B

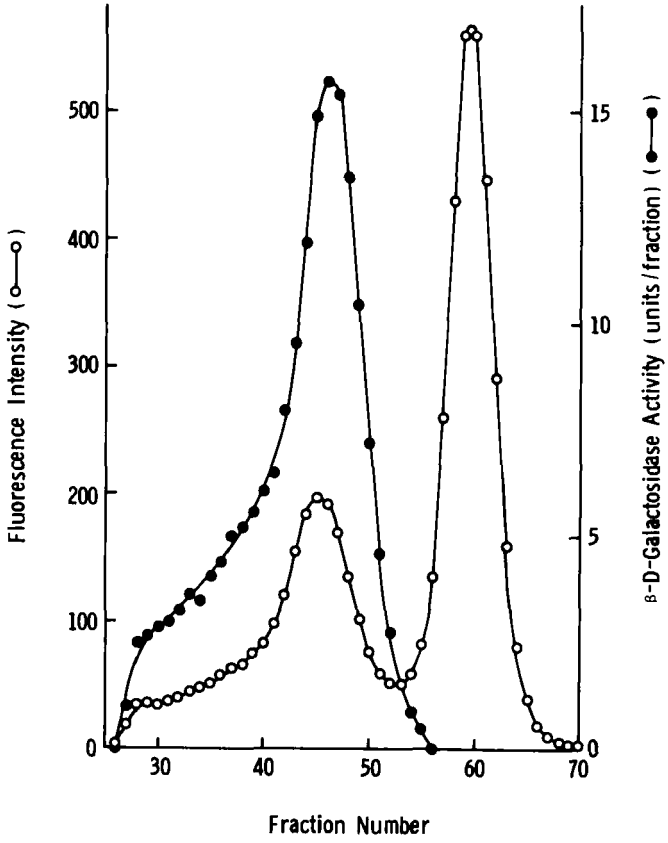


FIGURE V-3. Elution profile from a Sepharose 6B column of rabbit Fab'-ß-D-galactosidase conjugate prepared by the dimaleimide method (I). The amounts of ß-D-galactosidase and the maleimide-Fab' used for conjugation were 1.35 and 0.46 mg (2.5 and 10 nmol), respectively. The concentrations of ß-D-galactosidase and the maleimide-Fab' incubated for conjugation were 5 and 20 µmol/L, respectively. The column size was 1.5 x 45 cm, and the fraction volume was 0.96 ml. Fluorescence intensity of fluorescein-labeled Fab' was measured by adjusting that of 1 nmol/L fluorescein to a scale of 100. ß-D-Galactosidase activity was assay using 4-methylumbelliferyl-ß-D-galactoside. The proportion of Fab' incubated for conjugation was calculated from fluorescence intensity of fluorescein-labeled Fab' to be 41 % and the average number of Fab' molecules conjugated per ß-D-galactosidase molecule was 1.7.

column (IV-C) (35, 36, 52). This indicated that more than 99 % of the enzyme molecules in the conjugate was associated with at least one molecule of rabbit Fab' and that little free ß-D-galactosidase was present in the conjugate preparation.

b. Recovery of ß-D-Galactosidase and Fab'

As described above, more than 99 % of ß-D-galactosidase activity in rabbit Fab'-ß-D-galactosidase conjugate was adsorbed to a goat (anti-rabbit IgG) IgG-Sepharose 4B column, indicating that the recovery of the enzyme in the conjugate was more than 99 %.

The recovery of Fab' in the conjugate was calculated from the fluorescence intensity of fluorescein-labeled Fab' in the elution profile (Fig. V-3). It increased with the concentrations of ß-D-galactosidase and Fab' and increased as the molar ratio of Fab' to ß-D-galactosidase was reduced in the reaction mixture. In the dimaleimide methods, the recovery of Fab' in the conjugate was approximately 40 %, when more than 1.0 mg of Fab' were used and the concentration of the enzyme for conjugation was 5-10 µmol/L with a molar ratio of Fab' to enzyme of 3-5. In the pyridyl disulfide method with 4,4'-dithiodipyridine, the recovery of Fab' in the conjugate was 15-25 %.

c. Molecular Size of the Conjugate

The average number of Fab' molecules conjugated per enzyme molecule was calculated from the amounts of Fab' and ß-D-galacto-

sidase conjugated. The amount of Fab' conjugated was calculated from the amount of Fab' incubated for conjugation and fluorescence intensity of fluorescein-labeled Fab' in the elution profile (Fig. V-3), and the amount of enzyme conjugated was taken to be equal to that incubated for conjugation, since the enzyme and Fab' were almost completely recovered from a Sepharose 6B column and the enzyme was almost completely converted to the conjugate (35, 36, 52).

