Mycoplasmacidal activity of bovine milk for T-mycoplasmas

By J. BROWNLIE, C. J. HOWARD and R. N. GOURLAY

*Agricultural Research Council, Institute for Research on Animal Diseases,
Compton, Newbury, Berks. RG16 0NN*

(*Received* 24 *May* 1974)

SUMMARY

Normal bovine milk and whey was mycoplasmacidal for 6 of the 13 strains of bovine T-mycoplasmas examined. The *in vitro* assay used also demonstrated no killing of the human, canine and simian T-mycoplasma strains after 4 hr. incubation. However, there appeared to be some cow-to-cow variation in possession of this activity, and following *E. coli* endotoxin stimulation of the mammary gland the activity was considerably reduced.

Whey from three normal cows was fractionated on a Bio-Gel A 1·5 m. column and the mycoplasmacidal activity of the resulting five peaks assayed. Only the second peak, peak B, contained activity and was characterized as the only peak containing bovine IgA. The active component in whey, however, was found to be heat stable at 60° C. for 60 minutes and to pass through a dialysis membrane. This is inconsistent with it being immunoglobulin.

INTRODUCTION

Recently, certain T-mycoplasmas have been shown to cause experimental mastitis in cattle (Gourlay, Howard & Brownlie, 1972). These authors found variation both in the virulence of different T-mycoplasma strains and in the susceptibility of different cows (Howard, Gourlay & Brownlie, 1973). In an attempt to explain these variations, certain soluble factors in milk have been examined. The presence of soluble mycoplasmacidal factors has already been reported in serum from immune cattle (Priestley, 1952), in murine tissue extracts (Tully & Rask-Nielsen, 1967) and in rabbit and human neutrophil extracts (Dajani & Ayoub, 1969; Jones & Hirsch, 1971). Particularly pertinent to our own observations, to be reported here, was the finding that bull seminal fluid and serum could inhibit the growth of T-mycoplasmas (Taylor-Robinson, Thomas & Dawson, 1969).

In cow's milk, however, only bactericidal activity has so far been reported. Hesse (1894) was the first to report this effect and Hanssen, in 1924, called the responsible fraction 'lactenin'. Many observations have confirmed these findings with normal cow's milk, while Derbyshire (1964) demonstrated an even greater bactericidal activity in the milk of cows with a sterile inflammation of the mammary gland. This mastitis, induced by intramammary infusions of distilled water, was due to the pyrogens contained in distilled water (Shah & Morse, 1964).

The mycoplasmacidal activity of milk from the normal bovine mammary gland, from the gland after endotoxin stimulation, and after T-mycoplasma experimental infection was investigated in an attempt to explain the variable pathogenicity of T-mycoplasmas for the mammary gland.

METHODS

Cattle

The cattle were 3 to 6-year-old Friesian cross in the first 8–12 weeks of their second to fifth lactation. All animals studied were giving more than $2\frac{1}{2}$ gallons of milk a day. They had no recent history of mastitis and were designated as suitable animals if their total milk cell counts were below 100,000 cells/ml. of which neutrophil cell counts were only 10–15 %. Milk samples were stained by the 'single dip' method of Broadhurst & Paley (1939) and cells were counted by the technique of Pattison & Holman (1951).

Whole milk samples

Quarter milk samples from individual cows were collected in the afternoon, after an initial strip milking. They were cooled and used immediately or frozen at −20° C. for subsequent work.

Milk whey samples

Whole milk was centrifuged at 82,000 g for 60 min. and the clear whey pipetted from the casein and milk lipid layers before storing at −20° C.

Production of a sterile mastitis in the bovine mammary gland

The cows giving normal milk and described above were inoculated intra-mammarily with 1 μg. of *Escherichia coli* endotoxin. This inoculation will reliably produce a mild sterile mastitis and has been reported by Brownlie (1972).

Milk whey from experimental mycoplasma mastitis

Whey from the milk of a cow (B89) that had been inoculated with T-mycoplasma strain O13 and had mastitis at the time of sampling, was retained and stored at −20° C. This whey was assayed to examine the differences in mycoplasmacidal activity of wheys from endotoxin-stimulated and mycoplasma-infected mammary glands.

