Evidence for the Temporal Regulation of Protein Synthesis in
Synchronized Bacteriophage φX174 Infections

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SUMMARY

Virus-specific protein synthesis has been studied in unsynchronized and in
starvation-synchronized infections of u.v.-irradiated su-
host cells by φX174wt and a set of φX conditional lethal mutants. Several virus-specific protein species
were detected in association with the host cell membrane at various times during
the virus reproduction cycle; virus-specific proteins were also detected in the host
cell cytoplasm. The intracellular distribution, between the cell membrane fraction
and the cell cytoplasm fraction, of virus proteins was measured under a variety of
experimental conditions. The possible significance of the presence of virus proteins
on the host cell membrane is discussed.

INTRODUCTION

Despite the fact that Denhardt & Sinsheimer (1965a, b) showed a number of years ago
that the initiation of the virus reproduction cycle in φX174-infected cells can be synchronized
by either infecting cyanide-treated cells and then removing the cyanide, or by infecting
starved cells and then adding nutrients, no one has yet studied φX- specific protein synthesis
in synchronized infections. Such a study would be desirable for two reasons. Firstly to
confirm Godson's (1971a) observations that, in unsynchronized infections of u.v.-irradiated
cells, φX-specific protein synthesis becomes detectable at 5½ to 7 min after infection.
Godson's data indicate that although there is a different rate of synthesis for each of the
eight virus-specific proteins identified, the proteins become detectable virtually simulta-
neously, the rate of synthesis for each continues to increase for 25 to 30 min post-infection,
and then the rates begin to decrease, with detectable quantities of each protein being made
for as long as two hours after virus infection. Thus, there are no apparent 'early' or 'late'
φX-specific proteins. It should be noted that Godson used a multiplicity of infection of 15 in
his experiments, and while it may be argued that this high m.o.i. would tend to synchronize
the infections, Stone (1970) has shown that both host-cell macromolecular syntheses and
synthesis of φX-specific DNA, RNA and protein are severely depressed when high m.o.i.
are employed. It should also be noted that by 5½ to 7 min after infection, φX progeny RF
DNA synthesis, requiring at least the gene A protein(s), is already in progress (Sinsheimer
et al. 1962; Lindqvist & Sinsheimer, 1968), hence one must wonder why the virus gene A
products were not detected before 5½ to 7 min.

A second reason for a study of φX-specific proteins synthesized after synchronous initia-
tion of virus reproduction is to obtain information concerning the intracellular location of

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virus-specific proteins during the virus reproduction process. Although \( \phi \text{X RF DNA synthesis} \) occurs in association with the 'essential' host-cell membrane 'site', and progeny single-strand DNA synthesis occurs in the host-cell cytoplasm (Yarus & Sinsheimer, 1967; Knippers, Komano & Sinsheimer, 1968; Komano, Knippers & Sinsheimer, 1968), only Van Der Mei, Zandberg & Jansz (1972) have investigated the intracellular location of virus-specific proteins. The data of Van Der Mei et al. (1972) are difficult to relate to the data of Godson (1971a) and others because Van Der Mei et al. (1972) used chloramphenicol (35 \( \mu \text{g/ml} \)) to suppress host-cell protein synthesis; the host cells were not u.v.-irradiated. Van Der Mei et al. (1972) found that minor proportions (no calculations were presented) of the gene C, gene D and gene F proteins were associated with a lysed cell pellet fraction, while most of the gene H protein and all of the gene A protein (55,000 mol. wt.) were associated with the pellet fraction. All of the virus gene G protein was found in the supernatant fraction, as were the major proportions of the gene C, D and F proteins. The data of Van Der Mei et al. (1972) also indicate the presence of several unidentified polypeptide species in both the cell pellet and supernatant fractions. Godson (1971b) has, previous to the publication of Van Der Mei et al. (1972), presented experiments which indicate that the use of chloramphenicol severely depresses protein synthesis in \( \phi \text{X-infected} \) and in uninfected cells. His results indicate that \( \phi \text{X-specific proteins} \) cannot be detected unless the bacteria have been u.v. irradiated prior to the addition of 35 \( \mu \text{g/ml} \) chloramphenicol, followed by \( \phi \text{X infection.} \)

The experiments reported in this communication were carried out to study in some detail the timing of synthesis of \( \phi \text{X-coded proteins} \) in starvation-synchronized virus infections, and to study the intracellular locations of virus proteins at various times during the virus reproduction cycle, in an effort to gain additional information concerning the function(s) of virus proteins in the \( \phi \text{X infection process.} \) We have not attempted to resolve any existing disagreements over the identification of certain of the virus gene products. These disagreements are likely to continue until a study with a set of \( \phi \text{X deletion mutants} \) is possible; even then the severe polarity effects which have been observed (see Benbow et al. 1972 and Vanderbilt et al. 1972) may result in residual ambiguity.

**METHODS**

**Bacteria.** The following *Escherichia coli* strains were used. All were obtained from Dr R. L. Sinsheimer and have been used in the characterization of \( \phi \text{XI74 nonsense mutants} \) (Benbow et al. 1971).

C (BTCC 122) is the standard prototrophic su\(^-\) host. HF4714 and HF4726 are su\(^+\) hosts for \( \phi \text{X amber mutants} \). Su\(_{\text{nes}}\) is a su\(^+\) host for \( \phi \text{X amber and ochre mutants. CIT103} \) is a su\(^+\) host for \( \phi \text{X opal mutants. HF4704 thy}^-\), her\(^-\) is a su\(^-\) host for am, och, and op \( \phi \text{X nonsense mutants.} \)

**Bacteriophage.** \( \phi \text{XI74wt} \) and the following \( \phi \text{X nonsense mutants} \) were obtained from Dr R. L. Sinsheimer: am\(_{13}\) (E), am\(_{9}\) (G), am\(_{10}\) (D), am\(_{23}\) (H), am\(_{33}\) (A), am\(_{86}\) (A); och\(_{6}\) (C); op\(_{6}\) (F). The letters in parentheses indicate the \( \phi \text{X cistron} \) to which the mutants have been assigned (Benbow et al. 1971).

**Media and buffers.** Media and buffers in this study have been previously described (Loos, Tessler & Salivar, 1971).

**Chemicals.** Unlabelled purines, pyrimidines and amino acids were obtained from Sigma, as were SDS, Na\(_{2}\)-EDTA, urea, tris base, egg white lysozyme, 2-mercaptoethanol and dithioerythritol.
The following polyacrylamide gel electrophoresis reagents were obtained from Eastman Kodak Company: ammonium persulphate, acrylamide, \(N,N'-\)methylenbisacrylamide, \(N,N,N',N'\)-tetramethylethylenediamine and bromophenol blue.

Bacteriophage T4 lysozyme was obtained from Calbiochem.

Isotopes. A \([^{14}\text{C}]\)-labelled L-amino acid mixture (0.1 mCi/ml) and a \([^{3}\text{H}]\)-labelled L-amino acid mixture (1.0 mCi/ml) were obtained from New England Nuclear. Each of the mixtures contained 15 amino acids.

Unlabelled DNase I and pancreatic RNase, \([^{3}\text{H}]\)-RNase, \([^{3}\text{H}]\)-soybean trypsin inhibitor and \([^{3}\text{H}]\)-trypsin were purchased from Worthington Biochemical Corp.

