evidence that rabbits immunized with protein-conjugated sulfanil and dinitrophenyl groups develop splenic PFC specific for these haptens (*6*).

Previous unsuccessful attempts to employ protein-conjugated erythrocytes in the LHG technique may now be subject to review. The conditions imposed by antigen coupling or conjugation have frequently so damaged erythrocytes that they become useless for the LHG procedure. Also certain steric and conformational factors are likely to affect the orientation and subsequent activation of complement on the red cell surface. Hapten-conjugated erythrocytes may be functionally suitable in the LHG technique, by virtue of the relatively small dimensions of the attached haptenic groupings and their consequent close proximity to receptors indigenous to the erythrocyte, which are themselves normally involved in immune hemolysis. By analogy, cells producing antibody to protein or synthetic polypeptide antigens might be readily detectable with the LHG technique by using indicator erythrocytes conjugated with appropriate peptides or with amino acid copolymers.

The efficacy of simple haptens in the LHG technique adds a new dimension to investigation of the cytodynamics of the immune response. Despite their simple structure haptens elicit a complex antibody response. Nonetheless they are representative of the best defined and most extensively studied model systems in immunology. The present work demonstrates that these model immune systems are now amenable to sensitive analysis in the study of antibody-producing cells.

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11 April 1966

Influenza Virus Purification with the Zonal Ultracentrifuge

Abstract. *Sufficient amounts of influenza virus (density, 1.185; size, 722S) can be highly purified (22,000 chicken cell agglutinating units per milligram of protein) with a zonal ultracentrifuge, used first in a rate process followed by isopycnic banding, to permit its detailed biological and physical-chemical evaluation.*

Harvey (*1*) first used the isopycnic-zonal centrifugation procedure to separate biological materials by banding and Brakke (*2, 3*) originated the rate-zonal centrifugation technique. Reviews of these procedures (*3*) indicate their importance. As performed in swinging-bucket rotors, these microfractionation techniques should be considered analytical methods rather than preparative procedures. They lead to purified fractions, measured as drops, which usually can be assessed only by methods which are highly sensitive to the relatively low concentrations of material present (*4*). Anderson and his collaborators have reported the continuing development of several new zonal-ultracentrifuge rotors (*5*). These new instruments are of general interest because samples of much larger volume now can be processed conveniently to obtain highly purified material for new or more complete evaluation.

We now describe our use of the commercially available B-IV rotor (*6*) (1.7-liter volume) for virus purification. Influenza virus, including types A (PR-8), A_1 (Ann Arbor), A_2 (Taiwan), A_2 (Japan 170), A_2 (Japan 305), B (Maryland), and B (Great Lakes), was grown in embryonated chicken eggs. Harvested allantoic fluid was first subjected to differential centrifugation in a Sharples Supercentrifuge with subsequent low-speed clarification of the resuspended virus pellet (*7*). This procedure for commercial vaccine production concentrates virus tenfold with respect to allantoic fluid and purifies it approximately 100-fold with respect to protein content. This partially purified product, usually containing 2000 chicken-cell agglutinating (CCA) units (*8*) per milligram of protein (*9*), was employed as starting material for the density gradient centrifugations.

