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Characterization of the major antigens of *Haemophilus equigenitalis* (contagious equine metritis organism)

By M. J. CORBEL and R. A. BREWER

Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey KT15 3NB

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SUMMARY

Immunoelectrophoresis of ultrasonically disrupted *Haemophilus equigenitalis* (contagious equine metritis organism) cells against rabbit and equine antisera disclosed at least 11 precipitating antigens. Two of these, a polysaccharide and a lipopolysaccharide–protein complex, were of high molecular weight and located on the cell surface. The remaining antigens were intracellular and were small- to medium-sized proteins.

The surface antigens were the most significant in relation to the serological response in infected horses. They also reacted with sera from apparently healthy cattle, but the reason for this was not determined. No serological cross-reaction between *H. equigenitalis* and species of *Achromobacter* and *Moraxella* was detected.

INTRODUCTION

The bacterium implicated as the causative agent of contagious equine metritis has been identified as a new species *Haemophilus equigenitalis* (Taylor *et al.* 1978). The morphological, cultural, general bacteriological and pathogenic properties of this organism have been closely studied, particularly in relation to the development of procedures for its identification and for determining its taxonomic position (Platt, Atherton & Simpson, 1978; Rommel *et al.* 1978; Shreeve, 1978; Taylor *et al.* 1978; Timoney & Ward, 1978; Swaney & Breese, 1980).

In addition, a number of serological tests, including agglutination, antiglobulin, complement fixation and indirect haemagglutination procedures, have been developed for the detection of antibodies to *H. equigenitalis* for diagnostic purposes (Benson *et al.* 1978; Croxton-Smith *et al.* 1978; Fernie, Cayzer & Chalmers, 1979). Nevertheless, relatively little is yet known of the antigenic structure of the organism.

Recently, during the course of studies on the possible role of *H. equigenitalis* as an agent of reproductive failure in cattle, antibodies to this organism were detected in an unexpectedly high proportion of bovine serum samples (Corbel & Brewer, 1980).

The absence of an association of these antibodies with any recognizable disease pattern led to the conclusion that they probably arose from serological cross-reactions with other organisms. A similar conclusion has also been suggested as

an explanation of the occurrence of antibodies to *H. equigenitalis* in some human sera (Taylor & Rosenthal, 1978). Indeed, apparent cross-reactions between *H. equigenitalis* and *Moraxella* and *Acinetobacter* species have been briefly reported although not apparently confirmed (Smith & Young, 1978). [*Acinetobacter* and some strains of *Achromobacter* may be synonymous.] For this reason, an attempt was made to characterize the principal antigens of *H. equigenitalis*.

MATERIALS AND METHODS

Bacterial strains

H. equigenitalis strains Australia, E5, E15 and NCTC 61717 were provided by Mrs J. E. Shreeve of this laboratory. *H. equigenitalis* strain Cambridge was provided by Mr J. A. Benson, formerly of the Veterinary Investigation Centre, Cambridge. This strain was a recent isolate from a field case of contagious equine metritis. It had been re-inoculated intravaginally into a mare and then recovered from the cervical exudate. The strain was then lyophilized from the first sub-culture. It had received no more than four sub-cultures *in vitro* before use in the present work.

Achromobacter haemolyticus NCTC 10305 (syn. *Acinetobacter calco-aceticus*), *Achromobacter mucosus* NCTC 10303, *Moraxella liquefaciens* NCTC 7911 and *Moraxella non-liquefaciens* NCTC 10464 were all obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London.

Cultural methods

The *H. equigenitalis* strains were grown on layers of Eugon agar (BBL Division of Becton-Dickinson and Co., Cockeysville, Md. USA) containing 10% (v/v) chocolated horse blood and 100 μg/ml streptomycin, incubated at 37 °C in air + 10% (v/v) CO_2 for 7 days.

The other bacterial strains were grown on sheep blood agar incubated under similar conditions for 3–4 days. Organisms for antigen preparation were harvested off the agar layers by washing with the minimum volume of 0·15 mol/l NaCl and centrifuging the suspensions at 10000 *g* for 15 min. The deposited bacteria were then washed at least twice more by resuspension in fresh volumes of 0·15 mol/l NaCl. Agglutinating bacterial suspensions were prepared as described for *H. equigenitalis* by Benson *et al.* (1978). Suspensions of *H. equigenitalis* and the other bacteria for antigen extraction or for serum absorption were prepared in 0·15 mol/l NaCl buffered at pH 7·2 with 0·01 mol/l phosphate buffer (PBS) and killed by heating at 60 °C for 30 min.

Antigen extraction

Whole cell suspensions of killed *H. equigenitalis* or other bacteria containing approximately 10^{12} cells/ml were subjected to two periods of 30 min. ultra-sonication in a Branson Soniprobe (Dawe Instruments, London) tuned to maximum output. The suspension was cooled in ice and its temperature was not permitted to rise above 25 °C during ultra-sonication. Disrupted suspensions were clarified by centrifugation at 10000 *g* for 20 min and the supernatant extracts dried from the frozen state.

