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Phil. Trans. R. Soc. Lond. B 1976 **276**, 63-80
doi: 10.1098/rstb.1976.0098

Phil. Trans. R. Soc. Lond. B. **276**, 63–80 (1976) [63]
Printed in Great Britain

Structure and assembly of lipid-containing viruses, with special reference to bacteriophage PM2 as one type of model system

By R. M. Franklin, Rosmarie Hinnen, R. Schäfer and N. Tsukagoshi
Department of Structure Biology, Biocentre, University of Basel, Klingelbergstrasse 70, CH-4056 *Basel, Switzerland*

[Plates 16 and 17]

The simpler lipid-containing viruses (influenza, Semliki Forest, PM2) may have a phospholipid bilayer sandwiched between an outer shell of protein and an internal nucleocapsid possessing helical or icosahedral symmetry. Extensive physical and chemical studies have enabled us to form a more detailed picture of the structure of bacteriophage PM2 and controlled stepwise degradation of the virion has helped us to localize the four viral proteins. The surface protein (II) of PM2 is basic and interacts with the acidic phosphatidylglycerol of the bilayer to stabilize the membrane. The nucleocapsid protein (III) has proteolipid characteristics and may interact with the phospholipids in a hydrophobic fashion. The spikes are formed from protein I and the fourth protein (IV) is closely associated with the DNA. It is possible to reassemble the virus by reversing the degradation steps. Assembly has been especially useful in revealing the processes whereby the proteins and lipids interact to form the bilayer. Furthermore, results of *in vivo* studies of phospholipid synthesis and both *in vivo* and *in vitro* studies of viral protein synthesis have enabled us to form a reasonably complete picture of the biosynthesis of PM2.

In a recent description of the assembly of influenza virus, Compans and co-workers (1974) summarized the structural features of lipid-containing animal viruses which bud from the plasma membrane. The following summary is a paraphrase on that of Compans and co-workers (1974) and includes some differences and our own interpretations.

(1) Lipid content of 20–30 %. In one case (Harrison *et al.* 1971 *b*) the lipid has been shown to be arranged as a bilayer. In other cases (Landsberger, Lenard, Paxton & Compans 1971; Landsberger, Compans, Paxton & Lenard 1972; Landsberger, Compans, Choppin & Lenard 1973) the bilayer arrangement has been inferred from electron spin resonance studies, an inference which cannot be made in the cases studied where the rotational motion of the hydrocarbon chains is relatively slow. Indeed, under certain experimental conditions hexagonal arrays of lipid molecules may give rise to e.s.r. spectra similar to those obtained from viruses (Boggs & Hsia 1973). Only in cases where the lateral diffusion of the hydrocarbon chains is fast is it possible to distinguish a hexagonal phase from a bilayer (Seelig & Limacher 1974).

(2) Surface projections formed from one or more glycoproteins; these may penetrate the bilayer.

(3) The remaining polypeptides, chiefly found in the nucleocapsid (which can possess either icosahedral or helical symmetry), are non-glycosylated.

(4) The lipid composition of the virion is very similar, *but not identical*, to that of the plasma cell membrane (cf. Franklin 1974). The viral lipids are derived from the plasma cell membrane (cf. Franklin 1962). The viral structural proteins are, in general, viral-specific.

The lipid-containing animal viruses which bud from the plasma membrane form only one group of lipid-containing viruses, admittedly the largest group. But there are also lipid-containing animal viruses which bud from the nuclear membrane or from the membranes of the Golgi complex or other cytoplasmic membranes. Also there are lipid-containing animal viruses which form their lipid-containing components, assumed to be membranes, *de novo* and apart from any cellular membranes. And finally there are lipid-containing viruses of plants and bacteria. Therefore the above principles should be extended, as follows, to include all of the lipid-containing viruses.

(1′) The lipid content of some lipid-containing viruses may be as low as 9 or 10%. The important question with relation to lipid content is whether there is enough lipid to form a bilayer of the volume calculated to be that of the presumed bilayer. If this is not so, then there may be a considerable amount of protein in the bilayer, as in the case of bacteriophage PM2 (see below).

(2′, 3′) The surface of the virion may consist of a regular array, icosahedral in symmetry in the case of isometric particles. This may or may not appear in the form of surface projections and in some cases the proteins of the surface are not glycoproteins. In the icosahedral cytoplasmic deoxyviruses and in bacteriophage PM2 there do not seem to be *any* glycoproteins, for example.

(4′) The lipid composition of some lipid-containing viruses is very different from that of the host cell and in such cases is probably determined by interactions between lipids and viral proteins. Such lipid–protein interactions are probably quite general in biological membranes.

In this paper we will tell the story we know best, that of the structure and assembly of the lipid-containing bacteriophage PM2. We will attempt, however, to bring this story into the general one of the assembly of lipid-containing viruses and into the more general picture of the protein–lipid interactions involved in the assembly and stabilization of biological membranes.

The composition and structure of bacteriophage PM2

For most physical analyses, bacteriophage PM2 can be treated as a uniform spherical particle in solution. It has a hexagonal appearance in electron microscope projections with the suggestion of icosahedral symmetry elements (Silbert, Salditt & Franklin 1969). Unfortunately it has not been possible to unequivocally demonstrate morphological units on the surface of the particle and therefore it has not been possible to demonstrate the assumed icosahedral symmetry. The average diameter of the particle is 60 nm as determined by electron microscopy, low angle X-ray scattering and neutron diffraction (cf. Franklin 1974). The particle mass is between $(45.0 \pm 0.8) \times 10^6$ (Camerini-Otero & Franklin 1975) and $(45.1 \pm 1.8) \times 10^6$ according to calculations based on a number of equilibrium and transport physicochemical measurements (cf. Franklin 1974).

Before considering the structure of PM2 in detail it is necessary to discuss the chemical composition (table 1). The genetic material is a double-stranded circular superhelical DNA of molecular mass $(6.3–6.4) \times 10^6$. The molecular mass calculated by Camerini-Otero & Franklin (1975) is $(6.26 \pm 0.24) \times 10^6$ but $(6.3–6.4) \times 10^6$ is a more realistic value, considering the errors involved. The most recent calculation of the number of superhelical turns per native

PM2 DNA depends on the reevaluation of the unwinding angle ϕ_e as being 26° instead of 12° (Wang 1974). By using this value and the molar binding ratio of ethidium bromide per nucleotide of $\nu_c = 0.066$ (Wang 1974), then the superhelical density is $\sigma = -20\,\nu_c\,(\phi_e/360) = 0.095$ and the number of superhelical turns per DNA molecule is about 90.

TABLE 1. CHEMICAL COMPOSITION OF BACTERIOPHAGE PM2

DNA	14.3% by mass mol. mass $6.3–6.4 \times 10^6$ 42–43% GC content
lipid	12.6–14.0% by mass
phospholipid	64% phosphatidylglycerol, 27% phosphatidylethanolamine, 1% compound X
neutral lipid	7–8%
fatty acids	3.0% $C_{14:0}$, 12.0% $C_{16:0}$, 56.6% $C_{16:1}$, 7.3% $C_{17:cy}$, 13.9% $C_{18:1}$, 7.2% other
protein	72% by mass

The lipid content is approximately 13%, of which over 50% is phosphatidylglycerol. The exact composition, as given in table 1, is that found for PM2 grown in wild type cells on a synthetic medium (Braunstein & Franklin 1971; Camerini-Otero & Franklin 1972). This composition can change, however, depending on the fatty acid composition of the virion (Tsukagoshi, Peterson & Franklin 1975*a*, *b*; see below), but is always very different from that of the host cell which has about 70% phosphatidylethanolamine as its major phospholipid (Braunstein & Franklin 1971). Besides PG and PE there is also a minor phospholipid, originally called compound X (Braunstein & Franklin 1971). Compound X did not have the same R_f value as *B. subtilis* cardiolipin (Braunstein & Franklin 1971) or mammalian cardiolipin, but did have the same R_f value as *E. coli* cardiolipin (Tsukagoshi & Franklin 1975). Furthermore, if PG from *Pseudomonas* BAL-31 was incubated with a membrane fraction from *E. coli*, the product had the same R_f as compound X and *E. coli* cardiolipin (Tsukagoshi & Franklin 1975). These two observations lead us to imagine that compound X could be cardiolipin (cf. Franklin 1974). But no cardiolipin synthetase activity could be detected in either the total membrane fraction or the inner membrane fraction of *Pseudomonas* BAL-31 (Tsukagoshi *et al.* 1975*b*). Furthermore, alkaline hydrolysis of compound X yields glycerolphosphorylglycerol (Braunstein & Franklin 1971). Compound X seems to have the same R_f value as acyl phosphatidylglycerol from *S. typhimurium* in some solvent systems, but not in all (Diedrich & Cota-Robles 1974). Therefore compound X may be acyl phosphatidylglycerol but further chemical analysis will be necessary for an unequivocal proof.

