Cleavage of Virus-specified Polypeptides in Cells Infected with Semliki Forest Virus

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SUMMARY

Several new virus-specified polypeptides of high mol. wt. were detected in cells infected with Semliki Forest virus by use of pulse-chase methods in cells which had been either incubated at an elevated temperature in the presence of tosyl-L-phenylalanylchloromethane or treated with an inhibitor of glycoprotein synthesis, or an inhibitor of proteolytic enzyme activity, or five amino acid analogues, or an inhibitor of protein synthesis. The new polypeptides had estimated mol. wt. of 165,000, 127,000 and 105,000 and were shown to be converted to lower mol. wt. polypeptides in pulse-chase experiments. These polypeptides, together with known non-structural polypeptides of mol. wt. of 97,000, 78,000 and 63,000 are incorporated into a proposed cleavage mechanism for the synthesis of the virus structural proteins.

INTRODUCTION

A number of workers have investigated the synthesis and properties of virus-specified polypeptides in cells infected with alphaviruses. Strauss, Burge & Darnall (1969) reported the presence of the virus structural polypeptides and, in addition, a large number (12 to 16) of non-structural polypeptides in cells infected with Sindbis virus. They showed that a polypeptide of mol. wt. about 65,000 was possibly a precursor of the structural polypeptides, and Burrell, Martin & Cooper (1970), using a pulse-chase technique, showed that several polypeptides of high mol. wt. were precursors to the structural polypeptides. A larger polypeptide was found to accumulate in cells infected with a temperature-sensitive mutant of Sindbis virus (Scheele & Pfefferkorn, 1970), and large polypeptides were also found in infected cells treated with inhibitors of proteolytic enzymes (Pfefferkorn & Boyle, 1972) or incubated with canavanine (Ranki, 1972). In neither case was a movement of radioactive label from the postulated precursor to the product demonstrated. However, Waite (1973) has recently demonstrated such a movement of label in cells infected with Sindbis virus, although the large polypeptides were only partially resolved.

A previous paper (Morser, Kennedy & Burke, 1973) has described the detection of a number of virus-specified polypeptides from cells infected with Semliki Forest virus. In order of decreasing mol. wt., these polypeptides are NVP-97 (non-virion polypeptide of mol. wt. 97,000), NVP-78, NVP-63 and the virus structural proteins. The latter are now known to comprise two envelope glycoproteins (Schlesinger, Schlesinger & Burge, 1972; Simons, Keranen & Kääriäinen, 1973) and a core protein. Both NVP-97 and NVP-63 were shown to be glycosylated, while NVP-78 contained no sugar residues. NVP-63 was shown to resemble one or both of the envelope polypeptides by use of a specific antiserum and by
precipitation with concanavalin A, and recently NVP-63 has been shown to be a precursor of one of the envelope polypeptides, with the elimination of a small glycoprotein (Ranki, Kääriäinen & Renkonen, 1972; Schlesinger & Schlesinger, 1972). This present investigation had two aims. First to attempt to detect any other virus-specified polypeptides of higher mol. wt., and second to determine whether the high mol. wt. polypeptides were cleaved to form the low mol. wt. peptides.

METHODS

Tissue culture cells and virus. These have been described previously (Morser et al. 1973). Chick embryo cells or BHK-21 cells were used as indicated.

Measurement of macromolecular synthesis. The incorporation of [3H]-uridine, [3H]-valine, [3H]-glucosamine or [3H]-fucose was measured by the method of Skehel et al. (1967).

