

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

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Abogado
CARLOS R. OLARTE

ASUNTO	Radicación	09 024 344	
	Trámite	011	
	Evento	378	
	Actuación	519	E 1 3 0 6 2
	Oficio		
	Folios	14	

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (Fase Nacional)	☒	Capítulo I	☒ Capítulo II ☐
No. Solicitud Internacional	PCT/IB2007/002553		
Fecha de Presentación Internacional	30/Agosto/2007		

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 34 a 48	10/Marzo/2009
Reivindicaciones:	Folios 49 a 53	10/Marzo/2009
Prioridad:	US 60/843,665	11/Septiembre/2006

2. Objeto de la invención

Título de la solicitud: "Bacterinas tratadas con calor y vacunas de emulsión preparadas a partir de bacterinas tratadas con calor".

El problema planteado en esta solicitud consiste en la necesidad de suministrar vacunas de emulsión y métodos para estabilizar vacunas de emulsión.

Para resolver este problema técnico la solicitud en estudio proporciona bacterinas tratadas con calor, un método para producir bacterinas tratadas con calor y vacunas de emulsión preparadas a partir de estas bacterinas tratadas con calor. El método comprende calentar la bacteria a temperatura de aproximadamente 35 a aproximadamente 80°C para formar una bacteria tratada con calor.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/00, A61K 39/02.

3. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 se refiere a una bacterina, la cual corresponde a un producto, el cual sólo puede ser definido en términos de su proceso de fabricación si: el producto es nuevo e inventivo y no se puede definir por sus características esenciales. También se refieren a un valor de porcentaje de actividad lipasa, que es un resultado a alcanzar con el proceso realizado a la suspensión de bacterias muertas, no definen al producto porque la invención no queda definida por el resultado a alcanzar, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Teniendo en cuenta los capítulos descriptivo y reivindicatorio, se sugiere enfocar la materia reclamada a un procedimiento, puesto que un proceso de calentamiento corresponde a esta categoría y no a un producto.

Se observa en el pliego reivindicatorio que las reivindicaciones de producto y procedimiento se están definiendo con las mismas características, puesto que estas pertenecen a diferentes categorías, se sugiere definir cada uno por sus características esenciales y no definir a uno en términos del otro.

Las reivindicaciones 1 -6, 8 - 18 21 y 22 no son concisas porque se refieren a una bacterina sin definir de cual bacteria. En el capítulo descriptivo se encuentra soporte para *Leptospira canicola*, *Leptospira icterohaemorrhagiae*, *Leptospira grippityphosa*, *Leptospira hardjo* y *Leptospira Pomona*, por lo cual se sugiere restringir la solicitud a una razonable generalización de bacterinas según lo que se encuentra soportado incluyendo en las reivindicaciones independientes la bacteria de la cual es la bacterina.

Las reivindicaciones 2 – 7, 21 y 22 son reivindicaciones de producto, y sólo pueden ser definidas en términos de su proceso de fabricación si: el producto es nuevo e inventivo y no se puede definir por sus características esenciales. Se sugiere definirlo por sus características esenciales, estructurales o funcionales.

El término: "aproximadamente" de las reivindicaciones 2 – 6, 17, 18, 21 y 22 es impreciso, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto.

No se encuentran soporte descriptivo para los tiempos y temperaturas de las reivindicaciones 1 – 6, 17, 18 y 21, y por lo tanto, éstas parecen meras selecciones arbitrarias. Adicionalmente puesto que estas reivindicaciones se refieren a un proceso y sus condiciones se sugiere reclamarlo como tal, y no como un producto definido en términos de su procedimiento de obtención. Debido a la falta de soporte se sugiere restringir la solicitud a una razonable generalización según lo que se encuentra debidamente soportado.

No se encuentra sustento para las reivindicaciones 3 y 4, puesto que estas se refieren a procesos de calentamiento de 60 a 70°C durante un periodo de 5 a 10 horas, y al calentamiento a una temperatura de 65 a 70°C en un periodo de tiempo de 9 a 8 horas. En la descripción se encuentra que el proceso de calentamiento se realiza 60 70°C durante un periodo de 9 a 10 horas, y con calentamiento a una temperatura de 65 a 70°C de 5 a 8 horas (Pág. 6, líneas 18 – 23, Fl. 39). No es claro si es un error de transcripción. Se sugiere realizar las aclaraciones necesarias.

Las reivindicaciones 7, 19 y 20 se refiere a una bacterina que tiene una a cinco especies de *Leptospira*, sin embargo, en el capítulo descriptivo no se encuentra

soporte de ninguna combinación de leptospiras. Se sugiere restringir la solicitud a una razonable generalización de lo que se encuentra divulgado, puesto que solo se encuentra soporte cuando la bacterina tiene una leptospira.

Las reivindicaciones 8, 9, 11 y 12 no son concisas porque nombran una emulsión definiéndola en términos de clase de mezcla, virus vivos y virus muertos, definiéndolos solo por su condición de viabilidad y no por un ingrediente como tal. Se sugiere restringir la solicitud a una razonable generalización definiendo los ingredientes exactos de la composición.

Las reivindicaciones 10 y 16 hacen referencia a Quil A, que es una marca registrada y por tanto no define al producto porque su composición puede cambiar con el paso del tiempo aunque conserve el mismo nombre. Se sugiere aclarar cuáles son las características del producto en cuestión.

En las reivindicaciones 10, 13, 14 y 16 se reclaman vacunas que contienen Quil A, colesterol, el virus de la rinotraqueitis bovina (IBR) y el virus de la diarrea viral bovina (BVD) de tipo 1 y el virus de la diarrea viral bovina (BDV) de tipo 2 de las cuales no se encuentra ningún soporte descriptivo de su obtención ni prueba, por lo cual se sugiere eliminarlas del pliego reivindicatorio.

En el método de la reivindicación 18 se nombran como etapas "medir la actividad lipasa de la bacterina", "medir la actividad lipasa de la bacterina después del tratamiento de calor", "comparar la actividad lipasa de la bacterina antes de calentar con la actividad lipasa después de calentar", y "seleccionar una bacterina tratada con calor, donde la actividad lipasa después del tratamiento con calor es 50% o menos de la actividad lipasa de la bacterina antes del tratamiento con calor", estas etapas corresponden a etapas de análisis, control o selección no se consideran parte del procedimiento que permite la obtención de un producto, por lo cual se sugiere eliminarlas.

La expresión "tiempo suficiente" de la reivindicación 21 es imprecisa por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto. Además, se reitera, que esta reivindicación de producto se encuentra definida en términos de su proceso de obtención, lo cual no es adecuado.

Descripción (Art 28 D 486)

Se solicita definir la marca Quil A (Pág. 8, líneas 2 y 3, Fl. 41).

Se requiere al Solicitante hacer las aclaraciones necesarias.

4. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 11 de septiembre de 2006 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	POPE, V., Vol. 25, No. 2, Journal of Clinical Microbiology, Págs. 255 – 258	Effect of Heat or Chemical Treatment on Leptospiral Antigens	Febrero/1987
D2	PATEL, V., Journal of Bacteriology, Vol. 88,	Characterization of Leptospiral Lipase	Octubre/1964

Ítem	Documento	Título	Fecha de Publicación
	No. 4, Pág. 877 - 887		
D3	WO 2004/093904	Methods for Preventing Cattle Diseases	4/Noviembre/2004

D1 divulga (Pág. 255, Fl. 74) el efecto del tratamiento químico o térmico en antígenos de *Leptospira interrogans* serotipos *icterohaemorrhagiae*, *canicola*, *grippotyphosa*, *hardjo* y *pomona*.

