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TITLE: COXIELLA BURNETII VACCINE DEVELOPMENT:
LIPOPOLYSACCHARIDE STRUCTURAL ANALYSIS

PRINCIPAL INVESTIGATOR: VERNON N. REINHOLD

PI ADDRESS: Harvard University School of Public Health
#665 Huntington Avenue
Boston, Massachusetts 02115

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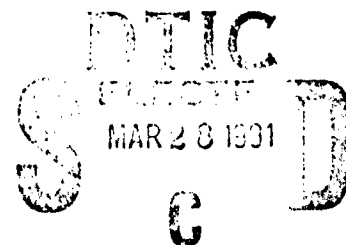
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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Analytical instrumentation and methodology is presented for the determination of endotoxin-related structures at much improved sensitivity and specificity using techniques in chromatography and mass spectrometry. These techniques include a newly synthesized reagent that has provided femtomole (10^{-15}) sensitivity for oligosaccharide detection; and a pre-analysis chemical approach for the determination of oligosaccharide linkage, branching, and sequence. These approaches are compatible with supercritical fluid chromatography-mass spectrometry (SFC-MS) and combine to provide the elements of a global approach to oligosaccharide structure. The utility of this approach has been demonstrated for glycolipids and N-linked glycans. The lipid-A structure from <u>Salmonella minnesota</u> has been demonstrated and the report outlines the currently understood structure of <u>Coxiella burnetii</u> lipid-A.						
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Vernon R. Bunk 2/20/91
PI - Signature DATE

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SUMMARY

This Final Report summarizes new analytical methodology developed over the contract period for the determination of endotoxin-related structures at much improved sensitivity and specificity. Reports, and their applications, are listed in Section IV. These papers have either recently appeared in press (7), are in press, (8,9) or manuscripts are in preparation (10). The final part of this report outlines the currently understood structure of Coxiella burnetii lipid-A.

One aspect of this contract was to improve the methodology for the analysis of glycoconjugate materials using techniques in chromatography and mass spectrometry. In this regard, an interface was designed (3) for supercritical fluid chromatography-mass spectrometry (SFC-MS); a reducing-end reagent was synthesized and conjugated to oligosaccharides providing femtomole (10^{-15}) sensitivity (7); conditions were established for the purification and detection of glycolipids (8); N-linked glycans (9); and, an approach was detailed for the determination of oligosaccharide linkage, branching, and sequence using periodate oxidation in combination with SFC-MS (10). These techniques combine to provide the elements of a global approach to oligosaccharide structure. The utility of supercritical fluid chromatography for a determination of lipid-A from Salmonella minnesota has been demonstrated.

I. INTRODUCTION

A lack of understanding endotoxin structure and the specific determinants that relate to antibody stimulation represents a major complication in vaccine development. Much of this difficulty is due to the complexity of glycolipid and oligosaccharide analytical methodology. Thus, each new endotoxin problem brings a concern on how to approach the structural problem. Should efforts be directed in areas that may lead to more effective methodology, or grind out results with archaic techniques that demand an inordinate amount of material and time.

We have been aware of the extreme difficulty for oligosaccharide and glycoconjugate structural determination and have tried to introduce alternative instrumental and methodological approaches. To evaluate these techniques they have been applied to two different lipid-A samples, a known structure from Salmonella minnesota, and the unknown lipid-A from Coxiella burnetii. In this Final Report we discuss these developments and apply the methodology to these and other glycoconjugate samples. As a final objective, this report concludes that the lipid-A obtained from Coxiella burnetii, and provided by Dr. Jim Williams, (USAMRIID), is based on a unique 2,3-diamino-2,3-dideoxy-D-glucose moiety with each of the four amino groups esterified with heterogeneous mix of aliphatic fatty acids.

II. METHODS DEVELOPMENT:

A. O-ANTIGEN DETECTION AT FEMTOMOLE SENSITIVITY

a.) Introduction.

Carbohydrates lack chromophoric groups and it is thus necessary to prelabel the sample or utilize specific detectors when trying to identify HPLC eluting components. Two methods have been frequently used: (i) pre-column reducing-end conjugation with fluorescent or UV-absorbing groups (1-5); and more recently, (ii) post-column amperometric detection of base ionized samples (6,7). Reducing-end conjugation has involved either reductive amination (2) or direct glycosylation (5). This latter technique has been extensively studied under this contract and provides improved column resolution, when compared to reduced samples (5), and other advantages consistent with subsequent chemical manipulations. Both conjugated products are well suited for analysis by fast-atom bombardment (FAB) because of terminal charge localization and improved sensitivity (3,4,8). This sensitivity, however, still falls short of that routinely available for sequencing DNA/RNA and proteins biopolymers. Moreover, the structural information available is wholly inadequate for full oligosaccharide characterization. For this reason alternative procedures were investigated to improve O-antigen and oligosaccharide detection.

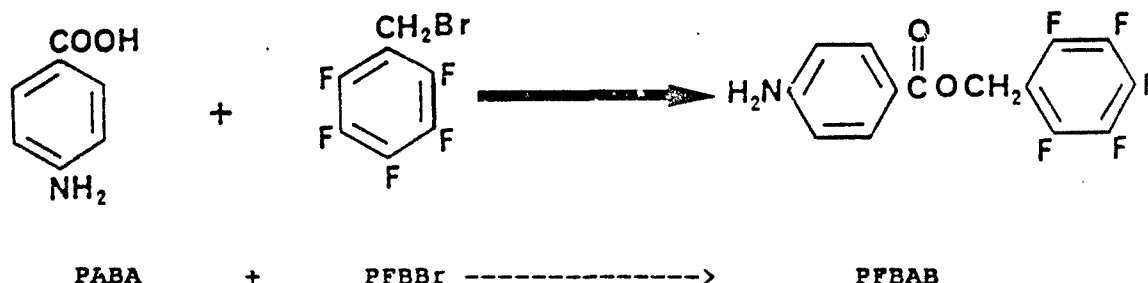
Halogenated materials, or samples prepared as such, have proven most successful in extending detection limits when coupled with negative ion chemical ionization (NCI). A specific example of this approach was demonstrated for derivatized prostaglandin samples, using electron capture detection (9-11) and NCI-MS (12-15). In an effort to

bring comparable sensitivity to the structural problems of *Coxiella burnetii* LPS, we have developed and describe a derivative which imparts improved chromatographic properties, appropriate chemistry for O-antigen and oligosaccharide conjugation, facile negative-ion chemical ionization (NCI) lability for maximum MS sensitivity, and aromatic character for anionic charge stabilization.

b.) Synthesis of Pentafluorobenzylaminobenzoate (PFBAB)

Summary (Details Provided in Appendix, Paper #7). Para-amino benzoic acid (PABA) was esterified with pentafluorobenzyl bromide in acetonitrile using diisopropylethylamine as a catalyst. The esterified product, PFBAB, was purified by preparative C-18 HPLC, using isocratic elution with acetonitrile and water.

These procedures were essentially those first reported by Wickramasinghe, et al., for the derivatization of prostaglandin F_{2α} (11).



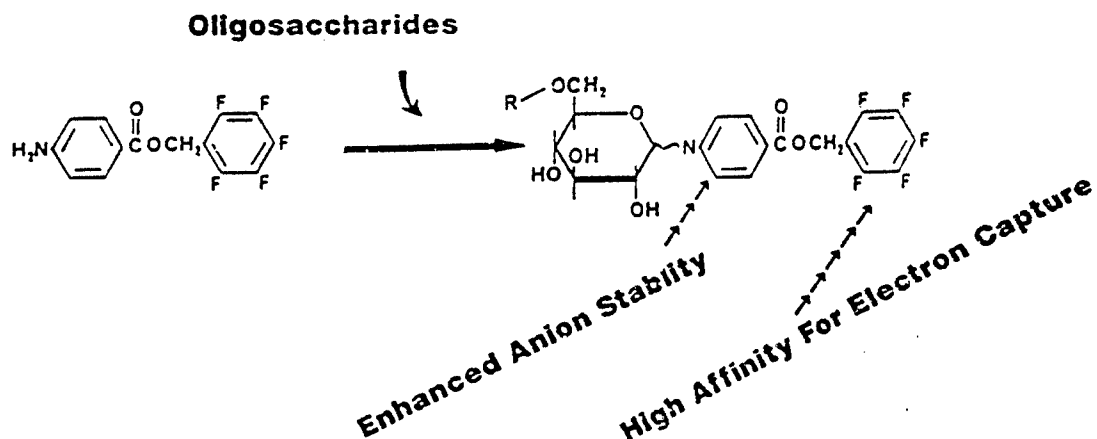
These workers modified the earlier derivatization procedures for phenols, mercaptans (9) and organic acids (10) by using acetonitrile as the solvent and introduced diisopropylethanolamine as the hindered base. Several recent reports have continued to use the same general derivatization procedures (12-15) using negative-ion chemical ionization mass spectrometry. The conditions described in this report are a simple scale-up of the earlier work (11) without heating and longer reaction times. The product yields were evaluated by both HPLC and GLC. Minor peaks appear in both chromatograms which were analyzed by mass spectrometry. The spectra of these minor fractions could be accounted for as pentafluorobenzylamine products of the free amine group on PFBAB and the unreacted free acid, PABA. These extraneous components were in such low concentration, and easily resolved by HPLC, that no attempt was made to diminish their accumulation by modifying the reaction conditions. The HPLC-purified PFBAB product exhibited a UV absorption maximum, $\lambda_{\max}$, at 293 nm with a minor red shift following glycosyl conjugation, (Fig. 1a). Using this absorption maximum, the extinction coefficient (ϵ) of PFBAB was determined to be $2.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $1.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for the fucose glycosylated derivative. The concentration of the latter derivative was determined by GLC. The UV detection limits were determined to be 1.6 μMolar , ($s/n = 10$). Using a similar approach, the pyridinylamine (PA) derivative of fucose was determined to have an $\epsilon = 0.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, indicating less sensitive detection limits.

The FA-fucose derivative was measured at its absorption maximum of 232 nm.

The PFBAB reagent was also found to be fluorescent. The fucose derivative provided a $\lambda_{\text{max}} = 350 \text{ nm}$ upon excitation, ($\lambda_{\text{ex}} = 296 \text{ nm}$), (Fig. 1b). Based on fluorescent scans of equal molar solutions, the PFBAB-fucose was a better fluorophor than FA-fucose by roughly two orders of magnitude. As expected, fluorescence monitoring extended the detection limits of PFBAB-fucose, in this case from $1.6 \mu\text{M}$ for UV monitoring, to 24 nM for fluorescence monitoring; an approximate 60 fold increase in detectability. Both detection limits were taken from fluorescence scans with signal to noise ratio of about 10/1. By using HPLC with UV detection the limits of detection of PFBAB-fucose was found to approximate 8 ng, (17 pmoles. The quantum yield (Q) of PFBAB was determined to be 0.04 using L-tryptophan as a reference standard, (Q = 0.13).

c.) Oligosaccharide Glycosylation With PFBAB

Oligosaccharides were solubilized in a reaction vial with methanol and acetic acid and a 10 molar excess of the PFBAB reagent in $25 \mu\text{l}$ of the same solvent was added and heated for 20 min at 65°C . Supercritical fluid chromatography, and peak area integration, provides a measure of yield as determined by reactant/product ratios, which under these conditions were always greater than 90%.



Preliminary conditions for PFBAB glycosylation were initiated using established procedures (5) and fucose as a model compound. The low molecular weight product could be acetylated for easy analysis by GLC. Extension of these conditions to oligosaccharides was facilitated by SFC which allowed both reactant and product evaluation via flame ionization detection. Increased product yields were a combination of achieving reactant solubility while avoiding product degradation. Although aqueous acidic solvents were most effective for solubilizing larger glycans, they were more prone to degradation with reaction time. Non-aqueous conditions with

1a

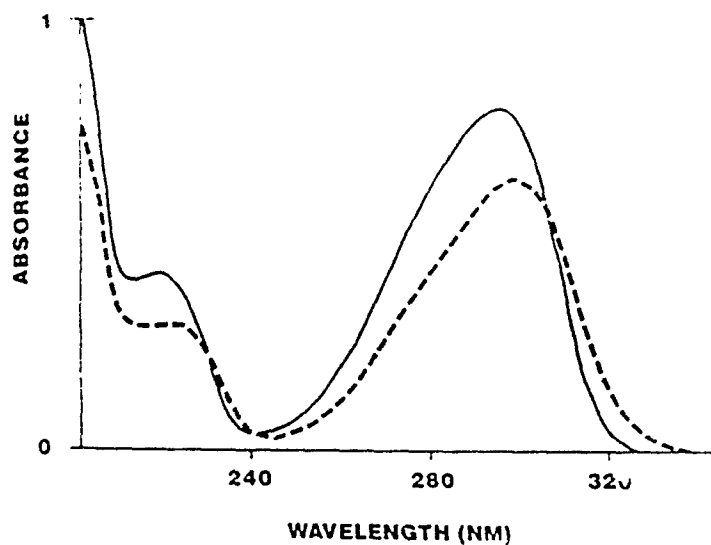


Figure 1a: UV Absorbance Scans of PFBAB (—), and PFBAB-maltoheptaose (---). Absorption maxima at 292 and 296 nm, respectively.

1b

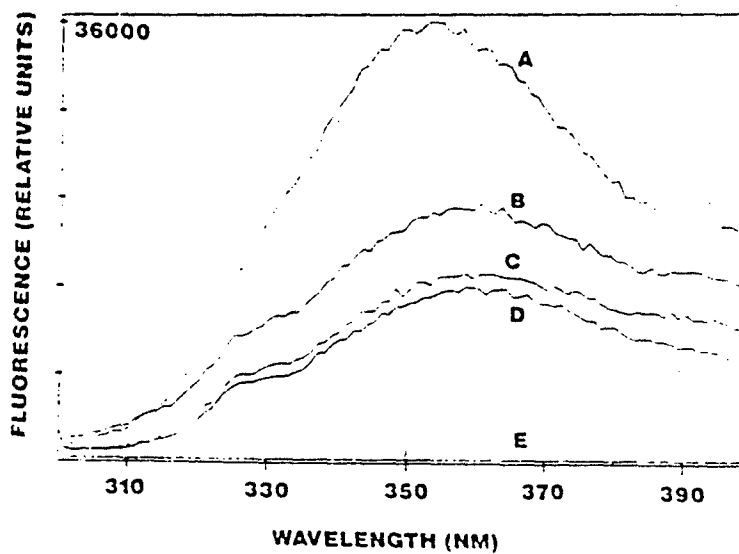


Figure 1b: Fluorescence Scans of PFBAB-fucose. Excitation wavelength = 296 nm. Scan rate = 1 sce/nm. A = 1.2 μ m; B = 24.0 nm; C = 6.0 nm; D = 12.0 nm; E = solvent blank.

acetic acid-methanol solvents proved most successful. The solvent polarity was adequate for bringing glycans into solution and, under the reaction conditions established, degradation was undetected. To evaluate this latter point, the loss of a labile group, (neuraminic acid), from glycans was evaluated. It was noted that this acid (both 3- and 6-linked) was not lost from the trisaccharide, neuraminyllactose, nor from α_1 -acid glycoprotein under the PFBAB derivatization conditions. The kinetics of PFBAB conjugation can be followed by SFC which allows a determination of products and reactants in each analysis using FID detection. For small saccharides (dp = 2 - 7) derivatization is quantitative, but with larger oligomers, 20 minutes was insufficient, (Fig. 2). When PFBAB glycosylation was applied to a mixture of N-linked high-mannose glycans obtained from a plant glycoprotein, the product yields decreased, but approached quantitation with longer reaction times and/or higher temperatures. Different classes of oligosaccharides will undoubtedly have to be evaluated separately to maximize yields. From these limited studies, however, it does appear that the chemistry of PFBAB glycosylation with hexoses and amino sugars proceeds satisfactorily, and under conditions that insure product stability. Although somewhat less than quantitative, the striking increase in sensitivity achieved by PFBAB glycosylation and NCI-MS (see below) greatly offsets these small losses in derivatization.

d.) Negative Ion Chemical Ionization MS Sensitivity

Min, et al., (12) using the PFB derivative and single-ion monitoring, $[(M - C_7H_2F_5)^-]$, NCI-MS had indicated sensitivities for prostaglandin $F2_\alpha$ approximating 1 pg (injected). This value was reported to be five times better than electron capture detection (EC-GLC) and twenty-five times better than a completely silylated derivative, $(M - TMSOH)^-$. Similar comparisons between NCI and positive EI have been reported and the striking stability of the carboxylate anion noted (15). In a preliminary effort, to access the limits of detectability, the PFBAB-fucose derivative was analyzed by SFC-NCI-MS using full mass spectral scans (100 - 1000 Da) (16). Using a series of dilutions, injection of 55 femtomoles provided an abundant signal for the $(M - C_7H_2F_5)^-$ fragment. No total ion current was detected at this level, however, an ion plot $[(m/z 408, M - C_7H_2F_5)^-]$ provided one major peak with a s/n = 10, Fig. 3. Sensitivity studies at higher mass has provided comparable data. Presented in Figure 4 is the total ion plot of a complex mixture of glycans isolated from a plant, labeled with PFBAB and analyzed by SFC-NCI-MS. Recessed to the right in this figure are a series of ion plots representative of each component comprising the mixture separated by these mass chromatograms.

