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Immunization by an Insoluble Fraction Extracted from *Brucella melitensis*: Immunological and Chemical Characterization of the Active Substances

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A peptidoglycan-containing fraction called fraction P.I. (phenol insoluble), extracted from *Brucella melitensis* and previously described by some of us, had immunogenic and protective properties and did not produce any allergic reactions. Since it is well known that bacterial peptidoglycans studied so far have immunoadjuvant properties, the isolation of the active factor(s) of *Brucella* was undertaken. By successive enzymatic and chemical treatments, a new, much more purified fraction, called "4A" (approximately 5% of fraction P.I.), is obtained, retaining the same properties as P.I. and giving better protection against infection by *Brucella*. Immunogenicity, immunoadjuvant activity, allergizing capacity, and specific and nonspecific protective effects of fractions P.I. and 4A are compared. Chemically, fraction 4A is constituted by a lipoprotein covalently linked to peptidoglycan and by a few (lipo)proteins that could be solubilized by hot sodium dodecyl sulfate. Intrinsic properties of peptidoglycan could not be studied, but it does not seem to be essential for the activity. In conclusion, fractions P.I. and 4A are not agglutinogenic and, since fraction 4A induces better protection against infection by *Brucella*, it could advantageously replace fraction P.I. as a vaccine for humans.

Like the cells of most gram-negative bacteria (9), *Brucella* cells are bounded by an envelope consisting of an inner or cytoplasmic membrane, an intermediate peptidoglycan layer (10, 21, 24), and an outer membrane; but the arrangement of these different constituents is not known as it is in the case of *Escherichia coli* (7) and some other bacteria (6).

The outer envelope is immunologically the most important structure because it is the part of the cell that interacts with the host defense mechanisms. Peptidoglycan also plays a role: it was first demonstrated as early as 1965 in gram-positive bacteria in which immunogenic properties of peptidoglycan were described (1), later the peptidoglycan of *Mycobacteria* was shown to be essentially responsible for the adjuvant properties of the whole cells (2), and now it is recognized that all the peptidoglycans studied so far have the same properties (16, 28, 38).

A lot of work has been devoted to the preparation of protective fractions from either living or killed *Brucella* in order to find out some effective substances able to protect cattle, persons at risk during their employment, or people in countries where brucellosis is endemic in farm livestock. Cell walls afford a better protection than intact cells (31), and *Brucella melitensis* give the highest degree of protection against homologous challenge (20, 33). In 1970, Gargani (14) published a review on vaccines used on cattle, but protection was often insufficient and most of the time there were side effects such as allergic reactions and delayed hypersensitivity.

Since 1970, some new preparations have been offered (30, 41), which unfortunately have the same drawbacks as previous ones. We have described an immunogenic phenol-insoluble fraction (fraction "P.I.") obtained by phenol extraction of delipidated *Brucella* cells, devoid of agglutinogenic activity; Rasooly et al. (33) and then McGhee and Freeman (23), as well as Renoux et al. (34), confirmed that phenol-extracted residues were highly immunogenic. Such a fraction was used in human vaccination by Roux et al. (37), giving good results.

Fraction P.I. is the best preparation we have obtained thus far to protect animals against *Brucella* infections. Therefore, we chose it as the starting material to prepare a better immunogenic fraction and to increase the activity by eliminating inert material. Fraction P.I. is a

very complex mixture, and our purpose was to obtain some information on the chemical nature of the substances implied in this protective activity. Peptidoglycan being present in this fraction, we first wished to learn if it plays a role in the observed properties of fraction P.I. Also, it was interesting to obtain some information on the linkages between the components of our preparation and their importance for biological activities.

By successive chemical and enzymatic treatments, a new insoluble fraction ("4A") was obtained, retaining the properties of the initial product (fraction P.I.) and inducing better protection. Some data on its chemical nature and its main biological properties are described in this paper.

(This paper includes part of a thesis submitted [1976] by A. L.-M. to the University of Toulouse in fulfillment of the requirements for a doctoral degree.)

MATERIALS AND METHODS

Chemicals. Silica Gel G (Merck) was used for thin-layer chromatography (TLC); ethylenediaminetetraacetate (Titriplex III, disodium salt) and tris(hydroxymethyl)aminomethane (Tris) buffer were also products of Merck. Ribonuclease (38,700 U/mg), papain, and L-cysteine were obtained from Fluka, deoxyribonuclease (43,000 U/mg; B grade) and lysozyme were from Boehringer, pepsin (3× crystallized; 25,000 U/mg) was from Nutritional Biochemicals Corp., and Pronase (750,000 tyrosine units/mg) was from Kaken Chemical Co. Sodium dodecyl sulfate (SDS), ammonium persulfate, hexamethyldisilazane, and trimethylchlorosilane were products of Touzart et Matignon. Acrylamide, *N,N*-methylene-bis-acrylamide, and *N,N,N',N'*-tetramethylethylenediamine were supplied by Eastman, Amberlite MB3 by British Drugs Houses, and Triton X-100 by Intertechnique. All solvents were redistilled before use, and chemicals were the best grade obtainable.

Bacteria and extraction of P.I. The strain of *B. melitensis* (type I, strain M15) and the culture conditions were the same as described previously (36). The preparation of P.I. from delipidated cells and its main properties were described earlier (C. Lacave, Ph.D. thesis, Univ. of Toulouse, Toulouse, France, 1969).