The fatty acid composition of PM2 is the same as that of the host cell where the presence of 57% $C_{16:1}$ is characteristic of marine pseudomonads (cf. Franklin 1974). The fatty acid composition of the host can be drastically altered by forcing individual fatty acids into an unsaturated fatty acid auxotroph of BAL-31 (Tsukagoshi *et al.* 1975*a*, *b*). The fatty acids of the viral phospholipids are also similar to those of the host in this case but the yield of virus is only 40–60% of the yield of virus grown in wild type cells having the normal spectrum of fatty acids (Tsukagoshi *et al.* 1975*b*).

There are four proteins in the virion and their molecular masses are presented in table 2. We will have more to say about these proteins later.

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Several enzymatic activities seem to be associated with the virion:

(1) The DNA-dependent RNA polymerase activity associated with the whole virion (Datta & Franklin 1972) is actually a rather unusual polynucleotide-dependent polynucleotide-pyrophosphorylase (Schäfer & Franklin 1975*b*). Protein IV, which is associated with the viral DNA (see below), is the protein responsible for this enzymatic activity, probably in the form of a dimer (Schäfer & Franklin 1975*b*). So far the function of this enzyme is not understood. Since the isolated viral DNA is infectious in spheroplasts (van der Schans, Weyermans & Bleichrodt 1971), the enzyme is not essential for initiation of infection, as are the viral-associated RNA-dependent RNA or DNA polymerases (cf. Baltimore 1971*a*, *b*). The enzyme probably does serve an important function in viral replication, however.

TABLE 2. MOLECULAR MASS OF PM2 PROTEINS

protein	no. AA†	mol. mass (AA)‡	mol. mass (p.a.g.e)§	moles protein per mole virion
I	398	43640	43000	80
II	246	27310	26000–27000	820
III	129	13250	12500	500
IV	61	6480	4650	300

† Number of amino acids based on a comparison of p.a.g.e. molecular masses with amino acid composition (Hinnen *et al.* 1975*b*).

‡ Molecular mass calculated from (*a*).

§ Molecular masses determined by SDS polyacrylamide gel electrophoresis (Schäfer *et al.* 1974).

(2) An endolysin activity, measured by the hydrolysis of ^{3}H-murein prepared from *E. coli* W945T3282 (a mutant requiring several amino acids and which is diaminopimelate decarboxylase negative) in the presence of ^{3}H-diaminopimelate, is also associated with purified virus preparations (Tsukagoshi & Franklin 1975). There is an increase in activity in frozen-thawed preparations and the activity is also found in the infected host cell but not in the uninfected control.

(3) An endonuclease activity which converts PM2 superhelical DNA into a linear form has also been reported to be associated with the virion (Laval 1974). The activity is the same in the presence or absence of non-ionic detergents and could well be a host cell contaminant. We have confirmed this supposition while testing virus in different stages of purification for endonuclease activity according to Laval (1974). We used our standard purification procedure (Hinnen, Schäfer & Franklin 1974). After the second CsCl gradient step, endonuclease activity could still be found in the virus band. After the last step in purification, however, no endonuclease activity remained associated with the virion fraction. In this step in which the virus is sedimented in a sucrose gradient in the presence of 3 M NaCl, all remaining host cell components, membrane fragments in particular, are separated from the virus fraction (Hinnen *et al.* 1974).

The structure of PM2 has been derived from a combination of physical and chemical studies (cf. Franklin 1974) and these are still continuing in an effort to obtain a fundamental understanding of the various macromolecular and protein–lipid interactions involved in the formation and stabilization of this rather complex virus particle (figure 1). The nucleocapsid extends to about 20 nm radius according to the electron density distribution obtained from low angle X-ray diffraction (Harrison, Caspar, Camerini-Otero & Franklin 1971*a*). The nucleocapsid shell is formed from protein III; protein IV is associated with the DNA (Hinnen *et al.* 1974).

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External to the nucleocapsid is the phospholipid bilayer, extending from 20 to 24 nm and external to this is the outer protein shell formed by protein II (Hinnen *et al.* 1974); this shell extends to a radius of 30 nm. Forked spike-like structures formed from protein I project from the vertices of the icosahedral-like shell. They are approximately 2–3 nm in diameter and 5–6 nm long. These structures are the sites of attachment of the virus to its host cell (Hinnen *et al.* 1974). Up to 30–50 % of the bilayer volume may be occupied by protein (Harrison *et al.* 1971*a*; Camerini-Otero & Franklin 1972) and this protein could be an extension of the nucleocapsid shell into the bilayer (see below). Protein II may also extend into the bilayer (see below).

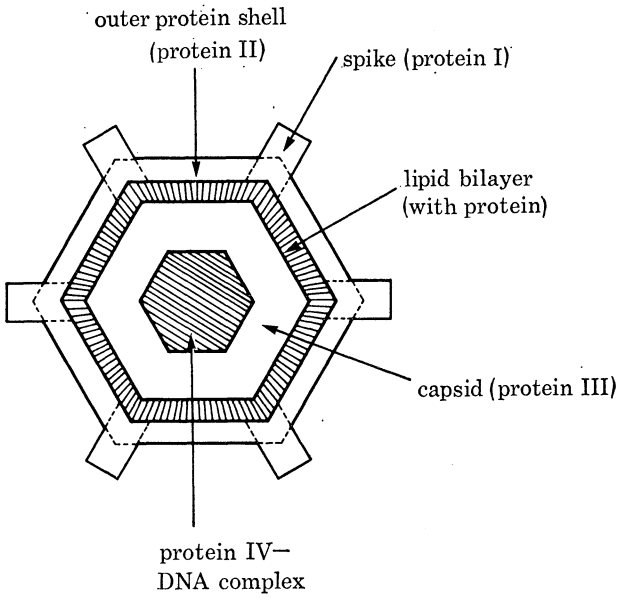

FIGURE 1. Model of the structure of bacteriophage PM2 showing the localization of the viral proteins.

PROTEIN-LIPID INTERACTIONS IN BACTERIOPHAGE PM2

Whereas the isoelectric points of the PM2 proteins I, III, and IV are all slightly acidic, protein II is strongly basic and the basicity is partly due to the presence of Ca^{2+} ions in the protein (Schäfer, Hinnen & Franklin 1974, cf. table 3). The isoelectric point of 12.3 is measured in the presence of 6 M urea plus 10 mM $CaCl_2$; the pI dropped to 12.1 when the $CaCl_2$ was replaced by 10 mM EDTA. After dialysis versus 6 M guanidine HCl plus 10 mM EDTA and then electrophoresis in 6 M urea plus 10 mM EDTA, the isoelectric point was 9.0. Thus protein II does not seem to be completely denatured in 6 M urea, certainly the Ca^{2+} binding sites do not seem to be affected in this solvent. The basicity of protein II depends on the amino acid composition as well as the tightly bound Ca^{2+}. Besides the amino acid composition, we have determined a sequence of the initial 39 amino acids from the *N*-terminus (Hinnen *et al.* 1975). In this sequence at least 7 of the amino acids are basic and of the 5 aspartates in this sequence, 4 are in the form of asparagine.

A great many Ca^{2+}-binding proteins are known and Ca^{2+} may play an extremely important role in regulation of certain physiological processes, many of which occur in membranes (cf. Kretsinger 1974, 1975). The only other known Ca^{2+} binding protein with a high pI is

the anthozoan luciferin binding protein (cf. Kretsinger 1975) and therefore PM2 protein II should be of general interest to those who are studying Ca^{2+} binding proteins.