D2 divulga (Pág. 877, Fl. 76) la caracterización de lipasa de leptospira y el efecto de la temperatura en su actividad.

D3http://worldwide.espacenet.com/publicationDetails/originalDocument?FT=D&date=20041104&DB=EPODOC&locale=en_EP&CC=WO&NR=2004093904A2&KC=A2&ND=4 divulga (Pág. 19, ejemplo 1) una vacuna de bacterina de *leptospira canicola*, *grippotyphosa*, *hardjo*, *icterohemorrhagia* y *Pomona*.

5. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en una bacterina tratada con calor, que comprende una suspensión de bacterias muertas que tienen una actividad lipasa de 50% o menos con respecto a la actividad lipasa de la bacterina antes del tratamiento con calor.

La reivindicación 21 consiste e una bacterina tratada con calor preparada:

- a) Formando una bacterina que tiene actividad lipasa; y
- b) Calentando la bacterina a una temperatura de aproximadamente 35 a aproximadamente 80°C durante un periodo de tiempo suficiente para reducir la actividad lipasa a un nivel de 50% o menor con respecto al nivel antes del tratamiento de calor.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Bacterina tratada con calor a una temperatura entre aproximadamente 35 y aproximadamente 80°C	Bacterina tratada con calor a una temperatura entre 50 y 80°C	Bacterina sometida a calentamiento a 37 Y 56°C

Las características esenciales de la bacterina de las reivindicaciones 1 y 21 habían sido divulgadas previamente en D1 y D2. El documento D1 enseña bacterinas calentadas a temperaturas entre 50 y 80°C, con calentamientos a 50, 56 y 80°C (Pág. 255, Fl. 74). El documento D2 revela una bacterina sometida a calentamiento a 37 y 56°C (Págs. 877 – 879, Fls. 76 y 77).

El documento D1 también enseña que los procesos de calentamiento a 50 y 56°C se realizaron por 30 minutos (Pág. 255, Fl. 74), que la bacterina es de *Leptospira interrogans* serotipos *icterohaemorrhagiae*, *canicola*, *grippotyphosa*, *hardjo* y *pomona* (Pág. 255, Fl. 74) y que las leptospiras se utilizan en vacunas (Pág. 257, Fl. 75)

Adicionalmente, el documento D2 enseña que el calentamiento a 37°C se realizó por 6 horas y a 56°C por 30 minutos (Pág. 878 y 879, Fls. 76 y 77), la preparación de la lipasa de leptospira fue emulsionada en aceite de olivas y tributirin (Págs. 877 y 879, Fl. 76 y 77).

En consecuencia, las reivindicaciones 1 y 21 carecen de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2, 7 y 22 no mencionan alguna característica que haga nuevas a las bacterinas de las reivindicaciones 1 o 21, por lo cual se considera que no son nuevas.

La reivindicación 17 consiste en un método para preparar una bacterina tratada con calor, que comprende calentar una bacterina que tiene actividad lipasa a una temperatura de aproximadamente 35 a aproximadamente 80°C durante un periodo de aproximadamente 20 minutos a aproximadamente 24 horas.

La reivindicación 18 consiste en un método para preparar una bacterina tratada con calor, que comprende

- a) Medir la actividad lipasa de la bacterina;
- b) Calentar la bacterina a una temperatura de aproximadamente 35 a aproximadamente 80°C durante un periodo de aproximadamente 20 minutos a aproximadamente 24 horas;
- c) Medir la actividad lipasa de la bacterina después del tratamiento con calor;
- d) Comparar la actividad lipasa de la bacterina antes de calentar con la actividad lipasa después de calentar;
- e) Seleccionar una bacterina tratada con calor, donde la actividad lipasa después del tratamiento con calor es 50% o menos de la actividad lipasa de la bacterina antes del tratamiento con calor.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Metodo para preparar una bacterina tratada con calor que comprende:	Metodo para preparar una bacterina tratada con calor que comprende:	Metodo para preparar una bacterina tratada con calor que comprende:
Calentar una bacterina con actividad lipasa a una temperatura entre aproximadamente 35 y aproximadamente 80°C durante un periodo de tiempo de aproximadamente 20 minutos a aproximadamente 24 horas	Calentar una bacterina a 50°C por 30 minutos y a 56°C por 30 minutos	Someter a calentamiento una bacterina a 37°C por 6 horas y a 56°C por 30 minutos

Las características esenciales del método para prepara una bacterina tratada con calor de las reivindicaciones 17 y 18 habían sido divulgadas previamente en D1 y D2. El documento D1 enseña un método que comprende calentar una bacterina a 50°C por 30 minutos y a 56°C por 30 minutos (Pág. 255, Fl. 74). El documento D2 divulga un método en el que se somete una bacterina a calentamiento a 37°C por 6 horas a y 56°C por 30 minutos (Págs. 877 – 879, Fls. 76 y 77).

El documento D1 también enseña que la bacterina es de *Leptospira interrogans* serotipos *icterohaemorrhagiae*, *canicola*, *grippotyphosa*, *hardjo* y *pomona* (Pág. 255, Fl. 74).

En consecuencia, las reivindicaciones 17 y 18 no son nuevas, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 19 y 20 no mencionan alguna característica que haga nueva a la composición de las reivindicaciones 17 y 18, por lo cual se considera

que no son nuevas.

Nivel Inventivo (Art18 D486)

La reivindicación 3 consiste en una bacterina tratada con calor de acuerdo con la reivindicación 1, donde el tratamiento con calor comprende calentar una bacterina que tiene actividad lipasa a una temperatura de aproximadamente 60 a aproximadamente 70°C durante un periodo de aproximadamente 5 a aproximadamente 10 horas.

La reivindicación 4 consiste en una bacterina tratada con calor de acuerdo con la reivindicación 1, donde el tratamiento con calor comprende calentar una bacterina que tiene actividad lipasa a una temperatura de aproximadamente 65 a aproximadamente 70°C durante un periodo de aproximadamente 9 a aproximadamente 8 horas.

La reivindicación 5 consiste en una bacterina tratada con calor de acuerdo con la reivindicación 1, donde el tratamiento con calor comprende calentar una bacterina que tiene actividad lipasa a una temperatura de aproximadamente 65 a aproximadamente 70°C durante aproximadamente 1 hora.

La reivindicación 6 consiste en una bacterina tratada con calor de acuerdo con la reivindicación 1, donde el tratamiento con calor comprende calentar una bacterina que tiene actividad lipasa a una temperatura de aproximadamente 55 a aproximadamente 65°C durante un periodo de aproximadamente 5 a aproximadamente 8 horas.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D5
Bacterinas tratadas con calor donde el tratamiento con calor comprende: Calentar a aproximadamente 60 a aproximadamente 70°C durante aproximadamente 5 a aproximadamente 10 horas; a aproximadamente 65 a aproximadamente 70°C durante un periodo de aproximadamente 9 a aproximadamente 8 horas; a aproximadamente 65 a aproximadamente 70°C durante aproximadamente 1 hora; o a aproximadamente 55 a aproximadamente 65°C durante un periodo de aproximadamente 5 a aproximadamente 8 horas.	Bacterinas tratadas con calor: Bacterina tratada con calor a una temperatura entre 50 y 80°C	Bacterinas tratadas con calor: Bacterina sometida a calentamiento a 37 Y 56°C

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en las reivindicaciones 3 - 6 porque divulgan bacterinas tratadas con calor. El documento D1 una bacterina que se somete a calentamiento a 50 o 56°C por 30 minutos y a 80°C por 10 minutos (Pág. 255, Fl. 74) y el documento D2 divulga bacterinas que se calientan a 37°C se realizó por 6 horas y a 56°C por 30 minutos (Pág. 878 y 879, Fls. 76 y 77).