Fab'-ß-D-galactosidase conjugate in the major peak eluted from a Sepharose 6B column consisted of one enzyme molecule and 1-4.5 Fab' molecules, when the concentration of ß-D-galactosidase for the conjugation was 1-10 µmol/L, and the molar ratio of Fab' to enzyme was 3-10. The number of Fab' molecules conjugated per enzyme molecule increased with the molar ratio. A minor component of the conjugate was eluted before the main peak and probably contained more than one enzyme molecule per conjugate molecule, due to the presence of dimerized or polymerized enzyme molecules in the original enzyme preparation. There was no significant dimerization or polymerization of ß-D-galactosidase when the concentration of ß-D-galactosidase used for conjugation was 1-10 µmol/L, but more conjugate was polymerized when higher concentrations were used.

d. ß-D-Galactosidase Activity in the Conjugates

There was no loss of ß-D-galactosidase activity in the dimaleimide and pyridyl disulfide conjugation methods.

e. Antigen-Binding Activity of the Conjugate

About 10 milliunits of rabbit (anti-human IgG) Fab'-ß-D-galactosidase conjugate in 0.1 ml of buffer A, pH 7.0 from the main peak fractions 40-50 (Fig. V-3) was applied to a column (0.3 x 1.5 cm) of human IgG-Sepharose 4B using buffer A, pH 7.0 (V-A), and the enzyme activity in the effulent was compared with that before the application. As a control, normal rabbit Fab'-ß-D-galactosidase conjugate was passed through the same column. The percentage of ß-D-galactosidase activity adsorbed to the column was 65-69 %, while the enzyme activity in normal Fab'-ß-D-galactosidase conjugate was fully recovered in the effluent. The average number of Fab' molecules conjugated per ß-D-galactosidase molecule was 4.0-4.3, and the content of anti-human IgG antibody (IgG) molecules in the IgG fraction used for the conjugate preparation was 22 %. From these values, statistically expected percentages of the adsorption of the enzyme activity on the column were calculated as follows :

$$(1-0.78^{n}) \times 100$$

where n = the number of Fab' molecules conjugated per enzyme molecule and 78 % = the content of non-specific IgG in (anti-human IgG) IgG fraction used. It may therefore be predicted that 63-66 % of these conjugate molecules would be associated with at least one anti-human IgG antibody (Fab') molecule, indicating that the ability of anti-human IgG (Fab') antibody to bind to human IgG was well preserved in the conjugates.

The average number of Fab' molecules conjugated per enzyme molecule in fractions 27-34 were 5.0-5.7, with a predicted 71-76 % of the enzyme activity in these fractions adsorbed to a human IgG-Sepharose 4B column. However, these fractions contained polymer enzyme conjugate and the Fab' molecules associated with each polymer conjugate molecule must have been at least twice as many as those conjugated per enzyme molecule. Therefore, the enzyme activity in these fractions would have to have been adsorbed at least 94 %, compared with the observed 86-88 %. This indicates that the activity of anti-human IgG in polymerized enzyme conjugates was partly lost.

f. Stability of the Cross-Link

Although thioether bonds formed by the reaction of thiol and maleimide groups are resistant to acid and alkaline hydrolysis, succinimides formed by the reaction of maleimide groups with thiol groups are not very stable. A careful examination by gel filtration of fluorescein-labeled Fab'-ß-D-galactosidase conjugate has, however, shown that little Fab' is released from the conjugate when stored at pH 6-7 at 4°C for at least 1 year. This has been confirmed in another laboratory (39). ß-D-Galactosidase conjugate of Fab' antibody to ornithine aminotransferase from rat liver was stored under the above conditions for 4 years and gave unchanged sensitivity in a sandwich enzyme immunoassay. The crosslinkages formed between human IgG and ß-D-galactosidase by the dimaleimide method were not split by incubation with undiluted serum at room temperature overnight (73).

g. Usefulness of the Conjugate

The use of Fab'-ß-D-galactosidase conjugates enabled us to measure 0.05 amol of human ferritin (1), 2 amol of human IgE (55) and 2 amol of human thyroid-stimulating hormone (56).

E. Maleimide Methods for Labeling IgG with ß-D-Galactosidase

Three methods are available. The dimaleimide method (I) uses N,N'-o-phenylenedimaleimide as a crosslinking reagent. IgG is reduced to generate thiol groups in the hinge portion and treated with excess of the dimaleimide to introduce maleimide groups. The maleimide-IgG obtained is allowed to react with thiol groups in the native form of ß-D-galactosidase from Escherichia coli. In the dimaleimide method (II), ß-D-galactosidase is treated with excess of N,N'-o-phenylenedimaleimide to introduce maleimide groups, and the maleimide-enzyme is allowed to react with thiol groups generated in the hinge of IgG by reduction. In the maleimide method (III), IgG is treated with N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate to introduce maleimide groups, and the maleimide-IgG obtained is allowed to react with thiol groups in the native form of ß-D-galactosidase.

The dimaleimide method (II) is more useful than the dimaleimide method (I) when the IgG to be labeled is available only in a small quantity (V-B). The maleimide method (III) is not recommended for labeling IgG (V-B). Polymerized conjugates are formed with increasing concentrations of IgG and ß-D-galactosidase, especially in the maleimide method (III).

a. Dimaleimide Method (I) for Labeling IgG with β-D-Galactosidase

Introduction of maleimide groups into IgG

Prepare the maleimide-IgG from 0.15-1.5 mg (1-10 nmol) IgG plus 4-40 μg fluorescein-labeled normal IgG in the same way as the maleimide-Fab' from $F(ab')_2$ in the dimaleimide method (I) for labeling Fab' (V-B-a). The average number of maleimide groups introduced into IgG was 2.6 per molecule.

