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PREPARATION OF JAPANESE ENCEPHALITIS VACCINE,
CHICK EMBRYO TYPE DRIED, FOR THE U.S.ARMY, 1947 *

by

The Staffs **

of the

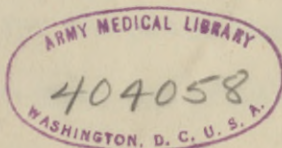
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* Information contained in this paper has already been reported to the Commission on Immunization, Army Epidemiological Board.

** Work reported here resulted from the combined efforts of numerous people at the Army Medical Department Research and Graduate School. It is not feasible to mention each individual and his exact contribution, since many provided part-time assistance in the preparation of vaccine in addition to performing all of their regular duties. This report was prepared by Joseph E. Smadel, Raymond Randall, and Joel Warren, August 7, 1947.



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In 1945 and 1946 military personnel in certain areas of Japan, Okinawa and Korea were vaccinated against Japanese encephalitis. A formalinized vaccine commercially prepared from infected mouse brain was used during both years and in addition, a formalinized vaccine prepared from infected chick embryos was employed in 1946. The latter had been prepared on a developmental basis at the Army Medical Department Research and Graduate School. Mouse brain (1) and chick embryo vaccines (2) induced in mice a comparable resistance to infection with the virus of Japanese encephalitis. Furthermore, antibodies capable of neutralizing the Japanese virus have been demonstrated in the sera of an equal proportion of human subjects following vaccination with either of the two types of vaccines (3). In the fall of 1946 it became evident that any vaccine used in the Pacific Theater during the 1947 season would of necessity be supplied by the Army Medical Department Research and Graduate School because commercial production had been discontinued early in 1946. Facilities for the manufacture of the mouse brain type were not available at the School. Japanese encephalitis vaccine, chick embryo type, fluid, was not entirely suitable since it was known to lose its immunogenic potency when stored at 5° C. for longer than one month (4). Various procedures were investigated in an attempt to find a means for retarding this deterioration. Only two of the methods tested provided encouraging results. Urea added to the crude vaccine was moderately effective as a stabilizing agent but vaccines containing 2% urea were no longer of acceptable potency after storage for two to three months. The second method, drying from the frozen state, appeared to offer the best solution to the problem of deterioration. This report deals with the methods employed for the production of lyophilized Japanese encephalitis vaccine, chick embryo type, and provides data on certain characteristics

of the final product.

PREPARATION OF THE VACCINE

General. The vaccine, prepared from the "Nakayama" strain, is an uncentrifuged 20% suspension of infected chick embryo tissue in buffered physiological saline solution containing sufficient formaldehyde to inactivate the virus. When the fresh vaccine is no longer infectious the residual formaldehyde is neutralized with sodium bisulfite and the fluid preparation is dried from the frozen state under carefully controlled conditions.

Preparation of Seed Inocula. The "Nakayama" strain of virus used for the preparation of vaccine was brought to the School in 1942 as infected mouse brain. For the past two and one-half years a line of this strain has been maintained by serial transfer in embryonated eggs. The seed virus consists of a 20% concentration of pooled infected embryos (usually three per pool) which are suspended in buffered saline, pH 7.8-8.0 (Sorensen's buffer), by mechanical shaking in a thick-walled bottle containing glass beads. The suspension, after light centrifugation, is tested for bacterial sterility and then frozen and stored at -70°C . The infectious titer of the seed preparation should be in the region of $10^{8.0}$ when fresh and should be close to this figure after thawed for use. The frozen seed virus is discarded if not used within ten days.

Shortly before the seed virus is employed, it is thawed and diluted with a solution of penicillin in such a manner that the final concentration of tissue is 10% and of penicillin 100 units per cc. Approximately 500 eggs are inoculated with each pool of seed virus.

