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Some Antigenic Characteristics and Immunologic Reactions of Horse Spleen Ferritin.* (29828)

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The usefulness of ferritin, the electron dense iron-storage protein, in various studies, particularly in the field of electron microscopy, suggested the need for additional information about some of its immunologic characteristics. Ferritin has a molecular weight of about 750,000 and exists as a protein shell covering an iron containing core(1). Anti-ferritin antibody had been prepared in rabbits(2) and goats(3). It may be stained with a suitable iron stain. The use of ferritin in labeling of antibodies recently has been reviewed(4).

The present studies describe the preparation of a solution of ferritin with a single antigenic component and its labeling with I^{131}. Studies of certain characteristics of rabbit and chicken anti-ferritin sera are illustrated, particularly utilizing the technique of precipitation of isotope labeled ferritin as a measure of the chicken and rabbit antibodies. A technique of localization of ferritin-anti-ferritin precipitin arcs by an iron staining procedure applied to immunoelectrophoretic preparations provides a means of study of types of antibodies against ferritin. The technique, similar to that of autoradiographic immunoelectrophoresis, may be used for study of immunoglobulins where ferritin can be used as the antigen without the necessity of an isotope label.

Methods and materials. Ferritin was obtained commercially (horse spleen ferritin, Pentex Laboratories, Kankakee, Ill.). The commercial ferritin was recrystallized 8 times by cadmium sulfate and reprecipitated 4 times by ammonium sulfate precipitation(5). The ferritin was finally purified by passage through Sephadex G-200 (Pharmacia, Uppsala, Sweden) in 0.1 M NaCl and 0.1 M TRIS pH 8.0 buffer. This purified ferritin was utilized in subsequent experiments and compared with the commercial and recrystallized preparations. Ferritin concentration in the gel filtration effluent was measured in a Beckman D.U. Spectrophotometer at 255 mμ, at which wave length maximal absorption by ferritin appeared to occur.

Three preparations of ferritin were studied. These were preparations A, B and C shown in Table I. All 3 were labeled with I^{131} ($I^{131}F$) using the method of labeling proteins described by Talmage *et al*(6).

Adult albino rabbits were immunized with commercial ferritin in incomplete Freund's adjuvant(7). Each rabbit received 50 mg ferritin subcutaneously weekly for 6 weeks. Rabbits were bled 8 days after the last injection. Adult White Leghorn roosters received commercial ferritin in 0.15 M NaCl. Because of toxicity of cadmium salts, immunization was carried out over a period of 2 days using divided (30 mg) doses intravenously to a total dose of 40 mg per kg body weight. Primarily immunized roosters were bled 7 days after initiation of immunization. Some roosters received a second immunization with ferritin 4 weeks after primary immunization and were bled one week later.

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TABLE I. Characteristics of Ferritin Preparations.

	Ferritin preparation		
	A	B	C
Gel diffusion precipitin bands*	5	5	1
Protein peaks after Sephadex filtration	2	2	1
% of yield of I^{131} trace labeling†	.7	.5	5
% of I^{131} ferritin precipitated by excess antibody	63	80	98

A. Commercial ferritin.
B. Commercial ferritin recrystallized with $CdSO_4$ 8 times and precipitated with $(NH_4)_2SO_4$ 4 times.
C. Commercial ferritin after gel filtration (peak I) dialyzed against distilled water.
* Using rabbit anti-commercial ferritin.
† $\frac{\text{Millicuries } I^{131} \text{ bound to ferritin}}{\text{Millicuries } I^{131} \text{ used to label}} \times 100.$

Immunoelectrophoresis of rooster antisera against ferritin was done by methods previously described(8). After completion of development of the immunoelectrophoresis pattern by rabbit anti-chicken serum, $I^{131}F$ was added to the antiserum trough and autoradiographs of the immunoelectrophoretic preparations made as previously described(8). In similar preparations, ferritin (238 μg N/ml) was added to the trough and incubation continued for 2 days. Slides were washed free of unbound ferritin and dried. Half of the slide was stained for the protein bands. The other half was stained for iron by immersion in 2% $K_4Fe(CN)_6 \cdot 3\ H_2O$ for 10 minutes, and differentiated in 2% HCl and washed with H_2O. Photographs were prepared as contact prints of the stained immunoelectrophoretic preparations or as exposed X-ray films(9) when $I^{131}F$ was used as antigen.

Precipitation of $I^{131}F$ by rabbit and chicken antisera was done by the method of Talmage and Maurer(10). Reactions using chicken antisera were done in 0.15 and 1.5 M NaCl(11).

