THE PROPERTIES OF SPECIFIC STAINS FOR ELECTRON MICROSCOPY PREPARED BY THE CONJUGATION OF ANTIBODY MOLECULES WITH FERRITIN

S. J. SINGER, Ph.D., and ANITA F. SCHICK, Ph.D.

From the Department of Chemistry, Yale University, New Haven, and the Virus Laboratory, University of California, Berkeley

ABSTRACT

In order to take full advantage of recent developments in the electron microscopic examination of cellular ultrastructure and composition, it is necessary to develop specific electron stains capable of identifying and localizing a wide variety of macromolecular components of cells. To this end, antibody conjugates have been prepared by chemically coupling the highly electron-scattering ferritin molecule to antibody. Antigen-antibody precipitations with these ferritin-antibody conjugates have demonstrated that under the appropriate conditions they retain the specific binding properties of the antibody from which they are prepared. An electron microscopic study has been made of aggregates of tobacco mosaic virus and its ferritin-conjugated antibody. The aggregates were prepared in solution and then sprayed onto specimen screens. The electron micrographs reveal that the conjugate specifically attached to, and delineated, the virus rods. The chemistry, structure, and resolving power of the ferritin-antibody conjugates, the specificity of their reactions with homologous antigen, and the nature of the problems to be faced in application of these conjugates to the study of the internal antigens of cells are discussed.

INTRODUCTION

In recent years, the development of techniques associated with electron microscopy has made it possible to visualize in fine detail the macromolecular contents of ultrathin sections of intact cells and cell organelles. More particularly, what is visualized is a map of the distribution of electron-scattering power of the fixed ultrathin section, and methods are now required for identifying and localizing individual macromolecular components on this map. An analogous situation exists in ordinary light microscopy, for which specific chemical stains have been developed which absorb or emit characteristic optical radiation. In electron microscopy, specific stains are required which scatter electrons in some characteristic manner. The most generally used and most highly specific of the light microscope stains is the recently introduced fluorescent antibody (Ab) conjugate (1). Such a conjugate is prepared by chemically coupling a fluorescent substance to an Ab which has been produced in response to, and is capable of specifically binding to, a particular antigen (Ag), generally some pure protein. When the fluorescent Ab conjugate is applied to a suitably fixed collection of cells, the specific Ag may be detected and localized at relatively low resolution by observation of the fluorescence.

In order to develop a similar specific staining

technique to function at the resolution available with the electron microscope, Ab conjugates must be prepared which, by virtue of their high electron-scattering power, can be distinguished within the macromolecular matrix of the ultrathin section. Ordinary unmodified Ab, having about the same electron-scattering power as the matrix, would not be so distinguished except under special circumstances. To prepare a suitable Ab conjugate, groups of large atomic number must be chemically coupled to the Ab molecule. We accomplished this by coupling ferritin, a highly electron-scattering protein molecule, to Ab; a preliminary note describing the method has been published (2).

Of possible methods for preparing suitable Ab conjugates, an obvious one is to use a small-molecule conjugate containing one or more heavy atoms, such as a compound containing iodine or mercury atoms, and to couple a large number of these over the surface of the Ab molecule. The preparation of such a conjugate has recently been reported (3). However, it is difficult to conceive that for general purposes sufficient electron-scattering power could be conferred upon an Ab molecule by this method without inactivating the Ab (4). It seemed to us more feasible to prepare a satisfactory Ab conjugate by coupling only one or two relatively large and highly electron-scattering molecules to an Ab molecule. Whereas the first method mentioned would confer a diffuse increase of electron-scattering power on the Ab molecule, the second method would attach one or two electron-scattering "tails" to it. In our search for a suitable "tail" to conjugate to Ab, it occurred to us that ferritin might serve the purpose. Ferritin is an easily prepared crystalline protein which can be obtained in large quantities from horse spleen (5). It has the unusual property of containing about 20 per cent iron, in the form of micelles of ferric hydroxide-phosphate situated within a protein shell (5, 6). Because of the extraordinary electron-scattering power conferred by the large iron content (about 120,000 atomic weight units of Fe per molecule), individual ferritin molecules may readily be seen in the electron microscope (6). The protein shell when freed of the ferric hydroxide is called apoferritin; it has a molecular weight of about 460,000, and both ferritin and apoferritin are nearly spherical molecules with a diameter of about 110 A. The ferric hydroxide micelles contained within the protein are arranged at the apices of a roughly tetrahedral lattice measuring about 55 A across (6, 7).

Since our preliminary note (2) describing the preparation of a ferritin-Ab conjugate was published, two communications have appeared (8, 9) reporting the use of our method in electron microscopic studies. In the meantime, we have explored the chemistry of the conjugation process intensively, and have developed an improved method. The details of these studies will be published elsewhere. In this paper, three experimental subjects are treated: A summary of the chemistry of the conjugation reactions is given insofar as it is essential to an understanding of the properties of the ferritin-Ab conjugates; the macroscopic Ag-Ab precipitation behavior of the conjugates is described; and an electron microscopic study of the reaction in solution of tobacco mosaic virus (TMV) with its specific ferritin-Ab conjugate is presented as a model system for exploring the feasibility of using ferritin-Ab conjugates to detect and localize specific antigen in cellular sections. Our more recent studies have been briefly reported (10).

MATERIALS AND METHODS

Once-recrystallized ferritin was prepared by a standard method (5) from freshly obtained horse spleens. The *m*-xylylene diisocyanate (XC) was the gift of the Carwin Company, North Haven, Connecticut, and the toluene 2,4-diisocyanate (TC) was kindly donated by Dr. V. E. Shashoua, of the DuPont Company, Wilmington, Delaware.[1] Tobacco mosaic virus (TMV, common strain), bushy stunt virus (BSV), and bacteriophage T6 were obtained from colleagues at the Virus Laboratory.

The sample of pure rabbit anti-TMV antibody used to prepare the XC conjugates was generously given to us by Dr. Irving Rappaport of the University of California at Los Angeles; the pure Ab used to prepare the TC conjugates was prepared from rabbit antiserum also provided by Dr. Rappaport, employing a procedure recommended by him. Antisera to bovine serum albumin (BSA) and bovine pancreatic ribonuclease (RNase), were preparations similar to those used in other studies (11). Normal rabbit serum was obtained from Microbiological Associates, Inc., Albany 6, California. For purposes of conjugation, the gamma globulin fraction was first isolated from

[1] Toluene 2,4-diisocyanate in 99 per cent purity may be obtained from National Aniline Division, Allied Chemical Corporation, 40 Rector Street, New York 6, N. Y.

serum by precipitation with 40 per cent saturated $(NH_4)_2SO_4$, and after one wash with this medium was stored as a precipitate at 4°C. until needed, whereupon a portion was dialyzed against the desired buffer.

The conjugation of ferritin to Ab by XC was carried out essentially by the procedure described earlier (2). To 5 ml. of a 1.5 per cent solution of ferritin in sodium borate–boric acid buffer, pH 9.5, $\Gamma/2$ 0.1 at 0°C., was added 0.10 ml. of XC. The XC is mostly insoluble in the aqueous phase. After vigorous magnetic stirring in an ice bath at 0°C. for ¾ hour, the mixture was centrifuged in a Servall instrument in a cold room for about ½ hour at a speed sufficient to pellet the unreacted diisocyanate without sedimenting any appreciable amount of ferritin. The supernatant solution was carefully removed by syringe to avoid contamination by diisocyanate scum at the meniscus, and was allowed to stand at 0°C. for an additional hour to allow any dissolved XC to react. This solution was then added to an equal volume of a 1.5 per cent solution of γ-globulin (containing specific Ab) in the above-mentioned borate buffer. (The 1.0 M NaCl added to this buffer as reported in our earlier note was found to be unnecessary.) A precipitate usually formed soon after mixing. After standing at 4°C. for 2 days, the mixture was dialyzed against 0.1 M $(NH_4)_2CO_3$, pH 8.8, to destroy any unreacted NCO groups on the conjugate. The precipitate was removed by centrifugation to leave a clear stable solution.

The preparation of the TC conjugates differed in some important details. To 5.0 ml. of a 1.5 per cent ferritin solution in phosphate buffer, pH 7.5, $\Gamma/2$ 0.1 at 0°C., was added 0.10 ml. of TC. The latter is solid when stored under refrigeration, and was melted to remove a small portion. After vigorous stirring in an ice bath at 0°C. for 25 minutes, the mixture was centrifuged as above to sediment the unreacted diisocyanate, and the supernatant was carefully removed and allowed to stand an additional hour at 0°C. The solution was then added to an equal volume of a 1.5 per cent solution of γ-globulin in borate buffer, pH 9.5, $\Gamma/2$ 0.1 at 37°C. The pH of the resultant mixture was close to 9.3. After 1 hour at 37°C., the mixture was dialyzed against 0.1 M $(NH_4)_2CO_3$. No precipitate formed at this stage in the conjugation reaction, in contrast to the result with XC as coupling agent.

