CHROMATOGRAPHIC PREPARATION OF PURIFIED STRUCTURAL PROTEINS FROM FOOT-AND-MOUTH DISEASE VIRUS

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Summary : After purification of an O type of Foot-and-mouth disease virus by caesium chloride centrifugation we obtain purified fractions of viral structural proteins by chromatography on DEAE Sephadex A 25. We point out a partial loss of the polypeptide VP1 during viral purification and present an easy separation of this polypeptide using its ionisation state at pH 8.6. These observations should be correlated with the biological role of VP1 when giving rise to neutralizing antibodies against the total virus.

Several pathogenic viruses belong to the picornavirus group : poliovirus, foot-and-mouth disease virus (FMDV), mouse-Elberfeld virus, bovine enterovirus... (1). The disorders induced by these viral agents in humans as well as in animals give them a considerable importance.

FMDV has been subdivided into seven distinct immunological types : O, A, C, Asia, Sat_1, Sat_2, Sat_3. The neutralizing antibody activity directed against one type does not cross react with those of other types, so animals must be immunized with polyvalent vaccines. The antigenic differences between these types could be a consequence of variations in their capsid structures.

The determination of molecular weights of constitutive viral proteins allows for the distinction of two groups of viruses (2) : one possessing four classes of polypeptides (O, Asia serotypes), the other possessing two classes of polypeptides (A, C, $Sat_{1, 2, 3}$).

The present experiments were performed with an O type mutant of FMDV, the capsid of which is constituted of four major polypeptides (VP1 to VP4).

Previous work showed that the polypeptide VP1 occupies an external position on the virus surface (3, 4). We postulated that these data are consistent with the immunogenicity of VP1 in the phenomena of viral neutralization.

In order to study the biological properties of each polypeptide, we isolated the different FMDV components. We report here the steps leading from whole cell lysate through virus to the purified polypeptides.

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METHODS

Virus production : the virus used for this study was a recently isolated clone from an O serotype of our laboratory ; the virus was grown in monolayers of BHK 21 cells (5) in Eagle's medium (6).

Virus titration : virus infectivity wasmeasured in BHK 21 clone S 13 according to Cooper (7) and La Bonnardière (8).

Virus labelling : virus containing labelled proteins was grown in monolayers of cells in Earle's saline Tris buffered medium, without serum, containing $[^{14}C]$ labelled aminoacids from a Chlorella hydrolysate (3 µCi/ml). Double labelled virus was obtained in the same conditions with $[^{3}H]$ labelled uridine (10 µCi/ml) and $[^{14}C]$ labelled aminoacids.

Virus concentration : infected cells were frozen and thawed three times. Cell debris were removed by centrifugation at 4000 rpm in a Sorvall centrifuge RC3, rotor HG-4L, for ten minutes. Solid PEG (Polyethylene glycol, Carbowax 6000) was added to the clarified supernatant to a 10 % concentration (w/v) ; after agitation for one hour at 4° the suspension was centrifuged at 5000 rpm for 15 minutes. The pellet was resuspended in Tris-HCl 0.080 M, NaCl 0.2 M, EDTA 5.10^{-3} M, pH 7.6 to 1/10 of the initial volume.

Virus purification : the density of the PEG concentrate was adjusted to 1.45 with caesium chloride (Merck); Virus was banded by centrifugating overnight (18 hours) in a SW 65 rotor (Spinco centrifuge model L2 65B) at 55 000 rpm at 4°C. Samples of 0.3 ml were collected with a density gradient fractionator (ISCO model 183).

Viral protein analysis by polyacrylamide gel electrophoresis : the process of disrupting purified virus in sodium dodecyl sulfate, urea and mercaptoethanol before application of the samples to be analysed as well as the processing and subsequent radioactive assay of the gels were previously described (4).

Desalting of purified virus : caesium chloride was taken off by G 25 Sephadex filtration in Tris-HCL 0.014 M buffer, pH 8.6.

