ocument 1.

Date _____

ETHICAL REVIEW COMMITTEE, ICDDR,B.

204

Principal Investigator S.Q. Akhtar

Trainee Investigator (if any) _____

Application No. 80-031

Supporting Agency (if Non-ICDDR,B) _____

Title of Study Investigations on the sistant and sensitive strains of brio cholerae.

Project status:
(✓) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

Source of Population: NA
(a) Ill subjects Yes No
(b) Non-ill subjects Yes No
(c) Minors or persons under guardianship Yes No

Does the study involve: NA
(a) Physical risks to the subjects Yes No
(b) Social Risks Yes No
(c) Psychological risks to subjects Yes No
(d) Discomfort to subjects Yes No
(e) Invasion of privacy Yes No
(f) Disclosure of information damaging to subject or others Yes No

Does the study involve: NA
(a) Use of records, (hospital, medical, death, birth or other) Yes No
(b) Use of fetal tissue or abortus Yes No
(c) Use of organs or body fluids Yes No

Are subjects clearly informed about: NA
(a) Nature and purposes of study Yes No
(b) Procedures to be followed including alternatives used Yes No
(c) Physical risks Yes No
(d) Sensitive questions Yes No
(e) Benefits to be derived Yes No
(f) Right to refuse to participate or to withdraw from study Yes No
(g) Confidential handling of data Yes No
(h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No

5. Will signed consent form be required: NA
(a) From subjects Yes No
(b) From parent or guardian (if subjects are minors) Yes No

6. Will precautions be taken to protect anonymity of subjects NA Yes No

7. Check documents being submitted herewith to Committee:
___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
___ Protocol (Required)
___ Abstract Summary (Required)
___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
___ Informed consent form for subjects
___ Informed consent form for parent or guardian
___ Procedure for maintaining confidentiality
___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

We agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

Rukaiya Akhtar
Principal Investigator

_____
Trainee

80-031
rec'd 29/7/80

SECTION I - RESEARCH PROTOCOL

(1) Title: Investigations on the resistant and sensitive strains of V. Cholerae.

(2) Principal Investigator: S.Q. Akhtar.

(3) Starting Date: June, 1980

(4) Completion Date: June, 1982

(5) Total Director Cost: $ 18,384

(6) Scientific Program Head:

This protocol has been approved by the **Host Defense** Working Group.

Signature of Scientific Program Head: WB

Date: 15/8/80

(7) Abstract Summary:

This study is designed to investigate drug resistant and sensitive strains of V. Cholerae. An extensive sub cellular level study of resistant and sensitive strains is expected to contribute towards immunological research and vaccine development. Study of cell components of two different types of strains might suggest some correlation of bacterial resis-

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basis with cellular components and might provide evidences of changes brought about by drugs. The investigations would be done mainly on the basis of comparative biochemical analysis of sensitive and resistant strains. All strains chosen for this study belong to the same bio- and serotype i.e. El Tor, Inaba. The criteria for choosing the resistant strains are (a) In vitro resistance - resistance transfered to sensitive cells from resistant cells of the same species (by transferring R. factor from resistant strains of V. cholerae to sensitive strains of V. cholerae) or from different species, e.g. from antibiotic resistant E. Coli to sensitive V. cholerae in the laboratory, (b) In vivo resistance - resistant strains obtained from patients reported in the ICDDR,B Hospitals. Strains chosen were either resistant to maximum number of commonly used antibiotics or sensitive to all of these antibiotics. All the strains would be grown maintaining identical cultural conditions. Culture supernatants would be tested for toxin by the existing methods. Different cell components e.g. proteins, lipids, carbohydrates, lipopolysaccharides would be extracted from the freeze dried cells or membranes by the established methods of extractions. Preliminary quantitative analysis of all these cell or membrane components would be done by following standard methods for the quantitative determinations of bacterial cell or membrane components. Further detail qualitative and quantitative analysis of all these components would be done by different chromatographic methods, e.g. column, thin layer, paper, gas liquid.

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(8) Reviews:

(a) Ethical Review Committee: _____

(b) Research Review Committee: _____

(c) Director: _____

(d) BMRC: _____

SECTION II -- RESEARCH PLAN

INTRODUCTION

1. Objective:

The objective of this study is to make a comparative investigation of drug sensitive and resistant strains of V. Cholerae which might make reasonable contribution to immunological researches. A sub cellular level study of these sensitive and resistant strains would give a better understanding of cell components, hence the correlation of cell components with bacterial resistance. Information obtained from the analyses of cell components of resistant sensitive strains is also expected to add information on sensitive cell becoming resistant. This investigations might also provide some evidence on the function of biological membranes, specially of the function of specific cell envelope lipids. The cell envelope lipids are reported to be correlated with bacterial resistance. An extensive comparative analyses of lipid components of sensitive and resistant strains has been designed in this study. The analyses is expected to reveal some difference in lipid contents of two different types of strains, specifically a significant increase in glycolipid content of resistant strains. A higher content of glycolipid in resistant cell membrane would indicate the possibility of resistant cells being L-forms of the parent strains. It has been reported in the literature that L-forms contain higher glycolipid than the parent strains. These L-forms have also been reported to be more effective than parent strains for vaccine development. The ultimate objective of this study would be to achieve a better understanding of resistant strains on sub cellular level and hance to contribute on the development of improved immunogens.

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2. Background:

There are reports in the literature regarding the causes of evolving resistant strains. Some of which suggested genetic change some stressed on changes in biochemical properties as an attempt to adapt with the adverse environment created by the presence of antibacterial substances. A correlation of bacterial lipid composition with antibiotic resistance has been reported by few workers. Dunnick and O'Leary (1970) reported consistant quantitative differences between the fatty acid compositions of sensitive and resistant strains of Gram-negative bacteria. Most significant fact shown was the decrease in cyclopropane acids in resistant strains as compared with sensitive strains. In all cases the antibiotid resistant Gram-negative organisms contained a higher concentration of unsaturated acids and a lower concentration of cyclopropane acids than was found in the corresponding sensitive strains. They also reported that the lipids of the resistant strains of a Gram-positive bacteria (S. aureus) contained more lipid than was found in the sensitive S. aureus strain. This investigation has explored the correlation of antibiotic resistance with lipid content and has shown a correlation between fatty acid content and antibiotic resistance in the Gram-negative bacteria, and between lipid content and antibiotic resistance in the Gram-positive bacteria. Hugo and Stretton (1966) also suggested that cellular lipids may have a role in the resistance of Gram-positive bacteria to penicillin. They also found increased lipid content in cells resistant to penicillin.Hugo and Davidson (1973) also studied the effect of cell lipid depletion in S. aureus upon its resistance to antimicrobial agents. From a comparison of the response of normal and lipid depleted cells of S. aureus to antimicrobial drugs a correlation of lipid depletion with an increased sensitivity to a number of antibiotics has been suggested. Tetracycline resistance has also been observed attributed to a decreased permeability of the drug (Benbough and Morrison, 1967; Franklin and Godfrey, 1965; Franklin, 1967; Izaki, et al 1966; Laskin and Chan, 1964; Okamoto and Mizuno,1962; Okamoto and Mizuno, 1964).

Ridg and O'Leary (1977) reported that quantitative differences which correlated with antibiotic resistance existed among the phospholipids and fatty acids of antibiotic-resistant and -susceptible members of the Enterobacteriacea. They observed a relatively higher concentration of [illegible]-positive phospholipid concomitant with a lower amount of phosphatidylethanolamine (PE) in antibiotic resistant strains of [illegible]. Bacterial strains which harbored R factor 222 had a higher ratio of phosphatidylglycerol (PG) to diphosphatidylglycerol (DPG) than their respective parent strains while those strains which were resistant to the polymyxin had a lower ratio of these phospholipids. Differences in the relative amounts of certain unsaturated and cyclopropane acids were observed between susceptible and resistant strains. Brown and [illegible] (1972) examined the lipids of polymyxin-resistant strains of Ps. aeruginosa and Klebsiella aerogenes and reported that although the total extractable lipid was greater in the resistant cells, the amount of phospholipid in the wall fraction of these cells was lower than compared with sensitive cells.

