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The Structural Proteins of Equine Arteritis Virus

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Equine arteritis virus (EAV) grown in Vero, BHK-21, and RK-13 cells was purified by pelleting, Sepharose 6B chromatography, and sucrose gradient centrifugation. Analysis of whole gradients by polyacrylamide slab gel electrophoresis and subsequent autoradiography revealed a large number of proteins in all fractions. Comparison of protein patterns of virus grown in the different cell systems showed that only three proteins with molecular weights of 12,000 (VP1), 14,000 (VP2), and 21,000 (VP3) were virus specific. VP1 is a phosphorylated core protein, while VP3 is a glycoprotein. These findings, together with data obtained earlier about morphology and RNA of the virion, lend further support to inclusion of EAV in the family Togaviridae with a possible relationship to lactic dehydrogenase virus.

INTRODUCTION

Based on morphological, physical, and chemical properties, equine arteritis virus (EAV) has been classified as a nonarthropod-borne member of the family Togaviridae (for a review see Horzinek, 1973b). Further support to this classification has been recently obtained in our laboratory from studies on the viral genome, which was shown to be an infectious colinear single-stranded RNA molecule of about four-million daltons (Van der Zeijst *et al.*, 1975). Analysis of purified EAV on polyacrylamide gels has resulted in the demonstration of nine proteins (Hyllseth, 1973) which would be at variance with the togavirus concept since only three structural polypeptides have been found for most members of the family. Because of these considerations and the exceptional ratio between the estimated coding capacity of the genome and the sum of the molecular weights of its nine reported polypeptides, the protein composition of EAV was reinvestigated.

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METHODS

The following methods have been described before (Van der Zeijst *et al.*, 1975): Culture of BHK-21 cells, preparation of high-titered virus stocks from BHK-21 clone 13 cells, plaque assay of the virus and preparation of labeled marker Semliki forest virus (SFV). Vero and RK-13 cells were grown in TC 199 medium supplemented with 10% calf serum. In this study a heat-resistant (HR) virus strain of EAV was used which had been prepared according to Burge and Pfefferkorn (1966) by three cycles of heating virus stocks at 65° for 3 min and regrowing survivors.

Preparation of radioactive virus. For labeling experiments in monolayers, the growth medium was replaced by 10 ml of Dulbecco's modification of minimal essential medium (DMEM) per 75 cm^2 Falcon flask with the following specifications: In the case of labeling with glucosamine, the glucose concentration was reduced to 450 mg/liter; for [^{35}S]methionine-labeling methionine was omitted from the medium; and for [^{14}C]amino acid-labeling an amino acid-free medium was used. Cells were infected at multiplicities around 10. Six hours later label was added (10 μCi/ml of [6-^{3}H]glucosamine, 13 Ci/mmol; 10 μCi/ml

of [^{35}S]methionine, 300 Ci/mmol; 10 μCi/ml of [^{14}C]protein hydrolysate, 55 mCi/mAtom, The Radiochemical Centre, Amersham, England). Virus was harvested at 20 hr after infection.

[^{3}H]Uridine-labeled virus was grown in cell suspensions as described by Van der Zeijst *et al.* (1975). ^{32}P-labeling was carried out accordingly using phosphate-free DMEM supplemented with 120 μCi/ml of carrier-free labeled phosphate (The Radiochemical Centre, Amersham, England). Cells were removed by low speed centrifugation from culture fluids and virus was pelleted at 100,000 *g* for 3 hr. Pellet material resuspended in 0.3 ml of TES buffer (0.02 *M* Tris–HCl, 1 m*M* EDTA, 0.1 *M* NaCl, pH 7.4) was passed through a 0.9 × 4.0-cm Sepharose 6B column and layered on top of a 20–50% (w/w) sucrose gradient in the same buffer. Centrifugation was at 50,000 rpm at 5° in a SW50L rotor for 3 hr. Gradients were sampled in 250-μl fractions.

