Kinetics and Characterization of the Proteins Synthesized during Infection by Bacteriophage PM2

By GREGORY J. BREWER* and MANJEET SINGH

Department of Medical Microbiology and Immunology, Southern Illinois University School of Medicine, Springfield, Illinois 62708, U.S.A.

(Accepted 4 December 1981)

SUMMARY

Using two-dimensional gel electrophoresis, we have examined the proteins whose synthesis is stimulated in Alteromonas espejiana by infection with the membrane-containing bacteriophage PM2. In addition to four virus structural proteins, 11 non-structural proteins have been resolved and identified by their apparent isoelectric points and molecular weights. The relative rate of synthesis of each of the proteins was determined during the course of infection. Synthesis of the earliest proteins began around 10 min after infection. Synthesis of the virus structural proteins as a group did not begin until about 25 min after infection. In contrast to these structural proteins, the rate of synthesis of most of the non-structural virus proteins began to decline between 30 and 35 min after infection. This time preceded the onset of cell lysis marked by ion leakage (47 min); it corresponded to the beginning of packaging of virus DNA, removing that DNA from replication and transcription. Protein processing could not be demonstrated by pulse-chase labelling. These 15 proteins account for all of the coding capacity of the virus DNA. The virus origin of 14 of these proteins was established in an in vitro transcription-translation system programmed by PM2 DNA.

INTRODUCTION

We are studying bacteriophage PM2 as a model for membrane biogenesis. PM2 infects Alteromonas espejiana (Chan et al., 1978), formerly Pseudomonas BAL-31 (Espejo & Canelo, 1968b), causing the assembly of new membranes and mature phage particles, followed by lysis of the host (Cota-Robles et al., 1968; Espejo & Canelo, 1968a). PM2 has four major structural proteins (Datta et al., 1971b; Brewer & Singer, 1974; Schafer et al., 1974). The major virus structural protein, sp27 (27000 mol. wt.) (Brewer & Singer, 1974; Hinnen et al., 1974), completely encapsulates the spherical phospholipid bilayer (Harrison et al., 1971). The vertices of the icosahedral capsid are composed of sp43. The other two structural proteins of the virus, sp13 and sp6-6, appear to have an as yet unspecified association with the virus membrane (Brewer & Singer, 1974) and possibly with the DNA of the virus (Marcoli et al., 1979; Satake et al., 1981). The amino acid compositions and partial sequences of sp6-6 and sp27 have been reported (Hinnen et al., 1976). The virus genome is double-stranded supercoiled DNA of mol. wt. 6.3 x 10^6 (Espejo et al., 1969; Camerini-Otero & Franklin, 1975). The coding capacity for protein is 315000 daltons. The four major virus structural proteins account for less than one-third of the coding capacity. Therefore, at an average mol. wt. of 23000, there could be eight non-structural virus proteins. Twelve classes of temperature-sensitive mutants of PM2 have been isolated (Brewer, 1978). Some of these mutants stimulate synthesis of proteins whose apparent mol. wt. are altered from those of cells infected with wild-type virus.
As a first step towards identifying proteins that may be involved in membrane biogenesis, we have sought to identify all the proteins whose synthesis is stimulated during virus infection. Utilizing two-dimensional gel electrophoresis, we have resolved from the background of host protein synthesis (Brewer, 1976b) 11 non-structural virus proteins in addition to the four structural proteins. Previous one-dimensional analyses suggested at least 14 polypeptides whose synthesis was associated with infection by PM2 (Datta et al., 1971a; Schafer & Franklin, 1978). However, resolution of proteins in these systems was insufficient for further analysis.

**METHODS**

**Cells and virus.** Culturing of PM2 and its host A. espejiana (formerly designated Pseudomonas BAL-31) have been described previously (Brewer, 1976b). Glucose and phosphate were added to minimal salts (MTS) to make MTG (Espejo & Canelo, 1968a). Cells were routinely grown in MTG at 29 °C to $3 \times 10^8$/ml and infected with PM2 at a multiplicity of 10 as indicated.

