Death and Lysis of Leptospiroae When Cultured in Asbestos-Filtered Growth Media

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Death and lysis of leptospiroae, when cultured in asbestos-filtered bovine albumin polysorbate 80 media, was quantitated. The pathogens (virulent and avirulent) required 2 x 10^6 cells/ml to initiate growth in such media, whereas inocula of 2 to 20 cells/ml grew in control medium. Saprophytic leptospiroae initiated growth from 2 cells/ml in asbestos-filtered medium as well as control medium. The adverse action of asbestos-filtered medium was not removed by storage of medium for 2 years at 25 C and was not diminished when such medium was frozen at -80 C. Washing with water, HCl and NaHCO3-NaCl, citric acid, and medium components did not remove the lytic activity associated with asbestos-filtered culture medium. Continuous subculture in asbestos-filtered medium was possible from large inocula; however, upon subsequent dilution and reinoculation into asbestos-filtered media, there was no evidence of acquired resistance, and all pathogens failed to grow.

Since 1916, the success in culturing leptospiroae has been aided by the use of serum-enriched media. Frequently questioned was whether the serum enrichment lacked either nutritive value, contained toxic materials, or both. Turner (12) has reported there was no medium that was optimal for the growth of all leptospiroae strains.

Difficulties developed in 1968 in the growth efficacy of BA-P80 medium (4) produced in the National Animal Disease Laboratory central medium laboratory. No such difficulty was experienced in our laboratory research module where the medium had been developed. The problem was considered to be one of (i) distilled water supply, (ii) albumin variability, (iii) shelf life and source of polysorbate 80, and (iv) procedures involving preparation of the medium.

The multiple role “water as a laboratory tool” plays has been cited by McGarvey (10), and because the National Animal Disease Laboratory distilled water supply involved softening, deionization, and distillation, water became a possible suspect in the problem. Water used for media production in the research module, in contrast, was derived from a single distillation of raw tap water.

Albumin as a medium component was a media variable considered because Hanson and Ballard (5) have shown it to vary in its contaminating micro-nutrients. In addition, albumins designated fatty acid-poor, fatty acid-free, and crystalline did not support the growth of diluted cell numbers of leptospiroae (H. C. Ellinghausen, submitted for publication).

Although manufactured by one company, the polysorbates popularly known as the Tweens (Atlas Powder Co., Wilmington, Del.) are distributed through many suppliers and are stored under various environmental conditions. Thus, the lipid source was considered as a possible problem.

Finally, media production procedures had to be considered, for at this point, all variables come to play in the finished medium.

Preliminary studies identified the problem of impaired media as related to filtering the complete medium through asbestos filters. House (6), Brown et al. (1), and Litterest and Lichtenstein (9) have documented problems associated with sterilizing tissue culture medium and serum through asbestos filters. Recently developed (5X) leptospiroae medium compounded by commercial houses may have been subjected to asbestos filtration, and when dealing with large volumes, individual investigators might elect to prepare further such media by asbestos filter sterilization.

The objectives of this study were: (i) to
quantitate the effect of media filtered through asbestos pads upon the growth of pathogenic leptospiral serotypes and saprophytes; (ii) to remove the adverse culture medium effect by various washing procedures of the pads; (iii) to determine if cross-agglutinating antigenicity is altered by continuous subculture in asbestos-filtered medium; and (iv) to assess if the growth process in such detrimental medium results in cell populations resistant to lysis.

MATERIALS AND METHODS

Media. The water used in liquid medium production was produced from the single distillation of community tap water by a Loughborough still (Bellco Glass, Inc., Vineland, N.J.) and stored in sterile glass carboys at 25 C. A similar still, but having a glass-encased heating element, was used, but no difference in the ability of the medium to support the growth of 1 cell/ml was observed.

BA-P80 was formulated as previously described (4), both in the research module and in the central media laboratory. This control medium was made by autoclaving basal medium at 121 C for 15 min and, upon cooling to 25 C, by adding membrane-filtered albumin. The term "control medium" throughout this report has been defined (H. C. Ellinghausen, J. Med. Microbiol., accepted for publication).

Organisms. Because all leptospirae are regarded as belonging to a single species, the organisms are designated by the serotype followed by the strain; e.g., pomona (DM2H) is a strain used frequently to test in asbestos-filtered media for factors resulting in the nongrowth and loss of viability of leptospirosis.

Inocula. All cultures tested were in the log phase of growth when used as inocula (72 h old; nephelometer readings averaging 25, approximating 200 x 10^6 cells/ml). No difference in lysis pattern was seen when older inocula cultures (4 to 10 days old; nephelometer reading approximately 50, approximating 600 x 10^6 cells/ml) were employed and appropriately diluted. Serotype pomona (DM2H) was used at 20.9 x 10^6 cells/ml or less to compare media made in the research module, central media laboratory, Difco E.M.I.H., and numerous lots of media subjected to asbestos filtration. Turbidimetric growth at the end of 21 days of static incubation at 29 C was measured, and the earliest appearance of macroscopically detectable growth was recorded. All cultures negative for growth were tested for their viability by using semisolid BA-P80 medium. Comparisons were made among the effect of total filtration of BA-P80 medium by (i) asbestos pad filtration (0.025 μm), (ii) membrane filtration (0.22 μm), and (iii) and (iv) by adding asbestos and membrane-filtered albumin to autoclaved basal medium.

Growth ability of control medium 100 and 80%, by volume, filtered through sterilizing and clarifying asbestos pads (0.025 and 1.0 μm), was tested with 22 x 10^6 cells/ml or less with pomona (DM2H). Cell numbers from 22 x 10^6 to 4.4 x 10^5/ml were inoculated into asbestos-filtered medium, and the developing turbidity was measured at 7 and 21 days; the developing macroscopic turbidity was noted; viability of cultures was tested at terminal incubation; and daily growth curves were performed on cultures initiated with 2 x 10^6 and 1.8 x 10^6 cells/ml in control and asbestos-filtered medium.

