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Isolation and properties of an RNA fraction present in *Brucella* culture supernatants

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SUMMARY

The supernatant fluids of batch and continuous cultures of *Brucella* strains contained up to 100 mg/l of soluble RNA which could be recovered by precipitation with lysozyme. This RNA fraction had many of the properties of ribosomal RNA and was single-stranded, sensitive to ribonuclease, with an approximate sedimentation constant of 5S, a molecular weight of about 35000 daltons and an adenine; guanine; cytosine; uracil content of 17·5; 26·5; 33; 23 mol % respectively. RNA fractions from lysozyme precipitates evoked high titres of *Brucella* agglutinins on injection into rabbits and induced acute inflammatory responses in guinea-pig skin. Highly purified RNA fractions prepared by phenol extraction of lysozyme precipitates did not evoke antibodies to *Brucella abortus*.

INTRODUCTION

During the course of growth *in vitro Brucella* organisms may release into the culture medium macromolecular products with a wide range of biological activities. Among those which have been described are components with antiphagocytic activity (Ellwood, Keppie & Smith, 1967), protective antigens (Paterson, Pirie & Stableforth, 1947; Smith *et al.* 1962), agglutinogenic lipid-polysaccharide-peptide complexes and their degradation products (Miles & Pirie, 1939*a*, *b*, *c*), phage receptors (Corbel, 1977) and allergens (Burnet, 1922; Renoux, 1964). Because of the presence of the latter components, crude *Brucella* culture supernatants have been used as the basis of skin test antigen preparations employed for diagnostic purposes (Alton & Jones, 1967).

Recently, during attempts to fractionate the biologically active components of *Brucella* culture supernatants, it was observed that concentrates of these consistently produced dense white precipitates on addition of lysozyme. Preliminary serological studies indicated that the precipitating factor did not correspond to any of the hitherto described antigenic components of *Brucella* cells. Accordingly, an attempt was made to characterize the lysozyme-reactive material and to assess its biological significance.

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METHODS

Organisms and growth conditions. The brucella strains used were all from the culture collection kept at this laboratory. They included the smooth strains *B. abortus* S19 and S99, *B. melitensis* 16M, *B. neotomae* 5K33 and *B. suis* 1330. The non-smooth strains used were *B. abortus* 45/20, *B. canis* RM 6/66, and *B. ovis* 63/290.

With the exception of *B. abortus* strains S19 and S 99, which were grown by continuous culture according to Boyce & Edgar (1966), all organisms were grown on layers of serum dextrose agar in Roux flasks incubated at 37 °C in air for up to 7 days. Supplementary CO_2 was supplied to those cultures requiring it.

Preparation of culture supernatants. The supernatant fluids were recovered from harvests of *B. abortus* S19 and S 99 by sedimenting the cells with 0·16 % (w/v) carboxymethylcellulose (Boyce & Edgar, 1966) followed by centrifugation at 20 000 *g* for 30 min. Culture supernatants from other brucella strains were prepared by washing the organisms off the agar layers by addition of phosphate buffered saline (PBS; 0·15 M-NaCl in 0·01 M phosphate buffer, pH 7) followed by centrifugation at 20 000 *g* for 30 min.

In both cases, the culture supernatants were dialyzed first against 0·15 M-NaCl, then against distilled water and concentrated by counter-dialysis against polyethylene glycol 20 M to about 2 % of the original volume. The resulting viscous solution was clarified by centrifugation at 20 000 *g* for 30 min. These and all subsequent operations were performed at 4 °C, except where stated to the contrary.

Fractionation of culture supernatants. A 1 % w/v solution of egg white lysozyme in 0·15 M-NaCl was slowly added to concentrated culture supernatant until no further precipitation occurred. The precipitate was recovered by centrifugation at 10 000 *g* for 15 min, washed with 0·15 M-NaCl and dissolved in 0·1 M-Na_3PO_4 adjusted to pH 11·0 with 0·1 M-HCl. D(+) glucose was added to a final concentration of 1 % (w/v) to act as a molecular weight marker and the solution applied to a 25 mm × 600 mm column of Sephadex G50 (Pharmacia, Uppsala) equilibrated with 0·1 M-Na_3PO_4 at pH 11·0. Fractions were eluted with the equilibrating buffer, monitored for absorption of ultraviolet radiation of 260 nm and 280 nm wavelength and restored to neutral pH by addition of 0·1 M-HCl. They were tested for precipitation with 1 % w/v lysozyme and for the presence of glucose by the glucose oxidase reaction using Clinistix (Ames Company, Slough).

