STUDIES ON AN ENDOTOXIN PRODUCED BY PSEUDOMONAS AERUGINOSA

SUMMARY

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The Introduction to the thesis is devoted to a review of the principal methods which have been used in the extraction of the endotoxins or somatic antigens of Gram negative bacteria, and the chemical nature of these substances. The part played by the lipid portion of the complex in the biological activity is discussed along with the effect on the biological activity of chemical modification of endotoxins.

The experimental work is in two main sections.

In the first of these, the isolation, purification and properties of the endotoxin from Pseudomonas aeruginosa are described and the results discussed.

The endotoxin was isolated as a lipopolysaccharide by a procedure which involved heating acetone-dried bacterial cells with aqueous phenol, followed by dialysis and high speed centrifugation of the aqueous extract. The lipopolysaccharide was shown to contain galactose, glucose, galactosamine, glucosamine and an aldoheptose. The lipid portion obtained after short acid hydrolysis contained ether-soluble and chloroform-soluble fractions in both of which glucosamine, several amino acids and a series of fatty acids were detected.

The lipopolysaccharide was shown to be antigenic and highly pyrogenic when injected into rabbits, and to be toxic to rabbits and mice.

The second experimental section is concerned with the effect of a combination of trichloracetic acid and phenol extraction on the Pseudomonas aeruginosa cells. Cold trichloracetic acid extraction by the Boivin techniqu

gave a low yield of endotoxin, but it was found that the residue after trichloracetic acid extraction could be treated with hot aqueous phenol to give appreciable amounts of an endotoxic material which was similar to the endotoxin obtained by direct treatment of the cells with hot phenol, but which was free from contaminating nucleic acid. By the use of the combined extraction technique, nucleic acid-free lipopolysaccharides were also obtained in good yield from Proteus vulgaris and Escherichia coli.

It was also found that the maintenance of low temperature during the trichloracetic acid extraction was not essential when using the double extraction method to obtain nucleic acid-free lipopolysaccharides.

A further series of experiments showed that even after multiple extraction with cold trichloracetic acid, a considerable amount of endotoxin remained unextracted and could be obtained from the residue by treatment with hot phenol.

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CONTENTS.

P R E F A C E.

A common feature among Gram -ve bacteria is the presence on the cell surface of an antigenic toxic and pyrogenic complex which forms the somatic antigen or endotoxin of the cell. Interest in these substances has developed rapidly since the work of Boivin in the 1930's and they have been the subject of a very large number of investigations reflecting the interests of chemists, immunologists and pharmacologists.

The aim of the work described in this thesis was to add to the general body of knowledge of these important biological substances, using an organism, Pseudomonas aeruginosa, which has not been the subject of extensive investigation. The relative unimportance of Pseudomonas aeruginosa as a human pathogen is probably the principal reason why this organism has not been the object of more detailed studies. It is interesting therefore to note reports of the increasing incidence of fatal generalized septicaemia resulting from Pseudomonas aeruginosa infections, and the fact that in some hospitals it has replaced staphylococci as the principal cause of septicaemia (1). There may well be some justification for the claim that Pseudomonas 'is making a bid for the role of "Ghengis Khan of the microbial world"' (2), and it seems likely that more attention will be paid to the toxic and antigenic properties of this group of bacteria.

INTRODUCTION AND REVIEW.

Among the first attempts to isolate the specific antigenic substances from Gram -ve bacteria were those of Pick (3) and Zinsser and Parker (4) involving saline extracts of typhoid bacilli. It was, however, with the work of Boivin and his associates that the first real progress was made in elucidating the nature and properties of the antigenic endotoxins. Following the introduction in 1932 of his cold trichloracetic acid (TCA) extraction procedure (5), Boivin examined the properties of the antigenic complexes isolated from a wide range of organisms including Bacillus aertryke (Salmonella typhi-murium), Proteus vulgaris, Bacillus pyocyaneus (Pseudomonas aeruginosa), B. paratyphi B and Shiga (6).

Several other extraction procedures were subsequently introduced by other workers. Raistrick and Topley (7) used tryptic digestion to isolate the antigenic and toxic somatic substance from Bact. aertryke (S. typhi-murium), and Miles and Pirie (8) extracted the somatic antigen from smooth strains of Brucella melitensis with 2% phenol. Diethylene glycol was used by Morgan (9) for the extraction of B. dysenteriae (Shiga), this solvent being chosen because it was neutral, dialysable and relatively specific in that it did not dissolve proteins or nucleic acids. In 1940, Palmer and Gerlough (10) utilized the protein-dissolving properties of phenol in the extraction of Eberthella typhosa (Salmonella typhosa). After treatment/

treatment of the acetone dried cells with 98% phenol, saline extraction of the residue yielded a toxic and antigenic substance which was almost protein-free. Westphal later modified the Palmer and Gerlough method, and by using mixtures of 90% phenol and water at higher temperatures obtained extracts which contained the endotoxic lipopolysaccharide essentially free from protein (11). This hot phenol method has been used successfully with a wide range of organisms and it appears to be a generally effective method of extracting endotoxins from Gram -ve bacteria.

Apart from these techniques, many other extraction methods have been introduced by other workers, and some of these are listed in Table 1 along with the methods mentioned above.

TABLE 1.

Procedure.	Authors.
Trichloracetic acid	Boivin (5)
Tryptic digestion	Raistrick and Topley (7)
Phenol (2%)	Miles and Pirie (8)
Diethylene glycol	Morgan (9)
Cold phenol (90%)	Palmer and Gerlough (10)
Urea (2.5M)	Walker (12)
Pyridine (50%)	Goebel et al. (13)
Hot Water	Roberts (14)

TABLE 1 (continued)

Procedure.	Authors.
Hot phenol (45%)	Westphal et al. (11)
Ether or dioxane	Ribi et al. (15)

(Table 1 is taken mainly from Westphal (16))

Apart from hot 45% phenol, most of the extraction methods listed in Table 1 seem to have a limited applicability. For example, diethylene glycol was found to be a good solvent for the extraction of the somatic antigen of Shigella dysenteriae, but it failed to extract the antigenic material from some strains of Shigella flexneri (13), and while 50% aqueous pyridine extracted the antigen of Shigella paradysenteriae (Flexner), it was not successful with Shigella sonnei Phase 1 and Phase 2 for which 50% glycerol and 7M urea respectively were found to be suitable solvents (18). Although trichloroacetic acid has been used successfully with many Gram -ve bacteria, it may not be a very efficient solvent for the somatic antigen (v.i.), and generally fails to extract the somatic antigens from Rough bacteria and from those organisms in which the antigen is covered by a surface protein component (19).

In recent years, the study of the surface anatomy of bacterial cells has added a further dimension to the work on somatic antigens and endotoxins.

The development of satisfactory methods for the separation of cell walls from cytoplasmic material, combined with the use of the electron microscope, has resulted in a considerable increase in our knowledge of the organization of the surface layers of bacterial cells (20). It is now believed that Gram -ve bacteria have a more complex surface structure than Gram +ve organisms - one of the reasons for this additional complexity being the presence on the surface of Gram -ve bacteria of the complex endotoxin or somatic antigen. The true anatomical relationship between the somatic antigen and the cell wall has been a matter of some debate. Wilkinson (21) considered that although closely bound to the cell wall, the antigen did not form an integral part of it, and he suggested that as it did not conform to the normal definition of a capsule (i.e. visible under the light microscope) it was best described as a microcapsule. He pointed out that the O antigenof Smooth Gram -ve bacteria could be lost by mutation without affecting cell viability, a typical property of capsules but not of cell walls. Davies, however, has suggested (22) that the Smooth to Rough variation may not involve the loss of an O antigen leaving the R antigen exposed, but may simply reflect a change in polysaccharide constitution. If this is so, then the loss of O specificity is not necessarily evidence for the microcapsular nature of the somatic antigen. The results obtained by Salton (23) on studies concerning the distribution of sugars in the isolated cell walls of several Gram -ve bacteria support the view that the/

the specific polysaccharides of Rough and Smooth antigens form an integral part of the lipid-polysaccharide-protein fraction of the cell wall and he concluded that the description of the polysaccharide complexes as 'microcapsular' was misleading and should be discontinued.

CHEMICAL AND BIOLOGICAL PROPERTIES OF ENDOTOXINS.

1. THE NATURE OF THE ISOLATED COMPLEXES.

The nature of the substances extracted by most of the methods listed in Table 1 has been adequately reviewed by Burrows (24) and by Westphal (25) and will not be considered in detail.

Boivin initially considered the endotoxic O antigens obtained by TCA extraction of Smooth Gram -ve bacteria to be protein-free glycolipids (26). It was subsequently shown however (27) that although they did not give the usual qualitative tests for proteins, they were in fact essentially similar to the substances which Morgan obtained from Shigella dysenteriae and Salmonella typhosa by extraction with Diethylene glycol and characterized by him as protein-polysaccharide-lipid complexes (28,29) of which the lipid was identified as a kephalin type (28,30).

Westphal later showed (56) that the polysaccharide contained an additional lipid component and introduced the terms lipid A and lipid B to distinguish between the/

the lipid associated with the polysaccharide (lipid A) and the kephalin-type lipid identified by Morgan (lipid B).

It is now clear that endotoxins can be isolated either as protein-polysaccharide-lipid complexes or as lipopolysaccharides depending on the extraction method employed.

The protein part of the complex plays an important role in the antigenicity of the endotoxin (16) and in the case of certain strains of Escherichia coli it is associated with bacteriocine activity (colicine K) (31). Apart from these effects, however, the protein component apparently plays no part in the general biological effects of endotoxins (16). The protein-free lipopolysaccharide shows all the biological properties of the endotoxin (32) and although it may be less potent than the protein complex in the stimulation of antibody formation, it contains the groups responsible for the specificity of the somatic antigen.

2. CONSTITUENTS OF THE LIPOPOLYSACCHARIDES.

Table 2 lists some of the chemical properties of lipopolysaccharides obtained from different organisms by extraction with hot 45% phenol.

TABLE 2.

Organism.	N (%)	P (%)	Sugar (%)	Lipid (%)	Hexosamine (%)	Reference
S. abortus equi	1.3-1.6	2.7-3.2	56-62	26	7.2	33
Ps. aeruginosa	3.5	3.7	30	+	5	34
P. pestis	1.6	2.2	30	37.5	15	19
N. gonorrhoeae	3.3	3.84	43	28.4	13.8	36

Acid hydrolysis results in a cleavage of the complex into lipid and polysaccharide. The lipid (lipid A) is normally isolated by heating solutions of the lipopolysaccharide in N mineral acid for 30-60 minutes.(37). These conditions, however, cause considerable breakdown of the polysaccharide, and for studies on the intact polysaccharide ('degraded' polysaccharide - Davies (40)) milder hydrolysis in 1% acetic acid as described by Morgan and Partridge (28), is more suitable.

A. SUGAR COMPONENTS OF THE LIPOPOLYSACCHARIDES.

The sugars present in the lipopolysaccharide can be identified by examination of hydrolysates of the degraded polysaccharide or hydrolysates of the lipopolysaccharide without prior separation of the polysaccharide. component. The conditions of hydrolysis used by different workers in the investigation of the component sugars show a considerable variation. Westphal (33), investigating the sugars present in the lipopolysaccharide from Salmonella abortus equi, found that the maximum reducing sugar value was obtained after 2 - 3 hours hydrolysis in N sulphuric acid and used these conditions for chromatographic identification of the sugars present. Davies (19) found that the maximum reducing value for the lipopolysaccharide from Pasteurella pestis occurred after 6 hours hydrolysis in 0.5N hydrochloric acid, but for chromatographic identification of the sugars he hydrolysed the material for 15 hours in N sulphuric acid at/

at 100°C. MacLennan has shown (39) the lipopolysaccharide from Bordetella bronchisepticus to be relatively resistant to the mild hydrolysis conditions normally used to determine total reducing sugars. The reducing value after 24 hours hydrolysis in 0.5N hydrochloric acid was 7%, yet the lipopolysaccharide was shown to contain 5% hexose (Dische), 18% aldoheptose (Dische) and 16.6% hexosamine (Rondle and Morgan).

Some of the sugars which occur as constituents of endotoxins (3:6-dideoxy hexoses - see below) are acid labile and may be destroyed by extended periods of hydrolysis, so it would seem therefore a wise precaution to use a range of hydrolysis conditions when attempting to identify the component sugars of a previously uninvestigated lipopolysaccharide.

Since the nature of the polysacharides of Gram -ve bacteria has recently (1960) been reviewed by Davies (40), while a wider coverage of the polysaccharides of micro-organisms is given in the recent book by Stacey and Barker (41), this Introduction will be concerned only with some of the more important aspects of the subject.

The complex heteropolysaccharides of Gram -ve bacteria may contain up to seven different sugar components. One or more of the common aldohexoses glucose, galactose and mannose along with glucosamine and/or galactosamine occur in most of the polysaccharides which have been/

been investigated. A wide range of additional sugars of different classes have, however, been identified and Table 3 lists sugars which have been found in somatic antigens or polysaccharide haptens.

TABLE 3.

Pentoses	Xylose	Di-deoxyhexoses	Colitose
	Arabinose		Abequose
	(Ribose)		Tyvelose
			Paratose
Aldohexoses	Glucose		Ascarylose
	Galactose		
	Mannose		
		Aldoheptoses	D-glycero-D-galacto-
Amino sugars	Glucosamine		D-glycero-D-manno-
	Galactosamine		L-glycero-D-mann0
	Fucosamine		

In the majority of cases, identification of sugar constituents has been by paper chromatographic examination of hydrolysates, a method which does not distinguish between D- and L- isomers.

Ribose has been reported as a constituent of the endotoxin of Pseudomonas aeruginosa (34). However, although polyribitol phosphate (teichoic acid) occurs as a major component of the cell walls of certain bacteria (43), and D-ribose, linked to a 2,6-diaminoaldohexose is found in the antibiotic Neomycin C (44), ribose has not been found in other /

other endotoxins, and the detection of it in hydrolysates is normally considered to be an indication of the presence of contaminating nucleic acid.

Hexuronic acids occur widely in capsular polysaccharides of Gram +ve bacteria, especially the pneumococci (41), but they have not been commonly reported as constituents of the somatic antigens of Gram -ve organisms. Uronic acid were found in the somatic antigens from 8 different species of Shigella flexneri (42), and Dzulynska and Mikulaszek (45) reported the probable presence of uronic acids in polysaccharides from 23 Gram -ve bacteria (including several Rough strains), but their chromatographic identification was rather unsatisfactory because they had no standard uronic acids for comparison. Galacturonic acid, however, has been detected by Davies in the degraded polysaccharide hapten from the somatic antigen of Chromobacterium violaceum strain 'Lewitus' (46), while Bordetella bronchisepticus lipopolysaccharide was found to have 20% uronic acid, based on the amount of CO_2 evolved on heating with 12% HCl and on the neutral equivalent obtained by titration in boiling 50% ethanol. However hexuronic acid could not be detected in the naphthoresorcinol or carbazole tests, nor on chromatograms sprayed with bromophenol blue (39).

Before the identification in 1952, of an aldoheptose in the lipopolysaccharide of Shigella sonnei Phase 2, (47), the only heptose /

heptose sugars known to occur in nature were ketoheptoses which had been identified in several plant materials (48) and as a phosphate ester in the products of yeast juice fermentation.(49)

Aldoheptoses have now been found in polysaccharides from many Gram -ve bacteria (see Davies (40)) and in several cases they have been isolated and identified. . It is interesting that, as yet, none of these sugars have been found outside this limited class of natural substances. The significance of the presence of these sugars in bacterial polysaccharides is not known. They may form up to 20-40% of the polysaccharide, but there is no evidence that they play any part in the antigenic specificity of the complex (50)

The other class of sugars of particular interest, the 3,6-dideoxy hexoses, are however known to play an important role in antigenic specificty. The presence of this type of sugar, previously unknown in nature, was first recognised in 1952, when Westphal (51) and Staub (52) detected, in hydrolysates of the polysaccharides of Salmonella abortus -equi and Salmonella typhosa, components which had R_f values greater than those of the 6-deoxyhexoses. These sugars were given the names abequose and tyvelose and were subsequently identified as 3,6-dideoxyaldohexoses (53). Three further examples of this class of sugar are now known: ascarylose, first found in the egg membrane of the worm Parascaris equorum (54), colitose from Escherichia coli 111 B4, and paratose from Salmonella paratyphi B. Table 4 - taken from Davies (40) -

lists these sugars and some of their properties.

TABLE 4.

Sugar.		D	R_{rh}
Abequose:	3,6-dideoxy-D-xylo-hexose	-3 to -4	1.16
Colitose:	3,6-dideoxy-L-xylo-hexose	+4	1.16
Ascarylose:	3,6-dideoxy-L-arabino-hexose	-25	1.29
Tyvelose:	3,6-dideoxy-D-arabino-hexose	+25	1.29
Paratose:	3,6-dideoxy-D-ribo-hexose	+10	1.25

Abequose and colitose and ascarylose and tyvelose are optical isomers; so far, no optical isomer of paratose has been found. Apart from Parascaris equorum, ascarylose has been identified in the specific polysaccharide of Pasteurella pseudotuberculosis Type 5 (55).

The rapid release of the dideoxy sugars from the polysaccharides on acid hydrolysis (32) suggested that they occupied terminal positions and as such might play an important part in the antigenic specificity. This has been confirmed in experiments which showed that these sugars could act as specific inhibitors of the precipitation of a polysaccharide by its homologous antiserum, tyvelose inhibiting the precipitation of S. typhose polysaccharide by its homologous antiserum (54), abequose inhibiting the precipitation of S. paratyphi B polysaccharide by/

by S. paratyphi B antiserum, and colitose inhibiting the precipitation of E. coli O111 polysaccharide by E. coli O111 antiserum (57). The specificity of the inhibition is shown by the failure of ascarylose, the optical isomer of tyvelose, to inhibit the precipitation of S. typhosa polysaccharide by S. typhosa antiserum (54) and the failure of abequose and colitose to inhibit the precipitation of E. coli O111 polysaccharide by E. coli antiserum and S. paratyphi B polysaccharide with S. paratyphi B antiserum respectively (57).

The Salmonella can be differentiated on the basis of their serological specificities, these specificities being symbolised in the Kauffmann-White scheme by numerals 1, 2, 3, 4, 5 etc. Up to four different specificities can be found in a single O antigen, and the numerous strains are arranged into broad groups depending on their O antigen formula, each group having a characteristic antigen - see Table 5.

TABLE 5.

Salmonella Group.	O antigens.
A	1, 2, 12
B	1, 4, 5, 12
D	9, 12
P	35

By inhibition studies (113) it has been shown that paratose is the determinant sugar of antigen 2 in Group A strains (e.g. S. paratyphi A), abequose the determinant of antigen 4 in Group B (S. paratyphi B) tyvelose of antigen 9 in Group D (S. typhi) colitose of antigen 35 in Group P (S. adelaide). Cross reactions between organisms such as S. adelaide and E. coli O111 B4 may thus be explained by the presence of a common antigen with colitose as the determinant group.

A more conclusive indication of the importance of the terminally linked 3, 6-dideoxy sugars in antigenic specificity has come from the preparation of artificial antigens containing colitose (58). The antigens were prepared by coupling diazotized p-amino-phenyl-α and -β-colito-pyranosides with purified bovine serum albumin or egg albumin. It was found that these antigens, when injected into goats, produced antibodies which were specific against colitose and which reacted against E. coli O111, causing agglutination of heated suspensions, precipitation of the specific polysaccharide and haemagglutination of erythrocytes sensitized with E. coli O111 lipopolysaccharide. The results showed that colitose itself can account to a great extent for the antigenic specificity of E. coli O111 (and serologically related organisms such as Salmonella O 35 and Arizona O 20. The antisera did not react strongly with another group of bacteria. (E. coli O55, Salmonella O50 and Arizona O9) which also contain terminally bound colitose - presumably because the structural arrangement of colitose in these organisms is different from that in the/

the artificial antigen and in the E. coli 0111 group. This emphasises the fact that identification of the component sugars of a polysaccharide is not in itself sufficient to provide a proper understanding of antigenic specificity. It is possible for two organisms to have the same sugars present in their polysaccharides and yet have quite different antigens. For example, Salmonella paratyphi B (Group B, antigens 1, 4, 5, 12) and Salmonella newport (Group C_2 antigens 6, 8) both contain glucose, galctose, mannose, rhamnose and abequose (59). Specificity is determined not only by the presence of certain sugars, but also by the manner in which they are organised.

B. CHEMISTRY OF LIPID A.

The first detailed studies of the composition of this lipid were carried out by Ikawa and Niemann (60) using a tumour necrotizing substance which theyisolated from culture filtrates of Escherichia coli. The isolated material consisted of a complex of polysaccharide with peptide and phospho-lipid from which a chloroform soluble lipid fraction could be obtained after heating in N sulphuric acid at 100°C for 1-2 hours. This fraction, which amounted to 25% of the weight of the complex, contained about 20% acetone-soluble material consisting mainly of fatty acids (61). Hydrolysis of the acetone-insoluble material in 5N hydrochloric acid for 10-16 hours led to the identification of the/

the following components:- lauric, myristic, palmitic and B-hydroxymyristic acids, D-glucosamine (probably N-acetylated), ethanolamine, aspartic acid, phosphoric acid and a previously unknown aliphatic amine, 4,5-diamino-eicosane, which they named necrosamine.

The components of the lipid from the highly purified lipopolysaccharide from Salmonella abortus equi were examined by Westphal (62). The lipid (lipid A) released after 30-60 minutes hydrolysis in N mineral acid at 100°C was soluble in chloroform and pyridine but only slightly soluble in other lipid solvents, and it represented 26% of the weight of the lipopolysaccharide. The products of hydrolysis of this lipid in 6N hydrochloric acid for 10-20 hours at 100°C included glucosamine, glutamic and aspartic acids, diaminopimelic acid, lysine, alanine, serine, ethanolamine and phosphoric acid. A series of long chain fatty acids was also obtained and by the use of reverse phase paper chromatography the following fatty acids were identified:- capric, lauric, myristic, palmitic, stearic, arachidic and lignoceric. There was an additional saturated acid with a high R_f value which was thought to be a β-OH acid and also four unsaturated acids. One of these had the same R_f as oleic acid but the other three were not identified (63). Necrosamine was not found in this lipid.

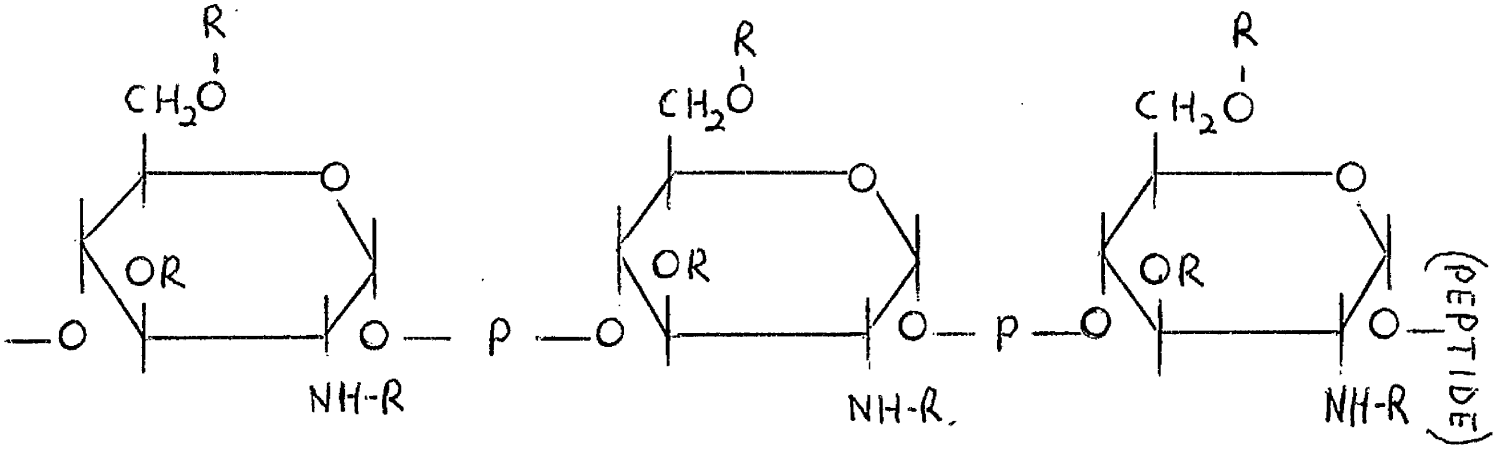
Westphal has compared the lipid components of lipopolysaccharides from several Gram -ve bacteria (25) and observed that although the amount/

amount present in the different lipopolysaccharides varied quite widely (from 10 to 30%), there was a close similarity in their analytical values. They all contained about 20% hexosamine, about 50% higher fatty acids and a peptide containing serine and amino-dicarboxylic acids (16). By paper chromatographic analysis, it was shown that lipopolysaccharides from eleven different species of Gram -ve bacteria all released the same series of long chain fatty acids on acid hydrolysis (63).

More detailed studies of lipid A have been carried out by Nowotny (64) who found that this lipid, obtained by acid hydrolysis from lipopolysaccharides, contained 30-40% free fatty acids which could be extracted with low molecular weight esters. Purification of the residue gave a mixture of three lipids present in varying amounts in different lipopolysaccharides. Complete hydrolysis of all three of these lipids yielded fatty acids, amino acids, glucosamine and phosphoric acid. The main difference between them appeared to be in the amount of peptide present. Using one of these lipids, FB 1 from Escherichia coli 08 (FB = firmly bound lipid) Nowotny found that after 12 hours hydrolysis in 3N hydrochloric acid, 55% was liberated as ether-soluble material and 45% as water-soluble. The ether-soluble fraction consisted mainly of fatty acids - C24, C22, C20, stearic, palmitic, myristic and β-hydroxymyristic. Examination of the water-soluble products by paper chromatography and high voltage paper electrophoresis showed the presence of glucosamine, aspartic and glutamic acids, valine, alanine/

alanine serine, arginine and lysine along with three glucosamine phosphates. The glucosamine phosphates were identified as D-glucosamine-4-phosphate, D-glucosamine-6-phosphate and 1-peptido-D-Glucosamine-4-phosphate, and it was then shown that the 6-phosphate was an acid-stable artefact produced during hydrolysis by transphosphorylation from the acid-labile 4-phosphate.

No glycerol, inositol or sphingosine could be detected in these lipids, and Nowotny has suggested that the structure is that of a glucosamine phosphate chain with the fatty acids present partly as esters through the hydroxyl groups on C3 and C6, and partly as amides. The postulated structure is



Burton, however, (65) found the purified lipid A from E. coli 0111 B4 to be free of amino acids and has proposed a different basic structure./

structure. After complete reduction of the lipid with sodium borohydride she found that half the original amino sugar was present as glucosamine and half as glucosaminol. She considered it unlikely that a lipid having a structure involving a Cl ester linkage as suggested by Nowotny could withstand the acid conditions used in the isolation of lipid A and that if any other diester linkage was involved the sodium borohydride would have reduced all the glucosamine to glucosaminol. On the basis of her results she proposed that the basic structure of lipid A consisted of two molecules of glucosamine joined by a glycosidic bond.

Burton also found that the lipid had reducing properties and considered this an indication that the lipid may be joined to the polysaccharide through the glucosamine. This suggestion had been advanced previously by Ikawa and Niemann (66) as a result of finding glucosamine in both the phospholipid and polysaccharide portions of the lipopolysaccharide from E. coli.

3. RELATIONSHIP OF THE STRUCTURE OF ENDOTOXINS TO THEIR BIOLOGICAL EFFECTS.

Apart from stimulating the production of antibodies, of a specificity determined by the nature of the polysaccharide portion of the complex, endotoxins also produce in experimental animals a syndrome of /

of additional biological effects. These effects, which have been reviewed by Burrows (67), Thomas (68), and Bennett and Cluff (69), are similar for all endotoxins, although there may be a variation in potency with different preparations. They are listed by Thomas as;

1. A profound vasomotor disturbance terminating in shock and characterized by intense generalized arteriolar constriction.
2. A metabolic disturbance consisting of hyperglycaemia followed by hypoglycaemia, abrupt depletion of liver glycogen and excessive amounts of lactic acid in the blood and tissues.
3. High fever sometimes followed by hypothermia.
4. Extreme polymorphonuclear leucopenia followed by leucocytosis.
5. Production of haemorrhagic necrosis in rapidly growing tumours.
6. With sublethal doses, the rapid appearance of a state of resistance against the same and other endotoxins.
7. Production of the local and generalized Shwartzman reactions.

Although a great deal is now known about these non-specific biological effects of endotoxins, there is still uncertainty about the precise mechanisms involved in their production. This can be illustrated by a consideration of the fever reaction - probably the most extensively studied of all these effects. It is known that after injection of endotoxin, there appears in the circulation an endogenous pyrogen similar to a fever producing substance which can be obtained from/

from polymorphonuclear leucocytes (for references see Bennett, I.L. (70). These facts have led to the suggestion that endotoxin fever results from a stimulation of the hypothalamus by the endogenous pyrogen released from leucocytes and is not due to a direct action of the endotoxin itself (71). This view of an entirely indirect effect of endotoxins has been challenged because of the observation that intrathecal injection of endotoxin results in a high fever without the production of detectable endogenous pyrogen (72), and also that during endotoxin fever endogenous pyrogen could not be detected in the sera of animals made leucopenic by prior injection with nitrogen mustard (72, 73). It has recently been shown however (74) that endogenous pyrogen can be detected in the sera of leucopenic animals if a sufficiently large volume is used. These workers considered that the decreased potency of such sera compared with those of normal rabbits injected with endotoxin is not convincing evidence against the hypothesis of the central role of endogenous pyrogen in endotoxin fever. The need for caution when applying conclusions reached as a result of experiments with rabbits to explain the mechanism of endotoxin fever in man is clear from the experiments of Cranston and his co-workers (75). They confirmed that a pyrogenic substance can be obtained from rabbit leucocytes by incubation at 37°, but they were, however, unable to detect any similar material in human leucocytes and concluded that the production of a pyrogenic substance from leucocytes themselves (as distinct from a pyrogen resulting from the interaction/

interaction of leucocytes with bacterial pyrogen) may not be essential in the production of fever. It is clear that even after a great deal of investigation, there is still uncertainty about the mechanism by which endotoxin fever is produced.

Westphal has suggested (16) that the great sensitivity of higher animals to endotoxins could be due to a type of anaphylactic reaction produced as a result of sensitization by endotoxin during the early years of life. This idea, which had previously been postulated by Stetson (76), when he drew attention to the similarities between the reactions of endotoxins and certain reactions of bacterial allergy, has received some support from the findings of Schaedler and Dubos (77). These workers found that mice raised and maintained free of ordinary pathogens and enteropathogens were highly resistant to the lethal effect of endotoxin, but susceptible to other effects- losing weight when treated with small (1 µg.) amounts, and succumbing rapidly to a fatal septicaemia when 1 ug or less of endotoxin was injected along with a suspension of St. aureus. After injection of heat-killed Gram -ve bacteria, the mice became highly susceptible to the lethal effects of injected endotoxin but showed an increased resistance to the infection-enhancing effect. Schaedler and Dubos concluded that there were at least two unrelated mechanisms involved in the pathological effects of endotoxins, one associated with what they called "primary toxicity" manifested by a loss of weight and an increased susceptibility to/

to infection, and a second reaction of a lethal nature which is of an immunological type resulting from prior sensitization of the animal by exposure to Gram -ve bacteria. These results, however, do not agree with those of Landy and his co-workers (78) who found that germ-free and conventionally reared mice showed no significant difference in their susceptibility to the lethal effects of endotoxins, nor are they supported by the results of experiments on guinea pigs carried out by Uhr (79) in which it was found that new-born guinea pigs were highly susceptible to the lethal effects of endotoxins and that this susceptibility decreased with age. Further reports which showed that chick embryos, new-born rabbits and germ-free rats were susceptible to the lethal effects of endotoxins are listed by Stetson (80). As Landy has pointed out (78) the impossibility of ensuring that a diet is antigen-free will probably prevent an accurate experimental assessment of the hypersensitivity theory, but the evidence available indicates that the variation in susceptibility to endotoxins is not controlled by the presence or absence of a bacterial flora.

The first progress in attempts to isolate biologically active portions of the endotoxin complex came from the work of Westphal and his co-workers who found that after coupling the lipopolysaccharide from S. abortus equi to casein, a 'lipocasein' could be obtained by heating the complex in 1% acetic acid for 1.5 hours. This lipoprotein, in which only the lipid was of bacterial origin was strongly toxic and pyrogenic/

pyrogenic in rabbits (25), while the isolated polysaccharide was inactive. Using this method, Westphal was able to prepare complexes which had up to 1/5th of the toxicity and pyrogenicity of the original material (38). When the lipid itself was injected into rabbits in the form of an aqueous suspension, it was pyrogenic at dose levels of 100 µg/kg to 1 mg/kg, whereas a suspension of the lipid in Tween was active at a level of 1µg/kg (38). Westphal subsequently reported the preparation of colloidal aqueous solutions of purified lipid A which had about 1/10th the biological activity of the original endotoxin.(82). Of special interest was the finding that injection of purified lipid A into mice produced an increase in non-specific resistance to infection without the initial decrease in resistance which occurs when the lipopolysaccharide is employed. On the basis of these results, Westphal has suggested that the lipid A portion of the complex is the carrier of endotoxic active groups (82) and that the degree of activity is determined by the extent to which it is dispersed - the most active preparations being those in which the lipid is combined with the water-solubilising polysaccharide (37).

Recently Westphal has reported (81) that the phosphomucolipid isolated from purified lipid A and characterized by Nowotny (64) is responsible for many of the biological effects of endotoxins.