Control medium, which had been shown capable of growing 1 cell/ml, was successively filtered four times through 5.7-cm asbestos filters (0.1 μm) and tested with inocula of 20 x 10^6 to 0.25 x 10^6 cells/ml.

Attempts were made to isolate and define the lytic factor conveyed to BA-P80 medium that was asbestos-filtered by various storage procedures of such media and by washing asbestos pads prior to use in filtration. Viability after 21 days was tested after media were inoculated with 20 x 10^6 cells/ml or less.

The length of time that pomona (DM2H) could be exposed to the action of lytic medium and still remain viable was examined over a 96-h period with 2 x 10^6 cells/ml or less.

Other serotypes. Thirty-seven leptospiiral strains, including pathogenic virulent and avirulent strains, saprophytic, and unclassified leptospirosis, were tested for their ability to grow from diluted cell numbers in asbestos-filtered and control medium. Where appropriate, the pathologic status (cells IP) for weaning hamsters is so designated. Included in this study were: Leptospirosa interrogans serotypes copenhageni, M20:avirulent (AV); icterohaemorrhagiae (VB- USDA: MLD 200); javanica, Veldrat-Bataviae 46; AV; canicola, Hond Utrecht IV; AV; (Moulton: MID 1.25 x 10^4), (NADL-A-13): MLD 26, (VB-USDA: MLD 26); ballum, S-102: AV; pyrogenes, Salinem: AV; sentot, Sentot: AV; australis, Ballico: AV; pomona, Pomona: AV; (DM2H: AV), (MLS-NADL-A-13): MID 2 x 10^4), (MLS-Neu, 100: MID 2 x 10^4), (MLS-Neu, acute: MID 2 x 10^4), (HCE: MLD 20), (B35: MID 2 x 10^4), (B-29: MID 2 x 10^4); grippotyphosa: Moskva V: AV, (I.C.S.: MID 200 x 10^4), (ISUSF: MID 2 x 10^4), (B-699 Univ. Ill.), andaman strains (Raccoon, Ga.), (Bobcat, Ga.), (Swamp rabbit, Ga.), (Cottontail rabbit, Ga.), (georgia, LT-117: AV; hardjo, Hardjooprajoeno: AV, (SC 1534: AV); medanensis, Hond HC; wolffi, 3705: AV; bataviae, Van Tienen: AV; ranae, (ICF: AV), patoc, Patoc 1: AV; biforma (SC #5: AV), (CDC: AV); sao-paulo (Sao-Paulo: AV); ilini (3055 hooked: AV); and ilini (3055 nonhooked: AV).

The inocula cultures in the log phase were adjusted to a nephelometer-reading turbidity of 25, approximating 200 x 10^6 cells/ml, and decimal dilutions to cell extinction were made in asbestos-filtered and control medium to estimate the minimal number of cells per milliliter resulting in growth in the respective media.

Turbidimetric growth. Thirteen serotypes and control strain ilini (3055) were measured for growth by turbidity over a 14- to 21-day incubation period at 29 C. Where no growth could be measured nephelometrically, cultures were tested for their viability by subculture of 1.0 ml amounts to 10 ml of BA-P80 semisolid medium. The starting inoculum approximated 20 x 10^6 cells/ml.

Saprophytes. Biflexa strains CDC, SC #3 and
After the inoculation, Patoc 1 at 20 × 10^6 cells/ml and less were inoculated into asbestos-filtered and control BA-P80 medium, and the growth developing from diluted inocula was measured at 7 and 14 days.

Continuous subculture. Twelve pathogenic serotypes and five saprophytic strains were serially subcultured in asbestos-filtered and control medium, and growth was measured at 7 and 14 days of incubation. After the 4th serial subculture, these organisms were again inoculated at the rate of 20 × 10^6 cells/ml or less into asbestos-filtered medium to see if they had acquired, during the growth process, any resistance to the lytic medium.

Antigenicity. Serotypes copenhageni, M-20, canicola, Hond Utrecht IV, gripotrophina (B-699), and hardjo, Hardjoprajitno, were tested for their cross-agglutinating activity by the microscopic agglutination test with various antisera after they were continuously subcultured in asbestos-filtered medium. Cross-agglutinating absorption studies were not performed.

Filtration methods. BA-P80 medium compounded as previously described (4) in 1,000- to 4,000-ml volumes was filtered through 1.0- and 0.025-μm asbestos pads or through 0.025-μm pads only (14 cm). Identical media had 100% of its volume sterilized by membrane filtration (0.22 μm, 14.2 cm without a prefiltre, Millipore Corp., Bedford, Mass.).

Albumin (5%) was sterilized by asbestos pads (1.0 and 0.25 μm) and membrane filters (prefiltre and 0.22 μm sterilizing membrane). Difco commercially prepared basal medium and albumin enrichment were also used (12).

An 800-ml portion of basal medium was filtered by using both 1.0- and 0.025-μm asbestos pads and the proper volume of membrane-sterilized 5% albumin added.

Medium storage. Previous studies (Ellinghausen, accepted for publication) had shown that BA-P80 liquid medium, stored 12 months at 25°C, would support the growth of inoculum of 1 cell/ml. Asbestos-filtered medium stored 24 months and 1 month at 25°C was tested to see if the medium lost its adverse properties during storage. Asbestos-filtered and control media were also frozen at −80°C to evaluate the possible effect of storage at this temperature on the media.

Washing of filters. Sterilizing asbestos pads (0.025 μm, 14-cm diameter) were washed with 2,500 ml of sterile distilled water, and BA-P80 complete liquid medium was processed by passage through the washed filter. Similar pads were treated with 500 ml of 1N HCl, followed by 500 ml of 5% NaHCO3 in 5% NaCl and three 500-ml volumes of distilled water, and finally BA-P80 medium sterilized. In addition, 2,500 ml of 10% citric-acid solution was also used as a pad-washing fluid.

