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Antileptospiral Agglutinins Produced in Rabbits *

S. GRAVES[1] & S. FAINE[2]

Agglutinating antibodies produced in an adult rabbit in response to intravenous injections of live Leptospira *sp. (serotype* biflexa, *strain Waz), consisted of a 19S component (present at day 6 after inoculation) and a 7S component (present at day 6 after inoculation). Both 19S and 7S antibodies persisted in the serum until the conclusion of the observations (day 216). The 19S antibody was completely destroyed by 2-mercaptoethanol but the 7S antibody was only partially sensitive to 2-mercaptoethanol.*

The serum proteins present in the 19S and 7S serum fractions were determined by immunoelectrophoresis and cellulose acetate electrophoresis. Elution of the serum components from cellulose acetate membranes directly confirmed that the β-migrating IgM was the 19S serum component with leptospiral agglutinating activity and that γ-migrating IgG was the 7S serum component with antileptospiral agglutinating activity.

Antibodies against leptospires have been described as mainly 19S. Hartmann et al. (1964) found the antibodies in a patient at an undefined stage in infection to be 19S, while Pike et al. (1965) and Hocker & Bauer (1965) found persistent 19S, with little 7S, in experimentally inoculated rabbits, although some serotypes also induced 7S antibodies. There is evidence that such chemically different antigens as proteins (bacterial flagella) and polysaccharides each give rise predominantly to antibodies of different and characteristic immunoglobulin classes in hyperimmune rabbit sera (Pike et al., 1965; Pike, 1967). Hence, the immunoglobulin participating in a serological reaction might indicate broadly the chemical nature of the corresponding antigen.

In this study, the immunoglobulin involved in agglutination of leptospires by rabbit antiserum has been examined closely throughout the immunization of a single rabbit.

MATERIALS AND METHODS

Leptospiral microscopic agglutination test

Serial twofold dilutions of the untreated whole serum, or Sephadex G-200 column fractions, were made in barbital buffer, pH 7.3, I = 0.15 (Kabat & Mayer, 1964) in duplicate or triplicate series by means of modified Takatsy microtitre pipettes, loops and trays.[3] An equal volume of viable *Leptospira* sp. (serotype *biflexa*, strain Waz) culture (0.025 ml), 4–9 days old, was added to each antiserum dilution and incubated at 30°C for 30–60 minutes. Each dilution mixture was examined by dark-ground microscopy and the end-point taken as the highest final dilution showing just less than 50% free (unagglutinated) leptospires (WHO Expert Group on Current Problems in Leptospirosis Research, 1967). Direct comparison with a 50% control assisted in the end-point determination. The titre is expressed as a reciprocal of the $\log_2$.

Reliability of the microscopic agglutination test

Replicate titrations of the same antiserum sample, using the same antigen suspension, showed the inherent variability within this test to be a variation of ±1 doubling dilution in the end-point determination. Other factors affecting the reliability of the titration were investigated and are presented in the Annex.

Treatment of sera with 2-mercaptoethanol

Sera were treated by the method of Adler (1965). Duplicate samples were adjusted to a final concentration of 0.1 M 2-mercaptoethanol, incubated for 1 hour at 20°C and then dialysed for 24 hours against 0.02 M iodoacetamide in 0.85% sodium chloride.

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[3] Supplied by the Cooke Engineering Co., Alexandria, Va., USA.

Control samples were dialysed only, without prior reduction with 2-mercaptoethanol.

Inoculation and bleeding of rabbits

A single rabbit used in this study received 7 intravenous injections of 5 ml of a culture of living leptospires (*biflexa*, Waz) on days 1, 21, 45, 74, 124, 191 and 212. Leptospiral cultures were grown in Korthof's medium containing 10% rabbit serum for 4–15 days at 30°C.

Serum samples were taken from the rabbit by marginal ear vein punctures at 2–3-day intervals.

Gel-filtration

A Sephadex G-200 column (90 cm by 2.5 cm), equilibrated with 0.05 M tris buffer, pH 8.0, in 1 M sodium chloride, was used. The absorption of the effluent at 280 nm was continuously recorded with an LKB Uvicord II absorptiometer. Column fractions of 6 ml were collected; sodium azide (0.1%) was incorporated into all buffer solutions. The column was run at room temperature (23°C) at a hydrostatic pressure of 20 cm and a flow-rate of 5 ml/hour. In each run 1.5 ml of undiluted serum was added to the column.

