STUDIES ON THE LIPOPOLYSACCHARIDE OF A MARINE BACTERIUM

by

Joseph M. DiRienzo

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for
the degree of Doctor of Philosophy

Department of Microbiology
Macdonald College of McGill University
Montreal, Quebec

October 1976

Short Title

LIPOPOLYSACCHARIDE FROM A MARINE PSEUDOMONAD

DiRienzo

A B S T R A C T

Ph.D. Joseph M. DiRienzo Microbiology

STUDIES ON THE LIPOPOLYSACCHARIDE OF A MARINE BACTERIUM

A smooth type lipopolysaccharide (LPS) was extracted from whole cells of a marine pseudomonad (ATCC 19855) with phenol-water. Carbohydrate analysis by gas-liquid chromatography (GLC) of the alditol acetate derivatives of the sugars present established that the LPS contained 3% heptose, 5% glucose, 8% galactose, 8% 2-amino-2-deoxyglucose, 12% 2-amino-2-deoxygalactose, 2% 2-amino-2,6-dideoxyglucose, and 1% of an unidentified monosaccharide. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis experiments demonstrated that the purified LPS isolated was an aggregate composed of three carbohydrate polymers and suggested that these polymers represented various molecular species of the LPS. Lipopolysaccharides isolated from *Escherichia coli* 0111:B4 and *Salmonella typhimurium* LT2, two bacteria which have been used extensively in cell wall studies, showed a similar heterogeneity in the polyacrylamide gel system. The three components from the marine pseudomonad, designated LPS I, II and III, in order of increasing electrophoretic mobility, were isolated and analyzed by GLC. Lipopolysaccharide III was composed of lipid A and the core sugars, heptose, galactose and 2-amino-2-deoxygalactose while lipopolysaccharides I and II represented smooth forms of the molecule each apparently having a different O-antigenic side chain.

It was deduced that the side chain in LPS I consisted of galactose, 2-amino-2-deoxygalactose, 2-amino-2-deoxyglucose, and 2-amino-2,6-dideoxyglucose. It was proposed that the side chain in LPS II was composed of galactose, glucose, and 2-amino-2-deoxygalactose. A homologous cross-reaction was obtained between LPS I and II during double immunodiffusion studies using a whole cell antiserum, however, no precipitin reaction was observed with LPS III. The LPS species were localized in the outer cell wall layers of the marine pseudomonad which represent the periplasmic region, the outer tripartite membrane, and material exterior to the outer membrane.

RÉSUMÉ

Ph.D. Joseph M. DiRienzo Microbiologie

ÉTUDE SUR LE LIPOPOLYSACCHARIDE D'UNE BACTÉRIE MARINE

Un type de lipopolysaccharide (LPS) lisse fût extrait à partir de cellules entières d'une bactérie du genre *Pseudomonas* d'origine marine (ATCC 19855), avec un mélange de phénol et d'eau. L'analyse de l'hydrate de carbone par chromatographie en phase gazeuse-liquide (GLC) des dériviés d'acétate d'alditol du sucre présent a établi que le LPS contient 3% d'heptose, 5% de glucose, 8% de galactose, 8% de 2-amino-2-deoxyglucose, 12% de 2-amino-2-deoxygalactose, 2% de 2-amino-2,6-dideoxyglucose et de 1% d'un monosaccharide non identifié. Des expériences d'électrophorèse employant un gel de polyacrylamide-sulfate dodécyl de sodium (SDS) ont montré que le LPS purifé était un aggrégat composé de trois polymères d'hydrates de carbone et suggèrent que ces polymères représentent diverses espèces moléculaires du LPS. Les lipopolysaccharides extraits de *Escherichia coli* O111:B4 et de *Salmonella typhimurium* LT2, deux bactéries qui ont été abondamment utilisées dans les études de l'enveloppe cellulaire, montrèrent une hétérogenéité similarie dans le système à base de gel d'amide polyacrylique. Les trois composants du LPS de la pseudomonade d'origine marine, désignés LPS I, II et III, par ordre croissant de mobilité électrophorétique, furent isolés et analysés par chromatographie en phase gazeuse-liquide. Le lipopolysaccharide III était composé d'un

lipide A et d'un centre à base de sucres d'heptose, galactose et 2-amino-2-deoxygalactose, alors que les lipopolysaccharides I et II représentaient des formes lisses de la molécule, chacun ayant apparemment une chaîne antigénique-O différente. On a déduit que la chaîne latérale dans le LPS I était composée de galactose, de 2-amino-2-deoxy galactose, de 2-amino-2-deoxyglucose et de 2-amino-2,6-di-deoxyglucose. Il fut proposé que la chaîne latérale de LPS II était formée de galactose, glucose et de 2-amino-2-deoxygalactose. Une réaction croisée homologue entre le LPS I et II fut observée durant des études d'immunodiffusion double utilisant un antisérum préparé à partir de cellules entières, et pourtant, il n'y a pas eu de réaction de précipitines observée avec le LPS III. Les différentes espèces de LPS furent localisées dans les couches extérieures de la paroi cellulaire de la bactérie d'origine marine, que représentent la region périplasmique, la membrane tripartite extérieure et une substance extérieure à la membrane elle-même.

TABLE of CONTENTS

A C K N O W L E D G E M E N T S

The author would like to thank Dr. R.A. MacLeod, Chairman of the Department of Microbiology, Macdonald College, for his interest and continued support during the course of this investigation and for his assistance in the preparation of this manuscript.

Generous appreciation is expressed to Dr. Carl F. Deneke for his helpful suggestions in the initial stages of this project and for stimulating discussions throughout. The author would also like to thank Dr. Jordan M. Ingram for his avid interest and advice.

Dr. Irving DeVoe and Dr. John D. Nelson are especially acknowledged for their generous gifts of *Neisseria meningitidis* lipopolysaccharide and marine pseudomonad whole cell antiserum, respectively.

Appreciation is extended to Dr. Malcolm B. Perry, of the National Research Council of Canada (NRC), Ottawa, for the identification of 2-amino-2,6-dideoxyglucose and Dr. Stanley Martin and the NRC for the use of their fermenter facilities.

The author is indebted to Dr. Charles E. Tanner of the Institute of Parasitology, Macdonald College, for his help and guidance during the immunological study.

Recognition is extended to Mr. James Gilchrist for performing all of the electron microscopy and for printing the electron micrographs.

My wife, Sharon, is warmly thanked for performing the polyacrylamide gel densitometry scans and for assistance and encouragement in the final preparation of this manuscript.

The author expresses sincere thanks to Mrs. Edna Rowell for proficiently and efficiently typing this manuscript and to Mr. J.E. Blais for his technical advice in the operation, maintenance, and construction of some of the equipment.

The author acknowledges the financial assistance of the National Research Council of Canada.

L I S T of T A B L E S

L I S T of F I G U R E S

C L A I M of C O N T R I B U T I O N S to K N O W L E D G E

1. The lipopolysaccharide (LPS), as extracted from whole cells of the marine pseudomonad, was an aggregate composed of three compositionally distinct species. Evidence was presented which implied that two of these species (LPS I and II) contained lipid A, core, and side chain regions while the other (LPS III) consisted of lipid A and core.

2. The LPS species were isolated by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and were shown to be completely homogeneous.

3. The carbohydrate compositions of the LPS species were determined by gas-liquid chromatography of the corresponding alditol acetate derivatives. It was deduced from the molar ratios of the carbohydrate components in the LPS species that the side chain repeating unit in LPS I consisted of a tetrasaccharide containing galactose, 2-amino-2-deoxygalactose, 2-amino-2-deoxyglucose and 2-amino-2,6-dideoxyglucose. It was also inferred from the data that the side chain repeating unit in LPS II was a trisaccharide composed of galactose, glucose and 2-amino-2-deoxygalactose. These conclusions suggested that two different O antigens existed in the same bacterium.

4. Compositional analysis of the LPS suggested that the unusual monosaccharide, 2-amino-2,6-dideoxyglucose, was an O-antigen-specific component.

5. A homologous cross-reaction was observed between LPS I and II when they were reacted with a whole cell antiserum. Consequently, there was a serological cross-reaction between two different O-antigens. No precipitation reaction was obtained with LPS III.

6. The LPS species were localized and quantitated in the periplasmic region, the outer tripartite membrane and in a loosely bound cell wall layer exterior to the outer membrane. They were also present in the growth medium in late logarithmic phase cultures.

7. Attempts were made to examine the biosynthesis of the LPS molecular species. Lipopolysaccharide I was synthesized rapidly and was closely followed, within several minutes, by the appearance of LPS II. Species III did not appear until cultures reached the late logarithmic phase of growth.

8. A system was developed for studying the biosynthesis of LPS in the absence of preformed cell wall. Mureinoplasts and protoplasts of the marine pseudomonad were metabolically active, pro-

ducing high molecular weight macromolecules which were found only in the growth medium.

9. An electron microscopic study of negatively stained samples demonstrated that the marine pseudomonad LPS was disrupted in the presence of SDS and was further dissociated during SDS-polyacrylamide gel electrophoresis.

10. Various electrophoresis buffers were examined for their effect on the migration of the marine pseudomonad LPS during polyacrylamide gel electrophoresis. The results indicated that the electrophoretic mobility of the LPS was due to the presence of SDS in the electrophoresis buffer. The migration of the lipid A and degraded polysaccharide portions of the LPS molecule in the SDS buffer was also tested. The results favored the existence of an SDS-lipid A complex in this system. It was shown that the migration rates of the LPS species were related to the relative amounts of lipid A that were present.

11. It was established that the marine pseudomonad LPS was extensively labeled in the polysaccharide chain when cells were cultured in a medium containing [^{14}C]galactose. The lipid A portion of the molecule was labeled only in the 2-amino-2-deoxyglucose residues. The degraded polysaccharide portion of the

LPS had a specific activity which was ten-fold higher than that of the lipid A.

12. The lipopolysaccharides from the marine pseudomonad variant 1 (smooth) and variant 3 (rough) were identical as determined by SDS-polyacrylamide gel electrophoresis. The spontaneous mutation from variant 3 to variant 1, that occurs with this organism, was not related to LPS structure as is the smooth to rough transition that is observed with some Gram-negative bacteria.

13. An efficient and simple method of removing SDS from non-sulfate-containing biological samples was developed based upon precipitation with $BaCl_2$.

14. It was demonstrated that *Neisseria meningitidis* LPS was homogeneous as visualized on SDS-polyacrylamide gels.

I N T R O D U C T I O N

Lipopolysaccharide (LPS) represents one of the major cell wall macromolecular components in Gram-negative bacteria and blue-green algae. An unusual and intriguing characteristic of many lipopolysaccharides is their complex heterogeneity or self-aggregation. The early studies on the heterogeneity of LPS were concerned with the effects that dissociation of the endotoxin had on its biological activity. For instance, Oroszlan and Mora (1963) discovered that a certain size aggregate of *Serratia marcescens* LPS was necessary to achieve tumor-damaging potential. Other investigators performed more detailed physical-chemical experiments on LPS extracted from the whole cells of a number of bacteria (Olins and Warner, 1967 and McIntire and co-workers, 1969). The results established the macromolecular heterogeneity of lipopolysaccharides from *Azotobacter vinelandii* and *Escherichia coli*. Knowing the chemical composition and probable structure of the components that comprised the LPS aggregate was essential in order to define LPS heterogeneity and to establish the relationship, whether it was biosynthetic or otherwise, among the components. Koeltzow and Conrad (1971) attempted to separate and characterize the LPS components in *Aerobacter aerogenes* and succeeded in showing the presence of a lipid A-core unit and a lipid-A-core side chain form of LPS in this bacterium. Although it was suggested that the rough type LPS was a precursor of the smooth form, convincing

evidence was not presented. Fuller *et al.* (1973) using an LPS isolated from a rough strain of *E. coli* demonstrated the existence of three core structures. One of the three core oligosaccharides appeared to be an LPS biosynthetic precursor, however, this remained to be experimentally established. Other studies, of major importance in LPS research, that were concerned with LPS biosynthesis and translocation from its site of synthesis to the outer cell wall, have completely ignored the possible existence of different species of LPS (Osborn *et al.*, 1972a and Kulpa and Leive, 1976).

It was the aim of the present study to carefully examine the nature of LPS heterogeneity in a marine pseudomonad (ATCC 19855). Besides attempting to isolate the LPS aggregate components and to determine their composition, a special effort was made to establish that LPS heterogeneity was not the result of degradation due to extraction or purification procedures, to localize the components and to demonstrate their *in vivo* biosynthesis. It was hoped that these findings would extend the observations of the other heterogeneity studies. The present study was a continuation of the LPS research begun in this laboratory. O'Leary *et al.* (1972) originally isolated LPS from whole cells of the marine pseudomonad and determined its chemical composition. Later, Nelson and MacLeod (submitted for publication) indicated that the marine pseudomonad LPS may be heterogeneous and they localized the whole cell LPS in the outer cell wall layers by double immunodiffusion experiments. This represented some

of the only available data concerning the composition and heterogeneity of lipopolysaccharides isolated from marine bacteria.

The immediate goal of isolating and characterizing the marine pseudomonad LPS aggregate components was realized and a definite correlation was demonstrated through a comparison of their carbohydrate compositions. Hopefully, this study supplied the sufficient techniques and analytical information necessary to achieve the long range goal of elucidating the biological basis of LPS heterogeneity.

The thesis is divided into four sections, the first section is concerned with characterizing the whole cell LPS. This LPS is termed "whole cell" to distinguish it from the isolated aggregate components designed LPS I, II and III. Part II is a transition section which bridges the study of whole cell LPS and that of its constitutive parts by establishing the methods used in the separation. The characteristics and relationship of the LPS components are examined in section III while the final section initiates the biosynthetic investigation of the LPS species emphasizing the methods that were employed.

LITERATURE REVIEW

BACTERIAL LIPOPOLYSACCHARIDES

Lipopolysaccharides are complex macromolecular components of the Gram-negative cell envelope and have long been recognized as the bacterial endotoxin. Consequently, a great deal of research was devoted to finding efficient methods of isolation and purification of this important compound. Detailed investigations into lipopolysaccharide (LPS) structure and biosynthesis were performed and its location within the cell envelope was studied due to its role as a permeability barrier and as a pyrogenic and immunogenic substance. These subject areas, involved with the study of LPS, will be discussed through an examination of the pertinent literature.

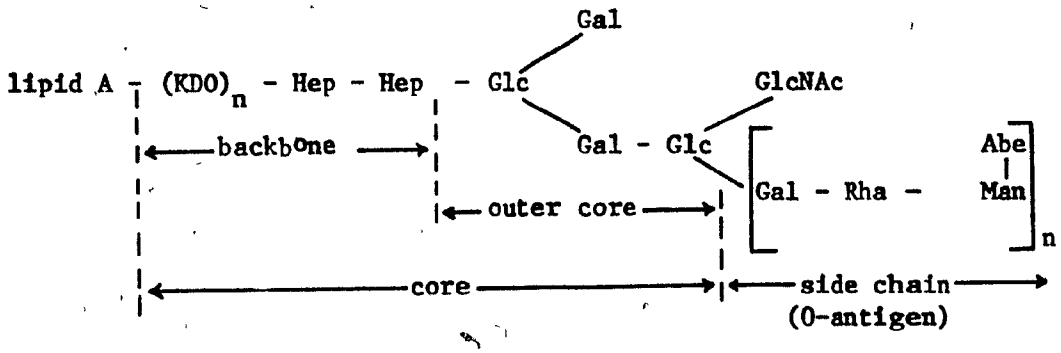
Isolation, purification, and structure

Boivin and Meserobeanu (1935) were the first to isolate a crude preparation of LPS, however, not until the phenol-water procedure of Westphal *et al.* (1952) was developed did a significant upsurge in the compositional and structural studies of LPS unfold. The advantage of this extraction procedure was that the LPS preparation was isolated relatively free of loosely bound protein and lipid. The LPS was extracted in the water phase while the protein and lipid was soluble in the phenol phase. The protein-free LPS preparations that were obtained by this method were more desirable for use in immunological studies; the Boivin-type antigen was too heterogeneous. Many varied extrac-

tion procedures were subsequently employed and a report by Nowotny and co-workers (1963) adequately reviewed and compared them. The phenol-water procedure as originally conceived, is still the most widely used method for the isolation of LPS with the only exception being the phenol : chloroform : petroleum ether procedure for the extraction of LPS from R mutants (Galanos *et al.*, 1969). The latter method was more convenient and less time consuming than the aqueous phenol procedure, but, it did not extract smooth forms of LPS.

When LPS is extracted from cell envelope preparations with aqueous phenol it is essentially pure, however, whole cell extracts are contaminated with nucleic acids because of the solubility of nucleic acids in the water phase. Generally, the nucleic acids were removed with nucleases, by ultracentrifugation, precipitation with cationic detergents such as cetyltrimethylammonium bromide or by a combination of these treatments (Westphal and Jann, 1965). A more recent method, which was discovered for the purification of LPS, was column chromatography on Sepharose gels (Romanowska, 1970 and Rubio and Lopez, 1971). The high molecular weight LPS was eluted in the void volume of the column and the lighter nucleic acids and inorganic components were retained in the included volume.

The general architecture of purified lipopolysaccharide appears to be as follows:



(Osborn, 1969)

The figure presented is of *Salmonella typhimurium* LPS, however, most lipopolysaccharides contain a general structure consisting of lipid A-core-side chain. Detailed structural studies regrettably were, for the most part, conducted on *Salmonella* species and *Escherichia coli*. In the latter, the lipid A region includes two 2-amino-2-deoxyglucose residues as a basal structure to which are attached acetylated long chain fatty acids and hydroxy acids (Burton and Carter, 1964). Although the fatty acids may differ in various strains the glucosamine basal unit appears to be a universal structure. A few exceptions exist, but, the substituted component has not been identified (Wilkinson *et al.*, 1973). In a later study Gmeiner *et al.* (1969) established that in *Salmonella* the lipid A contained a phosphorylated glucosaminyl-glucosamine disaccharide unit linked β (1,6). There was a great deal of speculation concerning the nature of the structural linkage between lipid A and the backbone region. Studies were complicated by the fact that 2-keto-3-deoxyoctulosonic acid (KDO) was extremely acid labile, therefore, it was usually destroyed when attempts were made

to isolate this material. Despite these difficulties, Dröge and co-workers (1970) were able to demonstrate that in *Salmonella* the bridge connecting lipid A to the core consisted of a KDO trisaccharide. Heptose was bound to the non-reducing terminal KDO and the KDO at the reducing end of the carbohydrate chain was ketosidically linked to the lipid A. The third KDO molecule formed a branch off of the reducing end of the disaccharide and it was also established that phosphorylethanolamine was attached to this KDO branch.

The structure of the core fraction was elucidated through the use of rough type mutants. Lüderitz *et al.* (1965) discovered that strains of *Salmonella* that gave rise to colonies having a rough morphology contained LPS which lacked O- specific side chains and they classified these cells as R (rough) forms. It was found that strains which had a smooth appearance contained side chains and were designated S (smooth) forms. Intensive immunological studies were performed on a series of *Salmonella* and *E. coli* mutant strains and the results suggested that the core region was usually species-specific (Lüderitz and co-workers, 1966). The core regions of LPS from various bacterial species were found to be compositionally but not structurally different. Structural investigations showed that the O-antigenic side chain of the LPS molecule was usually composed of repeating units of an oligosaccharide. Studies on this fraction were performed using chemical and immunological procedures and the serological specificity of the O-antigens from strains of *E. coli* and *S. typhimurium* has been

thoroughly reviewed by Lüderitz *et al.* (1966). The collection of information concerning the structure of the side chains from strains of these two bacterial species led to an elaborate system and listing of chemotypes which was of immense importance in clinical investigations.

The information which led to the formulation of the structure of bacterial lipopolysaccharides was obtained primarily from studies on *E. coli* and *Salmonella* species. Lipopolysaccharide research using other Gram-negative bacteria was devoted primarily to its chemical composition as opposed to its actual structure. The initial investigations on the composition of *Pseudomonas* lipopolysaccharides was confined to *P. aeruginosa* (Clarke *et al.*, 1967; Fensom and Gray, 1969; and Chester *et al.*, 1972). These studies established that the chemical composition of *Pseudomonas* LPS was basically the same as that found in enteric bacteria. In addition, Humphreys *et al.* (1972) demonstrated that the lipid A in *P. aeruginosa* was not unusual in composition and Chester and Meadow (1973) showed that the serological specificity of the LPS from this organism was based on the composition of the O-antigenic side chains. Wilkinson and co-workers (1973) published a comparison of the LPS compositions of a number of *Pseudomonas* species. The results showed that there was a marked absence of 2-deoxy sugars, as compared to enteric lipopolysaccharides, and there was also a greater abundance of 2-amino-2,6-dideoxy sugars. However, the overall composition was demonstrated to be similar to other bacterial

lipopolysaccharides.

Practically no information was available concerning the structure and composition of LPS from marine bacteria. In *Vibrio marinus* only lipid A, KDO, heptose and glucose were reported with no quantitation of these components (Deneke and Colwell, 1973). They did establish, however, that this LPS was of the R form. Mongillo *et al.* (1974) showed that the LPS from a marine bacterium contained lipid A, glucose and galactose but lacked KDO and heptose. It seems odd that they were able to isolate lipid A by a mild acid hydrolysis with no acid labile ketosidic linkage present. The data suggested that the LPS was an R type but the lack of the specific backbone sugars makes an interpretation difficult. O'Leary and co-workers (1972) performed detailed chemical analysis on a marine pseudomonad LPS and the results were similar to those obtained for other *Pseudomonas* lipopolysaccharides.

Heterogeneity

It has been known ever since the phenol-water extraction procedure was first developed that LPS preparations, isolated by this method and purified by the removal of nucleic acids, were amorphous aggregates of extremely large size (Westphal and co-workers, 1952 and Davies *et al.*, 1954). Numerous studies were subsequently initiated in an attempt to analyze and define these LPS aggregates. As will be evident, through a review of the literature, the question of LPS aggregation, or heterogeneity, was not easy to accurately answer. It had to be

established whether heterogeneity represented physical, chemical or biological differences, or a combination of these, within extracted LPS preparations.

It was proposed, in early studies, that a certain aggregate size, as determined by analytical ultracentrifugation, may be necessary for LPS pyrogenicity or tumor-damaging ability (Ribi *et al.*, 1960 and Mora and Young, 1961). These studies concluded that LPS from *Salmonella enteritidis* and *Serratia marcescens*, respectively, exhibited considerable heterogeneity. Oroszlan and Mora (1963) used the techniques of cesium chloride density gradient centrifugation and analytical ultracentrifugation to examine the LPS from *S. marcescens*. Two fractions were obtained from the cesium chloride gradient. The fraction which had the lower density produced tumor necrosis in mice while the higher density fraction did not. When the biologically active low density fraction was analyzed in sedimentation velocity experiments the schlieren pattern showed a large amount of fast sedimenting material (15.8s) and a relatively small amount of slowly sedimenting material (3.9s). However, when the experiment was repeated using the same fraction treated with sodium dodecyl sulfate (SDS) a single peak which had a sedimentation coefficient of 2.8s was obtained. The SDS-treated low density fraction did not produce tumor necrosis. If the SDS was removed the material reaggregated to form 3.5s and 18.8s peaks and regained tumor-necrosing ability. These workers concluded that *S. marcescens* LPS was dissociated by SDS to a "subunit" which was represented

by the 2.8s material. This "subunit" was responsible for tumor-necrosing ability but only in an aggregated state. The aggregated state consisted of the 15-18s material. The fact that SDS dissociated the LPS suggested that the aggregate was held together by hydrophobic bonds. The results can be interpreted in two different ways. According to the bioassay, tumor-necrosing ability, the LPS would have to be classified as heterogeneous since the low density fraction exhibited tumor necrosis but the high density fraction did not. However, based on the sedimentation velocity experiments performed in the presence of SDS, the LPS apparently consisted of a single "subunit" which simply aggregated when SDS was removed, to form the fractions having various buoyant densities. Depending upon which criterion was used the LPS could be termed heterogeneous or homogeneous.

The investigation by Oroszlan and Mora (1963) was extremely significant because it set the trend or direction that subsequent heterogeneity studies would follow. McIntire *et al.* (1967) treated an *E. coli* LPS with SDS, subjected the LPS to alkaline hydroxylaminolysis, or succinylated the LPS and, in each case, showed little or no evidence of heterogeneity in sedimentation velocity studies as well as in moving boundary electrophoresis and agarose gel permeation chromatography. Furthermore, SDS did not reduce the pyrogenicity of the LPS as measured in rabbits. Rudbach and Milner (1968) examined lipopolysaccharides from *E. coli* and *Salmonella enteritidis* by analytical ultracentrifugation and found that SDS dissociated the LPS preparations

from a 7s fraction to 1-2s fractions. However, LPS that was treated with concentrations of SDS ranging from 0.25 - 2% did not show any alteration in pyrogenicity as measured in rabbits. In another study *Shigella flexneri* LPS was treated by heating in citrate buffer (Hannecart-Pokorni and colleagues, 1970). Dissociation was determined, as in previous investigations, by analytical ultracentrifugation. A fraction which had a sedimentation coefficient of 113s at 85 C underwent a series of thermal transitions as the temperature was increased to 100 C. A similar dissociation occurred when the LPS was treated with sodium deoxycholate (NaD). It was shown that the smallest fraction that was obtained in the sedimentation velocity experiments still retained toxicity and the ability to act as a phage receptor. This thermal treatment may cause hydrolysis of some labile bonds within the LPS aggregate and must therefore be interpreted with caution. Olins and Warner (1967), in a detailed physical study, showed that LPS, isolated from *Azotobacter vinelandii*, was dissociated to a fraction of 134,000 daltons with ethylenediaminetetraacetate (EDTA). After dialysis the LPS reaggregated having a molecular weight of 873,000 daltons. When the LPS was treated with a mixture of EDTA and SDS a dissociated fraction with a molecular weight of 65,600 daltons was obtained. Thus, the original findings of Oroszlan and Mora (1963) were not extended by these subsequent studies. Most of these investigations did, however, confirm that LPS preparations could be dissociated to a single "subunit". There was no indication that this "subunit" could be

further separated by other analytical methods. Also, these latter studies did not substantiate the earlier conclusion that SDS-dissociated LPS loses its biological activity.

The observation that an LPS preparation could be dissociated and then reaggregated (Oroszlan and Mora, 1963) was also reported by several other workers. Ribi *et al.* (1966) extracted LPS from *E. coli*, *Bordetella pertussis* and *S. enteritidis* and found that these preparations were dissociated by NaD to approximately 2×10^4 dalton fractions. If the NaD was removed by dialysis the LPS preparations reaggregated to approximately $5 - 10 \times 10^5$ daltons. The dissociation and reaggregation of the LPS was confirmed by electron microscopy using negatively stained samples. Similar results were obtained with lipopolysaccharides which were isolated from several species of *Salmonella* (Hannecart-Pokorni *et al.*, 1973). These workers also used the methods of analytical ultracentrifugation and electron microscopy to observe the dissociation and reaggregation of the lipopolysaccharides.

The available information obtained from the previous studies still did not lend itself to a clear definition of LPS heterogeneity. In fact, it was difficult to discern whether LPS samples were heterogeneous. It appeared that LPS preparations were merely aggregates of a single species which was found when LPS was dissociated and then subjected to analytical ultracentrifugation. Physical studies alone could not be relied upon to answer the question of LPS heterogeneity;

chemical analysis of the isolated species was also necessary. Leive *et al.* (1968) found that 30 - 50% of the LPS in *E. coli* could be extracted from the cells by treating them with EDTA. The rest was removed by the phenol-water extraction procedure. The compositions of these two LPS fractions were extremely similar, containing equivalent molar ratios of KDO, heptose, glucose, galactose and colitose. The EDTA-extractable LPS was separated into two additional fractions by ultracentrifugation and analysis of the latter fractions indicated that there was a slight difference in the relative molar ratios of their carbohydrate components. The LPS fractions released by the two different extraction procedures probably indicate the existence of a free form and a bound form of LPS. The analytical ultracentrifugation analysis suggested that the total LPS (both EDTA and phenol-water extracted) may be heterogeneous, however, this analysis was not performed on phenol-water extracted LPS alone. This was the first study in which the separated LPS fractions were analyzed chemically. McIntire and co-workers (1969) chromatographed *E. coli* LPS in 1% NaD on a column of Sephadex G-100. Three major peaks were obtained and gel filtration studies indicated that they had different molecular weights. However, equilibrium sedimentation data indicated that they were approximately the same size, 113,000 - 118,000 daltons. Chemical analyses were performed on the materials from the three peaks and the analytical data showed that there were distinct quantitative but not qualitative differences among the neutral sugars, phosphorous,

lauric acid and β-hydroxymyristic acid. Koeltzow and Conrad (1971) also showed that LPS was heterogeneous, however, their work was interesting since they employed Triton X-100 as the dissociating agent. Many of the LPS heterogeneity studies utilized ionic compounds such as SDS and NaD. A crude LPS preparation from *Aerobacter aerogenes* was treated with 1% Triton X-100 and fractionated on a DEAE-cellulose column with the detergent present in the eluting buffer. Three LPS fractions were obtained, one of which contained very little total carbohydrate. Detailed chemical analyses were performed on the remaining two major LPS fractions demonstrating that they both contained lipid A portions, KDO and heptose It appeared that one LPS fraction represented a rough type LPS (containing lipid A and core only) and the other may be a smooth form (lipid A-core-side chain). It was hypothesized that the rough type LPS fraction represented a biosynthetic intermediate. This report was one of the first to attempt to define the components of an LPS aggregate in light of their structural relationship to the whole LPS. It would seem that a comparison of the carbohydrate and lipid A contents of the LPS fractions would be the only meaningful way to describe LPS heterogeneity. Since dissociated LPS preparations were separated into more than one species by gel permeation and ion exchange chromatography the resolution that was obtained in earlier studies, using the method of analytical ultracentrifugation, seemed to be inadequate.

In other LPS heterogeneity studies the technique of polyacrylamide gel electrophoresis was employed. Reports in the literature indicated that LPS could be electrophoresed on polyacrylamide gels (Rothfield and Pearlman-Kothencz, 1969 and Osborn *et al.*, 1972b). These experiments did not use extracted LPS but Gram-negative cell wall complexes and the results were not elaborated on for the purpose of demonstrating LPS heterogeneity. DiRienzo *et al.* (1974) used SDS treatment in combination with the technique of polyacrylamide gel electrophoresis to show that lipopolysaccharides from several bacteria were heterogeneous, thus confirming the results obtained in the previously mentioned studies. This investigation established that heterogeneity was not the result of extraction or preparative procedures. These results were later supported by Jann *et al.* (1975) who also implied that the LPS fractionation that was obtained on SDS-polyacrylamide gels was the same as that observed on Sephadex columns. Later, DiRienzo and MacLeod (1976),using LPS isolated from a marine pseudomonad, determined the carbohydrate compositions of the LPS fractions that were isolated from the SDS-polyacrylamide gels. The carbohydrate compositions suggested that the LPS from this bacterium was an aggregate containing three compositionally distinct species.

In a separate study, LPS from a rough strain of *E. coli* was hydrolyzed, then dephosphorylated and subjected to paper chromatography (Fuller and co-workers, 1973). The oligosaccharide mixture was resolved into three components which were exhaustively purified and structurally

analyzed. Two of the oligosaccharides that were isolated were shown to be involved in the process of core elongation in LPS biosynthesis, however, no role could be assigned to the remaining oligosaccharides. The results have to be carefully evaluated since hydrolytic procedures were involved in the isolation of the LPS components. Emphasis was now being placed on compositional and structural variation within LPS preparations, as isolated from whole cells. As more refined techniques were applied to separate LPS species a clearer picture of the heterogeneity of lipopolysaccharides was obtained.

Most of the studies on LPS heterogeneity have been conducted on LPS extracted from enteric bacteria. Lipopolysaccharide from *Pseudomonas aeruginosa* was also found to be heterogeneous. A protein-LPS complex from this bacterium was chromatographed on Sepharose 4B (Rubio *et al.*, 1973). The complex was treated with NaD and chromatographed with and without NaD in the elution buffer. If NaD was not included in the buffer three peaks were obtained and when it was present only one peak, corresponding to that of the lowest molecular weight, was observed. The chromatographic results were confirmed by electroimmunodiffusion experiments and equilibrium sedimentation analysis. Day (1973) examined the behaviour of *P. aeruginosa* LPS during isoelectric focusing in a 5% ampholine ampholite carrier (pH range 3 to 10). The LPS, dissolved in 6 M urea, spread over the entire range of the ampholite with the majority of the LPS dispersed from pH 7.0 to 10.

Many of the LPS preparations that were examined demonstrated heterogeneity which tends to invalidate any attempt to determine the molecular size of LPS which is extracted from whole cells. Davies *et al.* (1954) determined that whole cell aggregates were from 1 to 24 × 10^6 daltons. Lüderitz and co-workers (1966) reviewed some of the additional molecular size data for bacterial lipopolysaccharides and some of the methods that were used for LPS disaggregation in order to obtain more accurate analyses. For instance, alkali treatment causes disaggregation within a range of 2 × 10^5 daltons with a concurrent removal of fatty acids. Alkali cleaves long chain fatty acid esters which could be responsible for LPS aggregation through Van der Waals attraction. Sodium dodecyl sulfate, on the other hand, disrupts hydrophobic bonding within the lipid A portion of the LPS and hydrogen bonding within the polysaccharide chain. The molecular size of an LPS isolated from a rough strain of *Salmonella* was calculated as 10,300 daltons by sedimentation equilibrium analysis (Romeo *et al.*, 1970). The LPS was acetylated in order to make it soluble in benzene which was used as the solvent during measurements. It was established that the acetylation process did not cleave covalent bonds.

Biosynthesis

The most complete LPS biosynthetic scheme was elucidated using the genus *Salmonella*. The isolation of mutants which were blocked in the synthesis of specific nucleotide sugars facilitated the study of

LPS biosynthesis when it was discovered that these cells were also LPS-deficient (Fukasawa and Nikaido, 1961 and Nikaido, 1962). When an exogenous supply of the specific sugar was available a complete LPS was then synthesized by the cells. Osborn and co-workers (1962), using just such a mutant, demonstrated that galactose was added to the core through the action of a transferase enzyme. The enzyme activity was localized in the cell wall particulate fraction following sonication of the whole cells. The reaction was stimulated by the presence of Mg^{2+} or Mn^{2+}. In a series of investigations it was then shown that the remaining core sugars were added to the deficient LPS in a sequential manner by the action of a series of glycosyl transferase enzymes (Rothfield *et al.*, 1964 and Osborn and D'Ari, 1964). As each monosaccharide was added the newly elongated core acted as an acceptor for the next sugar therefore assuring that the sugars were added in a specified order.

The cell envelope preparations were active acceptors for the transferase enzymes, however, purified LPS was not. If cell membrane lipids were added back to the purified LPS preparation the acceptor activity was restored (Rothfield and Horecker, 1964). It was discovered that the lipid affected the strcture of the LPS and not the enzyme in the incubation mixture. The action of membrane lipid on the LPS biosynthetic mechanism has been thoroughly summarized by Rothfield and Romeo (1971). The major cell envelope phospholipid,

phosphatidylethanolamine (PE),was the active compound in the lipid fraction which was involved in the transferase system. The transferase enzymes bind to the LPS-PE complex which could be formed artificially by the heating and slow cooling of a mixture of LPS and PE (Rothfield and Pearlman, 1966). Monolayer experiments indicated that the phospholipid organized itself into a binary film which was easily penetrated by the LPS. This molecular arrangement was an active acceptor, without prior heating of the complex, when enzyme and substrate were added. These findings suggested that the binary LPS-PE complexes could represent a mixed bilayer in the cell envelope (Rothfield and Horne, 1967). The components of the system had to be added in a specific sequence in order to induce the transferase enzyme activity; that is, the LPS-phospholipid complex had to be formed prior to the addition of the enzyme. If enzyme was added to the film before LPS it appeared to block the incorporation of the LPS (Weiser and Rothfield, 1968).

The O-antigenic side chain of the LPS molecule was biosynthesized separately from the core by a completely different mechanism than that pertaining to core synthesis. As in the study of core biosynthesis, the literature pertaining to the synthesis of the side chain has been extensively reviewed by a number of workers (Osborn, 1969 and Rothfield and Romeo, 1971). A lipid was implicated as the carrier molecule (glycosyl carrier lipid; GCL) in the biosynthetic reactions

of the O-antigen (Wright *et al.*,1965 and Weiner and co-workers,1965). The single tetrasaccharide side chain unit was synthesized by the sequential transfer of nucleotide sugars to the phosphorylated GCL by a series of synthetase enzymes. First, uridine diphosphate-galactose was transferred to the carrier lipid with the release of uridine monophosphate. Consecutively, thymidine diphosphate-rhamnose, guanosine diphosphate-mannose and cytidine diphosphate-abequose (3,6-deoxy-D-galactose) were added to form a mannose-rhamnose-galactose-P-P-GCL unit.
abequose

The designation P-P-GCL represents the pyrophosphoryl derivative of the GCL. These units were polymerized by an O-antigen polymerase to form a complete side chain repeating unit with the subsequent release of P-P-GCL from each individual tetrasaccharide unit (Bray and Robbins, 1967). The O-antigen was then attached to the core oligosaccharide through the action of a LPS ligase (Osborn, 1969). Nikaido (1969) demonstrated that the site of attachment of the O-antigen to the core was the glycosyl residue rather than the terminal N-acetylglucosamine.

Little is known about the biosynthesis of the lipid A portion of the LPS molecule since a lipid A-deficient mutant has not yet been isolated. Recently a mutant strain of *Salmonella* was isolated which contains a defective lipid A which is under-acetylated and under-phosphorylated (Osborn, unpublished observations). Perhaps, the further study of this mutant will provide some insight into the biosynthesis of lipid A.

Biosynthetic information pertaining to the backbone region of the LPS is also incomplete, however, some data is available. Edstrom and Heath (1964) showed that cytidine monophosphate-KDO was the acceptor for the outer core sugars in *E. coli*. Rick and Osborn (1973) isolated a mutant of *S. typhimurium* which was defective in the synthesis of KDO. The mutant had an altered KDO-8-phosphate synthetase which, under normal conditions should convert D-arabinose-5-phosphate to KDO-8-phosphate. Analysis of the lipid A in this mutant showed a significant reduction in the degree of acetylation. Apparently the attachment of KDO to the deficient lipid A is necessary for the complete synthesis of lipid A.

During the study of heptose biosynthesis and the role it plays in core completion it was discovered that in several *Salmonella minnesota* R mutants, core elongation was prevented at the position where galactose should be added to the glucose residue even though the galactose transferase enzyme was present. It was found that the heptose residues were not phorphorylated and when phosphate was enzymatically added core elongation continued (Mühlradt *et al.*, 1968). Further investigations demonstrated that an ATP-dependent LPS phosphorylating enzyme was present and therefore the addition of phosphate to heptose had to occur before the complete core could be synthesized (Mühlradt, 1969). The heptose biosynthetic pathway was determined in *S. typhimurium*, however, the method by which it was linked to lipid

A-$(KDO)_3$- still remains unknown (Eidels and Osborn, 1974).

The localization of the LPS biosynthetic enzymes and the site of the biosynthesis of core and O-antigen have been obscure for some time. Some processes appeared to take place in the cell wall while other conditions favoured the cytoplasmic membrane. Procedures were perfected to effect the separation of the outer membrane from the cytoplasmic membrane (Osborn *et al.*, 1972_b). This procedure was used in conjunction with pulse-chase labeling experiments to localize the site of LPS biosynthesis and then to follow the translocation of LPS to its structural position in the cell envelope (Osborn *et al.*, 1972_a). By using a mutant strain of *S. typhimurium* in which the O-antigen could be specifically labeled with radiolabeled mannose it was shown that the side chain oligosaccharide was rapidly synthesized at the level of the cytoplasmic membrane and then, by an unknown mechanism, moved out into the cell wall. Attempts were also made to localize the O-antigen biosynthetic enzymes. Galactose-P-P-GCL synthetase, the first enzyme in the O-antigen biosynthetic pathway, O-antigen polymerase and O-antigen ligase were located only in the cytoplasmic membrane preparation. A second mutant was used in pulse-chase experiments to examine the biosynthesis of the core region. Again, as in the case of the side chain, the core was rapidly synthesized in the cytoplasmic membrane then, within two minutes, it traveled out into the cell wall. Two core biosynthetic enzymes, glucosyl transferase I and galactosyl

transferase I were difficult to localize. Since they were partially solubilized during the separation of the membrane and cell wall the activities of these enzymes were found in both of these cell envelope fractions. Additional labeling studies suggested that the core and core side chain were assembled at the cytoplasmic membrane and consequently were translocated as a complete unit. The fact that the O-antigen ligase was found to be firmly membrane bound supports the finding that core and side chain were assembled at the membrane level. Whether the completed LPS molecules were translocated in association with protein and phospholipid has not been established.

Translocation of lipopolysaccharide to the outer membrane

The actual mechanism of LPS translocation to its position in the outer cell envelope has not been elucidated. The current theory is that the LPS travels along specific attachment sites between the cytoplasmic and outer membranes. These contact sites were first identified in *E. coli* when plasmolyzed cells were fixed in the presence of sucrose and examined, in thin section, by electron microscopy (Bayer, 1968b). Cross sections of the contact sites showed them to be composed of the triple-layered membrane having a thickness of approximately 90Å, a dimension which was consistent with the thickness of the cytoplasmic membrane. The contact sites contained a central core with a diameter of 40Å. Evidence in support of the proposal that these areas of attachment could allow the transport of molecules stems from the

observations that bacteriophages T_1 to T_7 adsorbed almost exclusively to the areas of the bacterial wall which showed a connection with the cytoplasmic membrane (Bayer, 1968a).

Mühlrádt and co-workers (1973) combined the use of ferritin-labeling techniques with a mutant of *S. typhimurium* that could be specifically labeled in the LPS, to supply strong support for the concept that LPS translocation occurred only at these previously shown adhesion areas. The mutant cells that were used in the experiments synthesized only wild type LPS when exogenous galactose was added to the defined growth medium. By pulsing the cells with galactose for a short period of time and then examining the binding sites of ferritin-labeled antibodies, made against wild type LPS, it was found that the ferritin attached only to the cell surface areas that corresponded to the bridges formed between the two membranes upon plasmolysis. Because the antibody would not attach to the deficient LPS, it was established that these antibody binding sites represented translocation sites containing only newly formed LPS. It was determined that 86% of the ferritin patches on the cell surface were found over adhesion sites. It was proposed that the newly synthesized LPS was then dispersed over the bacterial surface by the process of lateral diffusion. If the experimental cells were incubated at 0° C the "new" LPS remained in clusters at the translocation sites, however, when the experiment was performed at 37 C the LPS spread, from these

sites, over the cell surface (Mühlradt *et al.*, 1974). This diffusion was also observed under conditions in which LPS biosynthesis was prevented and when cellular metabolism was blocked by azide poisoning. They proposed that the phospholipids of the outer membrane served as a medium for the lateral diffusion of the LPS. At 0° C the fatty acids of the phospholipids and the lipid A would be too rigid to allow for LPS mobility. In another study, which used a different approach, Kulpa and Leive (1976) made use of the density difference between deficient and complete LPS, produced in a mutant of *E. coli*, to show how LPS was translocated from the cytoplasmic membrane to the outer cell envelope. This mutant of *E. coli* synthesized a deficient form of LPS which lacked an O-antigenic side chain. When exogenous galactose was added to a culture of this mutant the galactose was specifically incorporated into the LPS. Only then could the LPS be completed by the addition of the side chain. This mutant was similar to the *S. typhimurium* mutant described by Nikaido (1962). An LPS molecule that contained a repeating side chain would be considerably more dense than one that did not. These two forms of LPS could then be separated on sucrose density gradients due to this difference in densities. The density of the outer membrane was examined when cells were grown without galactose. The culture was then pulsed with galactose and areas of the outer membrane rapidly became more dense. Removal of the exogenous galactose resulted in the disappearance of the dense outer

membrane. According to their interpretation of the results, the LPS must enter the outer membrane at specific regions in a non-random fashion. If the LPS entered the outer membrane in a random manner the sucrose density gradient profile would show a single peak, at any one time, that shifted gradually from a lower to a higher density. If the LPS entered the membrane at discrete regions then the high density peak would appear very rapidly in the gradient profile and both the high and low density peaks would be observed at the same time. The latter situation appeared to explain the results that were observed . However, the results are far from being conclusive and it would seem that a great deal of work is necessary before the mode of LPS translocation can be confirmed.

Localization and organization of lipopolysaccharide in the cell envelope

Until methods were developed for effectively removing the cell wall from Gram-negative cells and separating it from cytoplasmic membrane LPS was localized with the use of the electron microscope. *Veillonella* cells exhibited an outer double-track structure in thin section preparations. Following extraction of the cells with phenol-water the outer membrane was visually absent and the cells maintained their shape (Bladen and Mergenhagen, 1964). More detailed investigations using ferritin-labeled antibody demonstrated the presence of LPS on the inside of the outer membrane as well as on the cell surface

(Shands, 1966). A whole cell antiserum was used by Nelson and MacLeod (submitted for publication) to detect the presence of LPS in the isolated cell wall layers of a marine pseudomonad. They were able to localize the LPS in the periplasmic region, the outer double-track and in a loosely bound outer layer. Density gradient experiments suggested that the LPS may be associated with protein and phospholipid. The above mentioned studies support the organizational concept of the Gram-negative cell wall as illustrated by Costerton and co-workers (1974) in which LPS is found on the inside of the outer membrane extending into the periplasmic space. A more detailed view of the concept, in which the hydrophobic portion of the LPS molecule (lipid A) penetrates inside the outer membrane bilayer structure and the polysaccharide portion extends in a perpendicular arrangement from the bilayer, was experimentally supported by monolayer studies with purified LPS and phospholipid (Romeo *et al.*, 1970).

The molecular organization of the Gram-negative cell envelope remains to be established. It has been proposed that LPS exists *in vivo* as a complex with protein and phospholipid (Knox *et al.*, 1966 and Knox and co-workers, 1967). Extracellular complexes were believed to be excreted cell wall fragments. Rothfield and Pearlman-Kothencz (1969) analyzed a protein-LPS-phospholipid complex which was excreted into the growth medium by cells of *S. typhimurium*. They established that the protein that was excreted as part of the complex was only "old"

protein since a phospholipid-LPS complex continued to be excreted when protein synthesis was prevented with chloramphenicol or by amino acid starvation. Consequently, it was concluded that LPS and phospholipid were inserted into the cell envelope independently of wall protein. These results tended to rule out the possibility that the cell wall is assembled from protein-lipid-LPS subunits. Other investigations have demonstrated the existence of covalent protein-LPS complexes which could be extracted from whole cells. Just such a complex was released from *P. aeruginosa* cell walls by treatment with EDTA and it exhibited a single peak upon analytical ultracentrifugal analysis and gel filtration (Rogers and co-workers, 1969). Polyacrylamide gel electrophoresis of the complex revealed a single band which co-stained for both protein and carbohydrate. Rubio *et al.*, (1973) further characterized this protein-LPS complex and discovered that it was actually composed of three protein-LPS components. Rogers (1971) reported the isolation of an LPS-protein complex from *E. coli* by extraction of whole cells with 0.1 M Tris at 60 C and also presented evidence that the complex contained three protein-LPS fractions. Wu and Heath (1973) used 1% SDS to extract a protein-LPS complex from a mutant of *E. coli* 0111:B4. The protein had an approximate molecular weight of 14,000 daltons and was enriched in aspartate and glutamate. It was indicated that several of these complexes were observed in the wild type parent organism. A non-covalent complex composed of polysaccharide, protein, lipid and LPS was isolated from *Neisseria meningitidis* by Zollinger and co-workers

(1972) by treating the cells with EDTA at 60 C. An identical complex was located in the cell medium as well . Chemical analysis, electron microscopy and SDS-polyacrylamide gel electrophoresis of both complexes indicated that they were similar. Electron microscopic examination showed that the complex that was isolated from the medium had the same appearance as the outer trilaminar membrane from this bacterium.

An interesting relationship between LPS and periplasmic enzymes was proposed by Day and Ingram (1975). *Pseudomonas aeruginosa* alkaline phosphatase formed as artificial hydrophobic complex with LPS extracted from the organism. The activity of the complex, constructed from purified cell wall components, approached the activity of the crude complex that was found in the culture filtrate from *P. aeruginosa*. The associated release of alkaline phosphatase and LPS was observed in other bacteria as well (Lindsay *et al.*, 1973). A possible structural relationship between LPS and cell wall associated enzymes in the Gram-negative cell envelope has been proposed (Costerton and co-workers, 1974).

MATERIALS and METHODS

Cultures and media

The organisms used in this study were both variants 1 and 3 of a marine pseudomonad (ATCC 19855) which was designated B16 (Gow *et al.*, 1973) and was classified as *Alteromonas haloplanktis* by Reichelt and Baumann (1973). Additional cultures included *Escherichia coli* O111: B4, Macdonald College Culture Collection No. 168; *Salmonella typhimurium* LT2 (ATCC 19585); and *Pseudomonas aeruginosa* (ATCC 9027). The variants of the marine pseudomonad were cultured in a medium containing 0.8% nutrient broth (Difco), 0.5% yeast extract (Difco), 0.22 M NaCl, 0.026 M $MgCl_2.6H_2O$, 0.01 M KCl and 0.1 mM $Fe(SO_4)_2(NH_4)_2.6H_2O$. This medium was referred to as nutrient broth-yeast extract complex medium. In some experiments this complex medium was modified to contain 0.3 M NaCl and 0.05 M $MgCl_2$. Other experiments employed a medium containing 1% trypticase (BBL), 0.3 M NaCl, 0.05 M $MgCl_2$, 0.01 M KCl and 0.1 mM $Fe(SO_4)_2(NH_4)_2.6H_2O$ and was designated trypticase-salts medium. A chemically defined medium was used in mutant isolation studies and it consisted of 2% succinic acid (neutralized with concentrated NH_4OH), 0.05 M Tris-HCl, 0.3 M NaCl, 0.05 M $MgSO_4$, 0.01 M KCl, 1.0 mM $CaCl_2$, 1.0 mM $(NH_4)_2HPO_4$ and 26 µM $Fe(SO_4)_2(NH_4)_2.6H_2O$. In some cases these media were supplemented with 1% D(+) galactose (Sigma). Solid medium was made by adding 1.5% agar to the liquid medium. All the other organisms were grown in a medium composed of 0.8% nutrient

broth and 0.5% yeast extract. Unless specified, batch cultures were inoculated from agar slants into 10 ml of the appropriate medium contained in 50 ml flasks and were incubated on a rotary shaker for 8 h at 25 C for the marine pseudomonad and 30 C for the remaining bacteria. After the 8 h growth period the 10 ml cultures were poured into 250 ml of medium contained in 2 liter flasks. Cells were harvested between mid and late logarithmic phase of growth as determined with a Coleman Junior spectrophotometer at 660 nm. In order to obtain labeled LPS from the marine pseudomonad, [^{14}C]galactose, 0.05 µCi/ml of medium (63.5 µCi/µmole), was added to the 2 liter growth flasks immediately before inoculation.