Conjugation

Incubate 0.13-1.3 mg (0.87-8.7 nmol) of the maleimide-IgG and 86-860 μg (0.16-1.6 nmol) of β-D-galactosidase in the same way as in the dimaleimide method (I) for labeling Fab' (V-B-a). The concentrations of the maleimide-IgG and β-D-galactosidase in the mixture for conjugation should be about 25 and 5 μmol/L, respectively. Add 10 μl of 0.1 mol/L 2-mercaptoethylamine to the reaction mixture to block remaining maleimide groups, and perform gel filtration and further steps in the same way as in the dimaleimide method (I) for labeling Fab' (V-B-a).

b. Dimaleimide Method (II) for Labeling IgG

Preparation of the reduced IgG

Prepare the reduced normal IgG from 0.9 mg IgG plus 30 μg fluorescein-labeled IgG in the same way as described in III-B.

Preparation of the maleimide-β-D-galactosidase

Prepare the maleimide-β-D-galactosidase from 1.0 mg (1.9 nmol) of β-D-galactosidase as described in the section of V-B-b.

Conjugation

Incubate 86-860 μg (0.16-1.6 nmol) of maleimide-β-D-galactosidase and 0.13-1.3 mg (0.86-8.6 nmol) of the reduced IgG and perform further steps in the same way as in the dimaleimide method (II) for labeling Fab' (V-B-b). The remaining maleimide groups are blocked by adding 10 μl of 0.1 mol/L 2-mercaptoethylamine.

c. Maleimide Method (III) for Labeling IgG

Introduction of maleimide groups into IgG

1. Prepare 1.4 mg (9.3 nmol) of IgG in 0.5 ml of sodium phosphate buffer, pH 7.0, 0.1 mol/L.
2. Add to the IgG solution 50 μl (135 nmol) of 0.9 g/L N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate in N,N-dimethylformamide.
3. Incubate the mixture at 30°C for 30 min.
4. Apply the reaction mixture to a Sephadex G-25 (1.0 x 45 cm) using sodium phosphate buffer, pH 6.5, 0.1 mol/L. The average number of maleimide groups introduced is 3.2 per molecule.

Conjugation

1. Dissolve 1.5 mg (2.8 nmol) of β-D-galactosidase lyophilized or in a pellet (V-B-a) in 1.4 ml of sodium phosphate buffer, pH 6.5, 0.1 mol/L, containing 1.25 mg (8.3 nmol) of the maleimide-IgG so that the final concentrations of the enzyme and IgG are 2 and 6 μmol/L, respectively.

2. Perform further processes in the same way as in the dimaleimide method (I) for labeling Fab'.

F. Characterization of IgG-ß-D-Galactosidase Conjugate (35,52)

ß-D-Galactosidase used for conjugation was almost completely converted to the conjugate, when excess of IgG was used for conjugation, and there was little free enzyme in the conjugate preparation. This was shown by an almost complete adsorption of the conjugate to anti-IgG Sepharose 4B. Caution is required to minimize the proportion of unconjugated IgG eluted in the conjugate fractions, since the molecular weight of IgG (150,000) is closer to that of the enzyme than that of Fab'. The average number of IgG molecules conjugated per ß-D-galactosidase molecule was 1-2, and the recovery of IgG in the conjugate was 23-29 %. Although specific binding of IgG- and of Fab'-ß-D-galactosidase conjugates were similar, nonspecific binding of IgG conjugates was higher. Therfore, there is no reason to use IgG conjugtes unless sensitivity is not at issue or the antigen-binding activity is lost by pepsin digestion.

VI. LABELING WITH GLUCOSE OXIDASE

A. Assay of Glucose Oxidase

The assay of glucose oxidase is usually performed by measuring H_2O_2 which is produced by the catalytic action of glucose oxidase in the presence of glucose. Although a variety of

methods are available for measuring H_2O_2, peroxidase has been most widely used for this purpose. The method described below uses p-hydroxyphenylacetic acid as substrate for peroxidase. The sensitivities of 10 and 100 min assays using glucose oxidase from Aspergillus niger under the condition described below are 7.7 and 0.77 pg (50 and 5 amol), respectively. The assay with p-hydroxyphenylpropionic acid is slightly less sensitive.

Fluorimetric assay with p-hydroxyphenylacetic acid

1. Dissolve 50 mg p-hydroxyphenylacetic acid in 50 ml of sodium acetate buffer, pH 5.0, 50 mmol/L, to give a concentration of 1 g/L, and adjust the pH 5.0 with 10 mol/L NaOH.
2. Dissolve 1 mg of horseradish peroxidase (grade I, Boehringer Mannheim) in 50 ml of the substrate solution, pH 5.0.
3. Add 10 µl of enzyme in sodium phosphate buffer, pH 7.0, 10 mmol/L, containing 100 mmol/L NaCl and 1 g/L bovine serum albumin to 0.25 ml of the substrate-peroxidase solution, and incubate the mixture at 30°C for 5 min.
4. Start the enzyme reaction by adding 50 µl of 100 g/L glucose and continue the incubation at 30°C for 10-60 min in most experiments.
5. Stop the enzyme reaction by adding 2.5 ml of glycine-NaOH buffer, pH 10.3, 0.1 mol/L.
6. Measure fluorescence intensity using 320 nm for excitation and 405 nm for emission. Adjust the fluorescence intensity of 1 mg/L of quinine in 0.1 N H_2SO_4 to a scale of 100.

B. Maleimide Methods for Labeling Fab' and IgG with Glucose Oxidase (42, 53)

a. Maleimide Method (I) for Labeling Fab' with Glucose Oxidase

The principle for labeling Fab' with glucose oxidase is essentially the same as that with horseradish peroxidase. In the first step, glucose oxidase is treated with N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate to introduce maleimide groups. In the second step, maleimide groups introduced into glucose oxidase are allowed to react with thiol groups of Fab'.