Analysis of column fractions for agglutinins

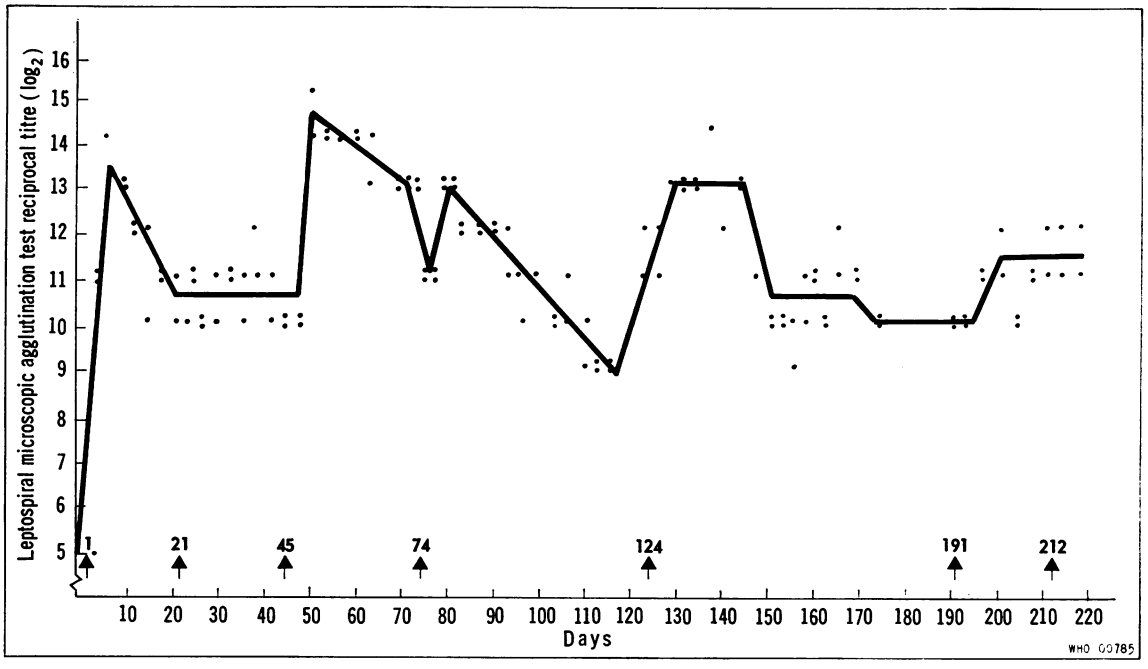
Since 1.5 ml of serum was added to the column and fractions containing 6 ml were collected, the minimal dilution of the serum during passage through the column was 1 : 4. For the purpose of the leptospiral microscopic agglutination test, undiluted column fractions were considered to be already at a dilution of 1 : 4; after addition of the leptospiral culture, the dilution became 1 : 8. Consequently, a negative reading in these titrations implied no agglutinating activity at a dilution of 1 : 8 or greater.

Immunoelectrophoresis

Column fractions to be examined by immunoelectrophoresis were concentrated 50-fold by overnight dialysis against Carbowax 4000. Electrophoresis was carried out in 1% Ionagar in barbital acetate buffer solution (pH 8.6), using barbital acetate buffer, pH 8.6, I = 0.1, as an electrolyte.

FIG. 1

TITRE OF ANTILEPTOSPIRAL AGGLUTININS PRODUCED IN RABBITS FOLLOWING INTRAVENOUS INOCULATION WITH 5 ML OF A CULTURE OF LIVING LEPTOSPIRES ON DAYS 1, 21, 45, 74, 124, 191 AND 212 (ARROWS) [a]



[a] Each point represents a titre obtained by the leptospiral microscopic agglutination test with an experimental error of 1 doubling dilution.

TABLE 1

AGGLUTINATING ACTIVITY OF ANTILEPTOSPIRAL SERA [a] TAKEN AT INTERVALS FROM RABBITS INOCULATED WITH LIVE LEPTOSPIRES

Days after inoculation [b]	Microscopic agglutination test reciprocal titres (log_2); duplicate titrations									
	Untreated sera		Sera treated with 2-mercaptoethanol [c]		Reduction in titre due to 2-mercapto-ethanol [c]	Sephadex G-200 peak				
						19S [c]		7S [c]		Ratio 19S : 7S
0	5	5 [d]	—		—	—		—		—
4	9	9	5	5	4	8	8	nil		all : none
6	10	11	8	8	2.5	9	9	6	6	9 : 1
10	11	11	10	10	1	8	8	8	8	1 : 1
18	10	10	9	8	1.5	—		—		—
23	8	8	7	8	0.5	—		—		—
42	9	8	9	8	0	—		—		—
51	10	10	9	9	1	9	10	9	9	1 : 1
80	11	10	9	8	2	—		—		—
117	9	9	9	9	0	—		—		—
138	11	12	10	11	1	—		—		—
154	10	11	10	10	0.5	—		—		—
175	10	10	9	8	1.5	—		—		—
202	12	11	11	11	0.5	—		—		—
216	11	12	10	11	1	10	10	11	11	1 : 2
						nil [e]		8	8 [e]	none : all [e]

[a] Sera were treated with 2-mercaptoethanol and or separated by gel-filtration on Sephadex G-200; agglutinating activity was titrated by the microscopic agglutination test.
[b] Rabbit inoculated on days 1, 21, 45, 75, 125, 191.
[c] — = Not done.
[d] "Natural" antileptospiral titre present in rabbit prior to inoculation.
[e] Day 216 sample treated with 2-mercaptoethanol prior to gel-filtration.