The extraordinary sensitivity developed with these techniques can be attributed to several factors. Most important, and as observed by others (12-15), is the unique character of the PFB moiety to serve as an efficient electron trap. The high electronegativity of F leads to a lowering of the lowest unoccupied molecular orbital energy and thus an increase of electron affinity. The resident negatively charged PFBAB residue is then stabilized by ester bond rupture to leave, in high abundance, the carboxylate anion. Secondly, is the level and distribution of fragmentation energy which

FUCOSE PFBAB Derivative

SFC-NCIMS (55 Femtomoles)

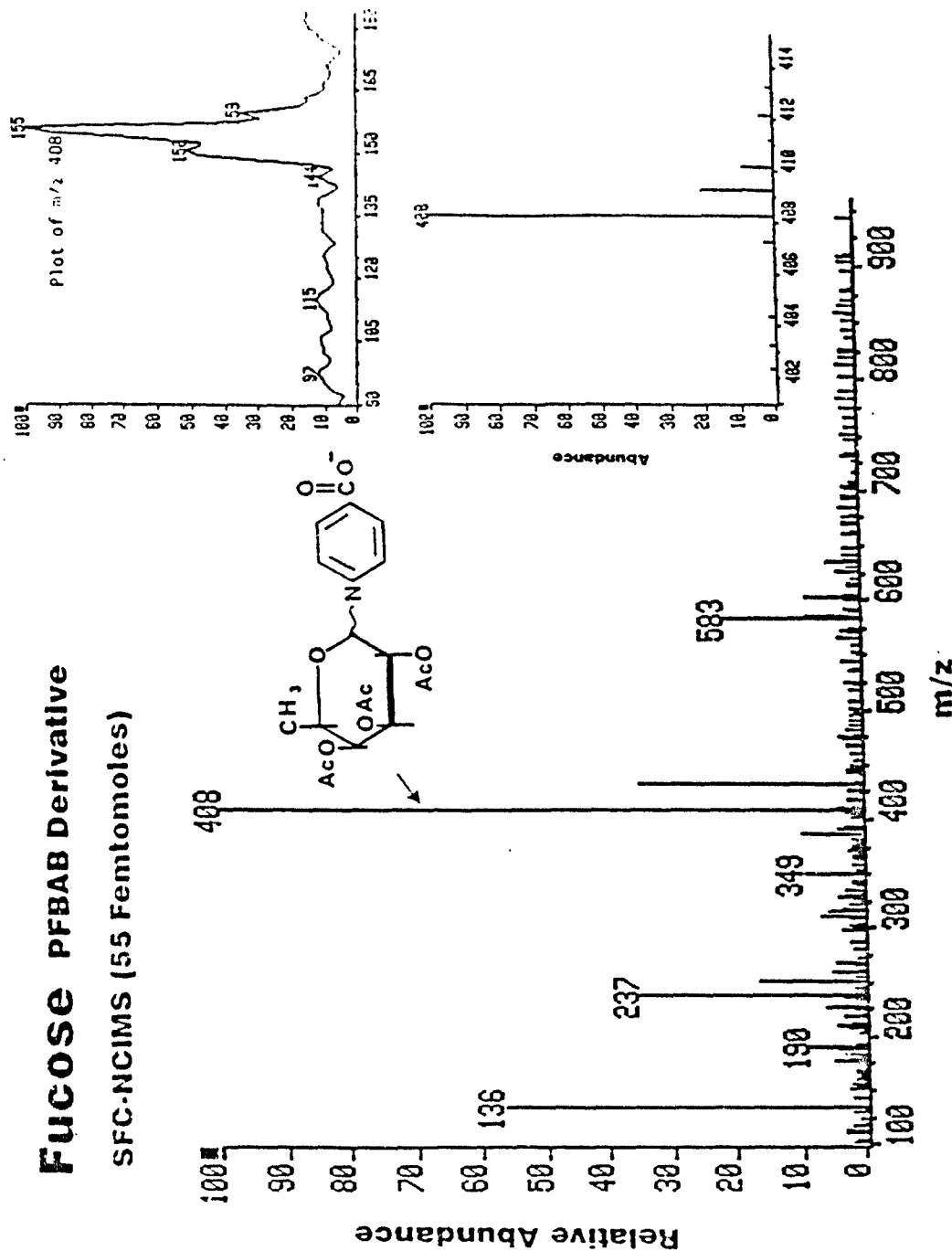


Figure 3: Sensitivity study using PFBAB labeled fucose. Product ion (m/z 408) loss of PFB group. Mass spectrum taken at dilution of 55 femtomoles. Inset showing mass chromatograph at m/z 408 and expanded spectral scan.

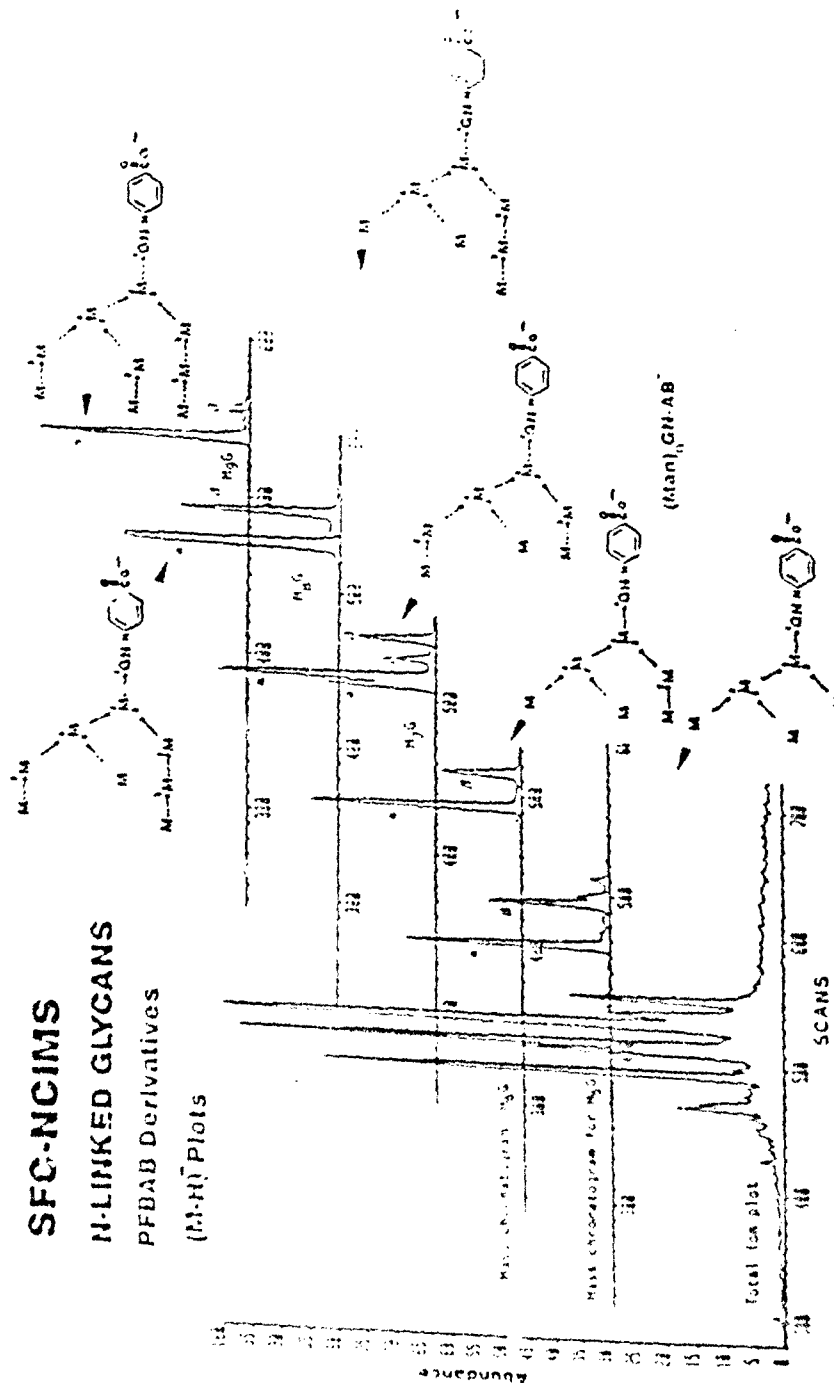


Figure 4: SFC-MS of PFBAB-labeled highly branched glycans obtained from plant lectin by endoglycosidase treatment. Foreground, total ion plot with mass chromatograms (ion plots) offset to the right for selected components in the complex mixture. M = mannose, G = glucosamine. Note resolution of branched isomeric doublet at M₇G.

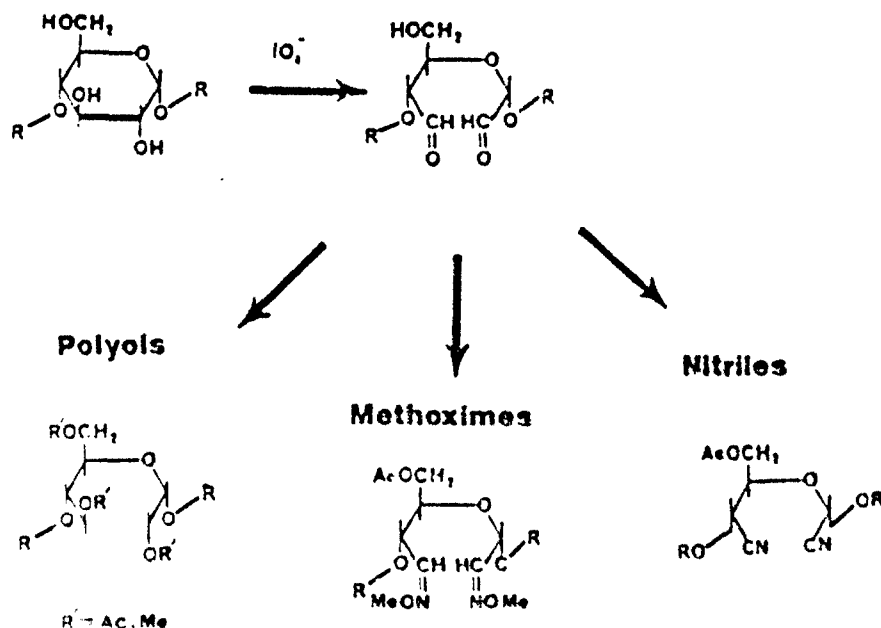
is adequate for ester rupture only and not dispersed into alternative fragmentation pathways. Thirdly, is the unique stability of the carboxylate anion. These factors combine to provide excellent detecting sensitivity.

B. LINKAGE, SEQUENCE & BRANCHING

The procedures described above, (which were developed to maximize sensitivity), provide a molecular weight analysis only with little structural detail. In fact, the soft ionization of SFC-NCI is a major factor that contributes to this excellent sensitivity. Sequence, linkage and branching detail, however, cannot be obtained from this analysis. Classically, this problem is approached by sample permethylation, hydrolysis and acetylation with the products analyzed by gas chromatography and mass spectrometry (e.g., alditol acetate procedure). Unfortunately, this approach provides only a composition of linkage type, not linkage data in a sequential array.

a.) Periodate Oxidation

To approach the problem of determining monomer linkage, we have introduced a series of chemical steps to impart greater molecular specificity.



From these structural modifications intervening moiety linkage can be determined. The reasons for this can be explained by considering the change in the residue molecular weight upon oxidation and aldehyde derivatization, Scheme 1. Inter-residue linkages that leave adjacent hydroxyl groups are subject to oxidation, thus.

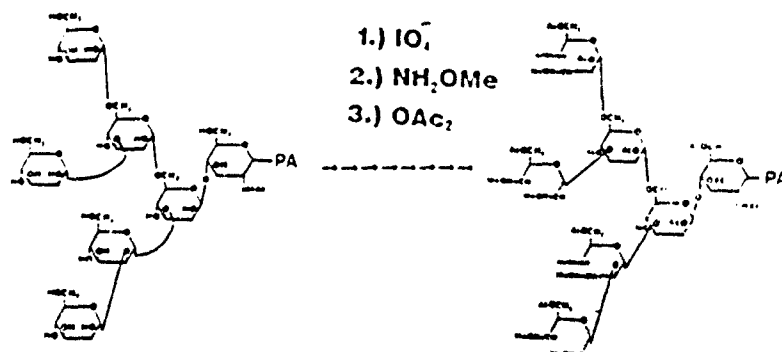
periodate oxidation of 2-, 4-, and 6-O-linked moieties, (3-O-linked unaffected) result in different structural products which contribute variations in structure and changes in glycan molecular weight. Periodate oxidation, reduction and permethylation has been utilized by Nilsson, et al., (17) for samples studied by FAB-MS. For the aldehyde derivatization step, we have investigated several derivatives to capture the products of periodate oxidation. At present, the chemistry appears to be more quantitative and the fragmentation following CID, (see below), more structurally informative when using the O-methyl oxime derivative. In addition to providing greater insight on linkage, there is information on furan/pyran structure. From the summation of residue molecular weight changes, a linkage composition analysis can be ascribed. The experimental conditions of oxidation and aldehyde derivatization has been established with di- and trisaccharides of different linkage type and more recently with highly branched N-linked glycans and oligosaccharides.

Fast atom bombardment mass spectrometry has been utilized to follow the chemistry. Presented in Figure 5 is the FABMS of a highly branched oligosaccharide periodate oxidized and conjugated with methoxamine. The spectral and molecular weight information provided by this analysis indicates the chemistry to be correct and quantitative. We have investigated other complex PFBAB-labeled oligosaccharides by SFC-NCI-MS with comparable results, Fig. 6. The application of this methodology to the O-antigen obtained from Coxiella burnetii will be a further consideration following installation of a new triple quadrupole instrument in May. This will allow MS/MS techniques as described below.

b.) Collision Induced Dissociation

An important follow-up of this chemical strategy would be to capture the inner-residue molecular weight changes in addition to the total molecular weights obtained by FAB. This can be done by collision induced dissociation (CID). We have previously reported on the structural determination of the O-antigen from Coxiella burnetii using CID. This technique allows the selection of parent ions and focuses these molecular weight-related materials into a collision cell that induces fragmentation at glycosidic linkages yielding oligomer sequence and branching. Combining CID with the periodate chemistry described above would provide for the first time an inner residue linkage assessment for oligosaccharides. As a summation of the chemical and instrumental techniques introduced in this Midterm Report, we would wish to indicate their potential significance when applied to a problem; the structural characterization of two highly branched oligosaccharide isomers, Man₇GN-PFBAB, Fig. 7. Although we have already resolved these structures by conventional methods, (using large amounts of material), and several weeks; it may now be possible to arrive at the same structural result with picogram amounts of sample and a few hours. The Figure illustrates the expected MS-CID spectra, that could be expected from each isomer following PFBAB-derivatization, periodate oxidation, aldehyde trapping and acetylation, (in this example we considered the sample to be prepared as the nitrile instead of the O-methyloxime....this would change the masses, but the relative shifts would be unchanged). The fragments speculated are limited to only those of labile glycosidic cleavages,

N-Linked Glycan



(Man)₆ GN-PA

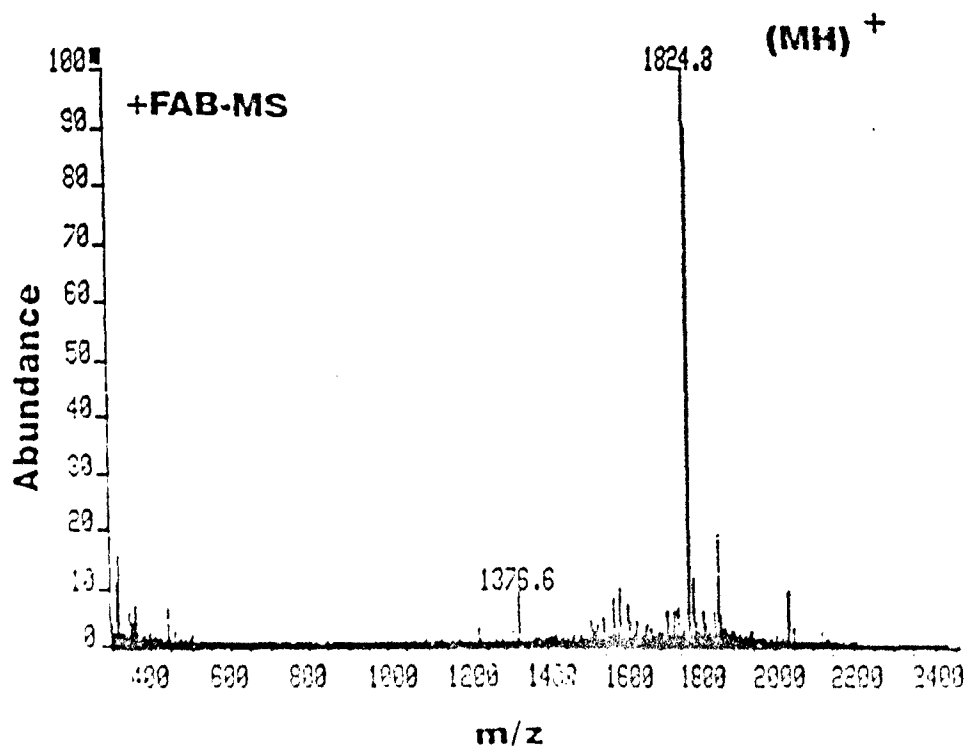


Figure 5: Positive ion fast atom bombardment mass spectrum of highly branched Man_6GN sample labeled with pyridinylamine. Previous to FABMS analysis sample was prepared for sequence, linkage and branching studies by periodate oxidized, methoximation, and acetate derivatization.