In studies of the precipitation behavior of ferritin-Ab conjugates (Tables I, II, and III), mixtures of the conjugates with Ag were prepared by dilution into phosphate or barbital buffers, and were incubated for ½ hour at 37°C. and then for 24 hours at 4°C. The precipitates, after washing with the appropriate buffer, were then analyzed. The precipitates of Table I were prepared in duplicate. One precipitate was digested for Nessler N determinations. The other of the pair was first treated with 1.5 N HCl for 1 hour at 37°C. to extract all the Fe, then was brought to 7 per cent in trichloracetic acid (TCA) to precipitate any dissolved protein, and a portion of the supernatant was analyzed for Fe with *o*-phenanthroline. Both kinds of analyses were performed on a single precipitate in the experiments of Tables II and III. The washed precipitate was treated with 1.5 N HCl and then in 7 per cent TCA as above. After centrifugation, a portion of the supernatant was analyzed for Fe with *o*-phenanthroline; the centrifuged precipitate was completely dissolved in 0.1 N NaOH, and the O.D. at 280 mμ was taken as a measure of the N content of the precipitate, after calibration with solutions of γ-globulin.

Solutions to be examined in the electron microscope contained only volatile buffer salts. One volume of a solution was mixed with an equal volume of an aqueous suspension of polystyrene latex (PSL) particles containing 3×10^{10} particles per milliliter. Immediately after the addition of the PSL, the mixtures were sprayed from a Vaponefrin nebulizer onto carbon-coated parlodion films. After air drying, and without washing or shadowing, the specimens were examined in an RCA Model EMU-2 electron microscope without an objective aperture.

A Perkin-Elmer Model 38A Tiselius apparatus was used for the electrophoresis experiments, and a Beckmann Model DU spectrophotometer for optical absorption measurements.

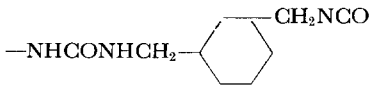
To free the solution of fer-TC-anti TMV of unmodified anti-TMV Ab, 2.0 ml. was diluted with 3.0 ml. of phosphate buffer, pH 7.5, $\Gamma/2$ 0.1, and the mixture was centrifuged in a Spinco Swinging Bucket rotor #39 at 35,000 r.p.m. for 2 hours. The supernatant was drawn off the pellet and discarded. The pellet was completely dissolved in 2.0 ml. 0.01 M $(NH_4)_2CO_3$ and dialyzed against the same solvent overnight to give the preparation fer-TC-anti TMV *a*.

CONJUGATION OF FERRITIN AND ANTIBODY

The chemical coupling of two protein molecules to each other to give a binary species has not received much careful investigation in the past. We considered that the most direct method was to employ some bifunctional small molecule whose functional groups were known to react with groups on protein molecules. The suitability of a reagent, and the appropriate conditions for its use, were first investigated by model experiments on the coupling of bovine serum albumin (BSA) and bovine gamma globulin (BGG), which we found to parallel the behavior of the ferritin-Ab system

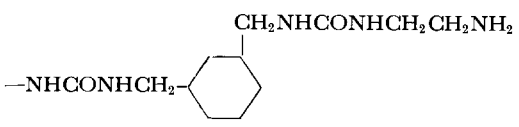
quite closely. In order to detect the occurrence of coupling, electrophoresis experiments were performed on the dialyzed reaction mixtures, any coupled mixed species appearing as new peaks with mobilities between those due to the uncoupled BSA and BGG.

In our initial experiments (2), *m*-xylylene diisocyanate (XC) was used as the coupling reagent. The coupling reaction was carried out in two stages in order to minimize the inactivation of the Ab. First BSA (or ferritin) was treated with XC in borate buffer, pH 9.5, Γ/2 0.1, under conditions which reacted about half the free amino groups of the protein, as determined by Van Slyke analyses. We presumed at the time that these reacted amino groups had been in large part converted to



groups, with the second isocyanate group available for further reaction. At this stage, after the excess XC was removed, the BGG (or Ab) was added in the same borate buffer. By the electrophoretic criterion, we did indeed find that BSA and BGG, and ferritin and Ab, could be coupled together in this manner. The ferritin-Ab conjugates precipitated with their homologous Ag, and exhibited at least partial specificity of precipitation in two unrelated Ag-Ab systems (2).

Before proceeding further with the use of these conjugates, however, we wished to determine whether the protein-protein coupling induced by XC was entirely by stable covalent linkage, or whether some kind of non-covalent and possibly reversible interaction was involved. The electrophoretic criterion for coupling clearly could not distinguish between these two mechanisms. To this end, the BSA which had been reacted with XC, but to which the BGG had not yet been added, was treated with a heavy excess of a primary amine, ethylene diamine, in a borate buffer at pH 9.5. We reasoned that the amine would react with any NCO groups on the surface of the BSA molecules to give the unreactive



product, which would then no longer be capable of combining with subsequently added BGG. If coupled species appeared in such mixtures that had been subjected to an intermediate treatment with ethylene diamine, their formation could be ascribed to a non-covalent type of linkage.

Parallel experiments on the conjugation of BSA and BGG by XC were therefore performed with and without intermediate treatment with ethylene diamine, and the final products of these reactions were examined electrophoretically both at pH 9.5 and at pH 7.5. From these experiments, which are reproduced in Fig. 1, several conclusions may be drawn. A considerable amount of non-covalent association did indeed occur in this system (Fig. 1 *b*, *d*) which was reduced at pH 9.5 as compared with pH 7.5, but was still evident at the higher pH. On the other hand, a substantial degree of covalent linkage was produced as well, as is clear from a comparison at pH 9.5 of the ethylene diamine–treated and untreated mixtures (Fig. 1 *a*, *b*). Similar results were obtained with ferritin-Ab conjugates prepared with XC; that is, the conjugates were in part covalently linked, in part non-covalently linked.

In view of these results, studies were next undertaken to produce an entirely covalently linked conjugate. Experiments on the chemistry of the XC reaction convinced us that the difficulty with XC was that its two functional groups have the same intrinsic reactivity, and that a very large number of XC molecules were therefore required to react with each BSA molecule in order to leave a sufficient number of NCO groups on its surface unhydrolyzed and available for reaction with BGG. The presence of so many aromatic XC residues on the surface of the BSA molecules apparently rendered the BSA capable of forming non-covalently bound aggregates with BGG. It may be inferred that the reason that this aggregation increased with a decrease in pH was the corresponding decrease in electrostatic repulsion of the BSA and BGG molecules.

A substantial improvement was achieved by utilizing a bifunctional reagent whose two functional groups are of different reactivity, toluene 2,4-diisocyanate (TC). In this compound, the NCO group ortho to the CH_3 is hindered and less reactive than the para one. Conditions were found in which the para group preferentially combined with BSA (or ferritin) in the first stage of the reaction, while the ortho NCO remained

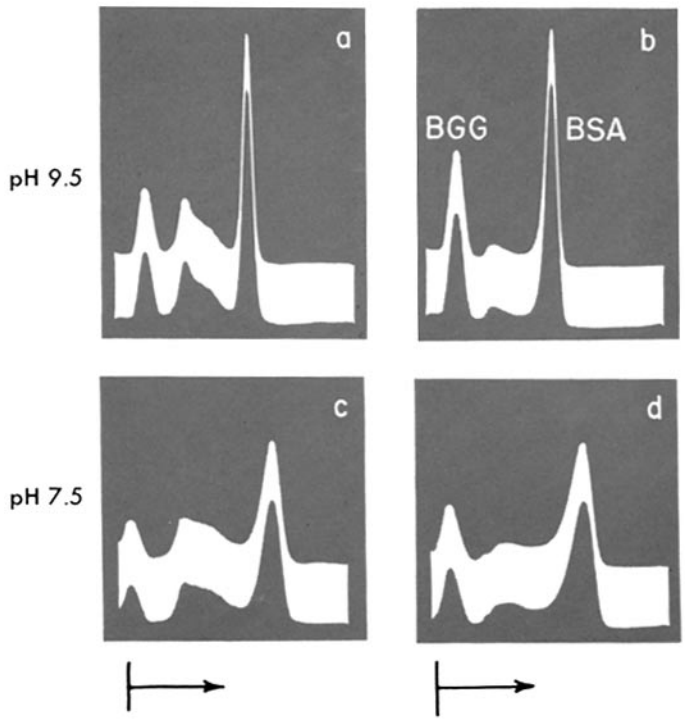


FIGURE 1

Electrophoresis patterns of the product of the conjugation by *m*-xylylene diisocyanate (XC) of bovine serum albumin (*BSA*) and bovine γ-globulin (*BGG*): *a* without, and *b* with, intermediate treatment with ethylene diamine, both examined in borate buffer, pH 9.5, Γ/2 0.1; *c* without, and *d* with, intermediate treatment with ethylene diamine, examined in phosphate buffer, pH 7.5, Γ/2 0.1. The direction of migration is indicated by the arrows. The fastest peak, labeled BSA, is due to uncombined, XC-modified BSA; the slowest, labeled BGG, to uncombined BGG; and the intermediate peaks are due to conjugates of the two.

in substantial part unreacted until BGG (or Ab) was added under a second set of conditions. The detailed reaction procedure is given above in the section on Materials and Methods. When parallel BSA-BGG conjugation experiments were performed using TC as the coupling agent, with and without ethylene diamine treatment after the first stage of the conjugation reaction, the results (shown in Fig. 2) were quite different from those obtained with XC as the coupling agent. There was no indication of any conjugation in the ethylene diamine–treated cases at pH 9.5 or pH 8.6, and only a trace at pH 7.5. On the other hand, considerable conjugation was produced in the case without intermediate ethylene diamine treatment, in amount independent of the pH at which the electrophoresis was performed. The conclusion is straightforward that the conjugation in this case is entirely by covalent linkage through reaction of BGG with the required NCO functions on the BSA molecules. With ferritin-Ab conjugates prepared using TC under the same conditions, similar evidence was obtained (Fig. 3) that exclusively covalent linkage had been produced between the two proteins.

