Virus-specific Polypeptides in Ascites Cells Infected with Encephalomyocarditis Virus

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SUMMARY

A study was made of the virus-specific proteins present in Krebs 2 ascites cells infected with encephalomyocarditis (EMC) virus. About 15 virus-specific polypeptides, mol. wt. varying from 13000 to 147000, were detected by acrylamide gel analysis of extracts from infected cells briefly pulsed with [3H]-amino acids. Three of the components were probably capsid proteins, while the fourth capsid protein (7200 mol. wt.) was not detected in the infected cell and possibly arose after maturation by cleavage of the largest capsid protein. Pulse-chase experiments showed that only two medium-sized (mol. wt. 36000 and 54000) and some smaller (mol. wt. 13500 to 17000) non-capsid proteins were stable in vivo; the remaining larger non-capsid proteins lost radioactivity during the chase with cold amino acids, and were probably intermediates in the formation of smaller polypeptides by some post-translational cleavage process. Various methods were employed to block this cleavage process, utilizing elevated temperatures, protease inhibitors and amino acid analogues.

INTRODUCTION

The spherical encephalomyocarditis (EMC) virus particles contain about 30% RNA of a mol. wt. of $2.7 \times 10^6$ (Burness, 1970; Burness & Clothier, 1970). This RNA should be able to code for polypeptides having a total mol. wt. of about $2.5 \times 10^6$. Interest in this virus and its proteins has grown recently following the demonstrations that its RNA can direct the synthesis of virus-specific proteins in cell-free protein-synthetic systems (Kerr, Cohen & Work, 1966; Mathews & Korner, 1970; Smith, Marcker & Mathews, 1970; Kerr & Martin, 1971; Dobos, Kerr & Martin, 1971). The EMC virus particle has been variously reported to comprise of two (Work, 1964), three (Rueckert, 1965), four (Burness & Walters, 1967) or five (Butterworth et al. 1971) polypeptides. Only recently have reports appeared describing some features of the virus-specific proteins in EMC-infected ascites and HeLa cells (Butterworth et al. 1971; Skarlat et al. 1971).

EMC virus closely resembles poliovirus in many of its structural features and in its mode of replication. It is clear from a number of studies that the genome of poliovirus is translated into large precursor polypeptides which are later cleaved into stable capsid and non-capsid proteins (Jacobson & Baltimore, 1968; Maizel & Summers, 1968; Summers & Maizel, 1968). A similar situation was reported to occur in cells infected with Coxsackie and Mengo viruses (Holland & Kiehn, 1968). Moreover, by the use of amino acid analogues and

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protease inhibitors to block cleavage, Jacobson, Asso & Baltimore (1970) were able to show that the primary gene product of poliovirus was a single large polypeptide which represented translation of the entire genome, and that this was cleaved after synthesis in a characteristic fashion to yield four to five capsid proteins and a small number of non-capsid proteins. More recently the gene order for the poliovirus genome was established using pactamycin to inhibit initiation of protein synthesis (Summers & Maizel, 1971; Taber, Rekosh & Baltimore, 1971; Rekosh, 1972). Using similar techniques Butterworth et al. (1971) have established the order of three groups of genes for EMC virus-specific polypeptides and determined some precursor-product relationships between the larger and smaller polypeptides.

The object of the work reported here was to study the synthesis of virus proteins in a cell infected with EMC virus to determine if polypeptide cleavage was involved, and also to provide information useful for the characterization of the products formed during the cell-free synthesis of virus proteins directed by EMC-RNA. To this end we have developed methods for the specific labelling of virus proteins in vivo and their separation and analysis on acrylamide gels. The results show that EMC virus proteins, like those of poliovirus, are synthesized as large precursor polypeptides which are broken down to produce the final functional capsid and non-capsid proteins. The breakdown pattern was found to be similar to that suggested by Butterworth et al. (1971) except that some larger intermediates in the breakdown process were detected.

METHODS

Materials. The following labelled amino acids were obtained from the Radiochemical Centre, Amersham, Bucks., England: [14C]-L-phenylalanine (475 Ci/mol); [14C]-L-isoleucine (312 Ci/mol); [14C]-L-leucine (270 Ci/mol); [14C]-L-valine (225 Ci/mol); 4,5-[3H]-L-leucine (23 Ci/m-mol); 2,3-[3H]-L-valine (19 Ci/m-mol); [35S]-L-methionine (20 to 30 Ci/m-mol). The protease inhibitors tosyl phenylalanyl chloromethyl ketone (TPCK), tosyl lysyl chloromethyl ketone (TLCK), phenyl methyl sulphonyl fluoride (PMSF) and phenyl boronic acid were purchased from Sigma Chemical Co., as were the analogues DL-p-fluorophenylalanine and DL-fluorotryptophan. Canavanine sulphate, L-azetidine-2-carboxylic acid and ethionine were supplied by Calbiochem Corporation. Actinomycin D was obtained through the courtesy of Merck & Co. Inc., Rahway, N.J.

