STUDIES ON THE ROLE OF OUTER MEMBRANE PROTEINS IN THE VIRULENCE OF NEISSERIA GONORRHOEAE

submitted by

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FACULTY OF MEDICINE

MICROBIOLOGY

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STUDIES ON THE ROLE OF OUTER MEMBRANE PROTEINS IN THE VIRULENCE OF *NEISSERIA GONORRHOEAE*

by Lloyd James

The role of surface proteins of *Neisseria gonorrhoeae* in the pathogenesis of gonorrhoea has been investigated, using colonial opacity variants having different combinations of outer membrane proteins.

The association of variants with leucocytes showed significant differences. In particular a variant containing one protein IIb (molecular weight 28,000) showed reduced association whereas another containing protein IIa (molecular weight 28,500) was greatly increased. Since variants containing protein IIa are also resistant to complement mediated serum killing and show enhanced attachment to epithelial cells, variant P9-13 (containing protein IIa as the only variable protein) was used in further attempts to determine the molecular mechanism involved in attachment.

Initially the major outer membrane protein (protein I, molecular weight 36,000) and protein IIa were isolated from outer membranes by an improved method in an antigenically reactive form. These could not however, be used in attachment experiments because it proved impossible to remove the detergent used in their extraction while retaining biological activity. As an alternative an attachment system was devised using intact outer membranes. Outer membranes of variants P9-13 (containing proteins I + IIa) and P9-1 (containing protein I only) were compared in a series of attachment experiments.

It was found that the presence of protein IIa on the surface of variant P9-13 played a significant role in increasing attachment. The surface of variant P9-13 was found to be both more hydrophobic and less negatively charged than that of variant P9-1, which would favour attachment. Evidence of a specific attachment mechanism was also found, involving protein IIa and a carbohydrate structure on the surface of buccal epithelial cells. The implications of these and other findings are discussed.

Abbreviations

SDS sodium dodecyl sulphate

LPS lipopolysaccharide

INTRODUCTION

Gonorrhoea is now epidemic in many of the world's communities, with 200 million cases reported annually (W.H.O. Annual Report, 1978). In England alone there has been a four-fold increase in the incidence of gonorrhoea since 1950. In the most susceptible age group (20 to 24 years) 27,450 new cases were reported in one year (Annual Report of the Chief Medical Officer, 1978). In a significant number of cases, the disease may become disseminated, giving rise to a wide spectrum of complications.

In one case study of infected women, 10.6% had developed salpingitis, and it was estimated that 500 women per annum are made infertile by gonococcal salpingitis in the U.K. alone (Rees and Annels, 1969).

The problem of gonorrhoea and it's complications is greatly amplified in the less medically sophisticated countries of the Third World. At one Nairobi family planning clinic, 17.5% of married and 26% of unmarried women were shown to have gonorrhoea (Hopcroft et al., 1973). In contrast, only 5.8% of women attending the Health Department Family Planning Clinic in Memphis-Shelby County, U.S.A. had gonorrhoea (Rendtorff et al., 1977).

The high incidence of gonorrhoea in Third World countries, combined with low level medical care, greatly increases the risk of disseminated infection. In the sub-Sahara region of Africa up to 50% of women never conceive due to pelvic inflammatory disease (Watt and Lambden, 1978). This situation could reach disaster proportions if the penicillinase producing gonococci, first reported by Phillips (1976) should ever become more widely disseminated. Penicillin is the most commonly used anti-gonococcal drug, because it is cheap, efficient and easily administered. Should penicillin be rendered ineffective, the recommended alternative is spectinomycin. This antibiotic is several fold more expensive than penicillin, and can only be administered parenterally.

An obvious alternative to chemotherapy for the control of gonorrhoea would be an anti-gonococcal vaccine. Provision of such an alternative has proven to be far from simple. The complexity of successful vaccine production was demonstrated when a vaccine composed of autolysed whole cell debris of three different strains of gonococci

was shown, not only to be non-protective, but was associated with an increased susceptability to infection (Greenberg et al., 1974).

It is clear that the successful production of an anti-gonococcal vaccine depends on a comprehensive understanding of the specific components involved in the virulence and immuno-biology of Neisseria gonorrhoeae.

Virulence of N. gonorrhoeae, and the pathogenesis of gonorrhoea

The gonococcus shows a high degree of species and tissue specificity. It is almost solely a human pathogen, and it's most common portal of entry is via the columnar epithelial cells of mucosal surfaces (Harkness, 1948). Detailed electronmicroscopy studies of urethral tissues and fallopian tube organ cultures infected by gonococci have illustrated many of the stages of attachment and invasion, and a generalised model for these processes has been proposed (Watt et al., 1978; Ward and Watt, 1972; Ward et al., 1975).

The initial stage of infection involves the attachment of gonococci to columnar epithelial cells, and is followed by cell penetration. It was suggested that the close proximity of the gonococcal cell to the surface of the host cell initiates phagocytosis which proceeds by a process involving ligands on the surface of the gonococcus interacting with receptors on the host cell surface, somewhat in the same way as has been suggested for ingestion by professional phagocytes (Griffin et al., 1975, 1976). The sequential binding interaction of ligands and receptors creates a zipper mechanism, eventually resulting in the gonococci being totally engulfed in the invaginated host cell membrane. No evidence was found of gonococcal penetration between epithelial cells, or passage to sub-epithelial tissues inside professional phagocytes. The direct erosion of host tissue cell-membrane was excluded by showing that cells remained impervious to heavy metal colloids throughout invasion. Following penetration, intracellular multiplication probably occurs and is followed by eventual cell rupture and invasion of sub-epithelial connective tissue. In the majority of cases, invasion is followed by an inflammatory response (Holmes, Eschenbach and Knapp, 1976) leading to symptoms typical of gonorrhoea; but a few are asymptomatic (3%).

Most clinical isolates of gonococci are serologically cross-reactive (Ward et al., 1978) and are susceptible to antibody-complement mediated killing by normal human serum (Ward et al., 1978). However in

those few cases which result in disseminated infection the organisms have been found to be resistant to the bactericidal action of human serum, presumably due to an alteration in surface components (Schoolnik, 1976).

In a few cases, host deficiencies in the complement pathway may also play a role (Petersen et al., 1979; Nicholson and Lepow, 1979).

Attachment The attachment of gonococci to the host cell surface is an essential early stage in the pathogenesis of gonorrhoea. Obviously, if this process is inhibited, resistance to further infection would be greatly enhanced. The preferential attachment of gonococci to columnar epithelial cells, highlights the highly developed nature of the gonococcal attachment mechanism. Columnar epithelial cells have a highly mobile surface due to their active secretion of mucus; and in sites such as the urethral mucosa, they are periodically scoured with urine. Yet despite such adverse conditions gonococci, unlike commensal bacteria, are able to adhere to, and colonise such sites (Mardh and Westrom, 1976). The identification of the surface component(s) involved in attachment would provide a strong candidate(s) for a vaccine.

Gram-negative bacteria have a cell wall structure which is both architecturally and functionally complex (DiRienzo et al., 1978). The gonococcal outer membrane is similar to that of other Gram-negative bacteria (Fig. 1), being composed of lipopolysaccharide (Fig. 2), phospholipids and a few proteins (Johnston and Gotschlich, 1974).

Ward et al. (1978) using purified gonococcal components to raise specific antibodies, found that anti-lipopolysaccharide was the most efficient in a bactericidal system using homologous and heterologous gonococci. However lipopolysaccharide is unlikely to be the significant outer membrane component in attachment of gonococci to mammalian cells. It has also been shown that lipopolysaccharide extracted from a wide variety of Neisseria species, some commensal (Johnson et al., 1975), gonococci (Stead et al., 1975) and meningococci (Jennings et al., 1973) is similar, suggesting that it is unlikely to be a specific virulence factor(s) of gonococci. Thus pili and the outer membrane proteins appear to be the most likely gonococcal adhesions.

