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CHARACTERIZATION OF THE PLASMA MEMBRANE OF *MYCOPLASMA LAIDLAWII*

IX. ISOLATION AND CHARACTERIZATION OF THE MEMBRANE POLYHEXOSAMINE

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SUMMARY

1. A simple method is described for extracting and purifying the hexosamine component from membranes of *Mycoplasma laidlawii* B.

2. This purified membrane carbohydrate contains *N*-acetyl-2-amino-2-deoxyglucose and *N*-acetyl-2-amino-2-deoxygalactose as the only sugars. Analysis reveals negligible amounts of amino acids or lipids.

3. Analytical ultracentrifugation, viscosity measurement, and reducing end-group determination show this hexosamine to be a high molecular weight polymer.

4. Elemental analysis for C, H, N, and O in the purified hexosamine agrees with the percentages calculated for polymerized *N*-acetylhexosamine. A determination of the ratio of galactosamine to glucosamine suggests a repeating unit of three galactosamines and one glucosamine.

5. The results of this study are that the membrane of *Mycoplasma laidlawii* B contains a high molecular weight polymer of *N*-acetylglucosamine and *N*-acetylgalactosamine.

INTRODUCTION

It has been reported in a previous paper of the series[1] that *Mycoplasma laidlawii* B plasma membranes contain glucosamine and galactosamine in significant quantities. The failure of these amino sugars from detergent-solubilized membranes to pass through dialysis tubing suggests they are either polymeric or bound to polymers. In this paper we report the purification and characterization of a high molecular wt polyhexosamine from membranes of *M. laidlawii*.

MATERIALS AND METHODS

Materials

The amino sugar standards and silylation reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Materials for gas–liquid chromatography came from

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Applied Science Laboratories, Inc., State College, Pa.. Radioactively labelled compounds were obtained through New England Nuclear, Boston, Mass., and were used without further purification.

Methods

Membrane preparation. Cells of *M. laidlawii* were grown at 37 °C on medium consisting of: 20 g Tryptose (Fisher Scientific Co.), 3.75 g Tris, 5.0 g NaCl, 10 g dextrose, 5 ml Difco PPLO Serum Fraction, and 1 l deionized water. Procedures for growing cells and isolating membranes are as previously described[2], using the above medium. A large batch (100 l of growth medium) was prepared with the same technique except a Sharples air-driven centrifuge was used for sedimenting cells and membranes from suspension. Membrane purification by sucrose gradient was done in a Spinco zonal rotor, Model Ti-15.

Analytical procedures. Hexosamine was quantitatively estimated using a modification of the Elson–Morgan procedure[3] reported previously[2]. Hydrolysis time for routine determination was 4 h at 100 °C.

To check extraction efficiencies some membranes were prepared with D-[1-^{14}C] glucosamine·HCl or [U-^{14}C]oleic acid using a previously reported procedure[2]. The [^{14}C]glucosamine is incorporated into the membrane hexosamine and the [^{14}C]oleic acid is incorporated into the membrane lipids.

The ninhydrin value of various samples was determined by the method of Moore and Stein[4] using 2-amino-2-deoxy-D-glucose·HCl as a standard for calibration.

Reducing equivalents of solutions were estimated using the method of Nelson[5] and 2-amino-2-deoxy-D-glucose·HCl as a standard. For determining the number-average weight by the endgroup the sub-micro method of Moyer and Isbell[6] using Na^{14}CN was used. Anhydrous D-glucose was used to calibrate the procedure. A high background was obtained but it was uniform with a standard deviation of 1.8 % so that no attempt was made to purify the cyanide. Radioactivity was determined using a liquid scintillation counter.

For a determination of total phosphate, samples were first digested by the following procedure: a sample containing up to 2.0 μmoles of phosphorus was placed in a 50-ml pyrex tube and dried. Then 1.2 ml of 2.5 M H_2SO_4 was added and the sample heated over a gas flame until the sample turned black and thick white fumes escaped from the tube. After adding 2 drops of concentrated HNO_3, heating was continued until no brown fumes were visible and the solution was clear. The tubes were then cooled slightly and a small amount of water was added. These samples were then brought to an equivalent volume and phosphate determined by the method of Fiske and SubbaRow[7].

Lipid ester groups were determined by the method of Antonis[8]. Determination of protein was by the method of Lowry *et al.*[9] with bovine serum albumin used as a standard.