La diferencia entre la invención, definida en las reivindicaciones 3 - 6 y las bacterinas de los documentos D1 y D2 es las temperaturas y los tiempos a las que se realiza el proceso de calentamiento.

El efecto que logran las temperaturas y tiempos en el procedimiento de calentamiento es la obtención de bacterinas con actividad lipasa de 50% o menos con respecto a la actividad lipasa de la bacteria antes del tratamiento de calor.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar las bacterinas conocidas en el documento D1 para reducir su actividad lipasa al 50% o menos".

Sin embargo, el documento D2 ya divulgaba que la actividad lipasa se ve modificada por la temperatura (Págs. 881 – 883, Fls. 78 y 79).

En consecuencia, la persona del oficio normalmente versada en la materia, conociendo que la actividad lipasa se modifica por la temperatura como se divulga en el documento D2 mediante procesos rutinarios de calentamiento evaluaría cual es la temperatura a la cual la actividad lipasa se ve disminuida en un 50% para llegar así al objeto de la reivindicación 3 - 6 de la solicitud. Por lo cual se considera obvia.

Conclusión:
Las reivindicaciones 3 - 6 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

La reivindicación 8 consiste en: una vacuna que comprende una emulsión y una bacteria tratada con calor de acuerdo con la reivindicación 2.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D2	D3
Una vacuna en emulsión que comprende:	Bacteria	Vacuna en emulsión que comprende:
Una bacteria tratada con calor de aproximadamente 35 a aproximadamente 80°C durante 20 minutos a 24 horas	Bacteria tratada con calor a 37°C por 6 horas y a 56°C por 30 minutos	Una bacteria.

Los documentos D2 y D3 constituyen el estado de la técnica cercano a la invención porque dan a conocer bacterinas tratadas con calor y vacunas de bacterinas. El documento D2 divulga bacterinas tratadas con calor, con calentamiento a 37°C por 6 horas y a 56°C por 30 minutos (Pág. 878 y 879, Fls. 76 y 77). El documento D3 da a conocer una vacuna con emulsión que comprende una bacteria (Pág. 17, líneas4 – 10; Pág. 19, ejemplo 1).

El documento D3 también enseña que la vacuna puede incluir virus vivos o inactivados de virus sincitial respiratorio bovino (BRSV), virus de parainfluenza (PIV3), virus de la diarrea viral bovina (BVDV) tipos 1 y 2, y las bacterias *Leptospira canicola*, *Leptospira grippotyphosa*, *Leptospira borggetersenii hardjo-prajitno*, *Leptospira icterohaemorrhagiae*, *Leptospira orpetersenuu hardjo-bois* y *Leptospira interrogans pomona* (Pág. 14, línea 24 – Pág. 15, línea 13), y que la vacuna puede incluir adyuvantes como lecitina, Quil A y colesterol (Pág. 17, línea 4 – 10).

La bacteria del documento D2 difiere de la vacuna de la reivindicación 8 en que la bacteria se encuentra formulada en una vacuna con una emulsión.

El hecho de formular la baterina sometida a calentamiento en una vacuna con una emulsión es la obtención de una formulación estable.

Por tanto, el problema técnico objetivo consiste en "cómo realizar una formulación de la bacterina del documento D2 para obtener una vacuna estable".

Sin embargo, el documento D3 ya divulgaba vacunas de bacterinas en emulsión (Pág. 17, líneas 4 – 10; Pág. 19, ejemplo 1).

La reivindicación es obvia, porque la persona del oficio normalmente versada en la materia, obtendría una vacuna en emulsión como se da a conocer en el documento D3 de una bacterina que ha sido sometida a calentamiento disminuyendo su actividad lipasa como la divulgada en el documento D2 para obtener una vacuna estable, logrando de esta forma objeto de reivindicación 8.

Por lo cual la vacuna de la Reivindicación 8 carece de Nivel Inventivo.

Puesto que las reivindicaciones dependientes 9 – 16 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:

Las reivindicaciones 8 – 16 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

6. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	POPE, V., Vol. 25, No. 2, Journal of Clinical Microbiology, Págs. 255 – 258	1, 2, 17, 18, 21, 22	Novedad
		3 – 6,	Nivel Inventivo
D2	PATEL, V., Journal of Bacteriology, Vol. 88, No. 4, Pág. 877 - 887	1, 2, 17, 18, 21, 22	Novedad
		3 – 6, 8 - 16	Nivel Inventivo
D3	WO 2004/093904	8 - 16	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JESÚS FERNEL GARCÍA GARCÍA

Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

27 JUL 2012

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Revisado por: Consuelo Leguizamón *CL*

Effect of Heat or Chemical Treatment on Leptospiral Antigens

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The use of bacterins is one of the primary methods in the control of leptospirosis in domestic animals, especially cattle, swine, and dogs. Bacterins have been made using chemically treated or heat-inactivated organisms. Many of the initial studies indicated that the bacterins gave complete protection, whereas later reports found that some inactivation methods resulted in bacterins that protect against death but not against kidney infection and renal shedding. This raised the possibility that some of the antigens had been altered. To investigate this possibility, we heat or chemically treated *Leptospira interrogans* serovar *icterohaemorrhagiae* CF-1, *L. interrogans* serovar *canicola* Moulton, *L. interrogans* serovar *grippityphosa* SC4397, *L. interrogans* serovar *hardjo* Hardjoprajitno, and *L. interrogans* serovar *pomona* M.L.S. Temperatures used to determine alterations in antigens were 50, 56, 80, 100, and 121°C. The chemicals used were formaldehyde, phenol, and thimerosal. Analysis was done using Laemmli gels and Western blots. We found that heating at 50 or 56°C had the smallest effect on antigens, whereas heating at 80 or 100°C caused the appearance of high-molecular-weight bands on Western blots. Heating at 121°C caused almost all the bands to disappear, in both the stained gel and the Western blot. Both phenol and formalin treatment altered proteins, whereas thimerosal treatment appeared to have little effect compared with heating at 50°C.

Leptospirosis is a zoonotic disease spread by contact with urine from an infected animal or contaminated moist soil. The disease results in abortions and infertility in livestock; in dogs and humans, the disease is primarily a febrile one. Wild rodents serve as the primary reservoir, with some animals remaining infective as renal shedders for life (10). The elimination of these animals is not feasible in the control of leptospirosis; therefore, the most practical approach has been to vaccinate susceptible domestic animals. Since 1950, leptospiruses for bacterins have been treated with phenol (2), thimerosal (11), formalin (1, 2, 9), or heat ranging from 50 to 121°C (2, 14). Live avirulent strains of leptospires have been used with moderate success (2, 5, 13) but have not been used routinely because of the possibility of reversion to virulence. Regardless of the bacterin used, all have been successful in preventing death, but efficacy in preventing renal infection and its subsequent renal shedder state has been variable (3). This strongly suggests alterations of some of the immunologically important proteins. To determine what changes occur, we treated various serovars of *Leptospira interrogans* with thimerosal, formalin, or phenol or temperatures ranging from 50 to 121°C and analyzed the proteins by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12) and Western blot (17).

