On the Disposition of the Proteins of the Membrane-Containing Bacteriophage PM2†

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ABSTRACT: The virion of bacteriophage PM2 has previously been shown to contain a lipid-bilayer membrane. This virus may therefore serve as a useful model system for studying the interactions of lipids and proteins in more complex biological membranes. To this end, we have analyzed the protein composition of PM2 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We have obtained preliminary information about the disposition in the viral particle of its six protein components by means of a variety of methods including: disruption of the virions by freezing and thawing followed by density gradient fractionation and analysis of the fragments; limited proteolysis of the virion; antibody production and antibody binding; enzyme-mediated labeling of the virion, using the [^{125}I]-lactoperoxidase method and a new method involving the enzyme transglutaminase; and the isolation and partial characterization of a small hydrophobic protein (protein 6) extracted from the virion by chloroform–methanol. We conclude that four of the proteins (1, 3, 4, and 5) are exterior to the lipid bilayer of the virion, proteins 1 and 3 being in a more peripheral location on the virion than proteins 4 and 5. Protein 3 is clearly the morphological unit of the icosohedral framework of the virion. The highly hydrophobic protein 6 is most likely an integral protein of the membrane. No evidence was found for a soluble DNA-bound core protein in this virus.

Most cell membranes are very heterogeneous in their protein and lipid compositions. It is also ordinarily very difficult to manipulate these compositions physiologically. Membrane-containing small viruses may therefore provide useful model systems for studying the structure and properties of membranes, because their protein composition is often relatively simple and, at least in principle, is subject to genetic manipulation; and their lipids, usually derived from host cell membranes, can be manipulated by variations in the host lipids. A number of small membrane-containing animal viruses have been extensively investigated, but we were particularly interested in the discovery several years ago of a membrane-containing bacteriophage, PM2, by Espejo and Canelo (1968a). The opportunities it presented for growing large quantities of virus, and for potential genetic manipulation of the virus and its *Pseudomonad* host cell (Espejo and Canelo, 1968b), appeared to offer great potential advantages in membrane studies. As a first step in such studies, we have now examined the proteins of PM2 and their disposition in the intact virion. During our investigations, the studies of Datta *et al.* (1971) on the protein composition of PM2 were published. To the extent that our compositional studies have overlapped theirs, there is general agreement of the results, but they did not present any direct information on the disposition of the viral proteins.

Methods presently available for studying protein disposition in noncrystalline viruses are not absolute or of high spatial resolution, and it is only by the concerted application of several independent methods that significant structural information can be obtained. Accordingly, we have combined the following methods in our studies of PM2: (1) freeze-thaw disruption of the virion and subsequent fractionation and analysis of the fragments; (2) limited proteolysis of the virion; (3) immunological approaches; (4) enzyme-mediated labeling of the proteins of the intact virion, including the use of a system new to membrane structure studies involving the enzyme transglutaminase (A. H. Dutton and S. J. Singer, to be published); and (5) organic solvent extraction. By these methods, we have obtained preliminary information about the dispositions of the six proteins of PM2 in the intact virion.

Materials and Methods

Growth, Purification, and Assay of BAL-31 and PM2. PM2 and host *Pseudomonas* BAL-31 (wild type and a thymidine auxotroph) were generously provided by Dr. Romilio T. Espejo. Growth, purification, and assay of *Pseudomonas* BAL-31 and PM2 were carried out according to Espejo and Canelo (1968a).

Preparation of Radiolabeled PM2. 1. [^{3}H]Leucine and [^{14}C]Leucine. Several milliliters of a saturated culture of *P.* BAL-31 was added to 40 ml of minimal medium plus an equal weight mixture of the 20 common L-amino acids omitting leucine, each at 20 μg/ml (Nutritional Biochemical Corp., Cleveland, Ohio). The leucine concentration was brought to 0.02 μg/ml and the culture was aerated at 29° until an OD_{440} of 0.25 was reached. At this time, either 0.2 ml of [^{3}H]leucine (55.2 Ci/mmol, New England Nuclear, Boston, Mass.) equaling 5 μCi/ml, or 0.08 ml of [^{14}C]leucine (262 Ci/mol, New England Nuclear) equaling 2 μCi/ml, was added. Aeration was resumed until an OD_{440} of 0.30 was reached, whereupon 8 $\times 10^{10}$ PFU of PM2 was added (multiplicity 10). Aeration was continued until lysis had occurred after which the OD_{440} decreased to less than 0.06. The culture was chilled on ice and was given a clearing spin of 15 min at 11,000 rpm (15,000*g*). The supernatant lysate bacteriophage were pelleted at 30,000 rpm (100,000*g*), for 1 hr. The pellet was resuspended in 0.3 ml of NT buffer[1] and layered over 4.7 ml of cesium chloride solution (in the same buffer) of a density of 1.26 at 4°. The bacter-

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[1] Abbreviations used are: NT buffer, 1.0 M NaCl–0.02 M Tris-Cl (pH 8.1); OD_{440}, optical density at 440 nm with a 1-cm path length; SDS, sodium dodecyl sulfate; PFU, viral plaque forming units.

iophage were the centrifuged to equilibrium density, $\rho = 1.28$, for longer than 15 hr in an SW 39 rotor at 39,000 rpm at 4°. The opalescent band was collected dropwise from the bottom of the tube. The major radioactive peak fractions (corresponding with infectivity) were pooled and dialyzed against NT buffer. The bacteriophage solution was finally layered over a 5–20% sucrose gradient in NT buffer and centrifuged at 39,000 rpm for 40 min, in an SW 39 rotor. Again, the opalescent band was collected and dialyzed. Typical specific activities were 2×10^{-5} dpm/PFU.

2. [^{3}H]THYMIDINE. A thymidine auxotroph of BAL-31 (Espejo *et al.*, 1971) was grown in 40 ml of minimal media plus an equal weight mixture of the 20 common L-amino acids each at 20 μg/ml plus thymidine at 20 μg/ml; 0.1 ml of [^{3}H]thymidine (22.3 Ci/mmol, New England Nuclear) resulting in an activity of 2.5 μCi/ml was added after an initial growth period as with the radioactive leucine. Further growth, infection, and purification of the virus were as described above. A typical viral specific activity was 2.5×10^{-4} dpm/PFU.

3. [^{3}H]PALMITATE. *P.* BAL-31 was grown on 40 ml of minimal media plus an equal weight mixture of the 20 common L-amino acids plus 0.05% fatty acid-poor bovine serum albumin (Calbiochem, San Diego, Calif.). At the indicated time, 0.1 ml of [^{3}H]palmitate (10 Ci/mmol, Schwarz/Mann, Orangeburg, N. Y.) was added resulting in an activity of 1.25 μCi/ml. Purification was as described above. A typical viral specific activity was 5.4×10^{-5} dpm/PFU.[2]

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis. Cylindrical gels of 0.5 × 20 cm size, containing 15% acrylamide (Matheson Coleman and Bell, Los Angeles, Calif.), 0.4% bisacrylamide (*N,N'*-methylenebisacrylamide, American Cyanamid Co., Wayne, N. J.), and 0.1% SDS (Matheson Coleman and Bell) were polymerized in buffers according to Laemmli (1970).

