Characterization of Cricket Paralysis Virus-Induced Polypeptides in *Drosophila* Cells

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Cricket paralysis virus purified from *Galleria mellonella* larvae was shown to be similar to virus purified from *Drosophila melanogaster* cells. Cricket paralysis virus contained three major structural polypeptides of similar molecular weight (around 30,000), had a buoyant density of 1.344 g/ml, and had a capsid diameter of 27 nm. Twenty virus-induced polypeptides could be detected in CrPV-infected *Drosophila* cells. Two major polypeptides found in the infected cells corresponded to two structural viral polypeptides (VP1 and VP3), whereas the third major intracellular polypeptide was the apparent precursor of the third viral structural polypeptide (VP2). Three of the primary virus-induced polypeptides had molecular weights of 144,000, 124,000, and 115,000. These and other polypeptides were chased into lower-molecular-weight proteins when excess cold methionine was added after a short [³⁵S]methionine pulse. Although cricket paralysis virus has a number of characteristics in common with the mammalian enteroviruses, the extremely fast processing of high-molecular-weight polypeptides into viral proteins seems atypical. Also, no VP4 (8,000 to 10,000 molecular weight) has been found in the virus particles.

Approximately 40 small RNA-containing viruses of insects have been isolated and partially characterized (4, 12, 15, 28, 41). The most studied of these are the honeybee (1, 3, 4, 23), *Drosophila* (9-11, 25, 26, 29, 44), and mosquito (30, 34) viruses. Several of these viruses are potential members of the Picornaviridae on the basis of their small size, the presence of single-stranded RNA, and a low number of structural polypeptides. However, several of the small RNA viruses of insects must be excluded from this group on the basis of having too few structural polypeptides (for example, as in *Nudaurelia* virus [43]) or a split RNA genome (for example, as in *Nodamura* virus [2, 19-22, 36, 37]). The most likely candidates for the *Enterovirus* genus of the Picornaviridae (6) are Kawino (30), *Drosophila* C (10, 27, 28), and cricket paralysis (27, 32, 33; C. Reinganum, M.S. thesis, Monash University, Melbourne, Australia, 1973) viruses as they have sedimentation coefficients of about 165S, buoyant densities of 1.33 to 1.34 g/ml in CsCl, and diameters of 27 to 28 nm and they contain single-stranded RNA. These viruses also contain three major polypeptides with molecular weights of around 30,000.

Cricket paralysis virus (CrPV) was first isolated from Australian field crickets by Reinganum (33; Reinganum, M.S. thesis), and it has since been demonstrated that this virus will grow in a fairly broad range of insect species (12, 27, 32; Reinganum, M.S. thesis). CrPV was shown

to grow in *Drosophila* cells by Scotti (39, 40), although Plus et al. (27) were unable to infect their *Drosophila* cell line with this virus.

This work was undertaken to compare the protein content of CrPV purified from insects and tissue culture and to investigate the virus-induced polypeptides in *Drosophila* cells to enable us to make an initial evaluation of this virus as a potential member of the *Enterovirus* genus.

MATERIALS AND METHODS

Reagents. Acrylamide and sodium dodecyl sulfate (especially pure grade) were supplied by British Drug Houses Ltd., Poole, England. *N,N'*-methylenebisacrylamide was purchased from Eastman Organic Chemicals, Rochester, N.Y. The molecular weight standards, obtained from Sigma Chemical Co., London, England, were as follows: β -galactosidase, 132,000; transferrin, 90,000; bovine serum albumin, 69,000; ovalbumin, 45,000; lactic dehydrogenase, 35,000; carbonic anhydrase, 31,000; α -chymotrypsinogen, 25,000; myoglobin, 16,900; and cytochrome c, 12,400. [³⁵S]methionine (specific activity 1,190 Ci/mmol) and [¹⁴C]-labeled protein hydrolysate (specific activity, 59 mCi/matom) were purchased from the Radiochemical Centre, Amersham, England.