Chromatography procedures. Amino acid analysis was performed on hydrolysates by the automated column chromatography method of Moore and Stein[10]. This gives an analysis of glucosamine and galactosamine as well as the amino acids. For determination of the galactosamine to glucosamine ratio carefully weighed samples of purified polyhexosamine were dissolved in constant boiling HCl. These samples were placed in small pyrex tubes which were evacuated, sealed and placed

in a 100 °C oil bath for hydrolysis. Hydrolysates were then dried in a vacuum dessiccator over NaOH pellets after which analysis was done on the amino acid analyzer.

Descending paper chromatography was done on Whatman No. 1 filter paper. The paper was presaturated in an atmosphere of solvent for 24 h. Sugars were separated using *n*-butanol–pyridine–water (6:4:3,v/v/v). The sugars were visualized using either Morgan–Elson reagent for amino sugars or sodium metaperiodate for other sugars[11]. R_X values were determined by measuring the leading edge of each spot and comparing with D-glucose on the same paper.

Gas–liquid chromatographic identification was done on the trimethylsilyl derivatives of the acetylated hexosamines prepared according to the method of Levvy *et al.*[12]. The chromatography procedures were those of Perry[13]. The instrument was a Perkin–Elmer model 990 equipped with a flame ionization detector optimized for response to column bleed. The hydrolysis procedure was performed with constant boiling HCl in vacuum at 100 °C. Hydrolysis times varied with the experiment. Tests with purified glucosamine and galactosamine showed little destruction of these sugars after 6 h under these conditions. After hydrolysis the sample was dried *in vacuo* over NaOH.

Infrared spectra. Infrared spectra were obtained on a Perkin–Elmer Model 337 grating spectrophotometer using a slow scanning speed and normal automatic slit adjustment. The samples were mixed 1 % (w/w) in dry powdered KBr and pressed under vacuum into pellets using a steel die. Air was used for the reference beam and the wavelength was calibrated using a 0.05-mm film of polystyrene.

Sedimentation coefficient. For this determination a sample of purified hexosamine was dialyzed against 0.5 M KCl. Centrifugation was carried out at 48 020 rev./min and 22 °C. Sedimentation coefficients were calculated from measurements taken on the photographic plates with a microcomparator.

Viscosity. The viscosity of a solution of purified hexosamine in deionized water was estimated using a Ubbelohde viscometer (Cannon No. 1B). A large water reservoir was used as a constant temperature bath.

RESULTS

Extractions and purification of the membrane hexosamine

Extraction with 75 % aqueous ethanol at 50 °C for 1 h solubilizes more than 90 % of the hexosamine along with most of the membrane lipid (Table I). Concentrating the ethanol solution 10-fold and adding 9 vol. of acetone precipitated most of the hexosamine after standing at 4 °C for 18 h (Table I). These two procedures formed the basis of a purification scheme which is outlined in Fig. 1. The final dialysis and centrifugation in this scheme was necessary to remove some amino acids which were carried through the rest of the procedure with the hexosamine. When done carefully this procedure results in a recovery of more than 50 % of the starting hexosamine in what appears to be a relatively pure form. The resulting material is white and fluffy in appearance and is soluble in water at low concentration.

Table II gives the results of some chemical tests performed on two separate preparations of the purified material. The agreement between the hexosamine and ninhydrin values of Preparation 2 is not in itself a demonstration of purity. The very low ninhydrin value of this material before hydrolysis indicates the nitrogen of the

