QUANTITATIVE STUDIES ON SALMONELLA ANTIGENS

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by

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ABSTRACT

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Virulence in Salmonellae is known to be associated with possession of a specific polysaccharide, which may carry several different O-antigenic determinants. Previous work in this department on two strains of Salmonella typhimurium, the virulent strain C5 and the avirulent M206, suggested that virulence in C5 might be associated with a heat-labile antigen, possibly O-antigen 5. As both strains contained this antigen it was suggested that the difference between them might be due to a quantitative difference in antigen content on the two bacteria. This thesis compares the two strains, with particular reference to the part played by antigen 5 in virulence.

Both C5 and M206 have the O-antigens 4, 5 and 12, but it was found, by absorption of specific sera against these antigens, that C5 contains 2 -4 times as much of each as M206. Labelling of bacteria with ^{131}I-labelled antibodies against the 5 antigen gave a similar result. Calculation from the absorption data of the number of sites available to antibody on the bacterial surface suggested that there were of the order of

10^3 - 10^4 per bacterium, with rather more 5 sites than 4 or 12. This figure represents approximately the number of antibody molecules which can be packed evenly on to one Salmonella, and suggests that the antigen-carrying O-specific polysaccharide is distributed on the surface in this way.

When the amount of O-polysaccharide was estimated by a chemical method it was again found that C5 had nearly twice as many sites as M206, but the total number of antigenic sites estimated by this method was much higher, of the order of 10^7. Similar determinations on a number of other Salmonellae of varying degrees of virulence in the mouse showed that at least one virulent strain had less O-polysaccharide than M206, indicating that there was not a simple relationship between polysaccharide content and virulence. On the other hand, a recombinant obtained from a genetic cross between a virulent and an avirulent strain, which had been selected for loss of virulence, showed a reduction in polysaccharide content to near that of M206.

Specific antisera against antigens 4, 5 and 12 were capable of fixing complement in both strains, sensitized M206 for complement-mediated killing in the <u>in vitro</u> reaction, and

opsonized C5 for phagocytosis and killing after injection into the normal mouse peritoneum. The anti-5 serum used was more effective than anti-4 or 12 in promoting both bactericidal reactions, and it is suggested that this may have been due at least in part to the greater number of antigenic sites with which it could react. Extrapolation of equilibrium constants calculated from absorption data to the two bactericidal systems indicated that the number of antibody molecules actually involved in the reaction at limiting dilutions was probably very small.

The conclusion was reached that antigen 5 is unlikely to affect virulence by protecting the organism from antibodies with other specificities, although it may well be that antibodies directed against it are particularly effective in protecting the host. It seems likely that protection of bacteria by polysaccharide is a function of its gross overall structure, rather than of the individual antigenic determinants which it carries.

ACKNOWLEDGMENTS

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To the best of the candidate's knowledge, this thesis contains no material previously submitted for a degree in any university, either by himself or by any other person, except where due reference is made in the text.

(signed) .

December 1969.

ABBREVIATIONS

The following abbreviations have been used.

abe	abequose (3, 6 dideoxy-D-galactose)
ACL	antigen carner lipid
AICAR	aminoimidazole carboxamide ribotide
AIR	aminoimidazole ribotide
BSA	bovine serum albumin
CA	common antigen of Kunin
CFA	complete Freund's adjuvant
col	colitose (3, 6 dideoxy-L-galactose)
EDTA	ethylenediaminetetra acetic acid
FA	fatty acyl
gal	galactose
glc	glucose
glc NAc	2-deoxy-2-amino-N-acetyl-glucose
hep	L-glycero-D-mannoheptose
HM	β-hydroxy-myristyl
HU	haemagglutinating unit
KDO	2-keto-3-deoxy-octonate
LD_{50}	lethal dose for 50% of test animals
LPS	lipopolysaccharide

man	mannose
NRS	normal rabbit serum
OD	optical density
P	phosphate
PBS	phosphate buffered saline
ppt	precipitate
res	residue
rha	rhamnose
supt	supernatant
tris	2-amino-2-hydroxymethylpropane-1, 3-diol (tris hydroxymethyl amino methane)
US	ultrasonicate
u.v.	ultra violet
VS	veronal buffered saline

PREFACE

Since the days of Hippocrates typhoid and paratyphoid have been known as severe, and sometimes fatal, diseases of man (Gay, 1918), and even in the 1960s they are still prevalent in many of the less wealthy areas of the world (World Health Organization, 1967). Many attempts in the last 75 years have been made to develop vaccines against them, but despite recent progress obtained by empirical methods, the principles underlying the production of an efficient vaccine are not understood. This thesis describes another attempt to obtain more information on the mechanisms of immunity to typhoid-like diseases.

The bacteriology of Salmonella diseases has been reviewed by Wilson and Miles (1964$_a$). They have been classified under three headings according to their severity and presumed mode of transmission. It seems likely that all Salmonellae, when ingested in sufficient numbers, can cause an acute enteritis. Normally this is limited to the gut, but a few species are able to pass from there and infect the blood and other organs to cause the enteric fevers known in man as typhoid and paratyphoid. These particular bacteria seem able to infect suitable organisms at much lower doses than most Salmonellae and for the purposes of this thesis are the only ones which will be regarded as virulent.

One of the most important characteristics of enteric fever bacteria is that many are relatively host-specific. This has made it very difficult to test anti-typhoid vaccines in the laboratory, because the only other animals which have contracted a typhoid-like disease when fed or injected with Salmonella typhi are anthropoid apes (Metchnikov and Besredka, 1911, Edsall,Gaines, Landy et al, 1960). Consequently most of the attempts to simulate human immunity to typhoid have had to be carried out on quite unsuitable experimental animals such as mice and guinea-pigs, and it has been necessary to try to reduce them to the level of higher primates either by looking at a very small part of the immune response, such as agglutinin production, or by trying to circumvent part of the immune mechanism by such tricks as injection of near-toxic doses of bacteria, or pre-treatment of the animal with hog gastric mucin to reduce the effectiveness of its phagocytic cells. Many of these methods were reviewed by Spaun and Uemura (1964), who concluded that none were of adequate predictive value for the development of typhoid vaccines.

Research on typhoid vaccines since the first attempts of Wright (1896) and Pfeiffer and Kolle (1896) has been concerned largely with attempts to recognise those antigens which are of particular importance in the development of immunity, and with experiments to find out how best they could be preserved in vaccines.

Three main antigens were considered, those known as H, O and Vi. The first two were named by Weil and Felix (reviewed by Felix, 1924), and it was shown that only the heat-stable O-somatic antigen appeared to be involved in protective reactions (Felix, 1924, Felix and Olitzki, 1926). Mutations which led to the loss of specific O-antigen and conversion of the Salmonella from smooth to rough also led to loss of the strain's effectiveness as a vaccine (Arkwright, 1927). In 1934 Felix and Pitt claimed to have shown that the apparently heat-labile Vi antigen was also of importance in immunity to S.typhi, (Felix and Pitt, 1934 $_{a,b}$) and in later attempts to produce effective vaccines an effort was made to include antigens which could give rise to a good anti-Vi response (Felix, 1941, Felix, Rainsford and Stokes, 1941, Landy, 1953).

In 1953 the World Health Organization instituted a series of field trials of different vaccines on normal human populations (Yugoslav Typhoid Commission, 1957). These included a number of different preparations of whole typhoid bacteria, and also several chemical vaccines, prepared from purified antigens (Hejfec, 1965, Polish Typhoid Committee, 1965). Table 1 lists some of these vaccines and shows their order of effectiveness.

TABLE 1

Relative effectiveness of typhoid vaccines in WHO-sponsored field trials in Guyana, Poland, the USSR, and Yugoslavia

Rank[a]	Method of preparation	Preservative	Ref[b]
1	formolized	phenol	1
2	acetone	dry	1,2,3,4
3	heat	phenol	1,2,3,4,5
4	alcohol	dry or alcohol	1,2 5
5	heated and frozen. autolysate, formolized adsorbed to Al $(OH)_3$ or $CaPO_4$	-	1,5
6	phenol-water extract adsorbed to Al $(OH)_3$ or added to a saline extract of acetone-treated bacteria (O and Vi antigen preparations)	-	1,5

(a) Ranking is only approximate, as the vaccines employed in the USSR and Poland were not identical with those employed in Guyana and Yugoslavia

(b) References

1 Polish Typhoid Committee 1965, 1966
2 Yugoslav Typhoid Commission 1957, 1962
3 Yugoslav Typhoid Commission 1964
4 Typhoid Panel, UK Department of Technical Cooperation, 1964
Ashcroft, Singh, Nicholson et al, 1967
5 Hejfec, 1965, Hejfec, Salmin, Lejtman et al, 1966

It was found that the order of effectiveness of these vaccines bore little relationship to the laboratory tests on which their preparation had been based (Spaun and Uemura, 1964) and the surprising fact emerged that the best test of a vaccine's effectiveness seemed to be its ability to give rise to H-agglutinins in man (Benenson, 1964). Apparently the role of antigens in stimulating the production of a suitable immune response to typhoid and paratyphoid bacteria was not properly understood. It seemed likely either that other unknown antigens were involved to a greater extent than had been expected or that the best ways of stimulating a protective response against the more familiar antigens were still not known. The following two chapters discuss first the chemistry and biology of some of these antigens, and then some of the reactions occurring in experimental animals which seem to be involved in combating typhoid-like diseases.

CHAPTER 1

SURFACE ANTIGENS OF SALMONELLAE

Introduction

The careful comparisons of White (1929_a) and Kauffmann of the cross-reactions of the antigens of a number of strains used in different laboratories were developed into a detailed system for the classification of Salmonellae by Kauffmann(1966_a). The Kauffmann-White scheme uses the cross-reactions of three types of antigen, the O and H antigens of Weil and Felix (reviewed by Felix, 1924) and the Vi antigen of Felix and Pitt ($1934_{a,b}$). These are the antigens most readily demonstrable on Salmonellae and are therefore those most frequently studied. However, as has been repeatedly stressed by Kauffmann (1966_b) they are not the only ones present. This chapter is an attempt to review the chemistry and biology of these and other antigens which may be associated with virulence in Salmonellae. Special attention is given to the antigens of S.typhimurium, on which were carried out most of the experiments described in this thesis. A very thorough review of the chemistry and biochemistry of cell-wall components of Gram-negative bacteria has recently appeared (Lüderitz, Jann and Wheat, 1968).

H flagellar antigens

The existence of a heat-labile antigen associated with motility in Salmonellae was recognised very early in the present century (Smith and Reagh, 1903, Beyer and Reagh, 1904, Felix, 1924). These antigens are carried by the flagellae and about 100 different forms have now been listed by Kauffmann(1966$_a$). Although they are regarded as heat-labile, Kauffmann(1966$_c$) recommends that vaccines be boiled for at least 2½ hours if they are not to give rise to anti-flagellar antibody. Heating for a shorter time at lower temperatures (70° for 20 minutes) makes flagellated bacteria inagglutinable by H antisera but does not destroy the immunogenicity of the H antigen (Beyer and Reagh, 1904). These antigens occur as proteins which are polymers of 40,000 molecular weight unit (McDonough, 1965).

Salmonella H antigens have the peculiar property of showing phase variation. Individual colonies on solid media from pure cultures of many species will show only one of two sets of antigen, and liquid cultures are likely to contain bacteria in both phases (Andrewes, 1922). It has been shown that the two specificities, known as phase 1 and phase 2, are coded on separate loci on the Salmonella chromosome (H_1 and H_2 fig 1) (Lederberg and Edwards, 1953). The first of these is homologous with the flagellar locus on Escherichia coli, while the other seems to be characteristic of the

Fig 1

Selected genes involved with antigens and virulence

adapted from

Krishnapillai and Baron (1964)

Mäkelä and Mäkelä (1966)

Sanderson (1967)

Sarvas (1967)

Stocker, Wilkinson and Makela (1966)

Markers

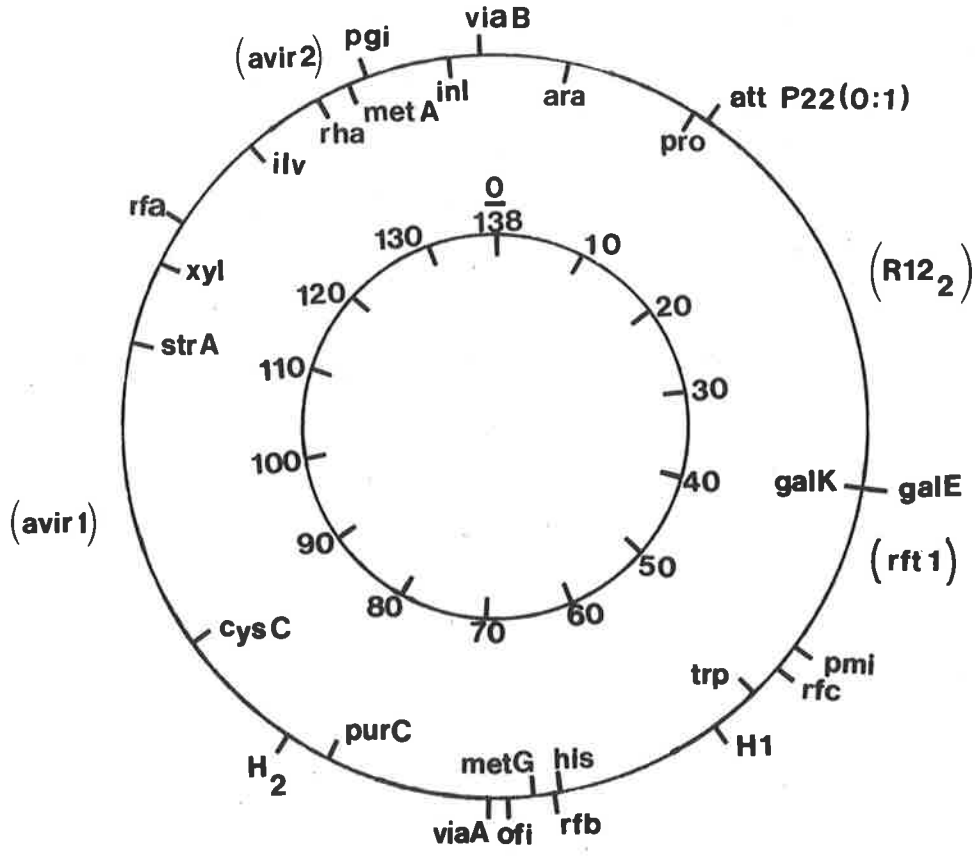
Gene	Position (min)	Enzyme or character
ara	4	arabinose utilisation
cys C	90	adenylyl sulphate kinase (cysteine synthesis)
gal K	38	galactose kinase
his	65	histidine synthesizing enzymes
ilv	122	isoleucine and valine synthesis
inl	135	inositol utilisation
met A	129	homoserine O transsuccinylase (methionine synthesis)
met G	67	methionine requirement
pro	12	proline synthesizing enzymes
pur C	79	AIR → AICAR (adenine requirement)
rha	127	rhamnose utilisation
str A	108	high level streptomycin resistance
trp	52	tryptophan synthesizing enzymes
xyl	113	xylose utilisation

continued

Fig 1 (continued)

Gene	Position (min)	Enzyme or character
att P22	13	phage PLT22 attachment site (O-antigen 1)
avir 1	89-109	avirulence
avir 2	127-4	avirulence
gal E	38	galactose-4-epimerase
H 1	55	phase 1 flagellar antigen
H 2	82	phase 2 flagellar antigen
ofi	68	O antigen 5
pgi	130	phosphoglucose isomerase
pmi	48	phosphomannose isomerase
rfa	116	LPS core-synthesizing enzymes
rfb	65	O-repeating unit synthesizing enzymes
rfc	50	O-repeating unit polymerisation
rft 1	38-50	T1 antigen production
R 12_2	12-52	antigen 12_2 variation
via A	69	Vi antigen
via B	137	Vi antigen

inner circle	approximate position on the chromosome (minutes)
inside outer circle	genetic markers
outside outer circle	genes involved in antigen synthesis and virulence
( )	genes not accurately mapped


viaB
pgi
(avir2)
inl
metA
ara
rha
att P22(0:1)
pro
ilv
0
138
rfa
130
10
(R12 2)
xyl
120
20
strA
110
30
100
40
galK
galE
(avir1)
90
50
(rft1)
80
70
60
cysC
trp
pmi
rfc
purC
H1
H2
metG
his
viaA
ofi
rfb

Salmonella group (Mäkelä, 1964), and is located close to a gene which controls which of the two is operative (Lederberg and Iino, 1956).

There is little evidence of a relationship between H antigens and virulence in Salmonellae (eg Tully, Gaines and Tigertt, 1963) and several of the best known virulent species, including S.typhi, S.enteritidis and the S.gallinarum-pullorum group, are deficient in the activity of one or both of the H genes. However Benenson's (1964) conclusion suggests that the capacity to stimulate production of anti-H antibody in a vaccine may fortuitously act as a marker for some other immunological property.

Vi antigens

Felix ($1952_{a,b,c}$) reported several Vi antigens, identified by their lability to heat, acids or alkali, but two of these, the Vi antigens of S.paratyphi A and S.paratyphi B are now considered to be respectively the Salmonella O-somatic antigens 2 and 5 (Kauffmann, 1966_{d}). However antigens similar or identical to the Vi antigen of S.typhi are found in S.paratyphi C and some strains of Citrobacter (Kauffmann, 1966_{e}). Felix and Pitt ($1934_{a,b}$) found that the presence of the Vi antigen of S.typhi prevents agglutination of bacteria by anti-O sera. Heating the bacteria makes the antigen dissociate from the surface and allows O antibodies to react (Spaun, 1952).

Chemical studies have shown the Vi antigen to be a highly acetylated polysaccharide containing large amounts of 1 - 3 linked galactaminuronic acid (Clark, McLaughlin and Webster, 1958, Heyns, Kiessling, Lindenberg et al, 1959, Heyns and Kiessling, 1967) acetylated on both the oxygen and the nitrogen groups (Gerfaux, Bernard and Staub, 1963). Two regions of the Salmonella chromosome are thought to be necessary for its appearance (via A and via B fig 1) (Johnson, Krauskopf and Baron, 1965, 1966), but the exact function of each gene is not understood. Nor is it clear what function, if any, these genes have in bacteria which do not produce a Vi antigen.

The question of whether the antigen is involved in virulence, and whether antibodies against it are of particular importance, has been the subject of some controversy. Felix and Pitt (1934_b) originally found that the LD_{50}s in mice of strains with Vi antigens were less than the LD_{50}s of those without. These results were obtained by injecting very large doses of organisms intraperitoneally, but other workers reached a similar conclusion when mice were injected intracerebrally with smaller doses (Landy, Gaines and Sprinz, 1957, Gaines and Tully, 1961). It was found that antisera against Vi strains were more effective in protecting mice against these

organisms than were sera against O-antigens (Felix and Pitt, 1934_a). Experiments of this nature were criticised on the grounds that the experimental situation in mice was very different from that in humans, in which it seemed that a very small dose, which itself was far from toxic, gave rise to the clinical disease (Ørskov and Kauffmann, 1936). More recent work suggests that although the Vi antigen is certainly not the only contributing factor to virulence in S.typhi, Felix and his co-workers were right (Tully, Gaines and Tigertt, 1962). In these experiments young chimpanzees were infected with S.typhi to give a disease which is similar to though probably less severe, than the human form. It was found that although a disease could be demonstrated when the animals were fed organisms of S.typhi lacking Vi antigen, very high doses had to be used, the resultant bacteraemia was very short, and the fever response was also very much reduced. Gaines, Landy, Edsall et al,(1961) demonstrated that the acetone-dried vaccine under test by the World Health Organization, which had originally been developed as a good Vi immunogen, was more effective than any other in preventing symptoms from appearing in immunised animals, and showed that of the more 'pure' vaccines, only Vi antigen had any demonstrable effect.

The mode of action of Vi antigen in promoting virulence is not known. Its ability to prevent O-agglutination seems to have led Felix and his fellow workers to believe that it simply prevented anti-O antibodies from reacting. They showed that antibodies against the Vi antigen had a direct effect on the bacteria and could act as opsonins promoting their phagocytosis by rabbit polymorphs (Bhatnagar, 1935, Felix and Bhatnagar, 1935). However Gaines, Tully and Tigertt(1961) have shown that it also has an 'aggressin'-like activity, and when injected simultaneously with them can promote faster growth of Vi negative strains of Salmonella and some other organisms in the mouse cerebellum, even though it seems unlikely that it is attached to the bacteria. Anti-Vi antibodies completely inhibit this effect. It seems likely that both effects are operative and one would expect that immunisation against the Vi antigen has some effect both in assisting in the disposal of bacteria and in inhibiting the aggressin effects of the antigen.

O polysaccharide antigens

Salmonella O antigens have been the subjects of extensive immunological and chemical research and have been implicated in virulence (reviewed by Roantree, 1967). Aspects of their chemistry, biochemistry and immunology have been reviewed on a number of

recent occasions (Osborn, Rosen, Rothfield et al, 1964, Horecker, 1966, Lüderitz and Westphal, 1966, Lüderitz, Staub and Westphal, 1966, Lüderitz et al, 1968, Nikaido, 1968).

Many methods have been used to obtain preparations with O-specificity from Enterobacteriaceae. They have included fractionation of trypsin digests or autolysates (Furth and Landsteiner, 1928, Raistrick and Topley, 1934, Miles and Pirie, 1939), extraction with cold trichloroacetic acid (Boivin and Mesrobeanu, 1933), extraction with diethylene glycol (Henderson and Morgan, 1938, Morgan and Partridge, 1940), acetic acid hydrolysis (Morgan, 1931, Freeman, 1942), phenol-water extraction (Jesaitis and Goebel, 1952, Westphal, Lüderitz and Bister, 1952) extraction with ether saturated water (Ribi, Hasking, Landy et al, 1961) and extraction with ethylene diamine tetraacetic acid (EDTA) at pH8 (Leive, 1965). It is interesting that the diethylene glycol and EDTA extractions release only about 50% of the antigens. On the other hand yields from acetic acid hydrolysis and Westphal et al's phenol-water extraction seem to be complete (Staub and Combes, 1951, Westphal and Jann, 1965). Most of the preparations yield a mixture of protein, lipid and polysaccharide, but phenol water extracts contain only polysaccharide and lipid, and Freeman acetic acid extracts only polysaccharide. The various preparations show

considerable physical as well as chemical variations (Ribi, et al, 1961, Ribi, Anacker, Brown et al, 1966).

Chemistry of rough antigens

Most Salmonellae isolated from infected animals have a smooth shiny colonial form. However it has been known for many years that they are liable to mutate to rough forms which appear more dull, give irregular-shaped colonies on agar, and tend to clump or precipitate from broth or saline (Arkwright, 1920). These rough bacteria, or R forms, can be shown to have had their specific O-antigens replaced by other groups which though not all identical, frequently share antigens which were not apparent in the parent strains (Schütze, 1921, Beckmann, Lüderitz and Westphal, 1964$_a$). The finding that colorimetric reactions for proteins could be carried out on the surfaces of some rough organisms but not on smooth forms and the identification of O antigenic specificity in a polysaccharide preparation led White (1929$_b$) to suggest that rough forms were lacking in carbohydrate. Chemical studies on purified antigens since 1960 have confirmed this suggestion. Identification of the sugars associated with polysaccharides isolated from a number of Kauffmann White groups showed that while certain specific sugars were associated with specific polysaccharides

from smooth organisms, polysaccharides from all smooth Salmonellae included a certain group of sugars. (Table 2) (Kauffmann, Lüderitz, Stierlin and Westphal, 1960, Kauffmann, Jann, Krüger, et al, 1962, Heath and Ghalambor, 1963, Osborn 1963).

TABLE 2

Selected Salmonella chemotypes (a)

Chemotype	Constituent sugars															O serotype
	D-galactosamine	D-glucosamine	2-keto-3-deoxy-octonate	L-glycero-D-manno-heptose	D-galactose	D-glucose	D-mannose	L-fucose	L-rhamnose	ribose	colitose	abequose	paratose	tyvelose		
I		+	+	+	+	+										J,V,X,Y,58
III		+	+	+	+	+	+									C_1,C_4,H,S
VI	+	+	+	+	+	+		+								G,N,U
XXV		+	+	+	+	+				+						52
X		+	+	+	+	+	+				+					O
XIII		+	+	+	+	+	+		+							E,F,54
XIV		+	+	+	+	+	+		+			+				B,C_2,C_3
XV		+	+	+	+	+	+		+				+			A
XVI		+	+	+	+	+	+		+					+		D

(a) abbreviated from Lüderitz et al, 1966.

These same sugars were found on many rough forms (Lüderitz, Kauffmann, Stierlin and Westphal,1960, Kauffmann, Krüger, Lüderitz and Westphal, 1961) and it was proposed that all smooth Salmonella lipopolysaccharides consisted of a rough core to which were attached sugars specific for the O-antigens (Lüderitz et al 1960). Subsequent progress in determining the structure of this core has depended on the preparation of a series of rough mutants which made different sorts of polysaccharides. The research has concentrated on rough forms from three species, S.typhimurium, S.minnesota, and S.ruiru, using mutants selected mainly on the basis of colonial form or phage sensitivity.

Fig 2 shows the currently accepted structure of core lipopolysaccharide. It has been found that the qualitative sugar compositions of lipopolysaccharides obtained from different S.minnesota mutants range from those in which all of the rough sugars are present to those which contain only N-acetyl glucosamine. As shown in Fig 2 they have been classified by Westphal and his fellow-workers as belonging to the chemotypes Ra to Re, depending on the complexity of the polysaccharides (Lüderitz et al, 1966). The antigenic types RI and RII of Beckmann and her colleagues (Beckmann et al ,1964$_a$, Beckmann, Subbaiah and Stocker ,1964$_b$) correspond to the first two of these chemotypes. It seems that

Fig 2

Chemical structure of rough Salmonella lipopolysaccharide

Abbreviations

gal	D-galactose
glc	D-glucose
glc NAc	2-deoxy-2-amino-N-acetyl glucose
hep	L-glycero-D-mannoheptose
HM	β-hydroxymyristyl (amide linkage)
FA	lauryl, myristyl and palmityl (ester linkage)
ethanolamine-P	ethanolamine phosphate

adapted from

Dröge and Lüderitz (1969)

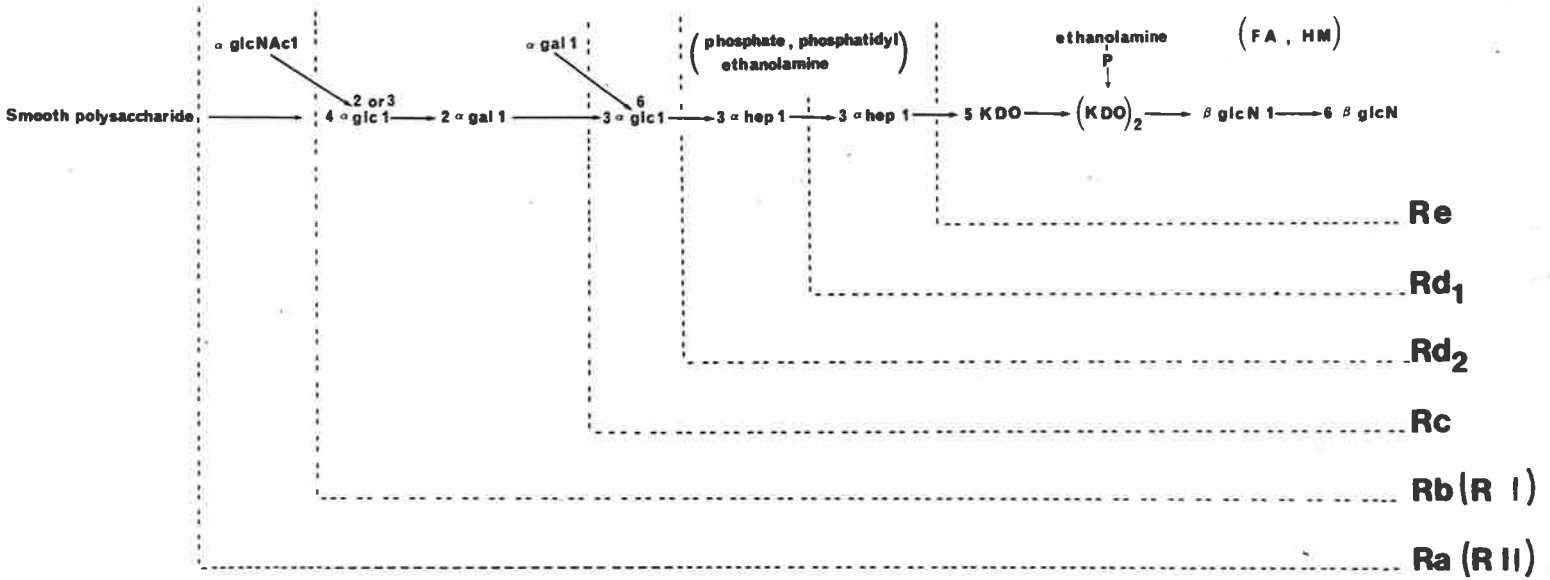
Dröge, Russmann, Lüderitz et al, (1968)

Gmeiner, Lüderitz and Westphal (1969)

Lüderitz et al, (1966)

Nikaido (1968)

Nikaido (1969)


α glcNAc1
2 or 3
α gal 1
6
(phosphate, phosphatidyl ethanolamine)
ethanolamine
P
(FA, HM)
Smooth polysaccharide
4 α glc 1
2 α gal 1
3 α glc1
3 α hep 1
3 α hep 1
5 KDO
(KDO)2
β glcN 1
6 β glcN
Re
Rd1
Rd2
Rc
Rb (R I)
Ra (R II)

rough lipopolysaccharide is synthesized by stepwise addition of monosaccharide units to the growing core. A lesion resulting in loss of the ability either to synthesize the activated monosaccharide precursor or to attach it to the core prevents the incorporation of all sugars distal to that linkage, and the chemotypes of Westphal's group correspond to mutations at some of these points. It should be noted in Fig 2 that although most of the chemotypes are well defined, chemotype Rb, because glucose and galactose occur twice in the same structure, includes organisms which can make several different varieties of rough polysaccharide. Most of the structure of the short branched terminal oligosaccharide distal to heptose now appears to be well established (see Nikaido, 1968) and its point of linkage with the O-specific polysaccharide is known (Nikaido, 1969). The structure of the proximal end is still not completely known. Mild acid hydrolysis cleaves lipopolysaccharide in the region of 2-keto-3-deoxy-octonate (KDO) to leave an insoluble residue known as lipid A (Westphal and Lüderitz, 1954) one of whose main constituents is a β1-6 linked glucosamine dimer to which are attached a number of medium to long chain fatty acids (Gmeiner, Lüderitz and Westphal, 1969). Three different phosphate groups have also been identified in the heptose-KDO region and a comparison of the

phosphate compositions of lipopolysaccharides from strains with mutations affecting this region has allowed the tentative assignments in Fig 2 (Dröge, Ruschmann, Lüderitz and Westphal, 1968, Dröge and Lüderitz,1969). It seems likely that there are cross-links between chains in this region, but there is little evidence on how they are formed. Oxidation of the amine group of phosphatidyl ethanolamine leads to an alteration in the molecular weight of the polysaccharide (Dröge and Lüderitz, 1969). It would be surprising if an ionic bond was involved in a linkage between two chains in a region which appears otherwise to be quite hydrophilic.

Most of the genes involved in synthesis of the rough core are located in a region known as rfa (Fig 1) (Stocker, Wilkinson and Mäkelä, 1966, Sanderson, 1967). These are the genes which appear to be concerned only with the synthesis of the rough core. Mutations in another region (rfb Fig 1) lead to the appearance of rough forms of serotype RII (Beckmann et al, 1964 $_{a,b}$). These affect the synthesis of the smooth O-specific polysaccharide which is attached to the core (Nikaido, Nikaido and Mäkelä, 1964, Stocker et al, 1966). A few other genes in other locations which can affect core synthesis are also involved in unrelated activities,

and are found in other parts of the chromosome. They include genes involved in the interconversion of glucose-6-phosphate and fructose-6-phosphate (pgi Fig 1) (Rothfield, Osborn and Horecker, 1964) and genes involved in the formation of UDP-galactose (gal E Fig 1) (Fukasawa and Nikaido, 1961).

Although all rough organisms are apparently avirulent, at least one strain is known to be able to exist within the host for a substantial period (Ushiba, Saito, Akiyama and Sugiyama, 1959). Dlabač (1968) has shown in studies on mutants from a virulent strain of S.typhimurium that the Ra and some Rb chemotypes are more resistant to the bactericidal action of serum than are lower chemotypes. It seems, therefore, that rough polysaccharide may give some protection from the host, and can function as more than a base for the attachment of specific polysaccharide. Complete protection, and the ability to multiply so rapidly that the host dies, depends on possession of smooth polysaccharide.

Smooth O-antigens

Species in the Kauffmann-White scheme have been defined on the basis of biochemical characteristics and antigenic structure. A further grouping is made on the basis of O-antigenic composition. Certain antigens, or groups of antigens are characteristic for each

group, and certain additional antigens may be present. There seems to be no relationship between the H antigens of a particular species and its corresponding O antigens. A number of Kauffmann's groups and sub-groups with their characteristic antigens are listed in table 3.

TABLE 3

Selected subgroups from the Kauffmann-White scheme[a]

Group or sub-group	Characteristic O-antigens	Optional O-antigens
A	2,12	1
B	4,12	$1,5,27_B$
C_1	6,7	1,25
C_2	6,8	-
C_3	(8)	20
C_4	9,12	1
D_1	9,12	1
E_1	3,10	-
E_2	3,15	-
E_3	(3),(15),34	-
E_4	1,3,19	-
O	35	-

(a) from Kauffmann 1966_a

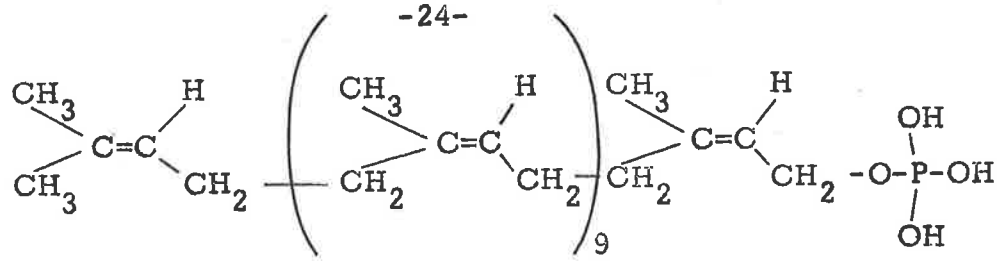
The chemical studies of polysaccharides on Kauffmann's various serotypes allowed a second classification to be made on the basis of chemotype (table 2) (Kauffmann et al, 1960, 1962). Selected qualitative analyses on a number of serotypes are shown in table 2. (from Lüderitz et al 1966). Bacteria with the same serotype all have the same chemotype, but the converse is not true, and a single chemotype may contain several serotypes. In table 2 sugars are quoted in increasing order of their lipophilic nature. Chemotypes were originally numbered in order of increasing complexity of their polysaccharides and the extent to which they contained the more lipophilic sugars. Since the addition of some rarer chemotypes this rule is no longer completely applicable.

The individual O-specific antigens of the Kauffmann-White scheme are characterised by specific immunodominant sugars. High concentrations of particular monosaccharides can at least partly inhibit precipitin reactions using specific antisera and purified O-specific degraded polysaccharide made by the Freeman method (Staub, Tinelli, Lüderitz and Westphal, 1959).

Thus the 3, 6, dideoxyhexoses paratose, abequose and tyvelose inhibit respectively specific antibodies against the O-antigens 2, 4 and 9 (Staub et al, 1959) and glucose inhibits some antibodies against 1 and 12 (Stocker, Staub, Tinelli and Kopacka, 1960, Staub, 1960). The immunodominant sugar does not constitute the whole antigen and its linkages with other sugars are important. Some pairs of antigens such as 1 and 12 (glucose) and 4 and 8 (abequose) have the same immunodominant sugar (Lüderitz et al, 1966), and oligosaccharides isolated after partial hydrolysis of O polysaccharides are sometimes much better inhibitors of precipitin reactions than the isolated monosaccharides (eg Staub, 1960). These results are in accord with those obtained using other polysaccharide systems (reviewed by Kabat, 1966). Kabat (1960) has shown that a polysaccharide antigenic determinant can be up to 6 hexose units in length. Inhibition studies can thus be used to obtain information not only on the varieties of immunodominant sugar involved, but also on their anomeric forms and even, to a limited extent, on the sugars to which they are attached (see Staub, 1960).

The specific part of the O-polysaccharide in subgroups E_1, E_2, and E_3 is itself a polymer of an O-specific tri- or tetra-saccharide (Robbins and Uchida, 1962), and since this discovery evidence has accumulated that the polysaccharides of groups B and D have a similar structure. Biosynthesis of the O specific part of lipopolysaccharide takes place separately from the rough core and the polymerised polysaccharide is added as a single unit (Nikaido, Nikaido, Subbaiah and Stocker, 1964, Weiner, Higuchi, Rothfield et al, 1965, Weiner, Higuchi, Osborn and Horecker, 1966). Mutations in the rfa region which prevent completion of the core also prevent incorporation of this polysaccharide, with the result that it accumulates and can be isolated separately (Beckmann et al, 1964_b, Kent and Osborn, 1968_a). It has been shown that this polysaccharide is synthesized in a part of the cell wall which becomes accessible only after treatment with EDTA and trishydroxymethyl amino methane (tris) at pH above neutrality (Kent and Osborn, 1968_a).

Synthesis of the O-specific polysaccharide appears to be closely connected with a phospholipid derived from a C_{55} polyisoprenoid alcohol known as antigen carrier lipid (ACL):



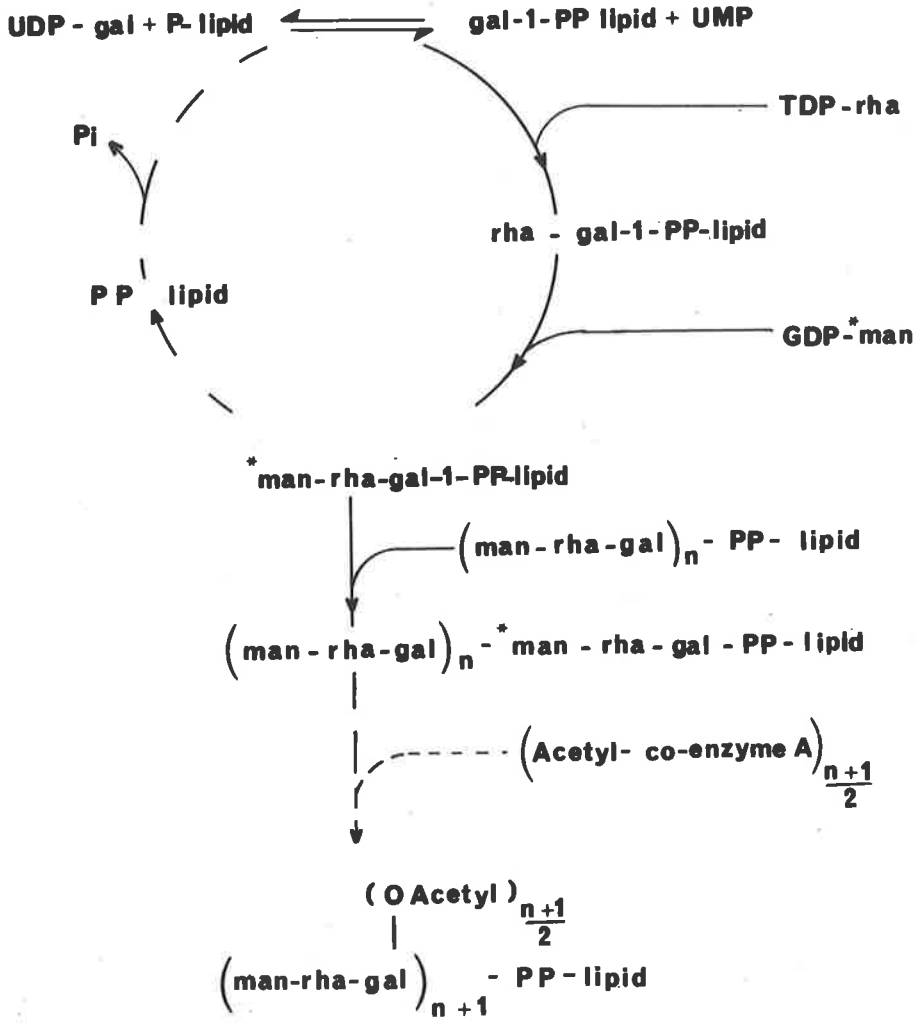
(Wright, Dankert, Fenessy and Robbins, 1967). Three apparently different classes of sugar derivative have been shown to be attached to this phospholipid. Synthesis of polysaccharides in groups B and E occurs by synthesis of an O-specific oligosaccharide sub unit of 3-5 monosaccharide units, and polymerisation of these units to form the completed polysaccharide, which is then attached to the rough core. The proposed series of reactions for the group E_1 Salmonellae, is shown in Fig 3. Both the oligosaccharide and polysaccharide are made by addition of sugars to a growing chain on a phospholipid carrier. It is likely that in vivo the polysaccharide obtained by Beckmann et al (1964_b) is attached to ACL, but that the pyrophosphate linkage is cleaved during phenol extraction (Weiner et al, 1965, 1966). Robbins and his colleagues have shown that chain growth procedes by addition of the polysaccharide to the lipid linked oligosaccharide, rather than vice versa (Bray and Robbins, 1967, Robbins, Bray, Dankert and Wright, 1967). There is some evidence that the enzyme involved in chain polymerisation remains associated with the chain throughout its period of growth (Robbins and Uchida, 1965).

Fig 3

Biosynthesis of O-specific polysaccharide in group E, Salmonellae

adapted from

Wright et al, (1967)


UDP - gal + P- lipid
gal-1-PP lipid + UMP
TDP-rha
Pi
rha - gal-1-PP-lipid
PP lipid
GDP-*man
*man-rha-gal-1-PP-lipid
(man-rha-gal)n- PP- lipid
(man-rha-gal)n-*man - rha - gal - PP - lipid
(Acetyl- co-enzyme A)n+1/2
(O Acetyl)n+1/2
(man-rha-gal)n+1 - PP-lipid

In a third ACL-linked reaction, described by Wright (1969), glucose for incorporation into the Group E lipopolysaccharide as the immunodominant sugar of antigen 34 is also carried on ACL. This reaction occurs only in the presence of the phage ε^{34}.

It is interesting that a similar lipid has been shown by Strominger and his colleagues to be involved in the biosynthesis of the mucopeptide of Gram-positive organisms (Higashi, Strominger and Sweeley, 1967), and by Scher and Lennarz, (1969) in the synthesis of a mannan in Micrococcus lysodeikticus. It may be that its main function is to localise metabolites in a particular area of the cell wall.

Most of the genes involved in the synthesis of O-specific polysaccharide are located in the region known as rfb (Fig 1) (Subbaiah and Stocker, 1964, Stocker et al, 1966). They include the genes for the synthesis of deoxy- and dideoxyhexoses (groups B and D) and genes involved in mannose metabolism (groups B, D and especially C_1) (Nikaido et al, 1966). The region maps as the main locus for antigen specificity in groups B, D and C, but there seem to be few homologies between the first two groups and the last (Mäkelä, 1966). A few other loci have been identified as affecting formation of O-specific polysaccharide.

They include the gene ofi (Fig 1) which is very close to rfa and codes for antigen 5 (Mäkelä, 1965, Johnson et al, 1966), a gene involved in synthesis of antigen 12_2 ($R12_2$ Fig 1) (Mäkelä and Mäkelä, 1966), and the site of attachment of the prophage PLT22 (att P22 Fig 1) which causes the appearance of antigen 1_{12} in groups A, B and D (Smith and Levine, 1965). A few genes involved in general sugar metabolism may also affect smooth polysaccharide (eg gal E Fig 1) (Fukasawa and Nikaido, 1961).

Semi-rough forms

An important group of Salmonella lipopolysaccharide mutants which have been discovered recently are known as semi-rough (Naide, Nikaido, Mäkelä et al, 1965). These are Salmonellae which possess at least some specific O antigens but are deficient in smooth polysaccharide and share many properties with rough bacteria. The most easily definable type of semi-rough Salmonella, known as type C, occurs in groups B, D, and E as a result of absence of the enzyme required to polymerize the O specific ACL- linked eligosaccharide to polysaccharide. Instead, short stubs of oligosaccharide are attached to the rough chain (Naide et al, 1965). Lack of the enzyme may result from mutation of the gene controlling its synthesis (rfc Fig 1), in

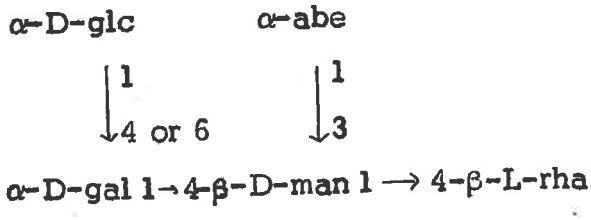
which case the bacteria are selected as apparently smooth colonies which are resistant to O-specific phage (Naide et al, 1965), or by recombination between Salmonellae of group C_1 with those of B or D (Mäkelä, 1966). Group C_1 bacteria do not appear to have a polymerase of this sort and as the rfc locus is fairly well separated from the rfa, recombinants which contain a new O-specific antigen from the rfa locus generally fail to obtain the corresponding rfc locus. (Mäkelä, 1966). The reciprocal conversion cannot be shown. Strains of S.montevideo (group C_1) into which the group B rfc locus has been introduced appear to make identical polysaccharides to their fellow unmated bacteria (Mäkelä, 1966). It seems, therefore, that there is either a fundamental difference between the methods of biosynthesis of lipopolysaccharide in the two groups, or that the gene in group C_1 corresponding to rfc in S. typhimurium is very closely linked to the rfa cluster. Fuller, Etievant and Staub (1968) suggested that there might be no need for a polymerase in group C_1 bacteria because the terminal sugar in both the rough and the O-specific oligosaccharide was N acetyl-glucosamine, so that the enzyme linking side chain to core would be carrying out virtually the same reaction as the polymerase and could act in both

capacities. However the recent demonstration by Nikaido (1969) that the O-specific polysaccharide in S.typhimurium is linked not to N-acetyl-glucosamine but to the adjacent glucose makes this explanation unlikely. The corresponding sugar in the O-specific oligosaccharide of group C_1 is D-mannose.

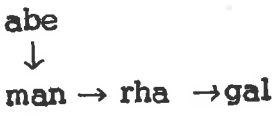
In semi-rough type C bacteria the rough polysaccharide appears to be fairly evenly covered by short stubs of specific polysaccharides. In contrast lipopolysaccharides synthesized by the semi-rough mutants which are known as type D appear to consist of a mixture of normal forms and uncompleted rough polysaccharide stubs (Naide et al, 1965). Most of these which were first found resulted from mutations in the rfa region (Naide et al, 1965). It appears that they are caused by leaky mutations in any of the normal lipopolysaccharide synthesising genes (Nikaido, 1968). This results either in low production of the relevant enzymes, or in the formation of partly defective enzymes which are unable to synthesize lipopolysaccharide at the required rates. Depending on the degree of seriousness of the lesion, semi-rough type D strains can appear either smooth or rough. It seems likely that they may in the past have been an important source of error in experiments where it was thought that either completely rough or completely smooth organisms were being used.