The inoculation schedule used for the fermenter grown culture of the marine pseudomonad was as follows. The culture was inoculated from an agar slant into 10 ml of medium contained in a 50 ml flask and was incubated on a rotary shaker for 8 h at 25 C. The contents of this flask were then poured into 300 ml of medium contained in a 2 liter flask and were incubated on a rotary shaker for 5 h at 25 C. The culture was added to a fermenter (New Brunswick) containing 6 liters of medium and the optical density was monitored at 600 nm until the cells reached mid logarithmic phase of growth (0.60 O.D.). This culture was used to inoculate the 100 liter fermenter. When the culture reached 0.62 O.D. units the cells were harvested in a Sharples centrifuge, frozen immediately in dry ice and lyophilized.

Complete salts solutions

The complete salts mixture that was used for washing or resuspending whole cells of the marine pseudomonad contained 0.22 M NaCl, 0.026 M $MgCl_2$ and 0.01 M KCl. Mureinoplasts and protoplasts were resuspended in a complete salts solution containing 0.3 M NaCl, 0.05 M $MgCl_2$ and 0.01 M KCl.

Extraction of lipopolysaccharides

For the isolation of lipopolysaccharides from variants 1 and 3 of the marine pseudomonad, the procedure of O'Leary *et al.* (1972) was employed with minor modifications. A final concentration of 0.05 M $MgCl_2$ was used in the 45% hot phenol and the extraction was performed at 65 to 68 C. The water phase was dialyzed against 0.05 M $MgCl_2$ for 48 h at 10 C to remove phenol. The homogenizations and final centrifugation at 27,000 x g were eliminated. The purified LPS pellet, after treatment with ribonuclease (RNase) and deoxyribonuclease (DNase) was dialyzed against distilled water to remove salts and then lyophilized. *E. coli* 0111:B4, *S. typhimurium* LT2 and *P. aeruginosa* lipopolysaccharides were extracted according to the hot 45% aqueous phenol method of Westphal and Jann (1965). The crude lipopolysaccharides were purified by treatment with RNase (50 µg/ml) and DNase (50 µg/ml) and repeated high speed centrifugations at 143,000 x g (Spinco 60 Ti rotor).

Isolation of lipid A and degraded polysaccharide

Lipid A and the degraded polysaccharide portions of the LPS mole-

cule were separated by hydrolyzing the LPS in either 0.1 N HCl (Burton and Carter, 1964) or 1% acetic acid (Gmeiner *et al.*, 1969) in a boiling water bath for 90 min (2-3 mg LPS/ml of acid). After hydrolysis the precipitated lipid A was removed by centrifugation and washed three to five times with glass distilled water (International Clinical Centrifuge, model CL). The lipid A pellet and the supernatant fluid combined with the water washes, which contained the degraded polysaccharide, were concentrated by lyophilization.

Isolation of core and core-side chain fractions

The procedure of Schmidt and co-workers (1969) was employed to separate LPS core from core-side chain oligosaccharide. Degraded polysaccharide (11 mg), isolated from the 1% acetic acid hydrolysis of LPS, was dissolved in 1 ml pyridinium acetate buffer at pH 5.4 (10 ml pyridine : 4 ml glacial acetic acid : 986 ml distilled water). The sample was applied to Sephadex G-50 which was packed in a 2.5 x 36 cm column which had been equilibrated with the same buffer. Two ml fractions were collected with an LKB Ultrorac fraction collector and were monitored for total neutral carbohydrate by the assay of Dubois *et al*, (1956). The column flow rate was maintained at 9 to 12 ml/h. Blue dextran (0.2%) was used to determine the void volume. Those fractions that corresponded to each carbohydrate peak were pooled and lyophilized.

Preparation of mureinoplasts and protoplasts

Mureinoplasts and protoplasts were prepared according to the

methods of DeVoe and co-workers (1970). Cells were harvested and washed once in a volume of complete salts equal to the volume of the growth medium (full volume). The cells were then washed three times in full volumes of 0.5 M NaCl, resuspended in one full volume of 0.5 M sucrose and incubated at 25 C for 30 min. The cells were collected and resuspended in a second full volume of 0.5 M sucrose and were recovered immediately by centrifugation. The resulting cell forms contained peptidoglycan as the only wall component and maintained their rod shape. Protoplasts were prepared by suspending mureinoplasts in a solution of lysozyme (150 µg/ml), complete salts and 1 mM Tris buffer (pH 7.5). In subsequent experiments mureinoplasts and protoplasts were maintained in a stable state by suspension in complete salts or complex medium.

Isolation of the outer cell wall layers and cytoplasmic membrane

The cell wall layers from the marine pseudomonad were isolated according to the method of Forsberg *et al.* (1970a) with several modifications as proposed by Nelson and MacLeod (submitted for publication). The cells were harvested from the growth medium and washed three times in volumes of complete salts equal to 1/3 of the volume of the growth medium. The cells were then washed with three successive volumes of 0.5 M NaCl, each equal to 1/6 of the volume of the growth medium. These NaCl washes were pooled and centrifuged at 143,000 x g (Spinco 60 Ti rotor) for 2 h to sediment the loosely bound outer layer. The cells were suspended in 1/6 volume of 0.5 M sucrose

and were incubated at 25 C for 30 min followed by resuspension in another 1/6 volume of 0.5 M sucrose and immediately centrifuged. The pooled sucrose washes were centrifuged at 73,000 x g (Spinco type 30 rotor) to sediment the outer double-track. The periplasmic layer was concentrated from the sucrose supernatant fluids. All of the cell wall layers were exhaustively dialyzed against distilled water at 10 C and lyophilized.

Cytoplasmic membrane was isolated and purified according to the procedure of Sprott and MacLeod (1972). Protoplasts were formed and were disrupted in a French pressure cell. The preparation was treated with DNase (50 µg/ml), RNase (50 µg/ml) and lysozyme (150 µg/ml) which was followed by centrifugation at 4,000 x g for 15 min. The supernatant fluid was then centrifuged at 75,000 x g for 35 min to sediment the membranes. The membranes were dialyzed against distilled water at 10 C to remove salts and were concentrated by lyophilyzation.

Isolation of medium material from whole cells, mureinoplasts and protoplasts

The cell medium material was collected by first centrifuging the medium at 35,000 x g (Spinco SS-34 rotor) for 20 min to remove any whole cells. The medium was then centrifuged at 143,000 x g (Spinco 60 Ti rotor) for 2 h to sediment the medium material. The pellet was washed twice in complete salts and then lyophilized. This procedure

was modified from that used by Nelson and MacLeod (submitted for publication). In order to isolate medium material from mureinoplast and protoplast preparations the same procedure was employed.

Polyacrylamide gel electrophoresis

The lipopolysaccharides and cell wall layers were electrophoresed in a buffer containing 0.05 M Na_2HPO_4, 0.05 M Na_2MoO_4 and 1% sodium dodecyl sulfate (SDS). In various experiments modifications of this buffer were examined. All of the electrophoresis buffers were adjusted to pH 7.0 with hydrochloric acid. The samples were electrophoresed at 5 mA/gel for 3 to 5 h or 10 mA/gel for 3 h with the lower electrode as the anode. Preparative gels were electrophoresed for 7 h at 10 mA/gel. Electrophoresis was performed in either a Model 6 Canalco or a Biorad Model 150 disc electrophoresis apparatus. A Shandon constant voltage/constant current or a Buchler power unit was used.

In preparation for electrophoresis the samples were solubilized in a solution adapted from Inouye and Guthrie (1969) containing 20% glycerol ($^{v}/v$), 2% SDS ($^{w}/v$) and the inorganic components at their concentrations in the particular electrophoresis buffer used. When Triton X-100 was substituted for SDS in the electrophoresis buffer, 0.2% Triton replaced the 2% SDS in the solubilizing solution. The samples were solubilized by heating in a boiling water bath for 5 to 10 min at a concentration of 1 to 2 mg of sample per 0.25 ml of

solubilizing solution to which was added 0.25 ml of glass distilled water. In some samples a drop of bromphenol blue solution (4 mg/ml) was added as tracking dye.

The gels were prepared using a slight modification of the method of Maizel (1966) and contained 5% recrystallized acrylamide, 0.13% recrystallized N,N'-methylenebisacrylamide as cross linker, 0.05% N,N,N',N'-tetramethylethylenediamine[1,2-bis(dimethyl amino)]ethane, 0.075% ammonium persulfate, 1% SDS and the inorganic components at their concentrations in the electrophoresis buffer used. When SDS was replaced by Triton X-100 in the gel electrophoresis buffer 0.1% Triton was used instead of 1% SDS. The gels were 7 or 9 cm in length and were cast in 5 or 6 mm I.D. acid-washed glass tubes. Preparative gels were 12 cm in length and 6 mm I.D. The gels were overlayed with electrophoresis buffer and polymerized for 30 min at room temperature. They were pre-run in electrophoresis buffer for 30 min at 10 mA/gel.

Staining reagents

For the staining of LPS gels, the Schiff reagent was prepared according to the Canalco technical bulletin. Basic fuchsin (0.5 gm) was added to 100 ml distilled water followed by 10 ml of 1 N HCl and 1 gm of sodium metabisulfite. This preparation was shaken at intervals for 3 h at the end of which time 0.5 gm activated charcoal were added. The solution was filtered and stored in a dark bottle in the cold.

Protein gels were stained with 0.25% Coomassie Brilliant Blue (Sigma) in 12% trichloroacetic acid - 50% methanol. The staining procedure of Inouye and Guthrie (1969) was used.

Fractionation and counting of radiolabeled gels

Polyacrylamide gels containing radiolabeled samples were fractionated into 1 mm slices with a Gilson Aliquogel fractionater. Gel fractions were allowed to swell overnight in Triton-toluene scintillation fluid and were then counted on a Nuclear Chicago Isocap/300 liquid scintillation counter.

Polyacrylamide gel densitometry

Polyacrylamide gels that were stained with the Schiff reagent were scanned on a Unicam SP 1800 ultraviolet spectrophotometer equipped with a densitometer attachment. Gels were scanned in 7.5% acetic acid at 540 nm using a slit width of 0.1 mm. Relative quantitation was obtained by comparing peak areas.

Scanning wavelength spectrophotometry

A Unicam model SP 800 ultraviolet spectrophotometer was employed for scanning wavelength analyses. All samples were read at room temperature in matched quartz cuvettes with a 1 cm light path.

Sepharose gel filtration

LPS (10 mg/ml) was solubilized in the SDS solubilizing solution used for polyacrylamide gel electrophoresis and was chromatographed

on Sepharose 4B and 6B (Pharmacia). The Sepharose was allowed to equilibrate in 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS buffer (pH 7.0) and was packed in K 25/45 columns (Pharmacia) fitted with flow adaptors. Columns were packed and operated at 25 ± 2 C. Samples were chromatographed by the technique of upward flow elution at a flow rate of 14 ml/h. The effluents were assayed by a Refractive Index monitor (Pharmacia) and 2 ml fractions were then collected by an LKB Ultrorac fraction collector. Fractions representing each peak were pooled and dialyzed against distilled water containing 0.02% sodium azide at room temperature. Sodium dodecyl sulfate was completely removed from the samples by precipitation with saturated $BaCl_2$ solution and the peak material was concentrated by lyophilization. Blue dextran (0.5%) and sucrose (10 mg/ml) were used to determine the void volume (V_o) and the included volume (V_i), respectively. Dextran preparations (10 mg/ml) of various molecular weight ranges were employed as standards. The molecular sizes of the LPS peaks were estimated by comparing their experimentally determined K_{av} values to those obtained with the standards. The formula used was $K_{av} = \frac{Ve - V_o}{V_t - V_o}$, where V_e is the elution volume, V_o represents the void volume and V_t is equal to the total bed volume.

Descending paper chromatography

LPS monosaccharides were separated by descending paper chromatography in ethylacetate-pyridine-water (120 : 50 : 40 v/v) at room

temperature on Whatman #3 chromatography paper (Smith, 1960). Chromatograms were developed for 20 - 24 h after which time they were dried and cut into strips for the detection of compounds with selective spray reagents or by counting for radioactivity. Hexoses were detected with analine phthalate and amino sugars with ninhydrin. Chromatograms containing [^{14}C]radiolabeled samples were cut into 1.5 cm^2 pieces which were placed in scintillation vials, moistened with 0.2 ml distilled water and counted with 10 ml Aquasol (New England Nuclear).

Hydrolytic procedures

For chromatographic analyses 2 - 5 mg LPS samples were hydrolyzed in sealed ampoules with 2 ml hydrochloric acid at the appropriate concentration and length of time required for the maximum release of the sugars being analyzed. Lipid A (1 mg) was hydrolyzed in the same manner after which the fatty acids were removed by extracting the hydrolysate with petroleum ether. All hydrolyses were carried out in an oven maintained at 100 to 105 C.

Gas - liquid chromatography

Monosaccharides were analyzed by gas-liquid chromatography of the corresponding trimethylsilyl (TMS) or alditol acetate derivatives. Trimethylsilyl derivatives were prepared and chromatographed according to the procedure of Kondo and Ueta (1972). Hydrolyzed LPS samples were treated with 0.5 ml pyridine-hexamethyldisilozane-trimethylchlorosilane (9:3:1)(Applied Science Laboratories) in teflon-capped reaction

vials. The reaction was allowed to proceed at room temperature and the derivatized samples were dissolved in chloroform or carbon disulfide for injection. Alditol acetate derivatives were formed by the method of Perry and Webb (1968) using methylene chloride as the solvent. D-glucoheptose was added to the samples as an internal standard, prior to the derivatization procedure, at a concentration of 2-3% ($^{w}/_{w}$).

Sample peaks were identified by comparing their retention times to known derivatized standards and quantitation was achieved by comparing peak areas of the unknown monosaccharides to that of the internal standard according to the equation A = h x w at 1/2 h, where A is the peak area in mm^2, h equals the peak height and w represents the peak width at half of the peak height.

A detector response curve and correction factor (K value) had to be determined for each monosaccharide standard chromatographed on both the HI-EFF and OV-275 columns. The flame ionization detector gives a linear response over a specific concentration range of each sugar, consequently, the relationship of peak area to sugar concentration is valid only within this range (Jones, 1970). The detector response curves were experimentally determined in the Results (Section I). Also, the flame ionization detector does not give the same response to equal concentrations of different monosaccharides and, therefore, the correction factor is necessary. The K values were calculated for each

sugar by using the formula $K_s = \frac{A_s \ W_{is}}{A_{is} \ W_s}$ in which A_s equals the area of the standard sugar, A_{is} equals the area of the internal standard, W_s the weight of the standard sugar, and W_{is} the weight of the internal standard. A known amount of each standard sugar was injected with a known amount of internal standard. Since W_s and W_{is} were known and A_s and A_{is} could be measured on the chromatogram, K_s could be calculated for each sugar. The LPS monosaccharides were then quantitated using the formula $W_u = \frac{A_u \ W_{is}}{A_{is} \ K_s}$ in which A_u and A_{is} equal the areas of the unknown sugar and internal standard, respectively (as measured on the chromatogram); W_{is} equals the weight of the internal standard (a known amount was added to the LPS sample); and K_s represents the correction factor which was previously calculated for each sugar (Sloneker, 1972).

Chromatography was performed on a Varian Aerograph series 1700 dual column chromatograph equipped with flame ionization detectors. Helium was used as the carrier gas. For the chromatography of the trimethylsilyl derivatives two columns were employed. Columns A and B were 3% SE-30 (a silicone gum rubber) and 3% OV-1 (a dimethylsilicone gum), respectively. Both phases were on 100/120 mesh acid-washed, dimethyldichlorosilane treated Chromosorb G in 6 ft. columns. The columns were operated at 180 C at a carrier gas flow rate ranging from 25 to 45 ml/min. Detector and injector block temperatures were 215 to 225 C and hydrogen and air flow rates were 13 to 14 and 500 to

600 ml/min, respectively. Alditol acetates were chromatographed on three columns. Column C was a 10% neopentylglycol sebacate (HI-EFF) liquid phase on an acid-washed, dimethyldichlorosilane treated Chromosorb W support, 80/100 mesh. The column was 5 ft. in length and was operated at 230 C at a carrier gas flow rate of 32 to 33 ml/min. Column D was a 3% cyanopropylmethylphenylmethylsilicone (OV-275) phase on acid-washed Chromosorb W 100/120 mesh. This 6 ft. column was run at 220 or 225 C with a carrier gas flow rate of 17 to 18 ml/min. Column E was a 3% ECNSS-M (an ethylenesuccinatecyanoethylsilicone copolymer) on 100/120 mesh acid -washed, dimethyldichlorosilane treated Chromosorb G (Crowell and Burnett, 1967). This column was 6 ft. in length and was operated at 200 C with a helium flow of 23 ml/min. The injector temperatures were maintained at 230 to 240 C for columns C and D and at 245 C for column E. The detector block was 270 C for columns C and D and 255 C for column E. The hydrogen gas flow rates for columns C and D were 20 to 22 ml/min and for column E, 24 ml/min. The flow rate of air was 400 to 463 ml/min for C and D and 500 ml/min for column E.

Preparation of whole cell antigen for immunization

Marine pseudomonad variant 3 was grown on nutrient broth-yeast extract agar plates overnight. Cells were washed off the agar with complete salts and were boiled for 1 h. The cells were washed in and then resuspended in complete salts containing 0.3% formalin to a concentration of 21×10^8 cells/ml (Kabat and Mayer, 1961). The antigen was used to inject, intravenously, New Zealand albino rabbits.

Collection and preparation of antiserum

Blood was collected by cardiac puncture, was allowed to clot at room temperature and was stored at 10 C overnight. The serum was decanted and any remaining erythrocytes were removed by centrifugation. The serum was sterilized by passage through a Seitz filter and was stored aseptically in serum bottles.

Determination of antibody titer

The antiserum was titered by passive hemagglutination according to the procedure of Leive *et al.* (1968). Lipopolysaccharide was treated with 0.02 N NaOH at 37 C for 18 h and was used to coat sheep red blood cells. Sheep erythrocytes were washed twice in 0.85% NaCl and were resuspended in saline to a concentration of 2.5% (v/v). The treated LPS was added to the suspension to obtain a final concentration of 100 µg LPS/ml of 2.5% sheep erythrocytes. The mixture was incubated for 2 h at 37 C while the serum was being inactivated at 56 C for 30 min. The sensitized red blood cells were washed twice in saline to remove excess LPS. Saline (50 µl) was added to dilution wells and 50 µl of the serum was serially diluted. The sensitized red blood cells (25 µl) were added to each well and the mixtures were incubated at room temperature.

Double immunodiffusion

The antiserum was concentrated for use in the double immunodiffusion studies. A saturated solution of $(NH_4)_2SO_4$ was added to the

antiserum (1:1 v/v) and the precipitate was collected by centrifugation. The precipitate was dissolved in 0.85% NaCl and dialyzed against saline to remove excess $(NH_4)_2SO_4$. The dialysate was reprecipitated and dialyzed two additional times. The final dialysate was adjusted to 60 mg protein/ml saline. This concentrated antiserum was used in all immunodiffusion experiments.

Clean glass microscope slides were precoated and layered with 1% Nobel agar (Difco) in physiological saline. Wells, 3 mm in diameter and spaced 9 mm apart (center to center), were made with a gel punch (Gelman Instrument Co.). The wells were filled with antibody or antigen suspensions and allowed to diffuse for 24 h at room temperature in a moist chamber. Precipitin lines were recorded and the slides were photographed following the incubation period. The slides were then immersed in saline for 48 h, with at least three changes, and dried under filter paper. The slides were stained with Amido black 10B for 1 h and destained in 2% glacial acetic acid (Uriel and Grabar, 1956).

Analytical methods

Total neutral carbohydrate was assayed either by the anthrone method as modified by Fairbairn (1953) or by the phenol sulfuric acid procedure (Dubois *et al.*, 1956). Total amino sugar was measured according to the Blix (1948) modification of the Elson-Morgan assay. Determination of 2-keto-3-deoxyoctulosonic acid (KDO) was by the

thiobarbituric acid assay (Weissback and Hurwitz, 1959) and was corrected for the interference of sialic acids (Warren, 1963). The procedure of Osborn (1963) was used in the determination of heptose. Protein was measured by the procedure of Lowry and co-workers (1951) using bovine serum albumin as the standard while phosphate was determined by the procedure of Chen *et al.* (1956) with KH_2PO_4 as the standard. The ash content of the LPS was determined as previously described (O'Leary *et al.*, 1972) and lipid A was quantitated by dry weight. The carbocyanin dye procedure was performed according to the method of Janda and Work (1971).

Dry weight determinations

Dry weights were determined in 10 or 20 ml glass beakers which had been acid-washed and chloroform-methanol (2:1) washed, heated in an 80 C oven for several hours, and allowed to equilibrate to constant weight over phosphorous pentoxide in a desiccator.

Scintillation fluid

Radiolabeled samples for liquid scintillation analysis were counted in a solution containing 0.5 gm 1,4-bis[2-(5-phenyloxazolyl)] benzene, 18 gm 2,5-diphenyloxazole, 1 liter Triton X-100 and 2 liters toluene. The efficiency of [^{14}C] counting in this fluid was 90%.

Preparation of glassware

All pipets and glassware used for chemical assays, gas-liquid

chromatographic sample preparation or in handling samples of any sort were acid-washed in sulfuric acid-nitric acid (2:1) and rinsed ten times in line distilled and three times in glass distilled water. Any glassware involved in the handling of lipids was chloroform-methanol (2:1) extracted.

Chemicals and radioactive chemicals

All enzymes, dextrans and monosaccharides were purchased from Sigma Chemical Co. Monosaccharide purity was established by gas-liquid chromatography of the corresponding alditol acetates. Acrylamide, 1-ethyl-2-[3-(1-ethylnaphtho[1,2d]-thiazolin-2-ylidene)-2-methyl propenyl]naptho[1,2d]-thiazolium bromide, and 2-thiobarbituric acid were obtained from the Eastman Kodak Co. N,N,N',N'-tetramethylethylene-diamine[1,2-bis(dimethylamino)]ethane (TEMED) and N,N'-methylene-bisacrylamide were products of K and K Laboratories, Inc. Other chemicals and solvents were purchased from Fisher Scientific or Canlab Chemicals and were reagent grade purity. Solvents were usually gas-liquid chromatographic and spectrophotometric quality. [^{14}C]galactose (uniformly labeled), dextran [^{14}C]carboxyl (molecular weight 15-17,000) and dextran [^{14}C]carboxyl (molecular weight 60-90,000) were purchased from New England Nuclear or Amersham/Searle Corporation.

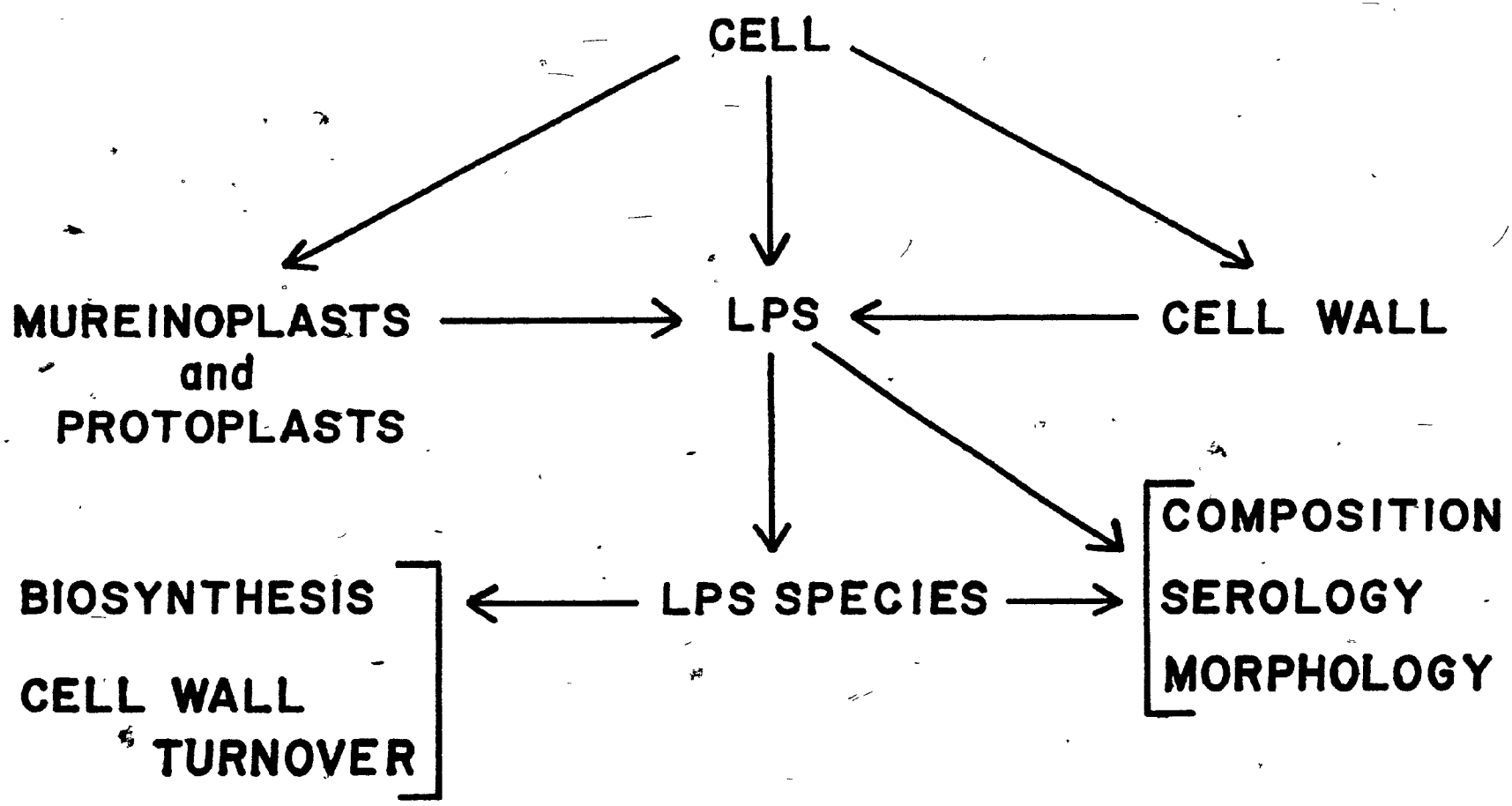
RESULTS

Introduction

The investigation into the characteristics and heterogeneity of the marine pseudomonad lipopolysaccharide (LPS) was approached according to the scheme presented in figure 1. The major emphasis of the work concentrated heavily on the experiments represented by the right side of the scheme because this aspect of the study had to be established prior to the more advanced experimental outline presented on the left. Briefly, experiments were designed to establish that LPS heterogeneity or aggregation was a real and important phenomenon and existed in LPS that was extracted from whole cells or cell walls of the marine pseudomonad. The relationship of the various components that contributed to this heterogeniety was examined by chemical analysis, immunological cross-reactivity and gross morphology. Once the nature of the LPS heterogeneity was established more intricate studies were initiated in an attempt to show the order and rate of biosynthesis of the LPS components *in vivo* and in an artificial cell system which does not contain any "old" LPS or outer cell wall.

FIGURE 1.

Fig. 1. Schematic representation of the approach used to study the heterogeneity of the marine pseudomonad lipopolysaccharide.


CELL
MUREINOPLASTS and PROTOPLASTS
LPS
CELL WALL
COMPOSITION
SEROLOGY
MORPHOLOGY
BIOSYNTHESIS
CELL WALL TURNOVER
LPS SPECIES

SECTION I

CHARACTERIZATION OF THE MARINE PSEUDOMONAD WHOLE CELL LIPOPOLYSACCHARIDE

Extraction from whole cells

The marine pseudomonad LPS was extracted by the procedure developed by O'Leary and co-workers (1972). During the course of initial extractions it became evident that several steps in the procedure resulted in variable yields of LPS. Several minor modifications were made and these can be listed as follows. The concentration of $MgCl_2$ that was used in the phenol-water extraction mixture was increased from 0.026 M to 0.05 M, the extraction temperature was increased from 60 C to 65 - 68 C, dialysis to remove phenol included 0.05 M $MgCl_2$, the homogenizations were eliminated and the cold acetone precipitation of the LPS was replaced by collecting the LPS by high speed centrifugation and concentrating by lyophilization. The increased Mg^{2+} concentration was an extension of the original observation of O'Leary that this ion resulted in a more efficient isolation of LPS in this bacterium than either Na^+, K^+ or a combination of all three ions. The extraction temperature was increased because phenol and water are much more miscible at the higher temperature and, for this reason, result in a better extraction (see Westphal and Jann, 1965). Dialysis in 0.05 M $MgCl_2$ simply was consistent with the extraction concentration and, in addition, the Mg^{2+} was found to be necessary for the sedimentation of the LPS probably through complex formation

as in the case of *Salmonella* (Osborn *et al.*, 1962). The homogenizations did not appear to increase the purity of the LPS preparations and seemed repetitious in view of the fact that several resuspensions and high speed centrifugations were employed in the procedure. The major difficulty in the O'Leary procedure was the final acetone precipitation. In some extractions, 1 volume of acetone would precipitate the LPS while in other experiments as many as 10 volumes did not cause precipitation. In some cases acetone will precipitate many inorganic components, consequently, it does not contribute to the purification of the crude LPS. When the modifications were employed, the LPS yield was reproducibly 2 to 3% of the dry weight of the bacterial cells and there was never an extraction in which no LPS was obtained. Table 1 is a comparison of the yields obtained in a series of LPS extractions.

Once it was established that the LPS could be isolated consistently and that the extraction procedure was trimmed to the essential steps it became advantageous to isolate, in a single extraction, enough LPS necessary to perform all chemical investigations. For this purpose a culture of the marine pseudomonad was grown in a 100 liter fermenter. Growth was monitored by following the optical density of the culture and the resulting growth curve is shown in figure 2. The cells were supposed to be harvested during late logarithmic phase, however, the culture entered stationary phase early and was collected at this time. Under batch culture growth conditions

TABLE 1.

Yield of lipopolysaccharide obtained by the O'Leary phenol-water extraction: Comparison of the original and modified procedures

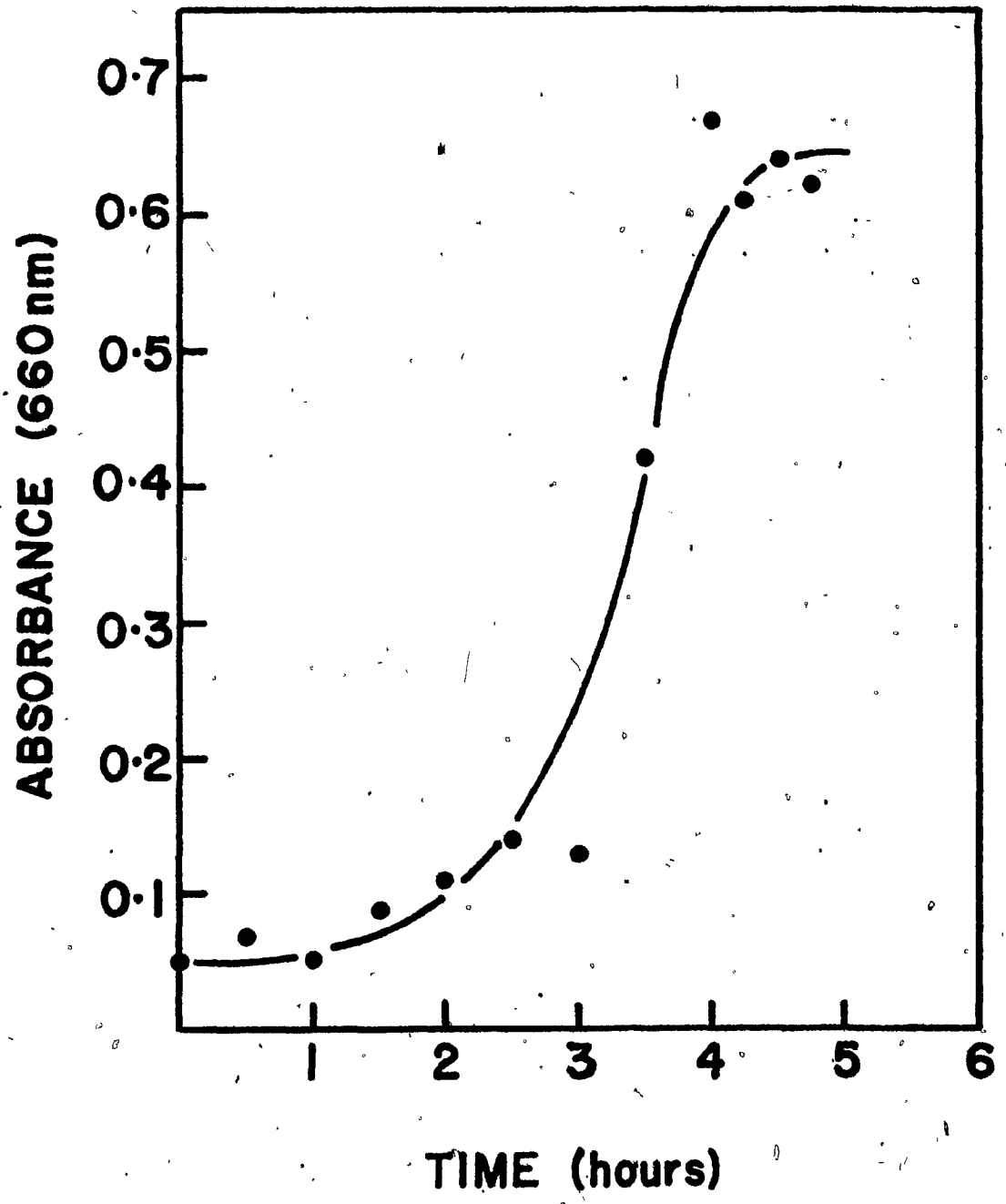
Experiment	Procedure	Mg material/gm of cells dry weight	LPS[a]
1	O'Leary *et al.* (1972)	20	+
2	O'Leary *et al.* (1972)	0	-
3	O'Leary *et al.* (1972)	1	-
4	modifications: (1) 0.05 M $MgCl_2$ (2) 5 volumes of acetone	20	+
5	modifications: (1) 0.05 M $MgCl_2$ (2) 10 volumes of acetone	0	-
6	modifications: (1) 0.05 M $MgCl_2$ (2) lyophilization	30	+
7	modifications: (1) 0.05 M $MgCl_2$ (2) lyophilization	30	+
8	modifications: (1) 0.05 M $MgCl_2$ (2) lyophilization	20	+

[a]The presence of LPS was determined by either a positive carbocyanin dye reaction, KDO assay, heptose assay, carbohydrate staining material on SDS-polyacrylamide gels or a combination of these procedures.

FIGURE 2.

Fig. 2. Growth curve of the marine pseudomonad cultured in a 100 liter fermenter.

The culture was grown in nutrient broth-yeast extract complex medium. Optical density was monitored with a Coleman Junior spectrophotometer at 660 nm.


ABSORBANCE (660nm)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
1
2
3
4
5
6
TIME (hours)

mid logarithmic phase is reached at 0.55 O.D. at 660 nm (Coleman Junior Spectrophotometer). The nutrient broth-yeast extract complex medium was used in the fermenter and it is not known whether nutrients or aeration was a limiting factor. Foaming did occur because only a small amount of antifoam was added. It was not known what effect, if any, antifoam would have on the bacterial cell wall. Aqueous phenol extraction of the cells from the fermenter grown culture yielded approximately 3% LPS by dry weight. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis demonstrated that this LPS gave the same electrophoretic pattern upon staining with the Schiff reagent as LPS extracted from batch cultures. For a more accurate comparison, the carbohydrate compositions of the two lipopolysaccharides were compared by gas-liquid chromatography of the trimethylsilyl derivatives. The chromatograms were qualitatively the same, however, it is obvious that there are a few quantitative differences among several of the monosaccharides (figure 3). Although the actual concentrations of the different sugars were not calculated, examination of the chromatograms demonstrates that the concentration differences are very slight. When radiolabeled LPS was needed, batch cultures were used for extractions.

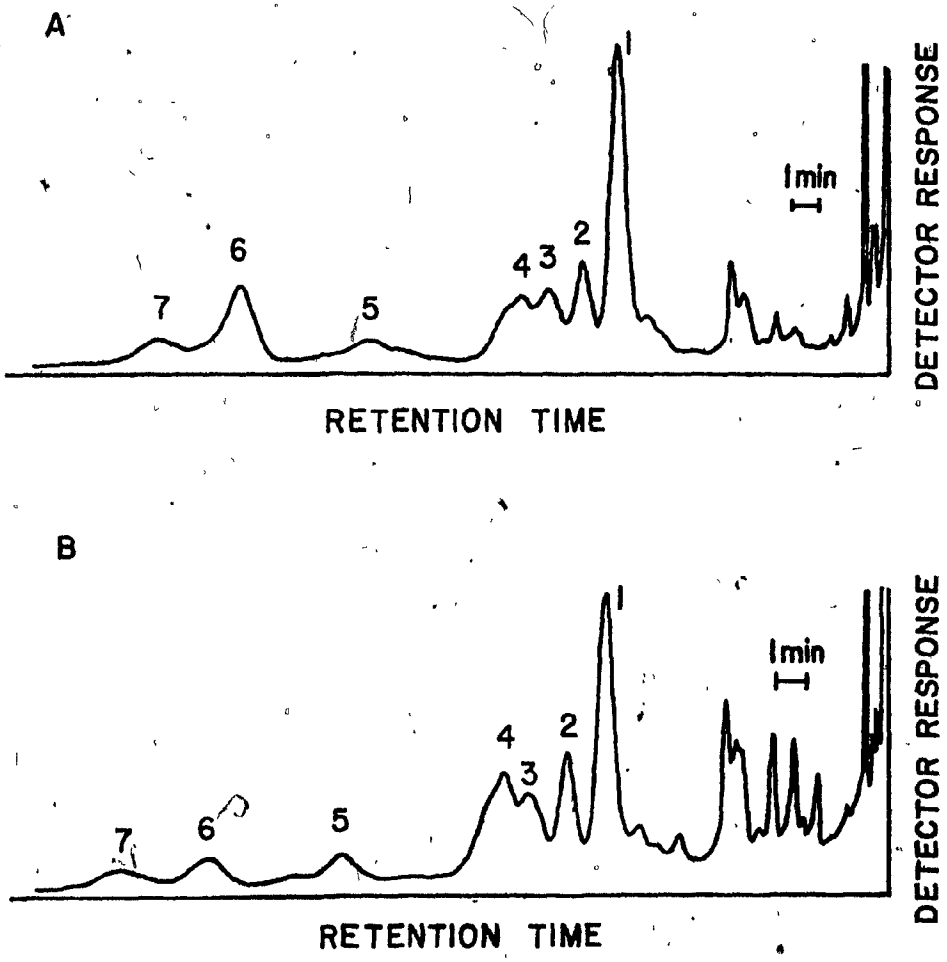
Determination of purity

The procedure of Janda and Work (1971), which is based on the occurrence of a predicted spectral shift when LPS is reacted with a

FIGURE 3.

Fig. 3. Comparison of the carbohydrate composition of the marine pseudomonad LPS extracted from: (A) fermenter grown cells and (B) batch culture cells.

The trimethylsilyl derivatives were chromatographed on column B at an oven temperature of 180 C. Peaks were identified as: 1. 2-amino-2-deoxygalactose, 2. 2-amino-2-deoxyglucose and galactose, 3. glucose, 4. galactose, 5. glucose, 6. 2-amino-2-deoxygalactose, and 7. 2-amino-2-deoxyglucose. The unlabeled peaks were not identified.


A
1
2
3
4
5
6
7
1min
DETECTOR RESPONSE
RETENTION TIME
B
1
2
3
4
5
6
7
1min
DETECTOR RESPONSE
RETENTION TIME

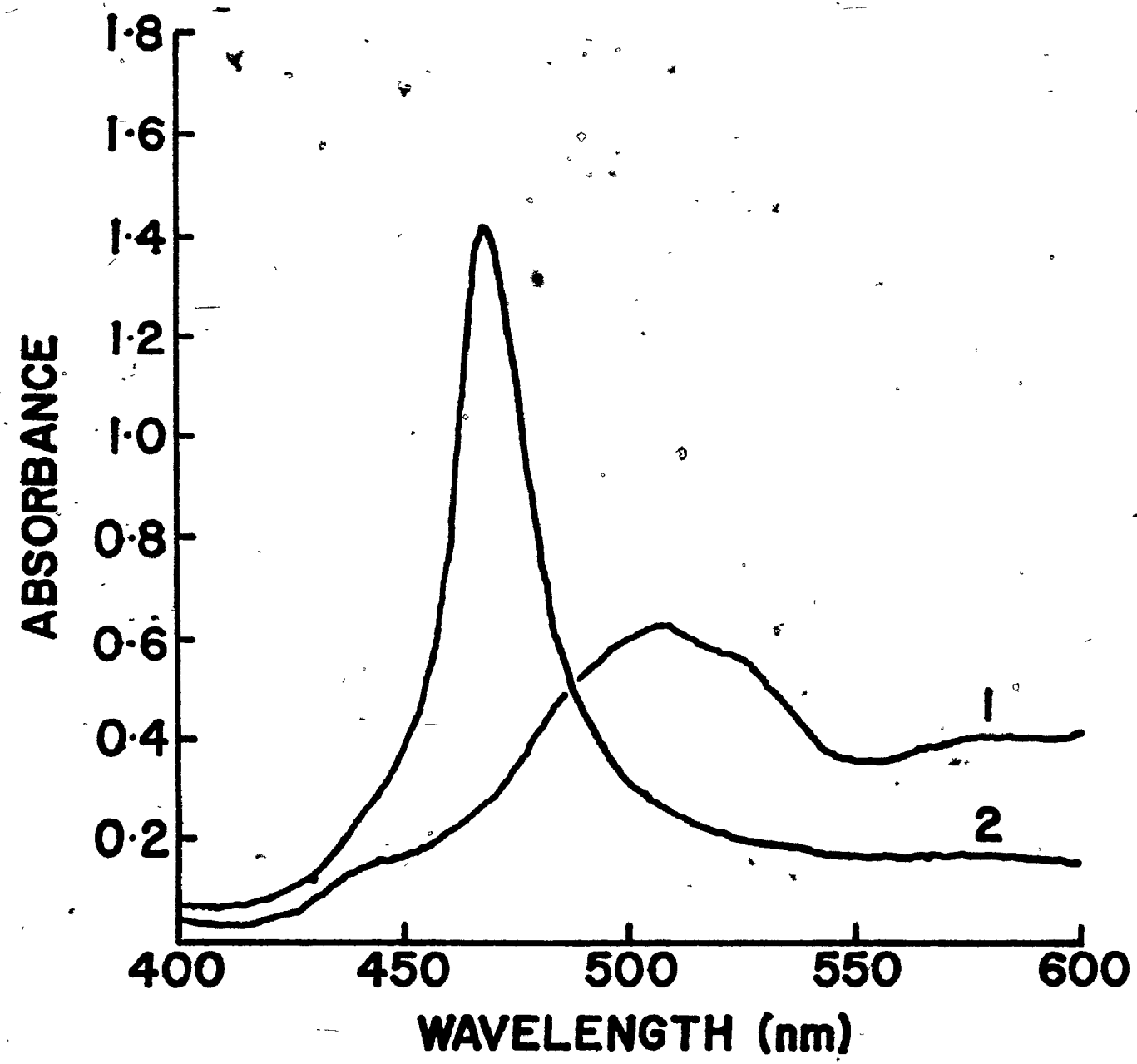
carbocyanin dye, was used as a measure of the purity of the marine pseudomonad LPS and to demonstrate its similarity to other bacterial lipopolysaccharides. The dye reagent has an absorption maximum at 510 nm and will undergo a shift to higher wavelengths when reacted with proteins, nucleic acids and acidic polysaccharides. However, a shift to lower wavelengths indicates the presence of LPS. Reaction of the marine pseudomonad LPS with the dye resulted in a spectral shift to 468 nm (figure 4). There were no additional absorbance peaks with this preparation from 510 to 600 nm suggesting that the LPS was relatively pure. Numerous attempts were made to develop this procedure into a quantitative assay, however, the results supported the findings of Zey and Jackson (1973) which stated that repeated trials to construct reproducible standard curves exhibited extreme variability if numerous experimental conditions were not rigidly controlled.

Chemical composition

A sensitive and quantitative method was needed to determine the carbohydrate composition of the LPS because of the complications involved in analyzing a complex mixture of monosaccharides particularly when they were often present in minute amounts of material. Paper chromatography did not afford the resolution and degree of sensitivity that was required for the analysis and quantitation by this technique was unreliable. Thin layer chromatography looked as if it

FIGURE 4.

Fig. 4. Carbocyanin dye scan of the marine pseudomonad LPS. Scan 1 represents the dye reagent and profile 2 is the dye reagent reacted with approximately 0.5 mg LPS.


ABSORBANCE
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
400
450
500
550
600
WAVELENGTH (nm)
1
2

would be a useful method and various solvent mixtures were examined for their ability to separate the LPS monosaccharides. However, the separation was exceedingly disappointing and quantitation posed the same problem as in paper chromatography. Gas-liquid chromatography appeared to satisfy the requirements of sensitivity, resolution of complex sugar mixtures and quantitative reproducibility. Initially, the trimethylsilyl derivatives were used and a typical separation of sugar standards is shown in figure 5A. Unfortunately, a separate peak is obtained for both the α and β anomers of a monosaccharide. The 2-amino-2-deoxy sugars sometimes resulted in as many as four peaks upon derivatization. This provided extremely complicated chromatograms on which sugar mixtures could not be resolved as evidenced by the chromatogram of LPS (figure 5B).

The trimethylsilyl derivatives were abandoned and the alditol acetate derivatives were considered as a replacement. This derivative had the advantage of yielding one peak for each monosaccharide which simplified the chromatogram. The separation of standards was excellent and all the monosaccharides analyzed could be separated on a minimum of two column packings (figure 6). In early studies the HI-EFF and ECNSS-M columns (column C and E respectively) were used for analysis of the alditol acetates. The peaks on the ECNSS-M column tended to tail which made accurate measurement of peak areas difficult. The OV-275 column (D) was far superior to column E in both separation and thermal stability. Consequently, column D was

FIGURE 5.

Fig. 5. Carbohydrate analysis of marine pseudomonad LPS by gas-liquid chromatography of the trimethylsilyl derivatives.

Gas-liquid chromatography was performed on column A at 180 C. Chromatogram A contains derivatized standards and chromatogram B represents LPS. Peak identification: (1) 2-amino-2-deoxymannose; (2) 2-amino-2-deoxygalactose and galactose; (3) 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose and galactose; (4) 2-amino-2-deoxymannose; (5) α,D(+)-glucose; (6) galactose; (7) 2-amino-2-deoxyglucose; (8) β,D(+)-glucose; and (9) N-acetylglucosamine. The remaining LPS peaks were not identified.

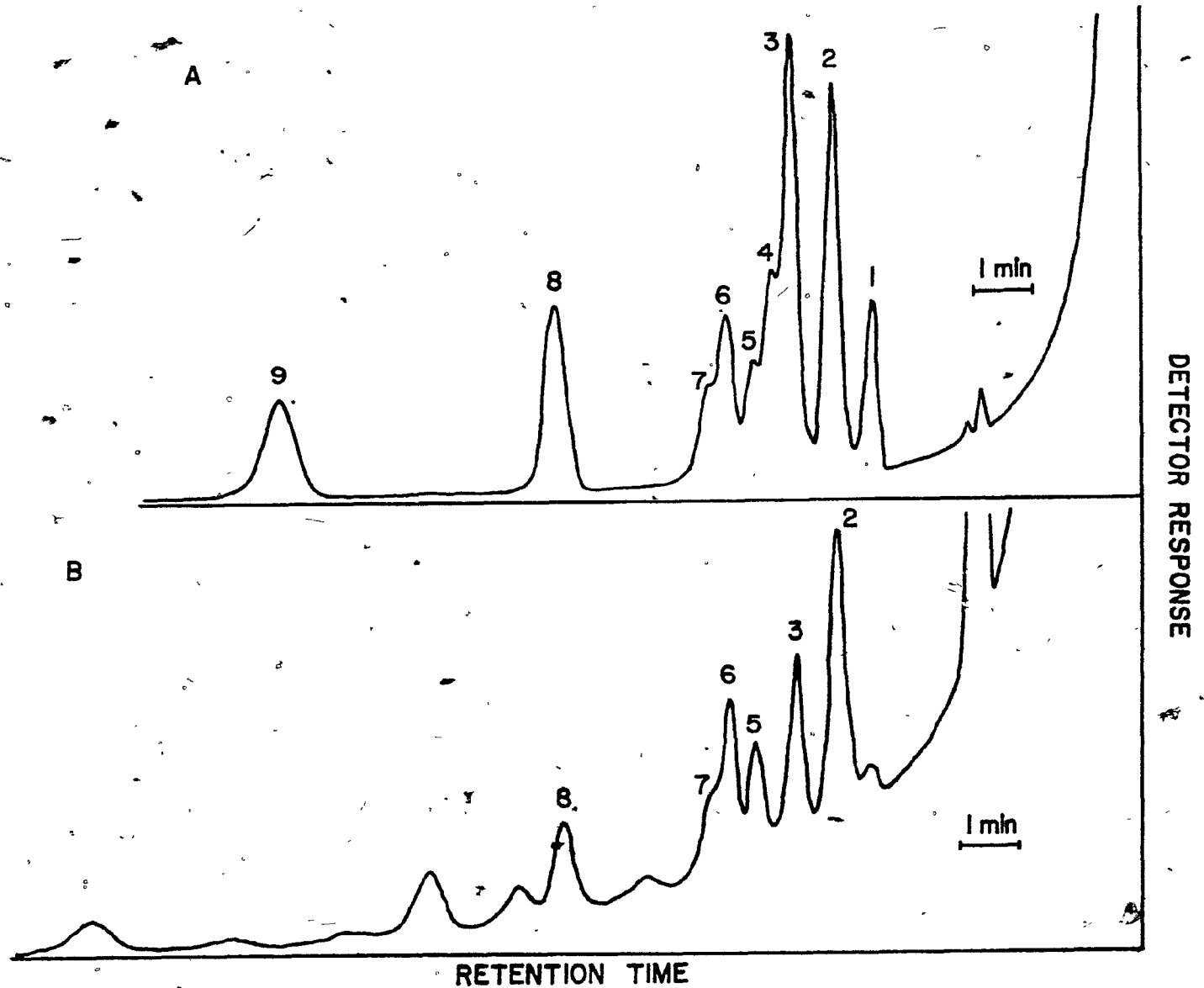
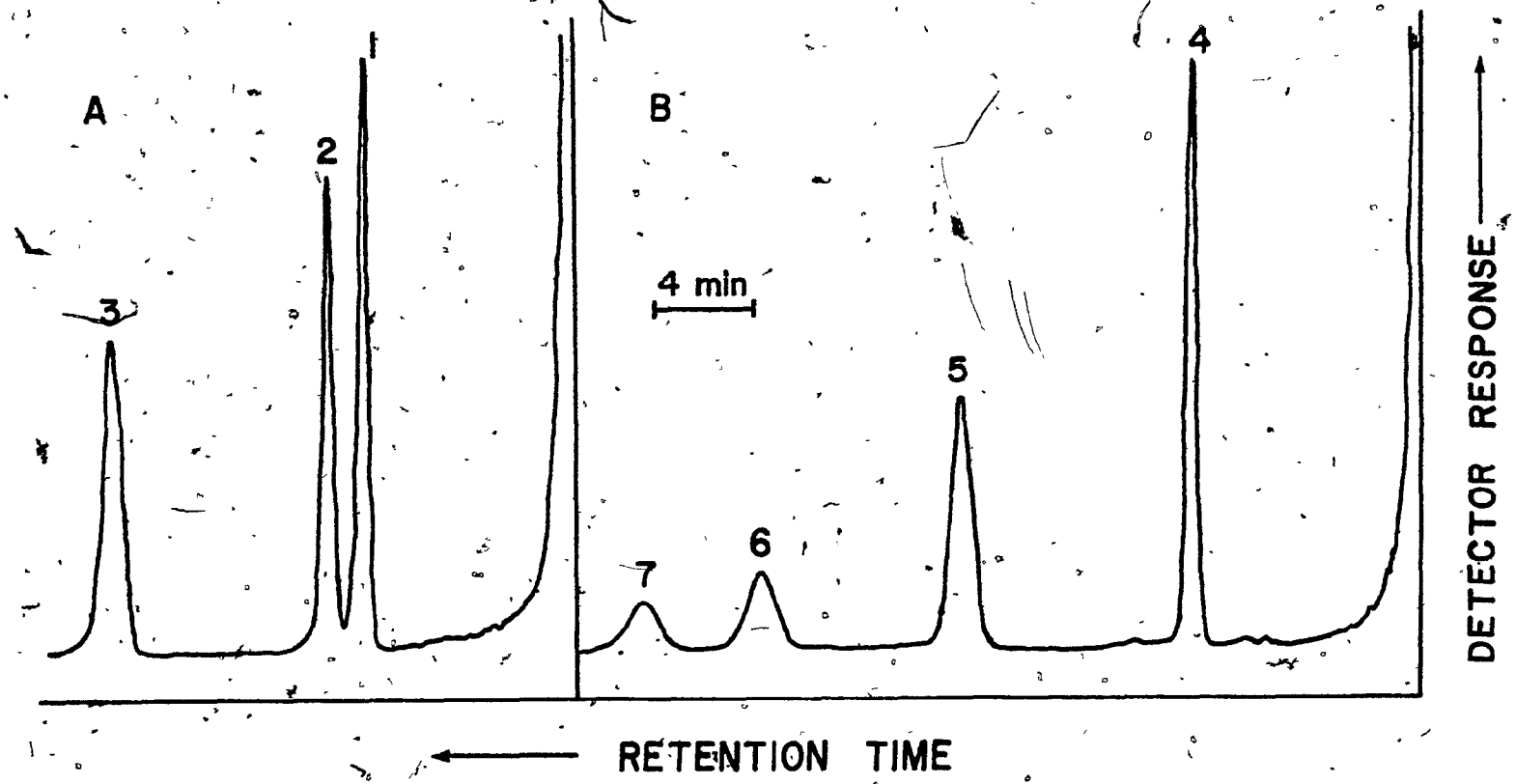

A
B
9
8
7
6
5
4
3
2
1
1 min
1 min
DETECTOR RESPONSE
RETENTION TIME

FIGURE 6.

