Nucleocapsid and Envelope Protein of Semliki Forest Virus as Affected by Canavanine

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(Accepted 16 December 1971)

SUMMARY
Cytoplasmic nucleocapsids of Semliki Forest virus were formed in BHK 21 cells treated with canavanine under such conditions that virus RNA synthesis continued but no infectious virus was released. On the other hand, envelope protein synthesis was strongly inhibited. The canavanine nucleocapsids formed sedimented at 140S and contained 42S virus RNA and a protein indistinguishable from the normal protein by polyacrylamide gel electrophoresis. The canavanine nucleocapsids were not released from the cells.

INTRODUCTION
Canavanine, the amino acid analogue of arginine, has been widely used as an inhibitor of growth in various micro-organisms. The earliest reports (Volcani & Snell, 1948; Schwartz & Maas, 1960; Schachttele & Rogers, 1965) show a uniform effect on bacteria. The findings of Schachttele & Rogers (1965, 1968) and Schachttele, Anderson & Rogers (1968, 1970) that canavanine causes an 'irreversible death' in Escherichia coli are very different from those obtained with other amino acid analogues. Host-induced canavanyl-proteins are made which tend to form complexes with DNA and the membranes responsible for the inhibition of genome replication. Similar complexes have been found recently in adenovirus-infected cells treated with canavanine (Neurath et al. 1970), as well as in polyoma-transformed BHK cells (Hare, 1970). Other viruses have also been shown to be canavanine-sensitive (Pilcher et al. 1955; Kundin, Robbins & Smith, 1959; Ackermann et al. 1966; Ranki & Kääriäinen, 1969).

Canavanine is a potent inhibitor of the growth of Semliki Forest virus (SFV) (Ranki & Kääriäinen, 1969). In cells infected with an RNA virus, e.g. SFV, host protein synthesis is efficiently inhibited (Strauss, Burge & Darnell, 1969), making the above-mentioned mechanism most unlikely. Nevertheless, canavanine has proved to be a valuable tool for studying different events during SFV replication. If it is present from the beginning of the growth cycle no replication occurs. Later addition arrests the formation of infectious virus and haemagglutinins. Addition during the first 2 hr of infection inhibits the synthesis of SFV RNA and consequently of all virus components. No RNA polymerase activity was detected under these conditions (Ranki & Kääriäinen, 1970).

On the other hand, if canavanine is added 3 hr after infection 42S and 26S virus RNA are found, but no infectious virus is released. The present study shows that under these conditions the synthesis of virus nucleocapsids proceeds fairly normally but that of the envelope protein is markedly inhibited. Some properties of the canavanine nucleocapsids have been characterized.
METHODS

Virus and cells. The origin of SFV, its cultivation, infectivity and haemagglutination assays have been described previously (Ranki & Kääriäinen, 1969; Kääriäinen, Simons & von Bonsdorff, 1969). BHK21 cells, clone WI-2, were cultivated in BHK21 medium (Gibco Manual, Grand Island, N.Y.) supplemented with 10 % calf serum and 10 % tryptose phosphate broth (Ranki & Kääriäinen, 1969).

Preparation of cytoplasmic extracts. Complete monolayers in 32 oz. prescription bottles or Petri dishes were washed with PBS and infected with SFV at a m.o.i. of 20. After the 1 hr adsorption at 37 ° the cultures were washed with warm medium, and Eagle’s minimum essential medium, usually deficient in arginine, supplemented with 0.2 % bovine serum albumin (Armour Pharmaceutical Co., England) containing actinomycin D (1 µg./ml.) (i.e. growth medium) was added. It has been shown that lack of arginine in the growth medium does not affect the virus titres (Ranki & Kääriäinen, 1969). This medium was replaced by growth medium containing canavanine (Calbiochem, California) at a concentration of 200 µg./ml., but no arginine. At the desired times the infected cultures were pulsed as follows: [3H]-uridine-5-T (25 C/m-mole) (The Radiochemical Centre, Amersham, England) was given, either alone or with [14C]-amino acids from Chlorella protein hydrolysate (54 mc/milli-atom carbon, Amersham) in the latter case in a medium containing one-tenth of the normal amino acid concentration. [3H]-(DL)-phenylalanine (1·59 C/m-mole, Amersham) was given in a medium lacking phenylalanine. The pulse length varied from ½ to 1 hr. To stop the incorporation of radioactivity the cultures were placed on ice, the radioactive medium sucked off, and cold PBS, containing a fourfold excess of the respective unlabelled amino acids or a 1000-fold excess of uridine, was added. After washing the cultures cells were allowed to swell in RSB (0·01 m-tris pH 7·4, 0·01 m-KCl, 0·0015 m-MgCl2), scraped off into the same fluid and disrupted in a Dounce homogenizer. The cytoplasmic extract was obtained after centrifuging the homogenate at 1000 rev./min. for 5 min. when the nuclei and debris were pelleted. Before analysis on polyacrylamide gels the cytoplasm pelleting with the nuclei was released by treatment with 2 % Nonidet P 40 (Shell Chemical Co.) in RSB and the supernatant fluid obtained after recentrifugation was added to the former extract.