A potential gradient of 3 V/cm was maintained for 3½ hours.

Commercial goat anti-whole-rabbit-serum (Hyland) was added to the antiserum trough for development of the precipitin arcs.

Cellulose acetate electrophoresis

Column fractions to be examined by cellulose acetate electrophoresis were concentrated 50-fold by overnight dialysis against Carbowax 4000. Millipore cellulose acetate strips (17 cm by 2.4 cm) were saturated in barbital acetate buffer, pH 8.6, I = 0.10. The sample was added with a double-wire applicator and submitted to electrophoresis for 3 hours, with a current of 1.5 ampere through each strip. After 3 hours the strips were either stained for protein with Ponceau S stain for 10 minutes and then washed 3 times in 5% acetic acid, or rapidly sliced transversely into strips 2 mm–3 mm wide. Each strip was added to 4 drops of leptospiral culture in a well in a haemagglutination tray for a direct qualitative observation of its agglutinating activity. The stained and eluted strips, when compared side by side, indicated the serum proteins possessing leptospiral agglutinating activity.

RESULTS

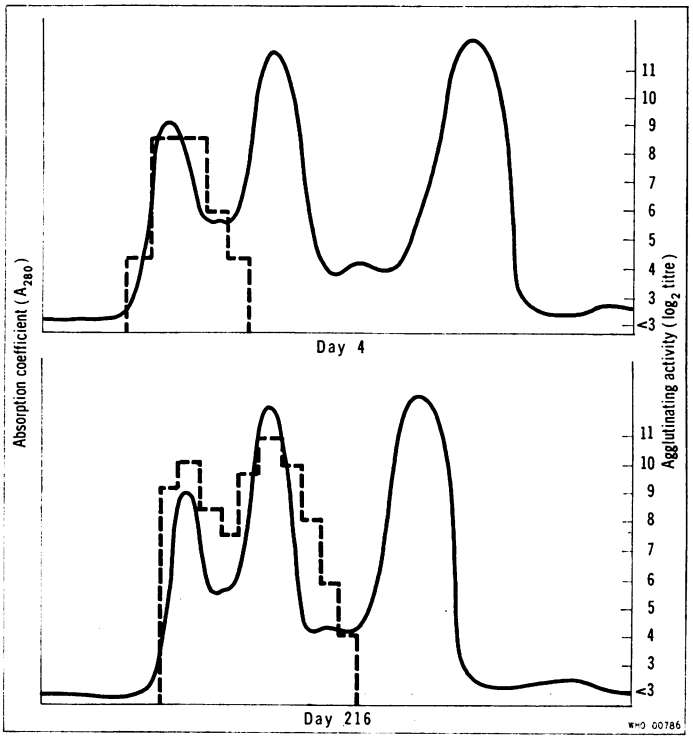
Production of antileptospiral agglutinins in the rabbit

Intravenous inoculation of the rabbit with living leptospires produced a high titre (2^{-13}) of agglutinating activity within a few days (Fig. 1). This level dropped, although subsequent boosts returned the serum titre to the initial high level.

Treatment of sera with 2-mercaptoethanol

Serum from the rabbi t, taken at various intervals (Table 1) after the initial inoculation, was treated

FIG. 2
GEL-FILTRATION OF RABBIT ANTILEPTOSPIRAL SERUM ON SEPHADEX-200 [a]



Protein concentration.
Antibody titre.

[a] A sample taken 4 days after the initial inoculation shows agglutinating activity only in the 19S fraction, while a serum sample taken 216 days after the initial inoculation showed agglutinating activity in both the 19S and the 7S fractions. The horizontal axes show the various fractions from the Sephadex column.

with 2-mercaptoethanol. Changes in the agglutinating titre, before and after treatment with 2-mercaptoethanol, were observed.

On days 4 and 6 after inoculation, the agglutinins were completely sensitive to 2-mercaptoethanol, while later agglutinins (days 10–216) were only partially sensitive, as shown by a reduction equal to 1 log_2 in the agglutinating titre (Table 1).