Preparation of bacteriophage stocks. Unlabelled bacteriophage stocks were prepared by the inoculation of a single plaque into a 10 ml broth culture of \(su^+\) cells grown to a titre of \(2 \times 10^8\) at 30 °C. Several of the \(\phi X\) nonsense chain termination mutants also have temperature-sensitive properties which preclude preparing stocks at 37 °C. The infected bacteria were incubated with aeration until lysis was complete, which required from 2 to 5 h. Particulate debris was removed from the medium by sedimentation at 10,000 g for 10 min. Approximately 2/3 of the progeny viruses attached to the debris; they were removed by resuspending the debris pellet in 1 ml of borate-EDTA, dialysing the resuspended pellet against 1 litre of borate-EDTA for 3 h at room temperature, and then pelleting the debris by sedimentation at 10,000 g for 10 min. The supernatant fluid containing the virus was combined with clarified growth medium from the first sedimentation and this was centrifuged at 24,000 rev/min for 3 h in the SW-27 rotor and a Beckman L2-65B ultracentrifuge. The supernatant fluid was discarded and the pelleted \(\phi X\) particles were resuspended in 1 ml of borate-EDTA. Titres of bacteriophage stocks ranged from \(1 \times 10^9\) to \(5 \times 10^{11}\). All of the \(\phi X\) mutants used in the experiments had a maximum of \(1 \times 10^4\) wt in the prepared stocks.

The preparation of \(\phi Xam3\) stocks was modified from the above procedure in that a second, high-titre stock was prepared by infecting \(su^-\) C bacteria with a multiplicity of 3 am3 from the primary stock. The sole defect in the am3 reproductive cycle is defective lysis of the su- host cell. The infected cells were incubated for 3 h, then pelleted at 10,000 g for 10 min. The cell pellet was resuspended in 1 ml of NET buffer and then 10 μg each of egg white lysozyme and phage T4 lysozyme was added. This mixture was dialysed for 18 h against borate-EDTA buffer, and then particulate cell debris was removed from the virus particles by sedimentation as described above. Typical am3 titres ranged from \(1 \times 10^8\) to \(5 \times 10^{18}\) with \(10^{-5}\) wt. Most am3 stocks were somewhat leaky on C and HF4704 su- indicators, such that very small, pinpoint plaques were observed with approx. \(5 \times 10^{-8}\) frequency.

The preparation of am3 stocks with either \([^{14}\text{C}]\)-labelled or \([^{3}\text{H}]\)-labelled capsid proteins was modified from the above procedure in that a second, high-titre stock was prepared by infecting C bacteria growing in PPA-supplemented minimal medium. 300 μCi of \([^{3}\text{H}]\)-amino acid mixture or 30 μCi of \([^{14}\text{C}]\)-amino acid mixture were added to 10 ml of infected cells 5 min after infection. Purification of the labelled am3 stocks was carried beyond the purification described above: the labelled virus stocks were sedimented to equilibrium in a CsCl density gradient buffered with borate-EDTA in the Spinco SW-50.1 rotor at 35,000 rev/min. Approx. 20 fractions were collected from 3-2 ml of gradient; the radioactivity and p.f.u. were coincidental in four to five fractions. Two or three of the peak fractions were recovered, dialysed against borate-EDTA, and then subjected to zone sedimentation through a 6 to 24% (w/v) linear sucrose gradient, buffered with NET, prepared in 1 × 3½" tubes and centrifuged for 6 h at 5 °C and 22,000 rev/min in the Spinco SW-27 rotor. Fractions of approx. 1 ml were collected and two or three fractions, which contained peak quantities of radioactivity and p.f.u., were pooled and dialysed against borate-EDTA. These radioactive viruses were
later disrupted and the labelled capsid proteins were used as electrophoretic markers (see Fig. 1).

**Preparation of host cells for virus infection and radioactive amino acid labelling.** Strain HF4704 bacteria was used in all of the experiments. The bacteria were grown to \(4 \times 10^8\) cells/ml in minimal medium which contained PPA and an additional 8 \(\mu\)g/ml thymine. Growth occurred at \(37^\circ\)C with aeration. In starved-host-cell experiments, the bacteria were washed twice with minimal medium salts and resuspended in minimal medium salts plus 1 mM-KH\(_2\)PO\(_4\), then incubated with aeration at \(37^\circ\)C for 90 min. This is a slight modification of the starvation procedure previously reported (Denhardt & Sinsheimer, 1965a; Salivar & Sinsheimer, 1969). Ten ml samples of starved or unstarved cells were placed in 100 mm diam. plastic Petri dishes, then u.v.-irradiated for 4 min with a G.E. 15T8 germicidal lamp situated 39.5 cm above the liquid surfaces. The lamp produced an intensity of 1800 ergs/s/cm\(^2\) at the 39.5 cm distance. The cells in the Petri dishes were continuously mixed during the irradiation.

**Virus infection and radioactive labelling.** U.v.-irradiated cells were incubated at \(37^\circ\)C with aeration for 10 min, then infected with a m.o.i. = 5 of the desired \(\phi X\) genotype. Ten min after the infection of starved, u.v.-treated cells, nutrients (glucose, NH\(_4\)Cl, PPA and thymine) were added to the cultures.

In experiments where the radioactive labelling interval extended for 30 min (or more) of the virus reproduction cycle, either 100 \(\mu\)Ci of \(^{3}H\)-amino acids or 20 \(\mu\)Ci \(^{14}C\)-amino acids were added per 10 ml of infected cells. The quantity added for shorter labelling intervals, such as 5 min, was 400 \(\mu\)Ci of \(^{3}H\)-amino acids or 80 \(\mu\)Ci of \(^{14}C\)-amino acids per 10 ml of infected cells.

The virus reproduction process and the incorporation of radioactive amino acids were halted at the desired times by the addition of KCN and NaN\(_3\) at concentrations of 10 mM to the cultures. This step was omitted for cell samples destined for transfer to non-radioactive medium for a 'cold chase'. The radioactive medium was removed from the samples by rapid filtration through a Millipore type HAWPO45 cellulose nitrate filter. The bacteria, trapped on the filter, were washed with 2 vol. of PPA+thymine-supplemented minimal medium which had been pre-warmed to \(37^\circ\)C, then removed from the filter and resuspended in 10 ml of pre-warmed, non-radioactive medium. This washing procedure consumed approx. 2 min on the average, a time which is not included in the chase times shown in the tables and figure legends. Recovery of cells from the filters after washing ranged from 80 to 90\%, based upon microscopic count, cell turbidity and radioactivity. The chase interval was terminated by the addition of KCN and NaN\(_3\) each at a concentration of 10 mM.

Upon the termination of labelling or chase intervals, the cultures were mixed with an equal vol. of ice-cold NET buffer, then centrifuged at \(10,000\) g for 10 min to pellet the cells. The cell pellet was resuspended in 10 ml of cold NET buffer and re-centrifuged. The cell pellet was resuspended in 0.8 ml of NET buffer and 10 \(\mu\)g each of egg white lysozyme and phase T\(_4\) lysozyme, in a total 0.2 ml vol. were added. This mixture was dialysed for 12 h at 25 \(^\circ\)C against NET buffer, during which time the cells lysed. The cell lysates were nearly water clear and somewhat viscous; no intact cells were observed upon microscopic examination and there were no viable, colony-forming cells.

**Fractionation of cell lysates and preparation of samples for electrophoresis.** The zone sedimentation technique described by Knippers & Sinsheimer (1968) and modified by Loos et al. (1971) was used to separate cell membranes and membrane-bound components from cell cytoplasm components. The membrane-associated material was recovered from the sucrose gradients in two to three fractions located at the CsCl underlayer-gradient inter-
Phage φX174 proteins

face, while cytoplasmic components were found in the top four to five fractions of the gradient.