Polyacrylamide gel electrophoresis. The slab gel technique described by Studier (1973) with the gel system of Laemmli (1970) was used. After electrophoresis (50 V, 6 hr) gels were stained using 1% Coomassie blue in acetic acid–water–methanol (7–43–50% by vol) for 2 hr. Gels were destained in the same solution, dried *in vacuo* on Whatman 3 MM filter paper, and autoradiographed on RP Royal X-omat film (Kodak, Rochester, N.Y.). Cylindrical gels (13%), prepared according to Maizel (1971), were fractionated and counted as described previously (Van der Zeijst *et al.*, 1975).

Preparation of viral nucleocapsids. Gradient-purified EAV material was mixed with two volumes 1.5% Triton X-100 in TES buffer and kept at room temperature for 5 min. Subsequently, nucleocapsids were separated from envelopes by centrifugation at 20° in an isokinetic sucrose gradient in TES buffer containing 0.1% Triton X-100. Centrifugation was carried out in a SW50L rotor at 46,000 rpm for 45 min. The gradient was calculated as described before (Van der Zeijst and Bloemers, 1975) for a top concentration of 15% (w/w) and an assumed particle density of 1.4 g/ml. Material from peak fractions was further concentrated by precipitation with 5% trichloroacetic acid; after washing the pellet with acetone it was resuspended in disintegration buffer according to Studier (1973).

RESULTS

Under the conditions described virus titers of approximately 3×10^7 PFU/ml were reached in BHK-21 cells at the beginning of cytopathic changes, 20 hr after infection. At this time a maximal amount of [^{35}S]methionine-labeled virus was found after concentration of the virus and purification in a sucrose gradient. Figure 1A shows that the peak of radioactivity in the sucrose gradient coincides with the maximum of infectivity. After 3 hr of centrifugation the virus had just reached its buoyant density position of 1.155 g/ml. Aliquots of every gradient fraction were analyzed by electrophoresis in a polyacrylamide slab gel (Fig. 1B). Autoradiographs revealed a large number of protein bands present in all fractions. Three proteins with molecular weights of 12,000, 14,000, and 21,000, however, were found accumulating at the position of the virus. These three proteins were also found in extracts of cells treated with 1 μg/ml of actinomycin D and labeled from 5 to 7 hr after infection with EAV, whereas they were absent from control cells (data not shown). It was concluded that at least these three proteins are present in the virion; however, other polypeptides, e.g., the prominent 32,000-daltons protein, also might be of viral origin, although they were not consistently encountered in the systems studied.

Further purification of the virus would have been the most direct way to detect other possible viral proteins which might be obscured by the cellular contaminants present in most of the gradient fractions. Because of significant losses a different approach was chosen. Since contaminants would differ in different cell systems, [^{35}S]methionine-labeled virus grown in BHK-21, Vero, and RK-13 cells were used in a comparative analysis. For virus grown in Vero and RK-13 cells the incorporation of label was comparable to BHK-21

