

## Properties of a cell-wall-defective variant of *Brucella abortus* of bovine origin

BY M. J. CORBEL, A. C. SCOTT

*Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory,  
New Haw, Weybridge, Surrey*

AND H. M. ROSS

*The North of Scotland College of Agriculture, Veterinary Investigation Centre,  
Drummondhill, Stratherrick Road, Inverness, IV2 4JZ*

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### SUMMARY

The properties of an atypical *Brucella* strain isolated from lymph node tissue of a cow slaughtered as a brucellosis reactor were examined. The organism was Gram negative and highly pleomorphic, existing as cocci, coccobacilli, rods, branched and irregular forms which stained with fluorescent antibody conjugates prepared against rough and smooth *Brucella abortus* strains. It produced lecithinase and required at least 15% v/v equine or bovine serum for growth. It did not need supplementary CO<sub>2</sub> for growth, produced H<sub>2</sub>S and was inhibited by brucella dyes and partially by *i*-erythritol. Growth inhibition or lysis was produced by brucella-phages. The organism was not pathogenic for guinea-pigs or mice but evoked antibodies mainly to rough *Brucella* antigens.

### INTRODUCTION

It is not uncommon for cattle giving positive reactions to *Brucella abortus* in serological tests to produce negative results on cultural examination at subsequent slaughter. Probably in many cases this results simply from failure to sample those portions of tissue actually containing the bacteria, especially in lightly infected animals. In other instances the serological reactions may have resulted from abortive infection, vaccination or exposure to the cross-reacting antigens of unrelated organisms (Corbel, 1975).

In a proportion of cases it is possible that active *Brucella* infection is present but that the organisms, although in a viable state, may have become modified as a result of growth *in vivo* to such an extent as to be incapable of propagation in ordinary culture media. Thus Nelson & Pickett (1951) reported that organisms described as cell-wall-defective forms of *Brucella* could be detected in cultures of some human blood samples. The properties of these organisms were similar to those described for the L-forms of other bacterial species and for *Brucella* spheroplasts induced artificially in special media (Hines, Freeman & Pearson, 1964*a*; Hatten, 1973).

Because the presence of organisms of this type in bovine tissues could explain some of the serological reactions encountered under field conditions, attempts were made to isolate such variants by using modified cultural procedures. During the course of this work, an organism was isolated whose properties resembled those described for *Brucella* L-forms by Nelson & Pickett (1951). The characteristics of this organism have been examined in detail.

#### MATERIALS AND METHODS

##### *Bacterial strain*

The isolate, designated 'Elgin', was cultured from homogenates of the retro-pharyngeal and internal iliac lymph nodes of a cow slaughtered as a reactor following routine serological testing for brucellosis. The herd had been accredited as free of brucellosis for the past 7 years and the only known exposure of the animal to live *Brucella* was calthood vaccination with *B. abortus* strain 19. The organism was isolated on serum dextrose antibiotic agar (Farrell, 1974) containing 15% v/v equine serum and incubated in air + 10% v/v CO<sub>2</sub> for 8 days. Subcultures were made on Eaton's mycoplasma agar prepared as described by Thorns (1978). Subsequent propagation was on this medium or on serum dextrose agar containing 20% v/v heated equine serum.

##### *Examination of cultural properties*

The ability to produce growth on standard *Brucella* culture media including those containing *i*-erythritol or the dyes basic fuchsin, thionin, thionin blue and safranin O, was determined as described by Corbel, Gill & Thomas (1978). Similar procedures were also used with media in which the serum concentration of the serum dextrose agar basal medium was increased to 20% v/v. Sterile heat-inactivated serum of bovine, chicken, equine, porcine or rabbit origin was used in some batches. For the examination of lecithinase activity and film production, 10% v/v egg yolk was incorporated into the medium in addition to the serum.

The procedures used for determining CO<sub>2</sub> dependence, H<sub>2</sub>S production, urease activity, catalase activity, nitrate reduction and fermentation reactions were as described by Corbel *et al.* (1978).