Quantitative precipitin tests were done by standard techniques. Nitrogen was determined by the Markham modification of the micro-Kjeldahl reaction(7).

Results. Preparation of a solution of ferritin with a single antigen. Commercial ferritin, further purified by recrystallization with $CdSO_4$ and $(NH_4)_2SO_4$ reprecipitation, was labeled with I^{131}. Since a maximum of only 80% of the radioactivity of this trace labeled preparation of $I^{131}F$ was precipitated by an excess of anti-ferritin antibody, the $I^{131}F$ was further purified by gel filtration through a Sephadex G-200 column. Results of the filtration of $I^{131}F$ through Sephadex G-200 are shown in Fig. 1. Two peaks in effluent protein concentration are seen; peak I is ferritin as demonstrated by iron staining of the protein using $K_4Fe(CN)_6 \cdot 3\ H_2O$. The passage of the ferritin through Sephadex was easily followed by the brown color of the ferritin. The second peak (II in Fig. 1) was colorless and radioactivity was precipitated by rabbit anti-ferritin but to a lesser extent than in the first peak. The initial peak contained one precipitin band with antiserum against crude ferritin by double gel diffusion precipitin analysis. Four antigen-antibody bands were found in the effluent from peak II. When the ferritin purified by Sephadex G-200 chromatography was trace labeled with I^{131} and refiltered through Sephadex G-200 a single peak of ferritin was found. Gel filtration of commercial ferritin without further recrystallization appeared to be a satisfactory method of purification of ferritin.

Characteristics of recrystallized ferritin or ferritin purified by gel filtration are summarized in Table I. The ferritin obtained as peak I after gel filtration contained one antigen and was the only preparation which was satisfactorily labeled with I^{131} for immunologic studies.

Rabbit and chicken anti-ferritin antibody. Rabbits immunized with ferritin not purified by Sephadex G-200 chromatography produced antiserum against the ferritin solution containing 5 precipitin bands demonstrated by double gel diffuson precipitin reaction. Four of these were not ferritin-anti-ferritin bands. A single precipitin band occurred against the Sephadex G-200 effluent containing ferritin, while the additional 4 bands present were against contaminants occurring in later samples of the gel filtrate effluent (peak II, Fig. 1).

The precipitation of $I^{131}F$ by rabbit anti-ferritin (Fig. 2B) is characteristic of the curve of precipitation of isotope labeled het-

erologous serum protein antigens by rabbit antiserum(10). Antibody content of the antiserum can be expressed in terms of μg N of antigen precipitated by 1 ml of antiserum. The isotope precipitin and quantitative precipitin reactions using rabbit anti-ferritin and $I^{131}F$ may be compared in Fig. 2B. The unusually long plateau of the ferritin-rabbit anti-ferritin quantitative precipitin curve has been described(2).

Circulating chicken anti-ferritin was present one week after intravenous immunization of chickens with ferritin. The precipitation of $I^{131}F$ by chicken anti-ferritin in 0.15 and 1.5 M NaCl (Fig. 2A) shows curves similar to rabbit anti-ferritin. The additional precipitation of antigen-antibody complexes at 1.5 M NaCl has previously been observed using quantitative precipitin reactions to measure chicken antiserum against heterologous serum protein antigen(11). Variation in concentration of NaCl from 0.15 to 1.5 M as medium for rabbit anti-ferritin resulted in no additional precipitation of $I^{131}F$.

Demonstration of anti-ferritin antibody by autoradiographic or chemical staining of immunoelectrophoretic preparations. Localization of anti-ferritin antibody in relation to other immunoglobulins was done by addition of $I^{131}F$ to the immunoelectrophoretic preparation and exposure of dried gel to X-ray film. Ferritin without an I^{131} label was added to similar immunoelectrophoretic preparations, followed by staining for iron. Ferritin-anti-ferritin bands stained blue. Such a preparation is shown in Fig. 3 using chicken anti-ferritin serum from a primary immune response. The localization of the immunoglobulins staining for iron demonstrated the presence of γ_2 globulin binding ferritin and two

