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JOHN A. M. SHAW

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Royal College of Science and Technology,
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STUDIES ON PYROGENIC FACTORS FROM PROTEUS VULGARIS

C O N T E N T S

PREFACE

The Gram-negative bacteria have, for more than half a century, attracted the attention of workers in various related fields because of the characteristic properties of the substances which can be extracted from their cells and from the culture medium in which the cells have been grown. The pronounced physiological changes produced in the animal body when these substances gain access to the blood stream are of interest to the physiologist and pathologist as a means of investigating the genesis of fever in infectious disease, to the immunologist in the study of non-specific resistance to infection and to the biochemist in studies of the relationship of chemical groupings to biological effects.

Although many workers have studied various properties of materials extracted from the bacterial cells and, to a lesser extent, those of the materials in solution in the culture fluid, no serious comparison of the relationship between the products from both sources appears to have been made. So far, it is not clear if the biological activity of the cell-freed culture fluid is due to the presence therein of an excretory product of bacterial metabolism, or

to a cellular component which has been produced by enzymic action or by mechanical removal from the cell surface.

The aim of the present investigations was to isolate, purify and characterise the physiologically active factors produced by Proteus vulgaris when grown in a simple fluid medium and to compare them with the active material obtained by extraction from the cells of the same strain of organism. The ability to produce fever in animals was mainly used as an index of physiological activity of the materials during their isolation and purification since this effect is probably the most sensitive of all the indices of such activity and certainly the easiest to measure.

Proteus vulgaris was chosen as the Gram-negative organism for study as it is relatively non-pathogenic, produces a highly pyrogenic cell-free culture filtrate and grows well in a simple medium. Moreover, the pyrogenic factors of this organism do not appear to have been studied in detail.

INTRODUCTION

'Pyrogen (πῦρ - FIRE + γεν - TO PRODUCE): a fever-producing substance; a hypothetical substance believed to exist in bacteria and to cause fever when present in the body.' The Illustrated Medical Dictionary, 20th edition, 1944. W. B. Saunders and Co. Ltd., Philadelphia.

Towards the close of the 19th century and during the early 20th century, when the intravenous route of administering medicine was being increasingly used, many substances were observed to produce fever on injection. That this property was assigned to the constituents of the injection is evident from the use of such terms as 'salt fever'[145] and 'Salvarsan fever'.[256] Subsequent investigations traced the cause of the fever to the diluent or vehicle used in preparing the injections and culminated in the work of Siebert[216,217] and Rademaker[190] who proved conclusively that the various injection fevers had a common cause, namely, the presence in the injection of a fever-producing factor produced by bacterial contamination. The name 'Pyrogen'[38] or 'Bacterial Pyrogen' was, and still is, used when referring to this fever-producing or pyrogenic substance, and it is now generally accepted that the

Gram-negative bacteria are the most fruitful source of such a substance,[34,115,262] although observations have been made of the ability of suspensions, culture filtrates[20,46,50,2] and lysates[223] of streptococci and other Gram-positive organisms to produce fever on injection.

Gram-negative bacteria, or their products, cause many reactions other than fever, and over the years there has accumulated a vast literature on the biological effects of toxic fractions from these bacteria. The name given to the toxic fraction in some cases reflects the particular biological phenomenon under investigation e.g. 'tumor-necrotising agent', 'Shwartzman toxin', while in others it relates to the chemical nature of the fraction e.g. 'lipopolysaccharide', 'glyco-lipid'. In recent years, it has become apparent that these biological effects can be attributed to a single type of component common to Gram-negative organisms and which is closely associated with the endotoxin.

Although there have been few studies aimed at correlating the various biological properties of endoxotins, it is generally assumed that the pyrogenic response reflects the general endotoxic properties as measured by other host reactions. Recently (1961), Landy and his co-workers[138]

have examined the febrile response as a method for the bioassay of endotoxins and have concluded that, especially in rabbits, this response as measured by the fever index provides a highly consistent assay procedure.

Evidence is available that substances other than those of bacterial origin may in themselves be pyrogenic. Thus colloidal calcium phosphate of certain surface charge and particle size[30] and methyl cellulose of certain molecular weight[367] produce a febrile response in the absence of bacterial contamination, and lysergic acid diethylamide has been shown to be pyrogenic in rabbits at a dose-level of 1-10$\mu g/kg$.[114] More recently, it has been reported that certain C_{19} and C_{21} steroids with a 3α-hydroxy, 5β configuration produce fever.[155] The pyrogenic potency of these materials is, however, low compared with that of materials obtained from bacteria.

Certain polysaccharides recently isolated from plant and animal tissues were reported to possess pyrogenic and other endotoxic activities[164] but later it was found that variations in the technique used in preparing the polysaccharides had permitted bacterial growth and only materials devoid of such activity were obtained under conditions in which exclusion of bacteria was demonstrated.[166]

The local inflammation and oedema produced by intramuscular injection or intrapleural instillation of turpentine is accompanied by a febrile response and extensive investigations of this phenomenon by Menkin[1,164] resulted in the isolation of a fever-producing euglobulin which Menkin termed 'Pyrexin' and which he postulated to be the active humoral mediator of fever associated with inflammation.[165] It is not certain, however, that the fever produced by 'Pyrexin' is not largely a result of contamination with extraneous pyrogenic substances introduced during the purification of the material.[21]

Since the early literature dealing with bacterial pyrogens has been adequately reviewed on several[4,20,70,228,26] occasions, the first part of this thesis will be concerned mainly with the more recent literature. An account will be given of the work which has resulted in the isolation and chemical characterisation of pyrogenic materials of considerable purity. The suspected mechanism of the febrile response to these materials will be discussed and reference made to some of their other biological properties.

The second part will consist of a report of the experimental work done by the author and will be followed by a discussion of the experimental results.

PART I

A REVIEW OF RECENT LITERATURE DEALING WITH THE ENDOTOXINS OF GRAM-NEGATIVE BACTERIA.

Section 1. The Chemistry of Bacterial Endotoxins.

From the investigations of Boivin and Mesrobeanu,[55,167] Morgan and Partridge,[173,175,190] Miles and Pirie[160] and Goebel,[9,28,99] it is now known that many species of Gram-negative bacteria contain a similar surface component made up of phosphorylated polysaccharide, protein and phospholipid residues. These complexes are referred to as endotoxins or 'O'-somatic antigens since, in addition to being toxic and pyrogenic, they are frequently the dominant immunological components of the cell surface. Westphal and his colleagues,[247,250] and others,[67,68] have shown that two different types of lipid are present, one of which, a toxic lipid referred to as lipid A,[247] is firmly bound to the polysaccharide moiety as a lipopolysaccharide and on liberation by acid hydrolysis is soluble only in chloroform and pyridine. The other, a biologically-inert, ether-soluble, cephaline-type lipid,[175] referred to as lipid B,[247] is easily removed by dissociation with formamide[175] and other solvents[67,160] without affecting any of the known immunological or toxic properties of the endotoxic complex.[67,68] If, after removal of lipid B, the protein-lipopolysaccharide (polysaccharide + lipid A) complex is dissociated by treatment with 90% phenol,[90,175,188] hot aqueous phenol[249]

or by alcohol fractionation in the presence of high salt concentrations,[237] the resulting protein-free lipopolysaccharide retains the toxic, pyrogenic and other related biological effects of the endotoxin[76,77,79,150,207,250] but is usually,[59,60,68,176] though not invariably,[151] only weakly antigenic as judged by its ability to stimulate the production of agglutinins and precipitins in rabbits, whereas the protein-lipopolysaccharide complex is a powerful antigen in this respect.[68] The protein component thus appears to be mainly concerned with antigenicity, although Goebel and Barry[98] have shown that colicine K, the bactericine of Escherichia coli K 253, is associated with the protein component of the 'O'-somatic antigen of this organism. The serological specificity of the 'O'-antigen is, however, in all cases retained by the lipopolysaccharide and is determined by the polysaccharide moiety.[55,175] Thus various workers[59,60,175,176,190,247] have demonstrated the ability of non-antigenic lipopolysaccharides to couple with proteins derived from heterologous Gram-negative bacteria, producing antigenic conjugates which carry the serological specificity of the homologous antigen. The only other biological property associated with the polysaccharide moiety is that of a bacteriophage receptor[239]; for example, the affinity of T4 phage for the lipopoly-

saccharide of Shigella sonnei appears to be determined by the polysaccharide moiety.[151] From the investigations of Goebel, Morgan, Westphal and their co-workers, it is clear, however, that while the antigenicity and serological specificity of the endotoxins are determined by the protein and polysaccharide components, the toxic and pyrogenic effects are associated with the lipid A moiety. Removal of this moiety from the complex also removes the toxic and pyrogenic properties leaving specific degraded polysaccharide and a simple amphoteric protein.

The existence of an active grouping (" T") responsible for the toxicity of the Shigella paradysenteriae endotoxin was first demonstrated by Goebel and his co-workers.[28,236] On acid hydrolysis of the endotoxin they obtained a non-toxic polysaccharide and a toxic protein (protein T), whereas by alcohol fractionation of an alkaline solution of the endotoxin they obtained a non-toxic protein and a toxic polysaccharide (polysaccharide T). The toxic factor " T" was thus associated with either polysaccharide or protein according to the degradative procedure and although Goebel did not study its chemical nature, subsequent investigations indicate that it was probably the lipid A moiety of the endotoxin.[246,247,266] Davies and Morgan[68,68]

for example, have shown that mild acid hydrolysis of the 'O'-antigen of Shigella dysenteriae yields a specific degraded polysaccharide and a lipoprotein, the lipid component of the latter resembling lipid A. On the other hand, extraction of the antigen with hot aqueous phenol[68] yields lipid A bound to the specific polysaccharide as lipopolysaccharide. Moreover, Westphal and his colleagues have been able to combine toxic pyrogenic lipopolysaccharides with inert proteins such as casein and serum albumin forming artificial protein-lipopolysaccharide complexes which, on acid hydrolysis under the same conditions as used by Goebel above, yield artificial toxic and pyrogenic lipoproteins in which only the lipid A moiety is of bacterial origin,[162,234,247] (Fig.1).

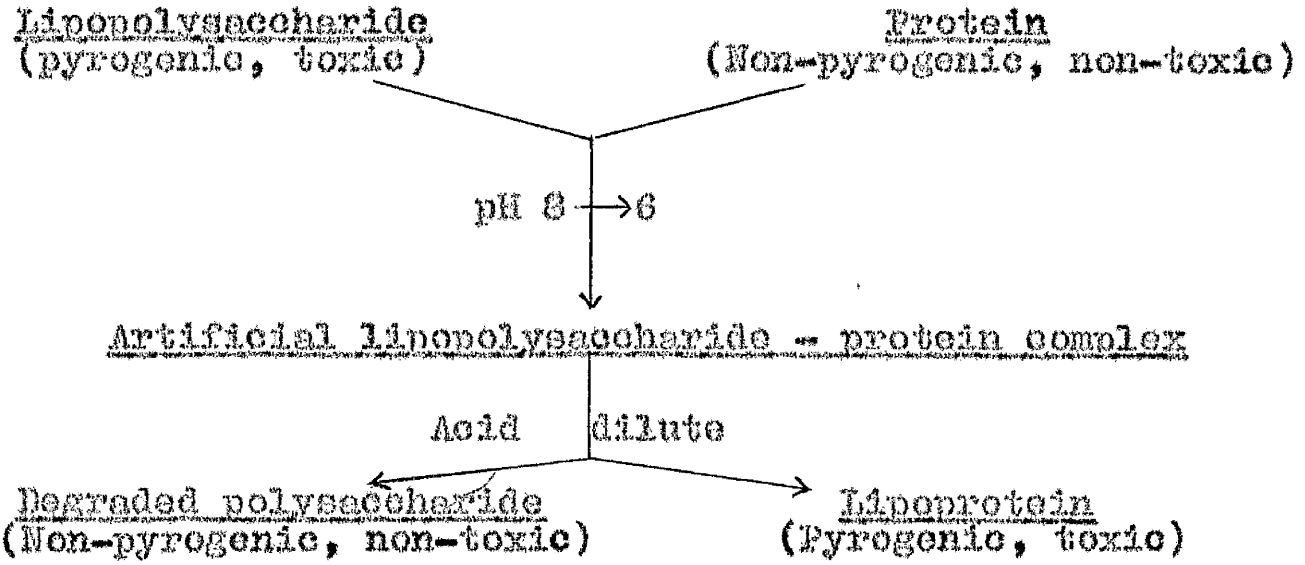


Fig.1. The combination of active lipopolysaccharide with an inert protein to form an artificial lipopolysaccharide-protein which, on acid hydrolysis, yields a pyrogenic and toxic lipoprotein.[162]

Since the degraded polysaccharide split off from the artificial complex during the acid hydrolysis is non-toxic and non-pyrogenic, it appears that the lipid component of the lipopolysaccharide is the important one for pyrogenic and toxic activity and that the polysaccharide, functioning only as a water-solubilising carrier, may be replaced by an otherwise biologically inert protein.

The evidence, therefore, suggests that in the isolated endotoxic complex the specific polysaccharide and protein components serve as water-solubilising carriers to disperse the otherwise water-insoluble active lipid A moiety; depending on the method of degradation of the complex, this moiety may remain associated with either the polysaccharide or the protein, giving toxic and pyrogenic lipopolysaccharide or lipoprotein. It has also been shown that most Gram-negative bacteria produce, in addition to the lipopolysaccharide-protein complex, some lipoprotein, probably as a result of a surplus in lipid A synthesis over carbohydrate production by the organisms, the surplus being bound to a protein carrier only.[85] These lipoproteins and the artificial lipoprotein referred to above are, however, much less active pyrogenically than the lipopolysaccharides, suggesting that the degree of activity is

related to the particle structure and that protein is a less suitable carrier for dispersing the lipid than is polysaccharide.

The influence of the degree of dispersion of lipid A on its pyrogenic activity is also shown if the lipid is separated by acid hydrolysis from either the lipopolysaccharide or the lipoprotein, when it occurs as a waxy material which, because of its insolubility, is only active at dose levels upwards of 100μg/kg. Dispersion of the isolated lipid in water by means of surface-active agent such as Tween[242,247] results in an increase in activity as shown in Table 1. A further increase in activity is obtained when lipid A is dispersed in a solution of low molecular weight dextrans or combined with protein as an artificial lipoprotein[243] (Table 1).

Dispersion of lipid A	Pyrogenic dose for rabbits
Suspension in water	100 μg/kg.
Dispersed in solution of Tween	1-10 μg/kg.
Dispersed in solution of low molecular weight dextrans	0.01 μg/kg.
Artificial lipoprotein	0.01 μg/kg.
Lipopolysaccharide	0.002 μg/kg.

Table 1. Effect of dispersion of lipid A on its pyrogenic activity.

Comparison of the pyrogenic potencies of the preparations given in Table 1 indicates that the lipopolysaccharide presents the lipid A moiety in a physico-chemical condition associated with maximum biological activity. Howard, Rowley and Wardlaw,[119] have also found that the isolated lipid A from Escherichia coli lipopolysaccharide, when dispersed in Aerosol OT or otherwise finely suspended in water, retains the property of the original lipopolysaccharide in stimulating the production of non-specific immunity, while the degraded polysaccharide is inert in this respect. Here too, the more effective dispersion of the lipid as a macromolecular lipopolysaccharide is indicated by the superior protective activity of the intact lipopolysaccharide over that of the artificially dispersed lipid A, the latter being about 1/10th as active on a weight basis. Again, Weter and his co-workers[180] have recently (1960) shown that the altered dermal reactivity of rabbits to epinephrine produced by Escherichia coli endotoxin is due to the lipid A component, the original lipopolysaccharide being approximately ten times more potent than the lipid A fraction obtained therefrom.

In recent years, the lipopolysaccharides of Gram-negative bacteria have been the subject of extensive

investigations since, in addition to their association with lipid A, they retain serological specificity in the polysaccharide moiety and furthermore they can be obtained in a highly purified state from a wide range of bacteria by the phenol-water process of Westphal and his colleagues.[161,249,250] In this process, the protein and lipid B components of the somatic antigen are dissociated, leaving a lipopolysaccharide which, because of its larger particle size, can be conveniently separated from ribonucleic acid by high-speed centrifuging. Lipopolysaccharides have been obtained in this way from a number of bacterial species, including Pasteurella pestis[59] from which diethylene glycol and trichloracetic acid generally fail to extract active materials, and it appears to be only method of extracting from rough strains, lipopolysaccharides which are as toxic and pyrogenic as those from corresponding smooth stains.[247]

Gentle acid hydrolysis of the lipopolysaccharide (or somatic antigen) results in the separation of acid - insoluble lipid A (or lipoprotein), leaving in solution a species-specific degraded polysaccharide hapten; the latter separated from solution and purified, is often employed in studies of polysaccharide constitution as it

is readily water-soluble and so more amenable to investigations of the homogeneity essential when relating chemical constitution to serological specificity.

The polysaccharides of Gram-negative bacteria, especially those of smooth strains, are generally more complex in composition than the specific polysaccharides of Gram-positive bacteria. In addition to the commonly occurring aldohexoses (D-glucose, D-galactose, D-mannose) and hexosamines (D-glucosamine, D-galactosamine), they frequently contain methylpentoses (L-rhamnose, L-fucose) and aldopentoses (D-xylose). With the development of new methods of extraction and purification of the polysaccharides and the application of modern analytical methods, especially paper chromatography, several additional monosaccharides have been identified. Aldoheptoses, for example, which until recent years were unknown in natural products have been identified in lipopolysaccharides and specific degraded polysaccharides from Shigella,[66,152,218] Bordatella,[176] Chromobacterium[38,61,177] Pasteurella,[59,60,93,178] and Salmonella[92] species, Escherichia coli strains[94,240] and in isolated Proteus vulgaris cell walls.[211]

Observations by Westphal[244,252] and Fon and Staub[198] of chromatographically fast-moving acid-labile sugars in the hydrolysates of specific polysaccharides from Salmonella species led to the discovery of a new series of sugars, the 3:6-dideoxyaldohexoses.[86,91,93,245] Five members of the series, representing three of the four possible configurations, have so far been isolated from polysaccharides of Gram-negative bacteria. Three of these, abequose, tyvelose and paratose have been found in specific polysaccharides of various Salmonella species[88,198,247,248] and their identity confirmed by synthesis.[89,91] Outside the Salmonella polysaccharides the only sources of these three sugars are the lipopolysaccharides from Pasteurella pseudotuberculosis group I (paratose), group II (abequose) and group IV (tyvelose).[56,60] Colitose, the optical isomer of abequose, has been isolated from the specific polysaccharide of an Escherichia coli strain,[100] and ascarylose, the optical isomer of tyvelose, from the specific polysaccharide of Pasteurella pseudotuberculosis group V.[66] The nomenclature and sources of these sugars are summarised in Table 2.

Sugar	Source of parent polysaccharide	Reference
3:6-dideoxy-D-ribo-hexose (paratose)	Salmonella paratyphi A.	69
	Pasteurella pseudo-tuberculosis Group I	56, 60
3:6-dideoxy-D-arabino-hexose. (tyvelose)	Salmonella Group D. e.g. S. typhosa	198, 247, 248
	S. enteriditis	248
	Pasteurella pseudo-tuberculosis Group IV.	56, 60
3:6-dideoxy-D-xylo-hexose. (abequose)	Salmonella Group B. e.g. S. paratyphi B.	247, 248
	S. abortus equi	247, 248
	Pasteurella pseudo-tuberculosis Group II.	56, 60
3:6-dideoxy-L-xylo-hexose. (colitose)	Escherichia coli strains.	160
3:6-dideoxy-L-arabino-hexose. (ascarylose - so called as it was first isolated from cell-walls of Ascaris eggs (parascaris equorum[sic]).	Pasteurella pseudo-tuberculosis Group V.	65

Table 2. Sources and nomenclature of the 3:6-dideoxyaldo-hexoses.

The rapidity with which these sugars are liberated from their polysaccharide by acid hydrolysis suggests that they occupy terminal positions in the polymer chain and thus

contribute to the serological specificity of the parent polysaccharides. It has been shown that abequose specifically inhibits the precipitation of Salmonella paratyphi B polysaccharide and tyvelose the precipitation of Salmonella typhosa polysaccharide by their respective homologous antisera;[69,221] furthermore, colitose effectively inhibits the precipitation of Escheriohia coli polysaccharide by its homologous antiserum, whereas no inhibition in this system is obtained with abequose, the optical isomer of colitose. The implication of the dideoxy-aldohexoses in serological specificity is also indicated in the recorded serological cross-reaction between Pasteurella pseudotuberculosis group II and Salmonella of Group B where abequose is a common sugar,[56,60] and between Pasteurella pseudotuberculosis group IV and Salmonella of Group D[230,231] where tyvelose is a common sugar.[56,60]

3:6-Dideoxyaldohexoses and 6-deoxyhexoses (rhamnose and fucose) appear to be characteristic of smooth strains since it has been shown that in the lipopolysaccharides derived from pairs of genetically related smooth and rough strains, these sugars where present in the 'Smooth' poly-saccharide are always missing in the polysaccharide of the respective rough variant.[60,65,72,248] The loss of

'O'-specific characteristics which occurs during the S → R change is generally assumed to be due to loss of Smooth 'O'-somatic antigen thereby exposing an underlying 'Rough' somatic antigen as a distinct chemical entity; being devoid of deoxy sugars it would thus have a different specificity. Davies, however, suggests that " it is likely that the 'Rough' polysaccharide is the skeleton of the 'Smooth' one, with side chains lacking"[60,64]

The presence of N-acetylglucosamine and N-acetylgalactosamine in the endotoxic lipopolysaccharides is well established, and recently a new aminodeoxyhexose, 2-amino-2:6-dideoxy-D-galactose(D-fucosamine), as the N-acetyl derivative, has been found in the specific polysaccharide of Chromobacterium violaceae.[65] Of these amino sugars, N-acetylglucosamine,which is found in all the lipopolysaccharides, is a constant constituent of lipid A but may on occasions be present also in the corresponding lipid-free degraded polysaccharide. In Salmonella typhosa lipopolysaccharide for example, this sugar is present in the lipid A component only, while in various Shigella lipopolysaccharides it is found in both the lipid A and degraded polysaccharide moieties.[90,247,261] On the other hand, where an amino sugar other than glucosamine is present

in the lipopolysaccharide, it appears to be associated with the polysaccharide moiety, since so far only glucosamine has been found in lipid A.[64]

In a recent review of the polysaccharides of Gram-negative bacteria, Davies has discussed the isolation and purification of the polysaccharides, identification of the monosaccharide units and the relationship of sugar constitution to immunological specificity. In addition, detailed tables are given of the known sugar components of the specific polysaccharides from the Enterobacteriaceae and other Gram-negative genera.[64]

Lipid A.

The lipid A component, which constitutes 25-45% of Gram-negative bacterial lipopolysaccharides,[59,136,152,169,247] is a complex phospholipid, readily soluble in chloroform and pyridine but slightly, if at all, soluble in other lipid solvents. Being firmly bound to be polysaccharide fraction of the lipopolysaccharide, it is only liberated from the latter by hydrolysis with dilute hydrochloride acid,[247] dilute acetic acid[90,175] or by treatment of the lipopolysaccharide with cationic ion exchangers.[183] The analysis of lipid A preparations from various lipopolysaccharides has revealed a close similarity between them;

they all appear to contain about 50% fatty acids, 15-20% D-glucosamine and 7-8% phosphoric acid esters, together with amino acids and other components.[345,347] Prolonged hydrolysis with a strong mineral acid (usually 8-10 hours with 5N hydrochloric acid) is required to set free the lipid constituents; extraction of the hydrolysate with ether separates the ether-soluble fatty acids from the water-soluble glucosamine, phosphoric acid esters and amino acids. Nieman and his co-workers[124] have shown that the phospholipid moiety of their Escherichia coli lipopolysaccharide, when hydrolysed in this way, also gives an ether and water-insoluble fraction which, on treatment with aqueous sodium hydroxide followed by solution in ether and subsequent precipitation with methanolic hydrogen chloride, yields the crystalline hydrochloride of the diamine, 4:5-diamino-N-eicosane. This diamine, to which Neimann has given the name 'necrosamine' and which has subsequently been synthesised,[126] is the first derivative of long-chain aliphatic hydrocarbons to be found in natural products.

Constituent fatty acids of Lipid A.

Nowotny and Westphal,[185] by chromatographic analysis of the fatty acids liberated by hydrolysis from lipopoly-

saccharides derived from eleven different species of Gram-negative bacteria, have shown that the same series of fatty acids is present in each case. In addition to the saturated fatty acids, capric, lauric, myristic, palmitic, arachidic, lignoceric and possibly behenic, the series includes oleic acid and a chromatographically fast-moving acid which appears to be a β-hydroxy acid. Niemann and his co-workers have also isolated a β-hydroxy acid from their Escherichia coli phospholipid, and confirmed its identity, as β-hydroxy-myristic acid, by comparison with synthesised acid.[185]

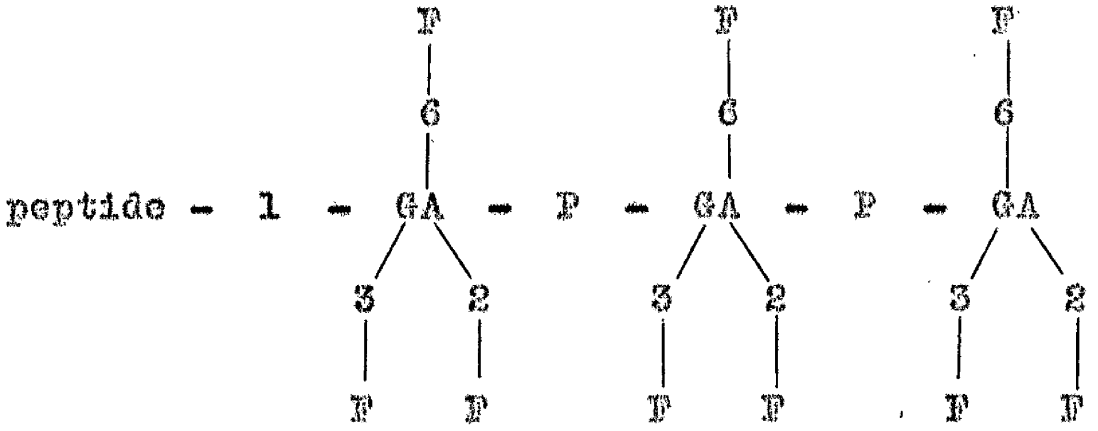
Water-soluble constituents of lipid A.

These include phosphoric acid, ethanolamine, D-glucos-amine and various amino acids especially the dicarboxylic acids, aspartic and glutamic.[184,247] In the water-soluble fraction of Escherichia coli and Salmonella abortus-equi lipid A hydrolysates, glucosamine is the major constituent, equivalent to 75% and 90% of the total nitrogenous constituents respectively.[247] Nowotny[184] has shown that in the case of lipid A from some Escherichia coli strains, the D-glucosamine is bound to the phosphoric acid in the form of D-glucosamine-4-phosphate. During the hydrolysis of the lipid with strong mineral acid, the D-glucosamine-4-phosphate is converted by transphosphorylation to the more stable

D-glucosamine-6-phosphate. D-Glucosamine-4-phosphate derivatives have also been found in the lipid fraction of Serratia marcescens, Pseudomonas aeruginosa and Neisseria gonorrheae lipopolysaccharides.[165]

Recently (1961), Nowotny has investigated the chemical structure of the purified isolated lipid component of several Salmonella lipopolysaccharides. Nowotny refers to this lipid as a phosphomucolipid,[165] because of its content of phosphorus (P, 1.9-2.2%), glucosamine (18-20%) and fatty acids (50-55%). Analysis of the water-soluble constituents of this lipid has revealed the presence of D-glucosamine, small amounts of several amino acids including aspartic and glutamic, D-glucosamine-6-phosphate, D-glucosamine-4-phosphate and D-glucosamine-4-phosphate substituted with a peptide chain in the 1-position. It appears that acid hydrolysis of the lipid liberates D-glucosamine-4-phosphate, 1-peptido-D-glucosamine-4-phosphate and D-glucosamine in equimolar amounts, and that the acid-stable D-glucosamine-6-phosphate found in the hydrolysate is produced by transphosphorylation from the D-glucosamine-4-phosphate derivatives during the hydrolysis. Nowotny has suggested that the D-glucosamine-phosphate units are probably linked, through phosphodiester bridges, in the form of a poly-D-

glucosamine-phosphate chain in which the glucosamine is esterified with fatty acids (Fig.2). Although the exact positions of the different amino acids in the chain has not yet been fully determined, the linkages appear to be through the amino group and the C_3 and C_6 hydroxy groups of the glucosamine.



F = Fatty acid

GA = D-Glucosamine

P = Phosphoric Acid

Fig.2 Tentative structure of the phosphomucolipid from Salmonella strains.[165]

Linkage of the protein, phospholipid A and polysaccharide components in the endotoxic complex.

Since glucosamine is always present in lipid A together with one or both of the dicarboxylic acids,

aspartic and glutamic, it has been suggested[124,247] that the principle moieties of the endotoxic complex are probably linked in the sequence polypeptide - lipid A - polysaccharide (Fig.3). Thus, gentle acid hydrolysis of the complex liberates the degraded polysaccharide hapten by splitting the acid-labile linkage between acetyglucosamine and other sugar residues.[65,66,68,242] Hydrolysis with alcoholic alkali, on the other hand, releases the protein component by splitting the alkali-labile protein aminodicarboxylic acid linkage.[28,226,248]

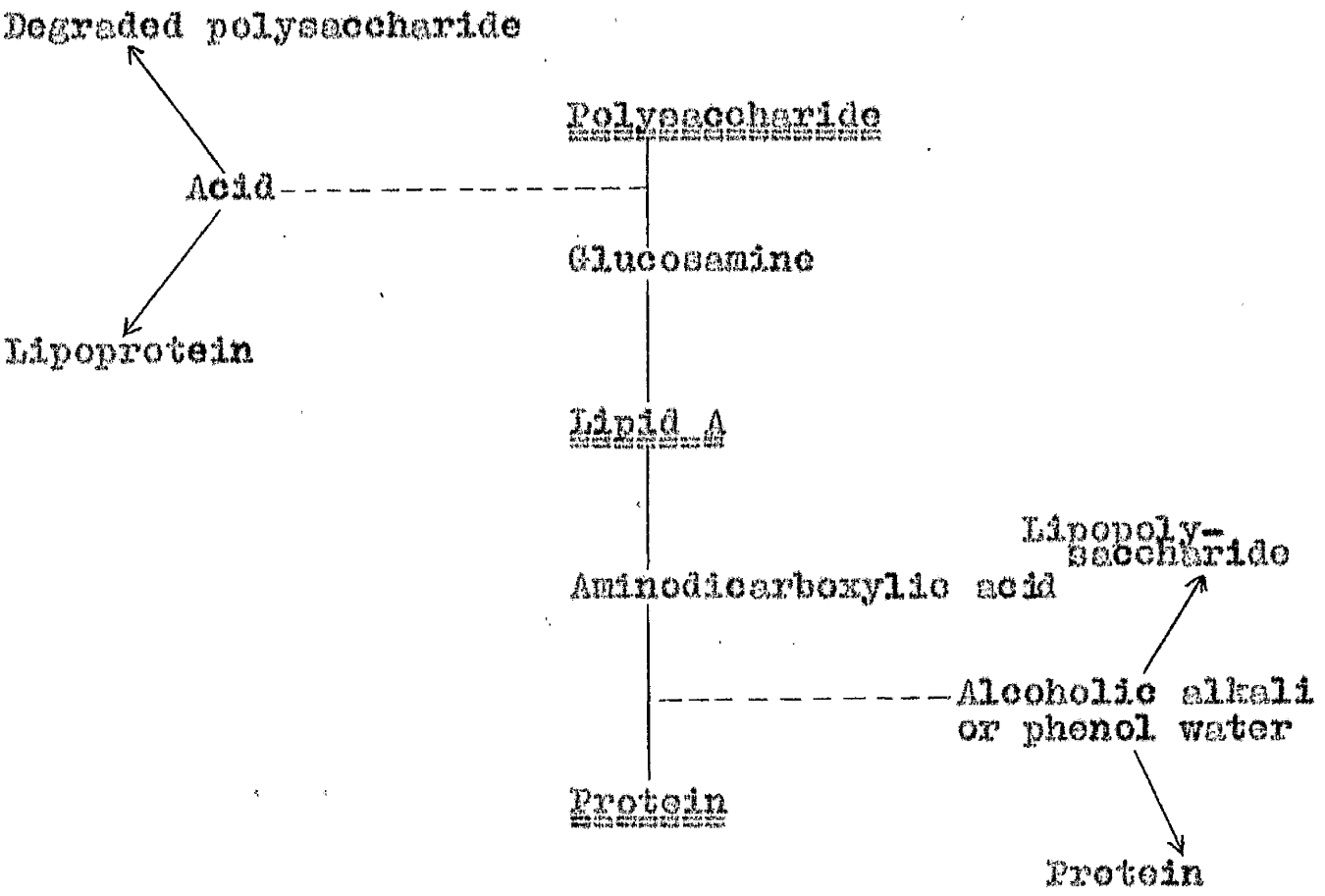


Fig.3 Probable linkage of the principle components in endotoxic complexes.[124,247]

Section II. Biological Effects of Endotoxins

" It is now known that these bacterial products (endotoxins) are a common denominator that explains seemingly unrelated observations and experiments carried out by microbiologists, physiologists, pathologists and clinicians over a period of almost a century". Bennett and Cluff, Pharmacological Reviews, 1957, 9, 427.

In studies of the biological effects of Gram-negative bacteria, different workers have employed endotoxin preparations varying from crude bacterial filtrates to the highly purified lipopolysaccharides described in the previous section. These endotoxins, irrespective of the species of Gram-negative bacteria from which they are derived, have in common the capacity to elicit, on injection into an appropriate host, such diverse reactions as fever, changes in circulating leukocytes, hyperglycaemia , metabolic disturbances, haemorrhagic necrosis of certain tumors, and the Shwartzman reaction.[24,227] In recent years these has been an increasing interest in the role of these substances in the pathogenesis of infection, especially in their ability, on intravenous injection, to stimulate a profound increase in non-specific immunity. In addition,

endotoxin fever, although artificial in that it involves the parenteral administration of a highly toxic purified foreign substance, has been invaluable in studies of the pathogenesis of fever generally.

Since the various physiological effects of endotoxins have been reviewed on several occasions,[4,20,52,70,227,228] and as recently as 1957,[24] the following account will deal mainly with present day views on the mechanisms underlying certain of these effects, and will indicate the trend of investigations which may open the way to a better understanding of the body's reaction to diseases of bacterial origin.

