Protein and RNA Composition of the Structural Components of Drosophila X Virus

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SUMMARY
Purified Drosophila X virus (DXV) particles have been analysed. They band at a density of 1.345 g/ml in CsCl. The virion proteins have been resolved into six major polypeptide species (mol. wt. 100,000, 50,000, 49,000, 44,000, 33,000 and 27,000) by polyacrylamide gel electrophoresis. The RNA sediments at 5S and 14S in sucrose gradients. The 5S RNA is sensitive to pancreatic RNase and the 14S RNA is resistant in its native form and sensitive after denaturation. The 14S RNA can be resolved into two equimolar fractions by polyacrylamide gel electrophoresis. The estimates of the mol. wt. of the two RNA species depends upon their structure. If they exist as double-stranded molecules their electrophoretic mobility compared to that of reovirus type 3 RNAs indicates for each species an average mol. wt. of 2.2 × 10⁸.

INTRODUCTION
Drosophila X virus (DXV) was identified as a contaminant in Drosophila cell cultures by Teninges et al. (1979). On a morphological basis this virus is very similar to the infectious pancreatic necrosis virus of the trout (Cohen et al. 1973), to Tellina tenuis virus (Hill, 1976) and to infectious bursitis virus (Nick et al. 1976). In order to characterize DXV further, we have undertaken a biochemical analysis of its protein and RNA constituents.

METHODS
Virus stock and propagation. The virus stock was a second passage in Drosophila of the original stock 347 (Teninges et al. 1979) and contained 10⁸ infectious units/ml. The virus was diluted 10⁻³-fold in TD saline solution (NaCl, 13 × 10⁻² M; KCl, 5 × 10⁻³ M; Na₂HPO₄, 7 × 10⁻⁴ M; tris, 8 × 10⁻³ M; pH adjusted to 7.6) and 0.5 μl per fly was injected into standard Drosophila as described by Plus (1954). After 5 days, when all the flies were sensitive to anoxia, they were used for DXV purification.

Purification of DXV. The procedure was adapted from that of Smith et al. (1969) for the purification of reovirus. Flies were crushed with a glass blender in ten times their weight of 0.15 M-NaCl, 0.01 M-tris, pH 7.4 (LSB). One half vol. Freon 113 was added and the suspension was homogenized on a Vortex-Genie mixer for 2 min. The aqueous and organic phases were separated by centrifugation at 3000 g for 15 min. The organic phase was re-extracted with 0.5 vol. of LSB. The combined aqueous phases were re-extracted in 0.25 ml of Freon 113. The resulting aqueous phase was layered on to 2 ml of 20% sucrose in a SW-41 rotor tube and centrifuged for 1 h at 150,000 g. The resulting pellet was resuspended in LSB and aggregates were dispersed by mild sonication. It was then added to a CsCl solution made up to obtain a final density of 1.34 g/ml. After centrifugation for 18 h in a
SW60 rotor at 200,000 g, a single virus band was observed at a density of 1.345 g/ml. The band was collected and diluted in at least 10 vol. of LSB and the virus was pelleted by centrifugation for 1 h at 150,000 g in a SW41 rotor. The pellet was resuspended in LSB and gently sonicated. Electron microscopy of this virus suspension revealed no detectable host material and that the virus particles were intact. The overall recovery of infectivity ranged around 20%.

Buoyant density determination. Purified virus was layered on top of pre-formed CsCl gradients and centrifuged for 6 h at 200,000 g in the SW60 rotor of a Beckman ultracentrifuge. The fractions were collected from the bottom of the tubes and the refractive index of the fractions measured in an Abbé refractometer. The absorbance at 280 nm was measured in a Beckman DB-G spectrophotometer.