Dialysis of whey samples

A variation in the mycoplasmacidal activity of whey samples was demonstrated after prolonged dialysis. Ten ml. of whey was dialysed against a barbitone buffer (complement fixation test diluent – Oxoid Ltd, London) in the 27/32 Visking dialysing tube of Scientific Supplies Company, London. The dialysis period varied from 1 h. to 24 hr. and all procedures were carried out at 4° C.

T-mycoplasma strains

The bovine strains T288, T95 and T488 were provided by Dr Livingstone (Texas, U.S.A.) whereas the remaining bovine strains were isolated at Compton. The virulence of these Compton strains has been assayed by intramammary inoculation (Howard *et al.* 1973) with the exception of strains GraX and D48.

The human strains 7, 23, 27, 58, 354, Pirillo, Cook and T960 were provided by Dr F. T. Black (Aarhus, Denmark) and have been described by him (Black, 1973). The remaining human strains CD573, CD408, CD343 and the canine strain Sp1701 and simian strain Sp1625A were supplied by Dr D. Taylor-Robinson (M.R.C. Clinical Research Centre, Northwick Park, London), and have been described previously (Howard *et al.* 1973).

Chromatographic fractionation of milk whey

Whey from cow (C14) was fractionated on Biogel A – 1·5 m. (100–200 mesh) in complement fixation test diluent. After concentration threefold in carbowax, 9 ml. of whey were applied to a column 2·5 cm × 90 cm (Pharmacia AB, Sweden) and the eluate recorded at 280 nm. The individual peaks were adjusted to 9 ml. and assayed for mycoplasmacidal activity.

The presence of immunoglobulins in these peaks was demonstrated by double diffusion plates with antisera to bovine globulins. (Antisera were kindly supplied by T. J. Newby, Department of Animal Husbandry, University of Bristol, Langford, Bristol.)

T-mycoplasmacidal activity of milk and whey samples and fractions

Overnight cultures of strains of T-mycoplasmas, grown in U2 broth (Gourlay, Brownlie & Howard, 1973) were used to determine mycoplasmacidal activity. Samples of milk, whey or fractions (1·8 ml.) were tested in duplicate by adding 0·2 ml. of culture, and the mixture was incubated at 37° C. on an orbital shaker at 120 rev./min. Duplicate 0·1 ml. samples were removed from the inoculation mixture at various times for the estimation of viable organisms and expressed as colour change units per ml. (c.c.u./ml.) (Gourlay *et al.* 1972).

RESULTS

The mycoplasmacidal activity of milk from normal and endotoxin-stimulated glands against 25 T-mycoplasma strains

Twenty-five T-mycoplasma strains from cattle and other animal species were incubated separately with normal bovine milk (cow A404). The viable organisms in the incubation mixture were assayed at timed intervals and results showed that killing was negligible at 1 hr. but evident at 4 hr. and more extensive at 24 hr. After 4 hr., normal milk was mycoplasmacidal to 7 of the 13 bovine strains (Table 1). The ten human strains and the canine and simian strains appear to resist any killing by normal milk over 4 hr. However, certain of the human strains Reow, CD408 and CD573 were killed within 24 hr. by the same milk sample.

Table 1. *Assay of mycoplasmacidal activity of milk from a normal and an endotoxin-stimulated mammary gland for 25 different strains of T-mycoplasmas*