MATERIALS AND METHODS

Leptospira organisms. *L. interrogans* serovar *canicola* Moulton, *L. interrogans* serovar *icterohaemorrhagiae* CF-1, *L. interrogans* serovar *grippityphosa* SC4397, *L. interrogans* serovar *hardjo* Hardjoprajitno, and *L. interrogans* serovar *pomona* M.L.S. were used. Laboratory-adapted cultures were grown in Tween 80-albumin (T80-Alb; 16); supplemented medium. When each culture reached the log phase of growth, 5×10^8 organisms, as determined by nephelometer readings (4), were put into each of nine

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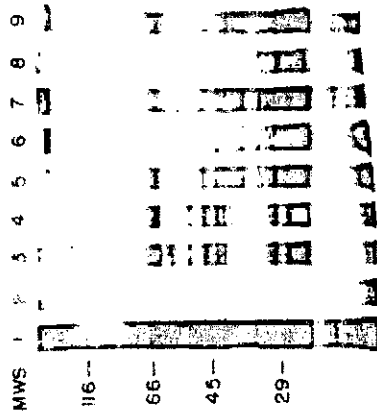


FIG. 1. Silver stain of heat- and chemical-treated *L. interrogans* serovar *Hardjoprajitno*. For each treatment, 5×10^8 cells were washed and then treated as follows. They were boiled for 5 min in SDS-PAGE sample buffer (lane 1), heated at 121°C in PBS for 10 min (lane 2), heated at 100°C in PBS for 10 min (lane 3), heated at 80°C for 10 min (lane 4), heated at 56°C for 10 min (lane 5), heated at 50°C for 10 min (lane 6), treated with phenol for 60 min (lane 7), treated with formalin for 60 min (lane 8), and treated with thimerosal for 60 min (lane 9). MWS, Molecular weight standards (10).

fixed in 50% methanol-10% acetic acid for 30 min and then in 5% methanol-7% acetic acid for approximately 18 h. The fixing solution was replaced with 10% glutaraldehyde for 30 min. Next, the gel was washed with 20% methanol for a minimum of 90 min with three changes of 30 min each. It was then silver-stained for 10 min with a solution of 2 ml of 20% AgNO_3 -10.5 ml of 0.36% NaOH -0.7 ml of NH_4OH -36.8 ml of deionized water. The stain was discarded, and the gel was rinsed with two quick rinses of deionized water and then developed in 100 ml of 0.005% citric acid-50 μl of 37% formaldehyde until the bands were distinct and the background relatively clear. It was then rinsed in several changes of deionized water and photographed on a light box.

Western blot. Electrophoretic transfer of proteins to nitrocellulose was based on the procedure of Towbin et al. (17), with blotting overnight at 100 mA in pH 8.3 Tris glycine buffer-20% methanol. Nonspecific-binding sites were blocked with 3% gelatin-Tris-buffered saline (TBS), with incubation for 45 min. Next was a 45-min incubation with 0.5% Tween 20-TBS (TBS-T20). The antileptospiral antibody was diluted 1:10 in TBS-T20 and incubated for 90 min. The blots were then washed twice, 5 min each, with TBS-T20. The anti-rabbit antibody conjugated to alkaline phosphatase (Cooper Biomedical, Inc., West Chester, Pa.) was diluted according to manufacturer directions in TBS-T20 and incubated for 90 min. This was followed by two 5-min washes with TBS-T20 and one wash with TBS. The substrate reaction mixture consisted of 40 μl of 2 M MgCl_2 , 2 ml of 0.1% Nitro Blue Tetrazolium in pyrogen-free distilled water, 2 mg of 5-bromo-4-chloro-3-indolyl phosphate in 400 μl of dimethylformamide, and 18 ml of 0.15 M sodium barbitol buffer. After blotting, the molecular-weight-standard lanes were stained with 0.1% India ink (8).

Antiserum preparation. Log-phase leptospiral organisms

RESULTS

The washed cells of all five *L. interrogans* serovars tested were either heat or chemically treated and assayed for changes in their protein profiles. Cells of each serovar were run on a 10.0% Laemmli gel and silver stained. A duplicate gel for each group was also run and transblotted to nitrocellulose sheets (0.45- μm pore size). Because the results with the five serovars were similar, *L. interrogans* serovar *hardjo* was arbitrarily chosen as representative of the group. The silver stain of the prewashed heat- and chemically treated *L. interrogans* serovar *hardjo* cells is shown in Fig. 1. Autoclaving (121°C) distinctly altered most of the proteins. Treatment of cells at 100°C appeared to cause major changes of the protein profile in the 90- to 100-kilodalton (kDa) range. Formalin treatment resulted in the loss of many of the protein bands and alterations in many of the remaining bands. Proteins of the thimerosal-treated cells had a pattern similar to that of the *L. interrogans* serovar *hardjo* cells treated at 50°C. The Western blot (Fig. 2) shows more dramatically the effect of heat on the antigens. Even heating at 56°C eliminated most antibody interactions with high-molecular-mass proteins. Phenol eliminated reactions with proteins above 90 kDa and greatly reduced the reactions

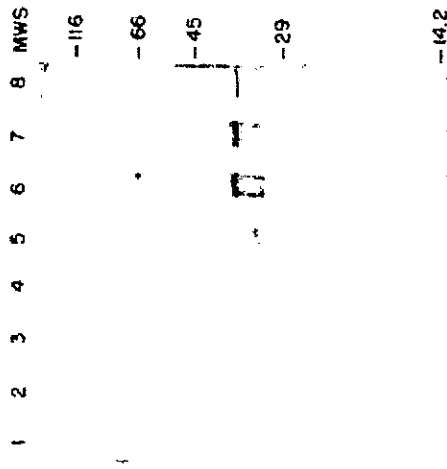


FIG. 2. Western blot of gel shown in Fig. 1. Rabbit anti-*L. interrogans* serovar *hardjo* antiserum and goat anti-rabbit immunoglobulin G (heavy and light chain) conjugated to alkaline phosphatase were used. See legend to Fig. 1 for description of lanes. MWS, Molecular weight standards (10).

with the proteins of about 20 kDa. There was an increase in the molecular mass of proteins that reacted with the anti-*L. interrogans* serovar *hardjo* antiserum in the Formalin-treated organisms. The banding patterns of thimerosal- and heat (50°C)-treated cells were very similar (Fig. 3), even when they were treated and then washed.

All sera were tested in the MAT against their native homologous antigens. Heat- and chemically treated *L. interrogans* serovar *hardjo* was also tested in the MAT. When live, unwashed, untreated antigens were used, the homologous *L. interrogans* serovar *hardjo* titer was 1:25,600. Leptospirae that had only been washed reacted at a titer of 1:6,400. Heating destroyed some of the reactivity, with decreases in titer corresponding to the increases in temperature. When heated at 100 or 121°C, there was no reactivity at the 1:1,000 dilution; at 80°C, the titer was 1:800; at 56°C, it was 1:3,200; and at 50°C, it was 1:6,400. There was no reactivity at the 1:100 dilution of the phenol-treated cells, whereas Formalin or thimerosal treatment resulted in a titer of 1:1,600. Agglutination was loose and lacy instead of compact when the leptospirae were treated with Formalin or thimerosal or with heat of 80°C.

DISCUSSION

It is known that heat or chemical treatment of leptospirae changes their effectiveness as vaccines. An ultrastructure study done in 1965 (18) demonstrated that phenol, Formalin, and thimerosal remove the outer envelope and damage the protoplasmic cylinder. Phenol also results in loose and broken periplasmic flagella. It is known that Formalin-treated antigens have lower sensitivity and greater cross-reactivity in the MAT (16). The effect of other treatments has not been tested in the MAT, and none of these has been previously analyzed using PAGE or Western blotting.

