EFFECT OF CARBON SOURCE ON LIPOPOLYSACCHARIDE PRODUCTION IN *PSEUDOMONAS FLUORESCENS*

By

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CHAPTER I

INTRODUCTION

Lipopolysaccharide is a macromolecular envelope component found in gram-negative bacteria. The functions of the lipopolysaccharide include acting as a phage adsorption site, giving endotoxic capabilities to the cell, and acting as O-antigenic determinants.

The lipopolysaccharide isolated from several microorganisms has been studied, including genera from both the Enterobacteriaceae and Pseudomonodaceae. Perhaps the best characterized lipopolysaccharide is that isolated from Salmonella typhimurium (Hellerqvist and Lindberg, 1971), although the lipopolysaccharide from Escherichia coli (Edstrom and Heath, 1967; Lieve, Shovlin, and Mergenhagen, 1968), and Pseudomonas aeruginosa (Wilkinson, 1967: Fensom and Gray, 1969; Chester, Gray, and Wilkinson, 1971) has also been studied extensively.

The O-polysaccharide, the R-core, and the lipid A are the three basic structural components of the lipopolysaccharide molecule. The O-polysaccharide and R-core region can be dissociated from lipid A by mild acid hydrolysis in Salmonella and E. coli (Luderitz, Staub, and Westphal, 1966; Nikaido, 1968). Fensom and Meadow (1970) recently isolated a lipid-free polysaccharide from P. aeruginosa which can be separated into two fractions corresponding to the O-polysaccharide and R-core regions of the enteric bacteria.

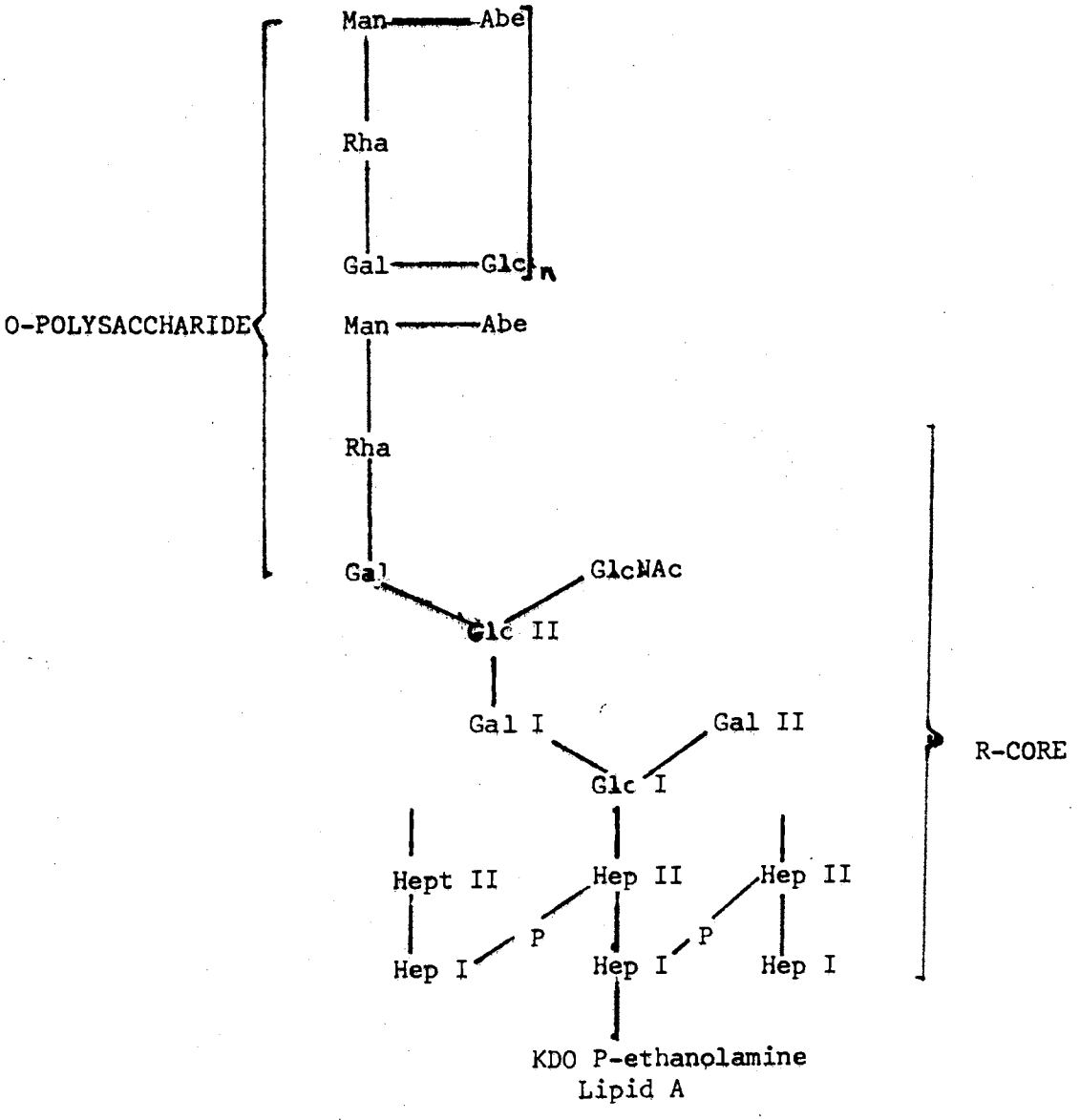
The O-polysaccharide is a heteropolysaccharide which shows a high degree of diversity in structure and composition in different organisms (Luderitz et al., 1966; Luderitz, Jann, and Wheat, 1968). Even within a genus, the O-polysaccharide is variable, giving rise to the serological classification of such organisms as Salmonella. In the genus Salmonella, there are approximately forty serogroups and organisms within each group that share at least one O-antigenic determinant (Kaufman, 1954).

The lipid A--R-core does not seem to vary among genera of the Enterobacteriacea (Luderitz et al., 1968). However, these areas of the pseudomonad lipopolysaccharide are qualitatively different in some respects from that of the Enterobacteriaceae.

Hellerqvist and Lindberg (1971) proposed a structure for the various components of the lipopolysaccharide of S. typhimurium (Fig. 1). The outer-most region is thought to be the O-polysaccharide, and this is the area which determines the O-antigenic specificity of the bacterial cell. The structure of the O-polysaccharide, the nature of the sugar components, and the linkage between sugar components determines the O-antigenic specificities. Uchida et al. (1965) states that both non-reducing terminal sugars and internal sugars are responsible for O-antigenicity. As indicated in Fig. 1, the common components of the O-polysaccharide of the enterics include several hexoses, N-acetylated hexosamines, deoxyhexoses, and dideoxyhexoses.

Nikaido (1969; 1970) has shown that the O-polysaccharide of S. typhimurium is linked to the R-core from the terminal galactose reducing group of the polysaccharide to the outermost glucose molecule of the R-core. Hammerling, Luderitz, and Wesphal (1970) confirm this

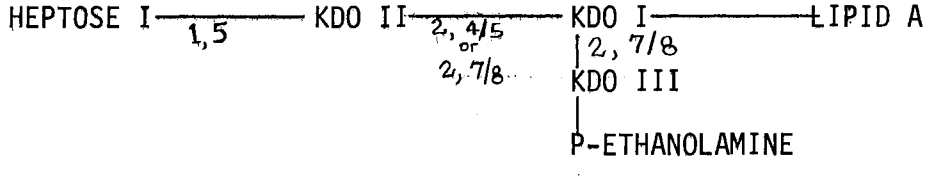
Figure 1. Structure of Salmonella typhimurium lipopolysaccharide
Hellerqvist and Lindberg (1971)
Abbreviations: Abe, abequose; Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Hep, heptose; KDO, 2-keto-3-deoxyoctulosonic acid; Man, mannose; P-ethanolamine, phosphatidylethanolamine; Rha, rhamnose; n represents repeating units


Man
Abe
Rha
Gal
Glc
n
O-POLYSACCHARIDE
Man
Abe
Rha
Gal
GlcNAc
Glc II
Gal I
Gal II
Glc I
R-CORE
Hept II
Hep II
Hep II
P
P
Hep I
Hep I
Hep I
KDO P-ethanolamine
Lipid A

as a 1,4 linkage.

Other studies involving the R-core have utilized mutants lacking the O-polysaccharide. In the Enterobacteriaceae, these are termed R-mutants to indicate rough colonial morphology. Studies of lipopolysaccharide composition and structure from R-mutants of E. coli and S. typhimurium have been made by Luderitz et al. (1966), Nikaido (1961; 1965), and Osborn et al. (1964). The components of the R-core include N-acetylglucosamines; the hexoses, glucose and galactose; L-glycero-D-mannoheptose; an eight-carbon sugar-acid, 2-keto-3-deoxyoctulonic acid (KDO); phosphate; and O-phosphorylethanolamine. The heptose of the pseudomonads is also the one common to the enteric bacteria lipopolysaccharide, L-glycero-D-mannoheptose (Chester et al., 1971). Fensom and Meadow (1970) report that the polysaccharide portion of the phenol-extractable antigenic lipopolysaccharide of P. aeruginosa can be separated into two polymers. One polymer contains glucose, heptose, and galactosamine, and the other most of the rhamnose, glucosamine, and mannose. Fensom and Meadow (1970) also report isolation of a mutant of P. aeruginosa which lacks the second polymer. The organism is more sensitive to some antibiotics and has an altered phage sensitivity, suggesting that it may have a defective polysaccharide side-chain like R-mutants of the Enterobacteriaceae.

