Salt—Isoelectric—Density Gradient (SID) Purification of Rabbit IgG* (33611)

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Criteria such as yield, speed, and number of steps, sample size, and equipment requirements determine choice of method for immunoglobulin isolation. Methods are categorized as *macro-* if the serum sample size is larger than 1.0 ml and *micro-* if the sample size is smaller. Methods in current use for 7 S gamma globulin (IgG) isolation are inconvenient for the quantitative multiple microisolations that are required for kinetic studies because they are either too time-consuming, and so are not practical when many samples must be processed, or they produce low-purity globulin. The problems involved with the various techniques include low purity of final product (salt fractionation, batch gel extraction), denaturation or failure to elute (column chromatography, disc electrophoresis), expense (analytical ultracentrifugation), low yield and/or lack of general suitability with various animal sera (batch gel extraction), as well as the general problem of difficulty in rapidly handling the many small samples characteristic of kinetic experiments.

The Salt—Isoelectric—Density gadient (SID) micromethod described herein produces good yield of high purity IgG which is directly visualized in gradient tubes without special optics or elution and assay; is suitable for simultaneous isolation of numerous small samples; and, perhaps most important, is practical for use in laboratories which lack analytical ultracentrifuges and numerous fraction collectors. The specific methods were worked out for rabbit serum hyperimmune to trypanosomatid antigens but the principles apply generally.

Methods. Antisera. Hyperimmune rabbit sera, prepared as previously (1, 2), were used throughout. Specifically, whole cells of the trypanosomatid flagellate, *Crithidia fasciculata* (*Culex* strain), ATCC 12857, were used as a source of mixed antigens. For hyperimmunization, each rabbit received a series of three subcutaneous injections at 1-week intervals. Each injection contained 0.2 ml packed whole flagellates and 0.3 ml saline (0.9% w/v NaCl) which was emulsified with 0.5 ml Freund's complete adjuvant (Baltimore Biological Laboratories) and used immediately after preparation. Two–5 ml of blood were collected before immunization and at 3–14-day intervals for 120 days beginning after the first subcutaneous injection of antigen. After the clot was removed, the serum was stored at −20° until use for SID purification or other purposes.

SID purification. The starting material was 0.5–1.0 ml of hyperimmune rabbit serum (Fig. 1A). To the serum was added an equal volume of saturated $(NH_4)_2SO_4$. The combination was shaken by hand for several minutes at room temperature, incubated for 1 hr at 4°, and then centrifuged at 5000*g* for 20 min at 4°. The precipitate was dissolved in 1.0 ml, 0.1 *M* phosphate buffer, pH 7.2 (this is considerably higher than the isoelectric point of IgG) and reprecipitated 4 more times in a like manner (Fig. 1B). Clearly, even after 5 salt precipitations, contaminating serum proteins are merely reduced, not eliminated.

The resultant crude globulin fraction, suspended in 0.1 *M* phosphate buffer, was quantitatively transferred to dialysis sacks and dialyzed for 24 hr at 4° with stirring and frequent changes of the same buffer. This exhaustive dialysis was essential to remove residual salt from the crude globulins: it obviated interference with formation of salt and

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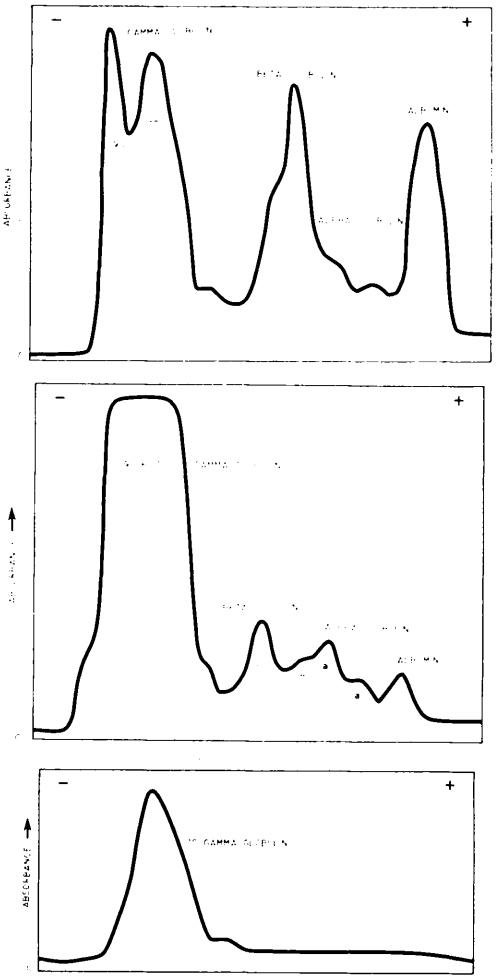

FIG. 1. Densitometer tracings of discontinous acrylamide gel electropherograms of rabbit hyperimmune serum against whole *Crithidia fasciculata* (*Culex* strain). A Serum before purification. B. Serum after 5 sequential $(NH_4)_2SO_4$ precipitations and dialysis. C. Serum after complete SID purification. This is the largest impurity ever noted.

pH gradients during subsequent density-gradient centrifugation.

