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**PURIFICATION AND PARTIAL DIFFERENTIATION
OF THE PARTICLES OF MURINE SARCOMA VIRUS
(M. MSV) ACCORDING TO THEIR SEDIMENTATION
RATES IN SUCROSE DENSITY GRADIENTS**

by F. SINOUSSE, L. MENDIOLA,
J.-C. CHERMANN, C. JASMIN' and
M. RAYNAUD

Service d'Immunochimie
INSTITUT PASTEUR
92380 Garches (France)

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SUMMARY

The viral particles from the Murine leukemia sarcoma complex MSV 78A₁ Moloney, can be partially differentiated according to their sedimentation rates in sucrose gradients. The viral particles isolated after rate zonal sedimentation do not show any apparent physical differences under microscopic observation. The same RNA dependent DNA polymerase activity was found in the region of the gradient where viral particles were found.

The viral particles separated by zonal centrifugation are able to cause focus formation in murine embryonic fibroblast tissue cultures, but the pool of viral particles partially differentiated by rate zonal centrifugation is more infectious.

Identical separation was observed with B_{XIV}₁₁ or B₃₀₁₁ zonal rotors. Thus, we described a very rapid method of purification of the murine leukemia viruses at the same time.

RESUME

Nous avons pu différencier partiellement les particules du complexe sarcomatogène murin selon leur vitesse de sédimentation dans un gradient de saccharose. Les particules que nous avons ainsi séparées ne présentent pas de différences physiques visibles par microscopie électronique.

Ces particules ont la même activité « RNA dépendant DNA polymérase » et sont capables d'induire la formation de foyers de conversion cellulaire.

Cependant, le mélange des particules ainsi séparées entraîne une augmentation du pouvoir transformant.

Le même type de séparation est observé avec les rotors B_{XIV}₁₁ et B₃₀₁₁. Il s'agit, par ailleurs, d'une méthode rapide de purification des virus sarcomatogènes murins.

INTRODUCTION

Murine leukemia viruses have been purified by differential centrifugation (1, 2) or by ultracentrifugation in density gradients of sucrose or salts (3, 4, 5, 6, 7). Since these viruses have a characteristic buoyant density of 1.16 g/ml, the most common procedure for their isolation has been through isopycnic centrifugation in density gradients.

I. Toplin has described a method for the large volume purification of tumor viruses. The virus fluids are subjected to a primary centrifugation

in continuous flow zonal rotor, then to a second centrifugation in a B₃₀₁₁ zonal rotor.

Thus we have seen that viral particles of MSV-78A₁ can be partially differentiated according to their sedimentation rates in sucrose density gradients.

We have observed the purity of these particles by electron microscopy. The RNA dependent DNA polymerase activity and the focus forming activity of the particles partially differentiated after rate zonal centrifugation were analysed.

MATERIAL AND METHODS

Virus :

The virus was obtained from culture supernatants of a continuous cell line (78A₁) of rat fibroblasts infected with murine sarcoma virus, Moloney (M-MSV), and regularly producing this virus (8).

Concentration of the virus with polyethylene glycol (PEG) :

The viral particles were concentrated from culture supernatants by precipitation of the clarified fluids with 1/10 volume of 50 p. cent polyethylene glycol (PEG) (9). The resulting precipitate containing the virus was then resuspended in a minimum volume of NTE buffer (0.15 M NaCl, 0.02 M Tris hydroxymethyl aminomethane, 0.001 M ethylenediaminetetraacetate, pH = 8.3).

Rate zonal centrifugation :

20 ml of viral suspension were introduced in a B_{XIV}₁₁ or B₃₀₁₁ zonal rotor (International Equipment Company) containing a linear gradient of 500 ml of 5 p. cent - 45 p. cent w/w sucrose buffered with NTE and a cushion of 45 p. cent w/w sucrose for B_{XIV}₁₁ zonal rotor or 10 p. cent

50 p. cent w/w sucrose buffered with NTE and a cushion of 50 p. cent w/w sucrose for B₃₀₁₁ zonal rotor.