Chemical structure of lipopolysaccharide from S.typhimurium and related strains

The chemical structure of a specific oligosaccharide from group B Salmonellae was established by Staub and her co-workers as follows:

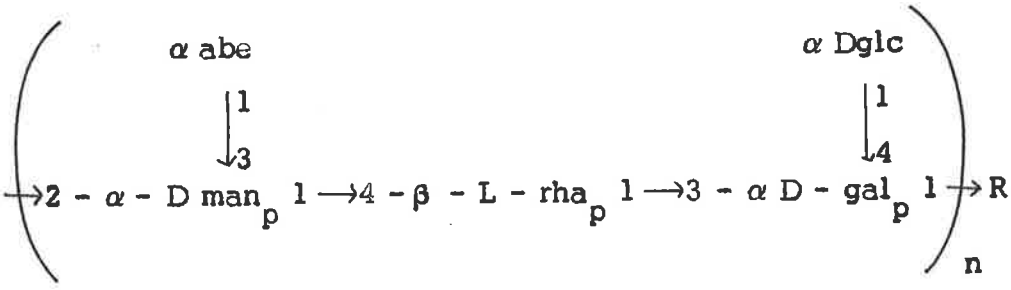


(Staub,1960, Bagdian, Lüderitz and Staub, 1966). The order of the sugars was determined by examination of oligosaccharides obtained from partial acid hydrolysates, and their linkages were suggested by the results of quantitative periodate oxidations and colorimetric reactions. Biochemical studies by Osborn and her colleagues showed that the naturally occurring oligosaccharide had the structure



(Weiner et al, 1965, 1966, Osborn and Weiner, 1968). Recently methylation studies and estimations of the amounts of each derivative obtainable by gas liquid chromatography have shown a different set of linkages (Hellerqvist, Lindberg, Svensson et al, 1968, 1969). It seems likely from the work of Muramatsu and Egami (1968) that mannose occurs as the α anomer. The currently accepted

structure of the repeating unit of the O-polysaccharide structure is shown below



Abequose or possibly mannose is the terminal non-reducing sugar and R represents the core polysaccharide (Fig 2). Group D Salmonellae are very closely related to group B. They share antigens 1_{12} and 12 (Stocker et al, 1960), give very similar oligosaccharides on partial acid hydrolysis, and react in an identical way with periodate (Tinelli and Staub, 1959). It seems likely that they have a similar structure to the one shown above with tyvelose in place of abequose. One might expect that group A polysaccharide would also be similar, although it behaves differently from the other two on periodate oxidation (Tinelli and Staub, 1959).

The O-antigens of S.typhimurium are 1_{12}, 4_1, 4_2, 5 and 12 (Kauffmann, 1966_a, Stocker et al, 1960, Staub and Bagdian, 1966).

Antigen 1_{12} is unusual in several respects. It is liable to considerable quantitative variation within a single strain. On the basis of agglutination behaviour and immunogenicity in rabbits Kauffmann (1940) classified single colonies grown on solid media from a single broth culture into those containing large quantities of 1 (1^{++} form), moderate amounts (1^{+}) and those in which it is poorly developed (1^{+}_{-}). When antigen 1 is present in certain groups other antigens also appear (antigen 19 in group E, 25 in group H, 37 in G). Amounts of these vary simultaneously with 1 (Kauffmann and Rhode, 1961).

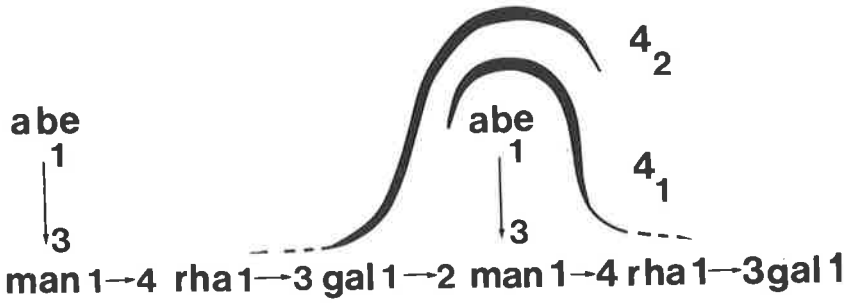
It has been found that antigen 1 in groups A, B and D occurs in bacteria with a specific prophage (Iseki and Kashiwagi, 1955, Zinder, 1957), and it seems likely that loss of the phage causes the variations observed by Kauffmann, The antigen is attributed to a 6-O-α-D-glucosyl galactosyl residue on the O-side chain (Stocker et al,1960).

Antigen 4, the antigen specific for group B, has been divided into 4_1 and 4_2 on the basis of its cross-reactions with Salmonellae of the type 4, 27 (Staub and Bagdian,1966). The structures of the relevant oligosaccharides in groups B_1 ($0{:}4_1,4_2$) and B_2 ($0{:}4_1,27_B$) are shown in Fig 4 together with

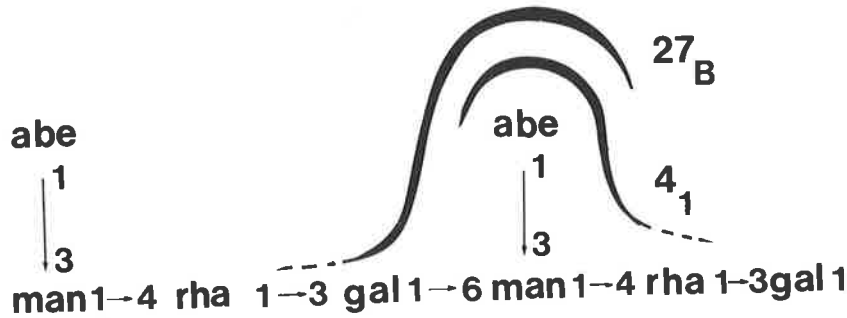
Fig 4

Chemical basis for differentiation between O-antigens 4_1, 4_2 and 27_B

modified from Bagdian et al, (1966)



ANTIGENS $4_1, 4_2$ (S. typhimurium)



ANTIGENS $4_1, 27_B$ (S. bredeney)

the areas with which antibodies against 4_1, 4_2 and 27_B are thought to react (Bagdian et al, 1966). One might expect that if antigen 1 or 12_2 was present the galactose part of antigens 4_2 and 27_B would be blocked, thereby causing a reduction in the amounts of antigens 4_2 and 27_B.

Antigen 5 is heat-labile, and as such has had a rather chequered career in and out of the O antigens. Felix (1952_c) referred to it as the Vi antigen of S.paratyphi B and Kauffmann (1966_f) has included it amongst the K antigens, a miscellaneous group of heat-labile antigens said to encapsulate Salmonellae. The notion of a capsule was attractive, as the presence of antigen 5 seemed to inhibit the actions of sera against 1, 4 and 12. In 1961 Kotelko, Staub and Tinelli established by inhibition of quantitative precipitin reactions that antigen 5 was a normal O-polysaccharide antigen whose determinant group was particularly sensitive to heat or alkali. By mild sulphuric acid hydrolysis and paper chromatography they isolated a hexasaccharide containing equal amounts of galactose, mannose and rhamnose which was able to inhibit the anti-5 serum. A chromatographically identical oligosaccharide was isolated from a strain lacking 5. However Kotelko et al showed that the

chromatographic spot with antigen 5 contained acetyl groups, while the spot lacking 5 had none. They therefore concluded that the immunodominant sugar for antigen 5 was O-acetylated. When they attempted to inhibit precipitation reactions with simple sugars they found that the best inhibitions, though still weak, were obtained from 2-deoxy-2-amino-N-acetyl-D-galactose (N-acetyl-galactosamine) and to a much lesser extent from galactose. They therefore concluded that the determinant sugar for antigen 5 was O-acetyl-galactose, probably acetylated in the 2 position.

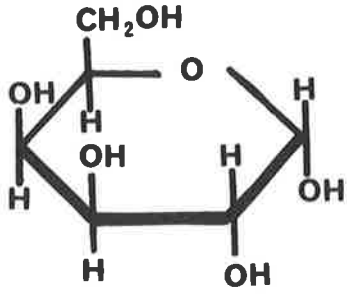
This structure left several puzzling biological problems. It did not well explain the inhibitory effect of antigen 5 on precipitation with antibody against 4. Furthermore it did not explain why antigen 5 occurred naturally only in group B, in spite of this group's close antigenic and chemical relationship with group D. Mäkelä (1965) found that recombinants which included both the group D rfb locus and the ofi antigen 5 locus were still unable to react with anti-5 antibodies. The alternative structure proposed by Hellerqvist *et al* (1968, 1969) is more in keeping with these properties. These workers succeeded in replacing every O-acetyl group in

the polysaccharide with a methyl group and found evidence only for the presence of 2-O-acetyl abequose. As abequose is the 3,6-dideoxy derivative of D-galactose it is not surprising that antibodies against the acetylated derivative should cross react with N acetyl galactosamine and acetyl galactose. From one surface, abequose and galactose are almost identical (Fig 5). However there is no obvious explanation for the apparent contamination of the hexasaccharide isolated by Kotelko et al with acetyl abequose, nor for their apparent failure to identify abequose in the eluate. It seems possible that strains other than the strain LT2 and 395MS examined by Hellerqvist et al have both abequose and galactose acetylated, or that during the manipulation of the polysaccharide in one or other system an acetyl transfer reaction took place. If such a reaction occurred in Hellerqvist et al's system it must have been quantitative, as they found no O-acetyl galactose.

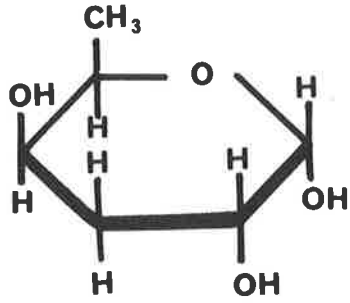
Antigen 12 is a complex antigen whose chemistry is not well understood. Three antigens are mentioned in the Kauffmann-White scheme, 12_1, 12_2 and 12_3. Organisms may have the antigenic formulae $12_1,12_2,12_3$; $12_1,12_2$ or $12_1,12_3$. This suggests that the chemical basis of 12_1 may be part of the chemical structure of both 12_2 and 12_3. These antigens can

Fig 5

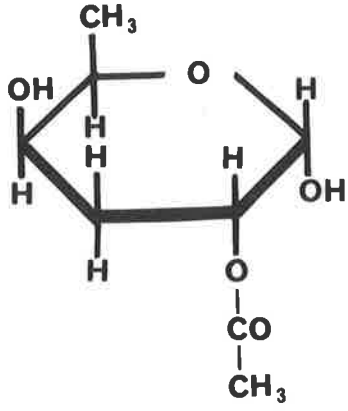
Haworth structures of 2-O-acetyl abequose and related sugars



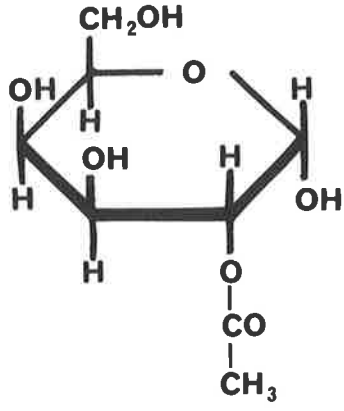
α-D-galactose



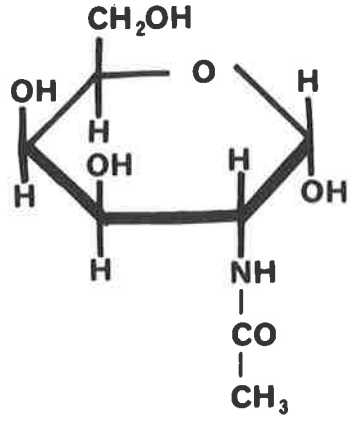
α-abequose



2-O-acetyl-α-abequose



2-O-acetyl-α-D-galactose



N-acetyl-α-D-galactosamine

occur in groups A, B and D. Staub (1960) showed that rabbit antisera against 12 were inhibited by glucose, while a horse antiserum was better inhibited by rhamnose. Antigen 12_2 has been shown to have the immunodominant sugar D-glucose connected by an α 1→4 linkage to galactose. It appears that the horse made an antibody directed primarily against the main polysaccharide chain. Phage P22 is said to be specific for antigen 12 but does not react with semi-rough type C mutants (Naide et al, 1965). Presumably antigen 12 by this definition involved the dimerised oligosaccharide, and at least the structure

$$\alpha\text{-D-gal } 1\rightarrow 2\text{-D-man}$$

Some antibodies against 12_2 react with semi-rough type C mutants, and it is clear that these antibodies do not need to react with mannose at the reducing side of galactose.

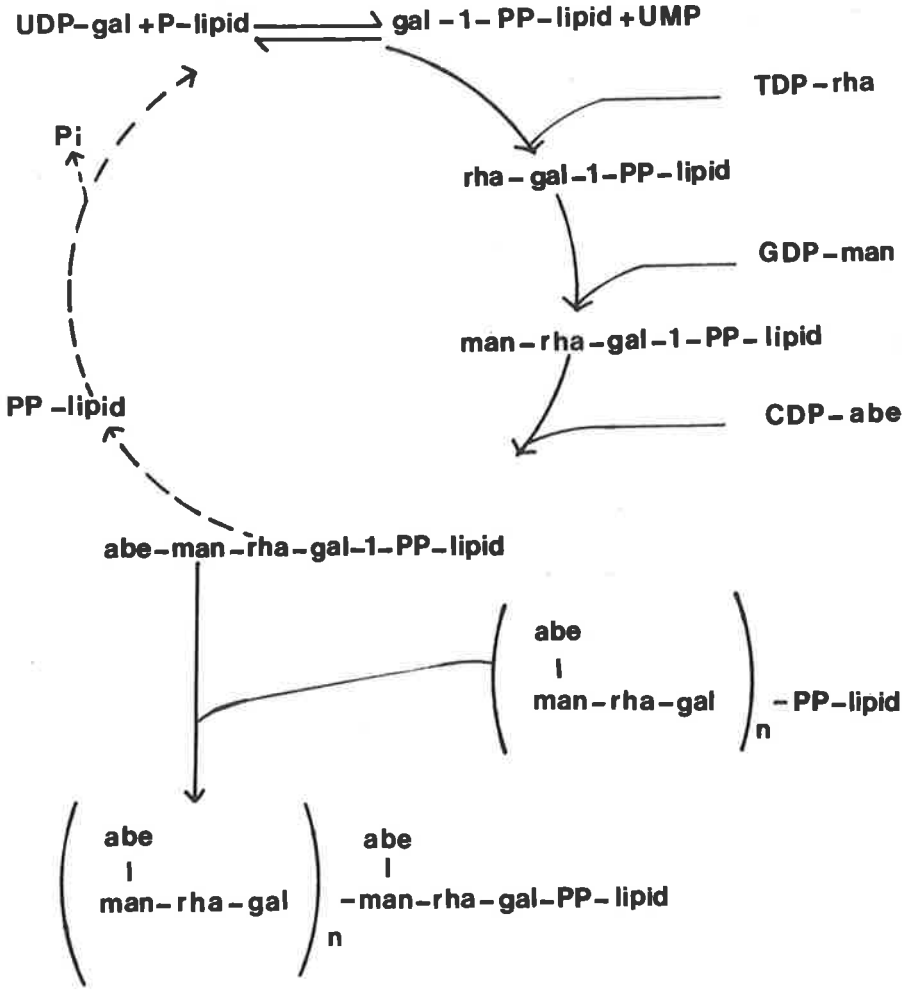
Like antigen 1, antigen 12_2 shows considerable variation in the amounts present in different colonies grown on solid media from a single liquid culture (Kauffmann, 1941). Control of this variation has been shown to rest in a gene mapping in the pro-trp region ($R12_2$ Fig 1) but there is no evidence for the involvement of a lysogenic phage (Mäkelä and Mäkelä, 1966).

Osborn and her colleagues have studied the series of reactions involved in the synthesis and polymerisation of S.typhimurium lipopolysaccharide shown in Fig. 6 (Weiner et al, 1965, 1966, Osborn and Weiner, 1968, Osborn and Tze-Yuen, 1968, Kent and Osborn, $1968_{a,b,c}$). Synthesis of both the O specific oligosaccharide and its polymer take place on ACL, the phospholipid identified by Wright et al (1967). The reactions are carried out in the order shown on a particulate fraction obtainable from the bacteria. Growing chains are not demonstrable on the bacterial surface either by agglutination by antibody or by absorption of the O specific phage P22 (Kent and Osborn, 1968_a). The polysaccharide, and presumably its site of synthesis, is exposed to the phage when the bacteria are treated with EDTA at alkaline pH (Kent and Osborn, 1968_a), a process which removes about 50% of the available lipopolysaccharide (Leive, 1965). Osborn and Weiner (1968) found that although incorporation of galactose, mannose and rhamnose was sufficient to allow polymerisation of the polysaccharide, the reaction was faster at lower temperatures (about 22°) if abequose was also added. The enzyme involved in the addition of this sugar was heat-labile, so that 87% of its activity was lost in 10 minutes at 37°. It showed some reactivity with CDP tyvelose as well as CDP

Fig 6

Biosynthesis of O-specific polysaccharide in group B Salmonellae

from Osborn and Weiner (1960)


UDP-gal + P-lipid
gal-1-PP-lipid + UMP
TDP-rha
rha-gal-1-PP-lipid
GDP-man
man-rha-gal-1-PP-lipid
CDP-abe
abe-man-rha-gal-1-PP-lipid
Pi
PP-lipid
abe
man-rha-gal
n
-PP-lipid
abe
man-rha-gal
n
abe
-man-rha-gal-PP-lipid

abequose, but would not react with CDP paratose.

The demonstration by Weiner et al (1965) that an in vitro system from S.typhimurium will polymerise a trisaccharide lacking abequose and glucose residues raises the question of how many potential antigenic sites actually contain abequose, glucose or an acetyl group. It might be expected that because the enzyme required to add abequose is labile in the in vitro system at 37° the maximum number of abequose residues would not be added at this temperature. However the studies of Hellerqvist et al (1968, 1969) on lipopolysaccharides synthesized in vivo showed that there were as many abequose residues as there were of mannose or rhamnose, and that in one case all of them were acetylated to make antigen 5. The situation with the glucose residues forming antigen 12_2 is different. In strain 395MS Hellerqvist et al showed that there was approximately one antigen 12_2 unit for every completed side chain (degree of polymerisation 11), while in LT2, with an average degree of polymerisation of about 7 the figure was closer to 3.5. There is no information as to whether these figures represent differences between individual clones of bacteria, which one might expect from the data of Kauffmann (1941) and Mäkelä and Mäkelä (1966) on 12_2 variation, or whether some

chains on a single bacterium have many antigenic glucose units while others have none or only a few. It is not necessarily certain that the 12_2 immunodominant sugar is incorporated before polymerisation of the oligosaccharide and a precedent exists for later addition as at least some antigenic determinants are incorporated in group E Salmonellae after polymerisation has occurred. O-acetylation of galactose in group E_1 bacteria, which appears to occur on only about 50% of the residues, can take place on isolated polysaccharides and their derivatives (P.W. Robbins, Keller, Wright and Bernstein, 1965). At the moment it seems that efficient addition of glucose to form antigen 34, which normally appears to be complete, occurs only if the acetylating enzyme is suppressed (Robbins and Uchida, 1965), is likely therefore to occur at the same time as, or subsequent to this reaction. The close similarities between the chemical structures of antigens 12_2 and 34 makes an equivalent mechanism not unlikely.

Role of O antigens in virulence

While it is established that only smooth bacteria are virulent (see review by Roantree, 1967), the role of antibody against O antigens in combatting Salmonella infections is much less clear. As noted previously Felix (1924) claimed a

correlation between O-agglutinins and favorable prognosis in enteric fever, and Felix and Olitzki (1926) showed that bacteria heated at 100^{o} for 2 hours could absorb out bactericidal antibodies against S.typhi and S.enteritidis, again suggesting that O-antibodies were important. Thibault (1939) found that isolated O antigen was effective in absorbing out bactericidal antibody against Shigella. However a Boivin type of antigen was used, and as this contains protein and lipid as well as carbohydrate it is not clear what the bactericidal antibody reacted with. An acetic acid extract, presumably containing only polysaccharide, was not effective in inhibiting the bactericidal action, suggesting that at least part of the activity was directed against lipid or protein.

Rowley and Turner (1966) showed that antisera containing opsonins for intraperitoneal killing of S.adelaide in mice could be absorbed out by phenol-water extracted lipopolysaccharides containing O-antigen 35. In this case it seems likely that antibody against lipopolysaccharide and only that antibody was involved in opsonization of these bacteria.

Using specific antisera Biozzi, Stiffel, Le Minor et al, (1963) showed that specific sera against O-antigens 1, 4 and 5 were effective in opsonizing formalin-killed S.typhimurium organisms, and that a 9, 12 antiserum was also effective as a source of opsonins.

Thus it seems certain that antibodies against O-polysaccharide antigens are effective as opsonins for Salmonellae. However the evidence that they are involved in in vitro bactericidal reactions is less clear. In either case it is possible that antibodies directed against cell components other than lipopolysaccharide O-antigens can also be effective.

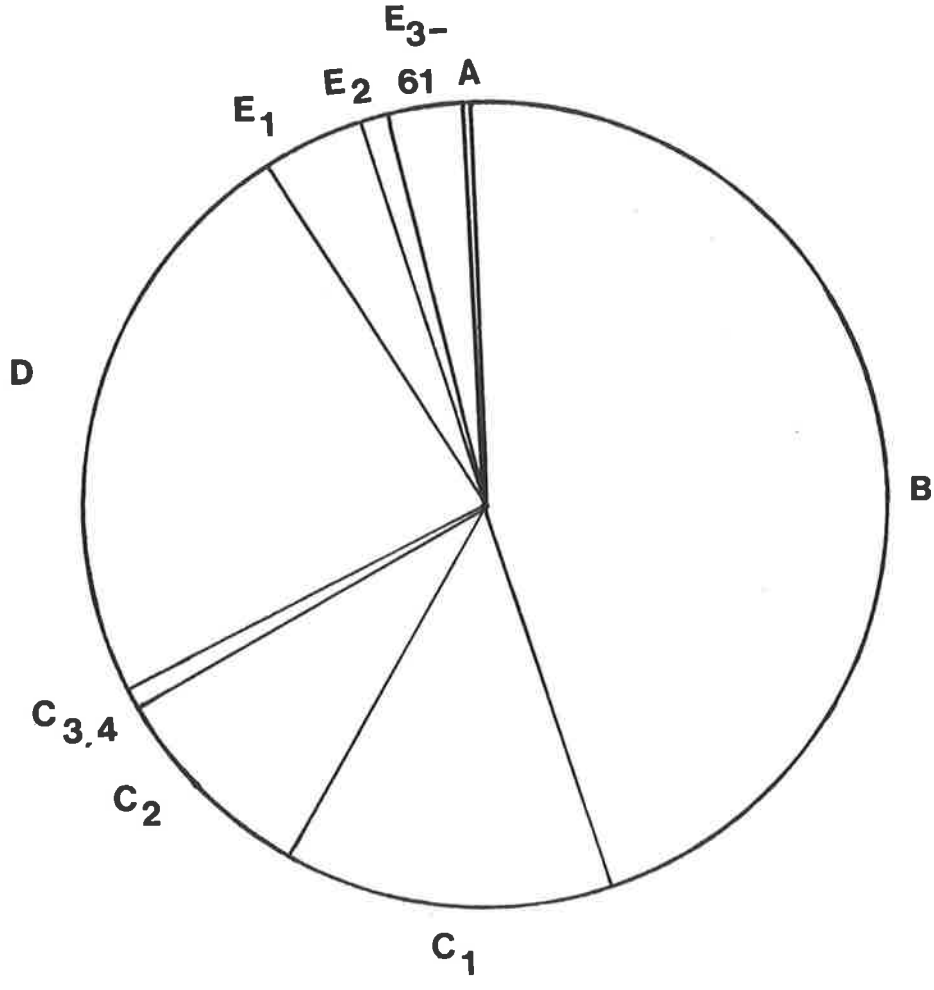
Recent studies on semi-rough forms show them to be phagocytosed either like rough forms (leaky rfa mutants) or in a manner intermediate between rough and smooth forms (semi-rough type C) (Nakano and Saito, 1968).

There appears to be some relationship between the structure of the smooth part of the lipopolysaccharide, virulence, and possibly host specificity. Most of the Salmonellae which are commonly isolated belong to only a few of the O antigen groups of Kauffmann. Fig 7, taken from Kelterborn (1967), shows the distribution amongst these groups of over 500,000 naturally occurring cultures from different parts of the world. It shows

Fig 7

Frequency of occurrence of the Kauffmann-White Salmonella groups

The diagram, from Kelterborn (1968), is constructed from records over 500,000 isolations in various parts of the world.


E3-
61
A
E2
E1
D
B
C3.4
C2
C1

that the 35 groups and subgroups from E_3 to 61 were represented in less than 4% of the total number of Salmonellae isolated, while more than 47% of them belonged to group B.

Although a few species, such as S.typhimurium, can infect very large numbers of different hosts, many of the more virulent species are more specific in their range (Wilson and Miles, 1964_b). Table 4 adapted largely from Wilson and Miles shows some better known species and their common hosts. Most of the organisms causing enteric fever in mammals belong to groups A, B, C and D. Group E Salmonellae are frequently isolated from humans and domestic stock but seem more prone to cause serious disease in birds than in mammals.

Kauffmann et al (1960) noted that most virulent Salmonellae belonged to higher chemotypes: groups C_2 and E were in chemotype XXII, B in XIV, A in XV and D in XVI (Table 2). These are characterised as containing the most complex lipophilic monosaccharide units, and Kauffmann et al suggested that virulence might be connected with the possession of a lipophilic coat. A notable exception to this rule is serotype C_1, including S.paratyphi C and S.cholerae suis, which is found in chemotype III.

TABLE 4

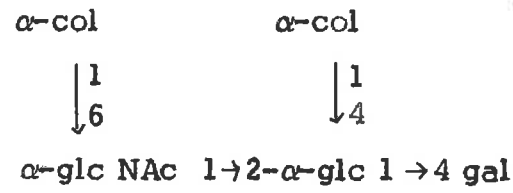
Selected Salmonella species and susceptible hosts[a]

Group	Species	Antigens	Hosts
A	S.paratyphi A	1,2,12	man
B	S.abortus equi	4,12	horse
	S.abortus ovis	4,12	sheep
	S.paratyphi B	1,4,5,12	man, (laboratory rodents)
	S.typhimurium	1,4,5,12	mouse, (frequent pathogen in many other animals)
C_1	S.cholerae suis	6,7	pig, man, rabbit, mouse
	S.paratyphi C	6,7,Vi	man, (mouse)
	S.typhi suis	6,7	pig
D	S.blegdam	9,12	man
	S.enteritidis	1,9,12	mouse, (man)
	S.dublin	1,9,12	cow
	S.gallinarum-pullorum	9,12	fowl
	S.moscow	9,12	man, (mouse)[b]
	S.sendai	1,9,12	man
	S.typhi	9,12,Vi	man,chimpanzee
E	S.anatum	3,10	poultry (intestinal disease)

(a) adapted from Wilson and Miles, 1964_b

(b) Melikova and Lesnjack, 1967

Medearis, Camitta and Heath (1968) studied the infection of mice with E.coli 0111 after they had been treated with hog gastric mucin. The O-polysaccharide of this organism is likely to be a polymer of the oligosaccharide



(Edstrom and Heath, 1967). A mutant which was unable to synthesize the colitose side branches had a very much increased LD_{50}. They concluded that virulence depended on the possession of branches on the smooth polysaccharide side chain. In terms of virulence this should mean that Salmonellae in groups E_1 and E_2, which lack such branches, should be avirulent in mammals. In this respect it is interesting that amongst the many records of isolation of group E Salmonellae in different parts of the world, and recorded in the book edited by Van Oye (1964), a smaller proportion of blood cultures of group E Salmonellae are recorded than there are of groups A, B and D. In particular, in a representative group from Senegal, Le Minor (1964) showed that 21% of group E Salmonellae isolated from humans were found in blood culture, as opposed to 49% of group B, 61% of group C,

95% of group D and 100% of group A. All of these figures are probably high because of the unlikelihood of minor Salmonella infections being diagnosed or identified. On the other hand group E Salmonellae are frequently found in the mesenteric lymph nodes of Senegalese pigs, and must therefore have at least some resistance to mammalian body fluids. This may simply be related to the rough polysaccharide (page 18), but it seems more likely that the smooth part also assists in preventing its removal by the host.

A variety of physiological effects have been attributed to preparations of O-antigen, which are frequently referred to as endotoxins (Wilson and Miles, 1964_c). Toxic activities can be found in lipopolysaccharides prepared by the phenol-water method of Westphal et al, and they are demonstrable in preparations with no smooth polysaccharide (Kim and Watson, 1967) and in preparations containing very little lipid (Ribi et al, 1961). It seems likely that toxic activity depends at least in part on the physical state of the preparations (Ribi et al, 1966).

Many of the symptoms of Salmonella infections are similar to those caused by endotoxin (Wilson and Miles, 1964_c), and it may be that endotoxin poisoning is the principle cause of death in this disease. However it has been shown that death occurs in

mice when the concentration of bacteria present is lower than the number of dead bacteria necessary to kill the animals (Meynell and Meynell, 1958), and it seems possible that the main toxic effects of Salmonellae are caused by other, heat-labile toxins (Mesrobeanu, Mesrobeanu and Mitrica, 1966, Kawakami and Mitsuhashi, 1965, Kawakami, Osawa and Mitsuhashi, 1966).

T antigens

Kauffmann (1956, 1957) discovered two sets of antigens on morphologically smooth Salmonellae which he called T antigens. The T2 antigen is still known only in a variant of S.bareilly, but the T1 antigen has been found in a number of different Salmonellae (Wheat, Berst, Ruschmann et al, 1967). At one time it was thought that the T antigens were intermediate in the conversion of rough forms to smooth. However Sarvas and Mäkelä (1965) succeeded in producing bacteria which had both T1 and O antigens. A gene controlling the synthesis of this antigen has been shown to map near rfc (rft 1 Fig 1) (Sarvas,1967). The T antigen is thought to be a polymer of ribose and galactose which is associated with the completed rough polysaccharide (Wheat et al, 1967). At least one strain of S.typhimurium which possesses the T1 antigen but lacks an O antigen is virulent in mice (Lüderitz, personal communication).

Little is known of the chemistry of the T2 antigen. It does not contain ribose (Wheat et al, 1967).

Fimbrial antigens

It is clear that other antigens exist on Salmonellae besides those used in the Kauffmann-White scheme. Of these the most important from the serologist's point of view are the fimbrial antigens (see Edwards and Ewing, 1962). Fimbriae are thread like processes which appear in cultures of some strains of Enterobacteriaceae under particular conditions. Two types have been distinguished on Salmonellae (Duguid, Anderson and Campbell, 1966), one of which causes attachment of bacteria to red blood cells of many vertebrate species and appears to assist growth in oxygen limiting conditions (Old, Corneil, Gibson et al, 1968). The ability to produce these structures is genetically determined (see Old et al, 1968).

In spite of the fact that they can cause attachment of bacteria to many different vertebrate cells there is no evidence that fimbriae act either as an aid or hindrance to infection (Duguid et al, 1966). Blaskett (1967) detected them by electron microscopy on preparations of the avirulent strain M206 of S.typhimurium but not on the virulent strain C5.

The occurrence of F factors in E.coli has been shown to be associated with the presence of small numbers of fimbriae which can be labelled specifically with F specific phages (Crawford and Gesteland, 1964, Brinton, Gemski and Carnahan,1964). It is likely that they possess a characteristic antigen (Ørskov and Ørskov, 1960). Although it is probable that similar fimbriae occur on Hfr strains of Salmonella, it seems most unlikely that they are directly associated with virulence.

Common antigen (CA)

One of the most studied antigens of the Salmonella-E.coli group is the common antigen (CA) of Kunin (Kunin, Beard and Halmagyi, 1962). Lipopolysaccharides or isolated lipid A appear to inhibit antibody production against CA when they are injected together (Suzuki, Whang, Gorzynski and Neter, 1964, Whang, Lüderitz, Westphal and Neter, 1965, Neter, Whang, Lüderitz and Westphal, 1966), so that it is not usually detected by antisera against normal bacteria. However when E.coli 0 14 is used as a vaccine, antibodies are produced which react with a large number of strains of E.coli, Salmonella, and other Enterobacteriaceae (Kunin et al, 1962).

Antibody against CA is ineffective as a bacterial agglutinin, although it can be shown by the use of fluorescein-labelled

antibodies that some CA is available on the bacterial surface (Aoki, Merkel and McCabe, 1966). The reasons for the ability of E.coli 0 14 to stimulate antibody production against CA are not clear, although CA appears to exist in two forms in this organism (Whang and Neter, 1965) and the fluorescence studies suggest that 0 14 reacts better with antibody against CA than do others. The inhibitory effect of lipid A on CA antibody production, and its apparent similarity with the situation using whole bacteria suggests that CA is usually associated with the deeper layers of lipopolysaccharide. CA can be separated from lipopolysaccharides in hot water extracts of bacteria by its solubility in 85% alcohol (Suzuki, Gorzynski and Neter, 1964). Little is known of its chemical nature.

It is likely that antibodies against CA are effective in promoting phagocytosis by rabbit polymorphs (Domingue and Neter, 1966).

Protein antigens (other than flagellin)

The success of the groups of Staub, Westphal and others in studying polysaccharide components of O-antigens has tended to obscure the importance of other antigens in O-agglutination (Barber, Vlădoianu and Dimache, 1966). Staub (1954) showed that rabbit

antisera against S.typhi contained a substantial proportion of agglutinins which were not directed against acid or alkali extracted polysaccharides. Barber and her colleagues have succeeded in extracting protein antigens from several species of Salmonella (Barber, Vlădoianu and Dimache, 1967, Barber, Eylan and Keydar, 1968). They have demonstrated that antisera against whole bacteria contain comparable amounts of antibody for both carbohydrate and protein antigens, and that antisera against carbohydrate-free proteins are still capable of causing O-agglutination (Barber et al. 1966). Unless particular care is taken, these proteins are likely to contaminate lipopolysaccharide preparations, even those prepared by the Westphal phenol-water method (Rowley, personal communication).

Kawakami et al. (1966) have extracted a toxic lipoprotein from S.enteritidis which is capable as a vaccine of giving some protection to mice challenged with 10 LD_{50}s of S.enteritidis. Collins and Mackaness (1968) obtained a protein antigen common to S.typhimurium and S.enteritidis against which a delayed hypersensitivity response occurs in the course of infection of mice by these organisms.

The relationship of these two proteins to other antigens is not known.

Cell wall proteins related to lipopolysaccharides have been studied in relation to their ability to act as colicins (Goebel and Barry, 1958, reviewed by Reeves, 1965), and some have been shown to be antigenic (Amano, Goebel and Miller-Smidth, 1958). Other workers have reported what may be additional antigens detectable in Salmonellae by in vitro techniques (eg Whiteside and Baker, 1962, Słuzewska, 1966, Engelbrecht, 1968). Most of these studies give little indication of the location of the antigens, their relationship to better studied antigens, whether they react with antibody while still associated with the bacteria, or whether antibodies against them have any significance in the appearance of immunity to those organisms which carry them.

Cell wall structure

Most research on bacterial cell walls has consisted of chemical and biochemical studies on extracted components (Salton, 1964, Lüderitz et al. 1968) and the way in which these fit together to form the cell wall is not yet well understood.

Three main techniques can be applied to the elucidation of the anatomy of the cell walls of Gram-negative bacteria. These are electron microscopic comparisons of cell walls before and after mild chemical treatments, and examination of the ability of

cell walls to react with highly specific biologically active molecules or viruses. In this last category come studies using antigen-antibody reactions. It is assumed that antibody can react only with antigen if the antigen is a surface component of the cell and is not blocked by some other substance. It is to be hoped that quantitative studies on the cell's ability to absorb antibodies will give an impression of how much of the total antigen is available on the cell wall surface.

A tentative cell wall structure deduced from studies with macromolecules is shown in table 5.

TABLE 5

Relative positions of antigens and lysozyme-sensitive components on the cell wall

outer surface	region exposed by EDTA and tris or after formation of protoplasts	inner region
Barber protein	mucopeptide	cell membrane
lipopolysaccharide	⟷ lipopolysaccharide	
Vi antigen	O-polysaccharide synthesis	
CA		

As bacteria can be agglutinated by antibodies against O or Vi antigens it seems reasonable that at least parts of both lie on the outer regions of bacteria. Vi antigen inhibits agglutination by O antibody (Felix and Pitt, 1934_a) and it seems likely that the Vi antigen overlies the O-antigen, or else associates with it so closely that antibody-combining sites directed against the relatively non-polar lipopolysaccharide antigens are repelled by its negative charge. The rapidity with which Vi antigen reacts with red cells and its ability to inhibit haemagglutination by anti-O antibodies (Spaun, 1952) suggest that it may have a considerable affinity for the highly branched polysaccharides of blood cells and Salmonellae.

O-agglutination by antibody against Barber's proteins (Barber et al, 1966) indicates that they are also on the outside of the bacterium. As antibody against Kunin's Common Antigen does not cause agglutination it seems likely that it is present on the surface only in very small amounts, or that it is situated in pockets on the surface such that a single attached antibody molecule is not capable of cross-linking with other bacteria. In this respect it should be noted that this antibody is still capable of opsonizing bacteria for reaction with rabbit polymorphs. It

seems likely, therefore, that the Salmonella surface normally exists as a mosaic of at least two chemically distinct antigens, one protein, the other carbohydrate, with which may be associated small amounts of a third antigen (CA), and which in some instances is overlayed by another polysaccharide (Vi antigen).

These conclusions are consistent with the electron micrographs of Shands (1965) who found by labelling O-antigens with ferritin-labelled antibodies that the antigen could be shown to exist in a wide layer of thickness about 800 Å around bacteria whose total diameter (excluding O-antigen) was only 5000 Å. These thicknesses are surprising. Murray, Steed and Elson (1965) showed that when smooth E.coli were centrifuged and resuspended they could approach to within 20-40 Å. De Petris (1967), using the rough E.coli B showed that the outer layers of the cell wall could lie against neighbouring cell wall without any intervening electron-transparent layer. It would seem from the results of these two groups, therefore, that smooth lipopolysaccharide protrudes from the surface of the cell wall, but that it has a thickness of about 10-20 Å. Even allowing for difficulties in interpretation of the thickness of structures shown by electron microscopy (see De Petris 1967) it seems that there is a large discrepancy between the

results of Shands and those of De Petris and Murray et al.

Shands' studies (1966) showed that although O-polysaccharide was normally demonstrable only on the outside of bacteria, when penicillin spheroplasts were subjected to osmotic lysis there was evidence for the presence of lipopolysaccharide in inner parts of the cell. Treatment of E.coli 0111:B4 cells with EDTA in tris buffer leads to the rapid release of about 50% of their lipopoly-saccharide, together with a certain amount of protein, phosphorus and other substances (Leive, 1965). The fact that about 50% remains on the bacteria suggests that it exists in two forms, only one of which is held in place by metal ions, and Levy and Leive (1968) have shown that two metabolically different populations do in fact appear to exist in the cell wall of a mutant of E.coli 0111:B4. Voss (1967) has shown that the action of EDTA in sensitizing bacteria to the bactericidal action of negatively charged surfactants depends on previous treatments with tris or other aliphatic amines, and it is likely that EDTA is capable of removing lipopolysaccharide from bacteria only after similar treatment (Jackson, personal communication). The combination of EDTA and tris seems to expose cell wall components which were not previously available to biologically active molecules. These include the rigid mucopeptide layer, which becomes susceptible to lysozyme

(Repaske, 1958) and the synthesizing system for O-specific polysaccharide (Kent and Osborn, 1968_c).

De Petris (1967) has shown that the mucopeptide layer of E.coli B can become sensitive to lysozyme after a short period of heating. Feingold, Goldman and Kuritz (1968) suggest that the mucopeptide layer is available even in normal cells but is not permanently damaged except when the cell wall is damaged by other treatments. Their evidence depends on the observation that cells in a system in which they were normally resistant to the bactericidal action of antibody and complement could be made susceptible by pretreatment with lysozyme. They offered no evidence of how much lysozyme attached to the cells had resisted washing and their results are directly contradicted by the careful studies of Glynn (1969); but it remains possible that although lysozyme attacks the cell walls of any Gram negative bacterium, its effects are not normally noticed because other components hold the broken pieces of mucopeptide in place until they can be repaired.

Chemical studies of isolated cell walls have shown that as well as lipopolysaccharide they contain large amounts of lipid (especially phosphatidyl ethanolamine) (Wardlaw, 1963), phenol-soluble protein (the lipoprotein of Martin and Frank, 1962), and a

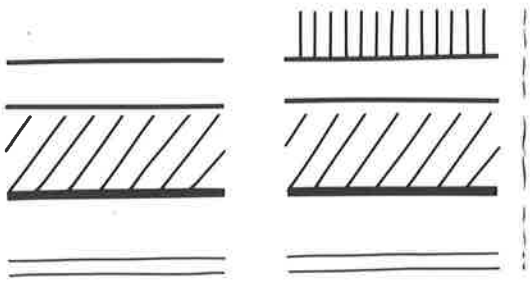
thin rigid mucopeptide layer, to which, in phenol extracted cell walls, are attached spheres of protein which give it a chain mail appearance. (Weidel, Frank and Martin, 1960). The convoluted surface observed by Bayer and Anderson (1965) and the possibility of complex interconnections between the various components make the interpretation of electron micrographs of normal cell walls difficult (De Petris, 1967). However exposure to some phages (Kellenberger and Ryter, 1958, De Petris, 1965) or to heat (De Petris, 1967) seems to cause some simplification of structure which allows electron micrographs to be interpreted in terms of layers of different components. De Petris (1967) (Fig 8) has shown that the cell wall of E.coli B consists of a flexible convoluted triple-layered outer membrane (the L layer) which shortens on heating and disappears on treatment with phenol, a process which removes lipopolysaccharide and some protein but does not affect the mucopeptide layer. Within this, and probably separated from it by protein, is a mucopeptide layer which seems to give the bacterium its shape (Murray et al, 1965), and is not normally convoluted, although it may appear slightly wavy (De Petris, 1967).

It seems likely that an assortment of degradative enzymes and also, possibly, some cell membrane transferases, are

Fig 8

Proposed structure for the cell wall of Gram-negative bacteria

from De Petris (1967)



ROUGH SMOOTH

S polysaccharide
L layer (LPS, lipid, protein)

G layer g_1 (protein)
g_2 (mucopeptide)
M layer (periplasm?)
cell membrane

associated with the cell wall (reviewed by Heppel, 1967). Some of these, including alkaline phosphatase, can be removed without prior treatment, but many are thought to be associated with a compartment known as the periplasm (Mitchell, 1961), and can be released only after insults such as treatment with EDTA and tris followed by either osmotic shock or warming to 48° (Heppel, 1967, Rogers, 1968). It seems likely that the periplasm corresponds to the electron-light gap named the M-layer by De Petris (Fig 8). The functions of the periplasm enzymes are not known, although it seems likely that one of them is to degrade certain metabolites to a state in which they can be absorbed (Mitchell, 1961). Loss of the enzymes is not fatal to bacteria in nutrient media, and they do not have to be completely replaced before multiplication can occur. (Heppel, 1967). The relationship between cell wall antigens and enzymes is not known. It would be interesting to find out, for example, if Barber antigens had any alkaline phosphatase activity or were involved in lipopolysaccharide synthesis, as seems likely from the recent note by Levy and Leive (1969).

CHAPTER 2

MECHANISMS OF IMMUNITY IN EXPERIMENTAL TYPHOID INFECTIONS

Introduction

The host specificity of Salmonella typhi makes it unsuitable for experiments on immunity to typhoid. Consequently most laboratory studies on typhoid have concentrated on similar diseases caused by related organisms in other hosts. Of these, one of the most suitable is the laboratory mouse. This animal has long been known to be susceptible to relatively small doses of a number of strains of S.typhimurium, S.enteritidis, S.paratyphi C and S.cholerae suis, and to a lesser extent to S.paratyphi B (Wilson and Miles, 1964_b). Recently it has also been reported to be highly susceptible to S.moscow, an organism which is pathogenic in man (Melikova and Lesnjack, 1967).

For a number of years Rowley and his colleagues have studied the immune response in mice to two strains of S.typhimurium, the virulent strain C5 and the avirulent M206. This work has been reviewed by Jenkin (1963_a), Jenkin and Rowley (1963) and Rowley (1966). This thesis describes a continuation of these studies, and where relevant, they will be referred to in the following sections.

The course of Salmonella infections in mice

The course of Salmonella infections in mice can be studied in much greater detail than in man. Where they can be compared the diseases appear to follow similar lines. Ørskov, Jensen and Kobayashi (1928) made a detailed examination of the course of the disease following oral infection, and stressed the importance of the phagocytic cells. Ingested bacteria had to compete with the natural flora of the mouse intestine and most of them died or were lost in the faeces. A few penetrated the intestine and escaped to the mesenteric lymph nodes, where they lived and multiplied. From there they again escaped, causing a transient bacteraemia, and were phagocytosed by cells in other parts of the body, notably the liver and spleen. Here multiplication continued. Bacteria in the liver infected the gall-bladder and from there poured back into the intestine, reaching the outside world in faeces. At the time of death nearly all of the organs contained bacteria and a bacteraemia had again set in. Mice which recovered often became carriers.

Infection by the intravenous route followed a similar pattern to the oral infection, but in this case commenced at the stage of the first transient bacteraemia following mesenteric lymph node infection.

It appears that when a dose of virulent Salmonellae near the LD_{50} is injected into a mouse only a few bacteria succeed in establishing infective loci (Meynell and Stocker, 1957). The others presumably are destroyed by specific or non-specific immune systems. These few bacteria grow much more slowly than would be expected of normal organisms in nutrient media (Maw and Meynell, 1968), but eventually they reach a stage where they are continually pouring bacteria into the blood. It is likely that most of the blood-borne bacteria at this time are destroyed (Mackaness, Blanden and Collins, 1966), at least until very near the time of death, but there appears to be a constant replenishment of the population from the foci from single organisms. When the total number of bacteria reaches a certain level, the host dies (Meynell and Meynell, 1958).

Immune mechanisms

It is obviously important that the growth of infecting organisms, which appears to be intracellular, is much slower than expected. Coupled with this are a number of mechanisms which appear to be significant in bringing about the actual destruction of the organism. The student of immunology rapidly becomes aware of the diversity of immune responses (reviewed by Humphrey and White, 1964 etc).

Several chemically distinguishable classes or sub-classes of human immunoglobulin are recognised, and studies in such phenomena as complement fixation suggest that there may be more. In addition there exists the phenomenon of delayed type hypersensitivity in which it has not been possible to prove the participation of any antibody. The injection of bacteria can give rise to non-specific reactions which assist the animal in destroying bacteria, but which make difficult attempts to assess specific immunity (Rowley, 1956, Mackaness, 1964). It seems very likely that any single infection is combated in a number of different ways. In these circumstances it requires considerable luck to be able to guess accurately one form of immunity which would be much superior to any other, and it is not surprising that there is some controversy over the mechanism of resistance to Salmonellae in mice. One reasonable approach is to study each identifiable aspect of resistance in detail, in the hope that eventually it will be possible to correlate findings in all of these aspects with studies on the course of the disease in whole organisms.

Two of the most commonly studied of the defence mechanisms against bacteria are phagocytosis and the serum bactericidal reaction. It seems that they may have certain features in common.

The bactericidal action of serum

Studies on C5 and M206 have shown that they differ markedly in their behaviour in both phagocytic and bactericidal systems. In a normal mammalian serum without added complement M206 is killed quite effectively. In contrast C5 is completely resistant to such a system and multiplies (Schwab and Reeves, 1966). It seems either that natural antibodies exist which react with M206 but not C5 or that C5 is for some reason resistant to antibody after it is fixed, whereas M206 is susceptible.