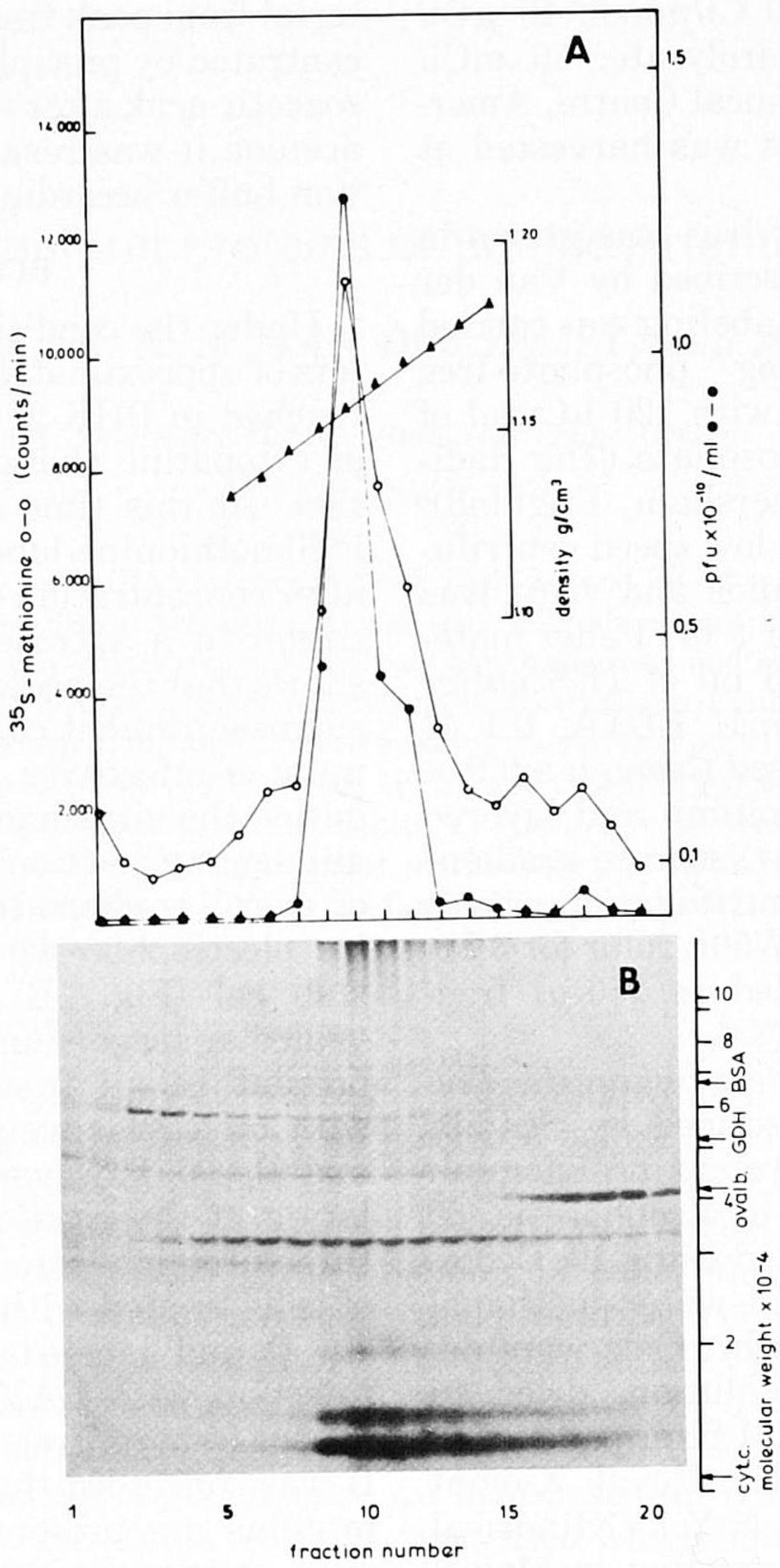

FIG. 1. Isopycnic banding and electrophoretic analysis of gradient fractions of EAV. (A) [^{35}S]methionine-labeled virus grown in BHK-21 cells was purified in a 20–50% sucrose gradient and assayed for hot trichloroacetic acid-precipitable radioactivity (○——○) and infectivity (●——●). The density of gradient fractions (▲——▲) was calculated from the refractive index. (B) Twenty-microliter aliquots of each fraction were analyzed by electrophoresis in a polyacrylamide slab gel. Molecular weights were determined using 2 μg of the following marker proteins: Bovine serum albumin (BSA, MW = 68,000), glutamate dehydrogenase (GDH, MW = 53,000), ovalbumine (MW = 43,000), cytochrome *c* (MW = 11,000).

grown virus; no other proteins than those with molecular weights of 12,000, 14,000, and 21,000 were concentrated in all three EAV preparations at the positions of viral infectivity (Fig. 2). We have designated these proteins as VP1, VP2, and VP3, respectively.