Droge et al. (1970), using S. minnesota mutants, postulated a structure for the covalent linkage of the R-core to the lipid A through a KDO unit in the R-core as follows:



This structure shows the arrangement of the KDO units and indicates that O-phosphorylethanolamine is linked to a KDO side chain through its phosphate group. The outermost KDO molecule (KDO II) is linked to a heptose unit (Cherniak and Osborn, 1966; Droge et al., 1968a; 1970).

Chester et al. (1971) reports that treatment of isolated lipopolysaccharide from P. aeruginosa with 0.1 M HCl releases the lipid A and 35-40 percent of the KDO. This indicates that KDO serves as the linkage between lipid A and the R-core. Osborn (1963) obtained similar results with E. coli; that is, about one-quarter of the total KDO serves as a link between the lipid A and R-core. Recent work on Salmonella (Droge et al., 1970) indicates approximately the same level of KDO involved in the linkage.

Characterization of the structure of the lipid A moiety has been difficult because no mutant has been isolated. Chemical studies have established that the basic structural component of lipid A is a diglucosamine unit substituted with fatty acids and phosphate groups on the free amino and hydroxyl groups (Burton and Carter, 1964). Gmeiner, Luderitz, and Westphal (1969) studied the composition and structure of the lipid A using heptoseless mutants of S. minnesota. The glycolipid of the mutant strain contains only KDO and lipid A. The structure of the diglucosamine unit was thought to be a glucosaminyl-β-1,6-glucosamine. However, no information regarding the overall organization of intact lipid A was given.

Adams and Singh reported structural features of lipid A preparations from Serratia marcescens (1969) and E. coli and Shigella flexneri (1970). Lipid A from S. marcescens consists of D-glucosamine units

linked 1,6 and probably in the same configuration as in S. minnesota. Lipid A isolated from E. coli and S. flexneri consists of D-glucosamine units linked 1,4, but again possibly in the β configuration.

In the enteric bacteria, β-hydroxymyristic acid is the major component of lipid A. It is not found in significant amounts in other lipids of the cell. The amino groups of the diglucosamine units are substituted with β-hydroxymyristic acid in some organisms (Luderitz et al., 1968).

Hancock, Humphreys, and Meadows (1970) report that lipopolysaccharide isolated from P. aeruginosa is unusual in that it lacks the β-hydroxymyristic acid characteristic of the enterics. Rather, hydroxydecanoic and hydroxylauric acids seem to be the major fatty acids found in the lipid A. Both 2- and 3-hydroxylauric acids are characteristic of the lipid A of several pseudomonads including P. aeruginosa, P. putida, P. acidovorans, and P. aminovorans. However, P. alkaligenes contains only 3-hydroxylauric acid.

Lieve et al. (1968), in studying the effect of ethylenediaminetetraacetate (EDTA) on E. coli, isolated two lipopolysaccharide fractions. The cells were initially treated with EDTA to release lipopolysaccharide and the residue left from the EDTA treatment was then extracted with the aqueousphenol method to remove residual lipopolysaccharide. Physical and chemical analyses of the two fractions indicate qualitative and quantitative differences in the sugars present. Lieve (1968) suggests that this indicates a heterogeneity in the lipopolysaccharide. Release of a lipopolysaccharide fraction by EDTA, which has a high level of antigenic side-chains, indicates that the colitose-rich fraction comprises the most

external layer of the lipopolysaccharide.

Many investigators observed that growth conditions can alter the phenotype of genetically identical cells. As indicated by Neidhardt (1963), the content of RNA, DNA, lipid, and carbohydrate may vary greatly. Only in more recent studies, however, have quantitative and qualitative differences been elucidated in any great detail. Much of the data presented in the literature deals with deprivation studies or the utilization of agents which block metabolic activities such as cell wall synthesis rather than with changes effected by varying the carbon source.

Veinblat and Bukhrakh (1970) studied the effect of growth conditions on the synthesis of mouse toxin, lipopolysaccharide, and capsular and somatic antigens by Pasturella pestis. The biosynthesis of antigens and toxins was not affected by the presence of galactose, mannose, xylose, or vitamins in the medium. The addition of glucose, however, inhibited production of capsular antigens. Increased production of toxin was noted in medium containing meat or blood hydrolysates when compared with hydrolysates of casein or fish meal. Changing the pH of the growth medium had no effect on toxin production. They noted that the quantity of lipopolysaccharide increases as the growth rate decreases.

The effect of pH has been studied by others including Lorian and Sabath (1970) who, in studying the effect of pH on antibiotic activity against gram-negative bacteria, found that an alkaline culture medium increases the effectiveness of erythromycin and streptomycin against the gram-negative group. Minimal inhibitory doses are significantly less at pH 8.0 than at 5.5. The test organisms included isolates of

P. aeruginosa, E. coli, and Proteus mirabilis.

Numerous studies involving modifications of wall structure both in gram-positive and gram-negative bacteria have been done. Wilkinson (1970) studied the effect of EDTA on P. aeruginosa and noted a lower phosphorous content and a higher carbohydrate content in walls from cells grown on nutrient agar than in walls from cells grown on tryptone-glucose extract agar. The nutrient agar grown cells were less sensitive to the action of EDTA than the tryptone-glucose agar grown cells.

In other studies, Patterson (1972) used cyanide sensitivity of respiration to gauge the effects of growth in varied carbon sources. Aerobic cultures of P. aeruginosa growing exponentially in a number of different carbon sources show varying ratios of three cyanide-sensitive components of the electron transport system. Respiratory particles prepared from cells with different proportions of the cyanide-sensitive components show quantitative differences in their capability to oxidize added substrates. Patterson suggests that these results reflect the variations in cytochrome content of P. aeruginosa seen under different cultural conditions as shown by Yamanaka and Okunuki (1963) and Azoulay (1964). Meyer and Wurtz (1968) also indicate an inverse relationship between cytochrome C and catalase activity in P. fluorescens; the activity relationship depends on the culture medium.

Young (1965) used Bacillus subtilis in his studies involving variations in chemical composition of cell walls in response to growth in different media. He reports that galactosamine content varies with the phases of the growth cycle and reaches a maximum when B. subtilis

168 reaches a competent condition. Decreasing the complexity of the medium is not conducive to development of competence.

Johnson and Campbell (1972) studied the effect of growth conditions on peptidoglycan structure and susceptibility to lytic enzymes in cell walls of Micrococcus sodenensis. Comparison of wall material from cells grown in a complex medium and a synthetic medium showed that the peptidoglycan strcuture was more complex in the walls of cells grown in complex medium. Qualitatively, no difference was shown between walls from cells grown in the two media since glutamic acid, glycine, alanine, and lysine were present in both. N-Acetylmuramic acid and N-acetyglucosamine were present in equimolar amounts. Cells grown in the synthetic medium contained twice as many hexosamine units as those grown in the complex medium. Digestion studies with lysozyme showed significant differences in susceptibility between walls from cells grown in the two media. Forty-nine percent of the dry weight of walls purified from cells grown in complex medium were solubilized by lysozyme as compared to seventy-five percent of the dry weight of walls from cells grown in synthetic medium.

It has also been reported (Grula, 1970) that cell length is dependent at least in part on carbon source. Several media were tested, and it was found that Erwinia species grown in a glucose-aspartate medium exhibited a greater cell length than cells grown in asparatate or mannose-aspartate media.

The purpose of this research was to correlate the effect of different carbon sources with lipopolysaccharide production in P. fluorescens. Quantitative and qualitative analyses were performed on lipopolysaccharide isolated from cells grown in glucose and asparagine

minimal medium to determine variations in the lipopolysaccharide as a result of changing the carbon source.

From reports in the literature, one would assume for the purpose of this study that the lipopolysaccharide of P. fluorescens would be similar to that of P. aeruginosa in containing two polysaccharide regions and a lipid area. The neutral monosaccharides and amino sugars should be similar.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The microorganism used throughout this study was a strain of Pseudomonas which was tentatively identified by Keudell (1967) as Pseudomonas fluorescens. The organism is a gram-negative, motile rod which forms smooth, raised colonies on nutrient agar. The organism produces fluorescein and pyocyanin when grown on Bacto-Pseudomonas F and Bacto-Pseudomonas P agar respectively.

Stock cultures of Pseudomonas fluorescens were maintained on either nutrient agar or glucose-salts agar slants and stored at 4 C.

