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PROTECTIVE ANTIGENS ISOLATED FROM *BR. ABORTUS*.

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MILES and Pirie (1939*a*) described a soluble antigen prepared from *Brucella melitensis*. In its most highly aggregated form it appeared to be a complex of at least two phospholipides with a protein-like material and the N-formyl derivative of a polyhydroxyamine. Mention was made (Miles and Pirie, 1939*a, b*) of a similar material obtained from *Br. abortus*, but the behaviour of this antigen was at that time irregular and no details of its preparation and properties were given. The differences that were observed in the biological behaviour of the melitensis antigen (Miles and Pirie, 1939*c*) in different states of aggregation, taken in conjunction with the general body of immunological experience, suggested that the relative inefficiency, as immunizing agents, of preparations made from *Br. abortus* by other workers, might be due to the fact that the antigens in them were not in the optimum state of aggregation. Working on this hypothesis many preparations of the abortus antigen have been made during the past seven years by different methods, and in this paper techniques are described whereby antigens capable of conferring a considerable degree of immunity to guinea-pigs can be prepared.

For the isolation of the antigen it is unnecessary completely to disintegrate the organisms, and for this and other reasons Miles and Pirie (1939*c*) suggested that in *Br. melitensis* it might exist as a capsule. The same arrangement of the antigen is probable in abortus organisms, and Huddleson (1940, 1941) has now presented histological evidence for a capsule.

One strain of *Br. abortus* organisms has been used throughout this work. In the earlier work on *Br. melitensis* (Miles and Pirie, 1939*a, b, c*) only one strain was used. The differences in chemical and physical properties to be described may, therefore, be strain and not species differences.

*Preparation of the Antigen.*

Suspensions were prepared in neutral 0.5 per cent phenol saline from 48–72 hours' liver agar growths of strain 544 (McEwen, Priestley and Paterson, 1939). This strain is virulent, smooth, and requires 5–10 per cent CO<sub>2</sub> for good growth. The cultures used as seed material were the second subcultures from a dried culture (the fourth subculture of the original isolation on 26/3/36), except where an early variant, able to grow without additional CO<sub>2</sub>, was chosen for comparative purposes.

Part of the antigen is extracted by the 0.5 per cent phenol, but this extract contains the greater part of the agar breakdown products and other materials that complicate the purification of the antigen. Unless economy of material is

essential, therefore, it is better to centrifuge the suspension and discard the fluid, or keep it for the preparation of largely disaggregated antigen by the methods described later. The bacterial mass left after centrifuging is suspended in 20 times its weight of neutral 2 per cent phenol, and the mixture is left, with occasional shaking, for 1-2 weeks. After centrifuging for an hour at 3500 r.p.m. the supernatant fluid is only slightly opalescent and contains most of the readily extractable antigen; further similar extraction with 2 per cent phenol brings out little more. There is no evidence that the use of stronger phenol increases the yield.

The antigen can be isolated by the direct ultracentrifugation of this extract, but this involves centrifuging inconveniently large volumes of fluid; it is, therefore, better to concentrate the extract first by ultrafiltration. "Cellophane" sausage skin tube 2.2 in. in circumference is suitable if it is protected from hydrostatic stretching by being surrounded by a cloth tube of the same diameter. The tube is knotted at one end, the other end tied firmly on to a rubber bung carrying a filling tube, and the whole is mounted in a bottle that can be exhausted and left clipped off. If the tube is kept filled and if its inside is at atmospheric pressure while its outside is at about 20 mm. pressure, the initial rate of ultrafiltration is about 1 ml. per hour per cm. length of tube. Slight clogging during filtration reduces the rate of flow, but this can be partly restored by releasing the vacuum and, with the tube full of fluid but flaccid, rubbing the two internal faces together gently. The fluid is ready for ultracentrifugation when there is a significant rise in its viscosity; at this stage it will have a solid content of 1 to 2 per cent. The pH is adjusted to 7, and it is centrifuged for 30 min. at 10,000 r.p.m. to get partial clearing.

An air driven ultracentrifuge of the type described by Masket (1941) was used to sediment the antigen; in general the centrifuge runs lasted for 30 min. at 30,000 r.p.m. (R.C.F. 50,000). The supernatant fluid is poured off from the turbid coherent pellets, and these are resuspended in water to give a fluid with a solid content of less than 1 per cent and ultracentrifuged again. On a third similar ultracentrifugation there is only a trace of un sedimented material in the supernatant fluid; no further fractionation is therefore possible by ultracentrifuging under these conditions. The pellets are resuspended in water, and cleared by centrifuging for 30 min. at 10,000 r.p.m. It is the resulting colourless opalescent fluid that is referred to as the antigen; solutions are stable at 0° C. for some weeks, but after some months part of the material flocculates. Bacteria do not grow in it readily, and it can be satisfactorily preserved with 0.5 per cent phenol.

