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Protein-Free and Low-Protein Media for the Cultivation of *Leptospira*

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A protein-free medium composed of charcoal-detoxified Tweens (polysorbates), vitamins B_{12} and B_1, inorganic salts, and an organic buffer is described that supports the growth and subculture of pathogenic and saprophytic *Leptospira*. Growth was initiated from small inocula, and cell densities of 10^9 organisms per ml were attained. Antigenicity and immunogenicity of *Leptospira* cultivated in this medium were similar to those of cells cultivated in serum-containing media. The protein-free medium was converted to a low-protein medium by the addition of 0.1% bovine serum albumin.

A number of chemically defined media for the cultivation of leptospires have been described previously (2, 9, 19, 22-24, 27). However, a protein-free (PF) medium that will grow a large variety of pathogenic serovars to high cell densities is not presently available. The most successful of the chemically defined media is that of Shenberg (22). She was able to serial-subculture 52 strains of pathogenic leptospires, initiate growth from small inocula, and obtain cell yields of 10^8 organisms per ml. However, serovars such as *ballum* and *javanica* could not be cultivated in the medium (22). Kida and Yanagawa (18) were able to grow serovars *autumnalis, australis, hebdomadis,* and *canicola* in Shenberg medium, but they were not able to grow *icterohaemorrhagiae* unless a mutant strain was used.

Leptospirosis is a zoonosis that is best controlled by vaccination (25). Vaccine production requires the use of large volumes of culture media, and the most expensive medium component is serum or serum albumin. The serum proteins are also the cause of adverse vaccine reactions. Moreover, variation in the suitability of serum and serum fractions used for the cultivation of leptospires occurs, and difficulties in sterilizing these media can be encountered. A PF medium circumvents these and other disadvantages of the protein-containing media.

This report describes a chemically defined, PF medium of simple composition that will grow virulent strains of *Leptospira* from small inocula to cell densities of at least 10^9 organisms per ml.

MATERIALS AND METHODS

Growth and maintenance of organisms. The serovars of *Leptospira interrogans* used in this study were maintained in the bovine serum albumin (BSA)-Tween 80 medium described below or in PLM 5 (prepared leptospire medium 5×, Reheis Chemical Co., Kankakee, Ill.). The primary test organisms were the: hamster-lethal *canicola,* Moulton; *icterohaemorrhagiae,* CF 1; *pomona,* H 10-414; and *grippotyphosa,* 11808; and the avirulent *hardjo,* LSU 91. Virulence of the first four serovars was maintained by periodic hamster passage (3). Cultures were incubated at 30°C under static or aerated conditions. Aeration of tubes (18 by 150 mm, containing 10 ml of medium) was accomplished by placing the tubes at a 70° angle in a reciprocating water bath (3.5-cm stroke distance at 112 strokes per min). Aeration of cultures was not initiated until the cell population had reached approximately 10^8 per ml, and, when desired, periodic additions of supplemental Tweens were made to attain maximal cell yields. Growth was monitored by nephelometry (Coleman nephelo colorimeter). The nephelometer was adjusted to a reading of 70 with a 20-U permanent turbidity standard (20). Direct counts were performed with a Petroff-Hausser counting chamber and correlated with the turbidity of the culture.

Preparation of BSA-Tween 80 medium. The following stock solutions were prepared (grams per 100 ml of distilled water): NH_4Cl (25.0), $ZnSO_4 \cdot 7H_2O$ (0.4), $MgCl_2 \cdot 6H_2O$ (1.5) and $CaCl_2 \cdot 2H_2O$ (1.5), $FeSO_4 \cdot 7H_2O$ (0.5), sodium pyruvate (10.0), glycerol (10.0), Tween 80 (10.0), thiamine·HCl (Sigma Chemical Co., St. Louis, Mo.) (0.5), and cyanocobalamin (0.02). The BSA supplement was prepared by adding 10 g of BSA FR V (Reheis Chemical Co., Kankakee, Ill.) to 50 ml of distilled water and, while the albumin solution was stirred, the following stock solutions were slowly added: $CaCl_2$ and $MgCl_2$ (1.0 ml), $ZnSO_4$ (1.0 ml), freshly prepared $FeSO_4$ (10 ml), cyanocobalamin (1.0 ml), and Tween 80 (12.5 ml). The pH was adjusted to 7.4, distilled water was added to bring the final volume to 100 ml, and the supplement was sterilized by filtration. The basal medium was prepared by dissolving in 996 ml of distilled water, Na_2HPO_4 (anhydrous) (1.0 g), KH_2PO_4 (anhydrous) (0.3 g), and NaCl (1.0 g). The following stock solutions were then added: NH_4Cl (1.0 ml), thiamine (1.0 ml), sodium pyruvate (1.0 ml), and glycerol (1.0 ml). The basal medium was adjusted to pH 7.4 and sterilized by autoclaving at