In a mutant of Rhizobium melitoti resistant to viomycin was reported to be accompanied by an accumulation of phospholipid in the cell envelope (Mackenzie and Jordon, 1970). Norrington and James (1970) observed that tetracycline-resistant strains of strep. pyogenes contained up to three-fold as much extractable lipid as did sensitive strains. Resistant cells of Ps. aeruginosa grown in presence or absence of chloranphenicol showed 28 % increase in total lipid content. Though no qualitative change in [illegible] component or fatty acids was observed, an increase in fatty acid quantity was observed (Anderes, Saudine and Elliker, 1971).

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Antibiotics and lipid metabolism:

A co-relation of bacterial resistance to antimicrobial substances have been reported in the literature. The view that the emergence of drug resistant bacterial strains as a result of adaption to adverse environment created with the extensive use of antibiotics is accepted. These suggestions may indicate some effect of antibiotics on lipid metabolism. Many antibiotics which effect bacterial cell wall are known to effect lipid metabolism in bacteria to some extent. Starka and Moravova (1970) observed that low doses of penicillin which inhibited division but permitted filament formation in E. Coli did not influence of total rate of phospholipid formation but altered the ratio of individual phospholipids to that characteristic of resting organism. The filament contained more cardiolipin and less phosphetidylglycerol than normal exponentially dividing organism. Simmler and Barbu (1970) reported changes in the proportions of phosphatidylglycerol (PG) and cardiolipin (DPG) which occur during transition from exponential to stationary phase also occur on addition of penicillin. The latter results in decreased cellular phosphorylating ability. Molenkamp and Veerkamp (1976) have reported a considerable enhancement of fatty acid incorporation in Bifidobacterium, bifidum, subsp. pennsylvanicus when treated with penicillin G, D-cycloserine and bacitracin. Shockman (1965), Miller (1967) have shown that S. falcalis, B. subtilis, B. cereus produce 2 to 3 times thicker cell walls when treated with chloramphenicol. Bishop et al (1973) also reported about some difference in the lipids of streptomycin sensitive and resistant bacteria. Bacterial strains resistant to polymyxin contain different lipids to that of susceptible strains (Imai, et al, 1975; Brown et al, 1972; Few, 1955).

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From these reports in the literature it is evident that antibiotics effect the lipid composition of bacteria. A significant effect is a tendency of bacterial cells to produce more lipid upon treatment with antibiotics which can be correlated with a higher lipid content of resistant strains.

Lipid changes on conversion to L-form:

There have been several reports on the difference in lipid composition of L-forms of bacteria and their parent organisms. According to Cohen and Panos (1966), the major membrane lipid differences between S. pyogenes and and its derived L-form were quantitative rather than qualitative. The L-form membrane contained more total lipid (35.6 %) than the protoplast membrane (15.3 %). The glycolipid content of L-form membranes was 22 % which was markedly greater than that of protoplast membranes, which was 11 %. The nonpolar lipid content of the total lipid extract was 23.4 % for the protoplast and 34.7 % for L-form membranes. Conversely L-form protoplast membrane [illegible] The phosphorus content of protoplast membrane lipid was 1.49 % which was almost two-fold to that of L-form membrane lipids (0.65 %). The L-form membrane fatty acid content was 57 % greater than protoplast membranes (Panos et al, 1966). cis-vaccenic acid was the predominating monoethanoid fatty acid of protoplast lipids but in the lipids of L-form membranes oleic acid which is an isomer of cis-vaccenic acid prevails. According to an earlier report by the same authers, conversion to the L-form resulted in a concomitent shift in its octadecanoic acid content. The average oleic acid content of of L-form membrane lipid fraction (26 %) was found to be more than twice that of the protoplast fractions (9.7 %). The C_{16} monolefinic fatty acid of the L-form fraction were approximately a quarter that of a similar fractions from the protoplast. They suggested that both these fatty acid changes are

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the increased glycolipid in the L-forms might be a consequence of its inability to synthesize a rigid cell wall. Similarly, the increased total lipid content found in L-form may also reflect a compensation for the lack of a rigid cell wall. These authors have previously reported (Panos et al, 1966) that the protein content of L-form (59 %)and protoplast membranes (68 %) was similar, which suggests that during conversion to L-form major changes are predominantly associated with lipid synthesis.

A study on the chemical composition of membranes of protoplasts L-forms of S. aureus (Ward and Perkins, 1968) also showd similar difference. Protoplast membranes were found to contain 22-25 % of lipid, whereas the L-form membranes contained 25-36 %. In both the L-forms they studied, derived from S. aureus 100 the amount of membrane glycolipid was about double that of protoplast membrane glycolipid and phospholipid content was 14 % (L_1) and 23 % (L_{20}) lower. L-forms of S.aureus H contained three times the amount of glycolipid present in protoplast membranes. The major phospholipids of the protoplast membranes were phosphatidy glycerol and some lipoamino acids, but in L-form membranes the chief phospholipid found was diphosphatidylglycerol, which indicates a qualitative change in lipid composition due to conversion in L-forms.

A characteristic feature of the organism lacking a rigid cell wall (e.g. Mycoplasma laidlawii)is an enhanced total lipid content and also a proportionately higher concentration of glycolipid content. Mycoplasma laidlawii contain appreciable quantities of glycolipid and its total lipid content is higher than the organisms possessing a rigid cell wall. A comparison of the lipid compositions of L-forms and their parent organisms clearly shows that on conversion to L-form increase in total lipid content with an increase in their

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glycolipid content takes place. Some L-forms of streptococci (Ward et al. 1958; Rolla et al, 1965) and staphyloccocci (Williams 1963; Molender, et al. 1964) were observed considerably more resistant to bacitracin than the parent organism. Resistance to antibiotics might be a result of higher lipid content of the L-form (Dunnick and O'Leary, 1970).

Drug sensitive bacterial strains including faecal streptococci show higher lipid content when grown in presence of antibiotics. Maximum increase in lipid content both in whole cells and in isolated cytoplasmic membrane was observed when treated with cell wall inhibitors, specially penicillin and its other derivatives. Initiation of L-forms by cell wall inhibitors may be the cause of higher lipid content as L-forms are reported to contain higher lipid than the parent organisms. Quantitative change has also been observed when treated with protein inhibitor, chloramphenicol or with serine, bacitracin, novobiocin. Some quantitative variations in fatty acid contents were also observed. All these quantitative variations are directly proportional to the amount of antibiotic present in the media (unpublished work). Higher lipid content of resistant cells and also of sensitive cells grown in presence of antibiotics may lead to a correlation of bacterial resistance with their lipid content and composition.

3. Rationale:

Evolution of drug resistant strains in the bacterial population has long been a problem. So a systematic study of resistant and sensitive strains is justifiable. An extensive comparative biochemical analysis of sensitive and resistant cells may provide some idea of correlation of cell components with resistance. Higher lipid content of resistant cells, as reported in the

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literature may indicate alteration of permeability of the cell membrane which may impart resistance to cells. Lipid is one of the major components of cell envalope and change in lipid content or composition of cells may bring about some alteration to other cell components, specially cell envalope components each as protein, lipopolysaccharides, carbohydrate. So an extensive investigation on such of these cell components may be worthwhile. This might lead to the development of improved, nontoxic immunogens. The presently available whole cell cholera vaccine has been proved to be unsatisfactory. So the study of cellular components from sensitive and resistant strain of V.cholerae for the development of better immunologen is desirable and justifiable. A successful completion of this study might also provide other information like function of biological membrane, difference between resistant and sensitive cell membrane. This might also give some information about resistant strains being L-forms of parent organisms, which are reported to be better used for vaccine development. This provides more justification to the proposed study.

B. SPECIFIC AIMS

1. To investigate on the causes of drug sensitive strains of V. cholerae and other bacterial pathogens becoming resistant drugs.
2. To study the cell components of sensitive and resistant cells.
3. To establish a correlation of bacterial cell component with resistance.
4. To compare the cytoplasmic membrane of drug sensitive and resistant cells.
5. To investigate in details the function of biological membranes and to provide evidences regarding the changes caused by drugs leading to resistance.
6. Though no electron microscopic examination of resistant cells have been planned, a higher content of glycolipid in resistant cell membrane would indicate the possibility of resistant cells being L-forms. If by further

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investigation it can be confirmed, this L-forms may be considered for vacoine development. It has been reported in the literature that L-forms may better be used for this purpose.

