Sonderdruck aus
Hoppe-Seyler's Zeitschrift für Physiologische Chemie
Walter de Gruyter & Co, Berlin · New York
Hoppe-Seyler's Z. Physiol. Chem. Printed in Germany
Bd. 356, S. 1123 - 1129, Juli 1975

Studies on the Asymmetric Arrangement of Membrane-Lipid-Enveloped Virions as a Model System

Wilhelm Stoffel, Robert Anderson and Jutta Stahl

(Received 7 April 1975)

Summary: Lipids of BHK 21 cells (baby hamster kidney) grown in tissue culture were labelled with radioactive fatty acids. The enveloped vesicular stomatitis virus was propagated in this host cell type. The virions were purified by density gradient centrifugation.

Neuraminidase treatment of the intact virions led to a complete transformation of hematoside [*N*-acetylneuraminosyl(α2-3)lactosyl(β1-1)ceramide] into lactosylceramide, with identical labelling of the ceramide portion in hematoside of the untreated virions and the lactosylceramide of the neuraminidase-treated particles. The morphology of the virions appeared unchanged in electron micrographs, but the neuraminic-acid-free virions had a strong tendency to aggregate.

The results of these studies are evidence that gangliosides are integrated exclusively into the outer lamella of the lipid bilayer in the viral envelope.

It is also evident that the viral envelope is a suitable model for studies on membrane asymmetry.

Untersuchungen zur asymmetrischen Anordnung der Lipide in membranhaltigen Viren

Zusammenfassung: Die Lipide von BHK 21 Zellen (baby hamster kidney) in der Gewebekultur wurden mit radioaktiv markierten Fettsäuren markiert. Vesicular Stomatitis Virus wurde in diesem Wirtszelltyp propagiert. Die Viren wurden durch Dichtegradientenzentrifugation gereinigt.

Neuraminidasebehandlung der intakten Viren führte zur vollständigen Umwandlung des in der Hüllmembran vorliegenden Hämatosids [*N*-Acetylneuraminosyl(α2-3)lactosyl(β1-1)ceramids] zum entsprechenden Lactosylceramid mit identischer Markierung des Ceramidteils. Elektronenmikroskopisch waren die Viren in ihrer Morphologie unverändert. Jedoch führt die Abspaltung der *N*-Acetylneuraminsäure zu starker Aggregation der Viren. Ansonsten war die Radioaktivitätsverteilung in diesen und den unbehandelten Kontrollviren identisch.

Diese Befunde beweisen, daß das Gangliosid ausschließlich in der äußeren Lipidschicht der Lipid-Doppelschicht der Virushülle angeordnet ist.

Ferner zeigen die geschilderten Untersuchungen, daß die Virushülle ein geeignetes Modell zum Studium der Membranasymmetrie ist.

Address: Prof. Dr. Dr. W. Stoffel, Institut für Physiologische Chemie der Universität zu Köln, D-5 Köln 41, Joseph-Stelzmann-Str. 52.

Enzyme:
Neuraminidase, acylneuraminyl hydrolase (EC 3.2.1.18).

Abbreviation: pfu = plaque-forming units.

The basic arrangement of amphiphilic lipids in natural membranes is the bilayer structure. In eucaryotic cell membranes, the polar head groups of phospholipids and glycosphingolipids are oriented toward the inner and outer surfaces of these bilayers, with the hydrocarbon chains of acyl residues and sphingosine bases forming the hydrophobic central part of the bilayer. The lipid composition is specific for each membrane type, not only quantitatively but also with regard to the lipid classes present. Whereas phospholipids are constituents of every membrane, glycosphingolipids and gangliosides occur predominantly in the plasma membrane and membranes derived thereof, such as lysosomal membranes[1,2].

Regarding the arrangement of lipid molecules in the bilayer areas of the membrane, two essential questions arise:

1) are the halves of the bilayer asymmetric? This means, is there a preferential arrangement of one or more lipid classes in the outer or inner monolayer?
2) Do particular lipid classes form clusters in the bilayer – or more precisely – in either lipid monolayer of the bilayer structure? These questions are closely related to the assembly of the bilayer and its physical properties.