Fractions precipitating with lysozyme were pooled, concentrated by counter-dialysis against polyethylene glycol 20 M and after addition of glucose to 1 % w/v, applied to a 25 × 450 mm column of Sepharose 6B (Pharmacia, Uppsala) equilibrated with 1·0 M-NaCl buffered at pH 7·0 with 0·1 M phosphate buffer. Elution was with the equilibrating buffer and the fractions were monitored as described for the Sephadex G50 separation. Those reacting with lysozyme in precipitation tests were pooled and concentrated by counter-dialysis against polyethylene glycol 20 M. Subsequent further purification was by ultracentrifugation at 100 000 *g* for 90 min.

Samples for chemical analysis were also purified by extraction with an equal

volume of 90% w/v aqueous phenol at room temperature for 1 h, followed by centrifugation at 10000 g for 30 min at 0 °C. The aqueous supernatants were then made 2% w/v with respect to sodium acetate and precipitated by addition of 2 volumes of ethanol at 4 °C. The precipitate was collected, washed free of phenol with ethanol:water (3:1, v/v) followed by diethyl ether and dried.

Analytical methods. Culture supernatants and fractions were monitored for precipitating activity by ring precipitin tests with a 1% (w/v) solution of lysozyme in 0·15 M-NaCl. Lysozyme enzymic activity was determined quantitatively by a radial diffusion assay using 0·05% (w/v) *Micrococcus lysodeikticus* cells (Sigma, London) incorporated in 1% (w/v) agarose-gel in PBS. The diameters of the zones of clearing formed after 1 h at 25 °C were compared with those produced by lysozyme solutions of standard concentration.

Disk electrophoresis in polyacrylamide gels at pH 8·6 was performed according to Davis (1964). Disk electrophoresis of phenol–acetic acid–water extracts was performed at pH 2·0 according to Morris (1973). Zone electrophoresis on cellulose acetate and hydrocellulose membranes (Chemetron, Milan) was carried out in 0·05 M barbiturate buffer at pH 8·6. The membranes were stained for protein using 0·5% (w/v) Amido Black IOB in methanol:acetic acid:water, 9:9:2 (v/v); for lipid using 0·1% Ciba 7B Fat Red in 0·05 M-NaOH in 60% (v/v) ethanol; for carbohydrate by the periodic acid–Schiff's reaction; for nucleic acids using 2% w/v acridine orange + 1% (w/v) lanthanum acetate in 15% (v/v) acetic acid and for RNA using 1% (w/v) methyl green in 0·1 M acetate buffer, pH 4·5.

Immunoelectrophoresis was performed in 1% (w/v) Oxoid No. 1 agar in 0·05 M barbiturate buffer pH 8·6, using the technique of Scheidegger (1955). Diffusion was carried out in a water-saturated atmosphere at 4 °C for 2–3 days, against antisera to *Brucella* spp. or 1% (w/v) solutions of lysozyme in 0·15 M-NaCl. Immunodiffusion tests against antisera to *Brucella* spp. were done according to Corbel (1973). Similar diffusion tests using 1% (w/v) solutions of lysozyme, trypsin, ribonuclease, poly-L-lysine, poly-L-arginine, poly-L-glutamic acid, poly-D-glutamic acid and normal rabbit and bovine sera were performed at 4 °C.

Carbohydrate was determined by the method of Dubois *et al.* (1956) using D(+) glucose as standard. Protein was estimated by the method described by Lowry (Layne, 1957) using bovine serum albumin as standard. Lipid was determined by the hydroxamic acid method (Snyder & Stephens, 1959) using glyceryl trioleate as standard. DNA was estimated according to Giles & Myers (1965) using calf thymus DNA as standard. RNA was estimated by the Bial-orcinol reaction using yeast RNA as standard (Mejbaum, 1939). Uronic acid was estimated by the carbazole reaction (Dische, 1947) using D-glucuronic acid as standard. The thiobarbituric acid reaction of Osborn (1963) was used for the estimation of 2-keto-3-deoxyoctulosonic acid (KDO).

RNA characterization methods. Estimations of sedimentation constant were made on 1% w/v solutions in 0·15 M-NaCl by the method of Martin & Ames (1961) using human haemoglobin, bovine IgG$_2$, bovine serum albumin and cytochrome C as reference markers. Molecular exclusion properties were examined on columns of Sepharose 6B equilibrated with PBS at 4 °C. D(+) glucose, lysozyme, haemo-

15-2

globin, bovine IgG and Blue Dextran 2000 (Pharmacia, Uppsala) were used as reference markers.