Bactericidal properties of serum have been studied since the late 19th century (eg Buchner, 1889), and it is known that for the killing of Gram negative bacteria all nine components of haemolytic complement are required (Inoue, Yonemasu, Takamizawa and Amano, 1968). Antibody and complement appear to have two actions on susceptible bacteria. In serum devoid of lysozyme they are capable of killing without causing drastic changes in shape of the bacteria (Muschel, Carey and Baron, 1959).

However when lysozyme is present in the serum lysis of bacteria occurs, and the killing may be more rapid (Amano, Inai, Seki et al, 1954). This has been shown in a number of varieties of Vibrio, E.coli and Salmonella (Amano et al, 1954, Muschel et al, 1959). It is probable, therefore, that complement is able to damage: outer parts of the cell wall, allowing attack on the mucopeptide layer by lysozyme, and that subsequently, in the absence of lysozyme, it is able to attack inner parts of the cell, probably the cell membrane (Muschel, 1965). Muschel and Jackson (1966) have shown that complement is bactericidal when it reacts with either spheroplasts of Gram negative bacteria or protoplasts of Gram positive organisms.

Complement has been shown to make what appear to be holes in the walls of sensitized red cells (Humphrey and Dourmashkin, 1965). Similar lesions have been shown to occur in the cell walls of E.coli treated with complement (Bladen, Evans and Mergenhagen, 1966) and in spherules of rough lipopolysaccharide isolated from mutants of S.minnesota (Mergenhagen, Gewurz, Bladen et al, 1968). It seems likely from these experiments that rough parts of lipopolysaccharides contain areas which can act as substrate for complement. The most likely candidate for a related structure in

the cell wall is the L-layer of De Petris (Fig 8), which probably includes parts of rough polysaccharide and lipid A. On this theory, it is postulated that the first effect of antibody and complement is the production of lesions in the L-layer allowing effective access of both complement and lysozyme to inner parts of bacteria.

Michael and Braun (1959) showed that bacteria were partly protected from complement by high concentrations of sucrose, unless they had been grown in penicillin, and Peingold, et al, have shown that in the absence of lysozyme, antibody and complement are ineffective in killing plasmolysed bacteria. They suggest that killing normally involves osmotic losses through the cell membrane after deleterious effects of complement. As most of the membrane has drawn back from the cell wall in plasmolysed bacteria, and as those pieces of membrane remaining are likely to be different from the rest (Bayer, 1968$_{a,b}$) it is also possible that in this system complement is fixed too far from its site of action to be effective.

The reasons for the sensitivity of some strains of Gram negative bacteria to complement mediated bactericidal reactions and the resistance of others are not understood. Michael and

Braun (1958) found an inverse relationship between penicillin and serum sensitivity in some strains of Shigella dysenteriae and E.coli, while Nelson and Roantree (1967) found that penicillin resistant mutants of a strain of S.typhimurium became susceptible to very high concentrations of antiserum. However it has been found that at least one of Nelson and Roantree's strains, P 173c, cannot be killed by concentrations of immune serum which are bactericidal for other sensitive strains in the presence of excess complement (Rowley, personal communication), so that the mechanism of killing may be abnormal. It has been shown that rough strains are all susceptible to the bactericidal action of serum (Rowley, 1956) and that conversion of a rough strain of S.typhimurium lacking UDP-galactose-4-epimerase to its smooth counterpart by provision of galactose in the medium results in its losing its sensitivity to serum (Dlabač, personal communication).

Rowley (1968) showed that antibodies bactericidal to a number of rough strains of S.minnesota can be raised by injection of a smooth serum-resistant strain. However the smooth strain was unable to absorb out the bactericidal antibody although this could be readily removed by the rough organisms. It seems likely, therefore, that smooth lipopolysaccharide covers certain antigens

common to smooth and rough organisms, and that if antibody and complement succeed in reacting with these antigens the bacteriae are killed.

Recently it has been demonstrated that bacteria previously thought to be resistant to the action of antibody and complement can be made susceptible by preincubation in a number of substances including tris and ethanolamine (Reynolds and Rowley, 1969). The mechanisms by which these substances act on cell walls is not clear, but it seems likely that they remove some components other than lipopolysaccharide from whole bacteria (Jackson, personal communication). The phenomenon may be analagous to the one studied by Chedid, Parant, Parant and Boyer, (1968), who found that normal mouse serum was able to render a previously resistant strain of Klebsiella sensitive to the action of antiserum against smooth Klebsiella pneumoniae or rough Salmonella.

Many smooth strains of Salmonella, including M206, are known to be susceptible to the bactericidal action of serum. The problem remains of how they differ from smooth resistant strains.

Phagocytosis

It is tempting to consider the bactericidal action of serum to be of great importance in resistance to disease. However, while it remains an interesting model for other bactericidal reactions, it is difficult to believe that it is of much importance in normal or immune resistance to Salmonella infections. Although by the use of suitable vaccines it is possible to protect some animals from Salmonellae it is often not possible to raise antibodies in these animals which are effective in the in vitro bactericidal reaction. In spite of the fact that all of the complement factors are required for bactericidal reactions laboratory mice lacking C^{5} seem no more susceptible than normal animals to bacterial infections under normal conditions (Caren and Rosenberg, 1966), although these authors were able to show a decrease in resistance to Corynebacterium infection. A comparison of the kinetics of the serum bactericidal reactions with those of phagocytosis and intracellular killing provides some explanation for its apparent lack of importance. In some circumstances the complement mediated bactericidal reaction can be very fast, causing more than 90% kill within 30 minutes. However it is frequently slow, and with some organisms may require up to 6 hours for equivalent killing (Šterzl, Pešák, Kostka and Jílek, 1964). In contrast, clearance of bacteria injected intra-

venously or intraperitoneally in the immunized animal is very rapid. Bacteria are associated with phagocytic cells within a few minutes and are thereafter, presumably, protected from serum (Benacerraf, Sebestyen and Schlossman, 1959, Whitby and Rowley, 1959). The important factor then becomes the ability of cells to phagocytose and kill bacteria.

One method of estimating rates of phagocytosis in mice is by measuring the rate at which a particle or organism disappears from the animal's blood after intravenous injection (Benacerraf et al, 1959). A phagocytic index has been defined which is a measure of the rate of removal of the organism. When C5 and M206 are compared in this way in a normal mouse it is found that M206 is rapidly cleared with a phagocytic index of about 0.2, but C5 is cleared only very slowly (phagocytic index 0.02) (Jenkin, 1962). If serum from certain other normal animals (eg pig) or serum from a mouse which has already been immunised with either M206 or C5 is mixed with C5 before it is injected, the bacterium can be cleared as rapidly as M206 (Rowley and Jenkin, 1962, Jenkin, 1962). This clearance promoting or opsonic factor for C5 can be reduced or removed from pig serum by absorption with C5, M206 or normal mouse cells (Jenkin, 1962), and it seems reasonable to

assume that there is an antibody directed against a component of the cell wall of C5 which cross reacts with both mouse cells and M206. M206 is cleared either because another antigen is available which is not available on C5 or because less antibody is required for the clearance of M206 than of C5.

Spiegelberg, Miescher and Benacerraf (1963) have shown that as well as antibody, mice require at least some of the factors of complement to assist in phagocytosis of bacteria. The requirement is probably limited to the first four factors. They allowed their bacteria to react with mouse complement in the cold, a process which inhibits the reaction of C'3 and C'5 in guineapig sera (Linscott and Nishioka, 1963). Furthermore it has been shown that mice which are deficient in C'5 are able to clear bacteria quite effectively (Stiffel, Biozzi, Mouton et al, 1964). It was suggested that one possible difference between C5 and M206 might therefore be an inability of antibody molecules on C5 to fix one of the first four complement factors.

Phagocytosis can also be studied after injection of bacteria into the mouse peritoneum and examination of fluid washed from it over the next 90 minutes. Here again M206 rapidly disappears and is presumably killed, while in the normal mouse C5 suffers at

worst a 50% drop in numbers and then begins to multiply. If C5 is treated with serum from an immunised animal it is rapidly phagocytosed and a large proportion of the bacteria are killed (Whitby and Rowley, 1959).

Phagocytosis has been studied in vitro using cells washed from the mouse peritoneum, which include a high proportion of macrophages. In this system once again, normal cells are able to phagocytose and kill M206, though with less efficiency than in the peritoneum, while C5 is not phagocytosed unless it is first treated with antiserum, or serum from animals other than mice (Whitby and Rowley, 1959). Cells from mice which have been infected with M206 two weeks previously are able to kill C5 without addition of antiserum. Rowley, Turner and Jenkin (1964) showed that this is due at least in part to the presence of a mercaptoethanol labile 19s molecule attached to the cells which they presumed to be IgM antibody or something very similar. Ushiba, Nakae, Akiyama and Kishimoto (1966) later extracted a similar substance from mouse cells immunised with a heat-killed smooth S.enteritidis vaccine but found its Svedberg constant to be 12.5s rather than 19s. Because it was not present on cells of mice immunised with a rough strain they considered it to be

antibody against O-polysaccharide.

Use of the in vitro phagocytic system has permitted the study of two separate components of phagocytosis - the association of bacteria with phagocytic cells and their subsequent intracellular killing. Jenkin (1963_b), using bacteria which were opsonised by antibody for a phage they were carrying, showed that even M206 was not killed when it was phagocytosed by normal cells in M206 - absorbed serum. It seems likely, therefore, that antibody is required for the intracellular killing of Salmonellae. This antibody has to be attached at a specific part of the bacterium, and is perhaps too far from sensitive parts of the cell when it is attached to phage P22. It is probable that antibody and complement are required for phagocytosis both in vivo and in vitro (Spiegelberg et al, 1963, Gigli and Nelson, 1968) and the requirement of specifically-placed antibody for intracellular killing implies that there is at least a strong possibility that complement is also required in this system. The fact that many bacteria which are readily phagocytosed and killed are also readily destroyed by the in vitro bactericidal system using either normal serum or diluted immune serum and complement suggests that there may also be some relationship between cellular and serum killing. It is interesting in this respect that certain enzymes

present in some phagocytic cells, including lysozyme, are able to enhance the serum bactericidal activity so that it occurs faster, is more quantitative, or causes more drastic dissolution of the bacteria (Amano et al, 1954, Amano, Seki, Kashiba et al, 1955).

The quality of the antibody response to Salmonellae

Differences in the properties of antibodies against different antigens have been recognised for a very long time. At present a large number of immunoglobulin types are known which are presumably antibodies and are chemically distinct in regions well separated from their combining sites (reviewed by Cohen and Milstein, 1967). It seems likely that the list is not yet complete. The biological functions of all of these different immunoglobulins is not understood. They have probably evolved in response to a variety of parasites and it may be predicted that their relative efficiencies in combatting different infections will vary. Some may be redundant, the vestiges of defences against long-extinct diseases. On the other hand the most successful must go unnoticed, as the 'parasites' against which they are directed will never be able to establish themselves. It is likely also that immunoglobulins are involved in other functions such as the removal of unwanted natural products of the body (see review by Boyden, 1963) or control of the immune response (reviewed by Uhr and Moller, 1968).

While there is general agreement that the various classes have different biological properties the available information on what these properties are is incomplete and sometimes contradictory. Biological properties of antibodies must depend to a very large extent on the affinity constant for the reaction between the combining site and the antigenic determinant. This has been shown to increase in the course of an immune response (Eisen and Siskind, 1964), so that the average properties of individual immunoglobulin classes may be expected to vary over this period.

Little can be said of IgA, one of the major immunoglobulin classes. It fixes some components of complement and can take part in bactericidal reactions (Adinolfi, Glynn, Lindsay and Milne, 1966). Most work appears to have concentrated on its secretory functions, but as secreted IgA appears to be made and secreted in situ as a dimer (Lawton and Mage, 1969), and serum frequently contains high concentrations of the monomeric immunoglobulin (Tomasi, Tan, Solomon and Prendergast, 1965) it is not unlikely that it has other functions. The two major antibodies which have been measured as directed against Salmonella O-antigens are IgG and IgM. Of these IgM appears to be the major antibody produced

under relatively normal conditions (Deutsch, Alberty and Gosling, 1946, LoSpalluto, Miller, Dorward and Fink, 1962, Pike and Schulze, 1964, Landy, Sanderson and Jackson, 1965). However IgG is produced in rabbits after prolonged or intensive stimulation (Weidanz, Jackson and Landy, 1964, J.B. Robbins, Kenny and Suter, 1965). It seems likely that most work on these two classes has been carried out on mixtures of subclasses which may vary in their properties. For example one class of mouse 7s (IgG?) antibody and possibly one of IgM either do not fix complement or fail to sensitize red cells for complement mediated lysis (Nussenzweig, Merryman and Benacerraf, 1964, Plotz, Talal and Asofsky, 1968, Plotz, Colten and Talal, 1968). Hence measurements of various biological properties are likely to underestimate the capacity of different classes either because the amount of active immunoglobulin is overestimated or because of actual competition for antigenic sites with inactive molecules.

IgG antibodies have been reported to have higher affinity constants than IgM when measured against isolated haptens (Onoue, Grossberg, Yagi and Pressman, 1968) but the rule does not necessarily hold for the reactions of whole molecules with complex antigens. Although J.B. Robbins et al. (1965)

reported that IgG was more readily absorbed than IgM from a mixed antiserum against Salmonella O-antigens, Greenbury, Moore and Nunn (1963) found that iodine-labelled rabbit IgM antibodies directed against human blood group A erythrocytes attached themselves more avidly than IgG. It seems likely that the avidity of an antibody is increased if it can react with more than one antigenic site. Greenbury, Moore and Nunn (1965) showed that monovalent fragments of rabbit IgG antibody had lower avidity than divalent or complete antibody molecules.

Several authors have shown IgM to be a more efficient agglutinin and haemagglutinin than IgG. Estimates of its superiority range from 50 to 1000 fold when measured in molecular terms (eg Greenbury et al 1963, Michael and Rosen, 1963, J.B. Robbins et al, 1965).

It is clear that two molecules of IgG but only one of IgM are required to fix one molecule of C'1 (Borsos and Rapp, 1965_a). One would expect IgM to be more efficient than IgG in complement-dependent reactions, and this in general appears to be the case. Mole for mole it is reported to be more efficient in promoting haemolysis (Stelos and Talmage, 1957), bactericidal reactions (Michael and Rosen, 1963, J.B. Robbins et al, 1965) and

phagocytosis (Rowley and Turner, 1966). The situation however is not a simple one. Möller (1966) published haemagglutinating, haemolytic and cytotoxic titres for 7s and 19s isoantibodies produced in several strains of mice against a particular strain of mouse red cell. It appeared that most strains of mice produced 19s antibody which, when related to haemagglutinating titre, was much better in haemolytic and cytotoxic activity than 7s. One strain, however, produced 7s and 19s antibodies which appeared to be equivalent in these activities. It is difficult to draw direct conclusions from the data because of absence of absolute measurements of the amounts of antibody used. However the experiment illustrates the difficulty of establishing exact quantitative relationships between the properties of the various immunoglobulin types. It seems likely that they depend on a number of factors as yet not properly understood.

The importance of labile antigens in immunity to Salmonellae

Several groups of workers have shown that live vaccines are superior to killed in stimulating immunity to Salmonellae (Kobayashi and Ushiba, 1952, Ushiba et al, 1959, Hobson, 1957, Mitsuhashi, Kawakami, Yamaguchi and Nagai, 1958). In most cases the immunising strain has apparently had similar antigens to the

challenge strain. Mitsuhashi and his colleagues, for example, immunized mice with strains of S.enteritidis which appear to have had the characteristics of semi-rough organisms (Mitsuhashi et al, 1958, Mitsuhashi, Harada and Kawakami, 1959) and live M206 is an effective immunizing strain for C5 (Jenkin, Rowley and Auzins, 1964). Sometimes, however, the immunizing strain does not apparently have the same O-antigens. S.enteritidis 11RX, a rough strain, is effective in provoking resistance to smooth Salmonellae (Ushiba et al, 1959, 1966, Rowley, Auzins and Jenkin, 1968), and it has even been shown that immunization with Listeria monocytogenes a Gram-positive intracellular parasite, can increase resistance to virulent Salmonellae (Blanden, Mackaness and Collins, 1966). It seems certain that the non-specific increase in resistance is due at least in part to an increase in the proportion of phagocytic cells which are capable of killing opsonized bacteria (Blanden et al, 1966, McIntyre, Rowley and Jenkin, 1967) and it has been suggested that in some instances resistance is increased by an adjuvant effect of the immunizing strain, which allows a rapid development of specific immunity immediately after challenge (Rowley et al, 1968). The findings of the various World Health Organisation field trials, with their evidence of the efficacy of gently killed vaccines under natural conditions suggest that there may be other important labile

antigens to be found on S.typhi (Benenson, 1964). A similar situation exists in studies of immunity to S.typhimurium. Springut in 1928 reported that immunisation of mice with unheated bacteria gave better protection against a subsequent S.typhimurium infection than did immunisation with heat-killed organisms. Rowley and his colleagues have more recently stressed the importance of heat-labile antigens in immunity to C5. Auzins and Rowley (1963) found evidence for a heat-labile antigen important in phagocytosis by examining the association of bacteria with mouse peritoneal cells. Bacteria were mixed with cells, incubated for up to 50 minutes and filtered through filter paper. Free bacteria passed through the filter while cells and their associated bacteria remained behind. It was found that normal cells reacted with M206 but not C5. However if a culture of C5 was heated at 100° for 1 hour the organisms became able to associate with macrophages. Streptomycin-killed C5 did not react and it was suggested that C5 behaved in this way because an antigen common to C5 and M206 was blocked by a heat-labile factor. It seemed reasonable that if antibody could be raised against this factor C5 would be phagocytosed. Jenkin and Rowley (1965) made vaccines from S.typhimurium or Citrobacter 396 (0:4,5) by a method which used

sodium dodecyl sulphate to extract the antigen and avoided the use of heat. This vaccine was fairly successful in protecting mice from infection by C5 as measured by reduced mortality after 28 days. Because live M206 was an effective vaccine for protection of mice against C5 it was suggested that both C5 and M206 had this protective antigen but that it was not present on M206 in sufficient quantity to protect it from antibody against other antigens.

The most obvious of the heat-labile antigens in S.typhimurium is antigen 5. As this was thought to have as its immunodominant sugar O-acetyl galactose (Kotelko et al, 1961),Jenkin, Karnovsky and Rowley (1967) carried out the experiment of acetylating a naturally occurring plant galactan and using it as a vaccine against S.typhimurium in mice. They found that it gave some protection against this strain, but none against S.enteritidis, which lacks antigen 5. Animals immunized with this antigen were able to destroy virulent S.typhimurium injected intraperitoneally and it was further shown that the antigen was capable of inhibiting such killing in mice immunized with S.typhimurium. It was therefore suggested that antibody against antigen 5 might be particularly important in the development of immunity to S.typhimurium. This hypothesis has two main implications.

First it suggests that antibody against antigen 5 is more effective than other antibodies in promoting phagocytosis and killing. Secondly it requires a difference between the state of antigen 5 relative to the other lipopolysaccharide antigens and also in the state of 5 in M206 from that in C5. The most likely difference between the two organisms was that C5 would have very much more antigen 5 than M206. The work reported in this thesis was undertaken in the hope that it would show whether these postulates were reasonable.

CHAPTER 3

MATERIALS AND METHODS

Bacterial strains

Unless otherwise stated bacterial strains were obtained from the collections of Professor D. Rowley and Dr. Nancy Atkinson of the University of Adelaide. Other workers who generously provided strains are listed below. The following organisms were used:-

Salmonella abony SW1444 (0:1, 4, 5, 12), an avirulent Hfr strain provided by Dr. V. Krishnapillai of the Department of Genetics, Monash University, Victoria,

S.abortus equi (0:4, 12),

S.adelaide (0:35),

S.berta (0:9, $12_{1,3}$),

S.bredeney (0:1, 4_1, 5, 12, 27_B),

S.derby (0:1, 4, 12),

S.enteritidis D10962 and D18956 (0:1, 9, 12), two strains originally isolated from human material, provided by Dr. K. Fahey, Department of Bacteriology, Melbourne University,

S.enteritidis (melbourne) (0:1, 9, 12), a strain, virulent in mice which was also provided by Dr. Fahey,

S.enteritidis 795 av (0:1, 9, 12), an avirulent mutant of a strain obtained from Dr. E. Ribi, Rocky Mountain Laboratory, Montana,

S.enteritidis 11RX (rough), an avirulent strain obtained from Dr. D. Ushiba, Keio Gijuku University, Tokyo,

S.minnesota 218 s (0:21, 26), a smooth serum-resistant strain obtained from Dr. O. Lüderitz, Max Planck Institut fur Immunbiologie, Freiburg,

S.reading (0:4, $12_{1,2}$),

S.typhi 0 901 (0:9, $12_{1,2,3}$) obtained from Mrs. H.M. Macdonald, Salmonella Reference Laboratory, Institute of Medical and Veterinary Science, Adelaide,

S.typhimurium C5 (0:1, 4, 5, 12) a virulent strain (Furness and Rowley, 1956),

S.typhimurium M206 (0:1, 4, 5, 12) an avirulent strain originally described by Jensen (1929),

S.typhimurium 11 (0:1, 4, 12), an avirulent strain lacking antigen 5 obtained from Dr. F. Kauffmann of the Staatens Seruminstitut, Copenhagen,

S.typhimurium P 173 c (0:1, 4, 5, 12) a virulent, serum-sensitive, penicillin-resistant strain derived from C5 (Nelson and Roantree 1967), obtained from Dr. R.J. Roantree, Department of Medical Microbiology, Stanford University, California,

S.typhimurium B11199 and B12347 (0:1, 4, 5, 12), two strains isolated from human material, provided by Dr. Fahey,

C5S (0:1, 4, 5, 12) a partially avirulent recombinant from a genetic cross between S.abony Hfr SW 1444 and a his-mutant of S.typhimurium C5. This strain contains the avir 1 gene of Krishnapillai and Baron (1964) and was described by these authors as C5 His^+ str r.

C5R (0:1, 4, 5, 12) an avirulent recombinant from S.abony Hfr SW 1444 and C5 (Krishnapillai and Karthigasu, 1969) containing both the avir 1 and avir 2 genes. Both the above strains were obtained from Dr. Krishnapillai of the Department of Genetics, Monash University, Victoria,

Citrobacter 396 (0:4, 5) is a strain which originated from Dr. Kauffmann's laboratory.

Culture and treatment of strains

Liquid media

Bacteria were generally grown in Difco nutrient broth at 37° overnight with shaking. Broth cultures (10 ml.) were shaken in 20 ml. screw-topped bottles.

Solid media

Bacteria were grown on 20 ml. Difco blood base agar in plastic $3\frac{1}{2}$" Petri dishes. When larger quantities of bacteria were required they were grown on 1 litre Roux bottles containing 100 ml. nutrient agar and plugged with cotton wool. Each bottle was seeded with 5 ml. overnight broth culture and incubated at 37° for 36 hours.

Preservation of strains

When a strain was received it was seeded into broth. The overnight culture was plated on nutrient agar and after a further 24 hours a colony of suitable morphology was selected and used to seed a second 10 ml. overnight culture. This culture was centrifuged on an MSE bench centrifuge for 30 minutes at 4,000 rev./min. and the supernatant decanted. To the residue was added 1 ml. of milk which had previously been centrifuged to remove cream and autoclaved. The bacteria were mixed with the sterile milk using a sterile Pasteur pipette and 2 drops were added to each of 6 sterile 4 x 1/4" tubes plugged with cotton wool. Cultures were freeze dried in a Speedivac Centrifugal freeze drier according to the manufacturer's instructions. Essentially the tubes were centrifuged while the pressure was reduced to about 200μ and held at that pressure over phosphorous pentoxide for

6 hours. When most of the moisture had been removed pressure was released, the cotton wool pushed about three-quarters of the way into the tube and a constriction was made about half way down the tube. Tubes were evacuated to a pressure of 30μ or less and held at that pressure over dry phosphorous pentoxide for 16 hours. They were then sealed at the constriction without releasing the vacuum.

Freeze dried cultures were stored at room temperature.

When a new freeze dried culture was used, a tube was broken at the level of the cotton wool, several drops of broth from a sterile Pasteur pipette were added, and these were used to seed an overnight broth culture. A sample was plated to check for purity and a suitable colony was used to seed a second 6 hour broth culture. About 0.1 ml. of this culture was dripped from a sterile Pasteur pipette on to a slope of 10 ml. nutrient agar in a 20 ml. screw-topped bottle. Ten slopes were set up. When a slope had been used 10 times it was discarded.

Animals

Mice used were a strain of Swiss White mice bred at the Waite Institute, South Australia. At the time of use they weighed 15-25 g.

Rabbits were an assortment of New Zealand White, Dutch, chinchilla, English lop and mixtures thereof, bred at the Waite Institute.

Guinea-pigs were a mixture of strains bred at the Waite Institute.

Estimation of virulence

Mice weighing 15-20 g. were injected within 10 hours of their arrival in the department's infected animal house. This procedure was adopted because it had been found that when animals were allowed to settle in the infected animal house for a few days their resistance to S.typhimurium C5 increased dramatically (Ielasi, personal communication). It has not been decided whether animals arriving in the department are particularly susceptible to Salmonella infections because of the trauma involved in transporting them, or whether a brief sojourn in the infected animal house is sufficient to immunize normal mice against these organisms.

Bacteria for use in virulence tests were grown in 10 ml. broth cultures and serial 10-fold dilutions were carried out in nutrient broth immediately before injection. In most experiments groups of 10 mice were injected with each dose and a total of 40 mice were used for each strain of bacteria. When doses of the order of 10^8

were to be used bacteria were precipitated from broth by centrifugation at 2,000 g. for 1 hour and resuspended in 10 ml. broth before dilution. Bacteria which were thought to be virulent were injected in the range 10^2 - 10^5, and presumed avirulent organisms in the range 10^5 - 10^8. An aliquot containing 0.1 ml. of a suitable dilution was plated for viable count at the same time as mice were injected. Deaths were recorded daily for 28 days and the LD_{50} of each strain was calculated by the method of Reed and Muench (1938).

Lipopolysaccharides

Lipopolysaccharides (LPS) were prepared by the method of Westphal et al (1952).

Bacteria grown in Roux bottles were rubbed free from the agar with a bent rubber-tipped glass rod, washed off with saline (about 30 ml. per bottle), filtered through glass wool to remove lumps of agar, and centrifuged at about 12,000 g for 15 minutes. The pellets of bacteria were resuspended in saline and washed twice more before being suspended in distilled water and stored at -20°.

To extract LPS the volume of the thawed suspension of bacteria was adjusted to give a bacterial concentration of about 30 mg. per ml. and warmed to 70°. An equal volume of 90% w/w phenol at

70° was added and the mixture stirred constantly for 20 minutes with the temperature maintained between 68° and 70°. The mixture was allowed to cool to room temperature and then left overnight at 4°. Centrifugation in glass bottles at about 12,000 g separated it into either 3 or 4 layers, of which the top opalescent aqueous layer contained LPS. This was carefully removed with a Pasteur pipette and added slowly, with stirring, to 5 volumes of alcohol. Generally a white flocculent precipitate appeared immediately. If this failed to develop, a few crystals of sodium acetate were added. After 30 minutes the precipitate was centrifuged at 1,200 g for 15 minutes. The opalescent supernatant was retained. Precipitates were resuspended in a smaller volume of distilled water and the process was repeated until the supernatant after centrifugation no longer appeared opalescent. Supernatants were combined and centrifuged in the 40 rotor of a Spinco model L centrifuge at 35,000 rev./min. (85,000 g) for 2 hours. This precipitates LPS and leaves nucleic acids in solution (Westphal and Jann, 1965). The precipitates were resuspended as described above and any material which would no longer remain in suspension during centrifugation at 2,000 g was discarded. After a second centrifugation at 85,000 g a u.v. spectrum of the resuspended LPS was determined between 220 and 300 mμ on an Optica CF4

spectrophotometer. The results of two such determinations on LPS after consecutive Spinco runs are shown in Fig 9. In general, two Spinco runs were sufficient to remove the nucleic acid peak between 240 and 285 mμ, but occasionally up to four runs were required. It was calculated that nucleic acid contamination of the LPS less than 0.3%.

Merthiolate at a final concentration of 1/10,000 was added to the purified LPS, which was dispensed into 5 ml. amounts and stored at -20°.

Preparation of bacterial ultrasonicates

Bacteria from overnight cultures on nutrient agar were washed once in saline, resuspended in 6 ml. of saline, cooled in ice, and then treated with ultrasound for 10 minutes. The suspension was centrifuged at 2,000 g at room temperature for 10 minutes and the supernatant at 37,000 g for 30 minutes in a model L Spinco. The pellet was suspended in glycine HCl buffer (0.1M, pH 3.0) for 30 minutes, centrifuged for a further 30 minutes at 37,000 g , resuspended in distilled water, and stored at -20° in this form. The yield of ultrasonicate from 4 agar plates was approximately 2.5 mg.

Fig 9a

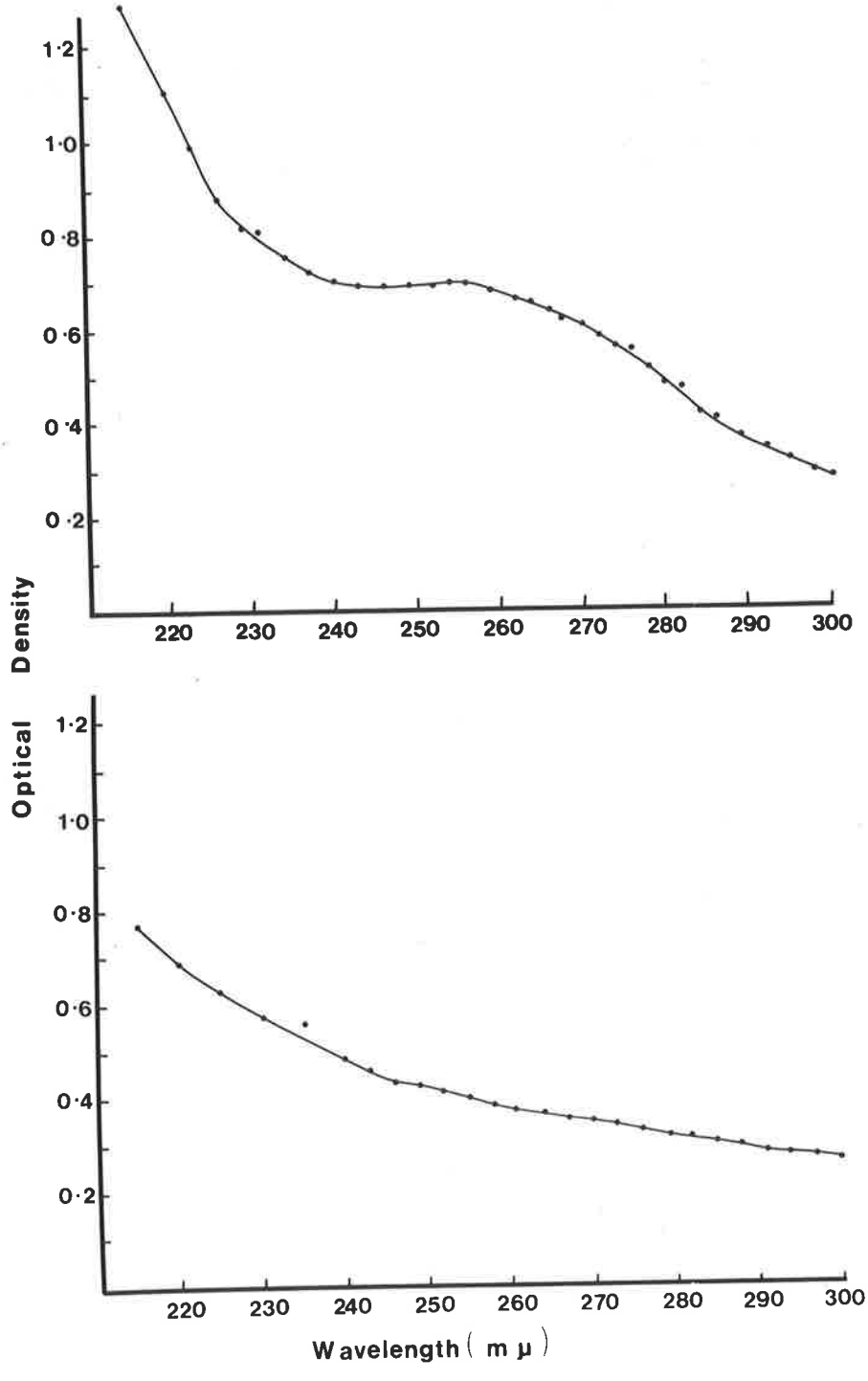
Ultra-violet spectrum of lipopolysaccharide from S.reading after one ultracentrifugation

Lipopolysaccharide was suspended in distilled water at a concentration of approximately 0.05 mg. per ml.

Fig 9b

Ultra-violet spectrum of lipopolysaccharide from S.reading after two ultracentrifugations

Conditions were the same as in Fig 9a.


Optical Density
1·2
1·0
0·8
0·6
0·4
0·2
220
230
240
250
260
270
280
290
300
Wavelength (m μ)

Haemagglutination of LPS sensitized cells

(1) Sensitization

The lipopolysaccharides employed did not normally require treatment to make them attach to red cells (Auzins, 1968). However, LPS treated with alkali by the method of Crumpton, Davies and Hutchison (1958) was occasionally used. This procedure had the important effect of destroying antigen 5. To 0.8 ml. solution containing up to 9 mg. LPS per ml. were added 0.2 ml. 0.2M sodium hydroxide. The mixture was incubated for 2 hours at 37° or left at room temperature overnight and then neutralized with 0.2M hydrochloric acid.

Whole citrated sheep blood was kept at 4° for up to 3 weeks. Cells used for sensitization were washed three times with about 10 volumes of saline and resuspended to give a final concentration of 4%. They were then mixed with an equal volume of saline containing 100 µg./ml. LPS and incubated with gentle rolling at 37° for 2 hours. After 3 further saline washes the cells suspended at a concentration of 0.5% were ready to use.

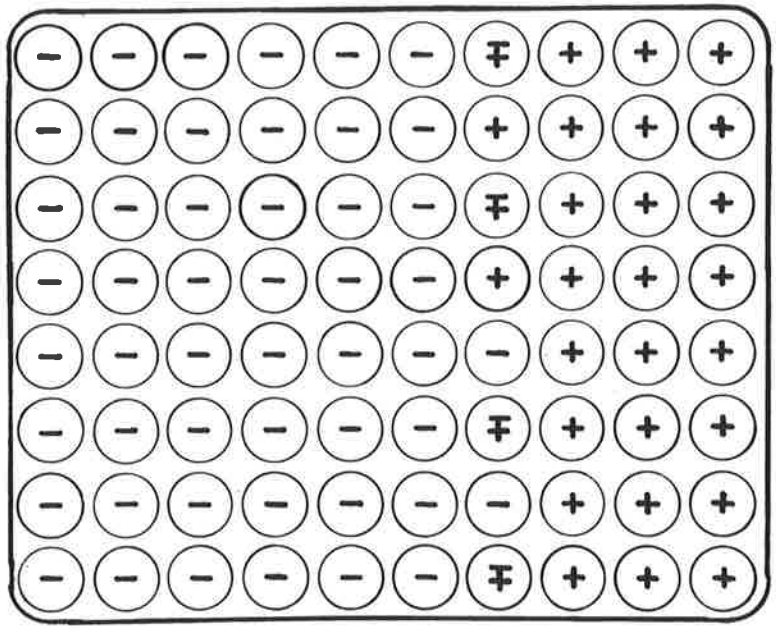
(2) Titration

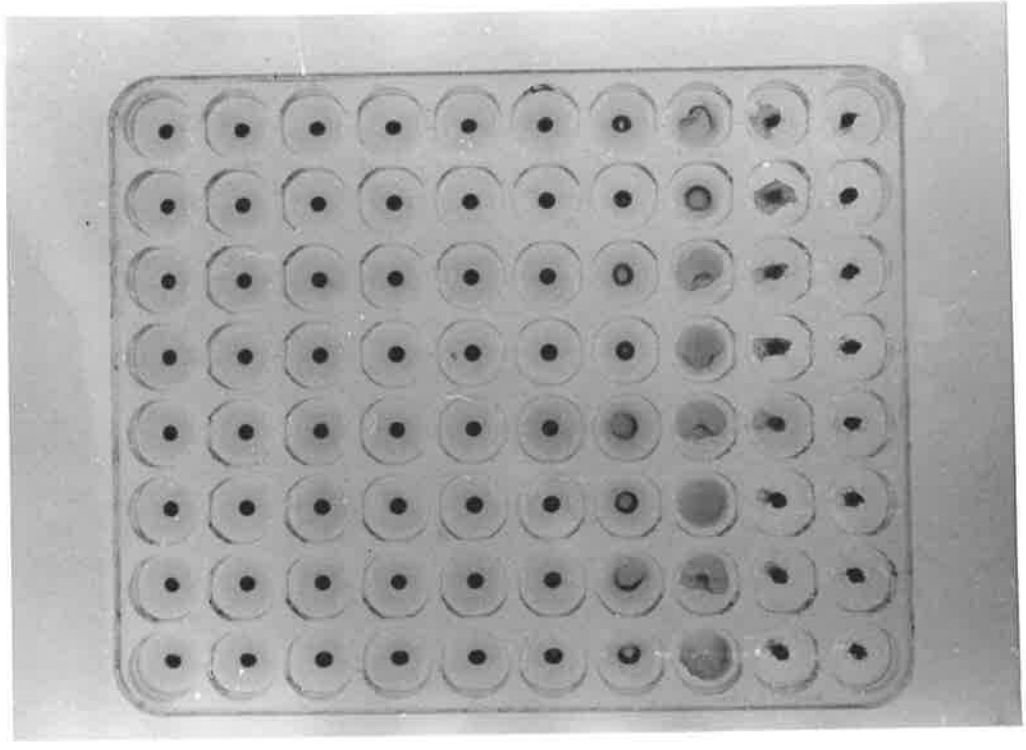
Titrations were carried out in Perspex haemagglutination trays with wells containing approximately 0.5 ml. Doubling dilutions of antiserum were made with a 0.2 ml. auto-zero pipette in 0.2 ml. saline containing 0.5% normal rabbit serum. Sensitized cells (0.2 ml.) were added to every well and the plates were covered and incubated for 1 hour at 37°. They were usually left at 4° overnight before final reading of haemagglutination, but for some experiments when preliminary results were needed quickly, they could be read to within 1 well about 2 hours after addition of red cells.

Titres were interpreted by the settling pattern of the cells (Fig 10). At the concentration used (a final 0.25%) there were not quite enough cells to fill a button at the bottom of each well. Instead, unagglutinated cells formed a thick compact ring about 3 mm. in diameter which was scored as -. When there was some slight agglutination the ring appeared larger, up to 5 mm. and was scored as $\overset{+}{-}$. When the ring appeared broken, or when cells formed a fairly even 'carpet' this was scored as +. Titres are quoted as the last dilution of antiserum which caused cells to give a + pattern.

Fig 10

Photograph of a haemagglutination tray and key to interpretation of the settling pattern





Haemagglutination inhibition titrations

This was a fairly rapid method for comparing the amounts of antigen in different preparations. First, serum was titrated against LPS-sensitized red cells as described above, and $1\frac{1}{2}$ hours after the addition of red cells the lowest concentration of antiserum able to cause complete agglutination was measured. This was defined as containing 1 haemagglutinating unit (HU) per 0.2 ml. A solution of antiserum containing 8 HU per 0.2 ml. was made. Doubling dilutions of the antigen to be tested were made in 0.1 ml. saline in Perspex haemagglutinating trays and to each well was added 0.1 ml. of antiserum containing 8 HU per 0.2 ml. (i.e. 4 HU were added and the volume was increased to 0.2 ml.) The trays were covered with metal foil and incubated at 37° for exactly 1 hour, and 0.2 ml. 0.5% sensitized cells were added to each well. Trays were incubated for a further hour and then left overnight at 4° before the haemagglutination pattern was read. The titre of an antigen was that weight which would completely inhibit haemagglutination by 4 HU of antibody.

It was found that it was important to incubate test antigen with antiserum for at least 1 hour before the addition of red cells. Shorter incubation periods appeared to lead to mixed agglutination,

particularly when the antigens were whole bacteria, and end points became very difficult to read. Longer incubation caused significant drying out of the wells.

Complement

Normal guinea-pig serum was used as a source of complement. About 10 ml. of blood was drawn from the heart of a guinea-pig under ether anaesthetic and allowed to clot at room temperature. After the clot had been dislodged from the side of the bottle it was incubated at 37° for 1 hour. Serum was withdrawn with a sterile Pasteur pipette and centrifuged at 2,000 g to remove the last red blood cells. Serum from several guinea-pigs was pooled, distributed in 1 ml. amounts in tight-fitting screw-capped bottles and stored for up to 1 month at -20°.

Complement titrations

These were carried out by a modification of the method described by Kabat and Mayer (1961_a). All titrations, dilutions of complement and final dilutions of cells were carried out in the diluent described below.

(1) Diluent

(a) Stock Ca^{++} and Mg^{++} solution.

This was made from solutions of calcium and magnesium chlorides, both of which form highly deliquescent salts. Stock solutions of calcium and magnesium chlorides containing respectively 34 g./100 ml. and 20 g./100 ml. of the wet salts were titrated against fresh silver nitrate solution using potassium chromate as an indicator. Analar sodium chloride was used as a primary standard in determining the molarity of the silver nitrate (Vogel, 1951). A stock solution of the two chlorides was made which was 0.5M with respect to magnesium and 0.15M with respect to calcium.

(b) Buffered saline.

Solution A contained:

NaCl	42.5 g.
Na 5, 5 diethyl barbiturate	1.875 g.

These were dissolved in 700 ml. distilled water.

Solution B contained:

5,5 diethyl barbituric acid	2.875 g.

This was dissolved in 250 ml. hot distilled water.

Solutions A and B were mixed, and cooled to room temperature.

(c) Concentrated diluent.

Stock calcium and magnesium solution (5 ml.) was mixed with the veronal buffered saline prepared as above and the resultant solution was made up to a final volume of 1 litre with distilled water. This solution was stored at 4°.

(d) Diluent.

Before use, concentrated diluent was diluted 1/5 with distilled water. The resultant solution, henceforth referred to as 'diluent', had the following characteristics:

pH (20°)	7.4
NaCl	0.15M
Ca^{++}	0.00015M
Mg^{++}	0.005M
5,5 diethyl barbiturate	0.005M

(2) Haemolysin sensitized cells

Sheep red blood cells between 1 and 3 weeks old were washed 3 times with cold saline, suspended in 18 volumes of cold (4°) diluent and filtered through a plug of cotton wool in a filter funnel. Cells (1 ml.) were added to 14 ml. 0.1% sodium bicarbonate and the optical density (O.D.) was measured at 541 mμ on a Unicam SP.600 spectrophotometer. The concentration of

cells was adjusted to approximately 10^9 per ml. using the formula:

$$V_{final} = V_{initial} \times \frac{O.D._{.541}}{O.D._{700}}$$

Suitably diluted rabbit anti-sheep red blood cell haemolysin (Commonwealth Serum Laboratories, Melbourne) was added dropwise to an equal volume of cells in a 200 ml. beaker or 50 ml. centrifuge tube with constant stirring.

(3) Direct complement titration

All dilutions were made in cold diluent prepared as described above. Cold diluted guinea-pig serum (0.2 ml.) was added to 0.2 ml. diluent in a haemagglutination tray and 8 doubling dilutions were made. Sensitized sheep red blood cells containing approximately 5×10^8 cells/ml. were diluted by 1/4 and 0.2 ml. cells were added to each well, giving a final concentration of 4.125×10^7 cells/ml. in a final volume of 0.4 ml. The tray was covered and incubated at 37° with occasional swirling. The well in which about 50% of the cells had lysed after 90 minutes was said to contain 1 unit of complement ($C'H_{50}$).

This method is very similar to that of Kabat and Mayer (1961_a). However, the final red cell concentration used in that publication is approximately 6.7×10^7 cells/ml.

(4) Selection of optimum haemolysin concentration

Haemolysin was supplied with an approximate estimate of its titre before addition of an equal volume of glycerine. Sheep red blood cells were sensitized with haemolysin at two-fold dilutions ranging from double the manufacturers estimate of its potency to $1/2^3$ of its estimated value. Guinea-pig complement was titrated against each batch of cells and the haemolysin concentration which gave rise to haemolysis at the lowest concentration of guinea-pig serum was found. This dilution was taken to be the titre of the particular batch of haemolysin and was used routinely for the sensitization of cells. The titre remained constant for periods of at least 2 months.

(5) Complement fixation tests

An aliquot of 0.2 ml. of antigen which had previously been mixed with antiserum was mixed with 0.2 ml. diluent in the first well of a haemagglutination tray and falling doubling dilutions in diluent were carried out using a 0.2 ml. auto-zero pipette. Guinea-pig serum (0.1 ml.) diluted to give a concentration of 4 $C'H_{50}$ units per 0.1 ml. was added to each well and the plate was incubated at 37° for 90 minutes with occasional shaking. Sensitized sheep red blood cells (0.1 ml.) containing 2.5×10^8

cells/ml. were then added to each well and the plate was incubated for a further 90 minutes with more shaking. In the well in which approximately 50% lysis had occurred it was assumed that 1 $C'H_{50}$ unit of complement remained, so that 3 units had been fixed. It was then possible, assuming direct proportionality, to calculate how much complement was fixed by a given weight of antigen mixed with a known amount of antibody. If there seemed to be either a sharp change from nearly complete inhibition of lysis to nearly complete lysis, or if adjacent wells showed considerable lysis, it was assumed that only a small amount of complement remained at the higher concentration of antigen and that the paradoxical amount of $4\frac{1}{2}$ units had been fixed.

Preparation of antisera

(1) Complete Freund's adjuvant

Complete Freund's adjuvant (CFA) was prepared by homogenising 7.5 ml. Arbocell A with 42.5 ml. Bayol and 25 mg. heat-killed dry Mycobacterium butyricum (kindly donated by Mr. J.D. Wetherall).

(2) Vaccines

Bacteria grown overnight in liquid culture media were washed 3 times in saline, resuspended at a concentration of 10^{10}/ml. and heated in a steam bath for $2\frac{1}{2}$ hours. After 2 further saline washes they were ready for suitable dilution as vaccines.

Bacteria used to prepare antibody against the heat-labile antigen 5 were killed by heating at 60° for 30 minutes.

(3) Immunization

Rabbits were given an initial intramuscular injection in the hind leg of 1 ml. bacteria at a concentration of 10^{9}/ml. thoroughly mixed with 1 ml. CFA. Further injections were given at intervals of 4 - 5 days in each of the other legs and subsequently intraperitoneally and intravenously. During the course of immunization the number of bacteria was increased gradually from 10^{9} to 10^{11} per ml. and 1 ml. injections were used throughout.

Rabbits were bled from the heart 10 days after the last injection. Blood was collected in 20 ml. glass screw-topped bottles and allowed to clot on the bench for 15 minutes. A Pasteur pipette was used to dislodge the clot from the side of the bottle, which was then incubated at 37° for 1 hour and left overnight at 4°. Serum was withdrawn from the bottle with a sterile Pasteur pipette and centrifuged for 10 minutes at 250 g. to remove the remaining red blood cells.

Serum was pooled from at least 5 rabbits and merthiolate added to a dilution of 1/10,000. The serum was dispensed in 2 ml. amounts in 5 ml. screw-topped bottles and stored at -20°.