The sample was followed by an overlay of NTE buffer (100 ml of NTE with B_{XIV}₁₁ or 70 ml of NTE with B₃₀₁₁). The gradient, cushion, sample and overlay were all introduced into the rotor while spinning at 2,500 rpm. The actual centrifugation was carried out at 25,000 rpm for 20 minutes at 5°C, after which time the rotor was decelerated to 2,500 rpm for unloading ($\int \omega^2 dt = 1.15 \times 10^8$). The contents of the B_{XIV}₁₁ rotor were recovered from the core by introducing 45 p. cent w/w sucrose through the rotor edge; the contents of the B₃₀₁₁ rotor were recovered from the edge by introducing water through the core. Ten milliliter fractions of the gradient were collected.

RNA dependent DNA polymerase activity of zonal fractions :

The reaction mixture, described by Baltimore (10) and modified by Peebles (personal communication), contained 0.05 M Tris-HCl pH = 7.9, 0.02 M KCl; 0.001 M dithiothreitol; 0.0005 M Mn (CH₃COO)₂ · 2.5 × 10⁻⁵ M HTTP (specific activity : 216 Ci/M).

Polymerase activity was measured using 10 μ l of every other fraction of the gradient per 65 μ l of reaction mixture. The reaction was stimulated by exogenous poly rA oligo dT in the presence of optimum concentration (0.04 p. cent v/v) of detergent Nonidet P₄₀. The incorporation of ³H-TTP into acid insoluble material was measured after 30 minutes incubation at 37° C, by trichloroacetic acid precipitation and counting on membrane filters.

Focus forming activity of zonal fractions

For murine leukemia-sarcoma complex MSV-78A, focus assays, murine embryonic fibroblasts were plated in Petri dishes at a concentration of 5×10^6 cells per dish and infected the following day with the different fractions of the gradient after dilution. Six days after infection, foci of transformed cells were counted under microscopic observation.

RESULTS

Figure 1 shows the sedimentation profile of a viral preparation subjected to rate zonal centrifugation with B XIV₁ rotor.

The same profile was observed in figure 2 with B₅₀ rotor.

The fractions corresponding to each different peak of absorbancy in the gradient (a-e) were pooled as indicated, and the presence of virus ascertained by subsequent electron microscopy.

The majority of viral particles was found in the regions of the gradient between 15 and 30 p. cent w/w sucrose corresponding to peaks marked b, c and d.

From the electron-photo micrographs (Fig. 3b-d), these fractions contained mainly typical spherical C-type particles.

No apparent differences in physical appearance could be discerned among the viral particles in these regions. There was no sign of aggregation of particles.

The fastest sedimenting peak (e) also contained virus particles, which were, however, mixed with large amounts of cell debris, the former presumably having been trapped by the latter (Fig. 3e).

Similarly, the starting sample zone (peak a) also contained some virus particles, but for the most part these fractions contained cell debris which under the conditions employed did not migrate into the gradient. Figure 3a shows an electron photomicrograph of the viral preparation before rate zonal centrifugation.

In another experiment, the fractions corresponding to regions b-d of the gradient (Fig. 1 or 2) were pooled, concentrated with 10 p. cent PEG and then subjected to a second zonal centrifugation under conditions approaching the density equilibrium of the virus particles. In the latter case, only one main peak could be seen, banding in the region of the gradient corresponding to a density of 1.14-1.15 g/ml (Fig. 4).