Virus and cells. The origin of the Krebs 2 mouse ascites tumour cells, their propagation in mice and the origin of the EMC virus have been described previously (Martin et al. 1961). The growth of the virus in ascites cells and its purification by calcium phosphate chromatography were performed as described by Kerr & Martin (1971). The preparation of EMC virus labelled with [35S]-methionine was as described by Dobos et al. (1971); a similar method was also used for the preparation of virus labelled with a mixture of [14C]-amino acids, replacing the methionine with 0.05 mCi each of [14C]-leucine, [14C]-valine, [14C]-phenylalanine and [14C]-isoleucine.

Infection of cells for the specific labelling of virus proteins. Krebs 2 ascites cells were harvested, washed, and suspended at $2 \times 10^7$ cells/ml in Earle's medium containing 3 µg/ml of actinomycin D. Usually 25 ml amounts of cell suspensions were incubated at 37 °C in 250 ml conical flasks which had been gassed with 5% CO₂. After 3 h incubation the cultures were infected with EMC virus at an added multiplicity of 10 to 15 p.f.u./cell. A small portion of the cells (e.g. 4 ml in a 25 ml flask) was incubated and infected 30 to 60 min prior to the main culture to monitor the rate of protein synthesis according to the method of Dobos et al. (1971). This way it was possible to determine precisely when virus protein synthesis had
reached a maximal rate in the main cultures (Fig. 3). At this time (usually 3.5 to 4 h after infection) radioactive amino acids (usually about 0.05 mCi of $[^{14}\text{C}]$-or 0.2 mCi $[^{3}\text{H}]$-amino acids) were added and incubation continued for the appropriate time. Ice-cold phosphate buffered saline (PBS) containing an excess of unlabelled amino acids was added to halt incorporation and the cultures were placed in crushed ice.

**Isolation of subcellular fractions containing labelled virus proteins.** The infected, labelled cells were washed three times with ice cold PBS and once with normal saline buffered with 0.02 M-tris, pH 7.4. The cells were then suspended in 6 mM-magnesium acetate and disrupted by Dounce homogenization. A volume equal to three-fifths of the packed-cell volume of a solution containing 1.5 M-sucrose, 0.21 M-tris-HCl (pH 7.6), 0.15 M-KCl, 0.06 M-2-mercaptoethanol, 0.006 M-magnesium acetate and 0.006 M-EDTA was added to the homogenate and the nuclei and debris were removed by centrifuging at 7000 g for 10 min. The nuclear pellet was washed once and the washings returned to the post nuclear supernatant fluid. This was then centrifuged at 13000 g for 20 min to yield a pellet of the large-particle ("mitochondrial") fraction; this pellet contained most of the virus specific proteins (see below). The supernatant fluid was discarded and the 'mitochondrial' pellet was suspended in 1 to 2 ml of 0.01 M-2-mercaptoethanol and sodium dodecyl sulphate (SDS) was added to a final concentration of 2% (w/v). The preparation was heated in a 90°C water bath until it became clear (about 5 to 10 min). It was dialysed for 16 to 24 h against 3l of 10 mM-sodium phosphate buffer (pH 7.3) containing 0.1% SDS, 0.5 M-urea and 0.1% (v/v) mercaptoethanol. The radioactivity of a 10 µl sample of the dialysed material was measured in a liquid scintillation counter and the sample was then stored at -20°C.

**Acrylamide gel electrophoresis.** A portion of the dialysed mitochondrial fraction (0.2 to 0.5 ml containing 20000 to 80000 cts/min of radioactivity) was mixed with the appropriate SDS-treated marker proteins (see below) and subjected to electrophoresis (16 h at 4 mA per gel) using 0.8 × 10 cm 5%, 7% or 10% polyacrylamide gels; the electrophoresis buffer was 0.1 M-sodium phosphate (pH 7.3) containing 0.1% (w/v) SDS. The gels were stained with 0.1% (w/v) amido black in acetic acid–methanol–water (5:40:55, v/v/v), destained with the latter solvent and cut into four longitudinal slices; the middle two slices were dried on cellophane and autoradiographed using ‘Ilfix’ X-ray film (Fairbanks, Levintal & Reeder, 1965) for 7 to 14 days. The developed films were then scanned for bands of radioactive protein with a ‘Chromoscan’ microdensitometer (Joyce Leob, Ltd.). When proteins labelled with $[^{3}\text{H}]$-amino acids were to be analysed, electrophoresis was as before except that the destained gels were scanned directly in the microdensitometer (to record the position of marker proteins) and sliced into discs of 1 mm thickness with a razor blade. The slices were digested in scintillation vials with 0.1 ml of 30% H$_2$O$_2$ for 3 h at 60°C. Ten ml of scintillation fluid (0.4% (w/v) 2,5-diphenyloxazole in a mixture of 2 parts xylene to 1 part ‘Triton X-100’) was added and radioactivity measured in a Beckman model LS 150 scintillation counter.

