A STUDY OF THE PYROGENIC LIPOPOLYSACCHARIDE OF *PROTEUS VULGARIS*

by

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SUMMARY OF THESIS

("A study of the pyrogenic lipopolysaccharide of *Proteus vulgaris*" by William Boyle.)

The major aim of this research was the isolation and characterisation of the heat-stable pyrogenic factor from the cells of *Proteus vulgaris* NCST 53, in order to compare it with the fever-producing factor, obtained by a colleague, from culture filtrates of the same organism. This was undertaken because a report on the fever induced by this bacterium in rabbits, claimed that there existed qualitatively different cell-associated and filtrate pyrogens. Recent studies had shown that the pyrogenic activity of Gram-negative bacteria was associated with the endotoxic complex and could most conveniently be obtained as the lipopolysaccharide component. However, no definitive study of the lipopolysaccharide of *P. vulgaris* had been reported nor was information available on a comparison of lipopolysaccharides obtained from cells and culture filtrates of any micro-organism.

The culture filtrate pyrogen had to be obtained from synthetic medium cultures but the quantity of cells from this source was small. Therefore, initially

crude pyrogenic lipopolysaccharide preparations were extracted with phenol from cells grown in synthetic medium and in nutrient broth cultures. Both cell extracts were shown to contain the same lipopolysaccharide on the basis of their similar elemental analysis, monosaccharide constitution, equivalent pyrogenicity and the serological identity of their major heat-stable component. Therefore, methods of purification and analysis were investigated using extracts from cells grown in nutrient broth cultures since they could be obtained in greater quantity.

The extract from nutrient broth culture cells was purified by differential ultracentrifugation. The purified lipopolysaccharide (LPS) had a reducing sugar value of 32%, and contained 16% hexosamine and 30-32% bound lipid. Monosaccharide constituents were glucose, galactose and an aldoheptose (probably D-glycero-L-mannoheptose), glucosamine and galactosamine. Protein could not be detected and nucleic acid contamination did not exceed 2%. Physical and chemical methods did not reveal heterogeneity in excess of 4% and immunological homogeneity was established. The lipid component contained all the saturated fatty acids of chain length C_{10} to C_{20} plus two long chain unsaturated

acids and β-hydroxy-myristic acid. The lipopolysaccharide was highly pyrogenic in rabbits; the Minimum Pyrogenic Dose was 0.002 μg/kg. body weight. The LD_{50} in mice was 1 mg. total dose, and in rabbits dosage in excess of 20 μg/kg. was toxic.

From synthetic medium culture cells a small quantity of lipopolysaccharide (LPI) was extracted, purified and analysed by the methods which had been used for the lipopolysaccharide (LPS) from nutrient broth culture cells. These lipopolysaccharides (LPI and LPS) were compared with a lipopolysaccharide (FPS) prepared by a colleague from synthetic medium culture filtrates. Their elemental analysis, carbohydrate and lipid contents were similar. Common constituents of all three preparations were the monosaccharides and fatty acids listed above. They gave serological reactions of complete identity and their pyrogenic activities were qualitatively and quantitatively alike. Minor dissimilarities in the relative content of two fatty acids was the only significant disparity among the products. In summary the same lipopolysaccharide was apparently being obtained from cells and from culture filtrates. Since the lipopolysaccharide (FPS) accounted for all the pyrogenic activity of the culture filtrate,

there seems no justification for belief in the hypothesis of the existence of distinct cell-associated and culture filtrate pyrogens. This hypothesis was based on the appearance of single or biphasic fever responses in rabbits, but it was found that all three lipopolysaccharide preparations could produce either type of fever dependent purely on the dosage administered.

The secondary aim of this research project was the pharmaceutical formulation of a standard reference pyrogen. A preparation was made by freeze-drying the lipopolysaccharide LPS with a mannitol carrier and its properties investigated. The preparation obtained appears to be a suitable reference standard because it is readily dispersable with full recovery of activity, can be terminally sterilised, is unaffected by the presence of a bacteriostatic agent and is stable on storage.

CONTENTS

INTRODUCTION.

The contamination of parenteral fluids by products of Gram-negative bacteria causes distressing symptoms on injection, the most noticeable of which is fever. The detection and removal of these contaminants, termed pyrogens, has concerned pharmacists for half a century with, so far, only limited success.

Recent findings have shown that the pyrogenic action can be attributed to the endotoxin, and many endotoxins have been isolated from Gram-negative bacterial cells and characterised chemically. This would appear to offer some hope of devising methods for removal of pyrogens based on knowledge of their chemical constitution. However, it has not yet been established that the pyrogenic factor released by autolysis of a bacterium in parenteral fluids would be identical with that obtained by extraction of the intact cell. In view of the findings of Wylie and Todd that the fever response of rabbits to injection of a cell-free culture filtrate differs qualitatively from that obtained by injection of the cells, it appeared necessary to obtain a strict comparison of the pyrogen extractable from the cell with that released into the culture fluid during growth. This was undertaken as a joint problem, and this thesis records my contribution

viz. the extraction, purification and chemical and biological characterisation of pyrogenic lipopolysaccharides obtained from cells of *Proteus vulgaris* grown in synthetic salt and nutrient broth media. These findings are compared with those obtained by my colleague, J. A. M. Shaw, on the pyrogenic factor isolated from the synthetic medium culture filtrates.

In the absence of universally applicable methods for removal of pyrogens, parenteral fluids must be subjected to an official limit test based on the fever response of rabbits. This test is greatly limited by the variation in response between rabbits, and to reduce this, the need for a standard reference pyrogen has been recognised. The World Health Organisation in 1953 decided to obtain a reference standard but up till now no product has been officially accepted. In an attempt to remedy this need, the formulation of a standard pyrogen preparation based on the *Proteus vulgaris* lipopolysaccharide was undertaken, and a report of this work forms the latter part of the experimental section.

The experimental section is prefaced by a review outlining the present views on the chemical, biological and pharmaceutical aspects of pyrogens of Gram-negative bacteria, and previous studies on polysaccharides and pyrogens of *Proteus* species.

REVIEW SECTION

PYROGENS OF GRAM-NEGATIVE BACTERIA

INTRODUCTION

Intravenous injection into man and other animals of sterile solutions frequently produces a febrile response. The substances which cause this fever are termed 'pyrogens' and were shown by Seibert [1] in 1925 to be heat stable, filterable products of micro-organisms. Gram-negative bacteria are regarded as the main source of pyrogens for although the gram-positive bacteria produce toxins with pyrogenic activity these are heat-labile and so cannot be the heat resistant factors in injection fluids. Fungi may produce pyrogenic factors [2] but the evidence for this is questionable.[3]

In this connection the pyrogens of Gram-negative bacteria have been of interest since the early part of the century but it is only within the past decade that their chemical nature has been determined. Much of the early research on pyrogens has been adequately reviewed [4-8] and will not be considered here. Instead attention will be directed towards the later work which has led to the isolation of "purified pyrogens" and established their relationship to the endotoxic complex.

CHEMICAL NATURE AND RELATION TO THE ENDOTOXIN COMPLEX.

The pyrogens which occur in injection fluids are present

only in microgram quantities and this may perhaps explain why little attempt was made in the early years of pyrogen research to isolate and characterise them. The first notable attempt was made as late as 1944 by Co Tui, Hope, Schrift, Powers, Wallen and Schmidt.[9] They obtained from the cell-free culture filtrate of Eberthella typhosa by ethanol precipitation followed by phenol extraction to remove protein, a pyrogenic preparation which contained only 1.5 per cent N, and 30 per cent reducing sugar. From these results they stated that the pyrogenic factor was polysaccharide in nature and did not require the presence of protein. These findings were corroborated by Robinson and Flusser[10] who obtained pyrogenic preparations from Pseudomonas aeruginosa, Salmonella typhi and Proteus vulgaris extracts by acetone precipitation and phenol extraction, which gave positive carbohydrate tests and were deemed to be protein-free because of the absence of nitrogen as determined by the sodium fusion test.

This was all that was known about the nature of bacterial pyrogens just over a decade ago. The considerable advance made since then has been due not only to studies on pyrogens per se, but also to endotoxin studies attempting to separate antigenicity and toxicity, to research on bacteriophage receptors, and investigations on tumour-necrotising substances derived from bacteria.

The relationship of these different fields of research can only be realised by a consideration of the composition of the active substances, and the purpose of this section will be to establish the common structural relationship of these substances and pyrogens.

The first extraction of the toxins of Gram-negative bacteria was carried out by Boivin and Mesrobeanu in 1933 by trichloracetic acid extraction of the Enteric group of organisms.[11] In the extracts they found a phospholipid-polysaccharide complex which accounted for the total toxicity of the organism and termed it the endotoxin. This "glyco-lipid" also appeared to be the complete dominant O-somatic antigen, for on injection into rabbits it produced antibodies which agglutinated the organism, and antisera to the organism precipitated the complex. This glyco-lipid complex gave negative reactions to protein tests. It could be extracted from Smooth variants only and strains which lacked it were non toxic.[12,13]

Raistrick and Topley [14] found in trypsin digests of Salmonella typhimurium a similar toxic antigenic complex of phospholipid and polysaccharide, which appeared to contain no protein but indicated the presence of peptide bonds by positive Biuret tests.

The work of Morgan and Partridge advanced considerably the knowledge of the endotoxic complex. They extracted

complexes from Shigella dysenteriae [15,16] and Bacterium typhosum [17] with diethylene glycol and showed that these were toxic and antigenic in a similar fashion to the complexes obtained by Boivin. They showed that there existed in the complexes phospholipid and polysaccharide moieties as described by Boivin, but they also detected a further component, a conjugated protein. They found that the phospholipid could be removed from the complex with cold formamide without affecting either the antigenicity or the toxicity. By splitting the protein and polysaccharide fractions with 90% phenol they were able to prove that the serological specificity of the complex was determined by the polysaccharide, but that this required to be joined to the protein fraction to be antigenic in rabbits. Their studies on the toxicity of the complex were less extensive but they suggested that it appeared "to be largely due to the undegraded polysaccharide." [17]

Similar lipid-protein-polysaccharide complexes were obtained from the Flexner group of organisms by Goebel, Binkley and Perlman using diethylene glycol or aqueous pyridine as the extracting solvents.[18,19,20] Tal and Goebel examined in detail one of these complexes, that from Sh. paradysenteriae type Z. By extraction with formamide in the presence of formic acid they corroborated Morgan's finding that the lipid fraction was not essential to toxicity.

They hydrolysed the remaining protein-polysaccharide conjugate with dilute acetic acid and produced a toxic conjugated-protein and non-toxic degraded polysaccharide. If the hydrolysis were carried out with alkali and ethanol the products obtained were a toxic polysaccharide and a non-toxic protein. From these studies they postulated the presence in the endotoxic complex of a further component responsible for the toxicity, the T-factor, which was neither the protein nor the polysaccharide alone since their different methods of hydrolysis showed that the toxicity could be attached to either one.[21]

In an attempt to elucidate the chemical nature of this toxic component they extensively degraded the endotoxic complex by sequential treatment with papain, formamide and formic acid, and with alkaline phosphatase. The product obtained (TM) was as toxic as the original complex but differed chemically as shown by the analytical figures (table 1).

TABLE 1

	C	H	N	P	Total lipid
Endotoxic complex	44.32	6.79	5.4	1.44	10.5
TM product.	47.0	7.2	2.2	1.1	5.0

Hydrolysis of TM in 1% acetic acid was accompanied by loss

of toxic properties within one hour, although no visible change occurred in the solution. However, on continued hydrolysis a precipitate was formed, and the material remaining in solution was shown to be degraded polysaccharide devoid of toxicity. The acid precipitate was ether-soluble but Tal and Goebel did not consider it was lipid because they believed the analytical figures viz.

C63.3 H9.5 N2.96 P3.3, especially the high nitrogen figure, were not typical of any known lipid.[21] It should be mentioned that Miles and Pirie[22] had found in the endotoxic complex of Brucella melitensis a bound lipid component which was released on acid hydrolysis, but since this was not tested for toxicity it was not considered as a necessary part of the complex at that time.

Thus, in 1952 the structure of the endotoxic complex was known to be phospholipid-protein-F factor-polysaccharide. It was against such a background of knowledge that the re-investigation of the chemical nature of pyrogens was begun by Westphal. Although endotoxins were known to produce many reactions in animals, including fever, pyrogens were still believed to be specific agents with no known relationship with other bacterial products.

However, one other field of research had recently produced a very potent pyrogen. In investigations into the tumour-necrotising action of bacteria, Shear and colleagues[23,2

isolated and purified an active fraction from the culture fluid of Serratia marcescens. Analysis of the purified material showed it consisted of 67% phosphorylated polysaccharide and 16% of a firmly bound lipid which could be released only on acid hydrolysis. Bock and Fisher [25] examined this material three years later and found it was the most potent pyrogen known. The Minimum Pyrogenic Dose, that is the dosage required to produce a rise of 0.6°C in the rectal temperature of rabbits, was of the order of 0.005 micrograms per kilogram rabbit body weight. This confirmed the earlier belief of the polysaccharide nature of pyrogens but was the first report of the occurrence of bound lipid material.

In 1952 Westphal and his group began an extensive series of investigations into the nature of bacterial pyrogens. They modified the phenol extraction method of Palmer and Gerlough [27] to produce protein-free pyrogens from Escherichia coli [28] and Salmonella abortus equi [29] but the method has since been applied to a wide range of organisms.[30,31,32] The dried bacterial cells were extracted with aqueous 45% phenol at 65°C and subsequent separation of the two phases by cooling and centrifuging yielded pyrogenic polysaccharide and nucleic acid in the aqueous phase, and amphoteric protein and formamide-soluble lipid in the phenolic phase.

The polysaccharide fraction obtained from Escherichia coli 08 was freed from nucleic acid by ultracentrifugation, and analysis detected 7½% phosphorylated polysaccharide and 12 - 13% lipid. These lipopolysaccharides were similar in activity to that produced by Shear et al,[25] their Minimum Pyrogenic Dose being 0.002µg/kg in rabbit and 0.001µg/kg in man.

The lipid was tightly bound to the polysaccharide, could not be removed by formamide but split off on acid hydrolysis leaving a pyrogenically inactive polysaccharide. This tightly bound lipid is termed lipid A to distinguish it from the loosely bound, formamide-soluble lipid described by Morgan, which is named lipid B. Suspension of the lipid A in Tween gave a pyrogenic response in rabbits which suggested it might be the active moiety. Further, by coupling the lipid A to proteins of non-bacterial origin which were non-pyrogenic and non-toxic, products were formed which were both toxic and pyrogenic, though at best, only one-fifth as active as the original lipopolysaccharide.[28,33]

The above facts suggested to Westphal that the lipidA might be the toxic T-factor of Tal and Goebel. By modifying their phenol treatment Westphal, Luderitz and Bister had been able to extract lipid A attached to protein, which then carried the toxicity, and the polysaccharide remaining showed little pyrogenic or toxic power.[26] These phenol treatments

bore a close similarity to the hydrolysis procedures of Tal and Goebel as shown in Fig. 1.

Analysis of the lipid A obtained by Westphal et al showed a close resemblance to that obtained by Tal and Goebel for their T-factor.[21] (See Table II below.)

Table II

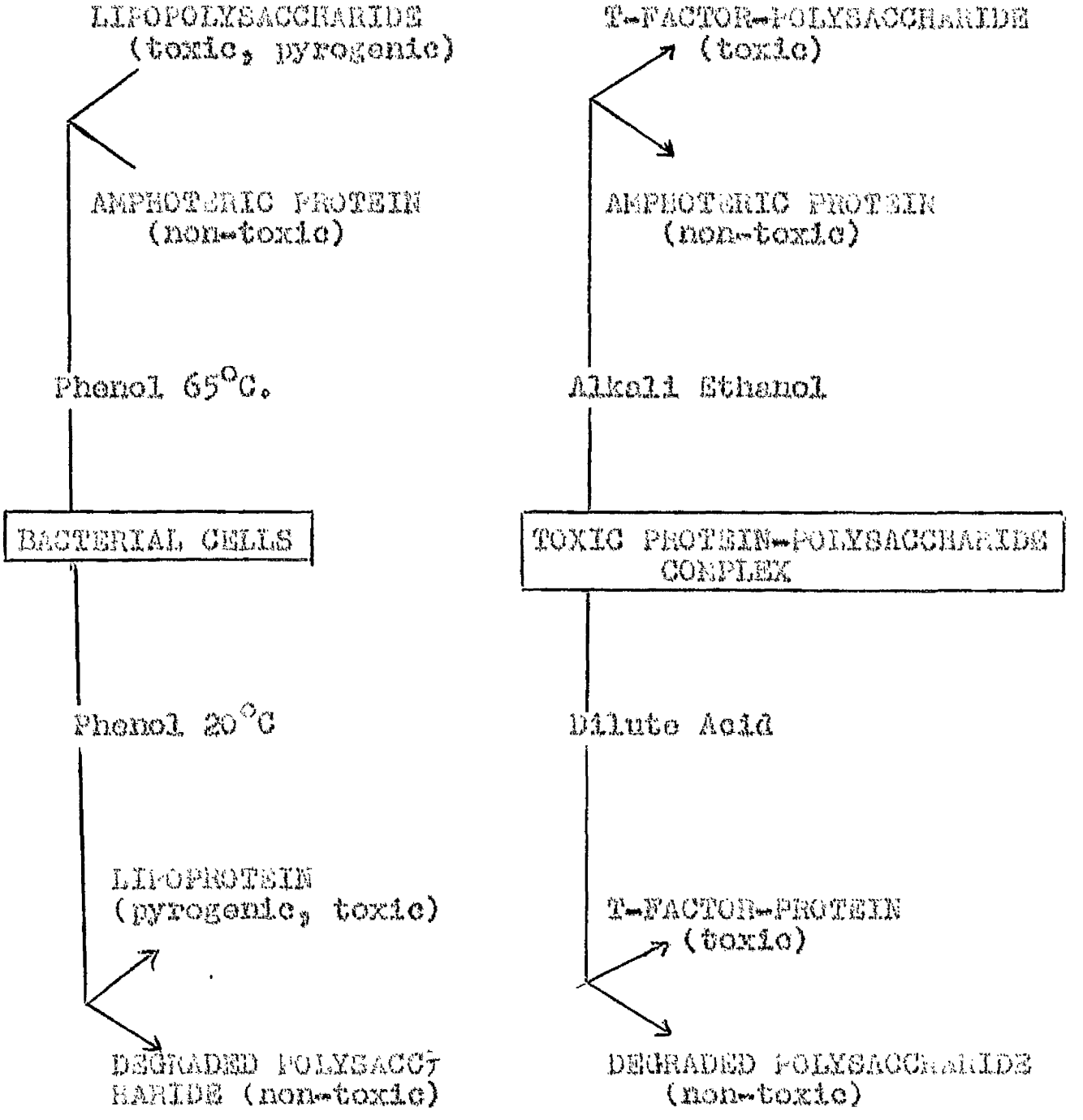
Preparation	Parent Organism	C %	H %	N %	P %	Reference
Lipid A	E.coli 08	61.1	9.4	1.9	2.3	28
T-Factor	Sh.paradysenteriae	63.3	9.5	2.7	3.3	21
Lipid A	S.abortus equi	63.0	9.4	2.0	2.0	28

Thus the gross structure of the endotoxic complex was shown to be lipid B-protein-lipid A-polysaccharide. The fact that lipopolysaccharides have been extracted from the endotoxic complex and found to produce many typical endotoxic reactions on injection into animals, including fever, is final evidence that they are part of the endotoxic complex.[31]

The endotoxic complex is estimated to form up to 20% of the dried weight of the bacterial cell and the relative concentrations of the various fractions are approximately as follows:

polysaccharide	45 - 60%
lipid A	5 - 15%

Fig.1.



WESTPHAL et al. (Ref.26)

TAL and GOEBEL(Ref.21)

protein 15 - 20% : lipid B 10%

The composition of any material possessing endotoxic activity will depend on the method of extraction employed. Table III lists a number of the common extraction solvents and indicates the type of product generally obtained. The extracts are usually contaminated with other cellular constituents and purification of the endotoxic fraction requires fractional precipitation with salts or organic solvents, or physical separations such as ultracentrifugation.

Table III

Extraction Procedure	Nature of Product	References
Cold Trichloracetic acid.	LPS-protein-lip B	12, 13
Tryptic digestion	LPS-peptide	14
Anhydrous diethylene glycol	LPS-protein-lip B	15, 16, 17.
90% phenol followed by distilled water	LPS	27
2.5% urea	LPS-protein-lip B	34
50% aqueous pyridine	LPS-protein-lip B	18
Hot distilled water	LPS-protein-lip B	35
Aqueous ether or dioxane	LPS-protein	36
45% phenol 65°C	LPS	26
2% phenol	LPS-protein-lip B	22

LPS = lipid A-polysaccharide
lip B = formamide soluble lipid B.

Extraction with urea, hot water, 2% aqueous phenol and aqueous ether have been used only in a limited number of instances, and the generality of their application is not known. Diethylene glycol extracts a good undegraded complex of high molecular weight from Shigella dysenteriae [15] and Shigella flexneri type 2 but failed to extract the complex from other strains of the latter organism.[18,19] Aqueous pyridine fulfilled this requirement, but also seems to have limited application since it would not extract the complex from Shigella sonnei.

Trichloracetic acid has been widely used to extract the complete complex from Smooth organisms but is not so effective with Rough organisms or those which have a surface protein layer.

Extraction with 45% aqueous phenol has proved to be of general application. The product is protein-free and has been the method of choice where a product is required with toxic activity but non-antigenic. Both Smooth and Rough organisms can be extracted by this method,[33] and Davies also found it effective in extracting Pasteurella pestis [37] which has a surface protein layer which interfered with other methods.

COMPONENT-ACTION RELATIONSHIP OF ENDOTOXIC COMPLEX.

The difference in the biological properties of products obtained by the above extraction methods, coupled with the

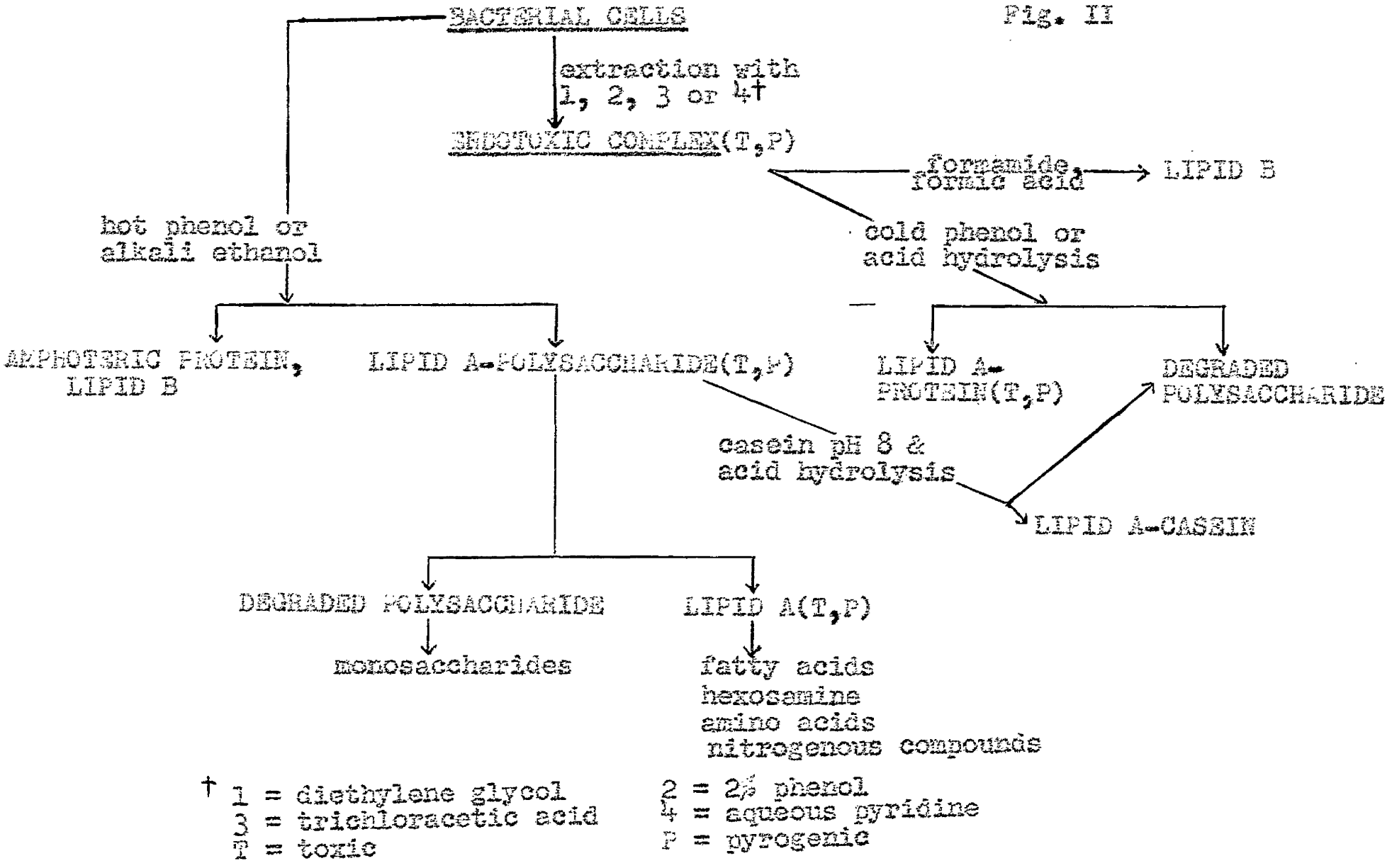
ability to degrade the total endotoxic complex by stages, has elucidated the relationship between the various parts of the complex and has gone some way in relating structure to function. Some of the relationships between the complex and its component parts are illustrated in Fig.II.

The lipid-polysaccharide-protein-complex carries all the endotoxic activity ascribed to the cell,[11] and also functions as the dominant O-somatic antigen.[15] The loosely bound lipid, lipid B, appears to play no role in either of these functions since it can be removed without impairing them.[17] In order to elicit the formation of antibodies to the complex in rabbits, the presence of the protein component is necessary, but the serological specificity resides in the polysaccharide fraction.[33] Artificial complexes composed of the lipopolysaccharide of one bacterial species coupled to the protein from another have been used to produce antibodies in rabbits, and the specificity of these is always directed towards the parent organism of the polysaccharide.[16,37]

It has proved more difficult to ascribe the toxic activity, including pyrogenicity, to any one part of the complex. The extraction experiments of Westphal which initially suggested lipid A as the toxic factor described by Tal and Goebal have already been summarised (cf. Fig.1).

When the lipid A-polysaccharide complex (hereafter

Fig. II



† 1 = diethylene glycol
3 = trichloracetic acid
T = toxic
2 = 2% phenol
4 = aqueous pyridine
P = pyrogenic

referred to as lipopolysaccharide) which is pyrogenic in rabbits at 0.002ug/kg body weight, was acid hydrolysed it produced water-insoluble, chloroform-soluble-lipid A and degraded polysaccharide. The degraded polysaccharide had no pyrogenic activity but if the lipid A was dispersed in water it showed pyrogenic activity, although this was only obtained at the relatively high dosage of 100ug/kg. This lipid A activity was considerably increased when dispersed in Tween 80 before injection, the dosage then being 1-10ug/kg. Further attempts to increase the dispersability of the lipid A by suspension in solutions of low molecular weight dextrans reduced the active dose to around 0.01ug/kg.[31,33,38].

