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Method for Rapid Separation of Immunoglobulin M from Immunoglobulin G Antibodies by Using Reorienting Gradients in Vertical Rotors

HANS J. WOLF,* GERT G. FRÖSNER, AND FRIEDRICH DEINHARDT

Max von Pettenkofer-Institut, University of Munich, 8000 Munich 2, Federal Republic of Germany

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The parameters for the use of reorienting gradients in vertical rotors for rapid separation of immunoglobulin M from immunoglobulin G on a preparative scale for the rapid diagnosis of infectious diseases are described.

The detection of immunoglobulin M (IgM) antibodies specific for defined antigens is of increasing interest for the diagnosis of acute infections. For all infections where methods for the detection of specific IgM antibodies such as immunofluorescence (7), radioimmunoassay (3), or enzyme-linked immune tests (1) are not available, a physical separation of IgM from IgG antibodies followed by testing of the fractions is necessary (2).

Separation of the antibody classes by column chromatography with molecular sieves is accurate but of limited usefulness for routine diagnosis because it usually involves considerable dilution of the fractions and is not convenient for investigation of a large number of samples in a short time. A frequently used method is separation by ultracentrifugation through sucrose gradients, using a swinging-bucket rotor. The disadvantages of this method are the limited number of samples (usually three, maximally six) which can be centrifuged at one time and a centrifugation time of almost 20 h (6). This does not permit the processing of a larger number of samples within a reasonable time, blocks the use of centrifuges for other work for extended periods, and does not allow quick answers in urgent diagnostic cases.

The amount of time required for sufficient separation of immunoglobulins depends on the distance the different proteins have to migrate to be clearly distinguishable. In the case of the swinging-bucket rotor the distance of migration of the protein fractions is the same as the distance the proteins are found relative to the meniscus of the gradient in its vertical position. The development of angle head rotors helped considerably to reduce the distance of migration for equilibrium density gradients. However, due to turbulence, these rotors were inappropriate for sedimentation velocity gradients. The introduction of angle head rotors with an angle of 90° (= vertical rotors) by Du Pont and later by Beckman Instruments solved that problem. These rotors accelerate the separation of heterogeneous mixtures considerably. In this case the distance of migration of the macromolecules is very short because the gravitational field parallels the length of the tube and not the tube diameter, which reduces the required time of centrifugation accordingly. The separation of the proteins is solely dependent on the relative position in the gravitational field of the separating chamber. Specially designed ultracentrifuges (e.g., Du Pont Instruments, Newton, Conn.; or Kontron, Zürich, Switzerland) or modification kits for conventional ultracentrifuges (Beckman Instruments, Inc., Palo Alto, Calif.) are necessary which can accelerate and slow down these rotors smoothly enough to allow the gradient and the sample to maintain their relative positions in the gravitational field and avoid disturbances of the gradient. During acceleration the densest part of the gradient moves from the bottom of the tube to the outer diameter, whereas the lightest part of the gradient moves from the top of the tube to the inner diameter. Upon deceleration this process is reversed. Because the length/diameter ratio of ultracentrifuge tubes is usually in the range of 5:1, the time required to achieve the same relative positions is considerably shorter. Specifically, we will show (Fig. 1 through 3) that 2 h are sufficient to effectively separate IgG from IgM. The generally used tables for the calculations of centrifugation times as presented by McEwen (4), or similar publications, cannot directly be applied to vertical rotors. In our laboratory, therefore, the conditions for the separation of IgG from IgM were derived empirically by centrifuging a pool of four sera containing IgG and IgM under various conditions.

The gradients were made up using sucrose for density gradients (Merck, Darmstadt, Ger-

many) in dextrose-gelatin-Veronal buffer (Flow Laboratories, no. 28-010). The gradients were mixed by using cylindrical interconnected chambers (diameter, 10 mm). The lighter solution was placed in the first chamber so that the light solution poured into the ultracentrifugation tubes first. The volumes for heavy and light solutions were corrected to give similar hydrostatic pressures in both chambers by using values for specific weights from the ISCO tables (5) to avoid distortions when starting the gradient. The nonwetting polyallomer centrifuge tubes (Du Pont or Kontron) were filled through a capillary reaching to the bottom of the tube. Up to eight gradients were centrifuged at a time at 4°C in a precooled TV 865 rotor (Du Pont) in a preparative ultracentrifuge TGA 50 or TGA 65 (Kontron) with the setting on "reograd" for reorienting gradients. The total volume of the gradients was 4.4 ml minus the sample volume (10 to 100 μl). When no monitoring of optical density was planned, the total volume was reduced to 4.2 ml, 200 μl of mineral oil was layered on top of the samples, and samples were harvested from the bottom.