**Estimation of protein mol. wt.** Estimation of the mol. wt. of capsid and non-capsid proteins was made by comparison of their mobility during electrophoresis with that of proteins of known mol. wt. (Shapiro, Vinuela & Maizel, 1967; Dunker & Rueckert, 1969). A linear relationship between log (mol. wt.) and distance moved was maintained only over a limited range of mol. wt., the range depending on the gel concentration (Dunker & Rueckert, 1969). Therefore, gels of different concentrations were used as follows: for mol. wt. less than 30,000, 10% gels; mol. wt. between 20,000 and 70,000, 7% gels; mol. wt. in excess of 60,000, 5% gels. The mol. wt. of proteins which overlapped these ranges were measured with both gel concentrations, and the results averaged. The following proteins, with their assumed
mol. wt. in parentheses, were used to calibrate the gels: insulin (5770), cytochrome C (13400), lysozyme (14500), human haemoglobin A (16250), myoglobin (17800), trypsinogen (23560), chymo-trypsinogen A (24700), lactate dehydrogenase (33500), creatin kinase (40000), ovalbumin (43500), glutamic dehydrogenase (50000), pyruvate kinase (57200), catalase (60000), bovine serum albumin monomer (66500), transferrin (88000), bovine serum albumin dimer (133000), myeloma 5563 protein (154000), bovine serum albumin trimer (199500), bovine serum albumin tetramer (266000). All protein markers were treated with 2% SDS and 0.1 M-mercaptoethanol at 90 °C for 5 min before use, except the myeloma protein and the serum albumin oligomers, which were added to the sample (containing SDS and mercaptoethanol) just before electrophoresis. The bovine serum albumin oligomers were prepared from the monomer by partial oxidation in the following manner: to 15 vol. of bovine serum albumin solution (6.6 mg/ml) was added 0.1 vol. of 0.001 M-potassium ferricyanide. Urea (0.6 g/ml) was added and the mixture kept at room temperature for 2 h. SDS (final concentration 2%, w/v) was added, the mixture heated at 90 °C for 5 min and then dialysed against 0.01 M-tris-HCl containing 0.1% (w/v) of SDS. After mixing of the radioactive proteins of unknown mol. wt. with the marker proteins (20 to 25 µg each) the relative mobilities of all proteins were measured after electrophoresis by microdensitometer scanning of the autoradiograms for the radioactive proteins (the origins of these had been marked with radioactive ink) or by scanning the dried gel slice for the stained marker proteins. A typical pair of such tracings is shown in Fig. 5a, where the stained proteins are those of the virus capsid. All estimates of mol. wt. were repeated at least four times, wherever possible using different combinations of marker proteins each time; only measurements where the calibration with marker proteins showed a linear relationship between distance moved and log (mol. wt.) over the appropriate range were used.

RESULTS

The proteins of the virus capsid

When purified EMC virus was heated with SDS and its proteins separated by acrylamide gel electrophoresis, two major (VP 2 and VP 3) and one minor (VP 4) stained protein bands were visible (Fig. 1a). If, however, the virus was labelled with radioactive amino acids before purification, and the radioautograms obtained after electrophoresis were examined, traces of a fourth minor component (VP 1) can be detected (Fig. 1b to e). The mol. wt. of these four components and their molecular proportions are given in Table 1. VP 2, VP 3 and VP 4 are obviously major components of the virus particle, occurring to the extent of 75 to 100 molecules/particle. On the other hand VP 1 is much less abundant (3.5 molecules/particle) and could well be a contaminant. Nevertheless, it was always present in approximately the same proportion despite extensive purification of the virus or prolonged incubation of the virus with trypsin. A similar minor component of approximately the same size has been detected in other picornaviruses such as poliovirus (Jacobson & Baltimore, 1968), ME virus (Rueckert, Dunker & Stoltzfus, 1969) and Mengo virus (O'Callaghan, Mak & Colter, 1970); it has also been found by others in EMC virus (Skarlat et al. 1971; Butterworth et al. 1971).