Westphal and his colleagues were also able to combine the lipopolysaccharide with inert proteins, such as casein or serum albumin, by incubating them together in weak alkaline solution.[38,39] The formation of a complex was indicated by the inability of trichloracetic acid to precipitate the protein from solution. On heating the artificial complex in dilute acid solution it was split to yield the lipid A attached to the protein and the lipid-free polysaccharide. These artificial lipoprotein complexes synthesized from non-pyrogenic proteins, were found to produce fever on injection into rabbits at 0.01ug/kg. body weight.

In an attempt to elucidate the nature of the moiety in the lipopolysaccharides responsible for the toxic and

pyrogenic actions, the lipopolysaccharide has been subjected to various treatments in the hope of selectively destroying separate activities.

Neter, Westphal, Luderitz, Gorzynski and Eichenberger[40] found that heating at 100°C for 2½ hours in pH 7.2 buffer did not destroy the toxicity for mice, pyrogenicity in rabbits, nor the reactivity with specific antiserum. However, heating in 0.25N NaOH at 56°C for one hour had no effect on the reactivity with antiserum, but destroyed the pyrogenic and toxic activities. Treatment with periodate destroyed the serological activities, reduced the toxicity to one-tenth of its original value but had no effect on the pyrogenicity.

Nowotny[41] has recently claimed that dry heat treatment destroyed the toxicity but had no effect on the serological reactivity, and in apparent further contradiction to the findings of Neter et al,[40] found that treatment with periodate destroyed both the toxicity and the serological reactivity.

Nowotny[42] claims that treatment of the complete antigenic complex with boron trifluoride or pyridinium formate cleaves the complex and from the resulting mixture ethanol precipitated a non-toxic but highly pyrogenic polysaccharide-rich fraction which was still capable of reacting with antiserum. The ethanol-soluble fraction was found to

contain most of the lipids which were originally present in the endotoxic complex.

On these findings it was accepted for a number of years that the lipid A was responsible for the endotoxic activity. Although the above experiments have involved measurement of endotoxic activity by pyrogenic reactions, this was used only because it is the most sensitive index of endotoxic activity, and in fact other typical endotoxic phenomena have also been produced in animals by dispersed lipid A alone.[31]

However, recently Ribi, Landy and Milner[43] reported on the endotoxic activity of lipopolysaccharides obtained by aqueous ether extraction of Salmonella enteritidis which were characterised by their low content of firmly bound lipid. They found comparable activity between these low-lipid-content materials, and phenol-extracted lipopoly-saccharides which usually contained up to ten times as much bound lipid. They postulated that if the lipid was the true endotoxic factor, then on isolation from the lipopoly-saccharide and with suitable dispersion it should in fact show an increased activity per unit weight over the intact lipopolysaccharide. The lipids they obtained by the acid hydrolysis procedure employed by Westphal were never found in pyrogenicity and mouse protection tests, to have more

than one-hundredth of the original activity even after dispersion in Tween solutions.

Ribi et al refluxed their aqueous-ether-extracted-lipopolysaccharides with firstly chloroform-methanol and then with monochlorobenzene-ethanol mixture. They obtained in the second solvent mixture extract, a chloroform-soluble material which was comparable in activity in pyrogen and mouse protection tests with lipid A preparations they had obtained by acid hydrolysis. However the lipopolysaccharide residue left after the non hydrolytic procedures described contained minimal per cent of lipid (3.9-4.7% total fatty acids) yet showed no reduction in pyrogenic or mouse protection potency. Hence they claim that although lipid A may have some endotoxic activity it is only minimal, and that the previous importance ascribed to it is due mainly to the comparison of its activity with the degraded polysaccharide produced along with it which has no activity.

They also conducted a series of experiments in which lipopolysaccharides were hydrolysed with acetic acid and samples were withdrawn at various time intervals and after neutralisation were tested for endotoxic activity. They were able to show that the loss of endotoxic activity occurred much earlier than could be accounted for by the release and precipitation of lipid A and recall the similar

findings of Tal and Goebel.[21] Westphal has reported that the endotoxic activity is due to a poly-glucosamine-phosphomucolipid fraction of lipid A,[44] but Ribi and his colleagues were unable to relate activity of lipid fractions obtained by various methods to their hexosamine content. Further, this active phosphomucolipid is reported to contain 30% of the fatty acids linked through the amino groups of glucosamine yet Ribi et al obtained lipid A preparations with average activity and could not detect any amide-linked fatty acid.

Ribi et al state that their experiments "provide no support for the concept that the lipid moiety is solely, or even chiefly, responsible for the biological activity of endotoxins." However, to add to the confusion, Westphal has examined a preparation of "lipid-free" lipopolysaccharide obtained from Ribi and found 15% lipid A present.[45]

Thus, although the experiments of Ribi et al are of a restricted nature, and in fact they have obtained their low-content lipopolysaccharides from only one organism, their results will cause re-examination of the role of lipid A.

However, it is clearly established even at this stage that for maximum pyrogenic potency, especially where it is wished to avoid the involvement of an immunological response in the host, the lipopolysaccharide fraction of the endotoxin

is the fraction of choice.

LIPOPOLYSACCHARIDES.

A considerable number of lipopolysaccharides have been extracted from Gram-negative bacteria for study of fever production,[28] induction of non-specific resistance to infection,[46] haemorrhagic necrosis of tumours,[23] specificity of bacteriophage receptors[47] and serological reactions,[32] and also for clinical use.[7] It would be impossible to consider here the chemical constitution of these lipopolysaccharides individually, but a review of their general composition will be undertaken. A number of reviews [31,32,38,48,49,50,51] on endotoxins and related products contain information on the lipopolysaccharides, and an exhaustive treatment of the polysaccharides of Gram-negative bacteria compiled by Davies[30] considers them fully.

Physical Properties

The lipopolysaccharides are soluble in water to varying degrees forming opalescent colloidal solutions. In alkaline solutions (buffer pH 8-9) in an electric field they move slowly towards the anode, due presumably to their phosphoric ester content.

Only a few preparations have been shown to be homogeneous by electrophoretic and ultracentrifuge studies, but from these they appear to be of high molecular weight.

Miles and Pirie[22] isolated from B.solitensis a lipopolysaccharide having a particle weight of about one million, while a similar product isolated by Shear from S.marcescens had a value ten times this.[23]

A lipopolysaccharide obtained from E.coli[28] was found to have a particle weight approaching twenty million by sedimentation studies, but optical measurements showed that the particles were aggregates of smaller units with particle weights of about one million. Aggregation of this lipopolysaccharide was shown to occur at high pH values and the process was only partially reversible on return to neutrality.[52]

Polysaccharide Fraction

The lipopolysaccharides obtained from Smooth strains of Gram-negative bacteria are heteropolysaccharides and are usually more complex than the corresponding specific polysaccharides of Gram-positive bacteria or the mucopolysaccharides of the cells of higher animals. Among the commonly occurring classes of monosaccharides found are aldohexoses, aldopentoses, methylpentoses, 3,6-dideoxyhexoses, aldoheptoses, and hexosamines.

The aldohexoses which have been found are limited to glucose, galactose, and mannose. The first two are most frequently encountered but mannose has been found extensively in the Salmonellae. Pentoses are usually represented by xylose, arabinose or ribose, but the latter may in many

instances be due to contamination of the product with nucleic acids. Methyl-pentoses (6-deoxyhexoses) found are rhamnose and fucose only.

A new class of sugar, 3,6-dideoxy-hexoses, were first detected in hydrolysates of lipopolysaccharides of Salmonellae[53,54] as fast moving components on chromatograms. Five 3,6-dideoxy-hexoses have now been isolated and characterised against synthesized products. These sugars are listed in Table IV along with their original bacterial source.

Table IV

Sugar	Original bacterial source	References
Abequose(3-deoxy-fucose)	S.abortus equi	53,55
Tyvelose(3-deoxy-D-rhamnose)	S.typhi	54,55
Ascarylose(3-deoxy-L-rhamnose)	P.pseudotuberculosis	58
Paratose(3,6-dideoxy-D-glucose)	S.paratyphi A	56
Colitose(3-deoxy-L-fucose)	E.coli O111	57

A method of determining the 3,6-dideoxy-hexoses in the presence of other monosaccharides has been developed[59] and they have been detected in a large number of lipopolysaccharides. They have been found most extensively in the Salmonellae[30,32] and have been shown to be involved in the serological reactions which form the basis of the Kauffmann-White classification.[60] For example, all Salmonellae of group A contain paratose, group B abequose, and group D

tyvelose. These sugars have not been found elsewhere in nature, except ascarylose which occurs in the membrane of the eggs of Parascaris equorum.[61]

Lipopolysaccharides of Gram-negative bacteria have also proved to be the first natural source of another class of monosaccharides, the aldoheptoses. The first report of an aldoheptose was by Jesaitis and Goebel in a lipopolysaccharide phage receptor of Shigella sonnei phase II.[47] The sugar was detected by the Dische reaction[62] and tentatively identified as D-glycero-L-mannoheptose by chromatography.

Although it has been shown that 12 of the 16 possible aldoheptose configurations can be separated chromatographically,[63] optical isomers cannot be resolved by this method so that in most cases this cannot be stated with certainty.

Stein and Schnell[64,65] isolated an aldoheptose from Sh.flexneri type 3, and by comparison of crystalline derivatives by X-ray diffraction, the sugar was recognised as L-glycero-D-mannoheptose. The same sugar was later isolated from the lipopolysaccharide fraction of the cell wall of E.coli by Weidel.[66]

Various Enterobacterial lipopolysaccharides have since been found to contain aldoheptoses and, although they have not been isolated, chromatographic identification has always suggested they are all of the glycero-manno configuration.

Aldoheptose has been found also in lipopolysaccharides of Pasteurellae,[63,67] Bordetellae[68] and Neisseria.[63]

Chromatographic separation of the monosaccharides of Chromobacterium violaceum 7917 showed an aldoheptose which behaved as a glycero-mannoheptose and this identification was strengthened by the production of mannose following periodate treatment and reduction of the lipopolysaccharide.[69,70] However, the lipopolysaccharide of a related organism Ch.violaceum BN produced galactose after similar treatment and isolation of the heptose showed it to be D-glycero-D-galacto-heptose.[70,71] This sugar has also been found in the Mrch strain of Ch.violaceum.[72]

The concentration of aldoheptoses varies greatly and values from 1% to over 50% have been found for different lipopolysaccharides.

Hexosamines reported to occur in bacterial lipopoly-saccharides have all been of the 2-amino-2-deoxy series. The commonest is D-glucosamine, but this is known to be part of the lipid A fraction and may not always be a constituent of the polysaccharide moiety. Other hexosamines found are D-galactosamine[64] and D-fucosamine,[73] but they are not known to occur in lipid A. Shigella sonnei phase II has been reported to contain an amino sugar different from those above.[74] The amino sugars are usually N-acetylated but an unidentified N-formylated sugar was reported in

B.melitensis.[75]

No structural studies have been carried out on these heteropolysaccharides but gentle hydrolysis procedures or use of monosaccharides to inhibit precipitation by specific antibody have been used to determine the terminal sugars in the polysaccharide chain, especially in the Shmonellae.[76,77]

Lipid A Fraction.

The lipid A fraction of lipopolysaccharides, although accepted for a number of years as the group responsible for the endotoxic activity, has not been so fully investigated as the polysaccharide part. A number of preparations have been given elemental analysis, as shown in Table V.

Table V. Analysis of lipid A preparations.

Bacterial source	% of LPS	C %	H %	N %	P %	Hexn. %	Ref.
Sh.somnei	29			1.4	1.3		47
Sh.flexneri	10	63.3	9.5	2.7	3.3		21
S.abortus equi	26	62.3	9.4	1.6	2.0	17.9	33
S.marcescens	16			1.9	3.1		24
E.coli	24	57.3	10.1	3.3	1.6	12	78
E.coli(Kroger)	13	61.1	9.4	1.9	2.3	17.8	28
B.melitensis	20-26	60-62	9.4-10	4.3-4.6	1.4-1.6		22

LPS = lipopolysaccharide. Hexn. = hexosamine.

The lipid A can be released from the lipopolysaccharide by hydrolysis with acetic or mineral acids or with cationic exchangers.[79] Mineral acid hydrolysis generally releases a greater weight of lipid than acetic acid but there is no evidence of qualitative difference between the products. The lipid A is usually insoluble in acetone and partially or completely soluble in ether or chloroform or both.[33]

Ikawa, Koepfli, Mudd and Niemann obtained lipid A from a lipopolysaccharide extracted from the culture filtrate of E.coli. Hydrolysis of the lipid with 5N HCl for 15 hours at 100°C followed by ether extraction produced a water-soluble, an ether-soluble and a water-and-ether-insoluble fractions. From the ether-soluble fraction lauric, myristic, palmitic and β-hydroxymyristic acids were isolated.[78]

Chromatography of the water-soluble fraction detected glucosamine, ethanolamine, probably aspartic acid and phosphoric acid. A number of other ninhydrin +ve spots were obtained, some of which reduced silver nitrate, but were not recognised.

The ether-and-water-insoluble material was finally crystallised as a picrate or dithiocarbamate and shown to be 4,5-diamino-n-eicosane, $C_{20}H_{44}N_2$, to which the name necrosamine was given. This was the first known detection of long chain aliphatic diamo compound in a natural product.[78]

Westphal, Luderitz, Eichenberger and Keiderling initially examined lipid A preparations obtained from E.coli[28] and S.abortu equi.[33] Inthe water-soluble fraction after hydrolysis they found the same substances as those described by Ikawa et al.[78] plus glutamic acid and glycerol.

Nowotny, Luderitz and Westphal[80] investigated the higher fatty acid constitution of lipopolysaccharides obtained from cells of six strains of E.coli and five Salmonellae. Paper chromatography of fatty acids of the lipopolysaccharide hydrolysates showed the presence of all the fully saturated acids of chain length C_{10} to C_{22} , two unsaturated acids and a fast moving component thought to be analogous to the β-hydroxymyristic acid found by Ikawa et al.[78] All the lipopolysaccharides examined were found to have qualitatively similar fatty acid spectrum, and in view of the fact that all lipopolysaccharides show similar physiological activity and cross tolerance to such activity,(in contradistinction to their serological reactivity which is specific for each lipopolysaccharide and ascribable to the polysaccharide part) this gave some weight to the belief that the lipid fraction was responsible for the biological activity.

Nowotny is reported to have found that lipid A was a mixture of sixteen components[44] and has described the gross structure of one of them, a phosphonucolipid.[79] Westphal has reported that all the endotoxic reactions of

the lipopolysaccharide can be ascribed to this phosphomuco-lipid.[44]

Phosphomucolipids have been isolated from six Salmonellae and had the general composition 18-20% glucosamine, 50-55% fatty acids, and 1-2% phosphoric acid.[79]

On hydrolysis of the phosphomucolipid with 3N hydrochloric acid at 100°C for ten hours, 65% of the total weight became ether-soluble and 50% remained water-soluble (the excess 15% is attributed to addition of water during hydrolysis). The ether-soluble fraction consisted almost exclusively of fatty acid(85% by titrimetric determination) and chromatography detected the full range of long chain fatty acids previously found in hydrolysates of the complete lipopolysaccharides.

High voltage electrophoresis of the water-soluble materials showed glucosamine as the main component, with small quantities of aspartic and glutamic acids, serine, alanine, valine and arginine.

A further three components detected on the electrophoretograms could not be related to known amino acids. They were separated on Dowex-50 columns and on subsequent analysis recognised as 6-phospho-D-glucosamine, 4-phospho-D-glucosamine and 1-peptido-4-phospho-D-glucosamine. Hydrolysis of the last product released all the amino acids already detected by electrophoresis above.

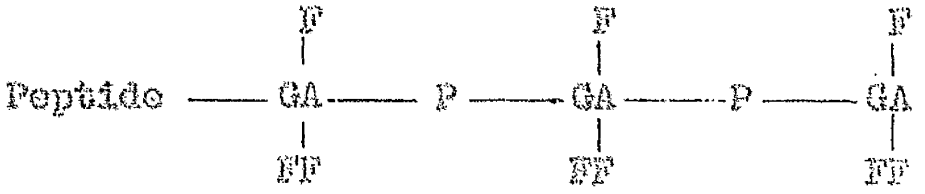
Studies of the rates of appearance and destruction of the glucosamine products during acid hydrolysis showed that the appearance of the acid-stable 6-phospho-D-glucosamine was coincident with the disappearance of the two 4-phospho-D-glucosamine derivatives. Thus the latter were believed to exist in the phospho-muco-lipid and that the 6-phospho-derivative was an artifact produced on hydrolysis.[79]

The two 4-phospho-D-glucosamine fractions and D-glucosamine were liberated in approximately equi-molar amounts during hydrolysis of the phosphomucolipid. Two possible types of linkage were thus envisaged : (i) direct linkage, or (ii) linkage through phosphate diester bridges. Alkaline and acid phosphatases failed to split free phosphate from the phosphomucolipid, and in view of this and of unpublished results obtained with venom diesterase, Nowotny suggests that the glucosamine phosphate units were joined by diester bridges.[79]

Neither glycerol nor sphingosine were found in the hydrolysates of the phospho-mucolipid and the attachment of the fatty acids had to be considered in other terms. Hydrazinolysis experiments showed that the amino groups of the glucosamine were acylated, approximately 30% of the fatty acids being bound in this way. Deacylation experiments showed that a further 35-45% of the fatty acids were bound

in labile ester linkage to the glucosamine, probably through the C(3) hydroxyl group. The remaining fatty acids were thought to be linked to the C(4) atom, but the exact position of the individual fatty acids has not been determined.[79]

Nowotny has thus given the following tentative structure to the phosphomucolipid :-



where GA = D-glucosamine, P = phosphoric acid, and F = fatty acid.[79]

Some discrepancies are noted when one compares the findings of Nowotny[79] and the Westphal group[28,33] on the lipid A of lipopolysaccharides obtained from cells with the findings of Ikawa and coworkers[78] on the lipopolysaccharide obtained from culture filtrate of E.coli. Reference to Table V (page23) shows that all the products obtained from bacterial cells, with the exception of the B.melitensis product,[22] have a N/P ratio between 0.77 and 1.07, while the two lipid A materials derived from lipopolysaccharides obtained from culture filtrates have much higher N/P ratio (1.7 for the S.marcescens product of Shear et al[24], and 2.0 for the E.coli product of Ikawa et al.[78]). While all the lipopolysaccharides obtained from cells which have been examined have contained all the saturated fatty acids of chain length from C_{10} to C_{22}, the only culture filtrate

lipopolysaccharide, that of Ikawa et al, appeared to contain only three higher fatty acids. Further, Ikawa et al reported the "probable absence" from their product of serine and glutamic acid, both of which have been found in lipopolysaccharides from cells. These differences may simply reflect a difference in the degree of analysis carried out on the cellular and culture filtrate lipopolysaccharides, but are noted because of the suggestion (see page 42) that the pyrogenic factor released into the culture medium during growth of Gram-negative cells differs qualitatively from the pyrogenic factor retained in the cell.

PYROGENS IN PARENTERAL SOLUTIONS.

The fever following the administration intravenously of sera, plasma, and other injection solutions has initiated considerable research into methods of detection and elimination of the substances responsible. Since Seibert's careful study[1] which showed conclusively that the fever-producing factors were products of bacterial contamination, much has been achieved but the problem has not yet been completely solved.

Following the work of Seibert, much more attention has been paid to the preparation of injection fluids under strict aseptic conditions and this has prevented the production of pyrogens during storage, but neither filtration[81,82] nor normal heat sterilisation[1,83] processes will remove

already-formed pyrogens.

Much can now be done to reduce pyrogen contamination during the preparation of synthetic injection fluids. The final containers and all glassware used in the preparation can be heat-treated (150°C for 2 hours)[84] to destroy adsorbed pyrogens. Pyrogen-free water for injection can be obtained by distillation in a suitably baffled still, collection in heat-treated containers and by sterilisation immediately following collection. However, a final preparation may still be pyrogenic because of contamination of the solid medicament employed, unless it is sufficiently stable to permit heat-destruction of the pyrogen. Also, natural products, such as sera and plasma, may be pyrogenic despite aseptic collection and processing, and here pyrogenicity can be a more serious problem because of the greater volumes administered and because the patient is likely to be in a condition where endotoxin shock would be more dangerous.[86-89]

Methods have therefore been sought to terminally destroy or remove pyrogens from parenteral solutions. Many destructive treatments have been tried, using for example heat[84,85], oxidising[90,91] or reducing agents[92], quinones[93], halogens[92,95], acid or alkali accompanied often by heat[9,96], or storage in presence of enzymes[97-99]. Most of these methods have been ineffective or too drastic to permit their use with many medicaments, but a few methods, such as the treatment of glucose solutions with quinones, have

had reported success in specific applications.[91,93,100,108]

The alternative approach has been to remove pyrogens by filtration or adsorption techniques. For this purpose a wide range of materials has been studied including glass[95,101], collodion[102], kaolin[96,103], celluloses[104], ion-exchange resins[105,-107], kieselguhr[92] and starch[92], but only asbestos and carbons appear to have general applicability. Asbestos has been used for the removal of pyrogens from solutions of dextrose[109,111,112], serum[106], inulin[110,113], plasma[106,114], protein hydrolysates[81], penicillin[85], and saline[82,111,112]. Because asbestos does not have as great an adsorbing capacity as many activated carbons[81,86], carbons are now favoured for commercial usage. They have been used for de-pyrogenising solutions of dextrose and saline[115,119], inulin[113], and various pharmaceutical preparations[95,101,116-118]. The high adsorbing capacity of the carbons, especially after acid treatment[108,115], permits the use of small quantities for the removal of pyrogens from solutions and thereby reduces the risk of concomitant removal of the medicament. However, even with the small quantities used, traces of colloidal materials will be taken up by the carbons and so limits their use to non-colloidal solutions.

The above methods of destruction or removal of pyrogens are all empirical in concept, designed to

eliminate a substance whose nature was unknown. The present knowledge of the physical and chemical character of pyrogens has not yet revealed a sufficiently specific property to allow design of a method for pyrogen elimination applicable to the wide range of parenteral solutions in which they might be found. It has thus been necessary to apply to parenteral solutions a terminal limit test for pyrogen contamination.

A number of chemical tests for the detection of pyrogens have been reported but they are non-specific.[91,92,96] On the basis of the present knowledge of purified pyrogenic lipopolysaccharides it is possible to speculate on the possibility of achieving a specific chemical test for the detection of pyrogens. A solution is termed pyrogenic if it will induce a rise of 0.6°C in the rectal temperature of a rabbit on injection of 10ml/kg. body weight. Purified lipopolysaccharides induce such a temperature rise at a dose of 0.002µg/kg. body weight. Hence a pyrogenic solution need contain only 0.0002µg/ml. This is below the level of detection of standard micro-analytical methods, even if one had a specific reaction for the total pyrogen. Although concentration of the pyrogen might be possible from simple salt solutions, it would be very difficult from complex colloidal solutions. So the hope of developing a chemical method of detection

appears slight.

It has therefore been necessary to employ a biological limit test for pyrogens in parenteral fluids. Many of the reactions of animals to endotoxin have been suggested as a basis for a pyrogen assay (for bibliography see ref.94); but the increase in rabbit rectal temperature following intravenous pyrogen injection has been the most widely studied, and was finally accepted as the basis for a limit test by the British Pharmacopoeia 1948, the XIIIth U.S. Pharmacopoeia and also appeared in the first issue of the International Pharmacopoeia.

Although extensive investigations of the test conditions have been carried out, the test at present is not completely satisfactory. The response of the rabbit is affected by environmental conditions, handling, method and timing of the temperature measurement, health of the rabbit, development of tolerance, but primarily, by the considerable difference in sensitivity of individual rabbits.

Attempts have been made to standardise the British official limit test by including weight and normal temperature limits on rabbits employed in the test, and by stating a minimal frequency of temperature recording. No direction has been given on restraint of the rabbits and temperature may be measured either by thermometers or thermocouples.

The least satisfactory feature of the test lies in the loose interpretation of the results. Borderline products cannot be classified unequivocally as pyrogenic or non-pyrogenic but must be tested again , and this repeated if necessary, until a final average of all the tests will give a definite answer. It has been felt for some time that the definition of an exact level of contamination at which a product could be classified definitely as pyrogenic would be more readily achieved if a standard reference pyrogen were available.

In 1950 the World Health Organisation Expert Committee on Biological Standardisation, during their recommendations to the Committee for the Unification of the Pharmacopoeias on pyrogen testing, noted that "a preparation of a pyrogenic substance was desirable as a standard for pyrogen tests". In the absence of definite information on the type of substance which should be used as a standard pyrogen, they authorised the collection of pyrogen standards in use in various laboratories for examination.[120]

Two years later they recommended that two preparations should be used for a collaborative study. The preparations were:-

Pyrogen " A "-- a partially purified extract of Proteus vulgaris prepared by fractional precipitation of a tryptic digest.

Pyrogen " B "... a purified lipopolysaccharide obtained from Serratia marcescens.

The intention was to determine the suitability of these preparations as standards, and to determine the sensitivity of rabbits used in pyrogen tests.

Freeze-dried samples of both preparations were sent to fourteen laboratories with the request to determine dose-response relationships over a wide range of dosage, and to compare them with local laboratory standards. The participants in the study were asked to compare the prep- by a four point assay, using a twin cross-over design, and to avoid the complication of any tolerance effects.

The results of the investigations were found to be so variable, and the slope of the log-dose determinations so small, that formal statistical analysis was impossible. The study did show however, that pyrogen B was more potent than pyrogen A, but the ratio varied between different laboratories from 1:1 to 20:1.

No formal report was published on the study and the only conclusions available were that the participants agreed on the need of a reference standard for pyrogen tests but could not agree which, if either, of the two preparations would be suitable.

Following the apparent failure of these two prep- arations, no further materials were offered for test

until 1959. This latest material is the O-somatic antigen or endotoxin complex of Shigella dysenteriae.[121] It has been well characterised chemically and physically and has been reported to have a Minimum Pyrogenic Dose of 0.003µg per kg.rabbit body weight[121], and to show a "reasonable log dose-response slope." [120]

This latest preparation appears to offer some advantages in that it is well characterised, so should be more readily replaceable, and is more water-soluble than lipopolysaccharides. However, it possesses one unwelcome feature in that it contains the protein fraction of the O-antigenic complex and so is fully antigenic in rabbits. It has been reported to induce the formation of antibody in rabbits with doses as low as 1µg. This could be a distinct disadvantage in studies of the development of pyrogen tolerance. A possible disadvantage of the preparation is its loss of pyrogenic activity on autoclaving[122] but if solutions of the preparation can be sterilised in some other way without loss in activity, it appears to be a useful reference preparation for many purposes. No results have yet been published for the preparation.

POLYSACCHARIDES AND PYROGENS OF PROTEUS SPECIES.

SPECIFIC POLYSACCHARIDES.