Cells, media, and virus. *Drosophila melanogaster* cells (38) were grown as monolayers at 28°C on 75-cm² plastic flasks (Falcon Plastics, Oxnard, Calif.) for virus growth and labeling and on 25-cm² plastic flasks for radioactive labeling of intracellular proteins. For cell growth, TC100 medium (7) containing 10% fetal calf serum, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml was used. TC100 medium minus

methionine containing 2% dialyzed fetal calf serum was used for labeling intracellular proteins in the presence of [35 S]methionine. (For some of the later work, *Drosophila* cells were grown in Schneider *Drosophila* medium [GIBCO-BIO-CULT Ltd., Glasgow, Scotland] as more confluent monolayers formed.) Pure CrPV, grown in *Antheraea pernyi* larvae, was obtained from C. Reinganum (Victoria Plant Research Institute, Burnley, Victoria, Australia).

Virus growth and purification. CrPV was initially grown in late instar larvae of the greater wax moth *Galleria mellonella*. Penultimate instar larvae were infected by injection of 1 to 4 μ g (protein) of purified CrPV. Infected larvae were maintained on a synthetic diet (C. F. Rivers, personal communication) at ambient temperature, and mortality occurred within 5 days. Larvae were harvested before mortality occurred (3 to 4 days postinfection) and either frozen at -40°C for future purification or extracted into ice-cold 10 mM phosphate buffer (pH 7.2) by thorough disintegration with a Colworth Stomacher. The extract was then squeezed through two layers of butter muslin, and the filtrate was shaken with an equal volume of ice-cold *n*-butanol-chloroform (1:1, vol/vol). The mixture was centrifuged at $2,000 \times g$ for 10 min, and the aqueous phase was removed. The substantial interphase and organic phase were mixed with an equal volume of ice-cold 10 mM phosphate buffer (pH 7.2) and centrifuged as described above. The pooled aqueous phases were centrifuged at $5,000 \times g$ for 30 min after the removal of residual organic solvents by gently bubbling nitrogen through the solution on ice. Virions were pelleted from the supernatant of the above centrifugation at $107,000 \times g$ for 2 h. The pellets were resuspended in a small volume of 10 mM phosphate buffer (pH 7.2), overlaid into a 10 to 40% (wt/vol) sucrose gradient in the same buffer, and centrifuged at $65,000 \times g$ for 90 min. Opalescent virus zones were removed, diluted four to fivefold with 10 mM phosphate buffer (pH 7.2), and pelleted at $107,000 \times g$ for 2 h. Resuspended virions were purified further by layering into 34% (wt/wt) cesium chloride in 10 mM phosphate buffer (pH 7.2) and centrifuging at $115,000 \times g$ for 16 h. The virus band was harvested and CsCl was removed by dialysis against 10 mM phosphate buffer (pH 7.2) at 4°C or by a large dilution in 10 mM phosphate buffer and pelleting as described above.

CrPV was adapted to growth in monolayer cultures of *Drosophila* cells by infecting a 25-cm² confluent monolayer of cells with 10 μ g of purified CrPV in 0.5 ml of filter-sterilized maintenance medium (TC100 medium plus 2% calf serum). After 1 h, another 5 ml of maintenance medium was added, and the cells were incubated at 28°C for 5 days, by which time a very marked cytopathic effect had occurred. A 100- μ l amount of the supernatant from this first passage was added to a second 25-cm² confluent monolayer as described above. Cytopathic effect occurred within 2 days, and the material from this passage was used to grow a large pool of virus in 75-cm² confluent monolayers of *Drosophila* cells. For virus growth, 75-cm² monolayers were infected with 0.5 to 1.0 50% tissue culture infective dose per cell for 1 h at 28°C . After 1 h at 28°C , the inoculum was removed and 5 ml of

maintenance medium was added. The cells were incubated at 28°C for 36 h, by which time all cells had disintegrated. Cell debris was removed by centrifugation at $2,000 \times g$ for 10 min, and the supernatant was stored in aliquots at -40°C .

The organic extraction step was unnecessary with purification of CrPV from tissue culture fluid. The fluids were clarified by centrifugation at $10,000 \times g$ for 30 min, and the virus was pelleted out of the supernatant from this step as described above. Purification was performed in sucrose rate and CsCl equilibrium gradients as previously described.

