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Purification and Electron Microscopy of Lactic Dehydrogenase Virus of Mice

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SUMMARY

Lactic dehydrogenase virus (LDV) was purified from infectious ascites fluid of mice bearing Ehrlich tumours using Sepharose gel filtration and rate zonal and isopycnic sedimentation. In glycerol gradients, a sedimentation coefficient of about 200S and a buoyant density of 1·14 g/ml was determined for the virus particle. Spherical particles with diam. between 62 and 80 nm, depending on the method of fixation and staining, have been identified electron microscopically. The virus particle consists of a spherical nucleocapsid wrapped into a double-layered envelope. The nucleocapsids, isolated by treatment with NP40 and purified by centrifuging on sucrose gradients had a sedimentation coefficient of 176S. Electron micrographs show spherical particles with a diam. of 35 ± 4 nm. Classification of LDV as a member of the togaviridae family is discussed.

INTRODUCTION

Lactic dehydrogenase virus is a small enveloped RNA virus infecting wild and laboratory mice. The infection does not produce overt disease in the animals but causes a five- to tenfold increase in the activity of lactic dehydrogenase in the plasma which may serve as a basis for detecting and titrating the virus (Notkins, 1965).

Referring to published evidence (de Thé & Notkins, 1965), LDV has been classified tentatively as a togavirus (Murphy, Halonen & Harrison, 1968; Melnick, 1969). Recent studies concerning the genome size and structural polypeptides support this taxonomic position (Darnell & Plagemann, 1972; Michaelides & Schlesinger, 1973; Niwa *et al.* 1973). The family togaviridae includes the established genera alpha- and flaviviruses whose members are arthropod-borne (Wildy, 1971) as well as several other animal and plant viruses which spread conventionally (Horzinek, 1973*a*, *b*). The aim of this series of studies (Horzinek & Mussgay, 1971; Horzinek, Maess & Laufs, 1971) is to provide comparative morphological and structural data on nonarbo togaviruses which might lead to a better definition and possibly subdivision of the family.

METHODS

Virus. The LDV strain employed in this study has been kindly provided by Dr B. W. J. Mahy, Cambridge, England; the virus was grown in randomly bred laboratory mice. Sindbis (SIN) virus was included for morphological comparison and [^{32}P]-labelled Semliki Forest (SF) virus served as a physical marker; both viruses were grown, assayed and purified using standard techniques (Pfefferkorn & Hunter, 1963).

Infectivity titration. Infectivity was assayed by intraperitoneal inoculation of several virus dilutions into mice which had been pre-checked for normal plasma lactate dehydrogenase levels. Five to eight days after infection blood samples were collected by retro-orbital puncture and enzyme levels were determined in individual sera using standard reagents and techniques (F.C. Boehringer & Soehne, Mannheim, West-Germany). Mice showing >1200 mU/ml were considered positive; infectivity titres were estimated employing the Kärber formula.

Starting material for virus purification. For growing the agent in quantity the procedure as described by du Buy & Johnson (1965) was followed. Ehrlich ascites carcinoma cells, freed from latent LDV by two passages in suckling rats were injected into groups of 120 to 150 mice by the intraperitoneal route. Ten to twelve days later the animals were inoculated with 0·2 ml LDV suspension containing about 10^6 ID_{50} units. Forty hours later the ascites fluids were harvested and 10 % of a 0·1 M-Na_2-EDTA solution, pH 7·2, was added. The pooled supernatant fluids obtained by centrifuging at low speed were kept at -20 °C in 100 ml samples. The average harvest was 8·5 ml per mouse containing $10^{8.8}$ ID_{50} units/ml.

Virus purification. Freshly thawed 100 ml pools of infectious mouse ascites fluid were centrifuged at 1000 ***g*** for 10 min; subsequently salmine sulphate (BDH Chemicals Ltd., Poole, England) dissolved in TEN buffer (0·05 M-tris-HCl, pH 7·9, 1·0 mM Na_2-EDTA, 0·1 M-NaCl) was added resulting in a final concentration of 5 mg/ml and the mixture was left overnight at 4 °C. After centrifuging further at low speed the supernatant fluid was applied to a 45×900 mm Sepharose 2B (Pharmacia, Uppsala, Sweden) column and eluted at a flow rate of about 40 ml/h. The fractions containing virus were pooled and spun for 4 h at 20000 rev/min in a Spinco 21 rotor (59000 ***g***). The resuspended pellets were treated with ultrasonic vibration at 20 kHz for 60 s in an ice bath and the resulting material was layered on top of a 10 to 38·5 % isokinetic glycerol gradient prepared in TEN buffer (van der Zeijst & Bloemers, 1974). After centrifuging for 150 min at 25000 rev/min in a Spinco SW 25.1 rotor, a distinct light-scattering band had appeared about halfway down the tube. The gradient was fractionated by top unloading and E_{254} recorded automatically (Uvicord, LKB-Produkter, Stockholm, Sweden). For further purification, the virus was diluted and layered on top of a linear 20 to 75 % glycerol gradient in TEN buffer. Band material, after equilibrium sedimentation for 18 h under the conditions mentioned above, constituted purified virus which was employed for electron microscopic study.

Purification of nucleocapsids. For isolation of nucleocapsids the virus was purified without previous chromatography using a linear sucrose gradient (15 ml, 15 to 30 %) with a 5·0 ml cushion of 50 % sucrose on the bottom of the tube; 17·5 ml of clarified infectious mouse ascites fluid were layered on top of the gradient and the tubes were centrifuged in a Spinco SW 27 rotor for 16 h at 25000 rev/min. After top unloading and E_{254} recording, fractions containing virus were pooled, diluted with an equal amount of TEN buffer and centrifuged in a Spinco 21 rotor for 5 h at 20000 rev/min. The pellet was resuspended in 0·5 ml of 0·2 % Nonidet P 40 (NP 40; Shell Chemical Company) in TEN and incubated for 10 min at 20 °C. The material was clarified by centrifuging at low speed and loaded on top of a 15 to