Preparation of cytoplasmic nucleocapsids. The cytoplasmic extract (usually treated with 1 % Nonidet P 40) was analysed on 15 to 30 % (w/w) sucrose gradients (ribonuclease free sucrose, Mann Research Laboratories, NY) in TN (0·1 m-NaCl, 0·05 m-tris pH 7·4), and centrifuged at 25,000 rev./min. for 3 hr at 4 ° in a Spinco Model L2-50 ultracentrifuge, rotor SW-27. One ml. fractions were collected from below and their extinction at 260 nm. was determined with a Zeiss Opton spectrophotometer. The sedimentation coefficient of the nucleocapsids was estimated by the method of Martin & Ames (1961) using 74s ribosomes as reference. The radioactivity in each fraction precipitated by cold 5 % trichloroacetic acid (TCA) was determined as described previously (Ranki & Kääriäinen, 1969). The size of the RNA was determined by centrifuging on 15 to 30 % (w/w) sucrose gradients in RSB, containing 0·1 % SDS in a SW-27 rotor at 24,000 rev./min. for 10 hr at 22 ° after treating the appropriate samples with 2 % sodium dodecyl sulphate (SDS) (Fluka), recrystallized three times according to Mandel (1964), 0·5 % Brij 58 (Atlas Chemical Industries, Delaware) and 0·5 mm-EDTA (Kääriäinen & Gomatos, 1969). For some experiments purified radioactive SFV was prepared as described by Kääriäinen & Söderlund (1971). This virus was treated with Nonidet P 40 and the nucleocapsids isolated after sucrose gradient centrifugation as above.

Density-gradient analysis. [3H]-uridine and [14C]-amino acid labelled cytoplasmic nucleo-
capsids after sucrose gradient centrifugation were fixed with 0.5% glutaraldehyde. They were then mixed with saturated CsCl-solution to an initial density of 1.4 g./cm.³. Centrifugation was performed in a Spinco SW-50 rotor at 37,000 rev./min. for 18 hr at 4°. Fractions of 15 drops were collected from below. Acid-insoluble radioactivity and density were determined from aliquots.

**SDS-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis was performed as described by Shapiro, Viñuela & Maizel (1967) and Weber & Osborn (1969). Cytoplasmic extracts were treated with 1% SDS and 1% 2-mercaptoethanol, warmed to 90° for 2 min. and subsequently dialysed against 0.01 M-sodium phosphate buffer containing 0.1% of both SDS and mercaptoethanol. The gels, 13 cm. long (7.5% acrylamide with 0.2% bisacrylamide), were pre-run for at least 1 hr before applying the samples. Electrophoresis was performed at pH 7, 3 v/cm. at room temperature. The radioactivity in the 2 mm. gel slices was determined in NCS-toluene (Caligui, Klenk & Choppin, 1969). Radioactive reference proteins: (a) thyroglobulin, mol. wt 165,000; (b) transferrin, mol. wt 77,000; (c) bovine serum albumin, mol. wt 68,000; (d) ovalbumin, mol. wt 44,500; (e) ribonuclease, mol. wt 13,700, were kindly supplied by Dr C. G. Gahmberg, Department of Serology and Bacteriology, University of Helsinki. They were prepared by methylation with [3H]-dimethyl sulphate as described by Kiehn & Holland (1970). When cytoplasmic extracts were analysed the protein concentration of the sample was determined as described by Lowry et al. (1951).