Virus titration. Virus infectivity was determined by a 50% tissue culture infective dose (31, 40). Serial dilutions (10 μ l) of CrPV in maintenance medium were overlaid on wells containing 5 μ l of 10^6 cells per ml in Falcon plastic microtest plates. Cytopathic effect, as demonstrated by cell fragmentation, was maximal at 4 days postinfection. Five determinations were made for each of the serial dilutions.

Radiolabeling of intracellular proteins. Monolayer cultures were infected with 25 or 100 50% tissue culture infective doses per cell at 28°C for 1 h. The inoculum was removed, maintenance medium was added (2 ml/25-cm² monolayer) postinfection, and the cells were incubated at 28°C . At various times postinfection, monolayers of cells were washed with TC100 medium containing no methionine. Cells were pulsed with 50 μ Ci of [35 S]methionine in 1 ml of TC100 medium without methionine for the times stated in the text. Postpulse monolayers were harvested into 5 ml of ice-cold maintenance medium and pelleted for 5 min at $2,000 \times g$. Cells were prepared for polyacrylamide gel electrophoresis by suspending each pellet from a 25-cm² monolayer in 250 μ l of solubilization buffer containing 2% (wt/vol) sodium dodecyl sulfate, 2% (vol/vol) 2-mercaptoethanol, 15% (vol/vol) glycerol, and 0.001% bromophenol blue tracking dye in 1/10-concentration electrophoresis buffer. Samples were boiled immediately at 100°C for 2 min and for another 30 s before electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were normally separated on 15-cm-long 12.5% polyacrylamide slab resolving gels with a 5% stacking gel, using the discontinuous Tris-glycine buffer system described by Laemmli (13). Other polyacrylamide concentrations were used, and these are mentioned in the text. Electrophoresis was performed for 16 to 18 h at a constant 100 V, and gels were fixed and stained with 45% methanol-7% acetic acid containing 0.5% Coomassie brilliant blue R250. Gels were destained by agitation in several changes of 45% methanol-7% acetic acid.

Alternatively, polyacrylamide gels were fixed in 7% acetic acid for 2 h, washed briefly with water, incubated in two changes of dimethyl sulfoxide for 30 min, and then impregnated with PPO (2,5-diphenyloxazole; 22% [wt/vol] in dimethyl sulfoxide) (14). The gels were then dried and exposed to Fuji X-ray film at -70°C .

Molecular weight determinations of viral structural and intracellular induced proteins were made by co-running nine marker proteins on the slab gel (see above) (42). The migration of radioactive viral proteins was determined from the autoradiographs by direct comparison with the corresponding stained gel.

Determination of buoyant densities of viral particles. A suspension of pure virus particles (1 mg/ml) was overlaid on 6 ml of 34% (wt/wt) CsCl in 10 mM phosphate buffer (pH 7.2), and the sample was centrifuged at $62,000 \times g$ in a 6×14 ml titanium swinging-bucket rotor (MSE Ltd., Crawley, England) for 20 h at 20°C. Fractions were collected from the top of the tube by displacement from the bottom with Fluorinert FC-48, using an ISCO density gradient fractionator (model 640). The gradient was scanned for protein at a UV absorbance wavelength of 254 nm with an ISCO absorbance monitor (model UA-5).

Electron microscopy. Purified virus preparations were dialyzed against several changes of 10 mM Tris buffer (pH 7.2) before negative staining for 1 min with 2% (wt/vol) uranyl acetate. Samples were examined on a AE1 EM6B electron microscope with an accelerating voltage of 60 kV.

Protein determinations. Protein concentrations in purified viral suspensions were determined by the method of Lowry et al. (16), using bovine serum albumin as a standard.

RESULTS

Polypeptide composition of CrPV and comparison of insect- and tissue culture-derived virus. Electron microscope examination of CrPV showed that the virus has a diameter of 27 nm and no distinct surface structure. Tissue culture- and insect-grown virions appeared to be identical by electron microscopy, and tissue culture-derived virus reacted with antiserum raised against virus purified from *G. mellonella* larvae. CrPV was grown in *Drosophila* cells in the presence of [35 S]methionine, and when equal amounts of purified radioactive virus and insect-derived virus were mixed and centrifuged on CsCl gradients, all of the radioactivity migrated with virtually all of the detectable protein. Both viruses had a buoyant density of 1.344 g/ml in CsCl gradients.

