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Partial Purification of the Virus of Epidemic Influenza by Adsorption on Calcium Phosphate.*

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The purpose of the present communication is to summarize certain of a series of experiments on the partial purification of influenza virus using calcium phosphate as the adsorbing agent. The procedure is simple and the amount of material that can be handled is limited only by the volume of infected lung suspension or chick embryonic tissue suspension available.

The procedure herein employed is a modification of a previously reported method[1, †] for the removal of bacteria from large quantities of broth culture. By the addition of calcium chloride and phosphate buffer, at pH 7.5, a precipitate of calcium phosphate is formed in the broth culture. The precipitate adsorbs the bacteria, settles quickly, and can readily be separated from the supernatant fluid. The bacteria are then "eluted" from the calcium phosphate by adjusting the hydrogen ion concentration of the resuspended precipitate to about pH 4.5. This causes the calcium phosphate to go into solution, leaving the bacteria suspended in a small volume of fluid from which they can be separated by centrifugation. Because of the instability of epidemic influenza virus when exposed to an acidity as high as pH 4.5,[2] this method for the removal of calcium phosphate could not be employed. Accordingly, the modification introduced was one in which the calcium phosphate was dissolved at neutrality by the use of the sodium salt of citric acid.

In this report, a typical experiment will be described in which a comparison is made between the infectivity, the immunogenicity, and the nitrogen content in the untreated and the partially purified preparation of a suspension of infected mouse lung. The amount of residual infective and immunogenic material which remained in the supernatant fluid after adsorption was also tested.

* This study was conducted under a grant from the International Health Division of the Rockefeller Foundation.

1 Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 228.

† Calcium phosphate has been employed for the purification of the diphtheria toxin (Roux, E., and Yersin, A., *Ann. Inst. Past.*, 1889, **3**, 273; Abt, G., *Ann. Inst. Past.*, 1928, **42**, 1336); and for the purification of the type specific polysaccharide of the pneumococcus (Felton, L. D., Kauffman, G., and Stahl, H. J., *J. Bact.*, 1935, **29**, 149).

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

2.8 grams of the lung tissue of mice infected with the PR-8 strain of influenza virus were ground with alundum and diluted with 11.2 cc of physiological salt solution and 16.8 cc of 0.05 M phosphate buffer (pH 7.8) to form a 10% suspension. This was centrifuged for 25 minutes in the Swedish Angle Centrifuge to remove gross particles of tissue. One portion was partially purified in the following manner involving 3 successive adsorptions, although it has since been found that only 2 are necessary. To 15 cc of the suspension in a centrifuge tube, 4.5 cc of 0.1 M calcium chloride and 4.9 cc of 0.1 M sodium hydroxide were added. The optimal hydrogen ion concentration for the precipitation and adsorption was found to be in the range of pH 8.0 to 8.4 as determined with a glass electrode. The reagents were introduced drop by drop, with constant mixing, in order that the precipitate might form evenly throughout the suspension. When the reagents were added in this manner, the precipitate that formed was very flocculent, settled quickly, leaving a crystal clear supernatant fluid. After spinning in the ordinary centrifuge, the separated supernatant fluid was again treated with 4.5 cc of 0.1 M phosphate buffer at pH 7.8, 4.5 cc of 0.1 M calcium chloride and 4.9 cc of 0.1 M sodium hydroxide, and centrifuged. To the second supernatant fluid the same volumes of phosphate buffer and calcium chloride and 4.5 cc of the sodium hydroxide were added. After the third centrifugation, the resultant supernatant fluid, which is ordinarily discarded, was set aside to be tested for residual infective and immunizing potency. The pH of 9.39 of the final supernatant fluid was corrected to pH 7.16 by the addition of 3.5 cc of 0.1 M hydrochloric acid. The precipitates of calcium phosphate were almost pure white and the supernatant fluid was bright red, indicating that little or none of the hemoglobin was adsorbed.

The 3 precipitates were pooled together and washed twice, with 30 and 20 cc, respectively, of 0.05 M phosphate buffer (pH 7.8). The washed precipitate was suspended in 2.5 cc of 1.0 M sodium citrate and 12 cc of 0.1 M hydrochloric acid. (In subsequent experiments citric acid has been used instead of hydrochloric acid.) The pH of the resulting mixture was approximately 6.5. A small amount of precipitate which remained undissolved was separated and treated with 0.7 cc of 1.0 M sodium citrate and 3.0 cc of 0.1 M hydrochloric acid. A small residue, insoluble in sodium citrate, was discarded and the 2 opalescent supernatant fluids were pooled. The final pH of 6.18 was changed to 6.61 by the addition of 4.0 cc of 0.1 M sodium hydroxide. This citrate solution was dialyzed in

TABLE I.
Comparison of Activity and Nitrogen Content of the Untreated and the Partially Purified Preparations.

