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Effects of Hydrostatic Pressure on a Membrane-Enveloped Virus: High Immunogenicity of the Pressure-Inactivated Virus

JERSON L. SILVA,[1]† PENG LUAN,[1] MICHAEL GLASER,[1] EDWARD W. VOSS,[2] AND GREGORIO WEBER[1]*

Department of Biochemistry[1] and Department of Microbiology,[2] University of Illinois, Urbana, Illinois 61801

Received 30 October 1991/Accepted 31 December 1991

A new approach to the preparation of antiviral vaccines relying on the inactivation of the virus particle by hydrostatic pressure is described. The enveloped virus vesicular stomatitis virus was utilized as a model; a pressure of 260 MPa applied for 12 h reduced infectivity by a factor of 10^4, and the antibodies against pressurized material were as effective as those against the intact virus when measured by their neutralization titer. Fluorescence measurements indicate that application of pressure results in perturbations of the particle interactions that permit binding of specific molecular probes. Electron microscopy showed that the membrane of the pressurized virus was partially preserved, presenting the spike pattern of the membrane G protein. Unlike the icosahedral viruses, dissociation into smaller particles was not observed, but a constant change in the morphology was the presence of a bulge in the surface of the pressurized virus, indicating a displacement of the capsid subunits, retained under the lipid and protein membrane.

Studies of the last few years have demonstrated the reversible dissociation of oligomeric proteins by hydrostatic pressure (for reviews, see references 32 and 33). Observations of comparable effects on proteins of many subunits and on viruses (8, 14, 18, 23–28) have engendered the idea of using hydrostatic pressure as a means to suppress virus infectivity, while preserving or perhaps improving the immunogenic properties. In most cases, effective immunization against viruses requires presentation of the whole virus particle to the immune system (7, 15, 16). This requirement coupled to the need for elimination of infectivity greatly limits the possibilities of preparation of appropriate vaccines.

The use of hydrostatic pressure as a virus inactivation method may fulfill the two requirements described above. To our knowledge, high-pressure methods have not been applied to produce vaccines. We found that brome grass mosaic virus is reversibly dissociated by pressure. Recovery of reassociated, but characteristically modified, virus particles decreases steeply at pressures greater than those that produce 75% dissociation (28). At higher pressures, unspecific aggregates of capsid proteins predominate over seemingly complete reassociated capsids. The formation of imperfect virus particles after a cycle of compression and decompression has also been demonstrated in simian virus 40 (28a) and rotavirus (20) by electron microscopy, gel filtration, and spectroscopy. In this article, we report the effects of pressure on a membrane-enveloped virus, vesicular stomatitis virus (VSV), a rhabdovirus that infects some animal cells, which we chose as an appropriate model for inactivation and immunogenicity studies (2, 29). Our results with VSV point to the possibility of utilization of hydrostatic pressure to prepare noninfectious whole virus particles that are highly immunogenic.

* Corresponding author.

† Permanent address: Departamento de Bioquimica, Instituto Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system to >10-MΩ resistance.

Virus preparation. VSV type Indiana was grown on BHK-21 cells (2, 4) in standard medium (Glasgow's modified Eagle's medium, 10% calf serum and 10% tryptose phosphate buffer). Nonpurified viruses were prepared by propagating in 100-mm-diameter dishes. The infection was monitored visually and typically allowed to proceed for 12 to 16 h at 37°C in a CO_2 incubator. The supernatant was collected and cleared of any cellular debris in a Sorvall centrifuge (5,000 rpm for 10 min in a GSA rotor). For the preparation of purified VSV, virus was propagated for 16 h in roller bottles that were placed on a roller apparatus in a 37°C warm room. After propagation, the supernatant was collected and cleared of the cellular debris. The supernatant was spun in a Beckman Ti45 rotor at 30,000 rpm for 2.5 h. The pellet was then resuspended in 3E buffer (0.12 M Tris, 0.06 M sodium acetate, 3.0 mM EDTA, pH 7.4) and layered onto a continuous 5 to 40% sucrose gradient (in 3E buffer) and spun for 1 h at 36,000 rpm in a Beckman SW-41 rotor. The virus band was collected and pelleted in a Beckman type 40 rotor (35,000 rpm for 1 hour) and resuspended in 10 mM Tris, pH 7.6. Viral stocks were kept at −70°C.