7. To observe the immune response of cell components from resistant and sensitive strains.

C. METHODS OF PROCEDURE

For this study 10 strains resistant in vivo (isolated from patients reported at the ICDDR,B Hospitals), 10 strains resistant in vitro (strains obtained by transferring resistant factor from a resistant V. cholerae strain to a sensitive V. cholerae strain or from a resistant E. coli to a sensitive V. cholerae strain) and 10 sensitive strains were selected. On the selection of in vitro resistant strains preference would be given to those resistant strains where resistance has been transferred from resistant strains included in this study to the sensitive strains chosen for this work.

All the strains selected for this study would be grown in a large volume maintaining identical conditions. Evan's medium with 0.2 % glucose would be used for the growth of all strains included in this study. Cultures would be grown aerobically with a moderate aeration (40/45 rpm). Each batch of culture should contain at least 1.5 litre. 100 ml culture would be grown in 500 ml flask. All media should be inoculated from the same pure overnight grown inoculum. Cells would be harvested at the stationary phase of growth (i.e. after 18 hrs of incubation). Temperature to be maintained for growth is 37°C. Cells at stationary phase would be spinned down with a Sorvall superspeed centrifuge at 12,000 rpm for 30 minutes at 4°C. Harvested cells should be washed to remove media contaminants first with normal saline and then with adequate quantity of distilled water. Cells would be freeze dried

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for biochemical analysis. A duplicate culture of each strain would be grown and analyse identically for more confirmatory results. Culture supernatant would be sterilized through milipore filter (0.45 um) and tested for toxin by the standard existing methods. Freeze dried cells would be divided into portions depending on the different quantities of the cell components within the cell. Approximately ⅓ of the dry cells would be used for the preparation of membranes as the yield of dry purified membrane is supposed to be 25 % of the whole dry cell.

Preparation of membrane:

V. cholerae is a Gram-negative organism and the cell envalope of this organism is structurally complex compared to the Gram-positives. The cell envalope of this bacterium is composed of three distinct layers: the outer membrane, the inner or cytoplasmic membrane and the peptidoglycan. The outer membrane contains phospholipids, proteins and lipopolysaccharides. In many Gram-negative bacteria a major protein called lipoprotein is present in both bound and free form. Recent findings by Geyer et al (1979) has suggested that some of the outer membrane proteins of Gram-negative bacteria possess antigenic properties by their presence on cell surface. They have isolated a LPS binding cell surface protein from Sal Minnesota which cross reacted with several enteric strains such as Sal typhimurium, E. coli and Shigella. The relative amounts of these components depend on growth conditions. For the preparation of outer membranes from V. cholerae cells the harvested cell pallet has to be suspended in 0.1 M Tris-HCl pH 7.8 (25 ml should be used for the quantity of cells obtained from 250 ml of cultures). The suspen-

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sion would then be sheared at 4°C in a Waring blender (19,000 rev/min, 45 sec) to remove flagella. The suspension would then be diluted sixfold in the Tris-buffer and centrifuged at 12,000Xg for 10 min. The cell pellet would be resuspended in cold 0.75 M sucrose-10mM Tris-HCl, pH 7.8 (0.7 ml sucrose solution per 10 A600 units of original culture). The suspension would then be transferred to an Erlenmeyer flask containing lysozyme 2 mg/ml of H_2O, 0.05 ml/ml of cell suspension) and the mixture incubated in ice for 2 minutes. The suspension would be diluted with 2 volumes of cold EDTA (1.5 mM) and kept at 4°C for 3 hr. The spheroplast would be lysed by the osmotic shock by slowly pouring the suspension into 4 volumes of cold water with magnetic stirring and will be stirred for 10 min. in the cold. The total membrane fraction would be recovered from the osmatic lysate first by centrifugation at 12,000Xg for 15 min. at 2 to 4°C to remove the unlysed cells and later by centrifuging the supernatant at 48,000Xg for 30 min. The pellet would be resuspended into 0.01 M Tris-HCl (pH 7.8) containing 10 mM EDTA by a syringe and to be washed once with Tris-HCl (0.01 M), pH 7.8 containing 5 mM $MgCl_2$. The inner membrane would be dissolved from the total membrane by extraction at 0°C for 20 min. with 2 % triton X-100 containing 5 mM EDTA described for E. coli by Schnaitman (1971). The outer membrane would be recovered as a pellet.

Analyses of cell or membrane components

Lipid: Lipids are a group of naturally occuring substances characterized by their insolubility in water and their solubility in organic or fat solvents such as ether, chloroform, boiling alcohol and benzene. Though individual members of this group show large individual variations in solubility as a class the lipids are readily distinguishable from other major groups of naturally occuring compounds - carbohydrates and proteins. They are limited to substances which are utilizable by the animal organism. Lipids in micro-organisms are mostly associated with cell wall and membrane structures. Lipids of Gram-positive bacteria are essentially membrane lipids (Weibull, 1957; Salton, 1960; Kolb et al, 1963), whereas Gram-negative organisms contain in addition lipids associated with the cell wall. Lipids may broadly be divided into two main classes, simple and complex lipids. Simple lipids are generally fatty acid esters of glycerol and include monoglycerides, diglycerides and triglycerides, compounds that differ in the number (1-3) of long chain fatty acid residues present. Complex lipids are polar phosphorylated compounds that include lecithin, phosphatidic acids, phosphatidyl serine etc.

The lipids common in bacteria are amphiphatic molecules, consisting of a polar and an apolar region and are usually derivatives of diacylglycerol in which long chain fatty acids are esterified to carbon atoms 1 and 2 of glycerol. The polar group linked to carbon atom 3 of glycerol may be phosphate ester phospholipid, or a carbohydrate derivative glycolipid.

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Lipids present in bacteria usually represent at least 3-5 % of dry cell weight but it can be much higher than that in some cases (Shaw, 1970). A noticeable difference in lipid content is found between cell walls isolated from Gram-positive bacteria and Gram-negative bacteria. Gram-positive organisms have very little extractable lipid in their walls (Salton, 1960) with the exception of the mycobacteria which have as much as 60 % of the dry weight of the wall composed as wax, phosphatides and other bound lipids.

Lipids present in *V. cholerae* 569B: The phospholipids that were identified as estimated in *V. cholerae* 569B (Inaba) were phosphatidylethanolamine, (PE), phosphatidylglycerol, lyso-phosphatidylethanolamine, phosphatidylserine and cardiolipin. Thin layer chromatography of the neutral lipid fraction showed the presence of free fatty acids only. The fatty-acid composition of whole cell extractable lipid, neutral lipid and phospholipid showed the presence of $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:0}$ and $C_{18:1}$ fatty acids: cyclopropane and hydroxy acids were absent from free lipids of *V. cholerae*; although hydroxy acids are present in lipopolysaccharide and lipid A (Raziuddin, 1976).

Extraction of lipids from bacteria: Dehydrating agents (ethanol, acetone, methanol) rupture lipid-protein linkages but since lipids may not be entirely soluble in these solvents, a non-polar solvent such as petroleum ether chloroform or diethylether is also included in solvent mixtures for extracting lipids from biological material.

Moist cells may be extracted directly with lipid solvents but it is more convenient to extract freeze dried bacteria. Extractions are usually

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carried out at room temperature simply by stirring the bacteria repeatedly with a large excess (Ca 30:1) of solvent and removing the defatted cells by filtration. It is possible to fractionate lipids by extracting bacteria with solvents of different polarity but in such cases considerable overlapping of fractions may occur. It is more useful to extract with a solvent that will extract all free lipids e.g. chloroform-methanol 2:1 (Folch et al, 1957) or butanol. So called bound lipids (e.g. lipopolysaccharides) require treatment with mild acids (e.g. phenol) for their liberation.

Solutions of lipids in organic solvents should be concentrated under reduced pressure at the lowest possible convenient temperature ($<37^{\circ}$) and the resulting lipids stored at low temperature.

Extraction of lipid from different strains of V.cholerae: Lipids were extracted by the modified Bligh and Dyer (1959) procedure described by Rizza, Tuker and White (1970). A suspension of bacteria (approximately 25 mg dry weight) in 50 mM-sodium phosphate buffer, pH 7.6 (50 ml), shaken vigorously and allowed to stand overnight. Chloroform (50 ml) and 50 ml of 1.0 M-KCl solution containing 0.4 % (V/V) glacial acetic acid were then added and the mixture shaken. After several hours, the mixture separated into two phases: the lower layer, containing the lipid was filtered through a Whatman No. 12 filter paper.

Column Chromatography:

For the purification of crude lipid and to separate their components column chromatography would be performed.