(4) Absorption of antisera

Bacteria used to absorb whole, non-specific antisera were grown in Roux bottles. Thirty-six hour cultures were washed from the bottles with saline, filtered through glass wool, washed twice in saline, heated at 100° for 1 hour, washed once more, and then suspended to give a concentration of about 1 Roux bottle equivalent of bacteria in 5 ml. of saline. Bacteria were then dispensed in 5 ml. amounts into 20 ml. screw-topped bottles and centrifuged at 2,000 g for 1 hour. The sedimented bacteria were mixed with 5 ml. of serum and gently rotated for 14 hours at 4°. Sera were then centrifuged twice at 12,000 g for 20 minutes and tested for residual activity against the absorbing organisms or their lipopolysaccharides.

(a) Anti-4 serum

An anti-S.reading serum was absorbed with S.typhi 0 901 and tested by slide agglutination on a boiled suspension of S.typhi 0 901. The absorption was repeated if any agglutination was observed when undiluted serum was used.

(b) Anti-5 serum

An anti-Citrobacter 396 serum was absorbed with S.reading and tested against sheep red blood cells which had been sensitized either with S.reading LPS or with C5 LPS which had previously been treated with 0.02N sodium hydroxide to destroy antigen 5. (Crumpton et al, 1958, Kotelko et al, 1961). No agglutination was observed at an antiserum dilution of 1/4.

(c) Anti-12 serum

An anti-S.reading serum was absorbed with Citrobacter 396 and tested against sheep red blood cells sensitized with Citrobacter 396 lipopolysaccharide. The absorbed serum gave no agglutination at a dilution of 1/4.

Serum bactericidal reactions

These were carried out by a modification of the method of Rowley (1968). Agar plates were heavily inoculated with the bacteria to be tested and incubated overnight. A few colonies were used to seed a 10 ml. broth culture which was grown with shaking for 4-5 hours. The rest were scraped from the agar, washed once in saline, mixed with up to 10 ml. of guinea-pig serum (complement), and kept at 4° for 1 hour. The serum was centrifuged twice at 12,000 g. for 20 minutes and then filtered

into a sterile 20 ml. bottle through a 0.45μ millipore membrane using a Sweeney adaptor. This apparatus could handle 2-4 ml. of serum before it became blocked by bacteria and other particles.

Sterile 3 x $\frac{1}{2}$" tubes with loose aluminium caps were set up to contain 0.9 ml. saline containing 0.02% magnesium chloride (Muschel and Treffers, 1956). A 1/10 dilution (0.1 ml.) of the antiserum to be tested was added to the first tube and 8 10-fold falling dilutions were made. A control tube containing no antiserum was always included. The freshly grown bacteria were diluted $5/10^6$ in magnesium chloride saline solution and mixed with an equal volume of a 1/10 dilution of complement. One ml. of the mixture was added to each tube. Samples for bacterial counts were immediately taken from randomly selected tubes. All tubes were then incubated at 37° for $2\frac{1}{2}$ hours.

Killing was measured in two ways. After a suitable time either 0.1 ml. samples from each tube were plated on nutrient agar and examined the following day to find out at which dilution 90% of the bacteria had been killed, or single drops from a standard sized Pasteur pipette were made on agar and these were inspected for a drastic fall in numbers the following day. The second method, while giving less information in molecular terms, was considerably

less expensive than the first, because one plate could be used for 5 separate counts, and was therefore used routinely.

Fig 11 shows the effect of treating S.reading with an anti-S.reading serum and following the bacterial counts over 4 hours. It will be seen that after a considerable delay, when apparently little happens, numbers of viable bacteria fall over a comparatively short period and then at least at high dilutions of serum begin to increase again. On the basis of this experiment counts were routinely made after $2\frac{1}{2}$ hours.

Hanks solution

Hanks solution was made according to the method of Weller, Enders, Robbins and Stoddard (1952).

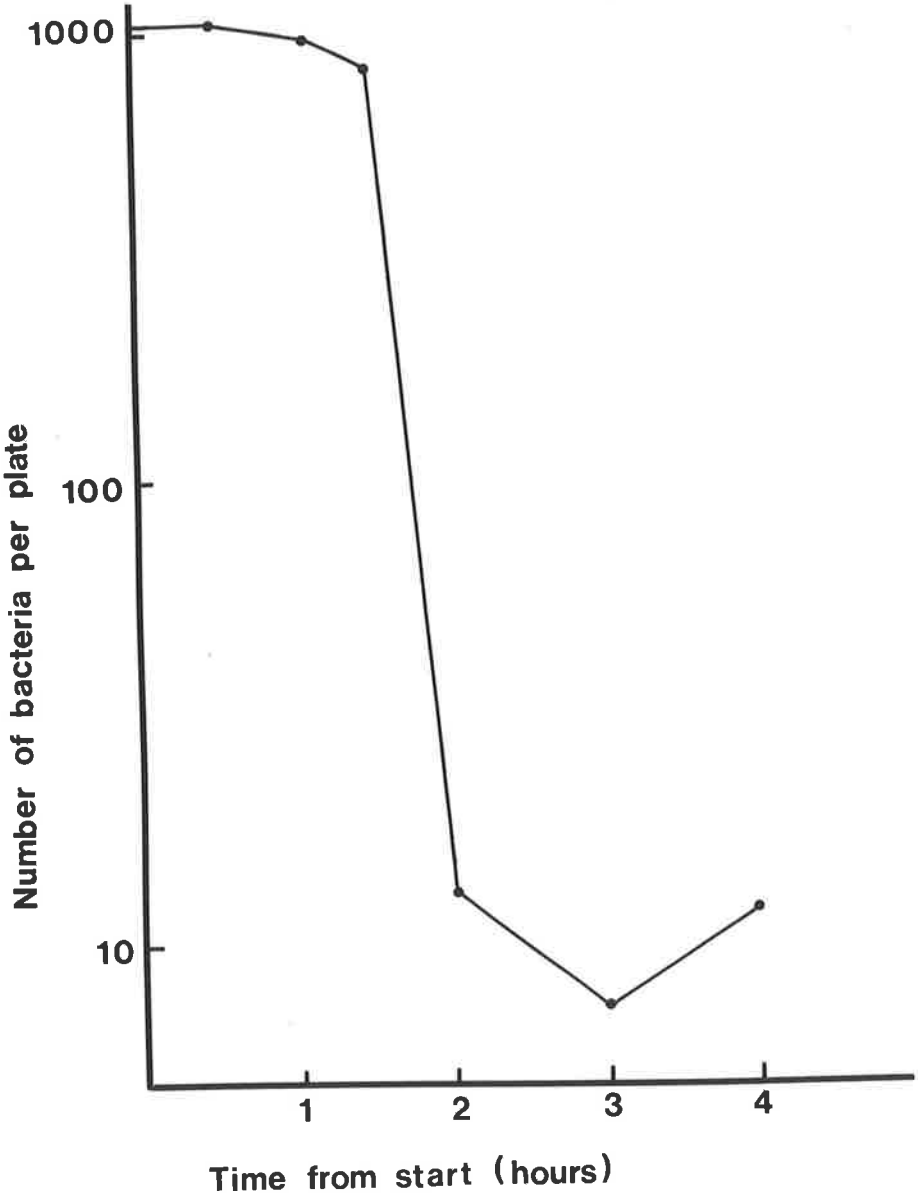
(a) Phenol red solution

Phenol red (1 g.) was placed in a 250 ml. volumetric flask and N/20 sodium hydroxide solution added with shaking until most of the powder was dissolved. Further additions were made slowly until the powder was completely dissolved and a deep red solution obtained. The volume was adjusted to 250 ml. with deionised water.

Fig 11

Effect of anti-S.reading serum and guinea-pig complement on S.reading

Antiserum was used at a dilution of $1/10^7$ and bacteria were counted after plating 0.1 ml. aliquots at appropriate intervals.


1000
100
10
Number of bacteria per plate
1
2
3
4
Time from start (hours)

(b) Solution A

Solution A contained:-

NaCl	160 g.
KCl	8 g.
$MgSO_4 7H_2O$	4 g.
$CaCl_2$	2.8 g.

These were made up to 1,000 ml. with distilled water.

(c) Solution B

Solution B contained:

$Na_2HPO_4.12H_2O$	3.04g.
KH_2PO_4	1.2 g.
Glucose	20 g.
Phenol red solution (0.4%)	100 ml.

These were made up to 1,000 ml. with deionised water.

For use, 1 volume of Solution A was mixed with 1 volume of B and 18 volumes of deionised water. The solution was dispensed in 90 ml. amounts and autoclaved. Immediately before it was used 10 ml. of 1.47% sodium carbonate solution which had been filtered through a sterile Millipore membrane was added to 90 ml. of the autoclaved solution and the orange yellow solution turned deep pink. Carbon dioxide was blown on to the surface of the solution

until the pink colour changed to orange, when the solution was ready to use.

Intraperitoneal killing of bacteria

The intraperitoneal killing of opsonized and unopsonized bacteria in mice was followed by the method of Whitby and Rowley (1959). Six hour cultures of bacteria grown on the shaker at 37° were diluted to a concentration of about 5×10^4/ml. and mixed with a suitable dilution of the antiserum to be titrated. After incubation for 30 minutes at room temperature 0.1 ml. of bacteria were injected into each of ten 20-25 g. mice. One mouse was immediately killed by cervical dislocation and pinned on its back on a cork board. After swabbing with alcohol the abdominal skin was cut longitudinally and pulled to the side, the peritoneal wall carefully lifted with fine forceps, and 1.5 ml. of sterile Hanks solution injected into the peritoneum using a 2 ml. syringe and 19 gauge needle. The peritoneum was gently pumped, and fluid was sucked up and down in the syringe to ensure thorough mixing. As much fluid as possible was drawn back into the syringe, which was then removed from the peritoneum. The plunger was withdrawn slightly and the syringe inverted several times to mix its contents. After a few drops had been

discarded to allow for fluid trapped in the needle during mixing, one drop was plated on nutrient agar and colonies were counted after overnight incubation at 37°. This procedure was repeated at roughly 9 minute intervals for 80-90 minutes. As it took about 4 minutes to carry out one washout on one mouse, it was possible to handle two batches of mice in one 90 minute period. When the abilities of two sera to opsonize bacteria for phagocytosis were being compared, equivalent dilutions of the two were used over the same 90 minutes. This helped to eliminate differences caused by variation in bacterial numbers and states of growth on different days.

Methyl pentose determinations

Methyl pentoses were determined by the methods of Dische and Shettles (1948).

(1) Reagents

Sulphuric acid: 6 parts concentrated sulphuric acid to 1 part water.

Cysteine hydrochloride: A 3% solution was prepared daily.

(2) Procedure

(a) Ten minute reaction

To 1 ml. of an ice-cold suspension of the sample were added

slowly with shaking 4.5 ml. sulphuric acid. Tubes were transferred to a rapidly boiling water bath and left there for exactly 10 minutes. When the tubes had cooled to room temperature 0.1 ml. cysteine hydrochloride was added to each. After 2 hours the spectrum between 380 and 430 mμ was measured in a Hitachi Perkin-Elmer 124 double beam recording spectrophotometer. Sulphuric acid digestion of bacteria leads to production of a pinkish-brown colour. Spectra were therefore read against a blank containing an identical sample to the original which had been treated in the same way except for the omission of cysteine hydrochloride. The methyl pentose content of a sample was estimated by subtraction of the O.D. obtained at 430 mμ from that at 396 mμ and comparison of this with the equivalent value obtained at the same time from a standard solution of rhamnose containing approximately 25 μg./ml.

(b) Three minute reaction

This method was used when it was hoped that hexoses as well as methyl pentoses would be measured. It was not suitable for this purpose when large amounts of rhamnose were present.

The reaction was carried out in the same way as the 10 minute reaction except that the sample was digested in sulphuric acid for exactly 3 minutes instead of exactly 10.

Protein determinations

Protein was determined by the method of Folin and Ciocalteu (1927) as modified by Lowry, Rosebrough, Farr and Randall (1951).

(1) Reagents

(a) Commercial Folin's reagent (By-Products and Chemicals Pty. Ltd., Sydney, N.S.W.) was diluted 1/3 in distilled water to bring it to a concentration of about 0.1N acid.

(b) Anhydrous sodium carbonate was dissolved to a concentration of 2% in 0.1N sodium hydroxide.

(c) 0.5% copper sulphate (hydrated) was dissolved in 1% potassium sodium tartrate. The complex crystallised out as pale flattened crystals and was redissolved by addition of a minimal amount of dilute sulphuric acid.

(d) 1 ml. of solution (c) was mixed with 50 ml. of solution (b).

(2) Procedure

The sample for protein determination (0.5 ml.) was mixed with 2.5 ml. of solution (d) and allowed to stand for 10 minutes at room temperature. Solution (a) (0.25 ml.) was added with shaking. Tubes were left for 30 minutes before the colour developed was measured by reading the O.D. at 750 mμ in either a Unicam SP 600 spectrophotometer or a Hitashi Perkin-Elmer 124 double beam

recording spectrophotometer. Colours were read against a saline blank which had otherwise been treated in the same way as the protein solution. Protein was estimated from a standard curve made with a commercial preparation of bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Victoria). A typical standard curve is shown in Fig 12. As the exact colour developed tended to vary from day to day, a few points on the standard curve were redetermined during each set of determinations.

Quantitative precipitin reactions

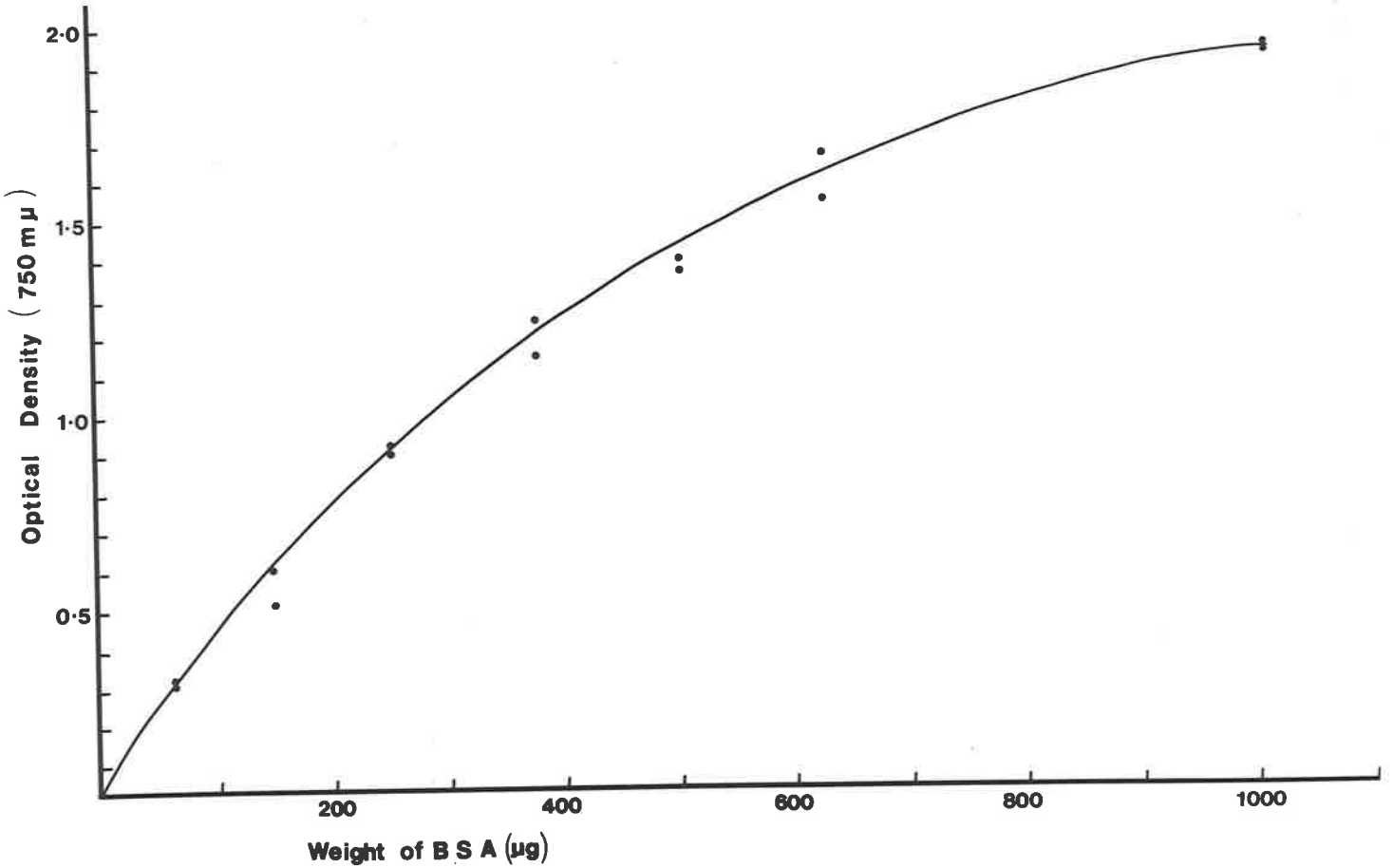
Quantitative precipitin reactions were carried out as described by Kabat and Mayer (1961_b).

Precipitation was carried out in 1 ml. conical glass tubes which had been soaked in chromic acid, rinsed in distilled water, and dried at 110°. Serum to be tested was heated at 56° for 30 minutes to destroy C'1q (Muller-Eberhard and Kunkel, 1961) and centrifuged for 1 hour at 20,000 g. Any fat which had risen was skimmed from the surface and the serum was carefully decanted. Serum (0.2 ml.) was added to tubes containing 0.1 ml. of C5 lipopolysaccharide in saline at concentrations of 14.5 to 455 µg/0.1 ml. The tubes were covered with Parafilm, their contents thoroughly mixed, and left to stand at 4° for 3 days, with further mixing each day. When it

Fig 12

Folin-Lowry protein determinations on standard amounts of bovine serum albumin (BSA) in saline

for details see text


2·0
1·5
1·0
0·5
Optical Density (750 mμ)
200
400
600
800
1000
Weight of B S A (μg)

was decided that the immune precipitates had formed completely they were centrifuged at 0°. Supernatants were carefully decanted into clean tubes and inspected for signs of precipitates. The tubes containing precipitates were inverted over absorbent paper, allowed to drain, and then tapped to remove the last drops of supernatant. Precipitates were resuspended in 0.2 ml. ice-cold saline and recentrifuged. This washing procedure was carried out twice.

On the same day that protein determinations were to be carried out 0.1 ml. of 1N sodium hydroxide was added to each tube and then 0.4 ml. saline. A Pasteur pipette was used to make certain that all the precipitate was dissolved and then it was transferred quantitatively into a 10 ml. graduated conical glass centrifuge tube with the assistance of the solution (d) described on page 108. The volume was made up to 3 ml. with solution (d) and protein was determined as described previously.

Labelling of antiserum with ^{131}I

Chloramine-T method. Antisera were labelled by the method of McConahey and Dixon (1966).

(1) Reagents

(a) Chloramine-T (1.41 mg./ml.) was dissolved in 0.15M borate buffer at pH 9.0.

(b) Potassium iodide was used at a concentration of 2×10^{-4}M (3.3 mg./100 ml.).

(c) Sodium metabisulphite (1.26 mg./ml.) was dissolved in borate buffer at pH 9.0.

(d) Iodine-131 was obtained as iodide in sodium thiosulphate solution from the Radiochemical Centre, Amersham, England (Code IBS.1).

(2) Procedure

Chloramine-T (0.1 ml.) was added to 0.2 ml. carrier-free iodine. This quantity had previously been determined as the amount required to neutralise the excess thiosulphate. After 1 minute there were added in order 0.1 ml. potassium iodide, 0.2 ml. chloramine-T, and 0.2 ml. specific antiserum. The mixture was left for 5 minutes before the reaction was stopped with 0.3 ml. sodium metabisulphite.

The radioactive mixture was transferred to a dialysis bag, dialysed against 1 litre of saline for 2 hours at room temperature, and then overnight at 4° against a further litre of saline.

Hydrogen peroxide method. This was essentially the method of Webster, Laver and Fazekas de St Groth (1962), with minor modifications suggested by Webster (personal communication).

(1) Reagents

(a) Hydrogen peroxide (100%)

(b) Potassium iodide (10^{-4}M)

(c) Sulphuric acid (1N)

(d) Carbon tetrachloride

(e) Iodine-131, obtained as carrier-free iodide from the Radiochemical Centre, Amersham, England (Code IBS.3).

(2) Procedure

Potassium iodide (1.5 ml.) was mixed with carrier-free iodine, the volume adjusted to 2.5 ml. with distilled water, and the mixture acidified with 0.2 ml. sulphuric acid. Addition of 0.2 ml. hydrogen peroxide caused liberation of free iodine. This was extracted into 1.0 ml. carbon tetrachloride, which turned a faint pink. The aqueous phase was carefully removed and the carbon tetrachloride washed 3 times with 2 ml. distilled water. Protein in 2.5 ml. 0.1M phosphate buffer, pH 7.0, was shaken with the carbon tetrachloride until the pink colour disappeared. The

aqueous phase was removed and dialysed for 2 hours against 2 litres of 0.15M saline at room temperature, and then overnight in the cold against a further 2 litres of saline.

CHAPTER 4

COMPARISON OF THE AMOUNTS OF ANTIGENS ON THE SURFACE OF S.TYPHIMURIUM STRAINS C5 AND M206

Introduction

The amounts of antigen in bacteria can be measured either by estimating the amount of a specific antigen which can be extracted from the bacteria, by measuring the amounts of antibody which react with whole organisms, or by chemical determination of the amounts of a specific antigen in the whole organisms.

It is possible to extract antigens from bacteria and titrate them against specific antisera which have already been standardised against known weights of antigen. This technique was used by MacLeod and Krauss (1950) in their studies on pneumococcal polysaccharides and has been used by Staub and Combes (1951, 1952) on the antigens of S.typhi using acetic acid degraded polysaccharide extracted by the Freeman (1942) method. In the hands of Staub and Combes the ratios of total polysaccharide to total nitrogen in the original bacteria gave a variation between determinations of bacteria from broth cultures of less than 4%. They found that Boivin antigen extracted from the same organisms was very variable and not suitable for this sort of study.

The occurrence of methyl pentoses in a number of Salmonellae has allowed the estimation of total amounts of polysaccharide by the determination of the total amounts of these sugars in these organisms (Webster, Sagin, Landy and Johnson, 1955, P.W. Robbins et al, 1965). Staub (personal communication) found that this method gave more variable results than her immunological method. In polysaccharide from one strain of bacteria the percentage of rhamnose in the polysaccharide was 19-20%. However, in extracts of whole bacteria the estimated rhamnose polysaccharide ratios ranged from 17-25%. The reasons for this variation are not understood.

These methods suffer from the disadvantage that they cannot measure amounts of different antigens located on the same molecule, the common situation with Salmonella O-polysaccharides. Furthermore, the possibility always exists that not all antigenic sites are available to antibody in the whole organism, and as both of the above techniques measure antigens extracted from disrupted cells, other methods are necessary if the availability of antigenic sites is to be measured. The simplest of these is to find the amounts of bacteria required to reduce the agglutinating titre of a standard serum by a standard amount.

If x times more of one strain of bacteria than another are required to absorb out a certain amount of monospecific serum activity, it is reasonable to conclude that that strain has only 1/x the amount of antigen available on the other. One must assume that all available antigenic sites have an equal chance of reacting with antibodies. This method is useful for comparing strains and, with suitable data, can even be used to measure the absolute amounts of antigen on the bacteria. Wilson and Miles (1932) successfully used this technique to differentiate between a number of strains of Brucella by the proportions of two antigens on the different strains. Felix used a similar technique to compare the amounts of Vi antigen on various Salmonellae. He made the interesting observation that the amount of either Vi antigen or S.typhi, or of O-antigen 2 (the 'Vi antigen of S.paratyphi A') present on an organism bore an inverse relationship to the titre to which that organism could be agglutinated by a standard antiserum (Felix and Pitt, 1936, Felix, 1952_b),incidentally demonstrating that simple tests involving direct agglutination of bacteria are quite inadequate as methods of assessing amounts of antigens.

Of the several techniques available for comparisons of the amounts of antigen on two strains of Salmonella, the simplest is

probably that of adsorption of a standard specific antiserum to a standard activity using a known number of organisms.

1. Adsorption of specific antibodies by C5 and M206

In the experiments described in this section bacteria were added to standard amounts of diluted 'monospecific' serum, mixed with it for some time, and then centrifuged. The supernatant serum was tested by haemagglutination of sheep red blood cells sensitized with lipopolysaccharide , a technique which it was hoped would provide added specificity to the determination because of its use of a purified antigen.

Early experiments were carried out with washed overnight cultures of C5 and M206. Bacteria were handled in the laboratory, and although both sera and bacteria were kept on ice throughout most parts of the experiments, many manipulations and centrifugations were carried out at room temperature. Although under these conditions the ratios of the amounts of C5 and M206 to absorb out a certain antibody activity were fairly constant, the absolute results obtained were very variable. One possible explanation was that the live bacteria used were capable of growing, and that this led to variable numbers of organisms at the end of the experiment. However, it was found that it was no easier to

obtain constant results with bacteria killed in 70% alcohol and washed with saline. The observation was made that the number of alcohol-killed bacteria required to absorb out a standard amount of agglutinating activity appeared to be less than 1/10 the number of live bacteria required to remove the same activity, but it was difficult to be certain of whether the effect was genuine because of the high background variability in results.

Two further factors seemed likely to be affecting the absorption results. It was possible that the bacteria cultures varied from day to day, or that in the handling of them a variable amount of lipopolysaccharide was lost. Secondly, it seemed likely that antibody was dissociating from the antigen/antibody complexes during manipulation. The laboratory was air-conditioned, but room temperatures were nevertheless rather variable, and it was probable that a different proportion of antibody was dissociated on each day. It was decided, therefore, that a standard preparation of bacteria should be made for use over a number of days, and that all manipulations and absorptions should be carried out in a 4° cold room or refrigerated centrifuge.

Preparation of bacteria

Bacteria were grown with shaking at 37° for 4 hours in 10 ml. broth in 20 ml. screw-topped bottles. Of these cultures 5 ml. were seeded into 100 ml. broth in 500 ml. flat-bottom gauze-stoppered flasks and incubated in the shaker for a further 12 hours. The bacteria were then centrifuged at 12,000 g , washed 3 times in saline, and resuspended in saline to give a concentration of about 10^{12} bacteria/ml. They were dispensed in 1 ml. amounts in 10 ml. screw-topped bottles and stored at -20°. Suitable dilutions of the thawed bacteria were counted in a Petrov-Hauser counter.

Quantitative absorption of anti 4, 5 and 12 sera

Conical polypropylene tubes, capacity 0.5 ml. were arranged to contain 0.4 ml. of serum at a final dilution of 1/40 together with varying numbers of C5 or M206. The tubes were rotated gently overnight at 4° and then centrifuged at 17,000 g for 20 minutes. Aliquots (0.2 ml.) of the supernatants were removed from each tube for assay of residual antibody by haemagglutination of sheep red blood cells sensitized with C5 lipopolysaccharide.

These experiments were carried out in two parts. A preliminary absorption study using 10-fold differences in the numbers of organisms in the range 10^7 - 10^{11} per tube was followed by absorptions evenly spaced over a suitable 10-fold range. It will be noted from Fig 13 that approximately 10 times as many bacteria were required to absorb out suitable amounts of antibody from the anti-4 and anti-12 sera as were required to absorb out the anti-5 serum. The final absorption was carried out in triplicate. Fig 13 shows the results obtained by absorbing the sera with both C5 and M206. Each point is an average of 3 determinations.

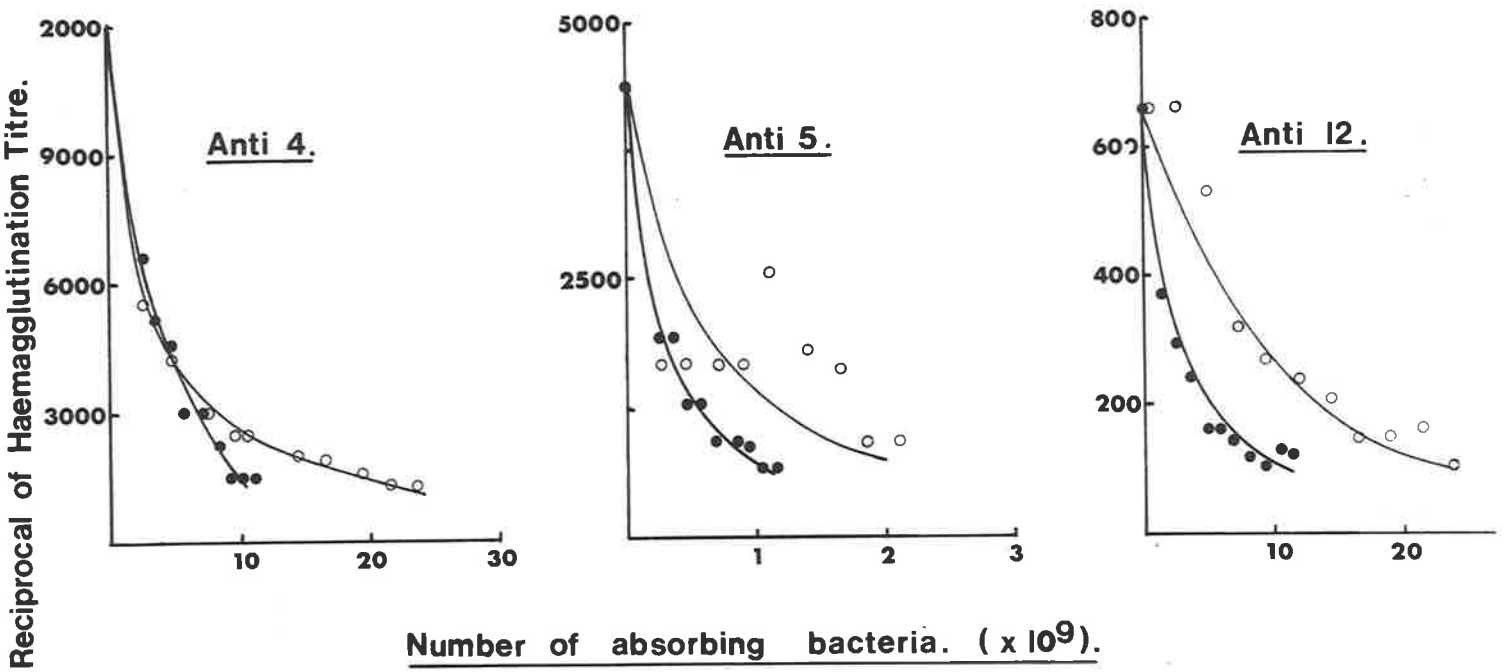
C5 contained more antigen than M206 whether measured by anti-4, anti-5 or anti-12 sera. It was hoped that the ratio of C5 to M206 required to absorb out a given amount of antibody would be constant. Unfortunately, the first 50% of antibody activity is absorbed out in a range in which it is difficult to estimate accurately how many bacteria are required, while in the lower part of the curve inconveniently large numbers of bacteria were required to give a detectable fall in agglutinating titre. The easiest parts of the curve to draw were in the region of absorption of 75% of the haemagglutinating activity. The ratio of the number

Fig 13

Effect on haemagglutinating activity against sheep red blood cells coated with C5 lipopolysaccharide of absorption of specific antisera by S.typhimurium C5 and M206

- ● - ● - C5

- o - o - M206


Reciprocal of Haemagglutination Titre.
2000
9000
6000
3000
Anti 4.
10
20
30
5000
2500
Anti 5.
1
2
3
800
600
400
200
Anti 12.
10
20
Number of absorbing bacteria. (x 10^9).

of organisms of C5 to the number of M206 required to absorb this amount of activity from a given antiserum was therefore taken as the reciprocal of the ratio of the amounts of antigen on C5 and M206. It will be seen that in all cases C5 had approximately twice as much antigen as M206 (table 6).

TABLE 6

Ratio of the number of organisms of S.typhimurium M206 to the number of organisms of C5 required to absorb out 75% of the haemagglutinating activities of specific antisera (from Fig 13).

Antiserum	Ratio M206/C5
4	1.3
5	2.0
12	3.8

Ability of antibodies to block uptake of antibodies of other specificities

Adler (1953) showed that the bactericidal action of some sera against Salmonellae could be increased by absorption with another strain and it was proposed that an antibody which inhibited the bactericidal action was absorbed out of the serum. As the specific structures for O-antigens 4, 5 and 12 were thought to be found on one pentasaccharide synthesized by S.typhimurium while an antibody

combining site can react with up to 6 monosaccharide units (Kabat, 1966), it seemed very likely that the reaction of antigen 5 with its antibody would be inhibited by antibodies against 4 and 12.

Methods

1 ml. of a suspension of C5 in saline containing 1.2×10^{12} organisms/ml. was mixed in a 5 ml. screw-topped bottle with 1 ml. rabbit antiserum. The anti-5 and anti-12 sera, whose passive haemagglutination titres were respectively 1/4,500 and 1/640 were used undiluted. Anti-4 serum with titre 1/10,000 was diluted 2/3 in saline. Bacteria and serum were mixed gently on a roller at 4° for 5 hours, centrifuged at 12,000 g for 20 minutes, taken up in 2 ml. saline at -13° and sonicated for 10 seconds to disrupt the pellet of agglutinated bacteria. Samples were diluted in the range 1/200 - 1/2,000 for quantitative absorption of anti-5 serum, and in the range of 1/20 - 1/200 for absorption of anti-12 serum. Antibody-laden bacteria (0.2 ml.) were mixed with 0.2 ml. 1/20 antiserum, giving a final serum concentration of 1/40. Sera were absorbed overnight and titrated against C5 LPS sensitized cells as described on page 90,

As controls, one batch of bacteria were carried through the whole procedure with the initial 'adsorption' carried out in saline instead of in the presence of a high concentration of antiserum. To ensure that the antibody measured was not adsorbed antibody which had been eluted from the bacteria during the overnight absorption, two extra tubes containing C5 coated with anti-4 were prepared. These were mixed with 0.2 ml. 1/20 anti-5 serum and left to rotate overnight. 0.2 ml. of the absorbed serum was titrated against sheep red blood cells sensitized with alkali-treated lipopolysaccharide. The titres obtained were both 1/60. As alkali destroys antigen 5, this titre must have been due to eluted anti-4 antibody. Most titres in other experiments were in the range 1/160 to 1/1280, so that the amount of antibody eluted was insignificant.

The results of this experiment are shown in Figs 14a and b. It is clear that antibodies against antigen 4, 5 and 12 all inhibit the reaction of antigen 5 with further antibody. As might have been expected, antibody against 5 is the best of these inhibitors. The reactions of antigen 12 with antibody are also inhibited by antibodies against 4, 5 and 12.

These results suggest that lipopolysaccharides are situated on the bacterial surface in such a way that several different

Fig 14a

Absorption of anti-5 serum by S.typhimurium C5 after coating with specific antiserum against 4, 5 and 12

—●—●— uncoated cells

—o—o— cells coated with anti-4 serum

—△—△— cells coated with anti-5 serum

—□—□— cells coated with anti-12 serum

Fig 14b

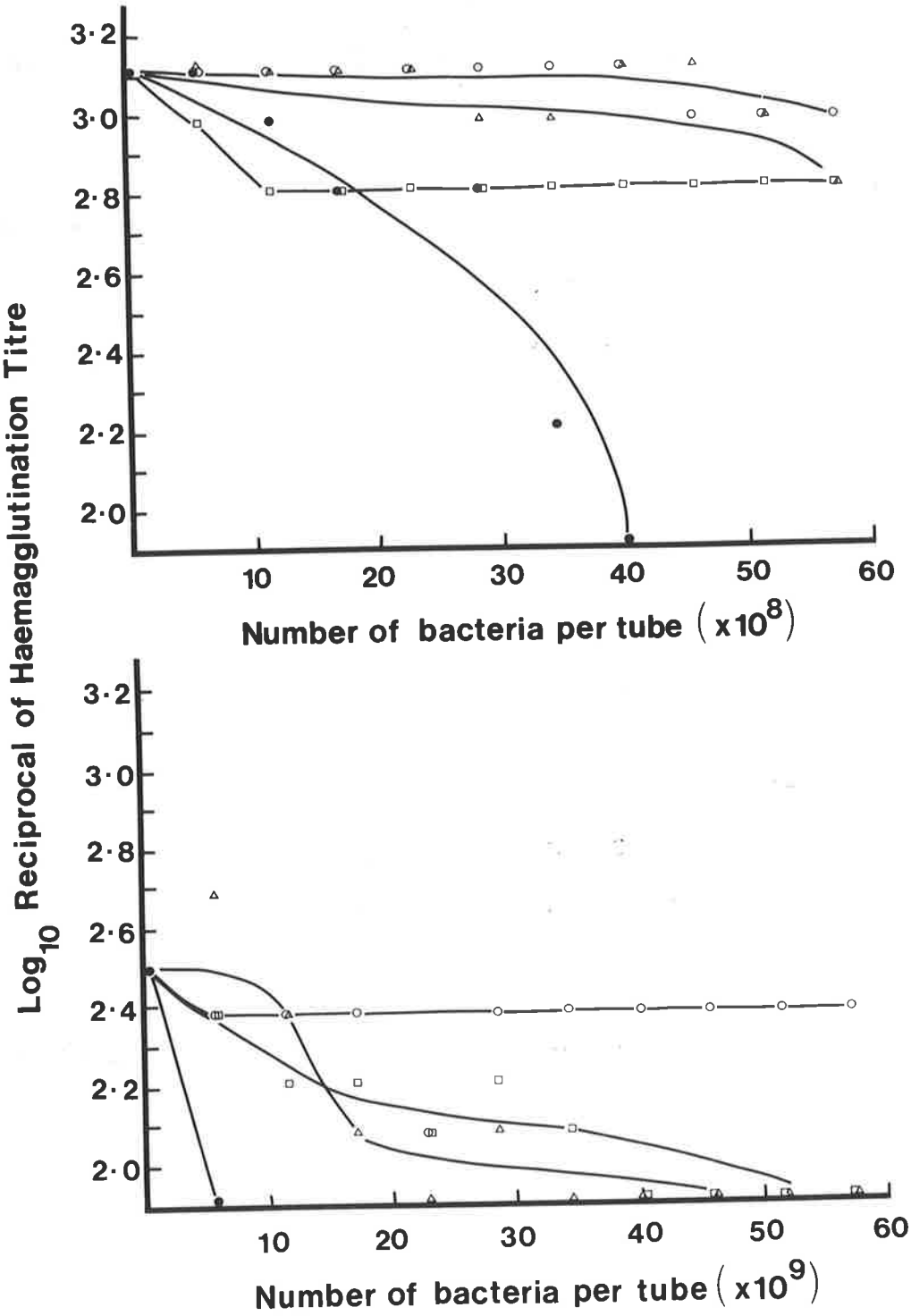
Absorption of anti-12 serum by S.typhimurium C5 after coating with specific antiserum against 4, 5 and 12

—●—●— uncoated cells

—o—o— cells coated with anti-4 serum

—△—△— cells coated with anti-5 serum

—□—□— cells coated with anti-12 serum


Log10 Reciprocal of Haemagglutination Titre
3·2
3·0
2·8
2·6
2·4
2·2
2·0
10
20
30
40
50
60
Number of bacteria per tube (x10^8)
3·2
3·0
2·8
2·6
2·4
2·2
2·0
10
20
30
40
50
60
Number of bacteria per tube (x10^9)

antigenic sites in a small area are simultaneously presented towards antibody molecules in solution. It is difficult to imagine how an antibody such as an anti-5 molecule specific for one of these sites can be more effective either in killing or as an opsonin than similar antibody directed against a different specificity only a few Angstroms from it.

Effects of various treatments on amount of available antigen

Because of the preliminary results suggesting that alcohol killed bacteria had up to 10 times more available antigen than untreated bacteria, and because it was known that tris buffer and EDTA have an effect on bacterial lipopolysaccharide (Leive, 1965), it was decided that it would be interesting to find what effect a number of different treatments had on the amount of antigen available on bacteria.

Bacteria were added to 5 ml. screw-topped bottles and treated as follows according to the protocol in table 7.

TABLE 7

Bottle No.	Volume used (ml) 1	2	3	4	5	6
C5 (1.15 10^9/ml.)	2	2	2	2	2	2
Saline	2	1	0.5	0.5	2	-
Tris HCl buffer 0.12M pH 8.0	-	1	1	1	-	-
EDTA 1.6 mg./ml.	-	-	0.5	0.5	-	-
Ethanol (50%)	-	-	-	-	-	2
Washed and resuspended	-	-	-	+	-	+
Heated 56° 1 hour	-	-	-	-	+	-
Final volume (ml.)	4	4	4	4	4	4

1. Untreated.
2. Tris/HCl buffer pH 8.0, final concentration 0.03M.
3. Tris/HCl buffer + disodium ethylenediaminetetra acetic acid (EDTA) pH 8.0, final concentration 0.2 mg/ml.
4. Tris/HCl + EDTA as in 3. Washed once in saline after 30 minutes.
5. Heated at 56°, 60 minutes.
6. Suspended in 50% alcohol for 30 minutes, washed once in saline.

Unless otherwise stated, the treated bacteria were diluted in saline at 4° to give concentrations in the range 2.3×10^{8} to 1.15×10^{9} bacteria/ml. and mixed with 0.2 ml. aliquots of 1/20 anti 5 serum. Serum was absorbed overnight and titrated against C5 LPS sensitized sheep red blood cells after centrifugation to remove the bacteria. The results of this absorption study are shown in Fig 15. It will be seen that in no case was there more than a twofold increase in the amount of available antigen 5. With the small number of determinations involved this difference cannot be regarded as significant. It was concluded, therefore, that treatment with ethanol, tris buffer, EDTA or heat did not expose much more O-antigen than was already available to antibody.

2. Reaction of bacteria with ^{131}I-labelled antibodies

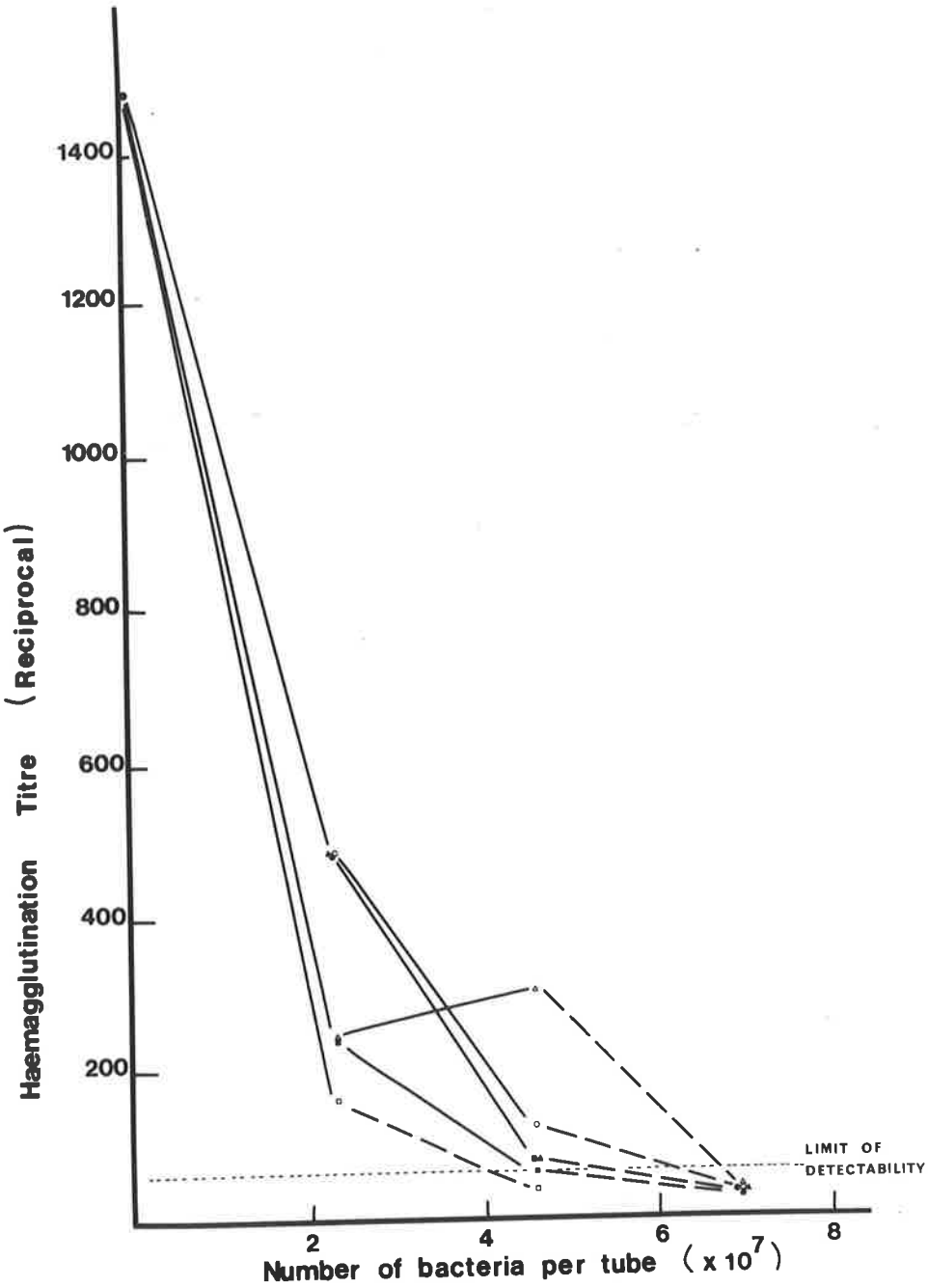
This section describes attempts to compare amounts of antigen by measuring uptake on the bacteria of radioactive labelled antibodies. Initial experiments used whole absorbed antisera labelled with ^{131}I by the chloramine-T method (page 110).

Most of the labelling experiments were carried out using modification of a standard technique.

Fig 15

Absorption of anti-5 serum by S.typhimurium C5 after various treatments

—●—●—	untreated cells
-■—■—	suspended in tris
-o—o—	suspended in tris + EDTA
—△—△—	suspended in tris + EDTA, washed and re suspended in saline
-▲—▲-	heated in saline at 56^{o} for 60 minutes
-□—□-	suspended in 50% alcohol for 30 minutes and washed once in saline


1400
1200
1000
800
600
400
200
Haemagglutination Titre (Reciprocal)
LIMIT OF DETECTABILITY
2
4
6
8
Number of bacteria per tube (x 10^7)

Suitable dilutions of ^{131}I-labelled antiserum mixed with the appropriate diluent or bacteria were filtered through 2 cm. membrane filters of pore size 0.45 μ with the assistance of a water pump. After washing, the whole filter membrane was removed, and inserted in a 3 x $\frac{1}{2}$" glass tube for counting on a Packard Auto Gamma well type sodium iodide crystal scintillation counter.

It was soon found that there was a very large non-specific uptake of radioactivity by the filter and bacteria. Consequently, a series of experiments were carried out which involved washing serum through filters to find some of the factors involved in this high background. These experiments were carried out over several weeks using several sera with different specific activities. For reasons which began to become apparent only towards the end of these studies, experiments carried out on different days did not give comparable results. Consequently the results below are quoted as pairs of experiments, each designed to measure one factor involved in non-specific adsorption of antiserum to membranes.

Effect of pretreatment of membrane

It seemed likely that protein was adsorbed non-specifically to the membrane. In these circumstances it should have been possible to block adsorption with unlabelled rabbit plasma or serum. Membranes were soaked in saline or in 10% heparinised normal rabbit plasma. 0.1 ml. of a 1/100 dilution of ^{131}I-labelled serum in saline was placed in the centre of a membrane filter, allowed to soak through it and washed under negative pressure with either saline or 1% normal rabbit plasma in saline. The results of this experiment are shown in table 8.