At neutral pH, which is the isoelectric point of the virion, protein II should be positively charged and therefore interact electrostatically with the negatively charged PG. This electrostatic interaction might be involved in both the assembly process and further stabilization of the virion. Ca^{2+} is required for PM2 virus synthesis and can be replaced by Sr^{2+} and Ba^{2+} but not by a number of other divalent cations (Snipes *et al.* 1974). The crystal ionic radii of these three cations are 97, 112, and 134 pm, respectively, but the hydrated ionic radii are considerably larger (118, 132, and 153 pm, respectively). Thus on the basis of size alone, it is difficult to understand why Sr^{2+} or Ba^{2+} can replace Ca^{2+} in the synthesis of bacteriophage PM2, as they can replace Ca^{2+} in the process of cell fusion induced by Sendai virus (Okada & Murayama 1966).

TABLE 3. PROTEINS OF PM2: COMPARISON WITH MYELIN†

protein	*N*-terminus	cysteine	absences	pI	⟨HΦ⟩‡	R1§	R2§	R3§
PM2-I	Thr	2	none	6.2	983	1.7	1.9	1.2
PM2-II	Met	2	none	12.3	1139	1.3	1.5	1.0
PM2-III	Phe	1	Tyr, His	5.8	1019	1.5	1.5	1.0
PM2-IV	Met	1	His	5.5	1241	1.1	1.2	0.7
myelin-PL	n.d.	12	none	n.d.	1186	0.88	1.0	0.52
myelin-Wolfgram	n.d.	4 or 5	none	n.d.	1002	1.69	1.88	1.31
myelin-Al	Ac-Ala	0	Cys	10.8‖	862	2.80	3.24	2.03

† Data on myelin from Barrantes (1973), Folch-Pi (1973), and Chan & Lees (1974). The number of cysteines in myelin-PL and myelin-Wolfgram proteins are based on amino acid analyses given by Barrantes (1973) and Folch-Pi (1973) and on molecular masses of approximately 30 000 for PL and 45 000 or 50 000 for the (2) Wolfgram proteins(s) as given by Chan & Lees (1974). The parameters ⟨HΦ⟩, R1, R2, and R3 for myelin are those calculated by Barrantes (1973). PL, proteolipid, Wolfgram, Wolfgram proteolipid, Al, basic protein of central nervous system myelin. In general the data refer to bovine white matter or central nervous system (c.n.s.) myelin; there are some differences when c.n.s. and peripheral nervous system myelin are compared and when species are compared (cf. Barrantes 1973; Eylar 1973; Folch-Pi 1973). n.d., not done.

‡ ⟨HΦ⟩ is the average hydrophobicity of the protein calculated from the Gibbs free energy of transfer of the individual amino acids from water to ethanol (cf. Tanford 1973; Bigelow 1967).

§ R1 is the ratio of hydrophilic to hydrophobic amino acids, R2 is the ratio of hydrophilic to apolar amino acids, and R3 is the ratio of total charged to hydrophobic amino acids

‖ Schäfer & Franklin (1975*c*), A1 protein isolated from human white matter.

Proteins I and IV are soluble in water whereas proteins II and IV are only soluble in high concentrations of urea. Proteins III and IV are soluble in methanol–chloroform–water (2:1:0.8, by vol.) where the water fraction is actually NTC, a buffered 1 M NaCl solution (Schäfer *et al.* 1974). Proteins soluble in chloroform: methanol (2:1, by vol.) in the presence of salt are called proteolipids (Folch & Lees 1951; Folch-Pi 1973). Since PM2 proteins III and IV are soluble in organic solvents, we also tested their solubilities under the conditions described by Folch & Lees (1951). Purified PM2 in NTC was mixed with chloroform–methanol in the ratio chloroform:methanol:NTC of 2:1:0.15 (by vol.). The soluble proteins and the proteins in the insoluble residue were identified by p.a.g.e. Proteins III and IV were soluble, proteins I, II, and the DNA, precipitated. Thus proteins III and IV can be called proteolipids. Protein III, the nucleocapsid protein, would be a likely candidate for hydrophobic interaction with the bilayer. *Thus our working hypothesis is that a combination of electrostatic and hydrophobic protein–lipid interactions contribute to stabilization of the PM2 bilayer; whereas protein II is primarily involved with electrostatic interactions, protein III may be involved in hydrophobic interactions.* The electro-

static interactions are selective whereas the hydrophobic interactions are relatively unselective (Schäfer & Franklin 1975*a*).

Electrostatic and hydrophobic interactions have also been postulated to be the major factors in stabilizing the myelin membrane (London & Vossenberg 1973; Eylar 1973). In this case the basic protein of myelin may play a role equivalent to that of PM2 protein II (Eylar 1973; Mateu *et al.* 1973). This protein has an isoelectric point of 10.8 in the absence of calcium (cf. table 3).

Several experiments support our hypothesis concerning the PM2 protein–lipid interactions.

(*a*) Urea, in increasing concentrations, dissociates the virus stepwise (Hinnen *et al.* 1974). In 1 M urea two structures are stable – a spikeless virus or a nucleocapsid with phospholipid. In 4.5 M urea the nucleocapsid without phospholipid is stable. This latter structure remains stable up to about 8 M urea, at which molarity the nucleocapsid swells. Urea denaturation of polypeptides is a consequence of the thermodynamically favoured solubilization of the peptide group and non-polar side chains in increasing concentrations of urea as compared with water (Nozaki & Tanford 1963; Tanford 1964). In the case of PM2 we are dealing with protein–protein and protein–lipid interactions. Low concentrations of urea disrupt the interaction between spikes and the outer protein shell as well as that between the individual protein II polypeptides of the outer protein shell. Some protein II remains bound to the lipid-containing nucleocapsid, however. This would suggest that the main factors in keeping the spikes and outer protein shell intact are weak hydrophobic interactions. Continuing with this argument, stronger hydrophobic interactions between the bilayer and protein III are disrupted in higher concentrations of urea. Some phospholipid probably remains bound to protein II under these conditions (see (*c*) below) and some probably forms micelles in the urea (cf. Tanford 1972). Perturbations in the nucleocapsid protein–protein interactions are manifested only at very high urea concentrations.

(*b*) In a mixed liposomal vesicle of PG and PE, the thermodynamically favoured distribution would place PG in the outer lamella (Israelachvili 1973) but it is not easy to predict the distribution in a natural protein–lipid bilayer. Electrostatic interactions between protein II and the bilayer would require, however, the PG to be localized in the outer lamella of the bilayer and this has been demonstrated by chemical labelling experiments (Schäfer *et al.* 1974).

(*c*) A direct demonstration of electrostatic interactions between protein II and PG was made by studying the distribution of PG and PE in a two-phase system, the phospholipids being in chloroform and the proteins in 1 M urea (Schäfer & Franklin 1975*a*). In the first case a mixture of proteins I and II were used and in the second case a mixture of proteins III and IV were used. Since protein II was present in a tenfold higher concentration than protein I in case 1, we can assume that the effects observed were chiefly due to the former protein. In case 2 there was approximately twice as much protein III as IV so that the effects could be due to the mixture of the two proteins. In case 1, PG was preferentially distributed in the urea phase in the presence or absence of NaCl (up to 0.5 M) and also in the absence of exogenous Ca^{2+} (all at pH 7, i.e. under conditions below the pI of protein II) but the distribution was non-preferential at pH 11.5, in the complete absence of Ca^{2+}, i.e. above the pI of the protein in this condition. In case 2 the distribution in the urea phase was non-preferential in 0.5 M NaCl or 0.01 M NaCl (both with added Ca^{2+}). These results clearly demonstrate the electrostatic interaction between protein II and PG and suggest that the interaction of protein III (and possibly protein IV) with the phospholipids is hydrophobic in nature.

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(*d*) Investigations of the effect of cross-linking reagents on the virus have also supported the view that both electrostatic and hydrophobic interactions play a role in stabilizing the viral bilayer (Schäfer *et al.* 1975*a*). Glutaraldehyde, dimethylsuberimidate, and tolylene-2,4-diisocyanate all reacted with proteins III and IV. Also, in all cases, PE became associated with the protein III, IV fraction. Not only do these experiments indicate an intimate contact between protein III and PE, but also suggest an intimate contact between proteins III and IV. Since the cross-linking reagents used would not be expected to react with PG, nothing can be said concerning a possible contact between PG and protein III. Furthermore contact between protein IV and the bilayer is not ruled out. Such contacts must result in predominantly hydrophobic interactions because of the proteolipid nature of proteins III and IV and the urea effects mentioned above. Over half the protein IV sequence has been determined (Hinnen *et al.* 1975). The clustering of basic amino acids near the *N*-terminus would serve for interaction of this protein with the DNA; the central hydrophobic sequence might be involved in interactions with protein III or possibly directly with the bilayer.

