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PHOSPHOLIPIDS IN NEWCASTLE DISEASE VIRUS INFECTED CELLS

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SUMMARY

Infection of chicken cells with Newcastle Disease Virus modifies phosphatidylserine and phosphatidylcholine synthesis in the host cell. The virion contains cellular phospholipids synthesized both before and after infection. Relative concentration of various labeled phospholipids in the virus differ from those in the corresponding cells and their surface membranes.

Late in infection, fragments of membranes with a distribution of labeled phospholipids similar but not identical to that of the virus can be found in the supernatant of infected cells.

The significance of these findings is discussed in relation to the origin of viral phospholipids and the intervention of the host cell membrane in the assembly of the viral envelope.

INTRODUCTION

When Newcastle Disease Virus (NDV) is released by budding from the plasma membrane of the host cell, it has already acquired an envelope. This envelope contains proteins, carbohydrates and lipids. Most proteins of the viral envelope are synthesized by the cell according to specifications provided by the viral genome. The origin of the lipids of the viral envelope is a subject of controversy. According to some authors [1–3] viral phospholipid composition reflects that of the host cell or the host cell membrane. According to others, viral phospholipid composition is determined by viral proteins but does not vary considerably between different viruses [4–7]. Quigley et al. [6] suggested that either the budding of virus during maturation or its subsequent adsorption to the host cells might impose restrictions on the phospholipid composition of the virus and that, therefore, a mechanism which regulates phospholipid composition of the viral envelope might exist. In any case, the phospholipid contained in the virus is synthesized in the cell. Various mechanisms for the transfer of cellular phospholipid to the viral envelope have been proposed: (1) the viral envelope, including its phospholipid, might be derived in toto from the host cell membrane. In this case, the segments of the host cell membrane which make up the

viral envelope, could be: (a) any part of the membrane with a phospholipid composition ressembling that of any other part; (b) a distinct part of the membrane with a phospholipid composition different from that of other parts of the membrane.

In either case, the plasma membrane could be modified or not after the cells were infected. If an indifferent part of the host cell membrane were incorporated into the viral envelope, viral phospholipid composition would reflect that of the cell membrane. Indeed, a viral phospholipid composition reflecting the phospholipid composition of different host cells has been described [3]. The main resemblance was a high sphingomyelin content of both membrane and virus as compared to the whole cell, but the relative amounts of other phospholipid did not vary proportionally. Furthermore, sphingomyelin and phosphatidylethanolamine content of the plasma membrane depended on the method used to prepare the membranes. When the method of Warren and Glick [8] was used, sphingomyelin content was higher and phosphatidylethanolamine content was lower than when the method of Wetzel and Korn [9], as modified by Heine and Schnaitman [10], was used for the preparation of membranes.

On the other hand, phospholipid composition of different viruses grown on the same cell line varied very little and was different from that of the host cell membrane [6].

(2) The phospholipid of the viruses might be "selected" by the viral proteins, either from the cellular phospholipid pool or from the cell membrane. Again, phospholipid composition of the cell or of its plasma membrane might or might not be modified by virus infection. If individual phospholipid were "selected" without modifications of the phospholipid composition of the host cell after infection, viral phospholipid composition would be independent of host cell phospholipid composition. But if host cell phospholipid composition were modified by virus infection, the modification might be reflected in viral phospholipid composition.

(3) Finally, viral envelopes could be made up from both membrane segments and from phospholipid individually selected. If this were the case, there would be no correlation between the phospholipid composition of the host cells or the host cell membranes and that of the viral envelopes.

This report deals with the distribution of labeled phospholipid in chorioallantoic cells, chick embryo cells, in the membranes of these cells and of NDV, at different times after infection and under different labeling conditions.

MATERIALS AND METHODS

Cells and virus

Chorioallantoic membrane cells were obtained from 11-day-old lymphomatosis-free embryonated eggs, cut into small pieces and washed in 0.14 M NaCl. They were then incubated in either a mixture of phosphate-free Eagles medium (45 %) and complete Eagles medium (45 %) supplemented with 10 % dialysed calf serum (phosphate-poor medium) or in complete Eagles medium supplemented with 10 % calf serum and 10 % tryptose phosphate broth (phosphate-rich medium) at 37 °C on a swirling machine [11]. Chick embryo cells were explanted from 11-day-old eggs, cultured in plastic Falcon bottles and passaged once before use. Surface membranes of cells were prepared as described elsewhere [12]. For infection, NDV (Kansas Loevenhorst strain) at a multiplicity of 3–5 p.f.u. was used. Virus was adsorbed for 1 h at 4 °C.