Pili Early studies with human volunteers showed that gonococci of piliated colonial type were more virulent than non-piliated types

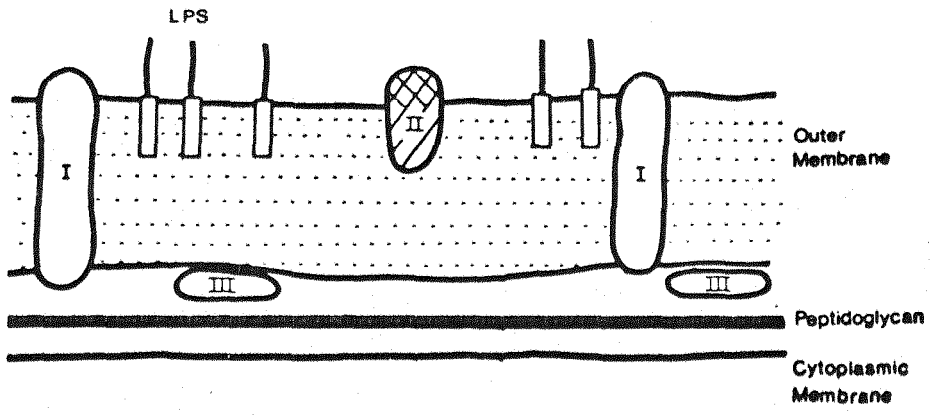


Figure 1 Model of the outer membrane of Neisseria gonorrhoeae (Courtesy of Dr. J.E. Heckels, Microbiology, Faculty of Medicine, University of Southampton)

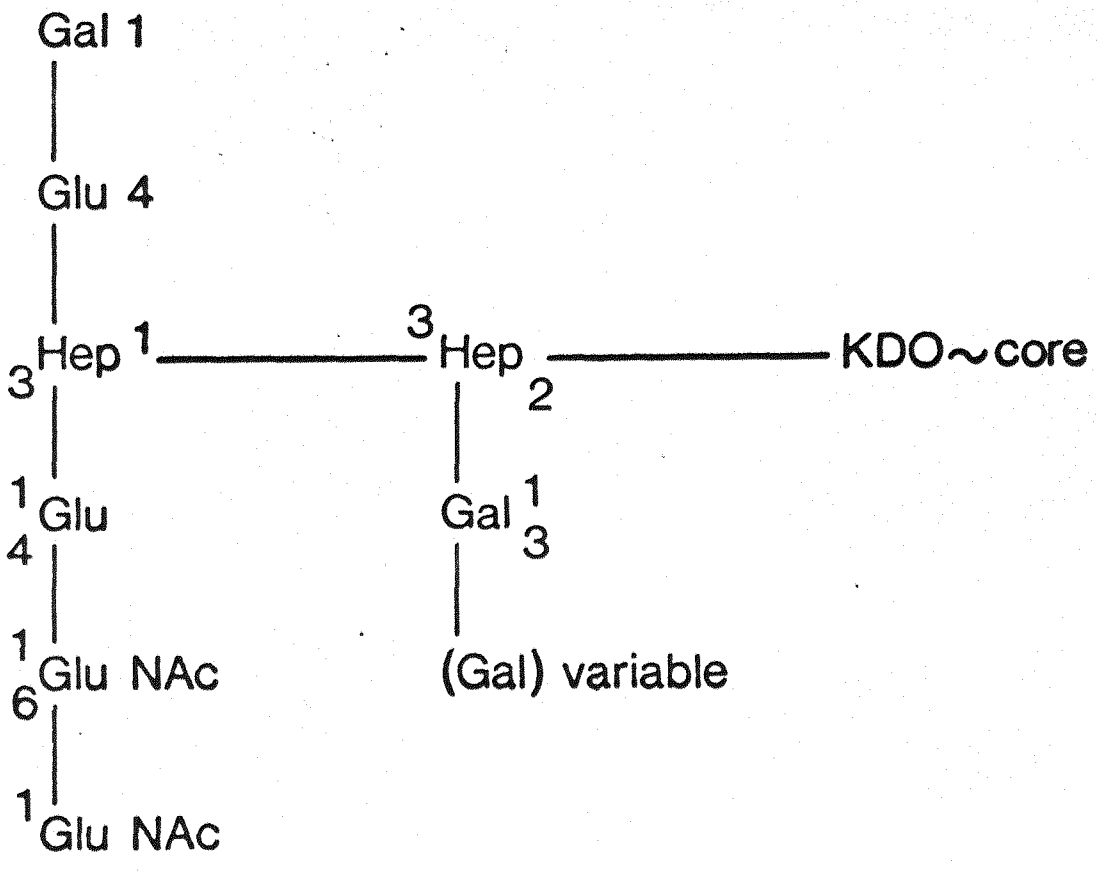


Figure 2 The chemical structure of gonococcal lipopolysaccharide (As reported by M.B. Berry at the San Francisco meeting of the American Microbiology Society, January 1978)

(Kellogg et al., 1968). In addition the possession of pili is correlated with increased adhesion to several epithelial cell types suggesting a mechanism for the increased virulence (Swanson, 1973). This was strengthened by the observation that gonococci appear to attach to epithelial cells via pili (Ward et al., 1975). A four-fold increase in attachment of piliated gonococci over non-piliated gonococci of the same strain, was abolished by blocking negatively charged groups on the gonococcal surface (Heckels et al., 1976). These observations led to a model relating increased virulence and piliation. The pilus seems to provide a functional means of penetrating the electrostatic-repulsive barrier between the negatively charged surfaces of mammalian cells and gonococci to make an initial contact, with or without specific host cell receptors at the pilus tip.

The gonococcus is constantly acted upon by random molecular and hydrodynamic forces which may combine with the restraining force of the attached pili to bring it into close contact with the host cell. Presumably close contact will involve the gonococcal outer membrane components. Although piliation has been shown to enhance attachment, non-piliated variants are nevertheless seen to attach to the same cells, and once attached are equally efficient at invasion (Watt et al., 1976). Close contact, high affinity binding of gonococci to the host cell surface, therefore, most likely involves outer membrane proteins (Watt, 1980)

Outer membrane proteins of gonococcal strain P9 Gonococcal outer membranes, like those of other Gram-negative bacteria, contain relatively few proteins. The composition of outer membrane proteins of colonial opacity variants of strain P9 has been studied in detail (Lambden and Heckels, 1979). One protein (protein I, molecular weight 36,000) present in all colonial variants is expressed on the surface of the gonococcus (Heckels, 1977, 1978), and is probably responsible for serospecificity (Johnston et al., 1976). A further protein (protein III, molecular weight 60,000) (Heckels and Everson, 1978) has been shown to be loosely associated with the internal surface of the gonococcal outer membrane, and is not expressed on the external surface (Heckels, 1978). In addition variants with opaque colonial phenotypes, produce one or more additional proteins in the molecular weight range 29,000 - 27,500 (proteins II to IId) (Lambden

and Heckels, 1979; Lambden et al., 1979). Similar proteins are also found in opaque colonial variants of other gonococcal strains (Swanson, 1978). Recent peptide mapping studies (J.E. Heckels, personal communication) have shown these proteins to be remarkably similar, with the greatest structural variation occurring in the surface exposed portion of the molecule. Possession of the variable proteins can be correlated with significant differences in biological properties. The variable proteins present in opaque colonial forms are more susceptible to trypsin digestion than protein I, which correlates with increased killing of opaque variants by proteolytic enzymes (Swanson, 1978). This may account for the selection of transparent colonial variants of gonococci by the protease rich environment of the cervix at menstruation (James and Swanson, 1978). A study of the variations in virulence related properties of colonial variants of strain P9 focused attention on protein IIa (molecular weight 28,500) (Lambden et al., 1979). It was found that variants having protein IIa in their outer membranes were the most resistant to serum killing; of the non-piliated variants, attached most avidly to buccal cells, and in the case of one variant P9-13 (containing IIa only) showed the highest leucocyte association.

The aim of this study was to attempt the purification of protein IIa in order to investigate it's role in the pathogenicity of gonorrhoea, and particularly in the virulence related attachment of Neisseria gonorrhoeae to mammalian cells.

MATERIALS AND METHODS

Materials

Carboyanine dye (Stainsall) was obtained from B.D.H., Poole, Dorset. Trypsin-T.P.C.K. was obtained from Worthington Biochemical Corp., Freehold, New Jersey 07728, U.S.A. Trypsin inhibitor (type 11L from lima beans) was obtained from Sigma Ltd., Fancy Road, Poole, Dorset. Mixed glycosidases (Lot ET4602) were obtained from Miles, Stoke Poges, Slough. Empigen BB was a generous gift from Albright and Wilson, Whitehaven, Cumbria.

