STUDIORUM PROGRESSUS

Chromatographic Evidence for Murein from the Bacteroid Symbiotes of *Periplaneta americana* (L)

Bacteria-like objects (bacteroids) have long been recognized in special cells (mycetocytes) in the fat body of cockroaches[1]. BROOKS and RICHARDS[2], reviewing literature dating back to 1896, point out that, based on staining reactions and treatment with UV-light, the bacteroids have been variously characterized as mitochondria, bacteria and products of cellular metabolism. There have been at least 9 claims of successful in vitro culture of the bacteroids interspersed chronologically with at least 7 denials of culture[3,4]. While electron microscopic studies[5-10] strongly support the bacterial nature of the symbiotes, the controversy regarding their identity is not resolved.

Since the isolation and characterization of α,ε-diaminopimelic acid (DAP)[11,12] and muramic acid (MurN)[13] (2-amino-3-O-[D-1-carboxyethyl]-2-deoxy-D-glucose) from bacteria, rapid progress has been made in deducing the biochemical nature of bacterial cell walls. It is now possible to conclude that MurN and glucosamine (GlcN) (2-amino-2-deoxy-D-glucose) are ubiquitous and indispensable components of the basal structure of the cell wall (murein)[14]. MurN is probably the most distinctive compound of bacterial cell walls and can be used as a reliable indicator of the presence of the murein complex[15].

Little is known about the biochemical nature of the bacteroid wall. Bacteroids are gram-positive. The walls are thin, seemingly encapsulating a cell membrane[8,9]. Lysozyme, an enzyme possessing a $\beta(1\text{–}4)$ *N*-acetylglucosaminidase activity, degrades the murein component of bacterial cell walls producing spherical protoplasts. Similarly, bacteroid protoplasts are produced by lysozyme[16] suggesting the presence of a $\beta(1\text{–}4)$ *N*-acetylhexosamine linkage and a murein basal structure.

In light of the biological significance of these objects, we sought positive evidence for their bacterial nature in the form of murein.

Methods. Last instar nymphs of *Periplaneta americana* (L.), selected without regard to sex, were chloroformed. After tying off the necks, the animals were placed individually in a stoppered vial containing 0.2% Hyamine (p-Diisobutyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium chloride) and shaken for several seconds. After 3 rinses each in fresh Hyamine and sterile distilled water the specimens were dissected in a sterile room. Aseptic techniques were observed throughout. The fat body was removed, taking care not to puncture the gut, weighed and placed in 100 volumes of cold, sterile 0.88 *M* sucrose solution for 12–16 h. Lysozyme, presumably present in fat body tissue[16], was discarded in the supernatant after collecting the tissues by centrifugation at 3830 *g* for 15 min at 0 °C. In the first centrifugation some fat body tissue remains at the surface of the sucrose solution and can be discarded with the supernatant fluid. Bacteroids, mycetocytes and other cell debris are in the residue. The residue was washed twice in fresh, cold 0.88 *M* sucrose solution and then placed in a 0.3% lipase solution in 0.88 *M* sucrose. With constant stirring for 30 min at room temperature the mycetocyte membranes are opened and the bacteroids are freed. The bacteroids, along with cell nuclei and other debris, are collected by centrifugation, washed 3 times in sterile 0.9% NaCl solution and frozen for storage. 3 preparations of bacteroids were obtained in this manner from (1) 3.589 g, (2) 8.315 g and (3) 6.599 g of fat body, respectively. The preparations were dried in vacuo at 60 °C giving dry weights of (1) 42.3 mg, (2) 37.2 mg and (3) 127.4 mg for the 3 preparations.

Bacillus subtilis was chosen for purposes of comparison since it contains MurN, GlcN and DAP in its wall murein. Cells were cultured in nutrient broth, harvested in the centrifuge and washed 3 times in 0.9% NaCl solution. 2 batches of *B. subtilis* yielded dry weights of (1) 77.9 mg and (2) 96.9 mg.

Bacteroid preparations (1) and (2) and *B. subtilis* (1) were placed in sealed hydrolysis vials containing 6 *N*-HCl for 24 h at 100 °C. Hydrochloric acid was removed in vacuo. The hydrolysates were dissolved in water and concentrated 3 times. Humis was removed by filtration through Whatman 40 paper discs.