Virus-specified protein synthesis. This was carried out as described previously (Morser et al. 1973), using medium containing 1 #g/ml actinomycin D for incubation on all occasions. Virus-specified proteins were extracted as follows. At the end of the labelling period, the Petri dishes were placed in a bath containing a freezing mixture of solid carbon dioxide in methanol. The fluids froze instantly, and 3 ml of 10 % (w/v) TCA was added which also froze. The dish and contents were allowed to thaw at room temperature, and the acid-insoluble material washed three times with 3 ml of 10 % (w/v) TCA, three times with 3 ml of ethanol and 3 ml of 80 % acetone containing 0.1 M HCl, before being allowed to dry. The sample was then dissolved in 0.4 ml of 5 mm-sodium phosphate pH 7.2, containing 1 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol, and 0.5 M urea, and boiled for 2 min before fractionation on 10 % polyacrylamide gels as described by Morser et al. (1973). Protein samples prepared in this way had a high specific activity, and gave sharper bands on gel electrophoresis than when obtained by the method described by Morser et al. (1973). In the gel electrophoresis system used in this work the two envelope proteins were not reproducibly resolved.

All other methods have been previously described (Morser et al. 1973).

Materials. DL-p-fluorophenylalaine, DL-ethionine, azotryptophan, N-acetyl glucosamine, were obtained from Sigma Ltd., London. Di-isopropylfluorophosphate was obtained from B. D. H. Ltd., Poole. N-fluororacetoyl glucosamine (FNAG) was a kind gift from Dr G. G. Butchard, Department of Biochemistry, Oxford University, Oxford. L-canavanine sulphate, L-azetidine-2-carboxylic acid and tosyl-L-phenylalaninyl chloromethane (TPCK), all grade A, were purchased from Calbiochem Ltd., London. Actinomycin D was the gift of Merck, Sharpe & Dohme Research Laboratories, Rahway, New Jersey, U.S.A. [3H]-d-glucosamine hydrochloride (2.3 Ci/m-mol), [14C]-l-leucine (300 mCi/m-mol), [4.5-3H]-l-leucine (19 Ci/m-mol), [14C]-l-lysine (300 mCi/m-mol), [14C]-l-methionine (50 mCi/m-mol), [14C]-l-phenylalanine (405 mCi/m-mol), [14C]-l-proline (265 mCi/m-mol), [14C]-l-threonine (208 Ci/m-mol), [14C]-l-tryptophan (50 mCi/m-mol), [14C]-l-tyrosine (507 mCi/m-mol), [5-3H]-uridine (3 Ci/m-mol), [2,3-3H]-l-valine (1.5 Ci/m-mol) and [14C]-valine (225 mCi/m-mol) were obtained from the Radiochemical Centre, Amersham, Bucks.

RESULTS

Amino acid composition of NVP-78 and NVP-63

Cultures of chick embryo cells, infected with SFV and treated with 1 #g/ml of actinomycin D, were labelled with [3H]-lysine and [14C]-valine simultaneously 6 to 7 h after infection. The proteins were extracted from both infected and non-infected cells, and analysed separ-
Fig. 1. Polyacrylamide gel electrophoresis on a 9 cm gel of the proteins extracted from (a) uninfected cells, and (b) infected cells which had been labelled with [3H]-lysine and [14C]-valine between 6 and 7 h after infection.

Fig. 2. Polyacrylamide gel electrophoresis on a 12 cm gel of the polypeptides extracted from infected chick cells incubated in the presence (△—△) or absence (●—●) of 0.5 mM FNAG. [3H]-valine was added to the FNAG-treated cells and [14C]-valine to the control cells between 6 and 7 h after infection.
Table 1. Amino acid ratios for NVP-78, NVP-63 and the virus structural proteins compared to tryptophan

<table>
<thead>
<tr>
<th></th>
<th>Envelope ((E_1 + E_2))</th>
<th>Core</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>4.24</td>
<td>5.26</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.12</td>
<td>5.34</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.08</td>
<td>6.71</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.44</td>
<td>3.02</td>
</tr>
<tr>
<td>Proline</td>
<td>4.25</td>
<td>6.65</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.84</td>
<td>8.76</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.44</td>
<td>3.85</td>
</tr>
<tr>
<td>Valine</td>
<td>4.61</td>
<td>7.08</td>
</tr>
</tbody>
</table>

* X: tryptophan.

