

PEROXIDASE-LABELED ANTIBODY A NEW METHOD OF CONJUGATION¹

PAUL K. NAKANE² AND AKIRA KAWAOI³

Department of Pathology, University of Colorado Medical Center, Denver, Colorado 80220

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A new method of conjugating horseradish peroxidase with proteins was developed. The carbohydrate moiety of fluorodinitrobenzene-blocked peroxidase was oxidized with sodium periodate to form aldehyde groups. The peroxidase-aldehyde was then bound to free amino groups of proteins unidirectionally at high efficiencies. Peroxidase-labeled immunoglobulin retained its immunologic as well as enzymatic activities.

Horseradish peroxidase (HRPO), when coupled to immunoglobulin G (IgG), has proven to be a useful marker for immunohistochemistry (13). In contrast to other immunohistochemical markers, such as fluorescein (7) and ferritin (20), HRPO may be used for both light and electron microscopy. It is especially suitable for the intracellular localization of antigens at the ultrastructural level since HRPO (40,000 molecular weight) is considerably smaller than ferritin (650,000 molecular weight); thus, HRPO-labeled IgG (HRPO-IgG) has superior penetration properties.

HRPO is usually coupled to IgG using bifunctional reagents. With earlier methods, the conjugation reactions were carried out in the presence of HRPO, IgG, and *p,p'*-difluoro-*m,m'*-dinitro-diphenyl sulfone (FNPS) (13), [1-cyclohexyl-3-(2-morpholinoethyl)] carbodiimide metho-*p*-toluenesulfonate (4), cyanuric chloride (1), bis-diazotized *o*-dianisidine (1) or glutaraldehyde (1), and resulted usually in more IgG polymers than HRPO-IgG. None of the investigators has published the yield of HRPO-IgG, but our past experience has indicated that only about 2% of the added HRPO was conjugated to IgG. Recognizing the low yield, several laboratories have recently modified the labeling procedures. For

example, HRPO was initially exposed to excess glutaraldehyde and then, following removal of the excess glutaraldehyde, the peroxidase-aldehyde was reacted with IgG (2). Clyne *et al.* (6) applied the two-step toluene-2,4-diisocyanate method, used by Schick and Singer (20) for the conjugation of ferritin and IgG, to the conjugation of HRPO and IgG. With both of these modifications, the randomness in the conjugation reactions has been reduced, but the efficiency of HRPO attachment to IgG, as estimated from the published chromatographic elution patterns, has not been significantly increased.

A possible reason for this low coupling efficiency is the blockage of the majority of α - and ϵ -amino groups and some hydroxy groups of commercial HRPO by allylthiocyanate (14). These groups are therefore not available for the coupling reaction. In order to increase the efficiency, another approach to the conjugation of HRPO to IgG was investigated.

HRPO II is known to be a glycoprotein with a carbohydrate content of approximately 18% (21). The carbohydrate portion is not required for enzymatic activity inasmuch as HRPO I, which contains little or no carbohydrate, has activity similar to that of HRPO II (15). It was thus recognized that the carbohydrate portion of HRPO II might be used for the formation of aldehyde groups by oxidation with sodium *m*-periodate (NaIO₄). Indeed, it was found that the oxidation of HRPO II yielded HRPO-aldehyde without significant loss of HRPO activity. Moreover, complete blocking of the remaining α - and ϵ -amino groups and hydroxy groups of HRPO with fluorodinitrobenzene (FDNB) (19)

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³Present address: Department of Pathology, Nihon University, School of Medicine, Tokyo, Japan.

prior to the oxidation prevented self-coupling, and allowed the HRPO-aldehyde to form Schiff bases with any protein having available α - or ϵ -amino groups with near perfect efficiency.

MATERIALS AND METHODS

Materials: Horseradish peroxidase (type VI, lot 111C-9340) from Sigma Chemical Company (St. Louis, Mo.), 1-fluoro-2,4-dinitrobenzene from Eastman Kodak Company (Rochester, N.Y.) or Sigma, sodium *m*-periodate from Matheson, Coleman and Bell (Norwood, O.), ethylene glycol from Fisher Scientific Company (Fair Lawn, N.J.), sodium borohydride from Sigma, *p*-cresol from Aldrich Chemical Company, Inc. (Milwaukee, Wis.) and hydrogen peroxide from Baker Chemical Company (Phillipsburg, N.J.) were used for these experiments.