Preparation and Harvesting of Vaccine. Fertile eggs from white Leghorn hens, free of infection with *S. pullorum*, are incubated for nine days at 39°C .

after which they are injected with the inoculum described above and incubated at 35° C. Prior to inoculation, one hole is made into the air sac and a second through the shell in the region between the equator and the air sac. A short, curved needle is introduced through the shell membrane underlying the second hole, 0.1 cc of the inoculum is injected, and the holes sealed. This technique, which had previously proved satisfactory in the preparation of vaccine for equine encephalomyelitis (5) almost invariably deposits the inoculum on the chorio-allantoic membrane without backflow of fluid. Inoculated eggs are candled daily and those with dead or sluggish embryos are discarded up to 48 hours. After incubation for 65-66 hours, at a time when approximately 40% of the embryos are dead and 30% are moribund, all of the embryos are harvested.

At the time of harvesting, eggs are dipped in a 3 per cent iodine tincture for five minutes, rinsed in a 1/2000 solution of Roccal, and drained. Under aseptic conditions the eggs are cracked on a breaking-bar in a manner so as not to rupture the amniotic sac. While still in the shell, the sac is ruptured and the embryo is removed with Allis forceps. This method was adopted from the procedure employed for equine encephalomyelitis vaccine (5). Embryos are pooled in lots of about 50 and then the eyes and feet are removed by dissection. Pools of embryos weighing 1600-1700 grams are ground in colloid mills (Eppenbach). The material is coarsely ground in the first of two mills and finely ground in the second. A sufficient amount of buffered physiological saline solution, pH 7.8, is added slowly during the process to make a final concentration of 20% embryo tissue. The entire grinding procedure requires 10-12 minutes for each batch of 1600 grams of tissue. A representative sample is taken from the first pool of ground tissue obtained on each day of harvest and its infectivity titered in mice. After the sampling, sufficient formaldehyde solution U.S.P. is added to the suspension during the milling process to

give a final concentration of 0.2%. The formalinized material is stored in the dark at 3-4° C. in nine liter bottles for 14-18 days; each bottle is shaken daily by hand to resuspend the sediment which forms. Between the 14th and 18th day the supernatant fluids from bottles of one or more batches are aspirated from the sedimented sludge and pooled. Residual coarse particles in the supernatant fluids are removed by passage through a fine mesh screen. Penicillin is now added to the pool in sufficient amounts to make a final concentration of 5 units per cc. At this stage a sample of each pool is taken for test of bacterial sterility, for safety test in animals and for an immunogenic assay.

Lyophilization of Vaccine. The lyophilization apparatus available had a capacity for drying 15 liters of vaccine in a 24 hour period. Therefore, complete lyophilization of the vaccine produced during one week would require 4-7 days. Approximately 16 liters of formalinized, pooled vaccine, an amount which was sufficient for each day's lyophilizing, was removed from storage and processed as outlined below.

a. Neutralization of formaldehyde. The formaldehyde in the pooled fluid vaccine is neutralized by means of sodium bisulfite. A 100 cc. sample is removed from the vaccine destined for drying on that day and the amount of sodium bisulfite solution required to neutralize the formaldehyde in this aliquot is determined. The technique previously employed with mouse brain vaccine (1) is used in the present procedure. This consists of adding sufficient bisulfite until the Schryver test formaldehydes become negative, after which the pH of the sample is adjusted to about 7.3. On the basis of the data obtained for the 100 cc. sample, a 16 liter volume is neutralized and adjusted. Samples of the larger pool after neutralization are retested

to insure that the added materials give the expected effect.

b. Bottling and Freezing. Sufficient merthiolate is added to bring the final concentration to 1:10,000. The fluid material is distributed in 25 cc. amounts in 60 cc. pyrex glass ampoules and immediately shell-frozen by mechanical rotation in a bath of alcohol and solid CO₂. The ampoules are then stored -70° C. for one or two hours when they are attached to the lyophilizing machine.