Media

Two synthetic media were used in this study to compare lipopolysaccharide production. Either asparagine or glucose was used as carbon source, but the minimal salts solution was the same for each. The synthetic minimal salts medium contained the following: 0.2 percent NaCl; 0.2 percent NH_4Cl; 0.32 percent KH_2PO_4; and 0.42 percent K_2HPO_4. The final pH of the media was adjusted to 7.0 before sterilization. Sterilization was accomplished by autoclaving for 15 minutes at 15 psi and 121^{o}C. The media were cooled to room temperature and 0.1 ml of mineral salts solution was added to each 100 ml of

medium. The mineral salts solution contained the following dissolved in 100 ml of glass-distilled water: 5 gm $MgSO_4 \cdot 7H_2O$; 0.1 gm $MnSO_4$; 1.0 gm $FeCl_3$; and 0.5 gm $CaCl_2$.

When glucose was used as the carbon source, it was autoclaved separately as a 10 percent (w/v) solution and then added to the sterile salts medium. Asparagine was added to the synthetic medium before sterilization. The final concentration of carbon source was 2.8×10^{-2}M except when otherwise indicated.

The phrase minimal sals buffer is used to designate the synthetic medium without mineral salts solution or carbon source.

Preparation of Cell Suspensions

The cell suspensions used in growth studies and for determination of relative amount of lipopolysaccharide produced were prepared as follows: nutrient agar or glucose-salts slants were inoculated and incubated 12-15 hours at 37C. The cells were suspended in 6 ml of either asparagine or glucose minimal salts medium. The starter cultures were incubated at 37 C for 8-10 hours and then used to inoculate the respective growth medium to an absorbance at 540 nm of approximately 0.1. All absorbancy readings were made at 540 nm on a Coleman Jr. II spectrophotometer using 18 x 150 mm Kimax tubes.

Growth Studies

Growth studies were conducted to determine the effect of different carbon sources on lipopolysaccharide production. Experiments involving assays performed on whole cells were done in 250 ml Erlenmeyer flasks with side-arms. The contents were incubated at

37 C on a reciprocal shaker. Absorancy was measured at 540 nm.

Experiments involving the withdrawal of larger samples were performed in 2 liter Fernbach flasks containing 500 ml of medium. The contents were incubated at 37 C on a reciprocal shaker and absorbancy was read at 540 nm.

Actinomycin D Sensitivity Studies

Actinomycin D (Merck, Sharp, and Dohme Research Laboratory) was dissolved in sterile glass-distilled water to give a concentration of 300 ugm/ml and stored at 4 C in the dark.

Sterile tubes (18x150 mm) containing 5 ml of medium were brought to final concentrations of 10, 20 or 30 ugm/ml of actinomycin D. The media were then inoculated with previously grown starter culture and buffer was added to bring the final volume to 6 ml. Growth was determined by following the increase in absorbancy of the cultures at 540 nm on a Coleman Jr. II Spectrophotometer.

Estimation of 2-keto-3-deoxyoctulosonate (KDO)

The thiobarbituric acid assay of Cynkin and Ashwell (1960) was used for the detection of KDO both in whole cells and isolated lipopolysaccharide. One ml of either a cell suspension or isolated lipopolysaccharide was hydrolyzed with 0.22 N H_2SO_4 for 30 minutes in a boiling water bath. The hydrolysate was clarified by centrifugation at 15,000 x g for 15 minutes, and 0.25 ml aliquots of the supernatant were taken for assay. Twenty-five hundreths ml of 0.025 M periodic acid in 0.125 N H_2SO_4 was added to each sample; the samples were placed in a 55 C water bath. After 25 minutes, 0.5 ml of 2 percent

sodium arsenite in 0.5 N HCl was added to the samples to end the periodate cleavage, and the transitory iodine dischange noted before the addition of 2 ml of 0.3 percent thiobarbituric acid. After heating for 12 minutes in a boiling water bath, absorbancies were read at 532 nm. One tenth ml of saturated NaOH was added to all tubes after the initial reading, and after 2 hours the absorbancies were again read at 532 nm. β-formyl pyruvate is the periodate cleavage product formed deoxyhexoses and it is stable to alkaline environments. Malonaldehyde is the cleavage product formed KDO and is not stable to treatment with strong base. The final absorbance at 532 nm two hours is taken as the corrected absorbence. The concentration of KDO in the samples is determined by comparison with a standard curve of authentic KDO.

Isolation of Lipopolysaccharides

Two methods were utilized to isolate the LPS from whole cells. The initial method utilized was an adaptation of the procedure of Westphal and Jann (1965) using aqueous phenol. 250 ml samples of the bacterial cultures were taken at points in the growth cycle.

The cells were centrifuged at 9,000 x g for 10 minutes, and the pellet was suspended in acetone at 0 C with stirring. The acetone was removed by centrifugation, and the pellet washed one time with acetone and dried. The residue was suspended in 10 ml water, and the mixture placed in a water bath at 68 C. Ten ml of 90 percent phenol at 68 C was added to the suspension, and the contents of the tube were incubated for 15 minutes at 68 C. The mixture was centrifuged for one hour at 0-4^{o}C to allow separation of the immiscible layers.

The phenol and gummy interface were extracted two more times, and the aqueous extracts were pooled and dialyzed at 4 C for 2 to 3 days against distilled water and lyophilized. The principle contaminant found in the isolated LPS is nucleic acid, and the LPS was further purified as follows: it was dissolved at a concentration of 20-30 mg per ml in 0.1 M NaCl containing 0.01 M $NaPO_4$, pH 7, and RNAse (25 ugm per ml final concentration) was added. This solution was then dialyzed at 25 C against the same buffer at 5-10 times volume and 5 changes. The non-dialyzable material was lyophilized and used for various assays.

Quantitative Analysis of Neutral Monosaccharides

Lipopolysaccharide samples (approximately 2 mg) were hydrolyzed in 0.8 ml of 2N H_2SO_4 in sealed tubes for 4 hours at 100 C. Mannose (2 ml of 0.25 umole/ml) was added to the hydrolysate as the internal standard. The hydrolysate was then passed through a column of Dowex 50-X4 (H+) (200-400 mesh) coupled to a column of Dowex 1-X8 (formate) (200-400 mesh) (Spiro, 1966). The effluent and wash fraction were combined and taken to dryness by lyophilization and dissolved in 10 ml of water. Xylose (0.5 ml of 0.25 umole/ml) was added to 4.0-5.0 ml aliquots of the suspension, and the mixtures were taken to dryness. The amount of neutral sugars present in the sample was determined by the Technicon automatic sugar chromatography system as described by Lee, McKelvy, and Lang (1969).

Quantitative Analysis of Aminosugars

Lipopolysaccharide samples (3 mg) were hydrolyzed in 1 ml of 4N HCl in sealed tubes for 6 hours at 100 C. To the hydrolysate was

added 1 ml of 0.25 umole/ml guanidinoalanine as internal standard, and the mixture was taken to dryness on a rotary evapomix at 50 C. The aminosugars present in the sample were determined on the short column of a Beckman 120C amino acid analyzer (Spackman, Stein, and Moore, 1958).

Heptose Determination

Heptose was determined by the method of Dische (1953) as modified by Osborn (1963) with seduheptulose as standard. Duplicate samples (0.5 ml) were placed in an ice-water bath, and 2.25 ml of H_2SO_4 (6 volumes conc. H_2SO_4 to 1 volume H_2O) were added and mixed by shaking in the cold. The mixture was placed at 20 C for 3 minutes, and heated in a boiling water bath for 10 minutes. After cooling, 0.05 ml of 3 percent cysteine-HCl was added to one sample, the other serving as blank. Absorbancies at 505 nm and 545 nm were determined 2 hours after addition of cysteine. Levels of heptose were determined by comparison with a standard curve of seduheptulose.

Phosphate Determination

Samples of isolated lipopolysaccharide were resuspended in water (1 mg/ml) and 0.01-0.05 ml were pipetted into test tubes. Five hundreths ml of ten percent $Mg(NO_3)_2$ in ethanol was added. The tube was heated over a strong flame with rapid shaking until the mixture evaporated and the brown flames disappeared. The test tubes were capped and heated in a boiling water bath for 15 minutes. After cooling, 0.7 ml of ascorbic molybdate mixture (1 part 10 percent ascorbic acid to 6 parts 0.42 percent ammonium molybdate $\cdot 4\ H_2O$ in

1N H_2SO_4) was added, and the samples were read after 20 minutes at 45 C at 820 nm (Ames and Dubin, 1960). Phosphate levels were determined by comparison with a standard curve of KH_2PO_4.

CHAPTER III

RESULTS AND DISCUSSION

Growth Studies

Experiments were conducted to determine the amount of lipopolysaccharide produced by P. fluorescens in glucose minimal salts and asparagine minimal salts medium. The carbon sources were used in equimolar concentrations of 2.78×10^{-2}M throughout the comparison growth studies.