Lipopolysaccharide–peptide complexes were extracted from whole organisms by the hot phenol–water method of Westphal, Lüderitz & Bister (1952). After removal of phenol by dialysis, the lipopolysaccharide–peptides were recovered from the aqueous phase by methanol precipitation.

Antisera

Equine antisera to *H. equigenitalis* were provided by Mr J. A. Benson. Rabbit antiserum to *H. equigenitalis*, *Achromobacter mucosus*, *A. haemolyticus*, *M. liquefaciens* and *M. non-liquefaciens* were prepared by intramuscular injection of emulsions of heat-killed organisms in Freund incomplete adjuvant, followed after 3 weeks by repeated intravenous injections of killed organisms suspended in sterile 0·15 mol/l NaCl. The serum was collected 7 days after the final injection and stored at −20 °C until required. Bovine sera from apparently healthy animals and cases of reproductive failure were collected as described previously (Corbel & Brewer, 1980).

Serological methods

The serum agglutination test (SAT) and Coombs antiglobulin test with *H. equigenitalis* antigen were done as described by Benson *et al.* (1978). Agglutination tests with *Achromobacter* and *Moraxella* species were performed by a similar procedure using killed suspensions of these organisms in 0·5 % (w/v) phenol saline standardized to the same optical density as the *H. equigenitalis* antigen.

Immunodiffusion tests were performed in 1 % (w/v) Oxoid No. 1 agar in either 0·15 mol/l or 1·0 mol/l NaCl containing 0·1 % (w/v) sodium azide. Reagent wells 5 mm in diameter were cut in isometric patterns with centres 7 mm apart. Antigen preparations were adjusted to a concentration which produced maximum precipitation with the homologous antiserum. Diffusion was allowed to proceed in a water-saturated atmosphere at room temperature for 1–3 days.

Immunoelectrophoresis was performed by the Scheidegger (1955) method in low electroendosmosis agarose (Sigma, London) gels made up to 1 % (w/v) in 0·05 M sodium diethyl–barbiturate buffer, pH 8·6 according to Laurell (1965). The antigen reservoirs were 4 mm in diameter and electrophoresis was performed at a potential gradient of 3 V/cm for approximately 1 h. as determined by the progress of phenol red and Blue Dextran 2000 markers. Parallel antiserum troughs were cut 2·5 mm to either side of the antigen well and antiserum added. Diffusion was allowed to proceed in a water-saturated atmosphere at room temperature for up to 5 days. The gels were washed 3 times in saline solution, followed by distilled water and then dried and stained. Staining for protein was with 1 % (w/v) Amido Black 10B in 7 % (w/v) acetic acid. Staining for lipids by the Sudan Black B method, for nucleic acids with acridine orange and for glycoproteins and polysaccharides by the copper–formazan reaction, were done according to Crowle (1973).

Analytical agarose gel electrophoresis was performed in a manner identical with immunoelectrophoresis except that the diffusion against antiserum and the subsequent washings were omitted.

Serum absorption was performed by mixing 3 vol. of serum containing 0·1 % (w/v) sodium azide with 1 vol. of packed, killed bacterial cells and incubating at room temperature overnight. The absorbed serum was recovered by centrifugation

at 10000 *g* for 20 min. In some cases the absorption process was repeated one or more times.

Inactivating agents

α-Amylase (Type IIA, bacterial), DNase (Type 1, bovine pancreas), RNase (Type XIIA, bovine pancreas), lipase (Type VII, *Candida cyclindracea*), pronase (*Streptomyces griseus* protease), trypsin (Type III, bovine pancreas), N-bromosuccinimide and N-ethyl maleimide was obtained from Sigma London Chemical Co. Ltd, Poole, Dorset. Dithiothreitol (DTT), 1-ethyl(2, 3 dimethyl aminopropyl)carbodiimide (ECDI), lysozyme and sodium periodate were obtained from BDH Chemicals Ltd, Poole, Dorset.

Glutaraldehyde was obtained from Koch-Light Laboratories Ltd, Colnbrook.

Other reagents used were of analytical quality.

Antigen characterization

The stability of individual precipitating antigens to pronase, trypsin, lipase, glutaraldehyde, 0·1 N NaOH, 0·1 N HCl, DTT, sodium periodate, N-ethyl maleimide, N-bromosuccinimide and ECDI was determined under the conditions described by Corbel (1977). Treatment with RNase (500 Kunitz units/ml) was at pH 6·0 and 37 °C for 1 h., and with DNase (10000 Kunitz units/ml) at pH 7·2 and 37 °C for 1 h. Incubation with lysozyme (2 mg/ml) was at pH 5·0 and 37 °C for 1 h. Treatment with α-amylase at 1500 units/ml was at pH 7·0 and 37° for 1 h. Stability to heat at 100 °C for 15 min was determined by heating at neutral pH in sealed ampoules.

