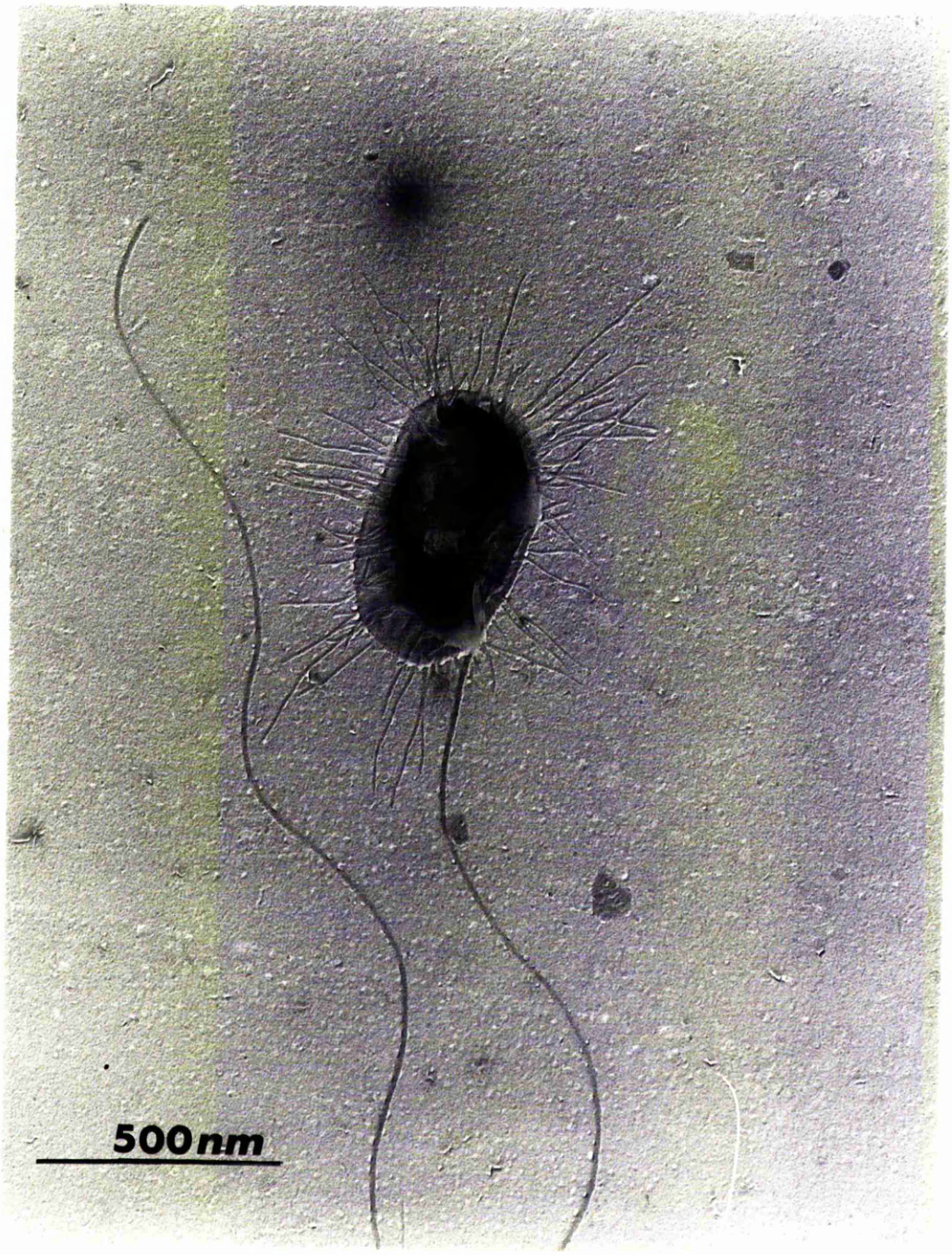
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FRONTISPIECE

An electron micrograph showing *Escherichia coli* 8623, shadowed with chromium.

Magnification X 70,000.

PREFACE

This thesis is the original work of the author. She wishes to acknowledge that the electron microscopy was carried out in collaboration with Dr. J.H. Freer and the polyacrylamide gel electrophoresis with Dr. Roger Parton.

Grace Sweeney

ADHESIVE PROPERTIES OF COMMON TYPE-1 BACTERIAL FIMBRIAE

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Presented for the Degree of Doctor of Philosophy in the Faculty of Science, University of Glasgow.

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October, 1977

ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor A.C. Wardlaw for permitting me to undertake this project and for affording me access to the excellent research facilities in the Microbiology Department of Glasgow University.

Profound thanks also to Dr. J.H. Freer for supervision of the research, for stimulating discussion, constructive criticism, endless patience and perseverance.

I gratefully acknowledge the assistance of all of the other members of the department for their help in various ways and to Mr. D.D. Whitelaw for arranging the regular supply of fresh horse erythrocytes. The skilful technical assistance of Mr. A. Ellis is also gratefully acknowledged.

Finally my sincerest thanks to Mrs. A. Strachan for her speedy and efficient typing of this manuscript.

Summary

The aim of this project was to investigate the nature, number and distribution of type-1 fimbrial agglutinins and their reaction with receptors on horse erythrocytes.

Optimal synthesis of fimbriae in Escherichia coli NCTC 8623, serotype O125, occurred under conditions identical to those described by Duguid et al. (1955), i.e. 48 h static, shallow nutrient broth cultures. The fimbriae prepared from such cultures agglutinated horse erythrocytes, Saccharomyces cerevisiae NCYC 366 and cell walls prepared from this strain. A method for recovery of isolated fimbriae from dilute solutions utilising their affinity for whole fixed yeast cells is described. Adsorbed fimbriae were eluted with α-methyl mannoside but under the conditions described no longer exhibited haemagglutinating activity.

A range of saccharides was examined for ability to inhibit agglutination between whole bacteria or isolated fimbriae and HRBCs. The most potent of the inhibitors tested was α-methyl mannoside, inhibiting the reaction at 5.5×10^{-7}M; αD-mannose caused inhibition at 2.0×10^{-6}M. Equilibrium dialysis showed that isolated fimbriae bound mannose with low affinity. Modal lengths were determined for freshly isolated fimbriae and fimbriae after five minutes exposure to ultrasound. The haemagglutination titre fell progressively during ultrasonic treatment indicating that haemagglutination was a function of fimbrial length and not the number of termini.

The results of experiments following the binding of ^{125}I-labelled fimbriae of differing lengths to erythrocytes indicated that fimbriae bind to the cell surface along their length rather than by their tips. An estimate of fimbrial receptors on HRBCs gives a minimum value of 4×10^6 sites per red cell.

Horse erythrocytes were shown to be susceptible to a variety of glycosidases including neuraminidase, by changes in lectin agglutinability. Only neuraminidase caused a decrease in fimbrial HA titre. Of five proteases tested, only crude papain treatment of HRBCs caused an increased fimbrial HA titre, whereas periodate oxidation caused a slight decrease. Isolated horse erythrocyte glycoproteins did not show any competitive inhibition in the fimbrial-HRBC agglutination system.

Neither non-specific ionic bonding nor hydrophobic interactions are solely responsible for fimbrial haemagglutination. A specific binding interaction between fimbriae and membrane receptors is most likely, with the possible involvement of a membrane glycolipid.

OBJECT OF RESEARCH

Common type fimbriae are frequently implicated in bacterial adhesion. Bacteria possessing these appendages stick to an extensive range of substrates including erythrocytes. The interaction between common type-1 fimbriae and horse erythrocytes was chosen for study because of a readily available supply of fresh horse blood. Bacteria possessing different types of common fimbriae agglutinate specific types of erythrocyte. This suggests that different receptor sites are involved.

The aims of this investigation were:

1. To prepare purified isolated fimbriae and ascertain whether or not these agglutinate the same group of substrates as the intact fimbriate bacteria.

2. To characterise the reaction between the common type-1 fimbrial agglutinin and its binding site in or on the horse erythrocyte.

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LIST OF ABBREVIATIONS

cm	centimetre
Col	Colicine plasmid
Col^+	production of colicine
ConA	Concanavalin A
cpm	counts per minute
DCP	dicetyl phosphate
DEAE-	diethylaminoethyl-
DNA	deoxyribonucleic acid
drd	derepressed
Ent	plasmid coding for enterotoxin production
Ent^+	enterotoxin production
EDTA	ethylenediamine tetraacetic acid
E_{620}^{10mm}	absorbance at 620 nm
EMS	ethyl methane sulphonate
F	prototype fertility factor
f+	male specific antigen
F^+	donor cell
F^-	recipient cell
Fim^+	genotypically and phenotypically fimbriate
Fim^-	genotypically and phenotypically non-fimbriate
$Fim^{(+)}$	genotypically but not phenotypically fimbriate
$Fim\sigma^+$	stably fimbriate, arising from Fim^+
$Fim\sigma^-$	stably non-fimbriate, arising from $Fim\sigma^+$
Fla^+	flagellate
Fla^-	non-flagellate

F_8	sex factor carrying galactose marker, i.e. F.gal.
F.lac	sex factor carrying lactose marker
F-like, R-plasmid	Resistance transfer plasmid coding for the synthesis of F-type fimbriae
FFHRBC	formaldehyde-fixed horse red blood cells
FBP	Fucose binding protein
F/RI	hybrid sex plasmid coding for two types of fimbriae
g	gravity
HA	Haemagglutination
HAI	Haemagglutination inhibition
Hfr	high frequency donor
HLY	plasmid coding for the synthesis of haemolysin
HLY^+	production of haemolysin
HRBC	horse red blood cells
$HU_b^{3\%}$	haemagglutinating unit of bacteria as titrated against 3% (v/v) HRBCs
$HU_f^{1\%}$	haemagglutinating unit of fimbriae as titrated against 1% (v/v) HRBCs
$HU_f^{2\%}$	haemagglutinating unit of fimbriae as titrated against 2% (v/v) HRBCs
h	hour
IO	inside-out
kV	killivolts
K88	plasmid which codes for the synthesis of proteinaceous K88 antigen
$K88^+$	production of K88 antigen
LIS	lithium 3,5, diiodosalicylate

MR	mannose resistant
mre	mannose resistant eluting
MS	mannose sensitive
mA	milliamp
mM	millimolar
min	minute
ml	millilitre
MIC	minimum inhibitory concentration
NCTC	National Collection of Type Culture
nm	nanometre
PAGE	polyacrylamide gel electrophoresis
PAS	Periodic Acid, Schiffs
PBS	Phosphate-buffered saline
PEG	polyethylene glycol
pH	negative logarithm of the hydrogen ion activity
pI	isoelectric point
PTA	phosphotungstic acid
RBC	red blood cell
RCA_{120}	Ricinus communis agglutinin$_{120}$
rpm	revolutions per minute
RNA	ribonucleic acid
RO	right-side out
R	Resistance transfer plasmid
s	second
SDS	sodium dodecyl sulphate
tra^-	transfer deficient mutant

traJ$^-$	transfer deficient mutant which can synthesise but not transfer F
μ	electrophoretic mobility
μg	microgram
μl	microlitre
μM	micromole
vol	volume
WGA	Wheat Germ Agglutinin

CONTENTS

INTRODUCTION

INTRODUCTION

I. GENERAL REMARKS

Bacteria possess two morphologically distinct types of surface appendages, i.e. flagella and fimbriae. The former are characterised by a diameter of 12-20 nm, a distinctive length of 3-12 μm and an undulating shape (Bode, 1973). Because removal of these organelles from bacteria eliminates motility, they are thought to be responsible for locomotion. By comparison, fimbriae are relatively shorter, thin, straight and less flexible. Due to the limited resolving power of the light microscope, fimbriae went undetected until the development of electron microscopy techniques in the 1940s. Anderson (1949) and Houwink (1949) using the gold-shadowing technique published micrographs of flagellated (Fla^+) strains of Escherichia coli and Pseudomonas pyocyanea which revealed small non-flagellar appendages on the surface of the cells. Controversy ensued regarding the origin of these structures; were they bacterial surface appendages or perhaps artefacts of the electron microscopy preparation process?

In 1950, Houwink & van Iterson published further micrographs showing several of the many organisms which they screened in their search for bacterial "filaments." Their results showed that many strains of Gram-negative bacteria, freshly isolated from natural environments possessed numerous appendages which they called "filaments." Noda & Wyckoff (1952) corroborated these findings by recording the existence of thread-like "filaments" on Fla^+ strains of E. coli. Metal-shadowed

preparations of a Fla^+ *Proteus* strain also revealed slender appendages of 10 nm diameter which were subsequently isolated from the bacteria by shearing and differential centrifugation (Weibull & Hedvall, 1953). These reports dispelled the original premise that the filaments were artefacts.

The vague term "filaments" was replaced by "Fimbriae" (Latin, threads or fibres) recommended by Duguid *et al.* in 1955. However, Brinton (1959) proposed that the term "Pili" (Latin, hairs or fur) should be used to describe these organelles. Although both terms are used synonymously throughout the literature, Duguid's proposal was generally accepted in Britain and Brinton's in the United States of America.

In a recent review Ottow (1975) highlighted this problem of terminology and reiterated the proposal of Meynell & Lawn (1967) and Meynell, Meynell & Datta (1968) that appendages with a genetic function be called pili, to distinguish them from the common type fimbriae which are known to be involved in adhesion.

Throughout this thesis, Duguid's terminology will be used since:

(a) a single, general descriptive term is adequate for all non-flagellar appendages. More than one leads to confusion in the literature
(b) appendages on the bacteria appear as fine fibres or threads as distinct from fur
(c) Duguid's terminology has temporal priority over Brinton's.

In a publication by Rhode et al. (1975) Duguid was quoted defining fimbriae as "organised filaments extruded radially from the bacterial cell-wall and present in this form in wet living bacteria."

Many of the original investigations performed by Duguid and his colleagues revealed fimbriae on Gram-negative organisms of the family Enterobacteriaceae especially Klebsiella aerogenes, E. coli, Salmonella and Shigella spp., freshly isolated from natural environments such as urinary tract infections and dysentery cases. The majority of these organisms can now be cultured under laboratory conditions, such that the production of fimbriae is favoured. These investigations and other published work on this family will be discussed further. However, recent reports indicate the existence of these organelles on other pro- and eukaryotic organisms.

Appendix A provides a very brief survey of the occurrence of fimbriae or fimbrial-like structures on organisms other than the Enterobacteriaceae. For a more comprehensive survey of fimbriate organisms, the reader is referred to the recent review by Ottow (1975).

II. CLASSIFICATION OF FIMBRIAE

Duguid et al. (1955) proposed the first fimbrial classification scheme. Appendages were assigned to two major types, viz.

(a) chromosomal gene controlled, present in large numbers on bacteria and lacking a genetic function - Common Type

(b) plasmid controlled, present in small numbers on the cells and possessing a genetic role - Sex Type.

Sub-division within the common type was based on number, distribution, diameter and affinity of the fimbriae for particular species of erythrocytes (Table 1).

Brinton (1959) proposed a slightly different classification which lacked definitive information on the types he proposed. Ottow (1975) reviewed fimbriate organisms and assigned them to groups with the common types of Duguid assigned to Group 1, subgroups 1-4. This system is presented in Table 2.

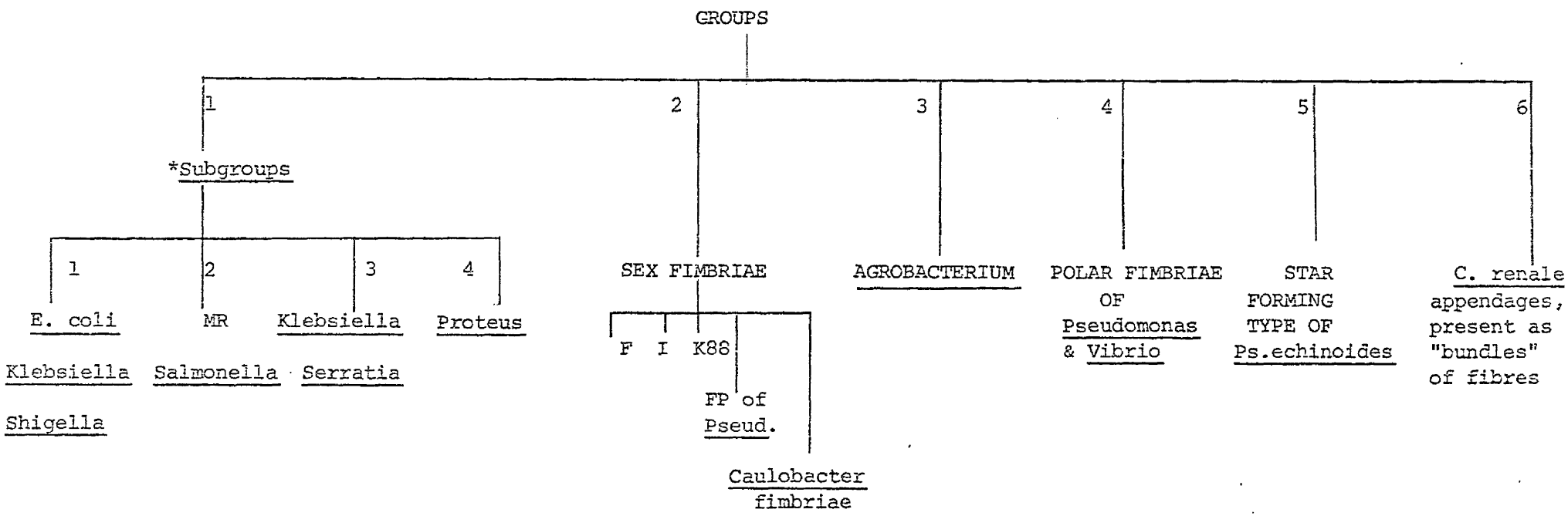
The more recently discovered fimbriae of Neisseria, Streptococcus and several fungal species (see Appendix) are not included in any of the schemes described.

Rhode et al. (1975) recently reported the isolation of "O" inagglutinable strains of S. enteriditis and S. typhimurium from pasta products. These bacteria possessed unique fimbrial appendages with a diameter of 2.7 nm, which appeared to be mutant forms of fimbrial protein. These and other types of fimbriae were isolated which did not fit into any of the classifications described above. There is an urgent requirement for an updated classification scheme which would permit the inclusion of freshly isolated types of fimbriae. A further complication may arise since, in addition to flagellar filaments, bacteria may possess two different kinds of common fimbriae either on the same or different organisms within the same population, e.g. Klebsiella spp., often possess types-1 and -3 borne on different bacteria in the same culture (Duguid, 1968).

Table 1 : Common type fimbriae (Duguid, 1968)

Type	Number per cell	Diameter (μm)	Length (μm)	Distribution	Mannose Sensitivity of Haemagglutinin	Erythrocyte Specificity	Typical Species	Adhesive Properties
1	50-400	7.0	0.25-1.5	Peritrichous	+	Guinea-pig, fowl, horse	E.coli, Sh.flexneri, S. marcescens, Kl.pneumoniae and some Salmonella serotypes except type-2	Adheres to epithelial cells and leukocytes. Strong adhesion for animal, plant and fungal cells.
2	100-200	7.0	0.2-1.5	Peritrichous	-	None	S. pullorum, S. gallinarum, S. paratyphi B	No affinity for animal or plant fungal cells.
3	50-1000	4.8	0.2-1.5	Peritrichous	-	Tanned erythrocytes	Kl. aerogenes, S. marcescens	Affinity for plant cells, cellulose fibres, fungal mycelium and leucocytes or erythrocytes heated to 70°C
4	50-1000	4.0	0.2-1.5	Peritrichous	-	Sheep, fowl	P.mirabilis, P.morganii, P.rettgeri, P.vulgaris	Adhesion for fresh untann RBCs of sheep and fowl. Different spectrum from type-1.
5	1-50	5.0	1.0	Monopolar	+	Sheep	Ps. echinoides	Possibly sex fimbriae. Phage-PO_4-isolated which adheres to Pseudomonas sp
6	4-40	10.0	10.0	Peritrichous	not known	not known	5% of Kl. ozaenae strains	May be sex fimbriae. Not enough information available on these structures.

Table 2 : Survey of fimbrial appendages (Ottow, 1975)



*Subgroups 1-4 are taken from Duguid's scheme - Common types 1-4.

III. COMMON TYPE FIMBRIAE

These appendages are characterised by the following features:

(a) are found on primary isolates from natural habitats

(b) are independent of motility or capsulation

(c) are produced predominantly in liquid media

(d) their presence on bacteria is marked by a drop in electrophoretic mobility (μ) (Brinton, 1959)

(e) are dispensible to the bacteria under laboratory conditions

(f) are not "cured" by acridine orange

(g) are immunogenic.

1. Type 1

Of the common type appendages, type-1 has been studied in most detail. These numerous, peritrichous organelles enable the bacterium to adhere to a diverse range of untreated erythrocytes and other eukaryotic cells. In particular, the bacterium may exhibit an adhesiveness for guinea-pig, fowl and horse cells which can be reversed by the addition of mannose, i.e. they display a mannose sensitive (MS) adhesin (Duguid et al., 1955).

In most strains possessing type-1 fimbriae (Fim^+), alteration of the culture conditions permits switching the synthesis of these appendages "off" and "on." Another feature of type-1 Fim^+ bacteria is that they grow as a surface pellicle in liquid medium.

a. Isolation: Fimbriae were originally isolated by Weibull & Hedvall (1953) from a Fla^+ Fim^+ strain of Proteus . Brinton in 1959 successfully

isolated these appendages from a Fla$^-$ E. coli B strain by blending the organisms and precipitating the fibres at their isoelectric point (pI) of 3.92, from the cell-free supernatant. Several cycles of precipitation using 0.1M $MgCl_2$ served to remove undesirable contaminants. Old (1963) also purified these filaments by applying them to a diethylamino ethyl (DEAE) ion-exchange column and eluting with a 2 molar salt gradient. The eluate was examined for haemagglutinating activity and active fractions were pooled and concentrated by polyethylene glycol.

b. Structure/Morphology/Chemistry: Several investigators including Brinton & Stone (1961), Old (1963) and Brinton (1965) have examined the chemical nature of type-1 fimbriae isolated from E. coli (strains unspecified). Analyses showed that these appendages were composed of protein, rich in acidic amino acid residues. Calculations based on X-ray diffraction data suggested a minimum molecular weight of 16,800 daltons for the fimbrial subunits. This compared favourably with the figure of 17,000 daltons obtained from amino acid analyses. Brinton (1965) proposed that these subunits polymerised into a right-handed α-helix of 7 nm diameter and pitch of 2.32 nm with 3.125 subunits per turn of the helix and a hollow core of 2-2.5 nm. Mitsui et al. (1973) also carried out X-ray diffraction studies on fimbriae isolated from a different strain of E. coli and concluded that the fimbrial protein subunits from their E. coli strain was significantly different from that of Brinton's because of published diffraction patterns of the isolated fimbriae and their reported isoelectric points (3.92 (Brinton) and 4.0 (Mitsui et al.)). This could be attributed to strain differences, methods of preparation or perhaps basic differences in the fimbriae.

Further work is required to substantiate one or other or both of these reports. Figure 1 illustrates the model of type-1 fimbriae (after Brinton, 1967).

Type-1 fimbriae are strongly hydrophobic and less electronegatively charged than the bacterial cell surface (Brinton, 1967). Neutralisation of the surface charge causes the fimbriae to aggregate in tightly packed, parallel sheets and since these exhibit a centre-to-centre distance of 63Å they must interlock laterally (Brinton, 1967).

In the presence of 0.1M $MgCl_2$ these fimbriae exhibit a characteristic streaming birefringence. Agents which break hydrogen bonds, i.e. 6M urea, glacial acetic acid and 1.5M guanidine hydrochloride cause depolymerisation of the fimbriae (Brinton & Huang, 1962). Dialysis against 0.2M KCl at pH 7.0, facilitates repolymerisation to give fibres indistinguishable from the untreated ones.

c. Synthesis: Freezing or the addition of 0.1M $MgCl_2$ in the cold, causes elongation of short pieces of fimbriae. These short lengths unite to form extremely long filaments which exhibit birefringence. This indicates that ATP or other energy sources are not required for elongation.

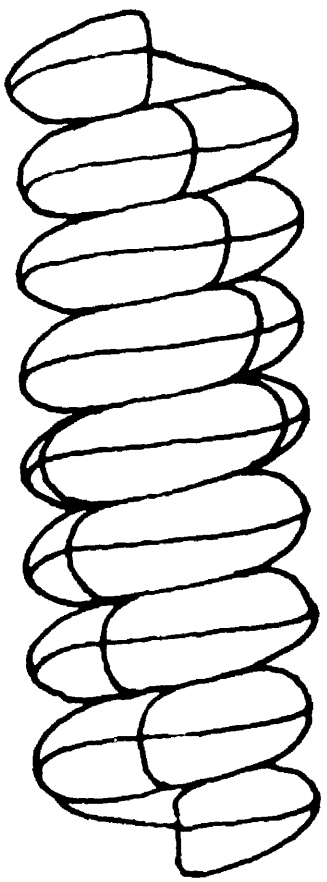
In 1959 Brinton reported rapid regrowth of fimbriae following their mechanical removal. Using standard conditions of volume (40 ml) temperature ($0^{\circ}C$) and type of blender (Omni-mixer, Sorvall), fimbriae were removed from an E. coli K-12 strain by blending in the cold at 9,200 rpm for 2 min. These vigorous blending conditions did not reduce the

Figure 1. Model of type-1 fimbriae (Brinton, 1967)

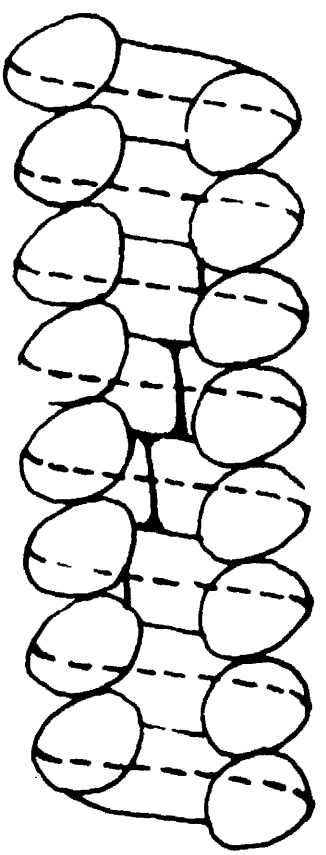
(a) end view

(b) longitudinal section

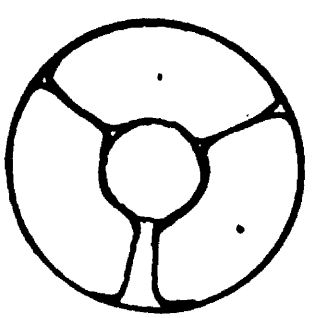
(c) transverse section



(a)



(b)



(c)

viability of the bacteria. Novotny and his colleagues (1969) recorded an average length of 0.48 μm for the fimbriae on the bacterial cell. Following removal of fimbriae by blending, the length returned to the pre-blended level within a period of 1-4 min. These investigators calculated that this elongation corresponded to a linear growth rate of 0.017 μm per min suggesting that presynthesised subunits lay within the cell envelope or cytoplasm (Brinton, 1965). Using penicillin treatment in hypertonic medium, Maccacaro & Turri (1959) prepared sphaeroplasts of Fim^+ E. coli K-12 which still retained fimbriae and haemagglutinating ability. From their results these authors proposed a cytoplasmic location for the site of synthesis of common type organelles.

d. Immunogenicity: The phenomenon of phase variation, characteristic of type-1, Fim^+ organism was used in the production of fimbrial antibody (Gillies & Duguid, 1958). Live Shigella flexneri of the fimbriate and non-fimbriate phases of the same strain were employed to raise antisera in rabbits. The non-fimbriate components were removed from the Fim^+ antiserum by absorption, leaving antibodies which inhibited the HA of the bacteria. The antibodies found in this antiserum - the anti-F antibodies - were distinct from those specific for the "H", "O" and "K" antigens of bacterial cells. The specificity of the serum obtained was tested in cross absorption with E. coli and Sh. flexneri strains.

Gillies & Duguid (1958) demonstrated that

i. E. coli possessed three antigens:

(a) one major coli-type specific antigen of which three types are recognised

(b) one or more flexneri-coli antigens

and

ii. Sh. flexneri possessed four antigens

(a) one major antigen shared by all other flexneri strains but not E. coli

(b) one or two minor antigens found in most E. coli strains

(c) one antigen - found only in a few E. coli strains.

Although fimbrial antiserum raised to either E. coli or Sh. flexneri cross reacted (due to component b), this preparation did not react with Salmonellae thereby indicating the lack of a common antigen in type-1 fimbrial strains. However the Salmonellae strains shared a common fimbrial antigen which was also found in Citrobacter (Duguid & Campbell, 1969).

Using isolated fimbrial filaments from a strain of E. coli B.(L)E, Brinton (1959) raised specific antiserum in rabbits, which agglutinated only Fim^+ strains. Fimbrial antibody was also raised to types-1 and -2 (common fimbriae) by Old (1963) and Old & Payne (1971). These investigations showed that types-1 and -2 were immunologically identical although the latter was non-adhesive.

Old (1963) prepared sera by both previously described methods. He concluded that antiserum obtained by the absorption method was not specific since it contained several components produced by the donor bacteria which were visualised by several precipitin lines on Ouchterlony plates.

e. Genetic control: Growth conditions such as temperature, type of

media and oxygen tension proved selective for either the fimbriate or the non-fimbriate phase (Duguid & Gillies, 1957). Bacteria cultured serially in aerobic static broth exhibit a preponderance of fimbriate cells whereas those grown on solid medium are predominantly non-fimbriate (Wilkinson & Duguid, 1960; Hashimoto et al., 1963a). However, some strains remain partly fimbriate on solid medium (Duguid et al., 1955) and complete suppression of fimbrial production has not been reported (Wilkinson & Duguid, 1960). Consequently the production of fimbriae is dependent on environmental factors which affect the expression of the genotype. In 1959, Brinton used interrupted mating experiments to establish the genetic locus for biosynthesis of fimbriae in strains of E. coli K-12 at a site between the threonine and β1 chromosomal marker. Using similar strains, Maccacaro & Hayes (1961) verified the location of the fim gene between threonine and thiamine loci. On a recent map of E. coli K-12 genome, the fim gene is located 88 min from the origin, between the glyV and hsm markers for glycine transfer RNAIII and DNA methylase host specificity, respectively (Taylor & Trotter, 1972).

During the original investigations on fimbriae, two main types of fimbriate bacteria were recognised:

(a) those which possess fimbriae or are capable of developing them under certain environmental conditions and

(b) those lacking fimbrial genes.