Two major structural polypeptides were detected by Coomassie brilliant blue staining when the proteins of CrPV purified from *G. mellonella* larvae were electrophoresed on 8, 10, and 12.5% discontinuous polyacrylamide slab gels. The molecular weights of the two proteins were found to be 28,000 and 30,500 on 12.5% polyacrylamide slab gels. Electrophoresis of the viral polypeptides on 17.5% gels resolved the higher-molecular-weight structural polypeptides into two discrete proteins (Fig. 1). By analogy with the mammalian picornaviruses, the proteins were called VP1, VP2, and VP3 in descending order of molecular weight. A minor polypeptide which reproducibly occurred above VP1 was termed VP0. Variable amounts of protein "contaminants" occurred with insect-derived CrPV, particularly a polypeptide with a molecular weight higher than that of VP3, as shown in Fig. 1.

FIG. 1. Coomassie brilliant blue-stained polypeptides of CrPV grown in (A) *G. mellonella* larvae and (B) *Drosophila* cells. Electrophoresis was on 17.5% polyacrylamide slab gels as described in the text.

There was no evidence of a structural polypeptide with a molecular weight of 8,000 to 10,000 (VP4) which occurs with mammalian picornaviruses.

Densitometer traces of the stained polyacrylamide slab gels on which purified tissue culture-produced virus was electrophoresed demonstrated the approximate equimolarity of the three major viral structural polypeptides. As shown in Fig. 2, tissue culture-derived virus proteins were electrophoresed on three different polyacrylamide gel systems. On gradient gels (Fig. 2A) and 17.5% slab gels (Fig. 2B), VP1 and VP2 were separated, whereas on 12.5% slab gels (Fig. 2C), these polypeptides comigrated. Partial separation was achieved with other electrophoretic runs on 12.5% polyacrylamide slabs. A small amount of VP0 was detectable above VP1 on all three polyacrylamide gels.

FIG. 2. Densitometer tracings of the Coomassie brilliant blue-stained polypeptides of CrPV electrophoresed on (A) 5 to 20% gradient, (B) 17.5% polyacrylamide slab gel, and (C) 12.5% polyacrylamide slab gel. On the gradient gel and 17.5% slab, VP1 and VP2 were separated. A small amount of an additional polypeptide (VP0) was apparent at a molecular weight higher than that of VP1 on the polyacrylamide gels.

Radiolabeling of the intracellular proteins in CrPV-infected *Drosophila* cells. When CrPV-infected *Drosophila* cells (treated postinfection with 2 μ g of actinomycin D per ml) were pulsed with [35 S]methionine at various stages postinfection, virus-specified proteins were apparent early in infection. Figure 3 shows the polypeptide pattern when cells were pulsed for 15 min at 1, 2, 3, and 4 h postinfection. At 2 h postinfection (Fig. 3B), the structural proteins VP1 and VP3, as well as comparatively large amounts of VP0, which appears as a shoulder on a major cellular polypeptide, were apparent. By 3 h postinfection, VP0, VP1, and VP3 were being synthesized in large amounts, and by 4 h, small amounts of high-molecular-weight virus-induced polypeptides were also apparent. There

was no evidence of a polypeptide corresponding to VP2 being labeled during a 15-min pulse. Figure 4 shows the densitometer traces of 35 S-labeled purified CrPV (Fig. 4B) and the intracellular polypeptides labeled at 5 h postinfection for 15 min (Fig. 4A). With the 17.5% polyacrylamide gel shown in Fig. 3 and 4, the structural polypeptide VP2 was resolved as shown with purified viral polypeptides in Fig. 4B. The virus-induced polypeptide occurred in very similar proportions at the various stages postinfection, as shown in Fig. 3 and 4. There was very little evidence of VP2 being synthesized when CrPV-infected cells were pulsed with either 14 C-labeled amino acids or [35 S]methionine for times of up to 1 h. In addition, with pulses of less than 10 min, there were always comparatively small amounts of nonstructural polypeptides of high molecular weight (larger than VP0) synthesized.