The presence of flagellar(H) and somatic(O) antigens

in Proteus species was demonstrated by Weil and Felix[123] in 1917, but although several classifications based on those antigens have since been attempted there is little chemical background to them.

Early reports on the presence of specific polysaccharides in Proteus organisms consisted mainly in demonstrating positive carbohydrate tests with crude cell extracts and the occurrence of precipitation when the extracts were mixed with anti-Proteus sera.[51]

The discovery that there occurred in the serum of typhus patients agglutinins for certain strains of Proteus, stimulated investigation of the strains involved, the X strains.[124]

Meisel and Mikulaszek extracted Proteus OX-19 with warm acetic acid and precipitated polysaccharides from the extracts with ethanol. Alternate acid and alkali extraction of the precipitates left residues which contained 58-80% reducing sugars and which precipitated with antisera to the homologous cells.[125]

White found that Seitz filtrates of boiled suspensions of Proteus OX-19 precipitated with both anti-Proteus sera and serum obtained from typhus patients. The filtrates on gentle treatment with alkali lost the reactivity with Proteus sera but retained the ability to precipitate with typhus serum. Also, extraction of cells with alkali produced extracts having only the typhus reactivity. From these results he postulated the existence on the surface of

Proteus X-19 of two receptors, the major or alkali-labile receptor responsible for the O-agglutination in anti-Proteus sera and a second alkali-stable receptor responsible for the Weil-Felix reaction, but playing only a minor role, if any, in the reactivity with homologous antisera.[126]

These findings were corroborated by Castaneda, who later separated the receptors by ethanol fractionation of Proteus X-19 extracts. The 'X' factor was precipitated at 50-70% ethanol concentration and reacted with both homologous antisera and typhus antisera, the latter reaction being unaffected by alkali treatment. Higher concentrations of ethanol (87-90%) precipitated the 'P' factor which reacted only with anti-Proteus sera and was destroyed by alkali. Both factors gave positive Molisch and negative Biuret tests, and were claimed to be polysaccharide because of their low nitrogen values ('X'; 1.4%, 'P' less than 1%).[127]

The only detailed investigation of Proteus O-somatic antigen was carried out by Bondich and Chargaff, using the OX-19 strain.[128] Extraction of the dried cells with trichloracetic acid (or less effectively with trypsin) followed by centrifugation of the extract at 30,000g, produced a sediment C-2, and from the supernatant fluid a fraction C-11 was precipitated with 67% ethanol. Both fractions were shown to be electrophoretically homogeneous and had similar mobilities in borate buffer. They were complexes

of protein, polysaccharide and lipid and differed mainly in that C-2 contained almost four times as much lipid as C-11. Analytical figures for the products are given in table VI.

Table VI. Percentage composition of Fractions C-2 and C-11 of *Proteus* OX-19.

Fraction	N	P	Acetyl	Reducing Value	Lipid	Hexos-amine
C-2	5.1	2.5	6.0	37.5	2.8	23.8
C-11	5.1	2.5	1.9	25.2	11.2	11.2

N-Acetylglucosamine, mannose and galactose derivatives were isolated from hydrolysates of the fractions. Fermentation experiments suggested that glucose was also a constituent of both fractions.

Antisera were prepared against the fractions. *Proteus* OX-19 was agglutinated by both types of antisera, but whereas the anti-C-2 serum also agglutinated suspensions of *Rickettsia prowazeki* (the agent of louse-born typhus) to a titre of 1:1280, the anti-C-11 serum gave doubtful agglutination even at 1:40. Partial removal of the lipid from C-2, even by mild treatment, destroyed the typhus specificity but had no effect on the reaction with anti-*Proteus* X-19 sera. Neither antiserum agglutinated a flagellate strain of *Proteus vulgaris*. The fractions were not tested for endotoxic or

pyrogenic reactions in animals.[128]

Mikulaszek and Dzulynska[129] reported on the monosaccharide constituents of polysaccharides obtained from smooth and rough variants of a Proteus X strain. No details were given of the preparation or of any serological or biological properties. The sugars were detected only by chromatography and they are listed in Table VII. It must be stated that these results were part of a general survey of the monosaccharide constituents of a number of micro-organisms. Their general finding was that xylose was a typical constituent of all Eubacterial polysaccharides but this sugar has seldom been reported in the large number of these polysaccharides examined by other workers.

Davies[30] quotes a personal communication from Luderitz in citing monosaccharide constituents of polysaccharides of four species of Proteus (see Table VII) but no details are given of preparation or biological actions.

Table VII. Monosaccharide constituents of polysaccharides of Proteus species.

Bacterial Source	Glu	Gal	Man	Gmn	Xyl	Hep	Reference
P.vulgaris	+	-	-			+	30
P.morganii	+	+	-			+	30
P.rettgeri	+	+	+			+	30
P.mirabilis	+	-	-			+	30
P.'X' Smooth	(+)	(+)	+	+	+		129
P.'X' Rough	(+)	(+)	-	-	+		129

Glu = glucose, Gal = galactose, Man = mannose,
Gmn = glucosamine, Xyl = xylose, Hep = aldoheptose.
(+) Recorded as either glucose or galactose.

EXOGENS

Robinson and Flusser[10] extracted dried Proteus vulgaris cells by heating them in water on a steam bath for two days, precipitated an active fraction from the dialysed soluble extract and deproteinised it with 95% phenol. The preparation had the following analysis:

C 35.83 H 6.06 N nil P 0.29 Ash 8.33

but the nitrogen value is based on a sodium fusion test. The preparation gave 'positive sugar tests' only after hydrolysis,

and was believed to be a polysaccharide. It was pyrogenic but calculation of a Minimum Pyrogenic Dose from their few figures gives a value between 0.5 and 1.0 µg/kg. body weight. This, by comparison with recent products obtained from other gram-negative bacteria,[31] suggests their product was grossly impure or activity was destroyed during isolation.

Dialysed tryptic digests of Proteus vulgaris were prepared by Ginger, Nesset, Riegel and Fitzsimmons[130] which were pyrogenic in rabbits. These extracts contained 30-40% nucleic acid, had a nitrogen value of 8% and gave a reducing sugar value of 9% after hydrolysis.

Cell-free culture filtrates of Proteus vulgaris show pyrogenic activity in rabbits[4,5,6] and also induce changes in the polymorphonuclear leukocytes[6] and small lymphocyte[5] populations in rabbits, reactions characteristic of endotoxins.

During investigations of the different types of fever curve that can be produced on injection of pyrogen, Wylie and Todd[131] noted that the type of curve produced on injection of Proteus vulgaris was dependant on the method of treatment of the culture before injection.

Injection of the complete autoclaved culture, or of the cell-free culture filtrate obtained by autoclaving the culture and subsequent removal of the cells, produced a double-peak fever response, the peaks occurring approximately 1½ and 3½ hours after injection. If the cells were first

removed and the cell-free filtrate then autoclaved, on injection this produced only a single-peak fever occurring 1½ hours after injection. Finally, injection of a suspension of well-washed organisms produced a single-peak fever, which corresponded in time of onset to the second peak of the double-peak response.

Wylie and Todd concluded that it was unlikely that three separate substances were involved and discounted the theory that the different curves were due to a simple dosage effect, since the removal of the cells from a complete autoclaved culture still resulted in the production of a double-peak effect. They found that the second peak was more readily diluted out than the first peak; and that autoclaving of the culture was not necessary to produce a double-peak, since injection of whole cultures which had been exposed to bacteriaides without heating also produced double-peak effects.

Their final conclusion was that there probably existed two types of pyrogen, one of which, in the viable culture, was dissolved in the medium and stimulates the single-peak fever of the immediate reaction type; the other is contained mainly in the bacterial cell and stimulates the single-peak fever of the delayed reaction type, a mixture of both causing the double-peak fever. Autoclaving or other killing of the cells was thought to release some of this second factor into the medium, and this explained the occurrence

of a double-peak response in the absence of cells when a filtrate of an autoclaved culture was used.[131]

EXPERIMENTAL SECTION

PRODUCTION OF CRUDE PYROGENS

GROWTH AND RECOVERY OF CELLS

Proteus vulgaris NCST53

This strain of Proteus vulgaris was selected because it had been used by Wylie and Todd in their investigations of the double-peak response in rabbits.[131] It was obtained from the Department of Microbiology, The Royal College of Science and Technology, where it is maintained as a stock culture (R.C.S.T. Collection Number 53). The morphological growth and biochemical characters of the strain are described in Appendix 1. From these tests the strain is a typical Proteus vulgaris Hauser as described by Bergey,[132] but differs from the Proteus hauseri type 1 of Kauffmann[60] by its inability to metabolise xylose. The serological character of the strain was unknown. During the period of this research the organism was maintained by weekly sub-culture on nutrient agar slopes.

Selection of Growth Media.

As previously stated this research formed part of a joint investigation to compare the pyrogenic factor extractable from the bacterial cell with that released into the culture fluid during growth. For a strict comparison it would have been ideal to use the same culture for both

isolation processes but this presented practical difficulties. Growth of the organism in nutrient broth produced higher yields of cells but greatly increased the difficulties in isolating the soluble factor from the culture fluid. With cultures in synthetic inorganic media the isolation of the culture fluid factor was eased but the yield of cells was small. Since the volumes of culture fluid that could be handled were limited, the highest yield of cells per unit volume was essential. It was finally decided to isolate the soluble factor from synthetic medium cultures and initially to produce cells from both types of medium. If the pyrogenic materials extracted from the cells from each medium were shown to be similar in activity and chemical characters then all subsequent growth of cells could be carried out in nutrient broth and comparison of the pyrogen extracted from these cells with the soluble pyrogen in synthetic culture medium would be valid.

Growth of cells in Synthetic Medium

The following medium was found to support growth of our strain of Proteus vulgaris:-

Ammonium Phosphate	$(NH_4)_2HPO_4$	4.0g.
Sodium Chloride	NaCl	1.0g.
Potassium Dihydrogen Phosphate	KH_2PO_4	1.0g.
Magnesium Sulphate	$MgSO_4.7H_2O$	0.7g.
Ferrous Sulphate	$FeSO_4.7H_2O$	trace.

D-Glucose	$C_6H_{12}O_6$	10.0g.
Nicotinic Acid	C_5H_4NCOOH	2×10^{-5}M
Water, apyrogenic distilled		to 1 litre

If the medium was autoclaved as a whole caramelisation of the glucose, and formation of a heavy precipitate of magnesium phosphate occurred and the resulting medium gave poor growth of cells. This was prevented by autoclaving the glucose and magnesium sulphate together at not more than 115°C. The remaining constituents were autoclaved together in solution at 120°C and to this the solution of glucose and magnesium sulphate were added aseptically.

The medium was inoculated by stepwise inoculation over 5-6 days and growth continued at 25°C for a further four days, with vigorous aeration.

The cells were recovered by passing the culture through a Sharples Super Centrifuge (13,000 g), washed twice with distilled water, and poured into ten volumes of acetone at -12°C. The cells were removed from the acetone after two days and finally dried in a vacuum desiccator over concentrated sulphuric acid. The dried cells were stored in sealed containers until required. Yield of dry cells obtained was 0.2 - 0.4g. per litre of culture fluid.

Growth of cells in Nutrient Broth

The constitution of the medium used was as follows:-

Peptone (Oxoid)	0.1g.

Lab Lemco (Oxoid)	0.03g.
Sodium Chloride NaCl	0.01g.
Distilled water	to 1 litre.

After autoclaving the medium, pH was adjusted to 7.0. Stepwise inoculation was followed by growth at the final stage for 18 hours at 25°C with vigorous aeration.

The cells were washed four times with distilled water and dried from acetone as previously described. Growth under these conditions produced 0.8-1.1g. dried cells per litre.

PHENOL EXTRACTION OF PYROGENS.

The dried bacterial cells obtained from both media were extracted separately with aqueous phenol (45% w/v) as described by Westphal, Luderitz and Bister.[26]

The cells were suspended in water by grinding in a mortar with water at 65°C followed by high speed mixing in an M.S.E. homogenizer. This produced a very fine suspension enabling intimate contact to be achieved with the extractive. The suspension was finally diluted with water so that one gram of dried cells was contained in 35ml. of suspension.

To the suspension immersed in a controlled temperature bath at 65°C was added an equal volume of aqueous phenol (90%w/v, 65°C). The unstable emulsion formed was maintained at 65°C for 30 minutes with continuous stirring to prevent

separation of the phases. The emulsion was then quickly transferred to beakers immersed in a freezing mixture at -12°C and allowed to cool, when the emulsion cracked. Centrifuging at 1,000g. for 1 hour separated an upper aqueous layer from a lower phenolic layer, and the residual cell debris was found at the interface.

The aqueous layer was siphoned off and to the phenolic layer plus debris, four-fifths of its volume of water was added and the mixture heated to 65°C with stirring. Immediately on reaching 65°C the emulsion was cooled as before, and the aqueous layer collected by centrifuging and siphoning.

The combined aqueous extracts were dialysed against running tap-water for four days until completely free from phenol. The dialysate was concentrated under reduced pressure till it contained about 1% solids, dialysed against distilled water till free of salts and phenol, filtered and freeze dried. Such freeze dried products are hereafter called "crude pyrogen".

From the phenolic layer a strongly Biuret positive material was precipitated with methanol and acetic acid. It was found to have little pyrogenic action and was not investigated further.

COMPARISON OF THE CRUDE PYROGENS

General Properties

In Table I are shown yields and analytical values of 3 batches of crude pyrogen isolated from cells grown in each type of medium.

Nitrogen was estimated by the Kjeldahl method using a Markham apparatus[133] and the indicator of Ma and Zuazaga,[134] and phosphorous by the method of Martland and Robison.[135] Nucleic acid was estimated on solutions in sodium hydroxide (0.01N) by measurement of the absorption at 260mu and reference to a Beer line constructed from similar measurements on standard solutions of a yeast nucleic acid reference preparation.

For subsequent comparison of the crude pyrogens obtained from cells grown in different media, samples of each pyrogen were chosen with similar nucleic acid content. These were, for nutrient broth grown cells, batch II, and for synthetic-medium-grown cells, batch I (see Table I). For brevity these fractions will be termed CPN (crude pyrogen from nutrient broth medium cells) and CPS (crude pyrogen from synthetic medium cells).

CPN and CPS were yellow-white powders which were difficultly soluble in cold distilled water to about 1%w/v, but in hot water 2%w/v stable opalescent, birefringent solutions were obtained.

In alkaline solution (0.01N NaOH) the opalescence and

Table 1. Yields and analysis of crude pyrogens.

Cell Source	Batch number	Weight of cells extracted(g.)	Weight of crude pyrogens(g.)	%age content of crude pyrogens		
				N	P	N/A
Nutrient broth medium	I	9.5	0.332	8.95	5.0	45
	II	11.2	0.583	8.33	4.9	36
	III	9.8	0.297	9.47	5.2	55
Synthetic salt medium	I	7.6	0.213	7.03	4.3	32
	II	8.4	0.304	8.71	4.8	43
	III	11.2	0.603	9.1	4.9	52

N/A = nucleic acid.

the birefringence were reduced, while in acid solutions (0.01N HCl) both products yielded a fine precipitate which flocculated slowly, leaving faintly opalescent supernatant solutions. On boiling the acid solutions both precipitates were increased and were partially soluble in ether and chloroform.

Both crude pyrogens gave positive Molisch and negative Biuret and Millon tests.

Pyrogenic Activity

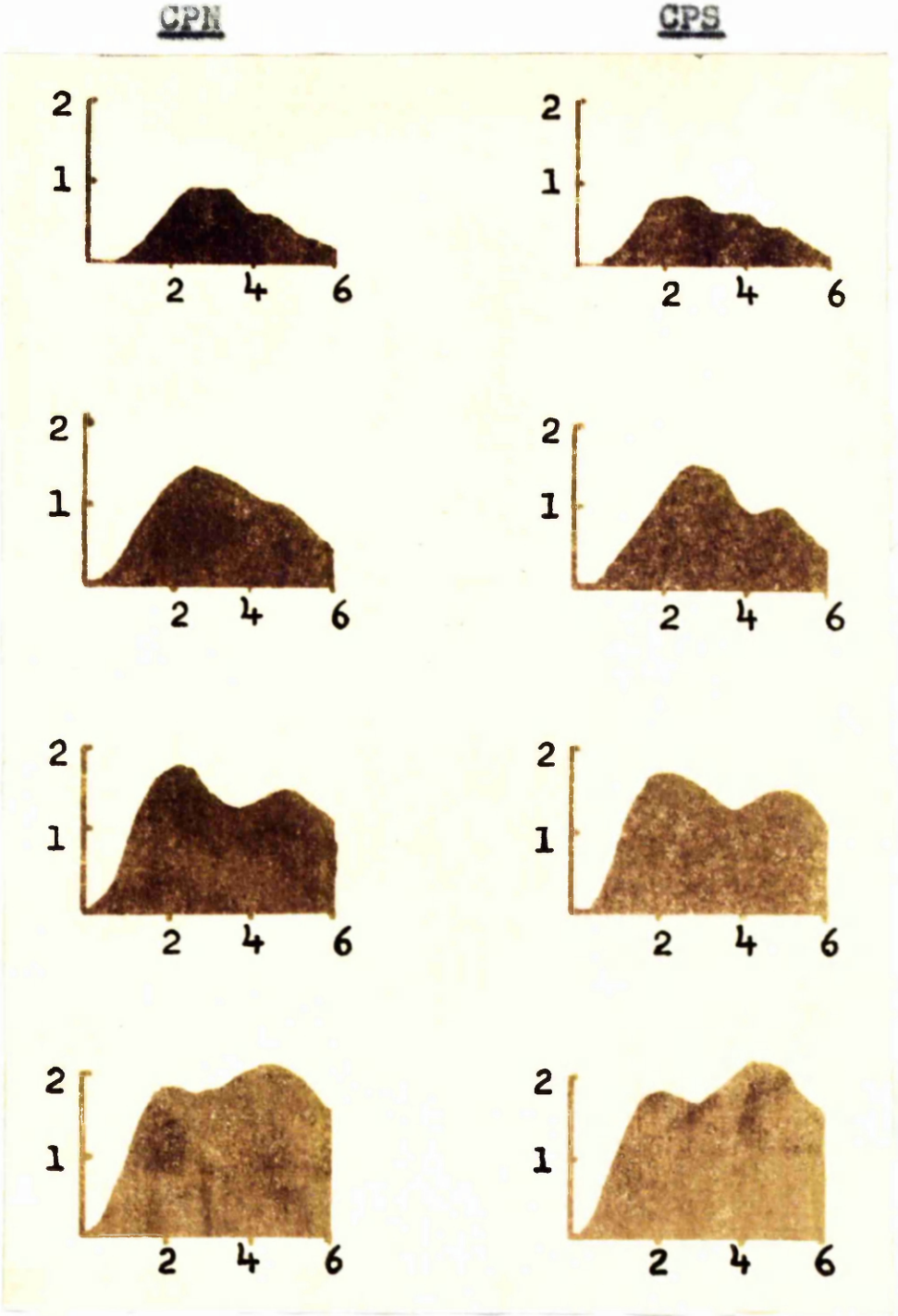
Intravenous injection of crude pyrogens into balanced groups of ten rabbits in dosage of 0.01, 0.05, 0.1 and 1.0 µg/kg. body weight produced the rises in rectal temperature shown in Fig. 1. From these results it appeared that the pyrogens extracted from cells grown in different media had similar activity.

From log dose-response curves the Minimum Pyrogenic Dose, (that is, the dose required to produce an average rise of 0.6°C in the rectal temperatures of a group of rabbits) was calculated as 0.005 µg/kg. body weight for both pyrogens, and the dose-response lines for the materials were parallel.

The details of the procedure and apparatus employed in these and all subsequent pyrogen tests throughout the thesis are described in Appendix II.

Figure 1.

Pyrogenic response of rabbits to injection of crude pyrogens obtained from cells grown in nutrient broth culture (CPN) and in synthetic medium culture (CPS).



Ordinates = Rise in rectal temperature in °C.
Abscissae = Hours after injection

Dose is the weight injected per kilo rabbit body weight.

Monosaccharide Constituents.

(a) Dische Reactions. Throughout the Dische tests described below, CPN and CPS were tested as aqueous solutions at 1 mg/ml. Authentic monosaccharides employed as positive controls were used at concentrations indicated in figures. In all experiments test and control samples were run in duplicate and results quoted are the average of these, but for ease of description the tests are described as single estimations.

(1) CPN and CPS were examined initially by the basic form of the reaction PCyR.[136] To each of two tubes containing 1 ml. of an aqueous solution of the substance under test, 4 ml. of concentrated sulphuric acid were added with mixing. During the addition of the acid the tubes were cooled by an ice-water mixture. To one tube of each pair was added 0.1 ml. of a 3% aqueous solution of cysteine hydrochloride, and to the other 0.1 ml. of water, and the contents mixed thoroughly. The second tube is included as a control since many substances of biological origin give coloured compounds with sulphuric acid alone.

After standing for 10-30 minutes, and again after 24 hours, the absorption spectra of both solutions were determined in a Unicam SP 500 Spectrophotometer between the wavelengths 350 mμ to 450 mμ.

The absorption spectra of CPN and CPS are shown in Fig.2(A and B). The spectra obtained with standard solutions of ribose and glucose similarly treated are also shown (curves C and D). Curve I represents in each case the spectrum obtained 10-30 mins. after addition of cysteine, curve II that after 24 hours, and curve III the readings of the control samples without cysteine. The latter did not change appreciably in 24 hours and only the earlier spectrum is included in the figure.

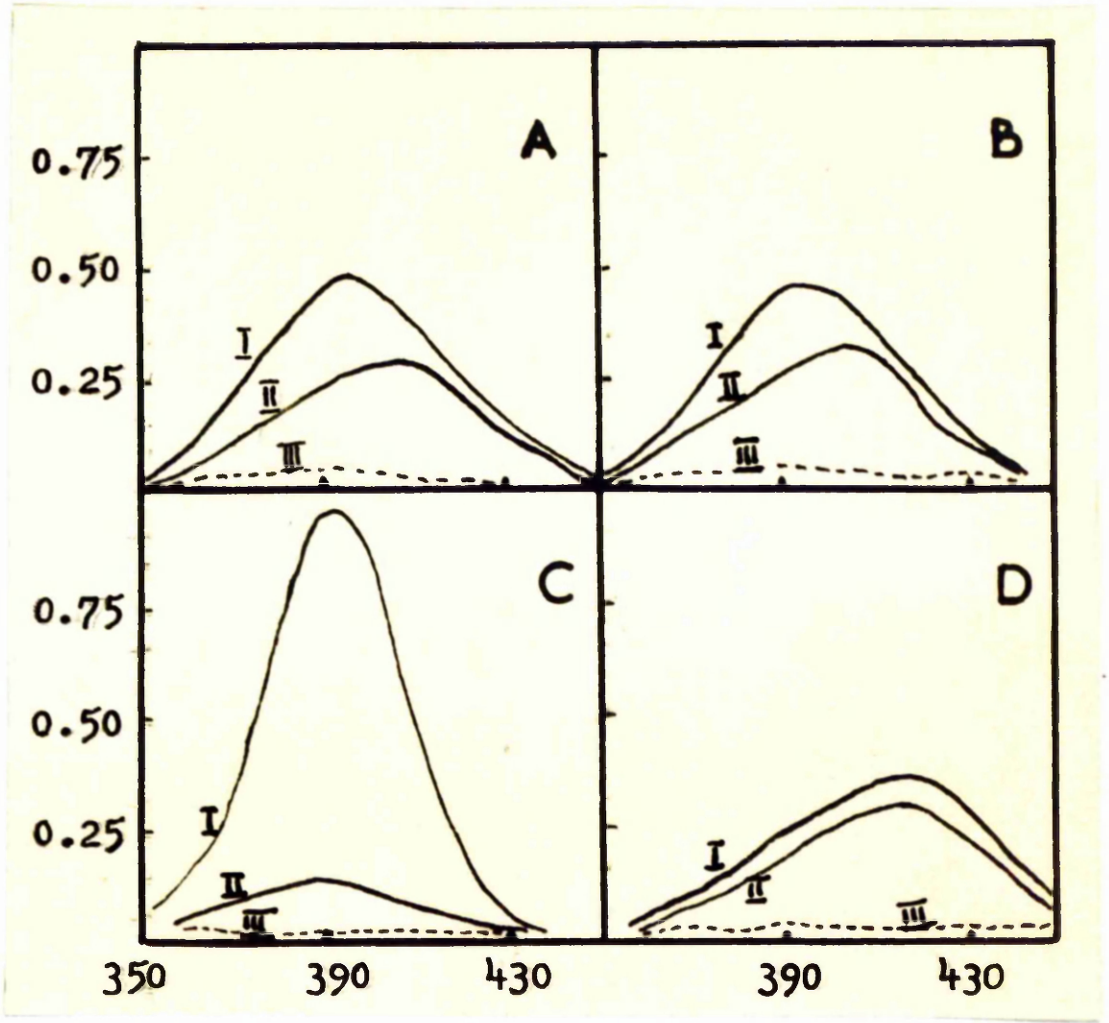
After 10-30 minutes the spectra of CPN and CPS show only one peak, with a broad absorption maximum at 390 mμ. This is due to pentose. It was not possible to determine the nature of the pentose present from this reaction since all members of the class absorb at the same wavelength.

The intensity of the pentose absorption in curves IA and IB obscured any maxima due to other monosaccharides, but the presence of hexose was established by measurement of the ratio $E_{440m\mu}:E_{360m\mu}$. The values for CPN and CPS were 1.02 and 1.13 respectively. In this test hexoses have a $E_{440m\mu}:E_{360m\mu}$ ratio greater than one, whereas pentose or methylpentose which have absorption maxima in this region, have values of zero or nearly zero.

The presence of hexose in CPN and CPS was confirmed by the absorption spectra after 24 hours (curves II). During this time the pentose reaction product rapidly

Figure 2.

The absorption spectra in the basic Dische test of CPN (A), CPS (B), glucose (D), and ribose (C).



Curves I = Sample + cysteine read after 10-30mins.

Curves II= Sample + cysteine read after 24 hours.

Curves III= Sample without cysteine read after 10- 30 minutes.

decreases to a minimum value while that due to hexose remains stable and its absorption maximum at 414 mμ becomes visible.

(ii) To test for methylpentose,[136] samples of CPN and CPS were treated as before, and 20 minutes after the addition of cysteine, the extinctions at 380 mμ and 400 mμ were read. To each tube 0.6 ml. of water was added and the extinctions read at the above wavelengths after 10 minutes and again after two hours.

The difference in optical density at 380 mμ and 400 mμ read 10 minutes after the addition of water is zero for pentoses, and positive for hexoses and methylpentose. After two hours the value for hexose decreases slightly but that for methylpentose shows a strong increase.

The values obtained for these readings with CPN and CPS are shown in Table 2, from which it can be seen that $E_{400m\mu}-E_{380m\mu}$ was positive 10 minutes after the addition of water but thereafter decreased slightly. This indicates that methylpentose is absent from CPN and CPS and also is further corroboration that both contain hexose.

Table 2. Effect of addition of water to cysteine reaction products of CPN and CPS.