The importance of lipid A in the biological activity of endotoxins has been questioned by several workers. Ribi et al (83) obtained a/

a Boivin type antigen from S. enteritidis (by aqueous ether extraction followed by dialysis and ethanol fractionation) which contained 2-12% bound lipid. 40-60% of this lipid could be removed by refluxing with ethanol or with chloroform-methanol followed by monochlorobenzene-ethanol-water, giving fractions containing as little as 1% lipid which were as toxic and antigenic as the starting material. The authors found that Tween suspensions of lipid A (obtained from acetic acid hydrolysates of the endotoxins) were of low toxicity compared with the material from which they were derived and they concluded that the toxicity of the endotoxin could be attributed mainly or entirely to the polysaccharide portion of the complex. These observations were extended in experiments carried out in collaboration with Landy (84, 85) in which it was again found that the results did not support the suggestion that the lipid moiety is solely or even chiefly responsible for the biological activity of endotoxin. None of the lipid fractions tested had a biological activity greater than 1% of that of the parent endotoxin. In a study of the changes in the biological activities of S. enteritidis endotoxin during hydrolysis with 0.1N acetic acid, Ribi, Haskins, Landy and Milner (86) observed a marked decrease in all the activities measured (non-specific resistance to infection, tumour damage, mouse lethality, dermal inflammation in rabbits and pyrogenicity) before there was a detectable release of chloroform-soluble lipid, indicating that reduction in biological potency was not the result of separation of /

of the lipid with consequent reduced solubility. They also pointed out that the pyrogenic activity after 90 minutes hydrolysis was of the same level as that of artificial lipoproteins prepared by themselves and Westphal by hydrolysis of casein-lipopolysaccharide complexes. This suggested that the activity of these lipoproteins was possibly not the result of solubilisation of the lipid by the casein.

Although the lipids tested by these workers exerted only a fraction of the activity of the intact endotoxins, they nevertheless could not be described as biologically inactive. Lipid extracted by non-hydrolytic methods had a level of activity similar to that of lipid A, although the 'delipified' endotoxin had not lost any of its original potency (84). This may mean that there are two active components present in endotoxins - an acid stable one present in the lipid, and another, sensitive to acids, which is responsible for the principal biological effects,

4. CHEMICAL MODIFICATION OF THE PROPERTIES OF ENDOTOXINS.

In addition to attempts to isolate biologically active portions of the endotoxin complex, there have been several investigations into the possibility of producing chemically modified endotoxins with altered biological properties. Apart from the insight such experiments may allow into the interdependence or otherwise of the various biological effects of endotoxins, they may also be of some practical/

practical value if they result in the selective retention of a desirable property such as immunological potency while at the same time destroying the toxicity of the complex.

In 1952, Westphal, Luderitz and Kiederling (87) reported some of the properties of an acetylated lipopolysaccharide from E. coli. Injection of 100 µg of this preparation into humans produced only a slight fever, while 200-500 µg caused a marked fever without any appreciable alteration in the total leucocyte count, Subcutaneous injection into humans did not result in the local inflammation produced by small doses of the untreated lipopolysaccharide.

Recently Freedman and his colleagues (88) described the production of an acetylated S. typhosa endotoxin which was not lethal in mice at a dose level of 0.9 mg, although this amount of the original endotoxin resulted in a 100% mortality. The pyrogenicity of the acetylated endotoxin was about 1/1000th that of the original material, while the degree of protection afforded to mice against a challenge of Ps. aeruginosa or E. coli was the same in both preparations. That the introduction of the acetyl groups was the factor responsible for the selective reduction in pyrogenicity and toxicity was shown by the restoration of the original level of toxicity and pyrogenicity by deacetylation with alkali. This does not, however, mean that the acetyl/

acetyl groups necessarily exerted a specific blocking action in the endotoxin molecule. It is clear from the paper that the acetylated endotoxin was much less soluble in water than the original material, and it is possible that the reduced activity was a result of this, since Davies has shown that the extent to which an endotoxin is dispersed in solution can have a profound effect on the level of the pyrogenic response (22).

Noll and Braude (89) have produced an interesting modified endotoxin from E. coli 0:113 by reduction with $LiAlH_4$. This treatment of the endotoxin resulted in the abolition or marked decrease of pyrogenicity in rabbits, lethality for mice, neutropenia in rabbits and intradermal inflammation in man. The reduced endotoxin was however still capable of stimulating tolerance to the pyrogenic (rabbits) and lethal (mice) effects of the unmodified material and it afforded the same significant degree of protection of rats against renal infection with E. coli as did the untreated endotoxin. The ability of this modified endotoxin to stimulate tolerance against the pyrogenic effect of the original endotoxin contrasts with the absence of this property in the acetylated endotoxin prepared by Freedman and his co-workers. Noll and Braude were unable to offer a complete explanation for the effect of $LiAlH_4$ on the biological properties of the endotoxin. The principal chemical result of the treatment was the reductive cleavage of ester linkages involving the fatty acids, with the release of a series of long chain aliphatic alcohols. However, although it was consistently found/

found that only those preparations which were ester-free were non-pyrogenic, it was clear that the reduction in toxicity was not simply the result of de-esterification. If the formation of insoluble aluminium bydroxide (from $LiAlH_4$ or LiAl alkoxide on the addition of water) was prevented with sodium potassium tartrate or if the reduction was affected with $LiBH_4$ or $NaBH_4$ there was a much smaller reduction in toxicity even although there was no difference in the extent of the cleavage of ester linkages. It was concluded that the toxic products remaining after $LiAlH_4$ reduction must be preferentially absorbed onto the aluminium hydroxide gel. This toxic fraction was not identified.

Noll and Braude found that heating with alkali (LiOH) resulted in the loss of toxic and immunogenic properties. Neter et al (90) had previously shown that treatment of the lipopolysaccharides from E. coli and S. abortus equi with 0.25N NaOH for short periods at 56° did not result in any alteration of their antibody-neutralising potency, toxicity or pyrogenicity, but increased their ability to modify erythrocytes for haemagglutination studies. Longer periods of alkali treatment resulted in a marked loss in toxicity and pyrogenicity. An interesting finding of these workers was that periodate oxidation of E. coli lipopolysaccharide gave a compound of low toxicity and high pyrogenicity. In this instance, the capacity to neutralise antibody was almost completely abolished.

These results demonstrate that not only is it possible to alter the immunological properties of endotoxins independently of their other biological effects, but that the toxic effects of lethality and pyrogenicity can be altered with respect to one another.

SECTION 1.

CHARACTERISTICS OF THE STRAIN OF PSEUDOMONAS AERUGINOSA USED IN THE PRESENT STUDIES.

The organism was one of several strains of Ps. aeruginosa available in this college, and it was selected after rough tests on autoclaved cultures showed that it elicited a marked pyrogenic response when injected into rabbits.

It was a motile Gram -ve rod showing a single polar flagellum, producing pyocyanin on Meader's medium and pyoverdin on Georgia and Poe's medium. It formed acid from glucose, sucrose and mannitol, but not from maltose. There was slight acid production from lactose. When grown in B.C.P. milk, acid was produced and a clot formed. The Methyl Red test was -ve after 3 days and the Voges-Proskauer test was -ve after 1 day. Gelatin liquifaction was -ve after 3 days and catalase production was +ve after 1 day. Nitrate was reduced rapidly to ammonia, no nitrite being detected.

When grown in liquid medium at 37°, the strain showed the following characteristics after 1 day.

Turbidity	-	+
Degree	-	Moderate
Nature	-	Uniform
Deposit	-	Present
Degree	-	Slight
Type	-	Powdery
Shaking	-	Disintegrates easily
Surface Growth	-	+
Nature	-	Pellicle
Degree	-	Fairly thick
Surface	-	Smooth
Shaking	-	Persistent
Odour	-	Slight

A streak on nutrient agar showed the following features when grown at 37° for 1 day.

Degree	-	Moderate
Form	-	Filiform
Opacity	-	Transparent
Structure	-	Granular
Edge	-	Entire
Colour	-	Greenish
Elevation	-	Raised
Surface	-	Moist
Lustre	-	Shining
Medium	-	Greenish

The subsequent work described in the experimental sections was carried out on a single batch of cells (634g) grown at the Antibiotics Research Station, Clevedon, as detailed below.

Seed Culture.

Seed Culture.

The organism was subcultured on 1 oz. nutrient agar slopes which were incubated at 37°.

Medium.

Unless otherwise stated the medium used throughout was the following:

Yeast extract (Difco)	0.5g
Sodium citrate	1.0g
Tap water	100ml

Medium adjusted to give pH 6.6-6.8

Shake Flask Cultures.

Two 250ml Erlenmeyer flasks containing 80ml medium were incubated at 30° on a reciprocating shaker for 16 hours. Both cultures were then used to inoculate a stirred aerated aspirator culture.

Stirred Aerated Aspirator Culture.

A 10 l. Pyrex aspirator containing 8 l. of medium was stirred at 300 rp.m. (large paddle), aerated at 3 l.p.m. and incubated at 35°. The incubation was continued for 8½ hours during which time a growth curve was obtained by measuring the optical density of the culture with a 'Spekker'. At the end of this period the aspirator was stored overnight in a refrigerator at 4°.

Final Fermentation.

A 100 gallon stainless steel fermentor containing 320 l. of medium was inoculated with 8 l. of aspirator produced culture. The vessel was stirred at 100 r.p.m., aerated at 6 cu. ft. and incubated at 37° for 8 hours. The pattern of growth was similar to that of the aspirator.

Preparation of Dried Cells.

The culture 320 l. was cooled with brine to 0° and held overnight. The next day it was passed through a Sharples centrifuge and the effluent discarded. The packed cells were macerated in acetone which had been cooled to approximately -5°, filtered through Green's 904 filter paper and then again subjected to a further treatment with acetone etc. The resulting cake was crumbled and allowed to dry overnight at air temperature. Finally, the cells were lightly ground in a mortar and pestle to break the larger portions and dried under vacuum over P_2O_5.

SECTION 2.

EXTRACTION AND INVESTIGATION OF THE LIPOPOLYSACCHARIDE.

The aim of the experimental work described in this Section was to isolate the lipopolysaccharide from the selected strain of Pseudomonas aeruginosa and to characterize it by examination of certain chemical and biological properties.

Isolation of the Lipopolysaccharide.

Acetone-dried cells (10g) were suspended in 350ml of distilled water and the suspension heated to 67°C. An equal volume of 90% phenol at the same temperature was added and the mixture stirred continuously for 30 minutes, the temperature being maintained at 65-67°C. After cooling to about 15° the mixture was centrifuged for 15 minutes at about 2500g, and this resulted in a separation into two layers - an upper aqueous layer and a lower phenol layer, the two being separated by a narrow band of insoluble material.

The aqueous layer was sucked off, and a further 250ml of water added to the Phenol. This was then heated with stirring until the temperature reached 67°, cooled and centrifuged as before, and the aqueous layer separated. The two aqueous layers were combined and dialysed against running water for 2-3 days. The opalescent solution thus obtained was concentrated under vacuum to about 1/5 of its volume, clarified of any/

any debris by centrifuging at 2500g for 15 minutes and freeze-dried.

The freeze-dried substance (fraction PE) was a white fluffy powder and was obtained in amounts equivalent to 14-18% of the weight of the acetone-dried cells; yields from four different batches were 1.41g, 1.84g, 1.60g and 1.74g.

Examination of Fraction PE.

Certain properties of fraction PE were examined to determine whether or not it contained the endotoxic lipopolysaccharide. These properties were constituent sugars, lipid content, pyrogenicity and reactivity in the agar precipitin test.

1. Sugars.

A sample (20mg) of PE was hydrolysed by heating for 3 hours in N sulphuric acid in a boiling water bath. The hydrolysate was neutralised with barium hydroxide solution, and after centrifuging to remove the precipitated barium sulphate, it was evaporated to dryness under vacuum. Two drops of distilled water were added and examination of the solution for the presence of sugars by circular paper chromatography showed that it contained large quantities of ribose along with galactose, glucose and mannose - Figure 1.

Full details of the chromatographic procedure are given in the Appendix.

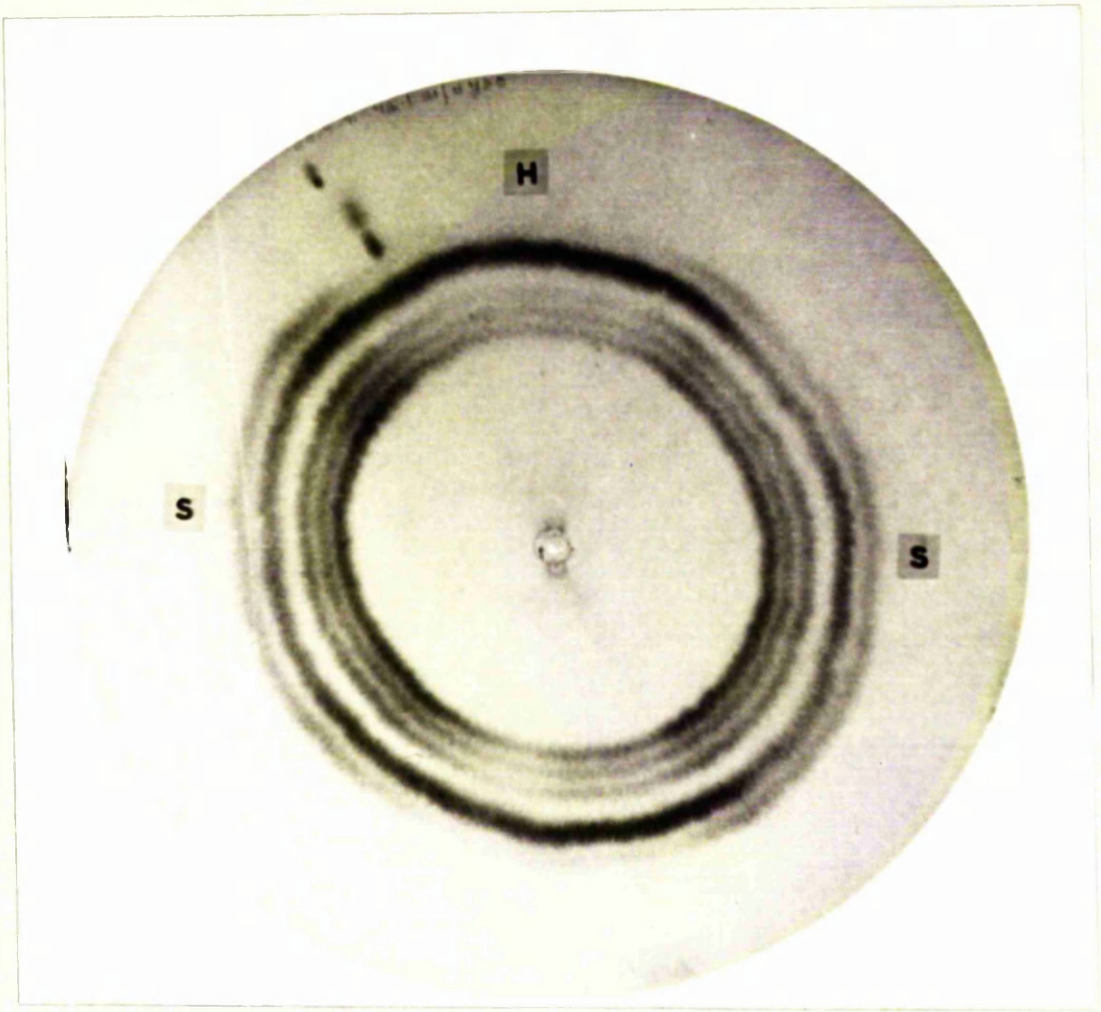


Fig. 1. Chromatogram of a 3 hour hydrolysate of fraction PE in N H_2SO_4 H - hydrolysate S- standard mixture (galactose, glucose, mannose, ribose and rhamnose - in order of increasing R_f)
Solvent - butanol, pyridine, water
Spray - aniline phthalate

2. Lipid Content.

The amount of lipid present was determined gravimetrically by hydrolysing 50mg of fraction PE for 1 hour in 10ml of N hydrochloric acid in a boiling water bath. This resulted in the formulation of a flocculant precipitate which was extracted with three 20ml portions of chloroform. After drying the combined chloroform extracts to constant weight at 80°, fraction PE was found to contain 13-14% chloroform-soluble material.

The hydrolysis was repeated with a further 50mg of PE and the solution extracted three times with 20ml of diethyl ether. The ether extracts were dried over anhydrous sodium sulphate then evaporated to constant weight at 80°. The amount of ether-soluble lipid present was 2.4mg (4.8%)

3. Pyrogenicity.

The effect of the material on the temperatures of rabbits was examined after injection of solutions in sterile pyrogen-free saline. An average maximum temperature increase of 1.0°C was elicited after injection of 0.01µg/kg into 10 rabbits. Details of the testing procedure are given in the Appendix.

4. Agar Precipitin Test. /

4. Agar Precipitin Test.

When an aqueous solution of PE was examined by the agar double diffusion technique against an antiserum prepared against the whole cells, three precipitin lines were formed - Figure 2.

The plate was photographed after standing for 24 hours at room temperature and observed for a further 7 days. No additional lines appeared. Details of the preparation of the antiserum and the agar plates are given in the Appendix.

5. Nucleic Acid.

In the use of the hot phenol, method for the extraction of bacterial endotoxins, it is commonly found that the product is contaminated with large amounts of nucleic acid. The detection of ribose in hydrolysates of fraction PE suggested the presence of RNA in this material, and when solutions were examined in an Optica CF4 spectrophotometer the absorption spectrum showed a marked peak at 260mμ - also indicative of the presence of nucleic acid - Figure 3.

From a calibration curve of yeast nucleic acid, the nucleic acid content of fraction PE was estimated to be about 54%.

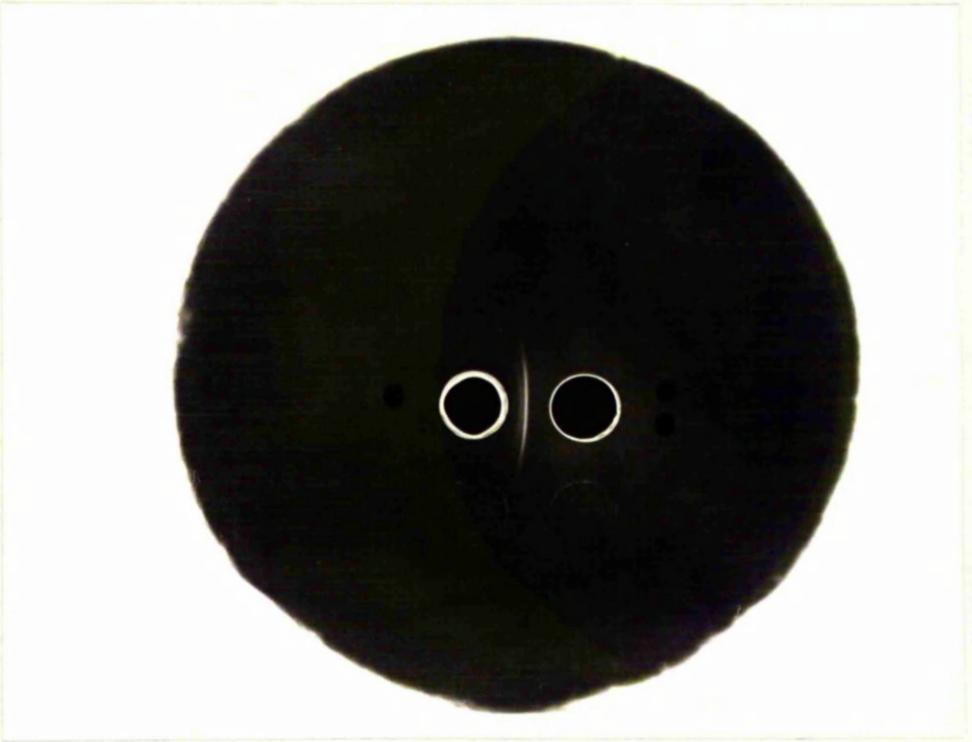


Fig. 2 Precipitin pattern produced by a 1% solution of fraction PE (0.2ml) and antiserum against acetone-dried Ps. aeruginosa cells (0.2ml)

● - fraction PE ⋮ - antiserum

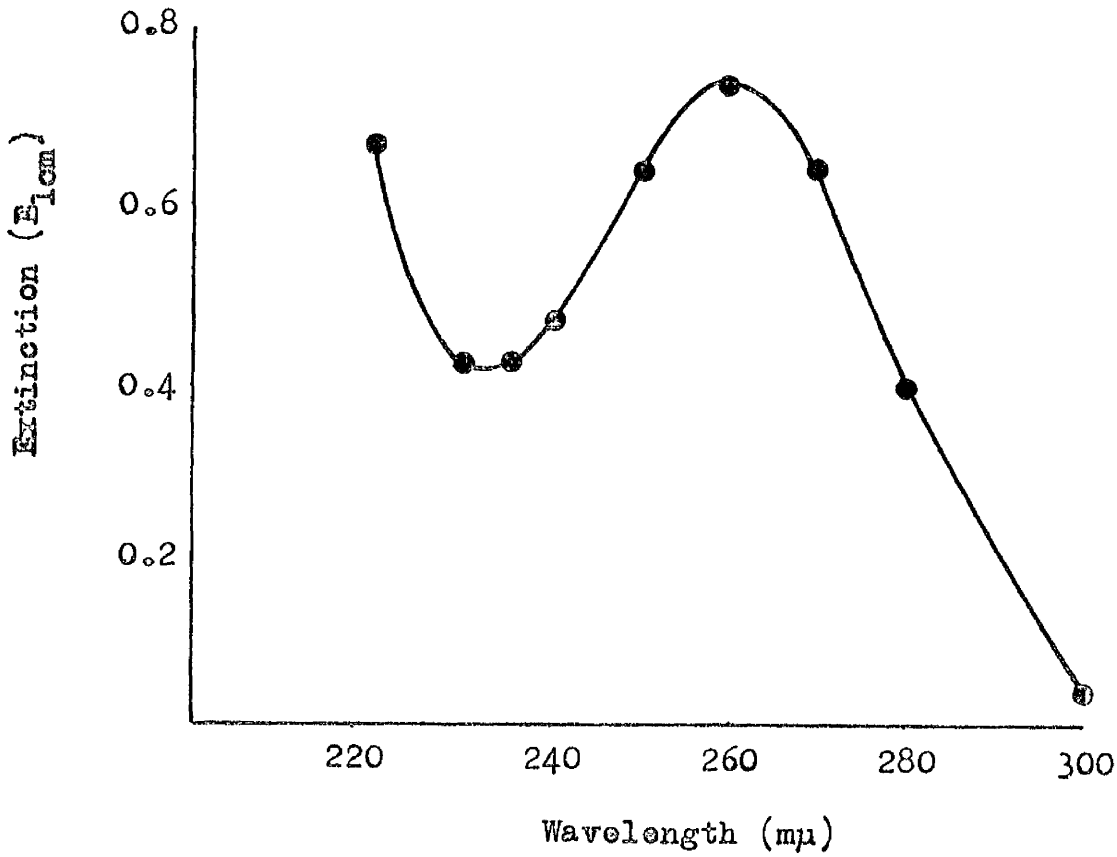


Fig. 3. Absorption spectrum of fraction PE 50μg/ml in N/100 NaOH.

Conclusion.

These results showed that the extract obtained by treating the cells with hot aqueous phenol had some of the characteristic properties of the lipopolysaccharides of Gram -ve bacteria and it was therefore considered a suitable material for further study.

PURIFICATION OF FRACTION PE

High-speed centrifugation has been used successfully by several workers as a method of separating lipopolysaccharide from the nucleic acid which is commonly present in extracts obtained by the hot phenol method, and it was decided to attempt a purification of fraction PE by this method.

A 1% solution of fraction PE was clarified by centrifuging for 30 minutes at 2500g, and the supernatant centrifuged for three hours at 105,000g in a Spinco Model L Ultracentrifuge. The deposit, which was in the form of a translucent pellicle, was resuspended in 50 ml of distilled water and 5ml of this solution was freeze-dried. The absorption spectrum of a solution of this freeze-dried material showed that the nucleic acid content had been reduced from 54% to about 40%

Repeated centrifugation resulted in a further decrease in the nucleic acid content of the deposit and it was found that after four sedimentations the nucleic acid had been removed completely (i.e. no peak at 260mμ) - Figure 4. The deposit after the fourth sedimentation was suspended in 100 ml of water and freeze-dried.

In several repeats of the extraction and purification procedure this nucleic acid-free fraction, which is subsequently described as fraction PE/SP, was obtained, in yields of 2-4% of the weight of the acetone-dried bacterial cells.

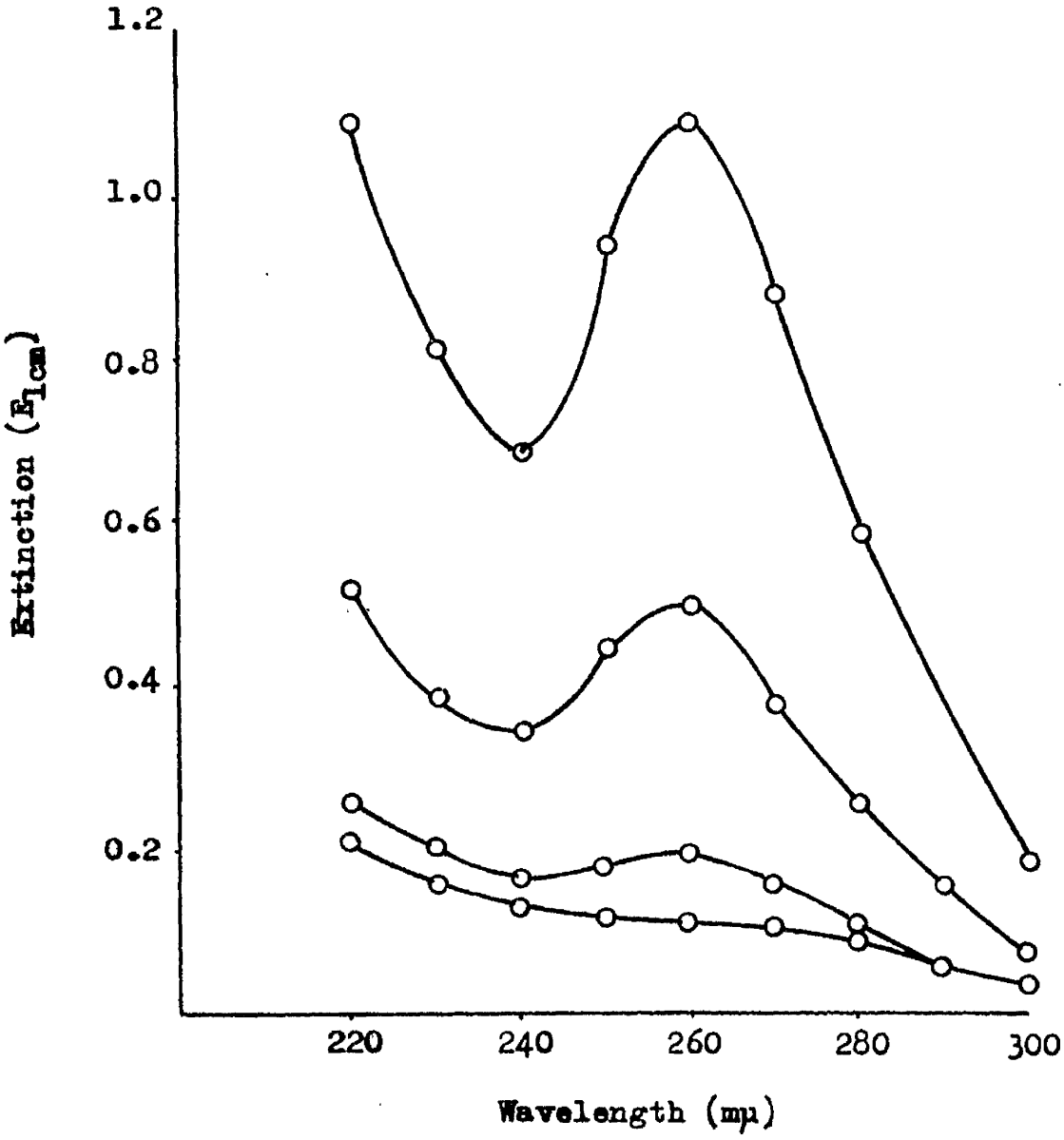


Fig. 4 Absorption spectra of the deposits from fraction PE obtained after 1,2,3 and 4 sedimentations in a Spinco Model L Ultracentrifuge at 105,000g for 3 hours.

The solutions contained 100μg/ml in N/100 NAOH

The estimates of the nucleic acid content were obtained from a calibration curve of yeast nucleic acid after subtracting the residual absorption at 260mμ of a solution of 100μg/ml of the purified material (PE/SP) from the absorption at 260mμ of 100μg/ml solutions of the various fractions. The assumption that the absence of a peak at 260mμ indicated freedom from nucleic acid was supported by the failure to detect ribose in hydrolysates of the purified material.

When the material in the supernatant from the first high-speed centrifugation was tested for pyrogenicity, amounts of 0.01μg/kg caused a temperature increase of 0.8° (average of 7 rabbits). Paper chromatographic examination of a sample (20mg) of the freeze-dried supernatant hydrolysed in N sulphuric acid for 3 hours showed that it contained mainly ribose, along with mannose and traces of galactose and glucose. - Figure 5. (the traces of galactose and glucose are not visible on the photograph). Traces (about 2%) of chloroform-soluble lipid were detected after hydrolysis of the supernatant material in N hydrochloric acid for 45-60 minutes.

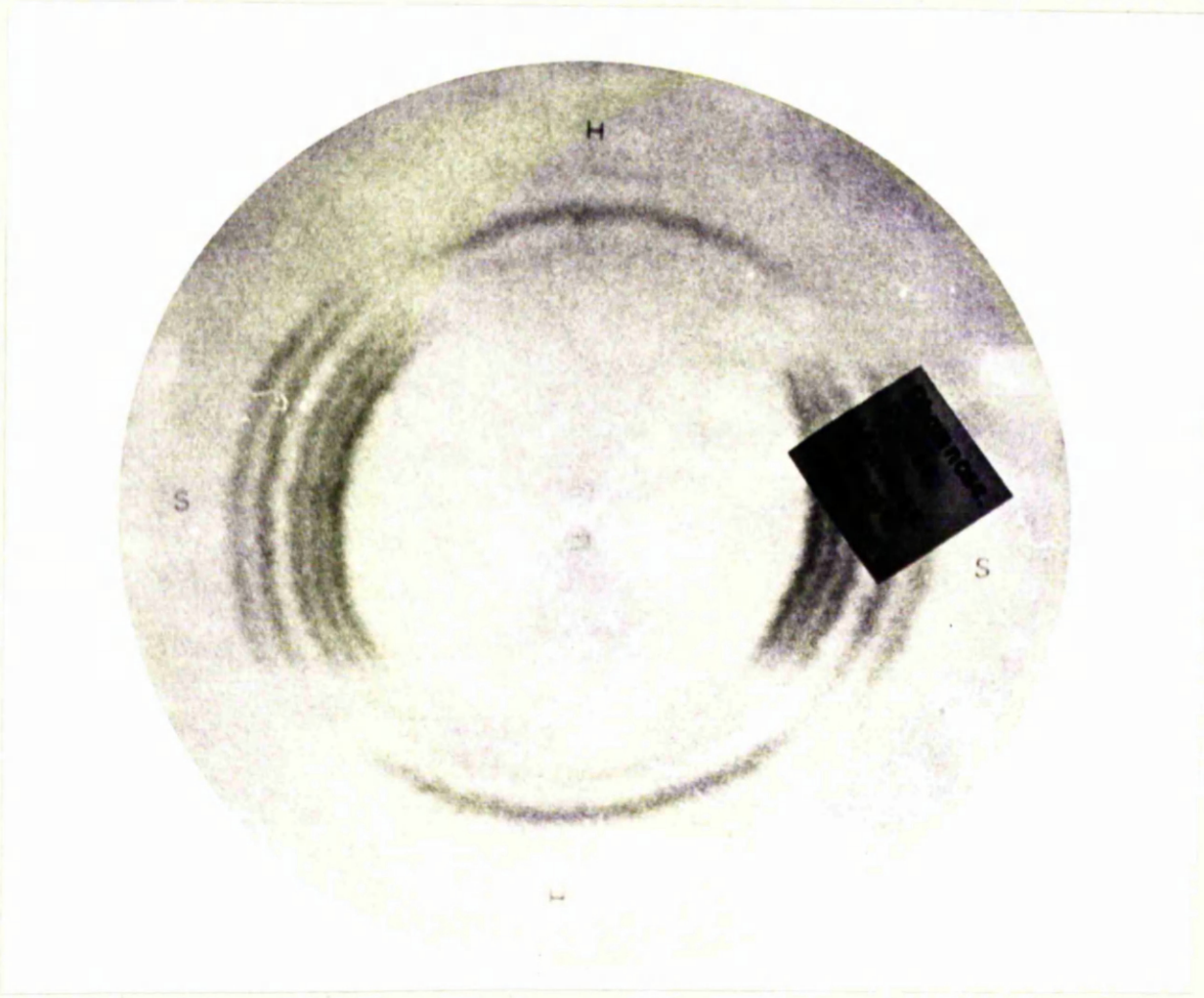


Fig. 5. Chromatogram of a 3 hour hydrolysate in N H_2SO_4 of 20mg of the material from the supernatant after centrifuging fraction PE at 105,000g for 3 hours

H - hydrolysate S - standard sugars

Solvent - butanol, pyridine, water

Spray - aniline phthalate

PROPERTIES OF FRACTION PE/SP

A. CHEMICAL PROPERTIES.

The values for nitrogen and phosphorus and the content of amino sugars, reducing sugars and chloroform- and ether-soluble lipid are shown in Table 6.