FIG. 3. Densitometer tracing of the autoradiogram of 35 S-labeled *Drosophila* cells infected with CrPV electrophoresed on a 17.5% polyacrylamide slab gel. The cells were pulsed with 50 μ Ci of [35 S]methionine for 15 min at (A) 1 h, (B) 2 h, (C) 3 h, and (D) 4 h postinfection. The viral structural polypeptides are indicated (VP1 and VP3), as is the major intracellular nonstructural polypeptide (VP0).

FIG. 4. Densitometer tracing of the autoradiogram of (A) ^{35}S -labeled *Drosophila* cells infected with CrPV and (B) ^{35}S -labeled polypeptides of purified CrPV. The CrPV-infected *Drosophila* cells were incubated in methionine-deficient maintenance medium from 4 to 5 h postinfection and pulsed for 15 min with 50 μCi of [^{35}S]methionine at 5 h postinfection. The major nonstructural virus-induced polypeptide is indicated (0), as are the major viral structural polypeptides (1, 2, and 3).

VP0, VP1, and VP3 were always present in approximately equimolar amounts. Virus-induced polypeptides were detectable in stained polyacrylamide gels of infected cells, as shown in Fig. 5, which shows a 17.5% slab of purified virus protein (channel 1) and mock-infected (channel 2) and infected (channel 3) *Drosophila* cells at 4.5 h postinfection. VP1 and VP3 were clearly apparent in the infected cells, with smaller amounts of VP2 also being present. VP0 comigrated with a cellular polypeptide and was not clearly resolved. There was no evidence of other nonstructural polypeptides on the stained gel.

Stability of CrPV-induced polypeptides in infected *Drosophila* cells. When CrPV-infected *Drosophila* cells were pulsed with [^{35}S]methionine at 4 h postinfection, a number of nonstructural polypeptides were apparent on the autoradiograms shown in Fig. 6. As shown before, the most obvious nonstructural polypeptide

was VP0 (labeled virus-induced polypeptide I in Fig. 6). Several high-molecular-weight nonstructural polypeptides, such as A, D, E, and F, were apparent. Virus-induced polypeptides B and C were not resolved on this gel due to the presence of large amounts of a major nonsuppressed host cell polypeptide. These two polypeptides could be separated from the interfering host protein on an 8% (or lower percent) polyacrylamide gel. Several low-molecular-weight polypeptides (P, Q, R, S, and T) were also apparent. They were more distinct, and clearly virus induced, when the polyacrylamide gel was exposed for longer periods to X-ray film. In Fig. 6, the structural polypeptides are indicated by M and O (VP1 and VP3, respectively).

When the CrPV-infected cells were pulsed for 10 min (Fig. 6, channel 2) and then excess cold methionine (50-fold) was added for increasing

FIG. 5. Coomassie brilliant blue-stained 17.5% polyacrylamide slab gel of (1) the polypeptides of purified CrPV, (2) mock-infected *Drosophila* cells, and (3) CrPV-infected *Drosophila* cells at 4.5 h post-infection. The viral structural polypeptides are indicated.

FIG. 6. Autoradiogram of ^{35}S -labeled (1) mock-infected *Drosophila* cells, (2) CrPV-infected *Drosophila* cells, and (3 to 6) cold methionine-chased CrPV-infected cells. At 3.5 h postinfection, the medium was removed, and 1 ml of methionine-deficient medium was added to each culture. At 4 h postinfection, this was replaced by another 1 ml of methionine-deficient medium, and 50 μCi of [^{35}S]methionine was added to each culture. One mock-infected culture (1) and one CrPV-infected culture (2) were harvested after 10 min. Four infected cultures were washed with medium containing 50-fold excess methionine and incubated in 5 ml of the same medium. Chased cultures were harvested after 10 min (3), 40 min (4), 110 min (5), and 140 min (6). Cells were immediately pelleted and suspended in 250 μl of solubilization buffer by boiling for 2 min at 100°C . Samples were electrophoresed on a 12.5% polyacrylamide slab gel. Virus-induced polypeptides are indicated. I is equivalent to VP0, and M and O indicate VP1 and VP3, respectively. There is partial resolution of VP2 (N) on this gel system.

