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WEAK ASSOCIATION OF GLUCOSAMINE-CONTAINING POLYMER WITH THE *ACHOLEPLASMA LAIDLAWII* MEMBRANE

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SUMMARY

1. Membrane-incorporated glucosamine does not seem to be covalently attached to membrane proteins as shown by polyacrylamide gel electrophoresis of native and pronase-digested membranes.

2. Membrane-incorporated glucosamine is readily released from membrane lipid and protein by brief sonication as shown by sucrose density gradient analysis.

3. Membrane-incorporated glucosamine is present in a polymeric form, as shown by comparing column elution profiles for free and membrane-bound glucosamine.

4. It is concluded that the polymer is weakly associated with the membrane and may reside on or near the exterior surface.

The plasma membrane of *Acholeplasma laidlawii* has been extensively studied as a model procaryotic membrane, due in large measure to its ease of isolation and to the lack of cell wall material common to other procaryotes[1]. Although no muramic acid-containing polysaccharides have been found in isolated membranes from this organism[2], about 4% of the membrane dry weight is made up of two amino sugars, galactosamine and glucosamine[3,4]. Previous speculation that this material represents a membrane-bound polysaccharide[4] has been confirmed by recent results demonstrating that polyhexosamine material can be extracted from these membranes with 75% aqueous ethanol[5]. In this note we investigate the relationship of this polyhexosamine to other membrane components and demonstrate the ease with which it is released by sonication.

A. laidlawii membranes can be conveniently labelled with radioactive glucosamine added to growth medium, either in a semi-defined medium or in broth. Such membrane-incorporated glucosamine can be recovered by acid hydrolysis and shown to be indistinguishable from native glucosamine by two-dimensional paper chromatography[4]. We wished to determine whether any or all of this label became covalently attached to membrane proteins, since glycoproteins have been reported as prominent constituents of other plasma membranes[6,7]. For this purpose we prepared membranes containing [^{14}C]glucosamine and ^{3}H-labelled protein and subjected part of the preparation to pronase digestion under conditions previously shown to promote extensive proteolysis[4]. This sample and a control were then dissolved in sodium dodecyl sulfate and subjected to electrophoresis on polyacrylamide gels as shown in