Measurements of ultra-violet absorbance were made on an Optica CF4N1 double beam recording spectrophotometer. Purified RNA preparations were examined for double-stranding by the methods described by Lampson *et al* (1967) These included sensitivity to low concentrations of ribonuclease at 22 °C, hyper-chromicity produced by incubation with formaldehyde or following thermal denaturation.

The purine and pyrimidine base composition of purified RNA samples was determined by the method of Bendich (1957).

Determination of biological activity. Purified preparations of the lysozyme precipitating compounds (LPCs) from *Brucella* culture supernatants were tested for antigenicity according to Corbel (1976a). Intradermal tests for delayed hypersensitivity were done as described previously (Corbel, 1976a) except that guinea-pigs sensitized by intramuscular injection of 10^6 viable *B. melitensis* Rev 1 organisms 4 weeks previously were used. Intradermal injections into uninfected guinea-pigs were done by an identical method except that the skin samples for histological evaluation were taken after 24 h.

RESULTS

Characterization of the lysozyme precipitation reaction

Addition of concentrated *Brucella* culture supernatants to aqueous solutions of lysozyme at neutral pH immediately resulted in the production of a dense white precipitate. The precipitate was relatively stable under mildly acid conditions but was reversibly dissociated at high pH values.

The precipitation reaction was independent of salt concentration in the range 0·01–1·0 M-NaCl. It was also relatively independent of temperature in the range 4–50 °C. The presence of chelating agents, including disodium EDTA and tri-sodium citrate, did not inhibit the reaction.

The precipitation reaction was not inhibited by the prior addition to the enzyme solution of the cleavage products released by lysozyme from muramyl-mucopeptide substrates or by related compounds. Thus addition of *N*-acetyl muramic acid, muramic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, *N*-acetyl-neuraminic acid, D-galactosamine, D-gluco-samine or D-mannosamine up to final concentrations of 10 % (w/v) did not prevent the formation of a precipitate on addition of *Brucella* culture supernatants to the lysozyme solutions.

The possibility of an ionic basis to the reactions was examined by adding con-centrated *Brucella* culture supernatants to 1 % (w/v) solutions of other basic proteins and polypeptides. All of those tested, including trypsin, ribonuclease, poly-L-lysine and poly-L-arginine gave positive precipitation reactions in both ring and agar diffusion tests. Solutions of compounds with a high negative charge, including poly-D-glutamic acid, poly-L-glutamic acid and poly vinyl sulphate, did not precipitate with *Brucella* culture supernatants. Furthermore, the lysozyme-

precipitating activity of the latter could be removed by prior precipitation with 1% (w/v) cetavlon.

Fractionation of the LPCs

The LPCs of the *Brucella* culture supernatants were initially fractionated by precipitation with an excess of lysozyme. The white precipitates consisted largely of protein with some carbohydrate and nucleic acid (Table 1). Disk electrophoresis of precipitates dissolved in phenol:acetic acid:water showed that the protein content was almost entirely attributable to the components of the lysozyme preparation used. One or two faintly staining bands possibly corresponded to proteins or polypeptides derived from the culture supernatants (Plate 1, fig. 1).

Fractionation of precipitate re-dissolved at alkaline pH, by gel filtration in Sephadex G50, resolved it into separate lysozyme-precipitating and lysozyme-containing fractions. The lysozyme precipitating activity was eluted in the fractions corresponding to the first ultra-violet absorbing peak of the elution profile. This produced greater extinction at 260 nm than at 280 nm in contrast with the second ultraviolet absorbing peak which corresponded to the lysozyme-containing fractions (Fig. 1).

Gel filtration of the partially purified LPCs on a column of Sepharose 6B resulted in the activity being eluted well after the void volume and only shortly before the glucose marker (Fig. 2). Fractions corresponding to the first ultra-violet absorbing peak in this case contained low concentrations of DNA, high molecular weight RNA, protein and carbohydrate.

Following ultracentrifugation at 100000 *g* for 90 min, most of the activity of the lysozyme-precipitating fractions recovered from Sepharose 6B remained in the supernatant. Only traces of activity were present in the small translucent gelatinous pellet.

The purified lysozyme-precipitating fraction was composed almost entirely of RNA, with only traces of protein and hexose. Minimal further purification resulted from extraction with aqueous phenol (Table 2).