Under these conditions, 90 % of the cells are infected, and 60 % of these cells produce virus during the first cycle [21]. Virus production was monitored by plaque titration on monolayers of chick embryo cells with an agar overlay [13]. Virus was partially purified by precipitation with $(NH_4)_2SO_4$, followed by centrifugation first on a sucrose cushion and then on a continuous sucrose gradient [14]. All material precipitated by $(NH_4)_2SO_4$ which did not sediment with the virus was collected and reprecipitated with $(NH_4)_2SO_4$ for further analysis.

Labeling

In order to distinguish between phospholipids synthesized before and affer infection, we used two labeling procedures.

(1) *Synthesis during infection.* Cells were washed with 0.14 M NaCl, infected and incubated in phosphate poor medium with 15 μCi ^{32}P/ml for 6, 7 or 17 h. Uninfected controls were labeled under the same conditions. In these experiments, biosyntheses of total phospholipids were evaluated by determinations of specific radioactivities. The distribution of newly synthesized phospholipids was determined by thin-layer chromatography and autoradiography.

(2) *Pre-infection synthesis and subsequent distribution.* Cells were incubated for 48 h in phosphate-poor medium containing 10 μCi ^{32}P/ml. They were washed in phosphate-buffered saline and then infected and incubated in phosphate-rich medium for 6, 7 or 17 h. Uninfected controls were labeled under the same conditions. Phospholipids were assayed as described under *Labeling* (1).

Phospholipid extraction and analysis

Phospholipids were extracted with a methanol–chloroform–water mixture (4/2/1; by vol.) according to Folch et al. [15]. The extracts were stored in chloroform at −20 °C for not more than 1 week and chromatographed on thin-layer silica gel plates (Merck) in two dimensions; first in chloroform–methanol–water (65/25/4; by vol.), then in *n*-butanol–acetic acid–water (3/1/1; by vol.). To both solutions 1 mg/ml of butylated hydroxytoluene was added to avoid oxydation of fatty acids. The plates were dried and a radiosensitive film (Kodak Definix Medical) was applied. The spots were located with the autoratiograph, scraped off, and the radioactivity determined in a scintillation liquid. Phospholipids were identified by co-chromatography with chromatographically pure commercial standards (General Biochemicals, Ohio). The standards were revealed by either a rhodamine or a fluorescine spray (Merck).

Specific radioactivity of total phospholipid was determined by evaporation of the chloroform extract and mineralization of the phospholipids according to Bartlett [16]. One aliquot was used to determine P_i according to Chen et al. [17], and a second to determine radioactivity. Protein content was estimated by measuring A at $\lambda = 280$ nm and at $\lambda = 260$ nm [18] with a Zeiss spectrophotometer model PMQ 2.

To obtain statistically significant results, the *t*-test [19] was applied when measured differences were small.

Electron microscopy

An aliquot of the suspension to be analyzed was filtered through a Millipore filter VCWP pore diameter 0.10 μm [20]. The filter was fixed for 1 h in 6 % glutaralde-

hyde, washed in 0.1 M phosphate buffer pH 7.4, fixed in 4 % OsO_4, washed in the same buffer, dehydrated and embedded in Epon. The thin sections were stained with 1 % uranyl acetate and lead citrate, stabilized with carbon and electron micrographs were made with a Philips EM 300 electron microscope [20].

RESULTS

(1) *Phospholipid synthesis in chorioallantoic membrane cells*

Fig. 1 shows the distribution of labeled phospholipids in chorioallantoic cells as a function of time of incubation. During the first 48 h, phospholipid distribution varies, due to different rates of synthesis of the individual phospholipids. At later time, this distribution is stable. Similar results have been described for chick embryo cells by Quigley et al. [6].