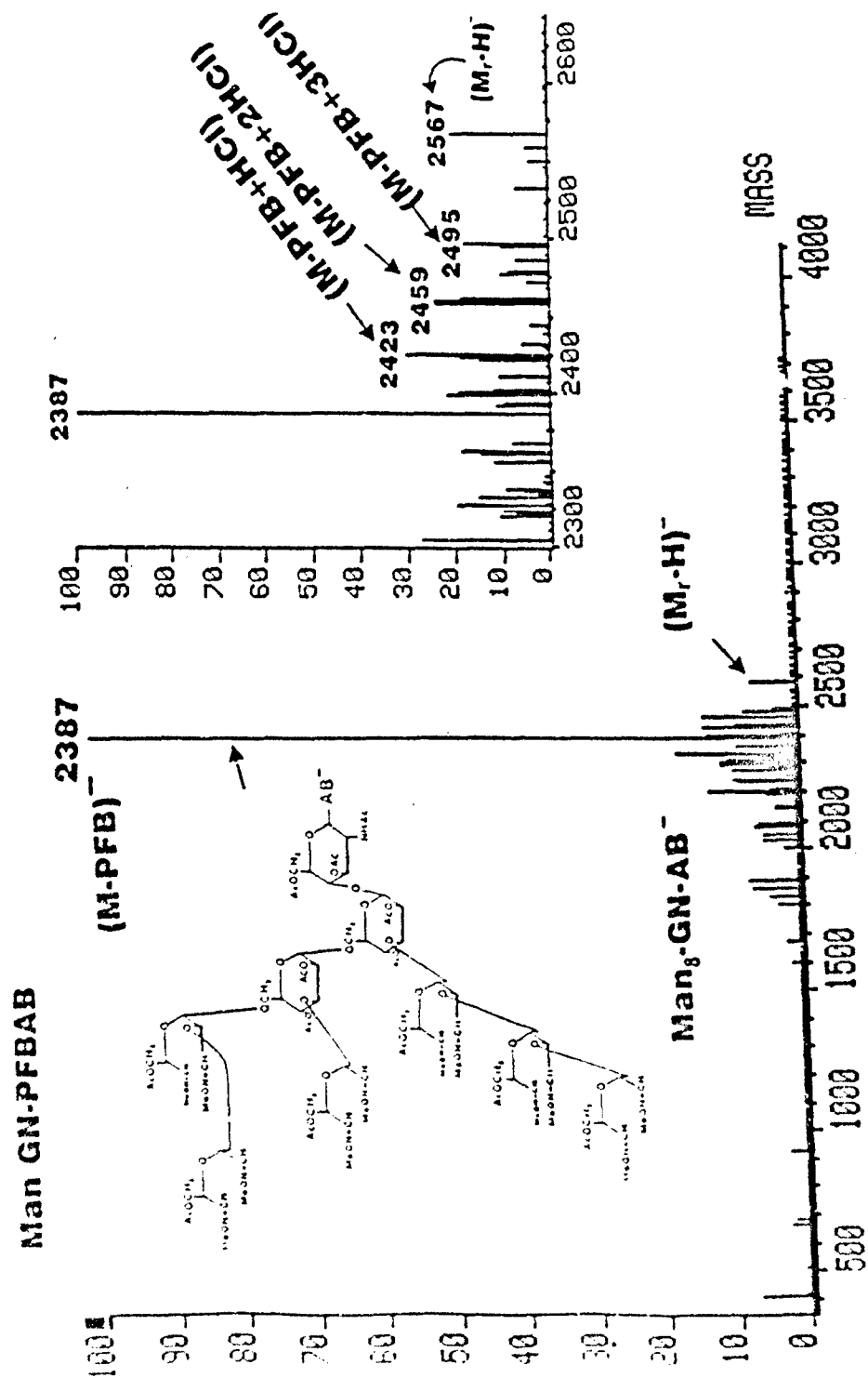


Figure 6: SFC negative ion chemical ionization mass spectrum (SFC-NCI-MS) of PFBAB labeled MangN isolated from plant lectin prepared for linkage, sequence, and branching studies by periodate oxidation and derivatization.

POLYNITRILE GLYCOSIDES MS-CID-MS

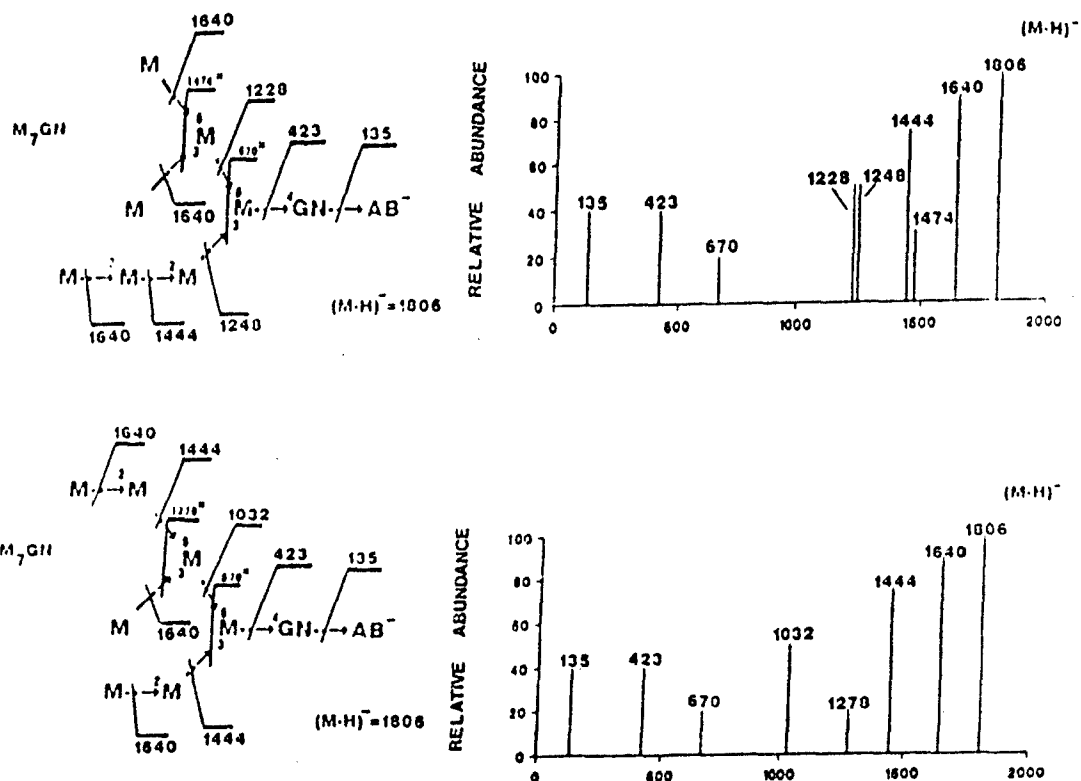


Figure 7: Expected results of combined SFC-NCI-MS-CID-MS analysis of complex branched glycan. Sample prepared as nitrile derivative. CID provides monomer mass intervals for sequence, periodate oxidation yields linkage and branching, and (M+H)⁺ ion yields M_r and supporting linkage and branching information.

and although it is easy to see spectral differences there is a wealth of information in each of the fragments indicating linkage, furan-pyran relationships and branching. Thus, these techniques introduce the possibility of glycan sequencing at enhanced sensitivity using periodate oxidation, reagent-labeling, and instrumental analysis by SFC-NCI and MS-CID.

C. ENDOTOXIN CHARACTERIZATION BY SFC

The characterization of lipid-A materials from Gram negative bacteria has, over the years, been a most demanding and challenging analytical problem. We introduced SFC to assist in the study of Coxiella burnetii LPS fractions, and with known glycolipid and oligosaccharide samples, developed separation parameters and a mass spectrometer interface for product characterization. The direct application of this instrumentation to Coxiella burnetii LPS has been thwarted by the earlier difficulties of partial degradation to release sufficient amounts of material for study. The value and power of this analytical approach is directly demonstrated in this section with the multi-component mixture obtained from Salmonella minnesota LPS. We have separated by TLC, extracted, and studied by FAB-MS all the entities comprising this lipid-A sample (18). This preliminary effort has aided in the development of chromatographic parameters to separate this complex mixture by SFC. Presented in Figure 8 is an SFC chromatogram of the lipid-A components detected in the partial hydrolysate of LPS obtained from Salmonella minnesota R595. The identification of these SFC peaks relates to our earlier study by TLC and FAB-MS. We will complete these studies by direct SFC-MS and SFC-MS-CID-MS with the new triple quadrupole.

III. COXIELLA BURNETII LPS CHARACTERIZATION:

A. EXPERIMENTAL, (unpublished results)

a.) Mass Spectrometry

Fast atom bombardment mass spectrometry (FABMS) was performed on a VG ZAB-SE instrument (VG Analytical, Manchester, UK) operated at 8 and 10 kV in the negative and positive modes, respectively. The Ion Tech gun was operated at 8 kV with 1 mA of current at the cathode; xenon was used as the FAB gas. Current controlled scans were acquired at a rate of 30 sec/decade, and 3-5 scans were summed as continuous data before converting to bar plots using the VG 11-250 data system. The resolution was typically 1:2000; CsI clusters were used for calibration. For positive FABMS, the matrix was a mixture of 1:1 meta-nitrobenzyl alcohol (MNBA) and thioglycerol, and for negative FABMS, 1:1 MNBA and triethanolamine was used. Direct chemical ionization mass spectrometry (DCIMS) was performed by depositing sample on a platinum wire, and heated at a rate of 16 mA/sec in an ammonia plasma with a source housing pressure of 1×10^{-4} mbar. Mass analyzed ion kinetic energy spectra (MIKES) were acquired by focusing the beam of FAB-generated ions into the collision cell in the second field-free region, and scanning the electric sector at a rate of 30 sec/scan. Helium was admitted into the collision cell at a pressure sufficient to attenuate the precursor beam to 30-50%.

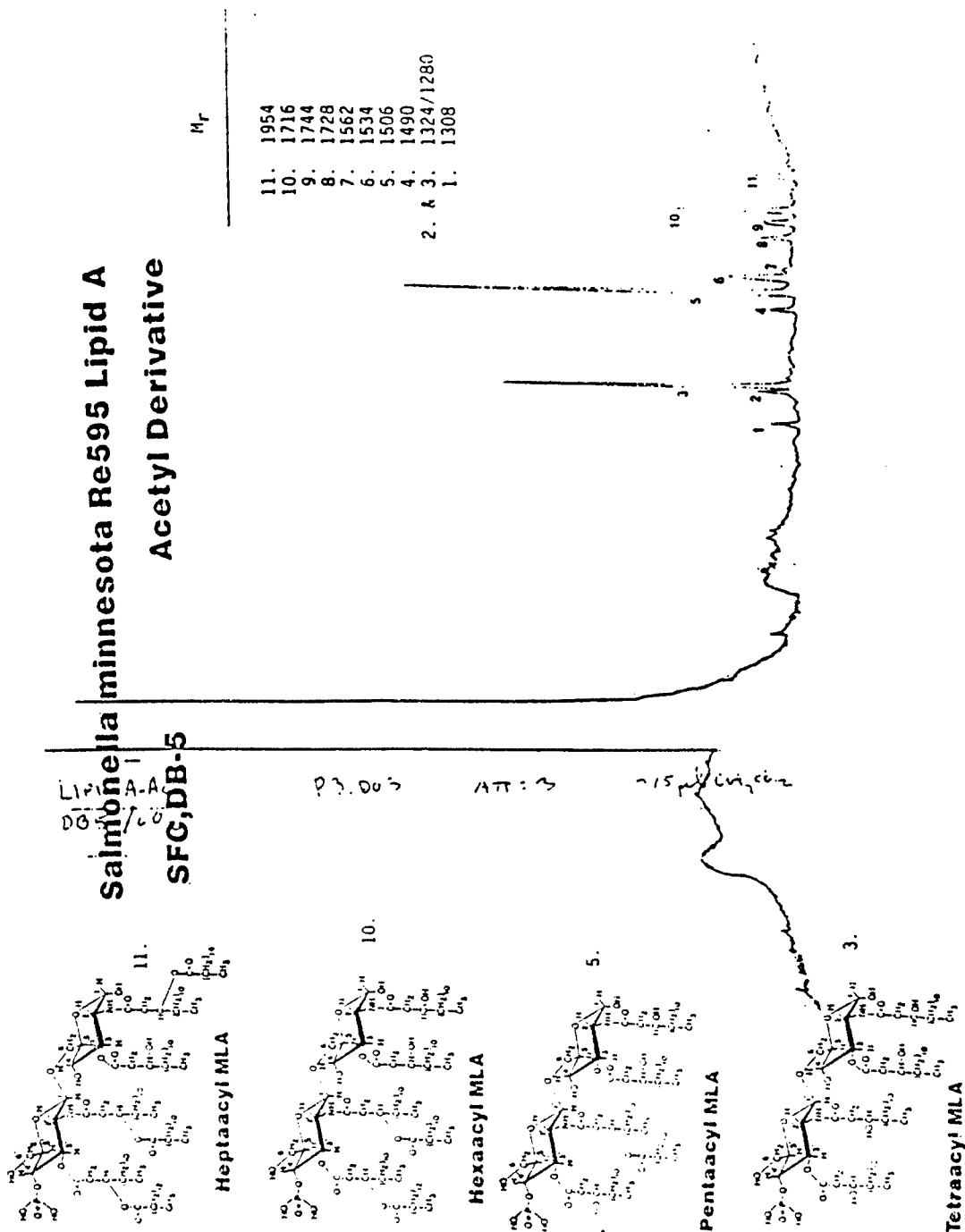


Figure 8: SFC of Lipid-A obtained from *Salmonella minnesota* Re595 sample prepared by acetylation. Cyanopropyl column with FID detection. Structure and peak correlation by isolated TLC fractions only; SFC-MS studies expected to be completed soon.

b.) Hydrolysis and Methanolysis of Lipopolysaccharides

Hydrolyses of the lipopolysaccharides (LPS) were carried out in 1:1 water:methanol (1-2 mg/ml) at HCl concentrations varying from 0.01 to 0.65 N HCl or 1% acetic acid (vol/vol) at 100 °C for two hours. A time study was also carried out using 0.04 N HCl in 1:1 water:methanol at 100 °C for 0.5, 2, and 6 hours. The same HCl acid concentration and time studies were also performed in anhydrous methanol. Methanolysis or methanolic hydrolysis products were extracted in 1:2:4 water (or 0.1 N HCl):methanol:chloroform. There usually were insoluble particulates at the organic-aqueous interface following centrifugation. This was somewhat greater in the methanolic hydrolysis, but was decreased by slight warming of the tubes and extracting with 0.1 N HCl rather than water.

c.) Gas Phase Reduction

A thin coating of the sample was dried onto the bottom centimeter of a capillary melting-point tube, and placed in a gas phase reaction vessel (19). A series of three gas phase reactions were carried out: (i) 0.25 N BH_3 (or BD_3) in THF for 1 hr at 45 °C; (ii) 0.5 N HCl in methanol for 3 hrs at 45 °C; and (iii) 1:1 30% H_2O_2 /3 N NH_4OH for 1 hr at 45 °C. The first reaction reduces amides to amines the second step solvates any boronic esters, and the last step oxidizes any carbon-boron bonds that form if any carbon-carbon double bonds are present (20).

d.) Composition Analysis

To characterize the lipophilic anchor and sugar backbone of this lipopolysaccharide, 5 mg of strain 9MIC7 LPS from *C. burnetii* was subjected to a 0.65 N HCl anhydrous methanolysis (1.25 mg LPS/ml) for 2 hours at 100 °C. The product was dried and extracted four times using 0.1 N HCl:methanol:chloroform 1:2:4, (discarding the top, aqueous layer each time), to insure no contamination from the core or O-antigen regions. To hydrolyze all of the amide bonds, this organic phase was dried and dissolved in 20 μl of THF, suspended in 2 ml of 6 N HCl and heated for 18 hrs at 100 °C. The hydrolysis products were dried, extracted two times with chloroform:0.1 N HCl, 1:1, and the aqueous fraction back extracted twice to remove trace residues of fatty acids. The components in the aqueous fraction were characterized using GLC as the N-acetyl, O-silyl derivative. These derivatives were prepared by dissolving the dried residue, obtained from acid hydrolysis, in 100 μl methanol, 50 μl pyridine, and 50 μl acetic anhydride and holding for 30 min at room temperature. The dried N-acetylated material was silylated in 2:1 pyridine:99% BSTFA + 1% TMCS at room temperature for 45 min. The products were separated on a SE-54 column (Alltech Econocap) using a HP 5890A gas chromatograph. The chromatogram showed three peaks that eluted between 265-275 °C, which were shown to be due to 2,3-diamino-2,3-dideoxy glucose by coinjection with a synthetic standard, (United States Biochemical Corp., Cleveland, OH).