Rabbit antimouse basement membrane serum was prepared as described previously (16). The IgG fraction of sheep antirabbit IgG serum was obtained by ammonium sulfate precipitation (22).

Polystyrene beads were obtained from Clifton Plastics, Inc. (Clifton Heights, Pa). Amino acid analyses were done on a JEOL JLC-6AH amino acid analyzer. Optical density measurements were made on a Beckman DB-G or DB-GT spectrophotometer using a 0.5-cm path length.

Assay methods:

HRPO activity: Twenty microliters of a suitable dilution of sample were mixed with 0.5 ml 14.3 *mM* *p*-cresol in 50 *mM* Tris-HCl buffer, pH 7.4. To this mixture, 0.5 ml 0.02% hydrogen peroxide in distilled water was added (17). After three minutes the enzymatic reaction was stopped by the quick addition of 2 ml 3.7 *mM* sodium cyanide in distilled water. Preliminary studies showed that sodium cyanide can completely inhibit the HRPO enzymatic reaction but does not interfere with subsequent spectrofluorometric readings. Fluorescence was measured at an activation wavelength of 320 nm and a fluorescence wavelength of 410 nm. The activity was expressed as a percentage of the original HRPO activity.

Immunologic and enzymatic activities of the conjugates: Initially, the conjugates were tested at the light microscopic level with conventional HRPO-labeled antibody methods on mouse kidney sections previously reacted with rabbit antimouse basement membrane serum (16). Later, the immunologic activity of the conjugates was tested using the immunoassay procedure whereby polystyrene beads were coated with normal rabbit serum according to the method developed by Catt (5). They were then washed overnight in 0.01 *M* phosphate-buffered saline (PBS), pH 7.2. Each conjugate (containing 5 mg IgG of which a variable amount was HRPO-labeled) was serially diluted with 2% normal sheep serum in PBS with 5 *mM* ethylenediaminetetraacetic acid (EDTA) (PBS-

EDTA). For the determination of the IgG saturation point of the serum-coated beads, the beads were exposed to these diluted conjugates (0.5 ml/bead) for 24 hr at 4°C, washed in PBS-EDTA overnight and assayed for HRPO activity by the HRPO assay outlined above. To determine the loss of immunologic activity of the conjugates, twice the concentration of conjugate required for saturation was competed with known amounts of unlabeled IgG for the binding sites on the beads. The loss of immunologic activity was then calculated by comparison of the actual HRPO bound with that which would have bound assuming no loss of immunologic activity.

RESULTS

FDNB treatment: Although the majority of α - and ϵ -amino groups in HRPO are blocked by allylthiocyanate (14), the facts that FNPS (13) and glutaraldehyde are able to cross-link HRPO to other proteins (3) and that FDNB is able to bind to HRPO (9) indicate that some free amino groups remain. In the present study, these free amino groups were reacted with FDNB to block their reaction with subsequently formed aldehyde groups.

The reaction with FDNB was carried out in 0.3 *M* sodium bicarbonate, pH 8.1 (19). Exposure of 5 mg HRPO in 1 ml sodium bicarbonate to 100 μ l of 1 or 5% FDNB in ethanol for 60 min reduced the HRPO activity by 35 and 50%, respectively. This reduction of activity was expected, inasmuch as the coupling of FDNB to α - and ϵ -amino groups and hydroxy groups of apoperoxidase results in production of hydrogen fluoride, which is an inhibitor of HRPO (21). Dialysis against 0.01 *M* sodium carbonate buffer at pH 9.5 overnight restored the lost enzymatic activity. The maximum absorption wavelength of HRPO was shifted slightly from 403 nm to a broader peak ranging from 395 nm to 403 nm. The RZ values of HRPO reacted with 1 or 5% FDNB were identical (RZ = 2.4); that of the unreacted HRPO was 3.1. For all subsequent experiments, 1% FDNB was used.

The amino acid composition of HRPO was analyzed before and after reaction with FDNB. The elution pattern of 2 of the 6 lysine residues/mole HRPO was affected by the FDNB treatment. This is in agreement with the finding of Francois (9) that FDNB binds to 2 lysine residues/mole HRPO.

Oxidation of HRPO: Sodium *m*-periodate in aqueous media has been used for the oxidation

of carbohydrates (10), glycoproteins (18, 23) and deoxyribonucleic acid (11). Oxidation conditions similar to those used for IgG (18) and deoxyribonucleic acid (11) were employed in the present work for the carbohydrate portion of HRPO.