Temperatures in excess of 56°C alter some of the antigens, as observed by SDS-PAGE with silver stain. Treating the leptospirae before washing (data not shown) appeared to preserve some of the proteins, because of the presence of bovine serum albumin from the medium, but it also became very difficult to wash the bovine serum albumin off the spirochetes, especially if the preparation was boiled first. Heating at 50°C appeared to be the least disruptive to the integrity of the antigen. Even an increase to 56°C resulted in a loss of some high-molecular-weight molecules.

The effects of the three chemicals on the leptospiral antigens were quite different. With phenol treatment, the high-molecular-mass antigens were altered. Formalin altered the antigens between 29 and 45 kDa, which may account for the lack of sensitivity and specificity in the slide MAT when formalinized cells are used. Thimerosal appeared to have the smallest effect on the antigens, both in their appearance in a silver stain and by Western blot technique. Not washing the cells before treatment seemed to have little effect. This is worth noting, because most bacterins are made with thimerosal-treated, nonwashed serovars grown in T80-Alb medium.

One vaccination study by Borge-Petersen (3) used *L. interrogans* serovar *icterohaemorrhagiae* treated with Formalin or heated, at 56 or 70°C, or washed and then heated at 70°C. All vaccines were effective in preventing death with an immunizing dose of 10⁶ organisms. No reference was made regarding the prevention of renal infection. With a dose of 10⁷ organisms, effectiveness fell with the heat-treated antigens only. Both the vaccines treated at 70°C were less efficient in preventing death than the vaccine treated at 56°C.

This phenomenon might be partially explained by the results we obtained on both SDS-PAGE gels and Western blots with the heat-treated leptospirae. Our results with formalinized antigen indicate that the antigens responsible for most of the protective immune response may be larger than 45 kDa or smaller than 29 kDa.

The data presented here, in conjunction with various vaccine studies, point out the need to use the least disruptive methods to inactivate leptospirae for use as bacterins. We do not know what effect the altered antigen has on the antibodies produced and their ability to prevent renal infection. Of the methods used here, heating at 50°C and inactivation by thimerosal appeared to alter the antigens the least. The use of a protein-free medium might be helpful to eliminate any possible masking effects of the leptospiral antigens by adherence of bovine serum albumin to the spirochete, because it is almost impossible to remove all of the albumin.

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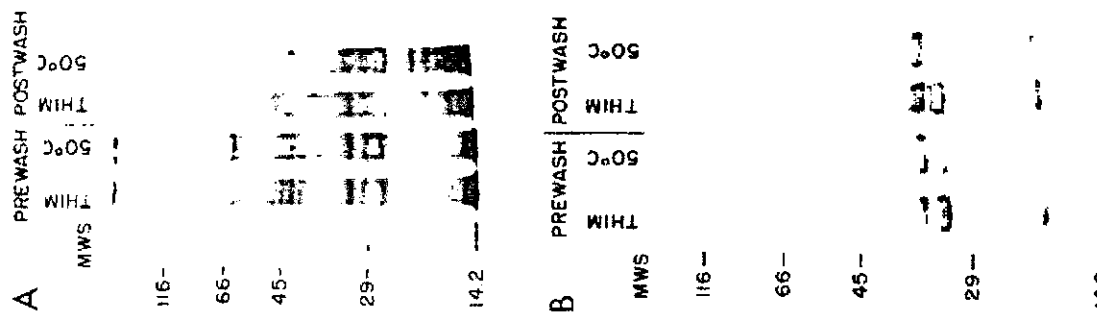


FIG. 3. *L. interrogans* serovar *hardjo* treated with thimerosal or at 50°C either after or before washing. Panels A and B were run on the same gel. The gel was then halved. The gel in panel A was silver stained, and the gel in panel B was Western blotted. Treatment was as follows: thimerosal for 60 min after washing (lane 1), heating at 50°C for 30 min after washing (lane 2), thimerosal for 60 min before washing (lane 3), and heating at 50°C for 30 min before washing (lane 4). MWS, Molecular weight standards (10³).

CHARACTERIZATION OF LEPTOSPIRAL LIPASE

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ABSTRACT

PATEL, VIRENDRA (University of Missouri, Columbia), HERBERT S. GOLDBERG, AND DONALD BLENDEN. Characterization of leptospiral lipase. *J. Bacteriol.* 88:877-884, 1964.—A technique for leptospiral lipase extraction which yielded a highly active, stable, and concentrated lipase preparation was developed. The chief characteristics of leptospiral lipase were determined and are summarized below. Leptospiral lipase was soluble in water and stable in both the dry state and in aqueous solution. Tributyrin was found to be the substrate upon which the enzyme was most active. With this substrate, leptospiral lipase was found to display optimal activity at pH 7 and at 30 C. The Michaelis constant of leptospiral lipase with tributyrin substrate was determined to be 4.76×10^{-4} M. The enzyme was not inhibited by low concentrations of mercury, iron, cobalt, or copper or by —SH blocking agents. Bile and calcium chloride in low concentrations were able to increase lipase activity at alkaline pH. The isoelectric point of leptospiral lipase was determined to be in the range of pH 5.2 to 5.4.

Recent investigations have established the presence of several enzyme systems in leptospires. Among these have been catalase (Faine, 1960), oxidase (Faine, 1959; Goldberg and Armstrong, 1959), transaminase (Markovetz and Larson, 1959), and lipase. Leptospiral lipase has aroused particular interest since its demonstration by several European workers (Kemenes and Lovrovich, 1959; Parnas, Koslak, and Kruska, 1960; Bertok and Kemenes, 1960).

Kemenes and Lovrovich (1959) were the first to study extensively the "fat-splitting" ability of various leptospiral cultures. These authors were able to show significant differences in extracellular lipase activity among 34 different strains of leptospires. An even more detailed study of leptospiral lipase activity was undertaken by Bertok and Kemenes (1960). They used a titrimetric method to assay lipase activity of whole cultures,

and found maximal activity against tributyrin substrate. Lipase activity of whole cultures was reported to increase in proportion to the growth of the organism and was associated with the growth curve.

None of the studies to date has attempted an isolation, nor have the physical and chemical characteristics of leptospiral lipase been established. This investigation, therefore, is concerned with attempts to concentrate leptospiral lipase and study its properties.

MATERIALS AND METHODS

Cultures. Fourteen strains of leptospires were used in this study. They included *Leptospira* *an-* *damum*, *L. manicula* Moulton, *L. canicola* Ruebush, *L. icterohaemorrhagiae* 351, *L. icterohaemorrhagiae* AB, *L. pomona* S-91, *L. pomona* IC-12, *L. alberti*, *L. autumnalis*, *L. biflexa* (all originally obtained from Walter Reed Army Institute of Research, Washington, D.C.), *L. biflexa* Patoc, *L. biflexa* Waz. P 438, *L. biflexa* Sao Paolo (from the Communicable Disease Center, Atlanta, Ga.), and *L. biflexa* L.S.U. (from Louisiana State University, New Orleans). They were all grown in Cox's (1955) liquid medium with 10% rabbit serum at 30 C for various incubation times as indicated.

Lipase activity. Lipase activity was assayed by titrating the free fatty acids liberated on incubation of the enzyme with various triglycerides or olive oil. Samples (1 ml) of leptospiral cultures were tested daily for 35 days for lipase activity. The procedure for the assay of lipase activity was as follows.

