of both rabbit and mouse brain tissue.

Comparative Antigenicities of Benzene and Ultraviolet Inactivated Vaccines. The relative antigenicities of benzene inactivated as compared with ultraviolet light inactivated vaccines, each made from the same lot of virus, are listed in Table I.

In most instances, when there were differences, the benzene inactivated vaccine showed the higher potency.

In addition to these comparisons showing benzene inactivated vaccines were as good as the ultraviolet inactivated vaccines, more than 20 other lots have been inactivated with benzene alone and have consistently produced good vaccines.

Discussion and Summary. The use of benzene-ether extraction as a preliminary step in eventual purification of rabies vaccines has been made a more practical procedure by the demonstration that inactivation of the virus by heating in benzene results in a highly potent vaccine. Thus the necessity for preliminary ultraviolet irradiation has been eliminated.

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Rabies Vaccine Freed of the Factor Causing Allergic Encephalitis.

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The preliminary phases of a program for the purification of rabies vaccine have been reported in earlier papers. These reports dealt with the problems of removal of lipids from brain tissue vaccines,[1] and the inactivation of rabies virus by heating the dried tissue in a lipid solvent (benzene).[2] Earlier attempts at the purification of rabies antigen by the usual chemical and physical fractionation procedures had met with failure. Brain tissue is rich in lipids and it was thought that these might have the effect of stabilizing the suspension of other constituents including virus, thus inhibiting sharp separation. The first step in separation of antigen, therefore, was the removal of lipids. For this purpose benzene and ether were used, and it was found that vaccines thus treated did not lose antigenicity. A corollary finding was the observation that the rabies virus could be rendered non-infectious by heating during the course of benzene extraction, thus obviating the necessity for preliminary inactivation by ultraviolet light or other agents.

Postvaccinal (rabies vaccine) encephalomyelitis in man, and its apparent counterpart, experimental allergic encephalitis in lower animals, are conditions which occur when brain tissue is injected parenterally.[3-7] The encephalomyelitis may be manifest as a paralysis of the Landry type. Pathognomonic lesions are found in the central nervous system.

A consideration of the etiology of this postvaccinal encephalomyelitis had led to the hope that removal of the lipid components of brain tissue might also eliminate the material which was responsible for the paralysis.[8] However, it was found that benzene-ether extracted vaccine was essentially unaltered in its

1 Wright, J. T., Bell, J. F., and Habel, K., *Science*, 1948, **108**, 118.

2 Habel, K., Wright, J. T., and Bell, J. F., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 455.

3 Freund, J., Stern, E. R., and Pisani, T. M., *J. Immun.*, 1947, **57**, 179.

4 Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117.

5 Kopeloff, L. M., and Kopeloff, N., *J. Immun.*, 1947, **57**, 229.

6 Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

7 Wolf, A., Kabat, E. A., and Bezer, A. E., *J. Neuropath. and Exp. Neurol.*, 1947, **6**, 333.

8 Alvord, E. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 459.

capability of producing paralysis in guinea pigs.

The present report will describe the further treatment of rabies vaccines which has resulted in antigens of high potency, yet freed of the factor which causes allergic encephalitis in experimental animals.

Materials and Methods. In each experiment, one lot of vaccine, or a pool of several lots was used, and comparisons of potency were made between various fractions of the one lot or pool. In every instance the various fractions were brought back to the volume of the original vaccine suspension before being compared. In some of the early experiments the potencies of the vaccines used were so great that no end-point of protection in mice was obtained, and therefore no comparisons of the potencies could be made. In later experiments, an attempt was made to select lots of vaccine which would protect mice when challenged with 100 to 10,000 LD_{50}, which figures are within the usual range of the end points of the mouse protection test. Both mouse and rabbit brain vaccines were used. In all of the tests for the factor causing allergic encephalitis, only rabbit brain vaccines were employed.