Digestion with commercial trypsin or autoclaving liberates further quantities of antigen from the bacterial mass left after extraction with 2 per cent phenol. After 3-4 days' digestion at pH 8 and 40° C. in the presence of chloroform as disinfectant the bacterial suspension is centrifuged and the antigen isolated by differential centrifugation as before. The residue from this treatment is resuspended and is autoclaved for 15 min. at 120° C. at an initial pH of 8.5. The pH is of some importance, for it falls during autoclaving and the antigen is destroyed below pH 6.5. If the suspension is made in 0.1 M phosphate buffer at 8.5 the final pH will not be below 7.5. This extract also is worked up as before. These two products should be kept separate from that made from the 2 per cent phenol extract, for one of them may be contaminated with constituents of the commercial trypsin, and the other is certainly contaminated with antigenically inert fragments of the bacterium. The bacterial bodies are partly disintegrated by

autoclaving, and if the fluid obtained by autoclaving a second time is fractionated ultracentrifugally, a product with the physical properties associated with the antigen can be separated. Its chemical properties are, however, clearly distinguishable from those of the antigen.

Batches vary somewhat both in the total yield of antigen and in the way in which it is distributed between the different extracts. Good yields, expressed as a percentage of the dry matter of the bacterial sediment in the original 0.5 per cent phenol suspension are :

	1 per cent from the 0.5 per cent phenol extract.
7	” ” 2 per cent phenol extract.
2.5	” ” trypsin extract.
2.5	” ” extract on autoclaving.

Under the best conditions the yield of antigen may thus be 13 per cent of the dry weight of the killed bacterium, and this yield is of the same order as that found with *Br. melitensis*. Yields may, however, fall to half of these values.

#### CHEMICAL AND PHYSICAL PROPERTIES OF THE ANTIGEN.

In its purified form the antigen is soluble over the whole pH range, but it readily precipitates from crude solutions along with any other material that is acid precipitable. Unlike solutions of the *Br. melitensis* antigen this material does not show anisotropy of flow. Solutions are apparently unaffected by heating for periods up to an hour at 100° C. in the neutral range, and 60 per cent of a preparation can be recovered by ultracentrifuging a solution that has been autoclaved for an hour at 120° C. and pH 7.

Preparations made by the methods described are ultracentrifugally inhomogeneous, as would be expected if they consist of particles derived by the more or less random fragmentation of a bacterial capsule. By centrifugation at an intermediate speed, such as 20,000 r.p.m., some fractionation is possible. The fractions do not differ from one another fundamentally in their chemical properties; their biological properties have not been compared.

After drying or freezing part of the antigen becomes insoluble, and all products that are to be used as immunizing agents or for serological studies have therefore been kept in solution. The antigen, unlike most other large molecules, cannot be satisfactorily dried by sublimation, for it forms a loose incoherent mass that is scattered by the wind from the evaporating ice. Analytical and biological data depend on dry weights measured by evaporating samples of the salt-free solution to dryness at room temperature over  $P_2O_5$  *in vacuo*. Conditions were such that the dried film was less than 0.1 mm. thick; drying is likely to have been complete throughout, and frequent checks showed no further loss of weight on drying at 100° C. These films are hygroscopic, but do not pick up water with the avidity of the more open textured mass that results from drying by sublimation. About 5 per cent of water is absorbed by the time weighing is finished; the results have not been corrected for this moisture content.

The nitrogen content is 4–5 per cent, but, as with other materials of this type, it is not a trustworthy index of contamination, for the probable contaminants

have similar nitrogen contents. Carbohydrate, estimated by an orcin method (Pirie, 1936), using glucose as standard, falls in the range 10–12 per cent, but the high lipid content makes this method somewhat uncertain on account of the slight turbidity of the final coloured solution. The main value of the determination is to detect contamination with agar; this gives a cherry red colour in the early stages of the estimation, and any preparation which gives this colour or contains more than 13 per cent of carbohydrate can probably be further purified by ultracentrifugation. The formyl content is a trustworthy index of the presence of antigen, for formic acid is not known to be liberated, under the conditions used, from any other substance except the melitensis antigen. 10–20 mg. lots of antigen are hydrolysed for 30 min. at 100° C. with 1–2 ml. of  $N H_2SO_4$ , and formic acid is estimated (Pirie, 1946) on an aliquot of the hydrolysate. Agar yields formic acid slowly when heated with acids, but the amounts that can remain in a purified preparation of antigen introduce a negligible error if the hydrolysis only lasts for 30 min. Values range from 4 to 5 per cent of CHO — Lower values are given by products made by autoclaving the bacterial residue because these are contaminated with fragments of the bacterium; higher values will be given by products from which the loosely bound phospholipid has been partially removed.