In 1959 however, Brinton recognised that in some strains of E. coli B, fimbriate cells (P^+) which gave rise to distinctive colonies, mutated irreversibly and at a high rate (4×10^{-4}/cell/generation) to non-fimbriate

cells (P^-). Subsequent investigations showed that Fim^+ K-12 gave two types of colony when plated out on to nutrient agar after a series of broth subcultures. The less common type of colony consisted of presumably fimbriate cells. Occasionally some of these latter colonies produced rough colonies containing non-fimbriate organisms which were unaffected by environmental factors. Maccacaro & Hayes (1961) designated these latter types stable mutants, i.e.

(i) those arising from Fim^+ strains which are stably fimbriate - Fim σ^+

(ii) those stable, non-fimbriate mutants arising from $Fim\sigma^+$ strains - $Fim\sigma^-$

and the relationship between these alleles is shown in Fig 2.

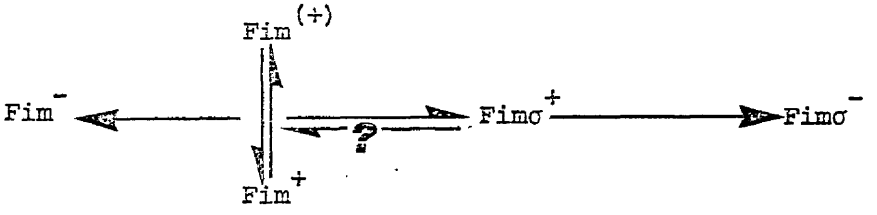
These researchers proposed that "the physiological and genetic differences between Fim^- and $Fim\sigma^-$" support the premise that two genetic determinants are involved in coding for the presence or absence of fimbriae. Genetic analyses have shown that this σ factor can be separated from the chromosomal fim locus by transduction with PI but not by recombination. The exact locus of σ on the genome awaits further investigation.

2. Types 2-6

Type-2 appendages are morphologically and immunologically identical to type-1 (Old & Payne, 1971). Because the former exhibit a mannose resistant (MR) haemagglutinin, these investigators concluded that a mutation had occurred in the gene coding for adhesiveness in type-2

Figure 2 : Relationship between forms of fimbriation

(Maccacaro & Hayes, 1961)



Fim^{+} } $Fim\sigma^{+}$ } difference between these is HA of $Fim\sigma^{+}$ is inhibited by peptone whereas Fim^{+} is not.

$Fim^{(+)}$ expression of fimbriation, inhibited by environmental conditions.

Fim^{-} } $Fim\sigma^{-}$ } difference between these lies in their oxidative and glycolytic ability, i.e.

Fim^{-} oxidative

$Fim\sigma^{-}$ glycolytic

appendages. This was supported by the observation that some strains of Salmonella with type-2 fimbriae mutated either spontaneously or following ethyl methane sulphonate (EMS) or nitrous acid treatment to give type-1 agglutination patterns (Duguid et al., 1955).

The red cell specificities and adhesive properties of type-3 differs from types-1 and -2. Pretreatment of erythrocytes with tannic acid is required before the type-3 agglutination occurs.

Thin fimbriae recognised on strains of Proteus by Shedden (1962) possessed a completely different spectrum of haemagglutinating activity against erythrocytes. This type is unique in that it agglutinates both fresh and tanned red blood cells. Coetzee et al. (1962) recognised that Proteus was perifimbriate, i.e. exhibited denser bundles of fimbriae at both ends of the bacterial cells. The 79 strains of Proteus studied by Shedden proved autoagglutinable in 0.10 - 0.85% saline. Each bacterium possessed 12-200 appendages of diameter 10 nm. Phase contrast microscopy was used to observe the adhesion of these strains to washed sheep erythrocytes, Candida albicans and glass surfaces.

The polar fimbriae of Pseudomonas species were assigned to type-5 in Duguid's scheme. At that time it was uncertain whether these were common or sex fimbriae. Bacteriophages were isolated recently (Bradley, 1973) which attach to some of these appendages, thus bringing them within the definition of sex fimbriae.

On approximately five per cent of Klebsiella ozaenae strains, very slender filaments were detected (Duguid, Anderson & Campbell, 1966).

These type-6 fimbriae showed little or no HA activity. Because of their scarcity - up to 40 per bacterium - Duguid (1968) proposed that they might be sex fimbriae. More information is required before these can be assigned to the common or sex groups.

IV. POSTULATED FUNCTIONS OF FIMBRIAE

1. Nutrition

The presence of fimbriae on bacterial cell surfaces results in a considerable increase in the surface area of the organism. Maccacaro & Angelotti (1955) suggested that fimbriae were structures responsible for the uptake of nutrients via the axial hole.

2. Respiration

The question of whether or not fimbriae aid respiratory activity is still unresolved. Wohlhieter et al. (1962) reported no correlation between fimbriation and respiration rate. However, Maccacaro (1958) and Dettori & Maccacaro (1959) found that Fim^+ E. coli K-12 increased their oxygen uptake for longer periods than Fim^- strains in shaken broths. Gillies & Duguid (1958) suggested that fimbriae played a role in concentrating Fim^+ organisms at the surface of the broth and that the increase in growth obtained was due to the excellent oxygen supply to the organisms in the pellicle. By inhibiting pellicle formation via 0.05% (v/v) Tween 80 or passing hydrogen through the culture, Fim^+ and Fim^- bacteria grew equally well. However, these E. coli K-12 organisms failed to exhibit haemagglutinating activity.

Gemski (1964) also reported a selective advantage of the Fim^+ cells when nitrogen and carbon dioxide gas were passed into the culture medium, pellicle formation was absent. Suboptimal conditions such as low oxygen tension and high cell density appeared to favour rapid growth of fimbriate phase organisms.

3. Twitching motility

Henrichsen (1972) proposed that this type of cell movement was a consequence of polar fimbriation.

The discovery that certain bacterial strains possessing polar fimbriae exhibited twitching motility motivated Henrichsen & Blom (1975) to screen almost 1,000 strains of prokaryotes for these features. Both polar fimbriation and twitching motility were discovered in strains of Acinetobacter, Cytophaga, Moraxella and Pseudomonas. A strain of Streptococcus sanguis also exhibited these features (Lautrob, 1961; Henriksen & Henrichsen, 1975). Ninety strains from the family Enterobacteriaceae possessing peritrichous fimbriae, did not exhibit this form of motility.

Twitching motility is a mechanism of translocation whereby bacterial cells move singly, with an intermittent, jerky movement over the surface of a moist agar plate which is supporting a critical thickness of liquid. Although these authors confirmed a correlation between the twitching motility and polar fimbriae, they experienced difficulty in differentiating between this type of movement and gliding motion. Because environmental conditions influence this translocation process,

twitching motility is only observed under certain conditions of media, temperature and moisture (Henrichsen, 1975).

All the strains possessing polar fimbriae were found by these investigators to exhibit twitching motility. However, the mechanism of such movement remains obscure.

4. Genetic role

Brinton et al. (1965) suggested that fimbriae may possess a genetic function. Common type appendages are excluded from this role but the sex type are believed to function in conjugation since their removal results in loss of the ability of the cell to undergo conjugation. Although there seems to be an obligate requirement for sex type fimbriae in conjugation, it appears that common type appendages are also required for efficient genetic transfer to take place (Mulcyz & Duguid, 1966; Meynell & Lawn, 1967).

5. Adhesion to:

a. Erythrocytes: Duguid & Gillies (1957) reported that agglutination of type-1 Shigellae to guinea-pig erythrocytes occurred between a pH range of 3-10 when borate or phthalate buffer was used to alter the pH. These researchers demonstrated the adhesiveness of Fim^{+} organisms to an extensive array of substrates. Brinton (1959) found that 0.5 µg/ml of isolated fimbrial protein was sufficient to obtain haemagglutination with 0.2% (v/v) chicken erythrocytes. Old (1963 & 1972) and Rivier & Darekar (1975) also prepared isolated fimbriae capable of agglutinating guinea-pig and horse erythrocytes. Evidence favouring

fimbriae as organs of attachment include the following:

(a) all Fim^+ bacteria are HA^+ except those with only type-2 organelles which are non-adhesive (Duguid & Gillies, 1957)

(b) with the exception of type-2, fimbriate organisms adhere in the fimbriate phase but not in the non-fimbriate phase

(c) isolated fimbriae agglutinate erythrocytes and other substrates (Brinton, 1959; Rivier & Darekar, 1975)

(d) the presence of anti-fimbrial antibody blocks the HA reaction (Gillies & Duguid, 1958).

These observations lend strong support to the view originally expressed by Houwink (1949) that fimbriae act as organelles of attachment. Membrane ghosts of guinea-pig erythrocytes prepared by saponin treatment also permitted adhesion of fimbriate bacteria. Duguid & Gillies (1957) verified the results of Collier & de Miranda (1955) when they found that mannose could inhibit the HA reaction at low concentrations, i.e. 0.01% (w/v). Although other saccharides were examined for their ability to inhibit the HA reaction - Haemagglutination Inhibition (HAI) - only α-methyl mannoside proved to be as effective as mannose (Collier & de Miranda, 1955; Duguid et al., 1955; Old, 1972; Rivier & Darekar, 1975). The specificity shown by mannose in effecting inhibition of the haemagglutination reaction may indicate a receptor on the erythrocyte structurally similar to mannose. Collier & de Miranda (1955), Duguid & Gillies (1957) noted that mannose did not bind irreversibly either to the erythrocytes or the bacteria since removal of the sugars via washing restored the HA activity. Old (1972) reported

that a specific configuration of saccharide was required for maximum binding to the fimbrial protein.

b. Other substrates: In addition to erythrocytes, fimbriate organisms adhere to an extensive array of substrates including whole cells of Candida albicans and Willia anomala, thrombocytes, tumour cells (Hashimoto et al., 1963b), leucocytes, spores of Penicillium notatum, and Trichomonas vaginalis (Duguid et al., 1966). Fim^+ organisms also adhere to epithelial cells of guinea-pig and ox colon (Duguid, 1959). This adhesiveness is correlated with the presence of fimbriae and HA activity. Hashimoto (1963b) also reported that the dye carmine red will cause fimbriate organisms to agglutinate. Isolated type-1 fimbriae from E. coli will also agglutinate in the presence of carmine red (Sweeney, unpublished observation).

V. FIMBRIAE-ERYTHROCYTE INTERACTION

1. Fimbrial agglutinin

Little is known about the location or nature of the agglutinin on the fimbrial filament, i.e. whether the binding site is located laterally on the fibre or situated solely at the tip. Whether these slender threads are monovalent, divalent, or polyvalent has not been established. To date, bacteriophages which adhere to these common type fimbriae have not been isolated. Gillies & Duguid (1958) and Old (1972) showed that the addition of formaldehyde to Fim^+ organisms did not alter the specificity of the HA reaction. This may suggest that the agglutinin is not solely protein. Brinton (1965) reported

that isolated fimbriae from E. coli K-12 F⁻ adhered by their tips to latex spheres. However, Poon & Day (1975) observed that Ustilago fibres though similar in protein nature and diameter (6-7 nm) to common type-1, did not behave in this manner.

Antisera raised to fimbrial types-1 and -2 of Salmonellae showed close antigenic similarity when examined by absorption tests (Old & Payne, 1971). However, the type-2 strains lacked the haemagglutination properties displayed by type-1 strains. Consequently these authors concluded that the agglutinin was a minor antigen and proposed the fimbrial tip as its locus. Duguid & Gillies (1958) detected lateral clumping of type-1 fimbriae of whole Sh. flexneri in the presence of specific fimbrial antibody. Following addition of the antibody, the ability of the bacteria to induce haemagglutination was abolished. These observations favoured the premise that the lateral surface of the filaments served as the loci for both the fimbrial haemagglutinin(s) and the fimbrial antigen(s).

Maccacaro & Dettori (1960), in a study of agents which promoted agglutination of fimbriated cells, examined aliphatic and aromatic compounds, antibiotics and chelating agents. Their results revealed that dipolar molecules, i.e. dicarboxylic acids, diamines and aminocarboxylic acids facilitated agglutination of Fim⁺ bacteria and they concluded that the position of the active groups - COOH, NH_2 and OH within the dipolar molecule was of prime importance.

2. Agglutinin binding sites on erythrocytes and other substrates

Except for the mannose sensitivity, little information is

available on the nature of the type-1 fimbrial receptor sites on erythrocytes or other substrates. Duguid et al. (1955) performed the original work on this subject when they screened sugars for ability to inhibit the haemagglutination reaction (HAI) in E. coli type-1. This line of investigation was pursued by Hashimoto and his colleagues (1963b) and Old (1972) who tested numerous saccharide moieties for HAI ability. Mannose, α-methyl mannoside and 1,5 anhydromannitol proved the most effective inhibitors. Although many other derivatives of mannose were surveyed, they proved to be non-inhibitory. Only sugars bearing unsubstituted hydroxyl groups in the C-2, C-3, C-4 and C-6 positions of the mannosyl ring were inhibitory. A specific requirement for the α-linkage was confirmed by the inability of β(1-4)-linked mannobiose and mannotriose to inhibit the HA reaction. Melizitose, a weak inhibitor, proved an exception to this rule.

In a later study, Duguid & Gillies (1957) treated C. albicans and guinea-pig erythrocytes with periodate and Vibrio cholerae receptor-destroying enzyme. On titration with Fim^+ organisms, the agglutination patterns they obtained remained unaltered. Pretreatment with other reagents, i.e. pepsin, trypsin, periodate, sodium hydroxide and hydrochloric acid on a strain of the yeast Willia anomala, prior to agglutination was investigated by Hashimoto et al. (1963b). Alteration of the titre was not observed except where complete abolition of the HA occurred following treatment with strong acid and alkali.

VI. ROLE OF FIMBRIAE IN PATHOGENICITY

Many conflicting reports are available on the role of fimbriae in pathogenicity. Some observations favouring the premise that fimbriae are not essential for pathogenicity are presented below.

(a) Saphrophytic *Klebsiella* strains are more richly fimbriated than the pathogenic ones (Duguid *et al.*, 1966).

(b) Fim^- strains of *Shigella* such as *Sh. dysenteriae*, *Sh. boydii* and *Sh. sonnei* are pathogenic (Duguid & Gillies, 1957).

(c) Some pathogenic *Salmonella* strains, i.e. *S. paratyphi*, *S. typhi* and *S. pullorum* are not fimbriate (Duguid *et al.*, 1966).

In contrast, several authors report that the infectivity of the organism is greatly enhanced when fimbriae are synthesised.

In 1961, Campbell (cited by Duguid, 1968) demonstrated that Fim^+ strains of *S. typhimurium* introduced orally into mice produced greater infectivity and virulence than Fim^- types. However, on intraperitoneal injection, this difference was not apparent. Darekar & Eyer (1973) showed that in mice challenged with Fim^+ and Fim^- *Salmonella typhimurium* via the oral and conjunctival routes, the Fim^+ organisms produced greater infectivity. Semenitz (1975) also observed that *E. coli* strains from human urinary tract infections were predominantly fimbriate in comparison to the Fim^- strains isolated from healthy humans. It therefore appears that fimbriae, although they may facilitate survival of the organism in a particular environment, are not essential for pathogenicity. The precise role of these organelles in pathogenicity remains to be established, but in some instances they do appear to increase virulence.

VII. SEX FIMBRIAE

1. Introduction

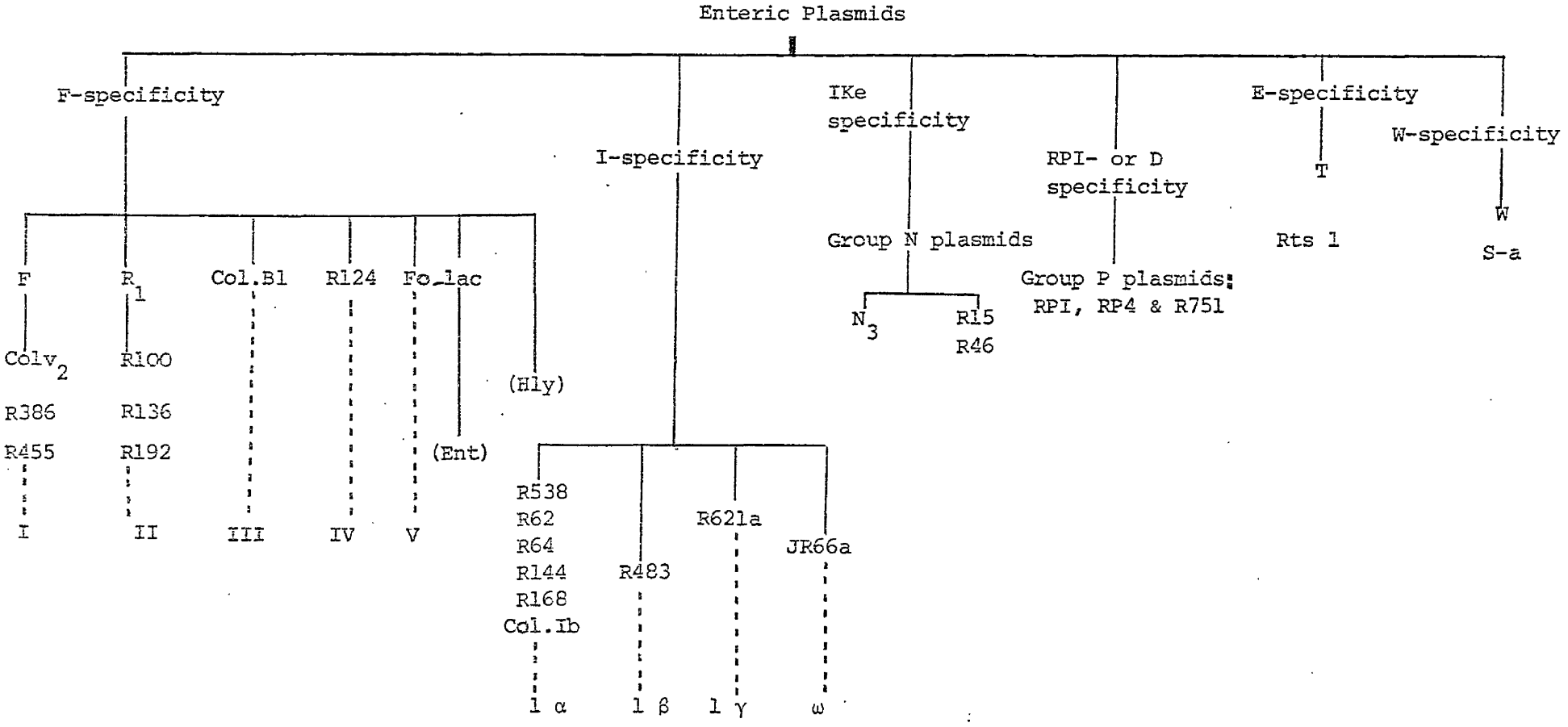
In 1960, Ørskov & Ørskov detected a proteinaceous surface antigen (f+) on donor strains of *E. coli* K-12, F^+ and Hfr. Four years later, Crawford & Gesteland (1964) reported fimbrial appendages on male strains of bacteria which absorbed R17 phage. Following the speculation of Sneath & Lederberg (1964) that this f+ antigen was the F fimbria, Valentine, Wedel & Ippen (1965) and Brinton (1965) demonstrated that susceptibility to male-specific phages, ability to conjugate and act as genetic donors, were related to the presence of fimbriae. The relationship was subsequently verified by the work of Ishibashi (1967), Nishimura *et al.* (1967) and Lawn (1967) all of whom prepared antiserum to donor strains and absorbed out antibacterial antigens using female strains. The residual antibodies agglutinated and showed specificity for the F fimbriae as demonstrated by electron microscopy. Recent work has revealed that these male specific fimbriae are produced by bacteria harbouring specific plasmids (Fig 3 and later sections).

2. General properties

In comparison with common type appendages, relatively few sex fimbriae (1-4) are synthesised by each bacterial cell. Investigations on these filaments are further complicated by the presence of flagella and common type fimbriae. Consequently, non-flagellate strains, lacking common type appendages and bearing derepressed (drd) sex determinants, are chosen for investigation where possible.

Figure 3 : Fimbrial specificity of plasmids

(modified from Novick, 1969)



Incompatibility groups : I - IV of F-specificity (Datta, 1974)

& 1α - ω of I-specificity (Hedges & Datta, 1973)

Sex fimbriae are found predominantly on Caulobacter species and members of the families Enterobacteriaceae and Pseudomonadaceae. In direct contrast to the common type, sex fimbriae are produced by bacteria cultured on solid medium (Lawn & Meynell, 1970).

The presence of terminal vesicles of 15-70 nm in diameter was recorded on sex appendages by Valentine & Strand (1965), Lawn (1966) and Pereverzer & Petrova (1969). In subsequent reports by Meynell & Aufrieter (1969), Lawn & Meynell (1970) and Brinton (1971), chemical treatments were shown to alter the slender filaments causing formation of vesicle-type structures, i.e. aggregates of fimbriin.

Another characteristic of sex fimbriae is that they act as receptors for bacteriophages. The isometric RNA phages f1 and MS2 attach laterally to the appendages (Brinton et al., 1964), whereas the filamentous DNA phages f2, fd and M13 adhere to the fimbrial tip (Caro & Schnos, 1966; Achtman, 1973). These differences in receptor site distribution suggest differences either in the chemistry of the shafts and tips or differences in the orientation of identical subunits at these two sites. Whatever the case may be, Meynell, Mathews & Lawn (1974) were able to raise antibodies which were specific for either the shafts or tips. This was achieved by sonicating partially purified fimbriae from bacteria carrying the hybrid sex factor F/R1 drd 19, whose shafts were of the F type. This sonicate was mixed with Freund's adjuvant and injected into rabbits. The antisera obtained was repeatedly adsorbed with F fimbriae until the antisera contained antibodies which specifically agglutinated fimbrial tips.

The characteristics which serve to differentiate F type from common type fimbriae are given in table 3.

a. Isolation: Beard et al. (1972) purified sex fimbriae from a strain of E. coli K-12, which also possessed common type appendages and a drd F-like, R plasmid. The purification process involved blending the bacteria, concentrating the cell-free supernatant following low speed centrifugation and collecting the appendages by centrifugation at high speed. The pellet obtained was then centrifuged in a caesium chloride gradient and a single band with buoyant density of 1.309 g/cm^3 was obtained. This band consisted of both common and sex fimbriae which were separated by isoelectric focusing into two components only one of which showed binding activity for male specific phage. Beard and his colleagues obtained a yield of 3-6 mg of fimbriae per 20-litre culture. The only details of Brinton's method (cited in Beard et al., 1972 and Tomoeda et al., 1975) indicate that his isolation method involved "precipitation of the fimbriae as crystalline aggregates in 10 mM tris-saline buffer (pH 8.5) and redissolution in 30% (w/v) sucrose." Purification of the filaments was achieved by cycles of low speed centrifugation and reprecipitation.

Tomoeda et al. (1975) purified sex fimbriae from E. coli carrying an F8 plasmid, by mixing them with polyethylene glycol (PEG) 6000. The appendages were harvested in 10 mM tris-HCl - 1 mM magnesium buffer (pH 7.2) and centrifuged at 10,000 x g for 20 min. A solution of 2% PEG - 0.5 M NaCl was added to the supernatant and the mixture stirred at $4^{\circ}C$ for 1 h. These researchers claimed 97% recovery of total

Table 3 : Comparison of fimbriae of *E. coli* and *C. renale*

(Kumazawa & Yanagawa, 1972)

	a Common type-1	b F sex fimbriae	c Type II of C. renale
Diameter (nm)	7.0	8.5	2.5-3.0
Length (μm)	0.25-1.5	0.5-2.0	2-15
pI	3.92	4.15	4.35[d]
Amino acid composition (moles/10^5 g protein)[e]			
Lysine	18	85	59
Histidine	12	0	15
Arginine	18	0	51
Aspartic acid	120	68	113
Threonine	120	68	85
Serine	60	93	40
Glutamic acid	78	34	117
Proline	12	0	46
Glycine	102	127	79
Alanine	205	127	90
½ Cystine	12	0	4[f]
Valine	78	178	74
Methionine	0	68	6[f]
Isoleucine	24	34	29
Leucine	60	76	50
Tyrosine	12	17	30
Phenylalanine	48	59	20
Tryptophane	0	17	5[f]

a. Brinton (1965)

b. Brinton (1971)

c. Kumazawa & Yanagawa (1972)

d. Ibid (1973)

e. results calculated by Kumazawa from data of Brinton (1965)

f. may be due to contamination according to authors in c.

F fimbriae following subsequent centrifugation of the mixture at 8,000 x g for 10 min.

b. Chemistry, structure and morphology: Results from studies on purified fimbriae from a transfer deficient mutant (tra^-) of *E. coli* K-12, led Brinton (1971) to suggest that F fimbriae consist of a single kind of fimbrial molecule with a subunit molecular weight - by SDS polyacrylamide gel electrophoresis - of 11,800, containing glucose and phosphate, i.e. a phospho-glyco-protein. Recent investigations of Beard (cited by Beard & Connolly, 1975) have verified Brinton's work and revealed that F fimbriae contains 1.56 moles of phosphate and 1.04 moles of glucose per mole of sex fimbriae. Brinton (1971) proposed that, unlike type-1 appendages, the F fimbriae consist of two parallel protein rods comprising an assembly of F monomeric subunits. The filament is 8.5 nm in diameter with a 2.5 μm hollow core.

The protein of F type fimbriae is rich in hydrophobic amino acids, readily disaggregated by trypsin and organic solvents (Wendt *et al.*, 1966; Brinton & Beer, 1967) and completely lacks cysteine, histidine, arginine or proline residues (table 3).

c. Synthesis: It seems reasonable to suppose that sex fimbriae are assembled from a pool of presynthesised subunits in the envelope or cytoplasm of the cell and evidence favouring this supposition comes from the work of Meynell (1973). Meynell & Lawn (1975) confirming earlier work, found that defimbriated bacteria rinsed in warm broth on cellulose acetate membranes re-synthesised sex fimbriae within 20-30 s.

Brinton (1965) also reported that within 5 min of blending, sex fimbriae returned to half their normal length. However, Novotny et al. (1969), Brinton (1971), Novotny & Lawn (1971), Meynell (1973) and Meynell & Lawn (1975) all failed to demonstrate the existence of presynthesised pools of subunits in the cytoplasm. This failure to demonstrate cytoplasmic pools by complement fixation or gel electrophoresis led Brinton (1971) to suggest a membrane location for synthesis, assembly and attachment of fimbriae. In support of this idea, he stated that in order to achieve such rapid resynthesis the subunits must be present, either in the membrane or be capable of very speedy incorporation to that site for assembly. Following the same line of reasoning Beard & Connolly (1975) examined the outer membrane fraction of E. coli K-12 envelopes for the presence of subunit pools. Using a transfer deficient mutant (tra J^-), i.e. a mutant able to synthesise but not transfer F, and carrying a drd F-like, R factor, they prepared inner and outer membrane fractions which were subjected to analyses by SDS polyacrylamide gel electrophoresis (PAGE). A component of molecular weight 12,500 $\pm$ 600 absent in cytoplasmic membranes was present in outer membrane fractions. Its absence from outer membrane fractions of plasmid-less mutants and its similarity in molecular weight to that reported for the subunits derived from isolated fimbriae led to the conclusion that it may represent the "pool" in the cell envelope from which assembly of the fibre originates. The absence of a precipitin reaction after this component was mixed with fimbrial antibody casts doubt on whether this isolated protein is fimbrial in nature, or a peptide from the cell envelope. In a recent publication, Flint &

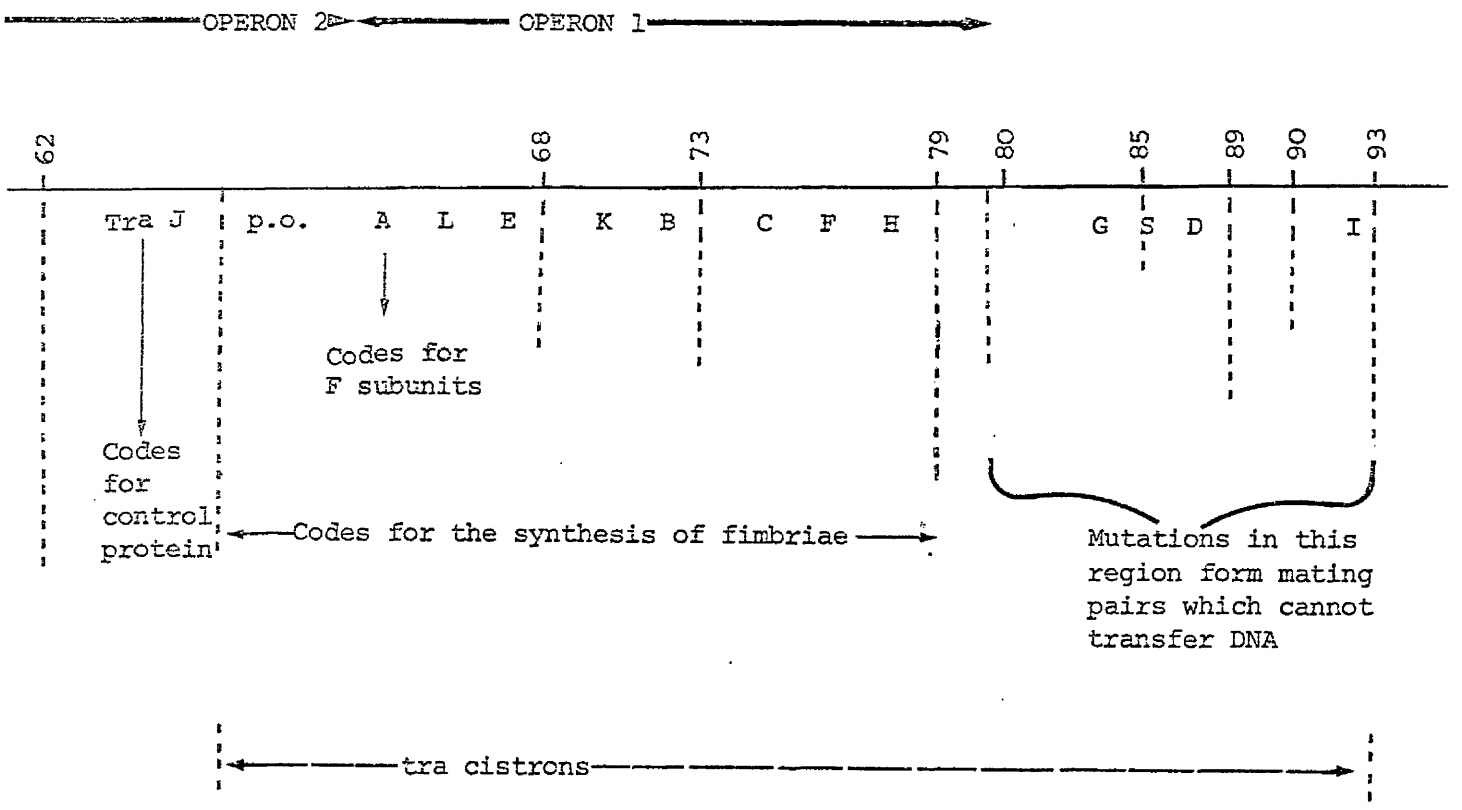
Maynell (1976) reported that F-like fimbriae treated with low concentrations of SDS or Triton X-100 failed to react with fimbrial antiserum. The identity of the component isolated by Beard & Connelly (1975) may be established by its reaction with antiserum prepared against SDS-treated F-like subunits.

d. Genetic control: Although Maccacaro (1955) failed to find a correlation between fimbriation and the possession of a fertility factor, there is now ample evidence favouring a close correlation between the F plasmid and the presence of fimbriae. Some bacteria, particularly those of the family Enterobacteriaceae harbour enteric plasmids (Gyles, 1972) which perform several functions including coding for the production of sex fimbriae. Novick et al. (1976) defined these as "replicons which are stably inherited in an extrachromosomal state. The naturally occurring plasmids of prokaryotes are generally dispensible."