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121°C for 20 min. The complete BSA-Tween 80 medium was prepared by adding 1 volume of BSA supplement to 9 volumes of basal medium.

Preparation of CT Tweens. Stock solutions of charcoal-treated (CT) Tweens were prepared by dissolving 20.0 g of Tween in 200 ml of distilled water. While the solution of Tween was stirred, 40.0 g of Norit A charcoal (J. T. Baker Chemical Co., Phillipsburg, N.J.) was added slowly to insure a homogeneous suspension of charcoal particles. This mixture was stirred slowly for 18 h at room temperature (22 to 25°C), after which it was allowed to settle for 18 h at 4°C. The Tween was then carefully decanted from the charcoal sediment, centrifuged (10,000 × *g* for 1 h), and subjected to thin-channel ultrafiltration with an XM 100 membrane (Amicon Corp., Lexington, Mass.) to remove any remaining charcoal from the Tween. The Tween concentrations of the CT Tweens were determined (24), and the stock solutions of the CT Tweens were stored at −20°C.

(Note: The patent for this method was applied for by the University of Minnesota.)

Preparation of the PF medium. The following BSA-Tween 80 medium stock solutions were used in the preparation of the PF medium: glycerol, $CaCl_2$-$MgCl_2$, $ZnSO_4$, $FeSO_4$, thiamine (heated at 121°C for 20 min), cyanocobalamin, and NH_4Cl. In addition, stock solutions of (grams per 100 ml of distilled water) KH_2PO_4 (anhydrous) (1.0), $MnSO_4 \cdot H_2O$ (0.1), and the CT Tweens (10.0) were necessary. The PF medium was prepared by dissolving in 50 ml of distilled water 1.2 g of *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES, Calbiochem, La Jolla, Calif.), 0.9 g of NaCl, and 0.2 g of sodium pyruvate. To this solution were added the following stock solutions (milliliters) with constant mixing: glycerol (1.0), CT Tween 60 (12.0), CT Tween 40 (3.0), $CaCl_2$ and $MgCl_2$ (1.0), $ZnSO_4$ (0.1), NH_4Cl (0.9), thiamine·HCl (1.0), cyanocobalamin (1.0), $MnSO_4$ (1.0), and KH_2PO_4 (1.0). After the pH of the medium was adjusted to 7.6 with 1 N NaOH, and distilled water was added to bring the volume to 100 ml, it was filter sterilized. The 1× PF medium was prepared by adding 1 volume of 10× PF medium to 9 volumes of sterile distilled water.

(Note: The patent for this method was applied for by the University of Minnesota.)

Organic buffers. When other organic buffers, *N*-2-hydroxyethyl piperazine-*N*-2-propanesulfonic acid (HEPS), *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES), or morpholinopropane-sulfonic acid (MOPS) (Calbiochem, La Jolla, Calif.), were added to the PF medium, they were substituted for TES and the media were formulated as described above. Concentrations of buffers ranged from 1 to 20 mM.

Amino acid and vitamin mixtures. Mixtures of amino acids and vitamins were prepared by the method of Shenberg (22). These mixtures were added in amounts varying from 0.1 to 2.0 ml/10 ml of medium.

