Structure and Chemical-Physical Characteristics of Lactate Dehydrogenase-Elevating Virus and Its RNA

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Lactate dehydrogenase-elevating virus (LDV) was purified from culture fluid of infected primary cultures of various mouse tissues (peritoneal macrophage, bone marrow, spleen, and embryo) and from plasma of infected mice. Electron microscopy of negatively stained virus and positively stained sections of LDV revealed spherical particles of uniform size with a diameter of about 55 nm, containing an electron-dense core with a diameter of about 30 nm. During sample preparation the envelope had a tendency to slough off and disintegrate to form aggregates of various sizes and small hollow particles with a diameter of 8 to 14 nm. Two strains of LDV exhibited a density of 1.13 g/cm³ in isopycnic sucrose density gradient centrifugation whether propagated in primary cultures of the various mouse tissues or isolated from plasma of infected mice. A brief incubation of LDV in a solution containing 0.01% Nonidet P-40 or Triton X was sufficient to release the viral nucleocapsid, whereas a similar treatment had no effect on Sindbis virus. The nucleocapsid of LDV exhibited a density of 1.17 g/cm³, was devoid of phosphatidylcholine, and contained only the smallest of the viral proteins, VP-1, which had a molecular weight of about 15,000. The envelope contained two proteins, VP-2 with a molecular weight of 18,000 and a glycoprotein, VP-3, which migrated heterogeneously (24,000 to 44,000 daltons) during polyacrylamide gel electrophoresis. When compared to the sedimentation rate of 29S rRNA, the RNAs of LDV and Sindbis virus sedimented at 48 and 45S, respectively, whether analyzed by zone sedimentation in sucrose density gradients containing low or high salt concentrations or denatured by treatment with formaldehyde. Our results indicate that LDV should be classified as a togavirus, but that LDV is sufficiently different from alpha and flaviviruses to be excluded from these groups.

The lactate dehydrogenase-elevating virus (LDV) is a lipoprotein-enveloped virus of intermediate size (see reference 27) containing a genome of single-stranded RNA with a mol wt of about 5×10^6 as estimated by zone sedimentation in sucrose density gradients (9). Although LDV resembles the togaviruses in certain of its characteristics, it has not yet been classified because of various uncertainties and controversies about its structure and physical-chemical properties. The present studies were designed to obtain further information about these properties, particularly with respect to the structure, stability, and density of the virions and their cores, the nature of the viral structural proteins associated with the core and the viral envelope, and the molecular weight and secondary structure of the viral RNA. A preliminary report of some of these results has been presented (M. B. Darnell, J. K. Collins, and P. G. W. Plagemann, *Annu. Meet. Am. Soc. Microbiol.* 1974, V121, p. 220).

MATERIALS AND METHODS

Materials. Materials were purchased as follows: [5-³H]uridine (26 Ci/mmol) and [³H]leucine (55 Ci/mmol) from Schwarz/Mann; D-[³H]mannose (611 mCi/mmol) from Amersham/Searle; D-[³H]glucosamine (18 Ci/mmol) and [methyl-³H]choline (20 Ci/mmol) from New England Nuclear Corp.; [carboxyl-¹⁴C]deoxycholate (3 mCi/mmol) from ICN; Nonidet P-40 (NP-40) from Shell Chemical Co. Buffer 12 (B12) was composed of 0.1 N NaCl, 0.05% (vol/vol) mercaptoethanol, 10 mM Tris-chloride (pH 7.4), and 1 mM EDTA. Buffer 6 (B6) was composed of 50 mM NaCl, 10 mM EDTA, 10 mM Tris-chloride (pH 7.4), and 0.5% (wt/vol) sodium dodecyl sulfate (SDS).

Cells and cell culture. Primary mouse peritoneal macrophage cultures were prepared as reported previously (5a). Briefly, macrophages were stimulated in adult Swiss mice by an intraperitoneal injection of a 1% (wt/vol) starch solution and were grown in medium 58B (9, 35) in 25-cm² plastic Falcon flasks.

Bone marrow cultures were prepared using mouse femurs, which had been dissected free of muscle and cut at the joints. The marrow was forced out with medium 58B, and cells were dispersed by pipetting

and seeded immediately. Spleen and mouse embryo cell cultures were prepared as described previously (35, 36). Spleen cells were grown in medium 58B, and mouse embryo cells were propagated in Eagle minimal medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics.

Viruses and preparation of unlabeled and radioactively labeled virions. Our strain of LDV was obtained in 1971 from the plasma of a C3H mouse carrying a transplantable tumor. Another strain of LDV was kindly supplied by A. L. Notkins. Pools of LDV were prepared as follows. Plasma was harvested from 24-h LDV-infected mice and diluted fivefold with medium 58B, filtered through a membrane filter (MA type, 450-nm pore diameter, Millipore Corp.), and stored at -80°C . The final suspensions contained approximately 10^9 median infectious units (ID_{50}) per ml. Virus titers were estimated by titration in mice as described previously (33).

West Nile virus (strain E 101) and Sindbis virus (strain SAAR 339) were propagated in brains of newborn hamsters as described previously (8). Brain pools of West Nile virus and Sindbis virus contained 10^8 to 10^9 and 10^{10} to 10^{11} PFU/ml, respectively, and were stored at -80°C . Plaque assays were conducted in LLC-MK2 cells and chicken embryo fibroblasts, respectively (8).

