Further work on this problem is in progress.

Summary. 1. Bile fistula rats show loss in blood coagulability and a decrease in the prothrombin level. 2. This condition can be relieved by administration of a vitamin K concentrate.

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The Loss of Specific Substance in Washing Phase I *H. pertussis* Vaccines.*

JOHN J. MILLER, JR. (Introduced by Harold K. Faber.)

From the Department of Pediatrics, Stanford University School of Medicine, San Francisco, California.

Recent field studies of immunization against pertussis have yielded conflicting evidence. This is not altogether surprising in view of the fact that there is no standard method of preparing vaccines of *Hemophilus pertussis.* Recently isolated, toxic, Phase 1 strains[1] of *H. pertussis* have been used in the majority of recent trials.[2-5] Stock strains, however, are still used in preparing vaccine.[6] Although the newer knowledge of the antigenic changes of *H. pertussis* contraindicates the use of stock strains, it is obvious that a proposed immunizing agent must be judged only by careful clinical trial. Such trials as have been made with stock strain vaccines[7, 8] have not been sufficiently comprehensive to permit comparison.

Vaccines made from recently isolated, toxic, Phase 1 strains have differed principally in the amount of washing to which they were subjected. It is a current view[9] that the efficiency of any bacterial vaccine depends on (a) its production from virulent, smooth bacteria, and (b) the presence of the surface antigens of the virulent,

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1 Leslie, P. H., and Gardner, A. D., *J. Hygiene,* 1931, **31**, 423.

2 Sauer, L., *Am. J. Pub. Health,* 1935, **25**, 1226.

3 Kendrick, P., and Eldering, G., *Am. J. Pub. Health,* 1936, **26**, 8.

4 Daughtry-Denmark, L., *Am. J. Dis. Child.,* 1936, **52**, 587.

5 Doull, J. A., Shibley, G. S., and McClelland, J. E., *Am. J. Pub. Health,* 1936, **26**, 1097.

6 Mishulow, L., Mowry, I., and Orange, R., *J. Pediat.,* 1936, **9**, 492.

7 Park, W. H., *J. Pediat.,* 1935, **7**, 690.

8 Shorr, E. Y., *J. Pediat.,* 1936, **9**, 49.

9 Topley, W. W. C., and Wilson, G. S., *The Principles of Bacteriology and Immunity,* 2nd Edition, Baltimore, 1936, p. 831.

smooth bacteria. Furthermore, Lawson[10] has reported that Phase 1 strains have a capsule, demonstrable only when smeared directly without emulsification in an aqueous solution. The amount of washing to which an *H. pertussis* vaccine is subjected would therefore seem to influence its antigenic potency.

The following data were obtained when *H. pertussis* vaccine washings were tested for the presence of specific substance.

Rabbits were given intravenous injections of an unwashed suspension of Phase 1 *H. pertussis* in 0.85% NaCl containing 0.5% phenol. The vaccine contained approximately 11 billion organisms per cc. The strains used were less than one month old and were grown on Bordet-Gengou media containing either horse or human blood. Intravenous injections were given every 4 to 5 days, with occasional rest period, for a duration of 5 months. An initial dose of 0.1 cc. was gradually increased to 2.5 cc. Two weeks after the last injection the rabbits were bled. The serum was inactivated and stored unfiltered at 10° C. without the addition of any preservative. These sera agglutinated a recently isolated strain of *H. pertussis* to a titer of 1:12,000.