Silicic acid column chromatography:

A slurry of silica gel (wt. of lipidx100) in chloroform is poured into glass column (1-2 cm. int. dia) fill with $CHCl_3$ and sealed at the bottom with cotton or glass wool and a layer of sand after the gel has settled a layer of sand is placed on top. The lipid sample is applied to the column in the minimum amount of chloroform and washed on with two further small portions of this solvent. A lipid insoluble in chloroform should be dissolved in $CHCl_3$-MeOH 2:1, acid-washed celite (545) added, the suspension evaporated to dryness and the lipid, adsorbed on the celite, placed on the column as a slurry in chloroform. The column is then eluted essentially according to Vorbeck and Marinetti:-

Chloroform	2x100 ml	neutral lipid
Chloroform-acetone 1:1	2x100 ml	glycolipids
Acetone	4x100 ml	

Chloroform-methanol 98:2	1x100 ml	
Chloroform-methanol 9:1	1x100 ml	Phospholipids
Chloroform-methanol 1:1	1x100 ml	
Methanol	2x100 ml	

Chloroform-acetone mixtures should be prepared well in advance since these solvents have a considerable heat of mixing. For the same reason the changes of eluants containing chloroform and acetone should not be sharp. A convenient procedure is to use a solvent reservoir at least twice as large as each batch of eluant being added and to keep this reservoir replenished; by this means mixing occurs in the reservoir and the column is not broken up by heat of solvent mixing. Such heat generated would also have the effect of causing premature desorption of adsorbed lipid components.

Fractions may be collected on a fraction collector or more simply in measuring cylinders. Each fraction should be evaporated under reduced pressure, transferred in the minimum amount of chloroform of chloroform-methanol (2:1) to tared 25 or 50 ml. conical flasks, the solvent evaporated and the lipid weighed. Thin layer chromatography of the separated fractions on a 20x20 cm. analytical plate enables combination of fractions having essentially the same composition. Fractions may also be investigated for phosphate hexoses etc.

Neutral lipids may be also fractionated on silicic acid (Marinetti p. 207) and the use of alumina (p. 221) should also be noted. Eluants are usually mixtures of petroleum ether and acetone. Argentation chromatography may be successfully employed on silicic acid columns. If a

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lipid mixture is adsorbed on celite and introduced as a slurry in petrol (in which the free lipid is insoluble) onto a silicic acid column and elution continued with petrol-acetone mixtures until pure acetone is reached (and then continuing as for polar lipid mixtures) it is possible to fractionate neutral and polar lipids in a single experiment Diethylaminoethyl (DEAE) cellulose column chromatograph. Lipid classess are separated by DEAE column chromatography by ion exchange and by difference in polarity provided by non-ionic groups, principally hydroxyl groups (Marinetti p. 120. Lowenstein P. 272). Lipids may for the purposes of discussion be divided into three groups, nonionic, ionic nonacidic and acidic. Nonionic lipids are eluted with chloroform containing small amounts of methanol (3-30 %). All nonionic (e.g. neutral lipids, glycolipids) and ionic nonacedic (e.g. phosphatidylethanolamine, PE) lipids are eluted with methanol, whereas acidic lipids (e.g. phosphatidylglycerol, PG) are not. Acidic lipids may be eluted and fractionated by incorporating increasing amounts of salt (e.g. ammonium acetate) in methanol as eluant. DEAE column chromatography therefore, has unique characteristics which make it different from silicic acid in which overlap of acidic and nonacidic lipid fractions occurs and is therefore a very useful alternative medium for the fractionation of lipids especially when large quantities of polar glycolipids are present.

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(a) Quantitative analysis

(i) Carbohydrate determination:

Lipid solutions of known concentration were prepared in chloroform/methanol (1:1, V/V). Aliquotes would be removed for hexose determination by phenol-sulphuric acid reaction described by Dubois, et al (1956). This is a general method for determination of pentoses, hexoses and deoxy aldoses and their derivatives.

(ii) Phosphorus determination:

Total phosphate

Total phosphate content of the lipid samples would be determined by the modified method of Chen, Toribara and Warner (1956).

Inorganic phosphate

Samples containing 1-5 µg of phosphorus are adjusted to 4 ml with water, 4 ml colour reagent is added and the mixtures are incubated at 37°C for 1.5 to 2 hrs. The solutions are cooled to room temperature and the extinctions read at 820 nm against a reagent blank. Compare with known concentration of inorganic phosphate similarly treated.

(b) Qualitative analysis:

(i) Deacylation - A sample of lipid was dissolved in a mixture of chloroform/methanol (1:1, V/V) and then treated with sodium methoxide in methanol (0.2N, 0.5 ml). The resulting solution was kept at room temperature for 0.5 hr. and then equal volumes of water and chloroform (5 ml: 5 ml) were added. The upper aqueous layer was separated and then

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passed through a short column of Dowex 50 resin (H^+ form). The elute was evaporated to dryness and investigated by paper chromatography. The chloroform layer was dried over anhydrous sodium sulphate and the solution of fatty acid methyl esters analysed by GLC.

(ii) Acid hydrolysis - A small quantity of lipid was hydrolysed at 100° in 2N HCl in a sealed tube for 2 hrs. The hydrolysates was dried in vacuo over KOH, the residue dissolved in the minimum quantity of water and analysed by paper chromatography.

Chromatography:

(i) Thin layer chromatography - Thin layer chromatography (t.l.c.) is an extremely sensitive and rapid technique for the analysis of a wide variety of organic compounds. Moreover, it may also be adapted for preparative work (p.l.c.) and under suitable conditions upwards of 100 mg of material may be rapidly purified. The support most widely used is silicic acid. For detailed and extensive analysis of lipid samples obtained from whole cells and membranes both one and two dimensional chromatographic analysis is desirable.

20X20 and 5X20 cm glass plates with a layer (0.4 mm) of silicic acid-Kieselgel G (Merck) are used for analysis (Marinetti, 1967).

Solvent: $CHCl_3$:MeOH:H_2O 65 : 25 : 4 is an excellent solvent for general use. This neutral solvent system would be used throughout for one dimensional thin layer chromatographic analysis of lipid samples. Acidic and basic modifications of this system are also useful:

i) $CHCl_3$: MeOH : HOAc : H_2O
65 : 25 : 8 : 4

ii) $CHCl_3$: MeOH : 7N NH_4OH
12 : 7 : 1

Solvents with a high concentration of acetone are useful for separating glycolipids e.g. acetone:HOAc:H_2O 100:2:9 which leaves phospholipids on the base line. For complex mixtures, two-dimensional thin layer chromatography may be advantageous (Marinetti - p. 145). For two dimensional thin layer chromatographic analysis (Minnikin and Abdolrahimzadeh, 1971) plates should be developed in the neutral system followed by the acidic system. Two-dimensional techniques are useful analytically (Marinetti p. 145); a system developed specifically for investigations of bacterial lipids has been described. This procedure utilises plates prepared from a slurry of 40 g silica gel H (preferred to PF_{254}) in 100 ml 0.2 % aqueous sodium acetate and dried at $<70^{\circ}$ overnight. The solvent for the first direction of development is $CHCl_3$ - MeOH - H_2O (65:25:4) and for the second direction is $CHCl_2$ - MeOH:HOAc:H_2O (65:25:8:4). The plate should be allowed to dry for 1 hr. at room temperature between developments. In an attempt to observe visually any quantitative changes in lipid samples obtained from different strains, spots of approximately identical concentration should be used.

Detection:

Two non-specific reagents are widely used:

i) Universal reagents (Lowenstein p. 542) plates spotted with lipid samples and run in tanks containing lipid solvent can be sprayed with 50 %

Contd.

H_2SO_4, followed by heating. Black spots of charred organic material provides a preliminary detection.

ii) Exposure to iodine vapour gives brown spots. Iodine vapour will complex with lipids and give brown spots on a pale background.

Specific reagents :

i) Ninhydrin reagents - 0.2 g ninhydrin and 1 ml glacial acetic acid in 100 ml of water saturated butanol. Spray paper and heat at 100° for 5 minutes to develop spots which appear purple or brown. Detects free amino-groups, e.g. phosphatidylethanolamine, lipo-amino acid esters.

ii) Molybdenum blue for phosphates (Hanes/Sherwood is not suitable for phospholipids -

Solution A : Boil 40.11 g of MoO_3 in 1 litre of 25N H_2SO_4 until dissolved.