##### *Morphological examination*

*Mycoplasma*-type colonies were examined by the agar block method using interference-phase contrast microscopy. Impression smears made of the agar blocks were stained by a modification of the Dienes method (Madoff, 1960) or, after ethanol fixation, with Giemsa stain, dilute carbol fuchsin or by Gram's method. Smears of bacterial-type colonies were ethanol-fixed and stained by Gram's method or with Giemsa stain or dilute carbol fuchsin, before light microscopy.

Preparations of organisms for electron microscopy were made by scraping the agar surface with a plastic loop, resuspending the material removed in a few drops of Albimi brucella broth and applying this to copper grids. After negative staining with phosphotungstate pH 6.6, the preparations were examined in the Philips EM 300 electron microscope.

*Serological examination*

Slide agglutination tests with antisera made monospecific for the A, M and R antigens of *Brucella* were done as described by Corbel *et al.* (1978). The organisms were examined for dissociation by testing their ability to form stable suspensions in 0.15 mol/l NaCl and 0.1% w/v acriflavine as described by Corbel *et al.* (1978).

Fluorescent antibody tests were performed on smears fixed with ethanol at room temperature for 15 min. The  $\gamma$ -globulin fraction of antiserum prepared against smooth *B. abortus* 544 was conjugated with fluorescein isothiocyanate as described by Corbel (1973). A fluorescent conjugate was prepared from the  $\gamma$ -globulin fraction of rabbit antiserum to rough *B. abortus* 45/20 by an identical procedure. The direct fluorescent antibody test was performed as described previously (Corbel, 1973).

*Determination of phage sensitivity*

The brucella-phages used were the Tbilisi (Tb), Weybridge (Wb), Berkeley (Bk2), R and R/O strains. The properties of these, with the exception of phage R/O, have been described previously (Morgan, 1963; Morris & Corbel, 1973; Corbel, 1977; Douglas & Elberg, 1976). Phage R/O was derived from phage R by serial passage in cultures of *Brucella ovis* 63/290. It showed a wide range of lytic activity towards non-smooth cultures of *Brucella* species with the exception of *B. canis*.

The phage preparations were used at concentrations corresponding to the routine test dilution (RTD) and  $10^4$  RTD as determined on their respective propagating strains. Phage sensitivity tests were performed by the standard procedure as described by Corbel *et al.* (1978) but using both RTD and  $10^4$  RTD concentrations. The plates were incubated at 37 °C for up to 7 days.

Cultures were examined directly for phage adsorption by scraping colonies off the agar surface with a plastic loop and resuspending in 0.1 ml volumes of phage suspension containing approximately  $10^9$  p.f.u. per ml. These were incubated at 37 °C for 30 min and samples were then applied to copper grids, stained with phosphotungstate pH 6.6 and examined in a Philips EM 300 electron microscope.

*Examination of animal pathogenicity*

Suspensions for inoculation were prepared by scraping colonies off the surface of plates showing near-confluent growth and suspending them in serum dextrose broth. Volumes of 1 ml of suspension, containing approximately  $10^9$  bacterial cells, were injected subcutaneously into groups of 12 specific-pathogen-free weaned guinea-pigs and 12 albino mice. The guinea-pigs were divided into 3 groups of 4 animals, each of which were killed at intervals of 10, 21 and 42 days after inoculation. The mice were killed 2 weeks after inoculation.

The spleens from all animals were homogenized in serum dextrose broth and replicate 0.2 ml volumes plated out on to Eaton's mycoplasma agar and incubated at 37 °C for 14 days. The serum from the guinea-pigs was examined for antibodies to rough and smooth *B. abortus* antigens using the serum agglutination test

(SAT), the complement fixation test (CFT), the Rose Bengal plate test (RBPT), the Coombs antiglobulin test (CAGT) and the immunodiffusion test (IDT) as described by Corbel & Bracewell (1976).