All treated antigen preparations and their respective controls were examined by immunoelectrophoresis against rabbit antiserum to *H. equigenitalis*. To minimize interference by residual enzymes or other reagents, diffusion was allowed to proceed at 4 °C for up to 7 days.

Estimates of antigenic molecular size were made by fractionating 1 ml of concentrated *H. equigenitalis* ultra-sonicate made 10% (w/v) in respect of D(+) glucose on a 30 mm × 300 mm column of Sepharose 6B (Pharmacia, Uppsala, Sweden) equilibrated with PBS containing 0·02% (v/v) sodium azide. Elution was performed with the same buffer at a flow rate of 18 ml/h and the column effluent monitored for absorption of ultraviolet radiation of 280 nm wavelength. Fractions were collected and pooled according to the elution profile, snap-frozen in liquid nitrogen and dried from the frozen state. The dried fractions were reconstituted in distilled water and examined by immunodiffusion and immunoelectrophoresis against bovine, equine and rabbit antisera to *H. equigenitalis*, and rabbit antisera to other organisms. The ultraviolet absorption spectrum of each fraction was also determined using an Optica CF4NI double-beam recording spectrophotometer.

The column was calibrated for molecular exclusion properties using Blue Dextran 2000 (MW 2×10^6 daltons), horse spleen ferritin (444000 daltons), catalase (232000 daltons), haemoglobin (68000 daltons) and lysozyme (14000 daltons). D(+) glucose was used as an internal marker and was detected with Clinistix (Ames Co., Ltd, Slough). The molecular weight of each antigen was estimated from its elution volume relative to the void volume of the column as described by Leach & O'Shea (1965).

RESULTS

Diffusion of ultrasonically disrupted cells of *H. equigenitalis* against antiserum to the homologous strain produced complex precipitation patterns comprising at least six distinct line pattern components. Essentially idèntical patterns were produced by extracts of all of the strains of *H. equigenitalis* examined and no evidence of strain-specific antigens was detected by immunodiffusion.

Diffusion of *H. equigenitalis* extracts against sera from mares convalescent from infection with this organism produced much less complex patterns, comprising only one or two precipitation lines. Similar results were given by sera from a small proportion of apparently healthy cattle with no known history of exposure to the organism. Comparison of the precipitation patterns produced by bovine and equine sera showed that these detected similar but not identical antigenic components. Thus, the equine sera gave reactions of identity with two of the precipitation lines produced by rabbit antiserum, whereas the bovine sera gave a reaction of partial identity with one of these (Plate 1*a*).

Attempts to attribute the activity of the bovine sera to cross-reacting antibodies produced in response to *Achromobacter*, or *Moraxella* species were unsuccessful. Thus, diffusion of extracts of *H. equigenitalis* against rabbit antisera to *A. haemolyticus* and *M. liquefaciens*, two precipitation lines against *M. non-liquefaciens* any precipitation lines. These antisera produced complex patterns with extracts of the homologous organism and also produced cross-reactions with the heterologous *Achromobactor* and *Moraxella* species. Thus, antiserum to *A. haemolyticus* produced a minimum of three precipitation lines on diffusion against extracts of *A. haemolyticus* and *M. liquifaciens*, two precipitation lines against *M. non-liquefaciens* extract and at least one precipitation line against *A. mucosus* extract (Plate 1*c*). Antiserum to *M. liquefaciens* produced at least two precipitation lines against its homologous extract and at least one confluent precipitation line against extracts of *M. non-liquefaciens*, *A. haemolyticus* and *A. mucosus* (Plate 1*d*). Essentially similar results were obtained with antisera to *A. mucosus* and *M. non-liquefaciens*. In contrast, antiserum to *H. equigenitalis* produced a complex precipitation pattern on diffusion against extracts of this organism but no precipitation with extracts of the other bacteria (Plate 1*b*).

Bovine sera occasionally produced single precipitation lines against *Achromobacter* and *Moraxella* extracts but these did not correspond with the precipitation lines produced against *H. equigenitalis* nor were they often given by the same sera.

Results consistent with these were obtained in cross-agglutination tests with these organisms. Thus agglutination titres of 1/1280 to 1/5120 against the homologous organism and titres of 1/160 to 1/640 against the heterologous *Achromobacter* and *Moraxella* species, were given by antisera to these bacteria but not to *H. equigenitalis*. Similarly, antiserum to *H. equigenitalis* produced high agglutination titres towards its homologous antigen suspension but displayed no significant activity towards the other antigens. Cross-absorption tests with the same antigens and antisera also failed to demonstrate any serological relationship between *H. equigenitalis* and the other organisms.