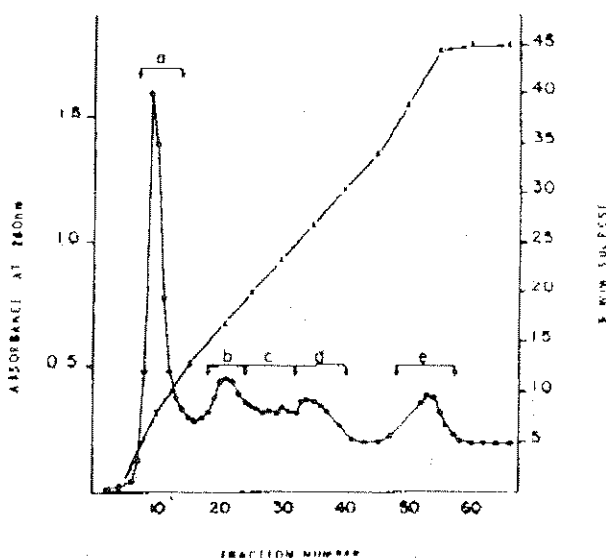


Figure 1. Absorbancy profile of a viral preparation of MSV-78A, subjected to rate sedimentation through a linear gradient of sucrose in the B XIV₁ zonal rotor. Twenty milliliters of viral suspension were introduced into the rotor under the conditions outlined in the text. Ten milliliter fractions were collected and the absorbancy as well as the refractive index of certain fractions was determined.

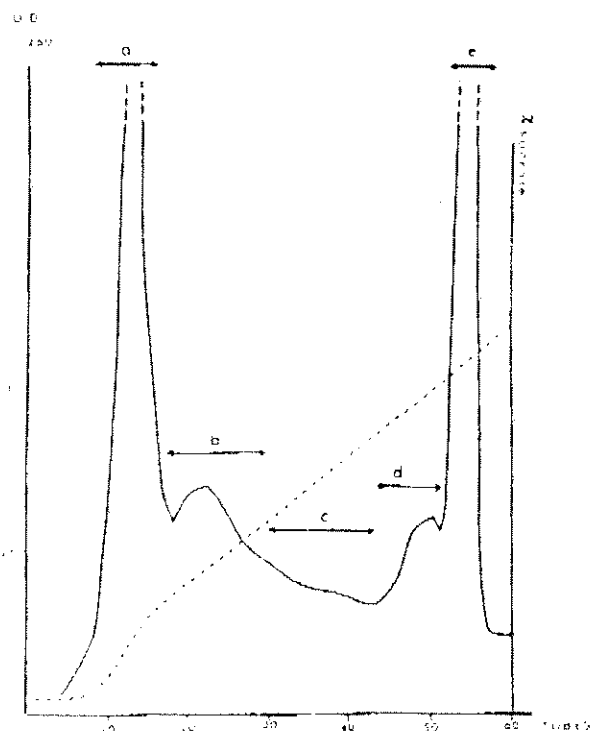


Figure 2. Rate separation of a viral preparation of MSV-78A, in the B₅₀ zonal rotor. Separation was similar to that in figure 1, the principal difference was the use of centrifugation in place of unloading from the edge.

In subsequent runs where the particles obtained from rate zonal sedimentation were subjected to equilibrium density centrifugation in sucrose gradients for 20 hours at 5° C, the virus was found again to band at a density of 1.14-1.15 g/ml.

