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Models of Structure of the Envelope of Influenza Virus*,†

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Abstract. Possible models of the structure of the influenza virus envelope are considered in terms of the known chemical composition. Models incorporating lipid in the form of a bimolecular leaflet are shown to be unlikely on geometrical grounds. A model having "inverted toadstool" protein units separated by spherical lipid micelles is favored, and is capable of explaining the appearance of the virus in the electron microscope and differences between normal and incomplete (von Magnus) forms of the virus.

Introduction. Examination of particles of influenza virus in the electron microscope shows that, despite considerable variation in particle shape and size with strain,[1–6] all have in common the morphological characteristics of a continuous bounding envelope carrying surface projections or spikes, and a helical inner component. "Incomplete" particles produced by infection at high input multiplicities show low infectivity and considerable pleomorphism,[7] but by electron microscopy are seen to have an envelope and spikes resembling those of infectious virus, although lacking some or all of the internal component. Both incomplete particles and "ghosts" or fragments produced by various disruptive techniques[8–10] exhibit to some degree the properties of hemagglutination and neuraminidase activity which are shown by intact infectious virus.[9, 11, 12] Viral proteins possessing these properties have been isolated from whole virus by a number of techniques,[12] and their approximate dimensions determined by electron microscopy[13, 14] and ultracentrifugation.[13, 15] The lipids of a number of myxo- and paramyxoviruses have been extracted and analyzed,[16–20] and the processes of viral maturation and release[1, 21] and viral attachment to and penetration of cell membranes[22, 23] have also been studied. The incorporation of the helical ribonucleoprotein internal component appears to be a largely passive process, and the extent of incorporation frequently determined by the size and shape of the membrane bud.[21]

Many properties of the virus thus seem to be determined by the nature of the envelope and the surface projections, and it is of interest to consider how the known envelope components may be organized. No complete analysis of all the components has yet been made, but a number of models have been proposed, largely utilizing information from electron microscopy.[2, 3, 13, 24] We propose to show, by consideration of geometrical factors, that certain types of model are invalid, and to present a number of models, some of which may rather better

explain some of the experimental observations of virus particle structure and stability.

In the absence of comprehensive data on the composition and properties of viral subunits, and since the virus itself, even in "highly purified" preparations, still shows variation in size and shape, we are unable to arrive at any indisputable structure. We feel, however, that most models so far put forward either conflict with some of the data, or do not take into consideration the physical and chemical properties of the constituent molecules. It is our hope that discussion of the models we propose will indicate experimental approaches capable of casting further light on the problem.

Composition of Influenza Virus. Gross composition: Although the amounts of each component vary with strain and method of extraction, most commonly accepted values are: protein, 70–75%; lipid, 20–24%; RNA, 0.7–1%; carbohydrate, 5%.[25] Particles of about 1000 Å diameter were found to contain 4.2×10^{-16} gm protein per particle.[26] Assuming a protein content of 70%, this gives a total dry weight of 6.0×10^{-16} gm.

Lipid classes present: The proportions of polar and neutral lipid classes (expressed as per cent of total dry weight of virus) found by Blough *et al.*[17] for the PR8 strain of influenza A are: total lipid, 23.8%; phospholipid, 10.3%; total cholesterol, 7.67%; cholesterol esters, 0.67%; total glycerides, 1.3%.

Size of genome: The viral RNA has been assigned a molecular weight of about 2×10^6.[27] All estimates are rendered difficult by the presence in most viral preparations of incomplete or multigenomic particles, but recent work by Pons and Hirst[28] indicates that the RNA corresponding to one genome normally exists in five or six separate pieces of total molecular weight $2.4–2.7 \times 10^6$. This is obviously not sufficient to code for a large number of late viral proteins; some structural role is therefore likely to be assumed by one or more of the functional envelope proteins.