These plasmids are assigned to particular groups according to the type of appendages which are synthesised (Novick, 1969). A diagram showing several of the 20 incompatibility groupings of plasmids (Datta, 1975) and the type of sex fimbriae for which they code, is presented in Fig 3. Investigations into the locus on the F-lac plasmid of E. coli coding for the synthesis of F fimbriae conducted by Nishimura et al. (1967), Ohtsubo et al. (1970), Willetts (1972), Helmuth & Achtman (1975), Miki & Horiuchi (cited by Tomoeda et al., 1972), revealed the involvement of at least 15 cistrons. From this data, a linear map of the locus is presented in Fig 4.

Figure 4 : Physical map of F operon of E. coli

(Helmuth & Achtman, 1975)


OPERON 2
OPERON 1
62
68
73
79
80
85
89
90
93
Tra J
p.o.
A
L
E
K
B
C
F
H
G
S
D
I
Codes for F subunits
Codes for control protein
Codes for the synthesis of fimbriae
Mutations in this region form mating pairs which cannot transfer DNA
tra cistrons

Because of the role in genetic transfer there is considerable interest in the F fimbriae of several members of the family Enterobacteriaceae, especially E. coli K-12 (Marvin & Hohn, 1969; Curtiss, 1969; Ou & Anderson, 1970; Achtman, 1973; Ou, 1973). The F plasmid of E. coli K-12 present as 1-2 copies per bacterial genome codes for F fimbriae (Willetts, 1972). Other plasmids, i.e. Enterotoxin (Ent), Colicine production (Col), Resistance Transfer Activity (R), Haemolysin (Hly) and K88 also code for fimbrial production and the characteristics of several of these are presented in table 4. In 1967, Stirm et al. detected and isolated slender filaments from agar cultures of two non-fimbriate, porcine Enteropathogenic E. coli strains (EEC) carrying the proteinaceous surface antigen K88 and showed that these were associated with a mannose resistant eluting (mre) haemagglutinin. Although these filaments differed from previously described E. coli types, they resembled the fragile types found on strains of Proteus and Vibrio. Evans et al. (1975) detected a plasmid coding for a colonising factor of human EEC strains, similar in function to the K88 antigen which is peculiar to calf strains. This factor is heat labile and also consists of fine surface filaments. Whether these colonising plasmids are of widespread occurrence awaits further study.

Smith & Linggood (1971) studied the effect of selective addition and removal of 3 plasmids, viz. haemolysin (Hly), Ent and K88 on the pathogenicity of porcine EEC strains. The introduction of $K88^{+}$ Ent^{+} into non-pathogenic strains converted them to pathogens. These authors concluded that the K88 antigen aided proliferation high in the pig

Table 4 : Comparison of some characteristics of plasmid-controlled fimbriae

Plasmid	Fimbrial Type	Diameter (nm)	Length (μm)	Distribution	No/cell	Phage Receptors	References
F	F	8.5-13.5	20	Peritrichous	1-4	f1, f2 MS2, M13 R17	Valentine, Ippen et al. (1969) Achtman (1973)
Ent	F	"	"	"	"		Smith & Linggood (1971); Stevens, Gyles & Barnum (1972); Jacks & Wu (1974); Gyles (1974a & b); Söderlind et al. (1974)
Hly	F	"	"	"	"		Smith & Halls (1967); Royer-Pokora & Goebel (1976)
Col.Ib	I	6.0-12.0	1	"	"	If_1 If_2	Meynell & Lawn (1968); Lawn & Meynell (1970); Howarth & Thompson et al. (1974)
Group N	Ike	thinner than F	-	"	"	Ike	Datta & Hedges (1971); Achtman (1973); Tomoeda et al. (1975)
K88	K88	7.0-11.0	0.1-1.5	"	Many	NR	Stirm et al. (1967a,b); Gyles (1972); Jones & Rutter (1972, 1974); Jones (1975)
*Pseudomonas-plasmid undetermined	NR	4.5-6.0	10	Polar	Many	PO_4	Bradley (1972, 1973); Weiss & Raj (1970)

*Known now, not coded for by plasmid FP2; NR - not reported.

intestine - an essential prerequisite in the pathogenesis of diarrhoea - and that the plasmids had a synergistic effect in eliciting disease.

K88 receptor

In 1972, Jones (cited by Jones & Rutter, 1974) postulated that K88 may attach to a glycoprotein receptor site in the intestinal mucosa of pigs. Treatment with sow colostrum blocked the adhesion of $K88^+$ strains to the small intestine of gnotobiotic pigs. A glycoprotein receptor was suggested since the inhibiting factor was heat-resistant, stable to proteases, insoluble in chloroform/methanol and susceptible to periodate oxidation. In an effort to identify this receptor, Gibbons, Jones & Sellwood (1975) fractionated monosaccharides, polysaccharides and glycoprotein fractions from sow colostrum and examined their ability to block the agglutination. Because none of the isolated fractions possessed blocking ability, they concluded that the receptor site involved more than a single molecule.

VIII. ROLE OF SEX FIMBRIAE

While no direct evidence is available for fimbriae as organelles participating in the process of genetic transfer, considerable circumstantial evidence does exist (Brinton, 1971). In 1964 this author proposed the "Conduction Theory" later amended (1971), which involved the conduction of single stranded DNA from the donor to the recipient bacterium via the fimbrial axial hole. Data thought to be favouring this theory was provided by Ou & Anderson (1970) who showed that genetic transfer occurred between some - but not all - cells which made cell-to-

cell contact. In a search for evidence relating to this model, Jacobson (1972) used electron microscopic autoradiography to examine F appendages for the presence of labelled DNA. However, no label was demonstrated in the fimbriae.

The "Retraction Theory" was proposed by Marvin & Hohn (1969) who suggested that retraction of the fimbriae occurred when they encountered RNA phages or recipient cells. The stimulus of contact triggered depolymerisation at the base of the fibres, drawing the two cells together. A variation on the latter theme was presented by Curtiss (1969) who suggested that cytoplasmic continuity in the form of a conjugation bridge was formed when the two bacteria came together. The bridge was thought to form as a result of cell-to-cell contact which in turn resulted in fimbrial contraction. This latter model is favoured by many researchers including Jacobson (1972), Novotny et al. (1972), Bradley (1972), O'Callaghan, Bradley & Paranchych (1973) and Novotny & Fives-Taylor (1974). However, Schreil & Christensen (1974) proposed that a conjugation tube from wall-to-wall is the more likely route for DNA transfer between cells.

In the presence of sodium cyanide (Novotny et al., 1972), arsenate (O'Callaghan et al., 1973) and on the addition of bacteriophage M13 (Bradley, 1973), F appendages are reported to disappear from the bacterial cell surface. Two explanations for this were proposed:

(a) the fimbriae fall off (O'Callaghan et al., 1973) or

(b) they retract into the host (Novotny et al., 1972).

Although Novotny & Fives-Taylor (1974) examined cell-free supernatants of

several strains of E. coli K-12 and B/r for the presence of detached fimbriae following treatment with NaCN, none was detected. Consequently they accepted the latter proposal.

Marvin & Hohn (1969) and Novotny & Fives-Taylor (1974) proposed a dynamic role for sex fimbriae, i.e. a balanced elongation and retraction in vivo. In agreement with this proposal, Lawn & Meynell (1972) reported a massive increase (x 25-fold) in the numbers of I-type fimbriae in E. coli K-12 strain 22 within 60 seconds of treatment with fimbrial antiserum. They concluded that the presence of the antibody prevented retraction of the appendages or that the attachment of antibody stimulated extrusion of fimbriae by upsetting equilibrium between free fimbrial molecules and polymerised subunits at the site of fimbrial assembly.

MATERIALS AND METHODS

MATERIALS AND METHODS

I. BASIC METHODOLOGY

1. Cultivation of bacteria

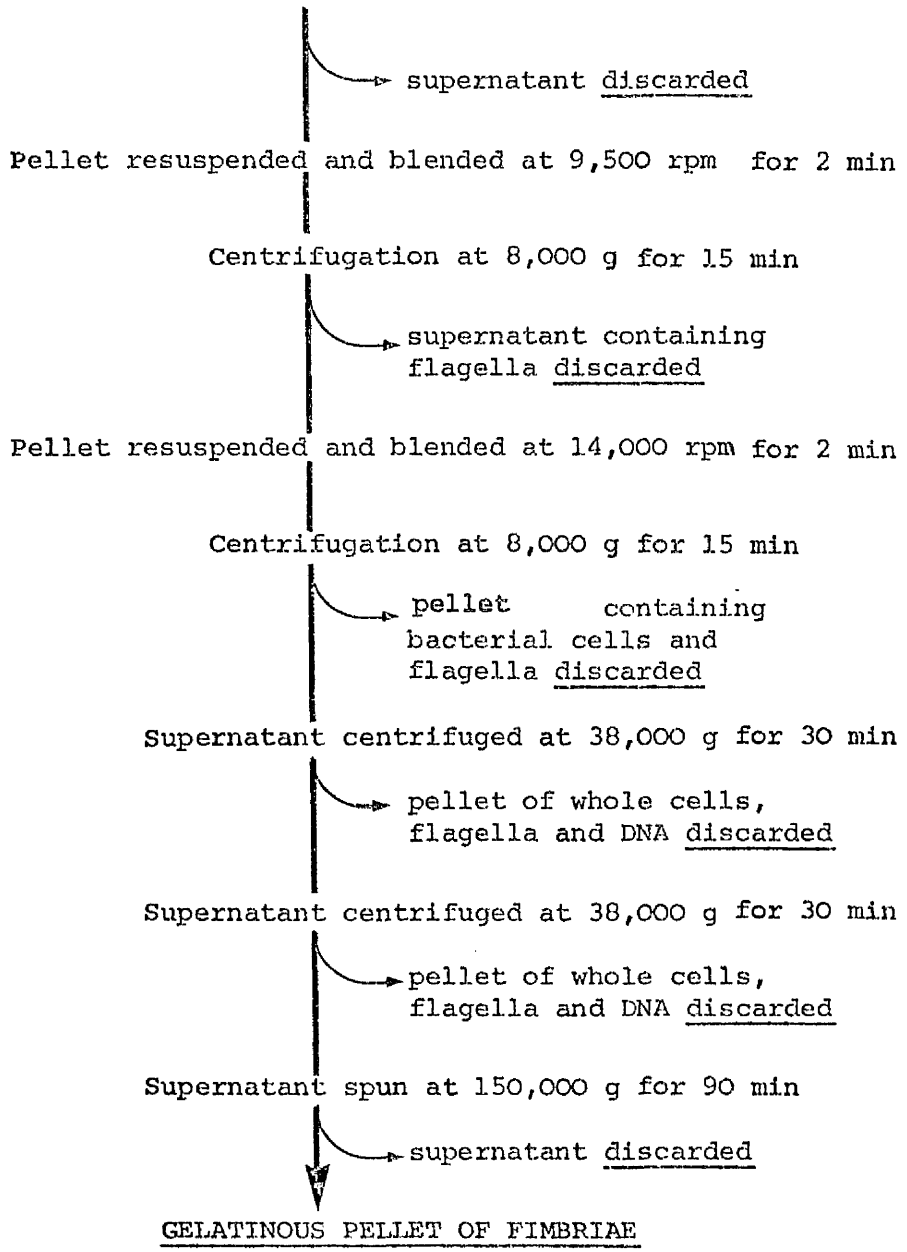
Escherichia coli NCTC 8623, serotype O125:K70(B15):19, a flagellate, fimbriate organism was grown by static incubation at 37°C for 48 h in nutrient broth (Oxoid, No. 2) dispensed into Fernbach flasks.

2. Preparation of fimbriae

Cultures were treated with 0.1% (v/v) formaldehyde, harvested by centrifugation and washed twice in sterile distilled water. Flagella and sex type fimbriae were released by blending the bacteria in the cold for 2 min at low speed (9,500 rpm) in an M.S.E. homogeniser (Novotny, Carnahan and Brinton, 1969). Following centrifugation at 10,000 x g the sedimented cells were resuspended in a small volume of pre-cooled distilled water and blended in the cold for a further 2 min at maximum speed (14,000 rpm) to release type-1 fimbriae. Centrifugation at 10,000 x g for 15 min sedimented the bacteria which were discarded. Contamination of the supernatant fraction with flagella, whole bacteria or high molecular weight DNA, was reduced by two cycles of centrifugation at 37,000 x g for 30 min. The clear supernatant from the second sedimentation step was then centrifuged at 150,000 x g for 2 h and the translucent gelatinous pellet obtained was gently suspended in sterile distilled water. A summary of the entire procedure is presented in Fig 5.

Figure 5 : Summary of the procedure employed in the isolation of type-1 fimbriae from *E. coli* 8623

FORMOLISED, 48 h BROTH CULTURE HARVESTED AND WASHED TWICE IN DISTILLED WATER (8,000 x g for 20 min)



II. NATURE OF RECEPTORS FOR TYPE-1 FIMBRIAE ON ERYTHROCYTES

1. Agglutination reactions

a. Haemagglutination tests (HA)

Haemagglutination tests were done using 1% (v/v) or 3% (v/v) suspensions of washed, horse erythrocytes in 0.85% (w/v) saline. Haemagglutinating activity of whole bacteria was assessed using formolised 48 h static cultures of *E. coli* 8623. Washed bacterial suspensions were adjusted to an absorbance of 8.0 at 620 nm in 10 mm cells using a Pye Unicam SP 600 spectrophotometer, this being equivalent to approximately 8×10^7 bacteria/ml. Tests using 1% (v/v) or 3% (v/v) horse erythrocyte suspensions and whole bacteria gave similar end points. Estimations of the number of erythrocytes in 3% (v/v) suspension were made using a Coulter counter model ZF (Coulter Electronics, Dunstable) with aperture of 16. Freshly prepared 3% (v/v) suspensions contained 4.1×10^8 RBC/ml.

200 µl aliquots of erythrocyte suspension were added to doubling dilutions in saline (200 µl) of standardised *E. coli* in WHO agglutination trays. Following the addition of erythrocytes, the plates were agitated for 5 min. Strong agglutination was indicated by visible clumping of the red cells within this mixing period. Smaller quantities of reagents were required in the microtiter system (Cooke Engineering Ltd.) and when this became available it was employed in preference to the former system.

Aliquots of isolated fimbriae were also assayed for haemagglutinating activity. A 1% (v/v) suspension of erythrocytes

provided a more sensitive indicator system and was used routinely in fimbrial HA assays. Replacement of fresh 1% or 3% (v/v) suspensions of erythrocytes by equivalent suspensions of formaldehyde-fixed horse erythrocytes (section II.1.c.) yielded similar HA patterns with whole bacterial cells or isolated fimbriae.

b. Haemagglutination inhibition tests (HAI)

A variety of sugars were examined for their haemagglutination inhibition activity between E. coli 8623 and a 3% (v/v) suspension of fresh horse erythrocytes. Solutions of sugars (10% w/v) dissolved in 0.85% (w/v) saline were prepared for testing in the microtiter system. Serial two-fold dilutions of the test sugar were made in 50 µl aliquots of 3% (v/v) horse erythrocytes. Aliquots (50 µl) of standard formolised bacterial suspensions were added to each of the wells in the tray. Duplicate titrations were prepared throughout a series of 24 wells. The trays were gently agitated for 5 min before being incubated at room temperature prior to reading. These HAI tests were also performed using a fixed aliquot of fimbriae and 1% (v/v) suspension of horse erythrocytes. The minimum inhibitory concentration of the potent inhibitors of both systems was recorded.

c. Fixation of erythrocytes by formaldehyde

Defibrinated horse red blood cells (less than two weeks old) were fixed in formaldehyde by the following procedure (Herbert, 1973). A 50 ml volume of horse erythrocytes (Oxoid) was washed five times with 0.85% (w/v) saline. The packed cell pellet (25 ml) was resuspended in 8 volumes of phosphate buffered saline (pH 7.2). A dialysis sac, 2/3 full

containing 1 volume of commercial formalin was immersed in the erythrocyte-buffer mixture, which was agitated gently for 3 h at room temperature. The remaining formalin was released by piercing the dialysis sac which was then removed from the mixture. The suspension was stirred overnight and the fixed erythrocytes filtered through muslin. Five or six saline washes were required to remove residual formalin.

A 25% (v/v) stock suspension of fixed red blood cells was prepared using physiological saline. With the addition of 10^{-5} M thiomersal as preservative, the formaldehyde-fixed,horse red blood cells (FFHRBC) were refrigerated until required.

2. Mannose binding via equilibrium dialysis

Lengths (200 cm) of 8/32" visking tubing (Scientific Instrument Centre) were heated at 80°C for 4 h in several 5-litre changes of distilled water. 70 mm lengths of tubing were rinsed carefully in distilled water before being tied at one end. Individual sacs were filled with 300 µl of one of the following:

Fimbriae, fresh horse erythrocytes, formaldehyde-fixed horse red blood cells or saline.

The sealed sacs were transferred to 100 ml conical flasks containing radioactive D-mannose-1-^{14*}C of specific activity 31.8 mCi/mmol (0.2 µCi/100 ml) in saline. To prevent growth of extraneous organisms during the experiment, 10^{-5} M thiomersal was added to the radioactive mannose. Twelve sacs,three of each type, were placed in each conical flask. A

control flask contained only 8 sacs of saline. To prevent leakage of the radioactive solution during mixing, the flasks were sealed using silicone bungs. The contents were stirred gently for 5 days at $4^{o}C$. At daily intervals a sac of saline was removed from the control flask along with a sample of the "external" buffer. The samples were measured for ^{14}C activity to establish whether equilibrium was attained. By day 5, triplicate samples from all sacs and flasks were counted in a Nuclear Enterprises NE 8312 scintillation counter. NE 260 scintillant (10 ml) was added to each sample before counting. To ascertain the degree of binding, varying concentrations of fimbrial protein were tested against different mannose concentrations.

3. Preparation of liposomes

Liposomes were prepared by mixing 17.5 µmoles of lecithin, 5.0 µmoles of dicetyl phosphate (DCP) and 2.5 µmoles of cholesterol in 5 ml of chloroform in a round bottomed flask and drying the lipids to a thin shell in vacuo using a rotary evaporator (Rotavapor R, Buchi). After complete removal of solvent, the lipids were resuspended by shaking in 3 ml of phosphate buffered saline (PBS) and allowed to swell for 1 hour at room temperature. Before use liposomes were sonicated for 30 s in an ultrasonic cleaning bath (Millipore Corporation, London) to promote dispersion. Agglutination reactions of isolated fimbriae were performed in the microtiter system using liposomes as substrates. The addition of 25 µl of 10 mM $CaCl_2$ to the liposome preparation provided a control pattern of agglutination.

4. Preparation of inside-out vesicles

Inside-out (IO) membrane vesicles were prepared from horse erythrocytes by the method of Steck (1974). After centrifugation of whole blood (600 x g for 5 min), the buffy coat layer was removed by aspiration and the erythrocytes washed three times in phosphate buffered saline (pH 8.0) each wash in 5 volumes of buffer. Aliquots (1 ml) of the final pellet of packed erythrocytes were lysed in 40 volumes of ice-cold, 5 mM sodium phosphate buffer (pH 8.0), in 50 ml centrifuge tubes. Repeated inversion of the capped tubes resulted in lysis of the erythrocytes. The stroma were sedimented by centrifugation at 20,000 x g for 15 min. Following aspiration of the supernatant, the tube was rotated gently to dislodge the membrane pellet and facilitate aspiration of the button of whole cells lying below. This process was repeated several times until a white pellet of ghost membranes was obtained. The clean membranes, resuspended in 40 ml of ice-cold 0.5 mM sodium phosphate buffer (pH 8.0), were kept on ice for 30 min and then collected by centrifugation at 20,000 x g for 30 min. The pellets were resuspended by vortex mixing in 1 ml of the same buffer. Subsequent incubation at 37^{o}C for 15 min served to "age" these erythrocyte membranes and aliquots (1 ml) of these were forced five times through a tuberculin syringe with a No. 26 gauge needle attached. This preparation contained predominantly inside-out vesicles.

a. Purification of IO vesicles

To agglutinate the contaminating unsealed ghosts and right-side out (RO) vesicles, 50 µg of Concanavalin A was added to 1 ml aliquots

of the vesicle preparation. The turbid suspension was agitated gently for 5 min prior to removal of agglutinated membranes by centrifugation at 600 x g for 10 min. The supernatant, carefully aspirated, was mixed with a second aliquot, 50 µg of Con A and the centrifugation step repeated. The supernatant was mixed with 500 µl of 0.002% (w/v) α-methyl mannoside to release any residual, bound Con A. The IO vesicles were washed in two, 10-fold volumes of 0.5 mM phosphate buffer (pH 8.0) collected by centrifugation at 20,000 x g for 30 min and resuspended in 1 ml of the same buffer. Haemagglutination tests were carried out to determine the agglutinability of these IO vesicles with isolated fimbriae.

5. Isolation of glycoproteins from erythrocytes

a. Preparation of erythrocyte ghosts

Fresh horse blood (or citrated horse blood) was centrifuged for 5 min at 600 x g and the plasma and buffy coat layer removed by aspiration. The packed erythrocytes were suspended in saline and washed by centrifugation and aspiration. This step was repeated a second time before the packed red blood cells were lysed by slow dropwise addition to 200 volumes of 10 mM tris-HCl buffer pH 8.0 made 0.1 mM with ethylene diamine tetraacetic acid (EDTA). During this step the mixture was kept stirring slowly and the temperature maintained at 4^{o}C. Ghost membranes were sedimented from the lysate (25,000 x g for 30 min) and were washed six times by resuspension in 20 volumes of buffer (described above) followed by centrifugation. Membrane protein was estimated by the method of Lowry et al. (1951) except that sodium dodecyl sulphate (SDS) was added to all samples (2% w/v final concentration) prior to estimation.

b. Glycoprotein isolation

i. Method of Hamaguchi & Cléve (1972)

Isolation of the glycoproteins from horse erythrocytes was carried out using a modification of the method used by Hamaguchi & Cléve (1972) for human erythrocytes.

To one volume of ghost suspension (2 mg ghost protein per ml suspension) nine volumes of chloroform:methanol (3:1) were added and the mixture stirred vigorously at room temperature for 30 min. Centrifugation at 1,700 x g for 10 min at 4°C yielded a clear supernatant containing the glycoprotein which was carefully decanted and evaporated to dryness under reduced pressure in a rotary evaporator (Rotavapor R, Buchi). Addition of distilled water yielded a clear solution indicating lack of contamination with interphase materials.

ii. Method of Marchesi & Andrews (1971)

Glycoprotein extraction: Fresh ghost membranes were resuspended to a final concentration of 25 mg membrane protein/ml in 0.05 M tris-HCl buffer, pH 7.5 made 0.03 M with lithium 3,5, diiodosalicylate (LIS) (Eastman Chemical Co.). The suspension was stirred at room temperature for 15 min after which time two volumes of ice-cold distilled water were added and stirring continued for a further 10 min at 4°C. The turbid suspension was then centrifuged at 45,000 x g for 1.5 h at 4°C and the sediment discarded. The supernatant was mixed with an equal volume of ice-cold 50% (w/v) aqueous phenol for 15 min at 4°C. Centrifugation (4,000 x g for 1 h) of the two-phase system gave a separated aqueous phase

which was carefully removed and dialysed against distilled water at 4°C for 24 h before freeze-drying.

Glycoprotein purification: The dried powder, resuspended in 100% ice-cold ethanol was mixed for 1-2 h at 4°C and centrifuged to collect the precipitate. This step was repeated three times. After overnight dialysis of the pooled precipitates against distilled water at 4°C the sac contents were centrifuged at 10,000 x g for 30 min in the cold and the supernatants contained the soluble glycoprotein.

6. Modification of the erythrocyte surface via chemical and enzyme treatments

a. Neuraminidase

Horse erythrocytes were treated with neuraminidase from Vibrio cholerae (Koch-Light Labs) or from Clostridium perfringens (Boehringer). Optimal enzymic activity of neuraminidase occurs between pH 5.0 - 5.5, a pH at which horse erythrocytes undergo considerable lysis. This can be prevented by a very brief exposure to low concentrations of protein cross-linking agents such as glutaraldehyde. Such fixation does not affect the haemagglutination of these cells by fimbriae or lectins. Washed erythrocytes were prepared from horse blood as described earlier. The red cells were fixed in 1.5% (v/v) solution of glutaraldehyde in PBS for 10 min followed by four washings each with 10 volumes of PBS (pH 7.3). Centrifugation at 500 x g for 10 min was used to collect the cells between washings. 1 ml aliquots of packed erythrocytes in a total volume of 5 ml were treated with 100 units of V. cholerae enzyme in 0.05 M

sodium acetate buffered 0.85% (w/v) saline (pH 5.5) containing 0.001 M $CaCl_2$ or 6 milliunits of Cl. perfringens enzyme in 0.1 M acetate buffer (pH 5.0) in total volume of 5 ml, at 37°C for 1 h. Treated erythrocytes were sedimented at 500 x g for 5 min and washed in three 10-fold volumes of PBS (pH 7.3). A 1% (v/v) suspension of each of the enzyme-treated samples of HRBCs was prepared for titration against a standard aliquot of isolated fimbriae (64 $HU_f^{1\%}$) and each of the lectins Con A, WGA, RCA_{120} and FBP.

b. Periodate oxidation

1 ml aliquots of packed formaldehyde-fixed erythrocytes were incubated in the dark with 0.05, 0.10 and 0.15 M sodium periodate in 5 ml of PBS buffer (pH 7.3) at 25°C for 1 h. The treated red cells were washed in three 5-fold volumes of PBS before being made up to a 1% (v/v) suspension which was used in titrations against a standard aliquot of fimbriae in the microtiter system.

c. Proteases

i. Enzyme treatment

Fresh horse erythrocytes were treated with one of five proteases, i.e. papain (Sigma), trypsin (Koch-Light), chymotrypsin A (Sigma), subtilisin (Koch-Light) or pronase (BDH). Because the optimum activity of two of these enzymes lay above pH 7.4, i.e. trypsin (pH 8.1) and chymotrypsin A (pH 7.8), these proteases were prepared in 0.041 M tris-buffered saline containing 0.015 M $CaCl_2$ (pH 8.1) and 0.08 M tris-HCl containing 0.1 M $CaCl_2$ (pH 7.8) respectively. Pronase and

subtilisin were prepared in PBS (pH 7.4) while PBS buffer containing 0.006 M mercaptoethanol, 0.005 M cysteine and 0.001 M EDTA (pH 6.5) was required for optimal activity of the papain. In order to prevent lysis of the erythrocytes at alkaline pH, each enzyme buffer was prepared in 0.85% (v/v) saline. The HRBCs were mixed with the enzymes in the ratio of 0.25 mg:1 ml of washed, packed erythrocytes in a total volume of 5 ml and the reaction mixtures incubated at 37°C for 1 h. Following protease treatment, the red cells were recovered by centrifugation at 600 x g for 5 min. Four 10-fold volumes of PBS at pH 7.4 were used to wash the treated erythrocytes, and 1% (v/v) suspensions of these were titrated with a standard aliquot of isolated fimbriae.

ii. Electrophoresis

Supernatant fractions from protease-treated HRBCs were dialysed, concentrated and subjected to SDS polyacrylamide gel electrophoresis using conditions described by Parton (1975).

d. Glycosidases

Fixed horse erythrocytes were treated with a range of glycosidases. Because the optimum activity of most of these enzymes lay between pH 4.0 - 5.5, fixation of the RBCs proved necessary to prevent cell lysis. Washed erythrocytes were fixed in 1.5% (v/v) glutaraldehyde in PBS (pH 7.3) for 10 min including centrifugation time. A minimum of four washes with 10-fold volumes of PBS buffer was required to remove the fixative. Aliquots (1 ml) of packed

erythrocytes were incubated with the particular glycosidase for 1 h at 37°C (in total volume of 5 ml). The number of units of enzyme used in each experiment is shown in Table 5, column i. Following enzyme treatment and extensive washing, the modified erythrocytes were sedimented by centrifugation at 600 x g for 5 min, adjusted to a 1% (v/v) suspension in 0.85% (w/v) saline and titrated against a standard aliquot of fimbriae.

The glycosidases employed in these investigations included:

α-L-fucosidase (E.C.3.2.1.51) in 0.2 M acetate buffer, pH 4.5

α-galactosidase (E.C.3.2.1.22) in 0.1 M phosphate buffer, pH 6.5

α-glucosidase (E.C.3.2.1.20) in 0.1 M sodium acetate buffer containing 1.35 mM EDTA, pH 6.0

α-mannosidase (E.C.3.2.1.24) in 0.1 M sodium citrate buffer, pH 4.5

α-D-N-acetyl galactosaminidase (E.C.3.2.1.49) in 0.1 M sodium citrate, pH 4.5

β-N-acetyl glucosaminidase (E.C.3.2.1.30) in 0.1 M sodium citrate buffer, pH 5.0

β-galactosidase (E.C.3.2.1.23) in 0.1 M potassium phosphate buffer, pH 6.5

β-N-acetyl hexosaminidase (E.C.3.2.1.52) in 0.1 M sodium citrate, pH 4.0.