For growth studies involving sampling at timed intervals, 500 ml of medium was placed in Fernbach flasks. The medium was inoculated with cells previously grown in the carbon source, and growth was followed by measuring absorbancy at 540 nm. Samples were taken at indicated time intervals, and KDO measurements were made on the cell pellet with the thiobarbituric acid assay (Cynkin and Ashwell, 1960). The KDO concentration was used as an indication of lipopolysaccharide level in the cell envelope.

The measurement of KDO concentration can be used as an indication of lipopolysaccharide concentration since KDO is found only in the core region of the molecule. It has been referred to as the backbone component so an increase in the KDO level would reflect an increase in the lipopolysaccharide level. The thiobarbituric acid assay is useful for measuring the levels of deoxy- and dideoxysugars in addition to measuring KDO concentration.

In order to measure only KDO, the assay was modified to negate the measurement of other compounds. β-formyl pyruvate, the periodate cleavage product of KDO, when reacted with thiobarbituric acid is stable under alkaline conditions. Malon aldehyde, the periodate cleavage product from deoxy- and dideoxysugars forms a chromagen which is alkaline labile (Waravdekar and Saslaw, 1959). The period of time for color fade of the unstable chromagens was determined to be two hours. Spectrophotometric readings were taken immediately after the assay was performed. One-tenth ml of saturated NaOH was then added, and after 2 hours at room temperature the samples were again read. The second reading is taken to be the concentration of KDO production.

P. fluorescens cells grown in glucose show an increase in lipopolysaccharide production as the age of the culture increases (Fig. 2). There is an initial doubling of the amount of lipopolysaccharide produced, but as the cells enter stationary phase a plateau value is reached.

When asparagine is used as the carbon source, the cells show an initial increase which does not quite double the amount seen in early logarithmic phase cells (Fig. 3). As the cells enter stationary phase, a plateau value is also reached.

Compared to cells growing in glucose, the asparagine grown cells do not reach the same level of lipopolysaccharide production. The glucose grown cells produce 50 percent higher levels of lipopolysaccharide than do the asparagine grown cells.

Figure 2. Growth Curve of P. fluorescens in Glucose Minimal Salts Medium and Measurement of KDO
Growth of P. fluorescens in 2.78×10^{-2} M glucose minimal salts medium and lipopolysaccharide production given as μgm KDO/mg dry cell weight vs. time of sample. ● = growth; ▲= KDO.

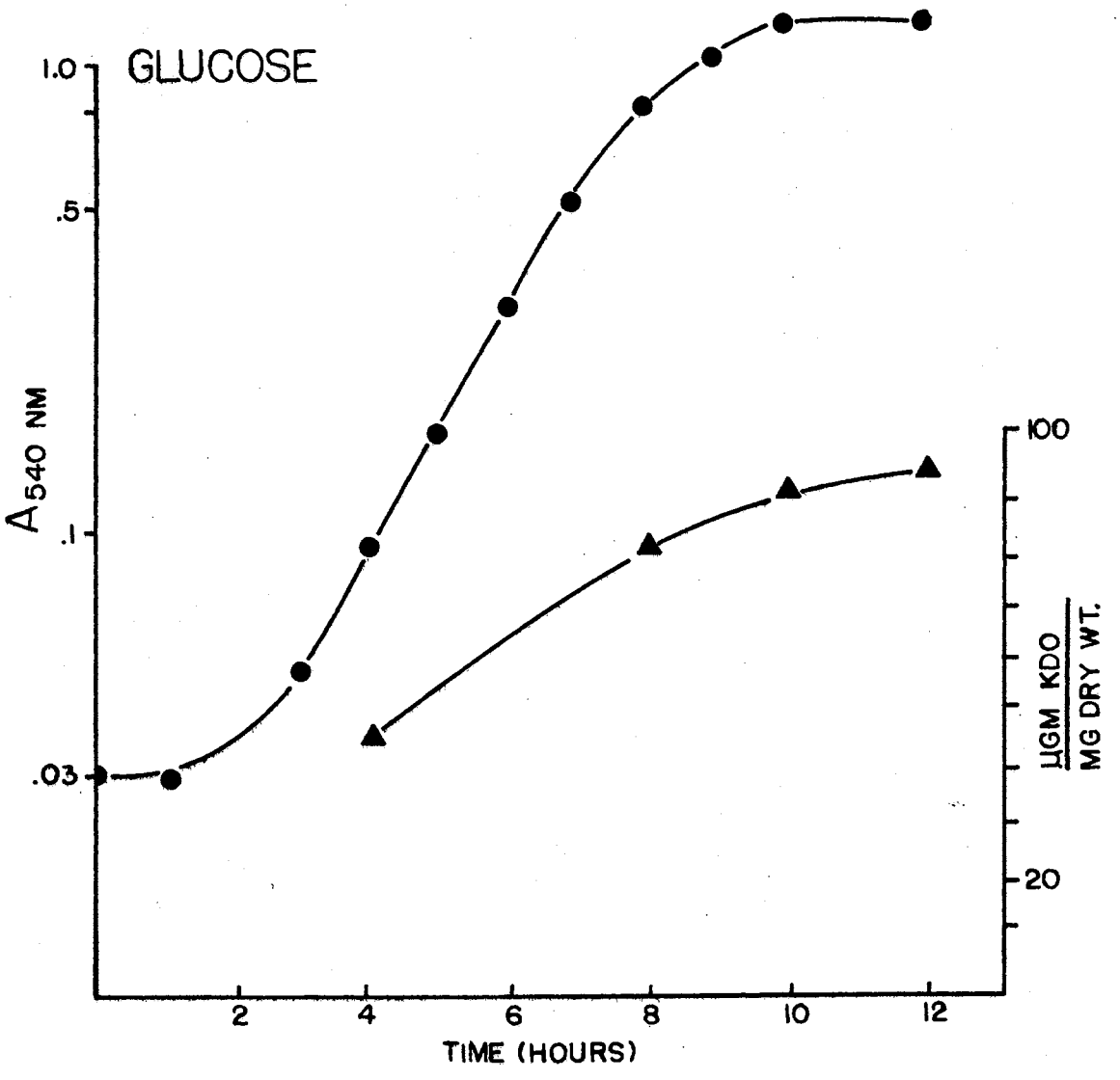
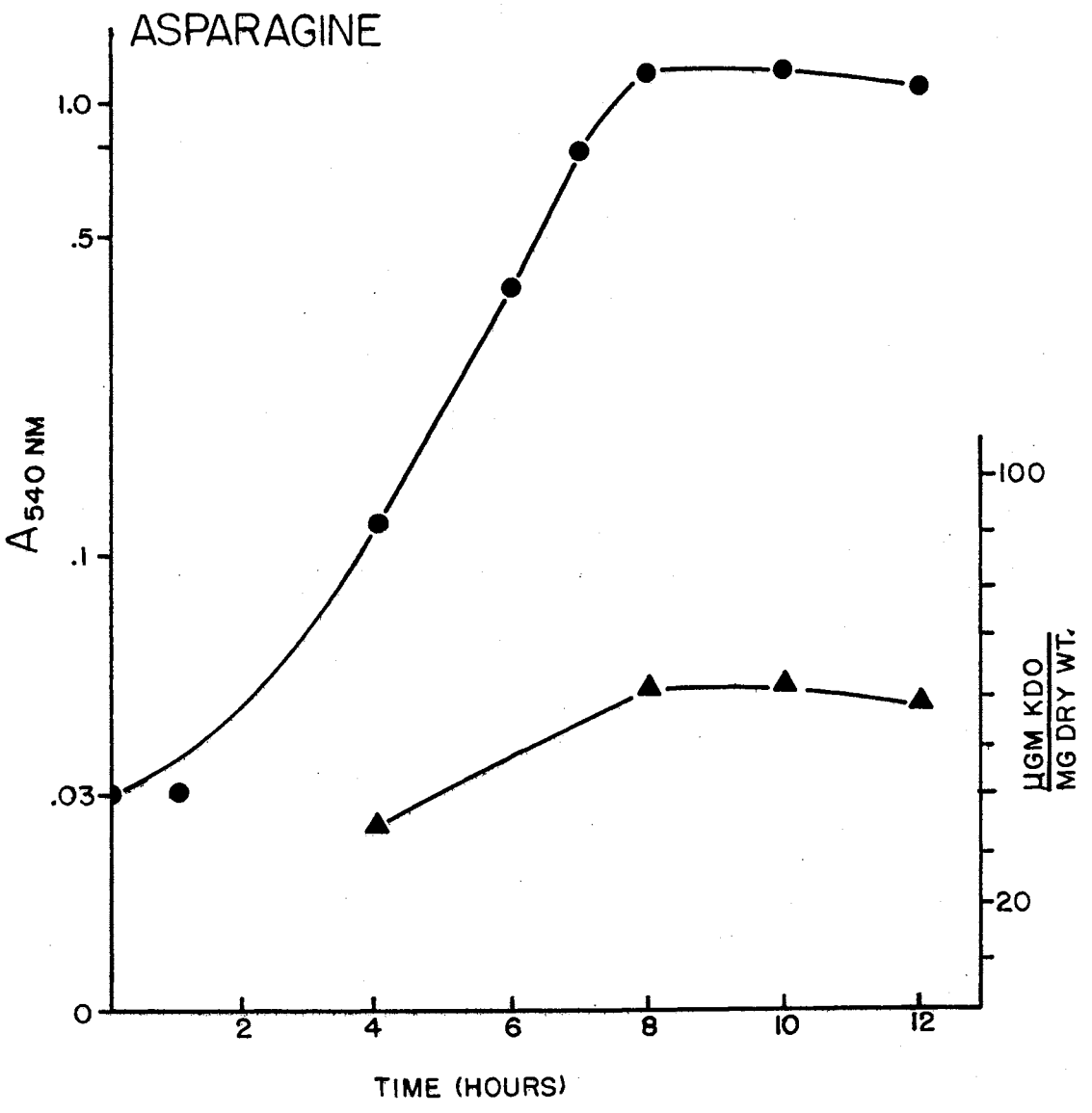