The results are the average of two series of experiments.

ately by polyacrylamide gel electrophoresis. In infected cells, the five virus-specific polypeptides contained differing ratios of \([\text{H}]\text{-lysine}\) to \([\text{C}]\text{-valine}\) counts, whereas in uninfected cells, which had also been treated with actinomycin, the ratio was constant along the length of the gel (Fig. 1). The different \([\text{H}]\)/[\text{C}] ratios of the five virus-specified polypeptides was also taken to reflect their different content of these amino acids. The experiment was repeated with seven other \([\text{C}]\)-amino acids, and in each case the results were represented as the ratio of \([\text{H}]-\text{lysine}\) to \([\text{C}]\)-amino acid counts for each of the polypeptides. Unfortunately, NVP-97 did not contain enough counts to give accurate results. Comparison of the ratios with those obtained from amino acid analysis of the envelope and core polypeptides (Kennedy & Burke, 1972) provided a correction factor which corrected for the effects of pool size, differential transport of the amino acids and different specific activities. Correction factors were obtained separately for both the core and envelope polypeptides and the mean of these two figures was used to correct the ratios for NVP-78 and NVP-63. This calculation gave absolute ratios with respect to lysine, which were then recalculated with respect to tryptophan, the amino acid present in the smallest amount.

The results (Table 1) show that the values for NVP-78 and core are very different from those for the two envelope polypeptides. NVP-78 does not contain enough lysine to account for the amount present in the core, and therefore cannot be a precursor of the core polypeptide.

The effect of N-fluoro-acetyl glucosamine (FNAG) on virus protein synthesis

FNAG has been reported to be a non-toxic inhibitor of glycoprotein synthesis (Kent, 1972). It was of interest to compare the specificity of its action with \(N\)-acetyl glucosamine (NAG) which has been observed to be an inhibitor of the multiplication of some viruses (Kaluza, Scholtissek & Rott, 1972), probably via an effect on the UTP pool (Scholtissek, 1972). Both FNAG and NAG inhibited SFV multiplication in chick cells (Table 2), although FNAG was more effective. The effect of either 0.5 mm-FNAG or 0.5 mm-NAG on the incorporation of radioactive precursors into RNA, protein and carbohydrate was investigated in infected cells. FNAG did not alter the rate of either RNA or protein synthesis, but inhibited the rate of glucosamine incorporation (Table 2), whereas the same concentration of NAG produced very little effect on any of these parameters or on virus yield. It was concluded that the data was consistent with FNAG being an inhibitor of carbohydrate and glycoprotein synthesis.

Chick embryo cells, infected with SFV, were incubated in the presence of 0.5 mm-FNAG,
Table 2. Effect of FNAG and NAG on RNA and protein synthesis in infected chick cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation* of [3H]-uridine</th>
<th>Incorporation* of [14C]-valine</th>
<th>Incorporation* of [3H]-glucosamine</th>
<th>Virus yield (p.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>377</td>
<td>5689</td>
<td>6836</td>
<td>--</td>
</tr>
<tr>
<td>Infected</td>
<td>482</td>
<td>4923</td>
<td>7514</td>
<td>7 x 10^7</td>
</tr>
<tr>
<td>Infected + 0.5 mM-NAG</td>
<td>479</td>
<td>4723</td>
<td>7263</td>
<td>6 x 10^6</td>
</tr>
<tr>
<td>Infected + 0.5 mM-FNAG</td>
<td>475</td>
<td>4691</td>
<td>2435</td>
<td>1 x 10^6</td>
</tr>
</tbody>
</table>

* Expressed as cpm/min in acid-insoluble material.

The isotopes were added between 6 and 9 h after infection, and virus yield measured at 9 h after infection. Actinomycin D (1 μg/ml) and either FNAG or NAG were added immediately after infection.

and [3H]-valine added between 6 and 7 h after infection. The proteins were extracted and mixed with a similar preparation from infected cells labelled with [14C]-valine, but which had not been treated with FNAG. The mixed extract was analysed by polyacrylamide gel electrophoresis (Fig. 2). In the material from treated cells there was less NVP-78, NVP-63, envelope and core polypeptides and more NVP-97 than in the material from control cells, but the amounts of NVP-63 and envelope polypeptides showed greater reduction than the others. This suggested that NVP-97 was converted into polypeptides of lower mol. wt., probably NVP-63 and the envelope polypeptides.