Fractionation of crude material. To fractionate the crude fraction P.I., we tried to eliminate most of the material by methods as mild as possible in order to retain biological activities. It is known that detergents modify conformation and, therefore, the activities of some enzymes (13); therefore, we did not use them at the beginning of the process. The fractionation procedure is given in Fig. 1, and yields of different steps are given in Table 1. P.I. is insoluble and therefore difficult to digest; unspecific proteases such as Pronase had to be used. P.I. was first ground in a mortar and suspended in Tris-hydrochloride buffer (10^{-2} M, pH 7.0); ribonuclease was added (1 mg/100 mg of P.I.) after incubation at 25 C for 30 min with stirring, the suspension was centrifuged, the sediment was washed three times with the same buffer to remove ribonuclease, and the insoluble residue was dried by an ethanol-ether mixture (1:1, vol/vol) and weighed. It was resuspended in Tris-hydrochloride buffer (10^{-2} M, pH 7.0) containing 5.10^{-3} M $MgSO_4$, and 1 mg of deoxyribonuclease was added. After incubation at 25 C for 60 min, centrifugation, and washing, the dried residue "1" was treated by different proteases (Fig. 1).

(i) Pepsin treatment. Pepsin (1%) in sodium acetate buffer (10^{-1} M, pH 4.0) was added. Incubation was at 37 C for 24 h.

(ii) Papain digestion. Papain (2%) in 10^{-1} M sodium phosphate buffer (pH 6.5) containing 10^{-3} M ethylenediaminetetraacetate and 5.10^{-3} M HCl-cysteine was used. Incubation was at 37 C for 24 h and then, after a 0.5% papain addition, for another 24 h.

(iii) Pronase. A 1-mg amount of enzyme per 50 mg of substrate was added to a suspension in 1.5×10^{-2} M calcium chloride (pH 8.5). Incubation was at 37 C for 24 h.

(iv) Lysozyme. A 3-mg amount of enzyme per 50 mg of substrate was added to a suspension in 5×10^{-2} M Tris-hydrochloride buffer (pH 7.6). Incubation was at 37 C for 24 h.

(v) Saponification. Samples were heated under reflux in 10% KOH (in methanol-water, 1:1, vol/vol) for 4 h. After acidification, lipids were extracted by ether. Hydrosoluble extracts were applied to an Amberlite MB3 column to remove the salts and then lyophilized before analysis by paper and gas chromatographies.

All the operations were performed in the presence of 10^{-3} M sodium azide, especially enzyme digestions, to prevent microbial contaminations. At each step, biological assays were carried out. Only results of the most active fractions, designated fractions 4A and 5, will be given hereafter.

Analytical methods. (i) Hydrolyses. All acid hydrolyses were performed in sealed tubes as described previously (C. Lacave, Ph.D. thesis).

(ii) Paper chromatography. Ascending paper chromatography was performed on Whatman no. 1 filter paper with the following solvent systems: A, *n*-butanol-acetic acid-water (4:1:1, vol/vol/vol); B, *n*-butanol-pyridine-water (6:4:3, vol/vol/vol). Amino acids were visualized by ninhydrin spray (0.2% in acetone), sugars by alkaline silver nitrate, and amino sugars by both reagents.

(iii) TLC. TLC was performed on Silica Gel G plates, which had been spread to a thickness of 0.25 mm. The following solvent systems were used: C, chloroform-methanol-water (65:25:4, vol/vol/vol); D, light petroleum (bp 55 C)-ether-acetic acid (70:25:1, vol/vol/vol); E, light petroleum (bp 55 C)-ether (9:1, vol/vol). Lipids were located under ultraviolet light after spraying plates with rhodamine B.

(iv) Gas-liquid chromatography. Gas-liquid chromatography was performed on an Aerograph gas chromatograph equipped with a 3% SE52 (on Chromosorb W) column (1.5 m) for trimethylsilyl derivates of sugars or a 10% SE30 (on chromosorb W)