TABLE 8

Effect of non-specific adsorption of counts by a filter membrane of prior washing of the membrane with saline or normal rabbit plasma

Treatment	cpm used (a)	cpm retained (a)	percent cpm retained
Saline (b)	72,132	15,084	21
NRS (c)	72,132	8,856	12

(a) Counts were carried out for 10 seconds.

(b) The membrane was washed with saline before addition of antiserum. Antiserum was subsequently washed with 100 ml. saline.

(c) The membrane was soaked in 10% normal rabbit plasma in saline. After addition of antiserum it was washed with 100 ml. 1% normal rabbit plasma in saline.

These results indicated that it was preferable to use a cold non-specific protein both to soak the filter and to wash through the serum. Normal rabbit plasma was used because it was freely available and seemed most likely to resemble the labelled protein. However, it proved difficult to store without clotting and further experiments used normal rabbit serum (NRS) at lower concentrations until it was found that bovine serum albumin (BSA) was at least as good as, and possibly better than, rabbit serum for prevention of non-specific adsorption. Filters were soaked in 0.35% BSA in saline and washings were carried out with the same solution.

Effect of particles in serum

It seemed very likely that during the storage, iodination and subsequent handling of the serum some denaturation of protein occurred, and that this protein was being filtered out in the membrane. To find out whether this was so, 0.1 ml. of 1/100 dilution of an anti-4 serum in saline was placed in the centre of a membrane filter previously soaked in 10% normal rabbit plasma, allowed to soak into it, and washed under pressure with 100 ml. of normal rabbit plasma in saline. The counts shown in table 9 are those left on the filter by unfiltered antiserum and by antiserum which had been filtered through a 0.45 μ membrane

filter in a Sweeney adaptor. It is clear that the percentage of counts held by the filter when filtered serum was used is substantially less than the percentage when the serum is not filtered. It was further found that if filtered serum was kept overnight at 4°, another filtration was required on the following day.

TABLE 9

Effect on non-specific adsorption of counts by a filter membrane of prior filtration of labelled antiserum

Serum treatment	cpm used (a)	cpm retained (a)	percent cpm retained
None	72,132	8,856	12.3
Filtered	41,706	870	2.1

(a) Counts were carried out over 10 second intervals.

Effect of rate of filtration

A major problem in the use of negative pressure to suck protein solutions through membranes is that considerable frothing occurs. It was felt that this could cause denaturation of protein and its deposition in the filter. A number of experiments were carried out in which the radioactive serum was allowed to soak on to the

filter and the first few mls of the washing fluid were permitted to drip through under gravity. Table 10 shows the effect of allowing the serum and first 5 ml. of washing fluid to pass through the filter under gravity. 0.1 ml. of 1/100 filtered anti serum was washed with 200 ml. 0.3% NRS through a membrane soaked in 5% NRS.

A preliminary slow wash without suction appeared to reduce the amount of non-specific adsorption.

TABLE 10

Effect of non-specific adsorption of counts by a filter membrane of permitting most of the labelled serum to pass through under gravity before application of suction

Treatment	cpm used[a]	cpm retained[a]	percent cpm retained
Suction[b]	37,704	1,538	4
Gravity[c]	37,704	534	1

(a) Counts were carried out over 1 minute.

(b) The filter was washed with 200 ml. 0.3% BSA under negative pressure.

(c) 0.5 ml. 0.3% BSA was allowed to drip through under gravity. This was followed by 200 ml. 0.3% BSA under negative pressure.

It was later realised, during alterations to the water supply of the laboratory, that the suction pressure applied to the filter and the amount to which the filter was allowed to dry between additions of washing fluid, had a very large effect on the amount of radioactivity left on the filter. Table 11 shows the difference in counts obtained when the filter was washed slowly with small amounts of washing fluid, allowing the surface to dry before the addition of the next batch of fluid, and when the filter was washed rapidly and the surface not allowed to dry between each wash. 0.1 ml.1/100 anti-9 serum in 10 ml. suitable diluent were allowed to soak on to a membrane filter soaked in 0.35% BSA and washed either quickly or slowly with 200 ml. veronal saline (VS) buffer (0.03M veronal in 0.09M saline). Slow washing in this case caused a considerable fall in the amount of radioactivity remaining in the filter.

TABLE 11

Effect of non-specific adsorption of counts by a filter membrane of the rate at which labelled serum was washed through the membrane

Rate	cpm used[a]	cpm retained[a]	percent cpm retained
Fast[b]	313,300	5,818	2
Slow[c]	313,300	650	0.2

(a) Counts were carried out over 2 minute intervals.

(b) 0.1 ml. 1/100 labelled antiserum was diluted in 10 ml. 0.35% BSA and washed through the membrane with 200 ml. veronal saline, pH 8.7.

(c) 0.1 ml. 1/100 labelled antiserum diluted in 10 ml. VS pH 8.7 was washed through the membrane with 200 ml. of the same buffer.

Effect of pH

Labelled, filtered, antiserum (0.1 ml.) was suspended in 10 ml. of one of 3 buffers, phosphate buffered saline (PBS) pH 4.9, PBS pH 7.2 and veronal saline (VS) pH 8.7, and washed through the filter with 200 ml. of the corresponding buffer. Membranes were treated with 0.35% BSA. The results in table 12 show that there was little difference in the effect of washing at neutral or at alkaline pH, but that at low pH the amount of uptake of radioactivity increased. This suggested that protein was denatured at low, but not high, pH.

TABLE 12

Effect on non-specific adsorption of counts by a filter membrane of washing through labelled serum with buffers of different pH

Buffer used	cpm used[(a)]	cpm retained[(a)]	percent cpm retained
PBS pH 4.9	313,000	10,864	3
PBS pH 7.2	313,000	7,388	2
VS pH 8.7	313,000	5,818	2

(a) Counts were carried out over 2 minute intervals.

Effect of iodide

The possibility that some of the counts remaining on the filter came from free radioactive undialysed iodide was checked by washing the filter with cold 0.15M potassium iodide (table 13). No significant reduction was found in the uptake of radioactivity by the filter.

TABLE 13

Effect on non-specific adsorption of counts by a filter membrane of washing through labelled serum with cold potassium iodide

Diluent	cpm used[a]	cpm retained[a]	percent cpm retained
VS pH 8.7[b]	313,300	650	0.2
KI[c]	313,000	622	0.2

(a) Counts were measured over 2 minute intervals.

(b) 0.1 ml. 1/100 antiserum was pipetted into 20 ml. diluent made from 10 ml. of 3% BSA and 10 ml. 0.15M potassium iodide solution. It was washed slowly through a filter with 40 ml. 0.15M potassium iodide solution followed by 150 ml. VS pH 8.7.

(c) 0.1 ml. 1/100 labelled antiserum was diluted in 10 ml. VS pH 8.7 and washed slowly through the membrane with 200 ml. of the same buffer.

Uptake of ^{131}I-serum by bacteria

As a result of the experiments on washing labelled serum through filters, the following procedure was adopted for measuring the uptake of radioactive antibody by whole bacteria.

0.1 ml. of serum suitably diluted in 0.35% BSA in saline were added to 0.1 ml. washed overnight cultures of bacteria and left for 15 minutes. The mixture was diluted into 10 ml. 0.35% BSA,

filtered through a membrane soaked in 0.35% BSA and washed slowly with 200 ml. phosphate buffered saline at pH 7.1. The filter was then placed in a 3 x 3/8" glass tube for counting on the scintillation counter. Control filtrations of antiserum without bacteria were carried out simultaneously and the amount of radioactivity on the bacteria was calculated by subtracting the counts left on the filter alone from those on the filter with bacteria.

Initial experiments involved measurement of the uptake of labelled anti-5 serum by Citrobacter 396 and S.reading. These strains were chosen to show the maximum difference between bacteria which reacted with the antibody and those which did not. The anti-5 serum, as an immune serum against Citrobacter 396 absorbed with S.reading, contained antibodies against other antigens on Citrobacter besides antigen 5 (appendix 2). On the other hand, it should not have reacted with S.reading. The results of a number of experiments are summarized in table 14. It is clear that Citrobacter 396 took up more radioactivity than S.reading, especially when higher numbers of bacteria were used. Uptake of radioactivity by S.reading was nevertheless still appreciable. Possible reasons for this included incorporation

TABLE 14

Uptake of ^{131}I-labelled anti-5 serum by Citrobacter 396 and S.reading

Bacteria	No. of bacteria used (approx.)	c.p. 5 min. used	Uptake by filter (c.p. 5 min.)	Uptake by filter + bact. (c.p. 5 min.)	Uptake by bacteria (c.p. 5 min.)	Percentage of total counts taken up by bact.
Citrobacter	10^9	8,933	186	361	175	2
	"	54,252	619	1,317	698	1
	"	515,068	2,469	10,215	7,746	1.5
Citrobacter	5×10^7	45,663	300	573	273	0.6
	"	450,000	2,722	4,177	1,455	0.3
S.reading	10^9	5,779	127	131	4	0.007
	"	50,603	230	500	270	0.5
	"	505,255	2,260	2,519	259	0.05
S.reading	5×10^7	45,663	300	498	198	0.4
	"	450,000	2,722	3,629	907	0.2

of labelled protein by growing bacteria, uptake of ^{131}I-labelled lipids by the bacteria, and non-specific adsorption of labelled proteins. Heat-killed Citrobacter proved to take up as much non-specific radioactivity as live. It was decided, therefore, to try to use purified specific antibodies to label the bacteria.

Determination of optimum conditions for preparation of pure specific haemagglutinins from rabbit anti-Citrobacter sera

The method used for preparation of specific antibodies was a modification of the technique used by Webster and Laver (1966).

Experiments were carried out to find optimum conditions for elution of antibodies from bacterial ultrasonicates (US) with glycine buffer as described diagrammatically in Fig 15.

Absorbed normal rabbit serum (absorbed NRS) was prepared by mixing 2 ml. NRS with 200 µg. C5 US prepared as described in page 89. Bacterial fragments were removed by centrifugation of the mixture at 45,000 g for 30 minutes.

Rabbit anti-Citrobacter serum was mixed with 200 µg. C5 US, left at room temperature for 15 minutes, and then on ice for 30 minutes. The sample was divided into four. One part (Column D, Fig 15) was used to test the value of separating ultrasonicate from serum by filtration on a 0.45µ Millipore filter, rather than the usual centrifugation technique. The other three parts (Columns A - C, Fig 15)

Fig 16

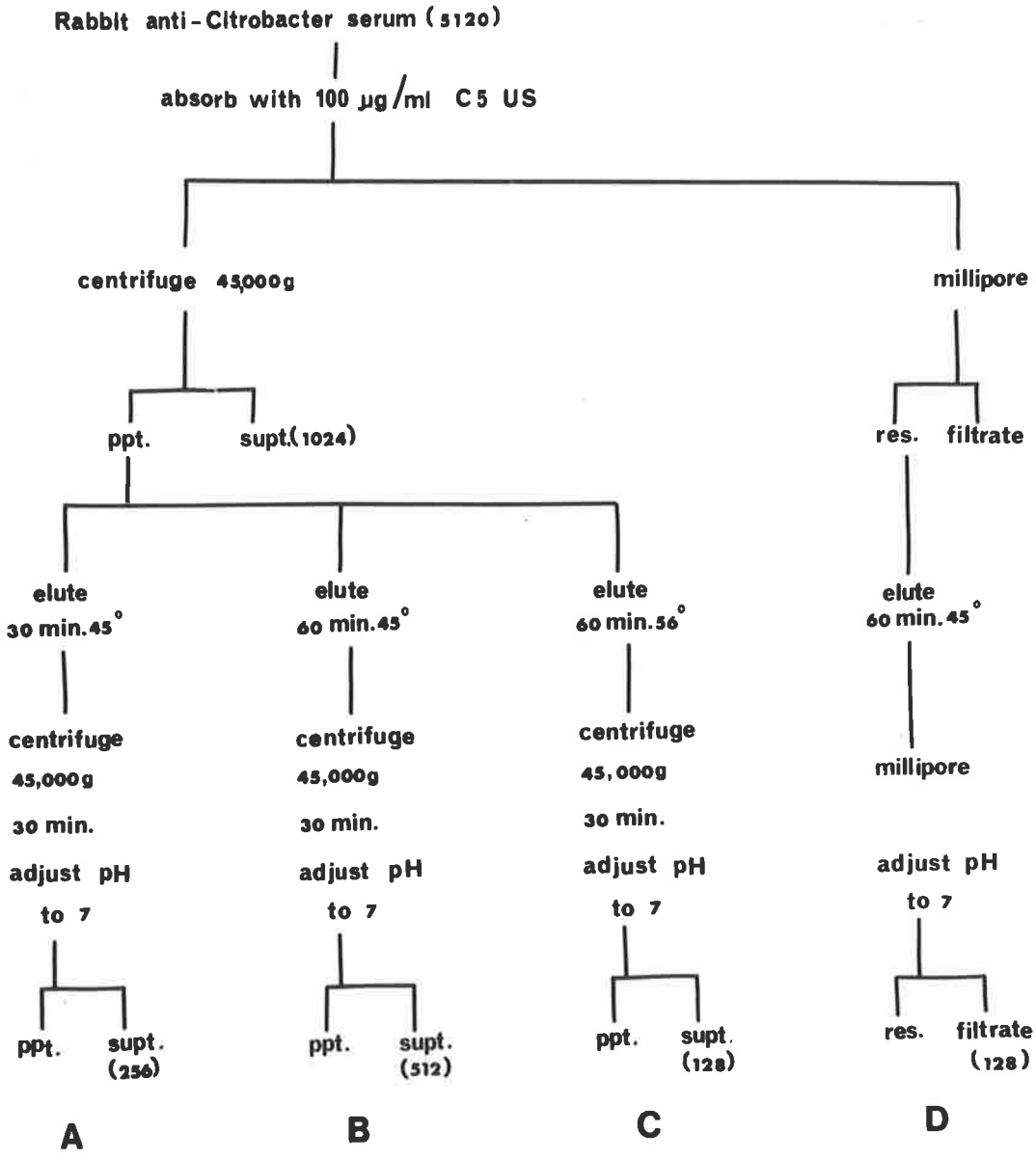
Test of methods for elution of haemagglutinins from bacterial ultrasonicates

For explanation see text.

Numbers in parenthesis represent haemagglutination titres against sheep red blood cells sensitized with untreated C5 lipopolysaccharide.

Abbreviations

US	ultrasonicate
ppt	precipitate
supt	supernatant
res	residue


Rabbit anti-Citrobacter serum (5120)
absorb with 100 µg/ml C5 US
centrifuge 45,000g
millipore
ppt.
supt.(1024)
res.
filtrate
elute 30 min. 45°
elute 60 min. 45°
elute 60 min. 56°
elute 60 min. 45°
centrifuge 45,000g 30 min.
centrifuge 45,000g 30 min.
centrifuge 45,000g 30 min.
millipore
adjust pH to 7
adjust pH to 7
adjust pH to 7
adjust pH to 7
ppt.
supt. (256)
ppt.
supt. (512)
ppt.
supt. (128)
res.
filtrate (128)
A
B
C
D

were used to test the value of different temperatures and times for elution of antibodies from ultrasonicates.

US was separated from serum by filtration or centrifugation at 45,000 g for 30 minutes in the Spinco model L centrifuge. Supernatants were retained for titration. The residues were suspended in 2 ml. glycine HCl buffer (0.1M, pH 3.0) containing 1% absorbed NRS and were incubated for the times and at the temperatures indicated in Fig 15. After elution ultrasonicates were again separated from the eluate by centrifugation or filtration and the eluates were immediately adjusted to pH 7 by addition of 2M disodium hydrogen phosphate. Eluates, the original serum, and the absorbed sera were titrated against sheep red blood cells sensitized with untreated C5 LPS with the results shown in parenthesis in Fig 15. It is clear that filtration offered no advantage over centrifugation in the preparation of specific haemagglutinins. Furthermore, elution for 1 hour at 45° was superior to any of the other methods tested.

Preparation of specifically labelled ^{131}I anti-5 antibody

Specific antibodies were finally isolated and labelled by a modification of a method described by R.G. Webster (personal communication).

Single factor anti-5 serum (1 ml.) containing 8,000 haemagglutinating units versus sheep red cells sensitized with native C5 lipopolysaccharide was absorbed with 400 µg. of C5 LPS in 1 ml. saline. After 30 minutes at room temperature the serum was centrifuged at 37,000 g for 40 minutes. By this means the haemagglutinating activity of the supernatant had fallen to 400 units, i.e. 95% of the activity had been absorbed. The minute pellet of lipopolysaccharide was resuspended with the help of 10 seconds treatment in the ultrasonic apparatus in 1 ml. of 0.1M glycine/HCl buffer containing 0.5% normal rabbit serum at pH 3, and the suspension was maintained for 1 hour at 45°. A further centrifugation at 37,000 g followed and the supernatant was found to contain 1,600 haemagglutinating units, i.e. 20% of the absorbed antibody had been eluted. This was labelled with 1 ml. of ^{131}I by the hydrogen peroxide method (page 112). The iodination mixture was dialysed against saline overnight, reabsorbed with 100 µg, of C5 lipopolysaccharide for 30 minutes at room temperature as before, centrifuged, and the deposit eluted with 1 ml. 0.1M glycine/HCl buffer containing 0.5% normal rabbit serum, exactly as in the first cycle. The final supernatant from centrifugation was adjusted to pH 7.2 with 2M phosphate.

Labelling of C5 and M206 with ^{131}I-labelled antibodies

The ^{131}I-labelled anti-5 serum contained 16-24 units of haemagglutinating activity and gave approximately 10^6 counts/ml./minute. It was absorbed with a culture of S.typhimurium strain ST11, which lacks antigen 5, to remove non-specifically absorbable protein which might have been formed during these procedures, and passed twice through millipore filters, 0.45μ pore size, previously saturated with 1% gelatine. These steps, although necessary, reduced the radioactivity to 3×10^5 counts/ml./minute, and the specific antibody content to 16 haemagglutinating units at the best. Different weights of washed saline suspensions of living C5 or M206 were measured into 3 x ½" glass tubes, together with a constant amount of the filtered labelled antibody solution and made up to a constant volume of 1 ml. in saline. These suspensions were left at room temperature for 30 minutes to allow absorption of antibody, and during this time all of the tubes were counted in the scintillation counter. The contents of each tube were then filtered through a 0.45μ 2 cm. diameter Millipore filter previously washed with 0.1% gelatine. The whole filter membrane was removed and inserted in a 3 x 3/8" glass tube

for counting. Control tubes with antibody and no organisms were included in each run and the average counts retained by the filter in the control tubes were subtracted from the counts on the other tubes to give a measure of the radioactivity taken up by the bacteria alone.

When the uptake of ^{131}I anti-5 antibody by C5 and M206 was measured by these methods, the results obtained were still unsatisfactory. Table 15 shows the results of one such experiment.

TABLE 15

Uptake of ^{131}I-labelled anti-5 serum by live C5 and M206 bacteria(a)

Strain	Weight of absorbing suspension mg.	Uptake (cpm)
C5	1.2	530
	0.7	187
M206	1.3	380
	0.8	96

(a) Each assay tube contained 0.08 haemagglutinating units of antibody labelled with 1,600 cpm.

It was consistently found that C5 had more antigen than M206, but results on different days were too variable to obtain equilibrium data.

The results obtained by measuring uptake of radioactive antibody complement those obtained by absorption. It seemed, however, that information on the absolute numbers of sites on bacteria was unlikely to be obtained by this method unless much more antiserum was available, and unless the filtration technique could be improved. It is likely that much of the variability encountered between results obtained on different days was attributable to variation in water pressure and in washing technique. If more reproducable results are obtained by the use of methods similar to the ones described here, it is likely that the filtration apparatus will have been altered to one using controlled positive pressure, as described by Fazekas de St Groth and Webster (1961).

Summary

The results presented in this chapter indicate that C5 has approximately twice as much of the three O-antigens 4, 5 and 12 as M206. These antigens are to a large extent situated close to each other on the bacterial surface. As they may all be parts of the same molecule this probably means that C5 has twice as much surface lipopolysaccharide as M206.

CHAPTER 5

ESTIMATION OF THE ABSOLUTE NUMBERS OF AVAILABLE ANTIGENIC SITES ON S.TYPHIMURIUM C5 AND M206

Introduction

The amount of antibody which can be absorbed from a given antiserum by a given number of bacteria is dependant on the absolute numbers of antigenic sites on these bacteria and on the avidity of the antibody for these sites. Theoretically it should be possible to use absorption data, such as the data used in Fig 13 of chapter 4 to calculate the exact number of antigenic sites and the equilibrium constants of the antibodies involved. This chapter describes an attempt to extract this information from data in chapter 4. The experiments were not specifically designed to provide this information, and the results obtained are very imprecise. However they shed a little light on the nature of the surface of S.typhimurium, and give some information on the nature of the antibodies which were used in these experiments, and for this reason the calculations are included.

Fazekas de St Groth (1961) using simple equilibrium considerations derived two equations which could be used to measure numbers of antigenic sites on a virus from absorption data. These equations are valid only if the reaction of antibody with an antigenic

site has no effect on adjacent sites. This is probably valid for the influenza viruses with which he was concerned, but is certainly not true for Salmonella O-antigens, in which the antigens are polymerised into large macromolecules and must lie very close to each other. A small modification to Fazekas de St Groth's equations (appendix 1) shows that they measure not the total number of antigenic sites available to antibody but the total number of antibody molecules which can be squeezed on to the bacteria if they are reacting with their specific sites. The number of surface antigenic sites on a bacterium estimated from these equations is therefore likely to be very much lower than the actual number present, and the number estimated when large antibody molecules such as IgM are used will be less than the number when smaller molecules such as IgG are employed. This may explain, for example, the finding of Greenbury et al (1963) that there were apparently only 1/5 as many antigen A sites on human red blood cells available to IgM antibodies as there were to IgG.

If antigens are packed closely on the bacterial surface it is likely that individual molecules will react at more than one site. This is unlikely to affect the validity of the equations of

appendix 1 in determining the number of antigenic sites available, as formally there is probably not a great deal of difference between the number of sites blocked by an antibody attached at one end, and the number blocked when it is attached by two or more ends. Combination at more than one site may in fact improve the validity of the equations by reducing the likelihood of cross-linking between bacteria. However attachment at more than one site must make a great deal of difference to the value estimated for the equilibrium constant of the reaction. While the rate of the initial reaction of antibody with bacteria will depend on the affinity of the antibody for the antigen and their concentrations in solution, subsequent reactions must occur in a position where the concentration of antigen is in effect greatly increased, and may be expected to occur much more rapidly. The equilibrium will shift considerably in the direction of combination between antibody and antigen, and the values obtained for equilibrium constants when whole bacteria are used must be related to the avidity of the whole antibody for the particular strain rather than to the affinities of individual antibody combining sites and antigenic determinants for each other. Avidities will thus tend to be higher than affinities determined for the reaction of antibody with oligosaccharide haptens. It is

interesting to note that this implies that the dependance of the equilibrium constant on the concentration of antigen on a bacterium can lead to errors in comparisons of amounts of surface antigen such as those of chapter 4 if the differences in these concentrations are very large.

These considerations show that in theory at least, intelligible results should be obtainable from absorption data. The practical problems in using the data of chapter 4 are still, however, considerable. The antisera used contained antibodies of more than one class. Quantitative precipitin data give an estimate of the total amount of antibody available. This presumably includes an undetermined amount of IgA, which cannot be assumed to play a part in haemagglutination even though it competes with other antibodies for sites on the bacteria. The results of quantitative precipitin determinations may therefore give too high an estimate of the amounts of antibody being measured by haemagglutination, while absorption data will underestimate the number of antigenic sites available. Even more serious is the use of a mixture of two haemagglutinating antibodies, IgG and IgM, one of which has a molecular weight about 6 times that of the other and, as discussed in chapter 2, is likely to have a very different avidity for the

bacteria. The problem of molecular weight may be less significant than first appears, because Greenbury et al's data suggest that the increase in the number of sites blocked by antibody of higher molecular weight may be roughly proportional to the increase in molecular weight. If it could be assumed, therefore, that the avidities of the two antibodies were the same, then there might be little difference in the estimated number of sites whether it was assumed that the antibody was all IgM or IgG. The problem of avidity is more difficult, as conflicting results have been reported in the literature. Where most of the haemagglutinating activity was due to IgM it was assumed that all of it was due to IgM. The major error here results from inhibition of IgM absorption by IgG molecules, so that the estimate of the number of combining sites is likely to be rather low. If almost equal amounts of IgM and IgG activity are present the most useful approach seems to be to accept the results obtained using Salmonellae (J.B. Robbins et al, 1965). It is assumed that the initial amount of antibody present is only half the amount measured, and the data is treated as if the antiserum contained only IgM. This is equivalent to saying that the first 50% of haemagglutinating activity to be absorbed is all due to high avidity IgG. As the

contribution of IgM to quantitative precipitin data is likely to be very low, the number of molecules of antibody used will be an overestimate, and the estimate of numbers of antigenic sites is likely to be too high.

No attempt is made here to test the problem of variability of avidity within a single class of antibodies. The measurements are neither sufficiently accurate nor extensive enough to warrant such refinements.

The equations of appendix 1 use a value α which is equivalent to the fraction of antibody which has reacted with the antigen. This value depends very heavily on the original estimate of the amount of antibody activity present. Here this estimate was made using a haemagglutination assay depending on doubling dilutions, in which a two-fold error can very easily occur. It was thought useful, therefore, to use the data in a series of calculations of the two regression lines derived in appendix 1 in which the initial titre was allowed to vary from half its original estimated value to double that value. In all, sixteen calculations were carried out on each equation for each set of data, using evenly spaced estimates of the initial titre. To assist in choosing the best calculated value obtainable from each experiment the product

moment correlation coefficient (R) was calculated for each line. This has a positive value if the gradient of the line is positive, and a negative value if the gradient is negative. Straight lines which describe perfectly the data for which they are calculated have correlation coefficients of +1 or -1. Both of the straight lines of appendix 1 should have positive gradients. The most suitable values of modified titre were selected as those which gave both lines with a correlation coefficient greater than +0.6.

Fazekas de St Groth (1961) showed that his equations were most reliable when more than 90% of the antibody activity was absorbed. If less was absorbed, errors in calculations became very high. Little of the data available in chapter 4 fits this requirement. To try to eliminate the greatest errors values of remaining activity of 50% of the original and over were omitted.

The calculation involved in estimating numbers of antigenic sites and equilibrium constants are long and tedious. For this reason Mr. A. Osmand very kindly designed a computer program which used the data available to calculate best fitting straight lines for both equations, and standard errors for their gradients and intercepts. It uses each line to estimate the number of antigenic sites and equilibrium constants, along with their

standard errors, and also calculates a value (K2) for the equilibrium constant which uses the gradients of both lines and does not require a rather inaccurately estimated intercept (appendix 1).

Estimation of total antibody used for absorption experiments

Quantitative precipitin reactions were carried out as described on page 109. The results of these reactions are shown in Fig 17 and table 16.

TABLE 16

Antibody precipitable by C5 lipopolysaccharide from specific antibody

Antiserum	Precipitable protein (BSA mg equivalents per ml)	Number of molecules of antibody per ml assuming a) all IgG	b) all IgM
4	1.54	5.78×10^{15}	9.32×10^{14}
5	1.32	4.95×10^{15}	7.92×10^{14}
12	1.44	5.40×10^{15}	8.64×10^{14}

Estimation of the proportion of each class of antibody involved in haemagglutination

The effect of 2-mercaptoethanol treatment on haemagglutination of sheep red blood cells sensitized with C5 lipopolysaccharide was measured.

Fig 17

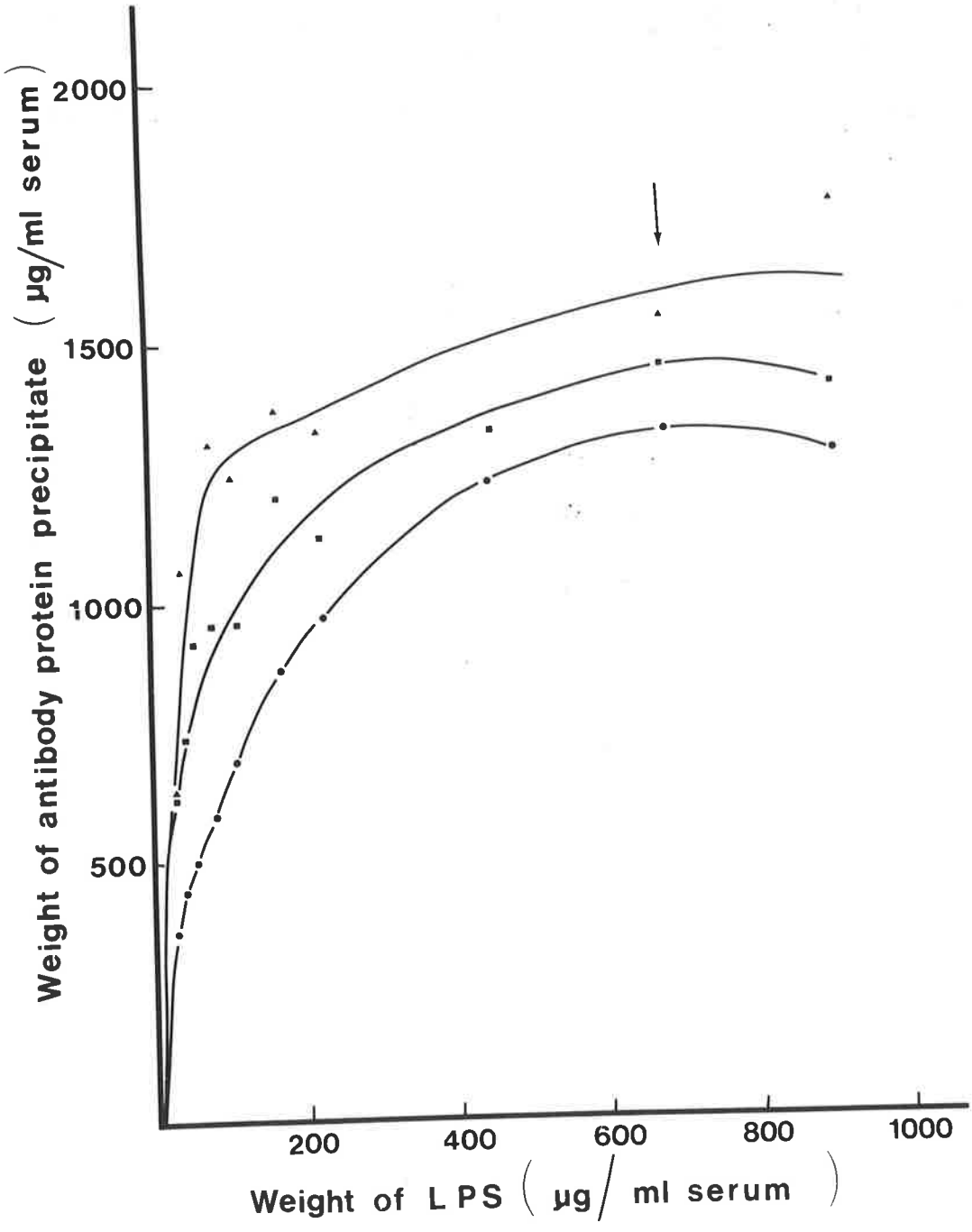
Results of quantitative precipitin tests on anti-4,-5 and -12 sera using S.typhimurium C5 lipopolysaccharide

Antiserum (0.2 ml.) was mixed with various dilutions of LPS to give a final volume of 0.3 ml. Precipitated antibody protein was measured by the Folin-Lowry method and compared with a standard curve prepared against bovine serum albumin.

—▲—▲— anti-4

—●—●— anti-5

—■—■— anti-12


Weight of antibody protein precipitate (µg/ml serum)
2000
1500
1000
500
200
400
600
800
1000
Weight of LPS (µg/ml serum)

Serum (0.3 ml.) was mixed in a 10 ml. screw-topped bottle with either 0.3 ml. saline or 0.3 ml. 0.2 M 2-mercaptoethanol in saline. The mixture was incubated for 1 hour at 37°, diluted with 5.4 ml. saline containing magnesium chloride (0.2 M) and used at that dilution for a haemagglutination titration as described on page 90. The results of this titration are shown in table 17.

TABLE 17

Haemagglutination titres[(a)] on C5 LPS sensitized sheep red blood cells of anti-4, 5 and 12 sera before and after treatment with 2-mercaptoethanol

Antiserum	Treatment	
	None	Mercaptoethanol
4	2560	1280
5	896	80
12	1280	640

(a) Results are quoted as geometric means from two determinations.

It is noticeable that the haemagglutinating activities of the antisera are different from those of chapter 4. This is probably because different batches of sensitized cells were used in each case.

Antisera against antigens 4 and 12 contained a much higher proportion of mercaptoethanol resistant haemagglutinating antibody (IgG) than did the antiserum against 5. It was decided to use the anti-4 and 12 sera as if they contained only IgM antibodies giving a titre of half the figure found experimentally, and the anti-5 also as if it contained only specific IgM.

Use of absorption data to calculate absolute numbers of antigenic sites

The computer program and likely results of variations in initial titre were first tested on the original data of Fazekas de St Groth (1961). He quotes an example in which a standard volume of antiserum containing 1.64×10^3 molecules of antibody was absorbed with a series of dilutions of virus from an original sample containing 8.15×10^{10} virus particles. Ten separate absorptions were carried out, all of them removing more than 90% of the activity of the serum as measured by a titration method. Standard statistical works show that the number of degrees of freedom available in calculating the intercepts and gradients of the lines are 8 and 9 respectively, corresponding to values of 2.31 and 2.26 for 5% probability. These are listed under TVEA and TVEB in the data input.

TABLE 18

Abbreviated computer print-out for estimation of the number of antigenic sites on a virus (data from Fazekas de St Groth, 1961) (using equation 10, appendix 1)

I	Modif.titre	Correl.coeff.	Equil.const.	Error	Antigenic sites	Error	K2
1	1774	.967	6.08E+11	-2.72E+11	2.00E+03	4.21E+02	
2	2129	.970	5.54E+11	-2.44E+11	2.10E+03	4.23E+02	
3	2484	.971	5.05E+11	-2.21E+11	2.17E+03	4.27E+02	
4	2838	.972	4.61E+11	-2.03E+11	2.22E+03	4.31E+02	
5	3193	.972	4.24E+11	-1.86E+11	2.27E+03	4.35E+02	
6	3548	.973	3.92E+11	-1.73E+11	2.30E+03	4.38E+02	
7	3903	.973	3.63E+11	-1.61E+11	2.33E+03	4.41E+02	
8	4258	.973	3.39E+11	-1.50E+11	2.35E+03	4.43E+02	
9	4612	.974	3.17E+11	-1.41E+11	2.37E+03	4.45E+02	
10	4967	.974	2.98E+11	-1.33E+11	2.39E+03	4.47E+02	
11	5322	.974	2.81E+11	-1.25E+11	2.41E+03	4.48E+02	
12	5677	.974	2.66E+11	-1.19E+11	2.42E+03	4.50E+02	
13	6032	.974	2.53E+11	-1.13E+11	2.43E+03	4.51E+02	
14	6386	.974	2.40E+11	-1.08E+11	2.44E+03	4.52E+02	
15	6741	.974	2.29E+11	-1.03E+11	2.45E+03	4.53E+02	
16	7096	.974	2.19E+11	-9.83E+10	2.46E+03	4.54E+02	

continued

TABLE 18 (continued)

Abbreviated computer print-out for estimation of the number of antigenic sites on a virus (data from Fazekas de St Groth, 1961) (using equation 11, appendix 1)

I	Modif.titre	Correl.coeff.	Equil.const.	Error	Antigenic sites	Error	K2
1	1774	.997	5.70E+11	1.42E+11	1.97E+03	1.12E+02	5.97E+11
2	2129	.997	5.33E+11	1.30E+11	2.07E+03	1.26E+02	5.47E+11
3	2484	.997	4.92E+11	1.20E+11	2.15E+03	1.38E+02	5.00E+11
4	2838	.997	4.54E+11	1.10E+11	2.21E+03	1.48E+02	4.58E+11
5	3193	.996	4.20E+11	1.02E+11	2.26E+03	1.56E+02	4.22E+11
6	3548	.996	3.89E+11	9.51E+10	2.29E+03	1.62E+02	3.90E+11
7	3903	.996	3.63E+11	8.89E+10	2.33E+03	1.68E+02	3.63E+11
8	4258	.996	3.39E+11	8.33E+10	2.35E+03	1.72E+02	3.39E+11
9	4612	.996	3.18E+11	7.84E+10	2.37E+03	1.77E+02	3.17E+11
10	4967	.996	3.00E+11	7.40E+10	2.39E+03	1.80E+02	2.99E+11
11	5322	.995	2.83E+11	7.00E+10	2.41E+03	1.83E+02	2.82E+11
12	5677	.995	2.68E+11	6.64E+10	2.42E+03	1.86E+02	2.67E+11
13	6032	.995	2.55E+11	6.32E+10	2.44E+03	1.89E+02	2.53E+11
14	6386	.995	2.43E+11	6.03E+10	2.45E+03	1.91E+02	2.41E+11
15	6741	.995	2.32E+11	5.76E+10	2.46E+03	1.93E+02	2.30E+11
16	7096	.995	2.21E+11	5.51E+10	2.47E+03	1.95E+02	2.19E+11

Table 18 shows an abbreviated version of the computer print-out when it was given Fazekas de St Groth's data.

The first 16 rows correspond to results obtained using the equation

$$\frac{1}{1-\alpha} = \frac{S.B_o}{K} \cdot \frac{d}{\alpha} - \frac{nA}{K}$$ (equation 10, appendix 1)

while the second 16 rows give results for the equation

$$1-\alpha = \frac{S}{n} \cdot \frac{B_o}{A} \cdot \frac{(1-\alpha)d}{\alpha} - \frac{K}{nA}$$ (equation 11, appendix 1)

where α represents the fraction of the original antibody which has reacted, S the number of antigenic sites on one bacterium, B_o the number of bacteria present in the original stock solution before dilution, d the dilution of bacteria used, n the number of antigenic sites blocked when an antibody molecule reacts with one site, and K the dissociation constant for the reaction of antigen with antibody (appendix 1). The column K2 is calculated using both equations.

The figures for I=6 correspond to those obtainable with an unadjusted titre, and in every instance correspond well with the values

$$S = 2.268 \pm 0.212 \times 10^{3}$$

$$K = 3.75 \pm 1.20 \times 10^{11}$$

calculated by Fazekas de St Groth. It is noticeable that even when adjusted values for the initial titre were used the correlation

coefficient (R) was still in the range 0.967 - 0.995. The best values for R were at I = 9 and 10, and did not correspond to the true value for the initial titre. In other words use of the correlation coefficient does not guarantee using the 'correct' value of initial titre. It merely helps to eliminate unacceptable values. Even after adjustment of the initial titre the estimates of the number of antigenic sites were all within the range 1.9 - 2.5×10^3 and the equilibrium constants in the range 2.2 - 6.0×10^{11}.

When data from chapter 4 was used in the same program the results obtained were much more variable and difficult to interpret. It is likely that this was primarily due to the use of data from absorptions in which much less than 90% of the activity was removed. Table 19 shows a typical set of calculations using the data summarized in Fig 13 obtained when anti-4 serum was absorbed by C5. Inspection of the correlation coefficients showed immediately that nonsensical results were obtained for values of I between 1 and 10. Reasonable values for R in both equations could be obtained only if the initial haemagglutination titre lay between 1/7687 and 1/10,250. Inspection of the size of the 5% probability limits in estimating the number of antigenic sites shows that they were of the same order as or slightly

TABLE 19

Abbreviated computer print-out for estimation of numbers of antigen 4 sites on S.typhimurium C5 (using equation 10, appendix 1)

I	Modif.titre	Correl.coeff.	Equil.const.	Error	Antigenic sites	Error	K2
1	2563	.696	-7.19E+12	-1.63E+12	-7.37E+01	-7.98E+01	
2	3075	-.245	-5.87E+12	-1.95E+12	7.30E+00	-3.03E+01	
3	3587	.252	-6.06E+12	-2.30E+12	-4.53E+01	-1.82E+02	
4	4100	.508	-5.23E+12	-1.56E+12	-8.33E+01	-1.48E+02	
5	4612	-.346	-3.99E+12	-1.16E+12	1.01E+01	-2.87E+01	
6	5125	-.491	-3.50E+12	-9.47E+11	6.74E+00	-1.26E+01	
7	5637	-.631	-2.11E+12	-7.26E+11	1.74E+02	-2.24E+02	
8	6150	-.512	-1.46E+12	-7.03E+11	2.93E+02	-5.15E+02	
9	6662	-.081	-2.27E+12	-1.68E+12	1.40E+02	-1.80E+03	
10	7175	.397	5.48E+12	6.40E+12	1.95E+03	4.72E+03	
11	7687	.628	2.50E+12	4.20E+12	1.36E+03	1.77E+03	
12	8200	.728	2.35E+12	5.00E+12	1.42E+03	1.40E+03	
13	8712	.780	2.43E+12	5.94E+12	1.54E+03	1.30E+03	
14	9225	.810	2.55E+12	6.68E+12	1.68E+03	1.27E+03	
15	9737	.830	2.67E+12	7.19E+12	1.82E+03	1.28E+03	
16	10250	.844	2.79E+12	7.50E+12	1.96E+03	1.30E+03	

continued

TABLE 19 (continued)

Abbreviated computer print-out for estimation of numbers of antigen 4 sites on S.typhimurium C5 (using equation 11, appendix 1)

I	Modif.titre	Correl.coeff.	Equil.const.	Error	Antigenic sites	Error	K2
1	2563	-.369	-8.55E+12	4.18E+12	-5.08E+01	1.34E+02	-4.95E+12
2	3075	-.042	-7.85E+12	4.25E+12	-1.31E+00	3.30E+01	1.06E+12
3	3587	-.428	-7.34E+12	3.00E+12	-6.07E+01	1.35E+02	-8.12E+12
4	4100	-.636	-5.90E+12	1.95E+12	-7.47E+01	9.50E+01	-4.69E+12
5	4612	.400	-4.80E+12	2.21E+12	8.00E+00	1.92E+01	-3.17E+12
6	5125	.681	-4.06E+12	1.61E+12	5.93E+00	6.70E+00	-3.08E+12
7	5637	.918	-2.29E+12	1.11E+12	1.76E+02	7.96E+01	-2.13E+12
8	6150	.965	-1.22E+12	8.43E+11	3.50E+02	9.96E+01	-1.75E+12
9	6662	.976	-5.01E+11	7.61E+11	5.20E+02	1.19E+02	-8.29E+12
10	7175	.974	6.43E+09	8.34E+11	6.60E+02	1.60E+02	1.86E+12
11	7687	.966	3.68E+11	9.88E+11	7.94E+02	2.22E+02	1.47E+12
12	8200	.955	6.25E+11	1.16E+12	9.13E+02	2.98E+02	1.51E+12
13	8712	.942	8.07E+11	1.33E+12	1.02E+03	3.82E+02	1.61E+12
14	9225	.928	9.33E+11	1.48E+12	1.11E+03	4.70E+02	1.69E+12
15	9737	.913	1.02E+12	1.61E+12	1.20E+03	5.60E+02	1.76E+12
16	10250	.899	1.07E+12	1.72E+12	1.27E+03	6.50E+02	1.80E+12

higher than the actual estimates, and that the magnitudes of the limits obtained using the first equations were greater than those using the second. The least unreliable estimates of the number of antigenic sites were therefore those obtained using the second equation.

As the percentage error in estimating the number of antigenic sites from the second equation was less than the error in estimating the equilibrium constant from the first the second value of the number of antigenic sites was used to calculate the value of the equilibrium constant from the gradient of the first line, and the results of the calculation are shown under column K2. The estimates of the number of antigenic sites range from 800-1200, with estimated 5% probability limits ranging from 25-50% of these values. The estimates for the value of the equilibrium constant is 14-18 x 10^{11}, with a 5% probability limit of $\pm$ more than 100% of that value.

Table 20 summarizes the results obtained by using similar arguments to extract data from the other absorption experiments of chapter 4. It was disappointing to find that the numbers of the modified titres (I) necessary to obtain straight lines from absorption data using M206 were invariably different from those guessed as

TABLE 20

Estimates from absorption of haemagglutinating activity of numbers of antigenic sites on C5 and M206 and equilibrium constants for the antigen/antibody reactions

Antiserum	Strain	Number of modified titre (I)	Number of antigenic sites ($\times 10^3$)		Equilibrium constant (K) ($\times 10^{12}$)
4	C5	11 - 16	1 - 2	($\pm$ 1-2)	1-2
4	M206	4 - 7	3 - 8	($\pm$ 1-9)	2-5
5	C5	2 - 3	6 - 10	($\pm$ 3-8)	2-3
5	M206	9 - 16	3 - 4	($\pm$ 2-3)	1-9
12	C5	8	0.7	($\pm$ 0.5)	4
12	M206	10 - 16	0.3 -2	($\pm$ 0.2-2)	2-5

suitable for the C5 data. There seems to be no reasonable theoretical explanation. The data are simply not good enough for it to be possible to draw good straight lines. Estimated errors are often more than the 100% of the estimated values. This means that lower limits to the number of available antigens often cannot be set, although upper limits may still be defined. In spite of these problems it seems reasonable to draw at least some tentative conclusions. The data suggest that the numbers of 5 sites on the two bacteria are of the order of 3-10,000, while the numbers of 4 and 12 sites are rather less, and may be in the hundreds. There are likely, therefore, to be slightly more antigen 5 sites than 4 on both bacteria, and more of both of these than 12.

The figure of just under 10,000 as a maximum number of available sites is interesting. It corresponds well with the value calculated by Muschel and Treffers (1956) for the maximum number of IgG antibodies which can be packed on to an organism of Salmonella typhi if it is assumed that the antibodies are evenly spread over a smooth surface. It seems very likely that this figure is obtained because the surface antigen is distributed in this way. It lends no support to the notion that there may be long streamers of antigenic lipopolysaccharide trailing from the bacteria.

The fact that the maximum possible number of antigenic sites seems to have been approached by both the 4 and 5 antigens suggests that there may in fact be differences in the amounts of each but that these are masked to a great extent by the numbers of antigens blocked when an antibody reacts. Appendix 1 shows that the estimate of the number of antigenic sites on the surface is not the true value, S, but that value divided by the number of sites blocked when antibody reacts. If the concentration of one antigen is twice that of another, then one may expect that an antibody reacting with that antigen will block twice as many sites as an antigen reacting with the other, so that the final estimate of the numbers of available sites will be the same, even though the avidity constants may be different. The magnitude of the error which can be obtained in this way depends entirely on the number of sites which can be blocked by one antigen antibody reaction.

The estimated errors in the dissociation constants are mostly larger than the actual values found. They give the impression, however, that the values for K are likely to be of the order of 10^{12}. It is useful at this point to consider what this value means in terms of the more frequently quoted avidity. K is derived from the

equation

$$K = \frac{[A]\,[S]}{[AS]}$$

where [A], [S], and [AS] represent the concentrations of antigen, antibody and antigen antibody complex in molecules per unit volume. In the absorption experiments described here it has units of molecules per 0.4 ml. and can be multiplied by 1/0.4 to bring it to a more conventional figure of molecules per ml. A K value of 1×10^{12} therefore corresponds to a figure of 2.5×10^{12} molecules per ml. This is equivalent to

$$\frac{2.5 \times 10^{12} \times 10^{13}}{6 \times 10^{23}} = 4 \times 10^{-7} \text{ moles per litre}$$

The avidity constant is the reciprocal of this value

$$\frac{1}{4 \times 10^{-7}} = 2.5 \times 10^{6} \text{ litres per mole.}$$

Values quoted for the affinities of antibodies for oligosaccharide antigens are of the order 10^5 (Karush, 1957). It seems likely that higher values are due either to a larger combining site, to multiple binding by antibody as was postulated on page 146 in the introduction to this chapter, or to the use of IgM antibodies, rather than the IgG antibodies which Karush seems to have employed.

