Myxovirus Envelope Proteins: A Directing Influence on the Fatty Acids of Membrane Lipids

Abstract. *Acyl chain compositions of the lipids of three strains of influenza virus show differences not anticipated from current theories of myxovirus assembly. Fatty acids of viruses with antigenically related envelope proteins show greater resemblance than those of an unrelated strain, which suggests that these proteins influence the composition of membrane lipids at the site of viral release.*

Analysis of the lipids from influenza B, the subtypes A_0 and A_2 of influenza A, and uninfected host-cell membrane fragments reveals differences of fatty acid composition not readily explained by current theories of myxovirus maturation (*1*). We suggest that the strain-specific envelope proteins of the virus are responsible for directing the species and amounts of lipids incorporated into the viral envelope. Benson and other workers (*2*) have suggested a model for membrane structure which consists of lipoprotein complexes in which the genetically controlled amino acid sequence (and hence tertiary structure) of membrane proteins determines the nature of the fatty acyl chains most stably bound into the membrane; such a specificity has also been demonstrated for mitochondrial structural protein (*3*). We consider that the myxovirus envelope offers a unique example of this type of specificity in an in vivo system.

Myxoviruses such as influenza consist of a loosely coiled ribonucleoprotein helix surrounded by a lipoprotein envelope, the lipid of which is derived from the host (*1*); embedded in the envelope are surface projections or spikes that contain strain-specific proteins (hemagglutinin and neuraminidase) (*4*). Influenza virus propagated in chorioallantoic membranes *in ovo* is released from the allantoic cell surface by a budding process (*5*). Examination by electron microscopy of whole mounts of cells infected with influenza virus shows spike-like projections on the cell surface at the site of release, prior to and during budding (*6*).

Salem (*7*) has shown that summation of dispersion forces between two adjacent hydrocarbon chains may lead to a total interaction of the same order of magnitude as ionic or hydrogen-bonding forces, and that this can rank among the major forces of cohesion of membrane components. Although developed for a lipid-bilayer membrane model (*8*), this type of calculation may be adapted to the case of binding between lipid chains and hydrophobic regions of proteins as in Benson's model (*2*). The magnitude of this van der Waals' attraction between nonpolar groups is very sensitive to changes in their separation (*7*) and is considerably altered by bending or twisting of acyl chains, as occurs with polyunsaturated fatty acids (*9*). Hence we may use the proportions of saturated, monounsaturated, and polyunsaturated fatty acid chains in the viral lipid extracts as a measure of the forces of cohesion within the membrane (that is, during viral assembly), without a more detailed analysis of the protein or lipid moieties.

The strains A_0/PR8/34, A_2/Jap/305/57 (*10*), and B/Lee/40 of influenza virus were propagated in the allantoic cavity of 11-day-old embryonated eggs. Eggs were inoculated with 100 EID_{50} (egg infective dose, 50 percent effective) of virus and the allantoic fluid harvested after incubation for 48 hours. The virus was purified by adsorption to and elution from a slurry of barium sulfate (*11*), followed by two cycles of differential centrifugation and, finally, banding on a preformed gradient of potassium tartrate (*12*). Isotope dilution studies show a very low level of contamination by cellular material after this purification procedure (*12*). The normal cell particles (NCP) described by Hoyle (*13*) were also prepared from the chorioallantoic membrane of 14-day-old uninfected embryonated eggs and purified by differential centrifugation (*14*). These particles consist largely of cell membrane surrounding fragments of cytoplasm; their lipid composition may be taken to represent that of the uninfected cell membrane. Lipids were extracted from lyophilized virus and NCP with a mixture of chloroform and methanol (2:1, by volume) (*15*), followed by extraction of the residue with boiling ethanol (*12*); the pooled extracts were separated into the neutral (NL) and polar (PL) lipid classes by silicic acid column chromatography (*16*). The methyl esters of the acyl groups in each of these classes were formed by transesterification with a mixture of boron trichloride and methanol (*17*).

The proportions of total saturated, monoenoic, and polyenoic acids are summarized in Table 1. Subtypes A_0

Table 1. Fatty acid composition of three strains of influenza virus and normal uninfected cell particles. Methyl esters of acyl groups from polar lipid (PL) and neutral lipid (NL) fractions were separated on 6-foot (1.8-m) columns of 17 percent ethylene glycol succinate polyester or 15 percent diethylene glycol succinate polyester on Anakrom ABS at 180°C; a Barber-Colman 5000 gas chromatograph with flame ionization detector was used. Detector response was determined by standard methyl ester mixtures (Applied Science Laboratories); peak areas were measured by planimeter. Results are expressed as percentage composition and are the mean of duplicate determinations, except for A_0 PL, which is the mean of four determinations made with three different methylation techniques. Corresponding peak areas in duplicate determinations differ by about 10 percent. Fatty acids are designated by the number of carbon atoms and the number of double bonds they contain. No attempt was made to use eggs from a single breed of hen; the four determinations made on A_0/PR8/34 were carried out over a 2-year period and presumably some variation of flocks took place in this time, but little difference was found in the analyses. Trace (Tr.) indicates less than 0.5 percent; N.D., not detected.