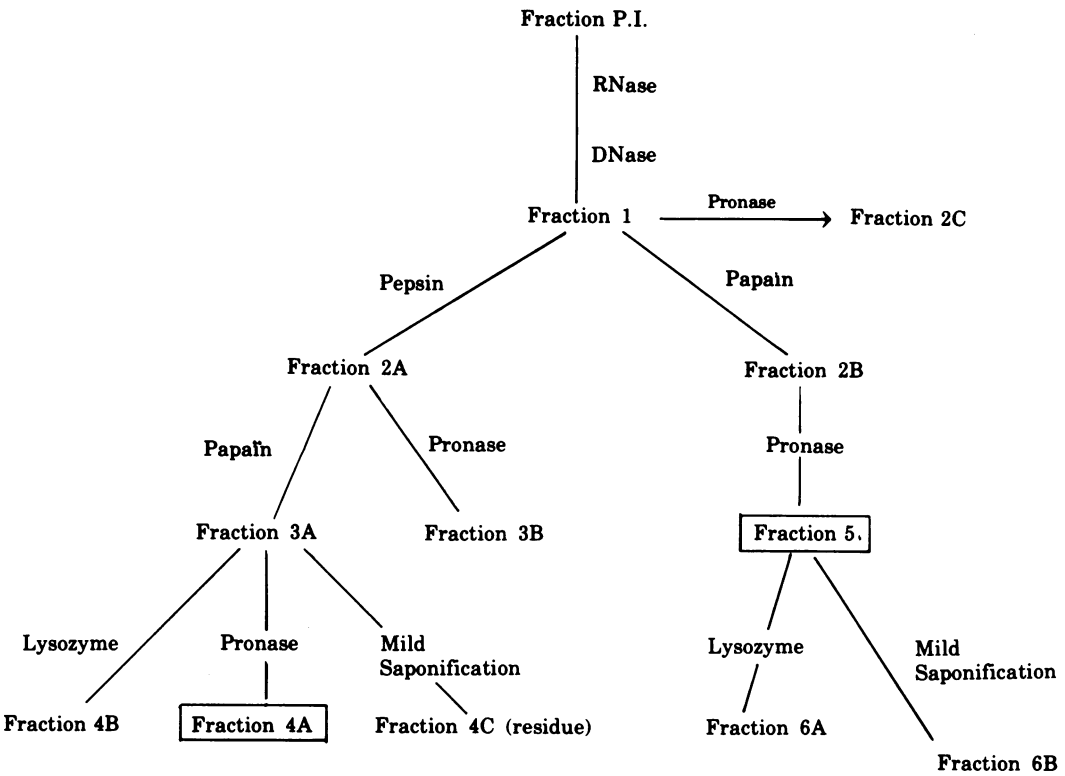

FIG. 1. *Fractionation procedure. RNase, Ribonuclease; DNase, deoxyribonuclease.*

TABLE 1. *Yields of the different steps of fractionation*

Extract no.	% Loss by extraction step	% Fraction P.I.
1	4	96.0
2A	50	48.0
3A	80	9.6
4A[a]	45	5.3

[a] Extract 4A represents less than 1% of delipidated cells.

column (1 m) for fatty esters. Nitrogen carrier flow rate was 20 ml/min.

(v) **Chemical analyses.** Protein determinations were performed by the method of Lowry et al. (22), with bovine serum albumin as standard; sugar determinations were done by the anthrone method and nucleic acid determinations by the orcinol method and spectrophotometric measurement (27). Fatty acids were identified by gas chromatography after esterification by means of an ether solution of diazomethane; sugars were identified by paper and gas chromatographies after transformation into trimethylsilyl derivates (2 ml of hexamethyldisilazane and 1 ml of trimethylchlorosilane in 4 ml of dry pyridine). Amino acids and amino sugars were analyzed after acid hydrolysis by an automatic Technicon apparatus with norleucine as internal standard. Phosphorus was determined by the method of Ames and Dubin (3).

(vi) **Polyacrylamide gel electrophoresis (in SDS).** Each gel contained 7.5% acrylamide and 2% SDS; the method used was essentially as described by Fairbanks et al. (11). All gels were prerun for 20 min before use. Samples to be analyzed (50 to 100 μg of protein per gel) were solubilized in Tris-glycine buffer (0.05 M, pH 8.3) containing 0.003% glycerol, and 2 to 4% SDS was added when stated; samples were boiled in SDS prior to analysis. Gels were run at room temperature for 30 to 45 min with bromophenol blue as marker (3 mA/gel). Proteins were fixed and stained with Coomassie blue; destaining was done non-electrically.

Biological tests. (i) Preparation of antigens. The immunizing antigens to be tested were suspended in physiological saline and homogenized by Potter grinding as described earlier (36). Adjuvant was never used with these preparations.

(ii) **Preparation of antisera.** For anti-whole bacteria sera, several rabbits were inoculated intravenously (ear vein) with 10^8 bacteria (*B. melitensis* M204). After 4 days, blood samples were collected every day for 10 days, then every 4 days for 1 month, and finally every week for 2 months. On every sample of serum, agglutinins were searched according to Wright's method, using an antigenic suspension of strain *B. suis* bacteria (Weybridge). Complement fixation and immunofluorescence tests were also performed on every sample of serum, as de-

scribed below. Immunoglobulin G (IgG) and IgM were differentiated by means of 2-mercaptoethanol (36).

For specific antisera rabbits were inoculated intravenously with 1 mg (a single injection) of the fractions to be studied and suspended as described above. Blood samples were collected every 4 days for 1 month. Also, mice were inoculated intravenously with either 1 or 10 μg (a single injection) of the suspensions to be tested. Blood samples were collected after 4, 7, and 10 days, and biological tests were carried out as described below.

(iii) Antigenic power. Every sample was studied as follows. Agglutination tests were done according to Wright's seroagglutination test. For complement fixation complement-fixing antibodies were searched by a Kolmer-type technique in veronal buffer. The antigens were the same as those used for Wright's seroagglutination test after twofold dilutions and addition of 2 complement units. For immunofluorescence test bacteria were *B. melitensis* 16M (Weybridge), and antiserum was supplied by the Pasteur Institute (rabbit antiglobulins labeled with fluorescein isothiocyanate [19]). For immunodiffusion Ouchterlony plates were prepared with 1.5% agar in saline. The center well contained antigen solution to be tested, and peripheral wells contained different antisera as described below. Plates were incubated at room temperature and observed daily for 3 days.

(iv) Immunizing activity. Preparation of bacterial suspensions for challenge infections were described previously (36). Groups of 20 mice (Swiss females; mean weight, 25 g) were immunized with suspensions of antigens in physiological saline. One, 10, and 100 μg were used for each antigen to be tested, and control mice received 0.5 ml of saline. After 20 days, infecting doses of *Brucella* were inoculated subcutaneously (10^6 viable bacteria/mouse; strain *B. suis* 1330, Weybridge) into vaccinated mice and control untreated groups. Twenty days after the challenge infection (the time previously established to be the peak of the response elicited by the immunization schedule), all mice were killed by ether. The organs (liver, spleen, ganglions) were removed under sterile conditions, ground, and plated on nutrient Albimi agar, after homogenization in a suitable amount of sterile saline. Colonies of *Brucella* were searched after incubation at 37 C for 3 days; protected animals were those that had no colony in any organ, and animals with only one colony in any given organ were considered to be unprotected. The degree of protection is expressed by: (number of infected vaccinated animals/number of infected control animals) $\times$ 100.