Analysis of virus polypeptides in polyacrylamide gels. Standard conditions for electrophoresis were as described by Spear & Roizman (1972): gels were formed in siliconized quartz tubes of 6 mm internal diam. The resolving gel (12 cm long) consisted of 8, 10 or 12 % acrylamide. The proteins were denatured and solubilized by the addition of concentrated reagents to yield a final concentration of 0.05 M-tris-HCl pH 7, 2 % SDS, 5 % β-mercaptoethanol and 0.005 % bromophenol blue followed by boiling for 2 min. Thirty to 100 μg of protein in vol. of 40 to 60 μl were subjected to electrophoresis at a constant current of 3 mA per gel cylinder. After electrophoresis, gels to be stained were fixed for 15 h in 20 % sulphosalicylic acid and soaked in 0.25 % Coomassie blue for 5 h. They were destained for 3 h in 7 % acetic acid in the presence of Dowex 50 W cation exchange resin. Densitometric tracings of the gels were made in an Isco recording spectrophotometer equipped with a linear transport mechanism. Gels in quartz tubes were scanned immediately after electrophoresis at 280 nm and stained gels were scanned at 640 nm.

Mol. wt. determinations were performed on 8 % acrylamide gels following the method of Shapiro et al. (1967), using the following polypeptides of known mol. wt. as references: albumin from bovine serum, albumin from hen's egg and chymotrypsinogen A from bovine pancreas. The markers were purchased from Boehringer–Mannheim (protein calibration kit Combithek). The values used for the reference mol. wt. were those given by Weber & Osborn (1969).

Labelling of DXV genome. An inoculum containing 10⁶ infectious units/ml of DXV was mixed with an equal vol. of ³²P-orthophosphate (1 mCi/ml, 200 mCi/mm; Amersham) and 0.5 μl of the mixture was injected into each fly. After 3 days' incubation at 25 °C, the flies were processed for virus purification.

Extraction of virus RNA. A purified virus suspension in LSB was adjusted to 0.05 M with EDTA. Pronase (Merck) was self digested for 30 min at 37 °C and then 10 μg/ml were added to the virus suspension. After incubation at 37 °C for 3 h the suspension was either loaded on to sucrose SDS–LiCl gradients according to the method described by Kolakofsky et al. (1974), or the RNA extracted by the phenol–SDS method (Bishop et al. 1967). In both cases, SDS was removed and RNA was concentrated by two successive precipitations in 80 % ethanol at −20 °C. The precipitate was pelleted by centrifugation for 30 min at 8000 g and dissolved in sterile LSB.

RNase digestion of virus RNA. A sample of ³²P-labelled virus RNA dissolved in LSB was divided into two aliquots. One was adjusted to 0.4 M-NaCl, the other to 0.05 M-NaCl, and both were heated at 100 °C for 3 min, then chilled in ice. Each aliquot was divided into two parts. One part received 20 μg/ml of pancreatic RNase (Merck). After incubation at 37 °C for 30 min, radioactivity precipitable by 5 % cold trichloroacetic acid in RNase-treated and control preparations was determined by liquid scintillation counting.

Analysis of virus RNA in polyacrylamide gels. Polyacrylamide gels containing 6 M-urea were prepared according to Martin & Zweerink (1972). Different concentrations of acry-
**RESULTS**

**Buoyant density of the purified virus particles**

Caesium chloride gradients of DXV gave the distribution of absorbance at 280 nm shown in Fig. 1. From the refractive index of the peak fraction, the buoyant density of DXV particles was estimated to be 1.345 g/ml.

**Virus polypeptides**

The average virus yield from an infected fly showing signs of anoxia sensitivity was 1 μg of virus protein. This is approx. 1/1000 of a fly's weight. Electrophoresis of solubilized DX virions in polyacrylamide gels revealed that this virus was composed of six major polypeptides. Fig. 2 shows the absorption profile at 640 nm of the polypeptides after...
Fig. 3. Data used for the calculation of the mol. wt. of the virus polypeptides, $P_1$ to $P_6$ (†). The standards used (●) in descending order of mol. wt. were: ovalbumin dimer, bovine serum albumin, ovalbumin, chymotrypsinogen.