DEAE Sephadex chromatography : solid urea was added to the desalted virus in the above buffer to a molarity of 8 moles/1, then the sample was layered on a DEAE Sephadex A 25 column (Pharmacia K 9/30) in 0.014 M Tris-HCI buffer, 6M urea, pH 8.6. Elution conditions are presented under Fig.3. After radioactive assay the fractions were pooled, concentrated by vacuum ultrafiltration through a collodion membrane (Sartorius type) and analysed on polyacrylamide gels after being treated as the total virus (4).

RESULTS

I - Virus purification

Cell lysate concentration : the first step of PEG precipitation reduced tenfold the volume of the initial solution ; no infectious particles were left in the supernatant ; after a careful resuspension of the precipitate a second step of concentration would again decrease the sample volume without any change in the total number of PFU.

Isopycnic centrifugation : in the first caesium centrifugation (Fig.1)

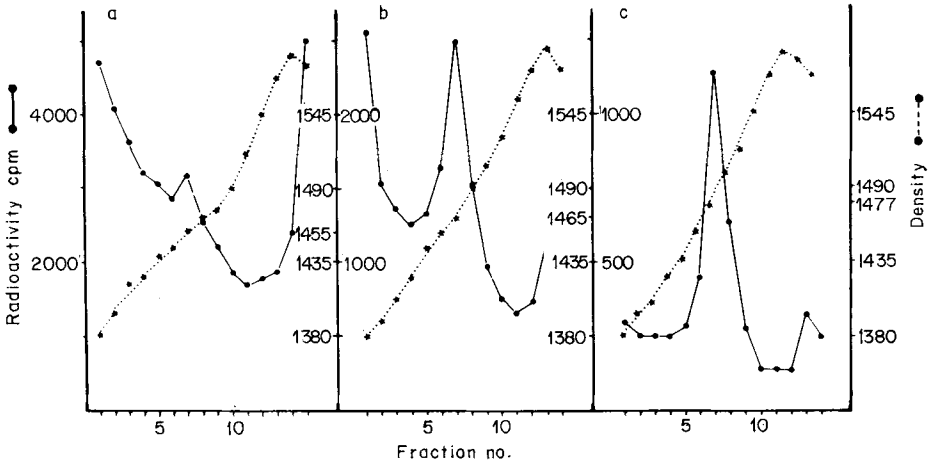

Figure 1 : Successive isopycnic centrifugations of FMDV.

The [^{3}H] uridine activities (●—●) were counted in 5 µl samples for each fraction of the first (a), second (b) and third (c) steps of centrifugation ; densities (✱·····✱) were determined from each refractive index. Virus is successively banded at 1.455, 1.465 and 1.477 g/ml.

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we observed one peak (density 1.46) containing numerous viruses when controlled in electron microscopy. An important part of the labelling was found in light material. Fractions 6 - 8 were pooled, adjusted to the density 1.45 and centrifuged again ; the peak detected in this second centrifugation was collected, adjusted to the density 1.45 and centrifuged a third time. The second and third centrifugations indicated a displacement of the virus peak to heavier densities (Fig.1). The electrophoregrams of the virus obtained after successive steps of centrifugation (Fig. 2a, 2b,