From the areas under the peaks of the electrophoresis patterns, it is apparent that about one-third of the BSA (or ferritin) and a similar fraction of the BGG (or Ab) in the TC conjugation products have been conjugated to each other, the rest remaining uncombined. In addition, ultracentrifuge studies demonstrated that with BSA-BGG conjugates prepared with TC, the predominant type consisted of one molecule of BSA attached to one of BGG, and that about one-third as many conjugates contained two BSA molecules attached to one BGG. There was only a negligible amount of still larger conjugates. It appeared that this

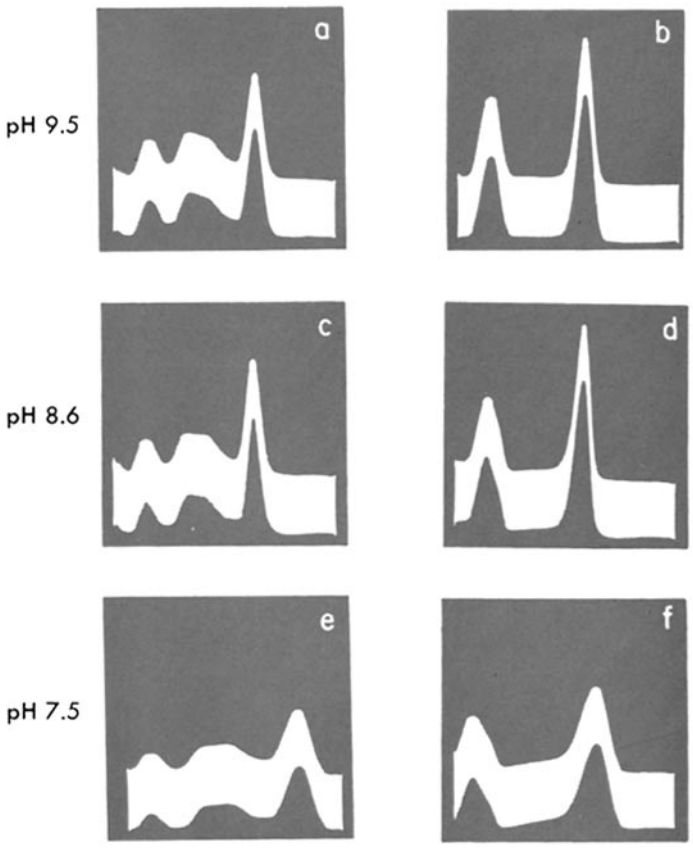


FIGURE 2

Electrophoresis patterns of the product of the conjugation by toluene 2,4-diisocyanate (TC) of bovine serum albumin and bovine γ-globulin: *a*, *c*, and *e* were not subjected to intermediate treatment with ethylene diamine, and were examined in borate buffer, pH 9.5, Γ/2 0.1, barbital buffer, pH 8.6, Γ/2 0.1, and phosphate buffer, pH 7.5, Γ/2 0.1, respectively; *b*, *d*, and *f* correspond to *a*, *c*, and *e*, respectively, except that they were treated with ethylene diamine. The peaks correspond to those in Fig. 1.

distribution was also at least qualitatively characteristic of ferritin-Ab conjugates prepared with TC, but the ultracentrifugal heterogeneity of ferritin did not permit unambiguous verification of this point.

To summarize this brief description of the chemistry of the preparation of ferritin-Ab conjugates: first, the use of *m*-xylylene diisocyanate as a coupling agent by the method previously reported (2) results in conjugates that are partly covalently and partly non-covalently linked; second, the extent of non-covalent linkage decreases with an increase in pH; third, toluene 2,4-diisocyanate as a coupling agent produces entirely covalently linked conjugates, under similar conditions. These considerations are of interest in connection with the behavior of these conjugates in their reactions with antigens, which is the major subject of this paper. A more detailed account of the chemistry will be published in the near future (Schick and Singer, data to be published).

PRECIPITATION OF FERRITIN-ANTIBODY CONJUGATES WITH ANTIGEN

In our earlier study (2), in order to investigate whether the properties of ferritin-Ab conjugates prepared with XC were satisfactory, the following experiments were performed. The γ-globulin fractions of antisera against bovine serum albumin (BSA) and bovine pancreatic ribonuclease (RNase) were conjugated with ferritin, giving preparations designated fer-XC-anti BSA and

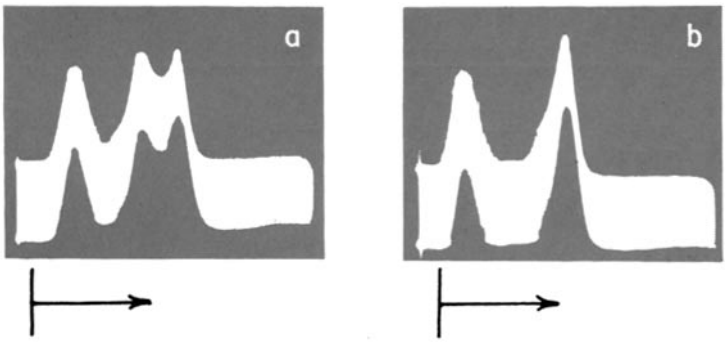


FIGURE 3

Electrophoresis patterns of the product of the conjugation by toluene 2,4-diisocyanate (TC) of ferritin and a rabbit γ-globulin containing antiribonuclease antibody, examined in borate buffer, pH 9.5, Γ/2 0.1: *a* without, and *b* with, intermediate treatment with ethylene diamine. The direction of migration is indicated by the arrows. The fastest peak is due to uncombined TC-modified ferritin, the slowest to uncombined γ-globulin, and the intermediate peak in *a* to the conjugate of the two.

fer-XC-anti RNase, respectively. (In these and all other experiments reported in this paper unless otherwise stated, the complete product of the conjugation reaction, containing the ferritin-Ab conjugate together with modified but uncoupled ferritin, unmodified Ab, unmodified non-antibody γ-globulin, and the ferritin conjugate of non-antibody γ-globulin, was used without further purification.) Fer-XC-anti BSA gave no precipitate with RNase, nor did fer-XC-anti RNase with BSA. Two mixtures were then prepared, one containing fer-XC-anti BSA plus unconjugated anti-RNase Ab; the other, equivalent amounts of fer-XC-anti RNase plus unconjugated anti-BSA Ab. Portions of each of these two mixtures were then treated with serial dilutions of either BSA or RNase, and the precipitates formed were analyzed for N and Fe. The precipitations were all carried out in phosphate buffer, pH 7.5, Γ/2 0.1. The results are presented again, in Table I, to facilitate comparison with those obtained in the following experiments.

In due course, the procedure for conjugating ferritin to antibody with TC was developed, and a set of experiments analogous to that in Table I was performed, also in phosphate buffer, pH 7.5, Γ/2 0.1, with the results shown in Table II. In addition, another similar set of experiments was carried out in barbital buffer, pH 8.6, Γ/2 0.1 (Table III), employing the same ferritin-TC-Ab conjugates used in the experiments of Table II, to determine whether increasing the pH would decrease the amount of non-specific precipitation.

A qualitative examination of the precipitation data, particularly of Tables I and III, indicates that under the appropriate conditions a considerable degree of specificity was exhibited by the ferritin-Ab conjugates in their reactions with Ag. In the first place, the conjugates, prepared by either XC or TC coupling, copiously precipitated with homologous, but not at all with heterologous, Ag. Secondly, in sections B of Tables I and III it is clearly demonstrated that the presence of fer-XC-anti BSA or fer-TC-anti BSA had little effect on the precipitation of RNase by anti-RNase, and that only a small amount of ferritin was brought down in any of those precipitates. Therefore, the substantial amount of ferritin in all the precipitates formed between RNase and fer-XC-anti RNase or fer-TC-anti RNase must have been bound to specific antibody. In BSA-anti BSA precipitations from solutions containing fer-XC-anti RNase or fer-TC-anti RNase (sections E, Tables I and III, respectively), only a small amount of ferritin was brought down non-specifically in precipitates made in Ag excess or close to equivalence (maximum precipitation zone), whereas in comparable precipitates formed between BSA and fer-XC-anti BSA or fer-TC-anti BSA (sections F, Tables I and III), a substantial amount of ferritin was incorporated, again indicating that specific precipitation had occurred under these circumstances. However, in BSA-anti BSA precipitates formed in Ab excess in the presence of the heterologous conjugate, the Fe/N

TABLE I

Precipitation of Ferritin-XC-Antibody Conjugates in Phosphate Buffer, pH 7.5, Γ/2 0.1