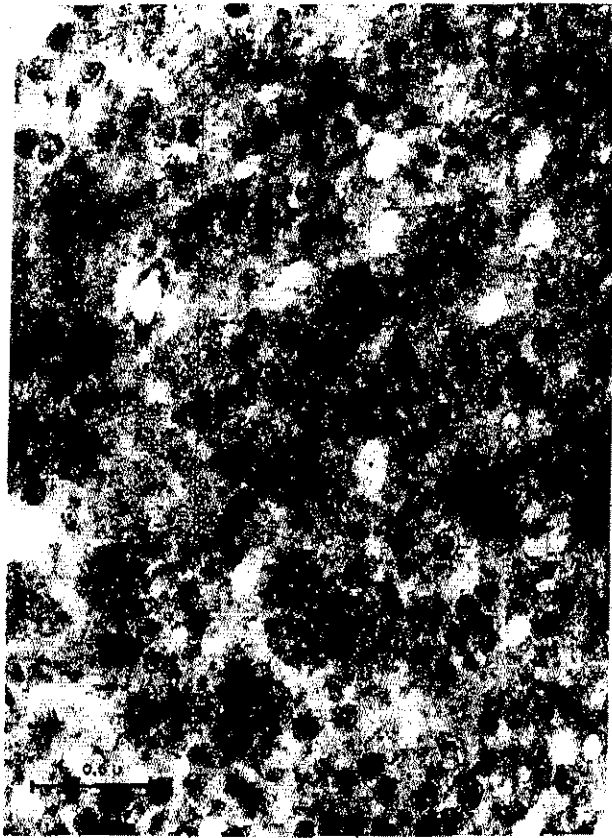


Figure 3a

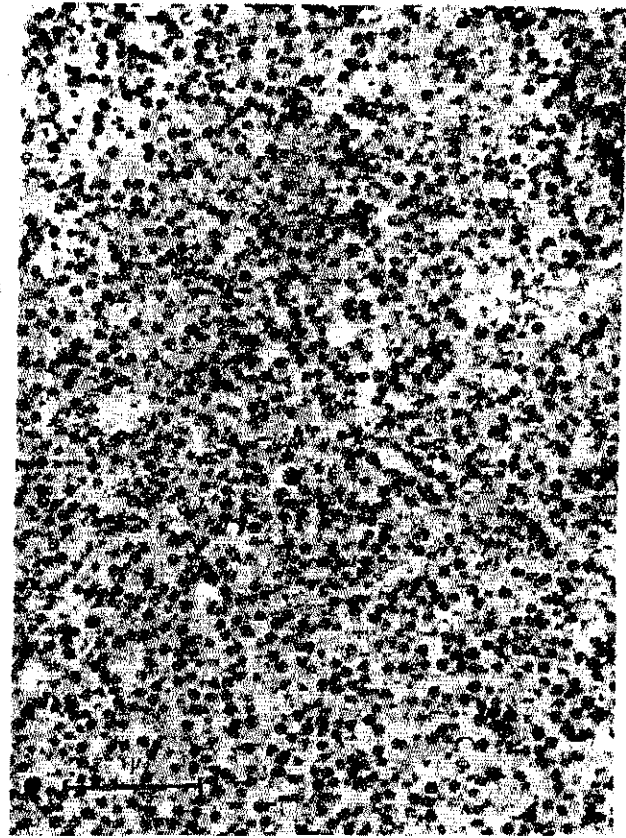


Figure 3b

In this case, rather small peaks of material were observed in the lighter and heavier ends of the gradient, indicating that the particles isolated under conditions of rate sedimentation considered mainly of virus particles and very little amounts of contaminating material.

Thus, we seem to have obtained evidence for a population of viral particles having the same equilibrium density, but which can be partially differentiated according to their sedimentation rates.

Since oncogenic viruses have recently been found to contain RNA dependant DNA polymerase (10), we decided to test every other fraction of the gradient for this enzyme activity.

Several peaks of polymerase activity were detected throughout the gradient, the peaks corresponding to peaks of absorbancy at 260 nm, as illustrated in fig. 5. Three partially resolved peaks of enzyme activity were seen in the region of the gradient (15-30 p. cent w/w sucrose) where essentially only viral particles were found before, again indicating the differentiation of these particles according to their sedimentation rates. There was significant activity in the sample zone and the fastest sedimenting peak, consisting mainly of cell debris. This enzymatic activity can be explained by the presence of some virus particles in these regions, and, since similar polymerase activity has been found in normal cells, it can be mainly ascribed to the cellular enzyme (11).