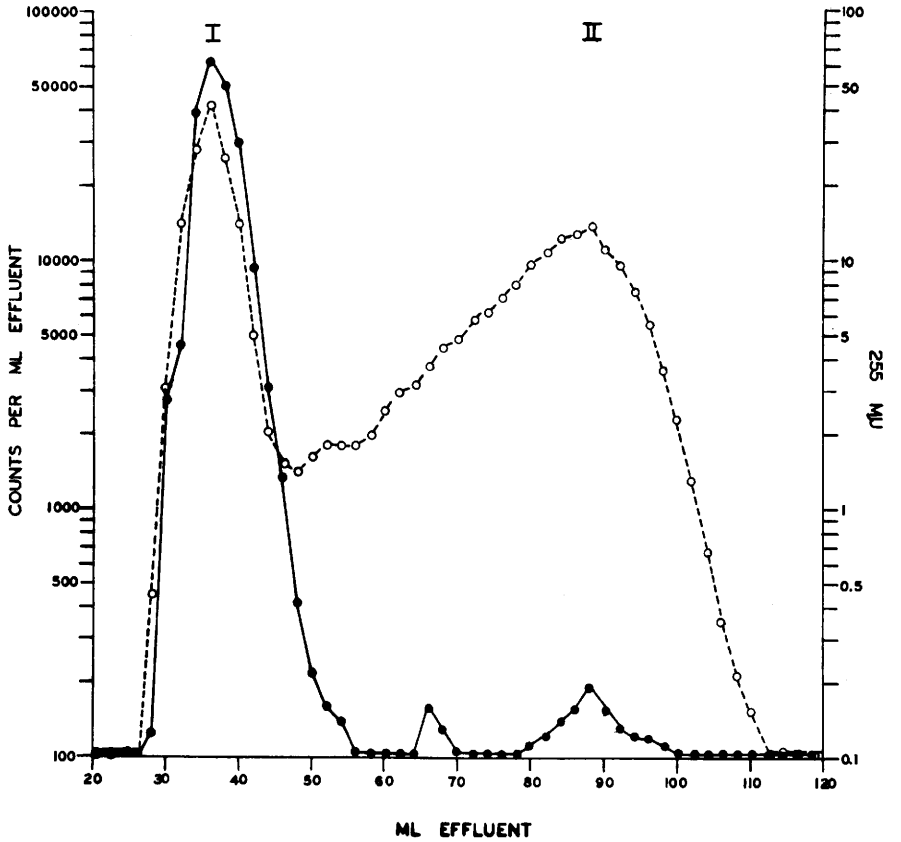

FIG.1. Purification of $I^{131}F$ by column chromatography using Sephadex G-200. Radioactivity of effluent solution recorded as counts per min per ml O——O. Optical density of effluent solution ●——●.

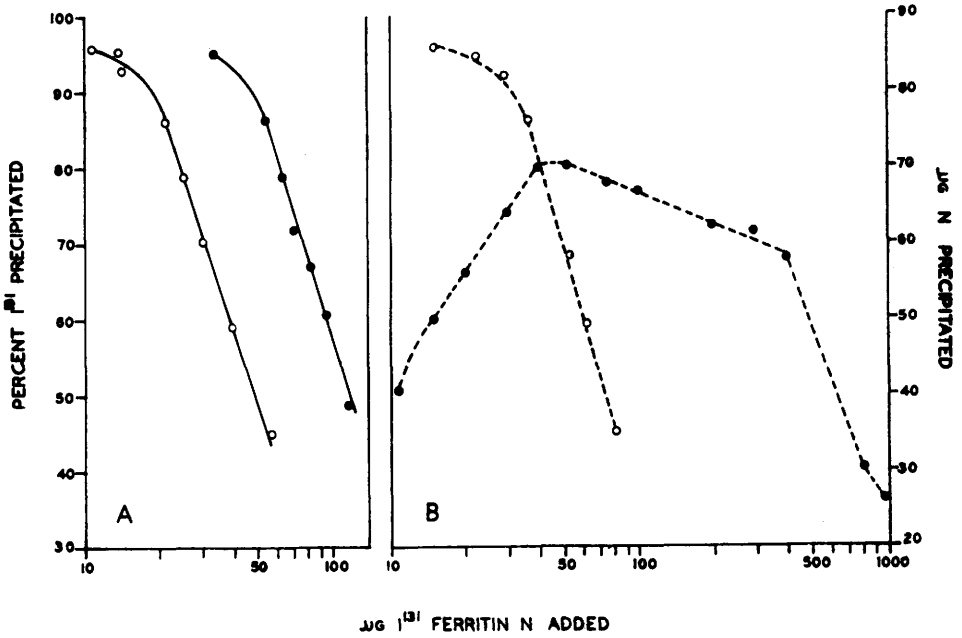

FIG. 2. Isotope precipitation curves of chicken anti-F (0.15 M NaCl) O——O; chicken anti-F (1.5 M NaCl) ●——●; rabbit anti-F O----O, expressed as percent radioactivity precipitated. Quantitative precipitin curve with rabbit anti-F, expressed as μg N precipitated ●-----●.

γ_1 globulins, perhaps one a macroglobulin, which stain for iron.