Strains	Source	Virulence	Inoculum log no. viable organisms/ml.	No. of logs reduction in viable organisms/ml. after 4 hr at 37° C.	
				Normal milk	Endotoxin-stimulated milk
SENSITIVE TO WHEY					
Vic9	Bovine lung	+ +	6·7	6·7	2·7
O13	Bovine eye	+ +	4	4	0
O12	Bovine urogenital tract	+ +	4	2	N.T.
A417	Bovine lung	+ +	5	3	1
GraX	Bovine lung	N.T.	3	3	1
T288	Bovine lung	N.T.	6	2	N.T.
RESISTANT TO WHEY					
Bu2	Bovine urogenital tract	—	5	1	0
B101	Bovine urogenital tract	—	6	1	1
T488	Bovine urogenital tract	N.T.	5	0	N.T.
M525	Bovine urogenital tract	+ +	4·7	0	N.T.
D20	Bovine lung	+	3·7	0	0
T95	Bovine lung	N.T.	6	0	N.T.
D48	Bovine lung	N.T.	4	0	N.T.
II	Human urogenital tract	N.T.	4	0	N.T.
III	Human urogenital tract	N.T.	5	1	N.T.
IV	Human urogenital tract	N.T.	5	0	N.T.
V	Human urogenital tract	N.T.	5	0	N.T.
VII	Human urogenital tract	N.T.	3	0	N.T.
Reow	Human urogenital tract	—	5	0	N.T.
T960	Human urogenital tract	—	5	0	N.T.
CD573	Human urogenital tract	—	5	0	N.T.
CD408	Human urogenital tract	—	5	0	N.T.
CD343	Human oral cavity	—	5	0	N.T.
Sp1701	Canine urogenital tract	—	5	1	0
Sp1625A	Simian throat	—	4	0	0

Virulence (Howard *et al.* 1973): + +, causes clinical mastitis; +, causes subclinical mastitis; —, avirulent; N.T. not tested.

Milk from an endotoxin-stimulated gland (cow A404) possessed reduced mycoplasmacidal activity against the 7 bovine strains killed by normal milk, and no activity against all the other strains examined (Table 1).

Comparison of mycoplasmacidal activity of whey obtained from milk of normal and endotoxin-stimulated mammary glands

The clear whey fraction obtained from milk by high-speed centrifugation contained most of the milk protein with the exception of sedimented caseins.

Assays of the mycoplasmacidal activity of normal milk and their respective wheys from 6 cattle demonstrated that whey contained an activity equal to the corresponding milk. This is particularly interesting in the case of whey from the

mastitis milk of endotoxin-stimulated glands of cows A404, D816, PL2 and C44. The activity of this whey is equal to the mastitis milk and is lower than whey from normal milk of the same cow. Similarly, the whey from milk of an experimental case of T-mycoplasma mastitis, cow M614, had a reduced activity compared with whey from its normal milk taken before inoculation.

Freezing milk and whey samples at $-20°$ C. for periods up to 3 months did not appear to alter this activity.

Comparison of mycoplasmacidal activity in the normal milk wheys from different cows

Coincident with the variation of whey mycoplasmacidal action for different T-mycoplasma strains, a cow-to-cow variation was expected. Accordingly, the wheys from five cows were incubated with five of the T-mycoplasmas, selected to include sensitive and resistant strains. The normal milk wheys from all five cows showed activity against strains Vic9 and A417, whereas against the remaining three strains Bu2, CD573 and Reow there was no demonstrable activity. The variation between cows was small. The wheys from a further 11 cows have been tested against the most sensitive of these T-mycoplasmas, strain Vic9. Only seven out of eleven gave reduction in viable count after 4 hr. incubation at $37°$ C., and thus a certain amount of animal variation was evident.

In order to determine heat lability of the mycoplasmacidal components whey samples from two cattle, C14 and D304, were heated to $56°$, $60°$ and $70°$ C. for 60 min. Both wheys retained all activity after being heated to $56°$ and $60°$ C. for 60 min., but had their 5-log activity reduced by 2-logs of viable organisms, after heating at $70°$ C. for 60 min.

Fractionation of milk whey

Biogel $A - 1.5$ m. chromatography. Whey from cow C14 was fractionated on Biogel $A - 1.5$ m. Five distinct peaks A–E were isolated and of six fractionations, Fig. 1 is the typical chromatographic profile. Peak A coincided with the exclusion volume and contained excluded molecules of approximately 1.5×10^6 daltons. The whey from the milk of three different cows C20, C30 and C14 was fractionated and gave similar chromatographic profiles to Fig. 1. All five distinct peaks following the three fractionations were assayed for mycoplasmacidal activity. Activity, equal to the original whey, was found to remain almost entirely in peak B, with reduced activity on only two occasions being detected in peak C.