In Figs. 1 and 2 it is shown that VP3 has incorporated little [^{35}S]methionine. This appeared not to be due to a low methionine content of this protein, since it is also poorly labeled using [^{14}C]amino acids (Fig. 4C). When assuming a comparable efficiency of labeling for each of the proteins the molar ratios between VP1:VP2:VP3 would be about 27:5:1 (Fig. 4C).

Nucleocapsids were isolated by centrifugation in a sucrose gradient after treatment with 1% Triton X-100. If prepared from [^{3}H]uridine-labeled virus they con-

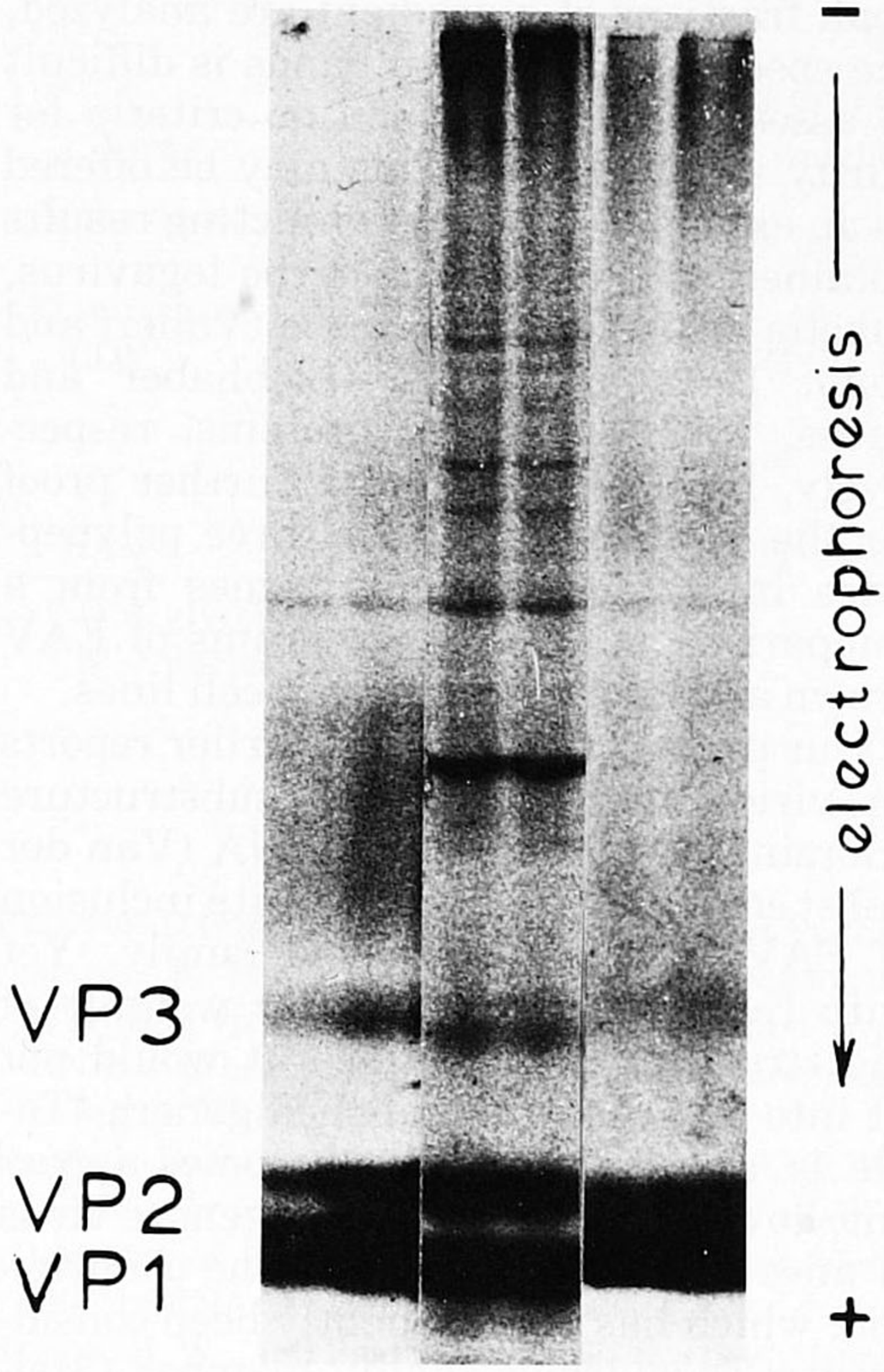

FIG. 2. Comparison of proteins present in EAV grown in Vero (A), BHK-21 (B), and RK-13 (C) cells. [^{35}S]Methionine-labeled virus was purified and analyzed as indicated in Fig. 1. Only fractions containing maximum virus titers are shown.

tained all the tritium label. EAV capsids sedimented slightly faster (158 S) than those of SFV (142 S) (Fig. 3). Nucleocapsids of virus labeled with [^{35}S]methionine contained 52–54% of the total radioactivity (Fig. 3). Electrophoresis of this material in a polyacrylamide slab gel revealed that VP1 is exclusively found in this structure and must therefore be regarded as the nucleocapsid protein (Fig. 4A). Electrophoresis of purified ^{32}P-labeled EAV in the same gel demonstrated that VP1 is phosphorylated. The radioactivity at the position of VP1 was not attributable to RNA or phospholipids since no label was lost after incubation for 2 hr at 37° with 1% Triton X-100, RNAse, and phospholipase C (20, respectively, 200 μg/ml, Boehringer, Mannheim, West Germany). Furthermore when nucleocapsids were prepared of ^{32}P-labeled EAV all radioactivity precipitable with cold trichloroacetic acid (50% of the total) was encountered in the 158-S structure. About 6% of the total phosphate label was precipitable with hot trichloroacetic acid.

When [^{3}H]glucosamine-labeled virus was analyzed together with [^{14}C]amino acid-labeled EAV serving as a reference, it was found that 50% of the radioactivity accumulated at the position of VP3 (Fig. 4D).

DISCUSSION

It has been demonstrated that EAV possesses three structural polypeptides with molecular weights of 12,000 (VP1), 14,000 (VP2), and 21,000 (VP3), respectively. Parallel electrophoretic analysis on polyacrylamide slab gels has shown that these proteins were confined to gradient fractions containing infectious virus, whereas several additional bands extended through the gradient in a random manner. If only