Two tests were run simultaneously, one for the leptospiral culture, and a second which consisted of Cox's medium with an equivalent amount of serum to act as a control. Into each of two Stender dishes were pipetted 2.5 ml of distilled water, 3.0 ml of emulsified olive oil lipase substrate (Sigma Chemical Co., St. Louis, Mo.), and 2.0 ml of tris(hydroxymethyl)aminomethane (tris) buffer at pH 8 (Gomori, 1946). The source of enzyme

was either the whole culture or the concentrated lipase. To one Stender dish, 1 ml of leptospiral culture was added. This dish was marked "test" and the other dish marked "blank." Both of these dishes were then incubated in a shaking water bath at 37 C for 6 hr. Immediately after starting incubation, 1 ml of leptospira culture was pipetted into a test tube marked "blank" and stored at 4 C. After 6 hr of incubation, the contents of the "blank" test tube were poured into the "blank" Stender dish. A 3 ml amount of 95% ethyl alcohol was added to each of the dishes to inactivate the lipase. The enzymatic solutions were titrated by use of freshly prepared 0.05 N NaOH to an end point of pH 10. The amount of NaOH in milliliters consumed by the "test" and the "blank" incubations were recorded. The "blank" NaOH reading was subtracted from the "test" reading. This value is expressed as the number of milliliters of NaOH required to neutralize the free fatty acids released owing to lipolysis.

All titrations were carried out with a Fisher Automatic Titrimeter (Model 36; Fisher Scientific Co., Pittsburgh, Pa.) equipped with a burette graduated to 0.02 ml.

Substrates. In addition to olive oil, the following ten saturated and unsaturated triglycerides were used as substrates in this investigation: triacetin, tripropionin, tributyrin, tricaproin, tricaprylin, tripalmitin, tristearin, and triolein. In addition, two esters, ethyl oleate and methyl linolenic acid, were also tested as substrates.

The method for the preparation and emulsification of substrates was as follows: A sterile 10% bile solution was made up in distilled water. Oxgall (Difco) was used as the source of bile. To the cooled solution were added 10 ml of glycerine for every 1 g of bile in the solution. To the bile-glycerol mixture was added enough substrate to make up a 1 M stock substrate mixture. The substrate was emulsified in a Sorvall high-speed omnimixer for 45 min with the rheostat setting at 35. This resulted in a finely dispersed substrate mixture which was diluted to the required concentration with tris buffer (pH 8) and distilled water. Although the substrate preparation could be safely stored at 3 C for 1 week, it had to be emulsified each day prior to use.

Concentration of leptospiral lipase. A modification of the procedure of Fiore and Nord (1950) for the extraction of lipase from *Parasium* *lim-*

was successfully carried out for leptospiral lipase. The procedure for the extraction and concentration of leptospiral lipase from cell free culture is described below.

Scheme 1. *L. pomona* LC 12 was grown for 24 days in Cox's medium, and was then centrifuged at $(10,000 \times g)$ for 60 min at 3 C (first centrifugation). The sediment was discarded. The cell-free supernatant fluid was assayed for lipase activity by use of tributyrin and olive oil substrates.

The supernatant fluid was cooled to 0 C, and 1 N acetic acid at 0 C was added with stirring until pH 5 was reached. The supernatant fluid was centrifuged at $2,000 \times g$ for 25 min at 2 C (second centrifugation). Both the supernatant fluid and the sediment were assayed for lipase activity against tributyrin and olive oil. The sediment, consisting largely of contaminating proteins, was discarded after assay.

The supernatant fluid was cooled to 0 C. It was extracted by adding slowly, with stirring, to three times the volume of 95% ethyl alcohol precooled to -50 C. The temperature of the mixture should not be allowed to rise above -10 C. It was centrifuged immediately at $2,000 \times g$ at 3 C (third centrifugation). The supernatant fluid was discarded.

The sediment was desiccated with acetone at -15 C and stirred gently. It was centrifuged for 20 min at $2,000 \times g$ at 2 C (fourth centrifugation), and the supernatant fluid was discarded. The sediment was placed in a vacuum desiccator over concentrated sulfuric acid at -15 C for 48 hr. The brown dried product was called the dry alcohol precipitate.

Scheme 2. A 2-g amount of the powdered scheme 1 product was dissolved in 100 ml of triple-distilled water at pH 7. The mixture was blended at 3 C until the dry alcohol precipitate was completely dissolved. The solution, cooled to 0 C, was rapidly strained through a double layer of muslin. The filtered solution was dialyzed against 4 liters of triple-distilled water at 3 C for 24 hr. The water was changed five times. It was very important that the water be completely free from metallic ions; otherwise, the preparation was inactivated. The dialyzed solution was extracted again with 3 volumes of 95% ethyl alcohol at -50 C, and the temperature was not allowed to rise above -15 C. The alcoholic solution was centrifuged at once for 20 min, and the

supernatant fluid was discarded. The sediment was lyophilized in a VirTis Freeze Dryer for 6 hr to yield a brown, active leptospiral lipase preparation.

This lyophilized scheme 2 preparation represents the concentrated leptospiral lipase used throughout this study. All fractions obtained in scheme 2 were assayed for lipase activity by use of tributyrin and olive oil substrates. To follow the results of the concentration schemes quantitatively, the protein nitrogen content of scheme 1 and scheme 2 preparations was determined by the method of Lowry et al. (1951). A diagrammatic representation of the scheme 1 and scheme 2 extraction procedures is given in Fig. 1.

Stability. A 20-mg amount of lipase was dissolved in 10 ml of tris buffer (pH 8) and subjected to various treatments, including boiling, from which the K_m was determined. A reciprocal

plot of velocity against substrate concentration was also used to determine the K_m .

Optimal pH. Two different buffers were used in the study of optimal pH. Sorensen's phosphate (pH 5.1 to 8.5; Koch and Hanke, 1948) and Mollvaline's (1921; pH 2.6 to 8.5). Each test solution contained 20 mg of lipase, tributyrin substrate (0.166 M, final concentration), and buffer at the required pH. A control was included for each test sample in which the enzyme was omitted. All samples were incubated in a water bath with shaking at 37 C for 2 hr, at the end of which 3 ml of 95% ethyl alcohol were added to each sample. The lipase activity at various pH levels was assayed ultravioletally by the method described earlier.

For comparative purposes, the optimal pH of a microbial fungal lipase (Mann Research Laboratories, New York, N.Y.) was similarly determined.

Optimal temperature. Leptospiral lipase was incubated at various temperatures from 3 to 57 C for 2 hr, and was assayed for activity. Each sample consisted of 20 mg of lipase preparation, 10 ml of Mollvaline buffer (pH 7), and emulsified tributyrin substrate (0.166 M, final concentration).

The optimal temperature of the microbial fungal lipase was determined by the same method, except that the pH of Mollvaline's buffer was 5.6. **Inhibitors and activators.** A series of experiments were performed to determine the effects of various compounds on the rate of activity of leptospiral lipase. Compounds which were investigated included metals and trace elements (mer-

cure chloride, copper sulfate, ferric sulfate, and cobalt chloride), and -SH inactivating compounds (*p*-chloromercuribenzoic acid, iodoacetic acid, and potassium ferrioxalate). Calcium chloride and bile as potential activators were also tested.

Inhibition or activation was calculated after determination of the rate of hydrolysis of tributyrin in the presence and absence of the various substances. Incubation was at 30 C, and these mixtures were buffered at pH 8 with tris buffer. Whenever necessary, the solutions of the different compounds were adjusted to the proper pH before addition to the enzyme. Prior to their addition to the other components of the digestion mixture, 1.5 ml of the compound under investigation were added to 20 mg of leptospiral lipase dissolved in 10 ml of tris buffer (pH 8), and allowed to remain in contact at 30 C for varying periods of time.