Since each phospholipid class and the glycosphingolipids have typical fatty acid substituents with different degrees of unsaturation and carbon chain length, a nonrandom distribution with structural implications beyond the polar head groups at either surface of the bilayer could result. In order to study these questions we have chosen the enveloped RNA vesicular stomatitis virus. Its envelope resembles a rather simple model of a natural membrane. It is well known that enveloped virions surround their nucleocapsid during the budding process with a host-specific lipid bilayer and with virus-specific glycoproteins projecting as "spikes" from the surface[3-5]. A detailed knowledge of the molecular arrangement and the interaction of their envelope components, lipids and glycoproteins, possibly would give insight into the process of organization, and might also allow conclusions to be drawn about the plasma membrane of the host cell.

The system described here has many advantages over the erythrocyte ghost, which so far has been used to study the problem of membrane asymmetry[6-14]. First of all, it is much simpler. This virion envelope contains only three glycoproteins. The virions can be purified in sufficient amounts. The envelope components can be labelled with suitable precursors in the lipid and/or protein components. The components are accessible to stepwise enzymatic degradation from the outside only. The envelope is not leaky. Therefore the sideness of the enzymic attack is unambiguous and avoids the objections raised in the case of the erythrocyte ghost[6-14]. In this first paper on the asymmetric arrangement of the lipids in the virus envelope, we give proof that the ganglioside hematoside [*N*-acetylneuraminosyl(α2-3)galactosyl(β1-4)glycosyl(β1-1)ceramide], which is the sole ganglioside in the virus envelope, is integrated exclusively into the exterior of the envelope.

Experimental

Materials

[1-^{14}C]Palmitic acid (specif. radioactiv. 0.73 μCi/μmol) and [9,10,12,13-$^{3}H_4$]linoleic acid (specif. radioactiv. 16.4 μCi/μmol) were synthesized in this laboratory by a $^{14}CO_2$ Grignard reaction with pentadecylbromide and a catalytic half reduction in an atmosphere of tritium, using Lindlar catalyst, respectively[15]. Neuraminidase was purchased from Behringwerke, Marburg/Lahn; pronase and trypsin from Merck, Darmstadt.

Preparation of labelled virions

BHK 21 host cells were prelabelled by growing to preconfluency in Dulbecco medium supplemented with 5% fetal calf serum in Roux flasks. The radioactive marker fatty acids were dissolved in absolute ethanol. 4 μmol [1-^{14}C]palmitic and 0.80 μmol [^{3}H]linoleic acid dissolved in 100 μl ethanol were added to each flask (60 ml medium). Cells were grown for another 24 h. They were then infected with vesicular stomatitis virions (1 pfu). The cytopathic effect was complete after 18 to 24 h. The medium was decanted and centrifuged at 1000 × *g* for 20 min. An equal volume of saturated $(NH_4)_2SO_4$ solution was added to the supernatant, and the precipitate was sedimented after 2 h. The pellet was suspended in a small volume of medium and the virions purified first by banding in a sucrose gradient (15 to 45%). The virus band was isolated, diluted with 1 vol. of phosphate buffered saline, sedimented in a 60 Ti rotor, again banded in a SW 27 rotor at 27000 × *g* for 150 min, and isolated as described before[3,16]. Purification was checked by negative staining with 2% phosphotungstic acid in a Philips EM 300.

Neuraminidase treatment of virus

0.5 ml of the virus suspension was incubated with 0.5 ml 0.05M sodium acetate buffer, pH 5.5, 100 µl 0.02M $CaCl_2$ and 0.5 ml of a neuraminidase preparation (200 neuraminidase units per ml) at 37 °C for 10, 20 and 40 min. The virus suspension was layered immediately on top of a potassium tartrate gradient (10 to 45% w/v) and banded in a SW 27 rotor at 27000 × *g* for 2 h. One single sharp band was obtained.

Combined pronase and neuraminidase treatment

0.5 ml of the virus suspension was mixed with 2 ml 0.1M Tris/HCl buffer, pH 7.8, 5mM $CaCl_2$, and 1 mg pronase, incubated for 20 min at 37 °C. Subsequently the pH was lowered to 5.5 with 0.1M acetic acid and treated with neuraminidase as described above.