Characterization of LPCs

Disk electrophoresis of aqueous and phenol:acetic acid: water solutions of purified LPCs at pH 8·6 and pH 2·0 respectively, failed to reveal any protein components. Zone electrophoresis on cellulose acetate and hydrocellulose membranes also failed to reveal any components reacting with stains for protein, lipid or carbohydrate. A single, discrete rapidly migrating band was detected by staining with acridine orange and methyl green, however. This corresponded to a component with an electrophoretic mobility slightly faster than that of the bromophenol blue reference marker.

Immunodiffusion tests on purified LPCs failed to produce any precipitation lines with antisera to *Brucella* antigens. In contrast, the crude concentrated culture supernatants produced numerous lines of precipitation on diffusion against these sera (Plate 1, fig. 2). These results were confirmed by the immunoelectrophoresis experiments. In these, the purified LPC failed to precipitate with antisera

Table 1. *Chemical composition of* Brucella *culture supernatants and their crude lysozyme precipitates*

	Chemical composition as % total dry weight				
Brucella strain	Protein	Carbohydrate	Lipid	DNA	RNA*
B. abortus S99					
Supernatant	26·04	11·98	1·40	0·6	3·39
Precipitate	24·44	0·70	< 0·70	0·47	0·32
B. abortus 45/20					
Supernatant	49·36	22·22	1·90	2·47	21·61
Precipitate	23·63	5·72	< 0·50	1·89	2·67
B. canis RM6/66					
Supernatant	48·08	27·89	1·92	1·78	3·37
Precipitate	20·83	5·36	< 0·45	0·63	1·79
B. melitensis 16M					
Supernatant	33·48	22·10	< 0·45	0·63	2·68
Precipitate	59·43	7·38	< 0·41	0·76	1·23
B. suis 1330					
Supernatant	47·87	22·34	1·30	2·66	10·64
Precipitate	9·05	4·63	1·00	0·63	2·01
B. neotomae 5K33					
Supernatant	64·82	20·37	1·85	1·85	15·74
Precipitate	12·07	25·69	1·39	2·15	4·17
B. ovis 63/290					
Supernatant	40·75	7·27	0·66	0·42	1·32
Precipitate	44·57	6·01	< 0·39	0·39	0·58

* As total pentose.
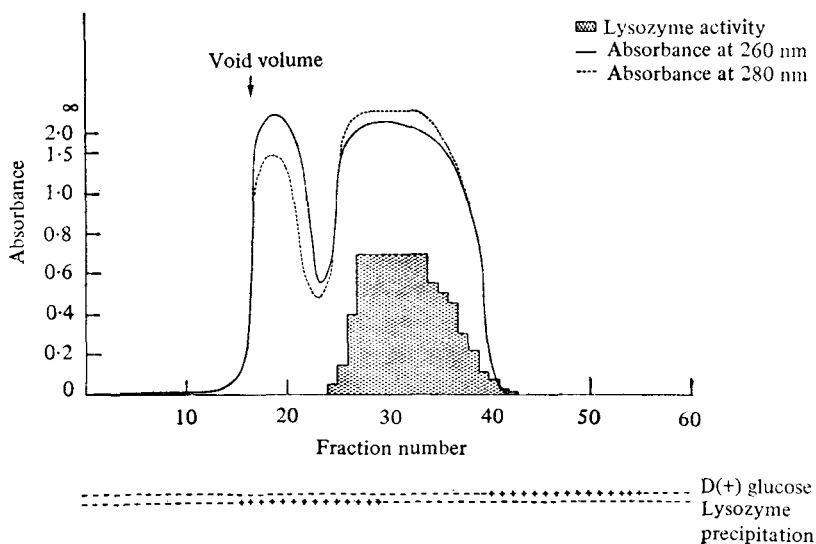
Fig. 1. Elution profile of crude lysozyme precipitate of *B. abortus* S99 culture supernatant fractionated on Sephadex G50 at pH 11·0.
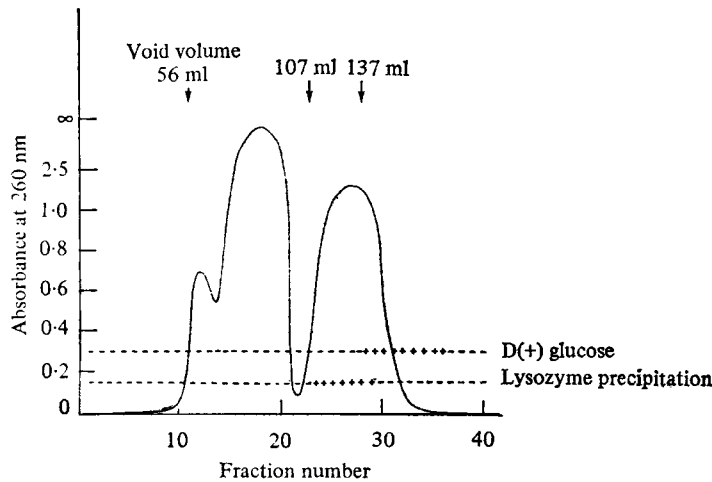
Fig. 2. Elution profile of Sephadex G50 peak 1 fraction of crude *B. abortus* S99
lysozyme precipitate fractionated on a Sepharose 6B column.