Lyophilizing Apparatus. A diagram of the apparatus used for drying the vaccine is presented in Figure 1. ~~Three units of the type illustrated were obtained from the International Health Division of the Rockefeller Foundation which had used them previously for drying influenza and yellow fever vaccines.~~ These were built according to designs modified from those previously published by Pickels and Bauer (6) who, in turn, modified the apparatus of Flosdorf and Mudd (7).

Process of Lyophilization. For the successful drying of Japanese encephalitis vaccine it is essential that the frozen biological not be permitted to thaw at any stage during the procedure. The initial period in the process is a highly critical one, and the interval between the removal of the ampoules from -70° C. storage and the time the frozen vaccine is subjected to a high vacuum must be short; in practice 15 minutes is required for loading and obtaining a vacuum of 30-50 microns with the International Health Division equipment. The drying cycle, which is completed in 24 hours, begins with a period lasting several hours during which the temperature of the air surrounding the outside of the ampoules of vaccine is in the region of -20° C. The air temperature slowly rises and reaches 0° C. 18 to 20 hours after starting the run. Then the temperature is elevated rapidly to + 30° C. and maintained here during the 22nd to 24th hours of the cycle. The temperatures

designated above are obtained in the early stage by mechanical refrigeration of the compartment wall which surrounds the ampoules (see Figure 1) and in the last stage by the use of electric hot air blowers (hair dryers). After the second hour, circulation of the air is maintained in each compartment by means of electric blowers (hair dryers with heating element switched off), thus insuring uniform air temperature throughout a compartment. At the end of the cycle the vacuum in the system is between $1\frac{1}{2}$ -3 microns of mercury. A graphic illustration of the temperature and pressure readings from a typical run is presented in Figure 2.

At the end of the cycle, the line to the vacuum pump is closed and "Super-dry", oil-pumped, nitrogen (moisture content less than 0.002%) is passed through a column of a chemical drying agent and thence into the evacuated system until atmospheric pressure is reached.

The manifolds with nitrogen charged ampoules attached are removed from the machine and the necks of the ampoules are sealed with a gas-oxygen torch. Ampoules of lyophilized vaccine are inspected and then stored at 5° C. while control tests are completed.

Shipment of Vaccine. Ampoules containing lyophilized Japanese encephalitis vaccine, chick embryo type, are supplied in individual boxes containing instructions on the method of rehydration together with a rubber stoppered bottle containing 25 cc. of sterile distilled water to be used for its rehydration. The dried vaccine is maintained at 5° C. Rehydrated vaccine is also stored at 5° C. when not in actual use and unused portions are discarded after eight hours.

Control Tests on Vaccine.

a. Safety and sterility tests. Tests for inactivation of virus and

other safety tests are made in accordance with the Federal Security Agency, National Institute of Health, Minimum Requirements, Japanese Encephalitis Vaccine, Chick Embryo Type, dried, August 7, 1947. Sterility tests are performed in general accordance with the accepted methods except that the media used for the bacterial cultures contains clarase as a source of penicillinase. This is added to destroy the penicillin which is incorporated in the vaccine at the time of pooling.

b. Residual moisture. Estimations of the residual moisture content are made on samples of dried vaccine from each day's run by weighing the materials before and after exposure to phosphorus pentoxide under partial vacuum at 37° C. for 48 hours. A moisture content of less than 1% is acceptable for a dried vaccine. The residual moisture content of 49 sub-lots averaged 0.62% with a maximum of 1.46% and a minimum of 0.15%.

c. Immunogenic potency test. The mouse protection test employed is that described in Federal Security Agency, National Institute of Health, Minimum Requirements, Japanese Encephalitis Vaccine, Chick Embryo Type, dried, August 7, 1947. Potency tests are performed on one or more sub-lots of each lot of dried vaccine. Freshly rehydrated material is used for each series of immunizing injections of mice. If a number of sub-lots are dried over a period longer than three days, then assays are performed on samples from the first and last sub-lot.