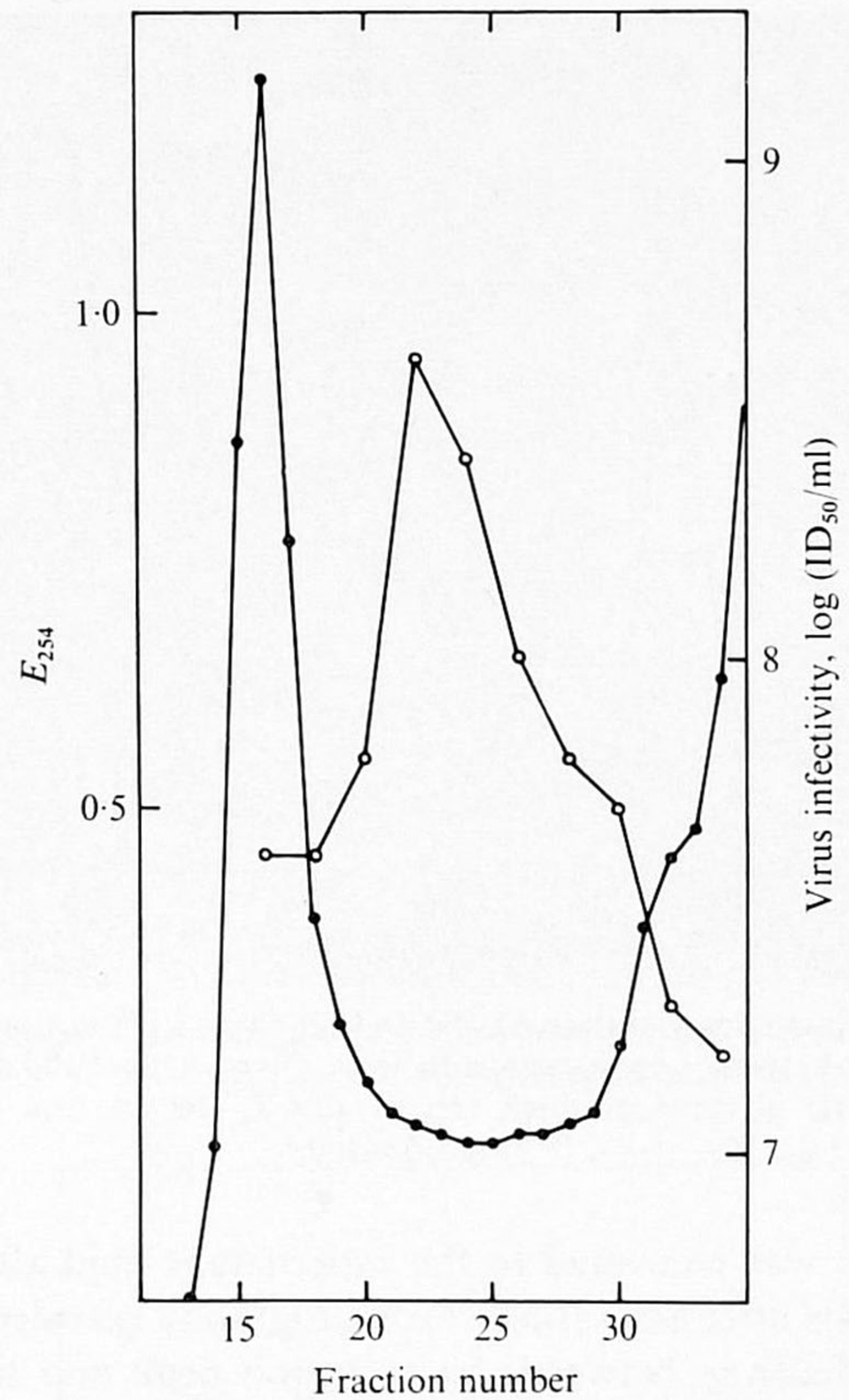

Fig. 1. Sepharose 2B chromatography of mouse Ehrlich ascites fluid containing LDV; the bulk of infectivity (○——○) was eluted between the exclusion and soluble protein peaks. ●——●, E_{254}.

27·5 % isokinetic sucrose gradient containing 0·1 % NP40 and 1 % BSA. After centrifuging for 50 min in a Spinco SW 50.1 at 45000 rev/min the gradient was fractionated and E_{254} recorded. Peak fractions were employed for electron microscopic study.

Electron microscopy. Negative staining was performed directly on the grid using aqueous solutions of 1 % unbuffered uranyl acetate (UA, pH about 4·5), 2 % phosphotungstic acid (PTA, adjusted to pH 5·0 with KOH), and 3 % ammonium molybdate (pH 5·2), respectively. For fixation, 0·2 % osmium tetroxide or 2 % glutaraldehyde in phosphate buffer, pH 7·2, were allowed to react with the virus for 10 min. A Phillips EM 200 or EM 301 electron microscope was employed in this study; the instrumental magnification was calibrated using a carbon replica of a diffraction grating (2160 lines/mm; E. F. Fulham). Particle sizes were measured from prints employing a Carl Zeiss Teilchengrössenanalysator TGZ3; mean values and standard deviations are given.

RESULTS

Purification of LDV

When native ascites fluid was applied to Sepharose 2B columns, formation of precipitates was noted at the meniscus which resulted in clogging of the gel and reduced flow rates. Although salmine sulphate precipitation did not significantly reduce the total protein concentration it improved gel filtration. In this series of experiments between 65 % and 110 %