The febrile response to endotoxins.

Characteristics of the fever reaction:- The endotoxins of Gram-negative bacteria, when introduced into the blood stream of man and experimental animals, cause a reproducible febrile response which is characterised by a well-established sequence of reactions.

1. A lag-period or latency of 15-30 minutes in rabbits, cats and dogs, and 50-90 minutes in man,[20,40,105] precedes the onset of an abrupt rise in body temperature. The

ensuing fever, after all but minimal doses, exhibit a biphasic pattern where a second fever peak follows an incomplete defervescence of the first;[20,24] the constancy of this biphasic response has proved to be an important point of discussion in relationship to the mechanism of endotoxin-induced fever. After ordinary doses of endotoxin the fever persists for 4-6 hours, but it may be much prolonged when large doses are administered.[103]

2. A pronounced leukopenia commences about 5 minutes after injection and persists 1-2 hours before giving way to a leukocytosis which attains a maximum 3-4 hours after injection.[4,5,20,71,202] The leukopenia is primarily due to granulocytopenia, attributable largely to retention of the leukocytes in the capillaries of the lungs and spleen;[71] in this respect, Wood and his co-workers[260] have recently shown that endotoxins cause circulating leukocytes to adhere to vascular endothelium. The rebound leukocytosis is maintained in the presence of a persisting and increasing lymphocytopenia and eosinopenia,[202] and appears to be associated with release of white cells from the lung and increase in bone marrow activity. Several workers have observed too, that leukocytosis can be elicited with sub-febrile doses of endotoxin.[202,254,265]

3. A state of tolerance, in which the febrile response is significantly depressed, is produced by repeated daily injections of endotoxins. This induced resistance is in no way specific, as animals rendered tolerant to endotoxin from one species of bacteria are also tolerant to those from heterologous species.[4,24] Moreover, resistance is developed not only to the febrile response, but also to other biological effects of endotoxins, such as tumor-necrosis,[2,12] leukopenia[11,24] and the lethal effect.[2,12,26] Tolerance is a relative, dose-dependent phenomenon in that animals refractory to the injection of a given dose may react normally to a higher dose while giving no response to a lower dose.[1,21] Furthermore, a complete refractory state is never achieved, as animals continue to respond to repeated daily doses with small but significant responses.[26]

While there is reason to believe that the development of resistance to the various biological effects of endotoxins involves the same basic mechanisms, the depression of the febrile response, in its ease of measurement, is a useful indicator in studies of the dynamics of the phenomenon. From the investigations of several workers it is now clear that the antigen-antibody reaction, in classical form, is not involved in the development of tolerance. It has been

shown, for example, that tolerance cannot normally be transferred passively by serum,[71,173] bears no relationship to circulating antibody levels[11,42,83,173,174] and can be produced in subjects with agammaglobulinaemia.[100] That the thermoregulatory centres do not become refractory to the pyrogenic action is evident from the failure of repeated daily instillations of endotoxins into the sub-arachnoid space to induce tolerance.[87]

There is, however, abundant evidence to suggest that the development of tolerance to bacterial endotoxins involves an increase in the phagocytic activity of the reticuloendothelial system, whereby injected endotoxin is removed more rapidly from the circulation with subsequent diminution of tissue injury and fever.[18,38,87] This is demonstrated, for example, by the abolition of tolerance following administration of reticuloendothelial " blocking" agents such as thorium dioxide (Thorotrast) or trypan blue; tolerant rabbits given these materials are again fully susceptible to the pyrogenic effect of the endotoxin.[12,13,16,82,161] Furthermore, it has been demonstrated that the injection of endotoxin into animals produces a transient depression of the reticuloendothelial system, followed by a phase of hyperphagocytic activity as measured by an increase in

the rate of clearance from the blood stream of intravenously administered colloidal carbon.[89] Recent studies show that the phase of stimulation is associated with an increase in the number of actively phagocytic cells in the liver, attributable to dormant cells development phagocytic properties.[117,180] Freedman (1960) has also found that tolerance to the pyrogenic effect of endotoxin in the rabbit can be achieved by passive transfer of plasma or serum from donor rabbits which had been rendered tolerant by the administration of a certain critical schedule of doses of endotoxin, and furthermore that the blood of the tolerant donor stimulates the reticuloendothelial system of the recipient, as measured by subsequent carbon clearance in the latter.[89] The observation that tolerance can still be induced in normal recipients by injection of plasma from donor animals in which tolerance has been abolished by administration of reticuloendothelial system 'blocking' agents,[89] and the finding that endotoxin when mixed with tolerant plasma and administered as single injection still produces fever,[60] suggest that tolerance or its transfer is not mediated by antibody to, or humoral inactivator of, endotoxin, but point rather to a causal relationship of the reticuloendothelial system to endotoxin tolerance. The

nature of the active substance in tolerant donor blood has not as yet been elucidated, but Freedman reports the isolation from the blood of tolerant but not normal donors of a fraction which confers protection against endotoxin and also stimulates the reticuloendothelial system in normal recipients.[88]

More recent investigations have, however, shown that in certain circumstances, mice possessing increased phagocytic activity, as measured by the rate of clearance of colloidal carbon from the blood stream, may be highly sensitive to the pyrogenic and toxic effects of endotoxins; for example, during the course of intracellular infection[2,118] or after administration of zymosan.[16] The reason for this has not yet been defined. Cooper and Stuart (1961)[48] have also shown that while glyceryl trioleate stimulates a phagocytic activity equal to that achieved with lipopolysaccharides, it renders the animals more susceptible to endotoxic activity. Moreover, depression of phagocytic activity by administration of ethyl stearate[33,34] does not appreciably enhance susceptibility to endotoxins as does 'blockade' of the reticuloendothelial system with Thorotrast.[40] On the basis of these observations, Cooper and Stuart[48] conclude that the development of resistance

to endotoxins may involve not only an increase in phagocytic cells, but perhaps also a stimulation of an intracellular mechanism of detoxification. Involvement of serum factors in the development of pyrogen tolerance will be discussed later.

Central site of action of the febrile response to endotoxins.

Central site of action of the febrile response to endotoxins.

the production of fever by bacterial endotoxins is the central nervous system,[24] the precise site of action in the system is not fully established. The observation by Isenschmid and Schnitzler in 1914[127] of the essential role of the hypothalamus in the regulation of body temperature, and the subsequent demonstration by Ranson and his co-workers[41,203,204] of the existence of a heat loss centre and a heat conservation centre in the region of the hypothalamus, suggested that fever could result from disturbance of the hypothalamic-regulatory mechanism and indicated the hypothalamus as a possible central site of the pyrogenic action of bacterial endotoxins. Investigations of this by earlier workers, who attempted to induce fever in animals following the placement of restricted and massive hypothalamic lesions, produced inconclusive and often conflicting results[40, 104, 204]

Recently (1959), however, Wood and his co-workers[359] have shown that connection of the lower brain stem to some hypothalamic area is essential for the production of fever in cats by intravenous injection of typhoid vaccine, and results obtained by Sheth and Borison (1960)[215] with intraventricular injection of endotoxin also support the hypothesis of a local hypothalamic action. Until the specific sites of action are fully defined, it is usual to refer to them as 'thermoregulatory centres'.

Mechanism of the febrile response.

While the participation of the central nervous system in the febrile response to bacterial endotoxin is well established, the train of events leading up to the triggering of the nervous system has not yet been fully elucidated. The period of time which elapses between injection of the endotoxin and the rise in body temperature suggested to Beeson[11] that the action of the toxin is indirect. The general concept that the fever accompanying various disease states is due to release of fever-producing substances from injured tissues and cells,[23] and the fact that endotoxins are capable of producing widespread cellular injury,[24] led Atkins and Wood[6] to suggest that the latent period is the time required for the endotoxin to cause

tissue injury with resultant release of a secondary factor of endogenous origin, which acts on the nervous system to produce fever. Following the observation of Grant and Whalen[105] that the serum of rabbits, as long as three hours after injection of typhoid vaccine, contains a component which produces a fever of rapid onset when injected into a second group of rabbits, Atkins and Wood have demonstrated the presence of two distinctly different pyrogenic factors in the sera of rabbits after intravenous injection of endotoxin. One of these is rapidly cleared from the blood stream and is biologically indistinguishable from the endotoxin itself. The other, which appears later, persists throughout the febrile response, and its concentration in the circulation is directly proportional to the intensity of fever.[7,8] This factor, referred to as endogenous pyrogen, differs biologically from endotoxin in that it produces a monophasic fever of short latent period in both normal and endotoxin-tolerant recipients but does not itself evoke tolerance on repeated injection.[7,8,194] Again, unlike endotoxin, it is thermolabile, being inactivated by heat at 90° for 30 minutes.[105] Petersdorf and Bennett have also shown the presence of endogenous pyrogen in the sera of dogs after administration of a number of different vaccines

and purified endotoxins.[101]

The previous demonstration by Bennett and Beeson[14,31,33] of a heat-labile fever-producing substance, biologically indistinguishable from endogenous pyrogen, in whole granulocytes, leukocyte extracts and sterile peritoneal exudates, and the pronounced leukopenia which invariably precedes fever after administration of endotoxin,[30,105] support the idea of Atkins and Wood that endogenous pyrogen is a product of damaged cells. The strong affinity of endotoxins for polymorphonuclear leukocytes,[57,240,264] the inability of dogs made leukopenic with nitrogen mustard to elaborate endogenous pyrogen,[101] and the finding that polymorphonuclear leukocytes release endogenous pyrogen into the cell-free fluid of inflammatory exudates in vivo and in vitro,[140] further indicate the polymorphonuclear leukocytes as an important source of this factor. In man, Cranston and his co-workers have shown that the leukocytes are essential for the fever of rapid onset produced by incubating endotoxin with the patient's own blood.[65,66]

These findings have led to the hypothesis that endotoxin-induced fever is mediated by an endogeneous pyrogenic factor released from leukocytes when injured by endotoxin. King and Wood,[141] have shown that this endogenous material acts directly on the thermoregulatory centres of the brain.

Attempts to elucidate the biochemical properties of this factor have so far been unsuccessful but it appears to contain both carbohydrate and protein, is non-dialysable through cellophane, and is thermolabile. Its pyrogenicity is unaffected by wide ranges of pH and it is resistant to the action of trypsin, chymotrypsin and ribonuclease.[21,200,260]

While King and Wood[141 200 261] believe endogenous pyrogen to be the sole factor responsible for the fever induced by moderate intravenous injections of endotoxin, recent studies by Bennett and his co-workers indicate that in some cases endotoxins may act directly on the thermo-regulatory centres.[19,25,27,191,194] Thus, for example, intrathecal administration of minute doses of endotoxin results in a high fever which is not associated with leukopenia or release of endogenous pyrogen;[27,136] no tolerance is evoked on repeated administration by this route, and endotoxin-tolerant rabbits show no decrease in reactivity to intrathecal injection but respond with prompt fever after little or no lag-period.[27] Again no endogenous pyrogen is found in the sera of rabbits fevered by injection of endotoxin into the subarachnoid space.[27] The disappearance of the second fever peak of the typical biphasic febrile

response to endotoxins referred to previously is usually the first sign of the development of tolerance, and appears to coincide with the failure of endogenous pyrogen to appear in the circulation.[194] Since both this peak and the endogenous factor reappear when, at this stage, a reticulo-endothelial blocking agent is administered, it has been suggested that endogenous pyrogen is probably responsible for the latter phase of fever and that the early phase, independent of the endogenous factor, is produced by direct action of the endotoxin on the thermoregulatory centres.[87,193,194] If this is the case, the early disappearance of the second fever peak may be explained in terms of increased phagocytic activity of the reticuloendothelial system, whereby injected endotoxin is removed from the circulation before tissue damage, with subsequent release of the secondary fever-producing factor, can occur. The concept of the direct action of endotoxin on the thermoregulatory centres producing the first fever phase also offers an explanation of the fact that animals never become completely tolerant, by presuming that some of the administered endotoxin, escaping clearance by the reticuloendothelial system, penetrates directly to these centres.

While criticisms of the interpretation of these findings as evidence of a direct action of endotoxin have been made on the grounds of the well-known sensitivity of the thermoregulatory centres to any noxious stimuli and the insensitivity of the passive transfer technique for detecting endogenous pyrogen,[261] the finding of significant amounts of unchanged endotoxin in the cerebrospinal fluid after intravenous injection of large doses of endotoxin[27] does indicate that in this case at least, endotoxin, by "spilling over" into the spinal fluid, may act directly on the brain. On the basis of these and other observations[21,25,27,191,195] Petersdorf and Bennett favour the hypothesis that endotoxin-induced fever is mediated by two distinct mechanisms:-

(i) An indirect action involving endogenous pyrogen and,

(ii) A direct action of the toxin on the brain by penetration into the cerebrospinal fluid.

The concept of Petersdorf and Keene[191] and Keene[156] that endotoxin penetrating into the cerebrospinal fluid affects the thermoregulatory centres is supported by the more recent (1960) demonstration by Vrana and his colleagues[253] of the high fever evoked in rabbits by intracerebral administration of minute doses of endotoxin. These workers, however, were

unable to confirm the finding of Bennett and Keeno[26] that the latent period is shorter by this route of administration, and they conclude from this that the fever is not in fact due to a direct action of the endotoxin of the thermoregulatory centres, but to the formation of endogenous pyrogen directly in the brain tissues in the immediate vicinity of these centres. While such a concept, in suggesting that the mechanism of the febrile response may in all cases be through mediators, would provide a basis for the uniform interpretation of fever, it presumes that endotoxins can, by reacting with nerve cells and brain tissues, release endogenous pyrogen and this has yet to be shown. In fact the only established source of endogenous pyrogen up to the present are the polymorphonuclear leukocytes,[19,260] and one of the main points of evidence quoted by Bennett and co-workers for a direct action of endotoxin on the thermoregulatory centres[27,198] has been the observation that a normal febrile response is elicited by administration of endotoxin to animals rendered granulocytopenic by means of mechlorethamine hydrochloride (nitrogen mustard.[21,25,86,191] Recently (1961), however, Herion, Walker and Palmer[111] have found that rabbits without circulating granulocytes develop no fever after injection

of endotoxin, and these workers suggest that the fever observed by Bennett and co-workers in mechlorethamine-treated animals was related to the degree of persistance of granulocytes.

The role of endogenous pyrogen in the pathogenesis of fever.

Atkins has recently shown that the febrile response to intrevenous injection of influenzal viruses is associated with a transferable endogenous pyrogenic factor the release of which into the circulation appears to be an essential step in the pathogenesis of viral fever.[6] The source of the endogenous material has not yet been established, but the absence of granulocytopenia in this type of fever[100,234] suggests the implication of cells other than polymorphonuclear leukocytes as precursors.

Evidence has also been presented that pyrogen of the endogenous type may be responsible for most, if not all, cases of fever associated with inflammatory conditions.[58,141] It has been established, for example, that the fever accompanying pneumococcal peritonitis in rabbits is due to endogenous pyrogen derived from the polymorphonuclear leukocytes in the peritoneal exudate and carried to the circulation via lymphatic channels emptying into the thoracic duct.[142] King and Wood have also demonstrated the presence of circulating endogenous pyrogen in animals with experimental streptococcal cellulitis.[143]

Effect of normal blood serum on the febrile response to endotoxins.

In the course of studies of the febrile response, various workers have shown that normal blood serum contains factors which, when they interact with endotoxins in vitro, may either enhance[44,81,98,102,103,158] or inactivate[40,105,106,107] the fever-producing properties of the latter. Enhancement of the pyrogenic action, characterised by a shortened latent period and an increased height of fever, has been demonstrated when endotoxin is mixed with normal rabbit and human blood serum for a short period before injection.[81,102,10] The mechanism of this effect has not been defined, and although the serum-altered endotoxin resembles endogenous pyrogen of leukocytic origin in several respects,[44,192,194] more recent investigations suggest that they are, in fact, different substances.[45] Cluff and his co-workers have also observed a close correlation between the appearance and disappearance of pyrogen tolerance and the disappearance and reappearance of the pyrogen-augmenting factors from rabbit serum, suggesting that these factors may be involved in the development of tolerance to endotoxins.[45] This effect would, however, appear to be secondary to the role of the reticuloendothelial system, since abolition of

tolerance by administration of Thorotrast is not associated with a reappearance of the augmenting factors in the serum.

Cluff and Bennett[44] have shown that whereas pyrogen augmentation is not dependent on the temperature of incubation of the endotoxin-serum mixture, inactivation of the pyrogen on the other hand is due to a heat-labile factor which is only evident after prolonged incubation at physiological temperatures. Under appropriate conditions, inactivation of all the other biological effects of endotoxins,[118,130,166,201,222] as well as alterations in their immunological properties,[45,106] may be obtained, and Hagemann[106] has suggested that the inactivating principle appears to be closely related to the properdin system. Recently (1961), Landy and his co-workers[137] have shown that the inactivation of the pyrogenic effect has the characteristics of an enzyme-catalysed reaction and is dependent upon the concentration of reactants and the pH of the reaction mixture as well as on the time and temperature of incubation. Complete pyrogenic inactivity of the endotoxin-serum mixture was not achieved, and the residual activity was shown to be due to unaltered endotoxin. Landy has discussed the possible origin of the endotoxin degrading enzyme in normal serum and has suggested that the presence of

such enzymes in phagocytic cells may prove to be of special significance in the defence of the host against endotoxins.[157]

Stimulation of non-specific immunity by endotoxins.

In addition to the acquired immunity to infection which, in the classical sense, depends upon the presence of circulating antibodies evoked in response to specific antigens, it is recognised that there also non-specific body defence mechanisms, which can be stimulated by the injection of various substances of bacterial and other origin.[35,144,203,214,268] Brandis, for example, has reported the rapid development in mice of a protective effect against experimental infections of Salmonella derby following the injection of a number of bacterial vaccines, an effect which he refers to as "proimmunity".[35] Again, Field, Howard and Whitby[85] have shown that, in addition to the specific immunity developed to Salmonella typhosa infections by the intravenous injection in mice of the homologous organism, an increased resistance to the infection is also rapidly produced by the administration of heterologous Gram-negative organisms, either living or dead. Although the level of non-specific resistance achieved in this way

is lower and more transient than the immunity which follows administration of specific immunising agents, it is nevertheless sufficient to indicate that it may play an important role in the general body defence against infection.

From the investigations of Rowley,[207] Landy and Pillimer[140,152,155] and Hurni[132] it is now clear that the component of the bacterial cell responsible for this activity is the lipopolysaccharide fraction of the 'O'-antigen, and it has been shown that lipopolysaccharides from virtually all species and strains of Gram-negative bacteria, including rough variants,[130,149] are equally effective in the respect. Furthermore, the resulting non-specific immunity appears to be effective not only against experimental infections by Gram-negative organisms[3,143] but to extend also to certain Gram-positive infections.[47,76]

The increased resistance to infection produced by these polysaccharides is evident 24-48 hours after intravenous or intraperitoneal injection into mice and is preceded by a short period of increased susceptibility immediately following injection.[130,152,155,206,245] Hurni has also shown that a second injection of lipopolysaccharide a short time after the first produces a more pronounced initial susceptibility in the course of which 1-10 Escherichia coli cells are

sufficient to kill the animals, whereas 24-48 hours later, when the augmentation of resistance is at a maximum, the lethal dose is of the order of 100 million organisms.[122] This illustrates the extent of the changes in resistance to infection which can be produced by lipopolysaccharides in the period of one day. The protective activity of the lipopolysaccharide is retained in the isolated lipid A component, although, in terms of minimal effective dose, the latter is less effective than the parent polysaccharide.[119] Westphal and his co-workers[245,255] have also shown that while the lipid A derived from Escherichia coli produces, when dispersed in dextran solution, a similar increase in non-specific immunity at dose levels of 100-1,000 µg. no phase of increased susceptibility follows the administration of the lower dose.

Mechanism of stimulation of non-specific immunity.

The observation by Rowley in 1956 that injection of lipopolysaccharides in mice produce an initial rapid decrease, followed by an increase in the bactericidal powers of the animals' serum led him to suggest that these changes in the bactericidal properties of serum are the cause of the changes in the immune state of the animal and that the

lipopolysaccharide contains the substrate on which the bactericidal system of serum acts.[207,208] This observation was extended by the work of Pillimer and his colleagues who demonstrated the presence in serum of a new serum globulin (properdin) which, together with the four components of complement and Mg^{++}, constitutes a bactericidal system effective against a wide range of Gram-negative bacteria.[128,195,235,236] Subsequently, these workers have shown that properdin combines with lipopolysaccharides in vitro[197] and, furthermore, that the rapid development of increased resistance to infection following injection of lipopolysaccharide is often accompanied by an elevation in serum properdin level which may be preceded by an initial depression.[149,152,155,197] There appears, however, to be no correlation between the stimulation of non-specific immunity and the serum properdin level at the time of challenge, since animals made resistant to infection by treatment with lipopolysaccharide at certain intervals prior to challenge may have normal or even subnormal properdin titres at the time of challenge.[130,148,152,153] Landy and Pillimer have observed that in such animals, properdin titres after challenge are maintained at levels close to normal, whereas in control animals, a progressive fall occurs up to the time of death.[153] On the basis of these findings the hypothesis has been advanced that increase

in non-specific immunity is associated with either elevated properidin levels or the maintenance of normal levels during the infectious process.[155] There are, however, certain aspects of the phenomenon which cannot be adequately explained solely on the basis of a humoral bactericidal mechanism involving the properidin system. Injection of zymosan, a yeast polysaccharide, for example, stimulates a marked elevation in mouse properidin levels[130,196] without significant increase in non-specific resistance.[130,145] Again, Howard, Rowley and Wardlaw[120] have found that a low properidin level during infection does not necessarily lead to death, as demonstrated by the survival of infected mice whose properidin levels were only 10% of normal.

As previously discussed under 'Tolerance', injected lipopolysaccharides produce an initial depression of the reticuloendothelial system followed by a phase of increased phagocytic activity as measured by the rate of clearance from the blood stream of intravenously administered colloidal carbon.[20] Rowley, Howard and Jenkin[210] have observed a similar biphasic change in the rate of clearance of isotope-labelled lipopolysaccharide following a previous injection of non-radioactive lipopolysaccharide, the labelled material becoming localised in the reticuloendothelial

system. Subsequent investigations have shown that these changes in reticuloendothelial function parallel the biphasic changes in non-specific resistance to infection and, furthermore, that activation of the reticuloendothelial system in this way is produced more regularly than elevation of the properdin level and is more closely correlated with the degree of resistance.[120] Although other colloids such as colloidal sulphur[145] can produce increased resistance to certain infections, their potency is low compared with that of lipopolysaccharide, and while reticuloendothelial cells preferentially take up colloidal particles of a limited range of particle size,[120,159,219] it is possible that optimum potency in stimulating immunity depends not so much on particle size,[87] as on the possession of lipid A or similar material distributed throughout the macromolecule.

Studies by Rowley and his co-workers[120] and Howard[117] of the mechanism whereby lipopolysaccharides stimulate the reticuloendothelial system have shown that the phase of hyperphagocytic activity occurring 48 hours after injection is associated with an increase in the number of actively phagocytic cells in the liver and spleen. An intensitification of the metalophilia of Kupffer cells of the mouse liver and an increase in the number of Kupffer cells

containing acid phosphatase were observed, the latter finding supporting the suggestion of Weiss and Fawcett[241] that the level of acid phosphatase in cells of the reticuloendothelial system reflects their phagocytic activity. The observed increase in the number of stellate (Kupffer) cells and the corresponding reduction in the number of endothelial-like cells in the liver sinusoids,[117] suggests that lipopolysaccharide activation of the reticuloendothelial system may be due to dormant cells developing phagocytic activity rather than mitosis of existing phagocytic cells.

Following the recent observation (1959) by Howard and Wardlaw[121] that the phagocytic activity of the reticulo endothelial system is dependent on serum opsonins, Rowley[200] has shown that the serum of mice previously injected with lipopolysaccharide is more active than normal mouse serum in preparing bacteria for phagocytosis by peritoneal macrophages. In addition, the macrophages themselves when treated with lipopolysaccharide were found to possess increased phagocytic activity, suggesting that the observed rapid clearance of viable bacteria from the peritoneum may be a dual mechanism involving both an increase in opsonic factors and a direct stimulation of the phagocytic activity of the peritoneal macrophages.

More recently (1961) Cooper and Stuart have found that while glyceryl trioleate and lipopolysaccharide are equally effective in stimulating phagocytosis in mice,[40] the lipopolysaccharide produces a much greater degree of protection against Streptococcus pneumoniae infection.[40] Since the properdin system, being active only against Gram-negative organisms,[128] cannot be involved, the lipopolysaccharide appears to stimulate, in this case, some other factor which causes a more rapid death of the organisms following their removal. Cooper and Stuart suggest that this may be either non-specific opsonin or an enhanced intracellular activity of the phagocytes.[49] The possibility of such stimulation by lipopolysaccharide, of the intracellular bactericidal mechanism of the phagocytes, has also been suggested by Rowley[130] to account for the observed susceptibility of mice to Salmonella typhimurium following stimulation of the reticuloendothelial system with other colloidal materials. Little is known, however, about the nature of the bactericidal mechanism within fixed phagocytes.[52]

Several recent investigations have been concerned with the humoral bactericidal aspects of non-specific immunity, especially the non-specific nature of the humoral components involved.[15,157] Benacerraf and Miesher, for example,[15]

have confirmed the observation by Rowley[209] of an increase in the level of non-specific opsonins in the sera of mice treated with endotoxin, and these workers suggest that the non-specific nature of these opsonins may be due to unsuspected cross reactivity between all endotoxins and which may be related to the lipid portion of the lipopolysaccharide molecule. Again Michael, Whitby and Landy (1961)[160] have shown that while the increased serum bactericidal activity following administration of endotoxins is effective against several unrelated strains of Gram-negative bacteria, absorption of the serum with one Gram-negative serotype removes the bactericidal activity for that strain but not for strains of other genera. This and other evidence presented indicates that the increased bactericidal effect of serum is due to the presence of a number of specific bactericidins having the functional characteristics of specific antibodies. Landy and co-workers suggest that the lipopolysaccharide probably acts on antibody-forming cells to either release preformed antibodies or to stimulate a temporary increase in the capacity of cells to produce antibodies.[160] No reference is made to a possible relationship of these bactericidins to the properdin system which, as already discussed, exerts a bactericidal effect on a wide range of Gram-negative bacteria and is also

usually elevated by administration of endotoxin, but certain characteristics of properdin such as its apparent lack of serological specificity and the requirement for Mg^{++} and complement are not compatible with classical ideas of specific antibody reactions.[180] Nelson,[179] however, from his findings that most sera from normal individuals contain antibody which is capable of sensitising zymosan to react in immune aggregation and complement fixation, has suggested that the properties ascribed to properdin may be explained on the basis of this antibody acting in conjunction with certain components of complement, and that the broad spectrum of reactivity and cross-reactivity of the antibody is probably due to the wide distribution of closely related polysaccharides in nature.

In summary, therefore, it appears that the development of non-specific resistance to infection following administration of endotoxins involves a stimulation of both the cellular and humoral defence mechanisms of the host. It remains for future work to integrate the various factors at play but it is evident that these, individually or collectively, may contribute significantly to the survival of the host during infection. Westphal has recently speculated that since all animals, including man, live in

close contact with Gram-negative bacteria, it appears possible that a perpetual flux of traces of endotoxin, provided especially by the intestinal flora, continually stimulates the defence functions of the host, which in the absence of such stimulation would succumb to the first bacterial infection.[245]

PART II

EXPERIMENTAL

Details of the materials and methods used in the experiments subsequently described are where possible contained in the Appendix.

Section 1. Isolation of crude pyrogenic material from Proteus vulgaris culture fluid.

The experiments reported here were performed with the object of reducing the bulk of the cell-free fluid of a largely inorganic medium culture of Proteus vulgaris, when the isolation of the active material therein could then be more easily effected. Methods used by other workers for reducing the liquid bulk include freeze-drying of the cell-free culture fluid[4] and removal of the active material from the culture fluid by adsorption of Seitz filter pads.[51,70] In the latter method the adsorbed material was subsequently eluted from the pads with an alkaline eluting fluid, and although the eluted material rapidly lost activity in contact with the eluate, a reasonable degree of concentration was claimed when the eluting fluid was sucked through the pads and the eluate immediately adjusted to a slightly acid reaction.[4] While these methods were probably satisfactory for concentrating relatively small volumes of culture fluid, evaporation under reduced pressure appeared to be a more practicable method of reducing the considerable volumes

required in the present investigations. Since, however, some workers have reported instability of the active material in the fluid medium to heat and concentration,[70,263] experiments were first undertaken to ascertain if a loss of potency occurred on concentrating the culture fluid by this method. No loss of potency was found under the conditions of evaporation used and it may, therefore, be assumed that the active factor is relatively heat stable.

(a) Preparation of cell-free culture fluid.

The growth from a 24-hour agar-slope culture of *Proteus vulgaris* was added to 75 ml. of the simple medium described in the Appendix, by washing in with the corresponding glucose-magnesium sulphate component of the medium, and incubated for 24 hours at 30°C. This was then added to 375 ml. of the medium and incubated for a further 48 hours at the same temperature. Finally, this culture was added to 9 litres of medium and incubated, with vigorous aeration, for 4 days.

After the four-day incubation the culture was clarified by passing it slowly through a Sharples continuous super-centrifuge and the clarified fluid sterilised by filtration through bacterial-grade Doulton filter candles.

(b) Concentration of the cell-free culture fluid by evaporation under reduced pressure.

For this purpose a circulatory cyclone evaporator was used in conjunction with a water-pump (Fig.4). It was possible by this means to distil off about 4 litres of water per hour at a temperature below 30°C. Two 9 litre volumes of cell-free culture fluid were prepared as previously described and divided into 4 x 4 litre volumes. After sampling and freeze-drying the samples for pyrogen tests, each 4 litre volume was concentrated to 500 ml. and the four concentrates combined; division of the culture fluid in this way avoided prolonged exposure to evaporation. The combined concentrates, representing an 8-fold concentration, were again sampled and the samples freeze-dried (PSC 1/8). Further concentration reduced the bulk to 1 litre (PSC 1/16) and finally to 500 ml. (PSC 1/32).

Pyrogenic activity of the concentrates. The contents of each ampoule were dissolved in sufficient apyrogenic saline to reconstitute the material to a volume equal to that of the original culture supernatant fluid from which it was obtained. Dilutions of the reconstituted solutions were then made and tested for pyrogenic activity at the dose-levels shown in Table 3. The results of the test on the

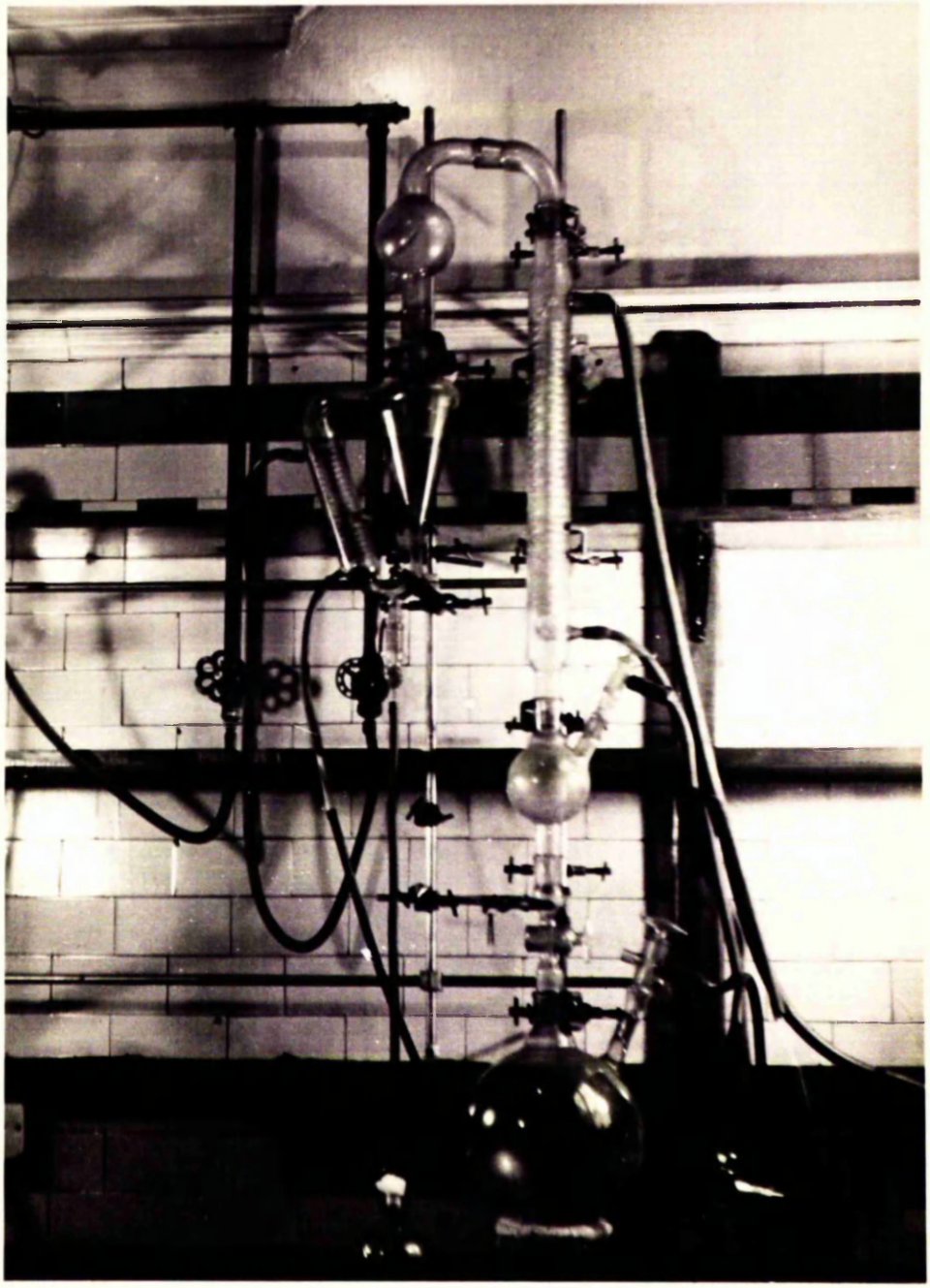


Fig.4 Circulatory cyclone evaporator used for concentrating the cell-free culture fluid. To avoid entrainment due to frothing, the normal stillhead supplied with the apparatus (Quickfit and Quartz Ltd.) was replaced by a splash head.

material which had been submitted to the greatest degree of concentration are shown in Table 3 where it is seen that no appreciable loss of activity had taken place. Tests on the other concentrates gave similar results, and repeated tests on a fresh batch of the culture fluid confirmed the findings.