**Ultraviolet treatment of cells to be infected.** Cells were exposed to u.v. light to inhibit host protein synthesis. Four 5 ml suspensions of exponentially growing cells were chilled to 4 °C and exposed as thin layers of suspension to u.v. light for 0, 15, 30 and 45 s. Cells were then kept in the dark. Subsequent plating for colony-forming ability indicated an exponential decline with time of u.v. exposure, after a lag of 6 s (extrapolated). Cell viability dropped to 50%, 6 s after the lag. Ultraviolet-treated cells were infected with PM2 at a multiplicity of 5 and incubated at 29 °C. Plaque assays 60 min after infection indicated an exponential decrease in production of infectious virus with no lag. Fifty percent loss of virus production occurred with 13 s u.v. exposure to the cells (extrapolated). The rate of protein synthesis was measured by incorporation of $[^{35}S]$methionine (10 μCi/ml, 437 μCi/nmol) 35 min after infection in 1 ml of u.v.-treated cells. The pulse-labelling proceeded for 2 min. After addition of unlabelled methionine to 1 mM, the cells were transferred to 5 ml 10% trichloroacetic acid (TCA) at 4 °C. TCA precipitates were collected by centrifugation (5000 rev/min, 5 min), washed twice with 2 ml 4 °C, 5% TCA and twice with 2 ml $-10$ °C, 100% ethanol. After drying, the residue was dissolved in 100 μl gel sample buffer (2% SDS, 62-5 mM-Tris-HCl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 0.002% bromophenol blue tracking dye), and heated to 100 °C for 1 min. Samples of 20 μl were applied to a stacking gel above a 17.5 to 12.5% acrylamide gradient slab gel in SDS as described by Laemmli (1970). Electrophoresis and autoradiography have been described previously (Brewer, 1976a). Apparent mol. wt. were obtained from a semi-logarithmic graph using the known mol. wt. of the structural proteins of PM2 (Hinnen et al., 1976). Total protein synthesis, measured as radioactivity in the TCA precipitates, decreased exponentially after u.v. treatment with a half-time of 18 s after a lag of 8 s.

**$[^{35}S]$methionine pulse-labelling.** From 20 ml of an infected culture, 2 ml of cells were added to $[^{35}S]$methionine (20 μCi, 660 μCi/nmol) at 0, 15, 20, 25, 30, 35, 40 and 45 min post-infection. After labelling for 2 min, 0.2 ml 10 mM-methionine was added. The cells were chilled on ice and pelleted for 3 min at 10000 rev/min at 4 °C. Previous analysis showed incorporation to be linear during the pulse. The pelleted cells were washed with 2 ml MTS at 4 °C and prepared for electrophoresis as described above.

**Two-dimensional gel electrophoresis.** Cells were prepared for electrophoresis by an adaptation of the method of Ames & Nikaido (1976). Each pellet of cells was resuspended in 60 μl 50 mM-tris–HCl pH 6.8, 0.5 mM-MgCl₂, at 4 °C. The suspension was sonicated for 1 to 2 min at room temperature (Bransonic 12, 80 W, Branson Instruments) and treated with DNase (50 μg/ml) for 5 min at 4 °C and then adjusted to 2% SDS, 5% β-mercaptoethanol and 10% glycerol. When two-dimensional gels were to be run in the absence of SDS, the
Proteins stimulated by PM2

samples were dissolved in 9 M-urea followed by 1.5 vol. 9.5 M-urea, 2% Nonidet P40 (NP40), 2% ampholytes, and 5% β-mercaptoethanol. Samples (2 × 10⁶ ct/min each) were loaded on to tube gels (125 × 2 mm internal diam.) containing urea, NP40 and 2% pH 3 to 10 ampholytes (Ames & Nikaido, 1976). Isoelectric focusing (IF) was performed at 0.07 W/tube constant power for 16 h. Gels were removed from the tubes, covered with plastic film and stored at -70 °C. A parallel tube containing no sample was cut in 5 or 10 mm pieces to determine the pH gradient (Ames & Nikaido, 1976). The second-dimension gel was in the form of a slab (1 × 144 × 140 mm wide) containing a 12.5 to 17.5% gradient of acrylamide with a 25 mm stacking gel in the SDS buffers described by Laemmli (1970). A small plastic insert created a well in the stacking gel for PM2 proteins as a standard. The tube gel was laid above the stacking gel and cemented in place with melted 0.5% agarose in the buffer of the stacking gel. Electrophoresis proceeded at 4.5 W/gel, constant power. Gels were radiographed after fixing and drying (Brewer, 1976b). To avoid quenching during scintillation spectrometry, the gels were not stained. The proteins specific for PM2 were cut out from the dried gel. Radioactivity of each protein was determined by incubation of the gel fragment at 37 °C overnight in 10 ml 94% Omnifluor, 5% Protosol (both from New England Nuclear), 0.75% water. Samples were counted in a Beckman LS-8000 scintillation spectrometer.