Bacteroid preparation (1) was dissolved in 3.0 ml of water. A 1.5 ml aliquot (21.15 mg calculated dry weight) was removed and streaked on Whatman 40 acid-washed paper beside MurN (1100 µg/1.5 ml) and DAP (1150 µg/1.5 ml) standards. Chromatograms were developed for 37 h in descending (collidine/2,4-lutidine/2,5-lutidine, 50:25:25) saturated with water (= solvent A).

Bacteroid preparation (2) and *B. subtilis* (1) were each dissolved in 2.0 ml of water. A 0.5 ml aliquot (9.3 mg calculated dry weight) of the bacteroid hydrolysate was streaked on Whatman 40 paper beside a streak of 0.25 ml (9.7 mg calculated dry weight) of *B. subtilis* (1), and the standards MurN (315.0 µg), DAP (217.5 µg) and GlcN (1500 µg). Chromatograms were developed in solvent A for 37 h.

The positions of ninhydrin reacting zones were located by cutting a narrow strip along the length of the paper (parallel to the solvent run) through each streak and spraying the strips with 0.25% ninhydrin in acetone. After locating the standards and similarly locating ninhydrin positive zones from the hydrolysates, the untreated zones were eluted in warm water and concentrated in vacuo. Considering the portion of the streak removed in the treated strip, the final concentration of the eluates was calculated. Bacteroid preparation (1) and the standards were concentrated to 1.0 ml so the MurN eluate contained 916.7 µg and the DAP eluate contained 958.0 µg. The 2 corresponding bacteroid (1) eluates contain unknown quantities of material. Bacteroid preparation (2) and *B. subtilis* (1) and standard eluates were concentrated to 0.4 ml so that the standard eluates contained

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respectively 236.3 μg MurN, 163.1 μg DAP and 1125.0 μg GlcN.

Several aliquots from each eluate were spotted on Whatman 40 paper (Table I). Preparation (1) and its standards were run in phenol/water, 80:20 (= solvent B) in an NH_3 atmosphere to the end of the paper (48 cm). Preparation (2) and *B. subtilis* (1) and their standards were run in methanol/10 *N*-HCl/water/pyridine, 80:2.5:17.5:10 (= solvent C)[17] to the end of the paper. After drying, the chromatograms were sprayed with 1.0% ninhydrin in 95% ethanol containing a 2% mixture of 5 volumes of glacial acetic acid and 1 volume of collidine. Before inspection the papers were placed in a 90 °C oven for 3 min.

A modification of a fractionation procedure designed to isolate murein[18] was employed with bacteroid preparation (3) and *B. subtilis* (2). Dry cells were suspended in 10 ml of glass-distilled water and to this was added 2.5 ml of 25% cold TCA (10 min at 0 °C). A residue was collected by centrifugation at 4000 *g* for 5 min. The supernatant was discarded. 15 ml of 80% ethanol were added to the residue and after 10 min the residue was collected in the same manner. The residue was next suspended in 10 ml of 5% TCA and heated 6 min at 90 °C, collected again, and finally placed in 9.5 ml of 0.05 *M*-NH_4HCO_3 containing 0.005 *N*-NH_4OH. To this was added 0.5 ml of a solution containing 1 mg/ml of crystalline trypsin. Trypsinization was allowed to proceed for 45 min at 37 °C. The remaining cell material was washed twice in buffer and then hydrolyzed in 6 *N*-HCl for 24 h at 100 °C. The hydrolysate was concentrated, as before, to a volume of 0.5 ml. Two aliquots (50 μl containing a calculated 3.2 mg of the original dry weight and 150 μl containing 9.6 mg) of the bacteroid hydrolysate (3), one aliquot of the *B. subtilis* (2) hydrolysate (30 μl containing a calculated 1.5 mg of the dry weight) and standards of MurN (26.3 μg), DAP

Table I. Aliquots spotted on Whatman 40 paper from the eluates of streaks developed in collidine/2,4-lutidine/2,5-lutidine (50:25:25) saturated with water

	Eluted zone	Range of size of several aliquots (μl)	Range (μg) in the aliquots
I	MurN standard	8– 30	7.3–27.5
	DAP standard	2– 10	1.9– 9.6
	Bacteroid (1) MurN zone	10–140	?
	Bacteroid (1) DAP zone	1– 80	?
II	MurN standard	20– 80	11.8–47.3
	DAP standard	6– 20	2.4– 8.2
	GlcN standard	10– 20	28.1–56.2
	Bacteroid (2) MurN zone	20–100	?
	B. subtilis (1) MurN zone	20–100	?
	Bacteroid (2) DAP zone	10– 80	?
	B. subtilis (1) DAP zone	10– 80	?
	Bacteroid (2) GlcN zone	20–100	?
	B. subtilis (1) GlcN Zone	20–100	?

