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DEPARTMENT OF THE ARMY
Fort Detrick

[illegible] -TILIZATION

[illegible] by Porath-Flodin [illegible] of the Institute for Research of Virus Diseases of Animals of the [illegible] Republic of Germany [illegible], Germany, which appeared in the German-language periodical [illegible] für Mikrobiologie (Archive of Bacteriology), Vol 182, pages 59-[illegible], 1961./

Recently Porath and Flodin (1) reported on a simple and rapid method of desalting biological material with the help of dextran gels. This method is based upon the well-known column chromatography technique in which the stationary phase is a new synthetic gel type. These dextran gels consist of hyrophile polysaccharide chains which are interlaced. The degree of interlattice is very constant within the individual gels. They have no active ion groupings, are nonsoluble in water, but have a stabilizing affinity for water. The degree of expansion of the gels in water is determined by the interlattice degree of the dextran. The polar character is brought about almost exclusively by the large number of hydroxyl groups of the gel.

The desalting as well as the fractionation of substances with dextran gels is based mainly upon the differences in their molecular size. When a substance mixture is filtered through a column packed with a dextran gel, the larger molecules migrate faster than those of smaller dimensions. In certain approximation and in the practical application of these methods, the molecular weight can be compared instead of molecular size. Therefore if the difference of the molecular weights of substances is sufficiently great, their complete separation is possible with this gel filtration. In this case, a molecule sieve effect is the main principle and the chromatographic process in its classical

(2,3).

[illegible] filtration technique (2-10). [illegible] undertake a change of the buffer system in [illegible] great loss or difficulty, as is often [illegible] chromatography or in electrophoresis. The Sephadex gel behaves [illegible] currently with regard to biological substances and biological activities [illegible] sustained (4-8).

Knowledge of this gel filtration method led us to conduct the following experiments on the use of this technique for desalting of various virus suspensions. The method seemed to us to be particularly useful with respect to the concentration of virus suspensions with ammonium sulphate and in the adsorption of the foot and mouth disease virus on aluminium hydroxide and subsequent elution with 1/3 m phosphate buffer, $P_H$ = 7.5 (11). We were looking for a full-scale replacement for the customary dialysis, which often leads to great losses in infectiousness and has certain other disadvantages as well. In order to obtain a broad basis, we examined the behavior of viruses of various origin, differing structuring and characteristics, and suspended in various saline solutions, during gel filtration. In addition to the Newcastle disease virus (NDV) and the virus of pigeonpox (TPV), our experiments dealt mainly with the contagious swine paralysis (polioencephalomyelitis enzootica suum) virus (Teschenvirus) and the virus of foot and mouth disease (FMD).

## Materials and Methods

Virus material: For examination of the smaller types of virus, we used mouse and culture viruses of the MMS strain "$O_2$-Brescia" as well as culture viruses of contagious swine paralysis (Teschener Disease), strain Konratice.

The MMS mouse virus came from a 10% extract in culture medium No. 2 (12), which was produced from heart and skeletal muscles of new-born mice of the 36th serial passage of the virus in mice. The MMS culture virus used was the 20th serial passage of virus in cultures of swine kidney cells. The virus medium used here was VM 3a (12). The virus material was stored at -20°C until the start of the experiment. The melted mouse material was always partially purified by diluting it with 50% chloroform (p.A.), shaking for 30 minutes at + 4°C and followed by slow centrifugation. The culture liquids were only centrifuged for 10 minutes at 7000 r.p.m. in order to remove cell materials. In both cases,

[illegible] virus was obtained from the MS virus from swine kidney cultures of the 70th serial passage of the virus.

Newcastle Disease virus (NDV) -- Somerset Strain -- and fibroma virus (TFV) were selected to represent the medium and large size virus types. NDV was available in the form of allantoic liquid from incubated chicken eggs (79th serial passage). TFV is culture virus (62nd serial passage in chicken fibroblast cultures) was used. These viruses were also stored until use at - 20°C and after thawing were cleared through slow centrifugation. Further details are mentioned in the text.

Preparation of dextran gel and column: In our experiments we used as the dextran gel Sephadex G 25 (Pharmacia, Uppsala, Sweden) with a tumefaction factor of 2.3 grams water/gram dry matter and a grain size (dry) of 50-270 mesh.

