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Protein-Free Suspensions of Virus: VI. Purification of Vaccine Virus by Adsorption and Elution.

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It was shown[1] that by repeated elution with N/500 $NH_4(OH)$ from the same adsorbate it was possible to obtain a potent phage suspension which gave negative protein and ninhydrin tests and contained only 1.4 to 2.0 mg. non-ammonia N per 100 cc. of eluate.

In the present paper experiments are reported showing that it is possible with the same procedure to obtain an equally pure active suspension of vaccine virus.

Technique. An infected rabbit testicle is removed on the 4th or 5th day after inoculation, ground in a mortar with sterile glass, and saline added slowly to give a 10% tissue suspension. After thorough trituration the tissue suspension is transferred to a centrifuge tube and centrifuged slightly to remove coarser particles. The material is handled aseptically to avoid contamination during the preparation. The heavy tissue suspension is then added to tubes containing 50% kaolin in saline, in the proportion of one part virus to one part kaolin suspension, thoroughly shaken, left in the icebox overnight and then treated in the manner previously described.[2]

The following experiments illustrate the details of the procedure employed and results obtained:

Experiment 1. The suspension was prepared in saline and adsorbed in buffered and unbuffered saline suspensions of kaolin. The adsorption was carried out in duplicate and each adsorbate eluted separately. The supernatant fluids after the kaolin adsorption as well as the individual eluates from the adsorbate were tested on rabbits by the injection of 0.1 cc. of the material intradermally. The results are summarized in Table I.

Adsorption with kaolin removed the greater part of the protein and virus from the suspension, and repeated elution with ammonia ultimately yielded a potent virus suspension which gave a negative protein reaction. The buffered kaolin adsorbed more completely but showed no difference in its response to elution. The first and second eluates still gave positive tests for protein and aminoacids; the third eluate, however, no longer gave positive Esbach and ninhydrin reac-

[1] Kligler, I. J., and Olitzki, L., *Brit. J. Exp. Path.*, 1934, **15**, 14.

Protocol. One gm. testis triturated and suspended in 10 cc. saline. After slight centrifugation, 8.0 cc. of the uniform suspension were used for adsorption. Adsorption with buffered (pH 5.6) and unbuffered kaolin; left in icebox 24 hours. The contents of each of the 2 tubes were eluted with: (I) 4 cc. N/100 NH_4OH, or (II) 4 cc. N/500 NH_4OH. Rabbits inoculated 11/23/33. Reactions recorded as —, +, ++, +++, according to area of induration.

TABLE I.

	Rabbit reaction*	Esbach	Ninhydrin
Supernatant fluid after adsorption:			
(a) with buffer I	+	+	+++
(b) " " II	±	+	+++
(c) without buffer I	+	+	+++
(d) " " II	++	+	+++
Eluate I:			
with buffer N/100 NH_4OH	+	+	+++
" " N/500 "	+	+	+++
without buffer N/100 NH_4OH	++	+	+++
" " N/500 "	++	+	+++
Eluate II:			
with buffer N/100 NH_4OH	+++	±	++
" " N/500 "	+++	±	++
without buffer N/100 NH_4OH	+++	±	±
" " N/500 "	++	±	±
Eluate III:			
with buffer N/100 NH_4OH	++	—	—
" " N/500 "	++	—	—
without buffer N/100 NH_4OH	++	—	—
" " N/500 "	++	—	—

*Reading 4th day after inoculation.

tions, although it still contained active virus. The second eluate contained the highest concentration of virus.

Experiment 2. This experiment was designed to ascertain the amount of virus eluted and the conditions most favorable for maximum elution. The original material as well as the respective eluates were titrated on the rabbit skin. To overcome the error due to variations in susceptibility of individual rabbits, a given dilution of each of the materials was inoculated into the same animal. Each animal was thus inoculated on 6 separate points, 5 rabbits being used for the experiment. This procedure also facilitated the comparison of the relative intensity of the reactions to different eluates, making the results as nearly quantitatively comparable as is possible in animal work. Nitrogen determinations were made on all the materials tested.*

It will be noted that the kaolin adsorbs about 80% of the protein and nearly all the virus. It is also of interest that the first eluate contains most protein and is weakest in virus; this is probably due to the neutralization of the NH_4OH by the buffer, thus

*The nitrogen determinations were made by Dr. Rosenberg.