I. Bacteroid preparation (1) and standards developed in phenol/water (80:20) in an NH_3 atmosphere. II. Bacteroid preparation (2), *B. subtilis* batch culture (1) and standards developed in methanol/10 *N*-HCl/water/pyridine (80:2.5:17.5:10).

Table II. Rf values of several chromatograms run in solvent B (phenol/water, 80:20, in an NH_3 atmosphere) or in solvent C (methanol/10 *N*-HCl/water/pyridine, 80:2.5:17.5:10)

	Eluate	Range	Mean
Solvent 'B'	MurN standards	0.584–0.593	0.589
	MurN bacteroids	0.571–0.603	0.587
	DAP standards	0.150–0.201	0.179
	DAP bacteroids (?)		
	1.	0.137–0.206	0.187
	2.	0.074–0.091	0.085
Solvent 'C'	MurN standards	0.787–0.818	0.807
	MurN bacteroids	0.787–0.815	0.802
	MurN *B. subtilis*	0.794–0.815	0.807
	DAP standards	0.337–0.407	0.373
	DAP bacteroids	none found	–
	DAP *B. subtilis*	0.357–0.402	0.372
	GlcN standards	0.741–0.766	0.753
	GlcN bacteroids	0.742–0.771	0.755
	GlcN *B. subtilis*	0.746–0.753	0.749

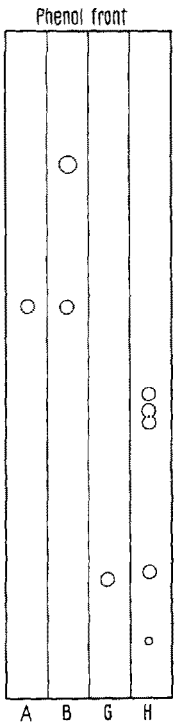

Fig. 1. Eluates of streaks developed in collidine run in descending phenol/water (80:20) in an NH_3 atmosphere. (A) muramic acid standard, (B) bacteroid preparation (1) eluate corresponding to the muramic acid streak in collidine, (G) diaminopimelic acid standard, (H) bacteroid preparation (1) eluate corresponding to the diaminopimelic acid streak in collidine.

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(2.3 μg) and GlcN (48.0 μg) were spotted on separate sheets of Whatman 40 paper and developed in 2 dimensions using first, solvent A and second, solvent C. After drying, the chromatograms were sprayed with the 1% ninhydrin reagent mentioned above and placed in the 90 °C oven for 3 min before inspection.

Results. In solvent A, zones of ninhydrin-reacting material from the bacteroid and *B. subtilis* streaks were found in positions identical to the MurN, DAP and GlcN standards. The bacteroid preparation (1) zone corresponding to MurN was found to contain 2 ninhydrin spots in the solvent B run, one of which behaves like the MurN standard (Figure 1). The mean Rf values for all ninhydrin-reacting spots are presented in Table II and here it can be seen that the MurN standard and the bacteroid MurN mean Rf values are nearly identical (± 0.002). 5 spots were obtained from material eluted from the DAP zone of bacteroid preparation (1) streaks. 2 of these are slow moving but neither matches precisely with the DAP standard (Figure 1). It is not possible to conclude unequivocally that DAP is present in these bacteroid hydrolysates.

Bacteroid preparation (2) and *B. subtilis* (1) eluates contained ninhydrin-positive material which, in solvent C, corresponded to MurN and GlcN standards (Figure 2). The bacteroids and *B. subtilis* produce the same patterns except that no DAP was found from the bacteroids. It is noteworthy that the MurN standard and bacteroid MurN eluate, when placed on the same spot, travel together producing a single spot in the vicinity of the MurN standard alone. In solvent A, the MurN and GlcN zones of the bacteroids and *B. subtilis* overlapped slightly, which explains the presence of some GlcN (broken circles in Figure 2) in the bacteroid and *B. subtilis* MurN eluates. Likewise, some MurN was found in the GlcN eluates.