## RESULTS

### *Cultural properties*

The organism was initially isolated on serum dextrose antibiotic medium enriched with 15% v/v equine serum and incubated in air + 10% v/v CO<sub>2</sub> for 8 days. Signs of growth did not appear until the sixth day of incubation and were visible to macroscopic examination only as areas of film on the medium. Under the stereomicroscope, colonies of typical *Mycoplasma* appearance were observed within these areas. These were minute granular 'fried egg' type colonies up to 0.05 mm in diameter. They and the adjacent agar surface were surrounded by a finely granular iridescent film of lipid material (Pl. 1, fig. 1). Under high-power magnification with interference-phase contrast illumination, the granular nature of the colonies and their lack of uniform consistency were apparent (Pl. 1, fig. 2). The colonies were firmly embedded within the agar surface and could not be removed with a loop.

Agar block preparations of colonies stained by the modified Dienes method took up the stain moderately well, but on examination under high-power magnification individual organisms of bacterial morphology could be seen which had adsorbed the stain rather poorly (Pl. 1, fig. 3).

The 'Elgin' isolate did not grow on serum dextrose agar containing 5% or 10% v/v equine serum. A minimum concentration of 15% v/v horse serum was required for growth. The optimum serum concentration was 20 to 25% v/v. The organisms were not inhibited by the antibiotics present in Farrell's serum dextrose agar nor by benzyl-penicillin or ampicillin at concentrations of up to 500 µg per ml. Growth on Eaton's mycoplasma medium was improved if the phenol-red indicator was omitted.

The organisms did not grow on serum dextrose agar or Eaton's mycoplasma agar in which the horse serum had been replaced by an equal concentration of rabbit, pig or chicken serum. Some growth developed on these media when 20% v/v bovine serum was used to replace the horse serum, but film was not produced around the colonies.

Because of their resemblance to *Mycoplasma* strains, the organisms were sub-cultured onto Eaton's mycoplasma agar. They grew fairly well on this medium, producing visible colonies after 48 h which were identical in appearance with those seen on the original plate after 6 or 7 days incubation at 37 °C in air + 10% v/v CO<sub>2</sub>.

During the course of subculture on Eaton's mycoplasma agar, a different colonial form appeared. These were small raised hemispherical colonies with a circular outline and an entire edge. They had a smooth shiny surface and were colourless, soft and easily emulsified. The colonies were approximately 0.1 mm in diameter after 3 days incubation at 37 °C and reached a maximum diameter of 0.2–0.3 mm

after 7 days. They resembled minute smooth *Brucella* colonies in appearance but they did not form stable suspensions in saline or acriflavine solutions. Furthermore, between 3 and 4 days after their appearance they became surrounded by film. The proportion of this colonial type increased with repeated subculture until, after about 20 serial passages *in vitro*, nearly all the colonies produced were of this type.

Growth was enhanced on Eaton's medium supplemented with 10% v/v egg yolk. Opacity developed very rapidly in the medium surrounding the colonies and film production was also increased. On prolonged incubation, there was a tendency for the opaque areas to clear slightly. The egg yolk could not replace horse serum as a growth requirement for the organism; growth was best on medium containing both materials. Film deposit scraped off the agar surface gave a positive reaction for phospholipids with the Dragendorff reagent, indicating that it was probably produced by the action of a phospholipase on lecithin and other phospholipids in the culture medium.

Growth was inhibited on serum dextrose agar + 20% v/v equine serum containing basic fuchsin at 1/50 000, thionin at 1/50 000, thionin blue at 1/500 000 or safranin O at 1/10 000. Growth was not inhibited on the same medium supplemented with *i*-erythritol at 1 mg/ml but was inhibited if the concentration was increased to 2 mg/ml.

The growth of the organism was not dependent upon the presence of supplementary CO<sub>2</sub> nor was it improved by this. Growth was improved if incubation was in a humidified atmosphere, equivalent to at least 70% saturation.

Growth was not produced in Albimi brucella broth, trypticase soy broth or serum dextrose broth, even if these were rendered hypertonic by addition of sucrose at 1 mol/l. Inconsistent results were obtained with Eaton's liquid medium, growth being obtained in only 2 out of 8 attempts. No growth was produced in thio-glycollate-containing media.