Immunoelectrophoresis of ultrasonicates of *H. equigenitalis* against rabbit antisera disclosed at least eleven distinct precipitating components (Plate 2*a*). The

Table 1. *Physical and chemical properties of* H. equigenitalis *antigens*

Inactivating agent	Stability of antigenic components										
	1	2	3	4	5	6	7	8	9	10	11
0·15 mol/l NaCl	+	+	+	+	+	+	+	+	+	+	+
Heat 100 °C/10 min	+	±	−	−	−	(+)	−	(+)	−	−	(+)
0·1 N HCl	+	(+)	−	−	(+)	(+)	−	−	+	−	+
0·1 N NaOH	−	+	−	−	−	−	−	−	−	−	−
α-Amylase	+	+	+	+	+	+	+	+	+	+	+
Pronase	+	±	−	−	−	−	−	−	−	−	+
Trypsin	+	+	+	+	+	(+)	(+)	(+)	+	−	+
Lipase	+	+	+	+	+	+	+	+	+	+	+
DNase	+	+	+	+	+	+	+	+	+	+	+
RNase	+	+	+	+	+	+	+	+	+	+	+
Lysozyme	+	(+)	+	+	+	+	+	+	+	+	+
ECDI	+	+	+	+	+	+	+	+	+	+	(+)
Glutaraldehyde	+	+	−	−	−	−	−	−	−	−	−
N-Bromosuccinimide	+	+	−	−	+	+	+	+	+	(+)	(+)
N-ethyl maleimide	+	+	−	−	+	+	+	+	+	(+)	(+)
DTT	+	(+)	−	−	−	(+)	(+)	(+)	(+)	(+)	(+)
$NaIO_4$	+	(+)	(+)	+	+	−	+	+	−	−	(+)
Composition	PS	LPS	P	P	GP	GP	P	P	P	BP	P
Estimated molecular weight ($\times 10^{-4}$)	110	18	5	5	5	5	5	5	5	5	5

− = antigen destroyed; + = stable; ± = antigen partially destroyed; (+) = physical properties modified; (±) = physical properties modified and antigen partially destroyed.

PS = polysaccharide; LPS = lipopolysaccharide; P = protein; GP = glycoprotein; BP = basic protein.

precipitation arcs corresponding to these were numbered sequentially 1–11 (Plate 2*b*). Immunoelectrophoresis against equine antisera to *H. equigenitalis* revealed two precipitating components giving reactions of identity with precipitation arcs 1 and 2 produced by the rabbit antiserum (Plate 3).

Absorption with washed, whole cells of *H. equigenitalis* removed the precipitating activity towards components 1 and 2 from rabbit antiserum and eliminated completely the precipitating activity of equine serum. The agglutinating activity and antibodies active in the Coombs antiglobulin test were also removed by this treatment. Provided compensation was made for dilution of the serum during absorption, precipitating activity towards components 3–11 was not removed from the rabbit antiserum by intact cell suspensions.

Staining reactions performed on the immunoelectrophoretograms indicated the presence of protein in all of the precipitation arcs produced with rabbit antiserum. Lipid was detected by Sudan Black B only in precipitation arc 2 which also was the only one to give a positive copper–formazan reaction for glycoprotein. Precipitating components 1 and 2 both gave positive copper–formazan reactions for polysaccharides. None of the precipitating components reacted with the acridine orange stain for nucleic acids when examined under ultraviolet illumination.

Similar staining reactions performed on agarose gel electrophoretograms of *H.*

equigenitalis ultrasonicate which had not been diffused against antiserum confirmed these results. Protein was distributed irregularly throughout the electrophoretogram, with most anodal to the antigen well. Lipid and glycoprotein were confined to the region adjacent to the antigen well and polysaccharide to this region and a position corresponding to the centre of precipitation arc 1. Nucleic acid was not detected.

Immunoelectrophoresis of lipopolysaccharide preparations extracted from *H. equigenitalis* by phenol–water disclosed two precipitating components reacting with either rabbit or equine antiserum to the homologous antiserum. One of the components was identified with precipitation arc 1 and the second with precipitation arc 2 produced with rabbit antiserum. Their staining properties indicated that the first component was a polysaccharide and the second a lipopolysaccharide.

Examination of the stability of precipitation arc-forming components 1–11 to inactivation by physical and chemical agents produced the results summarized in Table 1. Precipitating component 1 was unaffected by all of the treatments except exposure to alkali which completely eliminated its serological activity. In contrast, the precipitating activity of component 2 was enhanced by alkali treatment, but it was partially degraded by acid which also made it more diffusible. Heat also reduced the precipitating activity of component 2 but did not eliminate it. Digestion with pronase split off an antigenic fragment which formed an additional precipitation arc just inside the original one. Reduction with DTT increased the diffusibility and electrophoretic mobility of component 2 as did incubation with lysozyme. Periodate oxidation diminished the precipitating activity of this component but did not eliminate it.

Precipitating components 3 and 4 were similar in their susceptibility to inactivating agents. Both were degraded by heat, acid, alkali, pronase, glutaraldehyde, N-bromosuccinimide, N-ethyl maleimide and DTT but were unaffected by the other treatments. Components 5–10 were also susceptible to a greater or lesser extent to heat, acid, alkali, pronase and glutaraldehyde but varied somewhat in their behaviour with the other treatments. In general, these did not eliminate their serological activity but modified their electrophoretic mobility or their diffusibility. Component 11 was largely stable to most of the treatments except exposure to alkali. Its electrophoretic mobility was modified by reagents reacting with amino acid residues, although its serological activity remained intact.