Reading Number	Increment $E_{380m\mu}-E_{400m\mu}$	
	CPN	CPS
1	0.004	0.012
2	0.091	0.087
3	0.075	0.076

Reading Number 1 = Value before addition of water.
Reading Number 2 = Value 10 minutes after addition of water.
Reading Number 3 = Value 30 minutes after addition of water.

(iii) To determine the identity of the hexoses in CPN and CPS a further modification (CyR3)[62,136,138] of the Dische reaction was employed. To each of two tubes, held in an ice-water mixture, containing 4.5 ml. of a mixture of water and concentrated sulphuric acid in ratio 1:6 by volume, was added 1 ml. of an aqueous solution of the substances under test, and the contents of each tube thoroughly mixed. The tubes were transferred to a water bath at 25°C and when the solutions had reached this temperature, quickly transferred to a briskly boiling water bath for exactly three minutes. The tubes were then returned to the 25°C bath to cool. To one tube of each pair was added

0.1 ml. of 3% aqueous cysteine hydrochloride solution and to the other 0.1 ml. of water. After standing for 2 hours, and again after 48 hours, the absorption spectra were read throughout the range 350 mμ to 650 mμ.

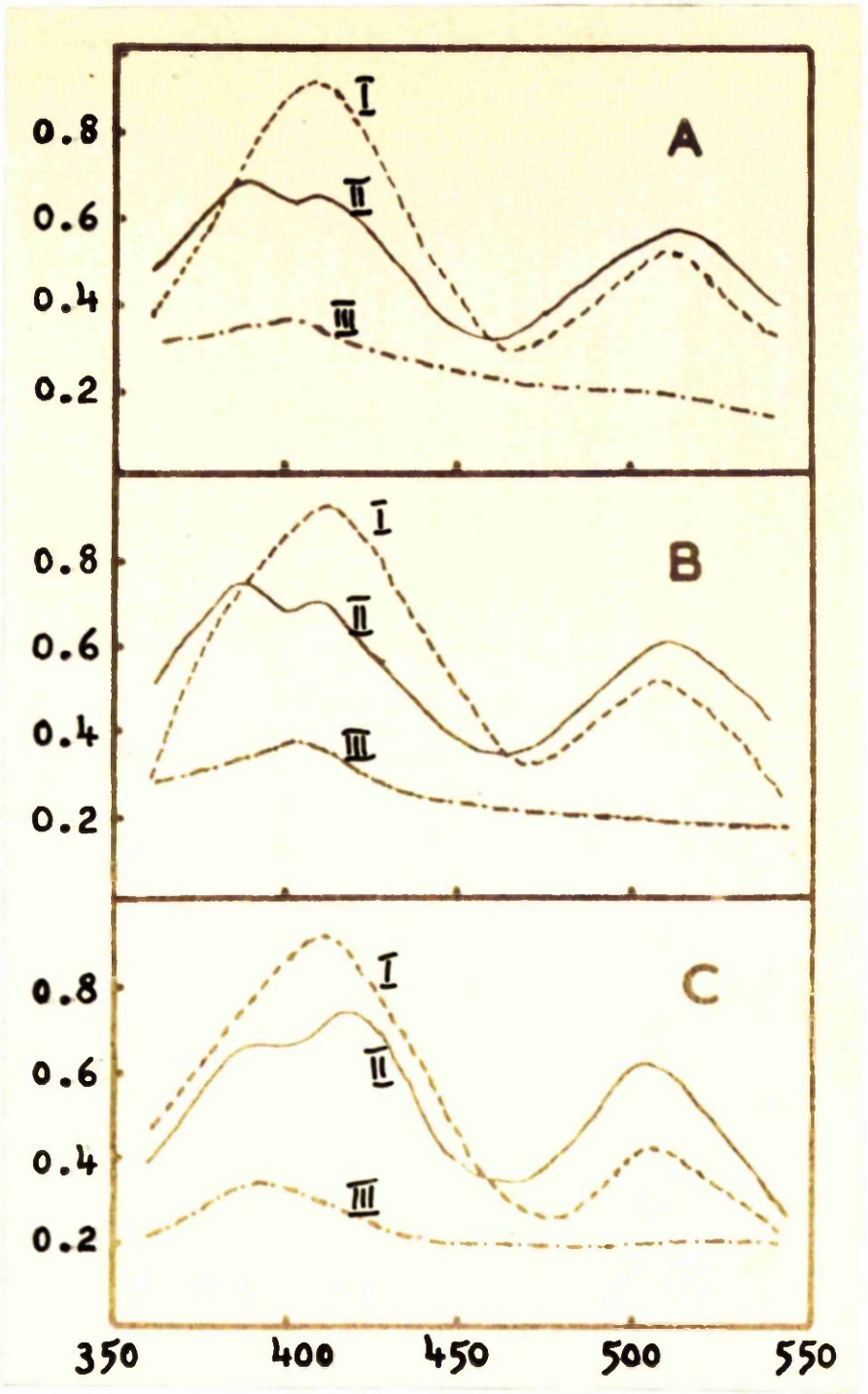
The spectra obtained for CPN and CPS are shown in Fig.3 (A and B). The spectrum of a standard mixture of D-galactose, D-glucose and D-glycero-L-glucoheptose treated similarly is also included in fig.3 (C). Curves I in each instance represent the spectral readings obtained 2 hours after cysteine addition and curves II those obtained after 48 hours. The samples without cysteine have their spectra shown in curves III. (This is the reading obtained at 48 hours which was almost exactly the same as the 2-hour reading.)

The spectra obtained after 2 hours show two absorption maxima, at 414 mμ and at 505 mμ, due to hexose and aldoheptose respectively. On standing for 48 hours the hexose peak becomes resolved into two, and the spectra now shows three maxima, at 388, 410 and 510 mμ. These are characteristic of galactose, glucose[62,136] and aldoheptose[136,138] respectively, and all three are present in both CPN and CPS.

The absorption spectra of the samples without cysteine (curves III) show a maximum at 400 mμ which is characteristic of aldoheptoses in sulphuric acid,[136,138] and offers

Figure 3.

Absorption spectra in the Dische CyR3 test of CPN (A), CPS (B), and standard sugar mixture (C).



Standard sugar mixture (C) contained glucose 50μg, galactose 50μg, and glucoheptose 100μg/ml.

further proof of the presence of this class of monosaccharide in CPH and CPS. The identity of the aldoheptose cannot be determined from this reaction.

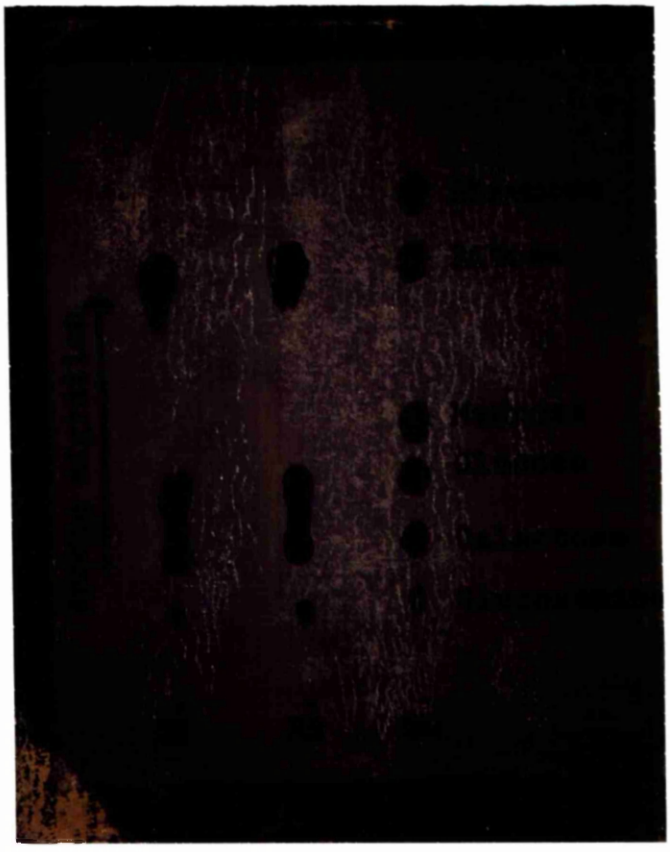
(b) Paper Chromatography. Samples of CPH and CPS (30 mg) were hydrolysed with 0.5N H_2SO_4 for 16 hours at 100°C, filtered, and neutralised with barum hydroxide. The supernatant fluids after neutralisation were concentrated by boiling under reduced pressure, and finally dried in vacuo over phosphorous pentoxide. The residues were redissolved in distilled water (100µl) and 10µl volumes applied to Whatman No.1 paper for chromatography. Mixtures of authentic sugars were run on the same chromatogram.

The chromatogram was developed by the descending method with butanol, pyridine, water (3:2:1.5). After 16 hours development at room temperature the chromatogram was dried by hanging in air, sprayed with 1% aniline phthalate[139] in moist n-butanol and heated at 100°C for ten minutes to reveal the sugar spots.

The stained chromatograms of both hydrolysates were similar, each showing three zones(see Fig.4). A slow-moving faint brown spot and a fast trailing pink spot co corresponded in position and colour to authentic samples of D-glucosamine and D-ribose respectively run on the same chromatogram. Between these a heavy brown zone covered an area which corresponded to control samples of

Figure 4

Section of chromatogram of hydrolysates of crude pyrogens CPN and CPS.



HN = Hydrolysate of crude pyrogen CPN

HS = Hydrolysate of crude pyrogen CPS

SM = mixture of standard sugars

D-glucose and D-galactose, but unlike the control sugars had not separated into two distinct spots yet from the intensity of the stained zone did not appear to be overloaded.

A second chromatogram was run as before with two samples of 30µl. of each hydrolysate in adjacent positions on the chromatogram and after development and drying was cut lengthwise into strips (each containing one hydrolysate spot) before staining. One strip of each pair was stained to localise the glucose-galactose zone and the corresponding area cut from the remaining strip and eluted with distilled water. Those eluates were tested by the Dische CyR3 reaction[62,136,138] and as well as glucose and galactose, aldoheptose was detected in both the CPN and CPS hydrolysates.

Serological Properties.

Antisera to Proteus vulgaris were prepared by injecting rabbits intravenously with a total of 7 mg. of acetone-dried cells. Injections were made twice weekly for three weeks as follows - 1st week 2 x 0.5mg, 2nd week 2 x 1mg. and 3rd week 2 x 2mg. doses. The rabbits were left for one week after the last injection before being bled for serum.

CPN, CPS and acetone dried cells were tested against such antisera by the Ouchterlony gel diffusion precipitin test.[140] The gel consisted of 1% w/v of washed agar in

phosphate-buffered saline (0.1M, pH 7.2) containing merthiolate (1:10,000).

Fig.5 shows a typical precipitation pattern obtained after 6 days incubation at room temperature. The acetone dried cells (20mg. per reservoir) reacted to give 7-9 lines with antiserum. CPS (3mg.) gave 4 lines, but CPN (3mg.) gave only 1 strong line and a very faint line not seen in photograph. The strong line given by CPN was completely confluent with a line gevon by the other three reactants and indicates a serologically identical constituent common to both crude pyrogens and to cells grown in both types of media.

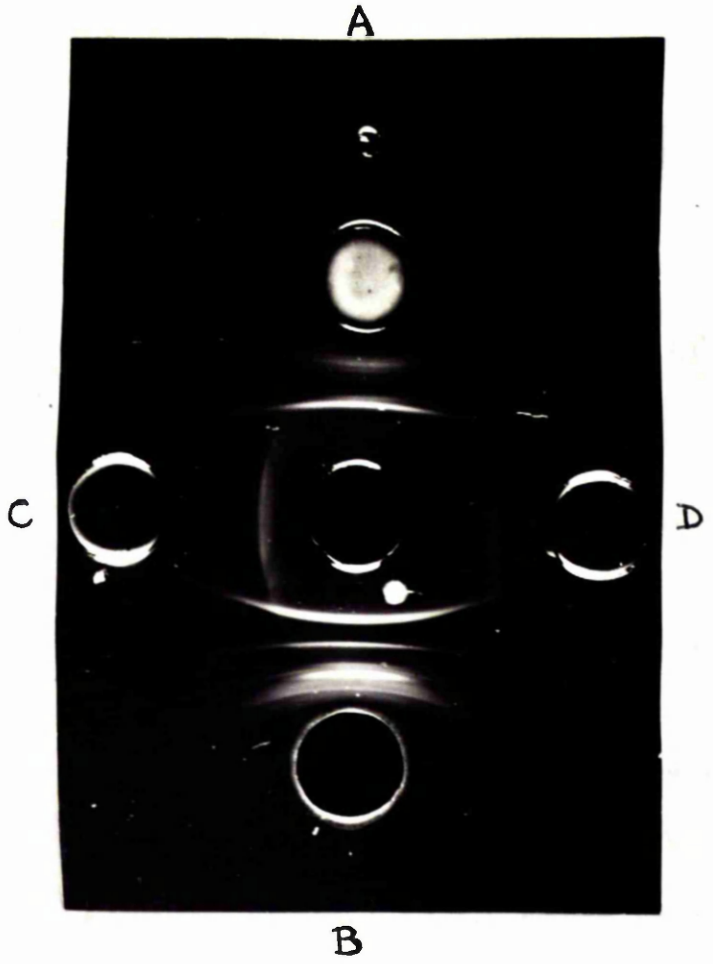
When the Ouchterlony test was repeated, but with the antigenic reactants heated at 100°C for 30 minutes before being put into the agar gel, only the precipitation line which had previously been shown to be common to all four antigen preparations appeared as strong as before, the others being absent or greatly diminished in intensity. This indicated that the common constituent of CPN and CPS possessed a heat stable antigenic specificity, a characteristic property of Gram-negative bacterial lipopolysaccharide.

Discussion.

The comparison of CPN and CPS had shown

Figure 5

Gel-diffusion-precipitin reaction of P.vulgaris cells and crude pyrogens CPN and CPS with antiserum to the cells.



Reservoirs A and B contain acetone-dried P.vulgaris cells obtained from nutrient broth and synthetic medium culture respectively.

ReservoirsC and D contain crude pyrogens CPN and CPS respectively. Central reservoir X contains rabbit antiserum to whole P.vulgaris.

'LPS line' indicates the heat stable line of precipitation common to all four products.

(i) both products were grossly similar in containing polysaccharide, lipid released only on heating in acid, and nucleic acids.

(ii) they possessed comparable pyrogenicity, similar monosaccharide constituents, and a serologically identical heat-stable antigen or hapten.

(iii) CPN contained fewer antigenic contaminants than CPS.

From (i) and (ii) it was concluded that both crude pyrogen preparations contained the same pyrogenic lipo-polysaccharide, and from (iii) that CPN offered a better source for its purification. Coupled to the latter point, cells could be obtained in greater yield from nutrient broth cultures. Thus it was decided to obtain all further crude pyrogen from nutrient broth grown cells.

II. METHODS OF PURIFICATION OF THE LIPOPOLYSACCHARIDE.

A number of chemical and physical methods have been employed to remove nucleic acids from bacterial lipopolysaccharide preparations which suggests that no single method is universally applicable, but the most generally successful method appears to be differential high speed centrifugation. Since an ultracentrifuge was not available till the latter part of this research programme other methods were tried. Of these, only ammonium sulphate fractionation was consistently successful and the details are given below. Other methods which were attempted will be described briefly.

Zone Electrophoresis.

Initial experiments showed that a good separation of nucleic acid and polysaccharide could be obtained by electrophoresis of 1% w/v solutions of crude pyrogen on glassfibre paper in borate buffer (0.1M, pH 9.3) at a potential of 30 volts/cm. applied for two hours. Attempts to convert this to a preparative procedure using the Shandon Continuous Electrophoresis apparatus were partially successful. Small amounts of polysaccharide with reduced nucleic acid content were obtained but the method was

cumbersome and unreliable since frequent burning of the paper curtain occurred.

Buffer Extraction.

In an attempt to make use of the known insolubility of nucleic acids at low pH values, samples of crude pyrogen were extracted with successive quantities of acetate buffers at pH values below 4. The soluble extracts were dialysed, freeze dried and tested for pyrogenicity. The best product obtained by this method had a nucleic acid content of 16% (starting material contained 60%) and had a Minimum Pyrogenic Dose of 0.003 µg/kg. but the freeze dried product was intensely hygroscopic and rapidly discoloured in air. Repeated dialysis did not effect this behaviour so the deterioration was regarded as not being due to the presence of acetate but was a result of some damage to the polysaccharide. This unstable product would not have been suitable as a permanent standard pyrogen so the method was abandoned.

Reversal of the procedure by extracting with borate buffers of pH greater than 8.5 gave no fractionation.

Protamine Sulphate.

Attempts to precipitate nucleic acid from solutions

of crude pyrogen in acetate buffer pH 5.0 by addition of solution of protemine sulphate at same pH were partially successful but the method was abandoned since complete removal of the protamine sulphate by subsequent pH change was not attained.

Ethanol Precipitation.

Addition of ethanol to aqueous solutions of crude pyrogen (1% w/v) precipitated nucleic acid at 50% v/v ethanol concentration. Very little material remained in the supernatant fluid but from this a small quantity of polysaccharide with only 8% nucleic acid and M.P.D. of 0.003µg/kg. was obtained by precipitation with six volumes of ethanol; on freeze drying it was intensely hygroscopic, similar to the acetate buffer product, and was discarded.

Ammonium Sulphate Fractionation.

Initial fractionation was carried out as follows:- to a 1% w/v aqueous solution of crude pyrogen was added slowly, with mixing, a saturated aqueous solution of ammonium sulphate, until the formation of a precipitate was indicated by an increase in opacity of the solution. The precipitate was allowed to flocculate by standing 3 hours at 20°C and removed by centrifuging. The addition

of ammonium sulphate to the supernatant fluid was continued until further precipitates were produced and they were recovered by centrifuging. In this way four fractions were obtained, precipitated at ammonium sulphate concentrations of 35, 40, 60 and 85% saturation. The precipitates were redissolved in water and dialysed till free of salts. Details of the weights and nucleic acid content of the fractions are given in Table III below.

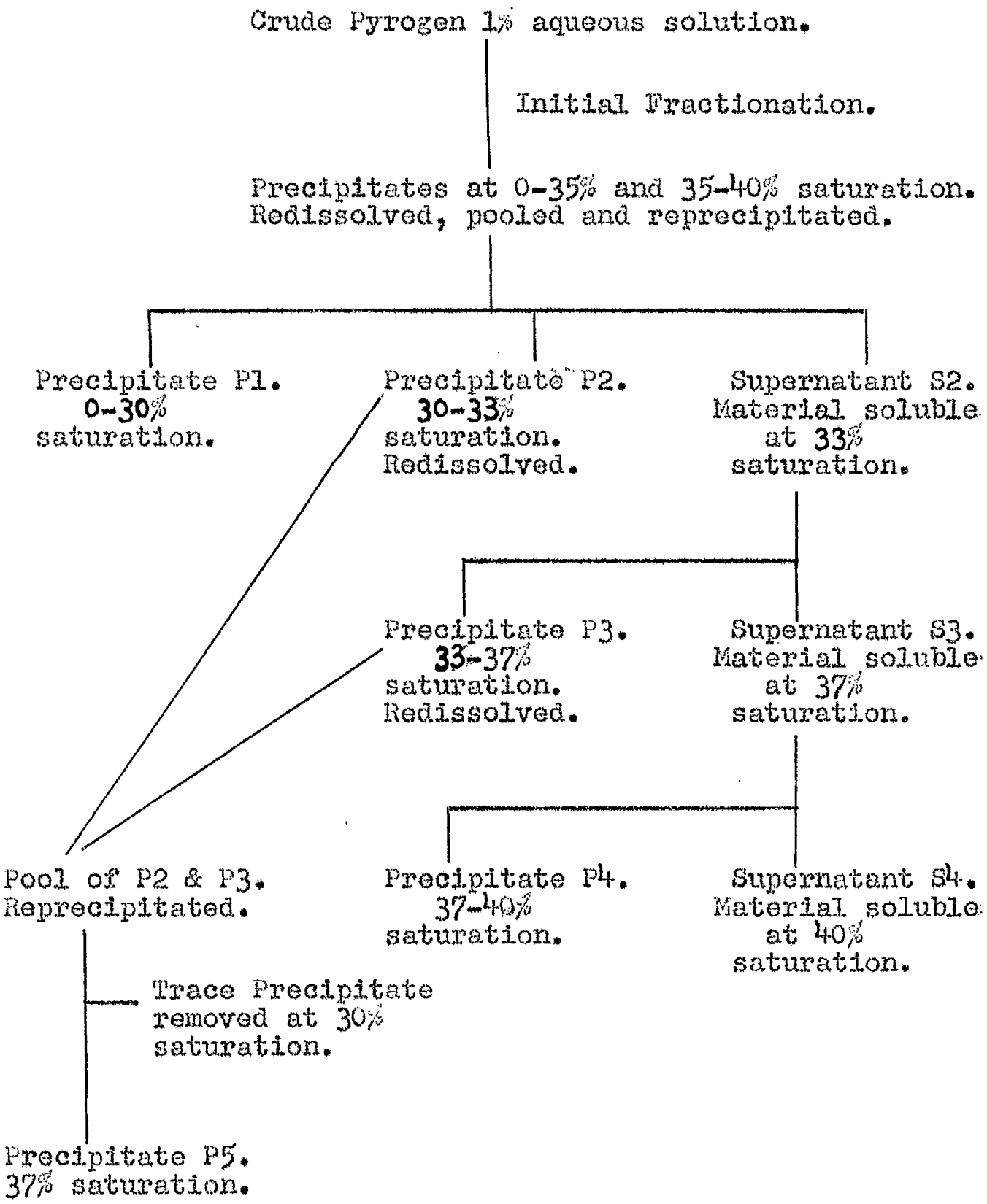
TABLE III

	Starting Material	Ammonium sulphate saturation			
		35%	40%	60%	85%
Fraction weight(mg)	350	170.4	66.5	22.1	15.7
Nucleic acid(%)	51	29	49	76	90

The 60% and 80% precipitates gave no fever when injected into rabbits at 0.05 µg/kg and were discarded. The 35% and 40% fractions retained the full pyrogenic activity of the original material so were pooled and the mixture refractionated with ammonium sulphate. (See Fig.6.)

Thirty percent ammonium sulphate saturation precipitated from the mixture only a trace of material (P1).

Fig.6. Ammonium Sulphate Fractionation of Crude Pyrogen.



Increasing the concentration to 33% gave a precipitate (P2) which was spun down, washed with 33% ammonium sulphate solution and redissolved in water. The washings of P2 were added to the 33% saturation supernatant fluid (S2) and the ammonium sulphate concentration raised to 37% saturation. This produced a precipitate (P3) which was removed, washed with 37% saturated ammonium sulphate solution and redissolved in water. To the supernatant (S3) containing material soluble at 37% saturation were added the washings of P3 and saturated ammonium sulphate solution added to give a final concentration of 40%. The precipitate occurring at this level (P4) was washed and redissolved.

After dialysis and freeze drying the precipitates P2 and P3 were strongly pyrogenic but P4 was inactive. P2 and P3 gave a coincident precipitin line when tested against antiserum to Proteus vulgaris cells in the Ouchterlony double-diffusion precipitin test,[140] and both contained glucose, galactose and heptose as shown by the Bische Cyh3 test.[136] P2 and P3 were therefore pooled and dissolved in water to 1% w/v concentration, a trace of precipitate produced by 30% ammonium sulphate saturation removed and saturated ammonium sulphate added to give 37% saturation. The precipitate obtained, P5, was washed twice with 37% saturated ammonium sulphate,

dissolved in distilled water, dialysed and freeze dried. This final product contained 4.5% nucleic acid but further reprecipitations did not reduce the figure below this value. It was highly pyrogenic (Minimum Pyrogenic Dose 0.003µg/kg) and contained glucose, galactose and aldoheptose.

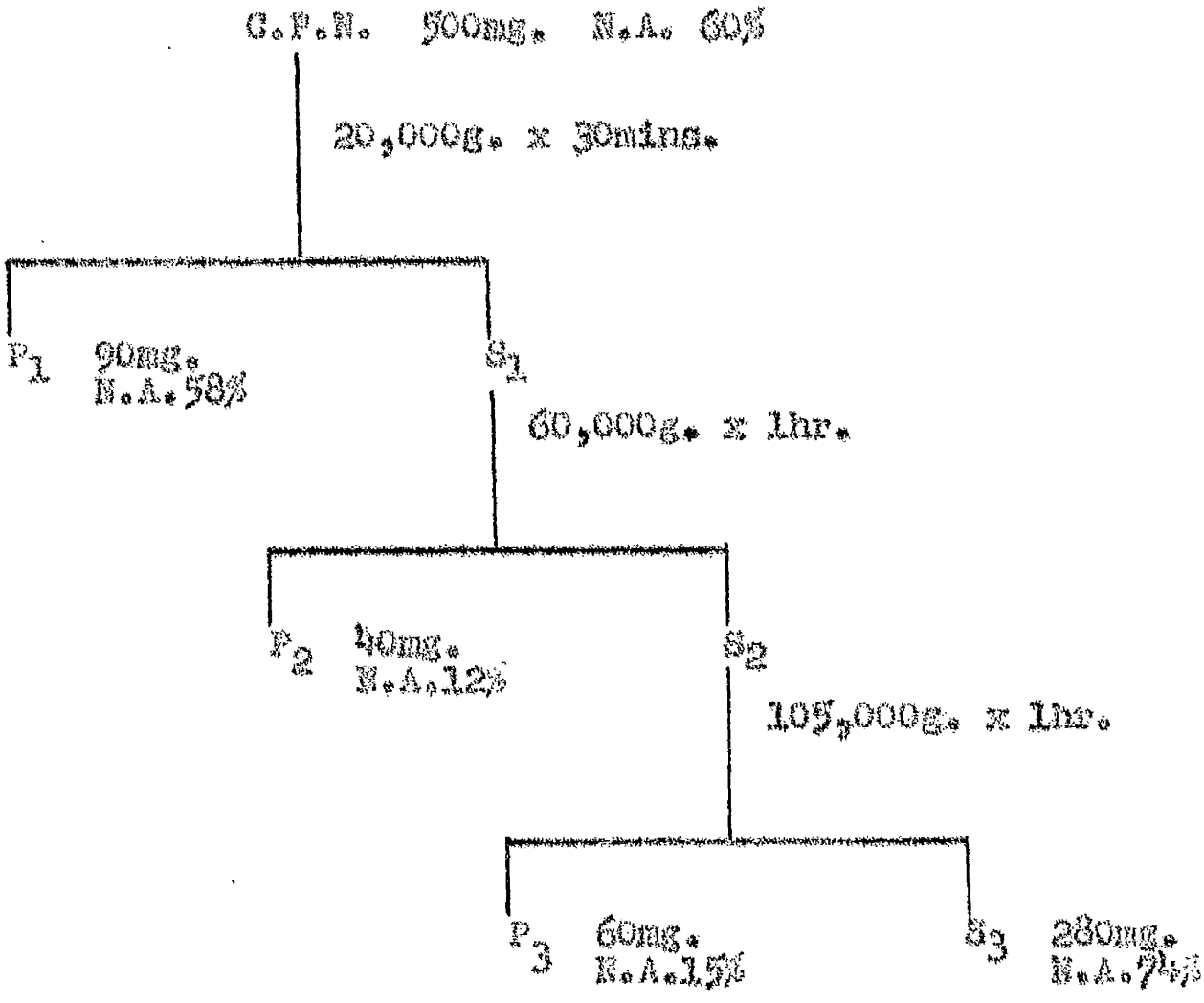
Ultracentrifugation.