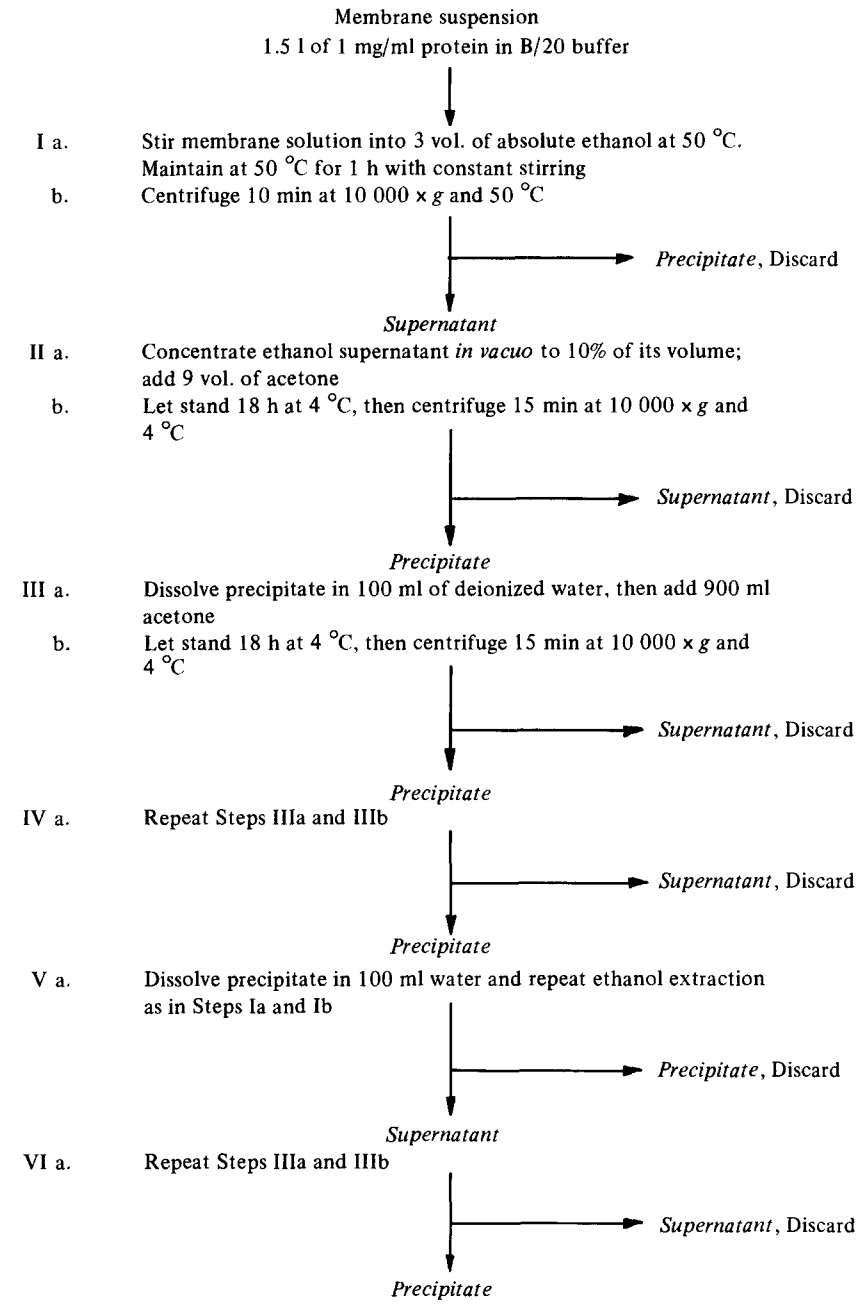

Fig. 1. A flow chart for the *M. Laidlawii* membrane hexosamine purification procedure.

TABLE I

EFFICIENCY OF ETHANOL EXTRACTION AND ACETONE PRECIPITATION OF *M. laidlawii* MEMBRANE HEXOSAMINE

A suspension of membranes in B/20 buffer was extracted 1 h with 75% ethanol at 50 °C. After 10 min of centrifugation at 10000 × *g*, aliquots of the supernatant and precipitate were assayed chemically for hexosamine. This extract of membranes labelled with [^{14}C]glucosamine or [^{14}C]oleic acid was then concentrated 10-fold and brought to 90% acetone. After standing at 4 °C for 18 h, aliquots of the resulting precipitate and supernatant were assayed for radioactivity with a liquid scintillation counter. A second fractionation was done on acetone Precipitate I dissolved in water.

Fraction	*Percentage of total hexosamine*	
Ethanol extraction		
Supernatant	94	
Precipitate	6	
	[^{14}C]*Glucosamine* (% *of total cpm*)	[^{14}C]*Oleic acid* (% *of total cpm*)
Acetone precipitation		
Ethanol residue	2	1
Acetone Supernatant I	8	94
Acetone Precipitate I	90	5
Acetone Supernatant II	2	4.5
Acetone Precipitate II	88	0.5

TABLE II

CHEMICAL CHARACTERIZATION OF THE PURIFIED HEXOSAMINE FROM *M. laidlawii* MEMBRANE

Two separate preparations of purified membrane hexosamine were dissolved in water and the listed determinations were carried out. Preparation I did not receive Step VII of the purification procedure (Fig. 1).