Gel-filtration of sera on Sephadex G-200

Sera taken at various intervals after initial inoculation were submitted to gel-filtration (Table 1; Fig. 2) and the column fractions were monitored for agglutinins by the microscopic agglutination test.

On day 4, only the 19S peak (Flodin & Killander, 1962) contained agglutinins. However, by day 6, the 7S peak (Flodin & Killander, *op. cit.*) also contained agglutinins. Both 19S and 7S agglutinins persisted until the completion of the observations (day 216).

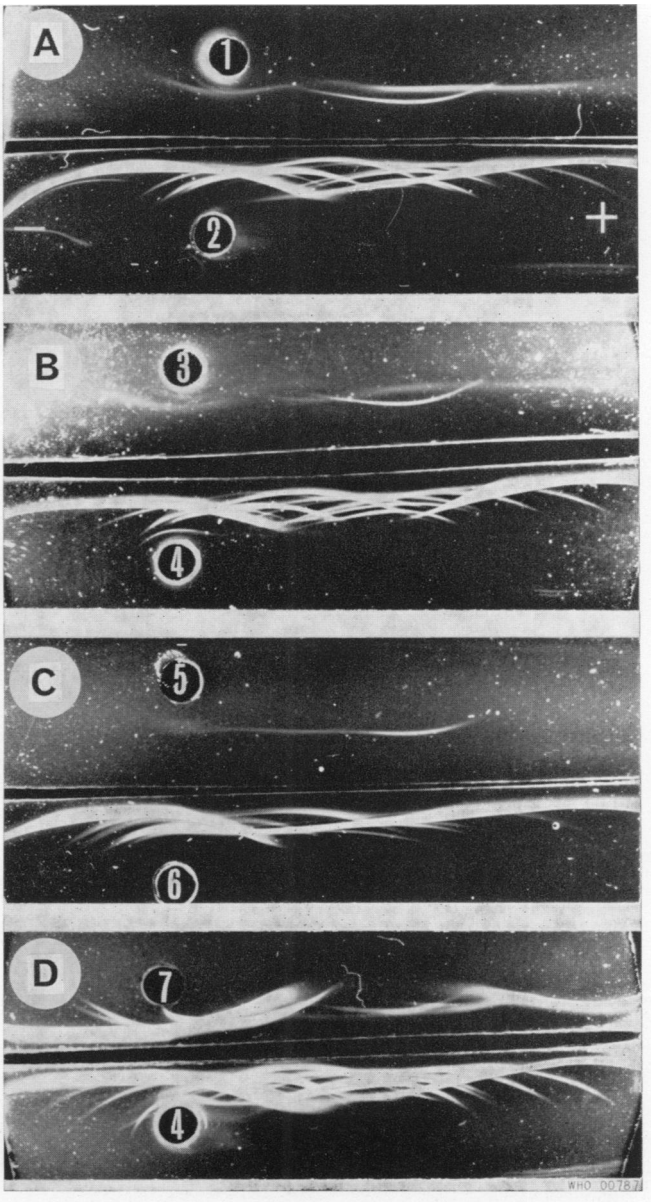
When the serum sample taken on day 216 was treated with 2-mercaptoethanol before gel-filtration, less agglutinating activity in the 7S peak and no agglutinating activity in the 19S peak, was seen, by comparison with the untreated sample of day 216 (Table 1). This indicated that 2-mercaptoethanol completely destroyed the 19S agglutinin and partially destroyed the 7S agglutinin activity.

Immunoelectrophoresis of Sephadex G-200 column fractions

In order to identify the serum proteins with antileptospiral agglutinating activity, column frac-

FIG. 3

IMMUNOELECTROPHORETIC COMPARISON [a] OF RABBIT ANTILEPTOSPIRAL WHOLE-SERUM WITH CORRESPONDING 7S AND 19S PEAK FRACTIONS [b] FOLLOWING GEL-FILTRATION ON SEPHADEX G-200 [c]



[a] The troughs contained goat anti-whole-rabbit-serum. Anode to the right, cathode to the left.

[b] A: 19S peak (1) compared with whole antileptospiral serum (2) on day 4 after inoculation.

B: 19S peak (3) compared with whole antileptospiral serum (4) on day 216 after inoculation.