periods of time (Fig. 6, channels 3 to 6), the high-molecular-weight nonstructural polypeptides disappeared or were seen in markedly reduced amounts. The major nonstructural polypeptide, VP0 (I in Fig. 6), also decreased with an increasing length of chase, and a leading shoulder (N) on VP1 (M) appeared. With increasing chase

periods, nonstructural polypeptides, such as polypeptide L above VP1 (M) and the low-molecular-weight protein, appeared. VP0 (I) was observed to be lost relatively slowly with an increasing length of chase, whereas the structural polypeptides VP1 and VP3 (M and O, respectively) remained stable. A total of 20 polypeptides, including the three major viral structural proteins, were induced in the *Drosophila* cells as a result of infection with CrPV (Fig. 6).

Determination of the molecular weight of virus-induced polypeptides. The molecular weights of the virus-induced polypeptides were determined on two different gel systems to achieve adequate separation of both the high- and low-molecular-weight polypeptides. The molecular weights of the smaller polypeptides were determined on 12.5% polyacrylamide gels, whereas the polypeptides which migrated above VP3 were estimated on 8% polyacrylamide gels (Fig. 7). To increase the accuracy of the determination of the molecular weights of polypeptides A, B, and C, we included the spectrin proteins (18) of erythrocyte membranes (kindly donated by R. Hunt, Department of Biochemistry, University of Oxford) as marker polypeptides on an 8% polyacrylamide gel on which the viral polypeptides were allowed to run off. The molecular weights of A, B, and C (Table 1) were slightly increased when these markers were included. The molecular weight of A was previously determined as 150,000 on a 5% polyacrylamide gel, and apparent molecular weights were

FIG. 7. Determination of the molecular weights of the CrPV-induced polypeptides in *Drosophila* cells. The molecular weights of the virus-induced polypeptides were determined from the log molecular weights versus electrophoretic mobility curve. The major polypeptides are indicated by letters, and the markers used were (1) β -galactosidase, (2) transferrin, (3) bovine serum albumin, (4) ovalbumin, (5) lactic dehydrogenase, (6) carbonic anhydrase, and (7) α -chymotrypsinogen. The molecular weights of polypeptides A to D were determined on 8% polyacrylamide slab gels.

TABLE 1. *Molecular weights of CrPV-induced polypeptides*

Polypeptide ^a	Mol wt ^b × 10 ⁻³
A	136.0 (144) ^c
B	122.0 (124) ^c
C	114.0 (115) ^c
D	63.0
E	58.0
F	54.0
G	43.8
H	43.2
I (VP0)	43.0
J	39.8
K	38.3
L	36.2
M (VP1)	35.0
N (VP2)	34.0
O (VP3)	30.0
P	22.8 ^d
Q	18.0 ^d
R	16.6 ^d
S	13.5 ^d
T	12.6 ^d

^a Refer to Fig. 6.^b Determined on 8% polyacrylamide slab gels, except for P, Q, R, S, and T.^c Determined using high-molecular-weight erythrocyte proteins as markers.^d Determined on 12.5% polyacrylamide slab gels.

found to increase with decreasing gel concentrations. When the 8% polyacrylamide gels were used to determine molecular weights, the viral polypeptides appeared slightly larger than those initially determined on 12.5% polyacrylamide slabs.