TABLE 6.

N (%)	P (%)	AMINO SUGAR (%)	REDUCING SUGAR (%)	LIPID (%) ETHER-SOL.	LIPID (%) CHLOROF-SOL
2.4	2.4	7.5	31	15	38

Nitrogen was estimated by the micro-Kjeldahl method, and phosphorus by the method of King (91). Amino sugars were determined as glucosamine hydrochloride by the method of Rondle and Morgan (92) on samples of the material which had been hydrolysed by heating in a sealed ampoule at 100°C in 6N hydrochloric acid for periods of 1,2,3,4,5 and 6 hours. The maximum value (7.5%) was obtained after four hours hydrolysis. Reducing sugars were estimated by the method of Shaeffer and Somogyi (93) on samples hydrolysed by heating in sealed ampoules at 100°C in N hydrochloric acid for 1,2,4,6,8, and 10 hours. Using glucose as a standard, a reducing sugar curve was drawn and is shown in Figure 6.

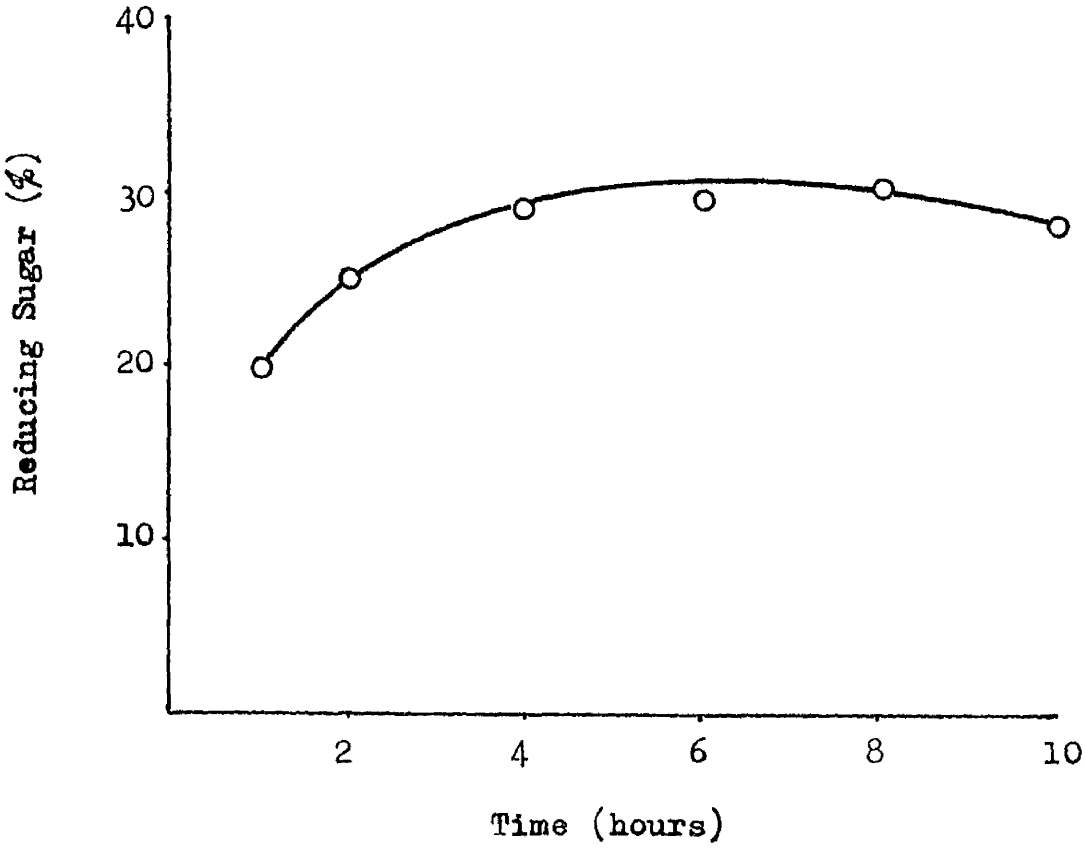


Fig. 6. Reducing sugar, calculated as glucose, released from fraction PE/SP during hydrolysis in N HCl

The ether and chloroform soluble lipid values were determined gravimetrically on 50mg samples as described above for fraction PE.

SUGAR ANALYSIS.

Chromatography.

When chromatograms of a 3 hour N sulphuric acid hydrolysate of PE/SP (20mg) were sprayed with aniline phthalate, two components corresponding to galactose and glucose were detected - Figure 7.

A 30 minute hydrolysate of PE/SP showed the presence of galactose and a small amount of glucose - Figure 8. There was also a faint band with an R_f less than that of galactose, and a more pronounced band which ran between mannose and ribose. This latter substance gave a red colour with aniline phthalate similar to that produced by the pentose sugars and it had an R_f which was very close to that of xylose.

Both hydrolysates gave negative results with the orcinol-trichloracetic acid reagent for ketosugars (94) and the vanillin-perchloric acid reagent for deoxy sugars (95).

Dische Reaction./

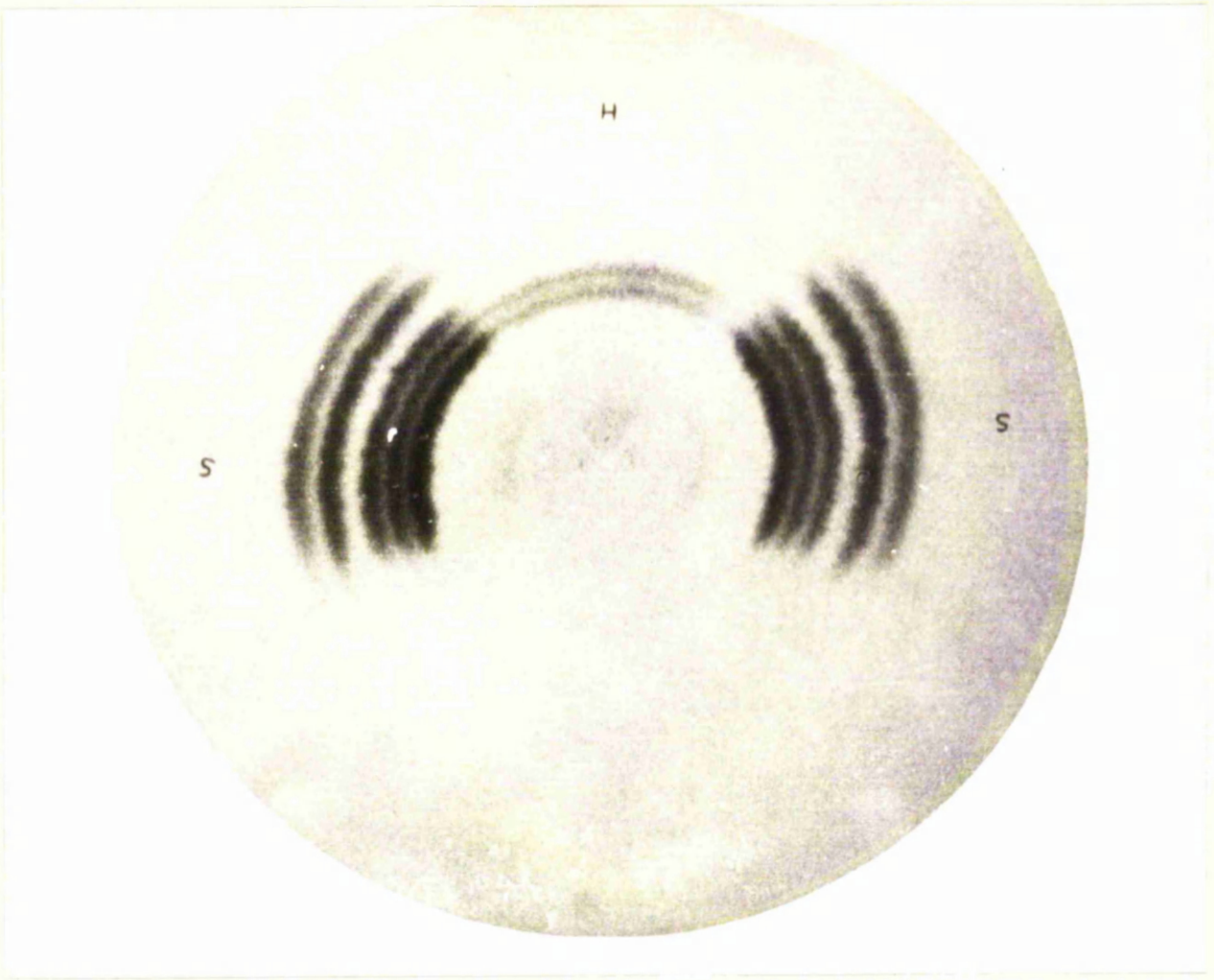


Fig. 7 Chromatogram of a 3 hour hydrolysate in N H_2SO_4 of fraction PE/SP

H - hydrolysate S - Standard sugars (glactose, glucose, mannose, ribose, rhamnose - in order of increasing R_f)

Solvent - butanol, pyridine, water

Spray - aniline phthalate

Fig. 8 Chromatogram of a 30 minute hydrolysate in N H_2SO_4 of fraction PE/SP

H - hydrolysate S - standard sugars

Solvent - butanol, pyridine, water

Spray - aniline phthalate

Dische Reaction.

A solution of fraction PE/SP containing 0.5mg/ml was examined by the sulphuric acid-cysteine reaction of Dische (96). 1ml of the solution was cooled on ice, then 4.5ml of strong sulphuric acid (sulphuric acid 60ml, water 10ml) were added. The mixture was kept for 3 minutes on ice, then for 3 minutes at room temperature and finally for 3 minutes in a boiling water bath. After cooling to room temperature, 0.1ml of a 3% aqueous solution of cysteine hydrochloride was added and the tube carefully shaken. The absorption of the solution in the range 370mμ to 550mμ was measured after 1 hour and 20 hours in a Unicam SP600 spectrophotometer against a blank containing acid, cysteine and water. Figure 9 shows the absorption spectra obtained. The absorption maximum at 410mμ indicated the presence of hexose, and the peak at 505-510mμ is characteristic of heptoses.

The chromatographic examination described above gave no indication of the presence of a heptose in the hydrolysates, but since some of the aldoheptoses have R_f values which are close to those of galactose and glucose, the possibility of the heptose being masked on the chromatogram by either of the hexoses was considered.

Descending strip chromatograms of the 3 hour hydrolysates were run using the butanol, pyridine, water solvent system, and by the use of guide strips stained with aniline phthalate, the area of the strip occupied by glucose and galactose was located. This area was cut from the paper, shredded, and the sugars eluted with 2ml of distilled water.

The/

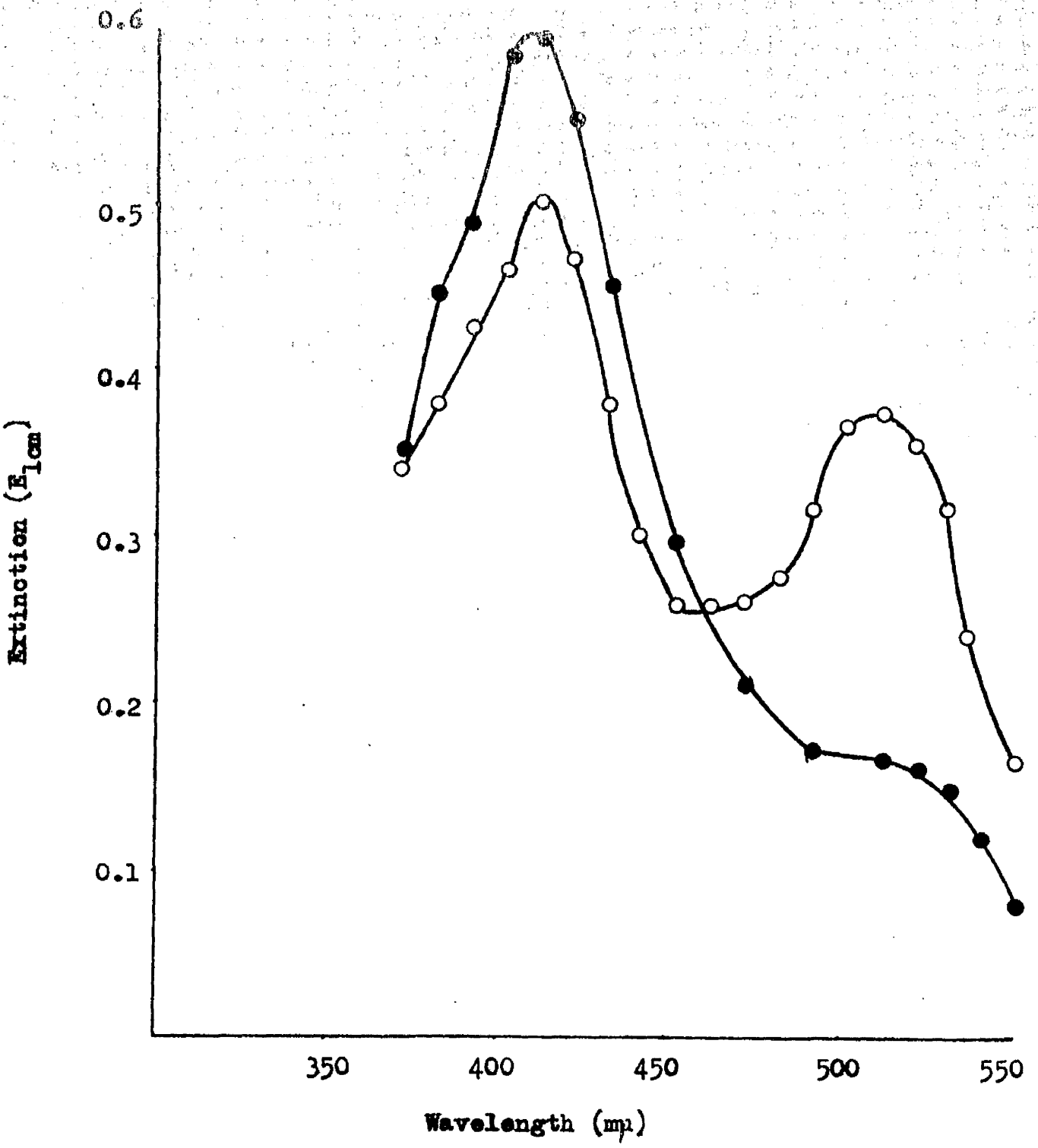


Fig.9. Absorption spectra of PE/SP (500μg) after treatment with H_2SO_4 - cysteine.

● - readings after 1 hour

○ - readings after 20 hours

The solution was passed through a sintered glass filter to remove paper fibres and examined in the sulphuric acid-cysteine reaction, using as a control an equal area of the paper which was free of hydrolysate material. Figure 10 shows that the combined glucose and glactose eluate gave the characteristic absorption spectrum of hexoses but showed no peak at 505-510mμ. When an eluate from the origin point of the chromatogram was treated in the same way there was a low level of chromogen formation in the Dische reaction, but a definite peak appeared at 505mμ between 1 hour and 20 hours. When the experiment was repeated with the 30 minute hydrolysate, an eluate of all the material within the range of galactose to ribose gave an absorption spectrum with a maximum at 410mμ but no peak at 505mμ.

The possibility was then considered that the duration of hydrolysis had not been long enough to release the heptose from the polysaccharide and 20mg amounts of fraction PE/SP were hydrolysed in N sulphuric acid for 5, 10, 15 and 20 hours. Although only two bands corresponding to; galactose and glucose could be detected on circular chromatograms of these hydrolysates, eluates prepared from strip chromatograms as described above and examained in the Dische reaction showed that with increasing hydrolysis time there was an increase in the release of heptose, and that the heptose was closely associated with glucose on the chromatograms- Figures 11-14.

As/

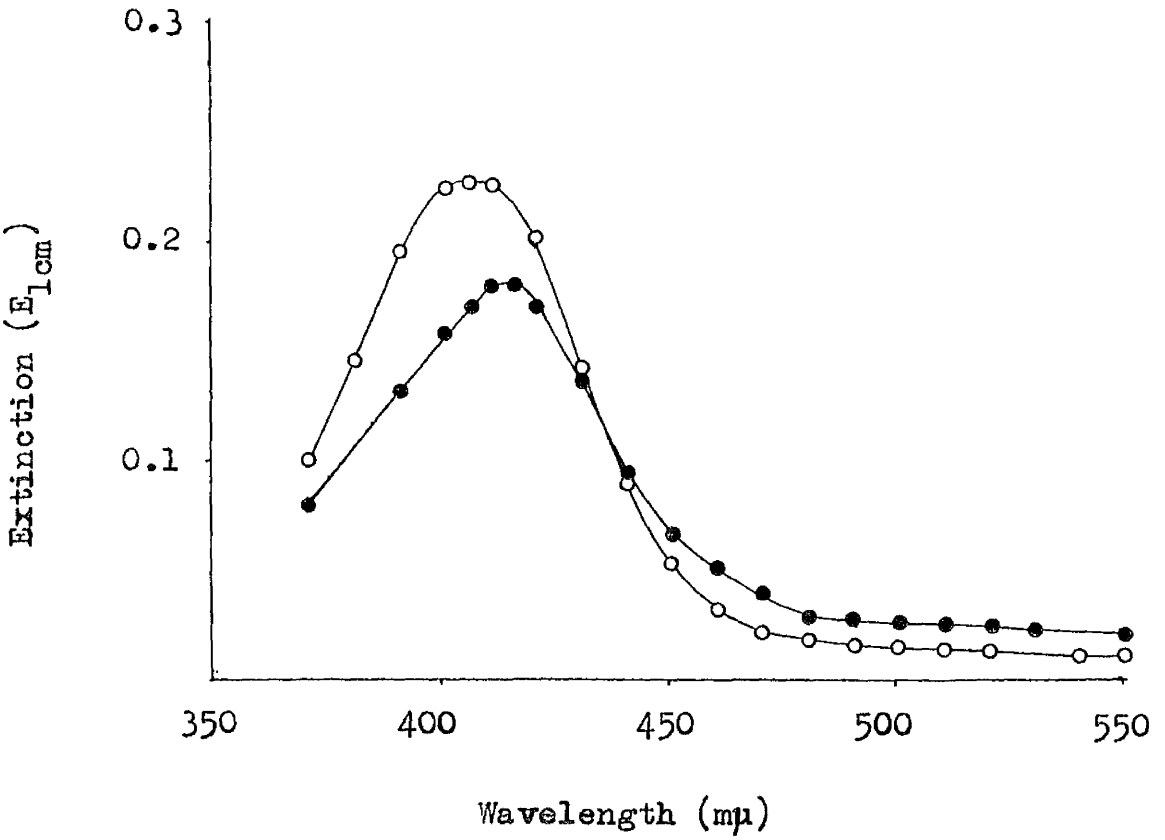


Fig. 10. Absorption spectrum, after treatment with H_2SO_4-cysteine, of the combined eluate of galactose and glucose from a chromatogram of a 3 hour hydrolysate of PE/SP in N H_2SO_4

o - Readings after 1 hour

● - Readings after 20 hours

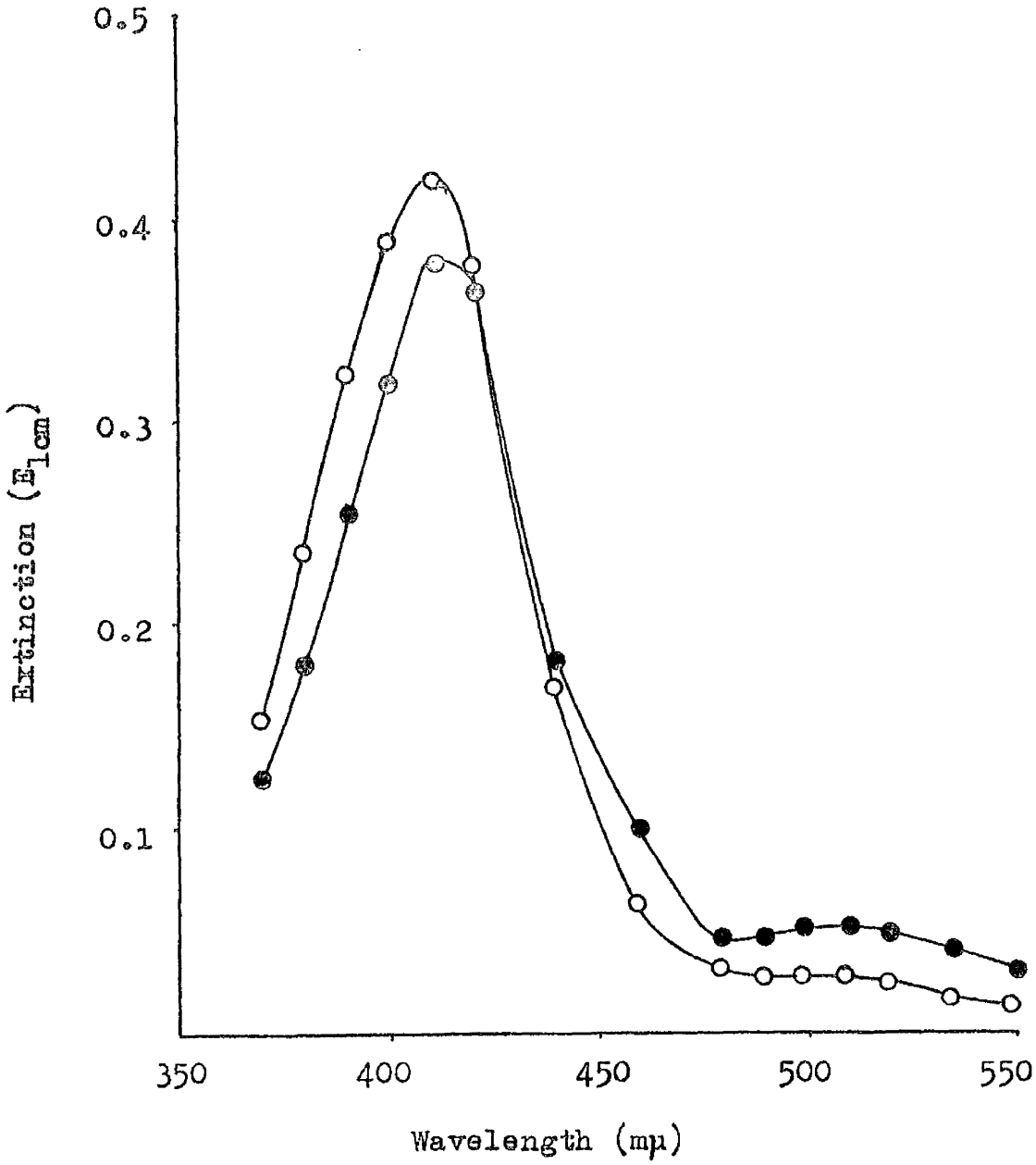
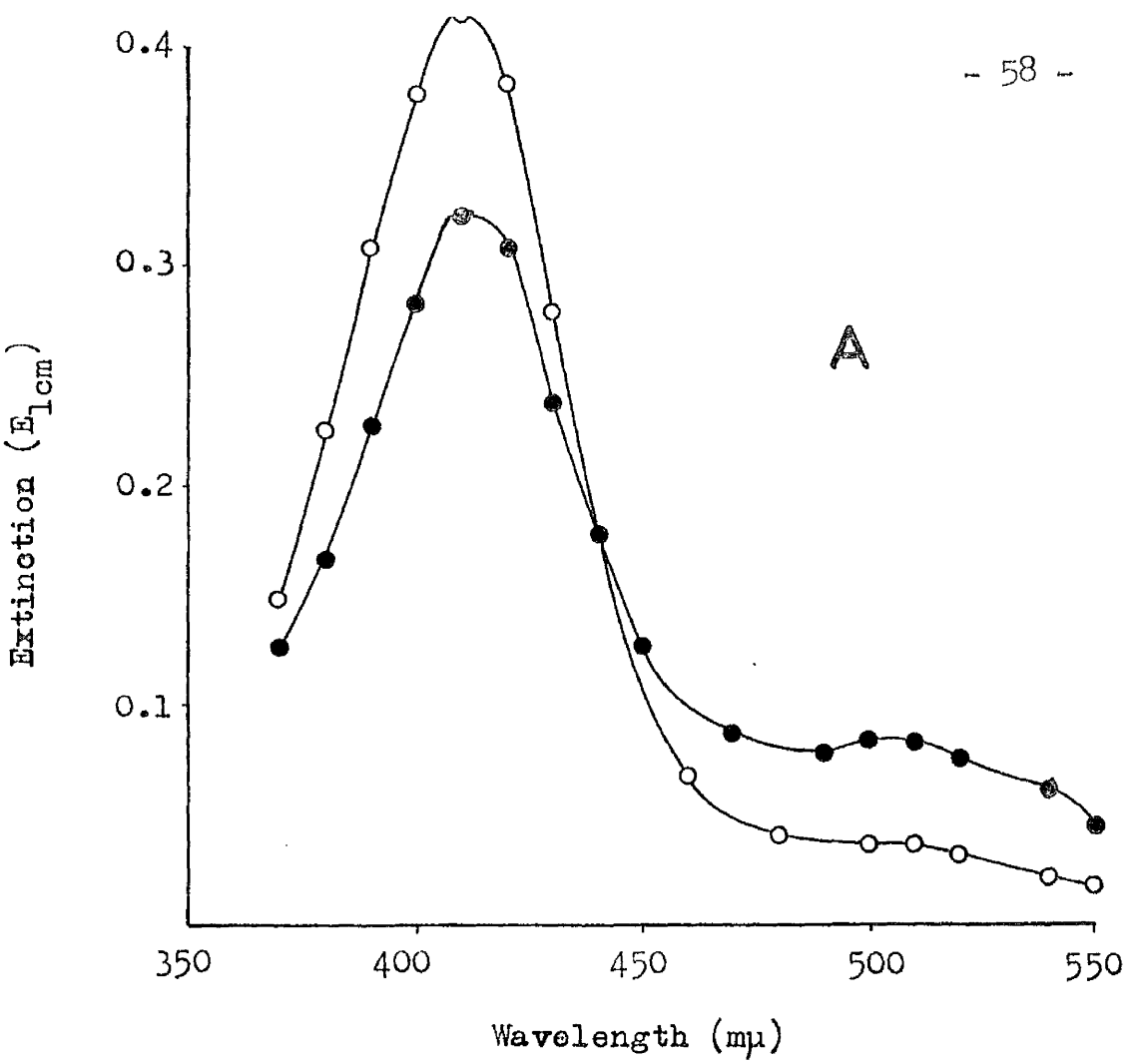


Fig.11. Absorption spectrum, after treatment with H_2SO_4-cysteine, of the combined eluate of galactose and glucose from a chromatogram of a 5 hour hydrolysate of PE/SP in N H_2SO_4

● – Readings after 1 hour

○ – Readings after 20 hours



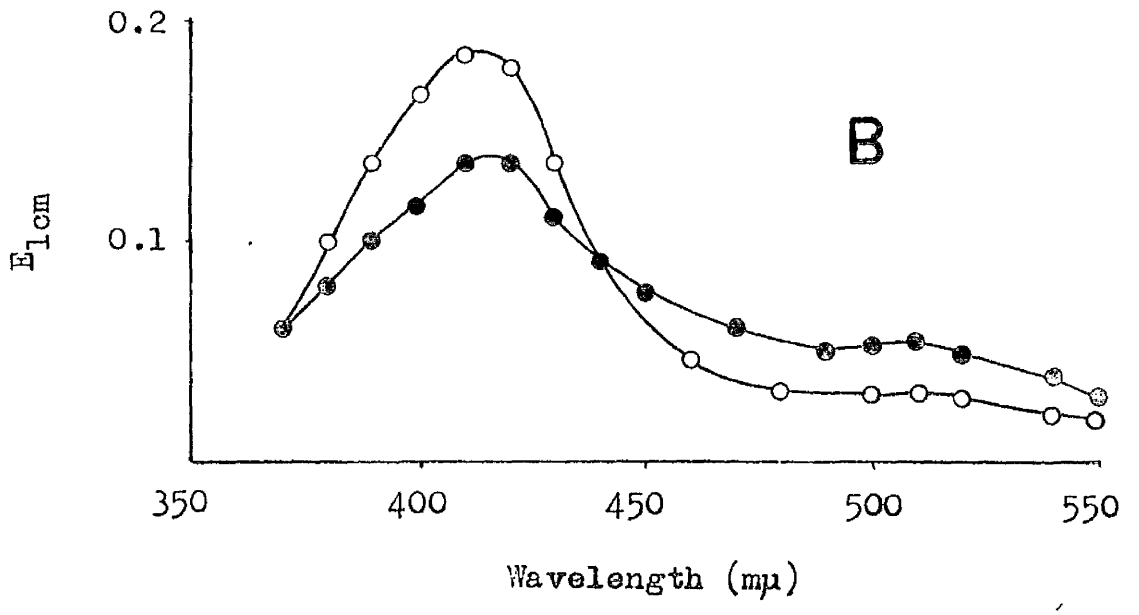


Fig. 12 Absorption spectrum, after treatment with H_2SO_4-cysteine, of the eluted material from a chromatogram of a 10 hour hydrolysate of PE/SP in N H_2SO_4

A - glucose B - galactose

o - Readings after 1 hour ○ - Readings after 20 hours

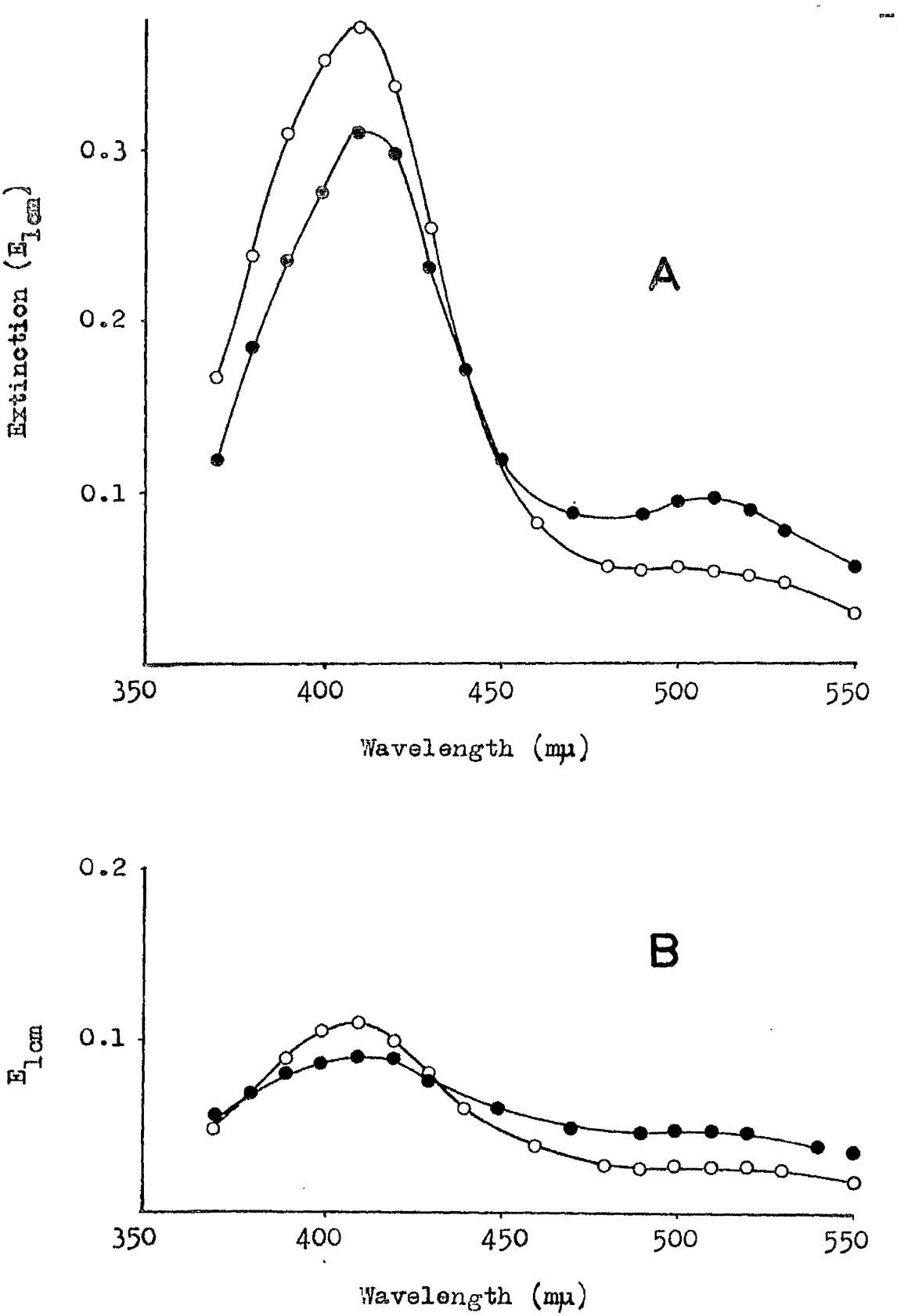


Fig.13. Absorption spectrum, after treatment with H_2SO_4-cysteine, of the eluted material from a chromatogram of a 15 hour hydrolysate of PE/SP in N H_2SO_4

A - glucose B - galactose

o - Readings after 1 hour ⊛ - Readings after 20 hours

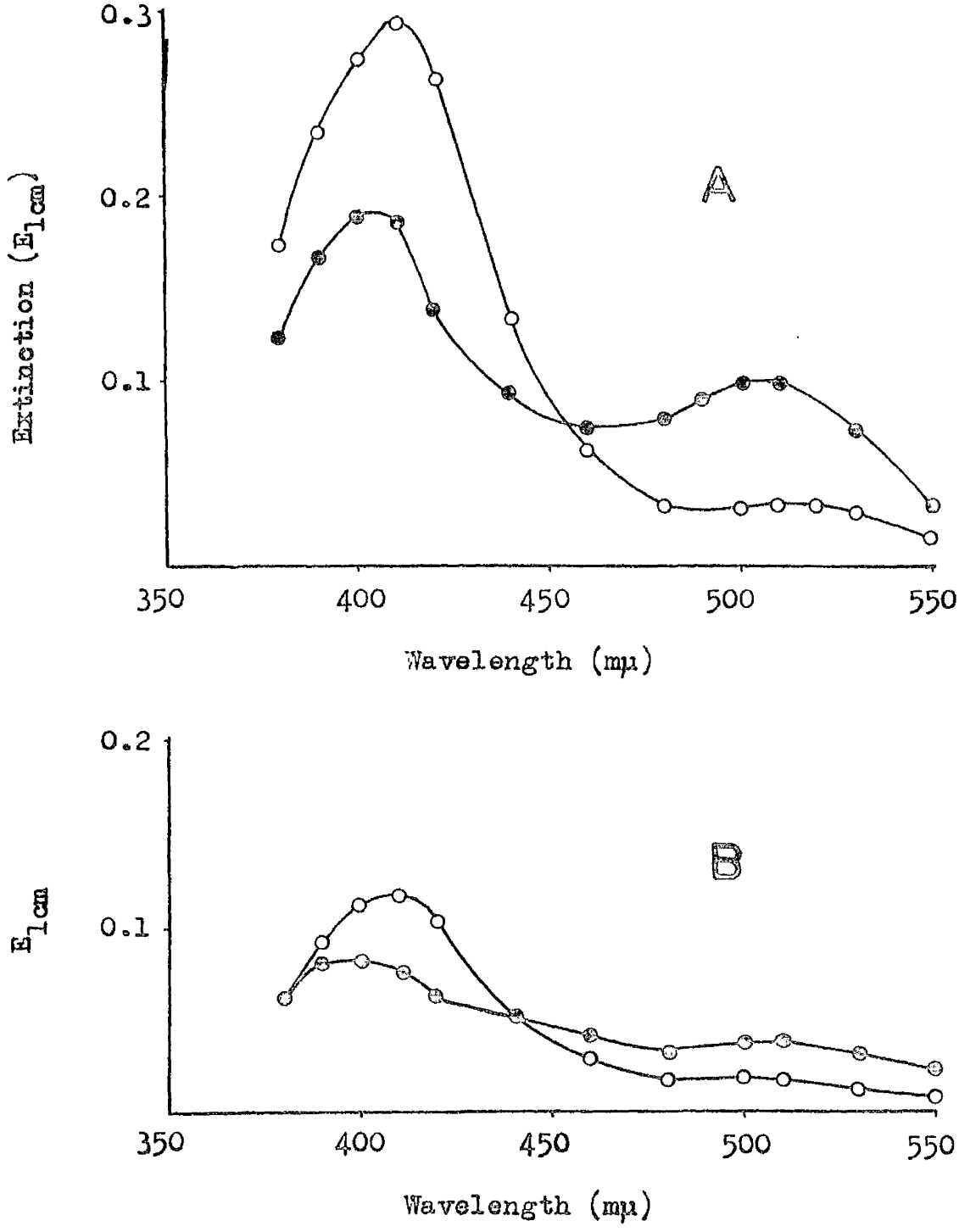


Fig. 14. Absorption spectrum, after treatment with H_2SO_4-cysteine, of the eluted material from a chromatogram of a 20 hour hydrolysate of PE/SP in N H_2SO_4

A - glucose B - galactose

o - Readings after 1 hour o - Readings after 20 hours

As the 20 hour hydrolysate still gave a negative reaction with the orcinol-trichloracetic acid reagent it was concluded that the heptose was an aldoheptose.