In a typical experiment (Fig. 1) we

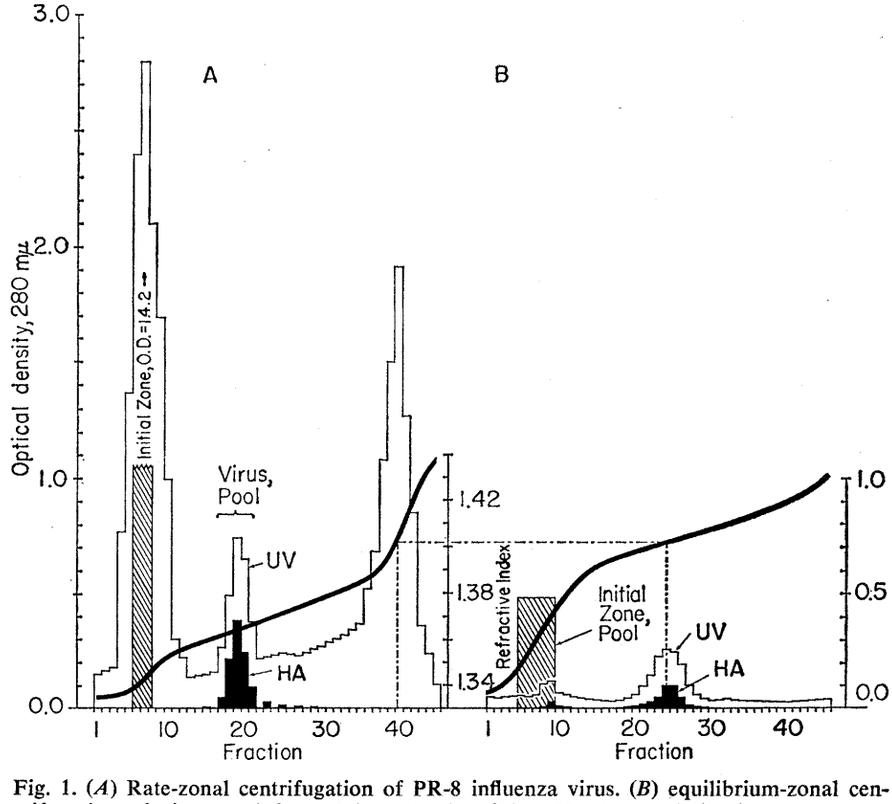

Fig. 1. (*A*) Rate-zonal centrifugation of PR-8 influenza virus. (*B*) equilibrium-zonal centrifugation of virus pool from (*A*). *UV*, ultraviolet; *HA* hemagglutination.

injected 100 ml of virus concentrate (10×) in 3 percent sucrose into the B-IV rotor spinning at 5000 rev/min. This initial zone, containing 1 mg of protein per milliliter, rested on a density gradient (1.2 liter) which was linear between 12 and 34 percent (by weight) sucrose (*10*), and was overlaid by 200 ml of buffer. This gradient rested upon a 200-ml cushion of 60 percent sucrose. We first made a rate-zonal separation at 25,000 rev/min for 30 minutes, unloading the rotor at 5000 rev/min and obtaining 45 fractions, each 40 ml (Fig. 1*A*). The bulk of the virus (S = 722) (*11*) was seen as a sharp ultraviolet-absorbing and hemagglutination zone (fractions 17 to 21) rising out of the microsomal background (*5*) (fractions 13 to 35) between the zone of soluble macromolecular impurity (fractions 4 to 12) and the zone of large particulate impurity (fraction 36 to 45). The latter is debris of chick tissue and bacteria. Virus fractions appear Tyndall blue. Typically, less than 10 percent of the total absorbancy at 280 $m\mu$ and of Lowry protein is found in the virus fractions. Unless a preparation is degraded, essentially all hemagglutinin is associated with virus.

As judged by electron microscopy the virus zone is contaminated with a few microsomes, which are larger than the virus and lack the typical spiked-surface morphology characteristic of myxoviruses embedded in phosphotungstate. Short filamentous forms of PR-8 were separated from the spherical forms by this rate-zonal process and were found in fractions 23 to 26 (Fig. 1*A*) by electron microscopy, as well as being discernible as a small shoulder in the ultraviolet and hemagglutinin profiles. The Ann Arbor strain repeatedly has given relatively more filamentous forms than have the other strains of egg-grown influenza viruses.

In order to remove the microsomal contaminant, we pooled the five 40-ml fractions containing the bulk of the rate-zonal purified virus and centrifuged it in the B-IV rotor, at 40,000 rev/min for 4 hours, in a 1.2-liter density gradient, linear between 34 and 50 percent sucrose. Analysis of these fractions (Fig. 1*B*) shows that the microsomes formed a band at buoyant density 1.090, and the virus formed a band at buoyant density 1.185 (*12*). The dashed horizontal line connecting both sides of Fig. 1 is drawn to illustrate the fact that the virus has approximately the same density as the bulk of the large-particle impurity. The initial rate-zonal process permitted effective sorting of the components according to size. If we had extended the time of that initial run, the virus band would then have been found with the large-particle impurity of the same density, as shown subsequently.