The phosphorus content varies in the range 0.7 to 2.0 per cent. Most of the phosphorus is present as phospholipid, and it can be separated from the antigen by treating an aqueous solution with 10 volumes of a mixture of alcohol and ether containing 0.5 per cent of concentrated hydrochloric acid (Miles and Pirie, 1939a). The defatted residue is fairly constant in its properties. It is now only partially soluble in water, and contains 0.5 per cent of phosphorus. Half of this is liberated as phosphate after hydrolysis for an hour at 100° C. in  $N/12 HCl$ ; the other half remains soluble after this treatment, but is present in the amorphous precipitate that separates on hydrolysis with stronger acid. This precipitate closely resembles the one prepared in the same way from the melitensis antigen and called PL2 (Miles and Pirie, 1939c).

The variability of the phosphorus content of the antigen is due to variations in the amount of phospholipid associated with it and in an extensive series of analyses products with satisfactory centrifugal behaviour and formyl content have always contained about 0.5 per cent of phosphorus not soluble in lipid solvents. Products made from the 2 per cent phenol extract have a higher phospholipid content than those made by the other three methods of extraction, and the more rapidly sedimenting fraction of a preparation generally has a higher phosphorus content than the more slowly sedimenting part. No sharp segregation into fractions of different phospholipid content has, however, been possible. Some of the nitrogen in the antigen occurs in these two phospholipid fractions, but the greater part remains soluble after acid hydrolysis, and this solution gives the Elson and Morgan (1933) colour reaction for amino-sugars. As with the corresponding product from *Br. melitensis* (Miles and Pirie, 1939d), the colour reaction is given without preliminary acetylation; there is, therefore, reason to think that the substance present is not one of the already known amino-sugars.

In the course of preparation the antigen is only subjected to ultracentrifugal fractionation, and this is designed to give a group of particles having sedimentation constants within a fairly broad range. Other methods of fractionation which might be of use both in the preparation and as criteria of homogeneity have

therefore been sought, but so far without success. Precipitation with alcohol or acetone removes part of the lipid and makes much of the antigen insoluble in water. Precipitation with ammonium sulphate at 40 per cent of saturation does not appear to alter the antigen if the precipitate is centrifuged off and redissolved in water in a few minutes, but if contact with ammonium sulphate of this concentration lasts for some hours much of the antigen is so altered as to be no longer soluble in water. Solubility can be restored by washing the precipitate free from ammonium sulphate and heating it in neutral aqueous suspension at 100° C. for some hours. Preparations that have been disaggregated by autoclaving or treatment with organic solvents can also be partially reaggregated by exposure to half saturated ammonium sulphate solution.

This behaviour with ammonium sulphate complicates the separation of the small amount of antigen from the original 0.5 per cent phenol extract of the bacteria. In a few experiments part of this extract has been directly ultracentrifuged, but the volume is too large for this to be a satisfactory routine procedure. Agar breakdown products in it precipitate with ammonium sulphate, but this precipitate does not redissolve unless the ammonium sulphate concentration is lowered below 1/10th of saturation. To separate the antigen, therefore, 360 g. of ammonium sulphate is added to each litre of extract; the precipitate is centrifuged off immediately and suspended in 4-5 times its bulk of water. After some hours it is centrifuged; the fluid contains much of the antigen, and this can be recovered by dialysis and ultracentrifugation. Alternatively the ammonium sulphate precipitate can be mixed with phenol or pyridine to disaggregate and dissolve the antigen; from this solution it is precipitated by alcohol, and can be purified by ultracentrifugation after partial aggregation with ammonium sulphate. By these procedures it is possible to recover only about half the antigen that can be separated by direct ultracentrifugation.

#### *Disaggregation of the Antigen.*

In some of the biological tests to be described later, disaggregated products have been used. A brief account of the methods used in their preparation is therefore necessary, but no conclusions about the nature of the differences between fractions with different physical properties have yet been reached.

Many disaggregating agents have been tried, but only concentrated phenol, pyridine and shaking with chloroform and amyl alcohol have been found to give reproducible results. In each case the treatment results in the separation of the antigen into two main fractions. The optically homogeneous solution that results when an aqueous solution of antigen is mixed with six parts of phenol or pyridine at room temperature gives a clear gelatinous layer when centrifuged at 3000 r.p.m. Disaggregated antigen can be recovered from this gelatinous layer by extraction with water, after removal of the reagent by washing with alcohol on the centrifuge. Similarly, part of the antigen, disaggregated, is present in the emulsion layer that is formed when the antigen, dissolved in acetate buffer at pH4, is shaken with chloroform and amyl alcohol (Sevag, 1934). The material soluble in phenol or pyridine can be precipitated by alcohol, and these precipitates dissolve freely in water. All the solutions thus obtained are clearer than those of the original antigen at the same concentration, and they are incompletely sedimented even when centrifuged at 40,000 r.p.m. A few comparisons have been made of the sedimentability of the original and disaggregated antigen at

24,000 r.p.m. Whereas 80–90 per cent of the former sediments into a coherent pellet in 30 min., only a third of the latter does so. There is no regular difference between products disaggregated by the three different methods, nor between the different fractions that result from each technique of disaggregation. In the biological tests reported only that part of a disaggregated preparation that sedimented at 24,000 r.p.m. was used.

