junction to a variable extent. Histologically, this uveitis appeared as hyperemia of the iris and ciliary body, usually with very little cellular exudate. The choroid and retina were not visibly affected after injection of either the eastern or western viruses into the anterior chamber.

Conclusion. The corneal edema and opacity resulting from intra-ocular injection of the virus of western equine encephalomyelitis in domestic rabbits occurs only in response to large amounts of virus. It appears to be analogous to the ocular response to influenza virus, which has been attributed to toxic properties of the virus. An entirely similar ocular reaction has been induced by injection of equine encephalomyelitis virus of the eastern type. Limited growth of equine encephalomyelitis virus may occur in the eye but is not a significant factor in the described reaction.

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A Vaccine Against Japanese B Encephalitis Prepared from Infected Chick Embryos.

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(Introduced by J. E. Smadel.)

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Most of the pathogenic viruses affecting man have been cultivated in the developing embryonated egg.[1] In addition the egg has provided a source of vaccine for immunizing human beings against such viruses as yellow fever,[2] influenza,[3] vaccinia,[4] and equine encephalomyelitis.[5] Although the virus of Japanese B encephalitis was shown to multiply in the embryonated egg,[6] growth of this agent in chick embryo tissue was not sufficiently luxuriant to offer promise of providing material for a potent vaccine against this disease. Indeed, the reported experiments regarding such a vaccine indicated that it possessed little immunizing capacity.[7]

The vaccines at present employed against Japanese B encephalitis are prepared from infected brain tissue of mice.[7] These are capable of eliciting neutralizing antibodies against the virus in vaccinated human beings and of inducing resistance to infection in experimental animals. Such vaccines have been prepared commercially and have been authorized for use in the U. S. Army. Although the mouse brain vaccine has desirable immunological properties, it possesses certain potentially objectionable features. For example, it is well known that vaccines containing brain tissue may on occasion produce a demyelinizing encephalopathy. In addition the technical difficulties inherent in the large scale manufacture of such a vaccine are considerable. Because of these disadvantages it seemed worth while to reinvestigate the possibilities of obtaining a potent vaccine from tissues of infected chick embryos.

1 Burnet, F. M., Med. Research Council, Special Report No. 220, 1936, London.

2 Theiler, M., and Smith, H. H., *J. Exp. Med.*, 1937, **65**, 787.

3 Burnet, F. M., and Lush, D., *Brit. J. Exp. Path.*, 1938, **19**, 17.

4 Goodpasture, E. W., Buddingh, G. J., Richardson, L., and Anderson, K., *Am. J. Hyg.*, 1935, **21**, 319.

5 Beard, J. W., Finklestein, H., Sealy, W. C., and Wyckoff, R. W., *Science*, 1938, **87**, 490.

6 Haagen, E., and Crodel, B., *Zent. Bakt.*, Abt. 1, orig., 1938, **142**, 269.

7 Sabin, A. B., Duffy, C., Warren, J., Ward, R., Peck, J. L., and Ruchman, I., *J. A. M. A.*, 1943, **122**, 477.

The present report describes the development and preparation of such a vaccine which, in its capacity to immunize mice, compares favorably with the commercial Japanese B vaccines currently made from infected mouse tissue.

Cultivation of the Virus. The "Nakayama" strain of virus was established and carried in embryonated eggs by the inoculation of 0.1 cc amounts of infected allantoic fluid or embryo suspension into the chorio-allantoic sac. Death of the embryo occurred in practically all the inoculated eggs between the fourth and the sixth day.

Several experiments were performed to determine the optimal conditions for growth of Japanese B encephalitis virus in the developing egg. The results may be summarized briefly as follows: The amount of virus in the inoculum and the route by which it was injected had no marked effect upon the yield of virus, provided the agent was inoculated beyond the chorio-allantoic membranes. The agent was present in all tissues and fluids of the inoculated embryo, but the embryo regularly yielded more virus than the fluids or membranes. The infectious titers obtained in one experiment illustrate this point; the results were, embryo $10^{-7.0}$, yolk sac $10^{-5.0}$, allantoic fluid $10^{-4.6}$, chorio-allantoic membranes $10^{-4.5}$, and yolk fluid $10^{-3.6}$. The maximum concentration of virus in the embryo or in the allantoic fluid of inoculated eggs was obtained after incubation at 35° C for 48 to 72 hours. It is of interest to note that during the course of serial passage of the virus in eggs, the infectivity of inoculated embryo increased. By the thirtieth passage, the infective titers of embryo suspensions ranged between $10^{-7.5}$ and $10^{-8.5}$, with an average value of about $10^{-7.8}$.