After mild glutaraldehyde treatment, under conditions which lead to the cross-linking just described, there is an increase in the order parameter calculated from e.s.r. spectra, physically equivalent to a temperature decrease of 5° in the native membrane (Schäfer *et al.* 1975*a*). These effects are more pronounced in the outer regions of the bilayer and can also be interpreted in terms of the protein–lipid interactions just discussed.

There are two further remarkable features of these cross-linking experiments.

(1) None of the reagents cross-linked protein II to anything although there are 15 lysines per protein II polypeptide and lysine is certainly one of the major reactive sites for glutaraldehyde (Korn, Feairheller & Filachione 1972), if not for the other two cross-linking reagents as well. All of these reagents, as a matter of fact, do react with primary amino groups (Habeb & Miramoto 1968; Davies & Stark 1970; Wold 1972). The lack of reaction with protein II may be due to the strong electrostatic interaction between basic amino acids such as lysine and the PG of the bilayer. The model proposed by Eylar for the interaction of the basic protein of myelin (A1 protein) with the bilayer may be relevant here (Eylar 1973). There is a further parallel in protein–lipid interactions in the case of the myelin A1 protein and PM2 protein II. In the isolated state both proteins are readily digested with proteolytic enzymes but are protected from the action of such enzymes when combined with acidic lipids or when in their respective intact structures (Schäfer & Franklin 1975*d*). This protection could be partly due to strong electrostatic interactions between the proteins and the lipids and partly to a possible burial of a portion of the polypeptide in the bilayer in the natural state.

(2) Dimethyl adipimidate, which is closely related to dimethyl suberimidate, will react both with erythrocyte membrane proteins and with haemoglobin within the intact erythrocyte (Niehaus & Wold 1970). But considering the charge of these imidoesters, it is hard to imagine that they could pass through a pure phospholipid bilayer. Indeed the erythrocyte membrane is known to contain at least one protein which spans the membrane (Bretscher 1971). There is also evidence that the glycoproteins of Semliki forest virus span the viral membrane (Garoff & Simons 1974, see below) and in this case dimethyl suberimidate can cross-link the glycoproteins to the nucleocapsid protein which is located on the inner side of the membrane. The reaction of dimethyl suberimidate with PM2 proteins III and IV would suggest, therefore, that at least protein III must penetrate deeply into the bilayer and possibly form protein channels to the outer protein shell. A similar argument can be made on the basis of the

glutaraldehyde reaction with proteins III and IV since this is also a rather polar molecule. Tolylene-2,4-diisocyanate, on the other hand, is relatively apolar. The proposed presence of proteins II (see (1) above) and III in the bilayer might be the basis for the high electron density there (Harrison *et al.* 1971) and the disparity between lipid volume and bilayer volume (Camerini-Otero & Franklin 1972).

(*e*) Although [^{35}S]sulphanilic acid diazonium salt (DSA) will only label protein II when the intact virus is treated under mild conditions, protein III is labelled in concentrations of LiCl greater than 0.75 M. Li^+ has a very high field strength resulting from its very small ionic radius (cf. Stein 1962) and has the corresponding highest hydration of the alkali metal ions (Glueckauf 1955). Thus we imagine that Li^+ will chiefly interact with the PG of the outer lamella, weakening the interaction between protein II and PG, thereby allowing the diazonium salt to interact with protein III. Because of the size and charge (zwitterionic) of the diazonium salt, it is not likely that it can penetrate very deeply into the bilayer under any condition and therefore we have further indication for channels of at least protein III in the bilayer.

(*f*) A comparison of the e.s.r. parameters of PM2 with those of egg-yolk lecithin provided further evidence for protein–lipid interactions (Scandella, Schindler, Franklin & Seelig 1974). The high polarity, as measured by the isotropic hyperfine splitting constant, might be due to extension of some of the viral protein into the hydrocarbon region of the bilayer. This is further suggestive evidence for hydrophobic interactions between protein and lipid in PM2.

(*g*) As already mentioned, changes in the fatty acid composition of PM2 can result in changes in the phospholipid composition (Tsukagoshi *et al.* 1974*a*, *b*). To cite one example, if the fatty acid is approximately 90% *cis* 16:1 then there is about 53% PG in the virion; if it is about 90% *trans* 16:1 then there is only 42% PG present in the virion. Certainly the packing of acyl chains will be different in these two cases, and different from the situation in virus growth in wild type cells where there is only about 50% *cis* 16:1 fatty acid. One interpretation of this result is the possible effect of the different packing of the *cis* and *trans* chains on the hydrophobic interactions, leading to a stronger selection of PE in the bilayer, which is presumed to interact hydrophobically with protein III. Reconstitution of viral-like particles with phospholipids containing *cis* or *trans* fatty acids has provided further information on this problem (see below).

(*h*) The chemical properties of the PM2 proteins are compared with those of myelin in table 3. Most striking is the high isoelectric points of PM2-II and myelin A1. From the various parameters describing hydrophobicity, PM2-II has more hydrophobic amino acids than myelin A1. PM2 proteins II, III, and IV are comparable in hydrophobicity to myelin proteolipid. These parameters are, of course, only the crudest way to investigate the possible hydrophobic nature of a protein; this can only be determined from amino acid sequences and configuration (cf. Segrest & Feldmann 1974). Protein I, parenthetically, has the lowest average hydrophobicity, and this may be correlated with its position on the periphery of the particle where it does not seem to interact with the bilayer at all.

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Reconstitution of bacteriophage PM2

The stepwise degradation of PM2 in increasing concentrations of urea suggested a method for reconstitution of the virus from its subunits (Schäfer & Franklin 1975*a*). In the first step proteins III and IV and the viral DNA, which are not associated in 8 M urea at pH 4.5, were dialysed to 4.5 M urea at neutral pH with the ensuing formation of a nucleocapsid which was identical to the nucleocapsid formed by degradation of the virion. All properties investigated were the same for these two nucleocapsids – these included densities in CsCl, chemical compositions, A_{260}/A_{280} ratios, and specific infectivity for spheroplasts of the DNA isolated from the particles by phenol extraction. The ultrastructure of the two nucleocapsids was also the same (figure 2, plate 16).

Reconstitution took place in 4.5 M urea, 0.5 M NaCl, 10 mM $CaCl_2$, 20 mM β-mercaptoethanol, and 25 mM tris buffer (pH 7.5 at 4° C). Ca^{2+} could not be replaced by other divalent cations, including Mg^{2+} or Mn^{2+}. Recently Sr^{2+} and Ba^{2+} were also tested and these were also ineffective as substitutes for Ca^{2+} in the reconstitution of nucleocapsid (Tsukagoshi, Schäfer & Franklin 1975*d*). These cations have not been tested as yet, however, in the reconstitution of virus from nucleocapsid. The yield of nucleocapsid was low (2.3 %) but could be improved over tenfold to 33 % by the addition of bovine serum albumin to the reconstitution mixture. This apparently diminished the non-specific aggregation of proteins III and IV (see above). Recently we have been able to reconstitute nucleocapsid from the individual highly purified polypeptides III and IV (Hinnen *et al.* 1975*a*) rather than from the mixture of proteins III and IV. In this case there was an excellent yield of nucleocapsid (35 %) and no bovine serum albumin was necessary (Tsukagoshi *et al.* 1975*d*). This exciting result demonstrates unequivocally that nucleocapsid contains only proteins III and IV and that these proteins, recovered from SDS gel filtration (Hinnen *et al.* 1975*a*), can be renatured to their native configuration. Under the conditions used for such a reconstitution, protein III alone does not form a nucleocapsid shell but rather a non-specific aggregate. It may be that the DNA–protein IV complex forms a centre of nucleation necessary for the formation of the capsid. We may consider this reconstitution as a process driven by the difference in free energy of solution of the polypeptides in high and low urea concentrations (Nozaki & Tanford 1963). Entropy differences resulting from changes in water structure compared with enthalpy differences arising from protein–protein interactions would give rise to the negative free energy difference needed to force assembly (cf. Lauffer 1975).