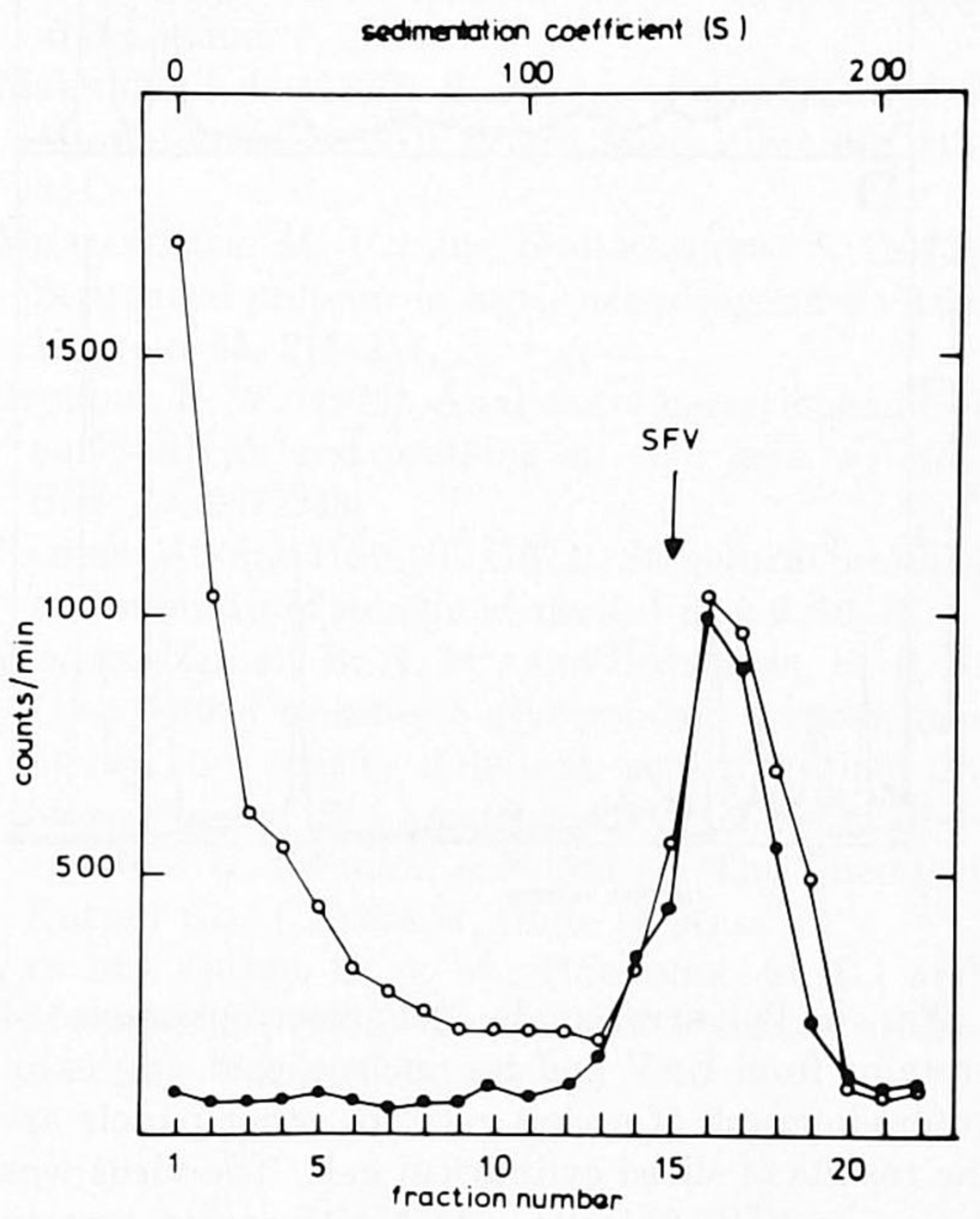

FIG. 3. Isolation of nucleocapsids of EAV. [^{3}H]uridine- (●——●) and [^{35}S]methionine- (○——○) labeled virus grown in BHK-21 cells was treated with Triton X-100 and capsids were purified by sucrose gradient centrifugation. The arrow indicates the position of SFV capsids in a parallel gradient.