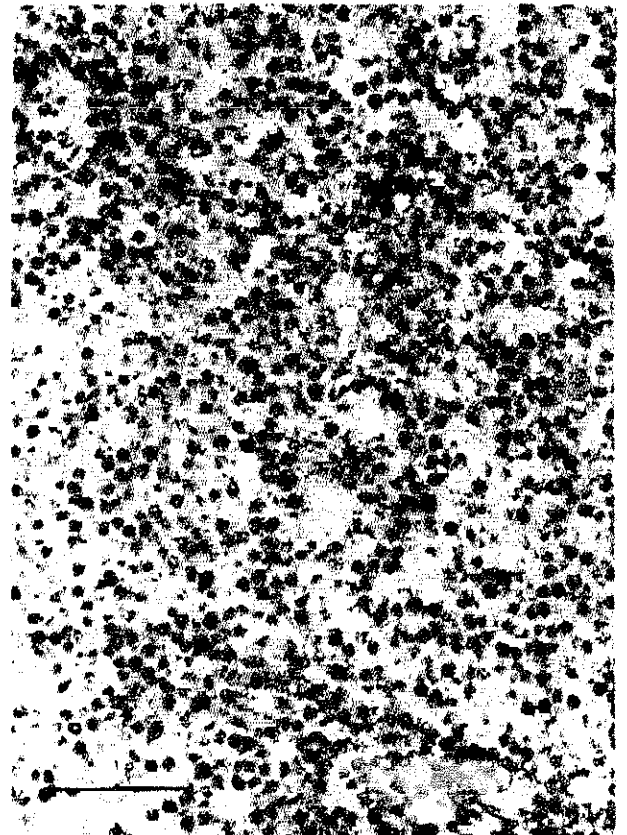


Figure 3c

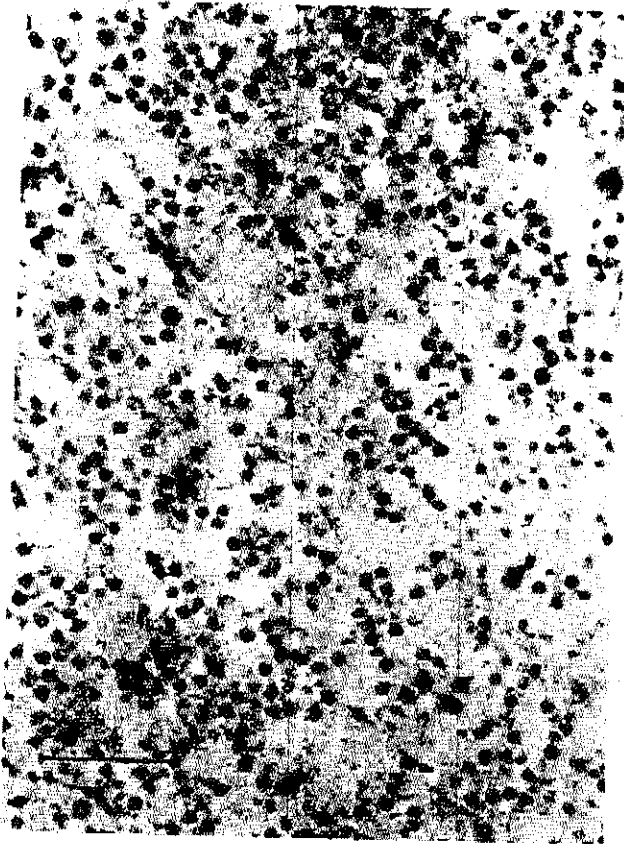


Figure 3d

Figure 3. Electron photomicrographs of the viral preparation of MSV 78A, before and after rate zonal centrifugation. The pooled fractions corresponding to the regions of the gradient as indicated in Figure 1 were concentrated by precipitation with PEG, resuspended in minimum volumes of NTE buffer and pelleted with 10 p. cent bovine albumin. An aliquot of the viral suspension before zonal centrifugation was similarly pelleted. The pellets were fixed with glutaraldehyde, embedded in Epon-Araldite and stained with uranyl acetate.