H<sub>2</sub>S was produced in small quantity, but because of the poor growth of the organism incubation had to be continued for at least 8 days before any change could be detected on lead acetate papers.

Urease production could not be assessed because no growth was produced on Christensen's medium. Similarly, insufficient growth was obtained for valid results to be produced in nitrate reduction or sugar fermentation tests. The organisms were catalase positive. The oxidase reaction was not determined.

### *Morphology*

It was difficult to prepare smears of the 'fried-egg' colonies, and impression smears were made of these by the agar block method. Individual organisms could be seen in these preparations after staining with Giesma stain, dilute carbol fuchsin or by Gram's method. The organisms were highly pleomorphic, occurring as clumps of cocci, coccobacilli, club shaped forms, branched forms, distorted rods and a variety of irregular shapes (Pl. 2, fig. 1). They varied in size from about 0.3 μm × 0.4 μm up to 0.8 μm × 2.0 μm. The cells were Gram negative but tended to be irregularly decolorized by acetone. They showed weakly acid-fast staining with dilute carbol fuchsin and were stained well by Giemsa stain. Irrespective of

the staining method, the organisms did not produce sharp images on microscopic examination but tended to have an indistinct outline. A similar effect was noted on electron microscopic examination.

In ethanol-fixed smears stained with the FITC-conjugated  $\gamma$ -globulin fraction of rabbit antiserum to smooth *B. abortus* 544 or rough *B. abortus* 45/20, clumps of fluorescent organisms were seen on examination under ultra-violet illumination. The morphology of these was similar to that of the cells seen in smears stained by tinctorial methods, with branched and irregular forms occurring frequently (Pl. 2, fig. 2). In negatively stained preparations examined in the electron microscope, these observations were confirmed. The organisms were highly pleomorphic and varied in appearance from cells virtually identical with those of normal *Brucella* strains to branched forms, 'collapsed balloon' structures and filamentous forms (Pl. 3, figs. 1, 2).

Many of the cells were extremely fragile and easily disrupted during preparation. Most showed rather indistinct outlines and were difficult to focus upon. This was exacerbated by their sparse growth and tendency to autoagglutinate in serum-free medium.

The morphological properties of the organisms from the small 'Brucella-like' colonies were identical with those of the organisms from the 'fried-egg'-shaped *Mycoplasma*-type colonies.

#### *Phage sensitivity*

In tests with brucella phage strains Tb, Wb, Bk2, R and R/O at 10<sup>4</sup> RTD, distinct areas of growth inhibition or lysis were produced on areas of near-confluent growth of the 'Elgin' culture (Pl. 4). When phage preparations standardized at RTD were used, the effects were less definite. Zones of semi-confluent lysis and small plaque formations were produced by phages R and R/O and to a lesser extent Tb. Little or no lysis was produced by the Bk2 and WB phages.

The phage interactions were confirmed by direct examination of phage + bacterium preparations in the electron microscope. These showed attachment of phage particles to the cell envelopes. The multiplicity of infection observed was very low in relation to the phage input, suggesting a reduction in the number of phage receptors relative to normal *Brucella* cells (Pl. 3, figs. 1, 2).

#### *Serological properties*

Stable suspensions of the organism could not be prepared in any medium tested. Rapid autoagglutination occurred in water, 0.15 mol/l NaCl, 0.1% w/v acriflavine and 0.1 mol/l phosphate buffer, pH 7.2. Agglutination occurred in rabbit antisera made specific for the A, M and R antigens of *Brucella* species. Fluorescent antibody staining reactions indicated the presence of antigens occurring in rough and smooth *B. abortus* organisms on the surface of the 'Elgin' strain cells. These reactions were confirmed by absorption tests.