Fig. 6. Separation of alditol acetate sugar derivatives on the HI-EFF and OV-275 columns.

Chromatogram A, standards on OV-275 at 225 C and chromatogram B, standards on HI-EFF at 230 C. Peak identification: 1, galactose; 2, glucose; 3, glucoheptose; 4, glucose and galactose; 5, glucoheptose; 6, 2-amino-2-deoxyglucose and 7, 2-amino-2-deoxygalactose.


A
B
1
2
3
4
5
6
7
4 min
DETECTOR RESPONSE
RETENTION TIME

employed thereafter instead of column E. Detector response curves were calculated for each available standard on columns C and D as relative to the internal standard, D-glucoheptose (figure 7). The response curves for the derivatives of the standard sugars were obtained by chromatographing increasing amounts of each standard with a constant amount of internal standard. The areas of the standard and internal standard peaks were measured and the ratio of these areas was plotted (Jones, 1970). In the case of the response curves for the internal standard, the peak areas had to be graphed directly (figures 7E and 7F). Linearity was obtained with sugar concentrations in the range of 0.005 to 0.05 μmoles. Table 2 lists the K values obtained for the alditol acetate standards. The K values for heptose and the two unknown sugars were arbitrarily chosen as 1.00 based on their relative retention times because standards could not be obtained for these components. The K values were used in the quantitation of the LPS sugars (see Materials and Methods section) and were dependent upon the type of column packing and detector. D-glucoheptose was chosen as the internal standard for the gas-liquid chromatographic analyses because: (i) it eluted in a central position in relation to the LPS monosaccharides; (ii) it could be separated from the LPS sugars in the columns employed, and (iii) it was easily obtainable in a relatively pure state. The originally purchased preparation demonstrated more than one peak when derivatized and chromatographed (figure 8A). One recrystallization in ethanol resulted

FIGURE 7.

Fig. 7. Gas-liquid chromatography detector response curves for the alditol acetate derivative standards on columns OV-275 and HI-EFF.

Graphs A and B depict the detector response curves for the alditol acetate derivatives of glucose and galactose, respectively. Chromatography was performed on the OV-275 column at 225 C. Graphs C and D represent the response curves for the derivatives of 2-amino-2-deoxyglucose and 2-amino-2-deoxygalactose, respectively. Chromatography was performed on the HI-EFF column at 230 C. Plots E and F represent the response curves for the derivative of glucoheptose (internal standard) on the OV-275 and HI-EFF columns, respectively. In graphs A-D, A_s/A_{is} is the ratio of the area of the standard sugar peak to the area of the internal standard peak. In E and F the area of the internal standard peak (A_{is}) is plotted directly.

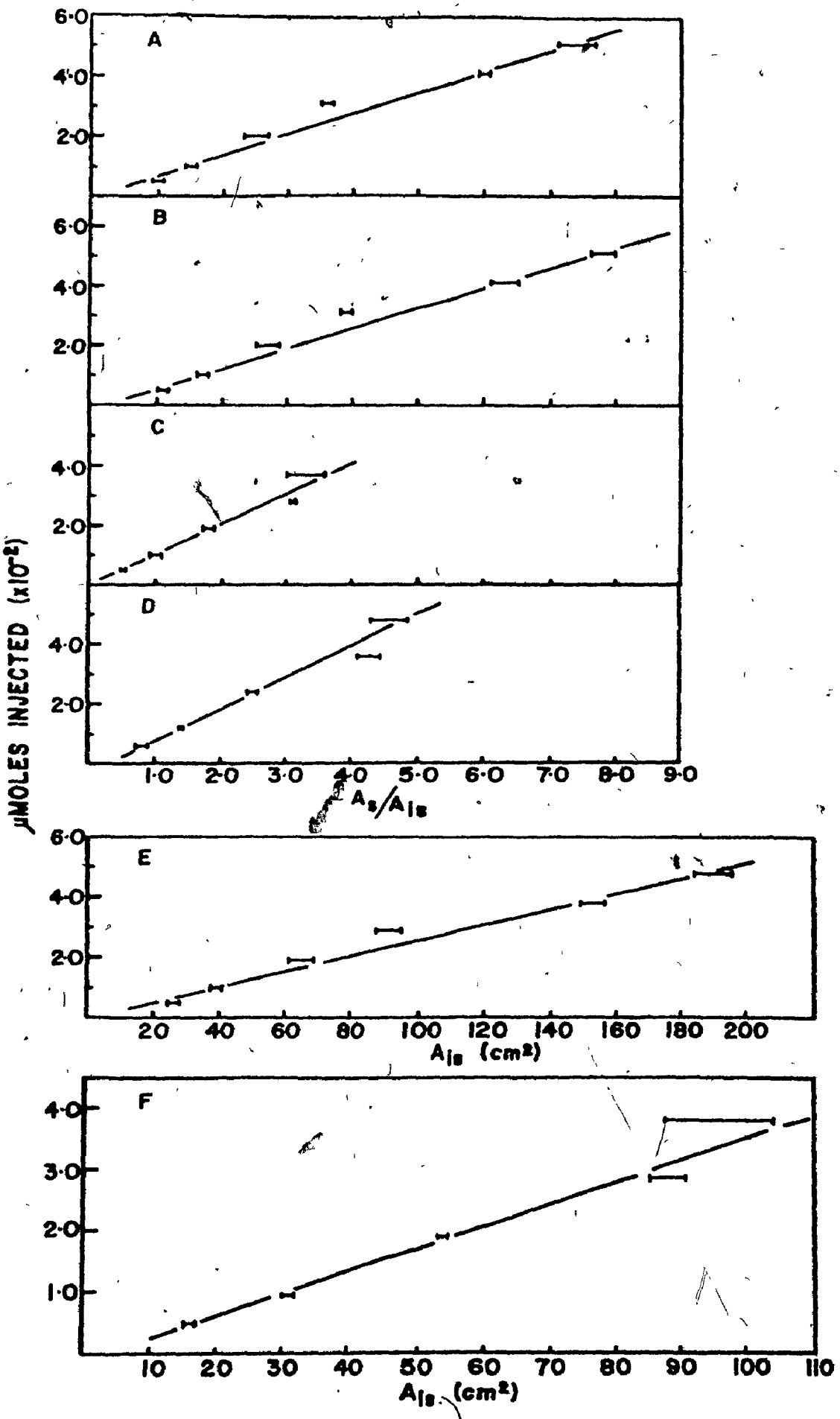

A
B
C
D
E
F
6·0
4·0
2·0
1·0
2·0
3·0
4·0
5·0
6·0
7·0
8·0
9·0
20
40
60
80
100
120
140
160
180
200
10
30
50
70
90
110
μMOLES INJECTED (x10⁻²)
A_s/A_{is}
A_{is} (cm²)
A_{is} (cm²)

TABLE 2.

Determination of the K values for the quantitation of monosaccharides by gas-liquid chromatography of the alditol acetate derivatives[a]

Monosaccharide	Molecular weight	K[b]	Standard deviation	Column
galactose	180.2	1.03	0.04	OV-275
glucose	180.2	0.96	0.02	OV-275
2-amino-2-deoxygalactose	215.6	0.59	0.03	HI-EFF
2-amino-2-deoxyglucose	215.6	0.54	0.04	HI-EFF
heptose[c]	210.2	1.00	--	HI-EFF
unknown 1 (2-amino-2,6-dideoxy-glucose)[c]	163.2	1.00	--	HI-EFF
unknown 2[c]	---	1.00	--	HI-EFF

[a]The internal standard, D-glucoheptose, was arbitrarily chosen as K = 1.00.

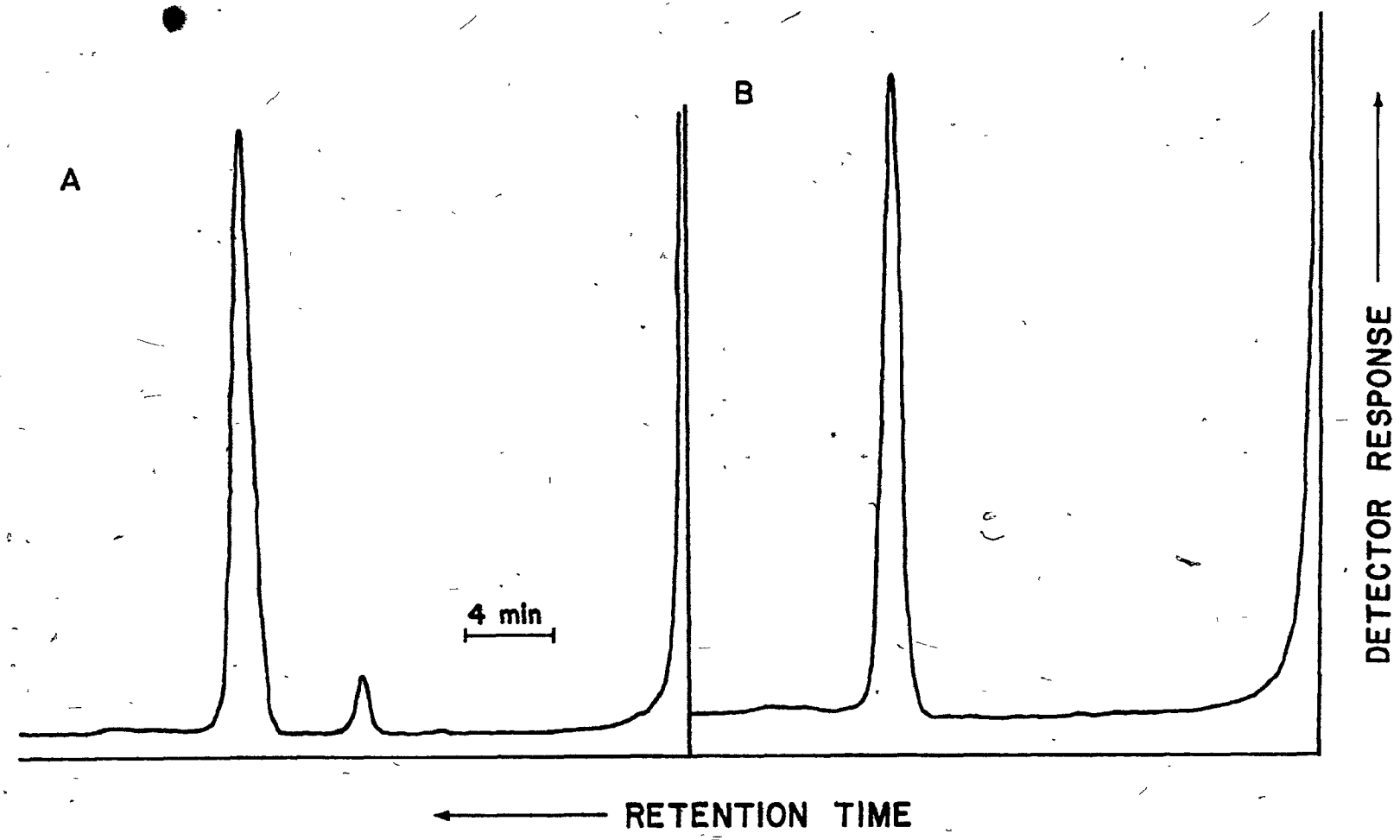
[b]$K_s = \frac{A_s \ W_{is}}{A_{is} \ W_s}$ (see Materials and Methods for explanation).

[c]Since no standards were available for these sugars the K values were determined from those standards that chromatographed the closest to these sugars.

FIGURE 8.

Fig. 8. Examination of the purity of the recrystallized preparation of D-glucoheptose by gas-liquid chromatography.

Glucoheptose hexaacetate was chromatographed on column C at 230 C. Chromatogram A, original D-glucoheptose and chromatogram B, D-glucoheptose following one recrystallization in ethanol.


A
B
4 min
DETECTOR RESPONSE
RETENTION TIME

in a pure preparation which was subsequently added to all samples as an internal standard (figure 8B).

For gas-liquid chromatography, the LPS was hydrolyzed under various conditions to determine the maximal release of the monosaccharides in each of the major carbohydrate groups present. The majority of the monosaccharides found in the marine pseudomonad LPS were either hexoses or hexosamines. This data is presented in Table 3. Maximal release of the neutral carbohydrate was achieved by hydrolysis in 0.1 N hydrochloric acid for 1 h at 100 to 105 C. Hydrolyzing the LPS in lower concentrations of acid resulted in broad peaks with very long retention times which could only be attributed to the presence of disaccharides. Hydrochloric acid (1.0 N) for 1 h at 100 to 105 C was necessary for the maximal release of the amino sugars. The neutral sugars were rapidly destroyed by this stronger hydrolysis while the amino sugars were much more stable to higher concentrations of acid. These results demonstrated that the marine pseudomonad LPS was considerably more acid labile than the lipopolysaccharides found in other species of *Pseudomonas*. Optimum conditions for the release of the carbohydrate from the fatty acids present in the lipid A portion of the LPS molecule were also examined for quantization purposes. Table 4 indicates that the maximum yield of 2-amino-2-deoxyglucose from lipid A was obtained when 4 N hydrochloric acid was used for 4 h at 100 to 105 C.

TABLE 3.

Determination of the hydrolysis conditions necessary for the maximum release of monosaccharides from the degraded polysaccharide portion of the marine pseudomonad lipopolysaccharide[a]

Hydrolysis conditions		% of degraded polysaccharide dry weight				
hydrochloric acid concentration	time (h)[b]	galactose	glucose	2-amino-2-deoxyglucose	2-amino-2-deoxygalactose	heptose
0.1 N	1	9.73±0.05	5.65±0.03	N.A.[c]	N.A.	3.16±0.03
0.5 N	0.5	8.22±0.10	5.10±0.03	2.31±0.09	9.94±0.10	2.91±0.01
0.5 N	1	7.44±0.36	4.28±0.24	5.49±0.06	11.09±0.08	2.37±0.02
1 N	1	7.34±0.09	4.11±0.04	6.25±0.03	12.00±0.08	1.92±0.01
1 N	2	3.24±0.08	1.75±0.06	6.07±0.03	11.22±0.06	0.76±0
2 N	1	2.48±0.06	1.33±0.03	5.75±0.14	9.62±0.20	0.49±0.03
2 N	2	0.94±0.02	0.41±0.01	6.07±0.12	9.95±0.27	0.16±0
2 N	4	0	0	6.87±0.10	10.28±0.07	0
4 N	4	0	0	5.97±0.12	8.92±0.21	0

[a] Quantitation was performed by gas-liquid chromatography of the alditol acetate derivatives.

[b] Hydrolyses were carried out in an oven maintained at 100 to 105 C for the specified length of time.

[c] N.A. = not assayed.

TABLE 4.

Determination of the hydrolysis conditions necessary for the maximum release of 2-amino-2-deoxyglucose from the lipid A portion of the lipopolysaccharide[a]

Hydrolysis conditions		2-amino-2-deoxyglucose
hydrochloric acid concentrations	time (h)	% of lipid A dry weight
4 N	2	11.38±0.12
4 N	4	13.51±0.14
4 N	6	3.88±0

[a]Experimental conditions were the same as those employed in Table 3.

With the hydrolysis conditions for the marine pseudomonad LPS established, gas-liquid chromatography was performed using LPS samples that were hydrolyzed in 0.1 N and 1 N HCl for 1 h. All LPS samples had to be hydrolyzed under both sets of conditions. The chromatograms in figure 9 illustrate the separation of the carbohydrate components in the degraded polysaccharide and lipid A portions of the LPS. All of the monosaccharides, except glucose and galactose, in the degraded polysaccharide portion of the LPS were cleanly separated on the HI-EFF column at 230 C. Glucose and galactose eluted as a single peak and, therefore, had to be separated on the OV-275 column. The amino sugars eluted exceedingly late on OV-275 resulting in broad, barely detectable peaks; consequently, the remainder of the chromatogram was not shown. The lipid A was chromatographed on the HI-EFF column which showed that 2-amino-2-deoxyglucose was the only identifiable component. The shoulders which are seen on the glucosamine and internal standard peaks could not be identified, however, they made an insignificant contribution to the total peak area on the chromatograms.

The chemical composition of the whole cell LPS is shown in Table 5. Total neutral carbohydrate was determined by the anthrone procedure rather than by the phenol-sulfuric acid assay. The latter method gave consistently higher values, a discrepancy which could not be explained. The lipid A portion of the LPS molecule was assayed by

FIGURE 9.

Fig. 9. Carbohydrate analysis of degraded polysaccharide and lipid A from the marine pseudomonad LPS by gas-liquid chromatography of the alditol acetate derivatives.

Degraded polysaccharide was chromatographed on HI-EFF (chromatogram A) and OV-275 (chromatogram B). Lipid A was chromatographed only on HI-EFF (chromatogram C). Peaks were identified as: 1, galactose; 2, glucose; 3, heptose; 4, glucoheptose; 5, glucose and galactose; 6, unknown 1; 7, unknown 2; 8, heptose; 9, glucoheptose; 10, 2-amino-2-deoxyglucose and 11, 2-amino-2-deoxygalactose. Att. equals attenuation.

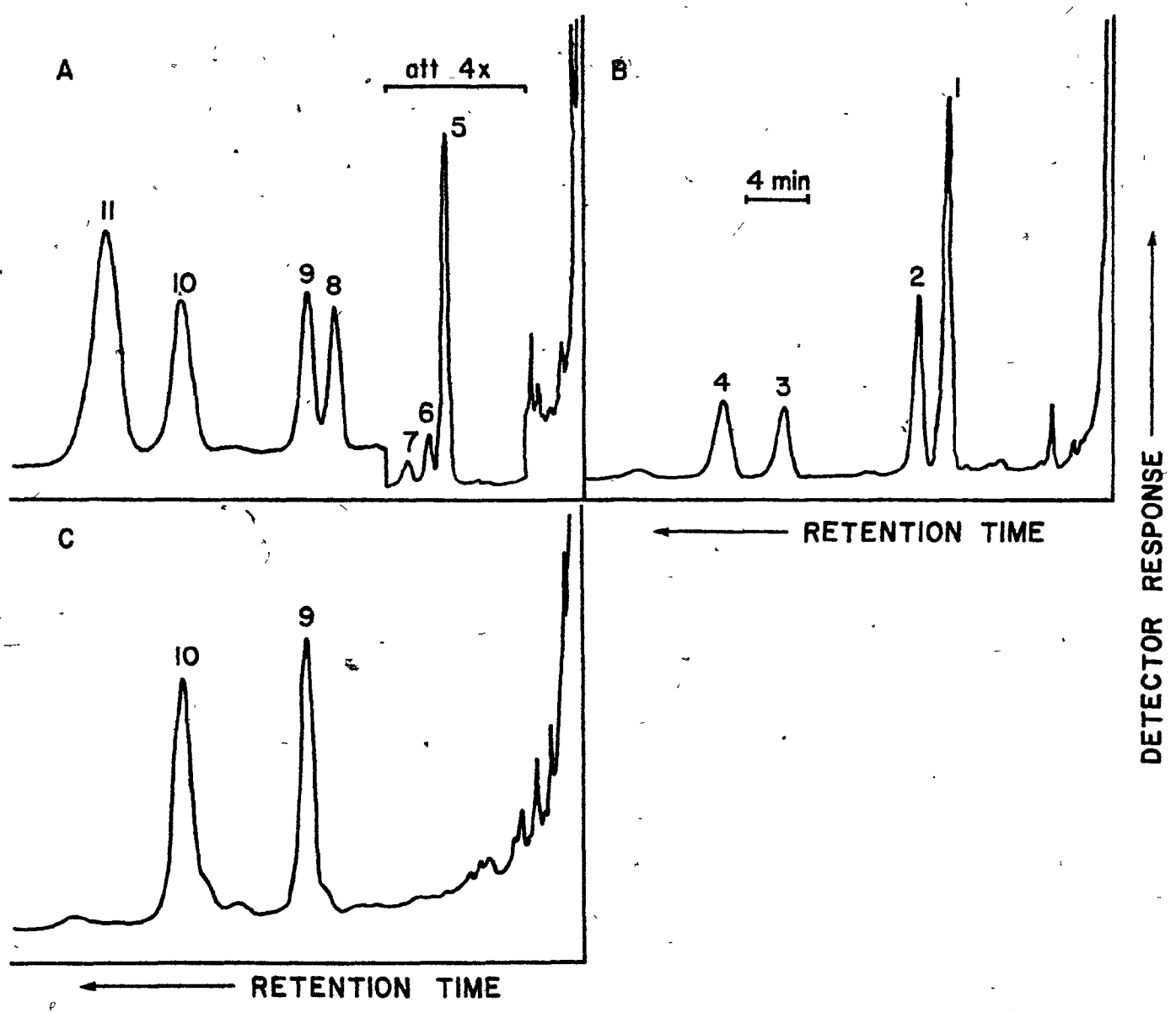

A
att 4x
11
10
9
8
7
6
5
B
4 min
1
2
4
3
RETENTION TIME
C
10
9
RETENTION TIME
DETECTOR RESPONSE

TABLE 5.

Composition of the lipopolysaccharide isolated from the marine pseudomonad

Components	% of LPS dry weight
Total neutral sugars	24.9 ± 6.2
glucose	4.64 ± 0.52
galactose	7.99 ± 0.90
heptose	3.06 ± 0.10
total amino sugars	27.7 ± 2.9
unknown 1[a]	1.71 ± 0.03
unknown 2	1.36 ± 0.09
2-amino-2-deoxyglucose	6.77 ± 0.27
2-amino-2-deoxygalactose	11.94 ± 0.21
lipid A	9.59 ± 0.91
2-amino-2-deoxyglucose	1.34 ± 0.13
2-keto-3-deoxyoctulosonic acid	trace
protein	1.0 ± 0.1
phosphate	3.0 ± 0.3
ash	19.2 ± 0.2

[a]This component was tentatively identified as 2-amino-2,6-dideoxy-glucose (quinovosamine).

the phenol-sulfuric acid procedure and gave a positive reaction. However, neutral carbohydrate was not detected when the lipid A was analyzed by gas-liquid chromatography. Total amino sugar, as determined by a modified Elson-Morgan, showed that the LPS consisted of a slightly higher percentage of total hexosamine than total hexose. The monosaccharides were identified and quantitated by gas-liquid chromatography of the corresponding alditol acetate derivatives as discussed previously. The dominant monosaccharide was 2-amino-2-deoxygalactose which along with 2-amino-2-deoxyglucose, galactose, and glucose composed the majority of the LPS. Unknown 1 was tentatively identified as 2-amino-2,6-dideoxyglucose (quinovosamine) by Dr. M.B. Perry (personal communication) using gas-liquid chromatography and ion exchange chromatography. Unknown 1 was found to be extremely stable to acid while unknown 2 was acid labile. O'Leary *et al.* (1972) discovered two unknown monosaccharides in the LPS from this bacterium and tentatively identified them as quinovosamine and a 4-amino sugar. The 4-amino sugars are known to be extremely acid labile (see review by Lüderitz and co-workers, 1966). Since there were not any standards available for these two sugars and due to the fact that O'Leary used descending paper chromatography, these results cannot be correlated with the gas-liquid chromatographic data. There is also some discrepancy between these results and those obtained by O'Leary and co-workers for the quantitation of the other monosac-

charides. Several factors should be noted: (i) O'Leary *et al.* extracted LPS from a mixture of the marine pseudomonad variants because they had not been distinguished at that time (Gow *et al.*, 1973). This study used a pure culture of variant 3. (ii) Lipopolysaccharides are known to undergo alterations in the concentrations of some components with changing cultural conditions (McDonald and Adams, 1971 and Frasch *et al.*, 1976). These analyses were performed on fermenter grown cells rather than on batch cultures.

Lipid A, isolated following the mild acid hydrolysis of LPS, was quantitated by drying to constant weight. It was soluble in $CHCl_3$-CH_3OH (2:1) but not in $CHCl_3$. Hydrolysis of the lipid A demonstrated that 2-amino-2-deoxyglucose was the only carbohydrate component. The fatty acids were identified as mainly C12 and C13 with lower concentrations of C10, C12 and C14 hydroxy fatty acids (C. Deneke, personal communication). Only a trace amount of KDO, usually less than 0.1%, could be detected by the thiobarbituric acid assay. The assay itself was very unreliable as already stated by Dröge *et al.* (1970). A small amount of contaminating protein was detected, by the Lowry procedure, as well as a low concentration of organic phosphate. No inorganic phosphate was detected. A high ash component was present and was detected following combustion of the LPS. The quantitative data for lipid A and noncarbohydrate components agreed with that reported previously by O'Leary *et al.* (1972).

Approximately 20 to 30% of the LPS could not be accounted for, which apparently is a common problem in LPS research (Nowotny, 1971). It was decided to examine the preparation for the presence of contaminating nucleic acids even though the LPS was treated with nucleases. The LPS, dispersed in distilled water with the help of gentle heating at 37 C, yielded a broad absorption peak at 255 nm which did not compare with an aqueous suspension of RNA which exhibited an absorption maximum at 260 nm. Also, there was no absorption peak observed at 280 nm which discounted the presence of large amounts of contaminating protein (figure 10). Yet, an unknown component still contributed ultraviolet absorption at 255 nm since purified LPS should not demonstrate any absorption maxima in the 210-300 nm region of the ultraviolet spectrum (Johnson *et al.*, 1975). Lipopolysaccharide was dispersed in 0.1% Triton X-100 and demonstrated a skewed peak again with a maximum absorbance at approximately 255 nm and a shoulder between 240 and 245 nm (figure 11). Lipid A alone, dispersed in 0.1% Triton X-100, absorbed ultraviolet light over the same wavelength range as the LPS but a shift was observed with the major peak now at 241 nm and a shoulder at 255 nm. When the LPS was read against the lipid A as a blank the 241 nm peak was lost with only the 255 nm peak remaining. Therefore, there are two LPS contaminants, other than nucleic acid or protein, one of which can be attributed to the lipid A portion and the other can be localized in both the lipid A and degraded polysaccharide.

FIGURE 10.

Fig. 10. Ultraviolet absorption scan of the marine pseudomonad LPS.

Curve 1, an aqueous suspension of LPS (550 µg/ml), curve 2, an aqueous suspension of yeast RNA (160 µg/ml) and curve 3 represents the distilled water baseline.

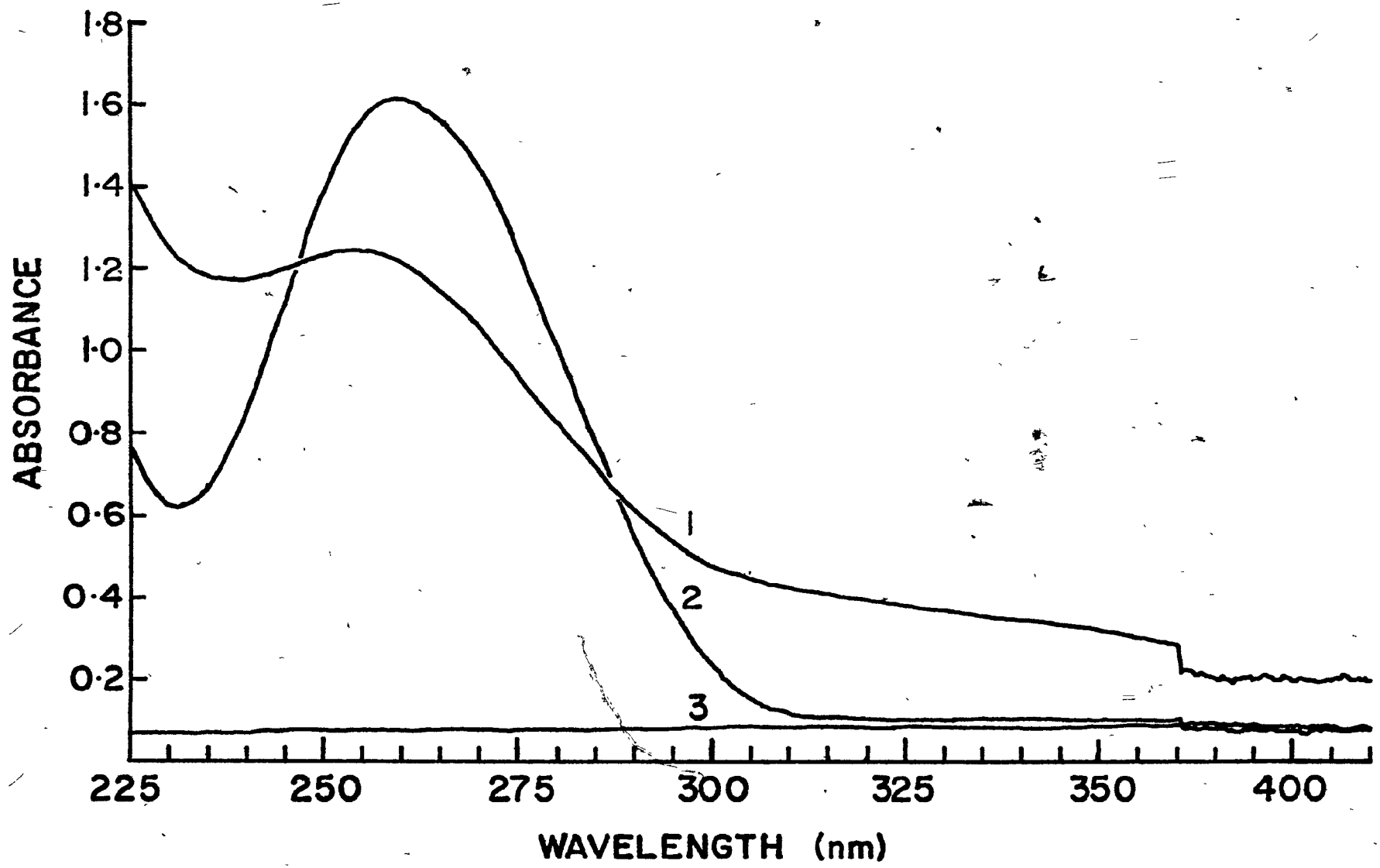
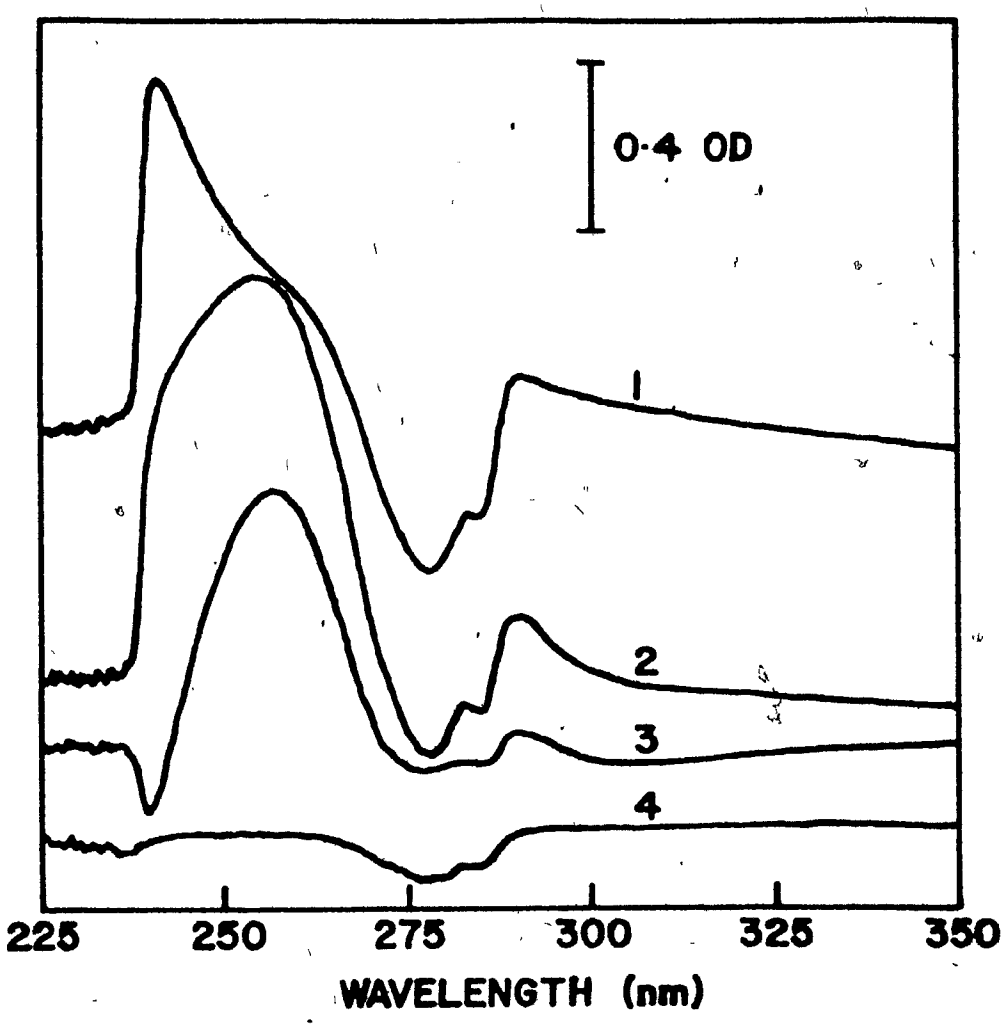

ABSORBANCE
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
225
250
275
300
325
350
400
WAVELENGTH (nm)
1
2
3

FIGURE 11.

Fig. 11. Ultraviolet absorption scan of LPS dispersed in the nonionic detergent, Triton X-100.

Curve 1 consists of lipid A in 0.1% Triton X-100 (250 µg/ml) and curve 2 represents LPS in 0.1% Triton X-100 (800 µg/ml). In curve 3, the LPS sample was read against the lipid A preparation as a blank. The Triton X-100 baseline is represented by curve 4.


0·4 OD
1
2
3
4
225
250
275
300
325
350
WAVELENGTH (nm)

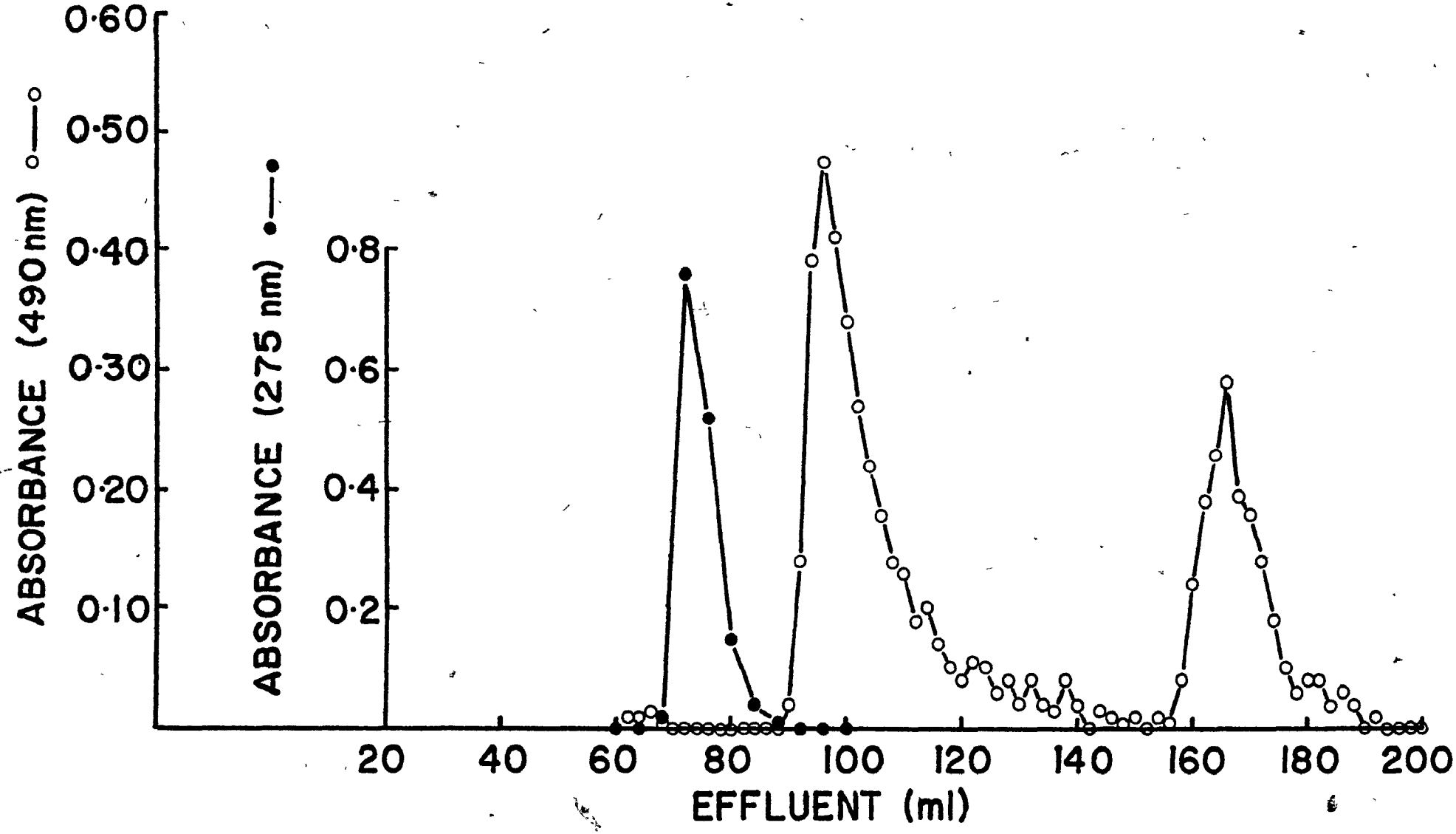
General structure

It was advantageous to gain information concerning the general form (the presence or absence of an O-antigen side chain) of the marine pseudomonad LPS. The LPS was hydrolyzed in 1% acetic acid and separated into lipid A and degraded polysaccharide. The degraded polysaccharide portion was dissolved in pyridinium acetate buffer (pH 5.4) and applied to a Sephadex G-50 gel filtration column. The sample was eluted in the same buffer and the effluent was monitored for the presence of total neutral carbohydrate. Two peaks were obtained, one eluted near the void volume and the second within the included volume of the column (figure 12). It appeared that there may be some very low molecular weight material eluting just behind the second carbohydrate peak. Chromatography of the degraded polysaccharide on Sephadex G-25 showed that the low molecular weight peak tailed slightly indicating the presence of some very light material (figure 13). This material could be monosaccharides or carbon fragments that were liberated during hydrolysis. Schmidt *et al*, (1969) established that two carbohydrate peaks were extracted from *E. coli* when chromatographed on Sephadex G-50 under the conditions used to obtain the results shown in Figure 12. The higher molecular weight peak represented a core-side chain fraction and the lighter peak consisted of core alone. Lipopolysaccharides which do not contain a side chain give rise to a single low molecular weight peak. Thus, the results in figure 12 support the conclusion that

FIGURE 12.

Fig. 12. Detection of core and core-side chain fractions by Sephadex G-50 gel filtration of the degraded polysaccharide portion of the marine pseudomonad LPS.

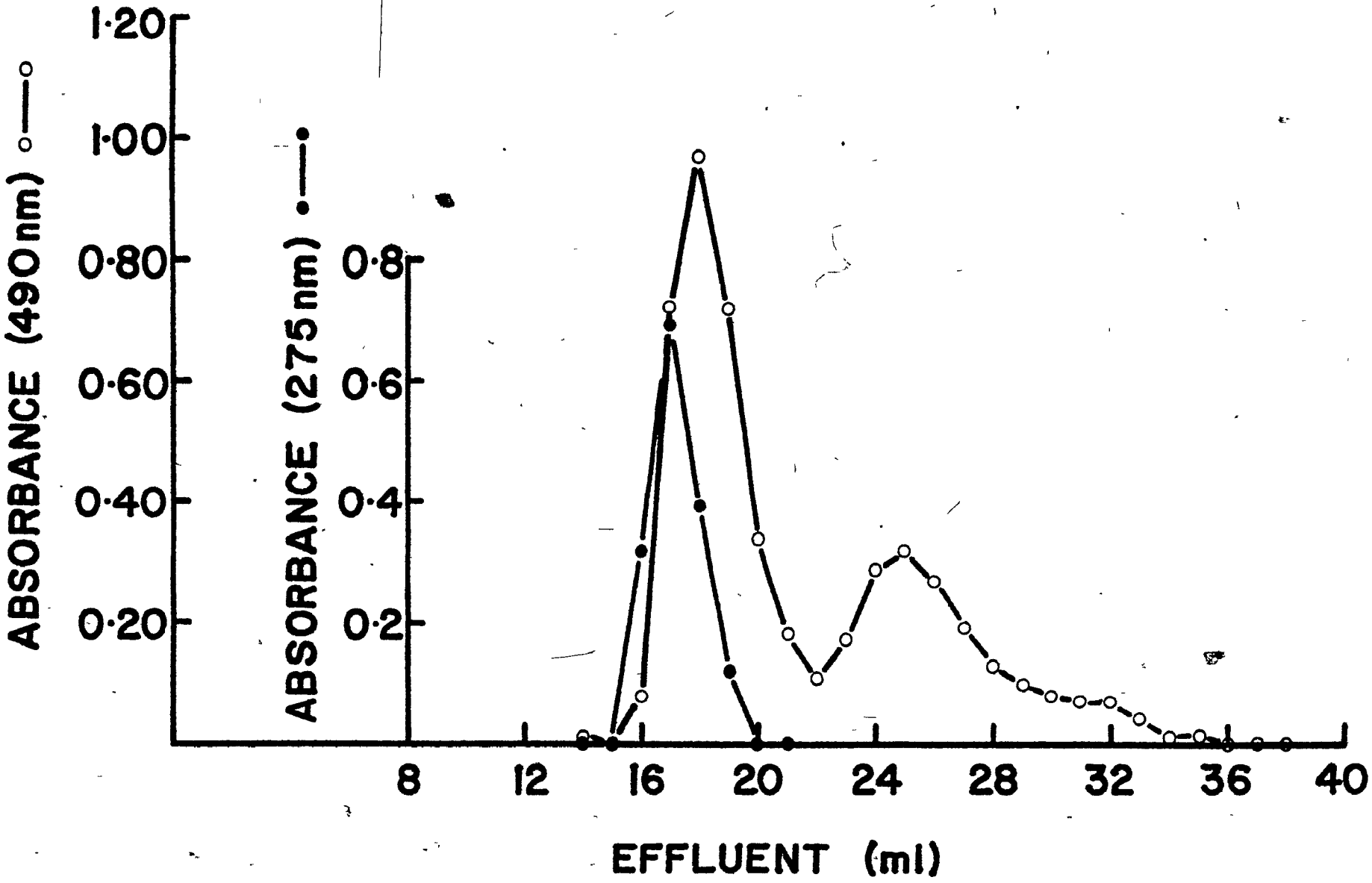
Degraded polysaccharide (11 mg/ml) was chromatographed in pyridinium acetate buffer (pH 5.4). The column effluent was assayed for neutral carbohydrate, O—O and the void volume of the column was determined with 0.2% blue dextran ●—●.


0·60
0·50
0·40
0·30
0·20
0·10
ABSORBANCE (490 nm)
0·8
0·6
0·4
0·2
ABSORBANCE (275 nm)
20
40
60
80
100
120
140
160
180
200
EFFLUENT (ml)

F I G U R E 13.

Fig. 13. Gel filtration of degraded polysaccharide on Sephadex G-25.

The experimental conditions were the same as those descrfbed for figure 12. Total neutral carbohydrate o—o. Blue dextran ●—●.


ABSORBANCE (490nm) o—o
ABSORBANCE (275nm) •—•
1·20
1·00
0·80
0·60
0·40
0·20
0·8
0·6
0·4
0·2
8
12
16
20
24
28
32
36
40
EFFLUENT (ml)

the LPS isolated from the marine pseudomonad contains both a core and side chain. Additional support for the results indicating that the marine pseudomonad LPS contained an O-antigenic side chain stemmed from the observation that LPS could not be extracted from this variant with the phenol-chloroform-petroleum ether extraction procedure of Galanos and co-workers (1969). These workers found that lipopolysaccharides that contain O-antigens cannot be extracted by this method.

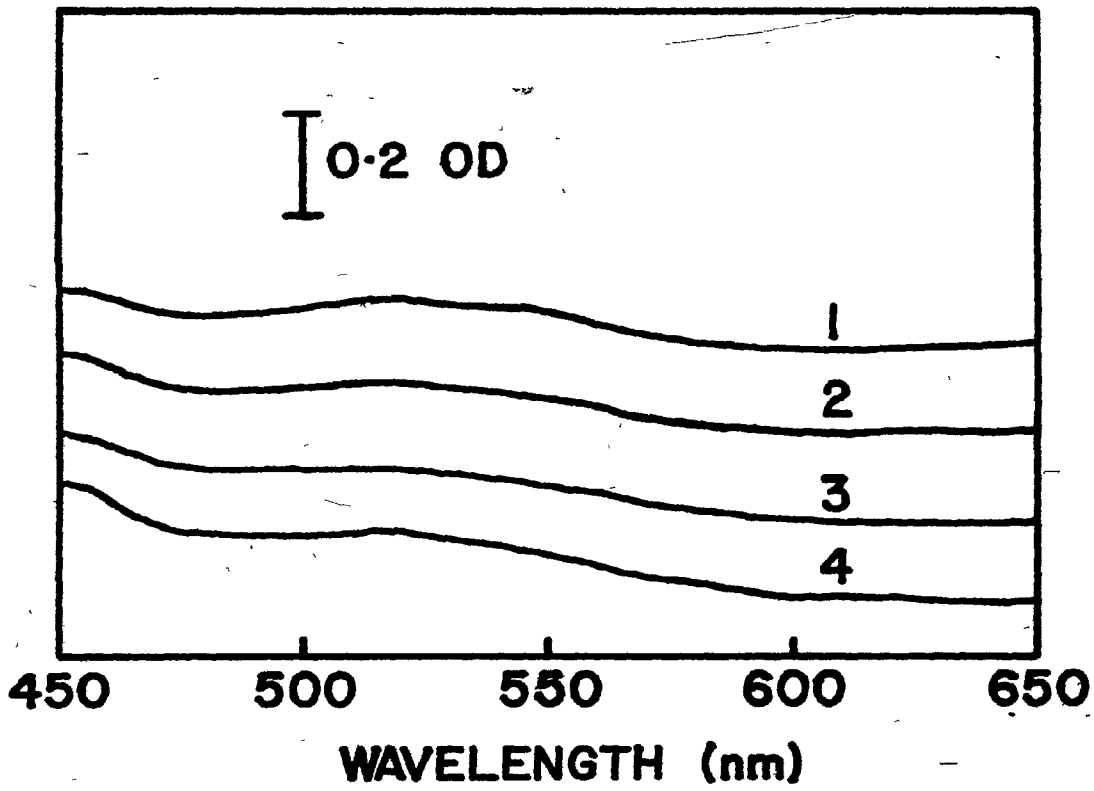
Localization

Nelson and MacLeod (submitted for publication) demonstrated, immunologically, the presence of LPS in the cell wall layers of the marine pseudomonad. Efforts were also made to demonstrate the presence of LPS chemically by analyzing for KDO and heptose in the cell wall layers. The amounts of these compounds present in LPS isolated from the marine pseudomonad are very low and when attempts were made to determine quantitatively the amounts of LPS in the wall layers based on the amount of KDO present discrepancies resulted. To re-examine these observations, LPS and the cell wall layers isolated from the marine pseudomonad were assayed for KDO and scanned for the presence of an absorption peak at 545 nm. Under these assay conditions a KDO peak was barely detectable in LPS and not detectable in the cell wall layers (figure 14). The heptose assay did not constitute much of an improvement over the KDO procedure since there were

FIGURE 14.

