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COMPARISON OF NON-IONIC DETERGENTS FOR EXTRACTION AND ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SENDAI VIRUS INTEGRAL MEMBRANE PROTEINS

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SUMMARY

The integral membrane proteins of Sendai virus haemagglutinin–neuraminidase (HN) and fusion protein (F) were extracted from purified virions with 2% of a non-ionic detergent, *i.e.*, polyoxyethylene alkyl ethers varying by 8–14 hydrocarbon units in the alkyl chain and by 4–8 ethylene glycol units in the oxyethylene chain. Triton X-100 and octyl glucoside were included as reference detergents. The hydrophile–lipophile balance (HLB) and the critical micelle concentration (CMC) of the detergents were determined. A decrease in length of the oxyethylate by 8–5 ethylene glycol units and an increase in the alkylate by 8–12 hydrocarbon units resulted in higher yields of extracted proteins. The highest yields were obtained for $C_{12}E_5$ with an HLB of 11.7. Yields of extracted protein could be correlated with the HLB values of the polyoxyethylene alkyl ethers. The structural integrity of HN and F was not affected during extraction by either detergent as measured by their reactivity with monoclonal antibodies directed against native HN and F. Extracts were subjected to anion-exchange high-performance liquid chromatography (HPLC) on a Mono Q column in the presence of 0.1% of the detergent used for extraction. Eluate fractions were analysed by sodium dodecyl polyacrylamide gel electrophoresis and recoveries of HN and F protein were determined by size-exclusion HPLC. The immunological activity of HN and F was tested in an enzyme-linked immunosorbent assay. The highest recoveries of HN and F (80%) were obtained with $C_{10}E_5$ in the elution buffer. HN and F were partially purified and the immunological activity was well preserved.

INTRODUCTION

Polyoxyethylene alkyl ethers are widely used as non-ionic detergents (surfactants)[1]. In biological research, they are employed for the solubilization of membrane

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proteins[2] and as additives in eluents for various modes of high-performance liquid chromatography (HPLC)[3-5]. Organic solvents which have been used for solubilization often cause denaturation of membrane proteins, whereas non-ionic detergents effective in solubilizing the protein allow their isolation in a biologically active form[2,6]. To study the enzymic, immunological as well as physico-chemical properties of hydrophobic proteins and for detergent removal or exchange in the preparation of membrane proteins, chemically pure and homogeneous non-ionic detergents are required[7]. Early studies were made with commercial products that best embodied the desired properties, but these products are often chemically impure and heterogeneous and hence may vary from one preparation to another. In ionic detergents the solubilizing power of the ionic group is fixed, and the hydrophobic group must be modified to alter the detergent properties. However, in non-ionic detergents the hydrophilic characteristic of the oxyethylate increases with increasing chain length and hence there is the possibility in oxyethylene-based compounds of modifying both the hydrophilic and the hydrophobic portions. Nowadays, many systematic changes in their structure have been made in order to produce pure and homogeneous detergents with extremely low toxicity, for a wide range of applications in biochemical research.

This paper reports a comparative study of a number of non-ionic detergents, *i.e.*, the polyoxyethylene (E_y) alkyl (C_x) ethers C_8E_5, C_8E_6, C_8E_7, $C_{10}E_5$, $C_{10}E_6$, $C_{10}E_7$, $C_{10}E_8$, $C_{12}E_4$, $C_{12}E_5$, $C_{12}E_6$, $C_{12}E_7$, $C_{12}E_8$, $C_{14}E_5$, Triton X-100 and octyl glucoside with Sendai virus envelope proteins as a model. Sendai virus is a paramyxovirus of mice. The viral envelope is composed of a lipid bilayer in which two integral membrane proteins are embedded, the fusion protein F (Mr = 65 000) and the haemagglutinin–neuraminidase protein HN (Mr = 68 000). Detergent extracts contain the F protein in a monomeric form and the HN protein in a tetrameric (HN_4), dimeric (HN_2) and occasionally a truncated form of the dimer without the anchoring region of the polypeptide chain (HN_2–)[8]. Both are hydrophobic proteins. The detergents were compared with respect to their ability to solubilize these proteins and the preservation of their immunological activity after extraction. In addition, a possible relationship between extraction properties and either the critical micelle concentration (CMC), *i.e.*, the concentration at which micelles begin to form, or the hydrophile–lipophile balance (HLB), *i.e.*, the ratio between the hydrophilic oxyethylene part and the hydrophobic alkyl part, was investigated.