Dose (ml./kg.)	Rise in rectal temperature (°C)	
	Non-concentrated culture fluid	P50/1 32
0.02	1.66	1.60
0.006325	1.46	1.38
0.002	1.14	1.09
0.0006325	0.58	0.69
0.0002	0.54	0.48

Table 3. Effect of concentration, by evaporation under reduced pressure, on the pyrogenic activity of Proteus vulgaris culture fluid. Each temperature rise shown in the Table was the average for 10 rabbits.

(c) Isolation of crude pyrogenically active material from the culture fluid by precipitation with alcohol.

For this purpose cultures were prepared in 9 litre

volumes, each batch consisting of 3-4 such volumes. After clarification, the cell-free fluid was concentrated in the cyclone evaporator; to avoid prolonged periods of exposure to evaporation, the concentrate in the cyclone arm was removed each time the distillate measured 4 litres, the volume in the cyclone arm being approximately 4.5 litres. This concentrate was passed slowly through the Sharples centrifuge, and the clear yellow solution further concentrated to 2 litres and dialysed against running water for 3 days. The dialysed fluid was then concentrated under vacuum to 100-150 ml., centrifuged at 3,000 rpm for 1 hour and the bright yellow clarified solution poured into 10 volumes of cold absolute alcohol. After standing at 0-4°C for 4 hours, the material which had precipitated was collected by centrifuging, washed twice with cold absolute alcohol and dried in a vacuum dessicator. The dry material obtained in this way from three such batches of culture fluid was extracted with 100 ml. of water by shaking for 1 hour; after centrifuging and removing the clear yellow fluid, the residue was again extracted for 1 hour with 50 ml. of water. The two extracts were mixed and freeze dried. From a total 18 batches, representing about 500 litres of the original culture fluid, 3.7 g. of a fawn-coloured material was obtained. Only part of the crude alcohol

precipitated material went into solution on extraction with water and the residue did not further dissolve on prolonged extraction. This suggests that some denaturation of the material had occurred during the treatment with alcohol and the subsequent drying in the vacuum dessicator.

The freeze-dried material, designated PSA/10 [Proteus supernatant, precipitated with alcohol (10 vols.)], was a highly active pyrogen; the results of pyrogen tests on the material are shown in Table 4.

Dose (μg./kg.)	Rise in rectal temperature
1.0	1.72°C
0.1	1.25°C
0.01	0.34°C

Table 4. Temperature response in the rabbit to various dose-levels of the material (PSA/10) obtained from Proteus vulgaris culture fluid by precipitation with alcohol. Each temperature rise given in the table was the average for 10 animals.

The material gave a positive Molisch reaction indicating its polysaccharide nature, but it appeared free

from reducing sugars as shown by negative Fehling and Benedict tests. The nitrogen value was 5.9% and a positive biuret reaction showed the presence of protein.

Section 2. Purification of the crude pyrogenic material from Proteus vulgaris culture fluid.

(a) - Phenol extraction.

The crude alcohol precipitated material was submitted to the deproteinisation procedure of Westphal[249] using hot aqueous phenol. The material (600 mg.) was shaken for several hours with 60 ml. of apyrogenic distilled water and centrifuged to remove the small amount of undissolved matter. The clarified solution was heated to 65°C and an equal volume of 90% w/v aqueous solution of phenol at 65°C added. The mixture was stirred at this temperature for 30 mins. and then rapidly cooled to 5°C, when a separation of the aqueous and phenol layers occurred which was hastened by centrifuging. The clear aqueous layer was pipetted off and the phenol phase again extracted by adding water (48 ml.) and heating to 65°C; the mixture was rapidly cooled as before, centrifuged and the aqueous phase removed. Both aqueous phases were dialysed against

running water for three days to free them from phenol, the dialysed solutions combined and concentrated under reduced pressure to approximately 60 ml. After clarifying by centrifuging, the concentrate was freeze-dried to yield 280 mg. of a fawn-coloured powder, equivalent to 47% of the weight of the starting material.

Tests showed that the product obtained in this way was highly pyrogenic in rabbits. It gave a strong Molisch reaction but was biuret-negative, and mild acid hydrolysis of a sample of the material resulted in the separation of a chloroform-soluble lipid. For a more complete examination, a stock of the material was then built up; to avoid the inconvenience of preparation in two stages, namely the isolation of the crude material in solid form and its subsequent treatment with phenol, further extractions proceeded direct from the dialysed concentrated culture supernatant fluid as follows:-

Batches of a four-day aerated culture, each consisting of 3-4 nine litre volumes, were clarified, concentrated and dialysed as described in Section 1 (c). The combined final concentrates from each three such batches, measuring about 300 ml., were extracted with warm aqueous phenol, the aqueous phase dialysed and the dialysed solution, after further concentration to 80-100 ml., centrifuged to remove any suspended

matter, and finally freeze-dried. The yield of freeze-dried material varied somewhat but was usually in the region of 8-10 mg./litre of original culture fluid. When a sufficient number of batches had been processed, the yields of product were combined and examined as follows; for convenience and brevity the material is referred to as PSPE (Proteus supernatant, phenol extracted).

Description. - A pale buff-coloured hygroscopic powder in the freeze-dried state which dissolved in water to give a pale-yellow solution; solution was usually somewhat difficult but could be facilitated to some extent by soaking the material in a little water overnight and then diluting.

Pyrogenic activity . - A significant pyrogenic response was evoked in rabbits with doses as low as 0.01 µg./kg. (Table 5).

Dose µg./kg.	Rise in rectal temperature
0.2	1.68°C
0.1	1.43°C
0.01	0.84°C

Table 5. Temperature response in the rabbit to various dose-levels of the polysaccharide material (PSPE) obtained from Proteus vulgaris culture fluid by extraction with hot aqueous phenol. Each temperature rise given in the table was the average for 10 animals.

Chemical composition. - Details of the analytical methods used are given in the Appendix. Analytical figures obtained were N, 4.7; total P, 0.8. Measurement of the ultraviolet absorption at 260 mμ. of the material dissolved in N/100 NaOH showed that some batches of PSPE appeared to be free from nucleic acid, while small amounts, not exceeding 3%, were present in others. (Fig.5). When examined by the H_2SO_4-cysteine reaction of Dische,[74,75] an absorption was found with a maximum at 505 mμ. which is characteristic of aldoheptose sugars; the heptose content, estimated as α-D-glucoheptose, was 1.4%. This is purely a relative value since different aldoheptoses give different colour intensities in the test,[62] and α-D-glucoheptose was the only aldoheptose available for use as an index sugar. The absorption curve illustrated in Fig.6 also shows a maximum at 410 mμ. which is characteristic of hexose sugars.

A sample of PSPE was hydrolysed with N-H_2SO_4 for four hours and the hydrolysate run on paper chromatograms (Whatman circles No.20) using butanol-pyridine-water as solvent. Spraying with aniline phthalate revealed the presence of galactose, glucose and mannose; a faint reddish-band was also apparent between galactose and glucose and due to the heptose sugar (Fig.7). No ribose was detected, confirming

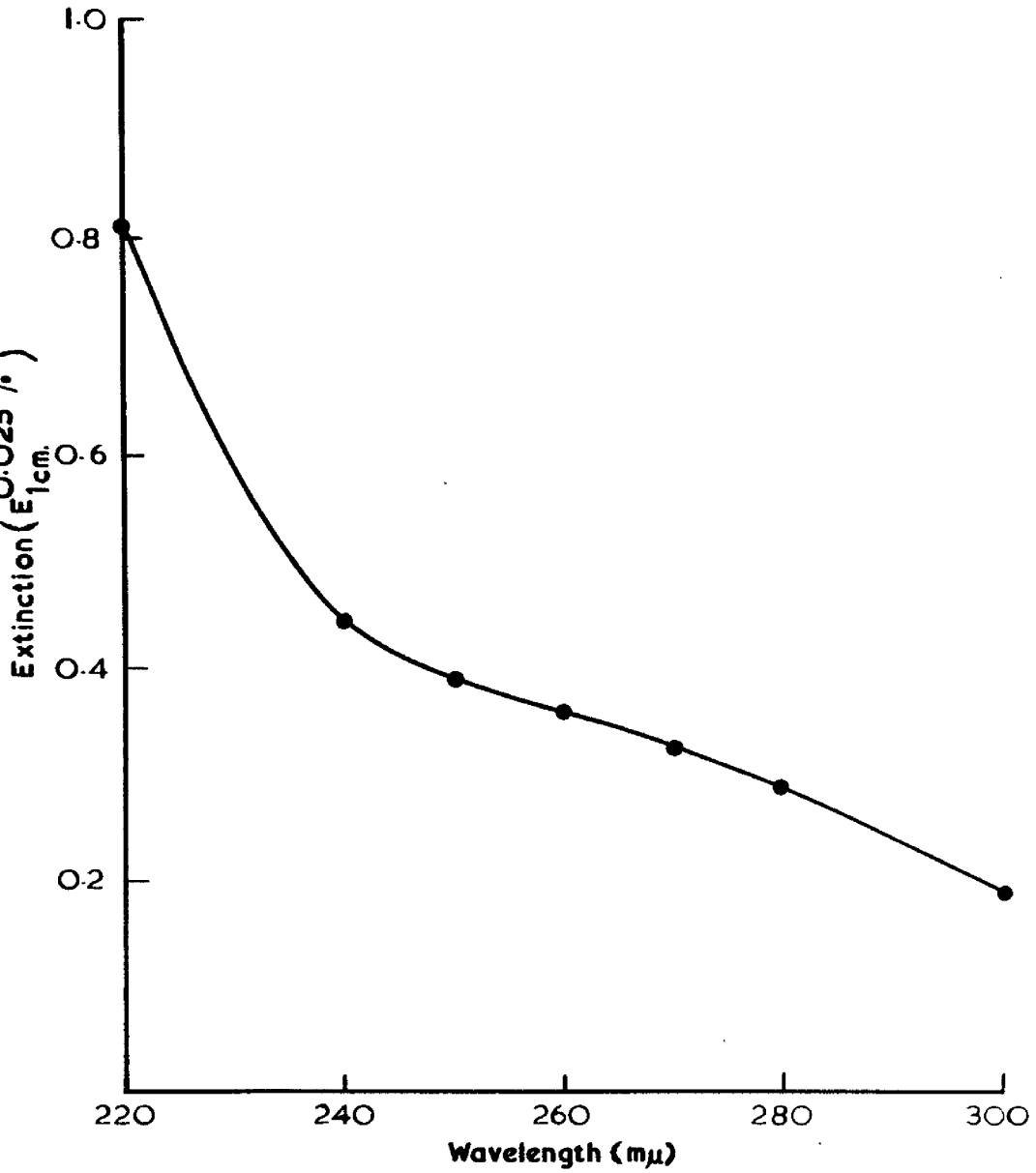


Fig. 5. The ultraviolet absorption spectrum of the active material (PSPE) obtained by phenol extraction of Proteus vulgaris culture fluid.

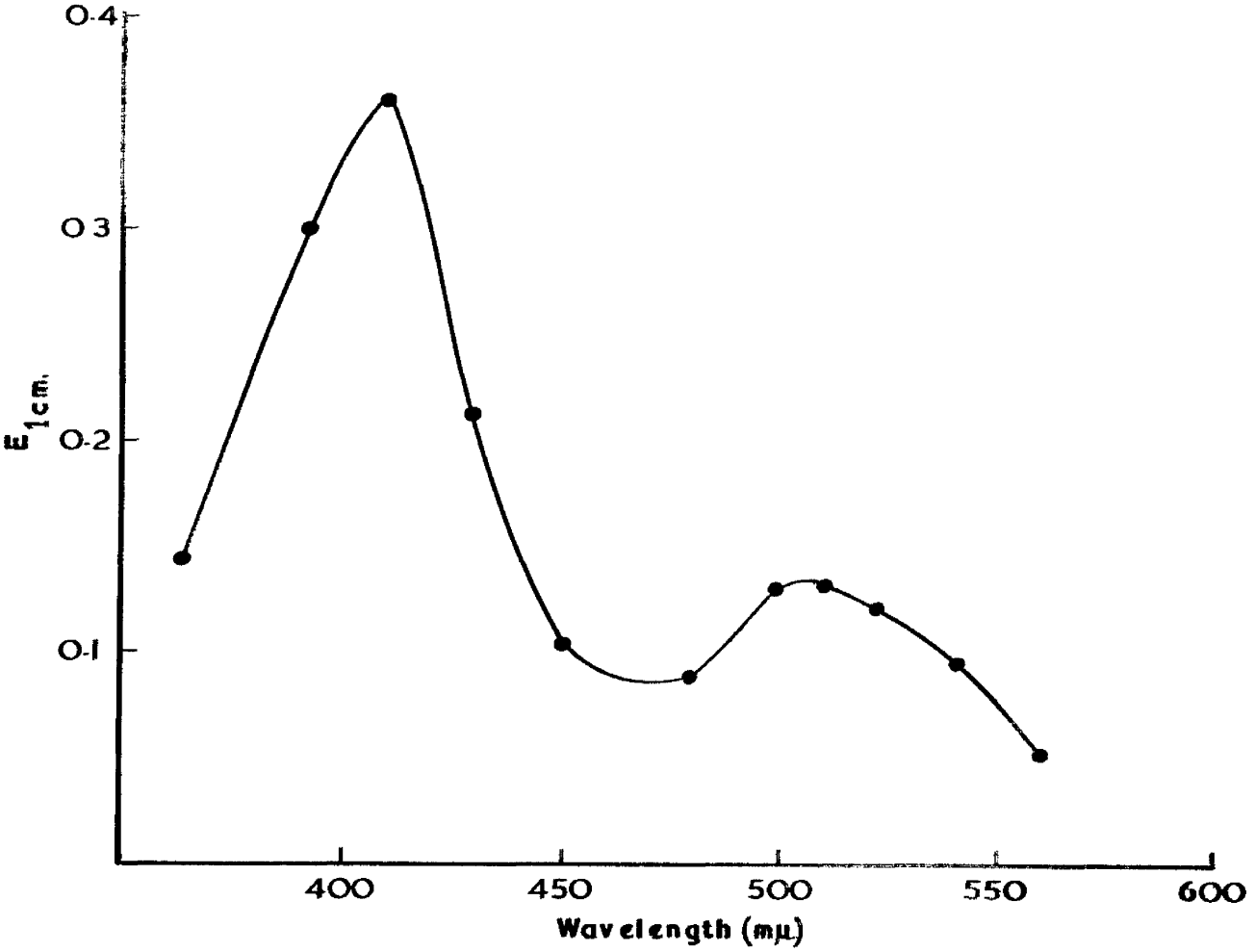


Fig.6. The Dische reaction on the active polysaccharide (PSPE) obtained by phenol extraction of Proteus vulgaris culture fluid. Absorption spectrum of the material (500 μg.) after treatment with H_2SO_4-cysteine: readings were made at 22 hours. The absorption maximum at 505 mμ. is due to aldoheptose.

the virtual absence of nucleic acid.

Another sample of the material was hydrolysed for 15 hours with 5N-HCl and the hydrolysate run on paper chromatograms (Whatman No.4) using butanol-acetic acid-water as solvent. When sprayed with ninhydrin-cupric nitrate indicator, several coloured bands were obtained. Two of these were identified, by comparison with the colours produced by this indicator with standard amino acids, as glutamic and aspartic acids, (Fig.8). In this solvent system it was found that standard glucosamine usually ran as a single band, whereas standard galactosamine usually gave two bands, one corresponding approximately to that of glucosamine and other inside this, as shown in Fig.8; this behaviour of the amino sugars will be referred to later in the section. In the chromatogram of the hydrolysate of PSPE a strong band corresponding to glucosamine was evident, and a weaker one near the position of the inner galactosamine band (Fig.8). To further identify the amino sugars, the hydrolysate was degraded with ninhydrin and run on paper chromatograms (Whatman circles No.20) with butanol-pyridine-water, when the presence of arabinose and lyxose, as revealed by spraying with aniline phthalate, appeared to confirm the presence of both glucosamine and galactosamine

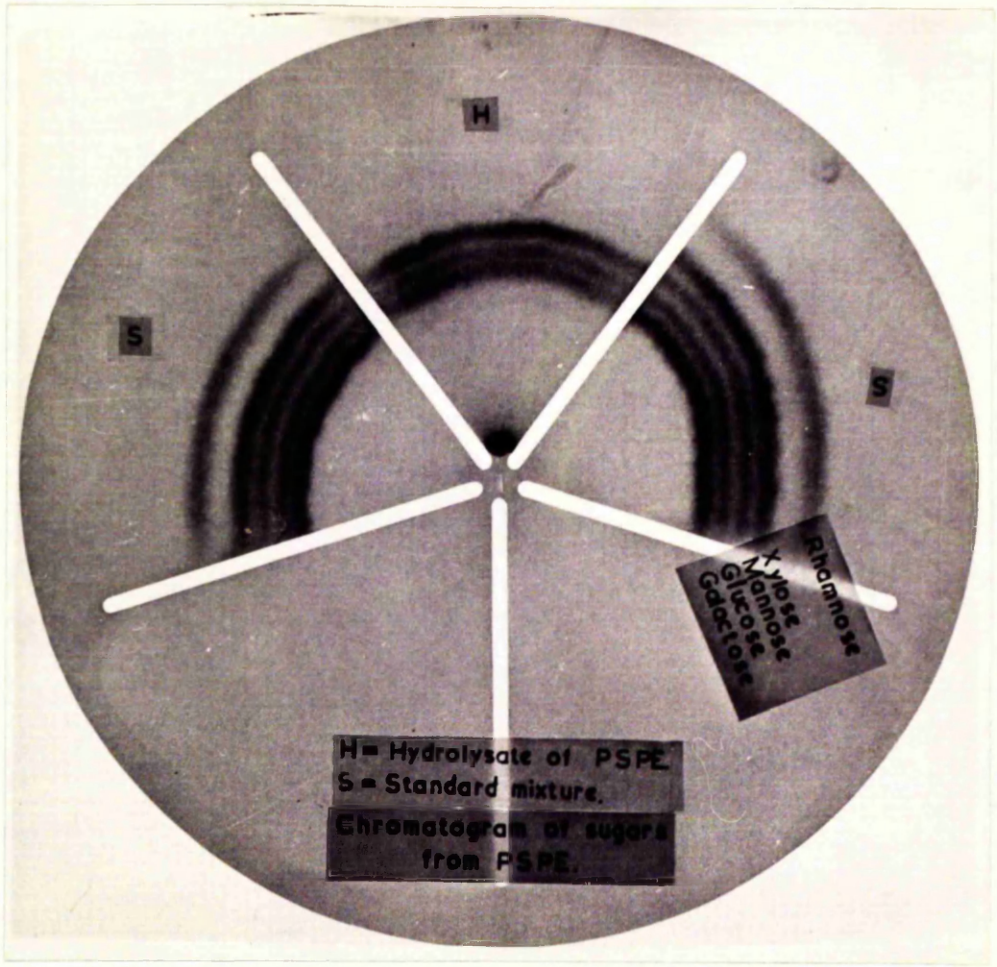


Fig.7 Chromatogram of the sugars in a 4 hr. N-H_2SO_4 hydrolysate of the active polysaccharide material (PSPE) obtained by phenol extraction of Proteus vulgaris culture fluid. Solvent, butanol-pyridine water; sprayed with aniline phthalate in moist butanol.

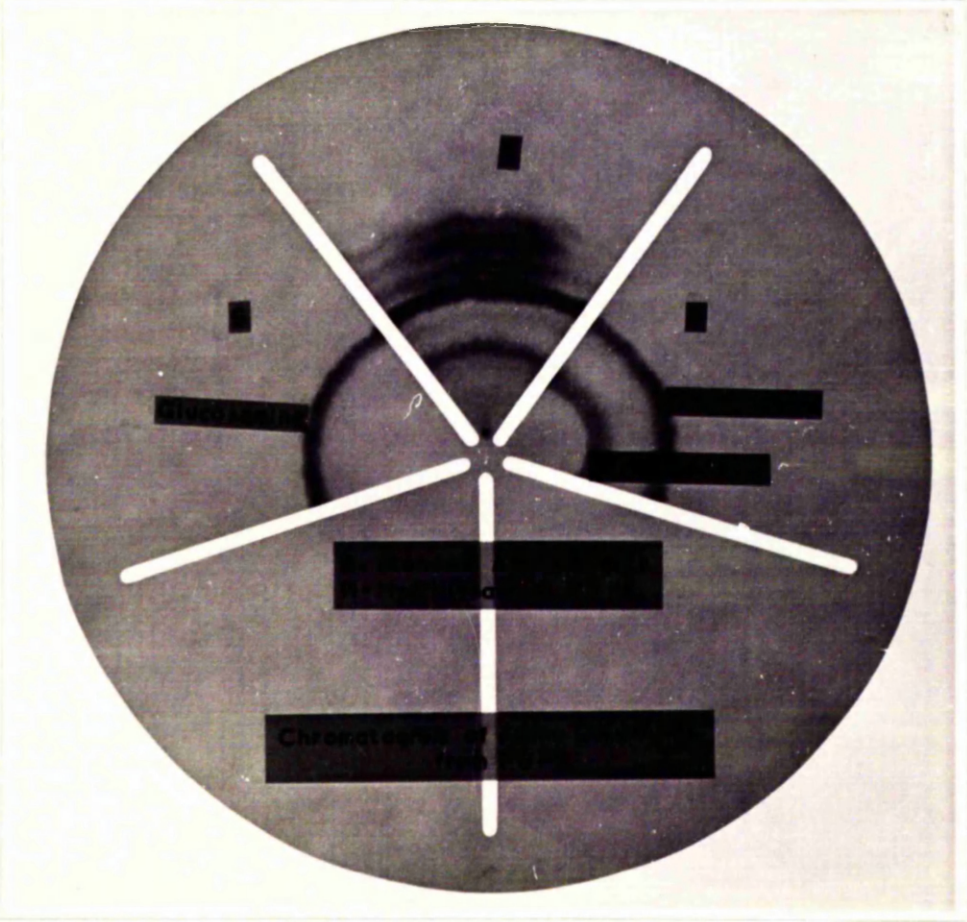


Fig.8 Chromatogram of the amino compounds in a 15 hr. 5N-HCl hydrolysate of the active polysaccharide material (PSPE) obtained by phenol extraction of Proteus vulgaris culture fluid. Solvent, butanol-acetic acid-water; sprayed with ninhydrin-cupric nitrate reagent.

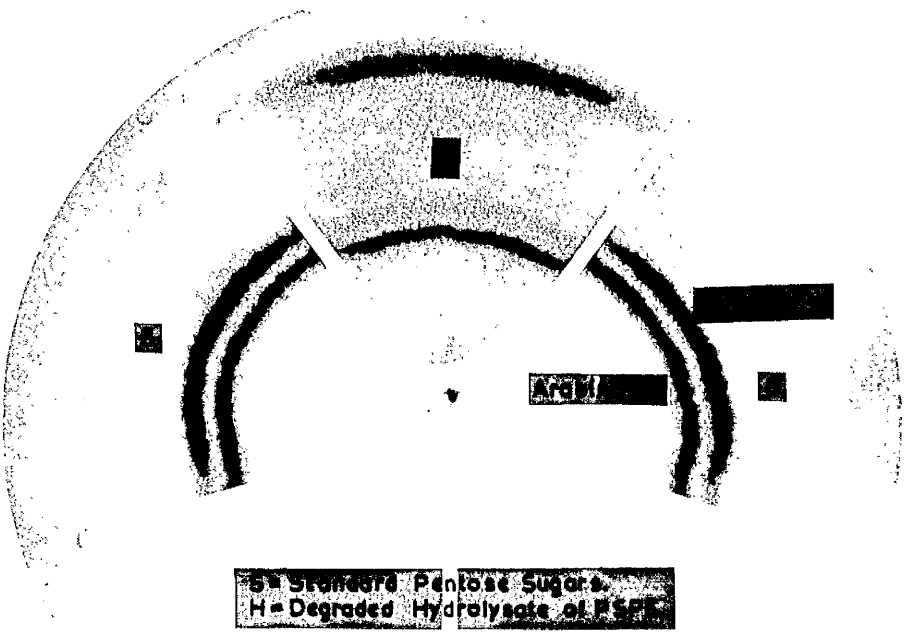


Fig.9 Chromatogram of the pentose sugars produced by ninhydrin degradation of a 15 hr. 5N-HCl hydrolysate of the active polysaccharide material (PSPE) obtained by phenol extraction of Proteus vulgaris culture fluid. Solvent, butanol-pyridine-water; sprayed with aniline phthalate in moist butanol.

in the original material (Fig.9).

During hydrolysis of PSPE with N-H_2SO_4, the mixture quickly went cloudy, and after about half an hour a flocculent precipitate began to form. When recovered by centrifuging this material was waxy in appearance and soluble in chloroform but not appreciably so in ether.

There was thus obtained by phenol extraction of Proteus vulgaris culture fluid, a highly pyrogenic lipopolysaccharide material, the component sugars of which were identified as galactose, glucose, mannose, an aldoheptose, glucosamine and galactosamine. Several other ninhydrin-staining components, including the dicarboxylic acids, glutamic and aspartic, were also present; these together with the apparently considerable amount of hexosamine, as judged by the intensity of its stain, would account for the relatively high nitrogen value (N, 4.7) of the material.

(b) Alcohol fractionation.

Further purification of PSPE was attempted by means of fractionation with alcohol. While the pyrogenic response as an index of increasing purity of a fraction was of little value due to the inability of the rabbit to react quantitatively over a wide dose range, it was possible

by pyrogen tests to eliminate those fractions showing little or no activity, and this procedure, together with measurement of increasing heptose content of the active fraction, was used to follow the purification of the material.

In a preliminary test, a solution of PSPE (350 mg.) in water (40 ml.) was cooled to 0-4°C and absolute alcohol at -10°C added slowly with continuous stirring. Slight clouding of the solution was observed when about 35 ml. of alcohol had been added but no precipitation occurred until the alcohol content of the mixture reached 50% v/v. After allowing to stand for several hours at 0-4°C, the material precipitating at this alcohol concentration was recovered by centrifuging redissolved in water and again precipitated by adding an equal volume of alcohol. The final precipitate was freeze-dried from aqueous solution (PSPE/AF50). The supernatant of the first alcohol precipitation was cooled to 0-4°C and the addition of cold alcohol continued when slight precipitation again occurred at 70% v/v alcohol concentration; this precipitate was also recovered by centrifuging and freeze-dried from aqueous solution (PSPE/AF70). Further increase in alcohol concentration to 80% v/v resulted in the separation of a

small amount of material; after recovery of this (PSPE/AF80), the supernatant fluid, containing material soluble in 80% alcohol, was concentrated to a small volume and freeze-dried (PSPE/AS80). The yields of the different fractions obtained were:-

PSPE/AF50	150 mg.	=	43% of PSPE
PSPE/AF70	31 mg.	=	9% of PSPE
PSPE/AF80	7 mg.	=	2% of PSPE

All fractions, including that soluble in 80% v/v alcohol, were tested for pyrogenic activity when a good response was obtained with the fraction PSPE/AF50 at a dose-level of 0.01 μg./kg. At this dose level the other fractions gave no response.

Since the main pyrogenic activity resided in the fraction PSPE/AF50 only this fraction was of direct interest. In subsequent fractionations, therefore, the material precipitating at 50% v/v alcohol concentration was recovered, and after reprecipitation, freeze-dried from aqueous solution; the supernatant solution of this precipitation, containing material soluble in 50% alcohol, was concentrated and freeze-dried (PSPE/AS50). Dry weight estimations carried out on these subsequent fractionations gave the yield of PSPE/AF50

as 40-45% based on PSPE. Several quantities each of about 300-500 mg. of PSPE were fractionated in this way to obtain a quantity of the active material and both main fractions examined as follows:-

Description.

PSPE/AF50. A pale buff coloured powder in the freeze-dried form which, like PSPE, was usually difficult to dissolve completely in water. At 1% w/v concentration the solution was somewhat viscous and opalescent.

PSPE/AS 50. This was darker in colour and readily soluble in water to give a clear yellow solution.

Pyrogenic activity. The results of pyrogen tests performed on both fractions at various dose levels are shown in Table 6.

Dose (µg/kg.)	Temperature response (°C)	
	PSPE/AF50	PSPE/AS50
0.003162	0.37	-
0.01	0.92	-
0.03162	1.10	-
0.1	1.67	0.15
1.0	-	0.19

Table 6. Temperature response in the rabbit to various dose-levels of the products obtained by alcohol fractionation of the phenol extracted material(PSPE from *Proteus vulgaris* culture fluid. Fraction PSPE/AF50 precipitated at 50% alcohol concentration; fraction PSPE/AS50 was soluble at this concentration of alcohol. Each temperature rise given in the table was the average for 10 animals.

The 50% alcohol soluble fraction was pyrogenically inactive at dose levels up to 1 $\mu g/kg$, but three rabbits receiving 20 $\mu g/kg$ of this material gave an average temperature rise of 1.16°C. A fever reaction at this comparatively high dose level does not necessarily mean, however, that the material was in itself pyrogenic, as due to the high activity of the 50% alcohol insoluble fraction, a small proportion of the latter present could confer pyrogenic properties.

Chemical composition.

PSPE/AF50. Analytical figures obtained were N,2.95; total P,1.3. Measurement of the ultraviolet absorption at 260 mμ. indicated that the material was free from nucleic acid (Fig.10). When examined by the H_2SO_4 - cysteine reaction, absorption maxima at 505 mμ. and 410 mμ. were obtained, showing the presence of both heptose and hexose sugars; the aldoheptose content, estimated as α-D-glucoheptose was 3.3% (Fig.11).

Hydrolysis of a sample of the material for four hours with N-H_2SO_4 liberated the sugars, galactose, glucose, an aldoheptose and mannose, as identified by paper chromatography (Fig.12). The chromatogram of a 15 hour 5N-HCl hydrolysate, when sprayed with ninhydrin - cupric nitrate reagent, showed a strong band corresponding to glucosamine, together

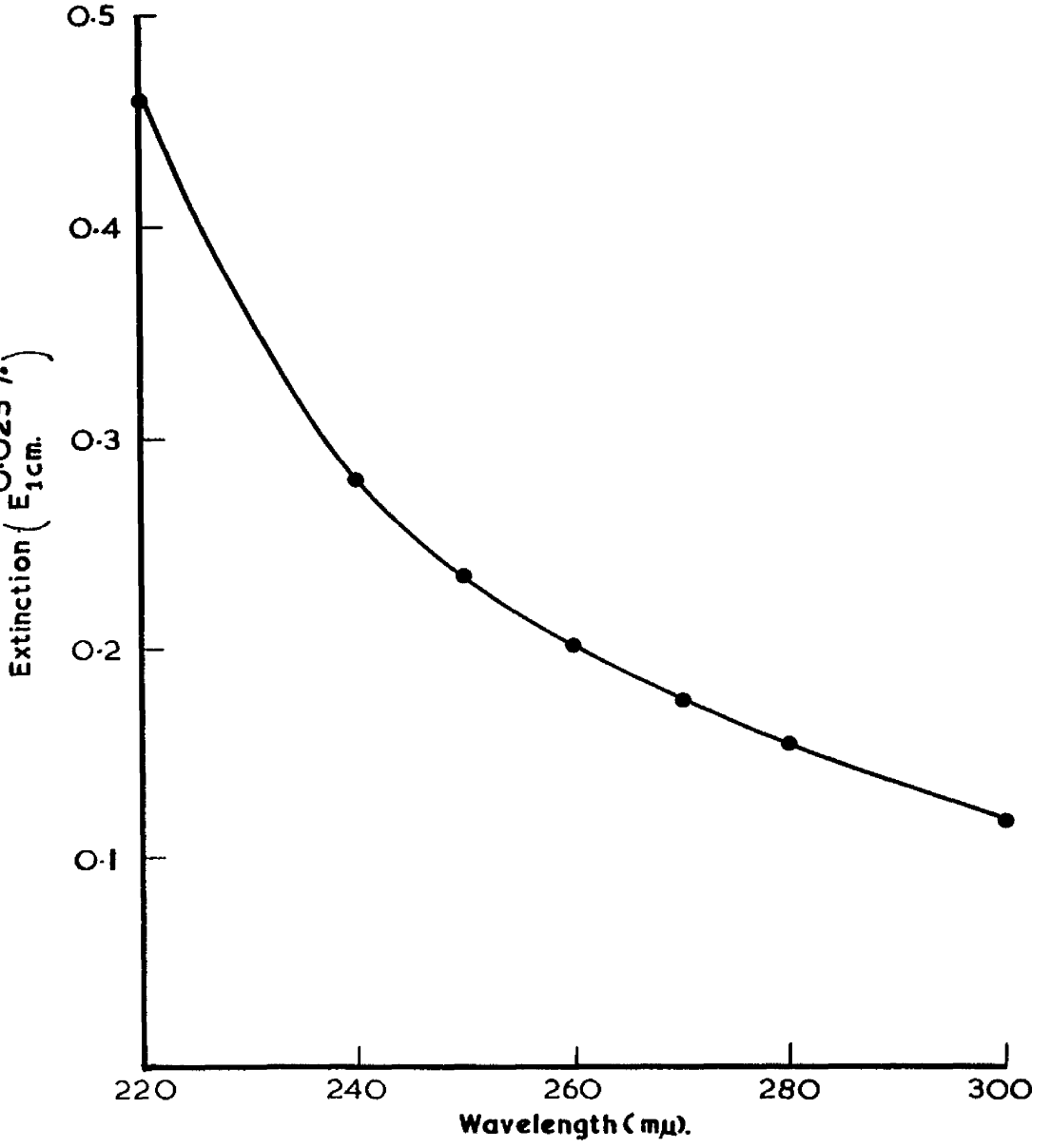


Fig. 10. The ultraviolet absorption spectrum of the active polysaccharide fraction (PSPE/AF50) obtained by alcohol fractionation of the phenol-extracted material (PSPE) from Proteus vulgaris culture fluid.