Fig. 14. Detection of KDO in LPS and the cell wall layers of the marine pseudomonad by scanning wavelength spectrophotometry following the thiobarbituric acid assay.

Curve 1, LPS; curve 2, loosely bound outer layer; curve 3, outer double-track and curve 4, periplasmic layer. In each case 1 mg of material was assayed. The absorbance maximum of KDO is 549 nm.


0·2 OD
1
2
3
4
450
500
550
600
650
WAVELENGTH (nm)

no detectable absorption peaks at the maximum wavelength of 505 nm (figure 15). The results supported Nelson's earlier observations which indicated that KDO and heptose could not be used as LPS indicators in the cell wall layers because of their low concentrations and the lack of reproducibility of the analytical results obtained.

The carbocyanin dye procedure gave an improved method of LPS detection in the cell wall layers as seen in figure 16, however, this procedure was not quantitative, as was described previously. The spectral shift to a lower wavelength was observed with each of the cell wall layers with a slight difference among the actual wavelength maxima. There was also the presence of large absorption peaks at the higher wavelengths which was to be expected since the wall layers contained protein and lipid in addition to the LPS. The material released into the growth medium also contained some LPS while the cytoplasmic membrane either failed to demonstrate any LPS or showed only a trace.

FIGURE 15.

Fig. 15. Detection of heptose in LPS and the cell wall layers by scanning wavelength spectrophotometry.

Curve 1, LPS; curve 2, loosely bound outer layer; curve 3, outer double-track; curve 4, periplasmic layer; and curve 5, D-glucoheptose (50 µg). The heptose assay was conducted on 1 mg of each of the other samples. The absorbance maximum of heptose is 505 nm.

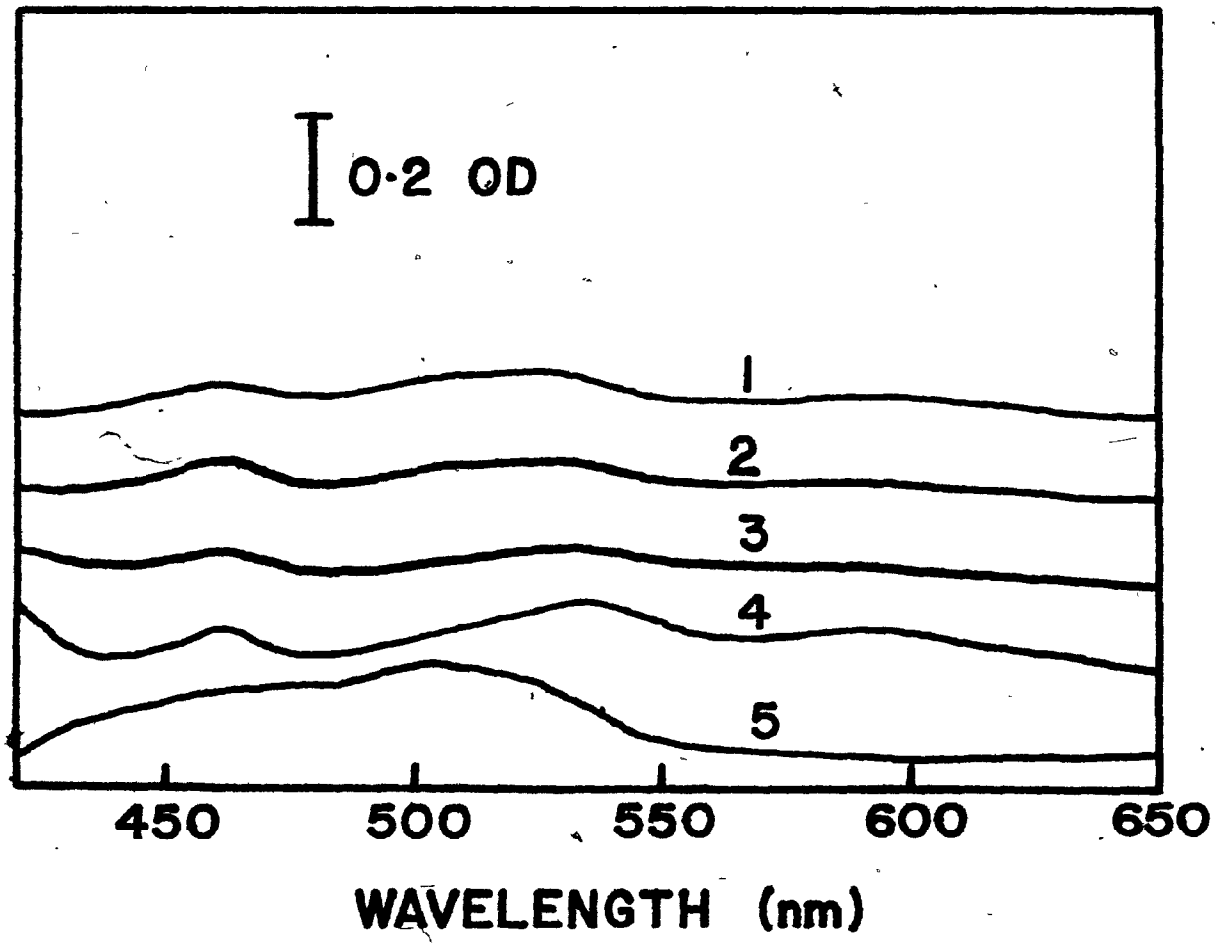
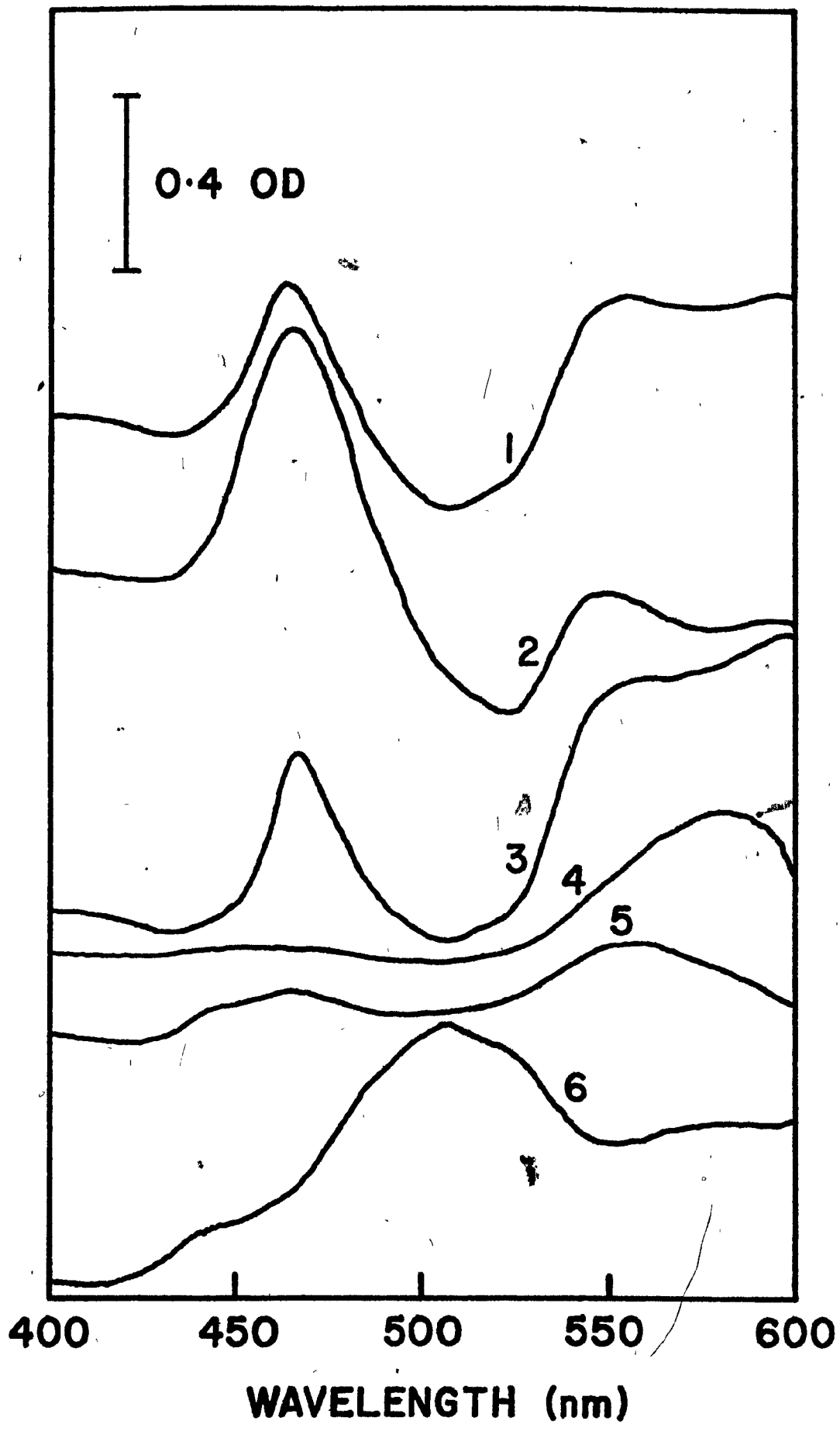

0·2 OD
1
2
3
4
5
450
500
550
600
650
WAVELENGTH (nm)

FIGURE 16.

Fig. 16. Localization of LPS in the cell wall layers and in the material released into the growth medium using the carbocyanin dye procedure.

The samples were reacted with the carbocyanin dye and scanned to observe the shift in absorbance maximum. Scan 1, 20 µg loosely bound outer layer; scan 2, 100 µg outer double-track; scan 3, 100 µg periplasmic layer; scan 4, 100 µg cytoplasmic membrane; scan 5, 100 µg material from growth medium; and scan 6, dye reagent.


0·4 OD
1
2
3
4
5
6
400
450
500
550
600
WAVELENGTH (nm)

SECTION II

HETEROGENEITY OF WHOLE CELL LIPOPOLYSACCHARIDE

Method for the determination of heterogeneity

The whole cell LPS, extracted from the marine pseudomonad and purified, was used for studies on LPS heterogeneity. Heterogeneity was examined using SDS-polyacrylamide gel electrophoresis which employed a buffer consisting of 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS. This buffer was a modification of that used by Shapiro *et al.* (1967) for the separation of polypeptide chains. It is well known that monosaccharides can be separated in an electric field in the presence of a buffer containing borate, molybdate or stannate (see reviews by Foster, 1957; Weigel, 1963 and Zittle, 1951). Migration is the result of complex formation between these anions and free hydroxyl groups on the carbohydrate and, for this reason, sodium molybdate was included in the electrophoresis buffer. Modifications of this buffer were examined: (i) to justify the presence of the individual buffer components and (ii) to determine the effect of the components on the electrophoretic mobility of the LPS.

Polyacrylamide gels that contained LPS samples were fixed and stained according to the procedure outlined in Table 6. This procedure was developed from that used by Zacharius and co-workers (1969) for the staining of glycoproteins. It was discovered that if the

TABLE 6.

Periodate-Schiff procedure used to stain lipopolysaccharides

Step[a]	Time
1. Fix gels in 20 ml 12% trichloracetic acid in 50% methanol	1 h or overnight
2. Rinse in distilled water	
3. Immerse gels in 20 ml 1% periodate in 3% acetic acid	3 h
4. Wash six times in 200 ml distilled water	10 min/wash
5. Immerse in 20 ml Schiff reagent in the dark	2 h
6. Wash gels three times in 50 ml freshly prepared 0.5% sodium metabisulfite	10 min/wash
7. Destain gels in 100 ml 7.5% acetic acid at 30 C with constant shaking	overnight
8. Store gels in 7.5% acetic acid	

[a]All procedures are carried out at room temperature unless otherwise noted.

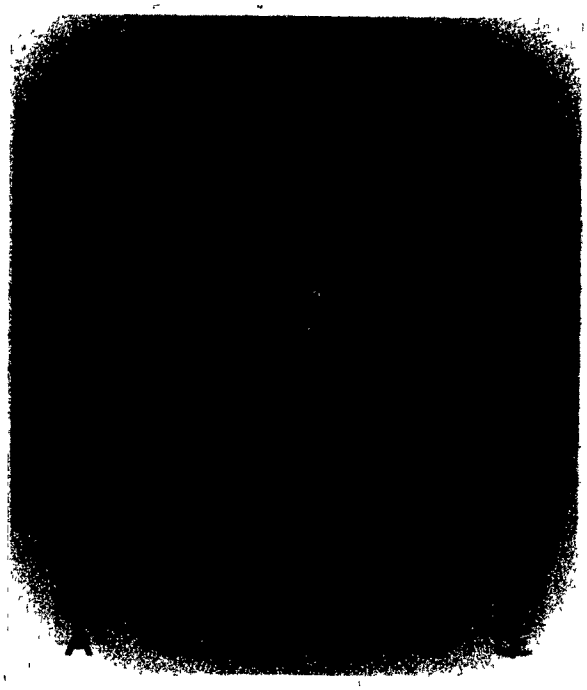
12% trichloroacetic acid was made up in 50% methanol more efficient fixation was achieved with complete fixation occurring in as short a period of time as 1 h. It was also found that maximum periodate oxidation did not occur until after 3 h and that the staining time had to be extended to 2 h. Due to the presence of SDS in the gels, destaining could not be performed in distilled water without discoloration of the gel. Instead 7.5% acetic acid was used for destaining the gels. Destaining could be completed overnight if constant mixing at an elevated temperature (37 C) was used.

Electrophoresis of the marine pseudomonad LPS extracted from variant 3 is shown in figure 17A. The LPS separated into three carbohydrate staining bands which were designated LPS I, II and III, as a matter of convenience, in order of increasing electrophoretic mobility. When compared to LPS extracted from variant 1 of the marine pseudomonad there did not appear to be any difference in the location or number of carbohydrate bands (figure 17B). It must be pointed out that the variants are distinguished on the basis of colony morphology (Gow *et al.*, 1973). *Escherichia coli* and *Salmonella typhimurium* lipopolysaccharides, after SDS-polyacrylamide gel electrophoresis, separated into two and three carbohydrate staining bands, respectively (figure 17C and 17D). The LPS from *Neisseria meningitidis* gave only a single homogeneous band which migrated at the position of the lowest carbohydrate band found in the other

FIGURE 17.

Fig. 17. Polyacrylamide gel electrophoresis of lipopolysaccharides extracted from various Gram-negative bacteria.

Electrophoresis was performed for 5 h at 5 mA/gel in a 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS buffer. The gels were 5% acrylamide and samples were electrophoresed towards the anode. LPS was detected by staining with the Schiff reagent after periodate oxidation. Gel A, marine pseudomonad (var. 3); gel B, marine pseudomonad (var. 1); gel C, *E. coli*; gel D, *S. typhimurium*; Gel E, *P. aeruginosa* and gel F, *N. meningitidis*. The amounts of LPS applied to the gels were: A and B, 207 µg; C, D, and F, 322 µg; and E, 338 µg.



bacterial lipopolysaccharides (figure 17F). *Pseudomonas aeruginosa* LPS (Figure 17E) repeatedly failed to stain clearly even when as much as 0.5 mg were applied to the gel. It is possible that this is due to the presence in the organism of LPS which is highly branched. An LPS which is highly branched would not be oxidized completely by periodate due to the lack of adjacent free hydroxyl groups in the molecule and, consequently, will not bind the stain. A faint band can be seen, however, at the position of the lowest bands in the other LPS gels.

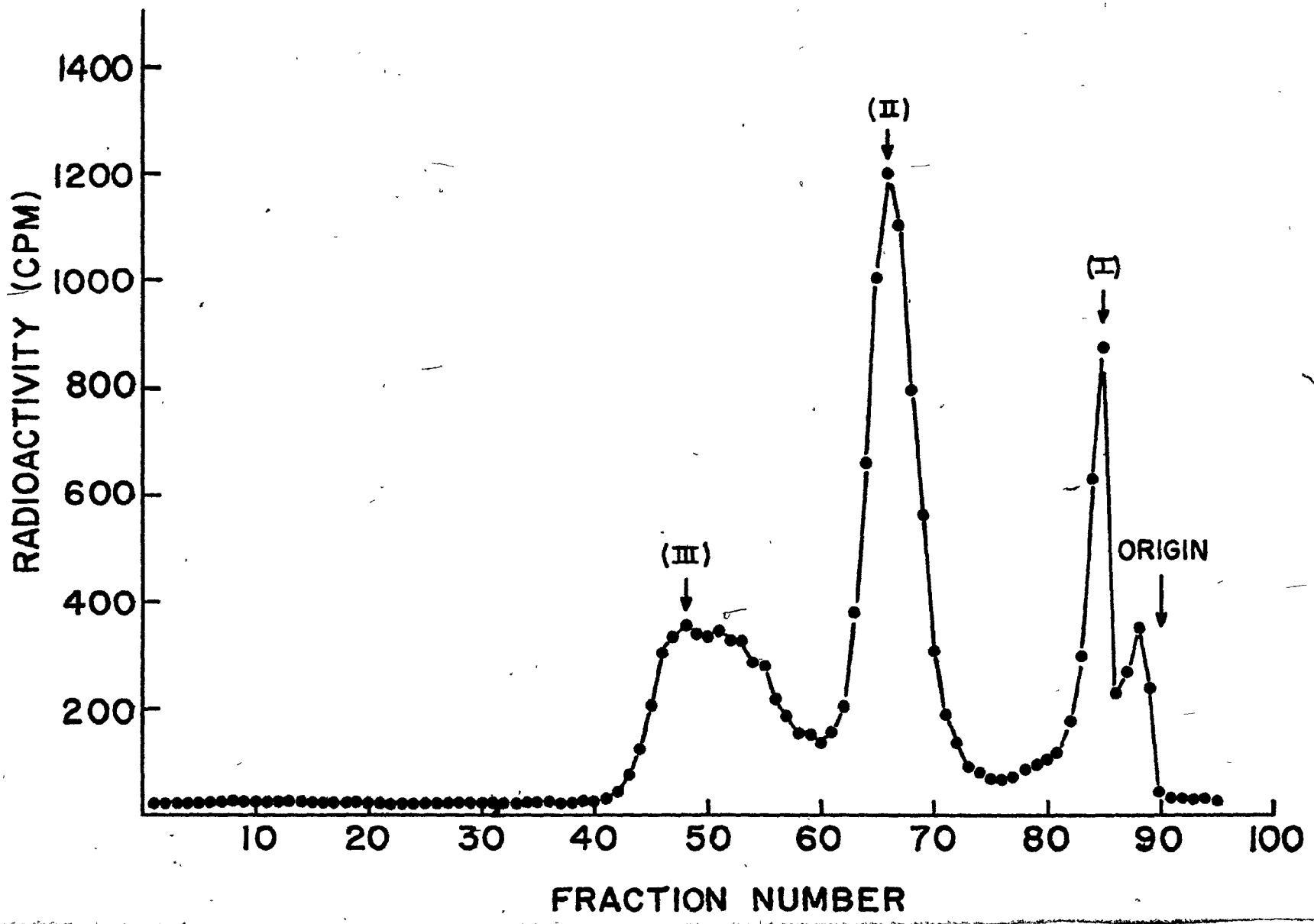
Characterization of [^{14}C]*radiolabeled lipopolysaccharide for use in heterogeneity studies*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was also performed on LPS labeled by growing variant 3 in the presence of [^{14}C]galactose. Figure 18 shows the separation of the [^{14}C]LPS components in the 0.05 M Na_2HPO_4- 0.05 M Na_2MoO_4 - 1% SDS electrophoresis buffer. The radiolabeled LPS was also stained for carbohydrate with the Schiff reagent after electrophoresis and the stained bands were compared with the radioactive profile and corresponded exactly. This gave two separate methods of detection of the LPS components, thus confirming the heterogeneity of aqueous phenol extracted LPS from the marine pseudomonad. Comparison of both methods also indicated that there was no selective staining of one or several of the LPS components by the Schiff reagent or specific labeling by [^{14}C]galactose.

FIGURE 18.

Fig. 18. Separation of [^{14}C]labeled LPS components by SDS-polyacrylamide gel electrophoresis.

The electrophoresis buffer consisted of 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS. The gel, was fractionated and counted for radioactivity with each fraction equivalent to 1 mm of gel. Electrophoresis time was 5 h at 5 mA/gel.


RADIOACTIVITY (CPM)
1400
1200
1000
800
600
400
200
(III)
(II)
(I)
ORIGIN
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

Usually 80 to 95% of the [^{14}C]LPS was recovered from the polyacrylamide gels.

The marine pseudomonad LPS could not be radiolabeled specifically because of unsuccessful attempts to isolate a proper mutant. Mutants were needed that lacked one of the specific transferase enzymes involved in core elongation during LPS biosynthesis (Nikaido, 1962). During attempts to isolate LPS deficient mutants, several problems became immediately evident. It was not known (i) if the LPS biosynthetic enzymes in the marine pseudomonad were the same as those characterized only in *E. coli* and *S.typhimurium*, and (ii) how to selectively screen for such a mutant in the marine pseudomonad. Several attempts to isolate mutants were made using ultraviolet irradiated cells, picking the minute colonies and replica plating onto succinate defined medium with and without galactose. Again, it was assumed that the marine pseudomonad might behave similarly to *E. coli* and *S. typhimurium* in that a mutant cell containing a defective LPS would grow without galactose but if galactose was present UDP-galactose would accumulate and after one or two generations the cells would lyse (Fukasawa and Nikaido, 1961). All colonies that were selected grew at an increased rate in the presence of galactose and never lysed.

It was already known that the LPS in variant 3 could be radiolabeled so it was decided to examine the disposition of the labeling. Lipopolysaccharide, labeled with [^{14}C] by adding [^{14}C]galactose during

growth of the cells, was hydrolyzed to release lipid A and degraded polysaccharide. The fractions were separated, dried to constant weight, and their specific activities were determined (Table 7). There was approximately a ten-fold difference between the specific activities of the degraded polysaccharide and the lipid A portions of the LPS molecule. The lipid A was hydrolyzed and separated into carbohydrate (water soluble) and fatty acid (petroleum ether soluble) fractions. Practically all of the label was discovered in the water soluble portion. Descending paper chromatography of the water phase demonstrated that 2-amino-2-deoxyglucose was the primary carbohydrate component and that most of the label was in this monosaccharide (figure 19).

Hydrolysis and chromatography of the degraded polysaccharide portion of the LPS molecule showed that most of the label was in 2-amino-2-deoxygalactose, 2-amino-2-deoxyglucose, and galactose with the remainder spread throughout the other monosaccharides (figure 20). This was the best separation that could be obtained on one chromatogram due to the presence of both hexosamines and hexoses in the same sample; thus, the overlapping of the label. Unlabeled degraded polysaccharide was also hydrolyzed and chromatographed (figure 21). The overlapping of the degraded polysaccharide carbohydrate components was more evident in this chromatogram. The degraded polysaccharide was hydrolyzed under conditions which favored the release of both

TABLE 7.

Specific activity of the marine pseudomonad whole cell lipopolysaccharide[a]

Compound or fraction	Specific activity (CPM/mg)	% of total CPM
LPS	2.98×10^5	
Degraded polysaccharide	2.40×10^5	
Lipid A[b]	2.20×10^4	
Water phase		99.3
Petroleum ether phase		0.7

[a]The cells were labeled with [^{14}C]galactose and the LPS was extracted and purified.

[b]The lipid A was hydrolyzed in 4 N HCl for 4 h and then phased with petroleum ether. The water phase contained the carbohydrate components and the ether phase contained the fatty acids.

FIGURE 19.

Fig. 19. Distribution of [^{14}C]labeled carbohydrate components in the lipid A portion of the LPS molecule as demonstrated by descending paper chromatography.

The hydrolyzed [^{14}C]lipid A was chromatographed in ethylacetate-pyridine-water (120 : 50 : 40) for 20 h at room temperature. Standards were detected by spraying with ninhydrin. Spots 1 and 2 represent 2-amino-2-deoxy-galactose and 2-amino-2-deoxyglucose, respectively. Each fraction of the chromatogram consists of a 1.5 cm^2 square which was counted for radioactivity. The radio-labeled profile was compared to the positions of the standard hexosamines.

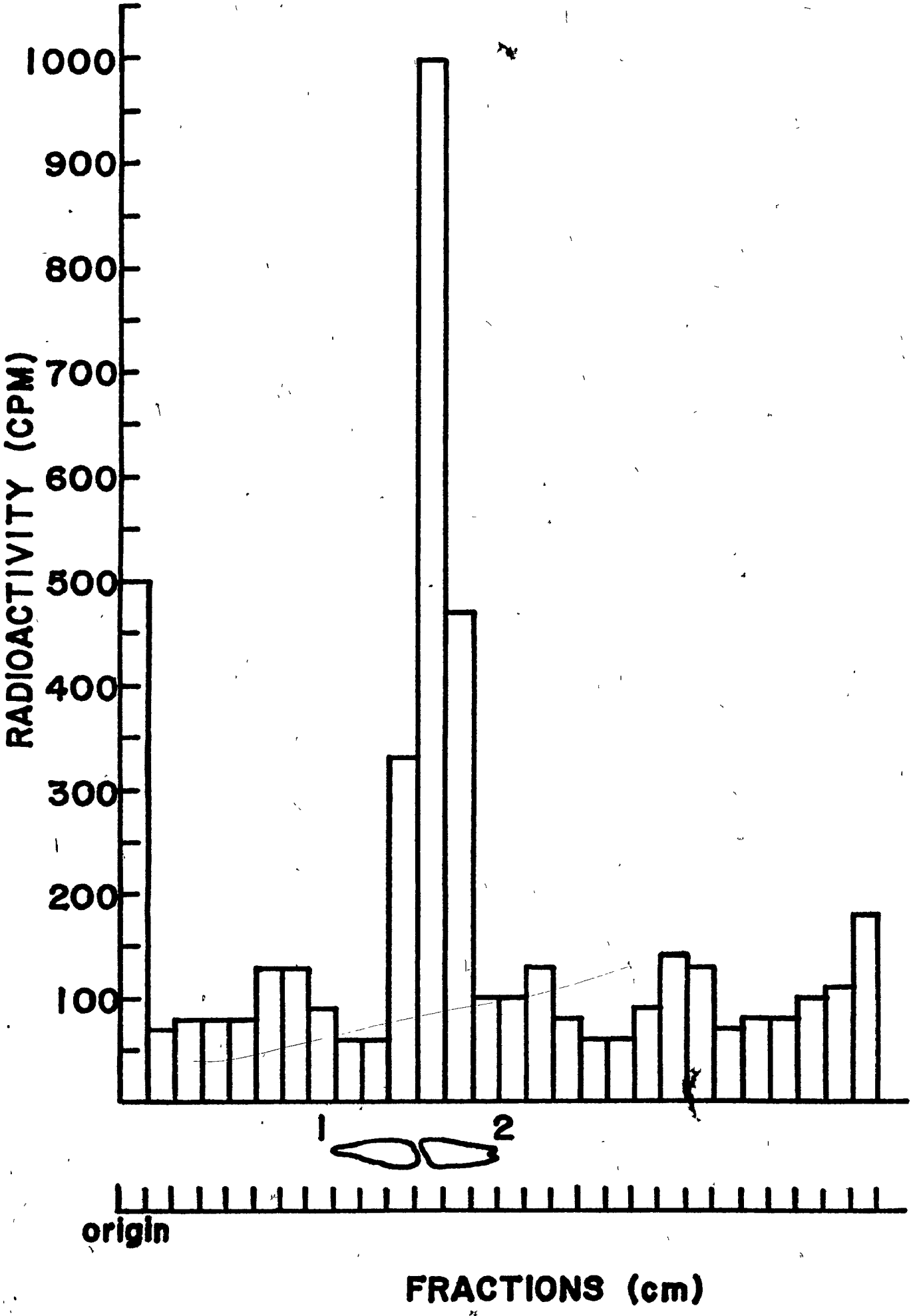

1000
900
800
700
600
500
400
300
200
100
RADIOACTIVITY (CPM)
1
2
origin
FRACTIONS (cm)

FIGURE 20.

Fig. 20. Distribution of [^{14}C]labeled carbohydrate components in the degraded polysaccharide portion of the LPS molecule as demonstrated by descending paper chromatography.

Experimental conditions were the same as in figure 19, except that analine phthalate was used in addition to ninhydrin for the detection of monosaccharide standards. Spot 1, 2-amino-2-deoxygalactose; spot 2, 2-amino-2-deoxyglucose; spot 3, galactose and spot 4, glucose.

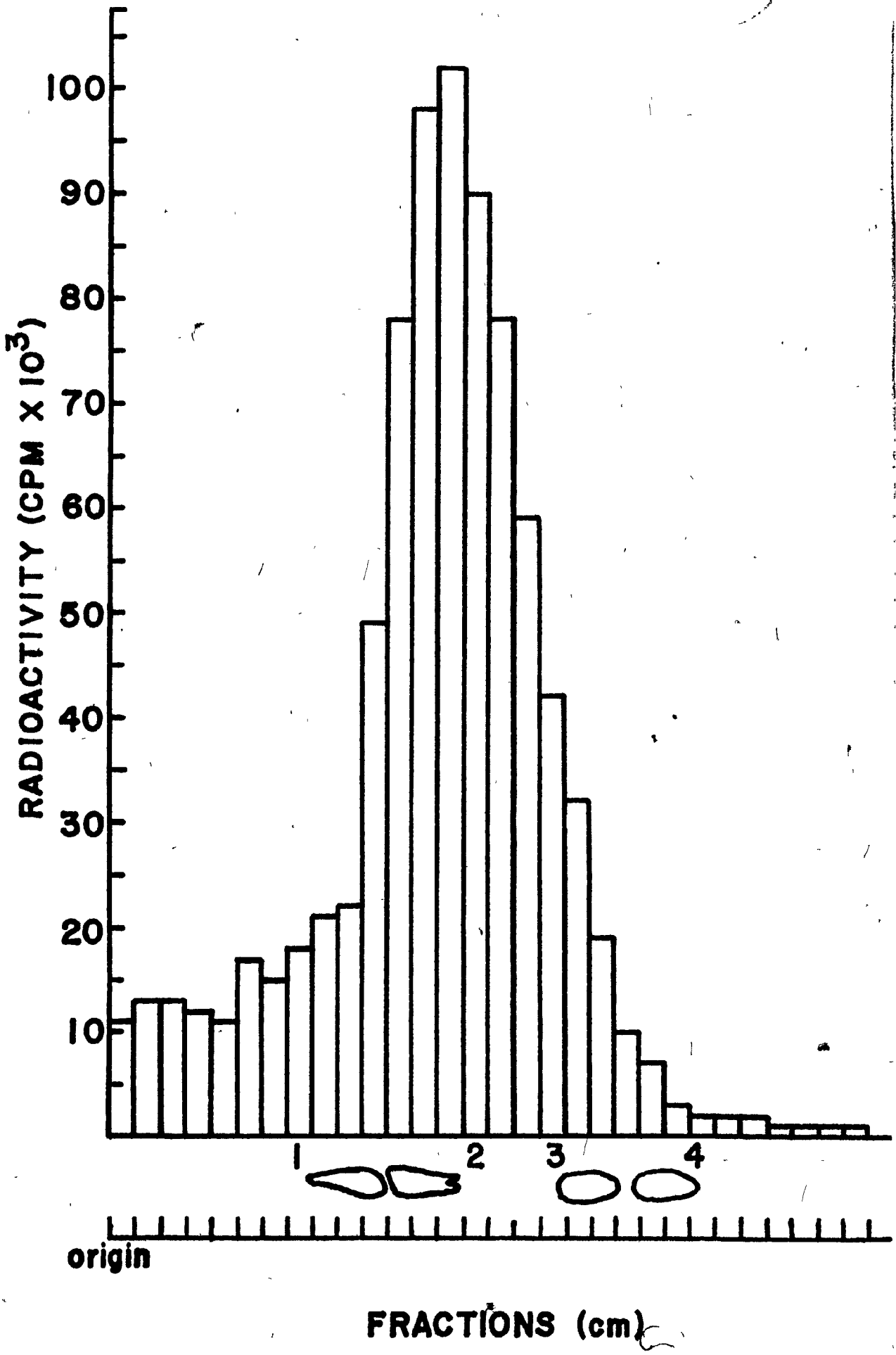
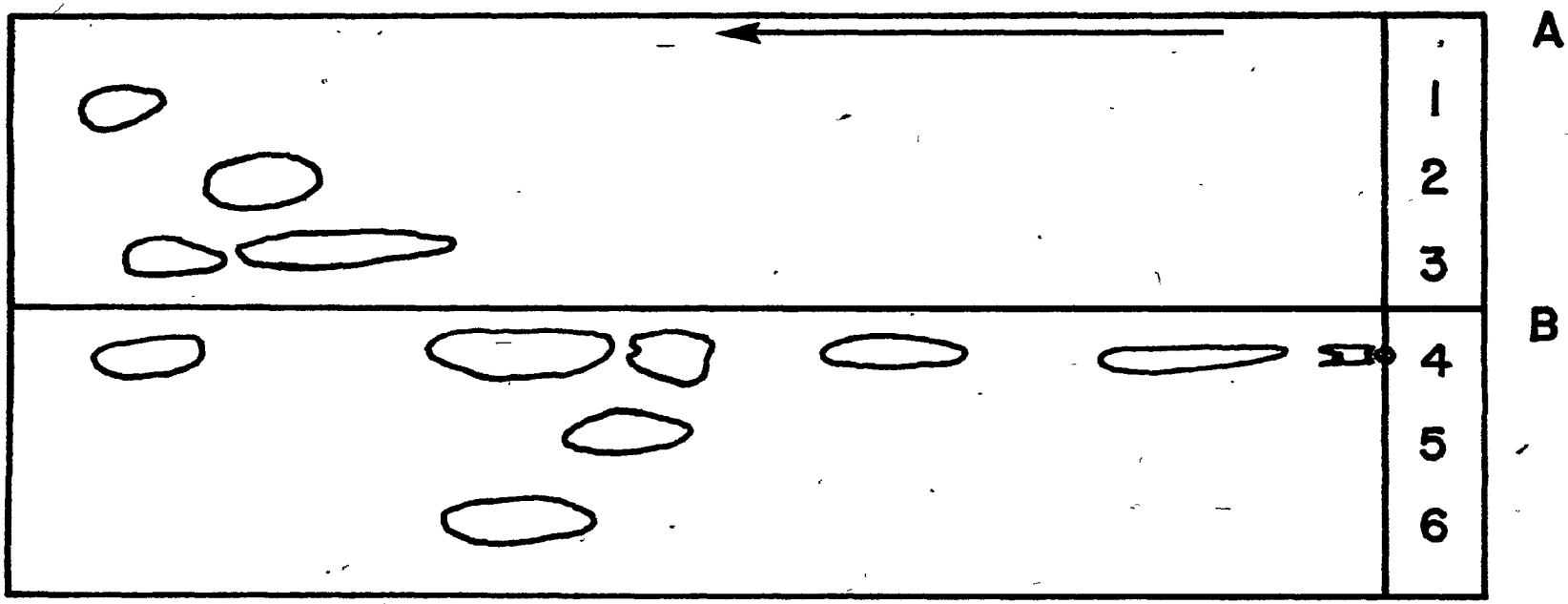

RADIOACTIVITY (CPM X 10^3)
100
90
80
70
60
50
40
30
20
10
1
2
3
4
origin
FRACTIONS (cm)

FIGURE 21.

Fig. 21. Separation of LPS carbohydrate components by descending paper chromatography.

Experimental conditions were the same as those in figure 20. The samples that were applied were: 1, glucose; 2, galactose; 3, 5 mg degraded polysaccharide (hydrolyzed in 0.5 N HCl for 30 min); 4, 5.3 mg degraded polysaccharide (hydrolyzed in 1 N HCl for 1 h); 5, 2-amino-2-deoxygalactose and 6, 2-amino-2-deoxyglucose. Chromatogram A was sprayed with analine phthalate and chromatogram B with ninhydrin.


A
1
2
3
B
4
5
6

neutral and amino sugars, consequently, two samples were chromatographed. The chromatogram was divided and one half was sprayed with analine phthalate in order to detect neutral carbohydrates (figure 21A) while the other half was sprayed with ninhydrin to detect amino sugars (figure 21B). Therefore, the degraded polysaccharide consisted of those components that were detected in both samples 3 and 4 on the chromatogram. Even though the sugars were separable they tended to streak together. What these experiments do demonstrate, however, is that the LPS was extensively labeled in the polysaccharide chain and in the carbohydrate portion of the lipid A.

Factors affecting the separation and electrophoretic mobility of the lipopolysaccharide components

The migration of the LPS samples, in variations of the electrophoresis buffer, was examined in order to determine those factors responsible for LPS mobility. Substituting 0.05 M Tris for Na_2HPO_4 in the original electrophoresis buffer did not alter the migration patterns that were shown in figure 17. The migration patterns of the lipopolysaccharides when electrophoresed in a buffer in which 0.05 M Tris replaced both Na_2HPO_4 and Na_2MoO_4 are shown in figure 22. The normal banding patterns occurred, but LPS remained on the surface of the gel in all cases suggesting that although the Na_2MoO_4 was not the primary factor involved in the electrophoresis of LPS it did contribute to the complete migration.

FIGURE 22.

Fig. 22. Electrophoretic mobility of various lipopolysaccharides upon polyacrylamide gel electrophoresis in a buffer containing 0.05 M Tris - 1% SDS.

Electrophoresis time was 2 h and 45 min at 5 mA/gel. Lipopolysaccharides were detected with the Schiff reagent. Gel A, marine pseudomonad (var. 3); gel B, marine pseudomonad (var. 1); gel C, *E. coli*; gel D, *S. typhimurium*; gel E, *P. aeruginosa*; and gel F, *N. meningitidis*. The amounts of LPS applied to the gels were: A, C, D and F, 300 µg; B, 200 µg; and E, 550 µg.


A
B
C
D
E
F

To examine the contribution that SDS makes to the electrophoresis of the lipopolysaccharides, the nonionic detergent, Triton X-100 was used in place of SDS in the electrophoresis buffer and the solubilizing solution. The concentration of Triton used was 0.1% since at 1% the Triton precipitated during the fixation and staining procedures. A Triton concentration of 0.1% was enough for complete solubilization of the LPS. It should be noted that the separation of the LPS components by electrophoresis could be achieved in 0.1% SDS without any changes in the migration pattern. However, since greater amounts of LPS could be solubilized in 1% SDS, a necessary requirement for preparative gels, it was decided to remain with this concentration of SDS in the analytical gel system as well. Figure 23 shows that there was no separation of the LPS components following electrophoresis in the Triton X-100 buffer. The *N. meningitidis* LPS was the only sample that completely entered the gel and remained as a single homogeneous band in a manner similar to that when electrophoresis was performed in the SDS buffer.

The lipopolysaccharides that were extracted from the various bacteria contained small amounts of contaminating protein (Table 8) The electrophoretic mobility of the LPS was not due to or affected by the migration of the protein because different batches of LPS extracted from the marine pseudomonad contained varying concentrations of protein yet the migration distances of the LPS components in these samples

FIGURE 23.

Fig. 23. Electrophoretic mobility of various lipopolysaccharides solubilized and electrophoresed in a buffer containing Triton X-100.

The electrophoresis buffer was composed of 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 0.1% Triton X-100. The time of electrophoresis was 5 h at 5 mA/gel. Samples were the same as those used in figure 22. The concentrations of LPS applied to each gel were: A, 200 µg; B, C, D, and F, 300 µg and E, 500 µg.

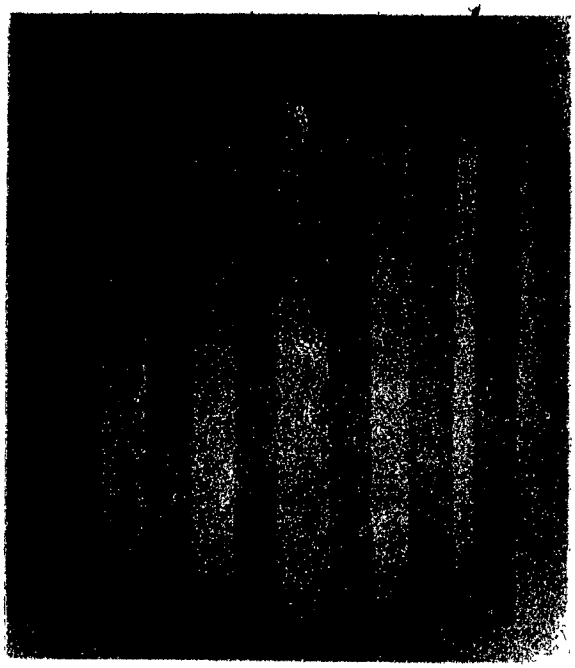


TABLE 8.

Protein concentrations of lipopolysaccharides extracted with phenol-water from various Gram-negative bacteria

Bacteria	Protein as % dry weight of LPS
Marine pseudomonad (variant 3)	2.7
Marine pseudomonad (variant 1)	2.8
Escherichia coli 0111:B4	4.6
Salmonella typhimurium LT2	4.5
Pseudomonas aeruginosa	2.0
Neisseria meningitidis	3.3

remained the same. Locating the peptide bands in the LPS gels was difficult because of the low concentrations of protein in the LPS samples. The gels had to be heavily overloaded with sample in order to apply enough protein for its detection by staining and even under these conditions localization of the peptide bands was not definitive (figure 24). In addition, the electrophoresis buffer used in this system for carbohydrate samples resulted in diffuse peptide bands because of the improper pH of the buffer for use with protein samples and the fact that stacking gels were not used.

A detergent had to be included in the electrophoresis buffer and corresponding solubilization mixture in order to disperse the hydrophobic lipopolysaccharides. It was found that heating the LPS in the presence of a detergent in a boiling water bath was not essential for solubilization. Mechanically shaking the LPS sample in the solubilization solution for several hours at room temperature yielded a clear suspension which, when compared to a sample heated in a boiling water bath for 5 to 10 min, exhibited an identical electrophoretic pattern (figure 25). Various heating times also failed to alter the banding pattern of the LPS and thus did not present the same problem as reported by Schnaitman (1973) for protein solubilization.

Since it was established that SDS was responsible for the electrophoretic mobility of the LPS the most likely site of SDS binding to the molecule was the lipid A. With this observation in mind, the

FIGURE 24.

Fig. 24. Presence of protein contamination in lipopolysaccharides extracted from several Gram-negative bacteria as determined by SDS-polyacrylamide gel electrophoresis.

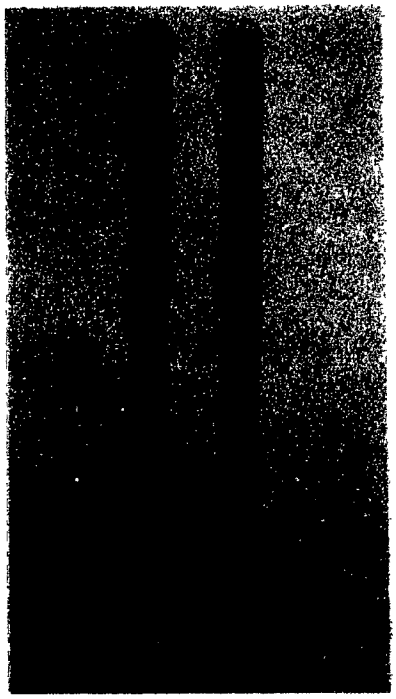
The lipopolysaccharides were electrophoresed in the 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS buffer and the gels were stained either with the Schiff reagent for carbohydrate or Coomassie Brilliant Blue for protein. Gels a and b contain LPS extracted from variant 3 of the marine pseudomonad and are stained for carbohydrate and protein, respectively. Gels c and d contain *S. typhimurium* LPS. Gel c is stained for carbohydrate while gel d is stained for protein. Gels e and f contain *E. coli* LPS and are stained for carbohydrate and protein, respectively.


a
b
c
d
e
f

FIGURE 25.

Fig. 25. Effect of varying the conditions used in the solubilization of LPS for SDS-polyacrylamide gel electrophoresis.

Gel A, LPS solubilized by heating in a boiling water bath for 5 to 10 min and gel B, LPS solubilized by mechanical agitation at room temperature for 2 h.

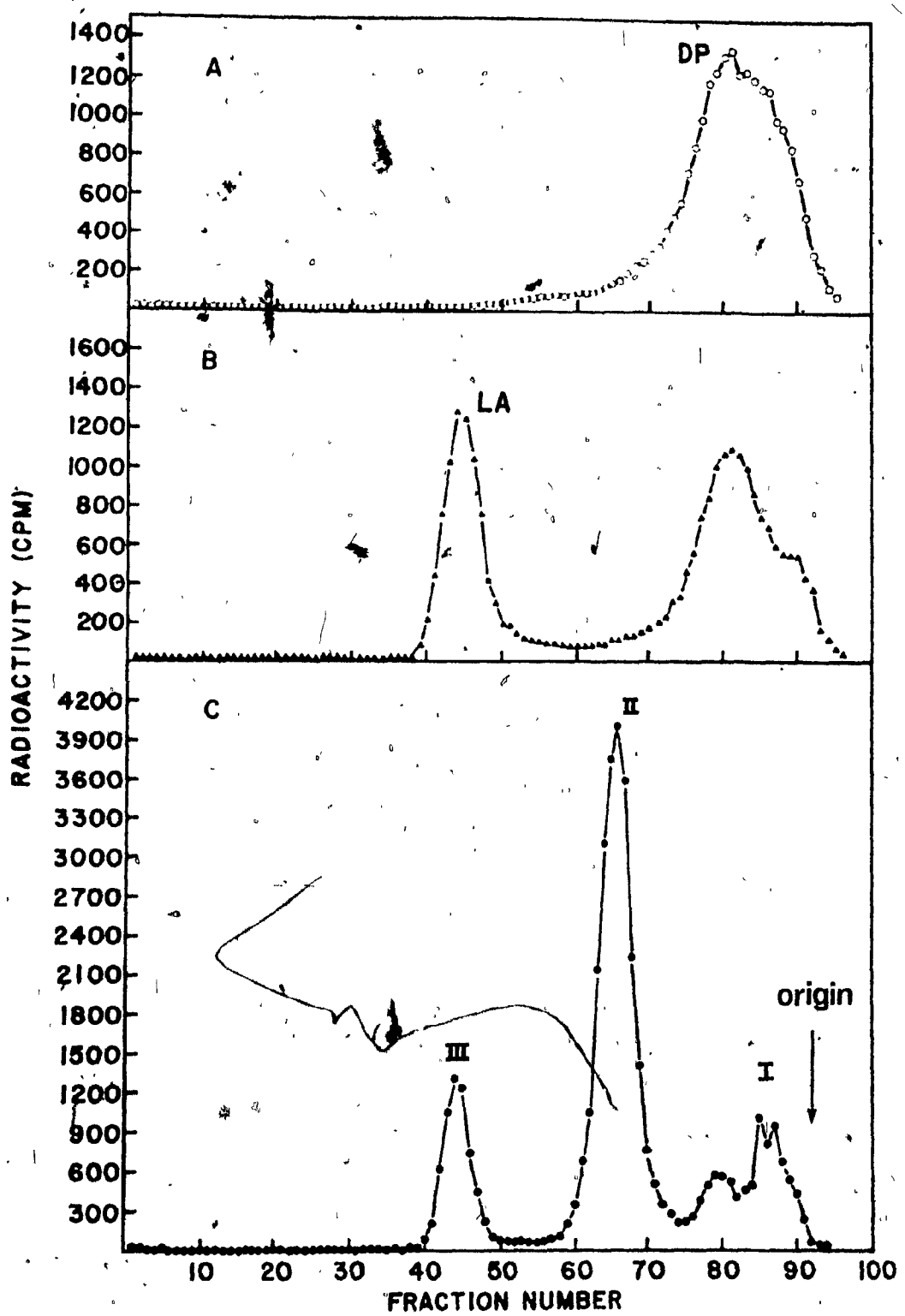


lipid A and degraded polysaccharide portions of the marine pseudomonad LPS molecule were isolated and examined in the gel electrophoresis system. Lipid A will not stain with the Schiff reagent consequently [^{14}C] labeled LPS was used in the study. The LPS was subjected to mild acid hydrolysis and the lipid A and degraded polysaccharide fractions were separated by centrifugation and solubilized. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the fractions in 0.05 M Na_2HPO_4- 0.05 M Na_2MoO_4 - 1% SDS gave the profiles illustrated in figure 26. The degraded polysaccharide fraction migrated as a broad peak which did not completely enter the gel (figure 26A). Only 69% of the degraded polysaccharide could be recovered in the gel. Two radiolabeled peaks were discovered following electrophoresis of the lipid A preparation (figure 26B). One of these peaks was found at the surface of the gel; thus, it had migrated to the same position as the degraded polysaccharide peak in figure 26 A. The other radiolabeled peak, in the lipid A preparation, migrated well into the gel. It would appear that the lipid A is represented by the peak which has the faster migration rate and that the preparation is contaminated with degraded polysaccharide. Figure 26C is the whole cell LPS control showing the relationship of the electrophoretic mobilities of the three LPS components to that of the lipid A. The lipid A peak migrated to the same position as that of LPS III, however, gas-liquid chromatographic analyses showed that these fractions were not similar

FIGURE 26.

Fig. 26. Electrophoretic mobility of the lipid A and degraded polysaccharide portions of the LPS molecule.

Lipopolysaccharide, labeled with [^{14}C], was hydrolyzed in 1% acetic acid and separated into lipid A and degraded polysaccharide by centrifugation. Gel profile A, [^{14}C]degraded polysaccharide; profile B, [^{14}C]lipid A preparation and profile C, unhydrolyzed LPS control. Lipid A and degraded polysaccharide are designated LA and DP, respectively.


A
DP
B
LA
C
II
III
I
origin
RADIOACTIVITY (CPM)
FRACTION NUMBER
1400
1200
1000
800
600
400
200
1600
1400
1200
1000
800
600
400
200
4200
3900
3600
3300
3000
2700
2400
2100
1800
1500
1200
900
600
300
10
20
30
40
50
60
70
80
90
100

in composition (see Results, Section III). The results emphasize that the lipid A component of the LPS molecule is the causative agent in LPS migration because once the lipid A is removed from the molecule migration is severely retarded. This information strongly supported the existence of a lipid A - SDS complex in the SDS-polyacrylamide gel system. The role of lipid A in LPS migration complicates the interpretation of the molecular size of the LPS components since the migration distance of each component is a function of both molecular size and amount of lipid A.

S E C T I O N III

CHARACTERIZATION OF THE LIPOPOLYSACCHARIDE COMPONENTS

Isolation

Marine pseudomonad LPS, 1.5 mg/gel was electrophoresed in 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS. One gel was stained by the periodate-Schiff procedure as a guide for locating the bands (figure 27). The remaining gels were sectioned between bands by cutting the gels with a razor blade. The corresponding gel sections were centrifuged at 12,000 x g (Sorvall SS-34 rotor) for 15 min to remove acrylamide and the supernatant fluids were dialyzed against distilled water containing 0.02% sodium azide for 48 h at room temperature. The dialysates were concentrated and lyophilized. The LPS bands that were isolated from the preparative gels were individually resolubilized in the SDS solubilizing solution by heating for 5 to 10 min. Each LPS band was electrophoresed on separate analytical gels and figure 28 illustrates that the resolubilized components maintained their homogeneity and could be isolated in the intact and pure state. The main problem with the procedure was that the recovery of the LPS was only 50 to 60%.