The various primary cultures of mouse tissues were infected with 20 to 50 ID_{50} of LDV or 30 PFU of Sindbis virus per cell 2 to 24 h after planting into 25-cm² Falcon flasks as described previously (5a, 8). LLC-MK2 cell monolayer cultures were infected in the same manner with 20 PFU of West Nile virus per cell. After 1-h periods of virus adsorption, the residual virus inocula were poured off and replaced with 5 ml of medium 58B in which the concentration of fetal calf serum was reduced to 2%. The medium was supplemented with 30 μCi of [³H]uridine, 25 μCi of [³H]leucine, 30 μCi of [³H]mannose, 10 μCi of [³H]glucosamine, or 10 μCi of [³H]choline per ml. In the case of choline, mannose, and glucosamine labeling, the cells were prelabeled for 3 h before infection. The medium containing the isotope was removed during virus adsorption and then added back to the cells. [³H]uridine and leucine were added to the medium 1 to 3 h after infection. When labeling with leucine, the normal concentration of unlabeled leucine in the medium was reduced 90%. The culture fluids were harvested 24 h after infection, clarified by sequential centrifugation at $500 \times g$ for 5 min and $15,000 \times g$ for 20 min, and then immediately layered in 5- to 8-ml amounts onto linear 0.5 to 1.5 M sucrose gradients made in B12. The gradients were centrifuged in an L-3 Beckman ultracentrifuge in an SW27 rotor at 22,000 rpm at 4°C for 14 h. One-milliliter fractions were collected with an ISCO gradient fractionator (model 183), which was attached to a continuously recording spectrophotometer and a fraction collector. Aliquots of each fraction or whole fractions were analyzed for radioactivity in acid-insoluble material (31) and/or infectivity. The density of gradient fractions kept at 4°C was measured directly by weighing 100- μl aliquots. For additional analyses (see below) fractions containing virus or viral

components were pooled and mixed with 2.5 volumes of ethanol at -20°C and small amounts of total cellular RNA from Novikoff rat hepatoma cells as co-precipitant. The precipitated material was collected by centrifugation after 24 to 48 h at -20°C .

LDV was also labeled *in vivo*. Mice were injected intraperitoneally with 500 μCi of [5-³H]uridine 4 h after an intraperitoneal injection on unlabeled LDV ($10^6 \text{ID}_{50}/\text{mouse}$), and their blood was collected by the retro-orbital bleeding technique 24 h after infection. The plasma from four mice was pooled, diluted fivefold with B12, and then clarified; LDV was isolated by isopycnic centrifugation on 0.5 to 1.5 M sucrose gradients as described above.

Isolation of viral RNAs and analysis by velocity sedimentation. RNA was isolated from virions or viral components by extraction with phenol-SDS-chloroform by the method of Perry et al. (29) or by deproteinization by incubation at 37°C for 5 min with vigorous mixing in B6 containing 2% (wt/vol) SDS. Samples of RNA were centrifuged in linear 0.15 to 0.9 M sucrose density gradients in an SW27 rotor at 20°C for 10 h. The salt composition of the gradient solutions varied and is indicated in the appropriate experiments. One-milliliter fractions were collected from the gradients and analyzed for radioactivity in acid-insoluble material as already described. Sedimentation rates of RNA were estimated by the method of Martin and Ames (20) using hot phenol-extracted 29 and 18S rRNA from N1S1-67 cells as standards (32).

Isolation of viral proteins and separation by gel electrophoresis. Samples of protein-labeled virus or viral components were supplemented with 1% (wt/vol) SDS, 1% (vol/vol) mercaptoethanol, and 0.5 M urea and incubated at 37°C for 1 h. The samples were concentrated to about 0.5 ml by vacuum dialysis against a solution composed of 10 mM sodium phosphate (pH 7.4), 0.1% (wt/vol) SDS, and 0.1% (vol/vol) mercaptoethanol. Samples of 0.2 ml of the concentrated solution were supplemented with 0.04 ml of a 60% (wt/vol) solution of sucrose and 5 μl of a 0.1% (wt/vol) solution of bromophenol blue. The proteins were separated by electrophoresis in 7.5% (wt/vol) SDS-polyacrylamide gels containing 0.2% (wt/vol) *N*, *N'*-methylene-bis-acrylamide as described by Maizel (19). Electrophoresis was in 20-cm glass tubes at 100 V for 8 h. The gels were sliced into 1-mm slices, and these were allowed to swell in 1 ml of water for 1 h at room temperature. Then the slices and eluates together were analyzed for radioactivity.

Radioactivity determination. Radioactivity was measured by liquid scintillation counting. All samples were mixed with 8 ml of a modified Bray solution described previously (34).

Electron microscopy. LDV samples prepared as described in the appropriate experiments were allowed to adsorb to charged copper grids covered with a Parlodian film. The excess liquid was syphoned off with a piece of filter paper, and a drop of 1% (wt/vol) phosphotungstic acid (pH 7.0) was put onto the grid. In some instances virus adsorbed to grids was fixed with 2% glutaraldehyde (pH 7.2) for 5 min and then rinsed twice with distilled water before staining with phosphotungstic acid. Pelleted LDV was prepared for

thin sectioning by sequential fixation with 2% (vol/vol) glutaraldehyde and 1% (wt/vol) osmium tetroxide in 0.1 M phosphate buffer (pH 7.2), followed by staining with 2% (wt/vol) uranyl acetate, dehydration in ethanol, and embedding in an epoxy resin mixture (Epon 812) according to the method of Luft (18). Sections were cut with a diamond knife in an LKB Ultratome and then mounted on copper grids covered with Parlodian film. The sections were stained with uranyl acetate (2%, wt/vol) and Reynold lead citrate. All preparations were examined with a Siemens Elmiskop electron microscope.