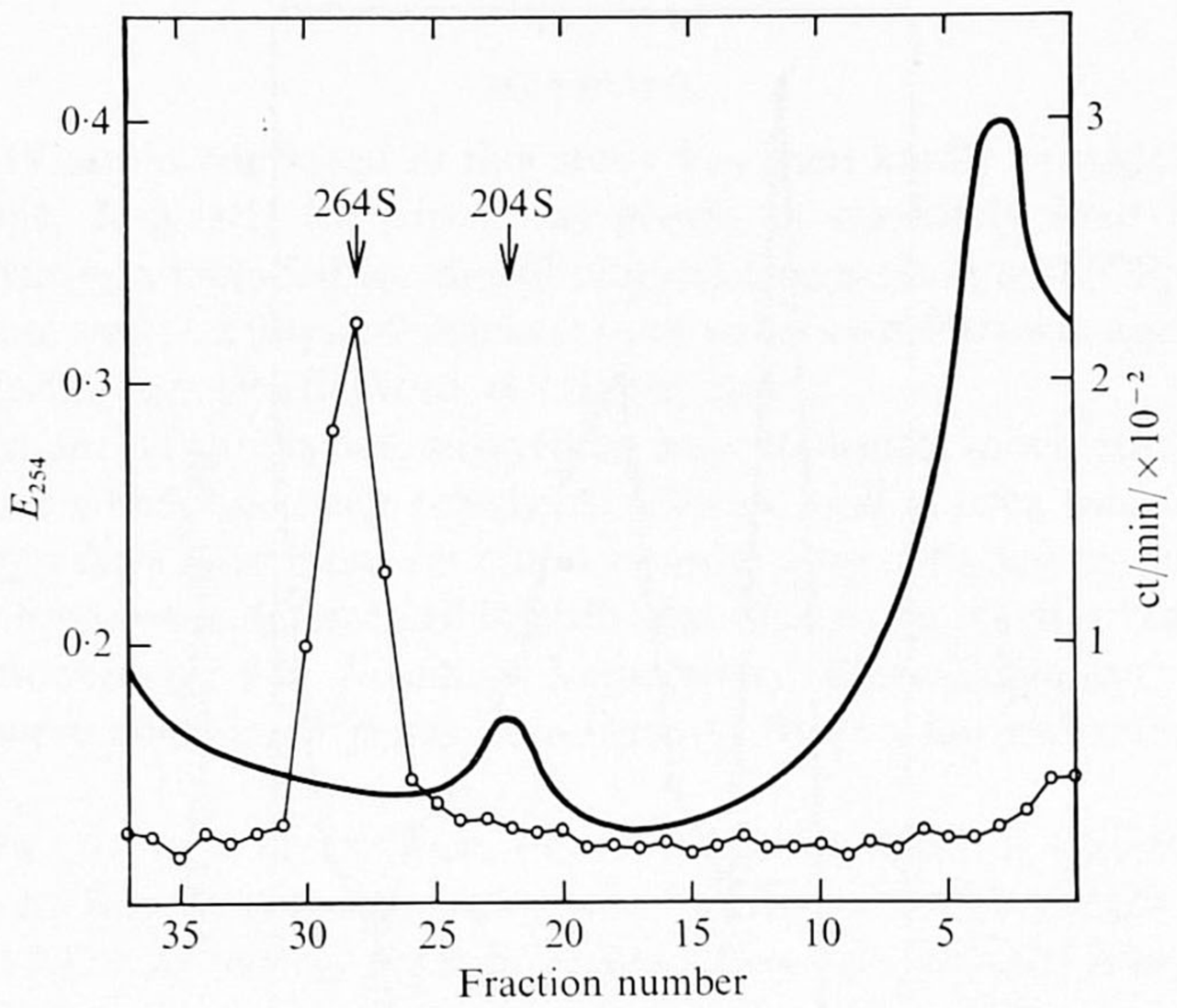

Fig. 2. Rate zonal co-sedimentation of native LDV and SF virus. LDV containing material from the Sepharose chromatography peak was concentrated and mixed with [^{32}P]-labelled SF virus; it was analysed on an isokinetic glycerol gradient (10 to 38·5 % for 150 min at 25000 rev/min in a Spinco SW 25·1). ——, E_{254}; ○——○, [^{32}P]-radioactivity.

of the original infectivity was recovered in the supernatant fluid after precipitation. Titration of individual fractions after Sepharose chromatography revealed that the bulk of infectivity was eluted about halfway between the exclusion peak and the low mol. wt. serum proteins (Fig. 1). Routinely, all the fractions eluted between the exclusion peak and the soluble protein peak were pooled; reduction of the ascites fluid protein content as determined by the Lowry method was between 95 % and 99 % with an accompanying fivefold dilution of the virus material but no measurable loss in infectivity. Further centrifuging removed between 70 % and 90 % of contaminating protein present in the column eluate.

Sedimentation coefficient and buoyant density of the virus particle

The results of a rate zonal sedimentation experiment are given in Fig. 2. Sedimentation coefficients between 197 and 204S were calculated from isokinetic glycerol gradients for material constituting the smaller light-scattering band in the gradient. In the electron microscope numerous particles similar in size and morphology were observed in material from the E_{254} peak (Fig. 3). Since more than 99 % of the original virus infectivity had accumulated in the ~ 200S peak it was concluded that the particles observed constitute lactic dehydrogenase virus particles. When, on the other hand, Sepharose eluate fractions from the exclusion and soluble protein peaks, respectively, were pelleted and subsequently analysed, only weak or no light-scattering bands could be detected in the 200S position. For [^{32}P]-labelled SF virus, serving as a marker in these experiments, a value of 264S was determined which is in agreement with data published for alphaviruses (see Horzinek, 1973*a*). After centrifuging of peak fractions from the zonal run under equilibrium conditions, light-scattering virus material accumulated at densities between 1·136 and 1·145 g/ml (Fig. 4).