Virus-specified proteins formed during long labelling periods

In all previous experiments, the radioactive precursor had been in contact with the virus-infected cells for 1 h. Comparison of the distribution of radioactivity between the polypeptides from infected chick cells labelled over 1 h (from 6 to 7 h after infection) and a longer labelling period (9 h, from 3 to 12 h after infection), showed that the envelope and core polypeptides made up a much higher proportion of the total protein during the long labelling period in comparison with the shorter labelling period (Fig. 3). NVP-97, NVP-78 and NVP-63 were all much more prominent after the short labelling period. Since it is probable that the only proteins released from the cell are the virus structural proteins, the relatively small amounts of the larger polypeptides must be due to their degradation, either by total breakdown or, more likely, by specific cleavage of a large intermediate to form virus structural proteins. Further evidence as to precursor-product relationships between these proteins was sought by pulse-chase experiments.

Virus-specified proteins formed during short pulses and pulse-chase experiments

Virus-infected BHK-21 cells were incubated with [3H]-leucine for 7 min at 6 h after infection (short-pulse experiment). The proteins were extracted and analysed by polyacrylamide gel electrophoresis. The results were compared with those from cells treated similarly, but which had been washed, and incubated for a further 53 min in the presence of 1 mg/ml of non-radioactive leucine. Finally, the polypeptide patterns obtained from both experiments were compared with that from cells which had been labelled for 1 h with [3H]-leucine (long-pulse experiment). The results, in Fig. 4, show that a movement of label from NVP-97 to the envelope polypeptides was taking place. In this experiment (Fig. 4a) and some others (e.g. Fig. 7a) the two envelope polypeptides appeared to be resolved to give two peaks at fractions 77 and 85.

The experiment was repeated using infected chick embryo cells instead of infected
Fig. 3. Polyacrylamide gel electrophoresis on a 9 cm gel of the polypeptides extracted from infected chick cells, after [PH]-valine had been present between 6 and 7 h after infection (●—●), or between 3 and 12 h after infection (□—□).

BHl-21 cells, since NVP-97 is much less prominent in chick cells than in BHK-21 cells (Morser et al. 1973). The results (Fig. 5) showed a reduction in size of the NVP-97 peak and a marked increase in size of the NVP-78 peak in the pulse-chase experiment, while in chick cells, but not in BHK-21 cells, there was an increase in the size of the core peak during the chase period. A new, high mol. wt., polypeptide peak was detectable in chick cells during the short pulse period. This peak (NVP-127) had an estimated mol. wt. of 127000 and is possibly identical with the protein of mol. wt. 130000 reported by Strauss et al. (1969) and by Waite (1973). This experiment suggests that a precursor-product relationship exists between NVP-127, NVP-97 and NVP-63 on the one hand and NVP-78 and the virus structural polypeptides on the other.

The effect of tosylphenylalanylchloromethylketone (TPCK) and diisopropylfluorophosphate (DFP) on virus-specified protein synthesis

Both TPCK and DFP are inhibitors of proteolytic enzymes (Walsh & Wilcox, 1970), TPCK being a specific inhibitor of chymotrypsin (Korant, 1972; Pfefferkorn & Boyle, 1972) and DFP a general inhibitor of serine proteases (Jacobson, Asso & Baltimore, 1970). Pfefferkorn & Boyle (1972) have reported that TPCK prevented the breakdown of NVP-97 in chick cells infected with Sindbis virus, while tosyllysylchloromethylketone (TLCK) did not. Since TLCK is an analogous inhibitor of trypsin, this suggests that cleavage was caused by an enzyme with a similar specificity to chymotrypsin. The effect of varying concentrations of both TPCK and DFP on the rate of incorporation of [3H]-valine into acid-insoluble material in BHK-21 cells was measured. TPCK, at the highest concentration used, had a smaller inhibitory effect on protein synthesis, than DFP at the lowest (Fig. 6). Both depressed the yield of infectious virus. Therefore TPCK was used in the following experiments.
Virus-specified polypeptides

Fig. 4. Polyacrylamide gel electrophoresis on 12 cm gels of the polypeptides extracted from infected BHK-21 cells to which [3H]-leucine was added 6 h after infection. Seven min later, polypeptides were extracted from one culture (a) short pulse and a second culture was washed and incubated with medium containing 1 mg/ml of non-radioactive leucine for a further 53 min before extraction of the polypeptides (b, pulse-chase.) A third culture was incubated with [3H]-leucine for 60 min before extraction (c, long pulse.)