Lipid extraction

The virus preparations were lyophilized and extracted twice with chloroform/methanol 2:1 and 1:2. The extracts were concentrated and the lipid mixture separated by silicic acid chromatography into a chloroform, acetone/methanol 9:1 and methanol fraction according to Vance and Sweeley[17]. The individual lipids of these fractions were separated by preparative thin-layer chromatography on silica gel H plates of 0.25 mm thickness. The following solvent systems were used:

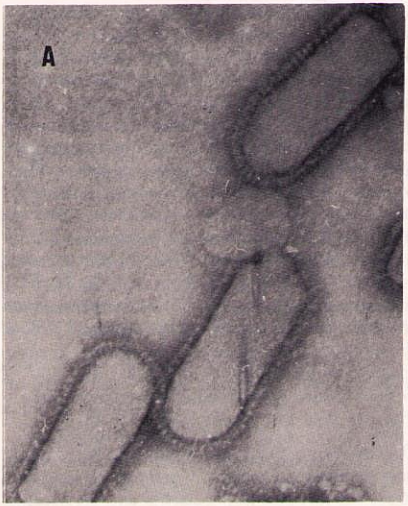
1) petroleum ether/ether/acetic acid 70:30:1 for neutral lipids (chloroform phase)
2) chloroform/methanol/water 100:42:6 for glycosphingolipids
3) chloroform/methanol/water 65:25:4 for phospholipids.

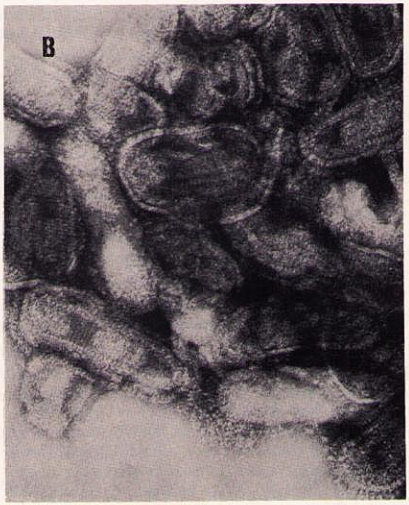
Radio thin-layer chromatograms were analyzed by a Berthold thin-layer scanner, model LB 2722 and a Packard scanner, model 7201. Sialic acids were released from hematoside purified from the virus lipid extract by silicic acid chromatography (acetone/methanol 9:1 fraction) by neuraminidase in a dialysis tube, and the dialysate was lyophilized and treated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide[18].

Results

BHK 21 (baby hamster kidney) cells were prelabelled with [1-^{14}C]palmitic acid and [9, 10, 12, 13-$^{3}H_4$]linoleic acid. They were then infected with vesicular stomatitis virions (1 pfu/cell). The virions of the supernatant were harvested after the whole cell monolayer showed a massive cytopathic effect. The virus purification was achieved by sucrose and tartrate gradient centrifugations according to established procedures[3,16]. Electron microscopic examination after negative staining of the virus preparation revealed no membranous contamination. The virus suspension was treated either with neuraminidase alone or first with pronase to remove the glycoprotein projections and then with neuraminidase. The enzyme-treated virions were again purified by density gradient centrifugation. The density of the intact virions was 1.1730, that of the neuraminidase-treated virions was 1.1683, and that of the particles treated with pronase and neuraminidase was 1.1590. The virus preparations were compared electron microscopically after negative staining, Fig. 1 A - C. The neuraminidase-treated virions (Fig. 1 B) tend to aggregate strongly, although their appearance was unaltered compared with the control virions (Fig. 1 A). The virions treated with pronase and neuraminidase had lost their spikes completely (Fig. 1 C).

The lipid extracts of the virus preparations were also compared. The $^{3}H/^{14}C$ ratio of the lipid extract was identical in the control and the neuraminidase-treated virions (1.035×10^7 dpm ^{3}H, 2.92×10^6 dpm ^{14}C; $^{3}H/^{14}C = 3.5$). Fig. 2 represents the radio thin-layer chromatogram of the total lipid extracts of untreated (control) and neuraminidase-treated virions. From both the radioscan and the analysis, it is apparent that hematoside (peak 1, Fig. 2 A) has completely disappeared after neuraminidase treatment of the virus preparation (Fig. 2 B). Hematoside comprises about 3% of the total radioactivity incorporated into the viral lipids. Its radioactivity, which is present in the ceramide part of the molecule, should appear in lactosylceramide present in the total lipid extract of the neuraminidase-treated virions. Therefore the lipid extract was separated by silicic acid chromatography[17] into a neutral lipid fraction (chloroform fraction), the glycolipid fraction (acetone/methanol 9:1 fraction), which also contains the hematoside if it is present in the lipid mixture (Fig. 3 A, B), and the phospholipid fraction (methanol fraction). It is apparent from these radioscans that the hematoside, present as the main glycosphingolipid in the acetone/methanol fraction (Fig. 3 A), is quantitatively transformed into lactosylceramide (Fig. 3 B), which is present in the control virion envelope only in trace amounts (Fig. 3 A). This hematoside molecule contains about 90% *N*-acetyl neuraminic