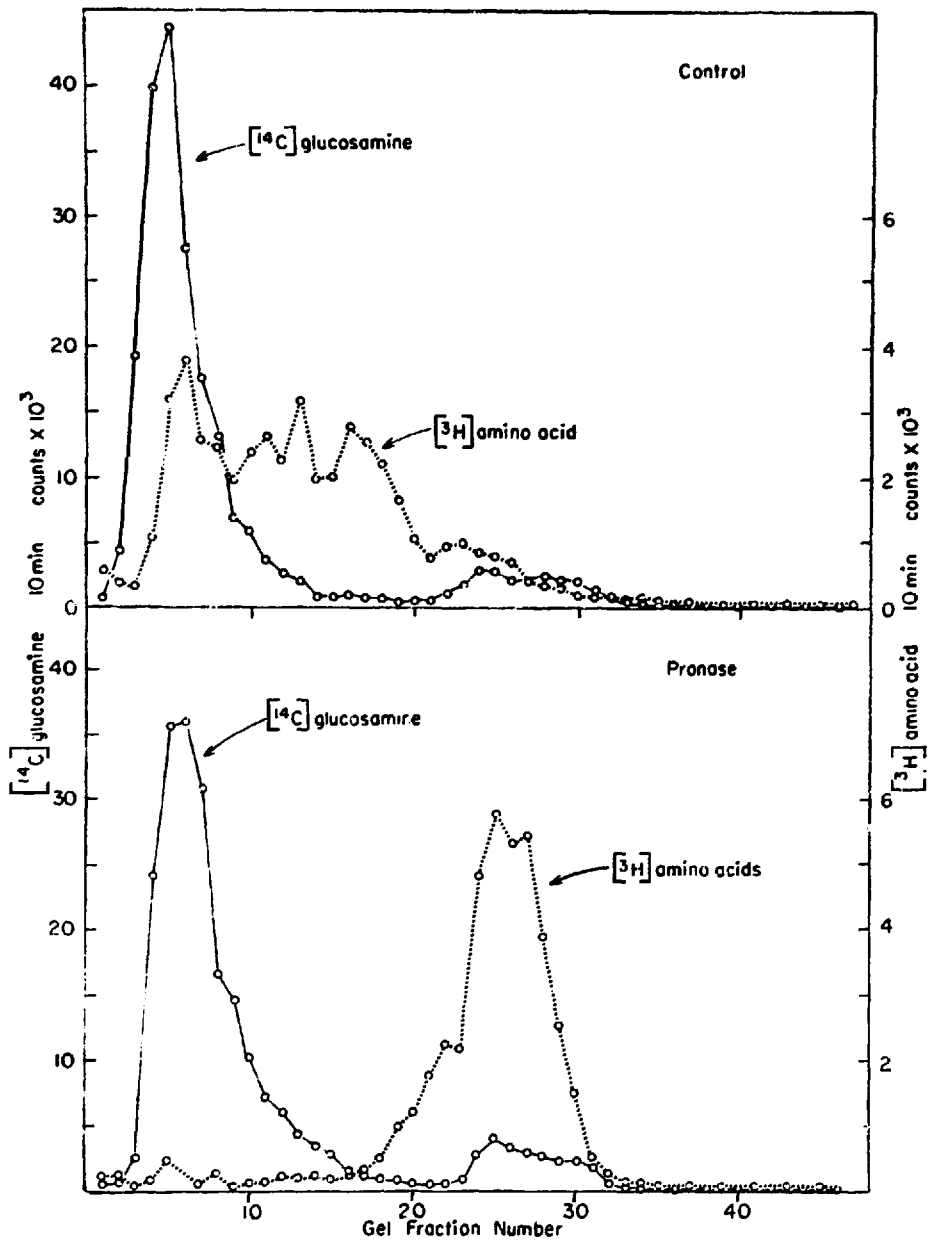

Fig. 1. Effect of pronase on the acrylamide gel distribution of labeiled glucosamine and labelled amino acids incorporated into *Acholeplasma* membranes. *A. laidlawii* B cells were grown to late logarithmic phase in a minimal medium[11] containing (per 100 ml) 25 μCi of D-[^{14}C]glucosamine and 100 μCi of ^{3}H-labelled amino acid mixture (both from New England Nuclear Corp.). Approximately 1% of each label was incorporated into cold trichloroacetic acid-insoluble material. Cells were washed in (0.156 M NaCl–0.05 M Tris–0.01 M 2-mercaptoethanol buffer in deionized water, adjusted to pH 7.4 with HCl), lysed osmotically in a 1:20 dilution of the above buffer in deionized water and purified by banding on discontinuous sucrose gradients as described previously[8]. Washed membranes in the buffer were incubated for 3 h at 37 °C with and without pronase (500 μg/ml; Calbiochem B grade). Membranes were then chilled, centrifuged at 30000 × *g* for 30 min, and the pellets resuspended in 0.005 M sodium phosphate (pH 7), 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. After boiling for 2 min, the solubilized membranes were applied to 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and electrophoresed as described previously[4]. After electrophoresis the gels were frozen and sliced laterally into thin slices 1 mm thick. Each slice was incubated for several days with 1 ml of 0.1% dodecyl sulfate in a scintillation vial, with two freeze–thaw steps to facilitate release of material from the gel, and finally counted on a Packard Tri-Carb liquid scintillation counter. Top: control membranes; bottom: membranes digested with pronase. Samples migrated towards the anode at right.

Fig. 1. In the control, protein was distributed in a series of peaks and glucosamine appeared predominantly as a single peak near the top of the gel, with a small broad peak further down the gel. After proteolysis, the protein peaks disappeared and all amino acid label appeared in a broad zone near the migration front; however, the position of labelled glucosamine remained unchanged. If all or some of this label had been covalently attached to proteins, we would have expected some glucosamine to remain associated with peptide fragments migrating further down the gel. Failure to observe any change in glucosamine distribution suggests that this sugar is not measurably incorporated into membrane glycoproteins.