Disaggregated antigen, from which the loosely bound phospholipid had been separated, was easily made from the *melitensis* antigen by treatment with 80 per cent acetic acid (Miles and Pirie, 1939a). This treatment is unsatisfactory with the abortus antigen because it is not soluble in strong acetic acid, and, although the treatment disaggregates part of the antigen, most of it is rendered insoluble in water. Disaggregated melitensis antigen was polydisperse, but it behaved towards precipitating agents like a single substance, and could be used as a convenient form of the antigen both in its isolation and in the study of many of its biological properties. It is clear from this survey that no such method of converting the abortus antigen into a water-soluble material of relatively low molecular weight has as yet been found.

#### BIOLOGICAL PROPERTIES.

##### *Inoculation of the Antigen into Normal Animals.*

(a) *Rabbits*.—Intradermal inoculation of 0.1 to 1 mg. produced small hard swellings at the site of inoculation at the 48th to 72nd hours. With certain preparations there was very slight necrosis of the superficial dermis at the 72nd hour, but all sites of injection had resolved by the 10th day without the skin breaking. In many animals, although not constantly with any given dose, a secondary oedematous inflammatory reaction was noted at the site of inoculation about the 9th–11th day. These swellings resolved in from 7–10 days, and presumably were due to circulating antibodies reacting with residual traces of antigen in the skin. Agglutinins were developed, reaching their maximum titre (1 : 640–1 : 1280) between the 6th and 11th days. By the 60th day they had dropped to 1 : 20–1 : 40. The inoculation of small single doses of the antigen by the intramuscular, intravenous or subcutaneous route provoked similar agglutinin responses.

The effect of repeated intradermal inoculation on the circulating agglutinins has been studied. In the first experiment groups of two rabbits were given a total dose of 0.6 mg. of the antigen as one, two and four doses at intervals of 28 days. Severe local reactions followed at the sites of the repeated inoculations. Necrotic areas up to 2 cm. in diameter developed, which subsequently sloughed. The titre continued to fall for four days after each repeat dose, and thereafter rose to 1 : 640 or 1 : 1280 during the course of a further 6–11 days, after which the titre again receded. Sixty days after the last portion of the total dose of 0.6 mg. had been inoculated the titres were at 1 : 20 to 1 : 40 level, at which height they remained for a further 60 days, when observation ceased.

In the second experiment a rabbit was given four doses of 0.3 mg. at intervals of 10–11 days. Three others were similarly inoculated with four doses of 0.03 mg., 0.003 mg. and 0.0003 mg. respectively. Local reactions were noted following the repetition of the 0.3 mg. doses, but not with those of smaller amount. The 0.3 mg. doses (repeated) at 10–11 day intervals maintained the titre at an almost constant level of 1 : 640–1 : 1280 from 7 days after the first dose until

10 days after the last. The rabbits inoculated with 0.03 mg. and 0.003 mg. developed titres similarly, but at a lower level, viz. 1 : 80–1 : 160. With 0.0003 mg. the titres failed to rise above 1 : 40.

In a third experiment three rabbits were given five doses of 0.3, 0.03 mg. and 0.003 mg. respectively at 7-day intervals, and the same treatment was applied to three rabbits at 3-day intervals. The effect of repeating these inoculations at short intervals was to increase agglutinin production slightly and to delay the maximum until about the 12th day. The rabbits inoculated with 0.3 mg. at 3- and 7-day intervals had maximum titres of 1 : 5000 on the 12th day after the first inoculation. The titres receded to 1 : 1280 by the 18th day. In the 3-day interval rabbit this recession was continuous but slow. The titre of the 7-day interval rabbit remained steady at 1 : 1280 from the 18th to the 44th day and then dropped slowly. At 85 days both rabbits had titres of 1 : 160. The agglutinin response in the rabbits inoculated with the smaller doses was in all respects similar to the above but on a lower level: with 0.03 mg. the maximum titre recorded was 1 : 640, and with 0.003 mg. 1 : 320 on the 10th and 12th days. Residual titres on the 89th day were 1 : 40–1 : 80.

The inoculation of larger amounts of antigen (3–10 mg.) caused transient inappetance and some loss of bodily condition for a few days. A large oedematous and erythematous swelling up to 5 cm. in diameter developed within 24 hours, and by the 72nd hour the centre of the swelling was necrotic (1 × 1 cm.) and umbilicated. About the 8th–10th day the necrotic centre sloughed away, releasing a variable quantity of pus. Healing took 4–6 weeks.

Repeated intramuscular inoculation of small doses did not cause any observable symptoms.

(b) *Guinea-pigs*.—Intradermal inoculation 0.1 to 1 mg. of antigen produced an evanescent local reddening. Inoculation of 3 to 10 mg. caused a more severe local reaction with necrosis, followed by rapid healing. In many animals secondary reactions were noted, as in rabbits.