Glucose oxidase has many amino groups which are reactive with N-hydroxysuccinimide ester, although horseradish peroxidase has only 2-3 amino groups. Therefore, the number of maleimide groups introduced into glucose oxidase cannot be easily controled, in contrast to the less than 2 groups per molecule of horseradish peroxidase even with excess reagent. As a result, the Fab'-glucose oxidase conjugate prepared by the maleimide method (I) is heterogeneous, consisting of conjugate molecules associated with various numbers of Fab' molecules, while the Fab'-peroxidase conjugate prepared by the maleimide method (I) is largely monomeric (IV-C). In addition, conjugates associated with different numbers of Fab' molecules and unconjugated glucose oxidase can barely be separated from each other by gel filtration, since the molecular weights of Fab' and glucose oxidase are 46,000 (64, 65) and 153,000 (74), respectively. Therefore, an excess of Fab' should be used to completely convert the maleimide-

glucose oxidase to conjugates. Losses of enzyme activity by conjugation with Fab' and IgG were 15 and 26 %, respectively.

Introduction of maleimide groups into glucose oxidase

1. Dissolve 6 mg (39 nmol) of glucose oxidase in 0.6 ml of sodium phosphate buffer, pH 7.0, 0.1 mol/L. Grade I preparations from Boehringer Mannheim are highly purified, while grade II preparations contain other proteins in significant proportions and have to be purified by gel filtration with Ultrogel AcA 34 before use. The ratio of absorbance at 280 nm to that at 450 nm for the purified glucose oxidase was 11, which is an indicator of purity. Calculate the amount of glucose oxidase from Aspergillus niger by taking the extinction coefficient at 280 nm and the molecular weight of the enzyme to be 1.67 $g^{-1}.L.cm^{-1}$ of (75) and 153,000 (74), respectively.
2. Dissolve 0.64 mg (1,950 nmol) of N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (m.w. 334.33) (Zieben Chemical Co., Tokyo or Pierce Chemical Co. Rockford, Ill.) in 20 µl of N,N-dimethylformamide.
3. Add 20 µl of maleimide reagent solution (50-fold molar excess) to 0.6 ml of glucose oxidase solution.
4. Incubate the mixture at 30°C for 30-120 min with continuous stirring.
5. Apply the reaction mixture to a Sephadex G-25 column (1.0 x 45 cm) using sodium phosphate buffer, pH 6.0, 0.1 mol/L.
6. For measuring maleimide groups, prepare the maleimide-glucose oxidase in 0.45 ml of sodium phosphate buffer, pH 6.0, 0.1

mol/L, with an absorbance at 280 nm of 0.7-1.0, and perform further steps in the same way as in labeling Fab' with horseradish peroxidase (IV-B). The average number of maleimide groups introduced under the above condition was 3.3-6.4 per enzyme molecule.

Conjugation

1. Prepare 3.0-3.8 mg (20-25 nmol) of the maleimide-glucose oxidase in 0.2 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L.
2. Prepare 3.7-4.6 mg (80-100 nmol) of Fab' plus 0.12 mg of fluorescein-labeled normal Fab' in 0.3 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 5 mmol/L EDTA (III-D). Fluorescein-labeled Fab' is added to monitor efficiency of the conjugation.
3. Mix the maleimide-glucose oxidase and Fab' solutions. The final concentrations of the maleimide-glucose oxidase and Fab' incubated for conjugation are 40-50 and 160-200 μmol/L, respectively.
4. Incubate the mixture at 4°C for 20 h.
5. Add to the reaction mixture 0.4 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L, and 10 μl of 100 mmol/L 2-mercaptoethylamine, and incubate the reaction mixture at room temperature for 20 min to block remaining maleimide groups.
6. Apply the reaction mixture to a column (1.5 x 45 cm) of Ultrogel AcA 34 using sodium phosphate buffer, pH 6.5, 0.1 mol/L.

7. 1) Read absorbance at 280 and 450 nm. 2) Measure fluorescence intensity of each fraction using 10 nmol/L fluorescein as a standard to monitor efficiency of the conjugation. Use 490 nm for excitation and 510 nm for emission. 3) Determine glucose oxidase activity of each fraction.
8. Store the Fab'-glucose oxidase conjugate at 4°C after adding NaN_3 to give a final concentration of 0.5 g/L and bovine serum albumin to give a final concentration of 1 g/L. Under these conditions, the cross-link formed in the conjugate molecule is stable for at least 1 year as shown by monitoring the release of fluorescein-labeled Fab' from fluorescein-labeled Fab'-glucose oxidase conjugate (42, 53) (IV-C) (Fig. VI-1).

b. Maleimide Method (III) and (IV) for Labeling IgG with Glucose Oxidase

Introduction of maleimide groups into glucose oxidase

Maleimide groups are introduced into glucose oxidase using N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate in the same way as in the maleimide method (I) for labeling Fab' with glucose oxidase. Thiol groups are introduced into IgG using S-acetylmercaptosuccinic anhydride in the same way as in the maleimide method (III) for labeling IgG with horseradish peroxidase (IV-G) or are generated by reduction of IgG with 2-mercaptoethylamine (III-B). The maleimide-glucose oxidase is allowed to react with the thiol groups of the mercaptosuccinylated IgG (Maleimide method III) or the reduced IgG (Maleimide method IV).