Methods

Growth and storage of bacteria. *Neisseria gonorrhoeae* Strain P9 was grown on 8.5 cm diameter plates of solid typing medium of the following composition:-

	per 500 ml
Thiotone (B.B.L. Cockeysville, U.S.A.)	3.75 g
Trypticase (B.B.L. Cockeysville, U.S.A.)	1.87 g
Soluble starch	0.5 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
NaCl	2.5 g
Agar (Oxoid No. 1)	5.0 g

Supplements were prepared containing:-

A (4 ml)

D-glucose	0.5
L-glutamine	50 mg
NAD (DPN)	1.25 mg
Cocarboxylase	0.5 mg
Thiamine-HCl	15 µg
Vitamin B12	50 µg
$Fe(NO_3)_3$	100 µg
p-aminobenzoic acid	65 µg

B (1 ml)

Adenine	5 mg
Guanine-HCl	0.15 mg
L-cysteine-HCl	0.13 mg

Supplements were filter sterilised and added to the medium just before it was poured.

Colonial variants were selected according to the method of Swanson (1978). The selection involved the examination of well-spaced colonies using a stereomicroscope equipped with a plane polished substage reflector adjusted to differentiate between transparent and opaque colonies. Two variants were used in this study, P9-1 and P9-13(Fig. 1). Colonies of P9-1 were large and flat, non-granulated, transparent, with an occasionally bluish tinge and well defined entire edges. In contrast colonies of P9-13 were large and very flat with an irregular outline. They were also granulated with a complex colouration and a bright, strongly defined but non-crenated edge. Variants were suspended in 1% (w/v) proteose peptone (Difco) containing 10% (v/v) glycerol as a cryoprotectant, and stored in a liquid nitrogen refrigerator (Ward and Watt, 1971).

Preparation of gonococcal outer membrane Bacteria for the production of outer membrane were grown on 27 cm x 37 cm plates of the typing medium described above. Bacteria were harvested into ice-cooled phosphate buffered saline and then recovered by centrifugation at 30,000 g for 30 min. The resultant pellet was placed in 0.2M lithium acetate solution pH 6.0 at 45°C (240 ml) and gently homogenised to disperse the organisms. The mixture was then shaken for 2½h at 45°C, and the bacteria removed by centrifugation at 30,000 g for 20 min. The supernatant solution was subjected to repeated centrifugation until only a trace of a bacterial pellet was seen.

Outer membrane was recovered from the supernatant solution by centrifugation at 100,000 g for 2.5 h, washed twice in 0.2M sodium acetate buffer pH 5.8 (Heckels, 1977). The crude membranes were resuspended in the same buffer containing 6M urea and incubated at 25°C for 30 min to remove protein III and other loosely associated proteins (Heckels and Everson, 1978). Outer membrane vesicles were recovered by centrifugation at 100,000 g for 2.5 h and washed twice in

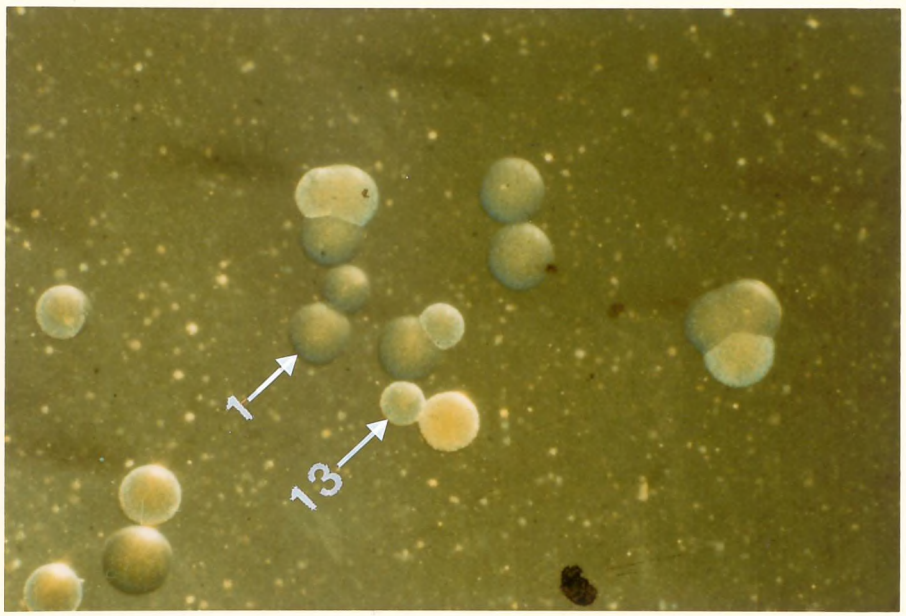


Figure 3 Opacity variant colonies of N. gonorrhoeae strain P9

water. The final product was suspended in water and stored at -20°C until required.

Protein determinations (Lowry et al., 1951) (1) Alkaline copper reagent was prepared by mixing 1% (w/v) copper sulphate (0.5 ml) with 2% (w/v) potassium tartrate (0.5 ml), and adding the resultant solution to 2% (w/v) sodium carbonate in 0.1N sodium hydroxide (50 ml). (2) Folin Ciocalteus phenol reagent was prepared by dilution to be equivalent to 1N acid.

Samples (containing 10 to 50 ug protein) were adjusted to 100 µl with water, alkaline copper reagent (1 ml) was added and incubated at room temperature for 15 min. Folin reagent (100 µl) was then added and the mixture was incubated for a further 30 min. The E_{750} of each sample was read against a reagent blank and the concentration of protein determined by reference to a calibration curve obtained using bovine serum albumin standards similarly treated.

Protein assays in the presence of sucrose and Triton X-100 were performed by the method of Markwell et al. (1978).

A modified alkaline copper reagent was prepared by adding 4% (w/v) copper sulphate solution (1 ml) to a solution (100 ml) containing 2% (w/v) sodium carbonate, 0.4% (w/v) sodium hydroxide, 0.16% (w/v) sodium tartrate and 1% (w/v) SDS immediately before use.

Samples (containing 10 to 50 µg protein) were adjusted to 300 µl with water, modified copper reagent was added and the mixture was allowed to incubate at room temperature for 30 min. Folin reagent (100 µl) was then added and after a further 45 min incubation the E_{660} of each sample was read against a reagent blank. The concentration of protein in the samples was determined by reference to a calibration curve obtained using bovine serum albumin standards similarly treated.

Carbocyanine dye assay for L.P.S. (Janda and Work, 1971) Carbocyanine dye reagent solution was prepared by dissolving carbocyanine dye (10 mg) in 1,4 dioxan (10 ml) and 0.03M sodium acetate buffer, pH 4.05 (10 ml), and the solution diluted with 0.03M sodium acetate buffer, pH 4.05 (80 ml). The resultant solution (15 ml) was further diluted with 0.03M sodium acetate buffer, pH 4.05 (10 ml) and water (25 ml), and 0.1M ascorbic acid (0.3 ml) was added just before use.

Samples (containing 1 to 10 μg lipopolysaccharide) were adjusted to 100 μl with water, carbocyanine dye reagent solution (1 ml) was added, and allowed to stand at room temperature for 5 to 10 min. The E_{472} of each sample was read against a reagent blank and the concentrations of lipopolysaccharide determined by reference to a calibration curve obtained using a standard lipopolysaccharide solution treated similarly.

Reducing sugar assay (Park et al., 1949) Samples (containing 1 to 8 μg reducing sugar) were adjusted to 100 μl with water, 0.05% (w/v) potassium ferricyanide solution (100 ul) and 0.065% (w/v) potassium cyanide in 0.53% (w/v) sodium carbonate solution (100 μl) were added, then boiled for 10 min. 0.025M sulphuric acid solution (500 μl) containing 0.15% (w/v) ferric ammonium sulphate and 0.1% (w/v) SDS was added to the cooled samples, which were allowed to incubate at room temperature for 15 min. The E_{690} for each sample was read against a reagent blank, and the concentration of reducing sugar determined by reference to a calibration curve obtained using glucose standards similarly treated.

Total carbohydrate determination: phenol method (Dubois et al., 1956) Samples (containing the equivalent of 20 to 100 μg glucose ml^{-1}) were adjusted to 200 μl with water. After adding 5% (w/v) phenol solution (200 μl) with mixing, concentrated "Analar" sulphuric acid (1 ml) was added, completely mixed, and the mixtures were allowed to stand at room temperature for 10 min, followed by 15 min incubation at 30°C. The E_{488} of each sample was read against a reagent blank, and the concentrations of carbohydrate equivalent to glucose determined by reference to a calibration curve obtained using a standard glucose solution treated similarly.