Table 1. *The serological response of guinea-pigs to rough and smooth B. abortus antigens following inoculation with the 'Elgin' isolate*

Animal number	Days after inoculation	Reciprocal titres in serological tests									
		Smooth <i>B. abortus</i> S 99					Rough <i>B. abortus</i> 45/20				
		SAT	CAGT	RBPT	CFT	IDT	SAT	CAGT	RBPT	IDT	
GPL 1	10	< 10	< 10	—	< 2	—	< 10	< 10	—	—	
GPL 2		< 10	< 10	—	< 2	—	< 10	< 10	—	—	
GPL 3		< 10	< 10	—	< 2	—	< 10	20	—	—	
GPL 4		< 10	10	—	< 2	—	< 10	20	—	—	
GPL 5	21	< 10	< 10	—	< 2	—	< 10	40	—	—	
GPL 6		< 10	< 10	—	< 2	—	< 10	40	—	—	
GPL 7		< 10	< 10	—	< 2	—	< 10	20	—	—	
GPL 8		< 10	< 10	—	< 2	—	< 10	40	—	—	
GPL 9	42	< 10	< 10	—	< 2	—	< 10	< 10	—	—	
GPL 10		< 10	< 10	—	< 2	—	< 10	10	—	—	
GPL 11		< 10	< 10	—	< 2	—	< 10	10	—	—	
GPL 12		< 10	< 10	—	< 2	—	< 10	< 10	—	—	

*Animal pathogenicity*

Inoculation of approximately  $10^9$  cells of the 'Elgin' strain into guinea-pigs and mice failed to produce any signs of disease. The organisms could not be recovered on culture of the tissues of animals killed between 10 days and 6 weeks after inoculation. No gross lesions were detected in the internal organs of the animals at post-mortem examination. Serological tests on the sera of the inoculated guinea-pigs indicated the presence of antibodies active against rough *B. abortus* 45/20 in the antiglobulin test. One of these animals also reacted with smooth *B. abortus* S99 antigen in the antiglobulin test. No complement fixing or agglutinating antibodies were detected to either rough or smooth *Brucella* strains (Table 1). Insufficient organisms were obtained to permit pathogenicity studies in domestic animals.

## DISCUSSION

Initially, the colonial morphology of the 'Elgin' isolate and its production of film on horse serum-enriched medium led to its presumptive identification as a *Mycoplasma* strain. This was discounted on microscopic examination of stained smears of the organism which disclosed its bacterial morphology. The bizarre appearance of many of the cells in the smear suggested that the organism might be a bacterial L-form, although the identity of the parent species was not apparent at that stage.

Because the serological status of the host animal indicated a recent exposure to *Brucella*, or organisms possessing serologically similar antigens, the isolate was examined with fluorescent antibody prepared against *B. abortus*. The extent of the cross-reaction with antisera prepared against both smooth and non-smooth *Brucella* strains suggested that the 'Elgin' culture was an anomalous *Brucella* isolate. This was confirmed by its sensitivity to lysis or inhibition by brucella phages. The sensitivity to Tb and R phages, considered in the context of the highly dissociated nature of the culture, was consistent with its identification as a *B. abortus* strain (Corbel *et al.* 1978).

The specificity of the phage sensitivity tests was confirmed by the results of electron microscopic examination of negatively stained preparations. These clearly demonstrated adsorption of the phage particles to the cell surface of the bizarre forms. The appearance of the bacterial cells in both the light and the electron microscope was similar to those described previously for *Brucella* L-forms isolated from clinical material and for *Brucella* spheroplasts induced artificially *in vitro* by antibiotics or glycine (Nelson & Pickett, 1951; Hines, Freeman & Pearson, 1964*a, b*; Roux & Sassine, 1971; Hatten, 1973; Peschkov & Feodorov, 1978).

Unlike artificially induced spheroplasts, the 'Elgin' isolate was not osmotically sensitive and in fact did not grow in the hypertonic medium which was nutritionally adequate for most *Brucella* strains. Its unusual serum requirement probably had a nutritional basis and may have reflected a high lipid turnover in the cell membrane, possibly required to compensate for an inadequate cell-wall structure. The bizarre morphology of the isolate was certainly consistent with a cell-wall



defect and was similar to that described for many bacterial L-forms (Mattman, 1974). The production of film from horse serum and egg yolk was clearly a result of lecithinase activity. Although extracellular lecithinase production is not a recognized characteristic of *Brucella* cultures most, if not all, micro-organisms contain membrane-bound phospholipases (Gatt, 1973). It seems possible that in this instance, the enzyme was released into the medium as a result of increased permeability of the defective cell-wall structure.