Antigen	Antibody-containing γ-globulin	Antigen-antibody precipitates		
		N	Fe	Fe/N
mg.	*mg.*	*mg.*	*mg.*	
A. RNase, 0.125	Anti-RNase, 3.0	0.044	0	0
0.063		0.100	0	0
0.031		0.083	0	0
0.016		0.046	0	0
B. RNase, 0.125	Anti-RNase, 3.0 + Fer-anti BSA, 4.0	0.039	0.008	0.21
0.063		0.097	0.009	0.09
0.031		0.090	0.008	0.09
0.016		0.051	0.007	0.14
C. RNase, 0.125	Anti-BSA, 2.0 + Fer-anti RNase, 6.0	0.009	0.015	—
0.063		0.043	0.048	1.1
0.031		0.149	0.119	0.80
0.016		0.084	0.068	0.81
0.008		0.049	0.046	0.94
D. BSA, 0.250	Anti-BSA, 2.0	0.058	0	0
0.125		0.113	0	0
0.063		0.089	0	0
0.031		0.046	0	0
E. BSA, 0.250	Anti-BSA, 2.0 + Fer-anti RNase, 6.0	0.008	0.003	—
0.125		0.134	0.034	0.25
0.063	—	0.125	0.064	0.51
0.031		0.095	0.064	0.67
0.016		0.061	0.057	0.93
F. BSA, 0.250	Anti-RNase, 3.0 + Fer-anti BSA, 4.0	0.009	0.005	—
0.125		0.067	0.049	0.73
0.063		0.133	0.098	0.74
0.031		0.078	0.052	0.67

ratio was larger, increasing with increasing Ab excess, and ferritin was non-specifically coprecipitated in appreciable amount. On the other hand, precipitates of BSA and fer-anti BSA conjugates exhibited a constant Fe/N ratio independent of the Ab/Ag ratio in the precipitates.

The fact that some non-specific coprecipitation of fer-anti RNase conjugates occurred in BSA-anti BSA precipitating mixtures appears to be disturbing at first sight, since it suggests that the ferritin in precipitates formed between BSA and fer-anti BSA conjugates might have been partly non-specifically and partly specifically carried down. However, quantitative considerations show that the specificity was nearly complete. Consider the data of Table III again. Note that in spite of a steady increase of Fe/N ratio in precipitates of increasing Ab/Ag ratio in section E, the Fe/N ratio in section F remains constant. If non-specific precipitation had occurred, the Fe/N ratios of section F would have shown the same trend as those of section E. Note also the equality of Fe/N ratios of sections C and F, involving two unrelated Ag-Ab systems. This result is exactly what is to be expected of the exclusively specific precipitation of a stoichiometric conjugate of ferritin and antibody. Similar considerations apply to the data of Table I. We therefore conclude that the degree of specificity exhibited by ferritin-Ab conjugates in their precipitation by homologous Ag was quite high.

To explain these results, we suggest that ferritin

TABLE II

Precipitation of Ferritin-TC-Antibody Conjugates in Phosphate Buffer, pH 7.5, Γ/2 0.1

Antigen	Antibody-containing γ-Globulin	Antigen-antibody precipitates		
		N	Fe	Fe/N
mg.	*mg.*	*mg.*	*mg.*	
A. RNase, 0.125	Anti-RNase, 3.0	0.061	0	0
0.063		0.196	0	0
0.031		0.153	0	0
0.016		0.098	0	0
B. RNase, 0.125	{Anti-RNase, 3.0 / Fer-anti BSA, 6.0}	0.067	0.048	0.72
0.063		0.269	0.080	0.30
0.031		0.233	0.078	0.34
0.016		0.133	0.066	0.50
C. RNase, 0.125	{Anti-BSA, 3.0 / Fer-anti RNase, 6.0}	0.347	0.210	0.61
0.063		0.375	0.285	0.76
0.031		0.360	0.228	0.63
0.016		0.257	0.168	0.65
0.008		0.179	0.115	0.64
D. BSA, 0.250	Anti-BSA, 3.0	0.047	0	0
0.125		0.170	0	0
0.063		0.134	0	0
0.031		0.097	0	0
0.016		0.055	0	0
E. BSA, 0.250	{Anti-BSA, 3.0 / Fer-anti RNase, 6.0}	0.044	0.015	0.34
0.125		0.295	0.114	0.39
0.063		0.273	0.130	0.48
0.031		0.207	0.127	0.61
0.016		0.105	0.101	0.97
F. BSA, 0.250	{Anti-RNase, 3.0 / Fer-anti BSA, 6.0}	0.139	0.079	0.57
0.125		0.338	0.212	0.63
0.063		0.270	0.173	0.64
0.031		0.230	0.130	0.56
0.016		0.175	0.098	0.56

coupled (either with XC or with TC) to anti-RNase Ab tends to attach non-covalently and relatively weakly to unmodified Ab in a BSA-anti BSA precipitating system. Since relatively more anti-BSA Ab is incorporated into the precipitates in Ab excess, there is relatively more opportunity for ferritin to bind non-covalently to these precipitates. Also as the pH is raised, increased electrostatic repulsions tend to exclude the fer-anti RNase conjugate from the BSA-anti BSA precipitate (section E, Table III, compared with section E, Table II). However, if fer-anti BSA conjugates are precipitated with BSA, essentially all the ferritin in the precipitate is specifically incorporated by virtue of its primary attachment to anti-BSA Ab, and we propose that any significant amount of secondary coprecipitation of ferritin, even if the precipitate contains a certain amount of unmodified Ab, is then excluded by steric or electrostatic repulsion by the ferritin which is specifically incorporated.

In these precipitation experiments, no radical difference in properties appeared as between XC and TC conjugates. (That the Fe/N ratios in Table I were generally larger than those of Tables II and III was primarily due to the larger iron content of the ferritin used in the first set of experiments.) Because the anti-BSA, anti-RNase,

TABLE III

*Precipitation of Ferritin-TC-Antibody Conjugates in Barbital Buffer, pH 8.6, Γ/2 0.1**

	Antigen	Antibody-containing γ-globulin	Antigen-antibody precipitates		
			N	Fe	Fe/N
	mg.	*mg.*	*mg.*	*mg.*	
A. RNase,	0.200	Anti-RNase, 5.3	0.065	0	0
	0.125		0.194	0	0
	0.063		0.213	0	0
	0.040		0.161	0	0
B. RNase,	0.200	Anti-RNase, 5.3 + Fer-anti BSA, 7.0	0.078	0.016	0.20
	0.125		0.200	0.024	0.12
	0.063		0.233	0.028	0.12
	0.040		0.183	0.024	0.13
C. RNase,	0.200	Anti-BSA, 2.7 + Fer-anti RNase, 7.0	0.025	0.002	—
	0.125		0.030	0.004	—
	0.063		0.173	0.078	0.45
	0.040		0.174	0.072	0.41
	0.025		0.134	0.050	0.37
D. BSA,	0.250	Anti-BSA, 2.7	0.026	0	0
	0.125		0.133	0	0
	0.063		0.116	0	0
	0.031		0.079	0	0
	0.016		0.045	0	0
E. BSA,	0.250	Anti-BSA, 2.7 + Fer-anti RNase, 7.0	0.025	0.001	—
	0.125		0.136	0.011	0.08
	0.063		0.139	0.027	0.19
	0.031		0.098	0.031	0.32
	0.016		0.077	0.027	0.35
F. BSA,	0.250	Anti-RNase, 5.3 + Fer-anti BSA, 7.0	0.041	0.004	—
	0.125		0.180	0.086	0.48
	0.063		0.164	0.080	0.49
	0.031		0.116	0.055	0.48
	0.016		0.082	0.035	0.43

* The same materials were used to obtain the data of Tables II and III. Precipitation of the unmodified Ag-Ag systems was somewhat reduced at pH 8.6 as compared with pH 7.5, the RNase-anti RNase system being more affected than the BSA-anti BSA.

and ferritin preparations used to obtain the data of Tables II and III all were different from those of Table I, any differences in properties may reflect factors other than the different conjugation procedures employed, and the two groups of results therefore do not bear too close a comparison.

Our primary objectives in performing the macroscopic Ag-Ab precipitation experiments with the ferritin-Ab conjugates were, first, to verify that the ferritin was indeed firmly bound to Ab in the conjugates; and, second, to demonstrate that under the proper conditions the conjugates would react with homologous Ag with a considerable degree of specificity. The data meet these objectives. It is clear, however, that if the ferritin-Ab conjugates are to serve as satisfactory stains for electron microscopy, the problem of the specificity of staining can ultimately only be answered at the electron microscopic level with each particular system to be studied. We therefore turn

now to consideration of some electron microscope experiments carried out to investigate further the properties of ferritin-Ab conjugates.

AN ELECTRON MICROSCOPIC STUDY OF THE REACTION OF TOBACCO MOSAIC VIRUS WITH FERRITIN CONJUGATES OF ANTIBODY TO TOBACCO MOSAIC VIRUS

The electron microscope experiments described here were intended to explore the ultimate feasibility of using ferritin-Ab conjugates to detect and localize specific Ag in cellular sections by examining first a model system which was relatively free of extraneous complications. Tobacco mosaic virus (TMV) was chosen as the Ag because of its distinctive rod-like morphology and ready identification in the electron microscope. TMV was added to a solution of its ferritin-conjugated rabbit Ab; the mixture was rapidly sprayed onto electron microscope specimen screens, and without washing or shadowing was examined in the microscope.