Solution B : Boil gently 1.5 g of powdered molybdenum in 500 ml of solution A for 15 minutes and after colling decant from any residue. The spray reagent is a solution of A:B:H_2O. The plates require no heating 1:1:2 and phospholipids give an immediate blue colour, may be used after ninhydrin spray.

iii) Periodate-Schiff reagents - Detects all lipids with -glycol groups.

a) 1 % aqueous sodium metaperiodate solution

b) 1 % para rosaniline hydrochloride solution decolourized with sulphurdioxide until a pale straw colour solution results. Spray paper

Contd.

with (1) and leave 5 minutes. Treat with sulphurdioxide until brown colouration initially formed disappears, spray with (2) and leave for spots to develop. The colour of the spot depends on the product of periodate oxidation, formaldehyde gives an immediate purple colouration, malondialdehydes yellow and other aldehydes blue in a half to two hours.

Glycolipids give a slow blue-grey coluur, phosphatidylglycerol gives a rapid purple colour due to liberation of HCHO; phosphatidyl nositol gives an initial yellow colour which turns grey-brown. After standing for 24 hrs. or longer most non-reactive components e.g. bis-phosphatidylglycerol can be observed as faint yellowish spots. As yet unidentified green spots at the solvent front, presumably neutral lipids have also been observed.

Typical R_f values :

Neutral lipids	-	solvent front
Diglycosyl diglycerides	-	0.6 - 0.7
Phosphatidylglycerol	-	0.5 - 0.6
Bis-phosphatidylglycerol	-	0.7 - 0.75.

Preparative thin layer chromatography :

Plates with absorbent 1 mm. thick are usually used. Depending upon the efficiency of the separation, 50 - 100 mg. of material may be introduced on to the base line of a 20 cm. plate as a thin band acreoss the total width of the plate (a mechanical device is available for this purpose). After development, the separated bands may be detected by spraying the edges of a masked plate with the appropriate reagent but it is preferable

Contd.

to spray both edges as the bands more often than not do not run parallel to the base line. Moreover they often dip in the centre so other methods of detection are preferable e.g. spraying the whole plate with water. Bands can also be detected by exposive of the plate to iodine vapour but this method should not be used where fatty acid analysis of the separated lipids is to be carried out. Special absorbents are available which incorporate phosphors to enable observation of the separated bands. Kieselgel PF_{254} contains an inorganic substance which shows up conjugated molecules at 254 m/μ and Kieselgel $PF_{254+366}$ contains in addition an organic material whose fluorescence is quenched by the presence of any organic compounds. Unfortunately the phosphor is eluted by polar solvents e.g. chloroform: methanol:water so these materials can only be used in non-polar solvents.

Additional strains for TLC. Rhodamine 6G for phospholipids.

0.05 % Rhodamine 6G in 80 % ethanol. Spray and exaime under U.V. while the plate is still damp.

Acidic phospholipids give a blue flourescence

Neutral phospholipids (phosphatidylethanolamine, phosphatidyl choline gives an orange/yellow flourescence. Basic lipids give a yellow/green flourescence.

Dichloroflourescein for phospholipids.

0.02 % 2,7-dichloroflourescein in 80 % ethanol. View under U.V. Flourescence as above, but colours not as distinct.

Iodine

This can be sprayed onto the plate as an 0.5 % solution in chloroform.

Ninhydrin

0.3 % W/V ninhydrin in acetone stains amino-phospholipids, develop at room temperature 1 hour or 100°C, 5-10 mins. pink spots.

Contd.

Chemical degradation of lipids :

Lipids may be degraded by both acid and alkaline hydrolysis. Acid hydrolysis causes practically complete breakdown into constituent sugars, polyols, or inorganic phosphate. Alkaline hydrolysis with aqueous sodium hydroxide is an efficient procedure for releasing free fatty acids and alkali-stable glycosides; phosphate esters may be extensively degraded by this reagent. Mild alkaline methanolysis is a more suitable procedure for breaking up lipids into their water-soluble glycosides or phosphate esters and has the advantage that fatty acids are obtained directly as their methyl esters.

Methanolysis of lipids with sodium methoxide.

Lipid material (1 to 20 mg) is dissolved in $CHCl_3$-MeOH, 1:1 (5 ml) - treated with a 0.2N solution of sodium methoxide in methanol (5 ml). The resulting solution is kept at room temperature for 0.5 hour, water (10 ml) added, neutralised carefully by treatment with Dowex 50 resin (H^+ form), either by direct addition or passage through a short column and evaporated to dryness. The resulting material is partitioned between $CHCl_3$ and water using Whatman 1 PS filter papers or a separating funnel. The aqueous layer is evaporated to dryness and investigated by paper chromatography for phosphate esters (n-propanol-ammonia-water, 6:3:1) and glycosides (butanol-pyridine-water, 6:4:3) and by gas liquid chromatography for glycosides (as TMSi / ether). The Chloroform layer is used for fatty acid methyl ester analysis.

Contd.

Gas-liquid chromatography (GLC) has been the most significant analytical innovation in the lipid field and is the method of choice for the analysis of almost all single-chain aliphatic compounds. The method was introduced by James and Martin (1952).

Gas-liquid chromatography involves the physical separation of a movi gas phase by adsorption onto a stationary phase consisting of an inert soli such as silica gel or inert granules of ground firebrick coated with a nonvolatile liquid (e.g. lubricating grease or silicone oils). In, practice a glass or metal column is packed with the inert solid and a mixture of the methyl esters of fatty acids is evaporated at one end of the column, the entire length of which is kept at temperature of 170-225°C. A constantly flowing stream of an inert gas such as argon or helium keeps the volatiliz esters moving through the column. As with other types of chromatography, separation of the vaporized fatty acid esters is dependent upon the differ affinities of the components of the gas mixture for the stationary phase. Gases which are strongly attached to the stationary phase move through the column at a slower rate and therefore emerge at the end of the column late than those that are relatively less attracted. As the individual fatty acid esters emerge from the column, they are detected by physical and chemical means and recorded automatically as a series of picks which appear at different times according to the tendency of each fatty acid ester to be retained by the stationary phase. The area under each pick is proportionate to the concentration of a particular component of the mixture. The identity of each component is established by comparison with the gas chromatographic pattern of a related standard mixture of known composition. A detector of radioactivity may also be incorporated into the gas stream, together with the mass detector. Thus, a measure of the specific radioactivity of each

Contd.

component separated is obtained. The advantages of gas-liquid chromatography are its extreme sensitivity which allows very small quantities of mixtures to be separated, and the fact that the columns may be used repeatedly. Application of the technic has shown that natural fats contain a wide variety of hither to undetected fatty acids.

The fatty acid compositions of the total lipids and of phosphatidyl ethanolamine, which is the main phospholipid of V. cholerae and if possible of other lipids obtained from different strains of sensitive and resistant V. cholerae be determined by gas-liquid chromatography of the methyl esters, prepared by trans-esterification of lipid using 10 % boron trifluoride (Morrison and Smith, 1964). Measurements were made a series 204 chromatograph, Pye Unicam.

Under the following conditions, column packing, 15 % (W/V) ethylene glycol succinate (ESS) polyester on chromosorb W (80 to 100 mesh); length, 5 ft. (118 cm), with 4 mm internal diameter; column temperature 190°C; detector temperature, 210°C; argon flow rate, 30 to 60 ml min. The fatty acid methyl esters were identified by comparing their retention times with those of standard compounds of known purity. Relative proportions of the fatty acids would be calculated from their respective peak areas, estimated from peak height X peak width at half height.

Protein:

Proteins have been defined as extremely complex nitrogen containing organic compounds of high molecular weight which are found in all living cells and is the most important of all known substances in the organic kingdom. Proteins constiture a major part of the living protoplasm. In bacteria protein represent about 50 % of the dry weight of the cells but varies considerably with the age of the culture. The amino acids which make up bacterial protein are the same as those which make up proteins of other living cells. Bacterial major cell components are either composed of pro or protein units associated with other macromolecules. The amino sugar, muramic acid, has not been found in any biological polymers other than peptidoglycan of the cell walls of bacteria and the closely related blue algae. The peptides also contain unusual amino acids, e.g. D-alamine and D-glutamic acid. Proteins in all living organism are essential for growth and repair and also indispensible for almost all metabolic activities. An extensive and systematic analysis of proteins of different sensitive resistant strains of V. cholerae may give a better understanding of its structure and function.