RESULTS

Density of LDV virions. Results from previous studies (9) indicated that LDV propagated in primary mouse macrophage cultures has an unusually low density for an enveloped animal virus (1.12 to 1.13 g/cm³). A similar value (1.14 g/cm³) has been reported for macrophage-propagated LDV by Michaelides and Schlesinger (21), whereas a density of 1.17 g/cm³ was reported for LDV propagated in mouse embryo cultures (25) or isolated from the plasma of infected mice (37). These different results suggested the possibility that the density of LDV might vary with the type of host cell and reflect differences in the lipid composition of the host membrane. Another possibility was that the differing results were due to the use of different strains of LDV. Our results negate both possi-

bilities. We have propagated LDV in primary cultures of mouse peritoneal macrophages, and of mouse bone marrow, spleen, and embryo cells, and have also isolated LDV from the plasma of infected mice. In each case LDV banded at a density of about 1.13 g/cm³ during isopycnic centrifugation in sucrose density gradients. Virus was located in the gradients by infectivity titrations or a radioactive label in the viral RNA or by both methods. Representative gradient profiles of LDV propagated in mouse embryo cultures and peritoneal macrophage cultures and of virus isolated from mouse plasma are illustrated in Fig. 1. Furthermore, a second strain of LDV, kindly supplied by A. L. Notkins, exhibited the same density when propagated in peritoneal macrophage cultures and analyzed in the same manner (Fig. 1B).

Detergent and proteinase treatment of LDV. Studies by Michaelides and Schlesinger (21) have shown that treatment of LDV with 0.2% NP-40 at room temperature for 1 h released two of the viral proteins in a soluble form. A similar treatment has been used to isolate the nucleocapsid of alphaviruses (13), but in the case of LDV the physical properties of the particles remaining after NP-40 treatment had not been studied in detail. The following results demonstrate that LDV is much more sensitive to detergent treatment than alpha

FIG. 1. Isopycnic sucrose density gradient centrifugation of two strains of LDV propagated in primary cultures of mouse embryo cells (MEC [A]), and macrophages (B), and isolated from plasma (C). The viruses were propagated in cell cultures and in mice in the presence of [³H]uridine as described in Materials and Methods. Samples of 6 ml of culture fluid or fivefold-diluted plasma were centrifuged through linear 0.5 to 1.5 M gradients of sucrose in B12 in an SW27 rotor at 22,000 rpm at 4 C for 14 h. Aliquots of the gradient fractions were analyzed for density, infectivity, and radioactivity in acid-insoluble material. Frame B is a composite of the gradient profiles of culture fluid from cells infected with our strain of LDV (A) or that of Notkins (B).

togaviruses. The results in Fig. 2A illustrate that a 10-min incubation of uridine-labeled LDV at 4 C in a solution containing as little as 0.01% (vol/vol) NP-40 completely converted the virions to particles which equilibrated at a density of about 1.17 g/cm³, with a concomitant loss of 99.9% of the infectivity. Incubation with 0.005% NP-40 had no effect on the density of the virions, but treatment with 0.1 or 0.2% NP-40 had the same effect as incubation with 0.01% (data not shown). Zone sedimentation of the RNA extracted from NP-40-treated particles by treatment with SDS demonstrated that this RNA was intact 48S viral RNA (Fig. 2B). The RNA within these particles, however, was completely degraded after a brief exposure of the particles to RNase, whereas the RNA within mature virions was resistant to such treatment (Fig. 2D). Incubation of LDV in solutions containing 0.01% (vol/vol) Triton X (Fig. 2E) or 0.05% (wt/vol) deoxycholate (data not shown) at 4 C for 10 min also resulted in its conversion to a particle with an approximate density of 1.17 g/cm³. In contrast, incubation of Sindbis virus in solutions containing these low concentrations of detergents did not affect its density (1.175 g/cm³; Fig. 2C). Additional studies have shown that a concentration of at least 0.1% NP-40 was required to quantitatively convert Sindbis virus to free nucleocapsids which had a density of 1.23 g/cm³ (data not shown).

In another experiment we incubated partially purified, uridine-labeled LDV in a solution containing 0.05% (wt/vol) [¹⁴C]deoxycholate at 4 C for 10 min and then analyzed the mixture by isopycnic centrifugation in a sucrose density gradient. No significant binding of labeled deoxycholate to the 1.17-g/cm³ particles could be detected. Less than 0.2% of the total radioactivity (70,000 counts/min) was recovered in the lower part of the gradient (see Fig. 2A); the remainder was present in the upper 8 ml of the gradient (data not shown).

Incubation of LDV in solutions containing 2.5 mg of trypsin per ml at 22 C for 30 min (Fig. 2E) or 1 mg of bromelain per ml at 22 C for 60 min (data not shown) had no effect on the density of LDV. Treatment of NP-40-treated particles with trypsin, on the other hand, resulted in complete destruction of these particles (Fig. 2E).

Lipid and protein composition of LDV and of NP-40-released particles. To obtain further information about the nature of the 1.17-g/cm³ particles released by NP-40 treatment, we propagated LDV in macrophage cultures in the presence of labeled leucine, choline, glucosamine, or mannose. The virus was purified and