SDS-polyacrylamide gel electrophoresis (Lambden and Heckels,1979)

Reagents:-

a) 50% (w/v) acrylamide solution containing 1.3% (w/v) bis-acrylamide

b) 2% (w/v) sodium dodecyl sulphate solution

c) Separating gel buffer (1.2M Tris-HCl) pH 8.8

d) Stacking gel buffer (0.25M Tris)HCl) pH 6.8

e) Running gel buffer (0.025M Tris) containing 0.19M glycine and 0.1% (w/v) SDS, pH 8.3

f) 0.5% (w/v) ammonium persulphate solution

g) Dissociating buffer (0.25M Tris-HCl) containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue

Method

A linear gradient of 10 to 25% (w/v) acrylamide was generated using a triple channel pump with solutions of the following compositions (16 ml of each solution) (Size = 20 x 14 cm).

	25% solution	10% solution
50% (w/v) Acrylamide	10 ml	4 ml
Separating buffer	5 ml	5 ml
Glycerol	2 ml	-
SDS	1 ml	1 ml
Persulphate	0.2 ml (add last)	0.4 ml
N,N!N'-tetramethylenediamine	0.01 ml	0.01ml
H_2O	1.8 ml	9.6 ml

A 3% (w/v) acrylamide stacking gel was then added of the following composition:-

30% (w/v) acrylamide	1 ml
Stacking gel buffer	5 ml
SDS solution	0.5 ml
Persulphate solution	1 ml
Water	2.5 ml

Sample application wells (24) were cast in the stacking gel by use of a toothed former. Samples (5 to 25 µg protein) were suspended in water (25 µl); dissociating buffer (25 µl) was added, the mixture was heated for 5 min at 100°C. The samples (50 µl) were applied to the gel and electrophoresis was carried out at 180V and 4°C for 24 h.

Fixing and staining gel (Fairbanks et al., 1971) The gel was placed in a staining solution of 0.05% (w/v) Comassie blue R250 in a solvent fixative of acetic acid; isopropanol and water in the relative proportions 1:2:7, for 24 h at 37 °C. The gel was destained in acetic acid; isopropanol and water of relative proportions 1:1:8 (6 to 9 h at room temperature) and then several changes of 10% (v/v) acetic acid until the background was fully destained leaving the stained protein bands visible. Standard proteins were run in parallel with the samples.

Before autoradiography could be performed on gels, they were dried. Cracking of gels during drying was greatly reduced by first immersing them in shrinking fluid containing 50% (v/v) methanol; 10% (v/v) glycerol in water for 4 h at room temperature. Gel drying took place using a 'Raven' gel drying apparatus under vacuum 16 to 20 h, or until examination showed the drying process to be complete.

Autoradiography Autoradiography of gels containing radioactively labelled proteins was carried out by placing the gel in contact with Kodak X-Omat H x-ray film. An optimal time exposure was determined (usually 6-24 h) and the film was developed in an X-Omat automatic film processor.

^{125}I-labelling of outer membrane protein vesicles

Reagents

a) 0.5M sodium phosphate buffer, pH 7.5

b) 0.05M sodium phosphate buffer, pH 7.5

c) Carrier-free Na^{125}I (1 mCi/10 µl) in 0.5M sodium phosphate buffer

d) Chloramine T (10 mg ml^{-1}) in the 0.05M sodium phosphate buffer

e) Sodium metabisulphite (2.5 mg ml^{-1}) in 0.05M sodium phosphate buffer

f) KI (5 mg ml^{-1}) in 0.05M sodium phosphate buffer

Method

Samples of outer membrane protein vesicles (containing 0.5 mg protein) were suspended in buffer 'a' (12.5 µl) and buffer 'b' (250 µl).

Iodine solution (5 µl) and chloramine T solution (12.5 µl) were added to react for 5 min at room temperature. The reaction was terminated by the addition of sodium metabisulphite (125 µl) and KI (125 µl) solutions. The membranes were recovered by centrifugation at 100,000 g for 2 h, washed three times with water, resuspended and stored at -20°C until required.

Preparation of negatively stained samples for transmission electronmicroscopy Samples (5 µl) of membrane protein vesicles were applied to 'Formvar' carbon coated E.M. grids. After one minute excess liquid was removed and replaced with 2% (w/v) uranyl acetate solution (5 µl), which after a further one minute was also removed and the grid was allowed to dry. Grids were examined using a Phillips 300 Transmission Electronmicroscope.

Preparation of antisera to outer membranes, and antibody assay Antisera to outer membranes were raised in New Zealand White Rabbits. Membranes (1 ml) were suspended in Freund's complete adjuvant (1 ml) to a final concentration of 50 µg protein/ml and injected into eight sites on the back and hind legs (approx. 0.5 ml per hind leg, and the remainder distributed equally between the other six sites). Booster immunisations were given in a similar manner on days 40 and 80, except that Freund's incomplete adjuvant was substituted. On day 90 blood (25 ml) was taken from a marginal ear vein and the serum stored at -20°C.

Double diffusion immuno-precipitation tests of purified protein I were performed against anti-outer membrane serum in 1% (w/v) purified agar (Oxoid) gels containing 0.02M barbitone buffer pH 8.5 and 1% Empigen-BB.

Standard method of attachment of outer membrane vesicles to buccal epithelial cells (Lambden et al., 1979) Standard attachment buffer was prepared according to the following composition:-

50 mM Tris-acetate buffer pH 7 containing:-
140mM NaCl
5mM $CaCl_2$
4mM KCl
2mM $MgCl_2$
Bovine serum albumin (1 mg ml^{-1})

In experiments carried out at different pH adjustments were made to the standard buffer by altering the quantities of Tris or acetic acid added to the solution (range pH 4 to pH 8.5).

Experiments were carried out in bovine serum albumin coated polypropalene vials prepared by soaking in phosphate buffered saline pH 7.4 containing 1% (w/v) bovine serum albumin and 0.005M EDTA for 16 to 20 h at 4°C. Immediately before use the vials were rinsed three times in phosphate buffered saline pH 7.4, and not allowed to dry.

Buccal cells were collected from human volunteers by scraping with a wooden spatula, pooled, suspended in phosphate buffered saline and centrifuged at 500 g for 5 min. The pellet was washed three times in phosphate buffered saline once in attachment buffer pH 7 and was finally suspended in the same buffer to a packed cell volume of 5%. ^{125}I-labelled outer membrane vesicles (1.74 ug protein) suspended in the same medium (200 µl) were added to the buccal cell suspension (400 µl) and incubated with gentle mixing at 37°C for 2 h. Samples of the mixture (4 x 100 µl) were then layered onto cushions (4 ml) of 6% (w/v) dextran in 9% (w/v) NaCl (Dextraven, Fisons) in polystyrene centrifuge tubes (75 mm x 12 mm). The tubes were centrifuged at 500 g for 1 min to sediment the buccal cells and adherent outer membrane vesicles. The dextran solution was then removed and the radioactivity associated with the sedimented buccal cells was determined using a LKB 1280 Ultragamma gamma counter. The percentage of vesicles adhering to the buccal cells was determined as:-

[100x (mean cpm in pellet) - (cpm pelleted in control without buccals)] / [total cpm applied to tube]

Modified attachment method for erythrocytes and substituted agarose gels (Lambden et al., 1979) To investigate different attachment systems the standard method was modified and materials substituted for the buccal cells were prepared as follows.

Erythrocytes used in attachment experiments were obtained from freshly collected, peripheral blood (containing 10I.U. ml^{-1} heparin) by centrifugation at 2,000 g for 10 min. The 'buffy' coat was removed and the pellet was washed three times in phosphate buffered saline, once in attachment buffer pH 6.5 and was finally suspended in the same buffer to a packed cell volume of 5% . Attachments to diethylaminoethyl (DEAE) agarose anion exchange gel (Biogel A, Biorad) (20% v/v) and alkyl substituted Agarose gels (10% v/v) was determined in a similar manner.