Acyl group	Strains of influenza virus						Normal cell particles	
	A_0/PR8/34		A_2/Jap/305/57		B/Lee/40			
	PL	NL	PL	NL	PL	NL	PL	NL
12:0	Tr.	N.D.	1.0	5.8	N.D.	14.2	1.5	N.D.
14:0	1.6	4.1	3.1	16.8	Tr.	14.8	3.5	2.8
16:0	15.1	28.5	17.5	23.7	20.9	25.0	23.2	22.3
16:1	5.2	6.6	1.1	3.2	0.8	1.1	3.1	5.9
18:0	14.8	18.8	9.7	6.2	16.9	16.9	17.3	8.5
18:1	15.8	22.0	20.5	10.3	17.0	9.6	17.1	35.5
18:2	4.3	4.6	7.0	0.9	4.2	6.5	3.9	12.3
18:3	0.9	Tr.	Tr.	0.8	2.8	4.8	1.1	0.6
20:0	8.4	6.1	5.0	3.1	3.9	1.9	0.9	0.9
20:1	Tr.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:4	4.2	4.9	14.4	14.1	13.4	1.6	12.9	4.0
22:0	13.7	4.4	9.6	4.7	7.3	2.0	1.2	Tr.
22:1	Tr.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
22:polyene	8.4	N.D.	1.1	5.6	N.D.	N.D.	6.5	3.7
24:0	6.8	Tr.	8.1	1.0	9.0	0.5	1.6	2.8
24:polyene	Tr.	N.D.	1.8	N.D.	N.D.	N.D.	N.D.	N.D.
Uncharacterized	0.6	—	—	3.9	3.8	1.1	6.3	0.5
Saturated	60	62	54	61	58	75	49	37
Monoenoic	21	29	21	14	18	11	20	41
Polyenoic	18	9	24	21	20	13	24	21

and A_2 have roughly similar total contents of saturated chains in both PL and NL fractions, and similar monoenoic contents in the PL fraction, but the PL fraction of A_2 is richer than that of A_0 in polyenoic acids. In addition, the ratio of monoenoic to polyenoic acids of NL is much higher for A_0 than for A_2. The PL fraction of influenza B has about the same level of total saturates as A_0 and A_2, but the NL fraction has much more, and the monoenoic levels are lower for both PL and NL fractions of influenza B than they are for either of the other viruses. The NCP shows much lower levels of total saturates than any of the viruses do and a much higher NL monoenoic level. It is possible that the NL fraction of the NCP is contaminated by egg-yolk lipids which contain large amounts of unsaturated acyl groups [notably 18:1 (18 carbon atoms: 1 double bond)] in the form of triglycerides, cholesterol esters, and free fatty acids. It is also not clear whether the fatty acid composition of NCP is representative of the overall host surface membrane, or whether localized and possibly transient fluctuations occur which may be related to release of NCP.

The greatest differences between the viruses are found in the NL fraction. The yield of lipid in this fraction is substantially increased after extraction with boiling ethanol; this suggests that some neutral lipid molecules are more strongly bound into the viral envelope, and the wide variations in NL fatty acid composition reflect distinct differences in the nature of these strong binding sites in each of the viruses.

Peptide maps of tryptic digests of the nucleoprotein (nucleocapsid) of two strains of type A influenza virus show but a single difference, whereas the surface antigens, for example, the hemagglutinin, of strains of the same type show many differences (*18*). Immunological cross-reactivity is not found for either nucleoprotein or surface antigens of types A and B influenza virus. We feel the observed variance in fatty acid composition reflects differences in the conformation, dependent upon the amino acid sequence, of the envelope proteins. If the nucleocapsid were the directing agent, we would expect similar compositions for A_0 and A_2; if no direct selection of lipids took place during formation of the envelope, all three viruses and NCP should have the same composition.

Since the viruses contain the same range of acyl groups as do NCP, the mechanism of selection of lipids may involve either rearrangement of existing membrane lipids in the vicinity of the area bearing viral envelope proteins, or exchange of acyl groups to achieve a more sterically favorable conformation of lipid molecules. The possibility exists that viral infection induces a specific enzyme (for example, an acyltransferase) that could produce these changes. Recent work has shown that many molecular species of lecithin exist in a single cell (*19*). It is possible, therefore, that a sufficient variety of lipids is already present in the cell to permit assembly of different enveloped viruses.

JOHN M. TIFFANY
HERBERT A. BLOUGH
Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia 19104

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Survival of Germfree Rats without Vitamin A

Abstract. *Weanling, germfree rats, transferred to a conventional animal room and fed a vitamin A-deficient diet, died in 23 to 54 days. In contrast, their littermates, kept germfree and on the same diet, survived for as long as 272 days. The rats kept in germfree conditions stopped growing after 1 to 4 months but responded to supplements of retinoic acid.*

In a previous study of vitamin A deficiency in the germfree rat, Beaver (*1*) noted no difference in the survival time of deficient rats, whether in the germfree or non-germfree condition. In contrast, we have found that germfree, vitamin A-deficient rats survive many months after their littermates, placed in a conventional animal room, have died.

A diet deficient in vitamin A (*2*) was fed to Sprague-Dawley strain females, kept in the germfree production unit of the National Institutes of Health, (i) at the beginning of pregnancy, and (ii) during the lactation period when the young were 10 days old. The young rats were weaned when they were 21 days old, and some were transferred to a metal germfree isolator, while the others (termed ex-germfree rats) were placed in a conventional animal room. All rats were caged singly on raised wire floors with free access to food and water. The vitamin A-deficient diet of the ex-germfree rats was steam sterilized under the same conditions (30 minutes at 121°C) as the diet fed to the germfree rats. Positive control rats received the same diet, with the addition of stabilized retinyl acetate (6 mg/kg).

In the first experiment [young rats whose mothers were fed the vitamin A-deficient diet at the beginning of pregnancy], both germfree and ex-germfree rats fed the vitamin A-deficient diet had slower rates of weight gain than the vitamin A-supplemented rats by the fourth week. The ex-germfree male deficient rats reached a weight plateau in 32 to 35 days (Table 1) and all were dead by 46 days. In contrast, the germfree male deficient rats continued to gain slowly. These rats, after about 70 days, developed nervous symptoms characterized by head wobble, slow gait, and hind-leg weakness. The latter was shown by the rat's difficulty in righting itself when placed on its side. This condition became more