TWO IMPROVED METHODS FOR PREPARING FERRITIN-PROTEIN CONJUGATES FOR ELECTRON MICROSCOPY

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ABSTRACT

Two improved procedures were developed for activating ferritin so that the ferritin could be covalently linked to antibodies. One procedure involved use of a water-soluble carbodiimide and N-hydroxysuccinimide to prepare ferritin-containing activated esters. The other involved activation of the ferritin with excess glutaraldehyde. The ferritin-antibody conjugates prepared with the two procedures were shown to have a number of properties which made them suitable for locating antigenic components in cells.

There has been considerable interest in determining the localization of components of cells by preparing antibodies to a selected component and covalently linking the antibodies to ferritin or to suitable marker enzymes (1–12). The "labeled" antibodies are then reacted with tissue blocks or sections under appropriate conditions to detect the distribution of the specific component. One of the limitations of this approach has been the difficulty of preparing cells so that the labeled antibody can enter and react with the antigenic component without loss of cell morphology or destruction of the antigenicity of the component. This problem is discussed in the accompanying paper (13). A second limitation of this approach has been the difficulty of preparing well-defined conjugates with the marker molecules linked to specific antibodies. Two improved methods for preparing such conjugates are presented in the present paper.

Marker enzymes have been linked to antibodies with a number of procedures and these have included the following reagents: *p,p'*-difluoro-*m,m'*-dinitrophenyl sulfone (2), water-soluble carbodiimides (2), cyanuric chloride (4), and glutaraldehyde (4, 12). In general, the reactions with these reagents are carried out in a mixture of antibody and the enzyme marker so that heterogeneous products are produced which, in addition to the desired antibody-enzyme conjugates, include enzyme-enzyme conjugates, antibody-antibody conjugates, and higher polymers of the antibody-enzyme conjugates. One exception to this is the peroxidase-antibody conjugates prepared with glutaraldehyde (12). Because peroxidase contains a limited number of lysyl residues, reaction of peroxidase with glutaraldehyde does not produce polymers but yields glutaraldehyde-activated peroxidase which can be separated from glutaraldehyde and subsequently coupled to antibody. However, the resolution of procedures involving peroxidase-labeled antibodies as well as other enzyme markers is limited by the tendency of the identifiable products of the enzymic reaction to diffuse away from the site of antigen-antibody binding.

The use of ferritin as a marker for electron

microscopy generally allows more precise definition of the localization of the antigen-antibody complex. A number of attempts were made to conjugate ferritin to antibody by directly reacting a mixture of two components with bifunctional coupling reagents such as *p,p'*-difluoro-*m,m'*-dinitrophenyl sulfone (14) or glutaraldehyde (15–17). However, the products were heterogeneous. In 1959, Singer introduced a two-step procedure for linking ferritin to antibody molecules with *m*-xylylene diisocyanate or toluene diisocyanate (1, 18). Toluene diisocyanate turned out to be the most useful of the two reagents. Because only one of the isocyanate groups in toluene diisocyanate reacted at pH 7.5, it was possible to activate the ferritin at this pH and then react the activated ferritin with antibody at pH 9.5. This two-stage reaction appeared to avoid the formation of ferritin-ferritin polymers but the yields of the desired product were low and inconsistent. In some cases, the antibody activity was greatly reduced or even lost during the conjugation procedure (19, 20). Kraehenbuhl and Jamieson (8) improved the Singer procedure for the synthesis of ferritin-labeled Fab, but their yields of the specific product were still low, and they did not demonstrate that the product was free of polymers of ferritin or of ferritin-Fab (8).

We here report two general procedures for activating ferritin so that it can be covalently linked to Fab or to IgG. In one procedure a water-soluble carbodiimide and *N*-hydroxysuccinimide are used to produce ferritin-containing activated esters. In the second procedure excess amounts of glutaraldehyde are used to activate ferritin under conditions in which the formation of ferritin-ferritin polymers is avoided.