Medium components in 2,500-ml volumes: KH2PO4, 87 mg/l; Na2HPO4, 664 mg/l; NaCl, 1,540 mg/l; MgCl2·6H2O, 152 mg/l; NaCl, 1,540 mg/l; MgCl2·6H2O, 152 mg/l; NH4Cl, 214 mg/l; and 0.1% (vol/vol) polysorbate 80 in the above phosphate buffer were used to wash the asbestos sterilizing pads. Complete medium was then sterilized through such pads and tested for ability to grow out diluted cell numbers of pomona (DM4H).

The 2,500-ml distilled water asbestos pad wash was used to make stock solutions, and this water was used as a source for making culture medium. Such basal medium was sterilized by autoclaving and membrane filtration in attempts to see if amounts of the lytic factor would solubilize and, if so, whether it was stable to autoclaving (121°C for 15 min).

RESULTS

Growth of serotype pomona. Growth of pomona (DM4H) developed from 2 to 20 cells/ml in media where autoclaved basal salts were supplemented with membrane-filtered albumin (Table 1). When total medium (salts and albumin) was asbestos filtered, the medium was unsatisfactory as seen by the nephelometric turbidity values and macroscopic turbidity. Growth was negative with inocula of 2 × 10^6 cells/ml or less in lot after lot of medium sterilized by asbestos filters. All negative growth cultures were nonviable when tested by subculture to semisolid BA-P80 medium.

Asbestos pads from the three leading manufacturers of asbestos filtering pads in the U.S. were studied. One of these prominent companies emphasized they control the pH and the acid treating of their pads throughout the manufacturing procedure. In addition, they report leachable calcium and iron content of less than 25 parts per million and suggested use of their pads as received; at the same time, they suggested washing pads prior to use with possibly distilled or deionized water without acid or with an acid solution of HCl of approximately 3% by volume. Pads (14 cm) available from two nationally known scientific supply houses were tested, though they were obviously derived from one of the three leading manufacturers of asbestos filtering pads. Twenty different lots of asbestos pads were tested.

No pad from any source, either as supplied or wash-treated as described in Table 6, was found satisfactory. Growth never developed from 2 × 10^6 cells/ml, this being true of all pads of every manufacturer and supplier.

As can be seen from the numbers in parentheses in Table 1, progressive growth was observed where autoclaved basal medium was supplemented with membrane-filtered albumin. Growth never developed progressively from diluted cell numbers when complete medium was asbestos filtered.

Photographs (Fig. 1) depict the effect of total filtration of BA-P80 medium by (i) asbestos pad filtration, (ii) membrane filtration, and (iii) the
addition of albumin sterilized by either asbestos or membrane filtration to autoclaved basal medium (Fig. 1c, 1d).

Growth was slow in membrane-filtered medium (Fig. 1b) from tubes having $2 \times 10^4$ to $2 \times 10^5$ cells/ml becoming macroscopically positive at 11, 13, and 14 days, respectively. Growth occurred from $2 \times 10^4$ to $2 \times 10^5$ cells/ml at 5, 7, and 10 days, respectively, in control medium. Growth never developed from 20 to 2 cells/ml in membrane-filtered medium (Fig. 1b), whereas growth routinely developed from 20 to 2 cells/ml in control medium (Fig. 1d). Sterilization of the 5% albumin supplement (in 2,000-ml quantities) by either asbestos or membrane filtration had little effect on medium efficiency (Fig. 1c, 1d).

**Sterilizing and clarifying filters.** The presence in asbestos sterilizing filters of a lysis-causing action upon diluted cell numbers of pomona (DM$_2$H) is shown (Table 2). Growth development was complete in control medium with $22 \times 10^8$ cells/ml or less. On day zero with 2.2 $\times 10^9$ cells/ml, such cultures were dark-field positive, whereas in the $10^{-6}$ to $10^{-8}$ dilutions, leptospiroval cells could not be found. After 28 days, these last dilutions had developed turbidities of 80, 68, and 61.

In asbestos-filtered medium, growth never developed from $22 \times 10^8$ cells/ml or less, the inoculum cells seen on day zero at 2.2 $\times 10^8$ to $2.2 \times 10^9$ cells/ml had disappeared, and this disappearance was verified by negative subculture results in semisolid medium.

**Cell inoculum necessary for growth.** The range for growth from $22 \times 10^8$ to $4.4 \times 10^8$ cells/ml of serotype pomona (DM$_2$H) in asbestos-filtered medium was determined in beginning increments of approximately $4 \times 10^8$ cells/ml. Maximum growth judged by turbidity

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### Table 1. Effect of inoculum size in asbestos-filtered medium with pomona (DM$_2$H) as measured by nephelometric turbidity and macroscopic turbidity

| Inoculum cells/ml | Nephelometric turbidity: 21 days at 29°C |
|-------------------|------------------------------------------|
|                   | Autoclaved basal$^a$ | Asbestos-filtered medium |
|                   | E$^b$ | CS$^b$ | CSEM$^b$ | 1 | 2 | 3 |
| $20.9 \times 10^5$ | 40 (0)$^c$ | 40 (0) | 43 (0) | 39 (0) | 60 (0) | 25 (0) |
| $2.09 \times 10^5$ | 40 (4) | 41 (4) | 43 (4) | 0 (0) | 48 (7) | 0 (0) |
| $2.09 \times 10^5$ | 40 (4) | 45 (4) | 48 (5) | 0 (−) | 0 (−) | 0 (−) |
| $2.09 \times 10^5$ | 41 (7) | 45 (7) | 49 (13) | 0 (−) | 0 (−) | 0 (−) |
| $2.09 \times 10^5$ | 41 (14) | 43 (13) | 40 (16) | 0 (−) | 0 (−) | 0 (−) |
| $2.09 \times 10^5$ | 17 (18) | 31 (16) | 22 (18) | 0 (−) | 0 (−) | 0 (−) |
| $2.09 \times 10^5$ | 20 (18) | 3 (20) | 0 (25) | 0 (−) | 0 (−) | 0 (−) |
| $2.09 \times 10^5$ | 2 (22) | 0 (22) | 0 (−) | 0 (−) | 0 (−) | 0 (−) |
| $2.09 \times 10^5$ | 0 (−) | 0 (−) | 0 (−) | 0 (−) | 0 (−) | 0 (−) |
| $2.09 \times 10^5$ | 0 (−) | 0 (−) | 0 (−) | 0 (−) | 0 (−) | 0 (−) |

$^a$ Supplemented with membrane-filtered albumin.

