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Preparation of Purified Influenza Virus.*

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Considerable effort has been directed, in recent years, toward the separation of viruses from the tissues within which they are propagated. This has met with variable degrees of success, and virus preparations ranging from complete to questionable states of purity have been reported by numerous investigators.

In the course of a study on active immunization against epidemic influenza conducted in this laboratory,[1,2,3] the obvious advantages of purified antigens for immunologic and serologic application prompted the investigation of methods for the purification of influenza virus.

Preliminary experiments indicated that influenza virus is in some respects rather labile and is readily affected by certain types of treatment which leave many other viruses relatively unimpaired. Attempts at salting out, extraction with ether or alcohol, mild hydrolysis, the addition of oxidizing or reducing substances and wide changes in pH, all reduced the activity of the virus quite drastically. Similar observations on some of these variables, studied in greater detail, were reported by Stock and Francis.[4] It became apparent, therefore, that other methods, less deleterious to the infectivity of the virus, would be required.

A technique of ultrafiltration, perfected by Bronfenbrenner and employed by him for the filtration of lytic filtrates of bacteriophage, was selected as suitable for this investigation. The details of the technique are given in the original report by Bronfenbrenner.[5]

The virus used for purification was obtained from 20% stock suspensions of influenza A virus prepared from pools of several hundred lungs of infected mice sacrificed 3-4 days after inoculation. The material was stored in a dry-ice cabinet at about —70°C, from which quantities were removed and thawed as required. These suspensions were diluted to 1% with saline or Tyrode's solution and subjected to a preliminary filtration through a Berkefeld V candle. Because of the variability of the results obtained with Berkefeld filters, due to frequent clogging, slow filtration and the occasional excessive loss of virus, filtration through paper pulp in a Buchner funnel was later substituted. There resulted a more rapid removal of the coarse particles, the loss of virus being on the average less than that following Berkefeld filtration.

The crude filtrate was then passed through the ultrafiltration apparatus in which an alundum thimble served as a support for a collodion membrane. Trials with collodion of various concentrations indicated that 2% collodion-acetate membranes were most consistently suitable for selective fractionation of the 1% virus filtrate. Membranes of this density, prepared within alundum thimbles, retained all of the virus while the greater proportion of inactive associated materials filtered through. However, the ease with which the separation was accomplished depended largely upon the degree of preliminary clearing.

Following ultrafiltration, the virus-containing residue was washed by permitting 200-400 cc of distilled water to pass through the

* Aided by a grant from the Metropolitan Life Insurance Company on the recommendation of the Influenza-Pneumonia Commission.

1 Siegel, M., and Muckenfuss, R. S., *Am. J. Hyg.*, 1941, **34** (Sect. A), 39.

2 Siegel, M., Muckenfuss, A. S., Schaeffer, M., Wilcox, H. L., and Leider, A. G., *Ibid.*, 1942, **35**, 55.

3 Siegel, M., Muckenfuss, R. S., Schaeffer, M., Wilcox, H. L., and Leider, A. G., *Ibid.*, 1942, **35**, 186.

4 Stock, C. C., and Francis, T., Jr., *J. Exp. Med.*, 1940, **71**, 661.

5 Bronfenbrenner, J. J., *Ibid.*, 1927, **45**, 873.

filter, resulting in an additional removal of inactive, diffusible substances. The residue was then recovered by removing the alundum thimble from the apparatus, adding small quantities of Tyrode's solution and dislodging the adherent material from the surface of the collodion membrane with the aid of a sterile swab.

Correcting for comparable volumes, assays were made of the relative infectivity and nitrogen content of the original 1% suspension, crude filtrate, wash water and concentrated residue. Titrations for infectivity were made in tenfold dilutions, each inoculated intranasally into 4 young Swiss mice under ether anesthesia, and the end-points determined by the 50% mortality method.[6] Nitrogen determinations were made by the micro-Kjeldahl method.

The results obtained in 10 experiments yielding substantially similar data are exemplified by Table I.

TABLE I.

Exp. No.	Material tested	Infectivity (Final dilution)	Nitrogen content (mg/cc)
1	1% crude suspension	$10^{-6.0}$	.318
	Berkefeld filtrate	$10^{-4.5}$	.277
	Ultrafiltrate	0	.182
	Wash water	0	.036
	Virus residue	$10^{-4.5}$	.014
2	1% crude suspension	$10^{-6.0}$	.377
	Paper pulp filtrate	$10^{-5.0}$	.229
	Ultrafiltrate	0	.091
	Wash water	0	.040
	Virus residue	$10^{-4.5}$	.021

From the data presented above it will be seen that while there was some reduction in nitrogen as a result of the preliminary crude filtration, there was also a considerable diminution in virus activity. From this point on, however, the bulk of the nitrogenous material was removed by ultrafiltration with relatively little or no loss in virus. Washing with water further reduced the nitrogen content so that the final residual virus recovered was depleted of 90-95% of the inactive and presumably non-essential associated nitrogen.