The dry Sephadex was suspended in a 1% solution of sodium chloride for steeping. After about one hour, we removed very fine grain material through repeated washing and decanting of the gel substance with distilled water. We then removed the small air bubbles clinging to the gel suspended in water through brief evactuation in a suction bottle. Chromatographic columns were then filled with this gel suspension. The column diameter was 1.5 cm and the column length 35-39 cm. In all experiments, we computed from the column diameter (d) and the column length (L) which the Sephadex gel filled, the total gel volume ($V_t = \pi \frac{d^2}{4} . L$). This value was controlled by measuring the volume of the column filled only with distilled water. In the main experiments, the computed and the measured total gel volumn was $V_t$ = 62-69 milliliter. In comparison tests with NDV, TFV and MKS viruses, we also used smaller columns with a value of $V_t$ = 25 milliliter. We determined the empty volume ($V_o$) of the columns in prior tests with hemoglobin, which, as a high-molecular protein (molecular weight 68,000), reacts indifferently to the Sephadex gel. Because of its brown-red color, hemoglobin is also a good indicator for the elutriation of the virus.

The hemoglobin was disolved to 0.1% in physiological, m/90 phosphate-buffered Sodium chloride solution, $p_H$ = 7.6 (phys NaCl-solution). After slow centrifugation, we brought 5 milliliter of the clear 0.1% hemoglobin solution drop-wise into a column packed with gel, which had been well washed with NaCl solution. After the hemoglobin solution had set, we eluted the blood pigmentation material and took off the eluent in fractions of 3 milliliter. These fractions were then measured individual in an ultraviolet sprectrophotometer at 280 millimicron. If the extinctions are plotted graphically to the elution volume in a graph, then

[illegible]

[illegible] which contained 0.1 [illegible] units of pendelmilch [illegible] liter of the virus [illegible] ed this with 5 milliliters [illegible] necting the column [illegible] lution, we started the elution. [illegible] cm, we obtained a flow velocity of 1 to 1.5 milliliter per minute. We collected the eluate in fractions of 5 to 5.5 milliliters. [illegible] ments were conducted at room temperature.

Disinfection of [illegible] Sephadex gel: The column [illegible] gel was filled with sterile, chloroform-saturated [illegible] on the day preceding an experiment. [illegible] forced this out of the gel quantitatively using chloroform-saturated NaCl solution, again taking precautions for sterility. [illegible] located at the lower end of our chromatographic column so that we could receive our fraction primary in a sterile condition. By observing sterile precautions, it was no difficulty to achieve a germ-free work.

We disinfected the Sephadex column following an experiment with virus using a 5% Formalin solution which we allowed to remain in the column over night.

Determining degree of infectiousness: We determined the infectiousness of the MM virus through intra-peritoneal immunization of 5-7 day old mice. The Teschen virus was titrated in swine kidney cultures, NDV and TPV in incubated chicken eggs by injection into the allantois cavity or the chorion membrane. All titrations were conducted with dilutions in stages of potentials of 10. We computed the titers according to Behrens and Kärber (13). They are based upon 0.1 milliliter of the starting material described under "experiments and results" ($MID_{50}$ = Mice-$ID_{50}$; $KID_{50}$ = Cultur-$ID_{50}$; $EID_{50}$ = Egg $ID_{50}$).

Analytical determination: The ammonium sulphate in the individual fractions was determined by distillation in a half micro-Kjeldahl apparatus. Titration was determined with n/70 hydrochloric acid in boric acid.

We performed the phosphate determination according to the molybdate method of Fiske and Subbarow (14), but used ascorbic acid as the reduction medium.

[illegible] virus, [illegible] such a [illegible] by [illegible]. [illegible] of the individual fractions [illegible] virus [illegible].

Number of [illegible]

Of particular interest to [illegible] virus suspensions with 1/3 [illegible] buffer, $p_H$ = 7.5, elution from aluminium hydroxide gel (Al), [illegible] sulphate. The experiments [illegible] problem. We started the [illegible] which, after [illegible] precipitate in distilled water, [illegible] logous virus material which was [illegible] 7.5.

Separation of MKS and Teschen virus [illegible] from ammonium sulphate:

Experiment MKS-1a. MKS mouse material purified with chloroform was diluted 1:100 with 1/3 m phosphate buffer, $p_H$ = 7.5, and to 300 milliliters of this suspension we added, drop-wise and during shaking, 300 milliliters of saturated ammonium sulphate, $p_H$ = 8.4. The $p_H$ value of the mixture (50% ammonium sulphate saturation) then amounted to 7.5. After one hour at + 4°C, there developed a strong clouding which sedimented after 90 minutes in rotor 21 of a preparation Spinco-Ultracentrifuge at 20,000 r.p.m. After this sediment had been absorbed in 6 milliliters of distilled water, we allowed it to sit over night at + 4°C and cleared it for 15 minutes in the laboratory centrifuge prior to the start of experiment in the morning. The clear virus-containing remnant (5 milliliters) with a $MID_{50}$ value of $10^{-5.90}$ was used as the starting material for the desalting tests with the Sephadex gel G 25.