Inocula. Inocula for all nutritional studies consisted of logarithmic-phase cells cultivated in the BSA-Tween 80 medium or in the PF medium. All inocula were diluted in PF medium devoid of the component being evaluated.

Immunogenicity determinations. Serovars evaluated for immunogenicity were cultivated in the PF medium until cell densities reached 10^9 organisms per ml. Outer envelope vaccines were prepared and evaluated for immunogenicity as previously described (3), except that cells in the PF medium were inactivated with 1 M NaCl. It was not possible to do this with BSA-Tween 80 medium.

Isolation of leptospires from blood of infected hamsters. Four hamster-lethal strains (*canicola*, Moulton; *icterohaemorrhagiae*, CF 1; *grippotyphosa*, 11808; and *pomona*, H 10-414) were used to evaluate the PF medium as an isolation medium. Blood was obtained from moribund hamsters by cardiac puncture and inoculated (0.05 ml) into 10 ml of growth medium. Leptospiral multiplication was monitored by microscopic observation.

Microscopic agglutination test. Antigenic stability of cells cultivated in PF, BSA-Tween 80, and rabbit serum media was evaluated by the microscopic agglutination test (5).

RESULTS

BSA-Tween 80 leptospire culture medium described by Johnson and Harris (13) was modified to improve its growth-supporting activity. Copper sulfate was deleted from the formulation, and the concentrations of calcium and magnesium were increased (see Materials and Methods). This medium was the starting point for the development of the PF medium.

The Tweens, such as Tween 80 (polyoxyethylene sorbitan monooleate), provide a relatively nontoxic, water-soluble source of long-chain fatty acids for the leptospires. In the BSA-Tween 80 medium without albumin, Tween 80 is an unsuitable substitute for the cultivation of *canicola* (Table 1). The anion-exchange resin, AG2-8X, the cation-exchange resin, AG50W-8X, and charcoal were evaluated as to their capacity to detoxify Tween 80. The anion-exchange resin, but not the cation-exchange resin, served as a substitute for albumin (Table 1). For the anion-exchange resin to function effectively, it must be incorporated into the medium. Attempts to detoxify Tween 80 with the resin before its addition to the medium were unsuccessful. Because of the inconvenience associated with the use of the ion-exchange resin, activated charcoal was investigated for its capacity to detoxify the Tween before incorporation into the medium. CT Tween 80 supported the growth of *canicola*, and activated charcoal in the finely powdered form (Darco or Norit A) was most effective for detoxifying the Tween 80. A slightly higher cell yield was achieved in a shorter period of time with the CT Tween 80 as compared with the Tween 80–anion-exchange resin combination, and this compares favorably with the BSA-Tween 80 control (Table 1). Of the five serovars tested (*canicola*, *icterohaemorrhagiae*, *pomona*, *grippotyphosa*, and *hardjo*), only *cani-*

TABLE 1. *Detoxification of Tween 80 for the cultivation of canicola, Moulton*[a]

Material used to detoxify Tween 80	Maximal no. of cells per ml × 10^7	Days of incubation required for maximal growth
None	0	—[b]
Anion-exchange resin (AG2-8X)	43	18
Cation-exchange resin (AG50W-8X)	7	9
Activated charcoal (Norit A)	50	12
BSA-Tween 80	56	12

[a] Tween 80 concentration, 1.25 mg/ml. Inoculum size, 10^4 organisms per ml, incubated statically. Culture medium, BSA-Tween 80 medium without BSA.

[b] —, No growth within 30 days of incubation.

cola could be cultivated in this CT Tween 80 medium.

CT Tween 60 (polyoxyethylene sorbitan monostearate) was more suitable for cultivation of the five leptospiral serovars than CT Tween 80. The CT Tween 60 medium supported growth of the virulent (lethal) *canicola* and *pomona*, the virulent (renaltrophic) *hardjo* and *grippotyphosa*, and the avirulent *icterohaemorrhagiae*. It did not support growth of the virulent (lethal) strains of *icterohaemorrhagiae* or *grippotyphosa*.