Chemical composition

Detailed carbohydrate analysis of LPS I, II and III was necessary to establish that these components were actual molecular species

FIGURE 27.

Fig. 27. Separation of the marine pseudomonad LPS components on preparative polyacrylamide gels.

Lipopolysaccharide (1.5 mg) was electrophoresed in 0.05 M Na_2HPO_4 - 0.05 Na_2MoO_4 - 1% SDS for 7 h at 10 mA/gel after which time the gel was stained by the periodate-Schiff procedure.

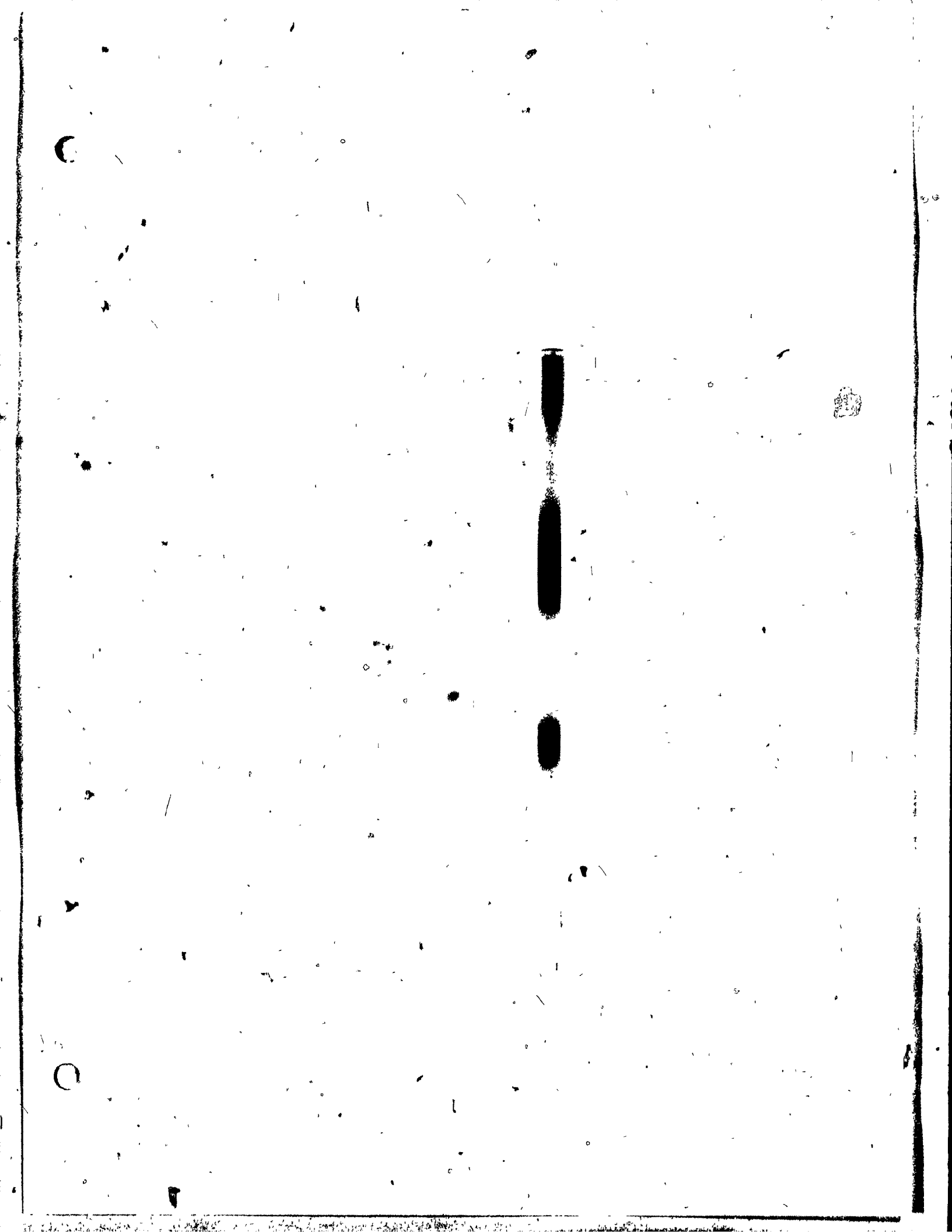
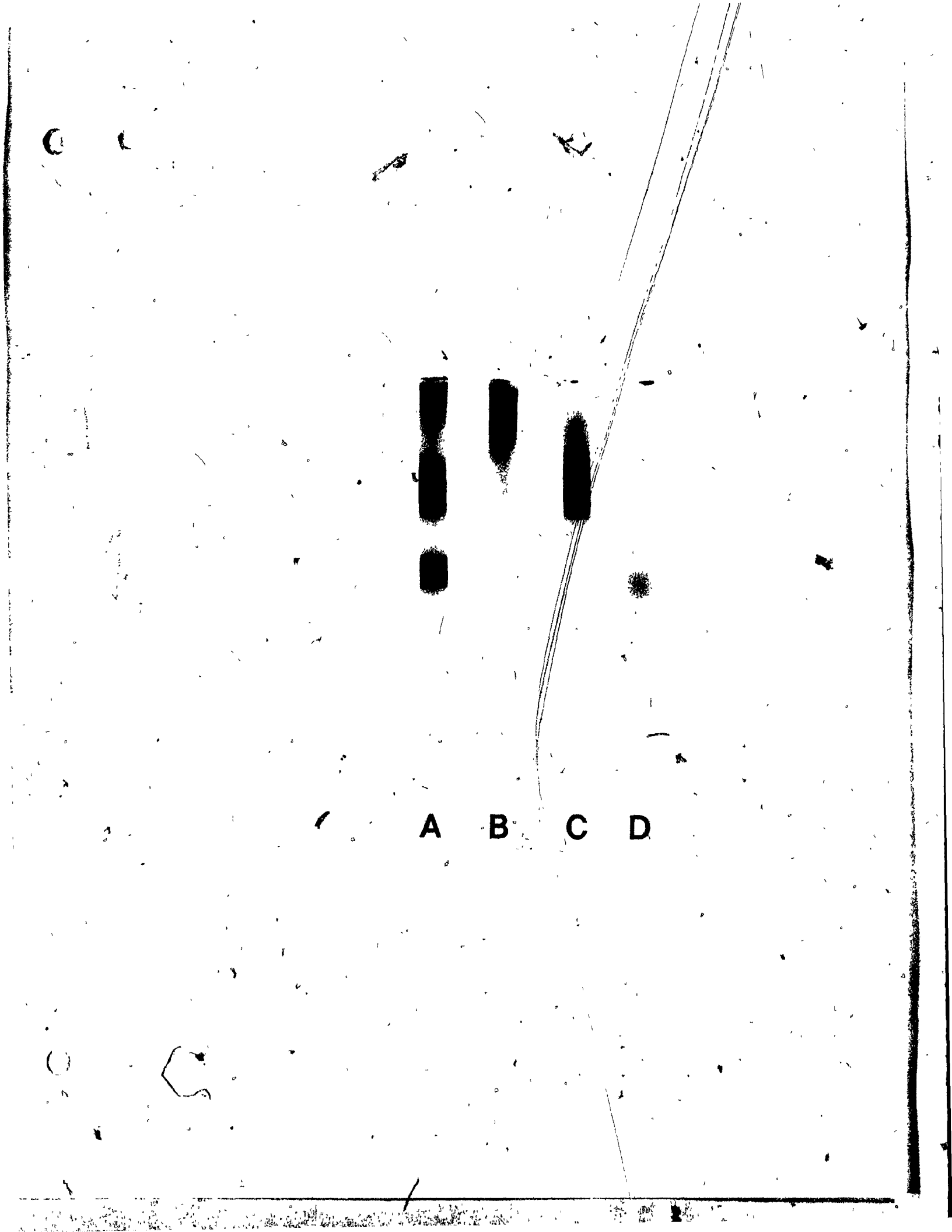


FIGURE 28.

Fig. 28. Homogeneity of the isolated LPS species as evidenced by re-electrophoresis on SDS-polyacrylamide gels.

Each LPS species was isolated from the preparative gels and resolubilized. Gel A, whole cell LPS; gel B, LPS I; gel C, LPS II and gel D, LPS III. Electrophoresis time was 3 h at 10 mA/gel.


A
B
C
D

of LPS. Their carbohydrate compositions were compared to those of LPS, core and core-side chain fractions of the molecule. Identification and quantitation of the carbohydrates were performed in the same manner as for the whole cell LPS. The retention times of the derivatives prepared from the sugars that were found in the LPS components were compared to those of the derivatives of the sugars in the whole cell LPS and of authentic standards. All conditions were equalized by measuring the retention times as related to the retention time of D-glucoheptose hexaacetate. The relative retention times, on both columns C and D, are shown in Table 9. The only major discrepancies occurred in the relative retention times of the derivatives of 2-amino-2-deoxygalactose found in the core region and in LPS III compared to the standard. Co-chromatography demonstrated that these peaks were the same sugar. The chromatograms of LPS I, II and III, core and core-side chain on columns C and D are illustrated in figures 29 and 30 respectively. The carbohydrate compositions of the LPS components, core and core-side chain fractions are listed in Table 10. The composition of whole cell LPS represents the concentration of each sugar in a mixture of the three LPS components. The core fraction from whole cell LPS lacked unknown 1 while both unknown sugars 1 and 2 were missing in LPS III. Since unknown sugar 1 was present in the core-side chain fraction, but not in the core alone, it can be classified as a characteristic side chain component in the marine pseudomonad LPS. Lipopolysaccharides I and II contained low levels

TABLE 9.

Comparison of the retention times of the alditol acetates of the sugars from the marine pseudomonad lipopolysaccharide fractions and molecular species relative to D-glucoheptose hexaacetate[a]

Compound	RT Glucoheptose							
	OV-275 column at 225 C[b]		HI-EFF column at 230 C[b]					
	galactose	glucose	glucose + galactose	unknown 1	unknown 2	heptose	2-amino-glucose	2-amino-galactose
derivatized standards	0.46	0.53	0.49	--	--	--	1.43	1.69
degraded polysaccharide	0.43	0.51	0.50	0.56	0.64	0.92	1.45	1.72
lipid A	--	--	--	--	--	--	1.45	--
core	0.43	0.50	0.50	--	0.64	0.90	1.44	1.77
core-side chain	0.43	0.50	0.50	0.56	0.64	0.90	1.44	1.70
LPS I	0.43	0.50	0.50	0.56	0.64	0.91	1.45	1.71
LPS II	0.43	0.51	0.50	0.56	0.64	0.91	1.45	1.71
LPS III	0.43	0.51	0.50	--	--	0.90	1.44	1.77

[a] D-glucoheptose hexaacetate = 1.00.

[b] Actual retention times of D-glucoheptose hexaacetate on OV-275 and HI-EFF are 24.25 and 17.50 min, respectively.

FIGURE 29.

Fig. 29. Gas-liquid chromatographic separation of the monosaccharides present in LPS I, II, III, core and core-side chain on the HI-EFF column.

The alditol acetate derivatives were employed. Chromatogram A, LPS I; B, LPS II; C, LPS III; D, core and E, core-side chain. The peaks were identified as: 1, glucose and galactose; 2, unknown 1; 3, unknown 2; 4, heptose; 5, internal standard; 6, 2-amino-2-deoxyglucose and 7, 2-amino-2-deoxygalactose. The column was operated at 230 C. Att. equals attenuation.

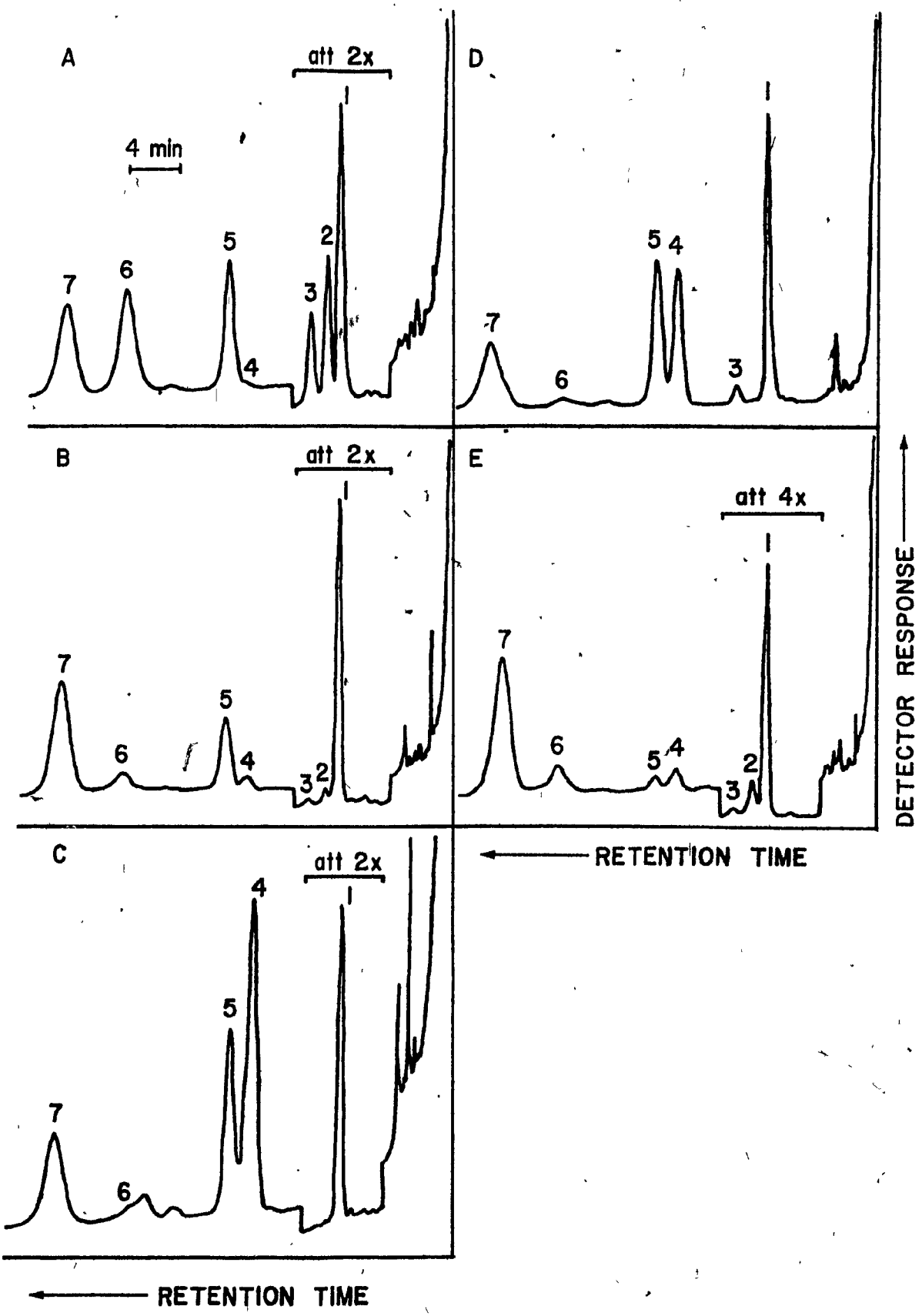

A
att 2x
4 min
7
6
5
4
3
2
1
D
7
6
5
4
3
1
B
att 2x
7
6
5
4
3
2
1
E
att 4x
7
6
5
4
3
2
1
DETECTOR RESPONSE
RETENTION TIME
C
att 2x
7
6
5
4
1
RETENTION TIME

FIGURE 30.

Fig. 30. Gas-liquid chromatographic separation of the monosaccharides present in LPS I, II, III, core and core-side chain on the OV-275 column.

The derivative that was used and the chromatographed material was the same as in figure 29. The peaks represent: 1, galactose; 2, glucose; 3, heptose and 4, internal standard. The remaining peaks were not identified. The column was operated at 220 C.

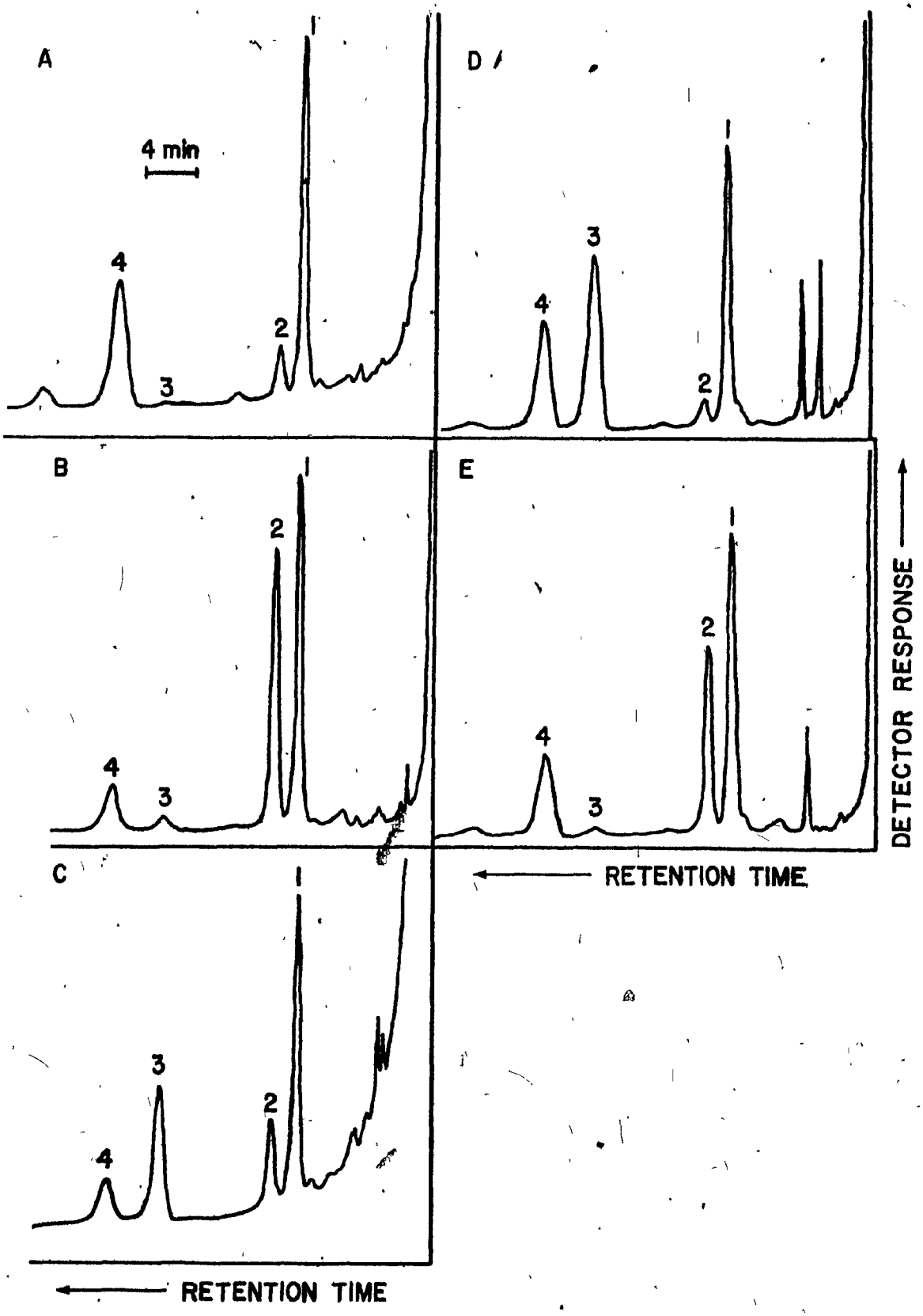

A
4 min
4
3
2
1
D
4
3
2
1
B
4
3
2
1
E
4
3
2
1
C
4
3
2
1
DETECTOR RESPONSE
RETENTION TIME
RETENTION TIME

TABLE 10.

Carbohydrate analysis of lipopolysaccharide fractions and molecular species from the marine pseudomonad.

Monosaccharide	% of dry weight					
	LPS	core	core-side chain	LPS I	LPS II	LPS III
glucose	4.64±0.52	1.00±0.02	7.11±0.32	0.44±0.03	6.80±0.16	0.65±0.40
galactose	7.99±0.90	7.02±0.16	9.95±0.98	3.04±0.38	6.71±0.45	1.97±1.07
heptose	3.06±0.10	8.29±0.09	1.08±0.12	0.17±0.03	0.72±0.02	1.67±0.86
unknown 1	1.71±0.03	0	4.35±0.10	1.92±0.29	0.49±0.06	0
unknown 2	1.36±0.09	0.64±0.03	3.34±0.11	1.65±0.33	0.36±0.05	0
2-amino-2-deoxyglucose	8.11±0.40	1.70±0.23	9.25±0.16	3.83±0.39	2.52±0.69	0.34±0.09
2-amino-2-deoxygalactose	11.94±0.21	8.16±0.16	23.49±0.04	3.33±0.50	10.37±1.98	1.05±0.43

of heptose and all the other monosaccharides that were present in whole cell LPS. The predominant monosaccharide in the core-side chain oligosaccharide was 2-amino-2-deoxygalactose. There was only 6% total carbohydrate in LPS III and replicate analyses showed a high degree of fluctuation in the monosaccharide concentrations as evidenced by the large standard errors. This non-reproducibility was a reflection of the small amount of LPS III that was present in the whole cell LPS and available for analysis.

The relationship among LPS I, II and III could be examined more readily when the molar ratios of the carbohydrate components were calculated (Table 11). There was an exact 1 : 1 : 1 molar ratio of heptose, galactose, and 2-amino-2-deoxygalactose in the core region of the LPS. The ratio of the sugars in LPS III is remarkably similar to that of the core region and thus appears to represent an LPS molecule in which the O-antigenic side chain has not been attached. The ratios of the sugars in LPS I and II are difficult to interpret. Assuming that the core region is basically the same in LPS I and II, as it is in LPS III, and that there is a 1 : 1 ratio of core to side chain in each LPS form, then, LPS I and II must differ from one another in the composition of their O-antigenic side chains. The side chain of LPS I would be expected to be composed of galactose, galactosamine, unknown 1 and glucosamine while that of LPS II would contain galactose, galactosamine and glucose. This conclusion was reached because the monosaccharides contained in the side chain

TABLE 11.

Molar ratios of the carbohydrate components from the lipopolysaccharide fractions and molecular species

Monosaccharide	Molar ratio					
	LPS	LPS I	LPS II	LPS III	core	core-side chain
heptose	1.00	1.00	1.00	1.00	1.00	1.00
unknown 1[a]	0.67	12.00	1.00	0	0	5.40
glucose	1.73	2.00	12.67	0.50	0.15	7.80
galactose	2.93	17.00	12.33	1.38	1.00	11.00
2-amino-2-deoxyglucose	2.53	18.00	4.00	0.25	0.21	8.60
2-amino-2-deoxygalactose	3.67	15.00	16.00	0.63	0.97	21.80

[a]Calculated as 2-amino-2,6-dideoxyglucose.

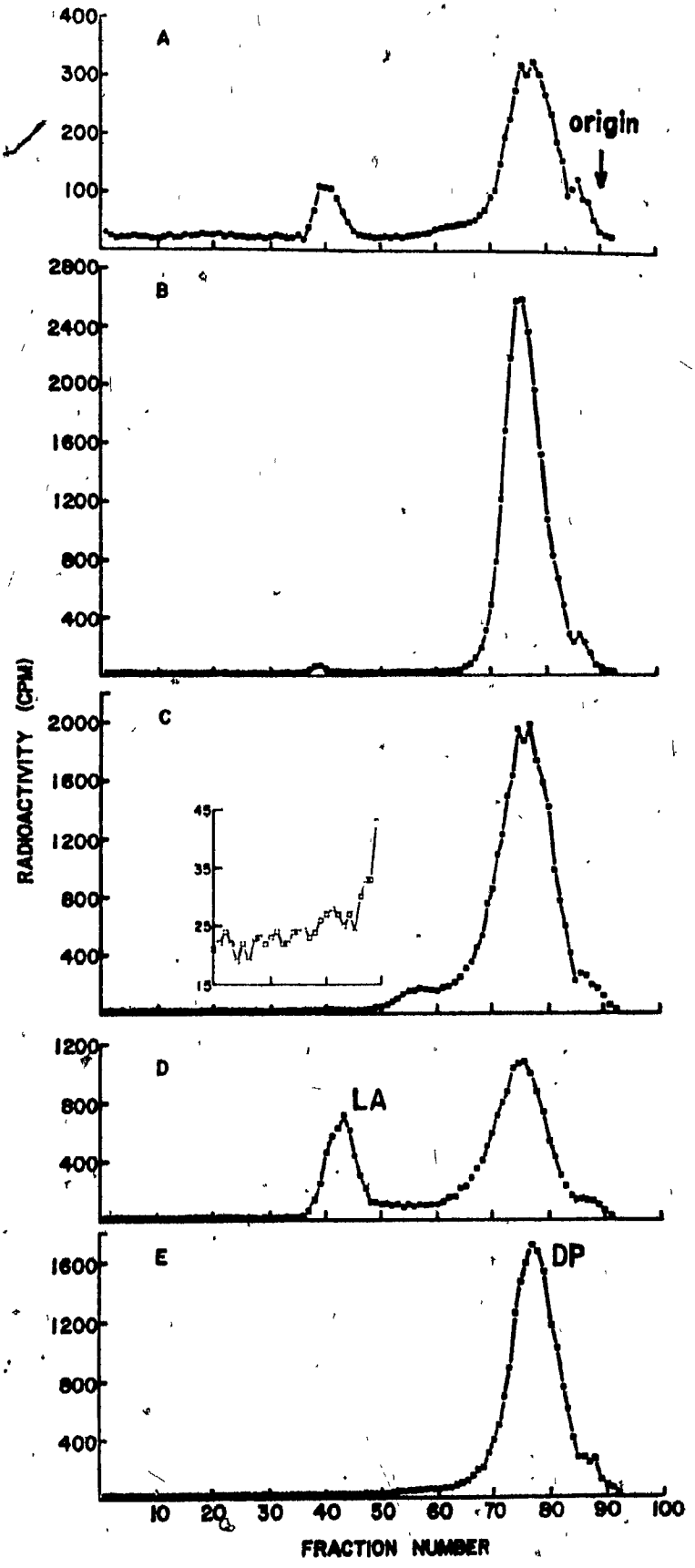
repeating units would be in a much higher ratio when compared with the sugars in the core structure. However, structural analysis of each of the LPS forms will be necessary to confirm these conclusions. In order to carry out structural analyses a more efficient method of preparative isolation of the LPS components is needed. To date no satisfactory method of isolation has been found.

It was difficult to establish the existence of lipid A in each of the LPS species because not enough of each species could be obtained to enable lipid A to be isolated and analyzed by gas-liquid chromatography. It has already been shown that the lipid A as well as the degraded polysaccharide fraction of the LPS incorporated radioactivity when the cells of the marine pseudomonad were labeled by growing the cells in the presence of [^{14}C]galactose. Consequently, the cells were labeled, the LPS was extracted, and the LPS species were isolated by procedures already described. The individual species were then hydrolyzed in 1% acetic acid and the hydrolysates were lyophilized. The concentrated samples were solubilized, run on SDS-polyacrylamide gels in the 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS buffer and the gels were fractionated and counted. Figure 31 shows the gel profiles of the hydrolyzed LPS species, lipid A and degraded polysaccharide. Lipid A was detected as the lower radiolabeled peak following electrophoresis of the lipid A precipitate from the mild acid hydrolysis of the LPS. There was considerable contamination of the lipid A material with degraded polysaccharide which was easily identifiable as a char-

FIGURE 31.

Fig. 31. Polyacrylamide gel electrophoresis as a method of detecting lipid A in the LPS species.

Profile A, LPS III; B, LPS II; C, LPS I; D, lipid A (LA); and E, degraded polysaccharide (DP). Each fraction represents 1 mm of the gel. The insert in profile C represents an expanded scale for the radioactivity in fractions 20-50.


A
origin
B
C
D
LA
E
DP
RADIOACTIVITY (CPM)
FRACTION NUMBER

acteristic peak at the surface of the gel (see Results section II). The lowest peak in the LPS III profile (figure 31A) contained a significant proportion of the total radioactivity suggesting that a major portion of LPS III is represented by lipid A. Lipopolysaccharide II (figure 31B) contained a trace of lipid A but considerably less than LPS III. From the data it appears that LPS I has no lipid A or trace amounts relative to the amount of degraded polysaccharide (figure 31C). Closer examination of the graphs showed that total counts were very low, especially in the case of LPS III, which was due to the small amounts of material obtainable, the repeated series of steps involved in the isolation and preparation of the samples, and the use of [^{14}C] galactose to label the LPS. Results previously discussed have shown that [^{14}C]galactose labels lipid A to only one tenth of the extent that it labels the degraded polysaccharide portion of the LPS molecule. In this case, the areas of the peaks that represent lipid A in figure 31 should be ten times greater when corrected for the difference in specific activities of the lipid A and degraded polysaccharide fractions. Therefore, LPS I, II and III contain considerably more lipid A than is actually depicted in the radiolabeled profiles. These experiments established that two of the three LPS species contained at least some lipid A. They do not, however, discount the possibility that LPS I contains lipid A.

By using a different approach it was indirectly demonstrated that

lipid A was present in LPS I as well as LPS II and III. Janda and Work (1971) have reported that lipid A alone causes a spectral shift of the absorbance maximum of the carbocyanin dye similar to that caused by whole LPS. Lipid A, core and core-side chain from the marine pseudomonad LPS were reacted with the dye and scanned from 600 to 400 nm. Lipid A produced a spectral shift from 510 to 458 nm, however, the core and core-side chain fractions did not; they absorbed maximally at the same wavelength as the carbocyanin dye (figure 32). When LPS I, II and III were reacted with the dye they all demonstrated a spectral shift which was identical to that obtained with the lipid A (figure 33).

Thus, the LPS species were shown to contain lipid A by several methods. (i) The lipid A was labeled directly. This method established the presence of lipid A in LPS II and III only. (ii) Lipopolysaccharide I, II and III demonstrated a spectral shift by reaction with the carbocyanin dye. (iii) All three LPS components migrated into SDS-polyacrylamide gels. It has already been established that when lipid A is removed from the LPS molecule the degraded polysaccharide portion does not completely enter the gel.

Proposed structures for the LPS species are shown in figure 34. The differences in the relative amounts of lipid A in each of the species is most probably a reflection of the increased amounts of polysaccharide in species I and II. The extensive side chain repeating

FIGURE 32.

Fig. 32. Carbocyanin dye scans of lipid A, core and core-side chain fractions from the marine pseudomonad LPS.

Scan 1 represents 3.0 mg lipid A, scan 2 is 1.9 mg core-side chain, scan 3 is 1.1 mg core, and scan 4 shows the dye reagent.

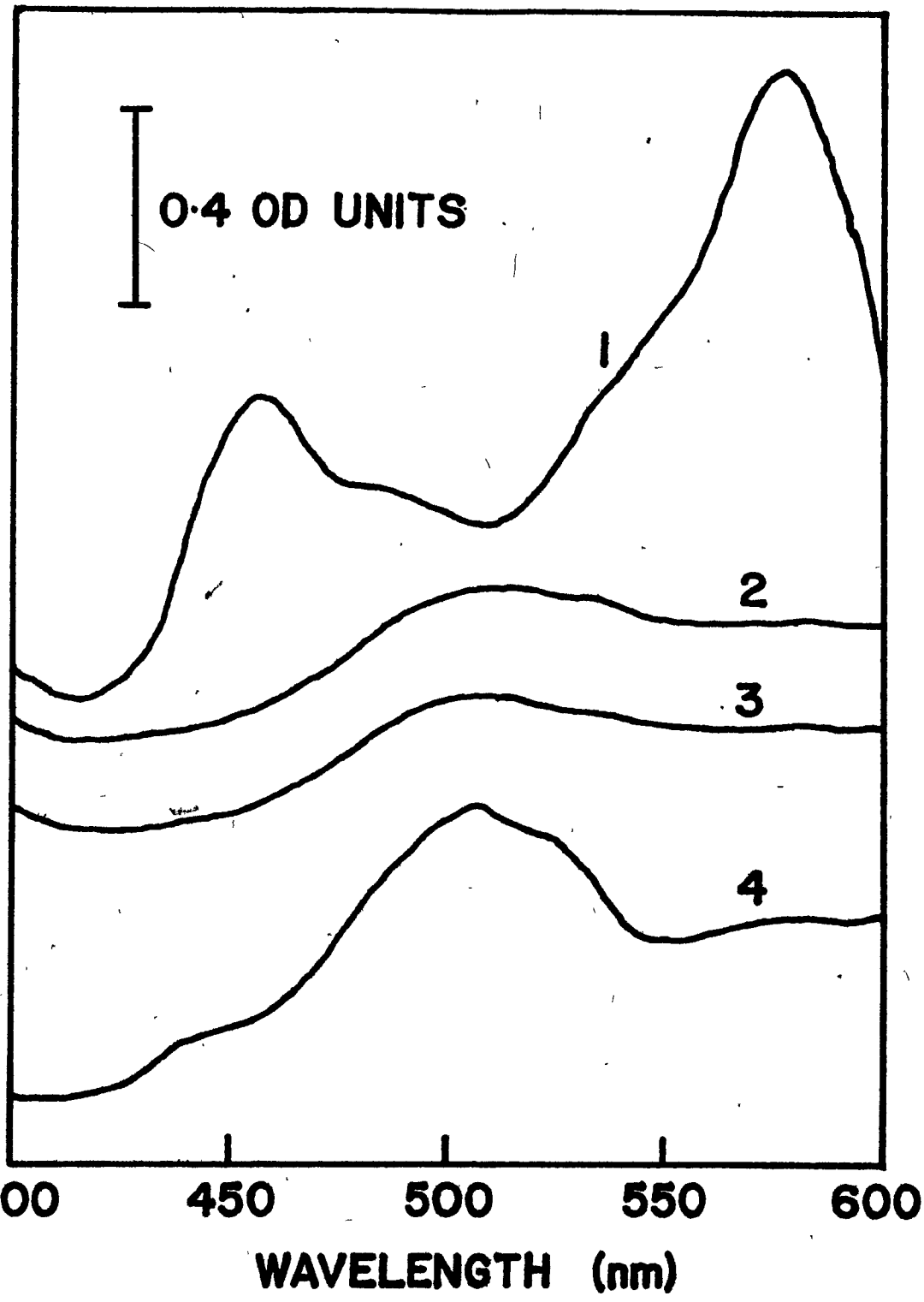

0·4 OD UNITS
1
2
3
4
400
450
500
550
600
WAVELENGTH (nm)

FIGURE 33.

Fig. 33. Carbocyanin dye scan of LPS I, II and III.

Scan 1, 2.0 mg LPS I; scan 2, 1.2 mg LPS II; scan 3, 1.7 mg LPS III and scan 4, dye reagent.

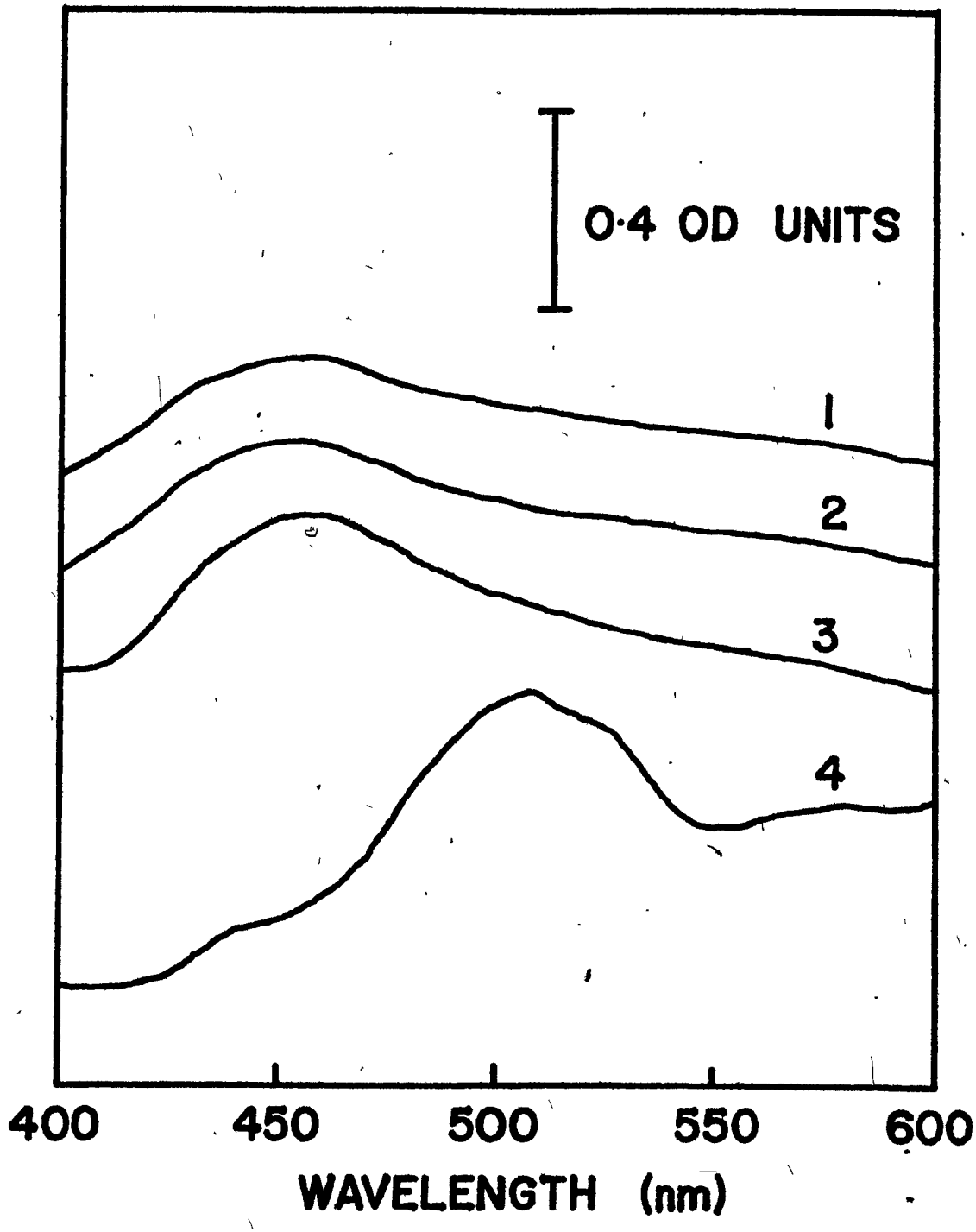
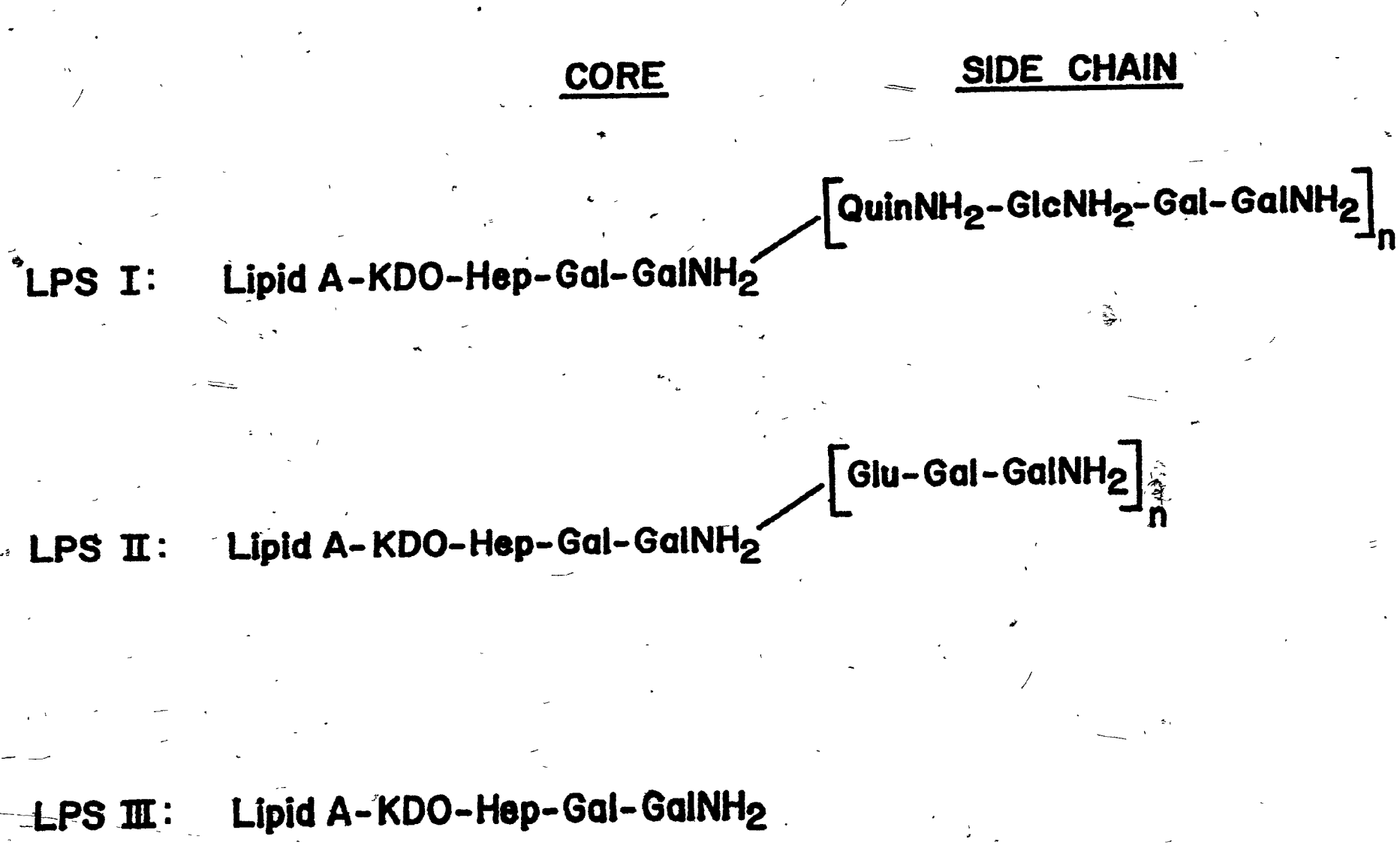

0·4 OD UNITS
1
2
3
4
400
450
500
550
600
WAVELENGTH (nm)

FIGURE 34.

Fig. 34. Proposed carbohydrate structure of the marine pseudomonad LPS species.


CORE
SIDE CHAIN
LPS I: Lipid A-KDO-Hep-Gal-GalNH2 [QuinNH2-GlcNH2-Gal-GalNH2]n
LPS II: Lipid A-KDO-Hep-Gal-GalNH2 [Glu-Gal-GalNH2]n
LPS III: Lipid A-KDO-Hep-Gal-GalNH2

unit in LPS completely dominates the smaller lipid A-core structure (Osborn, 1969). Although the general structure of the LPS species can be formulated from the present data, the sequence of the monosaccharides is not yet known.

Serology

In order to examine the serological cross-reactivity of the three LPS species the Ouchterlony double immunodiffusion technique was employed. A whole cell antiserum was obtained against the marine pseudomonad. The antigen that was used for injection was prepared according to the procedure outlined in Materials and Methods. Rabbits were injected according to the schedule shown in Table 12 and were bled on the 28^{th} day. The antiserum titer was determined by hemagglutination using sheep erythrocytes coated with whole cell LPS extracted from the marine pseudomonad and the highest reciprocal titer obtained was 512. This titer was too low for use in the double immunodiffusion studies so the antiserum was concentrated by ammonium sulfate precipitation and adjusted to a concentration of 60 mg protein/ml physiological saline. The concentrated antiserum was used in all subsequent experiments. The whole cell LPS, LPS species, core and core-side chain were isolated as described earlier and were suspended in physiological saline at a concentration of 1 mg/0.1 ml. Figure 35A demonstrates that purified whole cell LPS resulted in one precipitin line under the conditions employed in this method. Lipo-

TABLE 12.

Injection schedule for the immunization of rabbits in the preparation of a marine pseudomonad whole cell antiserum

Time	Amount injected[a,b]
Week 1	0.5 ml
	1.0 ml
	1.5 ml
Week 2	0.5 ml
	1.0 ml
	1.5 ml
Week 3	0.5 ml
	1.0 ml
	1.5 ml
Week 4	Rabbits were bled by cardiac puncture

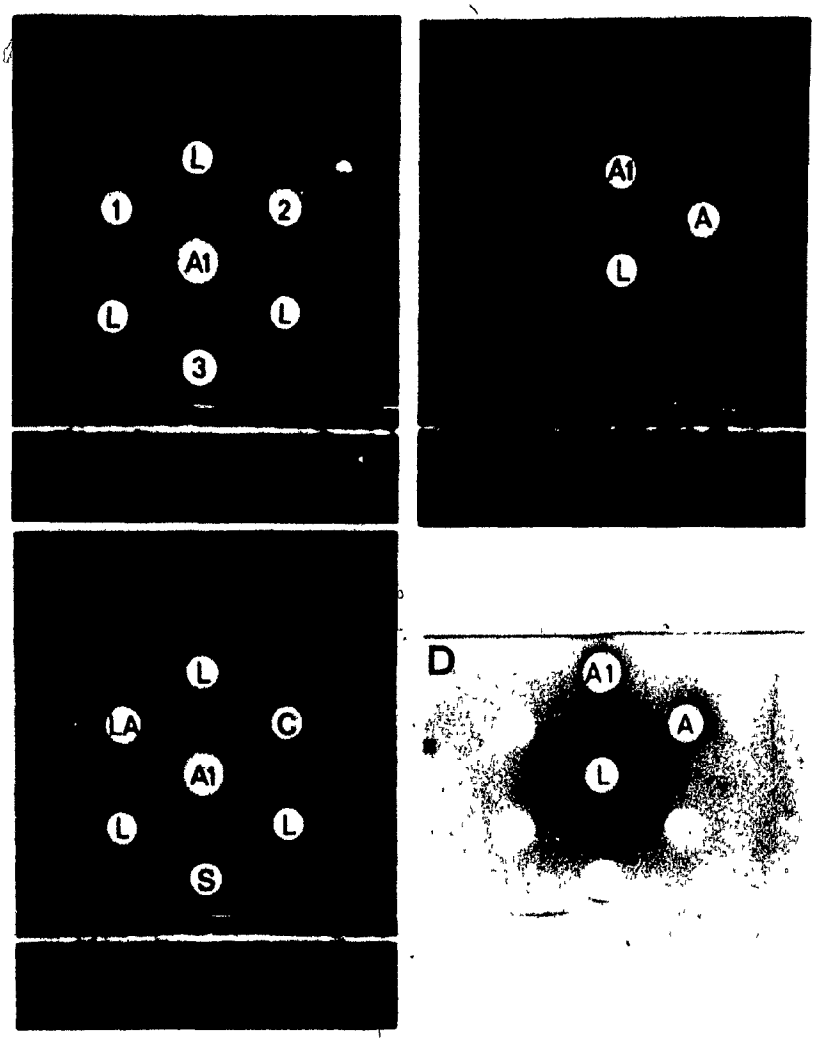
[a]Intravenous injections in the marginal ear vein.

[b]Antigen concentration, 21×10^8 cells/ml complete salts.

F I G U R E 35.

Fig. 35. Serological cross-reactivity of LPS I, II and III as determined by double immunodiffusion.

Slides A, B and C show the actual precipitin lines as recorded by dark field photography. Slide D is stained by the periodate-Schiff procedure. The wells contain: A_1, whole cell antiserum; A, Nelson's whole cell antiserum; L, LPS; 1, LPS I; 2, LPS II; 3, LPS III; LA, lipid A; C, core and S, core-side chain. All antigens were used at a concentration of 1 mg/0.1 ml saline.

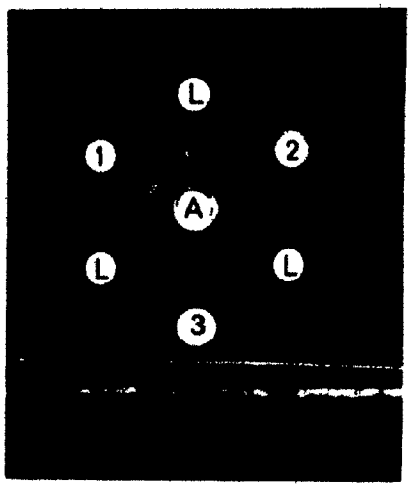

L
1
2
A1
L
L
3
A1
A
L
L
LA
C
A1
L
L
S
D
A1
A
L

polysaccharides I and II showed a homologous cross reaction with the whole cell LPS, but, LPS III did not give any precipitin reaction with the antiserum. Even when the LPS III antigen concentration was doubled no reaction was observed. Lipid A, core, and core-side chain fractions isolated from the whole cell LPS failed to exhibit a precipitin reaction (figure 35B). As a correlation with those studies performed by Nelson and MacLeod (submitted for publication), the whole cell antiserum prepared by Nelson was also tested in the double immunodiffusion experiments. The two antisera cross-reacted when exposed to the marine pseudomonad LPS (figure 35 C). The slide was stained by the periodate-Schiff procedure because a non-specific protein ring stained around the well of antiserum A_1. The agarose medium also stains during the periodate-Schiff procedure, yielding a dark background, however, the precipitin lines are clearly visible (figure 35 D). Nelson's antiserum also exhibited a homologous precipitin reaction among whole cell LPS, LPS I and LPS II (figure 36 A) with no precipitin lines observed with LPS III, lipid A, core, or core-side chain (figure 36B). Apparently, the antiserum had a higher antibody titer as evidenced by the stronger precipitin reactions. When these same slides were stained with Amido Black 10B, in each case, the same precipitin patterns were obtained (figure 36C and 36D). This information coupled with what is known about the carbohydrate composition of the LPS species, suggests that a complete LPS molecule

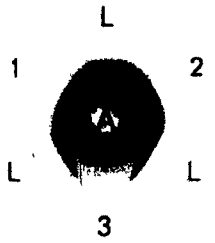
F I G U R E 36.

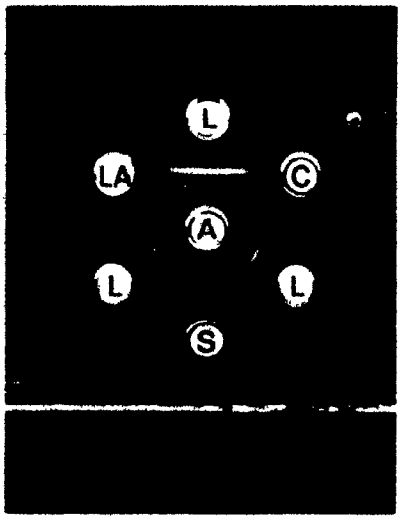
Fig. 36. Correlation of the immunological reactions of LPS I, II and III with those obtained against Nelson's whole cell antiserum.