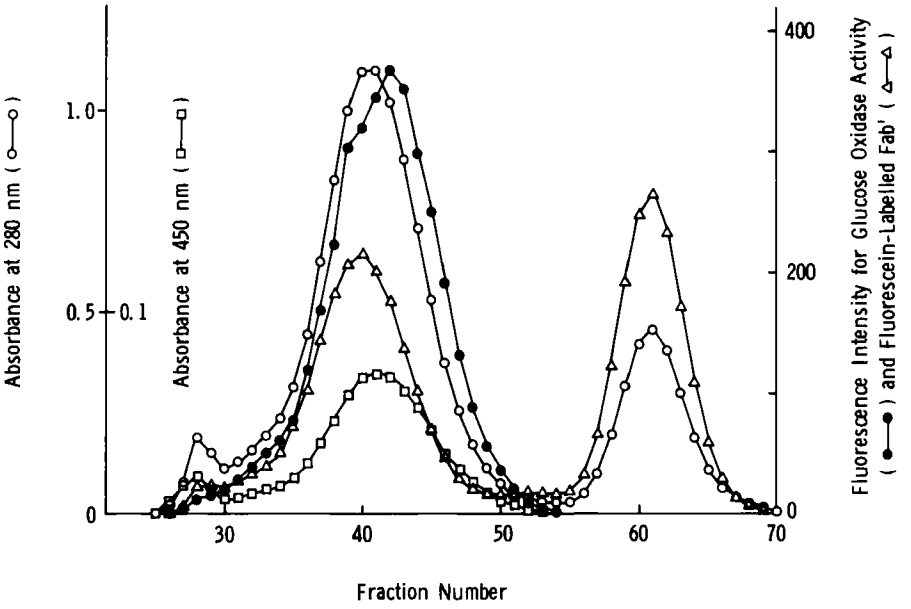


FIGURE VI-1. Elution profile from a Ultrogel AcA 34 of rabbit Fab'-glucose oxidase conjugate prepared by the maleimide method (I). The amounts of glucose oxidase and Fab' used were about 3 and 4 mg, respectively. The number of maleimide groups introduced per glucose oxidase was 5.5. The maleimide-glucose oxidase (0.042 mmol/L) and Fab' (0.166 mmol/L) were incubated at 4°C for 20 h. The column size used was 1.5 x 45 cm, and the fraction volume was 0.86 ml. Fluorescence intensity of fluorescein-labeled Fab' was measured by adjusting fluorescence intensity of 10 nmol/L fluorescein to a scale of 100. Glucose oxidase activity was determined by 10 min-assay using p-hydroxyphenylacetic acid. The recovery in the conjugate of Fab' incubated for the conjugation was calculated from fluorescence intensity of fluorescein-labeled Fab' to be 54.3 %. The average number of Fab' molecules conjugated per glucose oxidase molecule was calculated from fluorescence intensity of fluorescein-labeled Fab' to be 2.2.

Reduction of IgG

1. Prepare 5 mg of IgG plus 50 μg of fluorescien-labeled IgG in 0.54 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L.
2. Add 60 μl of 100 mmol/L 2-mercaptoethylamine-HCl, pH 6.0, containing 5 mmol/L EDTA.

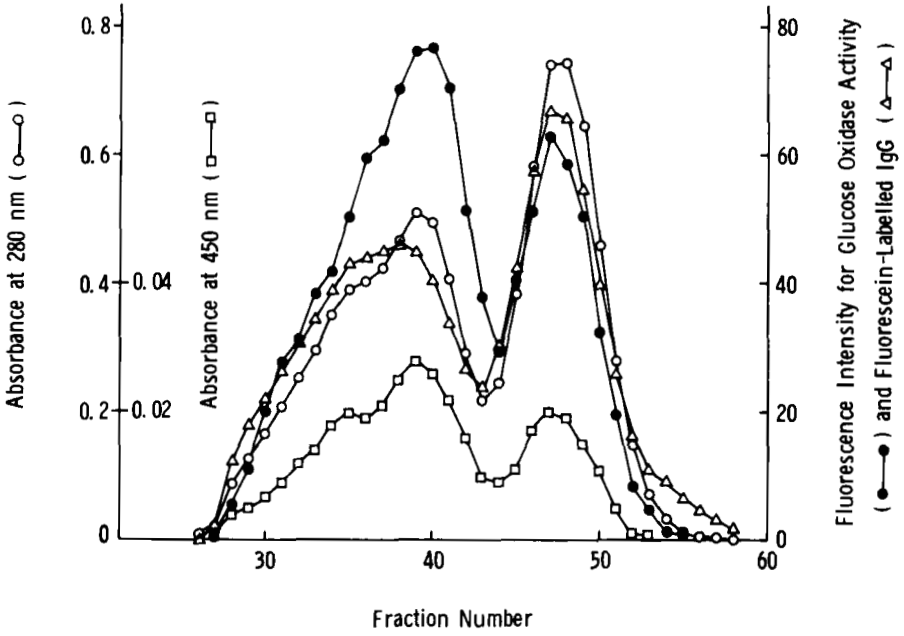


FIGURE VI-2. Elution profile from a Ultrogel AcA 34 column of rabbit IgG-glucose oxidase prepared by the maleimide method (IV). The amounts of glucose oxidase and IgG used were 2 and 4 mg, respectively. The average number of maleimide groups introduced per glucose oxidase molecule was 4.8. The maleimide glucose oxidase 0.027 mmol/L) and the reduced IgG (0.053 mmol/L) were incubated at 4°C for 20 h. The column size used was 1.5 x 45 cm, and the fraction volume was 0.86 ml. Fluorescence intensity of fluorescein-labeled IgG was measured by adjusting fluorescence intensity of 10 nmol/L fluorescein to a scale of 100. Glucose oxidase activity was determined by 10 min-assay using p-hydroxyphenylacetic acid. The recovery in the conjugate of glucose oxidase incubated for the conjugation was calculated from absorbance at 450 nm to be 73 %, and the recovery in the conjugate of IgG incubated for the conjugation was calculated from fluorescence intensity of fluorescein-labeled IgG to be 55 %. The average number of IgG molecules conjugated per glucose oxidase molecule was 1.5.