The closely related Mouse–Elberfeld virus has been reported to contain, in addition to components of a size similar to VP 1, VP 3 and VP 4 of EMC, two other major species of mol. wt. 30500 and 33000 in equimolar amounts (Rueckert et al. 1969); poliovirus also contains a similar pair of components (Jacobson et al. 1970). It therefore seemed likely that the major peak VP 2 (mol. wt. 31500) may be composed of two non-identical polypeptides.
Fig. 1. Electrophoresis of purified EMC virus proteins on SDS-acrylamide gels. a, microdensitometer tracing of 7 % gel stained with amido black. b–d, densitometer tracings of autoradiographs of [14C]-amino acid-labelled virus run on 5 % (b), 10 % (c) and 15 % (d) gels; e, tracing of autoradiogram of [35S]-methionine-labelled virus (5 % gel). The origin (Or.) is to the right of the tracings. Numbers 1 to 4 denote particle protein VP 1 to VP 4 as in Table 1.

Table 1. Molecular weights and proportions of EMC virus capsid proteins

<table>
<thead>
<tr>
<th>Capsid protein</th>
<th>VP 1</th>
<th>VP 2*</th>
<th>VP 3</th>
<th>VP 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean mol. wt.</td>
<td>38800</td>
<td>31100</td>
<td>23800</td>
<td>7300</td>
</tr>
<tr>
<td>S.E.M. (no. of determinations)</td>
<td>± 200 (13)</td>
<td>± 300 (15)</td>
<td>± 300 (15)</td>
<td>± 200 (7)</td>
</tr>
<tr>
<td>Relative proportions†</td>
<td>0.023</td>
<td>0.535</td>
<td>0.346</td>
<td>0.096</td>
</tr>
<tr>
<td>S.E.M. (no. of determinations)</td>
<td>± 0.001 (8)</td>
<td>± 0.020 (8)</td>
<td>± 0.012 (8)</td>
<td>± 0.008 (8)</td>
</tr>
<tr>
<td>No. of molecules per particle</td>
<td>3.5</td>
<td>101</td>
<td>85</td>
<td>77</td>
</tr>
</tbody>
</table>

* VP 2 could be resolved into two components of mol. wt. 32 500 and 30 000 (two determinations), they were in approximately equal proportions (Fig. 2).
† Obtained from acrylamide gel analysis of proteins labelled in vivo with a mixture of [14C]-amino acids.
‡ Calculated assuming a particle weight of 8.56 × 10⁶ for the EMC particle and 2.7 × 10⁶ for the mol. wt. of EMC RNA (Burness, 1970; Burness & Clothier, 1970).
of similar mol. wt. Despite repeated analysis of various EMC preparations, using acrylamide gel concentrations of from 5% to 15% (Fig. 1b, d), no separation of VP 2 into two individual protein species was observed. However, when the virus was subjected to prolonged electrophoresis on 25 cm long 10% gels, separation of VP 2 into two components was achieved (Fig. 2). Slicing of the gels in double labelling experiments also occasionally resolved VP 2 into two components VP 2A and VP 2B (Fig. 5b). Their mol. wt. were estimated to be 32,500 for VP 2A and 30,000 for VP 2B. The ratios of the individual capsid proteins given in Table 1 were those determined from virus labelled with a mixture of [14C]-leucine, isoleucine, valine and phenylalanine. When [35S]-methionine was the sole labelled amino acid, the molar ratio of VP 2A+VP 2B to VP 3 changed from 1.2:1 to 0.63:1, indicating that VP 3 contained a higher proportion of methionine (Fig. 1e).

**Virus protein synthesis in infected cells treated with actinomycin**

Like other picornaviruses EMC causes a profound inhibition of host protein synthesis (Martin et al. 1961; Martin & Kerr, 1968) and this provides conditions whereby the only protein synthesis taking place is that of the virus proteins. When ascites cells are incubated for 3 or more hours with actinomycin D, a modest inhibition of their protein synthesis occurs without their ability to support virus replication being impaired (Dalgarino, Cox & Martin, 1967). Addition of the virus inoculum to the cells then causes an almost immediate fall in the rate of total protein synthesis, which reaches a minimum 1 to 2 h later (Fig. 3). Thereafter there is a pronounced rise in the rate of total protein synthesis, reaching a maximum of about 3.5 h after infection; further incubation results in a rapid fall in protein synthetic rate.