Sodium dodecylsulphate (SDS) gel electrophoresis. Electrophoresis on 5 % SDS polyacrylamide gel of whey and the five isolated peaks following Biogel chromatography is shown in Plate 1. From peak A towards peak E there is a progressive increase in the smaller-molecular-weight components. Peak B contains several large proteins including the immunoglobulins. Traces of these large proteins can be seen in the gels of peak A but not in the remaining three peaks C, D and E.

The presence of immunoglobulins in peak B has been demonstrated with double diffusion plates (Fig. 2). Anti-IgM serum gave a precipitin line in peaks A and B. Anti-IgG serum gave a line in both peaks B and C while Anti-IgA serum gave a line in peak B only.
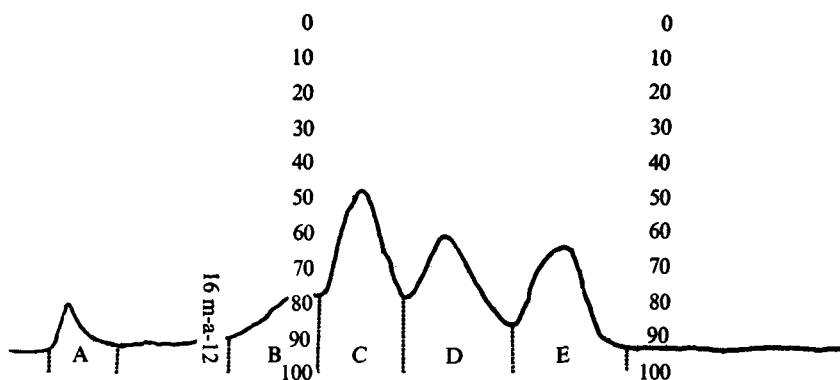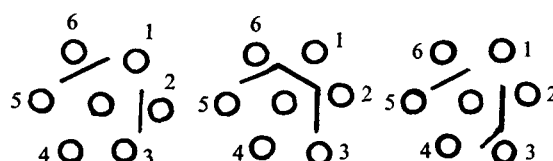
Fig. 1. Chromatographic profile at 280 nm. of whey from Cow C14 following fractionation on Bio-Gel A – 1·5 m.

Centre well	IgA	IgM	IgG
Peripheral wells	1 Peak A	2 Peak B	3 Peak C
	4 Peak D	5 Peak E	6 Whole whey

Fig. 2. Double diffusion plates of whey and whey fractions (Fig. 1) against bovine IgA, IgM and IgC, antisera.

Dialysis experiments. Dialysis of 1 volume of whey against 10 volumes of barbitone buffer resulted in no change in activity for the first two hours but, beyond that time, there was complete loss of activity. This loss of activity, following dialysis, was repeatable in eight subsequent experiments. No change was found when the knotted ends of the dialysis bag were above the solution. However, if the buffer volume for dialysis was equal to the whey volume, mycoplasmacidal activity could be demonstrated in both whey and buffer.

DISCUSSION

Certain bovine T-mycoplasma strains are capable of producing experimental mastitis in cattle whereas the human, simian and canine T-mycoplasma strains examined have had no effect when inoculated into the mammary gland (Gourlay *et al.* 1972; Howard *et al.* 1973). This *in vivo* variation in pathogenicity is probably due to both the virulence of the mycoplasmas and the activity of host local defence mechanisms. These latter mechanisms operate within the mammary gland, but little is known of their activity against mycoplasmas.

Our results show that six bovine T-mycoplasma strains, Vic9, O13, U12, A417, GraX and T288 are highly sensitive *in vitro* to both normal bovine milk and whey. However, the human, simian, canine and the remaining bovine strains in Table 1 were not so sensitive. These results do not accord with a simple explanation that the mycoplasmas which are capable of causing mastitis are more resistant to milk and whey mycoplasmacidal action. On the contrary, the virulent strains appeared to be the least resistant (Table 1).