Because of its unusual growth requirements and its dissociated state, it was not possible to determine the biotype of this *Brucella* isolate. Nevertheless the sensitivity of the culture to growth inhibition by dyes and *i*-erythritol was consistent with derivation from *B. abortus* strain 19. As this was the only known exposure of animals in this particular herd to live *Brucella*, it seems the most probable source of the isolate.

The factors responsible for the anomalous properties of the 'Elgin' isolate are not known, but the animal had a history of exposure to penicillin and to diethylstilboestrol. Penicillin is well recognized as an inducer of cell-wall-defective forms of many bacterial species, including *Brucella* (Hines *et al.* 1964*a*; Roux & Sassine, 1971; Hatten, 1973). Diethylstilboestrol and other hormones have been reported to induce development of *Brucella* L-forms *in vitro* (Meyer, 1976). Thus either or both of these factors may have been instrumental in inducing formation of cell-wall-defective forms from residual *B. abortus* strain 19 organisms persisting in the vaccinated cow.

A further possibility is that the cell-wall-defective organisms may have been produced as a result of exposure to the immune processes of the animal. Although only limited evidence of this is available (McGhee & Freeman, 1970), it is conceivable that such anomalous variants arise *in vivo* more frequently than has hitherto been realized. Such organisms would not be detected with the usual *Brucella* culture media. Whatever the source of the 'Elgin' isolate, the present results suggest that the tissues of *Brucella* reactors should be cultured on medium enriched with a high concentration of horse serum in addition to those normally used.

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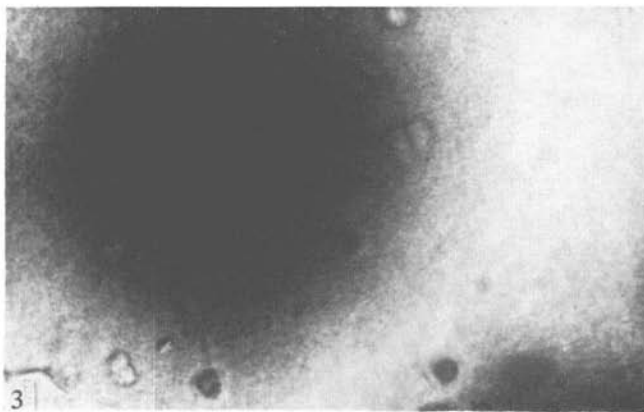
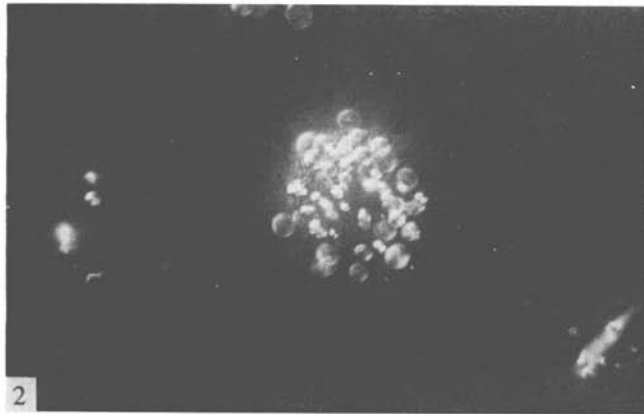
## EXPLANATION OF PLATES

## PLATE 1

Fig. 1. Appearance of colonies of the cell-wall-defective *B. abortus* isolate 'Elgin' on primary isolation. The colonies have a typical 'fried-egg' shape and a granular surface, resembling superficially those of mycoplasmas. A dense refractile deposit of lipid film produced by degradation of the culture medium is seen to the right of the picture. Interference-phase contrast,  $\times 100$ .