MATERIALS AND METHODS

Materials

Ferritin (six times recrystallized and cadmium-free) from horse spleen was purchased from Polysciences, Inc., Warrington, Pa. Rabbit IgG and goat IgG from goat antisera directed against rabbit IgG were purchased from Miles Laboratories Inc., Miles Research Div., Kankakee, Ill. Both of these IgG preparations were purified by *O*-(diethylaminoethyl)cellulose chromatography and their purity was established by immunoelectrophoresis. Gel filtration on 8% agarose (Bio-Gel A-1.5m, Bio-Rad Laboratories, Richmond, Calif.) demonstrated that both IgG preparations were essentially free of aggregates. Papain, a mercury-free suspension, was purchased from Worthington Biochemicals Corp., Freehold, N. J. Lactoperoxidase, purified from milk to an RZ value of 0.50 and approximately 35 purpurogallin U/mg of solid, was purchased from Sigma Chemical Co. St. Louis, Mo. Sodium [^{125}I]iodide, carrier-free, was obtained from New England Nuclear, Boston, Mass. Glutaraldehyde, as a 50% solution, was purchased from Polysciences, Inc. Succinic anhydride was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis., sodium succinate from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio, and *N*-hydroxysuccinimide from Sigma Chemical Co. The water-soluble carbodiimide used here was 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and was purchased from Calbiochem, San Diego, Calif.

General Methods

A minor modification of the method of Porter (21) was used to prepare the Fab fragments from purified goat IgG directed against rabbit IgG. Purified goat IgG, 100 mg, was digested with 1 mg of papain in 10 ml of 0.1 M sodium phosphate buffer, pH 7.3, containing 5 mM dithiothreitol (Sigma Chemical Co.) and 2 mM EDTA (J. T. Baker Chemical Co., Phillipsburg, N. J.). The digestion was carried out at 37°C for 18–24 h with gentle shaking. The products of the digestion were separated from the undigested IgG by gel filtration on a 2.5 × 90 cm column of 6% agarose (A-5m, 200–400 mesh) which was equilibrated and eluted at 4°C with either 0.1 M sodium phosphate buffer, pH 7.3, or 0.01 M sodium phosphate buffer, pH 7.0.

To separate the Fab from the Fc fragments in the papain digest, an immunoadsorbent was prepared with a minor modification of the procedure of Avrameas and Ternynck (22). Glutaraldehyde was diluted to a concentration of 2.5% in 0.1 M sodium phosphate buffer, pH 7.3, and 0.4 ml of the solution was slowly stirred into 2.0 ml of the phosphate buffer containing 100 mg of rabbit IgG. After 5 h at room temperature, the excess aldehyde groups were inactivated by suspending the gel in 50 ml of 1.0 M glycine in 0.1 M sodium phosphate buffer, pH 7.5, and the suspension was allowed to stand overnight at 4°C. The gel was homogenized in about 200 ml of 0.1 M sodium phosphate buffer, pH 7.3, and was washed several times with the same buffer. All procedures were carried out at 4°C and the absorbent was stored at 4°C. In order to isolate the Fab and Fc fragments, the immunoadsorbent gel was stirred at room temperature for 2 h in 76 ml of 0.01 M sodium phosphate, pH 7.0, containing the papain digest of 100 mg of goat IgG directed against rabbit IgG (see above). The adsorbent was isolated by centrifugation at 20,000 *g* for 10 min and then washed three times with 30 ml 0.1 M sodium phosphate, pH 7.3. The specific Fab was then eluted by stirring the gel in 10 ml of 0.01 M sodium phosphate buffer, pH 6.0, containing 3 M sodium thiocyanate (J. T. Baker Chemical Co.) (23). The elution was repeated

twice, and to avoid denaturation of the Fab, the combined eluates were immediately desalted on a 2.5 × 90 cm column of Sephadex G-25 (coarse, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and equilibrated with 0.1 M sodium phosphate, pH 7.3. In a typical experiment 12.8 mg of Fab was adsorbed by the gel and 85% was eluted and recovered after desalting.

The Fab and IgG were labeled with [^{125}I]iodine by using lactoperoxidase (24). The labeled samples were diluted with carrier Fab and IgG before each experiment.

