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TRYPSIN EXTRACTABLE PRECIPITATING ANTIGENS OF *LEPTOSPIRA INTERROGANS* (SEROTYPE *BIFLEXA*)

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Summary. Extraction of *Leptospira interrogans* (serotype, *biflexa*) with 0·2% trypsin yielded three precipitating antigens ('axial filament'; antigen 'd'; antigen 'e') as detected by immunodiffusion against rabbit antileptospiral serum.

In contrast to 'axial filament' antigen, antigens 'd' and 'e' could be detected only in stationary phase leptospires. Protein antigen 'd' was purified and gave rise to monoprecipitin sera in rabbits. Monoprecipitin sera against both antigen 'd' and 'e' were also produced by immunizing rabbits with specific immune precipitates in agar.

Rabbit anti-'d' and anti-'e' immunoglobulins were initially of the 19S type but subsequently became solely 7S.

INTRODUCTION.

The origins of this study stem from observations of Yanagawa and Faine (1966) on the precipitating antigens released from *Leptospira interrogans* (serotype, *biflexa*) after treatment with various chemicals, including enzymes. They identified the problems of the relationship between antigen and cellular components and the role, if any, of the antigens in infection with pathogenic leptospires.

These authors showed that the agglutinability of the leptospire was reduced as the envelope and axial filament were removed following treatment with 50% ethanol. This, and other treatments with deoxycholate, phenol or butanol, released from the cells antigenic material which could be detected by immunodiffusion against homologous antiserum. Precipitating antigens were also released with 0·2% trypsin.

This paper reports the properties of the unfractionated leptospiral 'trypsin extract' (TE), the purification of one of the precipitinogens (antigen 'd') and the production of monospecific antisera against the precipitating antigens.

MATERIALS AND METHODS.

Growth of leptospires.

Korthof medium (Alston and Broom, 1958), with the addition of thiamine (1 μg/ml) and cyanocobalamin (20 ng/ml) and containing 10% haemolysed rabbit serum, was used to grow cells for rabbit inoculations. Cultures were grown at 30°.

Korthof media containing 10% modified sheep serum (Faine, 1969) was used to grow large quantities of leptospires for trypsin extraction. For some experiments the leptospires were grown in a modified Ellinghausen-McCullogh/Johnson-Harris medium containing 6·2 g $Na_2HPO_4.2H_2O$; 1·5 g KH_2PO_4; 5·0 g NaCl; 1·25 g NH_4Cl; 25 mg Thiamine HCl; 0·5 g Glycerol; 10 μg Cyanocobalamin; 6·25 g Tween 80; 10 g Bovine Serum Albumin ('Pentex'); 10 mg $CaCl_2$; 10 mg $MgCl_2$; 50 mg $FeSO_4.7H_2O$. It was made up to 5 litres in distilled water, adjusted to pH 7·4 and sterilized by Seitz filtration.

Preparation of the leptospiral trypsin extract (TE).

The cells from 7-10 day (stationary phase) cultures were washed once in veronal buffered saline pH 7·3 (VBS) (Kabat and Mayer, 1964), and then made up to approx. 1/100th of the original culture volume in VBS. The culture was frozen and thawed three or four times and gently homogenized by hand using a teflon piston homogenizer to ensure an even suspension.

Trypsin (BDH) was added as a powder to a concentration of 0·2% (w/v) and the mixture was incubated for 4 h at 37° in a shaking water bath, centrifuged at 4,500 *g* for 20 min and the supernatant (TE) separated. The cellular sediment was designated 'trypsin treated cells'.

The TE was concentrated to approx. 10 mg/ml dry weight for use as TE antigen in immunodiffusion studies.

Preparation of sonicated leptospires.

Sonicated leptospiral suspensions were prepared from a 50-fold concentrated 7 day culture in rabbit serum Korthof medium, using an M.S.E. Ultrasonic Disintegrator at 20kHz for 45 sec. On examination by darkground microscopy no whole leptospires and only very small particles of disintegrated leptospires were visible.

Titration of antisera for agglutinating antibodies against leptospires.

Agglutinating titres of antileptospiral sera were determined by the leptospiral microscopic agglutination test (MAT), (WHO, 1967), using serial two-fold dilutions of the serum in VBS in 25 μl volumes in a microtitration system.

Total counts on leptospiral cultures.

The culture was diluted usually 1 in 2 or 1 in 4 with VBS and at least 400 leptospires counted in a Petroff-Hauser counting chamber.

Extractable antigens relative to age of culture.

(i) *Percentage of leptospires extracted with trypsin relative to age of culture.* The rabbit serum Korthof medium was inoculated with a 4 day culture containing $1·8 \times 10^8$ leptospires/ml to give an initial concentration of leptospires of approx. 9×10^6/ml.

On each of days 1, 2, 3, 4, 6, 8, 10 and 14 after inoculation an aliquot of 200 ml of the culture was taken on which total counts and dry weights were performed. The centrifuged leptospires from the 200 ml aliquot were freeze dried, and then extracted at a concentration of 10 mg/ml (dry wt.) by adding a volume of VBS containing 2 mg/ml trypsin. The dry weight of the extract was determined. The trypsin extracts were not dialysed prior to determining their dry weight.