Composition analysis was performed on the intact phase II LPS before and after dephosphorylation with HF (21). Methanolysis (0.65 N HCl) was carried out at 60 °C for 2 hrs, which is optimal for KDO analysis (22); samples were N-acetylated and silylated as described earlier.

e.) Acetylation and De-O-Acylation

Methanolic and methanolic hydrolysis products were peracetylated using 1:1 pyridine:acetic anhydride for 16 hrs at room temperature. Two methods of de-O-acylation were used - triethylamine (10 μ l) in water (300 μ l) at 100 °C for 15 min, or a saturated ammonia/anhydrous methanol solution at 65 °C for 16 hrs.

f.) Methylation, Diazomethylation, Deuteromethylation

Permethylation was carried out using the NaOH/DMSO method (23). Methylation with diazomethane and deuterodiazomethane was performed by first dissolving the sample in 100 μ l of THF and then adding an ether solution of the reagent until the yellow color appeared indicating completion of the reaction. Deuteromethylation was carried out using a deuterodiazomethane generating kit (Diazald, Aldrich Chem. Co., Milwaukee, WI).

B. Results

Isolated *Coxiella burnetii* LPS samples, hydrolyzed in 1-2% acetic acid or 0.1 N HCl to free lipid-A moieties, always resulted in large insoluble residues which appeared as a flocculate at aqueous-organic interfaces. Organic phase extracts of these hydrolysates, and mass spectrometric analysis using fast atom bombardment ionization, gave no useful fragments for structural characterization. However, when these fractions were highly concentrated and ionized by positive ion direct chemical ionization (DCI, methane as the reagent gas), major ions and fragments were detected at 14 mass unit intervals, which suggested the first clues to a lipid-A moiety, m/z 640, 658, and 672. No higher mass ions were detected, even at shorter hydrolysis time. This difference in ionization is significant in that the DCI approach is initiated by probe heating, suggesting that the major ions could be products of pyrolysis rather than a consequence of sample hydrolysis. The 14 mass unit interval was also troublesome because such alkane heterogeneity is not observed with lipid-A from other species. Within a single bacterial species, there are, however, different molecular entities that seem to serve equally as an LPS anchor, and this heterogeneity can usually be attributed to the presence or absence of specific acyl groups (24-27). Most important however is the observation that fatty acids methyl esters derived from *Coxiella burnetii* lipid-A fractions yield a distribution of C₁₄, C₁₅, C₁₆, & C₁₇ branched hydroxy fatty acids (33) and this ion distribution is comparable with these higher molecular weight samples.

The poor yields of this suspected lipid-A material focused our attention to alternative acidities and solvents for LPS degradation. The aqueous conditions currently used suggested that the classical conditions are inappropriate for *Coxiella burnetii*. It was soon observed that more lipophilic solvents greatly increased the abundance of the 658 ion. When these conditions are applied to the LPS obtained from *Coxiella burnetii*, ions at higher mass were obtained for the first time, m/z 766, 1405 and 2079 (Fig. 9a). At higher acid concentrations (1.5 N HCl), the ion at m/z 751 is predominant while lower concentrations favors the m/z 1405 ion, (Fig. 9b & 9c). Consistent with these differences, it has been reported that higher acylated homologs of lipid-A were detected from the LPS of *Salmonella typhimurium* when hydrolysis was carried out in aqueous methanol rather

- FAB FABMS

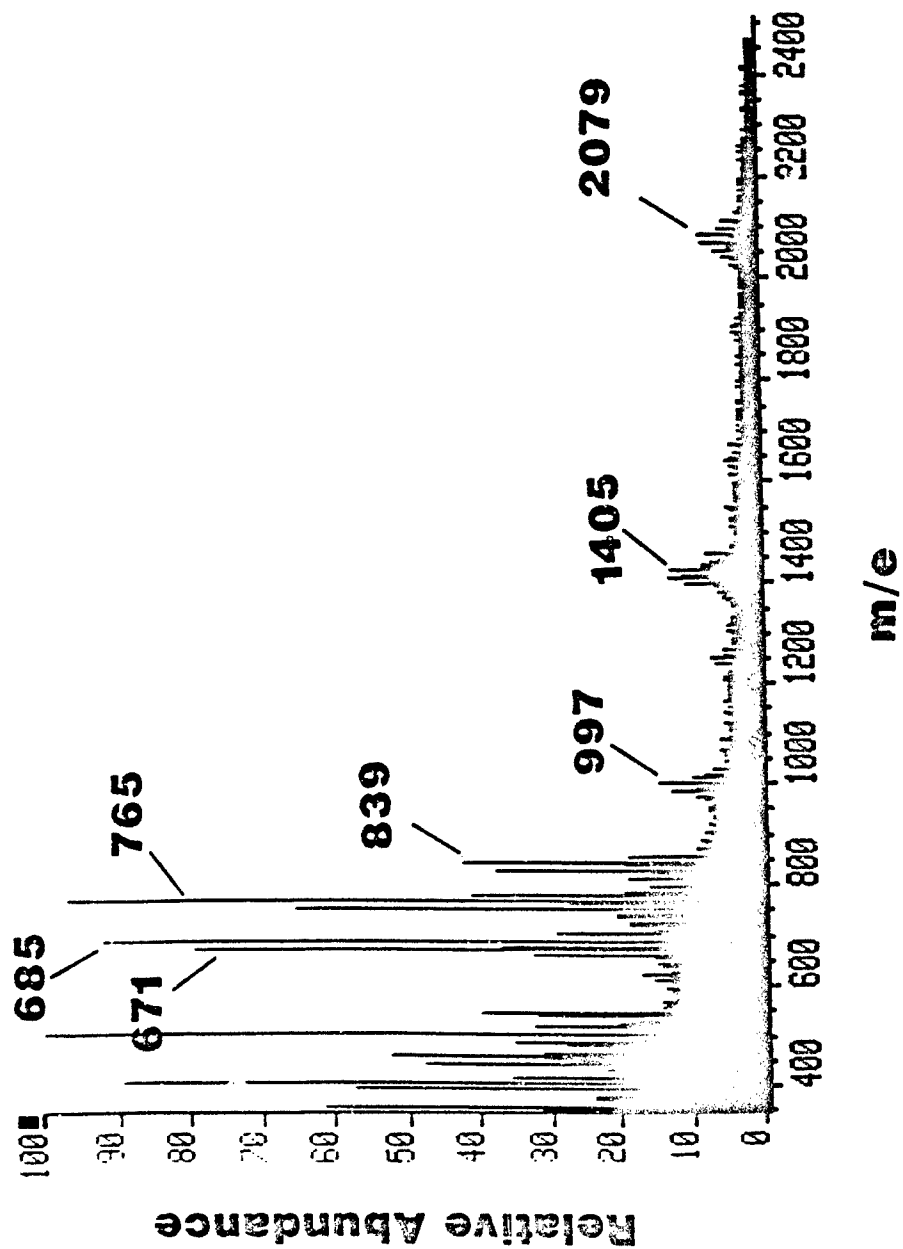


Figure 9a: Aqueous methanol hydrolysis of CB514 organic phase analyzed by negative ion FABMS.

- FABMS

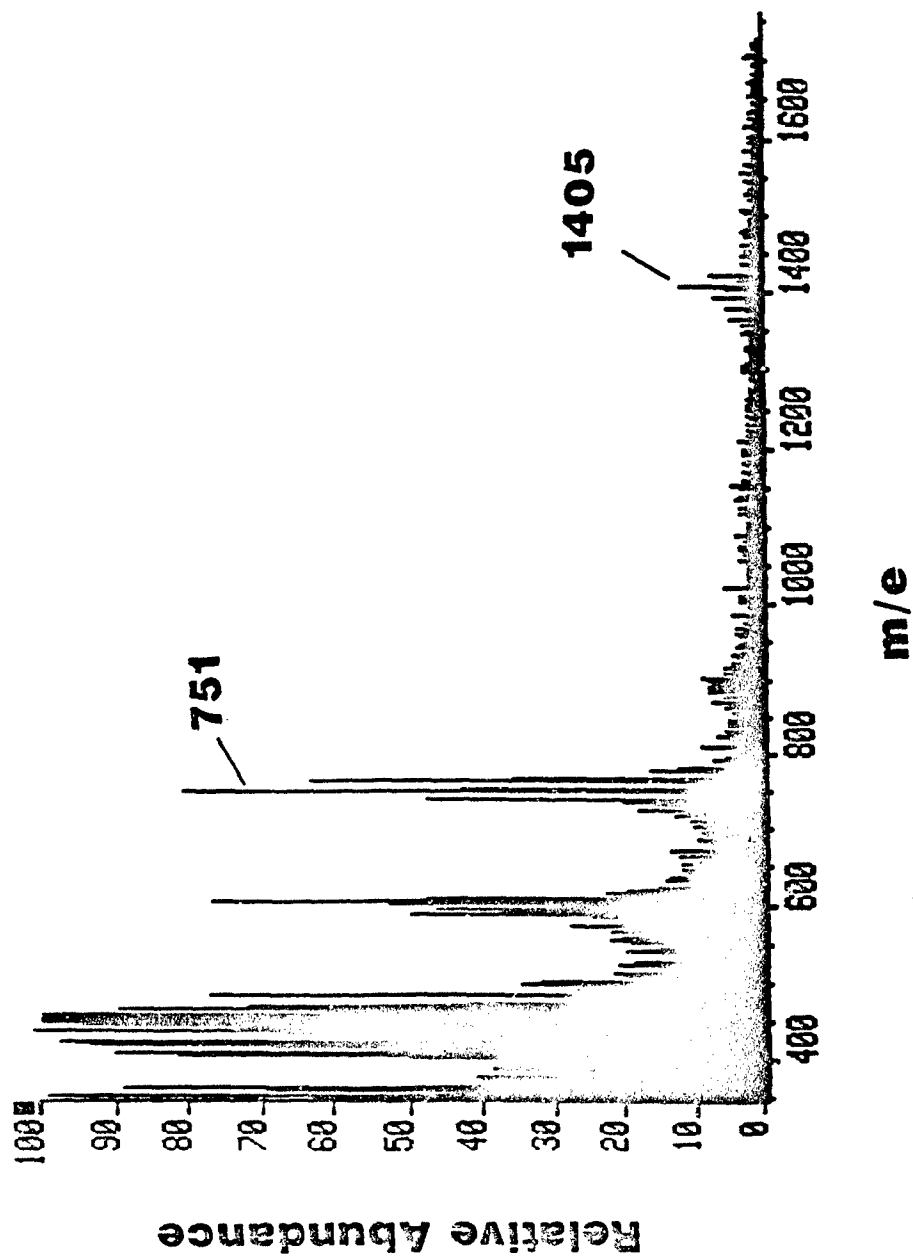


Figure 9b: Aqueous methanol hydrolysis (1.5N HCl) of CB9MIC7 organic phase analyzed by negative ion FABMS.

-FABMS

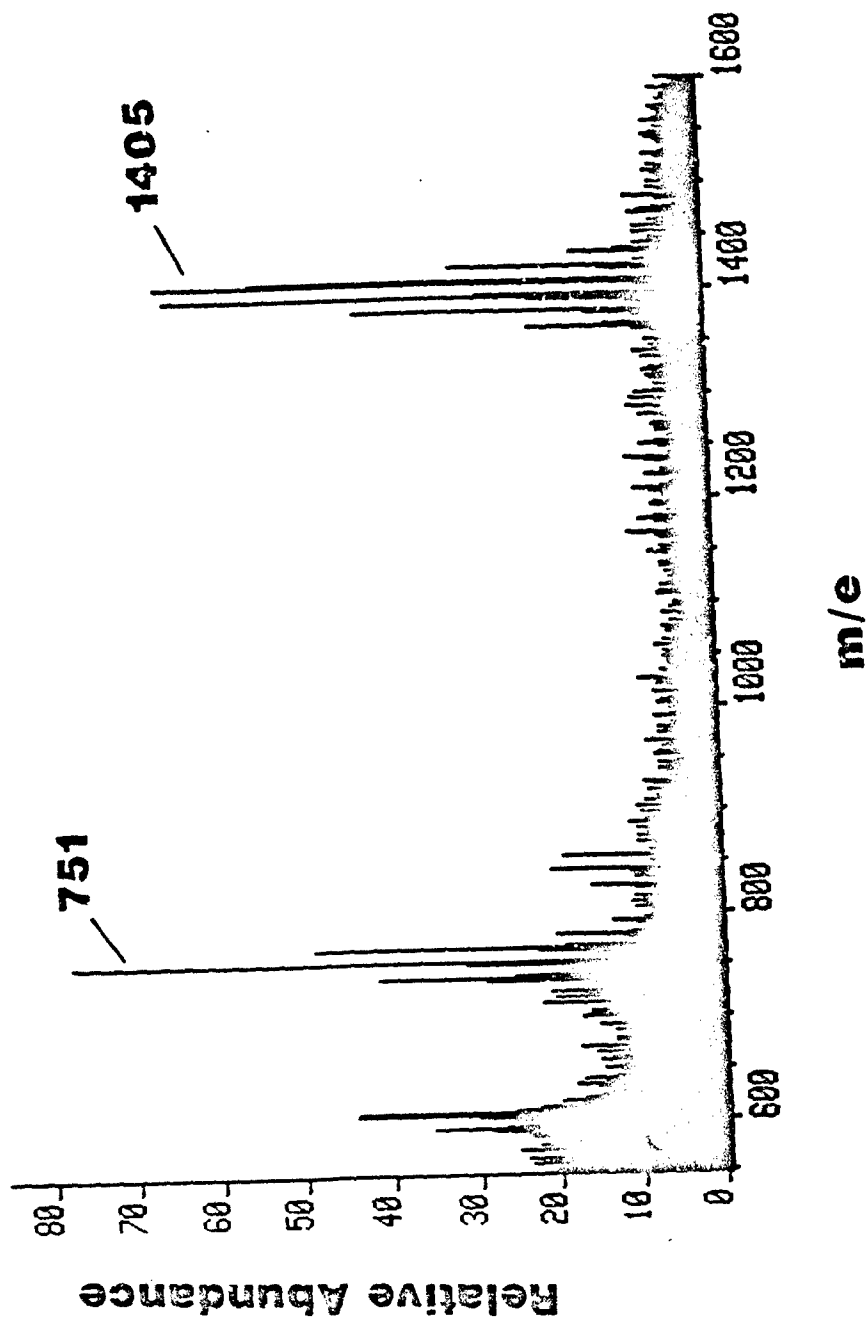
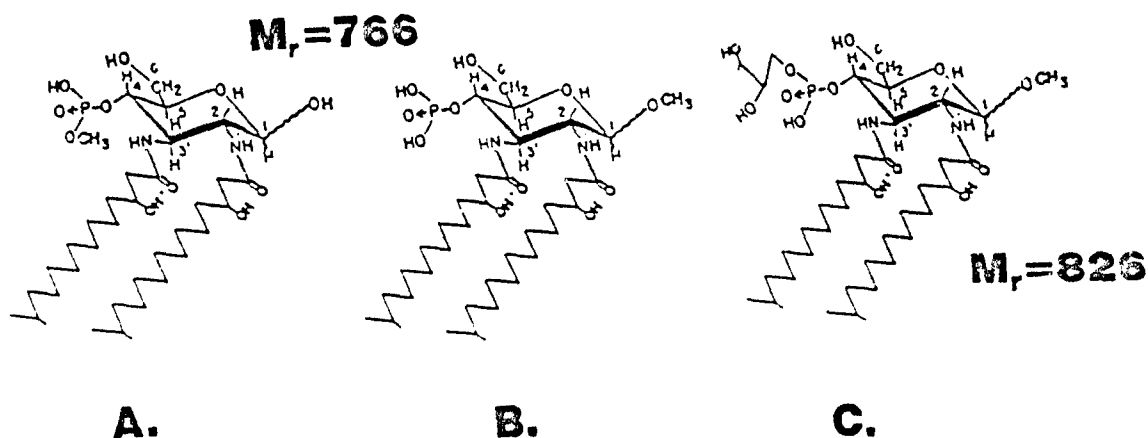


Figure 9c: Aqueous methanol hydrolysis (0.15N HCl) of CB9MIC7 organic phase analyzed by negative ion FABMS. Dimer area in high conc.

than 100% water (25).