Preparation and Assay of Vaccines. The seed virus to be used for inoculation of eggs was harvested and stored as a 10% suspension of infected embryo tissue in whole filtered chicken serum. Such seed suspensions withstood storage at -70° C in sealed ampoules for more than 1 month with no appreciable loss in titer. Both seed virus and material for vaccine were obtained from embryonated eggs which were 7 or 8 days old when infected via the chorio-allantoic sac. These were subsequently incubated at 35.0° C for 3 days and at the end of this time the embryos were harvested.

The present method for preparing formalinized vaccine from the infected embryo is now given in detail. The whole embryo was removed from groups of 20 to 200 infected eggs. The lens and retinal cup were dissected out and discarded. The remaining embryonic tissue was then pooled and homogenized in a Waring blendor for 5 minutes with sufficient physiological saline, buffered at pH 7.4, to make a 10 or 20% suspension by weight. The resulting suspension was strained through gauze. After samples were removed for sterility tests and for titration of infectivity, sufficient formaldehyde U. S. P. was added to give a final concentration of 0.2%.*

The material was then stored at 5° C for 2 weeks. It was then assayed for potency and a safety test was performed. For the latter test, 10 mice were inoculated intracerebrally with the vaccine and observed for signs of neurological disease for a period of 2 weeks in order to determine that inactivation of the virus had been complete. The protein nitrogen content of seven samples of 20% embryo vaccines was determined; the values ranged between 0.68 mg/cc and 0.95 mg/cc with an average of 0.84 mg/cc. (The protein nitrogen in a 10% suspension

* Experiments were performed to determine the comparative effectiveness of formaldehyde and ultra-violet light as inactivating agents on allantoic fluid vaccines. The minimal lethal concentration or exposure time was first determined for each agent. The ultra-violet source was a water-cooled mercury vapor lamp of the type described by Oppenheimer and Levinson.[8] Under the conditions of these experiments formaldehyde-treated vaccine yielded superior antigenic potency. The irradiation experiments were performed in collaboration with Dr. Fred Stimpert of the Parke, Davis Co., Detroit, Mich.

[8] Oppenheimer, F., and Levinson, S. O., cited in *J. Immunol.*, 1945, **50**, 317 (original paper unpublished).

TABLE I.
Japanese Encephalitis.
The Immunizing Capacity of Vaccines Prepared from the Chick Embryo.

Egg passage No.	Type of vaccine	Incubation temp., °C	Infectivity titer before inactivation	Minimal immunogenic dose; 50% end point cc
14	10% embryo	37.5	$10^{-7.0}$	0.57
17	" "	37.5	$10^{-7.4}$	0.043
22	" "	35.0	$10^{-7.5}$	0.038
24	" "	35.0	$10^{-7.3}$	0.060
28	" "	37.5	$10^{-7.0}$	0.057
31	" "	37.5	$10^{-7.5}$	0.038
32B-1	20% "	35.0	$10^{-8.4}$	0.006
32B-2	" "	37.5	$10^{-7.3}$	0.012
39A-1	" "	35.0	$10^{-7.5}$	0.009
39A-2	" "	37.5	$10^{-7.5}$	0.016
39	" "	35.0	$10^{-8.2}$	0.008
43	" "	35.0	$10^{-7.7}$	0.0048
43A	" "	35.0	$10^{-7.8}$	0.0043
Pool 50-52	" "	35.0	$10^{-7.2}$	0.121
50B	" "	35.0	$10^{-7.4}$	0.0023
51	" "	35.0	$10^{-8.0}$	0.0100
51A	" "	35.0	$10^{-7.3}$	0.008

of infected mouse brain simultaneously tested averaged 1.1 mg/cc.) The method employed in the assays of the Japanese B encephalitis vaccines was a simplified procedure used by A. B. Sabin[9] in routine laboratory tests on experimental vaccines. It differs from the standard method he developed for routine assay of mouse brain vaccines for commercial purposes in that fewer mice are used and the challenge dose of virus is not titrated intraperitoneally. However when assays on chick embryo vaccines were performed at the National Institute of Health using the standard method, the results agreed closely with tests on the same material done at the Army Medical School.