86Rb+ uptake (Brewer, 1976a). Cells (2.35 ml) were grown to a density of 2.7 × 10⁸/ml in MTG (KCl = 10 mM). 86RbCl (7.2 mCi/mg, 250 μCi/ml, New England Nuclear) was added to the culture. After 20 min, 1.2 ml of the culture was infected with PM2 at a multiplicity of 4. At the indicated times, 50 μl were removed and diluted with 1 ml warm MTS and filtered through a 0.4 μm pore, 25 mm diam. membrane filter (Nuclepore, Pleasanton, Ca., U.S.A.). The filter was washed twice with 2 ml warm MTS. Dried filters were counted in toluene-Omnifluor (New England Nuclear) by scintillation spectrometry using a wide-open window.

Pure [35S]methionine-labelled PM2. Ten min after infection of 10 ml cells, [35S]methionine was added to 5 μCi/ml (1.5 μM). Incubation was continued for approx. 90 min or until lysis. The culture was cleared of unlysed bacteria (8000 g, 5 min, 4 °C). Unlysed cells in the pellet were resuspended and treated for 5 min at 30 °C with 5 ml lysis medium containing 1 mg lysozyme (Brewer, 1978). All subsequent operations were at 4 °C. Bacterial debris was cleared by a second centrifugation. The combined supernatants were centrifuged at 80000 g for 45 min. The virus pellet was resuspended in 0.1 ml VSM (Brewer, 1978) and centrifuged in a 5 to 20% sucrose gradient containing VSM in a Beckman SW50.1 rotor at 36000 rev/min for 40 min. The visible virus band was collected automatically with an Isco gradient fractionator and dialysed for several hours against 1000 vol. VSM. The virus was further purified on CsCl-VSM (density 1.27 g/ml) in a Beckman 50Ti rotor at 30000 rev/min for 18 h. The virus band was similarly collected and dialysed overnight. Samples to be applied to polyacrylamide gels were subjected to ultrafiltration (Millipore PSAC filter) and dissolution of the residue in gel sample buffer.

Cell-free protein synthesis. Cell-free extracts were prepared from Escherichia coli K-12 strain CF 300 (recB21) (Yang et al., 1980) which was a gift of H. L. Yang (Department of Genetics, The Public Health Research Institute, New York, N.Y., U.S.A.). Cells were grown as described by Zubay (1973) and washed with buffer I (10 mM-tris-acetate pH 8.2, 14 mM-magnesium acetate, 60 mM-KCl, 6 mM-2-mercaptoethanol), followed by buffer II (buffer I containing 1 mM-dithiothreitol in place of 2-mercaptoethanol). They were stored as a pellet at -80 °C. Fifteen g of frozen cells were allowed to soften at 4 °C, were suspended in 15 ml buffer II and were disrupted using 80 g washed glass beads (0.10 to 0.11 mm) in a Sorvall Omni-Mix operated at maximum speed for three 90 s periods. From the disrupted cells, an S-30 extract was prepared by the method of Zubay (1973), except that the preincubation step was omitted. Washed ribosomes, a high-salt ribosomal extract and a supernatant fraction
were prepared from the S-30 extract (Kung et al., 1973, 1974). The supernatant was further fractionated by DEAE-cellulose chromatography (Kung et al., 1975). The peak which eluted with 0.25 m-potassium phosphate (0.25 m-DEAE fraction) contained aminoaoyl-tRNA synthetases, elongation factors and other factors required for protein synthesis. The peak which eluted with 1 m-potassium phosphate (1 m-DEAE fraction) contained RNA polymerase and L-factor.