Infectivity assays. Infectivity was studied by plaque assay as described previously (2). Confluent monolayers of BHK-21 cells on 60-mm-diameter dishes were infected with serial dilutions of VSV for 30 min at room temperature. After aspiration of the virus solution, 1% agarose in medium solution was added to each plate. The plates were left in a 37°C CO_2 incubator for 24 h. After the agarose was peeled off the plates, the cells were stained with crystal violet and plaques were counted.

Antibodies. Rabbits were injected with 1.0 ml of pressure-treated virus or control samples diluted 1:2 in complete Freund's adjuvant. After 3 weeks, the initial injection was followed by a booster injection and the rabbits were bled 2 weeks later. Gamma globulin fractions were prepared as previously described (30). After the rabbits were bled, sera were allowed to clot for 24 h. They were centrifuged in a Sorvall SS-34 rotor at 10,000 rpm for 15 min, and the

TABLE 1. Pressure inactivation of VSV

Condition	Titer (PFU/ml) of virus preparation[a]			
	A	B	C	D
Native virus (atmospheric pressure)	1.82×10^7	4.0×10^6	3.9×10^6	2.0×10^7
260 MPa, 3 h	1.41×10^5			
260 MPa, 5 h	1.80×10^4			
260 MPa, 12 h	1.80×10^3	$<2.5 \times 10^1$	$<2.5 \times 10^1$	75

[a] Virus preparations: A, nonpurified virus preparation; B, nonpurified virus preparation diluted 10-fold in 50 mM Tris-HCl (pH 7.5); C, Same as preparation of B, except that it was kept at room temperature for 2 weeks after the incubation at atmospheric pressure or 260 MPa; D, purified preparation of VSV in which the stock concentration was 3.4 mg/ml and it was diluted in 50 mM Tris-HCl (pH 7.5) to a final concentration of 100 μg/ml. All the pressure treatments were performed at 20°C.

TABLE 2. Neutralization assay of antibodies elicited by pressure-inactivated VSV[a]

Antibody dilution	Titer (PFU/ml)			
	Control	Preimmune	Anti-native VSV	Anti-pressurized VSV
1:2	5.0×10^8	5.3×10^8	$<2.5 \times 10^1$	$<2.5 \times 10^1$
1:20	5.8×10^8		$<2.5 \times 10^1$	$<2.5 \times 10^1$
1:200	5.7×10^8		1.6×10^6	1.0×10^6
1:2,000	5.8×10^8		1.6×10^8	1.4×10^8

[a] A high titer of VSV (5.0×10^8 PFU/ml) was incubated for 30 min with a gamma globulin fraction diluted as indicated. The stock concentrations of gammaglobulin were 4.8 (preimmune), 5.1 (anti-native VSV), and 4.9 (anti-pressurized VSV) mg/ml. The titer was measured as described in Materials and Methods.

supernatant was collected and treated with dextran sulfate and calcium chloride to remove serum lipoproteins. After centrifugation, the waxy pellet was discarded and the clear supernatant was treated with an equal volume of saturated ammonium sulfate. The resulting precipitate was allowed to form over a 24-h period at 4 to 5°C. After centrifugation, the precipitate was dissolved in 0.1 M potassium phosphate.

Electron microscopy. A JEOL 100 CX electron microscopy was used for observation of the samples. Negative staining was performed with ammonium molybdate or phosphotungstic acid.

High pressure and fluorescence. The high-pressure bomb has been described by Paladini and Weber (19). Fluorescence spectra were recorded on a SLM-AMINCO photon-counting spectrofluorometer.

RESULTS

Pressure inactivation of VSV. Table 1 shows the effect of 260 MPa of pressure on the infectivity of VSV. When high titers of VSV were utilized (1.8×10^8 PFU/ml), incubation for 12 h at 260 MPa promoted 10^4 decrease in the infectivity. Lower-titer preparations of VSV were inactivated down to levels at which the infectivity assay was no longer reliable (Table 1). The infectivity of the purified preparation of VSV decreased more than 5 orders of magnitude (Table 1), a pressure inactivation even higher than that obtained with the nonpurified preparation. Table 1 also shows that the virus remained inactive after 2 weeks.