Table I. Polypeptide species of Drosophila $X$ virus: their mol. wt. and distribution

<table>
<thead>
<tr>
<th>Polypeptide species</th>
<th>Mass % in virion*</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_1$</td>
<td>100,000</td>
<td>3 1 3 1 2 2</td>
</tr>
<tr>
<td>$P_2$</td>
<td>50,000</td>
<td>22 33 14 8 22 2</td>
</tr>
<tr>
<td>$P_3$</td>
<td>49,000</td>
<td>33 43 25 32 6</td>
</tr>
<tr>
<td>$P_4$</td>
<td>44,000</td>
<td>35 14 35 3 14 1</td>
</tr>
<tr>
<td>$P_5$</td>
<td>33,000</td>
<td>7 7 8 6 8 6</td>
</tr>
<tr>
<td>$P_6$</td>
<td>27,000</td>
<td></td>
</tr>
</tbody>
</table>

* Values were determined by weighing portions of absorbance tracings (average of five gels).
† From gels stained with Coomassie blue.
‡ From unstained gels scanned at 280 nm.

in a 12 % gel and staining with Coomassie brilliant blue. The six polypeptides were designated by numbers, $P_1$ for the largest to $P_6$ for the smallest. Polypeptides 2 and 3 appeared on the scanning profile as a single peak with a shoulder, while they appeared as two close but clearly distinct bands when the gels were examined visually. The same profile was obtained when an alternative purification procedure was used for the virus such as two successive sucrose gradients (10 to 30 % sucrose for 30 min at 150,000 g followed by 20 to 60 % sucrose for 2 h at 150,000 g in a SW41 Beckman rotor) instead of CsCl gradients. No protein band was visible when control uninfected flies were processed similarly.

In 12 % polyacrylamide gels, the migration of the mol. wt. marker proteins was not a linear function of log mol. wt. and therefore mol. wt. determinations were made from 8 % polyacrylamide gels in which this relation was linear. The relative electrophoretic mobilities of the virion polypeptides and of the markers are shown in Fig. 3, and Table 1 presents the estimated mol. wt. of the six virus polypeptide species.

The approximate mass percentages of the polypeptides in the virion were determined by weighing portions of densitometric tracings of gels, either stained with Coomassie blue and scanned at 640 nm or unstained and scanned at 280 nm (Table I). There is a discrepancy between the two estimations which mainly affects the species $P_5$. This may mean either that $P_5$ has a higher affinity for the stain and is therefore more basic, or that its absorbance coefficient at 280 nm is lower than that of the other polypeptide species.

Some minor proteins were also observed on heavily loaded gels but they represent less than 1 % of the total proteins of the virion. Their number and migration pattern varied, but two of them were regularly observed. Their mol. wt. were 105,000 and 75,000.
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Fig. 4. Sedimentation profile of DXV RNA. 32P-labelled virus was Pronase digested for 3 h at 37 °C and loaded on to a 5 to 20% sucrose gradient (0.1 M LiCl, 10 mM-Tris, pH 7.4, 4 mM-EDTA, 0.1% SDS). It was centrifuged for 16 h at 110000 g in a SW41 Beckman rotor. Fractions were collected from the bottom and Čerenkov radioactivity was counted directly. Chick cell ribosomal RNA was centrifuged on a parallel gradient. The superimposed positions of the cytoplasmic RNA markers are indicated by arrows. ▲ — ▲, 32P; ○ — ○, % sucrose.

Virus RNA

The percentage of RNA in the virus particles was determined in purified virus preparations by assaying the proteins according to the method of Lowry et al. (1951) and the RNA by the orcinol method (Mejbaum, 1939). The estimate of the percentage of RNA in the virion varied from 6 to 10%.