Summary

In this chapter, and in appendix 1, problems in estimating the total numbers of available antigens on Salmonellae are discussed. In spite of its basic unsuitability for the purpose, data from chapter 4 are used in an attempt to obtain an absolute value for the number of antigenic sites on C5 and M206. A computer program failed to solve the problems posed by inadequate data. However, the number of available 4, 5 and 12 sites were tentatively arranged in the order 12, 4 and 5, in increasing order of antigenic sites per bacterium, and the numbers of each seemed likely to be of the order 1-10,000. This was taken to mean that surface polysaccharide is more or less evenly spread in a single layer over the bacteria. Estimates of the equilibria between antigens and antibodies gave avidities which were rather higher than the usual range of affinities for polysaccharide antigens, suggesting that single antibodies were reacting at more than one antigenic site.

CHAPTER 6

ESTIMATION OF THE TOTAL AMOUNTS OF ANTIGEN ON A NUMBER OF SALMONELLAE

Introduction

The absorption studies described in chapter 4 showed that C5 had more antigen available on its surface than M206. They did not necessarily give a measure of the total amounts of antigen present on the 2 strains, as it is possible that much of the O-polysaccharide is prevented from reacting with antibody by other cell wall components. In a first attempt to compare the total amounts of O-antigen on C5 and M206 a comparison was made of the abilities of these two organisms to absorb antibody from serum after they had been treated with ultrasound (Archer and Rowley, 1969). The experiments indicated that C5 had twice as much O-antigen as M206. Absorption experiments could be criticised on the grounds that a cell wall component which blocked access of antibody to polysaccharide in the original cell wall was still likely to associate with polysaccharide in the mixture after treatment with ultrasound and thereby continue to inhibit the antigen/antibody reaction. Further, it was not known how much antigen was blocked by reaction with antibody. Because of this ambiguity it was decided to try to estimate the amount of

antigen by chemical determination in whole organisms of the amount of one of the monosaccharides which was characteristic of the O subunit. Rhamnose was chosen as a suitable sugar for a number of reasons. The structure of the polysaccharide in both group B and D salmonellae is such that there must be at least one rhamnose residue for every group of antigenic sites (Lüderitz et al, 1966). Furthermore, the Dische and Shettles colorimetric method for the estimation of methyl pentose is one of the most sensitive and specific methods known for measuring carbohydrate. Rhamnose is readily available commercially for use as a standard. Success of the method depends on the validity of the assumption that methyl pentose is not present in the bacteria in any form other than O-polysaccharide.

Method

Bacteria were grown overnight in 50 ml. broth cultures, centrifuged at 12,000 g for 15 minutes and resuspended in 5 ml. distilled water for immediate dry weight and methyl pentose determinations. The 10 minute Dische and Shettles method for estimating methyl pentose is described on page 106.

Test of the specificity of rhamnose estimations as a method of measuring O-antigen content in Salmonellae and related organisms

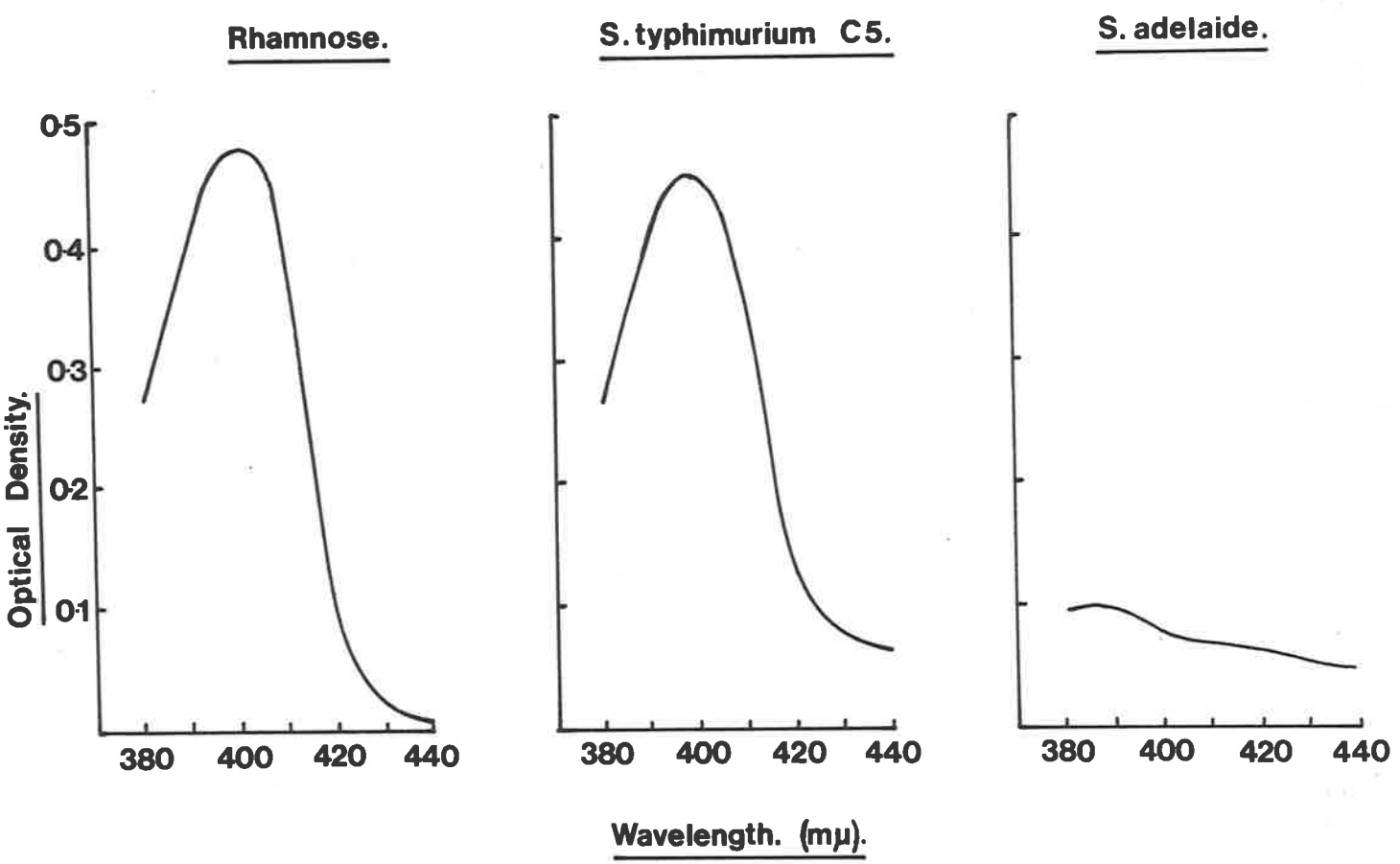
Rhamnose estimations were carried out on S.typhimurium C5, which contains rhamnose in its polysaccharide, and on two bacteria, S.adelaide (chemotype X), and Citrobacter 396, both of which lack methyl pentose in their O-antigens. (Kauffmann et al, 1960, Westphal, Kauffmann, Lüderitz and Stierlin, 1960). As a further control, rhamnose determinations were carried out on the rough S.enteritidis 11RX. This was because it seemed possible that rhamnose could be present in significant amounts in forms other than lipopolysaccharide in strains which contained the enzymes necessary to synthesize it. The strain is not entirely satisfactory as a control, as the nature of the lesion which makes it rough is not known and may possibly be in a gene affecting one of the rhamnose synthesizing enzymes.

Fig 18 compares the spectra obtained from S.typhimurium C5 and from S.adelaide with that of a standard rhamnose solution. Clearly, in spite of some broadening, the C5 spectrum is very similar to that of rhamnose, with a maximum at about 400 mμ. In contrast, the maximum in the spectrum obtained from S.adelaide is at about 385 mμ and is less distinct. The spectrum bears some resemblance to that reported for 2-deoxypentose (Dische and

Fig 18

Spectra obtained from rhamnose, S.typhimurium C5 and S.adelaide after methyl pentose determination by the ten minute cysteine hydrochloride method of Dische and Shettles

Weight of rhamnose	22.5 µg
Weight of S.typhimurium C5	2.5 mg
Weight of S.adelaide	2.2 mg


Rhamnose.
S. typhimurium C5.
S. adelaide.
Optical Density.
0·5
0·4
0·3
0·2
0·1
380
400
420
440
Wavelength. (mμ).

Shettles, 1948) so that the main contributor to its shape is probably bacterial DNA.

Table 20 lists the percentages of 'rhamnose' as determined by the differences in optical density at 396 and 430 mμ for each of the 4 bacteria. In no case did the apparent amount of rhamnose in a strain lacking rhamnose in its polysaccharide exceed 2% of the amount in C5.

TABLE 20

Methyl pentose contents of S.typhimurium C5 and 3 strains of Enterobacteriaceae lacking rhamnose in their lipopolysaccharides

Strain	Percent rhamnose w/w	Percent rhamnose relative to C5
S.typhimurium C5	0.706	100
S.adelaide	0.013	2
S.enteritidis 11RX	0	0
Citrobacter 396	0	0

Effect of bacteria on rhamnose determinations

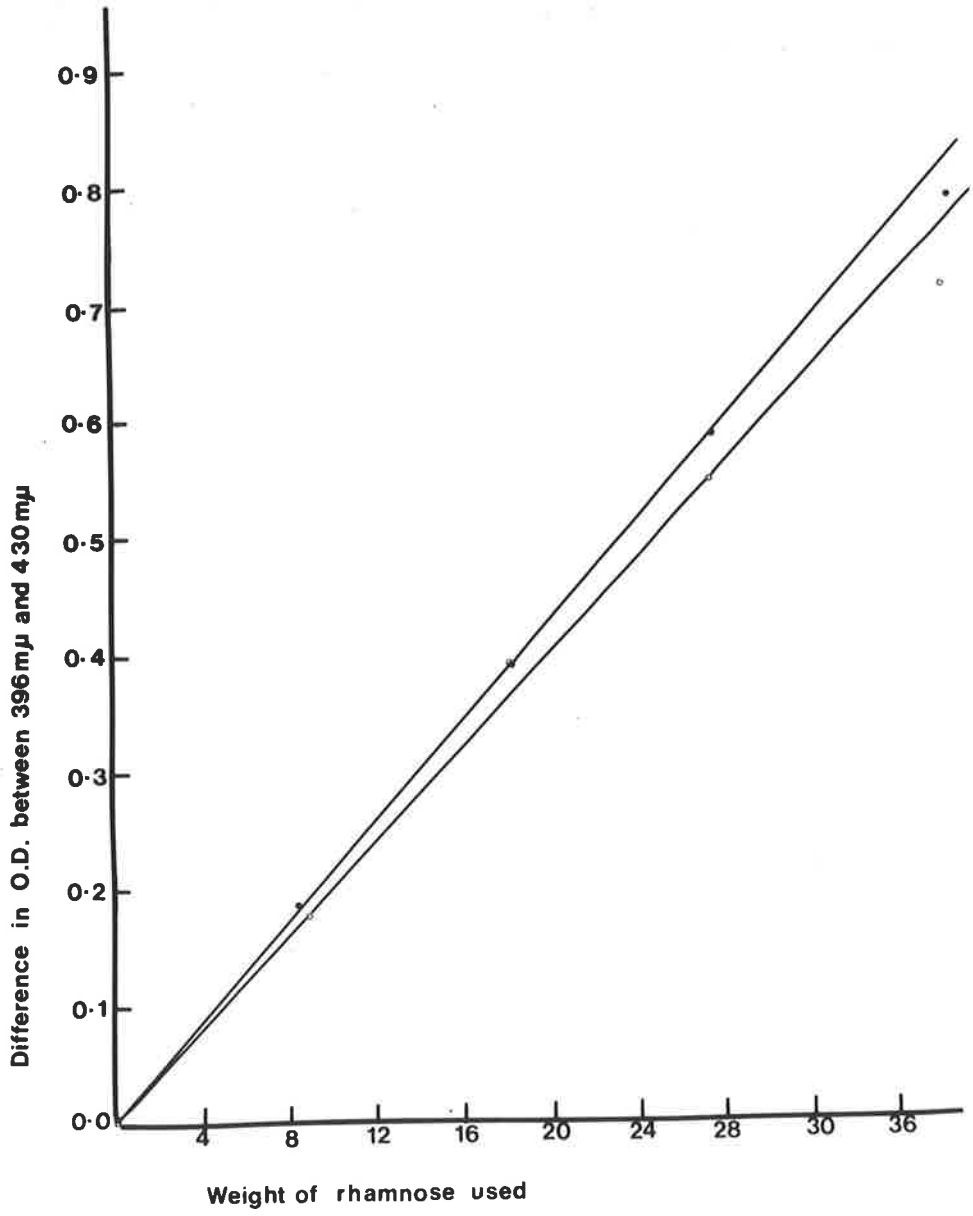
To find out whether bacterial products interfered with rhamnose estimates a series of determinations of known rhamnose solutions were carried out in the presence of S.adelaide. Fig 19 shows the

Fig 19

Effect of 2.25 mg. S.adelaide on the colour obtained when carrying out methyl pentose determinations on known amounts of rhamnose by the ten minute cysteine hydrochloride method of Dische and Shettles

- o - o - S.adelaide present

- ● - ● - S.adelaide absent


0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·0
Difference in O.D. between 396mμ and 430mμ
4
8
12
16
20
24
28
30
36
Weight of rhamnose used

graph of the difference in OD at 390 and 430 mμ after rhamnose determinations in the presence or absence of 2.25 mg. of this strain. It is clear that the difference between rhamnose estimates was never more than 5%. It was decided, therefore, that bacterial products other than rhamnose were unlikely to affect colour development in the Dische and Shettles reaction.

Variation of rhamnose content of S.typhimurium C5

Table 21 shows the estimated rhamnose contents from duplicate determinations grown on 4 different days. The result at no time varied from the mean by more than 5%.

TABLE 21

Rhamnose content of S.typhimurium C5 from a number of different cultures

Culture No.	Rhamnose content μg. rhamnose/mg. A	B	Variation from mean
1	7.6	7.4	1%
2	7.7	7.3	2%
3	7.1	-	5%
4	7.7	7.4	2%
Mean	7.5		

Washing the bacteria in physiological saline prior to estimations made no detectable difference to rhamnose contents.

Comparison of rhamnose contents of a number of virulent and avirulent Salmonellae

The availability of a rapid precise method for measuring total antigen content in some Salmonellae allowed estimation not only of the amounts on C5 and M206, but also on a number of strains from groups B and D which were of known virulence on mice. This made it possible to find out whether a low level of antigenic polysaccharide was a general occurrence in avirulent Salmonellae.

A number of strains of group B and D Salmonellae were tested for virulence as described on page 86 and their rhamnose contents were estimated as described above. The results of these experiments are shown in table 22. They confirm the previous conclusion that M206 contains about half as much total O-antigen as C5.

TABLE 22

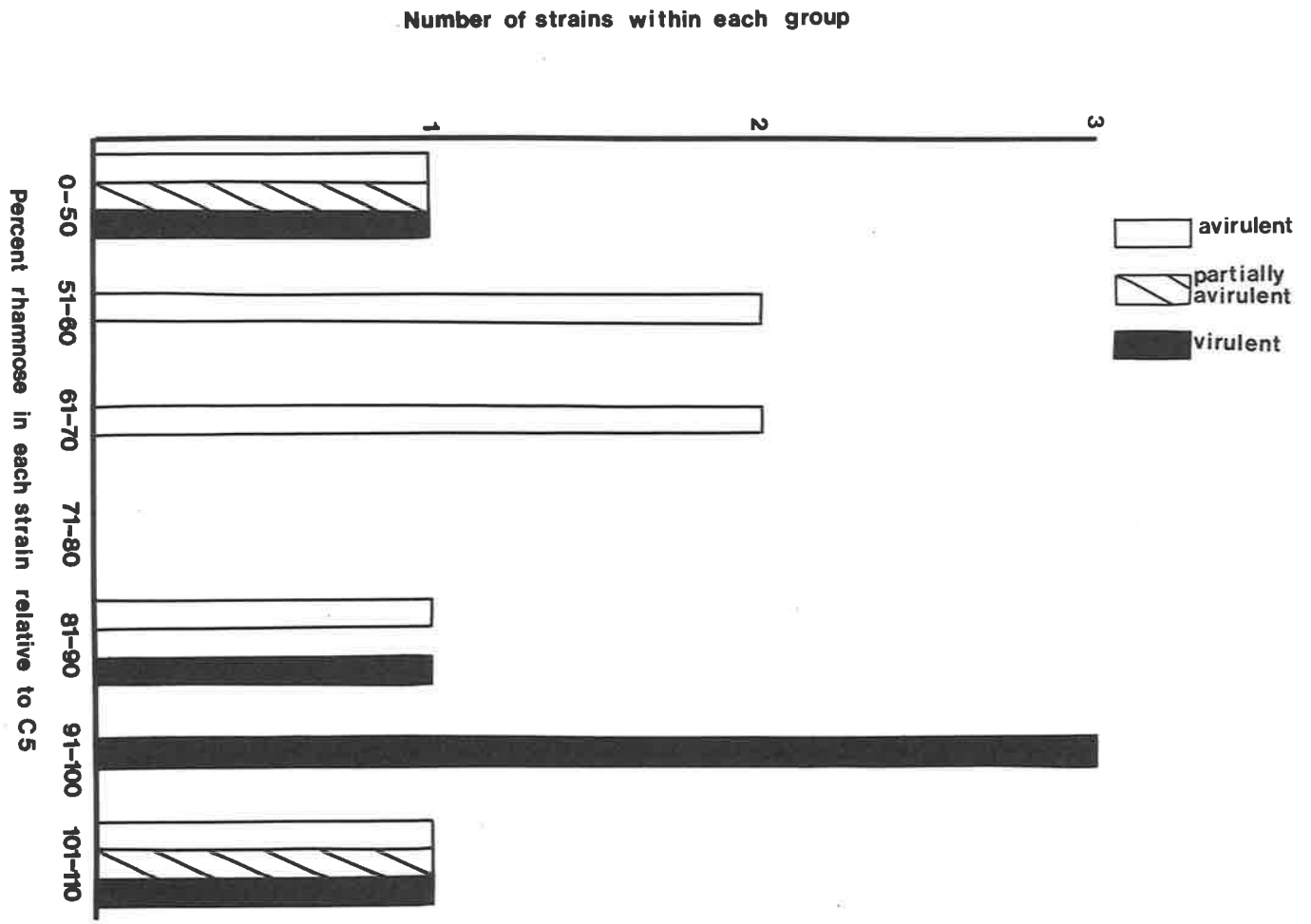
Rhamnose content and virulence in mice of a number of strains of Salmonella

Strain		Rhamnose (µg./mg. dry weight)	percent rhamnose relative to C5	LD_{50}
S.typhimurium	C5	7.5	100	$< 10^2$
	M206	3.9	52	2×10^9
	C5Sr(av)	4.6	61	$> 10^5$
	P173c	7.0	93	$< 10^2$
	ST11	2.0	27	3×10^6
	B11999	7.9	105	6×10^2
	B12347	6.1	81	6×10^2
S.abony	SW1444	5.2	69	8×10^7
Recombinant	C5S	7.8	104	8×10^5
	C5R	4.1	55	2×10^7
S.enteritidis	D10962	7.0	93	2×10^2
	D18956	8.0	107	$> 5 \times 10^5$
	795(av)	6.2	83	$> 10^5$
	melbourne	3.0	40	$< 10^2$
S.reading	-	0.6	8	2×10^8

If Krishnapillai and Baron's strain C5S is arbitrarily considered to be partially avirulent, then it is convenient to consider virulent bacteria to have an LD_{50} of less than 5×10^3 and avirulent one of more than 10^7 (or $>10^5$). Fig 20 has been constructed on the basis of these definitions. It summarizes data from table 22 and shows the number of strains containing given amounts of polysaccharide which could be regarded as being virulent, avirulent or partially avirulent. As bacteria may be avirulent for reasons independent of antigenic structure, such as inability to synthesize certain necessary metabolites (Bacon, Burrows and Yates, 1951, Furness and Rowley, 1956), it is not surprising that some avirulent bacteria have amounts of polysaccharide equivalent to those found in virulent strains. However, the converse of this is not true. If a certain amount of O-polysaccharide is necessary for virulence, then all virulent strains should have at least this amount. The comparatively small amount of rhamnose in S.enteritidis melbourne indicates either that the minimum amount of polysaccharide is less then was expected from the comparison between C5 and M206, or that this strain has a compensatory amount of another substance lacking rhamnose. It is possible that a T antigen could fill this role.

Fig 20

Incidence of virulence amongst Salmonellae with varying amounts of rhamnose relative to S.typhimurium C5.


Number of strains within each group
1
2
3
Percent rhamnose in each strain relative to C5
0-50
51-60
61-70
71-80
81-90
91-100
101-110
avirulent
partially avirulent
virulent

The results obtained using strains C5S and C5R are particularly interesting. These are recombinants obtained by Krishnapillai and Baron (1964) from a cross between S.abony Hfr SW1444 and a mutant of C5, and contain respectively the avir 1 locus and the avir 1 and avir 2 loci of these authors (Fig 1). It is clear that the avir 1 locus has no effect on polysaccharide content, so that at least one of the factors affecting the difference in virulence between C5 and S.abony SW1444 is independent of O-polysaccharide composition. However, in the recombinant containing both avirulence genes the rhamnose content is almost halved, and is close to that of the parent Hfr strain. The most likely explanation is that the avir 2 gene controls polysaccharide content in some way, and that the amount of polysaccharide on the bacterium has some significance in virulence. If this model is correct, then it must be assumed that the partially avirulent recombinants obtained by Krishnapillai and Baron containing only the gene avir 2 were also deficient in polysaccharide. Their results indicate that such a deficiency could cause a 100-fold decrease in virulence. Krishnapillai and Karthigasu (1969) found no difference in the clearance of C5 and C5R from normal mice, although C5R subsequently failed to multiply. This implies that the reduction in polysaccharide content in C5R had

no effect on its treatment by phagocytic cells, but may have had a profound influence on its subsequent survival. The avir 2 gene is located on a different part of the chromosome from any of the known genes affecting lipopolysaccharide synthesis except the gene controlling synthesis of phosphoglucose isomerase (pgi Fig 1). This gene is necessary for the production of mannose, required for synthesis of the smooth polysaccharide in both S.typhimurium and S.abony as well as for the formation of glucose and other hexoses involved in lipopolysaccharide synthesis from fructose when glucose is in limited supply. Clearly in in vitro conditions there is sufficient polysaccharide on C5R for it to be treated as a smooth organism by the phagocytic cells. It is possible, however, that when it attempts to multiply in the mouse it is compelled to extract food substances from unpromising sources, such as the inside of a phagocytic vacuole. Under these circumstances a minor deficiency in a lipopolysaccharide synthesizing enzyme could lead to major deficiencies in the lipopolysaccharide formed, so the organism after culture in vivo behaved as if it was rough. It is unfortunate that the position of the F factor on the SW1444 chromosome is so close to the avir 2 locus. This has necessitated the study of recombinants in which almost the whole of the donor chromosome has been

transferred, and it is difficult to be certain that the biochemical changes observed are due to alterations in the selected regions rather than cross-overs in other parts of the chromosome. The system would repay further study.

It is instructive to calculate the total number of rhamnose molecules on one bacterium. If we take the number of organisms of C5 in 1 mg. dry weight to be 10^9, then it can be shown that an amount of rhamnose of 7.5 μg./mg. corresponds to 2.7×10^7 molecules per bacterium. As there is one rhamnose molecule for every group of antigenic sites, this means that the maximum possible number of antigen 4, 5 or 12 sites is 2.7×10^7. Hellerqvist *et al's* data (1969) suggest that this should be close to the actual number, at least in the case of antigen 5. The figure calculated from the absorption data of chapter 5 was of the order of 10^3 or 10^4. We must conclude, therefore, that either only a small proportion of the potential antigenic sites are available to antibody, and that much of the O-antigen is in inaccessible parts of the cell wall, or that a single antibody molecule blocks a large number of antigenic sites.

A rough calculation can be made of the thickness of the polysaccharide outer layer, assuming that it consists of a solid layer and contains about half of the total polysaccharide present, corresponding to the 50% which can be released by EDTA. If C5 contains 7.5 µg. rhamnose per mg. dry weight, and rhamnose constitutes about 1/4 of the weight of one oligosaccharide unit, the weight of polysaccharide on one bacterium is approximately

$$\frac{7.5 \times 4}{2 \times 10^9 \times 10^6} \quad \text{g.}$$

Assuming a density of 1.2 g. per cc (see Kent and Osborn, 1968_a) this corresponds to a volume of

$$\frac{7.5 \times 4}{2 \times 10^9 \times 10^6} \times \frac{1}{1.2} \times \frac{1}{10^6} \quad \text{cu. m.}$$

If end corrections are neglected this can be regarded as forming a tube round a cylinder of length 2µ and radius 0.3 µ. Its volume (V) will be given by the formula

$$V = \pi h (r_1^2 - r_2^2)$$

where h is the length of the tube, r_1 its outer radius and r_2 its inner radius. In this instance

$$V = \pi \times 2 \times 10^{-6} \left[r^2 - (0.3 \times 10^{-6}) \right]^2$$

where r is the radius of the bacterium including polysaccharide.

Hence

$$\frac{7.5 \times 4}{2 \times 10^{9} \times 10^{6} \times 1.2 \times 10^{6}} = \pi \times 2x\, 10^{-6}\left[r^{2} - (0.3 \times 10^{-6})^{2}\right]$$

From this it can be calculated that the outer radius of the tube is 0.003μ giving a polysaccharide thickness of 3 mμ or 30 Å. The thickness of M206 should be about half of this value. The figures seem to correlate fairly well with the estimate of 10 - 20 Å by Murray et al. (1965), although it must be remembered that other components are also likely to be present on the surface. If the length of an oligosaccharide repeating unit is taken to be about 20 Å, this allows a thickness of 1.5 units at right angles to the surface of C5. Alternatively, if they lie parallel to the surface it may be possible to stack 5 or 6 chains in this thickness. Taking the first assumption, it can be calculated that the number of surface units covered when one antibody reacts is

$$\frac{2.7 \times 10^{7}}{2} \times \frac{1}{10^{4}} \times \frac{1}{1.5} = 900$$

As 10^{4} was a maximum value calculated for IgG molecules, the figure of 900 is probably a rather low estimate. The figure for IgM is more likely to be in the region of 4500.

Rhamnose content of C5 and M206 lipopolysaccharide

After the removal of merthiolate by dialysis against 2 litres of distilled water, the methyl pentose contents of C5 and M206 lipopolysaccharides were determined by the three minute method of Dische and Shettles (1948) as described on page 97. Table 23 shows the rhamnose contents of the two lipopolysaccharides.

TABLE 23

Methyl pentose content of C5 and M206 lipopolysaccharides

Strain	percent methyl pentose	percent LPS in whole organisms
C5	10.9	8.2
M206	7.9	6.2

The percentage of lipopolysaccharide in C5 and M206 calculated from the data in table 23 is surprisingly high in both cases. Protein estimations by the Folin-Lowry method described on page 108 subsequently showed that the M206 lipopolysaccharide preparation contained about 6% protein. However no protein could be detected in the C5 preparation by this method.

Summary

Evidence is presented which supports the earlier conclusion that C5 has twice as much O-antigen as M206. However, much more antigen can be demonstrated by the chemical methods employed in this study than were apparent when the reaction with antibody was examined. Antigen determinations on a number of other Salmonellae suggest that although O-polysaccharide content may be involved in virulence, the relationship is not a simple one.

CHAPTER 7

BIOLOGICAL EFFECTS OF SPECIFIC ANTISERA

Introduction

Implicit in the notion that a heat-labile antigen is important in the virulence of C5 and M206 is the assumption that antibodies against this antigen are much more effective than others in promoting immune reactions which are bacteristatic or lead to the destruction of bacteria. Two of these reactions were studied - the *in vitro* bactericidal reaction of antiserum in the presence of excess complement, and the *in vivo* bactericidal action which occurs after bacteria pretreated with antiserum have been injected into the mouse peritoneal cavity. As both of these reactions are complement mediated, complement fixation by antibody and bacteria was also studied.

1. *In vitro* bactericidal reactions

It is important to know whether the main antibodies involved in the bactericidal activity of an antiserum are directed against lipopolysaccharide or against other antigens. Table 24 shows the bactericidal titres of a number of rabbit antisera against several Salmonellae and *Citrobacter* 396, and the effect of absorption of these sera by various strains selected to make

the sera 'monospecific' for O-antigens according to the Kauffmann-White scheme. The rabbit antisera used were prepared as described in chapter 3. To prepare a monospecific serum, 5 ml. of serum were agitated overnight in the cold with 30-40 mg. bacteria which had been grown overnight on agar, washed once in saline, heated at 60° for 1 hour and again washed in saline. In vitro bactericidal reactions were carried out in the presence of excess fresh absorbed guinea-pig serum using the modification of the method of Rowley (1968) described on page 101.

Table 24 shows that the bactericidal activities of the sera studied are closely related to the antibodies against the lipopolysaccharide determinants which they are thought to contain. Unabsorbed sera gave titres of the order of $1/10^{7}$ - $1/10^{9}$ against homologous organisms, and of $1/10^{4}$ and over against heterologous organisms which shared O-antigens with the immunizing bacteria. When the sera were absorbed with heterologous organisms bactericidal titres against these organisms fell to $1/10^{2}$ or less, as did the titres against organisms whose only O-antigens shared with the immunizing strain were also shared with the absorbing strain. One exception is the killing of Citrobacter 396 by an anti-S.derby serum originally containing

antibodies against 1, 4 and 12, absorbed by S.reading with antigens 4 and 12. The resultant antiserum should have contained antibodies against only antigen 1, which is not found on Citrobacter 396. The titre of $1/10^3$ must be regarded as an indication either that the antiserum still contained a small amount of antibody against antigen 4, or that the serum originally contained unsuspected antibodies which cross-reacted with Citrobacter 396.

The table also shows that although M206 was susceptible to nearly all of the sera used, C5 was completely resistant. Several attempts were made to kill C5 with each of the antisera, and signs of killing were sought after 6 and 24 hours, as well as the usual $2\frac{1}{2}$ hours. In no case was there any indication that C5 could be killed in this system.

Many of the antisera used inhibited bactericidal activity when they were at concentrations of 1/100 and over. This prozone effect is the well-known Neisser-Wechsberg phenomenon (Neisser and Wechsberg, 1901). Titrations in which this was observed are denoted with a (p) in table 24.

TABLE 24

In vitro bactericidal titres [(a)] of rabbit antisera against Salmonellae and related organisms

Antiserum	Absorbing strain	Antibodies	S.reading	S.berta	S.derby	Citrobacter 396	C5	M206
S.reading	-	4, 12	8	4	6 (p)	4	<2	4
S.berta	-	9, 12	4	>8	3	2	<2	4
S.derby	-	1, 4, 12	9 (p)	3	8 (p)	4	<2	5
Citrobacter 396	-	4, 5	8	2	6	7 (p)	<2	7
S.derby	S.reading	1	<2	<2	2	3	<2	<2
S.reading	S.berta	4	8 (p)	<2	5 (p)	4	<2	4
Citrobacter 396	S.reading	5	<2	<2	2	5 (p)	<2	6
S.berta	S.reading	9	2	7	2	2	<2	2
S.reading	Citrobacter 396	12	6 (p)	3	5 (p)	2	<2	4
Normal serum	-	-	2	2	2	2	<2	2

(a) expressed as $\log_{10}$ (dilution at end point)

(p) denotes that a prozone effect was observed when the antiserum dilution was 1/100.

Absorption of serum in vitro bactericidal activity with lipopoly-saccharides

If the bactericidal action of serum is entirely due to antibody against O-antigen it should be possible to absorb out this activity with purified lipopolysaccharides. This proved extremely difficult. It can be calculated from the results summarized in table 25 that less than 50 mg. of bacteria are sufficient to absorb out almost completely bactericidal activity from 1 ml. of an antiserum with a titre of up to $1/10^9$. Assuming that there is about 5% lipopolysaccharide in these strains, one would imagine that if all the bactericidal antibodies are directed against lipopolysaccharide, then 2.5 mg. of lipopolysaccharide should be capable of absorbing almost completely the bactericidal activity of an equivalent antiserum. An attempt to show this was carried out using C5 lipopolysaccharide to inhibit the bactericidal action against M206.

Specific anti-4 and anti-5 sera prepared according to the method on page 98 were diluted 1/10. Diluted serum (0.15 ml.) was mixed in a 5 ml. screw-topped bottle with 1.35 ml. saline containing 0.01% magnesium chloride and 5 mg. C5 lipopoly-saccharide. The mixture was allowed to rotate for 2 hours at 4°, diluted 1/10 in magnesium chloride saline, centrifuged for 30

minutes at 27,000 g and filtered through a 0.45 μ Millipore membrane in a Sweeney adaptor. The filtered absorbed serum was tested for haemagglutinating activity against sheep red blood cells sensitized with C5 lipopolysaccharide and for in vitro bactericidal activity against M206. It was found in both cases that the haemagglutination titre fell from 1/1280 to less than 1/200. Results of the bactericidal assay are shown in table 25. Figures quoted are the number of viable bacteria present in one drop of the bactericidal system after $2\frac{1}{2}$ hours. It was found that the numbers of viable bacteria in 0.1 ml. of a control sample lacking antiserum was about 500 and it was assumed that those drops in which no killing had occurred contained about 100 organisms.

TABLE 25

Effect of absorption of antiserum with C5 lipopolysaccharide on survival of M206 in anti-4 and anti-5 sera and guinea-pig complement

Antiserum	Serum dilution						Titre
	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$	0	
4 (unabsorbed)	30	0	0	2	100	100	$1/10^5$
4 (absorbed)	4	60	100	100	100	100	$1/10^2$
5 (unabsorbed)	100	0	0	0	100	100	$1/10^5$
5 (absorbed)	0	0	14	100	100	100	$1/10^3$

It is clear that absorption of anti-4 serum with lipopolysaccharide leaves less than 0.1% of its original bactericidal activity. On the other hand, absorption of the anti-5 serum leaves just under 1% of its activity. Evidently anti-5 serum contains bactericidal antibodies directed against components other than lipopolysaccharide. This serum differs from the anti-4 serum in having been raised against bacteria which had been killed by being heated at 56° for 30 minutes, rather than boiled for 2 hours, and it may be that the unabsorbed activity was caused by antibody against an extra antigen susceptible to boiling. Nevertheless, in both cases more than 99% of the bactericidal activity could be attributed to antibody against lipopolysaccharide. If immunity in an animal depends on activities closely related to the in vitro serum bactericidal reaction it is unlikely that this extra antigen is of major importance in immunity in the immunized animal.

Comparison of the in vitro bactericidal activities of anti-4, 5, and 12 sera

The rest of the work described in this chapter was carried out with monospecific sera prepared from the same sera which were used for chapters 4 and 5.

To find out whether there was any evidence that anti-5 serum was better than anti-4 or anti-12 in in vitro bactericidal reactions against M206, titrations were carried out on all three sera. Furthermore, to ascertain whether these effects could be attributed to more than one class of antibodies the effect of treating the sera with 2-mercaptoethanol to destroy IgM antibodies was also measured.

Serum (0.3 ml.) was mixed in a 10 ml. screw-topped bottle with either 0.3 ml. saline or 0.3 ml. 0.2 M 2-mercaptoethanol in saline. The mixture was incubated for 1 hour at 37°, diluted with 5.4 ml. saline containing magnesium chloride (0.02M) and used at a further dilution of 1/5 for titration of in vitro bactericidal activity as described on page 101. Killing was estimated by measurement of the viable count in 0.1 ml. reaction mixture after $2\frac{1}{2}$ hours.

Fig 21 shows the amount of killing obtained using various dilutions of these sera and table 26 shows the end titres reached, together with the titres of similarly treated sera which had been titrated for haemagglutinating activity against sheep red blood cells sensitized with C5 lipopolysaccharide.

Fig 21

In vitro bactericidal activity against S.typhimurium M206 of specific antisera before and after 2-mercaptoethanol treatment

- ● - ● -	untreated serum
- o - o -	mercaptoethanol treated serum

Titrations of antisera were carried out as described on page 101.

Surviving bacteria were counted by plating 0.1 ml. aliquots on nutrient agar after $2\frac{1}{2}$ hours.

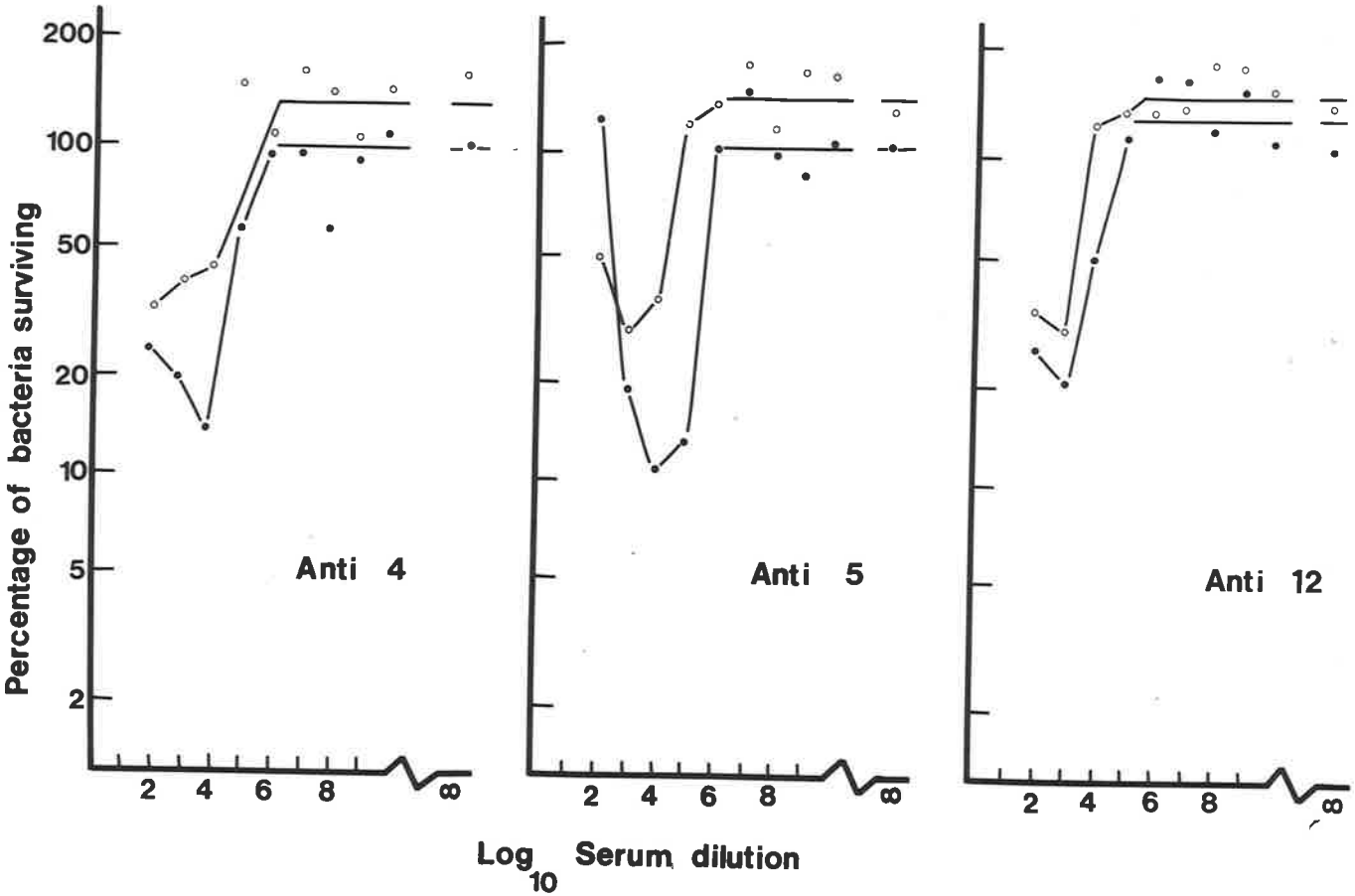

Percentage of bacteria surviving
200
100
50
20
10
5
2
Anti 4
Anti 5
Anti 12
2
4
6
8
∞
Log10 Serum dilution

TABLE 26

Comparison of bactericidal and haemagglutinating activities of anti-4, 5 and 12 sera and the effect of 2-mercaptoethanol treatment on these activities

Antiserum	Treatment			
	None		2-mercaptoethanol	
	H[(a)]	B[(b)]	H[(a)]	B[(b)]
4	2560	10^4	1280	10^4
5	896	10^5	80	10^4
12	1280	10^3	640	10^3

(a) H = reciprocal of haemagglutination titre (geometric mean of two determinations (from table 17 chapter 5).

(b) B = reciprocal of in vitro bactericidal titre.

Both Fig 21 and table 26 show that a major proportion (about 90%) of the activity of the anti-5 serum, both in haemagglutinating and in in vitro bactericidal reactions, was due to mercaptoethanol labile antibody, presumably IgM antibody. In contrast, both the anti-4 and anti-12 sera had considerable IgG activity in addition to IgM. It may be taken to count for about 50% of the activity in both titrations. This makes it difficult to compare results obtained with untreated anti-5 serum with those obtained from anti-4 and 12, because of the reports that IgM has a very

different efficiency from IgG in both haemagglutinating and bactericidal activities (see page 74). It is more meaningful to compare results obtained using mercaptoethanol treated sera in which only IgG activity has been measured. Table 27 shows the ratios of haemagglutinating to bactericidal activities of the sera used, and also the ratios of bactericidal activity to antibody protein originally present in the sera, assuming that all the sera originally contained 1,000 µg per ml. of antibody (page 151 chapter 5).

TABLE 27

In vitro bactericidal activities of anti-4, 5 and 12 sera relative to the total antibody content of the sera and their haemagglutinating activities

Antiserum	Ratio $\frac{B^{(b)}}{H^{(a)}}$	Ratio $\frac{B^{(b)}}{P^{(c)}}$
4 untreated	40	10
5 untreated	100	100
12 untreated	1	1
4 ME	10	-
5ME	100	-
12 ME	2	-

(a) H = reciprocal of haemagglutination titre

(b) B = reciprocal of bactericidal titre

(c) P = protein per ml. from quantitative precipitin reaction (= 1,000 µg.)

The relative efficiencies of the three antisera could be arranged in the order:

$$5 > 4 > 12$$

regardless of whether bactericidal activity was related to quantitative precipitin data or haemagglutination activity. Mercaptoethanol treated derivatives of these sera, in which apparently only IgG was measured, could be arranged in the same order. Without further information on the types of antibody in these sera and on their affinities for their respective antigens it is not possible to state categorically whether anti-5 antibody is better than antibodies of the other two specificities. However, it is interesting that the order of efficiency of antibodies found here is the same as the order of concentration of antigens on C5 and M206 tentatively deduced in chapter 5, and it seems reasonable to suggest that these antisera differ in efficiency at least partly because of the relative concentrations of their respective antigens on the bacteria. Appendix 3 shows that one can expect on theoretical grounds that a difference in efficiency of antibody can be caused in this way, and that in certain circumstances this difference should be measurable.

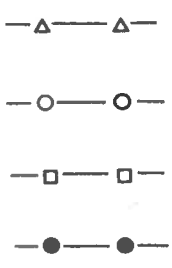
2. In vivo bactericidal activity of 4, 5 and 12 antisera

One of the original indications of the importance of a heat-labile antigen in immunity to C5 was the observation that C5 stuck to mouse peritoneal cells more efficiently after the culture had been heated (Auzins and Rowley, 1963). It was of interest, therefore, to find out whether an anti-5 serum was more effective than anti-4 or 12 sera in opsonizing C5 for killing in the mouse peritoneal cavity.

Bacteria were opsonized with serum at dilutions between $1/10^4$ and $1/10^6$ and in vivo bactericidal activities were measured as described on page 105. The results of these experiments are shown in Fig 22. It can be seen that all three antisera were equally effective in opsonizing bacteria at dilutions of $1/10^4$ and $1/10^5$. However, at $1/10^6$ dilution there was little evidence of opsonization except when anti-5 serum was used. With this serum the percentage of Salmonellae killed at a dilution of $1/10^6$ was roughly equivalent to the percentage killed at lower dilutions (about 95%). It appears from this experiment that the anti-5 serum was more effective than either the anti-4 or anti-12 sera as an opsonin for promotion of intraperitoneal phagocytosis and killing of C5 in mice.

Fig 22

Killing of S.typhimurium C5 in the mouse peritoneum after opsonisation with various dilutions of anti 4, 5 and 12 sera

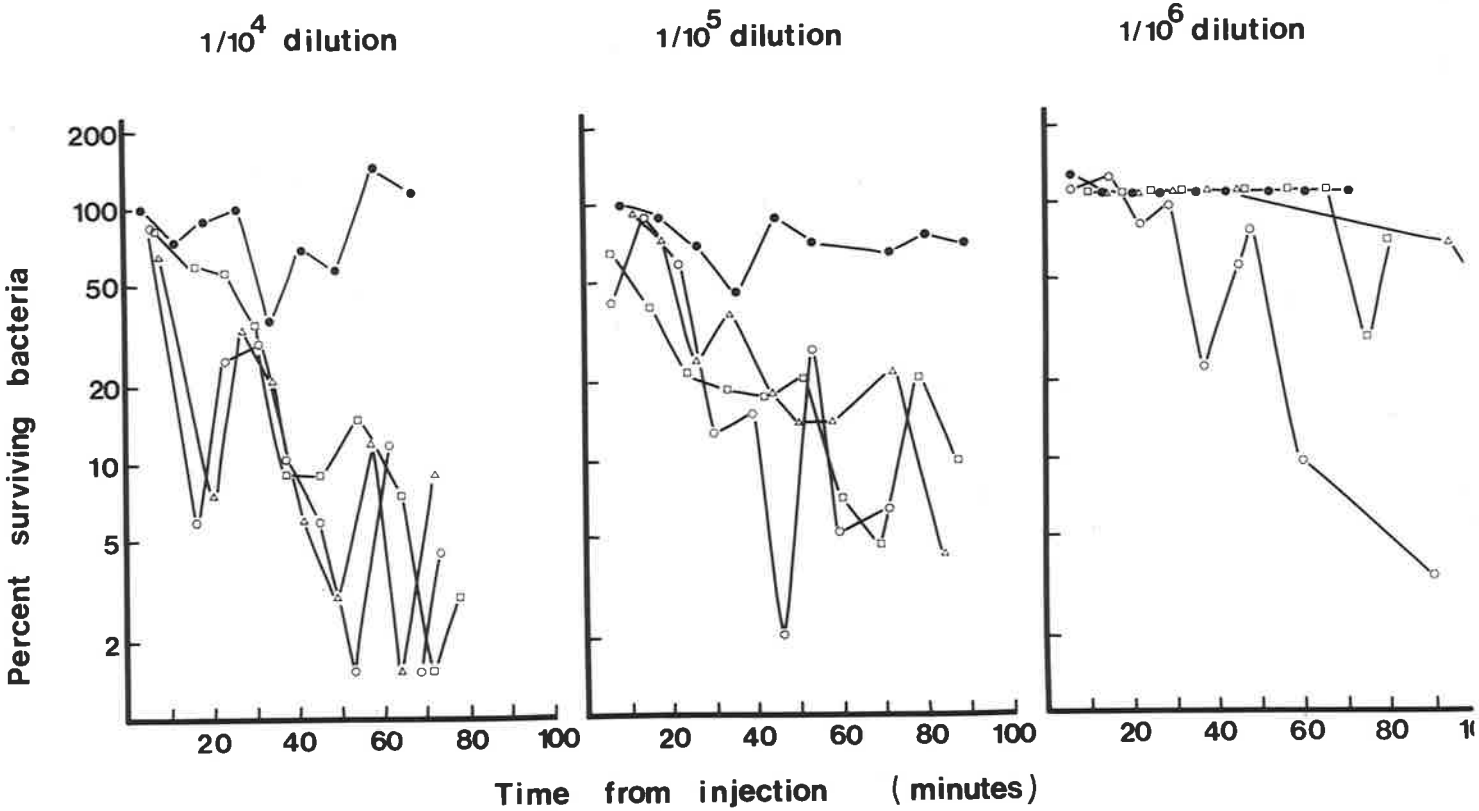


anti 4

anti 5

anti 12

saline


1/10^4 dilution
1/10^5 dilution
1/10^6 dilution
Percent surviving bacteria
200
100
50
20
10
5
2
20
40
60
80
100
Time from injection (minutes)

3. Complement fixation by antibodies against C5 and M206

As both the in vitro and in vivo bactericidal reactions depend on complement as well as on antibody, it was suggested that the difference in susceptibility between C5 and M206 might depend on the ability of complement to be fixed to these two organisms. No attempt was made to find out how well any of the individual nine known components of complement could react; but complement fixation tests using whole guinea pig serum were carried out.