The results also show that the mycoplasmacidal action of milk from normal glands containing less than 10^5 cells/ml. is greater than that of milk from endotoxin-stimulated glands of the same cow which contained 10^6 cells/ml. This contrasts with the results for bactericidal activity of normal milk which is always increased after pyrogen stimulation (Derbyshire, 1964). It may indicate that the mycoplasmacidal and bactericidal systems in milk are different. The reduction in mycoplasmacidal activity after endotoxin stimulation did not appear to be due to the release of active serum components in milk. These components will increase in milk because of the galactophoritis caused by endotoxin infusion (Brownlie, 1972), but addition of 30 % bovine serum to normal whey did not alter its *in vitro* activity.

One possible explanation for the reduction in activity might be the protection of mycoplasmas by milk cells. Neutrophils form over 90 % of the milk-cell population in endotoxin-stimulated milk (Brownlie, 1972) and in this context the study of mycoplasma-cell interaction by Simberkoff & Elsbach (1971) is particularly pertinent. They were unable to demonstrate killing of *Mycoplasma hominis* and *M. arthritidis* by rabbit neutrophils although the neutrophils were able to kill *Escherichia coli* and staphylococci. They suggested that neutrophils were unable to kill mycoplasma and this could explain the lack of killing in endotoxin-stimulated milk. Moreover, Lloyd & Trethewie (1970) found that mycoplasmas can survive in the presence of bovine macrophages. However, human macrophages have been shown to phagocytose and cause degradation of *M. pneumoniae*, *M. neurolyticum* and *M. gallisepticum* (Zucker-Franklin, Davidson & Thomas, 1966) and, similarly, murine macrophages are capable of phagocytosing and digesting opsonized *M. pulmonis* (Jones & Hirsch, 1971). Recently, Cole & Ward (1973) have demonstrated a protective effect by murine peritoneal macrophages of *M. arthritidis* against antibody.

An alternative explanation for the reduced activity in milk after endotoxin stimulation could be that with the increase in neutrophils there is either a concurrent increase in an inhibitor of mycoplasmacidal activity or adsorption of the active components by milk cells. Both mechanisms require the wheys from endotoxin-stimulated milk to have a reduced activity, compared with normal whey. This was shown, in the results, to be the case.

The retention of mycoplasmacidal activity in whey from normal milk, shows that soluble, and not cellular, components are responsible. Moreover, the assay of wheys from different cows demonstrated that there was some cow-to-cow variation of this soluble fraction.

The isolation of activity in peak B alone, after Biogel chromatography (Fig. 1),

indicates a molecular size of about 400,000 daltons, that of IgA. However, the SDS-polyacrylamide-gel electrophoresis of peak B (Plate 1), shows five protein-staining bands. Most of the protein is in two large molecular weight bands which are not found to any extent in the remaining four peaks. By elimination, these two bands may be associated with the mycoplasmacidal activity.

The question then arises, could the activity in milk be due to specific antibody? This would appear to be possible from the chromatographic evidence, but since mycoplasmacidal activity is not affected by heating to 60° C. for 60 min. complement is clearly not involved.

Finally, the dialysis experiment gave unexpected results. Activity was lost following the dialysis of one volume of whey in commercial Visking tubing against a tenfold volume of baritone buffer, and phosphate-buffered saline. However, the dialysis of equal volumes of whey and buffer appeared to allow an equilibrium to be established with activity in both whey and dialysing buffer. This indicates that components responsible for activity can pass through a dialysis bag. This contrasts with the chromatography where activity was only in the large-molecular-weight fraction of peak B.