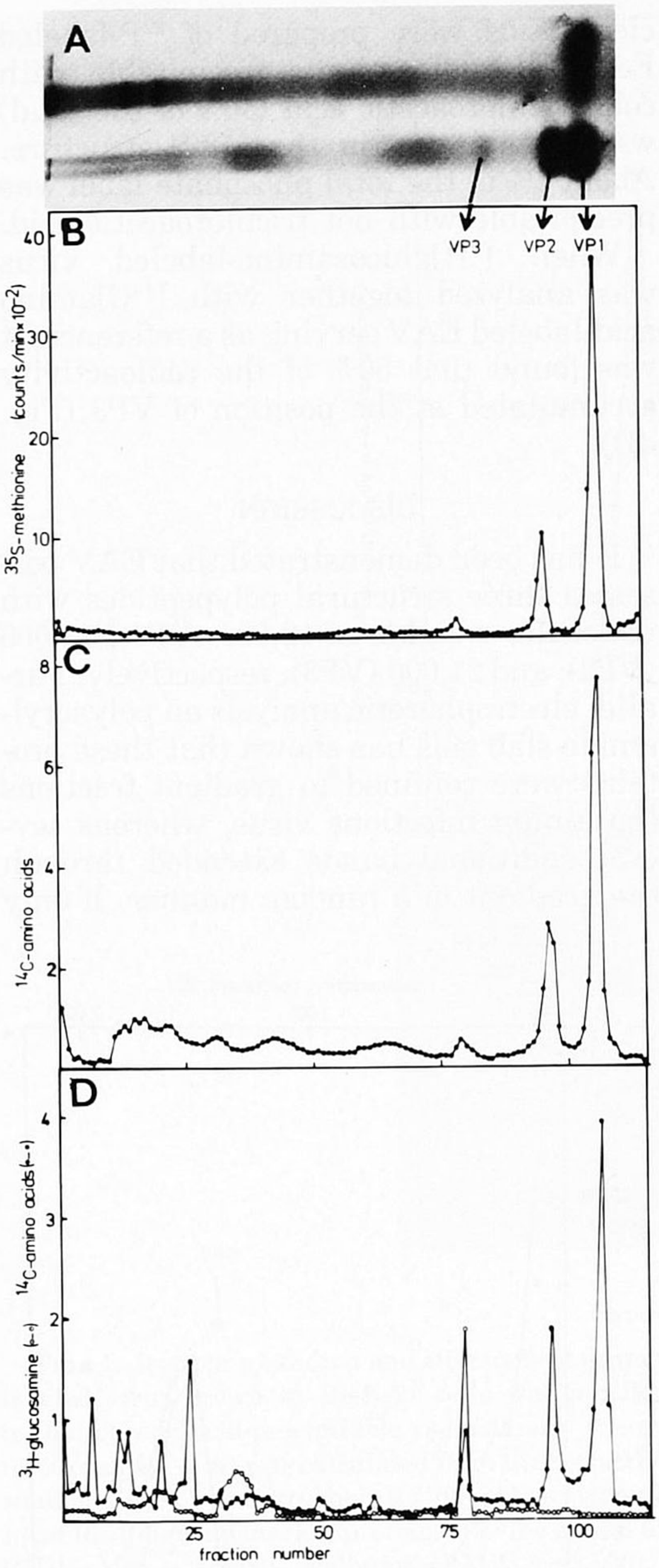

FIG. 4. Polyacrylamide gel electrophoresis of proteins from EAV and its nucleocapsid. (A) is an autoradiograph of a slab gel. The other panels are the results of sliced cylindrical gels. The virus was grown in BHK-21 cells. (A) Nucleocapsid protein from [^{35}S]methionine-labeled virus (lane 1), the intact virus (lane 3) and ^{32}P-labeled virus (lane 2). (B) EAV labeled with [^{35}S]methionine. (C) EAV labeled with [^{14}C]amino acids. (D) Coelectrophoresis of EAV labeled with [^{14}C]amino acids and [^{3}H]glucosamine.

peak fractions of a gradient are analyzed, the specificity of protein bands is difficult to assess, especially when no criteria for purity are available. This may be offered as an explanation to the conflicting results obtained with another nonarbo togavirus, rubella virus, for which three (Vaheri and Hovi, 1972) and eight (Liebhaber and Gross, 1972) structural proteins, respectively, have been claimed. Further proof for the viral nature of the three polypeptides in our experiments comes from a comparison of electropherograms of EAV grown and labeled in different cell lines.

Our present findings and earlier reports on virion morphology and substructure (Horzinek *et al.*, 1971) and RNA (Van der Zeijst *et al.*, 1975) permit definite inclusion of EAV in the Togaviridae family. Yet with respect to the molecular weights of the structural polypeptides, it would not fit into any of the established genera (Table 1). A close similarity becomes apparent, however, if lactic dehydrogenase virus of mice (LDV) is included in the comparison, which has independently been considered a Togaviridae family member (Melnick, 1970; Horzinek, 1973a) on the base of its particle structure, RNA and polypeptide composition (Horzinek *et al.*, 1975; Darnell and Plagemann, 1972; Brinton-Darnell *et al.*, 1975; Michaelides and Schlesinger, 1973; Brinton-Darnell and Plagemann, 1975). EAV and LDV share a number of additional properties which distinguish them from alpha- and flaviviruses (Horzinek, 1973a,b); further studies, particularly on a possible antigenic relationship must show whether these viruses should be assigned the taxonomic status of a genus.

