Protection of guinea pigs against Q fever by formalinized vaccines prepared from mouse, guinea pig, and yolk sac tissue has been demonstrated by several workers (Bengston, 1941; Smadel, Snyder, and Robbins, 1948). A formalinized vaccine, prepared from yolk sacs inoculated with the Henzenling strain of Coxiella burnetii, has been shown to elicit complement-fixing antibodies in approximately 50% of immunized humans and to provide protection against aerosol exposure even when demonstrable complement-fixing antibodies are not detected (Tigertt and Benenson, 1956). Because this disease has been an important factor in military operations in certain areas (Robbins, Gauld, and Warner, 1946) it would be important to have available a stockpile of safe, stable, effective vaccine. These studies were undertaken to develop a freeze-dried preparation of formalinized infected yolk sacs. The availability of some of the lot of liquid vaccine which had been used in human protection studies in 1955 provided a reference against which different methods of processing a vaccine could be compared.

METHODS AND MATERIALS

Strain. The 22nd egg passage of the Henzenling strain of C. burnetii received as a 20% yolk sac suspension from Miss E. Jackson, National Institutes of Health, was used as the seed strain. The seed suspension was stored at −70°C in rubber stoppered, aluminum sealed bottles. A 23rd egg passage to provide additional seed material was prepared at this laboratory.

Preparation of vaccines. Method A. Q fever vaccine was prepared by the method of Smadel et al., (1948) using an inoculation of 0.2 ml of a 10⁻³ dilution of the seed suspension, which produced a 20% mortality rate occurring between days 7 and 8. The yolk sacs were harvested from surviving embryos on the 8th day; excess yolk was permitted to drain off on a screen and the membranes were weighed and ground in an aerosol-free Waring Blender (Hilleman and Taylor, 1988) with sufficient buffered physiological saline (pH 7.2), containing formalin (Fisher, neutral) to make a 20% suspension with a 0.5% formalin content. The formalinized suspension was inactivated for 5 days at 4°C, then diluted with an equal volume of buffered physiological saline and centrifuged for 10 min at 700 × g. The midzone was collected and, after Merthiolate was added to a 1.10,000 concentration, was further processed by two ether extractions, each followed by a low speed centrifugation. The aqueous phase was collected in each instance and the final aqueous phase constituted the vaccine.

Method B. Eggs were inoculated and harvested as in method A. The yolk sacs were ground with sufficient distilled water to make a 60% suspension and then stored at −70°C overnight. The frozen suspension was thawed under cold running tap water and diluted with 0.85% saline to which sufficient formalin and phenol had been added to make a 30% tissue suspension containing 0.3% formalin and 0.75% phenol. This 30% suspension was held at room temperature (22°C) for 4 days, with shaking at least twice daily, for inactivation. The suspension was then diluted to a 10% tissue concentration with 0.85% saline and the pH adjusted to 5.7 with 10% HCl. One and one-half volumes of sulfuric ether were mixed with the suspension and the mixture was held at room temperature for 18 to 24 hr. Three layers developed and the aqueous bottom phase was collected. Ether was removed from the vaccine by placing the product under vacuum for 18 to 24 hr at room temperature. The pH was then raised to 6.8 with 1 N NaOH and Merthiolate was added to a concentration of 1:10,000 to constitute the final vaccine.

Guinea pigs. For comparisons of antigenicity,
Hartley strain guinea pigs, ranging in weight from 275 to 375 g, obtained from Fort Detrick, Maryland, were used. These were inoculated intraperitoneally with 1.0 ml of various dilutions of vaccine and bled 14 or 21 days later; complement-fixation determinations were performed on the individual sera.

Serological tests. Complement-fixation testing was performed by the method described by Smadel (1956), using 4 units of commercially prepared (Lederle) Nine Mile strain antigen, with overnight fixation. A single pool of immune guinea pig sera was included in each test as a control on sensitivity of the test system.

Seven-day-old embryonated eggs from White Leghorn hens raised on antibiotic-free feed were used in this study. Nitrogen content of the vaccines was determined by the micro-Kjeldahl technique followed by nesslerization. Formalin was measured by the Schiff test and phenol by the method described by Hawk, Oser, and Summerson (1947, par. 4). The rickettsiae were stained with Machiavello's stain and their number per ml of vaccine was determined by an adaptation of the method of Breed (1948). Safety tests on the vaccines were determined by the method described by Berman et al. (1960).

RESULTS

Comparison of methods of production. Several lots, prepared by both methods, were assayed for nitrogen, phenol, and formalin content, approximate number of rickettsiae per ml, antigenicity in the complement-fixation test and guinea pig complement-fixation response (immunogenicity). The results obtained with five consecutive lots of vaccine prepared by each method are given in Table 1.

No significant differences in the number of rickettsiae per ml, antigenicity, and immunogenicity were observed between vaccines prepared by either method. However, the lots of vaccine produced by method B were consistently lower in nitrogen content and considerably lower in formalin content than the lots produced by method A. It is to be noted that in method B, phenol is largely removed by the ether extraction. Since method B, a less involved procedure than method A, provided a product of comparable antigenicity with less nitrogen, these lots were used in the studies on preparation of a purified, dry vaccine.