to *Brucella* antigens but formed a single precipitation arc on diffusion against lysozyme solutions. The position of this arc corresponded to a component with an electrophoretic mobility greater than that of any of the antigens precipitating with anti-*Brucella* sera (Plate 1, fig. 3).

Purified LPC preparations produced an ultraviolet absorption spectrum with a maximum at 259 nm. The ratio of absorption at 260 nm to that at 280 nm was 1·663:1·00.

Chemical analysis of purified LPC preparations from various brucella strains showed that all were of similar composition and contained > 99% RNA. Lipid, protein and DNA were not present in detectable amount. The carbohydrate content could be largely accounted for by the RNA pentose. Heptose, hexose, methyl-pentose, dideoxyhexose and uronic acid could not be detected by the cysteine-sulphuric acid or carbazole reactions (Kabat & Mayer, 1961).

No lipopolysaccharide was detected by the carbocyanine dye assay of Janda & Work (1971), the purified LPC preparations producing a spectral shift characteristic of nucleic acids.

The average yield of lysozyme-precipitable RNA recovered from brucella culture supernatants was in the range 90–120 mg/l for continuous cultures.

The yield for batch cultures was much more variable but normally less than this.

Estimation of the sedimentation coefficient of purified lysozyme-precipitable RNA by the method of Martin & Ames (1961) gave a value of 5S. From this the molecular weight of this RNA fraction was estimated as about 35000 daltons. The purified RNA preparations were extremely sensitive to ribonuclease. Thus solutions containing 20 μg/ml RNA were completely hydrolyzed by incubation for 1 h at 25 °C with bovine pancreatic ribonuclease at a concentration of 0·2 μg/ml. Incubation with ribonuclease also abolished the ability of the RNA solutions to precipitate with lysozyme. The RNA preparations also showed a large increase in hyperchromicity after incubation with 1·5 % (w/v) formaldehyde at 37 °C for 1 h.
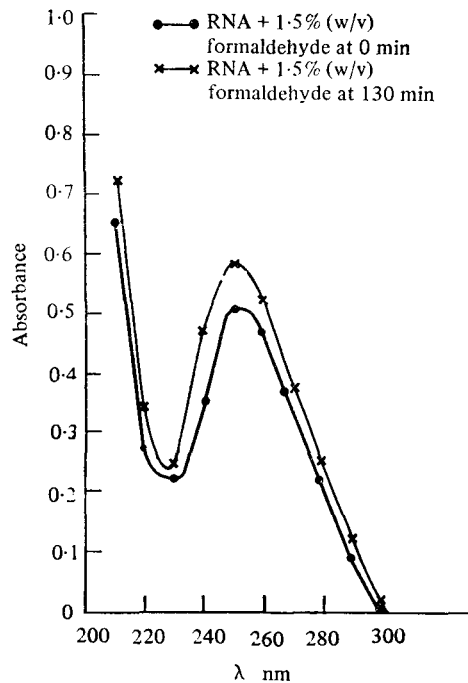
Fig. 3. Ultraviolet absorption spectrum of *B. abortus* S99 RNA purified from a lysozyme precipitate, showing the effect of formaldehyde treatment on absorbance.

(Fig. 3). No increase in hyperchromicity was produced by heating RNA solutions to 100 °C for 5 min and then cooling rapidly in ice.

Chromatographic examination of samples of RNA hydrolysed by heating with 1 M-HCl or 88% (w/v) formic acid showed that these contained cytosine, guanine, adenine and uracil but not thymine or detectable quantities of methylated pyrimidines. The adenine; guanine; cytosine; uracil ratios were estimated as 17·5:26·5:33:23 mol% respectively, by the method described by Bendich (1957).