The method we have employed is as follows: Graded amounts of vaccine are inoculated intraperitoneally into groups of 10 mice weighing 18 to 20 g each. The dose is repeated 3 days later. One week after the first dose of vaccine, the mice are injected intraperitoneally with a challenge inoculation consisting of 0.3 cc of a 10% suspension of infected mouse brain. In order for a test to be considered valid the challenge inoculum must have an intracerebral titer of $10^{-8.0}$ or more and must kill at least 80% of 20 non-vaccinated mice which are included in each test as a control. Test mice are observed for a period of 3 weeks and the deaths are recorded. The 50% end-point method of calculation is applied to the data to determine the amount of vaccine required to protect half of the mice against the challenge inoculation. This quantity is designated as the Minimal Immunogenic Dose.

The present standard of potency for Japanese B encephalitis vaccines which has been adopted by the National Institute of Health and by the Office of the Surgeon General, U. S. Army,[10] requires that a total inoculum of not more than 0.01 cc of vaccine protect at least 50% of the vaccinated mice.

Results. Vaccines against Japanese B encephalitis have been prepared from chick embryo which meet the standards acceptable for mouse brain vaccine, see Table I.

It will be noted that the vaccines which meet these requirements all contained 20% infected embryo tissue. Thus, 8 vaccines of a group of 11 which contained 20% tissue assayed 0.01 cc or less, while all 6 of the 10% vaccines were unacceptable. The 10% vaccines were prepared from eggs infected with early passage material, and the infectious

[9] Sabin, A. B., personal communication.

[10] Federal Security Agency, National Institute of Health, *Minimum Requirements: Japanese B Encephalitis Vaccine,* Revision of 5 February, 1945.

TABLE II.
Effect of an Adjuvant ("Falba" and Mineral Oil) on the Immunizing Capacity of Certain Japanese B Vaccines.

Vaccine tested	M.I.D. vaccine assayed in: Saline, cc	M.I.D. vaccine assayed in: 2% "Falba"-Min. oil cc
No. 22—10% chick embryo	0.038	0.017
No. 32B—20% chick embryo	0.006	0.0025
Pool II—20% chick embryo—crude	0.011	0.0029
Pool II—20% chick embryo—angle centrifuged	0.013	0.004
No. 43—10% mouse brain	0.040	<0.0012

titers of the tissues used in their manufacture were generally somewhat lower than the titers of tissues which were used for the 20% preparations. That there was some correlation between the infectivity of the embryo tissue and the potency of the vaccine prepared from it, is clearly demonstrated in Table I. Thus, all 5 of the 20% vaccines made from tissues with infective titers greater than $10^{-7.5}$ were acceptable when assayed while only three of the 6 similar preparations with infective titers before inactivation of $10^{-7.3}$ to $10^{-7.5}$ were satisfactory. Further evidence for the border line immunogenic properties of vaccines made from tissues with titers of $10^{-7.0}$ to $10^{-7.5}$ was found in the unsatisfactory results obtained with the 10% vaccines. Finally, several vaccines prepared from chorio-allantoic fluids with titers of $10^{-6.5}$ or less were unacceptable when assayed.

By means of adsorption, centrifugation, and the use of adjuvants, attempts were made to increase the immunizing capacity of the embryo vaccine. Concentration of the antigenic material of the vaccines could not be obtained with adsorption on calcium phosphate or potassium alum. High speed centrifugation in the Sharples† or the angle ultracentrifuge removed most of the antigen from the supernatant fluid of both allantoic fluid and 10% embryo vaccines but the sediment did not exhibit the expected increase in potency.

For example, the sediment obtained on high speed centrifugation of a 10% embryo vaccine was resuspended in sufficient saline solution to make 1/10th the original volume; this had a two- to three-fold increase in immunizing capacity over the original vaccine and contained about one and a half times the amount of protein nitrogen of that in the starting material. Thus, the immunizing agent had been concentrated in the ultra-sediment and perhaps somewhat purified.