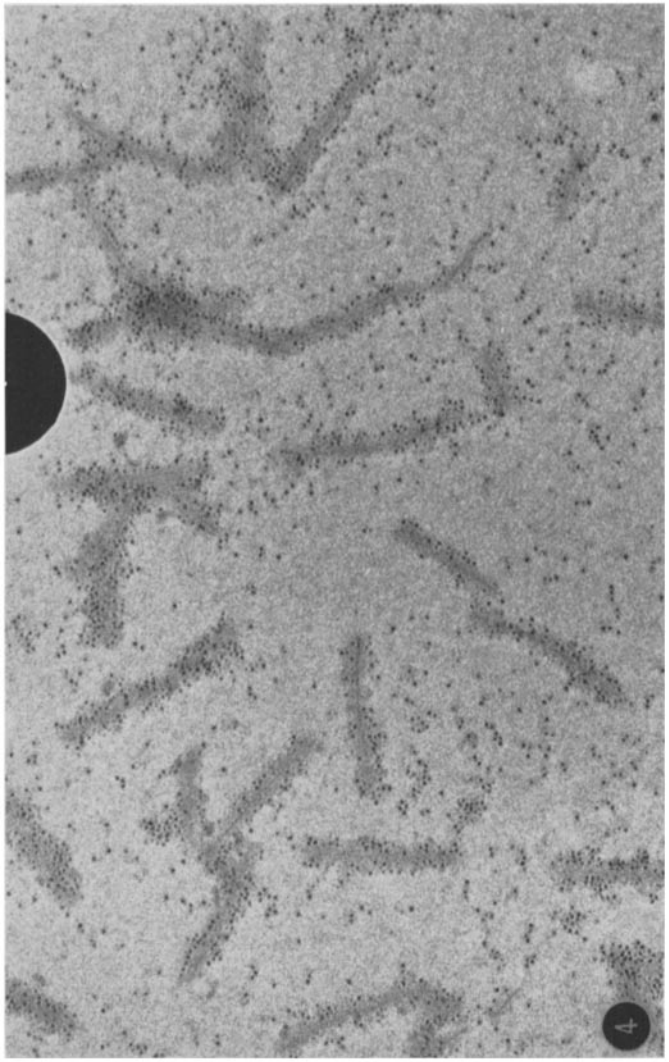
Our initial experiments were performed with a fraction of γ-globulin from rabbit antisera against TMV. The anti-TMV Ab content of the fraction was of the order of 2 per cent of the total protein. The ferritin conjugated to the non-antibody γ-globulin in this fraction produced such an intense background on the micrograph as to preclude any definitive observations on the association of the ferritin-Ab conjugate with the TMV rods. In order most easily to eliminate this difficulty, purified anti-TMV Ab preparations were subsequently used. The pure Ab was prepared by dissociation at pH 2.5 of a washed specific TMV-rabbit Ab precipitate, followed by removal of the TMV from the Ab in the acid solution by high speed centrifugation.

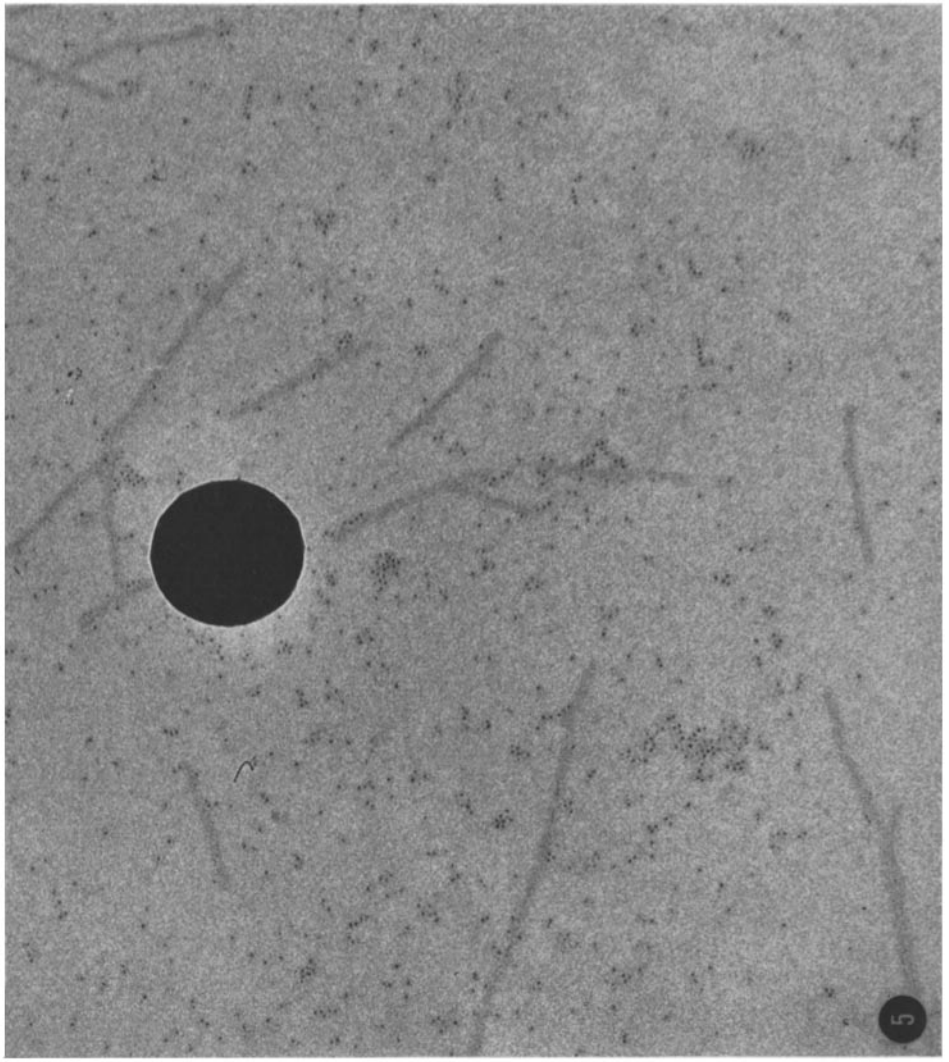
Using XC as the coupling agent, we then prepared a ferritin conjugate of pure anti-TMV (fer-XC-anti TMV) and of normal rabbit γ-globulin (fer-XC-NG). (These experiments were carried out before the chemical studies of the conjugation process described earlier had been performed, and before the use of TC as coupling agent had been introduced.) A mixture of fer-XC-anti TMV and TMV in a 0.2 M ammonium acetate–acetic acid buffer at pH 6.6 was prepared at 0°C., and within 1 minute after mixing, long before any visible precipitation had occurred, the solution was sprayed. An electron micrograph of this material is reproduced in Fig. 4. Most of the TMV rods in this field were singly dispersed and fairly uniformly coated with ferritin particles. A considerable amount of ferritin was not associated with the TMV rods, but the fact that most of the rods were singly dispersed and not aggregated signifies that the Ag-Ab reaction had not gone to completion in this particular spray droplet by the time it had dried, and therefore that some of the ferritin conjugate may not yet have had time to react with the rods. It should also perhaps be reiterated that the specimens were not washed before examination.

Several tests were made of the specificity of the association of the fer-XC-anti TMV with the TMV rods. A mixture of fer-XC-NG and TMV corresponding in composition to the specific mixture just discussed was similarly treated and sprayed, with the result shown in Fig. 5. Clearly there was no significant association of the ferritin conjugate of normal γ-globulin with the TMV rods. In other experiments mixtures of TMV with bacteriophage T6, and of TMV with bushy stunt virus (BSV), were similarly reacted with fer-XC-anti TMV. As is evident from Figs. 6 and 7, only the TMV was aggregated in these mixtures and the ferritin was specifically associated with the TMV rods. These tests therefore suggested that the association of fer-XC-anti TMV with TMV was satisfactorily specific.

On the other hand, our chemical studies had by this time shown that the XC conjugates of ferritin and Ab were partly covalently and partly non-covalently linked. We were therefore concerned as to whether the association of ferritin with the TMV rods observed in Fig. 4, though specific, might be partially reversible to the extent that non-covalent ferritin-Ab linkages were present. Furthermore, since the solutions of conjugates contained some unconjugated Ab, we wanted to determine whether the association of ferritin with the TMV rods was direct or indirect; that is, whether it occurred by virtue of the covalent attachment of ferritin to anti-TMV Ab, the covalent conjugate as a unit being bound to TMV (direct staining), or whether by virtue of a non-covalent attachment of ferritin to unconjugated anti-TMV Ab, the latter having first independently become bound to TMV (indirect staining).