	Dilutions	Untreated suspension	Partially purified fraction	Supernatant fluid
Titer of Infectivity	10^{-2}	4,4,++	4,4,4	8,++++,++
	10^{-3}			++,++,+
	10^{-4}	5,6,6	5,5,6	
	10^{-5}	6,6,6	++++,+++,++	
	10^{-6}	+++,++,+	++,++,+	
Nitrogen Content[3]	mg per cc 10% susp.	1.46	0.132	
	mg per M.I.D.*	7.3×10^{-7}	6.6×10^{-8}	

Numerals denote days of death of individual mice after inoculation with 0.05 cc of suspension, intranasally, while in ether anesthesia.
Survivors were autopsied on the 10th day after infection.
+ to ++++ represent increasingly greater degrees of pulmonary involvement in the survivors.
*M.I.D. refers to Minimal Infective Dose.

the ice-box for 1½ hours, against 3 changes of 10 volumes of distilled water. The dialyzed solution was then used as the partially purified virus preparation and tested for infectivity, immunogenic potency and nitrogen content. The crude and partially purified preparations and the adsorbed supernatant fluid were all diluted to comparable volumes before use. The results are shown in Table I.

It is clear from these data that the amount of virus activity remaining in the supernatant fluid after adsorption is very little when compared with the original. In fact it appears to be not more than 0.1 of 1% of the amount present in the crude suspension. However, the infective titer of the partially purified fraction compares quite favorably with the titer of the untreated suspension. Although there is a difference in the potency of the 10^{-5} dilutions in this experiment, this is not very great when it is considered that the course of a fatal infection in 2 of the 3 mice, in the group tested with the partially purified fraction, was interrupted by a termination of the experiment. The close similarity in infective titer of these 2 preparations, in view of the residual activity of the discarded supernatant fluid, suggests that little, if any, loss in virus activity occurs in the process of washing, elution, and dialysis. On the other hand, the nitrogen content of the partially purified fraction is distinctly less than that of the untreated, being about 9% of the original. Thus it has been possible to eliminate more than 90% of the nitrogenous impurities with a loss of very little of the virus activity. From a comparison of the nitrogen content of the minimal infective dose of the original

[3] Levy, M., and Palmer, A. H., *J. Biol. Chem.*, 1940, **136**, 57.

and the partially purified preparations it might be said that a purification of approximately 11 times has been effected. Preliminary tests for stability of the partially purified material indicate that the infectious titer is essentially unchanged after standing at ice-box temperature for 24 hours. Efforts toward further purification are in progress. One procedure being tested involves alternate rapid freezing and thawing of the citrate solution. By this means the finely suspended material coalesces and settles out as a precipitate. Approximately one-half of the remaining nitrogen-containing material can be removed in this fashion. However, the amount of reduction in activity which may result has not yet been determined.

Mice were immunized intraperitoneally with the crude material, the partially purified material, and the discarded supernatant fluid. A quantitative estimate of the immunogenicity of these preparations, made in the manner described by Francis,[4] revealed that the immunizing potency for mice paralleled the infective potency.

A comparison was made of the amount of nitrogenous material separated from both normal and infected lungs by the calcium phosphate method. No significant differences were observed. It seems, therefore, that the virus of epidemic influenza can be adsorbed by calcium phosphate in a manner similar to a fraction of normal tissue. This observation is in keeping with those reported by Kabat and Furth,[5] who studied the virus of leukosis and of sarcoma of fowls, using the ultracentrifuge; by Claude,[6] studying the agent causing mouse sarcoma and chicken tumor I; and by Taylor, Sharp, Finkelstein, and Beard,[7] working with chick embryonic tissue diseased with the virus of equine encephalomyelitis.

The degree of purification achieved in the present experiments, in which a chemical method was used, is of the same order as that obtained with the ultracentrifuge in investigations on certain other viruses.[5, 6, 8] More detailed studies are in progress and will be reported subsequently.

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[4] Francis, T., Jr., *J. Exp. Med.*, 1939, **69**, 283.

[5] Kabat, E. A., and Furth, J., *J. Exp. Med.*, 1940, **71**, 55.

[6] Claude, A., *Science*, 1940, **91**, 77.

[7] Taylor, A. R., Sharp, D. G., Finkelstein, H., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 462.

[8] Hughes, T. P., Pickels, E. G., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1938, **67**, 941.