Quantity of Vaccine Produced. During the period from early February to mid-May, 1947, two batches of 2600 eggs each were inoculated and harvested weekly and these together yielded about 90,000 cc. of a 20% suspension of chick embryo tissue. In general, the material produced each week was pooled and designated by a lot number. During this period 1,346,369 cc. of 20% tissue suspension were prepared. Of this amount 15% was discarded as sedi-

mented material during preliminary processing. The major portion of the formalinized vaccine cleared of the sediment was lyophilized; this amounted to 767,550 cc., (30,702 ampoules). Because of the limited facilities for lyophilization a variable amount of the excess fluid vaccine was treated with urea each week in order to retard antigenic deterioration. During the above period approximately 250,000 cc. were so treated, however, relatively little of this was subsequently used for the immunization of military personnel.

During the three and a half months of production one entire lot and two sub-lots were discarded because of bacterial contamination; these totaled 105,000 cc. Five lots, totaling 302,000 cc. produced during this period were discarded because of poor immunogenic activity. It may be noted that the infectivity of these five unacceptable lots ranged between $10^{-7.0}$ and $10^{-7.3}$, which is below the titer regarded here as a prerequisite for a potent vaccine. A total of 587,950 cc (23,518 ampoules) of lyophilized vaccine was shipped between 19 April and 26 June, 1947 for the immunization of military personnel in the Far East.

STABILITY OF THE VACCINE

Japanese encephalitis vaccine, chick embryo type, when in the fluid state deteriorates rapidly. The majority of such preparations are no longer of acceptable potency after 40 days at 5° C. When held at 37° C., three hours is sufficient to destroy most of the immunogenic material in the vaccine (See Table 1). In contrast, dried chick embryo vaccine is quite stable. It may be stored at 5° C. for at least 77 days without appreciable change in its assay value (See Table 2). Furthermore, dried vaccine stored at room temperature for two weeks displays no loss of activity, whereas when held at 37° C. for two weeks it shows some loss but still contains an appreciable amount of immunogenic activity, see Table 1.

RECOMMENDATIONS

The quantitative reproducibility of results with the present assay method employed for Japanese encephalitis vaccine leaves something to be desired. Potent vaccines with a low minimal immunogenic dosage usually have low values on repeated assays, and poor vaccines consistently give high values. Nevertheless, in the critical range near the present level of acceptability, marked variation occurs in repeated tests. Two examples among many may be cited. Sub-lot A of one lyophilized vaccine when tested on 23 April 1947 had an assay value of 0.031 cc. and when retested on 26 May the value was 0.015 cc; sub-lot C of this same vaccine had a value of 0.0095 cc. when tested on 29 April. Sub-lot A of another lyophilized vaccine when tested on 3 June and retested on 6 June gave values of 0.011 and 0.025 cc., respectively. Three assays performed on other sub-lots of this same vaccine had values of 0.012, 0.016 and 0.025 cc. In all 6 of the tests in which the above mentioned assays were included, the intraperitoneal titer of the challenge virus was between $10^{-1.95}$ and $10^{-2.2}$ and the mortality in the non-vaccinated control mice 100%. Further evidence indicating that minor variations in the range between 0.005 and 0.020 cc. in assay values on dried vaccines are of little consequence is found in studies on the capacity of two lyophilized chick embryo vaccine with assay values of 0.006 or 0.015 cc. to elicit neutralizing antibodies against Japanese encephalitis virus in human beings; little if any difference was observed (3). Efforts should be continued to improve the current method of assay of potency. In the meantime it is recommended that Japanese encephalitis vaccine, chick embryo type, dried, be considered acceptable if the minimal immunogenic dosage, as measured by the current method, is less than 0.020 cc.