Inactivation of the rickettsiae. The effect of time and temperature on the rate of inactivation of the rickettsiae in vaccines prepared by method B was determined. Prior to the addition of the formalin and phenol two aliquots were removed to serve as controls; one stored at -70 C and the other at +37 C. Five-tenths per cent formalin and 0.75% phenol were added to the remainder of the 30% infected yolk suspension and this was divided into 3 equal portions. One portion was held at +4 C, the second at +22 C, and the third at +37 C. Samples of each were removed on days 1, 2, 3, and 4 and held at -70 C. On the 5th day after the addition of the formalin and phenol, the frozen samples were thawed (including the control) and all samples were passed into 7-day-old embryonated eggs with blind passage of pooled yolk sacs of eggs surviving on the 12th day after inoculation (Berman et al., 1960).

As shown in Table 2, no rickettsiae were detected in the yolk sac suspensions held at +37 C for 2 days or more and none were detected in those suspensions held at +4 C and +22 C, respectively, for 3 days. The intermediate temperature was selected for vaccine preparation as a compromise between the advantage of more rapid inactivation at a higher temperature and the possible deleterious effect of a high temperature on the antigen. Four days of inactivation at this temperature is considered sufficient to ensure a vaccine free of residual, live rickettsiae.

Preparation of a partially purified, dry vaccine. High speed centrifugation sedimented the rickettsiae which could then be resuspended in a diluting fluid to original volume with no detectable loss in antigenicity. This method provided a vaccine essentially free of soluble and tissue nitrogen and of the residual formaldehyde and phenol left in the product after the ether extraction. A satisfactory dry product was obtained when the sediment was resuspended in saline containing 2% glucose to provide bulk and 0.05% formalin as the bactericidal agent to minimize contamination during the centrifuging and resuspending procedures. Based on tests on small numbers of guinea pigs, more consistent antigenicity was observed when the antigen was dried in the presence of glucose than when human serum albumin was used or the antigen was resuspended in buffered saline alone.

When 0.5% phenol was used as the bacterio-
static agent in place of formalin in the purified product, the antigenicity of the vaccine was lost in the drying process. The geometric mean complement-fixation titer of sera of guinea pigs inoculated with 1 ml of a $\frac{1}{4}$ dilution of the fluid phase before drying was 56.3; after drying the same dose elicited a geometric mean titer of only 4.6. This same reduction in geometric mean titer occurred when the antigen was resuspended in a phenol-saline medium (containing no glucose), but no significant loss in titer was observed when a saline-glucose medium was used.

Based on these results, the following procedure was adopted. Samples of several lots of the crude vaccine (method B), representing a 10% yolk sac suspension, were pooled and centrifuged at 22,600 × g for 75 min at 4 C. The supernatant was discarded and the sediment resuspended to the original volume in buffered physiological saline (pH 7.4) containing 2% glucose and 0.05% formalin. Prior to filling and drying, the formalin was neutralized by adding 35% sodium bisulfite and the pH readjusted to 7.0. The product was then dispensed in 10-ml volumes into 20-ml wide-mouth serum bottles, shell frozen, and dried in a chamber dryer. After drying, the bottles were stoppered with rubber stoppers and sealed under vacuum. The characteristics of the dried vaccine are shown in Table 3, in comparison with the method B liquid pool, a method A liquid pool, and the vaccine produced in 1954 by method A.

When reconstituted with sterile distilled water,
the nitrogen content of the dry product was approximately 5% that of the original pool while the formalin content was below detectable limits. The antigenicity and the response of guinea pigs on immunization were comparable to those obtained with the original pool. The purified preparation as well as the other vaccines were significantly more antigenic, as judged by the complement-fixation responses, than the vaccine prepared in 1954 and accordingly it was decided that the current liquid or dry vaccines could be diluted in half (to 5%) for human use.

On the basis of these studies the pool of vaccine produced by method B was processed with appropriate care to prepare a fluid and a purified and dried vaccine for human field trials.

Toxicity and anaphylaxis. Toxicity of the two types of vaccine (diluted to 5%) produced by method B was determined in Hartley strain guinea pigs (350 to 450 g). Three animals were inoculated intraperitoneally with 2.0 ml (twice the human immunizing dose) of each vaccine from final containers. The guinea pigs were observed daily for 14 days with daily rectal readings for the 1st week. A reading over 39.9 C was considered a fever. Of the 3 guinea pigs inoculated with the fluid vaccine, 1 animal had a reading of 40 C for 1 day (day 6); all animals showed an increase over initial weight on day 14. No elevated temperatures were observed with the guinea pigs inoculated with the dry product and the 3 guinea pigs showed a weight increase on day 14.