Protein content: Laver[9] determined that about 38% of viral protein was nucleocapsid and 37% was hemagglutinin. The remainder consisted of neuraminidase, but may also have included some denatured hemagglutinin or nucleocapsid. Molecular weights of 40,000 for nucleocapsid and 120,000 (possibly composed of two units of 60,000 each) for hemagglutinin were determined for these preparations, although more recent work[13] suggests that the molecular weight of hemagglutinin is 150,000. Kendal *et al.*[15] estimated that about 10% of total protein was neuramidase, with a molecular weight of about 220,000. Some doubt exists about the molecular weights of these proteins, depending on the method of isolation. Laver and Valentine[13] determined values of 7.5*S* and 8.5*S* for hemagglutinin and neuramidase, respectively, on detergent-stabilized preparations, which are apparently morphologically identical with surface projections of the intact virus. However, Eckert[10] has obtained envelope subunits as small as 2.2*S*, and in both cases[10, 13] reaggregation was detected on removal of the stabilizing agent.

Data used for calculations: Particles are found by electron microscopy to fall roughly into the spherical or filamentous classes; spherical or near-spherical, bean-shaped particles are typically 800–1200 Å in diameter, whereas filamentous particles may be of similar width but 1 μ or more in length.[5, 6] Since most analyses have been performed on preparations of spherical strains, we shall confine our attention to these. For subsequent calculations, we shall use the following values: spherical particles, over-all diameter 1000 Å; visible spike length 100 Å; spike diameter 40 Å;[13] spike spacing 65 Å, giving about 550 spikes per particle;[29] envelope thickness 90 Å, giving inner and outer envelope diameters of 620 and 800 Å, respectively; protein content 70% of dry weight,[26] of which 28% is nucleocapsid and the remainder hemagglutinin and neuraminidase in a ratio of about 4 to 1;[30] lipid content 20%, consisting largely of 10% polar lipids (mean mol wt 775) and 8% neutral lipids (cholesterol, mol wt 389), giving a cholesterol/phospholipid molar ratio of 1.5 and a mean molecular weight of 543; mass of protein and lipid per

particle, 4.2×10^{-16} gm and 1.2×10^{-16} gm, respectively; partial specific volume of proteins (of unknown degree of hydration), 0.7 cc/gm.

Earlier Models of Envelope Structure. Kates *et al.*[18] proposed a model in which lipid was used as an amorphous cement both in the ribonucleoprotein of the core and between envelope proteins, apparently ignoring both physical dimensions and amphipathic nature of the lipids. Hoyle[24] concluded from experiments on ether-extracted virus that the internal space not occupied by ribonucleoprotein was filled with hemagglutinin in a "gel-form," and that spikes of this projected through a lipoprotein shell to form the characteristic surface projections. Hoyle has also interpreted a globular substructure seen in some micrographs of negatively stained preparations[3] where stain has penetrated within the envelope, in which there is apparently a one-to-one correspondence of globules and spikes, as evidence that the spikes extend through the envelope region. Examination of the same micrographs suggests to us, however, that each globule underlies and acts as a base for the spikes. More recent studies by Laver[9] also indicate that the amount of hemagglutinin present is unlikely to be so large as to form a "gel" around the ribonucleoprotein within the envelope. Later models, based on positively stained thin sections of virus and proteolytic digestion[31, 32] have included a "nanogranular layer" of globular subunits approximately 40 Å in diameter, which on prolonged digestion disappear, leaving two concentric shells of smaller particles, apparently more resistant subunits of the "nanogranules."

Another model is based on the observation that during the budding process from the cell surface membrane there appears to be continuity of the triple-layered "unit membrane"[33] between the intact plasmalemma and the budding virus.[4] It has been deduced that the fundamental structural unit in the virus envelope is a lipid bilayer of the Davson-Danielli-Robertson type,[33] and that the viral surface antigens "float" on the outer surface of this bilayer, giving the uniform spike spacing remarked upon by Hoyle.[24] The model of Laver and Valentine,[13] although not specifically mentioning a lipid bilayer, is of this type.