Despite improvements in yield, the amount of nucleocapsid recovered was always very small. Since we had demonstrated the identity of the reconstituted nucleocapsid (n.c.) with that from the virion, we used the latter for the further steps in reconstitution. The next step was formation of a nucleocapsid with lipid (n.c.l.), formed by dialysing a mixture of nucleocapsid and phospholipids from the 4.5 M urea solution to a solution containing 1 M urea, 1 M NaCl, 0.01 M $CaCl_2$, and 25 mM tris buffer (pH 7.5 at 4 °C); n.c.l. was obtained in high yield with a lipid content 90 % of that expected on the basis of the lipid content of the virion. The lipid composition, however, was that in the incubation mixture, not that found normally in the virion. This is understandable on the basis of the postulated hydrophobic interactions between protein III and the bilayer. Furthermore n.c.l. particles could not be used for reconstitution of the infectious particle because protein II did not bind to such n.c.l. particles. In order to do this it was necessary to start with n.c. plus a mixture of phospholipids and proteins I

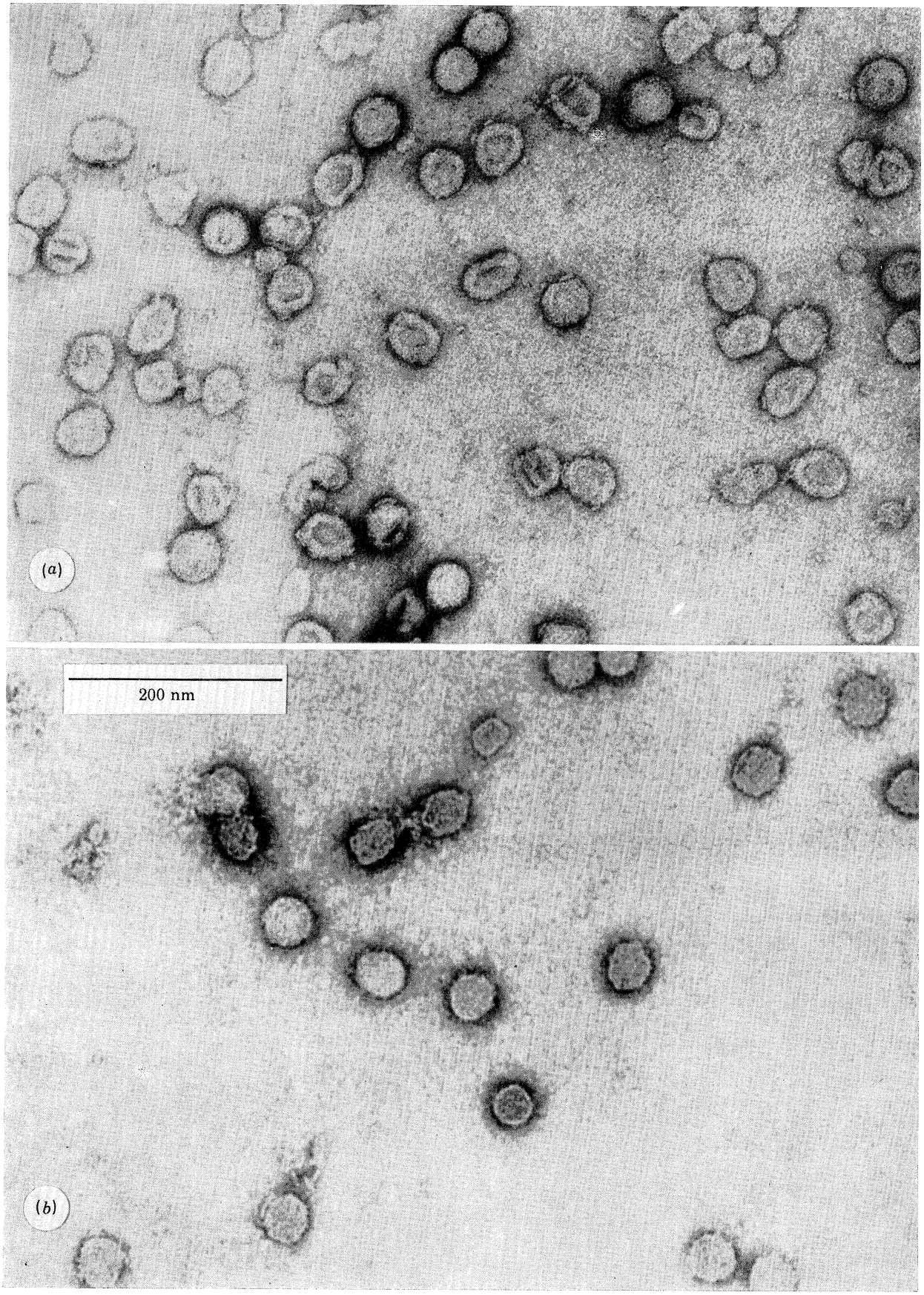

FIGURE 2. Nucleocapsid (*a*) prepared from virus by urea treatment and (*b*) reconstituted. In both cases the particles are unfixed, stained with uranyl acetate.

(*Facing p.* 72)

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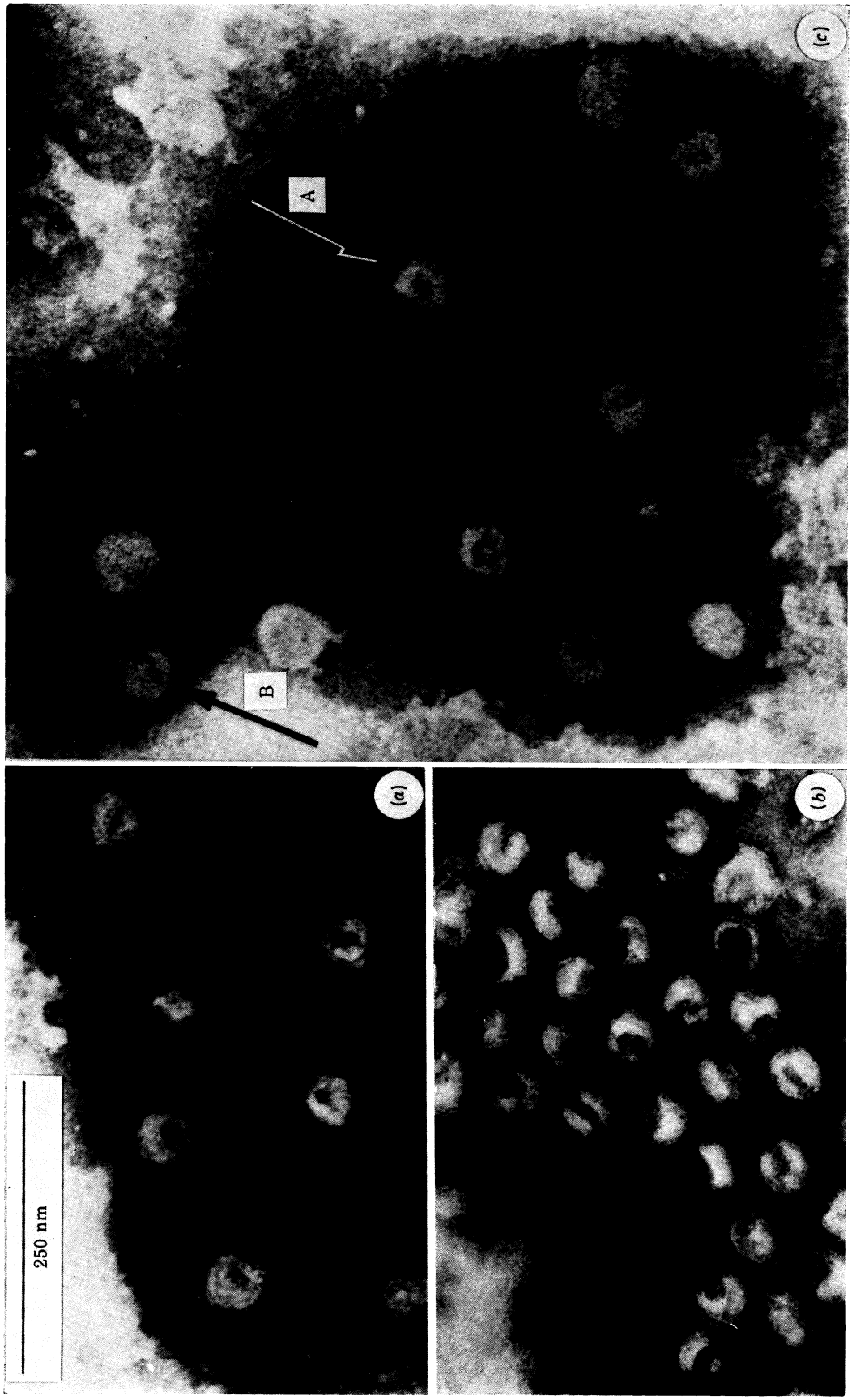

FIGURE 3. Virus and reconstituted virus-like particles. (*a*), (*c*) reconstituted particles. The arrows (A) and (B) in (*c*) point to particles where the spikes may be seen particularly well. (*b*) Standard preparation of purified virus. In all cases the particles are unfixed, stained with uranyl acetate.