Procedure I: Preparation of Fab-Ferritin or IgG-Ferritin with a Water-Soluble Carbodiimide and N-Hydroxysuccinimide

In the first step, the amino groups in apoferritin were acylated with succinic anhydride. In a typical experiment 101 mg of ferritin in 1.1 ml of water was mixed with 6 ml of water saturated with sodium succinate at room temperature. The mixture was cooled to 4°C, and it was reacted with 0.53 g succinic anhydride, an amount calculated to be 130 equivalents per NH_2 group in apoferritin. The reaction was allowed to proceed at 4°C for 1 h and then at about 22°C for 1 h. The sample was passed through the gel filtration column of 6% agarose described above, equilibrated, and eluted with 0.01 M sodium phosphate buffer, pH 7.0, at 4°C. The peak fractions containing monomeric succinyl ferritin were pooled and concentrated at 4°C to about 30 mg of ferritin per ml in an Amicon ultrafiltration cell with PM-30 membrane (Amicon Corp., Lexington, Mass.).

In the second step the succinyl ferritin was activated with the water-soluble carbodiimide and *N*-hydroxysuccinimide with a modification of the method of Cuatrecasas and Pakrikh (25). Succinylated ferritin, 15.2 mg in 0.5 ml of 0.01 M sodium phosphate buffer at pH 7.0, was cooled on an ice bath, and 37.0 mg of water-soluble carbodiimide and 23.4 mg of *N*-hydroxysuccinimide were added. After 3 h, the sample was passed through a gel filtration column (Sephadex G-25, coarse) which was 0.9 × 80 cm and which was equilibrated and eluted with 0.01 M sodium phosphate buffer, pH 7.0. The gel filtration step removed the excess water-soluble carbodiimide and *N*-hydroxysuccinimide from the activated succinyl ferritin. Because the active ester in the succinyl ferritin which eluted in the void volume was subject to hydrolysis, it was used for conjugation in the third step as soon as it was recovered from the column.

In the third step the active ester of the succinyl ferritin was linked to either Fab or IgG. For the conjugation with Fab the two peak fractions from the G-25 column (above), containing about 10 mg of activated succinyl ferritin in 5.6 ml, were mixed with 1.7 ml of 0.1 M sodium phosphate buffer, pH 7.3, containing 3.4 mg of [^{125}I]iodine-labeled Fab. The reaction was allowed to proceed at room temperature for 24 h and the product was isolated by gel filtration on a 6% agarose column which was 2.5 × 95 cm and which was equilibrated and eluted with 0.1 M Tris-HCl buffer, pH 7.5. The peak fractions containing the Fab-ferritin were concentrated to 2.1 mg of ferritin per ml in an Amicon ultrafiltration cell with a PM-30 membrane. The conjugation of ferritin with IgG was carried out under similar conditions, but it was found necessary to reduce the ratio of activated succinyl ferritin to antibody in order to minimize formation of polymers. In a typical experiment, 1.0 mg of activated succinyl ferritin in 2 ml of buffer was mixed with 4.4 mg of rabbit [^{125}I]IgG in 3 ml of 0.01 M sodium phosphate buffer, pH 7.0. The reaction was allowed to proceed for 18 h at 4°C. Because the solution was relatively dilute, the reaction was stopped by the addition of 400 mg of glycine, and the mixture was then concentrated to 0.8 ml in the Amicon ultrafiltration cell with a PM-30 membrane. The product was then isolated by gel filtration on a 6% agarose column, 0.9 × 60 cm, which was equilibrated and eluted with 0.1 M Tris-HCl buffer, pH 7.5.

Procedure II: Preparation of Fab-Ferritin or IgG-Ferritin with Glutaraldehyde

In the first step of the reaction, ferritin was activated with a 1,200-fold molar excess of glutaraldehyde per amino group in apoferritin. In a typical experiment 10 mg of ferritin in 2.0 ml of 0.1 M sodium phosphate buffer, pH 7.3, was mixed with 1 ml of 50% glutaraldehyde. The reaction was carried out with stirring at room temperature for 30 min. After centrifugation at 20,000 *g* for 10 min to remove a small amount of precipitate, the unreacted glutaraldehyde was separated from the activated ferritin by gel filtration on a Sephadex G-25 column (coarse) which was 2.5 × 90 cm and which was equilibrated and rapidly eluted at 4°C with 0.1 M sodium phosphate buffer, pH 7.3. The activated ferritin was immediately used to prepare the conjugate.