**Single-step purification of isotopically labelled virus.** A combination of velocity and isopycnic gradients was used, as described by Scheele & Pfefferkorn (1969), but proportionately larger quantities were used to give a volume of 29 ml. A 6 ml. sample was layered on to the gradient, which was centrifuged at 25,000 rev./min. for 3½ hr at 4° in a SW-27 rotor.

**Complement fixation test.** The titration procedures have been described previously (Mäntyläri, 1966). The immune serum was prepared in rabbit as follows: purified SFV was treated with 0.2% sodium deoxycholate (Mann Research Laboratories, NY) for 30 min., and mixed with Freund's adjuvant. Intramuscular injections were given three times at 10-day intervals. After three booster injections at 1 month intervals the rabbit was bled. The titre of the immune serum tested against whole SFV in the CF test was 320. The preparation of the antigens to be titrated is described in the text.

**RESULTS**

**Synthesis of SFV nucleocapsids in the presence of canavanine**

In a previous study it was found that SFV RNA synthesis continued if canavanine was added to infected cultures later than 3 hr after infection (Ranki & Kääriäinen, 1970). We have now studied synthesis of nucleocapsids in the cytoplasm of infected cells after canavanine treatment. Some of the cultures were canavanine-treated (200 µg./ml.) 3 hr after infection, others were untreated. A 1 hr pulse of [3H]-uridine and [14C]-amino acids was given 4½ hr after infection; cytoplasmic extracts were then prepared and subsequently analysed on sucrose gradients. In both cases a peak sedimenting at 140s, containing both [3H]- and [14C]-radioactivity was obtained (Fig. 1). These correspond to the nucleocapsid peaks of SFV isolated from the cytoplasm of infected cells reported by Acheson & Tamm (1970) and Friedman & Grimley (1969). The [14C]:[3H] ratios were identical in both peaks (value 1.38). In repeated experiments identical peaks sedimenting at 130 to 140 s were regularly found in infected cytoplasm after canavanine treatment.

After fixation with 0.5% glutaraldehyde samples from the nucleocapsid peak fractions were centrifuged on CsCl gradients. Densities of 1.46 (g./cm.³) were obtained for both control
and canavanine nucleocapsids, corresponding to the value obtained by Acheson & Tamm (1970). No release of infectious virus from canavanine treated cells was observed in any of the experiments.

**RNA and protein in the nucleocapsids**

To analyse the properties of these nucleocapsids in more detail their RNA and protein components were studied separately. The size of the RNA was determined from \(^{[\text{H}]}\)-uridine labelled nucleocapsids formed in the presence and absence of canavanine. The peak fractions in both cases were immediately treated with 2% sodium dodecyl sulphate and further analysed on 15 to 30% sucrose gradients. The RNA obtained was 42s RNA as in the intact virus. RNA from purified \(^{[\text{3P}]}\)-labelled virus was used as reference.

The nucleocapsids were further analysed on polyacrylamide gels, after labelling with \(^{[\text{H}]}\)-phenylalanine. Parallel experiments with proteins from purified SFV were performed. Control and canavanine nucleocapsids gave only one peak, which corresponded exactly to the nucleocapsid band from purified virus.

Earlier work has shown that canavanine is incorporated into the proteins of animal and bacterial cells in place of arginine (Kruse et al. 1959; Allende & Allende, 1964; Schachtele & Rogers, 1965). To see if this occurred in SFV nucleocapsids, infected cultures were pulsed with either \(^{[\text{H}]}\)-phenylalanine or \(^{[\text{14C}]}\)-canavanine and analysed on sucrose gradients as above. This experiment could not be performed under inhibitory conditions; nevertheless, it demonstrated the incorporation of canavanine in the nucleocapsid protein.
SFV protein synthesis and canavanine