GLUCOSE
1.0
.5
.1
.03
A_{540} NM
100
20
μGM KDO / MG DRY WT.
2
4
6
8
10
12
TIME (HOURS)

Figure 3. Growth Curve of P. fluorescens in Asparagine Minimal Salts Medium and Measurement of KDO
Growth of P. fluorescens in 2.78×10^{-2}M asparagine minimal salts medium and lipopolysaccharide production given as ugm KDO/mg dry weight vs. time of sample. ● = growth; ▲ = KDO


ASPARAGINE
1.0
.5
.1
.03
0
$A_{540\ NM}$
2
4
6
8
10
12
TIME (HOURS)
100
20
μGM KDO / MG DRY WT.

Concentration-Gradient Growth Studies

A second series of growth studies were done to determine the effect of the concentration of each carbon source on lipopolysaccharide production. Minimal salts medium was prepared with concentrations of 0.10, 0.25, 0.50, and 0.75 percent of the desired carbon source. Samples were taken at points during the growth cycle in each concentration of the carbon source. Absorbance at 540 nm was used to approximate comparable times in the growth cycle for each concentration tested.

Fig. 4 is a histogram representing levels of KDO production from cells in lograrithmic and stationary phase at the different concentrations of glucose. The initial levels of lipopolysaccharide production in all concentrations are approximately the same. At later sampling times, an increase in the lipopolysaccharide concentration from that in the early stage is seen in each instance. As the concentration of glucose is increased, a marked rise in lipopolysaccharide production is noted.

At 0.1 percent asparagine concentration, the amount of lipopolysaccharide remains constant and at a relatively low concentration (Fig. 5). An increase in lipopolysaccharide production is seen with increased culture age with the other three concentrations of asparagine. A slight increase in total lipopolysaccharide production is seen with increased asparagine concentration. The concentration effect is more pronounced when the concentration of glucose is varied than when the asparagine concentration is varied.

Figure 4. Glucose Concentration-Gradient Studies
P. fluorescens cells were grown in 0.1, 0.25, 0.5, and 0.75 percent glucose minimal salts medium. Whole cells were assayed, and the results are plotted as μgm KDO/mg dry weight of cells at the sample times.
Samples were taken during the logarithmic growth (log) and maximum stationary phase (stat).

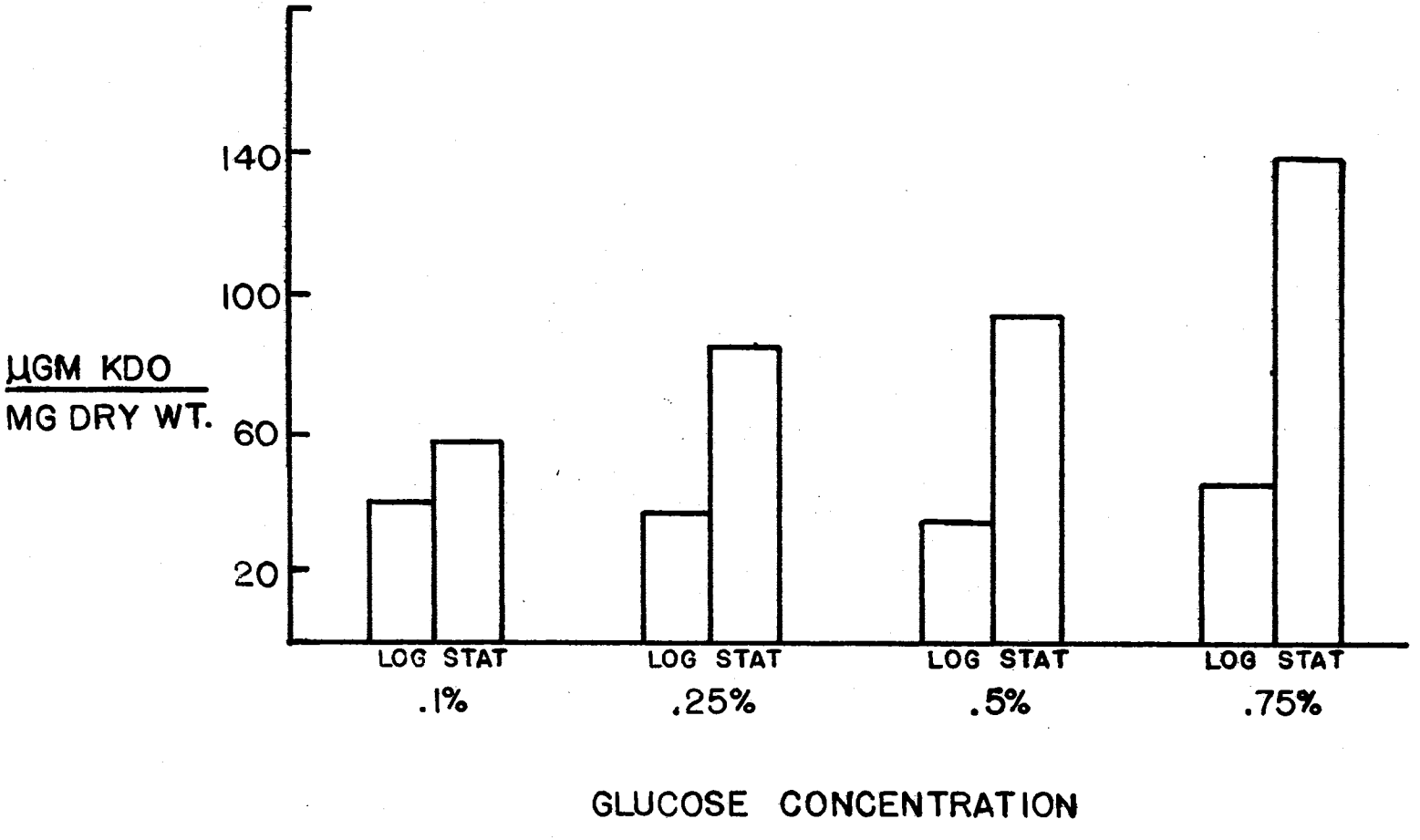
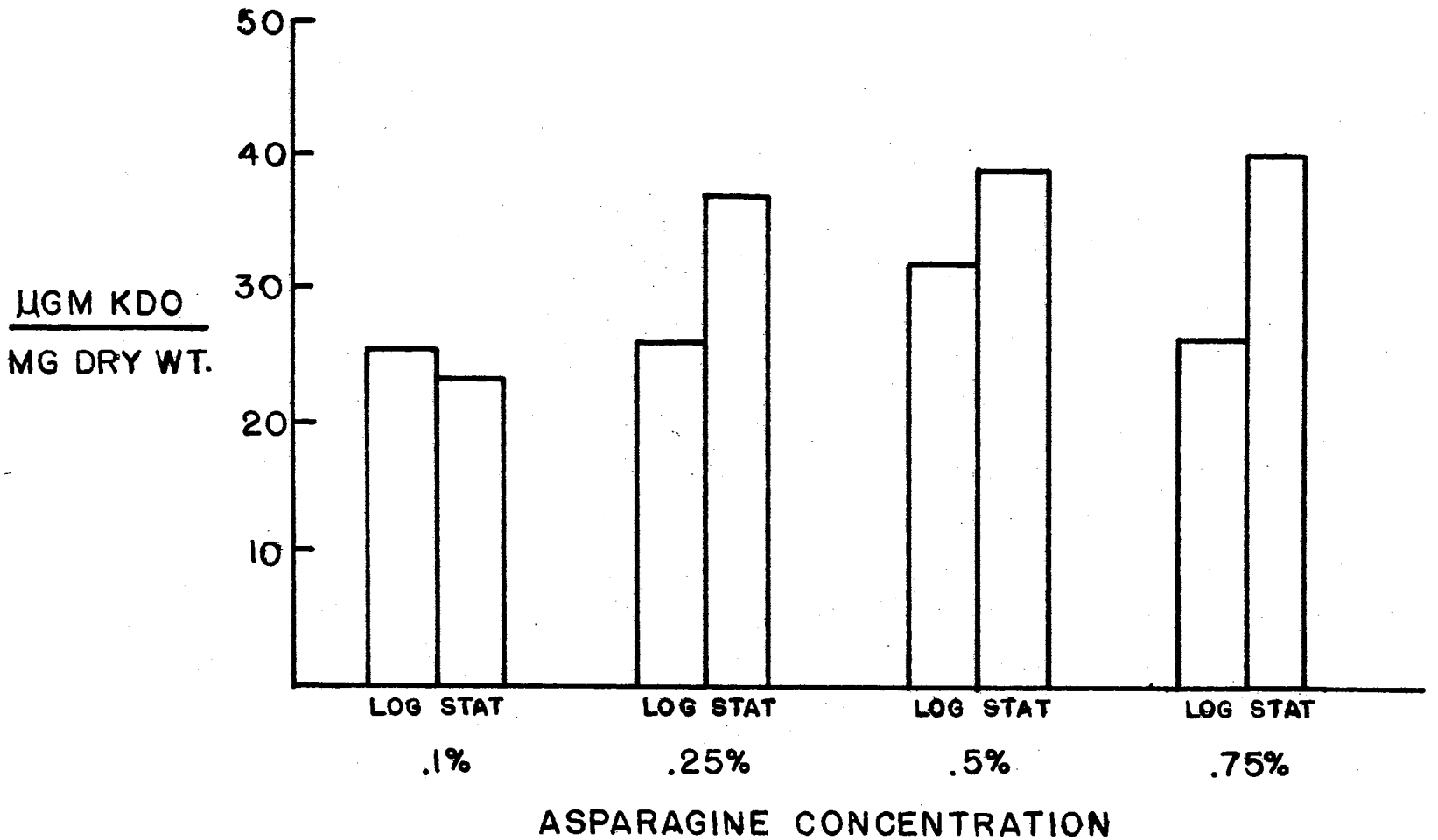