The basal medium used above contained a phosphate buffer. In an attempt to improve the CT Tween 60 medium, the organic buffers TES, HEPS, HEPES, and MOPS were substituted for the phosphate buffer. The CT Tween 60 medium with the organic buffer TES was superior to phosphate and MOPS buffers and slightly better than HEPS and HEPES buffers. Accordingly, the TES buffer was used in subsequent experiments. The growth response of the hamster-lethal, CF1, strain of *icterohaemorrhagiae* in the CT Tween 60 medium with the different buffers is seen in Table 2.

The CT Tweens 80, 60, and 40 were assayed for their growth-supporting activity in the TES-buffered medium. As was observed earlier, CT Tween 80 supported growth of *canicola* but not that of the other test serovars. The CT Tweens 60 and 40 supported growth of all five virulent serovars. A higher cell yield was obtained with CT Tween 60, but growth was initiated sooner with CT Tween 40. The combination of 1 part CT Tween 40 and 4 parts CT Tween 60 resulted in a medium that initiated leptospiral growth rapidly and produced a high cell yield. The addition of CT Tween 80 in a variety of combinations with CT Tween 60 and/or CT Tween 40 did not improve the medium. The growth response of *icterohaemorrhagiae* in the various Tweens is seen in Table 2.

Although the TES–CT Tween 40–60 medium was suitable for two to three transfers of *canicola*, serovars *pomona*, *icterohaemorrhagiae*, *grippotyphosa*, and *hardjo* could not be subcultured in it. The following additions to the medium did not allow subculturing of these serovars or improve their growth: amino acid mixture (1 to 100 μg/ml), Trypticase (1 to 500 μg/ml), yeast extract (1 to 500 μg/ml), glucose (1 to 50 mg/ml), sodium acetate (1 to 500 μg/ml), and the short-chain fatty acids isobutyric, caproic, and valeric (10 to 200 μM). However, the addition of manganese sulfate (1 μg/ml) to the medium did permit the four virulent serovars, *canicola*, *pomona*, *icterohaemorrhagiae* and *hardjo*, to be subcultured at least nine times, with maximal growth occurring with each transfer. Serovar *grippotyphosa* (hamster lethal) could not be transferred in this medium unless 0.1% BSA was present. The composition of the PF medium is described in Materials and Methods. All subsequent experiments were conducted with this PF medium. The growth response of *icterohaemorrhagiae*, CF 1, in the PF and BSA-Tween 80 media is seen in Fig. 1.

Limited nutritional studies on the hamster-lethal *canicola*, Moulton, and *grippotyphosa*, 11808, were performed in the PF medium with inocula of 10 cells per ml. The Tween fatty acids serve as the major carbon and energy source, and ammonium chloride serves as the source of nitrogen for the leptospires. If either of these compounds is deleted from the medium, growth

TABLE 2. *Effect of various buffers and CT Tweens on the growth of icterohaemorrhagiae, CF1*

CT Tween[a]	Buffer[b]	Maximal no. of cells per ml × 10^{8c}	Days of incubation required for maximal growth
60	Phosphate	NG[d]	—[e]
60	HEPS	7.8	11
60	HEPES	7.3	9
60	MOPS	3.0	7
60	TES	8.3	9
40	TES	7.4	7
60 + 40	TES	8.4	8
80	TES	NG	—

[a] Tween concentration, 0.9 mg/ml.

[b] Buffer concentration: Phosphate buffer, 8.3 mM; organic buffers, 5 mM.

[c] Inoculum size, 10^3 cells per ml; cultures were aerated.

[d] NG, No growth.

[e] —, No growth within 30 days of incubation.