then analyzed by isopycnic centrifugation in sucrose density gradients before and after incubation with 0.01% NP-40. About 30% of the total leucine label associated with intact LDV remained associated with the NP-40-treated particles equilibrating at 1.17-g/cm³, whereas the remainder was recovered in the upper portion of the gradient (Fig. 3A). In contrast, all choline-, glucosamine-, and mannose-labeled components of LDV were released in a soluble form by NP-40 treatment (Fig. 3B-D). The results suggest that the remaining particles were devoid of phospholipids (at least of phosphatidylcholine) and glycoproteins and therefore represented free viral nucleocapsids. This conclusion is supported by analysis of the leucine- and glucosamine-labeled proteins by acrylamide gel electrophoresis. Fig. 4B illustrates a typical profile of leucine-labeled LDV proteins. The profile is similar to that reported by Michaelides and Schlesinger (21) for LDV, except that our estimates of the molecular weights of the proteins are slightly greater than those reported by these investigators. The molecular weights, as estimated from the migration rates in 7.5% polyacrylamide gels relative to those of nine standard proteins, were 15,000 for Vp-1 and 18,000 for Vp-2 (Fig. 4C). A molecular weight for Vp-3 could not be estimated with accuracy because it was heterogeneous (24,000 to 44,000 daltons; Fig. 4B). The apparent heterogeneity was not an artifact of the method since Vp-3 from West Nile virus, a flavi togavirus, which possesses three proteins similar to those of LDV, migrated as a homogeneous fraction under similar conditions of analysis (Fig. 4A). Furthermore, a similar heterogeneity of Vp-3 from LDV was observed in 10% acrylamide gels (data not shown) and has also been observed by Michaelides and Schlesinger (21) using 15% acrylamide gels. Vp-3 was the only LDV protein labeled with glucosamine (Fig. 4E) or mannose and thus is the only glycoprotein of LDV. Polyacrylamide gel electrophoresis of the proteins from 1.17-g/cm³ particles remaining after NP-40 treatment (see Fig. 3A) showed that this particle contained only the smallest of the viral proteins, Vp-1 (Fig. 4D). The other two proteins, Vp-2 and Vp-3, were recovered from the upper portion of the gradient of NP-40-treated virions (Fig. 3A and D; Fig. 4D and E). LDV was quantitatively precipitated by concanavalin A. When a suspension of uridine-labeled LDV was supplemented with 800 µg of concanavalin A per ml, incubated at 4 C for 3 h, and then centrifuged at 5,000 × g for 20 min, over 90% of radioactivity was recovered in the pellet. The pelleted virus

contained intact viral RNA, as indicated by zone sedimentation of the RNA extracted from the pelleted material (not shown).

Properties of LDV RNA. Our previous estimate of the molecular weight of LDV RNA of 5×10^6 was based on a sedimentation coefficient of about 48S estimated by zone sedimentation in sucrose density gradients of moderate ionic strength, using 29 and 18S rRNA from Novikoff cells and various other viral RNAs as markers (9). An RNA of this size could contain 10 times more genetic information than is required for coding for the three structural proteins of LDV. The true molecular weights of many viral RNAs, however, are not known with certainty because their secondary structure or other properties may differ from those of the rRNA's. For instance, the RNAs of cardioviruses sediment significantly more rapidly (34S) than 29S rRNA in sucrose density gradients containing 0.1 M NaCl, whereas both types of RNA sediment at about the same rate in gradients containing 1 mM salt (22, 23). Similarly, Sendai virus RNA sediments at 32S in dimethyl sulfoxide gradients when compared to 29S rRNA, whereas it sediments at 47S in normal sucrose density gradients (16). The electrophoretic mobilities of the RNAs of Sendai virus and tobacco mosaic virus in formamide-polyacrylamide gels are also not as expected from their molecular weights when compared to the mobilities of 28 and 18S rRNA's from chicken cells (11). We have, therefore, investigated the effect of salt concentration and denaturation on the sedimentation behavior of LDV RNA. The results in Fig. 5 show, however, that LDV RNA sedimented at approximately 48S when compared to 29S rRNA, whether analyzed in sucrose density gradients containing high or low concentrations of salt. Furthermore, denaturation of LDV RNA

by preincubation with formaldehyde (5) also did not alter its sedimentation rate relative to that of rRNA (Fig. 6). Similarly, the sedimentation rate of Sindbis virus RNA (approximately 45S) in relation to rRNA was not affected under these experimental conditions (data not shown). The results suggest that the RNAs of both LDV and Sindbis virus exhibit secondary structure similar to that of the rRNA's of mammalian cells and that a molecular weight of 5×10^6 may be a valid estimate for the RNA of LDV. Notkins (26, 28) reported that butanol-, chloroform-, ether-, or phenol-extracted pellets obtained by high-speed centrifugation of plasma from LDV-infected mice were infectious when inoculated undiluted into mice and that this infectivity was destroyed by treatment with RNase. To confirm the conclusion drawn from these studies that LDV RNA is infectious, we have isolated RNA by phenol-SDS-chloroform extraction of purified, uridine-labeled LDV, purified the RNA by zone sedimentation through a sucrose density gradient (see Fig. 2B), and inoculated intracerebrally or intraperitoneally 0.1-ml aliquots of various dilutions of this RNA into groups of mice. The RNA was clearly infectious, but its titer varied with the route of inoculation. One milliliter of RNA solution extracted from 10^6 ID₅₀ of LDV contained 10^4 ID₅₀ of infectious RNA when inoculated intracerebrally and 10^2 ID₅₀ when inoculated intraperitoneally. Based on a 70% recovery of RNA from purified virus, as estimated from the recovery of radioactivity, a maximum ratio of the infectivity of LDV RNA to infectivity of intact LDV was estimated as about 10^{-5} .