Without further investigating these points with the XC conjugates, however, we turned our at-





tention to the chemically better defined TC conjugates which had since become available. Preparations were made with pure anti-TMV Ab (fer-TC-anti TMV) and with a rabbit γ-globulin fraction containing anti-BSA Ab (fer-TC-anti BSA). In all the experiments to be described involving mixtures of these TC conjugates with TMV and other substances, the mixtures were prepared in a buffer at pH 8.6 made from 1 volume of 0.1 M ammonium carbonate and 2 volumes of 0.1 M ammonium acetate. At this pH it had previously been found that non-specific coprecipitation of ferritin was diminished as compared with that at pH 7.5 (Tables II and III), and that Ag-Ab reactions, though considerably slower and somewhat less complete at pH 8.6 than at pH 7.5 (footnote, Table III), nevertheless occurred to an appreciable and sufficient extent.

A mixture of fer-TC-anti TMV and TMV at pH 8.6 was prepared at 0°C. After 1 hour it was sprayed and without washing was examined in the microscope (Fig. 8). A corresponding mixture of fer-TC-anti BSA and TMV was similarly treated (Fig. 9). The results were very similar to those obtained with the XC conjugates: specific association of the ferritin-Ab conjugate with the TMV rods was observed. In other experiments not reproduced here, no association was seen between fer-TC-anti TMV and either bacteriophage T6 or BSV particles.

In order to determine whether the observed specific binding of ferritin to the TMV rods was direct or indirect (see above), two additional types of experiments were performed. In the first, a mixture of fer-TC-anti BSA and of unmodified anti-TMV Ab was prepared, and was then added to TMV; subsequently, this preparation was treated in the same way as the samples just described. The quantity of unmodified anti-TMV Ab in this control experiment was equal to the total amount of anti-TMV Ab, conjugated and unmodified, in the direct experiment of Fig. 8, while the quantities of ferritin and TMV were the same. The opportunities for indirect staining were therefore even greater in this control experiment than in the direct experiment, since an even larger amount of unmodified anti-TMV Ab was present. On the contrary, however, the localization of ferritin on the TMV rods in this control (Fig. 10) was much less pronounced than in the direct experiment (Fig. 8).

In a second type of experiment, the unmodified anti-TMV Ab was first largely removed from the solution of fer-TC-anti TMV conjugate by high speed centrifugation. All the ferritin, both conjugated and unconjugated, after having been sedimented into a pellet in the bottom of the centrifuge tube and freed from the supernatant liquid (containing most of the unmodified Ab), was dissolved at the same concentration as the ferritin in the solution before ultracentrifugation. This preparation, designated fer-TC-anti TMV *a*, was shown by ultracentrifugal analysis to contain only 15 per cent of the unmodified anti-TMV Ab present in the original solution. Upon mixing fer-TC-anti TMV *a* with TMV under the same conditions, followed by the same treatment, as in the preceding experiments, we obtained electron micrographs represented by Fig. 11. There was certainly no decrease in the amount of ferritin associated with the TMV rods as compared with Fig. 8,

Figures 4 to 11 are all at the same magnification, × 80,000. The small black dots are due to the ferric hydroxide inner core (measuring 55 A across) of individual ferritin molecules. The large black spheres visible in some of the figures are polystyrene latex particles of 2600 A diameter.

FIGURE 4

Tobacco mosaic virus (TMV) and the ferritin-XC conjugate of pure anti-TMV antibody. Individual rods of TMV (3000 A long) are coated with ferritin particles.

FIGURE 5

TMV and the ferritin-XC conjugate of normal rabbit γ-globulin. No significant attachment of ferritin to the TMV rods is observed. Note that the rod thickness (150 A) is normal as compared with the rods in Fig. 4.

although 85 per cent of the unconjugated anti-TMV Ab had been removed from the mixture.

These experiments demonstrate that the localization of ferritin on the TMV rods was predominantly through specific attachment of fer-TC-anti TMV conjugates in these experiments, and that the ferritin staining of the rods was therefore direct.

DISCUSSION

The Properties of Ferritin-Antibody Conjugates: A first important result which is revealed by the electron micrographs is that the characteristic structure of ferritin is not significantly altered upon modification by XC or TC and conjugation to Ab. This is not immediately evident in the micrographs herein reproduced, but becomes apparent at higher magnifications. It is conceivable that such chemical modification might have disrupted the apoferritin protein shell, or the ferric hydroxide micelles, and rendered the conjugate useless; but on the contrary, even the tetrahedral arrangement of the ferric hydroxide micelles is preserved in the ferritin-Ab conjugates.

Two types of ferritin-Ab conjugates, made with either XC or TC as coupling agent, have been prepared and studied. Both types are capable of attaching to homologous Ag with a satisfactory degree of specificity. This is particularly seen to be true when it is realized that the tests of specificity applied in the experiments of Tables I, II, and III are more stringent than would arise in most future applications of the conjugates, since normally there would be no occasion to have a ferritin-Ab conjugate present together with an unconjugated Ab of different specificity. Of primary significance are the facts that with three different antigens, namely BSA, RNase, and TMV, neither ferritin itself nor a ferritin-Ab conjugate made with a heterologous Ab became attached to the Ag. Other investigators (8, 9) working with XC conjugates prepared by our method also found them to be specific in still other systems than those we have studied.

Our chemical investigations have subsequently revealed that ferritin-Ab conjugates prepared with TC are exclusively covalently linked under conditions where the XC conjugates are partly covalently and partly non-covalently linked. Though we have so far observed no significant differences between the behavior of XC and that of TC conjugates in their reactions with Ag, it seems clear that the TC conjugates are less likely to show nonspecific binding and other complications in the course of future studies. We have shown, furthermore, that under the appropriate conditions, the TC conjugates act as direct electron stains; that is, the ferritin becomes attached to an Ag only by virtue of the covalent linkage between the ferritin and Ab specific to that Ag, and not by some secondary or indirect process. This may also be true of the XC conjugates, but we did not attempt to prove it. For the purposes of our future studies we intend to concentrate on the use of the chemically better defined TC conjugates.

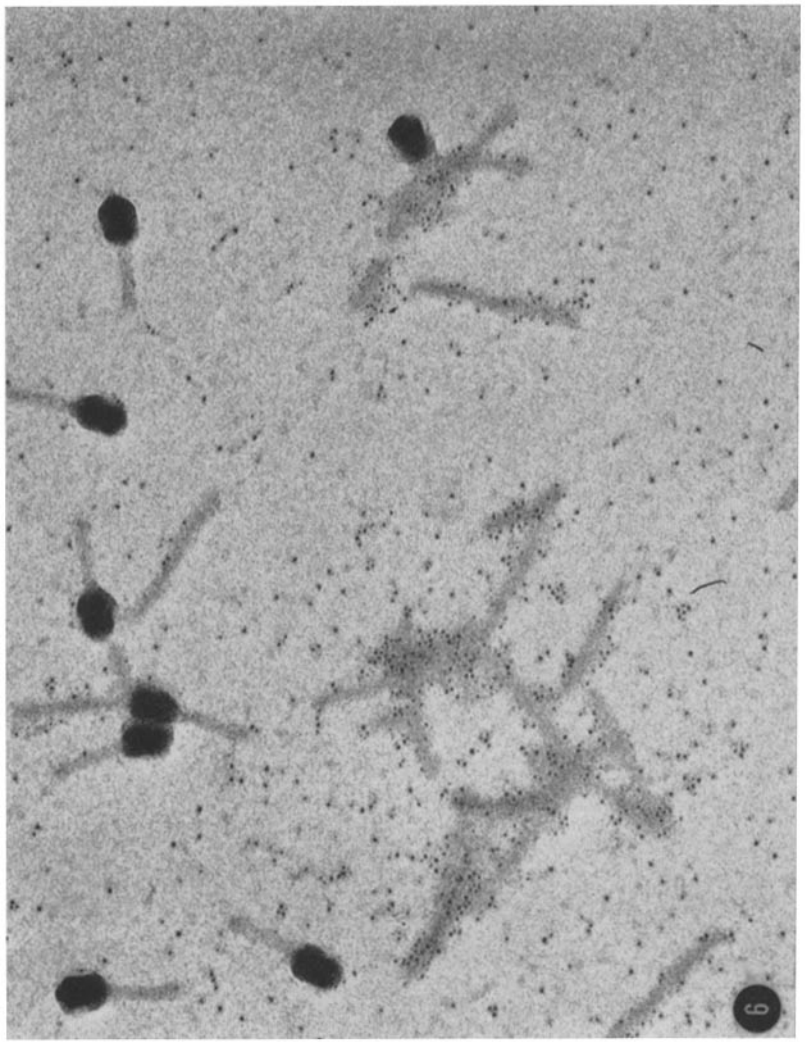
For many applications, it should be adequate to prepare and use a ferritin conjugate of a whole γ-globulin fraction, containing the particular antibody as some relatively small fraction of the total protein. If the conjugate of the inert γ-globulin could be washed free of the specimen, while the Ab conjugate was left attached to the specific Ag sites, the method would prove satisfactory. Where this method has so far been tried (8, 9), even though XC conjugates were employed, it has appeared to be successful. On the other hand, any increase in the relative amount of specific ferritin-Ab conjugate in a staining mixture should certainly improve the sensitivity of the method. One means of accomplishing this is to make a ferritin conjugate of a pure Ab. It is generally not difficult