Assuming these ions to be deprotonated molecules, $(M-H)^-$, a relationship between them can be deduced where two molecules of $M_r = 752$, minus water and a phosphate group equals the molecular weight 1406. The use of a milder acid during hydrolysis, (1% acetic acid rather HCL), resulted in an ion cluster 80 amu higher than the cluster at m/z 1405, $[(752 \times 2) - (18) = 1486]$, m/z 1485, (Fig. 10). Thus, from these data, an organic phase component appears to be a dimer associated with two phosphate groups, one somewhat more labile than the other. A decrease in the solvent polarity during hydrolysis followed by lipophilic extraction and FABMS, provided similar ion clusters extended to a tetramer region (Fig. 9a). Other than the higher masses detected, there are significant differences observed between aqueous vs nonaqueous methanolysis. These results can now be understood as a consequence of the addition of one or more methyl groups, e.g., the masses are shifted by ± 14 mass units. The shift from m/z 751 to 765 is quite apparent, and demonstrates the involvement of a hemiacetyl, carboxyl, or phosphate group, (Fig. 11). To define which of these groups are present the sample was reduced with $NaBH_4$. Analysis of these products indicated a 2 amu shift of the m/z 751 ion to 753, strongly suggesting a free reducing-end, (Fig. 12). Closer examination of this spectrum, however, shows incomplete reduction; note 2 amu lower satellite ions at m/z 751 and 765, but not for the sample lacking a phosphate group, m/z 671 and 685. This differential sensitivity to reduction may reflect isomeric structures as a consequence of methyl group location, as a methyl phosphate vs methyl glycoside. More interestingly, an associated pair of fragments incremented by 74 amu (m/z 825/839, see also Fig. 9a), also fails to shift upon reduction and is suggestive of an added glycerol moiety. This suggests that methanolysis yields three fragments; half of which are non-reducing methyl glycosides (Structure A, see below; also a small amount of a glycerol phosphate moiety, C), and the remaining half, a methyl phosphate residue that would provide a structure with a free reducing-end (A). These ion fragments can be accounted for by the following structures:



- FAB/MS

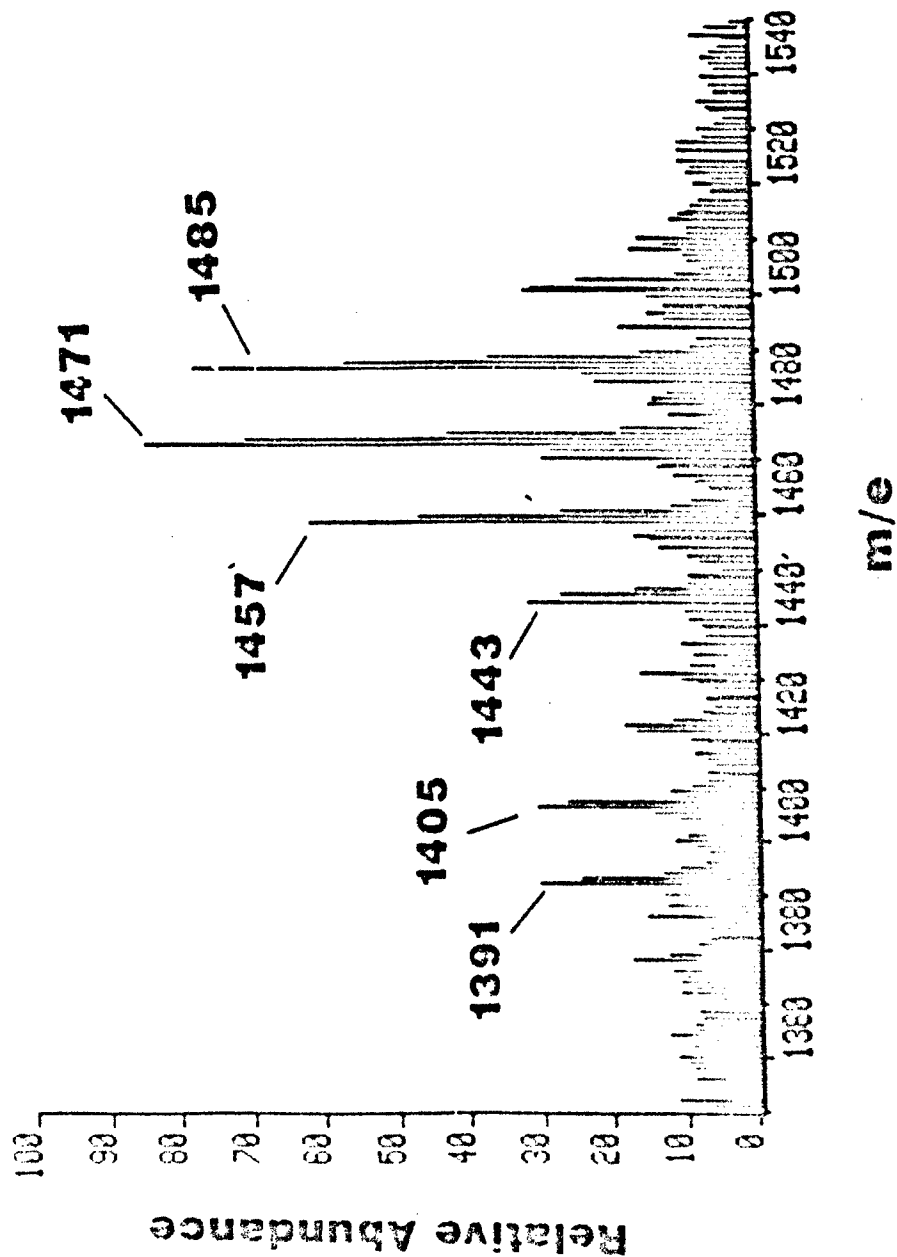


Figure 10: Methanolic hydrolysis (1% HOAc) of CB9MIC7 organic phase analyzed by negative ion FAB/MS. Added phosphoryl group.

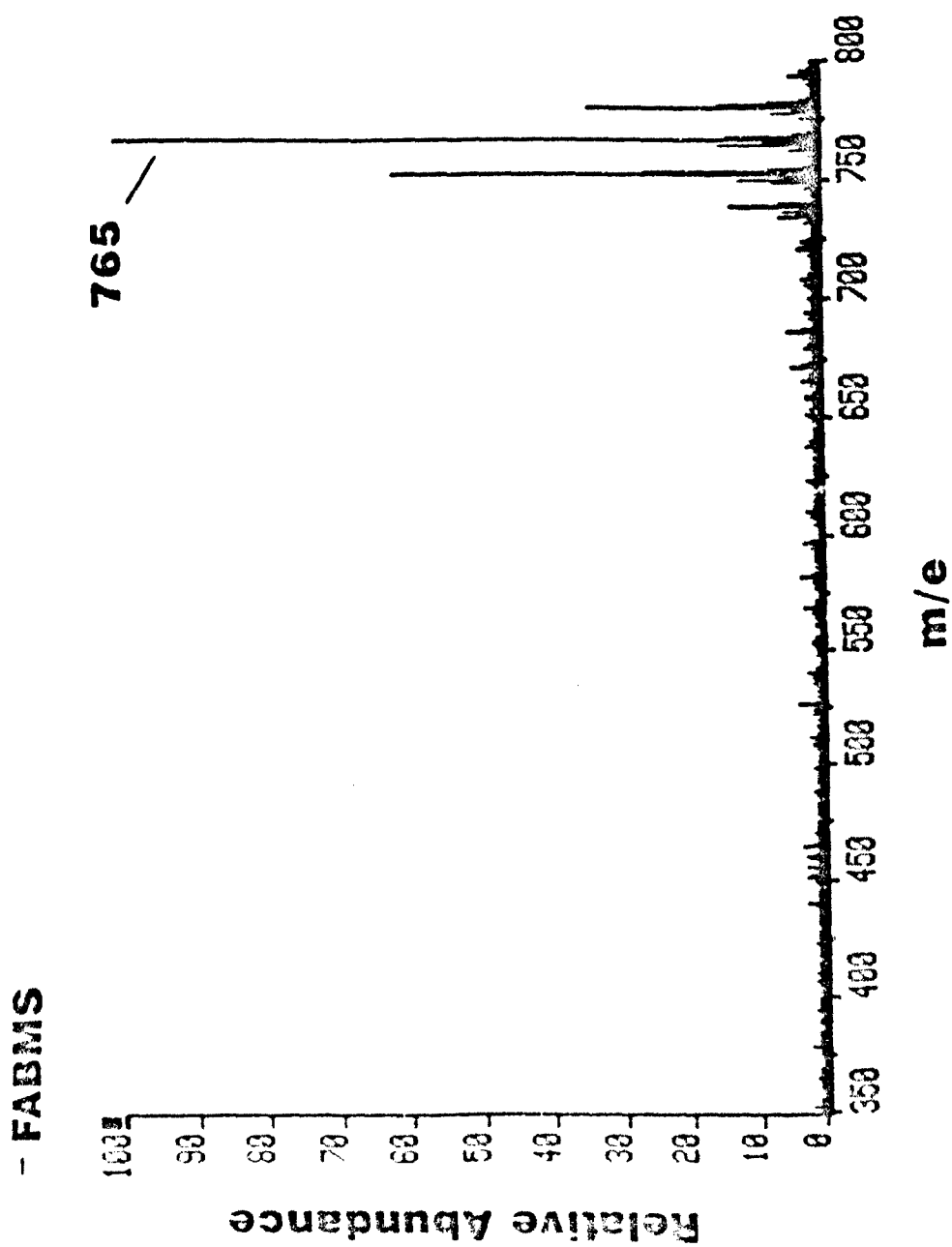


Figure 11: Negative ion FABMS of monomer prepared in 3N HCl methanol.
Two component isomeric mixture by CID, reduction, and acetylation.

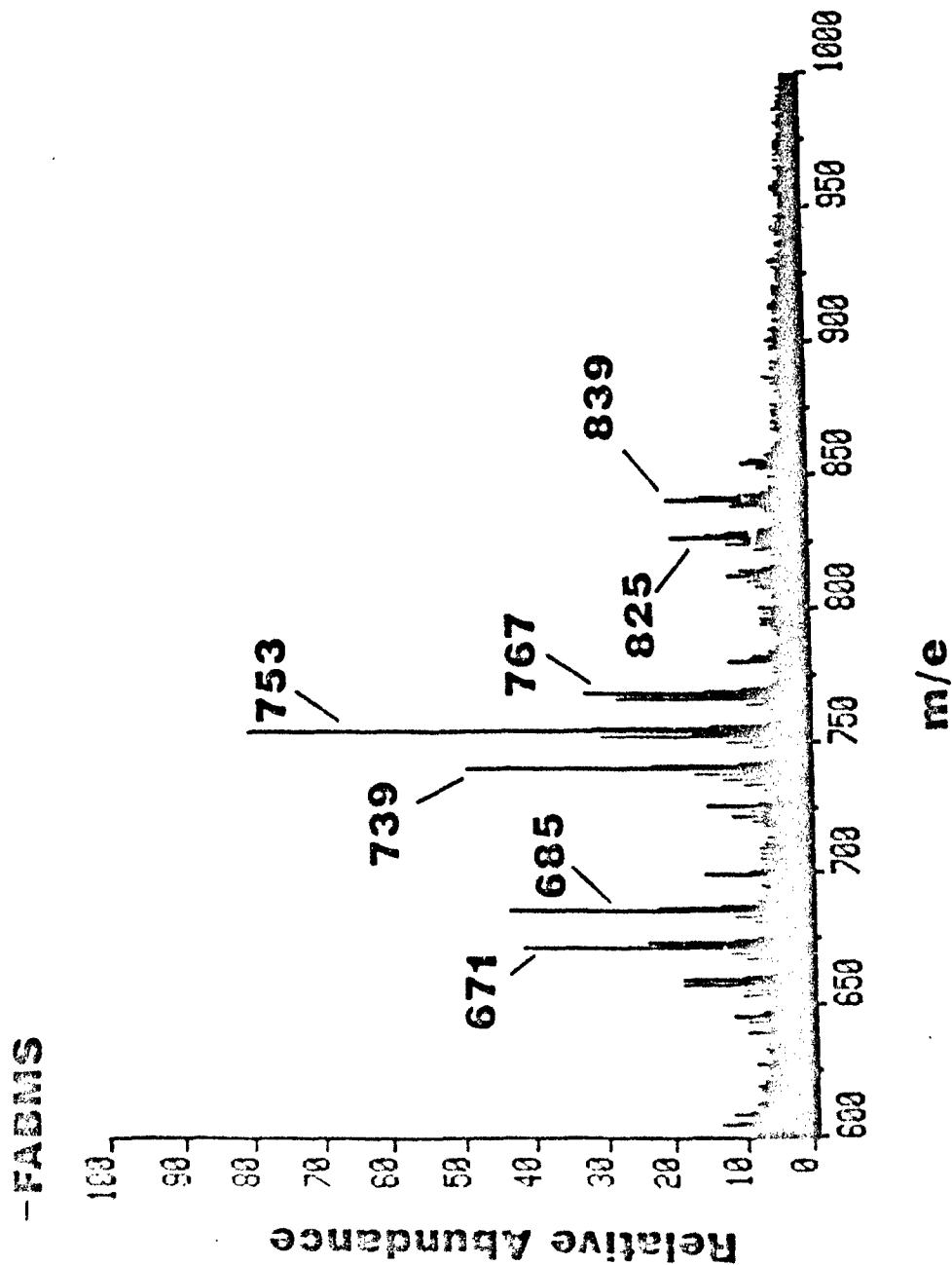
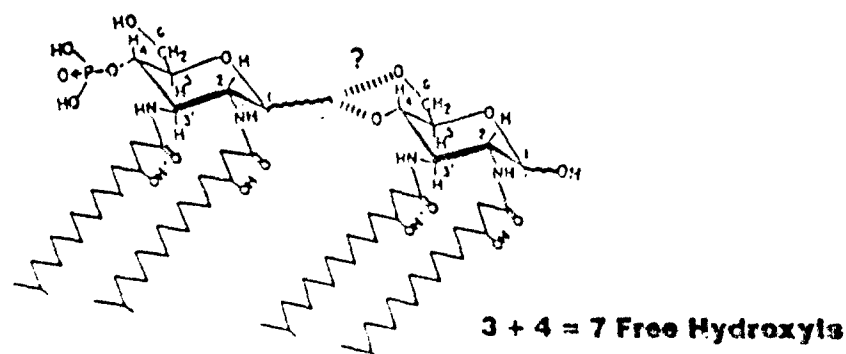


Figure 12: Negative ion FABMS of monomer and monomer phosphate prepared by reduction with NaBH_4 .

Further proof of these isomeric structures was approached by collision induced dissociation (CID) and peracetylation. In the former case ion focusing of m/z 765 and CID produced two daughter ion fragments in approximate equal abundance, (m/z 667 & 653), indicating an elimination of methyl phosphate and phosphate from the parent ion. These results corroborate the presence of the isomeric structures (A) and (B). Blocking the free hydroxyl groups and FABMS should also differentiate the isomeric m/z 765 structures by adding four (A) and three (B) groups. Peracetylation and negative ion FABMS provided the spectrum shown in Figure 13a. The mass shifts to m/z 891 and 933 represents the correct mass increments for the addition of three and four acetyl groups. The enhanced abundance for m/z 933 was not anticipated but could represent incremental acylation at the amide hydrogens, (this partial reactivity was also observed when the heptaacyl monophosphoryl lipid-A from *Salmonella minnesota* was peracetylated). The combination of CID, peracetylation, and reduction appears to support the two structures represented above.