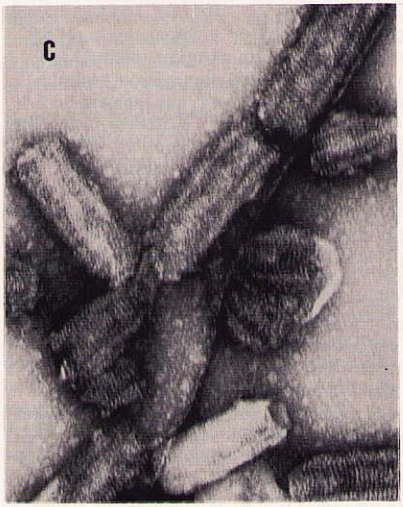


Fig. 1 A - C. Electron microscopy of negatively stained A) control virions, B) neuraminidase-treated virions, and C) virions treated with pronase and neuraminidase.

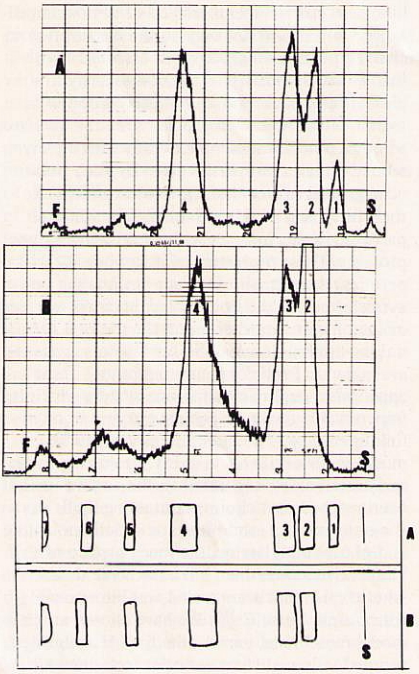


Fig. 2. Radio thin-layer scans of total lipid extract of virions grown in BHK 21 cells prelabelled with [1-^{14}C]-palmitic acid and [9,10,12,13-$^{3}H_4$]linoleic acid.

a) control virions, b) neuraminidase treated virions.

1 = hematoside, 2 = sphingomyelin, 3 = phosphatidylcholine, 4 = phosphatidylethanolamine, 5 = cardiolipin, 6 = cerebroside, 7 = triglyceride, ceramide. Solvent system: chloroform/methanol/water 65 : 25 : 4.

acid and 10% *N*-glycoloyl neuraminic acid, as shown by gas chromatography of the trimethylsilyl derivatives of the compounds released by neuraminidase. The time course of the hematoside transformation into lactosylceramide in the viral envelope was studied by sampling the virus after 10, 20 and 40 min of neuraminidase treatment. The sialic acid release was essentially complete within 10 min. The fatty acid composition of the hematoside of control virus and the lactosylceramide of neuraminidase-treated virions were almost identical. It consisted of about 70% palmitic acid, 20% stearic and 10% oleic acid.

On the other hand, the phospholipid composition of the viral envelope remains unchanged under the neuraminidase and pronase treatment, Fig. 4 A - C. The distribution of the radioactivity in the three labelled phospholipids sphingomyelin (1), phosphatidylcholine (2) and phosphatidylethanolamine (3) was identical, as were the isotope ratios as well. Identical results were obtained with virus preparations which had first been treated with pronase in order to digest the surface projections and then with neuraminidase.

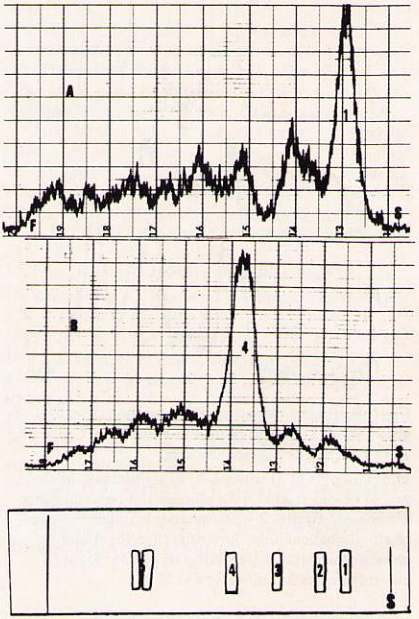


Fig. 3. Radioscan of glycosphingolipid fraction of a) control virus, b) neuraminidase-treated virions.