Preliminary studies showed that the presence of dialyzable compounds in the FDNB-treated HRPO did not interfere with the NaIO_4 reaction. Thus, the FDNB-treated HRPO was not dialyzed prior to reaction with NaIO_4 . Five milligrams FDNB-treated HRPO were oxidized by 1 ml 0–0.16 M NaIO_4 in distilled water for 30 min or by 1 ml 0.02 M NaIO_4 for 30–240 min at room temperature. The reaction was stopped by the addition of 1 ml 0.32 M ethylene glycol in distilled water (11). After one hour the samples were dialyzed against 0.01 M sodium carbonate buffer, pH 9.5.

The results of these studies are included in Table I. As the NaIO_4 concentration or the duration of the oxidation reaction was increased, there was a progressive loss in HRPO activity and RZ. The loss of RZ in the concentration study resulted primarily from decreased absorption at 403 nm, whereas the loss of RZ in the time study resulted primarily from increased absorption at 280 nm.

Coupling efficiencies: Five-milligram portions of the above HRPO-aldehydes were reacted with 5 mg lyophilized sheep antirabbit

IgG immunoglobulin for 3 hr at room temperature and then dialyzed against PBS. One-milliliter aliquots were used for the determination of total HRPO and immunologic activities of the conjugate mixture. Two-milliliter aliquots were chromatographed on a 82 x 1.5-cm column of Sephadex G-200. Fractions (1.5 ml) were collected and their absorbances at 280 and 403 nm were determined. The elution patterns of conjugate mixtures resulting from the reaction of IgG with HRPO activated with various concentrations of NaIO_4 are shown in Figure 1. The pooled peaks of conjugated (fractions 21–39) and unconjugated (fractions 40–60) HRPO were read at 280 and 403 nm and assayed for HRPO activity. The results are included in Table I.

The loss of HRPO activity which occurred during oxidation with NaIO_4 was almost completely recovered by reaction with IgG except in the cases of high NaIO_4 concentration or long oxidation time. By spectrophotometric measurements, the amount of HRPO bound to IgG increased as the NaIO_4 concentration or time of the activation reaction was increased. This increased HRPO binding was accompanied by a progressive decrease in enzymatic activity and IgG immunologic activity. An optimum conjugate was thus considered to be that formed between IgG and HRPO oxidized by 0.08 M NaIO_4 for 30 min.

Sodium borohydride (NaBH_4) treatment:

TABLE I
Studies on the HRPO and IgG Activities during the Preparation of HRPO-IgG Conjugate

Oxidation Conditions		HRPO-Aldehyde		HRPO/IgG Mixture ^a		HRPO-IgG Peak ^b	
Duration (min)	NaIO_4 concentration (M)	RZ ^c	HRPO activity ^d (percentage of original)	HRPO activity (percentage of original)	IgG activity ^e (percentage of original)	Absorbance at 403 nm (percentage of original)	HRPO activity (percentage of original)
30	0.00	2.44	100	102	100	1	1
30	0.02	2.31	94	101	98	22	21
30	0.04	1.97	89	100	98	49	47
30	0.08	1.55	68	98	92	73	68
30	0.16	1.25	57	80	57	81	63
60	0.02	1.97	91	95	100	36	36
120	0.02	1.70	86	98	102	58	55
180	0.02	1.51	62	88	95	62	52
240	0.02	1.31	56	79	59	68	43

^a Prior to chromatography on Sephadex G-200.

^b After chromatography on Sephadex G-200.

^c RZ, ratio of absorbance at 403 nm to absorbance at 280 nm.

^d Measured spectrofluorometrically as described in the text.

^e Measured by immunoassay as described in the text.