μGM KDO
MG DRY WT.
140
100
60
20
LOG STAT
.1%
LOG STAT
.25%
LOG STAT
.5%
LOG STAT
.75%
GLUCOSE CONCENTRATION

Figure 5. Asparagine Concentration-Gradient Studies
P. fluorescens cells were grown in 0.1, 0.25, 0.5, and 0.75 percent asparagine minimal salts medium. Whole cells were assayed, and the results are plotted as µgm KDO/mg dry weight of cells at the sample times. Samples were taken during the logarithmic growth (log) and maximum stationary phase (stat).


50
40
30
20
10
μGM KDO
MG DRY WT.
LOG STAT
LOG STAT
LOG STAT
LOG STAT
.1%
.25%
.5%
.75%
ASPARAGINE CONCENTRATION

Characterization of Isolated Lipopolysaccharide

Analyses were performed on lipopolysaccharide isolated from P. fluorescens cells grown in glucose and asparagine minimal salts medium. Samples were taken early in the logarithmic phase and in stationary phase of the growth cycle. Extractions were performed using the aqueous phenol methods. The lipopolysaccharide was analyzed for the presence of neutral monosaccharides, amino sugars, phosphates, and heptose.

Identification of Quantitation of Neutral Monosaccharides

Studies of the isolated lipopolysaccharide were done by chromatographing hydrolyzed samples prepared by suspending isolated lipopolysaccharide in distilled water and hydrolyzing in 4N HCl in vacuo for 2 hours on Whatman paper in a developing solvent of 1-butanol: pyridine:water (6:4:3) (Table I). Glucose and rhamnose were used as standards as these sugars have been found in most lipopolysaccharide samples studied to date. By comparison of R_f values, both glucose and rhamnose were shown to be present in the lipopolysaccharide isolated from cells growing logarithmically and in maximum stationary phase from both carbon sources.

Further chromatographic analyses were performed using column chromatography. Neutral sugars detected in isolated lipopolysaccharide from both sets of cells include rhamnose, glucose, fucose, and galactose (Table II).

TABLE I

PRELIMINARY CHROMATOGRAPHY OF MAJOR NEUTRAL SUGARS FROM ISOLATED LIPOPOLYSACCHARIDE

Standards	R_f	Glucose		ASP	
		Log	Stat	Log	Stat
Glucose	0.32	0.33	0.34	0.32	0.34
Rhamnose	0.59	0.58	0.55	0.58	0.59

Developing Solvent: 1-butanol:pyridine:water (6:4:3)

Abbreviations: Samples were taken during logarithmic growth (log) and maximum stationary phase (stat)

TABLE II

IDENTIFICATION AND QUANTITATION OF NEUTRAL MONOSACCHARIDES IN LIPOPOLYSACCHARIDE

Sample	Monosaccharide			
	Rhamnose	Glucose	Fucose	Galactose
Glu log	0.12	0.08	0.007	0.008
Glu stat	0.26	0.10	0.004	0.005
Asp log	0.20	0.15	0.005	0.007
Asp stat	0.18	0.13	0.005	0.008

Sugar concentrations are designated as umoles monosaccharide/mg isolated lipopolysaccharide.

Abbreviations: Samples were taken during logarithmic growth (log) and maximum stationary phase (stat).

Rhamnose is the predominant sugar isolated from the lipopolysaccharide of glucose-grown cells. The rhamnose concentration increases over two-fold from early logarithmic to stationary phase cells. Glucose is present in the next highest concentration and it increases by 38-40 percent during growth.

Fucose and galactose are present in much lower concentrations than either rhamnose or glucose, and both decrease as the culture grows rather than increasing as do rhamnose and glucose.

Rhamnose and glucose are present in the highest concentrations in lipopolysaccharide isolated from asparagine grown cells (Table III). Both sugar moieties remain constant during the growth cycle and the levels are much closer than those seen in lipopolysaccharide from glucose grown cells. Fucose and galactose are present in lower levels than rhamnose and glucose. These sugars also remain quite constant as the age of the culture increases.

Molar ratios of the monosaccharides are presented in Table IV. The ratios are based on rhamnose since it is the predominant sugar in all samples from the information given in Tables II and III to indicate changes within the lipopolysaccharide rather than a net increase or decrease of each individual sugar. In lipopolysaccharide isolated from glucose grown cells, the molar ratios of rhamnose to glucose, fucose, and galactose decrease. This would indicate that rhamnose is increasing more rapidly than the other three sugars during the same time interval.

The molar ratios of the sugars from asparagine grown cells indicate no change in the relationship among the sugars as the age of the culture increases.

TABLE III

MOLAR RATIOS OF NEUTRAL MONOSACCHARIDES
IN LIPOPOLYSACCHARIDE

Sample	Rha/Rha	Glu/Rha	Fuc/Rha	Gal/Rha
Glu log	1.0	0.61	0.05	0.06
Glu stat	1.0	0.40	0.02	0.02
Asp log	1.0	0.72	0.02	0.04
Asp stat	1.0	0.71	0.03	0.05

Abbreviations: Rha = rhamnose; Glu = glucose; Fuc = fucose;
Gal = galactose
Samples were taken during logarithmic (log)
and maximum stationary phase (stat).

The data presented in Tables II and III indicate that the major sugar species rhamnose and glucose are variable when glucose is used as the carbon source, but remain more nearly static when asparagine is used as the carbon source. The information lends supportive evidence for the growth experiments which indicate a greater level of lipopolysaccharide production in glucose grown cells than in asparagine grown cells.

Amino Sugar Determination

Glucosamine and galactosamine were identified chromatographically on the short column of the Auto Analyzer from samples of isolated lipopolysaccharide from glucose and asparagine grown cells.

An increase in glucosamine content is evident in lipopolysaccharide isolated from both glucose and asparagine grown cells (Table IV). In fact, the increase is the most noticeable change occurring in the lipopolysaccharide isolated from asparagine grown cells.

Galactosamine content increases by almost two-fold in the sample from glucose grown cells. Galactosamine remains constant in the sample from asparagine grown cells.

Molar ratios of glucosamine/galactosamine indicate that the relationship between the two amino sugars changes during the growth cycle on both carbon sources. The ratio in samples from glucose grown cells decreases and that in asparagine grown cells increases.

Heptose Determination

L-Glycero-D-mannoheptose has been identified as the heptose occurring in lipopolysaccharide of P. aeruginosa by Wilkinson (1970).

TABLE IV

IDENTIFICATION AND QUANTITATION OF AMINO SUGARS
IN LIPOPOLYSACCHARIDE

Sample	Glucosamine	Galactosamine	Molar Ratio NacGlu/NacGal
Glu log	0.021	0.024	0.87
Glu stat	0.035	0.046	0.75
Asp log	0.047	0.048	0.98
Asp stat	0.059	0.049	1.16

Amounts of amino sugars in umoles/mg isolated lipopolysaccharide.

Abbreviations: Samples were taken during logarithmic growth (log) and maximum stationary phase (stat).
NacGlu, n-acetyl glucosamine
NacGal, n-acetyl galactosamine

The heptose moiety is thought to play an integral role in the structure of the R-core region of the lipopolysaccharide, and analyses were performed to determine the relative amounts of heptose in lipopolysaccharide of P. fluorescens grown using glucose and asparagine as the carbon sources (Table V).