Surface modifications caused by enzymic treatments were detected by changes in the lectin induced agglutination patterns. Four lectins were used throughout these investigations:

1. Concanavalin A (Con A)
2. Fucose-binding protein (FBP)

Table 5 Range of enzyme units used in the glycosidase treatments

Glycosidase	Specific activity (units/mg)	Units of enzymes used in expts. (section II.6.d) i	ii	iii	iv
. α-L-fucosidase	2.0	0.4	0.4	0.8	0.4
. α-galactosidase	10.0	1.0	1.0	4.0	4.0
. α-glucosidase	50.0	1.0	2.0	4.0	10.0
. α-mannosidase	10.0	1.0	1.0	4.0	7.5
* α-D-N-Acetyl galactosaminidase	0.4	0.02	0.02	0.04	0.02
. β-N-Acetyl glucosaminidase	4.0	1.0	4.0	2.0	2.0
. β-galactosidase	30.0	-	-	-	3.0
* β-N-acetyl hexosaminidase	10.0	-	-	-	0.1

. purchased from Boehringer

* purchased from Miles-Yeda Ltd.

3. Ricinus communis agglutinin$_{120}$ (RCA$_{120}$)

4. Wheat Germ Agglutinin (WGA).

Con A was purchased from Sigma, the other lectins were purchased from Miles-Yeda Ltd. Erythrocytes, before and after enzyme treatment, were titrated against 100 µg/ml solutions of lectins in 0.85% (w/v) saline.

e. Combinations of enzyme treatments

i. Papain/glycosidases

1 ml aliquots of thrice washed, packed horse erythrocytes in 5 ml volume of papain buffer (see page 70) were treated with 400 µg of crude papain at 37°C for 1 h followed by three washes each of 10 volumes of PBS (pH 7.3). The cells, harvested at 600 x g for 5 min, were fixed in 1.5% (v/v) glutaraldehyde for 10 min including centrifugation time. The effect of each of the glycosidases on papain-treated fixed HRBCs was assessed after incubating the cells with the particular glycosidase for 1 h at 37°C (table 5) in a total reaction volume of 5 ml. The pattern of HA induced by fimbriae and lectins in a 1% (v/v) suspension of treated cells was recorded.

ii. Neuraminidase/glycosidases

Aliquots of HRBCs were pretreated with Cl. perfringens neuraminidase at a concentration of 10 µg of enzyme/ml of packed erythrocytes suspended in 5 ml of 0.1 M acetate buffer (pH 5.0) for 1 h at 37°C. The red cells were washed four times, each in 10 volumes of PBS (pH 7.3) and collected between washings by centrifugation at 600 x g

for 5 min. The cells were fixed in 1.5% (v/v) glutaraldehyde solution for 10 min, washed four times, each with 10 volumes of PBS (pH 7.3) and subsequently resuspended to 5.0 ml in the appropriate buffer (see page 71) and treated with the individual glycosidases at 37°C for 1 h using the concentration stated in Table 5, column (iii). Following four washes, each in 10 volumes of PBS, a 1% (v/v) cell suspension was prepared and titrated against fimbriae or lectins.

iii. Papain/neuraminidase/glycosidases

1 ml aliquots of packed whole erythrocytes were treated with crude papain (400 µg enzyme) in 5 ml volume of papain buffer (see page 70) for 1 h at 37°C followed by 4 washes, each in 10 volumes of PBS (pH 7.3). Cells were sedimented at 600 x g for 5 min and fixed in 1.5% (v/v) glutaraldehyde for 10 min including centrifugation time, followed by 4 washes, each in 10 volumes of PBS (pH 7.3). The erythrocytes were subsequently treated for 1 h at 37°C with Cl. perfringens neuraminidase (10 µg) in total volume of 5 ml of appropriate buffer (see page 69). Following four further washes, each of 10 volumes of PBS (pH 7.3) the erythrocytes were treated with glycosidases for 1 h at 37°C again in total volume of 5 ml of appropriate buffer (see page 71) using the enzyme units in table 5, column (iv). The cells were subsequently washed 4 times, adjusted to a 1% (v/v) suspension and titrated against fimbriae or lectins.

III. INVESTIGATIONS USING ^{125}I-LABELLED FIMBRIAE

1. Iodine labelling of isolated fimbriae

The labelling of isolated fimbriae was done using the method of Gow, Parton & Wardlaw (1976) a modification of the lactoperoxidase technique of Hubbard & Cohn (1972). The reaction mixture (total volume of 1 ml) listed in order of addition was:-

10 milliunits of lactoperoxidase (Sigma Chemical Co.)

10 milliunits of glucose oxidase (Sigma Chemical Co.)

10 µCi of carrier-free Na(^{125}I) (Amersham)

5 µM glucose (Sigma Chemical Co.)

1 - 2 mg of fimbrial protein.

Following incubation of the complete reaction mixture for 1 hour at 37^{o}C the preparation was dialysed against three, 5-litre volumes of distilled water containing 10^{-5} M sodium thiosulphate, over a period of 24 h. After dialysis was completed, the labelled fimbriae were transferred to a stoppered test tube and stored at 4^{o}C. The biological activity of the fimbrial preparation in terms of haemagglutinating units, was estimated by titration against 1% or 3% (v/v) formaldehyde-fixed HRBCs. The radioactivity of 100 µl of fimbriae was measured in a Nuclear Enterprises 8312 automatic, B-γ counter.

2. Saturation of horse erythrocytes with fimbriae

Increasing aliquots of ^{125}I-labelled fimbriae, i.e. ranging between 0-500 µg protein, were transferred to a series of fine capillary tubes each containing 11 x 10^{4} (100 µl) FFHRBC. The tubes were heat-sealed and the contents mixed by repeated inversion of the tubes before overnight incubation at room temperature. Centrifugation at 750 x g for 5 min in a swing-out rotor, sedimented the red blood cells. Using a fine

diamond-tipped marker, the capillaries were sheared at the RBC-supernatant junction and both samples transferred separately to glass scintillation vials before estimating the radioactivity.

This technique facilitated clean separation of the supernatant from the pellet because by sealing the capillaries by heat a vacuum was created inside each capillary. Subsequent shearing caused the liquid to be drawn away from the RBCs and the sheared end thus enabling transfer of each half of the capillary to individual vials without loss of contents. Measurement of the radioactivity in each of the pellets and supernatants was made on a Nuclear Enterprises β-γ-counter.

3. Estimation of the degree of binding of ^{125}I-labelled fimbriae to erythrocytes of various animal species

Increasing aliquots of ^{125}I-fimbrial protein were added to 100 µl amounts of formaldehyde-fixed erythrocytes from five animal species, i.e. rabbit, horse, dog, guinea-pig and sheep. An estimation of the number of erythrocytes present in a 1% (v/v) suspension from each species was made using a Coulter counter model ZF (Coulter Electronics, Dunstable). The erythrocytes and the fimbrial protein solution (total volume of 400 µl) were placed in small capillaries (prepared from pasteur pipette tips) and heat-sealed. These tubes, each of which was inverted five times, were incubated overnight at room temperature and centrifuged at 750 x g for 5 min to sediment the blood cells. Using a glass cutter, the capillaries were scored and broken at the junction between the erythrocyte pellet and the supernatant, each portion being transferred to a separate scintillation vial before estimating the radioactivity.

IV. STATISTICAL ANALYSIS ON THE LENGTH DISTRIBUTION OF FIMBRIAE ISOLATED BY MECHANICAL REMOVAL

1. Before sonication

Purified fimbriae were prepared by the method described in I.2, negatively stained with potassium phosphotungstate (pH 6.9) and examined by electron microscopy. Because some of these filaments exhibited a slightly curved appearance on the grids, a cartographer's wheel was employed to make accurate measurements of the length of individual fimbriae on photographically enlarged electron micrographs. Only appendages visible as entire filaments, i.e. both ends visible, were measured. From the length measurements of 1,000 fimbriae a histogram was prepared showing the length distribution of fimbriae isolated from *E. coli* 8623.

2. After sonication

Freshly isolated fimbriae were sonicated intermittently at 30 s intervals for a total period of 5 min at an amplitude of 7 mA using an exponential probe fitted to an MSE ultrasonic disintegrator. The sample was diluted and negatively stained with potassium phosphotungstate (PTA) and examined by electron microscopy. Length measurements were made on photographically enlarged electron micrographs. A histogram was prepared from this data illustrating the length distribution of the sonicated fimbriae.

V. AGGLUTINATION OF NON-ERYTHROCYTE SUBSTRATES INDUCED BY ISOLATED FIMBRIAE OR WHOLE, FIMBRIATE BACTERIA

1. Yeast - Saccharomyces cerevisiae

Whole E. coli 8623 and isolated fimbriae were tested for their ability to agglutinate whole boiled Saccharomyces cerevisiae NCYC 366, whole glutaraldehyde-fixed Sac. cerevisiae and isolated cell walls of this strain of yeast. The yeast was grown at 30°C in malt extract broth (Oxoid), dispensed in 500 ml aliquots in 2-litre shake flasks. Overnight cultures were harvested by centrifugation at 600 x g for 10 min and washed at least 5 times in 50-fold volumes of 0.85% (v/v) saline. The washed yeast suspension was dispensed into 3 aliquots and each of these treated separately.

The first aliquot was boiled for 10 min, cooled to room temperature and washed 6 times in 0.85% (w/v) saline. The cells were resuspended in a small volume of 0.85% (w/v) saline to which 10^{-5} M thiomersal was added prior to storage at 4°C.

The second aliquot of yeast was fixed in 3% (w/v) glutaraldehyde for 40 min at room temperature and washed extensively in 0.85% (w/v) saline. The fixed, whole yeasts were resuspended in 0.85% (w/v) saline containing 10^{-5} M thiomersal.

Isolated yeast cell walls were prepared from the third aliquot of yeast. A thick suspension of whole cells was mixed with glass beads and ruptured in a Braun cell homogeniser (Shandon Southern Instruments Ltd.) cooled with liquid CO_2. Maximum breakage occurred within 90-120 s. After breakage, the cell walls were harvested by centrifugation (515 x g for 20 min) and washed eight times with saline before

being shell frozen and stored at -4°C. Prior to use cell walls were resuspended in 0.85% (w/v) saline.

An attempt was made to isolate fimbrial appendages from suspension using as adsorbent, whole glutaraldehyde-fixed Sac. cerevisiae. 1 ml of fimbrial suspension (765 µg protein/ml) with an HA titre of 512/50 µl (against 1% v/v HRBCs) and a yeast suspension (2 ml) were mixed for 5 min at room temperature and incubated at 37°C for 30 min. Adsorption of the filaments to the yeast cells was detected by a reduction in HA titre of the supernatant following sedimentation of the fixed yeast at 600 x g for 10 min. Several aliquots of yeast suspension were added until a total of 21 x 10^9 cells were present in the suspension. At this stage the volume of mixture had increased to 8 ml and the HA titre was almost negligible, i.e. 2/50 µl. The Sac. cerevisiae sedimented from the solution as before, were washed twice in 10 ml aliquots of saline to dilute out, unbound fimbriae. Further centrifugation at 600 x g for 10 min served to pellet these eukaryotes. To elute the bound fimbriae, 2.0 ml of α-methyl mannoside was added to the pellet giving a final concentration of 0.01 M. Subsequent low speed centrifugation of this mixture served to sediment the yeast cells and leave the released fimbriae in the supernatant. The fimbrial-saccharide suspension was diluted to a volume of 10 ml and the fimbriae pelleted at 150,000 x g for 90 min in an MSE 65 ultracentrifuge. The gelatinous pellet of fimbriae was recovered and the washing procedure repeated on two further occasions. The fimbrial pellet recovered from the final centrifugation was resuspended and adjusted to a volume of 1 ml with 0.85% (w/v) saline prior to titration against a 1% (v/v) HRBC suspension.

2. Prokaryote - Micrococcus lysodeikticus

Micrococcus lysodeikticus (ML) (NCTC 2665) was cultured at 30°C in peptone water yeast extract (PWYE) broth which consisted of 5.0% (w/v) peptone (Difco Labs, West Molesey, Surrey), 0.1% (w/v) yeast extract (Ibid.) and 0.5% (w/v) sodium chloride at pH 7.4. Overnight cultures were harvested by low speed centrifugation (600 x g for 10 min) and washed four times in 0.85% (w/v) saline. Titrations were carried out using dilute saline suspensions of bacteria as substrate for adhesion by whole Fim^+ E. coli or isolated fimbriae.

RESULTS

RESULTS

I. AGGLUTINATION REACTIONS CAUSED BY WHOLE FIMBRIATE BACTERIA AND ISOLATED FIMBRIAE

1. Isolation of fimbriae

a. The optimal conditions of fimbrial production were those described by Duguid et al. (1955), i.e. shallow broth cultures incubated statically at 37°C for 48 h. Both overnight shake-cultures and static, oxygenated cultures in 20-litre carboys yielded cells with lower haemagglutinating ability and fewer fimbriae per cell. Fimbriae were isolated from the motile cells of E. coli. Either 20- or 40-litres of a formolised broth culture were used as a source of each preparation of fimbriae. Blending of the washed bacteria at relatively low speed (9,500 rpm) sheared the flagella, which could then be separated from the intact cells by centrifugation (8,000 x g for 15 min). The sedimented cells were then resuspended and blended at higher speed (14,000 rpm) when type-1 fimbriae were sheared. These were then separated and washed by differential rate centrifugation. Quantitative recovery of fimbriae was not achieved, since the higher blending speed required for near quantitative removal of fimbriae also caused release of significant quantities of lipopolysaccharide, seen as disc-like structures in negatively stained preparations viewed by electron microscopy.

The yield of fimbriae was similar to that recorded by Brinton (1959), i.e. 1-2 mg dry weight per litre of bacterial culture. The final product was adjusted to a protein concentration of 1-2 mg/ml in

sterile distilled water, prior to storage at 4°C. A sample of isolated fimbriae (shadowed with chromium) is presented in plate 1.

2. Inhibitory activity of sugars and sugar derivatives on fimbrial-induced haemagglutination

Approximately 40 of the 56 sugars screened by Old (1972) for HAI activity were examined in the passive HA system for their ability to block the agglutination of RBC induced by whole Fim$^+$ E. coli or isolated appendages. In the passive HA system, erythrocytes are mixed with whole bacteria which possess agglutinins and these adsorb to receptors on the RBCs resulting in visible agglutination of the complex. Covalent bonding is not involved in this process.

Cell surface agglutinins with specificities different from those of type-1 fimbriae are known to occur in Gram-negative bacteria and their presence can be demonstrated indirectly, by differences in sugar inhibition of haemagglutination induced on the one hand by whole cells and on the other by isolated fimbriae. The test sugars include many of those previously examined by Old (1972) in his study of the inhibition of the haemagglutinating activity of Shigella flexneri and Salmonella typhimurium (see Table 6). Those listed in section A of the table caused inhibition of the HA system. Where possible, the minimum inhibitory concentration (MIC) of the sugar was calculated and the results are summarised in Table 7. A direct comparison between MICs of isolated fimbriae and whole bacteria is not possible because of the indeterminate number of fimbriae per cell and their attachment to whole cells. The concentrations of erythrocytes employed in the tests were 3% (v/v) with

Plate 1 : Isolated fimbriae shadowed with chromium.

Magnification X 37,000.

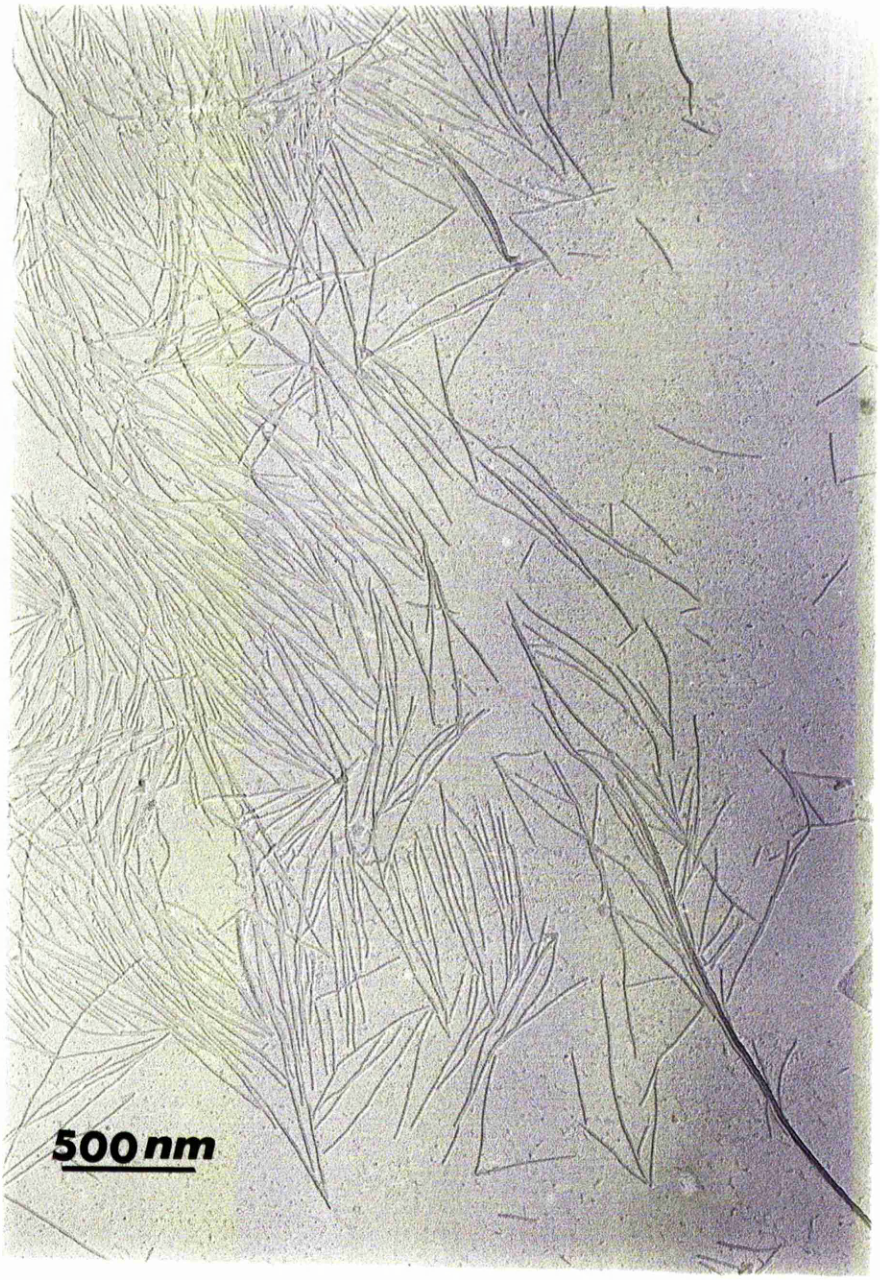

500 nm

Table 6 Saccharides assayed for inhibition of HA activity caused by whole bacteria or isolated fimbriae

A. INHIBITORY

D(+)Mannose	(A),(B)	Yeast mannan	(F)
α-Methyl-D-Mannoside	(F)	2-deoxy-D-Glucose	(D)
D-Mannoheptulose	(F)	6-deoxy-D-Mannose	(C)
α-D-Mannose-1-phosphate	(F)	D-Mannose-6-phosphate	(F)
D(-)Fructose	(A)		

B. NON-INHIBITORY

Melezitose	(C)	Ribitol (adonitol)	(F)
D-Mannosamine	(D)	Dulcitol	(B)
α-D-Mannoheptitol	(F)	Erythritol	(E)
D-Mannitol	(A)	D-Sorbitol	(F)
D-erythritol	(A)	Lactose	(A)
D-arabinose	(A)	Maltose	(D)
L(+)arabinose	(A)	Glycogen	(A)
D(-)Ribose	(A)	Inulin	(D)
2-deoxy-D-ribose	(D)	α-D-Melibiose	(D)
D(+)Xylose	(A)	D(+)Raffinose	(F)
D(+)Galactose	(A)	Sucrose	(A)
D-Glucose	(A)	Trehalose	(A)
2-deoxy-D-galactose	(F)	DL-Glyceraldehyde	(F)
Methyl α-D-Glucoside	(A)	Glycerol	(A)
α-D-Talose	(D)	Meso-Inositol	(A)
L-Fucose	(A)	Amygdalin	(F)
α-L-Rhamnose	(D)	Salicin	(D)
L(-)Sorbose	(D)		

*Source : A BDH
B Gurr
C Hopkins & Williams
D Koch-Light
E Merck
F Sigma

Table 7 Minimum inhibitory concentration (MIC) of saccharides in haemagglutination induced by whole cells or isolated fimbriae of E. coli

	Minimum inhibitory concentration (MIC*)	
ccharides	(a) whole bacteria	(b) isolated fimbriae
CH_3-mannoside	8.0×10^{-6}	0.5×10^{-6}
+)mannose	135.0×10^{-6}	2.0×10^{-6}
mannoheptulose	232×10^{-6}	145×10^{-6}
deoxy-D-glucose	38×10^{-3}	4.0×10^{-3}
D-mannose-1-P	48.0×10^{-3}	NT
D-mannose-6-P	48.0×10^{-3}	NT
ast mannan	7.8 µg/ml	0.06 µg/ml
ıctose	no inhibition	135×10^{-6}

*Molar, unless otherwise stated

NT - not tested

whole cells and 1% (v/v) with isolated fimbriae. The lower concentration of erythrocytes increased the sensitivity of the haemagglutination assay and was subsequently employed in routine experiments with isolated fimbriae.

One haemagglutinating unit of fimbriae ($HU_f^{1\%}$) is defined as the minimum concentration of fimbrial protein which caused agglutination of 50 µl of 0.5% (v/v) suspension of fresh horse erythrocytes. In contrast, one haemagglutinating unit of whole bacteria ($HU_b^{3\%}$) is the minimum number of bacteria from a 48 h, static, nutrient broth culture which produced agglutination of 50 µl of a 1.5% (v/v) horse erythrocyte suspension, i.e. approximately 156×10^2 organisms. From these definitions, it is apparent that direct comparison between $HU_b^{3\%}$ and $HU_f^{1\%}$ is not meaningful.

The results presented in Table 7 show that α-methyl-mannoside (α-M.M.) is a more effective inhibitor than α-D-mannose in both agglutination systems. These results are in agreement with those published by Old (1972) and by Rivier & Darekar (1975). These latter authors examined eleven sugars for their ability to inhibit haemagglutination of a 3% (v/v) suspension of guinea-pig erythrocytes induced by an enteropathogenic E. coli of serotype O125 - the same serotype as E. coli 8623. Two results taken from their publication are presented in Table 8 and show that α-M.M. is a more effective inhibitor of the type-1 HA system than is α-D-mannose. In the study reported here an increase in sensitivity of the assay system for HA was achieved over that reported by Old (1972), as is apparent from comparison of the MICs reported for

Table 8 : MICs of potent inhibitors of agglutination of guinea-pig erythrocytes induced by whole cells or isolated fimbriae of E. coli O125

(*after Rivier & Darekar, 1975)

	MIC of carbohydrate inhibiting HA caused by	
	whole bacteria	fimbriae
D-mannose (mg/ml*)	0.012	0.003
µmolar	67	16
α-CH_3-mannoside (mg/ml*)	0.006	0.003
µmolar	31	15

various sugars. This increased sensitivity was achieved by using isolated fimbriae, a 1% (v/v) horse erythrocyte suspension and carrying out the assays using the "microtiter system" manufactured by Cooke Engineering Ltd.

The polyol derivative 1,5,anhydromannitol was reported by Old (1972) to be the most potent inhibitor tested against haemagglutination induced by whole fimbriate cells of Shigella species but was not tested in this present study.

Fructose was reported by Old (1972) to be moderately inhibitory in the HA reaction caused by Salmonella and Shigella and in this present study it was inactive against whole cell induced HA. It was, however, shown to display inhibitory activity against HA induced by isolated fimbriae.

Although Old (1972) reported melizitose to be a weak inhibitor, it proved more effective in inhibiting the activity of the S. typhimurium haemagglutinin than that of the Sh. flexneri. This trisaccharide failed to inhibit HA induced either by whole E. coli 8623 or fimbriae isolated from this strain. The reason for these differences is not immediately apparent since all the organisms possess type-1 fimbriae. However the possibility of involvement of agglutinins other than type-1 fimbriae in the case of Salmonella and Shigella haemagglutination cannot be excluded.

Although the haemagglutination induced on the one hand by whole bacteria and on the other by isolated fimbriae cannot be directly equated with each other, there does appear to be a close similarity in

the order of potency of inhibition by the various saccharides tested in both systems. The order of potency of the saccharides shown in Fig 6 is similar for both fimbriae-induced haemagglutination and whole cell-induced haemagglutination.

3. Effect of isolated fimbriae and fimbriate bacteria on mannan containing substrates

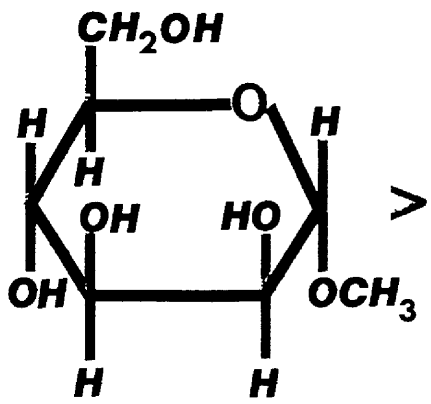
a. Agglutination reaction

Haemagglutination induced by isolated fimbriae (vide supra) or whole fimbriate bacteria (Old, 1972) shows certain similarities with that induced by the lectin concanavalin A, for example, its mannose sensitivity and reversal by α-methyl mannoside.

The possibility of using poly mannose-containing, particulate substrates as a selective adsorbent for harvesting fimbriae from dilute suspensions was investigated. Easily sedimentable particulate substrates used were whole cells of Micrococcus lysodeikticus, whole cells of Saccharomyces cerevisiae or their isolated cell walls. Both of these cell types contain mannan polymers in their surface layers and are not osmotically sensitive as is the case with erythrocytes. Selective adsorption to and specific elution from such substrates could provide a simple method for purification of fimbriae.

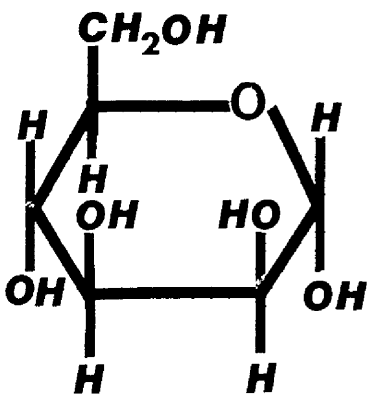
Table 9 shows the results obtained from tube tests where aliquots of substrate were mixed with either equal volumes of whole bacteria or fimbriae and shows that no agglutination occurred with whole cells of M. lysodeikticus.

Figure 6 : Steriochemical configurations of the potent inhibitory saccharides of the HA system



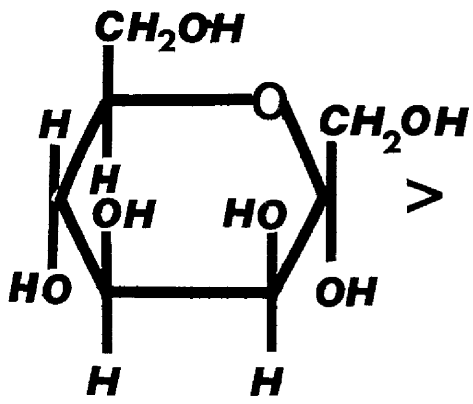
α-CH_3-MANNOSIDE.

>



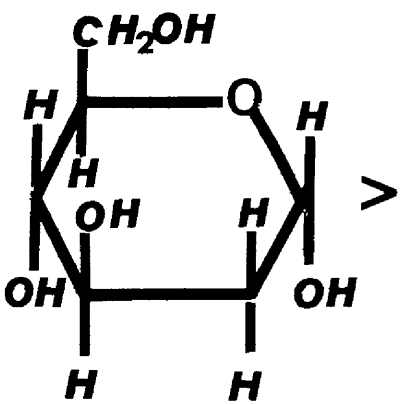
α-D-MANNOSE.

>



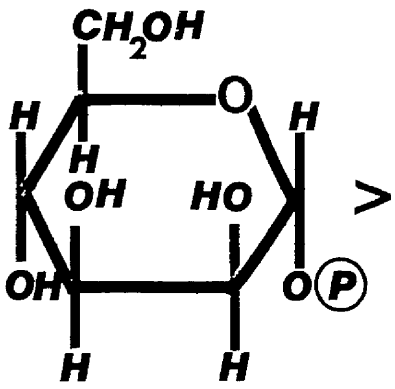
D-MANNOHEPTULOSE.

>



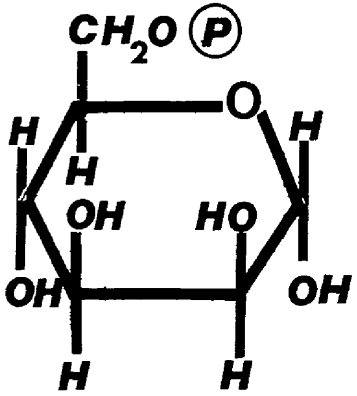
2-DEOXYGLUCOSE.

>



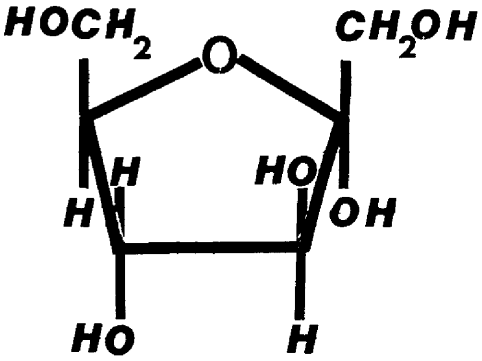
α-MANNOSE-1-Ⓟ .

>



α-MANNOSE-6-Ⓟ.

>



D-FRUCTOSE.

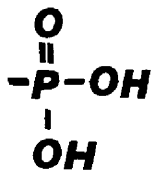
(Ⓟ = )

Table 9 : Agglutinability of non-erythrocyte substrates induced by whole cells and isolated fimbriae of E. coli 8623

Substrate	Haemagglutination caused by: whole bacteria	fimbriae
3% (v/v) horse erythrocytes	+++	+++
Whole Sac. cerevisiae - boiled for 10 min	+++	+++
Whole Sac. cerevisiae - glutaraldehyde-fixed	+++	+++
Isolated Sac. cerevisiae cell walls	++	++
Whole cells of Micrococcus lysodeikticus	-	-

With whole yeast cells, agglutination was detectable within a few seconds with both whole cells or isolated fimbriae. Also whole yeast cells, killed by either boiling for 10 min or treatment with 3% (v/v) glutaraldehyde for 40 min were agglutinated by both preparations. Even though agglutination of yeast cell walls was difficult to detect in the microtiter system, the tube tests established that agglutination had occurred with both whole bacteria and isolated fimbriae. A specimen of the washed fimbriae - yeast cell wall complex (shadowed with chromium) is presented in plate 2.