Samples for gel electrophoresis were prepared as follows. Whole bacteriophage were generally pelleted at 100,000*g* for 1 hr and dissolved in sample buffer containing 2% SDS, 62.5 mM Tris-Cl (pH 6.8), 10% glycerol, 0.002% Bromophenol Blue tracking dye, and 5% mercaptoethanol. Bacteriophage components were dialyzed by ultrafiltration with a PSAC membrane (Millipore Corp., Bedford, Mass.) to reduce the ionic strength, finally diluting the material retained with an equal volume of a twofold concentrated sample buffer. Material in the sample buffer was heated in a closed test tube in boiling water for 1 min, cooled and applied to a gel. Constant-current electrophoresis was performed at 1.5 mA/gel for 15 hr at room temperature with the cathode at the origin.

Radioactive gels were fractionated with a Maizel-type gel crusher (Maizel, 1966) effectively cutting 1.0–1.5-mm fractions. Where desired, fractions were treated with [^{14}C]dimethyl sulfate (New England Nuclear) to label the proteins, following the method of Kiehn and Holland (1968). Molecular weight marker proteins bovine serum albumin (crystallized, Armour Pharmaceuticals, Kankakee, Ill.), γ-globulin (Calbiochem, A grade, human 7s, La Jolla, Calif.), β-lactoglobulin (three times crystallized, Pentex, Kankakee, Ill.), cytochrome *c* (twice crystallized from *Candida krusei,* Calbiochem), and insulin (Calbiochem, B grade) were treated with [^{14}C]dimethyl sulfate and coelectrophoresed with differentially labeled PM2 proteins.

Scintillation counting was performed using a Triton detergent scintillation fluid (33% Triton X-100 (Atlas Chemical Co., San Diego, Calif.), 5% Liquifluor (New England Nuclear) in toluene).

Freeze-Thaw Disruption of PM2. A small volume of virus in a plastic tube (*e.g.*, 0.2 ml) was dipped in liquid nitrogen until frozen, whereupon the tube was transferred to a 30° water bath to thaw. In some cases this procedure was repeated a second time.

Proteolysis of PM2. To a solution of 1 ml containing 0.1 mM calcium chloride, 1.0 M sodium chloride, and 0.3 mg of protein as intact PM2 (unlabeled) and 10^5 cpm of [^{3}H]leucine labeled PM2 was added trypsin (Worthington twice crystallized) to a concentration of 4 μg/ml at zero time. Titration with 0.01 N sodium hydroxide was then carried out on a Radiometer pH-Stat at pH 7.7, 37°. At indicated times a 100-μl aliquot was removed, 1.0 μl of which was appropriately diluted and assayed for infectivity, and the remainder of which was mixed with 100 μl of ovomucoid trypsin inhibitor (50 μg/ml, Sigma Chemical Co.). To this inhibited mixture was added 30,000 cpm of [^{14}C]leucine labeled PM2 as internal standard. The solution was then concentrated and subjected to the SDS–polyacrylamide gel electrophoresis procedure.

Antibodies and Bacteriophage Inactivation. Protein 3 was purified as follows. Purified PM2 (approximately 8 mg of protein) was pelleted and resuspended in 1 mM sodium chloride–0.4 mM Tris-chloride (pH 7.6) (causing osmotic disruption) and was then homogenized in a Teflon–glass homogenizer. This material was layered over 18 ml, $\rho = 1.265$ cesium chloride in NT buffer in a cutoff SW 27 rotor centrifuge tube. Following centrifugation (27,000 rpm, 15 hr, 4°) the gradient was fractionated and scanned at 280 nm, and the fractions at the top of the gradient were pooled. This material was dialyzed overnight against 0.05 M sodium chloride–0.02 M Tris-chloride (pH 7.4)–2 mM EDTA, followed by dialysis against 0.15 M sodium chloride–60 mM phosphate (pH 7.4). According to SDS–polyacrylamide gel electrophoresis, the protein in this sample was more than 95% protein 3. Approximately 1 mg of this protein was added to an equal volume of Freund's complete adjuvant (Difco) and treated 1 min in a Virtis homogenizer, medium speed. The viscous emulsion was injected into the foot pads of a rabbit. Three weeks later a booster injection of 0.2 mg of protein was given intravenously. Approximately 40 ml of blood was collected from the ear vein on the fourth week as well as from the unimmunized control rabbit and the serum was collected and frozen. Tests for PM2 inactivation were conducted according to Adams (1959) in 0.15 M sodium chloride–5 mM Tris-chloride (pH 7.4), 40°. Ouchterlony double diffusion was performed in Hyland Immunoplates (Costa Mesa, Calif.).

Transglutaminase Labeling. Transglutaminase is an enzyme which catalyzes the exchange of the amide group of protein glutaminyl residues for any of a wide variety of primary amines (Clarke *et al.*, 1959). In our laboratory (A. H. Dutton and S. J. Singer, to be published) we have used this enzyme to label and to cross-link the proteins of erythrocyte and other membranes. In the experiments reported in this paper, guinea pig liver transglutaminase was used, purified according to the method of Connellan *et al.* (1971), and assayed by the colorimetric procedure of Folk and Cole (1966). The enzyme has a molecular

[2] When extracted with chloroform and methanol (Folch *et al.*, 1957) and run on thin-layer chromatography, the radioactivity cochromatographed with authentic phosphatidylethanolamine and phosphatidylglycerol. It is important to show that no [^{3}H]palmitate label is incorporated into PM2 protein. For this purpose, [^{3}H]palmitate labeled PM2 was mixed with [^{14}C]leucine labeled PM2 and the dissolved virus mixture was applied to an SDS–polyacrylamide gel. No tritium counts were found under the ^{14}C-protein peaks and all the tritium migrated near the included volume of the gel and the tracking dye as expected for low molecular weight species such as phospholipids.