RESULTS

Biological tests were used as a guide to choose the best fraction to study.

Biological activity of fraction 4A: immunogenic properties. Most of the fractions obtained according to Fig. 1 elicit agglutinating antibodies in mice and rabbits. Antibody titers were determined as described earlier, the highest titers being observed for fractions 4A and 5. Only results concerning fraction 4A will be given here.

In most of the biological tests (agglutination, complement fixation, immunofluorescence), the activity of fraction 4A is compared with the activities of killed whole bacteria from the same species and with the activity of fraction P.I. (in rabbits). Titers are much lower for fractions P.I. and 4A than for whole bacteria. It should be noted that no adjuvant was added, and it is impossible to compare these titers with those of other authors who used Freund adjuvant with similar preparations. Fractions P.I. and 4A gave close titers; their variations as a function of time were studied. Figure 2 summarizes the results of the agglutination test, and Fig. 3 summarizes those of complement fixation. Figure 2 shows that better responses are obtained with fraction 4A than with fraction P.I., but the differences are minor, the more purified substance being more active than the crude one. The responses became visible earlier with fraction 4A: maximum response was reached 5 days earlier with fraction 4A than with fraction P.I. In both cases, the first agglutinating antibodies that appear are IgM. The IgG are much lower and only transient. IgG are present earlier with fraction 4A than with fraction P.I. and remain a longer time but at a lower level. After injection of fraction P.I., the IgG are present between days 7 and 16, whereas after injection of fraction 4A they are found between days 18 and 35. These results are confirmed by the complement fixation test; Fig. 3 shows an earlier and higher response with fraction 4A than with fraction P.I. For both fractions, especially P.I., there was a renewed increase in complement-fixing antibodies after day 50.

Immunizing properties. Fractions 4A and 5 were chosen for the immunizing procedure and results are compared with those previously obtained with P.I. (36). Toxic reactions could never be observed even with doses as high as 1 mg/mouse: neither local dermal necroses, nor local necroses in the principal organs (liver, spleen, lungs, kidney), nor lethal toxicity.

(i) Specific protection. Immunization of mice was assayed by injection of fractions 4A and 5 (as described in Materials and Methods). With fraction P.I., we never obtained more than 65% protection, regardless of the immunizing dose (Table 2); maximum protection was observed with 1 mg of fraction P.I. With fraction 5 protection was better (75% protection with 100 μg) and with fraction 4A protection still increased, reaching 100% with 100 μg/mouse. Whatever the dose, no toxic effect could be detected. Protection was studied as a function of time for fractions P.I. and 4A. With P.I., protection

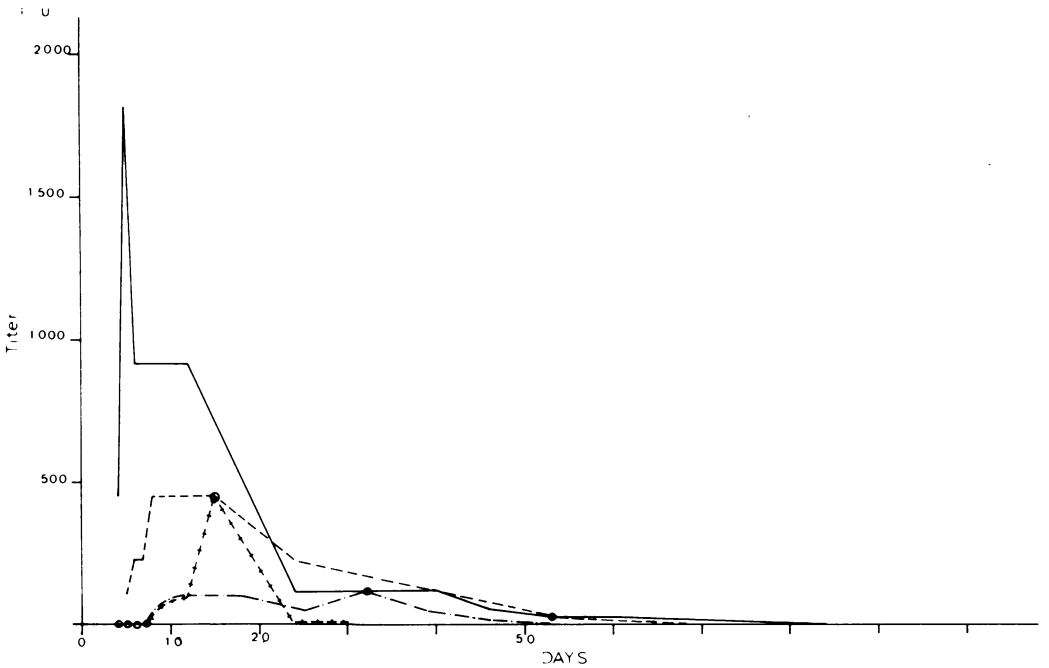

FIG. 2. *Serological response of rabbits inoculated with fractions P.I. and 4A; agglutination titers as a function of time. Fraction P.I.: (+++) IgG; (-----) total Ig's (IgG + IgM). Fraction 4A: (— · —) IgG; (— —) total Ig's (IgG + IgM).*