AMINO SUGARS AND AMINO ACIDS.

The lipopolysaccharide (PE/SO 20mg) was hydrolysed for 8 hours in 6N hydrochloric acid at 100°. The hydrolysate was centrifuged and the supernatant dried under vacuum. Distilled water (1ml) was added and the solution dried in a vacuum desiccator over sodium hydroxide. After the addition of two drops of water the hydrolysate was examined by circular paper chromatography using two solvent systems: butanol, pyridine, water (3:2:1.5 by volume), and butanol, acetic acid, water (4:1:5 by volume, upper layer). The papers were sprayed with 0.2% ninhydrin in water-saturated butanol then heated for 10 minutes at 95°C.

Several ninhydrin positive bands were obtained, two of which corresponded to standards of glucosamine and galactosamine - Figures 15 & 16. Ethanolamine was also detected along with the following amino acids: leucine, methionine, alanine, glycine, glutamic acid, aspartic acid and lysine. Two bands were not identified. In butanol, acetic acid, water, one of these ran between methionine and leucine, the other had an R_f less than lysine.

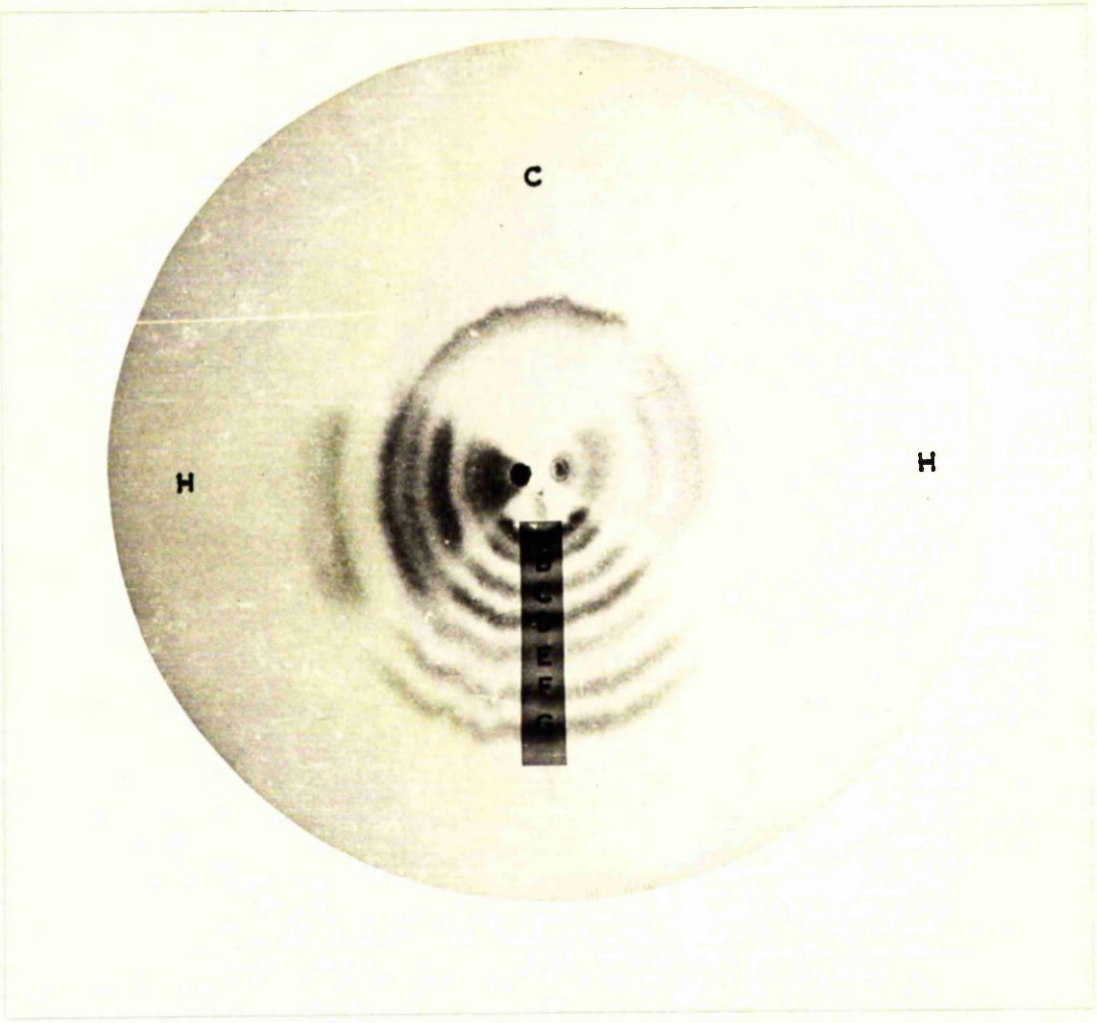


Fig. 15 Chromatogram of an 8 hour hydrolysate of the lipopolysaccharide (PE/SP) in 6N HCl.

H - hydrolysate C -galactosamine and glucosamine

Standard amino acids: A-lysine and aspartic acid, B-glutamic acid, C-glycine, D-alanine, E-ethanolamine, F-methionine, G-leucine

Solvent - butanol, pyridine, water

Spray - 0.2% ninhydrin in moist butanol

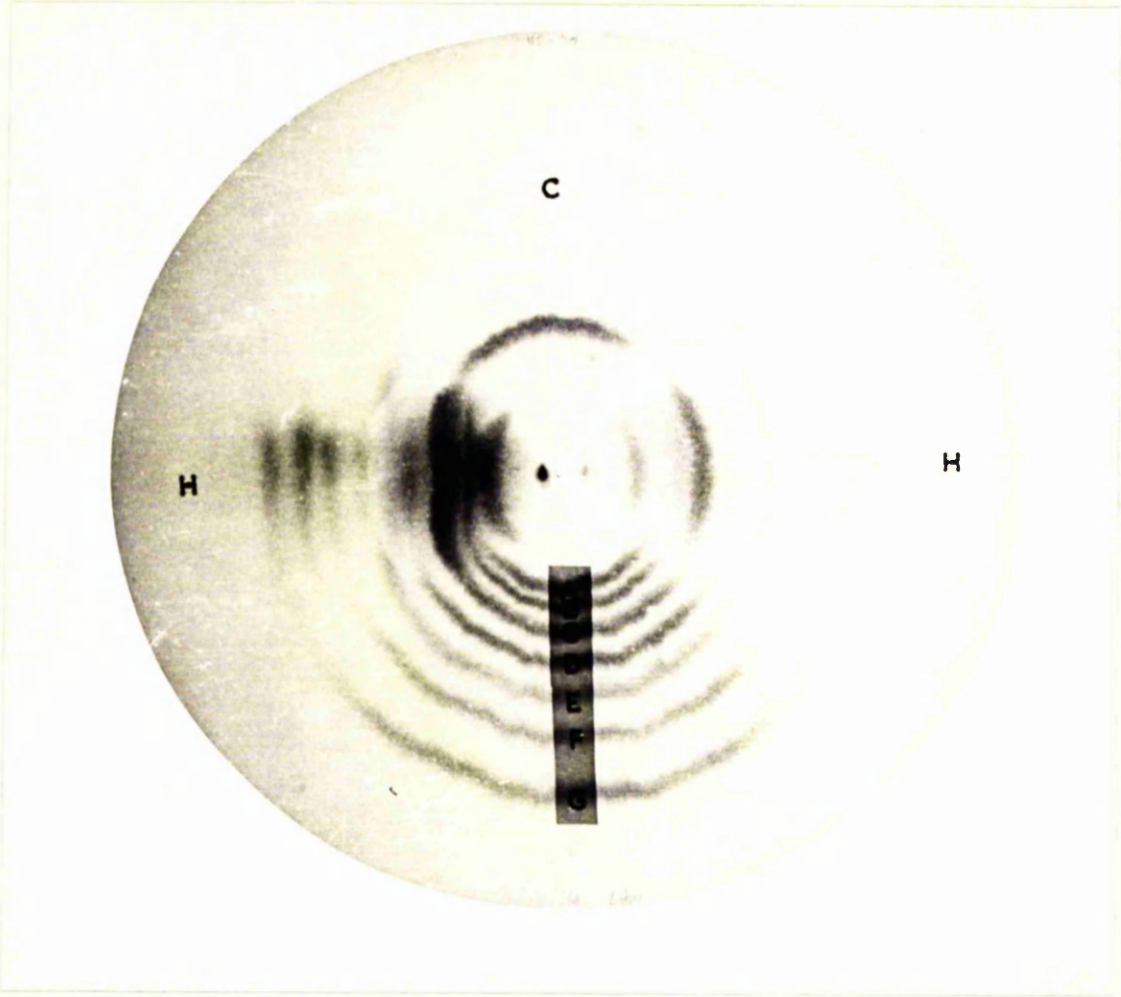


Fig. 16. Chromatogram of an 8 hour hydrolysate of the lipopolysaccharide (PE/SP) in 6N HCl

H - hydrolysate C - galactosamine and glucosamine

Standard amino acids; A-lysine, B-aspartic acid, C-glutamic acid and glycine, D-alanine, E-ethanolamaine, F-methionine, G-leucine

Solvent - butanol, acetic acid, water

Spray - 0.2% ninhydrin in moist butanol.

Because of the difficulty of achieving satisfactory resolution of glucosamine and galactosamine and also because of the danger of mistaking an amino acid for an amino sugar, the hydrolysate was subjected to the ninhydrin degradation procedure of Gardell (97) which converts the amino sugars to the corresponding pentoses. After the addition of 2.5ml of distilled water to the hydrolysate the solution was neutralised to pH 7 with pyridine. Approximately 4mg of ninhydrin hydrate were added, then the solution was heated for 30 minutes in a boiling water bath and evaporated to dryness under vacuum. Chromatograms of a solution of this product in two drops of distilled water showed the presence of arabinose and lyxose - pentose derivatives of glucosamine and galactosamine respectively - Figure 17.

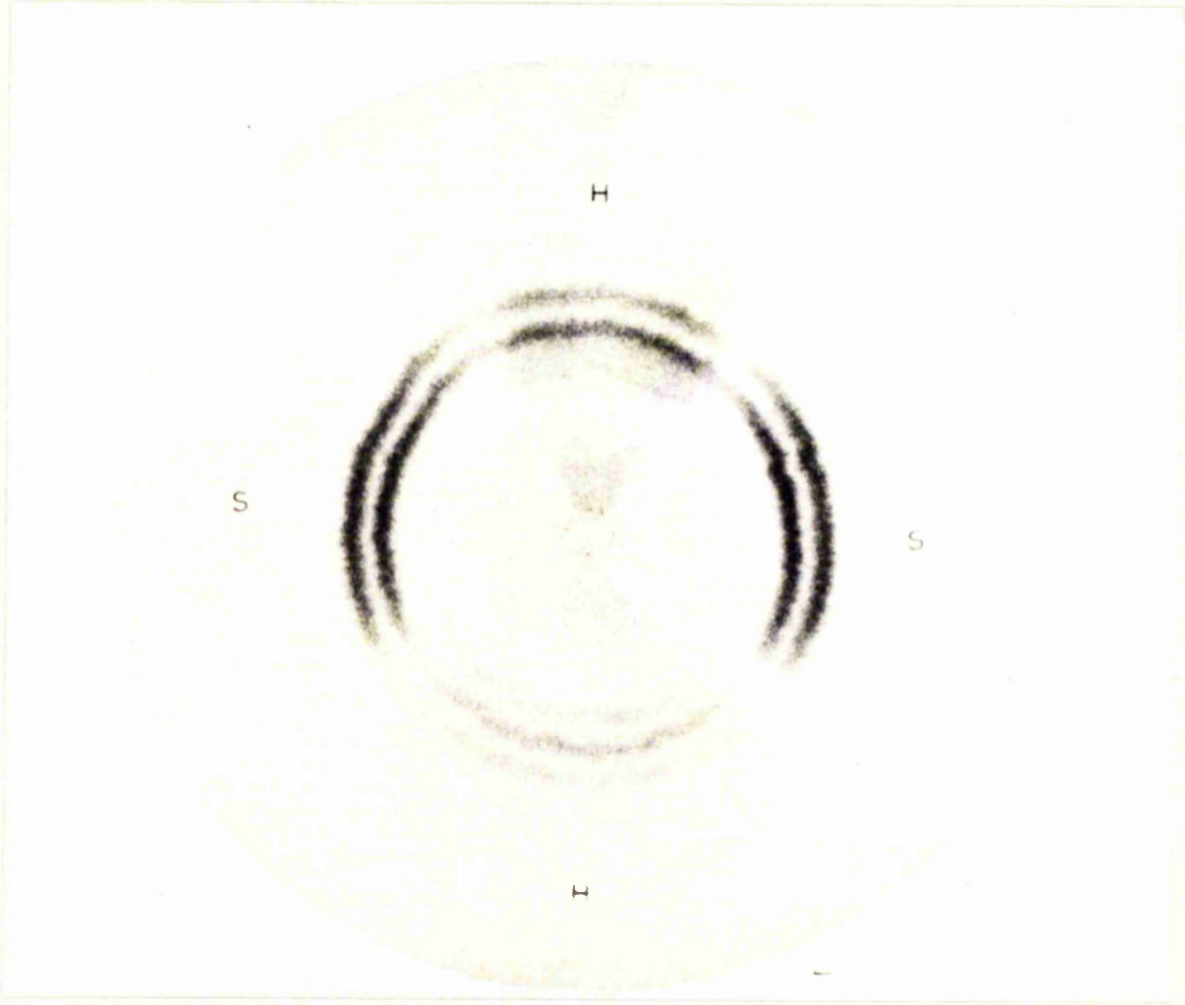


Fig. 17. Chromatogram of an 8 hour hydrolysate of the lipopolysaccharide (PE/SP) in 6N HCl after ninhydrin degradation (see text)

H - hydrolysate S - arabinose and lyxose

Solvent - butanol, pyridine, water

Spray - aniline phthalate

LIPID ANALYSIS.

1. CHLOROFORM SOLUBLE FRACTION.

30mg of the lipopolysaccharide (PE/SP) were hydrolysed for 1 hour with 5ml of N hydrochloric acid and the liberated lipid material extracted by shaking the hydrolysate with three 10ml portions of chloroform. The combined extracts were filtered and the chloroform removed by heating at 80°C. The residue was then hydrolysed by heating for a further 8 hours at 100°C with 5ml of 6N hydrochloric acid and, after cooling, the solution was extracted three times with 20ml of ether. The extract was evaporated to dryness and the residue dissolved in 0.2ml of benzene. This portion of the hydrolysate was examined for the presence of fatty acids by circular paper chromatography.

The aqueous part of the hydrolysate was evaporated to dryness under vacuum, dissolved in 1ml of distilled water then dried over sodium hydroxide in a vacuum desiccator. After the addition of two drops of distilled water this portion was examined for the presence of amino sugars and amino acids.

Fatty Acids.

Fatty acids were identified by paper chromatography using a modification of the copper-ferrocyanide method described by Newotny et al (63), in which the copper salts of the acids formed by immersing the paper in a solution of copper acetate were visualised by subsequently/

subsequently dipping the paper in a 0.1% aqueous solution of sodium diethyldithiocarbamate. The brown chelation product with the copper was found to give a better contrast than the red colour obtained when the copper salts react with ferrocyanide.

Unsaturated fatty acids were detected by the permanganate-benzidine reagent. Full details of the procedures are given in the Appendix.

Results.

Four main bands were found on development of the chromatograms - Figure 18. Three of these corresponded to arachidic, palmitic and myristic acids, while the fourth, which ran outside caprlc acid, had the same Rf as β-hydroxy myristic acid (not included in the standard mixture shown in the photograph). There were also faint lines corresponding to capric and lauric acids and a very slow moving component which may be lignoceric acid (no standard lignoceric acid was available for comparison).

When the chromatograms were developed with the permanganate-benzidine reagent for unsaturated acids, two bands appeared - a faint one with the same R_f as oleic acid and a more distinct band with an R_f slightly greater than that of linoleic acid.

The same fatty acid pattern was found when 20mg of PE/SP were/

Fig. 18. Reverse-phase chromatogram of fatty acids from an 8 hour hydrolysate in 6N HCl of the chloroform-soluble lipid from fraction PE/SP.
H - hydrolysate S - mixture of standard fatty acids (capric, lauric, myristic, palmitic, stearic and arachidic).
Stationary phase - Shellsol T
Moving phase - 90% acetic acid
Stained with copper acetate and sodium diethyldithiocarbamate (see text)

were hydrolysed for 8 hours in 6N hydrochloric acid without preliminary separation of the chloroform-soluble lipid.

Amino Acids and Amino Sugars.

Chromatographic examination of the aqueous portion of the hydrolysate showed the presence of glycine, aspartic acid, glutamic acid, lysine, alanine, methionine, leucine, ethanolamine and glucosamine. No galactosamine was detected, and after ninhydrin degradation only arabinose was present.

2. ETHER SOLUBLE AND ETHER INSOLUBLE, CHLOROFORM SOLUBLE FRACTIONS.

The experiments described above were carried out on the total chloroform-soluble material, but in view of the appreciable amount of ether-soluble material present, it was decided to repeat the experiments on the ether-soluble and ether-insoluble chloroform-soluble fractions.

50mg of PE/SP were hydrolysed for 1 hour in N hydrochloric acid and the solution was extracted three times with 10ml of ether. The combined ether extract was filtered, dried over anhydrous sodium sulphate and evaporated to dryness. The ether extracted solution was then shaken with three 10ml portions of chloroform and the chloroform extract filtered and dried at 80°. The ether-soluble lipid contained 0.8% P and/

and the chloroform-soluble fraction 1.7% P. The ether-soluble material at this stage was found to contain appreciable amounts of myristic, palmitic and B-hydroxymyristic acids when examined by reverse phase paper chromatography.

Both fractions were then heated in 5ml of 6N hydrochloric acid at 100° for 8 hours and examined for the presence of fatty acids, amino sugars and amino acids.

Fatty Acids.

The hydrolysate of the ether-soluble lipid contained arachidic, palmitic, myristic and β-hydroxymyristic acids along with a trace of lauric acid and possibily lignoceric acid. The chloroform-soluble fraction contained β-hydroxy myristic, myristic and arachidic acids with a trace of lauric and very faint traces of palmitic and lignoceric acids.

No quantitative estimations of the fatty acids were attempted, but when equivalent amounts of the hydrolysates of the three fractions (total chloroform-soluble, ether-soluble and ether-insoluble chloroform-soluble) were compared on the same chromatogram, it was clear that a considerable proportion of the myristic and β-hydroxymyristic acids, and almost all of the palmitic acid present in the total chloroform-soluble material was found in the ether-soluble fraction.

Amino Acids and Amino Sugars. /

Amino Acids and Amino Sugars.

The same range of amino acids were found in both the ether-soluble and the ether-insoluble chloroform-soluble fractions as were found in the total chloroform-soluble material. Glucosamine and ethanolamine were detected in both fractions, and after ninhydrin degradation of the hydrolysates, only arabinose could be detected.

B. BIOLOGICAL PROPERTIES OF PE/SP.

Pyrogenicity.

It has already been shown that the crude material isolated from the bacterial cells by phenol extraction (fraction PE - see above) caused an average maximum temperature increase of 1.0°C when injected into rabbits in amounts of 0.01µg/kg body weight. The same amount of purified lipopolysaccharide (PE/SP) caused an increase of 1.3°C (average of 25 responses). The fever curve obtained at this dose level was typically of the monophasic type (see Figure 19). Injection of 0.1µg/kg; produced a maximum temperature increase of between 1.5°C and 2.0°C, and at this dose level the curve usually showed a pronounced secondary peak which was reached about 3.5-4 hours after the injection - see Figure 19.

When larger amounts were injected, the responses of the individual rabbits showed a wide variation and no really typical fever curve emerged. This is illustrated in Figure 20, which shows the temperature responses in a group of nine rabbits following injection of 1.0µg/kg of PE/SP.

At a level of 10.0µg/kg the fever curves became more uniform throughout the group, and were normally of the type shownin Figure 19: a moderate increase in temperature followed rapidly by a prolonged period of hypothermia. At this dose level the animals began to show toxic symptoms, and two of the ten rabbits used in the test died within 24 hours/

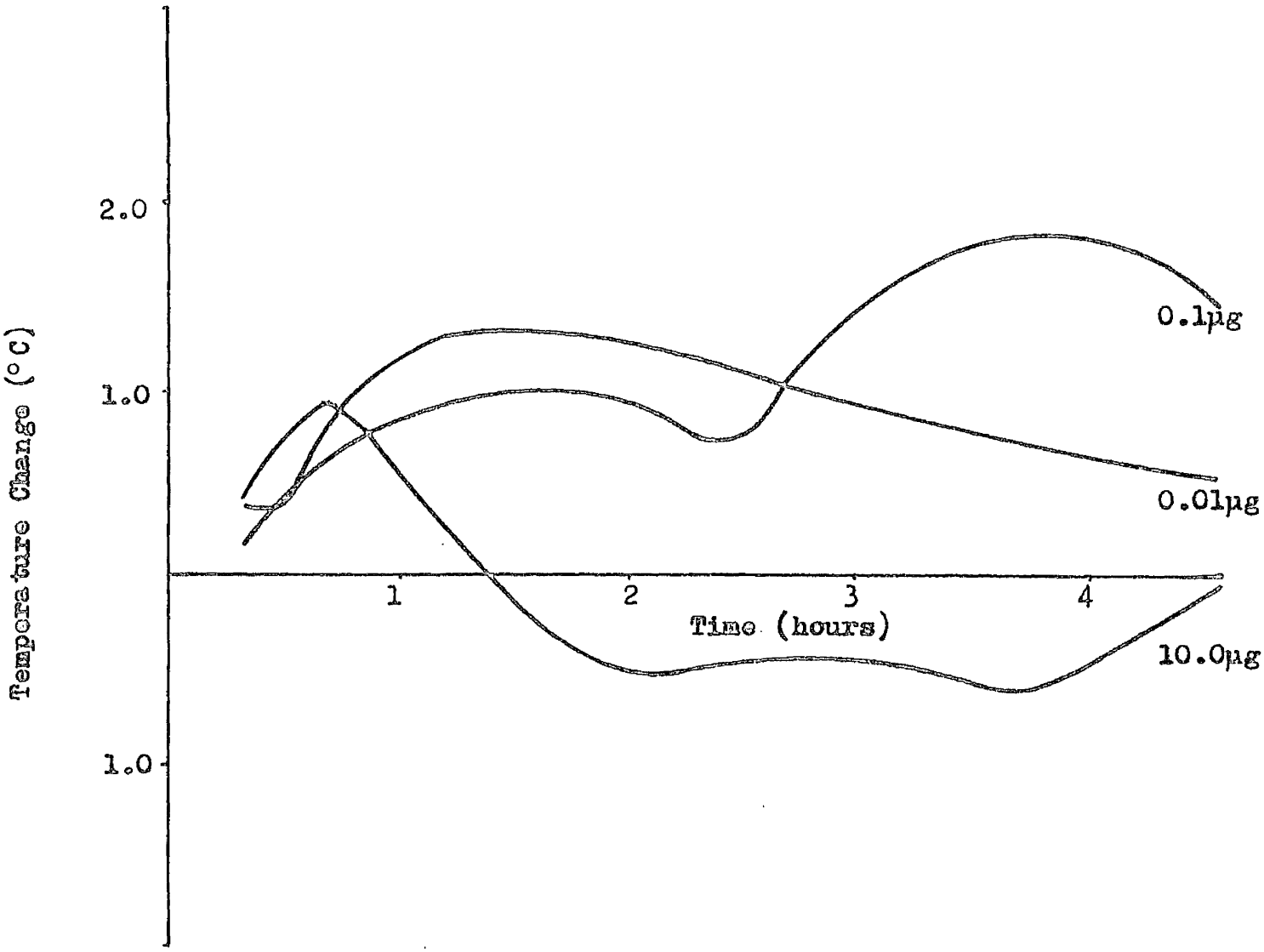


Fig.19. Temperature changes in rabbits after intravenous injection of 0.01µg/kg, 0.1µg/kg and 10.0µg/kg amounts of the lipopolysaccharide PE/SP

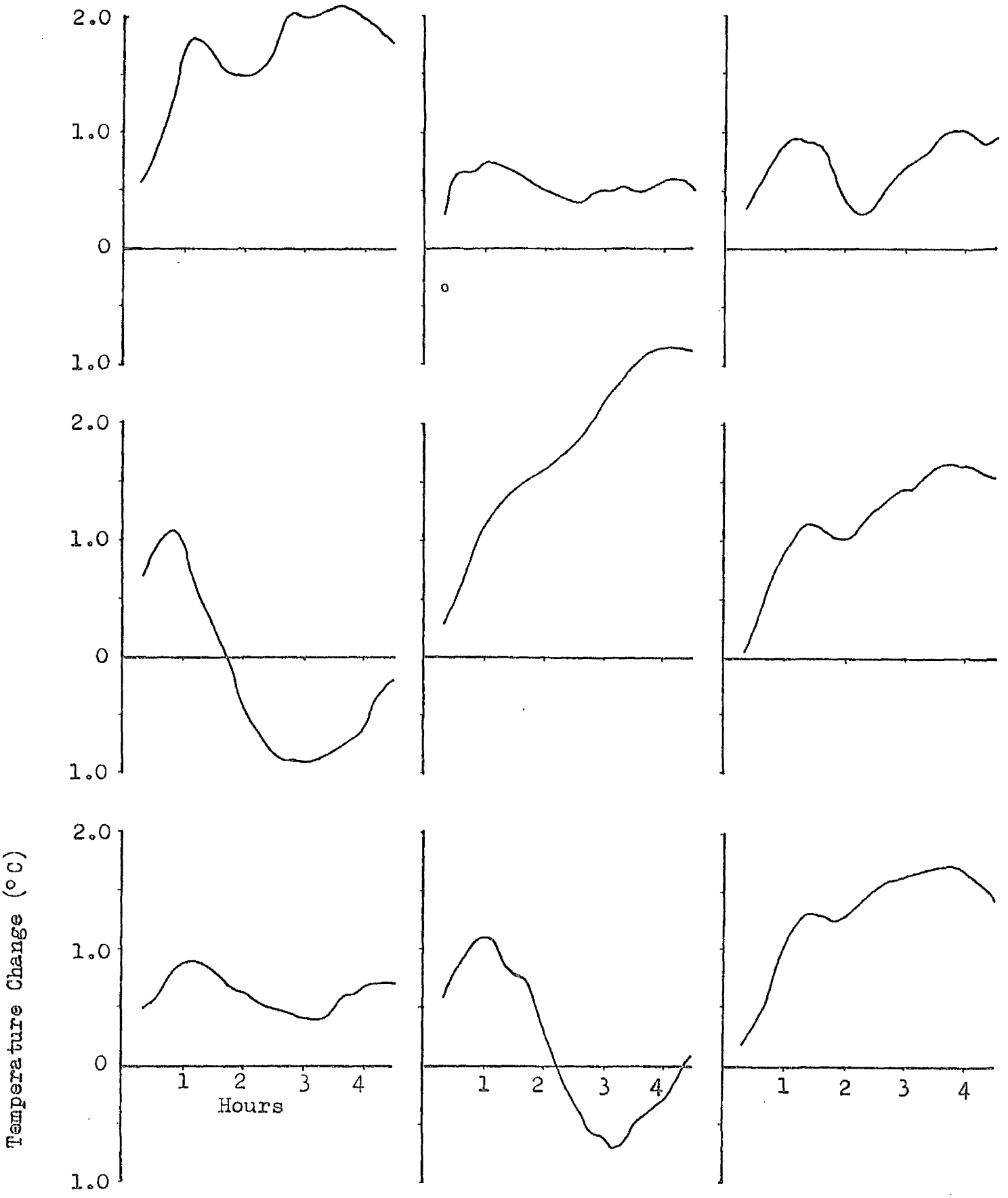


Fig.20. Individual temperature responses of nine rabbits following intravenous injection of 1µg/Kg of PE/SP

hours of the injection.

Toxicity.

Toxic symptoms appeared in rabbits at a dose level of 10.0µg/kg. The symptoms most commonly observed were excessive urination, partial paralysis of the rear limbs, and in those animals which survived, a loss of appetite which lasted for several days after the injection.

No accurate estimation of the toxicity of the lipopolysaccharide in rabbits was attempted, but in the few tests made at higher dose levels, it was found that the animals normally succumbed to amounts of 40-60µg/kg.

The figures for toxicity in mice are shown in Table 7. The mice, all three months old, were injected through the tail vein with 1ml of a solution of PE/SP in sterile saline, and the mortality figures were taken 48 hours after injection.

TABLE 7.

Amount of PE/SP injected (mg)	Deaths.
0.25	1/10
0.5	3/10
1.0	4/10
1.5	6/10
2.0	10/10

Some/

Some of the mice which survived 48 hours died within the following few days, but as these deaths could have been due to secondary or unrelated causes, they were not included.

Immunological Tests.

When 0.1%, 1.0% and 2% solutions of PE/SP were tested by the agar double diffusion method against an antiserum prepared against the whole cells, single precipitin lines were formed - Figure 21. The plate was photographed after 24 hours then observed for a further 7 days. No additional lines appeared.

To determine whether or not the lipopolysaccharide (PE/SP) was antigenic in rabbits, 1 µg/kg amounts were injected into two animals and blood samples withdrawn after 7 days. The sera obtained from these animals did not form a precipitin line when tested by the agar diffusion method against a 1% solution of PE/SP. Two rabbits were then injected three times a week with amounts of the lipopolysaccharide increasing from 1µg to 32µg. A week after the last injection blood was withdrawn, and when the serum was tested against a 1% solution of the injected material a single precipitin line was formed - Figure 22.

