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Biochemistry and Ultrastructure of Iridescent Virus Type 29

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Physical and chemical parameters of iridescent virus type 29, isolated from the mealworm, *Tenebrio molitor*, have been analyzed. The icosahedral capsid is 130–135 nm in diameter and is surrounded by a fringe of coarse filaments. The virus has a buoyant density in CsCl of 1.31 g cm^{-3} and contains 20 to 25 structural proteins as analyzed by isoelectric focusing and SDS–polyacrylamide gel electrophoresis. The DNA has a buoyant density in CsCl of 1.6874 g cm^{-3} indicating a G + C content of approximately 28%. The lipid components of this virus differ from those of the host cell; the virus contains about 80% cardiolipin and 20% phosphatidyl choline.

INTRODUCTION

Cryptic viral infections are widespread in natural insect populations. In response to appropriate stimuli, such as stress, the cryptic infections may become patent (Tinsley, 1975; Tanada, 1976). Little is known of the process itself or of the mechanisms controlling the transformation of cryptic infections into patent diseases.

Iridescent virus type 29, classified according to the scheme of Tinsley and Kelly (1970), was recently isolated from a colony of mealworms, *Tenebrio molitor*, at Colorado State University. Infections of this virus are rarely encountered in mealworm larvae but a bluish iridescence, characteristic of serious disease, is often seen in the pupae and adults. A similar developmental shift toward increased susceptibility to infection with maturation has been reported for another iridescent virus in *Tipula paludosa* (Carter, 1974). The present paper lays the basis for the future experimental analysis by describing certain properties of the virus itself.

As previously reported (Kelly et al., 1979), both gel electrophoresis and immunochemical data show that iridescent virus type 29 is distinct from previously isolated iridescent viruses. Immunodiffusion, complement fixation, and kinetic neutralization suggest that of the other known iridescent viruses, type 23 (from *Heteronychus arator*) is most closely related to type 29 (from *T. molitor*) (Kelly et al., 1979).

METHODS

Virus purification. Iridescent virus type 29 was isolated by a modification of the techniques of Matta (1970). Infected pupae of *Tenebrio molitor* were homogenized in 0.01 M phosphate buffer, *p*H 7.3, with a Dounce tissue grinder. The liquid was decanted off and subjected to two cycles of slow (5 min, 1500*g*)- and moderate (25 min, 30,000*g*)-speed centrifugation. The resulting blue pellet was resuspended and 0.4-ml fractions were applied to a phosphate-buffered (0.01 M, *p*H 7.3) sucrose gradient (6–54%, continuous) which was spun at 20,000*g* in a SW60 Ti rotor (Beckman L5-65 ultracentrifuge) for 20 min. Absorbance of successive 0.4-ml fractions was read at 260/280 nm in a Gilford Model 252 spectrophotometer, the three fractions with high optical density were pooled, and the virus particles were pelleted (50,000*g*, 30 min). The resulting preparation appeared homogeneous when viewed by electron micros-

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copy and as such contained no detectable contaminants.

CsCl density gradient centrifugation. Samples of iridescent virus type 29 were applied to a CsCl cushion (mean density, 1.30 g cm^{-3}) and centrifuged at 100,000g for 60 hr in a SW60 Ti rotor (Beckman L5-65 ultracentrifuge) to assess homogeneity and to determine buoyant density. The CsCl density of each fraction (0.2 ml) was read in an Abbe-3L refractometer (Bausch and Lomb) and the absorbance at 260/280 nm of each fraction was determined in a Gilford Model 252 spectrophotometer.

Sepharose CL 2B chromatography. The homogeneity of the virus preparation was tested by running samples of iridescent virus type 29 through a 25 × 1-cm Sepharose CL 2B column (Pharmacia) in 0.01 M phosphate buffer, pH 7.3. One-milliliter fractions were collected and absorbance read at 260 nm with an LKB 8300 Uvicord II UV analyzer.