Estimates of the molecular sizes of precipitating components 1–11 were made from their elution properties on Sepharose 6B (Fig. 1). All were assumed to behave as proteins except for components 1 and 2 which were treated as polysaccharides. On this basis, component 1 had an approximate molecular weight of about $1{\cdot}8 \times 10^5$ daltons, whereas that of component 2 was about $1{\cdot}1 \times 10^6$ daltons. Antigenic components 3–11 had molecular weights in the range $2{\cdot}5–5 \times 10^4$ daltons.

DISCUSSION

The immunodiffusion tests on ultrasonically disrupted *H. equigenitalis* extracts showed that these were antigenically complex. They also showed that the diffusible antigens of all of the *H. equigenitalis* strains examined were essentially similar. This indicated that if antigenic differences did exist between strains of this organism

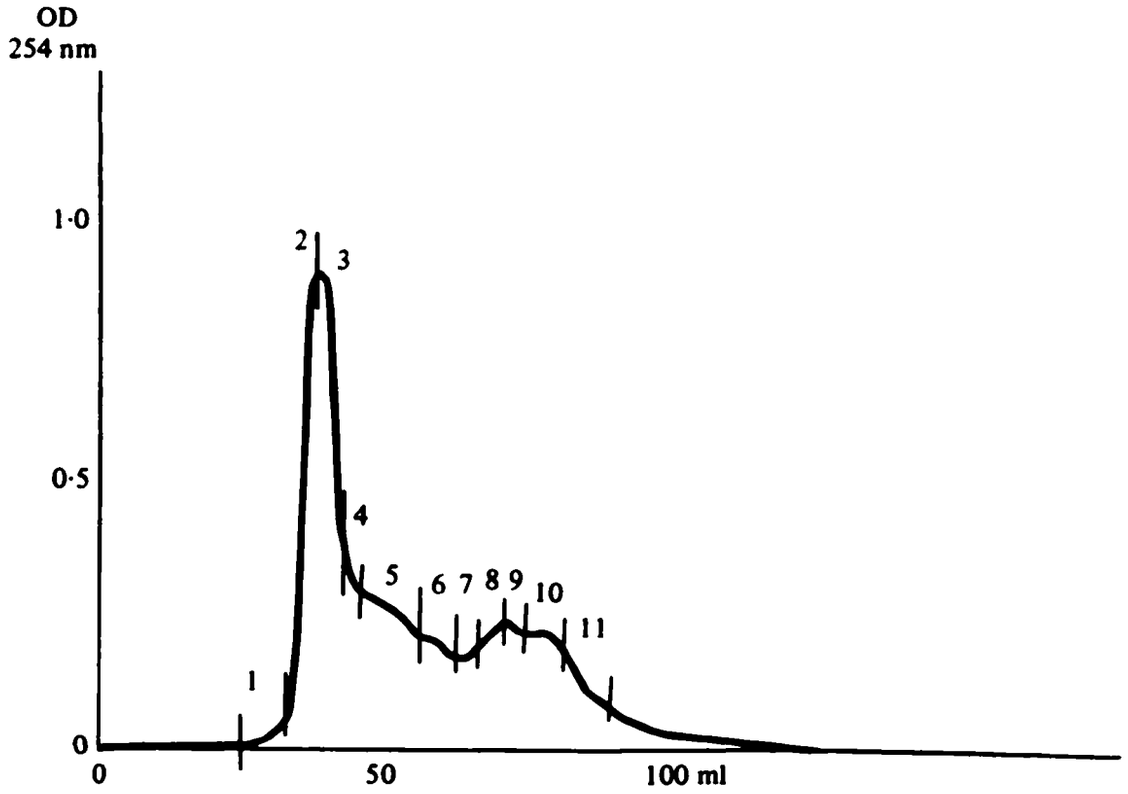

Fig. 1. Elution profile of *H. equigenitalis* ultrasonicate from a column of Sepharose 6B.

they were either too subtle to be detected by immunodiffusion methods or else involved non-diffusible antigenic components.

It was evident from both the immunodiffusion and the immunoelectrophoretic results that the antigenic components detected by rabbit antiserum also included those precipitating with bovine and equine sera. Furthermore, it was clear from the absorption studies that in the latter species only the surface antigens of *H. equigenitalis* elicited an antibody response detectable by the methods employed. The source of these antibodies in normal bovine sera was not elucidated. Indeed, no support was obtained for the previous report by Smith & Young (1978) of a serological relationship between *Achromobacter haemolyticus* and *M. liquefaciens* on the one hand and *H. equigenitalis* on the other, although the first two organisms were shown to share antigenic determinants with each other. The reactions of partial identity given by the bovine antisera with antisera to *H. equigenitalis* supports the suggestion that the activity of the former is attributable to cross-reacting antibodies but the identity of the provoking antigen is unknown.