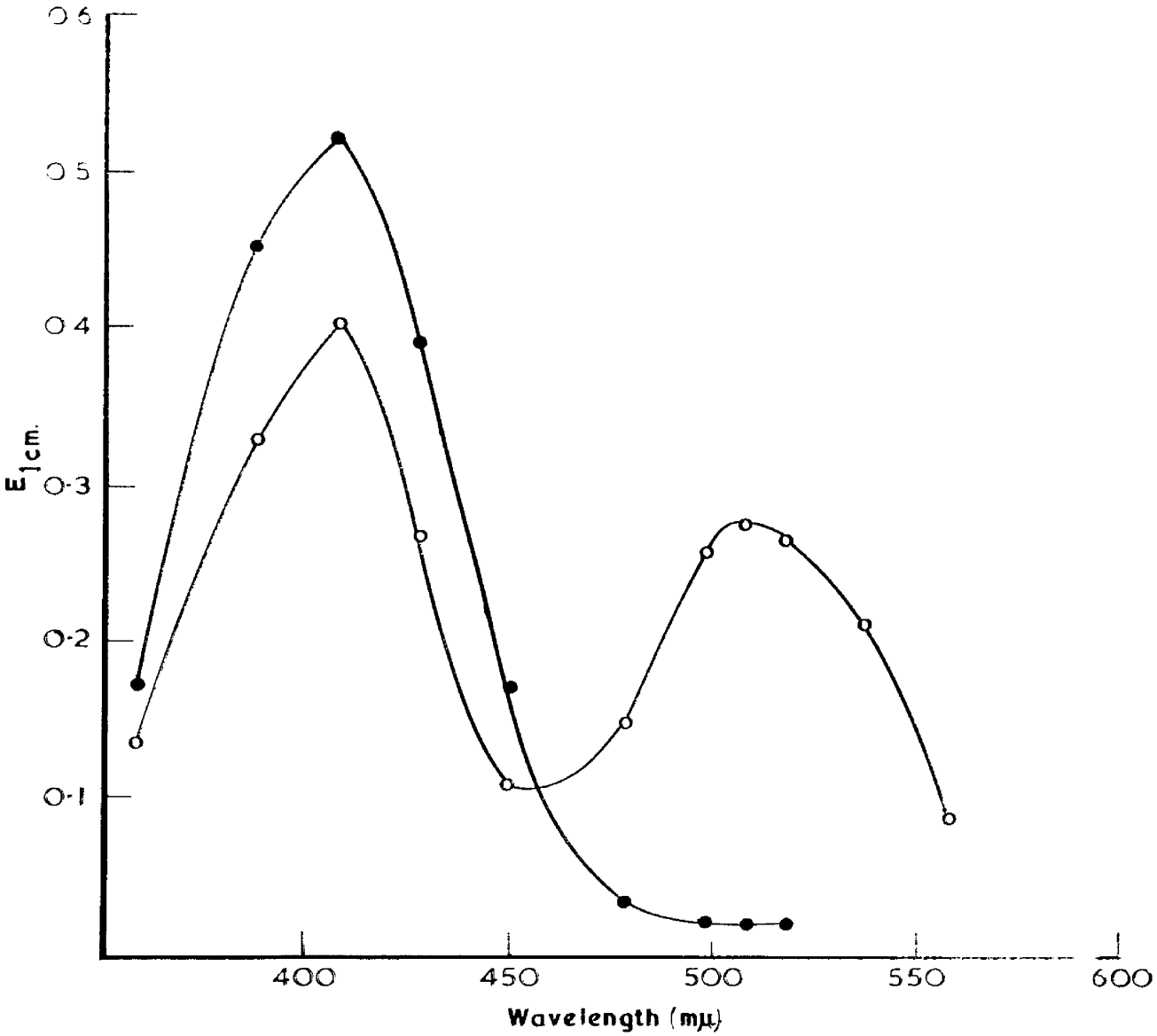


Fig. II. The Dische reaction on the products of alcohol fractionation of the phenol-extracted material from Proteus vulgaris culture fluid

o—o PSPE/AF50, precipitated at 50% alcohol concentration

●—● PSPE/AS50, fraction soluble at this alcohol concentration

Absorption spectra of the fractions (500 μg amounts) after treatment with H_2SO_4-cysteine: readings taken at 22 hours.

with other faint ninhydrin - staining bands one of which was identified as glutamic acid (Fig.13). When this hydrolysate was degraded with ninhydrin both arabinose and lyxose were obtained, indicating the presence of glucosamine and galactosamine in the original material (Fig.14).

As mentioned previously, it was found that whereas standard glucosamine usually ran as a single band in the solvent butanol-acetic acid-water, standard galactosamine usually gave two bands, one corresponding approximately to that of glucosamine and the other inside this (Fig.13). The development of more than one spot of chromatographing an amino sugar in an acidic solvent has been observed by other workers,[54] and is presumably due to formation of mixed salts of the base. Thus the two bands obtained with the standard galactosamine (applied as galactosamine hydrochloride) were probably due to its partial conversion to galactosamine acetate. Degradation of the hydrolysate of PSPE/AP50 with ninhydrin, however, gave both arabinose and lyxose indicating the presence of glucosamine and galactosamine in the undegraded hydrolysate, yet only a single band, corresponding to that of glucosamine, was observed on chromatographing the undegraded hydrolysate, using the same acidic solvent as for the standard galactos-

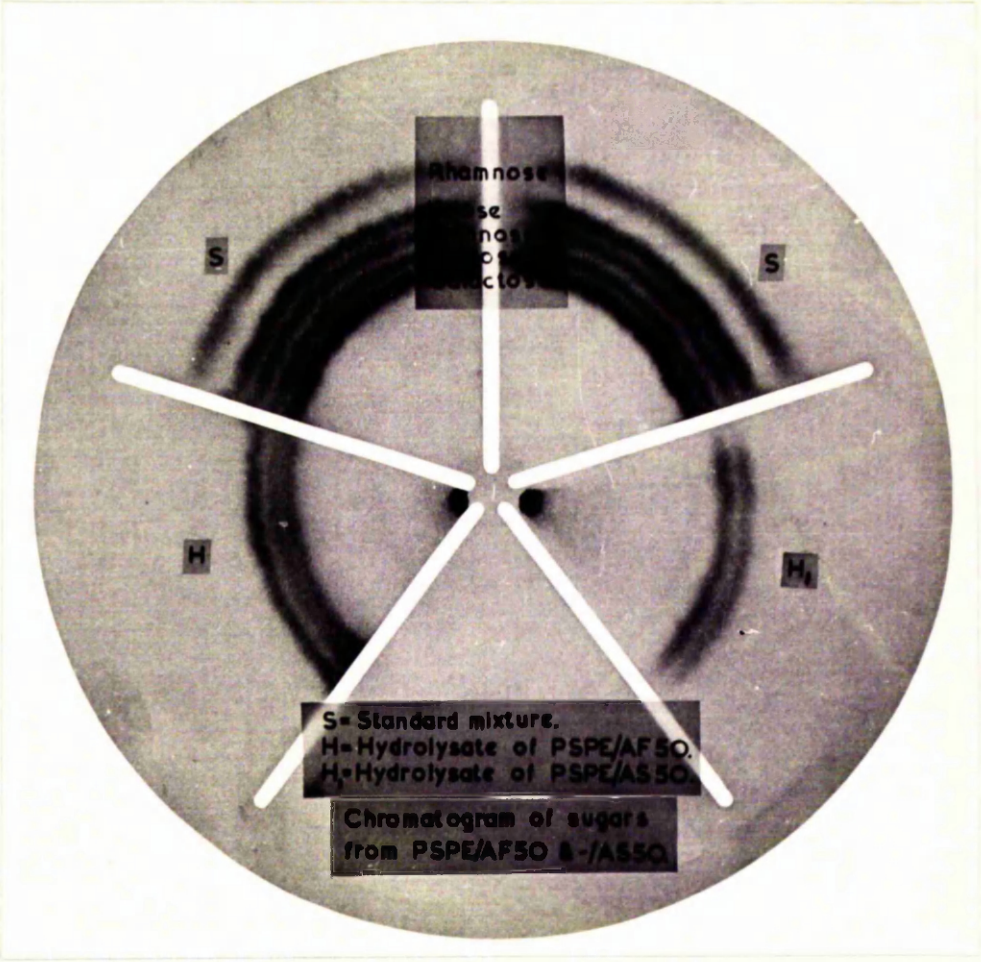


Fig.12 Chromatogram of the sugars in 4 hr. N-H_2SO_4 hydrolysates of the products obtained by alcohol fractionation of the phenol extracted-material from Proteus vulgaris culture fluid. Fraction PSPE AF50 precipitated at 50% alcohol concentration; fraction PSPE AS50 was soluble in this concentration of alcohol.
Solvent, butanol-pyridine-water; sprayed with aniline phthalate in moist butanol.

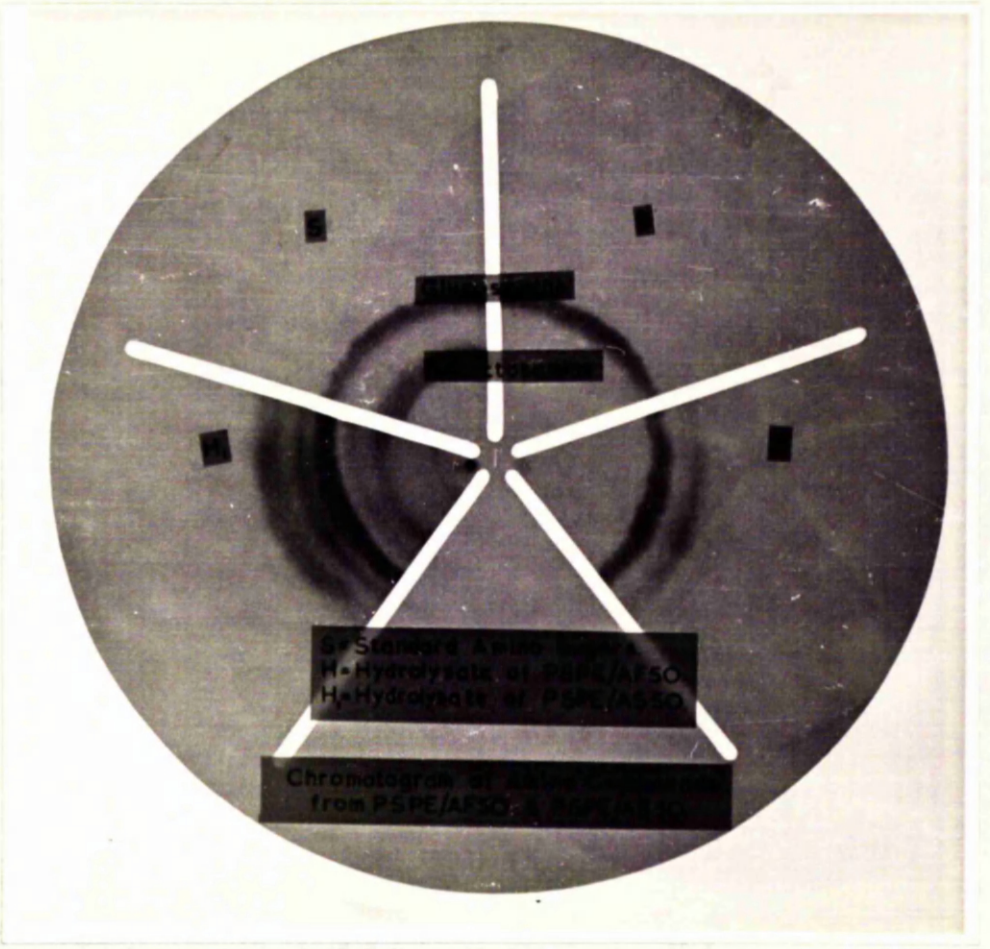


Fig.13 Chromatogram of the amino compounds in 15 hr. 5N-HCl hydrolysates of the products obtained by alcohol fractionation of the phenol-extracted material from Proteus vulgaris culture fluid. Fraction PSPE/AF50 precipitated at 50% alcohol concentration; fraction PSPE/AS50 remained in solution at this concentration of alcohol. Solvent butanol-acetic acid-water; sprayed with ninhydrin-cupric nitrate reagent.

Fig. 14 Chromatogram of the pentose sugars produced by degrading with ninhydrin, 15 hr. 5N-HCl hydrolysat of the products obtained by alcohol fractionation the phenol-extracted material from Proteus vulgari culture fluid. Fraction PSPE/AF50 precipitated at 50% alcohol concentration; fraction PSPE/AS50 remained in solution at this alcohol concentration Solvent, butanol-pyridine-water; sprayed with aniline phthalate in moist butanol.

amine. One possible explanation of these results is that the standard galactosamine hydrochloride may have contained some galactosamine base, so allowing ready formation of the acetate, whereas the hydrolysate obtained by hydrolysing PSPE/AF50 with 6N-HCl, certainly contained the amino sugars as hydrochlorides with possibly a trace of free hydrochloric acid, the latter preventing the conversion of the amino sugar hydrochlorides therein to acetates. The reason for using an acidic solvent in these experiments is its suitability for the separation of amino acids, and reliance was placed on the ninhydrin-degradation procedure to identify the amino sugars. It is, however, theoretically possible that the pentoses, arabinose and lyxose, could have been derived from mannosamine and talosamine, but the presence of these amino sugars is unlikely.

Hydrolysis of a sample of PSPE/AF50 with N-H_2SO_4 resulted in the separation of a chloroform soluble lipid.

PSPE/AS50. Analytical figures obtained were N,5.97; total P,0.4. When examined by the H_2SO_4-cysteine reaction only an absorption with a maximum at 410 mμ. was obtained, indicating the presence of hexose sugars but the absence of heptose sugars (Fig.11).

The sugars identified by paper chromatography were mannose and glucose (Fig.12); chromatography also revealed the presence of several ninhydrin-staining components, two of which were identified as glutamic and aspartic acids (Fig.13). Ninhydrin degradation of the 15 hour 5N-HCl hydrolysate yielded only arabinose, indicating the absence of galactosamine from this fraction (Fig.14).

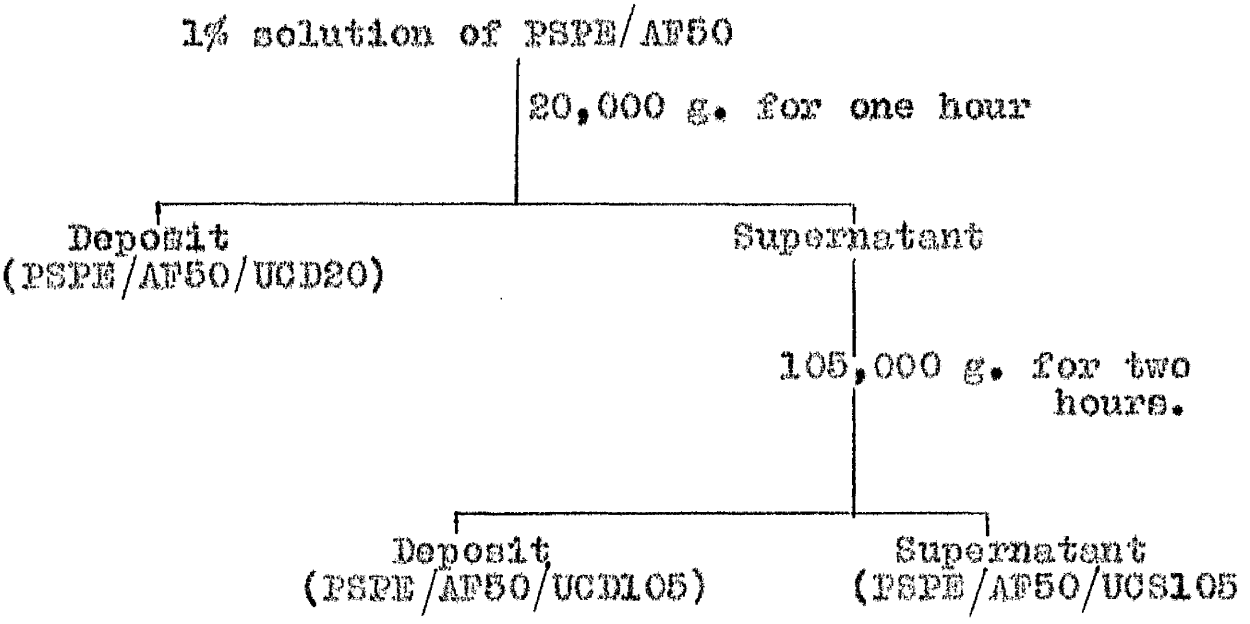
Acid hydrolysis did not result in the separation of lipid material even on prolonged heating; the solution merely darkened in colour.

There was thus obtained by alcohol fractionation of the phenol extracted material (PSPE), two main fractions in approximately equal amounts. That precipitating at 50% alcohol concentration was highly active and yielded on acid hydrolysis a chloroform-soluble lipid and the sugars galactose, glucose, an aldoheptose, mannose, glucosamine and galactosamine, while that remaining in solution at this alcohol concentration was relatively inactive, apparently lipid-free and contained as constituent sugars, glucose, mannose and glucosamine.

(c) Fractional Sedimentation of the ultracentrifuge.

Preparative ultracentrifuge runs were carried out in

a Spinco Preparative Ultracentrifuge, whereby sedimentations at values up to 105,000 g. were possible. Following the observation that a considerable amount of material sedimented when a solution of the active alcohol fraction (PSPE/AF50) was centrifuged at 20,000 g. for a short period, a fractional sedimentation was carried out as follows, the deposit in each case being redissolved in water and the solution freeze-dried; the final supernatant solution was concentrated under vacuum and also freeze-dried.



No further sedimentation occurred on continued centrifuging beyond two hours at 105,000 g.

Examination of Fractions. Estimations of nitrogen, phosphorus and aldoheptose contents were carried out on each

of the fractions with the results shown in Table 7. The material sedimenting at the two values of g. gave almost identical analytical figures, and the curves obtained in the H_2SO_4-cysteine reaction were practically superimposible, with peaks at 410 mμ. and 505 mμ. When hydrolysed with N-mineral acid each fraction gave a chloroform soluble lipid.

Fraction	N	P	Heptose	Lipid
PSPE/AF50/UCD20	2.10	1.72	7.0	+
PSPE/AF50/UCD105	2.08	1.70	6.8	+
PSPE/AF50/UCS105	3.42	0.90	-	-

Table 7. Analytical figures for the products of fractional centrifugationoof the active lipopolysaccharide material (PSPE/AF50) obtained by alcohol fractionation phenol extracted, Proteus vulgaris culture fluid. Fractions PSPE/AF50/UCD20 and PSPE/AF50/UCD105 sedimented at 20,000 g. and 105,000 g. resp. fraction PSPE/AF50/UCS105 remained in solution at 105,000 g.

The final centrifuge supernatant fraction, PSPE/AF50/UCS105, did not however, contain any detectable lipid, and in the H_2SO_4-cysteine reaction gave only an absorption with a maximum at 410 mμ, indicating the absence of aldoheptose sugar from this fraction. Tests showed that the two sediments

were equally pyrogenic.

From the close similarity of the analytical figures obtained with the two sedimented fractions (Table 7), it appeared that centrifuging at 105,000 g. had merely led to a complete sedimentation of the material already sedimenting after a short period of spinning at 20,000 g, in which case a somewhat longer period of spinning at the lower value of g would probably have been equally effective. Accordingly, a solution of PSPE/AF50 (1%) was centrifuged for four hours at 20,000 g and the supernatant fluid decanted from the deposit. Spinning of this superantant at 60,000 g for two hours resulted in only a trace of material, sedimenting, and this did not appear to increase in quantity when the value of g was increased to 105,000 for a further two hours. In subsequent purifications of the lipopolysaccharide, therefore, the active fraction was sedimented at 20,000 g.

Using 80-100 litre batches of culture fluid, the stages up to the fractionation with alcohol were as already described, except that the 50% alcohol insoluble fraction, PSPE/AF50, was not isolated in freeze-dried form but dissolved in 30-40 ml. of water immediately after precipitation. This solution was then centrifuged at 20,000 g for four hours, the supernatant fluid removed and the deposit

freeze-dried from aqueous solution (PSPE/AF50/UCD20). The supernatant fluid was centrifuged at 105,000 g for two hours, decanted from the minute residue, concentrated and freeze-dried (PSPE/AF50/UCS20). The yield of PSPE/AF50/UCD20 was approximately 45% based on PSPE/AF50. Both fractions were examined as follows.

Description.

PSPE/AF50/UCD20:- A very pale buff-coloured powder in the freeze-dried state which dissolved with difficulty in water; at 1% concentration the solution was somewhat viscous and opalescent.

PSPE/AF50/UCS20:- A pale brown powder dissolving readily in water to give a clear yellow solution.

Pyrogenic activity.

The results of pyrogen tests performed on the two fractions are shown in Table 8. The weak response obtained with the centrifuge supernatant fraction (PSPE/AF50/UCS20) at a dose level of 1 μg/kg. was probably due to the presence of a small proportion of the highly active fraction PSPE/AF50/UCD20, since centrifuging a solution of the former fraction at 105,000 g for two hours resulted in a loss of pyrogenic activity at a dose level of 1 μg/kg., although a

barely detectable amount of material sedimented.

Dose µg/kg.	Temperature response (°C)	
	PSPE/AF50/UCD20	PSPE/AF50/UCS20
0.005	0.67	-
0.01	1.12	-
0.05	1.43	-
0.1	1.69	0.37
1.0	-	0.86

Table 8. Temperature response in the rabbit to various dose-levels of the products of fractional centrifugation of the lipopolysaccharide material (PSPE/AF50) obtained by alcohol fractionation of phenol extracte Proteus vulgaris culture fluid. Fraction PSPE/AF50/UCD20 sedimented at 20,000 g; fraction PSPE/AF50/UCD20 remained in solution at values of g up to 105,000.
Each temperature rise in the table was the average for 10 animals.

Chemical composition.

PSPE/AF50/UCD20. Analytical figures obtained were C,43.07; H,7.86; N,2.1; total P,1.72. The reducing value (Somygyi), calculated as glucose, on hydrolysing with N-HCl, rose to a maximum of 30% in eight hours but reached 23% (76% of the maximum) in two hours (Fig.15). Hexosamine, determined as

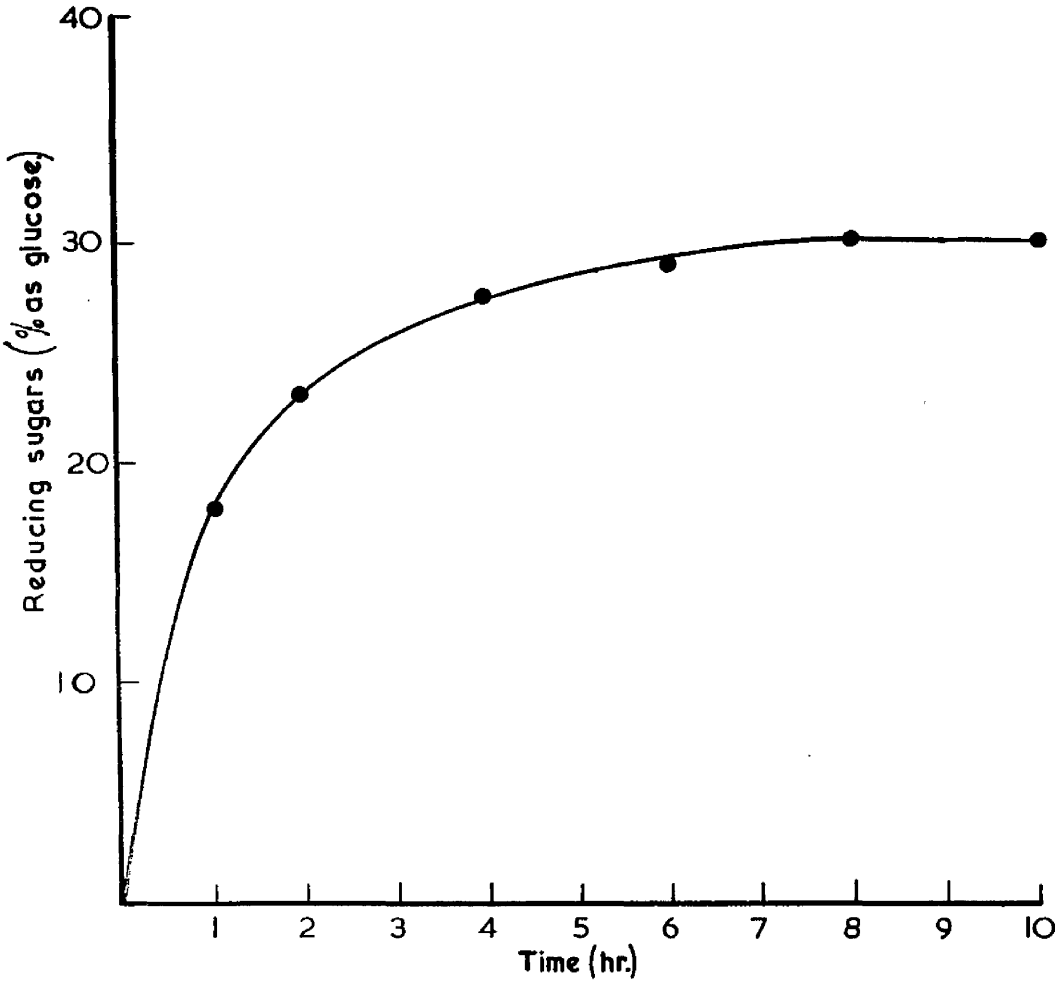


Fig. 15. Hydrolysis curve showing the release of reducing sugars, estimated as glucose, from the lipopolysaccharide (PSPE/AF50/UCD20) isolated from phenol-extracted Proteus vulgaris culture fluid by alcohol fractionation and fractional centrifugation.

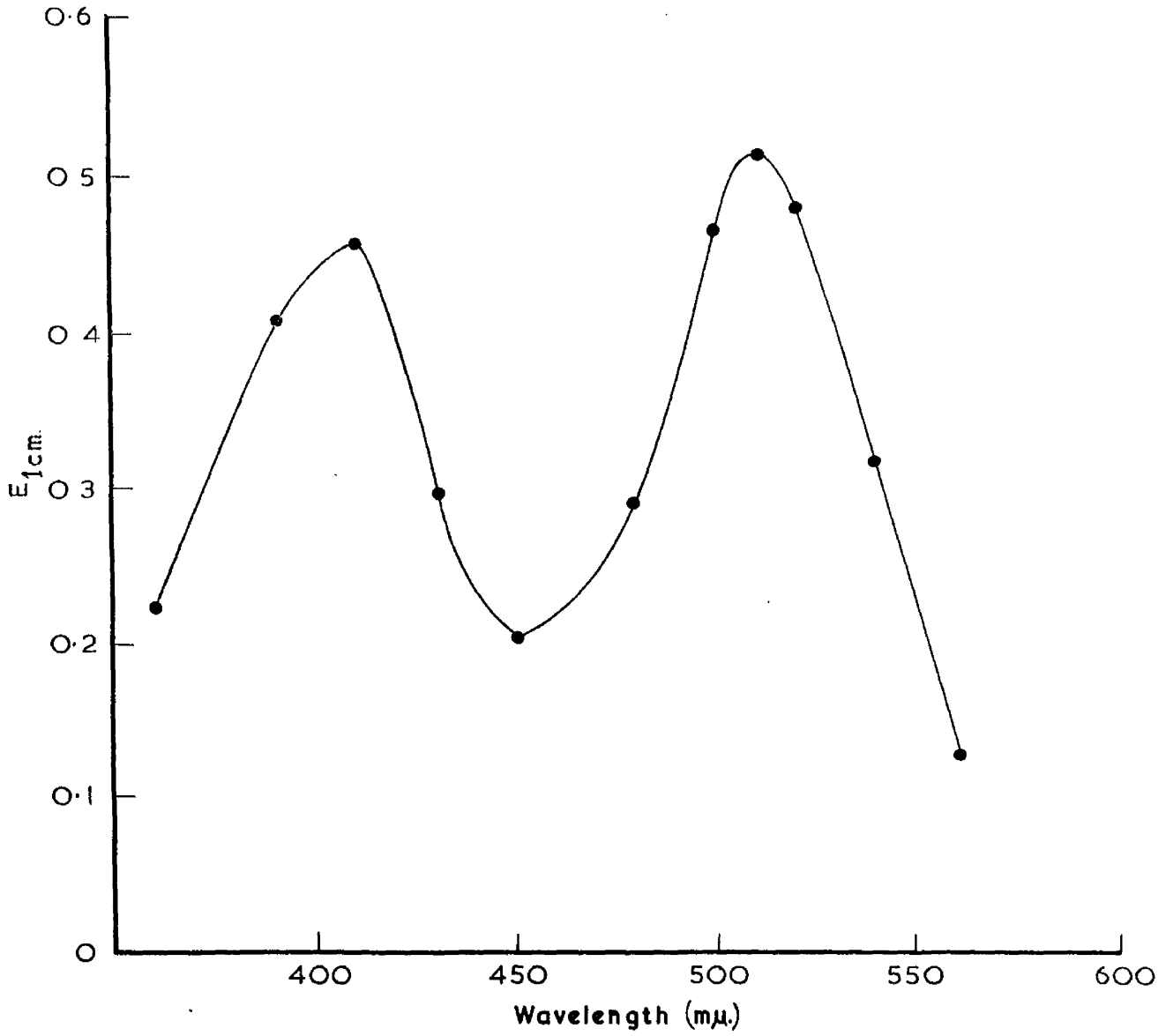


Fig.16. The Dische reaction on the lipopolysaccharide (PSPE/AF50/UCD20) obtained from phenol-extracted Proteus vulgaris culture fluid by alcohol fractionation and fractional centrifugation.
Absorption spectrum of the material (500 μg.) after treatment with H_2SO_4-cysteine: readings taken at 22 hours.

glucosamine by the Elson-Morgan method,[80,806] was estimated to compose 14% of the material. For the determination of chloroform-soluble lipid content, a sample of the material was heated with N-HCl for 3½ hours on a boiling water-bath, the flocculent precipitate recovered by centrifuging, dried and dissolved in chloroform. After removal of the solvent by heating in an atmosphere of nitrogen, the residue was weighed. From several such determinations, chloroform-soluble lipid was estimated to compose 32-34% of the original lipopolysaccharide. The aldoheptose content, calculated as α-D-glucoheptose was 7% (Fig.16).

Paper chromatography of an N-H_2SO_4 hydrolysate revealed the presence of glucose, galactose and heptose as the main sugars, together with a trace of mannose (Fig.17). The chromatogram of the hydrolysate obtained by hydrolysing a sample of the material with 5M-HCl showed a strong ninhydrin-staining band corresponding to glucosamine and a weak band which appeared to be glutamic acid (Fig.18); degradation of this hydrolysate with ninhydrin produced both arabinose and lyxose, indicating the presence of glucosamine and galactosamine in the original lipopolysaccharide (Fig.19).

PSPE/AF50/UCS20. Analytical figures obtained were N,3.4; total P,0.9. Acid hydrolysis did not liberate any lipid

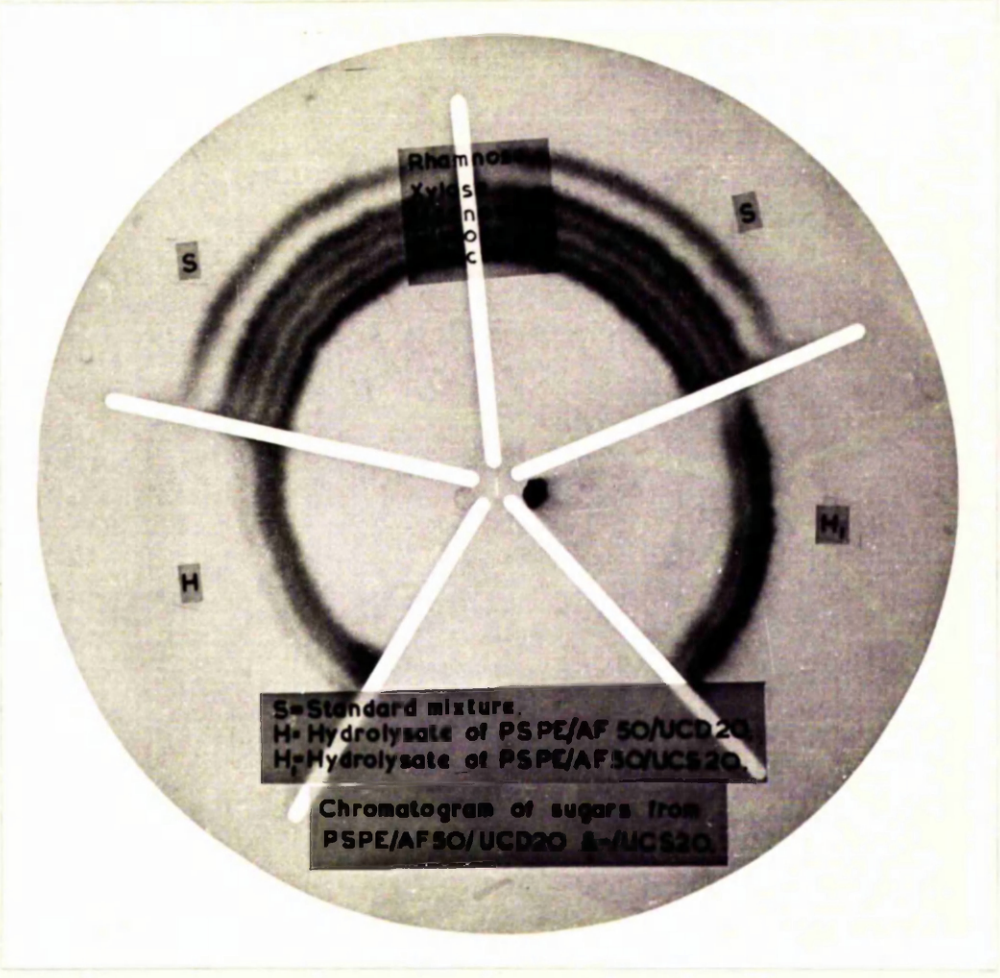


Fig.17 Chromatogram of the sugars in 4 hr. N-H_2SO_4 hydrolysates of the products of fractional centrifugation of the active lipopolysaccharide material (PSPE/AF50) obtained by alcohol fractionation of phenol-extracted Proteus vulgaris culture fluid. Fraction PSPE/AF50/UCD20 sedimented at 20,000 g; fraction PSPE/AF50/UCS20 remained in solution at values of g up to 105,000.
Solvent, butanol-pyridine-water; sprayed with aniline phthalate in moist butanol.