The membrane fractions were pooled, as were the cytoplasmic fractions, SDS was added at a concentration of 0.005%, then the fractions were dialysed against several changes of de-ionized water to remove CsCl, sucrose and the NET buffer salts. The addition of detergent was found to be necessary in preventing the binding of certain radioactive polypeptides to the dialysis membrane. Unfractionated, or whole cell lysates were also dialysed in this manner. The dialysed samples were dried, either by lyophilization or by incubation at 45 °C in a drying oven, then dissolved in 1 ml of a solvent consisting of: 25% (w/v) sucrose, 0.05 M-sodium citrate, 4 M-urea, 1.5 mM-Na₂-EDTA, 0.2% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. The pH of this solvent was approx. 7.2. The samples were heated to 90 °C for 3 min, cooled, then 5 μl was assayed for radioactivity. The heating process was repeated before samples were removed for electrophoretic analysis.

Polyacrylamide gel electrophoresis. Electrophoresis of radioactive, SDS-coated polypeptides was carried out in 6 mm diam. × 125 mm length gels. The composition of the commonly used gels included 12.5% (w/v) acrylamide (monomer) and a crosslink of N,N'-methylenebis-acrylamide at 0.425% (w/v). This crosslink (C) concentration amounts to 3.3% of the total gel concentration (monomer + C) and theoretically produces a ‘long fibre gel’ (Chrambach & Rodbard, 1971). The gels were buffered by a solvent consisting of 0.05 M-sodium citrate, 4 M-urea, 1.5 mM-Na₂-EDTA, 0.2% (w/v) SDS and 0.1% 2-mercaptoethanol, pH approx. 7.2. The polymerization agent was ammonium persulphate, prepared just before each use, and added to the buffer, monomer and crosslinking mixture at a final concentration of 3.3 mM.

The samples to be electrophoresed were layered, in a maximum total vol. of 60 μl, on to the gels. Volume sizes were determined by the requirement that a minimum of 30,000 [¹⁴C]-ct/min or 60,000 [³H]-ct/min was to be electrophoresed. A 5 μl vol. of bromophenol blue tracking dye was mixed with the sample, then tray buffer was overlaid to fill the remainder of the gel tube. Electrophoresis was performed at 25 °C at 100 V and 25 mA/gel for approx. 4 h, or until the tracking dye had moved to within 1.5 cm of the anode.

A Savant model AGD-30A autogeldivider was used to fractionate electrophoresed polyacrylamide gels after they had been removed from the glass support tubes. A mixing solvent of 1 mM-SDS was used in the autogeldivider. This solvent was added to the crushed gel particles at a 6/1 ratio. Fractions of 24 drops each were collected into glass scintillation counting bottles, each of which contained a Whatman GF/A 24 mm glass fibre disc. The fractions were slowly dried in an oven at 50 °C over a 16 h interval. Ten ml of a toluene-Omnifluor (New England Nuclear Corp.) scintillation counting fluid were added to each of the dried samples, which were then counted for [¹⁴C] and [³H] radioactivity in a Nuclear Chicago Mk I Liquid Scintillation System. Carbon-14 was detected with an efficiency of 53.4% while [³H] detection was 26.7% efficient.

Presentation of data in electropherograms. The raw electropherogram radioactivity data were processed by program in a Hewlett-Packard Model 9100B calculator, yielding radioactivity data which had been corrected for spectral overlaps and detection efficiency. The data were then refined further so that the [¹⁴C] or [³H] d/min in each electropherogram fraction was expressed as the percentage of the total [¹⁴C] or [³H] radioactivity, respectively, recovered from the electropherogram.
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RESULTS

Electrophoresis of virus capsid proteins

The electrophoretic profile of a mixture of the capsid proteins removed from purified \[^{14}C\]-\(\phi\)Xam3 and \[^{3}H\]-\(\phi\)Xam3 phages is presented in Fig. 1. There are four radioactive polypeptide peaks present and they are designated B, G, H and F, from left (anode of the electropherogram) to right, to indicate the probable virus cistron coding for each (see Benbow et al. 1971). The relative quantities and the \(R_f\), or mobilities, of the four SDS-coated virus capsid polypeptides are in good agreement with the results of others (Burgess & Denhardt, 1969; Gelfand & Hayashi, 1969; Godson, 1971a). The apparent mol. wt. of the four capsid polypeptides (and other virus and host-cell coded polypeptides to be described below) were determined by co-electrophoresing the \(\phi\)X capsid proteins or \(\phi\)X infected cell lysates with radioactive, known mol. wt. proteins. The standard proteins used were: bacteriophage M13 capsid proteins, soybean trypsin inhibitor, trypsin, and pancreatic RNase. The latter had considerable amounts of aggregates present and only the most mobile species was considered in calculations. A graph of log mol. wt. vs \(R_f\) of a given polypeptide provided a straight-line relationship over the range studied. These data (not shown) indicate that the apparent mol. wt. of the four \(\phi\)X capsid polypeptides are: B = \(10400 \pm 310\); G = \(18960 \pm 365\); H = \(36300 \pm 760\) and F = \(49000 \pm 1000\). The apparent mol. wt. are the averages from 10 or more electropherograms.

Identification of virus-specific proteins in \(\phi\)X-infected cell lysates

Several peaks of radioactivity are present in the electropherograms of \(\phi\)X infected cell lysates which are not present in the lysates of uninfected cells. This is illustrated in Fig. 2a, which contains the profiles of \[^{14}C\]-labelled polypeptides from an unsynchronized \(\phi\)Xwt infection of u.v.-treated HF4704 bacteria, labelled from 0 to 60 min during the virus reproduction process, and \[^{3}H\]-polypeptides from an uninfected, u.v.-treated culture of HF4704 cells labelled for 60 min. Areas of the electropherogram of interest are labelled I, II, B, D, G, A, H and F, from left (anode) to right. It can be seen that peaks I and II are present in the uninfected cell lysate in quantities which are proportionately equivalent to their amounts in the infected cell lysate.
Fig. 2. Electropherograms showing a comparison of intracellular φX174-specific [³°C]-labelled proteins synthesized during 60 min growth in radioactive medium with residual, host-cell-specific [³H]-labelled proteins synthesized during 60 min growth, and a comparison of the [³°C]-labelled intracellular φX174 proteins with [³H]-φam3 capsid proteins from purified phages. ○○○, [³°C]; ⋄⋄⋄, [³H]. (a) Whole cell lysates, containing both membrane and cytoplasmic fractions of [³°C]-virus-infected cells and [³H]-uninfected cells. (b) Whole cell lysate of [³°C]-virus-infected cells and [³H]-capsid proteins. (c) Membrane fraction of [³°C]-virus-infected cells and [³H]-capsid proteins. (d) Cytoplasm fraction of [³°C]-virus-infected cells and [³H]-capsid proteins.
Peaks I and II are present in the electropherograms of infected or uninfected cell lysates which have been prepared under a variety of experimental conditions: they are present upon the termination of short (1 to 2 min) or long (10 to 90 min) labelling intervals and they are apparently synthesized throughout the virus infection cycle, although they are in proportionately and absolutely greater quantities during the first 10 min of infection. The apparent mol. wt. of polypeptide I is 6900 and that of polypeptide II is 10,400. It is apparent from the Fig. 2a electropherogram that polypeptides I and II are of host-cell origin and are not virus-coded. We have been unable to develop any evidence that virus infection alters these species in any way other than to cause a decrease in the quantities synthesized during the latter stages of virus infection.

The electropherogram illustrated in Fig. 2b is the [14C]-labelled φXwt-infected whole-cell lysate, co-electrophoresed with [3H]-φXam3 capsid proteins. At least three of the virus capsid proteins, G, H and F, are present among the [14C]-labelled polypeptides in the cell lysate. The gene B capsid protein co-electrophoreses with the peak II polypeptide. We will describe in a section to follow an attempt to resolve the two species by isolation from a primary electropherogram and then re-electrophoresis.