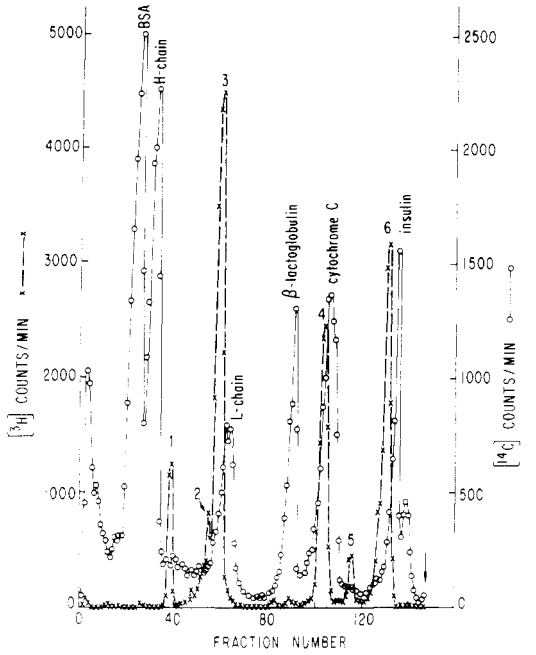

FIGURE 1: SDS-polyacrylamide gel electrophoresis of [^{3}H]PM2 proteins and ^{14}C-marker proteins. [^{3}H]Leucine labeled PM2 was mixed with ^{14}C-methylated marker proteins and coelectrophoresed. Electrophoresis in this and all other gels was from left to right. PM2 protein peaks are numbered; an arrow indicates the position of the tracking dye.

weight of 85,000 (Connellan *et al.*, 1971). The primary amine employed was [^{14}C]glycine ethyl ester (obtained from New England Nuclear), diluted to 2 Ci/mol. Conditions were found which produced essentially a saturating amount of labeling of PM2, as follows. In a volume of 0.2 ml was contained 100 μg of PM2 protein as intact virions and 7.5 μg of transglutaminase in 5 mM calcium chloride, 5 mM dithiothreitol, 100 mM Tris-chloride, pH 7.4, 0.15 M sodium chloride, and 5 mM [^{14}C]glycine ethyl ester. Incubation of this mixture was carried out for 1 hr at 37°. The reaction was terminated by the addition of 28 ml of cold NT buffer, and the virus was removed by centrifugation at 100,000*g* for 1 hr. The bacteriophage pellet was dissolved in gel sample buffer and subjected to the SDS-polyacrylamide gel electrophoresis procedure.

Lactoperoxidase Labeling (Phillips and Morrison, 1971). Only one set of experiments was carried out with this procedure. Intact PM2 virions (100 μg of PM2 protein) and 5 μg of lactoperoxidase (Calbiochem B grade) were present in a solution containing 100 mM Tris-chloride (pH 7.5), 150 mM sodium chloride, 250 μCi of potassium [^{125}I]iodide (25 Ci/mol) at a final volume of 0.2 ml. The reaction was initiated by the addition of H_2O_2 (J. T. Baker Chemical Co.) to a final concentration of 4 μM. Incubation was for 5 min at 37°. The reaction was terminated and the product analyzed as with the transglutaminase experiments described above.

Solvent Extraction and Characterization of Protein 6. Purified PM2 from a 15-l. culture was dialyzed against several changes of distilled water. Precipitated protein was collected by 100,000*g* centrifugation for 1 hr. The pellets were resuspended in a total of 2.5 ml of distilled water and combined with [^{3}H]palmitate labeled PM2 and [^{14}C]leucine labeled PM2 in 1 ml of their original buffer to serve as markers to follow the course of the purification. This starting material was extracted according to the chloroform–methanol method of Folch *et al.* (1957). The single phase mixture was "broken" by the addition of one-fifth volume of 10 mM Tris-chloride (pH 7.4) followed by vigorous shaking. After phase separation the lower phase was removed and rotary evaporated with a stream of nitrogen down to a few milliliters. More residual chloroform was removed by the addition of an equal volume of methanol and again evaporating down to 1.5 ml. This sample was loaded onto a Bio-beads SX-2 column in methanol (1 × 7 in., 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.). (The packing was washed with several volumes of methanol, followed by glacial acetic acid, and methanol, before sample application.) The loaded sample was eluted with 100 ml of methanol, followed by 100 ml of glacial acetic acid; 3-ml fractions were collected at a rate of 1.5 ml/min. Fraction aliquots were assayed for radioactivity and protein.

Glycoprotein Agglutination with Plant Lectins. Purified plant lectins were kindly supplied by Dr. Garth Nicolson of the Salk Institute for Biological Studies. *Ulex europaeus* lectin (specific for L-fucose, titre 16 *vs.* human O erythrocytes), ricin (specific for β-D-galactose, L-rhamnose, 0.5 mg/ml), wheat germ agglutinin (specific for *N*-acetylglucosamine, titre 4000 *vs.* rabbit erythrocytes), concanavalin A (specific for α-D-mannose, α-D-glucose, 10 mg/ml), and *Dolichos biflorus* lectin (specific for *N*-acetylgalactosamine, ~1 mg/ml) were tested for ability to agglutinate PM2 in a ring test. Approximately 10 μl of plant lectin was drawn into a 50-μl microcap pipet followed by about 10 μl of PM2 at 0.5 mg of protein/ml in 10% sucrose. The presence of an opalescent ring after several hours at room temperature was expected if the PM2 virions were agglutinated by the lectin. The contents of the capillary tubes were subsequently examined for aggregation under the light microscope. Three serial one to two dilutions of the lectins were also tested for PM2 agglutinating ability.

Amino Acid Analyses. These were kindly performed for us by Dr. Russell F. Doolittle on a Beckman Automatic Amino Acid analyzer. Details are given in Table IV.

Protein Assay. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard, calibrated by Kjeldahl nitrogen (performed by Dr. William Weigle's laboratory, Scripps Clinic and Research Foundation, La Jolla, Calif.) using 16% nitrogen for conversion.

Results

The Proteins of PM2. In order to learn about the relationship of protein to the membrane of PM2, it was first necessary to separate and determine the number of different proteins of the virus. This was most readily done by electrophoresis on 15% polyacrylamide–SDS gels which separates proteins according to molecular weight (Weber and Osborn, 1969). In order to determine the sizes of the PM2 proteins, internal standard proteins of known molecular weight were ^{14}C methylated with [^{14}C]dimethyl sulfate (Kiehn and Holland, 1968) and coelectrophoresed with differentially labeled [^{3}H]leucine PM2 proteins. As seen in Figure 1, four prominent and two less prominent protein peaks were observed. These gel patterns were not affected by repeated cesium chloride density gradient and sucrose velocity gradient centrifugations of the virus. Pulse-chase double labeling experiments (Brewer, 1972) also showed that no significant amount of host-derived protein was present in the purified PM2 virus preparations. The apparent molecular weights of the proteins based on comparison to the marker proteins are shown in Table I along with the results of Datta *et al.* (1971). With our techniques, proteins present at less than 0.3% (about five copies per virion) would not be detected above background. There may be four proteins present at just above this level, seen at gel fractions 48, 63, 83, and 98

TABLE I: The Proteins of PM2.