The suitability of some of the detergents as additives in ion-exchange (IE) HPLC eluents was investigated with respect to separation, recovery and immunological activity of the proteins after IE-HPLC.

EXPERIMENTAL

Detergent extraction of Sendai virus

Sendai virus was cultured in 10-day-old embryonated chicken eggs. Allantoic fluid was collected after incubation for 72 h at 37°C. Debris was removed by low-speed centrifugation (10 min at 2000 *g*) and virus was pelleted by ultracentrifugation (90 min at 70 000 *g*). Virus pellets were resuspended in 10 m*M* Tris–HCl (pH 7.2) containing 10% (w/v) sucrose and stored at −80°C. The amount of protein was determined[9]. Sendai virus suspensions containing 40 mg of protein were pelleted (90 min at 100 000 *g*) and resuspended in 1 ml of 10 m*M* Tris–HCl (pH 7.2). Detergent

extraction of F and HN proteins was performed by the addition of 1 ml of the same buffer, containing 4% (w/w) detergent [detergent to protein ratio = 1 (w/w)]. After 20 min at room temperature, extraction was terminated by ultracentrifugation (90 min at 100 000 *g*), and the supernatant, which contained the virus proteins HN and F, was stored in aliquots of 200 μl at −80°C. In some cases, detergent was removed by treatment with Amberlite XAD-2[8].

Detergents

Extraction and chromatography of the Sendai membrane proteins were performed with non-ionic detergents, *i.e.*, different polyoxyethylene alkyl ethers, Triton X-100 (BDH, Poole, U.K.) and 1-O-*n*-octyl β-D-glucopyranoside (octyl glucoside) (Boehringer, Mannheim, F.R.G.). The polyoxyethylene alkyl ethers C_8E_5, C_8E_6, C_8E_7, $C_{10}E_5$, $C_{12}E_5$ and $C_{12}E_8$ were a gift from Kwant-Hoog Vacolie Recycling and Synthesis (Bedum, The Netherlands). The polyoxyethylene alkyl ethers $C_{10}E_6$, $C_{10}E_7$, $C_{10}E_8$, $C_{12}E_4$, $C_{12}E_6$, $C_{12}E_7$ and $C_{14}E_5$ were obtained from Fluka (Buchs, Switzerland).

Determination of critical micelle concentration and hydrophile–lipophile balance

The CMC of each detergent was determined with magnesium 8-anilinonaphthalene-1-sulphonate (ANS)[10] (Fluka). The fluorescence of serially diluted detergent solutions in demineralized water was measured with ANS using a Perkin-Elmer LS-2 filter fluorimeter. The excitation wavelength was 375 nm and the emission wavelength was 490 nm.

The HLB of polyoxyethylene alkyl ethers was calculated as the weight percentage of the oxyethylene content divided by 5, as described by Becher[11].

Size-exclusion and ion-exchange HPLC

Chromatography was performed with an LKB 2150 pump (LKB, Zoetermeer, The Netherlands), a Rheodyne 7125 injector (Inacom, Veenendaal, The Netherlands), a Waters 441 detector (Waters, Etten Leur, The Netherlands) or an LKB 2151 detector connected with a Kipp BD 40 recorder (Kipp & Zonen, Delft, The Netherlands) and an LDC/Milton Roy Cl-10B integrator (Interscience, Breda, The Netherlands). Gradients were generated with an LKB 2152 LC controller and a LKB Ultrograd mixer driver.

Size-exclusion (SE) HPLC was performed on two tandem-linked Zorbax Bioseries GF 450 columns (250 × 9.2 mm I.D.) (DuPont, Wilmington, DE, U.S.A.) or on a TSK G4000SW column (600 × 7.5 mm I.D.) (LKB, Bromma, Sweden). Aliquots of 200 μl of virus envelope extracts were heated for 3 min in a boiling water-bath in the presence of 4% (w/w) sodium dodecyl sulphate (SDS) (electrophoresis grade, Bio-Rad Labs., Richmond, CA, U.S.A.) and injected into the HPLC system. The mobile phase was 50 m*M* sodium phosphate (pH 6.5) containing 0.1% SDS. The flow-rate was 1.0 ml/min and the absorbance was monitored at 280 nm. Yields of HN and F proteins were determined, using bovine serum albumin (BSA), ovalbumin (OVA) and trypsin inhibitor as a reference mixture with a concentration of 50 μg of each protein per 100 μl.