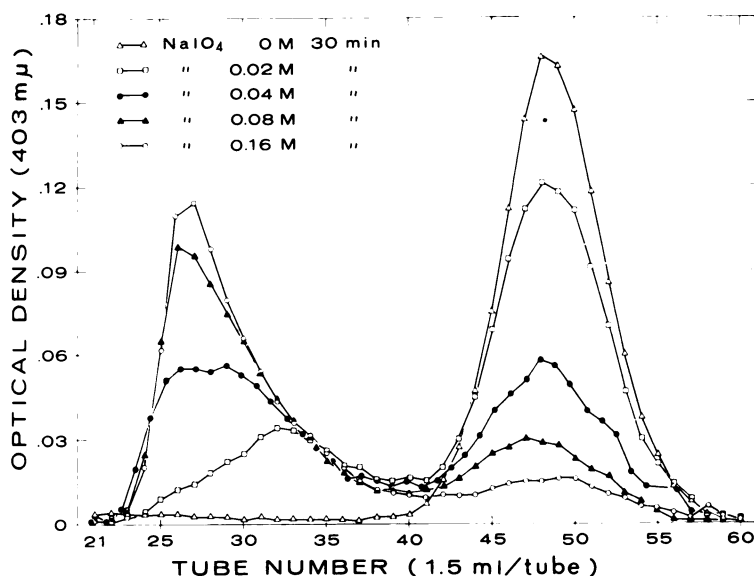


FIG. 1. Elution patterns of 3 mg sheep antirabbit IgG immunoglobulin conjugated with 3 mg HRPO activated by various concentrations of NaIO_4 for 30 min. The conjugates were chromatographed on a 82×1.5 -cm column of Sephadex G-200. Flow rate, approximately 5 ml/hr. Eluant, PBS. Optical densities at 403 nm were measured in cuvettes with 0.5-cm path length.

To stabilize the Schiff base formed by reaction of HRPO-aldehyde with IgG, NaBH_4 was used (11, 12). The effect of various concentrations of NaBH_4 on the enzymatic activity of HRPO-IgG was studied. The HRPO used for this conjugate was oxidized with 0.08 M NaIO_4 for 30 min. The conjugate was reacted overnight with NaBH_4 , ranging from 0 to 7 mg/mg HRPO in the conjugate. After dialysis against PBS, suitable dilutions were assayed for HRPO activity. The results are summarized in Figure 2. Eighty-two per cent of the HRPO activity remained following treatment with 1–5 mg NaBH_4 /mg HRPO. The loss was greater when 7 mg NaBH_4 /mg HRPO was used. No significant effect of NaBH_4 treatment was detectable when the conjugates were used immunohistochemically. Moreover, the NaBH_4 -treated conjugates were found to be extremely stable. After several months of storage at -20°C in the presence of 10 mg bovine serum albumin/ml, no loss of immunohistochemical staining properties of the NaBH_4 -treated conjugates could be detected.

Saturation point determination: To determine the maximum number of HRPO-aldehyde binding sites on sheep antirabbit IgG immunoglobulin, various amounts of HRPO-aldehyde were reacted with a fixed amount of IgG. The mixtures were chromatographed on a 85 x

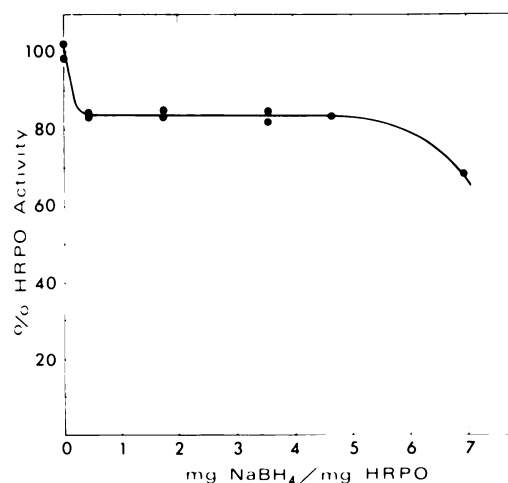


FIG. 2. Effect of NaBH_4 on HRPO activity of sheep antirabbit IgG immunoglobulin conjugated to HRPO activated with 0.08 M NaIO_4 for 30 min. Assay conditions are given in the text.

1.5-cm column of Sephadex G-100. On the basis of 403-nm readings of the total HRPO/IgG mixture and of the pooled peak of HRPO-labeled IgG, the amount of HRPO-aldehyde bound to IgG was calculated. These results are plotted in Figure 3. It appears that a maximum of 5–6 HRPO-aldehydes are capable of attaching to one IgG molecule. However, highly la-