EXPERIMENTAL AND RESULTS

Gonococcal/Leucocyte association Leucocytes were prepared essentially as described by King and Swanson (1978). Freshly collected peripheral blood (25 ml) was mixed with preservative free 'Heparin' (4 I.U. ml 1^{-1} final conc.) and added to an equal volume of 2% (w/v) gelatine in 0.9% (w/v) saline, mixed and allowed to stand for 30 min at 37°C to sediment the erythrocytes. Leucocytes were recovered from the resultant leucocyte enriched plasma by centrifugation at 50 g for 5 min and washed once in tissue culture medium of the following composition:-

TC199 (x10 concentration)	25 ml
L-glutamine	73 mg
Hepes	0.96 g
1M NaOH	2.525 ml
Preservative-free 'Heparin'	500 I.U.
Water to make 250 mls	pH 7

The washed cell pellet was suspended in 0.85% (w/v) ammonium chloride in 0.01M $KHCO_3$ buffer pH 7.4 containing 0.001M EDTA (4 ml) and incubated at room temperature for 5 min to lyse contaminating erythrocytes. Tissue culture medium (6 ml) was added and the cell suspension was centrifuged at 50 g for 5 min. The resulting leucocyte pellet was washed three times in modified tissue culture medium containing bovine serum albumin (25 mg/250 ml) but omitting heparin. A suspension (130 µl) containing 6.5×10^5 leucocytes was applied to a coverslip (13 mm diameter) and the cells allowed to adhere for 1 h at 37°C. The attached leucocytes were washed and stored in modified tissue culture medium.

Standardisation of gonococcal variants *N. gonorrhoeae* P9 variants (1, 2, 6, 9, 11, 13, 16 and 19) were suspended separately in phosphate buffered saline (1 ml) to yield a cloudy suspension which was centrifuged at 150 g for 1 min to remove aggregates of bacteria. The E_{560} of 1:10 dilutions of the resultant suspensions were determined. The suspensions were adjusted with modified tissue culture medium to give a final E_{560} equivalent to 0.05 (containing 1.4×10^8 cfu's ml^{-1}). The suspensions (100 µl) were added to the prepared leucocytes and incubated

Table 1

Association of variants of N. gonorrhoeae P9
with polymorphonuclear leucocytes

Variant	P9-1	P9-2	P9-6	P9-9	P9-11	P9-13	P9-16	P9-19
Variable proteins	-	piliated	II	IIa,IIb	IIa,IIa	IIa	IIb	II,IIc
association	10	8	21.5	20.5	20.2	32	4.6	21

in an enclosed container at 36°C on an orbital shaker rotating at 140 rev. min^{-1} for 20 min. The coverslips were washed, air-dried and stained with 0.25% (w/v) acridine orange (Smith and Romnel, 1977). Coverslips were coded and examined using an Ortholux II microscope (Leitz, Wetzlar, W. Germany) fitted with Ploem I incident ultraviolet illumination (barrier filter K570; excitation filter VP425 + 3 mm BG3 and a TK510 dichroic mirrow with a K515 suppression filter). Results (Table 1) were expressed as the percentage of polymorphs with associated gonococci and are the mean values from three separate experiments.

Differences in gonococcal-leucocyte association was seen between the variants. Outer membrane protein composition had a significant effect on leucocyte association, but piliation did not. Variant P9-16 showed decreased association, but other variants showed two to three-fold enhancement over the prototype P9-1.

Trial extraction of outer membranes with various detergents In order to attempt the purification of individual proteins, membranes were extracted with a range of detergents to determine the most efficient for solubilisation of outer membrane components. Membranes from variant P913 were suspended at a concentration of 4 mg protein ml^{-1} and separately extracted with the following detergent solutions: 1% (v/v) Triton X-100 in 0.05M Tris-HCl pH 7.4 containing 5mM EDTA; 1% (v/v) Empigen BB (Albright and Wilson, Whitehaven, Cumbria) in 0.1M glycine NaOH buffer pH 9.6; 1% (w/v) sodium cholate in glycine buffer pH 9.6 containing 5mM EDTA. The suspensions (150 ul) were gently shaken at 37°C for 1 h then centrifuged at 100,000 g for 15 min in a Beckman Airfuge centrifuge. The supernatant solution was removed and analysed for protein (Table 2). The results showed that Empigen-BB treatment though the most efficient in solubilising membranes, unlike cholate (Heckels, 1977) was non-selectively removing proteins I and IIa (Fig.4a).

A two stage extraction procedure was thus adopted for the purification of proteins I and IIa. Membranes were suspended in cholate-glycine buffer (1.5 ml),incubated at 37°C for 1 h and centrifuged at 100,000 g for 2 h. The residue was resuspended in Empigen BB-glycine buffer, re-extracted and insoluble material removed by centrifugation at 100,000 g for 2 h. Analysis at each stage (Table 3) showed that the bulk of the LPS together with 27% of the protein was removed by cholate treatment. SDS-polyacrylamide gel electrophoresis of the cholate soluble

Table 2

Analysis of detergent extracted protein

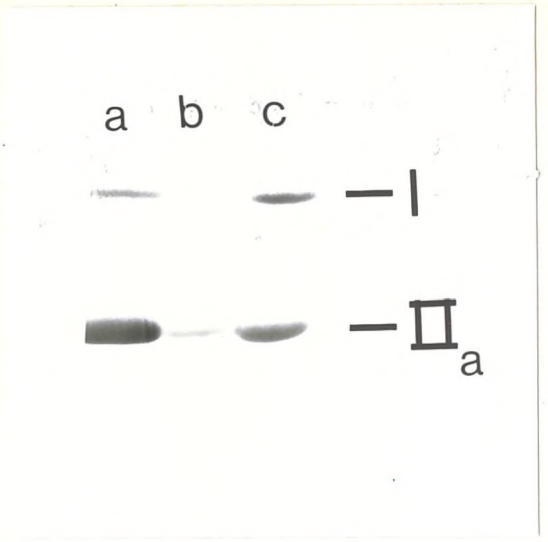
Solvent solution	Initial total protein	Total insoluble protein	Total soluble protein	Total recoverable protein	Percentage of total recoverable protein solubilised
1 (w/v) SDS	531 µg	250 µg	290 µg	540 µg	54
1 (v/v) Empigen BB	531 µg	145 µg	363 µg	508 µg	71
1 (w/v) Sodium cholate	531 µg	375 µg	163 µg	538 µg	30
1 (v/v) Triton X-100	531 µg	335 µg	188 µg	523 µg	36
NaCl	531 µg	575 µg	0 µg	575 µg	0
90 (v/v) n-Butanol	106 µg	65 µg	41 µg	106 µg	39

Table 3

Distribution of LPS and protein in fractions obtained on sequential extraction of outer membranes

	Cholate soluble fraction	Cholate insoluble/ Empigen soluble	Insoluble residue
LPS	90%	6%	4%
Protein	27%	55%	18%

(Results are expressed as percentage of the component originally present in membranes)



Fraction 4a SDS-Polyacrylamide gel electrophoresis of detergent extracts of outer membrane:
(a) original outer membrane
(b) cholate-soluble fraction
(c) Empigen-soluble fraction

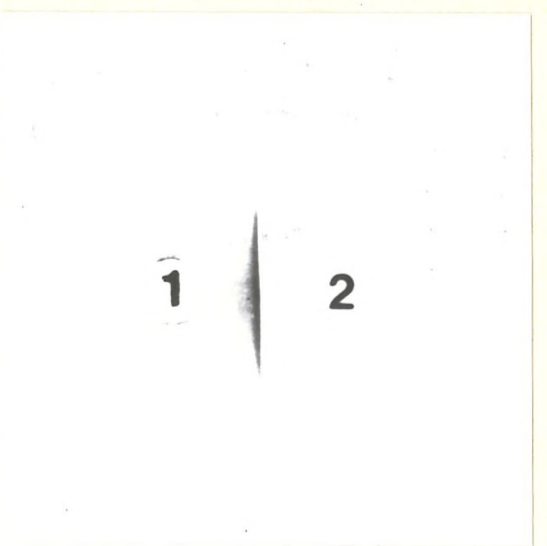


Figure 4c Ouchterlony immunodiffusion of purified protein I (well 2) against anti-outer membrane serum (well 1)