Fine structure of LDV. Electron microscopic examinations of crude preparations of LDV have suggested that LDV is bottle shaped or possesses tails and thus has a unique structure

FIG. 2. Isopycnic centrifugation of uridine-labeled LDV and Sindbis virus before and after treatment with detergents and/or trypsin. Uridine-labeled LDV and Sindbis virus were partially purified from culture fluid from infected cells by banding in isopycnic sucrose density gradients as described in Materials and Methods (see Fig. 1A). The fractions containing labeled virus were pooled and diluted threefold with B12. These virus suspensions were treated as follows: samples of LDV (A) or Sindbis virus (C) suspensions were supplemented with 0.01% (vol/vol) NP-40, mixed, and incubated at 4 C for 10 min. Duplicate samples of each remained untreated. (D) Two samples of LDV suspension were supplemented with 25 μ g of RNase A per ml, and one of them also with 0.01% NP-40, then mixed, and incubated at 22 C for 30 min. (E) One sample of LDV suspension was supplemented with 0.01% (vol/vol) Triton X, mixed, and incubated at 4 C for 10 min. Two other samples were supplemented with 2.5 mg of trypsin per ml, and one of them also with 0.01% NP-40, mixed, and incubated at 22 C for 30 min. All samples were analyzed by isopycnic centrifugation in 0.5 to 1.5 M sucrose density gradients as in Fig. 1. One-milliliter fractions were collected from the gradients, and aliquots thereof were analyzed for radioactivity in acid-insoluble material and density. In (A), fractions were also assayed for infectivity, and the total virus titers in each peak are indicated in the graph. (B) The remainders of the three peak fractions of the 1.13- and 1.17-g/cm³ particles in A (see bars) were pooled and mixed with 2 volumes of ethanol at -20 C. The precipitates were collected, treated with SDS, and analyzed by zone sedimentation in 0.15 to 0.9 M sucrose density gradients in B6 using 29 and 18S rRNA as markers as described in Materials and Methods. All frames are composites of independent gradients.

FIG. 3. Isopycnic centrifugation of untreated and NP-40-treated LDV labeled with leucine (A), choline (B), mannose (C), or glucosamine (D). LDV was propagated in peritoneal macrophage cultures in the presence of [^3H]leucine, [^3H]choline, [^3H]mannose, or [^3H]glucosamine and was partially purified by banding in sucrose density gradients as described in Materials and Methods. Fractions containing the labeled LDV were pooled and diluted threefold with B12. One sample of each labeled virus suspension was supplemented with 0.01% NP-40 ($\blacktriangle$) and another sample was not ($\bullet$). Both samples were incubated at 4 C for 10 min and then analyzed by isopycnic centrifugation in 0.5 to 1.5 M sucrose density gradients as in Fig. 1. One-milliliter fractions were collected, and aliquots thereof were analyzed for radioactivity in acid-insoluble material. The remainders of certain fractions in A and D were pooled (see bars) for further analysis by polyacrylamide gel electrophoresis (see Fig. 4).

(1, 10). Data from our electron microscopic examination of purified and semipurified preparations of LDV are not in agreement with this conclusion. Micrographs of positively stained sections of pelleted LDV purified from the plasma of infected mice by isopycnic centrifugation, followed by repeated differential centrifugation, revealed populations of spherical particles of relatively uniform size with an average diameter of about 55 nm, which contained an electron-dense core of about 30 nm in diameter. In some preparations which were obtained by twice pelleting LDV directly from plasma which had been diluted fivefold with B12, crystalline arrangements of LDV were observed (Fig. 7A and B). LDV purified from culture fluid harvested from 24-h infected macrophage cultures had the same size and structure, except that a proportion of the virions exhibited an elliptical shape (Fig. 7C and D). We believe, however, that this elliptical shape is due to distortion of the particles which results from purification of the virions and/or preparation of the samples for electron microscopy, since such particles were not observed in preparations of LDV obtained from plasma without extensive purification (Fig. 7A and B), and since LDV seems extremely unstable. We encountered great difficulties in preparing negatively stained LDV. Variations in the negative staining procedure and fixation with glutaraldehyde failed to yield satisfactory results. Intact virions were only observed in preparations twice pelleted from diluted plasma (Fig. 8A and B). The diameter of these particles was similar to that of particles observed in positively stained sections. On the other hand, when LDV preparations were examined in a similar manner after isopycnic centrifugation in sucrose density gradients, particles in various stages of disintegration were observed. Some of these micrographs, nevertheless, are of interest since they reveal certain features of the fine structure of LDV. For instance, Fig. 8C illustrates a loosening and sloughing off of the viral envelope, revealing the viral core structure with a diameter of about 30 nm. Figure 8D illustrates a later stage of disintegration. The larger particles, with a diameter of about 30 nm, are probably viral cores. The nature of the smaller particles of heterogeneous size is not clear, but they most likely represent various-sized aggregates of the sloughed off viral envelope, since little fibrillar material indicative of disintegration of the viral core was detectable on the grids. In addition, in some micrographs we have observed small hollow particles, which measured about 8 to 14 nm in

diameter (Fig. 8E). These particles resemble the substructure, which is faintly distinguishable in the envelope of whole virions (see arrow, insert Fig. 8B), and seem to become released from the virions during their disintegration (insert, Fig. 8E).

Lack of hemagglutination activity of LDV.

We have assayed suspensions of purified LDV containing 10^8 to 10^9 ID₅₀/ml for hemagglutinating activity at 37, 22, and 4 C using chicken, rabbit, mouse, and sheep erythrocytes and various buffers with a range of pH values found optimal for the agglutination of erythrocytes by different togaviruses (6). No agglutination of any of the erythrocytes under any of the experimental conditions was observed.