(c) *Cattle*.—Two normal yearling calves which were inoculated intradermally with 1 mg. of antigen developed hard, non-painful swellings which disappeared very slowly, some trace of them still being discernible after 6 weeks. The two animals developed their maximum titre (1 : 640) within 6 days after inoculation. Sixty days after inoculation their titres were 1 : 40.

#### *Inoculation into Sensitized Animals.*

Small doses of antigenic fractions of varying molecular size have been inoculated into rabbits and calves which had been sensitized 28 days earlier by subcutaneous inoculation, with suitable doses of living *Br. abortus*, for which purpose a smooth aerobic strain of moderate virulence (McEwen, 161) was used. The reactions of these sensitized animals were much more vigorous than those of normal animals.

In rabbits local oedema and reddening was observed at the 24th hour, and by the 72nd hour this had progressed so that a necrotic lesion approximately 1 cm. across was seen. This was followed in 4–7 days by scab formation. Subsequent healing was slow, taking 20–30 days, but there was no pus formation.

A series of nine sensitized and six normal control calves was inoculated intradermally into the skin of the neck region. The technique used in these experiments was essentially the same as that employed in carrying out the intradermal



test with tuberculin, the increase in the thickness of a fold of skin at the point of inoculation being determined by calipers. In the sensitized calves soft swellings developed, which were oedematous and painful, but they did not become necrotic. They reached their maxima at the 72nd hour, the skin thickness being then increased up to 12 mm. with the most active preparations. Resolution was slow, the skin not returning to normal for a period of 24–48 days. The swellings which developed in the control animals were hard and circumscribed and reached their maxima in 48 hours, fading greatly by the 72nd hour.

#### *Immunization Experiments in Guinea-pigs.*

Preliminary small scale experiments, that need not be reported in detail, showed that fractions made from the bacteria by many different procedures did, in varying degree, confer protection on guinea-pigs against subsequent parenteral infection with virulent organisms. These experiments also suggested that the strain, age, sex and weight of the guinea-pig affected the protection. In the experiments reported animals of the same strain and comparable age and weighing 300–400 g. were used. At the chosen interval after treatment with the substance under investigation, the group of animals was divided into three subgroups. Except in the experiment recorded in Table I these received, as test dose intramuscularly in the thigh, 1 ml. of 1 in  $10^{-5}$ , 1 in  $10^{-6}$ , 1 in  $10^{-7}$  dilutions respectively of a saline suspension of a 48–72-hour growth of strain 544 standardized by opacity to tube 3 on Brown's scale. In the experiment shown in Table I the doses were 1 ml. of 1 in  $10^{-6}$ , 1 in  $10^{-7}$  and 1 in  $10^{-8}$  dilutions. Immediately before injecting the test doses, viable counts were made on blood agar plates or by inoculating "roll" tubes made with liver agar. These counts showed that the test doses contained approximately 40,000, 4000, 400 and 40 organisms.

TABLE I.

Product.	Dose.	Infection rate.
10.3 mg. of washed phenol killed bacteria	$10^{-6}$	1/10 10%
	$10^{-7}$	5/10 50%
	$10^{-8}$	3/10 30%
		} 9/30 30%
0.3 mg. of a 2 per cent phenol extract precipitated by ammonium sulphate and fractionated by ultracentrifugation	$10^{-6}$	1/10 10%
	$10^{-7}$	3/10 30%
	$10^{-8}$	0/10 ..
		} 4/30 13%
0.3 mg. of a 2 per cent phenol extract fractionated by ultracentrifugation	$10^{-6}$	2/9 22.2%
	$10^{-7}$	7/10 70%
	$10^{-8}$	1/9 11.1%
		} 10/28 35%
Controls	$10^{-6}$	10/10 100%
	$10^{-7}$	10/10 100%
	$10^{-8}$	9/10 90%
		} 29/30 96.6%

*Notes.*—(1) In the above and in subsequent tables the denominator indicates the number of animals exposed to infection and the numerator the number infected.

(2) The test doses are expressed as dilutions of a suspension standardized by opacity to correspond with tube 3 on Brown's scale. The volume of the infecting suspensions employed was 1 c.c., the  $10^{-8}$  dilution containing approximately 4000 viable organisms.

Six weeks after infection all the animals were killed, the blood agglutinin titres determined, and the spleens ground up in 1 per cent broth-saline and cultured for *Br. abortus*. The presence of live organisms in the spleen has been

accepted as evidence that any immunity engendered had been overcome. The immunizing values of the various fractions or of different methods of immunization have been assessed by a comparison of the infection rates.

In Table I two products are compared with a quantity of killed organisms containing as much or more antigen; it is clear that the partially purified antigen is as effective as the killed organisms. Two products, derived from the same

TABLE II.