In order to elicit the signs and lesions of allergic encephalitis, guinea pigs were injected in the nuchal area with vaccines made up in mineral oil containing killed *M. tuberculosis* and Arlacel-A according to the method of Freund.[3,9] One injection of one ml of the adjuvant mixture was made subcutaneously in the nuchal area of the guinea pigs, and early signs of encephalitis usually ensued about 12 to 14 days later. The animals were observed for about 7 weeks after injection. Characteristic lesions of allergic encephalomyelitis sometimes occur in guinea pigs which remain asymptomatic. Therefore, random samplings, or in some cases, all of the guinea pigs in groups without signs of encephalomyelitis were killed at the termination of the experiments, and the brains and cords were examined for microscopic lesions.*

Calcium Acetate as a Precipitant for Virus Antigen. In the course of some experiments on the elution of live rabies virus from tissue it was observed that calcium acetate greatly reduced the titer of virus in the supernatant liquor of a centrifuged suspension of rabies brain. That this effect was not caused by killing the virus was proved by resuspending the sediment (in molar sodium chloride solution) and again centrifuging the suspension. The titer of virus thus obtained in this second supernatant fluid was as high as was ordinarily expected in the original. Further investigation revealed that the virus treated with calcium acetate, and apparently precipitated by it, could not be resuspended in water. Non-viable virus antigen likewise was precipitated by calcium acetate and remained insoluble in water. This precipitated antigen retained its antigenic potency.

In the first experiment rabies-infected brain was homogenized in a 1% solution of calcium acetate. The mixture was centrifuged and the titer of virus in the supernatant fluid was found to be low. Further experiments revealed that concentrations as low as M/100 (0.158%) of calcium acetate could be used with maximal effect. In these experiments the brain tissue vaccines were suspended in various concentrations of calcium acetate and filtered. The filtrates were then used as antigens in the mouse potency test.

It is evident here that calcium acetate in concentrations of M/100 or greater prevented passage of antigen through the filter.

The insolubility in water of virus antigen precipitated by calcium acetate is shown in Table II. In these experiments, vaccines which had been extracted by benzene and ether were suspended in water ("original"). Sufficient calcium acetate solution was added to the suspensions to make a final concentration of M/10 and the suspensions were centrifuged. (The supernatant fluid is referred to as "calcium acetate wash"). After decanting the supernatant fluid, the sediment was resuspended in a volume of distilled water equal to the first supernatant fluid, and the mixture was again centrifuged. (The supernatant

[9] Freund, J., Thomson, K. J., *et al., J. Immun.*, 1948, **60**, 383.

* The pathological studies were done by Dr. James Peers.

TABLE I.
Antigenic Potency of Filtrates of Vaccines in Various Concentrations of Calcium Acetate in H_2O, Expressed in LD_{50} Protection.

Exp. No.	M/10	M/100	M/1000	No Ca Ac
A 168	<22	<22	>220,000	220,000
A 179	<36	<36	1,500	20,000

TABLE II.
Antigenic Potency of Vaccine Fractions in LD_{50} Protection.

Exp. No.	Original	Cal. acetate wash	Water wash	Residue
A 187	69,000	420*	<42	11,000
A 188	2,900	<29	<29	360
A 195	1,200	<17	<17	1,100

* M/100 calcium acetate was used instead of M/10 and insufficient time for reaction was allowed.

TABLE III.
Comparative Potencies of Original and Washed Vaccines (LD_{50} Protection).

Lot	Original	Washed
A 142	>381,000	>381,000
A 152	470	1,200
A 158	> 66,100	17,800
A 163	> 32,400	> 32,400
A 177	>513,000	16,300

fluid is labeled "Water Wash"). The sediment was resuspended in distilled water to the original volume ("Residue").

The comparative antigenic potencies of other benzene-ether extracted vaccines and their washed calcium acetate residues are presented in Table III.