Each of the reaction vessels contained the following substances: 20 mg of lipase dissolved in 10 ml of tris buffer (pH 8) plus 1.5 ml of the compound under investigation and 2 ml of 1 M emulsified tributyrin (final concentration, 0.1484 M). A control was included for each test reaction. Bile was omitted in the emulsification of the substrate in the experiments on the effect of bile.

All the reaction vessels were incubated in a shaking water bath at 30 C, and the procedure for determining the rate of hydrolysis was identical with that previously described.

Results

Lipase activity of whole cultures. The lipase activities of 14 different strains of pathogenic and saprophytic leptospires are illustrated in Fig. 2. Lipase production of five saprophytic strains (*L. biflexa*) was generally less than that of pathogenic strains. It should be noted that *L. pomona* 1C-12 had the greatest lipase activity.

Results of comparative studies on lipase activities of virulent and avirulent strains of *L. icterohaemorrhagiae*, *L. canicola*, and *L. pomona* are also shown in Fig. 2. In each case, the virulent strain had greater lipase activity than the comparable avirulent strain.

Substrates. Since maximal lipase activity was exhibited by *L. pomona* 1C-12, all substrates were tested with the lipase of this serotype. Among the triglycerides tested, whole-culture lipase was most active against tributyrin (Table

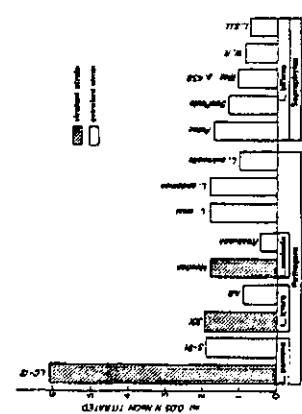
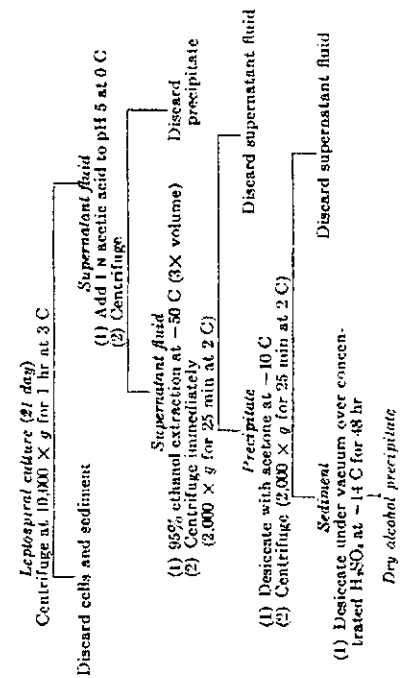


FIG. 2. Comparison of lipase activity in various strains of Leptospira.

SCHEME 1



SCHEME 2

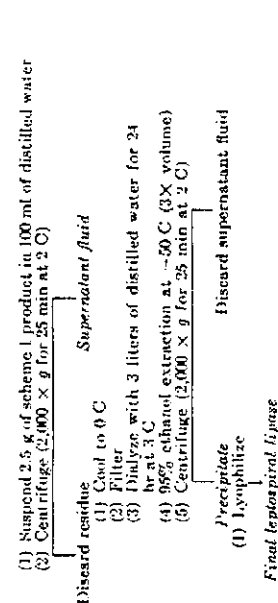


FIG. 1. Extraction and concentration of leptospiral lipase preparation.

TABLE 2. *Leptospiral* lipase (concentrated) activity with various substrates

Substrate (final concn 0.016 M)	Lipase activity*	Per cent of tributyrin activity
Triacetin	0.26	4.9
Tripropionin	2.45	46.7
Tributyryl	5.26	100.0
Triacrylyl	2.80	53.2
Trioleonin	2.80	53.2
Tripalmitin	1.43	27.0
Tristearin	1.21	23.0
Trilaidin	1.03	19.5
Linoleic acid, methyl ester	0.49	9.3
Methyl oleate	0.17	3.2

* Expressed as milliliters of 0.05 N NaOH required to neutralize the free fatty acids hydrolyzed by 20 mg of *leptospiral* lipase. Incubation, 6 hr at 30 C.

TABLE 3. Stability of *leptospiral* lipase at various temperatures

Material tested	Temp and duration	Per cent activity of normal rate*
Aqueous solution (20 mg)	3 C for 24 hr	90
	25 C for 24 hr	88
	56 C for 30 min	88
	100 C momentarily	0
Dry powder (20 mg)	0 C for 12 months	100
	-15 C for 12 months	100

* Standard control, untreated.

plotted against their respective substrate concentration (Fig. 4). The Michaelis constant of *leptospiral* lipase was calculated both from the reciprocal plot and from Fig. 4, and was found to be 4.76×10^{-2} M.

Optimal pH. The optimal pH of *leptospiral* lipase was determined with two buffer systems. *Leptospiral* lipase exhibited greatest activity at pH 7 with both Sorensen's phosphate buffer (Table 4) and with Mcllvaine's buffer (Fig. 5). **Optimal temperature.** Figure 6 shows the optimal temperature for *leptospiral* lipase. *Leptospiral* lipase was investigated at its optimal pH (7.0) with Mcllvaine's buffer, and optimal temperature was determined to be 30 C.

Inhibitors and activators. A large variety of substances have been reported to inhibit or activate

1). The activity of *leptospiral* lipase was found to decrease as the carbon chain lengths of the substrates increased.

In addition to testing substrate specificity of whole-culture enzyme, several additional triglycerides and their esters were tested to determine the specificity of concentrated *leptospiral* lipase. The results indicated that concentrated lipase was most active against tributyrin, and that lipase activity decreased as the carbon chain lengths increased (Table 2). The rate of hydrolysis of simple esters was very low.

Concentration of *leptospiral* lipase. Relatively good yields of *leptospiral* lipase were obtained by the extraction procedure described in Fig. 1. The yield of scheme 2 preparation was 4.6 g per 1,000 ml of starting material. The protein nitrogen content of scheme 2 final product was determined to be 68% with an activity of 5 units per 20 mg of protein, whereas scheme 1 product revealed 80% protein with an activity of 3 units per 20 mg of protein. The unit represents 1 ml of 0.05 N NaOH needed to neutralize free fatty acid in the method described. Each of these activities represents an average of five different sample preparations.

Stability. *Leptospiral* lipase was found to lose little activity at 3 and 25 C when stored for 24 hr, but was completely destroyed by boiling. Lipase was stable when stored in the dry form at 0 or -15 C for 12 months (Table 3).

Michaelis constant. The rate of lipase activity of whole-culture lipase and concentrated *leptospiral* lipase was determined; it was found that both exhibited a zero-order reaction rate (Fig. 3). The initial velocity of various substrate (tributyrin) concentrations was ascertained and

TABLE 1. *Leptospiral* lipase (whole culture) activity of *Leptospira pomona* LC-12 against triglycerides

Substrate (0.0065 M)	Lipase activity*	Per cent of tributyrin
Tributyryl	2.32	100
Triacrylyl	1.45	62
Tripalmitin	0.98	42
Tristearin	0.66	27

* Expressed as milliliters of 0.05 N NaOH required to neutralize the free fatty acids hydrolyzed by 20 mg of *leptospiral* lipase. Incubation, 6 hr at 30 C.

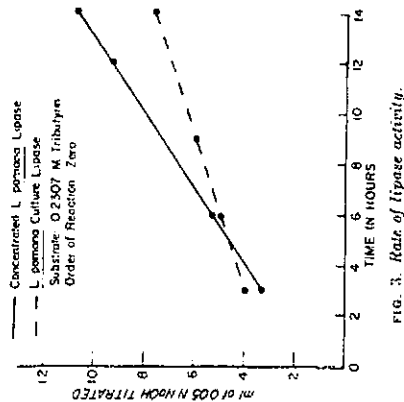


FIG. 3. Rate of lipase activity.