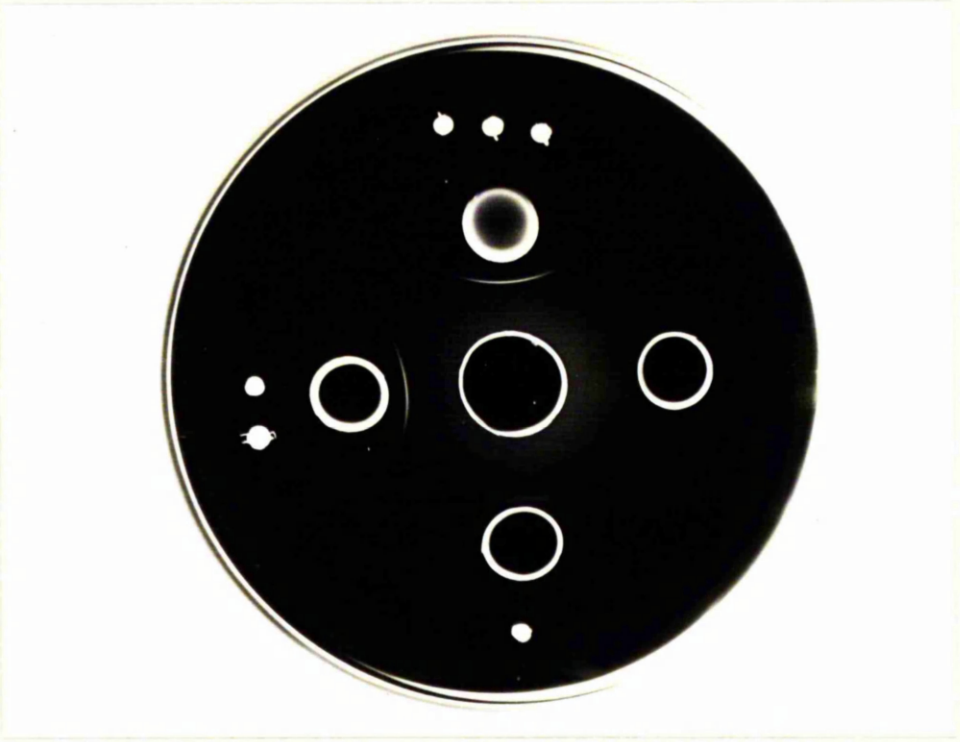


Fig. 21. Precipitin pattern in agar gel of 0.1%, 1.0% and 2.0% solutions of the lipopolysaccharide (PE/SP) with antiserum against dried Pseudomonas aeruginosa cells

Centre well - antiserum (0.2ml)

1 - 0.1% PE/SP (0.2ml), 2 -1.0% PE/SP (0.2ml)

3 - 2.0% PE/SP (0.2ml)

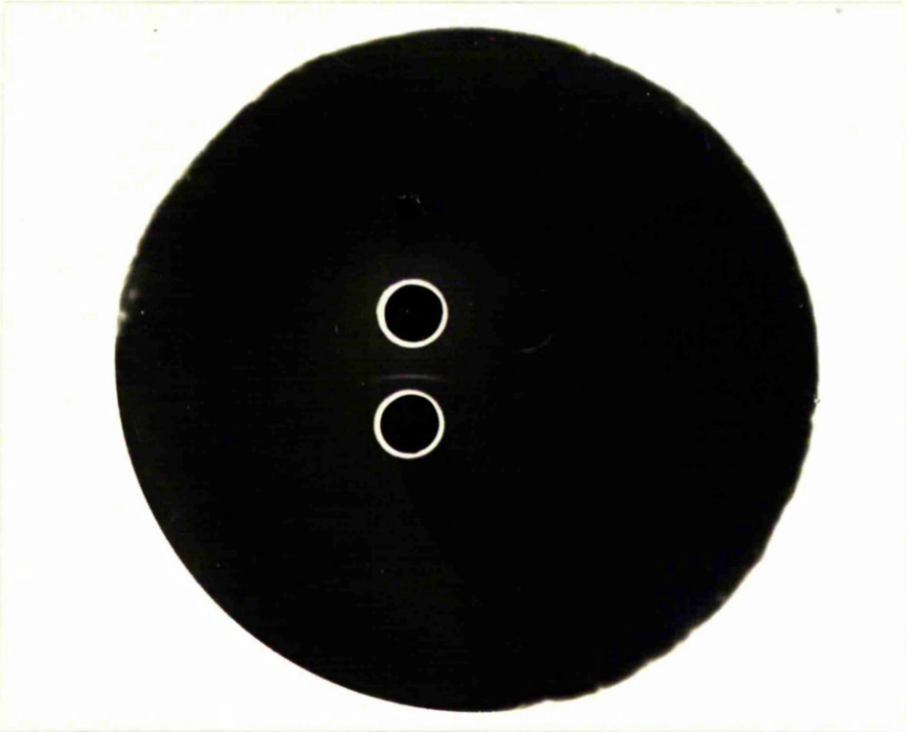


Fig. 22. Precipitin pattern in agar gel of the lipopolysaccharide (PE/SP 1%) with antiserum against PE/SP.

Unmarked well - PE/SP (0.2ml)

● - antiserum (0.2ml)

DISCUSSION.

The hot phenol procedure developed by Westphal (11) has been found to be the most generally effective of the methods used for the extraction of the endotoxins of Gram -ve bacteria, and in the present work, it has been employed successfully for the extraction of the endotoxin of Pseudomonas aeruginosa. Treatment of the acetone-dried cells with hot phenol, resulted in a fraction (PE) amounting to 14-18% of the cell dry weight and containing about 50% of RNA. The presence of nucleic acid in phenol extracts at this stage was noted by Westphal (11), and confirmed by other workers who have used the method (34, 39, 65). That the extract from Ps. aeruginosa also contained the endotoxin could be deduced from the pyrogenic reaction following injection of small (0.01µg/kg) amounts into rabbits, the liberation of chloroform-soluble lipid material on acid hydrolysis, and the formation of precipitin lines when solutions were tested onagar gels against antiserum prepared against the whole (acetone-dried) cells - all typical properties of the endotoxic lipopolysaccharides. The presence of polysaccharide material was confirmed by paper chromatographic examination of hydrolysates, which showed the presence of ribose, and the three aldohexoses galactose, glucose and mannose.

Repeated high speed centrifugation was found to be effective in separating the nucleic acid from the lipopolysaccharide, and by this/

this method, a nucleic acid-free lipopolysaccharide was obtained in yields of 2-4% of the cell dry weight.

This yield indicated that either the initial extract (PE), which amounted to 14-18% of the cell dry weight, contained additional components other than nucleic acid and lipopolysaccharide, or that a considerable proportion of the lipopolysaccharide was not sedimented during the high speed centrifugation. Burton (65) has found that the endotoxin extracted from Escherichia coli 0111 B4 contained two lipopolysaccharides, one of which was sedimented at 80,000g while the other remained in the supernatant liquid. In the present experiments, the freeze-dried supernatant contained about 2% of chloroform-soluble (released after 45-60 minutes acid hydrolysis) and it provoked an appreciable (0.8°) pyrogenic response in rabbits in amounts of 0.01µg/kg body weight, suggesting that even centrifugation at 105,000g did not sediment all the endotoxic material. However, no further experiments were carried out to investigate the nature of the pyrogenic substance remaining in the supernatant liquid.

Apart from lipopolysaccharide and nucleic acid, lower molecular weight polysaccharides may occur in phenol extracts of Gram -ve bacteria (19,22,38), and may remain in the supernatant along with nucleic acid after high-speed centrifugation. If these polysaccharides have the same composition as the polysaccharide portion of the lipopolysaccharide they can be regarded as having been split from the/

the antigenic complex during the extraction procedure. The findings detailed above however indicate the presence of two different polysaccharides in the strain of Ps. aeruginosa used in this work. Chromatograms of hydrolysates of the nucleic acid-free lipopolysaccharide (PE/SP) showed the presence of two bands, corresponding to galactose and glucose, but none of the mannose found in the crude extract (PE), while mannose was detected in hydrolysates of the freeze-dried supernatant along with ribose and smaller amounts of galactose and glucose. The presence of two different polysaccharides in a bacterial extract has been noted by other workers. Goebel and his co-workers for example (13), found that the diethylene glycol extract of Shigella flexner Type 5 (1a) contained as well as the somatic antigen, an immunologically inert polysaccharide which was apparently a glucan, while mannose was found in the crude extract from the culture fluid of Proteus vulgaris, but was not present in the purified lipopolysaccharide (106).

The immunological status of the mannose-containing polysaccharide detected in the present study remains undecided. The crude extract (fraction PE) formed three precipitin lines against a whole cell antiserum, while the purified lipopolysaccharide formed only. This, however, could have been due to the presence in the crude material of lower molecular weight specific polysaccharides of the same type as that present in the lipopolysaccharide, and does not necessarily mean that the mannose-containing material was immunologically active.

The purified material obtained in yields of 2-4% of the dry weight of the cells is fairly typical of the lipopolysaccharide endotoxins which have been extracted from other Gram -ve bacteria in both its overall chemical constitution and its biological properties. There were, however, two points of particular interest in the chemical nature of this material. Although MacLennan (39) found that the lipopolysaccharide from Bordetella bronchisepticus released about 13% ether-soluble lipid after acetic acid hydrolysis, the lipid material (lipidA) released from bacterial endotoxins after short acid hydrolysis is usually described as being soluble in chloroform and pyridine, but insoluble or not appreciably soluble in other lipid solvents (16). However, while short hydrolysis (45-60 minutes) of the Ps. aeruginosa lipopolysaccharide in N hydrochloric acid gave 38% of a chloroform-soluble material it was also found that 15% of the material released on hydrolysis was soluble in ether. It is clear that there can be a variation in the extent to which lipid A preparations from different lipopolysaccharides dissolve in solvents other than chloroform.

Nowotny has found 30-40% of the lipid A from E. coli to be made up of free fatty acids (64). The ether-soluble material from Ps. aeruginosa contained appreciable amounts of free myristic, palmitic and β-hydroxymyristic acids, but further hydrolysis resulted in the release of additional fatty acids indicating that it also contained intact lipid.

Both/

Both the ether-soluble and the ether-insoluble chloroform-soluble lipid fractions from Ps. aeruginosa were found to contain phosphorus, glucosamine and several amino acids as well as a series of long chain fatty acids. MacLennan also found hexosamine in both the ether-soluble and chloroform-soluble lipids from B. bronchisepticus, but apparently no amino acids were detected in hydrolysates of these substances. He found amino acids in the ether- and chloroform-insoluble residue and considered that these might account for all the ninhydrin positive substances (other than hexosamine) detected in hydrolysates of the total lipopolysaccharide. Nowotny however (64) detected amino acids associated with the purified lipid A fraction from E. coli, and Westphal has found that all the lipid A preparations which he has examined contained one or both of the amino dicarboxylic acids and one or two additional amino acids (37). Burton, on the other hand, considered that glucosamaine could account for all the ninhydrin positive material in hydrolysates of the purified lipid A from E. coli 0111 B4. These results need not be regarded as conflicting unless it is assumed that the lipid portions of lipopolysaccharides from different bacteria should have a close similarity. Nowotny has pointed out that the lipid A from E. coli contains several different components (64), so that even if there is a common lipid fraction which is present in all endotoxins and is responsible for certain of the non-specific biological effects, it is possible that the composition of the rest of the lipid shows a variation from one organism to another. More work will have to be/

be done on the analysis of lipid fractions of endotoxins before this question can be answered.

The other point of particular interest was the detection of an aldo-heptose in the lipopolysaccharide from Ps. aeruginosa. One of the dangers of paper chromatographic identification lies in the tendency to assume that a single spot or band represents a single substance, and this possibility is shown in the present work. Hydrolysates of the lipopolysaccharide consistently showed on paper chromatograms only two bands, corresponding to glucose and galactose. These bands separated quite cleanly and gave no indication of the presence of any other sugar, and yet when the intact lipopolysaccharide was examined in the sulphuric acid-cysteine test, the spectrum showed the absorption peak at 505mμ which is characteristic of heptoses. Extension of the hydrolysis period up to 20 hours showed that the heptose was released only slowly on hydrolysis and that on chromatograms it had an R_f value very close to that of glucose.

Masking of heptose on chromatograms by glucose and galactose was found by Salton (98) during an investigation of the cell wall polysaccharides from several Gram-ve bacteria, and an analogous situation has been reported with the lipopolysaccharide from Pasteurella pestis in which the glucose present in hydrolysates could not be detected on chromatograms because of the presence of large quantities of heptose (19). Although the hexoses and heptoses give characteristically /

different colours with reagents such as aniline phthalate and p-anisidine hydrochloride it is obviously possible for a minor component to remain undetected in the presence of an excess of a sugar of a different class.

The slow release of the heptose from the lipopolysaccharide is a rather unusual feature of this material. A high degree of resistance to acid hydrolysis would be reflected in the figure for total reducing sugars obtained after normal hydrolysis periods. This was found in the case of the lipopolysaccharide from B. bronchisepticus which was shown to contain 40% sugar (heptose, hexose and hexosamine) and yet gave a reducing sugar value of only 7% after 24 hours hydrolysis in 0.5N hydrochloric acid. 24 hour hydrolysates of this material in N hydrochloric or sulphuric acid gave only faint colour reactions for aldoheptose and hexosamine on chromatograms. The lipopolysaccharide from Ps. aeruginosa used in the present study, however, gave a maximum reducing sugar value after about 6 hours hydrolysis in N hydrochloric acid and yet the eluates from chromatograms examined by the Dische reaction showed that the amount of heptose released increased as the hydrolysis was extended to 10, 15 and 20 hours. This could result from different types of linkages involved in the polysaccharide structure, or possibly from the presence of two different polysaccharides. This latter possibility cannot be ruled out, because the presence of an additional major polysaccharide component could have escaped detection/

detection in the agar precipitin tests, either through a lack of reactivity (i.e. being immunologically inert), or through having the same diffusion rate as the other polysaccharide.

There have been comparatively few studies on the polysaccharide complexes from Pseudomonas aeruginosa. Akiya et al (99) isolated a polysaccharide from a strain of Ps. aeruginosa (Bacillus pyocyaneus) by the 50% pyridine method of Goebel (13) and found it to contain glucose and fructose, and Dzulynska and Mikulaszek (45) examined 5 polysaccharides from this species by paper chromatography and found galactose (and/or glucose), xylose (and/or ribose), rhamnose and uronic acid present in all five, while glucosamine was detected in four and mannose in two of the five. Warren and Gray (100) reported on a polysaccharide released from Ps. aeruginosa which was depolymerized by bovine testicular hyaluronidase but they gave no details of the chemical composition of this substance. In addition to these reports on polysaccharides, Jarvis and Johnston isolated , from cultures of Ps. aeruginosa, a crystalline lipid composed of L-rhamnose and β-OH-decanoic acid (101).

The only other detailed studies which have been carried out on the endotoxin of Ps. aeruginosa however are those of Naoi, Egami, Hamamura/

and Homma (34, 102, 103), who investigated the material from autolysed cultures and the extracts obtained from the bacteria by the use of the trichloracetic acid and hot phenol extraction methods. The lipopolysaccharide obtained after phenol extraction differed in several ways from that described in this work. The total reducing sugar value of both preparations was about the same, but the sugar pattern found by these workers was quite different from that found in the present investigation - the sugars identified being galactosamine, glucose, arabinose, ribose and rhamnose. That there should be differences noted in the polysaccharide composition is not in itself surprising. It is known (104, 105) that Ps. aeruginosa can be classified into at least six serological groups, so that variations in the composition of the polysaccharide portion of the antigen might be expected. It is surprising however to note the inclusion of ribose as a constituent sugar of the lipopolysaccharide. As mentioned in the Introduction, the detection of ribose is normally an indication of the presence of RNA in the extract, but Naio and his co-workers found that the UV spectrum of their lipopolysaccharide showed no sign of the presence of nucleic acid and they concluded that ribose formed part of the specific polysaccharide. These authors did not comment on this unusual finding, and it will be interesting to see if ribose is detected in the endotoxins from other strains of Ps. aeruginosa.

All lipopolysaccharides so far investigated have been found to /

to contain amino sugars, but although glucosamine and galactosamine may both occur in the complete lipopolysaccharide only glucosamine has been detected in the lipid, of which it appears to be a normal constituent(40). Both of the amino sugars were found in the lipopolysaccharide in the present investigation and glucosamine but not galactosamine was detected in hydrolysates of the isolated lipid fractions. Naio et al. however found galactosamine but no glucosamine in their lipopolysaccharide but they did not say whether or not this amino sugar was detected in the isolated lipid. The absence of glucosamine is particularly interesting because of its importance in the suggested structure of lipid A proposed by Nowotny (64).

The biological properties described in the present work show that the lipopolysaccharide from Ps. aeruginosa produces effects which are typical of bacterial endotoxins. Rabbits responded to intravenous injection of 0.01µg/kg with a temperature rise of 1.3° indicating a pyrogenic potency of the same high level as that of other preparations. The pattern of fever curve produced by different amounts of the lipopolysaccharide showed a definite variation. At 0.01µg/Kg the curve was of a monophasic type while at a higher dose level of 0.1µg/Kg a double peak was usually found. When the amount was increased to 10µg/Kg an entirely different type of corve was obtained in which a moderate increase in temperature was followed by an extended period of hypothermia. This pattern was regularly obtained when rabbits were/

were injected with this amount. At an intermediate dose level of 1µg/Kg the responses of the individual animals showed a wide variation and it was impossible to establish a typical fever curve. Two of the rabbits given 1 µg/Kg showed smaller temperature increases than any of the ten animals which received 0.1µg/Kg and the average maximum temperature of the 9 rabbits given 1.0µg/Kg was the same as the average of those given 0.1µg/Kg. This is not in agreement with Westphal's observation that the pyrogenic response to endotoxins becomes progressivly stronger as the dose is increased up to a level of 1.0µg/Kg (16).

No precise estimate of the toxicity of the lipopolysaccharide in rabbits was attempted, but it was found that the animals normally died after injection of amounts of 40-60µg/Kg. Toxicity experiments in mice showed that 100% mortality was not reached until the dose approached 2 mg. Endotoxins have generally been found to be lethal in rabbits at a level of 20-100µg/Kg., but normally the toxicity in mice is higher than that found in the present experiments. For example, the lipopolysaccharide from B. bronchisepticus was lethal to mice in amounts of 100µg (39), while the lipopolysaccharide from Ps. aeruginosa isolated by Naoi and his co-workers (34) had an LD_{50} in mice of 0.1-0.3mg. In view of the suggestion of Schaedler and Dubos (77) that variation in the sensitivity of mice to the lethal effects of endotoxins may be a feflection on the extent to which they have previously been exposed to ordinary bacterial /

bacterial pathogens, it is interesting that a lipopolysaccharide from Proteus vulgaris studies by Dr. Shaw in this college also showed a rather low toxicity in mice (LD_{50} 850µg) although rabbits died after receiving amounts of 20-40µg/Kg (106). This similarity between the endotoxin from P. vulgaris and that from Ps. aeruginosa may however be without any significance, and it should be remembered that the suggestion of Schaedler and Dubos has been challenged by other workers (78).

The lipopolysaccharide isolated in the present studies was found to be antigenic in rabbits, and was in this respect similar to the lipopolysaccharide from Ps. aeruginosa examined by Homma and his colleagues (34). There is obviously, however, a considerable variation in the antigenic potency of lipopolysaccharides isolated from different organisms. The lipopolysaccharide from P. pestis was considered to be a hapten and could be made antigenic by combination with the conjugated-protein component of the somatic antigen of Shigella dysenteriae, and the lipopolysaccharide from B. bronchisepticus was also thought to be non-antigenic. However, the lipopolysaccharides from P. multocida (35) and S. typhosa (17) were found to be antigenic. Although these results probably indicate inherent differences in the immunological potency of the various lipopolysaccharides, it should be pointed out that the failure to detect specific antibodies may be due to a lack of sensitivity in the methods employed. Davies for example(22) obtained/

obtained negative precipitin tests after injection of the lipopolysaccharide from Pasteurella pseudotuberculosis into rabbits, but when the more sensitive haemagglutination method was used specific antibodies were detected. Even the haemagglutination techniques, however, will only detect antibody present in amounts greater than 0.01ug/ml of serum (equivalent to about 10^{12} molecules of γ-globulin/ml) (107) so it is possible that substances considered non-antigenic may in fact be weak antigens and not true non-antigenic haptens.

SECTION 3.

INTRODUCTION.

One of the most widely used methods for the extraction of the O antigens of Gram -ve bacteria is the cold trichloracetic acid method introduced by Boivin thirty years ago (5). It is known, however, that this method may not remove all the antigen from the bacterial cell. Boivin found that a second, third and fourth extraction produced additional amounts of antigenic material (26), and by comparison with the tryptic digestion method of Raistrick and Topley (7) he estimated that a single trichloracetic acid extraction removed 60-70% of the antigen (26). Similar findings have been reported by more recent workers. Davies, for example, (108) found that trichloracetic acid extracted less of the O antigen of Shigella dysenteriae than did diethylene glycol, and Webster et al (109) showed that after extracting Salmonella typhosa cells with trichloracetic acid the residue was still active in the production of O agglutinins, and they estimated that trichloracetic acid removed only 20% of the O antigen.

If the residue of the cells left after trichloracetic acid extraction still contains an appreciable amount of the O antigen, it should be possible, by the use of the more drastic hot phenol method, to extract from it material which shows the endotoxic properties of the antigen. It/

It was decided therefore to investigate the effect of trichloracetic acid extraction on the strain of Pseudomonas aeruginosa used in this work, and also the effect the acid treatment of the cells had on the amount and nature of the material obtained by subsequent treatment of the residue with hot phenol.

TRICHLORACETIC ACID EXTRACTION.

Acetone dried cells (10g) were mixed for 3 hours with 100ml of 0.25N trichloracetic acid in an ice-water bath and the suspension was centrifuged for 10 minutes at 2500g. The sediment was washed with 100ml of distilled water, recentrifuged and stored at 4°C. The supernatant was dialysed for 2 days against running water, clarified by centrifugation then freeze-dried. The yield was 152mg (1.5%). This material was dissolved in 25ml of distilled water and fractionated by the addition of 2.5 volumes of cold ethanol. The flocculant precipitate obtained was deposited by centrifugation, reprecipitated at the same concentration of ethanol then separated, dissolved in a little distilled water and freeze-dried. The yield was 65mg (0.6%) - fraction TCA/ETH.

PROPERTIES OF TCA/ETH.

Sugars.

A sample of TCA/ETH (20mg) was hydrolysed for 3 hours in N sulphuric acid at 100°C, and the hydrolysate examined for the presence of sugars by circular paper chromatography as described in Section 2. The hydrolysate contained mainly mannose along with smaller amounts of galactose, glucose and ribose - Figure 23.

Fig. 23 Chromatogram of a 3 hour hydrolysate of Fraction TCA/ETH in N H_2SO_4 at 100°

H - hydrolysate S - standard sugars (galactose glucose, mannose, ribose and rhamnose)

Solvent - butanol, pyridine, water.

Spray - aniline phthalate

The spectrum obtained when 500µg samples of TCA/ETH were examined in the sulphuric acid-cysteine reaction showed an absorption maximum at 410mµ, but no peak at 505mµ - Figure 24.

PYROGENICITY.

A solution of TCA/ETH in sterile pyrogen-free saline caused an average maximum temperature increase of 1.0°C when injected into 10 rabbits in amounts of 0.01µg/Kg.

IMMUNOLOGICAL STUDIES.

When a 1% solution of fraction TCA/ETH was examined against a whole cell antiserum by the agar diffusion method, two precipitin lines were formed - Figure 25.

An antiserum against fraction TCA/ETH was prepared by injecting two rabbits twice a week for 4 weeks with amounts increasing from 1 µg to 32 µg. When the combined serum from these rabbits was tested on agar plates against a 1% solution of fraction TCA/ETH two precipitin lines were formed - Figure 25.

Two precipitin lines were formed when fraction TCA/ETH was tested against the antiserum prepared against the purified lipopolysaccharide (fraction PE/SP - Section 2) - Figure 26.

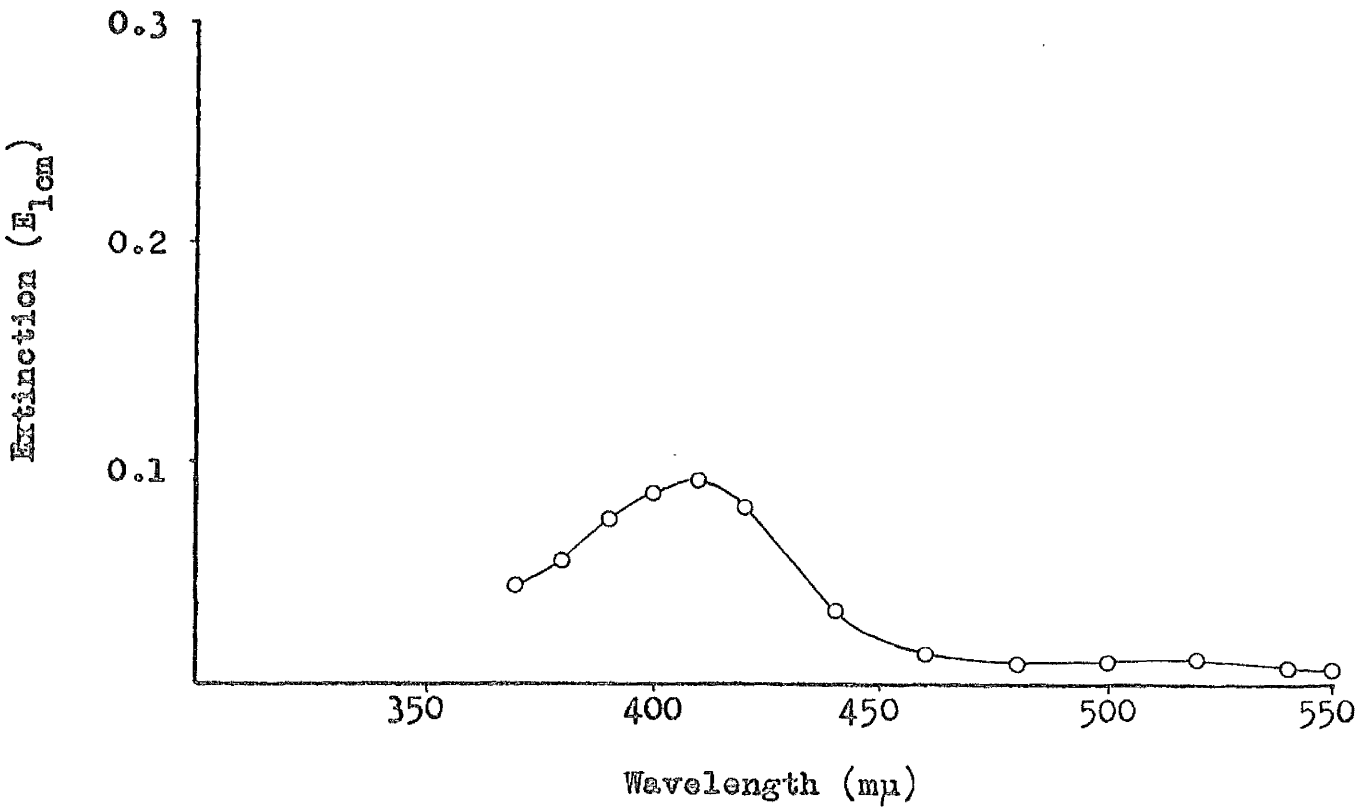


Fig.24 Absorption spectrum of TCA/ETH (500μg) after treatment with sulphuric acid-cysteine Readings taken after 20 hours

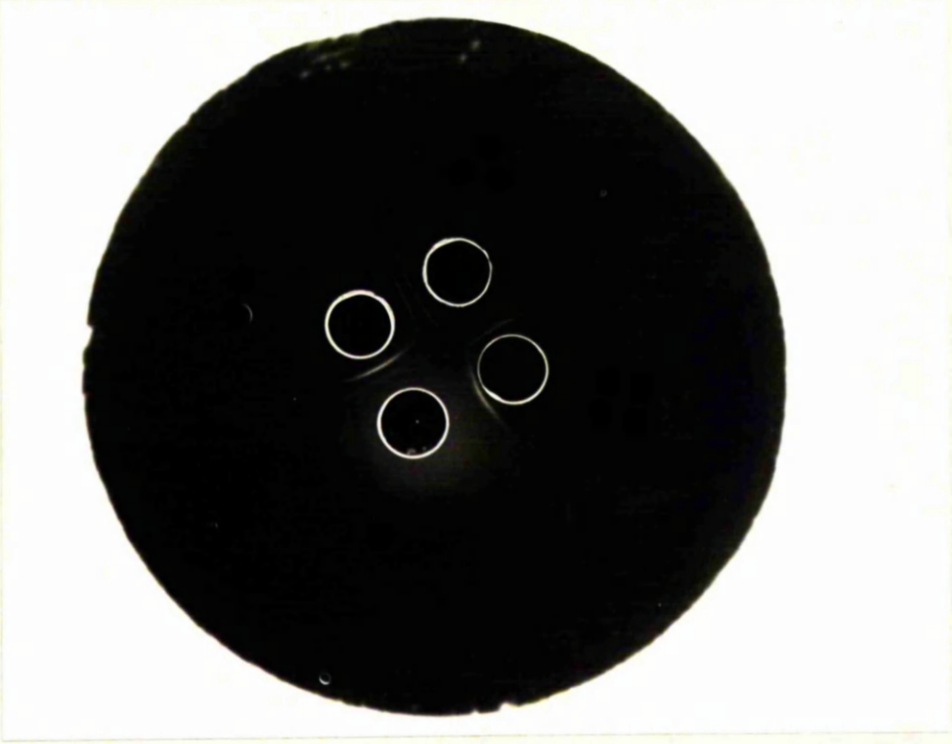


Fig. 25. Precipitin lines formed by 1% solutions of fractions TCA/ETH and TCA/PE/ETH (0.2ml.) with antiserum to TCA/ETH and antiserum to whole cells (0.2ml.)

1 - antiserum to whole cells

2 - TCA/ETH

3 - antiserum to fraction TCA/ETH

4 - TCA/PE/ETH

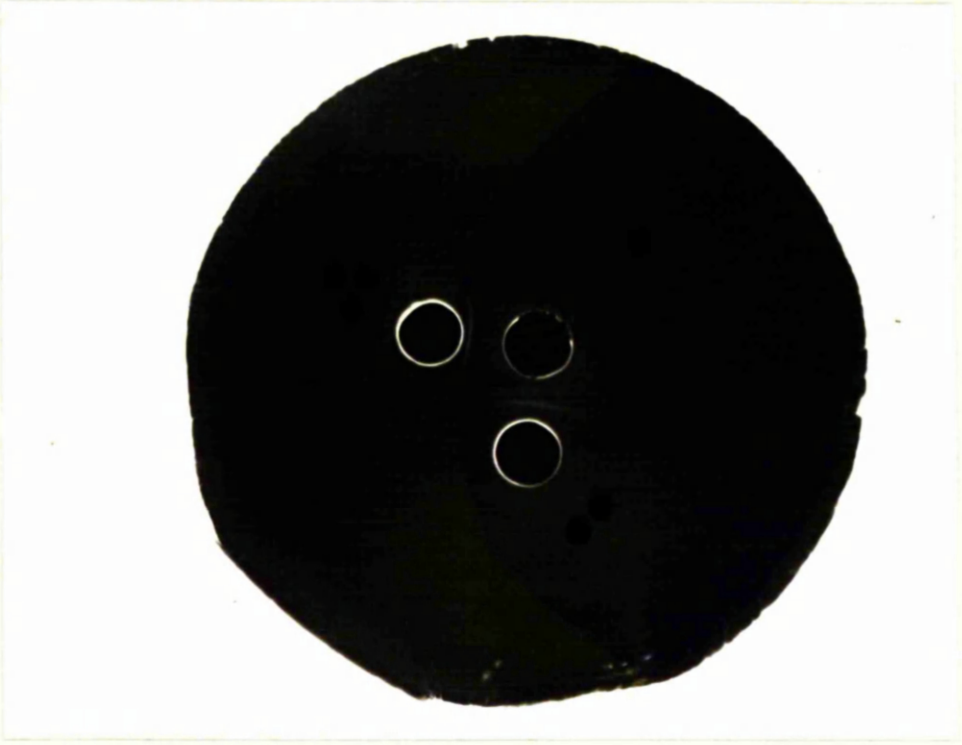


Fig. 26. Precipitin lines formed by 1% solutions of fractions TCA/ETH and PE/SP (0.2ml.) with antiserum to fraction PE/SP (0.2ml.)

1 - antiserum to PE/SP

2 - TCA/ETH

3 - PE/SP

The fact that trichloracetic acid extracted an amount of material equivalent to only 1.5% of the cell dry weight whereas the lipopolysaccharide could be obtained in yields of 2-4% of the cell weight (Section 2) indicated that an appreciable amount of the endotoxic substance was not extracted by the acid.

EXTRACTION OF THE RESIDUE.

The residue from the trichloracetic acid extraction of 10g of acetone-dried Ps. aeruginosa cells was mixed with 350ml of distilled water and treated with an equal volume of 90% phenol as described in Section 2. The aqueous solution obtained was dialysed against running water for two days, concentrated under vacuum to about 150ml, clarified by centrifuging and freeze-dried. The freeze-dried material (TCA/PE) weighed 320mg.

PROPERTIES OF TCA/PE.

Fraction TCA/PE contained 2.5% N and 34% chloroform-soluble lipid (estimated gravimetrically on a 50mg samples as described for PE/SP - Section 2). The absorption spectrum between 220mμ and 300mμ showed no peak at 260mμ - Figure 27.

Paper chromatography of a 20mg sample hydrolysed for 3 hours in N sulphuric acid at 100°C showed the presence of galactose, glucose and a trace of mannose. No ribose was detected.