H. pertussis vaccine washings were prepared in the following manner. A slightly modified Bordet-Gengou medium[11] containing 33% goat blood was inoculated with 2 or 3 strains less than 3 weeks old (and less than 5 transplants old). The 2-day growth on 6 petri dishes was scraped off with a glass rod into 10 cc. of 0.85% solution of NaCl. The 2-day growth on the remaining 6 petri dishes was scraped into 10 cc. of distilled water. The suspensions were agitated 5 minutes to break up the clumps of bacteria and immediately centrifuged for one hour at 3000 r.p.m. The supernatant washwater was pipetted off, and then recentrifuged 5 to 9 times for one hour each time at 3000 r.p.m. After each centrifugation the washwater was transferred to another centrifuge tube and chilled before being replaced in the centrifuge. Centrifugation was carried out twice after all traces of sediment disappeared. (It may be mentioned that after the second hour of centrifugation the supernatant was uniformly water clear.) After this prolonged centrifugation smears and cultures of the washwater were negative. When run in duplicate, saline and distilled water washwaters were centrifuged for the same period of time.

The washwaters were tested for the presence of specific substance

[10] Lawson, G. M., *Am. J. Dis. Child.*, 1933, **46**, 1454.

[11] Kendrick, P., Miller, J. J., and Lawson, G. M., *Am. Pub. Health Assn. Year Book*, 1935-36, p. 200.

by a simple flocculation test. 0.2 cc. of washwater were mixed with 0.1 cc. of rabbit antiserum in varying dilutions. At first 0.85% NaCl was used as a diluent. Later 0.2% NaCl as suggested by Dean[12] was employed because of the greater rapidity of flocculation. To obtain a uniformity of salt concentration when titrating saline and distilled water washings in duplicate, sufficient NaCl was added to the distilled water washing to make the salt concentration 0.85% and then 0.85% NaCl was used as a diluent for both washing and antiserum. The tests were read at room temperature within 15 minutes and again after incubation for one hour at 37°C. The appearance of opalescent floccules was considered evidence of a union of specific substance (hapten or antigen) with antibody. Flocculation occurred when antisera in dilutions of 1-3, 1-9, and 1-27 were mixed with washwater in dilutions up to 1-8. (Tables I and II.) With undiluted antiserum flocculation was less constant and often slower, indicating antibody excess in the tubes. Four dif-

TABLE I.
Distilled Water Washings.

	Undiluted	1-2	1-4	1-8	1-16	Saline Control*
Antiserum	xx x –	xx x –	xx x –	xx x –	xx x –	xx x –
Undiluted	3 2 0	0 1 2	1 1 1	0 0 3	0 0 3	0 0 4
1-3	7 0 0	2 2 1	1 4 1	0 1 5	0 0 5	0 0 7
1-9	8 0 0	4 1 0	3 2 1	0 4 2	0 1 4	0 0 7
1-27	5 1 2	3 1 1	1 3 2	0 1 5	0 0 5	0 0 7
Saline Control*	0 0 8	0 0 5	0 0 6	0 0 6	0 0 5	0 0 7

Results of 9 titrations of distilled water washings. 4 lots tested against 2 antisera.

Figures indicate number of tests at indicated dilutions. Line demarcates positive zone.

xx = Flocculation occurring within 15 minutes at room temperature.

x = Flocculation appearing after incubation for 1 hour at 37°C.

– = No flocculation.

*In some titrations 0.85% NaCl was used as a diluent, in others 0.2% NaCl was used.

ferent lots of distilled water washings varied somewhat in the end dilution to which they were flocculated. Two lots of saline washings prepared simultaneously and under the same conditions as two lots of distilled water washings were flocculated in essentially the same dilutions as the distilled water washings. It would seem that

[12] Dean, H. R., "A System of Bacteriology," *Medical Research Council, London*, 1931, **6**, 431.

saline and distilled water wash off about the same amount of specific substance.

TABLE II.
Saline (0.85% NaCl) Washings.