Product.	Test dose.*	Interval between test dose and killing.			Total infection rate.
		6 weeks.	9 weeks.	12 weeks.	
Aggregated, 3 mg. i.d.	10 <sup>-5</sup>	2/4	0/1		6/22 27%
	10 <sup>-6</sup>	2/4	1/4	0/1	
	10 <sup>-7</sup>	0/4	1/4		
Disaggregated, 1 mg. i.d.	10 <sup>-5</sup>	3/4	2/2		16/20 80%
	10 <sup>-6</sup>	3/4	3/4	1/1	
	10 <sup>-7</sup>	3/4	1/1		
Controls	10 <sup>-5</sup>	6/6	6/6	6/6	53/54 98%
	10 <sup>-6</sup>	6/6	6/6	6/6	
	10 <sup>-7</sup>	5/6	6/6	6/6	

\* The test dose was administered thirteen weeks after the vaccine dose.

TABLE III.

Product.	Dose in mg. dry matter.	Test dose.	Cambridge.	Weybridge.	Total infection rate.
2 per cent phenol extract precipitated with ammonium sulphate and redissolved by heating	0.3	10 <sup>-5</sup>	8/9	8/12	16/21 76%
	i.d.	10 <sup>-6</sup>	8/10	5/14	13/24 54%
		10 <sup>-7</sup>	3/10	1/13	4/23 17%
Extract from bacterial residue by trypsin digestion and autoclaving	0.3	10 <sup>-5</sup>	7/10	7/12	14/22 64%
	i.d.	10 <sup>-6</sup>	5/10	2/14	7/24 29%
		10 <sup>-7</sup>	0/8	1/13	1/21 5%
Controls	..	10 <sup>-5</sup>	10/10	12/12	22/22 100%
	..	10 <sup>-6</sup>	10/10	12/12	22/22 100%
	..	10 <sup>-7</sup>	9/10	12/12	21/22 95%
Number of viable brucella in 1 c.c. of 10 <sup>-6</sup> dose			3700	4360	

Note.—The products were prepared from strain 544A (aerobic).

batch of organisms, and which were fractionated by differential centrifugation so as to yield an aggregated and a disaggregated fraction were compared and the results are set out in Table II. It is clear that the former is the more effective. The efficacy of antigens prepared from the 2 per cent phenol extract of the bacteria and from the bacterial residue after this extraction by digestion with trypsin and autoclaving is shown in Table III to be comparable. These two products, unlike all the others for which the results of tests are given here, were made from a variant of strain 544 that did not need CO<sub>2</sub>.

A product prepared by digestion with trypsin and autoclaving a suspension of a CO<sub>2</sub> culture of 544 was selected for an investigation into the effect of varying the time interval between a single dose of the antigen and infection; of giving two doses of antigen and of giving a single larger dose.

TABLE IV.

Treatment.	Test dose.	Cambridge.	Weybridge.	Total infection rate.
<b>A</b>				
Single dose 8 weeks before infection. 0.3 mg. in 0.2 c.c.	10 <sup>-5</sup>	8/10	4/10	12/20 60%
	10 <sup>-6</sup>	6/10	3/10	9/20 45%
	10 <sup>-7</sup>	0/9	0/10	0/19 0%
} 21/59 36%				
<b>B</b>				
Single dose 4 weeks before infection. 0.3 mg. in 0.2 c.c.	10 <sup>-5</sup>	7/10	5/10	12/20 60%
	10 <sup>-6</sup>	3/10	2/10	5/20 25%
	10 <sup>-7</sup>	1/10	1/10	1/20 5%
} 18/60 30%				
<b>C</b>				
Single dose 2 weeks before infection. 0.3 mg. in 0.2 c.c.	10 <sup>-5</sup>	9/10	7/10	16/20 80%
	10 <sup>-6</sup>	6/10	2/10	8/20 40%
	10 <sup>-7</sup>	2/10	1/10	3/20 15%
} 27/60 45%				
<b>D</b>				
Two doses 6 and 2 weeks before infection, each of 0.3 mg. in 0.2 c.c.	10 <sup>-5</sup>	4/10	3/10	7/20 35%
	10 <sup>-6</sup>	1/10	0/10	1/20 5%
	10 <sup>-7</sup>	1/10	1/10	2/20 10%
} 10/60 17%				
<b>E</b>				
Single dose 2 weeks before infection. 0.6 mg in 0.4 c.c.	10 <sup>-5</sup>	7/8	9/10	16/18 89%
	10 <sup>-6</sup>	2/7	4/10	6/17 35%
	10 <sup>-7</sup>	0/7	1/10	1/17 6%
} 23/52 44%				
Controls	10 <sup>-5</sup>	10/10	10/10	20/20 100%
	10 <sup>-6</sup>	10/10	10/10	20/20 100%
	10 <sup>-7</sup>	10/10	10/10	20/20 100%
} 60/60 100%				
Viable count of 1 ml. of 10 <sup>-6</sup> dose . . . . . 4700 . 3970				

*Note.*—The product used in this experiment was from CO<sub>2</sub>-sensitive strain 544, and was made by incubating washed organisms with trypsin and autoclaving the residue.