Virus infectivity. The virus preparation contained particles which were infective to pupae of *T. molitor* and cells of *Spodoptera frugiperda* (IPLB-SF-21AE) (Vaughn et al., 1977). Pupae of *T. molitor* (from a virus-free colony, 1 to 3 days postecdysis) were injected between the fourth and fifth abdominal sternites with 1 μl suspension of iridescent virus type 29 in phosphate-buffered saline. Control animals were injected with phosphate-buffered saline. To prevent infection penicillin:streptomycin (1:1) was topically applied to the abdomen. All animals were incubated at 25°C for 6 to 7 days. The plaque assay used was a modification of Brown et al. (1978).

Electron microscopy. Infected tissue from adults and pupae of *T. molitor* were fixed in glutaraldehyde and osmium tetroxide, Epon embedded, and sectioned by the techniques described in Happ and Happ (1977). Assessment of virus preparation homogeneity was accomplished by staining virus particles from the sucrose gradient on Formvar v coated grids with 4% phosphotungstic acid, pH 6.0. Samples were viewed and photographed in an RCA EMA 3E or Philips 200 electron microscope.

Analysis of viral proteins. The proteins from iridescent virus type 29 were analyzed by one- and two-dimensional polyacrylamide gel electrophoresis. Three volumes of virus particles (protein concentration 5 μg/μl) were added to 5 vol SDS mix [4% (w/v) SDS; 20% (v/v) glycerol; 0.02% (w/v) bromophenol blue], 1 vol sample buffer (0.625 M Tris–HCl, pH 6.8; 0.01 M Na_2 EDTA), 1 vol β-mercaptoethanol, and heated at 100°C for 5 min. The sample (60 to 120 μg protein) was applied to 12% SDS–polyacrylamide slab gels (Laemmli, 1970) and run 3 to 4 hr at 100 V. Molecular weight standards were obtained from Sigma Chemical Company. Viral proteins were separated on isoelectric gels using broad range ampholytes (3-10 LKB) according to the procedure described by O'Farrell (1975). The proteins separated by isoelectric focusing were subjected to a second dimension SDS–polyacrylamide slab gel as described by Ames and Nikaido (1976). All protein determinations were made according to the method of Lowry et al. (1951).

Isolation and analysis of viral DNA. The DNA of iridescent virus type 29 was isolated by a modification of the technique of Marmur (1961). The virus preparation (15 to 20 mg total protein) was suspended in ten vol of TSE [0.01 M Tris–HCl (pH 8.0), 0.15 M NaCl, 0.01 M EDTA]; SDS was added to give a final concentration of 0.5%. An equal volume of phenol:TSE (7:3) was added, the aqueous phase was separated, and the phenol layer was reextracted with TSE. The aqueous layers were combined and extracted three times with equal volumes of phenol:chloroform: isoamyl alcohol (1:1:0.04). The DNA was spooled on cold glass rods following addition of 2 vol of cold (−20°) 95% EtOH. DNA was then dissolved in 5 vol of 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate); 0.1 vol 3.0 M sodium acetate, 0.001 M EDTA (pH 7.0) was added. DNA was precipitated at room temperature by adding 0.6 vol of isopropanol dropwise

with gentle stirring. The DNA pellet was dissolved in 0.1× SSC and treated with preincubated Pronase (30 μg/ml final concentration) for 2 hr at 37°C (Spelsberg and Hnilica, 1971). After digestion, the solution was extracted two times with equal volumes phenol:chloroform (1:1) and the DNA spooled onto glass rods. Final DNA was dissolved in 0.1× SSC and stored at −20°C.

Equilibrium buoyant density determinations were made by applying 5 μg of iridescent virus type 29 DNA and 3 μg of calf thymus DNA (standard, ρ = 1.700 g cm^{-3}) in 230 μl of 0.1× SSC to a CsCl cushion (mean density 1.60 g cm^{-3}) in the Beckman Model E analytical ultracentrifuge (Schildkraut et al., 1962).