	Undiluted	1-2	1-4	1-8	1-16	Saline Control*
Antiserum	xx x –	xx x –	xx x –	xx x –	xx x –	xx x –
Undiluted	2 1 0	1 1 1	1 0 1	0 0 3	0 0 3	0 0 3
1-3	3 1 0	3 1 0	1 1 1	0 2 2	0 0 4	0 0 4
1-9	3 1 0	3 1 0	0 3 0	0 1 3	0 1 3	0 0 4
1-27	2 2 0	2 2 0	0 1 2	0 1 3	0 0 4	0 0 4
Saline Control*	0 0 4	0 0 4	0 0 3	0 0 4	0 0 4	0 0 4

Results of 5 titrations of saline (0.85% NaCl) washings. 2 lots tested against 2 antisera.
For legend see Table I.

As a positive control the dilutions of sera were titrated against an extract of *H. pertussis* prepared by freezing and thawing in distilled water.[13] Antiserum 1-81 and lower dilutions flocculated this extract immediately.

As other control flocculation tests showed the presence of serum (from the media) in the washwater it was necessary to test the rabbit antisera for the presence of anti-goat serum antibody. Traces of goat serum flocculating antibody were found in one of the antiserums when it was used undiluted, but not in a dilution of 1-3. Benzidin tests showed the presence of hemoglobin in the washwater. The rabbit sera were then titrated against wide dilutions of hemoglobin. No flocculation occurred. These negative control tests indicate the specificity of the flocculation reactions described above.

After the preparation of one lot of distilled water washings in the manner described, the sedimented bacterial cells of the primary centrifugation were then resuspended in an equal volume of distilled water. This was centrifuged for one hour at 3000 r.p.m. The supernatants constituting the 1st and 2nd washings were then recentrifuged until sterile. Flocculation tests were done. Antisera in dilutions of 1-9 and 1-27 flocculated a 1-8 dilution of the first washwater whereas they flocculated only a 1-2 dilution of the second washwater. This would indicate that the first brief washing removed considerably more specific substance than the second. Undoubtedly not all was removed by both washings.

[13] Miller, J. J., *J. Immunol.*, 1934, **26**, 247.

A comparison was made of the agglutinability of *H. pertussis* bacterial cells before and after washing in distilled water. The cells were suspended in 0.85% NaCl and incubated for 20 hours at 37°C. Both unwashed and washed cells were agglutinated to a titer of 1-16,000. Apparently the washing in distilled water left sufficient surface antigen or hapten for combination with antibody.

It was found that Seitz filtration of the washwater removed all detectible specific substance. It has previously been noted that Berkefeld filtration of *H. pertussis* extracts removes the antigen which stimulates complement fixing antibodies in rabbits.[13]

The flocculation tests described above indicate that the Bordet-Gengou bacillus has a highly soluble surface which contains a specifically reacting substance. Whether this is a hapten or a complete antigen is not yet clear. Tests for the antigenicity of washings are in progress. It would seem certain, however, that a washed vaccine is not completely equipped with surface antigenic complex. On theoretical grounds, one must therefore agree with Sauer[14] in interdicting washing.

These findings are compatible with the favorable reports of Sauer[2] and Daughtry-Denmark[4] on the protective power of unwashed Phase I vaccine. They are also in line with the unfavorable report of Doull, Shibley and McClelland[5] on the use of a Phase 1 vaccine washed with distilled water. On the other hand these findings are less compatible with the evidence of Madsen[15] and Kendrick and Eldering[3] which indicates that a saline washed Phase 1 vaccine is an effective antigen. Apparently distilled water denatures the surface antigen in some way whereas saline simply removes a part of it. A comparison of the protective power in mice of saline washed and distilled water washed Phase 1 vaccine is being made.

Summary. Washing *H. pertussis* Phase 1 vaccine with either 0.85% NaCl or distilled water removes partially a specific substance which can be flocculated by hyperimmune rabbit serum. This finding constitutes grounds for omitting washing in the preparation of *H. pertussis* Phase 1 vaccine. It may explain in part the disparity in the reported results of immunization with Phase 1 vaccines.

[14] Sauer, L. W., *J. A. M. A.*, 1934, **102**, 1471.

[15] Madsen, T., *J. A. M. A.*, 1933, **101**, 187.