The heptose concentration doubles in the lipopolysaccharide isolated from glucose grown cells but remains constant in the lipopolysaccharide from asparagine grown cells.

Phosphate Determination

The data in the literature indicate that lipopolysaccharide molecules are linked through phosphodiester bridges (Droge, Luderitz, Westphal, 1968b). An assay of the relative amounts of phosphates present in each sample of isolated lipopolysaccharide would give information regarding the number of lipopolysaccharide units present. Table V presents the data from the isolated lipopolysaccharide samples. The isolated lipopolysaccharide from glucose-grown cells exhibit a 40 percent increase in the amount of phosphate present from logarithmic samples to stationary samples. The levels of phosphate in asparagine-grown cells remains constant.

Actinomycin D Sensitivity Studies

Walker (1973) noted that sensitivity of P. fluorescens to actinomycin D varied greatly with different carbon sources. To determine if actinomycin D sensitivity could be related to differences noted in lipopolysaccharide production in P. fluorescens grown in equimolar glucose and asparagine minimal medium, growth studies were

TABLE V

HEPTOSE AND PHOSPHATE DETERMINATION

Sample Time Carbon Source	μmoles heptose / mg LPS	μmoles phosphate / mg LPS
Glu log	1	40
Glu stat	2	66
Asp log	2	34
Asp stat	2	35

Abbreviations: Samples were taken during logarithmic growth (log) and maximum stationary phase (stat).

done. Two concentrations of each growth substrate were tested against three concentrations of actinomycin D.

The results indicate that 30 ugm/ml of actinomycin D produced a 50 percent inhibition of growth of P. fluorescens in 2.78×10^{-2}M glucose minimal medium (Fig. 6). Ten and 20 ugm/ml actinomycin D inhibited growth by 15 and 25 percent respectively. Decreasing the concentration of glucose to 5.5×10^{-3}M increased the inhibition of growth to 80 percent of the control at 30 ugm/ml actinomycin D concentration (Fig. 7).

Asparagine minimal medium 2.78×10^{-2}M containing 30 ugm/ml actinomycin D produced a 50 percent inhibition of growth (Fig. 7). However, at 5.5×10^{-3}M asparagine concentration and 20 and 30 ugm/ml actinomycin D complete inhibition of growth was seen. Ten ugm/ml actinomycin D inhibited growth by 90 percent (Fig. 7).

These data indicate that the concentration of each carbon source has a more significant influence on sensitivity of P. fluorescens cells to actinomycin D than does changing the growth substrate.

The concentration gradient experiments demonstrated that growth in lower concentrations of the carbon source lowered the final level of lipopolysaccharide production both in glucose and asparagine grown cells, and this correlated with the actinomycin D sensitivity.

Figure 6. Actinomycin D Sensitivity of *P. fluorescens* Grown in 2.78×10^{-2} M and 5.5×10^{-3} M Glucose Minimal Salts Medium

● = cells grown in glucose minimal salts medium without actinomycin D; ▲ = cells grown in glucose minimal salts medium with 10 μgm/ml actinomycin D; ■ = cells grown in glucose minimal salts medium with 20 μgm/ml actinomycin D; ● = cells grown in glucose minimal salts medium with 30 μgm/ml actinomycin D

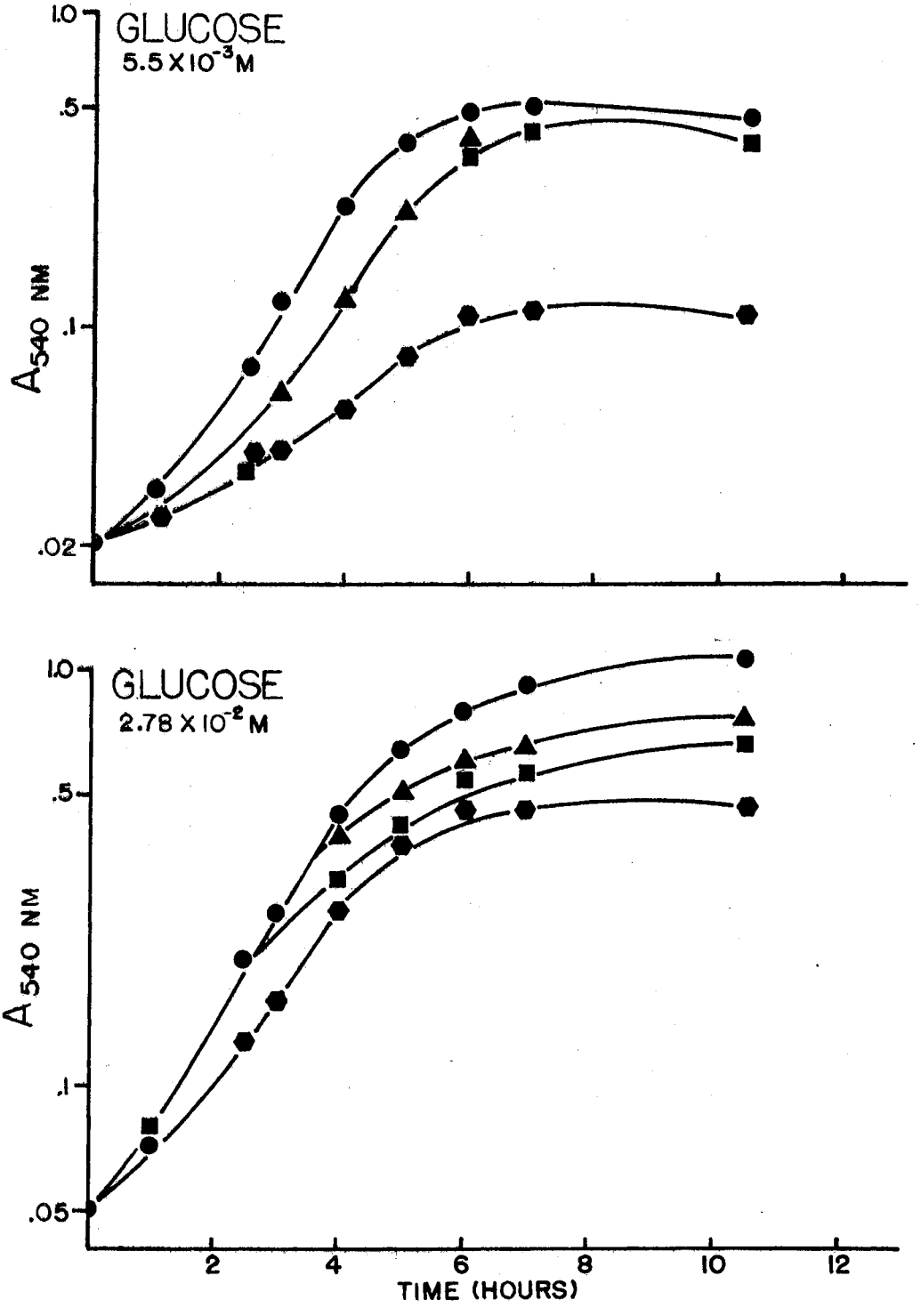
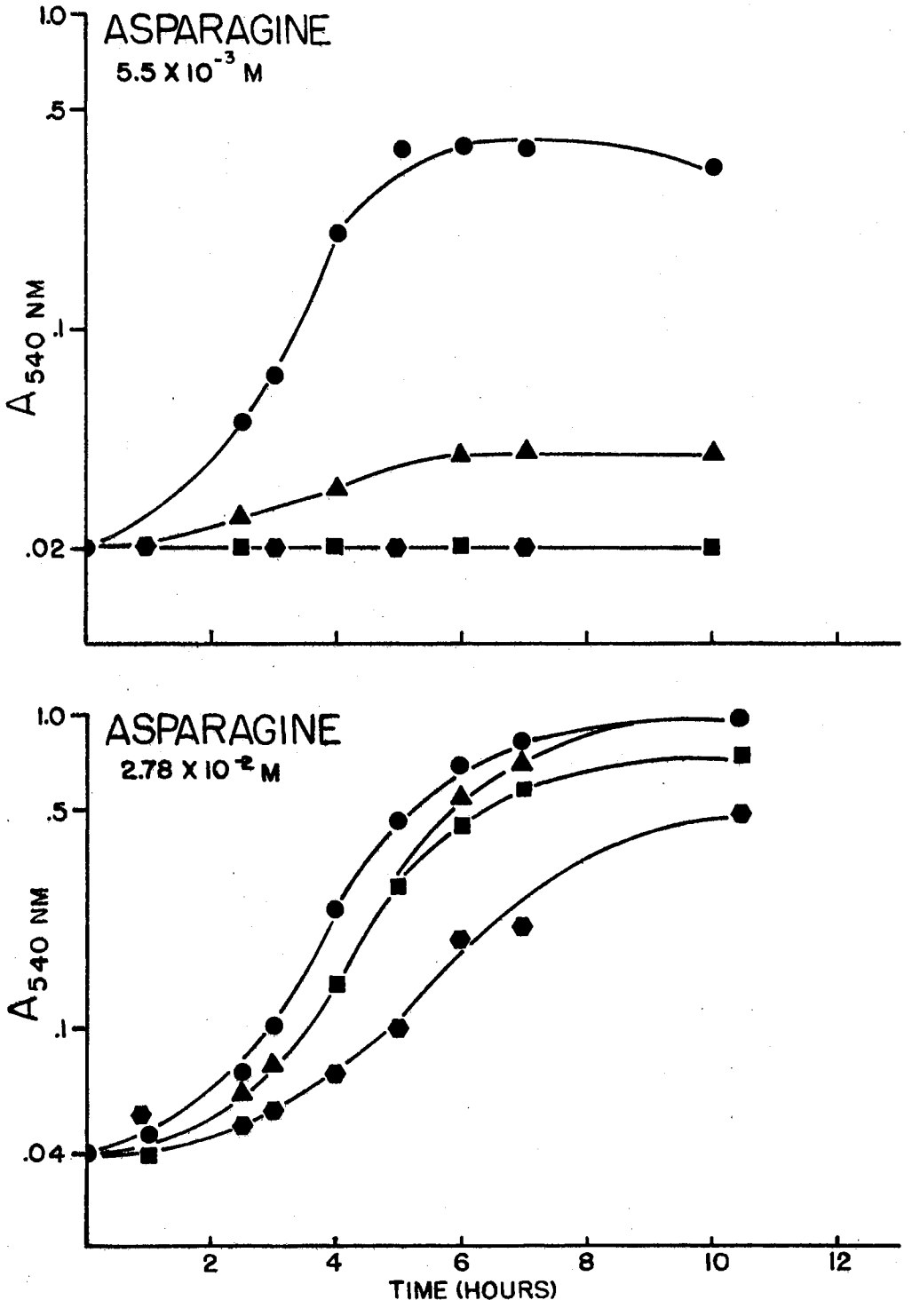