Analysis of phospholipids. Lyophilized virus particles and homogenates of virus-free insects were extracted three times with chloroform:methanol (2:1, v/v) at 50°C for 2 hr and the combined extracts evaporated to dryness under a stream of nitrogen. Lipids were subjected to a Folch wash (Folch et al., 1957) and aliquots of the lower chloroform phases were chromatographed on silica gel H (Merck) in chloroform:methanol:water (65:25:4). Standards were obtained from Sigma Chemical Company. Plates were sprayed with the molybdenum blue reagent of Dittmer and Lester (1964) to locate phospholipids. Parallel plates were exposed to iodine vapor, the spots excised, and assayed for total phosphorus by micromodification of the method of Lowry et al. (1954) and Keleti and Lederer (1974).

RESULTS

Virus Purification

Large quantities of iridescent virus type 29 could be isolated from relatively few pupae of *T. molitor,* e.g., each iridescent pupa, 110−140 mg fresh weight, typically yielded 275−325 μg of virus. In a typical isolation, the final virus preparation contained no detectable contaminants when analyzed by electron microscopy (Fig. 2), CsCl density gradient centrifugation, and Sepharose CL 2B chromatography (Fig. 1).

The virus preparation was biologically active; samples produced infection when injected into pupae and adults of *T. molitor* and produced distinct foci of infection in cells of *S. frugiperda*. The ID_{50} at 6 to 7 days for pupae of *T. molitor* was ca. 100 pg virus.

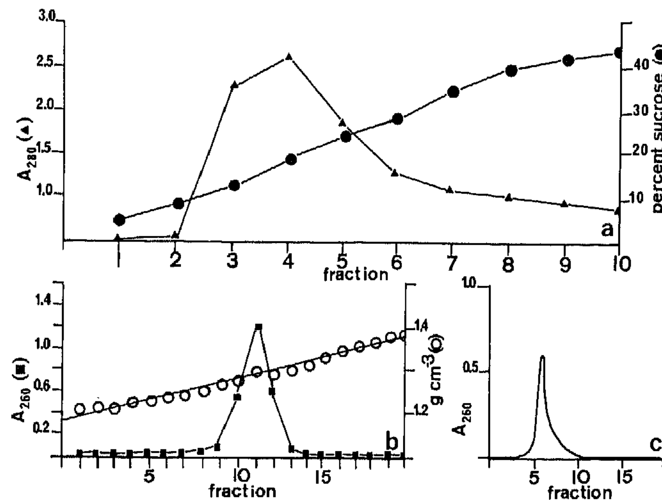


FIG. 1. Samples of iridescent virus type 29 applied to a phosphate-buffered sucrose gradient (a); fractions 3, 4, and 5 combined and analyzed by (b) CsCl density gradient centrifugation, and (c) Sepharose CL 2B chromatography.

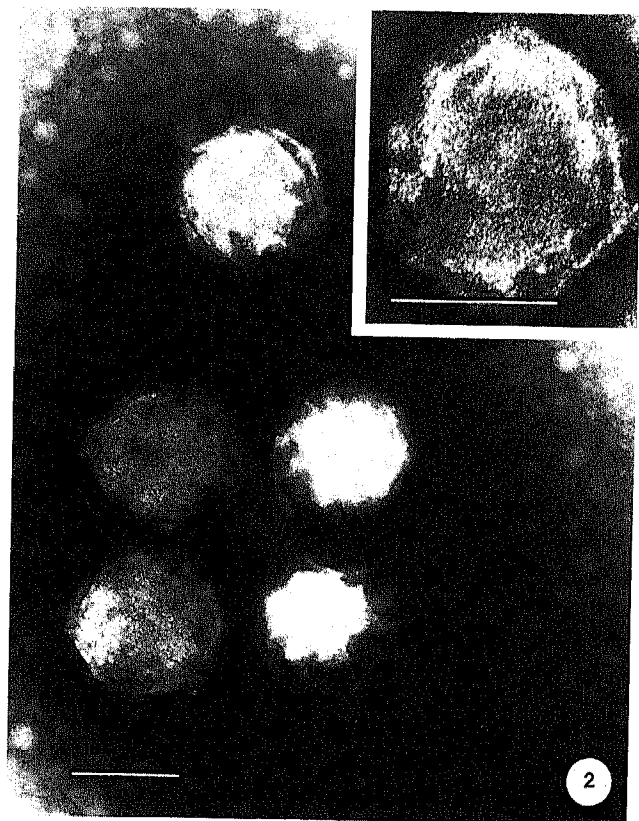


FIG. 2. Negatively stained particles of iridescent virus type 29 from sucrose gradient fractions 3, 4, and 5. Inset shows tertiary structure of virus particle (bars = 0.05 μm).