A mixture of "Falba"‡ and mineral oil has been used as an adjuvant to augment the immunizing capacity of certain bacterial and protozoal antigens[11] and of influenza vaccines for human beings.[12] We have obtained encouraging results with this adjuvant employed in conjunction with Japanese B embryo vaccines, see Table II.

A mixture of equal parts of "Falba" and mineral oil was prepared and a 2% emulsion of this material in physiological saline solution was used as a diluent for the vaccines throughout the assay procedure. Control assays were done on the same lots in the ordinary manner, *i.e.* dilutions were made in saline solution. It is evident from the results presented in Table II that the minimal immunogenic dose of a given vaccine was appreciably less when the adjuvant was employed. It may be pointed out that vaccines containing "Falba" and mineral oil elicit certain untoward reactions when injected into human beings.[12] For this reason studies are

† The Sharples centrifugation experiments were performed with the kind assistance of Dr. W. M. Stanley, Rockefeller Institute for Medical Research, Princeton, N.J.

‡ "Falba" is the proprietary name for a compound prepared from lanolin and composed of a mixture of "oxycholestrins and cholestrins."

11 Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548.

12 Henle, W., and Henle, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 179.

now in progress to determine whether other agents will serve as satisfactory adjuvants with Japanese encephalitis vaccine.

At the present time no information is available regarding the capacity of this vaccine either alone or with adjuvants to immunize man.

Summary. 1. Highly infectious suspensions of embryo tissue have been prepared from embryonated chick eggs inoculated with the Japanese B virus. Infective titers of $10^{-7.5}$ to $10^{-8.5}$ were regularly encountered under suitable conditions.

2. Formalinized vaccines made from 20% suspensions of infected chick embryo tissue are capable of immunizing mice against intraperitoneal infection with the virus of Japanese B encephalitis. Such vaccines are comparable in potency to the Japanese B encephalitis vaccines prepared from infected mouse brain and used by the U. S. Army.

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Cytotoxic Property of Mouse Cancer Antiserum.*

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In our investigations of the milk factor,[1] or the Bittner virus, associated with mammary carcinoma of mice, an antiserum prepared from rabbits has been tested for its toxicity for cancer cells. In his studies, Andervont[2] found that an antiserum prepared in rabbits by injecting as antigens the centrifugates of mouse tumor, neutralized the milk factor, while normal serums failed to do so. Green, Moosey and Bittner[3] likewise have shown that antiserums prepared from immunized rabbits, as well as from immunized rats, neutralized the agent. We have prepared additional control serums by immunizing rats and rabbits against normal mouse tissue centrifugates, and these have failed to neutralize the virus. This finding demonstrates that the milk factor is antigenically unlike mouse tissue and might more properly be considered a foreign virus. In the experiments here reported a rabbit antiserum prepared against the Bittner virus has been tested for its toxic properties for mammary cancer cells when mixed with them *in vitro*.

Three rabbits were immunized by 5 weekly injections of cancer centrifugate given intraperitoneally. A suspension of mouse cancer tissue was homogenized and subjected to fractional centrifugation. The first sediment, obtained by centrifugation for 1½ hours at 14,000 X gravity, was discarded. The supernatant suspension was again centrifuged 1 hour at 100,000 X gravity. This second sediment was collected, suspended in a 0.9% saline solution and injected in a dosage corresponding to 2 g of original tumor tissue. The rabbits were bled for serum 14 days after the fifth injection of centrifugate. An antiserum against normal lactating mouse mammary tissue was prepared in an identical manner by subjecting lactating breast tissue to the same processes. The normal breast tissue was obtained from mice susceptible to, but not carrying, the Bittner virus. An additional control serum was made from normal rabbit blood. Each of the 3 serums, as finally prepared, represented a pooled serum from 3 rabbits. The 3 serums were test-

* Aided by grants from the Cancer Research Fund, Graduate School of the University of Minnesota, and the Jane Coffin Childs Memorial Fund for Medical Research.

1 Bittner, J. J., *Science*, 1936, **84**, 162.

2 Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **5**, 143.

3 Green, R. G., Moosey, M. M., and Bittner, J. J., *Cancer Research*, 1945, **5**, 588.