Anion-exchange (IE) HPLC was carried out on a Mono Q HR 5/5 column (50 × 5 mm I.D.) (Pharmacia, Uppsala, Sweden), which was eluted with 20 m*M*

Tris–HCl (pH 7.8) containing 0.1% (w/w) of the same detergent as used for extraction. A 24-min gradient from 0 to 0.5 *M* sodium chloride was started after 10 min of isocratic elution. The flow-rate was 1.0 ml/min and the absorbance was monitored at 280 nm. The total amount of protein injected was between 0.95 and 1.05 mg for each detergent extract. Fractions of 2–3 ml were collected in 70 × 11 mm Minisorp tubes (Nunc, Roskilde, Denmark), and aliquots of 50 μl of each fraction were analysed by SDS-polyacrylamide gel electrophoresis (PAGE). The remaining part of the fractions was dialysed against demineralized water and freeze-dried. Each freeze-dried fraction was dissolved in 560 μl of water. Samples of 140 μl were made 5% with respect to SDS, heated for 3 min in a boiling water-bath and analysed by SE-HPLC to determine the amount of HN and F protein present in the fractions. The remaining part (420 μl) was analysed in an enzyme-linked immunosorbent assay (ELISA).

SDS-PAGE

The eluate fractions were analysed on 8% SDS-polyacrylamide gels[12], and polypeptide bands were revealed by silver staining[13], using phosphorylase b (M_r = 92 500), bovine serum albumin (M_r = 68 000), the heavy chain of immunoglobulin G (IgG) (M_r = 50 000) and chymotrypsin (M_r = 23 500) as reference proteins.

Enzyme-linked immunosorbent assay with monoclonal antibodies (Mabs) against Sendai virus HN and F protein

The immunological activities of HN and F protein (i) in the extract and (ii) in the fraction after IE-HPLC were determined in an ELISA[8] with a panel of six Mabs against Sendai virus HN protein (97, 135, 851, 852, 1.182 and 820) and two Mabs against Sendai virus F protein (1.017 and 1.216)[14]. Plates were coated with a serial dilution of the extracts and of the IE-HPLC fractions, starting at a protein concentration of 1–5 μg per well, and the ELISA was performed as described earlier[8]. The absorbance was monitored at 492 nm and reactions were considered to be positive at $A_{492} \geqslant 0.2$. An absorbance of 1.2 at 492 nm was used as an arbitrary measure of immunological activity. The immunological activity is inversely related to the amount of protein needed to obtain an absorbance of 1.2 at 492 nm.

RESULTS AND DISCUSSION

Extraction of Sendai virus membrane proteins with different detergents: yields of HN and F protein by SE-HPLC

Characteristics of the non-ionic detergents that have been used in this study are listed in Table I. The suitability of these detergent for extraction of the Sendai virus integral membrane proteins HN and F was investigated, and the amount of HN and F protein in the extracts was determined by SE-HPLC. It has been calculated by others[15,16] that the amount of HN and F protein accounts for *ca.* 39% of the total amount of protein present in Sendai virus particles. Extraction of Sendai virus with $C_{12}E_5$ gave the highest yields, which was about one quarter of the total amount of HN and F protein. For different virus preparations the amount of HN and F extracted per milligram of virus by $C_{12}E_5$ was slightly different. A second and third successive extraction can enhance the yield, but may cause partial disruption of the virus particles, resulting in a mixture of membrane and internal proteins. To allow compar-

TABLE I

CHARACTERISTICS OF NON-IONIC DETERGENTS; RELATIVE YIELDS OF HN AND F PROTEIN AFTER EXTRACTION OF SENDAI VIRUS

Detergent	*MW*[a] *(average)*	*HLB*[b]	*CMC*[c] *(mg/ml)*	*Micellar wt.*[d] *(aggregation number)*	*Relative yield*[e] *(%)*
C_8E_5	350	13.5	3.2		65
C_8E_6	394	14.3	3.4	15.5 (39)	64
C_8E_7	438	14.8	3.7		62
$C_{10}E_5$	378	12.5	0.26		82
$C_{10}E_6$	422	13.3	0.35	32 (76)	77
$C_{10}E_7$	466	14.0	0.41		74
$C_{10}E_8$	510	14.5	0.47		71
$C_{12}E_4$	362	10.7	0.017		14
$C_{12}E_5$	406	11.7	0.020		100
$C_{12}E_6$	450	12.5	0.029	47 (105)	90
$C_{12}E_7$	494	13.2	0.033		78
$C_{12}E_8$	538	13.7	0.036	65 (120)	74
$C_{14}E_5$	434	10.9	0.004		14
Triton X-100	628	13.5	0.15	90 (140)	63
Octyl glucoside	292	12.6	7.1	8 (27)	43

[a] Molecular weights in daltons.