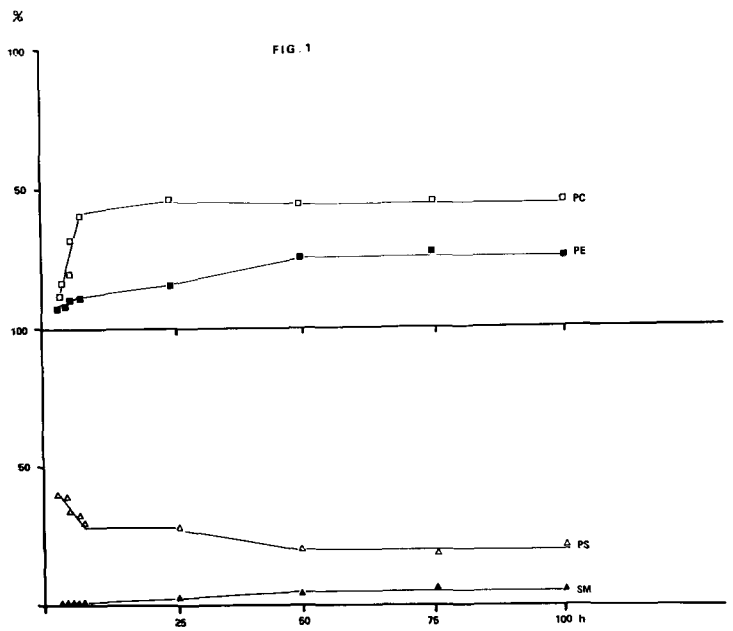

Fig. 1. Distribution of labeled phospholipids (expressed as percent of total cpm) in chorioallantoic membrane cells as a function of the time of incubation. SM, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

(2) *Phospholipid distribution in cells and in NDV labeled during infection*

Fig. 2 shows phospholipid synthesis in infected and uninfected cells, as determined by specific radioactivity of phospholipids. During the first 6 h (chorioallantoic cells) or 5 h (chick embryo cells) after infection there is no significant difference between the amounts of phospholipid synthesized by infected and uninfected cells.

At later times, infected cells synthesize less phospholipid than uninfected cells, presumably because virus producing cells are lysed.

Fig. 3 shows the distribution of labeled phospholipid in cells, at different

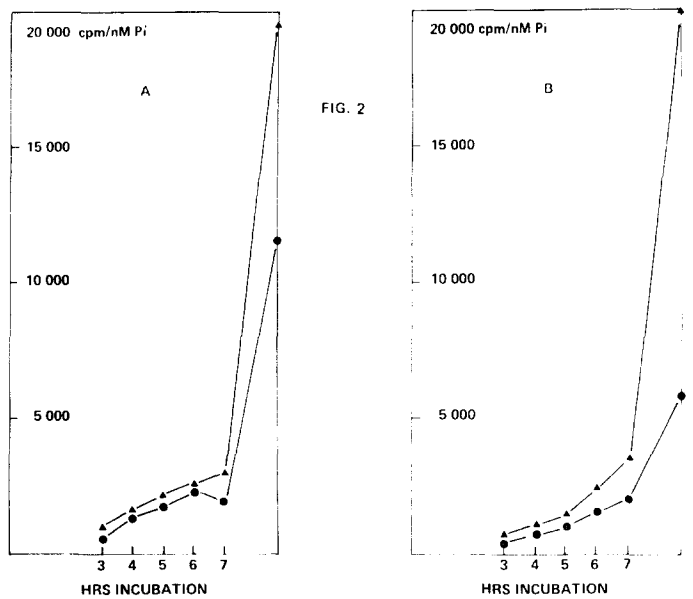

FIG. 2

Fig. 2. Synthesis of total phospholipids in whole cells. (A) Chorioallantoic cells; (B) chick embryo cells. ▲, uninfected cells; ●, infected cells.

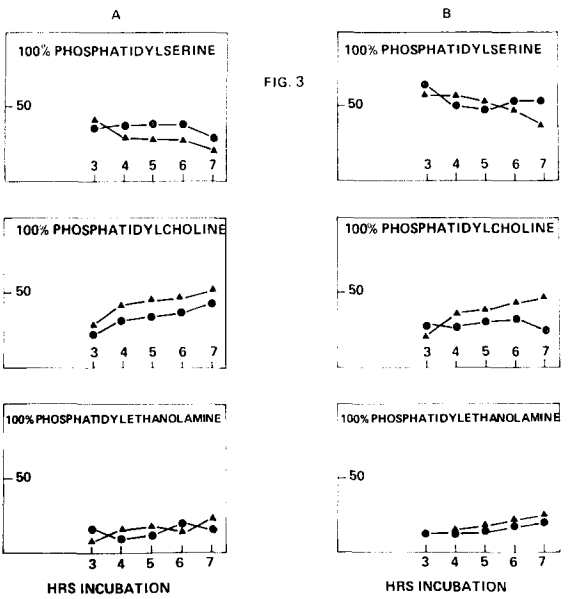

FIG. 3

Fig. 3. Synthesis of individual phospholipids in whole cells. (A) Chorioallantoic cells; (B) chick embryo cells. ▲, uninfected cells; ●, infected cells. %, cpm individual phospholipid/cpm total phospholipid.