Slides A and B show the actual precipitin lines as recorded by dark field photography. Slides C and D are the same slides after staining with Amido Black 10B. The well designations are the same as those in figure 35.

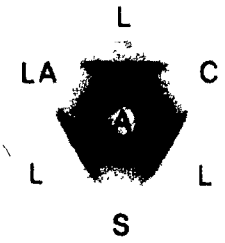


C





D



is necessary in order to obtain an immunological reaction. When the LPS molecule was fractionated the subsequent parts of the molecule did not show a precipitin reaction. Similarly, LPS III, which is lipid A-core, but is lacking a side chain, also failed to react with the antiserum. Lipopolysaccharides I and II which appear to contain most of, if not their complete side chains, demonstrated the same serological reaction as whole cell LPS. It would thus appear that it is the LPS species I and II which stimulate antibody formation when whole cells of this organism are injected into rabbits. Consequently, all of the LPS is not responsible for antigenicity since LPS III does not contribute to the overall reaction.

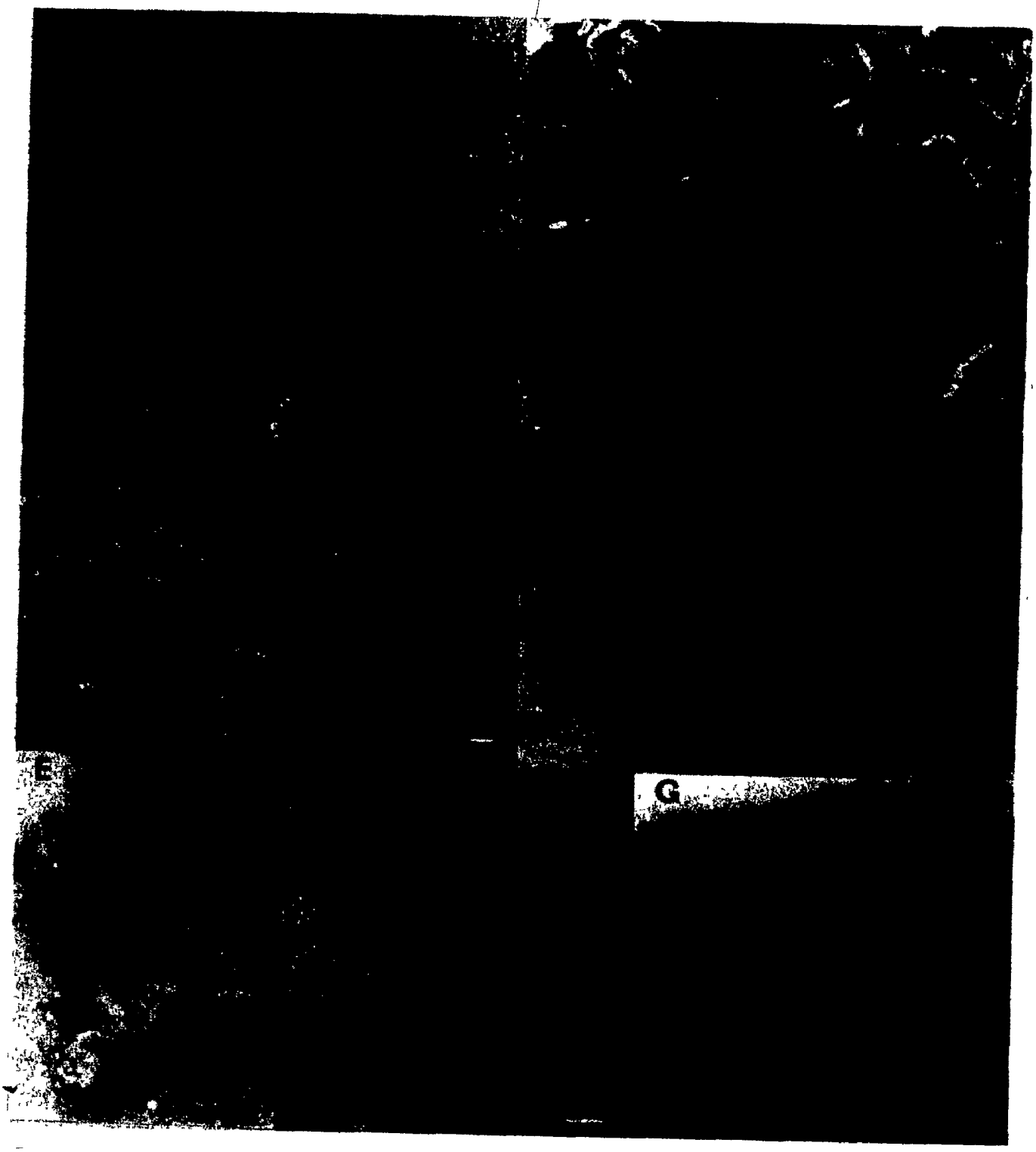
Morphology

The gross morphology of whole cell LPS and the LPS species was compared by electron microscopy. The samples were dispersed in distilled water by heating at 37 C for several minutes. In those samples that were examined for the effects of SDS on LPS structure, samples were dispersed in a 1% (w/v) solution of SDS. The preparations were stained with 1% phosphotungstic acid containing 0.01% bovine serum albumin (pH 7.5). The whole cell LPS was in the form of the typical ribbon-like structures that are usually seen in negative stains of this macromolecule (Shands *et al.*, 1967 and Hannecart-Pokorni *et al.*, 1973). In this preparation, however, the ribbons were contracted (figure 37A). In the presence of SDS the ribbons break down into

FIGURE 37.

Fig. 37. Morphology of whole cell LPS and the LPS species as determined by negative staining.

All samples were dispersed in distilled water at 37 C. A, whole cell LPS; B and C, whole cell LPS in 1% SDS (W/v); D and E, LPS I; F, LPS II and G, LPS III. Bar = 0.1 μm.

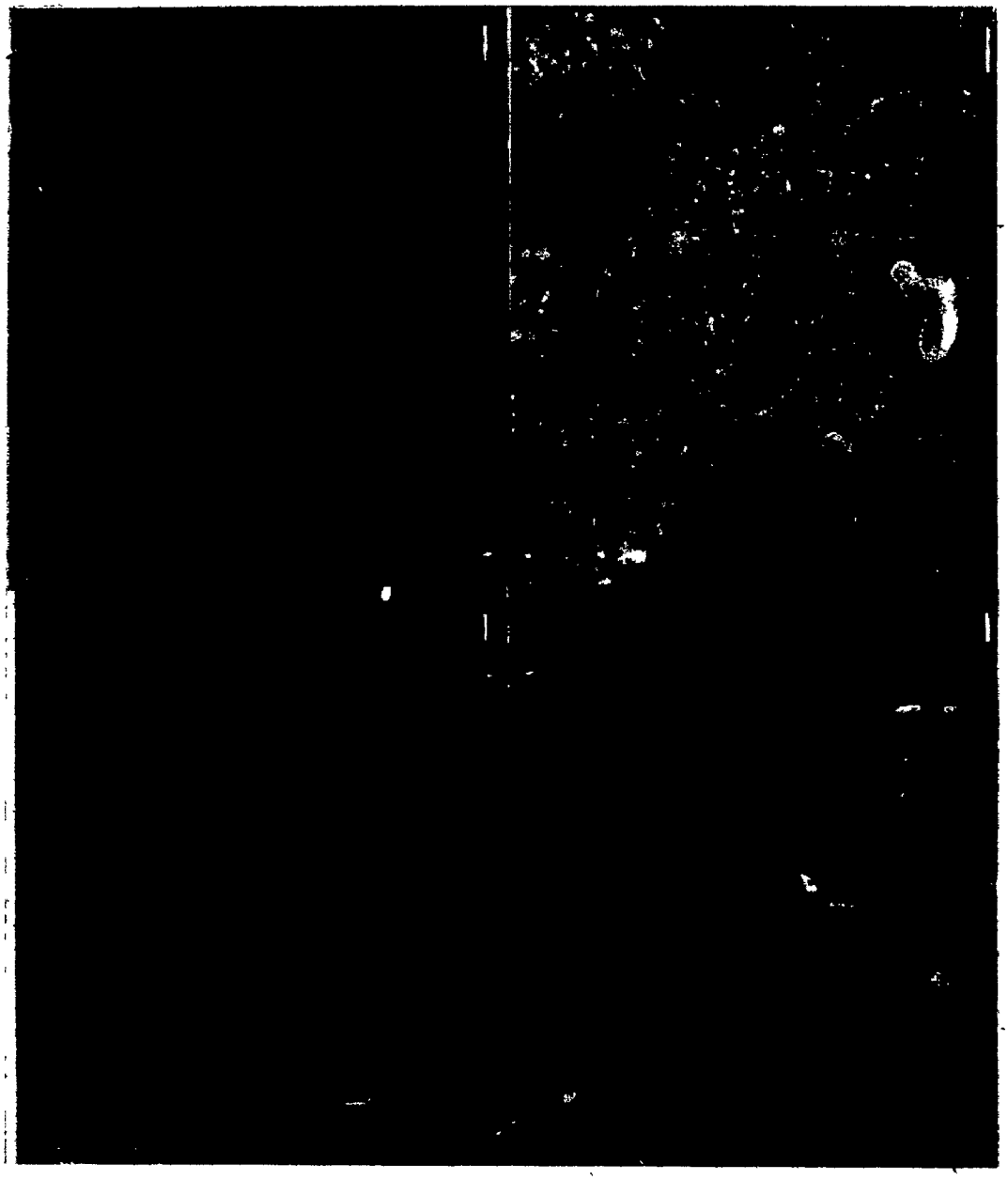

E
G

smaller vesicles, some with remnants of the ribbon-like structure remaining (figures 37B and 37C). The small amount of material that was found in preparations of LPS I looked remarkably similar to the structures seen in SDS treated whole cell LPS (figure 37D and 37E). Lipopolysaccharide II shows small spherical structures at high magnification indicating complete disaggregation (figure 37F). Figure 37G, which represents LPS III, contains little, if any material and no conclusions can be deduced from this photograph.

Lipopolysaccharide samples that were stained in a 0.05 M Na_2HPO_4-0.05 M Na_2MoO_4 buffer (pH 7.0) exhibited greater structural integrity (figure 38). The LPS ribbons appeared in both short and extremely long forms, some having bulbular ends. In the presence of SDS, short, layered structures were prevalent which have not been reported by other workers (figure 38C). Lipopolysaccharide II preparations contained very small spherical and rod-shaped structures. Lipopolysaccharides I and III, when negatively stained, did not show any discernable structures, thus, the electron micrographs were not shown. Overall, the negatively stained preparations of LPS buffered at pH 7.0 showed greater structural integrity than those which were not buffered. The SDS can be seen to dissociate the long ribbon-like aggregates and electrophoresis apparently separates the disaggregated components more completely.

F I G U R E 38.

Fig. 38. Morphology of whole cell LPS buffered at pH 7.0. All samples were dispersed in 0.05 M Na_2HPO_4 – 0.05 M Na_2MoO_4 at 37 C. A and B, whole cell LPS; C, whole cell LPS in 1% SDS (W/v) and D, LPS II. Bar = 0.1 µm.



Molecular weight determination

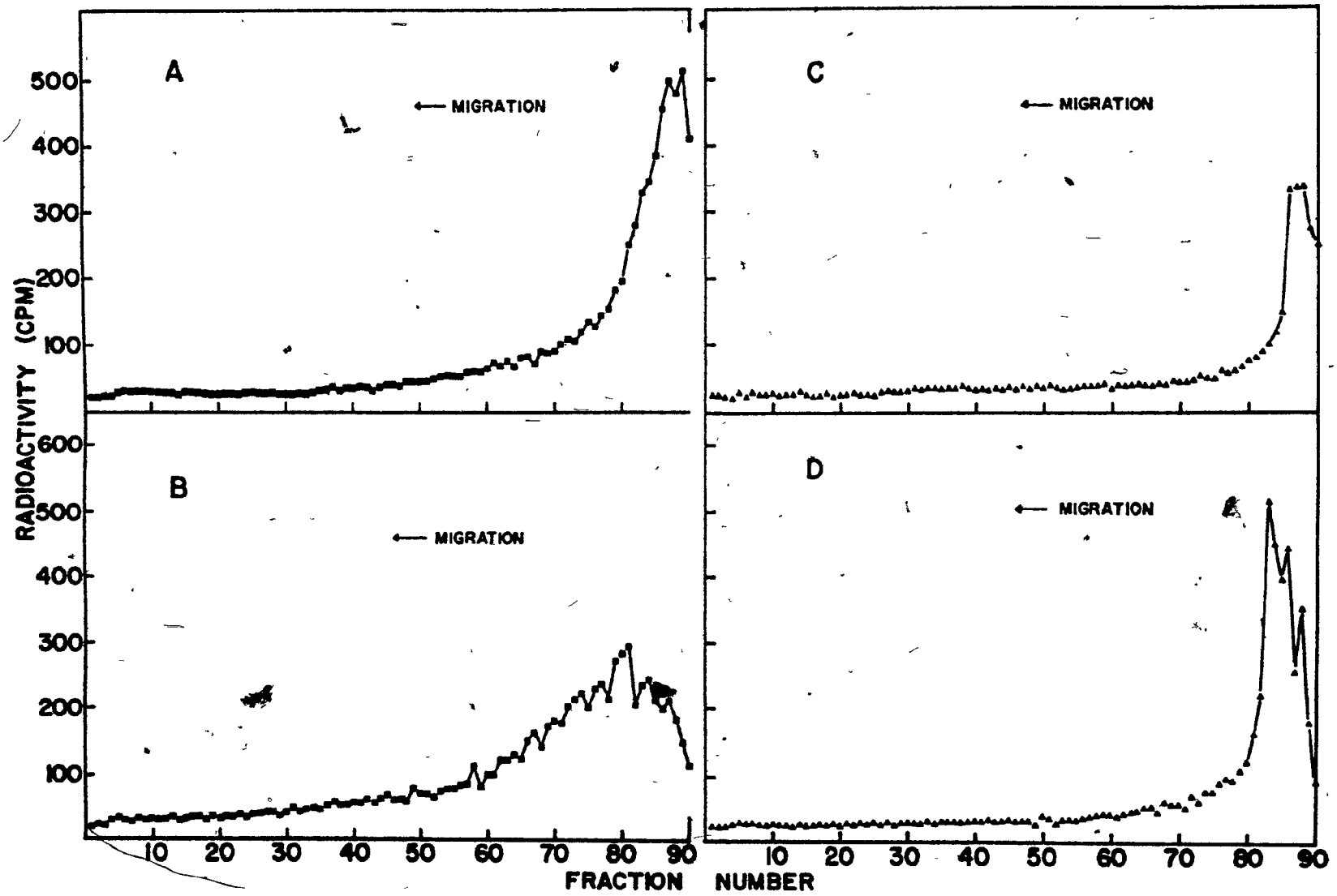
An attempt was made to determine the approximate molecular weights of LPS I, II and III by SDS-polyacrylamide gel electrophoresis. Several [^{14}C] dextran samples of different molecular weight ranges were examined for mobility in the buffer. The [^{14}C] dextrans, molecular weight range 15,000 to 17,000 and 60,000 to 90,000 daltons, respectively, were electrophoresed in 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS for 5 h (figures 39A and 39C). During this electrophoresis time, which was equivalent to the length of time routinely used for LPS electrophoresis, only 47% of the 15,000 to 17,000 molecular weight dextran and 16% of the 60,000 to 90,000 molecular weight sample entered the gel. When the electrophoresis time was increased to 16 h, 52% of the 15,000 to 17,000 and 25% of the 60,000 to 90,000 molecular weight dextrans were recovered (figures 39B and 39D). Consequently, dextrans cannot be used as molecular weight standards in polyacrylamide gel electrophoresis because of their lack of mobility. The fact that the dextrans will not migrate supports the concept that lipid is necessary for SDS binding. It was felt that the use of polypeptides as standards for LPS molecular weight determinations would be rather meaningless.

Dextrans could be useful carbohydrate molecular weight standards for column chromatography. The marine pseudomonad LPS was solubilized and applied to a Sepharose 4B column (exclusion limit 5×10^6 daltons)

FIGURE 39.

Fig. 39. Determination of the electrophoretic mobility of [^{14}C] dextrans for their use as molecular weight standards in SDS-polyacrylamide gel electrophoresis.

Electrophoresis was performed in the 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS buffer. Radioactive profile A, 15,000 to 17,000 molecular weight dextran after electrophoresis for 5 h at 5 mA/gel; profile B, same sample after electrophoresis for 16 h; profile C, 60,000 to 90,000 molecular weight sample electrophoresed for 5 h at 5 mA/gel and profile D, same sample after electrophoresis for 16 h. Each fraction represents 1 mm of the gel.


A
B
C
D
MIGRATION
RADIOACTIVITY (CPM)
FRACTION NUMBER
100
200
300
400
500
600
10
20
30
40
50
60
70
80
90

which was previously packed and equilibrated in the same 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS buffer that was used for the gel electrophoresis studies. The column was maintained at 25 ± 2 C in a constant temperature incubation room rather than at 10 C because SDS precipitates in the cold. Upward flow elution was employed and the column effluent was assayed by passage through a refractive index monitor (Pharmacia). The system was set up as in the schematic representation in figure 40. Two ml fractions were collected and those that corresponded to each peak were pooled and dialyzed for 36 h at room temperature to remove SDS. Dialysis removed only a limited amount of SDS therefore the remainder was eliminated by precipitation with $BaCl_2$. Following dialysis and concentration of the material eluted from the column, a saturated aqueous solution of $BaCl_2$ was added. The $BaSO_4$ precipitate was then removed by centrifugation. Pure LPS preparations did not precipitate when saturated $BaCl_2$ was added. The excess $BaCl_2$ and NaCl was then removed by dialysis which was checked for completeness by the addition of a saturated solution of $AgNO_3$ to the dialysis fluid. Formation of a white precipitate indicated the presence of AgCl. In this manner all of the SDS could be effectively removed.

The Sepharose 4B column profile shown in figure 41 demonstrates that the LPS separated into two peaks. When the material that was represented by these peaks was electrophoresed on SDS-polyacrylamide

FIGURE 40.

Fig. 40. Diagram of the Sepharose gel filtration system for the chromatography, detection, and collection of the LPS species.

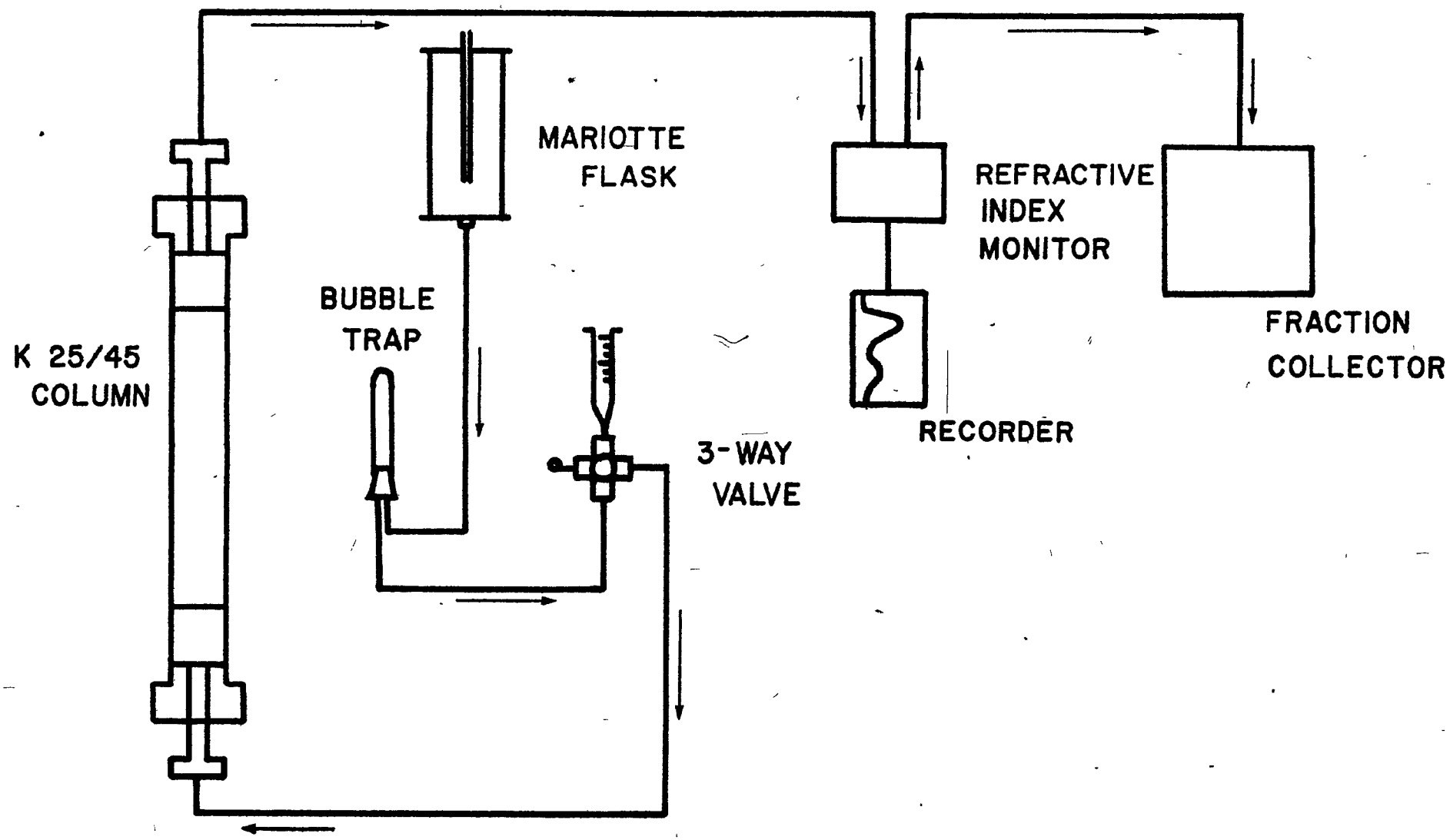
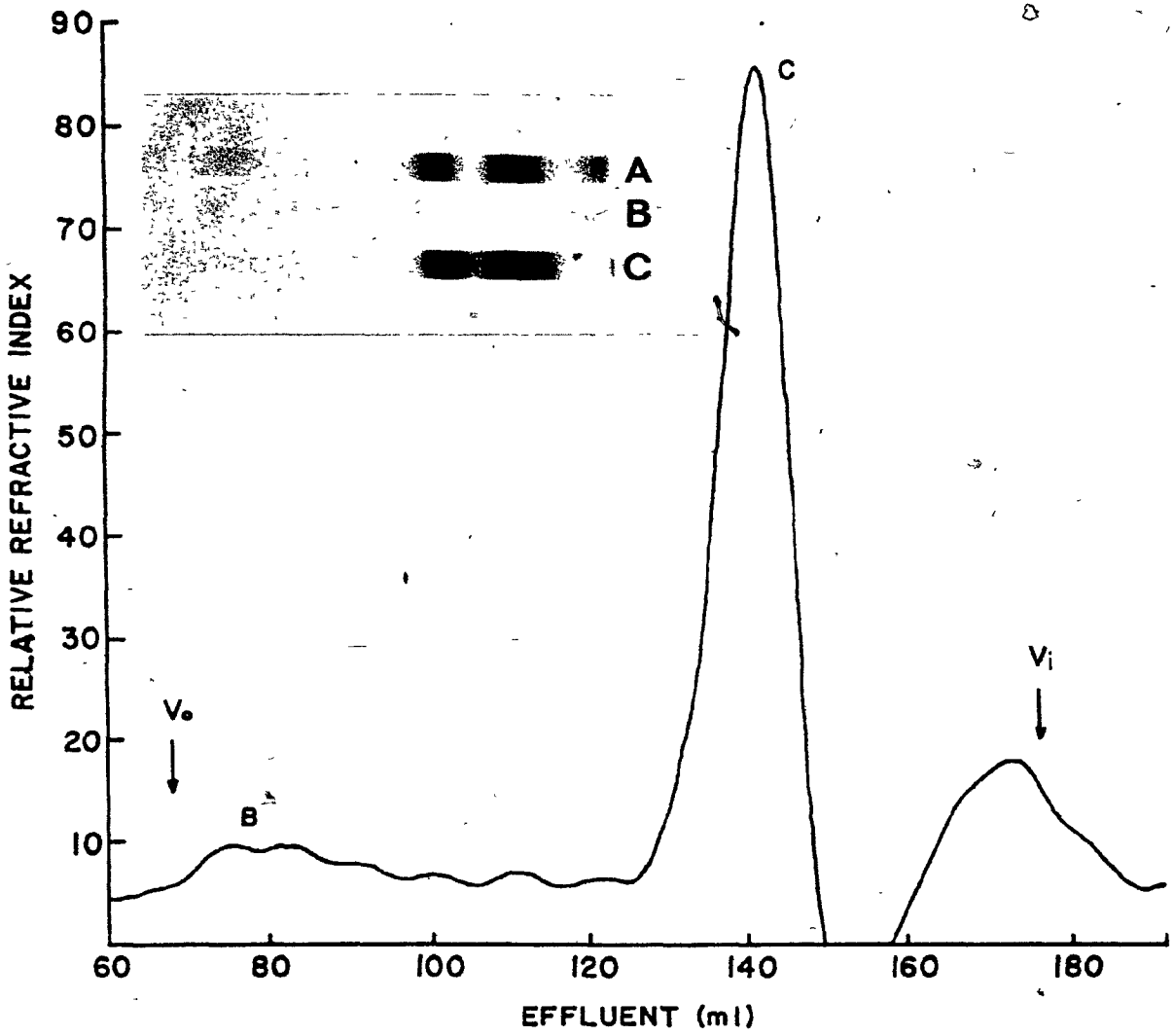

MARIOTTE
FLASK
REFRACTIVE
INDEX
MONITOR
FRACTION
COLLECTOR
BUBBLE
TRAP
K 25/45
COLUMN
3-WAY
VALVE
RECORDER

FIGURE 41.

Fig. 41. Gel filtration of whole cell LPS on Sepharose 4B.

The column was packed and the samples were eluted in 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS buffer. The material in peaks B and C was subjected to SDS-polyacrylamide gel electrophoresis. Insert: gel A, whole cell LPS control; gel B, material from peak B and gel C, material from peak C. The gels were stained by the periodate-Schiff procedure. The location of the void and included volumes are indicated by V_o and V_i, respectively.


RELATIVE REFRACTIVE INDEX
90
80
70
60
50
40
30
20
10
A
B
C
C
B
V_o
V_i
60
80
100
120
140
160
180
EFFLUENT (ml)

gels one peak was found to be LPS I and the second peak consisted of LPS II and III. When peak C, taken from the Sepharose 4B column was rechromatographed on Sepharose 6B (exclusion limit 1×10^6 daltons) a single peak was again obtained which contained LPS II and III (figure 42). Various molecular weight range dextrans were chromatographed on Sepharose 4B and the V_e and K_{av} values were determined (Table 13). The void volume (V_o) and the included volume (V_i) were measured by using blue dextran and sucrose. The molecular weight of the LPS material was determined from a semi-logarithmic plot of the K_{av} values and molecular weights of the dextran samples (figure 43). This method for elucidating the molecular weights of LPS I, II and III was no more fruitful than the gel electrophoresis system since only LPS I separated from the other two species. Sepharose gel filtration also proved not to be a useful technique for the preparative isolation of the LPS species, since only a limited amount of LPS could be applied to the column, and, again LPS II and III could not be separated.

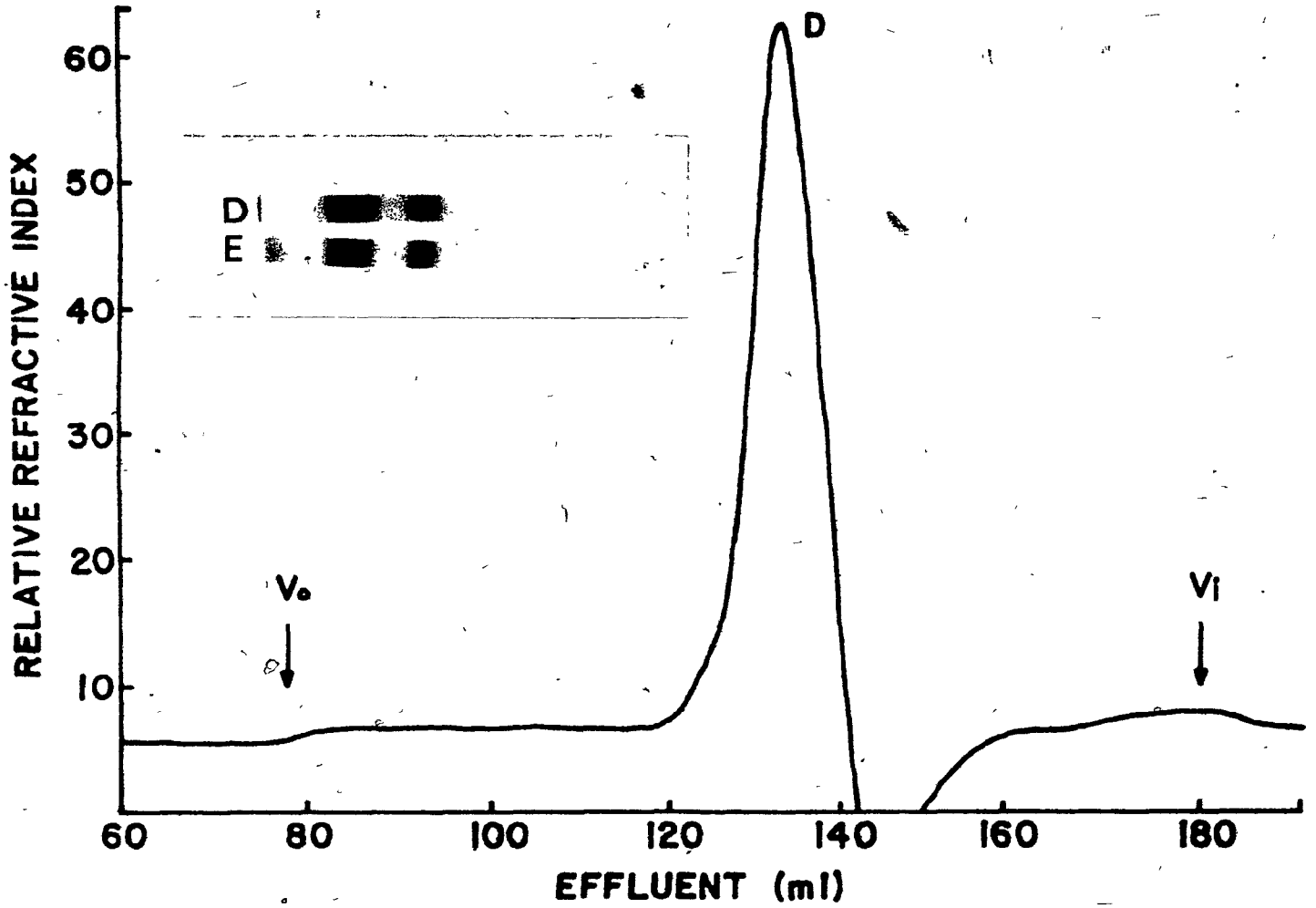
Localization

The LPS species were localized in the cell wall layers of the marine pseudomonad with the use of the SDS-polyacrylamide gel system. The outer cell wall layers of the marine pseudomonad were removed (see Materials and Methods), electrophoresed in the Na_2HPO_4 - Na_2MoO_4 - 1% SDS buffer and stained for carbohydrate. These cell wall layers

FIGURE 42.

Fig. 42. Gel filtration of LPS on Sepharose 6B.

The material from peak C of the Sepharose 4B column (figure 12) was rechromatographed on a Sepharose 6B column. The experimental conditions were identical to those in figure 41. Insert: gel D, material from peak D and gel E, whole cell LPS control.


RELATIVE REFRACTIVE INDEX
60
50
40
30
20
10
D
D
E
Vo
Vi
60
80
100
120
140
160
180
EFFLUENT (ml)

T A B L E 13.

Determination of K_{av} for standards and lipopolysaccharide on Sepharose 4B[a]

Applied sample	V_e (ml)	K_{av}
Dextrans[b]		
2×10^6 M.W.	70	0.02
5×10^5 M.W.	122	0.54
2.5×10^5 M.W.	144	0.76
4×10^4 M.W.	146	0.78
2×10^4 M.W.	158	0.90
1×10^4 M.W.	162	0.94
LPS I	74	0.06
LPS II & III	140	0.72

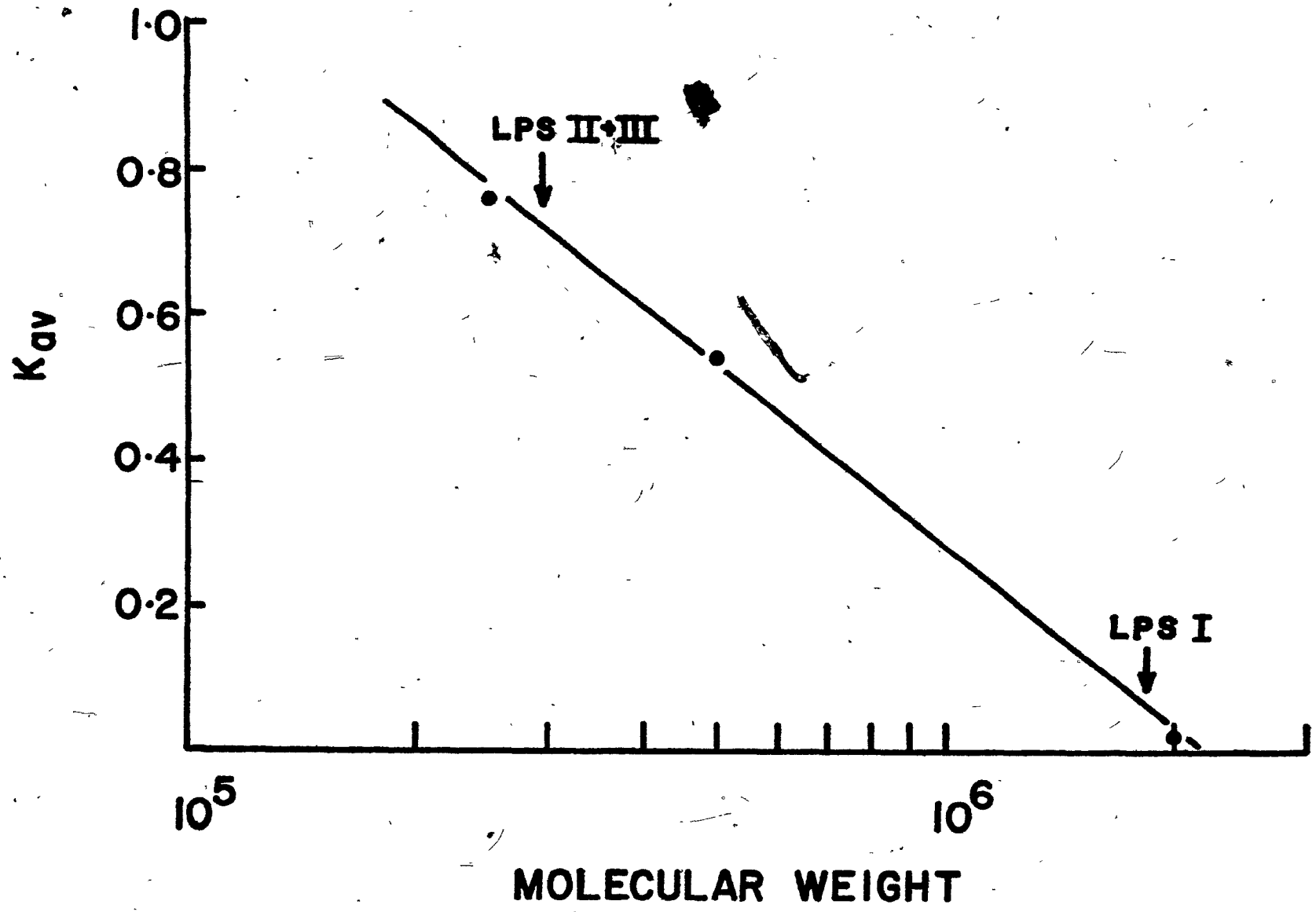
[a] $K_{av} = \frac{V_e - V_o}{V_t - V_o}$; where, V_o = 68 ml and V_t = 168 ml (See Materials and Methods for an explanation).

[b] Average molecular weights.

FIGURE 43.

Fig. 43. Molecular weight determination of the LPS species based on the K_{av} calculations from Sephrose gel filtration.

The K_{av} values were taken from Table 13 and the molecular weights of the dextran preparations were averages.


1·0
0·8
0·6
0·4
0·2
K_{av}
LPS II+III
LPS I
10^5
10^6
MOLECULAR WEIGHT

were solubilized in the same SDS solubilizing solution as was the LPS and were not subjected to any additional extraction or hydrolytic procedures. They represent regions of the cell envelope that correspond to the periplasmic space, the outer tripartite membrane and the material on the external surface of the outer membrane. They have been previously shown to contain protein, lipid, and carbohydrate (Forsberg *et al.*, 1970b). When compared to phenol-water extracted LPS from whole cells the three cell wall layers exhibited the same LPS species but these species varied in concentration within each layer (figure 44). The most notable differences occurred in the outer double-track and periplasmic layers in which LPS I and LPS III, respectively, appear to be severely reduced.

The absorbance maximum of the colorimetric product of the Schiff reagent was found to be 540 nm (figure 45) and densitometry scans of the gels containing the wall layer samples were made as shown in figure 46. The areas under the curves were calculated and used to quantitate each LPS species in the cell wall layers and whole cell LPS (Table 14). The findings proved that the LPS heterogeneity was not a result of the phenol extraction procedure and gave a third method for detecting the presence of LPS in the cell wall layers. (The other two methods were discussed in Results, Section I.) The high concentration of protein in the wall layers emphasized more dramatically the fact that LPS migration is unaffected by protein mobility.

FIGURE 44.

Fig. 44. Localization of the LPS species in the cell wall layers isolated from the marine pseudomonad.

The cell wall layers were electrophoresed in 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS for 4 h at 10 mA/gel. The gels were stained by the periodate-Schiff method. Gel A, 200 µg LPS; gel B, 400 µg loosely bound outer layer; gel C, 400 µg outer double-track and gel D, 400 µg periplasmic layer.

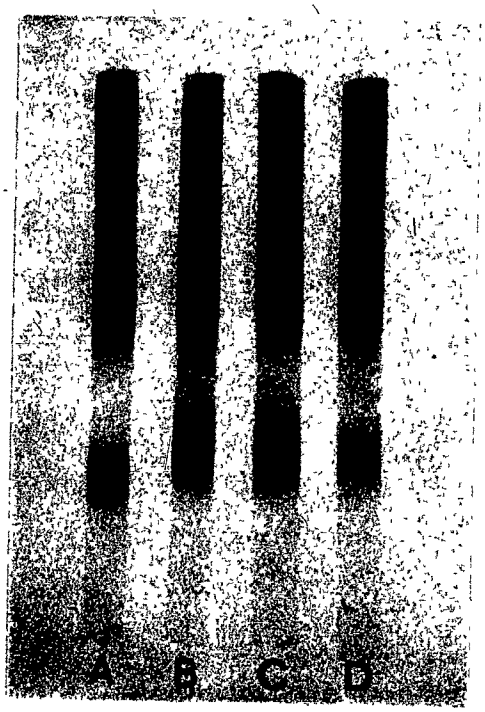

A
B
C
D

FIGURE 45.

Fig. 45. Determination of the absorbance maximum of the coloured aldehyde product of the Schiff reagent as used in polyacrylamide gel densitometry employing 7.5% acetic acid as the solvent.

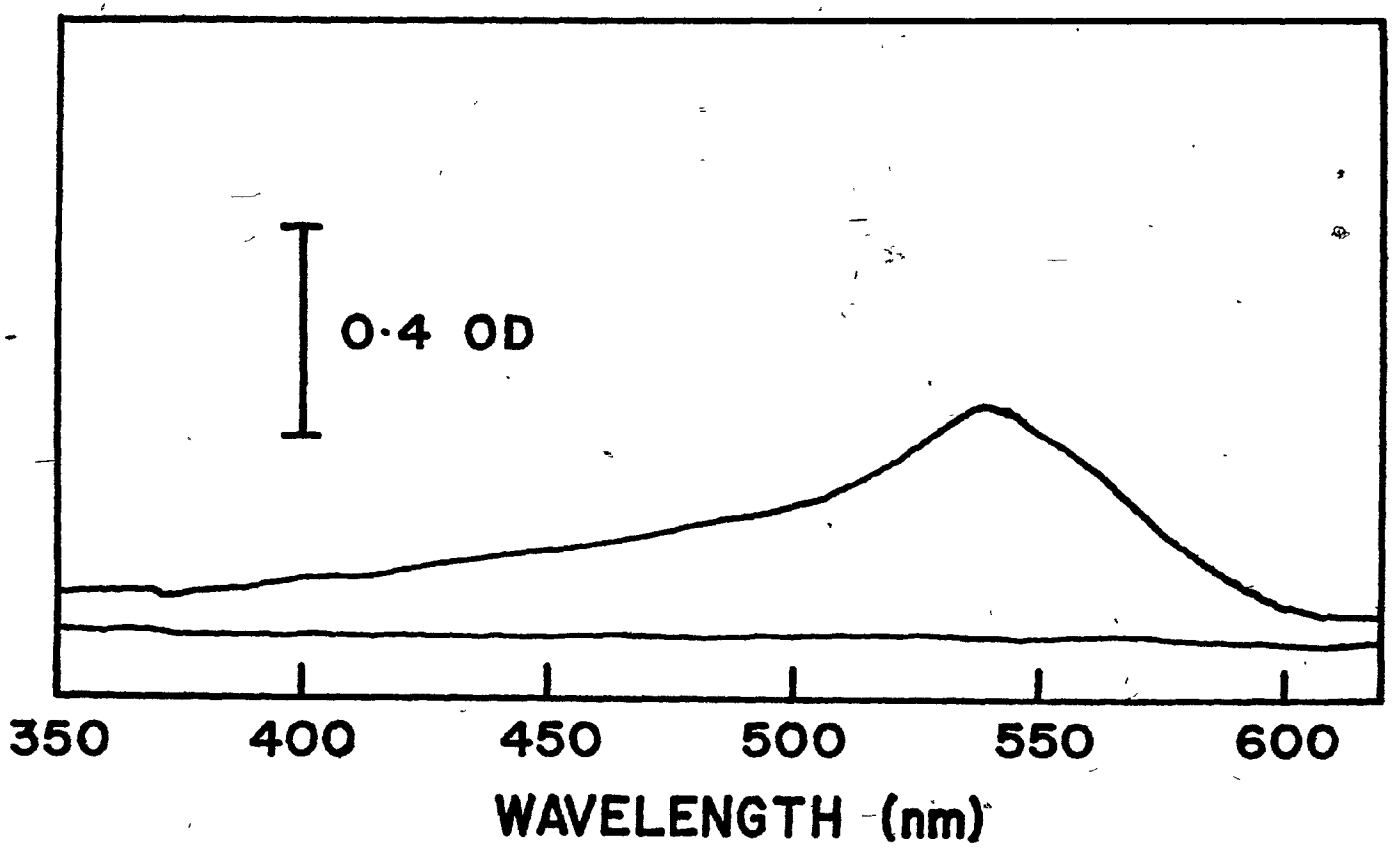

0·4 OD
350
400
450
500
550
600
WAVELENGTH (nm)

FIGURE 46.

Fig. 46. Densitometer tracings of the migration patterns of LPS and cell wall layers on SDS-polyacrylamide gels.

Gels were stained by the periodate-Schiff procedure and were scanned at 540 nm. Scan A, 200 µg whole cell LPS; scan B, 400 µg loosely bound outer layer; scan C, 400 µg outer double-track and D, 400 µg periplasmic layer. LPS I, II, III and the surface of the gel are represented by I, II, III and S, respectively.

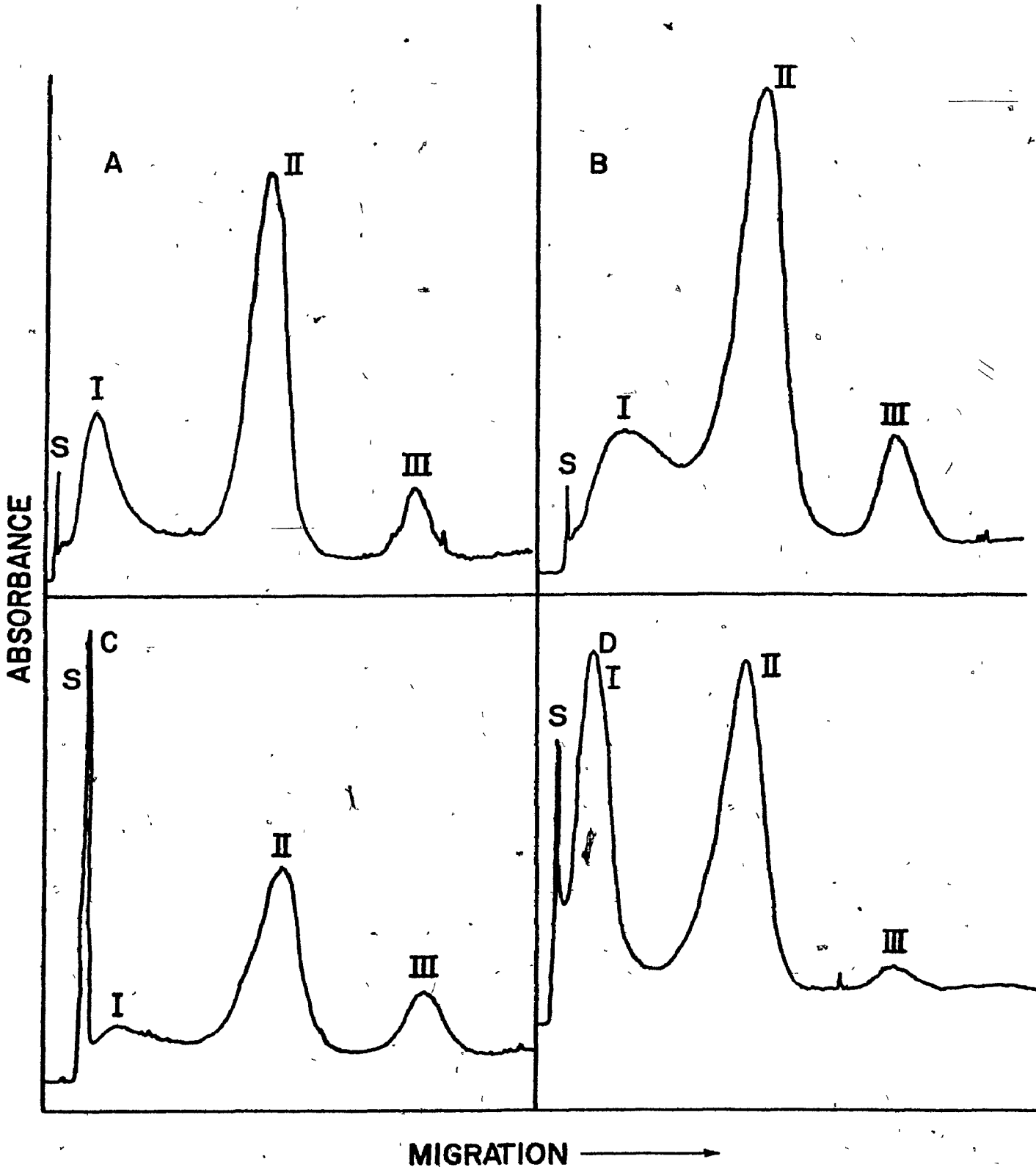

A
B
C
D
S
I
II
III
ABSORBANCE
MIGRATION

TABLE 14.

Quantitation of the lipopolysaccharide species in whole cell LPS and the cell wall layers of the marine pseudomonad as measured by densitometry

Sample[a]	LPS species	Peak area (mm^2)	% LPS species	% LPS in each layer	% of total LPS in each layer
Whole cell LPS	I	976	26.6		
	II	2385	65.1		
	III	304.5	8.3		
	total	3665.5			
Loosely bound outer layer	I	1222	21.5		
	II	3800	66.7		
	III	675	11.9		
	total	5697		77.7	45.6
Outer double-track	I	195	9.4		
	II	1463	70.5		
	III	418.5	20.2		
	total	2076.5		28.3	16.6
Periplasmic layer	I	2044.5	43.3		
	II	2484	52.6		
	III	192.5	4.1		
	total	4721		64.4	37.8

[a] Lipopolysaccharide (200 µg) and 400 µg of each of the cell wall layers were applied to the gels.

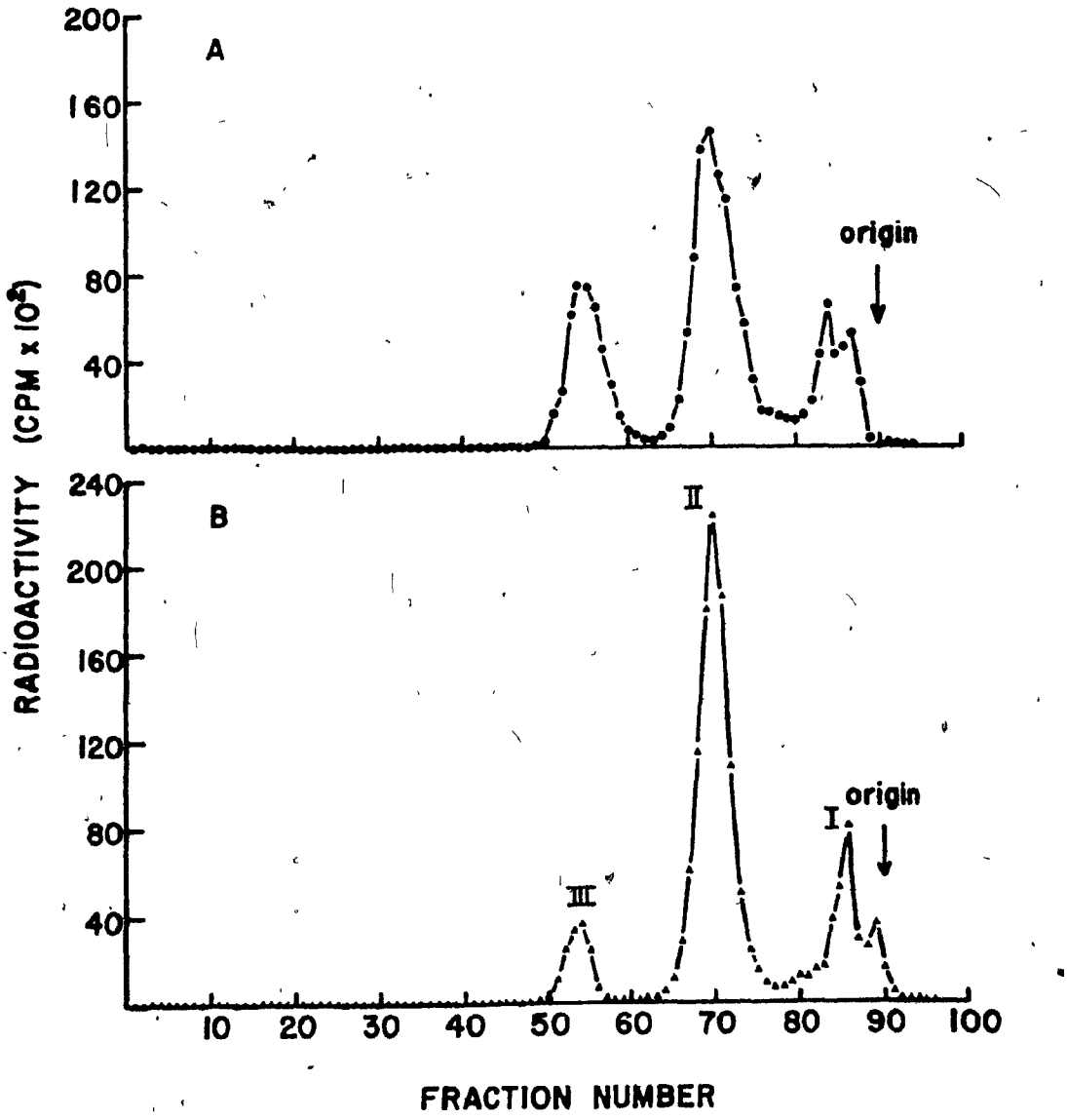
In figure 44 the LPS in gel A contained 1% protein whereas the cell wall layers contain 20 to 40% protein (Forsberg *et al.*, 1970b), however, the positions of the LPS bands were unaltered.