Viral Particles

Heavily infected cells contained an ordered array of virus particles (Fig. 3) each 130–135 nm in diameter spaced 180–200 nm center to center. The cytoplasmic viral assembly areas contained particles which appeared to be in various stages of completion. Associated with these assembly areas were degenerating cellular organelles, including short microtubules, profiles of rough and smooth endoplasmic reticulum, and ribosomes (Fig. 4). The filamentous fringe which surrounds each capsid (Fig. 5) may be responsible for the regular spacing. Individual particles were isometric and usually hexagonal in cross section (Figs. 3–7). In favorable sections, one or more pairs of electron-dense lines separated by a lucent space surrounded the capsid (Figs. 6, 7).

Isopycnic centrifugation in CsCl of isolated virus particles gave a single major peak at a buoyant density in CsCl of 1.31 g cm^{-3} (Fig. 1b).

Viral Proteins

One-dimensional gel electrophoresis of proteins from dissociated virus particles on SDS–slab gels or on isoelectric focusing gels separated 20–25 bands. The molecular weights of the proteins ranged from 15,000 to 72,000, and their isoelectric points were between 4.5 and 7.3. Many of the major proteins were detected on two-dimensional gels and thus their charges and molecular weights were correlated (Fig. 8). The pre-

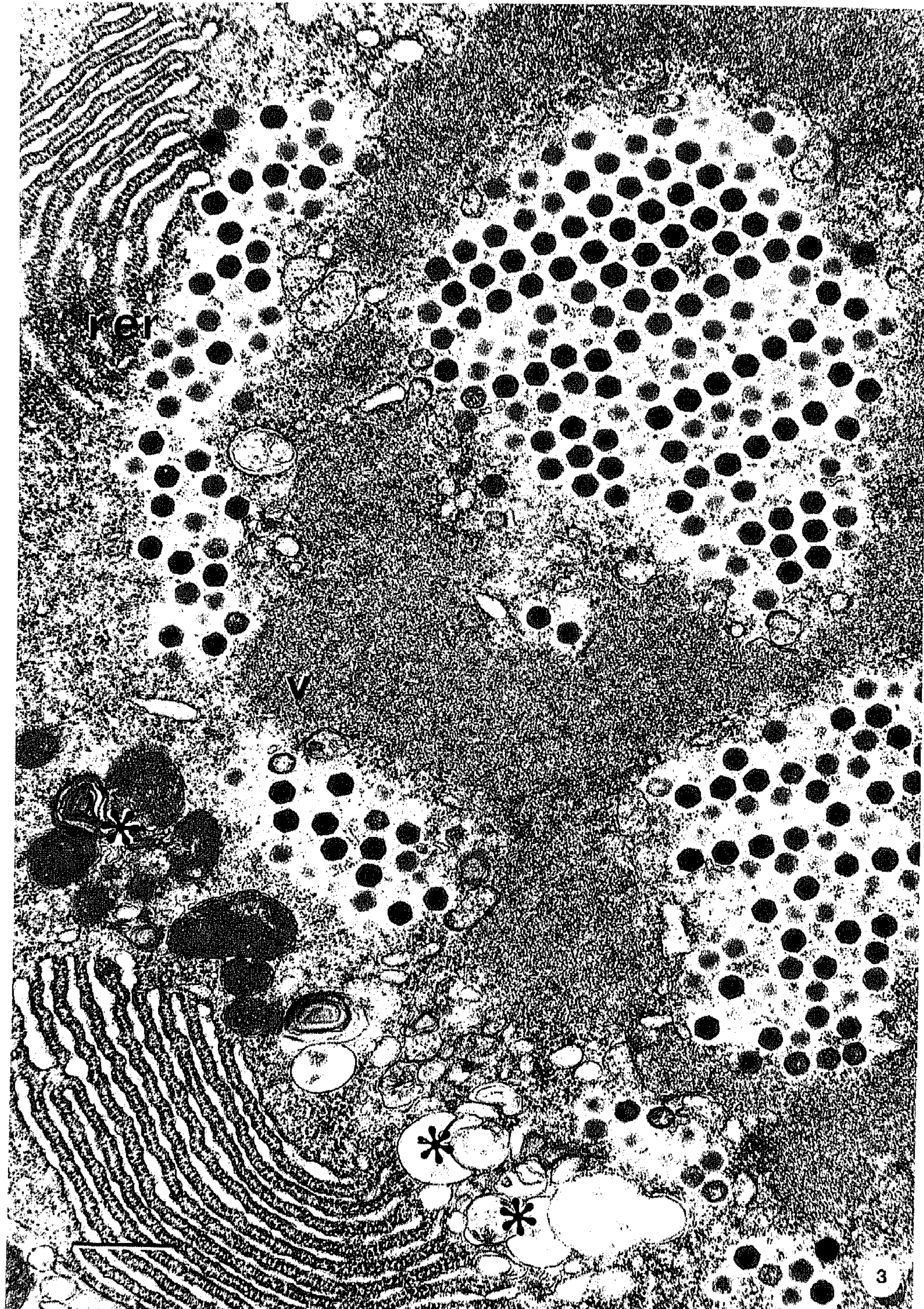
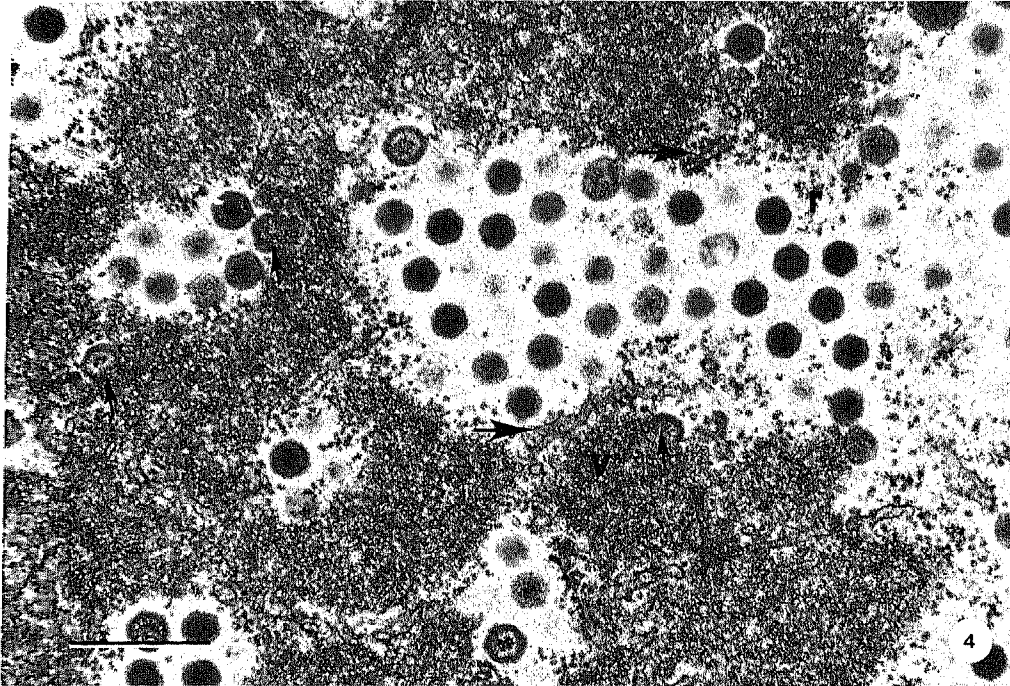
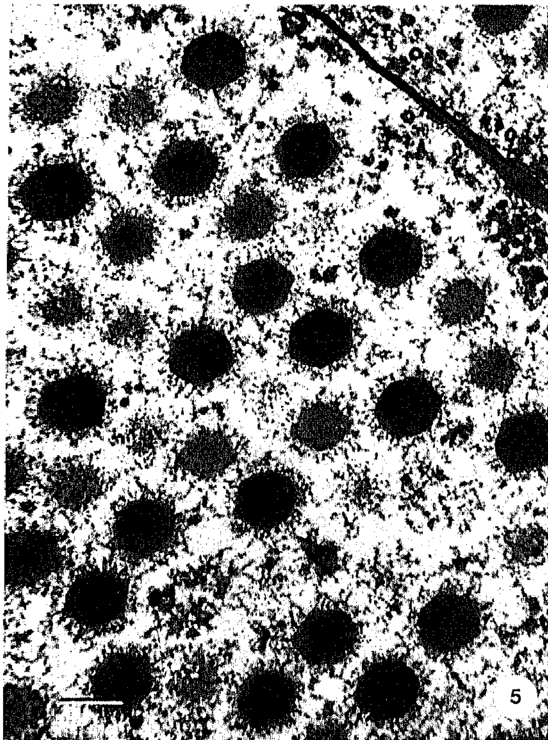
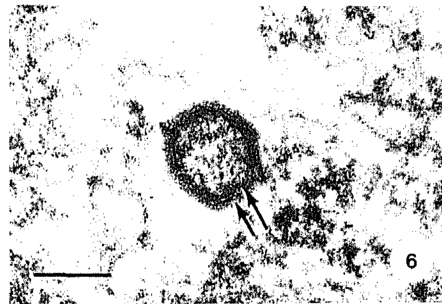