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and II in 4.5 M urea and dialyse this mixture to a urea-free buffer (0.5 M NaCl, 0.01 M $CaCl_2$, 10 mM β-mercaptoethanol, 25 mM tris buffer (pH 7.5 at 4 °C). Infectious virus was obtained in the presence of 30–50 mg/ml of bovine serum albumin, which prevented nonspecific aggregation (figure 3, plate 17). Since the ratio of physical to infectious reconstituted particles was about 3×10^{-7} (as compared to 0.6 for our standard virus preparation), we must designate the particles seen in the electron microscope as virus-like (figure 3). The yield of infectious particles depended on the PG/PE ratio in the reconstitution mixture. The yield was higher if this ratio was similar to that in the virion than when the ratio was similar to that in the host cell. In both cases, however, the PG/PE ratio in the virion was similar to that in native virus. Here is further evidence of the interaction of protein II with PG and may form the basis for a better understanding of the physico-chemical processes involved in both *in vitro* and *in vivo* (see below) assembly.

TABLE 4. RECONSTITUTION WITH *CIS* AND *TRANS* FATTY ACIDS

fatty acid	phospholipid†	input‡	particle§	infectivity
cis 16:1	PE	69.1	40.6	2.5×10^3 p.f.u.
	PG	29.8	57.0	
	X	1.1	2.4	
cis 16:1	PE	36.7	41.5	2.6×10^3 p.f.u.
	PG	62.3	56.2	
	X	1.0	2.3	
trans 16:1	PE	61.8	35.0	0
	PG	30.3	55.0	
	X	7.9	10.0	
trans 16:1	PE	30.5	36.2	0
	PG	61.2	52.0	
	X	8.3	11.8	

† PE, phosphatidylethanolamine; PG, phosphatidylglycerol; X, compound X.
‡ Input phospholipid composition (%).
§ Phospholipid composition (%) in the isolated reconstituted particles. Reconstitution and isolation of particles was carried out as described by Schäfer & Franklin (1975*a*).

In recent reconstitution experiments the role of compound X and the effect of *cis* versus *trans* fatty acids was investigated (Tsukagoshi *et al.* 1975*d*). NC derived from virus by urea treatment was mixed with proteins I and II, and various mixtures of lipids which were isolated from *Pseudomonas* BAL-31, strain UFA, our unsaturated fatty acid auxotroph, which had been grown in the presence of [^{32}P]phosphate and either *cis* 16:1 or *trans* 16:1 fatty acid (Tsukagoshi *et al.* 1975*a*, *b*). In the case of phospholipids containing approximately 90% *cis* 16:1 fatty acid, the yield of reconstituted infectious virus was the same in the presence or absence of compound X. In the comparison of *cis* versus *trans* fatty acids, particles which could be isolated by centrifugation were formed in both cases but only those formed with phospholipids containing *cis* fatty acids were infectious (table 4). In the experiment shown in table 4, the input percentage of compound X was high in the case of *trans* 16:1 in order to simulate the conditions found *in vivo* (Tsukagoshi *et al.* 1975*b*). In another experiment (not shown), an input percentage of 3% compound X was used and also in this case no infectious particles were formed. The ratio of PG to PE seemed to be controlled by protein II in both the *cis* and *trans* cases, as already described above. Also the amount of compound X in the particles was higher than in the input mixture and this might be due to electrostatic interaction between protein II and compound X.

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The lack of infectivity in the case of reconstitution with *trans* 16:1 particles would indicate that there may be problems in packing the *trans* fatty acids into the bilayer. These packing problems may be partially compensated for during *in vivo* assembly. They do not seem to be compensated for *in vitro* either below or above the temperature at which a phase transition occurs in *Pseudomonas* BAL-31 membranes containing *trans* 16:1 fatty acids since no infectious particles were obtained below or above the phase transition temperature.

Biochemical events in infected cells and some speculations concerning *in vivo* assembly of PM2

In relation to the difference in phospholipid composition between PM2 and its host cell BAL-31, it was of importance to investigate phospholipid metabolism in the infected cell. In initial experiments a trend towards an increase in PG synthesis and a decrease in PE synthesis was observed (Braunstein & Franklin 1971). This observation was confirmed and extended in later experiments (Tsukagoshi & Franklin 1974). PE present before infection is degraded and the rate of PE synthesis is reduced. There is no degradation of PG present before infection and its rate of synthesis increases. One third of the lipids found in the completed virion are derived from the pre-existing host cell lipids and the remainder are synthesized after infection. The enzymatic basis for these changes has been established (Tsukagoshi, Petersen & Franklin 1975*c*). Phospholipase A activity against PE increases about twofold during infection whereas activity against PG remains constant. Whether two different enzymes are involved and whether there is a viral specific phospholipase A has not been investigated. There is also an increase in PG synthetase activity during the first 30 min post-infection. PS synthetase and decarboxylase remain constant during this time; then the decarboxylase activity decreases, effectively blocking PE synthesis despite the rise in PS synthetase activity between 30 and 45 min p.i.

We call these metabolic changes *active* control processes as opposed to the *passive* control process of electrostatic interaction between protein II and PG. The reconstitution experiments which demonstrated an increased efficiency of reconstitution of the infectious virus in the presence of the viral PG/PE ratio as compared to reconstitution in the presence of the host cell PG/PE ratio would lead us to believe that the active process, increasing the PG content and decreasing the PE content of the cell, are actually of importance for an efficient *in vivo* assembly.

The intracellular assembly process does not seem to occur in conjunction with the plasma membrane of the host bacterium (Dahlberg & Franklin 1970). One might imagine a phospholipid exchange protein (Wirtz & Zilversmit 1968; Harvey *et al.* 1973; cf. Wirtz 1974) as playing a role in the transport of the lipids to an assembly site. Such a protein does seem to play a role in vaccinia virus assembly (Stern & Dales 1974) but no such protein has been found as yet in bacteria. Thus the *in vivo* events during assembly of PM2 are still rather obscure. The difficulties in handling the nucleocapsid, which aggregates even in low concentrations of urea, make it almost impossible to demonstrate such a structure in infected cells. Although we do foresee an even deeper understanding of the *in vitro* assembly process, which is amenable to physicochemical study and chemical manipulation, our prospects are not very good for rapid progress with *in vivo* assembly.

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Some other viruses with *de novo* assembly of the lipid components

Both the poxviruses and the icosahedral cytoplasmic deoxyriboviruses (ICDV) contain lipid components in the form of membrane structures which are assembled *de novo*, as compared with a utilization of pre-existing host cell membranes. The components of both types of viruses are synthesized in the cytoplasm and assembly of the virion also takes place there.

(1) *Vaccinia* has been used as a model for poxvirus multiplication. This virus has a lipid composition which differs significantly from that of several types of host cells (Dales & Mosbach 1968; Stern & Dales 1974). The phospholipid comparison was made with whole cells and since the phospholipid composition of the various cell membranes differs from the average (Weinstein, Marsh, Glick & Warren 1969) then it is conceivable that vaccinia lipids could have the same composition as some cellular membranes. The major difference, however, is the much smaller PE content in vaccinia and whereas the plasma membrane may have a PE content smaller than that of the whole cell (Weinstein *et al.* 1969), the PE content of the endoplasmic reticulum, which lies closest to the vaccinia viroplasmic foci and which is presumably the source of viral lipids, may be higher than that of the whole cell (Rouser, Nelson, Fleischer & Simon 1968). According to the present working hypothesis there is a *de novo* assembly of the vaccinia membrane (Dales & Mosbach 1968). Certain phospholipids are derived from those made before infection, others are partially derived from those made after infection (Stern & Dales 1974). Phospholipids can be transferred from liposomes to virion *in vitro* via a phospholipid exchange protein, but whether this process has any significance for vaccinia assembly is not clear (Stern & Dales 1974).