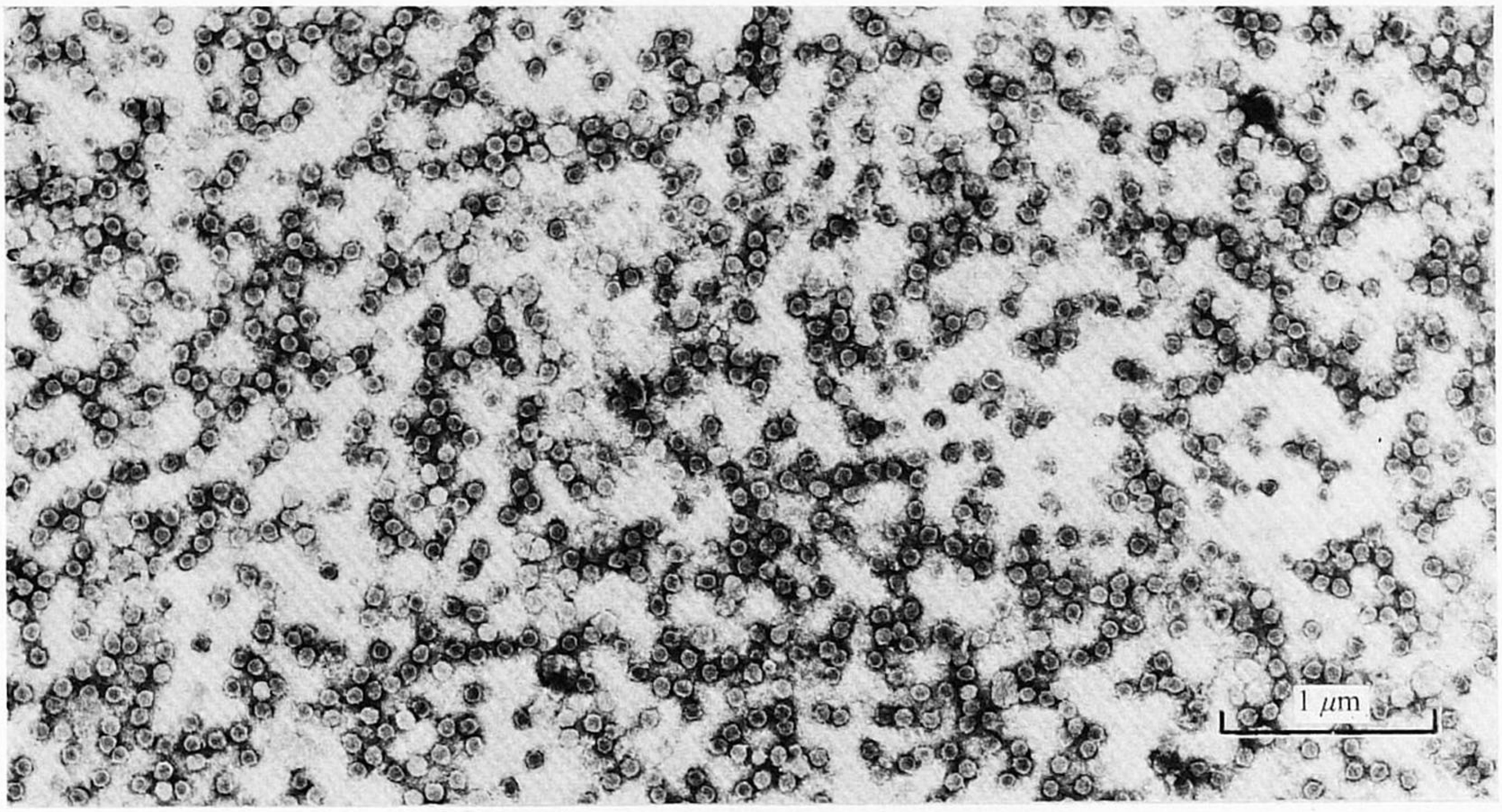

Fig. 3. Electron microscopic appearance of 204S material from the isokinetic glycerol gradient depicted in Fig. 2. Note the absence of rod-shaped particles (1 % UA, unfixed).

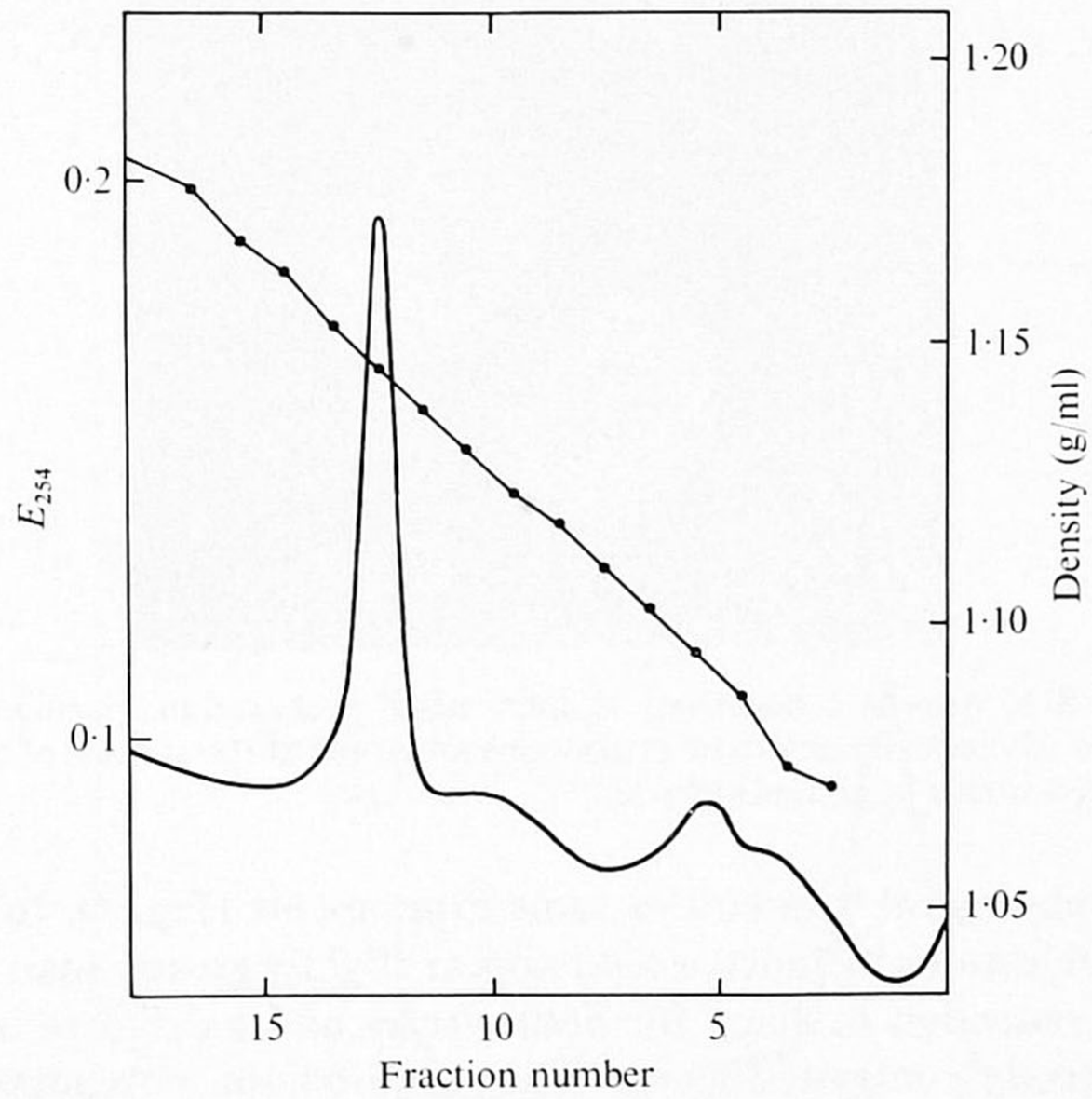

Fig. 4. Banding by isopycnic sedimentation in a glycerol gradient (20 to 75 % for 18 h at 25 000 rev/min in a Spinco rotor 25.1) of LDV pre-purified in an isokinetic gradient; the main E_{254} peak is located at a density of 1·14 g/ml. ——, E_{254}; ●——●, density.

