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Isopycnometric Serology: a New Technique based on Buoyant Density Changes in Latex Beads

WE wish to describe new serological techniques based on changes in buoyant density occurring when antibodies, or antigens, or both, are attached to latex beads. For the density change to be observed, the mass of the attached particles must be an appreciable fraction of the total mass. Using very small carrier beads less than one hundred antibody molecules or one virus particle per bead can be detected. The speed and sensitivity of the method make it directly applicable to the clinically important problem of rapid identification of infectious agents.

If the volume and density of one member of a hybrid pair (termed here the carrier) are known, then the positive or negative buoyancy of the second member (or load) can be determined from the buoyant density of the hybrid. If the buoyant density of the load is known, then its volume, and hence its mass, can be calculated. For the application of this principle to subcellular particles, viruses, and macromolecules, a series of carrier particles of different size is required, with a narrow size distribution within each sample. The carrier particles should be either more or less dense than any of the load particles which may be encountered. Most particles of biological interest, excluding lipids and lipoproteins, fall in the range between 1.19 and 1.7 g/cc[2]. Carrier beads lighter than 1.18 g/cc are preferable to those denser than 1.7 g/cc because less viscous gradients and, therefore, lower speed centrifugation may be used. Fortunately, very uniform polystyrene latex beads are available[1] which cover the lower portion of the size range of interest here. Immunoglobulin (IgG) has been found to attach to these after mild heating in a slightly alkaline environment[2], and flocculation of the resulting complex has been widely used to detect the human rheumatoid arthritis factor[3].

In all the experiments described here polystyrene latex with an average diameter of 0.109 μm (s.d. = 0.0027 μm, Dow latex batch No. LS1044–E) was used. In sucrose gradients these particles band at a density of 1.049–1.050 g/cc[4]. Using a small linear gradient mixer and a six channel peristaltic pump, six identical sucrose gradients were prepared and centrifuged in a Beckman SW50.1 swinging bucket rotor to study the banding behaviour of the particles. A band recovery apparatus[5] was used to observe and photograph bands and to recover samples. In some experiments plastic density-marker beads were used[5].

When the particles banded sharply, as little as 1 μg of latex could be seen and photographed. With diffuse bands, much larger samples (5 μg or more) were required for detection. Fig. 1 shows the variation in buoyant density observed when a fixed concentration of latex beads was incubated with varying concentrations of human IgG. Ten micrograms of beads was incubated with borate buffer[2] (*p*H 8.3) (tube 1), or with ten-fold dilutions of IgG in buffer ranging from 2,500 μg (tube 2) to 0.25 μg IgG (tube 6). The density of the large plastic marker bead used was 1.109 g/cc. After centrifugation at 28,000 r.p.m. for 60 min, samples of the bands were recovered and the sucrose concentration determined refractometrically.

The buoyant density of a hybrid particle is given by the equation:

$$\rho_H = \frac{V_C \rho_C + V_L \rho_L}{V_C + V_L}$$

where V and ρ refer to volume and density, and subscripts H, C, and L refer to hybrid, carrier, and load respectively. For a density of 1.35 g/cc for IgG, a bead volume of 6.78×10^{-16} cc, and a bead density of 1.05 g/cc, the mass of the IgG loads can be calculated using the equation:

$$M_L = \frac{V_C(\rho_H - \rho_C)}{1 - \rho_H/\rho_L}$$

The load masses fall in the range of 9.2 to 178×10^{-18} g. This is the low end of the range of virus mass.

The average number of IgG molecules per bead shown in Fig. 1 was calculated using a value of 2.56×10^{-19} g for one IgG molecule. Using the highest value of 695 molecules per bead, the average surface area per molecule is found to be 5367 Å^2, or equivalent to a square 73 Å on a side. IgG is a Y-shaped molecule[6] with a radius of gyration in solution of 76 Å[7]. The three segments of the molecule are joined by a hinge which allows considerable flexibility[8]. The area occupied by a molecule lying flat (occupying maximal area) is larger than that experimentally observed. There is no present evidence, however, that all molecules are bound in a similar fashion. Oreskes and Singer[9] measured the IgG contents of supernatants after centrifugal removal of the latex and estimated by difference the amount of IgG bound to two different sizes of polystyrene latex particles. For 0.802 μm particles, each adsorbed molecule occupied the equivalent of a 52 Å square, while for 0.220 μm particles the value equalled that of a 47 Å square in good agreement with the results obtained here. It should be noted, however, that our measurements were made on beads centrifuged through a protein-free density gradient, which may have produced some desorption (Fig. 2, tubes 2 and 3). Further

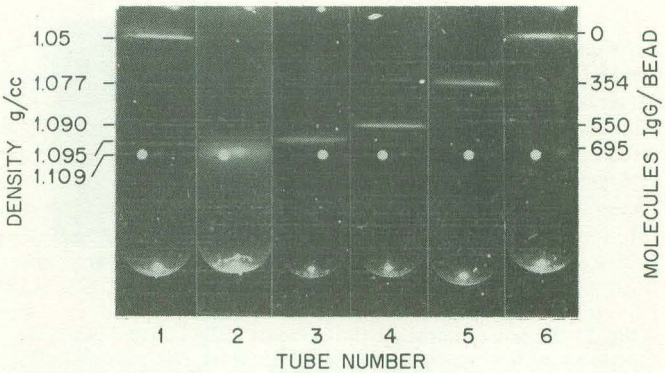

Fig. 1 Photographs of six gradients showing banded latex and large reference bead banding at 1.109 g/cc (the same reference bead was used for each tube). Tube No. 1 contains uncoated beads banding close to 1.05 g/cc, while the remaining tubes contain 0.109 μm latex beads incubated for 30 min at 56° C in ten-fold dilutions of a solution containing 2,500 μg human IgG (tube 2) and ranging down to 0.25 μg/ml. (tube 6). The banding densities and number of IgG molecules bound were respectively for tubes 2–6: 1.099, 695; 1.095, 629; 1.090, 547; 1.077, 354; and 1.053, with 31 molecules.

work is needed to characterize the mode of attachment of the antibodies to the beads. The negative charge on the latex seems to result primarily from a monomolecular soap film attached to the surface of the bead.