PM2 virus DNA was extracted by a modification of previous procedures (Espejo et al., 1969; Richardson, 1973). One ml freshly purified virus (2.7 x 10^{13} p.f.u./ml) was pelleted at 80000 g at 4 °C for 1 h. The pellet was resuspended in 0.5 ml 0.02 M-tris-HCl pH 8, 0.1 M-NaCl, 1 mM-EDTA. Sarkosyl NL 30 (Ciba-Geigy) was added to 0.5% final concentration. The solution was left at room temperature until clear (3 to 5 min), and then extracted twice with 1 vol. phenol saturated with the above-mentioned buffer. Phases were separated by centrifugation at 5000 rev/min at 25 °C for 5 min. Phenol was removed by dialysis (4 °C) against 0.02 M-tris-HCl pH 8, 0.05 M-KCl, 0.1 mM-EDTA, with three or four changes. DNA was stored frozen at −20 °C. Phenol had been distilled under vacuum and stored in the dark under N₂ in sealed ampules.

The complete system for protein synthesis (20 μl) contained 40 mM-tris-HCl pH 8, 60 mM-KCl, 7.5 mM-MgCl₂, 10 mM-dithiothreitol, 1 mM-ATP, 0.4 mM each GTP, UTP and CTP, 40 μg phosphoenolpyruvate, 1 μg pyruvate kinase, 0.1 mM each of 19 amino acids (minus methionine), 2 μg folic acid, 5 μg E. coli tRNA, 0.75 to 1.5 A₂₆₀ units of washed ribosomes, 0.25 M-DEAE fraction (35 μg protein), 1 M-DEAE fraction (4 μg protein), high-salt ribosomal extract (10 μg protein), and 10 μCi [³⁵S]methionine (typically 900 μCi/nmol). Reaction was initiated by addition of PM2 DNA (1 μg). After 50 min incubation at 37 °C, the reaction was stopped at 4 °C. Samples were prepared for electrophoresis by adding an equal volume of twice concentrated gel sample buffer as described above.

Chemicals. [³⁵S]methionine was from either Amersham or New England Nuclear; ampholytes, electrophoresis-purity acrylamide and urea were from Bio-Rad; N,N'-methylenebisacrylamide, agarose (type I), amino acids, dithiothreitol, DNase I (from bovine pancreas), folic acid, nucleotides, phosphoenolpyruvate (trisodium salt), pyruvate kinase, tRNA (E. coli) and tris base were from Sigma; especially pure SDS (BDH) was from Gallard-Schlesinger, Carle place, N.Y., U.S.A.; NP40 was from Particle Data, Elmhurst, Ill., U.S.A. Other inorganic chemicals were reagent grade from Mallinckrodt, St. Louis, Mo., U.S.A.

RESULTS

PAGE of cellular proteins stimulated by PM2 infection

In cells infected with PM2, intracellular protein synthesis was monitored by the incorporation of [³⁵S]methionine. Electrophoresis of proteins from cells infected with PM2 revealed more than 40 bands in a polyacrylamide slab gel containing SDS (PAGE) (Fig. 1). Many of these are host proteins whose synthesis is not inhibited during infection. In a brief pulse of incorporation of ³⁵S, the synthesis of the four major structural proteins (sp) of PM2 can be seen above the background of host proteins. In addition, proteins whose synthesis is stimulated by infection were evident at the apparent mol. wt. of 33000, 20000, 15000 and 90000 (p33, p20, p15 and p9). It was possible that some of these proteins were not unique but were derived by proteolytic processing of a precursor. Processing is indicated in a pulse-chase experiment if label in a larger peptide can be chased into a smaller peptide. Fig. 1 shows the resolution of proteins from cells 30 min after infection that were pulsed with [³⁵S]methionine for 40 s and then chased with excess unlabelled methionine for 0, 0.5, 1, 2, 3 and 5 min. Critical examination does not show a significant decline in any of these eight proteins. Nor was a rise seen in the intensity of these or other proteins. Similar results were
Proteins stimulated by PM2

Fig. 1. Pulse–chase analysis of proteins during infection with PM2. Thirty min after infection, cells were pulsed for 40 s with $^{35}$Smethionine before adding excess normal methionine. At the times indicated (min), samples were prepared for SDS–PAGE by TCA precipitation. An uninfected culture was treated similarly. Separate analysis of TCA precipitates of filtered aliquots indicated linear incorporation. The maximum radioactivity was reduced to 50% after 1 min of chase. P, Purified PM2 labelled with $^{35}$Smethionine. Proteins of interest are identified by their mol. wt. ($\times 10^{-3}$).

obtained when infected cells were pulsed at 25 or 40 min after infection. Thus, no evidence was obtained for processing of these virus proteins.