Protein[a]	Molecular Weight[b]	Protein Weight %[c]	Copies/ Virion[d]
1	45,200 ± 700 (43,000)	6.6 (10.9)	55 (90)
2	29,000 ± 300	5.9	80
3	26,300 ± 400 (35,000)	53 (65)	750 (710)
4	12,700 ± 300 (12,500)	12.5 (19)	370 (560)
5	10,000 ± 100	2.1	80
6	6,500 ± 40 (5,000)	16 (6)	900 (470)

[a] See Figure 1 for designations. [b] Mean and standard deviation of seven SDS-polyacrylamide gels with internal standards, as in Figure 1 for molecular weights with errors. Numbers in parentheses are from Datta *et al.* (1971). [c] Based on uniform carbon labeling from [^{14}C]glucose. Average of three gels from the same preparation. Estimated error 10%. Numbers in parentheses are based on uniform carbon labeling from [^{14}C]acetate by Datta *et al.* (1971). [d] Protein copies per virion = (PM2 MW) (fractional weight of total protein, 75%) (individual protein per cent of total protein)/(individual protein MW). Numbers in parentheses are from Datta *et al.* (1971), as was the value of 5×10^7 used for PM2 MW. See Discussion for an error analysis.

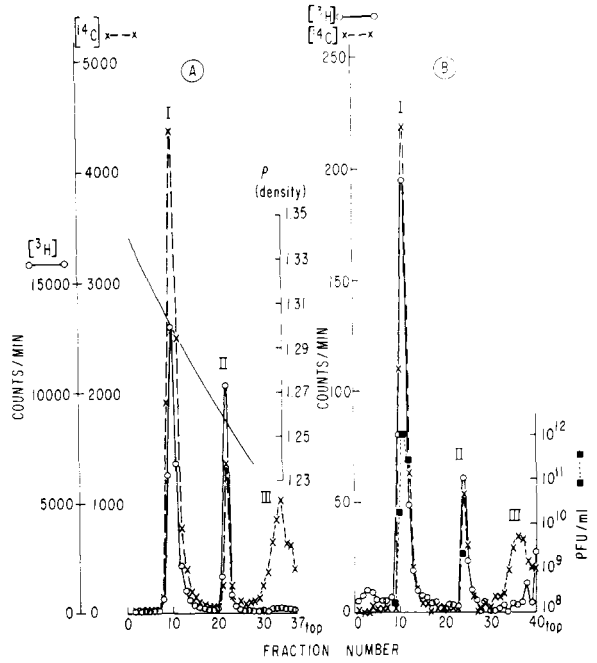

FIGURE 2: Cesium chloride density gradient of freeze-thaw disrupted PM2: (A) [^{3}H]palmitate labeled PM2 and [^{14}C]leucine labeled PM2 were mixed, frozen in liquid nitrogen, thawed, and centrifuged to equilibrium over ρ_4 = 1.24 cesium chloride in NT buffer; (B) [^{3}H]thymidine labeled PM2 and [^{14}C]leucine labeled PM2 were mixed and similarly treated.

corresponding to molecular weights of 35,000, 23,000, 18,500, and 14,000, respectively, but their significance is not clear.

In order to determine the number of copies of each of the six PM2 proteins in the virion, growth of the host and subsequent infection by PM2 were conducted in the presence of uniformly labeled [^{14}C]glucose as the sole carbon source. Purified bacteriophage then have their DNA, lipid, and protein uniformly carbon-14 labeled. Subjected to SDS-polyacrylamide gel electrophoresis, protein peaks were observed at the same positions as seen in Figure 1. The total label under each peak was taken as proportional to the weight fraction of that component in the virion. The weight per cent carbon in each protein was taken as 49%. From this information, and the molecular weights, the number of copies of each protein per virion was calculated (Table I).

Disposition of Proteins. 1. FREEZE-THAW DISRUPTION. In order to study the membrane of PM2 and any associated proteins, it was desirable to fractionate the virus to obtain a membrane-rich fraction. Freezing and thawing dilute virus solutions followed by density gradient centrifugation yielded three fractions as indicated in Figure 2. The radioactive peak at ρ_4 = 1.30 (fraction I) coincided with the infectivity, in contrast to fraction II which contained a 1000-fold less PFU/ml (Figure 2B). Fraction I showed the same SDS-acrylamide gel pattern as the original PM2, and may thus be identified with undisrupted virus. When [^{3}H]palmitate (lipid) labeled PM2 and [^{14}C]leucine (protein) labeled PM2 were mixed together and similarly treated, a coincidence of their labels was noted at a density ρ_4 = 1.255 (fraction II) (Figure 2A). Similarly, [^{3}H]thymidine (DNA) labeled PM2 showed a [^{3}H]thymidine peak coincident with a [^{14}C]leucine peak at ρ_4 = 1.255 (fraction II, Figure 2B). In order to determine whether specific proteins were associated with the lipid-protein-DNA complex, fraction II material from [^{3}H]leucine labeled PM2 preparation (without lipid or DNA labels) was electrophoresed on SDS-polyacrylamide gels (summarized in Table II). Diminished levels of proteins 1 and 3 and enrichment of proteins 4, 5, and 6 were observed in fraction II.

Fraction III material was then examined. No lipid or DNA was associated with fraction III (Figure 2). In Table II, it is shown that fraction III material was enriched in proteins 1 and 3, while a sharply diminished level of proteins 4 and 6 was observed.

Fraction II material was susceptible to DNase digestion in contrast to fraction I. As seen in Figure 3, 90–96% of the DNA thymidine label was digested, resulting in a shift in equilibrium density of the protein-lipid complex from ρ_4 = 1.255 to ρ_4 = 1.200. It was expected that if any core proteins were present as a soluble nucleoprotein complex, the DNA digestion would have released them into solution. Gel electrophoresis of the protein in the bottom and the top gradient fractions after DNase treatment, however, showed no enrichment of the major protein peaks over those in the undigested sample. Conversely, the major remaining protein-lipid band, now DNA free, showed no unique loss of specific proteins when observed on gels; the profile was essentially that of the undigested fraction II material. Therefore, no evidence for a DNA-associated core protein was obtained.

2. LIMITED PROTEOLYSIS WITH TRYPSIN. The results of the tryptic hydrolysis of PM2, carried out as described in the Methods section, are shown in Figure 4. Upon gel electrophoresis, the [^{3}H]leucine label was found in the usual six protein peaks, and no significant amounts in any others. From the ratios of ^{3}H to ^{14}C labels in each peak, the extent of proteolysis of each protein was calculated as a function of time. Proteins 1 and 3 appeared to show an initially rapid rate of proteolysis. Protein 4 was more slowly digested initially, while proteins 5 and 6 were not appreciably affected after 60-min incubation. Protein 2 was present in too small amount to yield significant data on its kinetics of proteolysis. Infectivity of the virus was slowly lost upon proteolysis following the kinetics of loss of protein 4 more closely than of the other proteins.