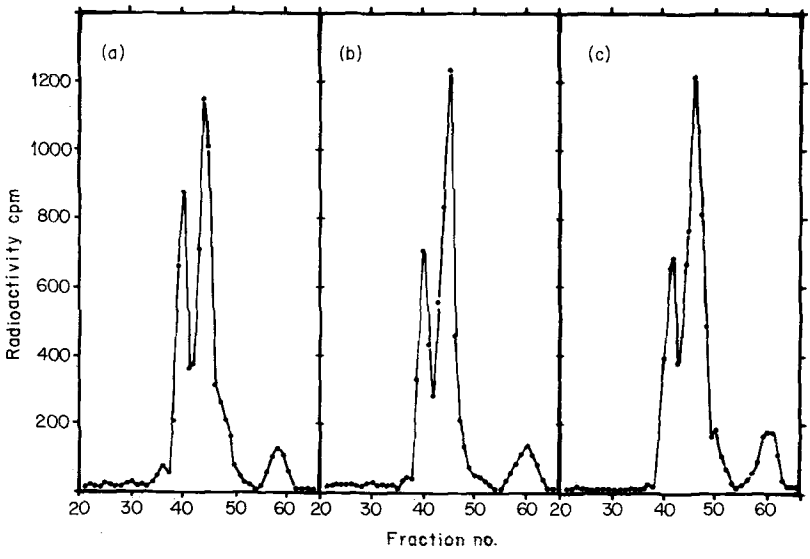

Figure 2 : Electrophoresis of polypeptides from virus purified by three steps of caesium chloride centrifugation. [^{14}C] labelled viral proteins were allowed to migrate in 10 % polyacrylamide gels, in 0.1 M sodium phosphate buffer pH 7.2,0.1 % SDS. The first twenty fractions contained no labelled material (migration from left to right). (a), (b), (c) : 1st, 2nd, 3rd steps of caesium chloride gradient purification.

2c) showed a difference in the distribution of the four polypeptides. Since proteins are supposed to be labelled uniformly with a mixture of [^{14}C] labelled aminoacids the peak areas▼ are representative of the polypeptide proportions in the virus. The calculation of the area ratios (Table 1) showed a decrease in the amount of VP1 with a relative increase of the other polypeptides.

II - Purification of structural polypeptides

Chromatography of 8M urea treated virus : double labelled [^{3}H] uridine [^{14}C] aminoacid virus was concentrated and purified by only one isopycnic centrifugation, desalted then disrupted in 8M urea and chromatographed on a DEAE Sephadex A 25 column as previously described (9). The elution gradient allowed for separation of the [^{14}C] labelled proteins from

▼ "area" is measured by summing up the [^{14}C] counts in the peak fractions.

	Molecular weight	First ccc.[1] Peak area (%)[2]	First ccc.[1] Molar ratio[3]	Second ccc.[1] Peak area (%)[2]	Second ccc.[1] Molar ratio[3]	Third ccc.[1] Peak area (%)[2]	Third ccc.[1] Molar ratio[3]
VP1	34 000	36	1.06	32.5	0.95	27	0.80
VP2-3	28 000	58	2.1	59.5	2.15	61	2.2
VP4	14 000	6	0.45	9.5	0.65	11.5	0.85

Table 1 : Polypeptide composition of purified virus.
1 : ccc = caesium chloride centrifugation
2 : peak area (%) = percentage of the polypeptide in total viral proteins = percent ratio of [^{14}C] counts in the corresponding peak to the total [^{14}C] counts in all the protein peaks.
3 : "peak area (%)" = "molar ratio" X "molecular weight" X 10^{-3}