To corroborate the number and type of carbohydrate-acyl linkages, the sample was investigated by base treatment. Two methods of basic hydrolysis were used, both known to induce complete de-O-acylate of carbohydrate samples and not effect N-acyl groups (25,29). Treatment of the peracetylated sample (Fig. 13a) with triethylamine in water, or ammonia in methanol and FABMS analysis of the products provided a spectrum identical with the starting material. This indicates that the added acetyl groups were O-linked and any other acyl groups are either amide- or ether-linked. Figure 13b shows the molecular ion region of the peracetylated methanolic hydrolysis product. The most abundant ions corresponds to the addition of seven acetyl groups, $[(7 \times 42) + 1406 = 1700]$ with a mass shift equal to two methylene residues caused by the incremental reactivity of the amide groups. This ion envelope does not show a split due to the difference in the number of acetyl groups, as was observed in Figure 13a for tri- and tetra-acetylated products. However, with such complexity it may be difficult to notice such detail. These results may be accommodated by the following structure:



This work suggested the alkane heterogeneity may be linked through acyl-amide linkages; a consideration directly investigated by methanolysis with 3N HCl, (Fig. 14). These conditions are expected to induce quantitative methyl glycoside formation and cause partial acyl-

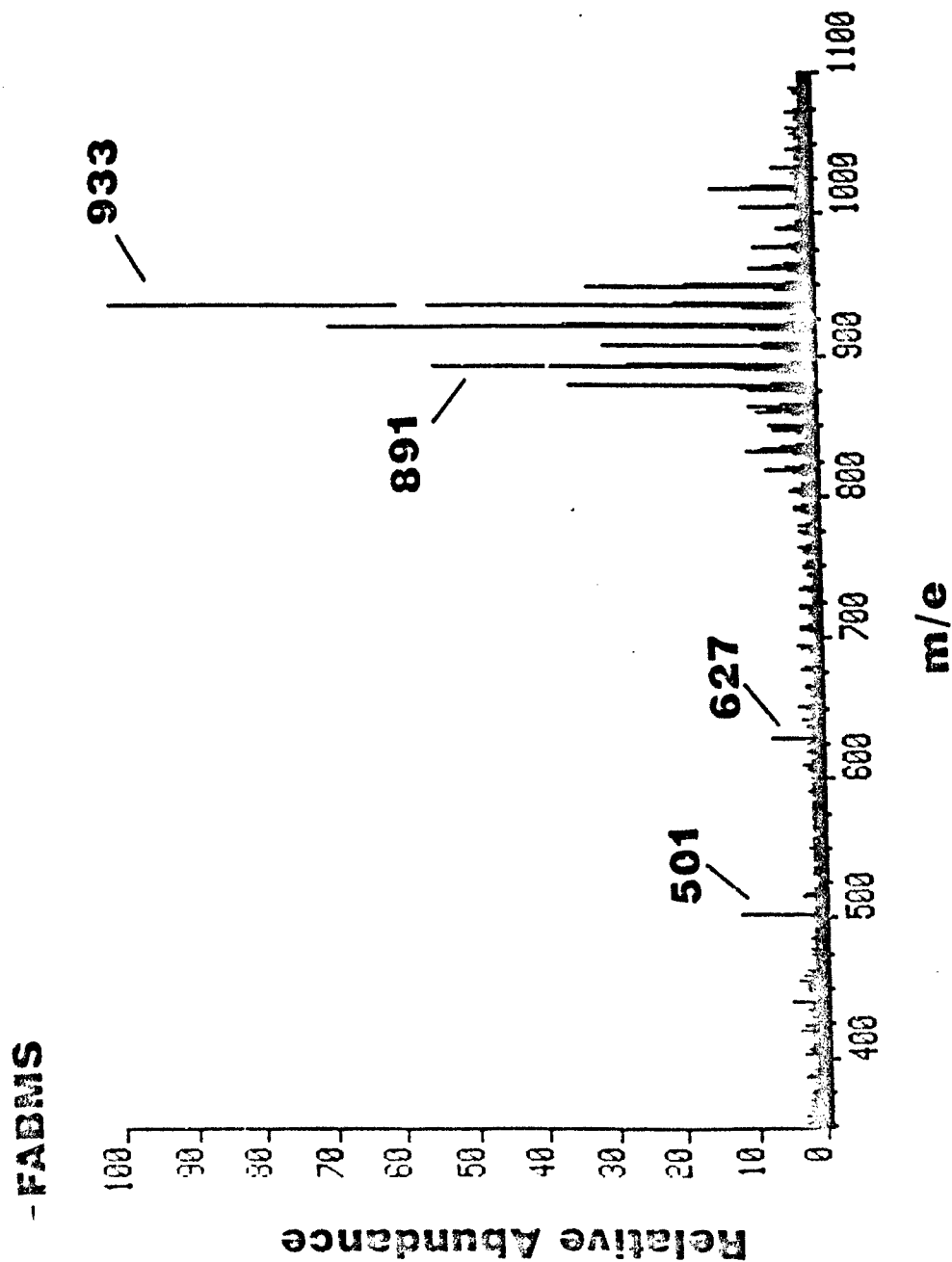


Figure 13a: Negative ion FABMS of monomer following peracetylation.
Determination of free hydroxyl groups.

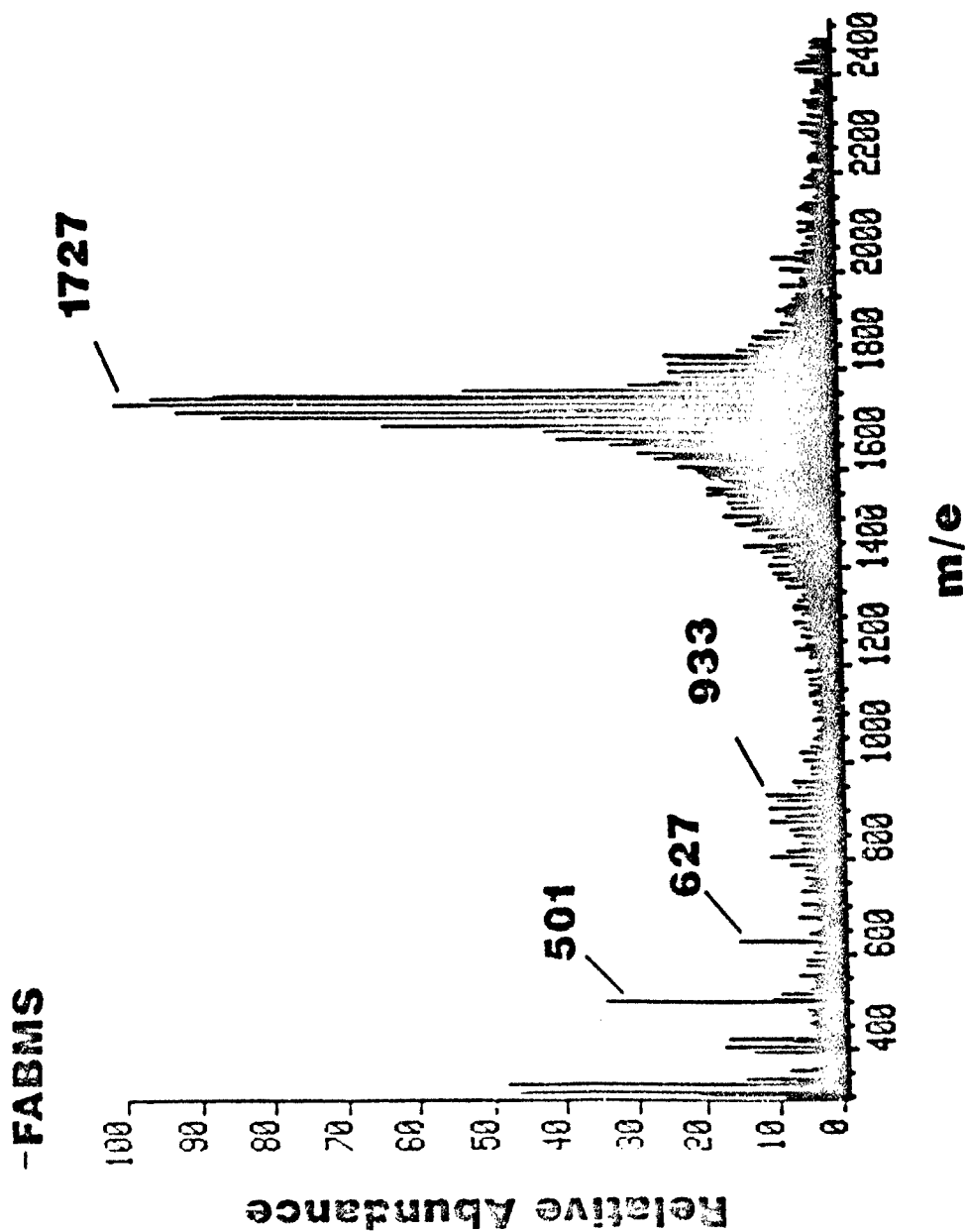


Figure 13b: Negative ion FABMS of dimer phosphate prepared by peracetylation; Determination of free hydroxyl groups.

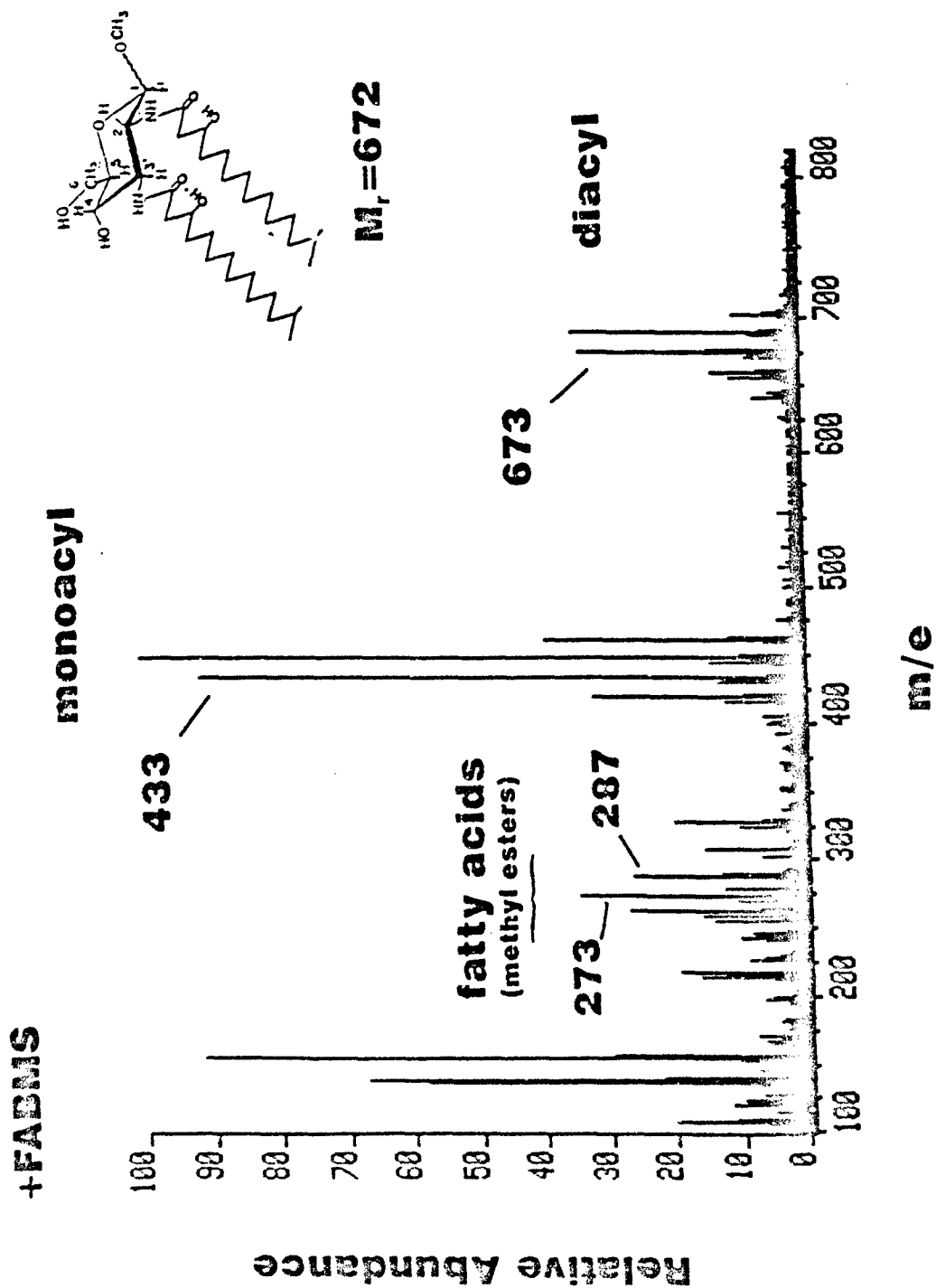


Figure 14: Positive ion FABMS of monomer following 3N HCl hydrolysis. Acyl-amide methanolysis to produce monoacyl monomer and fatty acid methyl esters.

amide degradation. As expected, the spectrum can be rationalized by considering de-N-acylation (monoacyl, m/z 433/457) and methyl esters of the released acyl groups (m/z 273/287). To further confirm the acyl-amide composition, the products m/z 657 and 737 were reduced and analyzed by +FABMS following treatment with diborane and deuterodiborane, procedures specific for amides (19,20). This procedure reduces amides to amines decreasing a compounds mass by 14 amu for each amide residue, (this procedure also hydrates carbon-carbon double bonds incrementing the weight by 18 amu). This technique was applied to the methanolysis hydrolysis products to ascertain mass shifts associated with the diacyl glycoside components, m/z 671/685, (Fig. 9a). The reduced material provided ions in the monomer area, m/z 645/659, (Fig. 15a), indicate a reduction in mass expected for the presence of two amide groups, (28 amu, incremented by 2 amu due to positive vs negative FABMS). Confirmation of this amide reactivity was supported with deuterodiborane reduction, where the same products $(M+H)^+$ appeared four mass units higher, m/z 649/663, (Fig. 15b). The methylene envelope in the $(M + H)^+$ region introduces isobaric problems (28 amu shift) and deuterodiborane was necessary for confirmation. The assignments proved to be correct for the monomer, but, four deuterium atoms were not introduced into the phosphorylated monomer (m/z 739/735, Fig. 15a) indicating incomplete reduction. Control experiments with lipid-A from *Salmonella minnesota* showed the same incomplete reduction. This lipid-A contains two amide groups, and only one reduced; thus, it would seem as though a proximal phosphate group on the terminal amino sugar somehow interferes with this reaction.

Fatty acids are not released under de-O-acylation conditions suggesting them to be either ether or amide linked. Reduction with BH_3 supports the latter possibility and the mass shift indicates two such groups. Ether linkages has been observed in archaebacterial membranes (12), but this data indicates acyl linkages to amide groups of a diamino hexose, as observed in other LPS's (27,31). Furthermore, the molecular weight of this subunit is in agreement with what would be expected given the amount and types of fatty acids previously described (32,33).

The above data has indicated two amide groups, linked with a homologous fatty acids series. Satellite clusters 80 amu above and below other prominent ion clusters suggest the presence of phosphate. Collision of the isobaric components, m/z 765, and daughter ion analysis have shown losses of 98 and 112 amu, a mass equal to phosphate and methyl phosphate, respectively. To obtain more direct evidence, samples were treated with diazomethane to methylate phosphoryl groups and the products analyzed by FABMS, (Fig. 16). Two clusters are observed, surrounding the ions m/z 687 and 795; the former cluster appears unchanged and appears at the expected mass for molecules not containing phosphate, (cf., m/z 673/685, Fig. 14). The latter group of ions shifted upwards by 28 amu, (cf., unmethylated, Fig. 11), expected for a dimethylphosphoryl ester. When the most abundant ion in this envelope, m/z 795, was mass selected and fragmented by CID an abundant fragment (daughter ion) was detected at m/z 669, Fig. 17. This can be accounted for by considering a

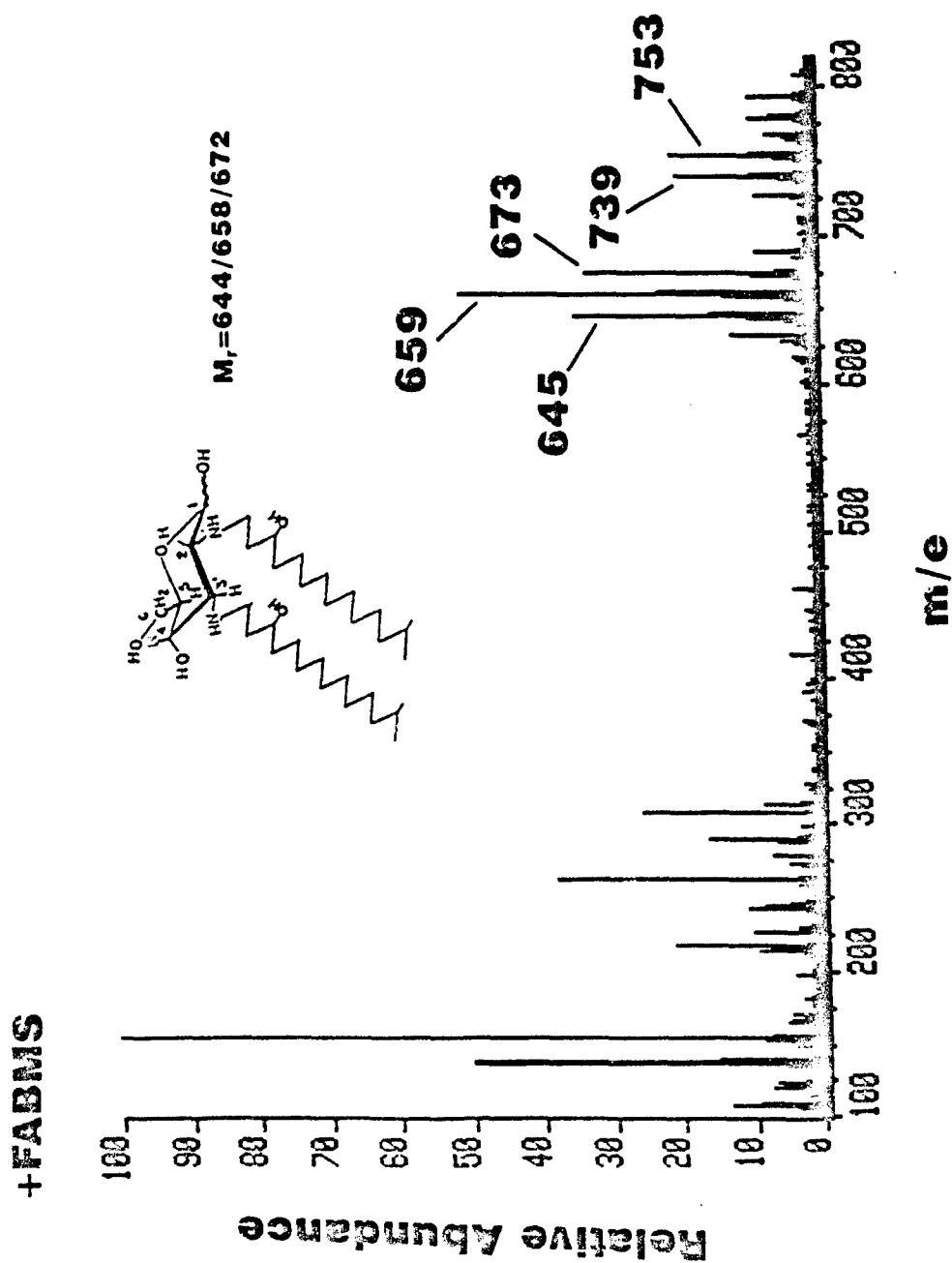


Figure 15a: Positive ion FABMS of monomer by BH_3 gas phase reduction of amides to corresponding amines.