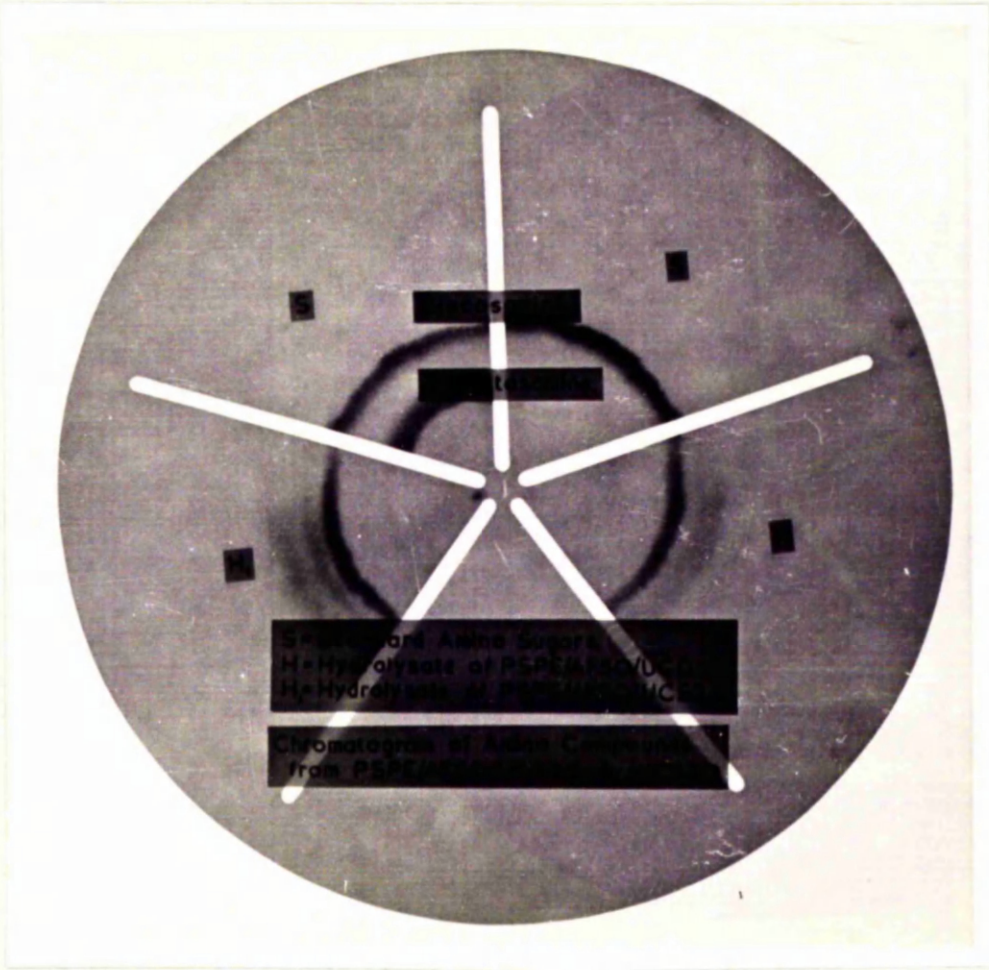


Fig.18 Chromatogram of the amino compounds in 15 hr. 5N-HC hydrolysates of the products of fractional centrifu ation of the active lipopolysaccharide material (PSPE/AF50) obtained by alcohol fractionation of phenol-extracted Proteus vulgaris culture fluid. Fraction PSPE/AF50/UCD20 sedimented at 20,000 g; fraction PSPE/AF50/UCS20 remained in solution at values of g up to 105,000.
Solvent, butanol-acetic acid-water; sprayed with ninhydrin-cupric nitrate reagent.

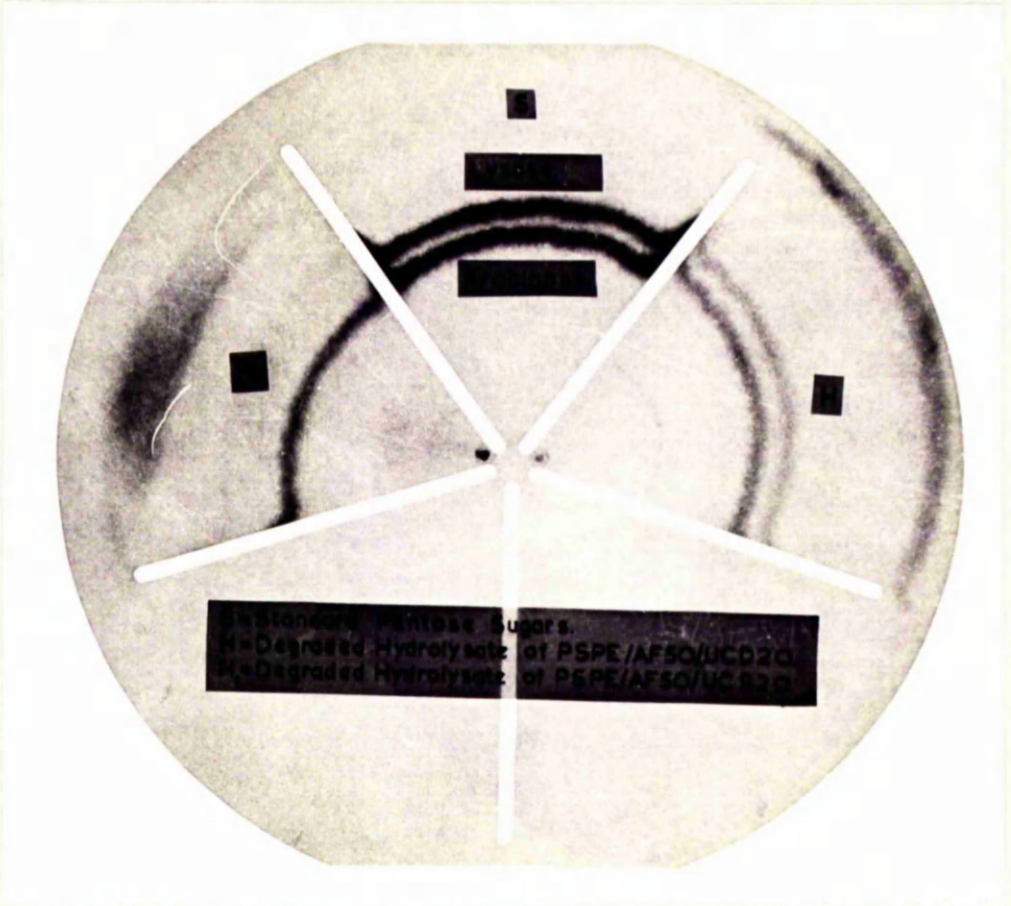


Fig.19 Chromatogram of the pentose sugars produced by degrading, with ninhydrin, 15 hr. 5N-HCl hydrolysat of the products of fractional centrifugation of the active lipopolysaccharide material (PSPE/AF50) obtained by alcohol fractionation of phenol-extract Proteus vulgaris culture fluid. Fraction PSPE/AF50 UCD20 sedimented at 20,000 g; fraction PSPE/AF50/ UCS20 remained in solution at values of g up to 105,000.

Solvent, butanol-pyridine-water; sprayed with aniline phthalate in moist butanol.

material and the H_2SO_4-cysteine reaction showed the absence of heptose. The constituent sugars were identified as glucose and mannose (Fig.17) together with glucosamine and several other ninhydrin-staining components (Figs.18 and 19).

In the foregoing experiments a combination of solvent fractionation and fractional sedimentation were used to separate relatively non-pyrogenic polysaccharides from the crude phenol-extracted material, leaving the active lipopolysaccharide. Since the relatively inactive fraction separated by alcohol fractionation (PSPE/A50) was soluble in 50% alcohol and readily soluble in water, it appeared to be of smaller particle size than the active fraction (PSPE/AF50) which was insoluble in 50% alcohol. As the inactive material present in the latter fraction did not sediment at 105,000 g, it seemed possible that high speed centrifuging of the aqueous phase of the phenol extraction might give as good a degree of purification, in the absence of preliminary solvent fractionation. Accordingly, the dialysed aqueous phase after concentration to approximately 1% w/v total solids was centrifuged at 20,000 g for four hours and the supernatant fluid removed. The sediment (PSPE/UCD20) was redissolved in the original volume of water and centrifuged in the same way a second and third time [PSPE/UCD20(1) and

PSPE/UCD20(2)]. The supernatant of the first centrifuging was spun at 105,000 g for four hours, decanted from the minute deposit and freeze-dried (PSPE/UCS20).

From the analytical results given in Table 9 it is seen that the lipopolysaccharide obtained by ultracentrifuging of the aqueous phase at 20,000 g (PSPE/UCD20) gave analytical figures similar to those of the lipopolysaccharide (PSPE/AF50/UCD20) obtained by alcohol fractionation and fractional sedimentation; a comparison of the two lipopolysaccharides is given in Table 10. Measurement of the ultraviolet absorption at 260 mμ. of a sample of PSPE/UCD20 in N/100 NaOH showed that the material was free from nucleic acid (Fig.20). The analyses given in Table 9 also show that resedimentation of PSPE/UCD20 at the same value of g did not significantly alter its analytical figures, but resulted in a somewhat decreased yield.

Examination of PSPE/UCS20.

The analytical figures obtained are given in Table 9. In the H_2SO_4-cysteine reaction only an absorption with a maximum at 410 mμ. was found indicating the absence of aldoheptose (Fig.21). The component sugars identified by paper chromatography were glucose and mannose together with glucosamine and other ninhydrin-staining components. The material did not evoke a pyrogenic response in rabbits at

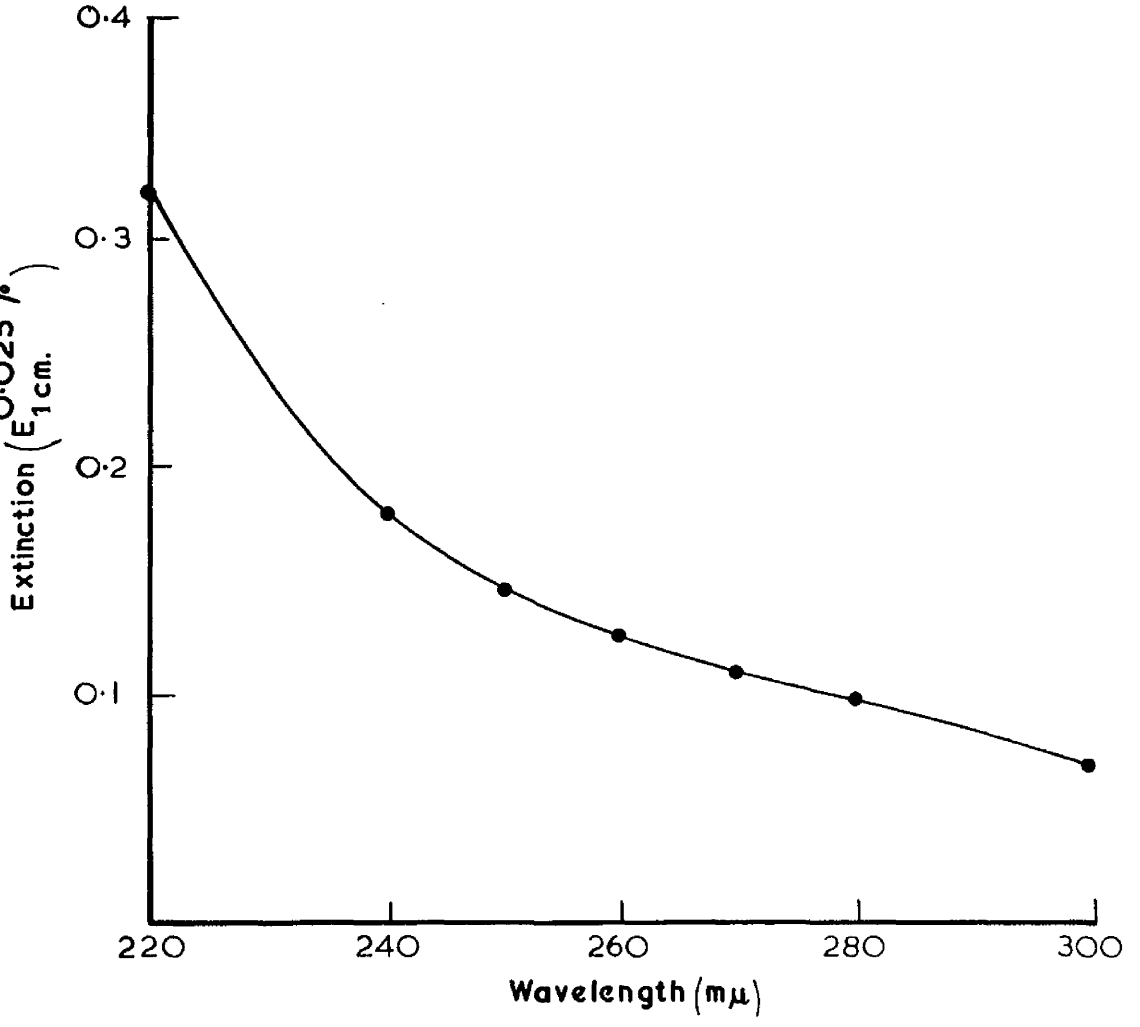


Fig. 20. The ultraviolet absorption spectrum of the lipopolysaccharide (PSPE/UCD20) obtained from phenol–extracted Proteus vulgaris culture fluid by sedimentation in the ultracentrifuge

Fraction	N	P	Yield as % of total solids in aqueous phase	Lipid
PSPE/UCD20	2.17	1.73	27	+
PSPE/UCD20(1)	2.14	1.68	23	+
PSPE/UCD20(2)	2.10	1.70	21	+
PSPE/UCS20	5.18	0.61	-	-

Table 9. Analyses of the products obtained by fractional centrifugation of phenol extracted Proteus vulgaris fluid. The fraction sedimenting at 20,000 *g* (PSPE/UCD20) was redissolved in water and resedimented a second (PSPE/UCD20.1) and a third time (PSPE/UCD20.2 fraction PSPE/UCS20 remained in solution at values of *g* up to 105,000.

Fraction	N	P	Heptose	Lipid
PSPE/AF50/UCD20	2.10	1.72	7.0	32-34%
PSPE/UCD20	2.17	1.68	7.0	30-34%

Table 10. Comparison of the analytical figures obtained for the lipopolysaccharides isolated from phenol extracted Proteus vulgaris culture fluid by alcohol fractionation and fractional centrifugation (PSPE/AF50/UCD20) and by fractional centrifugation without prior alcohol fractionation (PSPE/UCD20).

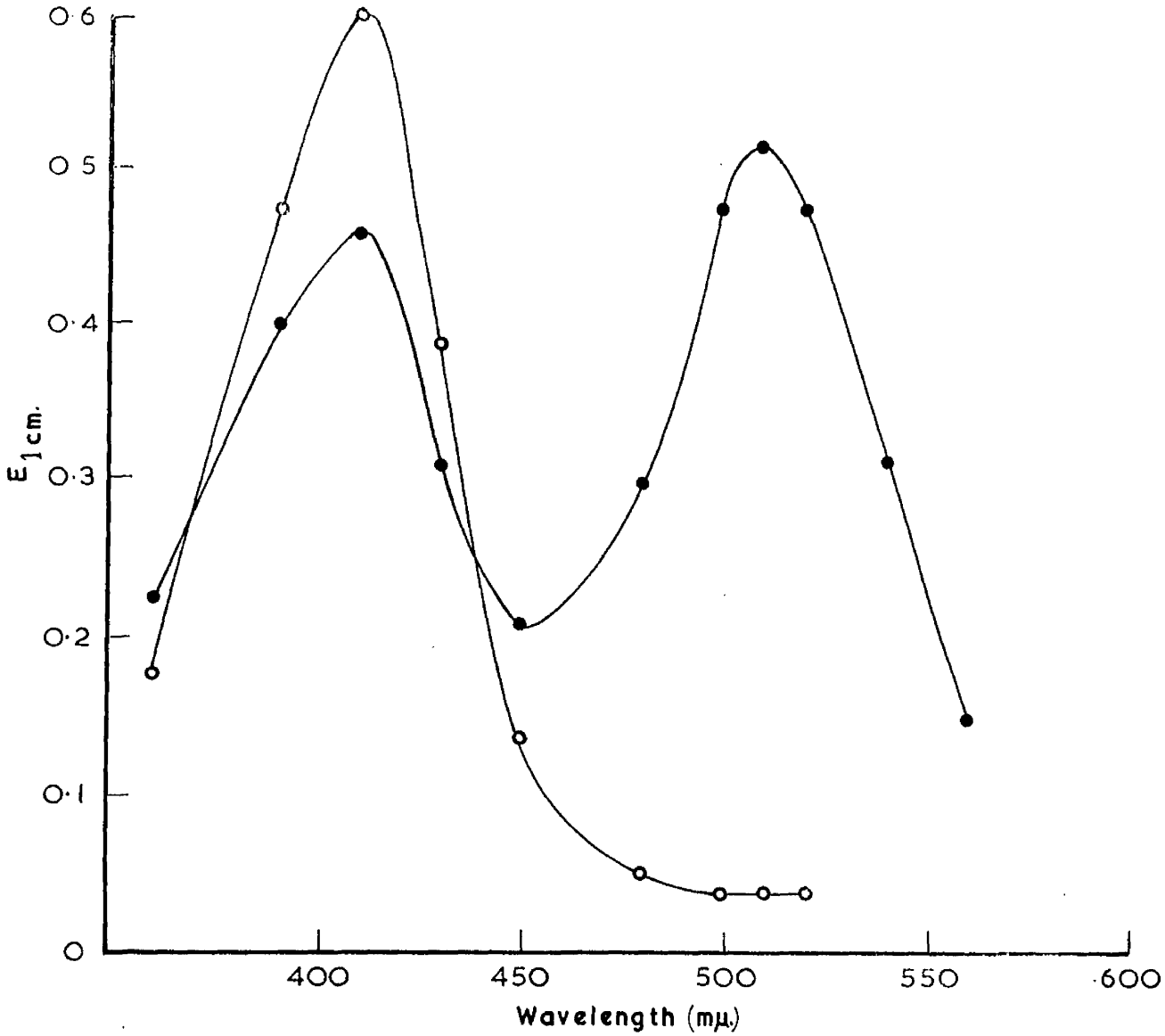


Fig. 21. The Dische reaction on the products of fractional centrifugation of phenol–extracted Proteus vulgaris culture fluid.

●—● Fraction PSPE/UCD20 sedimenting at 20,000g.

o—o Fraction PSPE/UCS20 remaining in solution at 105,000g.

Absorption spectra of the fractions (500 μg. amounts) after treatment with H_2SO_4–cysteine: readings taken at 22 hours.

dose-levels up to 1 μg/kg.

An unidentified sugar component of the lipopolysaccharide.

The usual hydrolysis time used in the chromatographic examination of the lipopolysaccharide for monosaccharides was 3-4 hours with N-H_2SO_4. Samples of the polysaccharide were also hydrolysed for periods of 5, 15 and 30 minutes and run on paper strips using the method of descending flow; the results are shown in diagrammatic form in Fig.22, since some of the spots were too weak to photograph. With the short periods of hydrolysis a sugar was detected running between ribose and rhamnose and staining reddish-brown with aniline phthalate. It was present after 5 minutes hydrolysis but could not be detected after 150 minutes hydrolysis, suggesting that it was an acid-labile sugar readily split off from the polysaccharide and probably occupying a terminal position in the polymer chain.

In the following sections, the abbreviation LPS(E) will be used to designate the lipopolysaccharide obtained from the culture fluid, whether purified by a combination of solvent fractionation and fractional sedimentation or by the latter process alone.

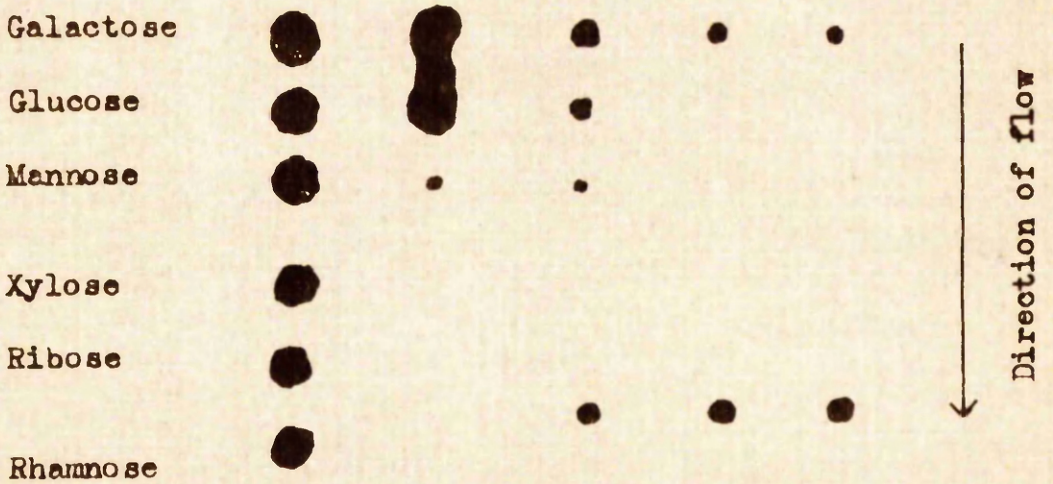


Fig. 22. Diagram of the chromatogram of reducing sugars from the lipopolysaccharide, PSPE/UCD20, after various periods of hydrolysis.

Solvent, butanol-pyridine-water; sprayed with aniline phthalate in moist butanol.

Section 3.

Component fatty acid of the lipopolysaccharide, LPS(E) from Proteus vulgaris culture fluid.

A sample of the lipopolysaccharide LPS(E) was hydrolysed on a boiling water-bath with 5N-HCl for 8 hours and the liberated fatty acids extracted from the hydrolysate with ether. After removal of the ether, the fatty acid residue was dissolved in benzene and chromatographed on paper as described in the Appendix. Whatman No.1 circles (26 cm. diameter) were used and the materials applied after cutting narrow slits in the paper to prevent diffusion of the spots on the stationary petroleum phase; by cutting six slits the paper was, in effect, divided into six segments, thus allowing the running of a sample of the hydrolysate together with the standard saturated fatty acids and the standard unsaturated acids on each half of the paper. On completion of the run the paper was dried and cut into two halves as shown in Fig.23. One half was then stained for total fatty acids and the other for unsaturated acids.

The acids identified by this means were capric, lauric, myristic and palmitic, the stains for the first two being too faint to be clearly visible in the photograph shown in Fig.23

Fig.23 Paper chromatogram of the fatty acids liberated from the lipopolysaccharide, LPS(E), by hydrolysing for 8 hours with 5N-HCl. Stationary phase, Shellsol T; mobile phase, 85% acetic acid.

H = Hydrolysate of the lipopolysaccharide.

M_1 = Standard mixture of saturated fatty acids in benzene solution, containing per ml.; 15 mg. capric acid (C10), 12 mg. lauric acid (C12), 12 mg. myristic acid (C14), 10 mg. palmitic acid (C16), 5 mg. stearic acid (C18) and 3 mg. arachidic acid (C20).

M = Standard mixture of unsaturated fatty acids in benzene, containing per ml.
15 mg. oleic acid (O) and 5 mg. linoleic acid (L).

Upper section stained with copper acetate and sodium diethyldithiocarbamate; lower section stained with benzidine reagent.

A dense stain at and near the point of application indicated the presence of higher acids, possibly C20 and upwards. The unsaturated acids were difficult to identify, but there appeared to be at least three, running close together; by varying the load and the duration of the run, one of these was identified as oleic acid.

Of special interest was the fast running acid which appeared, from the intensity of the stain, to be a major component (Fig.23). Nowotny, Luderitz and Westphal[185] also found a fast running acid in chromatograms of the fatty acids from the lipopolysaccharides of a number of organisms, and suggested that is was probably β-hydroxymyristic acid. This acid had previously been reported by Niemann and his co-workers[188] as a constituent of the lipid isolated from the lipopolysaccharide of Escherichia coli. To compare the acid obtained in the present work with β-hydroxymyristic acid, the latter was synthesised as described in the Appendix. When chromatographed on paper, the synthesised acid was found to run in the same position as the fast moving acid in the hydrolysate of LPS(E).

To establish further the identity of the fatty acids, the hydrolysate was submitted to gas-liquid chromatography

using argon as the gas phase and a polyester of ethylene glycol with adipic acid, cross-linked with pentaerythritol (LAC-2-R 446), as a stationary phase. The fatty acids were converted to their esters by means of diazomethane prepared as described in the Appendix. To the acids, dissolved in a little ether, the solution of diazomethane was added until slight excess, the solvent and excess diazomethane removed by distillation, and the residue of methyl esters stored in vacuum sealed ampoules until required for use.

A standard mixture of the saturated fatty acids, C10-C20, with the addition of synthesised β-hydroxymyristic acid, was methylated in this way and submitted to gas-liquid chromatography. The tracing obtained, Fig.24, shows the relative position of the β-hydroxy acid.

The fatty acids obtained by hydrolysis of LPS(E) were methylated and examined in the same way; to identify the elution peaks, the above standard mixture of methyl esters and a standard mixture of the methyl esters of oleic and linoleic acids were run before each analysis period. By comparison of the retention times from the air peak, the acids identified in the hydrolysate were:- capric, lauric, myristic, palmitic, stearic and β-hydroxymyristic, together with a small amount of oleic. The other acids were not

Voltage	1250v.
Argon flow	23ml./min.
Liquid phase	LAC-2-R446
Column length	4 ft.
Temperature	173°C.

C10 - Capric acid
C12 - Lauric acid
C14 - Myristic acid
C16 - Palmitic acid
C18 - Stearic acid
β-OH-C14 - β-Hydroxymyristic acid
C20 - Arachidic acid

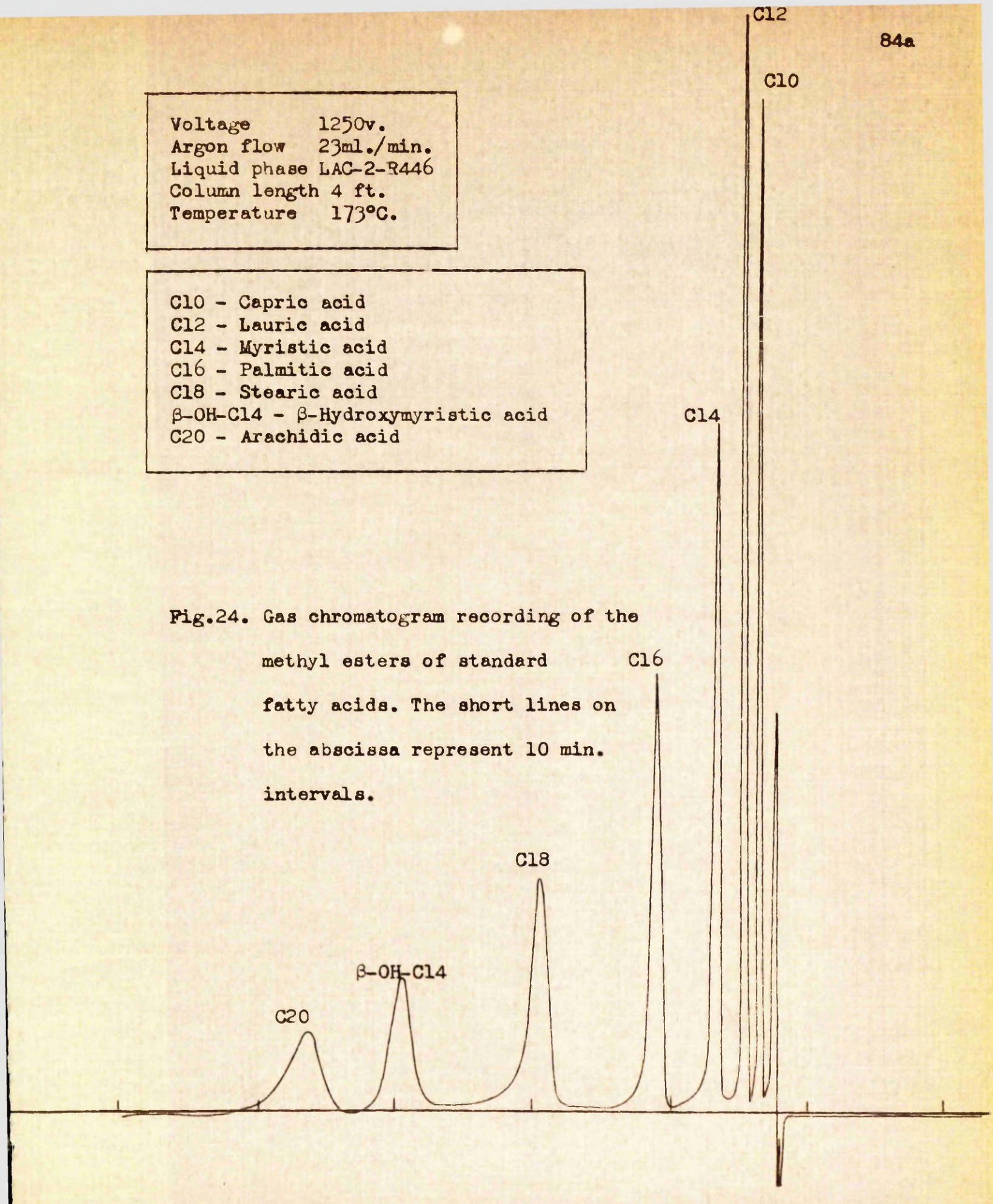


Fig.24. Gas chromatogram recording of the methyl esters of standard fatty acids. The short lines on the abscissa represent 10 min. intervals.

Voltage	1250v.
Argon flow	42ml./min.
Liquid phase	LAC-2-R446
Column length	4ft.
Temperature	176°C.

1. Capric acid
2. Lauric acid
3. Myristic acid
4. Palmitic acid
5. Stearic acid
6. β-Hydroxymyristic acid
A. Oleic acid
B. Probably palmitoleic acid

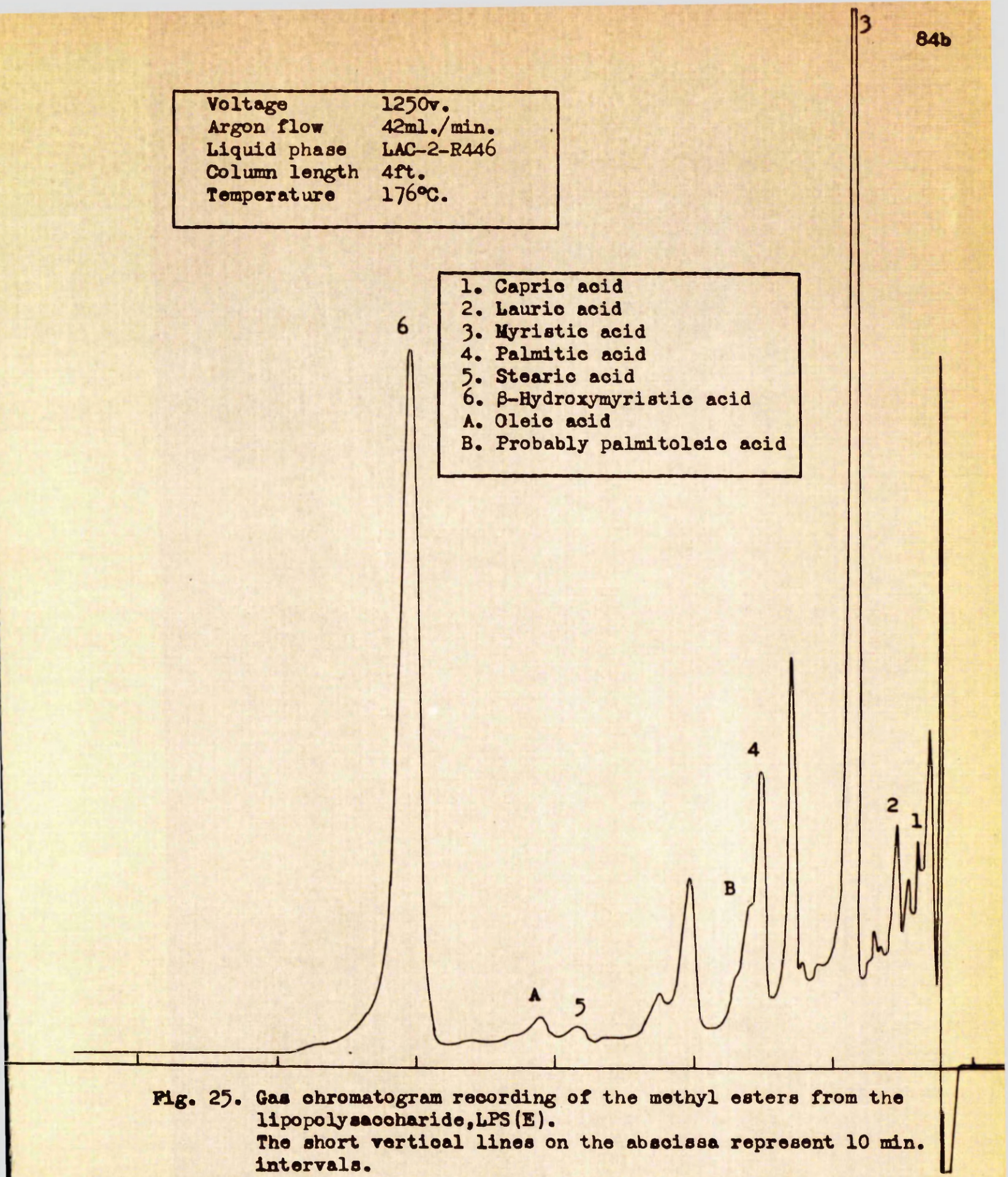


Fig. 25. Gas chromatogram recording of the methyl esters from the lipopolysaccharide, LPS(E).
The short vertical lines on the abscissa represent 10 min. intervals.

identified but one was probably palmitoleic and some of the others possibly odd-numbered carbon chain acids (Fig.25). Comparison of the gas-liquid chromatogram with the paper chromatogram, Fig.23, showed that the major identified acids present were myristic and β-hydroxymyristic with a lesser amount of palmitic. There did not appear to be any arachidic acid (C20) present and the amount of stearic was very small which would account for its apparent absence in the paper chromatogram (Fig.23).

Section 4.

Serological Reactions of the lipopolysaccharide, LPS(E).

Antisera.

(a) To Proteus vulgaris cells. - Antisera were produced in rabbits by intravenous administration of washed freeze-dried cells of Proteus vulgaris suspended in apyrogenic physiological saline at a concentration of 500 μg/ml. The animals were injected three times a week for eight injections with 0.5 ml./kg. of the suspension, followed by four injections of 1 ml./kg. Blood samples were then taken and the serum separated by centrifuging and stored at 0-4°C. Samples of serum were also

obtained from the animals before commencing the series of immunising injections.

(b) To lipopolysaccharide LPS(E). - Rabbits received 2.5 µg/kg. of LPS(E) intravenously three times a week for eight injections, followed by four injections of 5 µg./kg; blood samples were removed and the serum separated as before.