Non-pathogenic, aerobic, spore-forming bacteria frequently are found in

biological materials prepared from embryonated eggs. These generally resist the bactericidal action of 0.2% formaldehyde even if the biological is held at 5° C. for long periods of time. Fortunately, these organisms are highly susceptible to the action of small amounts of penicillin while the virus of Japanese encephalitis is not affected by such concentrations of the drug. Losses associated with contamination by organisms of this group are practically eliminated when penicillin is added to the seed virus and to the fluid vaccine. The addition of small amounts of penicillin to the seed virus is probably the more important step since it apparently inhibits the growth of these organisms in those occasional eggs in which bacteria are present or are introduced at the time of inoculation. The employment of an antibiotic for the control of bacterial contamination in the production of Japanese encephalitis vaccine has proved satisfactory and should be continued.

During the past year all lots of vaccine having an infectious titer of $10^{-7.4}$ or greater were satisfactory and none with titers below this level were acceptable. The average infective titer for 12 acceptable lots was $10^{-7.82}$. It is recommended that only lots with infective titers of $10^{-7.4}$ or greater be considered for further processing and testing.

Comparative studies with three other strains of virus, namely "Kalinina", "Matsunaga" and "Roum", (the latter isolated from a fatal human case in Korea in 1946 by members of the Virus and Rickettsial Disease Commission indicated that none of these strains multiplied in the chick embryo as well as did the "Nakayama". Furthermore, chick embryo vaccines prepared from these strains were of inferior potency when assayed in mice and by the standard method. Therefore, it is recommended that the "Nakayama" strain continue to be used for the preparation of Japanese encephalitis vaccine.

The immunogenic material in Japanese encephalitis vaccine is relatively labile and deteriorates under conditions which are not deleterious to many biologicals. Under proper conditions of lyophilization, none of the immunogenic activity of the fluid vaccine is lost in drying. Furthermore, the lyophilized product is of relatively stable potency. The method for lyophilization described in the text has given satisfactory results with Japanese encephalitis vaccine both in regards stability and residual moisture content. It is recommended that until more improved methods are developed the principles of drying embodied in the present work be used in subsequent manufacture of this vaccine. It is further recommended that a residual moisture content of less than 1% be required for dried Japanese encephalitis vaccine.

TABLE 1

Deterioration of Fluid and Dried Vaccines at 37° C.

Vaccine		Treatment	Assay
Type	Number		M. I. D.
Fluid	Lot 12	60 days at 4° C	0.0125 cc.
"	"	60 days at 4° C / 3 hrs. at 37° C	0.095 cc.
"	"	60 days at 4° C / 6 hrs. at 37° C	0.10 cc.
"	"	60 days at 4° C / 24 hrs. at 37° C	0.10 cc.
Dried	Lot 1A	30 days at 4° C	0.011 cc.
"	"	15 days at 4° C / 15 days at 25° C	0.015 cc.
"	"	15 days at 4° C / 15 days at 37° C	0.025 cc.
Dried	Lot 6A	35 days at 4° C	0.012 cc.
"	"	21 days at 4° C / 14 days at 25° C	0.016 cc.
"	"	21 days at 4° C / 14 days at 37° C	0.022 cc.