In the second step of the reaction the activated ferritin was coupled to Fab or rabbit IgG. In most cases, the reaction was carried out with about 1.5 mg of activated ferritin and about 0.4 mg of Fab or IgG per ml. These concentrations were achieved either by concentrating the reactants before they were mixed or by concentrating the mixture of the two reactants. In a typical experiment with Fab, the peak fractions of activated ferritin from the G-25 column were concentrated at 4°C to about 3 mg of ferritin per ml in an Amicon ultrafiltration cell with a PM-30 membrane. The concentrated, activated ferritin, 11.4 mg in 4.4 ml, was mixed with 3.0 mg of [^{125}I]iodine-labeled Fab dissolved in 2 ml of 0.1 M sodium phosphate buffer, pH 7.3. After gently stirring at room temperature for 3 h, the solution was cooled to 4°C and the reaction was allowed to proceed for an additional 65 h at 4°C. The conjugate was then separated from nonconjugated Fab by gel filtration on a 6% agarose column (A-5m, 200–400 mesh) which was 2.5 × 95 cm. In order to inactivate

residual aldehyde groups on the ferritin, the column was equilibrated and eluted with 0.1 M Tris-HCl buffer, pH 7.5 at 4°C. The product was concentrated to about 2 mg of ferritin per ml with an Amicon ultrafiltration cell and a PM-10 membrane, and it was stored at 4°C. In a typical experiment with IgG, the activated ferritin from the G-25 column (see above), 12.2 mg in 20 ml, was directly mixed at 4°C with 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.3, containing 3 mg of rabbit [^{125}I]iodine-labeled IgG. The mixture was then concentrated to 8.8 ml on the Amicon PM-30 filter and kept at 4°C for 67 h. The product was chromatographed on a 6% agarose column as described for the isolation of Fab-ferritin.

RESULTS

Procedure I: Preparation of Fab-Ferritin or IgG-Ferritin with Water-Soluble Carbodiimide and N-Hydroxysuccinimide

In the first step of the synthesis, the reaction with succinic anhydride converted reactive amino-terminal groups and ϵ-amino groups of lysine in the ferritin to *N*-succinyl groups. The reaction blocked the reactive amino groups so that the water-soluble carbodiimide would not produce ferritin-ferritin polymers. In addition, the reaction increased the number of carboxyl groups available for activation in the second step. Gel filtration of the product on 6% agarose (not shown) indicated that about 74% of the ferritin was recovered in monomeric form. The presence of dimers and higher polymers in the product was in part explained by the presence of such polymers in the commercial preparation of ferritin used for the reaction (14, 26) and the gel filtration step effectively removed the polymers from the product.

In the second step, active esters were introduced into the succinyl ferritin by reacting it with water-soluble carbodiimide and the *N*-hydroxysuccinimide. In order to measure the number of active esters produced, control experiments were conducted in which the product from the second step was reacted with [^{3}H]glycine instead of with Fab or IgG. [^{3}H]Glycine was used in these experiments because its smaller size allowed more complete reaction with the available active esters in the succinyl ferritin (see below). Succinyl ferritin was activated with the water-soluble carbodiimide and *N*-hydroxysuccinimide (see Materials and Methods), and 3 mg of the activated succinyl ferritin in 0.7 ml of 0.01 M sodium phosphate buffer, pH 7.0, was mixed with 18 mg of [^{3}H]glycine, 5.9×10^{7} cpm, in 1.3 ml of 0.01 M sodium phosphate buffer, pH 7.0. The reaction was allowed to proceed for 24 h at room temperature and the product was isolated on a 6% agarose column. Under these conditions about 34 mol of [^{3}H]glycine reacted with each mole of ferritin.