DISCUSSION

Our results support the view that LDV should be classified as a togavirus. Morphologically, both in fine structure and size, LDV clearly resembles togaviruses. Bottle-shaped or tailed particles were not observed in our studies, nor in another recent investigation (13a). Such pleomorphic particles probably arise due to distortion during preparation for electron microscopy because the envelope of LDV is unusually labile. In one case this effect may have been compounded by interaction with immunoglobulin used to concentrate the virions (1). The proposed new descriptive name for LDV indicating a bottle-shaped structure (1), therefore, is unwarranted. The release of aggregates of varying size and of small hollow particles upon sloughing off and disintegration of the envelope of LDV also seems typical for certain togaviruses. For instance, Smith et al. (40) observed 14-nm "doughnuts" and 7-nm subunits in dengue-2 virus preparations, and Bergold et al. (3) found numerous open ring-like structures in preparations of various togaviruses. Furthermore, like many togaviruses, LDV matures by budding through intracytoplasmic membranes (5a). The RNA of LDV is also like that of togaviruses in that it is single stranded, has a molecular weight of about 5×10^6 , and is a positive strand. Although the latter point has not been proved unequivocally, the fact that LDV RNA is infectious and that no viral RNA synthesis occurs in macrophages in the presence of inhibitors of protein synthesis (5a) strongly supports this view (see reference 2). In addition, preliminary evidence indicates that LDV RNA contains a segment of polyadenylic acid (Brinton-Darnell and Plagemann, unpublished data). The secondary structure of LDV RNA is similar to that of Sindbis virus RNA and rRNA,

but differs from that of the RNA of some picornaviruses (22, 23). In its possession of three structural proteins LDV also resembles togaviruses. The localization of the proteins of LDV in the core and envelope, however, differs from that of the proteins of alpha or flavi togaviruses. Alphaviruses possess one or two glycoproteins which are located in the envelope and one polypeptide in the core (24, 38). Flaviviruses, like LDV, possess one glycoprotein and two polypeptides, but the intermediate size protein rather than the smallest protein, as is the case with LDV, is the only polypeptide associated with the nucleocapsid (39). The glycoprotein of LDV also differs from that of flaviviruses in its apparent heterogeneity. It is not clear at present whether this variation in gel migration is due to microheterogeneity in the carbohydrate moiety associated with the protein or reflects the presence of more than one glycoprotein. Based on the assumption that the three LDV proteins contain about the same relative proportion of leucine, we estimate from the distribution of leucine radioactivity among the three proteins that Vp-1, Vp-2, and Vp-3 are present in LDV in a ratio of 2 to 3:1:1. This ratio differs from that (3 to 5:1:1) estimated by Michaelides and Schlesinger (21).

LDV differs from alpha and flavi togaviruses in other properties. First, its density is significantly lower than that of other togaviruses or any enveloped animal virus. Our present results indicate that the higher values reported previously (25, 37) are not due to propagation of LDV in different host cells or the use of different strains of LDV. More likely they resulted from differences in techniques. Our values seem accurate since two other viruses, a type C virus (9) and Sindbis virus, used as controls, exhibited densities of 1.16 and 1.175 g/cm³, respectively, which agree with those reported for these viruses by other investigators (12, 14, 30). Second, the envelope of LDV is more labile than that of

alpha or flavi togaviruses and tends to slough off. The lability of the envelope is indicated by the extreme sensitivity of LDV to detergent treatment and the fact that the virions tend to disintegrate upon preparation for electron microscopy. Whether this lability and the low density of LDV are related in some manner is not known. The viral core also exhibits an unusually low density (1.17 g/cm³), in spite of the fact that it seems devoid of phospholipids. This low density does not seem to be due to the binding of detergent used to remove the envelope. In agreement with this observation, it has been found that detergents tend to interact mainly with the glycoproteins of the envelope rather than with the polypeptides associated with the nucleocapsid of enveloped viruses (see reference 17). Third, LDV does not possess projections, which are present on the surface of both alpha (7) and flavi (15) togaviruses. The absence of surface projections is indicated by electron micrographs of untreated LDV and by the fact that the density of LDV is not affected and that no leucine or glucosamine label is released from the virions upon incubation with proteinases. Such treatment removes the surface projections of Sindbis virus and significantly decreases the density of the virions (7). These results indicate that the glycoprotein of LDV is more intimately associated with the lipid bilayer of the envelope than those of alpha- or flaviviruses. Whether this property is related to the lack of hemagglutinating activity of LDV is not known. The carbohydrate moiety of the LDV glycoprotein, nevertheless, is located on the outer surface of the envelope, since LDV, like Sindbis virus (4) and other enveloped viruses (41), is precipitated by concanavalin A. Our results, therefore, indicate that LDV does not belong to either the alpha or flavi togavirus groups. Additional studies are required to determine whether LDV is related to other as yet unclassified viruses.

FIG. 4. SDS-polyacrylamide gel electrophoresis patterns of proteins from untreated and NP-40-treated LDV and West Nile virus (WNV). Untreated leucine- and glucosamine-labeled LDV, and 1.17-g/cm³ particles and released proteins derived by treatment of LDV with NP-40, were isolated as illustrated in Fig. 3A and D. Leucine-labeled WNV was isolated in a similar manner. The suspensions were treated with SDS, and the proteins were separated by electrophoresis on 7.5% polyacrylamide gels as described in Materials and Methods. (A) Total proteins of [³H]leucine-labeled WNV. (B) Total proteins of [³H]leucine-labeled LDV. (D) [³H]leucine-labeled LDV virion proteins isolated from NP-40-treated, 1.17-g/cm³ particles (●) and from the NP-40-released material (▲). (E) Glucosamine-labeled protein from LDV (●) and NP-40-released material (▲). The molecular weights of the viral proteins were estimated from their migration rates relative to those of nine standard proteins (C): bovine serum albumin, 67,000; heavy chain of immunoglobulin G, 50,000; creatine phosphokinase, 40,000; DNase, 31,000; chymotrypsin, 25,000; trypsin, 23,000; myoglobin, 17,800; lysozyme, 14,000; and cytochrome c, 12,400. Rates of migration are expressed relative to that of the dye (in percent).