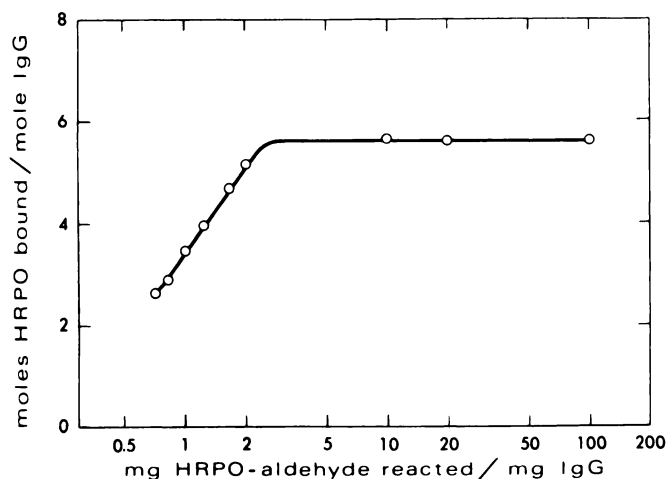


FIG. 3. Saturation of IgG by HRPO-aldehyde. HRPO was activated by 0.08 M NaIO₄ for 30 min. The amount of HRPO bound was determined spectrophotometrically as outlined in the text.

beled conjugates (e.g., greater than 4 HRPO/IgG) were unusable for immunohistochemistry because of high nonspecific affinity to tissue and low immunologic activity.

DISCUSSION

An ideal conjugation reaction would be the labeling of each IgG with one molecule of HRPO without loss of immunologic or enzymatic activities. Some aspects of this ideal reaction may be compromised, but others may not without loss of sensitivity of the method. For example, during the reaction HRPO should not lose its enzymatic activity since IgG labeled with enzymatically inactive HRPO will act as unlabeled IgG and compete for the antigenic sites. On the other hand, some loss of immunologic activity may be tolerated since the IgG without immunologic activity will not compete for the antigenic sites. More than one HRPO may be attached to some IgG molecules, although the size of the conjugate will be larger and will decrease its rate of penetration into tissues. However, if the conjugate is to be used to localize antigens at the light microscopic level or on ultrathin sections, the size of the conjugate is unimportant since penetration is not required.

The conventional conjugation reactions for HRPO and IgG involve a coupling agent and the amino groups of both HRPO and IgG. These methods have been plagued by problems of inefficiency and IgG self-coupling. Possibly, the

requirement for free amino groups in HRPO is not adequately satisfied. The majority of α - and ϵ -amino groups in commercially available HRPO are blocked by allylthiocyanate (14). Furthermore, it is likely that the carbohydrate moiety on the periphery of HRPO interferes sterically with reactions involving the protein core.

The conjugation reaction described in the present work, involving the carbohydrate moiety of HRPO and amino groups of IgG, overcomes the problems of IgG and IgG self-coupling. It does this without significant losses of enzymatic and immunologic activities and thus approaches an ideal conjugation reaction as discussed above. HRPO containing amino groups blocked by reaction with FDNB, and aldehyde groups formed as a result of NaIO₄ oxidation of its carbohydrate moieties, coupled unidirectionally to IgG. Using suitable reaction conditions, each IgG was essentially labeled with one or more HRPO.

Various oxidation reactions are known to produce aldehyde groups in carbohydrates. The periodate reaction was chosen because of the absence of side reactions, its high yield of aldehyde groups and the fact that it can be carried out in an aqueous solution at near neutral pH. Oxidizing agents such as lead tetraacetate which require acidic media (8) had to be avoided since in such media HRPO formed an insoluble gel and was denatured.