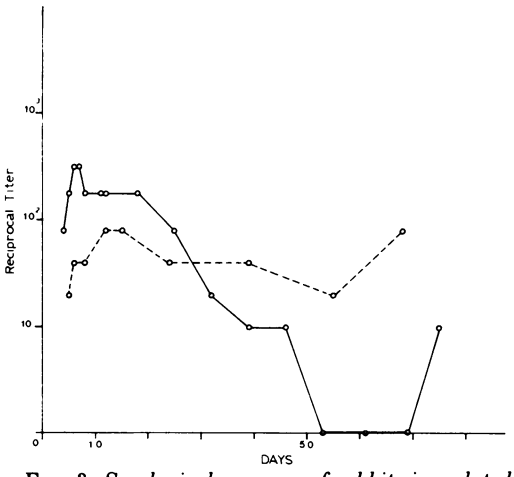

FIG. 3. *Serological response of rabbits inoculated with fractions P.I. and 4A; complement fixation test. Symbols: solid line, fraction 4A; broken line, fraction P.I.*

began to appear after 7 days and increased to reach 65% on day 30. Later on, protection seemed to decrease (30% after 60 days). With fraction 4A, protection was not immediate but began to appear after 11 days, to be complete on day 30. Figure 4 shows the variations of protection with this test as a function of time. After 30 days it seems that protection is decreasing, but many experiments must be performed with different infection challenges to reach a conclusion. Moreover, other tests such as clearance (12) were performed, showing that protection is still effective after 30 days (results to be given elsewhere). Probably other mechanisms of immunity play a role but these could not be seen with the first test.

TABLE 2. *Percentage of infection after immunization with indicated fractions*

Dose (μg)	Fraction		
	P.I.	4A	5
Control[a]	100	100	100
1	71	70	ND[b]
10	28	60	ND
100	65	0	25
1,000	35	0	25

[a] Control = 0.5 ml of saline.
[b] ND, Not determined.

Good protection is still observed against *B. abortus* and *B. suis* infections and it seems that fraction 4A, extracted from *B. melitensis*, is able to immunize animals against any species of *Brucella*. Challenge infections are generally practiced with a strain of *B. suis*, which is more virulent than the *B. melitensis* strain. Fraction 4A therefore contains antigens common to the three species.

(ii) Nonspecific protection. It was interesting

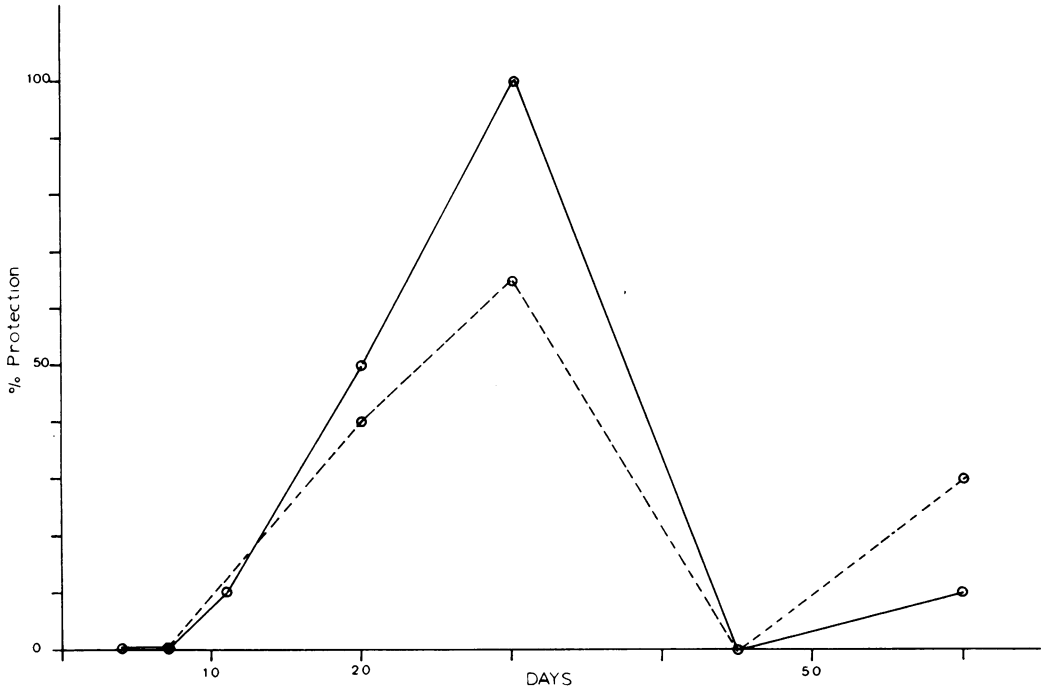

FIG. 4. *Vaccination of mice with fractions P.I. (1 mg) and 4A (100 μg); variations of protection as a function of time. Symbols: solid line, fraction 4A; broken line, fraction P.I.*

to learn if protection conferred by fraction 4A was specific or not, since it is known that peptidoglycan (an important constituent of fraction 4A) of most bacteria increases nonspecific resistance (25). Therefore, it might have been possible that the observed protection against *Brucella* infections was only due to peptidoglycan. In such a case, a similar protection should have been observed against other bacterial infections. A procedure of immunization was undertaken, and challenge injections were performed with *Listeria monocytogenes* (10 lethal doses, 10^5 bacteria/mouse) and *Salmonella typhimurium* (10 lethal doses, 5×10^5 bacteria/mouse). No protection could be registered against either *Listeria* or *Salmonella*. The same results were obtained with fraction P.I., except for a very short nonspecific protection 2 days after infection with *Listeria*. However, such an effect was described with wall preparations of many bacteria and seems to be completely different from the protection mechanism against *Brucella*.

Chemical analysis of fraction 4A. By progressive degradation, more than 90% of fraction P.I. was eliminated, making the composition of fraction 4A less complex (Fig. 1). However, this last fraction remains difficult to study because of its insolubility. As it still contains peptidoglycan, we tried to evaluate its quantitative importance in relation to others and to learn what relations might occur between the different components.

