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Benzene-Inactivated Rabies Vaccine.

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As a part of a program aimed at purification of rabies vaccine, an earlier report[1] was made on the benzene and ether extraction of ultraviolet inactivated rabies vaccine.

The technic described in our earlier paper involved the extraction of infected, dry brain tissue containing virus inactivated by ultraviolet light. Irradiation was used for inactivation because it was known to yield vaccines of high antigenic potency, and because cold benzene and ether did not inactivate all of the rabies virus. The present paper describes a method for inactivation of rabies virus by benzene during the course of extraction of lipids with that solvent.

Materials and Methods. The procedure of inactivating rabies virus by benzene differs only slightly from the method described for extraction of brain lipids.[1] A 20% infected brain suspension in distilled water is rapidly dried from a frozen state. To the dry powder is added sufficient benzene to equal 2 times the original volume. The suspension is vigorously agitated to break up clumps of tissue, poured into a screw-top bottle, and placed in a water bath at the desired temperature. After the appropriate period of time (see below) the benzene is removed by filtration through a sintered glass filter of "M" porosity by suction. The suction is then turned off and clean benzene is added to the residue on the filter. Resuspension is accomplished by stirring with a sterile applicator stick, and again suction is applied to remove the benzene. It has been established that the volume of benzene used for the first extraction is more than sufficient to dissolve the soluble lipids in the brain tissue. The first extraction is done, therefore, to dissolve lipids. The second extraction, of short duration, is done to flush out the benzene which contains lipids.

Following the extractions with benzene, the residue on the filter is resuspended in one volume of ether. The ether is permitted to filter without suction. A second resuspension in ether is done and this time the ether is removed by suction. The purpose of the ether extraction is twofold: to remove lipids—especially phosphatids which may not be soluble in benzene, and to flush out benzene not removed by filtration.

The small amount of ether which remains is removed by placing the entire filter in a vacuum chamber for a period of about 30 minutes. The dry powder is then added to a volume of distilled water equivalent to the original volume. Mixing and hydration are accomplished by churning in a Waring blender (a drop of caprylic alcohol is used to reduce the foam thus formed).

Antigenic potency of the vaccines is determined by the mouse protection test of Habel and Wright.[2]

The Temperature and Time Necessary for Inactivation of Rabies Virus (P M strain 980). The principle of using higher temperatures for the inactivation of rabies virus in benzene was derived from the early experiments in which the virus was treated with benzene at 5°C. It was observed at that temperature that the titer of live virus was markedly decreased, but the antigenic potency was not adversely affected. Subsequently, the vaccine was extracted at 37°C and no decrease in antigenicity was observed, but the titer of live virus was more markedly diminished.

It was subsequently found that 24 hours at 37°C would usually result in inactivation of the virus but occasionally viable virus could

1 Wright, John T., Bell, J. Frederick, and Habel, Karl, *Science*, 1948, **108**, 118.

2 Habel, Karl, and Wright, John T., *Public Health Rep.*, 1948, **63**, 44.

TABLE I.

Lot No.	Animal species and source of vaccine	Benzene inactivated			Ultraviolet inactivated, LD_{50} prot., × 1000
		Temp., °C	Time, hr	LD_{50} prot., × 1000	
1	Mouse	37	144	>417	289
2	"	37	72	>289	246
3	Rabbit	37	96	1.2	1.2
4	"	37	96	0.25	0.23
5	Mouse	56	8	35	35
6	Rabbit	56	8	1.7	2.2
7	"	56	9½	10	2.8
8	"	56	12	1.2	1.4

be detected. An obvious reason for this variability in inactivation seemed to be that some clumps of dry tissue were not broken up in benzene when simply shaken in that solvent. Subsequent extractions for 24 hours at 37°C using more finely divided tissues consistently yielded non-viable antigens.

For routine inactivation of the virus, however, it is desirable to submit the tissues to more than the bare minimum of exposure usually necessary for such inactivation, as long as antigenicity is not lost by this over-exposure. There has been no indication that over-exposure to benzene at 37°C results in any loss of antigenicity, and there is direct evidence that exposure for as long as 5 days at that temperature is not harmful.

Because benzene at 37°C was not inimical to the vaccine, even on prolonged exposure, it seemed probable that a higher temperature might be used for extraction. The following data compare the potencies of vaccines prepared from one lot of rabies infected brain emulsion exposed to temperatures of 37°, 45°, and 56°C for 72 hours during benzene extraction. All 3 vaccines were negative when tested for viable virus.

37°C	for 72 hrs	LD_{50} protection	>195,000
45°C	" " "	" "	>195,000
56°C	" " "	" "	11,400

It is apparent that 56°C for 72 hours caused a marked reduction in antigenicity, but did not destroy it completely.

The next step was to determine the minimum time necessary for rendering all the virus non-viable at 56°C. It was found that virus thus exposed for 9 hours was inactivated, but that at 5 hours sufficient live virus remained to infect one out of 6 mice when a 10% whole brain suspension was injected intracerebrally. One hour at 56°C was sufficient to effect a ten-fold drop in titer.

The effect of prolonged heating at 56°C in benzene upon the antigenicity of the virus is exemplified below:

56°C	12 hr	LD_{50} protection	11,300
"	24 "	" "	27,600
"	48 "	" "	<4
"	70 "	" "	<4

The vaccine extracted for 24 hours appears to have greater antigenicity than that heated for only 12 hours, but this difference is not significant. However, at some time between 24 hours and 48 hours the potency of the vaccine is almost completely destroyed.

From these data a procedure has been derived for the routine preparation of vaccines which accomplishes the two primary objectives of high antigenicity and non-viability. In ordinary use the virus has been exposed to benzene at 56°C for 12 hours. This treatment has resulted in uniformly high potency of various lots of vaccine, and has consistently yielded non-infectious vaccines.

The Lipid Content of Benzene-Treated Vaccines. In the previous report it was stated that benzene-ether extraction at 5°C effected a removal of 41% of the total dry weight of brain tissue. This statement was in error since subsequent studies have established by extraction of brain tissue with hot alcohol and ether that the lipid content of rabbit brain and of mouse brain are 50% and 40% respectively, of the total dry weight. After extraction with benzene and ether the residual lipids constitute about 25% of the dry weight

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.