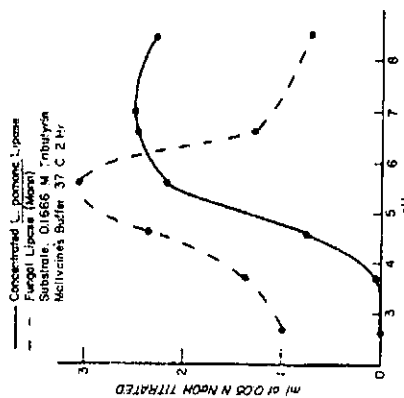


FIG. 5. Optimal pH of lipase.

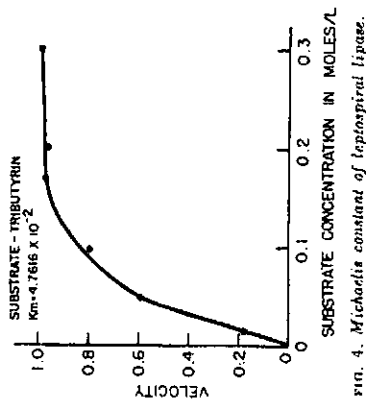


FIG. 4. Michaelis constant of *leptospiral* lipase.

TABLE 4. Optimal pH determination in Sorensen's phosphate buffer

pH	Leptospiral lipase activity	Microbial lipase activity
5.1	2.52	1.46
5.6	2.61	1.68
6.0	2.76	1.17
7.0	3.86	0.84
7.95	3.24	0.73
8.5	2.90	0.50

* Expressed as milliliters of 0.05 N NaOH required to titrate the free fatty acids liberated due to hydrolysis by 20 mg of lipase. Substrate, 0.166 M tributyrin; incubation, 2 hr at 37 C.

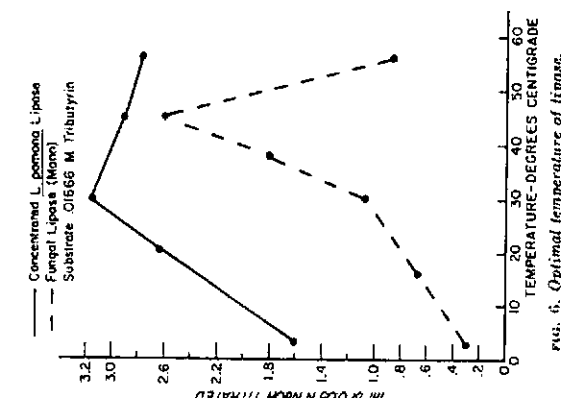


FIG. 6. Optimal temperature of lipase.

lipases. The effect of some of these substances on *leptospiral* lipase was investigated, with the following results. The effect of metallic compounds was dependent upon the specific metal and its concentration. In general, high concentrations (10^{-3} M) of mercury, iron, and cobalt were inhibitory. Increased activity usually resulted with

these metals at concentrations of 10^{-4} or 10^{-6} M. In these studies, copper exhibited little influence on lipase activity (Table 5). It is apparent from Table 6 that none of the three -SH inactivating agents that were studied (*p*-chloromercuribenzoic acid, iodoacetic acid, and potassium ferricyanide) influenced the activity of leptospiral lipase. Both calcium chloride and bile, when added to the substrate, resulted in increased leptospiral lipase activity.

DISCUSSION

Extreme variation in lipase production among different serotypes of *Leptospira* as reported by Parnas et al. (1980) and Bertok and Kenes (1960) was confirmed in this study. Furthermore, five saprophytic leptospires (*L. biflexa*) included in this work did not have as great lipase activity as the three saprophytic strains tested by Bertok and Kenes (1960). It seems unlikely, therefore, that this criterion could be used either to differentiate or to classify leptospires, as has been suggested. Although the results herein reported indicate that virulent strains have greater lipase activity than avirulent strains of the same serotype, only three pairs of serotypes were included. Many more of the 80 known serotypes would have to be tested to validate a relationship between lipase and virulence.

The concentration of an extracellular leptospiral lipase was carried out for the first time in this study. Although high yields of leptospiral lipase were obtained by the method described, a completely purified leptospiral lipase preparation is not yet a reality. Attempts are underway for further purification by zone electrophoresis, column chromatography, and gradient-density centrifugations.

TABLE 5. Effects of metallic compounds on leptospiral lipase after preliminary contact of 16 min

Final concn	Per cent activity of normal rate*			
	HgCl ₂	CuSO ₄	Fe ₂ (SO ₄) ₃	CoCl ₂
10 ⁻³ × 1.1	18	97	ppt.	04
10 ⁻⁴ × 1.1	69	110	122	121
10 ⁻⁵ × 1.1	100	104	134	124
10 ⁻⁶ × 1.1	100	98	145	135

* Standard control without metals.

TABLE 6. Effect of -SH inactivating reagents on leptospiral lipase

Reagent	Final concn	Per cent activity of normal rate*
<i>p</i> -Chloromercuri-benzoic acid	1.11 × 10 ⁻⁴	99
Iodoacetic acid	1.11 × 10 ⁻⁴	100
Potassium ferri-cyanide	1.11 × 10 ⁻³	100

* Standard control without -SH reagents.

A variety of triglycerides and esters were found to be hydrolyzed by leptospiral lipase. Since leptospiral lipase (both the purified preparation and in whole cultures) exhibited greatest activity against tributyrin, it is tempting to classify leptospiral lipase as a tributyrinase (Bertok and Kenes, 1960).

Unlike pancreatic and wheat germ lipases, leptospiral lipase was found to be heat-stable (56 C for 30 min) both in solution and in dry form. However, the optimal pH for leptospiral lipase lies in the range of pH 7.0 to 8.0, and is thus closer to pancreatic lipase (7.0 to 9.0) in this characteristic. The optimal pH of microbial fungal lipase was found to be 5.6.

It should be noted that the optimal temperature of 30 C for leptospiral lipase is similar to the optimal temperature for leptospiral growth in vitro. The optimal temperatures of most animal, plant, and microbial lipases are in the range of 37 to 40 C.

Leptospiral lipase, unlike pancreatic lipase, was not inhibited by low concentrations of mercury, copper, cobalt, or iron. Much higher concentrations were required to inhibit leptospiral lipase. Furthermore, leptospiral lipase was not affected by -SH blocking agents, and in this property is similar to the fungal lipase (Flora and Nord, 1950). Since only three -SH inhibitory compounds were included in this study, other compounds would have to be tested to determine conclusively the action of -SH blocking agents on leptospiral lipase.

Further characterization of leptospiral lipase indicates that this enzyme appears to have some common properties with lipases of animal, plant, and fungal origin. These include substrate speci-

ficity, method of extraction, reaction rate, optimal pH, and response to activators. As indicated above, differences have been observed in temperature optima, response to metals and -SH inhibitors, and heat stability.

Although the pathogenic manifestations of leptospirosis are well recognized today, very little is known about the virulence factors of leptospires. The significance of leptospiral lipase as a virulence mechanism is unknown and can only be postulated. Since fatty acids are the chief source of energy for leptospires and since extensive fatty degeneration of the liver is found in advanced leptospiral infections, it is quite conceivable that lipase plays a role in virulence. Further evidence not out of harmony with this theory is the fact that satisfactory conditions for optimal lipase activity are found in the mammalian hosts of this parasite. Nevertheless, further work is required to establish conclusively the role of lipase in leptospiral infections.

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