(ii) *Antigens 'd' and 'e' in TE relative to age of culture.* The leptospiral culture was similar to that in experiment (i). A 5% inoculum of 4 day culture was used to inoculate the preheated medium from which 100 ml aliquot were taken on days 1, 2, 3, 4, 6, 8, 10 and 14. Dry weight determinations were made and each aliquot was adjusted to a dry weight of

32 mg/ml in VBS. To 0·8 ml of each was added 0·2 ml of 1% trypsin in VBS, giving a final concentration of 25·6 mg/ml of leptospires and 0·2% of trypsin. After extraction the preparations were tested without further centrifuging and dialysis for antigen 'd' and 'e' by immunodiffusion against antileptospiral serum.

After the presence of antigens 'd' and 'e' in the TE had been confirmed, titrations were performed by adding to an antigen well 0·5, 0·75, 1·0 or 1·25 mg TE and 50 µl of antileptospiral serum in the central antiserum well. Antigens 'd' and 'e' were thus titrated simultaneously in TE from leptospires of different ages. The smallest amount of TE required to give a visible precipitin band after 24 h diffusion at room temperature was noted.

Staining of immunoprecipitin bands.

Lipid was stained by Sudan Black B (Crowle, 1961). As a positive control, a lipaemic rabbit serum reacted against goat antirabbit serum (Pentex) was stained. Polysaccharide was stained using Crowle's (1961) No. 2 polysaccharide stain, involving a p-phenylenediamine oxidation reaction. As a positive control Group C streptococcal carbohydrate was reacted against homologous rabbit antiserum and stained.

Treatment of TE with enzymes.

(a) *Treatment of TE with 'Pronase'* (Calbiochem, B grade, *Streptomyces griseus* protease) was used at a concentration of 0·8 mg/ml, at pH 7. The mixture was shaken for 40 h at 37°. A control sample without enzyme was similarly treated. At the end of this period the mixtures were concentrated and examined by immunodiffusion.

(b) *Treatment of TE with Papain.* Papain ('Sigma', 2 x crystallized) was used at a concentration of 3 mg/ml. A volume of 0·4 ml enzyme was mixed with 0·4 ml of a solution containing approx. 4 mg antigen and to it was added 0·4 ml of 0·009M cysteine, neutralized just prior to addition to the antigen. The final mixture consisting of 1 mg/ml papain in 0·003M cysteine and a control mixture, lacking papain, were incubated at 37° for 1 h in a shaking water bath, then concentrated and examined by immunodiffusion.

(c) *Treatment of trypsin extract with lipase.* Lipase (hog pancreatic lipase, 'Calbiochem' B grade) was used at 0·7 mg/ml in the presence of 0·005M $CaCl_2$, at pH 8. The mixture was shaken for 40 h in a 37° water bath. A control without lipase was also set up. After the reaction the mixture was concentrated and examined by immunodiffusion.

Chemical tests.

Carbohydrate was measured by the phenol-sulphuric acid method (Dubois *et al.*, 1956) as described by Williams and Chase (1968). Protein was measured by the biuret method (Williams and Chase, 1968).

Electron microscopy.

400 mesh, copper grids with parlodion support and carbon coating were used in a Siemens 1A electron microscope working at 80K volts with a pointed filament.

Separation of constituents of the TE by gel filtration.

A 90 cm column of Sephadex G-200 was used in conjunction with a high ionic strength phosphate buffer, pH 7·2 (1·305M NaCl, 3·6 mM KH_2PO_4, 11·4 mM Na_2HPO_4 and 0·1% NaN_3), at a hydrostatic pressure of 20 cm and a flow rate of 6 ml/h. Fraction volumes of 5·5 ml were collected and concentrated to 0·1 ml for examination by immunodiffusion.

Salt fractionation of the TE by $(NH_4)_2SO_4$.

The TE was rendered salt free by dialysis against running water for 72 h, then adjusted to the required salt concentration by mixing with a given volume of $(NH_4)_2SO_4$ solution saturated at 4°. After standing overnight at 4°, the mixture was spun at 30,000 *g* for 15 min and the sediment stored separately from the supernatant. The supernatant was then further adjusted to a different salt concentration if necessary. The sediment was suspended in a little water and dialysed against running water to remove salt. It was then concentrated and examined by immunodiffusion for the detection of precipitating antigens.

Isoelectric point precipitation.

This was performed on salt-free mixtures, using HCl to decrease the pH in steps of 0·5 pH unit. At each pH step the mixture was left to stand for 15 min, centrifuged at 30,000 *g* for 15 min and the sediment removed.

Production of antileptospiral serum.

One rabbit antiserum was used throughout. The rabbit was immunized as follows:

Day 1: immunized with trypsinized leptospires in Freund's complete adjuvant, at about 20 intradermal sites on back and flanks.

Days 35 and 45: intravenous injection of 0·1 ml of trypsinized leptospires following prior dialysis overnight against 0·85% saline.

Day 64: intravenous injection of 5 ml of normal 4 day old leptospiral culture.