Adsorption of fimbriae to Sac. cerevisiae was detected by a reduction in HA titre against a 1% (v/v) HRBC suspension, i.e. 256 to 2 per 50 µl of fimbrial suspension. Even though the fimbriae were shown to be adsorbed to the yeast cells by examination in the electron microscope and released by α-methyl mannoside, almost no haemagglutinating activity was obtained with the washed concentrated eluate, i.e. 4 per 50 µl of fimbrial suspension.

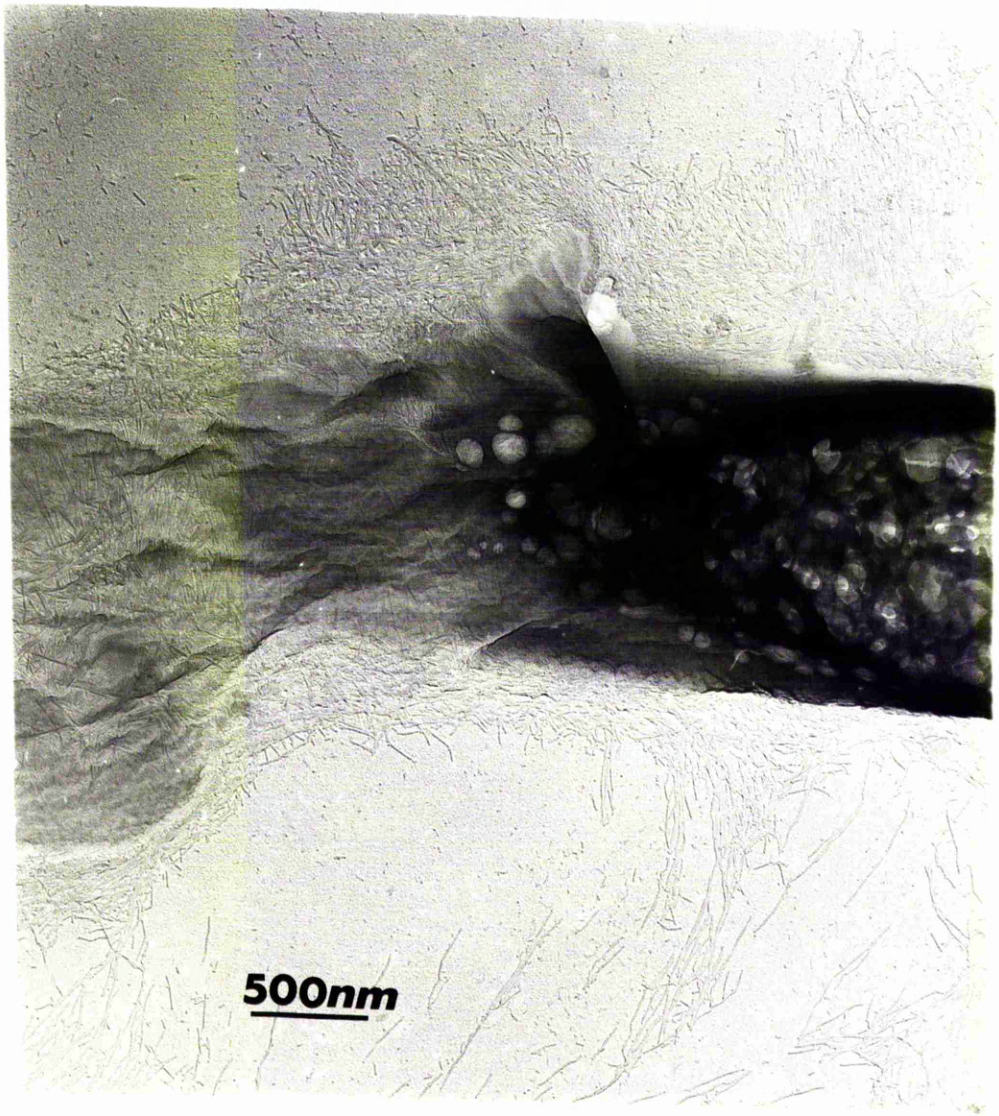
II. EXPERIMENTS WITH ISOLATED FIMBRIAE

1. Investigation on the binding of mannose via equilibrium dialysis

The reported mannose sensitivity of haemagglutination induced by whole fimbriate bacteria (Gillies & Duguid, 1957) also occurs in haemagglutination induced by isolated fimbriae. The mechanism of this observed interference by mannose and certain other sugars is not understood. A structure similar to mannose at the erythrocyte binding site has been proposed as a possible explanation (Old, 1972).

Plate 2 : Chromium shadowed preparation of fimbriae - yeast cell wall complex.

Magnification X 30,000.


500nm

In the following experiments the affinity between on the one hand ^{14}C-α-D-mannose and horse erythrocytes and on the other ^{14}C-α-D-mannose and isolated fimbriae was investigated using the technique of equilibrium dialysis. As previously described, four 100 ml conical flasks were filled with the following:

Flasks A, B and C - triplicate sacs of formaldehyde-fixed HRBCs, fresh HRBCs, fimbriae and saline

Flask D - 8 dialysis sacs of saline.

The 1 min counts of 250 µl aliquots of liquid withdrawn individually from inside and outside the sacs are presented in table 10. The ratio of the ^{14}C-label inside the sacs compared to that in the external liquid (Fig 7) reached a value of 0.99 at a sampling time of 93 h and 1.00 at 113 h. At this time 200 µl samples were withdrawn from triplicate sacs of the various samples in flasks A, B and C. Scintillation counts/min for these samples are shown in Table 11.

When subjected to analysis of variance and the 't' test, the results showed that in two out of three flasks, namely A and C, mannose binding to the fimbriae was significant at the 5% level. The anomalous result obtained in flask B, however, casts some doubt on the validity of the other results. Consequently a repeat experiment is necessary to validate the results obtained in flasks A and C. No binding was detected between mannose and the horse erythrocytes.

2. Measurement of the fimbrial length

Length measurements of isolated fimbriae taken from enlarged

Table 10 : Distribution of ^{14}C-α-D-mannose after various dialysis times

Sampline Time (h)	Radioactivity (counts/min) Saline(S)	Saline(E)	S/E
16	8285	9182	0.90
40	8769	9199	0.95
64	8785	9510	0.92
68	8927	9770	0.91
88	9297	9665	0.96
93	9628	9685	0.99
113	9504	9526	1.00

(S) = inside sac

(E) = outside sac

Figure 7 : Ratio of ^{14}C content of dialysis sac originally free of label (SAC) to the external buffer (EB) originally containing the radioactive label. Ratio at 93 h (0.99) indicates attainment of near equilibrium. The ratio at 113 h was 1.00.

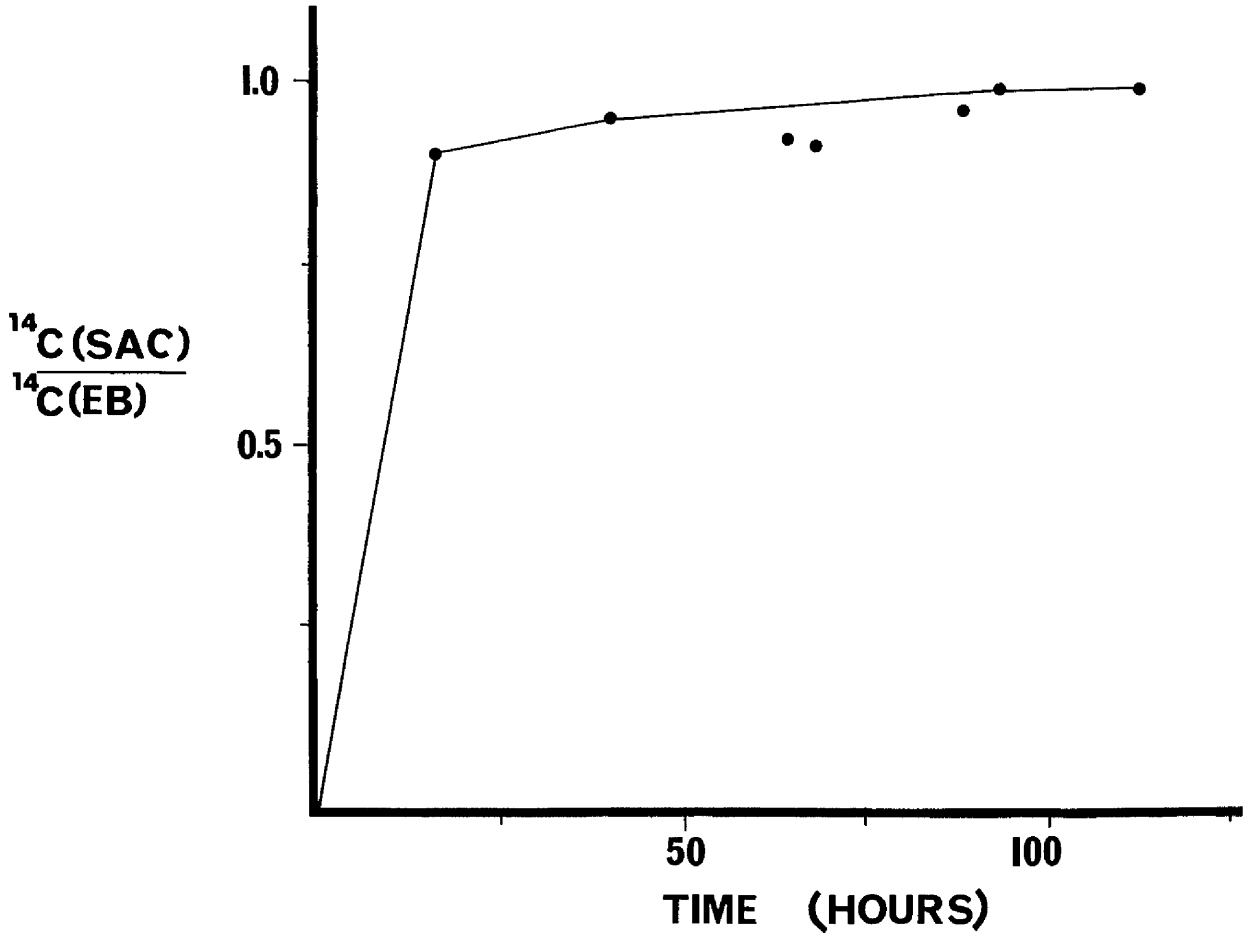

1.0
0.5
$^{14}C(SAC)$ / $^{14}C(EB)$
50
100
TIME (HOURS)

Table 11 : Distribution of ^{14}C-α-D-mannose after dialysis of fimbriae and horse erythrocytes against radioactive sugar

Flasks	Radioactivity (counts/min)				
	Fixed HRBC	Fresh HRBC	Fimbriae	Saline	External
A	7089	7148	8030	7562	8027
	7807	7049	7927	6659	7933
	7502	7165	8134	7724	7931
B	7171	7007	7500	8094	7977
	6994	7070	7405	7210	7931
	6319	6713	7490	7543	7521
C	4202	3654	4163	3937	3959
	3896	3642	4227	3895	4356
	3779	3768	3977	3638	4049

electron micrographs of negatively stained preparations (Plate 3) showed a frequency distribution as illustrated in Fig 8. The modal length of the filaments lay within the range 100-199 nm (midlength = 149.5 nm).

The molecular weight of fragments of fimbriae of this modal length can be calculated assuming a pitch distance of 23.2Å, 3.125 subunits/turn of the helix and a molecular weight for each subunit of 16,800 (data from Brinton, 1965).

Modal length = 1495Å

Pitch distance = 23.2Å

$\therefore$ Number of turns per filament = $\frac{1495}{23.2}$ = 64.44

Each turn contains 3.125 subunits

$\therefore$ Number of subunits for filament = 201.375

M.W. per subunit = 16,800

M.W. of filament = 3,383,100

= 3.4×10^6.

From this data, an estimation of the number of filaments of fimbriae in (a) 1 µg of protein and (b) one haemagglutinating unit of fimbriae (1 $HU_f^{1\%}$) was made (Fig 9).

3. Investigation of the agglutinating activity of sonicated fimbriae

Various batches of isolated fimbriae adjusted to the same protein concentration differed in their ability to agglutinate a 1% (v/v) suspension of HRBCs. This suggested that the length of individual fimbriae present in the sample was important in determining the ability

Plate 3 : Negatively stained (PTA) preparation of unsonicated fimbriae.
Magnification X 150,000.

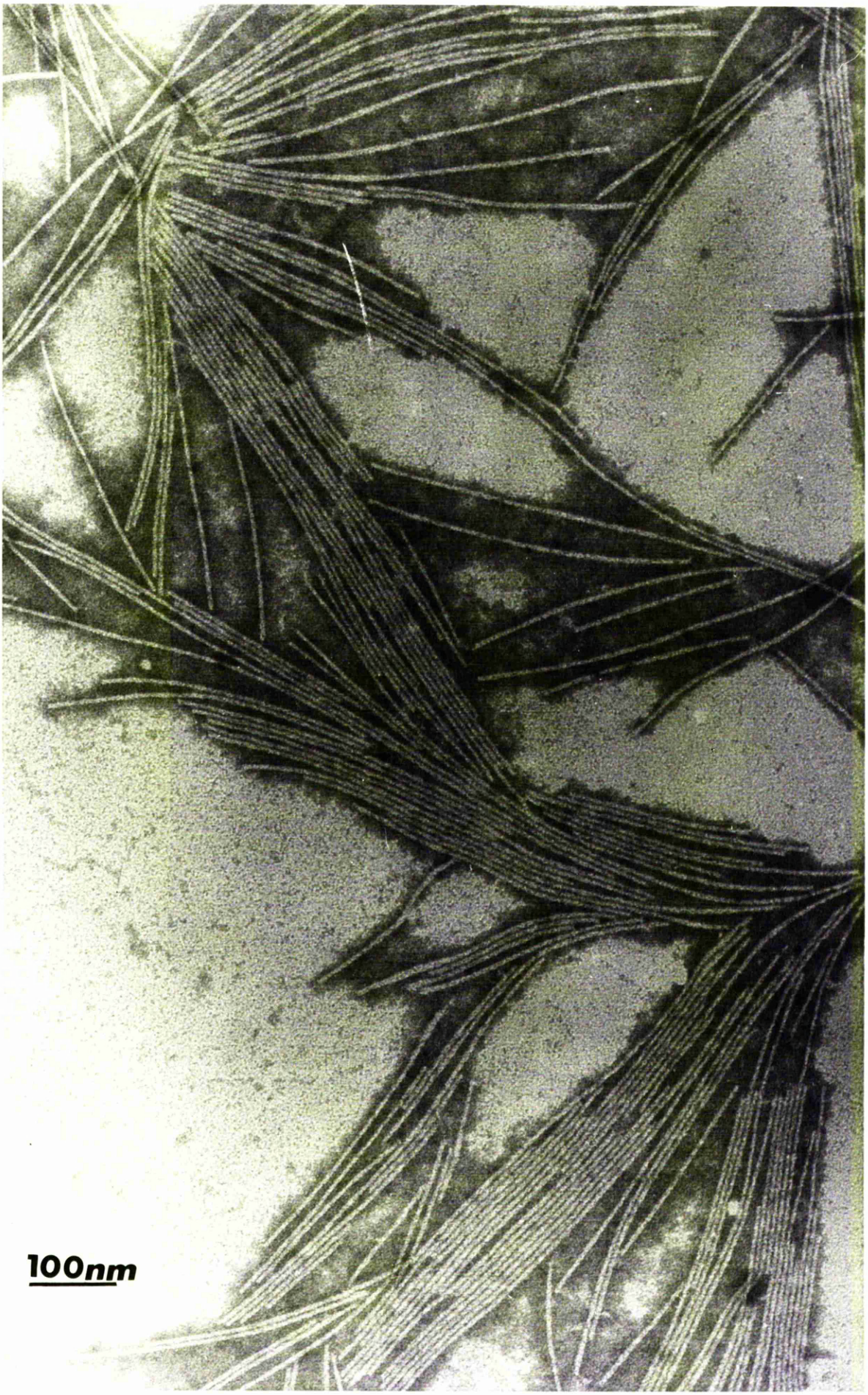
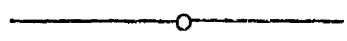

100nm

Figure 8 : Frequency distribution of lengths of isolated fimbriae measured from electron micrographs.

 illustrates the percentage of total protein in the functional sample

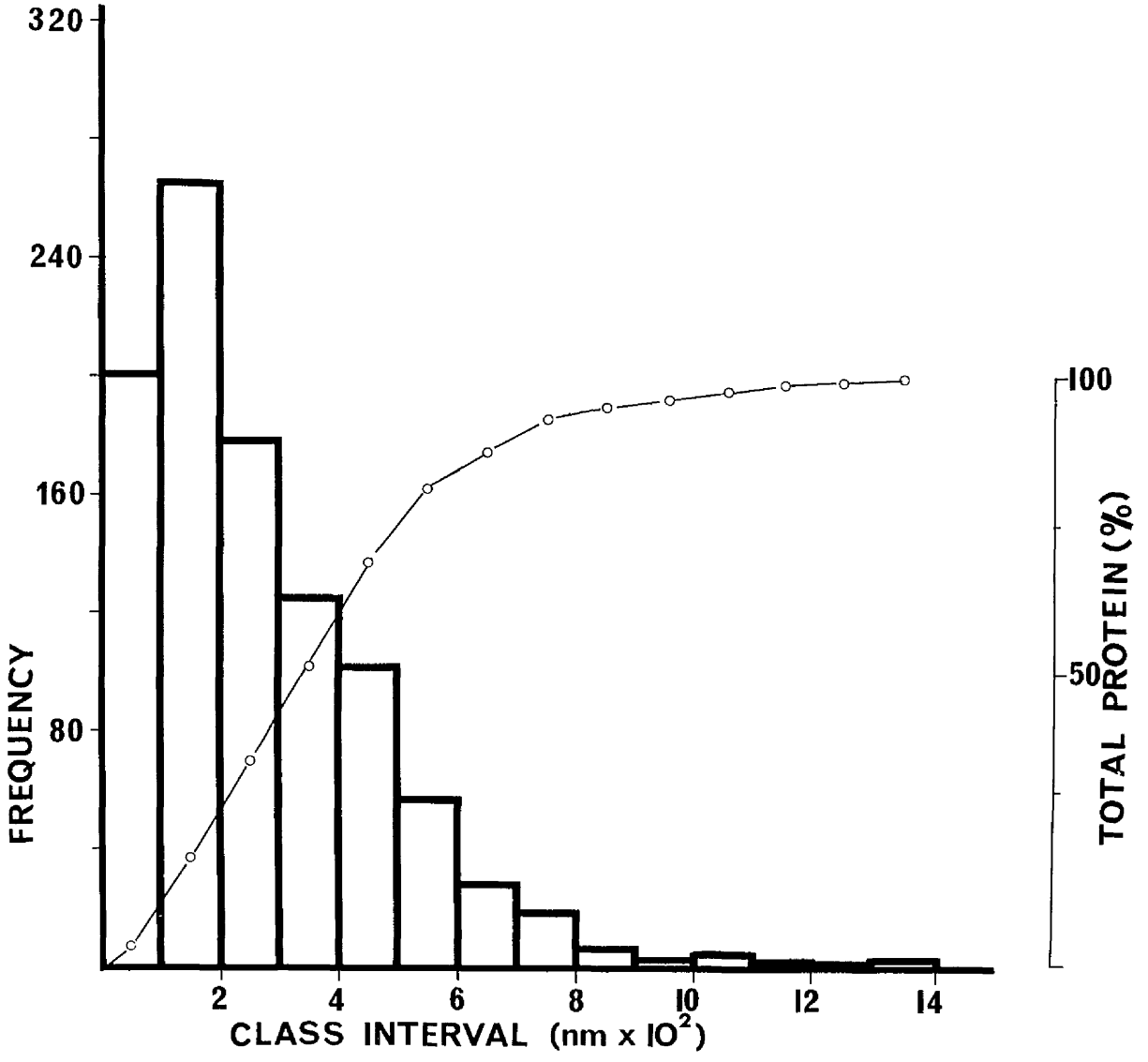

320
240
160
80
FREQUENCY
2
4
6
8
10
12
14
CLASS INTERVAL (nm x IO²)
100
50
TOTAL PROTEIN (%)

Figure 9 : Estimation of the number of molecules of fimbriae in 1 µg and 1 $HU^{1\%}$ of fimbrial protein

M.W. of fimbriae of modal length = 3.4×10^6 daltons (see page 98)

3.4×10^6 µg (1 mole) of fimbriae contains 6×10^{23} molecules

$\therefore$ 3.4×10^{-2} µg contains 6×10^{23} molecules

(i) $\therefore$ 1 µg contains $\frac{6 \times 10^{23}}{3.4 \times 10^{12}}$ = $\underline{17.6 \times 10^{10} \text{ fimbriae}}$

But 1 $HU_f^{1\%}$ $\equiv$ 0.06 µg $\equiv$ $0.06 \times 17.6 \times 10^{10}$

= 1.056×10^{10} fimbriae

(ii) $\therefore$ No. of fimbriae in 1 $HU_f^{1\%}$ = $\underline{1.056 \times 10^{10}}$

of the preparation to haemagglutinate. To investigate this possibility, a simple experiment was designed in which aliquots of fimbriae were sonicated for different times up to a maximum of 5 min (Plate 4) and these sonicated preparations examined for HA activity (Fig 10).

A decrease in HA titre was observed with fimbriae sonicated for periods as short as 60 s. Sonication for 5 min completely abolished the capacity of this preparation to haemagglutinate. However, a sonication time of between 80-100 s rendered the fimbriae capable of reduced HA activity. This suggested that a minimum length of appendage is necessary for HA activity and below this level, fimbriae are incapable of eliciting agglutination.

Length measurements of fimbriae sonicated for 5 min were made from enlarged electron micrographs of PTA stained preparations. The frequency distribution of these fragmented fimbriae presented in Fig 11, indicates that the modal length lay between 10-14.9 nm, i.e. midlength of 12.45 nm. From the modal length of the unsonicated (Fig 8) and sonicated appendages (Fig 11), a 91.6% reduction in modal length occurred following the 5 min period of sonication.

When an aliquot of unsonicated fimbriae was adjusted to 0.1 M with respect to $MgCl_2$, an increase in the length of fimbriae was observed (Plate 5) which was stable to repeated washing in saline. On subsequent titration against 1% (v/v) HRBCs, these elongated fimbriae produced a lower HA titre than the untreated preparation.

Plate 4 : Electron micrograph of fimbriae sonicated for 5 min and stained with phosphotungstic acid (PTA)

Magnification X 150,000.

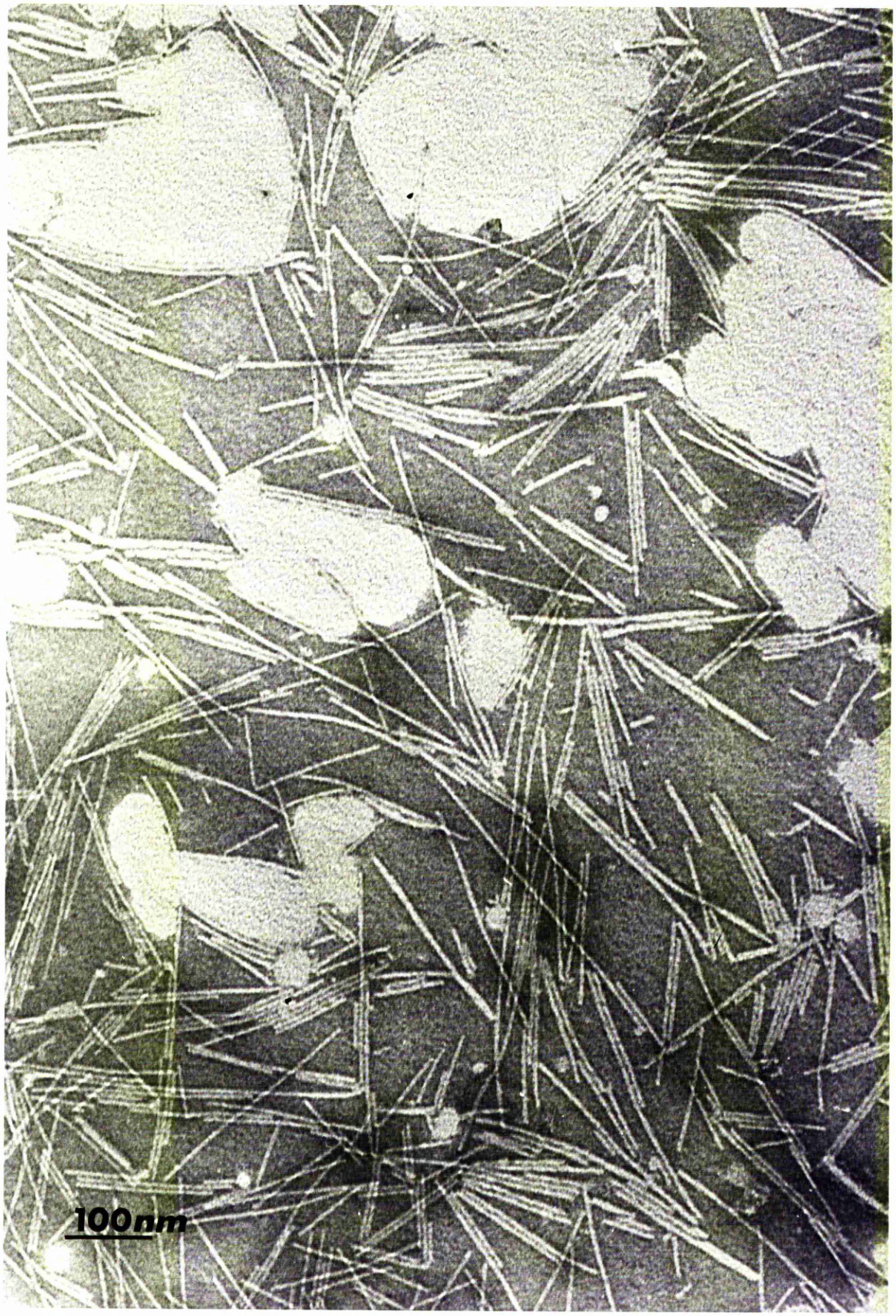

100nm

Figure 10 : Time course of loss of haemagglutinating activity of fimbriae during sonication

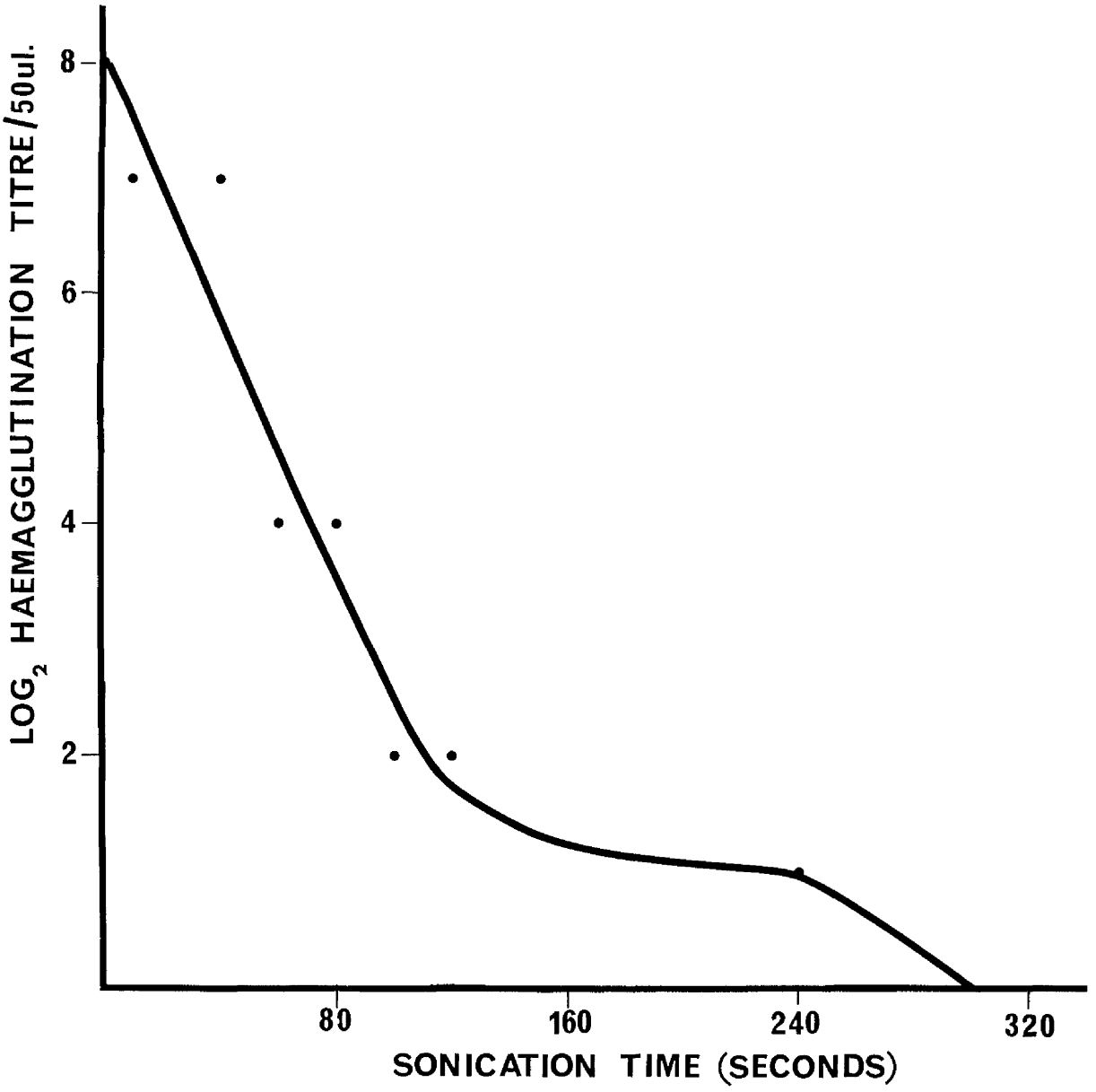
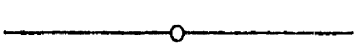

LOG2 HAEMAGGLUTINATION TITRE/50ul.
8
6
4
2
80
160
240
320
SONICATION TIME (SECONDS)

Figure 11 : Frequency distribution of the lengths of fimbriae sonicated for 5 min.