The anaphylactogenic potency of both the fluid and dried vaccine (5%) due to the residual yolk sac content was determined. One group of guinea pigs was inoculated intraperitoneally with 1.0 ml of the fluid vaccine and challenged intravenously 21 days later with normal yolk sac material (processed the same way as infected yolk sac). Ten animals were tested intravenously with 10% yolk sac material and of these, 5 showed no symptoms, 3 showed a reaction (coughing, weakness in legs) and 2 died within 5 min. Of 5 animals challenged with 1.0 ml of a 10⁻⁷ dilution of the 10% yolk sac material (the likely dilution of rickettsiae for use as a challenge dose in a protection test), no reactions were observed. Fifteen guinea pigs were immunized with the rehydrated dry vaccine and showed no reactions when tested with 1.0 ml of the normal yolk sac suspension, indicating that the purification procedure had reduced the content of yolk sac material below levels detectable by this method.

Human field trials with the fluid vaccine. In a limited human trial with three separate groups, fluid vaccine (5%) was administered to those who had no detectable antibodies. The number of conversions obtained with this vaccine are shown in Table 4, in comparison with a lot of vaccine (10%) produced in 1954 by method A and used in a study in 1955 and used again in 1958 to immunize personnel at this laboratory (Benenson, 1959).

The results with the fluid vaccine produced at this laboratory compare very favorably with the conversion rates obtained with the vaccine produced by method A.
TABLE 3
Chemical, antigenic, and immunogenic assays on the various Q fever vaccine preparations

<table>
<thead>
<tr>
<th>Vaccines (10%)</th>
<th>Bulk Complement Fixation</th>
<th>Guinea Pig Complement-Fixation Responses with Vaccine Dilution:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nitrogen</td>
<td>Formalin</td>
</tr>
<tr>
<td>Dry product method B</td>
<td>4 0.026</td>
<td>0.000</td>
</tr>
<tr>
<td>Pool method B</td>
<td>4 0.355</td>
<td>0.036</td>
</tr>
<tr>
<td>Pool method A</td>
<td>4 0.460</td>
<td>0.164</td>
</tr>
<tr>
<td>Method A (1954)</td>
<td>4 0.352</td>
<td>0.163</td>
</tr>
</tbody>
</table>

* Geometric mean.
† 10/10 guinea pigs converted to a positive complement-fixation response at a vaccine dilution of 1/2.

TABLE 4
Rate of conversion in man immunized with the fluid Q fever vaccines

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Dosage</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Positive*</th>
<th>Total</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Amt</td>
<td>Positive</td>
<td>Total</td>
<td>Positive</td>
<td>Total</td>
<td>Positive</td>
</tr>
<tr>
<td>Method A; lot #AMS541018</td>
<td>ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(fluid, 10%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1955 study†</td>
<td>3</td>
<td>1</td>
<td>30</td>
<td>59</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1958 study‡</td>
<td>3</td>
<td>1</td>
<td>9</td>
<td>16</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method B (fluid, 5%)</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>7 (1)</td>
<td>13</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>56</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>14</td>
</tr>
</tbody>
</table>

* A serum was recorded as positive if 2+ or greater complement fixation was observed at a serum dilution of 1/4 or greater. The figures in parentheses were sera positive at a 1/2 dilution and were not included in the total.
† Tigertt and Benenson (1956).
‡ Benenson (1959).

DISCUSSION AND CONCLUSIONS

Individual lots of Q fever vaccine with reproducible characteristics can be produced by either method A or method B. The vaccines produced by either method were similar antigenically, immunogenically, and in rickettsial content. However, the final fluid vaccines produced by method B were approximately 25 to 30% lower in nitrogen content and approximately 80% lower in formalin content than the vaccines produced by method A. Thus, the one ether extraction at adjusted pH (5.7) used in method B was more efficient (in terms of removing nitrogenous yolk sac material and formalin) than the two ether extractions in method A. Because of the lower nitrogen and formalin content of the final product, the room temperature inactivation and fewer manipulations required in method B, this was considered the method of choice.

Limited human trial of the fluid vaccine (method B) demonstrated this vaccine was as capable of eliciting complement-fixing antibodies...
in man as the vaccine produced in 1954 (method A). The purified dry product would provide a stable vaccine, with a minimum nitrogen content, equal in antigenicity and guinea pig immunogenicity to the liquid vaccine. A future human trial of this vaccine is anticipated.

An investigation is presently being considered on the advantage of replacing the glucose with other binders such as sucrose or human serum albumin for comparative purposes in a long term stability study on the dry product.

ACKNOWLEDGMENT

The authors are indebted to the Pitman-Moore Company for making available their techniques for the production of typhus vaccine, which were used in this study for the production of vaccine by method B.

SUMMARY

A modification of a procedure for the commercial production of typhus vaccines was shown to produce Q fever vaccines low in nitrogen content capable of eliciting high complement-fixation responses in guinea pigs and a satisfactory number of conversions in man.

A partially purified dry Q fever vaccine was prepared by the sedimentation of the rickettsiae with high speed centrifugation and resuspension of the rickettsiae in buffered saline containing 2% glucose and 0.05% formalin. The formalin was neutralized prior to drying and the resultant product was capable of eliciting complement-fixation responses in guinea pigs equal to those of the fluid vaccine from which it was made.

REFERENCES

Benenson, A. S. 1959 Q fever vaccine: efficacy and present status. In Medical science publication No. 6: Symposium on Q fever, pp. 47-60. Walter Reed Army Institute of Research, Washington, D. C.