3. IMMUNOLOGICAL APPROACHES. Only protein 3 was

TABLE II: Distribution of PM2 Proteins in Density Gradient Fractions Following Freeze–Thaw Disruption.[a]

Protein	Untreated PM2 % of Total	Membrane Fraction II			Top Fraction III		
		cpm	% of Total	Normalized Ratio[b]	cpm	% of Total	Normalized Ratio[c]
1	2.7	107	1.0	0.8	844	31.0	8.0
2	6.0	726	6.6	2.4	84	3.1	4.0
3	46.7	2,264	20.6	1.0	1622	59.6	1.0
4	17.6	3,279	30.8	3.8	70	2.6	0.15
5	1.4	279	2.5	4.0	18	0.7	0.31
6	25.6	4,356	39.6	3.4	84	3.1	0.13
Total		11,011			2722		

[a] Datta from SDS–polyacrylamide gel electrophoresis of disrupted PM2 fractions II and III (see Figure 2). [b] (Membrane fraction II %)/(untreated PM2 %), normalized to protein 3. [c] (Top fraction III %)/(untreated PM2 %), normalized to protein 3. Here, untreated PM2 % is slightly different from column 2 because of different fraction cutting (data not shown).

isolated in sufficient quantities for immunization. A rabbit antiserum against the purified protein 3 inactivated PM2 infectivity, with a K value of 77 min^{-1} at 40°. Normal rabbit serum had no effect. This antiserum gave the same single precipitin line with purified protein 3 as with a crude preparation of protein 3 containing also proteins 2, 4, and 6 (Brewer, 1972), demonstrating its specificity for protein 3. When this antiserum was precipitated with purified protein 3 at equivalence, its capacity to inactivate PM2 was decreased by a factor of about 600.

4. ENZYME-MEDIATED LABELING. (a) Transglutaminase Labeling. Under the conditions described in the Methods section, transglutaminase-mediated covalent labeling of intact PM2 virions with [^{14}C]glycine ethyl ester occurred with no loss of infectivity. Control experiments showed no labeling. For example, if the labeling reaction was carried out in the presence of 5 mM EDTA, which inhibits transglutaminase activity, no labeling occurred. From the specific activity of the glycine ethyl ester used, an average of about 2900 molecules were covalently bound to each PFU of bacteriophage in the labeling experiment. On gel electrophoresis, extensive labeling of proteins 3, 4, and 5 was observed (Figure 5). Proteins 1 and 2 appeared to be labeled, but because the total amount of these proteins was very small, the total amount of ^{14}C label was correspondingly marginal. Protein 6 was not significantly labeled. The moles of label/mole of polypeptide chain calculated from these results are given in Table III. In separate labeling experiments with PM2 virions that were first subjected to disruption by freezing and thawing, the most striking changes observed after gel electrophoresis was a marked labeling of protein 6 (> fourfold increase) and a considerable increase (twofold) in labeling of protein 5, when compared to protein 4.

(b) Lactoperoxidase Labeling. The distribution of ^{125}I label on the proteins from intact PM2 is shown in Figure 6. Proteins 1, 2, 3, 4, and 6 were labeled, protein 1 most extensively. Accompanying this labeling reaction, however, there was a 50% loss in infectivity. Furthermore, when the freeze-thaw disrupted PM2 was labeled in a parallel fashion, a similar distribution of label was found except for a significant (four times) increase in protein 3. Protein 5 was not labeled in either case; it may not have any tyrosine residue available for the labeling reaction. In separate experiments it was shown that the loss of infectivity of PM2 was due to the presence of the hydrogen peroxide togeth-

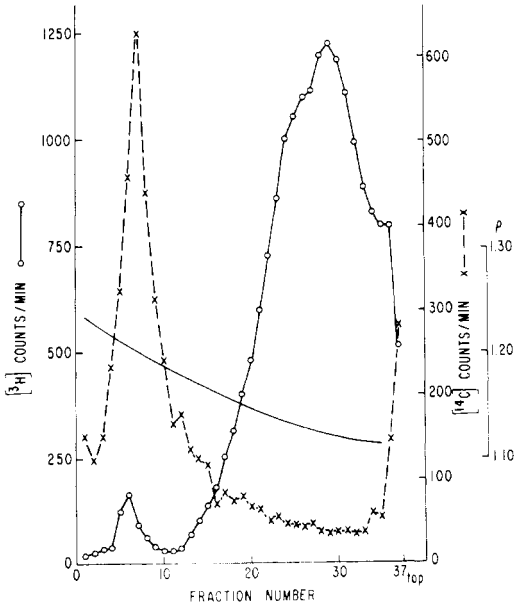

FIGURE 3: Sucrose density gradient fractionation of DNase-treated fraction II. A [^{3}H]thymidine-[^{14}C]leucine labeled PM2 mixture was treated as described in Figure 2. Fraction II was isolated, pelleted, and resuspended in 2.5 mM magnesium chloride. DNase (2 μg/ml) was added and digestion was followed at 260 nm, 37° to completion. Digested material was applied to a preformed gradient of sucrose in deuterium oxide (ρ_{25} = 1.22–1.10). Centrifugation (39,000 rpm, SW 39 rotor, 40 hr, 25°) was followed by fractionation, density measurement (25°, by weight of 100 μl), and radioactivity counting.

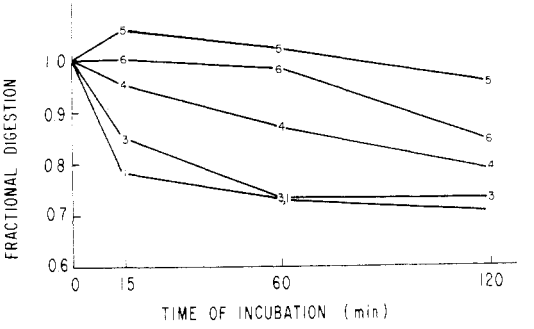

FIGURE 4: Proteolysis of PM2. Procedure as described in Methods. Protein peaks of SDS gels were integrated and normalized to zero time peak areas. Numbers correspond to PM2 proteins as seen in Figure 1.

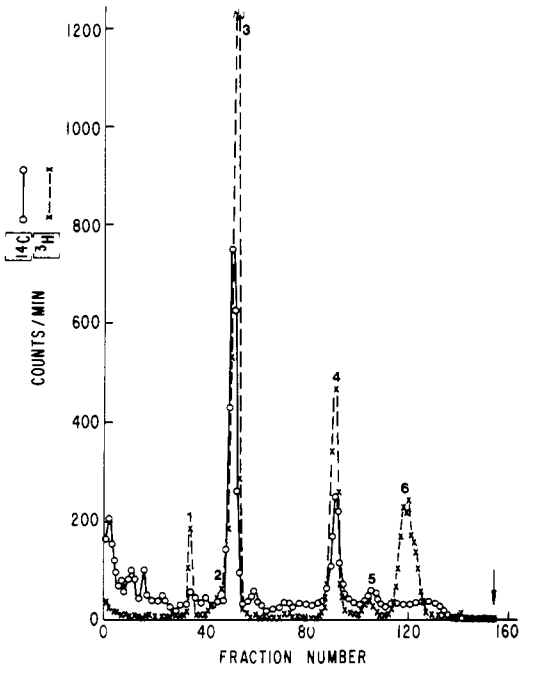

FIGURE 5: SDS-polyacrylamide gel electrophoresis of PM2 proteins after treatment of intact virions with [^{14}C]glycine ethyl ester and the enzyme transglutaminase. Following treatment as described in Methods, [^{3}H]leucine labeled untreated PM2 was added as an internal standard.