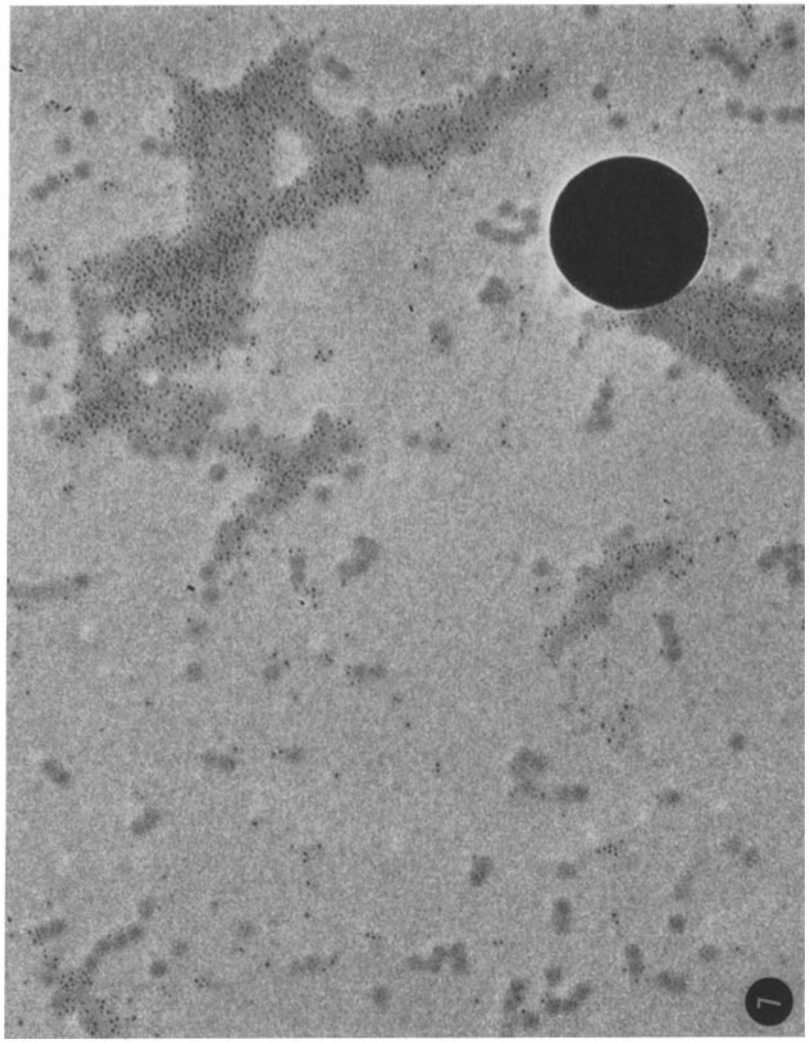
FIGURE 6

TMV and bacteriophage T6, mixed with the ferritin-XC conjugate of anti-TMV antibody. The ferritin particles are associated with the TMV rods, but not with the bacteriophage particles.

FIGURE 7

TMV and bushy stunt virus (BSV) mixed with the ferritin-XC conjugate of anti-TMV antibody. The ferritin particles are associated with the TMV rods, but the spherical BSV particles are essentially free of ferritin.





to isolate the relatively small amount of pure Ab required. With large antigens such as viruses, pure Ab may generally be prepared by acid dissociation of a specific Ag-Ab precipitate, followed by ultracentrifugal removal of the Ag. For protein Ag of the same order of size as Ab, a convenient general method for the isolation of pure Ab has recently been described (11). After the conjugation of ferritin to a pure Ab, the unconjugated Ab can be removed from the mixture by ultracentrifugation, as we have shown earlier, leaving in the staining mixture only ferritin-Ab conjugate and unconjugated ferritin.

Furthermore, with many systems involving antigens of moderate molecular weight it should be possible to isolate the ferritin-Ab conjugate even if the conjugation was initially carried out with a whole γ-globulin fraction containing only a small fraction of Ab protein. Addition of the homologous Ag to the ferritin conjugate mixture should precipitate the Ag and the ferritin-Ab conjugate together with the unmodified Ab; the non-antibody γ-globulin and its ferritin conjugate, as well as the unconjugated ferritin, should remain in the supernatant. The washed precipitate could then be dissociated at acid pH, and by subjecting this solution to ultracentrifugation under the appropriate conditions, the rapidly sedimenting ferritin-Ab conjugates should be completely freed of the relatively slowly sedimenting Ag and unmodified Ab, thereby being recovered in essentially pure state. Although we have not yet tried this procedure, it appears to be entirely feasible.

The resolution with which Ag sites can be located and differentiated by electron staining with a ferritin-Ab conjugate is illustrated by comparison of Figs. 4 and 5. When sufficient amounts of conjugate are employed, the ferritin spots themselves delineate the TMV rods, even without the electron density contributed by the rods. Whereas the individual TMV molecules are originally about 150 A thick, the ferritin-Ab bound to the rods is spread over a region corresponding to an average thickness of about 500 A (12). It is likely, therefore, that the ferritin conjugates can differentiate Ag structures which are separated by about 200 to 300 A or more.

Concerning the limiting resolution of which the method is capable, it may seem at first disadvantageous to use such a large molecule as ferritin as an electron-scattering conjugate. On the other hand, however, it should be realized that an Ab molecule itself is an elongated structure of about 250 A by 40 A (13), and the conjugation to it of a ferritin sphere of 110 A diameter does not radically decrease the resolving power. Any substantial improvement in resolution must involve the limiting factor, the size of the Ab molecule. In this connection, Porter (14) has demonstrated that fragments of Ab about one-quarter the mass of the original molecule may be obtained by limited papain digestion of Ab. These fragments contain

FIGURE 8

TMV and the ferritin-TC conjugate of pure anti-TMV antibody. The TMV rods are coated with ferritin particles.

FIGURE 9

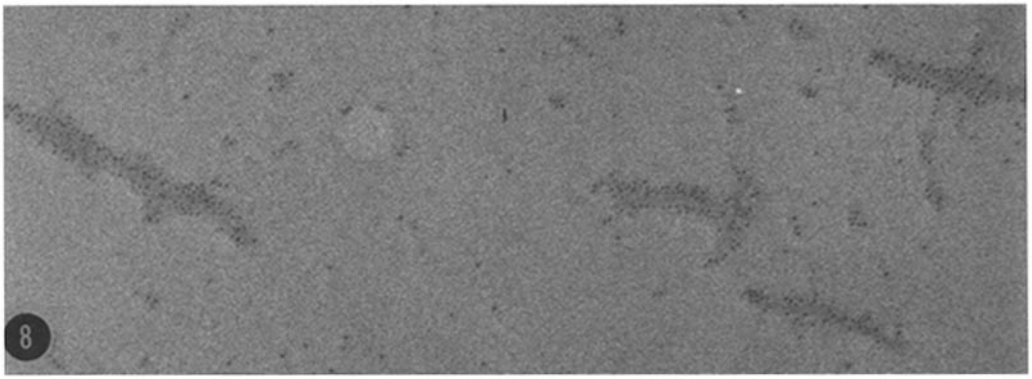
TMV and the ferritin-TC conjugate of a rabbit γ-globulin containing antibody to bovine serum albumin. No attachment of the ferritin conjugate to the TMV rods is observed.

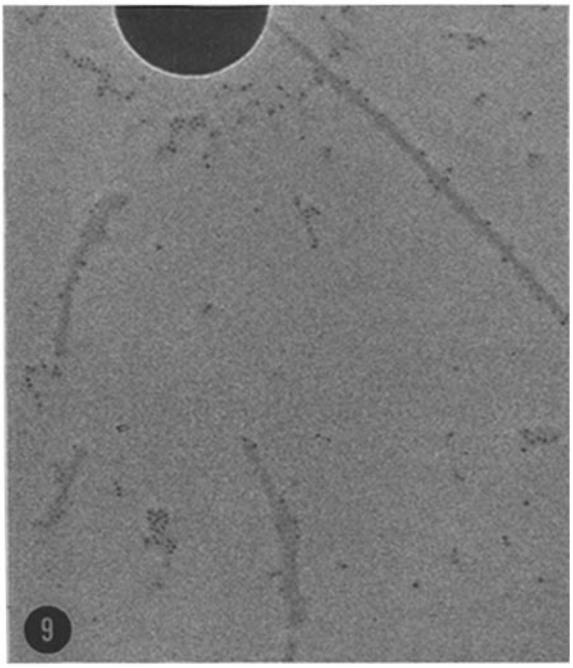
FIGURE 10

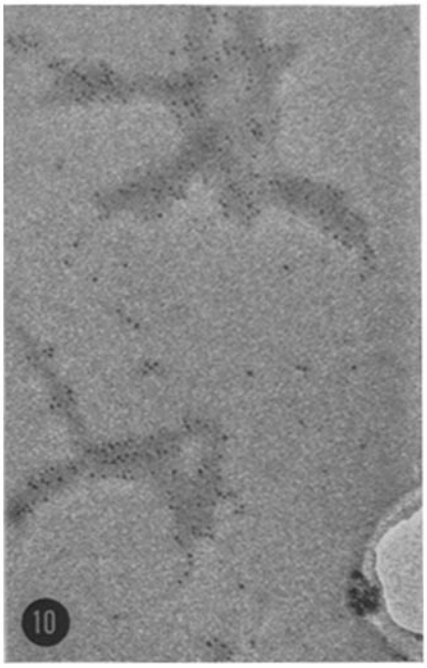
TMV and a mixture of unconjugated anti-TMV antibody and the ferritin-TC conjugate of antibody to bovine serum albumin. Although a significant amount of ferritin is associated with the TMV rods, it is considerably less than the amount in Fig. 8.