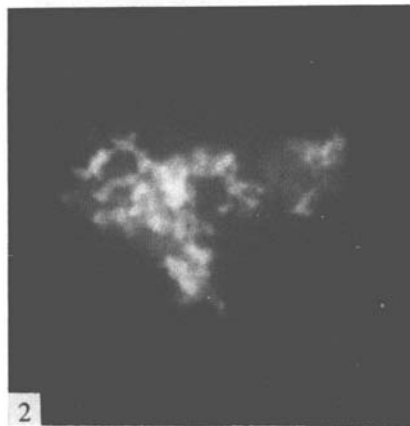
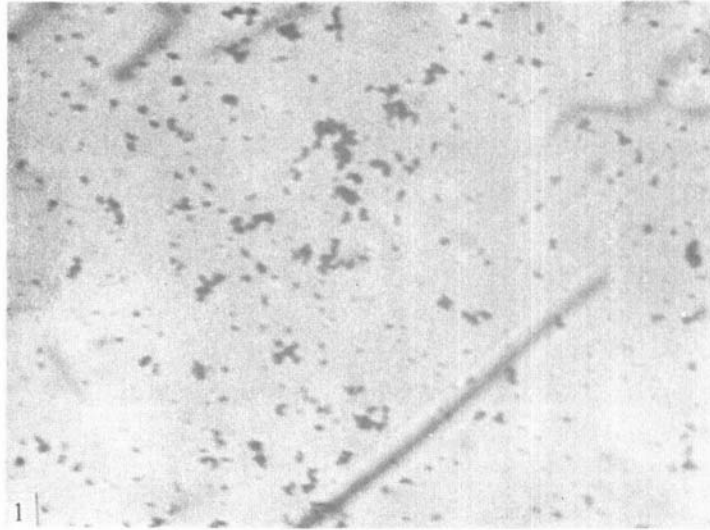
Fig. 2. Appearance of one of the colonies shown on Pl. 1, fig. 1, under higher magnification. The refractile spherical particles on the surface are probably lipid deposits. Interference-phase contrast,  $\times 250$ .

Fig. 3. Microscopic appearance of an agar block preparation of the culture shown in Pl. 1, figs. 1, 2 after staining with Dienes stain. The colony is composed of numerous lightly stained individual particles. Its appearance in this preparation is typical of a bacterial rather than mycoplasmal colony. Dienes stain,  $\times 600$ .

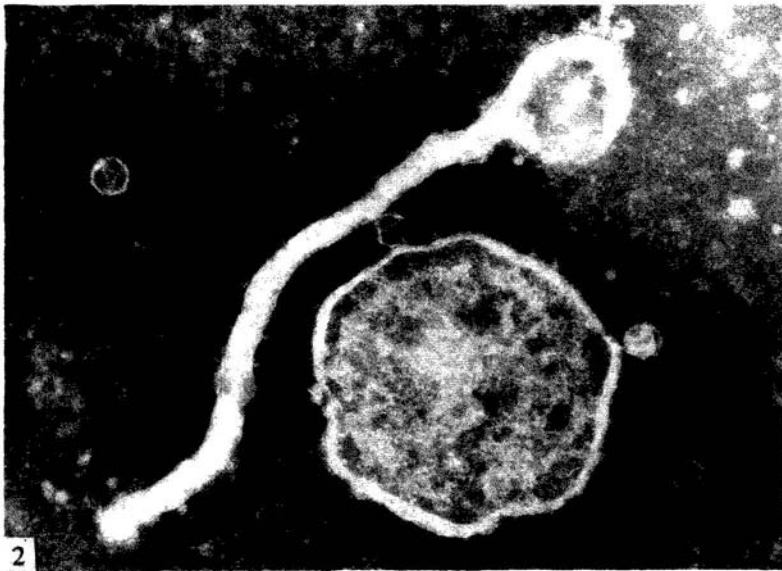
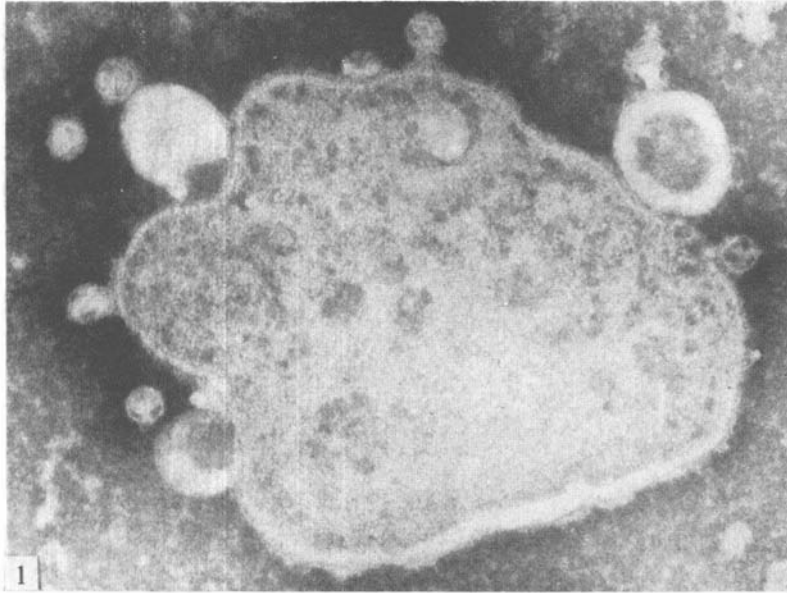