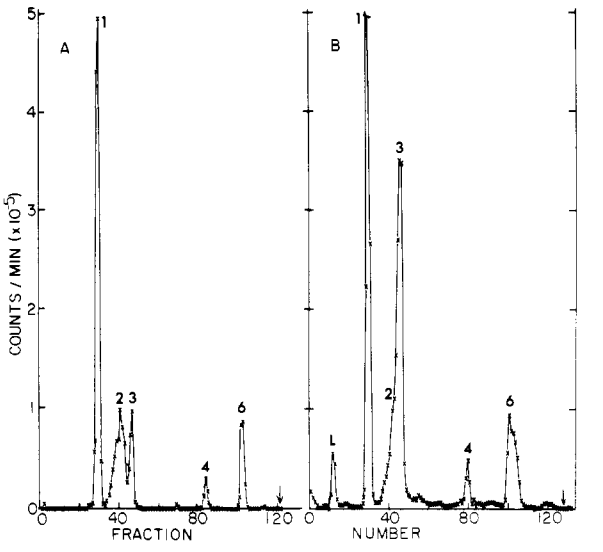

FIGURE 6: SDS-polyacrylamide gel electrophoresis of [^{125}I]lactoperoxidase treated PM2: (A) treatment of intact PM2; (B) treatment of PM2 disrupted by freeze-thaw. L indicates self-labeled lactoperoxidase.

er with lactoperoxidase and that the enzyme or peroxide alone had no such effect.

5. EXTRACTION WITH ORGANIC SOLVENTS. In a preliminary experiment, a preparation of [^{14}C]leucine labeled PM2 was subjected to the chloroform–methanol lipid extraction of Folch *et al.* (1957). It was thought that any hydrophobic protein might be extracted along with the lipids into the hydrophobic lower phase. This was found to be the case. Separation of the extracted bacteriophage lipid from the protein was carried out as described in the Methods section. When a mixture of [^{3}H]palmitate labeled PM2, [^{14}C]leucine labeled PM2, and unlabeled carrier PM2 was loaded on a cross-linked polystyrene bead column, the majority of the lipid could be eluted in the methanol void volume, and the protein in the glacial acetic acid void volume. Analysis of the protein on SDS-polyacrylamide gels (Figure 7) showed that only protein 6 was isolated by this procedure.

Preliminary amino acid analysis of protein 6 indicated that if pure it had only one methionine residue. Stoichiometric cleavage of the methioninyl bond with cyanogen bromide (Gross, 1968) should have revealed a single new N terminus if the original preparation was pure. Dansylation analyses (Hartley, 1970) for N-terminal amino acids gave no characteristic end group for intact protein 6 but indeed did reveal a single isoleucine N-terminal residue after the cyanogen bromide cleavage reaction. Preliminary attempts to separate and analyze the cyanogen bromide peptides by thin-layer chromatography or on Sephadex G-25 suffered from poor recovery, and were not further pursued.

A two-dimensional thin-layer cellulose chromatogram of protein 6 in a basic and an acidic solvent (Figure 8) showed a single spot detectable with ninhydrin and an arginine specific spray (Yamada and Itano, 1966). This is good evidence for homogeneity of the protein.

With the above indications of protein 6 purity, amino acid analysis was considered to be meaningful. This analysis is summarized in Table IV. Notable is the absence of proline, cysteine, and histidine, the preponderance of basic over acidic resi-

TABLE III: Summary of [^{14}C]Glycine Ethyl Ester–Transglutaminase Labeling of Intact PM2.[a]

Protein	cpm	No. of Tags/PFU[b]	No. of Tags/ Polypeptide[c]
1	105	70	1.3
2	(58)	(40)	(0.5)
3	2130	1500	2.0
4	871	600	1.7
5	130	90	0.9
6	0	0	0

[a] Derived from Figure 5. Figures in parentheses represent data of high uncertainty. [b] Calculated: (cpm/counting efficiency)(Avogadro's number)/(specific activity of label)(number of PFU at start). [c] Previous column divided by number of copies/virion from Table I.

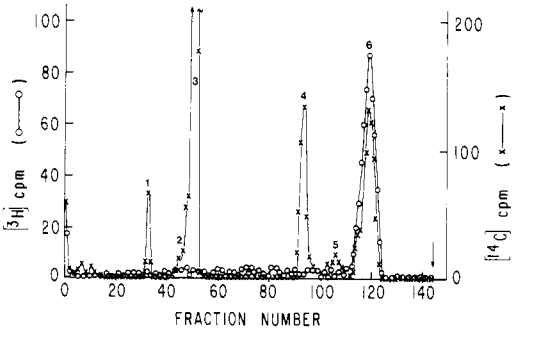

FIGURE 7: SDS-polyacrylamide gel electrophoresis of purified protein 6. [^{14}C]Leucine labeled PM2 was extracted with chloroform–methanol and purified as described in Methods. This material was then coelectrophoresed with the proteins of [^{3}H]leucine labeled PM2 as an internal standard.

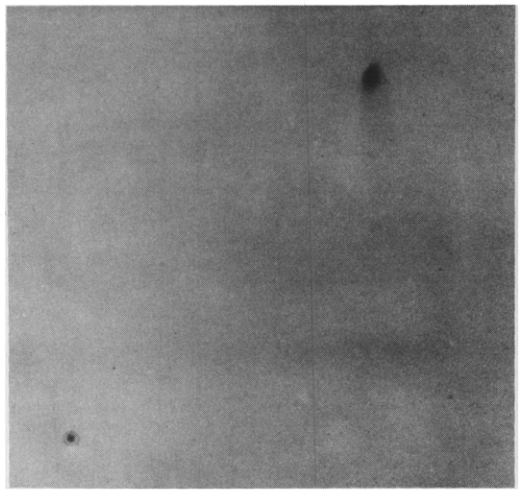

FIGURE 8: Two-dimensional thin-layer chromatogram of purified protein 6; 4–8 nmol of protein was applied to the lower left corner of a 6-cm^2 cellulose thin-layer chromatography plate (Chromagram, Eastman Kodak), run left to right in chloroform–methanol–28% ammonium hydroxide (6:3:1, v/v) and dried, and run bottom to top in diisobutyl ketone–glacial acetic acid (1:1, v/v). After drying detection was by ninhydrin spray (Waldi, 1965).

dues, and the presence of a disproportionate fraction of hydrophobic residues. The calculated molecular weight is 6591, in good agreement with the result of 6500 from SDS–polyacrylamide gels. Based on agarose gel permeation chromatography, Datta *et al.* (1971) estimated a molecular weight of 4650 for their comparable protein.