Figure 3 shows the results of 2-dimensional chromatograms of *B. subtilis* (2) hydrolysate, 2 runs of the bacteroid (3) hydrolysate, and a single run of the standards MurN, DAP, GlcN, leucines, valine and cystine. The patterns from these 4 papers have been superimposed to illustrate their similarities. The standards MurN, GlcN, the leucines and valine all have bacteroid and *B. subtilis* equivalents. The LL- and meso-isomers of DAP are separated here and correspond to the DAP of *B. subtilis*. The presence of DAP in the bacteroids is questionable. A ninhydrin spot (No. 12) from both bacteroid runs fell just short of LL-DAP. Its identity is unknown. None of the DAP spots, including standards, produced the common green color in reaction with ninhydrin. Rf values for this chromatogram are presented in Table III.

Our data reveal the presence of GlcN and at least one murein indicator, MurN, in the bacteroid symbiotes of *P. americana*, indicating a murein basal structure. From these data we conclude that the bacteroids are procaryotic microbes. Considering the presence of MurN in rickettsiae and psittacosis-type organisms[19] and the blue-green alga, *Phormidium uncinatum*[20], the discovery of MurN in the

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Table III. Rf values of 2-dimensional chromatograms developed in solvent A (collidine/2,4-lutidine/2,5-lutidine, 50:25:25, saturated with water) and solvent C (methanol/10 *N*-HCl/water/pyridine, 80:2.5:17.5:10)

Spot	Solvent 'A'	Solvent 'C'
Bacteroid preparation (3) A		
1	0.364	0.659
2	0.356	0.605
3	0.383	0.731
4	0.268	0.699
12	0.012	0.276
Bacteroid preparation (3) B		
1	0.366	0.664
2	0.354	0.603
3	0.412	0.739
4	0.279	0.697
12	0.014	0.281
B. subtilis (2)		
1	0.368	0.659
2	0.352	0.605
3	0.381	0.729
4	0.272	0.699
11	0.002	0.316
13	0.002	0.235
Standards		
1 MurN	0.387	0.670
2 GlcN	0.350	0.603
3 leucines	0.375	0.737
4 valine	0.272	0.702
11 LL-DAP	0.014	0.302
13 meso-DAP	0.014	0.227
14 cystine	0.141	0.273

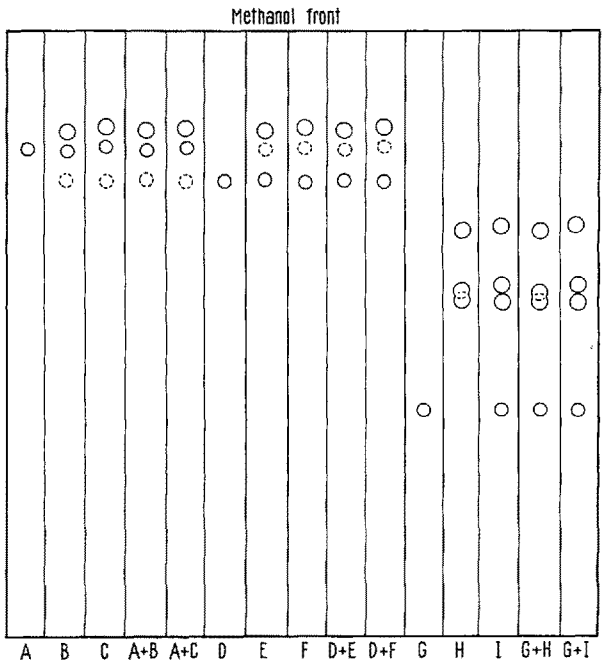

Fig. 2. Eluates of streaks developed in collidine run in descending methanol/10 *N*-HCl/water/pyridine (80:2.5:17.5:10). (A) muramic acid standard, (B) bacteroid preparation (2) eluate corresponding to the muramic acid streak in collidine, (C) *B. subtilis* (1) eluate corresponding to the muramic acid streak in collidine, (D) glucosamine standard, (E) bacteroid preparation (2) eluate corresponding to the glucosamine streak in collidine, (F) *B. subtilis* (1) eluate corresponding to the glucosamine streak in collidine, (G) diaminopimelic acid standard, (H) bacteroid preparation (2) eluate corresponding to the diaminopimelic acid streak in collidine, (I) *B. subtilis* (1) eluate corresponding to the diaminopimelic acid streak in collidine.