3. Incubate the mixture at 37°C for 90 min.
4. Apply the reaction mixture to a Sephadex G-25 column (1.0 x 30 cm) using sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 5 mmol/L EDTA.

Conjugation

1. Prepare about 2 mg (13 nmol) of the maleimide-glucose oxidase in 0.2 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L.
2. Prepare about 4 mg (25 nmol) of the reduced IgG in 0.3 ml of sodium phosphate buffer, pH 6.0, 0.1 mol/L, containing 5 mmol/L EDTA.
3. Mix the two solutions, and incubate the mixture at 4°C for 20 h.
4. The subsequent steps are the same as for the preparation of Fab'-glucose oxidase conjugate (Fig. VI-2).

C. Other Methods

In place of N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate used in the maleimide method (I), Fab'-glucose oxidase conjugates can be prepared using other reagents such as N-succinimidyl m-maleimidobenzoate (IV-E-a) and N-succinimidyl 3-(2-pyridyldithio)propionate (IV-E-c).

VII. LABELING WITH ALKALINE PHOSPHATASE

A. Assay of Alkaline Phosphatase

Both alkaline phosphatases from calf intestine and Escherichia coli are commercially available, but the former has

almost always been used as label, probably because its specific activity is higher (38). The enzyme contains zinc ion and is inactivated in the presence of chelating agents such as EDTA. It is not very stable and loses its activity in very dilute solution and by treatment with various cross-linking reagents. The enzyme activity is protected by the presence of other proteins such as serum and egg albumin and is inhibited by inorganic phosphate. The enzyme can be stored in the presence of NaN_3 as a preservative. The sensitivities of 10 and 100 min fluorimetric assays of alkaline phosphatase from calf intestine under the condition described below are 1.0 and 0.1 pg (10 and 1.0 amol), respectively.

Fluorimetric assay with 4-methylumbelliferyl phosphate

1. Prepare enzyme samples in a total volume of 0.1 ml of glycine-NaOH buffer, pH 10.3, 0.1 mol/L, containing 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, 0.5 g/L NaN_3, and 250 mg/L egg albumin.
2. Dissolve 2.6 mg (0.01 mmol) of 4-methylumbelliferyl phosphate (m.w. 256.2) in 33.3 ml of glycine-NaOH buffer, pH 10.3, 0.1 mol/L to give a concentration of 300 μmol/L.
3. Incubate the sample solution at 30°C for 5 min, and start the enzyme reaction by adding 50 μl of substrate solution.
4. Continue the incubation at 30°C for 10-60 min in most experiments.
5. Stop the enzyme reaction by adding 2.5 ml of K_2HPO_4-KOH buffer, pH 10.4, 0.5 mol/L, containing 10 mmol/L EDTA.

6. Measure fluorescence intensity using 100 nmol/L 4-methylumbelliferone in the same buffer as a standard, using 360 nm for excitation and 450 nm for emission.

B. Maleimide Methods for Labeling Fab' and IgG with Alkakine Phosphatase

Glutaraldehyde has been used for labeling with alkaline phosphatase, but the enzyme is polymerized even in the two-step method (13). We describe here a maleimide method in which alkaline phosphatase is conjugated with Fab' using thiol groups in the hinge without self-conjugation of the enzyme or Fab'. Judging from the results with horseradish peroxidase, Fab'-alkaline phosphatase prepared by the maleimide method may be more useful with better retention of antigen-binding activity and lower nonspecific binding than the corresponding glutaraldehyde conjugate, although this remains to be shown.

Introduction of maleimide groups into alkaline phosphatase

1. Dissolve 4 mg (40 nmol) of alkaline phosphatase from calf intestine in 0.5 ml of sodium borate buffer, pH 7.6, 50 mmol/L, containing 1 mmol/L $MgCl_2$ and 0.1 mmol/L $ZnCl_2$, and dialyse the enzyme solution against the same buffer. The enzyme may be obtained in a pellet by centrifuging the enzyme suspension in 3.2 mol/L $(NH_4)_2SO_4$ containing 1 mmol/L $MgCl_2$ and 0.1 mmol/L $ZnCl_2$. Calculate the amount of the enzyme from its absorbance at 280 nm by taking the extinction coefficient

at 280 nm and molecular weight of the enzyme to be 0.99 $g^{-1}.L.cm^{-1}$ (76) and 100,000 (77), respectively.