$^b$ Fabrication source: E, research lab; CS, central media lab; EMJH, Difco.

$^c$ Symbols: (0), macroscopically positive on day of inoculation; incubation day of earliest positivity in parenthesis after nephelometric turbidity; (−), negative growth, turbidity never developed.

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**Fig. 1. Effect of 100% asbestos (a) and membrane filtration (b) upon growth of pomona (DM$_2$H) (20 $\times$ 10$^8$ to 2 cells/ml) in BA-P80 medium and partial filtration of albumin supplement (c) by asbestos and (d) membrane filtration and their addition to autoclaved basal medium (200 ml of 5% albumin plus 800 ml of basal medium).**

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**APPL. MICROBIOL.**
TABLE 2. Adverse effect of total medium filtration with asbestos sterilizing and clarifying filters upon growth of pomona (DM₄H)

| Basal medium (800 ml) | Albumin (200 ml) | Total ml of medium asbestos filtered | Asbestos pads (used in filtration process) | Macropscopic day pos.* | Nephelometric-reading turbidity for 28 days at 29°C (cells/ml) |
|----------------------|-----------------|-----------------------------------|------------------------------------------|------------------------|--------------------------------------------------|
|                      |                 |                                   | Clari-fi-yzing | Sterili-zing | 2.2 × 10⁶ cells/ml | 2.2 × 10⁷ cells/ml | 2.2 × 10⁸ cells/ml | 2.2 × 10⁹ cells/ml | 22 | 2 |
| Autoclaved           | Membrane filtered | 0                                | ...         | ...         | 1d                  | 60               | 61               | 71               | 77 | 66 | 80 | 68 | 61 |
| Asbestos-filtered    | Membrane filtered | 1000                             | 1.0 μm      | 0.025 μm   | 22d                 | 53               | 32               | 0                | 0  | 0  | 0  | 0  | 0  |
| Asbestos-filtered    | Membrane filtered | 1000                             | ...         | 0.025 μm   | 6d                  | 53               | 61               | 0                | 0  | 0  | 0  | 0  | 0  |
| Asbestos-filtered    | Membrane filtered | 800                              | 1.0 μm      | 0.025 μm   | 6d                  | 51               | 57               | 0                | 0  | 0  | 0  | 0  | 0  |
| Asbestos-filtered    | Membrane filtered | 800                              | ...         | 0.025 μm   | 3d                  | 50               | 59               | 0                | 0  | 0  | 0  | 0  | 0  |

* Day zero: 10⁻⁴ dilution with 2000 cells/ml was dark-field positive, whereas 10⁻⁴ dilution with 200 cells/ml was dark-field negative.

... Not used.

developed from 22 × 10⁶ to 2.2 × 10⁹ cells/ml after 21 days as judged by maximum growth turbidities of 66.

Growth neither macroscopically nor nephelometrically measurable developed from 1.98 × 10⁶ to 4.4 × 10⁸ cells/ml, and such cultures were nonviable when subcultured to semisolid medium.

The growth pattern of pomona (DM₂H) was immediate in control medium, inhibited in asbestos-filtered medium, and did not develop from an initial inoculum level of 1.8 × 10⁹ cells/ml (Fig. 2). Some variation was seen in asbestos-filtered medium inoculated at 2 × 10⁶ cells/ml. Lag periods were protracted sometimes (Fig. 2), this lag always being related to the lot of medium, but growth never developing from 2 × 10⁶ cells/ml or less.

When 400-ml volumes of complete BA-P80 medium were successively passed through four individual asbestos serum-sterilizing pads (5.7 cm, 0.1 μm), severe inhibition of growth was evident with 20 × 10⁶ cells/ml when growth was measured after 6 days of incubation (Table 3).

Attempts to define lytic factor. The influence of various storage conditions and asbestos pad washing procedures upon the ability of BA-P80 media to support growth of pomona (DM₂H) is shown in Table 4. Storage for various lengths of time at various temperatures did not alter the characteristics of asbestos-filtered medium’s lytic action for pathogenic leptospirae. All attempts to remove the asbestos filter-associated lytic effect failed when asbestos pads were subjected to various washing procedures.

Viability in media and exposure time. Viability of pomona grown in the media of Table 4 is shown in Table 5. In the nine instances of asbestos-filtered medium, viability could not be demonstrated where 2 × 10⁸ cells/ml or less were used, after 30 days of incubation at 29°C. Time of exposure of pomona (DM₂H) to asbestos-filtered medium influenced viability. With increasing time, diluted cell numbers were sensitive to asbestos-filtered medium. A total of 200,000 cells/ml of inoculated cultures could be shown viable at 72 h after exposure to lytic medium, but was not shown viable after 76 h.
When $2 \times 10^4$, $2 \times 10^3$, $2 \times 10^2$, and $2 \times 10^1$ cells/ml were exposed to asbestos-filtered medium, viable cultures could be grown out at 48, 24, 8, and 4 h, respectively. With 2 cells/ml at zero h, the culture could not be recovered.

**Minimal inocula for serotypes.** All 32 pathogenic leptospiral strains were sensitive to the lytic effect of asbestos-filtered medium (Table 6). Growth in control medium could be initiated from few cell numbers. There was no correlation on whether virulent strains (noted under organisms) were more sensitive to the lytic action than avirulent laboratory-adapted pathogenic strains. Saprophytic strains in Table 6 were clearly resistant to the lytic action always observed for the pathogens in asbestos-filtered medium.

**Turbidimetric growth in asbestos-filtered medium.** Growth turbidity values of representative serotypes of the genus in asbestos-filtered and control media are presented in Table 7. With the pathogens, diluted cell numbers failed to grow in asbestos-filtered medium, whereas excellent growth developed in control medium. The illini serotype of either the hooked or straight variety was not lysed by the asbestos-filtered medium.