Immunogenic properties of pressure-treated VSV. The experimental protocol consisted in subjecting a VSV preparation to 260 MPa of pressure for 12 h. The preincubation titer was 4×10^6 PFU/ml, and the infectivity decreased to less than 25 PFU/ml after pressure treatment (same condition as column B of Table 1). Pressure-treated virus and control samples were injected into rabbits with the addition of complete Freund's adjuvant. Rabbits injected with the pressurized virus showed no additional side effects. Neutralization of native VSV by the gamma globulin fraction obtained from the immunized rabbits is shown in Table 2. The pressure-inactivated VSV elicited neutralizing antibodies as well as native VSV particles did. The 1:2 and 1:20 dilutions could neutralize a high-titer preparation of VSV (5×10^8 PFU/ml), decreasing the number of PFU by a factor greater than 10^7. The steep decline in neutralization observed between the serum dilutions of 1/20 and 1/200 is expected for the region of antigen excess, if a number of immunoglobulin molecules is necessary for the neutralization of infectivity of a single virus particle.

Table 2 also shows that neutralizing antibodies were not present in the sera of rabbits before immunization (preimmune sera). The titer of the neutralizing antibody appears to be the same for the anti-native VSV and anti-pressurized VSV, because an antibody dilution of 1:2,000 reduces the infectivity in both cases to 25%. We do not believe that the similarity of the immune response obtained with equal numbers of virus particles inactivated to the extent of 99.99% and with intact viruses can in anyway be attributed to the 0.01% of survivors. Neither the mass action that governs the chemical reactions in and out of organisms nor previous immunological experience would authorize such a conclusion. These results suggest that hydrostatic pressure promotes alterations that cause suppression of the infectivity without affecting the ability to elicit neutralizing antibodies.

Electron microscopy. Observations of VSV samples incubated for 12 h either at atmospheric pressure or at 260 MPa were performed. The samples incubated at atmospheric pressure or at 260 MPa were stained either by ammonium molybdate (Fig. 1) or phosphotungstic acid (Fig. 2). These figures show that the general morphology of the pressurized virus was not altered. In the pressurized virus, the membrane was preserved and the spike pattern of the membrane G protein could be visualized. A very consistent alteration in the virus surface was the presence of a bulge. This surface protrusion could be observed in all the pressure-treated viruses, whether they were stained with ammonium molybdate (Fig. 1) or phosphotungstic acid (Fig. 2) but not in the native particles. We surmise that this alteration is linked to the molecular processes under pressure that lead to inactivation (see Discussion).

Binding of an anionic fluorescent probe. Silva and Weber (28) showed that the fluorescent probe 4-4′-bis-1-phenylamino-8-naphthalenesulfonate (10, 22) at a concentration of 2 μM was poorly bound to brome mosaic virus at atmospheric pressure, as shown by the small increase in fluorescence over that of the free probe in water. An enhancement of the fluorescence was observed to begin at 150 MPa and reach a maximum of a factor of 10 over the initial fluorescence at 300 MPa. Figure 3 shows the fluorescence emission of a 2 μM solution of bis(8-anilinonaphthalene-1-sulfonate) (bis-ANS) in the absence (spectrum c) or presence of VSV at atmospheric pressure (spectrum b) and in the presence of VSV incubated at 260 MPa (spectrum a). Figure 4 shows the fluorescence enhancement by VSV as a function of pressure. Comparison of the fluorescence observed when the same