The electrophoretic profiles of [14C]-labelled proteins from the membrane fraction and the cytoplasmic fraction of the φXwt-infected cell lysate are illustrated in Fig. 2c and Fig. 2d, respectively. Each has been co-electrophoresed with [3H]-φXam3 capsid proteins. There are present several virus-specific polypeptides and also polypeptides I and II in both electrophoretic profiles.

The distribution of φX-specific polypeptides between the membrane and cytoplasm of cells infected with φXwt or with φX mutants was measured in a series of experiments which are summarized in Table I. The data given in this table are the averages of three electropherograms for each of two infected cell lysates of each virus genotype used.

One general feature of the data contained in Table I is that the gene C polypeptide was not detected in any of the profiles, whether the origin of the protein samples was from cells in which a normal infection occurred (line 1, φXwt and lines 8 to 11, am3), or from cells in which the infections were abnormal because of virus mutation (lines 2 to 7). A second general feature is that the ubiquitous polypeptide peak I and II species were generally predominantly membrane-associated. Also of interest is the fact that the gene A product was detected in only four instances. (1) The φXwt profile, in line 1, where 6% of the total radioactivity is possibly the A protein; in this case all of it was found to be membrane-associated. (2) In the am10 profile, in which the A protein equals 9% of the total, with somewhat over one-third of it being membrane-associated. (3) In the op6 profile in line 7, in which 5% of the total recovered radioactivity is the A protein. In this experiment, all of the gene A protein was membrane-associated. (4) In the am9 profile, line 4, 8% of the total, all cytoplasmic in location, was accounted for as the A protein.

It is generally agreed that the polypeptide products of virus genes D and F are the two predominant species in the electrophoretic profiles of φX-specific polypeptides. Our results confirm these prior observations. The D protein amounted to 25 to 40% of the total radioactivity; in our experiments the major proportion of it was always in the cytoplasm fraction. From 15 to 27% of the total radioactivity was identified as the gene F protein in our experiments, but the intracellular distribution was variable. An examination of the am3 10 to 20 min label experiment in line 8 and the am3 10 to 20 min label with 90 min chase experiment, in line 9, reveals that some of the D and F polypeptides which were found with the membrane upon the termination of labelling were later chased into the cell cytoplasm. This release pattern is also true for the gene G protein in these experiments.
Table 1. The relative amounts and the intracellular distribution between membrane and cytoplasm of $\phi X$-specific proteins from cell lysates of unsynchronized infections with $\phi X$wt and with $\phi X$ mutants*

<table>
<thead>
<tr>
<th>$\phi X$ genotype and reproduction effect</th>
<th>Protein labelling interval (min)</th>
<th>% of total cellular acid-insoluble radioactivity associated with:</th>
<th>The fraction of total radioactivity recovered from electropherogram found in these proteins, and their intracellular distributions (expressed as % membrane-associated/% cytoplasmic)</th>
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<tr>
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<td>I</td>
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<tr>
<td>1 wt (none)</td>
<td>0-60</td>
<td>60 ± 1.3</td>
<td>60 ± 1.3</td>
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<tr>
<td>2 am10 (D) (SS DNA)</td>
<td>0-90</td>
<td>49 ± 2.2</td>
<td>51 ± 2.2</td>
</tr>
<tr>
<td>3 och6 (C) (?)</td>
<td>0-90</td>
<td>38 ± 1.9</td>
<td>62 ± 1.9</td>
</tr>
<tr>
<td>4 am9 (G) (spike)</td>
<td>0-90</td>
<td>60 ± 1.1</td>
<td>40 ± 1.1</td>
</tr>
<tr>
<td>5 am86 (A) (RF DNA)</td>
<td>0-90</td>
<td>48 ± 2.4</td>
<td>52 ± 2.4</td>
</tr>
<tr>
<td>6 am23 (H) (spike)</td>
<td>0-90</td>
<td>38 ± 0.9</td>
<td>62 ± 0.9</td>
</tr>
<tr>
<td>7 op6 (F) (capsid)</td>
<td>0-90</td>
<td>50 ± 1.2</td>
<td>50 ± 1.2</td>
</tr>
<tr>
<td>8 am13 (E) (lysis)</td>
<td>10-20</td>
<td>40 ± 2.7</td>
<td>60 ± 2.7</td>
</tr>
<tr>
<td>9 am13 (E) (lysis)</td>
<td>10-20</td>
<td>26 ± 3.1</td>
<td>74 ± 3.1</td>
</tr>
<tr>
<td>10 am13 (E) (lysis)</td>
<td>30-40</td>
<td>33 ± 3.2</td>
<td>67 ± 3.2</td>
</tr>
<tr>
<td>11am13 (E) (lysis)</td>
<td>60-70</td>
<td>22 ± 2.7</td>
<td>78 ± 2.7</td>
</tr>
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</table>

* Average of 3 electrophorograms for each of 2 infected cell lysates.
† Represents a 90 min chase in non-radioactive medium.
‡ The gene H polypeptide made by am23-infected cells is smaller than that made by wt-infected cells.
Table 2. The relative amounts and the intracellular distribution between membrane and cytoplasm of \( \phi X \)-specific proteins synthesized at various times during unsynchronized infections with \( \phi X_{\text{wt}}^* \)

<table>
<thead>
<tr>
<th>Post-infection labelling interval (min)</th>
<th>Virus reproduction halted (min of development)</th>
<th>Total acid-insoluble radioactivity incorporated ([\text{cpm}], \times 10^6)</th>
<th>% total cellular acid-insoluble radioactivity associated with:</th>
<th>The fraction of total radioactivity recovered from electropherogram found in these proteins, and their intracellular distributions (expressed as % membrane-associated/% cytoplasmic)</th>
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<tr>
<td>I</td>
<td>II</td>
<td>D</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>1 0–3</td>
<td>3 1.76</td>
<td>$57 \pm 2.1$</td>
<td>$43 \pm 2.1$</td>
<td>$20 \pm 2.5$</td>
</tr>
<tr>
<td>2 3–6</td>
<td>6 3.38</td>
<td>$54 \pm 2.3$</td>
<td>$46 \pm 2.3$</td>
<td>$16 \pm 1.8$</td>
</tr>
<tr>
<td>3 3–6</td>
<td>11 2.75</td>
<td>$59 \pm 3.2$</td>
<td>$41 \pm 3.2$</td>
<td>$15 \pm 0.7$</td>
</tr>
<tr>
<td>4 5–8</td>
<td>8 3.15</td>
<td>$52 \pm 2.4$</td>
<td>$48 \pm 2.4$</td>
<td>$15 \pm 1.2$</td>
</tr>
<tr>
<td>5 8–11</td>
<td>11 5.04</td>
<td>$51 \pm 1.8$</td>
<td>$49 \pm 1.8$</td>
<td>$8 \pm 1.4$</td>
</tr>
<tr>
<td>6 11–14</td>
<td>14 7.14</td>
<td>$51 \pm 2.2$</td>
<td>$49 \pm 2.2$</td>
<td>$6 \pm 1.7$</td>
</tr>
<tr>
<td>7 14–17</td>
<td>17 10.24</td>
<td>$45 \pm 1.6$</td>
<td>$55 \pm 1.6$</td>
<td>$6 \pm 0.7$</td>
</tr>
<tr>
<td>8 17–20</td>
<td>20 10.52</td>
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<td>$63 \pm 1.3$</td>
<td>$6 \pm 1.1$</td>
</tr>
<tr>
<td>9 20–23</td>
<td>23 10.28</td>
<td>$38 \pm 2.5$</td>
<td>$62 \pm 2.5$</td>
<td>$5 \pm 0.3$</td>
</tr>
</tbody>
</table>

* Average of data from 4 electropherograms of each sample.