FIG. 5. Zone sedimentation of LDV RNA in low- and high-ionic-strength sucrose density gradients. RNA was isolated from partially purified, uridine-labeled LDV, mixed with ¹⁴C-labeled rRNA from Novikoff cells, and analyzed by zone sedimentation in 0.15 to 0.9 M sucrose density gradients as described in Materials and Methods. In (A) sucrose was dissolved in 1 mM EDTA, 1 mM Tris-chloride (pH 7.4), and 0.5% SDS, and in (B) in 20 mM EDTA, 20 mM Tris-chloride (pH 7.4), 100 mM NaCl, and 0.5% SDS. Fractions from the gradients were analyzed for ³H and ¹⁴C in acid-insoluble material.

FIG. 6. Effect of formaldehyde pretreatment on the sedimentation rate of LDV RNA and rRNA. One mixture of [³H]uridine-labeled LDV RNA and ¹⁴C-labeled rRNA's was supplemented with 6% (vol/vol) formaldehyde, incubated at 63 C for 15 min, and then quickly cooled (▲). A duplicate mixture remained untreated (●). Both samples were sedimented through 0.15 to 0.9 M gradients of sucrose in B6 in an SW27 rotor at 22,000 rpm at 20 C for 10 h. Fractions from the gradients were analyzed for ³H and ¹⁴C in acid-insoluble material. The graph is a composite of the gradient profiles of ³H-labeled formaldehyde-treated and untreated RNAs. The positions of the rRNA's (¹⁴C) are indicated by the arrows.

FIG. 7. Electron micrographs of positively stained thin sections of pelleted LDV. (A and B) Plasma was collected from mice 24 h after infection with LDV, diluted fivefold with B12, and then centrifuged at $100,000 \times g$ at 4 C for 2.5 h. The pellet was resuspended in B12 and recentrifuged in a small conical tube floated on 70% glycerol. (C and D) LDV was partially purified from culture fluid harvested from 24-h infected macrophage cultures by banding in isopycnic sucrose density gradients. Peak fractions were pooled (see Fig. 1), diluted fivefold with B12, and pelleted twice as described above for the plasma LDV. Virus pellets were fixed, stained, and sectioned, and the sections were examined in the electron microscope as described in Materials and Methods. Magnifications: (A) $\times 30,000$, (B) $\times 120,000$, (C) $\times 120,000$, (D) $\times 240,000$.

FIG. 8. Electron micrographs of negatively stained LDV. (A and B) Plasma collected from mice 24 h after infection with LDV and (C-E) LDV from culture fluid of 24-h infected macrophage cultures was partially purified by isopycnic sucrose density gradient centrifugation. Mouse plasma and LDV containing gradient fractions were diluted fivefold with B12, clarified by low-speed centrifugation, and then centrifuged at $100,000 \times g$ at 4 C for 2.5 h. The pellets were resuspended in small amounts of B12. Samples thereof were added directly onto grids. The grids were stained with phosphotungstic acid and examined in the electron microscope as described in Materials and Methods. Magnification: (A) $\times 45,000$; (B-E) $\times 240,000$.

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LITERATURE CITED

1. Almeida, J. D., and C. A. Mims. 1974. The unique morphology of the lactic dehydrogenase virus. *Microbios* 10:175-180.
2. Baltimore, D. 1971. Expression of animal virus genomes. *Bacteriol. Rev.* 35:235-241.
3. Bergold, G. H., L. A. G. Marquez, R. Mazzali, and K. Munz. 1969. Structure of nine arboviruses. *J. Gen. Virol.* 5:135-140.
4. Birdwell, C. R., and J. H. Strauss. 1973. Agglutination of Sindbis virus and of cells infected with Sindbis virus by plant lectins. *J. Virol.* 11:502-507.
5. Boedtker, M. 1968. Dependence of the sedimentation