[b] Hydrophile–lipophile balance, calculated according to Becher[11].

[c] Critical micelle concentration, determined according to De Vendittis *et al.*[10].

[d] Micellar weights in kilodaltons, from refs 2, 3 and 19.

[e] The yield obtained with $C_{12}E_5$, which was *ca.* one quarter of the total amount of HN and F protein, was taken as 100%.

ison between different detergents, the highest yield obtained with extractions performed with $C_{12}E_5$ was taken as 100%. The relative yields of HN and F protein calculated for the different detergent extracts are shown in Table I. An increase in the number of ethylene glycol units in the oxyethylene chain from 5 to 8 (at a fixed alkyl chain length) and a decrease in the number of hydrocarbon units in the alkyl chain from 12 to 8 (at a fixed oxyethylene chain length) decreases the yields of HN and F protein in the extracts. An increase in the alkyl chain length to C_{14} or a decrease in the oxyethylene chain to E_4 resulted in very low yields (14%), and these detergents ($C_{14}E_5$ and $C_{12}E_4$) dissolved poorly in aqueous solutions and therefore were not suitable for IE-HPLC. Extracts of Sendai virus obtained with Triton X-100 and with octyl glucoside (included as reference detergents) gave protein yields of 63% and 43%, respectively.

In Fig. 1, the yields of protein after extraction are related to the HLB values of the detergents. The highest yields were obtained with polyoxyethylene alkyl ethers with HLB values ranging from 11.5 to 12.5. Detergents with HLB values below 11.5 are probably not suitable because of their insolubility in aqueous solutions. Umbreit and Strominger[6] reported optimum HLB values of detergents ranging from 12 to 14. They investigated other types of non-ionic detergents for the extraction of D-alanine carboxypeptidase from *Bacillus subtilis* and phosphoacetylmuramylpentapeptide translocase and succinate dehydrogenase from *Micrococcus luteus*. Presumably, opti-

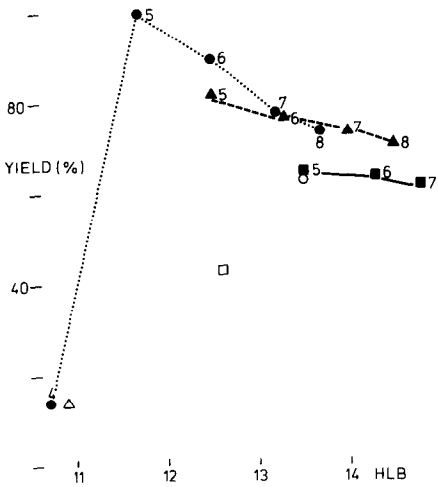

Fig. 1. Relative yields of Sendai virus membrane proteins after extraction with 2% of a non-ionic detergent. The highest yield was obtained with $C_{12}E_5$ and this was taken as 100%. Yields were estimated from SE-HPLC elution patterns. Either a TSK 4000SW or two tandem-linked Zorbax GF 450 columns were used. The mobile phase was 50 m*M* sodium phosphate (pH 6.5) containing 0.1% SDS. Yields are plotted against the HLB values of the detergents investigated: (■—■) C_8E_{5-7}; (▲- - -▲) $C_{10}E_{5-8}$; (●· · ·●) $C_{12}E_{4-8}$; (△) $C_{14}E_5$; (○) Triton X-100; (□) octylglucoside. The numbers indicate the number of ethylene glycol units in the oxyethylene chain.

mum HLB values depend on the hydrophobicity of the protein to be solubilized, although different types of non-ionic detergents may also differ in their solubilizing properties. In the study reported by Umbreit and Strominger[6], the yield could be correlated with the length of the oxyethylene chain but did not correlate with the chemical nature of the hydrophobic portion of the detergent. In other studies, an increase in the solubilizing ability of the detergent with an increase in the hydrophobic moiety of the detergent has been described[17,18]. In our study, solubilization of HN and F protein depends on the length of the oxyethylene chain and, to a larger extent, on the length of the hydrocarbon chain. It may be possible that this correlation only applies to non-ionic detergents within the group of polyoxyethylene alkyl ethers.