Centrifugation at different 'g' values. A one percent aqueous solution of crude pyrogen prepared by high speed mixing in the MSE homogenizer, was centrifuged at 20,000g. for 30 minutes and the resulting sediment P_1 and supernatant fluid S_1 separated. S_1 was then centrifuged at 60,000g. for 1 hour to give a sediment P_2 and supernatant fluid S_2. S_2 was further centrifuged at 105,000g for 1 hour yielding a sediment P_3 and a supernatant S_3, which were separated.

Sediments P_1, P_2 and P_3 were separately redissolved in water and nucleic acid and dry weight estimations performed on the solutions. S_3 was similarly tested. The figures obtained on the four fractions are included in the fractionation scheme in fig.7.

Examination of P_1, P_2 and P_3 by the Dische (yn3 test[136] showed the presence in all three of glucose, galactose and a heptose, and the hexose:heptose ratio was similar in each case.

Fig.7. Initial Ultracentrifugal Fractionation of CPN.



N.A. = Nucleic Acid.

The high nucleic acid content of P_1 suggested it was a difficultly soluble portion of the original crude pyrogen and it was not examined further.

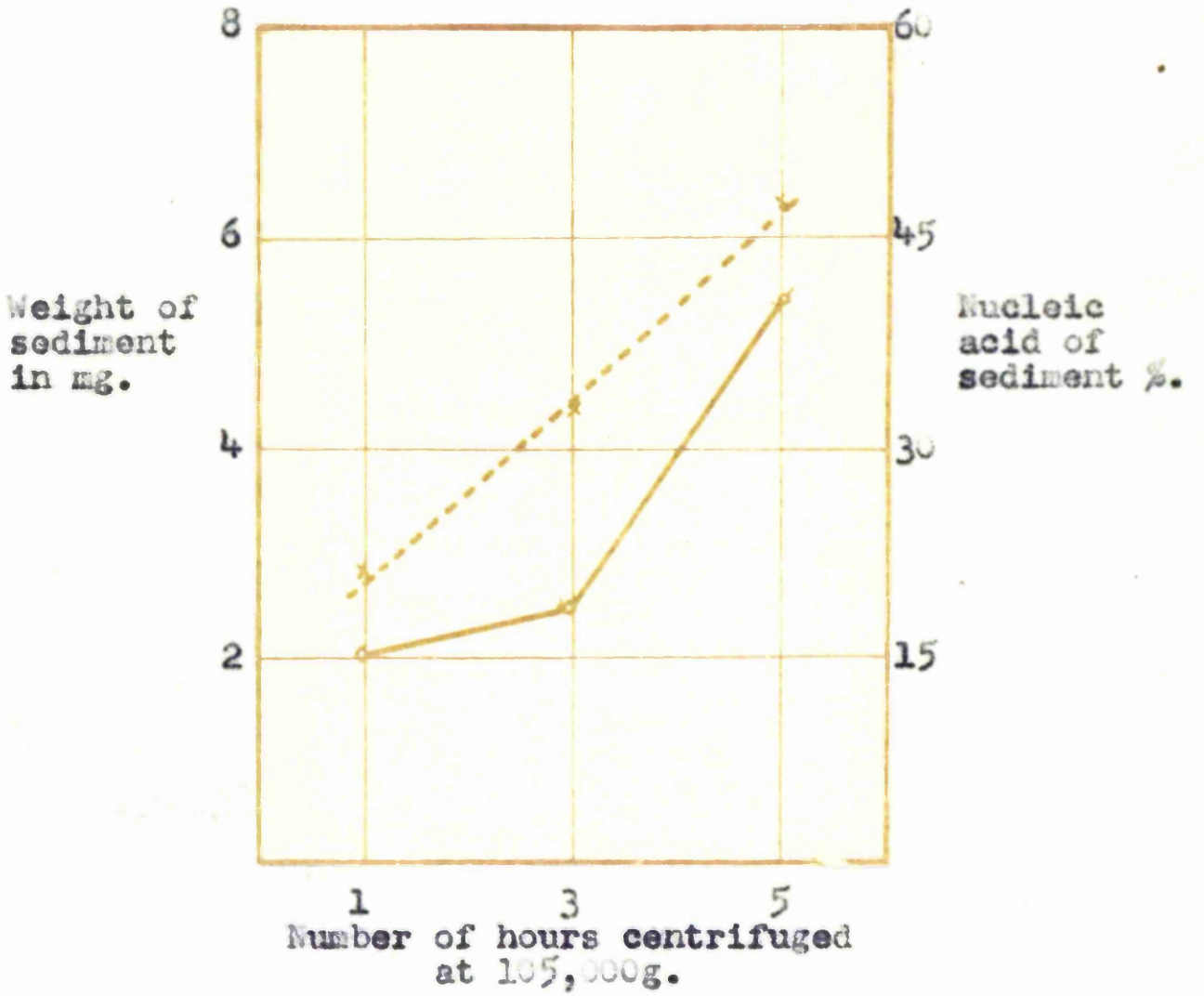
P_2 and P_3 were pyrogenic to a similar degree and on examination by the Ouchterlony technique previously described, they exhibited a common dominant precipitin line against homologous antiserum to Proteus vulgaris cells. These results, plus the similarity in sugar content as shown by the Dische CyR3 test[136] were accepted as evidence that the same polysaccharide was being sedimented at 60,000g and 105,000g. Since the higher 'g' value gave a higher yield of polysaccharide this was taken as a suitable level for purification of the polysaccharide. Centrifugation at 105,000g for different times. A two percent aqueous solution of crude pyrogen was prepared by stirring for 48 hours and cleared of insoluble matter by centrifuging at 20,000g for 30 minutes.

Samples of the supernatant fluid obtained were spun at 105,000g for periods of 1, 3 and 5 hours. The sediments obtained were redissolved in water and dry weight and nucleic acid estimations performed. The results are shown in Fig.8.

The Dische CyR3 test[136] showed the hexose:heptose ratio was similar in the sediments obtained after 1 and 3 hours, but was higher in the five hour sediment. Also

Figure 8.

Results of centrifugation of a solution of crude pyrogen at 105,000g for different periods of time.



-----= Weight of sediment per ml. of solution centrifuged (left ordinate).

———= Per cent nucleic acid content of sediment (right ordinate).

since the nucleic acid content rose markedly from 19% to 40% between the 3 and 5 hour sediments, it was decided that the 3 hour period was most suitable for a purification procedure.

All centrifuge runs were carried out in a Spinco Model 'L' Preparative Ultracentrifuge at 5°C. The rotor heads used were of the enclosed angle type and as a result the centrifugal force applied differs at different levels in the tube. The figures quoted in this section refer to the 'g' values exerted at the centre of the tubes but full details are given in Table IV.

TABLE IV

Centrifuge runs at	Rotor Head No.	Speed r.p.m.	'g' value exerted at:- Top of tube	Centre of tube	Bottom of tube
20,000g	30	15,000	12,566	19,600	26,390
60,000	30	26,500	39,130	60,900	82,173
105,000g	40	40,000	67,910	105,400	144,700

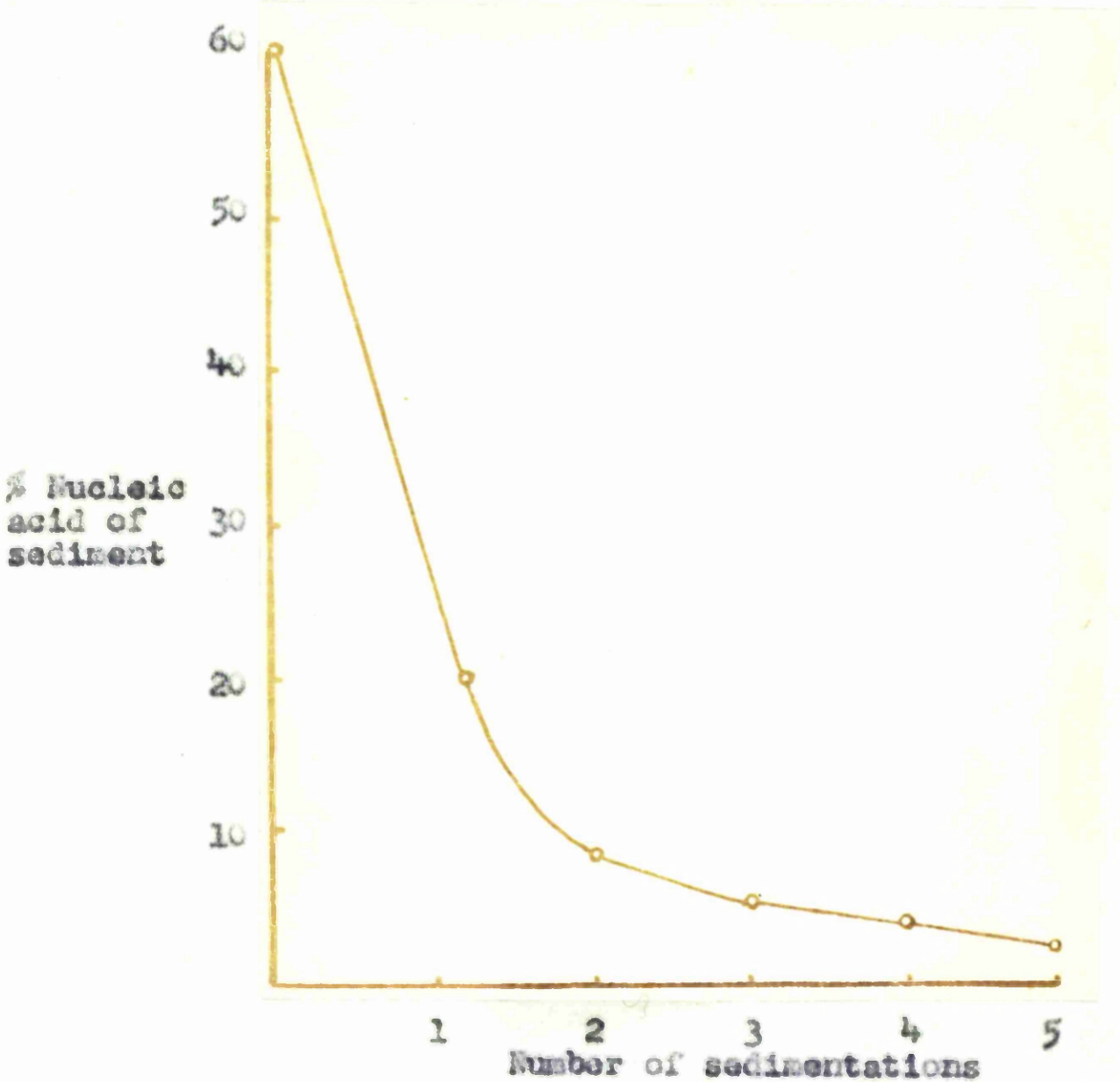
Effect of repeated ultracentrifugation. A two percent aqueous solution of crude pyrogen prepared as before and cleared by centrifuging at 20,000g for 30 minutes was centrifuged at 105,000g for three hours. The sediment obtained was redissolved in water and spun at 105,000g

for three hours. The sediment obtained was treated as before and the process of redissolving and respinning was carried out 5 times in all. Estimates were made of nucleic acid content and total weight for each sediment. These figures which show the removal of nucleic acid are plotted in Fig.9.

As can be seen from Fig.9, after five three hour periods of centrifuging the nucleic acid has been reduced to not more than 2% and this figure could not be reduced by further periods of centrifuging. The final sediment was strongly pyrogenic (Minimum Pyrogenic Dose 0.003µg/kg), gave a single line of precipitation when tested against antisera to P.vulgaris in the gel-diffusion precipitin test, and contained glucose, galactose and aldoheptose[136].

Figure 9.

Removal of nucleic acid from crude pyrogen by repeated sedimentation at 105,000g.



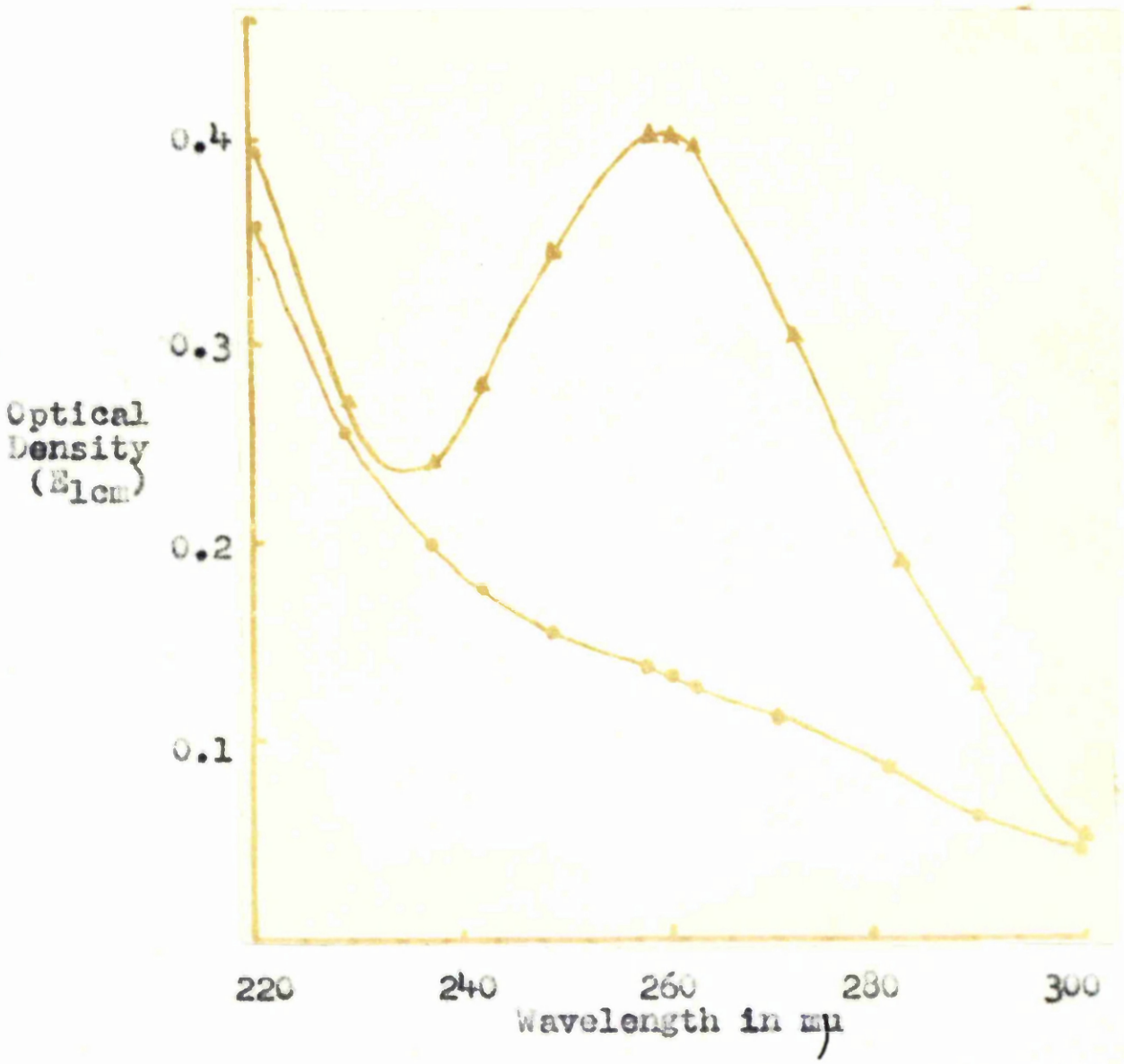
III. LARGE SCALE PRODUCTION OF PURIFIED LIPOPOLYSACCHARIDE.

From bulk cultures of Proteus vulgaris RCST53 in nutrient broth medium 250g. acetone dried cells were obtained. Five extractions were made, each employing 50g. cells in 1,750ml. water to which was added an equal volume of aqueous phenol (90% w/v). The aqueous phases of the five separate extractions were bulked, dialysed till free of salts and freeze dried. The crude pyrogen so obtained (6.7g.) contained N 8.42%, P 4.8%, nucleic acid 43%, and had a Minimum Pyrogenic Dose of 0.005µg/kg. rabbit body weight.

An aqueous solution of the crude pyrogen (2% w/v) was cleared by centrifugation at 20,000g. for 30 minutes and from the supernatant the lipopolysaccharide deposited by ultracentrifugation (105,000g. x 3 hours). Purification of the lipopolysaccharide was completed by four further sedimentations at 105,000g. (as described on p.67). The final sediment was suspended in distilled water, dialysed free of salts and freeze dried. This product, total weight 790mgm., contained not more than 2% nucleic acid (see Fig.10) and is hereafter described as purified lipopolysaccharide (LPS).

Figure 10.

Ultraviolet absorption spectra of crude and purified lipopolysaccharide obtained from [illegible] nutrient broth culture cells.



Homogeneity.

Ultracentrifugation.

An aqueous solution (0.5% w/v) of the lipopolysaccharide (LPS) was examined at intervals during sedimentation in a Spinco Model 'E' Analytical Ultracentrifuge at speeds up to 80,000g. and photographs obtained at intervals are shown in Fig. 11.

The material appeared monodisperse since only a single peak of sedimentable material was found, and the opalescence of the solution was completely sedimented along with the peak. After sedimentation of the peak shown in photographs the supernatant fluid was further centrifuged up to 250,000g. but failed to reveal any other sedimentable component.

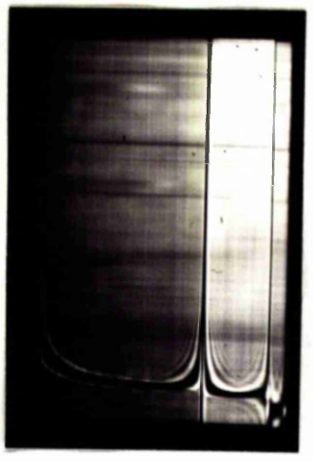
An exactly similar experiment was carried out and when the single peak shown in photographs had reached the bottom of the cell, the supernatant fluid was carefully removed and the sediment resuspended. Pyrogen and gel diffusion-precipitin tests showed that both activities were contained only in the sediment.

Fractional Solubility Test.

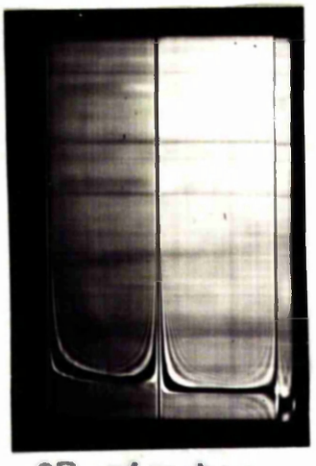
A sample of the lipopolysaccharide (100mg.) was shaken with 10ml. distilled water and when some solution

Figure 11

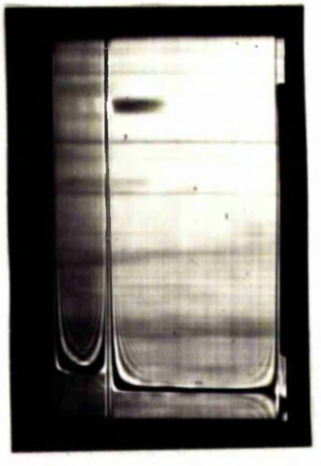
Examination of an aqueous solution of the lipopolysaccharide in the analytical ultracentrifuge.



18 minutes



27 minutes



36 minutes

Concentration of lipopolysaccharide solution in water 0.5% w/v.

Phototgraphs show sedimentation from right to left, and were taken at the times stated after reaching 80,000 x g.

appeared to have taken place the suspension was centrifuged. The supernatant was removed and retained, and a further 10ml. distilled water added to the sediment, shaken and centrifuged as before. By repetition of the process four extracts were obtained and these and the residue were dried and analysed. The results are shown in Table V.

Table V. Analysis of fractions obtained in fractional solubility test of lipopolysaccharide.

Fraction Number.	Yield mg.	N %	P %
1	26	2.24	1.87
2	17	2.19	1.92
3	23	2.31	1.75
4	14	2.21	1.93
5	7	2.30	1.81

Recovery 87%

The variation in figures obtained for the N and P values are within the normal limits of error of the estimations[133,135]. The results do not show any evidence of gross chemical heterogeneity within the

product.

Zone Electrophoresis.

Samples of the lipopolysaccharide were electrophoresed on cellulose acetate strips (12 x 2.5cm.) using borate buffer (0.07M pH9.0) at a constant current of 0.4mA/cm width of strip, as described by Kohn[141]. The cellulose strips after drying were stained with nigrosine or light green dyes to detect protein, and by a periodic acid-Schiff technique for carbohydrate.[142]

A single band with a slight anodic mobility was detected by the polysaccharide stain when 50µg. samples were employed. When 200µg., the maximum loading possible, was used, no further bands were seen.

No reaction was obtained for protein even when 200µg. was used, although the stains employed were able to detect a 5µg. sample of bovine serum albumin run as control.

Extraction of Lipid Materials.

Samples of lipopolysaccharide (50mg.) were extracted for eight hour periods in a soxhlet apparatus with diethyl ether, petroleum ether 40° - 60°C, or methanol. The extracts obtained by the first two solvents represented 2 and 1.3% of the starting weight, but the methanol extracted 4.5%. Two-thirds of the latter extract was, however, soluble in

distilled water and the remainder in ether.

Further evidence of homogeneity is suggested by the results of the serological reactions of the lipopolysaccharide described later (page 89).

PHYSICAL PROPERTIES.

After freeze drying, the lipopolysaccharide (LPS) was difficultly soluble in cold water, but stable strongly opalescent suspensions could be obtained at 1% w/v in water at 37°C by high speed mixing for short periods in the M.S.E. homogenizer. In salt solutions 1% w/v suspensions were obtainable but settled out on standing.

Measurement of the optical rotation of an aqueous ½% w/v solution gave $[\alpha]_D^{20}$ of +9 but the accuracy of the reading was impaired by the opacity of the solution.

CHEMICAL ANALYSIS.

Elemental Analysis.

Elemental analysis gave the following percentage figures:-

C 41.21 H 7.28 N 2.28 P 1.9 Ash 10.86.

Nitrogen was determined by the Kjeldhal method using the Markham distillation apparatus[133] and the mixed methylred-methylene blue indicator of Ma and Zussage.[134] Phosphorous was determined on samples containing 5-25γ/mg. P by the method of Martland and Robison.[135]

The figure quoted for Ash represents the non-combustible material remaining after the estimation of carbon.

Total Reducing Sugar.

Samples (2mg.) were hydrolysed in hydrochloric acid (1 N) for periods of up to 10 hours and the reducing value of the hydrolysates estimated by the copper reduction method of Somogyi.[143] The reducing value rose to a maximum of 32% in 6 hours but reached 28% (87% of final figure) in two hours. The figures quoted are as anhydrous glucose. The reducing curve obtained for the lipopolysaccharide is shown in Fig.12.

Hexosamine.

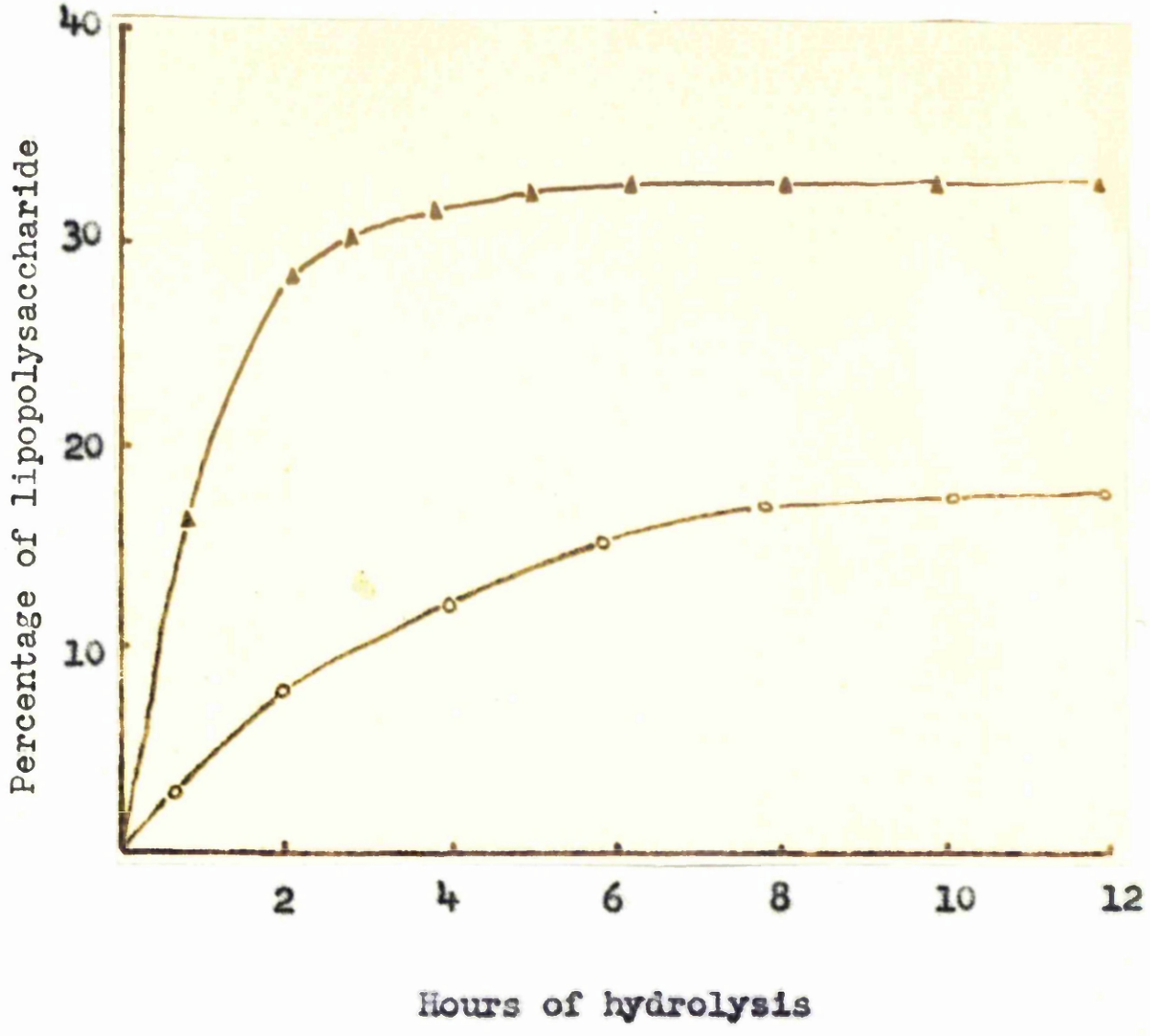
Hexosamine content of the lipopolysaccharide was determined by the method of Rondle and Morgan[144] on 5 mg. samples hydrolysed in 1N HCl for periods up to 16 hours. The curve showing the rate of release of hexosamine is shown in Fig. 12. The maximum content of hexosamine estimated was 16% (calculated as glucosamine base).

Paper Chromatography and Electrophoresis.