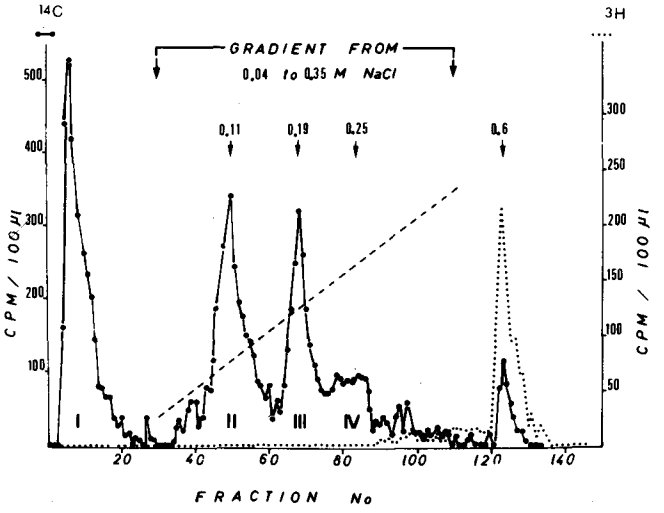

Figure 3 : DEAE-Sephadex chromatography of 8M urea treated double labelled virus.
Column volume : 15 ml, diameter 0.9 cm ; sample load : 1,5 ml ; fraction volume : 1 ml ; flow rate : 20 ml/hour.
After draining out of the unadsorbed fraction I with 30 ml of 6M urea Tris buffer, pH 8.6, a linear gradient from 0.04M to 0.35M NaCl was applied in 80 ml of the same buffer, then 20 ml of 0.6M NaCl eluted the remaining material.
The [^{14}C] and [^{3}H] activities of the fractions were counted on a SL 30 Intertechnique liquid scintillation spectrometer and computed with an Intertechnique programme on the Multi 8 Intertechnique computer.

the [^{3}H] uridine labelled nucleic acid (Fig. 3) ; a peak of protein (I) passed unadsorbed through the column ; a gradient in sodium chloride concentration from 0.04 M to 0.35 M NaCl allowed for elution of three peaks of proteins : II, III, IV at 0.11, 0.19, 0.25 M NaCl. Viral RNA was just eluted at 0.6 M NaCl. Higher molarities released no more labelled material.

Characterization of the fractions : after concentration of the pooled fractions, the labelled material of each peak was analysed by gel electrophoresis (Fig. 4). The unadsorbed material, fraction I, gave a well defined radioactive peak (Fig. 4a). The fraction II was composed of two distinct populations of proteins (Fig. 4c). The fraction III was constituted of one peak with probably a minor component (Fig. 4e). The polypeptide in the

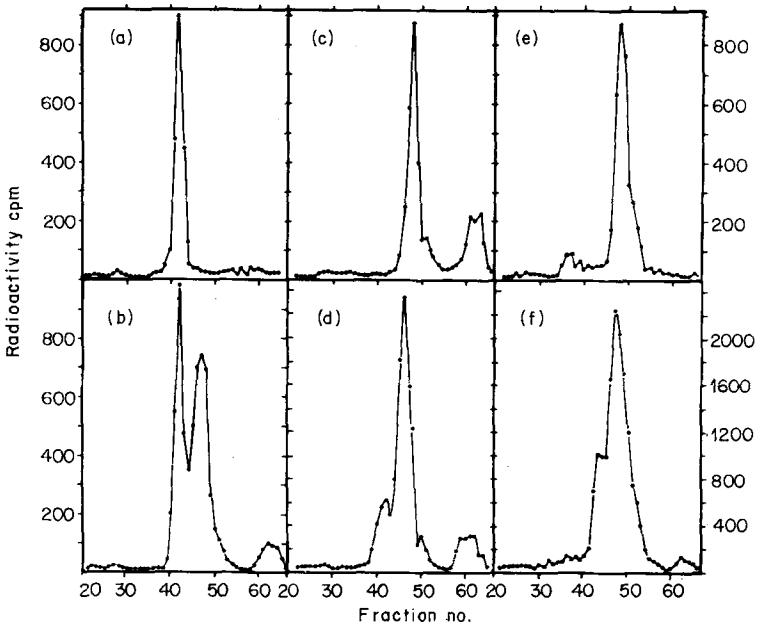

Figure 4 : Co-electrophoresis of DEAE-Sephadex chromatography fractions and twice centrifugated virus.
Electrophoresis was performed as under Fig. 2 ; migration from left to right. (a), (c), (e) : fractions I, II, III alone ; (b), (d), (f) : mixtures of twice centrifugated virus (see Fig.2b) and respectively fractions I, II and III.

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fraction I seemed to migrate slower than those of fractions II and III. The fraction IV, poorly resolved by chromatography and weakly labelled, appeared as a mixture of viral polypeptides (not represented).