When 32P-labelled virus was prepared by injecting flies with 32P-orthophosphate, the specific activity of purified virus particles was only 800 ct/min/µg of virus protein (liquid scintillation counts). Attempts to increase this specific activity by starving the flies during virus incubation resulted in a very low yield of complete virus and a high yield of a light material sedimenting at the top of the CsCl gradients. It had the appearance of empty virus particles (in negative-contrast electron microscopy) and the same polypeptide profile as complete virions. Such material was at no time observed using the usual incubation conditions.

Following the usual SDS-phenol extraction procedure of purified 32P-labelled virus, no more than 50% of the counts were released in the aqueous phase. Incubation with Pronase prior to SDS-phenol extraction increased the amount to 95%. This method was therefore adopted.

The sedimentation profile on sucrose gradients of DXV RNA showed two peaks with approximate sedimentation coefficients of 4 to 5S and 14S (Fig. 4). Each of these RNA species was isolated and submitted to pancreatic RNase digestion in the native state and after denaturation. The reaction conditions and results are shown in Table 2. While the
Table 2. Sensitivity of virus RNA to pancreatic RNase*

<table>
<thead>
<tr>
<th>Virus RNA†</th>
<th>32P ct/min</th>
<th>% RNase resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− RNase</td>
<td>+ RNase</td>
</tr>
<tr>
<td>Native</td>
<td>2950</td>
<td>122</td>
</tr>
<tr>
<td>4-5S</td>
<td>2130</td>
<td>48</td>
</tr>
<tr>
<td>Denatured</td>
<td>8616</td>
<td>8098</td>
</tr>
<tr>
<td>Native</td>
<td>8724</td>
<td>262</td>
</tr>
<tr>
<td>14S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denatured</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 32P-labelled virus was Pronase digested and centrifuged on a 5 to 20% SDS-containing sucrose gradient (see Fig. 4). Each RNA species, 4 to 5S and 14S, was recovered from the corresponding fractions by ethanol precipitation and dissolved in LSB.

† Aliquots to be tested in the native state were adjusted to 0.4 M-NaCl. Those to be denatured were adjusted to 0.05 M-NaCl, heated at 100 °C for 3 min and chilled in ice. RNase treatment was 20 μg of pancreatic RNase/ml at 37 °C for 30 min.

4 to 5S material is RNase sensitive in its native state, the 14S material is RNase resistant and becomes RNase sensitive only after denaturation.

Analysis of DXV RNA in polyacrylamide gels was performed with different gel concentrations and times of electrophoresis. When DXV particles were incubated for 1 h in 2% SDS at 37°C for dissociation and then subjected to electrophoresis on gels containing 6% acrylamide and 6 M-urea, most of the material remained at the top of the gel. A small fraction with the electrophoretic mobility expected of a 4 to 5S RNA species moved through the gels. When the gels were loaded with Pronase-digested virions plus SDS or with purified virus RNA, the virus genome could be resolved into two close peaks of RNA in equimolar amounts (Fig. 5). In the electrophoresis conditions used to obtain this resolution, the 4 to 5S RNA species went off the gel. No further resolution was obtained by increasing the gel concentration up to 7% and the time of electrophoresis up to 70 h.

DXV RNA was co-electrophoresed with different markers to estimate the mol. wt. of the segments. Fig. 6 shows the relative electrophoretic mobilities plotted against log mol. wt. When the double-stranded RNA segments of reovirus type 3 were used as markers (Fig. 6a) the two RNA segments of DXV migrated between the L and M RNA species of this virus [mol. wt. 2.5 × 10⁶ and 1.4 × 10⁶ (Shatkin et al. 1968)] and their mol. wt. were therefore estimated to be approx. 2.2 × 10⁶.