The above non-structural proteins, together with the structural proteins of the virus, still account for only half of the coding capacity of the virus genome. In order to identify other virus proteins, we attempted to lower the background of host proteins by pretreatment of the cells with u.v. light. This approach resulted in inhibition of synthesis of virus proteins to the same degree as that of host proteins. Thus, resolution was not improved.

Two-dimensional polyacrylamide gel electrophoresis

Due to the difficulty in resolving virus-specific proteins in one-dimensional electrophoresis, we turned to a system in which proteins are separated according to their isoelectric points (pI) in one dimension and subunit mol. wt. in a second dimension. This system covered the range of pI values from 4 to 7. For reference, the structural proteins of PM2 were subjected to two-dimensional electrophoresis (Fig. 2). The virus structural protein sp43 showed two isoelectric forms around pH 5·4 in the first dimension. The major virus capsid protein, sp27, was present as three charged forms (pI = 6·8, 6·4 and 6·2). The most abundant form had the highest pI. The other virus proteins, sp13 and sp6·6, did not enter the isoelectric focusing gel most likely because of their hydrophobic nature (Brewer & Singer, 1974) but were observed at the edge of the second dimension gel. The faint indication of material at mol. wt. 13000 with the same isoelectric properties as sp43 suggests aggregation of sp43 and sp13 in the isoelectric gel.
When subjected to two-dimensional gel electrophoresis, protein spots were seen in infected cells which were absent from uninfected cells. At least 21 polypeptides were identified whose synthesis was stimulated by virus infection (circled in Fig. 3). In relation to locally identifiable patterns of host proteins, these spots were highly reproducible. By averaging data from a number of these gels, apparent isoelectric points and molecular weights were assigned (Table 1). The proteins in the distorted region at the acid edge were seen in every gel. The localization of sp43 amongst a large background of host proteins was more difficult but was possible based on less exposed autoradiograms and its isoelectric point seen in Fig. 2. Multiple isoelectric forms were found for sp43, sp27 and p23.

**Kinetics of protein synthesis**

The radioactivity in a spot in Fig. 3 is proportional to the rate of incorporation of methionine into that particular polypeptide during the period of pulse-labelling with $[^{35}\text{S}]$methionine. By performing this analysis at various times after infection, we observed the kinetics of synthesis of these proteins before cell lysis at 50 min after infection. As a control, the rate of synthesis of a bacterial protein (dashed circle in Fig. 3 b) remained nearly constant until 40 min after infection (Fig. 4a). In Fig. 4, the radioactivity of each virus protein was corrected for its background activity in the uninfected preparation (Fig. 3a, zero time) and
Proteins stimulated by PM2

Fig. 3. Two-dimensional PAGE of proteins synthesized in (a) uninfected cells or in (b) cells infected for 40 min with PM2. Samples pulse-labelled for 2 min with [35S]methionine were first subjected to isoelectric focusing in a tube gel. Further processing is described in Fig. 1 and Methods. Differences in background are due to photography. The intensities were nearly identical on the original autoradiograms. A partially purified PM2 standard was run in parallel on the left as a mol. wt. marker. Circled proteins are specific for infection. Dashed circle is the host protein used for the data in Fig. 4.

Table 1. Proteins whose synthesis is stimulated by infection with PM2: apparent molecular weights, isoelectric points and kinetic parameters*