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TABLE 1

COMPARATIVE LISTING OF TOGAVIRUS STRUCTURAL POLYPEPTIDES

Virus	Molecular weights × 10^{-3}					References
Alphaviruses	53–50 G[a]	32–30 N[b]				
	53–50 G					see Horzinek, 1973
Flaviviruses	60–50 G			14–13 N	9–8	
LDV		28 G	17	13 N		Michaelides and Schlesinger, 1973
		44–24 G	18	15 N		Brinton-Darnell and Plagemann, 1975
EAV		21 G	14	12 N		Present report

[a] G = glycoprotein.
[b] N = nucleocapsid protein.

REFERENCES

BRINTON-DARNELL, M., COLLINS, J. K., and PLAGEMANN, P. G. W. (1975). Lactic dehydrogenase-elevating virus replication, maturation and viral RNA synthesis in primary mouse macrophage cultures. *Virology* **65**, 187–195.

BRINTON-DARNELL, M., and PLAGEMANN, P. G. W. (1975). Structure and chemical-physical characteristics of lactic dehydrogenase-elevating virus and its RNA. *J. Virol.* **16**, 420–433.

BURGE, B. W., and PFEFFERKORN, E. R. (1966). Isolation and characterization of conditional-lethal mutants of Sindbis virus. *Virology* **30**, 204–213.

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

HORZINEK, M., MAESS, J., and LAUFS, R. (1971). Studies on the substructure of togaviruses. II. Analysis of equine arteritis, rubella, bovine viral diarrhoea and hog cholera viruses. *Arch. Gesamte Virusforsch.* **33**, 306–318.

HORZINEK, M. C. (1973a). Comparative aspects of togaviruses. *J. Gen. Virol.* **20**, 87–103.

HORZINEK, M. C. (1973b). The structure of togaviruses. *Progr. Med. Virol.* **16**, 109–156.

HORZINEK, M. C., VAN WIELINK, P. S., and ELLENS, D. J. (1975). Purification and electron microscopy of lactic dehydrogenase virus of mice. *J. Gen. Virol.* **26**, 217–226.

HYLLSETH, B. (1973). Structural proteins of equine arteritis virus. *Arch. Gesamte Virusforsch.* **40**, 177–188.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685.

LIEBHABER, H., and GROSS, P. A. (1972). The structural proteins of rubella virus. *Virology* **47**, 684–693.

MAIZEL, J. V., JR. (1971). Polyacrylamide gel electrophoresis of viral proteins. *In* "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), Vol. 5, pp. 179–246. Academic Press, New York and London.

MELNICK, J. L. (1970). Summary of classification of animal viruses, 1970. *Progr. Med. Virol.* **12**, 337–341.

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

STUDIER, F. W. (1973). Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J. Mol. Biol.* **79**, 237–248.

VAHERI, A., and HOVI, T. (1972). Structural proteins and subunits of rubella virus. *J. Virol.* **9**, 10–16.

VAN DER ZEIJST, B. A. M., and BLOEMERS, H. P. J. (1975). 4698 Isokinetic glycerol and sucrose gradients for density gradient centrifugation. *In* "Handbook of Biochemistry and Molecular Biology" (G. D. Fasman, ed.). 3rd ed. The Chemical Rubber Co., Cleveland, Ohio, in press.

VAN DER ZEIJST, B. A. M., HORZINEK, M. C., and MOENNIG, V. (1975). The genome of equine arteritis virus. *Virology* **68**, 418–425.