There is one glycoprotein in vaccinia and the amino sugar is probably only glucosamine, present as one or more *N*-acetylglucosamine residues per polypeptide (Garon & Moss 1971). This is a situation quite unlike that found in viruses which incorporate preformed cellular membranes into their structures during budding. In those cases the composition of the carbohydrate portion of the viral glycoprotein(s) is similar to that found in the host cell and varies with variation in the host cell (Burge & Strauss 1970; Burge & Huang 1970; Compans, Klenk, Caliguiri & Choppin 1970; Keller, Spear & Roizman 1970). In these latter cases membrane-localized cellular glycosyltransferases are utilized and the carbohydrate portions are coupled to viral specific polypeptides. Such membrane bound enzyme systems do not seem to be available during the *de novo* formation of the vaccinia membrane.

(2) *Frog virus* 3 (FV3) and some *insect iridescent* viruses have been studied as representatives of ICDV (cf. McAuslan & Armentrout 1974). Budding of FV3 from the membranes of infected cells has been reported (Darlington, Granoff & Breeze 1966; Lunger & Came 1966) but membrane-like structures have been reported in the cytoplasmic foci of replication of FV3 (Lunger & Came 1966) and insect iridescent viruses (Stoltz 1973). FV3 has a phospholipid composition different from that of the host cell (Willis & Granoff 1974) but the criticism concerning an interpretation of this finding, mentioned in relation to vaccinia virus, must hold here also. The phospholipid composition of FV3 was, on the other hand, very similar in two types of cells, fathead minnow cells and chick embryo fibroblasts, which have somewhat different phospholipid compositions (Willis & Granoff 1974). Furthermore, FV3 has a low content of cholesterol and sphingomyelin, uncharacteristic of the host cell membranes.

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PM2 seems to be similar to ICDV in structure but, except for the small plant ICDV, most viruses in this class are much larger than PM2 (cf. Kelly & Robertson 1973). Furthermore, the number of polypeptides reported to be present in the larger ICDV varies between 16 and 19 (Tan & McAuslan 1971; Kelly & Tinsley 1972) but many of these may arise by aggregation. Two strains of mosquito iridescent virus were reported to contain 9 polypeptides but the number was reduced to 4 after carboxymethylation and it was possible to account for the other 5 by various combinations of the 4 found after carboxymethylation (Wagner, Pashcke, Campbell & Webb 1974). None of these proteins reacted with Schiff's reagent, suggesting the absence of a glycoprotein in these viruses. The smaller cauliflower mosaic virus has been reported to have only two structural proteins (Tezuka & Taniguchi 1972). Therefore it may be justifiable to place PM2 in the ICDV group.

Viruses utilizing host cell membranes

The majority of the lipid-containing viruses are animal viruses which are assembled as they bud through some cellular membrane, deriving the lipid components directly from the particular host cell membrane involved (cf. Franklin 1962; Klenk 1973). In this article we can only discuss a few salient features of such viruses and mention some problems currently under study.

In general, the proteins of these viruses, as well as those of most viruses, are virus-specific. During the budding process the nucleocapsid, formed in the cytoplasm, associates with a special region of the host cell membrane in which the cellular membrane proteins have been replaced by one or several viral specific proteins (cf. Choppin *et al.* 1971). We do not understand the replacement mechanism, particularly since there is little or no disturbance of the arrangement of cellular lipids during this process. The viral-specific proteins which are found in the cellular membrane are the so-called envelope proteins. They are synthesized in the endoplasmic reticulum and then migrate to the plasma membrane in those cases where the budding occurs there (cf. Klenk 1974). They are never found free in the cytoplasm, rather they are always associated with a cellular membrane system. As mentioned above, those that are glycoproteins are glycosylated by a cell-specific enzyme system after the viral-specific polypeptide is synthesized. The hydrophilic carbohydrate portion of membrane glycoproteins is, in general, exposed at the cell surface and at least part of the polypeptide contains an unusually large proportion of non-polar amino acids which interact hydrophobically with the bilayer (cf. Hughes 1973). Treatment of influenza virus (Brand & Skehel 1972; cf. Klenk 1974) and Semliki forest virus (Utermann & Simons 1974) with certain proteolytic enzymes results either in the digestion of the glycoprotein(s) or cleavage of the glycoprotein(s). The remaining fragment or one of the cleaved products is a hydrophobic polypeptide. This polypeptide seems to penetrate the bilayer and be closely associated with the nucleocapsid protein in the case of Semliki forest virus (Garoff & Simons 1974). A working hypothesis for assembly of budding viruses has been based on these observations (Garoff & Simons 1974). The viral glycoproteins are oriented in the plasma membrane as in the virion with the polar carbohydrate portion directed towards the outside and the hydrophobic portion projecting towards, or possibly into, the cytoplasm. This would provide a recognition site for the nucleocapsid and a nucleation centre where more of the viral glycoproteins would form the final viral membrane structure. A further understanding of the physicochemical and biochemical processes involved in the incorporation of

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the viral proteins into the cellular membrane with accompanying displacement of cellular proteins is desirable and necessary.

Unlike PM2, the lipids of budding viruses closely resemble those of their host cell and vary accordingly when grown in host cells of different lipid composition (cf. Klenk 1974). The viral lipid compositions are, however, *not identical* with that of the host cell, suggesting that also in these cases *protein–lipid interactions might play a slight role in determining the lipid composition of the virion*. In one of the most recent careful studies of this problem, the lipid composition of Sindbis virus was compared with that of the plasma membrane of its host cell, the chick embryo fibroblast (Hirschberg & Robbins 1974). There was more phosphatidylserine and sphingomyelin, less phosphatidylcholine and phosphatidylinositol, and the same amount of phosphatidylethanolamine in the virion as in the plasma membrane. Also there was more cholesterol in the virion (cholesterol/phospholipid ratio of 0.8 compared with 0.6 in the plasma membrane). The same species of glycolipids in approximately the same proportions were found in the virion and its host cell. Curiously, the authors of this study concluded that there was *no* selection of lipids during viral assembly (Hirschberg & Robbins 1974). Further studies of protein–lipid interactions will be necessary before this problem is settled.

Conclusion

As with so many other problems of molecular biology, viruses are being used very profitably in studies on the structure and synthesis of biological membranes. Some lipid-containing viruses can be readily obtained in large amounts in a highly purified form amenable to physical and chemical analysis. Infection of the host cell triggers a series of biochemical changes leading to the appearance of either a new membrane system or a modified membrane system. The viral membrane is admittedly a very special membrane, but what membrane is not?

This work was supported in part by grant number 3.8530.72 SR from the Swiss National Fund. Valuable technical assistance was provided by Miss E. Zoumbou. Advice and discussion concerning certain aspects of this paper were kindly given by Professor J. Seelig and Dr M. Zulauf.