FIG. 3. A hemocyte of *Tenebrio molitor* infected with iridescent virus type 29. Although some rough endoplasmic reticulum (rer) persists, many unattached ribosomes (r) are clustered together. The viral assembly area (v) contains zones of ordered hexagonal virus profiles. Asterisks indicate degenerating mitochondria and cytoplasmic membranes (bar = 0.5 μm).







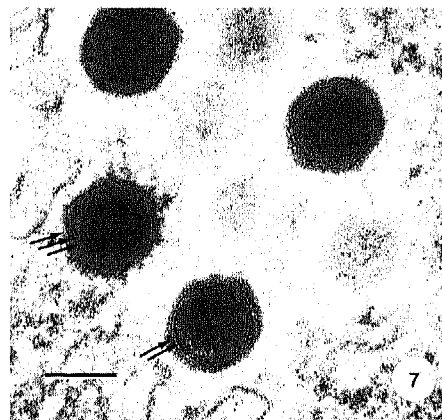


FIG. 4. A viral assembly area with hexagonal particles of varying core densities and with several apparently incomplete viral shells, indicated by the small vertical arrows. Ribosomes (r) and cytoplasmic membranes (large horizontal arrows) are also found in this assembly area (bar = 0.5 μm).

FIG. 5. Each viral particle is surrounded by a filamentous fringe (bar = 0.1 μm).

FIG. 6. A viral shell with electron-dense lines (arrows) in its walls (bar = 0.1 μm).

FIG. 7. Viral particles with electron-dense lines in their walls (bar = 0.1 μm).

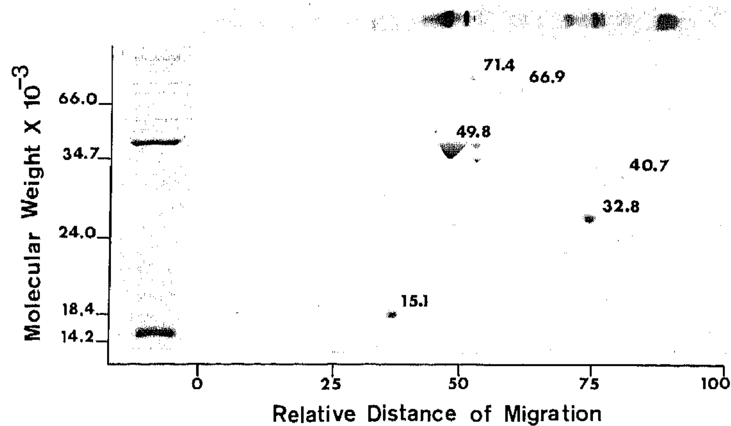


FIG. 8. Iridescent virus type 29 protein, separated on the basis of molecular weight (vertical dimension, SDS–polyacrylamide gel) and isoelectric point (horizontal direction); *p*H gradient from 8.6 (left) to 3.5 (right).