DISCUSSION

On 12.5% polyacrylamide slab gels, CrPV was shown to contain two major structural polypeptides of molecular weights 28,000 and 30,500, with a molar ratio of 2:1. The 30,500-molecular-weight polypeptide was sometimes partially resolved into two polypeptides on this gel system. A minor protein component, which had a molecular weight approximately 10,000 higher than the 30,500-molecular-weight complex, was also present. With the increased resolution obtained with 17.5% polyacrylamide gels and 5 to 20% gradient gels, a third major structural polypeptide was clearly resolved. Quantitation of the three polypeptides gave a molar ratio of 1:1:1. This is similar to the mammalian picornaviruses, which normally have three polypeptides of an approximate equimolar ratio with molecular weights around 30,000 (35). In addition, with the mammalian picornaviruses, a minor structural protein (VP0) with a molecular weight approximately 8,000 to 10,000 higher than that of VP2 is often found. With CrPV, the molecular weight

of this minor protein was estimated to be 11,000 higher than that of VP2 on 12.5% polyacrylamide gels and 9,200 higher on 8% slab gels. This minor structural protein is a small amount of the "nonstructural" immediate precursor of VP2 and VP4 with mammalian picornaviruses. However, with CrPV, no evidence of VP4 (8,000 to 10,000 molecular weight) was found with short electrophoretic runs of the viral polypeptides on high-percentage polyacrylamide gels. The obvious possibility that VP4 is lost during virus purification can probably be excluded as virus which had been partially purified (i.e., pelleted, banded on sucrose, and dialyzed) did not appear to contain this protein. Another possible explanation for the absence of VP4 is that VP0 is cleaved to give VP2 and the residual 8,000- to 10,000-molecular-weight piece is then further degraded. Hence, the mature virions do not contain a recognizable VP4. However, it is possible that it is present in such small amounts that staining (or ³⁵S labeling) of the protein is inadequate.

The virions produced from insects (*G. mellonella* larvae) had a protein composition very similar to those produced from tissue culture. Invariably, the insect-derived virions contained at least one additional protein between VP2 and VP3 on polyacrylamide gels. The virions from tissue culture and insects were serologically indistinguishable and had the same protein capsid diameters and the same buoyant densities.

When CrPV was used to infect *Drosophila* cells in the presence of actinomycin D, three major virus-induced polypeptides were labeled with [³⁵S]methionine or ¹⁴C-amino acids. Two of these polypeptides comigrated with VP1 and VP3 of the mature virions, whereas the third major induced polypeptide (VP0 by analogy with the mammalian picornaviruses) comigrated with the minor viral structural polypeptide. Surprisingly, with short pulses of [³⁵S]methionine, VP0, VP1, and VP3 were present in very large amounts, and there was a low content of high-molecular-weight precursor polypeptides. If CrPV is a classical enterovirus, we expect to find, with short pulses, a relatively small accumulation of the viral structural polypeptides and a considerable amount of high-molecular-weight uncleaved precursors of the viral polypeptides (5, 8, 17, 35). There was no evidence for the presence of VP2 with short pulses, although it was shown to be slowly formed on chase of the ³⁵S-labeled polypeptides with excess cold methionine. The most likely candidate for the immediate precursor of VP2 is the major virus-induced protein that we have prematurely called VP0, as this protein is slowly cleaved during the pulse-chase.

A total of 20 induced polypeptides were detectable in CrPV-infected *Drosophila* cells with a combination of polyacrylamide gel systems, different exposure periods of the gels to X-ray film, and the visualization of additional polypeptides during the chase period. Several high-molecular-weight polypeptides were unstable during the chase with cold methionine and disappeared with increasing times.

From the evidence presented here, CrPV appears to have a number of intracellular characteristics in common with the mammalian enteroviruses. There are a large number of proteins present in the infected cells, some being of a considerably higher molecular weight than those of the virus structural polypeptides. The radio-labeled high-molecular-weight polypeptides can be chased by the addition of excess cold amino acid as with the mammalian enteroviruses. There is a protein present in the infected cell equivalent to VP0 which can be chased into VP2. The major difference between the intracellular events with CrPV and the mammalian picornaviruses is that with CrPV the processing of the proteins is extremely asymmetrical. There is very fast formation of VP1 and VP3, with a much slower cleavage of VP0 into VP2. We are currently studying the effect of protease inhibitors such as iodoacetamide and amino acid analogs to attempt to stop the initial fast cleavage. In addition, we are investigating the intracellular events with *Drosophila C* virus in *Drosophila* cells, and initial data appear to demonstrate that the processing of the precursor proteins to virus-sized proteins is also extremely fast.

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