*INCORPORATION OF BRANCHED-CHAIN FATTY ACIDS INTO MYXOVIRUSES**

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Abstract and Summary.—The incorporation of free fatty acids into myxoviruses was shown, using branched-chain fatty acids as molecular markers. The presence of isostearic or phytanic acid was detected by gas-liquid chromatography in the phospholipid fraction of the A_0/PR8/34 strain of influenza virus. Uptake of free fatty acids into the virus varied from 8 to 11 per cent and was accompanied by a shift in the fatty acid profile. Infected allantoic fluids from eggs treated with branched-chain acids possessed higher hemagglutinin activity when compared to fluids infected under normal conditions. Attempts to detect branched-chain acids in Sendai virus were unsuccessful. Shifts in acyl chain composition persisted after three passages of modified viruses in the absence of branched-chain acids. Force-area curves at an air-water interface revealed the cross-sectional area of branched-chain acids to be greater than their straight-chain homologs. It is suggested that hydrophobic interactions can alter the configuration of envelope proteins. Such changes may have an important role in the selection of fragments of influenza viral genome and can conceivably alter viral genotype.

Myxoviruses such as influenza and Sendai viruses acquire their lipoprotein envelope at the surface membrane of the host cell by a process analogous to budding.[1, 2] The lipids of these viruses comprise 20–28 per cent of the total weight of the virion,[3, 4] and it has been suggested that the composition of these lipids is determined by viral envelope proteins, environmental factors, and the biosynthetic properties of the host cell.[3–5] It has been established that, with the exception of phosphatidic acid, the glycerophosphatide moiety of phospholipids of the virion is derived from preformed host cell elements,[6] but the origin of the fatty acyl chains has not been ascertained. Studies in this laboratory have shown that vitamin A alters the phenotype of enveloped viruses and this is accompanied by changes in the phospholipid and acyl chain composition of the virion.[3] To date, specific incorporation of exogenous lipid components into an enveloped virus has not been demonstrated. The purpose of this investigation is to show that branched-chain fatty acids, not normally found in avian membranes,[5] are incorporated into influenza virus. The influence of free fatty acids at an air-water interface will be correlated with the effect they may exert in biological membranes. A preliminary report of this work has been presented elsewhere.[7]

Materials and Methods.—Free fatty acids: 16-Methyl-hexadecanoic acid (MHD) was obtained by alkaline hydrolysis of the methyl ester purchased from Applied Science Laboratories, State College, Pa. 3,7,11,15-Tetramethylhexadecan-1-oic acid (phytanic acid) was obtained from Analabs, Inc., Hamden, Conn. Both fatty acid preparations were found to be >99% pure by gas-liquid chromatography.

Virus: The A_0/PR8/34 strain of influenza virus, a murine strain of parainfluenza 1

virus (Sendai), and the Italy-Milano-1945 strain of Newcastle disease virus (NDV) were used.

Propagation and purification of virus: 50 μg of either MHD or phytanic acid were inoculated intra-allantoically into 150 eleven-day-old embryonated eggs. These eggs were inoculated with 100 EID_{50} of influenza virus 4–5 hr later. Virus grown in the absence of branched-chain fatty acid was used as a control. Influenza virus was purified by adsorption to and elution from a slurry of $BaSO_4$, followed by two cycles of differential centrifugation and banding in a preformed gradient of potassium tartrate.[3] Isotope dilution studies with [^{32}P] have shown that contamination by host cell material was less than 1% by this method.[3]

Analyses of fatty acids: Virus lipids were extracted with $CHCl_3$-CH_3OH (2:1, v/v) and boiling ethanol; the neutral lipids and polar lipids were separated by silicic acid column chromatography. These fractions were methylated by catalytic transesterification with BF_3- or BCl_3-CH_3OH.[3] The fatty acid methyl esters were assayed by gas-liquid chromatography (GLC) on 6-ft columns of 15% ethylene glycol succinate polyester (EGS) or 3% Apiezon-L on Anakrom ABS in a Barber-Colman 5000 instrument with a flame ionization detector.