Day 72: bled.

Production of monoprecipitin antisera against antigen 'd' or 'e'.

An antigen-antibody complex, immunogenic with respect to its antigen component, was used to produce antisera specifically directed against the antigen in the complex.

The required precipitin band of either 'd' or 'e' specificity was excised from a number of individual immunodiffusions and washed in daily changes of 0·85% saline for one week to remove un-precipitated material. A control of agar was similarly treated. The agar precipitates were then homogenized in saline and mixed with an equal volume of Freund's complete adjuvant. One ml of the mixture was injected intradermally in doses of 50 µl at each of about 20 sites on the rabbits' shaved back and flanks. The rabbits were bled by marginal ear vein puncture twice a week. If serum antibody was not detectable after 6 weeks, the rabbits were re-immunized with the same antigen preparation in incomplete Freund's adjuvant, intramuscularly into both hind legs.

Purified antigen 'd' was inoculated with Freund's complete adjuvant, intradermally into rabbits (as above).

Preparation and use of immunoadsorbent.

An antiserum prepared as above directed specifically against a precipitating antigen of the TE was converted into an insoluble immunoadsorbent by cross-linking with either ethylchloroformate or glutaraldehyde (Avrameas and Ternynck, 1969). The serum proteins precipitated as a cross-linked gel which was then extensively washed by shaking followed by centrifugation with the following solutions seriatim (a) phosphate buffered saline (PBS), (b) 0·1% Na_2CO_3, (c) more PBS, (d) glycine HCl pH 2·2 buffer (until there was no further absorption at 280 nm, indicating no residual soluble protein), and finally (e) more PBS (pH 7·2).

The antigen mixture was mixed with the immunoadsorbent for 2 h at room temperature, with occasional stirring, and then centrifuged at 10,000 *g* for 10 min. The supernatant comprising the unadsorbed fraction was collected, concentrated and examined by immunodiffusion. The immunoadsorbent was washed with PBS and treated with 5·5M KI in pH 8·2 TRIS buffer to elute the antigen. The eluted fraction was cleared of particles of insoluble protein by filtration through a cellulose acetate membrane. After dialysis and concentration, the eluted fraction was examined by immunodiffusion.

Alternatively, the antigen was eluted from the immunoadsorbent with 0·1M glycine-HCl buffer, pH 2·8.

In some preparations the immunoadsorbent was regenerated immediately and reused in an attempt to produce a pool of antigen.

Immunoglobulin classes associated with anti-'d' and anti-'e' sera.

This was determined by gel filtration of monospecific antisera on Sephadex G-200.

Separation of serum constituents by gel filtration.

Sephadex G-200 was swollen in distilled water for 3 days, with occasional changes of water. It was finally washed several times in the eluting buffer comprising 0·05M 'TRIS', pH 8·0 in 1M NaCl with 0·1% sodium azide added. A column of 2·5 cm diameter was packed up to 90 cm, giving a total volume of 450 ml, and washed with 500 ml buffer before use at a hydrostatic pressure of 15-20 cm and a flow rate of 6 ml/h. Fractions of 5·5 ml were collected and monitored for absorption at 280 nm by an LKB Uvicord II.

Fractions were concentrated by negative pressure dialysis and examined for precipitin activity by immunodiffusion.

Leptospires.

The leptospire used in this study is a serotype *biflexa*, which reacted to a titre of 1:6,400 with antisera against *biflexa* LT 430 and *biflexa* Waz Reiden, and to a titre of 1:1,600 with antisera against *biflexa* Waz. It did not cross-react significantly with other strains in the *biflexa* group such as Patoc, Sao Paulo, CDC or Semaranga (Alexander, personal communication). It was designated 'strain 22' in this laboratory.

RESULTS.

Precipitating antigens of TE.

Precipitating antigens of the TE were detected by immunodiffusion against rabbit antileptospiral sera. One antiserum was used throughout the study. In immunodiffusion against TE this antiserum produced three distinct precipitin bands:

(i) 'axial filament' (AF) antigen (Chang and Faine, 1970)
(ii) antigen 'd'
(iii) antigen 'e' (Fig. 1).

Variations in the precipitating antigens present in the TE occurred when the cells were grown in a Tween-albumin medium. An additional precipitin line is shown in the immunodiffusion of TE from leptospires grown in Tween-albumin medium compared with the TE from leptospires grown in modified sheep serum Korthof medium.

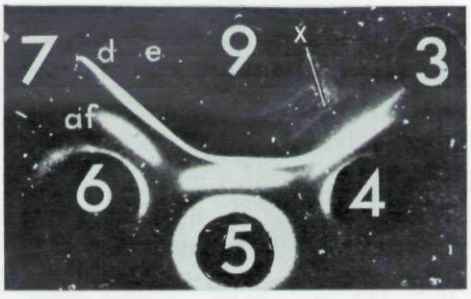

Fig. 1. Immunodiffusion of leptospiral trypsin extract (4, 5, 6) against antileptospiral serum (9) showing precipitin bands against axial filament antigen (af), antigen 'd' and antigen 'e'. The trypsin extract from leptospires grown in Tween-albumin medium (4) shows an additional antigen (x) compared with the trypsin extract from sheep serum Korthof medium (5, 6). Wells 3 and 7 contained saline.