In another experiment we examined the sucrose density gradient distribution of membrane-bound glucosamine, lipid and protein after brief sonication. Control membranes reach equilibrium as a single band with an isopycnic density of about 1.18 g/cm^3. All glucosamine label is found in this region (Fig. 2a) along with lipid and protein[8]. Sonication of such membranes for 5 s causes virtually all glucosamine label to appear at the top of the gradient (Fig. 2b); similar results are seen after longer sonication periods. By contrast, lipid and protein labels, although more spread out than in the control, are not significantly present at the top of the gradient under these conditions (Figs 2c, d); only after prolonged sonication do these components

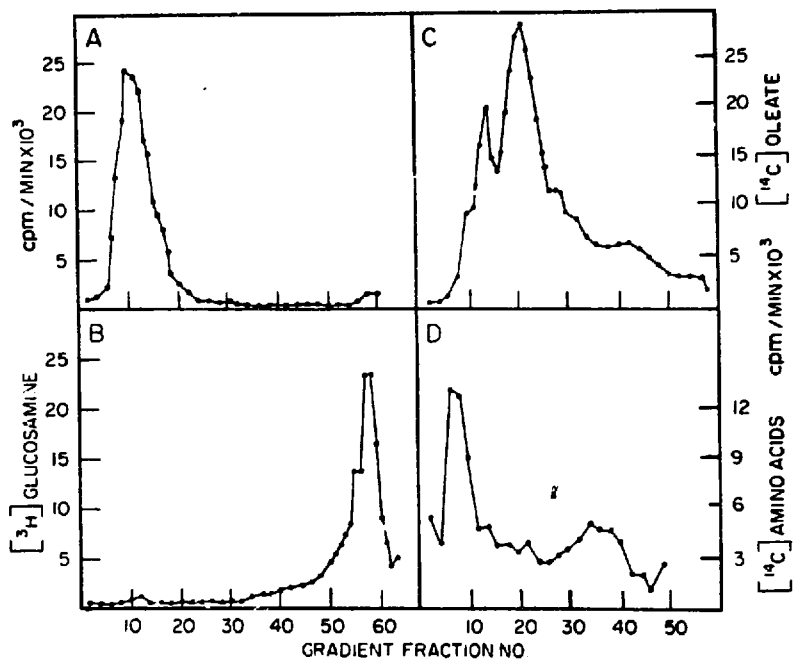

Fig. 2. Distribution of membrane-bound glucosamine, lipid, and protein on density gradients after brief sonication. *A. laidlawii* cells were grown to late logarithmic growth phase on a tryptose–glucose–bovine serum albumin medium[12] supplemented with one of the 3 following labels (per 2000 ml): 500 μCi of [^{3}H]glucosamine, 50 μCi of [^{14}C]oleic acid, or 100 μCi of ^{14}C-labelled amino acid mixture (ICN Corp.). Membranes were prepared as described in the legend to Fig. 1. 0.3-ml aliquots of washed membranes containing 2 mg protein in the buffer described in the legend to Fig. 1 were sonicated in a Branson model LS75 Sonifier at position 4 for 5 s. Samples were cooled in an ice bath during sonication. They were then applied to continuous gradients of 25–50% (w/w) sucrose in a 1:10 dilution of the same buffer and centrifuged for 18 h at 35000 rev./min and 4 °C in an IEC 283 rotor. Gradients were unloaded by bottom puncture and fractions assayed for radioactivity in a Packard Tri-Carb scintillation counter. Graphs show: (a) distribution of labelled glucosamine in unsonicated control membrane; (b) distribution of labelled glucosamine in sonicated membranes; (c) distribution of labelled lipid in sonicated membranes; (d) distribution of labelled protein in sonicated membranes. Direction of sedimentation is to the left.

remain at the top of the gradient[9]. Thus sonication appears to dissociate glucosamine from lipid and protein.