Table 1. Titre of the cytoplasmic nucleocapsids in the CF test

<table>
<thead>
<tr>
<th>Antigen (protein content as mg./ml.)</th>
<th>Titre in the CF test</th>
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<tbody>
<tr>
<td>Purified SFV (1.8 mg./ml.)</td>
<td>200</td>
</tr>
<tr>
<td>Nucleocapsid from purified SFV (0.16 mg./ml.)</td>
<td>40</td>
</tr>
<tr>
<td>Cytoplasmic nucleocapsid at 3 hr (0.1 mg./ml.)</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Cytoplasmic nucleocapsid at 5½ hr (0.13 mg./ml.)</td>
<td>8</td>
</tr>
<tr>
<td>Cytoplasmic nucleocapsid at 5½ hr in the presence of canavanine (0.12 mg./ml.)</td>
<td>8</td>
</tr>
</tbody>
</table>

The titres of the different antigens were tested by box-titration using rabbit-anti-SFV serum.

CF-titre of the canavanine nucleocapsids

It was possible that canavanine would alter the gross antigenic properties of the nucleocapsids. The CF test was used to check this possibility. The nucleocapsid preparations were made as follows: 5 x 10^6 cells were infected with SFV; half of them received canavanine 3 hr after infection. One culture from both series was pulsed with ^3H^-phenylalanine from 4½ to 5½ hr infection time, for identification of the nucleocapsids to be collected. Cytoplasmic extracts were made from all cultures at 5½ hr, the labelled and unlabelled extracts were pooled and subsequently centrifuged as above. The same quantity of infected cells was collected, dialysed against veronal-buffered saline and subsequently used as antigens in the CF test. The extinction at 260 nm. of the ribosomes was used as quantitative indicator of cytoplasm concentration and hence nucleocapsid content. In the cells collected at 3 hr no distinct nucleocapsid peak was seen, but the corresponding fractions were collected and used as a control for the antigen level before canavanine treatment. Purified SFV and nucleocapsids isolated from purified virus by treatment with Nonidet P 40 were used as controls. The antigen titres in each case were determined by box-titration, using anti-SFV rabbit immune serum. Identical titres were obtained for both canavanine and control cytoplasmic nucleocapsids (Table 1).

Release of SFV nucleocapsids from the infected cells

The next step was to determine whether the canavanine nucleocapsids were released as non-infectious virus. Infected cultures received canavanine 3 hr after infection, as above. ^3H^-phenylalanine was given from 5 to 5½ hr to both canavanine-treated and untreated cultures. At 5½ hr the cultures were washed, the radioactive medium being replaced by a chase medium containing a fourfold excess of phenylalanine. The incubation with the chase medium was continued for 1 hr with canavanine present on the appropriate cultures; 6½ hr after infection the growth medium was harvested, since Scheele & Pfefferkorn (1969) have shown that pre-labelled nucleocapsids are maximally released during the hour immediately following the pulse. The culture fluids of two similar cultures were pooled, the cells washed with PBS and this added to the pool. The virus in the medium was purified on a single step purification gradient. From the normally infected cells a virus band was obtained, in which both the HA activity and radioactivity coincided (Fig. 2). In the presence of canavanine no radioactive peak was detected, either in the region where whole virus was found in the controls, or elsewhere in the density gradient section. In both cases ^3H^-labelled cytoplasmic nucleocapsids were synthesised, as in the preceding experiments.
Fig. 2. SFV released from infected cells after a ½ hr pulse with [3H]-phenylalanine (50 µc/dish) and subsequent chase for 1 hr with unlabelled phenylalanine. Only the density section of the purification gradient used is illustrated in the figure: • counts/min.; △, HA titre, controls; ○, counts/min.; △, HA titre, in the presence of canavanine.