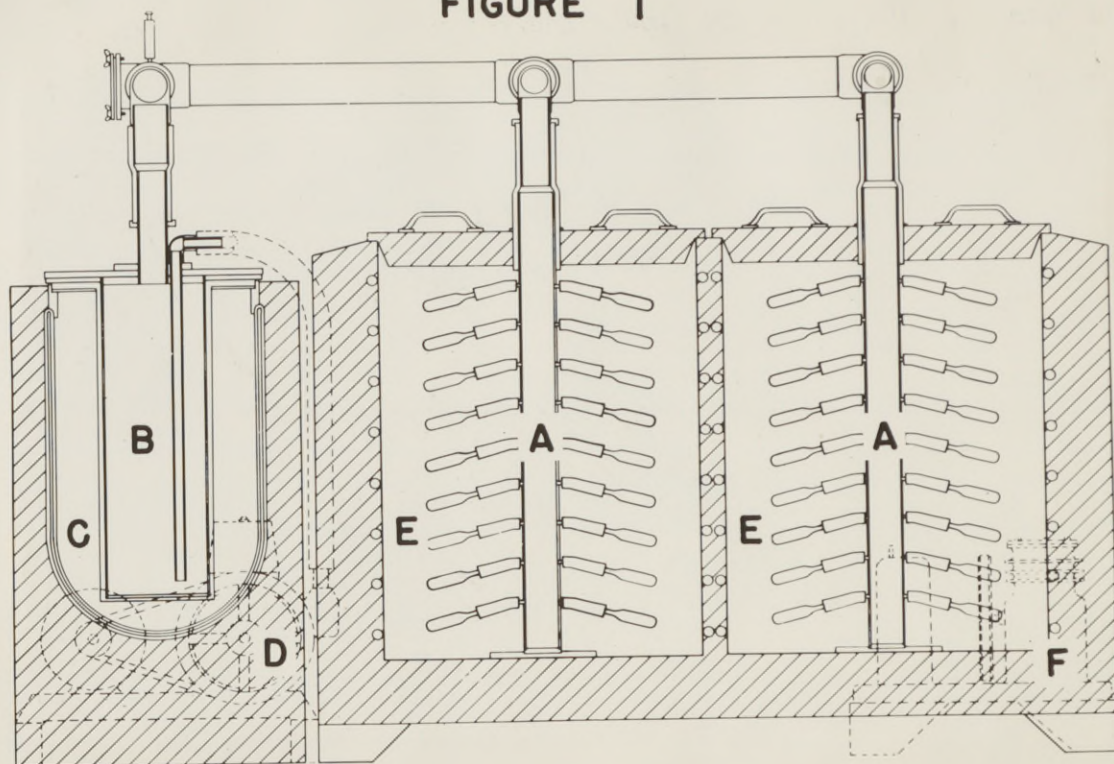
TABLE 2

Stability of Vaccines Stored at 4° C.

Vaccine Lot	Interval Between Time of Drying and Assay	Assay M.I.D.	Vaccine Lot	Interval Between Time of Drying and Assay	Assay M.I.D.
E-66	8 days	0.015 cc.	3D	3 days	0.015 cc.
"	60 days	0.0098 cc.	"	28 days	0.019 cc.
1A	1 day	0.006 cc.	"	120 days	0.015 cc.
"	30 days	0.011 cc.	6A	4 days	0.0083 cc.
"	77 days	0.015 cc.	"	35 days	0.012 cc.
"	135 days	0.019 cc.	9A	1 day	0.008 cc.
3A	7 days	0.009 cc.	"	25 days	0.018 cc.
"	124 days	0.019 cc.			

Vaccines were lyophilized 18-22 days after harvest of the infected embryos.

FIGURE 1



Description of Lyophilizing Apparatus (Bauer & Pickels)