Qualitative determination of protein:

(i) The biuret method:

The Robinson-Hagden biuret method with a simple modification by Stickland (1951) is applicable to whole microbial cells or to insoluble cell fraction such as cell walls, ribosomes etc.

(ii) Lowry method:

Folin-Ciocalteu (1927) is a more sensitive method for the determination of total protein content of microorganism. This requires as little as 50 µg of cells. A modification of this method by Lowry et al. (1951) may be adapted for the analysis of whole micro-organisms. This method is simpler to carry out than the biuret method and about sixty times sensitive and allows amounts of protein down to 10 µg to be determined. Even then it can not be considered an absolute method since different proteins give widely differing colour values and so rather less reproducible. In the method of Lowry et al. (1951) soluble protein are treated with a $NaOH-Na_2CO_3-CuSO_4$-tartrate reagent before adding the Folin-Ciocalteu reagent, while insoluble proteins are first dissolved in NaOH and then treated with a $Na_2CO_3-CuSO_4$-tartrate reagent.

Qualitative analysis:

The individual amino acids present in the extracts of micro-organisms can be identified by paper, thin layer or column chromatography. The presence of peptides is detected by comparing the number of amounts of amino acids present before and after hydrolysis with 6N hydrochloric acid for 16 hours at 105°C in a sealed tube and removal of the acid in vacuo; or unidentified spots on the chromatograms are eluted, hydrolysed and rechromatographed. Hot water extracts for chromatographic analysis are freed from cells by centrifugation and/or filtration through bacterial filters (simfered glass, Oxoid or Millipore membrane filters), desalted and concentrated. Removal of salts can be achieved by an electrolytic method but losses of certain amino

acids and the formation of ninhydrin-positive compounds may occur (Stein and Moor, 1951) and treatment with ionexchange resins is perhaps to be preferred. Mandelstam, (1958) passed aqueous extracts of E. coli through a column of Zeo-Karb 225 (H^+ form), washed the column thoroughly with water and eluted the amino acids with 1.5N ammonia; the elutes were taken to dryness, the residue dissolved in a small volume of water and chromatographed on Whatman No. 3 paper. (Block et al, 1955; Mangold et al, 1964; Randerath, 1963). Qualitative and quantitative analysis of amino acids in the pool of staph. aureus is described by Hancock (1958) who chromatographed concentrated extracts on columns of Dowex 50.

Qualitative and quantitative analysis of protein from all the strains under study using gas liquid chromatography may also be performed dependi on the facilities available.

Carbohydrate:

Carbohydrate is one of the essential components of bacterial cells. This carbohydrate content varies from about 4 to 24 percent, the concentration being markedly dependent on carbon excess in the medium. In addition to starch and glycogen, a variety of polysaccharide gums are formed, and complex polysaccharides, either free or associated with protein and lipids, are commonly present in the surface layers of the cells. These polysaccharides, which are in the form of highly polymerized units, are of considerable practical importance, conferring immunological specificity to the cell. Such polysaccharides have also been shown to be antigenic.

Quantitative determination:

All the common methods for the determination of total carbohydrate content of bacterial cells are calorimetric. They involve heating the material with strong (20N or more) sulphuric acid and a colour develope which is usually an aromatic amine or phenol. Two general methods would be followed for the determination of total carbohydrates of all strains under study. The anthrone method (Dreywood, 1946) approximates to a tota hexose method, giving identical colours (though not of equal intensity) with all hexoses, while other sugars give different and much weaker colo... The phenol method (Dubois, et al, 1956) approximates to a universal re for all types of sugar, including the carbohydrate residues of neuclei acids; this also gives identical colours with all hexoses. Usually the results obtained by the phenol reagent are always higher than those ob by the anthrone reagent, presumably the former reacts strongly with m sugars to which the latter is relatively insensitive.

Analysis of sugars by chromatographic procedures:

Complete analysis of the complex mixture of sugars requires separati of individual sugars by paper or column chromatography (Whistler and BeMiller, 1961; Hough, 1954; Hough and Jones, 1961; Lemienx, 1961; Cardel , 1958). Determination of sugars by gas liquid chromatography may be perfor d following the references by Kircher (1961) and Sweeby et al, (1966).

Lipopolysaccharide :

The cell wall Lipopolysaccharide of Gram-negative bacteria are acidic amphipathic macromolecules and function as a permeation barrier. This endotoxic component shows a wide spectrum of toxic and immunological properties They induce a number of metabolic, cellular and vascular changes in animals. According to recent report on the activities of Lipopolysaccharide from V. cholerae biotype El Tor, it was found responsible for pyrogenicity, lethal toxicity, local Shwartzman reaction and limulus lysate gelation (Raziuddin, 1978). Lipid A, is biologically the most active component of lipopolysaccharide from these bacteria and various biologicals characteristics of lipopolysaccharide are dependent on its lipid A moiety (Galanos, 1972; Galanos, 1977). It is a ubiquitous structure in bacterial endotoxins (Lipopolysaccharide) and most of the pathoplysiological reactions reside in the lipid A moiety. Many activities including lethal toxicity, pyrogenicity, complement inactivation, adjuvanticity and B lymphocyte mitogenicity has been demonstrated with free isolated lipid A. This covalently bound, ubiquitous lipid component was termed lipid A by Westphal and Luderitz in 1954 in order to differentiate it from loosely associated phosphatidyl ethanolamine (PE), termed lipid B. Recently it was shown that lipid A, when exposed on the surface of bacterial cells, acts as an immunogen, giving rise to the production of specific antilipid A antibodies. According to the structural similarities of lipid A among Enterobacteriaceaae, antilipid A antibodies cross react with lipid A obtained from otherwise serologically unrelated Lipopolysaccharide. These findings prompted investigations into the biological significance of lipid A antisera. Lipid A can be characterised by 3 main constituents: glucosamine, phosphate and long chain fatty acids. Glucosamine and phosphate constitute the hydrophilic backbone whereas

Long chain fatty acids give the hydrophobic property to the lipid A molecule. These long chain fatty acids remain attached to the hydrophilic backbone. Removal of fatty acids lead to preparations of reduced endotoxic activities.

No definite correlation of these biological activities of the lipopolysaccharide of these Gram-negative bacteria, in vivo and in vitro with the structure or function has yet been established. Neither the lipopolysaccharide of all these pathogenic bacteria has been characterized properly.

Extraction:

All strains under study extracted for lipopolysaccharide preparation by the phenol-water extraction method (Westphal, Jaun, 1965). The dry bacteria (10g) are suspended in 175 ml water warmed to 65°C. An equal volume of 90 % (W/V) aqueous phenol at the same temperature is then added and the mixture stirred vigorously for 5 minutes. It is then cooled rapidly and centrifuged at 3000g for 30 minutes at 0°C to permit phase separation. The upper aqueous layer is carefully removed and dialysed for 48 hours against running tap water to remove phenol. The non-diffusible solution contsins lipolysaccharide RNA, glycogen and lipopolysaccharide. The last of two can be removed by ultracentrifugation at 100,000g for 4 hours, when it is found as a clear viscous gel at the foot of the centrifuge tubes. The lipopolysaccharide is obtained as a clear viscous gel after ultracentrifugation at 100,000g for 4 hours. This material is carefully removed with a spatula, suspended inaa small volume of water and lyophilized. At this stage contaminating glycogen and nucleic acid are still present. Further purification can be obtained by dissolving the lipopolysaccharide in water to give a 1% (W/V) solution and repeating the ultracentrifugation. The gel is again recovered and lyophilized. One further repeatition of the ultracentrifugation procedure, making three in all, yields an almost pure product.

The polysaccharide may be obtained free from lipid by mild acid hydrolysis. The lipopolysaccharide is dissolved in 1 % (V/V) acetic acid to give a concentration of 1 % (W/V). The solution is gassed with nitrogen and hydrolysed under a atmosphere of nitrogen in a sealed vessel for 4 hrs at 100°C. The lipid A is deposited by low speed centrifugation. Contd.

The polysaccharide containing supernatant solution is dialysed and lyophilized. The product is essentially pure and comprises about 40 % of the lipopolysaccharide starting material. The lipopolysaccharide content of bacterial cells is much less variable in quantity than are the other cell components. Yields obtained by the phenol extraction technique are of the order of 0.5 - 2.0 % of the dry weight. In terms of pure lipid-free polysaccharide the yield is 0.2 - 0.8 % dry weight.