Biological properties: Infectivity, hemagglutination, and neuraminidase were assayed as previously described.[8]

Electron microscopy: Purified virus particles were examined by the negative contrast technique, with 2% ammonium molybdate at pH 6.8, in a Siemens Elmiskop at an accelerating voltage of 80 kv and with an instrumental magnification of 40,000×.

Surface spreading of free fatty acids: The lowering of surface tension at an air-water interface due to an oriented monomolecular film of free fatty acid was measured by the Wilhelmy dipping-plate technique.[9] The aqueous phase was permanganate-distilled water or 0.01 *N* HCl and measurements were done at 25°. Surface pressure, numerically equal to the lowering of surface tension, was determined as a function of the area per molecule in the spread film.

Results.—Analyses of the phospholipid fraction of influenza virus grown in the presence of branched-chain fatty acids revealed incorporation of both MHD and phytanic acid into the virion (Table 1). The incorporation of these fatty acids into the virion was accompanied by a shift in other acyl chains. MHD led to an increase in monoenoic ($C_{16:1}$ and $C_{18:1}$) and dienoic acids as well as short-chain saturated fatty acids ($C_{12:0}$), with a concomitant decrease in long-chain polyunsaturated fatty acids ($C_{20:4}$ and $C_{22:6}$). Influenza virus propagated in the presence of phytanic acid had a decrease of most long-chain fatty acids (Table 1). Attempts to detect the uptake of phytanic acid into a member of the paramyxovirus group (Sendai virus) under similar conditions were unsuccessful. To test the effect of passage on the biological properties and chemical composition of influenza virus, PR8 initially propagated in the presence of MHD (PR8-MHD) was submitted to three additional passages *in ovo* in the absence of MHD (MHD-P_0^3). It can be seen (Table 1) that the acyl chain composition of MHD-P_0^3 did not resemble that of the first passage material (PR8-MHD) or control virus. Residual MHD was not detected in MHD-P_0^3, although distinct shifts in the fatty acid profile of this virus were evident; especially prominent were large increases in medium- and long-chain unsaturated acids (Table 1).

Influenza virus propagated in the presence of MHD or phytanic acid was pleomorphic; in contrast to virus grown in the presence of vitamin A, filamentous forms were rare. Pretreatment of eggs with phytanic acid or MHD resulted in increases in hemagglutinating (HA) activity for influenza virus; infectivity and

TABLE 1. *Acyl chain composition of phospholipid fractions of A_0/PR8/34 strain of influenza virus grown in the presence of iso-stearic acid (MHD) and phytanic acid.**

Fatty acid	Percentage Composition			
	PR8-Control	PR8-MHD	PR8-MHD-P_0^3†	PR8-Phytanic
12:0	—	5.7	0.4	Trace
14:0	1.6	5.6	Trace	3.3
16:0	15.1	23.9	18.1	19.4
16:1	5.2	3.0	0.8	1.3
MHD	—	7.7	—	—
Phytanic	—	—	—	11.4
18:0	14.8	13.3	13.4	25.6
18:1	15.8	21.7	24.1	17.3
18:2	4.3	5.3	7.7	8.2
18:3	0.9	—	—	—
20:0	8.4	2.3	4.6	2.2
20:4	4.2	1.3	12.2	2.5
22:0	13.7	5.5	7.5	1.8
22:1	—	—	—	1.0
22:polyene	8.4	2.6	1.3	—
24:0	6.8	2.3	5.4	Trace
24:1	—	Trace	—	—
24:2	Trace	—	—	0.6
Uncharacterized	0.6	—	4.5	5.1

* For the purpose of this paper, fatty acids with a carbon number of 12 or less will be referred to as short; C_{14}-C_{18}, medium; C_{20} or greater are long-chain. Detector linearity and response for fatty acids of different molecular weights were determined with quantitative methyl ester standards (Applied Science Laboratories).

† Virus grown in presence of MHD, followed by three passages without MHD.