2. Dissolve 0.67-1.34 mg (2,000-4,000 nmol) of N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (m.w. 334.33) (Zieben Chemical Co., Tokyo or Pierce Chemical Co., Rockford, Ill.) in 30-50 μl of N,N-dimethylformamide.
3. Add 30-50 μl of reagent solution to 1.0 ml of dialysed enzyme solution and incubate the mixture at 30°C for 0.5-1 h with continuous shaking.
4. Apply the reaction mixture to a Sephdex G-25 column (1.0 x 45 cm) equilibrated with Tris-HCl buffer, pH 7.0, 0.1 mol/L, containing 1 mmol/L $MgCl_2$ and 0.1 mmol/L $ZnCl_2$.

   Do not use NaN_3 as a preservative, since it accelerates the decomposition of maleimide groups. Measure maleimide groups in the same way as in the maleimide method (I) for peroxidase. The extinction coefficient at 280 nm and molecular weight of alkaline phosphatase from calf intestine are 0.99 $g^{-1}.L.cm^{-1}$ (76) and 100,000 (77), respectively. The average number of maleimide groups introduced per enzyme molecule under the above conditions was calculated to be 1.6-6.2, and 20-40 % of the enzyme activity was lost by the introduction of maleimide groups.

Conjugation

1. Prepare 1 mg (10 nmol) of the maleimide-alkaline phosphatase in 0.5 ml of Tris-HCl buffer, pH 7.0, 0.1 mol/L, containing 1 mmol/L $MgCl_2$ and 0.1 mmol/L $ZnCl_2$.

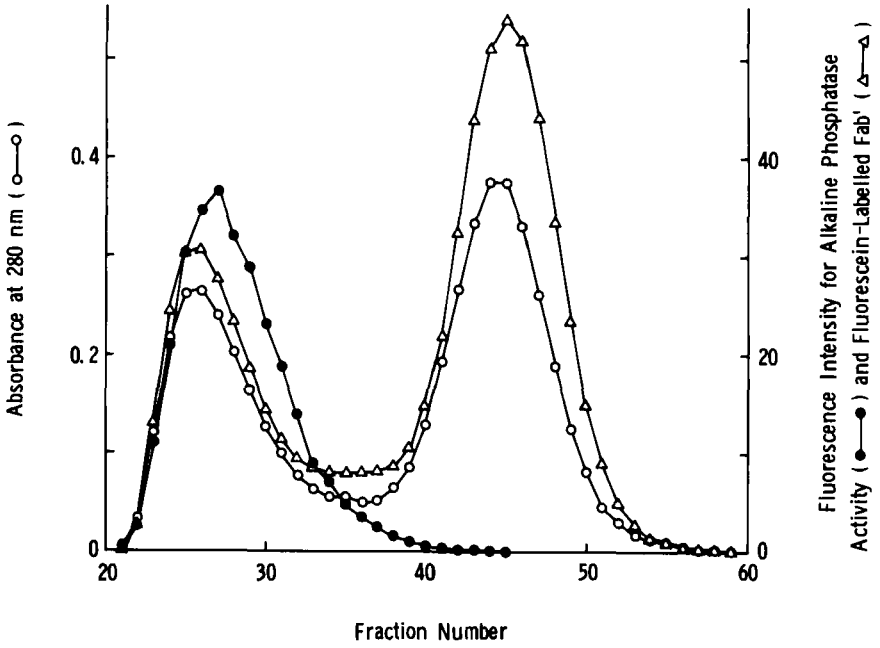


FIGURE VII-1. Elution profile from a Sephadex G-200 of rabbit Fab'-alkaline phosphatase conjugate prepared by the maleimide method (I). The amounts of alkaline phosphatase and Fab' used were about 1 and 4.6 mg, respectively. The number of maleimide groups introduced per alkaline phosphatase was 5.0. The maleimide-alkaline phosphatase (0.01 mmol/L) and Fab' (0.1 mmol/L) were incubated at 4°C for 20 h. The column size used was 1.5 x 45 cm, and the fraction volume was 1.07 ml. Fluorescence intensity of fluorescein-labeled Fab' was measured by adjusting fluorescence intensity of 10 nmol/L fluorescein to a scale of 100. Alkaline phosphatase activity was determined by 10 min-assay using 4-methylumbelliferyl phosphate. The recovery in the conjugate of Fab' incubated for the conjugation was calculated from fluorescence intensity of fluorescein-labeled Fab' to be 34 % and the recovery in the conjugate of alkaline phsphatase incubated for the conjugation was calculated from activity of alkaline phosphatase to be 96 %. The average number of Fab' molecules conjugated per alkaline phosphatase molecule was 3.5.

2. Prepare 4.6 mg (100 nmol) of Fab' plus 0.2 mg of fluorescein-labeled Fab' in 0.5 ml of sodium acetate buffer, pH 5.0, 50 mmol/L (III-D).
3. Mix the maleimide-enzyme and Fab' and incubate the mixture at 4°C for 20 h.
4. Add to the reaction mixture 10 μl of 100 mmol/L 2-mercaptoethylamine, and incubate the reaction mixture at room temperature for 20 min to block remaining maleimide groups.
5. Apply the reaction mixture to a Sephadex G-200 column (1.5 x 45 cm) equilibrated with Tris-HCl buffer, pH 7.0, 10 mmol/L, containing 0.1 mol/L NaCl, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, and 0.5 g/L NaN_3. Store the fraction containing the conjugate at 4°C after adding 1/99 volume of 100 g/L bovine serum albumin (Fig. VII-1).