dominant protein had a molecular weight of 49,800 and an isoelectric point of 6.6; also prominent proteins were at MW 71,400, p*I* 6.5; MW 66,900, p*I* 5.7; MW 40,700, p*I* 4.5; MW 32,800, p*I* 5.4; and MW 15,100 p*I* 7.3.

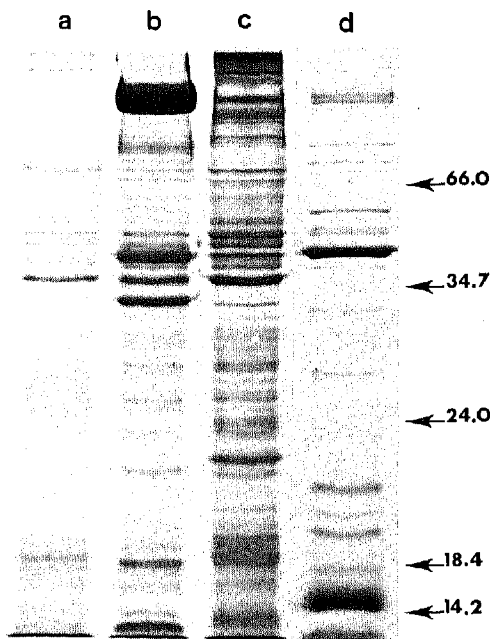


FIG. 9. SDS–polyacrylamide slab gel of iridescent virus type 29 proteins (d) and host (*Tenebrio molitor*) proteins: tubular accessory gland (a), ovaries (b), and bean-shaped accessory gland (c). Arrows indicate molecular weight standards ($\times 10^{-3}$).

The strong band at MW 52,400 was not well defined on the two dimensional gels. The viral proteins appear to be distinct from major host proteins (from ovaries, bean-shaped accessory glands, and tubular accessory glands) as shown in Figure 9.

Viral DNA

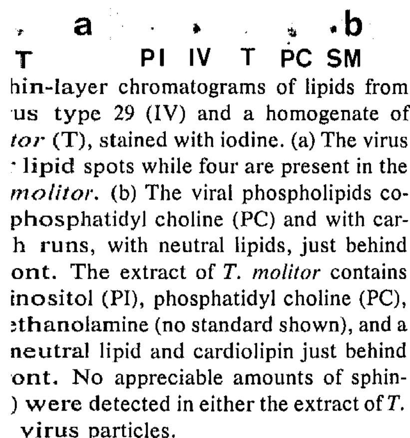
The buoyant density of the isolated DNA was determined on CsCl gradients as 1.6874 ± 0.006 g cm^{-3}. Guanine–cytosine content was calculated as 29.4% (Rolfe and Meselson, 1959) or 27.9% (Schildkraut et al., 1962).

Viral Lipids

Lyophilized virus particles contained significant quantities of two phospholipids: cardiolipin and phosphatidyl choline (Fig. 10). Their relative concentrations are shown in Table 1. The proportions of phospholipids in the host insect had a distribution more typical of animal cells (Table 1).

DISCUSSION

The size of iridescent virus type 29, 130–135 nm, places it in the class of small insect iridescent viruses (Kelly and Robertson, 1973). A quite similar (perhaps identical?) iridescent virus has recently been isolated from *T. molitor* in France (Thomas and Gouranton, 1978). Our data



hin-layer chromatograms of lipids from us type 29 (IV) and a homogenate of *tor* (T), stained with iodine. (a) The virus · lipid spots while four are present in the *molitor*. (b) The viral phospholipids co-phosphatidyl choline (PC) and with car-h runs, with neutral lipids, just behind ont. The extract of *T. molitor* contains inositol (PI), phosphatidyl choline (PC), ethanolamine (no standard shown), and a neutral lipid and cardiolipin just behind ont. No appreciable amounts of sphin-) were detected in either the extract of *T.* virus particles.