In testing for serological activity, antigen-antibody precipitation in agar-gel plates was used.[187] The agar (Difco was dissolved in water at 2% concentration by heating in a steamer for one hour with occasional swirling, and the solution filtered three times through filter paper pulp in a preheated Buchner Funnel, using gentle suction to prevent frothing. This was found to give a sufficiently clear agar-gel without recourse to the more elaborate methods of clarification which have been recommended by others.[84] After filtration, the agar was distributed in 50 ml. volumes into clean, sterile flasks which were then sealed by means of several thicknesses of grease proof paper secured with elastic bands. When cooled the flasks were stored at 0-4°C.

Petri dishes of 6.5 cm. internal diameter, after examination to ensure freedom from scratches and abnormalities in the glass, were washed with a chromic acid cleansing mixture, rinsed with distilled water and dried. Treatment of

the inside bottoms of the plates with a liquid silicone preparation (Repelcot, Hopkin and Williams Ltd.) effectively prevented under running of the reactants.

A flask of agar was melted by heating in a steamer for one hour and carefully mixed with an equal volume of double strength phosphate-citrate buffer, pH 7.6, containing 0.4% sodium azide and which had previously been filtered hot several times through the same Whatman No.1 filter paper. The agar solution was then distributed into the petri dishes placed on a flat levelled board, using 8 ml. of agar solution to each dish, and the board covered with a large inverted glass tank to provide a relatively air-tight seal. This metho is preferable to covering each dish individually as in the latter case there is a tendency for condensed water to drop on to the agar.[73] One or two petri dishes containing water were placed under the cover to prevent undue evaporation of water from the agar.

When solidified, wells were cut in the agar by means of Feinberg Agar Gel Cutters (Shandon Scientific Co.) and the cut agar discs removed by means of a piece of glass tubing attached to a water pump and passed down through each cutting shaft. The wells were numbered by pressing small lead shot into the agar. Photographic records of the precipitation line

were made as described in the Appendix.

The LPS(E) used as "antigen" was prepared as previously described except that at no stage was the material freeze-dried; the deposit after ultracentrifuging was dissolved in distilled water by shaking for several hours and, after dry-weight estimations of solids content, adjusted to 1% w/v concentration. This was necessary because of the poor solubility of the lipopolysaccharide after freeze-drying.

During the stages in the purification of LPS(E) each fraction was tested against the bacterial-cell antiserum; with the crude phenol extracted material (PSPE) several lines were obtained, two of which are clearly seen in Fig.26 (well 1). The pronounced band about midway between the wells and actually consisting of more than one line, was given by the weakly pyrogenic fraction (PSPE/UCS20) obtained by fractional sedimentation of PSPE (Fig.26, well 4) but not by the active LPS(E) (well 2.). The single line given by the lipopoly-saccharide indicated that the material was immunologically homogeneous, although in some experiments, a second faint line was obtained.

No precipitation was apparent when LPS(E) was tested against its own antiserum (Fig.27) suggesting that the lipopoly saccharide was a hapten and not a complete antigen. It is

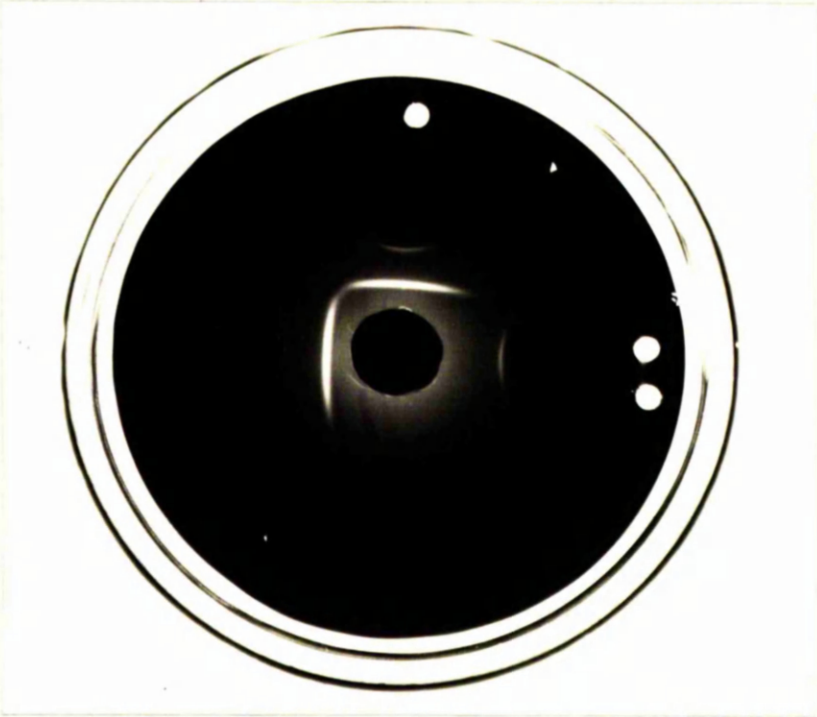


Fig.26 Agar-diffusion precipitin pattern of polysaccharide fractions from Proteus vulgaris culture fluid when reacting with homologous bacterial-cell antiserum. Centre well, bacterial-cell antiserum; well 1, 2% solution of the phenol-extracted material, PSPE (0.1 ml.); well 2, 1% solution of the lipopoly-saccharide, LPS(E) (0.1 ml.); well 4, 2% solution of the fraction PSPE/UCS20, remaining in solution during fractional centrifugation of the phenol-extracted material (0.1 ml.).

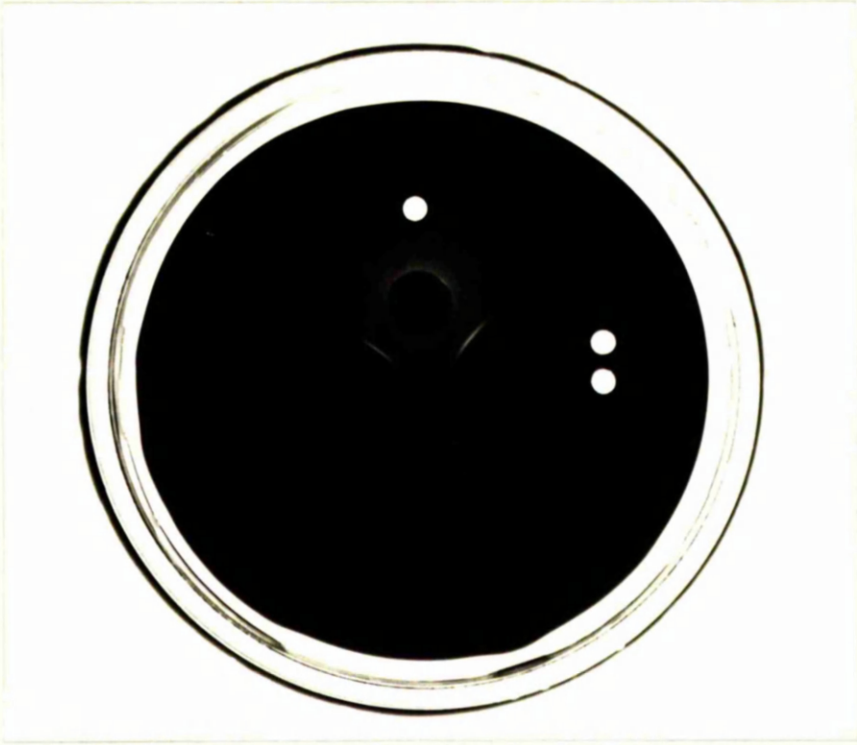


Fig.27 Agar-diffusion precipitin pattern produced by the lipopolysaccharide LPS(E), from Proteus vulgaris culture fluid when reacting with homologous bacterial-cell antiserum and LPS(E)-antiserum. Well 1, bacterial-cell antiserum; well 2, lipopolysaccharide (1% solution, 0.1 ml.); well 3, lipopolysaccharide antiserum; well 4, as well 2.

pointed out, however, that very small doses were used in the immunisation procedure; higher doses were tried but the animals did not survive the full course of injections.

When tested against LPS(E), no reaction was obtained with any of the sera taken before immunisation.

While the foregoing results indicated that the protein-free lipopolysaccharide obtained from the culture fluid was not a complete antigen, earlier investigations had shown that the crude material isolated from the culture fluid before phenol extraction (PSA10 Section 1) gave a positive test for protein. Attention was next directed to the isolation and purification of the protein-containing material and the investigation of its immunological properties.

The cell-free supernatant fluid from a four-day aerated culture of Proteus vulgaris was concentrated under reduced pressure as described in Section 1(c). After dialysis the concentrated solution was further concentrated to a small volume and clarified by centrifuging at 10,000 rmp for 15 minutes. The clarified solution was centrifuged at 105,000 g for four hours and the supernatant fluid decanted from the deposit and freeze dried (PSC/UCS105 - Proteus supernatant concentrated; ultracentrifuge supernatant 105,000 g). The deposit obtained during the ultracentrifuging was

redissolved in water and recentrifuged for four hours at 105,000 g. The sedimented material was then dissolved in water and adjusted, after dry-weight estimations of solids content, to 1% concentration. Some of this solution was set aside for serological tests and the remainder freeze-dried (PSC/UCD105 - Proteus supernatant, concentrated; ultra-centrifuge deposit 105,000 g). Pyrogen tests on both materials show that the pyrogenic activity resided in the fraction PSC/UCD105 (Table 11); the yield of this fraction was approximately 3 mg./litre of culture fluid.

Fraction	Dose µg/kg.	Rise in rectal temperature.
PSC/UCD105	0.1	1.80°C
	0.01	1.25°C
PSC/UCS105	0.1	0.65°C
	0.01	0.15°C

Table 11. Temperature response in the rabbit to 0.1 µg/kg. and 0.01 µg./kg. of the products obtained by fractional centrifugation of concentrated, dialysed Proteus vulgaris culture fluid. Fraction PSC/UCD105 sedimented at 105,000 g; fraction PSC/UCS105 remained in solution at this value of g. Each temperature rise given in the Table was the average for 10 rabbits.

Chemical properties of PSC/UCD105. - The presence of both protein and polysaccharide was shown by positive Biuret and

Molisch reactions, and acid hydrolysis liberated a chloroform-soluble lipid. Repeated ultracentrifuging at 105,000 g. did not significantly alter the nitrogen value (N, 4.99) suggesting that the material was in fact a protein-lipopolysaccharide complex.

Serological reactions of PSC/UCD105. - Antisera to the material were produced in rabbits by the procedure given above for LPS(E), and examined by the agar-gel diffusion precipitin technique. As illustrated in Fig. 28, a single line of precipitation was obtained between PSC/UCD105 (wells 2 and 4) and the specific immune serum prepared against it (well 3) and this line was continuous with that obtained between PSC/UCD105 and bacterial-cell antiserum (well 1); a faint line inside the latter line indicated that the material was not completely immunologically homogeneous. When LPS(E) was tested against both the antiserum to PSC/UCD10 and that to the bacterial cells, one line of precipitation was obtained in each case; the arc formed by the two lines (Fig. 29) demonstrated the specific identity of the antibody reacting from both antisera.

The results of these experiments showed that the highly pyrogenic material, existing in the culture fluid as a protein-lipopolysaccharide complex, was fully antigenic in

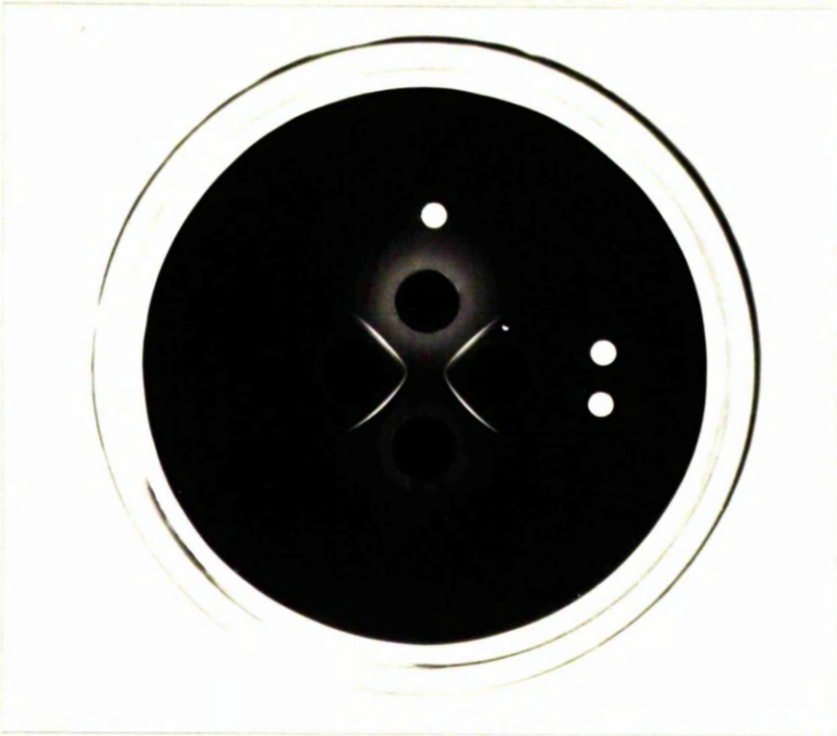


Fig.28 Agar-diffusion precipitin pattern produced by the protein-lipopolysaccharide complex (PSC/UCD105) from Proteus vulgaris culture fluid when reacting with homologous bacterial-cell antiserum and antiserum prepared against the complex.
Well 1, bacterial-cell antiserum; wells 2 and 4, 1% solution of the protein-lipopolysaccharide complex (0.1 ml.); well 3, antiserum prepared against the complex.

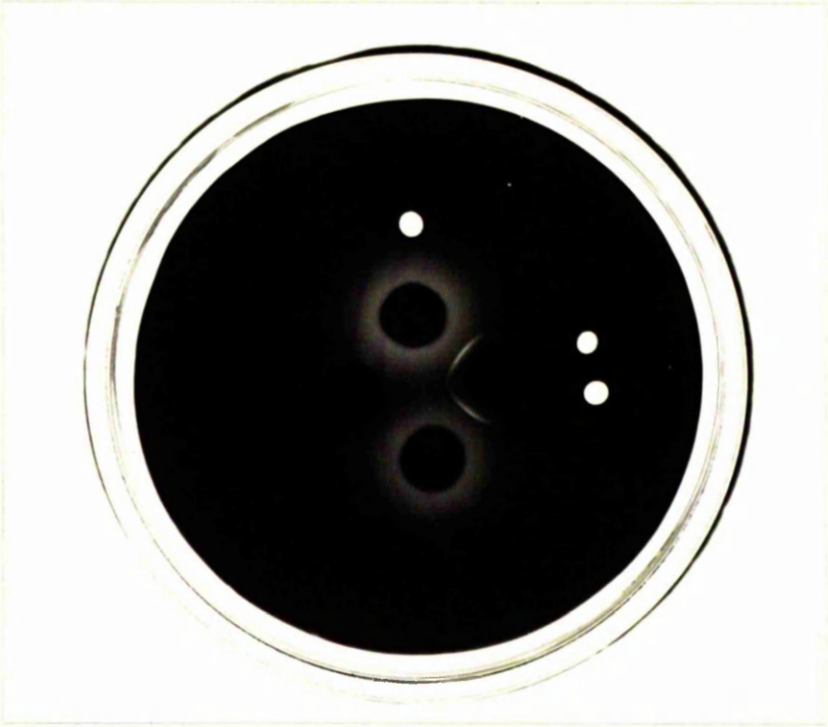


Fig.29 Agar-diffusion pattern produced by the lipopolysaccharide [LPS(E)] from Proteus vulgaris culture fluid when reacting with homologous bacterial-cell antiserum and antiserum prepared against the complex of LPS(E) with protein (PSC/UCD105).
Well 1, bacterial-cell antiserum; well 2, 1% solution of the lipopolysaccharide (0.1 ml.); well 3, protein-LPS(E) antiserum.

stimulating the production of precipitating antibodies in rabbits; removal of the protein component by phenol extraction resulted in a loss of full antigenic properties but the specificity of the antigen was retained in the liberated lipopolysaccharide hapten.

Section 5. Pyrogenic and toxic properties of the lipopolysaccharide, LPS(E).

The numerical average response of groups of rabbits to doses of 0.005-0.1 μg./kg. of the lipopolysaccharide have already been given in section 3. A dose of 0.01 μg./kg. elicited a typical fever response in which the body temperatur rose, after a latent period, to a maximum 1½ - 2½ hours after injection and thereafter gradually fell back to normal (Fig.30). When the same dose was repeated daily for eight days, the animals developed a degree of tolerance as evidenced by a lowered fever response, although none of them became completely refractory (Table 12). A similar type of fever curve was obtained with 0.01 μg./kg. of the protein lipopolysaccharide complex (PSC/UCD105) and again repeated injection of the same dose produced a notable degree of tolerance.

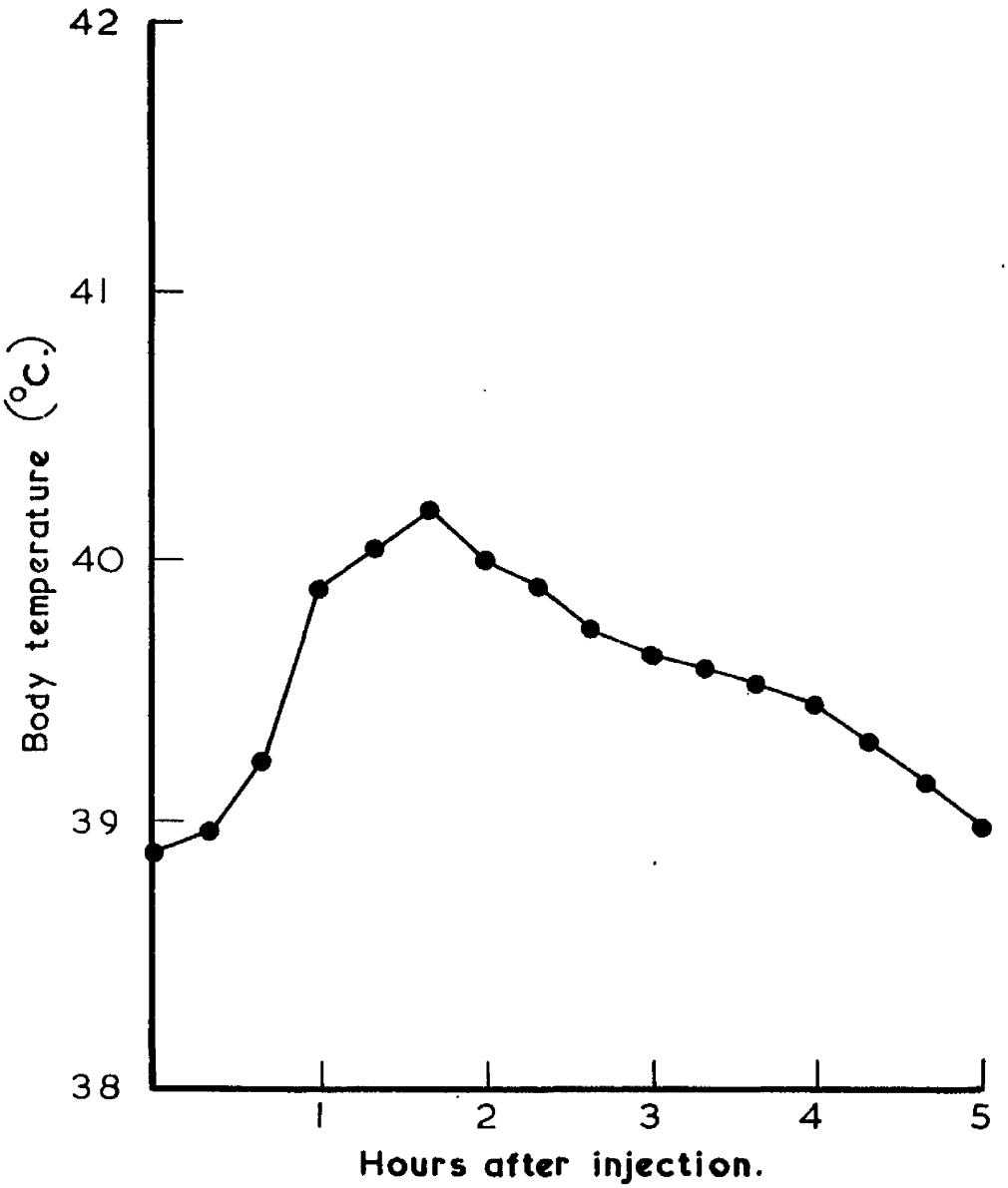


Fig. 30. Typical fever response in the rabbit to intravenous administration of 0·01 μg./kg. of lipopolysaccharide, LPS(E).

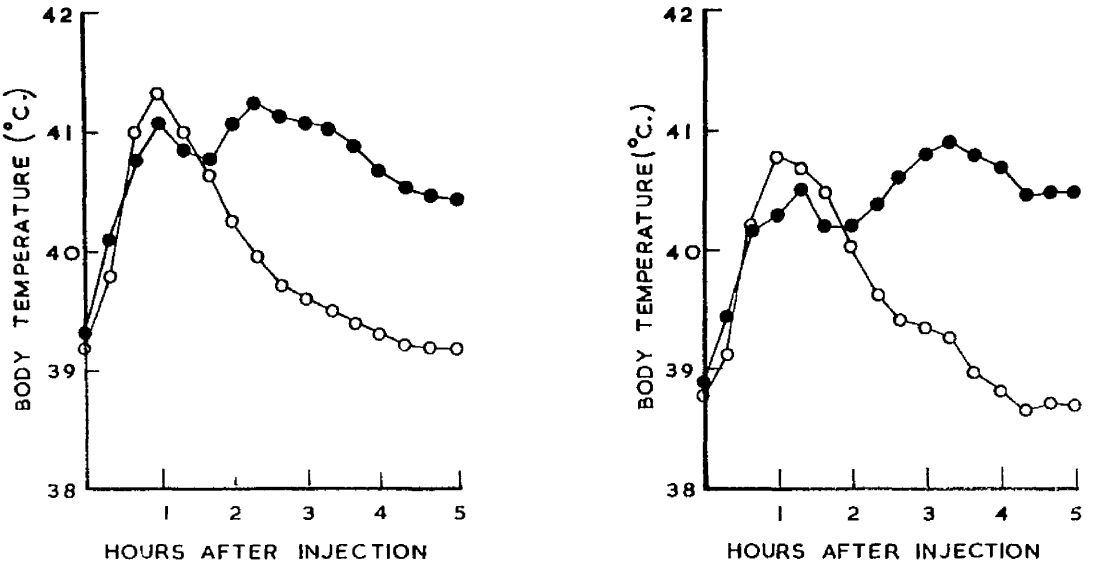
Day	Rabbit No.								
	211	212	213	214	215	216	217	219	221
	LPS E, 0.01 µg/kg					Protein lipopolysaccharide complex (PSC/UCD/20/ 0.01 µg/kg			
1	1.25	1.15	1.15	1.35	1.10	1.2	1.2	1.40	1.25
2	1.15	1.10	1.05	1.30	1.10	1.10	0.85	1.20	1.15
3	1.10	0.90	0.80	1.20	0.95	0.90	1.15	0.80	1.25
4	1.05	1.05	0.55	1.10	0.95	0.60	0.70	0.65	0.85
5	1.10	1.00	0.55	1.20	0.60	0.65	0.70	0.50	0.95
6	1.05	0.75	0.90	1.15	0.75	0.40	0.70	0.65	0.65
7	0.95	0.80	0.80	1.05	0.90	0.60	0.45	0.55	0.65
8	0.95	0.95	0.80	1.15	0.70	0.60	0.55	0.65	0.60

Table 12. Effect of daily repeated injections of the lipopolysaccharide and the protein - lipopolysaccharide complex on the body temperature of rabbits.

Although there appeared to be a greater degree of tolerance developed to the protein-lipopolysaccharide complex than to the lipopolysaccharide, this was probably not significant because of the small number of animals used; it is generally accepted that the development of tolerance is not associated with antibody formation (Part I, Section 2).

After the eighth injection, blood samples were removed from all the animals and the sera tested against LPS(E) by the agar-gel diffusion method. A faint line of precipitation was detected in the case of the sera from those animals which had received the protein-lipopolysaccharide complex, thus illustrating the pronounced antigen properties of this material. No reaction was obtained with the sera of the animals which had received the lipopolysaccharide.

When the dose of the lipopolysaccharide was increased to 0.1 μg./kg. a biphasic temperature response was produced in most cases, the second peak being often higher than the first, and the temperature usually returned to normal more slowly than in the case of the lower dose. The same dose (0.1 μg./kg.) repeated the following day resulted in a virtual disappearance of the second fever peak as shown in Fig.31; similar results were obtained with the same dose of the protein-lipopolysaccharide complex.



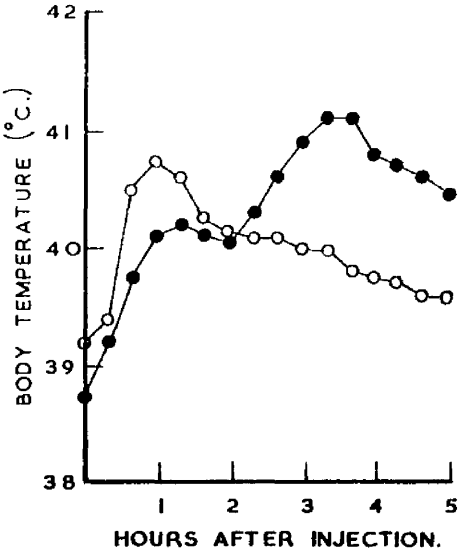


Fig.31. Effect of a second intravenous injection of the lipopolysaccharide (LPS(E)) on the typical biphasic fever response in rabbits.

●—● 0·1 µg./kg. of LPS(E) — 1st. day.

o—o 0·1 µg./kg. of LPS(E) — 2nd. day.

Further increase in the dose of LPS(E) to 1 μg./kg. did not result in an increased height of fever; in fact a lower average temperature response was obtained (Table 13). A considerable variation in response was observed, and in some cases a fall in temperature to below normal followed the initial rise; frequent urination and defecation by several of the animals occured during the test.

Dose (μg./kg.)	Rise in body temperature (°C)
0.1	1.61
1.0	1.37

Table 13. Temperature response in rabbits to 0.1 μg./kg. and 1.0 μg./kg. of lipopolysaccharide LPS(E). Each temperature rise was the average for 15 rabbits.

Administration of doses of 5 μg. and 10 μg./kg. produced even more variable temperature responses (Fig.32), and about 1-2 hours after injection most of the animals showed signs of general collapse, in which the hind limbs were apparently paralysed, and there was no control over urination and defecation; during this state, which lasted for one to three hours, the animals were unable to stand and the head could only be moved slightly. Following the test none of the animals showed any inclination to feed and in

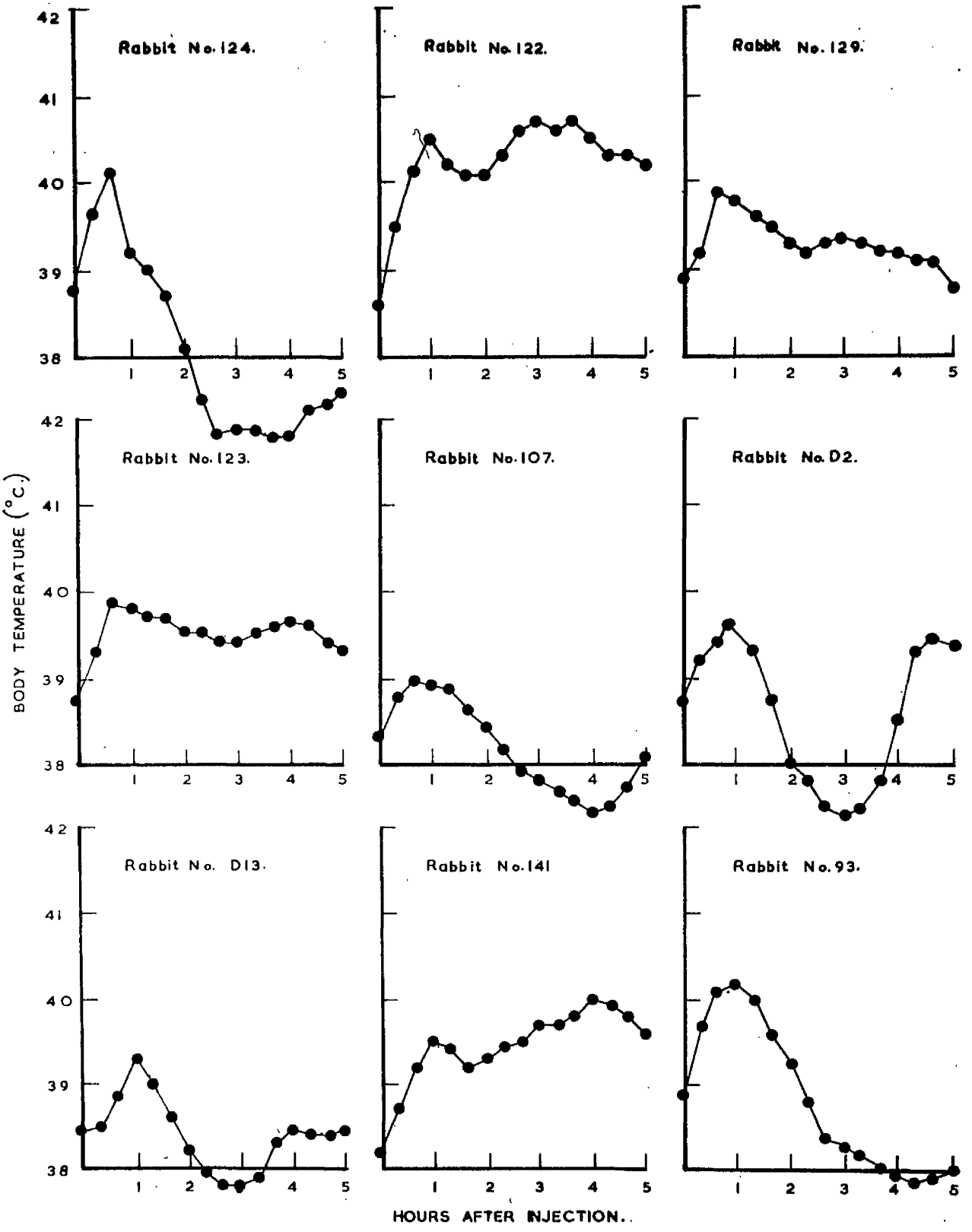


Fig. 32. Examples of individual fever responses in rabbits to administration of 10·0 µg./kg. of lipopolysaccharide (LPS(E)).

many cases the appetite did not return for several days.

At a dose level of 20 µg./kg. of the lipopolysaccharide death, preceded by general collapse and rapid and laboured breathing, occurred in about 20% of the animals. Post-mortem examination revealed slight punctuate haemorrhages of the kidneys, the lungs were congested and the right heart soft; the gut, liver and spleen appeared normal.

Of a group of fifteen rabbits receiving 40 µg./kg, eleven died. Death, preceded by the usual symptoms of toxicity, occurred in some instances within a few hours after injection while in others the animals survived one to two days. Post-mortem examination revealed renal congestion with usually small punctuate haemorrhages visible on the outer surface; the lungs were again congested, often severely so, and the right heart flabby; the liver, spleen and intestinal tract appeared normal. From these post-mortem findings it appeared that death was due to right cardiac failure secondary to pulmonary congestion. In many cases a fall in temperature followed injection without any initial rise while in others a slight rise preceded the fall. That the toxicity was only associated with the active lipopoly-saccharide fraction was shown by the absence of toxic symptoms in rabbits receiving 50 µg./kg. of the ultracentrifuge

supernatant fraction, PSPE/UCS20; two rabbits receiving this material reacted with a monophasic temperature response giving an average rise of 1.60°C and which returned to normal after four to five hours.

The mouse was found to be relatively refractory to the lipopolysaccharide. Albino mice weighing approximately 25 g. were used, and administration of the lipopolysaccharide was via the tail vein. From the results shown in Table 14, the calculated LD50 for a 25 g. mouse was approximately 850 μg.

Dose/25 g. mouse (μg.)	No. of mice	No. dead	% mortality
1625	6	6	100
1200	6	6	100
1000	6	5	83
800	9	3	33
600	6	1	17
400	9	1	11

Table 14. Toxicity of the lipopolysaccharide LPS(E) for mice.

Section 6.

Extraction of lipopolysaccharide from Proteus vulgaris cells.

Lipopolysaccharide was extracted by means of hot aqueous phenol from cells of the same strain of Proteus vulgaris used in the previous experiments. The organisms were grown in nutrient broth, the cells removed by centrifuging and dried with acetone.

Phenol extraction. - The acetone-dried cells (10 gm.) were suspended in 350 ml. of water and stirred until thoroughly dispersed. The suspension was heated to 65-67°C and an equal volume of 90% w/v aqueous phenol at 65°C added; the mixture was stirred at this temperature for 30 minutes and then rapidly cooled to 0-5°C, whereupon a separation of the aqueous and phenol phases occurred which was completed by centrifuging. The aqueous phase was pipetted off, water (280 ml.) added to the phenol phase, the mixture heated with stirring to 65°C and rapidly cooled as before. After centrifuging, the aqueous phase was removed, added to the first aqueous phase and dialysed for four days against running water. The dialysed solution was concentrated under reduced pressure, clarified by centrifuging and freeze-dried. Examination of the ultra-violet-absorption

spectrum of a sample of the material revealed the presence of about 40% nucleic acid (Fig.33).

Purification of the lipopolysaccharide. - The crude lipopolysaccharide from the phenol extraction was dissolved in water at 1% concentration, the solution centrifuged at 105,000 g for 4 hours and the supernatant fluid removed. The deposit was redissolved in water, the sedimentation repeated twice and the final deposit freeze-dried from aqueous solution. Although the lipopolysaccharide so obtained, still contained a considerable amount of nucleic acid (Fig.33) it was judged to be sufficiently pure for the purpose of comparison, as described in the next section, with the lipopolysaccharide isolated from the culture fluid.

For convenience and brevity, and to distinguish it from the lipopolysaccharide isolated from the culture fluid [LPS(E)], the lipopolysaccharide isolated from the bacterial cells as above will be referred to subsequently as LPS(S).