The lipopolysaccharide (30mg.) was heated in sulphuric acid (1N, 5ml.) at 98°C when a heavy white precipitate of fat-like material formed which flocculated

Figure 12

Release of reducing sugar and hexosamine during acid hydrolysis of lipopolysaccharide.



completely within one hour. This precipitate was removed by centrifuging, washed with acid and the washings and supernatant solution heated together at 98°C for a further 4 hours. The hydrolysate was neutralised with barium hydroxide to pH 7, centrifuged and the supernatant fluid after concentration to about 1 ml. applied to a column of Zeo-Karb 225 resin (The Permutit Co. Ltd., London).

Neutral sugars were eluted from the column with the necessary volume of distilled water (previously determined by calibration of the column with standard glucose solution) and the column was then washed with an equal volume of dilute hydrochloric acid (0.33%) to obtain the amino sugars.

(a) Neutral Sugars. The aqueous column eluate was concentrated by boiling and finally dried over phosphoric oxide under vacuo. The residue obtained was redissolved in water and used for paper chromatography and electrophoresis.

Paper chromatography was performed on Whatman No.1 paper (22" x 10") and development of the chromatogram was by descending flow of a n-butanol-pyridine-water mixture (3:2:1.5v/v) for 16 hours. The chromatograms were then dried in air and returned to the tank for a further 16 hours development with the same solvent mixture. The

chromatograms were finally dried in air, sprayed with 3% p-anisidine hydrochloride in water-saturated n-butanol,[145] and heated at 120°C for 3 minutes to obtain the maximum differentiation of different classes of monosaccharides.[63]

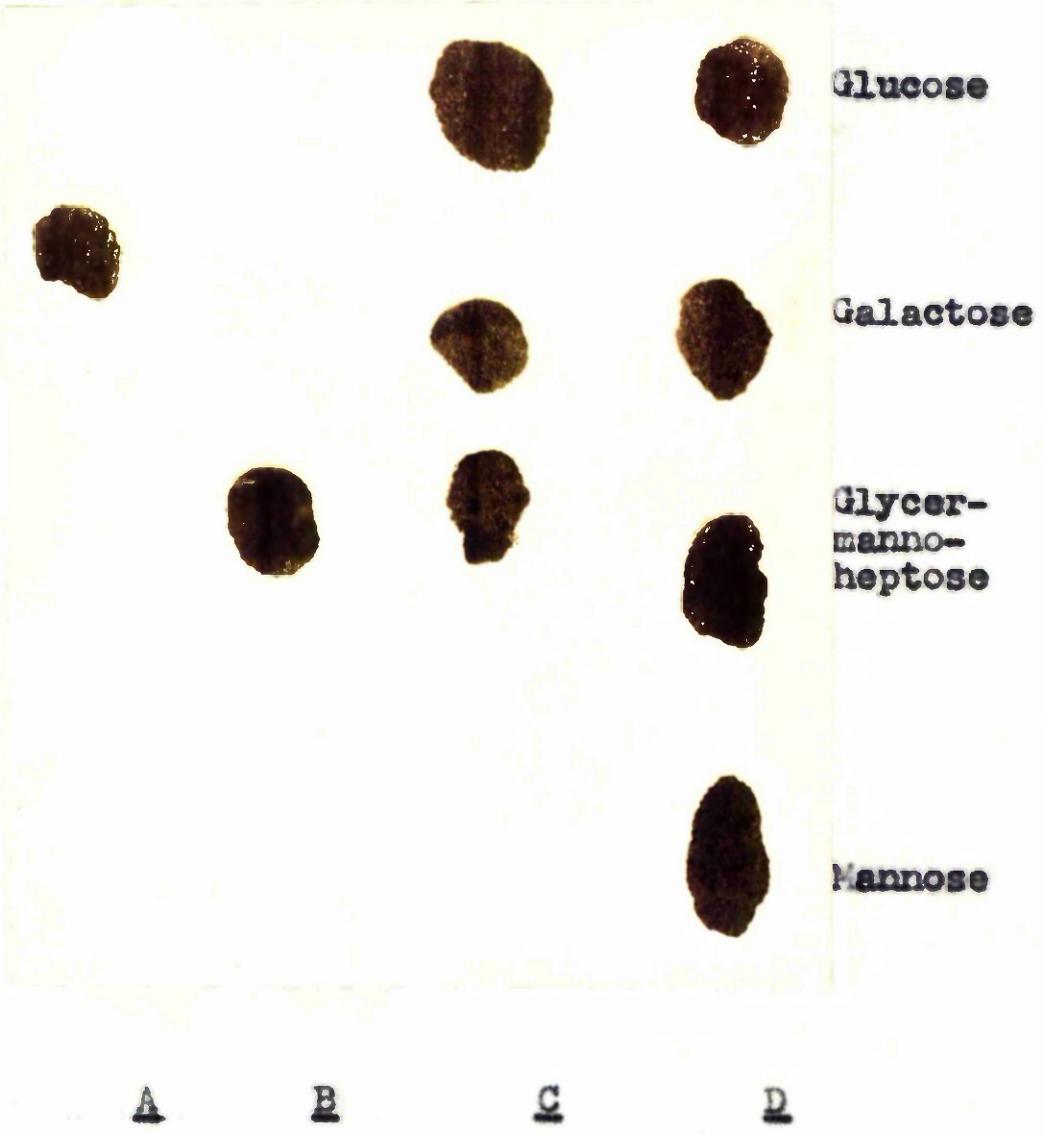
Three components were detected after heating with p-anisidine. Two of these showed the typical brown colour due to hexoses and corresponded in position to authentic samples of D-glucose and D-galactose run on the same chromatogram. The third component gave the characteristic greenish-purple colour of aldoheptoses[63] and occupied a position between glucose and galactose on the chromatogram. Under these chromatographic conditions three aldoheptoses are known to run between glucose and galactose, namely, D-glycero-D-guloheptose, L-glycero-D-manoheptose, and D-glycero-L-mannoheptose.[63]

A sample of the neutral sugar mixture was electrophoresed on Whatman No. 3MM paper (22" x 5" strips, origin 5" from cathode) in borate buffer (0.1N, pH 9.2) for two hours at 35 volts per cm. length. The electrophorerogram was dried and stained with p-anisidine hydrochloride as previously described.

Fig.13 shows the stained electrophorerogram. This corroborates the presence of glucose and galactose found by chromatography. It also eliminates D-glycero-L-

Figure 13.

Section of electrophorerogram of hydrolysate of lipopolysaccharide.



A = standard D-glycero-L-guloheptose
B = standard D-glycero-L-mannoheptose
C = hydrolysate of lipopolysaccharide
D = standard sugar mixture as labelled

Direction of anodic migration is from bottom to top of page.

guloheptose as a possible constituent of the lipopolysaccharide and show that the aldoheptose must be D-glycero-L-mannoheptose, or its optical enantiomorph, L-glycero-D-mannoheptose, which cannot be resolved by this technique.

(b) Amino Sugars. The hydrochloric acid eluate of the Zeo-Karb column, containing the amino sugars, was reduced in volume by boiling and repeatedly dried in vacuo in the presence of sodium hydroxide pellets. The final neutral residue was dissolved in water and chromatographed as described for the neutral sugars, except that only a single 24 hour descending development was used.

The dried chromatogram was stained with ninhydrin reagent[146] and revealed five spots. Two of these spots also gave a positive reaction with Ehrlich's reagent[146] and corresponded in position with control samples of D-glucosamine and D-galactosamine on the chromatogram. A third slow-moving component also gave a positive Ehrlich reaction and might be a hexosamine-containing oligosaccharide due to incomplete hydrolysis. The remaining two spots gave a weak reaction with ninhydrin, failed to react with Ehrlich reagent and were not recognised.

To confirm the presence of glucosamine and galactosamine samples of the amino acid eluate were degraded

with ninhydrin[147] and samples of D-glucosamine and D-galactosamine were similarly treated as controls. Chromatography of the degradation products as described above and staining with p-anisidine hydrochloride revealed arabinose and lyxose, these sugars being found also on chromatograms of the ninhydrin degredation of standard D-glucosamine and D-galactosamine respectively.

Dische Tests.

The lipopolysaccharide in aqueous solution (at 250µg, 500µg and 1mg/ml) was examined by the Dische CyR3 test[62,136,13(] as described previously (page 55). After 48 hours incubation following the addition of cysteine, the lipopolysaccharide showed three peaks of maximum absorption at wavelengths of 388, 412 and 510mµ, characteristic of glucose, galactose and aldoheptose respectively.

Standard mixtures of D-glucose, D-galactose (both at 40 µg/ml) and D-glycero-L-mannoheptose (at concentrations from 10-100µg/ml) were prepared and examined in the CyR3 test. It was found that the optical density increment $E_{510m\mu} - E_{540m\mu}$ gave a straight line when plotted against aldoheptose concentration from 10 to 80µg/ml and was not affected by the presence of glucose or galactose. The $E_{510m\mu} - E_{540m\mu}$ value for the lipopolysaccharide was

determined and by reference to the standard graph obtained with the synthetic sugar mixture, the aldoheptose content was estimated as 18% (as D-glycero-L-mannoheptose).

The absorption maxima of glucose and galactose in the Dische test were clearly distinguishable but for quantitative estimation of the two sugars in admixture a correction had to be determined for the contribution of either sugar at the absorption maximum of the other, since both absorption curves overlapped.

Tests were therefore carried out on aqueous solutions of D-glucose (10 to 100µg/ml.) and D-galactose (10-100µg/ml) and mixtures of both sugars at similar concentrations. Investigation of the absorption curves in the CyR3 test showed:-

(i) Galactose absorption curve had maximum at 388mµ., and was symmetrical, so that $E_{412m\mu}$ was exactly equal to $E_{364m\mu}$.

(ii) Glucose absorption curve had maximum at 412mµ. and was symmetrical, so that $E_{388m\mu}$ exactly equalled $E_{436m\mu}$.

(iii) Absorption curves of the two sugars in mixture were summations of the individual absorption curves of each sugar.

(iv) Plotting galactose concentration against $E_{388m\mu}-E_{436m\mu}$ gave a straight line even in the presence of four-fold excess of glucose.

(v) Plot of glucose concentration against $E_{412m\mu} - E_{364m\mu}$ gave a straight line even in presence of four-fold excess of galactose.

(vi) Glucose and galactose concentrations could be independently determined in mixture by using values $E_{412m\mu} - E_{364m\mu}$ and $E_{388m\mu} - E_{436m\mu}$ respectively, when tested on synthetic standard mixtures of the two sugars.

Before applying this method of estimation to the lipopolysaccharide a further correction had to be made. In the control samples (lipopolysaccharide and sulphuric acid but without cysteine) the aldoheptose constituent gave an absorption maximum at 400mμ. while in the test sample the presence of cysteine caused complete transformation to a new chromogen with absorption maximum at 510mμ. Thus the control sample, which was included to determine non-specific background colour due to reaction with sulphuric acid, had an "artificially" high absorption between 375mμ. and 425mμ. and subtraction of the control readings from the test sample readings for estimation of glucose and galactose resulted in low values for these sugars.

Absorption spectra of solutions of D-glycero-D-gluco-heptose (3-100μg/ml) were determined for samples with and without cysteine. These showed that the absorp-

tion spectra crossed at 465mμ, and that for the sample to which cysteine was added the absorption curve remained horizontal from 465mμ. to 365mμ. From this it was decided that a more accurate value of lipopolysaccharide "non-specific" background absorption could be obtained by using the $E_{465m\mu}$. value of control samples and subtracting this from the readings for the test sample (cysteine present).

The galactose and glucose values of the lipopolysaccharide were therefore estimated by the values

$$\left[\text{Test } (E_{388m\mu} - E_{436m\mu}) - \text{Control } (E_{465m\mu})\right] \quad \text{and}$$

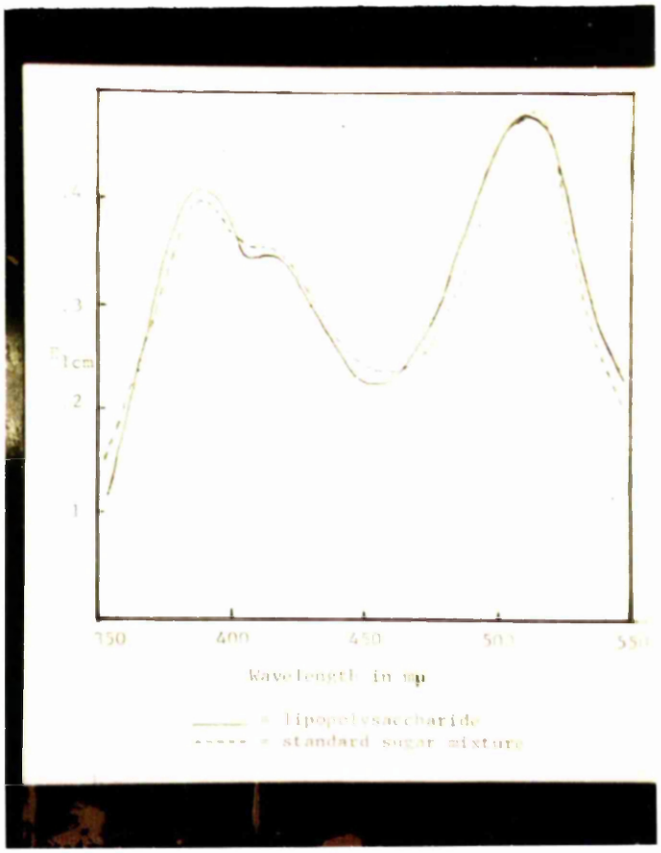
$$\left[\text{Test } (E_{412m\mu} - E_{364m\mu}) - \text{Control } (E_{465m\mu})\right] \quad \text{respectively.}$$

This gave values of 8% and 4% respectively for the galactose and glucose content of the lipopolysaccharide.

As a check on the validity of these measurements for estimation of the monosaccharide content of the lipopolysaccharide, Dische CyR3 tests were carried out on the lipopolysaccharide solution (500μg/ml) and on a synthetic mixture of D-glucose, D-galactose and D-glycero-L-mannoheptose (at 20, 40 and 90μg/ml respectively). The synthetic sugar mixture was constructed to contain the sugars in the same concentration as had been estimated for the lipopolysaccharide. Fig.14 shows the almost superimposable absorption curves obtained for the sugar solution and the lipopolysaccharide and offers proof of the accuracy of the method of estimation employed.

Figure 14.

Absorption spectra in the Dische CyR3 test of lipopolysaccharide (LPS) and standard sugar mixture (SM).



LPS - lipopolysaccharide at 0.5 mg/ml.

SM - mixture of glucose, galactose and glycero-mannoheptose at 20, 40 and 90 μg/ml respectively.

Standard modifications of the Dische tests were employed to test for the presence in the lipopolysaccharide of mannose[136], hexuronic acids[137], methylpentose[62] and pentose[136] and showed that all were absent.

DEGRADATION PRODUCTS.

The lipopolysaccharide (50mg.) was heated at 100°C in acetic acid (5ml. of 1%w/v) for 4 hours, according to the method of Morgan and Partridge.[16] After centrifuging, the supernatant fluid was carefully removed, and the precipitate washed with 2 x 3ml. volumes of 1% acetic acid. The washings and supernatant fluid were bulked, dialysed till free of acetic acid and freeze dried. This is degraded polysaccharide.

The precipitate was washed twice with acetone, dissolved in chloroform and after drying with anhydrous sodium sulphate, the chloroform solution was dried in a stream of nitrogen. The residue obtained is lipid A.

Degraded Polysaccharide.

The degraded polysaccharide (N1.1,P0.91%) was obtained in yield of 52% of the original weight of lipopolysaccharide. Examination by chromatography and Dische tests, as already described, detected the same sugars as found in the lipopolysaccharide.

Pyrogen tests showed that the product was inactive. Fig.15 shows the response obtained on injection of 1µg/kg rabbit body weight and includes for comparison the response in the same rabbits obtained with one-twentieth of this dose of lipopolysaccharide.

When tested by agar diffusion-precipitin tests[140] the degraded polysaccharide failed to produce a line with antiserum to the homologous cells, even at concentration of 50mg/ml. (cf. lipopolysaccharide, page 95).

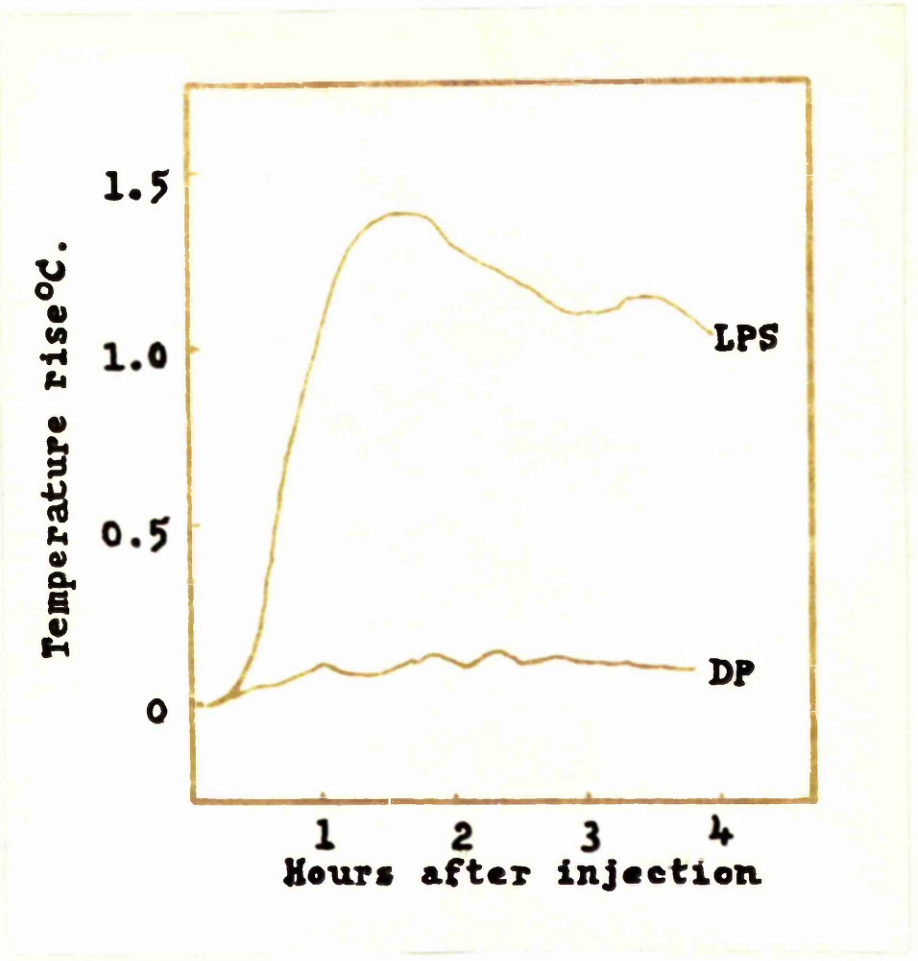
Lipid A.

This product (N 1.9, P 2.1%) was obtained in yield of 28% by weight of the lipopolysaccharide. Hydrolysis of lipid A with HCl(6N) at 100°C for 15 hours followed by three extractions with an equal volume of ether, separated the hydrolysate into ether- and acid-soluble components which were examined by chromatography.

(i) Acid Soluble Components. The acid soluble fraction was repeatedly dried over phosphorous pentoxide in a vacuum desiccator in the presence of sodium hydroxide pellets till the residue on suspension in distilled water gave a neutral pH reaction. This solution was chromatographed on Whatman No.1 paper by two dimensional technique in phenol-ammonia and butanol:acetic acid mixtures as described by Dent.[148] The developed chromatogram was sprayed with ninhydrin revealing seven spots. Aspartic

Figure 15.

Average rectal temperature response of five rabbits to injection of degraded polysaccharide.



LPS = lipopolysaccharide control 0.05µg/kg.

DP = degraded polysaccharide 1µg/kg.

acid, alanine, and probably glutamic acid, were recognised by comparison with separation of known amino acid mixtures but no positive identification of the other spots was possible.

(ii) Ether-soluble Components. The ether solution was evaporated to dryness under nitrogen and the residue obtained resuspended in benzene for chromatography. This was carried out by the horizontal method using 24cm. circles of Whatman No.1 paper which had previously been rendered fat-free by ether washing. The paper circles were immersed for a few minutes in a solution of 12% w/v 'Pink Paraffin' in petroleum ether (40-60°C). The papers were removed, blotted lightly and dried by hanging in air for one hour.

The benzene solution of the fatty acids liberated by acid hydrolysis was applied to the chromatogram and also included on the same chromatogram was a standard mixture[80] of known long chain fatty acids of chain length C_{10} to C_{20} inclusive.

After development with aqueous acetic acid (85%w/v) for 18 hours, the chromatogram was removed from the tank and immediately transferred to a bath containing a solution of copper sulphate (0.5%w/v) in sodium acetate (0.1N).[80]

After a few minutes the chromatogram was withdrawn and the excess copper salts removed by washing in a running water bath for three hours. The papers were finally immersed in an aqueous solution of sodium diethyldithiocarbamate (1%w/v). The areas containing fully saturated fatty acids appeared as brown rings on a white background.

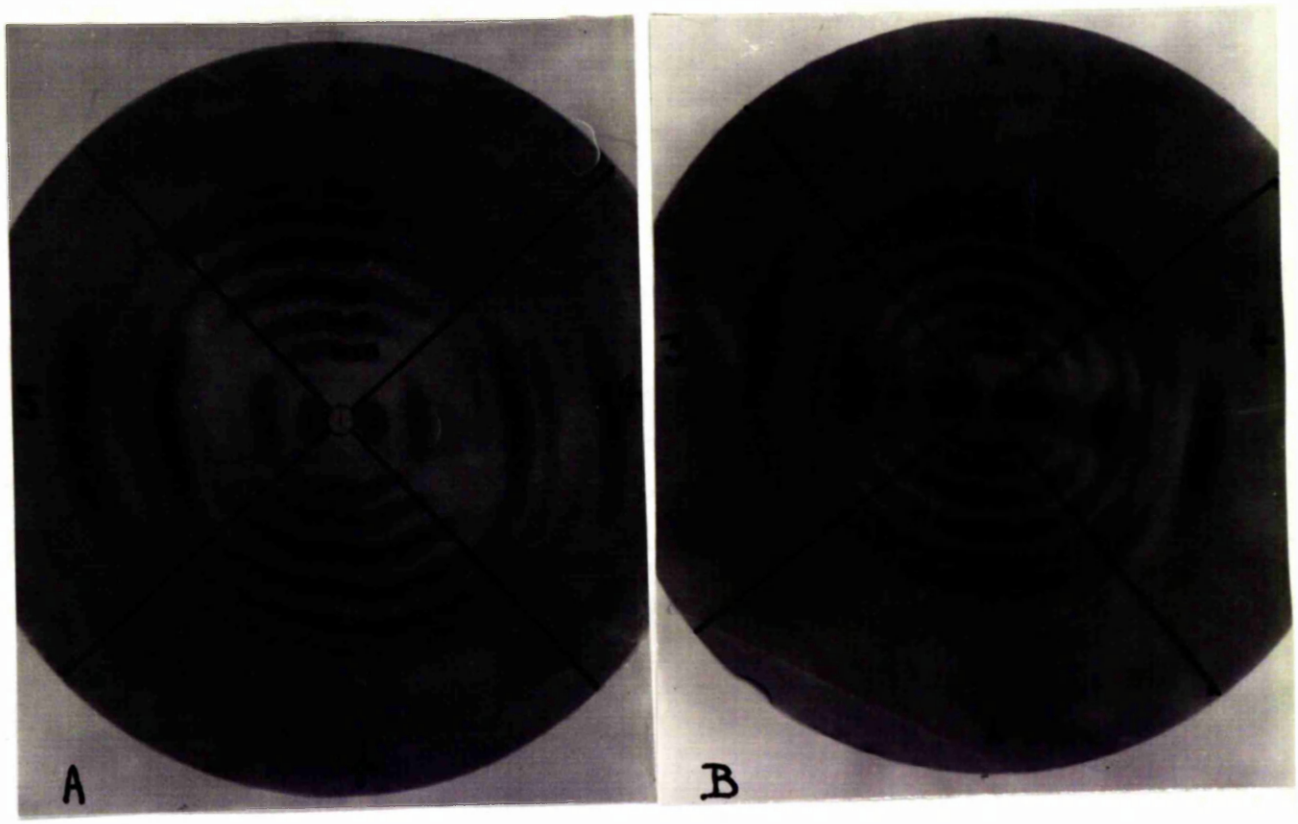
The developed chromatogram is shown in Fig. 16. Sections 1 and 2 are patterns produced by standard fatty acid mixture. This shows the acids separated radially in order of decreasing chain length. Sections 3 and 4 show the fatty acids of the lipid A component. It can be seen that the strongest reactions are given for arachidic acid (C_{20}) and myristic acid (C_{14}). Positive reactions were also given for capric (C_{10}) Lauric (C_{12}) palmitic (C_{16}) and a faint reaction not seen in photograph for stearic acid (C_{18}).

Two other bands appeared, one faster moving than capric acid which was later compared with synthetic β-hydroxy-myristic acid and gave a coincident band. The second band is slow moving and is in the position lignoceric acid (C_{24}) would be expected to occupy, but no sample was available for comparison.

When higher concentrations of hydrolysate were applied to chromatograms a further faint band appeared

Figure 16

Chromatography of the higher fatty acids of lipid A component of the lipopolysaccharide.



Sections 1 and 2 contain a standard mixture of fatty acids of chain length C-10 to C-20.

Sections 3 and 4 contain the ether-soluble fraction of the acid hydrolysate of lipid A component of the lipopolysaccharide.

On chromatogram B a heavier load of lipid A hydrolysate has been applied than on chromatogram A.

between the slow moving band described and arachidic acid. This is possibly due to behenic acid (C_{22}) but if present would appear to be in smaller amount than any of the other acids.

When chromatograms were exposed to osmium tetroxide vapour in a sealed container for 15 minutes two black-blue bands appeared in positions representing palmitoleic acid and another unrecognised unsaturated acid.

BIOLOGICAL PROPERTIES.

Pyrogenicity.