Size and morphology of the virus particle

Particle diam. were measured from electron micrographs of purified and concentrated LDV preparations stained with UA and PTA, respectively, with or without previous fixation using osmium tetroxide (Table 1); SIN virus was included for comparison and used

Table 1. *Effect of negative stain and fixation on diameter values of LDV and SIN virus*

	LDV				Sindbis virus (projections included)			
	Light centre*		Dark centre		Light centre		Dark centre	
	n	nm	n	nm	n	nm	n	nm
Uranyl acetate	250	80 ± 6	250	62 ± 4	200	74 ± 4		—†
Pre-fixed	201	65 ± 5		n.d.‡	250	71 ± 5		n.d.
Phosphotungstate		n.d.	250	69 ± 5		n.d.	150	74 ± 4
Pre-fixed	78	63 ± 4	78	63 ± 8	250	72 ± 5		—

* Refers to predominantly negatively stained virus particles (light centre) or particles which had been penetrated by the stain (dark centre).

† No size measurements made.

‡ No particles detected in significant amounts.

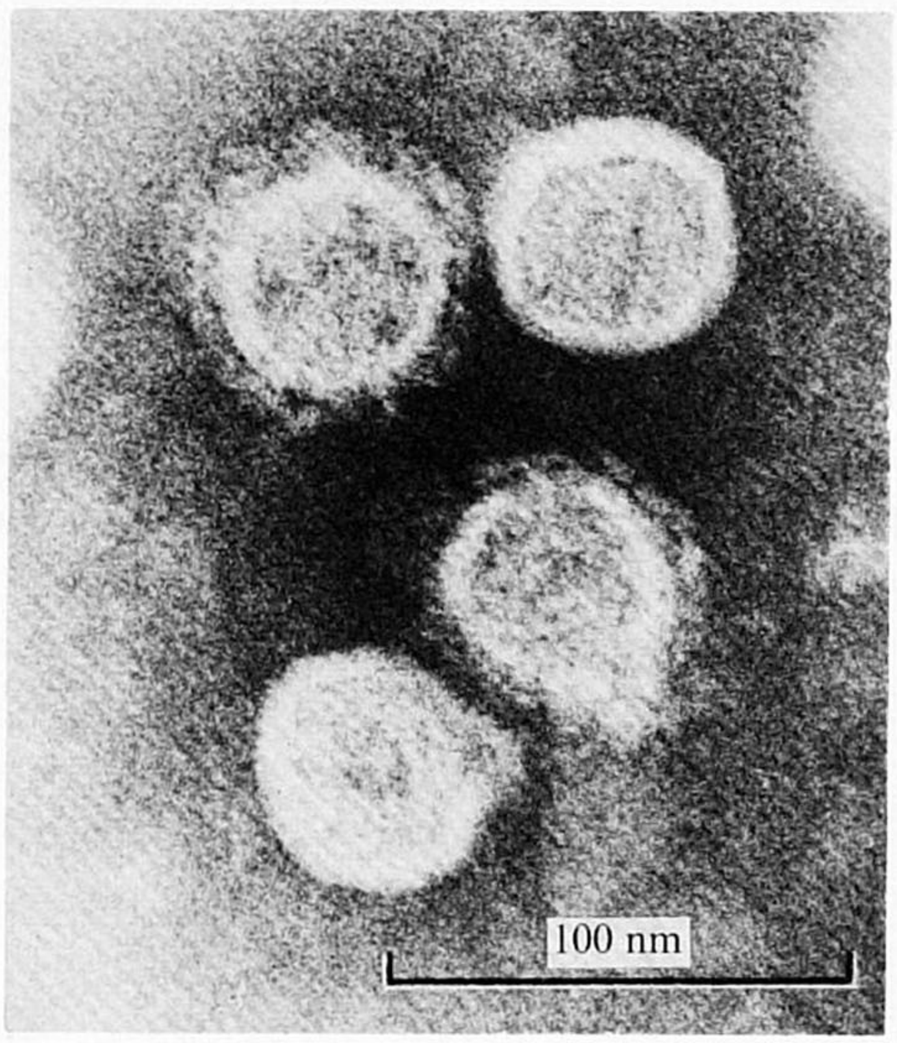

Fig. 5. LDV and SIN virus in a negatively stained mixed preparation; purified LDV appears as a smooth structure whereas characteristic projections are noted at the surface of SIN virus particles (2 % PTA, pre-fixed with 2 % glutaraldehyde).

as an internal morphological standard in some experiments (Fig. 5). In general, SIN virus with its fringe of projections included would appear slightly greater than LDV. Fixation was accompanied by a reduction in diam. for both viruses and a decrease in the percentage of particles with positively contrasted cores. When ammonium molybdate was employed to pre-fix preparations (not included in the table) the diam. values of LDV were significantly smaller (50 ± 3 nm; $n = 250$).

Orientating experiments using the platinum shadowing technique as well as stereoscopic pictures of pre-fixed, negatively stained virus particles indicated that the LDV particle is essentially spherical. However, when PTA was used with unfixed material, many distorted virus particles could be detected and a polymorphous particle shape was prevalent, especially when sucrose had been used in the gradients instead of glycerol.

The LDV particle consists of a spherical core considered as the nucleocapsid and an