**Saprophyte growth in asbestos-filtered medium.** Turbidimetric values for three saprophytic strains are presented in Table 8. When growth was measured after 7 days, it was shown to be stimulated in asbestos-filtered medium, although this varied with the strain, the level of inoculum, and the length of incubation period.

**Continuous subculture in asbestos-filtered medium.** When maximum growth cultures of

### Table 3. Effect of four successive asbestos filtrations of total medium upon growth of serotype pomona (DM2H)

| Successive filtrations* | Nephelometer reading (Inoculum-cells/ml x 10^6) |
|------------------------|-----------------------------------------------|
|                        | 20    | 16    | 12    | 8     | 4     | 2     | 1     | 0.5   | 0.25 |
| 1                      | 46    | 43    | 42    | 39    | 34    | 31    | 28    | 20    | 4    |
| 2                      | 42    | 40    | 36    | 35    | 29    | 25    | 19    | 5     | 0    |
| 3                      | 40    | 36    | 33    | 30    | 27    | 21    | 9     | 0     | 0    |
| 4                      | 2     | 1     | 0     | 0     | 0     | 0     | 0     | 0     | 0    |

* 5.7-cm, 0.1-μm asbestos filters; 400-ml filtered volume; nephelometer readings after 6 days of incubation at 29°C.

### Table 4. Influence of various storage conditions and asbestos pad washing procedures upon the ability of BA-P80 media to support growth of pomona (DM2H)

| Medium                        | Medium pH | Nephelometer readings for 21 days at 29°C (Inoculum-cells/ml) |
|-------------------------------|-----------|---------------------------------------------------------------|
|                               |           | $2 \times 10^6$ | $2 \times 10^5$ | $2 \times 10^4$ | $2 \times 10^3$ | $2 \times 10^2$ | $2 \times 10^1$ | 20 | 2   |
| Lytic (2 yr at 25°C)          | 7.2       | 33             | 20             | 0               | 0               | 0               | 0               | 0  | 0   |
| Lytic (1 mo at 25°C)          | 7.28      | 29             | 18             | 0               | 0               | 0               | 0               | 0  | 0   |
| Lytic (-80°C)                 | 7.32      | 30             | 19             | 0               | 0               | 0               | 0               | 0  | 0   |
| Control (-80°C)               | 7.28      | 50             | 51             | 52              | 51              | 57              | 52              | 46 | 50  |
| Control (25°C)                | 7.25      | 50             | 49             | 50              | 51              | 55              | 52              | 49 | 50  |
| Membrane filtered             | 7.4       | 38             | 32             | 40              | 51              | 37              | 27              | 0  | 0   |
| Filter washes                 |           |                |                |                 |                 |                 |                 |    |     |
| Water                         | 7.12      | 42             | 44             | 0               | 0               | 0               | 0               | 0  | 0   |
| Acid-alkaline                 | 7.12      | 45             | 51             | 0               | 0               | 0               | 0               | 0  | 0   |
| Phosphate                     | 7.12      | 57             | 57             | 0               | 0               | 0               | 0               | 0  | 0   |
| NaCl, MgCl₂                   | 7.12      | 51             | 53             | 0               | 0               | 0               | 0               | 0  | 0   |
| NaCl,MgCl₂, NH₄Cl              | 7.12      | 52             | 56             | 0               | 0               | 0               | 0               | 0  | 0   |
| 0.1% T-80, PO₄                 | 7.12      | 53             | 55             | 0               | 0               | 0               | 0               | 0  | 0   |
| 10% citric acid               | 7.34      | 58             | 57             | 22              | 0               | 0               | 0               | 0  | 0   |
| Asbestos-filtered distilled water used in medium |           |                |                |                 |                 |                 |                 |    |     |
| Control medium                | 7.3       | 48             | 47             | 49              | 51              | 49              | 52              | 50 | 47  |
| Membrane filtered             | 7.4       | 37             | 37             | 31              | 34              | 41              | 0               | 0  | 0   |
| Vol asbestos filtered         |           |                |                |                 |                 |                 |                 |    |     |
| (1 to 4 liters)               | 7.25      | 29             | 35             | 0               | 0               | 0               | 0               | 0  | 0   |
LEPTOSPIRAE IN ASBESTOS-FILTERED MEDIA

Table 5. Viability of serotype pomona after 30 days of incubation in various media

| Medium                               | Inoculum-cells/ml |
|--------------------------------------|-------------------|
|                                      | 20 × 10⁴ | 2 × 10⁵ | 2 × 10⁶ | 2 × 10⁷ | 2 × 10⁸ | 2 × 10⁹ | 20 | 2 |
| Lytic (2 yr)                         | V         |        |        |        |        |        | V  |   |
| Lytic (1 mo)                         | V         |        |        |        |        |        | V  | V |
| Lytic (−80 C)                        | V         |        |        |        |        |        | V  | V |
| Control (−80 C)                      | V         | V      | V      | V      | V      | V      | V  | V |
| Control (25 C)                       | V         | V      | V      | V      | V      | V      | V  | V |
| Membrane filtered                    | V         | V      | V      | V      | V      | V      | V  |   |
| Filter washes                        | V         |        |        |        |        |        | V  | V |
| Water                                | V         |        |        |        |        |        | V  | V |
| Acid-alkaline                        | V         |        |        |        |        |        | V  | V |
| Phosphate                            | V         |        |        |        |        |        | V  | V |
| NaCl, MgCl₂                          | V         |        |        |        |        |        | V  | V |
| NaCl, MgCl₂, NH₄Cl                   | V         |        |        |        |        |        | V  | V |
| 0.1% T-80, PO₄                       | V         |        |        |        |        |        | V  | V |
| Asbestos-filtered water used         | V         | V      | V      | V      | V      | V      | V  | V |
| for medium                           | V         | V      | V      | V      | V      | V      | V  | V |
| Heat sterilized                      | V         | V      | V      | V      | V      | V      | V  | V |
| Membrane sterilized                  | V         | V      | V      | V      | V      | V      | V  | V |

* Symbols: V, viable; —, nonviable.

copenhageni, M20; canicola, Hond Utrecht IV; sentot, sentot; pomona (DM₄H); grippotyphosa, Moskva V, (B699); andaman, (Cottontail rabbit); georgia, LT-117; hardjo, Hardjoprajitno; wolfii, 3705; bataviae, Van Tienen; ranae (ICF); illini (3055H); and illini (3055 NH) were subcultured at 7 or 14 days of incubation, all grew continuously and well in either asbestos-filtered or control medium. At the end of the 4th subculture when rediluted to 2 × 10⁶ cells/ml or less and inoculated into asbestos-filtered medium, no resistance had been acquired to the lytic action of asbestos-filtered medium.