All these models, except that of Kates *et al.*,[18] assume that all the viral lipid is localized in the envelope. Let us consider the probable extent of the bilayer formed by the observed quantity of lipid, relative to that required for a 1000 Å spherical particle using the data given above. For the mean area per molecule in a close-packed monolayer or bilayer we take de Bernard's value of 50.3 $Å^2$ for the egg-lecithin/cholesterol system at this molar ratio.[34] 1.2×10^{-16} gm of lipid is then capable of covering 6.7×10^6 $Å^2$ as a monolayer, or 3.35×10^6 $Å^2$ as a bilayer. The mean diameter of the envelope is 710 Å, and the required area of bilayer is hence 1.58×10^6 $Å^2$. Thus there is more than twice the amount of lipid required to form a lipid bilayer within the envelope. A similar calculation for the paramyxovirus SV5 shows that this also contains twice as much lipid as can be accounted for by a bilayer model.[20] It should be noted that many of the values used in this calculation are open to question as leading to too small an estimate of the total lipid area; the mean area per molecule will be larger if the cholesterol/phospholipid ratio is lower (as in a number of strains other than PR8), or if the repulsive effect of the charged groups in acidic phospholipids such

as phosphatidyl ethanolamine is taken into consideration. We have also considered only 20 per cent lipid content, although figures as high as 26 per cent have been recorded.[16]

Ji and Benson[35] have shown that spinach chloroplast proteins (mol wt ca. 23,000) are capable of binding up to about 15 phospholipid molecules per protein molecule. We estimate the total molecular weight of nonspike envelope protein to be 6.0×10^7, and if comparable with chloroplast protein this would bind 5.1×10^{-17} gm of lipid per particle, out of a total lipid content of 1.2×10^{-16} gm. It is therefore just possible that the lipid not used in forming a bilayer within the envelope could be bound hydrophobically to envelope proteins. However, if these lipoprotein complexes were distributed on either side of the bilayer, we should observe a much thicker envelope by electron microscopy. The thicknesses of lamellar protein-lipid bilayers are found to be in the region of 60 to 70 Å.[36] The volume of nonspike envelope protein is about 1.07×10^8 Å^3 per particle. This gives a protein layer about 33 Å thick on either side of the bilayer, and an envelope thickness of about 130 Å, much thicker than actually observed. Again, if the spike proteins are embedded in a lipid matrix (presumably replacing one half of the lipid bilayer in some regions) as suggested by Laver and Valentine,[13] even less nonspike protein would be accommodated. Other objections to the bilayer hypothesis can be made, relating to the difficulty of transporting viral proteins to the outer side of such a membrane during assembly. An assembly system involving local enlargement of the cell membrane is easier to visualize if it involves the successive insertion of preassembled lipoprotein building blocks[37] into a region composed of similar units, rather than the transport of individual protein molecules through a hydrophobic region, since this latter process is thermodynamically unfavorable.[38] Electron micrographs showing continuity of the "unit membrane" from the host cell membrane into the viral envelope during budding[4] must be treated with caution; the localization of stain in these preparations is still ill-defined,[39] and Robertson[40] has shown that tilting of the section during observation can make globular structures give a trilamellar appearance. Others have suggested that the lipid undergoes a phase change into the lamellar form on preparation for microscopy.[41]

Models Involving Lipoprotein Complexes. Our inability to account for the observed lipid content by a bilayer structure led us to consider a number of other possible structures, all based on a protein building block extending through the envelope and carrying the spikes. The problem is hence one of determining a way of distributing the lipid and protein to fit the observed dimensions of the envelope and to conform to the required number of structural subunits. The models considered below correspond to the lettered segments of Figure 1.

(*a*) Modified Benson model: Benson[42] has suggested a structure in which the basic units are protein, with lipid molecules inserted between adjacent loops of polypeptide and bound hydrophobically between nonpolar regions of protein and lipid. Polar groups of the lipid lie on the surface of the protein unit and contribute to its surface charge. Where such structures form an extended sheet or membrane, binding between adjacent units may be largely by nonpolar forces between externalized hydrophobic regions of protein. Lipid would then largely be