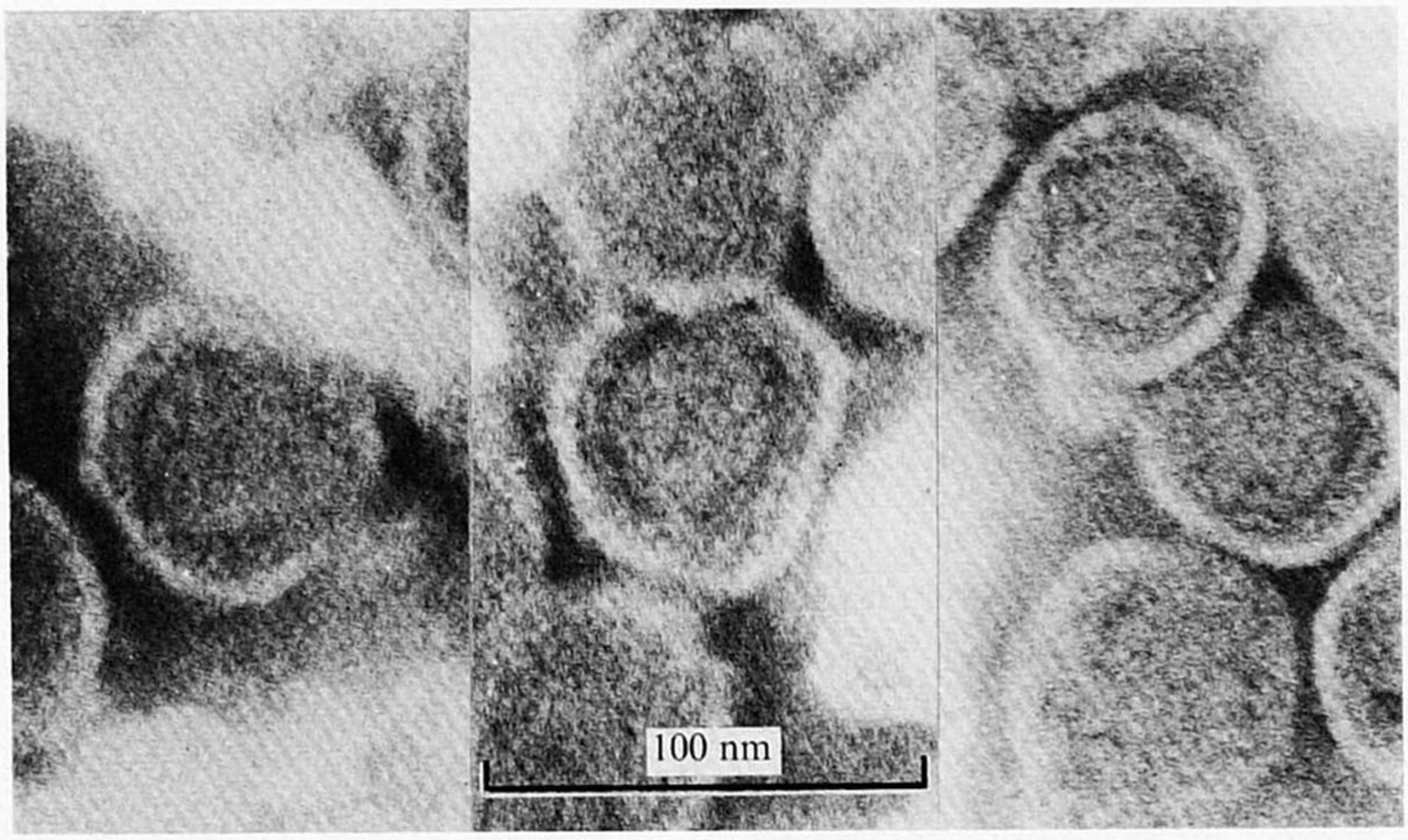

Fig. 6. LDV particles in which the membrane had been penetrated by negative stain to show an intermediate layer (2 % PTA, unfixed).

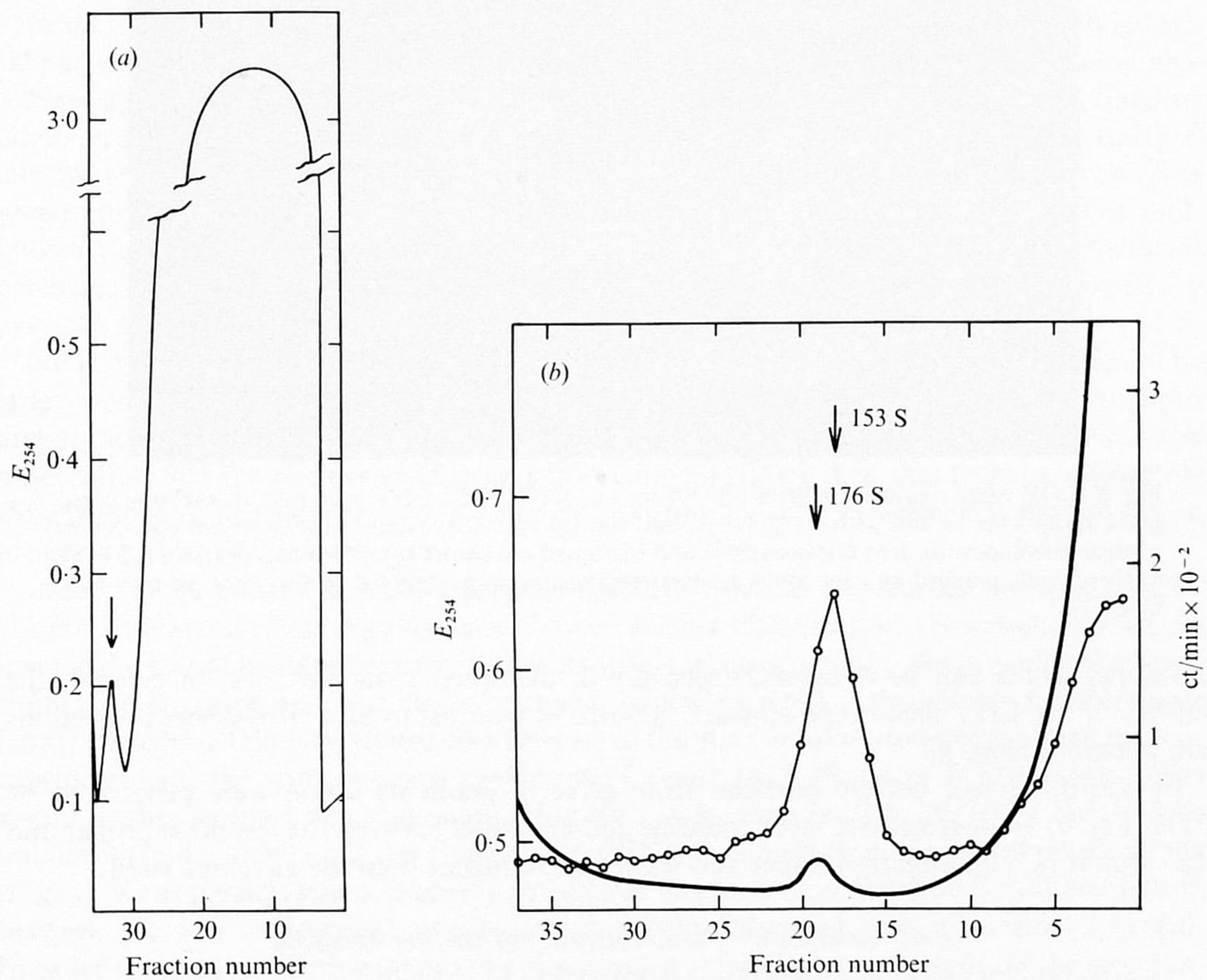

Fig. 7. (*a*) Sucrose gradient sedimentation of mouse Ehrlich ascites fluid containing LDV. 17·5 ml of clarified fluid was layered on top of a sucrose gradient (15 ml, 15 to 30 %; 5 ml, 50 %). After centrifuging (16 h at 25000 rev/min in a Spinco SW 27 rotor) virus particles slightly contaminated with membrane fragments are present in fractions 32, 33 and 34 (arrow). (*b*) Rate zonal co-sedimentation of LDV and [^{32}P]-labelled SF virus, treated for 30 min with 0·1 % NP40 at 21 °C. The material was analysed on isokinetic glycerol gradients (10 to 38·5 % for 150 min at 25000 rev/min in a Spinco SW 25.1 rotor at 4 °C). ——, E_{254}; ○——○, [^{32}P]-radioactivity.