- Picture A = material before rate zonal centrifugation
- Picture B = peak b \times 32000
- Picture C = peak c \times 40000
- Picture D = peak d \times 40000
- Picture E = peak e \times 80000

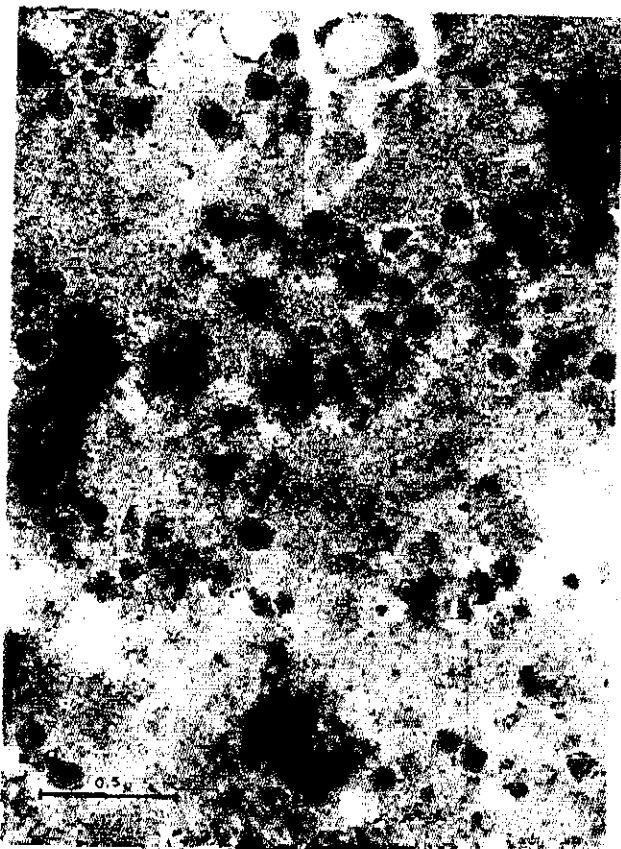


Figure 3e

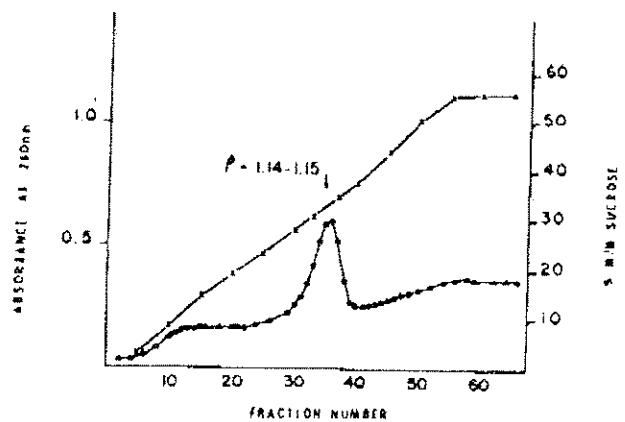


Figure 4. Absorbancy profile of the viral particles previously isolated by rate sedimentation in sucrose gradients and subsequently subjected to a second density gradient centrifugation under conditions approaching density equilibrium of the particles.

The fractions corresponding to the regions marked b - d of the gradient in Figure 1 were pooled, concentrated by precipitation with PEG and resuspended in 10 ml of NTE buffer. The material was then introduced into a B XIV₁₁ zonal rotor containing 550 ml of a gradient of 10 - 55 p. cent w/v sucrose buffered with NTE followed by an overlay of 100 ml NTE. Centrifugation was carried out at 40000 rpm for 5 hours at 5° C ($f \omega^2 r = 3.13 \times 10^{11}$).