One explanation of these results could be that the small active molecules aggregate and therefore chromatograph in peak B. On dialysis, there is disaggregation and the active components form an equilibrium across the membrane. Reaggregation in the dialysing buffer may occur. Alternatively the activity might be due to a small-molecular-weight component which in whey is bound to large molecules like immunoglobulins. On dialysis, the small active component separates from its carrier molecule and moves through the membrane forming an equilibrium between whey and buffer. If the volume of buffer is small, then activity can be demonstrated in the buffer with our assay technique. These dialysis results clearly distinguish this dialysable activity from the non-dialysable factor of Taylor-Robinson *et al.* (1969), from lactenin (Wilson & Rosenblum, 1952), and from lactoferrin (Oram & Reiter, 1968). It is hoped that further work will establish the nature of the mycoplasmacidal activity. Small basic proteins with non-specific antimicrobial activity have already been demonstrated (Hirsch, 1960; Brownlie & Hibbitt, 1972) and it may be a molecule of this nature that is responsible.

REFERENCES

Black, F. T. (1973). Modifications of the Growth Inhibition Test and its application to human T-mycoplasmas. *Applied Microbiology* **25**, 528–33.

Broadhurst, J. & Paley, C. (1939). A single dip stain for the direct examination of milk. *Journal of the American Veterinary Medical Association* **94**, 525.

Brownlie, J. (1972). The isolation and characterization of antimicrobial proteins from bovine milk. Ph.D. Thesis, University of Reading.

Brownlie, J. & Hibbitt, K. G. (1972). Antimicrobial proteins isolated from cervical mucus. *Journal of Reproduction and Fertility* **29**, 337–47.

Anode

Origin

Fraction

Whole whey   A   B   C   D   E
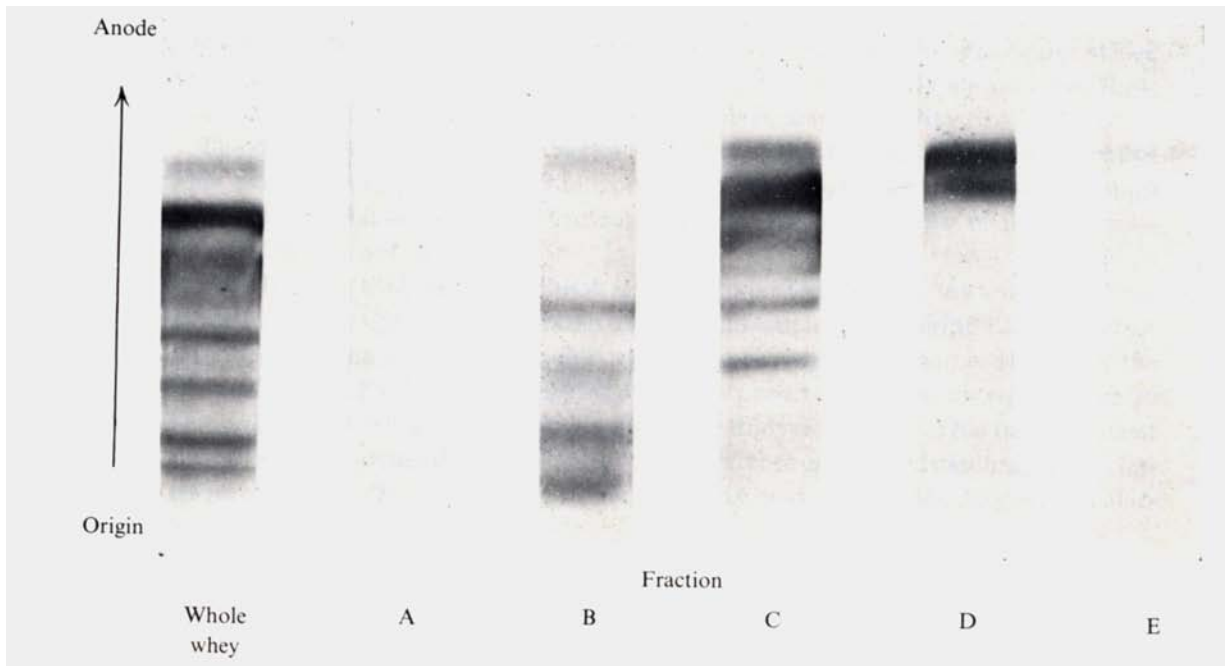
COLE, B. C. & WARD, J. R. (1973). Interaction of *Mycoplasma arthritidis* and other myco-plasmas with murine peritoneal macrophages. *Infection and Immunity* 7, 691–9.