During the growth of the marine pseudomonad, material was found in the medium. It was electrophoresed on SDS-polyacrylamide gels after release from [^{14}C] labeled cells and contained three peaks which were compared to the three species found in cellular LPS (figure 47). The material isolated from the medium migrated to the same positions in the SDS-gels as did LPS I, II and III and no additional radiolabeled peaks were observed. On this basis, the material which was released into the cell medium appeared to represent excreted cellular LPS. The medium material was found to be present at extremely low levels and obtaining enough material for gel electrophoresis was difficult. These results appeared to substantiate those of Nelson and MacLeod (submitted for publication) in which the release of LPS to the medium was demonstrated using double immunodiffusion techniques.

FIGURE 47.

Fig. 47. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of the material released into the medium when cells of the marine pseudomonad were grown in the presence of [^{14}C]galactose.

Electrophoresis time was 3 h at 10 mA/gel in 0.05 M Na_2HPO_4 - 0.05 M Na_2MoO_4 - 1% SDS. One gel fraction corresponds to 1 mm of the gel. Gel profile A, the material released into the medium and B, whole cell LPS.


A
B
200
160
120
80
40
240
200
160
120
80
40
origin
origin
I
II
III
RADIOACTIVITY (CPM x 10²)
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

SECTION IV

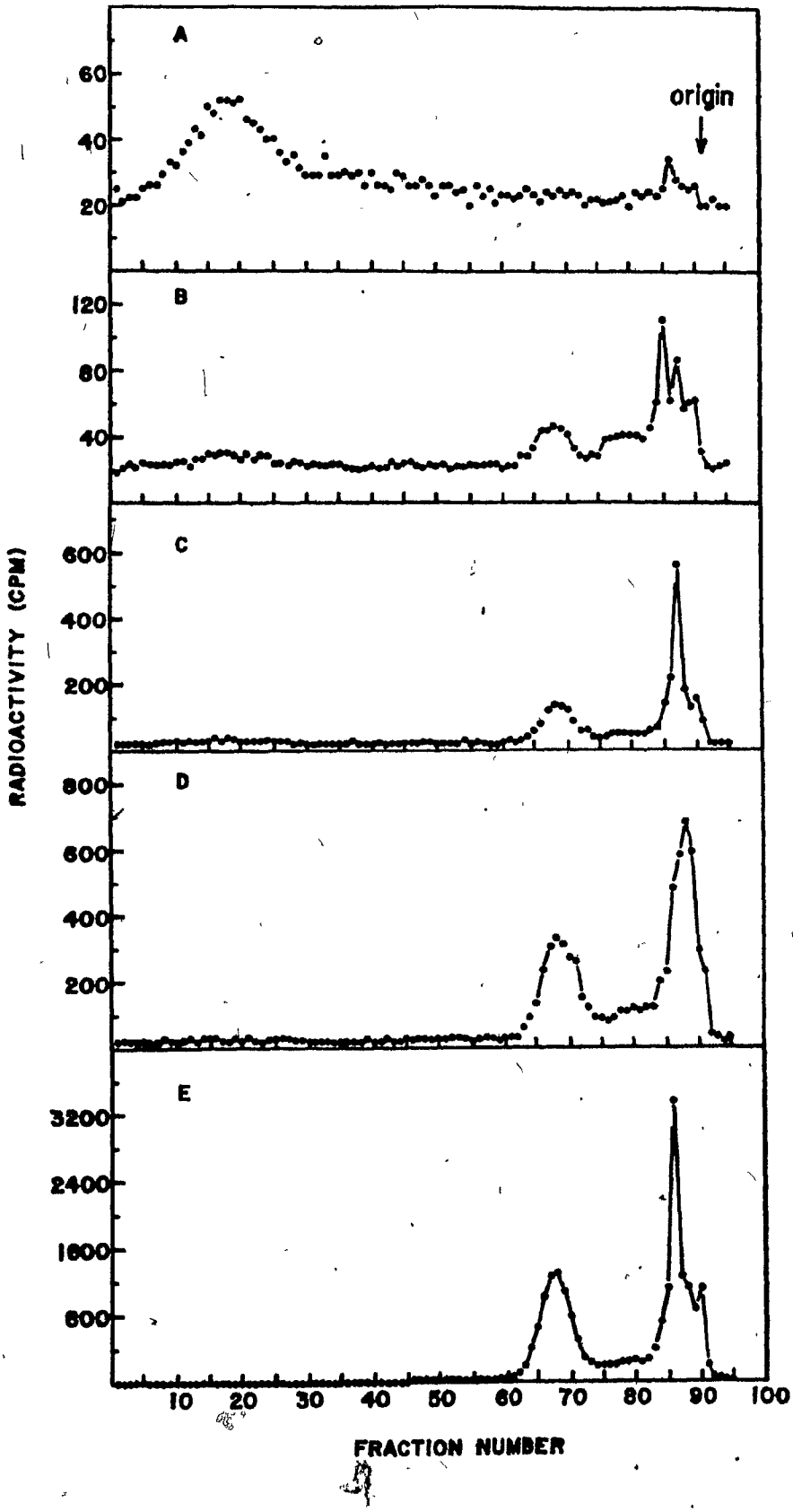
STUDIES INVOLVING THE BIOSYNTHESIS OF THE LIPOPOLYSACCHARIDE SPECIES

Order and rate of biosynthesis

The ability to detect the LPS species by gel electrophoresis supplied an excellent assay system for the study of their biosynthesis. The pulse-labeling technique used by Osborn and co-workers (1972a) was adapted for use in these experiments. Cells of the marine pseudomonad were grown to early logarithmic phase and at an optical density approximately 0.40, 0.2 μCi/ml of [^{14}C]galactose (43 μCi/μmole) were added. Aliquots (5 ml) were removed at specific time intervals following the addition of the isotope and were pipetted directly into 5 ml of preheated 90% phenol to prevent the further incorporation of label. The extraction and purification of LPS was performed on each cell sample and the LPS was then electrophoresed on SDS-polyacrylamide gels in the Na_2HPO_4 - Na_2MoO_4 - 1% SDS buffer. The gels were fractionated and counted and the profiles are illustrated in figure 48. In the first sample, in which the cells were labeled for 2 min, LPS I can be seen. There was a very low molecular weight peak near the bottom of the gel, but, it is not known what this peak represents. After labeling the cells for 5 min, LPS II was evident while LPS I continued to incorporate label and the low molecular weight peak began to diminish. During an additional 10 to 40 min of labeling both LPS I and II continued to incorporate label with no

FIGURE 48.

Fig. 48. Pulse-labeling of the marine pseudomonad LPS. Cells pulsed with [^{14}C]galactose were phenol-water extracted and the isolated LPS was electrophoresed for 3 h at 10 mA/gel. Labeling times were: profile A, 2 min; profile B, 5 min; profile C, 10 min; profile D, 20 min; and profile E, 40 min.


A
origin
60
40
20
B
120
80
40
C
600
400
200
D
800
600
400
200
E
3200
2400
1600
800
RADIOACTIVITY (CPM)
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

appearance of LPS III. The generation time of the marine pseudomonad when grown in nutrient broth-yeast extract complex medium, under the conditions used in these experiments, was approximately 60 min (figure 49). Therefore, the cells were allowed to grow for slightly less than one generation after first exposure to the label. During the time that the cells were exposed to label they were in the logarithmic phase of growth. In this situation LPS I and II were rapidly biosynthesized in exponential phase cells while LPS III was not. The experiment was repeated and the cells, after inoculation into a medium containing [^{14}C]galactose, were incubated until the culture reached early stationary phase of growth. Aliquots of the culture were removed after two, three and four generations of exposure to label and the LPS was extracted and electrophoresed. Thus, the experiment followed the incorporation of label into the LPS species over the entire growth cycle of the culture. At the end of the second generation, LPS II was incorporating the radioisotope at a faster rate than LPS I (figure 50A). After three generations this rate change was confirmed and LPS III had still not appeared (figure 50B). The final sample was removed after four generations and the results show that LPS III was present in these cells (figure 50C). Figure 51 demonstrates that the cell samples that were collected after exposure to label for two, three and four generations were at early logarithmic, mid-logarithmic and late logarithmic to stationary phases of growth, respectively.

FIGURE 49.

Fig. 49. Logarithmic plot of the growth of the marine pseudomonad in nutrient broth-yeast extract complex medium. Optical density was monitored with a Bausch and Lomb Spectronic 20 spectrophotometer at 660 nm.

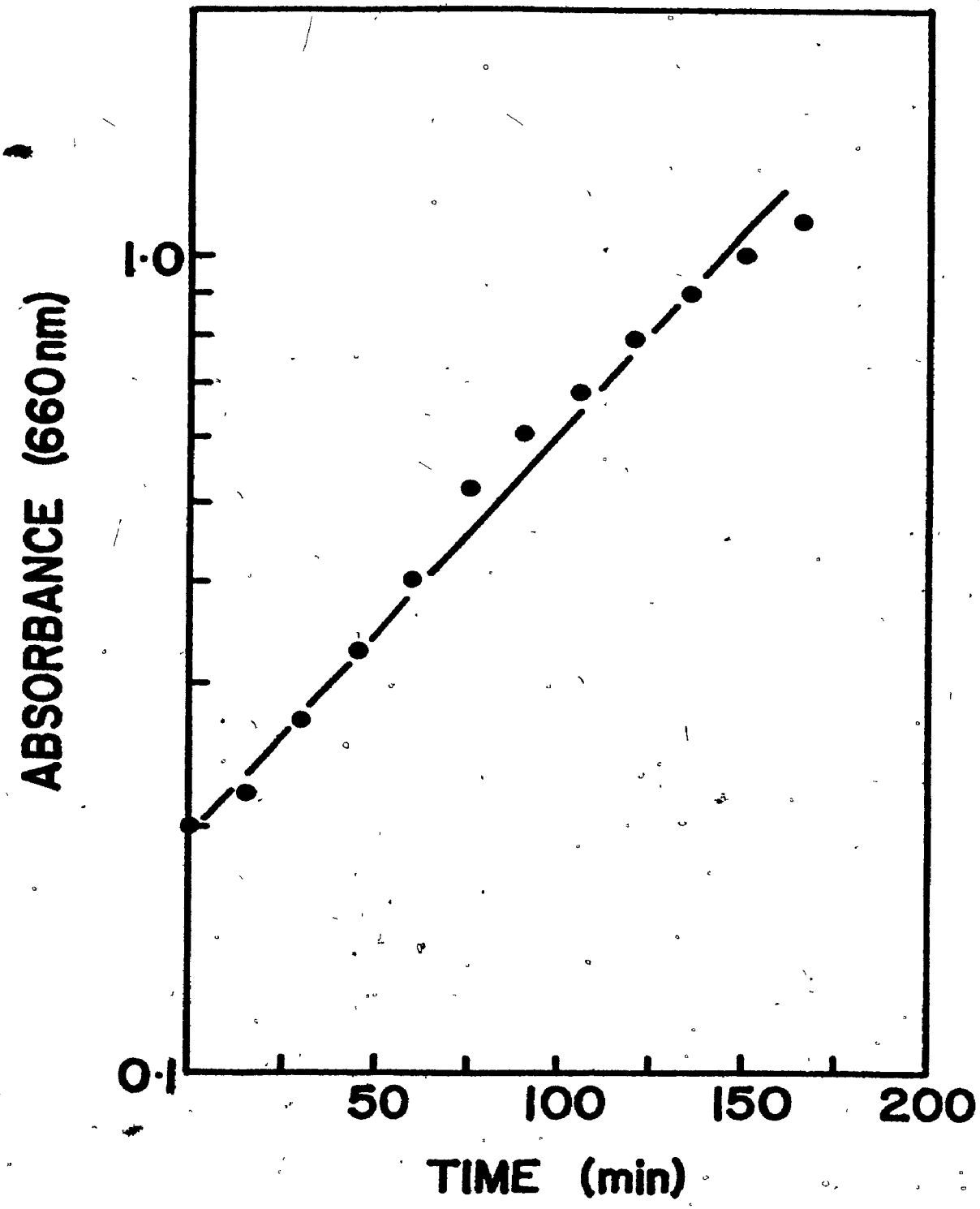

ABSORBANCE (660nm)
1·0
0·1
50
100
150
200
TIME (min)

FIGURE 50.

Fig. 50. Pulse-labeling of the marine pseudomonad LPS over the entire growth cycle.

The experiment was performed as in figure 49. Samples were electrophoresed after A, 2 generations; B, 3 generations; and C, 4 generations of labeling.

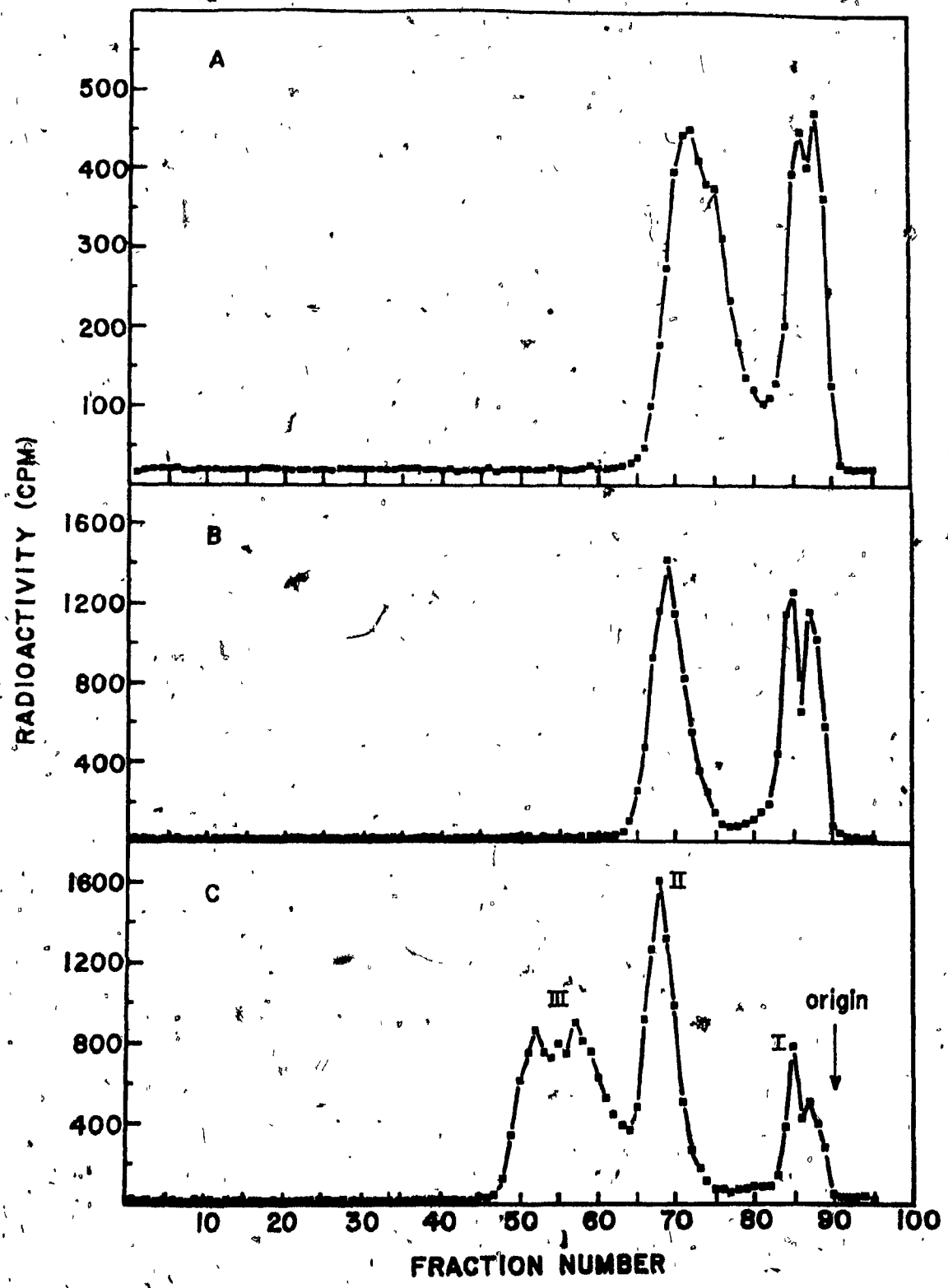

A
B
C
500
400
300
200
100
1600
1200
800
400
1600
1200
800
400
II
III
I
origin
RADIOACTIVITY (CPM)
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

FIGURE 51.

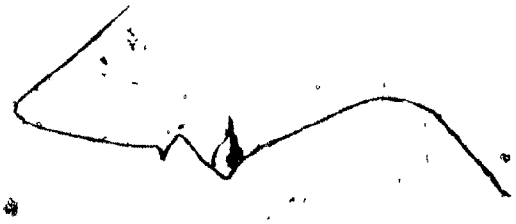
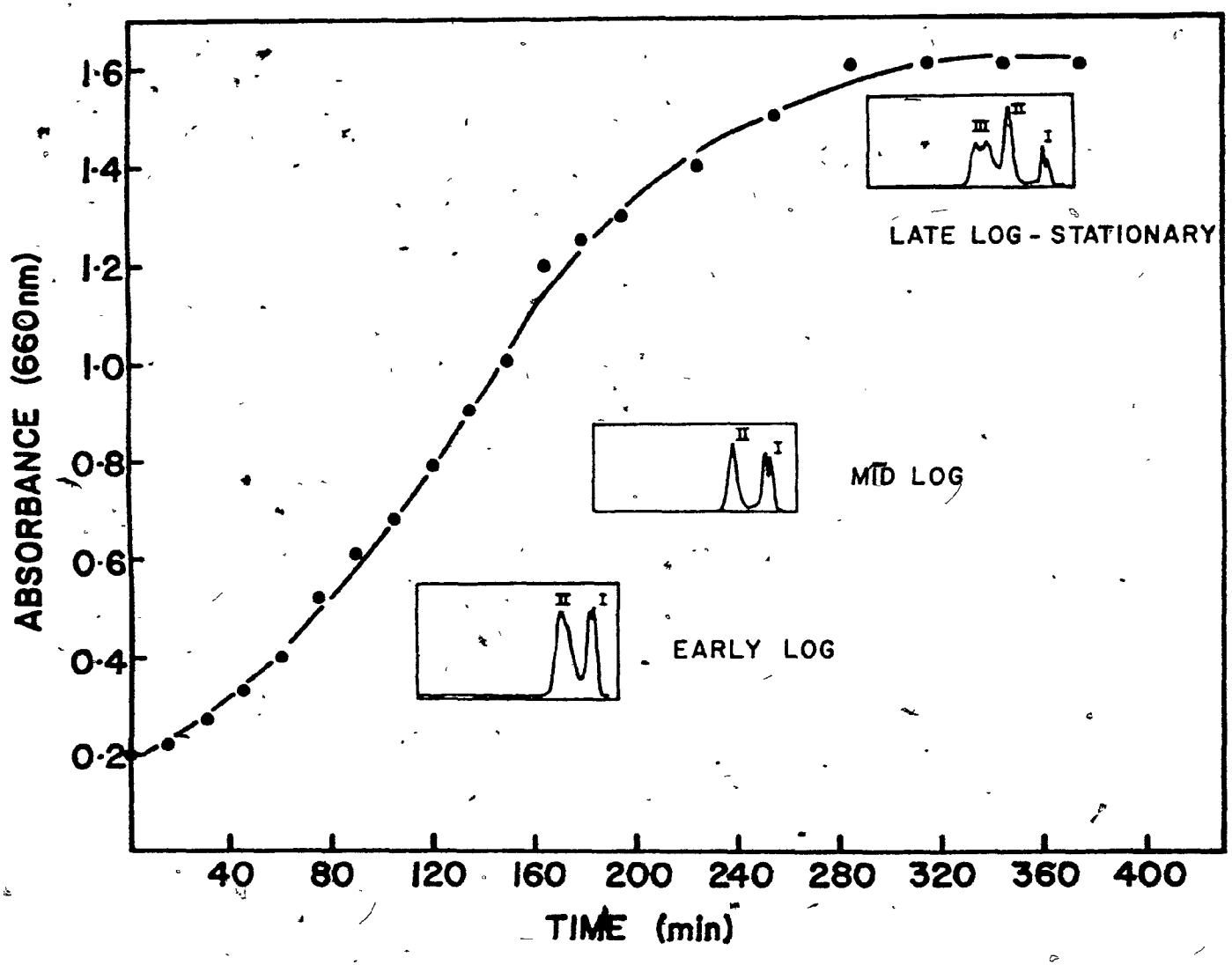


Fig. 51. Correlation between the appearance of [^{14}C]radiolabeled LPS species and growth phase of the culture.

The inserts represent the gel profiles from the experiment in figure 50.


ABSORBANCE (660nm)
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
40
80
120
160
200
240
280
320
360
400
TIME (min)
III
II
I
LATE LOG - STATIONARY
MID LOG
EARLY LOG

Pulse-chase labeling experiments were attempted on a number of occasions to determine whether one or more of the LPS species were biosynthetic precursors. In this type of experiment the cells were grown in the presence of [^{14}C]galactose until LPS I was labeled then cold galactose was added to the medium to reduce the specific activity. Incubation of the cells was continued and aliquots were taken at various times. The LPS species were then separated by gel electrophoresis to detect any transfer of label from LPS I to lipopolysaccharides II and III. Complications in the technical aspects of the experiment resulted in repeated failures. An adequate method of preventing the continued incorporation of the radioisotope from the cell medium into the LPS, during the chase part of the experiment, was not found. When isotope incorporation was stopped in some experiments no transfer of label from one LPS species to another was observed. Additional experiments on the effect of metabolic inhibitors on the marine pseudomonad are necessary before this type of experiment can be performed satisfactorily.

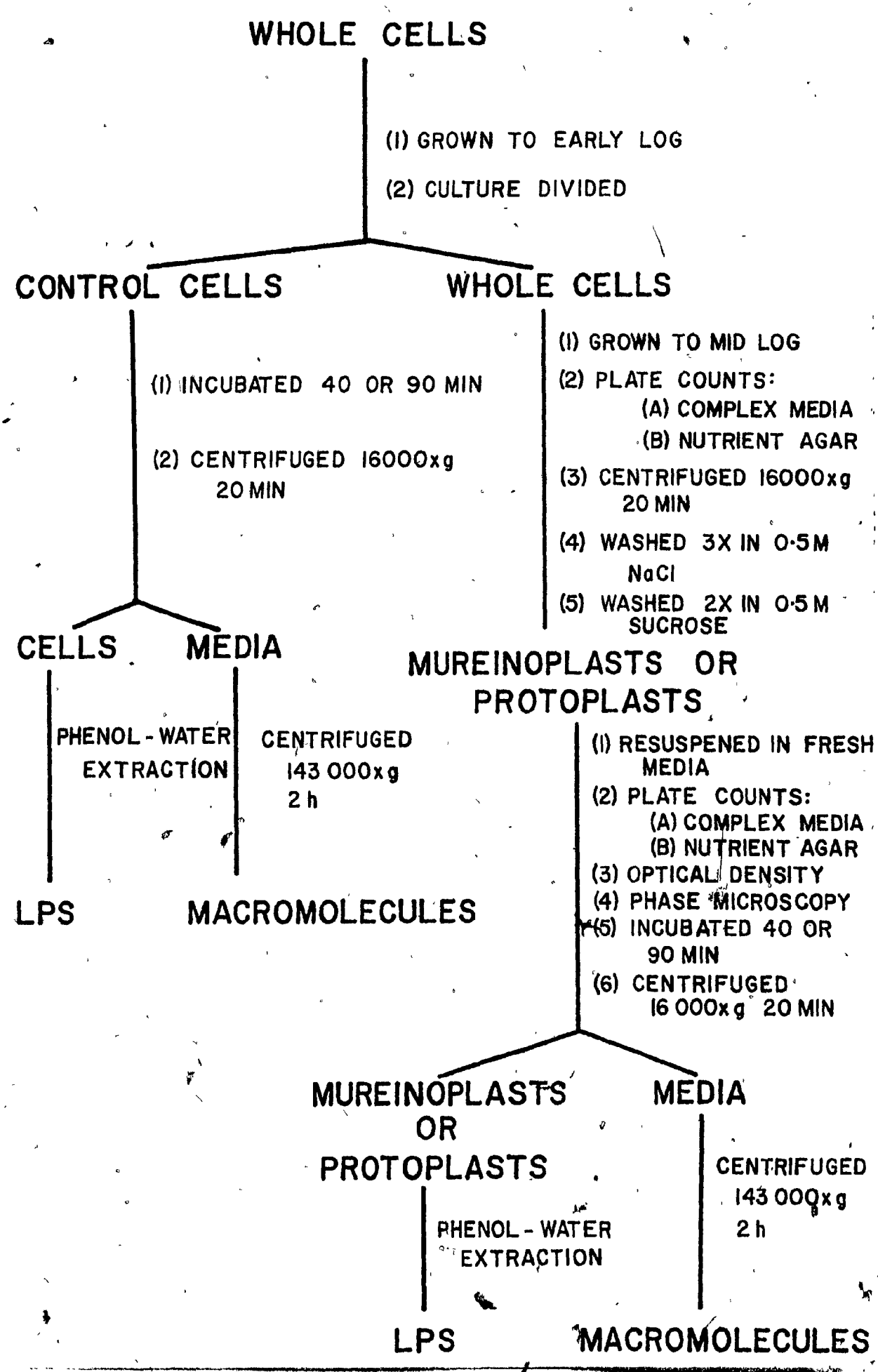
Biosynthesis in mureinoplasts and protoplasts

DeVoe *et al.* (1970) demonstrated that the mureinoplasts and protoplasts that can be formed from the marine pseudomonad were stable in a salts mixture, containing 0.3 M NaCl, 0.05 M $MgCl_2$ and 0.01 M KCl, for up to 40 min and 90 min, respectively. This afforded a method for the study of LPS biosynthesis in a system in which the outer cell

wall layers have been removed. The usefulness of this type of experiment, in which wall-bound enzymes and LPS are absent will be explained later (see Discussion). The protocol for these experiments is shown in figure 52. The bacterial culture was divided in half so that the biosynthesis of LPS by whole cells could be used as a direct comparative control. Mureinoplasts or protoplasts were formed from the remaining half of the original culture according to the procedure of DeVoe and co-workers (1970) which employed full wash volumes of 0.5 M NaCl and 0.5 M sucrose. All experimental procedures were carefully controlled. Preparations were checked for the presence of whole cells of the marine pseudomonad that remained during the formation of the mureinoplasts and protoplasts. Viable counts were made on nutrient broth-yeast extract complex medium as a measure of whole cell contamination. Plate counts were also made on nutrient agar to insure that aseptic conditions were maintained. The stability of the mureinoplasts and protoplasts was monitored by following the optical density of the preparation during the 40 or 90 min incubation period. The preparations were also examined by phase microscopy to be sure that the mureinoplasts did not prematurely lyse or form spheres. When mureinoplasts were used, their formation was checked by the occurrence of protoplasting upon the addition of lysozyme. Any LPS that was biosynthesized during the experiment was isolated from the control cells and mureinoplasts

FIGURE 52.

Fig. 52. Experimental design for the study involving the biosynthesis of LPS by mureinoplasts and protoplasts.


WHOLE CELLS
(1) GROWN TO EARLY LOG
(2) CULTURE DIVIDED
CONTROL CELLS
WHOLE CELLS
(1) INCUBATED 40 OR 90 MIN
(2) CENTRIFUGED 16000xg 20 MIN
(1) GROWN TO MID LOG
(2) PLATE COUNTS:
(A) COMPLEX MEDIA
(B) NUTRIENT AGAR
(3) CENTRIFUGED 16000xg 20 MIN
(4) WASHED 3X IN 0·5M NaCl
(5) WASHED 2X IN 0·5 M SUCROSE
CELLS
MEDIA
MUREINOPLASTS OR PROTOPLASTS
PHENOL - WATER EXTRACTION
CENTRIFUGED 143 000xg 2 h
(1) RESUSPENED IN FRESH MEDIA
(2) PLATE COUNTS:
(A) COMPLEX MEDIA
(B) NUTRIENT AGAR
(3) OPTICAL DENSITY
(4) PHASE MICROSCOPY
(5) INCUBATED 40 OR 90 MIN
(6) CENTRIFUGED 16 000xg 20 MIN
LPS
MACROMOLECULES
MUREINOPLASTS OR PROTOPLASTS
MEDIA
PHENOL - WATER EXTRACTION
CENTRIFUGED 143 000xg 2 h
LPS
MACROMOLECULES

or protoplasts by extraction with phenol-water and was removed from the media by centrifugation at 143,000 × g in the presence of Mg^{2+}. The experiment was designed so that the LPS and any other cellular products were collected during the period of logarithmic growth of the cells. If labeling studies were performed then 0.21 μCi/ml [^{14}C]-galactose (43 μCi/μmole) were introduced at the beginning of the 40 or 90 min incubation period. Table 15 demonstrates the stability of the mureinoplasts under the experimental conditions that were employed. Mureinoplasts were stable for a minimum of 40 min and occasionally as long as 55 min before lysis began. Sometimes the mureinoplasts would lyse prematurely, but these experiments were discarded. Once the mureinoplasts started to lyse, after 40 min of incubation, optical density readings could not be taken due to the appearance of white, stringy cell debris. Whole cells of the marine pseudomonad that remained in the preparation were routinely 0.1% or less of the population and, on occasion, they reached a level of up to 0.3%. Laboratory contamination of the mureinoplast preparation never occurred. All centrifuge tubes, salts solutions and glassware were autoclaved and 0.5 M sucrose solutions were filter sterilized.

The mureinoplasts were resuspended in the nutrient broth-yeast extract medium to supply an energy source and to maintain favorable conditions for metabolism. Therefore, the salts concentration of the medium had to be increased from 0.22 M NaCl and 0.026 M $MgCl_2$ to

TABLE 15.

Stability of mureinoplasts during incubation in a complete salts solution[a]

Incubation time (min)	Optical density (660 nm)	Phase contrast microscopy
0	0.85	rods; phase dark
10	0.80	
20	0.80	
30	0.80	rods; phase dark
35	0.80	
40	0.80	
45	0.80	
50	0.80	rods; phase dark
55	0.77	rods and cell debris; addition of 150 µg/ml lysozyme yielded spheres
60	--[b]	rods swell and sphericalize without the addition of lysozyme; cell debris

[a]The complete salts solution contains 0.3 M NaCl, 0.05 M $MgCl_2$ and 0.01 M KCl.

[b]As mureinoplasts lyse, white stringy material collects and renders optical density readings impossible.

0.3 M NaCl and 0.05 M $MgCl_2$ to insure the stability of the mureinoplasts. There was no difference in the growth curves of the marine pseudomonad when these salts mixtures were compared (figure 53).

The first experiments to be performed employed radioactive isotopes in order to examine whether the mureinoplasts actually incorporated label and biosynthesized cellular products. The material that was extracted from the mureinoplasts and whole cells and from their corresponding media were electrophoresed on 5% SDS-polyacrylamide gels (figure 54). Practically no LPS could be extracted from the mureinoplasts, however, significant amounts of radiolabeled products were found in the mureinoplast medium. These products were spread throughout the gel with the majority of the material remaining in the top half of the gel; an indication that the material was of a large molecular size. The material isolated from the mureinoplast medium could not be correlated with the control cell LPS or material from the control cell medium, based solely on the radiolabeled profiles, because the material spread throughout the gel.

Protoplasts were also examined for their ability to biosynthesize the LPS species in experiments conducted in the same manner as with mureinoplasts. The protoplasts did not contain any bound LPS but the medium contained radiolabeled material which had an electrophoretic mobility similar to that exhibited by the material from the mureinoplast medium (figure 55).

FIGURE 53.

Fig. 53. Comparison of the growth of the marine pseudomonad in medium containing the increased salts concentrations necessary for the stability of murinoplasts and protoplasts with growth in the presence of salts at their normal concentration.

Growth curve A was determined in nutrient broth-yeast extract complex medium containing the normal concentrations of NaCl (0.22 M) and $MgCl_2$ (0.026 M). Growth curve B was determined in the same medium in which the concentrations of NaCl and $MgCl_2$ were increased to 0.3 M and 0.05 M, respectively. The cultures were maintained at 25 C and optical density readings were made with a Bausch & Lomb Spectronic 20 spectrophotometer at 660 nm. The growth curve for each salts concentration was determined on duplicate cultures.

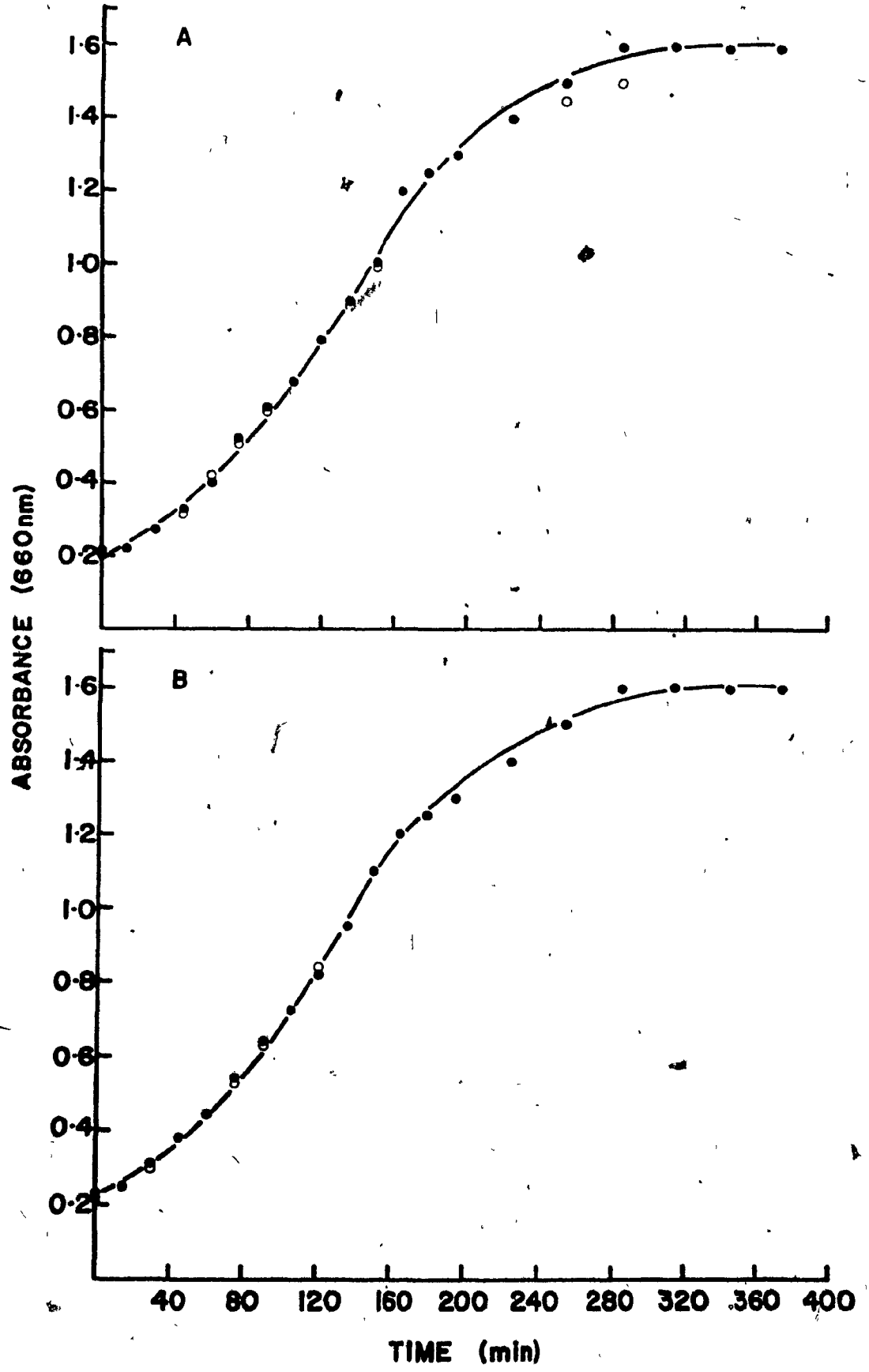

A
B
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
ABSORBANCE (660nm)
40
80
120
160
200
240
280
320
360
400
TIME (min)

FIGURE 54.

Fig. 54. Incorporation of [^{14}C]radiolabel into cellular products during incubation of mureinoplasts in nutrient broth-yeast extract complex medium.

Polyacrylamide gel electrophoresis and fractionation of the radiolabeled gels were performed as in previous experiments. Electrophoresis time was 3 h at 10 mA/gel. Profile A, LPS extracted by phenol-water from mureinoplasts; profile B, material recovered from medium after removal of mureinoplasts; profile G, material recovered from medium after removal of control cells; and profile D, phenol-water extracted LPS from control cells.

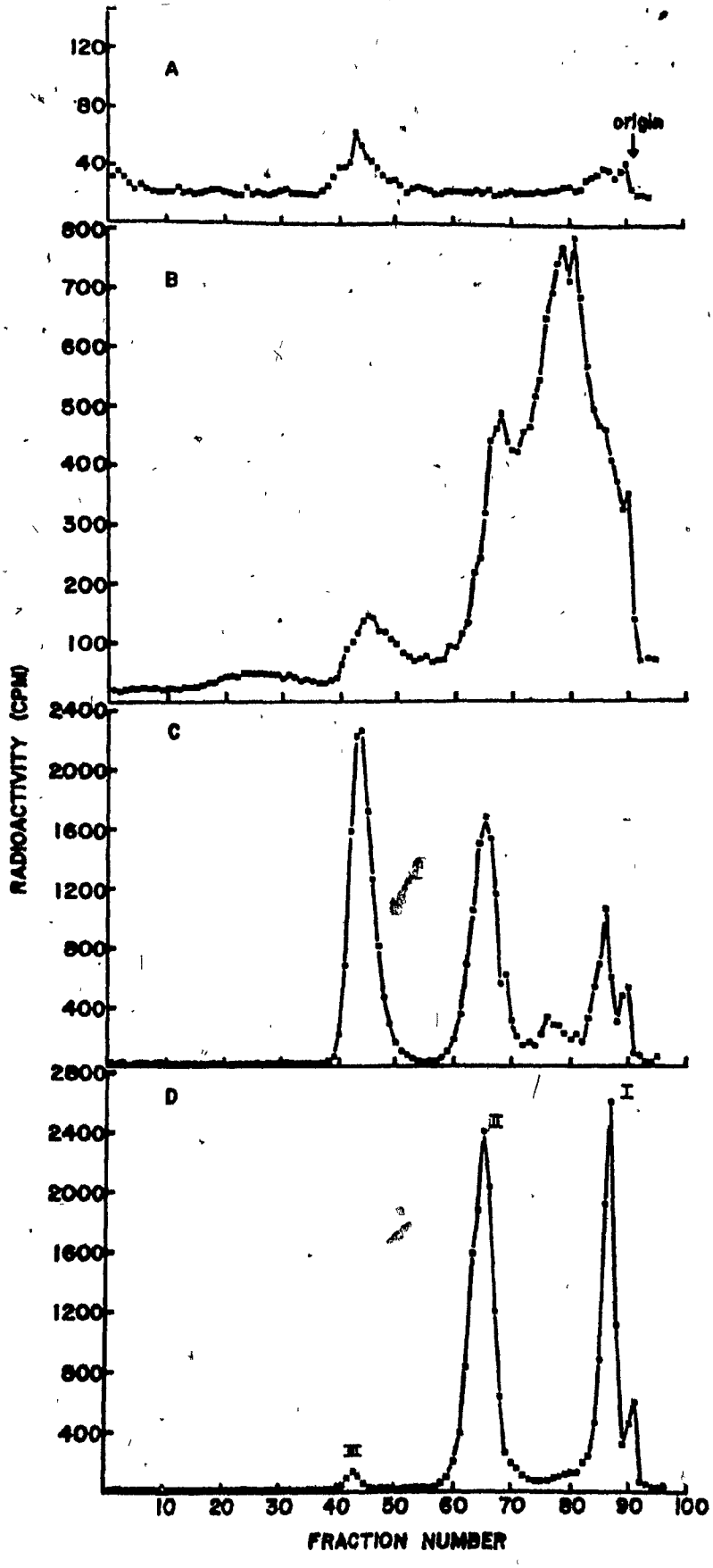
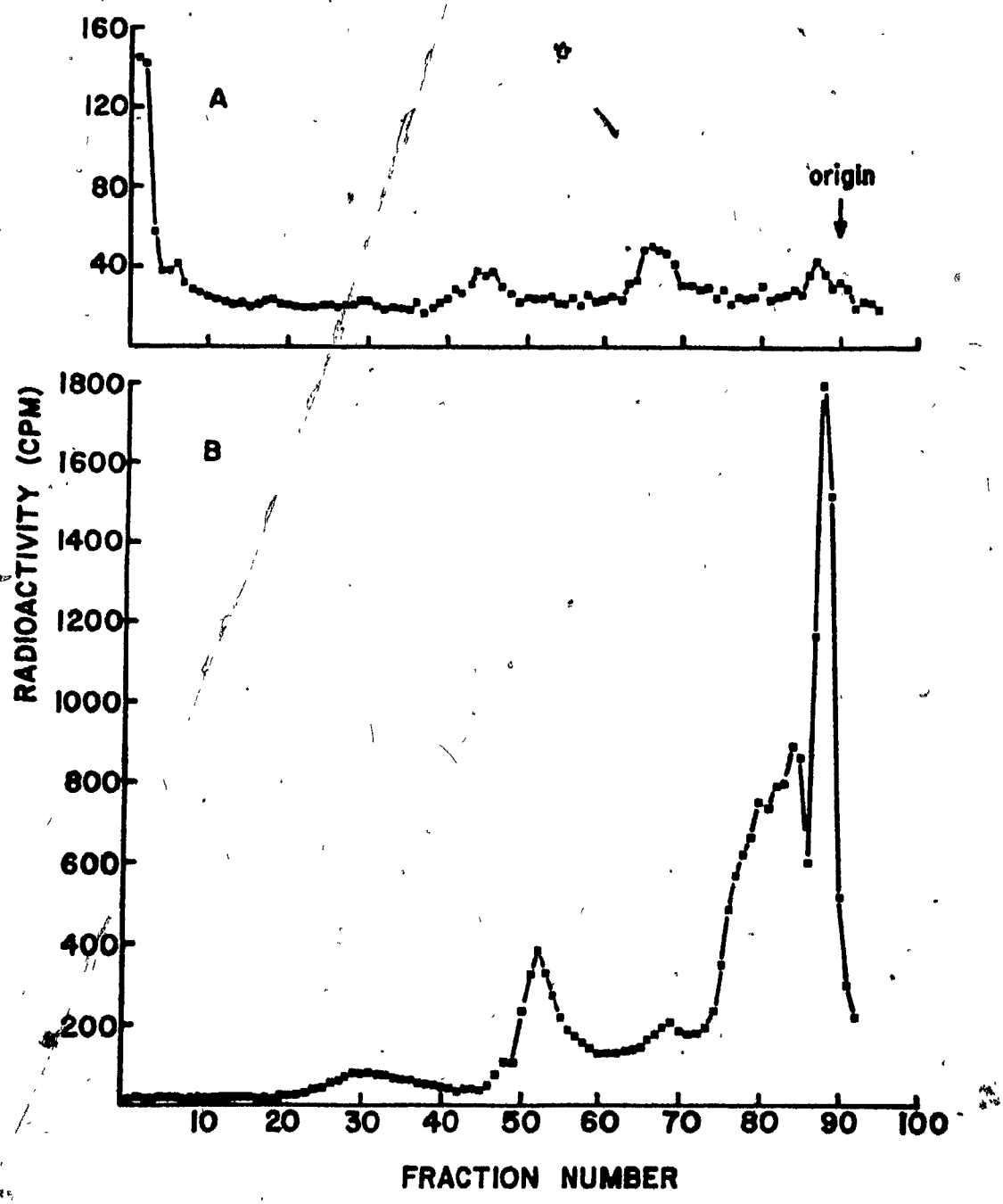

A
origin
B
C
D
I
II
III
RADIOACTIVITY (CPM)
FRACTION NUMBER

FIGURE 55.

Fig. 55. Incorporation of [^{14}C]radiolabel into cellular products during incubation of protoplasts in nutrient broth-yeast extract complex medium.

All experimental conditions were the same as those in figure 53. Profile A is LPS extracted by phenol-water from protoplasts and profile B is material recovered from the medium after removal of protoplasts.


160
120
80
40
A
origin
1800
1600
1400
1200
1000
800
600
400
200
B
RADIOACTIVITY (CPM)
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

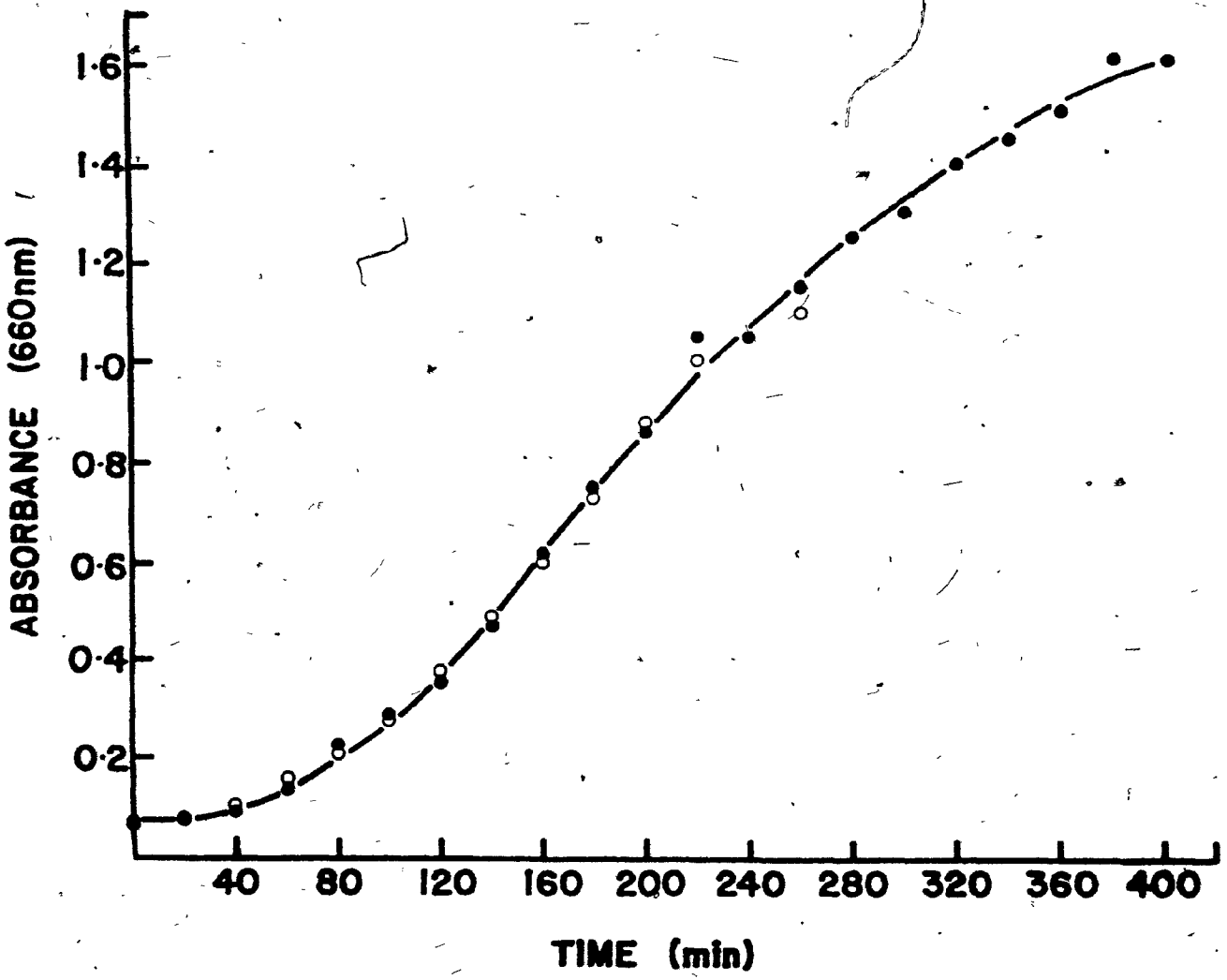
The products that were isolated from the mureinoplast and protoplast media were not subjected to phenol-water extractions because the amounts isolated were very low. It was expected that their radiolabeled gel profiles would be similar to the simple profile obtained with the material isolated from whole cell medium, and there was a possibility that these materials would be cell wall complexes composed of protein, lipid and LPS. Any type of complex would have been completely disrupted by extraction with phenol-water. Efforts were made to assay the materials from the mureinoplast and protoplast media in order to characterize them chemically rather than by SDS-polyacrylamide gel electrophoresis. Unfortunately, the nutrient broth-yeast extract contained a carbohydrate fraction which pelleted at 143,000 × g along with the labeled material. This interfered with any attempts at chemical characterization. Even though the contaminating carbohydrate would not be labeled it is not known if it could be separated from the mureinoplast and protoplast products.

Drapeau and co-workers (1966) employed a trypticase-salts medium for use in transport studies involving the marine pseudomonad. This medium was found to be carbohydrate free and, therefore, was considered for use in these experiments. The marine pseudomonad grew well in the trypticase-salts medium which had been corrected to contain the concentrations of Na^+ and Mg^{2+} necessary for the maintenance of mureinoplast stability (figure 56). A slightly longer generation

FIGURE 56.