Discussion. Preparation of ferritin for immunologic studies by fel filtration is a rapid and effective method of purification which results in a single antigenic component. This technique is simpler than $CdSO_4$ recrystallization. The color of ferritin and the ease of staining of ferritin iron both aid in this method of preparation of pure ferritin. Ferritin prepared by $CdSO_4$ precipitation may contain sufficient cadmium salts to be toxic when used for immunization of animals and the cadmium may be removed by the same Sephadex G-200 filtration which removes contaminating antigenic proteins.

Precipitating antibody against horse spleen ferritin can be obtained with ease by immunization of rabbits with alum precipitated ferritin(2) or ferritin in Freund's adjuvant. Chickens produce precipitating antibody in one week after intravenous injection of ferri-

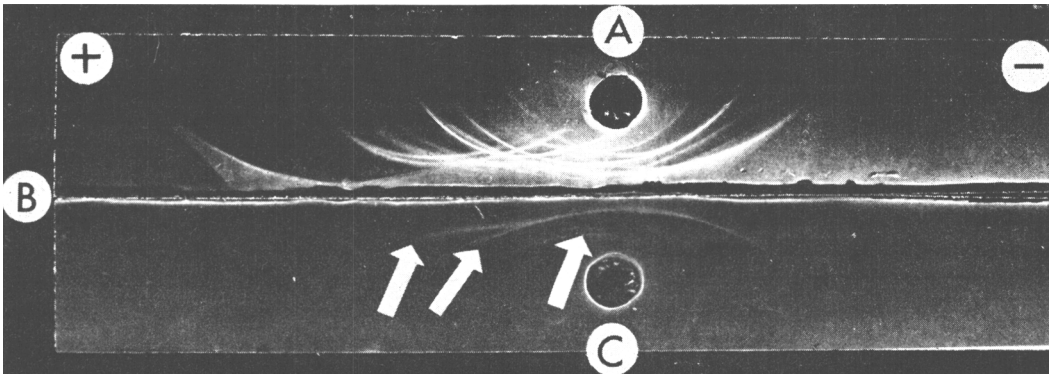

FIG. 3. Immunoelectrophoretic preparation of localizing chicken anti-ferritin antibodies. A, Chicken anti-ferritin serum; B, rabbit anti-chicken serum; C, chicken anti-ferritin serum. Top section stained for protein. Bottom section immersed in ferritin solution, washed free of unbound ferritin and strained for iron.

tin. The quantitative precipitin curves obtained with rabbit anti-ferritin and ferritin are characterized by a sharp rise in N precipitated by increasing addition of N in the antibody excess zone, and a very slow decline in N precipitated in the region of increasing antigen excess. This type of curve, previously described(2) and confirmed in these experiments, makes determination of the zone of equivalence more difficult. The quantitative isotope precipitin curve provides an advantageous method of antibody determination with chicken or rabbit anti-ferritin systems since the anti-ferritin content of a serum can be expressed as μg N of antigen precipitated per ml of antiserum.

Chicken anti-ferritin contains 3 immunoglobulins which react with ferritin in precipitin arcs, demonstrated by iron staining or $I^{131}F$ autoradiography. This heterogeneity of chicken antisera has been previously described (8) in the chicken anti-insulin system. Attempts to separate these antibodies for further characterization are in progress. The use of iron staining of complexes of anti-ferritin immunoglobulins and ferritin provides a method of study of antibodies similar to autoradiographic immunoelectrophoresis without involving the use of radioactive labeled antigens or antibodies.

Summary. Gel filtration was used to obtain a preparation of horse spleen ferritin apparently free of antigenic contaminants. The purified ferritin was labeled with I^{131}. Quantitative isotope precipitin curves with rabbit and chicken anti-ferritin are described. The staining of ferritin iron in ferritin reacting with immunoglobulins of electrophoretic preparations was used to show the heterogeneity of chicken antibodies in the primary and secondary response against ferritin.

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Absorption, Distribution and Excretion of Pentaerythritol and Pentaerythritol Tetranitrate by Mice. (29829)

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Although pentaerythritol tetranitrate (PETN) has been used extensively for many years to prevent angina pectoris, there is a dearth of information about its metabolism. One of the difficulties has been the lack of assay methods for the intact PETN molecule. Previous investigators demonstrated PETN absorption by the difference between the dose administered to rats and the subsequent nitrate content of the gastrointestinal tract (1) and by nitrate and nitrite assays of the blood of humans who ingested the drug (2,3,4). Since these approaches omit the intermediary metabolism of PETN, they preclude furtherance of our interest in elucidating the mechanism of action of this drug.