the percentage of total protein in the sonicated sample

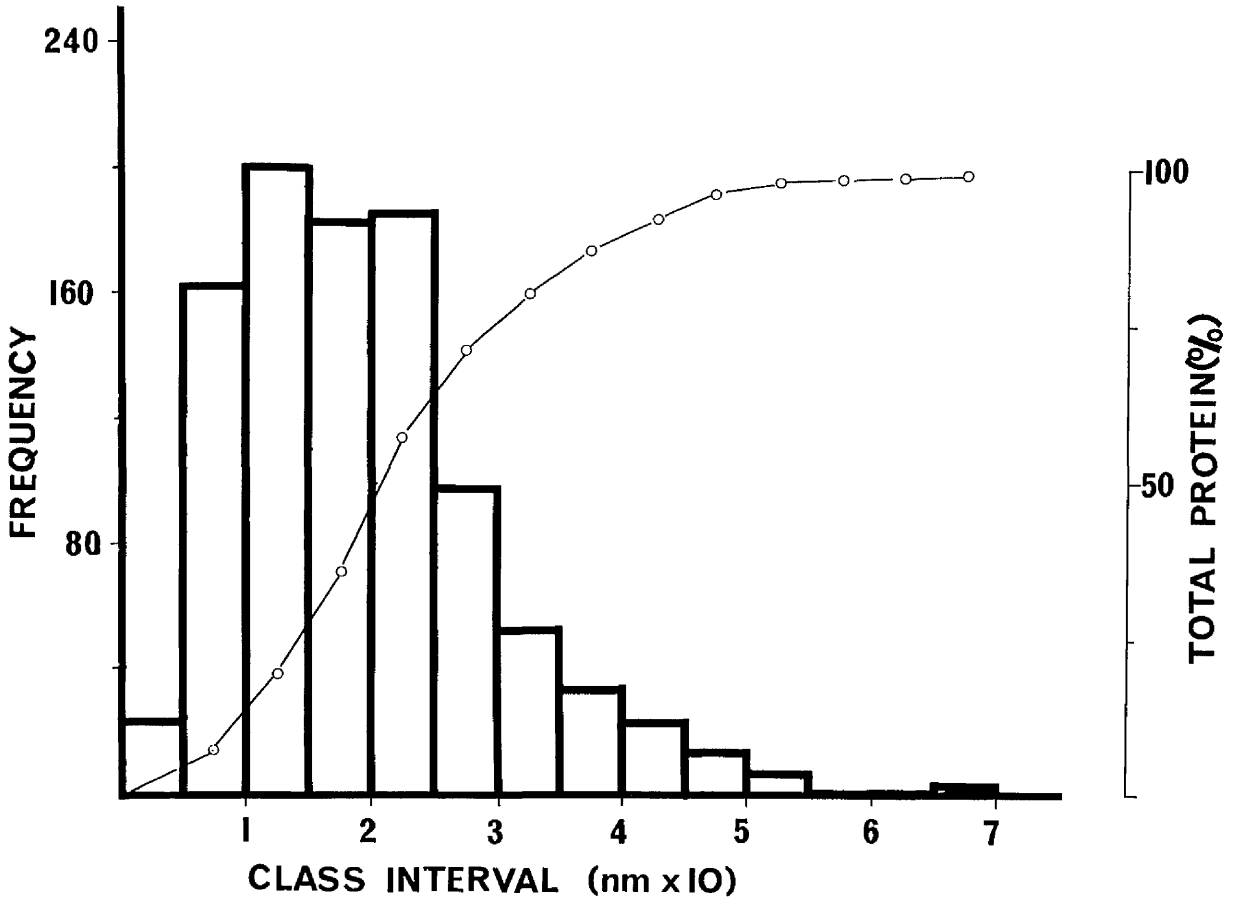
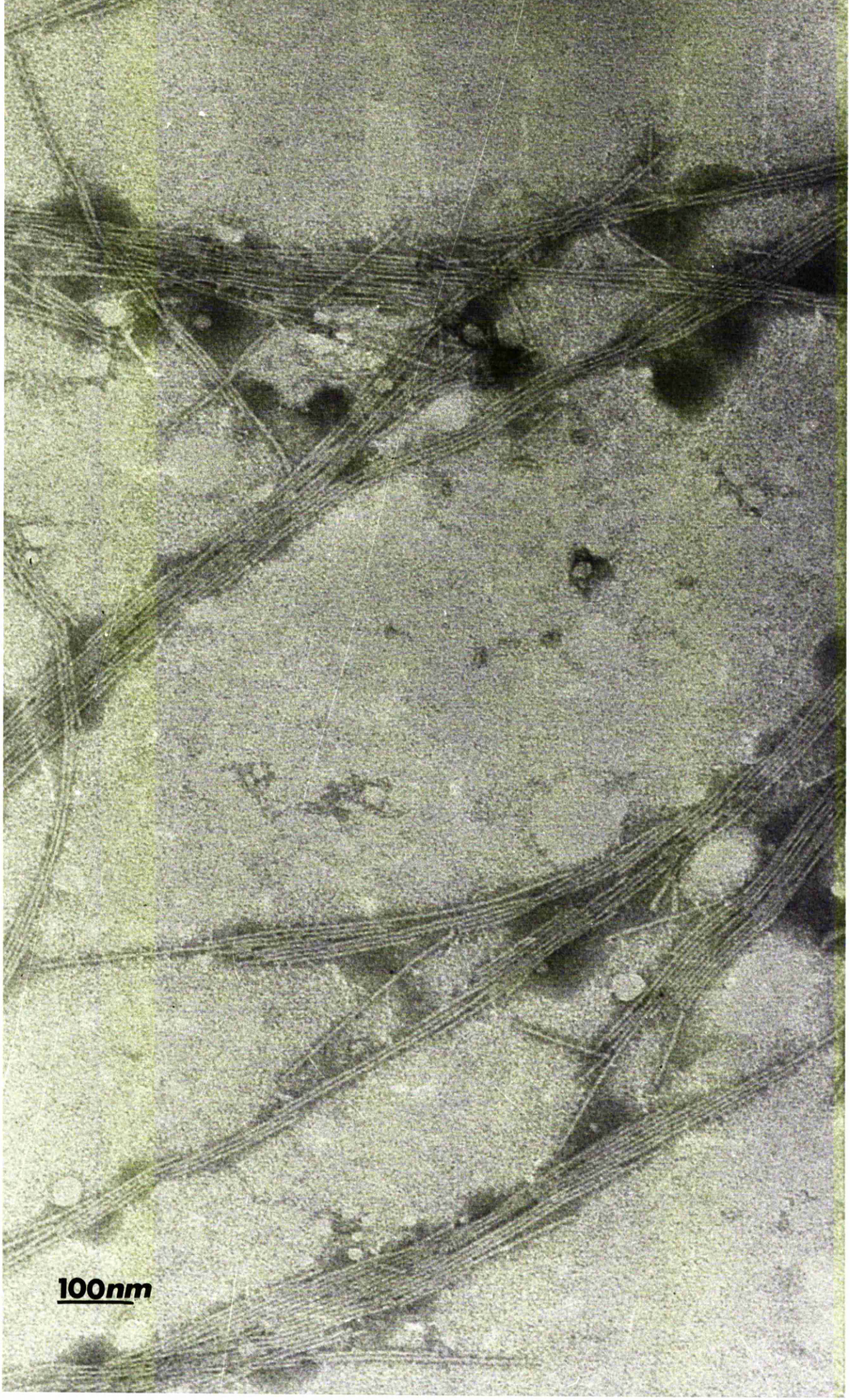

240
160
80
FREQUENCY
100
50
TOTAL PROTEIN(%)
1
2
3
4
5
6
7
CLASS INTERVAL (nm x IO)

Plate 5 : Negatively stained (PTA) preparation of unsonicated fimbriae after the addition and removal of 0.1 M $MgCl_2$.

Magnification X 150,000.


100nm

4. Binding of sonicated fimbriae to erythrocytes

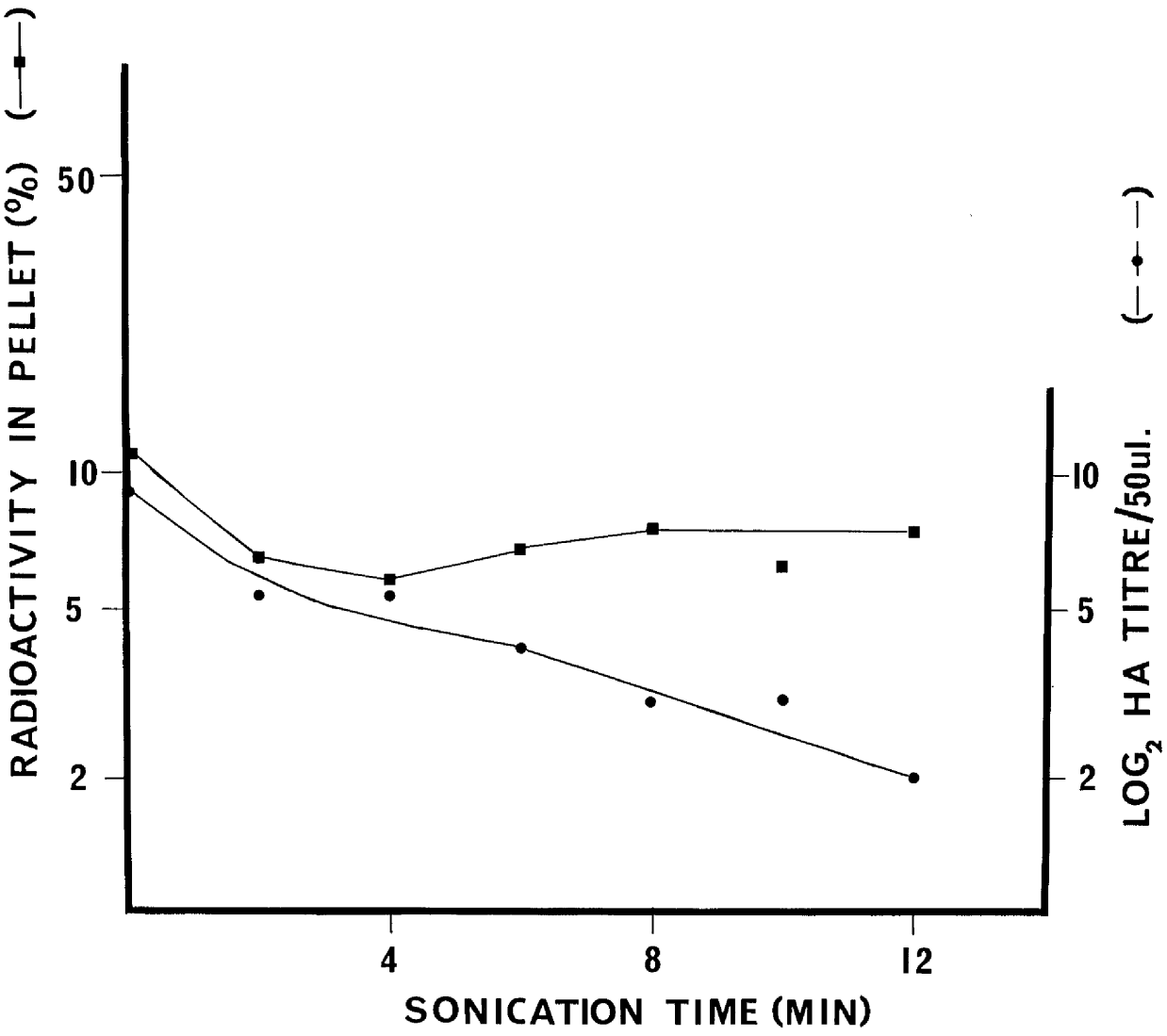
Short fragments of isolated appendages were produced by sonication (Plate 4, Fig 11). The following experiment was undertaken to ascertain whether the quantity of fimbrial protein bound to erythrocytes altered, following sonication of the appendages.

An aliquot of ^{125}I-labelled fimbriae was sonicated for varying time intervals (0-12 min) and a sample withdrawn from each titrated against 1% (v/v) HRBCs. Duplicate 100 µl aliquots from each of the sonicated preparations were mixed with 80 µl portions of a 1% (v/v) HRBC suspension. After heat-sealing the tubes and mixing their contents, these were incubated overnight at room temperature. The erythrocytes were sedimented by low speed centrifugation (2,000 rpm for 5 min) in an MSE minor. The "micro-tubes" or capillaries were sheared at the erythrocyte-supernatant junction and radioactive counts made on both samples. Fig 12 illustrates the relationship between the haemagglutinating capacity of the sonicated fimbriae and the amount of ^{125}I-labelled fimbriae bound to the RBCs. With decreasing HA ability, the percentage radioactivity in the pellet remained at an approximately constant level following the initial drop in binding at 4 min. This general trend obtained in several experiments, suggested that a constant proportion, i.e. 6-7% of the sonicated fimbrial preparations added, was binding to the erythrocytes.

5. Influence of substrate size on agglutinating activity of fimbriae

This experiment was designed to investigate what influence

Figure 12 : The relationship of the haemagglutinating capacity of sonicated fimbriae to the amount of fimbrial protein bound to horse erythrocytes


RADIOACTIVITY IN PELLET (%) (—■—)
50
10
5
2
LOG2 HA TITRE/50ul. (—•—)
10
5
2
4
8
12
SONICATION TIME (MIN)

the size of substrate has on HA titre. Previous work (Section I.3) showed that isolated appendages from E. coli 8623 agglutinated horse erythrocytes and whole yeast cells.

50 µl of a suspension of fimbriae titrated against an arbitrarily chosen, 2% (v/v) of HRBCs produced an HA titre of 8. This fimbrial preparation was diluted in 0.85% (w/v) saline by a factor of 8 to yield a suspension containing 1 HU of fimbriae with respect to 2% (v/v) equine erythrocytes, i.e. 1 $HU_f^{2\%}$.

This $HU_f^{2\%}$ was titrated against doubling dilutions of an arbitrarily chosen suspension of glutaraldehyde-fixed Sac. cerevisiae of E_{620}^{10mm} 8.0 in order to obtain the most sensitive and readily detectable concentration of yeast cells which would agglutinate with this quantity of fimbrial protein. Because this range lay between E_{620} 0.5 - 1.0, tube tests were prepared using 1 $HU_f^{2\%}$ of fimbriae and dilutions of yeasts starting with a suspension of E_{620}^{10mm} of 2.0. The optimum concentration of yeast was E_{620}^{10mm} of 0.8, i.e. 4.5×10^7 cells/ 50 µl (table 12).

One $HU_f^{2\%}$ of fimbriae was titrated against an arbitrarily chosen 16% (v/v) suspension of HRBCs. The most sensitive and readily detectable concentration of erythrocytes lay between 0.25 - 2% (v/v). Again, dilutions of 4% (v/v) HRBC suspension were prepared in tubes and titrated with 1 $HU_f^{2\%}$ of fimbrial protein. The results of these titrations presented in table 13, show that the optimal HRBC concentration was 1% (v/v), i.e. 6.67×10^6 RBC/50 µl. One $HU_f^{2\%}$

Table 12 : Agglutinations of whole yeast cells caused by 1 $HU^{2\%}$ of fimbriae

a. Plate Test

Yeast suspension E_{620}^{10mm} 16.0

Yeast dilution	E_{620}^{10mm} yeast	HA reaction	
1/2	8.0	+++	
1/4	4.0	+++	
1/8	2.0	+++	
1/16	1.0	+++	most sensitive range
1/32	0.5	+	
1/64	0.25	+	
1/128	0.13	+	

b. Tube Test

E_{620}^{10mm}	Agglutinin	HA reaction	
2.0	1 $HU^{2\%}$ fimbriae	-	excess of yeast
1.8	"	-	
1.6	"	±	
1.4	"	+	
1.2	"	+	
1.0	"	+	optimum yeast concentration
0.8	"	++	
0.6	"	+++	
0.4	"	+++	
0.2	"	+++	

Table 13 : Agglutinations of HRBCs caused by 1 $HU^{2\%}$ of fimbriae

a. Plate Test

Erythrocyte suspension			
RBC dilution	% (v/v) HRBC	HA reaction	
1/2	8.0	-	excess of RBCs
1/4	4.0	+	most sensitive
1/8	2.0	++	range
1/16	1.0	++	
1/32	0.5	++	
1/64	0.25	+ -	lack of RBCs
1/128	0.13	-	

b. Tube Test

% (v/v) RBC	Agglutinin	HA reaction	
4.0	1 $HU^{2\%}$ of fimbriae	-	not detectable
3.5	"	-	due to RBC
3.0	"	-	excess
2.5	"	-	
2.0	"	-	
1.5	"	+	optimum
1.0	"	+++	concentration
0.5	"	++	of RBCs
0.25	"	+	

was added to each well of a series of doubling dilutions of a 1% (v/v) HRBC suspension or a yeast cell suspension of $E_{620}^{10mm} = 0.8$. The end point, that is the highest dilution showing agglutination in each series was determined visually. An end point of 1/32 for the yeast dilutions and 1/8 for the 1% HRBC dilutions was evident.

The ratio of HRBC to yeast cells bound by 1 $HU_f^{2\%}$ was 3:5. This ratio was determined by calculating from the dilution factor the number of cells in each end point well as follows:

1% HRBCs end point 1/8

Number of cells in 50 µl of original suspension = 6.67×10^6

Number of cells in end point well = $\frac{6.67 \times 10^6}{8}$

= 8.34×10^5

Yeast suspension end point 1/32

Number of cells in 50 µl of original suspension = 4.5×10^7

Number of cells in end point well = $\frac{4.5 \times 10^7}{32}$

= 1.4×10^6

∴ the ratio of HRBCs (mean diam 6.0 µ) to yeast cells (mean diam 10-15 µ) bound by fimbriae is 1:1.68 or approximately 3:5.

III. THE NATURE AND FREQUENCY OF RECEPTORS FOR FIMBRIAE IN/ON THE ERYTHROCYTE MEMBRANE

The following experimental approaches were used in an attempt to obtain information on the nature and frequency of fimbrial receptors on the erythrocyte membrane.

1. The investigation of agglutinability of whole erythrocytes, inside-out membrane vesicles derived from erythrocyte ghosts, and artificial membrane vesicles (liposomes) with isolated fimbriae.
2. The influence of isolated membrane glycoproteins on the fimbrial agglutination of whole erythrocytes.
3. The ability of fimbriae to haemagglutinate erythrocytes with surface modifications induced by chemical or enzymic treatments.
4. The binding kinetics of erythrocytes with ^{125}I-labelled fimbriae.

1. a. The effect of inside-out membrane vesicles on the haemagglutinating activity of isolated fimbriae

Because of the possibility of lysis of fresh HRBCs when mixed with vesicles, a 1% (v/v) suspension of formaldehyde-fixed erythrocytes was used in this experiment. From the results obtained, the presence of inside-out (IO) vesicles derived from horse erythrocytes in the HA system did not alter the HA titre of fimbriae (i.e. no inhibition). Using IO vesicles as substrates, an identical settling pattern was observed in wells containing vesicles with or without the

addition of fimbriae, indicating that fimbriae did not agglutinate IO vesicles. However haemagglutination of whole horse erythrocytes - these being equivalent in membrane orientation to right-side-out vesicles - was observed. Consequently fimbrial binding sites in/on the red cell membrane are located solely on the exterior of the erythrocyte plasma membrane.

b. The effect of liposomes on the haemagglutinating activity of isolated fimbriae

The agglutinating activity of isolated fimbriae against artificial membrane vesicles was investigated using liposomes as substrates or as competitive inhibitors, i.e. mixed with FFHRBCs in the HA system. Liposomes were not agglutinated by fimbriae, nor did their presence in the HA system alter the fimbrial HA titre.

2. Investigation of the effect of isolated erythrocyte membrane glycoprotein on fimbrial agglutination of whole erythrocytes

Glycoproteins of horse erythrocyte membranes, prepared by the methods of Marchesi & Andrews (1971) and Hamaguchi & Cléve (1972) were each taken up in 1 ml of distilled water. The protein concentrations of these preparations were 0.2 mg/ml and 4.0 mg/ml respectively. When these samples were electrophoresed in a 1% (v/v) SDS polyacrylamide gel system, one major glycoprotein band and at least three other bands containing protein and carbohydrate were visualised by staining with Coomassie Blue and Periodic Acid-Schiffs (PAS) Reagent. These results are in accordance with those published by Fujita & Cléve (1975) on

horse erythrocyte membrane glycoproteins. The influence of glycoproteins on the haemagglutinating activity of isolated fimbriae is presented in table 14. These isolated glycoproteins did not inhibit the HA reaction.

3. Modification of the erythrocyte surface induced by chemical and enzymic treatments

a. Periodate

The effects of varying concentrations of sodium periodate on the agglutinability of formaldehyde-fixed horse erythrocytes was investigated and the results are presented in table 15. Changes in haemagglutination titre were monitored using isolated fimbriae or concanavalin A, where the HA is known to be dependent on the presence of α-D-mannose or α-D-glucose residues.

After treatment with 0.05 M periodate, the HA titre induced by fimbriae was reduced, whereas that induced by concanavalin A was abolished. Increasing the molarity of periodate to 0.1 M produced a further reduction in the fimbrial-induced HA titre, but additional increases in concentration had no further effect on titre.

Two effects may be responsible for the observed results:

(1) α-D-glucose or α-D-mannose residues are involved but are not solely responsible for fimbrial HA

(2) the periodate is having a non-specific effect on the receptor site, which does not necessarily involve α-D-mannose and α-D-glucose residues.

Table 14 : Haemagglutination of horse erythrocytes induced by isolated fimbriae in the presence of erythrocyte glycoproteins

	Concentration of glycoprotein (µg/50 µl) in 1% (v/v) horse suspension	HA titre
	Control	32
(a)	200	32
	100	32
	50	32
	25	32
	12.5	32
	6.2	32
(b)	10	32
	5	32
	2.5	32
	1.2	32
	0.6	32
	0.3	32

(a) Glycoproteins prepared by Hamaguchi & Cléve (1972)
(b) Glycoproteins prepared by Marchesi & Andrews (1971)

Table 15 : Agglutinability of formaldehyde-fixed horse erythrocytes after treatment with periodate

Periodate concentration	HA titres (a) fimbriae	(b) Con A
Nil (control)	32	32
0.05 M	16	0
0.10 M	8	0
0.15 M	8	0

b. Proteases

i. Effect on fimbrial haemagglutination titre

Horse erythrocytes were modified by the action of several proteases and the resultant changes in agglutinability with fimbriae are presented in table 16. The significant feature of these results is the 16-fold increase in HA titre obtained with papain-treated erythrocytes and isolated fimbriae.

One feature worthy of note is that the trypsin buffer consisting of 0.015 M $CaCl_2$ in 0.041 M tris-buffered saline at pH 8.0, in the absence of enzyme, induced a high HA titre with horse erythrocytes.

ii. Release of PAS-positive material

Although the gels containing isolated glycoprotein from HRBCs contained one major and three minor PAS-positive bands, the gels containing the supernatant fractions from protease-treated HRBCs, lacked stained material. This indicated that none of the proteases tested released glycoprotein from the intact HRBC.

c. Glycosidases

The degree of agglutinability of untreated HRBCs with fimbriae or lectins is presented in table 17.

i. Neuraminidases

The effects of neuraminidase treatment of horse erythrocytes on the fimbrial haemagglutination titre were investigated

Table 16 : Agglutinability of protease-treated horse erythrocytes by fimbriae

Protease treatment	HA titre	Factor difference
Nil (PBS buffer)	512	
Papain	4096	+ 16
Papain buffer	256	
Trypsin	1024	- 4
Trypsin buffer	4096	
Pronase	1024	+ 2
Pronase buffer	512	
Subtilisin	512	0
Subtilisin buffer	512	
Chymotrypsin	512	0
Chymotrypsin buffer	512	

Table 17 : Agglutinability of untreated horse erythrocytes by isolated fimbriae and lectins

Substrate	HA titres				
	(a) Fimbriae	(b) Lectins			
		Con A	FBP	RCA_{120}	WGA
Untreated horse erythrocytes	32	32	0	0	0

Con A - Concanavalin A

FBP - Fucose binding protein

RCA_{120} - Ricinus communis agglutinin 120

WGA - Wheat germ agglutinin

using enzymes from two sources. Both enzymes, one isolated from Vibrio cholerae and the other from Clostridium perfringens are reported to have the same substrate specificity (Müller, 1974). These glycosidases remove terminal sialic acid residues from glycoproteins and glycolipids by cleaving the α-O-ketosidic bond (Seaman & Uhlenbruck, 1963). Although horse erythrocytes contain 90% N-glycolyl-neuraminic and 10% N-acetyl-neuraminic acids (Yamakawa, 1960), both acids are susceptible to the action of neuraminidase (Müller, 1974).

Both enzymes produced a slight reduction in titre with fimbriae but no significant change in titre with concanavalin A or fucose binding protein (table 18). In both cases, however, an increase in titre was observed for Ricinus communis agglutinin$_{120}$.

Agglutination of untreated erythrocytes (1% v/v suspension) occurred with concanavalin A at concentrations as low as 10-50 µg/ml. Untreated erythrocytes did not agglutinate in the presence of RCA or WGA (table 17) but cells pretreated with V. cholerae enzyme were agglutinated by both lectins (table 18). In comparison, treatment with the Cl. perfringens neuraminidase produced agglutination only with RCA$_{120}$. This suggests that the former preparation was less specific in its action and contained enzymic activities absent from the Cl. perfringens preparation.

ii. Other glycosidases

Although treatment with the other glycosidases did not markedly alter the haemagglutination titre with fimbriae (table 19) it

Table 18 : Agglutinability of horse erythrocytes after treatment with neuraminidase

Enzyme treatment	HA titres (a) Fimbriae	(b) Lectins			
		Con A	FBP	RCA_{120}	WGA
Nil (control)	32	64	0	0	0
Neuraminidase (*V. cholerae*)	8	128	2	4096	32
Neuraminidase (*Cl. perfringens*)	8	64	0	128	0
Lectin specificities		D-mannose, L-fucose D-fructose, D-glucose		β-D-galactose, β-N-acetyl galactosamine	N-acetyl glucosamine

Table 19 : Agglutinability of glycosidase-treated horse erythrocytes with Concanavalin A

Glycosidase used	HA titres	
	(a) Fimbriae	(b) Lectin Con A
Nil (control)	32	64
α-fucosidase	64	8
α-galactosidase	32	4
α-glucosidase	64	8
α-mannosidase	32	4
α-D-N-acetylgalactosaminidase	64	8
β-D-N-acetylglucosaminidase	32	4

produced a decrease in titre with Con A in all instances. Consequently, modification of erythrocytes with glycosidases did not affect the fimbrial receptors on the erythrocytes but destroyed the Con A receptors. Pretreatment of HRBCs with glycosidases did not affect the HA titre with the other lectins.

The particularly significant feature of these results is that treatment with mannosidase - the effect of which lies in removal of terminal mannose residues and all saccharides on the non-reducing terminal side of the mannose residue - did not alter the capacity of the erythrocytes to agglutinate with fimbriae.

d. Sequential enzymic treatment

Treatment of erythrocytes with combinations of enzyme in sequence, followed by titration with fimbriae and lectins was done in order to overcome possible restrictions of enzyme accessibility to substrates due to blocking by other surface groups. The haemagglutination titres after sequential enzyme treatments could then be compared with those after treatment of erythrocytes with single enzymes.

i. Neuraminidase/glycosidases

Pretreatment of red cells with neuraminidases resulted in a decrease in agglutinability with fimbriae as previously reported. Subsequent treatment with four of the glycosidases did not alter this result (table 20). However α-galactosidase and α-fucosidase modified the erythrocytes such that fimbrial agglutinability was restored.

Table 20 : Agglutinability of horse erythrocytes treated with neuraminidase and glycosidases

Treatment	HA titres (a) Fimbriae	(b) Lectins			
		Con A	FBP	RCA_{120}	WGA
Nil (control)	32	64	0	0	0
Neuraminidase (Cl. perfringens)	8	64	0	128	0
Neuraminidase + α-fucosidase	32	128	0	128	0
" + α-galactosidase	32	128	4	64	0
" + α-glucosidase	8	128	0	128	0
" + α-mannosidase	8	128	0	256	0
" + α-D-N-acetyl galactosidase	8	128	2	256	0
" + β-D-N-acetylglucosaminidase	8	128	2	256	0

Neuraminidase treatment of erythrocytes produced a control titre of 64 with Con A. Further treatment with glycosidases served to raise this level by a factor of 2, i.e. 1 well, the significance of which is doubtful. A puzzling feature however, is that treatment with glycosidases alone produced a consistent reduction in Con A titre whereas sequential treatment with neuraminidase and the glycosidases produced no detectable change. Modification with neuraminidase and galactosidase increased the agglutination titre with FBP. Removal of sialic acid and galactose residues therefore permitted access of this lectin to a sufficient number of fucose residues, resulting in binding and finally in FBP agglutination.

ii. Papain/glycosidases

Although pretreatment of erythrocytes with crude papain increased markedly the titre with fimbriae, the Con A titre was unaffected (table 21). Subsequent treatment with glycosidases reduced the fimbrial titre and some of the Con A titres. These results suggest that papain exposes additional fimbrial receptors which are then sensitive to the glycosidases but were not sensitive without papain treatment.

Alteration in agglutination patterns with the three remaining lectins was not observed with this enzyme combination.

iii. Papain/neuraminidase/glycosidases

Treatment with crude papain significantly increased the fimbrial HA titre but no effect was observed on the Con A titre with this protease (table 22).

Table 21 : Agglutinability of horse erythrocytes treated with papain and glycosidases

Treatment	HA titres (a) Fimbriae	(b) Lectins			
		Con A	FBP	RCA_{120}	WGA
Nil (control)	32	64	0	0	0
Papain	512	64	0	0	0
P + α-fucosidase	32	16	0	0	0
P + α-galactosidase	32	64	0	0	0
P + α-glucosidase	16	32	0	0	0
P + α-mannosidase	64	16	0	0	0
P + α-D-N-acetylgalactosaminidase	32	16	0	0	0
P + β-D-N-acetylglucosaminidase	32	32	0	0	0

Table 22 : Agglutinability of horse erythrocytes treated with papain, neuraminidase and glycosidases

Treatment	HA titres (a) Fimbriae	(b) Lectins			
		Con A	FBP	RCA_{120}	WGA
Nil (control)	32	64	0	0	0
Papain	512	32	0	0	0
P + neuraminidase	16	64	0	64	0
P,N + α-fucosidase	128	32	0	0	64
P,N + α-galactosidase	16	128	0	128	0
P,N + α-glucosidase	32	64	0	128	0
P,N + α-mannosidase	32	64	0	256	0
P,N + α-D-N-acetyl galactosaminidase	32	64	0	64	0
P,N + β-D-N-acetylglucosaminidase	32	128	0	128	0
P,N + β-galactosidase	16	8	0	512	0
P,N + β-N-acetylhexosaminidase	16	4	0	256	0

With the exception of α-fucosidase, all of the triple enzymic modifications unmasked receptors for RCA_{120} viz β-D-galactose or β-N-acetyl galactosamine and this was reflected in the HA titres obtained with RCA_{120}. Alterations by N-acetyl hexoseaminidase produced a sharp decrease in Con A titre.