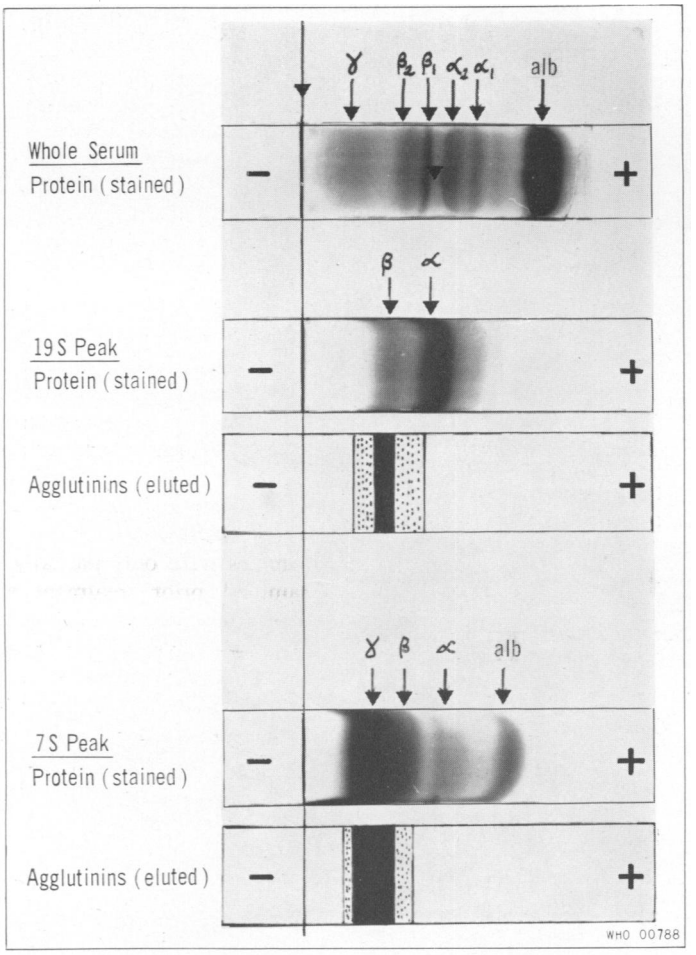
C: 19S peak after reduction with 2-mercaptoethanol (5) compared with whole antileptospiral serum on day 216 after reduction with 2-mercaptoethanol (6).

D: 7S peak (7) compared with whole antileptospiral serum on day 216 (4).

[c] 1 % agar, pH 8.6, 3 V/cm for 3½ hours.

FIG. 4

ELECTROPHORESIS OF RABBIT ANTILEPTOSPIRAL SERA AFTER GEL-FILTRATION ON SEPHADEX G-200 [a]



Strong leptospiral agglutinating activity.

Weak leptospiral agglutinating activity.

No leptospiral agglutinating activity.

[a] The protein peaks (19S and 7S) were examined by cellulose acetate electrophoresis and subsequent elution of proteins from the unstained cellulose acetate strip by slicing it into thin strips. Proteins with leptospiral agglutinating activity were thereby localized and identified. Anode to the right, cathode to the left.

tions representing the 19S and 7S peaks of the gel-filtration profile were concentrated and examined by immunoelectrophoresis against anti-rabbit serum (Fig. 3).

The 19S peak contained α_2M globulin, IgM and lipoprotein. The IgM was not present in the immuno-electrophoresis pattern of sera previously treated with 2-mercaptoethanol.

The 7S peak contained at least 8 serum proteins of various electrophoretic mobilities. The γ-migrating IgG was very strong. Although some serum components were not present in sera previously treated with 2-mercaptoethanol, the IgG arc was not visibly affected by prior treatment with 2-mercaptoethanol.

Cellulose acetate electrophoresis and elution of Sephadex G-200 column fractions from cellulose acetate strips

Column fractions representing the 19S and 7S peaks of the gel-filtration profile (and with anti-leptospiral agglutinating activity) were concentrated and examined by cellulose acetate electrophoresis. One strip was stained to show localization of protein and the duplicate strip was sliced transversely into small pieces. Each piece was qualitatively examined for antileptospiral activity (Fig. 4).

In the 19S fractions, activity was β-migrating, corresponding to IgM and not to the α_2M globulin. In the 7S fractions, activity was γ-migrating and probably corresponded to the IgG, while activity was absent from the α and β globulins and the albumin.

DISCUSSION

In humans, leptospiral infection has been shown to give rise to IgM (β_2M) 19S antibody (Pike et al., 1965; Hartmann et al., 1964; van Dalen et al., 1967; Lataste-Dorolle et al., 1964), although infection with some serotypes (e.g., *sejroe*) also produced 7S antibody (Pike et al., 1965). The stage of infection (acute or convalescent) at which the serum sample was taken is important when considering the types of antibody present.

In rabbits, initial 19S response followed by a variable degree of 7S agglutinin, depending on the infecting serotype, was described by Pike et al. (1965) and Pike & Schulze (1965). In some cases, no 7S antibody was found (Hocker & Bauer, 1965).