Methods

Before it was used in complement fixation reactions, guinea-pig serum prepared as described in chapter 3 was absorbed for 2 hours at 4° with 1 overnight agar plate culture of M206 per 2 ml. of guinea pig serum, centrifuged twice at 15,000 g in a refrigerated centrifuge, filtered through a 0.45 μ Millipore membrane in a Sweeney adaptor, and stored at -20° in 0.1 ml. aliquots in 5 ml. screw-topped bottles. Frozen absorbed complement was kept for up to 2 weeks. Before use it was titrated against haemolysin sensitized cells as described in chapter 3. Freezing and thawing caused a drop of about 50% in complement activity, but storage for up to 2 weeks seemed to have little effect.

Preliminary experiments showed that the anti-4, 5 and 12 sera were all strongly anti-complementary even before addition of bacteria. When these sera were dialysed against saline to remove merthiolate, centrifuged twice at 27,000 g and then filtered successively through Millipore membranes of 0.45 and 0.22 μ pore size the anti-complementary activity disappeared. It was assumed, therefore, that this activity was caused by a combination of merthiolate inactivation, and fixation of complement by aggregated antibody molecules and residual antigen antibody aggregates remaining from absorptions. As the process of preventing a serum from being anti-complementary was rather complex, and it was found that centrifugation was necessary on each day that the antiserum was used, a method was adopted in which centrifuged serum was allowed to react with bacteria, removed by centrifugation, and the ability of the bacteria to fix complement then measured.

In a typical complement fixation experiment antisera were centrifuged at 27,000 g for 20 minutes before use. A 100 ml. shake culture of bacteria was centrifuged at 12,000 g for 15 minutes, washed once in saline, and suspended in 1.5 ml. distilled water. Dry weight determinations were carried out on

duplicate 0.1 ml. samples and then 1.3 ml. 0.3M sodium chloride solution was added to bring the concentration of the suspending medium to that of physiological saline. After suitable dilutions in saline, 0.1 ml. bacteria were mixed with 0.8 ml. antiserum diluted if necessary in saline. The experiments were carried out in such a way that a number of dilutions of antiserum were tested against a fixed number of bacteria. Antiserum and bacteria were mixed in the cold for 1 hour and then centrifuged in a Beckman 5/16" dia. x 1-15/16" cellulose nitrate tubes (No. 303369) at 12,000 g for 15 minutes. The pellet of bacteria was suspended in a known volume (0.4 - 1 ml.) of complement diluent (chapter 3) and mixed as well as possible. This was difficult at some serum concentrations because of agglutination of bacteria by antibody. Complement fixation tests using these antibody-laden bacteria were carried out as described on page 97.

Complement fixation by sera absorbed with lipopolysaccharide

When serum was absorbed with C5 lipopolysaccharide and centrifuged at 20,000 g for 30 minutes, a fall in complement fixing titre with bacteria of about 75% was obtained. While this showed that at least 75% of the complement fixing ability of both

C5 and M206 was due to the reactions of antibodies with lipopolysaccharides it left the question of the remaining 25% of activity unclear. This could have been due to fixation of complement by other antibodies attached to non-lipopolysaccharide antigens, or it could have been the result of complement fixation to antibody-lipopolysaccharide complexes being carried down with the bacteria.

Complement fixation by anti-4 and 12 sera

Approximately 1 mg. of bacteria in 0.1 ml. saline was mixed with 0.4 ml. of anti-4 or 12 serum at appropriate dilutions and complement fixation was carried out as described above.

In the final complement fixation test about 1/4 of the bacteria (250µg.) were present in the first well of the haemagglutination tray. The value for the amount of complement fixed was an adjusted value denoting the amount which could have been fixed by the bacteria in the first well, assuming that there was strict proportionality between the number of bacteria present and the amount of complement fixed.

Fig 23 shows the results of complement fixation tests on C5 and M206 using anti-4 and anti-12 sera. The method was not sufficiently precise to show differences as small as the two-fold

Fig 23

Complement fixation by S.typhimurium C5 and M206

(a) using anti-4 serum

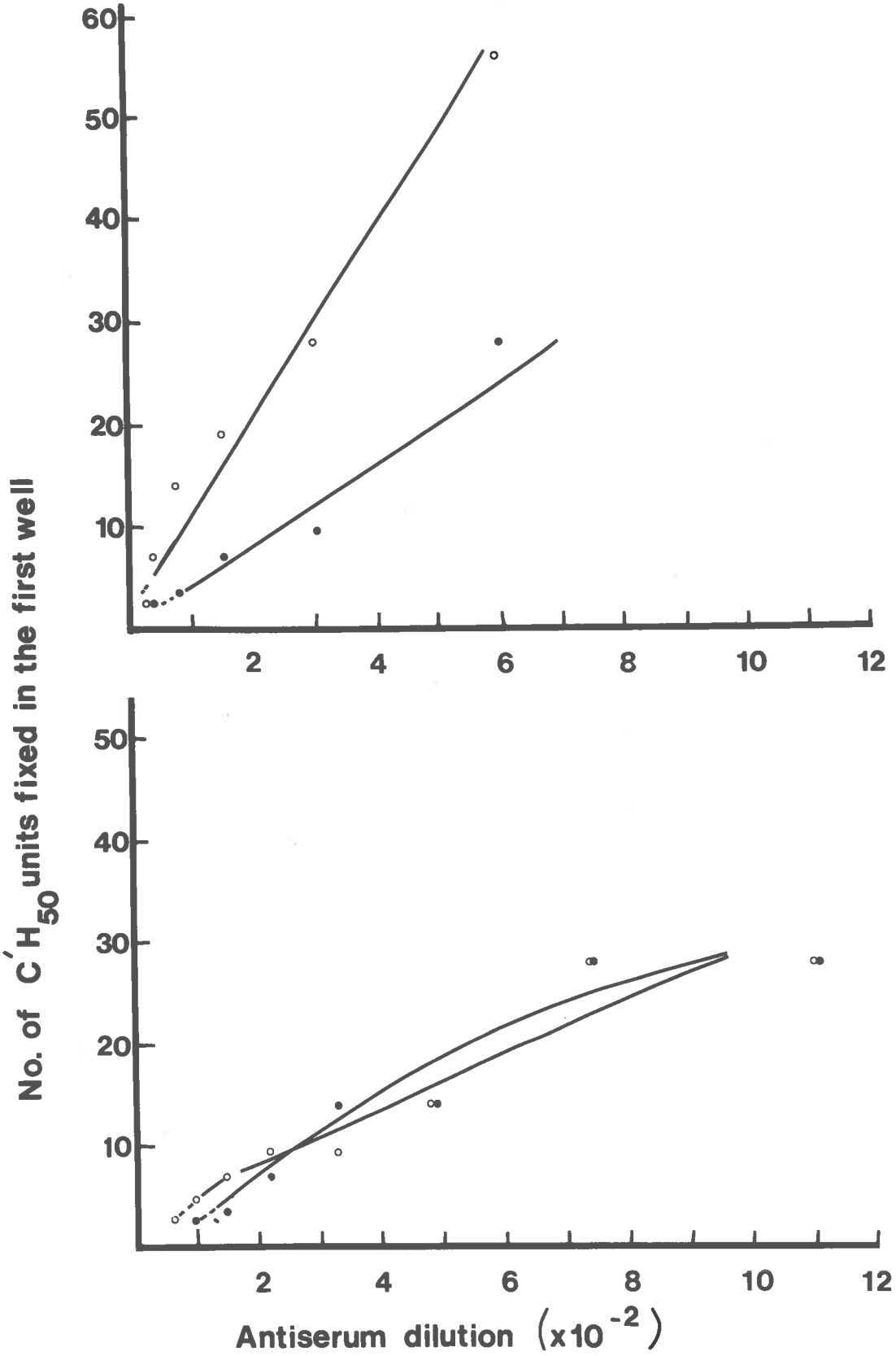
- ● - ● - C5

- o - o - M206

(b) using anti-12 serum

- ● - ● - C5

- o - o - M206


60
50
40
30
20
10
2
4
6
8
10
12
50
40
30
20
10
2
4
6
8
10
12
No. of C´H50 units fixed in the first well
Antiserum dilution (x10-2)

differences in antigen content demonstrated in chapter 4. It is clear, however, that complement fixed almost equally effectively in both C5 and M206 at the concentrations of antiserum which were used.

The number of complement fixing sites on antibody-sensitized bacteria

To try to obtain more information on the actual number of sites on each bacterium which could fix complement, an attempt was made to set up a mathematical model which would describe complement fixation in the system used in this chapter (appendix 2A). This treated the antigen/antibody reaction as an equilibrium reaction between 1 antigenic site and 1 antibody combining site. Complement was treated as a single molecule, and it was assumed that the number of molecules fixed was proportional to the number of sites formed by antigen/antibody reactions. The assumption was made that only 1 molecule of antibody was required to fix 1 molecule of complement. The equations obtained suggested that if the model was reasonable, the graph of the reciprocal of the number of C^1H_{50} units fixed against the reciprocal of the antiserum dilution should give a straight line which intersected the ordinate at a positive value

equal to the reciprocal of the maximum number of $C'H_{50}$ units which could be fixed by the number of bacteria used. The equation also had the property that at high antibody concentrations if equal numbers of two particles, each with different numbers of identical antigenic sites, were used, then the ratios of both their intercepts of their gradients were equal to the reciprocals of the ratios of the total numbers of antigenic sites which each contained. For reasons explained in appendix 2A, the value for the maximum number of $C'H_{50}$ units fixed could be used to calculate only the minimum number of sites available for complement fixation on the bacteria.

When data from the previous section were plotted to fit the equation it was found that results from a number of experiments (e.g. anti-4 v. C5 and M206) seemed to give reasonably straight lines, but that these lines passed very close to the origin. This led to the suspicion that the experiments might have been carried out in a region of antigen excess where there was no guarantee of a correlation between the amounts of antigen on each bacterium and the amount of complement fixed (equation 12, appendix 2). To test these equations further, and in the hope that a better comparison could be made of the numbers of comple-

ment fixing sites on C5 and M206, two experiments were carried out using high concentrations of anti-5 serum.

Method and results

An attempt was made to use standard preparations of bacteria and complement as well as of antiserum. Bacteria were grown overnight in 6 x 100 ml. liquid culture, washed once in saline, suspended in about 10 ml. distilled water for dry weight determinations and then mixed with an equal volume of sterile 0.3M saline. The bacteria were stored in 2 ml. aliquots at -20°. Before use they were diluted in saline to give a concentration of 2.0 mg. per ml. Anti-5 serum was centrifuged at 31,000 g for 20 minutes before use. Aliquots of 0.1 ml. bacteria were mixed with 0.9 ml. of various concentrations of serum at dilutions of up to 1:100 in saline. Bacteria and antiserum were mixed thoroughly, left in the cold for 1 hour and then centrifuged at 12,000 g for 15 minutes. The pelletted bacteria were suspended in 0.4 ml. diluent and titrations of complement fixing ability were carried out as described above.

It was found that bacteria which were mixed with antiserum at dilutions greater than 1:10 formed rather firm aggregates

which were difficult to disperse evenly. Hence results obtained at dilutions of antiserum between 1:10 and 1:100 were particularly unreliable and had to be discarded.

The results of the first experiment were most encouraging but the conclusions reached could not later be substantiated. Fig 24 (A) shows the effects of plotting the reciprocal of the number of $C'H_{50}$ units fixed against the reciprocals of the serum concentrations. Straight lines and the errors of the intercepts at the 5% level were fitted by the method of least squares. A sample calculation is shown in appendix 2B. Results of these calculations are summarized in table 28 (A).

Fig 24

Complement fixation by S.typhimurium C5 and M206 using high concentrations of anti-5 serum

A first experiment

- ● - ● - C5

- o - o - M206

B second experiment

- ● - ● - C5

- o - o - M206

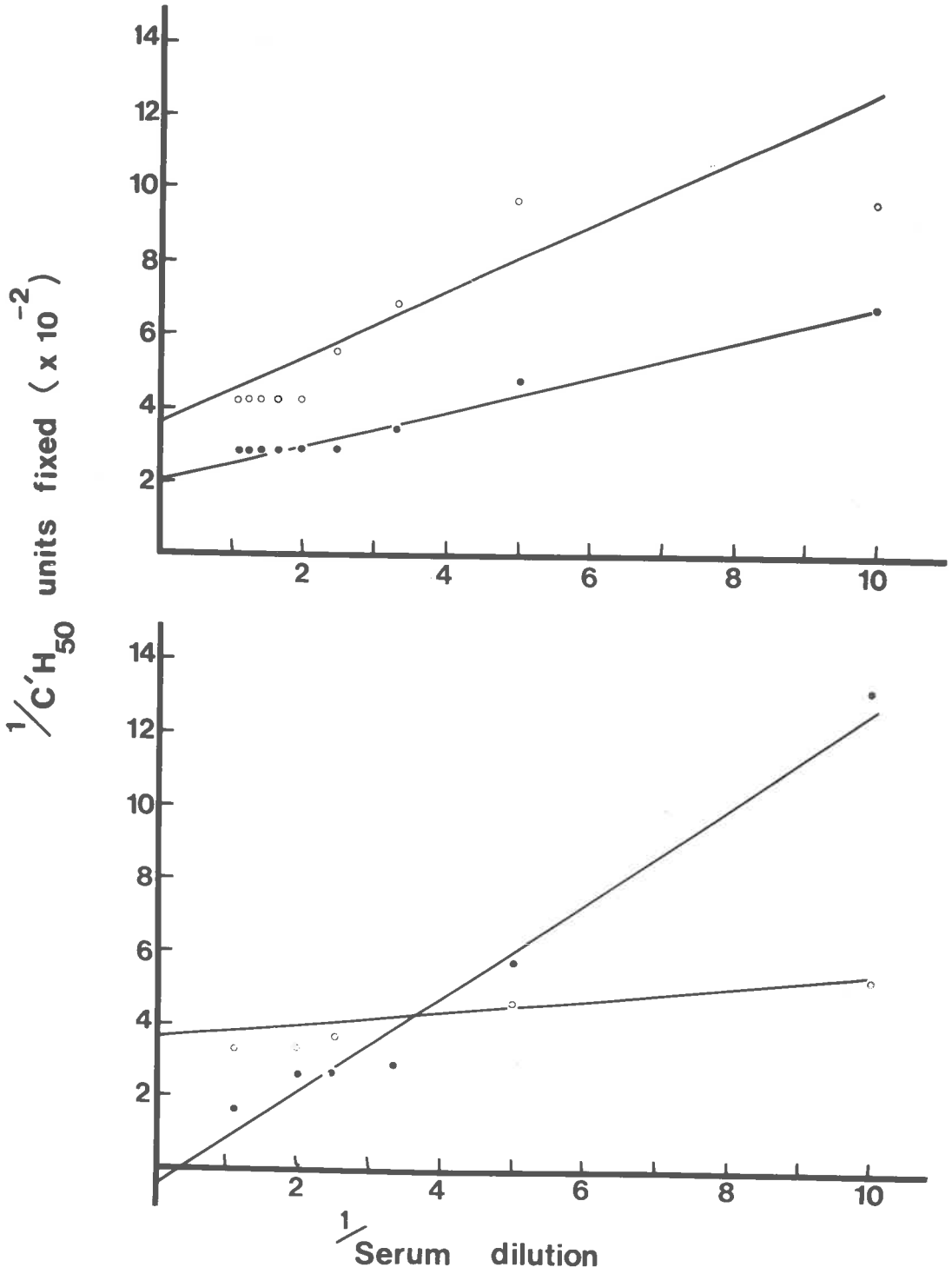

14
12
10
8
6
4
2
2
4
6
8
10
1/C'H50 units fixed (x 10-2)
1/Serum dilution

TABLE 28

Parameters of the equation $y = bx + a$ [a] calculated by the method of least squares from complement fixation experiments using 0.5 mg. C5 and M206 with high concentrations of anti-5 serum

Strain	a	b	r [b]
(A) First experiment			
C5	0.020(0.005) [c]	0.005(0.002)	0.982
M206	0.036(0.014)	0.007(0.001)	0.883
(B) Second experiment			
C5	-0.005(0.001)	0.013(0.003)	0.989
M206	0.03 (0.02)	0.002(0.001)	0.950

(a) $y = 1/C'H_{50}$ units fixed

b = constant/maximum number of $C'H_{50}$ sites

x = 1/relative serum concentration

a = 1/maximum number of $C'H_{50}$ sites

(b) r = product moment correlation coefficient

(c) Figures in parenthesis are 5% confidence limits

Inspection shows that there were not enough experimental points to decide whether the equation was reasonable. It could be shown, for example, that a straight line was obtainable when the reciprocal of the square of the serum concentration was plotted against the reciprocal of the number of $C'H_{50}$ units fixed. The product moment correlation coefficient (r) calculated for both sets of points was close enough to +1 to show that all of them could be plotted as fairly good straight lines, but the situation was complicated by the fact that the antiserum contained 2 types of antibody. Whereas γM antibody should have reacted with C'1a according to the first equation used, γG should have reacted in a much more complex manner (Borsos and Rapp, 1965). It seemed from the experimental points that the lines intercepted the ordinate at a positive value. Table 29(A) shows the nature of the gradients and intercepts of each line found from corresponding values obtained using C5 and M206. It is interesting that these ratios corresponded well with the ratio (0.52) of the amounts of polysaccharide found in the two bacteria as described in chapter 6.

TABLE

Estimation of the ratios of complement fixing sites and minimum numbers of sites on C5 and M206 after treatment with anti-5 serum

Experiment	$\frac{a_{C5}}{a_{M206}}$	$\frac{b_{C5}}{b_{M206}}$	Minimum No. of C' fixing sites on C5	Minimum No. of C' fixing sites on M206
(A)	0.56	0.67	17 ± 4	10 ± 3
(B)	-0.16	5.7	-	10 ± 4

Abbreviations are the same as those in table 29.

As these results seemed to agree fairly well with equation (9) of appendix 2A, the values obtained from the intercepts were used to calculate minimum values for complement fixing sites on each bacterium. The numbers of bacteria present in the first well were equivalent to 0.05 mg dry weight, or 5×10^7 bacteria. The number of complement 'molecules' was calculated from the number of $C'H_{50}$ units as follows:

In each well there were approximately 2.5×10^7 red blood cells. One $C'H_{50}$ unit was sufficient to lyse 50% of these cells. The single hit theory as described by Mayer (1961) predicts that for a given amount of complement, the fraction

of the total number of cells with 0, 1, 2, 3 .... n ... etc. lesions is given by the successive components of the expression

$$e^{-z}\left(1 + z + \frac{z^2}{2!} + \frac{z^3}{3!} + \ldots\ldots \frac{z^n}{n!} + \ldots\ldots\right)$$

where z is the average number of sites present on each cell. As 50% of the cells did not lyse we know from the first term that:

$$e^{-z} = 0.5$$

$$-z = \log_e(0.5) = 2.3 \log_{10}(0.5) = -0.69$$

Hence the total number of complement lesions represented by one $C'H_{50}$ unit in this system is:

$$0.69 \times 2.5 \times 10^7 = 1.7 \times 10^7 \text{ lesions}$$

This represents a minimum estimate for the number of complement 'molecules' represented by one unit, and was used in the calculation of the fourth and fifth columns in table 29 A. It seemed likely that the minimum value lay between 10 and 20, but as the wastage of individual complement components can be very high during complement fixation the actual number could be very much more, perhaps as high as the total number of antibody molecules present on the bacteria.

A subsequent experiment failed to confirm this interpretation. The results in Fig 24B and tables 28(B) and 29(B) were calculated from the geometric means of triplicate determinations carried out in the same manner as the previous experiment. The lines obtained differed too much from those previously calculated for it to be reasonable to try to explain them in terms of random variation. Bacteria and sera were frozen aliquots from the same samples and it seemed unlikely that they were the cause of the differences. However, a more careful examination of the results obtained in different haemagglutination trays showed that there seemed to be considerable systematic errors resulting from the use of different trays. It is likely, therefore, that something was wrong with either the trays or the procedure used for washing them. This difference could easily have caused the differences noted between different sets of results and probably also those obtained when the different strains of bacteria were used. At this stage, unfortunately, experiments had to be discontinued.

An over-optimistic interpretation was required to make either of the equations of appendix 2A seem reasonable descriptions of the experimental situation, to suggest that the amounts of comple-

ment which can be fixed by C5 and M206 are proportional to the number of antigenic sites on the two organisms, or to make rather uninformative the estimate of 10-20 as the minimum number of complement fixing sites on these bacteria. Even after solution of the problem of the differences between haemagglutination trays, it seems improbable that much more helpful results will be obtained without a more precise method for measuring complement fixing ability than one based on doubling dilutions of antigen. Better estimates of numbers of complement fixing sites are likely to be obtainable by the use of the C'1 transfer reaction of Borsos and Rapp (1965_b), and the equations of appendix 2A are more likely to prove valid for studies involving this reaction alone than for the very complicated complete complement reaction. However, it is at least clear from the results using all three anti-sera that there is not an enormous difference between the number of complement fixing sites on C5 and M206. It is unlikely that the difference in virulence between C5 and M206 has anything to do with a difference in the ability of their respective O-antigens to fix complement.

Discussion

The experiments reported in this chapter were intended to investigate the relative efficiencies of the antibodies against heat-stable and heat-labile antigens. All of the antisera used were directed against fairly stable antigens which could at least survive heating at 56° for 30 minutes, and two were against antigens which could withstand prolonged boiling. It is clear that all of these antisera were effective not only in killing the avirulent M206 in the in vitro bactericidal reaction, but also as opsonins for C5 in the intraperitoneal killing test. The latter conclusion is important. It means that even if antibody against heat labile antigens is an important factor in immunity to this strain of the unimmunised mouse as is suggested by the work of Auzins and Rowley (1963), there is no reason to suggest that this antigen in any way renders ineffective opsonic antibodies against heat stable lipopolysaccharide components. This conclusion is in agreement with the findings of other workers (Biozzi, et al, 1963). In spite of their failures at the quantitative level, the complement fixation experiments lead to results which support this conclusion. They show that antibody reacting with O-antigen 4, 5 and 12 on

C5 is apparently just as capable of reacting with guinea-pig complement as is similar antibody on M206. Two possibilities remain as likely reasons for the differences between the resistances of C5 and M206 to antibody and complement. Either C5 is able to repair lesions caused by complement before they result in the death of the organism, or complement, although it fixes to C5, is unable to cause lesions. The possibility exists at the moment that even an M206 complement fixation leads to cell death only when it fixes at a limited number of sites on the lipopolysaccharide.

It is not possible from the information in this chapter to calculate how many antibody molecules are required to kill one M206 organism by the serum bactericidal reaction or to opsonize one C5 for phagocytosis and killing. However, limits can be set which show the maximum number of molecules that are required if it is assumed that all of the precipitable antibody is either IgM or IgG, and that this is equal to approximately 1 mg. per ml. in each serum (chapter 5). Results of these calculations are set out in table 31.

TABLE 30

The molecular efficiencies of anti-4, 5 and 12 sera in the killing of M206 by the in vitro bactericidal reaction and of C5 in the in vivo bactericidal reaction after opsonisation

Antibody specificity	Ratio at final dilution of: Number of antibody molecules initially present / Number of bacteria initially present			
	Assuming 1 mg/ml. IgM before diln.		Assuming 1 mg/ml. IgG before diln.	
	in vitro[a]	in vivo[a]	in vitro[b]	in vivo[b]
4	1×10^7	1×10^5	9×10^7	7×10^5
5	1×10^6	1×10^4	9×10^7	7×10^4
12	1×10^8	1×10^5	9×10^8	7×10^5

(a) Estimates based on quantitative precipitin reaction of chapter 5 and bactericidal titres of whole sera from table 26 (initial number of bacteria = 4×10^3) and Fig 22 (initial number of bacteria = 5×10^4).

(b) Estimates based on quantitative precipitin data from whole sera (chapter 5) and the bactericidal activity of mercaptoethanol treated sera (table 26) (initial number of bacteria = 4×10^3).

The table shows that invariably more antibody was required than could be fitted on to the bacteria whether it was assumed that all of the antibody was IgM or IgG. In no case was there any approach to the efficiency of antibody action found by Rowley and Turner (1966) in opsonization for intraperitoneal killing.

Similarly, the apparent number of antibody molecules required to kill one bacterium never appeared to be as low as 30 molecules of IgM or 600 of IgG as can be calculated from the data of Šterzl and his colleagues (Šterzl, Kostka and Lanc, 1962, Šterzl, 1963), nor even the figure of 700 to 900 calculated by Muschel and Treffers (1956) for the apparent number of rabbit IgG antibody molecules required to kill one susceptible organism of S.typhi. The requirements for antibody and the other components of these systems are very poorly understood, and the validity of equating numbers of reactive antibody molecules present in a given system with the numbers required to cause cell death is questionable. Only maximum values can be set. This is because it has been observed that the end point of an in vitro bactericidal reaction titration is frequently independent of the number of bacteria used in the test (Weidanz and Landy, 1963). These authors showed, for example, in one set of titrations using techniques similar to those described here, that the number of bacteria used could be varied from 10^2 to 10^6 without affecting the titre of a given antiserum. This could arise if a comparatively small fraction of antibody was fixed at the time that the reaction took place. Suppose, to

take a very simplified example, that killing occurs when one molecule of antibody (A) reacts with one bacterium (B) to form a sensitized bacterium (AB) which can be killed by complement, and that the titre depends on the number of these sensitized bacteria which are present at a certain moment. We can write the equation:

$$A + B \rightleftharpoons AB \qquad (1)$$

The law of mass action allows us to write:

$$\frac{[A][B]}{[AB]} = K \qquad (2)$$

where K is the equilbrium constant of the reaction, so that:

$$\frac{[B]}{[AB]} = \frac{K}{[A]} \qquad (3)$$

If the reaction is carried out in unit volume and the initial concentration of (B) is written B, then:

$$[B] = (B - [AB])$$

$$\therefore \quad \frac{(B - [AB])}{[AB]} = \frac{K}{[A]} \qquad (4)$$

$$\therefore \quad \frac{B}{[AB]} - 1 = \frac{K}{[A]} \qquad (5)$$

$$\therefore \quad \frac{B}{[AB]} = \frac{K}{[A]} + 1 \qquad (6)$$

$$\therefore \quad \frac{B}{[AB]} = \frac{K + [A]}{[A]} \qquad (7)$$

Inverting, we have:

$$\frac{[AB]}{B} = \frac{[A]}{K + [A]} \qquad (8)$$

At high concentrations of antibody, when $A \gg K$, this can be written:

$$\frac{[AB]}{B} \simeq \frac{[A]}{[A]} = 1$$

i.e. all of the bacteria are sensitized for killing and the actual number killed depends on other factors.

The titre of the serum is defined as the maximum dilution which can lead to the killing of a defined percentage of bacteria. That is $\frac{[AB]}{B}$ is fixed. In general, the end point will be reached when [A] is of the same order as K. For an end point of 90% killing, for example, if AB is completely converted to killed bacteria, it can readily be shown, by substituting in equation (8) that [A] = 9K. As we have assumed that only one molecule of antibody per bacterium is required for sensitization, A can be written as (A - [AB]), where A is the initial number of antibody molecules present. This will be almost constant if A is very much greater than AB. In this case, at the end point, A = 9K.

In chapter 5 a dissociation constant of about 10^{12} molecules/ml. was calculated for the reaction of anti-5 antibody with bacterial lipopolysaccharide antigen on bacteria and it was pointed out that this represented an antibody of unusually high avidity if the reaction was compared with those of oligosaccharide haptens. If we assume that there are 10^4 antigenic sites on each bacterium, then the corresponding constant for the dissociation of antibody from bacteria will be:-

$$\frac{10^{12}}{10^4} = 10^8,$$

i.e. assuming that the same antibodies are involved, at the end point,

$$A = 9 \times 10^8.$$

The number of bacteria used in these experiments is of the order of only 10^3 and we originally assumed that the end point was reached when only one antibody molecule reacted per bacterium, so that the number of unreacted molecules is far in excess of the number which react at the end point of this titration. In this case, we estimate the excess number of antibodies per bacterium at the end point as being of the order of:-

$$\frac{9 \times 10^8}{10^3} = 9 \times 10^5.$$

The estimated range (table 31) varied from 10^6 to 9×10^7.

In view of the uncertainties of the assumptions involved in deriving this equation, and the estimates of antibody efficiency and equilibrium constant, the correlation is much better than could reasonably have been expected. The fact that an equation such as the one above can be set up and made to show even a vague correspondance with experimental data implies that the phenomenon described by Weidanz and Landy can be explained in more or less simple equilibrium terms. It also explains why different authors will reach widely discordant estimates of the efficiencies of antibodies in causing the in vitro bactericidal reaction unless high concentrations of bacteria or antibodies with very high avidity are used. In the examples under discussion here it can be assumed that one reason for the apparently greater efficiency of Muschel and Treffer's sera is that their reactions were carried out using a concentration of bacteria of about 5×10^7 per ml. It is probable that the conclusions derived from this equation apply also to phagocytosis, although it must be assumed that another limiting factor in this situation will be the ability of the mouse to phagocytose very large numbers of opsonized bacteria.

The question now arises of whether antibody against the relatively heat-labile antigen 5 is more effective than antibodies against other lipopolysaccharide components. Although the anti-5 serum used throughout most of these studies was more effective than anti-4 or 12 in both the bactericidal reactions tested, much of this superiority was probably due to the higher proportion of IgM antibody which it contained. 2-mercaptoethanol-resistant antibody was not tested in the killing reaction against C5. However, it seemed that when it was used against M206, anti-5 still appeared to be more effective than 4 or 12. It has not been established whether this superiority was due to a difference in the avidity of the antibody or to some difference between 5 and the other two antigens on the bacterial surface. The most likely difference, if it is important, is that there is probably more 5 antigen available than 4 or 12. It has been shown that this is likely to lead to a difference in efficiency of antibodies against 5 compared with other antibodies, but that this difference will only be apparent if the antibodies used are of relatively low affinity or if the experimental conditions are such that very low concentrations of bacteria are used. (Appendix 3). Biozzi et al. (1963) found little difference between antibody against 5

and other anti-lipopolysaccharide antibodies in opsonising S.typhimurium for clearance from the mouse circulation. All of the problems referred to in this discussion in distinguishing effects of different class and affinity apply with equal force to the findings of these workers. The results available must, therefore, be regarded as inconclusive.

In the course of the studies on the in vitro bactericidal reaction it was frequently noticed that at high concentrations of antiserum (dilutions of 1/100 and less) the bactericidal action was inhibited. The phenomenon is particularly noticeable in the results shown in Fig 21. The effect is specific and is not due to an anti-complementary effect of absorbed serum, as can be seen from its distribution amongst the titrations of table 24, where inhibition at high concentrations of serum occurred only when bacteria which were antigenically closely related to the original immunizing strain were used. The graphs in Fig 21 also show that the effect is caused at least in part by mercaptoethanol-stable antibodies or antibody fragments, but do not exclude further participation by whole IgM antibodies. Availability of rough estimates of the number of antigenic sites on bacteria and the approximate equilibrium constant for the

antigen/antibody reaction allows calculation of the number of antibodies attached when the prozone appears. In a suspension containing 10^3 bacteria/ml. each with 10^4 antigenic sites, the total concentration of antigenic sites is 10^7 sites/ml. At a dilution of 1/100 the concentration of anti-5 antibodies is approximately 6×10^{12} molecules of IgM/ml. The concentration of antigen/antibody complexes present when the system has reached equilibrium may be calculated by substituting into the equation:

$$\frac{[A]\ [B]}{[AB]} = K$$

i.e.
$$\frac{(6 \times 10^{12} - [AB])\ (10^7 - [AB])}{[AB]} = 10^{12}$$

It can be shown that [AB] in this equation has a value of the order of 10^7, the total number of antigenic sites which were originally present. In other words, a prozone occurs when the antibody molecules are present on the surface in such numbers that they are jostling each other for position and almost completely cover the organism.

Several highly speculative mechanisms can be proposed to account for this phenomenon. It may be, for example, that antibody molecules form a protective layer and prevent access of complement to the inner parts of the cell wall, or even that an antibody, to be effective, fixes to the outside and then slithers, by successive reactions with different antigenic sites, to a more effective position nearer the cell membrane and is prevented from doing so by the high concentration of adjacent antibodies. Alternatively, it is possible that complement fixation depends very precisely on the way in which antibody is fixed. If, for example, it cannot occur unless the antibody reacts at more than one site, then the reaction may be made very difficult at high antibody concentration because of competition between individual molecules. This may be difficult, even with antibodies such as IgM, in which single molecules are capable of fixing complement. Borsos and Rapp (1965$_a$) have shown that two molecules of IgG are required to fix one of C'1, and in this case the chances of two adjacent molecules both being able to combine at two sites may be even less. This suggests that IgG antibodies may be more prone to cause a prozone than IgM. Unfortunately complement fixation studies at the dilutions of bacteria used in these studies are difficult.

Summary

It has been shown that antibodies directed against antigens 4, 5 or 12 are all effective in the in vitro bactericidal reaction against M206, the in vivo reaction against C5 and in complement fixation, but that at least in the first case other antibodies may also be effective. There is no conclusive evidence relating to the relative efficiencies of antibodies of the three specificities The effects on antibody titres and on the Neisser-Wechsberg phenomenon of the number of bacteria used in a system, and of the concentration of antigen on these bacteria are discussed.

CHAPTER 8

DISCUSSION

The work in this thesis was primarily undertaken to find out whether there was any reason to believe that antigen 5 content contributed to virulence in Salmonellae, and in particular, to find out if antigen 5 gave any special protection to S.typhimurium strain C5 in the mouse. The short answer seems to be that it does not. As was shown in chapter 4 antigen 5 is situated almost identically to its fellow O-antigens 4 and 12, and the results of chapter 7 demonstrate that antibodies against any of these three components are able, under suitable conditions in the presence of complement, to lead to the killing of either C5 or M206. There is thus nothing magical about the presence of antigen 5. It does not prevent the other lipopolysaccharide antigens from reacting, nor inhibit the immune disposal mechanisms after the reaction of antibody.

One qualification must be made. The evidence is based entirely on the reactions of sera from hyperimmune rabbits. It is also possible that antigen 5 may affect the immune response of an animal. Auzins and Rowley (1969) have shown, for example, that one highly susceptible inbred strain of mice is apparently unable to synthesize antibody against 5 and it may well be that

this failure is associated with the increased virulence of Salmonellae such as M206 in these animals. However animals from the outbred colony of Swiss white mice used in the virulence tests referred to in table 22, which are highly susceptible to C5, appear to be quite capable of producing anti-5 antibodies (Auzins and Rowley, 1969), so that the virulence of C5 in this animal which was used as a model in the studies described here, is unlikely to be associated with antigen 5. On the other hand there does appear to be some evidence in chapter 7 to suggest that anti-5 antibodies are rather more effective in dealing with C5 and M206 than are antibodies against the closely related antigens 4 and 12. The reasons for this apparent difference in efficiency are better discussed after some consideration of what we mean by 'specific' antibodies,

One of the recurring problems found in the course of this work was that of deciding what was a specific antibody, or a specific antigenic determinant. The definitions of the Kauffmann-White scheme are perfectly adequate for serological typing in epidemiological studies, the purpose for which they were designed, and it seemed, from the work of the early 1960s (eg Staub, 1960) that these definitions could be simply translated into chemical terms

in which the antigen was described as a specific sugar or sequence of sugars. This is, at least in theory, adequate to describe the bacterium or its isolated lipopolysaccharide. Problems arise, however, as soon as we try to introduce antibodies into this second definition. It is clear that most 'specific' antisera contain a heterogeneous mixture of molecules, probably directed against a number of qualitatively different combining sites of variable size (Kabat, 1966). It was obvious from Staub's inhibition studies that although single monosaccharide units could be regarded as providing much of the specificity of the individual antigens, complete inhibition of her antisera could not be obtained without the use of larger oligosaccharide units. The observation that the linkages between monosaccharide units contribute to the specificities defined in the Kauffmann-White scheme also shows the importance of groups of sugars rather than single monosaccharides. In spite of these complications the assumption was made at the time that this work was begun, that quantitative absorption studies would measure how much of each individual sugar, or at least pair of sugars, was available on the bacterial surface. Anti-4 serum could be used to measure how much abequose was available, anti-5 how much acetyl galactose, and so on. Hellerqvist et al's (1968) results make this approach more than questionable. They showed that

the presence of antigen-5 was attributable to acetylation of antigen 4, and that in one strain was, as well as they could measure, complete. While this explained a number of observations, it raised the important question of what constitutes antigen-4 in a bacterium with antigen-5. There appear to be two possibilities. First, there may be a number of non-acetylated abequose residues on each bacterium. Hellerqvist et al's data would probably permit up to 5 percent. As there are about 10^7 potential antigenic sites on each bacterium (chapter 6) this would still allow 5×10^5 sites which could be described chemically as antigen-4, and such a small percentage might arise either from an inefficient acetylating enzyme, or from wear and tear on the labile group of the O-antigen after it had been formed. An alternative hypothesis arises from knowledge of the heterogeneity of the antibody response. As the oligosaccharides corresponding to antigens 4 and 5 differ only in the presence of an acetyl group it is likely that a number of antibodies which react with 4 will also be able to react with 5. This means that when a specific anti-4 serum is made to react with a bacterium which contains, chemically, only antigen-5,considerable cross-reactions may be observed, so that the bacterium is said to contain antigen-4 as well as 5. In this case it will appear,

formally, as if there are as many antigen 4 sites as 5 on the bacterium. This interpretation is inconsistent with the conclusions of chapter 5, but in view of the difficulties of interpreting results using antisera containing antibodies of heterogeneous class and avidity and the use of measurements outside the optimal range it cannot be said to have been disproved. A combination of both effects seems likely. However, as one involves the reaction of an antibody with its homologous antigen while the other requires a reaction with a larger, heterologous antigen, one would expect affinities of antibodies involved in the former effect to be greater than those involved in the latter, which may partly explain why differences were found in chapter 5.

It is interesting that the converse argument of cross-reaction with antigen 4 by a specific anti-5 serum cannot be applied, as all anti-5 sera which have been raised against bacteria have been thoroughly absorbed with organisms containing only antigen 4. This means that when we compare the reactions of antisera against 4 and 5 on a bacterium with both antigenic specificities, we are probably comparing antibodies which are highly specific for a very small antigenic determinant with a population of antibodies containing a fairly large proportion which are less specific for the antigen, and which may well be directed

against a larger site. Kaplan and Kabat (1966) have shown in a system using human blood group A substance that IgM antibodies appeared to be directed against small, and IgG against larger sites, and it may well be that the absorption techniques used to prepare specific antisera tend also to cause some fractionation of antibodies into their different classes.

Two factors probably contribute to the apparent increased efficiency of anti-5 serum over anti-4. One is the greater number of antigen 5 sites than 4 on the bacteria; the other is an artefact, resulting from absorption techniques involved in making specific antisera. A third possibility can also be suggested from the work of Hellerqvist **et al.** Abequose, the determinant of antigen 4, is a rather hydrophobic sugar, and when combined in lipopolysaccharide has only two free hydroxyl groups. Acetyl abequose, however, with only one free hydroxyl, is even more hydrophobic. As it seems likely that antibodies can combine better with hydrophobic than with hydrophilic groups (see, for example, Eisen and Siskind, 1964, Singer, 1965) this means that it should be possible for an animal to make antibodies which bind more strongly to antigen 5 than to antigen 4. In other words the average affinity constant of antibodies against antigen 5 would be expected to be greater than those against 4. Their

avidities would depend on the geography of the bacterial surface, and again might be expected to be greater than those of anti-4 molecules, especially if the concentration of 5 sites is greater than that of 4. The values for the equilibrium constant (K) in chapter 5, although consistent with this view, cannot be regarded as providing strong evidence for it. Antibody affinities depend on many factors, including the class of antibodies involved, the period of immunisation, the size of the immunogenic dose and so on.

For a definitive answer the question would have to be investigated using large numbers of preferably inbred animals, a range of carefully planned immunization schedules, and a test method using free rather than bound haptens.

Other authors have suggested that antibodies against certain O-antigens are particularly effective. Daguillard and Edsall (1968), using antisera against S.typhi 0 901, found that preparations of anti-12 antibodies were more effective than anti-9 in the in vitro bactericidal reaction. This result was obtained whether IgG or IgM preparations were used. Antibodies were prepared by absorption and elution from S.haarlem (0:(9),46) to obtain anti-9 antibodies and from S.paratyphi B (0:1,4,5,12)

to prepare anti-12. It seems unlikely that these results can be explained by a difference in numbers of antigenic sites, as has been proposed in this thesis for the antigen 5 case, or by a difference between terminal and internal residues in the polysaccharide, as was originally proposed by Daguillard and Edsall. In the first case, if biosynthesis of polysaccharide on S.typhi 0 901 follows a similar course to the corresponding series of reactions described for S.typhimurium by Osborn and her colleagues (see chapter 1) it seems unlikely that there will be less antigen 9 sites (immunodominant sugar tyvelose) than 12 (whose specificity appears to arise partly from the structure of the main chain and partly from a glucose side branch). It is likely that the terminal sugars are tyvelose or mannose, and not glucose and galactose, as suggested by Daguillard and Edsall. It may be that there are common, unsuspected antigens shared by S.typhi 0 901 and S.paratyphi B, as was also suggested by these authors, or that, for other reasons, there is a closer affinity between the 12 antigens of S.typhi 0 901 and S.paratyphi B than there is between the 9 antigen of S.typhi 0 901 and (9) of S.haarlem.

It is clear from the foregoing discussion that there is no evidence from the quantitative data or from tests of biological activities to suggest a connection between the possession of antigen 5 and virulence. If anything, antigen 5 should be expected to be a disadvantage to the organism, as one might expect the avidity of antibodies directed against it to be higher than their avidity against other antigens. If the amount of antigen 5 is of no significance in affecting the avirulence of M206, we must return to the question of deciding whether we know anything of the organism's chemistry which is likely to be important. It is probable, in fact, that more than one factor contributes (Furness and Rowley, 1956). This is hardly surprising. Jensen (1929) originally obtained the strain by subculturing a virulent strain 206 times on nutrient media, and in the circumstances mutations to avirulent forms were more than likely to occur.

One difference between C5 and M206 found in the course of this work was that C5 contained almost twice as much O-specific polysaccharide as M206, whether this was measured as available antigen (chapter 4) or as total polysaccharide (chapter 6). At first sight this seems to be too small a difference to be important.

However, it is interesting to look at the analogy with pneumococcal strains. With these organisms there is little doubt that protection and resistance can be conferred by specific anti-polysaccharide antibody (Wilson and Miles, 1946). MacLeod and Krauss (1950) compared the type specific polysaccharide contents of virulent and avirulent smooth pneumococcal strains and found with two strains differing in LD_{50} in mice by a factor of 10^7, that the virulent strain had only 50 percent more polysaccharide than the avirulent smooth strain of the same type. In a series of strains examined in this way they found that the virulent members always had more polysaccharide than did the smooth avirulent members of the same type, but that the difference in polysaccharide content were always small. Hence it is not unlikely that the small difference observed is a significant attribute of the difference in virulence between C5 and M206.

The results of the estimations of total polysaccharide content in chapter 6 give a rather equivocal confirmation of this conclusion. There seems to be a preponderance of bacteria with large amounts of polysaccharide amongst the virulent strains, and the results of the determinations on the avirulent and partially avirulent recombinants derived from C5 indicate

that it is more than likely that a reduction in polysaccharide content can cause a fall in virulence. However the discovery of a highly virulent strain with even less polysaccharide than M206 raises the question of what sorts of reduction can lead to a fall in virulence and what sorts have no effect.

In the discussion to chapter 7 it was shown that it is probable that only a very small number of IgM antibody molecules need be bound to a single bacterium to cause killing either by the direct action of complement or through phagocytosis in the mouse peritoneum, and it may even be that one molecule is sufficient. It is unlikely, therefore, that any sort of cooperation occurs between complement fixing sites. If, as seems likely, the quantity of O-antigen affects the efficiency of the complement mediated reactions, the effect must be explained in terms of how lipopolysaccharide prevents complement at a single binding site from killing the bacterium.

Two main ways in which O-specific polysaccharide can affect susceptibility to serum, and perhaps virulence, have been proposed. Both rely on the assumption that they cause fixation of antibody and complement at too great a distance from the bacterial surface to be effective. In the first mechanism

(Archer and Rowley, 1969) polysaccharide is treated as if it forms a homogeneous fluid, or at least a three-dimensional net, covering the bacterial surface, and assumes that antibody attaches only at the outer surface while complement fragments can pass through. It is suggested that distance itself is sufficient to prevent access of complement to sensitive surfaces. The assumption is made that complement acts by releasing a shower of active molecules from the point of attachment of antibody, and that if these can reach a certain concentration on the bacterial surface, lesions and death result. The number of complement factors reaching a given area of cell wall within a certain time would be expected to vary more or less inversely with the square of the distance of antibody from the surface, and if the factors are also labile, an inverse cube relationship could result. Such a relationship could lead to a surprisingly large difference in the effect of antibodies attaching at varying distances from the complement sensitive surface.

Although this mechanism has attractive features, notably the simplicity of its view of the bacterial surface, and its correlation with the findings that at least two complement factors, C'4 and C'3 appear to be able to release active fragments which are able

to act at a distance from the original site of antibody fixation (see review by Muller-Eberhard, 1968), a number of findings are difficult to explain. It is unreasonable to suggest, for example that doubling the thickness of a thin cylinder of lipopolysaccharide, which is the difference between C5 and M206 found by the chemical method, would lead to a doubling of the area of the cylinder, which would be necessary to explain the absorption results of chapter 4. Furthermore it is difficult to explain the occurrence of highly virulent bacteria with low polysaccharide content on this model. If the Melbourne strain of S.enteritidis is to fit then one must postulate that it contains another antigen which can take the place of the missing O-antigen. At present, as suggested in chapter 6, the only plausible alternative would seem to be the T_1 antigen.