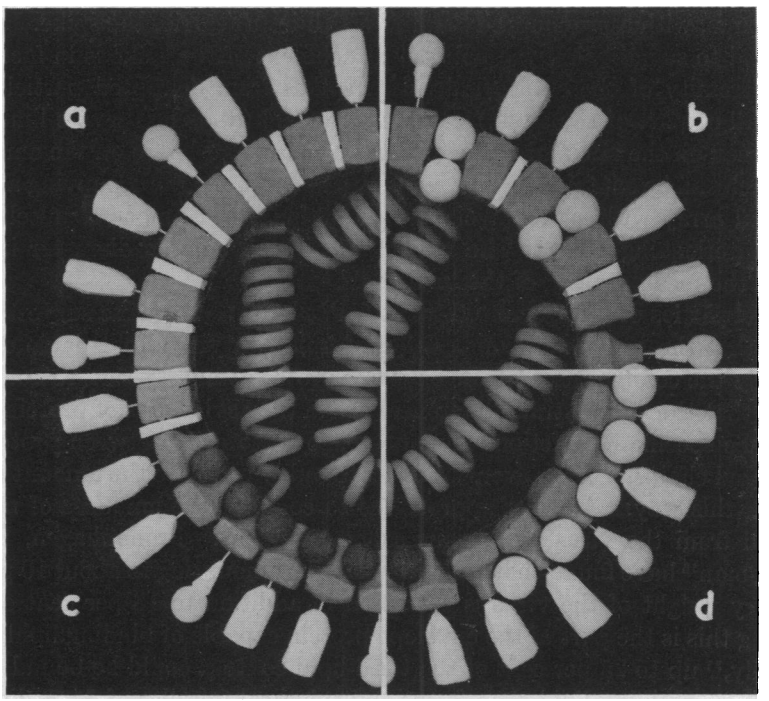

FIG. 1.—A model of the influenza virus envelope showing the possible lipoprotein subunit structures (*a*) to (*d*) referred to in the text. Protein units are grey, separated by lipid bilayer (white spacers) or spherical micelles (white or grey spheres). The two types of spike are intended only as rough representations of hemagglutinin and neuraminidase, and not necessarily in the correct ratio.

inserted from the free membrane surfaces, and by electron microscopy give a unit membrane appearance. However, we have calculated that nonspike envelope protein is capable of binding at most half the available lipid in this way. If 90 Å thick, the 550 protein units can take the form of cylinders about 52 Å in diameter, leaving about 40 per cent of the envelope surface unoccupied, capable of accommodating only part of the remaining lipid.

(*b*) **Micellar Benson model:** If certain of the areas of lipid bilayer in model (*a*) are replaced by small spherical lipid micelles two deep, considerably more lipid can be incorporated, since two such micelles would incorporate approximately four times as much lipid as bilayer occupying the same area of membrane surface. The 40 per cent of envelope not taken up by protein could then be divided almost equally between bilayer regions and micellar regions. Although capable of accounting for all or most of the components present, this would require a wider spacing of spikes than is normally observed.

(*c*) **and** (*d*). **"Toadstool" and "inverted toadstool" models:** These represent a further modification which seems capable of fulfilling many of the requirements mentioned above. Some of the lipid is bound hydrophobically with envelope protein, and the rest is in the form of spherical micelles either on the inner (*c*) or outer (*d*) face of the envelope. The protein units thus have a "toad-

stool" shape, with a pronounced "stalk" which in (*d*) carries the spike. (We do not intend to suggest that the point of attachment of the spike is in fact at the outer extremity of the stalk; individual spikes from disrupted particles may include all or part of the stalk.) We favor model (*d*) as less severe limitations are imposed on the size and shape of lipid micelles, and hence on the amount of lipid which can be contained. We envisage the protein units occupying a largely hexagonal array within the envelope,[43] with the same number of micelles in a similar interlocking array. The maximum possible micelle diameter is hence equal to the mean spacing of the toadstools.

Characteristics of the "inverted toadstool" model: We have suggested elsewhere[45] that the acyl chain composition of influenza virus lipids is determined in part by the configuration of the envelope proteins, and it seems reasonable to suppose that the major strain differences are in the acyl chains of protein-bound lipid, since specific interactions have been shown to exist between protein and certain acyl chains.[45] However, we cannot readily estimate the amount of lipid bound in this way. Another approach is to consider the amounts of material extracted from the virus by various solvent systems. Frommhagen, Knight, and Freeman[16] have found that a protein fraction amounting to about 10 per cent of the dry weight of the virus is extracted in addition to 18 per cent of lipid. Assuming this is the part of the envelope protein capable of binding lipid hydrophobically,[35] up to 25 per cent of the total lipid content could be bound to protein. The remaining lipid then forms 550 spherical micelles of 54 Å diameter. The volume available in the envelope for protein is then about 9.8×10^7 Å,^{3} whereas the volume calculated from the mass of nonspike envelope protein is 1.07×10^8 Å.^{3} This may be taken as fair agreement, since we had no definite values of partial specific volume. We have also considered the possibility that the micelles project slightly from the envelope; it is found in electron microscopy of negatively stained virus preparations[46] that the stain penetrates so deeply around the bases of the spikes that the attachment points cannot be seen, and the spikes appear separated from the envelope by about 10 Å. Such a 10 Å projection of micelles, as indicated in Figure 1(*d*), still retaining the envelope protein thickness of 90 Å, would permit deep penetration of negative stain around the spikes, and better agreement with the calculated volume of envelope protein.