<table>
<thead>
<tr>
<th>Mol. wt. (x 10^-3)†</th>
<th>+SDS pI‡</th>
<th>−SDS pI‡</th>
<th>Maximum rate (ct/min x 10^-5/2 min pulse)§</th>
<th>Start (min)‖</th>
</tr>
</thead>
<tbody>
<tr>
<td>p54</td>
<td>4.3</td>
<td>4.2</td>
<td>191</td>
<td>29</td>
</tr>
<tr>
<td>sp43</td>
<td>5.4, 6.2</td>
<td>6.2</td>
<td>73</td>
<td>15</td>
</tr>
<tr>
<td>sp43</td>
<td>5.0, 6.2</td>
<td>ND</td>
<td>105</td>
<td>15</td>
</tr>
<tr>
<td>p33</td>
<td>4.8</td>
<td>1</td>
<td>38</td>
<td>8</td>
</tr>
<tr>
<td>sp27</td>
<td>6.8</td>
<td>6.8</td>
<td>1122</td>
<td>23</td>
</tr>
<tr>
<td>sp27</td>
<td>6.4</td>
<td>6.4</td>
<td>139</td>
<td>23</td>
</tr>
<tr>
<td>sp27</td>
<td>6.2</td>
<td>6.2</td>
<td>36</td>
<td>28</td>
</tr>
<tr>
<td>p35</td>
<td>7.0</td>
<td>1</td>
<td>56</td>
<td>15</td>
</tr>
<tr>
<td>p23</td>
<td>6.2</td>
<td>6.2</td>
<td>68</td>
<td>24</td>
</tr>
<tr>
<td>p23</td>
<td>5.8</td>
<td>ND</td>
<td>81</td>
<td>23</td>
</tr>
<tr>
<td>p20</td>
<td>4.6</td>
<td>ND</td>
<td>184</td>
<td>15</td>
</tr>
<tr>
<td>p18</td>
<td>5.4</td>
<td>ND</td>
<td>77</td>
<td>22</td>
</tr>
<tr>
<td>p15</td>
<td>5.8</td>
<td>6.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>p14</td>
<td>5.3</td>
<td>5.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>sp13</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>p11</td>
<td>6.6</td>
<td>6.6</td>
<td>9</td>
<td>28</td>
</tr>
<tr>
<td>p9</td>
<td>5.2, 6.2</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>p8</td>
<td>5.2</td>
<td>1</td>
<td>36</td>
<td>11</td>
</tr>
<tr>
<td>sp6-6</td>
<td>1</td>
<td>1</td>
<td>44</td>
<td>25</td>
</tr>
<tr>
<td>Host-13</td>
<td>6.6</td>
<td>6.6</td>
<td>29</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data were taken from Fig. 4 and the averages of a number of analyses of gels like Fig. 3(b). Note that recorded averages may be different from those values apparent in any one gel such as Fig. 3.

† Average apparent molecular weight of proteins (p) and structural proteins (sp).

‡ Average apparent isoelectric point when first-dimension electrophoresis was conducted in the presence of SDS (+SDS) or in its absence (−SDS): i, insoluble, i.e. did not enter IF gel but migrated along border through second-dimension gel.

§ Maximum rate of incorporation used to normalize Fig. 4.

‖ Extrapolated start of synthesis from Fig. 4 (start ± 5 min).

ND, Not determined.
Fig. 4. Kinetics of protein synthesis of PM2-stimulated proteins. The proteins circled in Fig. 3 and those at other indicated times after infection were cut out in order to determine the amount of radioactivity present. (a) Host protein circled in Fig. 3 with radioactivity at 0 min normalized to 1.0. In the following panels, the background radioactivity of the corresponding region in the uninfected gel was subtracted from each point. These numbers were normalized to the rate of incorporation at the time of its maximum (Table 1). (b) p33 (●) and p8 (○); (c) isoelectric forms 5.4 (●) and 5.0 (○) of sp43, p25 (×), and p20 (□); (d) isoelectric forms 6.2 (□) and 5.8 (●) of p23, and p18 (×); (e) isoelectric forms 6.8 (●) and 6.4 (×) of sp27, sp13 (○) and sp6-6 (□); (f) p54 (○), isoelectric form 6.2 of sp27 (○), and p11 (×). Lines connecting the points were drawn 'by eye' and for the sake of clarity follow only selected proteins.

Fig. 5. 86Rb+ uptake in infected (●) and uninfected (○) cultures. Cells to be infected received virus at 0 min. Lines were fitted by linear regression analysis. Squared correlation coefficients were 0.88 for infected culture before 45 min [ct/min = 3770 + 119 (min)] and 0.96 after 45 min [ct/min = 22800 - 288 (min)], and 0.89 for uninfected culture [ct/min = 3030 + 125 (min)].

normalized to the rate of incorporation at the time of its maximum (Table 1). It is appropriate that the major capsid protein which is required in greatest abundance is the protein whose rate of synthesis is the highest.