Fig. 56. Growth of the marine pseudomonad in the trypticase-salts complex medium.

Growth curves were determined on duplicate cultures and optical density was monitored with a Bausch & Lomb Spectronic 20 spectrophotometer at 660 nm.


ABSORBANCE (660nm)
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
40
80
120
160
200
240
280
320
360
400
TIME (min)

time was observed in this medium. It has to be fully established that all of the controls operate properly and that mureinoplasts and protoplasts can be formed from cells grown in this medium. Although a great deal of work remains to be performed in order to complete this study, the initial experiments demonstrate the feasibility of using true mureinoplasts and protoplasts for Gram-negative cell wall biosynthetic studies and show that these cell forms biosynthesize macromolecular components.

D I S C U S S I O N

The question of the heterogeneity of bacterial lipopolysaccharides has become a fundamental aspect of LPS research as a result of the recent concentrated emphasis on the structure, function, biosynthesis and biological activity of this unusual macromolecule. In the study of whole cell lipopolysaccharides, if heterogeneity existed and was not considered, then the data on composition could only be interpreted as representing average values for a mixture of lipopolysaccharides. The evidence presented in this study demonstrated that the marine pseudomonad LPS (i) is a complex heterogeneous aggregate which is composed of three distinct LPS species, (ii) is qualitatively the same when extracted from batch culture and fermenter grown cells, and (iii) has a carbohydrate composition which is similar to the composition of lipopolysaccharides isolated from other *Pseudomonas* species.

O'Leary *et al.* (1972), using LPS extracted from batch cultures of the marine pseudomonad, reported its chemical composition. In the present study large amounts of LPS were needed for the isolation and characterization of the LPS species and therefore extractions were performed on fermenter grown cells. For this reason the chemical composition of the whole cell LPS was re-examined. Gas-liquid chromatography demonstrated that the lipopolysaccharides extracted from the cells grown in a fermenter or batch culture were qualitatively similar with minor quantitative differences among several monosaccharides.

The sensitivity and resolution of the gas-liquid chromatographic method allowed for the quantitation of some of the minor sugar components that were tentatively identified by O'Leary *et al.* (1972). The similarity between the lipopolysaccharides isolated from cells grown under two different conditions justified the use of fermenter cultures for extractions.

Several of the apparently unusual characteristics of the marine pseudomonad LPS were also noted in other bacterial lipopolysaccharides. For example, the rare sugar, 2-amino-2,6-dideoxyglucose was reported in *Pseudomonas stutzeri* and *P. syncyanea,* as well as in three strains of *P. aeruginosa* (Wilkinson *et al.*, 1973 and Wilkinson and Galbraith, 1975). This rare dideoxy hexosamine was found not only in *Pseudomonas* species but was also reported in *Proteus vulgaris, Salmonella* groups S and 58 and *Arizona* 1.33 (Lüderitz *et al.*, 1968); an *Achromobacter* species (Smith, 1964) and *Vibrio cholerae* strains Inaba and Ogawa (Jann and co-workers, 1973). An extremely low concentration, less than 0.1%, of 2-keto-3-deoxyoctulosonic acid (KDO) was found in the marine pseudomonad LPS in the present study and in two strains of *P. rubescens* as shown by Wilkinson and co-workers (1973). The complete absence of KDO was reported in another marine bacterium (Mongillo *et al.*, 1974). In the marine pseudomonad LPS, which was examined in the present study, the remainder of the carbohydrate components were common hexoses and hexosamines with only a single unidentified sugar which was present in

a low concentration. The only exceptional characteristic of the LPS was that its structure did not seem to be related to the colony morphology. That is, in the marine pseudomonad, cells forming rough colonies contain LPS apparently possessing an O-antigenic side chain. In the Enterobacteriaceae cells which form colonies having a rough appearance contain LPS which lacks an O-antigenic side chain while those cells that form smooth colonies contain completed LPS. There is also a spontaneous mutation from smooth to rough colony types in the Enterobacteriaceae (Lüderitz *et al.*, 1966). However, in the marine pseudomonad the rough variant 3 mutates to the smooth variant 1 (Gow *et al.*, 1973). Sodium dodecyl sulfate (SDS)-polyacrylamide gel profiles of LPS isolated from both variants 1 and 3 were identical suggesting that even though there is a transition in colony appearance the LPS remains the same structurally. Therefore, it should be noted that the relationship between colony morphology and the presence or absence of an O-antigen does not hold true for all Gram-negative bacteria.

Lipopolysaccharide heterogeneity was assayed by the technique of SDS-polyacrylamide gel electrophoresis. Previous investigators observed that LPS could be electrophoresed on SDS-polyacrylamide gels but they were concerned with the examination of cell envelope complexes rather than with the heterogeneity of purified LPS (Rothfield and Pearlman-Kothencz, 1969 and Osborn *et al.*, 1972b). Zollinger and co-workers

(1972) electrophoresed purified *N. meningitidis* LPS and failed to observe any heterogeneity. This was due to the fact that many Neisserial lipopolysaccharides do not contain O-antigenic side chains and consequently yield single carbohydrate polymers upon gel electrophoresis as was demonstrated in this study and by Johnson *et al.* (1975). The SDS-polyacrylamide gel electrophoresis system appeared to be a superior method for the detection of LPS aggregate components and afforded the sensitivity, resolution and specificity that other methods, such as gel filtration, lacked. Two methods, periodate-Schiff staining and [^{14}C]radiolabeling, were used to locate the LPS components in the gels in order to eliminate the possibility that one method might not detect all of the sample. The major concern was that the radioisotope was not dispersed throughout a major portion of the LPS. Results obtained by descending paper chromatography experiments demonstrated that the degraded polysaccharide portion of the LPS was extensively labeled when cells were grown in the presence of [^{14}C]galactose. Three labeled carbohydrate polymers were obtained upon gel electrophoresis and they exhibited co-migration with the stained LPS components. Apparently there was no selective staining or specific labeling of any of the LPS aggregate components, an important point to establish because labeled LPS was used exclusively in the pulse-labeling experiments. An additional advantage of determining the extent of the radiolabeling in the LPS was the finding that the

2-amino-2-deoxyglucose in the lipid A incorporated the radioisotope, thus permitting the localization of this portion of the LPS molecule alone on SDS-gels.

Lipopolysaccharide heterogeneity, as determined by SDS-polyacrylamide gel electrophoresis, was not limited to the marine pseudomonad but was also apparent in *Escherichia coli* O111:B4 and *Salmonella typhimurium* LT2. These two bacteria were employed extensively in LPS and Gram-negative cell wall studies (see review by Osborn, 1969). The finding that *E. coli* and *S. typhimurium* lipopolysaccharides were heterogeneous emphasized that LPS heterogeneity was a prevalent phenomenon that should not be ignored in the study of the Gram-negative cell envelope.

A close examination of the electrophoresis buffer established which factors contributed to the electrophoretic mobility of the carbohydrate polymers and what intrinsic differences resulted in their separation. The experimental evidence suggested that an SDS-lipid A interaction was responsible for LPS migration with secondary effects due to the action of molybdate. These findings were supported by the fact that (i) almost complete migration was achieved in an electrophoresis buffer containing SDS but not molybdate; (ii) no ordered migration pattern was obtained when Triton X-100 replaced SDS in the electrophoresis buffer; (iii) purified lipid A exhibited mobility in the SDS electrophoresis buffer whereas only 65% of the degraded

polysaccharide entered the gel remaining instead near the gel surface and (iv) dextran polymers had a migration pattern similar to degraded polysaccharide. The premise that an SDS-lipid A complex contributed to the electrophoretic mobility of LPS was also proposed by Jann *et al.* (1975), however, the report did not supply any convincing evidence to support the proposition.

The carbohydrate composition of each of the LPS components isolated by SDS-polyacrylamide gel electrophoresis were determined by gas-liquid chromatography. The monosaccharide composition of LPS III was remarkably similar to that of the core region of the LPS molecule in that the sugars heptose, galactose and 2-amino-2-deoxygalactose were present in a 1:1:1 molar ratio. This would make the simplest core structure a trisaccharide. Lipopolysaccharide II contained all of the core sugars, however, glucose, galactose and 2-amino-2-deoxygalactose were in much greater concentrations than the other monosaccharides which suggested that this species had a side chain composed of a repeating trisaccharide unit consisting of these monosaccharides. Similarly, LPS I contained 2-amino-2,6-dideoxyglucose, 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose and galactose in high proportions suggesting a tetrasaccharide repeating unit. In addition it was concluded from the molar ratios that LPS I and II contained core regions and that these had the same composition as the core region in LPS III. However, the core regions in the three species may not necessarily have the same

structure since it has been shown that heterogeneous core oligosaccharides exist in a rough strain of *E. coli* (Fuller *et al.*, 1973). These core oligosaccharides differed from one another in both the number and type of monosaccharides that were present in each. The possibility that the LPS species, isolated from the marine pseudomonad, contained different core structures could explain why in LPS I the sugars 2-amino-2,6-dideoxyglucose, 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose and galactose had a molar ratio of 12:18:15:17 and in LPS II, glucose, galactose and 2-amino-2-deoxygalactose had a molar ratio of 13:12:16 (see Table 11). If, as was proposed, the tetrasaccharide and trisaccharide represent the side chains in LPS I and II, respectively, then they should each contain sugars in an equal (1:1:1:1) molar ratio. The extra monosaccharides could be part of the core region in each species. The actual number of repeating side chain units in LPS I and II is not known but would appear to be at least 11 or 12 in both species.

An important question in establishing the relationship between the LPS species was whether or not they all contained lipid A. The presence of lipid A in each species was established using the SDS-polyacrylamide gel technique, in the case of LPS I and LI, and the carbocyanin dye procedure for all three. The demonstration of the presence of lipid A as well as heptose in LPS I and II complimented the data that was obtained by gas-liquid chromatography and showed

that these compounds were complete LPS molecules and did not represent free side chain oligosaccharides. The term "complete" refers to an LPS molecule that is composed of lipid A, core and side chain. The difference in the relative amount of lipid A that was detectable in these two LPS species was probably a reflection of the greater amounts of side chain oligosaccharide in LPS I and II. The existence of several different types of complete LPS in the same bacterial strain has not been reported in the literature. It was reported that a strain of *Aerobacter aerogenes* contains two forms of LPS, one of which consists of lipid A, core and side chain and the other contains only lipid A and core (Koeltzow and Conrad, 1971). In other studies, it was suggested that several different side chain-containing lipopolysaccharides, when present in the same cell represent various degrees of polymerization of the LPS molecule (Jann *et al.*, 1975). In the above investigation, which used a *Citrobacter* strain as a model, it was assumed that the LPS bands visualized on SDS-polyacrylamide gels were identical to the carbohydrate peaks obtained on Sephadex G-50 columns. Carbohydrate analysis was performed only on the material collected from the Sephadex column. In one case, the LPS was electrophoresed without prior treatment while the gel filtration studies used degraded polysaccharide that was isolated from hydrolyzed LPS. The gel bands were equated with the column peaks simply on the basis of position and number. In the present study the marine pseudo-

monad whole cell LPS was not subjected to a hydrolysis or any harsh chemical treatments prior to the separation of its aggregated components. Subsequently, chemical analyses were performed directly on the material that was separated on the SDS-polyacrylamide gels.

It was hoped that the double immunodiffusion experiments would demonstrate LPS heterogeneity in the marine pseudomonad, however, the results were not what was expected and they did not add information regarding the chemical differences between the LPS species. Lipopolysaccharides I and II exhibited a homologous cross reaction even though their antigenic side chains appear to be somewhat different. Both species however, contained galactose and 2-amino-2-deoxygalactose in their side chains and if these sugars were the determinant groups on the antigen, due to the presence of the same terminal linkages, they could be responsible for the identical serological responses and block the reaction of the remaining side chain sugars. O-antigen structural groups which are similar can be responsible for cross-reactivity and yet may be only a part of the whole antigen structure (Lüderitz *et al.*, 1966). Therefore, even though the side chains differ in one or two monosaccharides, they could belong to the same serogroup. There was no precipitin reaction between LPS III and the whole cell antiserum which can be explained by the possibility that in whole cells the extensive O-antigens on LPS I and II obscure the LPS III core and prevent it from being available during the formation of

antibody, as long as the cell envelope is not degraded. The O-antigens probably extend to the cell surface while the LPS core does not. *Escherichia coli* and *Salmonella typhimurium* lipopolysaccharides which are composed of only lipid A and core are known antigens (Lüderitz *et al.*, 1966). Therefore, LPS III which is lipid A-core may also be an antigen but is blocked in the cell envelope of the marine pseudomonad. The isolated lipid A, core and core-side chain fractions of the LPS molecule did not react with the whole cell antiserum. The core-side chain fraction would be expected to give a positive reaction with whole cell antiserum because it contains the O-antigenic side chains found in LPS I and II. Since the core-side chain was isolated by subjecting the whole cell LPS to a mild acid hydrolysis it is conceivable that this fraction could have been partially degraded and consequently not be recognized by the antibody.

Identical serological reactions were obtained with two different antisera which were prepared at two different times, the results of which supply strong verification of the data in the face of the notorious variability that is known to occur in immunological systems. Previously, two precipitin lines were obtained when the marine pseudomonad LPS and a whole cell antiserum were reacted during double immunodiffusion experiments (Nelson and MacLeod, submitted for publication). When Nelson's antiserum was reacted against the whole cell LPS, isolated and purified in this study, a single precipitin line was observed.

Apparently, there was an additional component in Nelson's LPS preparation which reacted with the antiserum. This component did not correspond to any of the LPS species detected in this study.

Attempts to differentiate the LPS species morphologically were not successful, but evidence was presented which established that the marine pseudomonad LPS (i) formed typical ribbon-like structures in negative stained preparations; (ii) was dissociated by SDS, that is, the ribbon-like structures were disrupted to shorter fragments which then rearranged into stacked sheets (in Na_2HPO_4 - Na_2MoO_4 buffer) or vesicles (in distilled water), and (iii) was more stable in a neutral pH environment. The morphology of the whole cell LPS was similar to that of *S. typhimurium* LPS as reported by Shands and co-workers (1967). It was difficult to detect the trilaminar appearance in the ribbon-like forms of the marine pseudomonad LPS which could be due to the use of phosphotungstic acid instead of uranyl acetate for staining. The loss of the structural integrity of the whole cell LPS which occurred when the LPS was stained in a non-buffered, ion-free environment has not been observed elsewhere. In a buffer at a neutral pH, SDS appeared to completely dissociate the LPS yet produced only partial disruption when the LPS was present in distilled water. The trilaminar structures were apparent in buffered SDS preparations with reaggregation occurring, in some instances, in the form of stacked sheets (figure 38C). The results would indicate that this reaggregation phenomenon

was probably caused by a combination of effects due to pH and the presence of free molybdate anions which interact with the SDS-LPS complexes. The morphology of isolated LPS I appeared to be very similar to that of whole cell LPS in an aqueous solution of SDS, but little information was obtainable from the negatively stained preparations. Lipopolysaccharide II stained as small rods and doughnut-shaped forms which looked like the SDS dissociated LPS from *S. typhimurium* (Hannecart-Pokorni *et al.*, 1973). A good negative stained preparation of LPS III was not obtained. It appeared, upon an examination of the negative stains,that LPS treated with buffered SDS was further dissociated during electrophoresis. The stacked sheet structures that were observed in the electron micrograph of whole cell LPS treated with buffered SDS were not present in the LPS II preparation which was separated and isolated by SDS-polyacrylamide gel electrophoresis. Instead, rod-shaped and doughnut-shaped forms were found in the negatively stained sample of LPS II. These forms were considerably smaller than the stacked sheet structures.

Forsberg *et al.* (1970a) established that the cell envelope of the marine pseudomonad could be separated into layers which represented the periplasmic region, the outer tripartite membrane and a material exterior to the outer tripartite layer. This ability to isolate the outer cell wall layers made it possible to determine the distribution of LPS in the various layers of the Gram-negative cell envelope. The

presence of LPS I and II in each of the cell wall layers would be expected considering the fact that LPS is biosynthesized at the cytoplasmic membrane. In the marine pseudomonad it appears that the LPS is translocated to the outer layers through the periplasmic region. This seems reasonable considering that a large percentage of the periplasmic layer is LPS I and II; relative to the outer double-track layer (see Table 14). If LPS III is a degradation product of the other LPS species, as the experimental evidence would seem to indicate, then it should also be found in all of the wall layers. More information concerning the structure of the Gram-negative cell envelope is necessary to determine how these different forms of LPS contribute to the overall configuration.

An interesting observation that stems from these results was the large concentration of LPS found in the periplasmic region of the marine pseudomonad, a finding which is seldom reported in other Gram-negative cells. Shands (1966), however, did show the presence of LPS in the periplasmic region in an electron microscopic study using ferritin-labeled antibody. It was proposed (R.A. MacLeod, personal communication) that this periplasmic LPS is normally discarded with the supernatant fluid during the formation of spheroplasts. This would explain why LPS has not been localized in the periplasmic region by chemical means. The presence of LPS in the periplasm would tend to refute the mechanism of LPS translocation from the cytoplasmic

membrane to the outer double-track by means of discrete attachment points between these two membranes as was proposed by Mühlradt and co-workers (1973). Ferritin-labeled antibody was used by these workers in order to detect the appearance of newly formed LPS on the bacterial surface. They concluded from the results obtained that the LPS was found in patches on the cell surface only in areas where the cytoplasmic and outer membranes were in close proximity. It does not seem that the resolution of this electron microscopic technique was fine enough to permit this conclusion.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments indicated, in the present study, that whole cells of the marine pseudomonad release LPS into the medium during growth of the cells. Radiolabeled gel profiles of the material isolated from the medium correspond to the three radiolabeled peaks that are obtained with LPS extracted from whole cells. It is not unusual to find LPS in the medium and many reports have been published (Crutchley *et al.*, 1967; Lindsay *et al.*, 1973 and Johnson and co-workers, 1975). Normally, low levels are found unless the LPS release is induced under artificial conditions such as lysine starvation (Bishop and Work, 1965) or treatment of the cells with chloramphenicol (Rothfield and Pearlman-Kothencz, 1969). In all cases reported, medium LPS has been shown to be identical to the cellular type and appears to represent old cell wall that is cast off during the normal turnover process (Knox *et al.*, 1966 and Taylor and co-workers, 1966).

The detection of the LPS species in the cell wall layers by the method of SDS-polyacrylamide gel electrophoresis led to several other important conclusions. First, since the wall layers were solubilized and electrophoresed without prior LPS extraction the results established that the whole cell LPS was not degraded by the phenol-water extraction and subsequent purification procedures. Second, the high concentration of protein in the wall layers did not affect the migration of the LPS which established that the small amounts of contaminating protein or peptide, found in whole cell LPS preparations, were not responsible for the electrophoretic mobility of the LPS.

One of the characteristics of the SDS-polyacrylamide gel electrophoresis system that allows its use for the determination of molecular weights is the fact that the SDS masks all native charges on the sample thus making everything negatively charged. This means that samples should separate on the basis of molecular size and not charge differences (Shapiro *et al.*, 1967). In the case of LPS, it was found that the amount of lipid A also plays a role in the mobility of the LPS apparently due to the binding of SDS to the lipid A. This observation and the non-mobility of dextran molecular weight standards made it impossible to use the gel electrophoresis system for the determination of the molecular weights of the LPS species. Sepharose gel filtration had been used previously for the analysis of detergent treated LPS (Rubio *et al.*, 1973) and the availability of carbohydrate standards

made this an appealing method for the estimation of the molecular sizes of the LPS species. The molecular weight values of 1.8×10^6 daltons for LPS I and 3.0×10^5 daltons for a mixture of LPS II and III appeared to be exceedingly large for dissociated LPS units when whole cell LPS aggregates were shown to have molecular weights in the range of 1 to 24×10^6 daltons (Davies and co-workers,1954). In the present study the extent of SDS binding to the LPS was not known but this should contribute little to the overall molecular weight. The reason why LPS II and III were not separable by gel filtration was not determined. Perhaps their separation by polyacrylamide gel electrophoresis reflected the relative proportion of lipid A in the species rather than their molecular size. An additional problem that arose during the gel filtration experiments, besides the one of not being able to separate LPS II and III, was the difficulty of completely removing SDS from the material eluted from the columns. This problem was overcome by precipitating the SDS with barium chloride. The actual mechanism of the reaction is not known, however, the most feasible lattice arrangement is one in which the barium ions are shared between the sulfate groups on neighboring SDS molecules. This afforded an easy and efficient method for the removal of SDS from non-sulfate containing compounds. Other procedures such as dialysis, chloroform extraction, or ion exchange chromatography (Lenard, 1971) either did not remove all of the detergent or were extremely tedious.

The biosynthesis of the LPS species remains as the next major effort in the study of LPS heterogeneity. Osborn and co-workers (1972a) elucidated the steps involved in the biosynthesis of *S. typhimurium* LPS without considering the existence of several different molecular forms in the same cell. The SDS-polyacrylamide gel electrophoresis system used in the present investigation supplies an assay system for use in the study of the biosynthesis of various LPS species in the cell. The resolution of the sucrose gradient system used by Osborn was probably not good enough to obtain the separation of various LPS forms. Although the work presented in this study merely initiated an examination of the problem, it is hoped that the methods that were developed will be refined to permit the continuation of this investigation in the future. The rapid appearance of LPS I, within two minutes of the addition of label, followed by the presence of LPS II within another three minutes indicated that the process that was being monitored was indeed biosynthesis rather than degradation. It was unusual that LPS I, which had the higher molecular weight, should be synthesized first. However, this observation, coupled with the following facts, lent strong support to the possibility that LPS I and II were biosynthesized independently and that LPS II was not a precursor of LPS I: (i) The composition of the side chains in LPS I and II appear to be different. (ii) LPS I seems to have a side chain which contains repeating tetrasaccharide units while LPS II has a side chain composed of repeating

trisaccharide units. Lipopolysaccharide III, on the other hand, was not detected in the labeling experiments until late in the logarithmic phase of growth. It appears, based on this information which was obtained from the pulse-labeling experiments, that LPS III may be a degradation product of LPS I, II or both. Degradation could be a result of normal cell wall turnover processes due to the action of an autolytic enzyme. Environmental conditions during the late stages of the growth cycle of the culture may favor an increase in the activity of an autolytic enzyme. This may explain why LPS III does not appear until late in the logarithmic phase of growth. Even though LPS degradation enzymes have not been found in Gram-negative cell walls, one has been discovered in a coliphage (Reske *et al.*, 1973). Not enough information is yet available to confirm this possibility and a great deal of work remains to be completed before the LPS species biosynthetic scheme is clear. The present studies should provide the necessary approach and do demonstrate that the LPS species can be studied *in vivo*.

The mureinoplast and protoplast experiments were developed to attempt to answer many of the questions that the previously described labeling study could not. In addition, the mureinoplast and protoplast study can be used to investigate many of the problems that have been encountered in the study of the biosynthesis and structure of the Gram-negative cell wall.

(i) The method of LPS translocation from its site of synthesis at the cytoplasmic membrane to its position in the outer cell wall is not known. If an LPS carrier molecule is involved or if LPS is translocated as a cell wall subunit in a complex with protein or lipid it could be easily isolated from the mureinoplast medium. This material is found only in the medium; that is, it is not found in mureinoplasts which suggests that the carrier may not be able to release the LPS if no cell wall acceptor site is available or if there is no attachment site for an LPS-protein wall subunit. The native complex would simply be translocated into the medium following biosynthesis.

(ii) Osborn *et al.* (1972a) indicated that some LPS biosynthetic enzymes may be cell wall bound. If mureinoplasts and protoplasts manufacture LPS then all LPS biosynthetic enzymes must be membrane associated.

(iii) As was discussed earlier, Mühlradt and co-workers (1973) proposed that newly formed LPS was transported via discrete attachment sites between outer and cytoplasmic membranes. They also suggested that the LPS was distributed on the cell surface by lateral diffusion from these attachment sites (Mühlradt *et al.*, 1974). Both of the above investigations relied upon electron microscopic studies using ferritin-labeled antibody. Mureinoplasts could

be used, under favorable conditions, to establish whether or not the outer membrane functions as an anchor point for newly synthesized LPS.

(iv) It has never been established whether or not autolytic enzymes exist in the Gram-negative outer cell wall. If they exist they may play a role in cell wall or LPS turnover. If the LPS species are biosynthesized by mureinoplasts and protoplasts then LPS degradative enzymes cannot be present in the marine pseudomonad cell wall, provided that LPS III is a product of LPS I or II. Lipopolysaccharide heterogeneity then could not be the result of digestive enzymatic processes.

Hopefully, this investigation has emphasized that the structure of LPS is extremely complex and that this should be considered in any study on the composition, structure or biosynthesis of LPS. The reason why some bacteria contain several structurally different forms or species of LPS remains unknown. It has been postulated that one function of LPS is to create and maintain favorable environments for the protection and operation of cell wall associated enzymes (Costerton *et al.*, 1974). Perhaps structurally distinct lipopolysaccharides are associated with specific enzymes and, in this manner, various regions of specific hydrogen ion concentration and degree of hydrophobicity are maintained in the same cell wall.

BIBLIOGRAPHY

Bayer, M.E.
1968a Adsorption of bacteriophages to adhesions between wall and membrane of *Escherichia coli*. J. Virol. 2:346-356.

Bayer, M.E.
1968b Areas of adhesion between wall and membrane of *Escherichia coli*. J. Gen. Microbiol. 53:395-404.

Bishop, D.G. and E. Work.
1965 An extracellular glycolipid produced by *Escherichia coli* grown under lysine-limiting conditions. Biochem. J. 96:567-576.

Bladen, H.A., and S.E. Mergenhagen.
1964 Ultrastructure of *Veillonella* and morphological correlation of an outer membrane with particles associated with endotoxic activity. J. Bacteriol. 88:1482-1492.

Blix, G.
1948 The determination of hexosamine according to Elson and Morgan. Acta Chem. Scand. 2:467.

Boivin, A. and L. Meserobeanu.
1935 Researches sur les antigènes somatiques et sur les endotoxines des bactéries. I. Considérations générales et exposé des techniques utilisées. Rev. Immunol. 1:553-569

Bray, D. and P.W. Robbins.
1967 The direction of chain growth in *Salmonella anatum* O-antigen biosynthesis. Biochem. Biophys. Res. Commun. 28:334-339.

Burton, A.J. and H.E. Carter.
1964 Purification and characterization of the lipid A component of the lipopolysaccharides from *Escherichia coli*. Biochemistry 3:411-418.

Chen, P.S. Jr., T.Y. Toribara, and H. Warner.
1956 Microdetermination of phosphorous.
Anal. Chem. 28:1756-1758.

Chester, I.R., G.W. Gray, and S.G. Wilkinson.
1972 Further studies of the chemical composition of the lipopolysaccharide of *Pseudomonas aeruginosa*.
Biochem. J. 126:395-407.

Chester, I.R. and P.M. Meadow.
1973 The relationship between the O-antigenic lipopolysaccharides and serological specificity in strains of *Pseudomonas aeruginosa* of different O-serotypes.
J. Gen. Microbiol. 78:305-318.

Clarke, K., G.W. Gray, and D.A. Reaveley.
1967 The extraction of cell walls of *Pseudomonas aeruginosa* with aqueous phenol. The insoluble residue and material from the aqueous layers.
Biochem. J. 105:759-765.

Costerton, J.W., J.M. Ingram and K.-J. Cheng.
1974 Structure and function of the cell envelope of Gram-negative bacteria.
Bacteriol. Rev. 38:87-110.

Crowell, E.P. and B.B. Burnett.
1967 Determination of the carbohydrate composition of wood pulps by gas chromatography of the alditol acetates.
Anal. Chem. 39:121-124.

Crutchley, M.J., D.G. Marsh and J. Cameron.
1967 The endotoxin.
Nature 214:1052.

Davies, D.A., W.T.J. Morgan, and W. Mosimann..
1954 Studies in immunochemistry. 13. Preparation and properties of the O somatic antigen of *Shigella dysenteriae* (shiga).
Biochem. J. 56:572-581.

Day, D.F.
1973 Alkaline phosphatase and the cell envelope of *Pseudomonas aeruginosa*.
Ph.D. Thesis, McGill University, Montreal, Canada.

Day, D.F. and J.M. Ingram.
1975 *In vitro* studies of an alkaline phosphatase-cell wall complex from *Pseudomonas aeruginosa*.
Can. J. Microbiol. 21:9-16.

Deneke, C.F. and R.R. Colwell.
1973 Lipopolysaccharide and proteins of the cell envelope of *Vibrio marinus*, a marine bacterium.
Can. J. Microbiol. 19:1211-1217.

DeVoe, I.W., J. Thompson, J.W. Costerton, and R.A. MacLeod.
1970 Stability and comparative transport capacity of cells, mureinoplasts, and true protoplasts of a Gram-negative bacterium.
J. Bacteriol. 101:1014-1026.

DiRienzo, J.M., C.F. Deneke, and R.A. MacLeod.
1974 Fractionation of lipopolysaccharide by SDS-polyacrylamide gel electrophoresis.
Abstr. Am. Soc. Microbiol. 74:181.

DiRienzo, J.M. and R.A. MacLeod.
1976 Three forms of lipopolysaccharide in a marine bacterium.
Abstr. Am. Soc. Microbiol. 76:151.

Drapeau, G.R., T.I. Matula, and R.A. MacLeod.
1966 Nutrition and metabolism of marine bacteria. XV. Relation of Na^+-activated transport to the Na^+ requirement of a marine pseudomonad for growth.
J. Bacteriol. 92:63-71.

Dröge, W., V. Lehmann, O. Lüderitz, and O. Westphal.
1970 Structural investigations on the 2-keto-3-deoxyoctonate region of lipopolysaccharides.
Eur. J. Biochem. 14:175-184.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith.
1956 Colorimetric method for determination of sugars and related substances.
Anal. Chem. 28:350-356.

Edstrom, R.D. and E.C. Heath.
1964 Sugar nucleotide transferases in *Escherichia coli* lipopolysaccharide biosynthesis.
Biochem. Biophys. Res. Commun. 16:576-581.

Eidels, L. and M.J. Osborn.
1974 Phosphoheptose isomerase, first enzyme in the biosynthesis of aldoheptose in *Salmonella typhimurium*. J. Biol. Chem. 249:5642-5648.

Fairbairn, N.J.
1953 A modified anthrone reagent. Chem. Ind. (Lond.) 53:86.

Fensom, A.H. and G.W. Gray.
1969 The chemical composition of the lipopolysaccharide of *Pseudomonas aeruginosa*. Biochem. J. 114:185-196.

Forsberg, C.W., J.W. Costerton, and R.A. MacLeod.
1970a Separation and localization of cell wall layers of a Gram-negative bacterium. J. Bacteriol. 104:1338-1353.

Forsberg, C.W., J.W. Costerton and R.A. MacLeod.
1970b Quantitation, chemical characteristics, and ultrastructure of the three outer cell wall layers of a Gram-negative bacterium. J. Bacteriol. 104:1354-1368.

Foster, A.B.
1957 Zone electrophoresis of carbohydrates, p. 81-115. *In* M.L. Wolfrom (ed.), Adv. Carbohydr. Chem., Vol. 12, Academic Press, New York.

Frasch, C.E., R.M. McNelis, and E.C. Gotschlich.
1976 Strain-specific variation in the protein and lipopolysaccharide composition of the group B meningococcal outer membrane. J. Bacteriol. 127:973-981.

Fukasawa, T. and H. Nikaido.
1961 Galactose-sensitive mutants of *Salmonella*. II. Bacteriolysis induced by galactose. Biochim. Biophys. Acta 48:470-483.

Fuller, N.A., M-C. Wu, R.G. Wilkinson, and E.C. Heath.
1973 The biosynthesis of cell wall lipopolysaccharide in *Escherichia coli*. VII. Characterization of heterogeneous "core" oligosaccharide structures. J. Biol. Chem. 248:7938-7950.

Galanos, C., O. Lüderitz, and O. Westphal.
1969 A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. 9:245-249.

Gmeiner, J., O. Lüderitz, and O. Westphal.
1969 Biochemical studies on lipopolysaccharides of *Salmonella* R mutants. 6. Investigations on the structure of the lipid A component. Eur. J. Biochem. 7:370-379.

Gow, J.A., I.W. DeVoe, and R.A. MacLeod.
1973 Dissociation in a marine pseudomonad. Can. J. Microbiol. 19:695-701.

Hannecart-Pokorni, E., D. Dekegel, and F. DePuydt.
1973 Macromolecular structure of lipopolysaccharides from Gram-negative bacteria. Eur. J. Biochem. 38:6-13.

Hannecart-Pokorni, E., D. Dekegel, F. DePuydt, and J. Dirkx.
1970 Study of the structure of *Shigella flexneri* O-antigen. I. Chemical degradation. Biochem. Biophys. Acta 201:155-166.

Humphreys, G.O., I.C. Hancock, and P.M. Meadow.
1972 Synthesis of the hydroxyacids in lipid A of *Pseudomonas aeruginosa*. J. Gen. Microbiol. 71:221-230.

Inouye, M. and J.P. Guthrie.
1969 A mutation which changes a membrane protein of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. 64:957-961.

Janda, J. and E. Work.
1971 A colorimetric estimation of lipopolysaccharides. FEBS Lett. 16:343-345.

Jann, B., K. Jann, and G.O. Beyaert.
1973 2-amino-2,6-dideoxy-D-glucose(D-quinovosamine): a constituent of the lipopolysaccharides of *Vibrio cholerae*. Eur. J. Biochem. 37:531-534.

Jann, B., K. Reske, and K. Jann.
1975 Heterogeneity of lipopolysaccharides. Analysis of polysaccharide chain lengths by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Eur. J. Biochem. 60:239-246.

Johnson, K.G., M.B. Perry, I.J. McDonald, and R.R.B. Russell.
1975 Cellular and free lipopolysaccharides of some species of *Neisseria*.
Can. J. Microbiol. 21:1969-1980.

Jones, R.A.
1970 Interpretation of the chromatogram. A. Quantitative Analysis, p. 77-91. *In* An Introduction to gas-liquid chromatography, first edition, Academic Press, New York.

Kabat, E.A. and M.M. Mayer.
1961 Preparation of antigens for immunization and injection schedules, p. 871-872. *In* Experimental immunology, second edition, Charles C. Thomas, Springfield, Ill.

Knox, K.W., J. Cullen, and E. Work.
1967 An extracellular lipopolysaccharide-phospholipid-protein complex produced by *Escherichia coli* grown under lysine-limiting conditions.
Biochem. J. 103:192-201.

Knox, K.W., M. Vesk, and E. Work.
1966 Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *Escherichia coli*.
J. Bacteriol. 92:1206-1217.

Koeltzow, D.E. and H.E. Conrad.
1971 Structural heterogeneity in the lipopolysaccharide of *Aerobacter aerogenes* NCTC 243.
Biochemistry 10:214-224.

Kondo, E. and N. Ueta.
1972 Composition of fatty acids and carbohydrates in *Leptospira*.
J. Bacteriol. 110:459-467.

Kulpa, C.F.,Jr. and L. Leive.
1976 Mode of insertion of lipopolysaccharide into the outer membrane of *Escherichia coli*.
J. Bacteriol. 126:467-477.

Leive, L., V.K. Shovlin and S.E. Mergenhagen.
1968 Physical, chemical and immunological properties of lipopolysaccharide released from *Escherichia coli* by ethylenediaminetetraacetate.
J. Biol. Chem. 243:6384-6391.

Lenard, J.
1971 Rapid and effective removal of sodium dodecyl sulfate from proteins.
Biochem. Biophys. Res. Commun. 45:662-668.

Lindsay, S.S., B. Wheeler, K.E. Sanderson, and J.W. Costerton.
1973 The release of alkaline phosphatase and of lipopolysaccharide during the growth of rough and smooth strains of *Salmonella typhimurium*.
Can. J. Microbiol. 19:335-343.

Lowry, O.H., N.J. Rosebrough, A.L. Farr,and R.J. Randall.
1951 Protein measurement with the folin phenol reagent.
J. Biol. Chem. 193:265-275.

Lüderitz, O., J. Gmeiner, B. Kickhofen, H. Mayer, O. Westphal, and R.W. Wheat.
1968 Identification of D-mannosamine and quinovosamine in *Salmonella* and related bacteria.
J. Bacteriol. 95:490-494.

Lüderitz, O., H.J. Risse, H. Schulte-Holthausen, J.L. Strominger, I.W. Sutherland, and O. Westphal.
1965 Biochemical studies of the smooth-rough mutation in *Salmonella minnesota*.
J. Bacteriol. 89:343-354.

Lüderitz, O., A.M. Staub, and O. Westphal.
1966 Immunochemistry of O and R antigens of *Salmonella* and related Enterobacteriaceae.
Bacteriol. Rev. 30:192-255.

Maizel, J.V., Jr.
1966 Acrylamide-gel electrophorograms by mechanical fractionation: Radioactive Adenovirus proteins. Science 151:988-990.

McDonald, I.J. and G.A. Adams.
1971 Influence of cultural conditions on the lipopolysaccharide composition of *Neisseria sicca*. J. Gen. Microbiol. 65:201-207.

McIntire, F.C., G.H. Barlow, H.W. Sievert, R.A. Finley, and A.L. Yoo.
1969 Studies on a lipopolysaccharide from *Escherichia coli*. Heterogeneity and mechanism of reversible inactivation by sodium deoxycholate. Biochemistry 8:4063-4067.

McIntire, F.C., H.W. Sievert, G.H. Barlow, R.A. Finlay, and A.Y. Lee.
1967 Chemical, physical, and biological properties of a lipopolysaccharide from *Escherichia coli* K-235. Biochemistry 6:2363-2372.

Mongillo, A., K. Deloge, D. Pereira, and G.P. O'Leary.
1974 Lipopolysaccharide from a Gram-negative marine bacterium. J. Bacteriol. 117:327-328.

Mora, P.T. and B.G. Young.
1961 Interaction of an endotoxin with cationic macromolecules. J. Gen. Microbiol. 26:81-95.

Mühlradt, P.
1969 Biosynthesis of *Salmonella* lipopolysaccharide. The *in vitro* transfer of phosphate to the heptose moiety of the core. Eur. J. Biochem. 11:241-248.

Mühlradt, P.F., J. Menzel, J.R. Golecki, and V. Speth.
1973 Outer membrane of *Salmonella*: site of export of newly synthesized lipopolysaccharide on the bacterial surface. Eur. J. Biochem. 35:471-481.

Mühlradt, P.F., J. Menzel, J.R. Golecki, and V. Speth.
1974 Lateral mobility and surface density of lipopolysaccharide in the outer membrane of *S. typhimurium*. Eur. J. Biochem. 43:533-539.

Mühlradt, P.F., H.J. Risse, O. Lüderitz, and O. Westphal.
1968 Biochemical studies on lipopolysaccharides of *Salmonella* R mutants. 5. Evidence for a phosphorylating enzyme in lipopolysaccharide biosynthesis.
Eur. J. Biochem. 4:139-145.

Nikaido, H.
1962 Studies on the biosynthesis of cell-wall polysaccharide in mutant strains of *Salmonella*, I.
Proc. Natl. Acad. Sci. U.S.A. 48:1337-1341.

Nikaido, H.
1969 Structure of cell wall lipopolysaccharide from *Salmonella typhimurium*. I. Linkage between O side chain and R core.
J. Biol. Chem. 244:2835-2845.

Nowotny, A.
1971 Chemical and biological heterogeneity of endotoxin, p. 309-329. *In* G.Weinbaum, S. Kadis, and S.J. Ajl (eds.), Microbial Toxins, Vol IV, Academic Press, New York.

Nowotny, A.M., S. Thomas, O.S. Duron, and A. Nowotny.
1963 Relation of structure to function in bacterial O antigens. I. Isolation methods.
J. Bacteriol. 85:418-426.

O'Leary, G.P., J.D. Nelson, Jr., and R.A. MacLeod.
1972 Requirement for salts for the isolation of lipopolysaccharide from a marine pseudomonad.
Can. J. Microbiol. 18:601-606.

Olins, A.L. and R.C. Warner.
1967 Physiochemical studies on a lipopolysaccharide from the cell wall of *Azotobacter vinelandii*.
J. Biol. Chem. 242:4994-5001.

Oroszlan, S.I. and P.T. Mora.
1963 Dissociation and reconstitution of an endotoxin.
Biochem. Biophys. Res. Commun. 12:345-349.

Osborn, M.J.
1963 Studies on the Gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*.
Proc. Natl. Acad. Sci. U.S.A. 50:499-506.

Osborn, M.J.
1969 Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. 38:501-538.

Osborn, M.J. and L. D'Ari.
1964 Enzymatic incorporation of N-acetylglucosamine into cell wall lipopolysaccharide in a mutant strain of *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. 16:568-575.

Osborn, M.J., J.E. Gander, and E. Parisi.
1972a Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Site of the synthesis of lipopolysaccharide. J. Biol. Chem. 247:3973-3986.

Osborn, M.J., J.E. Gander, E. Parisi, and J. Carson.
1972b Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. 247:3962-3972.

Osborn, M.J., S.M. Rosen, L. Rothfield, and B.L. Horecker.
1962 Biosynthesis of bacterial lipopolysaccharide, I. Enzymatic incorporation of galactose in a mutant strain of *Salmonella*. Proc. Natl. Acad. Sci. U.S.A. 48:1831-1838.

Perry, M.B. and A.C. Webb.
1968 Analysis of 2-amino-2-deoxyhexoses by gas-liquid partition chromatography. Can. J. Biochem. 46:1163-1165.

Reichelt, J.L. and P. Baumann.
1973 Change of the name *Alteromonas marinopraesens* (ZoBell and Upham) Baumann *et al.*, to *A. haloplanktis* (ZoBell and Upham) comp. nov. and assignment of strain ATCC 23821 (*Pseudomonas enalia*) and strain C-Al of DeVoe and Oginsky to this species. Int. J. Syst. Bacteriol. 23:438-441.

Reske, K., B. Wallenfels, and K. Jann.
1973 Enzymatic degradation of O-antigenic lipopolysaccharides by coliphage Ω 8. Eur. J. Biochem. 36:167-171.

Ribi, E., R.L. Anacker, R. Brown, W.T. Haskins, B. Malmgren, K.C. Milner, and J.A. Rudbach.
1966 Reaction of endotoxin and surfactants. I. Physical and biological properties of endotoxin treated with sodium deoxycholate.
J. Bacteriol. 92:1493-1509.

Ribi, E., B.H. Hoyer, K.C. Milner, T.D. Perrine, C.L. Larson, and G. Goode.
1960 Physical and chemical analysis of endotoxin from *Salmonella enteritidis*.
J. Immunol. 84:32-47.

Rick, P.D. and M.J. Osborn.
1973 Properties of a mutant of *Salmonella typhimurium* defective in synthesis of 3-deoxy-D-mannooctulosonate (KDO).
Fed. Proc. 32:481.

Rogers, D.
1971 Release of a lipopolysaccharide-protein complex from *Escherichia coli* A by warm-water treatment.
Biochem. Biophys. Acta 230:72-81.

Rogers, S.W., H.E. Gilleland, Jr., and R.G. Eagon.
1969 Characterization of a protein-lipopolysaccharide complex released from cell walls of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid.
Can. J. Microbiol. 15:743-748.

Romanowska, E.
1970 Sepharose gel filtration method of purification of lipopolysaccharides.
Anal. Biochem. 33:383-389.

Romeo, D., A. Girard, and L. Rothfield.
1970 Reconstitution of a functional membrane enzyme system in a mono molecular film. I. Formation of a mixed monolayer of lipopolysaccharide and phospholipid.
J. Mol. Biol. 53:475-490.

Rothfield, L. and B.L. Horecker.
1964 The role of cell-wall lipid in the biosynthesis of bacterial lipopolysaccharide.
Proc. Natl. Acad. Sci. U.S.A. 52:939-946.

Rothfield, L. and R.W. Horne.
1967 Reassociation of purified lipopolysaccharide and phospholipid of the bacterial cell envelope: electron microscopic and monolayer studies.
J. Bacteriol. 93:1705-1721.

Rothfield, L., M.J. Osborn and B.L. Horecker.
1964 Biosynthesis of bacterial lipopolysaccharide. II. Incorporation of glucose and galactose catalyzed by particulate and soluble enzyme in *Salmonella*.
J. Biol. Chem. 239:2788-2795.

Rothfield, L. and M. Pearlman.
1966 The role of cell envelope phospholipid in the enzymatic synthesis of bacterial lipopolysaccharide. Structural requirements of the phospholipid molecule.
J. Biol. Chem. 241:1386-1392.

Rothfield, L. and M. Pearlman-Kothencz.
1969 Synthesis and assembly of bacterial membrane components. A lipopolysaccharide-phospholipid-protein complex excreted by living bacteria.
J. Mol. Biol. 44:477-492.

Rothfield, L. and D. Romeo.
1971 Role of lipids in the biosynthesis of the bacterial cell envelope.
Bacteriol. Rev. 35:14-38.

Rubio, N. and R. Lopez.
1971 Purification of *Pseudomonas aeruginosa* endotoxin by gel filtration on Sepharose 4B.
J. Chromatogr. 57:148-150.

Rubio, N., A. Portoles, and R. Lopez.
1973 Immuno-physical properties of *Pseudomonas aeruginosa* protein-lipopolysaccharide complex treated with surfactants.
Arch. Mikrobiol. 94:149-158.

Rudbach, J.A. and K.C. Milner.
1968 Reaction of endotoxin and surfactants. III. Effect of sodium lauryl sulfate on the structure and pyrogenicity of endotoxin.
Can. J. Microbiol. 14:1173-1178.

Schmidt, G., B. Jann, and K. Jann.
1969 Immunochemistry of R lipopolysaccharides of *Escherichia coli*. Different core regions in the lipopolysaccharides of O group 8.
Eur. J. Biochem. 10:501-510.

Schnaitman, C.A.
1973 Outer membrane proteins of *Escherichia coli*. I. Effect of preparative conditions on the migration of protein in polyacrylamide gels.
Arch. Biochem. Biophys. 157:541-552.

Shands, J.W.
1966 Localization of somatic antigen on Gram-negative bacteria using ferritin antibody conjugates.
Ann. N.Y. Acad. Sci. 133:292-298.

Shands, J.W., Jr., J.A. Graham, and K. Nath.
1967 The morphologic structure of isolated bacterial lipopolysaccharide.
J. Mol. Biol. 25:15-21.

Shapiro, A.L., E. Vinuela, and J.V. Maizel, Jr.
1967 Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels.
Biochem. Biophys. Res. Commun. 28:815-820.

Sloneker, J.H.
1972 Gas-liquid chromatography of alditol acetates, p. 20-24. *In* R.L. Whistler and J.N. BeMiller (eds.), Methods in carbohydrate chemistry.
Vol. VI, Academic Press, New York.

Smith, E.J.
1964 The isolation and characterization of 2-amino-2,6-dideoxy-D-glucose (D-quinovosamine) from a bacterial polysaccharide.
Biochem. Biophys. Res. Commun. 15:593-597.

Smith, I.
1960 Sugars and related compounds, p. 248. *In* I. Smith (ed.), Chromatographic and electrophoretic techniques, Vol. 1, Interscience Publishing, Inc., New York.

Sprott, G.D. and R.A. MacLeod.
1972 Na^+-dependent amino acid transport in isolated membrane vesicles of a marine pseudomonad energized by electron donors.
Biochem. Biophys. Res. Commun. 47:838-845.

Taylor, A., K.W. Knox, and E. Work,
1966 Chemical and biological properties of an extracellular lipopolysaccharide from *Escherichia coli* grown under lysine-limited conditions.
Biochem. J. 99:53-61.

Uriel, J. and P. Grabar.
1956 Emploi de colorants dan l'analyse electrophorétique et immunoelectrophorétique en milieu gel ifié.
Ann. Inst. Pasteur 90:427-439.

Warren, L.
1963 Thiobarbituric acid assay of sialic acids, p. 463-464.
In S.P. Colowick and N.O. Kaplan (eds.), Methods Enzymol., Vol. VI, Academic Press, New York.

Weigel, H.
1963 Paper electrophoresis of carbohydrates, p. 61-97.
In M.L. Wolfrom (ed.), Adv. Carbohyds. Chem., Vol. 18, Academic Press, New York.

Weiner, I.M., T. Higuchi, L. Rothfield, M. Saltmarsh-Andrew, M.J. Osborn, and B.L. Horecker.
1965 Biosynthesis of bacterial lipopolysaccharide. V. Lipid-linked intermediates in the biosynthesis of the O-antigen groups of *Salmonella typhimurium*.
Proc. Natl. Acad. Sci. U.S.A. 54:228-235.

Weiser, M.M. and L. Rothfield.
1968 The reassociation of lipopolysaccharide, phospholipid, and transferase enzymes of the bacterial cell envelope. Isolation of binary and ternary complexes.
J. Biol. Chem. 243:1320-1328.

Weissback, A. and J. Hurwitz.
1959 The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. I. Identification.
J. Biol. Chem. 234:705-709.

Westphal, O. and K. Jann.
1965 Bacterial lipopolysaccharides: Extraction with phenol-water and further applications of the procedure, p. 83-91. *In* R.L. Whistler (ed.), Methods in Carbohydrate Chemistry, Vol. V, Academic Press, New York.

Westphal, O., O. Lüderitz, and F. Bister.
1952 Über die extraktion von bakterien mit phenol/wasser. Z. Naturforschungen 7(b):148-155.

Wilkinson, S.G. and L. Galbraith.
1975 Studies of lipopolysaccharides from *Pseudomonas aeruginosa*. Eur. J. Biochem. 52:331-343.

Wilkinson, S.G., L. Galbraith, and G.A. Lightfoot.
1973 Cell walls, lipids, and lipopolysaccharides of *Pseudomonas* species. Eur. J. Biochem. 33:158-174.

Wright, A., M. Dankert, and P.W. Robbins.
1965 Evidence for an intermediate stage in the biosynthesis of the *Salmonella* O-antigen. Proc. Natl. Acad. Sci. U.S.A. 54:235-241.

Wu, M-C. and E.C. Heath.
1973 Isolation and characterization of lipopolysaccharide protein from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 70:2572-2576.

Zacharius, R.M., T.E. Zell, J.H. Morrison and J.J. Woodlock.
1969 Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. 30:148-152.

Zey, P. and S. Jackson.
1978 Conditions that affect the colorimetric analysis of lipopolysaccharide from *Escherichia coli* and *Treponema pallidum*. Appl. Microbiol. 26:129-133.

Zittle, C.A.
1951 Reaction of borate with substances of biological interest, p. 493-527. *In* F.F. Nord (ed.), Advances in Enzymology, Vol. 12, Interscience Publishers, New York.

Zollinger, W.D., D.L. Kasper, B.J. Veltri, and M.S. Artenstein.
1972 Isolation and characterization of a native cell wall complex from *Neisseria meningitidis*. Infect. Immun. 6:835-851.