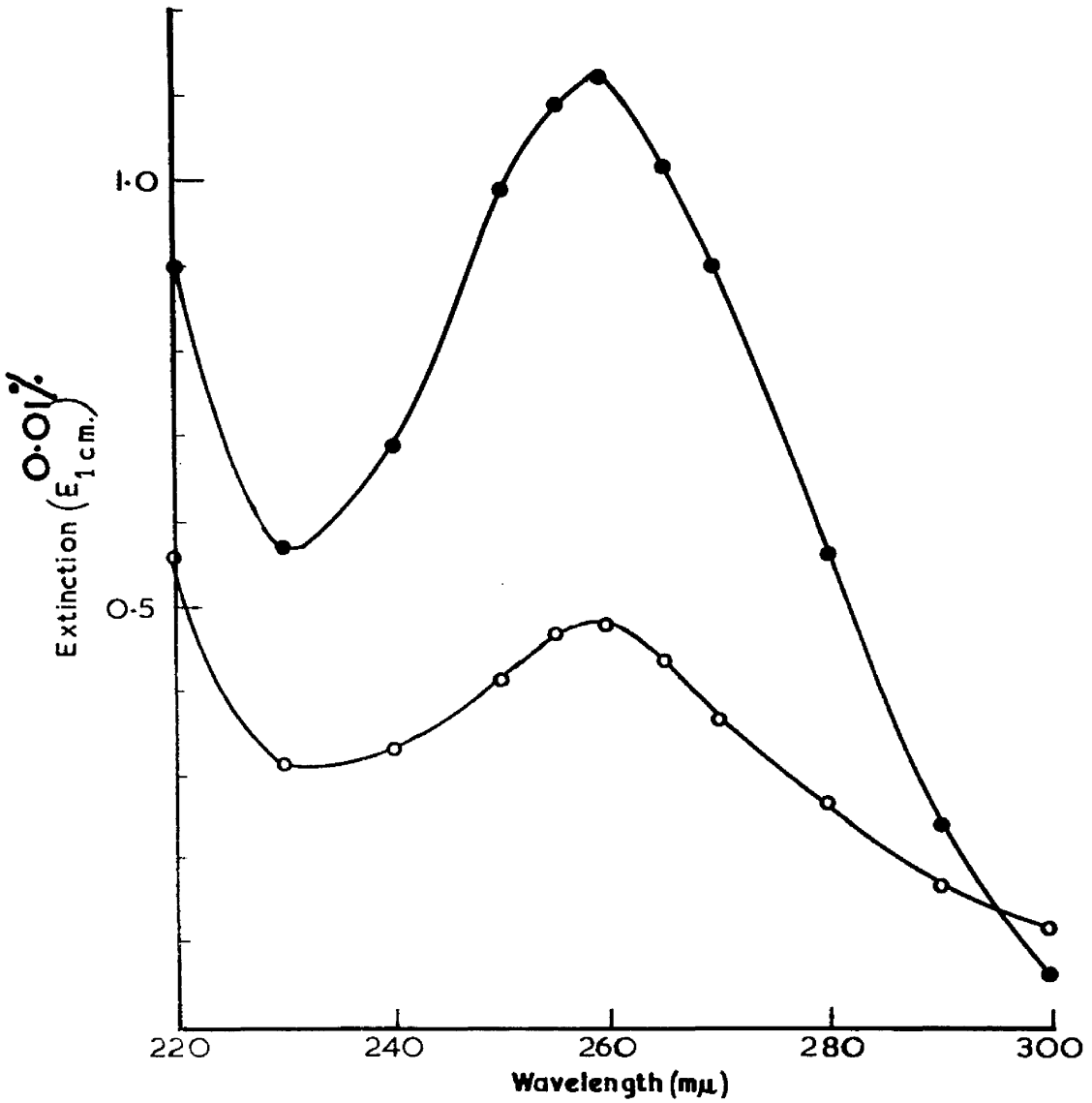


Fig. 33. Ultraviolet absorption spectra of lipopolysaccharide from Proteus vulgaris cells.

●—● Crude lipopolysaccharide extracted from the cells with hot aqueous phenol.

o—o The lipopolysaccharide after repeated sedimentation in the ultracentrifuge.

Section 7.

Comparison of the lipopolysaccharide, [LPS(S)] from Proteus vulgaris cells with that from the culture fluid [LPS(E)].

Analytical figures obtained for LPS(E) have been compared with those obtained for a purified LPS(S) prepared by a previous colleague.[229] These have indicated a close similarity between the two materials (Table 15).

COMPARISON OF LPS(S) AND LPS(E)

	C	H	N	P	Reducing sugars as glucose	Lipid	Minimum pyrogenic dose
LPS(S)	41·21	7·28	2·28	1·95	34 per cent	30–32 per cent	0·005 μg./kg.
LPS(E)	43·07	7·86	2·10	1·72	30 per cent	32–34 per cent	0·005 μg./kg.

Table 15. Comparison of the analytical figures obtained for lipopolysaccharides from Proteus vulgaris cells and culture fluid.[229]

In addition, the absorption spectra in the H_2SO_4-cysteine reaction showed a similar pattern and indicated a similar ratio of hexose to heptose in both compounds;[229] the curves obtained were practically superimposible. Using the LPS(S) prepared as described in the previous section,

some further comparisons of the materials were made as follows:-

Solubility - LPS(S) dissolved easily in water to give a stable opalescent colloidal solution which at 1% concentration was somewhat viscous. LPS(E) on the other hand was very difficult to dissolve and although soaking the material in a little water overnight before diluting facilitated solution to some extent, centrifuging of this solution at speeds as low as 2,500 rpm resulted in material sedimenting. The method of purification of the materials also reflects the difference in solubility; centrifuging at 105,000 g was necessary to completely sediment LPS(S) whereas 20,000 g sedimented the total LPS(E).

Component Sugars - Both lipopolysaccharides contained galactose, glucose and an aldoheptose, as revealed by paper chromatography (Fig.34), but whereas LPS(E) also contained a trace of mannose, this sugar was apparently absent from LPS(S). The presence of mannose in LPS(E) was probably due to incomplete removal of some of the inactive fractions during purification of the lipopolysaccharide; reference to the chromatogram of the ultracentrifuge supernatant fraction, PSPE/AF50/UCS20, (Fig.17) shows the high proportion

Fig.34 Chromatogram of the sugars liberated from Proteus vulgaris lipopolysaccharides by hydrolysing with N-H_2SO_4 for 4 hours. LPS(E) = Lipopolysaccharide obtained from the culture fluid. LPS(S) = Lipopolysaccharide obtained from the bacterial cells. Solvent, butanol-pyridine-water: sprayed with aniline phthalate in moist butanol.

of mannose in that material. The band outside glucose in the chromatogram of LPS(S) was due to ribose derived from the nucleic acid present (Fig.34).

Lipid Components - Gas-liquid chromatographic examination of the fatty acids obtained from the lipid of LPS(S) gave the tracing shown in Fig.35. This differed in some detail from that obtained with the fatty acids from LPS(E) (Fig.25). The identified common acids were capric, lauric, myristic, palmitic, stearic, ß-hydroxymyristic, and oleic and many of the unidentified acids were common to both lipopolysaccharides. LPS(S), however, contained arachidic acid and a considerable proportion of stearic acid, whereas in LPS(E) the former appeared to be absent and the latter present in only a small amount. Overall, however, both lipopolysaccharides appeared to be predominately rich in myristic and ß-hydroxymyristic acids.

Serological reactions - In the agar-gel precipitation test, purified lipopolysaccharide isolated from Proteus vulgaris cells gave with antiserum prepared to the homologous organism, a single line of precipitation which could not be differentiated from that given by LPS(E) with the same antiserum.[229] Further to this, the LPS(S) prepared as described

Voltage	1250v.
Argon flow	42ml./min.
Liquid phase	LAC-2-R446
Column length	4ft.
Temperature	176°C.

1. Capric acid
2. Lauric acid
3. Myristic acid
4. Palmitic acid
5. Stearic acid
6. β-Hydroxymyristic acid
7. Arachidic acid
A. Oleic acid
B. Probably palmitoleic acid

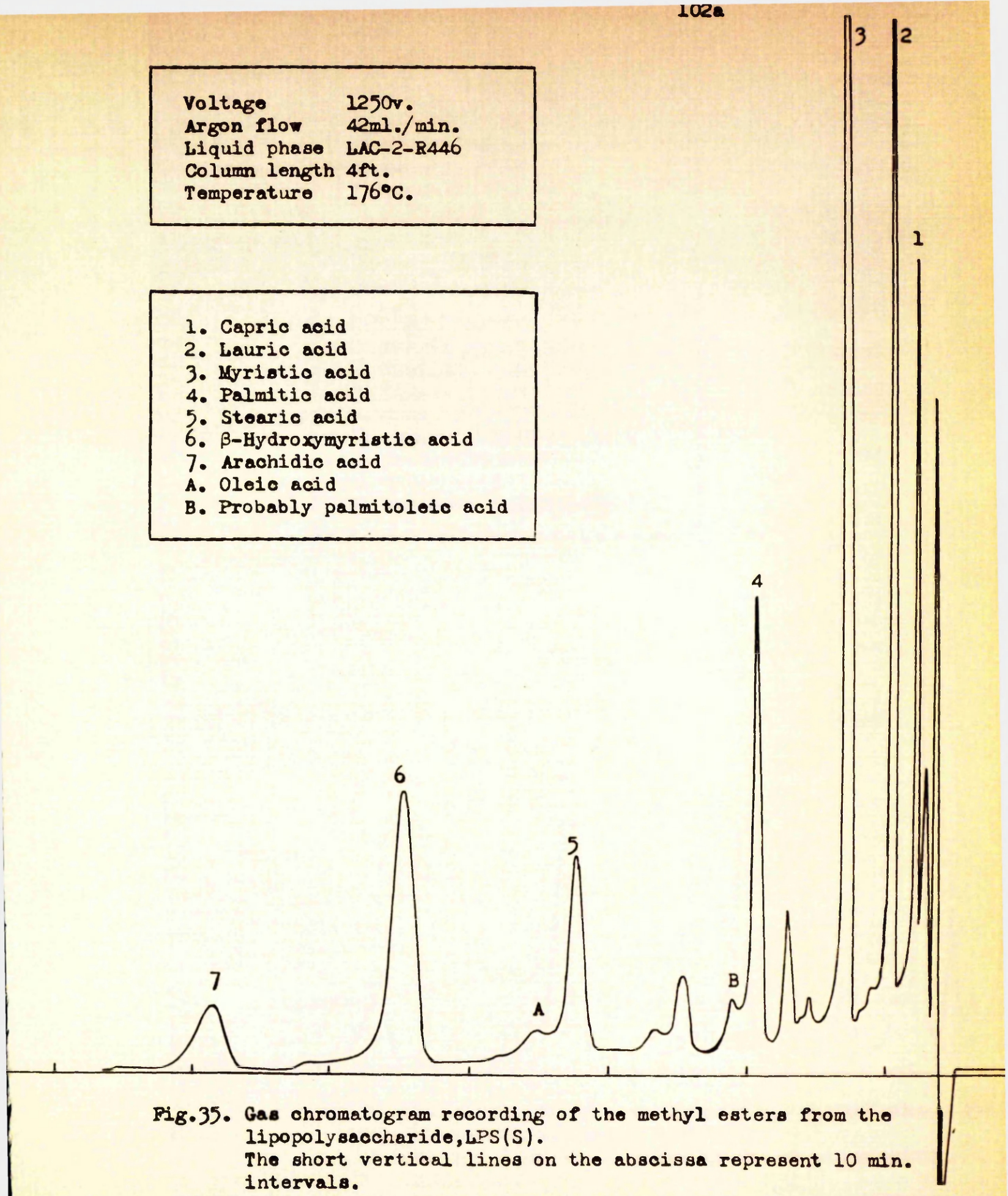


Fig.35. Gas chromatogram recording of the methyl esters from the lipopolysaccharide, LPS(S).
The short vertical lines on the abscissa represent 10 min. intervals.

in the previous section was tested against the serum of rabbits immunised with the protein-lipopolysaccharide complex isolated from the culture fluid (PSC/UOD105) when a single line of precipitation was obtained which was continuous with that formed between LPS(S) and the antiserum to the homologous organisms (Fig.36). The serum of rabbits given a series of immunising injections of LPS(S) or LPS(E) gave no reaction with either LPS(E) or LPS(S), illustrating the hapten nature of both materials. Immunological tests did not, therefore, distinguish between the two lipopolysaccharides but indicated a close relationship between them.

Toxicity - The lethal effect of LPS(S) for rabbits was similar to that of LPS(E); doses of 40 µg./kg. killed a large proportion of the animals and post mortem findings were common to both. LPS(S), however, appeared to be more toxic to mice, the calculated LD50 being approximately 450 µg. as compared with 850 µg. for LPS(E) (Table 16).

Dose/25 g. mouse (µg.)	No. of mice	No. of dead	% mortality
1056	6	6	100
768	6	6	100
480	6	3	50
288	6	2	33
96	6	0	0

Table 16. Toxicity of the lipopolysaccharide, LPS(S) for mice.

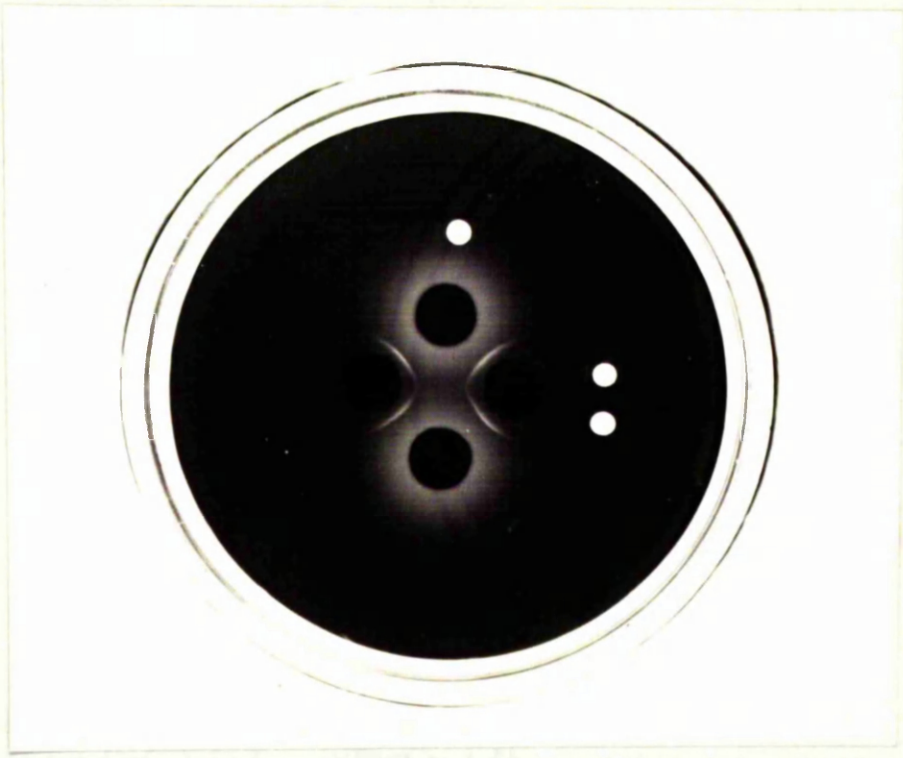


Fig.36 Agar-diffusion precipitin pattern produced by Proteus vulgaris lipopolysaccharides when reacting with homologous bacterial-cell antiserum and antiserum prepared against the protein-lipopolysaccharide complex (PSC/UCD105).
Well 1, bacterial-cell antiserum; well 2, 1% solution of the lipopolysaccharide [LPS(S)] from Proteus vulgaris cells (0.1 ml.); well 3, antiserum to the protein-lipopolysaccharide complex; well 4, 1% solution of the lipopolysaccharide [LPS(E)] from the culture fluid (0.1 ml.).

DISCUSSION

It is now clear that the ability of Gram-negative bacteria to produce, on injection into man and experimental animals, a wide variety of physiological changes can be attributed to the endotoxins of these organisms. The ubiquity of these bacterial products and their biological potency have not only necessitated the routine screening of all fluids and medicaments prepared for parenteral administration but have also on occasions led to misinterpretation of experimental results in various fields. The report by Landy and Shear[104] in 1957, for example, of the isolation from mammalian and plant tissues of lipopolysaccharides which elicited host responses similar to those produced by bacterial endotoxins was regarded by several workers as a major contribution to a better understanding of diseases not only of bacterial origin but of other etiologies,[34,103,200] in that it suggested that varied stimuli such as bacterial endotoxins or viruses might serve merely to liberate naturally occurring endotoxin-like substances into the circulation. More recently (1960), however, it has been shown that only materials devoid of such activity are obtained under conditions in which bacteria are excluded.[180]

As described in Part I, Section I, the endotoxin may

be isolated as a specific somatic antigen, which in undegraded form consists of a protein-lipopolysaccharide (specific polysaccharide + lipid A) - inert lipid B complex, or as the lipopolysaccharide fraction of the complex. The toxic, pyrogenic and related biological effects of these preparations appear to be associated with the lipid A moiety while serological specificity is determined by the specific polysaccharide fraction. Although the complete structure of lipid A is as yet unknown, the preparations of this component which have so far been examined are all of very similar if not identical composition. A constant composition of the lipid A components of various endotoxins would explain the non-specific nature of the biological effects produced. The monosaccharide constituents of the specific polysaccharides have also been discussed, especially the more recently discovered 3:6-dideoxyaldohexoses which appear to play an important role in the specificity of the parent polysaccharides.

Much of the work done on the endotoxins of Gram-negative bacteria has been confined to the genera Shigella, Salmonella and Escherichia, while fewer contributions have been made to the isolation and study of such materials from Proteus. For this reason, and the fact that we were anxious

to obtain adequate supplies of active lipopolysaccharides from relatively nonpathogenic sources, attention in the present instance was devoted mainly to Proteus vulgaris.

Investigations by various workers of the antigenic components of Proteus vulgaris strains have been concerned mainly with Proteus OX19 because of the serological cross-reaction of this strain with Rickettsia prowazeki, the causative agent of typhus fever (the Weil - Felix reaction). Early work on this subject by White[256] showed that the antigenic material obtained by tryptic digestion of Proteus OX19 cells contained two receptors, one of which was alkali-labile and produced in rabbits an antiserum containing homologous agglutinins, while the other was stable to alkali and appeared to be the factor responsible for the Weil-Felix reaction. The results of Castaneda[59] agreed with those of White and showed the presence in the antigenic material of an alkali-labile polysaccharide ('P' factor) which was specific to Proteus OX19 only, and an alkali-stable polysaccharide ('X' factor) which was common to both Proteus OX19 and Rickettsia prowazeki.

More detailed information on these antigens has been provided by the work of Bendich and Chargaff[17] who found that

the antigenic material obtained from Proteus OX19 by tryptic digestion or trichloracetic acid extraction could be resolved by high speed centrifuging into two fractions. The fraction sedimenting (C_2) reacted with both Proteus OX19 and typhus antisera, while that remaining in solution (C_{11}) possessed only Proteus specificity. Both fractions were electrophoretically homogeneous and being composed of lipid and phosphorylated polysaccharide-protein complexes were analogous to the 'O' somatic antigens of Gram-negative bacteria. Although qualitatively both fractions were of similar chemical composition, there were certain quantitative differences; for example, the heavy fraction (C-2) contained much more lipid. The identified polysaccharide components were N-acetyl-D-glucosamine, as the major sugar constituent, together with galactose, a small amount of mannose and possibly also a small amount of glucose.

While the foregoing work was concerned with the isolation and examination of antigenic substances from Proteus, few investigations have been made of Proteus as a source of simple pyrogenic materials. Robinson and Fluesser[205] in 1944 extracted a pyrogenic material from Proteus vulgaris by heating an aqueous suspension of the cells on a steam bath for one to two days. Following dialysis and removal of the

cells, the soluble material in the cell free fluid was precipitated with acetone and the precipitate deproteinised by the Palmer and Gerlough phenol method.[188]

The final material, in contrast to the well defined lipopolysaccharides which can be extracted by modern techniques contained no detectable nitrogen, had a very low phosphorus content and required a comparatively high dose to elicit a significant febrile response, which suggests that it was composed mainly of a simple polysaccharide. By a modification of this method, Dare[57] obtained from Proteus vulgaris a pyrogenic preparation which although more potent than that of Robinson and Flusser was nevertheless very impure. Ginger and his co-workers[97] have also prepared a pyrogenic concentrate from these organisms using tryptic digestion of the cells, but the analytical data presented, in showing the presence of desoxyribonucleic acid (10%) and ribonucleic acid (4%) together with much nitrogenous material (N 8%), indicate the impurity of the preparation.

Anderson[4] attempted the isolation of pyrogenic materials from Proteus vulgaris on the assumption that the cell-free supernatant fluid from an inorganic culture medium would provide a source of such material with the minimum of contaminating substances requiring removal. The cell-free

dialysed culture filtrate was concentrated by freeze-drying and the residue purified by reprecipitations from aqueous solution using acetone. Spectrophotometric examination showed the absence of nucleic acid while acid hydrolysis liberated reducing sugars. In the present work a fuller investigation of the nature of this material was undertaken and a comparison made with pyrogenic material extracted from the cells of the organisms.

Since freeze-drying, as used by Anderson for comparatively small volumes of culture fluid, is not suitable for reducing the volumes necessary to obtain a reasonable amount of the material therein, evaporation under reduced pressure was adopted in the present investigations. It was first established that no appreciable loss in pyrogenic activity occurred during evaporation.

The addition of alcohol (10 volumes) to the dialysed concentrated cell-free culture fluid precipitated a pyrogenically active material containing both polysaccharide and protein. Deproteinisation procedure carried out on this crude precipitate with hot aqueous phenol (Westphal process) gave a product which yielded on acid hydrolysis a chloroform-soluble lipid and the sugars galactose, glucose, mannose, aldoheptose, glucosamine and galactosamine. Spectrophotometric

examination revealed that while some batches of the product appear to be quite free from nucleic acid, small amounts were present in others. Although a negative Biuret reaction was obtained, the more sensitive method of chromatography showed the presence of several amino acids which were not removed by prolonged dialysis and were thus presumably present in protein-like form. Furthermore, since fractionation of the product with alcohol yielded a lipopolysaccharide which, apart from traces of glutamic acid, was substantially free from amino acids, it must be assumed that in the crude material from the phenol extraction this protein-like material is not associated with the lipopolysaccharide. While this suggests that the phenol-water extraction process may be inadequate to remove all extraneous protein, the isolation of the lipopolysaccharide as a bound complex with protein by high speed centrifuging of the concentrated culture fluid without prior phenol extraction, as will be subsequently discussed, shows that the process does, however, effectively dissociate the protein component of the complex. The traces of glutamic acid present in the lipopolysaccharide, and persisting throughout subsequent purification procedures, was presumably derived from the lipid component.

The distribution of the sugar constituents during the alcohol fractionation of the phenol-extracted material is noteworthy. The highly active lipopolysaccharide fraction precipitating at 50% alcohol concentration contained galactose, glucose, mannose, aldoheptose, glucosamine and galactosamine, while the weakly active and apparently lipid-free fraction remaining in solution at this alcohol concentration yielded on hydrolysis only glucose, mannose and glucosamine. The absence of aldoheptose in the latter fraction was also shown by the H_2SO_4-cysteine reaction.

When the lipopolysaccharide fraction obtained by alcohol fractionation was further separated in the ultracentrifuge two main fractions were obtained, one sedimenting at 20,000 g and the other remaining in solution at values up to 105,000 g. The highly active lipopolysaccharide sediment thus obtained contained the sugars galactose, glucose, aldoheptose, glucosamine, galactosamine and a trace of mannose, while the ultracentrifuge supernatant fraction was only weakly pyrogenic, appeared to be free from lipid and contained glucose, mannose and glucosamine. A lipopolysaccharide of the same degree of purity could also be obtained by high-speed centrifuging of the phenol-extracted material without prior solvent fractionation. Inaddition to the

above mentioned sugars, chromatography also revealed the presence of an unidentified acid-labile sugar, the rapid release of which during hydrolysis suggested that it might occupy a terminal position in the polymer chain. From its rate of movement on the chromatogram it did not, however appear to be related to the acid-labile 3:6-dideoxy sugars discussed in Part I Section I.

Although the lack of precise information on the polysaccharide materials obtained from Proteus by other investigators provides little basis for comparison, the component sugars of these preparations where known are given in Table 17, along with those of the lipopolysaccharide LPS(E) as found in the present work.

Species	Component sugars						Ref.
	Galactose	Glucose	Mannose	Xylose and/or Ribose	Heptose	Hexos-amine	
Proteus vulgaris OX19	+	+	+	+	-	+	17
Proteus vulgaris	-	+	-	-	+	-	64
Proteus vulgaris (X) (smooth)	+	+	+	+	-	+	78
Proteus vulgaris (X) (rough)	+	+	+	-	+	+	78
LPS(E)	+	+	+	-	+	+	

Table 17. Sugar components of polysaccharide from Proteus.

Preliminary examination of the chloroform-soluble lipid, composing 32-34% of the lipopolysaccharide LPS(E), revealed as major fatty acid constituents, myristic and β-hydroxy-myristic together with a lesser amount of palmitic. Several minor acid components were also identified. The presence of β-hydroxymyristic acid, detected in this work by chromatographic examination (using for comparison the acid synthesised for the purpose), was first reported by Ikawa[125] in the tumor-necrotising lipopolysaccharide from Escherichia coli, and later provisionally identified by Nowotny[126] in lipopolysaccharides from several strains of Escherichia coli and species of Salmonella. Although the full series of saturated fatty acids, C10 to C20, was present in all the lipopolysaccharides examined by Nowotny, and in the lipopolysaccharide extracted from Proteus vulgaris cells during the present investigations, the lipopolysaccharide LPS(E) from the culture fluid did not appear to contain any arachidic acid (C20) and only a trace of stearic (C18).

While insufficient to the lipopolysaccharide, LPS(E), was available for a complete investigation of the water-soluble hydrolysis products of the lipid component, the traces of ninhydrin-positive constituents and at least some of the glucosamine, detected on paper chromatograms of the lipopoly-

saccharide when hydrolysed with 6N-HCl, were probably derived from this component. Ikawa,[124] for example, found several ninhydrin-positive water-soluble constituents, including D-glucosamine and aspartic acid, in the hydrolysate of the lipid from Escherichia coli lipopolysaccharide, and more recently Nowotny[105] showed the presence of glucosamine, D-glucosamine-phosphate derivatives and small amounts of several amino acids in the isolated lipids from several Salmonella lipopolysaccharides (Part I Section I). The galactosamine obtained on hydrolysing the lipopolysaccharide, LPS(E), was probably derived from the specific polysaccharide portion; Davies[64] has pointed out that although glucosamine seems to be a constant feature of lipid A and may or may not be also present in the specific polysaccharide obtained from the lipopolysaccharide by acid hydrolysis, when an amino sugar other than glucosamine is present it has proved to be always in the polysaccharide moiety.

The lipopolysaccharide, LPS(E), appeared to be immunologically homogeneous, giving one precipitin line when examined by the agar-gel diffusion technique against an antiserum prepared to the homologous organisms. No detectable precipitating antibodies were found in the sera of rabbits given repeated intravenous injections of the lipopolysaccharide

suggesting that it is a hapten and not a complete antigen.

The foregoing findings suggest that the pyrogenically active material obtained by phenol extraction of the concentrated cell-free fluid from a culture of *Proteus vulgaris* in a simple inorganic medium is a lipopolysaccharide similar in composition to the well-defined lipopolysaccharides obtainable by extraction from Gram-negative bacterial cells generally, and comparative analytical figures given in Table 18 confirm this.

Bacterial species	C	H	N	P	Hexos-amine	%lipid	Ref.
Shigella sonnei	45.4	7.1	2.8	3.9	8.3	29	132
Salm. abortus-equi	48.6	7.3	1.3	2.8	8.7	26	247
" *typhi*	-	-	1.6	3.0	8.8	-	247
" *paratyphi*	48.4	7.1	1.4	2.6	7.7	-	247
E. coli			1.9	1.4-1.7	17-18	21-25	123,12
Serratia marcescens	47.5	7.1	2.2	1.1	-	16	10,11
Pasteurella pestis	46.0	7.6	1.6	2.2	15	46	59
LPS(E)	43.07	7.86	2.10	1.72	14	32-34	

Table 18. Comparison of analytical figures obtained for LPS(E) with those of lipopolysaccharides from other Gram-negative bacteria.

Investigations were next directed to determining the form in which the pyrogenically active material exists in the culture fluid. Preliminary examination had revealed the presence of protein in the crude pyrogenic material precipitated by alcohol from the concentrated cell-free culture fluid. Subsequently, a purified protein-containing material was isolated from the fluid by means of the ultracentrifuge. Since repeated sedimentations in the ultracentrifuge did not significantly alter the nitrogen content of this material, the protein appeared to be a bonded moiety and not a persistant impurity, thus suggesting that the purified material consisted of a protein-lipopolysaccharide complex. This complex was a potent pyrogen and fully antigenic in stimulating the production of precipitating antibodies in the rabbit. Although agar-gel diffusion tests indicated that the complex was not completely immunologically homogeneous since it gave a second fainter precipitation line when tested again antisera to the homologous organisms, the major line corresponded to the single line given with the specific antiserum prepared against the complex, thus suggesting that the fainter line was probably due to another substance of a hapten nature present along with the principle complex. The relationship of the complex to the lipopoly-

saccharide, LPS(E), was shown by the single line of precipitation given when the lipopolysaccharide was tested against the specific antiserum, and which corresponded to the line obtained between the lipopolysaccharide and the antiserum to the homologous organisms. This established the identity of the complex as an antigen having the specificity of the polysaccharide component of the lipopolysaccharide, LPS(E).

It may thus be concluded that the actively pyrogenic material exists in the culture fluid as an antigenic lipopolysaccharide-protein complex and in this respect is analogous to the somatic antigens of Gram-negative bacteria. A similar conclusion was reached by Ikawa[125] who isolated a tumor-necrotising polysaccharide-polypeptide-phospholipid complex from the culture fluid of Escherichia coli by evaporation under reduced pressure and subsequent purification by alcohol fractionation, and the tumor-necrotising agent isolated by Shear and his co-workers[110] from cultures of Serratia marcescens was probably also of similar nature as is indicated by the fall in nitrogen content from 4% to 2% after tryptic digestion. Ikawa[125] found that a greater yield of the polysaccharide-polypeptide-phospholipid complex could be obtained from Escherichia coli culture filtrates

when the whole culture was concentrated prior to removal of the cells, and he suggested that this was presumably due to autolytic breakdown of the cells during the concentration. Similarly, Wylie[362] found that a seven-day culture of Proteus vulgaris was approximately 75 times more potent than a two-day culture, indicating that the bulk of the soluble pyrogenic material was produced during the decline phase of growth. On the other hand, Mondolfo and Houni,[171] in 1947, observed that 50% of the total pyrogen produced by a culture appeared in the suspending fluid during the logarithmic phase of growth and they suggested from this that the pyrogen may have been an excretory product of bacterial metabolism rather than a cellular component. Observations have been made, however, that in ordinary broth cultures the total number of organisms generally exceeds the number of viable organisms over during the logarithmic phase,[154] so that in Mondofo and Houni's experiments a considerable number of dead bacteria were probably present.

Anderson, using the ring test, failed to detect precipitating antibodies in the sera of rabbits given repeated injections of a pyrogenic material obtained by simply freeze-drying the dialysed cell-free fluid from a culture of Proteus vulgaris in an inorganic medium. From

the results of the present investigations it would appear certain that the pyrogenic activity of Anderson's material was due to the complex described above, in which case an immunological response would have been expected. The failure to obtain such a response may have resulted from the very small doses of material administered by this worker, although rabbits gave specific anti-polysaccharide sera containing detectable amounts of precipitating antibodies after eight daily injections of 0.01 µg/kg. of the complex isolated in the present work.

It is generally agreed that the pyrogenic activity of bacterial lipopolysaccharides is associated with the lipid A component. In these investigations the high pyrogenic potency of the protein-lipopolysaccharide complex was retained in the lipopolysaccharide fraction when the complex was split with phenol, while other polysaccharide fractions obtained during purification of the lipopolysaccharide showed little activity and contained no detectable lipid. The slight activity in these cases was probably due to the presence of a small proportion of lipopolysaccharide. Of interest also was the absence of heptose, galactose and galactosamine from all fractions except the lipopolysaccharide, although it is extremely doubtful if the polysaccharide component plays any

part in pyrogenic activity apart from being a suitable carrier for the lipid.

A small dose of the lipopolysaccharide, LPS(E), (0.01 $\mu g/kg.$) elicited in rabbits a typical fever response consisting of a latent period followed by a rise in rectal temperature reaching a maximum $1\frac{1}{2}$ - $2\frac{1}{2}$ hours after injection and thereafter falling back to normal. Repeated daily injections of the same dose resulted in the development of a degree of tolerance although the animals did not become completely refractory. The present day views on the mechanism of tolerance development have been discussed in detail in Part 1, Section 2 and it has been pointed out that the failure of repeated injections of endotoxins to produce a completely resistance state has been quoted by Bennettand co-workers as evidence of a direct action of these toxins on the thermoregulatory centres since the residual fever is said to occur in the absence of demonstrable amounts of circulating endogenous pyrogen.[87]

At a higher dose level [0.1 $\mu g/kg$ LPS(E)] the temperature curve was biphasic and the temperature usually fell back to normal more slowly than in the case of the lower dose. Repeated injection of the same dose the following day resulted in a disappearance of the second peak. This

biphasic febrile response and early disappearance of the second fever peak on repeated injection are well recognised phenomena which have been noted by various workers and have proved to be important factors in investigations of the mechanism of endotoxin-induced fever. Bennett and his co-workers[193,194] have provided evidence to suggest that endogeneous pyrogen is probably responsible for the second fever peak, while the early fever phase, independent of the endogeneous factor, is due to a direct action of the endotoxin on the thermoregulatory centres. At first sight it would appear questionable that the early peak is due to a direct action of the toxin, as penentration into the nervous system is slower for the large endotoxin molecule than it is for the endogeneous material, but the point has been made by Petersdorf and Bennett[193] that endotoxin is always present in the blood stream before endogeneous pyrogen appears and this head-start could make up for the difference in rates of penentration. The early disappearance of the second fever peak may thus be explained as a stimulation, by the first injection, of reticuloendothelial system activity whereby the subsequently injected endotoxin is cleared from the circulation before the endogeneous factor can be formed. At the same time some endotoxin,

escaping clearance by the reticuloendothelial system, penetrates directly to the thermoregulatory centres so producing the first fever peak. It is also interesting to note that a number of years ago (1949) Wylie[365] came to the conclusion that there were two pyretic substances present in bacterial filtrates, one stimulating the first fever peak and the other the second, but the existence of endogeneous pyrogen was unknown at that time.