DAJANI, A. S. & AYOUB, E. M. (1969). Mycoplasmacidal effects of polymorphonuclear leukocyte extract. *Journal of Immunology* 102, 698–701.

DERBYSHIRE, J. B. (1964). The multiplication of *Staphylococcus aureus* in the milk of cows with mild mastitis. *Journal of Pathology and Bacteriology* 87, 137–44.

GOURLAY, R. N., BROWNLIE, J. & HOWARD, C. J. (1973). Isolation of T-mycoplasmas from goats, and the production of sub-clinical mastitis in goats by the intramammary inoculation of human T-mycoplasmas. *Journal of General Microbiology* 76, 251–4.

GOURLAY, R. N., HOWARD, C. J. & BROWNLIE, J. (1972). The production of mastitis in cows by the intramammary inoculation of T-mycoplasmas. *Journal of Hygiene* 70, 511–21.

HANSSEN, F. S. (1924). The bactericidal property of milk. *British Journal of Experimental Pathology* 5, 271–80.

HESSE, W. (1934). Ueber die Beziehung e zwischen Kuhmilch und Cholerabacillen. *Zeitschrift für Hygiene und Infektionskrankheiten* 17, 239–71.

HIRSCH, J. G. (1960). Antimicrobial factors in tissue and phagocytic cells. *Bacteriological Reviews* 24, 133–40.

HOWARD, C. J., GOURLEY, R. N. & BROWNLIE, J. (1973). The virulence of T-mycoplasmas isolated from various animal species, assayed by intramammary inoculation in cattle. *Journal of Hygiene* 71, 163–70.

JONES, I. G. & HIRSCH, J. G. (1971). The interaction in vitro of *Mycoplasma pulmonis* with mouse peritoneal macrophages and L-cells. *Journal of Experimental Medicine* 133, 231–59.

LLOYD, L. C. & TRETHEWIE, E. R. (1970). In *The Role of Mycoplasmas and L-forms of Bacteria in Diseases* (ed. J. T. Sharp). Springfield, Illinois: Charles C. Thomas.

ORAM, J. D. & REITER, B. (1968). Inhibition of bacteria by lactoferrin and other iron chelating agents. *Biochimica et biophysica acta* 170, 351–65.

PATTISON, I. H. & HOLMAN, H. H. (1951). Studies on experiments with streptococcal mastitis. *Journal of Comparative Pathology* 61, 26–37.

PRIESTLEY, F. W. (1952). Observations on immunity to contagious bovine pleuropneumonia with special reference to the bactericidal action of blood. *British Veterinary Journal* 108, 153–61.

SHAH, P. C. & MORSE, E. (1964). Studies on albumin in 'mastitic' milk: factors affecting the presence of blood-serum albumin in 'mastitic' milk. *American Journal of Veterinary Research* 25, 714–19.

SIMBERKOFF, M. S. & ELSBACH, P. (1971). The interaction *in vitro* between polymorphonuclear leukocytes and mycoplasma. *Journal of Experimental Medicine* 134, 1417–30.

TAYLOR-ROBINSON, D., THOMAS, M. & DAWSON, P. C. (1969). The isolation of T-mycoplasmas from the urogenital tract of bulls. *Journal of Medical Microbiology* 2, 527–33.

TULLY, J. G. & RASK-NIELSEN, R. (1967). Mycoplasma in leukemic and non-leukemic mice. *Annals of the New York Academy of Sciences* 143, 345–52.

WILSON, A. T. & ROSENBLUM, H. (1952). The antistreptococcal property of milk – the effect of lactenin on haemolytic streptococci of several serological groups. *Journal of Experimental Medicine* 95, 25–38.

ZUCKER-FRANKLIN, D., DAVIDSON, M. & THOMAS, L. (1966). The interaction of mycoplasmas with mammalian cells, HeLa cells, neutrophils and eosinophils. *Journal of Experimental Medicine* 124, 521–42.

EXPLANATION OF PLATE

SDS gel electrophoresis of whey and whey fractions (Fig. 1).