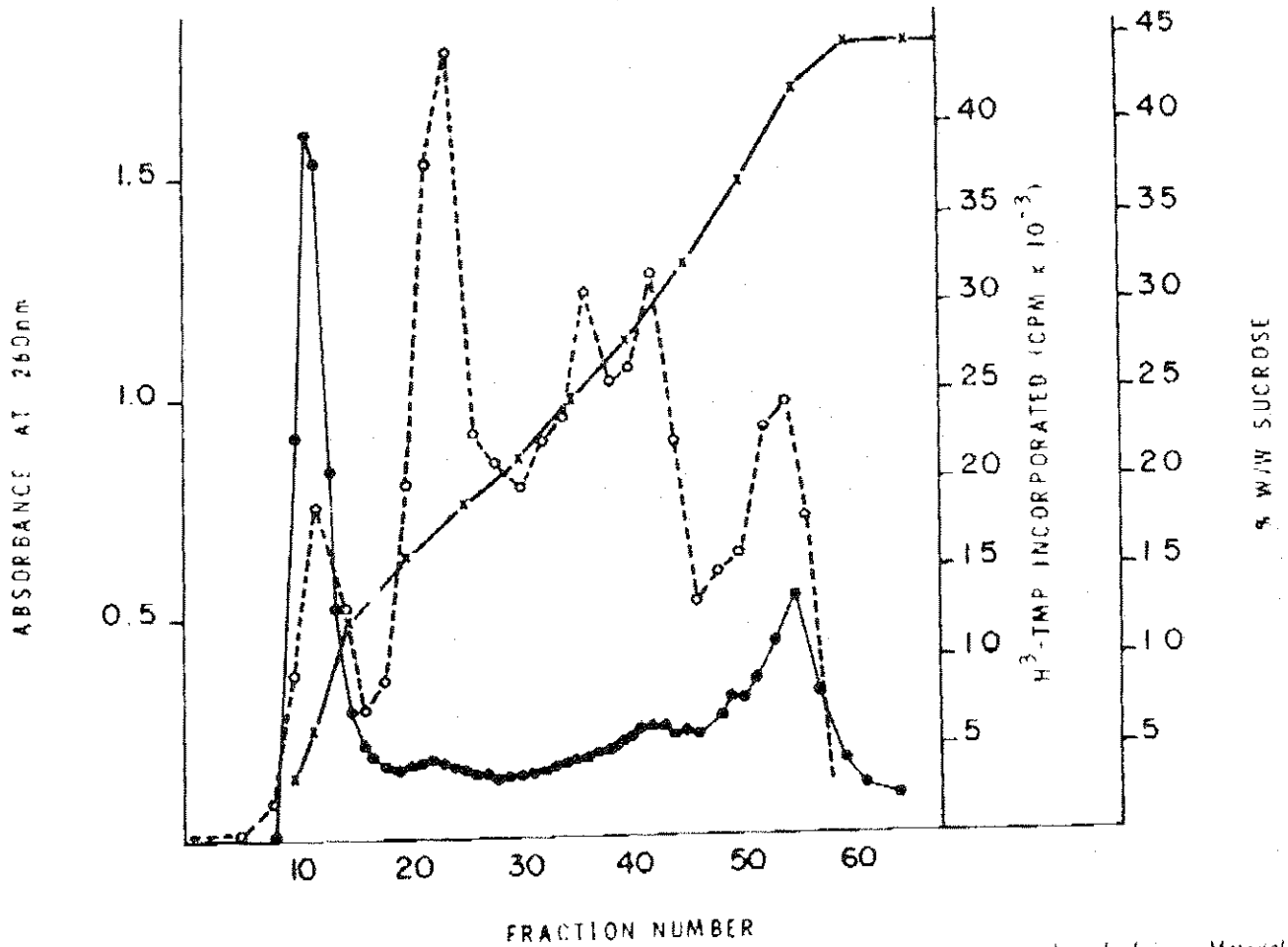


Figure 5. Absorbancy and polymerase activity profile of a viral preparation (20 ml) subjected to rate zonal sedimentation in sucrose gradients under conditions identical to those described in Figure 1.

Assay for polymerase activity was described in "Material and Methods". Absorbancy, closed circles, polymerase activity, open circles.