In order to show that labelled glucosamine remained in polysaccharide form after sonication (and to show that membrane-bound label was in fact incorporated into polysaccharide), we compared chromatographic column elution profiles for membrane-bound glucosamine released from sonicated membranes (Fig. 3, Peak a), membrane-bound glucosamine extracted from native membranes with 75% ethanol[5] (Fig. 3, Peak b) and free glucosamine (Fig. 3, Peak c). The porous glass column matrix (see legend to Fig. 3) has an exclusion limit of approximately 15000 daltons (ref. 13 and G. Hawk, personal communication). Peaks a and b both elute with the void volume, whereas free glucosamine is retained on the column (Peak c). Thus the effective molecular weight of the polymers prepared by solvent extraction or sonication is greater than 15000. This lower limit accords with the molecular weight estimates of 17000, 60000 and 90000 by three different methods[5].

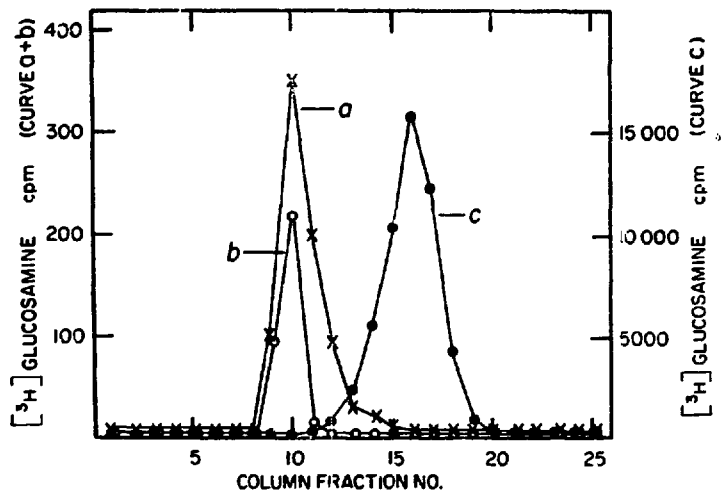

Fig. 3. Column elution profiles of free glucosamine (c) and membrane-incorporated glucosamine prepared either by 75% aqueous ethanol extraction (b) or by sonication (a). Membranes containing [^{3}H]glucosamine were prepared as described in the legend to Fig. 2. One aliquot of these membranes was sonicated for 30 s and centrifuged on a density gradient as described in Fig. 2b. Fractions corresponding to the peak at the top of the gradient were pooled, concentrated by evaporation in a dialysis bag, and used as column Sample a. Another aliquot of membranes was extracted with 75% aqueous ethanol[5] at 50° for 1 h, then filtered through Whatman No. 1 filter paper. The filtrate was evaporated to 1/10th the original volume, then restored to the original volume by the addition of acetone. After 18 h at 4 °C, the precipitate was collected by centrifugation at 30000 × *g* for 20 min (4 °C), dissolved in a small volume of H_2O, and used as column Sample b. An aliquot of the original [^{3}H]glucosamine stock constituted Sample c. Each sample was applied to a column of polyethylene glycol-treated porous glass (Corning Glass Works, CPG 10-125, actual pore diameter 138 Å ± 6.5%, 120–200 mesh)[13]. The bed dimensions were 1.5 cm × 20 cm. Samples were eluted with 0.1 M Tris–HCl, pH 7.5, using a polystaltic pump and collected in 2-ml fractions with an LKB fraction collector. Each fraction was counted on a Packard Tri-Carb scintillation counter. The column void volume was experimentally determined with blue dextran.

From these data we conclude that membrane-associated glucosamine is contained in some polymeric structure which is not significantly disrupted upon sonication. The polymer is not covalently attached to protein and can be released from membranes by sonication, under conditions where lipid and protein remain sedimentable. This polymer is probably identical to the polyhexosamine described recent-

ly[5]. Other data indicate that this polyhexosamine is the major antigenic determinant of these membranes[10]. Given the present evidence for weak association of this polymer with the membrane, it is reasonable to speculate that the polyhexosamine is localized on or near the exterior face of the plasma membrane.

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