VIII. EVALUATION OF DIFFERENT ENZYMES AS LABEL

A. Sensitivity of Enzyme Assays

The sensitivities of the assays for the four enzymes which have been used in enzyme immunoassay and immunohistochemistry are shown in Table VIII-1. The fluorimetric assay of ß-D-galactosidase from Escherichia coli with 4-methylumbelliferyl ß-D-galactoside is the most sensitive, being able to measure 0.2 and 0.02 amol of the enzyme in 10 and 100 min assays, respectively. The fluorimetric assays of horseradish peroxidase with p-hydroxyphenylpropionic acid and alkaline phosphatase from calf intestine with 4-methylumbelliferyl phosphate can measure 5-10 and 0.5-1

TABLE VIII-1

Sensitivity of Enzyme Assay

Enzymes	Substrates	Sensitivities	
		10-min assay	100-min assay
		amol	
ß-D-Galactosidase (Escherichia coli)	4-Methylumbelliferyl ß-D-galactoside[a]	0.2	0.02
	o-Nitrophenyl ß-D-galactoside	1,000	100
Peroxidase (horseradish)	p-Hydroxyphenyl-propionic acid[a]	5.0	0.5
	o-Phenylenediamine	25	25
Alkaline phosphatase (calf intestine)	4-Methylumbelliferyl phosphate[a]	10	1.0
	p-Nitrophenyl phosphate	10,000	-
Glucose Oxidase (Aspergillus niger)	p-Hydroxyphenyl-acetic acid[a]	50	5.0

[a]Assay conditions are described in the text (IV-A, V-A, VI-A and VII-A).

amol in 10 and 100 min assays, respectively. The fluorimetric assay of glucose oxidase from Aspergillus niger is less sensitive.

The specific radioactivity of carrier-free ^{125}I (R) is calculated from its half life (60.2 days) as follows.

$$R = \frac{A}{N} \text{ (Ci/gram atom)}$$

$$= \frac{3.7 \times 10^{10} \times 60 \times 6.02 \times 10^{23}}{1.44 \times 3.7 \times 10^{10} \times T} \text{ (dpm/gram atom)}$$

$$= 4.82 \times 10^{18} \text{ dpm/gram atom} = 4.82 \text{ dpm/amol}$$

A: Avogadro's number = 6.02×10^{23}

N: number of radioisotopic atoms per Ci

T: half life in sec (60.2 x 24 x 60 x 60)

1 Ci = $3.7 \times 10^{10} \times 60$ dpm

In the same way, the specific radioactivities of other radioisotopes were also calculated (Table VIII-2). Judging from these values, the fluorimetric assay of β-D-galactosidase is more sensitive than the measurement of these radioisotopes, and the

TABLE VIII-2

Specific Radioactivity of Carrier-Free Radioisotopes

Radioisotopes	Half lives	Specific radioactivities
	days	dpm/amol
^{131}I	8.06	36.1
^{125}I	60.2	4.8
^{35}S	87.4	3.3
^{3}H	12.262 x 365	0.06
^{14}C	5,570 x 365	0.00014

fluorimetric assays of peroxidase and alkaline phosphatase are as sensitive as the measurent of ^{125}I.

B. Nonspecific and Specific Bindings of Antibody-Enzyme Conjugates

Nonspecific binding to normal rabbit IgG-coated polystyrene balls of normal rabbit Fab' conjugates with peroxidase, alkaline phosphatase and glucose oxidase prepared by the maleimide methods using thiol groups in the hinge differed little and was lower than that of normal rabbit Fab' conjugate with ß-D-galactosidase. Nonspecific binding with normal rabbit Fab'-enzyme conjugates was lower than with the corresponding IgG-enzyme conjugates (Table VIII-3).

Specific bindings of Fab' and IgG conjugates with these enzymes were similar when compared by calculating the number of conjugate molecules bound per antigen molecule on a polystyrene ball.

As a result, the sensitivities of sandwich enzyme immunoassay with the Fab'-peroxidase conjugates prepared by using thiol groups in the hinge of Fab' are fairly close to those with the Fab'-ß-D-galactosidase conjugates (IV-F-f and V-D-g), whereas the assay of ß-D-galactosidase is 25-fold more sensitive than that of peroxidase (Table VIII-1). Furthermore, serum interference tends to be less severe with the Fab'-peroxidase conjugates than with the Fab'-ß-D-galactosidase conjugates, and Fab' can be more efficiently conjugated with peroxidase (IV-C) than with ß-D-galactosidase (V-D).

TABLE VIII-3

Nonspecific Bindings of Nomal Rabbit Antibody-Enzyme Conjugates to Normal Rabbit IgG-Coated Polystyrene Balls

Antibody-enzyme conjugates		Nonspecific binding
Enzymes	Antibodies	
		%
ß-D-Galactosidase (*Escherichia coli*)	(free enzyme)	0.065-0.075
	Fab'	0.055-0.066
	IgG	0.10-0.15
Peroxidase (horseradish)	(free enzyme)	0.000-0.001
	Fab'	0.010-0.012
	IgG	0.10-0.11
Alkaline phosphatase (calf intestine)	(free enzyme)	0.0014-0.0035
	Fab'	0.015-0.022
Glucose oxidase (*Aspergillus niger*)	(free enzyme)	0.0035-0.0045
	Fab'	0.015-0.016
	IgG	0.023-0.026

Nonspecific bindings are the enzyme activities nonspecifically bound expressed as percentages of those added.

A more critical evaluation of enzymes as labels in enzyme immunoassay will be described elsewhere.

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