6. THE POSSIBLE PRESENCE OF GLYCOPROTEIN.ee. With the properties of several animal viruses in mind, the presence of structural glycoproteins in PM2 was investigated. First, saccharide-binding plant lectins were added to attempt agglutination of PM2 virions. Using concanavalin A, ricin, *D. biflorus*, wheat germ, and *Ulex* agglutinins, no detectable agglutination was observed either in a ring test or by observation under the light microscope. In view of the studies of Camerini-Otero *et al.* (1971) claiming incorporation of [^{3}H]glucosamine into PM2, attempts were made to repeat this finding. These attempts were unsuccessful, despite the host incorporation of trichloroacetic acid precipitable [^{3}H]glucosamine. Furthermore, no glucosamine above 0.1 mol/mol of protein 6 was detected upon amino acid analysis of protein 6 (Table IV).

TABLE IV: Amino Acid Composition of Protein 6.[a]

	Whole PM2 mole %[f]	Protein 6 mole %	No. of Residues/ 55[g]	Nearest Integer
Asx	10.3	5.75[d]	3.16	3
Thr	5.5	9.20[c]	5.06	5
Ser	12.8	8.46[c]	4.66	5
Glx	11.9	1.15[d]	0.63	1
Pro	3.7	0		
Gly	12.1	4.27[b]	2.35	2
Ala	9.8	10.69[d]	5.87	6
Cys/2	0.5	0		
Val	5.6	13.89[d]	7.64	8
Met	2.3	2.22[e]	1.22	1
Ile	4.2	10.52[d]	5.79	6
Leu	7.3	15.20[d]	8.35	8
Glucosamine[i]	0	0		
Tyr	1.0	Trace		(1)[j]
Phe	2.2	Trace		
His	1.4	0		
Lys	6.0	12.58[d]	6.92	7
Arg	2.8	6.16[b]	3.39	3
Try				4[h]
				59

[a] Samples of pure protein 6 or whole PM2 were hydrolyzed 24, 48, and 72 hr. Samples were analyzed by the laboratory of R. F. Doolittle employing a Beckman automatic amino acid analyzer, Model 117, using single column analysis. [b] Average of 24-, 48-, 72-hr hydrolyses. [c] Extrapolated to zero time. [d] Extrapolated to infinite time. [e] 24-hr value. [f] Average of two 24-hr hydrolyses. [g] Chosen for a least-squares best fit (smallest deviation from integral values) between 44 and 60 total residues (not including tryptophan). [h] Determined colorimetrically (Spande and Witkop, 1968) in glacial acetic acid. [i] Camerini-Otero *et al.* (1972) reported the presence of glucosamine or mannosamine in their preparation of PM2 associated with protein 6, but did not carry out amino acid analyses of the protein. Subsequent to their report, the possible presence of these amino sugars was examined in our PM2 protein amino acid analyses. Dr. Russell F. Doolittle showed that authentic glucosamine was found to elute slightly before tyrosine on a single column analysis. No corresponding peak was found in hydrolysates of protein 6 or the whole virus, indicating that less than 0.1 mol of amino sugar was present/mol of protein 6. [j] Although tyrosine was never present in greater amount than 0.5 mol/mol of protein in these analyses, the ^{125}I labeling of protein 6 with lactoperoxidase (Figure 6B) suggests the presence of at least one residue of tyrosine in protein 6 (see also Brewer, 1972), especially in view of the total absence of histidine.

Discussion

The six proteins of PM2 bacteriophage discussed in this paper are all components of the virion by the criteria that their distribution is unaltered upon repeated purification of the virus and that pulse-chase double labeling experiments (Brewer, 1972) show that none of these proteins is host derived. The molecular weights of the major PM2 proteins, although estimated in a different type of polyacrylamide gel system, generally agree with those of Datta *et al.* (1971) as noted in Table I. Their proteins I, II, III, and IV correspond to proteins 1, 3, 4, and 6, respectively, described here. The better resolution of the Laemmli gel system consistently demonstrated protein 2 as a high molecular weight shoulder of protein 3 and protein 5 well resolved between proteins 4 and 6. The DNA of PM2 at 6.9 × 10^6 daltons (Espejo and Canelo, 1969) can code for 420,000 daltons of protein. All the proteins listed in Table I, including four questionable trace components (Brewer, 1972), total 220,000 daltons, or only half of the genome potential. Quantitative differences exist between the numbers of copies of each major protein per virion calculated from our data and by Datta *et al.* (1971). These differences are compounded of differences in the apparent molecular weights and in the weight percentages of each protein. The molecular weight and the weight percentage values may be sufficiently precise in each case, but they are not absolute values. The former may vary with the type of gel used, and the latter with the internal label em-

ployed. As both the gels and the internal labels were different in our experiments and in those of Datta *et al.* (1971), the apparent discrepancies may be methodological, and this suggests that the calculated stoichiometries of the components be employed with caution.

Some comments are in order concerning the methods we have used to investigate the disposition of these proteins in the intact PM2 virion. Several of the methods (immunological approaches, transglutaminase and lactoperoxidase labeling, and proteolysis) reflect upon the *accessibility* of regions of the individual proteins of the intact virion to short-range interaction with a soluble large protein (antibody or enzyme). The intactness of the virion during such an experiment is critical to an unambiguous interpretation of the results. In the binding of antibody to protein 3 by the intact virion, and in the use of the enzyme transglutaminase for covalent labeling with [^{14}C]glycine ethyl ester without any inactivation of the virus, the virion most likely remained intact. Another important feature of the transglutaminase labeling method is the extensive labeling produced (A. H. Dutton and S. J. Singer, to be published). With levels of 1–2 mol of label/mol of protein (Table III), the possibility is precluded that only a minor (perhaps disrupted) fraction of the virus preparation was labeled. In our limited experiments with lactoperoxidase labeling, however, we found the virus to be 50% inactivated. It is not certain whether the virions had remained intact during this reaction, and we have therefore not placed much weight on these experiments in regard to the disposition of the ^{125}I-labeled proteins. In the proteolysis experiments, infectivity was progressively lost as the reaction proceeded; we therefore consider as significant only those results obtained at initial stages of the proteolysis.

With these considerations in mind, the following inferences about the dispositions of the proteins of the PM2 virion are consistent with the experimental results we have presented.