A light modification of the method described above for the preparation of lipopolysaccharide may also be attempted for a clarification of extraction. After the bacterial cultures had grown for a desired length, the culture is sterilized by addition of formaline to 0.4 % (final concentration, V/V). Leave the culture at 37°C for 3 - 4 hours, and then preferably in the cold for an additional 18 hours. The addition of formaline besides killing the bacteria serves to denature and seal the cytoplasmic membrane thereby reducing the risk for co-extraction of contaminating nucleic acids and glycogen.

The culture is palleted at 4,000 - 6,000 X g at +4°C for 15 minutes. The supernatant is discarded. The pellet is resuspended in phosphate buffered saline (PBS) and centrifuged again. The procedure is repeated twice. Finally the pallet is suspended in distilled water to about 100 mg/ml (wet weight) corresponding to about 20 mg/ml (dry weight). Then equal volumes of bacterial suspension and 75 % phenol (W/V), preheated to 68°C, are mixed and kept in a waterbath at 68°C for 30 minutes with occasional shaking. Cool to +4°C. The extraction mixture can be left at +4°C over night. Usually there is a partition in a top water and a bottom phenol phase - if not separation can be achieved by centrifigation at 6,000 X g

for 20 minutes at +4°C. Siphon off the water phase, which contains the lipopoly-saccharide, to a separation funnel.

Residual phenol in the water phase is removed by extraction with diethyl ether - equal volumes of ether and water phase are mixed and shaken for about 30 seconds. The ether phase is discarded. Repeat the procedure 3-6 times. Traces of ether the water phase are removed by bubbling air through the water phase. The water phase is dialysed against distilled water (1:10 ratio) with 2-3 changes at +4°C during 12-18 hours. The waterpphase is lyophilized and weighed.

An extensive analysis of lipopolysaccharide obtained from different strain under study is desirable. Quantitative determination of purified and crude lipo-polysaccharide from different strains would be followed the quantitative determination of free polysaccharide obtained by mild acid hydrolysis of lipopolysac-charide. Detail qualitative analysis of lipopolysaccharide, free polysaccharide, lipid A by column, paper, thin layer and gas liquid chromatography would be performed.

D. SIGNIFICANCE

An extensive biochemical investigation of resistant and sensitive strains of V. cholerae may provide some clue to sensitive cells becoming resistant to commonly used drugs. Study of individual components of two different types of strains may possibly reveal some correlation of bacterial resistance with cellular components. This study might help establishing a correlation between drug resistance of microbial organisms with their cellular lipid content and composition. A better understanding of their cell components leading to the function of individual components may also be expected. Detail information of resistant cells may also be helpful for immunological researches. If resistant strains are L-form, those may be better used for vaccine preparation. Rabbit immunization with cell components like LPS, lipid A, total lipid is also expected to show higher level of antibodies.

E. FACILITIES REQUIRED

1. Office space: None
2. Laboratory space: None
3. Hospital resources: None
4. Animal resources: 120 rabbits
5. Logistical support: None
6. Major items of equipments:
   a. Ultracentrifuge
   b. Gas liquid chromatography
   c. Polyacrylamide gel electrophoresis
   d. Spectrophotometer
   e. Chromatography kits
      i. Thin layer chromatography
      ii. Paper chromatography
      iii. Column chromatography

   Most of these equipments are available in this laboratory, except a few chromatography kits which has to be bought.
7. Other specialized requirements: Appointment of a new Research Technician.

F. COLLABORATIVE ARRANGEMENTS

A collaborative research program has been set with the Microbiological Chemistry Research Laboratory, University of Newcastle Tyne, England to perform a part of the work, which can best be done in that laboratory. This laboratory in England is very well equipped for analytical work of bacterial cell components. This laboratory also has a special set up

Contd.

for lipid methodology which is a major part of this protocol. Guidance and suggestion from the senior members of that laboratory would also help on the successful completion of the proposed study.

SECTION III - BUDGET

A DETAILED BUDGET

1. PERSONNEL SERVICES

NAME	POSITION	% TIME USED	ANNUAL SALARY	TAKA	DOLLARS
S.Q. Akhtar	Assistant Scientist	25 %	56,016	14,004	-
Dr. Hamidur Rahman	Veterinarian	10 %	42,824.23	4,282.4232	-
New employee	Research Assistant	100 %	30,808.8	30,808.8	-
Mr. Abdul Huq	Res. Technician	25 %	36,460.7	9,365.175	-
			Sub total	58,460.398	

2. SUPPLIES AND MATERIALS

Reagents, chemicals	20,000	-
Sub total	20,000	

3. EQUIPMENT

Chromatography Kits

(a) Thin layer chromatography kits - 5 $ 250 each	1,250
(b) Chromatographic columns (complete systems) - 5 $ 100 each	500
(c) Tanks for thin layer and paper chromatography	300
(d) Precoated analytical and preparative TLC plates and few other small chromatography tools	750
Sub total	2,800

Contd.

4. PATIENT HOSPITALISATION

None

5. OUTPATIENT CARE

None

6. IC DTR.B TRANSPORT

None

7. TRAVEL AND TRANSPORTATION OF PERSONS

	TAKA	DOLLARS
Per diem for P.I. to work in England 60 days		3,000
Expected date of travel November-December, 1980	Sub-total	$ 3,000

8. TRANSPORTATION OF THINGS

None

9. RENT, COMMUNICATIONS AND UTILITIES

Postage	Tk. 2,000
Telephone - None	
Cable - None	
Rent - None	
Sub-total	Tk. 2,000

10. PRINTING AND REPRODUCTION

Publication costs	Tk. 5,000
Sub-total	Tk. 5,000

Contd.

11. OTHER CONTRACTUAL SERVICES

None

12. CONSTRUCTION, RENOVATION AND ALTERATIONS

None

13. ANIMAL REQUIREMENT

120 rabbits		Tk. 15,000
	Sub total	Tk. 15,000

B. BUDGET SUMMARY

	CATEGORY	Year - 1		Year - 2	
		Taka	Dollar	Taka	Dollar
1.	Personnel	58,460	-	64,306	-
2.	Supplies	20,000	-	20,000	-
3.	Equipment	-	2,800	-	-
4.	Patient Hospitalisation	-	-	-	-
5.	Outpatient care	-	-	-	-
6.	ICDDR,B Transport	-	-	-	-
7.	Travel Persons	-	3,000	-	-
8.	Transportation things	-	-	-	-
9.	Rent/Communication	2,000	-	2,000	-
10.	Printing & reproduction	5,000	-	2,000	
11.	Contractual service	-	-	-	
12.	Construction	-	-	-	-
13.	Animal requirement	15,000	-	-	-
	Total	100,460	5,800	88,306	
			6,697		5,88
	Grand Total		12,497		5,88
			12,497		
			5,887		
	Total for 2 (two) years		$ 18,384		

REFERENCES

Anderes, E.A., W.E. Sandine, and P.R. Elliker, (1971). Can. J. Microbiol. 17:1357-1365.

Bauer, A.W., W.M. Kirby, J.C. Sherris, and M. Truck. (1966) Am. J. Clin. Pathol. 45:493-496.

Benbongh, J.E. and G.A. Morrison, (1967). J. Gen. Microbiol. 49:469-485.

Bishop, E.A. and M.A.C. Berminghan, (1973). Antimicrob Agents Chemother. 4:378-379.

Bligh, E.G., and W.J. Dyer, (1959)Can. J. Biochem. Physiol. 37:911-917.

Block, R.J., Durrum, E.L., and Zweig, G.(1955). A Manual of Paper Chromatography and Paper Electrophoresis Academic Press, London.

Brown, M.R.W. and Wood, J.M. (1972) J.Phar. Pharmac. 24:215-218.

Brundish, Shaw, N. and Baddiby, J. (1966).

Cho, K.Y. and M.R.J. Salton. (1966) Biochim. Biophys. Acta. 116:73-79.

Cohen, M. and Panos, C. (1966). Biochem. 5:2385-2392.

Dean, A.G., Ching, Y.C., William, R.G., Harden, L.B. (1972) J. Infect. Dis. 125:407-411.

De Pamphilis, M.L. and T. Adler, (1971). J. Bact. 105:376-385.

DiRienzo, J.M., K. Nakamura and M. Inouya. (1978). Ann. Rev. Biochem. 47:481-532.

Dittmer, J.C. and R.L. Lester (1964). J. Lipid Res. 5:126-127.