Envelope protein synthesis in SFV infected cells treated with canavanine

Canavanine apparently had little effect on the synthesis of the nucleocapsid, but it could perhaps inhibit virus production through its effect on the envelope protein. For this reason envelope protein synthesis was analysed. Infected cell cultures, some treated with canavanine 3 hr after infection, others left untreated as controls were pulsed with [14C]-amino acids from 5 to 6 hr, and the cytoplasmic extracts prepared for analysis on polyacrylamide gels. Four distinct protein peaks were obtained in both cases: the nucleocapsid (mol. wt 34,000), the envelope protein (mol. wt 53,000), one protein of a mol. wt of about 68,000 and another of 95,000. A still bigger protein could usually be detected as a broadening of the last protein (mol. wt 95,000) peak. In spite of the qualitative similarity differences were observed in the amounts of envelope and the 95,000 mol. wt proteins produced. In the experiment presented (Fig. 3) the envelope protein production in the presence of canavanine was only 25% of that in the control, whereas the nucleocapsid protein production was 80% of that in the control. In addition to this some increase was seen in the amount of the 95,000 mol. wt protein in the presence of the analogue. That the two structural proteins of SFV clearly and consistently differ in their sensitivity to canavanine may be seen from Table 2.

**DISCUSSION**

The present study shows that virus nucleocapsids are produced in the presence of canavanine and the properties of these nucleocapsids are very similar to the normal ones in spite of the analogue being incorporated into the nucleocapsid protein. These canavanine nucleocapsids are not released from the cells.

The different proteins identified by polyacrylamide gel electrophoresis were similar to
Fig. 3. Polyacrylamide gel electrophoresis of proteins synthesised in SFV infected cells labelled with $[^{14}\text{C}]-\text{amino acids (1 \mu g/dish)}. \text{A, Untreated infected cells; B, infected cells treated with canavanine. The arrows indicate [PH]-marker proteins electrophoresed with the samples. (a) thyroglobulin, (b) transferrin, (c) bovine serum albumin, (d) ovalbumin, (e) ribonuclease. Radioactivity expressed as counts per min. per mg. protein.}

Table 2. Polyacrylamide gel electrophoresis of proteins synthesised in SFV infected cells labelled with $[^{14}\text{C}]-\text{amino acids}

<table>
<thead>
<tr>
<th>Counts/min. per mg. protein</th>
<th>(i)</th>
<th>(ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mol. wt 95,000</td>
<td>3,700</td>
<td>6,750</td>
</tr>
<tr>
<td>%</td>
<td>41</td>
<td>10</td>
</tr>
<tr>
<td>Mol. wt 68,000</td>
<td>9,400</td>
<td>6,240</td>
</tr>
<tr>
<td>%</td>
<td>10</td>
<td>9.4</td>
</tr>
<tr>
<td>Envelope</td>
<td>25,400</td>
<td>6,650</td>
</tr>
<tr>
<td>%</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>Nucleocapsid</td>
<td>20,100</td>
<td>16,900</td>
</tr>
<tr>
<td>%</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td>Total incorporation</td>
<td>89,300</td>
<td>66,600</td>
</tr>
</tbody>
</table>

those reported to occur in SFV and Sindbis virus infected cells (Hay, Skehel & Burke, 1968; Friedman, 1968; Strauss et al. 1969). However, if canavanine was present, a marked reduction was seen in the amount of the protein migrating at the position of the envelope protein. There are at least two possible explanations to this selective inhibition: if the
envelope protein is a breakdown product of a larger precursor protein canavanine could inhibit specific cleavage; this has been shown to happen in poliovirus proteins in the presence of several amino acid analogues (Jacobson & Baltimore, 1968; Maizel & Summers, 1968; Summers & Maizel, 1968). Similar experiments in which cells infected with Group A arboviruses were exposed to the analogues have not shown the existence of a comparable precursor product relationship (Friedman, 1969; Scheee & Pfefferkorn, 1970). However, in our experiments we have used a longer treatment with canavanine. Another possibility, of course, is that canavanine or canavanine-containing proteins specifically interfere with the translation of envelope protein. These possibilities are under investigation.

I thank Dr Leevi Kääriäinen for his help and valuable criticism. This work was supported by the Emil Aaltonen Foundation, Sigrid Jusélius Foundation and The Association of Finnish Life Assurance Companies, Finland. The technical assistance of Miss Sirkka Keränen and Miss Ritva Saarelmo is gratefully acknowledged. Actinomycin D was a gift from Merck, Sharp & Dohme.

REFERENCES


SFV protein synthesis and canavanine


(Received 11 October 1971)