The presence of TE precipitating antigens in untrypsinized leptospires was demonstrated by reactions of complete identity between the TE precipitins and their counterparts in the sonicated leptospiral suspension. This indicated that the TE antigens were not trypsin-induced artifacts.

Chemical studies on the TE.

(i) *Dry weight determinations.* Trypsin extraction of leptospires yielded approx. 21% of the dry weight of the organisms. Five per cent of the dry weight was released merely by shaking the cells for 4 h at 37° in VBS (non-trypsin dependent release).

Of the material extracted with trypsin, 72% was lost during 18 h dialysis and 80% lost during 48 h dialysis, leaving only 20% of the extracted material, including the precipitating antigens, as the non-dialysable fraction.

Thus, following adequate dialysis, the TE contained only 4% of the dry weight of the leptospires.

(ii) *Protein and carbohydrate determination.* The protein content of different leptospiral TEs varied between 73% and 101% and carbohydrate content from between 10% and 12%.

(iii) *Destruction with enzymes.* The precipitating antigens of TE were examined for sensitivity to various enzymes. The axial filament antigen and antigen 'e' were insensitive to the enzymes tested. Antigen 'd' was partially destroyed by papain but not by either of two preparations of the proteolytic enzyme Pronase. Further trypsin treatment up to 48 h did not affect any of the trypsin extracted antigens.

(iv) *Heat susceptibility.* The precipitating antigens of the TE were tested for heat lability by heating at 100° for 1 h in a water bath. Both the 'd' and 'e' determinants were destroyed.

(v) *Histochemical staining of immunoprecipitin bands.* Sudan Black B, a lipid stain, stained a control preparation but not the immunoprecipitates 'd', 'e' or axial filament, indicating that they did not contain lipid at concentrations detectable by this technique.

Crowle's (1961) polysaccharide stain No. 2 was used to detect polysaccharide in the immunoprecipitates but neither 'd', axial filament nor 'e' took up the polysaccharide stain any more strongly than the background agar.

Microscopy of TE.

Leptospires were washed once with VBS and then treated with 0·2% trypsin for 4 h at 37° in VBS. There was a reduction of approx. 30% in the number of cells recognizable as leptospires by darkground microscopy. A reduction of only 8% in the numbers of leptospires occurred if trypsin was added directly to the culture medium, without first resuspending the cells in VBS. Much cellular particulate matter was also visible by darkground microscopy after trypsin treatment.

By electron microscopy, the morphology of leptospires was seen to have been affected by an apparent loss of the outer cellular material (Fig. 2).

The TE (Fig. 3) contained mainly axial filaments, cellular debris and structures similar to striated tubes (Chang and Faine, 1970). No whole leptospires were seen.

Antigen content of leptospires as a function of age of culture.

This investigation was done in two parts each using a separate, but similar leptospiral growth experiment from which leptospires were harvested at different times. (i) the percentage of the leptospiral dry weight extracted with trypsin was determined, as a function of the age of the leptospiral cells, (ii) the amounts of antigens 'd' and 'e' measured serologically were determined for a given concentration of TE obtained from leptospiral cultures of different age.

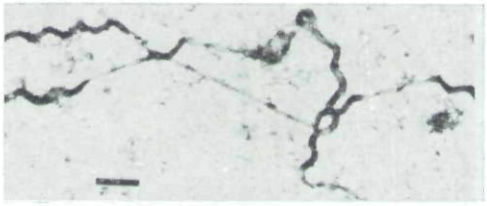

Fig. 2. Electron micrograph of trypsin-treated leptospires showing uneven degradation of the cell along the length of the leptospires. Stained with 0·5% uranyl acetate. Magnification: x 25,000. Bar: 400 nm.

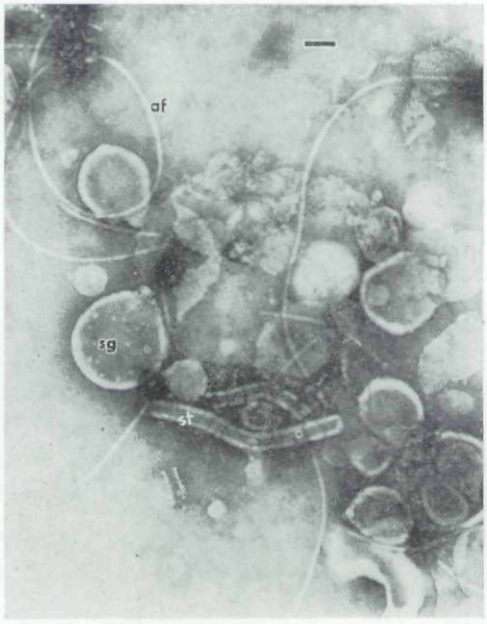

Fig. 3. An electron micrograph of the leptospiral trypsin extract showing axial filaments (af), the 'striated tube' structures (st), large spherical globules (sg) and other cellular debris. Stained with 0·5% uranyl acetate. Magnification: x 100,000. Bar: 100 nm.