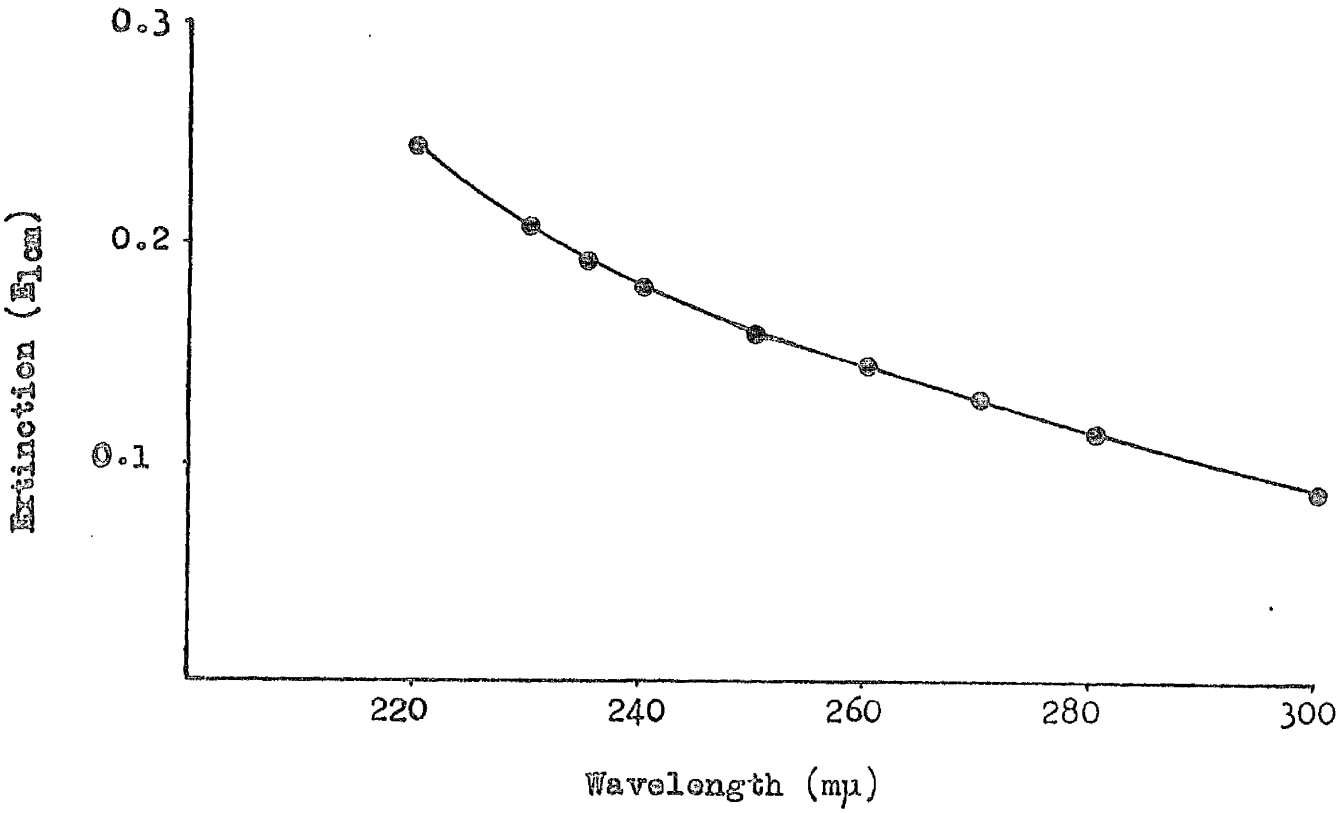


Fig. 27 Absorption spectrum of fraction TCA/PE 100μg/ml. in N/100 NaOH

Chromatograms of a samples of 25mg hydrolysed for 15 hours in 6N hydrochloric acid showed that the fraction contained glucosamine and galactosamine and the same amino acids as were found in PE/SP (Section 2).

PYROGENICITY.

Fraction TCA/PE in amounts of 0.01µg/Kg caused an average maximum temperature increase of 1.0°C when injected into a group of 10 rabbits.

PRECIPITIN REACTION.

When a 1% solution of TCA/PE was examined by the agar gel diffusion method against an antiserum against the whole cells, three precipitin lines were formed.

FRACTIONATION OF TCA/PE.

A few fractionation experiments were carried out on TCA/PE using cold ethanol (-10°C). It was found that a concentration of 45-50% precipitated about 70% of the material. Some of the properties of this fraction - TCA/PE/ETH are given below.

PROPERTIES OF TCA/PE/ETH.

The fraction contained 2.4% N, and 2.6% P. The reducing sugar value was 28% and it contained 35% chloroform-soluble lipid.

SUGARS./

SUGARS.

Paper chromatographic examination of a 20mg sample of TCA/PE/ETH hydrolysed for 3 hours at 100° in N sulphuric acid showed only two bands, corresponding to galactose and glucose, and no evidence of the mannose present in fraction TCA/PE. The reaction with sulphuric acid-cysteine (Dische) gave an absorption spectrum with peaks at 410mu and 505mu - Figure 28.

PYROGENICITY.

Injection of 0.01µg/Kg of fraction TCA/PE/ETH, caused an average maximum temperature increase of 1.2° in 6 rabbits.

PRECIPITIN TEST.

When a 1% solution of fraction TCA/PE/ETH was tested against the whole cell antiserum on agar gel, a single precipitin band was formed rapidly and a second faint precipitin line developed after standing for two or three days - Figure 25. (The faint line is not visible in the photograph). Fraction TCA/PE/ETH also reacted with the antiserum against fraction TCA/ETH - Figure 25.

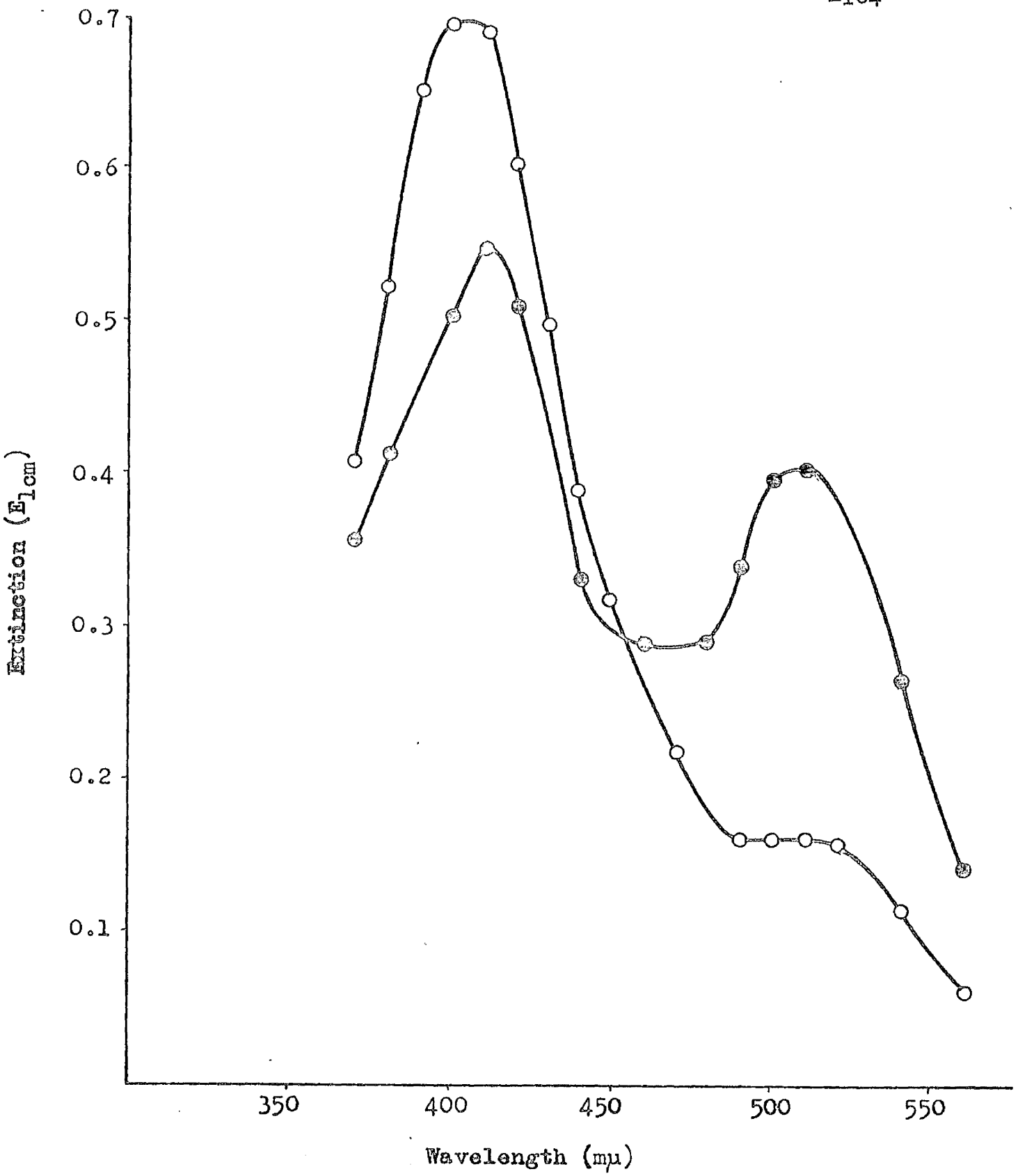


Fig. 28 Absorption spectrum of TCA/PE/ETH (500) after treatment with sulphuric acid - cysteine

○ - readings after 1 hour

● - readings after 20 hours

THE NUCLEIC ACID CONTENT OF TCA/PE

The absence of nucleic acid from this fraction, shown by the failure to detect ribose in hydrolysates and by the absence of an absorption peak at 260mμ when a solution was examined in the spectrophotometer, was of considerable interest as it has been shown in Section 2 that the material found in the aqueous layer after direct phenol extraction contained large quantities (c 50%) of nucleic acid.

Examination of the trichloracetic acid-soluble fraction (fraction TCA) showed that it contained only about 5% of nucleic acid, but in an experiment in which the acid extract was dialysed for 24 hours against distilled water, it was found that the dialysate contained material with an E_{max} of 260mμ. No quantitative estimation of these dialysable nucleic acid fractions was attempted, but that they did not account for all the cell nucleic acid was shown in an experiment in which 10g of acetone-dried Ps. aeruginosa was subjected to repeated extraction with trichloracetic acid. Table 8 shows the amounts and nucleic acid contents of the successive fractions obtained by thirteen extractions. (The purpose of this experiment was to see if repeated extraction with trichloracetic acid would remove all the endotoxin from the cell)

TABLE 8. /

TABLE 8.

TCA Extraction	1	2	3	4	5	6	7	8	9	10	11	12	13
Weight (mg)	156	36	58	90	95	145	134	107	84	46	58	35	23
Nucleic acid (%)	6	14	27	58	48	71	71	80	72	80	60	54	

The dialysed acid extracts were freeze-dried without prior fractionation. The nucleic acid content was estimated on solutions of 50 or 100µg/ml from a calibration curve of yeast nucleic acid, assuming the absorption at 260mµ to be due solely to the nucleic acid present in the fraction.

As shown in Table 8, the non-dialysable nucleic acid content of the trichloracetic acid-soluble fractions increased after the first extraction, indicating that a single treatment of the dried cells with acid did not result in the release of the total cell nucleic acid as dialysable fractions.

The residue left after the 13th extraction was washed with distilled water and stored in the refrigerator.

From these results, it appeared that when the residue left after a single trichloracetic acid extraction of the cells was treated with hot phenol, the nucleic acid must either have been present in the aqueous phase as dialysable fractions, or have been retained in the phenol layer.

When the aqueous phase (after removal of the phenol by shaking with diethyl ether) was examined in the spectrophotometer before dialysis, there was no peak at 260mμ, and no ribose could be detected in hydrolysates of the undialysed aqueous layer.

According to Westphal (11), the treatment of Gram -ve cells with hot 45% phenol results in a separation of the deoxyribonucleic acid from the ribonucleic acid - the RNA appearing with the lipopolysaccharide in the aqueous phase while the DNA remains in the insoluble residue in the phenol layer. If, therefore, as a result of prior extraction of the cells with trichloracetic acid, the RNA remained in the phenol layer, it should be possible to show this by the presence of ribose in hydrolysates of the material present in the phenol. The following experiment was carried out to check this possibility.

Acetone-dried cells (5g) were extracted with trichloracetic acid, as described above and the residue treated with phenol as described in Section 2. A second sample of 5g was treated with phenol without prior extraction with trichloracetic acid. After careful removal of the aqueous phase, the phenol layers were thoroughly stirred and 5ml portions of each were mixed with 5ml of N sulphuric acid and heated at 100° for one hour. After cooling, the phenol layers were separated and the acid solutions extracted with diethyl ether then neutralised and prepared for chromatography.

Chromatograms of these solutions showed that when the bacterial cells were treated with phenol alone, only a trace of ribose occurred in the hydrolysates of the material present in the phenol layer, but when they were first extracted with trichloracetic acid, the phenol layer contained large quantities of ribose - Figure 29.

It was also shown that in the latter case, the ribose was present in bound form, since it could not be detected without prior hydrolysis.

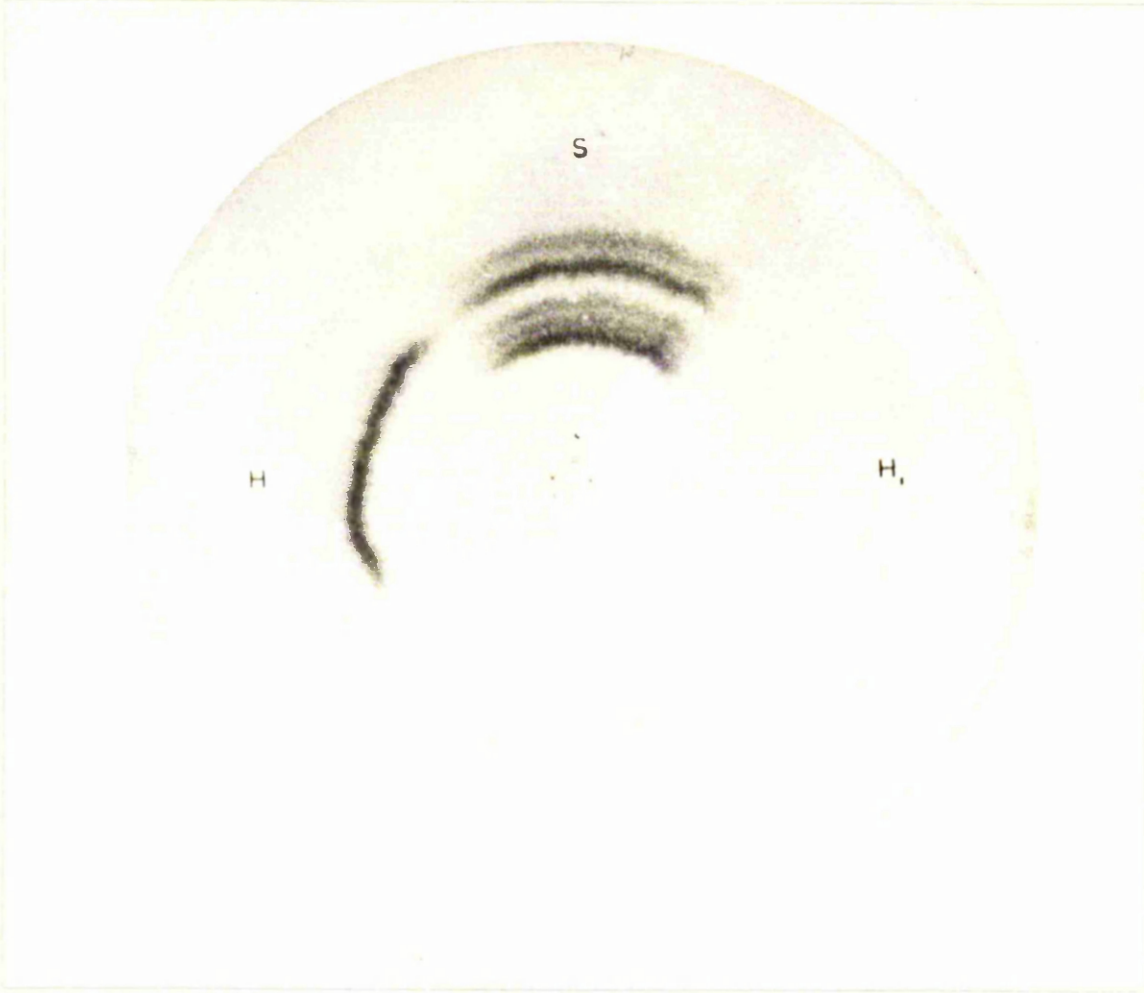


Fig. 29 Chromatogram of hydrolysates of the material from the phenol phase after phenol extraction

H - cells previously extracted with TCA

H_1 - cells treated with phenol alone

S - standard sugars (galactose, glucose, mannose, ribose and rhamnose)

Solvent - butanol, pyridine, water

Spray - aniline phthalate

THE EFFECT OF COMBINED TRICHLORACETIC ACID EXTRACTION AND PHENOL EXTRACTION.

ON ESCHERICHIA COLI AND PROTEUS VULGARIS.

The results given above with Pseudomonas aeruginosa showed that by the use of combined trichloracetic acid and phenol extraction it was possible to obtain an endotoxin which was not contaminated with large amounts of nucleic acid. As the complete separation of nucleic acid from the lipopolysaccharides obtained by phenol extraction alone can be a lengthy procedure involving repeated high speed centrifugation, salt or solvent fractionation, it was considered that this was a useful modification and a few experiments were carried to see if similar results were obtained when other species of Gram -ve bacteria were subjected to the combined extraction procedure.

Samples (10g) of dried Proteus vulgaris and Escherichia coli O111 were extracted with trichloracetic acid then with phenol as described above. The aqueous layers from the phenol extractions were dialysed, clarified and freeze-dried. Table 9 shows some of the properties of these extracts.

TABLE 9. /

TABLE 9.

	Yield (mg)	Nucleic acid (%)	Pyrogenicity *
P. vulgaris	270	0	1.4
E. coli 0111	260	0	0.8

* Average maximum temperature increase of 10 rabbits after i/v injection of 0.01ug/kg.

When the procedure was repeated on a second batch of 10g of Proteus vulgaris the yield was similar but in this case there was a trace (c 2%) of nucleic acid in the extract.

When these bacteria were extracted with phenol without prior treatment with trichloracetic acid, the extracts in both cases contained 40-50% of nucleic acid.

The results obtained with these three species are sufficiently similar to permit the suggestion that the combined trichloracetic acid-phenol extraction procedure may be generally effective as a method of obtaining nucleic acid-free endotoxins from Gram -ve bacteria (110).

FURTHER INVESTIGATION OF THE TRICHLORACETIC ACID - PHENOL PROCEDURE.

As already mentioned in the Introduction to this Section, Boivin/

Boivin found that a second, third and fourth extraction with trichloracetic acid removed additional amounts of antigenic material. He made no observations, however, on the endotoxic activities of the residues. In this Section, it has been shown that a single extraction with trichloracetic acid left a considerable amount of endotoxin in the residue, and it seemed of interest to investigate the effect of multiple extraction with trichloracetic acid.

The experiment above was, therefore, carried out in which 10g of dried Ps. aeruginosa cells were extracted thirteen times with cold trichloracetic acid. This procedure extracted a total of 1.07g of material (10.7%) of which approximately half was nucleic acid (see Table 8).

Chromatograms of hydrolysates from 20mg samples of fractions 1, 3, 5, 7, 8 and 9 in N sulphuric acid for 3 hours showed that in the first extract mannose was the principal sugar, in the third only a trace of mannose was present along with galactose, glucose and ribose, while subsequent extracts contained galactose, glucose and increasing amounts of ribose but no mannose.

When tested individually for their fever producing effects, only the first extract was found to be markedly pyrogenic - an amount of 0.01µg/kg causing a temperature increase of 1.0° (average of 10 rabbits) - Table 10.

TABLE 10.

Pyrogenic Effect of Fractions Obtained by Repeated Extraction of *Ps. aeruginosa* with Cold Trichloracetic Acid.

Extract	Temperature Increase (°C)
1	1.0
2	0.6
3	0.4
4	0.65
5	0.65
6	0.55
7	0.6
8	0.6
9	0.5
10	0.6
11	0.6

All fractions tested at dose level of 0.01µg/kg.

Fractions 1 - 7 average maximum temperature increase of 10 rabbits.

Fractions 8-11 average maximum temperature increase of 5 rabbits.

Fractions 12 and 13 were nottested.

However, pyrogenic material was still present in the residue left after the final trichloracetic acid extraction. This was shown when the residue was suspended in 300ml of distilled water and treated with an equal volume of 90% phenol as described in Section 2. After dialysis, the aqueous layer was clarified by centrifugation and freeze-dried. The weight of the material (fraction TCA-13/PE)was 284 mg (2.8% of the acetone-dried cells). It contained about 10% of nucleic acid and about 35% of chloroform-soluble lipid.

A three hour hydrolysate in N sulphuric acid showed the presence of galactose, glucose and ribose. On injection into rabbits, a dose of 0.01µg/Kg, caused a temperature increase of 0.9° (average maximum increase of 7 rabbits).

When tested on an agar plate with an antiserum against the whole cells fraction TCA-13/PE formed two precipitin lines.

It is clear therefore, that even after thirteen extractions with trichloracetic acid, a certain amount of the endotoxin remained unextracted in the residue. Whether or not further extraction would eventually have removed all the endotoxin is not known, but this seems unlikely, since Table 8 shows that by the thirteenth extraction, the yield had dropped to 0.2% of the dry weight of the cells.

THE EFFECT OF DIFFERENT TEMPERATURES DURING TRICHLORACETIC ACID EXTRACTION. /

THE EFFECT OF DIFFERENT TEMPERATURES DURING TRICHLORACETIC ACID EXTRACTION.

To obtain the most satisfactory antigen extracts by the trichloracetic acid method, it is apparently important to carry out the extraction in the cold (109, 112). In the present experiments, however, in which the procedure was being used as a preliminary to phenol treatment for the extraction of the endotoxin, it seemed possible that rigid control of temperature may not be so necessary.

Experiments were therefore carried out to compare the results given above, i.e. when the trichloracetic acid extraction was carried out at 2°, with those obtained when the extraction was carried out at room temperature (c 18°) and at 60°.

Two 10g amounts of dried cells were extracted with 0.25 N trichloracetic acid, one extraction being carried out at room temperature, and the other in a water-bath at 60°. The solutions were centrifuged as before and the acid extracts dialysed, clarified and freeze-dried. The residues were washed once with distilled water then extracted with hot phenol as described in Section 2. Table 11 shows the weights of material found in the acid extract (TCA) and after phenol extraction (TCA/PE) under the two sets of conditions.

TABLE 11. /

TABLE 11.

Temperature	TCA	TCA/PE.
18°	130mg	532mg
60°	602mg	377mg
(2°)	(152mg)	(360mg(

(For comparison, figures are given of the yields obtained when the trichloracetic acid extraction was carried out at 2°.)

The absorption spectra of these four fractions in the range 220mμ to 300mμ are shown in Figures 30 and 31.

The absence of nucleic acid in the fraction obtained when the acid extraction was carried out at 60° (fraction TCA-60), and in the material from the phenol extraction of the residue left after the acid extraction was carried out at room temperature (fraction TCA-18/PE) was confirmed by the absence of ribose in hydrolysates of these fractions - Table 12.

TABLE 12./

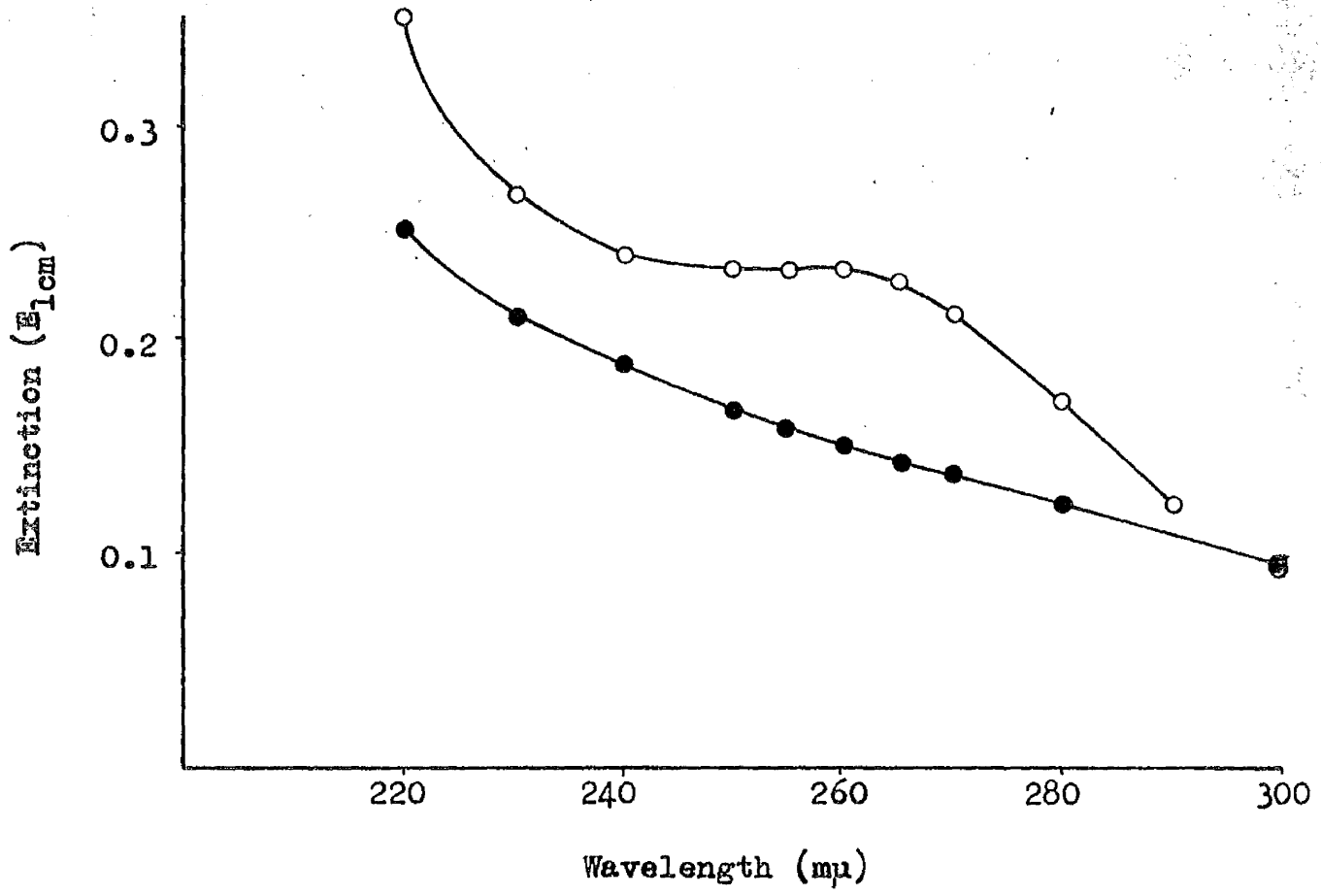


Fig. 30 Absorption spectra of TCA-60 (●) and TCA-60/PE (○)
Both solutions 100μg/ml in N/100 NaOH

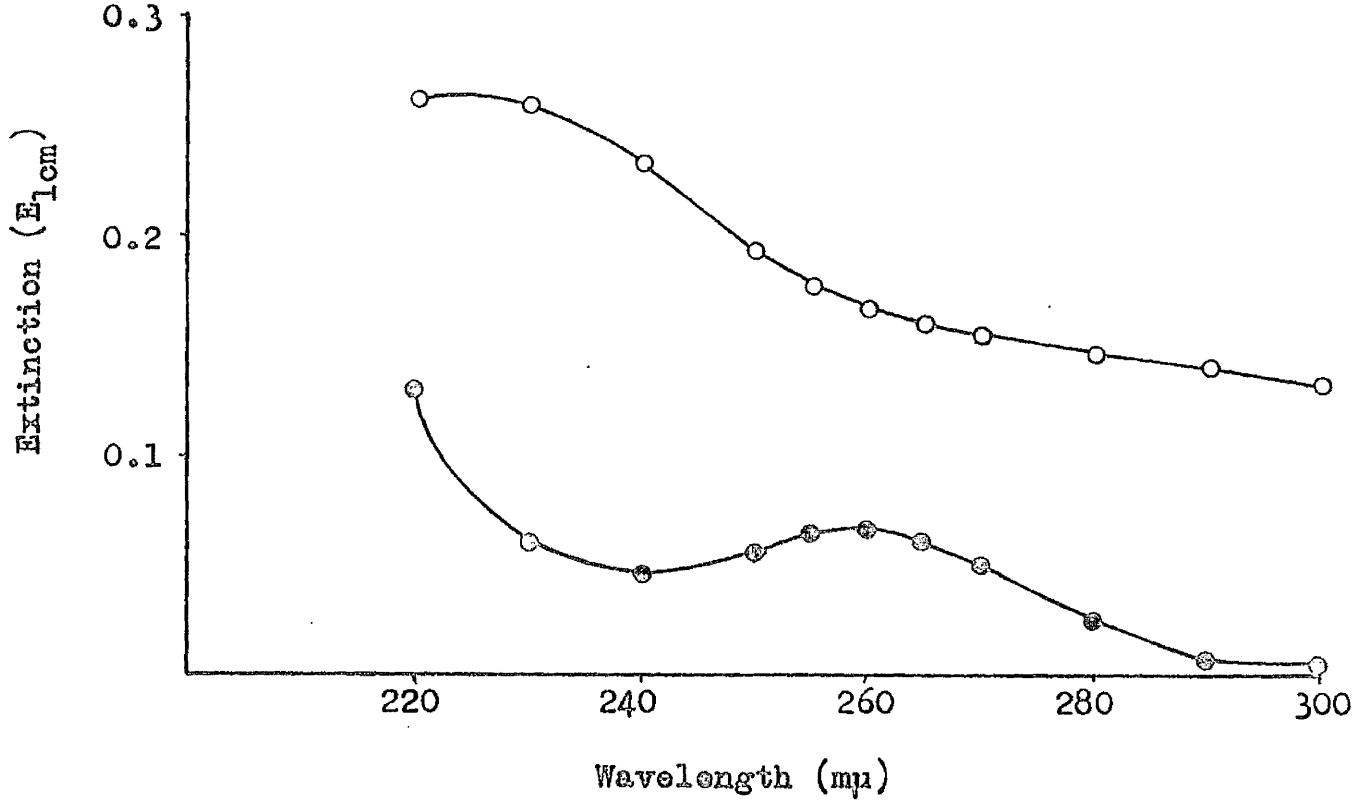


Fig. 31 Absorption spectra of TCA-18 (○) and TCA-18/PE (○)
Both solutions 100μg/ml in N/100 NaOH

TABLE 12.

Fraction	Sugars.
TCA-18	Mannose, (Galactose, Glucose)
TCA-18/PE	Galactose, Glucose
TCA-60	Galactose, Glucose (Mannose)
TCA-60/PE	Galactose, Glucose, Ribose

The sugars were identified chromatographically from hydrolysates of 20mg samples (3 hours in N sulphuric acid at 100) - Figures 32 and 33. Minor components are given in brackets. In the hydrolysate of TCA-60, an unidentified band appeared with an R_f less than that of galactose.

Figures 34 and 35 show the absorption spectra obtained when the four fractions were examined in the sulphuric acid-cysteine reaction.

Table 13 lists some other properties of these fractions.