Confirmation of the identities of the fraction I, II, III poly-

	2^{nd} ccc virus + F I			2^{nd} ccc virus + F II			2^{nd} ccc virus + F III		
	Peak area (%)[1]	Molar ratio (a)	(b)	Peak area (%)[1]	Molar ratio (a)	(b)	Peak area (%)[1]	Molar ratio (a)	(b)
VP1	40.5	1.20	1.40	19.0	0.65	0.95▼	15.0	0.45	0.95▼
VP2-3	52.0	1.85	2.15▼	67.5	2.40	4.1	81.0	2.45	6.50
VP4	7.5	0.55	0.65▼	13.5	1.00	1.70	4.0	0.30	0.65▼
→	Fraction I = VP1			Fraction II = VP2 or VP3+4			Fraction III = VP2 or VP3		

Table 2 : Characterization of the polypeptides from the DEAE Sephadex chromatography fractions.

1 : see Footnote 2 on Table 1.

(a) : "Molar ratio" as deduced from the electrophoregram ; see Footnote 3 on Table 1.

(b) : "corrected value" : for each mixture the molar ratio of the most obviously unchanged peak (underlined) was taken as equal to the reference value in the virus alone (see Table 1, second ccc). This corrected value points out the close adjustment or distorsion with the composition of virus alone ; ▼signals the numbers in close agreement with those in the purified virus alone.

peptides was obtained from co-electrophoresis experiments with twofold caesium chloride purified virus (see Fig. 2b) and from calculation of the different peak ratio variations (Table 2). The polypeptide isolated in the fraction I migrated as VP1 (Fig. 4b). The polypeptides in the fraction II had the same positions as VP 2 or 3 and VP4 (Fig. 4d). The major polypeptide of the fraction III corresponded with VP 2 or 3 (Fig. 4f).

DISCUSSION

The existence of several classes of polypeptides in FMDV was shown by different investigations : N-terminal aminoacid analysis (9), C-terminal aminoacid determination (10), molecular weights (11). Their purification could lead to the understanding of their biological properties.

The obtainment of the polypeptides needed processing of large volumes of cell lysate ; so in the first step it was necessary to decrease the sample volume with minimal loss of active material. Methods involving ammonium sulfate or cold methanol precipitation give poor yields in infectious material. Polyethylene glycol precipitation appears to be a more efficient procedure since PFU yields approach 100 % (12). Such a high output has already been described with leucemogene virus (13).

Preliminary virus purification eliminated most cellular proteins before chromatographic fractionation of the viral polypeptides. In order

to obtain very pure viruses, successive caesium chloride centrifugations were performed. The increase in buoyant density observed during these steps, as previously noticed for acidolabile picornaviruses (14), is compatible with the loss of protein material. Gel electrophoresis analysis confirmed the partial loss of VP1. Brown (2), studying an O type virus, presented the molar ratios of the four polypeptides as 1-1-1-0.5, as in other picornaviruses, and their molecular weights as following : VP1 : 34 000, VP2 : 30 000, VP3 : 26 000 and VP4 : 14 000. Our gel technique did not allow us to separate VP2 from VP3, so calculations were done on the basis of an average molecular weight of 28 000 for each. The molar ratios between the different polypeptides passed from 1.1-2.1-0.45 for the onefold purified virus to 0.8-2.2-0.85 for the threefold one. The loss of VP1 during centrifugation as well as its cleavage by trypsin (3,11) and its preferential labelling in iodination experiments (3, 4), is in agreement with the external position of this polypeptide ; these observations support the localization of VP1 at the vertices of the icosahedron as suggested by Brown from Rueckert's model (15).

The close variations between molecular weights of the viral polypeptides made their purification difficult on size criteria only, but their ionisation state at pH 8.6 allows for easy separation of VP1 from the other components.

The study of the biological properties of the viral components has been undertaken with the purified polypeptides. The major importance of VP1 in producing neutralization antibodies is known, since trypsination inhibits the acquirement of neutralizing immunity (16). In our conditions does VP1 alone support this immunogenic activity ? Recent results obtained with piglets indicate that this protein actually induces the apparition of such an activity in animal sera with a booster effect (17). The fraction II containing VP 2 or 3 and VP4 does not produce a similar effect (unpublished results).

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