Dubois, M., Gilles, K. et al. (1956). Anal. Chem. 28:350.

Dunnick, J.K. and W.M. O'Leary. (1970). J. Bacterial. 101:892-900.

Few, A.V. (1955). Biochim Biophys. Acta. 16:137-145.

Folch, J.M., Lees, and G.H. Sloane stanley. (1957). J. Biol Chem. 226: 497-507.

Franklin, T.J. (1967). Biochem. J. 105:371-378.

Franklin. T.J., and A. Godfrey. (1965). Biochem. J. 94:54-60.

Gabrielli, G. (1956). Nuovi Ann 1g. 7:146.

Galanos, C., Freudenberg, M., Hase, S., Jay, F., Ruschmann, E. In D. Schlessinger (ed). Microbiology 1977. Am. Soc. Microbiol. Washington D.C. 1977. p. 269-276.

Galanos, C., O.Luderitz, E.T. Rietschel and O.Westphal. International Review of Biochemistry. Biochemistry of Lipids II.Vol. 14. Edited by T.W. Goodwin. Copy right 1977. Univ. Park Press, Baltimore.

Galanos, C., Rietschel, E.T., Luderitz, O., Westphal, O., Kim, Y.B., Watson, D.W. (1972). Eur. J. Biochem. 31:230-233.

Gallin, J.E., D. Kaye, and W.M. O'Leary. (1969). New Eng. J. Med. 281: 1081-1086.

Geyer, R., C. Galanos, O. Westphal, J.R. Gotecki (1979). Eur. J. Biochem. 98:27-28.

Guerrant, R.L., Brunton L.L., Schnaitman L., Rebhum 1. Gilman A.G.

Hancock, R. (1958). Biochim. Biophys. Acta 28:402-412.

Hugo, W.B., and R.J. Stretton. (1966). J. Gen. Microbiol. 42:133-138.

Hugo, W.B. and J.R. Davidson. (1973). Microbios. 8:63-72.

Hugo, W.B. and 1. Franklin. In Abstract of papers presented at the 9th International Congress for Microbiology, Moscow. No. A2/10. 1966. p.21.

Huq M.I., and Ellinga, G. (1978). Cholera Res. Lab. Bangladesh. Sci. Report No. 15.

Imai, M., Inoue, K., and Nojima, S. (1975) Biochim. Biophys. Acta. 375: 130-137.

Izaki, K., K.Kiuchi, and K. Arima. (1966). J. Bacteriol. 91:628-633.

Kabir, S. ( ). Microbios. 20:47-62.

Kabir, S. (1980). ICDDR,B. Protocol.

Kennedy, J.R. and S.H. Richardson (1969). J. Bacteriol. 100:1393-1401.

Kligler, 1. J. (1917). Amer. J. Pubb. Helh. 7:1042.

Laskin A.1 and W.M. Chan. (1964). Biochim. Biophys. Res. Comm. 14:137-142.

Lowry, Rosebrough, Farr and Randall. (1951) J. Biol. Chem. 193:265.

Luderitz, O., Galanos, C., Lehmann, V., Nurmineu, M., Rietschel, E.T., Rosenfelder G., Simon, M., Westphal, O. (1973). J. Infec. Dis. 128 (Suppl). S17-S29.

Mackel, D.C., Langley, L.F. and Venice, L.A. (1961). Ann. J. Hug. 73: 219-223.

Mangold. H.K., Schmä, H.H.O., and Stahl, E. (1964). Meth. Biochem. Anal. 12:393-451.

Marinetti, G.V. (1964). In New Biochem. Separations. ed. A.M. James and L.J. Morris London. Van Nostrand.

Maxwell and Williams. (1967). J. Chroma. 31:62.

Metcalfe, L.D., and A.A. Schmitz. (1961). Anal. Chem. 33:363-364

Miller, 1. L., Zsigray, R.M. and Landman, O.E. (1967). J. Gen. Microbiol. 49:513-525.

Molander, C.W., Kagen, B.M., Weinbergey H.J., Heimlich, E.M. and Busser, R.J. (1964). J. Bacteriol. 88:591-594.

Molenkamp, G.C. and Veerkanp, J.H. (1976). Antimicrob. Agents Chemother. 10:786-794.

Monsur, K.A. (1961). Trans. Roy, Soc. Trop. Med. and Hug. 55:440-445.

Norrington, F.E. and A.M. James. (1970). Biochim. Biophys. Acta. 218: 269-277.

Okamato, S. and D. Mizuno. (1962). Nature (London). 195:1022-1023.

Okamoto, S. and D. Mizuno. (1964). J. Gen. Microbiol. 35:125-133.

O'Leary, W.M. (1967). The chemistry and metabolism of microbial lipids. The world Publishing Co. New York.

Panos, C., Cohen, M. and Fagan, G. (1966). Biochem. 5:1461-1468.

Randerath, K (1963). Thin layer chromatography. Academic Press, London.

Raziuddin, S. and T. Kawasaki. (1976). Biochem. Biophys Acta 431:116-12

Raziuddin, S. and Ambegaokar, S.D. (1976). Indian. J. Biochem. Biophys 13:57-61.

Raziuddin, S. (1978). Immunochem. 15:611-614.

Raziuddin, S. (1979). J. InfoDis. 140:590-595.

Raziuddin, S. (1977). Biochem. J. 167:147-154.

Redmond, J., Lehman, V., In. D. Schlessinger (ed). Microbiology, 1977. Am. Soc. Microbiol. Washington D.C. p. 262-263.

Rietschel, E.T., Galanos. C., Luderitz, O. In D. Schlessinger (ed). Microbiology - 1975. Am. Soc. Microbiol. Washington D.C. 1975. p. 307-31

Rietschel, E.T., Kim, Y.B., Watson, D.W. Galanos, C., Luderitz, O., Westphal, O., (1973). Infec. Immun. 8:173-177.

Rietschel, E.T., Hase, S.,King.M.,

Rietschel, E.T., Galanos, C., Tanaka, A., Ruschman, E., Luderitz, O., Westphal. O., (1971). Eur. J. Biochem. 22:218-224.

Robinson, H.W. and Hogden, C.G. (1940). J. Biol. Chem. 135:707-725.

Rotta, J., Karakawa, W.W. and Krause, R.M. (1965). J. Bacteriol. 89: 1581-1585.

Salton, M.R.J. (1964). The bacterial cell wall. Elsevier Publishing Co. New York.

Schnaitman, C.A. (1971). J. Bacteriol. 108:545-552.

Sereny, B. (1955). Acta. Microbiol. Acad. Sient. Hungarical. 2:293-296.

Shaw, N. (1968). Biochim. Biophys. Acta. 164:435-436.

Shockman, G.D. (1965). Bacteriol. Rev. 29:345-357.

Siakotos and Ronser, (1965). J.A.OC.S. 42:913.

Simmler, C. and Baru, E. (1970). Ann. Inst. Pasteur. 119:289-301.

Smith and Freeman (1959). J. Dairy Sci. 42:1450.

Smith, H.L. and Goodner, K. (1958). J. Bacteriol. 76:662-663.

Starka, J. and Moravova, J. (1970). J. Gen. Microbiol. 60:251-257.

Stickland, H.L. (1951). J. Gen. Microbiol. 5:698-703.

Sulong, W.J. and W.M. O'Leary. (1977) Can. J. Microbiol. 23:1045-1051.

Sulong, W.J. and W.M. O'Leary (1975). Antimicrobiol. Agents chemother. 8: 334-343.

Vaczi, L., J. Szila, and V. Cilleszky. (1957). Acta. Microbiol. Acad. Sci. Hung. 4:437-445. (Abstracted in Biol. Abst. 32:41517:1958).

Ward, J.B. and Perkins, H.R. (1968). Biochem. J. 100:391-399.

Ward, J.R., Madoff, S. and Dienes, L. (1958) Proc. Soc. Expt. Biol. Med. 97:132-135.

Wells and Dittmer (1963). Biochem. 2:1259.

Westphal, O., Jann. K. In R.L. Whistler (ed) Methods in carbohydrate Chemistry. Vol. 5 Academic Press. New York. 1965. p. 83-85.

White House, M.W., Bresler, A.E. and Staple, E. (1958). J. Chromatogra. 1:385.

Williams, R.E.O. (1963). J. Gen. Microbiol. 33:325-334.

Woodford, F.P. and C.M. Van Gent. 1960. J. Lipid Res. 1:188-190.