Biological properties

Intradermal injection of purified lysozyme-precipitated RNA into uninfected guinea-pigs provoked an acute inflammatory reaction with doses of 50 μg or greater. The reaction was characterized by an erythema which became readily apparent after 6 h and attained maximum development with accompanying oedema 12 h–18 h after injection. Histological examination of skin samples from the reaction sites excised 24 h after injection showed local congestion of blood vessels and infiltration of the dermal and subcutaneous tissues by numerous polymorphonuclear leucocytes (Plate 2, fig. 1).

Injection of similar doses of RNA into the dermis of guinea-pigs sensitized by inoculation with *B. melitensis* Rev 1 produced identical reactions. However on injecting more dilute preparations, the sensitized guinea pigs developed low grade inflammatory reactions at RNA concentrations which produced no response in unsensitized animals. RNA doses in the range 1 to 20 μg produced this effect.

Table 3. *Antigenic activity of* B. abortus *lysozyme precipitating component in rabbits*

Preparation	Reciprocal titres in serological tests*				
	Serum agglutina-tion test	Coombs anti-globulin test	Complement fixation test	Rose Bengal plate test	Immuno-diffusion test
B. abortus S99					
Crude lysozyme	640	2560	800	512	+
precipitate	640	1280	800	512	+
Purified lysozyme	160	640	800	128	+
precipitating fraction†	80	640	400	64	+
Purified lysozyme	< 10	10	< 2	—	—
precipitating fraction after phenol extraction	< 10	< 10	< 2	—	—

* Four weeks after subcutaneous injection in Freund incomplete adjuvant.
† Fractionated on Sephadex G50, Sepharose 6B and by ultra-centrifugation.

Histological examination of skin samples from these animals showed a mixed cellular response with infiltration of both mononuclear and polymorphonuclear leucocytes at 24 h after injection (Plate 2, fig. 2).

Following injection of doses of 50–100 μg of RNA with or without adjuvant, rabbits produced moderate to high titres of agglutinins and complement fixing and precipitating antibodies to *B. abortus* (Table 3). The RNA preparations from rough brucella strains produced low titres of antibodies reacting with smooth *B. abortus* strains but higher titres reacting with the homologous antigen.

RNA which had received a final purification by phenol extraction showed little or no antigenicity in rabbits (Table 3). These preparations did still induce low-grade non-specific inflammatory reactions in guinea pigs after intradermal injection of doses of 50–100 μg however.

DISCUSSION

Examination of the effects of pH, temperature, ionic composition and high concentrations of muramyl-mucopeptide cleavage products on the precipitation reaction between lysozyme and *Brucella* culture supernatants indicated that this was not the result of a specific enzyme-substrate interaction. This conclusion was confirmed by the demonstration of similar precipitation reactions using other basic proteins, including some devoid of enzymic activity, and the cationic detergent cetavlon.

These results suggested that the lysozyme precipitation reaction was the result of an ionic interaction between the positively charged protein molecules and negatively charged components of the culture supernatants.

Immunoelectrophoretic analysis showed clearly that the lysozyme precipitating material did not correspond to any of the *Brucella* antigens detected but to a

rapidly migrating fraction of high negative charge. The staining reactions of the material fractionated by zone electrophoresis indicated that the lysozyme precipitating activity was associated with the nucleic acid fraction.

Although chemical analysis of crude lysozyme precipitates of culture supernatants showed that they were heterogeneous, examination of the lysozyme precipitating fractions recovered from these indicated RNA as the major component. Furthermore the activity of the purified RNA fraction in tests with lysozyme and other cationic compounds was identical with that of the crude culture supernatants.

The sensitivity of the purified RNA to low concentrations of RNase, the hyperchromicity produced on incubation with formaldehyde and the absence of demonstrable strand separation on heating were consistent with a single-stranded structure (Fraenkel-Conrat, 1954; Lampson *et al.* 1967). The estimated sedimentation coefficient of 5S and the purine and pyrimidine base composition were consistent with the low molecular weight fraction of ribosomal RNA.

These properties indicated that the components of *Brucella* culture supernatants largely responsible for co-precipitating with lysozyme probably corresponded to a ribosomal RNA fraction.