Antigenicity in asbestos-filtered medium. Substantial changes in the cross-agglutinating activity of serotype copenhageni, M-20 were not seen in the microscopic agglutination test where the final serum dilutions were of 10-fold nature (Table 9). Similar observations were made with serotypes canicola, Hond Utrecht IV; pomona (DM₂H); grippotyphosa, Moskva V; and hardjo, Hardjoprajitno.

DISCUSSION

The demonstration of the lytic action of an asbestos-filtered medium upon leptospiral pathogens and the resistance of leptospiral saprophytes has practical and theoretical importance. Whole serum and isolated proteins used in the preparation of media are often filtered and aseptically added to basal medium. This situation is adequate if the nonprotein portion of the medium formulation can withstand heat sterilizing procedures. In other cases, the entire medium is filtered. Dilution can be a critical factor in either small- or large-scale operations related to sample size or the important problem of sterility. One can speculate that such a situation would be important in the large-scale production of leptospiral bacterins and attempts to develop 5 × BA-P80 medium by various media manufacturers.

Nutritional research with leptospirae heavily depends on the use of liquid medium where either turbidimetric measurements of growth or direct cell counts are employed. Anything such as asbestos filtration of culture medium that would impair such cultivation would be undesirable. Liquid medium affords a means of propagating leptospiral isolates for serological identification. The transition from a semisolid growth environment to adverse-quality liquid medium could be impeded. Where 10% by volume inoculation of maximum growth serological antigens prevail, the problem will be less acute.

Although semisolid medium is considered to be the most useful type of media when attempting isolation of leptospirae from blood, urine, liver, kidney, lung, and brain tissue, this requirement is not absolute. There are various times where estimates of the degree of infection of body fluids and diluted tissues are significant.
in studies of pathogenesis. This fact further emphasizes the critical nature of having liquid medium capable of growing out one cell and free of undesirable growth properties.

It would seem appropriate to revise some of the concepts related to asbestos as used in biological filters. Asbestos, as cited in the Merck Index (11), is stated to be a calcium-magnesium silicate useful as an inert filter medium in laboratory and commercial situations. Chrysotile asbestos can be filtered into silty fibers and can be shown by chemical analysis to be silica (SiO₂) 40%, alumina (Al₂O₃) 0.7%, ferrous oxide (FeO) 1.0%, ferric oxide (Fe₂O₃) 1.5%, calcium oxide 0.2%, MgO 42.4%, CO₂ 0.2%, and water of crystallization 14% (14). In addition, organic matter and metals are found in various types of asbestos; primary oils and secondary oils may be present; amino acids can be extracted from virgin chrysotile; and jute oil can be a common contaminant (14). The lytic action seen with leptospires is not a simple process nor can it be probably attributable to one single chemical entity.

It has been commonly considered that asbestos filtering pads will leach calcium, iron, copper, and magnesium. In our studies, citric acid, known to have value in minimizing these leachables, did not significantly improve the use of asbestos filters and subsequently the quality of leptosporidial media; neither did hydrochloric acid followed by a bicarbonate wash. In addition, water and individual medium components as wash fluids for asbestos filter pads were to no avail in removing the leptosporidial lytic activity. It will be interesting to identify absolutely what is the specific phenomenon in asbestos-filtered albumin polysorbate 80 medium responsible for the lysis and death of pathogenic leptosporidiae.

The loss of viability of pathogenic leptosporidiae in asbestos-filtered medium appears as a numerical ratio of inoculum cell surface to the lysis and death action. Because heavy growth of leptosporidiae in lytic medium is possible with dense inocula, a given number of cells is probably attacked by the lytic activity, and cells so attacked have binding activity for the lytic agent. This is verified by the fact that after secondary subculture in lytic medium when cell numbers are again diluted, lysis again occurs. A metabolic mechanism appears not to exist, and some other explanation seems feasible.

Hutner (7), House (6), Brown et al. (1), and Litterest and Lichtenstein (9) have reported their observations related to the use of asbestos pads in biological filtration procedures.

Hutner (7) observed that saprophytic mycobacteria were stimulated in their growth when medium was filtered through bacteriological asbestos-type pads. The material responsible was not extracted by cold ether or alcohol, but was extracted by hot water and saline, found soluble in dilute HCl, and identified as Ca⁺⁺. Commercial forms of BA-P80 medium (8) contain 10 µg of CaCl₂ per ml; however, such formulations in our studies have not been found lytic for growth of leptosporidiae from diluted cell numbers.