For individual proteins, [35S]methionine was maximally incorporated at different times between 25 and 40 min after infection. The proteins could be roughly grouped into classes depending on their kinetic profiles (Fig. 4), although the assignment of the minor proteins is equivocal due to experimental variability. The proteins whose rates showed the greatest scatter were those for which the absolute rate of incorporation was low. Putative non-structural virus proteins p33 and p8 appeared to be the first virus proteins made in the infected cell (Fig. 4b). Extrapolation to zero rate suggests that their synthesis began around 10 min after infection (Table 1). After reaching a maximum at 25 min, their rate of synthesis declined to near zero by the end of infection.

The kinetic profiles of sp43, p25 and p20 were remarkably similar (Fig. 4c). Their synthesis began shortly after 15 min, peaked between 30 and 35 min and then declined to
Proteins stimulated by PM2

near zero with the exception of the 5.0 isoelectric form of sp43. The synthesis of the other virus structural proteins (Fig. 4e) continued at a substantial rate until virus assembly was complete. Their continued synthesis would supply these proteins at maximal levels during the period of virus assembly, 35 to 45 min after infection (Cota-Robles et al., 1968; G. J. Brewer, unpublished observations). The kinetic profiles of p18 and p23 were similar in starting synthesis around 22 min after infection, peaking at 30 min, and declining thereafter (Fig. 4d). With a shift to times 5 min later, the same profiles were characteristic of p54, p11 and the 6.2 isoelectric form of sp27 (Fig. 4e).

Does ion leakage inhibit protein synthesis?

It was interesting that the synthesis of some of the proteins nearly stopped in the middle of the infectious cycle (p8, p18 and p25) while others continued at near maximal rate until the completion of virus maturation (sp6.6, sp13 and two forms of sp27). An explanation was sought for the break in the rate of protein synthesis that occurred between 30 and 35 min after infection. Possibly, at this time, cells became leaky to ions such as K⁺ and Na⁺ whose concentrations are known to affect protein synthesis (Ennis & Artman, 1972; Egberts et al., 1977; Carrasco, 1978). We tested this hypothesis by measuring the uptake of the K⁺ analogue, ⁸⁶Rb⁺. As seen in Fig. 5, Rb⁺ uptake continued throughout the infectious cycle at the same rate as seen in uninfected cells. The observed scatter is typical for uptake/transport studies. The rate did not decrease until the onset of cell lysis at 47 min post-infection. Thus, no evidence was found for changes in major intracellular ions being responsible for the decreased rates of protein synthesis.

Effects of SDS on pI values

The anionic detergent SDS was used to achieve solubilization of hydrophobic proteins prior to electrophoresis. Nevertheless, sp13, sp6.6 and some p9 did not enter the isoelectric focusing gel where excess NP40 bound the small amount of SDS present in the sample. Not until these proteins were re-exposed to SDS in the second-dimension gel were they solubilized and rendered mobile. Conversely, some of those proteins that remained soluble may have done so with residual bound SDS whose charge altered the true isoelectric point. We conducted sample preparation and isoelectric focusing in the absence of SDS and found that the pI values of sp43 and p15 were significantly higher than when they had been exposed to SDS (Table 1). Assuming a pKa for SDS of 2.08 (Hejl, 1965), the shift in pI could be explained by the stoichiometric binding of 36 dodecyl sulphate ions per molecule of sp43. The other pI of 5.0 is explained by the binding of 56 dodecyl sulphate ions per molecule of sp43. This latter figure is about 0.4 g SDS/g protein that is observed for many proteins (Reynolds & Tanford, 1970). The pI of sp43 obtained in NP40 detergent using ampholytes agrees well with that of Schafer et al. (1974) who used extrapolation to zero mobility after electrophoresis at various pH values in 6 M-urea in the absence of detergent. Insoluble in NP40–9 M-urea were p33, p25, sp13, p9, p8 and sp6-6; they migrated at the edge of the second dimension gel. These proteins may be hydrophobic proteins.