Experiment Teschen-3a. To 0.5 milliliter Teschen culture virus we added, drop-wise, 1.5 milliliter saturated ammonium sulphate, $p_H$ = 7.4. This virus suspension was centrifuged slowly for ten minutes for clearing. 5 milliliter of the clear, virus-containing remnant with a $MID_{50}$ value of $10^{-6.5}$ was used as the starting material for the desalting.

The two experiments MKS-1a and Teschen-3a are graphically portrayed in Illustration 1. The separation of the virus components from the saline gradients can be clearly seen in the elution diagram. The initial value of $ID_{50}$ is reached in one fraction for each of the two types of virus. If the virus titer of the individual fractions is compared to the final volume, it can be seen that no loss of virus occurred in the desalting. The agreement between the two tests, which was also shown in numerous other tests, is clear. The peak of the virus titer is also identical

volu[illegible].

[illegible]

$p_H$ = [illegible]

[illegible] to the [illegible]. [illegible] carried the [illegible] virus suspension [illegible] centrifugation and used 5 ml [illegible] for desalting with Sephadex gel.

[illegible] determined the initial titer [illegible] for the EMC virus and a $MID_{50}$ of $10^{-6.0}$ [illegible]. The Sephadex columns from the ammonium sulphate solutions [illegible] ing disinfection and sterilization which will be described later.

The elution diagrams of experiments 5c and 4c [illegible] again show the clear separation of the virus [illegible] from the salting gradients. No virus loss can be seen. [illegible] of the virus gradients and the peaks of the ammonium sulphate gradients in tests 5c and 4c is identical with the differences in the above-described tests 1a and 3a. This means that the distribution coefficients for ammonium sulphate and the secondary sodium phosphate/primary sodium phosphate (phosphate buffer, $p_H$ = 7.2) have the same values.

Comparison tests with EBV, TPV and [illegible] virus

In order to determine that the small viruses such as [illegible] virus and Teschen virus were no different, but that the lipoid-containing large viruses also behaved indifferently to Sephadex gel and that they were also identical in this respect, we compared the EMC virus with EBV and TPV in additional tests. For this we used columns of larger volume ($V_t$ = 25 ml). This time the pre-treatment and elution of the gel was done with distilled water which was brought to a $p_H$ value of 7.2 with a few drops of 1/10 n NaOH. The saline gradients, which here are certain electrolyte mixtures (culture mediums and body liquids), were determined through conductivity measurements of the individual fractions.

Illustration 3 shows the results of these comparison tests. All three types of virus have the same elution volume. Their peaks are identical and no loss of virus occurred during the desalting. This demonstrated that the desalting is independent of the virus size and virus structure.

[illegible] both plotted in a linear fashion.

Discussion

Gel filtration with Sephadex as the carrier material [illegible] above all the separation of substances of varying molecular [illegible] and varying molecular size. This has been convincingly shown previously in numerous publications (1-10). Up to now, however, no virus suspensions have been purified or desalted with the help of this method. The desalting of virus suspensions without loss of infectiousness is not possible in most cases by means of the usual dialysis in cellophane membranes, particularly in the case of very labile viruses such as that of MKS. Furthermore, it is time consuming and not suitable for small volumes. Gel filtration with Sephadex G 25 as the carrier medium proved to be very practical in these cases and offered other possible advantages in other processes.

We have shown with our experiments that such labile viruses as that of MKS can be desalted very well and without loss of infectiousness. Other types of virus, such as the Teschen virus, Newcastle Disease virus and the virus of pigeon pox retained their full activity during desalting. Since the elution volume of these viruses (Illustration 3) are equal to each other and also hemoglobin as determined in pre-tests, it can be concluded that Sephadex gel G 25 does not react with the viruses. The peak of the elution volume for each virus component corresponds to the empty volume $V_0$. The MKS and the Teschen viruses are among the smallest presently known viruses whereas NKV and TPV vary from those first named in molecular weight by a factor of about $10^2 - 10^3$. Furthermore, the chemical composition of NDV and TPV is much more complex than that of MKS and Tischen virus. In that we examined widely differing viruses, it can be concluded that the gel filtration technique can be used for the desalting of all types of virus, irregardless of their molecular size and chemical structure.