The dialyzed crude globulins, in 0.5–1.0-ml samples, were then layered on top of a steep sucrose gradient (discontinuous or continuous) in which the sucrose was prepared by solution in 0.01 *M* phosphate buffer pH 6.0 (this yielded a gradient which ranged significantly below the isoelectric pH of IgG as shown in Fig. 2) and centrifuged for 16 hr at 35,000 rpm in a SW39 rotor of a model L or a SW65 rotor of an L2-65 Spinco ultracentirifuge. Extension of centrifugation past 16 hr had no effect on the position of the IgG band.

During centrifugation, pH and salt (PO_4) gradients form. It is essential that the isoelectric and isodensity areas of the gradient coincide so that the IgG reaches both its isoelectric pH and isodensity area of the gradient simultaneously. With a properly constructed SID gradient, the IgG band appears as a distinct, white-opaque, compact band in the 25–32% sucrose area of the gradient (rather than the diffuse, transparent band produced in simple gradients).

This band was easy to visualize even through somewhat opaque polyallomer centrifuge tubes. To recover the IgG fraction, the clear upper portion of the gradient was first removed with a Pasteur pipette or syringe attached to a needle with most of the bevel removed. Using a fresh pipette (or syringe and needle) the opaque IgG band was then removed from the *top* of the gradient tube. Removal of the IgG band through the bottom of the tube via the usual tube-puncture method led to contamination by IgM whereas IgM contamination is impossible when the top-of-tube removal method is used.

The protein content of the opaque IgG band (and the other fractions recovered from the gradient tube) was assayed by either the method of Lowry (3) or more usually that of Waddell (4) since the latter is more accurate and does not destroy the product during assay. When Waddell's (4) method was used, the IgG was first dialyzed against buffer to remove the sucrose which interfered with the spectrophotometric determination.

Discontinuous electrophoresis was done by the standard acrylamide technique of Davis (5) and the stained proteins were diagrammatically represented by analyzing the gels with a Joyce-Loebl Chromoscan (Fig. 1).

Results. Gamma globulin prepared by the SID method routinely showed one brand in the analytical ultracentrifuge and on disc electropherograms. Infrequently a sample exhibited a minor band on disc electropherograms (Fig. 1C). Since analytical ultracentri-