The CMC values may be an important parameter for detergent removal or detergent exchange[2,7,19], and they may serve as an indication of the degree of homogeneity of a detergent[1,2], but CMC values do not correlate with the solubilizing properties of the detergent, and nor do other physical parameters such as micellar weight, size, shape and aggregation number.

IE-HPLC of detergent extracts of Sendai virus membrane proteins

As detergent extracts of Sendai virus obtained with C_8E_5 $C_{10}E_5$ and $C_{12}E_5$, gave a relatively high yield of HN and F protein, they were selected for further studies, together with the more commonly used detergents octyl glucoside and $C_{12}E_8$. IE-HPLC was performed with 0.1% of the detergent used for extraction, added to the elution buffer. The amount of HN and F protein injected was between 0.95 and 1.05 mg for each chromatographic run. Fig. 2 shows the IE-HPLC elution pattern of a $C_{10}E_5$ extract of Sendai virus and 0.1% $C_{10}E_5$ in the elution buffer.

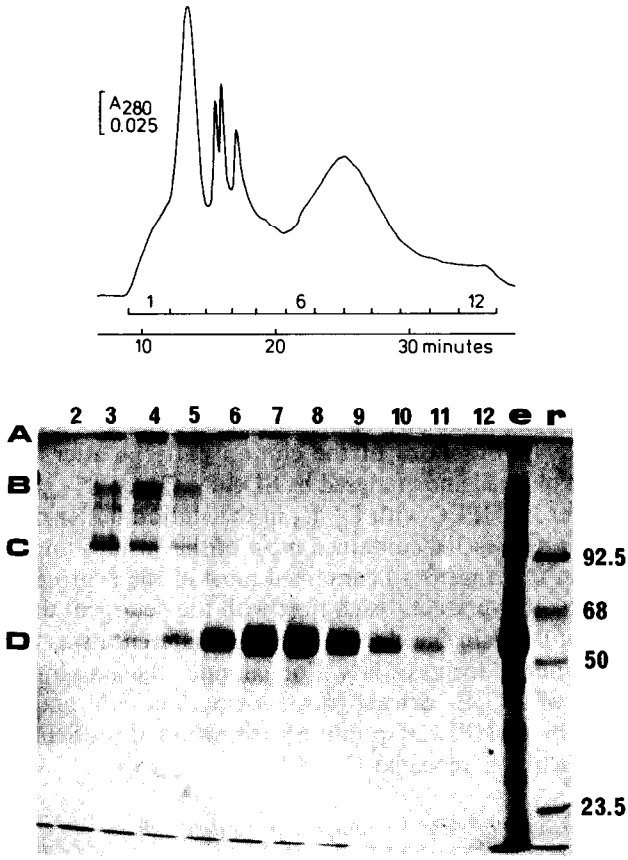

Fig. 2. IE-HPLC of a $C_{10}E_5$ extract of Sendai virus membrane proteins on a Mono Q column. Elution was performed with a 40-min gradient from 20 m*M* Tris–HCl (pH 7.8), containing 0.1% $C_{10}E_5$, to 0.5 *M* NaCl in the same buffer. The flow-rate was 1 ml/min and the absorbance was monitored at 280 nm. Fractions were collected as indicated, and samples were analysed by SDS-PAGE (8% gel) under non-reducing conditions. Polypeptides were revealed by silver staining. Lane numbers of the gel refer to IE-HPLC fractions. A, B, C and D are the tetrameric, dimeric and truncated form of HN protein and F protein, respectively. r, reference proteins; molecular weights are given in kilodaltons. e, the $C_{10}E_5$ extract prior to chromatography.

Recoveries of HN and F protein after IE-HPLC were determined by SE-HPLC of the fractions collected, as indicated in Fig. 2, and are listed in Table II. The highest recovery for both HN (83%) and F (80%) was obtained with $C_{10}E_5$ in the eluent. IE-HPLC in the presence of other detergents resulted in lower yields: 61% and 44% (C_8E_5), 64% and 46% ($C_{12}E_5$), 64% and 48% ($C_{12}E_8$) and 31% and 4% (octyl glucoside) for HN and F protein, respectively (see Table II). All detergents were used at a concentration of 0.1%. For some of them (C_8E_5 and octyl glucoside), this is below the CMC, and it could be argued that for these detergents higher concentrations should be used[5,17]. However, the recoveries of the HN and F protein after IE-HPLC with 0.1% C_8E_5 and 0.1% $C_{12}E_5$ were similar and ranged from 44 to 46% for the F protein and from 61 to 64% for the HN protein (Table II), despite the

TABLE II

RECOVERIES OF HN AND F PROTEIN AFTER IE-HPLC

Detergent[a]	*Recovery (%)*	
	HN	*F*
C_8E_5	61	44
$C_{10}E_5$	83	80
$C_{12}E_5$	64	48
$C_{12}E_8$	64	46
Octyl glucoside	31	4

[a] Sendai virus C_8E_5 extracts were subjected to IE-HPLC with the same detergent in the mobile phase as used for extraction.