The characteristics of the proteins being synthesized at the time of maximum protein synthesis (3.5 h) were examined and compared with those present in uninfected cells (Fig. 4). A major shift in the pattern of newly synthesized proteins had occurred, with a more rapid synthesis of far fewer different species of proteins taking place. In uninfected cells there was an appreciable synthesis of very high mol. wt. proteins, but by 3.5 h after infection this had ceased entirely. Instead, a pattern of 11 to 14 polypeptides characteristic of the infected cell developed. These newly synthesized proteins did not obviously correspond to any synthesized before infection (Fig. 4). Polypeptides IC 31, IC 36, IC 39 were frequently difficult to resolve from each other by either microdensitometry or gel slicing; however, they could be distinguished by visual observation of the autoradiograms (e.g. Fig. 7g). It seems reasonable to assume that the synthesis of host proteins has been entirely shut off by 3.5 h and that the proteins which are being synthesized are virus-specific. Further evidence that this was so was given in a previous report describing a comparison between the methionine containing tryptic peptides derived from purified virus and virus proteins labelled in vivo at 3.5 h after infection (Dobos et al. 1971).
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Fig. 3. Effect of EMC virus infection on rates of protein synthesis in Krebs ascites cells treated with actinomycin D, measured by 10 min incubations with [3H]-leucine. O--O, uninfected cells; ●--●, cells infected with EMC virus at an input multiplicity of 6 p.f.u./cell.

The pattern of 11 major protein species was always seen when infected cells were incubated for 20 min with radioactive amino acids, and it was unaltered during the course of infection from 1.5 to 5 h. The proportions of the individual protein species, as determined by microdensitometry, remained approximately constant during this period, indicating that the synthesis of the individual proteins was co-ordinated and that all major species were made at all times.

Subcellular localization of virus protein synthesis

Dalgarno et al. (1967) had observed that virus-specific proteins in EMC-infected ascites cells were probably synthesized on large polysomal aggregates attached to endoplasmic reticular material which sedimented at low speeds and that the virus RNA polymerase, together with newly synthesized virus RNA and much of the mature virus, were largely localized in the large particle (i.e. 'mitochondrial') fraction (Dalgarno & Martin, 1965). It therefore seemed likely that this fraction rather than the microsomal or soluble fractions would be the major site of virus protein synthesis. This was in fact found to be the case when ascites cells were pulsed 3.5 h after infection with labelled amino acids, disrupted and subjected to the usual differential centrifuging procedures for the separation of the subcellular components. In the post-nuclear supernatant fluid, infection caused a marked change in the distribution of newly synthesized proteins when compared with uninfected cell fractions. In the control cells only 40 to 50% of the total labelled proteins from the post-nuclear supernatant fluid were found in the large particle fraction; in contrast in infected cells up to 90% of the radioactivity was present in this fraction. Acrylamide gel analysis of the proteins in these various fractions showed that all polypeptides present in the nuclear and post-mitochondrial fractions were also present in the mitochondrial fraction, although the first two fractions were depleted in the highest mol. wt. species of polypeptides (IC 117, IC 103, IC 81, IC 68). Gel analysis of labelled proteins from the whole
cell homogenate and the larger-particle fraction were also compared and no significant qualitative differences between the two were found. The use of the large-particle fraction rather than the whole cell had obvious technical advantages in characterizing the virus proteins, and for this reason it was used in the studies reported here.

Characterization of the virus-specific proteins labelled in vivo

When the length of the amino acid pulse was reduced to 10 to 15 min a total of 15 bands of virus-specific radioactive proteins was routinely seen (e.g. Fig. 5a); this included two proteins larger in size than those evident in Fig. 4. The mol. wt. of each of these polypeptides was measured by comparison of their electrophoretic mobilities with those of reference proteins, and the results are summarized in Table 2. They vary in mol. wt. from 147,000 to 13,000.

Fig. 5 also shows an attempt to determine which of the proteins labelled in vivo corresponded to those present in the virus capsid. The two types of protein were co-electrophoresed, using either stained but unlabelled virus proteins (Fig. 5a) or by double labelling (Fig. 5b). These results suggested that VP1 and IC 39 corresponded in position, as did VP3 and IC 24, as would be expected from the estimates of their mol. wt. VP2 (A+B) probably corresponded with IC 31, although the latter polypeptide band was often difficult to resolve from IC 36 and IC 39 by either gel slicing or microdensitometry. These three bands can be distinguished on Fig. 7g. In neither type of experiment was the small capsid component VP4 ever detected amongst the proteins labelled in vivo. Although three of the four capsid proteins could be detected in the infected cell, their proportions were obviously
Fig. 5. (a) Densitometer tracings of autoradiograph showing radioactive proteins in ascites cells 3.5 h after EMC infection, pulsed with [14C]-amino acids for 15 min and subjected to acrylamide gel electrophoresis (upper tracing). The lower profile is the densitometer tracing of the stained dried gel slice used for the autoradiogram and shows the bands of virus capsid proteins added as markers (VP 2 and VP 3). The numbers indicate the polypeptide species listed in Table 2.