The results of the staining reactions and the inactivation tests showed that the surface antigens, identified as precipitating components 1 and 2, were composed of polysaccharide and lipopolysaccharide–protein respectively. The latter component clearly corresponded to the somatic 0 antigen complex of the organism. The precise identity and function of component 1 was not determined but its general properties and estimated molecular weight of about $1{\cdot}8 \times 10^5$ daltons would be consistent with a sub-unit or precursor of the lipopolysaccharide–protein complex.

The greater diffusibility of the lipopolysaccharide–protein complex following exposure to lysozyme suggested that it may have been associated with mucopeptide residues derived from the inner layer of the bacterial cell wall. This was consistent with the observation that the phenol-extracted lipopolysaccharide serologically

identical with precipitating component 2 was more diffusible than that released by ultrasonic disruption.

The other antigenic components detected by rabbit antiserum, although not at present considered significant in relation to the serological diagnosis of contagious equine metritis, were identified as small- to medium-sized proteins. Antibodies to these might be detectable by more sensitive methods, such as radio-immunoassay, in the sera of naturally infected animals. At present the function of these antigens in relation to the properties of *H. equigenitalis* remains obscure.

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EXPLANATION OF PLATES

PLATE 1

(*a*) Immunodiffusion of (1) bovine serum, (2) rabbit antiserum to *H. equigenitalis* strain Cambridge, (3) equine convalescent serum, against *H. equigenitalis* strain Cambridge ultrasonicate in the central well. The bovine serum has given a reaction of partial identity with one of the precipitation lines common to the rabbit and equine antisera.
(*b*) Immunodiffusion of rabbit antiserum to *H. equigenitalis* (in the central well) against ultrasonicates of (1) *H. equigenitalis* strain Cambridge, (2) *M. liquefaciens* NCTC 7911, (3) *M. non-liquefaciens* NCTC 10464, (4) *A. mucosus* NCTC 10303 and (5) *A. haemolyticus* NCTC 10305 Precipitation lines have been produced only by the *H. equigenitalis* extract.
(*c*) Immunodiffusion of rabbit antiserum to *A. haemolyticus* NCTC 10305 in the central well against the antigens in wells (1), (2), (3), (4) and (5) used in Plate 1*b*. All of the extracts except that of *H. equigenitalis* have produced precipitation lines against the antiserum.
(*d*) Immunodiffusion of rabbit antiserum to *M. liquefaciens* NCTC 7911 against the antigens in wells, (1), (2), (3), (4) and (5) used in Plates 1*a* and *c*. As in Plate 1*c*, precipitation lines have been produced by all of the extracts except that of *H. equigenitalis*.

PLATE 2

(*a*) Immunoelectrophoresis of *H. equigenitalis* strain Cambridge ultrasonicate against homologous rabbit antiserum.
(*b*) Diagrammatic representation and numerical identification of the precipitation lines produced by *H. equigenitalis* antigens on immunoelectrophoresis.

PLATE 3

Immunoelectrophoresis of *H. equigenitalis* strain Cambridge against serum from a horse convalescent from infection with the organism.