(i) *Percentage of leptospires extracted with trypsin relative to age of culture.* Larger amounts of TE (26-30% of dry weight) were extracted from younger cultures aged 2-6 days than older cultures aged 8-14 days (19-22% of dry weight). This finding indicated that the chemical composition of leptospires changed quantitatively as the culture aged.

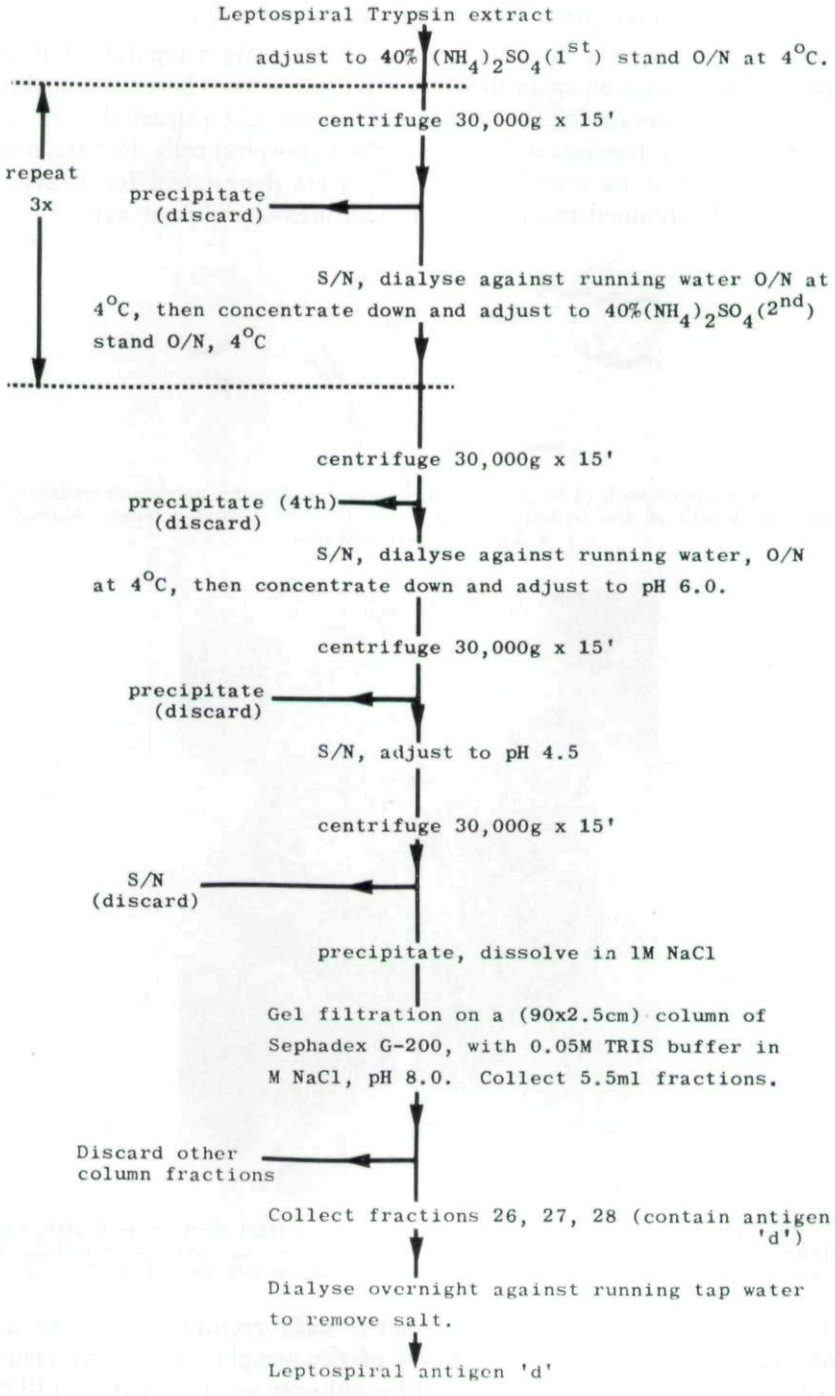

Fig. 4. Flow-sheet diagram for the preparation of leptospiral precipitating antigen 'd', free of other trypsin extract antigens.

(ii) *Antigens 'd' and 'e' in TE relative to age of culture.* Antigens 'd' and 'e' occurred in much lower concentration or were possibly absent in rapidly growing leptospires (day 1 and 2) and were present maximally in cells of the stationary phase (day 6-10). As the culture entered the decay phase, the concentration of both 'd' and 'e' in the cells also started to decline.

Changing cellular concentrations of antigens 'd' and 'e' were not reflected by changes in their concentration in the culture supernatant. Only axial filament antigen was detected in 50-fold concentrates of culture supernatants from 8 and 16 day cultures.

Purification of antigen 'd'.

The procedure used for preparing antigen 'd' is shown in Fig. 4. Adjustment of the TE to 40% saturation with $(NH_4)_2SO_4$ resulted in the precipitation of approx. 50% of the dry weight of the TE and containing axial filament (Fig. 5). Three further precipitations were necessary to completely remove all traces of axial filament antigens from the 40% $(NH_4)_2SO_4$ supernatant.