Fig. 5. A similar experiment to that shown in Fig. 4 but using chick embryo cells, and (a) a 7 min pulse, (b) a 7 min pulse followed by a 53 min chase and (c) a 1 h pulse.

TPCK (20 μg/ml) was added to infected cells 5½ h after infection, which were then incubated at 37 °C for 30 min before addition of [3H]-leucine for a short pulse, a pulse-chase and a long (1 h) pulse as described in the previous section. TPCK was present throughout the pulse and chase. The proteins were extracted and analysed by polyacrylamide gel electrophoresis with the results shown in Fig. 7 (for BHK-21 cells) and Fig. 8 (chick embryo cells). NVP-127 could now be detected in infected BHK-21 cells as well as in infected chick cells, and in addition two new high mol. wt. polypeptides were detected in infected chick cells which were assigned mol. wt. of 165,000 (NVP-165) and 105,000 (NVP-105). The mol. wt. of NVP-165 may not be very accurate since the value was estimated by extrapolation from a graph in which β-galactosidase (mol. wt. 135,000) was the highest point. In both types of infected cells there was a substantial increase in the amount of core polypeptide labelled during the chase, and smaller increases in the amount of the envelope polypeptides. In BHK-21 cells, but not in chick cells, there was also an increase in the amount of NVP-63. After the chase NVP-165, NVP-127 and NVP-105 could no longer be detected while the amount of NVP-97 was substantially reduced. These results demonstrate that NVPs -165, -127, -105 and -97 are precursors of NVP-78, NVP-63 and structural polypeptides, although the formation of NVP-78 in the chase experiments is restricted to chick cells.
Fig. 6. The effect of TPCK (Δ—Δ) and DFP (○—○), added at 5.5 h after infection, on the incorporation (ct/min x 10^-2) of [3H]-valine into acid-insoluble material in BHK-21 cells between 6 and 7 h post-infection. The yield (p.f.u.) of infectious virus at 9 h after infection was also measured when TPCK (Δ—Δ) or DFP (○—○) had been present since 5.5 h after infection.

Fig. 7. The effect of TPCK on virus-specified protein synthesis in BHK-21 cells. TPCK (20 µg/ml) was added 5.5 h after infection, and at 6 h a pulse-chase experiment was carried out as described in the legend to Fig. 4. The samples from (a) the short pulse, (b) the pulse-chase and (c) the 1 h pulse were analysed on 12 cm polyacrylamide gels. TPCK was present throughout.

Fig. 8. A similar experiment to that shown in Fig. 7, but using chick embryo cells, showing (a) a 7 min pulse, (b) a 7 min pulse followed by a 53 min chase and (c) a 1 h pulse.
Fig. 9. The effect of TPCK and five amino acid analogues on virus-specified protein synthesis. The analogues and TPCK were added 5 h 40 min after infection, and at 6 h the cells were washed and [3H]-leucine was added. The samples from (a) the short pulse, (b) the pulse-chase and (c) the 1 h pulse were analysed on 12 cm polyacrylamide gels. TPCK was present throughout.