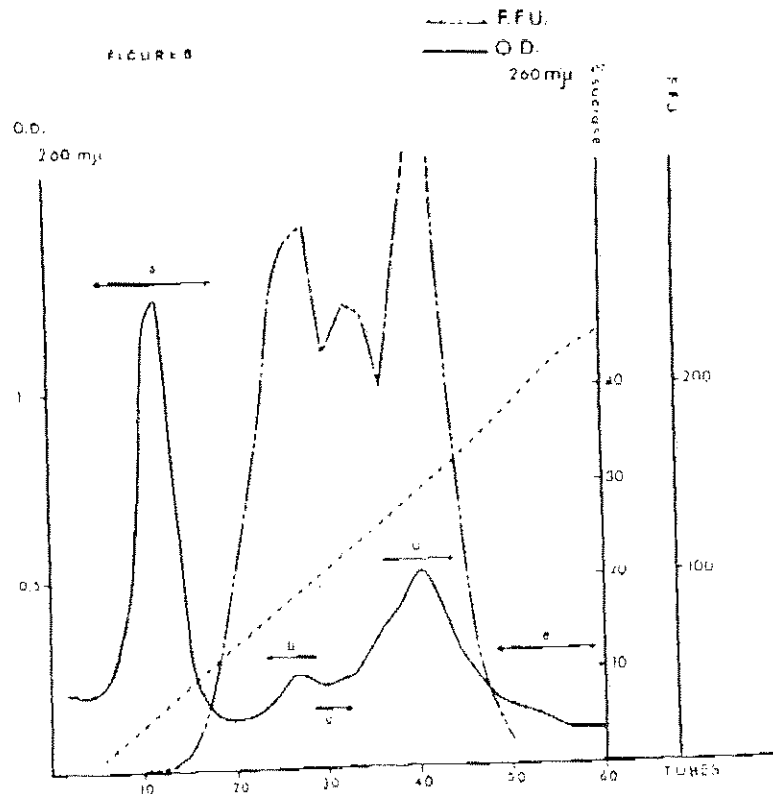


Figure 6. Absorbancy and focus forming activity of a viral preparation subjected to rate zonal separation in sucrose gradient.

Conditions of focus forming assay are described in the text. Absorbancy = unbroken line, focus forming activity = broken line.

On the other hand, we tested ability of the different fractions of the gradient to induce transformation of murine embryonic fibroblasts as described in « Material and Methods ».

Figure 6 shows the infectivity profile of the different fractions in terms of their ability to cause focus formation. Three peaks of focus forming

activity were observed in the region of the sedimentation profile where electrophotomicrograph shows essentially virus particles.

Preliminary experiments seem to show that pool of fractions corresponding to region b and d, after PEG concentration was more able to induce cellular transformation than either those of regions b or d.

DISCUSSION

In this paper, we described a very rapid mean for the purification of Murine Sarcoma Virus by rate zonal centrifugation with B XIV₀ or B₃₀ rotor.

On the other hand, our results demonstrate that virus particles which band at essentially the same density can be partially differentiated according to their sedimentation rates in sucrose gradients. It is known that stocks of MSV consist of at least two different viral particles, murine leukemia viruses (MLV) and defective murine sarcoma virions (MSV) (12). Recently, the existence of a third component, consisting of competent murine sarcoma virions capable of inducing cellular transformation without the intervention of helper leukemia virus has been postulated (13). Competent MSV was found to have a higher sedimentation coefficient in sucrose gradients than defective MSV particles (14). On the other hand, it has been reported that competent MSV particles had a higher equilibrium density in sucrose gradients and could thus be separated from MLV and defective MSV (15).

So, it will be interesting to determine the differences of biological activity of the viral particles in order to make any conclusions on the exact relationship of the particles having different sedimentation rates in sucrose gradient with the components of the murine leukemia.

In preliminary experiments, the enhancement of foci forming activity by the mixture corresponding to regions b and d of the gradient suggest a differentiation of the biological activity of these two peaks.

Further studies are being carried out to substantiate this condition. Two cell assays which detect presence of leukemia virus are being used: sarcoma positive-leukemia negative cell assay described by R. Bassin (16) and XC cell assay described by P.W. Rowe (17).

These studies should make it possible for us to draw conclusions as to the nature of viral particles differentiated according to their sedimentation rates in sucrose density gradients.

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