Table 3 compares the overall chemical compositions of fractions P.I. and 4A, which are very similar. No significant difference appears between them in spite of a loss of 90% of the material in fraction 4A; however, the amount of lipids and carbohydrates is much higher in fraction 4A than in fraction P.I., in which they

TABLE 3. *Comparison of principal constituents in fractions P.I. and 4A*

Fraction	Constituent (%)						
	P	Nucleic acid	Lipids, fatty acids	Proteins	Sugars	Glycerol	Peptidoglycan
P.I.	1.0	5	Trace	70–75	Trace	+	1–2
4A	0.6	2	3	60–65	3	+	25–30

were almost undetectable. Qualitative analysis of the different components of these fractions was performed on acid hydrolysates by paper chromatography (solvent B for sugars, solvent A for amino acids and amino sugars), and quantitative determinations were carried out as outlined above. Free lipids were extracted by chloroform-methanol and chromatographed on thin-layer plates of silica gel (solvent C); no free lipids could be detected and bound lipids could only be released after acid hydrolysis. They were detected as free acids by TLC (solvent D) and also after methylation (with an ether solution of diazomethane) by TLC (solvent E) and gas chromatography. Apart from a small amount of nucleic acids (spectrophotometric and orcinol determinations) that could not be removed by different treatments (even with nucleases after action of proteolytic enzymes), fraction 4A is essentially composed of peptidoglycan and proteins plus a small quantity of lipids and carbohydrates, which are also probably linked to peptidoglycan or proteins or both.

(i) Peptidoglycan. All the peptidoglycan of the wall appears to remain in fraction 4A, and it has already been seen that quantitatively it represents a very small percentage of the cell wall of *Brucella* (<1%) (C. Lacave, Ph.D. thesis). Moreover, after acid hydrolysis and amino compound analysis, it could be noticed (Table 4) that only one-half of the muramic acid residues of peptidoglycan was substituted by peptidic chains, since there is a higher molar content of amino sugars (muramic acid and glucosamine) than of peptidic substituents (alanine, glutamic acid, and diaminopimelic acid); this fact confirms preceding results (C. Lacave, Ph.D. thesis).

(ii) Proteins. At least in part, proteins seem to be covalently linked to peptidoglycan, but also to lipids and carbohydrates. Amino acid composition was studied after acid hydrolysis of fraction 4A to evaluate the quantitative importance of peptidoglycan and proteins in subfractions; Table 4 gives the results for fractions P.I., 4A, and 5. There is no significant difference between the overall chemical compositions of these three fractions. Although biological activity of fraction 4A is higher than that of fraction 5, the former contains less amino acids, but their ratios are very similar in both fractions. Content of peptidoglycan strongly increases in subfractions of P.I.: instead of 1.5% of the total proteins in fraction P.I., it reaches 35 and 42%, respectively, of the total proteins in fractions 5 and 4A. A high content of glutamic acid must be mentioned: Ivanova (18) had already noticed this quantitative importance of glutamic acid in some *Brucella* strains. Glutamic acid content was already important in fraction P.I., and generally most of it is extracted, with proteins constituting both fractions. Yet it must be pointed out that in some cases the high amount of this acid is not found after acid hydrolysis of fraction 4A; this fact could be an indication that glutamic acid does not belong to the proteins but rather to an insoluble polymer [poly(L-glutamic acid], as in the case of *Mycobacteria* (42, 43). Glycine, valine, and aspartic acid are the most important acids, whereas aromatic amino acids are very low so that determinations of proteins according to Lowry et al. are probably too weak. It is noteworthy that there is neither cysteine nor methionine nor histidine, but there is a small amount of ornithine.

TABLE 4. *Amino acid compositions of fractions P.I., 4A, and 5*[a]

Amino acids	Amt (moles) in fraction:		
	P.I.	5	4A
Asp	7	1	0.6
Thr	5	0.5	0.5
Ser	6	1	0.5
Muramic acid	1	2	1.75
Glu	12	4	3.5–8
Pro	2	0.3	0.3
Gly	5	1	1
Ala	6	3	2.5
Glucosamine	1	2	1.5
Val	5	0.5	0.5
Ile	4	1	0.75
Diaminopimelic acid	1	1	1
Leu	6	1	0.75
Tyr	2	0.2	0.15
Phe	4	0.5	0.3
Orn			0.1
Lys	3	0.4	0.25
His	1	0.15	0.1
Arg	4	0.5	0.25

[a] Two hydrolyses were performed on each sample with the same quantity of material (2 mg): one to determine the amount of amino sugars (4 N HCl for 4 h); one to determine the amount of amino acids (6 N HCl for 18 h). Moles relative to diaminopimelic acid = 1.

Proteins found in both fractions (4A and 5) could be linked to peptidoglycan. In the cell wall of *E. coli* (17), it was shown that trypsin has the property of selectively splitting the linkages between peptidoglycan and lipoprotein (5). Our first assays with this enzyme do not bring evidence that the linkage is split, but for a definitive conclusion it would be necessary to remove other proteins.

(iii) Carbohydrates. Carbohydrates are iden-

tified after acid hydrolysis of fraction 4A by paper chromatography; glucose, galactose, arabinose, and xylose are identified as well as glycerol. For confirmation of these results, hydrolysates were passed through a Dowex-50 column to remove amino sugars and amino acids which could interfere in gas chromatographic determinations of trimethylsilyl derivates. These same four sugars were found in addition to glycerol.