times after infection. From 4–7 h after infection, chorioallantoic cells synthesize more phosphatidylserine and less phosphatidylcholine than uninfected controls; infected chick embryo cells synthesize less phosphatidylcholine than uninfected controls.

Distribution of labeled phospholipid was determined in uninfected and in infected whole cells and in their surface membranes 6 or 7 h after infection. Virus release begins at this time, but the cells are not yet lysed. The distribution was also determined 17 h after infection when virus release is terminated and when most infected cells are lysed. These distributions were compared with those of labeled phospholipid in partially purified virus, and in the particulate debris found in the supernatant of infected cells 17 h after infection. Electron micrographs of the material found in the supernatant of infected cells showed that this was essentially membraneous material of either cellular or viral origin (Plate 1). The ratio p.f.u./$A_{280\ nm}$ of this material was 10–50 times lower than the ratio p.f.u./$A_{280\ nm}$ of partially purified virus. At most, 50 % of the membraneous material was clearly of cellular origin, so that at most 50 % may be of viral origin. Supernatants of control cells contained very little cellular material precipitable with $(NH_4)_2SO_4$ and the distribution of labeled phospholipid was the same as that of the uninfected cells.

The supernatant of the $(NH_4)_2SO_4$ precipitable material contained very little phospholipid, and its distribution reflected that of the corresponding cells.

Surface membranes prepared from the cells remaining 17 h after infection had the same distribution of labeled phospholipid as the surface membranes from non-infected cells. This is probably because only inefficiently infected cells remained in the culture.

Tables I and II summarize the results of these experiments. Variation of labeled phospholipid distribution is seen as due to: (a) the cell type, (b) the duration of

TABLE I

Phospholipid distribution before virus release, cells labeled during infection (expressed as percentage of total cpm). A, chorioallantoic cells 7 h after infection; B, chick embryo cells 6 h after infection. Others, phosphatidylinositol, phosphatidylglycerols, phosphatidic acid and lysophosphatidylcholine.

Phospholipid	Whole control cells (%)	Difference with infected cells	Surface membranes from controls (%)	Difference with surface membranes from infected cells
A				
Sphingomyelin	1.4	–	2	–
Phosphatidylserine	20.1	+ ($P = 0.10$)	17.9	–
Phosphatidylcholine	50.5	– ($P = 0.05$)	49.0	–
Phosphatidylethanolamine	17.0	–	15.2	–
Others	11.0	–	15.9	–
B				
Sphingomyelin	1.3	–	2.6	–
Phosphatidylserine	33.2	–	31.4	–
Phosphatidylcholine	39.2	– ($P = 0.10$)	39.7	–
Phosphatidylethanolamine	20.6		20.6	