One additional advantage of this method is the rapid and exact

[illegible]

Our [illegible]
viruses [illegible]
not mentioned [illegible]
for desalting [illegible]
ful experiments [illegible]
virus nucleic [illegible]
move the [illegible]
extraction. [illegible]
virus material must also be [illegible]
which are used in [illegible]
cess described.

A minor dilution effect occurs [illegible] particles from the Sephadex gel. [illegible] action between virus and gel [illegible] diffusion of the virus particles in the [illegible] portance. In the most unfavorable case, a dilution [illegible] obtain, and this usually falls within the [illegible] the infectiousness used. Our experiments have shown that [illegible] factor is unimportant, for in a [illegible] particularly with high saline concentration of the dialysis material, a considerable increase in the volume of the dialysate occurs [illegible]. In gel filtration, this increase in volume is independent of the saline concentration and depends solely upon the gel volume. It would seldom exceed the dilution factor of 1:2.

It was seen, as we reported earlier (16), that a saline solution saturated with chloroform shows bactericide effects. At that time, we determined that vegetative forms of bacteria, molds and yeasts were killed with certainty in 1-3 hours. Bacteria spores are very resistive against chloroform, however. Since pathogenic spore formations very seldom occur, the treatment of Sephadex gel with chloroform-saturated table salt solutions has sufficed. Also, as mentioned before, we work under antibiotica protection.

The 5% Formalin solution which serves to disinfect the columns after an experiment does not change the characteristics of the gel in any way. It can also be quantitatively eluted. We have conducted more than twenty experiments on such disinfected columns and have always obtained

[illegible] such as the fowl and mouse adapted virus, the Lansing virus, the virus of Newcastle disease and the pigeon pox virus were tested in different saline solutions. During the quantitative drying process, no loss of virus was observed in these virus-salt mixtures.

[illegible]


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/Note: Miss Hella Ludwig and Miss Gerda Förstel receive our thanks for their technical support in the conduct of the experiments./

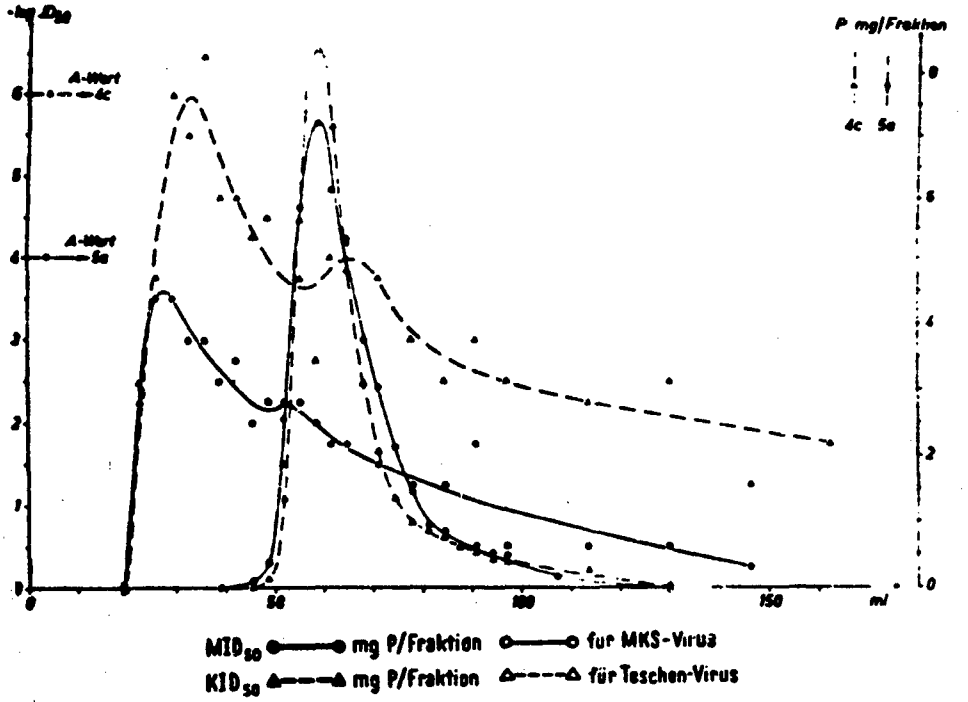


Illustration 2. Elution diagram of MKS and Teschen culture viruses in 1/3 m phosphate buffer, $p_H$ = 7.5