To establish the method, the optical density profile of the gradients was measured after centrifugation times as given in the figures in an ISCO fractionator (model 184, Instrumentation Specialties Co., Lincoln, Neb.). The tubes were pierced at the bottom, and 60% (wt/wt) sucrose in water stained with some bromophenol blue was pumped into the tubes with an LKB Varioperpex peristaltic pump (setting 1.8 × 10; 3-mm inner tube diameter) from the bottom. At least 20 fractions per gradient were collected for analytical runs, using drop counts in a Redi Rack fraction collector (LKB, Uppsala, Sweden) interconnected to an ISCO UA 5 optical unit with a 5-mm-optical pathway flow cell, 280-nm filter set, paper advance of 150 cm/h, and optical density setting which varied from 0.5 to 2. The fraction collector was custom modified to allow event marking with the ISCO recorder. A 5-μl amount of each fraction was tested on IgG Partigen and IgM S-Partigen plates (Partigen and S-Partigen plates, Behringwerke AG, Marburg, Germany). The amounts of IgG and IgM, respectively, present in the fractions were calculated according to the instructions supplied with the plates.

Figure 1 shows a good separation of IgG from IgM with sharp bands for a 10-μl sample and a gradient with 5 to 35% (wt/wt) sucrose. Figure 2 shows somewhat inferior separation with broader bands of the same sample on a standard 5 to 20% (wt/wt) gradient. Figure 3 shows that the same gradient as used in Fig. 1 (5 to 35%

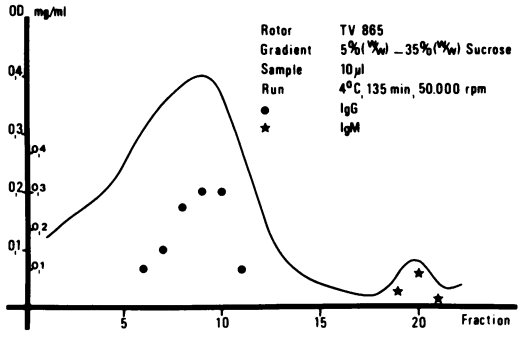

FIG. 1. *Optical density (OD) and content of IgG and IgM of the fractions from a 10-μl serum sample centrifuged through a 4.4-ml gradient with 5 to 35% (wt/wt) sucrose for 135 min at 50,000 rpm and 4°C. All fractions of the gradient were tested for IgG and IgM. Only values above the margin of significance (0.1 mg/ml for IgG and 0.01 mg/ml for IgM) are shown on the figure.*

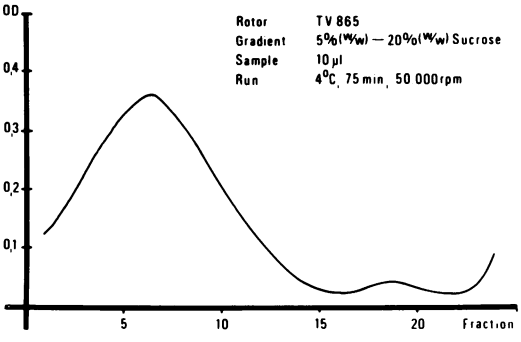

FIG. 2. *Same as Fig. 1, except that a 5 to 20% (wt/wt) sucrose gradient was used, fractions were not tested for IgG and IgM, and the time was reduced to 75 min.*

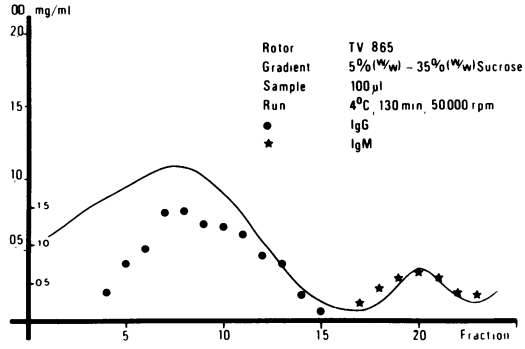

FIG. 3. *Same as Fig. 1, except that 100-μl samples were applied and the centrifugation time was 130 min.*

[wt/wt] sucrose) still yields separate IgG and IgM bands with 100 μl of sample applied. Gradients using higher concentrations of sucrose (not shown) did not yield considerably sharper bands nor did they increase the separation of the bands; they did, however, require longer times of centrifugation.

Sucrose gradients ranging from 5 to 35% (wt/wt) were chosen for routine work because they gave good separation of the immunoglobulins in a relatively short time. The resolution of IgM and IgG is best if only 10 to 50 μl of serum is applied. This amount of serum is usually sufficient for testing for specific antibodies with sensitive tests such as radioimmunoassays. One hundred-microliter samples are required if the fractions have to be tested by a less sensitive test such as hemagglutination inhibition, which is usually used in the diagnosis of rubella. Even in this case the resolution is still acceptable (Fig. 3) and the results are obtained rapidly and accurately.

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