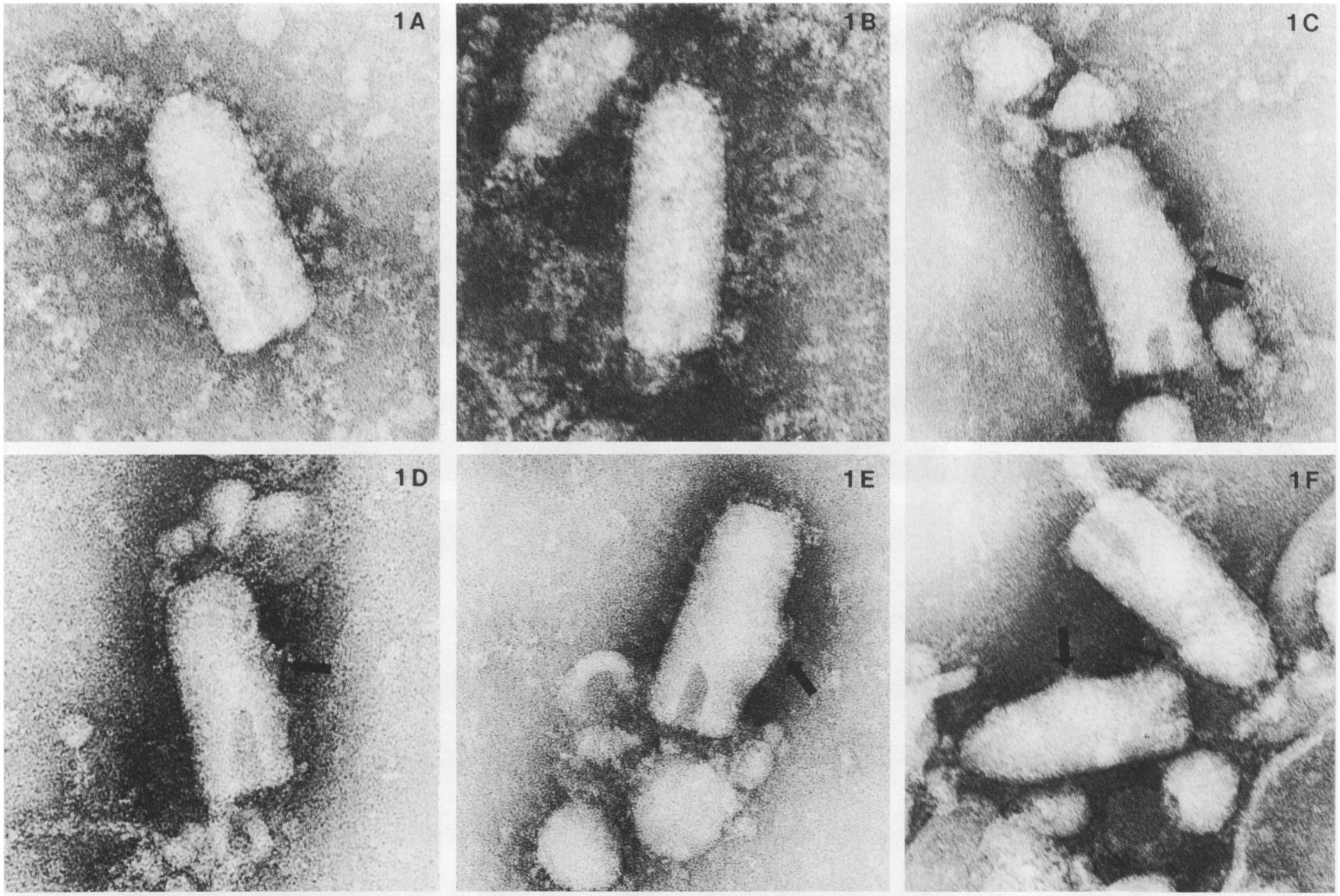

FIG. 1. Electron microscopy of pressurized VSV negative stained with ammonium molybdate. VSV (10^7 PFU/ml) was incubated for 12 h at atmospheric pressure (1A and 1B) or at 260 MPa (1C to 1F). The temperature was 20°C. The samples were negative stained with ammonium molybdate. The arrows indicate the protrusions in the pressurized virus. Magnification, ×276,000.