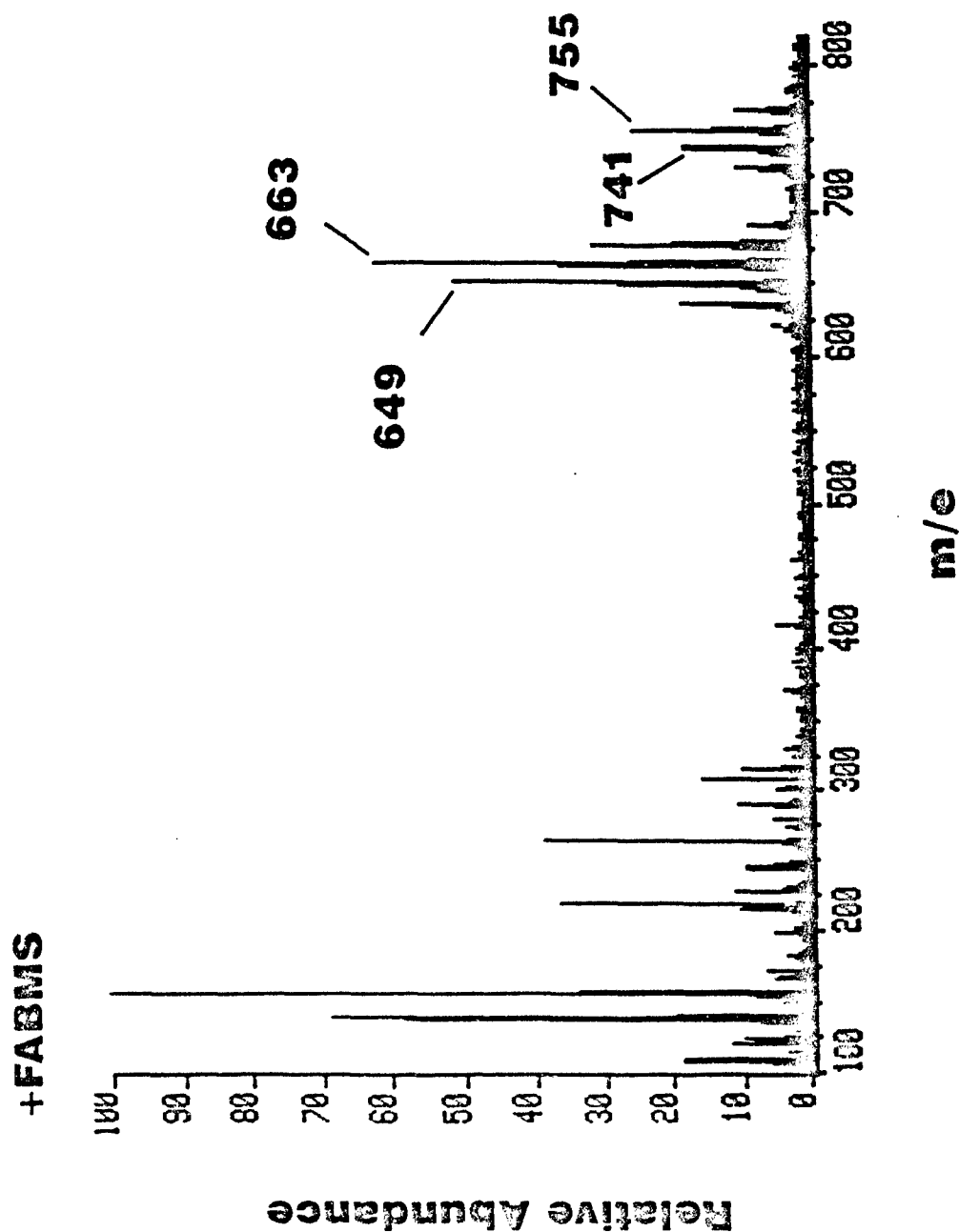


Figure 15b: Positive ion FABMS of monomer by BD₃ gas phase reduction of amides to corresponding amines.

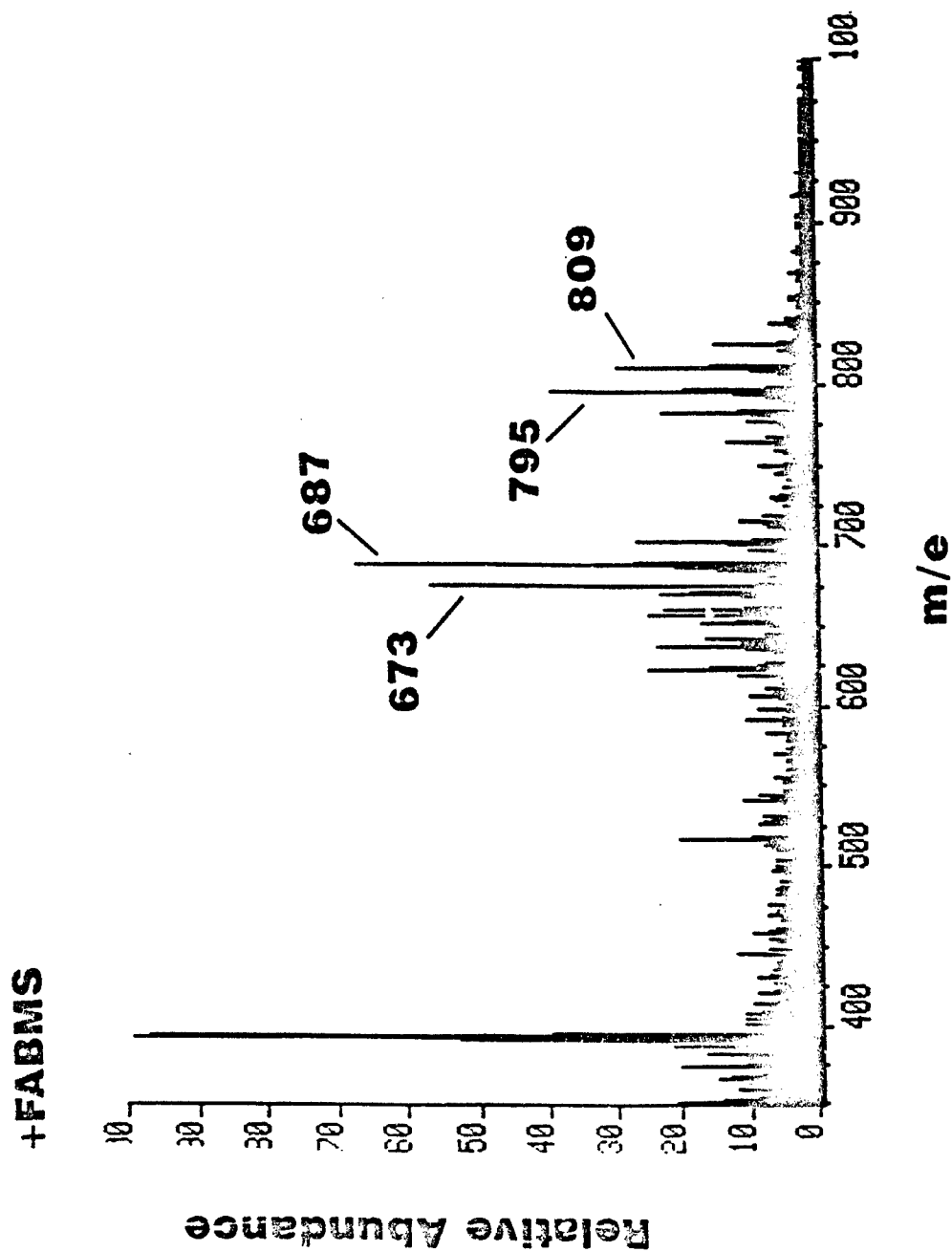


Figure 16: Positive ion FABMS of monomer and monomer phosphate following diazomethylation for phosphate methylation analysis.

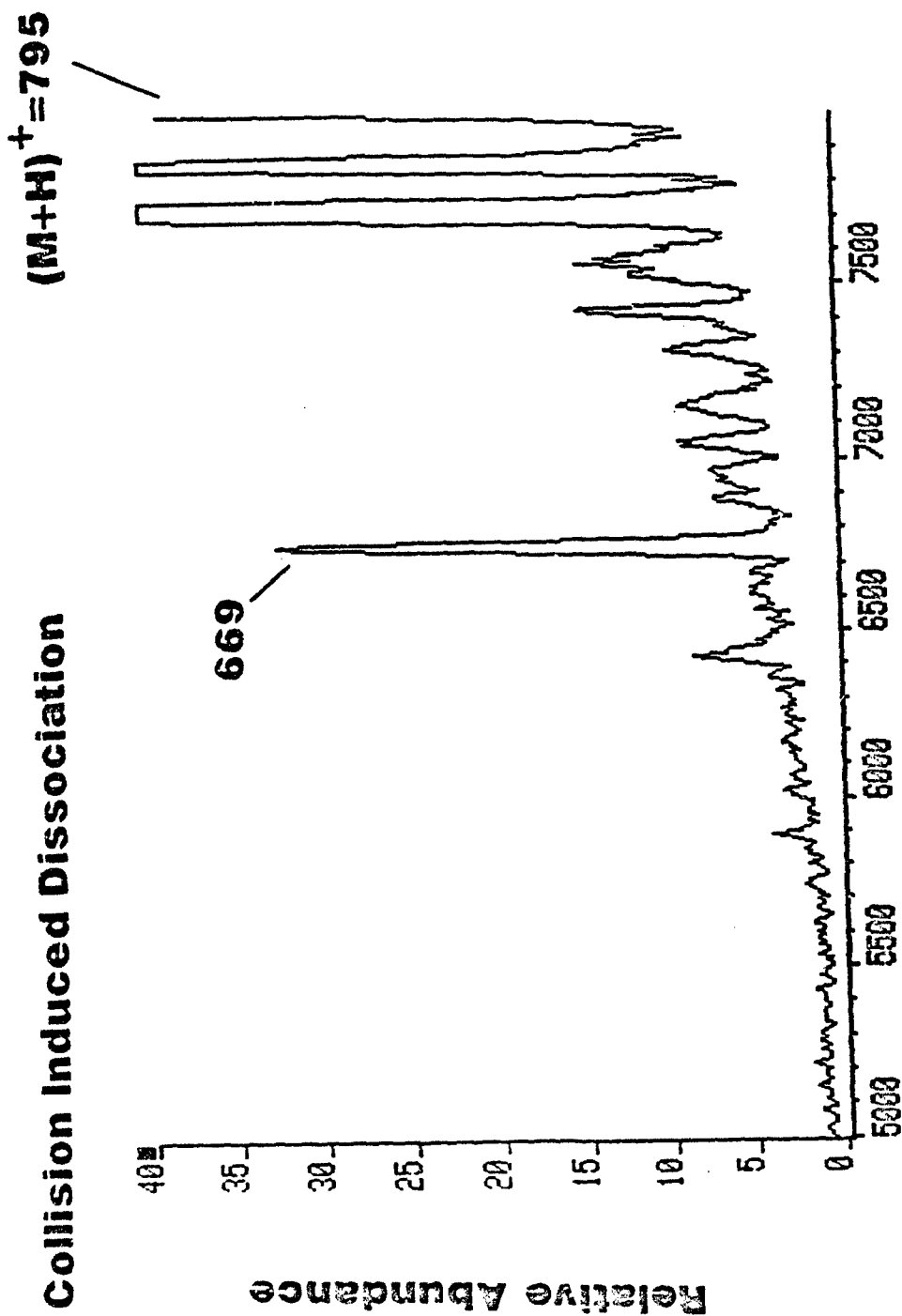


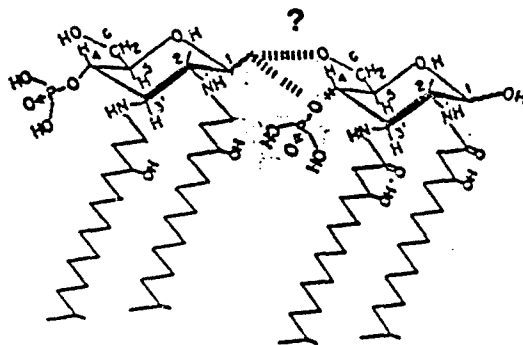
Figure 17: Collision induced dissociation spectrum of monomer phosphate indicating methyl phosphate elimination.

dimethylphosphate elimination.

Two amide groups, an aliphatic fatty acid series, and a phosphate group are components of the monomer structure. The location of the phosphate group was investigated by reduction, periodate oxidation, aldehyde trapping with methoxime and acetylation, (see Page 8, Section B. a). A methanolic hydrolysis of lipid-A was carried out to obtain higher yields of the monomer ($M_r = 752$) (Fig. 9a). This sample was reduced to the alditol with NaBH_4 and the product periodate oxidized, methoximated, and acetylated. Analysis by -FABMS provided an ion at m/z 875 (Fig. 18) which can be accounted for by considering periodate cleavage of a 5,6-diol, placing the phosphate group at position 4, (Scheme II, A = 6-O-, B = 4-O-Phosphate).

The 4-O-phosphoryl-diacyl-diamide monomer was suspected to be 2,3-diamino-2,3-dideoxy glucose, a component previously identified in other lipid-A samples (27). To check this possibility, a lipid-A fraction was extensively washed and hydrolyzed under very harsh conditions (6 N HCl at 100 °C for 18 hrs) to remove all amide-linked fatty acids. The released fatty acids were extracted and the aqueous fraction dried, N-acetylated and O-silylated and compared with a commercially available sample. The *Coxiella burnetii* derived sample was identical to that of a standard 2,3-diamino-2,3-dideoxy-D-glucose sample, as determined by gas chromatography after derivatization.