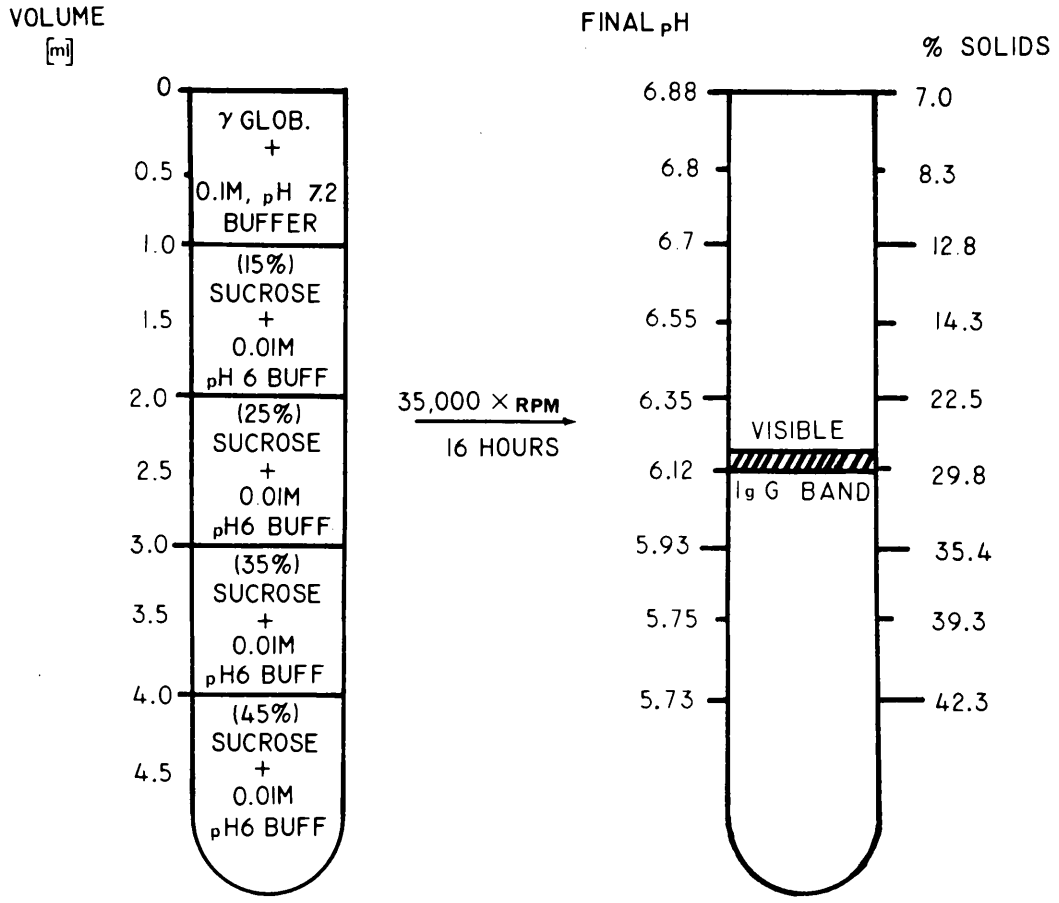

FIG. 2. Diagrammatic representation of formation of pH and continuous sucrose gradient from a SID discontinuous sucrose gradient with a 1.0-ml sample. Percentage of solids was determined with a Bausch and Lomb model Abbe L-2 refractometer and pH determined with a Corning expanded scale pH meter equipped with a combination microelectrode.

fuge and disc electrophoresis results checked well, we elected to rely solely on the latter method for purity checks since it is more rapid, cheaper, and, in our hands, more sensitive.

Our results show that increase in protein in the IgG fraction preceded detectable agglutinating activity and that the IgG fraction was almost exclusively responsible for agglutinating activity beginning about 3 weeks after the first dose of antigen (Fig. 3). Notably, the serum protein levels of IgG and IgM alone do not completely account for their agglutinating activity (compare Fig. 3A and 3B). That is to say, the primary peak in IgM protein concentration occurred about 7 days after the first antigen injection (Fig. 3B) but peak IgM agglutinating activity did not occur until about 21 days after the first antigen injection. Similarly, the primary peak in IgG protein concentration occurred about 12 days after the first antigen injection (Fig. 3B) when IgG agglutinating activity was just beginning to be detected (Fig. 3A). Peak IgG agglutinating activity did not occur until about 34 days after the first antigen injection. At this time (34 days after the first antigen injection) there was little contribution by IgM to the total serum-agglutinating titer (Fig. 3A); the serum titer could be explained solely on the basis of the IgG contribution.

Discussion. It is impractical to separate IgG by direct application of serum on sucrose gradients because several serum proteins

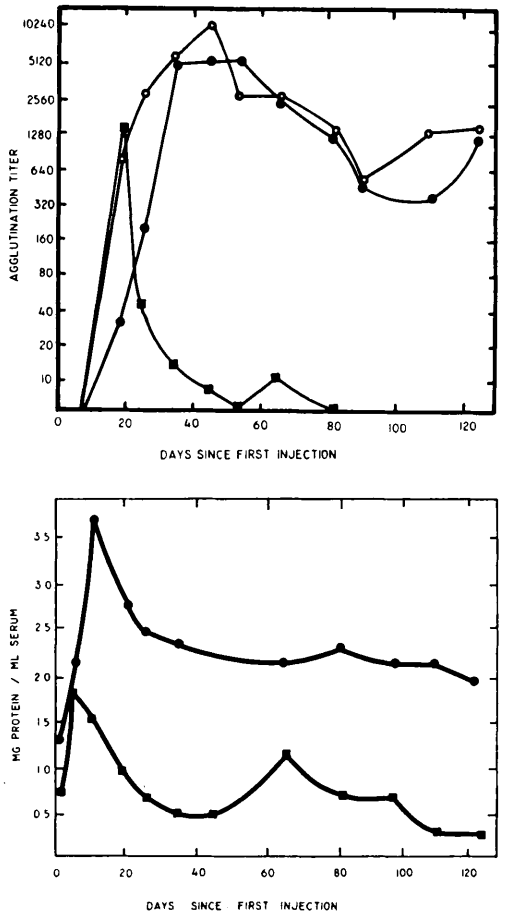

FIG. 3. Typical results of protein analyses and agglutination tests during the course of the hyperimmune response of rabbits to *Crithidia fasciculata* (*Culex* strain). The animals received antigens at days 0, 7, and 14. A. Homologous agglutinating titers for whole serum and purified globulins: (○) whole serum; (●) IgG; (■) IgM. B. Protein content of immunoglobulin fractions (●) IgG; (■) IgM.

have similar sedimentation constants and samples are often contaminated with varying amounts of hemoglobin. Although the sedimentation constant of hemoglobin is below 5, it is often present in sufficient amounts to contaminate the adjacent 7 S, IgG region (which is a diffuse band in ordinary density gradients).