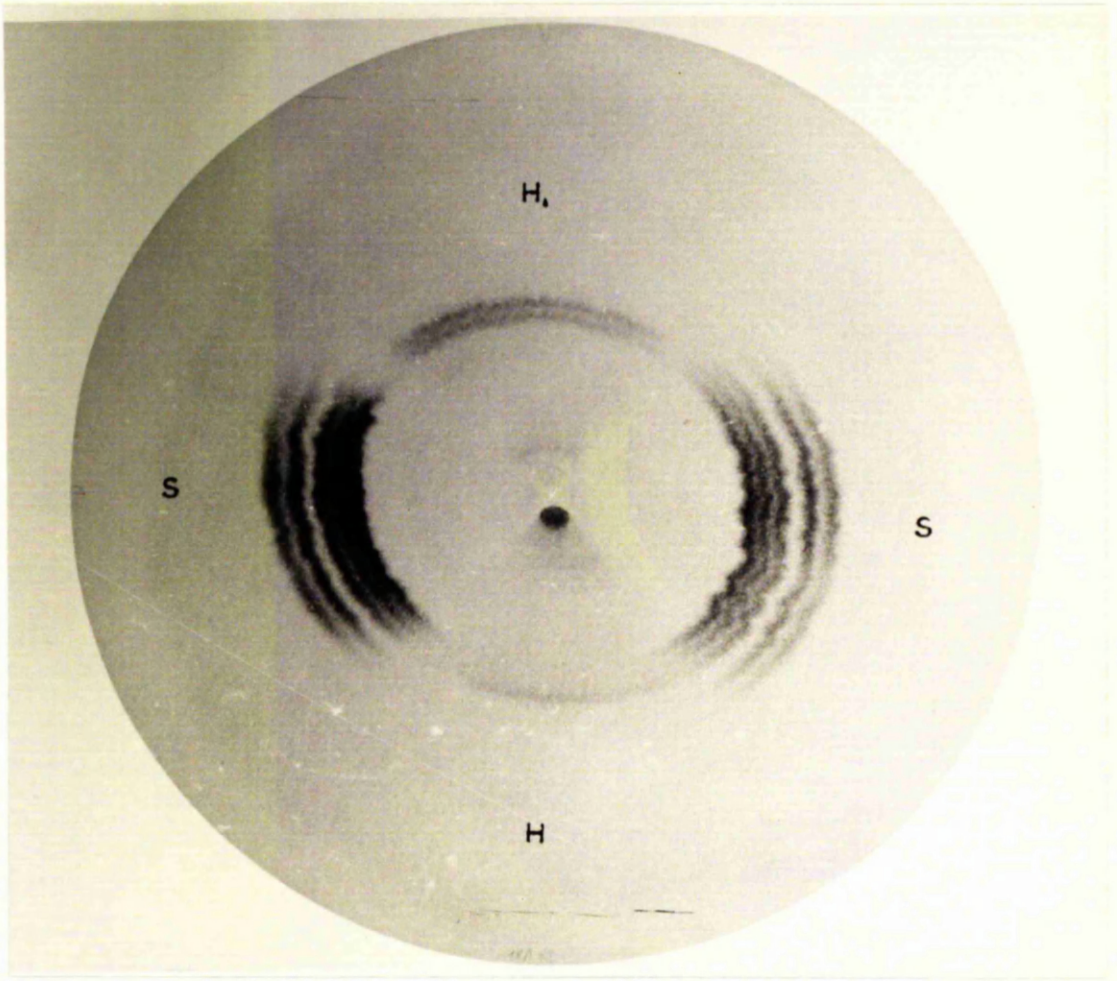


Fig. 32 Chromatogramof hydrolysates of fractions TCA-18 and TCA-18/PE

H - TCA-18

H_1 - TCA-18/PE

s - standard sugars (galactose, glucose, mannose, ribose and rhamnose)

Solvent - butanol, pyridine, water

Spray - aniline phthalate

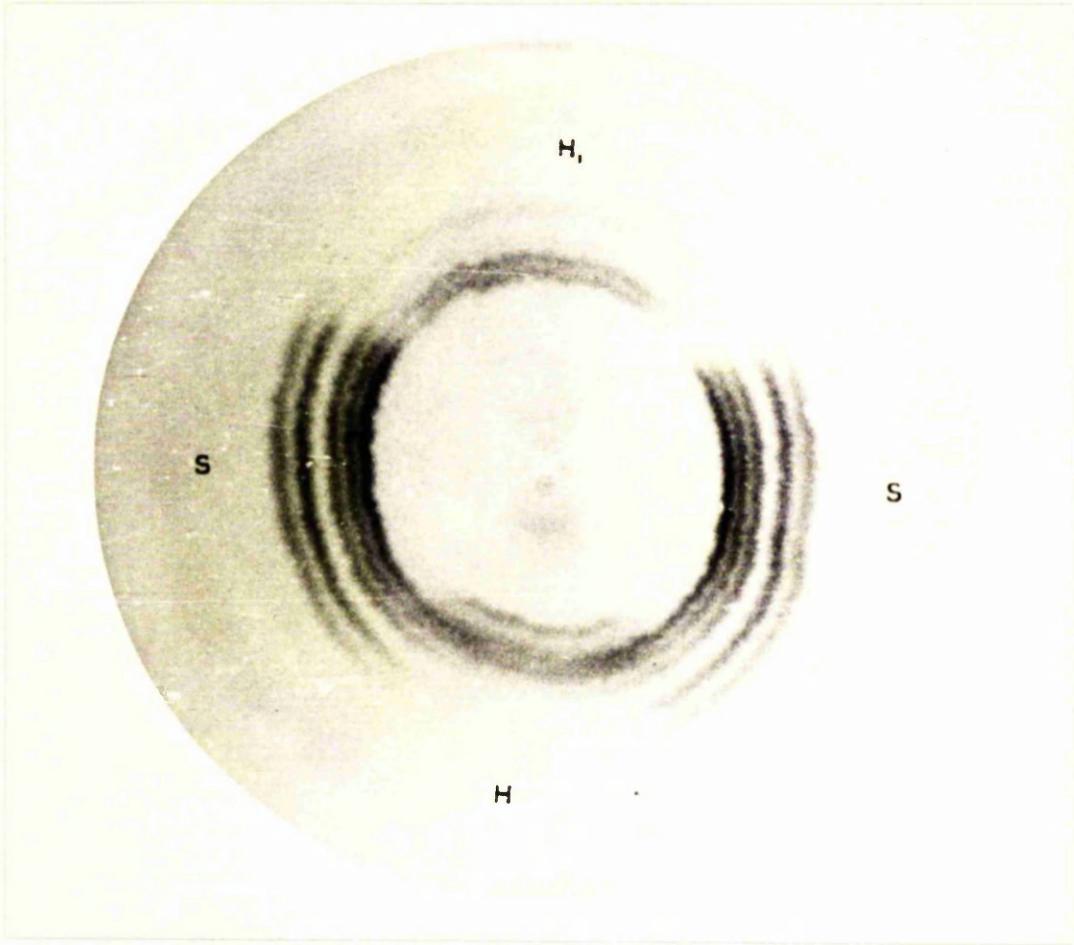


Fig. 33 Chromatogram of hydrolysates of fractions TCA-60 and TCA-60/PE

H - TCA-60

H_1 - TCA-60/PE

S - standard sugars (galactose, glucose, mannose, ribose and rhamnose)

Solvent - butanol, pyridine, water

Spray - aniline phthalate

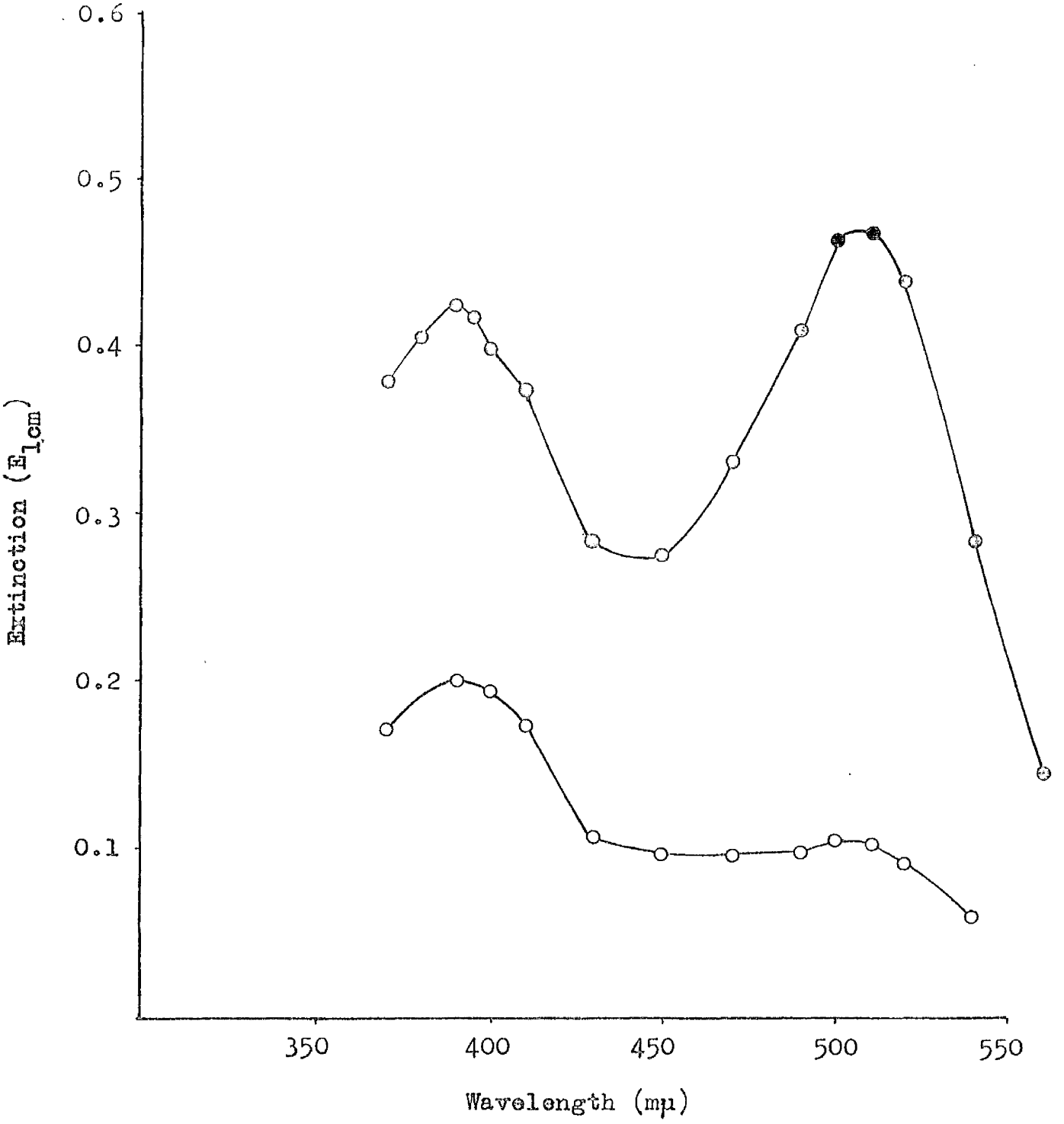


Fig. 34 Absorption spectra of TCA-18 (O) and TCA-18/PE (○ after treatment with sulphuric acid-cysteine

Each solution contained 500μg

Readings taken after 20 hours

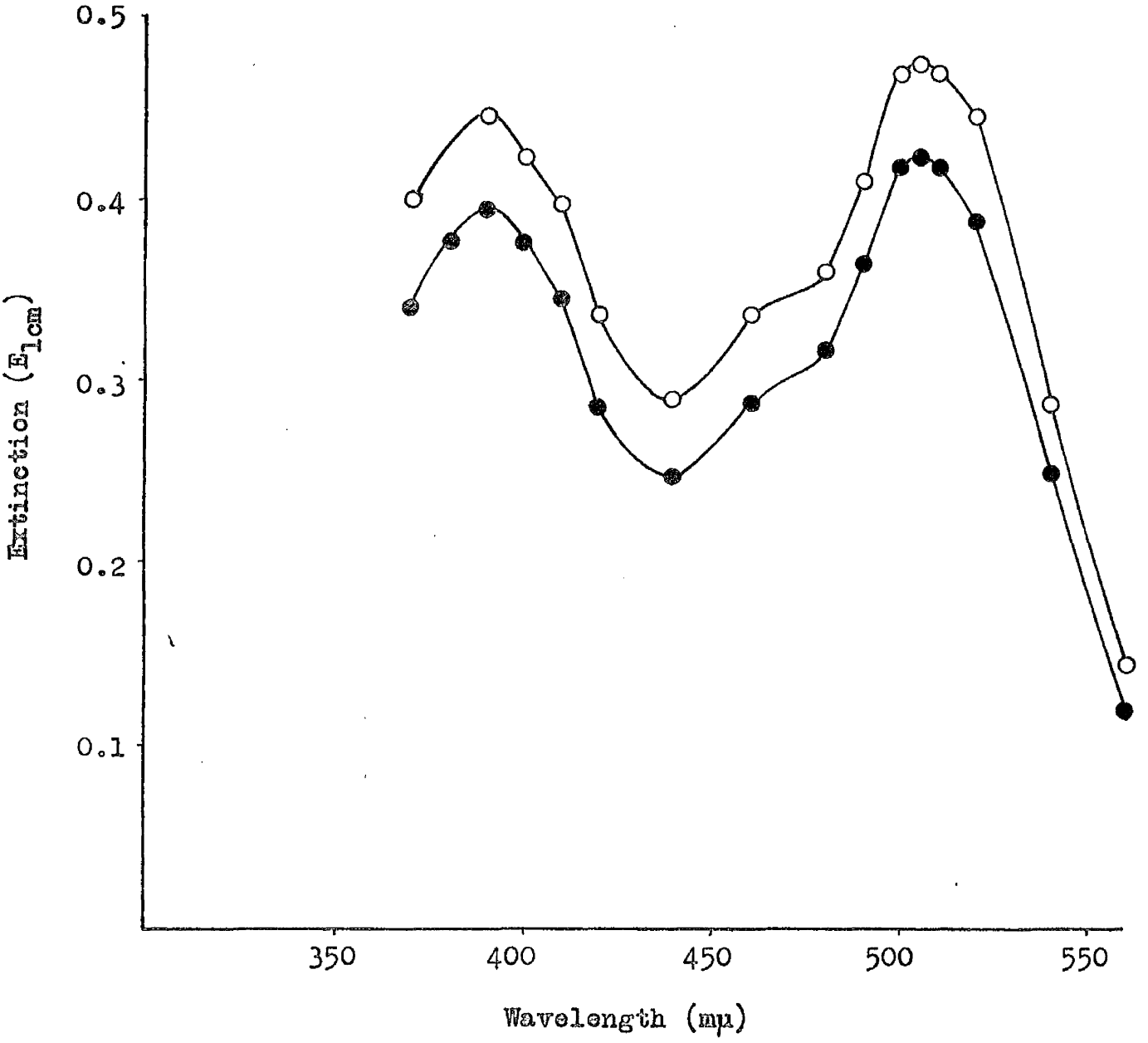


Fig. 35 Absorption spectra of TCA-60 (○) and TCA-60/PE (●) after treatment with sulphuric acid-cysteine

Each solution contained 500μg

Readings taken after 20 hours

TABLE 15.

Fraction	Nitrogen (%)	Lipid (%) *	Pyrogenicity **
TCA-18	2.88	6	0.9
TCA-18/PE	2.37	38	1.0
TCA-60	5.7	38	1.0
TCA-60/PE	2.55	37	0.4

* Chloroform-soluble lipid estimated as before on 50mg amounts.

** The average maximum temperature increase of 10 rabbits after injection of 0.01µg/kg.

DISCUSSION.

In Section 2, it was shown that the crude extract obtained after treating the bacterial cells with hot aqueous phenol contained two different polysaccharides, one of which, containing mannose, remained in the supernatant after high-speed centrifugation. The results of cold trichloracetic acid extraction of Pseudomonas aeruginosa suggest that the mannose-containing polysaccharide is more accessible than the polysaccharide containing galactose, glucose and heptose, since chromatographic examination of hydrolysates of the TCA-soluble fraction (TCA/ETH) showed that it contained mainly mannose along with smaller amounts of glucose and galactose, while no heptose could be detected in the sulphuric acid-cysteine test. Boivin noted (26) that cold trichloracetic acid extraction removed the somatic antigen from Gram -ve bacteria without disintegrating the cells, and in the present study microscopic examination of the Ps. aeruginosa cells after trichloracetic acid extraction showed that they were still intact rods. It seems probable therefore that the mannose-containing polysaccharide overlies the material containing heptose.

The agar gel precipitin studies on fraction TCA/ETH presented a rather confusing picture. The fraction formed two lines when tested against a whole cell antiserum and two when tested against its homologous antiserum. This could be the result of the presence in the fraction of different antigens giving rise to two different types of precipitating antibodies, or it could be due to the/

the presence of two components with the same antigenic specificity but with different diffusion rates. The formation of two lines with the antiserum against the purified lipopolysaccharide (fraction PE/SP - Section 2) suggests however that fraction TCA/ETH contains two components with the same antigenic specificity, since the antiserum against fraction PE/SP formed only one line with fraction PE/SP and presumably therefore contained only one type of precipitating antibody.

The reaction of the antiserum against the lipopolysaccharide (fraction PE/SP) with fraction TCA/ETH and of the antiserum against fraction TCA/ETH with the lipopolysaccharide obtained by combined trichloroacetic acid and phenol extraction (fraction TCA/PE/ETH) shows a common immunological specificity between the different fractions. Since fraction TCA/ETH contained no heptose, and fractions PE/SP and TCA/PE/ETH contained no mannose, these two sugars can play no part in the common specificity.

As mentioned in the Introduction to this Section, the cold trichloracetic acid procedure of Boivin can be an unsatisfactory method of extracting the maximum amount of the somatic antigen from Gram -ve bacteria, and in the present study it was found that the residue left after acid extraction could be treated with hot aqueous phenol to yield further appreciable amounts of an endotoxic lipopolysaccharide (fraction TCA/PE) which had chemical and biological properties similar to those of the purified lipopolysaccharide (PE/SP - Section 2), obtained by/

by phenol extraction without prior treatment with trichloracetic acid. Precipitin studies on agar gels however showed that the extract (TCA/PE) contained three components, and even after fractionation with 45-50% ethanol, two precipitin lines were formed against a whole cell antiserum. This fractionation did, however, remove the trace of mannose detected in hydrolysates of TCA/PE and the only sugars which could be detected on chromatograms were galactose and glucose, although, as with PE/SP (Section 2) the sulphuric acid-cysteine reaction showed that the polysaccharide contained a heptose sugar.

The most interesting feature of these results was the absence of nucleic acid in fraction TCA/PE. The removal of nucleic acid from the lipopolysaccharide extracts obtained from Gram -ve organisms by the hot phenol method can be a time-consuming process involving the use of fractionation procedures or high-speed centrifugation, and the fact that the combined trichloracetic acid and hot phenol treatment of Ps. aeruginosa yielded appreciable amounts of the endotoxin without the nucleic acid contaminant was considered a useful modification of the extraction technique. The similar results obtained when samples of Proteus vulgaris and Escherichia coli were subjected to the double extraction procedure suggest that it may be generally effective as a method of obtaining nucleic acid-free lipopolysaccharides from Gram -ve bacteria.

A/

A similar observation has been noted in a Japanese journal in a paper on the antigens of Pasteurella pseudotuberculosis rodentium, but the author (111) apparently did not remark on the possible usefulness of this extraction technique.

The experiments which were carried out in an attempt to explain this effect of trichloracetic acid showed that only about 5% nucleic acid was present in the acid extract itself (fraction TCA), and although the undialysed solution contained more material with an E_{max} at 260mu, these dialysable nucleic acid fragments did not account for all the nucleic acid present in the Pseudomonas aeruginosa cells. It was clear, therefore, that the nucleic acid remaining must have been affected by the trichloracetic acid in such a way as to prevent the appearance of RNA along with the lipopolysaccharide in the aqueous phase after phenol extraction. Since examination of the aqueous layer before dialysis showed that it contained no material with an absorption maximum at 260mμ it appeared that the RNA must be associated with the phenol layer. This suggestion received support from the observation that substantial amounts of ribose could be detected in hydrolysates of the material present in the phenol layer when the bacterial cells had previously been extracted with trichloracetic acid, whereas only traces of ribose were found in a similar hydrolysate when the cells had not been extracted with the acid .

The effect of the hot phenol treatment on the nucleic acids of the bacterial cells was described by Westphal (11) who found that the/

the DNA occurred as an insoluble deposit in the phenol layer while the RNA was found in the aqueous phase along with the lipopolysaccharide. A possible explanation of the results obtained in the present work is that extraction of the bacterial cells with trichloracetic acid causes a denaturation of the ribonucleoprotein which prevents the liberation of RNA into the aqueous phase, when the cells are heated with 45% phenol.

The results of the experiments in which the bacterial cells were extracted thirteen times with cold trichloracetic acid showed however that the effect of TCA on the nucleic acid content of the phenol extract could be at least partially reversed by prolonged contact between the acid and the cells, since fraction TCA13/PE was found to contain about 10% nucleic acid. This series of experiments also showed that even after multiple extraction with cold trichloracetic acid, the Ps. aeruginosa cells still contained appreciable amounts of endotoxin which could be recovered by phenol extraction of the residue (fraction TCA13/PE) This appears analogous to the results reported by Westphal (37) when he found that after Sh. dysenteriae had been extracted repeatedly with diethylene glycol until no further material weht into solution, the residue could be treated with hot aqueous phenol to yield additional amounts of material similar to the O antigen. The fraction obtained by the phenol method in this case, however, had glucose present in addition to the sugars found in the diethylene glycol extracts. These results are probably a reflection on the difference between milder/

milder extraction procedures such as trichloracetic acid or diethylene glycol, and the more drastic hot phenol method.

The individual fractions obtained during the repeated extraction with trichloracetic acid were not examined in detail, but it was found that only the first extract was markedly pyrogenic. The first extraction apparently removed almost all of the mannose-containing polysaccharide, for although mannose was the principal sugar detected in hydrolysates of this fraction, the third extract contained only a trace of mannose along with galactose, glucose and ribose, and in subsequent fractions no mannose could be detected on chromatograms. The increasing ribose content of the hydrolysates was due to the sharp increase in the amount of nucleic acid present in the fractions.

The failure of these fractions, apart from the first, to provoke a marked fever in rabbits at the normal dose levels suggests that only the first trichloracetic acid extract contained the endotoxin. The nucleic acid content of some of the non-pyrogenic fractions was very high, but since none of them had a pyrogenic potency equal to that of the crude product from phenol extraction (fraction PE - Section 2), which contained about 50% nucleic acid, the low pyrogenic potency could not have been due to a simple 'dilution' of the endotoxin by nucleic acid. A possible explanation of these results is that in Ps. aeruginosa the endotoxin is present in the surface layers in two forms, differing in location or in structure, only one of which can be extracted with/

with trichloracetic acid. Repeated acid extraction removes additional polysaccharide and nucleic acid but leaves a certain amount of endotoxin which can be recovered by treating the residue with hot phenol. It should be pointed out that although a single extraction with trichloracetic acid left the cells as apparently intact rods, repeated extraction resulted eventually in their breakdown - a process which might have been expected to facilitate the extraction of the endotoxin by trichloracetic acid if the limiting factor were simply one of availability.

The results obtained when the trichloracetic acid extraction was carried out at higher temperatures suggest that in the use of the combined trichloracetic acid-phenol procedure as a method of extracting endotoxin, the temperature during the initial trichloracetic acid extraction may not be as important as it apparently is when the Boivin technique is used to obtain extracts of the somatic antigen. By extracting with trichloracetic acid at room temperature (c 18°C) and at 60°C and treating the residues in each case with hot aqueous phenol, four fractions were obtained - TCA-18, TCA-60, TCA-18/PE, and TCA-60/PE.

The yield of TCA-18 was similar to that from the cold acid extraction and the two fractions did not differ greatly in the properties which were examined. Mannose was the principal sugar detected in hydrolysates of TCA-18, and the sulphuric acid-cysteine reaction indicated the presence of only a small amount of heptose. Although a solution of TCA-18 showed/

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Extraction of Nucleic Acid-free Lipopolysaccharides from Gram-negative Bacteria

THE extraction of Gram-negative bacteria by the hot phenol method of Westphal[1] yields a lipopolysaccharide along with considerable amounts of nucleic acid. The nucleic acid can be separated from the high molecular weight lipopolysaccharide by high-speed centrifugation, but to reduce the nucleic acid to a level of less than 2 per cent may require four or five sedimentations.

It is known that when the trichloracetic acid method of Boivin is used to extract the somatic antigen, a considerable amount of the antigen may remain in the residue[2]. Working with a *Pseudomonas* species, we have found that the residue left after the dried cells have been treated with trichloracetic acid, can be extracted with phenol to yield a lipopolysaccharide virtually free from nucleic acid.

10 gm. of the acetone-dried cells were mixed for 3 hr. in 100 ml. of 0·25 *N* trichloracetic acid at a temperature of 2–4° and then centrifuged. The deposit was washed with 100 ml. of distilled water and again centrifuged. This deposit was then extracted with 45 per cent phenol at a temperature of 65–67° and, after cooling, the aqueous phase was separated, dialysed, centrifuged at 4,000 r.p.m. for 10 min. to remove debris, and the supernatant was then freeze dried. The yield of extracted material from different batches was between 300 and 400 mgm. This compares favourably with the yield of lipopolysaccharide obtained after removal of the nucleic acid by high-speed centrifugation.

The lipopolysaccharide obtained by this method contained no detectable amounts of nucleic acid and was highly pyrogenic in rabbits in doses of 0·01 μgm./kgm. body-weight. Preliminary analysis showed the presence of galactose and glucose and 45–50 per cent of a lipid soluble in chloroform.

Similar results have been obtained with *E. coli* 0111 (supplied by Dr. O. Lüderitz) and with *P. vulgaris*. In both cases the modified extraction procedure yielded highly pyrogenic, nucleic acid-free lipopolysaccharides in amounts equivalent to 2–3 per cent of the dry weight of the bacteria.

The results with these three organisms suggest that pre-treatment with trichloracetic acid before phenol extraction may be a general method of obtain-

ing nucleic acid-free lipopolysaccharides without the lengthy procedure of high-speed centrifugation. This suggestion is supported by unpublished observations made by D. A. L. Davies (personal communication) working with different bacteria.

One of us (G. J. O'N.) acknowledges the award of a Cross Trust research scholarship during the tenure of which this work was done.

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Zur Immunchemie der O-Antigene von Enterobacteriaceae

V. Die Antigenfaktoren in isolierten Salmonella-O-Antigenen

Von

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Das O-Antigen einiger *Salmonella*-Serotypen kann bekanntlich durch den Nachweis mehrerer Spezifitäten serologisch differenziert werden. Diese Spezifitäten sind im Kauffmann-White-Schema [7] durch arabische Ziffern — 1, 2, 3, 4, 5 usw. — symbolisiert. Jede Species (= Serotyp) ist hinsichtlich des O-Antigens durch eine oder mehrere Zahlen charakterisiert, welche die O-Antigen*formel* ergeben. Die O-Antigene gramnegativer Bakterien enthalten als wesentliche Komponente stark verzweigte Polysaccharide, die, soweit untersucht, für die O-Spezifität der Keime verantwortlich sind.

Zur Isolierung der O-Antigene sind verschiedene Verfahren ausgearbeitet worden, die zu den spezifischen Polysacchariden oder deren Komplexen mit Lipoid oder Lipoid und Protein führen [20]. In den letzten Jahren wurden sie aus vielen gramnegativen Bakterien, insbesondere aus Enterobacteriaceen, dargestellt und analysiert. So sind die Zuckerbausteine der spezifischen Polysaccharide von Vertretern sämtlicher bislang bekannter *Salmonella*-Gruppen ermittelt worden [8]. Es wurde gefunden, daß die *Sero*typen jeweils bestimmten *Chemo*typen zugeordnet werden können [8].

Bei der serologischen Analyse von Salmonellen sind in einigen O-Antigenen bis zu vier verschiedene Spezifitäten aufgefunden worden, so z.B. in der *Salmonella*-Gruppe B die Antigenfaktoren 1, 4, 5 und 12, denen in *Salmonella* B-Antiseren vier verschiedene Antikörper-Fraktionen — Anti-1, Anti-4, Anti-5 und Anti-12 — entsprechen. Für die Spezifität 12 konnte überdies nachgewiesen werden, daß sie aus wenigstens drei differenzierbaren Teilfaktoren — 12_1, 12_2 und 12_3 — zusammengesetzt ist [6]. Basierend auf den Prinzipien der Immunchemie wissen wir heute, daß diesen Spezifitäten oder Antigenfaktoren des Kauffmann-White-Schemas verschiedene *determinante Gruppen* in den betreffenden Antigenen zugrunde liegen [18]. Den determinanten Gruppen in den O-antigenen Polysacchariden entsprechen im allgemeinen oligosaccharidische Strukturen von wenigen Zuckerbausteinen, soweit bisher untersucht in der Größenordnung von 2—5 Zucker-Einheiten.

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Es erhebt sich damit die Frage, inwieweit die einzelnen Antigenfaktoren des Kauffmann-White-Schemas tatsächlich am jeweiligen Polysaccharid des O-Antigen-Komplexes lokalisiert sind und ob beim Vorliegen von mehr als einer Spezifität in O-Antigenen einzelner Salmonellaspecies die entsprechenden determinanten Gruppen an ein und demselben Polysaccharid oder an mehr oder weniger heterogenen Mischungen von Polysacchariden verankert sind, ob also Salmonellen und andere Enterobacteriaceen zum Aufbau ihrer Zellwand *ein einziges* komplexes Polysaccharid mit allen Antigen-Faktoren *oder mehrere* prinzipiell trennbare Polysaccharid-Antigene bilden.

Zur Klärung dieser Frage haben wir verschiedene isolierte *Salmonella*-O-Antigene, welche entsprechend dem Kauffmann-White-Schema mehrere O-Antigen-Faktoren enthalten, mit solchen kreuzreagierenden *Salmonella*-Antiseren präcipitiert, bei denen die betreffende Kreuzreaktion durch nur eine Art spezifischer Antikörper bedingt ist. Die erhaltenen Präcipitate wurden isoliert und anschließend hinsichtlich des Antikörper-Gehaltes sowie eines charakteristischen Zuckerbausteins analysiert. Außerdem haben wir aus den Präcipitaten die präcipitierten O-Antigene vom Antikörper präparativ getrennt und ihre Zuckerbausteine mit denjenigen des ursprünglich eingesetzten O-Antigens verglichen.

Als isolierte O-Antigene verwendeten wir die hochgereinigten Lipopolysaccharide der untersuchten *Salmonella*-species [8]. Die Lipopolysaccharide der Enterobacteriaceen sind die biologisch wirksame Untereinheit der O-antigenen Endotoxin-komplexe in ihren Zellwänden. Während die Lipoidkomponente (Lipoid A [22]) für die akut-endotoxischen Manifestationen wesentlich und von Keim zu Keim sehr ähnlich wenn nicht identisch zusammengesetzt ist, sind die Polysaccharidkomponenten *species*-spezifisch und, soweit untersucht, Träger der determinanten Gruppen des betreffenden O-Serotyps. Bei serologischen Präcipitationen mit O-Antiseren reagieren demnach bestimmte Gruppen der *Polysaccharid*-Komponenten. — Auf die mit hydrolytischen Mitteln mögliche Abspaltung der Lipoidkomponente aus den Lipopolysacchariden haben wir bewußt verzichtet, um sicher zu sein, daß nicht etwa polysaccharidische Bruchstücke (Untereinheiten) infolge unübersichtlicher hydrolytischer Spaltungen am genuinen Polysaccharid-Antigen zur Analyse kamen.

Die Versuche haben ergeben, daß die Zuckerbaustein-Analyse der untersuchten O-antigenen Lipopolysaccharide *vor und nach* Kreuzpräcipitation stets die gleiche war, gleichgültig mit welchem der O-Antigenfaktoren im eingesetzten Lipopolysaccharid die verwendeten Antiseren mit selektiver Spezifität reagierten. Die Analysen haben keinerlei Anhaltspunkte für das Vorliegen von heterogenen O-antigenen Polysaccharid-Mischungen erbracht; sie unterstützen vielmehr die früher

schon diskutierte Vermutung, wonach verschiedene Antigenfaktoren (Spezifitäten) eines O-Antigens am gleichen Polysaccharid-Molekül verankert sind.

Material und Methoden

O-Antigene. Die Lipopolysaccharide verschiedener *Salmonella*species wurden mit Hilfe des Phenol/Wasser-Verfahrens [21] extrahiert, aus der wäßrigen Phase isoliert und durch Ultrazentrifugation in üblicher Weise gereinigt [2, 8]. Die Durchführung der *Zuckerbaustein-Analyse* aus Hydrolysaten wurde früher bereits eingehend beschrieben [2, 8]. *Tab. 1* gibt eine Übersicht über die verwendeten Serotypen, ihre O-Antigenformel nach dem Kauffmann-White-Schema und die Zuckerbausteine der Polysaccharid-Komponente, wobei hier Hexosamin(e) und Heptose(n) nicht berücksichtigt sind. In der vorliegenden Arbeit wurden nur Hexosen, 6-Desoxy- und 3,6-Didesoxyhexosen als typische Bausteine der bakteriellen Polysaccharid-Antigene analysiert.

Für die Präcipitations-Versuche mit kreuzreagierenden Antiseren verwendeten wir 1%ige Lösungen in 0.9%igem NaCl, denen zur Sterilhaltung Merthiolat (1:10000) zgesetzt war. Die Lösungen wurden bei 4°C zentrifugiert (10 min, 3000 TpM), um eventuelle Spuren ungelöster Partikeln, welche in die Präcipitate gelangen könnten, zu entfernen.

Antiseren. Bei den Antiseren handelte es sich um Kaninchen-Testseren aus Statens Seruminstitut, Internationale *Salmonella*- und *Escherichia*-Centrale, Kopenhagen. Je 30 ml Antiserum wurden mit 30 ml 0,9%iger NaCl-Lösung verdünnt und ebenfalls vor Beginn des Versuchs bei 4°C zentrifugiert. Von dem absorbierten O 5-Serum standen 5 ml zur Verfügung, die mit 5 ml 0,9%iger NaCl-Lösung verdünnt wurden.

Die Kaninchen wurden entsprechend den Angaben von Kauffmann („Enterobacteriaceae", E. Munksgaard, Kopenhagen, II. Edit., 1954, S. 74) 5mal in Zeiträumen von 4—5 Tagen mit Bouillon-Kulturen, die $2^1/_2$ Std im Kochschen Dampftopf auf 100°C erhitzt waren, i.v. injiziert. Die Dosen betrugen 0,25, 0,5, 1,0, 1,5, 2,0 ml Kultur.

Präcipitationen. 60 ml Antiserum (Verdünnung 1:2) wurden mit 0,5—2 mg Lipopolysaccharid (1%ige Lösung) versetzt. Nach mindestens 48 Std bei 4°C wurde das gebildete Präcipitat abzentrifugiert und zum Überstand weiteres Lipopolysaccharid hinzugefügt, bis keine Präcipitation mehr eintrat. Dies war im allgemeinen nach Zusatz von 2—10 mg Lipopolysaccharid erreicht. Anschließend wurde zum Überstand das Lipopolysaccharid eines anderen Serotyps, wiederum in Portionen von 0,5—2 mg, zugefügt bis keine Präcipitation mehr eintrat. Auf diese Weise wurden nacheinander sämtliche Antikörper-Quoten des Serums mit den entsprechenden Lipopolysacchariden selektiv zur Reaktion gebracht. Präcipitate des gleichen Lipopolysaccharids mit der gleichen Serumprobe wurden im allgemeinen vereinigt, 3mal mit 0,9%iger NaCl gut gewaschen und analysiert.