(iv) Lipids. After saponification of fraction 4A under normal conditions and extraction, it was impossible to find any fatty acid and acid hydrolysis was necessary. Such behavior would indicate the presence of amide linkages between fatty acids and amino compounds rather than ester linkages. The main fatty acids found were palmitic acid (42% of total fatty acids), *cis*-vaccenic acid (25%), and very-long-chain fatty acids (ca. 30 carbon atoms, 10%); small amounts of stearic acid (3.5%) and short-chain fatty acids (4.5% were also detected, as well as 11% of long-chain fatty acids (between 20 and 30 carbon atoms).

The next point was to separate the different constituents of fraction 4A to determine the composition of the mixture and the kind of linkages between the different entities and to resolve several problems. Is the biological activity due to a peculiar constituent? Is it possible by degradation and removal of peptidoglycan to get an active substance? Has peptidoglycan alone a protective activity or is the mixture of peptidoglycan and other constituents necessary?

Further studies on fraction 4A: isolation and subfractions. Since fraction 4A contains peptidoglycan and protein-like material, we tried on the one hand to eliminate the peptidoglycan to get the protein material intact and, on the other hand, to remove as much protein material as possible from the peptidoglycan-containing insoluble product.

(i) Degradation of peptidoglycan. Lysozyme alone has very little action on fraction 4A, and the procedure described by Schnaitman (40), using lysozyme after detergent treatment, was adopted. A 2% solution of Triton X-100 in phosphate buffer (10^{-2} M, pH 7.4) containing 5 mM ethylenediaminetetraacetate is stirred at room temperature for 1.5 h, and after partial solubilization of the material lysozyme is added. Under such conditions, peptidoglycan is strongly degraded and solubilized: only traces of muramic acid and diaminopimelic acid could be detected in the insoluble residue after lysozyme digestion (Table 5). No biological activity could be detected in the insoluble residue obtained after such a treatment (after washing and drying, Triton did not remain in this fraction). This might be explained by the disappearance of peptidoglycan; moreover, linkages between constituents could be indispensable for biological activity.

(ii) Partial solubilization of proteins. Solubi-

TABLE 5. *Amino acid compositions of subfractions from fraction 4A and comparison with E. coli lipoprotein (6)*[a]

Amino acids	Insoluble triton + lysozyme	Insoluble SDS + lysozyme	SDS		*E. coli* lipoprotein (6)
			Insoluble	Soluble	
Asp	11.5	15	1.3	1.7	15
Thr	10	8	0.75	1.2	2
Ser	11	8	0.8	1.4	6
Muramic acid	1.5	0	8	0	0
Glu	13	7.5	8 (6–13)	1.3–3	6
Pro	3.5	1	0.6	0.6	0
Gly	25	17	2.8	3.6	0
Ala	23	15	9	2.3	9
Glucosamine	23	25	8	0	0
Val	9.5	5	1.4	3.1	4
Met	0	0	0	0	2
Ile	12.5	6	1	1.6	1
Diaminopimelic acid	Trace	Trace	4	0	0
Leu	9.5	11	1.7	2.2	4
Tyr	5	2	2	0.6	1
Phe	11	8	0.7	1.2	0
Orn	0	4	0.7	2	0
Lys	6	4	0.4	0.8	5
His	2	1	0.1	0	0
Arg	6.5	6	0.75	1	4

[a] The values for amino acids are expressed as micromoles per 10 mg (dry weight). For evaluation of amino sugars, another hydrolysis (4 N HCl for 4 h) was performed on the same quantity of material.

lization of noncovalently linked proteins was performed by means of SDS. Rosenbusch (35) described solubilization of proteins by using 4% SDS solutions at 100 C; after checking that we got the same results at 100 and 80 C (amino acid analyses and electrophoretic patterns on acrylamide gels), we chose the second temperature, which seemed less drastic for the biological activity. A suspension of fraction 4A is stirred for 2 h at 80 C in the presence of 4% SDS; two subfractions are separated. The insoluble fraction (45% of the initial fraction) contains peptidoglycan with strongly bound (lipo)protein(s); carbohydrates are also present. The soluble fraction (55%) contains (lipo)proteins which were initially only loosely associated with other constituents. These two subfractions were hydrolyzed and analyzed; Table 5 gives their amino acid composition and shows that all the peptidoglycan stays in the insoluble fraction, since neither muramic nor diaminopimelic acids can be detected in the soluble fraction. The principal results are summarized as follows. Peptidoglycan represents approximately 70% of the insoluble fraction (I.F.). Amino acids are always present and belong to some polypeptidic material linked to peptidoglycan. The most important amino acids are aspartic acid, glycine, and valine, and the presence of ornithine is noteworthy.

No free lipids are present, but after hydrolysis it is shown that palmitic and vaccenic acids represent almost all the fatty acids; vaccenic acid is liberated by saponification and could be linked by an ester bond, whereas palmitic acid is only liberated by acid hydrolysis (amide linkage probable). After saponification, the hydrosoluble fraction is examined for the presence of glycerol and carbohydrates by paper and gas chromatographies. All the carbohydrates of fraction 4A are found here, and even after digestion of peptidoglycan by lysozyme carbohydrates are still found in the insoluble residue.

Protective activity was examined; about 20% protection is registered with 100 μg of this I.F., but the optimum of activity with this fraction has so far not been studied.

The soluble fraction only contains proteins and lipids. Nature and ratios of amino acids resemble those found in the I.F., except for the absence of ornithine (Table 5). As well as in I.F., lipids are only partially liberated by saponification (vaccenic acid). Palmitic and very-long-chain fatty acids detected in whole fraction 4A are present in this subfraction and probably linked by amide linkage, since an acid hydrolysis is necessary to liberate them. The nature of these long-chain fatty acids is under investigation.