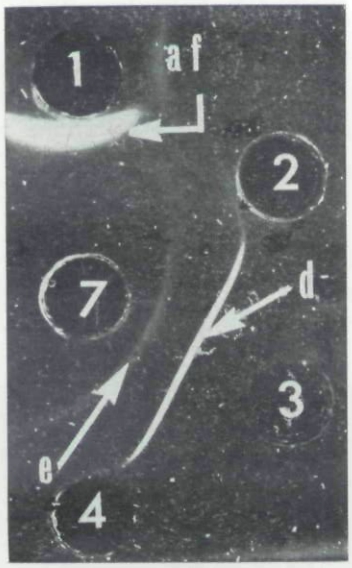

Fig. 5. Immunodiffusion of ammonium sulphate fractions of trypsin extract (TE) against antileptospiral serum. 1: 40% saturation $(NH_4)_2SO_4$ precipitate of TE. 2: 50% saturation $(NH_4)_2SO_4$ precipitate of TE after removal of the 40% saturation precipitate. 3: 60% saturation $(NH_4)_2SO_4$ precipitate of TE after removal of the 40% and 50% saturation precipitates. 4: 70% $(NH_4)_2SO_4$ precipitate of TE after removal of the 40%, 50% and 60% saturation precipitates. 7: Rabbit antileptospiral serum: af = axial filament antigen line, d = antigen 'd' line, e = antigen 'e' line.

The final 40% $(NH_4)_2SO_4$ supernatant contained antigens 'd' and 'e', although some antigen 'e' was lost in the 40% $(NH_4)_2SO_4$ precipitate. Both 'd' and 'e' were precipitated at 60% $(NH_4)_2SO_4$ saturation (Fig. 5). Some non-antigenic material present in the 40% $(NH_4)_2SO_4$ supernatant was precipitated by adjusting the supernatant (after dialysis against water and concentration) to pH 6·0.

When the pH was reduced further no precipitation occurred until pH 4·5 when antigens 'd' and 'e' precipitated from solution. A further precipitate which appeared at pH 4·0 did not contain antigens 'd' or 'e'.

Gel filtration of the antigen 'd' and 'e' mixture from the above preparation on a 90 cm column of Sephadex G-200 enabled some antigen 'd' to be isolated free of antigen 'e', but samples of antigen 'e' were always contaminated with antigen 'd'. Although antigen 'd' was eluted from the column first, only 8 ml after the void volume, elution was completed only after the passage of another 88 ml of buffer.

Antigen 'e' was eluted 30 ml after the void volume in a volume of 17 ml comprising 3 fractions, each 5·5 ml, which consequently contained both antigens 'd' and 'e'. In order to obtain purified antigen 'd', only the first three fractions containing antigen 'd' alone (3 x 5·5 ml) were collected (Fig. 4). It was not possible to obtain purified antigen 'e' free of antigen 'd'.

The molecular weight of antigen 'd' was calculated as approx. 430,000 daltons and antigen 'e' as approx. 280,000 daltons, using the formula of Determann (1968), applied to the elution data from the Sephadex G-200 column.

Immunogenicity of antigen 'd'.

Antigen 'd', prepared as shown in Fig. 4, with Freund's complete adjuvant, was immunogenic for rabbits. Anti-'d' precipitating antibodies appeared within 2 weeks of immunization.

Anti-'d' immunoadsorbent.

Anti-'d' serum, cross-linked to form an insoluble immunoadsorbent specifically absorbed antigen 'd' from the TE. However, attempts at subsequent use of the regenerated immunoadsorbent resulted in non-specific absorption of antigen 'e'.

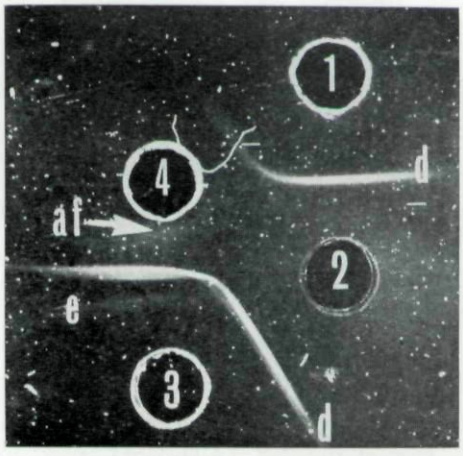

Fig. 6. The production of specifically anti-'d' antibodies by a rabbit immunized with 'd' anti-'d' immune precipitate in agar. 1: Rabbit anti-'d' serum—rabbit immunized with 'd' anti-'d' immune precipitate in agar. 2: Antigen 'd' (purified). 3: Rabbit anti-'whole leptospire' serum. 4: Leptospiral trypsin extract (TE), containing antigens 'd', 'e' and 'axial filament'.

Production of monospecific antisera.

Rabbits immunized with 'd' immune precipitates in agar produced 'd' specific precipitins (Fig. 6) and rabbits immunized with 'e' specific precipitin bands produced 'e' specific precipitins (Fig. 7).