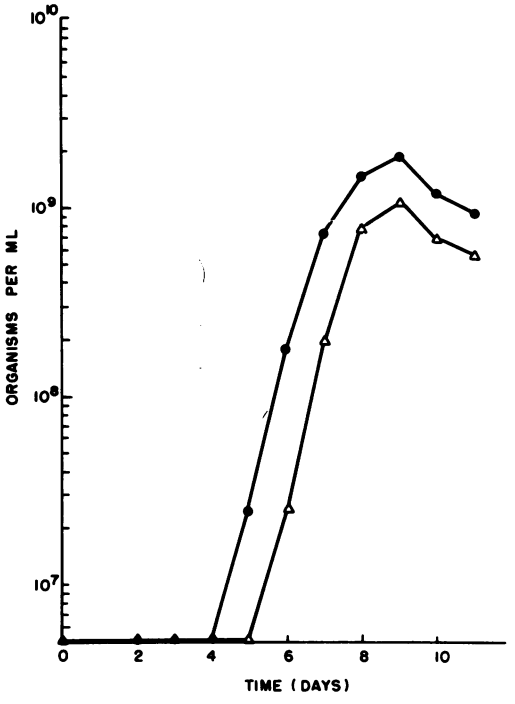

FIG. 1. *Growth of icterohaemorrhagiae, CF 1, in PF medium (△) and in BSA-Tween 80 medium (●). The inoculum size was 10^3 cells per ml, and cultures were incubated aerobically. PF medium contained 0.8 mg of CT Tween 60 per ml, and the BSA contained 1.25 mg of Tween 80 per ml.*

does not occur. Both cyanocobalamin and thiamine are required vitamins, and when either is deleted from the medium the leptospires can not be subcultured. In the PF medium containing thiamine but not cyanobalamin, the leptospires cannot be subcultured more than once or twice. When the situation is reversed, the leptospires can be subcultured three to four times with limited growth occurring. No other vitamins were required by these leptospires. Magnesium and manganese were necessary for continuous subculturing of the leptospires. Calcium, although not required for subculturing of these organisms, was necessary for achieving maximal cell yields. Without calcium only one-half to one-fourth the usual cell yields were obtained. Although definite requirements for iron, zinc, potassium, and pyruvate could not be established, their presence in the PF medium enhanced leptospiral growth. Other components of the medium were not evaluated.

The suitability of the PF medium for cultivation of a wide variety of leptospiral serovars was investigated. A total of 17 serovars (27 strains) was grown in the PF medium and carried through at least nine transfers (Table 3). The maximal cell yield and the time required to attain it did not change significantly for any of

TABLE 3. *Leptospires cultivated in the PF medium*[a]

Serovar	Maximal no. of cells per ml × 10^8	Days of incubation required for maximal growth
australis, Mal 1556	4.0	7
fort-bragg, Fort Bragg	8.7	7
ballum, Mus 127	9.0	7
bataviae, Swort	9.0	7
biflexa, Codice	9.0	6
canicola, Moulton, Hond Utrecht IV	10.0–11.0	6
celledoni, Celledoni	5.0	8
copenhageni, M 20	9.0	7
coxi, Cox	4.0	7
grippotyphosa, 11808,[b] SC 4397, Moskva, V Mal 1540	7.0–8.3	6–8
hardjo, 11601, LSU 91	3.0–8.0	7–8
icterohaemorrhagiae, CF 1, SC 1157	9.0	8
illini, 3055	9.8	6
javanica, SB 33	2.0	10
patoc, Patoc I	9.0	7
pomona, H 10-414, Pomona, Wickard, BJ1	5.4–9.0	5–7
pyrogenes, Salinem	8.0	9
sao paulo, Sao Paulo	5.0	9

[a] Inocula, 10^5 cells per ml.
[b] Requires 0.1% albumin for serial transfers.

TABLE 4. *Initiation of leptospiral growth from small inocula in BSA-Tween 80 medium and PF medium*[a]

Serovar	Culture medium[b]	Maximal no. of cells per ml × 10^8	Days of incubation required for maximal growth
icterohaemorrhagiae, CF 1	ALB	15.5	14
	PF	13.0	16
canicola, Moulton	ALB	15.8	13
	PF	15.0	14
pomona, H 10-414	ALB	14.0	14
	PF	12.0	15
grippotyphosa, 11808	ALB	10.0	14
	PF	9.2	17
hardjo, LSU 91	ALB	18.3	13
	PF	17.8	14

[a] Inoculum size, 10 organisms per ml, incubated aerobically; CT Tween concentration, 0.8 mg/ml.
[b] ALB, BSA-Tween 80 medium.

the test organisms during the transfer period. The addition of 0.1% albumin to the PF medium was necessary to subculture the hamster-lethal *grippotyphosa*, 11808, but not the renaltropic or avirulent strains.