(b) Co-electrophoresis of radioactive proteins labelled for 90 min with [3H]-amino acids (●—●) in ascites cells 3.5 h after EMC infection and purified virus protein labelled with [14C]-amino acids (○—○). The numbers indicate the polypeptide species listed in Tables 1 and 2.
Table 2. Molecular weights of virus-specific polypeptides present in EMC-infected ascites cells

<table>
<thead>
<tr>
<th>Protein peak designation*</th>
<th>Mean mol. wt. ( (\times 10^{-3}) )</th>
<th>S.E.M. (no. of determinations)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC 147</td>
<td>146.5</td>
<td>± 1.6 (12)</td>
<td>Labelled only after short pulses</td>
</tr>
<tr>
<td>IC 133</td>
<td>132.7</td>
<td>± 1.1 (9)</td>
<td></td>
</tr>
<tr>
<td>IC 117</td>
<td>117.2</td>
<td>± 0.8 (19)</td>
<td></td>
</tr>
<tr>
<td>IC 103</td>
<td>102.9</td>
<td>± 0.8 (19)</td>
<td></td>
</tr>
<tr>
<td>IC 91</td>
<td>90.6</td>
<td>± 0.8 (12)</td>
<td>Short pulses only</td>
</tr>
<tr>
<td>IC 81</td>
<td>80.7</td>
<td>± 0.7 (17)</td>
<td></td>
</tr>
<tr>
<td>IC 68</td>
<td>67.7</td>
<td>± 0.7 (14)</td>
<td></td>
</tr>
<tr>
<td>IC 54</td>
<td>54.4</td>
<td>± 0.3 (17)</td>
<td></td>
</tr>
<tr>
<td>IC 47</td>
<td>46.9</td>
<td>± 0.7 (4)</td>
<td></td>
</tr>
<tr>
<td>IC 39</td>
<td>38.7</td>
<td>± 0.6 (4)</td>
<td>Probably VP1</td>
</tr>
<tr>
<td>IC 36</td>
<td>35.8</td>
<td>± 0.6 (4)</td>
<td>Probably VP2A + VP2B poorly</td>
</tr>
<tr>
<td>IC 31</td>
<td>31.0</td>
<td>± 1.0 (4)</td>
<td>Resolved from IC 36</td>
</tr>
<tr>
<td>IC 24</td>
<td>23.9</td>
<td>± 0.3 (6)</td>
<td>Probably VP3</td>
</tr>
<tr>
<td>IC 17</td>
<td>17.2</td>
<td>± 0.7 (4)</td>
<td></td>
</tr>
<tr>
<td>IC 13</td>
<td>13.1</td>
<td>± 0.3 (5)</td>
<td></td>
</tr>
</tbody>
</table>

* IC = virus-specific protein in infected cells.

Stability of virus-specific proteins in pulse-chase experiments

The sum of the mol. wt. of the virus-specific proteins listed in Table 2 is in excess of \( 9.5 \times 10^3 \), yet EMC virus RNA can only code for protein with a total mol. wt. of about \( 2.5 \times 10^3 \). Therefore it is probable that the larger polypeptides are precursors for the smaller proteins, as is the case with other picornaviruses. The increase in the number of larger proteins with shorter labelling periods (cf. Figs. 4, 5) is consistent with this view. To examine this proposal further, infected cells were incubated briefly with labelled amino acids and then subsequently incubated for various periods with unlabelled amino acids. The results of one such experiment are illustrated in Fig. 6, which shows the densitometer tracings of the radioactive proteins obtained from infected cells pulsed with labelled amino acids for 15 min then chased with cold amino acids for 15, 30 and 90 min. Estimates of the proportions of each species of protein were made by measurement of the areas under each peak of the tracing, and the changes in these proportions are also plotted in Fig. 6. It is apparent that the four largest polypeptides (IC 117, IC 103, IC 81 and IC 68) are not stable, and are broken down during the chase with cold amino acids. On the other hand many of the smaller proteins (IC 13 to IC 54) gain radioactivity during the chase, and are presumably cleavage products of the larger proteins. The behaviour of the group of proteins which includes IC 39 and IC 36 is equivocal and both gain and loss of polypeptide material may be occurring.

Attempts to find larger precursors

The results summarized in Fig. 6 and Table 2 indicate that the stable virus-specific proteins arise from the breakdown of larger precursors. However, these experiments give no indication as to the size of the primary product of the translation of the genome; that is, whether it is a single polypeptide of mol. wt. about \( 2.5 \times 10^3 \). That this was the case with poliovirus was shown by Jacobson et al. (1970), who were able to render the precursor more resistant to protease cleavage by incorporating into it a number of amino acid
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analogues. It seemed desirable to carry out similar experiments with the EMC-infected cell to determine whether precursors larger in size than IC 147 could be detected.