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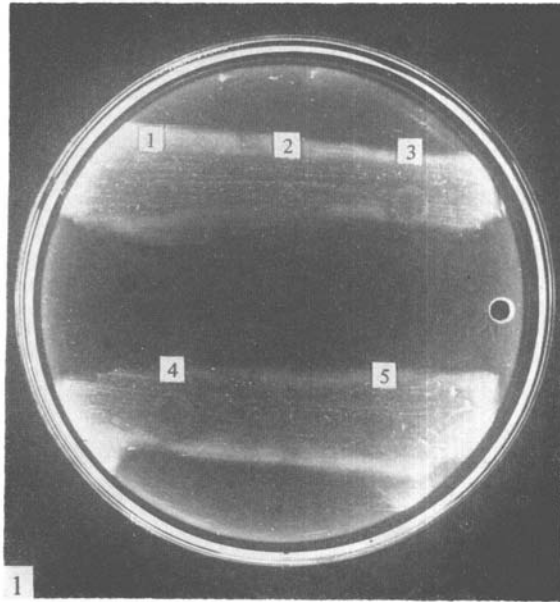
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PLATE 2

Fig. 1. Impression smear of a colony shown in Pl. 1, fig. 1. This shows clumps of pleomorphic bacteria varying from small cocci to club-shaped rods and other irregular forms. Gram stain,  $\times 1000$ .

Fig. 2. Ethanol-fixed impression smear of a colony of the 'Elgin' isolate stained with fluorescent antibody to rough *B. abortus*. The organisms are visible as clumps of bacteria resembling those shown in Pl. 2, fig. 1. Anti-*B. abortus* 45/20-FITC,  $\times 1000$ .

PLATE 3

Fig. 1. Electron micrograph of a negatively stained suspension of the 'Elgin' isolate after incubation with brucella phage R, showing an irregularly shaped organism with several phage particles adsorbed to its surface. Neutral potassium phosphotungstate,  $\times 80000$ .

Fig. 2. Electron micrograph of a negatively stained suspension similar to that shown in Pl. 3, fig. 1. This shows a coccoid organism and a filamentous 'bizarre form' each with a brucella phage adsorbed to its surface. Neutral potassium phosphotungstate,  $\times 80000$ .

PLATE 4

Fig. 1. The effect of brucella phages Tb (1), Wb (2), Bk2 (3), R (4) and R/0 (5) at  $10^4$  RTD on the 'Elgin' isolate growing on Eaton's agar medium. All of the phages have produced either lysis or growth inhibition of the bacterial strain. The Wb phage has produced the least effect, with only an area of partial lysis at the site of application. The areas of bacterial growth are surrounded by a zone of opacification produced as a result of degradation of the medium by extracellular phospholipase.