1 = hematoside, 2 = sphingomyelin, 3 = trihexosylceramide, 4 = lactosylceramide, 5 = monohexosylceramide. Solvent system: chloroform/methanol/water 100 : 42 : 6.

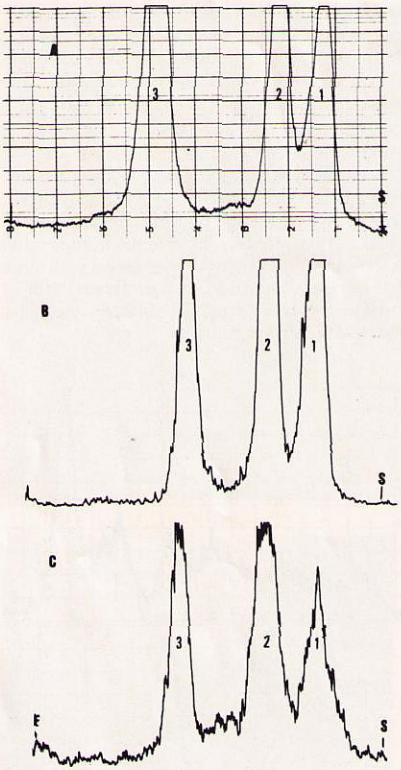


Fig. 4. Radioscan of phospholipid fraction of virions *grown in BHK 21 cells prelabelled with [1-^{14}C]palmitic* and [9,10,12,13-^{3}H$_4$]linoleic acid.

Phospholipids of a) control, b) neuraminidase-treated and c) virions treated with pronase and neuraminidase 1 = sphingomyelin, 2 = phosphatidylcholine, 3 = phosphatidylethanolamine. Solvent system for A and B: chloroform/methanol/2N NH_4OH 60:35:8; for C: chloroform/methanol/water 65:25:4.

Discussion

The question whether cholesterol and the complex phospho- and glycosphingolipids form bilayers with asymmetric distribution in the lipid bilayer of natural membranes is of utmost importance, with regard not only to the distribution of charged polar head groups, but associated with this, to an asymmetry of the nonpolar hydrophobic fatty acyl residues in the hydrophobic region. Other unsettled problems are the questions whether patches of complex lipids with closely related physical properties given by their polar head groups and/or the degree of saturation in their fatty acid chains are formed, or whether particular lipids interact with integral membrane proteins. These problems are now open for experimental treatment. One approach is the enzymic attack of the lipid molecules from one side of the bilayer. Another one is the method of cross linking lipid molecules. (So far the only reagents available are for linking amino groups.) These approaches require a membrane system which is impermeable to the respective enzyme or cross-linking reagent. The most intensively studied model is the erythrocyte ghost. From enzymatic and cross-linking experiments, the conclusion has been reached that choline-containing lipids prefer the outer surface, and phosphatidylethanolamine and phosphatidylserine, the inner surface of the bilayer. However, there has been some doubt whether the ghosts are sealed and impermeable for phospholipase C[10]. We have chosen another model membrane, namely the lipid bilayer of enveloped virions such as vesicular stomatitis virion. Carbon-13 studies with ^{13}C-labelled fatty acids and choline precursors have demonstrated that the hydrophobic region of the lipid bilayer in the envelope of vesicular stomatitis virions is rather rigid, due to the high cholesterol content and the glycoproteins interacting with the lipid in the central region of the bilayer. The glycoprotein projections do not influence the polar head groups of phosphatidylcholine in the bilayer to a significant extent[19]. In this paper we have given the experimental evidence that the ganglioside hematoside (GM_3), which is the only ganglioside present in these virions and their host cell BHK 21, is exclusively located in the outer monolayer of the envelope. The *N*-acetyl neuraminic acid of hematoside is completely released by brief neuraminidase treatment, as shown in the kinetic study. Since we prelabelled the ceramide part of hematoside, the resulting lactosylceramide has been recovered and analyzed.