Larger polypeptides could not be found in extracts from infected cells when the period of labelling was reduced to 1 min or by pulse-labelling the cells as early as 2 h after infection. When cells were incubated for 15 min with a mixture of canavanine (9 × 10⁻⁴ M), ethionine (8 × 10⁻⁴ M), p-fluorophenylalanine (8 × 10⁻⁴ M) and azetidine-2-carboxylic acid (20 × 10⁻⁴ M) 4 h after infection, then pulsed with a mixture of [¹⁴C]-amino acids for 20 min, the result shown in Fig. 7b was obtained. When compared with the untreated infected cells (Fig. 7a), which contained no virus-specific proteins larger than IC 117, treatment with the analogues caused the appearance of IC 133 and IC 147 and markedly decreased the amount of radioactivity in the smaller polypeptides. No precursor larger than IC 147 was seen, even when the concentration of the analogues was doubled (Fig. 7c). In pulse-chase experiments in the presence of analogues no radioactivity could be chased from any of the larger components (IC 105 to IC 147). Thus, although the analogues had prevented the cleavage of this group of large polypeptides, they had neither affected the breakdown of any larger precursors nor enabled the presumptive primary gene product ('IC 250') to be detected.

In an attempt to demonstrate the existence of this primary product other methods were employed. In one series of experiments virus protein synthesis was synchronized by treatment with sodium fluoride followed by release from this inhibition (see Smith et al. 1970;
Fig. 7. Effects of various treatments which might inhibit cleavage of the synthesis of virus polypeptides in ascites cells infected for 3.5 h with EMC virus. The Fig. shows autoradiographs of acrylamide gels after electrophoresis of proteins from cells incubated for 15 min with [14C]-amino acids or [35S]-methionine. a, untreated infected cells; b, after treatment for 15 min with ethionine canavanine, p-fluorophenylalanine and azetidine-2-carboxylic acid; c, as for b except that drug concentration was doubled; d, after treatment with sodium fluoride, washing and labelling for 5 min; e, after incubating infected cells for 15 min at 41 °C; f, as for e, except that treatment was at 43 °C; g, after incubating infected cells for 20 min with phenylboronic acid; h, after 20 min incubation with phenyl methyl sulphonyl fluoride; i, after 20 min incubation with tosylamide lysyl ethylchloromethyl ketone; j, after 20 min incubation with tosylamide phenyl ethylchloromethyl ketone.
Dobos et al., 1971). When infected cells were pulsed for 5 min with $^{[35]S}$-methionine after this treatment only proteins IC 117, IC 103, IC 81 and IC 68 contained appreciable radioactivity (Fig. 7d); longer pulses resulted in patterns similar to the control (Fig. 7a). Again, no presumptive primary precursor was seen. Attempts were also made to block the cleavage by incubating the cells at elevated temperatures, a treatment which has been successful in demonstrating large precursors in poliovirus-infected cells (Garfinkle & Tershak, 1971).

At 41.5 °C total protein synthesis in both infected and uninfected ascites cells was stimulated two- to threefold compared to that at 37 °C, but at 43 °C the synthetic rate fell to about one-third of the 37 °C rate within 30 min. Infected cells were incubated at 37 °C for 3.5 h and then at temperatures of either 41.5 °C (Fig. 7e) or 43 °C (Fig. 7f) for 15 min, when the cells were pulsed for 15 min with $^{[14]}$C-amino acids. Analysis of the radioactive proteins showed an accumulation of radioactivity in IC 147, IC 133 and especially in IC 117 and a reduced incorporation into the smaller virus proteins (cf. Figs 7e, a). Radioactivity could not be chased from these larger polypeptides into smaller proteins by subsequent incubation with unlabelled amino acids at either 37 °C or at the elevated temperature. The results were therefore essentially similar to those obtained with the amino acid analogues and no primary gene product was detected.

In a final series of experiments an attempt was made to block cleavage with protease inhibitors as suggested by B. D. Korant (personal communication). To cultures of cells infected for 4 h, one of the following protease inhibitors was added, each at a final concentration of $2 \times 10^{-3} \text{M}$: TPCK, TLCK, PMSF or phenylboronic acid. The cells were incubated for a further 20 min, then pulsed with $^{[35]}$S-methionine; the results of acrylamide gel analyses are shown in Figs 7g–j. Phenylboronic acid (Fig. 7g) had little effect on the pattern of radioactive polypeptides. At the concentration used TPCK and TLCK were toxic to the cells and caused an appreciable inhibition of total protein synthesis; nevertheless, even under these circumstances appreciable cleavage took place and the largest polypeptide present was IC 147 (Figs 7i, j). The effects of PMSF were variable. In some experiments (e.g. Fig. 7h) it produced patterns which showed an appreciable accumulation of radioactivity in IC 147, IC 133, IC 117, IC 103 and IC 81 but no convincing evidence of the presence of larger precursors. However, on one occasion two more species of polypeptides were observed, a major protein band of mol. wt. 164000 and a fainter band with a mol. wt. of approximately 250000. The faint band of the highest mol. wt. may represent the primary gene product but it cannot be taken as proof of its existence.