In this study, both 19S and 7S agglutinins were produced by the rabbit in response to leptospires (*biflexa*, Waz). The 19S agglutinin was produced sooner than the 7S agglutinin (4 days compared with 6 days) although this may have merely reflected a lower threshold and greater sensitivity for detecting 19S antibody when an agglutination test was used. IgM antibodies are more readily detected than IgG antibodies by cellular agglutination (Pike, 1967; Pike et al., 1966).

The presence of "natural" leptospiral agglutinins (titre 2^{-3}) prior to inoculating possibly explains the rapid attainment of a high agglutinating titre after 1 inoculation. The nature of the natural agglutinin was not investigated; there is no evidence that the natural antibody carried specificity against the same antigens as those which react in the microscopic agglutination test. Hocker & Bauer (1965) likewise found no increases in agglutinating antibody on subsequent boosting.

Differences in microscopic agglutination test titres performed on sera before and after treatment with 2-mercaptoethanol measure the proportion of serum agglutinins sensitive to 2-mercaptoethanol. The very early samples (days 4 and 6 after initial inoculation) were completely sensitive, while samples from day 10 and subsequent serum samples were only partially sensitive. In the latter samples, prior treatment with 2-mercaptoethanol gave a reduction in the agglutination end-point of 1 $\log_2$. This may not be confidently concluded as being a 50% reduction in agglutinating antibody concentration, since the inherent variation in the microscopic agglutination test itself is equal to ± 1 doubling dilution (see Annex) and a 1 $\log_2$ reduction in end-point may not, therefore, be statistically significant. However, an average reduction of 1 $\log_2$ in titre occurred consistently in sera taken after day 10 (Table 1). The 1 $\log_2$ reduction probably represents a summation of the complete destruction of the 19S agglutinin and the partial destruction of the 7S agglutinin. Adler (1965) and Hong & Nisonoff (1964) have shown that rabbit 7S IgG is partially sensitive to sulfydryl bond reduction with 2-mercaptoethanol and this is presumably the situation with rabbit antileptospiral 7S agglutinins.

Sephadex G-200 gel-filtration separates the serum proteins into 3 broad groups, based mainly on molecular size and shown to correspond to sedimenting coefficients of 19S (and larger), 7S and 4S (Flodin & Killander, 1962; Loewi & Nind, 1967). The 19S peak contained mostly α-macroglobulin, which has been shown to consist of 80% α and 20% α_2 globulin (Bloth et al. 1968). Also present in the 19S peak were IgM and lipoprotein. The IgM can be

separated from the α-globulin by electrophoresis on either polyvinyl chloride (Bloth et al., op. cit.) or on cellulose acetate, as was done in this study. In both cases, antibody activity was found only in the IgM region. Purified rabbit IgM has been shown to consist of 2 species, 26S and 19S, in the ratio 1 : 4 (van Dalen et al., 1967).

These experiments (Fig. 4) have shown directly, by elution of separated serum protein from cellulose acetate, that IgM is the 19S antileptospiral agglutinating antibody. The 7S antibody was shown to be γ-migrating and probably IgG, with a possible IgA involvement. Only some rabbits produce serum IgA (Onoue et al., 1964). In our study, no conclusions could be made concerning IgA agglutinins, since activity in the 7S peak was spread broadly over the γ-migrating region (Fig. 4), due to IgG, and presumably would mask any IgA activity, were it present.

Protein antigens (e.g., bacterial flagella), usually give rise to a 19S antibody response which gradually abates and is followed by a persistent 7S response. Somatic antigens of Gram-negative bacteria, with polysaccharide determinants, usually induce a predominant and persistent 19S response (Pike, 1967). The persistent nature of both the IgM and IgG agglutinins in the rabbit after inoculation with viable leptospires may suggest that both protein and polysaccharide antigens are involved in the production of agglutinins in rabbits. The protein axial filament (axistyle, flagellum) of the leptospires, in an isolated and apparently purified preparation, will induce agglutinins in the rabbit (A. Chang and others, personal communication). However, other antigens are almost certainly involved also in the induction of antileptospiral agglutinating antibodies. Thus the agglutinating titre of any antileptospiral serum would be the manifestation in a microscopic agglutination test of the sum of the immunogenic effects of a complex mixture of antigenic determinants, surface and subsurface, protein and polysaccharide, type-specific and pan-specific.

RÉSUMÉ

AGGLUTININES ANTI-LEPTOSPIRES PRODUITES CHEZ LE LAPIN

On a administré à un lapin sept injections intraveineuses, étalées sur une période de 212 jours, de 5 ml d'une culture de *Leptospira* sp. (sérotype *biflexa*, souche Waz), afin d'étudier la nature des anticorps agglutinants anti-leptospires. Des échantillons de sérum ont été recueillis à intervalles variables pendant 216 jours après l'inoculation.