In the third step of the reaction, the activated succinyl ferritin was coupled to Fab or to IgG. In order to follow this reaction [^{125}I]iodine-labeled Fab or IgG was used (see Materials and Methods). The results indicated that the products contained little if any polymeric ferritin and that essentially all of the ferritin applied to the column was recovered in a monomeric peak (Figs. 1 and 2). Calculated on the basis of specific activity of the [^{125}I]iodine-labeled Fab and the specific absorbance of the ferritin at 410 nm (not shown), the molar ratio of Fab to ferritin in the monomeric peak was 1.3 in the experiments with Fab (Fig. 1). In comparable experiments with IgG, the ratio of IgG to ferritin in the monomeric peak was also 1.3 (Fig. 2). With either Fab or IgG about 30% of the initial Fab or IgG was recovered in the conjugate-containing monomeric ferritin.

In the course of developing the procedure, several of the conditions were varied to improve the yield of conjugate. Acetylation of the ferritin was tried in place of succinylation, but the yield of Fab-ferritin was lower than with the procedure involving succinylation. Also, the pH of the cou-

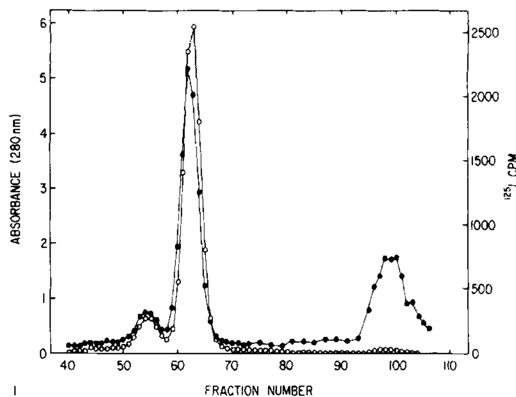

FIGURE 1 Gel filtration of [^{125}I]iodine-labeled Fab-ferritin conjugate prepared by procedure I. The conditions were described in Materials and Methods. The column of 6% agarose was 2.5×95 cm and the fraction volume was 4.2 ml. The void volume appeared in fraction 43 and the inclusive volume appeared in fraction 107. As indicated, monomeric ferritin was recovered in fractions 61–67. Symbols: O—O—O = absorbance at 280 nm; ●—●—● = counts per minute.

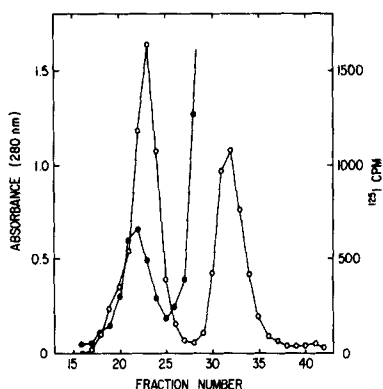

FIGURE 2 Gel filtration of [^{125}I]iodine-labeled IgG-ferritin conjugate prepared by procedure I. The conditions were as described in Materials and Methods. The column of 6% agarose was 0.9×60 cm and the fraction volume was 1.15 ml. The void volume appeared in fraction 17 and the inclusive volume in fraction 42. As indicated, monomeric ferritin was recovered in fractions 20-25. Symbols: O—O—O = absorbance at 280 nm; ●—●—● = counts per minute.

pling step was increased to 8.5 with no improvement in the [^{3}H]glycine to ferritin molar ratio (see above). Similarly, increasing the time of the coupling reaction to 48 h had no effect on the yield. In two series of experiments it was established that the linkage between ferritin and IgG or Fab occurred through the active esters produced in the ferritin. In one type of experiment, the active esters in succinyl ferritin obtained in step two (above) were hydrolyzed by adjusting the pH of the sample to 8.0 with 0.1 M sodium hydroxide and incubating the sample at 22°C for 24 h. The product was examined by gel filtration on 6% agarose (Bio-Gel A-5m) and a peak of *N*-hydroxysuccinimide was recovered in the total volume of the column. Based on absorbance at 260 nm the amount of *N*-hydroxysuccinimide recovered was about 50 mol/mol of ferritin, indicating that about this number of active esters had been introduced into the ferritin in step two. In a second series of experiments it was found that if the water-soluble carbodiimide was omitted in step two, no [^{3}H]glycine was linked to ferritin. If the *N*-hydroxysuccinimide was omitted in step two, the amount of [^{3}H]glycine subsequently linked to ferritin was reduced by 60%. These observations indicated that most of the bonds linking the ferritin to Fab were derived from active esters and few if any of the bonds were derived directly from labile anhydrides generated in the first step.