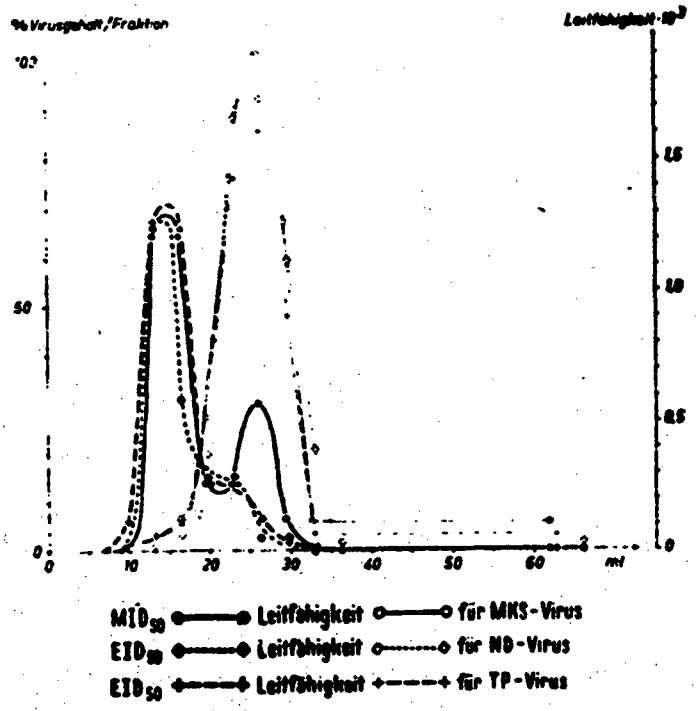


Illustration 3. Elution diagram of Newcastle disease, pigeon pox and MKS virus from physiological environments with distilled water, $p_H$ = 7.6.

Legend: virusgehalt/Fraktion = virus content/fraction
Leitfähigkeit = conductivity

FIGURE APPENDIX

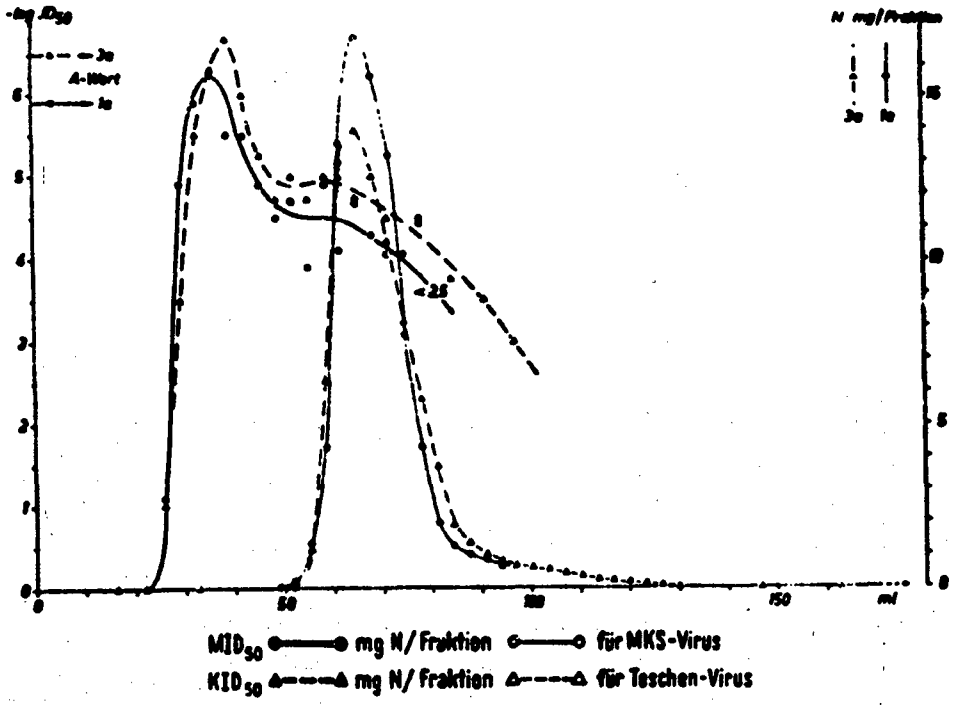


Illustration 1. Elution diagram of MKS mouse virus and Teschen culture virus in ~15% ammonium sulphate solution (experiments 1 a and 3 a).

Legend: A-Wert = initial value
Fraktion = fraction
für = for