**DISCUSSION**

*EMC virus capsid proteins*

Acrylamide gel analysis of the virus capsid proteins routinely revealed four components (Fig. 1), but one of these (VP2) could be resolved into two polypeptide species of similar size (Fig. 2). Thus EMC virus is composed of five non-identical polypeptides, four of which are in approximately equimolar proportions while the fifth (VP1) is a minor component: in this respect EMC closely resembles the related picornavirus Mouse–Elberfeld (Reuckert et al., 1969).

Reuckert et al. (1969) have proposed that the virus particle of Mouse–Elberfeld virus is assembled from an equimolar mixture of the 41000, 33000 and 25000 mol. wt. species, and that the largest is subsequently split in the maturation to give rise to the 30500 and 10000 mol. wt. species, while a similar scheme has been proposed for EMC virus by Butterworth et al. (1971). According to this scheme the pro-virus particle would consist of VP1, VP2A
and VP3 with VP1 being split subsequently to VP2B and VP4. This sequence of events would explain the relative abundance of IC 39 (i.e. VP1), the lesser abundance of IC 31 (i.e. VP2A) and the absence of VP4 in the patterns of virus-specific proteins found in the infected cell (Figs 4, 5).

The origin of the capsid and non-capsid proteins

Acrylamide gel analysis of the radioactive proteins present in extracts of EMC-infected ascites cells briefly pulsed with labelled amino acids reveals at least 11 polypeptides which are not identifiable as capsid proteins. Four of these (IC 54, IC 36, IC 17 and IC 13) appear to be quite stable and are presumably end products of the cleavage of the larger precursors. Their functions can only be guessed at; presumably one or two of them represent the virus-specific RNA polymerase. The remainder (IC 147, IC 133, IC 117, IC 103, IC 91, IC 81 and IC 68) were not stable to chasing with unlabelled amino acids and are probably precursors of the smaller proteins; the stability of IC 47 is not known as the band was too faint to obtain an accurate estimation of its concentration during chasing.

The pattern of virus-specific polypeptides found in EMC-infected ascites cells is basically similar to that reported by Butterworth et al. (1971) for HeLa cells infected with EMC virus. At least eight of the proteins described by them (A, B, C, E, F, G, and H plus I) are probably identical to the proteins we have designated IC 103, IC 91, IC 81, IC 54, IC 36, IC 17 and IC 13 (assuming we have not resolved all of the smaller protein species, see below). It is also probable that the remaining protein (D, mol. wt. 76000) may be identical to the protein we refer to as IC 68. The major difference in the patterns we have obtained with infected ascites cells is the appearance of larger mol. wt. proteins (IC 147, IC 133 and IC 117). It is possible that cleavage to the level of IC 103 and smaller polypeptides occurs more rapidly in the HeLa cells.

It is probable that some of the components represent mixtures of two or more different protein species. For example, IC 68 has always appeared as rather a broad band on acrylamide gels with an appreciable range of estimates of mol. wt., and hence could be two components. Also, the molar proportions of the two smallest proteins (IC 17 and IC 13) are two- to fourfold higher than any of the other proteins, particularly when the pulse of labelled amino acids exceed 30 min. This suggests that these components may be a mixture of breakdown products rather than two homogeneous protein species.

Butterworth et al. (1971) have proposed that the EMC genome is initially translated into three gene products (A, C, and F, equivalent to IC 103, IC 81 and IC 36), and that the largest of these (IC 103) is cleaved sequentially (via IC 91) to one molecule each of VP1, VP2A, VP2B, VP4 and IC 13. Our results are in agreement with this observation although other schemes are also possible to explain the cleavage process.

Recently, Butterworth & Rueckert (1972) established the gene order of EMC virus with the use of pactamycin which blocks initiation of translation but allows polypeptide chain elongation to proceed. Their experiment lends strong support to the proposal that the primary gene product of picornaviruses is a single high mol. wt. polypeptide representing the entire coding region of the virus RNA. In our system polypeptide IC 147 and IC 133 may represent earlier cleavage products of the primary gene product.

A comparison of tryptic peptides of precursor and stable polypeptides should firmly establish the cleavage mechanism operating in EMC infected cell.
REFERENCES


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