The capacity of the partially purified RNA fraction to elicit antibodies to *Brucella* agglutinogenic antigens was of interest in relation to previous observations on the properties of *Brucella* ribosomes (Corbel, 1976*a*, *b*). Thus both the RNA and the ribosomal fractions elicited high titres of *Brucella* agglutinins in rabbits, even though the lipopolysaccharide-protein agglutinogen could not be demonstrated in the preparations by direct methods. Indeed it seems improbable that the purified RNA preparations could have contained intact agglutinogen as their estimated molecular weight of 35000 daltons was approximately 100 times less than that of the lipopolysaccharide-protein complex. Nevertheless, it is quite possible that these preparations were contaminated with partially degraded agglutinogen which could have formed an RNA complex. Under these circumstances the RNA could have acted as an immunological adjuvant (Merritt & Johnson, 1965).

That the RNA was not antigenic *per se* was demonstrated by the failure of highly purified phenol-extracted material to evoke antibodies. In contrast, DNA has been reported as essential to the serological specificity of some *Brucella* antigens (Philips, Braun & Plescia, 1958; Plescia *et al.* 1961; Freeman, McGhee & Baughn, 1970).

The capacity of the RNA to form complexes with antigens and haptens and to elicit *agglutinating antibodies and non-specific inflammatory reactions on injection* into animals, suggested that its presence would be undesirable in diagnostic skin test antigens.

Recently, the need to exclude lipopolysaccharide endotoxin from *Brucella* skin test preparations has received considerable emphasis (Jones, Diaz & Taylor, 1973). The results of the present study suggested that RNA should also be excluded from these diagnostic reagents.

From a practical point of view this would probably be of greater significance in relation to allergens intended for veterinary use than those intended for human

diagnosis. This is largely because of the much greater quantities of antigen required to elicit positive intradermal reactions in cattle compared with man.

The means whereby substantial quantities of ribosomal RNA accumulated in *Brucella* culture supernatants was not determined. Nevertheless, it seems most probable that it was released during the course of autolysis of *Brucella* cells. This would also account for the presence of other macromolecular components, including a number of antigens, in the culture supernatants. Such autolysates would also be expected to contain enzymes of cellular origin, including endogenous nucleases capable of degrading RNase-sensitive RNA. That such degradation evidently did not occur could have been attributable to the binding of these enzymes to subcellular particles, thus reducing their activity on RNA in free solution. Evidence has been presented to show that virtually all of the cellular RNase of *Escherichia coli* is bound to the 30S ribosomal sub-units (Tal & Elson, 1963) but it is not known if this is so in *Brucella* cells.

An additional possibility, although one for which no direct evidence is available, is that the RNA was released by actively growing *Brucella* cells. If such a process was to occur *in vivo*, it might confer some protection against the cationic proteins of phagocytic cells. These have been reported to exert an inhibitory effect on ingested *Brucella* organisms (Ralston & Elberg, 1961). It is not known however, if *Brucella* cells release soluble RNA *in vivo* and clearly further studies are required to elucidate the biological role, if any, of this material.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Disk electrophoresis of phenol:acetic acid:water solutions of (a) lysozyme, (b) lysozyme precipitate of *B. abortus* S99 culture supernatant.
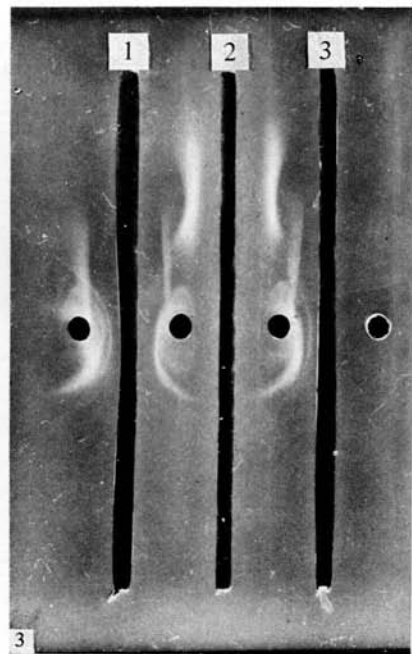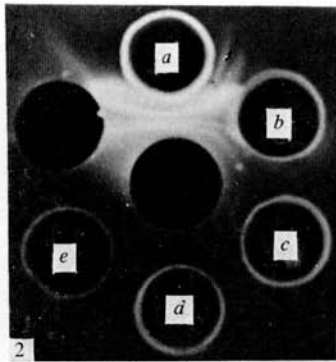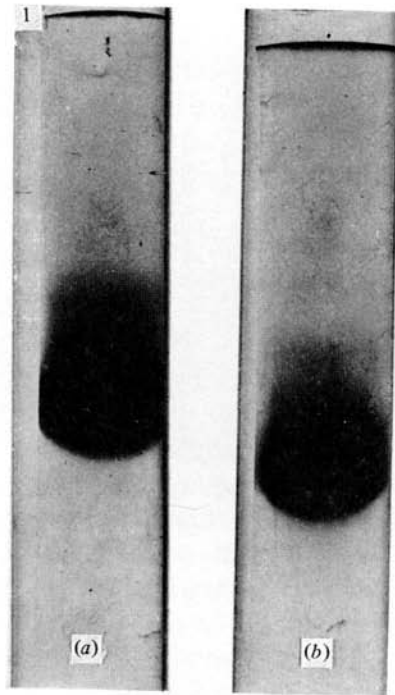
Fig. 2. Immunodiffusion of (a) concentrated culture supernatant of *B. abortus* S99, (b) Sephadex G50 peak 1 fraction of lysozyme precipitate of (a), (c) Sepharose 6B peak 3 fraction of (b), (d) ultra-centrifuged supernatant from (c), (e) phenol-extracted supernatant from (d). Rabbit antiserum to *B. abortus* 544 is in the centre well. Precipitation lines have formed only against wells (a) and (b).