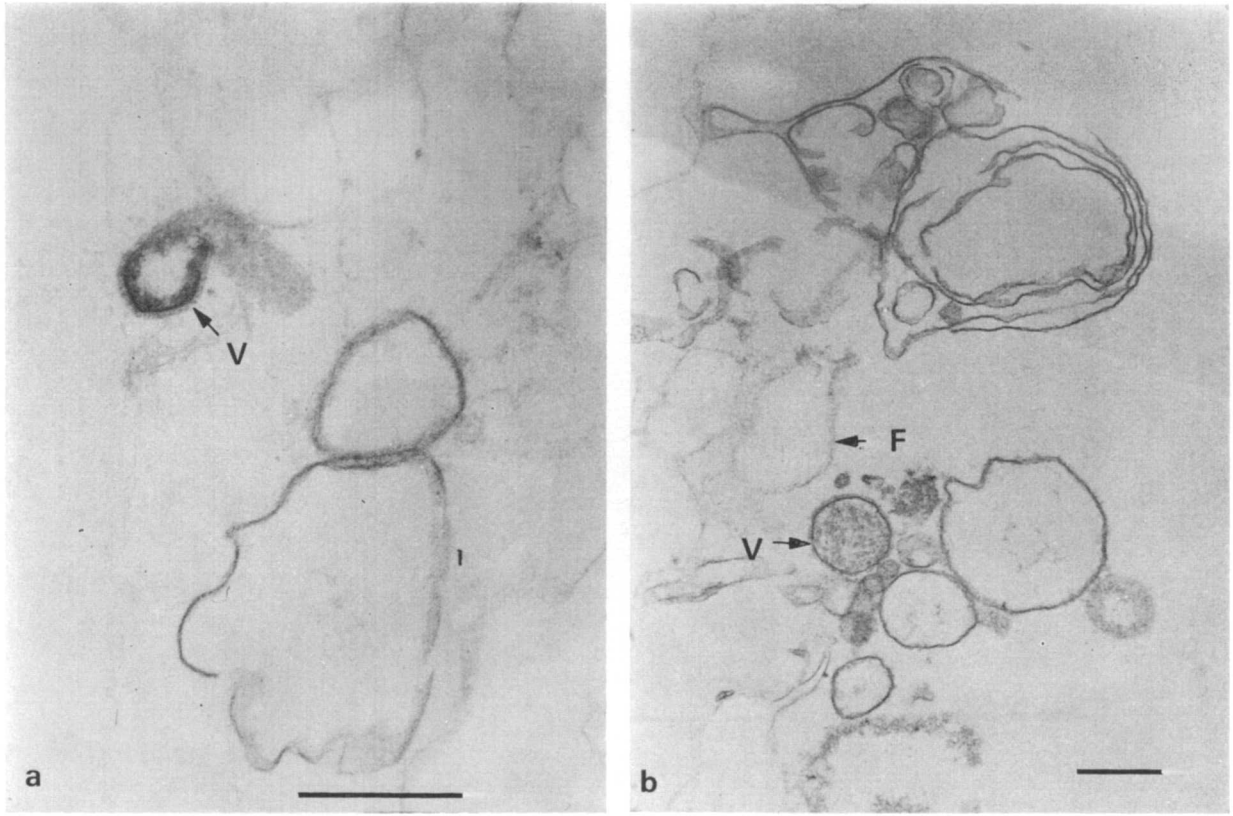

Plate 1. Membraneous material (cf. Material and Methods) retained by a 0.1 μm Millipore filter. Scale lines are 0.25 μm. (a) On the top of the filter, two membraneous profiles adjoin a NDV particle easily distinguishable from the cell membranes by its characteristic structure and the presence of external projections. The internal content of the virus particle is extruding. (b) Membrane vesicles of various size are retained at the surface of the Millipore filter (F), with a NDV particle (V).

TABLE II

Phospholipid distribution (expressed as percentage of total cpm) after virus release, cells labeled during infection. A, chorioallantoic cells 17 h after infection; B, chick embryo cells 17 h after infection Others, phosphatidylinositol, phosphatidylglycerols, phosphatidic acid and lysophosphatidylcholine.

Phospholipid	Whole cells		Uninfected surface membranes (%)	Particulate debris (%)	Virus released (%)
	Control	Infected			
A	(2 Expts)	(3 Expts)	(2 Expts)	(4 Expts)	(3 Expts)
Sphingomyelin	3.4	2.7	4.0	9.4	15.7
Phosphatidylserine	18.0	21.1	16.1	20.5	16.3
Phosphatidylcholine	51.2	49.0	52.4	29.6	25.8
Phosphatidylethanolamine	22.6	21.6	21.7	33.4	34.1
Others	4.8	5.4	5.8	7.1	8.1
B	(1 Expt)	(2 Expts)	(1 Expt)	(2 Expts)	(2 Expts)
Sphingomyelin	2.6	4.5	4.8	2.2	4.3
Phosphatidylserine	15.0	16.0	14.0	18.3	17.2
Phosphatidylcholine	54.6	42.4	49.4	40.3	40.6
Phosphatidylethanolamine	22.4	28.1	24.1	33.8	37.6
Others	5.4	9.0	7.7	5.4	0.3

labeling, and (c) the component examined (whole cells or surface membranes). Early in infection, infected cells synthesize more phosphatidylserine and less phosphatidylcholine than uninfected controls.