Proteins 1 and 3 appear to be peripheral proteins (Singer, 1971) at the outer surface of the virion. Protein 3 is the major protein by weight and is easily cleaved from the virus and isolated in a pure state (Brewer, 1972). The fact that the intact virion absorbed the antibody specifically directed to purified protein 3 strongly points to the exterior location of that protein on the virus particle. Protein 3 was also heavily labeled in the transglutaminase reaction with the intact virus (Figure 5). Protein 1, on the other hand, is a relatively minor protein of the virion. It was not labeled in the transglutaminase reaction either with intact or freeze–thaw disrupted virions, which results are therefore uninformative about the location of the protein. However, protein 1 showed closely similar behavior to protein 3 in the freeze–thaw experiments and in proteolysis. Both proteins were solubilized in molecularly intact form by the mild freeze–thaw treatment, whereas the other proteins remained associated with the membranous structure of the virus (Figure 2); and both proteins were more rapidly lost than the other proteins in the early stages of tryptic digestion of the virion (Figure 4).

Proteins 4 and 5 are accessible on the outer surface of the lipid bilayer, since they were both labeled by the transglutaminase reaction with the intact virion (Figure 5). On the other hand, they appear to be more deeply situated in the virion than are proteins 1 and 3. They remained associated with the membrane fraction in the freeze–thaw experiments, and they were more slowly digested by trypsin treatment of the virus than were proteins 1 and 3. In electron micrographs of PM2, the outer 60 Å of the virus is observed to be permeable to negative stains (Harrison *et al.*, 1971); it is therefore possible for a protein to be situated between the outer surface of the lipid bilayer and an external protein layer and still be accessible to the enzyme transglutaminase.

Protein 2 cannot be localized on the virion by these experiments. It is difficult to carry out quantitative studies with protein 2 since it is a minor component of the virus and it is difficult to resolve in gel electrophoresis from the large peak due to protein 3. In fact, its presence was not detected by Datta *et al.* (1971). It was not clearly fractionated in the freeze–thaw experiments, nor markedly labeled in the transglutaminase experiments. Protein 2 was, however, labeled with high specific activity in the lactoperoxidase experiments (Figure 6) and if it were possible to carry out such experiments without inactivation of the virus (perhaps by the use of milder H_2O_2 generating procedures (Hubbard and Cohn, 1972)), useful information about the localization of protein 2 might be obtained.

Protein 6 was isolated by the procedure described in the Methods section, and appeared to be a homogeneous polypeptide. It migrated as a single component in two-dimensional thin-layer chromatography (Figure 8), and had no detectable free N-terminal residue. Upon cyanogen bromide cleavage, however, it exhibited a single isoleucine N terminus, consistent with the amino acid analyses which indicated that protein 6, if pure, contained a single methionyl residue. Its amino acid analysis (Table IV) is consistent with its apparent molecular weight of 6500 determined from SDS–polyacrylamide gel electrophoresis experiments. Protein 6 is very likely an integral protein of the viral membrane; *i.e.*, it is probably at least partly embedded in the lipid bilayer (Singer, 1971; Singer and Nicolson, 1972). This would be consistent with its unusually hydrophobic characteristics: its solubility in chloroform–methanol (independently discovered by Camerini-Otero *et al.*, 1972), and its markedly hydrophobic amino acid composition (Table IV). In fact, compared to the presently known membrane proteins (Guidotti, 1972), it is the most hydrophobic in composition[3] with the single exception of the C_{55}-isoprenoid alcohol phosphokinase of *Staphylococcus aureus* membranes (Sanderman and Strominger, 1971). Protein 6 was also inaccessible to transglutaminase labeling in the intact virion (Figure 5), but was labeled in the freeze–thaw disrupted virion; presumably the single Glx residue of the protein (Table IV) is a glutaminyl residue at which this labeling occurs.

Camerini-Otero *et al.* (1972) claim that this protein is a glycoprotein, and that the label of [^{3}H]glucosamine can be incorporated into the protein during viral synthesis. To account for their results, given the specific activity of the [^{3}H]glucosamine they used, the glucosamine would have had to be diluted at least 2000-fold in an internal pool in the infected host cell. In our amino acid analyses of protein 6 (Table IV), the presence of as little as 0.1 mol of glucosamine, mannosamine, or galactosamine/mol of protein would have been detectable, and none was detected.

If protein 6 is a more-or-less globular molecule, it is too small to span the lipid bilayer. If it is an amphipathic molecule (Singer, 1971; Singer and Nicolson, 1972), with a hydrophobic end embedded in the lipid bilayer, and a hydrophilic end (containing most or all of its ionic amino acid residues) protruding from the bilayer, we have no direct evidence as yet to say which face of the bilayer it protrudes from. Assuming that the protein 6 molecule were a sphere, it would have a radius of about 12 Å; 900 copies of it would occupy about nine-tenths of the area of

[3] A hydrophobicity index can be calculated (Hatch, 1965) from the percentage of apolar amino acids in the protein. PM2 protein 6 has 56% apolar residues, bovine rhodopsin 52%, *S. aureus* phosphokinase 60% (Guidotti, 1972).

the inner surface of the viral membrane (at r = 200 Å; Harrison *et al.* (1971) and about six-tenths of the outer surface (at r = 240 Å), so the former position is unlikely. That some significant amount of protein is embedded in the lipid is suggested by the fact that the measured lipid content of the virion appears to be enough only to fill 65% of the volume occupied by the bilayer shell (Harrison *et al.* 1971).

Our evidence indicates that there is no significant amount of protein associated with the DNA as a soluble nucleoprotein complex inside the lipid bilayer of the PM2 virion. It is therefore not clear how the excess negative charge of the DNA is neutralized in the intact virion.

The X-ray diffraction and electron microscopic evidence (Harrison *et al.*, 1971) is that PM2 is an icosahedral virion of about 300-Å equivalent radius, with its noncrystalline lipid arranged as a bilayer centered at 220-Å radius. The information we have reported about the protein disposition in the virion is generally not precise enough to assign the proteins to specific locations in the icosahedral structure, except for protein 3. From its abundance and external location protein 3 is probably the main protein of the icosahedral shell. For icosahedra with triangulation numbers of 12 or 13 (Caspar and Klug, 1962), 720 or 780 morphological units, respectively, would be expected in the virion. The number of copies of protein 3 per virion is 750, according to our measurements (Table I); this number is therefore consistent with each copy of protein 3 being a morphological unit, and with either triangulation number. From molecular weight and composition data, but without direct evidence for the external location of protein 3 in the virion, Datta *et al.* (1971) made the same structural assignment. Protein 1, which also has an external location but many fewer copies per virion than protein 3, may be a component of the spikes seen by electron microscopy at the 12 vertices of the viral icosahedron (Harrison *et al.*, 1971). This would predict that an antibody made to a purified protein 1 would react with the intact Pm2 virion, and might be observed to localize at the icosahedral vertices.

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