Accordingly, most of the serum proteins and hemoglobin were first cleared from the immunoglobulin fraction by several sequential $(NH_4)_2SO_4$ precipitations (Fig. 1A and 1B). Usually 3 or 4 precipitations were sufficient to remove all hemoglobin (the precipitate turned white rather than pink) but our standard 5 precipitations cleared even the most contaminated sample. After the fifth precipitation, the gamma globulins were enriched but contaminating albumin, and alpha and beta globulins were still detectable (Fig. 1B).

The heart of the SID separation is the next step in which the last $(NH_4)_2SO_4$ precipitate, which is composed mostly of gamma globulins, is dissolved in a relatively concentrated buffer, whose pH is above the isoelectric pH of IgG, and layered onto a steep sucrose gradient prepared in buffer tenfold lower in molarity than the sample buffer. Addition of the buffer to sucrose yields a gradient whose pH ranges below the isoelectric point of IgG (Fig. 2). The difference in molarity between the sample and gradient buffers facilitates final gradient mixing and retards premature precipitation of the IgG.

When imperfect gradients are made so that IgG reaches the area of isoelectric pH *before* that of isodensity, large clumps of protein form which rapidly precipitate to the bottom of the tube, or IgG reaches its isodensity area before the pH of that area is at the IgG isoelectric pH. Then, the IgG area usually remains as a transparent diffuse band.

Usually IgM (19 S globulin) is isolated directly from serum on a simple density gradient if it is the only immunoglobulin wanted. As long as the IgM is removed from the bottom of the gradient tube, it will be free of contaminents. Similarly, one may choose to isolate IgM from an SID gradient, after removal of IgG, by puncturing the bottom of the tube and draining the IgM which is in the bottommost portion of the tube.

The total time for SID isolation is 48–50 hr but this time may be shortened by as much as 20–24 hr if the 24-hr buffer dialysis which precedes density centrifugation is replaced with the rapid, but more expensive, dialysis against a molecular sieve.

One may choose to isolate IgM and IgG from one serum sample using only the SID method but we have not yet established the

percentage of recovery for IgM thus isolated. We suggest instead a more time-consuming two-gradient procedure which becomes practical when the total amount of serum available is severely limited. This method consists of direct isolation of IgM from serum using a simple sucrose gradient which ranges up to 45% (w/v) sucrose. The IgM is then removed from the bottom of the gradient tube. The rest of the gradient (protein, sucrose, salts) is dialyzed and concentrated. The IgG in the concentrate then can be purified using the complete SID method.

The SID method also holds promise for the isolation and direct visualization of one enzyme from a mixture of proteins. This application requires that the pH gradient is set up in such a way that no protein reaches its isoelectric pH while other proteins in the mixture are still passing through that segment of the gradient.

Summary. A high-purity micromethod called Salt—Isoelectric—Density Method (SID) for simultaneous isolation of multiple IgG (or IgG and IgM) samples is described. Kinetics of the immune response to mixed flagellate antigens isolated by the SID method is described.

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The Influence of Three Analogs of Isoleucine on *in Vitro* Growth and Protein Synthesis of Erythrocytic Forms of *Plasmodium Knowlesi** (33612)

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Recently we reported that L-isoleucine is essential for *in vitro* growth of erythrocytic forms of *Plasmodium knowlesi* (1). In the present communication it was shown that L-isoleucine was more avidly incorporated into plasmodial protein than seventeen other radiolabeled amino acids. On the basis of these observations, two isoleucine antagonists, L-*O*-methylthreonine and alloisoleucine, known to inhibit *in vitro* growth of certain organisms (2, 3), were examined for their effect on DNA and protein synthesis in erythrocytic forms of *P. knowlesi in vitro*. *N*-Acetylisoleucine, thought on a structural basis to act as an antagonist of isoleucine, was similarly tested. It was found that L-*O*-methylthreonine (L-OMT) strongly inhibited *in vitro* growth of *P. knowlesi*. Certain aspects of the mode of action of L-OMT were investigated and discussed.

Materials and Methods. The methods of preparing plasmodial cultures, dialyzing serum, and determining DNA have been described (1).

Incorporation of isotopically labeled amino acids into protein. Plasmodia in the trophozoite stage were incubated for 15–16 hr in Eagle's basal medium containing 0.2 mmole of each L-amino acid, including L-alanine, L-aspartic acid, L-glutamic acid, glycine, L-proline, and L-serine and supplemented with 10% dialyzed human AB serum. One half or 1

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