The same result was obtained when the surface projections were hydrolyzed with pronase. Neither the proteolysis of the glycoprotein in conjunction with the neuraminidase treatment nor the neuraminidase alone had any effect on the phospholipid pattern. Therefore, the bilayer of the virus envelope remained intact. Our experiments point out that the spikes do not prohibit the free access of the neuraminidase to the carbohydrate group of the glycosphingolipid of the bilayer. The release of *N*-acetyl neuraminic acid from hematoside is a rapid one. The elimination of the *N*-acetyl neuraminic acid from the glycoprotein and the hematoside causes aggregation of the virions, because the repulsive negative charges on the surface of the virion are no longer present, and virus aggregation may be caused by polar interaction between the carbohydrate side chains or by hydrophobic interactions between the proteins. If we assume that the budding process of the virus from the plasma membrane does not lead to a disturbance of the lipid bilayer structure of the plasma membrane area of the host cell, it is also reasonable to localize the hematoside in the outer surface of the host cell bilayer. This conclusion is supported by the observation that the reverse process, namely the incorporation of the plasma membrane during pinocytosis, localizes its gangliosides in the inner surface of the secondary lysosomes, as demonstrated by colloidal iron staining[20,21].

These studies demonstrate also that the virus envelope is a suitable model for study of the details of the lipid bilayer structure, provided a suitable labelling technique has been applied to ensure reliable analytical data, and that purified hydrolytic enzymes are available to make such studies feasible.

We are indebted to Dr. *Dennis Brown*, Institut für Genetik der Universität Köln, for carrying out the electronmicroscopic controls of the virus preparation.

The support by the *Deutsche Forschungsgemeinschaft* is gratefully acknowledged.

Literature

1 Stoffel, W. (1971) *Annu. Rev. Biochem.* **40**, 57 - 82.
2 Henning, R. & Stoffel, W. (1973) *this J.* **354**, 760 - 770.
3 Klenk, H. D. & Choppin, P. W. (1969) *Virology* **38**, 255 - 268.
4 Klenk, H. D. & Choppin, P. W. (1971) *J. Virol.* **7**, 416 - 417.
5 Lenard, J. & Compans, R. W. (1974) *Biochim. Biophys. Acta* **344**, 51 - 94.
6 Woodward, C. B. & Zwaal, R. F. A. (1972) *Biochim. Biophys. Acta* **274**, 272 - 278.
7 Colley, C. M., Zwaal, R. F. A., Roelofsen, B. & Deenen, L. L. M. van (1973) *Biochim. Biophys. Acta* **308**, 74 - 82.
8 Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D. & Deenen, L. L. M. van (1973) *Biochim. Biophys. Acta* **323**, 178 - 193.
9 Zwaal, R. F. A., Roelofsen, B. & Colley, C. M. (1973) *Biochim. Biophys. Acta* **300**, 159 - 182.
10 Low, M. G., Limbrick, A. R. & Finean, J. B. (1973) *FEBS Lett.* **34**, 1 - 4.
11 Gordesky, S. E. & Marinetti, G. V. (1973) *Biochem. Biophys. Res. Commun.* **50**, 1027 - 1031.
12 Marinetti, G. V., Sheeley, D. S., Baumgarten, R. & Love, R. (1974) *Biochem. Biophys. Res. Commun.* **59**, 502 - 507.
13 Bretscher, M. S. (1972) *J. Mol. Biol.* **71**, 523 - 528.
14 Kahlenberg, A., Walker, C. & Rohrlick, R. (1974) *Can. J. Biochem.* **52**, 803 - 806.
15 Stoffel, W. (1964) *Liebigs Ann. Chem.* **673**, 26 - 36.
16 Cartwright, B. (1969) *J. Gen. Virol.* **5**, 1 - 10.
17 Vance, D. E. & Sweeley, C. C. (1967) *J. Lipid Res.* **8**, 621 - 630.
18 Donike, M. (1969) *J. Chromatogr.* **42**, 103 - 104.
19 Stoffel, W. & Bister, K. (1975) *Biochemistry*, submitted for publication.
20 Gasic, G. J., Berwick, L. & Sorrentino, M. (1968) *Lab. Invest.* **18**, 63 - 71.
21 Henning, R., Plattner, H. & Stoffel, W. (1973) *Biochim. Biophys. Acta* **330**, 61 - 75.