Distributions of labeled phospholipids in virus is different from that in cells: virus contains more sphingomyelin and phosphatidylethanolamine and less phosphatidylcholine than the corresponding cells.

Distribution of labeled phospholipid in debris resembles that in virus but is not identical to it. The values for phosphatidylethanolamine are the same in the virus and in the debris, and higher than in the cells, whereas the values for sphingomyelin are higher and those for phosphatidylcholine are lower in the virus than in the debris. This suggests that the debris are not mixtures of unmodified membrane fragments with complete or incomplete virus, because were this the case the values found for each phospholipid would of necessity have a mean value of those for surface membranes and virus.

In Table III two sets of comparisons are indicated, after 17 h of labeling. The specific radioactivity of phospholipid extracted from uninfected membranes is compared with whole cells, and cellular debris is compared with released virus. No significant difference exists between the specific radioactivities of uninfected cells and that of their surface membranes. However the specific radioactivity of virus is higher than that of debris. Thus, as the virus is indeed labeled, then at least some of the phospholipid must have been synthesized during infection, whereas the remaining phospholipids were present in the cell before infection.

(3) *Phospholipids in cells labeled prior to infection*

In these experiments, unlabeled phospholipid newly synthesized after the

TABLE III

Specific radioactivity of phospholipids of (a) membranes and (b) virus, expressed as percent of (a) uninfected cells and (b) cellular debris.

Chorioallantoic cells labeled for 17 h Sample	cpm/nM P
(a) Uninfected cells	100.0
Uninfected surface membranes	92.4
(b) Cellular debris	100.0
Virus released	105.2
Chick embryo cells labeled for 17 h	
(a) Uninfected cells	100.0
Uninfected surface membranes	90.1
(b) Cellular debris	100.0
Virus released	138.2

chase and infection were found to replace labeled phospholipid at a rate characteristic of the phospholipid and of the cell type.

7 h after infection chorioallantoic cell surface membranes contain less labeled sphingomyelin and more labeled phosphatidylserine than surface membranes from uninfected controls (Table IV). In Table V, the phospholipid distribution in infected cells, in their surface membranes in remaining infected cells, in particulate debris and in virus, are compared. Whereas there is no significant difference between uninfected cells, their surface membranes and infected cells, distribution of labeled phospholipids is different in particulate debris and in virus. The sphingomyelin and phosphatidyl-

TABLE IV

Phospholipid distribution before virus release, prelabeled cells (expressed as percentage of total cpm). A, chorioallantoic cells 7 h after infection; B, chick embryo cells 6 h after infection. Others, phosphatidylinositol, phosphatidylglycerols, phosphatidic acid and lysophosphatidylcholine.

Phospholipid	Whole control cells (%)	Difference with infected cells	Surface membranes from controls (%)	Difference with surface membranes from infected cells
A				
Sphingomyelin	3.2		4.1	(P 0.10)
Phosphatidylserine	21.1		14.8	(P 0.05)
Phosphatidylcholine	50.0		55.2	
Phosphatidylethanolamine	20.3		13.0	
Others	5.3		12.9	
B				
Sphingomyelin	4.4		7.6	
Phosphatidylserine	15.8		16.1	
Phosphatidylcholine	42.6		41.5	
Phosphatidylethanolamine	31.7		29.9	
Others	5.5		4.9	

TABLE V

Phospholipid distribution (expressed as percentage of total cpm) after virus release, prelabeled cells. A, chorioallantoic cells 17 h after infection; B, chick embryo cells 17 h after infection. Others, phosphatidylinositol, phosphatidylglycerols, phosphatidic acid and lysophosphatidylcholine.