Group Assayed	*μmoles/ml*	
	Preparation 1	*Preparation 2*
Hexosamine	3.18	0.90
Ninhydrin		
before hydrolysis	—	Trace
after hydrolysis	4.15	0.90
Lipid esters	—	<0.05
Phosphate	<0.01	<0.02
Reducing equivalents	<0.02	<0.01

hexosamine is bonded in such a way as to be unreactive with ninhydrin. Low values for phosphorous and lipid esters show an insignificant content of membrane lipid. The low reducing equivalent of this material suggests that the anomeric carbons of the hexosamines are bound and the material is polymeric.

Characterization

Separation by three chromatographic procedures coupled with comparison of the retention of known standards served to identify the hexosamine in this material

TABLE III

CHROMATOGRAPHIC IDENTIFICATION OF THE HYDROLYSIS PRODUCTS OF PURIFIED HEXOSAMINE FROM *M. laidlawii* MEMBRANE

Samples of the purified hexosamine were hydrolyzed for 4 h *in vacuo* for paper and gas–liquid chromatography and 20 h *in vacuo* for the amino acid analyzer. For gas–liquid chromatography the hydrolysates were acetylated and trimethylsilylated before separation.

	Chromatography system		
	Paper chromatography ($R_{glucose}$)	*Amino acid analyzer* ($R_{time\,(min)}$)	*Gas–liquid chromatography* ($R_{solvent}$)
Component 1	0.63	188	9.8
Component 2	0.73	166	11.9
2-Amino-2-deoxy-D-galactose·HCl	0.62	189	9.8
2-Amino-2-deoxy-D-glucose·HCl	0.74	166	11.9
Amino acids		< 5 % of total	

(Table III). On paper chromatography two spots were visible with the same retention as standard 2-amino-2-deoxy-D-glucose–HCl and 2-amino-2-deoxy-D-galactose·HCl. These also gave a positive reaction using the Morgan–Elson spray reagent which is specific for amino sugars. No other spots were visible using a periodate reagent except at the origin, which may represent non-hydrolyzed material. The amino acid analyzer showed two peaks which gave identical retention times as 2-amino-2-deoxy-D-glucose·HCl and 2-amino-2-deoxy-D-galactose·HCl using standard column conditions. On gas–liquid chromatography of the trimethylsilyl derivatives, two major peaks were present with the same retention times as the derivatives of the standard glucosamine and galactosamine.

The infrared spectrum of the membrane hexosamine in Fig. 2 shows a broad –OH absorption and distinct Amide I and Amide II absorptions. This result together

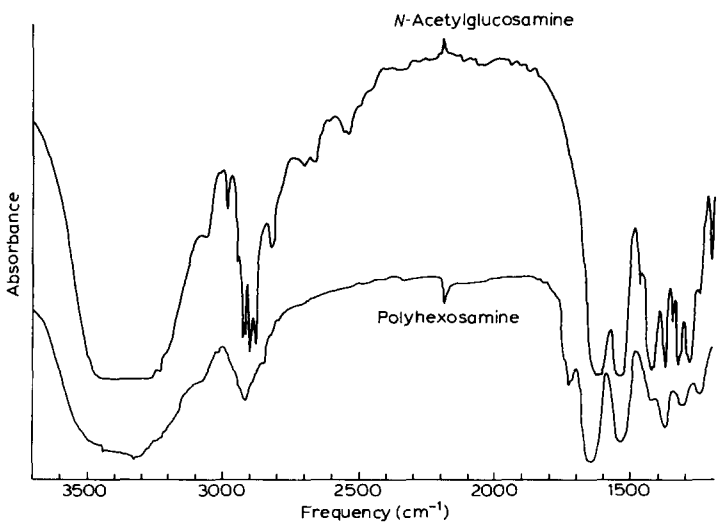

Fig. 2. Infrared spectra of purified membrane hexosamine and standard *N*-acetylglucosamine.

with the low ninhydrin value of the non-hydrolyzed material indicates the hexosamine is acetylated on the amino group.

A sedimentation coefficient of 7.6 was calculated from a sedimentation velocity experiment on the analytical ultracentrifuge. The exposure shown in Fig. 3 indicates a single sedimenting substance with no very large or very small compounds in the solution.