Cell-free protein synthesis

In order to demonstrate the viral origin of the proteins whose synthesis is stimulated in vivo, we established an in vitro transcription–translation system programmed by PM2 DNA. Earlier attempts to prepare cell-free extracts from A. espejiana yielded little protein synthesis above background (Schafer & Franklin, 1978; Grobovsky & Brewer, 1979). Synthesis using E. coli extracts was also low until an exonuclease was eliminated by the preparation of extracts from a recB mutant (Yang et al., 1980). Seventeen proteins were synthesized in vitro using PM2 DNA (Fig. 6). With the exception of p37, p19 and p16, these appear to be the
Fig. 6. Cell-free protein synthesis. Samples were prepared and incubated as described in Methods. Incubations contained PM2 DNA (lanes 3 and 6) or no DNA (lanes 1 and 4). Samples were applied to a polyacrylamide–SDS slab gel along with pure virus labelled with $^{35}$Smethionine (lanes 2 and 5). The exposure of the autoradiogram in lanes 1, 2, and 3 was six times that of lanes 4, 5 and 6.

same proteins synthesized in vivo. In vitro, p54 was not detected. Only the larger form of sp6.6 was made in vitro. Schafer & Franklin (1978) also demonstrated the in vitro synthesis of sp43, p37, p33, sp27, sp13, p9, p8 and sp6.6.

**DISCUSSION**

Using two-dimensional PAGE, we have identified 15 proteins whose synthesis is stimulated in *A. espejiana* by infection with bacteriophage PM2. They are characterized by discrete molecular weights and isoelectric points. The multiple isoelectric forms of sp43, sp27 and p23 could result from deamination of glutamines or other post-translational modifications of charge (Bobb & Hofstee, 1971). Omitting multiple isoelectric forms as not uniquely coded, the sum of mol. wt. of these 15 proteins is 320000. Assuming that none of these proteins is a cleavage product or host-derived, they account for all of the 315000 dalton coding capacity of the virus DNA. The cell-free synthesis of all but one of these proteins (p54) is strong evidence that they are in fact coded by the DNA of PM2.

The kinetic parameters of synthesis of the virus-stimulated proteins were distinct from those of host proteins. The earliest proteins to be synthesized after infection were p33 and p8, followed by p25, p20, sp43, p23 and p18. This order may represent the order of transcription. Also, the grouping of these proteins into sets of kinetic profiles may be a consequence of the
Proteins stimulated by PM2

operons from which they are transcribed. Mutants of PM2 blocked at an early stage of infection (Brewer, 1978) may be defective in one of the early proteins or may be defective in control of protein synthesis. These proteins are candidates for early virus functions such as regulation of DNA replication. There appears to be control of gene expression to delay the synthesis of the virus structural proteins until late in infection. Because the synthesis of p11 and p54 was also delayed, these proteins may participate in the late maturation of the virus. However, differences in grouping for different isoelectric forms may be more indicative of experimental variability.

The break in the rate of synthesis that occurs for most of the virus proteins at 30 to 35 min after infection apparently is not due to a change in intracellular ion concentration. This time in the maturation cycle is precisely the end of virus eclipse and the start of appearance of infectious progeny (Franklin et al., 1969; G. J. Brewer et al., unpublished observations). Thus, this is the time when packaging of virus DNA depletes the pool of DNA being transcribed; levels of messenger RNA begin to decrease due to turnover. The continued synthesis of the virus structural proteins and p54 probably reflects the stability of their RNAs but may also be due to translational control.

Although proteolytic cleavages occur in the morphogenesis of other viruses (for example, Laemmli & Favre, 1973), by pulse-chase studies, we have failed to detect proteolytic processing of PM2 proteins. No proteolytic cleavages occur during the morphogenesis of phage P22 of Salmonella (Botstein et al., 1973). With the possible exception of sp6.6, the in vitro synthesis of proteins of the same mol. wt. as those synthesized in vivo suggests that proteolytic processing is not required for assembly of PM2.

We thank James McIntosh, Jr. for perfecting the two-dimensional technique in our laboratory and David Warren for technical assistance. We are grateful for the comments of Drs Michael Lai and Raymond Mosteller in the preparation of this manuscript. We also thank Drs William Wickner and Herbert Weissbach for assistance with in vitro protein synthesis. This work was supported by grants from the Robert E. and May R. Wright Foundation, the National Science Foundation (PCM 77-02733), and the National Institutes of Health (AI17679).

REFERENCES


(Received 1 October 1981)