Fig. 3. Immunoelectrophoresis of concentrated culture supernatant of *B. abortus* S99. Antiserum to *B. abortus* 544 is in troughs 1 and 3 and a 1 % (w/v) solution of lysozyme in trough 2. It is evident that the culture supernatant component precipitating with the lysozyme does not correspond to any of the antigens reacting with the antiserum.
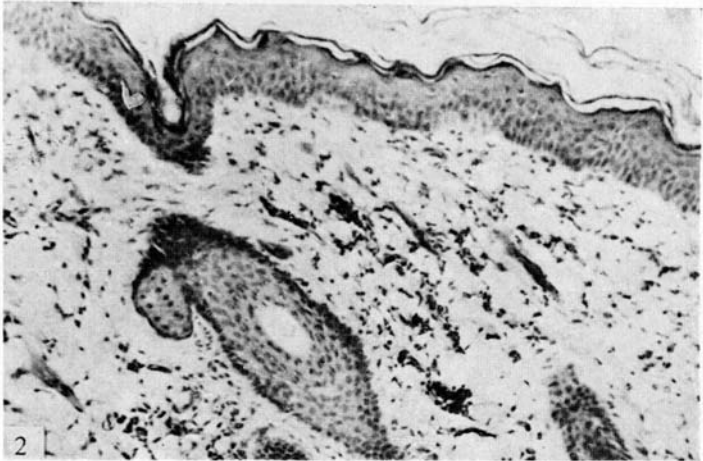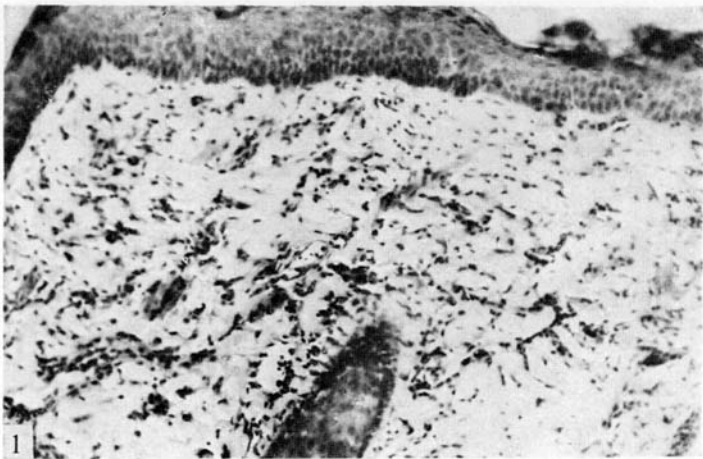
PLATE 2

Fig. 1. Section of skin from an unsensitized guinea-pig taken 24 h after local injection of partially purified *B. abortus* RNA. There is evidence of a non-specific inflammatory reaction involving mainly polymorphonuclear leucocytes. Haematoxylin and eosin, × 100.

Fig. 2. Section of skin of a guinea-pig sensitized with *B. melitensis* Rev 1 taken 24 h after local injection of partially purified *B. abortus* S99 RNA. There is evidence of a low grade inflammatory response involving both polymorphonuclear and mononuclear leucocytes. Haematoxylin and eosin, × 100.

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