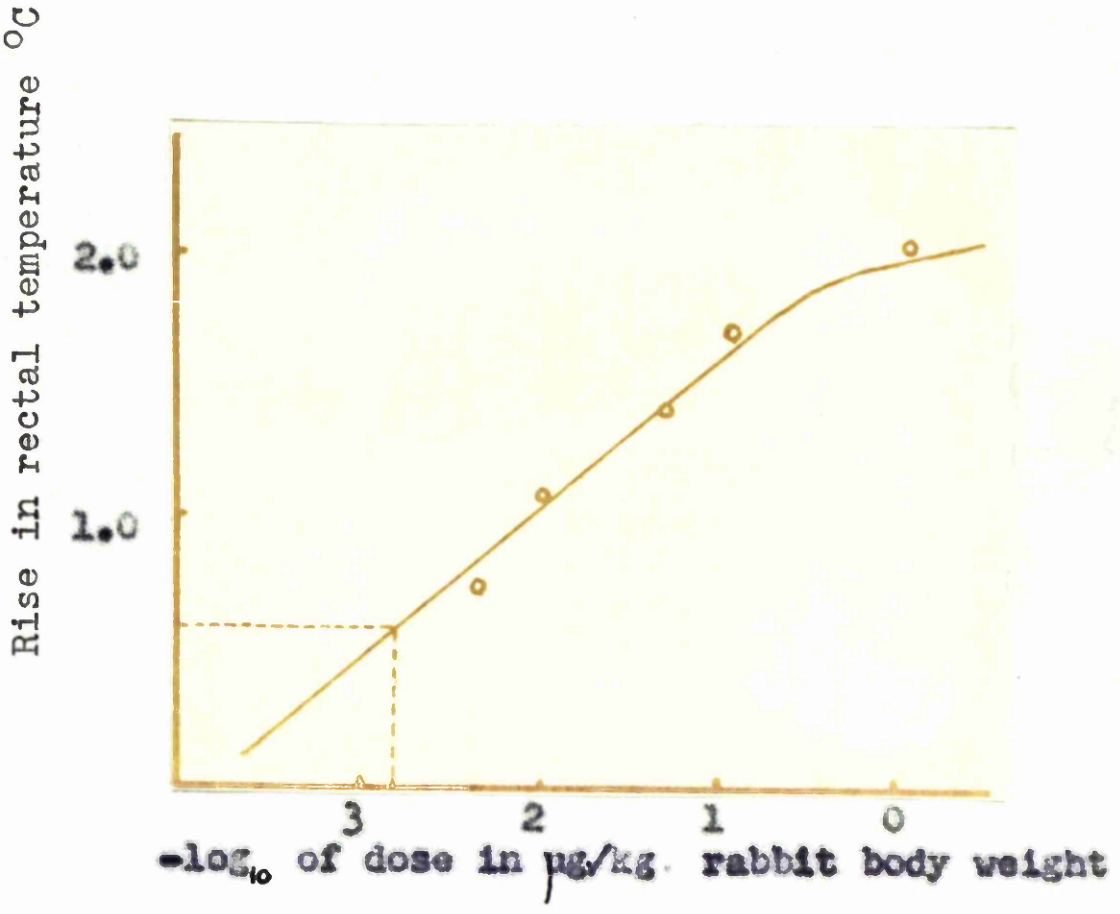
The pyrogenic activity of the lipopolysaccharide was determined in groups of ten rabbits as outlined in Appendix II. The lipopolysaccharide was tested at dose levels of 0.005, 0.01, 0.05 and 0.1 and 1.0µg/kg rabbit body weight. The average rise in temperature obtained for each dose was plotted against the log-dose as shown in Fig.17 from which the Minimum Pyrogenic Dose (that is, the dose causing an average rise in rectal temperature of $0.6^{o}C$ in a group of rabbits) was estimated to be 0.002 µg/kg. rabbit body weight.

Toxicity.

(1) Rabbits. Pairs of rabbits (2-3kg.) were injected intravenously with the following single doses of 10, 20,

Figure 17

Pyrogenicity of the lipopolysaccharide.
Log-dose-response curve.



40,or 50µg. Lipopolysaccharide per kg. body weight.

The two rabbits which received the 10µg/kg. dose appeared mildly discomforted, produced long-lasting fevers but showed no effects the following day.

All rabbits injected with dosage above 10µg/kg. after one hour displayed laboured breathing, tremors and diarrhoea. In both rabbits which received the 50µg/kg. dose and in one which received 40µg/kg. the initial reaction was followed by convulsive seizures, bleeding at the nostrils, prostration and death 5-6 hours after injection.

In one rabbit of the 20µg. and the second of the 40µg. injected pairs the convulsions were weaker, but both eventually became prostrate and died after 24 hours. The remaining 20µg. injected rabbit showed similar prostration, but did not die. It remained ill for 48 hours, during which time it refused food but full recovery was obtained after four days.

Post mortem examination showed typical endotoxin lesions. The most obvious macroscopical changes were congestion and haemorrhage in the lungs and haemorrhage and muscular collapse of the right auricle. The small intestine showed petechial haemorrhage and distension. The liver, spleen and kidney were oedematous and haemorrhaged. The overall picture was of widespread

vascular damage and it appeared that death was due to respiratory failure following collapse of the right auricle.

The range of toxic dosage, 20 to 50µg., is wide and the different susceptibilities of individual rabbits could not be explained as functions of weight, sex, or breed.

(ii) Mice. Initial experiments showed that the LD50 dose of lipopolysaccharide in white mice was in the region of 1 to 2mg. Consequently six groups of ten mice (male, 18-22 grams body weight) were given intraperitoneal injections of the lipopolysaccharide in saline at doses of 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6mg. in 0.5ml. volume. Each group of ten mice received one of the above doses. The number of deaths occurring in each group are shown in Table VI below, from which the LD50 for mice was calculated to be 1mg. total dose.

Serological Reactivity.

To induce antibody against the lipopolysaccharide and to detect any antigenic contaminants, rabbits were injected according to the following schedules:-

(a) lipopolysaccharide in Freund's complete adjuvant was injected intramuscularly in six 100µg. doses at weekly intervals.

(b) a lipopolysaccharide-protein conjugate was prepared

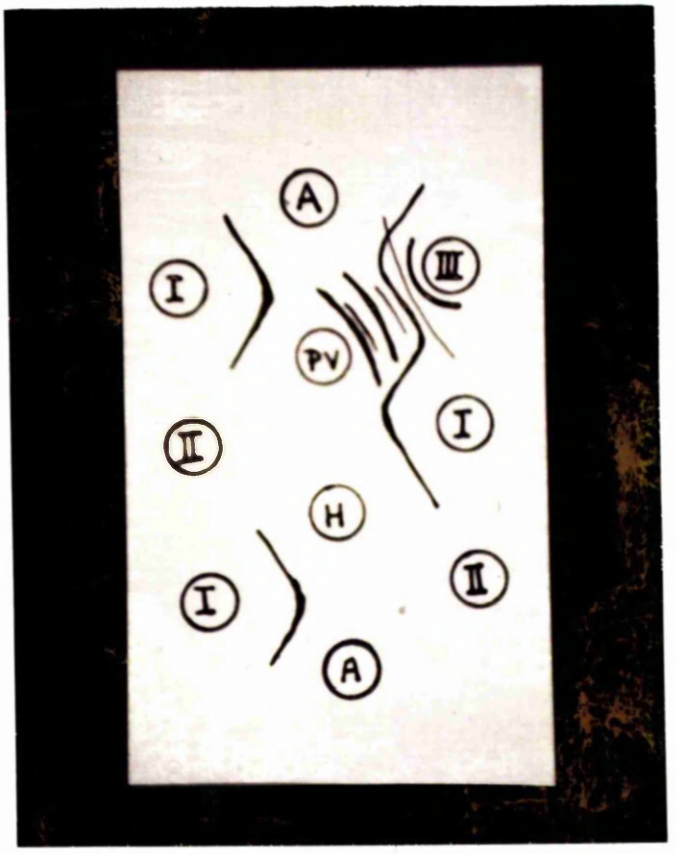
Table VIII.

Toxic effect of lipopolysaccharide LPS in mice.

Dose per mouse (mg.)	Number of mice injected.	Number of mice killed.	% age mortality.
1.6	6	6	100
1.4	6	6	100
1.2	6	5	84
1.0	6	3	50
0.8	6	2	33
0.6	6	0	0

Figure 18

Diagram of the agar-diffusion-precipitin reaction of P.vulgaris cells and lipopolysaccharide.



Reservoir PV contains acetone-dried P.vulgaris cells. Reservoir A containslipopolysaccharide and reservoir H the same after heating at 100°C for 30 minutes.

In reservoirs I, II, and III, respectively, are sera obtained from rabbits immunised with lipo-polysaccharide-protein conjugate, lipopolysaccharide alone and P.vulgaris cells

by coupling the lipopolysaccharide to the conjugated-protein component of the O-somatic antigen complex of Shigella dysenteriae by the method of Partridge and Morgan[16] as described by Davies.[37] Six intramuscular injections of the conjugate, each of 200µg., were given in Freund's complete adjuvant at weekly intervals.

In both schedules, rabbits were bled for antiserum ten days after the last injection.

The lipopolysaccharide was tested against these antisera and against antisera to whole Proteus vulgaris cells by the Ouchterlony diffusion-precipitin analysis in agar.[140]

In Fig.18 are shown the reactions obtained with lipopolysaccharide (1mg.), lipopolysaccharide heated at 100°C for thirty minutes (1mg.), and acetone-dried cells of P. vulgaris (20mg.) against the antisera. The reaction of lipopolysaccharide with antiserum against P.vulgaris cells to give a single strong line of precipitation which was unimpaired by heating the lipopolysaccharide, indicates the absence of other native antigens of P.vulgaris from the lipopolysaccharide preparation. More critical corroboration of this apparent immunological homogeneity is shown by, (i) the lack of reaction of antiserum to untreated lipopolysaccharide when tested against lipopolysaccharide or cells, and (ii) by the formation of only a single confluent line by both lipopolysaccharide

(unheated or heated) and cells when reacting with antiserum to the lipopolysaccharide-protein conjugate.

IV. LIPOPOLYSACCHARIDE FROM CELLS GROWN IN SYNTHETIC MEDIUM.

Acetone-dried cells (20g.) of Proteus vulgaris RCST 53 which had been collected as a by-product during the isolation of the pyrogenic factor from synthetic medium culture filtrates, were extracted with 45%w/v aqueous phenol.[26] The freeze dried product obtained in 3.9% yield of the weight of dried cells had values of N 7.23% and P 4.3%, and contained 42% nucleic acid.

The product was freed of nucleic acid by the ultra-centrifugation procedure which had been employed for the purification of lipopolysaccharide from cells grown in nutrient broth medium. The final product contained not more than 4% nucleic acid and the yield was 0.2% of the dried cells by weight. This product (LPI) was analysed by the methods already described for the lipopolysaccharide obtained from cells grown in nutrient broth (LPS). The results for these two products are compared with the factor isolated from culture filtrates in the next section.

V. COMPARISON OF LIPOPOLYSACCHARIDES OBTAINED FROM CELLS WITH LIPOPOLYSACCHARIDE FROM CULTURE FILTRATE.

The culture filtrate lipopolysaccharide pyrogen (FPS) was isolated by my colleague, J.A.M. Shaw, with whose permission the details of the product are reported here. The cell-free culture filtrates of synthetic medium growths of Proteus vulgaris RO5T 53 were concentrated under reduced pressure in a Quickfit cyclone evaporator to a total solids concentration of about 1%. The concentrate was added to an equal volume of cold ethanol and the resulting precipitate separated, resuspended in water and freeze-dried. The freeze-dried product was extracted with 45%w/v aqueous phenol by the Westphal method and the aqueous layer product finally purified by repeated ultracentrifuging in a process analogous to that already described for the lipopolysaccharide obtained from cells(LPS).

(1) General Analytical Values.

Tables VII and VIII summarise and allow comparison of the analysis of the two products obtained from cells with that from culture filtrate.

Table VII. Elemental Analysis of Proteus vulgaris pyrogens.

Product	C	H	N	P	N/P	Ash
LPS	41.21	7.28	2.28	1.95	1.20	10.8
LPI			2.10	1.69	1.24	
FPS	43.07	7.86	2.10	1.72	1.22	7.89

Table VIII. Percentage Composition of Proteus vulgaris pyrogens.

Product	Reducing(a) Value	Hexosamine(b)	Lipid A
LPS	32	16	30-32
LPI	29	-	27
FPS	30	13	32-34

(a) - calculated as anhydrous glucose.

(b) - calculated as glucosamine base.

(ii) Monosaccharide Constituents.

Table VIII shows that the three products had similar reducing sugar and hexosamine contents. Glucose and galactose were detected by chromatography as common constituents, and an aldoheptose with similar Rf value was also detected.

The Dische Cy(3 test[136] detected these same sugars in the products and gave similar absorption spectra. From the absorption spectra the products were quantitatively similar, the total hexose:heptose absorption ratios($OD_{4.8m\mu}$: $OD_{505m\mu}$) being LPS 0.801, LPI 0.831, and PPS 0.824.

Glucosamine and galactosamine were detected in the three preparations by chromatography of hydrolysates, and corroborated by chromatography of the ninhydrin degradation products of the hydrolysates.

Mannose was detected in small quantity in some preparations of PPS and in the sample of LPI, but was not found in LPS, and is regarded as a probable contaminant.

(iii) Lipid A.

The contents of lipid A are similar in the three products, as shown in Table VIII. Examination of the fatty acid spectrum of the lipid A hydrolysates showed that the same acids (β-hydroxy-myristic, capric, lauric, myristic, palmitic, stearic, arachidic and a further unidentified

higher saturated homologue, plus palmitoleic acid and one other unsaturated fatty acid) were present in the three products. The synthetic medium products FPS and LPI appeared to contain less arachidic and stearic acid than the nutrient medium cell product LPS but otherwise the fatty acid contents were similar.

(iv) Serological Reactions.

The products LPS, LPI and FPS were non-antigenic in rabbits but they reacted in the Ouchterlony diffusion-precipitin test against Proteus vulgaris RCSF 53 antisera to produce a single confluent line of precipitation. Even on prolonged incubation there was no appearance of spur formation between the products which indicates their complete serological identity. When the products were heated at 100°C for thirty minutes the three products still reacted to give the same confluent line, showing the thermostability characteristic of lipopolysaccharides.

With LPI and FPS there was the formation in the Ouchterlony test of a faint second zone which was also heat stable. This may be due to slight contamination of the products, but instances have been recorded in the literature of lipopolysaccharides which were homogeneous by all the physical criteria but which formed two lines in diffusion-precipitin tests.[68]

When the products were tested against antisera to

the artificial LPS-conjugated-protein complex, or anti-sera to the culture filtrate product before phenol extraction, only a single common line was formed.

(v) Pyrogenicity.

The average response of groups of 10 rabbits to the intravenous injection of LPS, LPI and FPS are summarised in Table IX.

Table IX. Fever Response of Rabbits on Injection of Proteus vulgaris pyrogens.

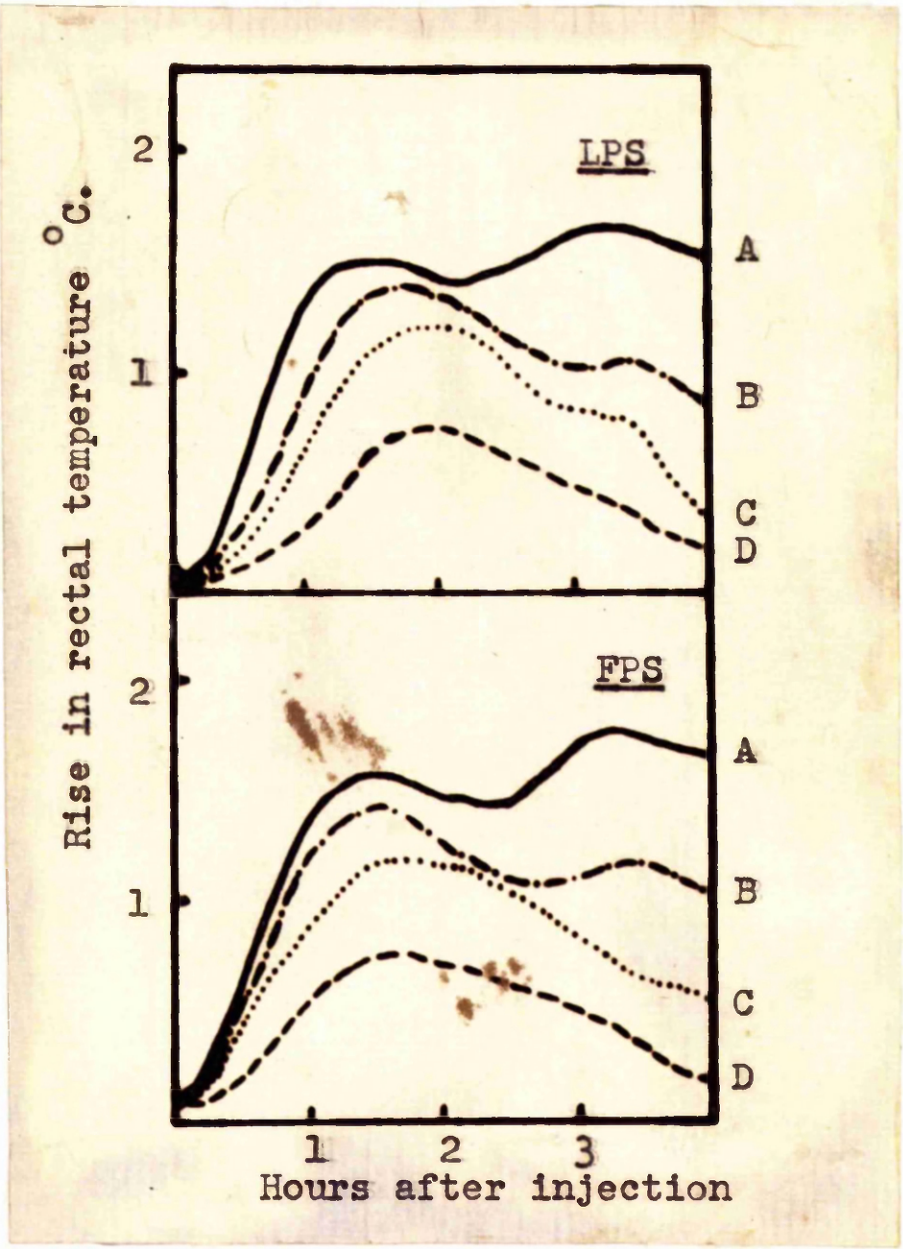
Product	Increase in rectal temperature °C at dosage of (µg per kg. body weight)				
	0.005	0.01	0.05	0.1	1.0
LPS	0.75	1.10	1.39	1.70	2.05
LPI		1.05	1.44	1.66	
FPS	0.68	1.12	1.35	1.72	1.93

The difference in response obtained for the products are within the error of the test system.

The type of fever curve obtained for the cellular and filtrate products LPS and FPS are shown in Fig. 19. From this it can be seen that either a single or a double peak fever response can be obtained for either the cellular or the filtrate product depending on the dose

Figure 19

Fever response of rabbits to injection of lipopolysaccharide obtained from nutrient broth culture cells (LPS), and of lipopolysaccharide obtained from cell-free filtrate of synthetic medium culture(FPS) of P.vulgaris



Dosage in µg. per kg. rabbit body weight:-

A - 0.1, B - 0.05, C - 0.01, D - 0.005.

level employed.

VI. FORMULATION OF A STANDARD REFERENCE PYROGEN.

Effect of Freeze Drying with Mannitol.

For preliminary investigation two solutions of lipopolysaccharide LPS (0.05µg/ml) and mannitol (50mg./ml.) were aseptically prepared using sterile, pyrogen-free phosphate buffers, one of pH 5.5, the other of pH 8.

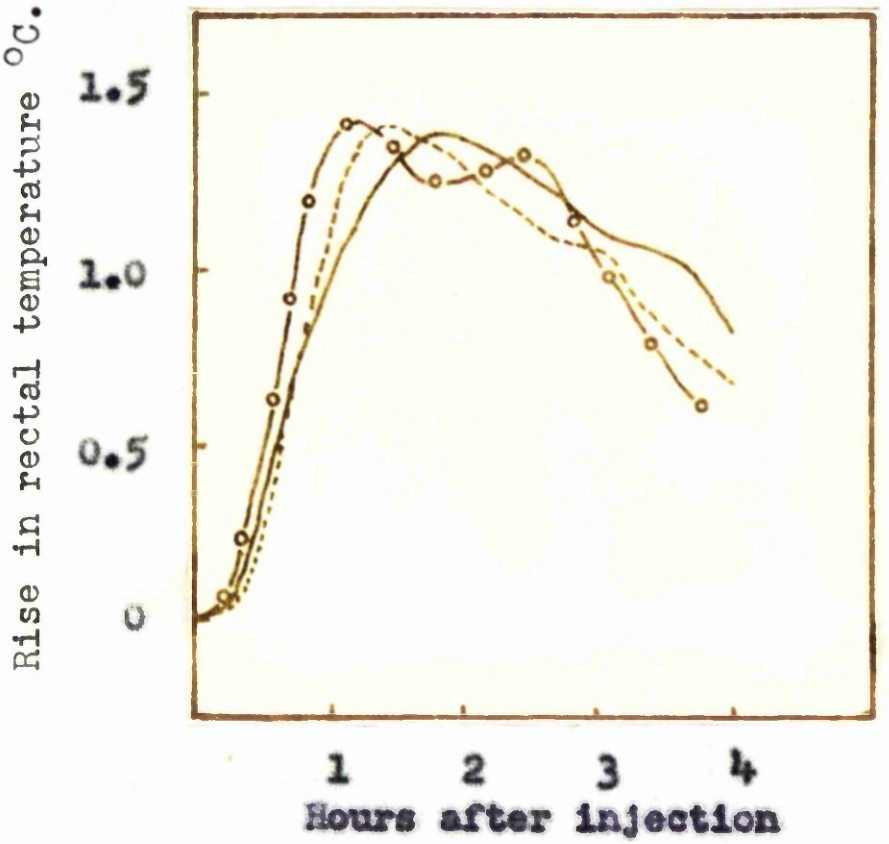
Experience had shown that pyrogens were more stable in slightly acid solution;[5] pH 5.5 was chosen to satisfy these conditions without causing undue irritation on injection.

It was not known if the lipopolysaccharide LPS could be autoclaved without destruction. The second solution was prepared at pH 8.0 since this was known to produce a much less viscous solution and would more likely permit sterilisation by filtration.

Samples of both solutions were freeze-dried without further treatment and the products redissolved in pyrogen free water. Fig.20 shows the results of pyrogen tests on these solutions and includes for comparison the fever response produced by an aqueous solution of the lipopolysaccharide LPS alone at the same dose level (0.05µg/kg. rabbit body weight). Control solutions of 5%w/v mannitol in the buffers employed produced no

Figure 20

Effect on pyrogenicity of freeze-drying lipopolysaccharide with mannitol.



——o—— lipopolysaccharide-mannitol pH5.5

—— lipopolysaccharide-mannitol pH 8.0

- - - - - lipopolysaccharide alone

Dosage in all cases 0.05µg/kg. rabbit body weight.

temperature rise on injection into rabbits. From the results shown in Fig. 20 it was assumed that mannitol had no effect on the pyrogenicity of the lipopolysaccharide LPS. (In all the figures in this section each point recorded on the graphs is the average of 15-20 determinations.)

Methods of Sterilization.

Samples of solutions of mannitol-lipopolysaccharide at both pH values were sterilized by autoclaving or by filtration through sintered glass bacterial filters (porosity 5/3).

Fig. 21 shows the results obtained with the pH 5.5 solutions. Filtration removed a considerable portion of the LPS, but autoclaving at 105°C for thirty minutes completely sterilised the product without impairing its pyrogenicity.

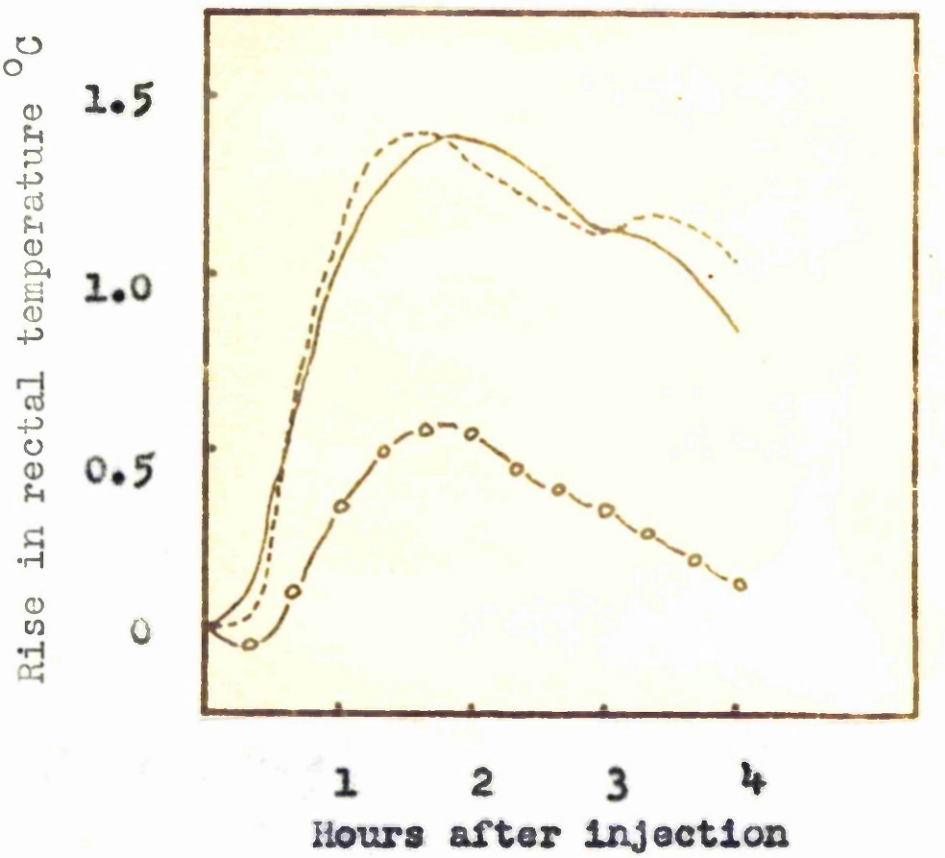
With the pH 8.0 solution, filtration sterilized the product and only slightly reduced the activity but autoclaving caused considerable destruction. These results are summarised in Fig. 22.

From the above results it was decided to produce vials of the mannitol-LPS mixture at pH 5.5 which could be sterilized by autoclaving.

Since these standard pyrogen preparations might

Figure 21.

Effect of sterilization on the pyrogenicity of lipopolysaccharide-mannitol preparation pH 5.5.



Lipopolysaccharide-mannitol preparation pH5.5

——— untreated

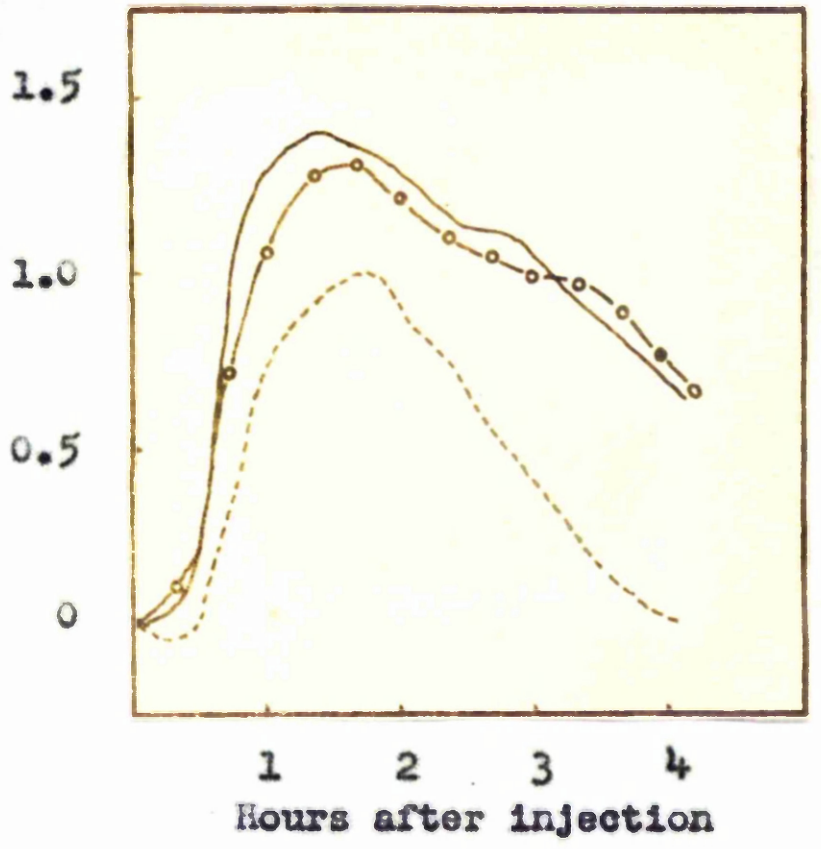
----- autoclaved at 105°C x 30minutes

o——o sterilized by filtration

Dosage in all cases 0.05µg/kg. rabbit body weight.