Can the IgG attached to the beads selectively bind other proteins and increase the banding density of the bead–protein complex further? Serum from rheumatoid arthritis patients contains IgM which reacts with aggregated IgG and with IgG attached to latex beads. The series of experiments shown in Fig. 2 illustrate the effect of adding rheumatoid factor (*a*) to IgG coated latex beads in the presence of excess dissolved heated IgG (as is the case when the test is run in the usual manner on a slide), and (*b*) to gradient-washed IgG coated beads. When washed beads are used the buoyant density is very markedly increased (Fig. 1, tube 5) in the presence of rheumatoid factor positive serum, but not with negative serum (tube 6). A small increase in density is seen in tube 4 in which the rheumatoid factor seems to be partially complexed by the excess heated IgG in solution. For isopycnometric measurements, washed beads are preferable. Using the equations given, the washed beads used bound approximately 500 IgG molecules per bead, and the excess density observed after reaction with rheumatoid factor corresponds to an additional 130 molecules of IgM per bead.

Other experiments have shown the applicability of this technique to the detection of T_2 and T_4 phage particles, and

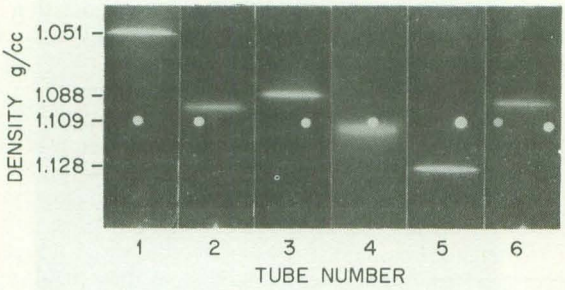

Fig. 2 Binding of human arthritis factor to IgG coated beads in presence and absence of excess IgG. Note reference density bead in each tube banding at 1.109 g/cc. Tube 1, uncoated beads; tube 2, latex beads sedimented directly out of incubation mixture containing excess IgG. Tube 3, latex from tube 2 reban-ded. Tube 4, latex banded directly out of mixture of 10 μg beads, 0.25 mg IgG, and 20 μl. of arthritis factor positive serum in a total serum of 520 μl. Latex and IgG incubated at 56° C before adding serum. Tube 5, washed IgG coated beads (10 μg) plus 20 μl. of arthritis factor positive serum in volume of 520 μl. layered over gradient. Tube 6 identical to tube 5 except that arthritis negative serum was used. Gradients contained the borate buffer of Singer and Plotz[2] and extended linearly from 13 to 25% w/w sucrose with a small cushion of 66% sucrose at the bottom. Centrifugation was for 1 h at 27,000 r.p.m. at 20° C.

their identification one from the other. Present results indicate that isopycnometric methods are applicable to various problems ranging from the detection of incomplete antibodies to rapid identification of virus and bacteria, and to studies on subcellular particles and whole cells.

To exploit this technique fully, however, antibodies purified by immuno-absorption may be required. If a given species of antibody represents only a very small fraction of the antibody present, the number of antigen molecules bound per bead will be correspondingly low. This problem is less serious when the bound antigen is large; for example, only a few antibody molecules are required to attach one virus particle.

If the same antibodies are attached to two species of beads with different buoyant densities, dissimilar beads held together by an antigen will band at an intermediate position. This variation of the technique may ultimately prove to be the most sensitive.

Two particles with different densities will be subject to a separative force when banded in a centrifugal field. By adjust-ing the force field and by a suitable choice of bead size and density, it is possible to dissociate loosely bound particles and retain only those whose binding energy exceeds a determined level, thus decreasing the effect of weak non-specific binding.

If the particles are truly at an equilibrium, dissociation will be observed during centrifugation since particles momentarily dissociated would tend to move apart.

In this work a minimum of 1.4×10^9 latex beads have been used per test. Because light scattering measurements have been made on single beads somewhat larger than those studied here[10], and because single beads of the size used in this study have been counted in separate experiments by detecting scattered near ultraviolet laser light, the method is potentially capable of measuring a very small number of antigen-bead complexes separated isopycnically from the main mass of beads. Intensive efforts are in progress to adapt isopycnometry to the very rapid detection and identification of bacterial and viral pathogens using a higher speed version of the GeMSAEC fast analyser[11] to allow a large number of measurements to be made during centrifugation. We are also trying to produce fluorescent or scintillator containing latex particles to which antigens or antibodies may be covalently linked. By incorporating a radioisotope, scintillator beads may serve as their own light sources and be detected by quantum counters.

We believe that isopycnometry using small well-defined carrier beads will make possible for the first time routine mass measurements in the 10^{-12} to 10^{-16} g range.

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