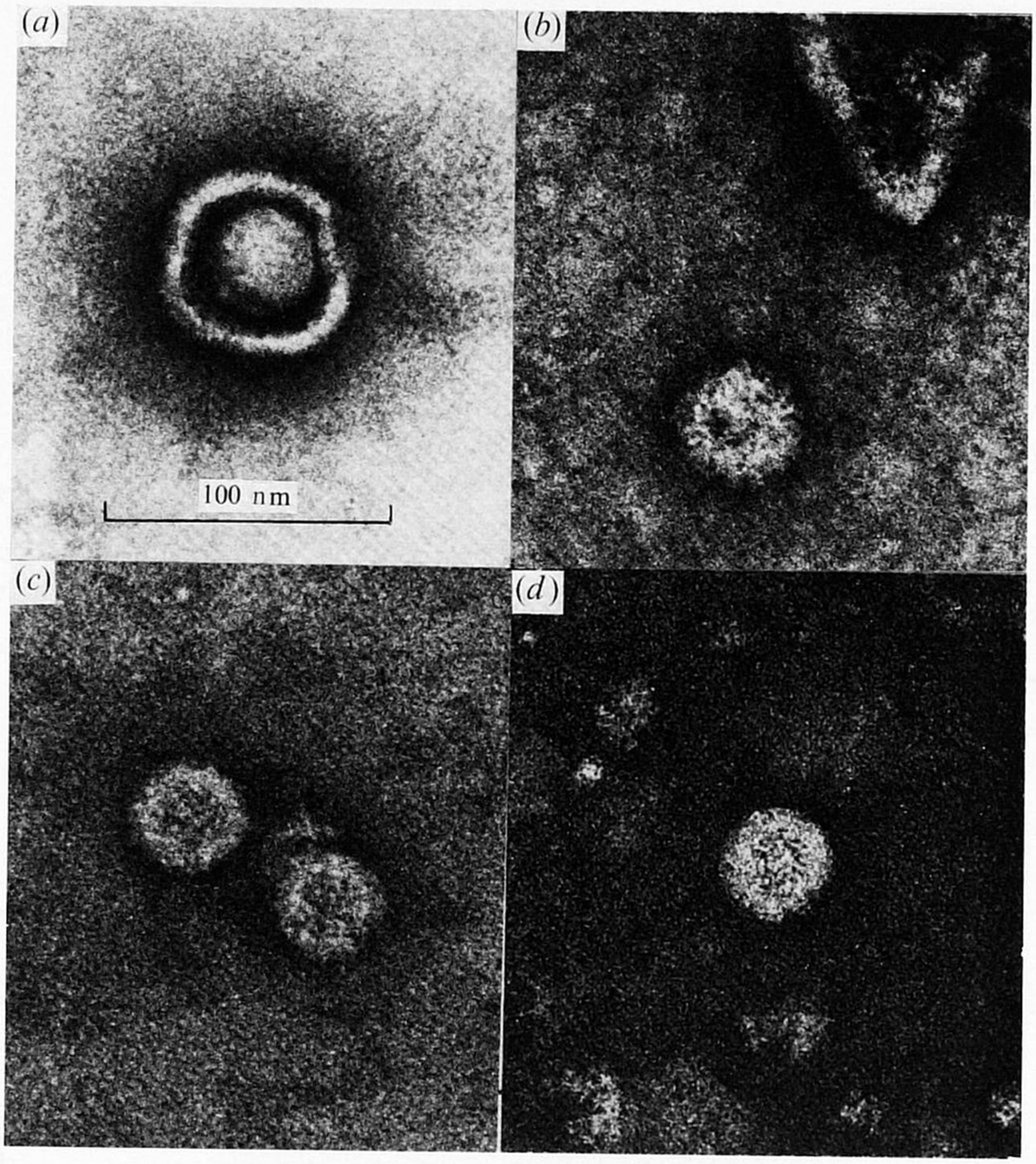

Fig. 8. LDV after treatment with 0·1 % NP40. (*a*), (*b*) Purified LDV mounted on carbon-coated grids, treated for 10 min with 0·1 % NP40, and stained with UA. Nucleocapsids within remainders of their envelopes (*a*), free nucleocapsids and disrupted envelopes (*b*) were observed. (*c*), (*d*) LDV nucleocapsids purified by rate zonal gradient sedimentation as denoted in Fig. 7(*b*), stained with UA.

envelope, which can be discerned especially in disrupted virus particles. In general, the surface of the LDV membrane appears smooth, in contrast to SIN virus where projections are apparent (Fig. 5).

In well-preserved, unfixed particles from glycerol gradients which were penetrated by PTA (Fig. 6) an intermediate layer could be demonstrated between the envelope proper and the virus core. It is slightly thinner and less electron-lucent than the envelope itself.

Purification and characterization of the nucleocapsid

Treatment with the non-ionic detergent NP40 has been shown to yield the virus nucleocapsid and the envelope proteins of LDV (Michaelides & Schlesinger, 1973). For this series of experiments the virus was purified on sucrose gradients directly from mouse ascites fluid (Fig. 7*a*). After disruption of purified LDV using NP40, a collection of fragmented envelopes and free nucleocapsids appeared in the electron microscope. Also nucleocapsids within remainders of their envelopes were observed (Fig. 8*a*, *b*). Identical results were

achieved using 0·05 % sodium deoxycholate as detergent (results not shown here). For further characterization, the nucleocapsids were purified from NP40-treated virus preparations by rate zonal sedimentation. In isokinetic glycerol as well as sucrose gradients a sedimentation coefficient of 176S was determined (Fig. 7*b*). The peak radioactivity of the SF virus nucleocapsid marker sedimented corresponding to a coefficient of 153S. In the electron microscope negatively stained purified LDV nucleocapsids appeared as spherical particles measuring 34 ± 4 nm in diam. ($n = 80$) (Fig. 8*c*, *d*).