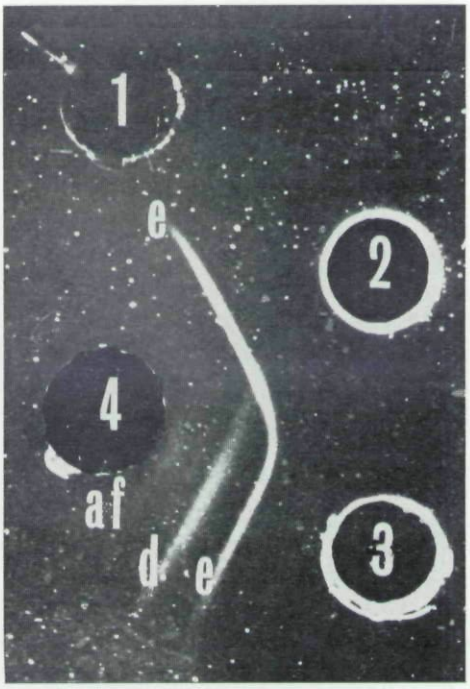

Fig. 7. The production of specifically anti-'e' antibodies by a rabbit immunized with 'e' anti-'e' immune precipitate in agar. 1: Rabbit anti-'e' serum, after immunization with 'e' anti-'e' precipitate in agar, but before second immunization (no antibody detectable). 2: Same as 1, but after second immunization of 'e' anti-'e' precipitation (anti-'e' antibody detectable). 3: Rabbit anti-'whole leptospire' serum, showing anti-'e' and anti-'d' antibodies. 4: Leptospiral trypsin extract (TE) containing antigens 'd', 'e' and 'axial filament'.

Immunoglobulin classes present in anti-'d' and anti-'e' sera.

Table 1 shows that in the case of the anti-'d' serum, both 19S and 7S immunoglobulin types were present on day 9 after initial immunization. Subsequently, however, the immunoglobulins became completely 7S. In the anti-'e' serum 19S immunoglobulins were also present initially, but without a 7S component. By 4 weeks after initial immunization, however, the antibodies were all 7S.

DISCUSSION.

Yanagawa and Faine (1966) attempted to correlate morphological destruction of leptospires with antigens released following chemical treatment by ethanol, deoxycholate, phenol, butanol, ethyl ether and trypsin. Amongst the limitations of this approach are: (i) the released antigens detectable serologically

TABLE 1.

Immunoglobulins associated with precipitating antisera directed against leptospiral antigens 'd' and 'e' at different stages of the immune response. Separation was on a 90 cm Sephadex G-200 column.

Days after immunization of rabbit with antigen	Specificity of monospecific antiserum			
	anti-'d'		anti-'e'	
	19S	7S	19S	7S
9	+	+	+	−
27	+	+	−	+
56	−	+	−	+

Key: − antibody of this type not present
+ antibody of this type present.

may be only a small part of the material released chemically. The removal of antigen may be either a consequence or a cause of the chemically induced morphological changes, (ii) chemical treatment may have caused chemical artifacts identified as antigens, or else an antigen of the whole cells may have been destroyed or reduced to something not detectable by the serological procedures used.

Few analyses of purified leptospiral antigen preparations have been made by immunodiffusion and immunoelectrophoresis (Yanagawa and Faine, 1966). Schricker and Hanson (1963) compared the serogroups pomona, autumnalis, sejroe and biflexa for common and specific precipitating antigens. They also isolated a precipitinogen ('S fraction') common to all strains of pomona tested. It was water soluble, heat labile (100° for 1 h) and contained 24% protein and 1% carbohydrate. Klatt (1964) compared 12 leptospiral serotypes by immunodiffusion with homologous and heterologous antisera using various types of antigenic preparations.

In this study the TE, taken as the starting material for the antigenic analysis, represented a reduced spectrum of leptospiral antigens, since trypsin-sensitive protein antigens would have been destroyed.

Chemical tests on the TE demonstrated both protein and carbohydrate. The extract contained 21% of the dry weight of the organism. However, about one quarter of this material (5% dry weight) was very loosely bound to the cell and was removed by shaking the leptospires in buffer without trypsin for 4 h. Thus, a simple mechanical method released some leptospiral material, possibly 'envelope' or other superficial components of the cell. Furthermore, 80% of the TE was lost in the preliminary purification step of dialysis, showing that much of the extract comprised small molecular weight compounds which were probably not antigenic. However, no change in the precipitinogens before and after dialysis was detected by immunodiffusion.

Antigen 'd' appears to be a protein because it was destroyed by papain and by heating at 100° for 1 h. Orskov and Orskov (1970) define the K antigens of *E. coli* which are destroyed by 100° for 1 h as 'heat labile'. They claim that such antigens are probably protein.

Histochemical staining of the 'd' anti-'d' immune precipitate in agar for carbohydrate did not reveal any carbohydrate. However, the sensitivity of this method is not known, and is further reduced because the specific carbohydrate stain was taken up slightly by the agar, itself a polysaccharide.