Figure 2 shows representative electron micrographs of the original 10× concentrate before and after purification by zonal ultracentrifugation. These micrographs were made under identical conditions for quantitative electron microscopy by a modification of Sharp's procedure (*13*).

Essentially all of the virus of this experiment was recovered in the isopycnic pool, as determined by hemagglutination and particle count by electron microscopy. The electron microscope, which can reveal impurities of about 1 percent when properly employed (*13–15*), showed this to be a pure virus preparation. Dividing our Lowry protein determination (0.16 mg/ml) by our particle count (3.8×10^{11} viruses per milliliter) we obtain the following value, 4.2×10^{-16} g of protein per virus. Accepting that 75 percent of this virus is protein and using Avogadro's number, we can calculate directly that the molecular weight for this virus would be approximately 3×10^8, a value in agreement with previous determinations (*15*) and suggesting a high degree of purity (*14*).

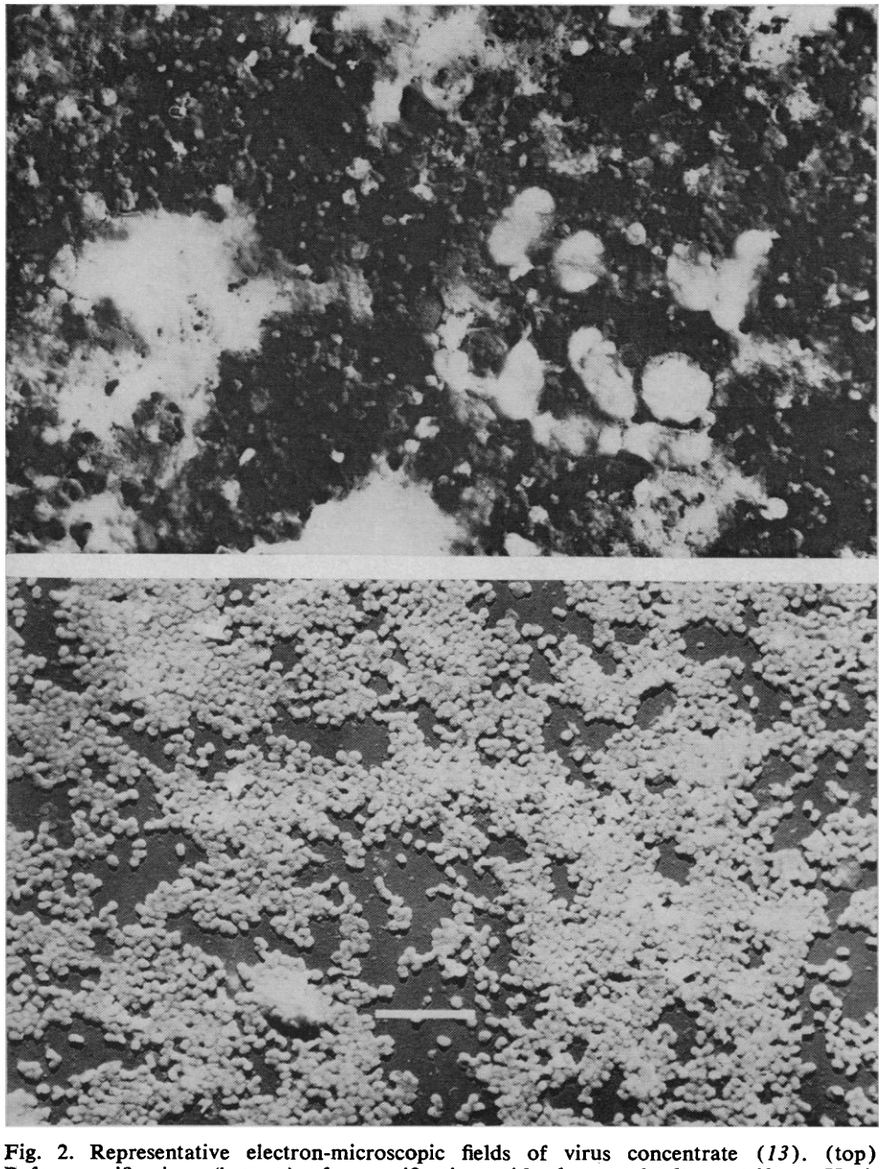

Fig. 2. Representative electron-microscopic fields of virus concentrate (*13*). (top) Before purification; (bottom) after purification with the zonal ultracentrifuge. Horizontal mark denotes 1.0 micron.