The virulence of *canicola* and *pomona* subcultured nine times in the PF medium and the BSA-Tween 80 medium was compared. Groups of seven hamsters were injected intraperitoneally with 10^8 organisms from the test cultures. All infected hamsters died within 3 to 4 days, indicating that a large proportion of the cells cultivated in both media retained their virulence for hamsters.

The PF medium was also evaluated for its suitability for isolating leptospires from blood, initiating growth from a small inoculum, and producing high cell yields. Hamster-lethal strains of *canicola, pomona, icterohaemorrhagiae,* and *grippotyphosa* were isolated from the blood of infected hamsters by using the PF medium and the BSA-Tween 80 medium. Growth (10^8 cells per ml) occurred within 4 to 6 days in both media. With a known small inoculum (10 cells per ml), growth was initiated several days sooner in the BSA-Tween 80 medium than in the PF medium. However, high cell yields were obtained in both media (Table 4). The cell yields achieved with the PF medium could be substantially increased by supplementing the culture medium with additional CT Tween 60 once the culture was in the logarithmic phase of growth (Table 5).

The antigenicity and immunogenicity of leptospires cultivated in the PF medium were evaluated. The first test serovars were cultivated in PF, BSA-Tween 80, and rabbit serum media. The antigenicity of these cells was then compared by the microscopic agglutination test. Regardless of the culture medium used, the agglutination titers obtained with hyperimmune rabbit serum to each serovar were not significantly different (within one dilution). Moreover, no major differences in titers were observed with cells from the first subculture and those from the ninth subculture in these media (Table 6). The immunogenicity of the outer envelopes from *canicola, icterohaemorrhagiae,* and *pomona* cultivated in the PF and the BSA-Tween 80 media was evaluated in hamsters. The PD_{50D} of the outer envelope preparations ranged from 0.014 to 0.083 μg, and the PD_{50k} ranged from 0.013 to 0.3 μg (Table 7; for definitions of PD_{50D} and PD_{50k}, see footnotes *c* and *d*). Although the outer envelope from cells cultivated in the BSA-Tween 80 medium were of slightly higher immunogenicity than the outer envelope from cells grown in the PF medium, both media were suitable for producing cells of high immunogenicity.

TABLE 5. *Effect of supplementing the PF culture medium with additional substrate during the logarithmic phase of growth*[a]

Serovar	No. of cells per ml × 10^8	
	Without supplement	With supplement[b]
icterohaemorrhagiae, CF 1	9.3	32.0
canicola, Moulton	9.2	34.0
pomona, H 10-414	9.1	33.0
grippotyphosa, 11808	9.0	28.0
hardjo, LSU 91	9.3	35.0

[a] Inoculum size, 10^3 cells per ml, incubated aerobically.

[b] CT Tween 60 (0.6 mg/ml) added to medium on days 7, 10, and 13.

TABLE 6. *Antigenicity of leptospires cultivated in different culture media*

Serovar	Culture medium[a]	Microscopic agglutination titer[b]	
		First transfer in medium	Ninth transfer in medium
icterohaemorrhagiae, CF 1	RS	5,120	2,560
	ALB	10,240	5,120
	PF	5,120	2,560
canicola, Moulton	RS	5,120	5,120
	ALB	10,240	5,120
	PF	5,120	5,120
pomona, H 10-414	RS	640	640
	ALB	640	640
	PF	640	640
grippotyphosa, 11808	RS	10,240	5,120
	ALB	10,240	5,120
	PF	10,240	5,120
hardjo, LSU 91	RS	2,560	2,560
	ALB	5,120	5,120
	PF	5,120	2,560

[a] RS, Rabbit serum medium; ALB, BSA-Tween 80 medium.

[b] Reciprocal of highest dilution of antiserum that agglutinated 50% of the cells.