If a comparison of the infective titers and nitrogen contents were to be made between the crude suspensions and final virus residues, it might be concluded that essentially no purification was effected but that the result could easily be duplicated by mere dilution. But since, strictly speaking, purification begins with ultrafiltration, it will be observed that using the crude filtrates as the starting point, considerable amounts of nitrogen were removed with little or no loss of virus.

At about the time that these data were obtained, Salk reported the partial purification of influenza virus by adsorption on calcium phosphate.[7] Following the same technique results comparable to those described by Salk were obtained. However, if instead of the final dialysis, as prescribed by Salk, the material was subjected to the ultrafiltration and washing described above, it was possible to obtain a product containing only about 1.5% of the original nitrogen; but a slight depletion in the infectivity of virus occurred during this procedure. The results are illustrated in Table II.

TABLE II.

Material	Infectivity (Final dilution)	Nitrogen content (mg/cc)
10% crude virus susp.	$10^{-6.0}$	3.520
Virus susp. after adsorption on calcium phosphate and resusp. in sodium citrate and hydrochloric acid	$10^{-6.0}$	0.273
Adsorbed and resuspended virus after ultrafiltration, washing and final resuspension in Tyrode's solution	$10^{-5.5}$	0.048

Besides yielding a more purified product, the combination of these two procedures with the substitution of ultrafiltration for dialysis has the added advantage of permitting the washing of the residue with large amounts of water and subsequent concentration to any desired volume with relative rapidity.

Tests of the comparative immunizing properties of the original virus suspensions and purified products have shown that no ap-

[6] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

[7] Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 709.

parent impairment of antigenicity occurs as a result of the purification. The solid protection against 1000 minimal infective doses induced in groups of mice inoculated intraperitoneally with 0.5 cc of the crude virus suspension, was produced as well in others with 0.5 cc of the purified preparations restored to their original volumes.

That a further step may be available for the production of more completely purified virus is indicated by some preliminary experiments entailing the use of proteolytic enzymes for the splitting off of associated protein material. While the details have not been worked out thoroughly, evidence was obtained that the infectivity of influenza virus is not affected by the separate action of trypsin, chymotrypsin and ribonuclease on suspensions of infected mouse lungs. When 1.8 cc amounts of a 10^{-3} dilution of mouse lung suspension were mixed with 0.2 cc of each of the crystalline enzyme solutions† containing 5 mg per cm^3 in N/200 HCl (except ribonuclease which was made up in distilled water), the mixtures adjusted to a pH of 7.6 and placed in the refrigerator at 4°C for 24 hr, there was no apparent diminution in the virus content.‡ In fact, the infective titers of the digested virus appeared to be about 10 times higher than those of the undigested virus controls kept under the same conditions. Ultrafiltration of one sample of the digested material resulted in a reduction of about 99% of the original nitrogen content while the virus activity remained unaltered. On the basis of nitrogen content and virus concentration of the final product as compared to the original crude suspension, this procedure appeared to give the most satisfactory purification results thus far obtained.

Summary. While the lability of influenza virus limits the types of chemical and physical procedures to which it can be subjected, several methods are available for the purification of the virus. Either adsorption on calcium phosphate or ultrafiltration through collodion membranes results in preparations free of 90% or more of the nitrogenous material contained in the crude suspensions of infected mouse lungs. When these two techniques are combined, the residual virus recovered is freed of about 99% of the original nitrogen content. The loss of virus activity is only slight and its immunologic properties are apparently unimpaired.

Preliminary experiments with the proteolytic enzymes, trypsin, chymotrypsin and ribonuclease, indicate their possible use as a further step in the purification of influenza virus. A suspension of virus ultrafiltered after preliminary digestion contained but 1% of the original nitrogen, while the virus was concentrated tenfold.

† The crystalline enzymes made available by Dr. J. J. Bronfenbrenner were originally obtained from Dr. John Northrop.

‡ Control tubes consisting of 0.1% casein and enzymes, and mouse lung suspension and enzymes indicated that the enzymes were acting effectively under these conditions.

The author wishes to express his appreciation to Dr. J. J. Bronfenbrenner for his advice and suggestions and particularly for his generosity in making available the facilities of his laboratory at Woods Hole, Mass., where this work was begun during the summer of 1940.