In einigen Fällen präcipitierten wir das betreffende Lipopolysaccharid in Gegenwart eines Überschusses eines anderen Lipopolysaccharids von einem *nicht*-kreuzreagierenden Serotyp, welches ein oder mehrere charakteristische Zuckerbausteine enthielt, die dem präcipitierten Antigen fehlten. Auf diese Weise versicherten wir uns in mehreren unabhängigen Versuchen, daß *keine* Co-Präcipitation erfolgte.

Analyse der Präcipitate. Die gewaschenen Präcipitate wurden in dest. Wasser unter Zusatz von etwas Alkali gelöst.

1. Proteingehalt. Ein aliquoter Teil der Lösung wurde nach Folin-Ciocalteau [5] analysiert. Als Vergleich verwendeten wir Standard-Lösungen von kristallisiertem

Rinderserum-Albumin. Die erhaltenen Antikörperprotein-Werte sind als Milligramm Rinderalbumin angegeben.

2. Quantitative Rhamnose-Bestimmung. Nahezu alle hier untersuchten Salmonella-O-Antigene enthalten Rhamnose (siehe Tab. 1) als Baustein des Polysaccharids. Ein aliquoter Teil der Präcipitat-Lösung sowie eine Lösung des ursprünglichen Lipopolysaccharids wurden gleichzeitig der Rhamnose-Bestimmung nach GIBBONS [4] unterworfen. Aus den erhaltenen Werten berechneten wir die mit dem ursprünglichen Lipopolysaccharid zum Serum zugefügte sowie die präcipitierte Menge an Rhamnose.

3. Zuckeranalyse des präcipitierten Lipopolysaccharids. Die restliche Präcipitat-Lösung wurde neutralisiert und zur Entfernung des Proteins mit Phenol (1:1) extrahiert [21]. Die wäßrige Phase, welche das präcipitierte (Lipo)-polysaccharid enthielt und frei von Protein war, wurde dialysiert, im Vakuum auf ein kleines Volumen konzentriert und nach Hydrolyse in üblicher Weise papierchromatographisch analysiert [8]. Zum Vergleich dienten die Hydrolysate der entsprechenden Lipopolysaccharide, welche jeweils bei dem betreffenden Präcipitations-Ansatz verwendet worden waren.

Ergebnisse

Die Versuche wurden an sechs verschiedenen *Salmonella*-Antiseren ausgeführt, von denen jedes Serum, mit Ausnahme des absorbierten O 5-Serums, mehrere gegen verschiedene Antigenfaktoren (Spezifitäten)

Tabelle 1. *Die verwendeten O-antigenen Salmonella-Lipopolysaccharide*

Salmonella-species (Serotyp)	O-Gruppe	Antigen-formel	Zuckerbausteine (8)						
			Galaktose	Glucose	Mannose	Rhamnose	Abequose	Paratose	Tyvelose
S. paratyphi A	A	1, 2, 12	+	+	+	+		+	
S. abortus equi	B	4, 12	+	+	+	+	+		
S. java	B	4, 5, 12	+	+	+	+	+		
S. schleissheim	B	4, 12, 27	+	+	+	+	+		
S. cholerae suis	C_1	6, 7	+	+	+				
S. newport	C_2	6, 8	+	+	+	+	+		
S. sendai	D_1	1, 9, 12	+	+	+	+			+
S. typhi	D_1	9, 12	+	+	+	+			+
S. senftenberg	E_4	1, 3, 19	+	+	+	+			

gerichtete Antikörper-Quoten enthielt. Diese Seren wurden in bestimmter Reihenfolge mit verschiedenen Lipopolysacchariden aus im Ganzen neun *Salmonella*-Serotypen von unterschiedlicher Antigen-Formel (Tab. 1) präcipitiert. Versuchsanordnung und Ergebnisse sind in *Tab. 2* aufgeführt.

Wie aus Tab. 2 hervorgeht, wurde zur Präcipitation jeweils das Lipopolysaccharid eines Serotyps ausgewählt, bei dem *nur ein* Antigenfaktor des O-Antigens nach Kauffmann-WHITE mit dem verwendeten Antiserum reagieren konnte.

Tabelle 2. *Ergebnisse der Präcipitationen von Salmonella-Antiseren mit den O-Antigenen kreuzreagierender Salmonella-Serotypen (Species)*

Vers.	Antiserum (Antikörper-Quoten)	Zur Präcipitation verwendetes Lipopolysaccharid (LPS)				Präcipitat-Analyse									
		aus Serotyp (Antigenformel)	reagierender Antigen-Faktor	zugefügte		mg Antikörper*	mg Rhamnose	Zucker (chromatographisch)							
				mg LPS	mg Rhamnose			Galaktose	Glucose	Mannose	Rhamnose	Abequose	Colitose	Paratose	Tyvelose
a	Anti-A 1, 2, 12	S. abortus equi (4, 12)	*12*	3	0,25	2,4	0,19	+	+	+	+	+			
		S. senftenberg (1, 3, 19)	*1*	3		2,8		+	+	+	+				
		S. paratyphi A (1, 2, 12)	*2*	4	0,47	5'6	0,14	+	+	+	+			+	
b	Anti-B 4, 5, 12, 27	S. typhi (9, 12)	*12*	3	0,27	0,6	~0,05	+	+	+	+				+
		S. abortus equi (4, 12)	*4*	4	0,33	8	0,33								
				2	0,16	1,05	0,10								
				2	0,16	0,78	0,12	+	+	+	+	+			
				2	0,16	0,67	0,09								
				2	0,16	0,40	~0,02								
		S. java (4, 5, 12)	*5*	2	0,14	2,44	0,10								
				2	0,14	2,00	0,11	+	+	+	+	+			
				2	0,14	0,40	~0,03								
		S. schleissheim (4, 12, 27)	*27*	2	0,20	0,70	0,06	+	+	+	+	+			
				2	0,20	0,60	~0,03								
c	Anti-05 (abs.)	S. java (4, 5, 12)	*5*	3,5	0,24	1,6	0,04	+	+	+	+	+			

d	Anti-C_1 6, 7	S. newport (6, 8)	*6*	4 8	0,32	0,88 0	~0,04 0	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)			
		S. cholerae suis (6, 7)	*7*	4		6,7		+	+	+					
e	Anti-$C_{1,2}$ 6, 7, 8	S. sendai (1, 9, 12)	∅	6		0	0	(+)	(+)	(+)	(+)				(+)
		S. newport (6, 8)	*6, 8*	4		Präcipitat		+	+	+	+	+			
				10		0	0	(+)	(+)	(+)	(+)	(+)			
		S. cholerae suis (6, 7)	*7*	4		Präcipitat		+	+	+					
f**	Anti-D 9, 12	S. abortus equi (4, 12)	*12^1*, 12_3	4,5	0,37	6	0,31	+	+	+	+	+			
		S. sendai (1, 9, 12)	*9*, 12_2	4,5	0,30	8,8	0,30	+	+	+	+				+
g	Anti-D 9,12	S. adelaide (35)	∅	6		0	0	(+)	(+)			(+)			
		S. sendai (1, 9, 12)	*9, 12*	6		Präcipitat		+	+	+	+				+

+ = Zucker im Chromatogramm eindeutig nachgewiesen; (+) = Zuckerbausteine des *nicht* präcipitierten Antigens; * Als Rinderserumalbumin bestimmt; ** Das hier verwendete Serum enthielt neben der Anti-9-Quote noch die Antikörper-Quoten Anti-12_1, -12_2 und -12_3. Das zur Präcipitation benutzte Lipopolysaccharid war aus *S. abortus equi* mit der Antigenformel 4, 12_1, 12_3 isoliert worden. Nach vollständiger Absorption verbleiben daher in diesem System noch Antikörper gegen die Faktoren 12_2 und 9; das absorbierte Serum reagiert dann mit dem *S. sendai*-Lipopolysaccharid (Antigenfaktoren 1, 9, 12_1, 12_2, 12_3) auf Grund der Faktoren 9 und 12_2.

Da die Lipopolysaccharide gegen Ende des Versuchs jeweils in geringem *Überschuß* zugefügt waren, kann angenommen werden, daß jeweils alle gegen den entsprechenden Antigenfaktor gerichteten Antikörper in Reaktion getreten und somit aus dem System entfernt worden waren.

Tab. 2 zeigt, daß in allen Versuchen, bei denen auf Grund der Antigenformel des betreffenden Serotyps Kreuzreaktion erwartet werden konnte, eine Präcipitation des Lipopolysaccharids mit Antiserum erfolgte. So konnte für die Antigenfaktoren 1, 2, 4, 5, 6, 7, 9, 12 und 27 demonstriert werden, daß diese Spezifitäten in den entsprechenden Lipopolysacchariden verankert sind. Eine Übersicht gibt *Tab. 3.*

Tabelle 3. *Antigenfaktoren (Spezifitäten), welche auf Grund der Kreuzpräcipitationen (Tab. 2) in den gereinigten Lipopolysacchariden nachgewiesen wurden*

Salmonella-species (Serotyp)	Antigenformel des Serotyps	Im Lipopolysaccharid nachgewiesener Antigenfaktor
S. paratyphi A	1, 2, 12	2
S. abortus equi	4, 12	4 und 12
S. java	4, 5, 12	5
S. schleissheim	4, 12, 27	27
S. cholerae suis	6, 7	7
S. newport	6, 8	6
S. sendai	1, 9, 12	9 und 12_2*
S. typhi	9, 12	12
S. senftenberg	1, 3, 19	1

* Siehe Fußnote ** unter Tab. 2.

Das Ergebnis der Zuckeranalysen der Präcipitate (siehe Tab. 2) ist sehr einheitlich: es wurden stets sämtliche Zucker-Bausteine nachgewiesen, die bereits früher als Bausteine der entsprechenden hochgereinigten Lipopolysaccharide aufgefunden worden waren. Die Reaktion einzelner Antigenfaktoren mit spezifischen Antiseren führt demnach zu Präcipitaten, in denen das präcipitierte Lipopolysaccharid aus den gleichen Zuckern besteht wie das entsprechende zur Präcipitation benutzte Lipopolysaccharid.

Auch hinsichtlich der relativen Quantitäten der Zucker zueinander konnten wir bei Betrachten der Chromatogramme der aus den Präcipitaten isolierten und jener der ursprünglich eingesetzten Lipopolysaccharide keine Unterschiede auffinden.

Wie die Rhamnosebestimmungen zeigen (Tab. 2), erscheint in einigen präcipitierenden Systemen nahezu die gesamte Rhamnose, die dem Serum mit dem ursprünglichen Lipopolysaccharid zugefügt wurde, im Präcipitat — offensichtlich immer dann, wenn mit einem nicht zu großen Überschuß an Lipopolysaccharid präcipitiert wurde. So fand sich z. B. praktisch die gesamte Rhamnose des Lipopolysaccharids von *S. abortus equi* im Präcipitat wieder, gleichgültig ob mit Anti-4 oder mit Anti-12 Antiserum präcipitiert wurde (Versuche b und f in Tab. 2). Gleiches gilt für die Rhamnose im *S. sendai*-Lipopolysaccharid vor und nach Präcipitation mit einem absorbierten Anti-9 Antiserum (Versuch f) sowie *S. java-*

Lipopolysaccharid (Versuch b). Da beide Präcipitate des *S. abortus equi*-Lipopolysaccharids mit Anti-4 oder Anti-12 außerdem die gleichen Zucker in gleicher relativer Verteilung (chromatographisch) enthielten wie das ursprünglich eingesetzte Lipopolysaccharid, kann für dieses Antigen angenommen werden, daß die selektive Präcipitation *eines* Antigenfaktors zur Fällung des gesamten Antigenkomplexes (Lipopolysaccharids) führt.

Um auszuschließen, daß bei der spezifischen Präcipitation serologisch nicht verwandte, aber chemisch ähnliche Lipopolysaccharide unspezifisch co-präcipitiert werden, führten wir in einigen Versuchen (d, e und g, Tab. 2) die Präcipitation in Gegenwart eines nichtkreuzreagierenden Lipopolysaccharids aus. Die anschließende Zuckeranalyse des Präcipitats zeigte *in jedem Fall*, daß *nur* das spezifische Antigen präcipitiert wurde.

So erscheint in Versuch d bei der Präcipitation des O-Antigens von *S. cholerae suis* keine Rhamnose und Abequose im Präcipitat, also kein *S. newport*-Lipopolysaccharid. In Versuch g findet man keine Colitose im Präcipitat, was heißt, daß *S. adelaide*-Antigen (mit Colitose) nicht copräcipitiert wurde. Im Versuch e (Tab. 2) erfolgte die Präcipitation von *S. cholerae suis*-Lipopolysaccharid (mit den O-Antigenfaktoren 6, 7) bei Gegenwart der O-Antigene von *S. sendai* (Faktoren 1, 9, 12) und *S. newport* (Faktoren 6, 8) mit absorbiertem Anti-7-Serum *selektiv*: das Präcipitat enthielt weder Tyvelose (*S. sendai*-Antigen) oder Abequose (*S. newport*-Antigen) noch Rhamnose (beide nichtverwandten Antigene), sondern nur die für *S. cholerae suis* typischen Hexosen [8] Galaktose, Glucose und Mannose (siehe Tab. 1).

Die Antigenfaktoren 5 und 27 wurden bislang nur in einigen Serotypen der *Salmonella*-Gruppen B, aber nie gemeinsam, aufgefunden. Da die Lipopolysaccharide der Gruppe B sämtlich von den gleichen Zuckern aufgebaut sind, also dem gleichen Chemotyp angehören [8], war es in diesem Fall nicht möglich, die Präcipitate auf Grund der Zuckeranalyse zu differenzieren. Wir haben die Reaktionen der Lipopolysaccharide in Versuch b (Tab. 2) mit Anti-5 bzw. Anti-27 sowie die Absorption der betreffenden Antikörper eingehender verfolgt. Die fraktioniert gefällten Präcipitate wurden gesondert isoliert und Protein und Rhamnose bestimmt. Das sukzessive Absinken bei der Reaktion mit dem ersten Lipopolysaccharid und erneute Ansteigen der Werte nach Zugabe eines anderen Lipopolysaccharids zeigt die Erschöpfung der ersten Antikörper-Quote (hier Anti-4) bzw. die Reaktion der zweiten (Anti-5) und endlich der dritten Quote (Anti-27) an.

Diskussion

Die Frage des chemischen Status der Antigenfaktoren, d.h. die Lokalisierung der serologisch spezifischen Strukturen in isolierten O-Antigenen von Enterobacteriaceen wurde früher schon von verschiedenen Arbeitskreisen mittels serologischer Methoden bearbeitet.

So haben Furth u. Landsteiner [3] schon 1929 gezeigt, daß die verschiedenen Spezifitäten eines *Salmonella*-O-Antigens am gleichen Molekül verankert sind. Diese Ergebnisse wurden jedoch mit relativ rohen Bakterienextrakten gewonnen. Das gleiche gilt für die Versuche von Meyer [10], Cohen [1] sowie Nakaya u. Fukumi [12], die an Protein-Lipoid-Polysaccharid-Komplexen der O-Antigene ausgeführt

wurden. In der vorliegenden Arbeit wurden gereinigte proteinfreie Lipopolysaccharide untersucht.

Auf der Basis selektiver Kreuzreaktionen wurden in den isolierten Lipopolysacchariden aus neun verschiedenen *Salmonella*-Serotypen folgende Spezifitäten (O-Antigenfaktoren) nachgewiesen: 1, 2, 4, 5, 6, 7, 9, 12 und 27 (siehe Tab. 3). Es sind dies sämtliche Faktoren, welche bislang in den *Salmonella*-Gruppen A, B, C_1 und D_1 serologisch erkannt wurden und die in verschiedener Kombination bei den Serotypen dieser Gruppen auftreten (siehe Tab. 1 u. 3).

Im Lipopolysaccharid aus *S. abortus equi* wurde die Anwesenheit der Faktoren 4 und 12 bewiesen. Bei den übrigen untersuchten Lipopolysacchariden wurde auf Grund der Versuchsanordnung jeweils *ein* Antigenfaktor identifiziert. Es besteht kein Zweifel, daß alle spezifischen Polysaccharide der Gruppen A, B, C_1 und D_1 und darüber hinaus wahrscheinlich auch der übrigen *Salmonella*-Gruppen Träger der Spezifitäten sind, wie sie durch die O-Antigenformeln des Kauffmann-White-Schemas zum Ausdruck kommen.

Die Resultate der Präcipitat-Analysen zeigen, daß es nicht gelingt, das Lipopolysaccharid einer Species in Fraktionen zu zerlegen, die sich hinsichtlich ihrer Zuckerbausteine unterscheiden. Mit welchem Antigenfaktor selektive Präcipitation erfolgt, stets enthalten die Präcipitate sämtliche Zuckerbausteine des eingesetzten Lipopolysaccharids.

Aus diesen Ergebnissen allein kann noch nicht mit Sicherheit geschlossen werden, daß die Lipopolysaccharide aus *einheitlichen* Molekülen bestehen. Die folgenden Ergebnisse sprechen indessen gegen die Auffassung der isolierten O-Antigene als chemisch heterogene, prinzipiell trennbare Substanzen.

Ebenso wie die chemische hat auch die serologische Fraktionierung der Lipopolysaccharide nicht zu Fraktionen von unterschiedlicher Bausteinanalyse geführt. — Beim Vergleich der Chromatogramme von Hydrolysaten der präcipitierten und der zur Präcipitation eingestzten Lipopolysaccharide konnten hinsichtlich der relativen Intensität der Zuckerflecke keine Unterschiede festgestellt werden. — Es zeigte sich, daß bei der Präcipitation einiger rhamnosehaltiger Lipopolysaccharide die Rhamnose nahezu quantitativ im Präcipitat erscheint. Beim Lipopolysaccharid aus *S. abortus equi* (mit den Antigenfaktoren 4, 12) führte die selektive Reaktion sowohl mit Anti-4 wie mit Anti-12 zur Ausfällung der gesamten eingesetzten (gebundenen) Rhamnose.

Für einzelne, in den stark verzweigt gebauten Lipopolysacchariden terminal gebundene Zucker konnten STAUB et al. [15] auf Grund von Hemmungs-Experimenten zeigen, daß sie an der Struktur der determinanten Gruppen bestimmter Antigen-Faktoren wesentlich beteiligt sind, z. B. Abequose bei Faktor 4 oder Tyvelose bei Faktor 9 [16]. Das Auftreten von Abequose nach Präcipitation des abequosehaltigen *S. abortus equi*-Lipopolysaccharids mit Anti-5-Serum spricht dafür, daß

mit dem Faktor 5 gleichzeitig auch Faktor 4 präcipitiert wurde. Entsprechendes gilt für die Koppelung der Faktoren 4 und 12, 9 und 12 sowie 4 und 27.

STAUB u. Mitarb., welche mit lipoid-freien Polysacchariden arbeiteten, haben unabhängig mittels immunologischer Reaktionen gezeigt, daß die Spezifitäten 9 und 12 [14], 4 und 1 [17] sowie 4 und 5 [13] jeweils am gleichen Polysaccharid-Molekül verankert sind.

Das Vorkommen von mehr als einer Spezifität im gleichen Antigenmolekül ist an sich nicht unerwartet. Es gibt hierfür sowohl Beispiele künstlicher als auch solche natürlicher Antigene. So enthält die Blutgruppensubstanz AB des Menschen die Spezifitäten A und B am gleichen Mucopolysaccharid [11]. Durch Präcipitation mit Anti-A-Seren gelangt auch die B-Spezifität in das Präcipitat, und umgekehrt. Werden dagegen die serologisch reinen A- und B-Substanzen von Personen der Blutgruppen A bzw. B *gemischt*, so lassen sich aus dieser Mischung reine A- oder B-Substanz selektiv mit Anti-A- oder Anti-B-Seren präcipitieren und auf diese Weise trennen. — Prinzipiell gleiche Absorptionsexperimente mit entsprechenden Ergebnissen wurden an künstlichen (chemospezifischen) Antigenen mit zwei Spezifitäten durchgeführt.

Auch in reinem menschlichen Serumalbumin ließen sich mehrere Spezifitäten am gleichen Molekül nachweisen [9]. — Man kann annehmen, daß bei natürlichen Antigenen, welche sämtlich hochmolekulare Substanzen sind, im allgemeinen viel häufiger *mehr als eine Spezifität* ausgebildet ist, so daß Antiseren gegen das betreffende Antigen mehr als eine spezifische Fraktion enthalten. Es ist hauptsächlich eine Frage der serologischen Technik, die verschiedenen determinanten Gruppen in Antigenen zu diagnostizieren.

Das Auftreten verschiedener determinanter Strukturen (Spezifitäten) am gleichen bakteriellen Polysaccharidmolekül bedeutet, daß entsprechend viele Enzymsysteme bei der Polysaccharidsynthese wirksam sind.

Da einzelne determinante Gruppen bei verschiedenen *Salmonella*-Serotypen und auch bei kreuzreagierenden *E. coli*-, *Arizona*- oder *Pseudotuberculose*-Stämmen vorkommen können (siehe z.B. [19]), muß man schließen, daß die an der Ausbildung dieser serologischen Merkmale beteiligte genetische Ausrüstung der betreffenden Bakterien *als Ganzes* bei der Selektion der Serotypen und Genera erhalten blieb oder übertragen wurde. Die O-antigenen Polysaccharide der bakteriellen Zellwand und das Studium ihrer Biosynthese sind daher auch unter genetischen Aspekten von Interesse.

Herrn Professor Dr. F. KAUFFMANN, Statens Seruminstitut Kopenhagen, Internationale *Salmonella*- und *Escherichia*-Zentrale, sind wir für die Züchtung der in dieser Arbeit verwendeten Bakterien und die Bereitstellung standardisierter Antiseren sowie auch für Diskussion der Ergebnisse und wertvolle Anregungen zu besonderem Dank verpflichtet.

Zusammenfassung

Die polysaccharidischen O-Antigene zahlreicher *Salmonella*-Serotypen sind häufig durch das Vorhandensein mehrerer Spezifitäten charakterisiert. Diese Serotypen sind im Kauffmann-White-Schema, das die Salmonellen serologisch klassifiziert, durch die Kombination von Antigenfaktoren — die sogenannte Antigenformel — gekennzeichnet.

Neun isolierte *Salmonella*-O-Antigene (Lipopolysaccharide) der *Salmonella*-Gruppen A, B, C_1, C_2, D_1 und E wurden mit selektiv absorbierten oder geeigneten kreuzreagierenden Antiseren präcipitiert und die erhaltenen Präcipitate hinsichtlich Antikörper-Protein und Rhamnose (soweit vorhanden) quantitativ, sowie auf Zuckerbausteine der bakteriellen Lipopolysaccharide qualitativ analysiert.

Durch die Präcipitationsversuche konnte die Verankerung der Antigenfaktoren 1, 2, 4, 5, 6, 7, 9, 12 und 27 an den betreffenden Lipopolysacchariden demonstriert werden.

Die Zuckerbaustein-Analysen der Lipopolysaccharide *vor und nach* Kreuzpräcipitation war stets die gleiche, gleichgültig mit welchem der Antigenfaktoren die Antiseren selektiv reagierten. Die Analysen haben keinen Anhaltspunkt für das Vorliegen heterogener O-antigener (Lipo)-Polysaccharide erbracht. Entsprechend früher schon diskutierter Vermutung, erscheinen die verschiedenen Antigenfaktoren des O-Antigenkomplexes eines Serotyps am gleichen Polysaccharidmolekül verankert. Es ist demnach wahrscheinlich, daß die untersuchten *Salmonella-species nur ein* O-antigenes Polysaccharid in ihrer Zellwand bilden.

Summary

The polysaccharidic O antigens of many *Salmonella* serotypes frequently carry more than one immunological specificity. These serotypes are classified in the Kauffmann-White scheme and characterized by the combination of antigen factors, the so-called antigen formula.

9 purified *Salmonella* O antigens (lipopolysaccharides) of *Salmonella* groups A, B, C_1, C_2, D_1 and E were precipitated by selectively absorbed or suitable cross-reacting antisera. The precipitates so obtained were quantitatively analyzed for antibody protein and rhamnose (if present). They were further analyzed qualitatively for the known sugar constituents of the lipopolysaccharide used.

On the basis of these experiments the location of the antigen factors 1, 2, 4, 5, 6, 7, 9, 12 and 27 on the respective lipopolysaccharides was demonstrated.

The sugar analyses of lipopolysaccharides *before and after* crossprecipitation gave always identical results, no matter what antigen factor was involved in the reaction. There was no indication for any heterogeneity

of the O-antigenic (lipo)-polysaccharides used for the serological precipitations. According to earlier conclusions, the various antigen factors of the O antigen complex of a given serotype appear to be linked at one and the same polysaccharide molecule. It is reasonable therefore that the *Salmonella* species investigated produce only one single O-antigenic polysaccharide as a constituent of their cell wall.

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showed an absorption maximum at 260mμ, the total absorption at this wavelength corresponded to a nucleic acid content of less than 2%, and no ribose could be detected on chromatograms.

Extraction of the residue from this experiment with hot phenol gave fraction TCA-18/PE in a yield of 532mg (5.3%). This material was pyrogenic; it contained no nucleic acid and only two bands corresponding togalactose and glucose could be detected on chromatograms although the sulphuric acid-cysteine reaction showed the presence of appreciable amounts of a heptose sugar. It appears, therefore, that an endotoxin extract can be obtained by phenol treatment of the residue from room temperature trichloracetic acid extraction which is as satisfactory as that obtained by the bombined procedure when the trichloracetic acid extraction is carried out at low temperatures.

No immunological studies were made on fraction TCA-18/PE, but by analogy with fraction TCA/PE it seems probable that the material was not homogeneous.

When the trichloracetic acid extraction was carried out at 60°, the acid extract (fraction TCA-60) amounted to 602mg (6%), a very much higher figure than those obtained at 2° or 18°. This fraction was nucleic acid-free and it contained galactose, glucose and a trace of mannose, plus heptose (shown by the Dische test). It had the same pyrogenic potency as fraction TCA-18/PE. Extraction of the/

the residue with hot phenol inthis case, however, gave a fraction (TCA-60/PE) of almost negligible pyrogenicity, containing galactose, glucose and a heptose along with a small amount of nucleic acid. The use of a temperature of 60° therefore seems to have resulted in the trichloracetic acid extracting a greater amount of the endotoxin but reduced the advantage of phenol extraction of the residue. It is not known if fraction TCA-60 retained the full antigenic potency normally found with Boivin extracts, but in view of other workers' experience with this method, it seems likely that the higher temperature would have had an adverse effect on this property.

The chloroform soluble lipid (lipid A) content of the four fractions listed in Table 13 shows that there is no apparent co-relation between the amount of lipid and the pyrogenicity of the extracts. Fraction TCA-18 contained only 6% lipid and was quite strongly pyrogenic, while TCA-60/PE contained 37% and was virtually devoid of pyrogenicity in rabbits. These results would appear to support the suggestion made by Ribi (84, 85) that the endotoxic properties of lipopolysaccharides are not due to the lipid A fraction of the complex as postulated by Westphal (82).

The overall results of these experiments with Ps. aeruginosa show therefore that in the use of the combined trichloracetic acid-phenol process for the recovery of the endotoxin, the maintenance of low temperature during the trichloracetic acid extraction is not essential, in

in that satisfactory results can be obtained when the extraction is carried out at room temperature. Elevated temperatures may improve the yield in the trichloracetic acid extract, but have an adverse effect on the value of the combined extraction.

SUMMARY.

In the Introduction, the principal methods which have been used for the extraction of the endotoxins or somatic antigens of Gram -ve bacteria have been considered and the nature of these substances reviewed, The biological importance of the lipid portion of the complex has been discussed along with the effects of chemical modification of endotoxins.

The experimental work is in two main sections.

SECTION 1. The isolation, purification and properties of the endotoxin from Pseudomonas aeruginosa are described and the results discussed.

The endotoxic lipopolysaccharide was isolated from the bacterial cells by the hot aqueous phenol method and purified by ultracentrifugation. It was shown to contain galactose, glucose, glucosamine, galactosamine and a heptose which was masked on chromatograms by glucose. The lipid material which was released from the lipopolysaccharide after short acid hydrolysis was shown to contain ether-soluble and chloroform-soluble fractions in both of which glucosamine, several amino acids and a series of fatty acids were detected.

The lipopolysaccharide was shown to be antigenic and highly pyrogenic when injected into rabbits, and to be toxic to rabbits and mice.

SECTION 2. The effects of trichloracetic acid extraction and of combined trichloracetic acid and hot phenol extraction on Pseudomonas aeruginosa are described and the results discussed.

It was found that the residues of the cells left after cold trichloracetic acid extraction could be treated with hot phenol to give an extract containing the endotoxic lipopolysaccharide without the nucleic acid contamination which is found when hot phenol alone is used, and it was shown that after this double extraction procedure the RNA is found in the phenol layer of the cooled phenol-water mixture. The combined extraction procedure was also found to be an effective method of obtaining nucleic acid-free lipopolysaccharides from Proteus vulgaris and Escherichia coli. These studies were extended to consider the effects of carrying out the initial trichloracetic acid extraction at higher temperatures, and the results showed that rigid temperature control during the acid extraction may not be necessary when using the combined TCA-phenol method to obtain nucleic acid-free lipopolysaccharide. A further series of experiments showed that after multiple extraction with trichloracetic acid, appreciable amounts of endotoxin could be obtained from the residue by treatment with hot phenol.

APPENDIX.

EXPERIMENTAL METHODS.

CIRCULAR PAPER CHROMATOGRAPHY.

The solvent was placed in a 6 cm. diameter glass petri dish lying inside the cover of a 15 cm. diam. petri dish. The paper was placed on top of the dishes with a wick from the centre dipping down into the solvent, and covered with a 30 cm. diam. glass trough. The whole system rested on top of sheet rubber covered with aluminium foil so that when a weight was placed on top of the glass trough, an adequate seal was obtained. This simple arrangement was found to give better results than the Shandon 'Kawerau' circular chromatography unit in which the paper is held between the flanges of the two halves of the tank.

The paper used throughout was 24 cm. Whatman No. 1 filter paper. This was chosen in preference to the slotted circular chromatography paper (Whatman) because of the tendency found with the latter for the solvent to flow at slightly different rates in the different segments.

Sugars.

The aniline phthalate spray used for the detection of sugars was prepared as follows:

Aniline	0.91 ml.
Phthalic acid	1.66g
Water saturated butanol	100ml

After spraying, the paper was heated for 10 minutes at 95-100°.

Fatty Acids.

The following method was adopted for paper chromatography of fatty acids. The paper was immersed for one minute in a high boiling point petroleum fraction (Shellsol T - Shell Petroleum Co. Ltd.), pressed between sheets of blotting paper, then hung at room temperature for 45 minutes. The solutions of fatty acids in benzene were spotted onto the circumference of a 1 cm. diameter circle marked in the centre of the paper after small slits had been cut in the circle at right angles to the circumference to prevent intermingling when the solutions were applied. The paper was then placed in the apparatus described above with a wick dipping into a dish containing 90% acetic acid saturated with Shellsol T, and allowed to run overnight. After removal from the tank, the paper was dried in a warm oven (c 45°) for 30 minutes, dipped for one minute in a solution of copper acetate (0.5% copper acetate in 0.05N sodium acetate) and washed for 45 minutes in running water. At this stage in the original method (63) the paper was immersed for 30 minutes in ferrocyanide solution (0.5% potassium ferrocyanide in 0.1N hydrochloric acid) to visualize the copper salts of the fatty acids. In the present experiments, however, it was found that a better contrast was obtained if, after the copper acetate treatment, the paper was dipped for 15 minutes in a 0.1% aqueous solution of sodium diethyldithiocarbamate, then washed in running water for 30 minutes. The fatty acid/

acid salts then appeared as brown bands on a faint brown background.

The benzidine reagent used for the detection of unsaturated fatty acids was

Benzidine (recrystallised from aqueous alcohol)	1.0g
Trichloracetic acid	8.0g
Glacial acetic acid	20ml
Water	20ml
Absolute ethanol	160ml

The chromatogram was prepared and run as described above, and after drying, it was immersed in the reagent for 5 minutes.

PYROGENICITY TESTS.

The rabbits used for pyrogenicity testing were healthy adults of both sexes which had previously been accustomed to the procedure by being placed several times for periods of a few hours in the restraining boxes with the rectal thermocouples in position. The groups used in the tests were formed after all the rabbits had been tested with a standard pyrogen. After rejection of any individuals with unusually high or low responses, the remainder were arranged in groups of ten.

Food was withheld from the animals for a 24 hour period preceeding a test. The temperatures were measured by means of rectal thermocouples attached/

attached to a direct reading electric universal thermometer (Type TE-3, Elektrolaboratoriet, Copenhagen). Rectal temperatures were measured at 10 minute intervals from 1 hour before until about 4½ hours after the injection. The test material, in pyrogen-free saline, was heated to 37° before being injected by a marginal ear vein, and the solutions were made up so that the dose was administered in volumes of 1 ml/Kg body weight.

PRECIPITIN TESTS

The agar plates for the precipitin tests were made up from 1% Ionagar No. 2 (Oxoid) in 0.9% saline containing 0.1% sodium azide. Wells were cut in the plates by means of Feinberg Agar Gel Cutters (Shandon Scientific Co.), and after drying overnight in an incubator (25°) the bottom of each well was sealed with a drop of molten agar.

Serum was prepared by withdrawing blood by cardiac puncture. The blood was kept at room temperature until a clot had formed, then placed in a refrigerator overnight. The serum was then decanted from the contracted clot, centrifuged free of cells and stored in the deep freeze.

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