HRPO-aldehydes, made under various condi-

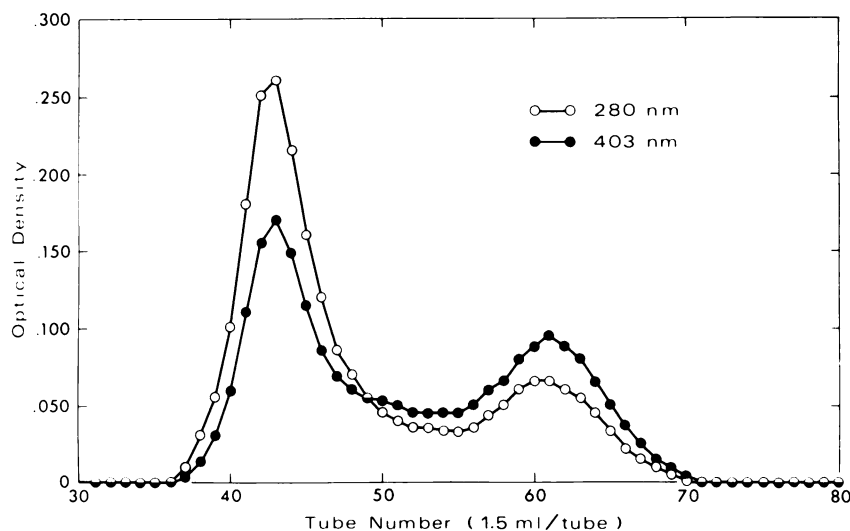


FIG. 4. A typical elution pattern of 5 mg sheep antirabbit IgG immunoglobulin conjugated with 5 mg HRPO activated by 0.08 *M* NaIO₄ for 30 min. Chromatography was carried out on a 85 x 1.5-cm column of Sephadex G-100. Flow rate, approximately 10 ml/hr. Eluant, PBS. Optical densities were measured in cuvettes with 0.5-cm path length.

tions of NaIO₄ concentration and reaction time, were assessed for their ability to form useful conjugates with IgG. Although the formation of HRPO-aldehyde inhibited HRPO activity, most of the loss was recovered upon reaction with IgG. The use of highly activated HRPO-aldehydes or large proportions of HRPO-aldehyde to IgG resulted in highly labeled HRPO-IgG conjugates. However, such reactions were accompanied by significant losses (as measured chemically and immunohistochemically) of the HRPO or IgG immunologic activities.

A maximum of five to six HRPO-aldehyde molecules were bound to one molecule sheep IgG when HRPO-aldehyde formed by oxidation with 0.08 *M* NaIO₄ for 30 min was used. Only conjugates labeled with less than 4 moles HRPO/mole IgG were useful immunohistochemically. Conjugates containing an average of 2 moles HRPO/mole IgG were excellent immunohistochemically since, in addition to high IgG immunologic activity, they had very low nonspecific tissue affinity. Such conjugates have been found to have an approximate RZ of 0.6.

One current method for the preparation of HRPO-labeled IgG is given in the appendix that follows this discussion. A typical elution pattern of the conjugate mixture on Sephadex G-100 is given in Figure 4.

The availability of a method for conjugation of HRPO to IgG with high efficiency should expand the use of HRPO as a marker, not only for IgG but for proteins in general. For example, various proteinaceous antigens may be labeled with HRPO and used in immunoassay systems in place of radiolabeled antigens. This use of HRPO avoids the hazards and complicated handling of radioisotopes since the enzymatic activity of HRPO can be detected using commonly available laboratory equipment. It also avoids the inconveniences of radioisotope decay since HRPO-labeled antigens can be stored without loss of activity. The sensitivity of such an immunoassay system should be comparable to that of radioimmunoassay, since one can detect as little as 10 picograms of HRPO with reliable reproducibility by increasing the reaction time of the assay used for the current study.⁴

METHOD OF HRPO-LABELED IgG PREPARATION

Our current laboratory procedure for the preparation of HRPO-labeled IgG is given below. We use the same procedure for labeling sheep, rabbit, goat, guinea pig and human IgG fractions as well as their Fab' derivatives. The NaIO₄ concentration required for an optimally

⁴Nakane PK, Kawaoi A: Unpublished observation.

activated HRPO aldehyde will depend on the particular lot of HRPO used.

1. Dissolve 5 mg HRPO (Sigma HRPO, type VI, RZ approximately 3.0) in 1.0 ml freshly made 0.3 M sodium bicarbonate, pH 8.1.

2. To the above solution, add 0.1 ml 1% FDNB in absolute ethanol. Mix gently for 1 hr at room temperature.

3. Add 1.0 ml 0.04–0.08 M NaIO₄ in distilled water. Mix gently for 30 min at room temperature. The color of the solution will become green-yellow.

4. Add 1.0 ml 0.16 M ethylene glycol in distilled water. Mix gently for 1 hr at room temperature.

5. Dialyze the solution against three 1-liter changes of 0.01 M sodium carbonate buffer, pH 9.5, at 4°C.

6. Add 5 mg IgG to 3 ml HRPO-aldehyde solution. Mix gently for 2–3 hr at room temperature. The IgG may be dissolved in 1 ml carbonate buffer prior to mixing or may be added in powder form free of salt.

7. Add 5 mg NaBH₄. Leave at 4°C 3 hr to overnight.

8. Dialyze at 4°C against PBS. A small amount of precipitate may form and should be removed by centrifugation.

9. Apply the sample to a 85 x 1.5-cm column of Sephadex G-100 or G-200 equilibrated in PBS. Read the absorbance of the fractions at 280 and 403 nm. Pool the fractions of HRPO-labeled IgG, the first peak (Fig. 4).