† Radioactivity present in 1.0 ml of cell lysate containing 4.0 x 10^6 lysed cells. The lysates were then fractioned by zone sedimentation.
Phage φX174 proteins

In non-synchronous infections

Viable progeny phages become detectable intracellularly at approx. 20 min after infection of u.v.-irradiated bacteria by φXwt. In order to study the relative amounts of φX-specific proteins synthesized, and their intracellular distributions during the replication events which led to the appearance of progeny viruses, a 100 ml culture of HF4704 bacteria was u.v.-irradiated, then infected with φXwt. At various times after infection, 10 ml samples of cells were provided with [3H]-amino acids for 3 min intervals. The labelling (or non-radioactive chase) was halted with KCN-NaNO3, the cells were washed, lysed and separated into membrane and cytoplasm fractions. The radioactive polypeptide profiles were then analysed electrophoretically; a summary of the information obtained is contained in Table 2. Inspection of columns 4 and 5, which contain data on the gross protein radioactivity associated with the cell membrane and with the cell cytoplasm fractions, respectively, reveals that a majority of the radioactivity, from 51 to 59% of the cellular total, is associated with the membrane until the 14 to 17 min labelling interval. There is then a transition, so that by the termination of the final, 17 to 23 min labelling interval, 62% of the total cellular radioactivity is found in the cytoplasm fraction.

There is no detectable virus-specific protein synthesis during the first 0 to 3 min labelling interval. There is detectable polypeptide I, and a majority 85% of this species is associated with the cell membrane. Twenty per cent of the recovered radioactivity is in the polypeptide I peak, with the remainder of the radioactivity present in approximately equal quantities/fraction of the electropherograms. Virus-specific proteins become detectable upon the termination of the second, 3 to 6 min labelling interval. The products of φX genes D, F and G appear first. Synthesis of the D protein reaches a maximum level during the 14 to 17 min labelling interval and, prior to this, from 1/3 to 1/2 of the total gene D protein radioactivity found at the termination of each labelling interval is associated with the membrane fraction. Thereafter, up to 81% of the total is found in the cytoplasm fraction. Synthesis of the gene F capsid protein appears to reach a maximum level during the 11 to 14 min labelling interval and thereafter declines slightly. There is a transition in this case also, such that the major amounts of gene F protein synthesized after the 14 to 17 min interval are found in the cytoplasm fraction. The profile of the gene G polypeptide synthesis is similar to that of the D protein, but in the G polypeptide profile only about 1/3 of the total is membrane-associated early in the infection. The gene C polypeptide, whose function is unknown, is first detected during the 8 to 11 min labelling interval. All of the gene C protein, which ranged from 1 to 5% of the total radioactivity recovered, was found in the cytoplasm. The gene H protein was first detected during the 5 to 8 min labelling interval and at the termination of this interval nearly 1/2 of the total was membrane-associated. The total amount of H protein synthesized during each of the labelling intervals remained nearly constant as a proportion of the total radioactivity recovered, but the proportion of cytoplasmic gene H protein became predominant at later times.

The gene A product was not detected until the 17 to 20 min labelling interval, which is a surprising result, since this gene product is required for progeny RF DNA synthesis, which begins very early in φX reproduction. The relative amount of the gene A product detected was quite small, 2 to 3% of the total. All of the gene A product detected was membrane-associated.

The data in line 3 of Table 2 contain the results of a non-radioactive chase, in which a sample of the cells labelled from 3 to 6 min was washed, then incubated in cold medium for
5 min. These data show that, upon termination of the chase, there is an increase in the proportions of the D and F polypeptides found with the membrane, while the relative total intracellular amounts of the two species have remained essentially constant. Thus, some of the cytoplasmic gene D and gene F proteins have migrated to the membrane. The converse is true of the gene G polypeptide: all of the radioactive G protein synthesized during the 3 to 6 min interval was found in the cytoplasm at the termination of the chase interval. These data also show that no virus gene A, C or H proteins have materialized at the end of the chase, an observation which rules out the possibility of other, identified polypeptides serving as material precursors for any of these species. Note also that the relative amount and the intracellular distribution of polypeptide I has not changed in the chase sample, as compared to the 3 to 6 min label interval sample.

In starvation-synchronized \(\phi X\) infections

The patterns of virus-specific polypeptide synthesis which have been observed in the preceding experiments might not provide an accurate portrayal of the timing of synthesis of the individual \(\phi X\) gene products in any single infected cell. Under normal physiological conditions, each host cell can support the replication of as many as four parental RF DNA molecules and the sites of virus reproduction activity are not necessarily synchronous, even within each infected cell. \(\phi X\) DNA replication can be synchronized within a large population of infected cells by starving the cells of carbon and nitrogen sources prior to infection, then providing the infected cells with nutrients. In such experiments, an average of 1.5 \(\phi X\) parental RF DNA molecules/infected cell are replicated, and replication is synchronous within the population of infected cells (Denhardt & Sinsheimer, 1965a, b; Yarus & Sinsheimer, 1967; Salivar & Sinsheimer, 1969).

The electropherograms shown in Fig. 3 contain the profiles of radioactive polypeptides from the membrane and cytoplasm fractions of cell lysates in which \(\phi X\)wt and \(\phi X\)am86 (gene A) infections were synchronized by starving the host cells for 90 min prior to u.v.-irradiation and virus infection. In this experiment, proteins synthesized during the interval from 0 to 5 min after initiating virus reproduction were radioactively labelled: \(\phi X\)wt-infected cells were labelled with \([14C]\)-amino acids, am86-infected cells were labelled with \([1H]\)-amino acids, and uninfected cells were labelled with \([3\text{II}]\)-amino acids. The electropherogram shown in Fig. 3a is that of a mixture of membrane-associated proteins from the \(\phi X\)wt-infected cell lysate and the uninfected cell lysate, while the electropherogram in Fig. 3c is a mixture of the membrane-associated proteins from the \(\phi X\)wt- and am86-infected cell lysates. In both electropherograms, the two most prominent species are polypeptide peaks I and II; in Fig. 3a there is the possibility of small quantities of \(\phi X\)wt-coded gene D and gene F proteins. If indeed this identification is correct, the amounts of the D and F proteins which are membrane-associated at 5 min of virus reproduction are 10% or less of the total intracellular amount of each gene product. The electropherograms in Fig. 3b and Fig. 3d contain, respectively, the cytoplasmic proteins of \(\phi X\)wt-infected and uninfected cell lysates, and \(\phi X\)wt-infected and am86-infected cell lysates. In addition to polypeptides I and II, the \(\phi X\)wt-specific profile contains species we identify as the products of virus genes D, G, A, H and F. The am86-specific profile (Fig. 3d) does not contain a peak which we believe is the gene A protein. We were unable to detect the gene C protein in this experiment.