Figure 22.

Effect of sterilization on the pyrogenicity of lipopolysaccharide-mannitol preparation pH 8.0.



Lipopolysaccharide-mannitol preparation pH 8.0.

——— untreated

------ autoclaved at 105 °C x 30 minutes.

o——o sterilized by filtration

Dosage in all cases 0.05µg/kg. rabbit body weight.

require long periods of storage the effect of inclusion of a bacteriostatic agent was examined. Phenol at 0.5% concentration was introduced into the mannitol-LPS pH 5.5 solutions and the sterilization procedure repeated. Pyrogen tests showed that the phenol had no destructive effect on the fever-producing activity. Inclusion of 0.5% phenol also permitted sterilization of the solutions to be achieved by heating at 98°C according to the British Pharmacopoeia method for sterilization in the presence of a bacteriostat.

For production of large quantities of mannitol-LPS preparation in vials, the following method was finally adopted. For 1,000 vials, each containing 2.5µg LPS, dissolve in 900ml. of freshly distilled water:

Sodium dihydrogen phosphate anhydrous	10g.
Mannitol	5g.

Adjust the pH to 5.5 by the addition of 1N NaOH(c6.25ml.) bring the volume to 1 litre and sterilize immediately by autoclaving at 115°C for 30 minutes. After cooling add 5g. phenol. When the phenol has dissolved the volume is again adjusted to 1 litre to replace the water lost on autoclaving.

A sample of the LPS (2-2.5mg.) is accurately and rapidly weighed to prevent absorption of moisture from the atmosphere.

Under a sterile hood the calculated quantity of

solvent required to prepare a solution of 2.5mg LPS per ml. is measured out and the LPS is dispersed in about 30 ml. When a suitable dispersion has been achieved the contents of the container are transferred to a sterile flask and the container washed with solvent to ensure complete transfer of the LPS to the flask. The remaining solvent is added to the flask and heated at 98°C for thirty minutes. Cross-contamination having been eliminated by the earlier autoclaving of the solvent this lower temperature in the presence of phenol is sufficient to render the product sterile.

After the flask has cooled, 1 ml. quantities are filled aseptically into 5 ml. siliconised vials and freeze dried. After drying the vials are capped with butyl rubber caps and sealed.

Where facilities are not available for the aseptic filling of ampoules the procedure can be modified. The final solution is introduced into the vials and these can then be autoclaved and freeze dried and the final product satisfies the required standards of sterility and activity achieved by the original procedure.

Storage Tests.

A considerable number of vials prepared by the above procedure were stored at room temperature for 6 months

and tested at intervals to determine the effect of storage. The results obtained in some of these tests are shown in Table X.. from which it appears that no loss of pyrogenic activity occurred in this time.

Table X.. Effect of Storage at Room Temperature on the pyrogenic activity of the linopolysaccharide-mannitol pH 5.5 preparation.

Period of Storage in Months.	Fever Response °C.	Period of Storage in Months.	Fever Response °C.
0	1.42	3.5	1.39
1	1.40	4.0	1.41
1.5	1.39	4.5	1.45
2.0	1.45	5.0	1.36
2.5	1.43	5.5	1.46
3.0	1.37	6.0	1.44

Dosage in every test 0.05 mg/kg. body weight.

VII. GENERAL DISCUSSION.

The major aim of this investigation was to isolate and characterise the pyrogenic factor from *P.vulgaris* cells in order to compare it with the pyrogen obtained from cell-free culture filtrates of the same organism. This was undertaken because of the suggestion by Wylie and Todd that a Gram-negative bacterial culture contained two distinct pyrogenic factors, viz., a fast-acting pyrogen which occurred in the cell-free filtrate, and a slow-acting cell-associated pyrogen.[131]

Proteus vulgaris NCST53 was used here because it was the organism most fully investigated by Wylie and Todd. This strain offered other advantages since it was easily grown in nutrient or synthetic media, was a good source of pyrogen, and being only a mild pathogen gave minimal risk in handling large volumes of culture fluids.

It was shown in the review section that the pyrogenicity of Gram-negative bacteria is a property of the endotoxic complex, and that a number of methods were available that would extract an endotoxic fraction with pyrogenic activity. Phenol extraction was chosen for this study because with other Gram-negative bacteria it had been the most widely applicable method and yielded

the most pyrogenic product. Such products were protein-free and poorly, if at all, antigenic in rabbits. This was an advantage, for the secondary aim of this investigation was the production of a purified reference pyrogen which should preferably be non-antigenic.

Since the culture filtrate pyrogen was already being isolated from synthetic medium cultures, it would have been ideal to have used the same culture as a source of the cellular pyrogen, but the yield of cells was low. A much higher yield of cells was obtainable from nutrient broth cultures but it was not known if the cells would contain the same pyrogenic factor if grown in different media. Therefore, phenol extracts were prepared from both types of cell and compared. The comparative tests were designed to establish:- (i) did each extract contain pyrogenic lipopolysaccharide, and (ii) was it the same lipopolysaccharide in both.

The yield, elemental analysis and overall composition of both phenol extracts were comparable with those reported for crude lipopolysaccharide preparations from other Gram-negative bacteria. The lipopolysaccharide nature of both preparations was confirmed by the release on acid hydrolysis of monosaccharides and a chloroform-soluble material. Possibly the strongest evidence for presence of lipopolysaccharide in the extracts was their strong

pyrogenicity; such a degree of pyrogenic activity is found only in Gram-negative bacterial lipopolysaccharide preparations.

The presence of the same monosaccharides in similar ratios, similar general character and comparable pyrogenicity over a range of doses, suggested the same lipopolysaccharide was present in both cell extracts. The detection, by gel diffusion-precipitin tests, of a heat-stable serologically identical antigen (or hapten) in both products strengthened the evidence in favour of a common lipopolysaccharide, since such heat stability is characteristic of lipopolysaccharides. These results were accepted as sufficient evidence that the synthetic medium and nutrient medium cells contained the same lipopolysaccharide. This permitted investigation of methods of purification and analysis to be carried out on extracts from the more readily available cells from nutrient broth cultures.

Of the purifications methods tried, ultracentrifugation was the best, but where an ultracentrifuge was not available ammonium sulphate precipitation would be the method of choice. Some of the methods that failed have had reported success elsewhere, but perhaps the existence of such a range of methods for nucleic acid removal is an indication that no single method can expect universal success.

The chemical and physical tests applied did not reveal heterogeneity in excess of 4% in the purified lipopolysaccharide prepared from nutrient medium grown cells. However, these tests would fail to detect contamination with a closely similar substance. Serological tests demonstrated the immunological homogeneity of the lipopolysaccharide. The absence of contamination of the lipopolysaccharide by any native P.vulgaris antigens was shown by the failure to induce antibody against any P.vulgaris antigens by injection of the lipopolysaccharide into rabbits. A further critical test of the serological homogeneity was when the lipopolysaccharide-protein complex induced antibody which gave only a single line of precipitation with P.vulgaris cells or lipopolysaccharide. These tests, however, would fail to reveal any non-immunological contaminants.

In its overall composition, analysis, serological and biological properties, the lipopolysaccharide preparation was comparable with lipopolysaccharides from other Gram-negative bacteria.

The lipopolysaccharide contained only monosaccharides which had previously been reported in other Gram-negative bacteria. Since the monosaccharides were not isolated their identification is tentative. However, the probability of the identification being correct is strengthened

by the fact that each was detected by at least two independent methods. The recognition of the aldoheptose as D-glycero-L-mannoheptose, or its optical enantiomorph is weak because the chromatographic and electrophoretic data on which it was based are available for only twelve of the aldoheptose isomers. However, it should be noted that glycero-mannoheptose is the only aldoheptose so far detected in the Enterobacteriaceae.[30]

Summation of the percentage contents of monosaccharides, organic phosphate and lipid, accounts for only about 80% of the total lipopolysaccharide, but this is typical of values quoted for other lipopolysaccharides.(Ref. 24, 28, 38, 68).

The lipopolysaccharide LPS also behaved typically on hydrolysis with acetic acid, yielding degraded polysaccharide and lipid A. Although the degraded polysaccharide contained all the monosaccharides of the intact lipopolysaccharide LPS, some damage to it must have been caused by the gentle hydrolysis procedure since it did not retain the serological specificity of the lipopolysaccharide. This loss of specificity is unusual in Enterobacterial products[149] but has been reported with a Bordetella pertussis lipopolysaccharide.[68]

Phenol extraction and ultracentrifugation had

apparently yielded a purified typical lipopolysaccharide from cells grown in nutrient broth culture (LPS). The same methods were therefore applied to a small batch of P.vulgaris cells grown in synthetic medium culture. The product obtained, although not so fully analysed because of shortage of material, was used for comparative purposes as the equivalent purified lipopolysaccharide (LPI) from cells grown in synthetic medium culture.

Comparison was made of the cell extracted lipopolysaccharides LPS and LPI with the culture filtrate lipopolysaccharide (F.P.S.). Elemental analysis, overall chemical constitution, monosaccharide and fatty acid content, biological and serological properties, demonstrated a close similarity for all three lipopolysaccharides. Against such similarity some points of difference were found. Traces of mannose were found only in lipopolysaccharides LPI and FPS, but since all preparations of FPS did not contain mannose it is regarded as a trace contaminant. The second faint line of precipitation shown by LPI and FPS only in the gel-diffusion-precipitin tests may have been due to the mannose-containing contaminant but it could also have been a reflection of the physical state of the products. Of the eight higher fatty acids recognised in all three products, two (arachidic and stearic acids) appeared from chromatograms to be present in smaller amount in lipopolysaccharides LPI and FPS

than in lipopolysaccharide LPS. This would appear to be a reflection of the medium used for the cell growth.

In the review section attention was drawn to the fact that Ikawa et al[78] isolated only four fatty acids from a culture filtrate lipopolysaccharide while Nowotny et al[80] by chromatography showed the presence of all the higher fatty acids from C(10) to C(20) in a number of lipopolysaccharides obtained from cells. It was mentioned that these results might indicate a difference in culture filtrate and cellular lipopolysaccharides or just be due to a difference in sensitivity of the two techniques. The latter explanation now seems likely since studies of lipopolysaccharides LPI, LPS and FPS have shown that all three contain all the fatty acids from C(10) to C(20).

The pyrogenic activity of the lipopolysaccharides LPS, LPI and FPS were similar over a range of dosage. Of importance was the demonstration that each of the lipopolysaccharides induced in rabbits a single early-peak (c.1.5 hours post injection) fever at low dosage, and a double-peak fever (c.1.5 and 3.5 hours post injection) at high dosage. From these results, and from the similarity in composition already described, it was deduced that the cellular and culture filtrate pyrogens were the same or closely similar.

This conclusion disagrees with the postulate of Wylie

and Todd that there exists distinct cellular and culture-filtrate pyrogens. Their postulate was based on the following findings :-

(a) injection of cell-free filtrate of a viable culture produced only a single early-peak fever.

(b) injection of an autoclaved whole culture or of a filtrate of an autoclaved culture induced a double-peak fever.

(c) injection of well-washed cells produced only a late-peak fever.

The culture filtrate lipopolysaccharide FPS, which accounts for all the pyrogenic activity of the filtrate, has been shown to give a similar fever to that described in experiment (a). However, if given at higher dosage it induced a double peak effect. Wylie and Todd tested the culture filtrate at only one dose level.

From experiment (b) Wylie and Todd said that autoclaving released a slow-acting cellular pyrogen and this caused the second fever. On the basis of the present studies it could be explained equally well by the release from the cell of more of the same lipopolysaccharide. It has been shown that following injection into rabbits, lipopolysaccharides are almost immediately sequestered by the leucocytes but that about 20% always remains free

in the serum.[150] A recent theory of pyrogenic action[151] contends that this residual lipopolysaccharide causes the initial early fever. The leucocytes which have taken up the rest of the lipopolysaccharide migrate to the reticulo-endothelial system. If sufficient dosage has been given, there is a later release of pyrogenic material and this induces late-fever peak. This would explain the single- or double-peak effect which has been obtained with each of the lipopolysaccharides LPI, LPS and PPS, depending on the dosage used. It also offers an explanation why only a late-peak fever was found by Wylie and Todd on injection of cells. They state their cells were well-washed, so would be free of any soluble pyrogenic factor. Bacterial cells are known to be completely cleared from the bloodstream within minutes after injection. It seems possible, then, that in the absence of any soluble pyrogenic factor they obtained no early peak, but later release of pyrogen from the entrapped cells in the reticulo-endothelial system caused the late fever.

Because of the need for a suitable reference pyrogen preparation, mainly to help eliminate the deficiencies in the limit test for pyrogens in injection fluids, the formulation studies in the last part of the experimental section were undertaken. The lipopolysaccharide from

nutrient-medium-grown cells appeared to be a suitable material for a standard pyrogen since it had been characterised sufficiently to be replaceable, was highly pyrogenic, non-antigenic and had a high toxic:active dose ratio in rabbits.

Lipopolysaccharides retain their pyrogenic activity for longer periods as freeze-dried powders than in solution, but to present the lipopolysaccharide as a freeze-dried standard posed a number of problems. It was known to be difficultly soluble in water or salt solutions, was strongly adsorbed to glass surfaces and because of its high potency the quantity contained in a single-dose or small-number-dose vial would scarcely be visible. It was felt that the combination of these factors would have caused difficulty in ensuring complete solution of the contents of any ampoule, and in consequence administration of accurate dosage would have been uncertain.

To overcome these problems it was decided to freeze-dry the lipopolysaccharide with a considerable quantity of soluble inert substrate, which would serve three purposes:-

(i) the lipopolysaccharide would be distributed in a finely dispersed form throughout the substrate, thus greatly assisting resolution,

(ii) it would be preferentially adsorbed onto the substrate rather than the glass surface of the container, and

(iii) the material in each vial would be visible and so its solution could be seen.

Mannitol was chosen as the substrate because it was water-soluble even after freeze-drying, was non-pyrogenic per se, would withstand autoclaving and in the dosage employed caused no detectable pharmacological reaction on injection into rabbits.

From the studies described on the mannitol-lipopolysaccharide preparation, it was decided that the pH 5.5 product was the most suitable. It should be noted that this preparation could also be made with inclusion of a bacteriostatic agent without affecting the pyrogenicity. This would be of some advantage if the products had to be stored for long periods. However, recent interest in method of action of pyrogens has involved increased use of intracerebral methods of injection. The British Pharmacopoeia forbids the inclusion of a bacteriostatic agent where intracerebral injection is planned so it was of importance that the preparation, without bacteriostatic agent, could be autoclaved without destruction.

The storage tests have shown that over a six-month period no loss could be detected, even though the storage was at room temperature and with no precautions taken except the exclusion of light.

This mannitol-lipopolysaccharide preparation appears

to be a suitable reference standard, but its widespread use could only be recommended after a much more rigorous trial than could be performed in one laboratory. What is suggested is that the methods used here could be applied to any lipopolysaccharide preparation. Even the present product distributed by the World Health Organization, the O-somatic antigen complex of Shigella disenteriae, is not effectively presented. It is offered in milligram quantities in a single ampoule which requires redistribution by each laboratory into suitable number of test samples, and no recommendation has been made for preparation of such samples, their sterilization or their storage. Different methods of handling the preparation could introduce errors into any comparative tests between laboratories. This could be offset by the provision of the material in such a form as the mannitol-lipopolysaccharide preparation described, terminally sterilised in suitable dose containers, and with directions for storage.

An alternative application of the mannitol-lipopolysaccharide formulation is that, even in laboratories supplied with the World Health Organization preparation, there will be the need for a local standard calibrated against the W.H.O. product. Such local standards could conveniently take the form of the mannitol-lipopolysaccharide preparation.

VIII. Appendix I. GROWTH AND CULTURAL CHARACTERISTICS OF PROTEUS VULGARIS NCTC253.

1. Morphology.

After growth on nutrient agar for one day at 37°C the organisms were straight, parallel-sided, round-ended rods approximately 1 x 3μ in size. These Gram-negative rods were actively motile by peritrichous flagella, and occurred singly or in pairs, but occasional filaments were seen. No spore formation was observed.

2. Cultural Characters.

(a) The following characteristics were noted on solid agar medium:

	Colonies on agar at 37°C	Film on agar at 37°C
Shape	Circular	"
Size	0.1-1.5mm.	"
Degree	"	Moderate-Abundant
Form	"	Spreading
Opacity	Translucent	Transparent
Structure	Amorphous	Amorphous
Edge	Entire	Undulate
Colour	Greyish-white	Greyish-white
Elevation	Low convex	Raised
Surface	Smooth	Smooth
Lustre	Shining	Shining
Consistency	Butyrous	Butyrous
Emulsifies	Easily	Easily
Differentiation	None	"
Medium	Unchanged	Unchanged

The characters described above for both types of growth are those noted after one day. Examination after six days growth showed that the colonies had become opaque and the streak translucent, but in both cases the characters were otherwise unchanged.

(b) Growth characters in liquid medium:

	Period of growth in nutrient broth medium at 37°C.	
	1 day	6 days
Turbidity	++	
Degree	Abundant	
Nature	Uniform	
Deposit	+	
Degree	Moderate	
Type	Flocculent-viscid	Granular
Shaking	Disintegrates with difficulty	
Surface Growth	+	
Nature	Pellicle	Pellicle + ring
Degree	Slight & thin	
Surface	Smooth	Otherwise as for 1 day.
Shaking	Easily	
Odour	Rather faecal	
Medium	Unchanged	

3. Growth-Temperature Relationship.

Growth of the organism on nutrient agar was determined at various temperatures and these are summarised in table below:

Temperature °C.	Degree of growth after 1 day.
18	Slight
25	Slight
30	Slight-moderate
37	Moderate
55	No growth

4. Metabolism-Biochemical Tests.

(a) The methyl-red test was positive, but the Voges-Proskauer test was negative after six days incubation at 37°C. In the same period the organism gave weak production of hydrogen sulphide but was unable to metabolise Kosar's Citrate.

Production of indole and catalase, destruction of urea and reduction of nitrate to nitrite were all positive after one days growth.

In the gelatin stab test liquefaction was obvious after two days incubation either at 37°C or 25°C.

On a potato plug a yellowish-grey growth was produced and on McConkey agar non-lactose fermenting colonies were produced within 24 hours.

(b) Sugar metabolism. This is summarised in the following table.

Carbohydrates tested	Number of days incubation at 37°C.	
	1	6
Glucose	A	Ag
Sucrose	Ag	AG
Lactose	O	O
Mannitol	O	O
Dextrin	O	O
Fructose	a?	Ag
Galactose	AG	AG
Glycerol	O	AG
Maltose	AG	AG
Mannose	O	O
Salicin	Ag	Ag
Xylose	O	O

a = slight acid production.
A = strong acid production.
g = slight gas production.
G = strong gas production.

Throughout the above tests where 'agar' is mentioned this means nutrient Lab-Lemco agar medium. Similarly, 'broth' means nutrient Lab-Lemco broth medium.

IX. Appendix II. APPARATUS AND PROCEDURE EMPLOYED IN FEVER TESTS.

The apparatus employed was basically as described by Wylie[4]. Essentially it consisted of copper-constantan thermocouples, for rectal insertion into rabbits, which could be connected in sequence into circuit with a similar reference thermocouple, the end of which was immersed in a water-bath. The water-bath was set around 35°C and the temperature controlled to within ± 0.01°C by means of a mercury-toluene regulator connected through a Sunvic relay to the electrical bath heaters. The current which flowed as a result of the temperature difference between the test and reference thermocouples was detected by the inclusion in the circuit of a galvanometer of 50 ohms internal resistance.

The thermocouples were initially made extra long and after careful calibration at a number of temperature levels, were cut to size, so that a 1°C difference between the test and reference thermocouple was expressed as a 10-division change in the galvanometer reading. This allowed direct plotting of the galvanometer readings on to 1-inch, 10-division square graph paper and each inch rise in the graph could be read off directly as a 1°C

rise in temperature.

Rabbits. Mixed breeds of rabbits were employed and were accepted for testing if the following conditions were satisfied viz.,

(i) weight was between 1.5 and 3 kilograms,

(ii) normal temperature fell within the range 38.9 - 39.8°C,

(iii) on injection with pyrogen-free saline they did not show fever, but on injection with a known dose of pyrogen their fever response was not outwith the value of mean response of all the rabbits plus the standard deviation.

Conditioning of Rabbits. New rabbits required to be conditioned to the test procedure before being used in tests. This consisted of placing the rabbits in the restraining boxes for increasing periods of time each day until they were used to remaining in the boxes for five to six hours, the normal duration of a test. After this the rabbits were conditioned to the insertion and retention of the rectal thermocouples by a similar graded method.

Grouping of Rabbits. All the rabbits in the colony were tested three times at each of three dose levels with Escherichia coli lipopolysaccharide. The doses used were chosen to fall on the steep part of the known log-dose response curve for the lipopolysaccharide.

The average values of the fever responses for each dose level were calculated for each rabbit, and compared with the average values for the whole colony. Any rabbits which showed excessively high or low responses were rejected. The remaining rabbits were divided into groups of five. The groups were determined from the fever responses to the E.coli lipopolysaccharide, such that each group of five had a similar average fever response to any of the other groups. The differences in the mean fever responses of each group was less than the standard deviation for all groups. Thus, it was valid to compare the pyrogenicity of any two products by their responses in different groups, and a twin-cross-over test was not required.

Fasting. The rabbits were denied solid food for fifteen hours preceding and during the test. This diminished any metabolic temperature changes, and also reduced the risk of dislodgement of the thermocouple by defaecation during the test.

Test Procedure. Pyrogen tests were carried out in the room in which the rabbits were normally housed, but in a partitioned section. The apparatus allowed simultaneous testing of eighteen rabbits, but in general only ten rabbits were used at one time. The availability of

extra thermocouples was, however, an advantage, since the not infrequent breaking of thermocouples did not necessitate termination of the test.

Rabbits were placed in the restraining boxes on racks facing away from the operator and the recording apparatus. After about ten minutes thermocouples were inserted into the rectums to a depth of 6 centimetres, the distance being fixed by a rubber stop on the thermocouple, and tied lightly to the tail with wire. After forty-five minutes the recording of temperature was begun and continued at ten-minute intervals for forty minutes or until a stable normal temperature was reached.

Test samples were dissolved in pyrogen-free saline at room temperature and injected into an ear vein in a volume equivalent to 1 ml. per kg. body weight. Temperature readings were taken immediately following injection and at ten-minute intervals throughout a 4½ - 5 hour period.

During the test, noise and movement in the test room were kept to a minimum. The rabbits were checked at intervals to ensure the thermocouples were properly in place and that the rabbits were not drowsy.

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Abstract

The major aim of this research was the isolation and characterization of the heat-stable pyrogenic factor from the cells of Proteus vulgaris NCIB 53, in order to compare it with the fever-producing factor, obtained by a colleague, from culture filtrates of the same organism. This was undertaken because a report on the fever induced by this bacterium in rabbits, claimed that there existed qualitatively different cell-associated and filtrate pyrogens. Recent studies had shown that the pyrogenic activity of Gram-negative bacteria was associated with the endotoxic complex and could most conveniently be obtained as the lipopolysaccharide component. However, no definitive study of the lipopolysaccharide of P. vulgaris had been reported nor was information available on a comparison of lipopolysaccharides obtained from cells and culture filtrates of any micro-organism.

The culture filtrate pyrogen had to be obtained from synthetic medium cultures but the quantity of cells from this source was small. Therefore, initially crude pyrogenic lipopolysaccharide preparations were extracted with phenol from cells grown in synthetic medium and in nutrient broth cultures. Both cell

extracts were shown to contain the same lipopolysaccharide on the basis of their similar elemental analysis, monosaccharide constitution, equivalent pyrogenicity and the serological identity of their major heat-stable component. Therefore, methods of purification and analysis were investigated using extracts from cells grown in nutrient broth cultures since they could be obtained in greater quantity.

The extract from nutrient broth culture cells was purified by differential ultracentrifugation. The purified lipopolysaccharide (LPS) had a reducing sugar value of 32%, and contained 16% hexosamine and 3-32% bound lipid. Monosaccharide constituents were glucose, galactose and an aldoheptose (probably D-glycero-D-manoheptose), glucosamine and galactosamine. Protein could not be detected and nucleic acid contamination did not exceed 2%. Physical and chemical methods did not reveal heterogeneity in excess of 4% and immunological homogeneity was established. The lipid component contained all the saturated fatty acids of chain length C_{13} to C_{22} plus two long chain unsaturated acids and β-hydroxy-myristic acid. The lipopolysaccharide was highly pyrogenic in rabbits; the minimum pyrogenic dose was 0.002 µg/kg. body weight. The LD_{50}

in mice was 1 mg. total dose, and in rabbits dosage in excess of 20 µg/kg. was toxic.

From synthetic medium culture cells a small quantity of lipopolysaccharide (LPI) was extracted, purified and analysed by the methods which had been used for the lipopolysaccharide (LPS) from nutrient broth culture cells. These lipopolysaccharides (LPI and LPS) were compared with a lipopolysaccharide (PPS) prepared by a colleague from synthetic medium culture filtrates. Their elemental analysis, carbohydrate and lipid contents were similar. Common constituents of all three preparations were the monosaccharides and fatty acids listed above. They gave serological reactions of complete identity and their pyrogenic activities were qualitatively and quantitatively alike. Minor dissimilarities in the relative content of two fatty acids was the only significant disparity among the products. In summary the same lipopolysaccharide was apparently being obtained from cells and from culture filtrates. Since the lipopolysaccharide (PPI) accounted for all the pyrogenic activity of the culture filtrate, there seems no justification for belief in the hypothesis of the existence of distinct cell-associated and culture filtrate pyrogens. This hypothesis was

based on the appearance of single or biphasic fever responses in rabbits, but it was found that all three lipopolysaccharide preparations could produce either type of fever dependant purely on the dosage administered.

The secondary aim of this research project was the pharmaceutical formulation of a standard reference pyrogen. A preparation was made by freeze-drying the lipopolysaccharide (A) with a mannitol carrier and its properties investigated. The preparation obtained appears to be a suitable reference standard because it is readily dispersable with full recovery of activity, can be terminally sterilised, is unaffected by the presence of a bacteriostatic agent and is stable on storage.