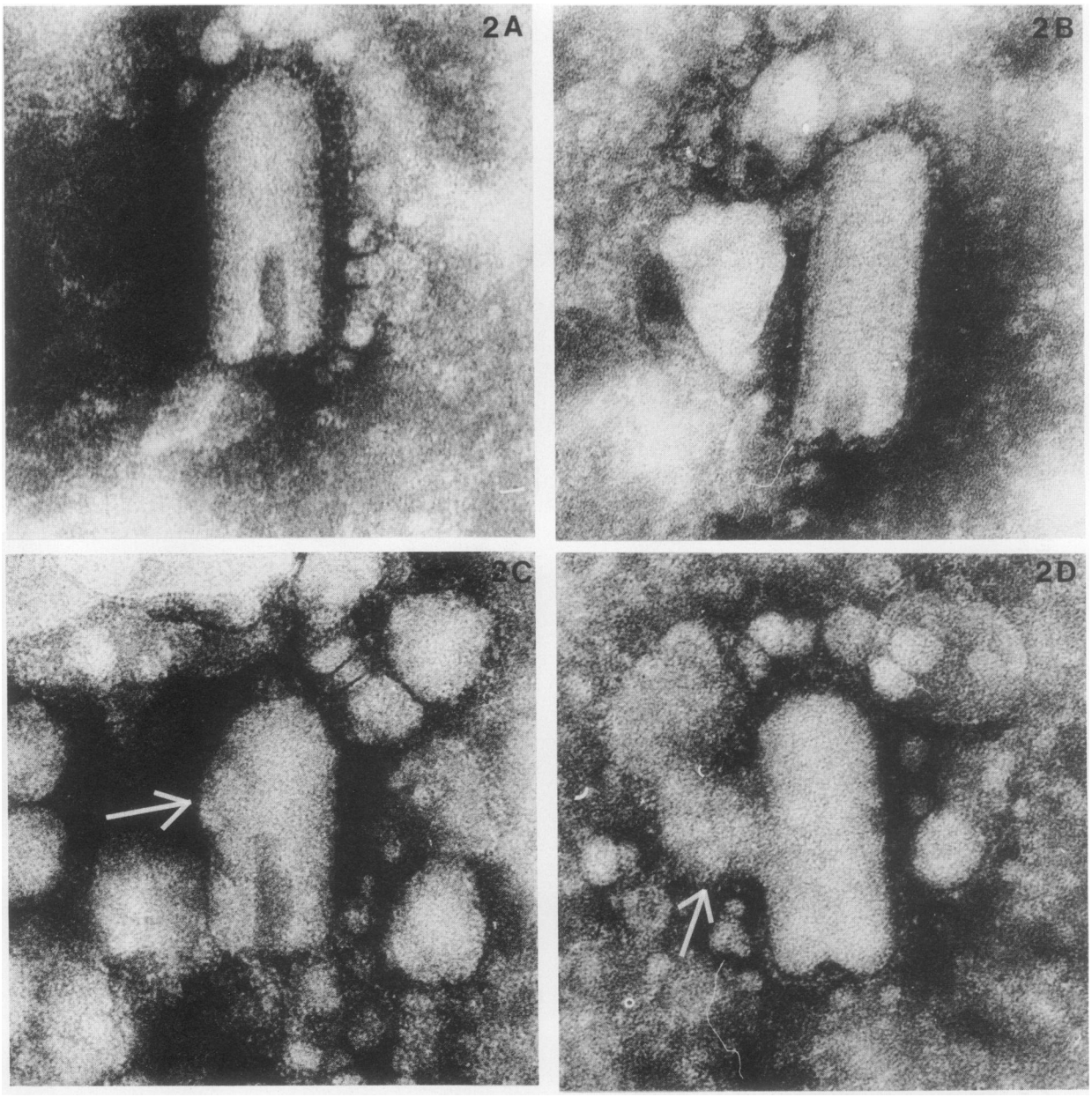

FIG. 2. Electron microscopy of pressurized VSV negative stained with phosphotungstic acid. VSV (10^7 PFU/ml) was incubated for 12 h at atmospheric pressure (2A and 2B) or at 260 MPa (2C and 2D) before negative staining with phosphotungstic acid. The arrows indicate the protrusions in the pressurized virus. Magnification, ×300,000.

concentration of bis-ANS is added to suspensions of brome mosaic virus (Fig. 10 of reference 28) and VSV show that the fluorescence intensity is considerably higher in the latter case. The appreciably greater fluorescence signal at atmospheric pressure in comparison with brome mosaic virus is undoubtedly due to the partition of some bis-ANS into the membrane. The further increase between the pressures of 100 and 240 MPa is to be attributed to the disruption in both viruses of the interactions between subunits or between protein and nucleic acid, and the appearance of sites (probably of basic character) at which bis-ANS is bound. Similar effects were observed employing the related fluorescence probe 4′-butyl-1-anilino-8-naphthalenesulfonate (butyl-ANS). Fluorescence observations like these appear useful in providing a simple means of assessing the magnitude and the range of pressure at which disruption of the particles takes place.

DISCUSSION

The experiments described above provide the first demonstration that hydrostatic pressure can inactivate a membrane-enveloped virus while preserving the immune response. The effects observed in VSV must be interpreted by reference to the extensive studies of the last few years on the high-pressure effects upon oligomeric proteins and icosahe-