Neither the use of CT Tweens in the BSA-Tween 80 medium nor the addition of 0.1 to 1% albumin to the PF medium resulted in a medium obviously superior to the BSA-Tween 80 medium. With the original medium formulation containing phosphate buffer and CT Tween 60, leptospiral growth could not be initiated from a 10^3-cell-per-ml inoculum when the albumin concentration was reduced from 1 to 0.1%. In con-

trast, the PF medium could be converted to a low-protein medium by the addition of 0.1% BSA. Growth is initiated from low inocula sooner, and higher cell yields are obtained in the low-protein medium as compared with the PF medium (Table 8).

DISCUSSION

The leptospires have relatively simple nutritional requirements. An albumin or serum medium containing long-chain fatty acids (11, 26), vitamins B_1 (21), and B_{12} (1), and inorganic salts (8, 10, 12) will support good growth of these spirochetes (7, 13). Pyruvate, although not an essential nutrient, augments the initiation of growth of parasitic leptospires (16, 24). Our results confirm these observations.

Fatty acids are the major carbon and energy source for leptospires (11, 15). In addition, long-chain fatty acids are essential nutrients because the leptospires are unable to synthesize these acids de novo (14). Although they can decrease the length and desaturate fatty acids, the leptospires cannot increase the length of these compounds (14). Because free fatty acids are toxic to the leptospires, serum or serum albumin is incorporated into culture media to bind the fatty acids in an available but nontoxic form (11, 17). Anion-exchange resins also bind fatty acids, and they can serve as a substitute for albumin for the cultivation of *Leptospira* (17).

A relatively nontoxic, water-soluble source of fatty acids is the polysorbates (Tweens). The polysorbates are mixtures of molecules containing varying ratios of sorbitan, fatty acids (mixture of fatty acids with one acid predominating), and ethylene oxide (average composition of 1:1:20, respectively). Tween 80 (polyoxyethylene sorbitan monooleate) contains 22.5% fatty acids, with approximately 3% of the acids in the free, unesterified form (6). Because of the free-fatty acid content and possibly Tween molecules that contain more than one fatty acid per sorbitan molecule, Tween 80 must be combined with albumin to provide a nontoxic substrate for leptospires (7, 13).

Staneck et al. (24) reported that albumin was not necessary for cultivation of *canicola* and *pomona* if the Tween 80 was passed through an anion-exchange column to reduce its free-fatty acid content. They also reported that treatment of the Tween with the anion-exchange resin was not essential if an adequate concentration of iron was present in the medium. We were unable to detoxify the Tweens with iron. However, low concentrations of iron did stimulate leptospiral growth. Our attempts to detoxify the Tweens by passage through an anion-exchange column were unsuccessful, but incorporation of the anion-exchange resin into the medium with the Tweens rendered them satisfactory for the cultivation of the leptospires. The major disadvantage of using the resins in this manner is that they must be separated from the leptospires when they are harvested. Charcoal, which is well known for its adsorptive properties, readily adsorbs fatty acids and is used for the production of fatty acid-poor albumin (4). We found that the Tweens could be effectively detoxified with finely powdered, activated charcoal before their

TABLE 7. *Immunogenicity of outer envelope vaccines prepared from cells cultivated in BSA-Tween 80 and PF media*

Serovar	Culture medium[a]	Challenge inoculum LD_{50}[b]	PD_{50D}[c]	PD_{50k}[d]
icterohaemorrhagiae, CF 1	ALB	2.0×10^5	0.06	0.3
	PF		0.083	0.3
canicola, Moulton	ALB	4.3×10^4	0.016	0.023
	PF		0.032	0.05
pomona, H 10-414	ALB	3.6×10^4	0.014	0.013
	PF		0.04	0.05

[a] ALB, BSA-Tween 80 medium; cells from third transfer in media.

[b] LD_{50}, 50% lethal dose.

[c] PD_{50D}, Micrograms (dry weight) of outer envelope per animal which provides 50% protection against death.