The effect of amino-acid analogues on virus-specified protein synthesis

If the specific cleavage of NVP-165, NVP-127 and NVP-97 is due to a protease which recognizes a particular amino acid sequence or three-dimensional configuration of the polypeptide, then cleavage might be prevented by replacement of appropriate amino acids with analogues. An experiment was therefore performed with five amino-acid analogues, all of which are known to be incorporated into proteins of eukaryotic cells. The analogues were p-fluorophenylalanine (FPA) which is an analogue of phenylalanine (Levintow et al. 1962), canavanine which is an analogue of arginine (Kruse et al. 1959), azetidine-2-carboxylic acid which is an analogue of proline (Fowden & Richmond, 1963), ethionine which is an analogue of methionine (Rabinovitz, Olson & Greenberg, 1957) and azatryptophan which is an analogue of tryptophan (Friedman, 1969). The five amino acid analogues (3·4 mm-azetidine carboxylic acid, 2 mm-azatryptophan, 3·3 mm-canavanine, 1·8 mm-ethionine and 2·5 mm-FPA) plus TPCK (20 μg/ml) were added to infected chick embryo cells 5 h 40 min after infection and [3H]-leucine was added 20 min later for a short pulse (7 min), a pulse-chase (7 min + 53 min) or a 1 h pulse as described earlier. Polyacrylamide gel electrophoresis of the extracted proteins showed that NVP-165, NVP-127 and NVP-97 (or NVP-105) could be labelled during the short pulse (Fig. 9a). During the chase (Fig. 9b) the amount of NVP-78, NVP-63 envelope and core polypeptides increased, the amount of NVP-97 decreased, while both NVP-165 and NVP-127 were no longer detectable. The peak of NVP-97 was very broad in Fig. 9a and was resolved in the 1 h pulse (Fig. 9c) into two peaks, NVP-97 and NVP-105. These experiments added more weight to the hypothesis that NVP-165, NVP-127, NVP-97 and NVP-63 are precursors of NVP-78 and the virus structural proteins.
The effect of a temperature jump on virus-specified protein synthesis

D. Baltimore (personal communication) showed that raising the temperature at which HeLa cells, infected with poliovirus, were incubated caused an inhibition of virus-specified protein synthesis. After the release of such inhibition, there was build up of precursors. To measure the effect of temperature on SFV-specified protein synthesis, [3H]-leucine was added to BHK-21 cells 6 h after infection, and the Petri dishes immediately placed in a water bath at either 42.5 °C or 44 °C. At 2 min intervals, the incorporation of isotope into acid-insoluble material was determined. Since cellular protein synthesis was almost completely inhibited by this time after infection, this was a measure of virus-specified protein synthesis. At both temperatures, incorporation was halted (Fig. 10), but at 44 °C it was almost immediate while at 42.5 °C inhibition took 10 min to be complete. When the temperature was lowered to 37 °C, protein synthesis resumed in the cells which had been incubated at 42.5 °C, after a short lag, at control rates. However, protein synthesis was considerably depressed in cells which had been incubated at 44 °C.

The incubation temperature of infected BHK-21 cells was increased from 37 °C to 42.5 °C 5½ h after infection. After incubation at 42.5 °C for 30 min, with or without the addition of TPCK (20 µg/ml), the temperature was dropped to 37 °C and [3H]-leucine added. After a further 10 min incubation at 37 °C the proteins were extracted and analysed by polyacrylamide gel electrophoresis (Fig. 11). In the absence of TPCK, the major product was NVP-97 (which was not resolved from NVP-105 in this experiment). There were also small amounts of NVP-63, envelope and core polypeptides but less than in the control (not shown). In the presence of TPCK, it was possible to detect NVP-165 and NVP-127 as well as NVP-105, NVP-97 and NVP-63 but there were only small amounts of the envelope and core polypeptides. It was concluded that NVP-165, NVP-127, NVP-105 and NVP-97 were precursors of the structural proteins.

The effect of inhibitors of protein synthesis on virus-specified protein synthesis

The effect of three inhibitors of protein synthesis on the course of virus-specified protein synthesis was investigated. The inhibitors were sodium fluoride, n-butanol and aurintricarboxylic acid (ATA), all inhibitors of initiation (Ravel, Mosteller & Hardesty, 1966; Freedman, Hori & Rabinovitz, 1967; Stewart, Grollman & Huang, 1971). The inhibition due to sodium fluoride has been reported to be readily reversible, and when protein synthesis is restored, the first proteins to be formed will be the primary transcripts of the RNA, which will then be broken down into the final products. Wunner & Pringle (1972) have used the method to investigate the way in which the proteins of vesicular stomatitis virus were synthesized.