L'immunisation a provoqué l'apparition rapide d'agglutinines à des titres élevés. Les anticorps comportaient une fraction 19S, décelable 4 jours après l'inoculation, et une fraction 7S apparaissant à partir du 6e jour. Les agglutinines 19S et 7S ont été retrouvées dans le sérum jusqu'au dernier jour d'observation (216e jour). Le traitement du sérum par le 2-mercaptoéthanol a eu pour résultat de détruire complètement la fraction 19S et d'inhiber partiellement l'activité des anticorps 7S. L'immunoélectrophorèse a montré que la fraction 19S contenait de l'α_2 M-globuline, de l'IgM et une lipoprotéine, et la fraction 7S au moins 8 protéines dont une IgG à mobilité électrophorétique γ. L'électrophorèse sur acétate de cellulose a confirmé que dans la fraction 19S l'activité agglutinante était portée par l'IgM à mobilité β et que dans la fraction 7S elle devait être attribuée à l'IgG.

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Annex

FACTORS AFFECTING RELIABILITY OF TITRATION

Various factors, other than serum antibody, affecting the titres obtained in the leptospiral microscopic agglutination test were examined; they were as follows:

(1) The inherent variation within the test itself, using the same serum sample and same leptospiral culture.

(2) The age of the leptospiral culture used.

(3) The concentration of leptospires (Borg-Petersen & Fagraeus, 1949).

(4) The proportion of non-viable leptospires in the culture.

(5) The incubation period of the test.

The results of the investigations were as follows:

(1) The inherent variation in the test itself was ± 1 doubling dilution variation in the end-point.

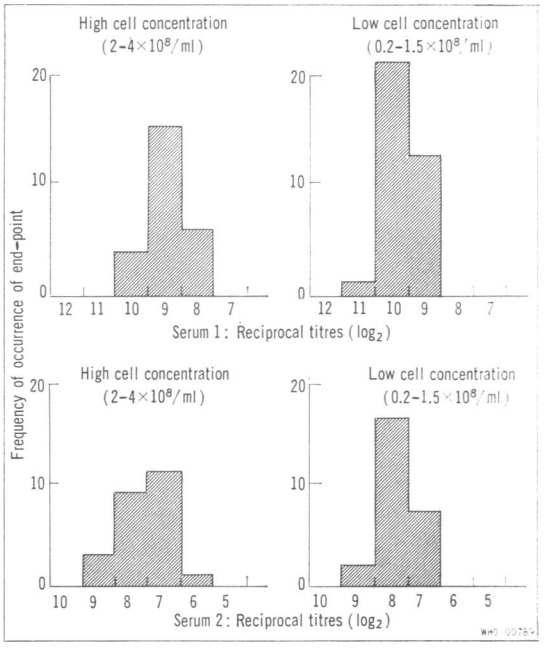
(2) Cultures between 2 and 15 days old did not cause systematic variations in titre (Table 2). Variations observed were within ± 1 doubling dilution of the mean titres and could, therefore, be explained as "inherent variation".

(3) Concentrated cultures ($2\text{–}4 \times 10^8$ organisms/ml) gave titres consistently 1 doubling dilution lower than dilute cultures (i.e., $0.2\text{–}1.5 \times 10^8$ organisms/ml) (Fig. 5). This observation was statistically significant (χ^2 test) at the 1% level.

(4) The proportion of viable cells in the antigen suspension could fall to 60% before end-point readings were affected (Fig. 6).

(5) Maximum agglutination in the test occurred after only 20 minutes incubation at 30°C. The end-point remained constant for 2 hours thereafter (Fig. 7).

FIG. 5
HISTOGRAMS OF RELATIONSHIPS BETWEEN CONCENTRATION IN THE ANTIGEN SUSPENSION AND THE END-POINT OF THE LEPTOSPIRAL MICROSCOPIC AGGLUTINATION TEST



In this laboratory, microscopic agglutination tests involving comparisons of serum titres are performed, wherever possible, with the same leptospiral culture, thus minimizing variations due to cell densities and different "agglutinating sensitivities" of the cells. Nevertheless, differences in titre of only 1 doubling dilution are not considered significant unless obtained consistently.