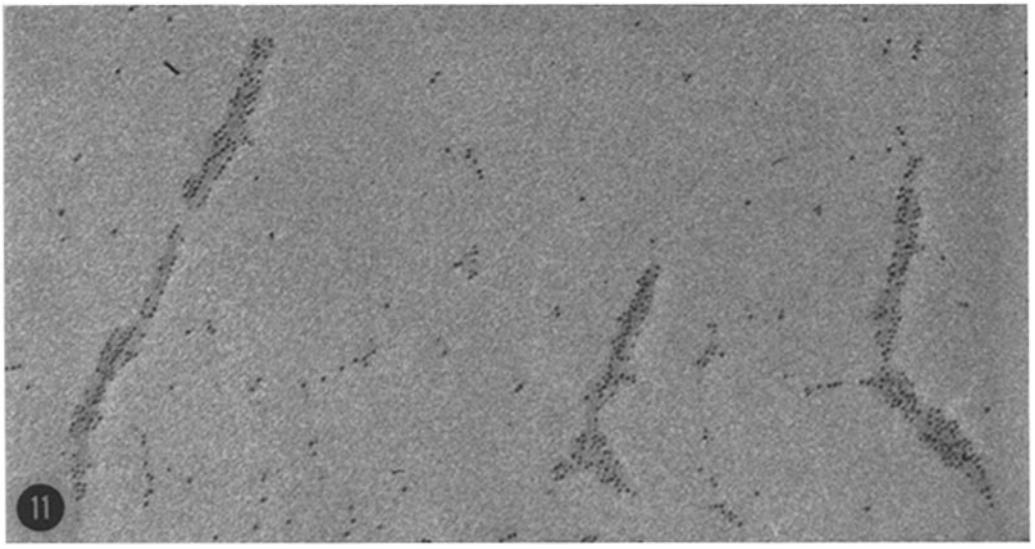
FIGURE 11

TMV and the ferritin-TC conjugate of anti-TMV antibody from which 85 per cent of the uncombined antibody had first been removed. Note that the association of ferritin with the TMV rods is at least as extensive as in Fig. 8.









in intact form, and with full binding capacity for Ag, one of the two active regions of the original Ab molecule. For electron-staining purposes, one specific active site on an Ab fragment should be adequate. To such a fragment it would be desirable, however, to conjugate some electron-dense "tail" smaller than ferritin. Such a "tail" might be prepared by exhaustively coupling to a small protein molecule such as ribonuclease a large number of groups containing heavy atoms (without regard to possible "denaturation" of the ribonuclease). Other potential electron-dense conjugates include derivatives of inorganic heteropoly acids, such as phosphotungstic acid, and low molecular weight synthetic polymers containing heavy atoms. By the use of TC or some more appropriate reagent, such small "tails" might be conjugated to an Ab fragment. For the time being, however, the resolution of which the ferritin-Ab conjugates appear to be capable is more than adequate for investigating a number of interesting problems.

Antigens in Cellular Matrices: Though there are some problems to which the ferritin-Ab conjugates can be directly applied, for example the estimation of the number of antigenic sites on a large Ag molecule by determining the maximum number of ferritin-Ab conjugates capable of attaching to it, or the localization of antigens on the external membranes of cells, the most important problems involve antigens present within cells or cellular bodies. The properties of ferritin-TC-Ab conjugates which we have described are entirely consistent with their possible use as specific electron stains for intracellular antigens. However, serious auxiliary problems arise in connection with the preparation of such stained cells in a condition suitable for electron microscopy. The membranes of living cells are generally impermeable to Ab.[2] Therefore, the ferritin-Ab conjugate would not function as an internal stain for intact living cells. Though for special purposes other less demanding techniques may be adequate, it appears that in general it will be necessary to fix and embed cells and cut ultrathin sections of them *before* applying the ferritin-Ab conjugate, in order to allow access of the conjugate to internal antigens.

The problem then arises whether the particular antigenic determinant of interest can undergo the treatment involved in fixation and embedding for electron microscopy, and retain its antibody-binding capacity. We have recently shown (17) that the cell wall antigens of *E. coli* and *S. pyogenes* survive the commonly employed techniques of osmium tetroxide fixation and methacrylate embedding, and are capable of specifically binding fluorescein-Ab conjugates after this treatment. This is certainly an encouraging development. On the other hand, there may well be encountered a considerable number of more readily inactivated antigenic determinants, and for these fixation by freeze-drying (18) or freeze-substitution at low temperatures (19), followed by embedding in an ultraviolet-initiated, low-temperature-polymerized methacrylate resin (20), might provide a suitable procedure.

The ferritin-Ab staining technique should be applicable to the study of a wide variety of problems of interest in cell biology. These problems generally require a resolving power and a sensitivity far beyond that of the fluorescent-Ab technique. The processes of viral development, and the sites of origin of viral antigens (21); the localization of the different antigenic components within viruses, particularly the larger ones such as vaccinia (22); the localization of enzymes and other proteins within cell organelles (23); the persistence of antigens, and the early stages of antibody synthesis (1); the distribution of specific protein within the ultrastructure of muscle cells (24)—these are only a few of the areas in which this technique may make eventual contributions.

[2] By appropriate stimuli (15) certain cells can be made to undergo pinocytosis (16), an engulfment and incorporation of surrounding fluid into their cytoplasm. Although this process might be used to introduce a ferritin-Ab conjugate into the cells, which might then be fixed, embedded, and sectioned by any conventional procedure, it is not yet a general enough phenomenon to utilize except in special circumstances.

The studies reported in this paper were carried out while the senior author was a Guggenheim Foundation Fellow at the University of California, Berkeley, on leave from the Department of Chemistry, Yale University. The senior author wishes to express his gratitude to Drs. W. M. Stanley and R. C. Williams for extending to him the hospitality and facilities of the Virus Laboratory, and for stimulating discussions during these studies. He is also greatly indebted to Dr. W. R. Adams, of the Yale School of Medicine,

with whom initial unpublished electron microscope studies of ferritin conjugates were carried out. This research was supported in part by Grants E-1204 to S. J. Singer and C-2245 to R. C. Williams from the U. S. Public Health Service.

This paper is Contribution No. 1647 from the Department of Chemistry, Yale University.

Received for publication, December 18, 1960.

REFERENCES

1. Coons, A. H., *Int. Rev. Cytol.*, 1956, **5**, 1.
2. Singer, S. J., *Nature*, 1959, **183**, 1523.
3. Pepe, F. A., Finck, H., and Marshall, J., Jr., *Anat. Rec.*, 1960, **136**, 257.
4. Habeeb, A. F. S. A., Cassidy, H. G., Stelos, P., and Singer, S. J., *Biochim. et Biophysica Acta*, 1959, **34**, 439.
5. Granick, S., *Chem. Rev.*, 1946, **38**, 379.
6. Farrant, J. L., *Biochim. et Biophysica Acta*, 1954, **13**, 569.
7. Harrison, P. M., *J. Molecular Biol.*, 1959, **1**, 69.
8. Smith, C. W., Metzger, J. F., Zacks, S. I., and Kase, A., *Proc. Soc. Exp. Biol. and Med.*, 1960, **104**, 336.
9. Rifkind, R. A., Hsu, K. C., Morgan, C., Seegal, B. C., Knox, A. W., and Rose, H. M., *Nature*, 1960, **187**, 1094.
10. Singer, S. J., and Schick, A. F., Abstr. of meeting of American Chemical Society in New York, September, 1960, p. 35C, 51C; *Chem. Eng. News*, 1960, **38**, 55.
11. Singer, S. J., Fothergill, J. E., and Shainoff, J. R., *J. Am. Chem. Soc.*, 1960, **82**, 565.
12. Anderson, T. F., and Stanley, W. M., *J. Biol. Chem.*, 1941, **139**, 339.
13. Hall, C. E., Nisonoff, A., and Slaytor, H. S., *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 407.
14. Porter, R. R., *Biochem. J.*, 1959, **73**, 119.
15. Rinaldi, R. A., *Exp. Cell Research*, 1959, **18**, 70.
16. Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1931, **49**, 17.
17. Spendlove, R. S., and Singer, S. J., *Proc. Nat. Acad. Sc.*, 1961, **47**, 14.
18. Sjöstrand, F. S., and Baker, R. F., *J. Ultrastruct. Research*, 1958, **1**, 239.
19. Feder, N., and Sidman, R. L., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 593.
20. Müller, H. R., *J. Ultrastruct. Research*, 1957, **1**, 109.
21. Kellenberger, E., Séchaud, J., and Ryter, A., *Virology*, 1959, **8**, 478.
22. Smadel, J. E., and Hoagland, C. L., *Bact. Rev.*, 1942, **6**, 79.
23. Subcellular Particles, (T. Hayashi, editor), New York, Ronald Press, 1959.
24. Huxley, H. E., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 631.