TABLE 2. *Biological activity of various myxoviruses grown in presence of branched-chain fatty acids.**

Virus	HA titer per 0.2 ml	Neuraminidase activity (units/mg protein)	Infectivity Log_{10} (EID_{50}/0.2 ml)
Influenza:			
PR8-control	1024–2048	1.03	8.88
PR8-MHD	8196–16394	1.23	8.72
PR8-MHD-P_0^3	16392	1.42	8.65
PR8-phytanic	16392	1.18	8.60
Sendai control	1024	N.D.	9.4
Sendai-phytanic	256	N.D.	9.0
NDV (Milano):			
Control	1024	0.94	8.04†
NDV-phytanic	1024	0.79	8.12†

HA and infectivity were assayed on crude allantoic fluid and neuraminidase on purified virus.

* N.D.: not done; P_0^3 represents third passage in the absence of MHD of PR8-MHD virus.

† PFU/0.2 ml on monolayers of primary chick embryo fibroblasts.

neuraminidase were unchanged (Table 2). Additional passage in the absence of MHD (P_0^3) *in ovo* revealed that the progeny virus possessed higher levels of HA and enzymic activity and were still pleomorphic. With the exception of a slightly reduced HA in Sendai virus, biological activity of paramyxoviruses propagated in phytanic acid was unchanged (Table 2).

Discussion.—This study demonstrates that exogenous-free fatty acids are incorporated into the phospholipids of the influenza virion and this incorporation leads to alterations in the biological properties and morphology of the virion.

This uptake is not surprising, since other workers have shown that free fatty acids, including 3,3,12,12-tetramethylmyristic acid,[10] can be incorporated into mammalian cells of various species.[11, 12]

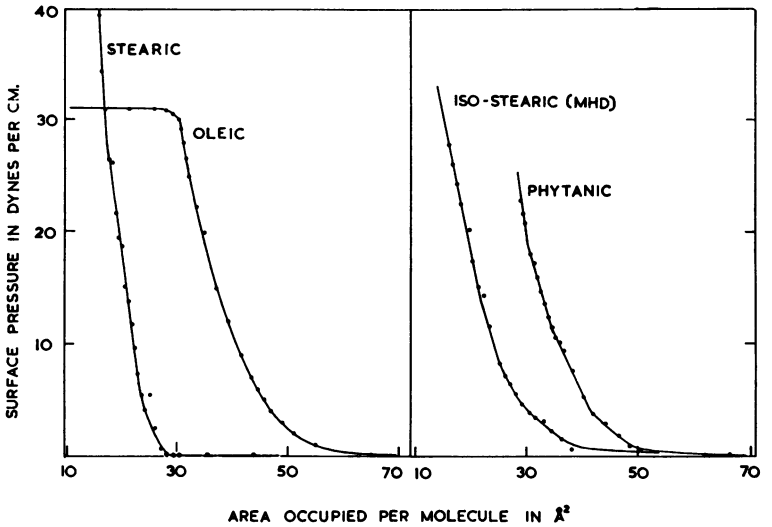

FIG. 1.—Force-area curves of various fatty acids at an air-water interface. Extrapolation to zero surface pressure of the steepest portion of the curves gives the average area occupied by the molecule in its close-packed orientation: stearic acid, 20.5 Å²; iso-stearic acid, 28 Å²; phytanic acid, 36 Å².

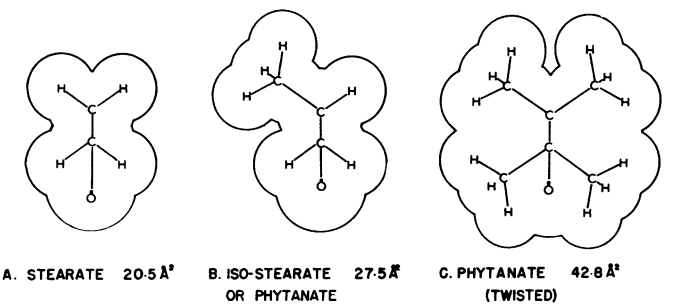

FIG. 2.—Plan projections perpendicular to the long axis of the molecule of three fatty acids,[23] determined from the published data of van der Waals' radii of the atoms.[24] The projection of the zig-zag carbon skeleton is indicated by the line joining the two central carbon atoms. Lines between atoms show bond directions and not valency fulfillments. There is good agreement between these calculated areas and those obtained from the data in Fig. 1 for stearic and iso-stearic acids. Two extreme configurations are shown for phytanic acid; the observed area is 36 Å²/mol, indicating a slight twisting of the chain.