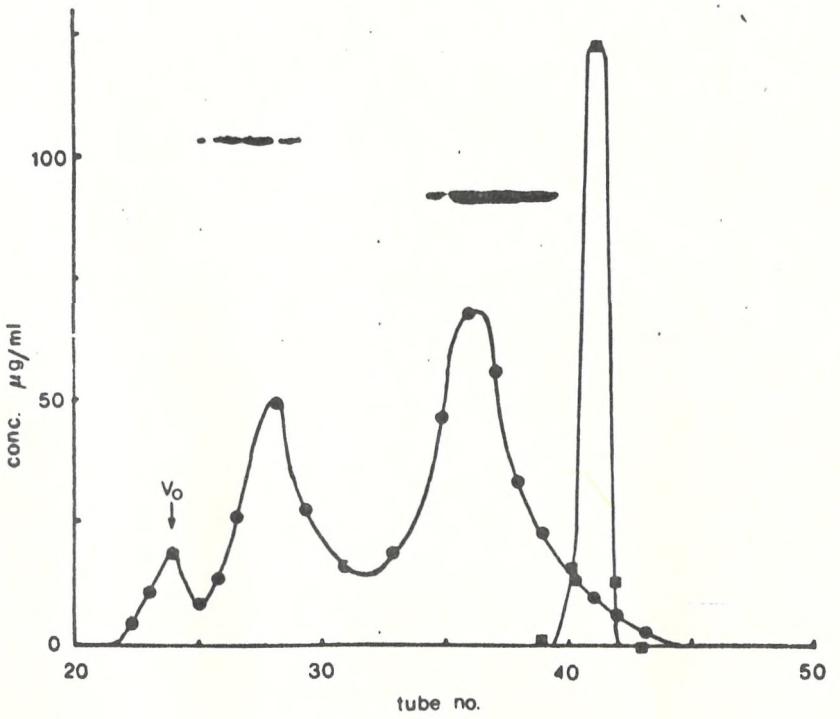


Figure 4b Chromatography of Empigen-soluble material on Sephadex G-200. Fractions were analysed for protein and LPS . Samples of the protein containing fractions were subjected to SDS-polyacryamide gel electrophoresis (figure shows gel superimposed)

material (Fig.4a) showed a selective enrichment of protein IIa. Analysis of the Empigen-soluble material showed proteins IIa and I and 6% of the LPS in solution.

The Empigen-soluble material was applied to a column (640 x 15 mm) of Sephadex G-200 and eluted with Empigen-glycine buffer at a flow rate of 0.12 ml/min. Fractions (2 ml) were collected and analysed for protein and LPS, which showed two well separated peaks of protein and one peak of LPS (Fig.4b). SDS-Polyacrylamide gel electrophoresis of each fraction showed the first peak contained pure protein I and the second peak contained pure protein IIa (Fig.4b). A third peak containing that LPS not solubilised by cholate treatment was eluted after the protein IIa.

That protein I was still antigenically active at the completion of the purification process was shown by the strong line of precipitation obtained on double diffusion against antiserum raised in rabbits to the unfractionated outer membrane complex (Fig. 4c).

Attempted preparation of water soluble vesicles of proteins I and IIa

In order to study the role of proteins I and IIa in experimental systems it was necessary to remove detergent and present the proteins in the correct orientation. An attempt was thus made to prepare water soluble vesicles, essentially as described by Simons et al. (1978) for the removal of Triton X-100 from amphiphilic membrane proteins.

Proteins I (200 μg 500 μl^{-1}) and IIa (50 μg 300 μl^{-1}) were suspended in 0.1M glycine-NaOH buffer pH 9.6 containing 1% (v/v) Empigen-BB. Samples were applied to linear gradients of 20% (w/v) to 50% (w/v) sucrose in 0.2M acetate buffer pH 6 and centrifuged at 100,000 g for 22 h and 10°C in a 6 x 13 ml swing-out rotor. Fractions (0.5 ml) were collected by puncturing the base of the centrifuge tube and displacing the gradient upwards on a cushion of 60% (w/v) sucrose solution. The E_{280} of the fractions were determined. The only protein detected remained at the top of the gradient and did not sediment as the discrete band expected (Fig. 5).

Since the original method of Simon et al. (1978) involved Triton X-100, this was substituted for Empigen BB in purified fractions containing proteins I and IIa. Triton X-100 (5 μl) was added to samples of proteins I and IIa and the samples were repeatedly dialysed against 50mM Tris-HCl buffer pH 7.4 containing 0.1M NaCl for 16 h at 4°C. The

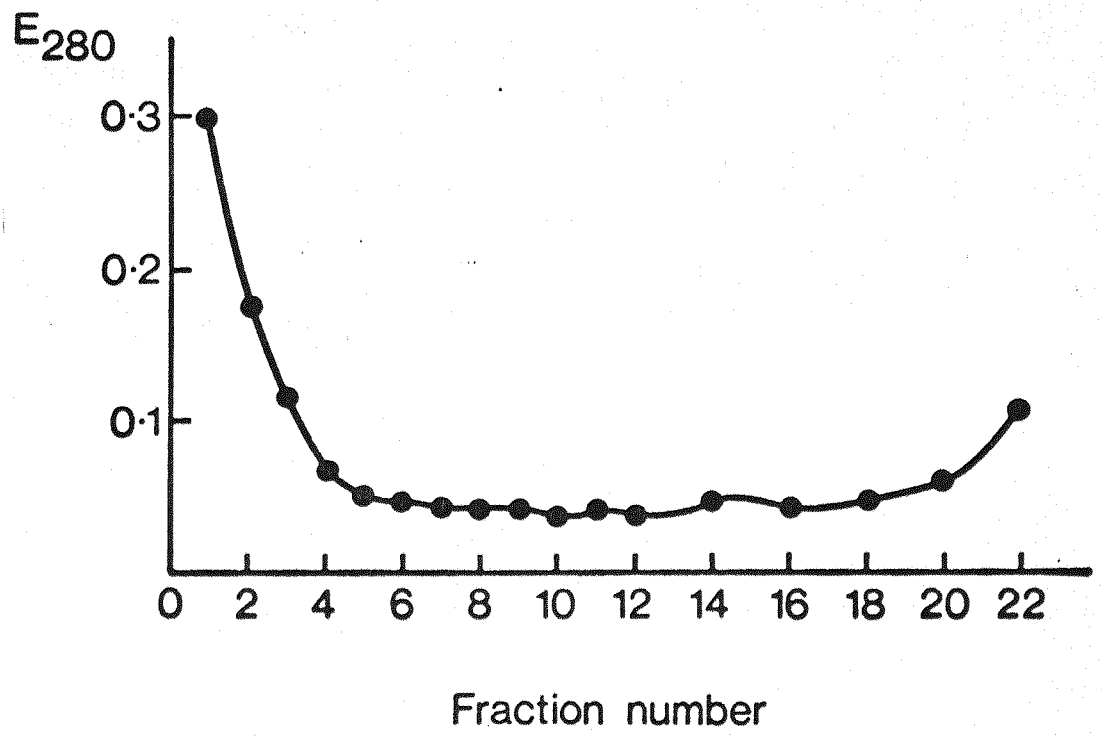


Figure 5 Distribution of outer membrane protein after density gradient centrifugation

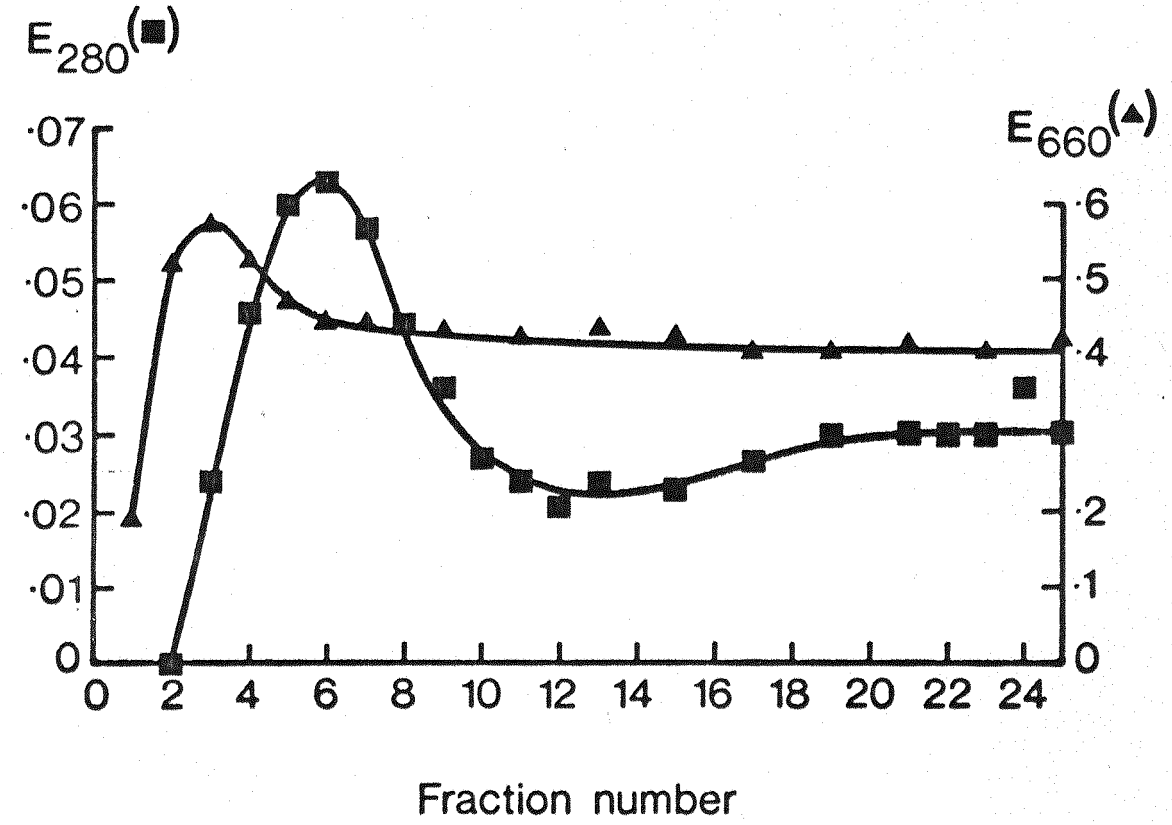


Figure 6 Partial separation of outer membrane protein (▲) and Triton X-100 (■) after density gradient centrifugation