Procedure II: Preparation of Fab-Ferritin or IgG-Ferritin with Glutaraldehyde

In the first step of the reaction, the ferritin was activated with a large molar excess of glutaraldehyde so that a monovalent reaction occurred and the reactive amino groups in the ferritin were rapidly linked to glutaraldehyde, but few if any polymers were formed.

To examine the number of reactive groups introduced by the activation step, ferritin was activated with glutaraldehyde and the activated ferritin was reacted with [^{3}H]glycine as described above in the control experiments with activated succinyl ferritin. Ferritin, 3.3 mg/ml, was activated with 17% glutaraldehyde, as described in Materials and Methods, and 4 mg of the resulting activated ferritin was reacted with 24 mg of [^{3}H]glycine, 5.0×10^7 cpm, in 1.6 ml of 0.1 M sodium phosphate buffer, pH 7.3. The reaction with [^{3}H]glycine was carried out at 4°C for 15 h and the product was isolated on a 6% agarose column (see above). About 60% of the [^{3}H]glycine-ferritin was recovered in the peak of monomeric ferritin and the remainder in the peak containing dimeric ferritin. The molar ratio of [^{3}H]glycine to ferritin in the product was about 190. When the ferritin was activated with 0.05% glutaraldehyde instead of 17%, the molar ratio of [^{3}H]glycine to ferritin in the product decreased to 9, indicating that fewer amino groups were activated when a lower concentration of glutaraldehyde was used. It was noted that the activated ferritin obtained with the lower concentration of glutaraldehyde tended to form polymers on standing.

In further experiments the activated ferritin was reacted with [^{125}I]iodine-labeled Fab or IgG. The results indicated that most of the ferritin in the product was in a monomeric form (Fig. 3). Some polymeric ferritin was also present but the polymer was readily separated from the monomer in the gel filtration step.

In the experiments with [^{125}I]iodine-labeled Fab, about 15% of the initial [^{125}I]iodine-labeled Fab was recovered in the monomeric ferritin peak and in the experiments with IgG about 25% of the [^{125}I]iodine-labeled IgG was recovered in a comparable peak. The results therefore indicated that the conjugation with IgG was slightly more efficient than the conjugation with Fab, apparently because the molar amount of IgG used for conjugation was less. With both the Fab and IgG conjugates the

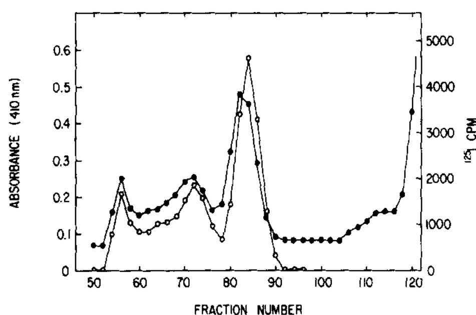

FIGURE 3 Gel filtration of [^{125}I]iodine-labeled Fab-ferritin prepared by procedure II. The conditions were as described in Materials and Methods. The column was the same as in Fig. 1, except that the fraction volume was 3.2 ml, the void volume appeared in fraction 56 and the inclusive volume appeared in fraction 140. As indicated, monomeric ferritin was recovered in fractions 78-90. Symbols: O—O—O = absorbance at 410 nm; ●—●—● = counts per minute.

molar ratio of antibody to ferritin in the peak of monomeric ferritin was about 0.8.

The gel filtration chromatograms indicated that the Fab-ferritin or IgG-ferritin conjugates did not contain free Fab or free IgG (Figs. 1-3). To examine the question of whether the product contained polymers of IgG which might co-chromatograph with the ferritin conjugates, the product was examined by immunoelectrophoresis under conditions in which IgG had no electrophoretic mobility. The results (Fig. 4) demonstrated that less than 5% of the IgG in the isolated conjugate was present as free IgG or polymers of IgG.