It is evident from this experiment that virus protein synthesis in synchronized virus infections does begin within the first 5 min of virus reproduction, and the majority of each of the virus proteins synthesized is not membrane-associated.
Fig. 3. The electrophoretic profiles of φX-specific proteins synthesized during the first 5 min after initiating development of φXwt-infected or φXam86-(gene A) infected, starvation synchronized u.v. irradiated HF4704 bacteria. O---O, φC<sub-ln</sub>-label and membrane fraction of infected cell lysate, 0 to 5 min [3H]-label. (b) Cytoplasm fraction of φXwt-infected cell lysate, 0 to 5 min [3H]-label and cytoplasm fraction of φXam86-infected cell lysate, 0 to 5 min [3H]-label. (c) Membrane fraction of φXwt-infected cell lysate, 0 to 5 min [3H]-label and cytoplasm fraction of φXam86-infected cell lysate, 0 to 5 min [3H]-label.
The estimated size of the gene A protein is 29000 ± 1000; this value is 5000 to 6000 smaller than the estimate of Linney, Hayashi & Hayashi (1972). We note that, characteristically, the A protein peak is unusually broad, indicating the possibilities of contamination or heterogeneous size. Also, we have no evidence for the existence of the A' protein (60 to 65000 mol. wt.). In order to test the possibilities that the gene A protein is (a) heterogeneous in size and therefore gives rise to a broad electrophoretic peak, and/or (b) can aggregate with itself or with another protein species to give rise to the larger gene A' protein, a series of experiments were carried out in which a number of variables were tested. Among these were: altering of the buffer in which the proteins were dissolved and in which electrophoresis was carried out; and altering the concentration (pore size and structure) of the polyacrylamide gels. In addition, each of the φX-specific polypeptides was isolated from an electrophogram, freed of polyacrylamide and undesirable ions, lyophilized and then re-dissolved in a desired buffer in a sufficient quantity to allow several subsequent electrophoretic analyses. The standard buffers chosen for these experiments were the pH 6.8 phosphate buffer described by Godson (1971a) and used by Linney et al. (1972), and the citrate buffer, pH 7.2, we described above.

Electropherograms illustrating the results of these experiments are shown in Fig. 4. The overall results can be summarized by stating that in none of the electrophoretic profiles were we able to demonstrate the existence of the gene A' protein, either as the result of aggregation of purified gene A protein, or by its presence in infected cell lysates. In the profiles shown in Fig. 4a and 4b, isolated gene A protein was electrophoresed through a citrate-buffered gel and through a phosphate-buffered gel, respectively. The acrylamide concentration in each of the gels was 12.5%. The A protein in each gel migrated as a single species. Thus, even though the A protein peak which was isolated from the primary electropherogram was quite wide, upon re-electrophoresis there is no detectable division into two or more polypeptide species. In the electropherogram shown in Fig. 4c we have re-electrophoresed the isolated polypeptide II + gene B capsid protein peak through a 15% acrylamide gel, buffered with citrate, in an attempt to resolve the two polypeptide species. The [14C]-labelled reference proteins co-electrophoresed in this experiment were φX capsid proteins. It can be seen that the gene B capsid protein and the polypeptide II species remain unresolved. The electropherogram in Fig. 4a contains the profiles of isolated gene F protein co-electrophoresed with φX capsid proteins through a 10% gel buffered with citrate, pH 7.2. Electrophoresis in this experiment was carried out for a longer time interval, in order to allow the gene F protein to migrate a greater than normal distance into the gel. In this experiment there is no division of the gene F polypeptide into two or more species. We wish to note also that, when the F protein was isolated from the primary electropherogram, we included in the pooled material fractions from the electropherogram which would have been expected to contain the gene A' protein (the calculated expected position of the gene A' protein in our electropherograms is from fraction 83 to 87). The profiles of re-electrophoresed virus gene D, G and H proteins, the electropherograms of which are not shown, were also indicative of a single polypeptide species in each case. We do note that, however, when samples which contain the virus gene D, C and G polypeptides together (as in infected cell lysates) are electrophoresed through gels of 7.5% acrylamide concentration, the peaks of the D and G polypeptides appear such that G is the heavy shoulder of the D peak, and the C polypeptide is lost between the two.

Based upon the post-infection timing of φX RF DNA replication, single-strand DNA synthesis and progeny virus assembly-maturation, one might intuitively expect that not all of the virus genes would be translated simultaneously and continuously throughout the
Fig. 4. Electrophoretic profiles of individual φX-specific polypeptides isolated from the standard 12.5% polyacrylamide gel electropherograms and then electrophoresed a second time in the standard gel + buffer system, or in an altered gel + buffer system. ○···○, [14C]; ○···○, [3H]. (a), [14C]-gene A polypeptide from a φXwr-infected cell lysate, 0 to 10 min [14C] label after starvation synchronization and φXam86-infected whole cell lysate, 0 to 5 min [14C] label after starvation synchronization. Electrophoresed in standard 12.5% gel with citrate-based buffer, pH 7.2. (b) As in (a), but electrophoresed in 12.5% gel with Godson's (1971a) phosphate-based buffer, pH 6.8. Both the [14C]-gene A protein and the [3H]-am-86 intracellular proteins were placed in phosphate-based sample solvent prior to electrophoresis. (c) [3H]-gene B+ peak II protein(s) from an unsynchronized φXam3 whole cell lysate, 0 to 90 min [3H]-label and [14C]-φXam3 capsid proteins. Electrophoresed in 15% gel with standard citrate-based buffer. (d) [3H]-gene F protein from an unsynchronized φXam3 whole cell lysate, 0 to 90 min [3H]-label and [14C]-φXam3 capsid proteins. Electrophoresed in 10% gel with standard citrate-based buffer.
virus reproduction process. Thus, for example, might not the gene A protein be (one of) the
first to appear, since it is required for progeny RF DNA replication? If progeny RF replication
is drastically curtailed, or even halted, at 12 to 15 min after infection, why should
there be a need for gene A protein synthesis beyond this time?

In order to explore this possibility and other possible differences in the timing of \(\phi X\) gene
translation, experiments were conducted with synchronized \(\phi X_{\text{wt}}\) infections, in which
virus-specific polypeptide synthesis was monitored by labelling with \([\text{H}]\)-amino acids
during: (1) an early stage of virus reproduction (2 to 7 min after initiating virus reproduc-
tion), (2) 10 to 12 min after initiating virus reproduction, and (3) 15 to 17 min after initiating
virus reproduction. In order to study the possible flow of nascent radioactive polypeptides
from host-cell cytoplasm to the cell membrane, or \textit{vice versa}, and the possible turnover of
radioactive polypeptides, samples from the labelled synchronized infections were trans-
ferred to non-radioactive medium for cold ‘chases’. The results of these experiments are
presented in Table 3, which contains data derived from electropherograms of \([\text{H}]\)-labelled
polypeptides recovered from the membrane and cytoplasm fractions of cell lysates. An
inspection of Table 3 reveals several interesting patterns.

The rate of total protein synthesis by the infected cells increased with time over the
intervals studied, as shown in column 3. With respect to the destination of the incorporated
radioactivity, the data in columns 4 and 5 show that the early, 2 to 7 min labelling resulted
in 71 % of the incorporated radioactivity being membrane-associated. The proportion of
membrane-associated radioactivity dropped as virus reproduction proceeded, so that only
49 % of the radioactivity incorporated during the 15 to 17 min interval was membrane-
associated. It should be noted that when a sample of cells which was labelled from 2 to 7 min
was chased in non-radioactive medium for 22 min, the proportion of membrane-associated
radioactivity dropped to 49 % of the total incorporated label. Non-radioactive chases of
samples of infected cells labelled from 10 to 12 min or 15 to 17 min resulted in slight
changes in the membrane/cytoplasm distribution of radioactive proteins. Thus, it appears
that the most marked changes in the distribution of nascent proteins occurred during the
first 10 to 12 min of virus reproduction.

It is evident from the data in Table 3 that the virus D, G, A, H and F proteins (from left
to right in this table) were synthesized during the 2 to 7 min labelling interval, and that the
entire detectable quantity of each protein was located in the cell cytoplasm. The major
species which were membrane-associated at this time are polypeptides I and II. The major
proportions of each of these host-cell coded proteins were found associated with the cell
membrane at all times during this experiment. The relative quantities synthesized during the
labelling intervals appeared to drop as virus reproduction progressed.