The results of this experiment (Table IV) clearly indicated that the injection of two doses spaced by a month was superior to any of the single injection methods, and that probably the best time for a single injection was about four weeks before injection of the test dose. There was no indication that doubling the dose had any beneficial effect when given 14 days before infection. In a preliminary experiment we found that increasing the dose five times (to 1.5 mg. i.d.) did not give increased protection when the interval between vaccination and infection was 28 days.

There was, however, one feature of this experiment which calls for mention.

Earlier in this paper attention has been drawn to the severe reactions following the intradermal inoculation of the antigen into sensitized rabbits and guinea-pigs. One of the effects of the initial dose of 0.3 mg. in group D was to render a proportion of the guinea-pigs sensitive, and the inoculation of the second dose was followed by a marked local reaction. Twenty-four hours after inoculation the site was hot, red and painful, and about 2 cm. in diameter. By the 72nd hour it had enlarged to between 1.5 and 2.0 cm. The raised centre of the swelling was yellow, becoming necrotic. By the fifth day the centre of the swelling was purple, necrotic and definitely umbilicated. This necrotic tissue sloughed out. Healing was fairly rapid, usually being complete by the 15th day.

A further experiment was carried out in which the antigen was a mixture of aggregated products from several different lots of bacteria. Treatments B and D of Table IV were repeated. In order to find out whether the local reaction which follows a second injection could be avoided without reducing its effectiveness, by using the intramuscular route, groups were set up in which the primary doses were intradermal and subsequent doses intramuscular. It was also decided to observe the effect of the larger dose when given at what we believed to be the optimum time before infection, viz. one month. The effect of dividing this larger dose into four equal parts was also studied.

The intramuscular injections were performed in one of the hind limbs, the other being reserved for the inoculation of the test dose. The interval between infection and killing was approximately 42 days.

The local reactions to the second inoculation in Group B were severe, but in Groups E and F the local reactions to the second or subsequent inoculations were less marked. Rarely was necrosis and sloughing noted, but soft, raised, reddened swellings were always produced.

No apparent ill-effects followed the intramuscular inoculations.

The results, which are set out in Table V, showed again that there was an advantage in dividing the immunizing dose into two injections at an interval of 28 days, the last two weeks before test inoculation, but that the division of the immunizing dose into four parts had no particular advantage over two. The use of the intramuscular route for doses other than the first did not reduce the level of protection. The inoculation of 0.6 mg. had no advantage over 0.3 mg. when given as a single dose 28 days before infection.

#### DISCUSSION.

\* The studies of Miles and Pirie (1939*a, b, c*) and the work presented here show that *Br. melitensis* and *Br. abortus* contain antigens of a broadly similar nature which can be separated from the bacterial cell in relatively pure states. They have well defined physical and chemical characteristics, which can be used as criteria in estimating their probable value as immunizing agents.

Brucella extracts have been used by various workers. A trichloroacetic acid extract prepared by Boivin's method from *Br. melitensis* was used by Roman (1938), who claimed some immunizing properties. The antigen was said to be non-protein in character and was called a glucolipid antigen. Priestley (1940) and Stahl and Hamann (1941), however, were unable to protect guinea-pigs with trichloroacetic extracts of *Br. abortus*. Priestley's extract was antigenic, heat-stable and slightly toxic, but it failed to elicit skin reactions in infected guinea-pigs. He was also unable to obtain an antigen in this way from rough strains.

TABLE V.

Treatment.	Test dose.	Cambridge.	Weybridge.	Total infection rate.
A				
Single dose 0.3 mg. i.d. 4 weeks before infection	10 <sup>-5</sup>	8/8	9/10	17/18 94%
	10 <sup>-6</sup>	4/10	5/10	9/20 45%
	10 <sup>-7</sup>	0/8	4/10	4/18 22%
} 30/56 54%				
B				
Two doses 0.3 mg. i.d. 6 and 2 weeks before infection	10 <sup>-5</sup>	7/9	7/9	14/18 78%
	10 <sup>-6</sup>	8/10	2/10	10/20 50%
	10 <sup>-7</sup>	0/7	0/10	0/17 0%
} 24/55 44%				
C				
One dose 0.6 mg. i.d. 4 weeks before infection	10 <sup>-5</sup>	7/7	9/10	16/17 94%
	10 <sup>-6</sup>	7/10	5/10	12/20 60%
	10 <sup>-7</sup>	4/10	2/10	6/20 30%
} 34/57 60%				
D				
0.3 mg. i.d. 6 weeks before and 0.3 mg. i.m. 2 weeks before infection	10 <sup>-5</sup>	8/9	1/10	9/19 47%
	10 <sup>-6</sup>	6/10	1/10	7/20 35%
	10 <sup>-7</sup>	3/10	1/10	4/20 20%
} 20/59 34%				
E				
4 doses each of 0.15 mg. i.d. given 14, 10, 6 and 2 weeks before infection	10 <sup>-5</sup>	7/9	5/10	12/19 63%
	10 <sup>-6</sup>	6/10	0/10	6/20 30%
	10 <sup>-7</sup>	2/9	1/9	3/18 17%
} 21/57 37%				
F				
2 doses 0.15 mg. i.d. 14 and 10 weeks before infection followed by 2 doses 0.15 mg. i.m. 6 and 2 weeks before infection	10 <sup>-5</sup>	6/9	4/10	10/19 53%
	10 <sup>-6</sup>	5/9	4/10	9/19 47%
	10 <sup>-7</sup>	0/9	2/9	2/18 11%
} 21/56 38%				
Controls	10 <sup>-5</sup>	7/7	9/10	16/17 94%
	10 <sup>-6</sup>	9/9	10/10	19/19 100%
	10 <sup>-7</sup>	9/10	8/10	17/20 85%
} 52/56 93%				
Viable count of 1 ml. 10 <sup>-6</sup> dose		4120	5300	