[d] PD_{50k}, Micrograms (dry weight) of outer envelope per animal which provides 50% protection against kidney infection.

TABLE 8. *Growth of leptospires in low-protein medium and PF medium*[a]

Serovar	Medium[b]	Days of incubation required for maximal growth	Maximum no. of cells per ml × 10^8
canicola, Moulton	PF	14	11.0
	LP	12	14.0
icterohaemorrhagiae, CF 1	PF	16	12.3
	LP	14	14.6
grippotyphosa, 11808	PF	18	9.8
	LP	15	11.0
hardjo, LSU 91	PF	14	17.0
	LP	12	20.2
pomona, H 10-414	PF	14	11.8
	LP	13	14.4

[a] Inoculum, 10 cells per ml.

[b] LP, PF medium containing 0.1% BSA.

addition to the culture medium. Coarser particles of charcoal were less satisfactory for this purpose.

Unsaturated fatty acids are more toxic to the leptospires than are the saturated acids in protein-free media (22, 24). However, Tween 80 (major fatty acid, oleic acid) is superior to Tweens 60 and 40 (major fatty acids, stearic and palmitic, respectively) in the 1% BSA medium. In contrast, CT Tweens 60 and 40 are more suitable substrates in the PF medium than is CT Tween 80. Of the five test serovars, *canicola* was the most tolerant of unsaturated fatty acids.

In the phosphate-buffered medium containing CT Tween 60, high cell yields of virulent (hamster-lethal) *canicola* and *pomona*, virulent (renaltrophic) *hardjo* and *grippotyphosa*, and avirulent *icterohaemorrhagiae* could be realized. The immunogenicity of *canicola* and *pomona* cells cultivated in this medium was equivalent to that of cells grown in the BSA-Tween 80 medium. The immunogenicity of the other serovars was not evaluated. The CT Tween phosphate-buffered medium was "toxic" for the hamster-lethal strains of *grippotyphosa* and *icterohaemorrhagiae*. Even when large inocula (10^6 cells per ml) were used, the cells failed to grow and became nonviable within several days. Replacement of the phosphate buffer with an organic buffer resulted in a medium that would support the growth of all virulent and avirulent strains of leptospires tested. Difficulty in serial subculturing of the leptospires was encountered with this medium. The addition of manganese to the medium resolved this deficiency for all of the test organisms except the hamster-lethal strain of *grippotyphosa*. The addition of 0.1% BSA to the PF medium allowed serial subculturing of this strain.

Incorporation of 0.1% BSA into PF medium converts it to a low-protein medium that is superior to the PF medium for cultivation of leptospires. Compared with the PF medium it has the disadvantage of containing protein but has the advantage of containing one-tenth the BSA present in the BSA-Tween 80 medium. In the phosphate-buffered medium it is not possible to reduce the BSA concentration to this level and obtain satisfactory growth of the leptospires. Acetate, which has been reported to improve the utilization of Tween 80 by leptospires (2), was without growth-supporting activity in the PF medium.

One of the major advantages of the PF medium over protein-containing media is the production of high yields of leptospires free from contaminating proteins for vaccines and for physiological studies of these spirochetes. Aeration of the PF medium, once cells are in the logarithmic phase of growth (ca. 10^8 cells per ml), coupled with the periodic addition of supplemental CT Tween, results in cell yields of 2×10^9 to 4×10^9 leptospires per ml. It is inexpensive and can be conveniently prepared as a 10× concentrate, which is stable for at least 12 months at 5°C. In addition, the antigenicity and immunogenicity of leptospires cultivated in the PF medium are comparable to those cultivated in the BSA-Tween 80 and rabbit serum media. The PF medium has the additional advantage of readily being converted to a low-protein medium by the addition of 0.1% BSA. Growth can be initiated from small inocula in the PF medium, and the generation time of cells cultivated in this medium is about the same as that of cells grown in the protein-containing media. Isolation of the outer envelope (3) from leptospires is simplified when using the PF medium. The cells can be inactivated by NaCl while in the medium, whereas it is necessary to remove the cells from the BSA-Tween 80 medium before they can be processed.

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