The virus A and D proteins, at 7 % of the total recovered radioactivity each, were the
most abundant virus-specific proteins synthesized during the 2 to 7 min interval. Synthesis
of the gene F capsid protein amounted to only 1 % of the total during the 2 to 7 min labelling
interval. Synthesis of the F protein appeared to be accelerating with time, and reached 6 %
of the total during the 10 to 12 min interval and 10 % during the 15 to 17 min interval. The
origin of the F protein is probably the cell cytoplasm, inasmuch as the first detected evidence
of this protein was in the cytoplasm. There is evidence in this experiment that there is
transfer of the F protein from the cytoplasm to the membrane, at least in the case of protein
which was synthesized early.

Synthesis of the gene D protein appeared to accelerate with time during this experiment,
the relative values increasing from 7 to 14 to 23 % of the total recovered radioactivity in the
three labelling intervals. The origin of the D protein also appears to be the cell cytoplasm,
Table 3. The relative amounts and the intracellular distribution between membrane and cytoplasm of \( \phi X \)-specific proteins synthesized during synchronized infections with \( \phi X wt^* \)

<table>
<thead>
<tr>
<th>Post-infection labelling interval (min of development)</th>
<th>Virus reproduction halted (min of development)</th>
<th>Total acid-insoluble radioactivity incorporated (PH, ct/min ( \times 10^6 ))</th>
<th>% of total acid-insoluble radioactivity associated with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Membrane</td>
</tr>
<tr>
<td>Expt. 1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1a 2–7</td>
<td>7</td>
<td>1.58</td>
<td>71 ± 1.4</td>
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<tr>
<td></td>
<td></td>
<td>35 ± 1.2</td>
<td>19 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>92/8</td>
<td>65/35</td>
</tr>
<tr>
<td>1b 2–7</td>
<td>17</td>
<td>1.51</td>
<td>58 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36 ± 1.6</td>
<td>18 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96/4</td>
<td>69/31</td>
</tr>
<tr>
<td>1c 2–7</td>
<td>29</td>
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<td>49 ± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28 ± 1.1</td>
<td>23 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86/14</td>
<td>48/54</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a 10–12</td>
<td>12</td>
<td>3.68</td>
<td>67 ± 2.0</td>
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<tr>
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<td></td>
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<tr>
<td></td>
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<td>89/11</td>
<td>67/33</td>
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<tr>
<td>2b 10–12</td>
<td>17</td>
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<td>66 ± 1.9</td>
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<td>32 ± 0.9</td>
<td>10 ± 0.6</td>
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<td></td>
<td>92/8</td>
<td>69/31</td>
</tr>
<tr>
<td>2c 10–12</td>
<td>22</td>
<td>3.56</td>
<td>68 ± 1.8</td>
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<td>Expt. 3</td>
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<td>3a 15–17</td>
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<td>58/42</td>
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<td>3b 15–17</td>
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<td>92/8</td>
<td>59/41</td>
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The fraction of total radioactivity recovered from electropherogram found in these proteins, and their intracellular distributions (expressed as % membrane-associated/% cytoplasmic)

<table>
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<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>D</th>
<th>C</th>
<th>G</th>
<th>A</th>
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<th>F</th>
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<tbody>
<tr>
<td>1a</td>
<td>35 ± 1.2</td>
<td>19 ± 2.1</td>
<td>7 ± 0.9</td>
<td>0</td>
<td>4 ± 0.3</td>
<td>7 ± 0.6</td>
<td>3 ± 0.3</td>
<td>1 ± 0.1</td>
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<tr>
<td>1b</td>
<td>36 ± 1.6</td>
<td>18 ± 0.9</td>
<td>10 ± 1.3</td>
<td>2 ± 0.8</td>
<td>6 ± 0.8</td>
<td>6 ± 1.5</td>
<td>6 ± 0.2</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>1c</td>
<td>28 ± 1.1</td>
<td>23 ± 0.7</td>
<td>10 ± 0.6</td>
<td>1 ± 0.3</td>
<td>4 ± 0.2</td>
<td>8 ± 0.2</td>
<td>5 ± 0.5</td>
<td>2 ± 0.1</td>
</tr>
</tbody>
</table>

* Average of data from 4 electropherograms of each time point in a duplicate experiment.
† Radioactivity present in 1.0 ml of cell lysate containing \( 4.0 \times 10^6 \) lysed cells. The lysates were then fractionated by zone sedimentation.
although 27 to 30% of the protein labelled during the 2 to 7 min interval was subsequently chased to the membrane. The ability to chase nascent D protein from the cytoplasm to the membrane decreased markedly as virus reproduction progressed, so that by the 15 to 17 min labelling interval, a maximum of 2% of the labelled D protein was transferred to the membrane during a 10 min chase.

The pattern of synthesis and subsequent intracellular distribution of the gene G protein was similar to those parameters observed with the D and F proteins, while the gene H protein appeared to be synthesized at a constant (or slightly accelerating) rate. Approximately one-half of the nascent H protein synthesized during each of the 2 to 7 min and 10 to 12 min labelling intervals was transferred from cell cytoplasm to cell membrane during a 10 min non-radioactive chase.

Evaluation of the synthetic pattern and the intracellular distribution of the gene C protein is difficult. No C protein was detected upon termination of the 2 to 7 min labelling, but a small amount was detected in the cell cytoplasm of the two chase portions of this experiment. No detectable C protein was synthesized during the 10 to 12 min labelling, but again, following the 15 to 17 min labelling and in the two chase portions of the experiment, a small amount of C protein was observed in the cell cytoplasm. While our data certainly cannot rule out either a pattern of discontinuous C protein synthesis or the generation of C protein from a pre-existing protein species, a more plausible explanation is that we cannot always detect a small amount of this protein when it is flanked in electropherograms by larger amounts of the D and G proteins.

The pattern of synthesis of the gene A protein in this experiment indicates that the protein is synthesized at a maximal rate early in virus reproduction, with the origin of this protein being the cell cytoplasm. The translation of gene A is turned-off by 15 to 17 min of virus reproduction. Transfer of nascent A protein to the membrane was observed only in the extended (22 min) chase of the 2 to 7 min labelled protein.

**DISCUSSION**

The analysis we have made of the φX174 capsid proteins is in good agreement with published results of others (Burgess & Denhardt, 1969; Gelfand & Hayashi, 1969; Borrás Vanderbilt & Tessman, 1971; Godson, 1971a; Benbow et al. 1972). We believe that the general agreement on the number, molecular size and relative abundance of the φX capsid proteins provides a valid calibration of our methods.

We were unable to confirm several other reported results, however. Benbow et al. (1972) suggested that the φX gene A product may be in the form of two polypeptides: one 14000 mol. wt. and a second 67000 mol. wt. Linney et al. (1972) agree with this with respect to the larger polypeptide, which they designated the A' protein. Linney et al. (1972) believe that the other gene A product, the A protein, is 35000 mol. wt. The gene A protein we have identified has a mol. wt. of approx. 29000, although the protein appears to be somewhat heterogeneous in size. Re-electrophoresis of the A protein isolated from a primary electropherogram resulted in a single polypeptide of 29000 mol wt. with respect to reference φX capsid proteins. We obtained this result using both our standard electrophoresis system, and that described by Godson (1971a) and Linney et al. (1972). We were unable to generate the A' protein from the A protein, suggesting that, in our experimental conditions, the A protein is not a material precursor of the A' protein. We were also unable to identify the A' protein by re-examination of material which might be migrating with or near the gene F capsid protein.
We estimate that the mol. wt. of the gene C protein is 17150. This is at variance with the results obtained by Borrás et al. (1971), who believe that the mol. wt. of the C protein is 10000. If they are correct and we are wrong, then the gene C protein would co-electrophorese with the host-cell coded polypeptide II and with the virus gene B protein in our experiments. As it is, we have had considerable difficulty in finding the C protein consistently because of its relative paucity compared to the D and G proteins, which flank it in electropherograms. It should be noted that Burgess & Denhardt (1969) believed that a 10,000 mol. wt. virus-coded protein was the gene E product (virus lysozyme), but Borrás et al. (1971) disputed this. We observed no differences between the electrophoretic profiles of labelled polypeptides from φXwt infected cells and φXam3 (gene E) infected cells.