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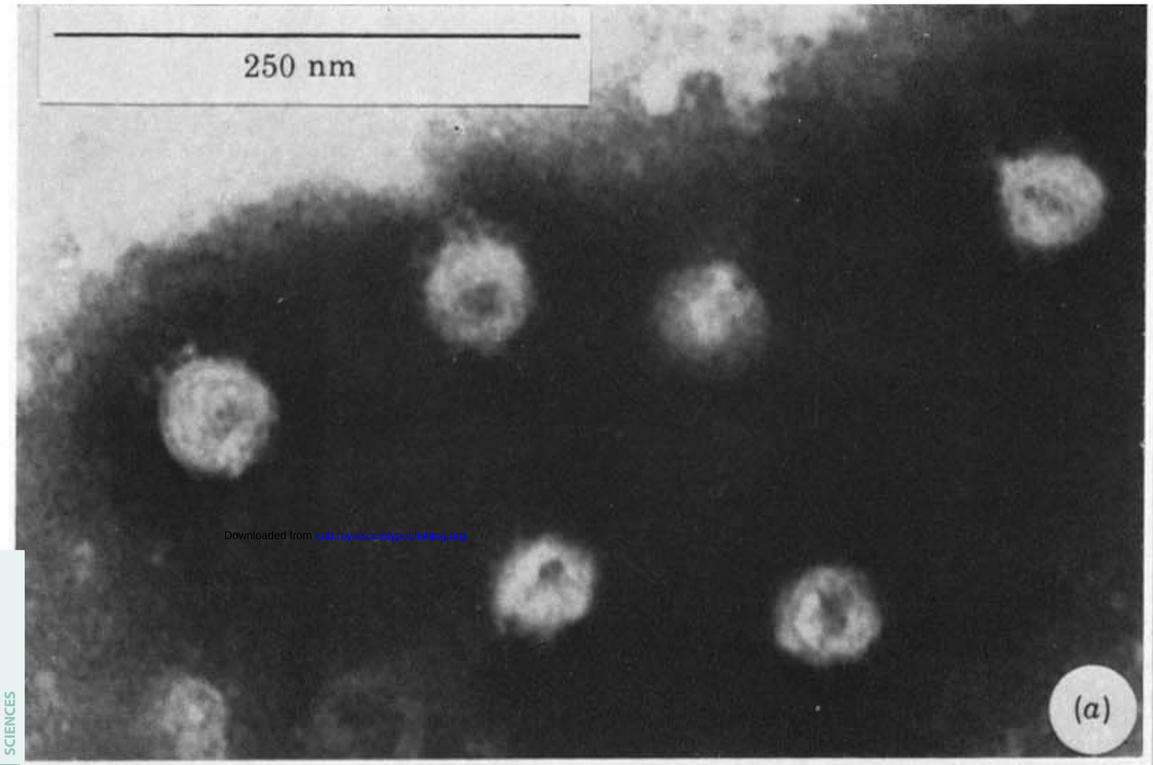
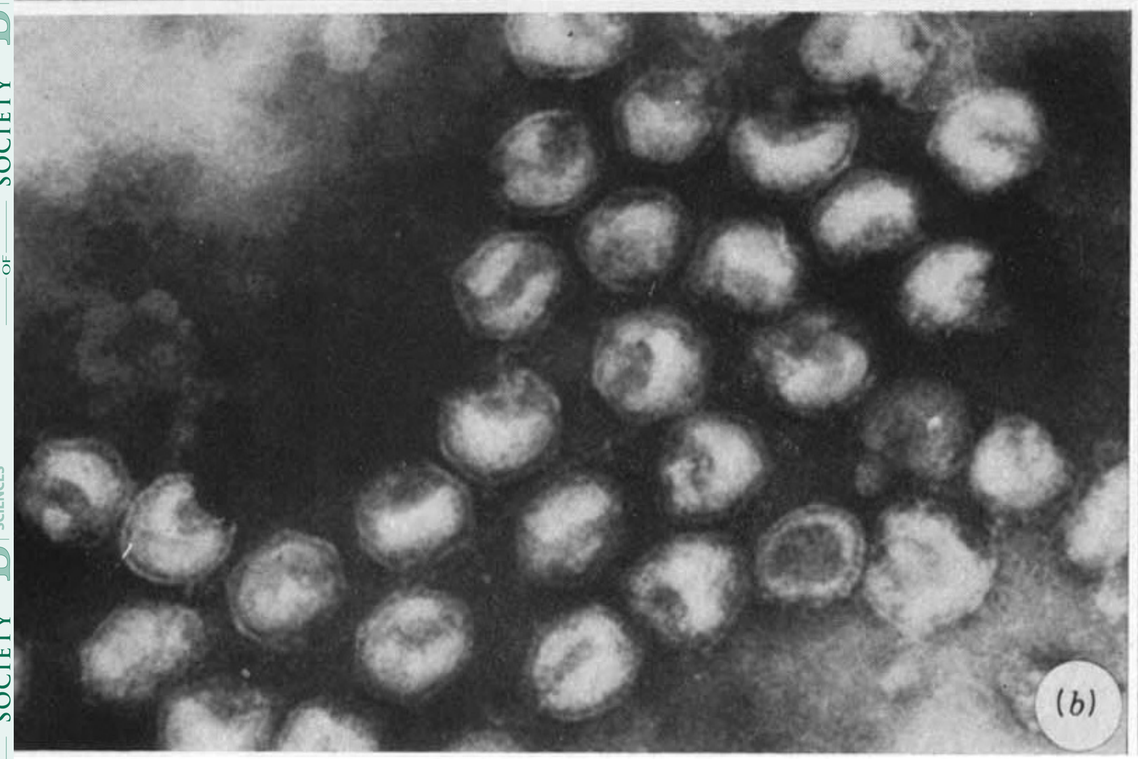
FIGURE 2. Nucleocapsid (*a*) prepared from virus by urea treatment and (*b*) reconstituted. In both cases the particles are unfixed, stained with uranyl acetate.





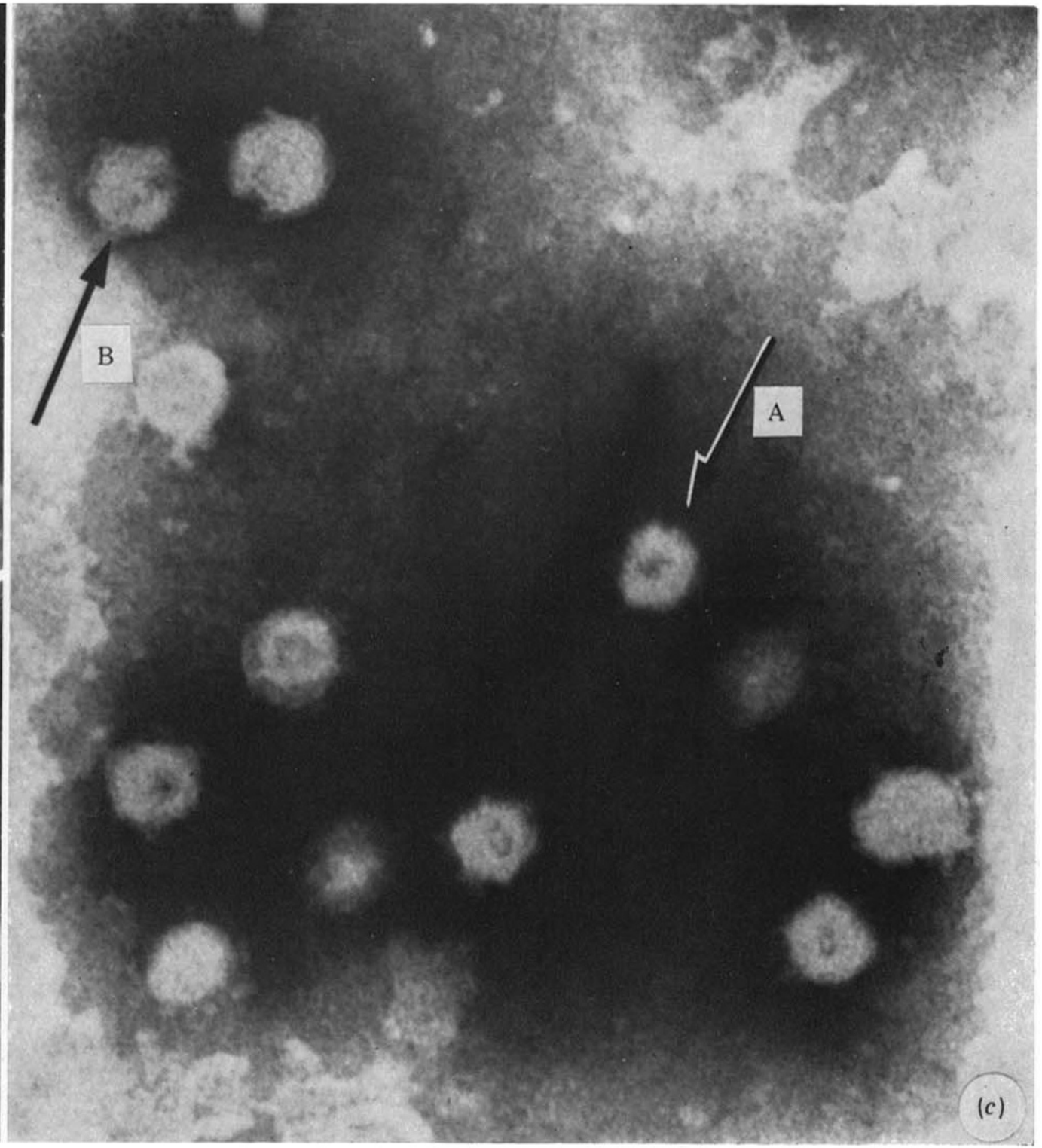


FIGURE 3. Virus and reconstituted virus-like particles. (*a*), (*c*) reconstituted particles. The arrows (A) and (B) in (*c*) point to particles where the spikes may be seen particularly well. (*b*) Standard preparation of purified virus. In all cases the particles are unfixed, stained with uranyl acetate.