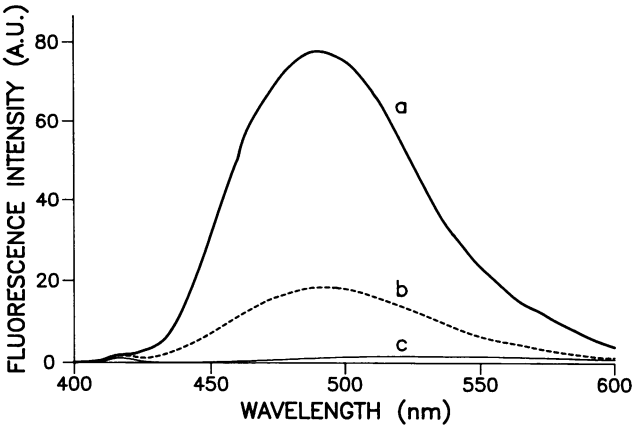

FIG. 3. Fluorescence spectra of 2 μM bis-ANS free in solution (line c) or in the presence of VSV at atmospheric pressure (line b) or at 260 MPa (line a). The excitation wavelength was 360 nm. The VSV concentration was 50 μg/ml. The temperature was 20°C. A.U., arbitrary units.

dral viruses. In the oligomeric proteins and some viruses, dissociation of protein subunits takes place under pressure and is followed by complete or partial reassociation on decompression. The differences observed in the reversibility of the dissociation and in regaining the native properties are determined by the number of subunits and the complexity of their interactions in the original aggregates. Thus, the rapid reappearance of the enzyme activity on decompression, independently of the temperature is the rule in dimers (enolase [18], β_2 tryptophan synthase [25], hexokinase [23], and Arc repressor [26]). In tetramers (lactate dehydrogenase [14] and glyceraldehyde phosphate dehydrogenase [24]), the reappearance of activity and spectral properties upon decompression is much slower, and depending on time and magnitude of the previous pressure application, it may take hours to days. It is even possible to indefinitely delay the recovery of enzyme activity in tetramers by keeping the preparations at 0°C (24). In oligomeric proteins of many subunits, reassociation is very slow upon decompression, particularly if sufficiently high pressures are reached and kept for an appropriately long time (27). All these observations are qualitatively explained on the assumption that when the subunits are out of contact with each other, they undergo structural changes that have been termed "conformational drift" (14, 23–28, 31, 32). Reversal of the conformational drift takes place progressively more slowly the higher the structural requirements and the number of contacts of each subunit with its neighbors in the original particle.

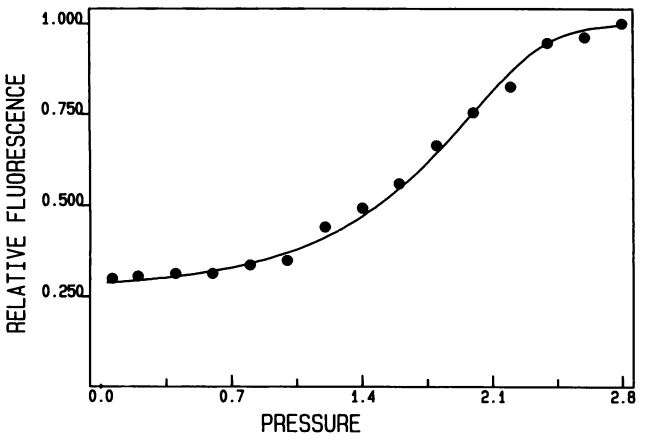

FIG. 4. Effect of pressure (10^2 MPa) on the fluorescence of bis-ANS bound to VSV. Other conditions are as described in the legend to Fig. 3.

Several icosahedral viruses are uniformly dissociated by pressure. In brome mosaic virus, imperfect reassembly occurs depending on the extent of dissociation (28). In simian rotavirus (SA11), only the outer protein capsid shell is dissociated by pressure and return to atmospheric pressure is followed by reassembly, ensuing a noninfectious particle (20). Imperfect assembly occurs also in the papillomavirus simian virus 40 after a cycle of dissociation and reassociation (28a). All the pressure studies performed on viruses reveal a peculiar characteristic: the pressure effects, when present, occur in the range 100 to 250 MPa and are virtually independent on virus concentration. Oligomeric proteins that are made up of many subunits like erythrocruorin (27) and hemocyanin (12) show similar independence of pressure effects upon the concentration. The only explanation so far envisioned is that virus particles and large aggregates are extremely heterogeneous, resulting in a "thermodynamic individuality" (9, 27) of the particles. This individuality of the particles arises in differences in conformation and needs to be distinguished from that owing to genetic variations of the virus. Existence of the latter type has been recently confirmed by observations on a slightly more complex multisubunit system. Brauch et al. (1) were able to select pressure-resistant variants of T4 phage by replating the phage that survived repeated pressure applications.