House (6) noted a reduced cloning efficiency of hamster fibroblast cells in culture medium collected at different time sequences of filtration with asbestos pads; yet his data suggested that introduction of a toxic factor was the problem and not the removal of an essential nutrient. A similar effect was noted when calf serum alone was asbestos pad-filtered. The

| Serotype (strain) | Minimum no. of cells/ml resulting in growth |
|-------------------|---------------------------------------------|
|                   | Filtered       | Non-filtered            |
| copenhageni, M-20 | 2.12 x 10⁴     | 2.12                    |
| icterohaemorrhagiae, (VB-USD) | 1.7 x 10⁴ | 1.7                     |
| javanica, Veldrat-Batavia 46 | 2.62 x 10⁴ | 26                     |
| canicola, Hond Utrecht IV | 2.06 x 10⁴ | 2.06                    |
| canicola, (Moulton) | 1.25 x 10⁴     | 1.25                    |
| canicola, (NADL-A13) | 2.6 x 10⁴     | 2.6                     |
| canicola, (VB-USD) | 2.6 x 10⁴     | 2.6                     |
| ballum, S-102      | 2.4 x 10⁴     | 2.4                     |
| pyrogenes, Salinem  | 2.0 x 10⁴     | 30.8                    |
| pomona, pomona     | 2 x 10⁴       | 2                       |
| pomona, (DM,H)     | 1.6 x 10⁴     | 1.6                     |
| pomona, (MLS-NADL-A13) | 2.46 x 10⁴ | 0.24                    |
| pomona, (MLS-Neb, 100) | 2.76 x 10⁴ | 0.27                    |
| pomona, (MLS-Neb, acute) | 2.70 x 10⁴ | 0.02                    |
| pomona, (HCE)      | 2.12 x 10⁴    | 2.1                     |
| pomona, (B-35)     | 1.42 x 10⁴    | 1.42                    |
| pomona, (B-29)     | 2.24 x 10⁴    | 2.2                     |
| grippotyphosa, Moskva V | 2.6 x 10⁴ | 20                      |
| grippotyphosa, (I.C.S.) | 2.85 x 10⁴ | 2.8                    |
| grippotyphosa, (I.S.U.S.F.) | 2.7 x 10⁴ | 2.7                    |
| grippotyphosa, (Baccon, Ga.) | 2.56 x 10⁴ | 2.5                    |
| grippotyphosa, (Bobcat, Ga.) | 2.96 x 10⁴ | 2.9                    |
| grippotyphosa, (Swamp rabbit, Ga.) | 2.1 x 10⁴ | 2 x 10⁴ |
| grippotyphosa, (Cottontail rabbit, Ga.) | 2.2 x 10⁴ | 2 |
| sentot, Sentot     | 2 x 10⁴       | 2                       |
| georgia, LT-117    | 3.46 x 10⁴    | 34                      |
| hardjo, Hardjoprajitno | 3 x 10⁴     | 30                      |
| hardjo, (SC 1584)  | 2.8 x 10⁴     | 28                      |
| medanensis, Hond HC | 3.0 x 10⁴    | 33                      |
| wolffi, 3705       | 3.4 x 10⁴     | 3.4                     |
| bataviae, Van Tienen | 3.9 x 10⁴    | 3.9                     |
| ranae, (I.C.F.)    | 2.58 x 10⁴    | 0.25                    |
| patoc, Patoc 1     | 2             | 2                       |
| biflexa, (S.C. 3)  | 2             | 2                       |
| sao-paulo, (Sao-Paulo) | 2             | 2                       |
| illini, (3065H)    | 2             | 2                       |
| illini, (3066 NH)  | 2             | 2                       |
TABLE 7. Growth of leptospiral pathogenic serotypes in asbestos-filtered total medium

| Serotype (strain) | Medium* | 20 x 10^3 | 2 x 10^3 | 2 x 10^4 | 2 x 10^5 | 2 x 10^6 | 20 | 2  |
|------------------|---------|-----------|-----------|-----------|-----------|-----------|----|----|
| copenhageni, M20  | Filtered | 45 47    | —         | —         | —         | —         | —  | —  |
|                  | Control  | 42 42    | 44 44     | 46 46     | 47 44     | —         | —  | —  |
| canicola, (NADL-A13) | Filtered | 33 42    | 49 47     | 48 33     | 22 2     | —         | —  | —  |
|                  | Control  | 27 27    | 26 26     | 32 32     | 3 2       | —         | —  | —  |
| javanica, Veldrat Batavia 46 | Filtered | 38 41    | —         | —         | —         | —         | —  | —  |
|                  | Control  | 55 58    | 63 56     | 60 55     | 36 2      | —         | —  | —  |
| ballum, S-102     | Filtered | 42 37    | —         | —         | —         | —         | —  | —  |
| pyrogenes, Salinem | Control  | 55 58    | 63 56     | 60 55     | 36 2      | —         | —  | —  |
| canicola, (VB-USDA) | Filtered | 45 52    | 52 56     | 60 71     | 71 3      | 0         | —  | —  |
|                  | Control  | 42 47    | —         | —         | —         | —         | —  | —  |
| sentot, Sentot    | Filtered | 33 42    | 49 47     | 48 33     | 22 2      | —         | —  | —  |
|                  | Control  | 64 64    | —         | —         | —         | —         | —  | —  |
| australis, Ballico| Filtered | 35 33    | —         | —         | —         | —         | —  | —  |
|                  | Control  | 34 46    | 47 55     | 60 60     | 58 32     | —         | —  | —  |
| pomoza, (H.C.E.)  | Filtered | 52 64    | 76 80     | —         | —         | —         | —  | —  |
|                  | Control  | 33 44    | 47 56     | 4 2       | 0         | —         | —  | —  |
| grippotyphis, Moskva V | Control  | 51 58    | 62 61     | 63 45     | 5 2       | —         | —  | —  |
|                  | Filtered | .  .      | —         | —         | —         | —         | —  | —  |
| georgia, (LT-117) | Filtered | .  .      | —         | —         | —         | —         | —  | —  |
|                  | Control  | .  .      | —         | —         | —         | —         | —  | —  |
| wolffi, 3705      | Filtered | .  .      | —         | —         | —         | —         | —  | —  |
|                  | Control  | .  .      | —         | —         | —         | —         | —  | —  |
| bataviae, Van Tienen | Filtered | .  .      | —         | —         | —         | —         | —  | —  |
|                  | Control  | .  .      | —         | —         | —         | —         | —  | —  |
| Controls          | Filtered | 112 120  | 107 107   | 107 85    | 85 85     | —         | —  | —  |
| illini, (3055H)   | Control  | 87 87    | 87 83     | 90 92     | 84 8 —    | —         | —  | —  |
| illini, (3055NH)  | Filtered | 73 65    | 72 72     | 130 112   | —  —      | —         | —  | —  |
|                  | Control  | 70 71    | 70 74     | 85 74     | 77 77     | —         | —  | —  |

* . . , Not performed.
* Filtered means asbestos pad (0.025 μm) filtered. Control means autoclaved basal plus membrane-filtered albumin.