The significant result obtained from these experiments is that changes in HA titre for Con A, FBP, RCA_{120} and WGA after these enzyme treatments do not parallel the changes recorded in the fimbrial HA titre.

IV. INVESTIGATIONS USING ^{125}I-LABELLED FIMBRIAE

1. The saturation of fimbrial receptors on horse erythrocytes using ^{125}I-labelled fimbriae

The aim of this experiment was to determine the amount of fimbrial protein required to saturate receptors on horse erythrocytes, thus allowing an estimate of the minimum number of fimbrial receptors per red cell. The data obtained from the addition of increasing aliquots of ^{125}I-labelled fimbriae to a fixed quantity of RBCs are tabulated (table 23). A graph of the "bound" fimbriae plotted against increasing amounts of added ^{125}I-fimbriae is shown in Fig 13.

These results revealed that the saturation point of the erythrocytes was reached and an estimate of the number of receptor sites per RBC was calculated (Fig 14).

Table 23 : Saturation of horse RBCs with ^{125}I-labelled fimbriae

Radioactivity added (cpm)	Radioactivity in supernatant (cpm)	Radioactivity in pellet (cpm)
	Radioactivity (counts per minute)	
50216	25972	24244
73590	38280	35310
86715	40807	45908
102484	55402	47082
132900	68415	64485
164244	74689	89555
264672	117565	147107
271421	155181	116240

Reaction mixture consisted of 100 µl of 3% (v/v) HRBCs and 200 µl of radioactive protein

Figure 13 : Graph of the amount of ^{125}I-protein added against the amount of ^{125}I-protein bound, for horse erythrocytes.

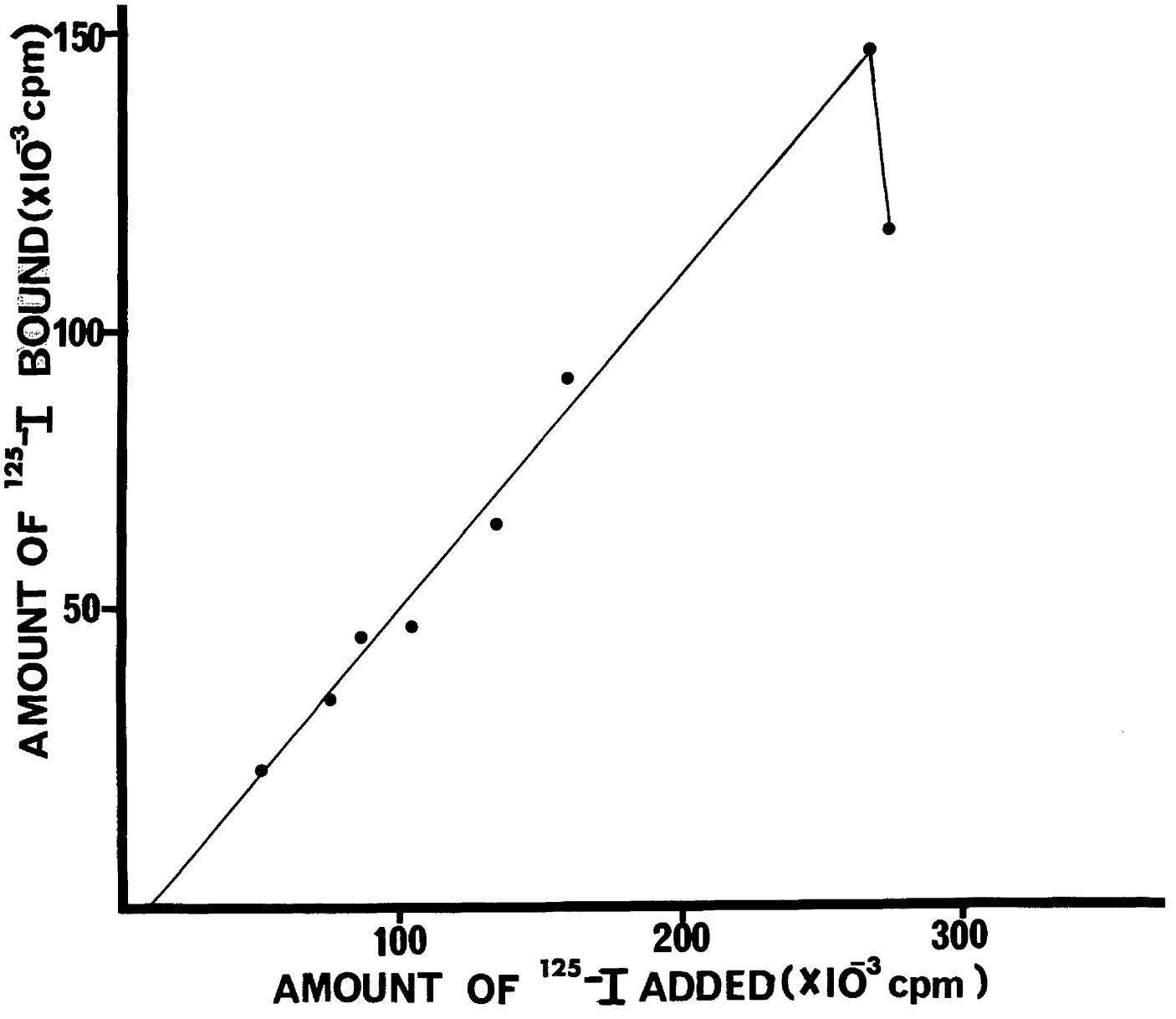

AMOUNT OF ^{125}-I BOUND(x10^{-3}cpm)
150
100
50
100
200
300
AMOUNT OF ^{125}-I ADDED(x10^{-3}cpm)

Figure 14 : Estimations of the number of fimbrial receptor sites on horse erythrocytes
(from data in Table 23 and Fig 13)

Concentration of fimbrial protein at saturation 288.0 µg

(This is equivalent to 2.3 pgm fimbriin per erythrocyte)

Number of erythrocytes present at saturation 12.3×10^7

If 1 µg of fimbrial protein contains 17.6×10^{10} filaments (*assuming they all have M.W. of 3.4×10^6) then the minimum number of sites per erythrocyte

$$= \frac{\text{Number of fimbriae present at saturation}}{\text{Number of erythrocytes present at saturation}}$$

$$= \frac{288.0 \times 17.6 \times 10^{10}}{12.3 \times 10^7}$$

$\therefore$ minimum number of sites per red cell = 4.11×10^5

2. Comparison of the degree of binding of ^{125}I-fimbriae to horse, dog, guinea-pig, sheep and rabbit erythrocytes

Although type-1 fimbriae adhere preferentially to guinea-pig, fowl and horse erythrocytes, they also agglutinate red cells from dog, sheep and rabbit. The object of this experiment was to estimate the degree of binding of ^{125}I-fimbriae to RBCs of various species. From this data the number of fimbrial receptors and their relationship to the surface area of the cells in question could be determined.

The results of adding increasing aliquots of γ-labelled fimbriae to 100 µl portions of RBCs from horse, dog, guinea-pig, sheep and rabbit are presented in table 24. Graphs of the ^{125}I bound to the erythrocytes against the amount of ^{125}I added were prepared for each species of erythrocyte (Fig 15).

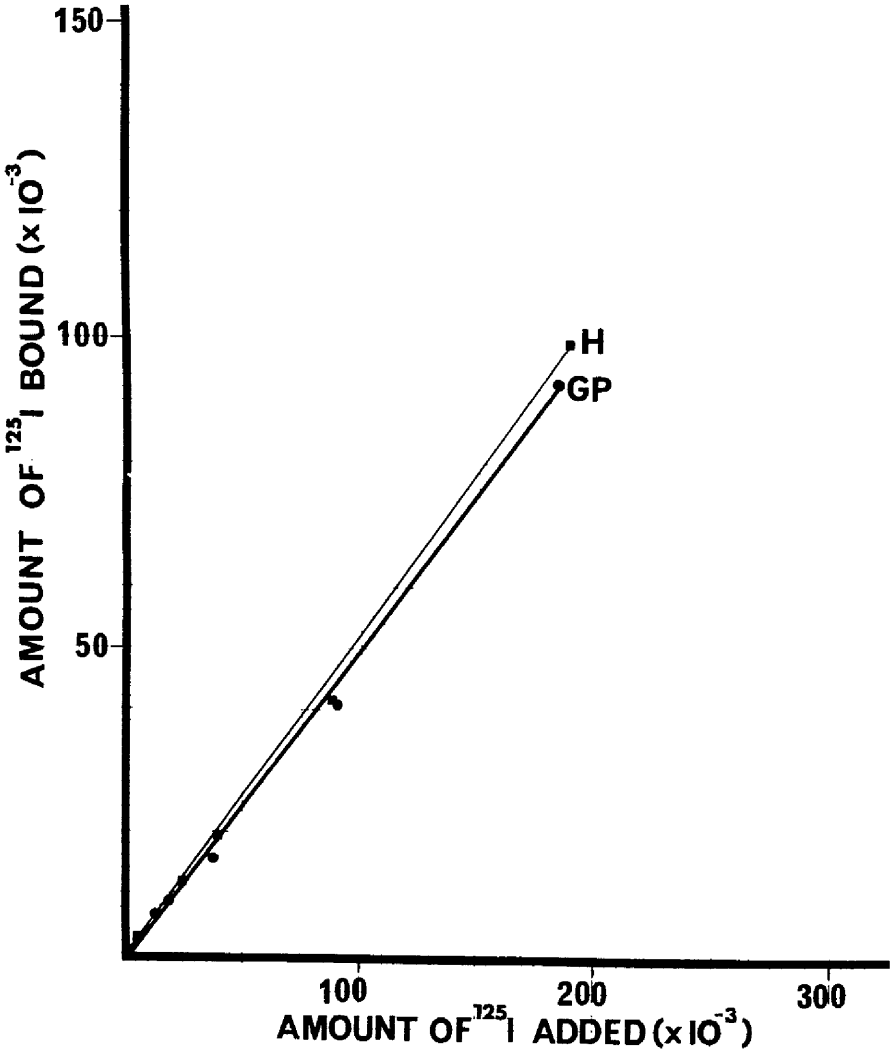
The rates of binding are remarkably similar considering the differences in susceptibility of the erythrocyte species to agglutination by fimbriae. The degree of binding is related but not directly proportional to the surface area of the cells, i.e. rabbit > horse > dog > guinea-pig > sheep, for both surface area and binding. In all cases the amount of protein bound lay between 40-60% of the amount added. From the graphs presented on page 135, it appears that saturation of the erythrocytes with fimbriae was not reached in any of the experiments. However, results from the previous experiment (page 131) show that at least in the case of HRBCs the last point on the graph probably represents saturation. In all cases the maximum protein added to the erythrocytes was 288 µg.

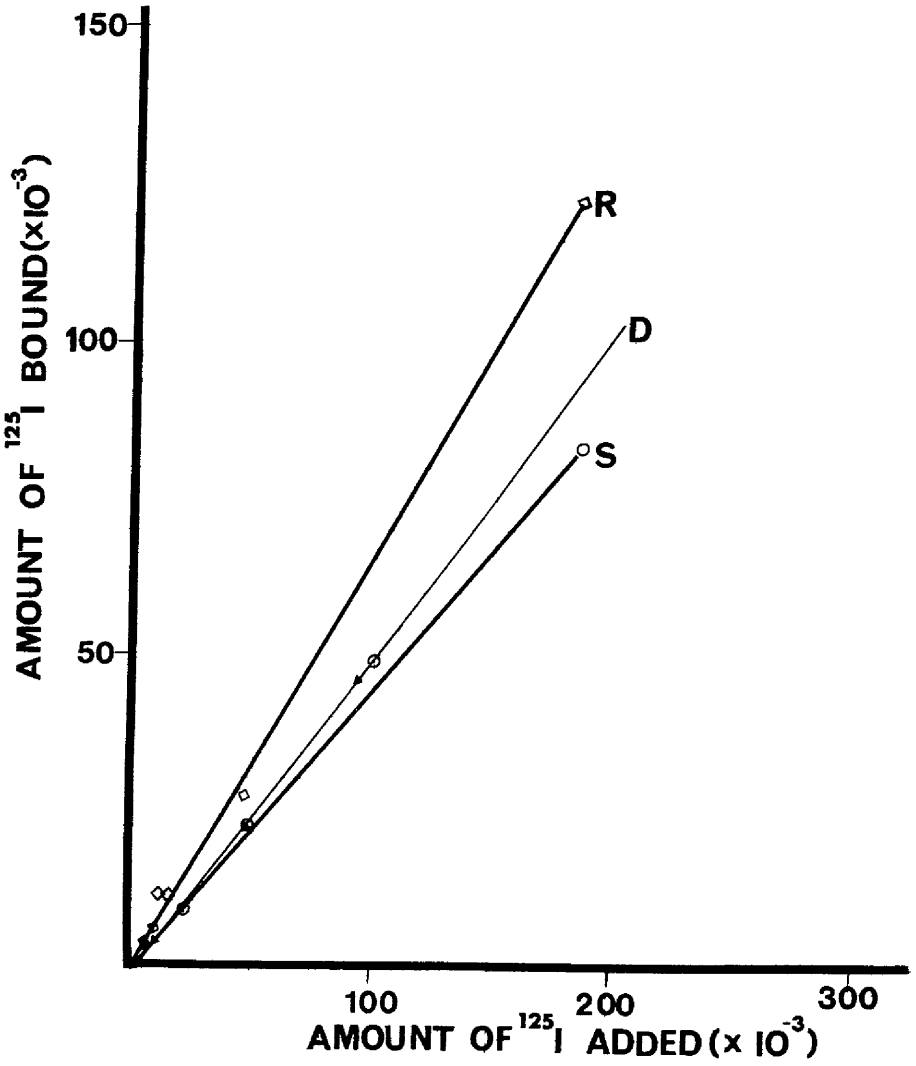
Table 24 : Radioactive counts obtained from the addition of increasing aliquots of ^{125}I-fimbriae to horse, dog, rabbit, sheep and guinea-pig erythrocytes

	Radioactivity (counts per minute)		
	Radioactivity added	Radioactivity in pellet	Radioactivity in supernatant
Horse	1935	1489	446
	1951	1087	864
	4966	2323	2643
	10789	5710	5079
	21679	12150	9529
	39805	19939	19866
	88048	42289	45753
	187178	99195	87983
Rabbit	629	309	320
	1910	1265	645
	5115	2748	2367
	11381	7687	3694
	15018	7719	7299
	46237	27499	18738
	62455	34684	27771
	179329	123348	55982
Dog	1252	626	626
	1669	764	905
	5053	2351	2702
	9262	4675	4587
	21330	11467	9863
	47461	22505	24956
	93478	46020	47458
	----------tubes broken----------		
Guinea-pig	1300	699	601
	205	158	47
	3693	2071	1622
	12165	7201	4964
	18647	8825	9822
	37830	15803	22027
	90143	41083	49060
	182048	92984	89064
Sheep	1399	772	627
	2147	1296	851
	5514	2314	3200
	10669	4367	6302
	22032	8895	13137
	50318	22894	27424
	99297	49197	50100
	185744	82666	103078

Reaction mixture consisted of 100 µl of RBCs and 300 µl of radioactive protein

Figure 15 : Graph of the amount of ^{125}I-protein added against the amount of ^{125}I-protein bound for each of the five species of RBC tested


150
100
50
AMOUNT OF ^{125}I BOUND (x IO^{-3})
H
GP
100
200
300
AMOUNT OF ^{125}I ADDED (x IO^{-3})


150
100
50
AMOUNT OF ^{125}I BOUND(xIO^{-3})
R
D
S
100
200
300
AMOUNT OF ^{125}I ADDED (x IO^{-3})

DISCUSSION

DISCUSSION

Although the presence of fimbriae on bacteria was demonstrated by electron microscopy almost thirty years ago, the function of these organelles is not fully elucidated. From electron micrographs, these appendages were seen to radiate in all directions from the bacterial cells whereas the flagella appeared drawn to one side, i.e. in the direction of removal of water from the collodion coated grid. On the basis of a suggestion made by Houwink & van Iterson (1950) that fimbriae served as organs of attachment, Duguid *et al.* (1955) and Constable (1956) showed that fimbriate organisms adhered to various species of red blood cells. To date little is known about the fimbrial receptor sites on erythrocytes or on other eukaryotic cells and the mechanism of this fimbrial adherence is not understood. An investigation of the properties of the haemagglutinin(s) of type-1 fimbriae and factors affecting their adherence to receptor sites in/on equine erythrocytes is reported in this thesis.

I. FIMBRIATE BACTERIA AND ISOLATED FIMBRIAE

E. coli NCTC 8623 possesses type-1 fimbriae and resembles the Group 1 strains described by Duguid *et al.* (1955) in that it shows great affinity for guinea-pig, fowl and horse red blood cells but does not agglutinate human erythrocytes. Although *E. coli* 8623 adheres preferentially to guinea-pig and fowl RBCs, horse erythrocytes were used in this study since these were readily available.

Following blending of whole bacteria, short stumps of fimbriae remain on the bacterial cells as shown by electron microscopy. Although these pellets of blended, washed bacteria retain the capacity to agglutinate horse RBCs, this is probably due to large sheets of fimbrial filaments entangled within the clumps of whole bacteria. This observation led to the conclusion that many fimbriae are lost during the isolation and purification process and that the low yield of 1-2 mg of fimbrial protein/litre of bacterial culture does not reflect the amount present in the culture. However this low yield is similar to that obtained by Brinton (1959) and Rivier & Darekar (1975), indicating that a more efficient method of recovery of fimbriae is required. An attempt was made to recover isolated fimbrial appendages from dilute suspension using as the substrate whole, glutaraldehyde-fixed yeasts, viz. Sac. cerevisiae. The addition of fixed yeast to a dilute fimbrial suspension resulted in the production of fimbrial-yeast complexes, which were subsequently recovered by low speed centrifugation. Following washing of the pellet, release of fimbriae from the yeast was achieved by the addition to the complex of α-methyl mannoside (αMM). A further low speed spin served to harvest the yeasts while leaving the fimbriae free in suspension. Two 50-fold volumes of saline were used to dilute out the residual carbohydrate. The final fimbrial pellet did not induce agglutination of horse erythrocytes and so this line of investigation was abandoned.

Several saccharides, including mannose, were reported to inhibit the HA reaction of type-1 fimbriate bacteria, by Collier & de Miranda (1955); Duguid & Gillies (1957); Old et al. (1968) and Old (1972).

The inhibitory effect of a range of sugars on agglutination of horse RBCs induced by whole E. coli or isolated fimbriae was assessed. By microtitration α-methyl mannoside was shown to be the most effective inhibitor of HA causing inhibition at a concentration of 0.1 μg/ml (5.5×10^{-7} M). α-D-mannose was also a potent inhibitor being effective at a concentration of 0.36 μg/ml (2.0×10^{-6} M). Collier & de Miranda (1955) reported that D-glucose, L-sorbose, sucrose and trehalose caused inhibition of the HA reaction between fimbriate E. coli (whole cells) and guinea-pig erythrocytes. Although the results reported here with E. coli 8623 conflict with those of Collier & de Miranda (1955), they concur with the more recent studies of Old (1972). In comparison with the weak HAI of melizitose with Salmonella spp., no inhibition was detected using this sugar with either fimbriate E. coli or isolated fimbriae. The inhibition pattern obtained using isolated fimbriae differs slightly from that obtained using whole E. coli, particularly with the monosaccharide D-fructose. This sugar inhibited HA caused by isolated appendages but not by whole organisms. This finding suggests that whole bacterial cells possess agglutinin(s) - not fructose sensitive, i.e. fructose resistant, which mask the fimbrial reaction. To minimise false reactions caused by cell surface-associated agglutinins, subsequent investigations were made with isolated fimbriae rather than fimbriate bacteria.

There are numerous reports on the ability of fimbriate organisms to stick to an extensive array of substrates including many types of eukaryotic cells and dyes. Brinton (1959) also claimed that isolated fimbriae adhered to latex beads.

II. FIMBRIAL HAEMAGGLUTININ(S)

In agreement with the work of Brinton (1959), isolated type-1 fimbriae were found to agglutinate erythrocytes which were agglutinated by the whole fimbriate bacteria from which they were derived. Exposure of either the fimbriate bacteria, the fimbriae or erythrocytes to 0.1-0.25% formaldehyde prior to haemagglutination did not alter the specificity of the haemagglutination. From electrophoretic and spectrophotometric analyses, Brinton & Gemski et al. (1961) claimed that fimbriae were composed of 100% protein. These techniques would be inadequate for the detection of low levels of carbohydrate, i.e. 1% or less of dry weight which may be present in the fimbriae. Although these investigations have not demonstrated the presence of carbohydrate, common type fimbriae may yet prove to be chemically similar to sex fimbriae, i.e. glycoprotein in nature.

In the fimbriae-erythrocyte agglutination complex, the presence of low concentrations of mannose causes dissociation of this complex, resulting in HAI. Since this mannose inhibition is readily alleviated by washing either fimbriae or erythrocytes in buffer, it suggests that the sugar is not tightly bound to the fimbrial protein or erythrocytes. Using low concentrations of ^{14}C-mannose and two different amounts of fimbrial protein, i.e. 250 and 500 µg, two out of three equilibrium dialysis experiments showed that isolated fimbriae bound mannose at the 5% significance level. As indicated by the results obtained from these investigations (page 97) fimbrial protein exhibited a low affinity for mannose. In this experiment the ratio of sugar

molecules to fimbrial filaments was estimated from available data to be between 3-600:1. If one assumes that only 1% of these sugar molecules is bound then 3-6 mannose residues would be bound to each protein molecule (filament).

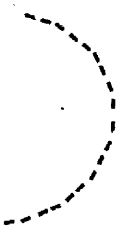
Haemagglutination induced by fimbriae or whole bacteria occurs as a result of binding between fimbriae and the red cell surface. Several questions arise relating to the geometry of binding. The concept of a unique chemical structure responsible for the binding site, at the distal end of a filament is not tenable, since if this were so no cross-linking of substrates would be expected in the case of isolated fimbriae. Since cross-linking (i.e. agglutination of substrates) does occur with isolated fimbriae then it seems reasonable to assume that fimbriae are composed only of identical subunits. In the case of haemagglutination induced by isolated fimbriae binding could occur in two different ways:

(a) by virtue of a binding site occurring at each tip of a filament due to specific areas of subunits exposed at the tips of filaments which are masked by subunit interactions along the length of the fibre (Fig 16)

(b) by every subunit carrying a binding site exposed at the surface of the filament (Fig 17).

The sole evidence favouring the former viewpoint was presented in the form of electron micrographs of metal-shadowed fimbriae and latex beads which Brinton (1967) described as showing "pili sticking to polystyrene-latex spheres solely by their tips." However this observation may be

Figure 16 : Binding site located solely at the tip of the fimbriae

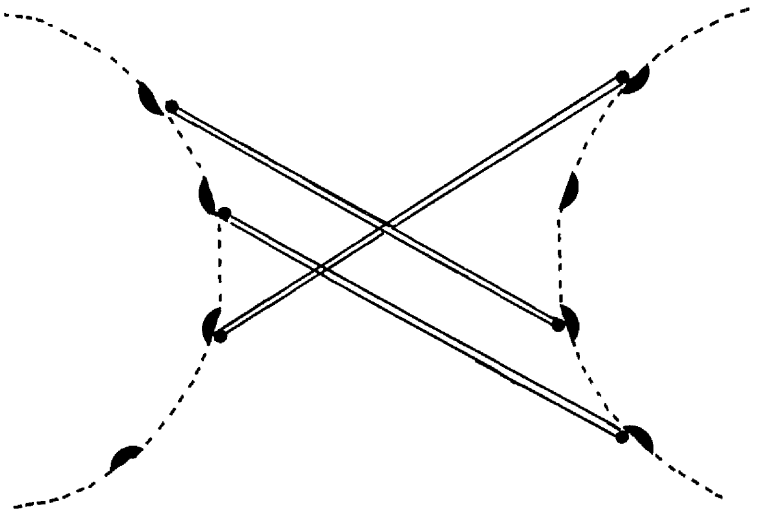


HRBC

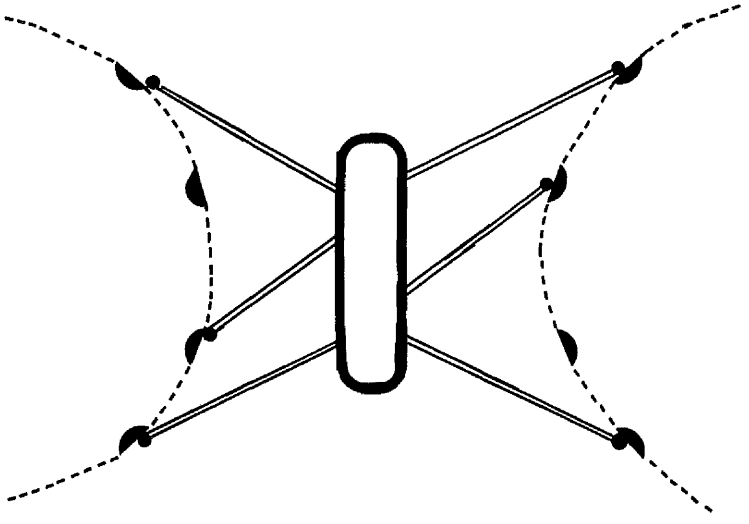
fimbrial receptor sites on HRBC

fimbrial appendages

fimbrial haemagglutinin



ISOLATED
FIMBRIAE



WHOLE, FIMBRIATE
BACTERIA

BINDING SITE AT TIP OF FIMBRIAE.

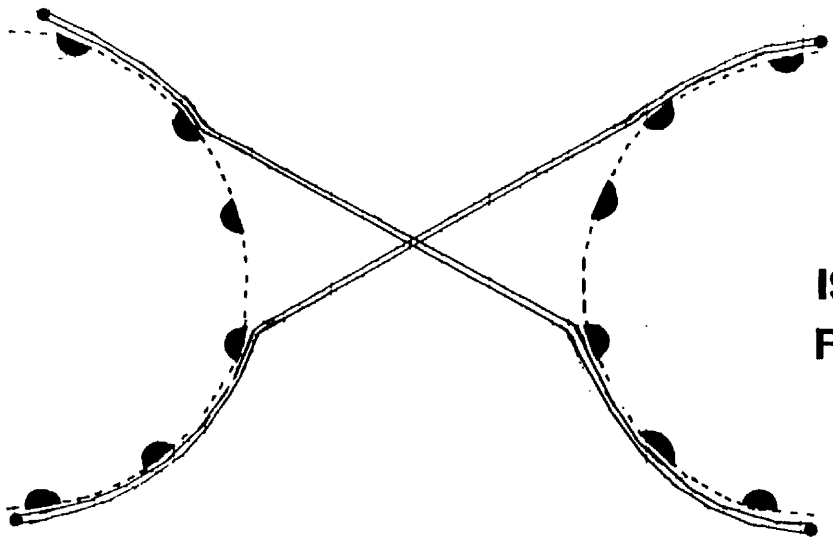
Figure 17 : Binding sites on each subunit of the fimbriae

HRBC

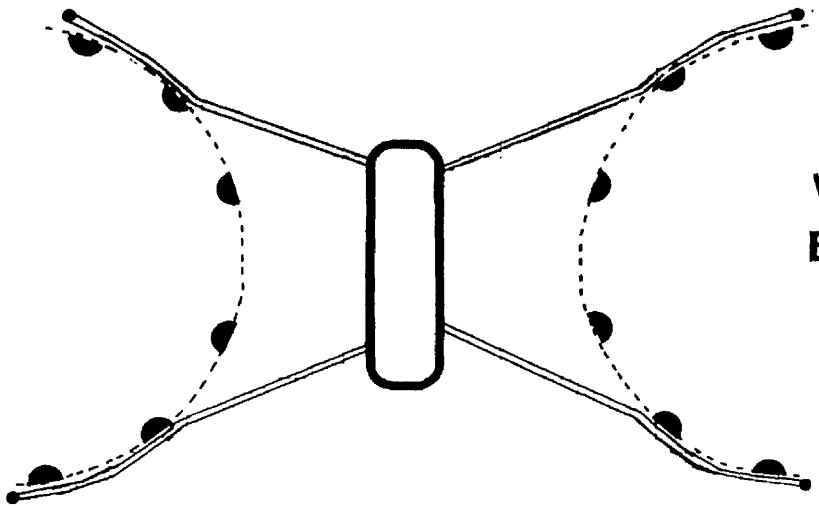
fimbrial receptor site on HRBC

fimbrial appendages

fimbrial haemagglutinin



ISOLATED
FIMBRIAE



WHOLE, FIMBRIATE
BACTERIA

BINDING SITES ON EACH SUBUNIT.

misleading in the following sense. If the fimbrial subunits are held together in the filament by hydrophobic interaction, then a break in the filament could expose at the tips relatively hydrophobic regions. Since polystyrene is a non-polar molecule, then in an aqueous environment the most favourable orientation of filaments from a thermodynamic point of view would be with the tips associated with the non-polar surface of the beads. This arrangement may be quite unrelated to the specific binding mechanism observed in the haemagglutination reactions. Attempts by Duguid (personal communication) and myself to repeat this finding failed.

A proposal that the fimbrial tip was the haemagglutinin site was presented by Old & Payne (1971). These investigators studied the fimbrial antigens of Salmonella spp., and found that some species of this genus possessed type-1 fimbriae, e.g. S. typhimurium - these were adhesive to guinea-pig erythrocytes - while others had type-2 appendages, e.g. S. gallinarum - these were non-adhesive to guinea-pig erythrocytes. By cross absorption of antisera, Old & Payne (1971) showed that both species of Salmonella carried the major genus-specific fimbrial antigens, i.e. F2, F3, F4, F5 (Campbell, 1961). Because the F1 antigen was detected in both adhesive and non-adhesive Salmonella strains this antigen was eliminated as a candidate for the adhesin. The possibility that one or other of the remaining fimbrial antigens represented the adhesin was subsequently dismissed by these investigators after the discovery that both type-1 and type-2 fimbriae of S. paratyphi B contained the same fimbrial antigens. Old & Payne (1971) concluded that the haemagglutinin was not one of the detectable antigens and was

therefore "present in small amounts at the surface of the fimbriae." From this conclusion they proposed the fimbrial tip as the haemagglutinin locus. Other interpretations of their data are equally possible, for example that the haemagglutinin is a poor antigen.