non-diffusible fractions were reduced to 500 µl by negative pressure dialysis and were centrifuged on sucrose gradients as described above. Gradient fractions (0.5 ml) were analysed for protein, and for Triton X-100 by determining the E_{280}. Only partial separation of protein and Triton X-100 was found (Fig. 6) but this was not sufficient to enable a pure protein fraction to be obtained. In addition considerable losses of protein had occurred, therefore it was decided to use intact outer membrane vesicles to study attachment.

Influence of pH on the attachment of outer membrane vesicles

Experiments were performed over a range of pH values (pH 4.5 to pH 8.5) to determine the optimum pH for attachment of outer membrane vesicles of variants P9-1 and P9-13 to buccal cells and erythrocytes. Buccal cells were equilibrated by washing three times in attachment buffer at the appropriate pH. Outer membrane vesicles in the relevant attachment buffer were then added to the buccal cells. The attachment of outer membrane vesicles to erythrocytes was determined in a similar manner.

Results showed (Fig. 7) that P9-13 outer membrane vesicles attached to buccal cells more avidly than those of P9-1 and that the separation between them was greatest over the pH range 6.5 to 7.5. The standard pH for attachment experiments was therefore set at pH 7. In contrast vesicles attached less well to erythrocytes than to buccal cells with P9-1 attaching over a wide pH range with greater avidity than P9-13 (Fig. 8). Similar results have been previously reported using whole cells of gonococcal variants (Lambden et al., 1979).

Attachment of outer membrane vesicles of variants P9-1 and P9-13 to buccal cells In order to determine the optimum concentration of outer membrane vesicles, attachment experiments were carried out with increasing amounts of membrane added. Results showed (Fig. 9) increasing counts bound as the membrane concentration increased. In addition the attachment of P9-13 membranes was 2 to 3 fold greater than that of P9-1 at each concentration. In order to determine the relative affinity of each membrane for the buccal cells, adsorption isotherms (Gibbons et al., 1976) were plotted (Fig. 10; Table 4).

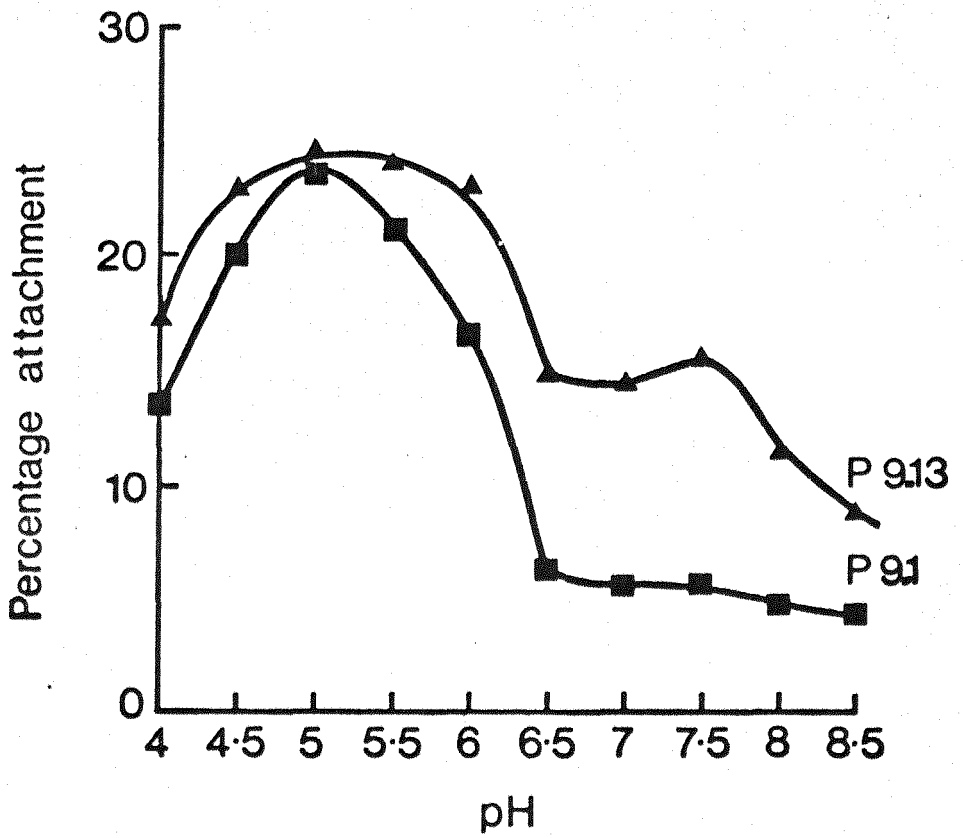


Figure 7 The effect of pH on the attachment of vesicles of outer membrane of variants P9-1 (■) and P9-13 (▲) to buccal epithelial cells

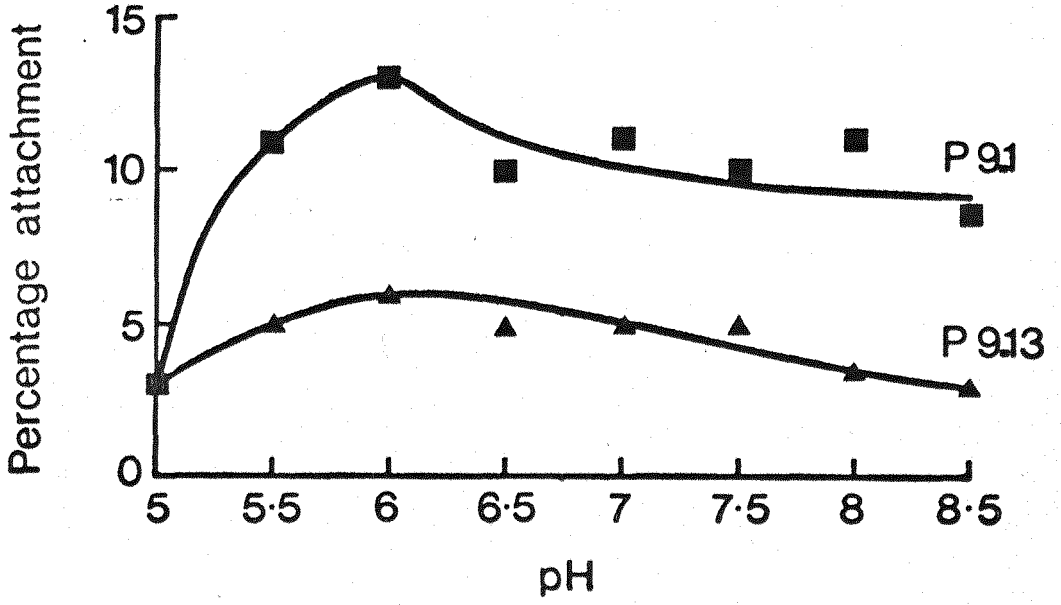


Figure 8 The effect of pH on the attachment of vesicles of outer membrane of variants P9-1 (■) and P9-13 (▲) to erythrocytes