10. Store aliquots of the conjugate in the presence of 10 mg bovine serum albumin/ml at –20°C.

With the above procedure, approximately 70% of the HRPO should couple with IgG and approximately 99% of the IgG should be labeled with HRPO. No significant losses of either IgG immunologic or HRPO activities occur.

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LITERATURE CITED

- Avrameas S: Détection d'anticorps et d'antigènes à l'aide d'enzymes. *Bull Soc Chim Biol* 50:1169, 1968
- Avrameas S, Lespinats G: Enzymes couplées aux protéines: leur utilisation pour la détection des antigènes et des anticorps. *C R Acad Sci [D] (Paris)* 265:1149, 1967
- Avrameas S, Ternynck T: Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochimistry* 8:1175, 1971
- Avrameas S, Uriel J: Méthode de marquage d'antigènes et d'anticorps avec des enzymes et son application en immunodiffusion. *C R Acad Sci [D] (Paris)* 262:2543, 1966
- Catt KJ: Radioimmunoassay with antibody-coated discs and tubes, Karolinska Symposia on Research Methods in Reproductive Endocrinology. 1st Symposium: Immunoassay of Gonadotrophins. Edited by E Diczfalussy. Periodica, Copenhagen, 1969, p 222
- Clyne DH, Norris SH, Modesto RR, Pesce AJ, Pollak VE: Antibody enzyme conjugates: the preparation of intermolecular conjugates of horseradish peroxidase and antibody and their use in immunohistology of renal cortex. *J Histochem Cytochem* 21:233, 1973
- Coons AH: Fluorescent antibody methods, General Cytoplasmic Methods. Edited by JF Danielli. Academic Press, New York, 1958, p 399
- Fieser LF, Fieser M: Reagents for Organic Synthesis. John Wiley & Sons, New York, 1967, p 537
- Francois D, Oriol R, Binaghi RA: Immunoperoxidase localization of antihapten antibodies in rats responding unequally to antigenic stimulation. *J Histochem Cytochem* 20:527, 1972
- Jackson EL: Periodic acid oxidation. *Organic Reactions* 2:341, 1944
- Khym JX: The reaction of methylamine with periodate-oxidized adenosine 5'-phosphate. *Biochemistry* 2:344, 1963
- Kraehenbuhl JP, Galardy RE, Jamieson JD: Preparation and characterization of an immunoelectron microscope tracer consisting of a heme-octapeptide coupled to Fab. *J Exp Med* 139:208, 1974
- Nakane PK, Pierce GB: Enzyme-labeled antibodies: preparation and application for localization of antigens. *J Histochem Cytochem* 14:929, 1966
- Ornstein L: Discussion on "Enzyme-labeled antibodies for light and electron microscopic localization of antigens" by PK Nakane et al. *J Histochem Cytochem* 14:790, 1966
- Paul KG: Heme compounds in enzyme catalysis, The Enzymes. Edited by PD Boyer, H Lardy, K Myrback. Vol 3. Ed 2. Academic Press, New York, 1960, p 305
- Pierce GB, Jr, Midgley AR, Jr, Ram JS, Feldman JD: Parietal yolk sac carcinoma: clue to the histogenesis of Reichert's membrane of the mouse embryo. *Am J Pathol* 41:549, 1962
- Roth M: Fluorimetric assay of enzymes, Methods of Biochemical Analysis. Edited by D Glick. Vol 17. Interscience Publishers, New York, 1969, p 236
- Rothfus JA, Smith EL: Glycopeptides. IV. The

- periodate oxidation of glycopeptides from human γ -globulin. *J Biol Chem* 238:1402, 1963
19. Sanger F: The free amino groups of insulin. *Biochem J* 39:507, 1945
20. Schick AF, Singer JJ: On the formation of covalent linkages between two protein molecules. *J Biol Chem* 236:2477, 1961
21. Theorell H: The iron-containing enzymes. B. Catalases and peroxidases. "Hydroperoxidases," *The Enzymes*. Edited by JB Sumner, K Myrback. Vol II. Part I. Academic Press, New York, 1951, p 397
22. Williams CA, Chase MW: *Methods in Immunology and Immunochemistry*. Vol 1. Academic Press, New York, 1967, p 319
23. Yasuda Y, Takahashi N, Murachi T: The composition and structure of carbohydrate moiety of stem bromelain. *Biochemistry* 9:25, 1970