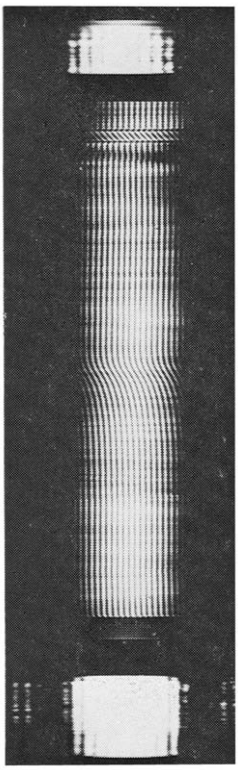

Fig. 3. Exposure of interference pattern from analytical ultracentrifuge. This exposure was taken 57 min after attaining speed and is representative of all the exposures taken.

The viscosity measurement was made on a single dilute solution. Centrifugation to remove any large aggregates in the sample did not markedly affect the time for the solution to flow through the capillary. As the viscosity of liquids is proportional to the time for a given volume to flow through the capillary[14] the times may be used in the Einstein–Simha equation to calculate the viscosity increment[15].

$$\frac{t/t_0 - 1}{\phi} = \nu \qquad \eta = kt$$

η, viscosity; ν, viscosity increment; ϕ, volume fraction of solute. Approximating the partial specific volume of glucosamine as 0.66, gives a viscosity increment of approximately 200. This figure is an estimate but as such indicates the molecule is in the shape of a long rod and is consistent with an extended linear polymer of approximately 60 000 molecular weight.

TABLE IV

VISCOSITY MEASUREMENTS ON SOLUTION OF *M. laidlawii* MEMBRANE HEXOSAMINE

A sample of purified membrane hexosamine weighing 8.8 mg was dissolved in 10.0 ml of deionized water and the time for flow through a capillary tube measured using an Ubbelohde viscometer. The solution was centrifuged at 10000 × *g* for 30 min and the supernatant used for the second measurements. Each number is the mean of 8 determinations.

Sample	*Time* (*s*)
Water	17.77
Hexosamine	
(a) before centrifugation	19.73
(b) after centrifugation	19.98

TABLE V

REDUCING END-GROUP DETERMINATION OF THE *M. laidlawii* MEMBRANE HEXOSAMINE USING $^{14}CN^-$

A known amount of membrane hexosamine and D-glucose were reacted for 24 h at 55 °C with $Na^{14}CN$ in 0.6 M NH_4Cl. After removing unreacted cyanide the samples were counted by liquid scintillation counting. Figures in table are the means of three determinations.

	Weight (*μg*)	*cpm*	*Reducing group* (*nmoles*)	% *S.D.*
Hexosamines	450	17 754	26.5	1.4
D-Glucose	1.05	38 669	57.8	2.3

The experiment shown in Table V gives a quantitative estimate of the reducing end group and together with the amount of acetylated hexosamine estimated from the weight of polymer used gives a minimum number-average molecular weight of approximately 17000.

The ratio of galactosamine to glucosamine in the purified polymer was estimated using the amino acid analyzer. Known weights of purified polyhexosamine were

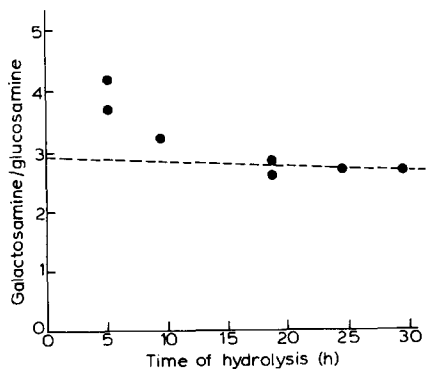

Fig. 4. Ratio of galactosamine and glucosamine in the polyhexosamine from *M. laidlawii* membranes. Samples of purified polyhexosamines were hydrolyzed by standard conditions for different times. The resulting hydrolysates were quantitatively analyzed using the amino acid analyzer. Response for glucosamine and galactosamine was determined using carefully dried standards.

hydrolyzed for various times under carefully controlled conditions. The response of the analyzer was found to be the same for glucosamine and galactosamine standards. Results of these determinations (Fig. 4) show that hydrolysis is not complete in the shorter hydrolysis times. An extrapolation of the longer hydrolysis times to zero time indicates a ratio for galactosamine:glucosamine of 3:1. The negative slope of this line indicates that the galactosamine is destroyed more quickly than glucosamine as reported elsewhere.[16]

TABLE VI

ELEMENTAL ANALYSIS OF PURIFIED HEXOSAMINE FROM *M. laidlawii* MEMBRANE

A sample of 2 mg of purified carbohydrate from *M. laidlawii* membranes was sent to Dr Franz Pascher, Mikroanalytisches Laboratorium, 53 Bonn, Buchstrasse 54, West Germany, for elemental analysis.