Force-area curves of various fatty acids at an air-water interface (Fig. 1) reveal that branched-chain acids occupy a larger cross-sectional area than their straight-chain homologs and thus behave in a fashion more like unsaturated fatty acids. The projected cross-sectional areas of various fatty acids and the influence they may exert on lipid packing are depicted in a model system of phosphatidylcholine containing various fatty acyl chains (Figs. 2, 3). It can be seen that substitution

of additional CH_3 groups in the fatty acid, twisting of the phytanate molecule, or substitution of unsaturated fatty acids in the phospholipid molecule constitute further obstacles to the compact organization of lipid films (Figs. 2, 3). Physicochemical studies of close-packed films of fatty acids reveal that the energy of

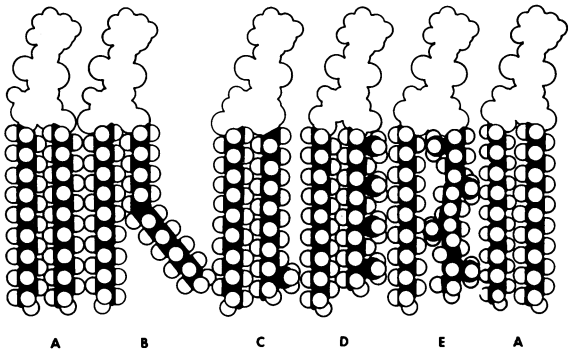

FIG. 3.—The effect of incorporation of branched-chain fatty acids on the packing of a monolayer of a stearoyl-phosphatidylcholine with the following acyl chains in the β-position. (*A*) Stearoyl, (*B*) oleoyl, (*C*) isostearoyl, (*D*) phytanoyl, and (*E*) phytanoyl (twisted).

interaction between two adjacent paraffinic chains is considerably reduced by the addition of a terminally substituted methyl group.[13] Such changes will also reduce the cohesion between acyl hydrocarbon chains and hydrophobic regions of proteins and allow for a more expanded and fluid viral envelope. It is suggested that the inclusion of branched-chain fatty acids may alter certain hydrophobic binding sites (of envelope proteins) in a fashion similar to that of certain organic solvents.[14] Hydrophobic reactions may cause the exclusion of water from certain areas of the peptide chain,[15] and hence change the secondary and tertiary structure of the viral envelope proteins. This fits with the postulate of Green *et al.*[16] that the structure of membranes is determined by hydrophobic rather than by electrostatic interactions and is consistent with the model membrane system composed of lipoprotein complexes as proposed by Benson.[17]

The basis of the high hemagglutinin-infectious particle ratio is at present unknown. We speculate that the altered structure of the envelope affects the biological properties of the virion in one of two ways. First, altered envelope protein may select a population of genomic fragments[18-21] coding for a number of hemagglutinin molecules of increased activity, and on subsequent passage these breed true. If, on the other hand, one assumes that branched-chain acids alter the surface projections of the virion so that additional sites are exposed for HA, this may explain the high hemagglutinin titer observed in infected allantoic fluids of first-passage virus (e.g. PR8-MHD) but not in subsequent passages (P_0^3). Assuming that the influenza genome consists of several small, independently replicating pieces of various sizes,[18-21] it is possible that a small fragment of genome acts as a regulating element in hemagglutinin synthesis (with no apparent effect on infectivity). Changes in the configuration of the envelope proteins might prevent the binding of this genomic fragment to the inner surface of the virion. This would behave as a deletion mutation on subsequent passage and may account for the high hemagglutinin and neuraminidase titers observed in P_0^3. Further evidence that these envelope proteins are altered is apparent from the acyl chain data which have been shown to be a sensitive index of differences in

the structural proteins of the virion.[4, 5] The reason for the failure to detect phytanic acid in Sendai virus is at present unknown, but it may be related to the large percentage of unsaturated fatty acids in that virus[4] and involve competition between these acids and phytanic acid for binding sites.[22]