Acrylamide gel electrophoresis gives an idea of the complexity of the mixture; three bands could be detected in the presence of SDS (after boiling for 3 min in SDS prior to analysis). The soluble fraction was studied by immunodiffusion against different sera: the anti-whole bacteria of the three species (*B. suis* S6, *B. melitensis* M807, and *B. abortus*) and antisera prepared with fractions P.I., 4A, and I.F. Figure 5 represents the most important results. Three bands are common to all the antisera except to the anti-I.F. serum in which only one band could be detected, this band being one of the three preceding ones. Two bands fuse completely with each other; for band 3 it is necessary to increase the concentration of the antiserum to see the fusion. Since one band is common to soluble and insoluble fractions, the same protein is present in the two subfractions of fraction 4A.

As with the I.F., some biological activity (about 20% protection) is observed, but in spite of different treatments (dialysis, Diaflo membranes, gel filtration on Sephadex G15) some SDS is always present and may have an inhibitory effect on the biological response, the conformation of proteins being modified.

DISCUSSION

Using as starting material fraction P.I. (previously described), we tried to fractionate it with a double purpose: firstly, to obtain better protection, since with fraction P.I. only 65% of the animals seemed to be protected, and, secondly, to isolate and identify the active sub-

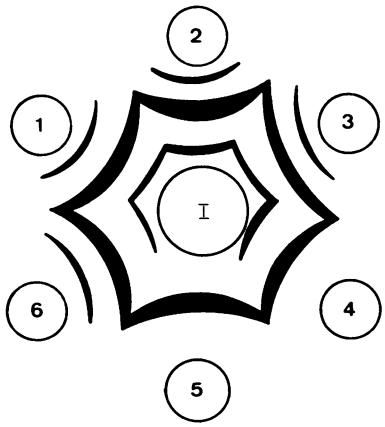

FIG. 5. *Schematic representation of immunodiffusion patterns. Center well contains antigen solution (I), fraction soluble in SDS from fraction 4A. Peripheral wells contain antisera: wells 1-3, sera anti-whole bacteria (1, anti-B. melitensis; 2, anti-B. suis; 3, anti-B. abortus); well 4, serum anti-fraction P.I.; well 5, serum anti-fraction I.F. (insoluble SDS from fraction 4A); well 6, serum anti-fraction 4A.*

stances from the crude extract and to dissociate the action of peptidoglycan from that of other constituents. Indeed, many papers describe properties of peptidoglycan, such as immunogenicity, immunoadjuvant properties (2, 28), and stimulation of nonspecific resistance (25), which are essentially nonspecific, and, if they are the same in *Brucella*, the protection effect had to be due to other substances. In *Brucella*, induction of nonspecific resistance was ascribed to a surface protein (4) and, more recently, to whole bacteria (32). It was plausible that fraction P.I., containing many different constituents, could exhibit various properties. Our first findings showed that active substances with agglutinogenic properties and immunization activity against brucellosis might be extracted from fraction P.I. and a more simple fraction could be obtained with the same properties (fraction 4A). This fraction is essentially made of peptidoglycan and proteins plus a small quantity of strongly bound lipids and carbohydrates. Some nucleic material could not be removed by enzymatic treatment. We cannot assert whether these minor components play a role in the biological activity (such properties were attributed to a deoxyribonucleic acid fraction in *Brucella* by some authors [8, 29]).

A covalent linkage between peptidoglycan and some (lipo)protein(s) is made probable by the fact that amino acids remain present with peptidoglycan after heating fraction 4A with SDS. A similar covalent linkage was described between the components of the envelope of *E. coli*, but the composition of the protein material of fraction 4A is different from that of the lipoprotein of *E. coli* (7) and membrane proteins from some other gram-negative bacteria (35). Moreover, trypsin does not seem to split the bond between peptidoglycan and proteins as in the case of *E. coli* (5). The only analogy with the structural envelope protein of *E. coli* is the absence of histidine. Schnaitman (39) thought that the absence of histidine was a feature of structural protein, and it is possible that the insoluble protein plays a similar role in *Brucella* in which peptidoglycan constitutes only a small percentage of the cell wall; a protein matrix constituting a two-dimensional lattice could be postulated. However, besides this possible structural role, the material isolated from *Brucella* has important biological properties, and this does not seem to be the case, to our knowledge, for the lipoprotein of *E. coli*.

Some lipids and carbohydrates are also linked to the other constituents, as lipoproteins and polysaccharides, as in *Mycobacteria* in which polysaccharides and mycolic acids are linked to peptidoglycan, or as insoluble lipopolysaccharides (linked or not to the other compound). Palmitic and long-chain fatty acids could only be liberated by strong acid hydrolysis from whole fraction 4A, and it is only after solubilization of 55% of the components of the fraction that vaccenic acid could be liberated by saponification. As well as in the lipoprotein of *E. coli* (15), fatty acids are linked by ester and amide linkages to the protein core.

Solubilization of some protein components decreases the activity, but we cannot choose between a synergistic mechanism of the various entities and an inactivation produced by the use of hot SDS or Triton X-100. All attempts to isolate pure peptidoglycan have failed, and we cannot give its intrinsic properties.

The material isolated from *Brucella* is able to induce immunogenicity and protection against homologous infections. This new fraction, which gives good results in immunization of mice and rabbits without addition of adjuvants, could therefore be considered as a brucellosis vaccine for humans.

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