From the volumes of toadstool and spike, we calculate that the molecular weight of the toadstool is about 167,000 and that of the spike is about 108,000. Laver and Valentine's[13] most recent estimate for the molecular weight of isolated hemagglutinin is about 150,000, suggesting that the spike they visualize includes the toadstool stalk, giving a total length of about 140 Å and mol wt about 160,000. If the stalk is attached to the toadstool base by protein-protein hydrophobic interaction (as in the membrane model proposed by Green and Perdue[47]), this might explain Laver and Valentine's observations of solubilization by sodium dodecyl sulfate and reaggregation of the isolated spikes on removal of detergent.[13] It also seems likely that the isolated neuraminidase units, if occupying separate spikes in the intact virus, incorporate some part of the toadstool stalk. A spike of the dimensions given for the neuraminidase unit[13] has never

been observed by electron microscopy of intact virus particles, possibly implying that the 50 × 85 Å "head" may be differently oriented before extraction (e.g., as a spike 50 Å wide and 85 Å long, with the narrow fiber lying alongside and partially coiled underneath and, together with the 40 Å terminal globule, forming all or part of the toadstool stalk). Extraction with detergent may permit unfolding into the "dumb-bell" configuration, and the molecule apparently does not revert to its original form on removal of detergent. The molecular weight, from the dimensions given, appears to be about 187,000, that of the head alone being 130,000.[13] On protease treatment of whole virus, Kendal *et al.*[15] showed release of active neuraminidase of molecular weight about 200,000; in this case part of the toadstool base may also be released.

The inverted toadstool model can explain some of the effects of staining for electron microscopy, both with and without protease treatment. It seems likely that regions of protein complexed with lipid will both accept metallic stains less readily, and be more resistant to proteolytic digestion, than lipid-free proteins. Thus the "nanogranules" described by Apostolov and Flewett[31] may represent the toadstool bases, with poor definition of the stalks, and the double granular layer following digestion[32] indicates the regions of the bases stabilized by associated lipid. These are probably at the vertices between adjacent toadstools, rather than the base of the toadstool. Under appropriate conditions of staining, an apparent bilayer structure may be produced.

Different strains of influenza virus vary in their resistance to agents such as lipid solvents, lipases, and proteases, indicating that our postulated structure must incorporate some flexibility of composition to explain these differences. Influenza virus is partially disrupted by treatment with ether, probably largely by removal of the micellar lipid, but enough remains for parts of the envelope to retain local continuity and form rosettes.[8] Extraction by chloroform-methanol removes much of the hydrophobically bound lipid, except that which is most strongly bound or least accessible, for which ethanol extraction is required.[17, 44] We have not hitherto considered a role for glycolipids in the viral envelope. These may be incorporated into the micelles and protect the exposed lipid surfaces from attack by lipases through a shielding action of their bulky polar regions. Similarly, protease activity may be resisted by some regions of hemagglutinin such as the carbohydrate moiety,[16, 25, 48] or by specific regions of the structural proteins complexed with lipids.

The morphology of viral envelopes constructed according to the toadstool model is sensitive to changes in either the lipid classes or the acyl chain composition. Recent work[49, 50] shows that the lipids of incomplete virus differ from those of normal virus in containing a higher proportion of acidic phospholipids and fewer medium- and long-chain saturated acyl groups. Changes of this type can lead to a more irregular conformation of lipid within the micelles and weaker binding between lipid and proteins, resulting in a more fragile and pleomorphic particle.

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