FIG. 6

TITRES OBTAINED FROM READING THE LEPTOSPIRAL MICROSCOPIC AGGLUTINATION TITRATION OF A RABBIT SERUM SAMPLE USING LEPTOSPIRAL CULTURES WITH A KNOWN PERCENTAGE OF VIABLE CELLS AS ANTIGEN

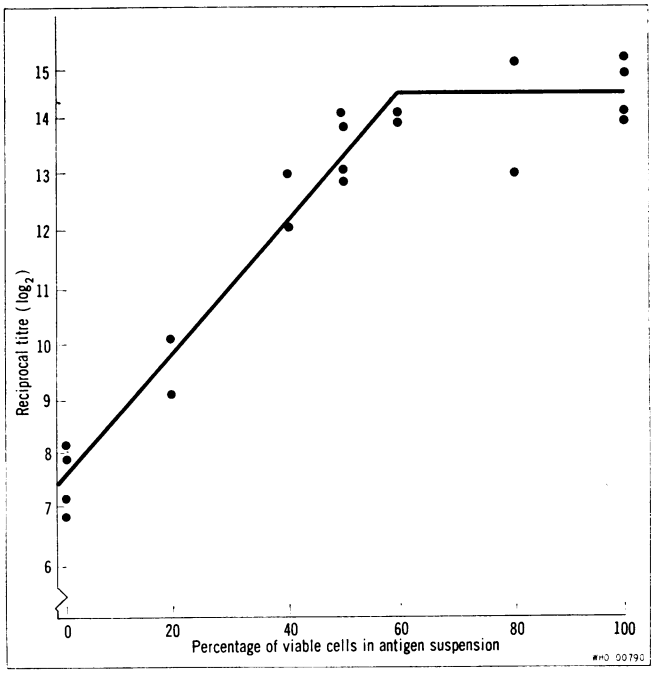
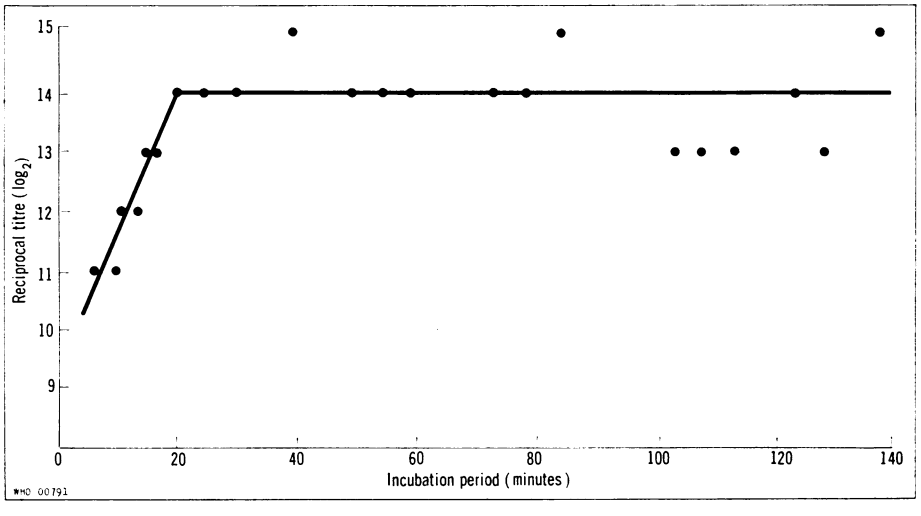


FIG. 7

TITRES OBTAINED FROM READING THE LEPTOSPIRAL MICROSCOPIC AGGLUTINATION TITRATION OF A RABBIT SERUM SAMPLE AT VARIOUS INTERVALS AFTER THE ADDITION OF THE LEPTOSPIRAL SUSPENSION [a]



[a] Incubation at 30°C.

TABLE 2

LEPTOSPIRAL MICROSCOPIC AGGLUTINATION TEST TITRES OBTAINED WITH TWO RABBIT ANTILEPTOSPIRAL SERA, USING LEPTOSPIRAL CULTURES OF DIFFERENT AGES

Age of leptospiral culture (days)	Reciprocal titres (log_2) [a]									
	Serum 1				Average	Serum 2				Average
2	11	10	9	10	10	7	7	8	8	7.5
3	9	9	10	10	9.5	7	7			7
4	9	8	10	10	9.3	7	7	8	8	7.5
5	9	10	9	9	9.3	8	8	8		8
6	8	9	9	9	8.8	7	8	8	7	7.5
7	10	9	9	10	9.5	8	8	8	9	8.3
8	10	9	9	10	9.5	9	9	9	8	8.7
9	9	9	9	10	9.3	8	9	8	8	8.3
10	9	8	10	10	9.3	8	7	8	8	7.8
11	9	9	10	10	9.5	8	7	8	8	7.8
12	9	8	9	9	8.8	7	7	7	7	7
13	9	10	10	10	9.7	6	8	7	7	7
15	8	8	9	10	8.8	7	7	8	8	7.5
Average	9.3					7.7				
Range	8–10					7–9				

[a] Each serum was examined in 4 replicate titrations. The average is the arithmetic mean of the values obtained.