100-fold difference in CMC (Table I). Fractions of the elution patterns after IE-HPLC, as indicated in Fig. 2, were collected and analysed by SDS-PAGE on 8% gels. The gel pattern of the fractions after IE-HPLC with $C_{10}E_5$ in the elution buffer of a $C_{10}E_5$ extract is shown in Fig. 2. Fractions 3–6 contained most of the various multimeric forms of the HN protein, and fractions 6–10 contained most of the F protein. Similar patterns were found for the other extracts, each time with the detergent used for extraction at a concentration of 0.1% in the eluent. The only difference was the relative amount of the various multimeric forms of HN. For example, the amount of HN_2– was higher after IE-HPLC of a C_8E_5 extract (not shown). The HN and F protein were only partially purified by IE-HPLC. Optimum purification of the Sendai virus proteins HN and F possibly requires repeated IE-HPLC.

Immunological activity in ELISA of Sendai virus membrane proteins

As a measure of the intact conformation of HN and F protein, the immunological activity of these proteins was determined in an ELISA with conformation-dependent Mabs against Sendai virus HN and F protein[8,14]. ELISA was performed with (i) Sendai virus HN and F proteins extracted with C_8E_5, $C_{10}E_5$, $C_{12}E_5$, $C_{12}E_8$, Triton X-100 and octyl glucoside and (ii) fractions after IE-HPLC with 0.1% of C_8E_5, $C_{10}E_5$, $C_{12}E_5$ and $C_{12}E_8$ in the eluent. ELISA of the extracts was performed with and without detergent removal. The amount of protein necessary to obtain an absorbance of 1.2 at 492 nm (A_{492}) was measured for each extract. These amounts ranged from 4 to 11 ng for HN and from 2 to 7 ng for F (Table III). When detergent was not removed, only $C_{12}E_5$ and $C_{12}E_8$ interfered with protein coating at a concentration of 0.004% detergent. Lower concentrations of the detergents did not affect protein coating and, for this reason, ELISA of the IE-HPLC fractions could be performed without removing the detergent. ELISA of the IE-HPLC fractions was performed with the same panels of Mabs against Sendai virus HN and F protein. In all fractions (with exception of the first fractions which did not contain protein), HN and F protein could be detected ($A_{492} \geq 0.200$). The amounts of HN and F necessary to obtain an A_{492} of 1.2 were estimated, and varied from 16 to 39 ng for HN and from 4 to 9 ng for F protein (Table III). For instance, 4 ng of the HN protein in the $C_{12}E_5$ extract (the starting material for IE-HPLC) was required to obtain an A_{492} of 1.2. A larger amount (39 ng) of HN protein was necessary to obtain the same absorbance

TABLE III

IMMUNOLOGICAL ACTIVITY OF HN AND F PROTEIN IN THE DETERGENT EXTRACTS AND AFTER IE-HPLC

Detergent	*HN Protein*[a]		*F Protein*[a]	
	Extract	*IE-HPLC*	*Extract*	*IE-HPLC*
C_8E_5	10	16	2	9
$C_{10}E_5$	11	36	6	6
$C_{12}E_5$	4	39	5	3
$C_{12}E_8$	4	30	7	4

[a] Immunological activity, amount of protein in ng needed to obtain an absorbance of 1.2 at 492 nm.

after it had been subjected to IE-HPLC. However, after denaturation of the HN protein by boiling in 4% SDS, more than 1000 ng of protein was necessary to measure an A_{492} of 1.2. This means that there is less than a 3.5% loss of immunological activity of the HN protein after it had been subjected to IE-HPLC, which indicates that the conformation of the protein is still largely intact.

The results in Table III show that the immunological activity of F protein is not affected by IE-HPLC in the presence of the various detergents. The results show that polyoxyethylene alkyl ethers can be used for adequate solubilization of integral membrane proteins of Sendai virus and that the conformation of the proteins remains intact during extraction and after IE-HPLC in the presence of the detergent used for extraction.

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