TABLE 1
QUANTITATIVE PHOSPHOLIPID DATA FOR IV29 AND FOR *Tenebrio molitor*

Lipids	Molar %	
	T. molitor	Virus
Phosphatidyl inositol and sphingomyelin	11.1	Trace
Phosphatidyl choline	47.1	21.2
Phosphatidyl ethanolamine	29.7	—
Cardiolipin	12.1	78.8

he buoyant density of iridescent !9 is like that reported for irides-:ype 1 (1.32 g cm^{-3}) and type 9 $^{-3}$) (Kelly and Robertson, 1973) lower than the value of 1.354 g ted for iridescent virus type 3 0).

ranging in molecular weights ,000 and 100,000 with a major ;0,000–52,000 are characteristic of insect iridescent viruses, including type 29 (Kelly and Tinsley, 1972; Wagner et al., 1974; Carey et al., 1978). High-molecular-weight proteins (>100,000) were not observed in iridescent virus type 29 although they have been reported in other iridescent viruses (Kelly and Tinsley, 1972). Our data on the isoelectric points of iridescent virus type 29 proteins are the first to be reported from iridescent viruses.

The filamentous fringe surrounding each iridescent virus type 29 capsid is probably analogous to that reported in other icosahedral cytoplasmic deoxyriboviruses (Zwillenberg and Wolf, 1968; Stoltz, 1971). Kelly and Robertson (1973) have suggested that the fibers might "cause the interplanar spacings in crystals of these viruses." To our knowledge, the present study provides the first published micrographs of such a fringe in an array of insect iridescent virus particles as they become ordered in the host cell.

Buoyant density in CsCl of the DNA of iridescent virus type 29, 1.6?74 g cm^{-3}, is very close to those of iridescent virus types 1, 2, and 6 (Bellet and Inman, 1967; Kelly and Avery, 1974) and unlike that of iridescent virus type 9 (1.702 g cm^{-3}) (Kelly and Avery, 1974). Similarity among buoyant densities of the DNA of iridescent viruses does not reflect close relationships since serological analyses show only a mod-

erate relationship between type 29 and types 2, 6, or 9 (Kelly et al., 1979).

Despite the lack of a membranous envelope derived from host cell membranes, phospholipids have been reported in iridescent virus types 2 and 6 (Kelly and Vance, 1973). In their electron micrographs of iridescent virus type 2, Kelly and Vance (1973) show internal dense lines suggestive of lipoid membranes within the protein shell of the capsid. We also find laminate staining, perhaps reflecting layers in the wall of the capsid of iridescent virus type 29, but our micrographs do not show a consistent pattern of staining and thus we do not feel confident in equating "electron opacity" with an internal lipid membrane.

Similarities in ultrastructure and patterns of synthesis have been drawn between iridescent viruses and the bacteriophage PM2 (Franklin, 1976). Both iridescent virus type 29 and PM2 contain only two major phospholipids. Almost 90% of the phospholipids of iridescent virus type 29 is cardiolipin (or diphosphatidyl glycerol) while almost 70% of the phospholipid of PM2 is phosphatidyl glycerol, a precursor of cardiolipin. The second major phospholipid or iridescent virus type 29 and PM2 is phosphatidyl choline and phosphatidyl ethanolamine, respectively. Cardiolipin is present in trace amounts (1%) in PM2.

The ratios of phospholipids of iridescent virus type 29 are quite different from the ratios found in pupae of *T. molitor* (Table 1). The high amount of cardiolipin (80%) in these virus particles is especially interesting since this particular lipid is mostly found in the inner mitochondrial membranes of eukaryotes (Comte et al., 1976) and in prokaryotes. It may be that the assembling capsids of iridescent virus type 29 sequester lipids from degenerating mitochondria such as are seen near the assembly areas (Fig. 3). Association between viral replication sites and degenerating mitochondria has also been reported for frog virus 3, another icosahedral cytoplasmic deoxyribovirus (McAuslan and Smith, 1968).

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