DISCUSSION

The evidence for regarding the particulate structures described as LDV is based mainly on the observation that their appearance and frequency in the electron microscope correlated with the results of infectivity tests in mice. The density of 1·14 g/ml in glycerol found in our experiments is the same as reported by Michaelides & Schlesinger (1973) for sucrose gradient analyses; values between 1·12 g/ml (Darnell & Plagemann, 1972), 1·17 g/ml (Notkins, 1971) and 1·17 to 1·20 g/ml (Riley *et al.* 1964) have been published for the virus particle.

In the electron microscope, purified and concentrated LDV preparations contain spherical particles with diam. between 62 and 80 nm, depending on the fixation and staining procedures applied. In our experiments elliptical or rod-shaped virus particles as described by others in thin sections of pellets from viraemic mouse plasma (de Thé & Notkins, 1965) or infected peritoneal macrophages (Prosser & Evans, 1967) did not appear in negatively stained preparations or in pellets of purified LDV (F. Spies, personal communication). The well-known togavirus artefacts (Horzinek, 1973*a*, *b*) such as 'blebs', distorted and disrupted particles, which predominated in PTA preparations could be practically eliminated by previous fixation, as already suggested by Bladen & Notkins (1963).

NP40 treatment of LDV revealed membrane fragments and intact nucleocapsids. The latter were studied *in situ* and after purification by rate zonal sedimentation. In both preparations the nucleocapsids appeared as isometric particles 35 ± 4 nm across. The observation that the nucleocapsid from LDV sediments faster than that from an alphavirus (Michaelides & Schlesinger, 1973) could be confirmed in our experiments. Studies are in progress to identify its substructure.

Based on the present results we propose that LDV should be regarded as an additional nonarbo togavirus. The arguments in favour of this classification (Horzinek, 1973*a*, *b*) include the size of the virus particle and of its spherical nucleocapsid, the presence of a lipid-containing envelope and of a single-stranded, infectious RNA. Michaelides & Schlesinger (1973) consider LDV as flavivirus-like because of the mol. wt. of its non-glycosylated nucleocapsid protein; the sedimentation coefficient of about 200 S (Boutlon & Westaway, 1972) would further support this suggestion. However, in its virus particle size LDV clearly does not differ from a member of the alphaviruses (SIN), as demonstrated in mixed preparations (Fig. 5). Virus particle size is generally considered as a structural criterion for differentiating between the two arthropod-borne togavirus genera (Mussgay, 1964; Horzinek, 1973*a*). In order to avoid conflicting taxonomic situations it is felt that more details on the structure and antigenic composition must be known before assigning serologically unrelated nonarbo togaviruses to established genera or creating new subgroups within the togavirus family.

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REFERENCES

BLADEN, H. A. & NOTKINS, A. L. (1963). Electron microscopic demonstration of the lactic dehydrogenase agent. *Virology* **21**, 269–271.

BOULTON, R. W. & WESTAWAY, E. G. (1972). Comparison of togaviruses: Sindbis virus (group A) and Kunjin virus (group B). *Virology* **49**, 283–289.

de BUY, H. G. & JOHNSON, M. L. (1965). Some properties of the lactic dehydrogenase agent of mice. *Journal of Experimental Medicine* **122**, 587–600.

DARNELL, M. B. & PLAGEMANN, P. G. W. (1972). Physical properties of lactic dehydrogenase-elevating virus and its ribonucleic acid. *Journal of Virology* **10**, 1082–1085.

HORZINEK, M. C. (1973*a*). The structure of togaviruses. *Progress in Medical Virology* **16**, 109–156.

HORZINEK, M. C. (1973*b*). Comparative aspects of togaviruses. *Journal of General Virology* **20**, 87–103.

HORZINEK, M. C., MAESS, J. & LAUFS, R. (1971). Studies on the substructure of togaviruses. II. Analysis of equine arteritis, rubella, bovine viral diarrhea and hog cholera viruses. *Archiv für die gesamte Virusforschung* **33**, 306–318.

HORZINEK, M. C. & MUSSGAY, M. (1971). Studies on the substructure of togaviruses. I. Effect of urea, deoxycholate, and saponin on the Sindbis virion. *Archiv für die gesamte Virusforschung* **33**, 296–305

MELNICK, J. L. (1969). Summary of classification of animal viruses, 1969. *Progress in Medical Virology* **11**, 451–453.

MICHAELIDES, M. C. & SCHLESINGER, S. (1973). Structural proteins of lactic dehydrogenase virus. *Virology* **55**, 211–217.

MURPHY, F. A., HALONEN, P. E. & HARRISON, A. K. (1968). Electron microscopy of the development of rubella virus in BHK 21 cells. *Journal of Virology* **2**, 1223–1227.

MUSSGAY, M. (1964). Growth cycle of arboviruses in vertebrate and arthropod cells. *Progress in Medical Virology* **6**, 193–267.

NIWA, A., YAMAZAKI, S., BADER, J. & NOTKINS, A. L. (1973). Incorporation of labelled precursors into the RNA and proteins of lactic dehydrogenase virus. *Journal of Virology* **12**, 401–404.

NOTKINS, A. L. (1965). Lactic dehydrogenase virus. *Bacteriological Reviews* **29**, 143–160.

NOTKINS, A. L. (1971). Enzymatic and immunologic alterations in mice infected with lactic dehydrogenase virus. *American Journal of Pathology* **64**, 733–746.

PFEFFERKORN, E. R. & HUNTER, H. S. (1963). Purification and partial chemical analysis of Sindbis virus. *Virology* **20**, 433–445.

PROSSER, P. R. & EVANS, R. (1967). An electron microscopic study of lactic dehydrogenase virus in cultures of mouse peritoneal macrophages. *Journal of General Virology* **1**, 419–424.

RILEY, V., CAMPBELL, H. A., LOVELESS, J. D. & FITZMAURICE, M. A. (1964). Density gradient centrifugation and molecular sieve studies of lactic dehydrogenase-elevating virus-like agents. *Proceedings of the American Association for Cancer Research* **5**, 53.

de THÉ, G. & NOTKINS, A. L. (1965). Ultrastructure of the lactic dehydrogenase virus (LDV) and cell-virus relationships. *Virology* **26**, 512–516.

WILDY, P. (1971). Classification and nomenclature of viruses. *Monographs in Virology* **5**.

van der ZEIJST, B. A. M. & BLOEMERS, P. (1974). 4698 Isokinetic glycerol and sucrose gradients for density gradient centrifugation. In *Handbook of Biochemistry*. Edited by G. Fasman. The Chemical Rubber Co.

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