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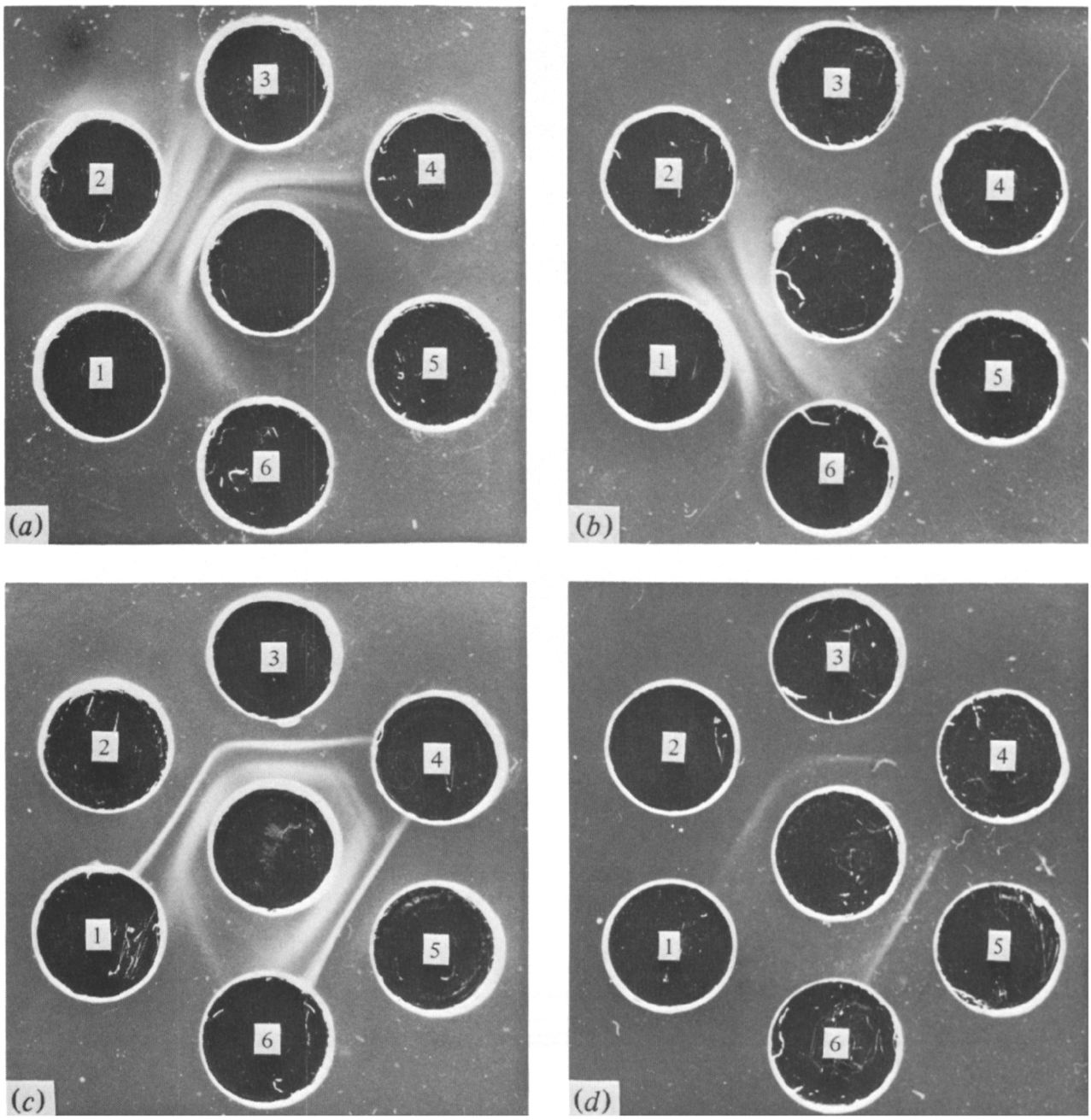

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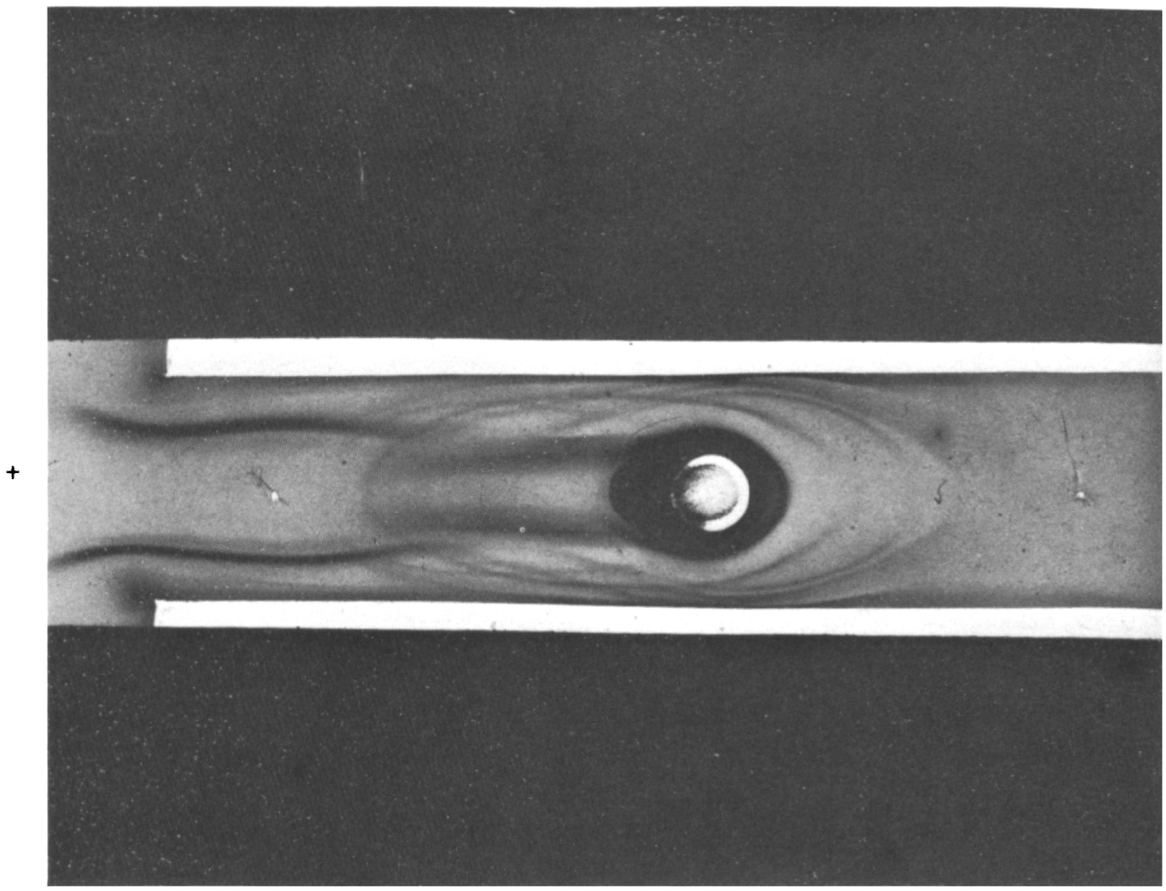
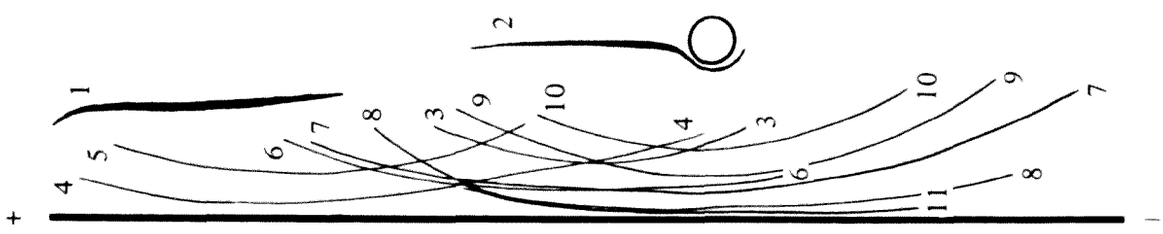