The effects of sodium fluoride, n-butanol and ATA on protein synthesis in infected BHK-21 cells were measured first (Fig. 12a). Then, after washing the cells twice, the recovery of protein synthesis was measured (Fig. 12b). Sodium fluoride was the only one of the three inhibitors that proved to be almost completely reversible. It was therefore added to infected BHK-21 cells 6 h after infection. Then 30 min later, the cultures were washed, and protein synthesis resumed. The proteins synthesized during the next 10, 30 or 60 min were compared by analysis on polyacrylamide gels, and the areas under the peaks integrated and plotted with respect to time (Fig. 13). Immediately after resumption of protein synthesis, NVP-97 (which was not resolved from NVP-105) was the major polypeptide detectable. In contrast to the other proteins, its contribution then fell while the proportions of NVP-78, envelope and core polypeptides increased continuously during the 60 min labelling period.
Fig. 10. (a) The incorporation of [\textsuperscript{\textit{H}}]-leucine into acid-insoluble material in infected BHK-21 cells, after raising the incubation temperature to 42.5 °C (\(\square\)) or 44 °C (\(\triangle\)), compared with incubation at 37 °C (\(\bigcirc\)).

(b) The incorporation of [\textsuperscript{\textit{H}}]-leucine into acid-insoluble material at 37 °C after infected BHK-21 cells had been incubated for 30 min at 42.5 °C (\(\square\)) or 44 °C (\(\triangle\)), compared with cells incubated throughout at 37 °C (\(\bigcirc\)).

Fig. 11. The effect of a temperature jump, with or without TPCK, on virus-specified protein synthesis. The incubation temperature of infected BHK-21 cells was raised from 37 °C to 42.5 °C 5.5 h after infection, with or without the addition of TPCK (20 μg/ml). After 30 min, the incubation temperature was dropped to 37 °C, and [\textsuperscript{\textit{H}}]-leucine was added for 10 min before extraction of the proteins and analysis by polyacrylamide gel electrophoresis on 12 cm gels. (a) In the presence of TPCK. (b) In the absence of TPCK.
Fig. 12. The effect of 5 mM-ATA (□—□), 5 mM-n-butanol (△—△) and 10 mM-sodium fluoride (●—●) on virus-specified protein synthesis, compared with controls (○—○).
(a) The inhibitors were added at 6 h after infection and the effect on incorporation of [3H]-valine measured.
(b) The cells were treated as above, washed free of inhibitor at 6.5 h after infection, and the effect on incorporation of [3H]-valine again measured.

Fig. 13. The synthesis of virus-specified polypeptides after removal of sodium fluoride. After removal of the inhibitor, [3H]-leucine was added and the proteins extracted and fractionated after different times. The results are plotted as the percentage of total protein synthesis contributed by NVP-97 (○—○), NVP-78 (△—△), NVP-63 (□—□), envelope (●—●) and core (▲—▲) polypeptides.
DISCUSSION

These results clearly show the presence of several polypeptides of high mol. wt. in cells infected with Semliki Forest virus. The polypeptide of mol. wt. 165,000, which has not been reported before, was readily detected in cells treated with TPCK, in cells treated with amino acid analogues plus TPCK, or in cells incubated at an elevated temperature plus TPCK. The polypeptide of mol. wt. 127,000 has previously been detected by Strauss et al. (1969), and by Waite (1973), who also detected polypeptides of mol. wt. 112,000 and 95,000. The latter is probably identical with our NVP-97. Scheele & Pfefferkorn (1970) also reported the presence of a large polypeptide of mol. wt. 90,000, although it is not certain which polypeptide this corresponds to in our nomenclature. With the exception of this present work, all these large polypeptides were detected in cells infected with temperature sensitive mutants, and therefore it was not certain whether they were synthesized by normal wild-type virus. This has now been shown to be so. In addition we have detected another polypeptide of mol. wt. 105,000 which was not always resolved from NVP-97.