It has been observed that the most effective way of adding calcium acetate to the vaccine is by the addition of a solution of the chemical to a suspension of the vaccine. (Other methods such as the addition of the calcium acetate to the vaccine before drying and extracting with benzene and ether, adding the dried extracted powder to a solution of calcium acetate, or adding the chemical to a vaccine resuspended after extraction were tried. The last procedure was satisfactory but less consistent in results.)

It will be noted in the experiments recorded in Table II that the potencies of the residues are less than those of the control vaccines. While variations of less than three-fold may be within the limits of error of the mouse potency test, some of the apparent loss of potency seems to be the result of the poor dispersion of the precipitated antigen when resuspended. This belief is substantiated by the decrease in antigenicity caused by the addition of calcium acetate directly to vaccine as compared to the same whole emulsion vaccine without calcium acetate. (See Table IV.)

This effect could be interpreted as an actual diminution of antigen, but the lack of toxicity of calcium acetate to live virus, and the relative constancy of the effect without regard to time of exposure or concentration of the salt have led us to believe that the difference is caused by poorer dispersion of the precipitated antigen.

The Nitrogen and Calcium Contents of Washed Vaccine. Repeated analyses of vaccine before and after washing with M/10 calcium acetate followed by water have revealed that the first wash (*i.e.*, with calcium acetate) removes about 45% of the total nitrogen, and the subsequent washing with water removes about 5%. The residual washed vaccine then contains about 50% of the total nitrogen of the original vaccine. A small portion of the calcium added to the vaccine is retained by the insoluble tissue components. Most of this is removed by

TABLE IV.
Effect of M/10 Calcium Acetate on Antigenic Potency* of Crude Rabies Vaccine.

Vaccine	Without calcium acetate	With calcium acetate
A 183	>355,000	60,300
A 188 K	10,300	2,900
A 188 L	2,900	1,100

* Potency is expressed in LD_{50} protection.

TABLE V.
Pathogenic and Antigenic Potencies (LD_{50} Protection) of Crude and Washed Vaccines.

			Allergic encephalitis	Antigenic potency
A188	Unextracted	Original vacine	9/10	1,100
		Cal. acetate wash	1/8	<29
		Washed vaccine	2/9	130
	Extracted	Original vaccine	4/10	2,900
		Cal. acetate wash	9/10	<29
		Washed vaccine	0/9 *	360
A195	Unextracted	Original vaccine	5/8	1,400
		Cal. acetate wash	5/10	<17
		Washed vaccine	7/10	1,200
	Extracted	Original vaccine	7/10	1,200
		Cal. acetate wash	8/10	<17
		Washed vaccine	0/10†	1,000

The denominator indicates the number of animals injected. The numerator indicates those specifically affected.

* Five animals were killed and the brains and cords were submitted to histological study. None showed lesions of allergic encephalitis.

† All of the animals in this series were killed and the brains and cords were found free of the lesions of allergic encephalitis.

washing with water. The final calcium content of the washed vaccine (10% vaccine) is then about 0.005%.

Extraction of the Factor Causing Allergic Encephalomyelitis. When it was discovered that much water soluble material could be removed from the vaccine without loss of antigen by the use of the calcium acetate it seemed worth while to test the various fractions thus obtained for the encephalitic effect. Consequently, a portion of a lot of vaccine, after washing with calcium acetate, was then treated with molar saline in an attempt to elute antigen from the sediment. The original lot of vaccine, the calcium acetate wash liquor, the molar saline wash liquor, and the final residue were each mixed with adjuvant and injected into guinea pigs. The results were as follows:

Original vaccine (Lot A169)	2 of 8 guinea pigs paralyzed
Calcium acetate wash	7 of 10 " " "
Saline wash	4 of 10 " " "
Residue	None of 9 " " "

It was obvious then that one washing with calcium acetate was insufficient to remove all the factor. However, it was equally evident that the factor was soluble in the calcium acetate solution. That guinea pigs injected with the second wash liquor were also affected could readily be explained by the fact that not all of the calcium acetate solution could be removed from the residue by drainage. That this encephalitic factor can be completely removed by a second washing with water is demonstrated by the experiments reported in Table V.