Organisms possessing type-1 fimbriae include E. coli, Sh. flexneri, Klebsiella aerogenes and some species of Salmonella. Since all of these type-1 species stick to the same range of substrates and all possess the MS adhesin characteristic of type-1 it would seem probable that they possess adhesin antigens which are either identical or very similar.

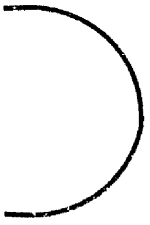
A lateral location for the fimbrial haemagglutinin was suggested by Gillies & Duguid (1958) who showed that the addition of fimbrial antibodies to Sh. flexneri caused inhibition of the HA reaction. From electron microscopic observations they found that these antibodies adhered specifically to the sides of the fimbrial filaments as opposed to the tips. The results obtained from agglutination of erythrocytes with sonicated fimbriae, where a decrease rather than an increase in HA titre was observed, also favoured this latter site. If individual filament(s) possessed haemagglutinins located solely at each tip of the filament sonication of the fimbriae would be expected to expose new haemagglutinin sites and titration of the sonicated fimbriae would produce an increased HA titre over the unsonicated preparation.

Changes in amount of bound fimbrial protein and HA titre after fimbriae were subjected to varying periods of ultrasound are reported in this thesis and favour a lateral location for the haemagglutinin.

With progressive shortening and fragmentation of the ^{125}I-labelled filaments, there is a progressive loss of HA titre yet no significant change in cell bound radioactive protein. If fragments were bound to receptors by their tips then a progressive diminution of radioactivity bound to the RBCs would be expected probably accompanied by a reduction in HA titre. Each receptor site should still be binding a single filament by the tip but the filament length and hence the radioactivity would be much reduced (Fig 18a). This was not found to be the case as far as bound radioactivity is concerned. A constant amount of radioactivity being bound with a reducing HA titre is in keeping with lateral binding sites on fimbriae. If all cell receptor sites are coupled with haemagglutinin, then the actual length of the filaments should not greatly influence the amount of protein bound, as was found to be the case (Fig 18b).

Various estimates of the length of fimbriae attached to whole bacteria were made by Duguid et al. (1955), i.e. 0.3-1.0 μ, Hashimoto et al. (1963a) and Duguid (1968), i.e. 0.2-1.5 μ. Results of length measurements made on isolated fimbriae from E. coli 8623 yielded a modal length within the range 100-199 nm (midlength 149.5 nm). When measurements were made on enlarged electron micrographs, all fimbriae showing two ends were measured on each electron micrograph. Selected areas of the micrographs were the PTA-stained appendages were clearly visible as individual filaments rather than clumps or sheets were chosen for photographic enlargement. Bearing in mind that these selections introduced a bias into the system, various calculations were made from the measurement data. The molecular weight of a fimbria of mid-modal

Figure 18 : Fimbrial haemagglutinin : tip vs lateral site



HRBC

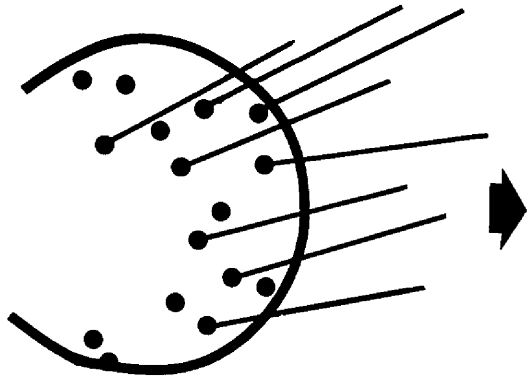
fimbrial receptor on HRBC

fimbriae

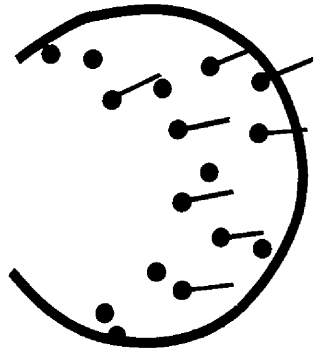
(a) HA site at tip

(b) HA site on lateral locus

(a)

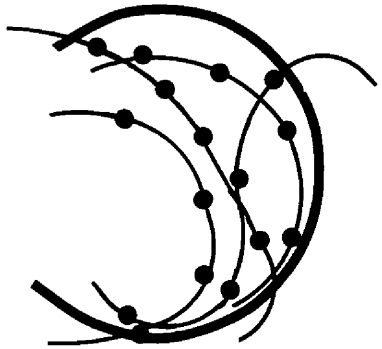


n.cpm

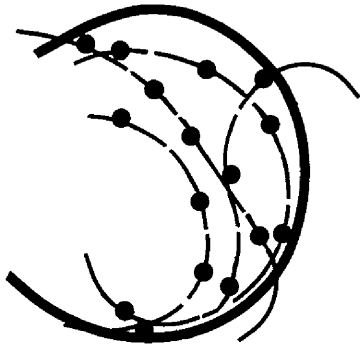


$\frac{n}{4}$ ·cpm (*Assuming length reduced 4x)

(b)



n.cpm



n.cpm (length reduced 4x)

length, i.e. 149.5 nm was calculated to be approximately 3.4×10^6 daltons (page 98), and the number of filaments in 1 µg of fimbrial protein was estimated at 17.6×10^{10} (Fig 9).

Published work by Brinton (1959) and Rivier & Darekar (1975) revealed that microgram quantities of fimbrial protein, i.e. 0.5 µg fimbrial protein and 1.0 µg fimbrial protein respectively, were sufficient to induce agglutination of erythrocytes. The results reported here using 1% (v/v) horse RBCs and isolated fimbriae concur with the former publications and further show that one haemagglutinating unit of fimbriae (1 $HU_f^{1\%}$) is equivalent to approximately 0.06 µg of fimbrial protein per 50 µl of red cell suspension. Assuming a mid-modal length of 149.5 nm, the number of molecules of fimbriae in 1 $HU_f^{1\%}$ was estimated to be approximately 1.06×10^{10} (Fig 9).

From the sonication experiments it was shown that an optimum length of fimbrial appendage is required for haemagglutination and that above or below this level the HA ability decreased. Other evidence favouring this conclusion is:

a) that the addition of $MgCl_2$ to fimbrial preparations caused elongation of the filaments (detectable by electron microscopy - Plate 5) with a concomittant decrease in HA titre
b) that sonication of fimbriae caused a simultaneous reduction in length and HA activity
c) that different batches of fimbriae of almost identical protein concentration gave different HA titres against a standard 1% (v/v) HRBC suspension.

These indicate that HA activity of the fimbriae is a function of the length of the filaments present as opposed to the protein concentration of the preparation.

III. FIMBRIAL RECEPTORS ON EUKARYOTES

The number, nature or location of type-1 fimbrial receptor molecules on eukaryotic substrates have not been established. A minimum value for the number of fimbrial receptor sites on HRBC was calculated from the amount of fimbrial protein required to saturate the binding sites on the cell. Assuming a single cell surface binding site for each filament bound, then the red cell carries at least 4×10^5 receptor sites (see page 132 for results). However, from the results presented on the binding of fimbriae after exposure to ultrasound (page 108, Fig 12) no appreciable reduction in bound fimbrial protein occurs even though the modal length is reduced by a factor of ten. This means that each filament must have had at least 10 binding sites prior to the treatment with ultrasound. Therefore the previous estimate of 4×10^5 receptor sites can be increased tenfold and still be a minimum estimate.

The role of mannose in preventing adhesion suggests a binding site at least part of which structurally resembles mannose. Two approaches to studying the nature of the fimbrial receptors on horse erythrocytes are possible:

1. Chemical alteration of the fimbrial haemagglutinin.
2. Chemical modification of the fimbrial receptor molecules.

This latter approach was chosen by Hashimoto et al. (1963b) in studying fimbrial receptors using whole fimbriate bacteria and cell suspensions of the yeast Willia anomala as substrate. The yeast cells were treated with chemical agents including 0.001 - 0.01 M periodate, 0.001 - 0.1 N HCl and NaOH, 0.25% (v/v) formalin, 95% (v/v) alcohol and proteases, i.e. trypsin and pepsin, prior to titration using whole bacteria. These investigators found that treatment of the substrate with either 0.1 N HCl or 10 mM periodate or trypsin (10 mg/ml) at pH 5.0 caused abolition of the bacterial-induced haemagglutination whereas acid or alkali did not destroy the fimbrial receptors on the substrate. In the present work, erythrocytes were exposed to a range of chemicals or enzymes and their effects on HA assessed. Treatment of RBCs with neuraminidase from either source consistently reduced but did not eliminate the fimbrial agglutination titres, did not alter the Con A titre yet increased the titre with the RCA_{120}. Neuraminidase removes accessible terminal sialic acid residues and exposes underlying subterminal saccharides which then become available for agglutination with lectins. The reduction in fimbrial titre observed with neuraminidase treatment would suggest that a reduction in the number of fimbrial receptor sites on the cell surface had occurred. If NANA or NGNA (the latter residue accounting for 90% of the sialic acids on HRBCs (Eylar et al., 1962; Hudson et al., 1964)) were the receptor molecules for fimbriae, then treatment with neuraminidase should eliminate the titre, providing that both substrates are susceptible to the enzyme and that all the binding sites are accessible to the enzyme. The fact that a reduction and not an elimination of the fimbrial titre occurred after enzyme treatment may

be due to the fact that NANA residues on glycolipids are resistant to neuraminidase treatment (Yamakawa et al., 1960). The lack of any parallel in the titres for fimbriae and for Con A after neuraminidase treatment does not support the concept of mannose being an essential component in the fimbrial binding site. The increase in RCA_{120} titre after neuraminidase treatment indicates the generation of new binding sites involving β-D-galactose or N-acetyl galactosamine. In view of the evidence for the resistance of at least a fraction of glycolipid neuraminic acid residues to neuraminidase (Yamakawa et al., 1960), this finding suggests that the subterminal residues on some of the susceptible glycoproteins or glycolipids are β-D-galactose or N-acetyl galactosamine. The subterminal residue on the sialoglycolipid described by Klenk & Padberg (1962) is indeed β-galactose. It is interesting to note the complete resistance of the agglutination reaction to α-galactosidase, which would be expected if the only exposed galactose residues were β-linked, was reported (Klenk & Padberg, 1962).

Goldstein et al. (1965) and Old (1972) inadvertantly misquoted the findings of Sumner & Howell (1936) when they reported that horse RBCs did not agglutinate with Con A. In the original 1936 publication, Sumner & Howell stated that Con A agglutinated six species of erythrocyte. Included in this group was horse cells which agglutinated with Con A "in high dilution." Receptors for this particular lectin include α-D-glucopyranose and α-D-mannopyranose (Lis & Sharon, 1973), the presence of which were reported in horse erythrocytes by Hudson et al. (1975). The former saccharide was also detected in horse glyco-

sphingolipids by Handa & Yamakawa (1964). Consequently receptors for Con A are present on HRBCs and from the results presented here (Table 19) are sufficiently accessible for lectin to bind and cause agglutination of unmodified cells.

Old (1972) highlighted the strong similarity in sugar inhibition patterns between fimbrial haemagglutinin - RBC and Con A - Dextran system. However, similarity also exists between fimbrial adhesin - HRBC and Con A - HRBC systems. Goldstein et al. (1965) showed that unmodified groups at C-3, C-4 and C-6 of the α-D-glucopyranose or α-D-mannopyranose were essential for binding to concanavalin A. Although no actual data on the binding of sugars to fimbriae was presented, Old (1972) reported that "unmodified hydroxyl groups at C-2, C-3, C-4 and C-6 of the D-mannose molecule seemed to be required for maximum binding to the fimbrial protein." Certainly from the results presented here and those of Old (1972) the most potent inhibitors of fimbrial haemagglutination have unsubstituted hydroxyls on C-2, C-3, C-4 and C-6.

The investigations reported here reveal differences between receptors for Con A and fimbrial agglutinins on horse erythrocytes. Periodate (0.05 M) treatment of HRBCs abolishes the Con A agglutinability but not the fimbrial one. Modification of horse cells with glycosidases including mannosidase - but with the exception of neuraminidase - also reduced the Con A titre without altering the fimbrial titre. The effects of treatment with either glycosidases or with periodate suggest that hexoses are not an essential part of the

fimbrial receptor on the cell membrane. This conclusion is at variance with that of Salit & Gotschlich (1976).

There are conflicting reports published concerning the action of proteases on the components of horse erythrocyte membranes (Triplett & Carraway, 1972; Fletcher & Lo, 1974; Carraway et al., 1975). The technique most commonly used for monitoring the effects of proteases involves treatment of washed erythrocytes with the enzyme, followed by the preparation of ghost membranes which are then subjected to SDS polyacrylamide gel electrophoretic analysis. Changes in the position, disappearance, or appearance of peptides and glycopeptides after enzyme treatment are detected by comparison of stained gel patterns with those obtained from untreated erythrocyte ghosts and isolated glycoproteins. This procedure was adopted by Triplett & Carraway (1972) to study the effect of trypsin and α-chymotrypsin on horse erythrocytes, resealed ghosts and isolated membranes. Their results indicated that enzymic cleavage of the major glycoprotein had not occurred on intact HRBCs. Fletcher & Lo (1974) performed similar experiments using the proteases trypsin, pronase and papain. While pronase was reported to degrade a minor fraction of the major membrane glycoprotein, cleavage by trypsin was not detected. However, papain cleaved this major glycoprotein, as demonstrated by the disappearance of the periodic acid - Schiff (PAS), staining band on the gel. Subsequent investigations by Carraway et al. (1975) involving digestion of whole HRBCs or horse erythrocyte ghosts by trypsin, chymotrypsin or pronase revealed no cleavage in intact cells with any of the proteases. The membrane proteins of isolated ghosts, however, proved susceptible

to proteolytic action of each of these enzymes as demonstrated by the appearance of low molecular weight bands which stained with coomassie blue but not with PAS.

An alternative procedure consists of electrophoresing supernatant fractions after appropriate concentration, from enzyme-treated horse erythrocytes and staining for protein and carbohydrate. Results reported in this thesis indicate that none of these proteases cleave the major membrane glycoprotein in intact cells. This is in agreement with the findings of Triplett & Carraway (1972) and Carraway et al. (1975). Although there is no published data on the effect of subtilisin on horse erythrocytes, results presented here show that this enzyme has little or no effect on the membrane proteins or glycoproteins of the intact erythrocyte. It is apparent that the presence of anionic groups on the glycoprotein may influence its susceptibility to protease cleavage. Carraway et al. (1975) reported that horse glycoproteins are resistant to proteases or ^{125}I-labelling unless the erythrocytes are pretreated with neuraminidase. Iodination of HRBCs performed by the lactoperoxidase method resulted in the iodine label being found in protein component III but absent from the major membrane glycoprotein as shown by a gel scan of labelled ghosts. However pretreatment of HRBCs with neuraminidase resulted in the major glycoprotein being labelled. The general conclusion from these studies is that the proteases tested did not cleave the major glycoprotein in intact cells. However papain was reported to cleave the glycoproteins in isolated membranes.

Of the proteolytic enzyme treatments of HRBCs reported here, only crude papain stimulated an increase in HA titre induced by isolated fimbriae. Since this increase in HA titre was not observed after treatment of HRBCs with the purified enzyme, it is possible that a contaminant present in the protease preparation could be responsible for this effect. However this increased agglutinability of HRBCs was found to be mannose-sensitive. Fractionation of the crude papain should be carried out in order to reassess this finding and determine the nature of the active constituent.

An alternative explanation for increased fimbrial agglutinability of erythrocytes following papain treatment is that the enzyme caused a reduction in surface charge density of HRBCs thereby facilitating agglutination. Indeed, Fletcher & Lo (1974) reported that papain caused the release of 50% of the sialic acid residues of intact horse erythrocytes yet Seaman & Uhlenbruck (1963) found that papain did not alter significantly the cationic character of such cells nor the electrophoretic mobility. In contrast, treatment of cells with neuraminidase released approximately 50% of the sialic acid residues and reduced the electrophoretic mobility by approximately half. In the present study, papain treatment of intact HRBCs resulted in a marked increase in susceptibility to fimbrial induced agglutination yet no detectable release of PAS positive material, nor any increased tendency to autoagglutinate.

During these experiments a marked increase in agglutinability of HRBCs with fimbriae was observed in "trypsin buffer" which contained

only 0.015 M $CaCl_2$ in 0.041 M tris-buffered saline (pH 8.0). This was attributed to the combination of calcium ions present and the alkaline pH favouring the formation of ion bridges between cell surface groups and fimbriae, thereby producing a marked increase in agglutinability of the erythrocytes.

Recently, Salit & Gotschlich (1976) reported that treatment of monkey kidney monolayer cells with either trypsin, pronase or galactosidase did not alter the attachment of whole Fim^+ E. coli to this substrate. However they did obtain increased attachment of Fim^+ bacteria to kidney cells pretreated with neuraminidase. Although this result conflicts with the effect of neuraminidase on receptor activity on HRBCs reported in this thesis, Salit & Gotschlich's work is in agreement with the publication of Fischer (1948) cited by Duguid (1968) which stated that guinea-pig RBCs treated with cholerae RDE, remained fully agglutinable with whole bacteria. The former workers showed this agglutinability to be mannose sensitive. Fischer (1948) reported that periodate-treated RBCs remained fully agglutinable by E. coli cells. This conflicts with the results of Hashimoto et al. (1963b) who reported loss of agglutinability of cells after periodate oxidation. The action of periodate on HRBCs as reported in this thesis caused reduction in agglutinability with fimbriae as opposed to complete elimination of the reaction. The effect of periodate treatment on HRBCs is unclear. Because of the effects of periodate and the fact that adhesion of isolated fimbriae from their E. coli strain was inhibited by αMM, Salit & Gotschlich (1976) concluded that fimbriae

bound to "mannose-like residues" on the kidney monolayer surface. Using horse RBCs as substrate we found that the receptor on the cell surface was partially sensitive to periodate oxidation, but that it was resistant to treatment with α-mannosidase, whereas agglutination by concanavalin A was, as expected, sensitive to this enzyme. Thus our evidence does not favour α-linked mannose residues being involved in the fimbrial receptor.

General Discussion

In investigations of cell surface components, one of the most informative approaches to the biochemist is by the use of enzymes as specific probes. They have been used widely and with a considerable amount of success in investigations of the accessibility and distribution of membrane components and the problems of "sidedness" of membranes.

The main difficulties associated with their use are

(1) defining purity and specificity

(2) demonstrating activity on cell surface components

(3) differentiating direct from indirect changes induced by enzyme treatment, e.g. activation of endogenous enzymes.

Ideally, the activity and purity of the enzymes should be confirmed before they are used for such studies as those described in this thesis. However, the high cost of these reagents and the time involved in defining the above mentioned properties are such that it is not always

possible to do so. The specificity can be assessed by recording changes in lectin agglutinability of treated cells. However, the possibility of indirect effects resulting from enzymic hydrolysis of membrane components such as activation of endogenous membrane proteases or perturbation of membrane structure by binding or insertion of enzyme molecules or hydrolysis products cannot be easily excluded. Modification of surface components using chemical procedures also presents similar uncontrollable hazards. With these limitations in mind, both enzymes and chemical procedures offer a means of investigating the cell surface and has been used here in an attempt to identify the fimbrial receptor on the horse erythrocyte surface.

Several mechanisms are possible for the adhesion of fimbriae and fimbriate bacteria to the cell surface. These are:

(a) a non-specific reaction involving either ionic interaction (electrostatic bonding either with or without the participation of cations)

(b) binding due to hydrophobic interaction between membrane components and fimbriae either with or without accompanying changes in configuration.

Both the above mechanisms could also be aided by hydrogen bond formation and London - van der Waal's forces.

(c) a specific interaction (which may involve the above two mechanisms) between an agglutinin site on the fimbriae and a receptor site on the cell surface resulting in the

formation of a binary complex similar to an enzyme/substrate complex or an antigen/antibody complex. The essence of this type of bonding is its relatively high degree of specificity.

With respect to mechanism (a), that is a non-specific ionic interaction, the evidence obtained in this thesis and elsewhere does not generally favour such a proposal. The most likely candidates for interaction on the cell surface (which carries a net negative charge at neutral pH) are the sialic acids which are largely responsible for the cells net charge. Enzymic removal of sialic acids from the cell surface has been claimed by several investigators not to eliminate or reduce the HA titre using whole E. coli (Fischer, 1948; Salit & Gotschlich, 1976). However, apparently not all sialic acids are removed by enzyme treatment since those bound to lipid are reported to be resistant to enzymic removal (Honda & Yamakawa, 1964).

The sialic acids of the HRBC surface while not appearing to play a dominant role in a non-specific electrostatic bonding mechanism may, however, be implicated in a mechanism similar to type (c) mentioned above. Indeed the reduction in fimbrial titre obtained after neuraminidase treatment of HRBCs reported in this thesis could be taken as evidence in favour of this proposal. However, other explanations are also possible, for example contaminating glycosidase activities in the enzyme preparation.

If an electrostatic mechanism is involved then it should be sensitive to pH, ionic strength and modification of -COOH and $-NH_2$- groups

on the cell surface by succinylation, carbamylation etc. However there is no positive method of showing that haemagglutination induced by fimbriae depends solely on a non-specific electrostatic bonding mechanism, since any modification of cell surface charged groups may change the properties of specific receptor sites.

As far as mechanism (b) is concerned relevant evidence presented in this thesis does not favour a solely hydrophobic interaction for the fimbrial haemagglutination mechanism. No agglutination of liposomes was observed using isolated fimbriae, nor were inside-out vesicles prepared from HRBCs susceptible to these agglutinins.

If hydrophobic interaction is responsible for fimbriae-erythrocyte binding, then agglutination of liposomes or inside-out vesicles would be expected to occur in the presence of fimbriae. The lack of reaction with inside-out vesicles derived from susceptible erythrocytes favours the third mechanism proposed above (c) and shows the polarity across the membrane with respect to receptor sites. It is also impossible to reconcile mechanisms of type (a) or (b) with the known specificity of fimbrial haemagglutination towards erythrocytes of different species.

A specific receptor site is most likely to involve either a membrane protein or a sugar-containing molecule (glycoprotein or glycolipid) since specificity in the lipid components of membranes lies largely in the hydrocarbon moieties which are not accessible to hydrophilic ligands but are embedded in the apolar interior of the membrane.

The pronounced sensitivity of haemagglutination induced by fimbriae or fimbriate cells to D-mannose or α-methyl mannoside suggests that the receptor site involves a mannose or substituted mannose residue. This narrows the possible membrane receptor molecules to either glycoprotein or glycolipid components.

Results presented in this thesis show that:

(1) neuraminidase-treated erythrocytes have a reduced susceptibility to agglutination by fimbriae

(2) the susceptibility of glycosidase-treated erythrocytes to fimbrial-induced agglutination remains unaltered yet the Con A-induced agglutination titre is reduced by all glycosidases tested

(3) the susceptibility of periodate-treated erythrocytes to fimbrial agglutination is slightly reduced whereas the Con A-induced agglutination is abolished

(4) extracted glycoproteins from erythrocytes show no competitive inhibition when added to the fimbrial-erythrocyte agglutination system

(5) treatment of erythrocytes with proteases, apart from crude papain, does not alter their susceptibility to fimbrial-induced agglutination

(6) treatment of erythrocytes with crude papain increases the susceptibility to fimbrial-induced agglutination by sixteen-fold.

The significance of the reduced titre after neuraminidase treatment is doubtful in view of the lack of competitive inhibitory activity of isolated sialic acids in this system reported by Rivier & Darekar (1976). Lack of a reduction in fimbrial-induced HA titre after treatment of HRBCs with glycosidases or when titration was done in the presence of isolated glycoproteins, points to a receptor other than glycoprotein. However, a slightly reduced fimbrial-induced HA titre was obtained after periodate treatment of HRBCs, suggesting the involvement of a sugar or sugar derivatives in the receptor site. The most likely candidate being a glycolipid, possibly a ganglioside of the type reported by Klenk & Padberg (1962). Another possible explanation of this reduction may involve some non-specified effects of periodate, other than the oxidation of vicinal hydroxyl groups. A systematic examination of the competitive inhibitory activity of purified glycolipids from HRBCs in the fimbriae-erythrocyte system would resolve this question.

Results of studies on the fimbriae involving ultrasound favour a lateral location for the haemagglutinin rather than a terminal site. No initial increase in titre of the preparation was seen after brief exposure to ultrasonic radiation but a progressive loss in titre occurred with time during five minutes exposure to ultrasound. A concomittant 92% reduction in length of fimbriae occurred during the exposure yet there was no significant reduction in bound fimbrial protein to the erythrocytes. These results favour a lateral locus for the haemagglutinin. A terminal site would have resulted in a

transient increase in titre after brief sonication, where sites would be doubled for every break of a filament. Also a reduction of bound protein would have been expected as the filaments were shortened. There does appear to be a minimal length of filament for effective cross-linking of RBCs.

From the results of equilibrium dialysis experiments, fimbriae appear to have an affinity for mannose, whereas HRBCs do not. This affinity presumably reflects the sensitivity of fimbrial haemagglutination to inhibition by mannose.

Since the receptor site is partially sensitive to periodate, but resistant to the glycosidases tested, the mechanism of mannose reversal of HA could involve a mannose-like residue on a glycolipid, the environment of which modulates its susceptibility to enzymes. Alternatively the mannose may be acting on the membrane receptor site in an allosteric manner and may not be directly involved in the geometry of the receptor ligand interface. An understanding of the exact mechanism of mannose sensitivity should result from elucidation of the chemical nature of the membrane receptor.

ADDENDUM

Since the preparation of this manuscript, OFek *et al.* (1977) have published a report on investigations into the effect of enzymes and chemical agents on fimbrial substrates. These authors found that treatment with 10 mM periodate abolished the adhesiveness of oral epithelial cells for whole fimbriate *E. coli*. This is in agreement with the work published by Hashimoto *et al.* (1963b) and Salit & Gotschlich (1976) who also used whole *E. coli* as a source of haemagglutinin.

Although OFek *et al.* (1977) verified that mannan and yeast cells (*Sac. cerevisiae*) agglutinated Fim^+ *E. coli* in this project it was shown that agglutination of these substrates also occurred with isolated appendages. In direct contrast however, OFek *et al.* (1977) concluded that mannose acted as a receptor for Fim^+ *E. coli*. No evidence in favour of this conclusion is presented in this thesis.

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APPENDIX

DISTRIBUTION OF FIMBRIAE IN NATURE

1. PROKARYOTES

a. Gram-negative bacteria.

Genus	Comments	Reference
Bordetella	20Å diam filaments, exhibit HA activity but distinct from type-1.	Morse & Morse, 1970
Bacteroides	Surface filaments resemble type-1 appendages in that they are resistant to proteases. HA occurs with sheep, guinea-pig and horse RBCs and this is lost if bacteria heated to 80°C/10 min	Okuda & Takazoe, 1974
Caulobacter	Polar appendages 1-8/cell - probably sex fimbriae	Schmidt, 1966
Neisseria	Found on T_1 + T_2 type colonies, i.e. pathogenic ones but not on T_3 + T_4 (non-pathogenic). T_1 + T_2 fimbriae have MR HA on human erythrocytes	Kellog et al. 1963 Swanson et al. 1971 Wistreich & Baker, 1971 Ward & Watt, 1972 Kraus & Glassman, 1974 Novotny & Turner, 1975 Buchanan, 1975 Novotny, Short & Walker, 1975 Koransky, Scales & Kraus, 1975 Buchanan & Pearce, 1976

Moraxella	Some exhibit "twitching motility" probably sex fimbriae	Bovre & Froholm, 1972 Pedersen et al. 1972 Simpson et al. 1976
Pseudomonas	Polar type appendages. Bacteriophages now recognised which have receptor on these fimbriae .˙. are sex type	Fuerst & Hayward, 1969 Weiss, 1971 Weiss & Raj, 1972 Bradley, 1972a,b
Vibrio	1-9 fimbriae reported on strains of Vibrio cholerae biotype El Tor which exhibits HA. This is partially inhibited by mannose	Barua & Chatterjee, 1964 Tweedy & Park, 1968 Bhaskaran et al. 1969
	b. Gram-positive bacteria	
Streptococci	Fringe of material detected around a strain of group A strep. Although related to M antigen they were called fimbriae. Not true fimbrial appendages. Polar appendages found on Strep. sanguis, which exhibit twitching motility	Swanson & McCarty, 1969 Swanson & Gotschlich, 1973 Beachey & Ofek, 1976
Corynebacterium	First report of these fimbriae on Gram-positive bacteria. Same strains weakly agglutinated sheep RBCs	Yanagawa & Otsuki, 1970 Kumazawa & Yanagawa, 1972 Ibid, 1973 Honda & Yanagawa, 1974 Ibid, 1975 Yanagawa & Honda, 1976
2. EUKARYOTES		
Ustilago violaceae	Fimbriae similar to type-1 E. coli. 60-70Å dia and 1-10 μ in length. However pronase causes digestion of fimbriae	Poon & Day, 1974 Day & Poon, 1975
Saccharomyces scotti Sac. cerevisiae Hansenula wingei	Fimbriae appear not to play role in conjunction since pronase treatment digests fimbriae but does not eliminate pairing ability	Day, Poon & Stewart, 1975

Some Properties of Isolated Fimbriae from Escherichia coli

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Common type fimbriae have been isolated and purified from an enteropathogenic strain of E. coli (NCTC 8623) and shown to cause agglutination of guinea-pig and horse erythrocytes. The fimbrial haemagglutination is inhibited by α-D-mannose at 5 μM. Other inhibitors include α-methyl mannoside and mannoheptulose. The same sugars were reported to inhibit haemagglutination by whole fimbriate cells of Shigella flexneri at approximately 1 mM by Old (Journal of General Microbiology 71, 149-157; 1972).

The lectin-like binding characteristics of fimbriae to erythrocytes indicated that saccharide moieties on the erythrocyte membrane may function as receptors. However, treatment of erythrocytes with N-acetyl-neuraminidase prior to titration with fimbriae did not affect the haemagglutination titre. A sixteen-fold increase in fimbrial haemagglutination titre was observed with papain-treated erythrocytes. This haemagglutination was also inhibited by mannose. The effects of proteolytic enzyme treatment of erythrocytes on fimbrial haemagglutination will be discussed in relation to possible fimbrial receptors in the erythrocyte membrane.

Proceedings of the Society for General Microbiology (1976) vol. III part 4, p. 175.