TABLE 8. Growth of leptospiral saprophytes in asbestos-filtered total medium

| Culture  | Days  | Medium* | 20 x 10^3 | 2 x 10^3 | 2 x 10^4 | 2 x 10^5 | 2 x 10^6 | 20 | 2  |
|----------|-------|---------|-----------|-----------|-----------|-----------|-----------|----|----|
| biflexa, (CDC) | 7     | Filtered | 61 (23)* | 53 (51) | 39 (95) | 24 (100) | 5 (..) | — | —  |
|          |       | Control  | 47 26    | 47 2    | —         | —         | —         | —  | —  |
|          |       | Filtered | 65 69    | 78 75   | 83 63     | 40 27     | 22        | —  | —  |
|          |       | Control  | 51 51    | 62 58   | 35 35     | 18 1     | —         | —  | —  |
| patoc, (Patoc 1) | 7*    | Filtered | 89 (8)  | 74 (8)  | 63 (24)  | 57 (50)  | 45 (89)  | 36 21 | 6  |
|          |       | Control  | 81 68    | 48 29   | 4 4       | —         | —         | —  | —  |
|          |       | Filtered | 82 90    | 93 93   | 92 83     | 95 90     | 80        | —  | —  |
|          |       | Control  | 72 85    | 80 91   | 78 55     | 33 2      | —         | —  | —  |
| biflexa, (SC #3) | 7     | Filtered | 79 (10) | 81 (10) | 86 (20)  | 83 (25)  | 85 (44)  | 76 (53) | 61 (57) | 46 |
|          |       | Control  | 71 73    | 69 61   | 48 36     | 27 3      | —         | —  | —  |
|          |       | Filtered | 72 77    | 80 75   | 83 87     | 80 82     | —         | —  | —  |
|          |       | Control  | 56 60    | 55 73   | 76 73     | 80 82     | —         | —  | —  |

* Symbols: . . , not calculated; —, no growth nephelometrically measurable as turbidity.
* Filtered means asbestos pad (0.025 μm) filtered. Control means autoclaved basal plus membrane-filtered albumin.
* Numbers in parentheses indicate percentage of growth stimulation at 7 days.
The mechanism of lysis of leptospiral pathogens by asbestos-filtered medium may have some application in the field of human health. Rajan et al. (13) reported that, in 8 days, human parietal pleurae are adversely affected by the presence of asbestos fibers, which contrasts to the long periods of time necessary to produce a similar effect in vivo.

It has become a common practice in the pharmaceutical industry to employ fiber catchers at the end of filtration procedures involving human injectables. This factor suggests that fragmentation of asbestos fibers takes place in the filtration process. It remains to be conclusively proved that the lytic activity for pathogenic leptospires in asbestos-filtered medium is caused by either a soluble or particulate entity, or both, associated with such pads.

Still unanswered is the major difference surrounding the leptospiiral pathogens and saprophytes and their different reaction in growth and viability in asbestos-filtered medium.

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**TABLE 9. Antigenicity of *Leptospira interrogans* copenhageni (M-20) grown in asbestos-filtered and control medium**

| Antiserum | Growth medium | MA titer |
|-----------|---------------|----------|
| copenhageni, M-20 | | |
| Asbestos | 2+ | 3+ | 4+ | 1+ | 2+ | 2+ |
| Cellulose | 3+ | 4+ | 3+ | 1+ | 2+ | 2+ |
| Control | 2+ | 1+ | — | — | — | — |
| australis, Bal- | | |
| Asbestos | 4+ | 2+ | 1+ | — | — | — |
| Cellulose | 2+ | 1+ | — | — | — | — |
| Control | 2+ | 1+ | — | — | — | — |
| sentot, Sentot | | |
| Asbestos | 2+ | 1+ | — | — | — | — |
| Cellulose | 2+ | 1+ | — | — | — | — |
| Control | 2+ | 1+ | — | — | — | — |
| canicolana, Hond | | |
| Asbestos | 4+ | 3+ | 2+ | — | — | — |
| Cellulose | 4+ | 3+ | 2+ | — | — | — |
| Control | 4+ | 3+ | 2+ | — | — | — |
| Utrecht IV | | |
| Asbestos | 2+ | 1+ | — | — | — | — |
| Cellulose | 2+ | 1+ | — | — | — | — |
| Control | 2+ | 1+ | — | — | — | — |
| autumnalis, | | |
| Asbestos | 4+ | 3+ | 2+ | — | — | — |
| Cellulose | 4+ | 3+ | 2+ | — | — | — |
| Control | 4+ | 3+ | 2+ | — | — | — |
| Akiyami A | | |
| Asbestos | 3+ | 1+ | — | — | — | — |
| Cellulose | 3+ | 1+ | — | — | — | — |
| Control | 3+ | 1+ | — | — | — | — |
| pyrogenes, Salinem | | |
| Asbestos | 4+ | 3+ | 2+ | — | — | — |
| Cellulose | 4+ | 3+ | 2+ | — | — | — |
| Control | 4+ | 3+ | 2+ | — | — | — |
| ballum, S-102 | | |
| Asbestos | 3+ | 2+ | 2+ | — | — | — |
| Cellulose | 3+ | 2+ | 2+ | — | — | — |
| Control | 3+ | 2+ | 2+ | — | — | — |
| pomona, (J) | | |
| Asbestos | 4+ | 2+ | — | — | — | — |
| Cellulose | 4+ | 2+ | — | — | — | — |
| Control | 4+ | 2+ | — | — | — | — |

* — No agglutination.

AF, Asbestos filtered.