Summary.—The uptake of free fatty acids into the phospholipid fraction of myxoviruses was studied by gas-liquid chromatography using branched-chain fatty acids as molecular markers. The incorporation of branched-chain acids into the A_0/PR8/34 strain of influenza was accompanied by changes in the fatty acid profile of the virion. Infected allantoic fluids of eggs treated using branched-chain fatty acids had enhanced hemagglutinin activity when compared to fluids infected under normal conditions. Shifts in the fatty acid profile and altered biological activity persisted after three passages of modified virus in the absence of branched-chain acids. Attempts to detect phytanic acid in Sendai virus propagated under similar conditions were unsuccessful. Force-area curves reveal that these fatty acids occupy a larger area than their straight-chain homologs. It is suggested that the incorporation of branched-chain fatty acids alters the configuration of envelope proteins by hydrophobic bonding reactions. Such changes may play an important role in selecting out fragments of the genome and may be responsible for a mutational event.

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[1] Morgan, C., H. M. Rose, and D. H. Moore, *J. Exptl. Med.*, **104**, 171 (1956).

[2] Berkaloff, A., *J. Microscopie*, **2**, 633 (1967).

[3] Blough, H. A., D. B. Weinstein, D. E. M. Lawson, and E. Kodicek, *Virology*, **33**, 459 (1967).

[4] Blough, H. A., and D. E. M. Lawson, *Virology*, **36**, 286 (1968).

[5] Tiffany, J. M., and H. A. Blough, *Science*, in press.

[6] Kates, M., A. C. Allison, D. A. J. Tyrrell, and A. T. James, *Biochim. Biophys. Acta*, **52**, 455 (1961).

[7] Tiffany, J. M., and H. A. Blough, *Federation Proc.*, **27**, 616 (1968).

[8] Blough, H. A., *J. Bacteriol.*, **92**, 266 (1966).

[9] Wilhelmy, H., *Ann. Physik*, **119**, 177 (1863).

[10] Spector, A. A., D. Steinberg, and A. Tanaka, *J. Biol. Chem.*, **240**, 1032 (1965).

[11] Geyer, R. P., A. Bennett, and A. Rohr, *J. Lipid Res.*, **3**, 80 (1962).

[12] Elsbach, P., *Biochim. Biophys. Acta*, **84**, 8 (1964).

[13] Kavanau, J. L., in *Structure and Function in Biological Membranes* (San Francisco: Holden-Day, 1965), vol. 1, p. 98.

[14] Kauzmann, W., *Advan. Protein Chem.*, **14**, 1 (1959).

[15] Guzzo, A. V., *Biophys. J.*, **5**, 809 (1965).

[16] Green, D. E., H. D. Tisdale, R. S. Criddle, and R. M. Bock, *Biochim. Biophys. Res. Commun.*, **5**, 81 (1961).

[17] Benson, A. A., *J. Am. Oil Chemists' Soc.*, **43**, 265 (1966).

[18] Hirst, G. K., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 27 (1962), p. 303.

[19] Pons, M., *Virology*, **31**, 523 (1967).

[20] Duesberg, P. H., and W. S. Robinson, *J. Mol. Biol.*, **25**, 383 (1967).

[21] Pons, M., and G. K. Hirst, *Virology*, **34**, 386 (1968).

[22] Laurell, S., *Biochim. Biophys. Acta*, **152**, 75 (1968).

[23] Vold, M. J., *J. Colloid Sci.*, **7**, 196 (1952).

[24] Pauling, L., *The Nature of the Chemical Bond* (Ithaca, New York: Cornell University Press, 1960), 3rd ed.