Protocol, Exp. 2. Material not centrifuged before adsorption. Tissue emulsion 1/10 mixed with equal amounts of kaolin suspension. All elutions made with N/500 NH_4OH. B = with buffer; W = without buffer.

TABLE II.

	Original material	Supernatant fluid after adsorption		Eluates (undiluted)							
				I		II		III		IV	
		B	W	B	W	B	W	B	W	B	W
(a) Test of Virus.											
Rabbit Test*	+++	++	++	++	+++	+++	+++	+++	+++	++	++
Non NH_3 N (mg. %)	280	45.8	33.4	5.12	9.1	4.72	6.31	3.95	5.44	2.68	1.04
Esbach Reaction	+++	++	++	±	+	±	±	tr.	tr.	—	—
Ninhydrin Reaction	+++	++	++	±	+	±	±	±	±	—	—

(b) Titration of Virus.

	Dilution tested	Original suspension	Supernatant Fluid	Eluates I	II	III	IV
Rabbit 1	1:100	+++	±	+	+	+	±
" 2	1:1,000	++	±	+	++	++	++
" 3	1:10,000	+++	—	±	+	++	++
" 4	1:100,000	++	—	±	+	+	±
" 5	1:1,000,000	+	—	—	±	+	±

*—, +, ++, +++ indicate relative intensity of the reaction. The rabbit results are the readings on the 5th day after inoculation.

reducing its eluting capacity. The third eluate was the most potent, but the fourth eluate was still active in a 1:1,000,000 dilution, the highest dilution tested. The protocol also brings out clearly the differences in the reactivity of animals, the 1:1,000 dilutions producing milder lesions than the 1:10,000.

Experiment 3. The previous experiment was repeated with the purpose of carrying the dilutions to the maximum and comparing the virus content in the respective eluates with that in the original suspension. The procedure was the same as before. The results are summarized in Table III.

The titration experiments yielded paradoxical results. The eluates were active in as high a dilution as the original tissue emulsion; at the same time, in the lower dilutions, the reactions produced by the tissue suspension were more severe than those produced by the eluates. The greater severity of reaction produced by the original tissue suspension is ascribable to the phenomenon observed by Duran-Reynals.[2] The testicular extract increases the invasiveness

[2] Duran-Reynals, F., *J. Exp. Med.*, 1929, **50**, 327.

Protocol. Infected testes were triturated and a 10% suspension made in saline. The adsorption was made as above by mixing equal portions of the suspension with buffered kaolin. The elution was carried out with N/200 NH_4OH. Four successive elutions were made. The eluates as well as the original suspension were titrated on the rabbit skin. The readings were made on the 4th and 5th day after inoculation. The table gives the results at the last reading.

TABLE III.

Virus Dilutions	Original Suspension	Eluates			
		I	II	III	IV
10,000	+++	++	++	++	++
100,000	+++	+	++	++	++
1,000,000	+++	+	+	±	++
10,000,000	±	±	±	±	++
Esbach reaction	+++	+	+	tr.	—
Ninhydrin	+++	++	+	±	—

The signs ±, +, ++, etc., indicate intensity of reaction; tr. = trace.

of virus by increasing tissue permeability, thus causing a more extensive lesion. Normal testicular extract added to the eluates increases the extent and severity of the lesion.

Summary. Experiments are presented showing that it is possible by adsorption with kaolin and subsequent successive elutions with ammonia to obtain a potent suspension of vaccinia virus giving negative Esbach and ninhydrin tests and containing 1.0 to 2.7 mg. non-ammonia *N* per 100 cc. of fluid. The severity of the reaction produced by a given dilution of the original testicular suspension is always greater than that produced by an equal volume of the same dilution of the eluted pure virus. In most instances the eluates were active in as high a dilution as the original suspension. The vaccinating efficiency of the pure virus has not yet been tested, but it is anticipated that it may prove useful for intradermal vaccination, particularly because of the milder character of the reaction produced by the purified virus.