According to Pinder et al. (1974) migration in formamide–polyacrylamide gels after melting the RNA by heating in formamide, provides analysis of RNA molecules in standard conformations. Therefore the migration of DXV RNA in formamide gels was compared with
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Fig. 6. Electrophoretic mobilities of DXV RNA (arrows) relative to different markers: rRNA species from chick cells and double-stranded RNA species from reovirus type 3. (a) DXV RNA was co-electrophoresed with that of reovirus type 3 in 6% polyacrylamide 6 M-urea gel at 2 mA for 19 h. (b) Purified DXV RNA and chick rRNA were melted in formamide and electrophoresed in 4% polyacrylamide-formamide gel at 1 mA (100 V for 4 h). The gels were scanned at 280 nm immediately after the run.

chick cell ribosomal RNA species as markers (Fig. 6b). In the electrophoresis conditions used, the two RNA segments of DXV were not resolved and they appeared in a single peak migrating a little slower than the 28S cell RNA. The virus 4 to 5S fraction was still present in the gel and its migration was slower than that of the chick cell RNA. In these conditions, the mol. wt. estimation is \(1.8 \times 10^6\) for the long genome segments and \(6 \times 10^4\) for the 4 to 5S fraction.

DISCUSSION

The results presented here demonstrate that more than 99\% of DXV proteins consist of six polypeptide species. Further experiments are necessary to determine whether all of them are primary products or whether some species are cleavage products of the others. The minor polypeptide species observed occasionally are likely to be either host products or degradation products from the major polypeptide species.

The analysis of the genome confirms that DXV is an RNA virus. The easy release of the 4 to 5S fraction from SDS-treated virions observed in polyacrylamide gel electrophoresis experiments contrasts with the need of protease digestion for releasing the rest of the virus RNA. Whether the 4 to 5S species are host products or products from the virus-associated polymerase is not known (J. Bernard & A. M. Petitjean, unpublished results). The resistance to RNase of the 14S fraction in its native state indicates that it is composed either of double-stranded RNA or highly ordered single-stranded RNA molecules.

Polyacrylamide gel electrophoresis shows that this RNA can be resolved into two peaks with very close electrophoretic mobilities and in equimolar amounts. When these RNA species are melted in formamide and co-electrophoresed with chick cell rRNA species in formamide-containing gels, the chains are devoid of secondary structure and we obtained a mol. wt. of \(1.8 \times 10^6\) if the RNA is single-stranded, or \(3.6 \times 10^6\) if it is double-stranded with no covalent link between the two complementary chains. The estimation derived from the electrophoretic mobilities of DXV RNA and of reovirus double-stranded RNA species is lower, at \(2.2 \times 10^6\). If we assume that each of the two peaks observed contains a single RNA species, the mol. wt. of the whole genome will consequently be \(3.6 \times 10^6\) if single-stranded or between \(4.4 \times 10^6\) and \(7.2 \times 10^6\) if double-stranded. If the six major polypeptides are primary products of the virus genes, their synthesis requires a coding capacity corresponding to a minimum of 9000 nucleotides or 9000 nucleotide pairs, implying mol. wt. either \(3 \times 10^6\) (ss) or \(6 \times 10^6\) (ds). In the single-strand model a unique RNA species in each
peak is sufficient for coding all six polypeptides. In the double-strand model, the adjustment is more problematic: a mol. wt. of $4.4 \times 10^6$ cannot code for all six polypeptides. In this case, each peak contains either two RNA species or only one RNA species and some polypeptides have to be cleavage products of others. If we consider the mol. wt. estimation derived from the electrophoretic mobility in formamide gels to be $7.2 \times 10^6$, then the coding capacity of a unique RNA species in each peak is sufficient for all six polypeptides.

The results presented here confirm the very strong similarity between DXV and IPN virus for which an extensive analysis has provided strong evidence that the virus genome is composed of two segments of double-stranded RNA with mol. wt. $2.5 \times 10^6$ and $2.3 \times 10^6$ (Dobos, 1976; MacDonald & Yamamoto, 1977; Dobos et al. 1977). The RNA of two other viruses: infectious bursal disease virus and Tellina tenuis virus which share identical morphological features with IPN virus, also presented the same electrophoretic properties (Nick et al. 1976; Underwood et al. 1977).

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REFERENCES


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