In these experiments the effects of the washing procedures on antigenicity as well as the encephalitic factor of the vaccines were tested. Both benzene-ether extracted and unextracted irradiated vaccines were subjected to the calcium acetate treatment. The extracted and unextracted vaccines of each lot were suspended in M/10 calcium acetate solution and samples were removed for testing ("Original"). The remainder was centrifuged and the supernatant fluid ("calcium acetate wash") replaced by an equal volume of distilled water. The sediment was resuspended in water and again centrifuged. After decanting and measuring the supernatant fluid, the sediment was suspended in the same quantity of M/1 saline. Antigenic potency as recorded in Table V in the row marked "washed vaccine" was determined for this resuspended sediment. However, for the detection of the factor causing allergic encephalitis in the washed vaccines only the liquid phase of the mixture was used.

The data indicate that the factor which causes encephalomyelitis was present in the original vaccines, both extracted and irra-

TABLE VI.
Absence of the Encephalitic Factor from Washed Vaccines (Whole Washed Sediments).

	Vaccines	Encephalitis	Antigenicity (LD_{50} protection)
A 200	Original vaccine	8/10	4,080
	Washed vaccine	0/10	1,000
A 201	Original vaccine	3/5	340
	Washed vaccine	0/5	120

diated, and that it was soluble in the calcium acetate washes. After washing once with calcium acetate solution, and once with distilled water, no more of the factor could be demonstrated in the vaccines which had previously been extracted with benzene and ether. However, the method does not appear to be effective with vaccine which has not been previously extracted.

Further evidence that the factor has been completely removed from washed vaccine is presented in Table VI where the whole washed sediment was tested.

Discussion. By a process of extraction with calcium acetate solution, rabies vaccine may be freed of the factor which causes allergic encephalitis. Absence of the encephalitic factor from the washed vaccine was first indicated by the fact that it was not present in the liquid phase of the final washed vaccine. Further proof was obtained by injecting the whole washed vaccine into guinea pigs without producing paralysis.

In the experiments recorded here calcium acetate has been used because it has been found to give satisfactory results. No doubt other salts of calcium, and salts of other alkaline earth elements may be substituted. In fact, calcium chloride has been found to react very similarly.

Whether the allergic encephalomyelitis as produced in guinea pigs is identical with that occasionally seen in man following rabies vaccination is not known, but the signs and lesions, together with the common factor of development of the syndromes following the injection of brain tissue suggest that the conditions are actually similar.

The results of our experiments have been reported here because of their possible application to the production of safe rabies vaccine for human use. It is presumed that washed rabies vaccines which no longer produce allergic encephalitis in experimental animals will likewise not cause post vaccinal paralysis in man. Further purification of vaccine for human use is desirable, and the problem is being studied.

Summary. A method has been presented for the removal from rabies vaccine of the factor which causes allergic encephalomyelitis. This factor is not removed by extraction with benzene and ether, but preliminary treatment of the vaccine with these solvents facilitates separation by subsequent treatment. The presence of calcium acetate prevents the loss of antigen when the vaccine is washed. It does not prevent removal of the encephalitic factor which appears to be water soluble. About one-half of the total nitrogen of the vaccine is removed by this washing process. The technic may be briefly summarized as follows:

1. A suspension of infected brain in water is dried from the frozen state.
2. The dried brain is extracted with benzene followed by ether (Live virus may be killed in this stage by heating in benzene).
3. After removal of the ether the dried brain is suspended in distilled water.
4. Sufficient solution of calcium acetate is added to make final concentration of M/10 calcium acetate, and the suspension is permitted to stand in the cold for an hour or two.
5. The calcium acetate solution is removed by centrifugation or filtration and the sediment is resuspended in distilled water to the original volume with agitation (clumps of sediment must be broken up).
6. The distilled water is removed by centrifugation or filtration.
7. The sediment is resuspended in distilled water or saline and homogenized. This is the washed vaccine.