The 10× Sharples concentrate had 1800 CCA units while the final product had 22,000 CCA units per milligram of protein. Similar results are obtained with formalin-inactivated or with infectious virus.

In contrast to the relatively impure starting material used in these studies, the highly purified virus preparations passed through Millipore filters (0.45 μ) without loss of virus.

The purified, formalin-inactivated virus, given as a single immunizing inoculation, protected mice challenged intranasally to the same extent as impure virus did when given at the same virus concentration. The mice protected by purified egg-grown virus developed neutralizing antibodies against influenza virus adapted in tissue culture and against virus adapted in mice. The pure virus, in contrast to the starting material, was nonpyrogenic in rabbits. To date, these properties, as well as hemagglutinin (standard CCA test) have been stable for at least 8 months at 4°C. Therefore, this purification process is not detrimental to these biological characteristics of the virus.

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28 March 1966

Pentagonal Aggregation of Virus Particles

Abstract. *Virus aggregates with a unique fivefold axis have been observed in the electron microscope.*

Bagley (*1*) has described a dense structure with fivefold symmetry (Fig. 1, left) which results from the continued packing of hard spheres on a pentagonal bipyramidal nucleus. The growth of such a nucleus was proposed as a possible mechanism to account for a number of observations of apparent fivefold symmetry in crystals (*2*). These had earlier been explained as quintuple twins about a common axis (110) of face-centered cubic individuals, with lattice strain or imperfections making up the angular deficit of 7°20′.

During the preparation of bacteriophage f1 a contaminant phage fraction was isolated by differential centrifugation. Because our laboratory is involved in the large-scale growth of R17, we believe it to be the contaminant. Negatively stained electron-microscope preparations of both the contaminant and R17 phages appear identical. In an electron micrograph of an area in which both the contaminant virus concentration and the specimen thickness were so great as to approach the limits of electron transmission, we were surprised to find two pentagonal groups of viruses (Fig. 2) which we had not seen on the electron-microscope viewing screen. Although we have been unsuccessful in finding further examples of this type of virus packing, the chance observation

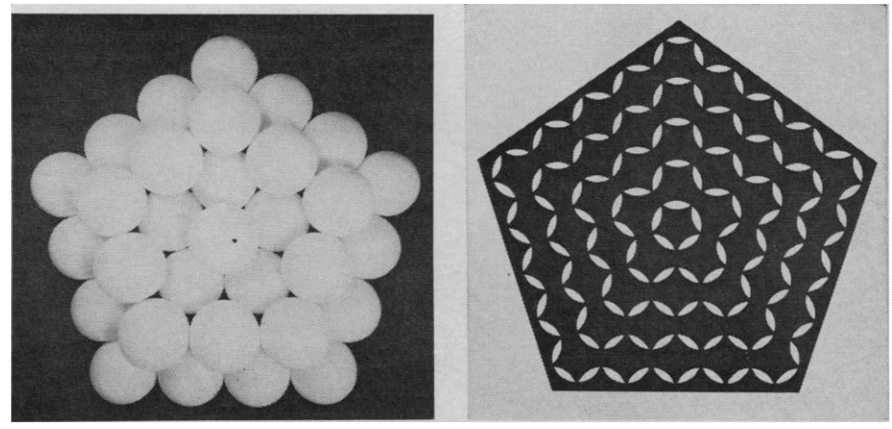

Fig. 1. (Left) Model of the packing system proposed by Bagley (*1*). (Right) Staining pattern predicted if stain fills the gaps between virus particles arranged at the left.