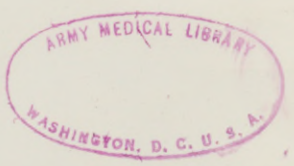
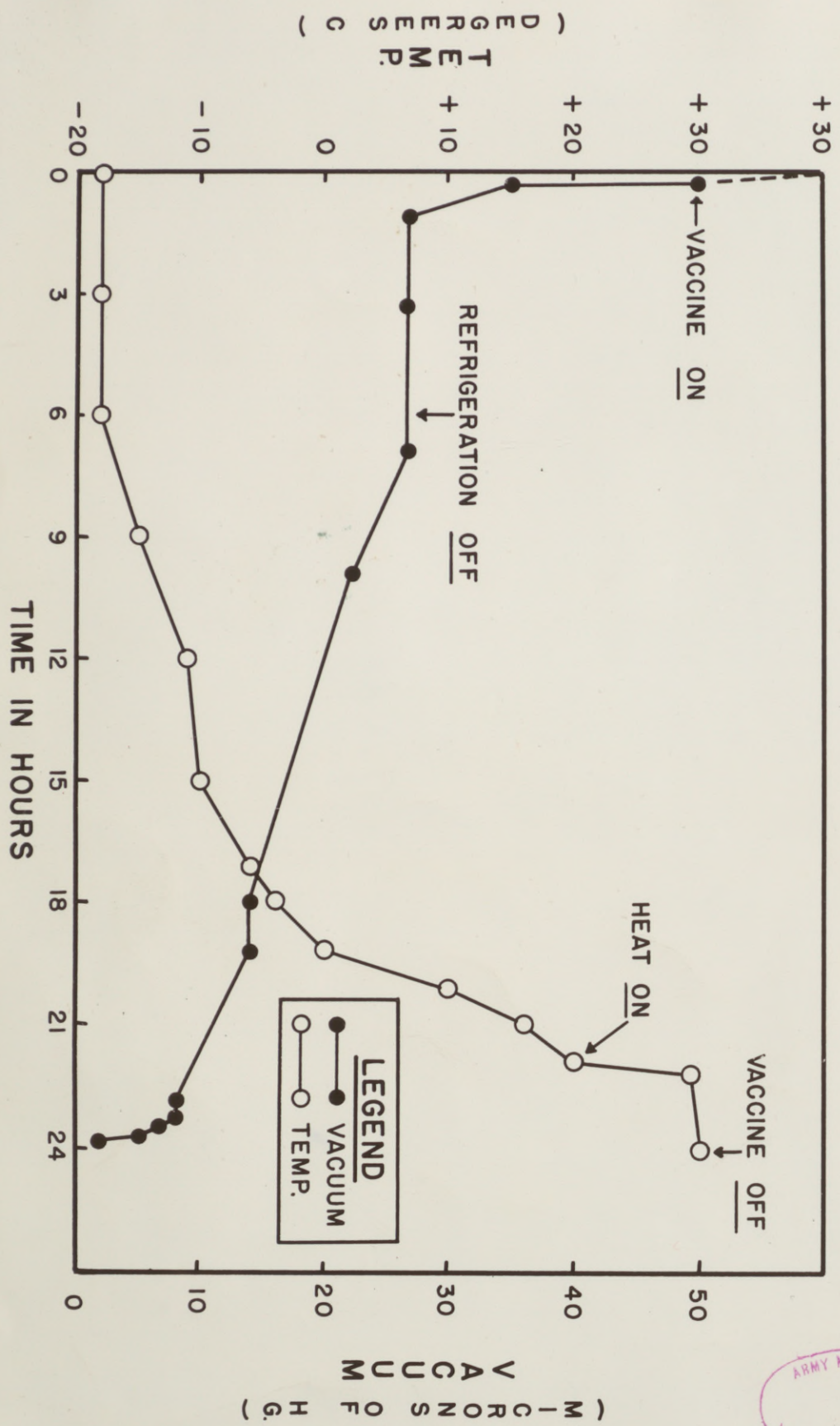
The brass manifolds (A) are attached to a condenser (B) which is immersed in a large Dewar flask (C) containing ethyl alcohol and solid CO_2 . A high vacuum rotary pump (D) is attached to the condenser and serves to evacuate the system. The manifolds are fitted with nipples to which the ampoules are attached by rubber tubing. The manifolds and ampoules are placed within an insulated compartment (E) which can be cooled by mechanical refrigeration (F) to -20°C . Drawing reproduced with permission of the International Health Division of the Rockefeller Foundation.

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TEMPERATURE AND VACUUM DATA OBTAINED DURING
 LYOPHILIZATION OF JAPANESE ENCEPHALITIS VACCINE,
 CHICK EMBRYO TYPE (Lot 114C, Unit 2, 6-4-47)

FIGURE 2



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