(*a*)



(*b*)

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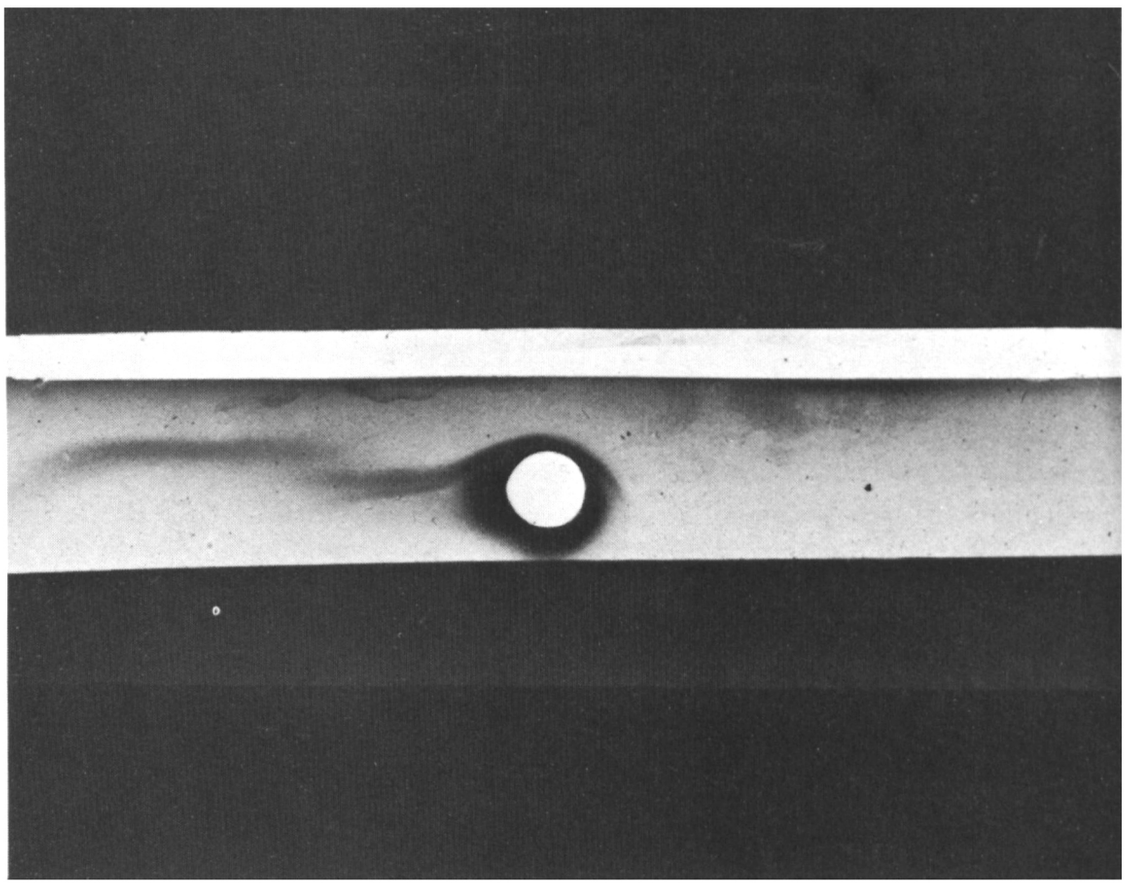

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