A specific stain for lipid failed to identify lipid in either the 'd' anti-'d' or the 'e' anti-'e' immune precipitate.

It was difficult to understand why neither of two different preparations of the broad spectrum proteolytic enzyme Pronase destroyed antigen 'd'. However, Pronase was reported not to destroy the axial filament which is known to be protein (Nauman *et al.*, 1969).

The effects of trypsinization as seen by electron microscopy of trypsin-treated leptospires and of the TE itself were to:

1. Remove outer layers of the leptospire including in some cases material apparently responsible for the maintenance of helical structure (the 'perimural layer' of Yanagawa and Faine, 1966). The extent of chemical dissection seemed to vary along the length of the leptospire and from one cell to another.

2. Reduce the numbers of leptospires seen by darkground microscopy more effectively when the cells were suspended in VBS, than in the original culture medium.

3. Release axial filaments from the cell (Chang and Faine, 1970).

4. Release leptospiral 'striated tube' structures of uncertain origin and antigenicity (Chang and Faine, 1970).

A comparison of the antigenic content of the leptospiral cells relative to the age of the culture could help to determine the nature and role of the antigens in the whole cell. Kasarov and Addamiano (1969) noted that the phospholipase activity of leptospiral cultures varied with the age of the culture, although it was not clear whether this activity was corrected for the number or dry weight of leptospires. In other studies of gram negative bacteria, immune lysis was affected by the metabolic state of the bacteria (Melching and Vas, 1971).

In this study it was shown that more antigen was extracted from actively growing leptospires with trypsin than from older cells, indicating some chemical changes in the leptospires as they age. On the other hand, antigens 'd' and 'e' were not detectable in logarithmically growing cells, but only in stationary phase leptospires. Their apparent absence in rapidly growing cells makes it unlikely that they would be essential cellular components or even enzymes. They are more probably by-products of sub-optimal growth conditions. In a similar situation *E. coli* growing rapidly contained only 3% glycogen but when growing slowly due to limiting nitrogen contained 23% glycogen (Holme, 1957). The absence of antigens 'd' and 'e' from the culture supernatants of both young and old cultures indicates that they are not released into solution on lysis and degradation of the old cells. They must therefore be part of the structure such as cell wall or membrane in cells of old cultures. An electron microscopic study might detect morphological differences in leptospires taken from log phase and stationary phase of growth.

It was necessary to separate the leptospiral precipitating antigens present in the TE to investigate their individual properties. Antigen 'd' was separated from the other antigens of the TE but antigen 'e' was not successfully purified.

Ammonium sulphate precipitation and isoelectric point precipitation removed axial filament antigen and non-antigenic material from the TE. Antigen 'd' was purified by subsequent gel filtration on Sephadex G-200 although fractions containing antigen 'e' always contained antigen 'd' as well.

Other separation procedures were attempted unsuccessfully. The leptospiral antigens failed to attach to the CM-cellulose column, and were so strongly bound to DEAE-cellulose that 0·2M NaCl would not elute them. Higher salt concentrations removed the antigens but they had apparently been irreversibly altered and would not precipitate with antiserum to form bands in the normal manner. Antigens 'd' and 'e' were both negatively charged at pH 7·3. By lowering the pH this charge was neutralized until the isoelectric point was reached at pH 4·5. Since antigens 'd' and 'e' could not be separated by ion exchange chromatography and both antigens precipitated at pH 4·5, their overall electrical charge was presumed to be very similar.

Although the final preparation of antigen 'd' gave only one precipitin line in immunodiffusion and immunoelectrophoresis with antileptospiral sera, there is no other evidence that it was a pure preparation of one macromolecular compound free from non-antigenic material.

The formula of Determann (1968) for calculating the molecular weight of a globular protein on the basis of its retention by Sephadex G-200 was used to estimate the molecular weight of the antigen 'd 'as approximately 430,000 daltons and antigen 'e' as approximately 280,000 daltons. This procedure for estimation of molecular weight assumes the molecule to be spherical and to have no chemical interaction with the gel material. To make a more accurate estimate of molecular weight, molecules of known size may be used to standardise a particular column. Immunoadsorbents prepared with either glutaraldehyde or ethylchloroformate cross-linked immune monospecific sera were used in this study. The technique was of limited value and was eventually discarded because of non-specific adsorption or other antigens and presumably of non-antigens on to the immunoadsorbent and their subsequent elution. Furthermore, the technique was unsuitable for preparative production of antigens using reasonable quantities of antiserum because such small quantities of antigen were adsorbed each time.

The elution of antigen 'e' from the anti-'e' immunoadsorbent and its use to immunise a rabbit gave rise to an antiserum that initially contained only anti-'e' antibodies. However, the antigen 'e' preparation was contaminated with other antigens since the rabbit subsequently produced other precipitin lines. Nevertheless, the change in anti-'e' immunoglobulin type was observed (Table 1). Both anti-'d' and anti-'e' antibody were initially 19S and gradually changed to 7S, with no persistent 19S component. This change is characteristic of protein antigens, in contrast to antigens with polysaccharide determinants, which generally give rise to a persistent 19S response (Pike, 1967).

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