*Note.*—The product used in this experiment was a mixture of still aggregated extracts from several lots of bacteria (CO<sub>2</sub>-sensitive).

Huddleson (1943) reported moderate protection of guinea-pigs by means of an extract obtained by passing *Br. abortus* through a wet bacterial mill. The extract was extremely unstable, its immunizing capacity being destroyed by 56° C. or 0.5 per cent phenol, and it seems unlikely therefore that the active constituent is related to the material we find effective. Protection of guinea-pigs with an extract was also reported by Live, Sperling and Stubbs (1945) using a sonic method, which is believed not to denature labile somatic antigens (Chambers and Flosdorf, 1936). They describe their product as containing protein. There is reason to think that it may contain material resembling the antigen described by us.

In our preliminary experiments on immunization, fractions which had been disaggregated by exposure to phenol or pyridine did not induce such a high degree of resistance as those which were still in the aggregated state. Our observations on the skin reactions in sensitized animals, on the other hand, showed that the largest swellings were provoked by the disaggregated products. It would appear, therefore, that there are different optimal particle sizes for skin testing and immunization. It is also clear that an intracutaneous method for the detection of sensitized cattle might be elaborated. It would be of practical value only if the skin reaction can be elicited by material so far disaggregated that it no longer leads to the development of agglutinins in normal animals.

Having shown in the earlier experiments that particle size was of importance, and having tested products prepared in a variety of ways, but all of which were thought likely to have good immunizing properties (Tables I, II and III), attention was next directed to mode of administration and dosage. The finding (Tables IV and V) that division of the inoculum into two injections with an interval between was better than increase of the size of a single dose was not unexpected. Further division (Table V) appears of no advantage.

The variation found in successive experiments with products thought likely to have similar protective properties was often greater than expected. There is no doubt, however, that the fractions prepared have, taken as a whole, considerable protective properties. This is shown not only by the figures for positive and negative post-mortem findings recorded here, but also by the less severe infection and the marked reduction in the numbers of organisms recovered in spleen counts from vaccinated animals as compared with the unvaccinated controls.

The degree of protection is best expressed by comparison with other well-known immunizing agents, such as the American Strain 19, now in general use in the U.S.A. and Great Britain. It is now well established (Weybridge results, unpublished) that living vaccines prepared with S. 19, which confer a high resistance on cattle, fail to protect approximately 15, 20 and 50 per cent of guinea-pigs respectively against the three test doses generally used in the work reported here (i.e. except in the experiment in Table I). de Ropp (1945) has already published somewhat similar results using the same methods. Comparison of the results obtained with our fractions shows that the level of protection was sometimes similar to, or once (with two doses), greater than that conferred by S. 19, but that with single doses it was usually definitely lower.

The relative values of antigens prepared from CO<sub>2</sub>-sensitive and aerobic strains have not been adequately investigated, but such evidence as we have does not indicate that the former are greatly superior.

Such questions as the duration of immunity and the possibility of combining

the antigen with living (avirulent) vaccine have not yet been considered, and further work is needed on particle size, but we feel that the evidence that has been accumulated regarding these antigens should be presented at this stage.

#### SUMMARY.

The methods employed to isolate an antigenic substance from suspensions of *Br. abortus* are described together with its physical and chemical properties.

The inoculation of the antigen by various routes, in doses as low as 0.0003 mg., into normal animals causes the production of circulating agglutinins. When large doses are inoculated intradermally symptoms of toxæmia develop in addition to massive local necrosis at the site of inoculation.

The intradermal inoculation of small doses of antigen into sensitized animals is marked by local oedema and swellings which tend to persist for lengthy periods.

As judged by guinea-pig tests, the antigen possesses considerable immunizing powers. The resistance conferred by a single dose of 0.3 mg., or by larger doses, was however usually lower than that produced by one dose of living *Br. abortus* of low virulence, e.g. S. 19. The results with two doses of 0.3 mg. or with four doses of 0.15 mg. varied, but more nearly approached those obtained with strain 19.

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