Phospholipid	Whole cells (%)		Uninfected surface membranes (%)	Particulate debris (%)	Virus released (%)
	Control	Infected			
A	(2 Expts)	(7 Expts)	(4 Expts)	(5 Expts)	(7 Expts)
Sphingomyelin	8.3	6.8	7.7	15.1	14.0
Phosphatidylserine	13.7	15.8	15.8	15.6	18.8
Phosphatidylcholine	46.2	48.5	48.5	33.0	25.7
Phosphatidylethanolamine	25.3	24.8	24.8	34.2	35.0
Others	6.5	4.1	4.1	2.1	6.5
B	(2 Expts)	(5 Expts)	(2 Expts)	(1 Expt)	(5 Expts)
Sphingomyelin	9.4	10.6	11.4	15.8	15.9
Phosphatidylserine	9.2	10.1	14.7	14.1	15.4
Phosphatidylcholine	43.8	42.6	41.2	31.7	24.0
Phosphatidylethanolamine	27.9	29.9	27.1	34.5	38.5
Others	9.7	6.8	5.6	3.9	6.2

ethanolamine contents are higher, and the phosphatidylcholine content is lower in virus and in particulate debris. Particulate debris contains relatively more phosphatidylcholine than the virus, whereas the values for the other phospholipids are equivalent, confirming the corresponding findings in cells labeled during infection.

Table VI shows the specific radioactivities of phospholipids in cells, in surface membranes, in debris and in virus. The specific radioactivities of cell homogenates and surface membranes do not differ, the particulate debris is higher than virus. However, as the virus is labeled this shows that some viral phospholipids had been synthesized before infection, either during the 48 h labeling or before the cells were isolated. When the specific radioactivities of particulate debris and of virus in cells

TABLE VI

Specific radioactivity of phospholipids of (a) membranes and (b) virus, expressed as percent of (a) uninfected cells and (b) cellular debris

Sample	cpm/nM P
Pre-labeled chorioallantoic cells	
(a) Uninfected cells	100.0
Uninfected surface membranes	116.0
(b) Cellular debris	100.0
Virus released	25.7
Pre-labeled chick embryo cells	
(a) Uninfected cells	100.0
Uninfected surface membranes	97.0
(b) Cellular debris	100.0
Virus released	38.7

labeled prior to infection are compared it can be seen that virus contains more newly synthesized phospholipids than the cellular debris.

DISCUSSION

The results described above show that: (1) Infection with NDV modifies the synthesis of phospholipid by increasing the synthesis of phospholipid and decreasing that of phosphatidylcholine. (2) The virus contains phospholipid synthesized both before and after infection: (a) a virus grown on cells labeled prior to infection is labeled in the phospholipid. (The turnover rate of the phospholipid, in particular that of sphingomyelin, is too slow to account for this labeling by neosynthesis from breakdown products). (b) Virus grown on cells labeled during infection is also labeled. (3) Distribution of labeled phospholipid of the virus differs from that of uninfected and infected whole host cells and from that of their surface membranes, at least until the onset of cell lysis. Late in infection a membraneous material can be found in the supernatant of infected cells, with a distribution of labeled phospholipid similar but not identical to that of the virus and different from that of the host cell or its surface membrane. This material contains less newly synthesized phospholipid than the virus. (4) The turnover rate differs for various phospholipid and cell types.

In view of these results and assuming that phospholipid metabolism and distribution in virus infected cells do not differ between productive and non-productive cells, we can formulate the following hypothesis concerning the origin of viral phospholipid and the intervention of the host cell surface membrane.

A part but not the total of the viral phospholipid is derived from the host cell membrane, modified as a consequence of infection. Membrane fragments with a phospholipid composition resembling that of the virus can be found. As these membrane fragments have a phospholipid composition similar to but not identical with the phospholipid composition of the virus, it seems likely that some viral phospholipid have other origins. Concomitantly, cellular phospholipid metabolism is modified after infection and this modification parallels virus phospholipid composition.

It appears that the assembly of the viral envelope is a complex process. The viral phospholipids are apparently not derived at random, either in toto from the surface membrane or from the host cell. They could be derived from the modified host cell membrane and/or the modified cellular phospholipid pool. If the surface membrane were the exclusive source of viral phospholipid, "budding sites" should exist with a phospholipid compositions identical with that of the virus. However, there is no evidence for such sites, though membrane fragments with a phospholipid composition similar if not identical to that of the virus were found late in infection. A process involving viral proteins as regulators of cellular phospholipid metabolism after infection and as "selecting agents" for viral phospholipid may be imagined.

If phospholipid metabolism and distribution is not the same in productively and unproductively infected cells it could be argued that the observed modifications are due exclusively or partially to either of the cell populations. For instance, the modification of phospholipid metabolism could be triggered by infection without virus production (as seems to be indicated by the early appearance of this modification), whereas redistribution of cellular phospholipids could be a consequence of virus assembly and therefore be restricted to the virus producing population.

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