The other hypothesis suggests that polysaccharide can form a complete surface on the bacterium through which it is impossible for either antibody or complement to penetrate. This situation would occur in C5 and also, perhaps, on S.enteritidis (melbourne). In some organisms, including, presumably, M206, the surface is uneven and patchy, allowing access of complement to sensitive internal membranes. This

is likely to occur if at some parts of the cell wall O-specific polysaccharide does not form, so that at least some of the lipopolysaccharide is incomplete. In this case the organism would correspond to one of the semi-rough forms described by Naide et al (1965). In this respect it is interesting to note that Krishnapillai and Karthigasu (1969) recently reported that M206 was susceptible to a number of phages which are normally specific only for rough forms, and Krishnapillai (personal communication) has also shown that ST11, a partially avirulent strain which was shown in chapter 6 to have a low polysaccharide content, is also susceptible to a phage which normally attacks only strains of the Rb chemotype. It seems very probable, therefore, that M206 is a semi-rough form in which not all of the surface is covered with polysaccharide. The rather surprising possibility also emerges that S.abony SW1444 is also semi-rough, although this does not appear to have been detected. It is obvious that semi-rough is a quality which can have many shades of meaning, and it may be that the deficiency of the relevant gene in SW1444 and C5R is only obvious under conditions of severe stress, as when it is at the focus of an active immune response in the mouse. It may be interesting to look for rough lipopolysaccharide in both these strains using the recently described method of Galanos, Lüderitz and Westphal (1969).

If S.typhimurium C5 and S.enteritidis (melbourne) are both evenly covered with O-specific polysaccharide some other difference must be found between the two strains. At the moment the most likely appears to be in the average length of the O-specific polysaccharide side-chain. In spite of advances in knowledge of the gross structure and immunological properties of Salmonella O-polysaccharide, little seems to be known of the factors which determine chain length. It seems likely that length is variable both between strains and within a single organism. Hellerqvist et al (1969) showed in comparisons between two strains of S.typhimurium by measurement of the ratio of terminal to internal mannose, that the average number of O-specific oligosaccharides in one strain was 11 per chain, while in the other (LT2) the average degree of polymerisation was only 7. This second figure is at variance with the figure obtained by Kent and Osborn (1968_a), who estimated the chain length of the ACL-bound O-polysaccharide hapten synthesised by LT2 mutants which could normally synthesize neither complete core nor sidechain without the supply of additional sugars. Using the ratio of reducing terminal to internal galactose in the O-polysaccharide hapten they found a degree of polymerisation of about 30. This variability suggests that the enzymes involved in synthesis and polymerisation of oligosaccharides on

the antigen carrier lipid are not arranged like those of the C_{16} fatty acid synthesizing system, which make polymers up to a certain size and then release them (see Vagelos, 1964). Furthermore the enzyme linking O-hapten to the rough core also seems relatively non-specific over chain length, as is shown by the occurrence of semi-rough type C mutants with a degree of polymerisation of one. It seems likely, therefore, that chain length in group B, D and E Salmonellae depends on competition between the polymerizing enzyme which lengthens the chain and the polymerase which attaches it to the core. Mutants like those studied by Kent and Osborn, which lack a completed core, would be expected to make longer hapten chains than normal bacteria in which this competition was possible. This suggests that the number of core stubs in M206 may be rather more, and the number of side-chains rather less than the number one might expect from the fact that it contains half as much polysaccharide as C5. In comparisons between the two virulent strains it must be assumed that the ratios of the relative efficiencies of the oligosaccharide polymerizing to the polysaccharide completing enzymes must be greater in C5 than in S.enteritidis melbourne.

The most important question raised by this thesis is whether by accepting the picture of M206 as an organism which presents a surface which is either susceptible or permeable to antibody and complement, sparsely covered by strands of smooth lipopolysaccharide, it becomes possible to develop a more complete theory of immunity to S.typhimurium. It is probable that this surface is antigenic, and likely that its relevant antigens are found on many other strains of bacteria. They are likely to consist of both the rough lipopolysaccharide antigen, which is thought to be common to all Salmonellae, and Barber proteins, which show a different set of antigenic cross-reactions from those recognised in the Kauffmann-White scheme. As these antigens are more likely to be met than the O-specific antigens, it is probable that most organisms will have a permanent low level of 'natural' antibodies directed against them. When M206 is treated in the presence of complement with 'normal' rabbit serum at a dilution of 1/100 it is generally killed (table 24). It is likely that these antibodies are not directed against smooth polysaccharide, and that similar antibodies account for the natural opsonin level for M206 which is found in mouse serum. C5 is generally protected from this opsonin, but when it is heated, or when metal ions are withdrawn from the protein-

lipopolysaccharide outer layers, it seems likely that the surface becomes disrupted, allowing access of antibody and complement to the inner parts of the cell wall. This may explain the sticking of heated C5 to normal mouse peritoneal macrophages (Auzins and Rowley, 1963) and the susceptibility of the organism to antibody and complement in conditions where magnesium is limiting (Reynolds and Rowley, 1969).

Much confusion seems to have resulted from the assumption that the antibody composition of the serum of an immunized animal is a specific magnified version of serum composition in the 'normal' individual. It seems much more likely that most of the antibody in the normal animal is directed against common antigens, access to which is difficult in most virulent Salmonellae, while in the immunized animal, antibody is directed mainly against specific O-polysaccharides, and also possibly Barber proteins which are located at the outside of the cell wall. These antibodies may be expected to be effective in promoting complement fixation and phagocytosis but are likely to fail to promote serum bactericidal action except when there are holes in the polysaccharide coat.

The other observation which led to the assumption that 5 might be an unusually important antigen in immunity was the observed superiority of live of alcohol or acetone-killed Salmonella vaccines over heat-killed and other forms (chapter 2). One reason for the increased resistance of recently infected animals to subsequent infection is a non-specific effect due to an increase in the proportion of macrophages which are capable of killing bacteria and it is possible that in the case of Salmonellae a further increase results from production of antibody against a heat-labile toxin (see chapter 2). It also seems likely that the immune response is different depending on how the antigen is presented (Stern, Davidsohn and Masaitis, 1956, Uhr and Möller, 1968). This could well be due to possession by the host of natural antibodies directed against components which are normally concealed. One may expect an alcohol-treated C5, or acetone dried S.typhi organism with its common antigens still covered by smooth lipopolysaccharide, to be treated differently from a heated organism which is immediately recognised by the host as being something to which it already possesses an adequate supply of antibody. The first may be recognised as foreign but unknown, and give rise to a very different response to the second, which is likely to be rapidly

removed and digested. The distinction here is probably drawn too sharply, and features of both responses will be observed in each case. Nevertheless, quantitative differences between the two types of response are likely to occur, and these will be reflected in the ultimate resistance attained by the host.

The model to explain the apparent importance of heat-labile antigens in immunity to Salmonellae can be summarised as follows. It is proposed that one of the essential features of a virulent organism is its possession of a complex coat of specific polysaccharide and perhaps protein which is impervious to antibody and complement. A comparative lack of specific antibody to this coat in the normal animal allows the organism to grow for some days before an immune response can be developed, and by this time the number of bacteria present is so great that they are able to overwhelm the host before they can be completely destroyed. However if very gently killed bacteria are injected in this form they are treated as unknown foreign antigens and give rise to a particular type of immune response directed mainly against the outermost antigens. Treatment of the bacteria by heat or other means frequently used in the preparation of vaccines exposes antigens which are not normally apparent.

The availability of 'naturally occurring' antibody to these vaccines causes the organisms to be treated differently from live or very gently killed organisms. They may either be rapidly destroyed or give rise to a population of antibodies which, although directed against the same antigens, contain antibodies of various classes distributed in a different, less effective, way. This suggests that one of the more important considerations in the development of new vaccines may be the provision of the antigen in a form in which it will be treated as 'unknown' rather than 'known', and the avoidance of associated antigens to which the host may already have been immunised.

The work in this thesis still leaves questions in two important fields. We wish to know the nature of the antigens on S.typhimurium, and the ways in which responses to these antigens can be modified. There is still little information available on the factors which control the amount of type-specific polysaccharide on a bacterium. In particular the avir 2 gene appears to affect lipopolysaccharide content but is apparently not associated with the better-known lipopolysaccharide synthesizing enzymes. More precise quantitative data are required to distinguish between the two hypotheses for the form of antigen 4 in bacteria containing 5.

The non-lipopolysaccharide antigens which it is assumed are available on M206 and heated C5 are poorly defined. It is likely that antibodies against them will be available in relatively high titre in hyperimmune sera which have been absorbed with acetic acid, or even phenol-extracted polysaccharides, and this may make a useful starting point for further studies. Such a serum may well also prove of interest in the other field. It would be interesting, for example, to find out whether passive transfer of this antibody could affect the production of different classes of antibody against M206, an organism in which it is suggested that the antigen is already available. The response in terms of different classes of antibody against S.typhimurium should in any case be investigated, and it may be wiser not to try to make artificial distinctions between different components of the same molecules in these studies.

In the last twenty years advances in the knowledge of the antigenic structures of Salmonellae have been considerable. It is clear, however, that even with all the information now available about the antigens of the Kauffmann-White scheme we still cannot explain virulence or the host specificity of Salmonellae. We need to know more about the non-polysaccharide somatic antigens and their role in the immune response.

APPENDIX 1

Use of quantitative absorption experiments to determine the number of antigenic sites on a particle

Fazekas de St Groth (1961) developed a set of equations which could be used to estimate the number of antigenic sites on a virus by use of quantitative absorption techniques. While these equations are valid for a system in which the reaction of a single antigen has no effect on adjacent sites, they must be modified if they are to describe a situation such as the one found with Salmonella O-antigens in which it is likely that individual sites are packed so closely together that antigen antibody reactions interfere with each other. Under these circumstances it will be shown that only a minimum number of antigenic sites can be determined.

The following model follows closely the model of Fazekas de St Groth (1961). If we consider a situation on which the antibody reacts at only one site denoting antibody and antigen by A and S respectively, we can write $A + S \rightleftharpoons AS$

If K is the equilibrium constant for this reaction

$$K = \frac{[A]\ [S]}{[AS]} \qquad (1)$$

If A is the number of antibody molecules initially present per unit volume, B the number of bacteria and S the number of antigenic

sites present on each bacterium, the proportion of antibody which reacts is α, so that the proportion remaining is $(1-\alpha)$, and we assume that the reaction of 1 antibody with 1 site in fact blocks n sites, we can write, by substituting into equation (1)

$$K = \frac{(1 - \alpha)A \ (SB - n\alpha A)}{\alpha A} \tag{2}$$

Cancelling A in equation (2) we have

$$K = \frac{(1 - \alpha) \ (SB - n\alpha A)}{\alpha} \tag{3}$$

Dividing (3) throughout by $K(1 - \alpha)$

$$\frac{1}{(1 - \alpha)} = \frac{S}{K} \cdot \frac{B}{\alpha} - \frac{n \alpha A}{K\alpha} \tag{4}$$

$$\therefore \quad \frac{1}{(1 - \alpha)} = \frac{S}{K} \cdot \frac{B}{\alpha} - \frac{nA}{K} \tag{5}$$

This equation indicates that the graph of $\frac{1}{(1-\alpha)}$ against $\frac{B}{\alpha}$ should give a straight line of gradient $\frac{S}{K}$ and intercept $\frac{-nA}{K}$.

Also, expanding (3) we can write

$$K = \frac{(1 - \alpha) \ SB}{\alpha} - \frac{(1 - \alpha) \ n\alpha A}{\alpha} \tag{6}$$

$$\therefore \quad K = \frac{(1 - \alpha) \ SB}{\alpha} - (1 - \alpha) \ nA \tag{7}$$

Dividing throughout by nA

$$\frac{K}{nA} = \frac{(1 - \alpha) \ SB}{\alpha nA} - (1 - \alpha) \tag{8}$$

$$\therefore \quad (1 - \alpha) = \frac{(1 - \alpha)}{\alpha} \ \frac{B \ \ S}{nA} - \frac{K}{nA} \tag{9}$$

In this case the graph of $(1 - \alpha)$ against $\frac{(1 - \alpha)}{\alpha}$ B should give a straight line of gradient $\frac{S}{nA}$ and intercept $- \frac{K}{nA}$

If a value for α, the fraction of antibody used, can be found, and if A is determined independently, then it is theoretically possible to use either equation (5) or equation (9) to find values for $\frac{S}{n}$ and $\frac{K}{n}$.

The equations do not give absolute values for these parameters unless, as in Fazekas de St Groth's original example, $n = 1$.

As in all the experiments carried out in the work described in this thesis the number of bacteria used was varied by adding standard volumes of various dilutions of a standard bacterial suspension, it was convenient to plot the dilution d of the standard suspension containing B_o bacteria per unit volume. Equations (5) and (9) then become respectively :

$$\frac{1}{(1 - \alpha)} = \frac{S.B._o.d}{K\alpha} - \frac{nA}{K} \qquad (10)$$

and

$$(1 - \alpha) = \frac{B_o.S.}{nA} \cdot \frac{(1 - \alpha)d}{(\alpha)} - \frac{K}{nA} \qquad (11)$$

Because the mathematical manipulation required to use experimental data in these equations was considerable, and furthermore it was considered desirable to obtain relatively unbiased estimates of intercept and gradient, a Fortran computer program was designed by Mr. A. Osmand which could use the experimental data to calculate best fitting straight lines by the method of least squares using both equations (10) and (11).

It should be noted that this statistical method is not strictly valid for the equations used here because α, the dependent variable, appears on both sides of the equation. It has been pointed out by Mr. W. Venables, of the Department of Mathematical Statistics of Adelaide University, that a mathematically more reasonable practice is to use the curve obtained by plotting α against d, and calculate standard errors from that curve. This would have the added advantage that all of the data could be used. The practice adopted in this appendix may, however, be used to obtain approximate values for the constants $\frac{S}{n}$ and $\frac{K}{n}$.

One of the problems in the technique used to measure antibody was that a method of doubling dilutions was used, and each estimate of antibody activity was rather inaccurate.

This was a particularly serious problem in the estimations of the initial titre before absorption by bacteria. An error of 50% in this estimate could lead to enormous systematic errors in α and $(1 - \alpha)$. For this reason the program was designed to calculate values of $\frac{S}{n}$ and $\frac{K}{n}$ for a series of values of initial titre ranging from half its original measured value to twice that value. A value for the product moment correlation coefficient was also calculated. This should have the value +1 if the equation used is that of a perfectly straight line with positive slope, and this value was used to help to select the best value of initial titre to cause the rest of the data to fit Fazekas de St Groth's equations. The program written by Mr. Osmand reads as follows :

```
RUN(S,,,,,,1000)
LGO.
'       END OF RECORD
        PROGRAM ANTIBUG(INPUT,TAPE1=INPUT,OUTPUT)
        REAL K,JTITRE(99),JD(99),MX,MY,MTITRE(16),K2
        DIMENSION TB(16),B(16),X(99),Y(99),ALPHA(99)
    5   READ 10,ANTIS,ABPT,ITITRE,NBUG,NTEST,BACTNO,TVEA,
            TVEB
   10   FORMAT(A4,E10.3,I6,A4,I2,EI0.3,F5,F5)
        IF(EOF,1)100,15
   15   %=0.4
        DO 20 I=1,16
        %=%+0.1
        MTITRE(I)=ITITRE*%
   20   CONTINUE
        PRINT 25,ANTIS,ABPT,ITITRE,NBUG
   25   FORMAT(1H1,///,20X,*ANTISERUM *,X,A4,5X,*ANTIBODY
            CONTENT =*,X,E10
     B.3,5X,*INIT.TITRE =*,X,I6,5X,*ABS. BACTERIA*,2X,
            A4,///)
        PRINT 26
   26   FORMAT( 6X,*I*,2X,*MODIF.TITRE*      ,5X,*CORREL.
            COEFF*,5X,*EQUIL.
      BCONST.*,4X,*ERROR*,9X,*ANTIGENIC SITES*,5X,*ERROR*,
            9X,*K2*,//)
        READ 30,(JTITRE(J),JD(J),J=1,NTEST)
   30   FORMAT(6(F6,F6))
        DO 40 I=1,16
        SUMX=0 $ SUMY=0 $ SUMXSQ=0 $ SUMYSQ=0 $ SUMXY=0
        DO 35 J=1,NTEST
        ALPHA(J)=(MTITRE(I)-JTITRE(J))/MTITRE(I)
        Y(J)= 1/(1-ALPHA(J))
        X(J)= JD(J)/ALPHA(J)
        SUMX=SUMX+X(J)
        SUMY=SUMY+Y(J)
        SUMXSQ=SUMXSQ+X(J)**2
        SUMYSQ=SUMYSQ+Y(J)**2
        SUMXY=SUMXY+X(J)*Y(J)
   35   CONTINUE
        SXX=SUMXSQ - (SUMX**2)/NTEST
        SYY=SUMYSQ - (SUMY**2)/NTEST
        SXY=SUMXY - SUMX*SUMY/NTEST
        VONE=(SXY**2)/SXX
        VTWO=(SYY-VONE)/(NTEST-2)
        B(I)=SXY/SXX
```

```
      TB(I)=SQRT(VTWO/SXX)
      EB=TVEB*TB(I)
      MX=SUMX/NTEST
      MY=SUMY/NTEST
      A=MY-B(I)*MX
      TA=SQRT(VTWO*(1/NTEST+(MX**2)/SXX))
      EA=TVEA*TA
      R=SXY/(SQRT(SXX*SYY))
      K =-ABPT/A
      EK= ABPT/(A+EA) +K
      S=K*B(I)/BACTNO
      ES=K*EB/BACTNO
      PRINT37,I,MTITRE(I),R,K,EK,S,ES
   37 FORMAT( 5X,I2,5X,F6,12X,F6.3,10X,E10.3,3X,E10.3,
           8X,E10.3,3X,E10.3)
   40 CONTINUE
      DO 50I=1,16
      SUMX=0 $ SUMY=0 $ SUMXSQ=0 $ SUMYSQ=0 $ SUMXY=0
      DO 45J=1,NTEST
      ALPHA(J)=(MTITRE(I)-JTITRE(J))/MTITRE(I)
      X(J)=JD(J)*(1-ALPHA(J))/ALPHA(J)
      Y(J)= 1-ALPHA(J)
      SUMX=SUMX+X(J)
      SUMY=SUMY+Y(J)
      SUMXSQ=SUMXSQ+X(J)**2
      SUMYSQ=SUMYSQ+Y(J)**2
      SUMXY=SUMXY+X(J)*Y(J)
   45 CONTINUE
      SXX=SUMXSQ - (SUMX**2)/NTEST
      SYY=SUMYSQ - (SUMY**2)/NTEST
      SXY=SUMXY - SUMX*SUMY/NTEST
      VONE=(SXY**2)/SXX
      VTOO=(SYY-VONE)/(NTEST-2)
      B2-SXY/SXX
      TB2=SQRT(VTOO/SXX)
      EB=TVEB*TB2
      MX=SUMX/NTEST
      MY=SUMY/NTEST
      A=MY-B2*MX
      TA= SQRT(VTOO*(1/NTEST+(MX**2)/SXX))
      EA=TVEA*TA
      R=SXY/(SQRT(SXX*SYY))
      K=-A*ABPT
      EK=EA*ABPT
```

```
      S=B2*ABPT/BACTNO
      ES=EB*ABPT/BACTNO
      K2=S*BACTNO/B(I)
      PRINT47,I,MTITRE(I),R,K,EK,S,ES,K2
   47 FORMAT( 5X,I2,5X,F6,12X,F6.3,10X,E10.3,3X,E10.
           3,8X,E10.3,3X,E10.3,
     B4X,E10.3)
   50 CONTINUE
      GO TO 5
  100 STOP
      END
      END OF RECORD
```

APPENDIX 2A

The relationship between the amount of complement fixed on a bacterium and the amount of antibody initially present in solution

The reaction between antibody and complement is extremely complex and attempts to obtain detailed information on mechanism from kinetic or equilibrium data derived from observations of complete reactions are likely to fail (see Rosenberg, 1965, Borsos and Rapp, 1965_a). Nevertheless, in the hope that at least some estimate might be made of the number of complement fixing sites on bacteria, a pair of equations were developed to describe the action of complement with antigens which had been pre-treated with antibody. It was assumed that the number of complement haemolytic units fixed was proportional to the number of suitable antigen/antibody complexes available. Hence, if the number of 'molecules' of complement fixed was calculated, a minimum value for the number of fixing sites could be found.

To develop this equation it was assumed that complement reacts after one antibody molecule (A) reacts with one site (S). Borsos and Rapp (1965_a) have reported this to be the situation when the antibody molecule is IgM and so the situation may bear some

relationship to the one which occurs when the anti-5 serum of chapters 4, 5 and 7 is used.

$$A + S \rightleftharpoons as$$

where as is the antigen antibody complex.

The equilibrium equation may be written

$$K = \frac{[A]\,[S]}{[as]} \tag{1}$$

Let the fraction of total antibody fixed be α, and let the initial numbers of antibody molecules and antigenic sites per unit volume be A and S respectively and the final concentration of antigen/antibody complexes be as 'molecules' per unit volume

$$[as] = \frac{1}{K}\,[A]\,[S] \tag{2}$$

$$\therefore \quad as = \frac{1}{K}\,(A - \alpha A)\,(S - \alpha as) \tag{3}$$

$$\therefore \quad as = \frac{A}{K}\left[S(1 - \alpha) - as\,(1 - \alpha)\right] \tag{4}$$

Transferring all values of as to the same side

$$as\left[1 + \frac{A}{K}\,(1 - \alpha)\right] = \frac{A}{K}\,.S(1 - \alpha) \tag{5}$$

$$\therefore \quad as = \frac{\frac{A}{K}\cdot S(1 - \alpha)}{1 + \frac{A}{K}\,(1 - \alpha)} = \frac{A.S.(1 - \alpha)}{K + A(1 - \alpha)} \tag{6}$$

Inverting, we have

$$\frac{1}{as} = \frac{K}{A.S\ (1-\alpha)} + \frac{A\ (1-\alpha)}{AS\ (1-\alpha)} \qquad (7)$$

$$\frac{1}{as} = \frac{1}{A}.\ \frac{K}{S\ (1-\alpha)} + \frac{1}{S} \qquad (8)$$

When A is very much larger than S, then only a small fraction will be used and $1-\alpha \triangleq 1$

Then $$\frac{1}{as} = \frac{1}{A}.\ \frac{K}{S} + \frac{1}{S} \qquad (9)$$

Hence the graph of 1/ as against 1/A should give a straight line of gradient K/S and intercept on the Y axis of 1/S. A can be replaced by a serum concentration as long as the antibodies involved can be regarded as a homogeneous population, as its absolute value is irrelevant to the estimation of 1/S. A problem arises because as cannot be estimated directly. It is recorded as a number of $C'H_{50}$ units fixed. By assuming that a red cell can be lysed when only one complement lesion has been made it it not difficult to use the Poisson distribution to calculate the total number of complement 'hits' at the end point of a given haemolytic system (Mayer, 1961). This value is likely to be proportional to a number of complement 'molecules' fixed. As complement components can be wasted and do not always eventually lead to lysis (Müller-Eberhard, 1968) the figure

obtained will be lower than the actual number of sites involved.

It is interesting to know the form of this equation when antigen is in excess. In this case, denoting the fraction of antigen which has reacted as γ, and assuming that antibody still reacts at only one site, from equation (2), by a similar series of operations we find

$$as = \frac{S.A\,(1-\gamma)}{K + S\,(1-\gamma)} \tag{10}$$

$$\frac{1}{as} = \frac{1}{A}\left(\frac{K}{S\,(1-\gamma)} + 1\right) \tag{11}$$

When antigen is in great excess, $(1-\gamma)$ is approximately equal to 1, so that

$$\frac{1}{as} = \frac{1}{A} \cdot \left(\frac{K}{S} + 1\right) \tag{12}$$

The graph of $\frac{1}{as}$ v $\frac{1}{A}$ is a straight line passing through the origin with gradient $\left(\frac{K}{S} + 1\right)$. The amount of information obtainable from it will depend on whether or not the ratio $\frac{K}{S}$ is significantly greater than 1 and even in that case further information is required to calculate S.

Fig 25 shows the general shape of the curve to be expected when 1/as is plotted against 1/A over a wide range of values of A. Clearly the most useful results are obtained at the highest concentrations of A, and graphs which look likely to pass through the origin must be viewed with suspicion.

Fig 25

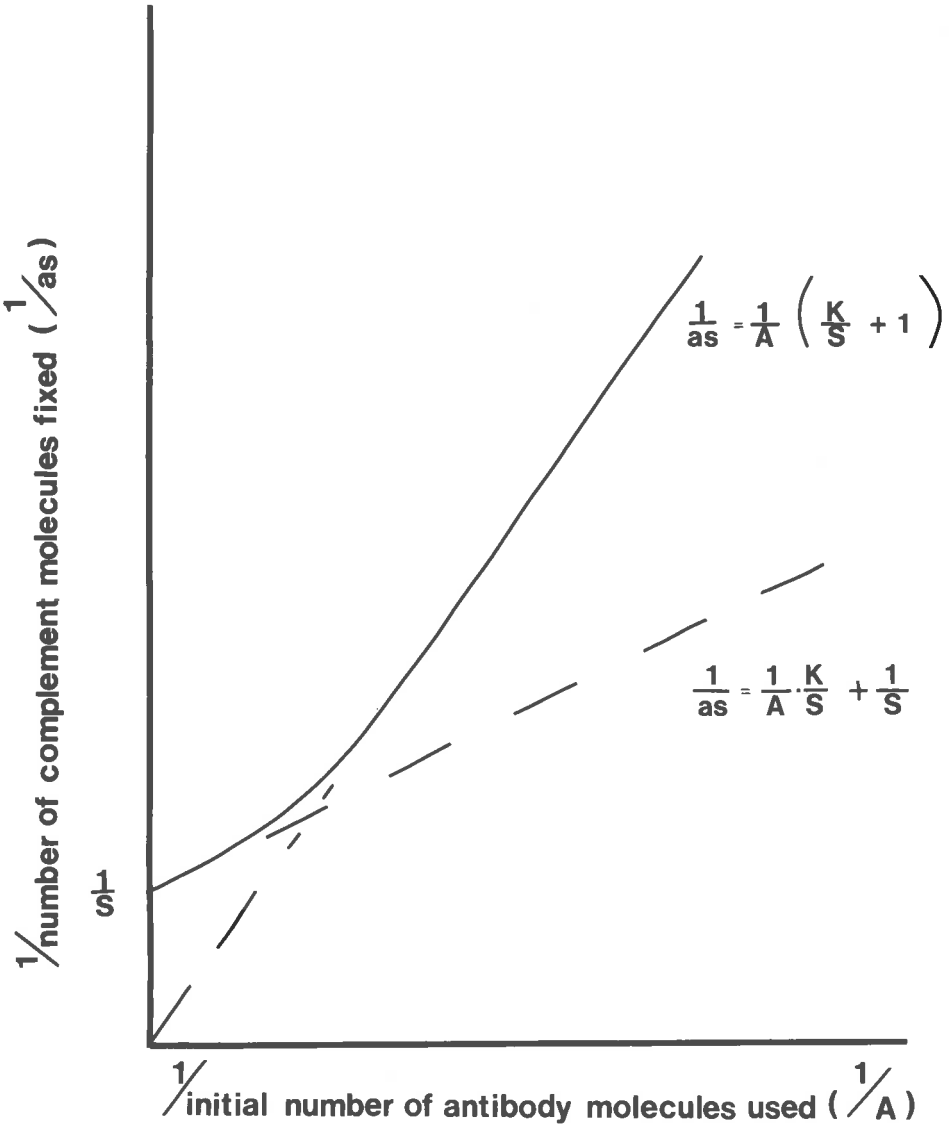
Form of the proposed relationship between the amount of antibody used to treat bacteria and the amount of complement fixed by them

A = concentration of antibody used

S = number of complement fixing sites available

as = number of complement molecules fixed

K = constant


1/number of complement molecules fixed (1/as)
1/S
1/initial number of antibody molecules used (1/A)
1/as = 1/A (K/S + 1)
1/as = 1/A · K/S + 1/S

APPENDIX 2B

Example of the use of complement fixation data in the calculation of a line of best fit and 5% errors by the method of least squares

This experiment was carried out as described on page 198. The statistical methods are described in Mather (1964) and the notation is adapted from Fazekas de St Groth (1961).

Serum conc.	$\frac{1}{\text{Serum conc.}}$ x	x^2	$C'H_{50}$ fixed	(a) $\frac{1}{C'H_{50}}$ fixed y	y^2	xy
0.1	10.00	100.00	14.7	0.068	0.004624	0.680
0.2	5.00	25.00	20.8	0.048	0.002304	0.240
0.3	3.33	11.10	29.4	0.034	0.001156	0.113
0.4	2.50	6.25	36.0	0.028	0.000784	0.070
0.5	2.00	4.00	36.0	0.028	0.000784	0.0560
0.6	1.67	2.90	36.0	0.028	0.000784	0.0468
0.7	1.43	2.00	36.0	0.028	0.000784	0.0400
0.8	1.25	1.60	36.0	0.028	0.000784	0.0350
0.9	1.11	1.20	36.0	0.028	0.000784	0.0311
TOTALS	28.29	154.05	-	0.318	0.012788	1.3119

(a) Data obtained using S.typhimurium C5.

$$\bar{x} = 3.14333$$

$$\bar{y} = 0.03533$$

$$\begin{array}{rl} & \Sigma x^2 \quad 154.05 \\ - & \frac{(\Sigma x)^2}{n} \quad 88.9249 \\ \hline \Sigma (x-\bar{x})^2 = S_{xx} = & 65.12 \end{array}$$

$$\begin{array}{rl} & \Sigma y^2 \quad 0.012788 \\ - & \frac{(\Sigma y)^2}{n} \quad 0.011236 \\ \hline \Sigma (y-\bar{y})^2 = S_{yy} = & 0.001552 \end{array}$$

$$\begin{array}{rl} & \Sigma (xy) \quad 1.3119 \\ - & \frac{(\Sigma x \Sigma y)}{n} \quad 0.9996 \\ \hline \Sigma (y-\bar{y})\ (x-\bar{y}) = S_{xy} = & 0.3123 \end{array}$$

Variance due to regression $V_1 = \frac{(S_{xy})^2}{S_{xx}} = 0.0014975$

Error Variance in y $V_2 = \frac{S_{yy} - V_1}{n - 2} = 0.0000077$

Gradient of line $$b = \frac{S_{xy}}{S_{xx}} = \frac{0.3123}{65.12} = 0.004795$$

Standard error of b $$= \sqrt{\frac{V_2}{S_{xx}}} = \sqrt{\frac{0.000028}{65.13}}$$

$$= 0.0006557$$

Number of degrees of freedom for b = n-1 = 8

Value of t for 5% probability and 8 degrees of freedom = 2.31

Hence 95% probability limits for b

$$d_b = \pm 2.31 \times 0.0006557$$

$$= 0.0015$$

Intercept of line on the Y axis

$$a = \bar{y} - b\bar{x} = 0.03533 - 0.0048 \times 3.1433$$

$$= 0.02024$$

Standard error of a $$= \sqrt{V_2 \left(1/n + \frac{\bar{x}^2}{S_{xx}}\right)}$$

$$= \sqrt{0.000028 \quad \left(0.111 - \frac{9.881}{65.13}\right)}$$

$$= 0.0021345$$

Number of degrees of freedom for a = n - 2 = 7

Value of t for 5% probability and 7 degrees of freedom = 2.37

Hence 95% probability limits for a, $d_a = \pm 0.002134 \times 2.37$

$$= \pm 0.0051$$

i.e. the line has the equation $y = 0.0006x + 0.0021$

product moment correlation coefficient

$$= \frac{S_{xy}}{S_{xx} S_{yy}}$$

$$= \frac{0.3123}{65.12 \times 0.001552}$$

$$= 0.9827$$

APPENDIX 3

Effect of differences in antigen content on the efficiency of specific antibodies

The remarks made on the difficulties of relating equilibrium date to mechanism in studies on complement apply with even greater force when we try to describe in vivo biological effects of antibodies. In any attempt to explain differences in the effectiveness of different antibodies in terms of the concentrations of their respective antigens, however, it is important to know what form the equations relating efficiency to antigen concentration are likely to take. This information should give at least a qualitative idea of what other factors are likely to affect results.

Two situations can be considered. Either an immune reaction (such as phagocytosis or the serum bactericidal reaction) requires fixation of a given number (n) of antibodies, or the reaction occurs after a given antibody has reacted with n sites.

In the first case we have the reaction $nA + nS \rightleftarrows A_n S_n$ where A represents antibody, S antigen and $A_n S_n$ an antigen antibody complex of sufficient size to promote the reaction. If we make the simplifying assumption that the end point in a titration depends on antigen antibody complex reaching a certain concentration in solution, we can write :

$$K = \frac{[A_n S_n]}{[A]^n [S]^n} \qquad (1)$$

$$\text{so that } [A_n S_n] = K[A]^n [S]^n \qquad (2)$$

where K is related to the affinity constant of a single antigen antibody reaction. If A is taken to represent the number of antibody molecules per unit volume and B the number of bacteria, s the number of antigenic sites on one bacterium and p the proportion of all available antigenic sites which have reacted at the end point we have :

$$[A_n S_n] = psB \qquad (3)$$

and from equation (2)

$$[A_n S_n] = K(A - psB)^n (sB - psB)^n$$

$$= s^n B^n K(A - pB)^n (1 - p)^n \qquad (4)$$

Consider another antibody G reacting with an antigen which exceeds the number of sites of s by a factor of m. If the antibodies A and G have identical affinity constants we can write, by analogy with equations (3) and (4)

$$[G_n S_n] = q\,m\,s\,B \qquad (5)$$

$$[G_n S_n] = m^n s^n B^n K(G - qmsB)^n (1-qm)^n \qquad (6)$$

where q is the proportion of the total available sites with which G has reacted at the end point. If the mechanism of the immune reaction is identical in both cases, then at the end point :

$$A_n S_n = B_n S_n \qquad (7)$$

Hence from (4) and (6)

$$s^n B^n K(A - psB)^n (1 - p)^n = m^n s^n B^n K (G - qmsB)^n (1 - qm)^n \qquad (8)$$

which simplifies to :

$$(A - psB) (1 - p) = m (G - qmsB) (1 - qm) \qquad (9)$$

Also, from equations (3) and (5)

$$psB = qmsB$$

$$p = qm \qquad (10)$$

In reactions such as phagocytosis and killing by antibody and complement it has been shown that comparatively few antibody molecules per bacterium are required to cause the reaction (Šterzl et al, 1962, Šterzl, 1963, Rowley and Turner, 1966). Near the end point of a titration the proportion of antigenic sites which have reacted must be very small compared with the total number of sites available.

Hence $(1 - p) = (1 - q) = 1 \qquad (11)$

Substituting these values from equations (10) and (11) into equation (9)

$$(A - qmsB) = (G - qmsB) \quad (12)$$

qms may be written as σ, the average number of sites occupied per bacterium at the end point of a titration. This is defined by the selection of the end point.

$$\text{Thus } (A - \sigma B) = m\,(G - \sigma B) \quad (13)$$

One way to show what this equation means in practice is to substitute into it specific values for G and σ.

It has been estimated from the data of Šterzl et al, (1962) that as few as 30 molecules of IgM antibody were required to kill one bacterium in their system. If we take the initial number of molecules of G to be 30 B and the corresponding number of molecules of A to be a B

$$(aB - \sigma B) = m\,(30B - \sigma B) \quad (14)$$

which can be written :-

$$a = (30 - \sigma)\,m + \sigma \quad (15)$$

In this equation, σ must lie between 1 and 30. If $\sigma = 30$, $a = 30$, so that there is no difference between the initial numbers of antibody A and antibody G required to give the same titre. This situation would occur if the antibodies used had a very high affinity for the antigen or very large numbers of bacteria were used, so that all of the available antibodies reacted. At the other

extreme (as in the model described in the discussion of chapter 7) if only 1 molecule of antibody G reacts with each bacterium (i.e. the antibodies have lower affinity or fewer bacteria are used)

$$a = 29\,m + 1$$

In this case the relative efficiencies of antibodies are almost proportional to the amounts of each antigen on the bacteria, and one may reasonably hope to show differences in suitably designed experiments.

A second model is plausible because all well-defined antibodies have more than one active site. If we assume that an antibody molecule is ineffective unless it has reacted with at least n sites, using the previous notation and similar assumptions it can be shown that the relationship between the initial number of antibody molecules required at the end point of a titration and the number of antigenic sites per bacterium is :

$$(A - psB) = m^n (G - qmsB) \qquad (16)$$

$$\text{so that } A = m^n (G - qmsB) + pS \qquad (17)$$

Because of the existence of antigen antibody complexes which include less than n antigenic sites it is not possible to equate p with q m. p must in fact be greater than qm. It is clear, however, that as in the previous example, when the

affinity constant is very high, A and G have identical efficiency. If it is low, however, the differences between them will be even greater than in the first example because they depend on the n^{th} power of m instead of the 1st.

Both of the examples set out above lead to the conclusion that the success of any experiment designed to compare the efficiencies of antibody molecules of different specificity reacting in the same area on the bacterium depends on the use of antibodies with 'relatively' low affinity, or on the use of 'relatively' dilute suspensions of bacteria. Neither of these relatives has been defined. It seems reasonable to assume, however, that the results of experiments by different workers will not be comparable unless some account of these parameters is taken.

APPENDIX 4

Lipopolysaccharide antigens of Citrobacter 396

In the course of the studies described here Citrobacter 396 (0: 4, 5) was used in the preparation of anti-5 sera. In Salmonella, O-antigen 4 has not been reported except when accompanied by one of the 12 antigens (Kauffmann, 1966_a). The Citrobacter strain also lacks rhamnose in its LPS and thus belongs to a different chemotype from the group B Salmonellae. Clearly Citrobacter 396 LPS is very different from known Salmonella lipopolysaccharides and it is not surprising that evidence was found for an additional antigen which was absent from the Salmonellae used here. Some evidence was also obtained which suggested that Citrobacter 396 might share an antigen with S.reading which was absent from S.typhimurium. Evidence for the occurrence of both these antigens is presented here.

(1) Antigens present in anti-5 serum

Early evidence for the presence of an antigen other than 4 or 5 on Citrobacter 396 was obtained from haemagglutination inhibition titrations as described on page 92. Attempts were made to inhibit the reaction of an anti-Citrobacter 396 serum with alkali-sensitized Citrobacter LPS coated cells. As alkali destroys

antigen 5, only antigen 4 should have been present. When LPS from S.reading (0: 4, 12) was used to inhibit haemagglutination of these cells, no inhibition was obtained when 11.5 mg. LPS per 4 haemagglutinating units serum were used. In contrast, S.reading LPS could inhibit the reaction of its homologous serum with cells sensitized with alkali-treated S.reading LPS when any concentration greater than 1 mg. per 4 HU was used.

While this seemed indicative of the presence of an alkali-stable antigen other than antigen 4 on Citrobacter 396 this evidence could not be regarded as positive proof for its occurrence. Further evidence came from use of quantitative precipitin reactions. Initially these were carried out using an anti-5 serum and two preparations of lipopolysaccharide S.typhimurium C5 and M206, as well as preparations kindly provided by Professor O. Westphal of lipopolysaccharide from Citrobacter 396 and another strain of S.typhimurium. Lipopoly-saccharides were suspended in saline and solubilised by treatment with ultrasound for 1 minute. Quantitative precipitin reaction tubes contained 0.5 ml. centrifuged antiserum mixed with a suitable amount of LPS and made up to 5 ml. with saline. Precipitation was allowed to continue for 6 days and the

approximate size of the precipitate was noted on each day. The first precipitates appeared after 48 hours and seemed to give a maximum in the region of 150 μg. LPS. Precipitates in the presence of high concentrations of LPS began to form later in the week but it was by no means certain that maximum precipitation at the highest concentrations of LPS was obtained. The precipitates were eventually washed three times in 2 ml. cold saline and dissolved in 0.1 ml. NaOH. Protein determinations were carried out using the Folin Lowry method described on page 108.

The results of quantitative precipitin reactions are shown in Fig 26. It is clear that at their maxima, all 3 preparations of S.typhimurium LPS precipitated approximately the same amount of antibody. However, Citrobacter 396 LPS precipitated more than 3 times as much protein. This was taken as rather stronger evidence that it contained an additional antigen. In an attempt to confirm this, three dilutions of C5 LPS (not treated with ultrasound) were mixed with 0.5 ml. anti-5 serum at a final volume of 5 ml. and allowed to precipitate in the cold for 7 days. The precipitates were centrifuged out of the serum, washed twice with 2 ml. cold saline and protein contained in them was

Fig 26

Quantitative precipitin reactions using anti-5 serum and lipopolysaccharide obtained from Citrobacter 396 and three strains of S.typhimurium

—●—●— Citrobacter 396

—○—○— S.typhimurium C5

—△—△— S.typhimurium M206

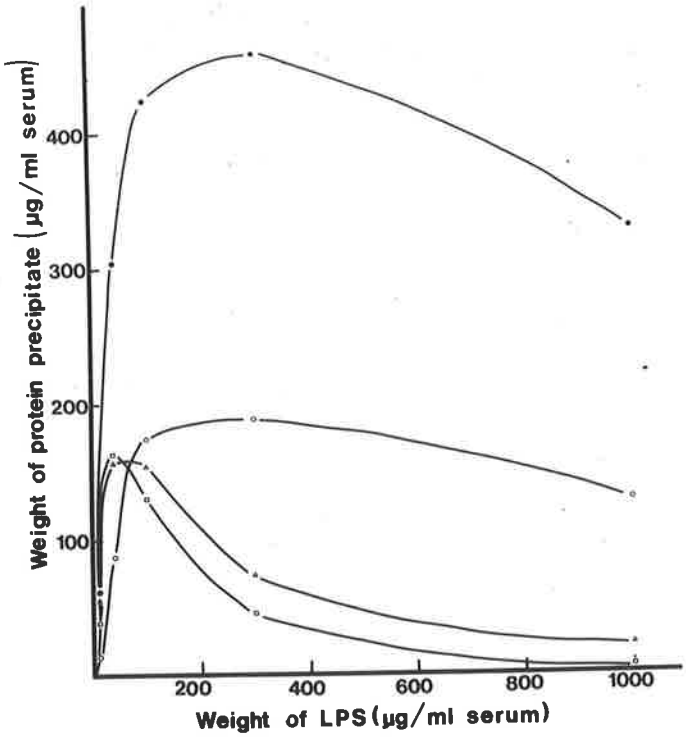
—□—□— S.typhimurium (Westphal)

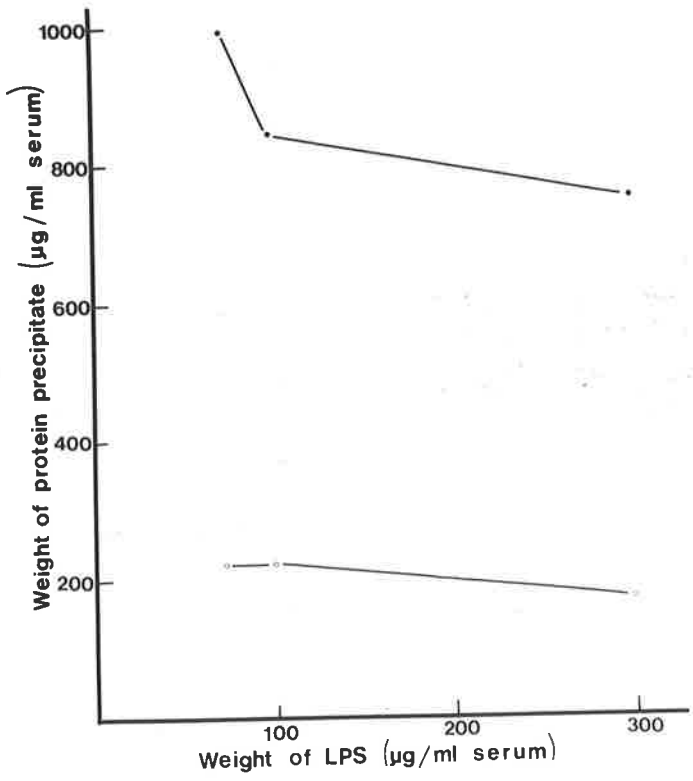
Fig 27

Sequential quantitative precipitin curves obtained by precipitating a standard amount of anti-5 serum with varying amounts of S.typhimurium C5 lipopolysaccharide and precipitating the resultant supernatants with a standard amount of Citrobacter 396 lipopolysaccharide

-○-○- S.typhimurium C5

-●-●- Citrobacter 396


Weight of protein precipitate (µg/ml serum)
400
300
200
100
200
400
600
800
1000
Weight of LPS (µg/ml serum)


Weight of protein precipitate (µg/ml serum)
1000
800
600
400
200
100
200
300
Weight of LPS (µg/ml serum)

measured as described above. To each supernatant were added 100 µg. Citrobacter LPS which had previously been treated with ultrasound. Precipitates formed over 7 days and proteins in them were determined in the same way that was used for the C5 LPS precipitates.

The results of this experiment are shown in Fig 27. On this occasion C5 appears to have precipitated less protein than before, suggesting either that precipitates dissolved during washing, that the reaction had still not quite reached completion in spite of the fact that unsonicated lipopolysaccharide was used, or that sonication had, in fact, exposed antigens not previously available. The results therefore cannot be regarded as completely satisfactory. Nevertheless, it is clear that C5 lipopolysaccharide at its optimum concentration still failed to precipitate most of the anti-Citrobacter antibody in an anti-5 serum. This was taken as further evidence that Citrobacter 396 has a major LPS antigen which is not shared by either S.typhimurium C5 or S.reading, the strain used to absorb the original anti-5 serum.

(2) Cross-reactions between Citrobacter 396 and S.reading

Antigen 4 has been divided into 2 specificities by Staub and Bagdian (1966) on the basis of cross-reactions between S.typhimurium (0: 1, 4_1, 4_2, 5, 12) and S.bredeney (0: 1, 4_1, 27_B, 5, 12). (see page31,Fig 4). Antigen 4_1 includes abequose and possibly mannose and rhamnose, while antigen 4_2 includes at least parts of galactose and mannose, as well as abequose.

In an attempt to prepare a 'specific' anti-4_2 serum 0.4 ml. of anti-4 serum was treated with the growth from 1 Roux bottle of washed S.bredeney cells as described on page 100. The resultant absorbed serum was titrated against sheep red blood cells sensitized with either C5 or Citrobacter 396 LPS. The results of titrations with absorbed and unabsorbed sera are shown in table 31.

TABLE 31

Haemagglutination titres of anti-4 serum before and after absorption with S.bredeney on sheep red blood cells sensitized with LPS from S.typhimurium C5 and Citrobacter 396

Sensitizing LPS	Antiserum Unabsorbed (4_1, 4_2)	Antiserum Absorbed (4_2)
S.typhimurium C5	5120	<40
Citrobacter 396	640	160

It seems that while a combination of S.typhi 0 901 and S.bredeney were sufficient to absorb from an anti S.reading serum almost all the agglutinating activity against C5 LPS they were not sufficient to absorb out all of the activity against Citrobacter 396. This suggested that the Citrobacter shared an antigen with S.reading which was absent from the other two strains. The failure of C5 to react with anti-4_2 serum was puzzling. It is possible that the differences lie in different amounts of substitution on the O-side chain galactose.

It seems unlikely that the occurrence of either of the Citrobacter antigens affected the results reported in this thesis. Most work was carried out using an anti-5 serum which by definition contained no antibodies reacting with S.reading. While it is clear that this antiserum probably contained considerable amounts of another unspecified antibody, its failure to precipitate with either C5 or M206 LPS suggests that it would not have affected results obtained using these two bacteria.

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