The appearance of a defective virus particle after pressure treatment is analogous to the formation of oligomers that had undergone conformational drift (28, 32). Virus particles present added complexities absent in the oligomeric proteins, namely, the interactions of the capsid with the nucleic acid and interactions of the capsid with the associated lipids in the membrane-enveloped viruses. As a result, we expect virtually complete irreversibility of the pressure effects in membrane-enveloped viruses.

In the assays of pressurization as well as in the studies of immunogenicity, we used high densities of virus particles (10^6 to 10^8 particles per ml) because only such high densities permit a reliable estimate of the reduction of the infectivity by the pressure and the determination of measurable antibody concentrations in a reasonably short time. We are aware that infectivity was not yet reduced to the extent necessary in a vaccine that could be safely applied to humans, but there is reason to believe that this can be achieved by either repeated or lengthier pressure application or by a combination of pressurization and chemical treatments that are ineffective at atmospheric pressure. These studies are being presently pursued.

The long time necessary to achieve complete inactivation (hours) is presumably related to the need of time-dependent structural fluctuations that cooperate with the hydrostatic pressure in disrupting the normal subunit association. For a membrane-enveloped virus like VSV, these necessary fluctuations will involve the contacts of membrane proteins with lipids and RNA, besides the interactions of the capsid subunits of the simpler icosahedral viruses. It has been demonstrated that the VSV membrane and probably the G protein are bridged to the capsid protein N through a basic

protein (M) (11, 34). These interactions seem to be of electrostatic nature, which cause them to be sensitive to pressure dissociation.

The high titer of the neutralizing antibodies elicited by pressure-inactivated VSV (Table 2) indicates that hydrostatic pressure can be used to prepare whole-virus immunogens. Pressure-inactivated rotaviruses were also able to elicit a high titer of neutralizing antibodies (20). In both cases, hydrostatic pressure reduces dramatically the infectivity, with minimal effects on the overall structure and hydrodynamic properties of the particles.

In some cases, chemical inactivation by formalin has provided highly efficient immunization (Salk's poliovirus vaccine), but in other cases it has resulted not only in insufficient immunity protection but in more severe forms of the disease upon exposure to native virus (e.g., measles virus [3] and respiratory syncytial virus [13]) which apparently occurred because formalin treatment destroyed one of the antigens. These problems are unlikely to arise when pressure is used. As the inactivation of the viruses results exclusively from the altered physical disposition of the parts, without changes in covalent chemistry one would not anticipate a loss of the immunogenic properties. For a membrane-enveloped virus, an enhancement of immunogenity of the whole particle after pressure treatment may exist because of the exposure of antigenic sites buried in the membrane or hidden by strong subunit interactions. Generally we expect that natural selection favors the virus varieties in which antibody-eliciting epitopes are masked and that the irregular conformational drift of the subunits will lead to their unmasking, resulting in an increase in desirable immunogenic properties. Both rotavirus and VSV are already strongly immunogenic as intact particles, which means that the question of the effect of conformational drift on immunogenicity remains open. To clarify this question will require a study of the immune response to poorly immunogenic viruses before and after pressurization.

Hydrostatic pressure is known to promote a decrease in lipid fluidity of biological membranes (5), which would favor both extrusion from the membrane and changes in the association of integral membrane proteins. Exposure to high pressures has been claimed to promote tumor antigenicity in EL-4 leukemia cells (21).

The high-pressure approach would have important advantages over other methods of vaccine preparation. The traditional attenuation approach has the drawback that the attenuated viruses, as distinct from fully inactive ones, may cause the disease that they are intended to prevent or worsen the actual disease. Immunization with isolated subunits is another method for vaccines that has been recently developed. To date, such method has been successful with hepatitis B and influenza virus vaccines (16). One disadvantage of the subunit procedure is that one or a few viral proteins may fail to elicit an adequate antibody response because the immune system recognizes the isolated antigen less effectively than the whole virus. For example, in poliovirus, the isolated capsid protein VP1 elicits only low titers of neutralizing antibodies (6). A more dramatic instance in which vaccination with a recombinant virus that carried only one of the antigenic proteins caused the disease was recently reported (17).

The observations on VSV reported in this paper indicate that hydrostatic pressure provides an efficient means of virus inactivation without loss of immunogenicity. Studies are in progress to test the applicability of this conclusion to other viruses. If generally valid, the high-pressure method could provide a simple and inexpensive means to produce effective antiviral vaccines.

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