Benbow et al. (1971) have tentatively identified the protein products of φX genes I and J as polypeptides of 7000 or 34000 (I) and 9000 (J) mol. wt. It is unlikely that polypeptides I and II which we report in this communication correspond to either the I protein or the J protein, because of evidence that polypeptides I and II are synthesized by u.v.-treated uninfected bacteria.

We have presented evidence that, among the proteins associated with the rapidly sedimenting host-cell membrane component, are several φX-specific polypeptide species. Any assessment of the importance to the virus reproduction process of this virus protein-host cell membrane association must be based upon evidence that these proteins are functionally associated with the membrane, and are not merely sticking to it because of environmental conditions during and after cell lysis.

All of the evidence for a functional association that is available now is indirect. In reconstruction experiments, [14C]-amino acid-labelled φX-specific proteins were recovered from zone sedimentation gradients and identified as cytoplasmic proteins. Samples of the [14C]-labelled proteins were mixed with (1) [3H]-labelled, φX-infected cells which had been lysed with lysozyme and dialysed against NET buffer for 18 h; (2) [3H]-labelled, φX-infected cells to which lysozyme had just been added. This mixture was then dialysed against NET buffer for 18 h; (3) [3H]-labelled, φX-infected cell membrane fraction isolated by zone sedimentation and dialysed against NET buffer. Each of the three mixtures was analysed by zone sedimentation, isolation of membrane and cytoplasm fractions and finally the virus-specific [3H]-labelled proteins were electrophoresed. In each case, all of the [14C]-labelled radioactivity was found in the cytoplasm fractions, indicating that there was no binding of it to the membrane. In a second experiment, T-M buffer (50 mM-tris+10 mM-EDTA, pH 7.0) was substituted for NET buffer and the above procedures were repeated. Again, no [14C]-radioactivity was found with the cell membrane fractions. In a third experiment, [14C]-labelled φX virus particle proteins from heat-disrupted am3 phages (heated to 98 °C for 10 min in borate-EDTA buffer) were substituted for the [14C]-labelled cytoplasmic proteins in an NET-buffered system. No association of [14C]-labelled virus capsid proteins with the membrane fractions was found. These results suggest that virus proteins do not become non-specifically bound to the host cell membrane. Such a conclusion must be provisional, however, because of the lack of certainty that experimentally added virus proteins have equivalent access to the cell membrane’s interior surfaces as the intracellular virus proteins.

A second line of evidence for the functional association of virus proteins with the host cell membrane can be inferred from the experimental observations that in φXwt infections the first detected virus proteins are located in the cell cytoplasm, then a portion of these proteins are later transferred to the cell membrane. At times which correspond to peak levels of membrane-localized virus RF DNA replication, one finds the greatest relative amounts of
several virus-specific proteins bound to the cell membrane. Later in the virus replication process, at times which correspond to cytoplasm-localized single strand DNA synthesis, the major proportions of these same virus-specific protein species are found in the cell cytoplasm.

Additional indirect evidence comes from the observations that, in a number of abortive infections with \( \phi X \) conditional lethal mutants, the intracellular distributions of virus-specific proteins are significantly different from the distributions of the same protein species in \( \phi X wt \)-infected or in \( am3 \)-infected bacteria. Thus, for example, all of the gene F capsid protein synthesized during a 90 min infection of \( sur^- \) host cells by the gene D mutant, \( am110 \), remains associated with the cell membrane, as compared to 58% of the wild-type gene F protein being membrane-associated in a parallel experiment (see Table 1, lines 1 and 2). Another example, in Table 1, line 7, shows that 100% of the gene G spike protein which was synthesized during 90 min by \( su^- \) cells infected by \( op6 \) (gene F) was found in the cell cytoplasm, while in \( \phi X wt \) infected cells, 26% of the gene G protein was membrane-associated (Table 1, line 1).

The data we have obtained from experiments in which virus reproduction was synchronously initiated suggest that there is, indeed, a temporal regulation of \( \phi X \)-specific protein synthesis and also a temporal regulation of the intracellular location of at least some of the virus-coded proteins. If our estimation that virus-specific proteins are synthesized in the cell cytoplasm is correct, then the following picture of \( \phi X \) replication emerges: virus messenger RNA is translated in the cell cytoplasm, with the gene A protein(s) and the gene D protein being the predominant ‘early’ proteins. The gene A protein(s) are required for parental RF DNA replication at the membrane-essential ‘site’, hence their transfer to the membrane. The difficulty we have had in showing efficient transfer of the gene A protein might be explained if the association between membrane-bound parental RF DNA and A protein is very transient or of a low binding energy. Also, if only one molecule of A protein is required per replicating parental RF DNA, then the opportunity to discover this association would be further reduced. After a pool of some 20 progeny RF DNAs has been synthesized and released into the cell cytoplasm, progeny single-strand synthesis commences. At this time (12 to 15 min of virus reproduction), there is little or no need for the gene A protein, so synthesis of A protein ceases. The need for an early and accelerating synthesis of D protein might be due to the need for a D protein molecule to associate with each nascent progeny RF DNA as it is formed at the essential ‘site’. This association might be protective (nuclease attack) or it might cause or aid in the release of the nascent progeny RF DNA from the membrane-essential ‘site’. Because the cytoplasmic progeny RF DNA are re-used in progeny single-strand DNA synthesis, there may be a sufficient need to establish a ‘late’ pool of D protein molecules in the cell cytoplasm, to ensure that single-strand DNA synthesis will continue efficiently.

The pattern of synthesis of the gene F protein is consistent with that of a ‘late’ protein. The F protein is the major capsid sub-unit, so large quantities are needed, in the cell cytoplasm, late (after 15 min) in the virus reproduction cycle. A dilemma arises because of the observation that some of the ‘early’ F protein could be chased to the membrane in our experiment. We suggest that, in addition to the D protein, the F protein (and probably the G and H proteins also) are required for protective or RF DNA-release purposes. Thus, a nascent progeny RF DNA molecule is envisioned as being released from the essential ‘site’ as a complex with at least 1 molecule each of the D, F, G and H proteins. Inasmuch as the function(s) of the gene C protein remain unknown, it is not possible to place the C protein into this scheme. It is, perhaps, of significance that we observed the C protein only in the
Phage φX174 proteins

cell cytoplasm. It should be noted that, while we indicate that φX protein synthesis occurs in the cell cytoplasm, this protein synthesis could well be accommodated by membrane-associated polysomes, but the conditions of our experiments (5 mM-EDTA) would be expected to release polysomes from the cell membrane, with concomitant disruption of them.

We are presently testing certain facets of the proposed model, and have been able to demonstrate the presence of virus gene A, G, H and F proteins in the replication complex (consisting of a sub-membrane fragment + φX parental RF + nascent progeny RF) released from the membrane of φX infected cells by the method described by Loos et al. (1971). We have not been able to demonstrate the presence of the virus gene D protein in these complexes, but for posterity, polypeptides I and II remain ubiquitous.

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