This data confirms the amino sugar present in *Coxiella burnetii* lipid-A to be a 2,3-dideoxy-2,3-diamino-D-glucose with each amino group acylated to provide a monomer molecular weight of 672 daltons. These acyl-amide residues impart an envelope of molecular ions differing by 14 amu characteristic of the aliphatic methyl ester determined earlier (33). Of the two phosphate groups, one is located at position C'-4 on the non-reducing amino sugar monomer, and the second is located on the reducing amino sugar at either position C-4 or C-6. Dimer linkage analysis has caused continuous complications and is still undergoing studies with model compounds according to the diagram presented in Scheme III. Placement of this linkage would establish, by default, the position of this second phosphate group. Consistent with other Gram-negative species, this O-antigen membrane anchor appears to be an amino sugar dimer providing a molecular weight equal to 1486 daltons. A summary of these results are presented below:



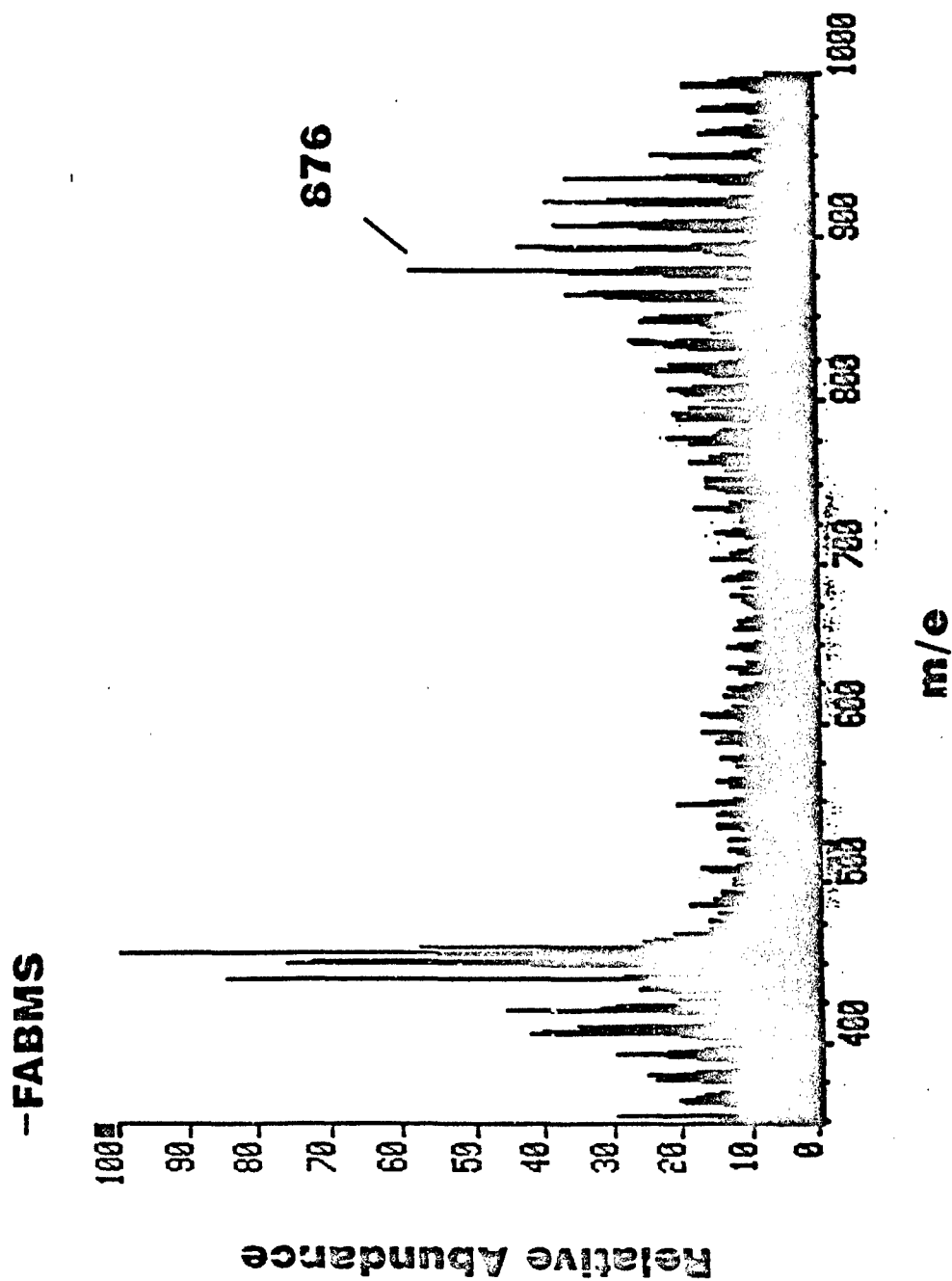
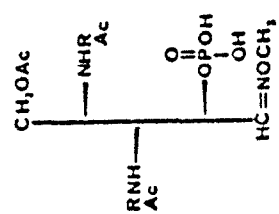
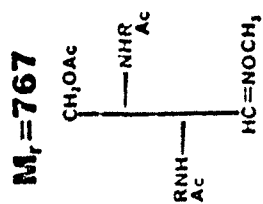
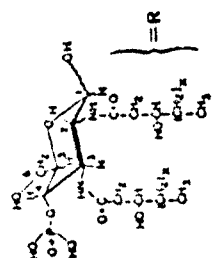
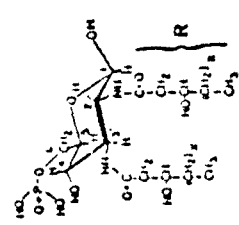


Figure 18: Negative ion FABMS of monomer phosphate following reduction, periodate oxidation, aldehyde trapping, and acetylation.

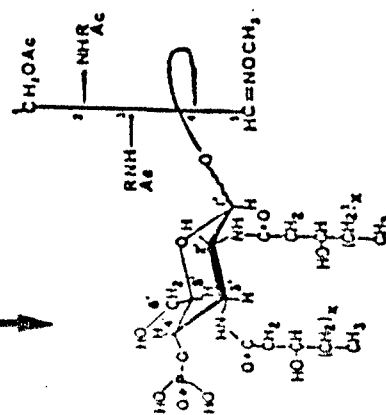
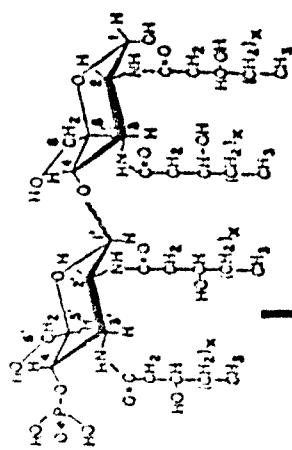


- 1.) BH₄⁻
 2.) IO₄⁻
 3.) H₂NOMe
 4.) Ac₂O/Pyr

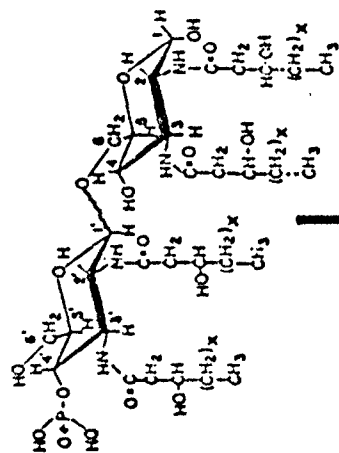


Scheme II

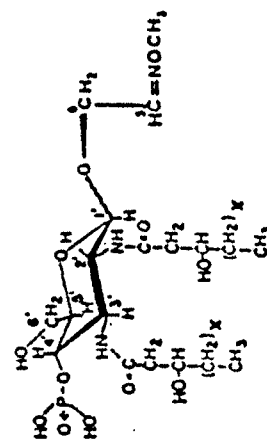
$M_r=1406$



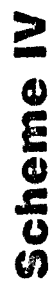
$M_r=1406$



- 1.) BH_3^-
- 2.) IO_3^-
- 3.) H_2NOMe
- 4.) Ac_2O/Pyr



Scheme III



An interesting finding, in the unraveling of this LPS structure, is the apparent alkane heterogeneity that exists in this lipid-A. Fatty acid methyl ester analysis of lipid-A preparations isolated earlier suggest this heterogeneity originates with these acyl groups, but since these are total compositional analysis, site specific conjugation remains to be determined. Moreover, since successful attempts at extraction and isolation have always involved methanol, a technical question must be considered whether this apparent alkane heterogeneity (14 amu intervals) may in fact be due to partial ester formation on phosphates or glycoside formation on hemiacetyls (also providing 14 amu intervals). To address these questions we have carried out two experiments with stable isotopes. The first by methanolysis in the presence of CD_3OD and, the second involve reduction and perdeuteriomethylation of the monomer, m/z 751.5 (Fig. 9b). Three strains from phase I, phase II, and the intermediate phase (strains 9MIC7, 9MIIC4, and RSA514, respectively) were each subjected to both methanolysis and methanolic hydrolysis, examined by negative FABMS, and all gave similar spectra (Figs. 9 and 10). These results seem to suggest that the differences in the virulent and avirulent phases are due to the oligosaccharide portion of the LPS and not in the lipid-A moiety.

The term lipid-A does not define a number of completely identical chemical structures but is used for all the various lipid moieties of LPS which occur in the outer membrane of gram-negative bacteria and cyanobacteria. The structural work presented above with Salmonella lipid A represents a general motif for other enterobacterial genera, and even in bacteria taxonomically quite distant from Enterobacteriaceae. Salmonella has been taken as the prototype of the lipid A structure where the backbone consists of $\beta(1 \rightarrow 6)$ linked glucosamine disaccharide, and substituted by phosphate groups on each end at the C-1 and C'-4 positions. Amine groups each carry 3-hydroxy fatty acids. The hydroxyl groups of the disaccharide (except the C-4) carry ester-linked 3-hydroxy fatty acids. Amide and ester linked 3-hydroxy fatty acids may carry saturated fatty acids which are substituted on the 3-hydroxy group, thus forming 3-acyloxyacyl residues. We and others (34,35) have precisely located the fatty acids and substituted fatty acids on the central core disaccharide. One technique allows the liberation of amide (and to a minor extent ester) linked 3-acylated-3-hydroxy fatty acids as methyl esters. They can directly be analyzed by GC-MS (35,36). The positional analysis of amide-linked fatty acids in lipid-A can be carried out by means of a technique recently described (37). Free lipid-A devoid of the glycosidic phosphate is reduced and then oxidized by periodate. After a second reduction (with $NaBH_4$), the N-acylated 2-aminoglycerol formed is permethylated and analyzed by GC-MS. This technique makes it possible to recognize the amide-linked substituent of GlcN I (37,38). The residual GlcN II is not destroyed by periodate, and its amide-linked fatty acid can be determined separately. The third technique allows the determination of the number and position of unsubstituted OH-groups in lipid A, by applying a silica gel-catalyzed methylation (39), which operates at neutral pH. Thus, only unsubstituted hydroxyl groups in free lipid-A are methylated and the linkage point of KDO may be recognized (40-42). By the use of these techniques and additional physicochemical methods, such as FABMS (43,44) and 1H -, as well as ^{13}C -, and ^{31}P -NMR (45, 46), the structure of Salmonella lipid A now seems to have been completely established. KDO, the link between sugar chains and the lipid A moiety, is ketosidically attached to position 6' of the lipid A backbone.

Several observations are of considerable interest and are summarized below. These points will be considered in subsequent studies: (a) Lipid-A may carry additional sugar constituents glycosidically attached to the 4-position of GlcN I or the 4'-position of GlcN II; (b) The amide-linked fatty acid(s) is not of the usual (R)-3-hydroxy type, but of the rare 3-(or 4-)oxo fatty acid type; (c) D-glucosamine as backbone sugar is replaced by another amino sugar, 2,3-diamino-2,3-dideoxy-D-glucose; and, (d) Most of these unusual lipid-A's are nontoxic. They have first been discovered amongst the purple phototropic bacteria, mainly of the Rhodospirillaceae family.

As a corollary lipid-A from Rhodopseudomonas viridis F is obtained by splitting with 1% acetic acid (1.5 h, 100°C). It is free of phosphate and of D-glucosamine but also contains 2,3-diamino-2,3-dideoxy-D-glucose (47) and was the first report of this sugar in a natural product. Only one of the two amino groups appears to be substituted, (probably in the 2-position), by a 3-hydroxy fatty acid (3-OH-14:0). The other amino group seems to be N-acetylated judging from mass spectrometry using chemical ionization. Also, the reducing end of the 2,3-diamino-glucose is substituted in Rhodopseudomonas viridis, and even under fairly harsh conditions it was not possible to obtain lipid A in a reduced form with the amide-linked constituents still attached. We have had similar problems with Coxiella burnetii. In contrast, lipid A of Rhodopseudomonas viridis is free of ester-linked fatty acids. A methylation analysis of the free lipid A yielded the 4,6-di-O-methylated-2,3-diamino sugar, which was released under severe hydrolytic conditions (4 N HCl, 18 h, 100°C).

As with other lipid A's, KDO seems to be linked to the 6-position of free lipid A in Rhodopseudomonas viridis F. In accordance with these major differences in structure between lipid A of Salmonella and that of Rhodopseudomonas viridis, only a very weak serological cross-reactivity was found between these two lipid A species in passive hemolysis and in its inhibition (48). The likewise low cross-reactivity of Rhodopseudomonas viridis lipid A with those of Rhodopseudomonas gleatinosa and Rhodospirillum tenue was also to be expected (48). LPS of Rhodopseudomonas viridis is about 250 times less toxic (lethal toxicity in adrenalectomized mice) than that of Salmonella, and its pyrogenicity is more than 10,000 times lower. The complement-inactivating capacity is as high as that of Salmonella endotoxin (48,49). LPS containing 2,3-diaminoglucose were found in twelve different strains of Rhodopseudomonas palustris which, like Rhodopseudomonas viridis have budding-like cell division (50). With two strains (8/1 and 11/1) of this species, it was shown that the 2,3-diaminoglucose (DAG) was the only amino sugar in the respective lipid-A preparations (51), and it can be assumed that the same holds true for the other strains. Since LPS of Rhodopseudomonas palustris is nearly exclusively extracted into the phenol phase upon phenol-water extraction and since it is co-extracted with phospholipids upon chloroform-methanol extraction, so far no definitive data on the fatty acid composition of lipid A of Rhodopseudomonas palustris can be given. Nevertheless, 3-OH-14:0 was the predominating fatty acid in all LPS-containing fractions. The additional presence of 3-OH-16:0 in addition to typical phospholipid fatty acids (unsaturated fatty acids) should be noted. Extraction with phenol-chloroform-petroleum ether

(PCP-extraction (52) of strain 8/1 of Rhodopseudomonas palustris yielded LPS with 3-OH-14:0, 16:0, some 3-OH-16:0 (and Δ^2 -14:1; the latter is an artifact formed from 3-OH-14:0 during hydrolysis) as predominant fatty acids (40). As expected, no serological cross-reaction with Salmonella and Rhodopseudomonas gelatinosa lipid A, but a strong one with that of Rhodopseudomonas viridis (48) was observed. The LPS of Rhodopseudomonas palustris, like that of Rhodopseudomonas viridis, was not lethally toxic to mice and not pyrogenic for rabbits, but had a high complement-inactivating capacity (49).

Rhodopseudomonas sulfoviridis, is taxonomically closely related to Rhodopseudomonas viridis (53), and also contains DAG instead of d-glucosamine in lipid A (54). Like that of Rhodopseudomonas viridis, the lipid A of this species was essentially free of phosphorus, and 3-OH-14:0 was the only fatty acid present in significant amounts. On the basis of these similarities in composition, structural identity with lipid A of Rhodopseudomonas viridis can be assumed (55).

An increasing number of reports describe the finding of DAG with cocomitant lack of glucosamine in lipid A's of nonphototrophic bacteria. This sugar occurs in lipid A from two closely related Pseudomonas diminuta and Pseudomonas vesicularis (55,56), in two Nitrobacter species (Nitrobacter winogradskyi and Nitrobacter hamburgensis (57,58), formerly designated as Nitrobacter X₁₄ (59) and in two strains (E and K₂) of a group of not yet classified gram-negative soil bacteria which are able to degrade the herbicide chloridazone (60).

All of these nonphototrophic bacteria lack phosphate in their lipid A's: they differ from each other characteristically in the number and nature of amide- and in some cases also ester-linked fatty acids. An unusual amide-linked oxo-fatty acid has been found in Pseudomonas diminuta and Pseudomonas vesicularis. It is not clear whether this oxo-fatty acid observed with the two Pseudomonas species is 4-oxo-14:0 or 3-oxo-2-methyl-13:0, (H. Mayer and S.G. Wilkinson, unpublished). These two fatty acids are not easily differentiated by mass spectrometry of their methyl esters. Ester-linked fatty acids are present in trace amounts in N. hamburgensis, but they occur in significant amounts in strain E of the above mentioned soil bacteria (60). In strain E, the two ester-linked fatty acids are 12:0 and (R)-3-hydroxy- Δ^5 -cis-12:1. Ester- and amide-linked acyloxyacyl residues have been observed. Methylation analysis carried out with free lipid A reduced by NaBH₄ showed that KDO is linked to the 6-position of the DAG. The reducibility of free lipid A of strain E, in contrast to free lipid A from Rhodopseudomonas viridis and viridis, shows that different substituents are occupying the reducing groups in the Rhodospirillaceae and in strain E.

Recent studies on the phylogenetical relationship of bacteria by determining the extent of homology of their 16S ribosomal RNA (expressed as S_{AB}-values) or of their cytochrome C₂ structure

(61,62,63), include a number of phototrophic purple bacteria (64,65). As a result of these studies it was realized that these bacteria - as ancient microorganisms - are phylogenetically diverse and that their genealogical relationships do not agree well with their present taxonomical classification (66,67). Three distinct species-groups can now be distinguished within the photrophic purple bacteria: one comprises Rhodopseudomonas gelatinosa, Rhodocyclus purpureus and Rhodospirillum tenue (60,61), another includes various species, such as Rhodopseudomonas sphaeroides, Rhodopseudomonas capsulata, Rhodopseudomonas viridis, Rhodomicrobium vannielii, Rhodopseudomonas palustris and Rhodospirillum rubrum. R. sphaeroides and R. capsulata form a close subgroup among this large species group; the three budding species R. viridis, R. vannielii and R. palustris also form a subgroup, but a less close one. The third group is so far only represented by Chromatium vinosum (60).

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V. PERSONNEL SUPPORTED FROM CONTRACT
A. TECHNICIANS, AND POSTDOCTORAL FELLOWS

Dr. Vernon N. Reinhold, P.I.
Dr. Zamas Lam, Postdoctoral Fellow
Mrs. SY Chan, Technician
Mr. Keyes B. Linsley, Technician
Dr. Richard S. Johnson, Postdoctoral Fellow