GLUCOSE
5.5 X 10⁻³ M
GLUCOSE
2.78 X 10⁻² M
A540 NM
1.0
.5
.1
.02
.05
2
4
6
8
10
12
TIME (HOURS)

Figure 7. Actinomycin D Sensitivity of P. fluorescens Grown in 2.78×10^{-2} M and 5.5×10^{-3} M Asparagine Minimal Salts Medium

● = cells grown in asparagine minimal salts medium without actinomycin D; ▲ = cells grown in asparagine minimal salts medium with 10 μgm/ml actinomycin D; ■ = cells grown in asparagine minimal salts medium with 20 μgm/ml actinomycin D; ⬢ = cells grown in glucose minimall salts medium with 30 μgm/ml actinomycin D


ASPARAGINE
5.5 X 10⁻³ M
1.0
.5
.1
.02
A 540 NM
ASPARAGINE
2.78 X 10⁻² M
1.0
.5
.1
.04
A 540 NM
2
4
6
8
10
12
TIME (HOURS)

CHAPTER IV

SUMMARY AND CONCLUSIONS

Growth studies using P. fluorescens suggest that changing the carbon source causes a difference in the levels of lipopolysaccharide production. At early logarithmic stage, glucose grown cells show a higher lipopolysaccharide level than do asparagine grown cells; and, with continued incubation, the glucose grown cells produce approximately 40 percent more total lipopolysaccharide than do the asparagine grown cells. Both sets of cells exhibit an increase in lipopolysaccharide from initial to late sampling time and the same rate of increase is seen. When the cells reach the logarithmic phase of growth, the lipopolysaccharide levels remain constant, but as the cells enter the stationary phase, an increase in the lipopolysaccharide levels is seen.

Growth studies in medium containing different concentrations of each carbon source indicate that levels of lipopolysaccharide production reflect not only changes of carbon sources, but also changes in the concentration of each carbon source. From these studies it appears that the concentration of glucose has a more pronounced effect on lipopolysaccharide production than that of asparagine. A two-fold or greater increase is seen from samples taken during logarithmic growth and maximum stationary phase at 0.25, 0.5, and 0.75 percent carbon source. A very slight increase is seen from cells

growing logarithmically when compared to cells in stationary phase 0.1 percent glucose grown cells. This perhaps indicates that as the organisms are grown in substrate levels which exceed the amount needed for metabolism, the "over-abundance" is shuttled into lipopolysaccharide production.

In the asparagine-gradient growth studies, the final levels of lipopolysaccharide production do not reach that attained when glucose is used as growth substrate at any concentration. When asparagine is used as the carbon source, the lipopolysaccharide remains constant at the 0.1 percent concentration, but increases in cells growing logarithmically to cells in stationary phase on the three other concentrations.

At no concentration of the carbon source in the comparative growth studies do the final levels of levels of lipopolysaccharide produced in asparagine grown cells reach the same level as that produced in the glucose grown cells. At the highest concentrations of growth substrate tested, the level of lipopolysaccharide production is 3.5 times greater in the glucose grown cells than in the asparagine grown cells. At the other concentrations, the final level of lipopolysaccharide production is two fold greater in the glucose grown cells than in the asparagine grown cells.

Studies on isolated lipopolysaccharide indicate that the lipopolysaccharides from cells grown on each substrate are qualitatively similar but quantitatively different (Table VI). Rhamnose, glucose, fucose, and galactose are the neutral sugars present in the isolated lipopolysaccharide from both sets of cells at both time intervals tested. The levels of each of the sugars present in the two sets of

TABLE VI

SUMMARY OF QUALITATIVE AND QUANTITATIVE DIFFERENCES IN ISOLATED LIPOPOLYSACCHARIDE

Component	Growth Medium	
	Glucose	Asparagine
Rhamnose	Increase (200%)	N.C.
Glucose	Increase (38-40%)	N.C.
Fucose	Decrease (45%)	N.C.
Galactose	Decrease (38%)	N.C.
Glucosamine	Increase (40%)	Increase (21%)
Galactosamine	Increase (200%)	N.C.
Heptose	Increase (100%)	N.C.
Phosphate	Increase (34%)	N.C.

The lipopolysaccharide was analyzed for both glucose and asparagine grown cells; samples were collected at the early logarithmic and stationary growth phases; the lipopolysaccharide analyzed and differences in the components at the two intervals determined.

N.C. indicates no change.

lipopolysaccharides, however, differ quantitatively both in the initial samples and as they change through the growth cycle.

The lipopolysaccharide isolated from glucose-grown P. fluorescens cells show a two-fold increase of rhamnose from early to late sampling times. In asparagine grown cells, the rhamnose level remains constant. Glucose increases in the glucose grown cells by 38-40 percent. No change is seen in glucose content from early to late sampling times in the asparagine grown cells. Fucose decreases by 45 percent and galactose by 38 percent in the glucose grown cells. The levels of fucose and galactose remain constant in the asparagine grown cells.

Amino sugar analyses indicate that glucosamine and galactosamine are present in both sets of isolated lipopolysaccharide. Glucosamine increases 40 percent and galactosamine increases 200 percent from early to late sampling times in the glucose grown cells. In asparagine grown cells glucosamine increases by 21 percent, but no change is seen in the levels of galactosamine with increased culture age.

From the data quantitating the neutral sugars and amino sugars, as well as the phosphate and heptose determinations, it is evident that much greater changes occur in the lipopolysaccharide when glucose is used as the carbon source than when asparagine is used as the carbon source. The levels of the constituents of the lipopolysaccharide isolated from asparagine grown cells remain in a static state as the age of the culture increases. Glucose grown cells exhibit a greater ability to change the lipopolysaccharide molecule through the growth cycle. This may reflect the relative ease with which glucose is metabolized by the organism. More energy would be needed to convert

asparagine to intermediates that might be placed in the lipopolysaccharide molecule. Therefore, a higher level of lipopolysaccharide is seen in the glucose grown cells.

Since the portions of the lipopolysaccharide molecule that have been investigated comprise the lipopolysaccharide molecule that have been investigated comprise the polysaccharide moieties of the R-core and the O-polysaccharide, the conclusion can be drawn that changing the carbon source may drastically alter the molecular architecture of these two regions. Since antigenic specificities are based on the nature of the sugars in the O-polysaccharide and the linkage between them, changing the carbon source could change the antigenic specificity of the molecule. The serological classification of gram-negative bacteria is based on the O-polysaccharide region (Kaufman, 1954), therefore, one should interpret the serological data with caution.

It has also been shown that lipopolysaccharide acts as a permeability barrier for some molecules such as antibiotics. Changing the growth substrate has been implicated in the sensitivity of P. fluorescens to actinomycin D, and thus the change in carbon source from glucose to asparagine might be expected to change the permeability of the cell envelope. Growth experiments with P. fluorescens in equimolar concentrations of growth substrate show that asparagine grown cells are completely inhibited by the addition of 10 ugm/ml final concentration of actinomycin D. Glucose grown cells, on the other hand, are not inhibited when 30 ugm/ml (final concentration) of actinomycin D is added to the growth medium.

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