A comparison of the pyrogenic activity of the lipopolysaccharide LPS(E) with that of several well-defined lipopolysaccharides from unrelated organisms is shown in Table 19. Extrapolation of the log dose-response graph obtained with dose-levels of 0.1 µg., 0.05 µg., and 0.01 µg. of LPS(E) gave an MPD (dose required to give a temperature rise of 0.6°C) of approximately 0.002 µg/kg., although a group of ten

Source of lipopolysaccharide	MPD (µg/kg.)	Ref
Escherichia coli	0.002	250
Serratia marcescens	0.005	10
Salmonella typhosa	0.0002	150
Shigella dysenteriae	0.002	68
Pasteurella pestis	0.007	59
LPS(E)	0.002-0.005	

Table 19. Minimum pyrogenic doses of various lipopolysaccharide.

rabbits receiving 0.005 µg/kg responded with an average rise of 0.67°C. In table 19 therefore, the MPD for LPS(E) is given as 0.002 - 0.005 µg/kg.

When the dose of LPS(E) was increased to 1 µg/kg. in rabbits, a higher fever response was not obtained but the fever curve tended to become flat and protracted. Doses of 20-40 µg/kg resulted in the death of many of the animals, presumably due to pulmonary congestion leading to circulatory failure since post-mortem examination revealed right heart collapse and severe congestion of the lungs. The only other post-mortem finding was renal hyperaemia. The liver and intestinal tract appeared normal although other workers have reported that the organs become congested and oedematous and show scattered areas of haemorrhage and necrosis.[68,387,400] The changes following a lethal dose of endotoxins are not well understood but there is evidence that blood is trapped in the lungs by vasoconstriction; Kuida and co-workers, for example, have shown that there is an increase in the vascular resistance of the isolated perfused lung when bacterial endotoxin is added to the perfusing fluid.[140]

The fact that these workers were only able to obtain the effect when the perfusing fluid contained blood, suggests the involvement of an endogeneous factor rather than a direct action of the toxin.

The lethal dose of the lipopolysaccharide, LPS(E), for rabbits was similar to that of lipopolysaccharides obtained by other workers from various species of Enterobacteriaceae, as illustrated in Table 20. A recent Russian paper,[81] however, has described the preparation from Proteus vulgaris of a pyrogenic polysaccharide which was non-toxic for rabbits in doses up to 500 μg/kg. The method of preparation consisted of washing the cells from solid agar cultures and purifying the material from the cell-free filtrate, a method adopted 'in order to avoid admixture of endotoxin'. In view of the relatively high dose (1 μg/kg.) of the product required to elicit a pyrogenic response it would appear probable, on the basis of the present investigations, that it consisted of a small proportion of lipopolysaccharide in an otherwise inactive material; agar from the cultures may have contributed to the latter.

Source of lipopolysaccharide	Lethal dose (rabbits)	Ref.
Escherichia coli	20-50 µg/kg.	250
Serratia marcescens	20-100 µg/kg.	250
Salmonella typhosa	20-50 µg*	150
LPS(E)	20-40 µg/kg.	

* Rabbits weighing 2.1-2.4 kg.

Table 20. Lethal dose of various lipopolysaccharides for rabbits.

As in the case of pyrogenic activity, the lipid component of the lipopolysaccharide appears to be the factor responsible for toxicity. Westphal and his colleagues, for example, showed that a short period of alkali degradation of their lipopolysaccharide reduced the molecular weight from 1×10^6 to 200,000, and although some fatty acids were split off during the process, the product still contained lipid and possessed the toxic properties of the original lipopolysaccharide; further alkali treatment reduced the lipid and resulted in a marked decrease in toxicity.[161] In the fractions obtained in the present work only that containing detectable lipid showed pronounced toxic activity.

Mice were relatively refractory to the lipopolysaccharide, LPS(E), the LD50 being in the region of 850 μg/25 g. mouse. The LD50 of the lipopolysaccharide, LPS(S), obtained from Proteus vulgaris cells, however, appeared to be about half that of LPS(E), the difference being possibly due to the poor solubility of the latter, especially noticeable in preparing the relatively concentrated solution required for the test. This resistance of mice to endotoxin has been noted by other workers; Goebel and his co-workers, for example, found that Shigella endotoxin was frequently lethal in rabbits at a dose of 20 μg, whereas about 500 μg were required to kill mice[99] and the lipopolysaccharide isolated by Landy and Johnstone[159] from S. typhosa was lethal in rabbits at 20-50 μg, whereas the LD50 for 15 g. mice was approximately 250 μg.

As already mentioned, the pyrogenic material as isolated from the culture fluid by fractional sedimentation was a protein-lipopolysaccharide complex and fully antigenic in stimulating the production in the rabbit, of precipitating antibodies. Removal of the protein resulted in the loss of full antigenic properties although the protein-free lipopolysaccharide behaved as a hapten and retained the specificity of the antigen. A similar loss of antigen properties, on

removing the protein component of the antigen of Shigella dysenteriae, was observed by Morgan and his co-workers[165] and Davies[59] found that the lipopolysaccharide from Pasteurella pestis did not give rise to detectable antibodies unless combined with a conjugated protein. On the other hand Landy[161] has provided evidence that the lipopolysaccharide from Salmonella typhosa is the immunologically active form of the 'O' antigen of this organism.

It may be noted that, because of its toxicity, only relatively small doses of the lipopolysaccharide, LPS(E), (2.5 μg increasing to 5 μg) could be given when carrying out immunisation procedures; however, with repeated doses of as little as 0.01 μg/kg of the protein-lipopolysaccharide complex, precipitating antibodies were detected.

The other polysaccharide fractions, of relative high nitrogen values, obtained during purification of the lipopolysaccharide precipitated with immune sera prepared against the homologous organism but their identity as heptens or antigens was not investigated. Goebel,[99] however, found specific polysaccharide heptens along with the protein-polysaccharide complex he isolated from Shigella flexneri by extraction with diethylene glycol or pyridine.

One of the objects of the present investigations was to compare the lipopolysaccharide, LPS(E), obtained by phenol extract from the culture fluid of Proteus vulgaris with that extracted by the same process from cells of the same strain of organism [LPS(S)]. Analytical figures indicated a close similarity between the two materials and the H_2SO_4-cysteine reaction revealed a similar ratio of hexose to heptose in both. Acid hydrolysis of LPS(S) liberated the sugars galactose, glucose, an aldoheptose and hexosamine while the same sugars with the addition of a trace of mannose were obtained from LPS(E). The presence of mannose in LPS(E) does not necessarily indicate a distinction between the two materials as, being only a trace, it could be accounted for by an incomplete removal, during fractional sedimentation, of the ultracentrifuge supernatant fraction (PSPE AF50 UCS20) which appeared to contain a high proportion of this sugar. Whereas the full series of saturated fatty acids, C10-C20 were present in the lipid from LPS(S), that from LPS(E)did not contain any arachidic acid (C20) and only a small amount of stearic (C18). The significance of this difference in fatty acid composition has not been established, although it may be pointed out that the cells used for extraction of LPS(S) were grown in a nutrient broth medium while LPS(E) was obtained from a simple inorganic-glucose medium. In both

lipids, however, the major acids appeared to be myristic and β-hydroxymyristic and many of the unidentified acids were common to both.

Further evidence to the close identity of the two lipopolysaccharides was provided by immunological examination when each gave a single line of precipitation with both the antiserum to the homologous organism and that prepared against the complex of LPS(E) with protein. Both materials were equally pyrogenic and toxic in rabbits.

While the culture medium may provide a useful source of small quantities of pyrogenic materials, the present investigations show that there does not otherwise appear to be any advantage in using this source over direct extraction from the bacterial cells. In either case considerable fractional procedures are required to give a pure material, and the advantage of freedom from nucleic acid in the culture filtrate is offset by the difficulties of handling the large volumes of culture medium required. From 10 gm. of Proteus vulgaris cells, for example, 10-20 mg. of pure lipopolysaccharide can be obtained, whereas 10 litres of culture filtrate are required to give the same amount.

Summary

1. A survey has been made of the recent literature dealing with the chemical and biological properties of bacterial endotoxins.

2. The pyrogenic constituent present in the culture filtrate of Proteus vulgaris has been isolated as an antigenic protein-lipopolysaccharide complex, analogous to the 'O' somatic antigens of Gram-negative bacteria.

3. The lipopolysaccharide, N,2.1; P,1.7, extracted from the isolated complex, or from the concentrated culture filtrate, by means of hot aqueous phenol, contained about 30% of a chloroform soluble lipid. It was soluble with difficulty in water and toxic in rabbits at doses of 20-40 μg/kg. but much less toxic in mice (LD50 850 μg.). The material possessed the immunological specificity of the protein-polysaccharide complex but was non-antigenic in inducing the formation of precipitins in rabbits; it was a powerful pyrogen, active at 0.005 μg/kg. in rabbits. The sugar composition of the polysaccharide component and a preliminary analysis of the fatty acids of the lipid component are given.

4. The lipopolysaccharide constituted 20-25% of the crude material extracted with phenol from the concentrated culture filtrate; the remaining 75-80% was obtained as two main fractions:-

(a) a lipid-free weakly pyrogenic polysaccharide, N,5.9; P,0.4, soluble in 50% alcohol, giving amino acids on hydrolysis and containing the sugars glucose, mannose and glucosamine. It gave a precipitin reaction with immune sera prepared against the homologous organisms, distinguishable from that given by the lipopolysaccharide, and probably contained more than one component.

(b) a lipid-free weakly pyrogenic polysaccharide, N,3.4; P,0.9, precipitating along with the lipopolysaccharide at 50% alcohol concentration but remaining in the supernatant during fractional sedimentation at 20,000 g. The predominant reducing sugar found was mannose along with some glucose; glucosamine was present and several amino acids were obtained on hydrolysis.

5. The lipopolysaccharide obtained from the culture filtrate [LPS(E)] was compared with that obtained from cells of the same strain of Proteus vulgaris [LPS(S)]. Chemically they differed in that LPS(E) contained a trace of mannose which was absent from LPS(S) and the lipid from LPS(E) did not appear to contain arachidic acid and only a small amount of stearic acid which were notably present in the lipid of LPS(S). Physically, LPS(E) was more difficult to dissolve in water. Pyrogenicity and toxicity in rabbits were similar

with both materials but LPS(S) appeared more toxic to mice.

Serological tests did not distinguish between the two substances.

6. The effect of various doses of the lipopolysaccharide, LPS(E), on the pyrogenic response in rabbits was investigated Post-mortem findings following administration of lethal doses are recorded.

7. The construction of an apparatus for the photographic recording of the results of agar-diffusion precipitin tests is described (Appendix).

Acknowledgements.

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Appendix.

Materials and Methods.

Organisms.

The strain of Proteus vulgaris used was chosen for its high pyrogenic potency. It had been maintained for a number of years in the department by subculture of agar slopes, and was periodically checked for identity and purity.

Morphology and staining. - Straight rods; 1 μ by 3 μ; occurring singly and in pairs with occasional filaments; actively motile by means of peritrichous flagella (Fig.37); non-sporing; Gram-negative.

Cultural characteristics. - Aerobe and facultative anaerobe; optimum temperature 37°C but grows well at 30°C.

Agar streak incubated at 37°C for 24 hours. - Produces a moderate to abundant, moist, transparent, greyish-white growth which spreads over the surface of the agar; consistency butyrous; emulsified easily.

Colonies on agar. - Circular, translucent, greyish-white colonies; 1-1.5 mm. in diameter; consistency butyrous; emulsifies easily; surface smooth; lustre shining.

Fig.37 Electron micrograph of Proteus vulgaris X1200.

The plates were well dried before innoculation to prevent spreading.

Broth after incubation at 37°C. - Markedly turbid with moderate deposit which disintegrates with difficulty; thin surface pellicle; odour rather faecal.

Horse blood agar. - After incubation for 24 hours at 37°C shows β-haemolysis.

Gelatine stab. - Shows stratiform liquifaction.

Potato plug. - Yellowish-grey growth.

MacConkey plate. - Non-lactose fermenting colonies in one day.

Biochemical reactions. -

Glucose	+
Sucrose	+
Lactose	+
Mannitol	+
Dextrin	-
Fructose	+
Galactose	+
Glycerol	+
Maltose	+
Mannose	-
Salicin	+
Xylose	-

Table 21.

Methyl-red reaction	+
Voges-Proskauer reaction	-
Citrate utilisation (Koser)	-
Indole	+
Catalase	+
Urease	+
Nitrate $\longrightarrow$ nitrite	+
H_2S	weak +

Table 22.

In table 21, a + opposite the substance signifies acid and gas formation.

Medium.

To avoid complex substances requiring removal, the following simple medium, in which the above strain of Proteus vulgaris grows well, was used.

Ammonium Phosphate, $(NH_4)_2HPO_4$	4.0 g.
Sodium Chloride	1.0 g.
Potassium Dihydrogen Phosphate KH_2PO_4	1.0 g.
Magnesium Sulphate	0.7 g.
Ferrous Sulphate	trace
Glucose	10.0 g.
Nicotinic Acid	2×10^{-5} M
Water, apyrogenic distilled to	1 litre

If the ingredients are all dissolved together and autoclaved, caramelisation of the glucose occurs and a heavy precipitate, probably magnesium phosphate, is deposited. To overcome this the glucose and magnesium sulphate were dissolved together in some of the water and the solution autoclaved. The other ingredients were dissolved in the remainder of the water and sterilised by autoclaving. When required for use the two solutions were mixed.

Nitrogen. Total nitrogen was determined by the micro-Kjeldahl method using a mixed bromocresol green-methyl red indicator.[163]

Phosphorus. This was determined by a method based on that of Kuttner and Lichtenstein,[147] as described by Holden and Pirie,[118] using samples containing approximately 10 μg. of P. The material was ashed by means of sulphuric acid with the aid of one drop of 60% perchloric acid, and the blue colour developed by reduction with stannous chloride after the addition of ammonium molybdate. The standards were put through the same ashing procedure.

Nucleic acid. Estimations were made by measuring, in a Uvispec spectrophotometer, the absorption at 260 mμ. of the material dissolved in N/100 NaOH. A purified yeast nucleic acid was used as a reference standard.

Sugars. Hexosamine was estimated as glucosamine by the method of Elson and Morgan[80] as modified by Rondle and Morgan.[206]

Aldoheptose was estimated by the H_2SO_4-cysteine reaction of Dische.[62,74,75] Optical densities were measured in a Unicam SP600 spectrophotometer after 22 hours and the figures stated for heptose content of the materials were calculated as α-D-glucoheptose.

Reducing sugars were measured by the copper method of Somygyi[84] on samples hydrolysed for the times stated.

Chromatography.

Monosaccharides. - A sample of each polysaccharide fraction (20 mg.) was hydrolysed in a sealed tube at 100°C with N-H_2SO_4 (6 ml.) for the time stated and the hydrolysate neutralised with Ba $(OH)_2$ solution. After centrifuging to remove Ba SO_4, the neutral solution was concentrated by vacuum distillation and finally dried under vacuum over solid NaOH. The dry material was dissolved in one or two drops of water and the solution applied to paper chromatograms; most of the chromatograms described were run on circulat papers (Whatman No.20) using as solvent butanol-pyridine-water (3:2:1.5).[116,150] On completion of the run the papers were dried at room temperature in a fume cupboard for 40 mins. and then in an oven at 100°C for 5 mins. After spraying with a solution of aniline phthalate in water saturated butanol, the papers were heated at 105°C for 5 mins. to reveal the positions of the sugars. With this reagent aldopentoses appear reddish brown while methyl pentoses and aldhexoses appear brown.

Amino Compounds. - The method used was a modification of a procedure described by Gardell and co-workers[95] for amino sugars. A sample of the materials (20 mg.) was hydrolysed for 15 hours with 5N-HCl (5 ml.) and after centrifuging to remove carbon the hydrolysate was dried in vacuo over solid NaOH. To the dry material 0.5-1.0 ml. of water was added, the solution centrifuged and again dried over solid NaOH. The residue redissolved in a drop of water was then applied to paper chromatograms (Whatman circles No.4) and run with butanol-acetic acid-water (4:1:5) as solvent.[189] After drying at 104-110°C for 5 mins., the papers were sprayed with the following indicator and placed in an oven at 105°C for 15 mins.

Ninhydrin - Cupric nitrate indicator.[170]

Solution I.	Ninhydrin	0.2%
	Absoluto ethanol	50 ml.
	Glacial acetic acid	10 ml.
	2:4:6-Collidine	2 ml.
Solution II.	Cupric nitrate (1%) in absolute alcohol.	

The two solutions were combined in the ratio of 25 volumes of solution I to 1.5 volumes of solution II

immediately before use. Amino acids give characteristic coloured complexes with this reagent while the amino sugars, glucosamine and galactosamine, appear as brown stains.

To the remainder of the hydrolysate, 2 ml. of water were added, the solution neutralised with pyridine to pH 7, approximately 4 mg. of ninhydrin added and the mixture heated at 100°C for 30 minutes. After drying in vacuo the residue was redissolved in a drop of water, applied to paper chromatograms (Whatman circles No.20) and run in butanol-pyridine-water along with standards of lyxose and arabinose. The chromatograms were then developed with aniline phthalate in moist butanol.

The first chromatogram, sprayed with the ninhydrin-cupric nitrate reagent, shows the amino sugars together with any other amino compounds. Boiling with ninhydrin degrades the hexosamines to pentoses, glucosamine to arabinose and galactosamine to lyxose, and these separate easily. A chromatogram was also run of the hydrolysate, before degradation, on Whatman No.20 circles in butanol-pyridine-water and developed with aniline phthalate to test for the possible presence of arabinose and lyxose in the original material.

Fatty acids - The method used was a modification of that described by Nowotny, Luderitz and Westphal.[185] A sample of lipopolysaccharide (20 mg.) was hydrolysed for 8 hours with 10 ml. of 5N-HCl on a boiling water-bath and the hydrolysate extracted three times with 20 ml. volumes of ether in which the long chain fatty acids are soluble while lipid A is insoluble. The ether extracts were combined, dried over anhydrous sodium sulphate, evaporated to dryness and the residue dissolved in 0.2 ml. of benzene. Paper circles (Whatman No.1) were dipped in a petroleum fraction, 200-210°C (Shellsol T), for one minute, pressed between sheets of filter paper and dried at room temperature for one hour. The benzene solution of the fatty acids was applied to the papers in the usual way, except that narrow slips were cut in the paper to prevent diffusion of the spots on the stationary petroleum phase, and the chromatograms run in 85% acetic acid. On completion of the run the papers were dried at 80°C and immersed in a copper acetate solution (0.6% cupric acetate in 0.05N sodium acetate) for 2 mins. After washing in running water for 1 hour, followed by several changes of distilled water for 15 minutes, the papers were finally dipped in a 0.1% solution of sodium diethyl-dithiocarbamate,[186] washed with several changes of distilled

water and dried at room temperature.

In the above method the positions of the fatty acids, both saturated and unsaturated, are revealed as brown stains; for unsaturated acids only, the following method was used.[226] After completion of the run the paper was dried as before, dipped into a 1% solution of sodium metaperiodate, allowed to drain for about 4 mins. and then dipped in a 0.5% solution of potassium permanganate for 1 minute. When washed free from permanganate, unsaturated compounds are visible as brown stains. The chromatogram was then dried in an oven at 100°C, dipped in benzidine reagent and dried between sheets of warm filter paper, when the original brown stains now appeared as bright blue bands.

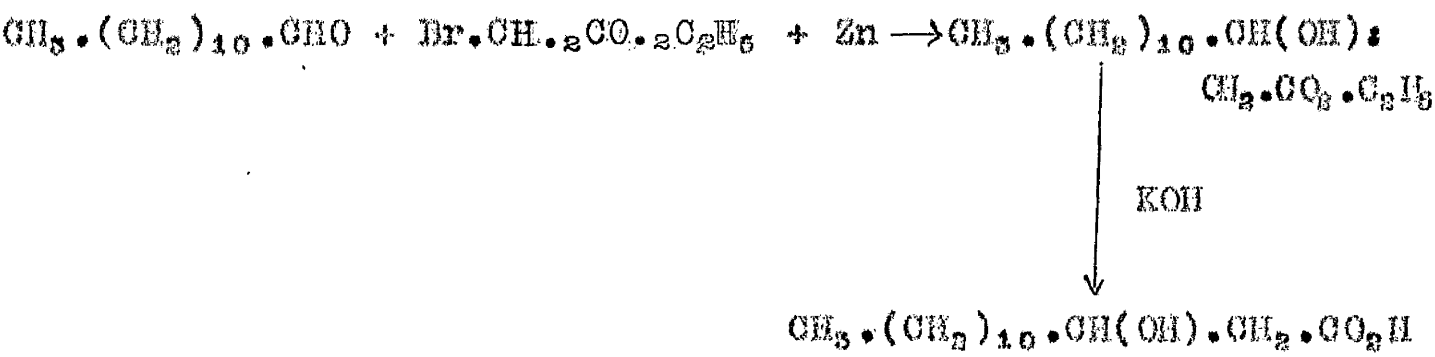
Benzidine Reagent.

Benzidine (recrystallised from aqueous alcohol)	1.0 gms.
Trichloracetic acid	8.0 gms.
Glacial acetic acid	20.0 ml.
Water	20.0 ml.
Absolute alcohol	160.0 ml.

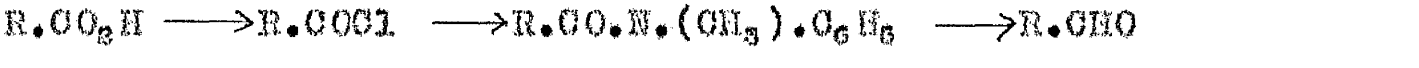
Synthesis of β-hydroxymyristic acid.

A Reformatsky reaction[216] was carried out between lauraldehyde and ethyl bromoacetate to give ethyl-β-hydroxy-

myristicate, which was then hydrolysed to the acid:-



The aldehyde was prepared from lauric acid by the following reactions:-[182]



Acid Acid chloride N-methylanilide aldehyde

$(R = CH_3.(CH_2)_{10}-)$

<u>Lauroyl chloride:-</u> Lauric acid (40 g.) was mixed with 52 g. of redistilled thionyl chloride and boiled under reflux for one hour. The excess thionyl chloride was distilled off and the residual acid chloride distilled under reduced pressure and collected at 92-98°C (0.04 mm Hg). Yield, 38 gms.

<u>N-Methylanilide:-</u> Lauroyl chloride (38 mgs.) was dissolved in 100 mls. of benzene, cooled in ice and a solution of 22.4 gms. of redistilled methylaniline in 25 ml. of pyridine slowly added. The mixture was kept at 20°C for half an hour,

water added and extracted with benzene. The benzene solution was washed twice with 2N-HCl, twice with water and dried over anhydrous sodium sulphate. After removal of the benzene, the reaction product was distilled under reduced pressure and the yellow liquid distilling at 153-156°C (0.05 mm hg.) collected. Yield, 35 gms.

Analytical figures required for $C_{19}H_{21}O$: N,4.8; C,78.6; H,10.7

Found : N,5.2; C,78.1; H,10.15

Aldehyde:- A solution of lithium aluminium hydride (1.5 gms.) in dried ether was added slowly with constant stirring to a solution of the N-methylanilide (30 gm.) in dry ether at 0°C. Stirring was continued for a further 3 hours and the excess lithium aluminium hydride then destroyed by the addition of water. 2N-HCl was added, the mixture extracted with ether, the extract washed thoroughly with 2N-HCl, then with water and dried over anhydrous sodium sulphate. After removal of the ether, the product was distilled under pressure and the fraction distilling at 88-100°C (0.05 mm Hg.) collected to give a solid of melting point 42-44°C.

Yield, 10.0 gms.

2:4 Dinitrophenylhydrazone - Melting point, 103°C.

Analytical figures required for $C_{18}H_{20}N_4O_4$: N,15.3; C,59.34; H,7.69

Found : N,14.9; C,59.44; H,7.67

β-Hydroxy acid:- Aldehyde (8 gms.) and ethyl bromoacetate (9 gms.) were dissolved in 20 ml. of sodium dried ether and 5 ml. of dry benzene. A little of the solution was added to 4.5 gms. of zinc wool and warmed until reaction started when the mixture was stirred and the remainder of the solution slowly added. When all the solution had been added, refluxing was continued for an additional half hour and the reaction product after cooling in ice was poured into ice cold 10% H_2SO_4 with stirring. The acid layer was drawn off, the benzene solution extracted twice with 20 ml. portions of ice cold 5% H_2SO_4, then washed once with 10 ml. of cold 10% sodium carbonate, 10 ml. of cold 5% H_2SO_4 and finally with two 10 ml. portions of water. The combined acids solutions were washed with two 20 ml. portions of ether and the combined ether and benzene solutions dried over anhydrous magnesium sulphate. After filtration, the solvents was removed by distillation and the residue fractionated, the fraction distilling at 120-130°C (0.05 mm Hg.) being collected.

Yield, 2.8 gms.

The ester was hydrolysed by adding 10 ml. of N-alcoholic KOH and heating under reflux for 30 mins. After cooling, in ice, the precipitated potassium salt was

filtered off, washed with cold ethanol and dried. The salt was then dissolved in water, the solution cooled in ice, acidified to Congo red with 15% HCl and the precipitated acid filtered off, washed with water, dried over P_2O_5 and recrystallised from ligroin.

Yield, 1.8 gms. M.Pt. 73°C.

Analytical figures required for $C_{14}H_{30}O_8$: C,68.8; H,11.47

Found : C,67.9; H,11.47

Preparation of Diazomethane.

This was prepared by the action of KOH on nitrosomethylurea.[aaa]

Nitromethylurea:- Concentrated HCl was added to 20 gm. of 25% aqueous methylamine solution until acid to methyl red, the acidified solution diluted with water to bring the total weight to 50 gm., 30 gm. of urea introduced and boiled under reflux for three hours. The mixture was then cooled to room temperature, 11 gms. of sodium nitrite added, cooled to 0°C and slowly poured with stirring into a mixture of 60 gms. of crushed ice and 10 gms. of concentrated H_2SO_4, cooled in an ice-salt bath. The precipitated nitrosomethylurea was filtered off and dried in vacuo.

Diazomethane. - Nitrosomethylurea (4.12 gms.) was added to

12 ml. of 50% aqueous KOH and 40 ml. of ether and the evolved diazomethane distilled into 10 ml. of ether, containing 10% methanol, cooled in an ice salt mixture. The solution of diazomethane so obtained was immediately placed in a deep freeze until required for use.

Photographic recording of the results of agar - diffusion precipitin test.

Methods of recording the precipitin lines have been discussed by Dike.[73] These include free hand drawing, drying the agar plates onto lantern slides and photographic methods based on the capacity of the precipitin bands to reflect incident light. To overcome the disadvantages attending these methods, and the need, in many cases, for expensive apparatus, Dike has described a method which claims dimensional accuracy with high contrast and definition while being cheap and easily carried out. His method uses an ex-RAF continuous processing unit enlarger, although a disused half or whole plate camera or projector could equally well be used. The apparatus used in the present work was based on that of Dike but was constructed of readily available components. A sketch of the apparatus is shown in Fig.38.

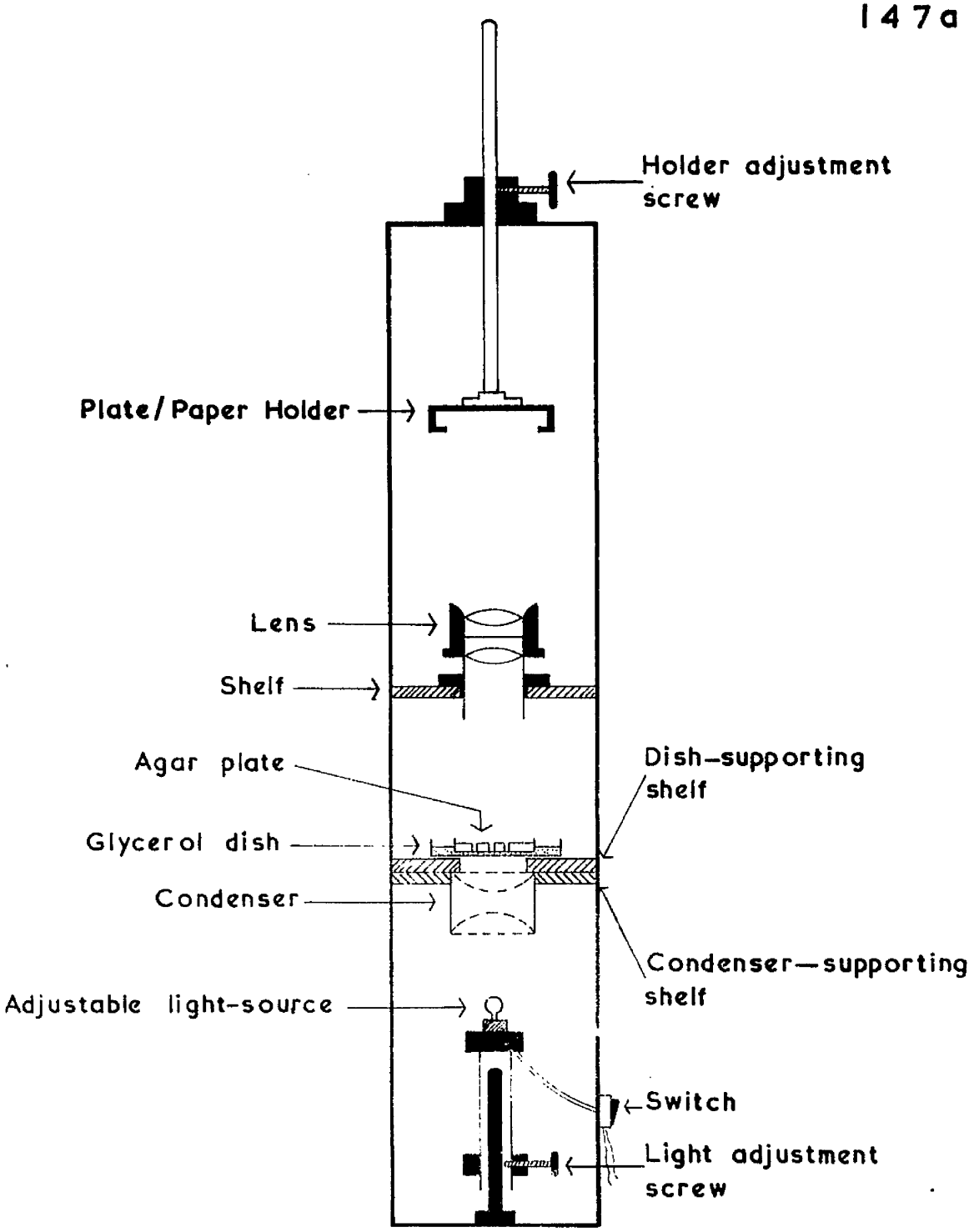


Fig. 38. Apparatus for the photographic recording of the results of agar-diffusion precipitin tests.

The carcase of the apparatus consisted of a wooden box, 4 ft x 9 ins x 9 ins painted flat black on the inside. The light source was a 75 watt enlarger bulb attached to the end of a short length of brass tubing, and made adjustable by sliding the latter onto a brass rod attached to the inside centre of the base on the box; a screw held the tube at any desired height. The plate paper holder was constructed from plywood and strips of Tufnol and the dimensions were such as to take $4\frac{1}{2}$ ins x $3\frac{1}{2}$ ins photographic plates or papers. A brass rod attached to the holder and passing through the centre of the top of the box allowed the holder to be adjusted in the vertical plane. The condenser unit (set of $4\frac{1}{2}$ ins optical condensers) was mounted in the lower shelf as shown in Fig.38, and a screen, consisting of $\frac{3}{4}$ ins plywood with a circular aperture slightly larger than the agar plates, was fixed to the upper surface of the condenser - supporting shelf, and supported the glycerol dish. The lens (5 ins ex WD f4) was attached to a short length of brass tube and the latter fitted through the centre of the upper shelf, a brass flange providing a sliding fit and allowing sufficient adjustment of the lens. Close fitting doors enclosed the two lower compartments of the apparatus.

Procedure. - The agar plate was carefully washed with distilled

water from a wash bottle and the agar covered with a thin layer of water. The dish was lower into glycerol contained in a large petri dish, avoiding the formation of bubbles in the glycerol, and placed on the dish - supporting shelf of the apparatus. The light source was adjusted to give a parallel beam incident on the petri dish; with a piece of white paper in the plate/paper holder, the latter was adjusted to give a sharp image of the precipitin lines on the paper. Photographic plates were used in some experiments but it was found more convenient to use photographic paper (Kodak bromide No.3) to give "negative" prints. To keep the paper flat in the holder, it was sandwiched between two pieces of thin vulcanite sheeting, the lower piece having a circular apperature to suit the image size. With the photographic paper in position the apparatus was stopped down and the light switched on for a predetermined exposure time. The paper was then developed in a contrast developer, fixed, washed and dried.

The only advantage claimed for the apparatus described above is that it is easily constructed from readily available materials and being containèdein a closed box is easily portable.

Testing of materials for pyrogenic activity.

Healthy rabbits weighing 2-2.5 Kg. were used and all animals were conditioned to the procedure to be adopted in the tests by being made to sit in the test boxes for a few hours at a time on several days, with rectal thermocouples inserted. Rabbits conditioned in this way are more co-operative and less easily upset during the first few tests than animals unaccustomed to the test procedure. For routine testing of different fractions, to avoid widely differing responses, several groups each of five rabbits were used. This grouping was done by injecting all the animals on three consecutive occasions with the same dose of a standard pyrogenic material and recording their responses. From the results of these tests balanced groups were worked out.

The test material, dissolved in apyrogenic physiological saline, was injected intravenously via the marginal ear vein and the dose volume administered was kept the same throughout (1 ml./Kg) so that no matter what the dose of pyrogen, each animal received the same volume of solution on each occasion. This removes the possibility of an effect due to administration of different volumes of solution. All injections were warmed to 37°C before administration.

To decrease the amount of defecation, thereby

minimising possible expulsion of the thermocouples due to defecation, the animals were not fed on the day prior to the test.

The animals were placed in their boxes at 8.30 a.m. and the thermocouples inserted. Preinjection temperatures were then taken at 10 minute intervals from 9.15 a.m. and by about 10.15 a.m. these temperatures were usually steady and the animals well settled down. Injections were normally completed by 10.30 a.m. and rectal temperatures were recorded at 10 minute intervals for the next 5-6 hours.

At first, temperatures were recorded by the method developed by Wylie and Todd[262,263] using a system of copper-constantin thermocouples, a selector switch and a reference junction immersed in a controlled water bath. The readings were taken on a Cambridge "Spot" Galvanometer, previously calibrated. Later this unit was replaced by a direct reading electric universal thermometer (type TES, Electrolabatororiet, Copenhagen) which operates in a similar manner but in which a reference junction is unnecessary as the instrument automatically compensates for variation in ambient temperatures. Each scale division represents 0.1°C and up to 30 animals can be tested simultaneously. The thermocouple applicators are adjusted by the manufacturers

so that no calibrations are required, and rubber rings on the applicators ensure insertion to a uniform depth.

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