We have demonstrated movement of radioactivity from the high mol. wt. polypeptides (NVP-165, -127, -105 and -97) to polypeptides of lower mol. wt. including the virus structural polypeptides. Recently Schlesinger & Schlesinger (1972) and Ranki et al. (1972) have shown that NVP-63 is converted to one of the envelope proteins, possibly with the elimination of a glycoprotein (G-20). It therefore appears that the alphavirus produce their structural (and some non-structural, i.e. NVP-78) polypeptides by post-translational cleavage of a high mol. wt. precursor. The following facts serve as clues in elucidation of the cleavage mechanisms.

1. Two species of virus RNA, of mol. wt. $2.4 \times 10^6$ and $1.8 \times 10^6$ (Levin & Friedman, 1971) are found associated with virus polysomes (Kennedy, 1972). Rosemond & Sreevalan (1973) also found two RNA species (a 28 S and an 18 S and 15 S species) associated with polysomes and Moshowitz (1973) found the $1.8 \times 10^6$ mol. wt. species plus a little of the $2.4 \times 10^6$ mol. wt. species, plus some virus particle RNA. These RNA species are probably the messenger RNAs for virus structural protein synthesis. An RNA of mol. wt. $1.8 \times 10^6$ would produce an initial transcript of about $1.8 \times 10^6$ mol. wt. The species of $2.4 \times 10^6$ mol. wt. is only present in relatively small amounts and is unlikely to make a major contribution to the polypeptide products.

2. NVP-97, NVP-63 and the two envelope polypeptides are glycoproteins; NVP-78 and the core polypeptide are not. NVP-97, NVP-63 and the two envelope polypeptides are the only polypeptides precipitated with concanavalin A. NVP-63 and the envelope polypeptides share a common antigenic determinant.

3. There is not enough lysine in NVP-78 for it to be a precursor of the core polypeptide, and the evidence from the pulse chase data suggests that it is a terminal polypeptide in chick cells.

4. Schlesinger & Schlesinger (1973) have recently provided evidence that the protein which accumulates in chick embryo cells infected with a ts mutant of Sindbis virus contains [$^{14}$C]-arginine tryptic peptides present in the three virus polypeptides. This protein is probably identical with the protein shown by Waite (1973) to have a mol. wt. of 130,000 and with out NVP-127. Schlesinger & Schlesinger (1973) also showed that a slightly smaller
Fig. 14. A possible cleavage mechanism for the production of the virus-specified polypeptides. The glycosylated polypeptides are starred. The direction of translation is from left to right.

protein, which is detected in BHK cells infected with wild type Sindbis virus contains the [\(^{14}\)C]-arginine tryptic peptides of the two envelope proteins but not those of the nucleocapsid protein. This protein is almost certainly identical to our NVP-97.

(5) Recent results obtained with pactamycin (Schlesinger & Schlesinger, 1972) suggested that the nucleocapsid polypeptide may be N-terminal rather than the envelope polypeptides.

Using these clues we can suggest a partial cleavage scheme (Fig. 14), in which the initial transcript is NVP-165, which is then cleaved to form NVP-127. This is, in turn, cleaved to form the core polypeptide and NVP-97. The latter is glycosylated, presumably as it crosses the endoplasmic reticulum membrane, and serves as the precursor of all the glycosylated polypeptides – either directly to yield \(E_1\) or via NVP-63 to yield \(E_2\) plus G-20. This scheme does accommodate a number of findings reported in the literature but it does not explain what happens to the material removed when NVP-165 is cleaved to form NVP-127, for there was no evidence for a polypeptide of about the correct mol. wt. (~ 40000) in any of our experiments. Nor does it explain the formation of NVP-105 and NVP-78. These might be derived by cleavage of NVP-165, but it cannot be excluded that they are derived from the minor species of messenger RNA (mol. wt. \(2.4 \times 10^6\)). Further work is clearly necessary before the complete cleavage scheme is worked out.

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REFERENCES


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