To examine the question of how much of the Fab in the isolated Fab-ferritin conjugates was active, the goat Fab-ferritin was tested with an immunoadsorbent containing rabbit IgG. As indicated in Table I, 40-51% of the Fab in the conjugate was bound to the immunoadsorbent, indicating that this fraction of the Fab remained active after conjugation to the ferritin. The remainder was presumably inactivated as a result of the conjugation. As a control for the immunoadsorbent experiment, the goat Fab-ferritin was eluted from the immunoadsorbent with 5.7 mg/ml of goat IgG and was isolated by gel filtration on a 6% agarose column. The purified goat Fab-ferritin was then shown not to be adsorbed onto a similar immunoadsorbent prepared with goat IgG. However, the goat Fab-ferritin which was isolated and tested in this manner was subsequently readsorbed by the original immunoadsorbent containing rabbit IgG (not shown).

In the experiments presented in Table I, 25-29% of the ferritin was bound to the immunoadsorbent and the ratio of Fab to ferritin was higher in the adsorbed material than in the initial conjugate. The latter observation indicated that 25-29% of

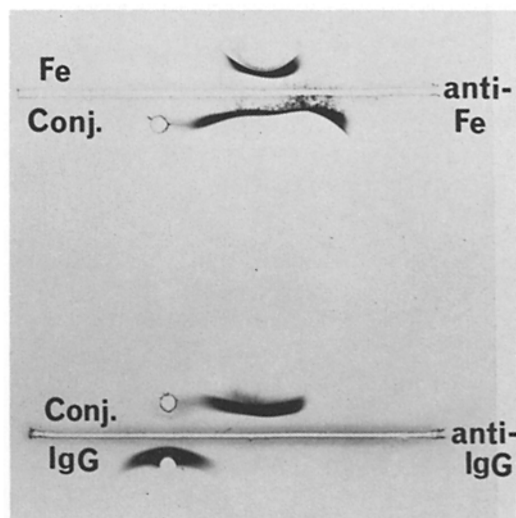

FIGURE 4 Immunoelectrophoresis of IgG-ferritin conjugates prepared by procedure II. In the upper frame, the trough contained antiserum to ferritin (anti-Fe) prepared in rabbits. As indicated, the antiserum produced a single immunoprecipitin line with ferritin (1 mg/ml in upper well). The antiserum produced two arcs in a reaction of identity with the IgG-ferritin conjugate (4.4 mg of ferritin and 0.75 mg of IgG per ml in lower well), indicating that the sample contained both IgG-ferritin conjugate and some free ferritin. In the lower frame, the trough contained antiserum directed against rabbit IgG (anti-IgG) prepared in sheep. The antiserum produced a single immunoprecipitin line with the conjugate (4.4 mg of ferritin and 0.75 mg of IgG per ml in the upper well). Since essentially no immunoprecipitate was seen at the origin, the results indicated that the conjugate did not contain monomers or polymers of IgG not linked to ferritin. The lower well in the lower frame contained 0.6 mg/ml of purified rabbit IgG. Other experiments (not shown) demonstrated that 35 μg/ml of IgG were detectable in the system. The immunoelectrophoresis was carried out in 1% agarose and in 0.05 M sodium barbital buffer, pH 8.6. The electrophoresis was run for 90 min with 85 V and 18 mA. After immunodiffusion for 24 h, the gels were strained with 0.5% Coomassie Brilliant Blue R in ethanol:acetic acid:water (45:10:45) and destained in the same solvent (32).

TABLE I
Characterization of Fab-Ferritin Conjugates by Adsorption with an Immunoadsorbent Gel Containing Rabbit IgG

	Preparation of Fab-Ferritin conjugate	
	Procedure I	Procedure II
Ferritin in sample (μg)		
Initial	7,030	400
Adsorbed	2,067	99
Fab* in sample (cpm)		
Initial	240,000	4,500
Adsorbed	96,500	2,280
Molar ratio Fab*:ferritin		
Initial	1.3	0.8
Adsorbed	1.8	1.6

* Fab was labeled with [^{125}I]iodine as described in Materials and Methods.

the ferritin was bound to active Fab and the remaining 71–75% was present either as ferritin bound to inactive Fab or free ferritin.