- coefficient on molecular weight of RNA after reaction with formaldehyde. *J. Mol. Biol.* **35**:61-70.
- 5a. Brinton-Darnell, M., G. K. Collins, and P. G. W. Plagemann. 1975. Lactate dehydrogenase-elevating virus replication, maturation and viral RNA synthesis in primary mouse macrophage cultures. *Virology* **65**:187-195.
 6. Clarke, D. H., and J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am. J. Trop. Med. Hyg.* **7**:561-573.
 7. Compans, R. W. 1971. Location of the glycoprotein in the membrane of Sindbis virus. *Nature (London) New Biol.* **229**:114-116.
 8. Darnell, M. B., and H. Koprowski. 1974. Genetically determined resistance to infection with group B arboviruses. II. Increased production of interfering particles in cell cultures from resistant mice. *J. Infect. Dis.* **129**:248-256.
 9. Darnell, M. B., and P. G. W. Plagemann. 1972. Physical properties of lactic dehydrogenase-elevating virus and its ribonucleic acid. *J. Virol.* **10**:1082-1085.
 10. De Thé, G., and A. L. Notkins. 1965. Ultrastructure of the lactic dehydrogenase virus (LDV) and cell-virus relationships. *Virology* **26**:512-516.
 11. Duesberg, P. H., and P. K. Vogt. 1973. Gel electrophoresis of avian leukosis and sarcoma viral RNA in formamide: comparison with other viral and cellular RNA species. *J. Virol.* **12**:594-599.
 12. Fuscaldo, A. A., H. G. Aaslestad, and E. J. Hoffman. 1971. The biological, physical, and chemical properties of eastern equine encephalitis virus. I. Purification and physical properties. *J. Virol.* **7**:233-240.
 13. Harrison, S. C., A. David, J. Jumblatt, and J. E. Darnell. 1971. Lipid and protein organization in Sindbis virus. *J. Mol. Biol.* **60**:523-528.
 - 13a. Horzinek, M. C., P. S. van Wierink, and D. J. Ellens. 1975. Purification and electron microscopy of lactic dehydrogenase virus in mice. *J. Gen. Virol.* **26**:217-226.
 14. Kindig, D. A., R. Karp, and W. H. Kirsten. 1968. Further characterization of L-cell virions. *Proc. Natl. Acad. Sci. U.S.A.* **59**:1103-1109.
 15. Kitano, T., K. Suzuki, and T. Yamaguchi. 1974. Morphological, chemical, and biological characterization of Japanese encephalitis virus virion and its hemagglutinin. *J. Virol.* **14**:631-639.
 16. Kolakofski, D., and A. Bruschi. 1973. Molecular weight determination of Sendai RNA by dimethyl sulfoxide gradient sedimentation. *J. Virol.* **11**:615-620.
 17. Lenard, J., and R. W. Compans. 1974. The membrane structure of lipid-containing viruses. *Biochim. Biophys. Acta* **344**:51-94.
 18. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409-414.
 19. Maizel, J. V. 1969. Acrylamide gel electrophoresis of proteins and nucleic acids, p. 334-362. In K. Habel and N. P. Salzman (ed.), *Fundamental techniques in virology*. Academic Press Inc., New York.
 20. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236**:1372-1379.
 21. Michaelides, M. C., and S. Schlesinger. 1973. Structural proteins of lactic dehydrogenase virus. *Virology* **55**:211-217.
 22. Miller, R. L., and P. G. W. Plagemann. 1974. Effect of ultraviolet light on mengovirus: formation of uracil dimers, instability and degradation of capsid, and covalent linkage of protein to viral RNA. *J. Virol.* **13**:729-739.
 23. Montagnier, L., and F. K. Sanders. 1963. Sedimentation properties of infective ribonucleic acid extracted from encephalomyocarditis virus. *Nature (London)* **197**:1178-1181.
 24. Morser, M. J., and D. C. Burke. 1974. Cleavage of virus-specified polypeptides in cells infected with Semliki Forest virus. *J. Gen. Virol.* **22**:395-409.
 25. Niwa, A., S. Yamazaki, J. Bader, and A. L. Notkins. 1973. Incorporation of labeled precursors into the RNA and proteins of lactic dehydrogenase virus. *J. Virol.* **12**:401-404.
 26. Notkins, A. L. 1964. Recovery of an infectious ribonucleic acid from the lactic dehydrogenase agent by treatment with ether. *Virology* **22**:563-567.
 27. Notkins, A. L. 1965. Lactic dehydrogenase virus. *Bacteriol. Rev.* **29**:143-160.
 28. Notkins, A. L., and C. Scheele. 1963. An infectious nucleic acid from the lactic dehydrogenase agent. *Virology* **20**:640-642.
 29. Perry, R. P., J. LaTorre, D. E. Kelly, and J. R. Greenberg. 1972. On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes. *Biochim. Biophys. Acta* **262**:220-226.
 30. Pfefferkorn, E. R., and H. S. Hunter. 1963. Purification and partial chemical analysis of Sindbis virus. *Virology* **20**:433-445.
 31. Plagemann, P. G. W. 1968. Mengovirus replication in Novikoff rat hepatoma and mouse L cells: effects on the synthesis of host-cell macromolecules and virus-specific synthesis of ribonucleic acid. *J. Virol.* **2**:461-473.
 32. Plagemann, P. G. W. 1970. Temperature and phenol-induced alterations in sedimentation rates of 29-S and 18-S ribosomal RNA's from Novikoff hepatoma cells. *Biochim. Biophys. Acta* **244**:451-457.
 33. Plagemann, P. G. W., K. F. Gregory, H. E. Swim, and W. Chan. 1963. Plasma lactic dehydrogenase-elevating agent of mice: distribution in tissues and effect on lactic dehydrogenase isozyme patterns. *Can. J. Microbiol.* **9**:75-86.
 34. Plagemann, P. G. W., and H. E. Swim. 1966. Replication of mengovirus. I. Effect on synthesis of macromolecules by host cells. *J. Bacteriol.* **91**:2317-2326.
 35. Plagemann, P. G. W., and H. E. Swim. 1966. Relationship between the lactic dehydrogenase-elevating virus and transplantable murine tumors. *Proc. Soc. Exp. Biol. Med.* **121**:1142-1146.
 36. Plagemann, P. G. W., and H. E. Swim. 1966. Propagation of lactic dehydrogenase-elevating virus in cell culture. *Proc. Soc. Exp. Biol. Med.* **121**:1147-1152.
 37. Riley, V., M. A. Campbell, J. D. Loveless, and M. A. Fitzmaurice. 1964. Density gradient centrifugation and molecular sieve studies of lactic dehydrogenase-elevating virus-like agents. *Proc. Am. Assoc. Cancer Res.* **5**:53.
 38. Schlesinger, M. J., and S. Schlesinger. 1972. Identification of a second glycoprotein in Sindbis virus. *Virology* **47**:539-541.
 39. Shapiro, D., and K. A. Kos. 1973. Japanese encephalitis virus glycoproteins. *Virology* **56**:88-94.
 40. Smith, T. J., W. E. Brandt, J. L. Swanson, J. M. McCown, and E. L. Buescher. 1970. Physical and biological properties of dengue-2 virus and associated antigens. *J. Virol.* **5**:524-532.
 41. Stewart, M. L., and J. V. Maizel. 1974. Rapid analysis of oncornaviral RNA employing agglutination of virions with concanavalin A. *Virology* **59**:595-599.