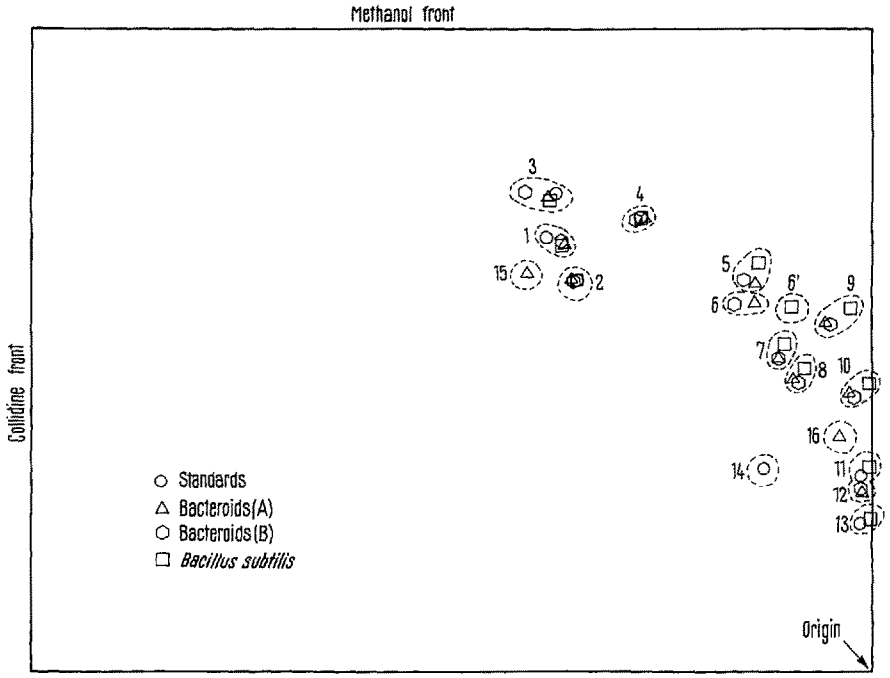

Fig. 3. 2-dimensional chromatograms of standards and bacteroid and *B. subtilis* hydrolysates superimposed here for purposes of comparison. (1) muramic acid, (2) glucosamine, (3) leucines, (4) valine, (11) LL-diaminopimelic acid, (13) meso-diaminopimelic acid, (14) cystine. Identity of 5–10, 12 and 15 is unknown.

bacteroids does not restrict them to the Eubacteria. However, considering these data together with studies of bacteroid fine structure, we are left with no doubt as to the bacterial nature of these cockroach symbiotes[21].

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Zusammenfassung. Papierchromatographisch wurde Murein in symbiontischen Bakteroiden der *Periplaneta americana* nachgewiesen. Die Murein-Komponente Glukosamin und der Murein-Anzeiger Muramic-Säure wurden mittels Bakteroid-Hydrolysaten identifiziert. Das Vorhandensein von α-ε-Diaminopimelic-Säure jedoch konnte nicht einwandfrei festgestellt werden.

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PRO EXPERIMENTIS

Methods for Increasing Sensitivity in Immunoelectrophoresis

Introduction. In the course of studies of the antigens of the vertebrate lens it was found advantageous to develop methods of immunoelectrophoresis giving increased resolution in 2 respects: firstly, the resolution of antigens whose arcs are superimposed, and secondly, the resolution of extracts available only in very small quantities. The superimposition of arcs in immunoelectrophoresis is due to a number of factors, primarily the possession of identical electrophoretic mobilities by different protein fractions and overlapping zones of optimal combination of several antigen/antibody complexes. Several methods of overcoming these difficulties and increasing the resolution of components in a mixture have been reported[1–9]. The majority of these methods rely on the use of an additional supporting medium for electrophoresis, which has the effect of gel filtration. However, this may so alter the position of antigens in the run that comparison with standard immunoelectrophoresis becomes difficult. In order to improve resolution but permit recognition by mobility, regions were cut from an electrophoresed gel and rerun at a different pH, thus resolving antigens whose electrophoretic mobility in the first run was similar.

Methods have also been devised for the immunoanalysis of small quantities of starting material, e.g. immunoelectrophoresis on cellulose acetate[10]. However, this is not ideal in that it is not possible to follow the progress of arc formation and it was considered that a method which would prevent lateral diffusion of the electrophoresing material would increase the likelihood

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