A separate series of experiments (not shown) suggested that when conjugates of IgG-ferritin instead of Fab-ferritin were prepared, a larger fraction of conjugated antibody molecules remained immunologically active. When IgG directed against prolyl hydroxylase was linked to ferritin with procedure II and the conjugate was assayed with a passive hemagglutination test with erythrocytes coated with prolyl hydroxylase, the IgG-ferritin had the same antibody activity per milligram IgG as the initial unconjugated IgG. In control experiments it was shown that the same IgG-ferritin conjugates did not agglutinate erythrocytes coated with the collagenase-resistant fragment of procollagen (27).

In a final series of experiments IgG-ferritin conjugates were stored at 4°C for 8 mo and then analyzed by gel filtration on 6% agarose. The elution pattern indicated that the conjugate was unaltered and there was no evidence of free IgG or of the formation of aggregates during storage. The stability of the conjugates is probably explained by the demonstration that the cross-links produced in proteins by glutaraldehyde are largely the result of stable Michael-type adducts formed between amino groups and α,β-unsaturated aldehydes (28, 29).

DISCUSSION

There are several criteria which can be used to evaluate ferritin-antibody conjugates and the procedures used to synthesize them. One criterion is that the antibody in the conjugate should retain most of its immunological activity. A second criterion is that the conjugate should be relatively free of antibody which is not linked to ferritin. Otherwise, the unconjugated antibody would bind to antigenic sites in the specimen and competitively prevent the binding of the labeled antibody. A third criterion is that the product should be free of ferritin-ferritin polymers, since such polymers tend to be relatively insoluble and to adsorb nonspecifically to tissue or cell preparations. A fourth criterion is that the conjugation procedure should be efficient so as not to waste specific antibody which may be in short supply. A fifth criterion is that the molar ratio of antibody to ferritin in the conjugate should be about 1. A higher molar ratio would provide a conjugate which might bind well to cellular antigens but have relatively little ferritin. A sixth criterion is that the amount of free ferritin in the conjugate should not be excessive. Otherwise, it will be difficult to prepare the active conjugate in a high enough concentration for adequate binding to the cellular antigen during incubation with cells or tissues.

The two procedures presented here meet most of these criteria. With both procedures 50–51% of the Fab in the ferritin conjugates was still immunologically active. When IgG-ferritin was prepared with procedure II, all the IgG in the conjugate appeared to retain its immunological activity as tested with a passive hemagglutination inhibition assay. The conjugates did not contain any free Fab or free IgG because these were effectively removed by the gel filtration after the coupling reaction. The gel filtration step also removed the ferritin-ferritin polymers from the final product. Immunoelectrophoresis of the IgG-ferritin conjugates demonstrated that they did not contain significant amounts of polymeric IgG not bound to ferritin. The yields of the desired conjugates were relatively high and under specific conditions employed 15–30% of the initial Fab and 25–30% of the initial IgG were incorporated into the conjugate containing monomeric ferritin. Also, a large proportion of the Fab or IgG used as starting materials and not conjugated were recovered and if necessary, could be re-used for the synthesis of additional conju-

gate. With both procedures it was possible to obtain conjugates in which 1-2 mol of Fab or IgG were covalently attached to each mole of ferritin. Although free ferritin was present in the final products, the amount was low enough so that it did not present any problem in using IgG-ferritin for labeling of cells (see accompanying paper [13]).

On the basis of the criteria listed above, the two procedures presented appear to have a number of advantages over previous procedures (1, 8, 15-20) for preparing antibody-ferritin conjugates. As indicated in the accompanying paper (13) and elsewhere (30, 31), conjugates prepared with these procedures have been successfully used to determine the location of procollagen and prolyl hydroxylase in fibroblasts.

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