	Calculated elemental composition for polymerised N-acetylglucosamine (*wt* %)	*Experimental analysis* (*wt* %)
Carbon	47.28	47.23
Hydrogen	6.46	6.24
Nitrogen	6.89	6.23
Oxygen	39.36	39.48

An elemental analysis for carbon, hydrogen, oxygen and nitrogen was performed on the purified material by a private laboratory. The results (Table VI) compare closely to the amounts of these elements calculated for a polymerised *N*-acetylglucosamine. This shows that very little of any other carbon-base compounds could be in this preparation as well as confirming that the group attached to the amine is an acetyl group.

DISCUSSION

A combination of ethanol extraction and acetone precipitation appears to produce a very clean preparation of the hexosamine from the *M. laidlawii* B membrane. The degree of purity is indicated by the low amounts of amino acids and lipids found in the final preparation as well as the close agreement of the elemental analysis with the composition calculated for a polymerised *N*-acetylglucosamine. The radioactive labelling of the hexosamine and lipid shows that acetone precipitation gives a good separation of fatty acid-containing lipids from the membrane hexosamine and repeated precipitations should result in a reduction of the lipid content of the hexosamine to insignificant amounts for further characterization. Attempts to determine degree of purity by determining chemically reactive hexosamine in known dry weights did not give comparable results either by the Elson–Morgan reaction or ninhydrin reaction as done on the amino acid analyzer. This is thought to be due to problems associated with the hydrolysis of the polymeric hexosamine[17]. Either method requires complete cleavage of the polymer without destruction of the monomeric units. The ninhydrin method of detection also requires complete cleavage of

all acetyl groups from the amine. The results do indicate that the purity of the polyhexosamine as prepared is sufficient for further characterization and structural determination.

Three different methods indicate that the purified material is a relatively high molecular weight polymer. A careful determination of reducing end group using $^{14}CN^-$ gave a number-average molecular weight of 17 000, or approximately 80 hexosamine residues assuming the polymer is all hexosamine. This represents a minimal estimate since the reaction was carried out at alkaline pH which may disrupt some of the glycosidic linkages. The estimated viscosity increment of 200 would indicate an approximate axial ratio for a prolate ellipsoid of 300:1. If the hexosamine dimensions are assumed to be 10 Å × 10 Å, this corresponds to a linear polymer of 300 hexosamine residues or a molecular weight of 60 000. The sedimentation coefficient of $S = 7.6$ is consistent with a molecular weight on the order of 90 000 assuming the diffusion coefficient is independent of concentration. Since the concentration of polymer was low this is a reasonable first approximation. These results do not provide precise quantitative values for the molecular weight of this polymer but serve to point out the macromolecular character of the polyhexosamine.

The chromatographic procedures and the elemental analysis give reasonable certainty that the carbohydrate moieties in this polymer are 2-*N*-acetyl-D-galactosamine and 2-*N*-acetyl-D-glucosamine. The infrared spectrum of the polymer is consistent with this interpretation. The ratio of galactosamine to glucosamine as determined on the amino acid analyzer is three to one. This assumes that all the hexosamines in the polymer have equal probability of being cleaved or destroyed by the acidic hydrolysis for the long-time reactions. A likely possibility from this measurement is a repeating unit of four hexosamines. This possibility is supported by X-ray diffraction measurements which will be included in a subsequent report on the structure of this polymer (D. H. Engelman, J. M. Gilliam and H. J. Morowitz, unpublished results).

In present models of membrane structure the role of carbohydrates is usually ignored even though these are generally present in all membranes. The lack of a suitable model for the structural role of polysaccharides and the complexity of membrane systems makes it difficult to propose an adequate model. This report shows that the membrane from *M. laidlawii* B contains a macromolecular polyhexosamine which comprises approximately 5 % of the membrane dry weight. Such a system may provide a useful model for the role of carbohydrates in membrane structure.

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