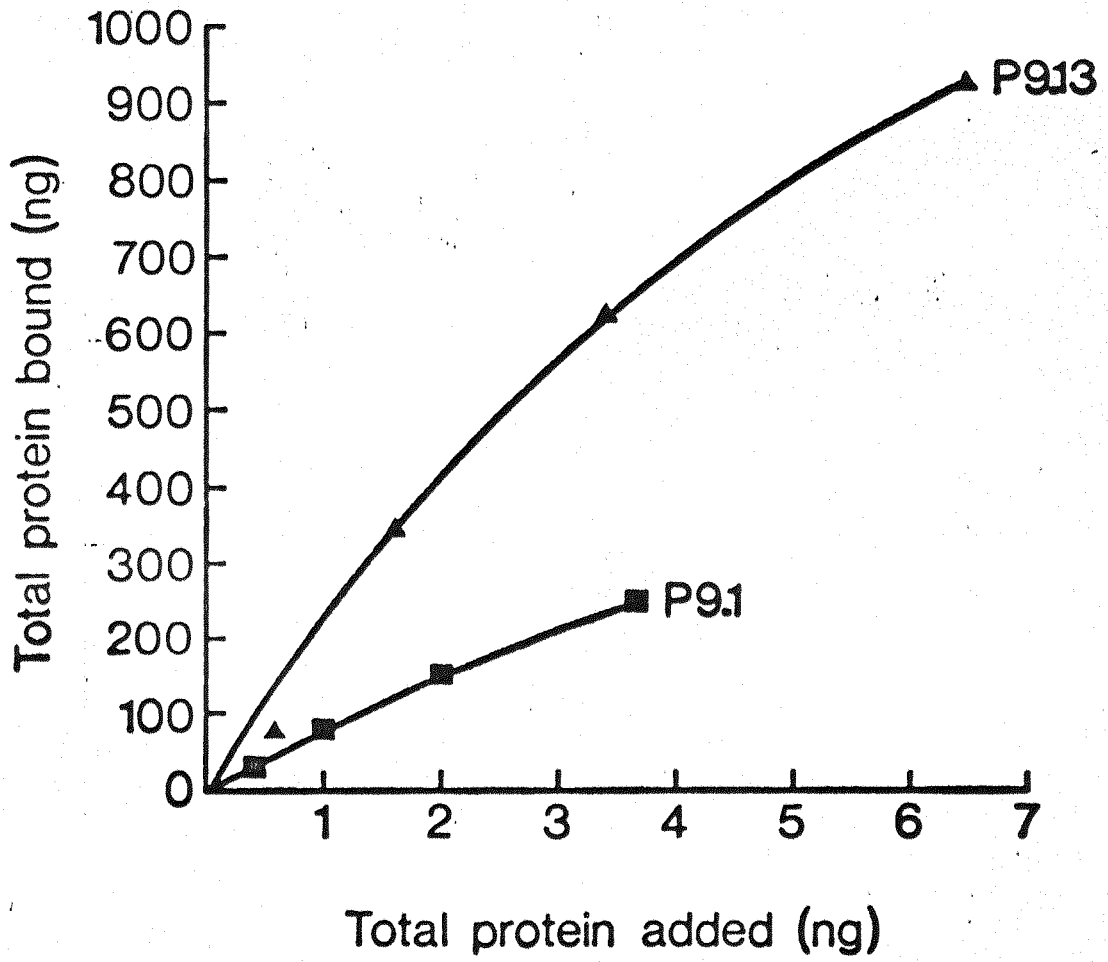


Figure 9 Outer membrane protein bound to buccal epithelial cells plotted against total protein added

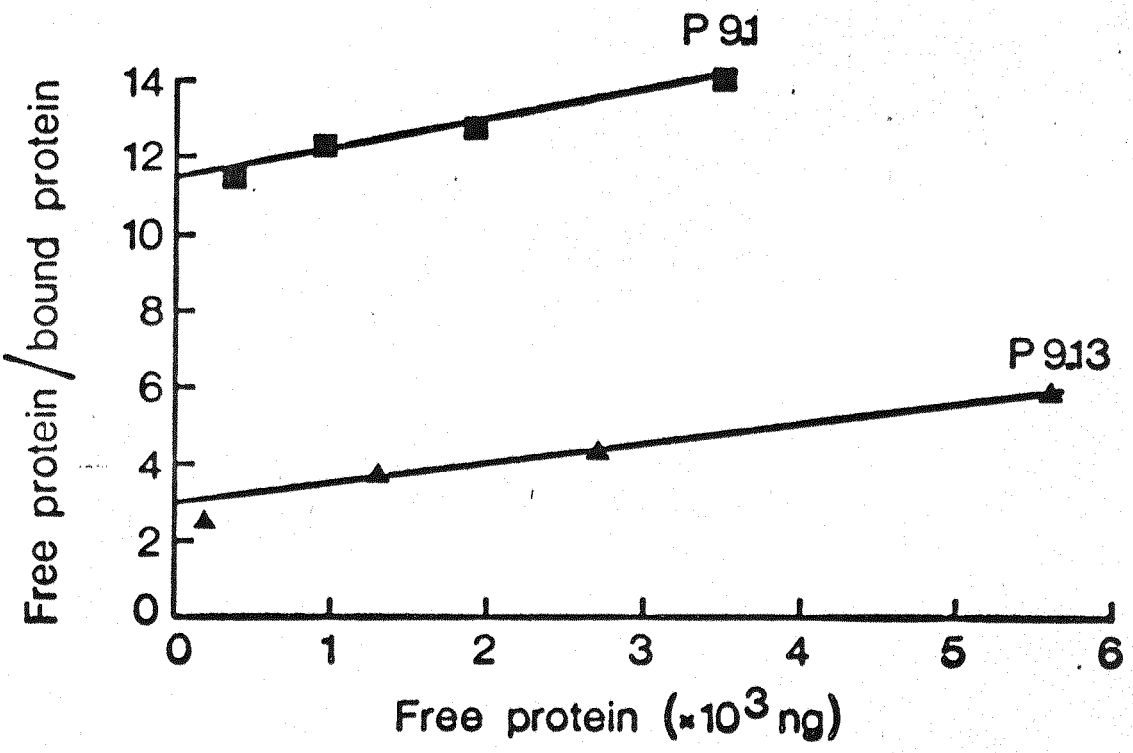


Figure 10 Langmuir adsorption isotherms

Table 4

Attachment of outer membrane vesicles at various concentrations to buccal cells

P9-1

Total protein (ng)	403	1044	2019	3706
Bound protein (ng)	32	78	146	246
Free protein (ng)	371	966	1873	3460
Free/bound	11.5	12.3	12.8	14

P9-13

Total protein (ng)	610	1638	3359	6517
Bound protein (ng)	176	349	630	928
Free protein (ng)	434	1289	2729	5589
Free/bound	2.5	3.7	4.3	6

Since for reversible attachment with no steric hindrance:-

$$\frac{C}{Q} = \frac{1}{KN} + \frac{C}{N}$$

where,

C = concentration of free particles

N = the maximum number of free binding sites

Q = the number of particles adsorbed

K = the affinity constant for the system at a given temperature

A plot of "cpm free/cpm bound" VS "cpm free" has a gradient $\frac{1}{N}$ and an intercept $\frac{1}{KN}$. Values obtained for N and K (Table 5) confirm the greater affinity of P9-13 vesicles for binding sites on the surface of buccal cells as compared with those of P9-1.

In subsequent attachment experiments the amount of protein used was standardised at 1.74 μg.

Time course of attachment of outer membrane vesicles to buccal cells Experiments were performed for different periods of time in order to determine the optimum time required for attachment of outer membrane vesicles to buccal cells at pH 7. Experiments were terminated after 0, 10, 20, 40, 60, 120, 240 and 360 mins by immediate centrifugation on "Dextraven" at 500 g for 1 min.

Results (Fig. 11) showed that maximum attachment of both variants had occurred by 2 h, and this time was selected as standard in further experiments. In addition some decrease in attachment was seen on prolonged incubation.

Statistical analysis of attachment Having determined the optimum conditions for further experiments, statistics were calculated from ten replicate attachment experiments of each variant to buccal cells (Table 6).

Table 6

Variant	Mean percentage attachment	Standard deviation	Percentage coefficient of variation
P9-1	23.5	2.66	11.6
P9-13	48	8.4	17

Table 5

Adsorption isotherm analysis data

	P9		P9
N (c. min^{-1})	1218		940
Affinity constant (K) (c^{-1} min)	7×10^{-5}		36×10^{-5}
Relative number of binding sites $\frac{N_{13}}{N_1}$	1	:	0.77
Relative affinity of binding $\frac{K_{13}}{K_1}$	1	:	5.1

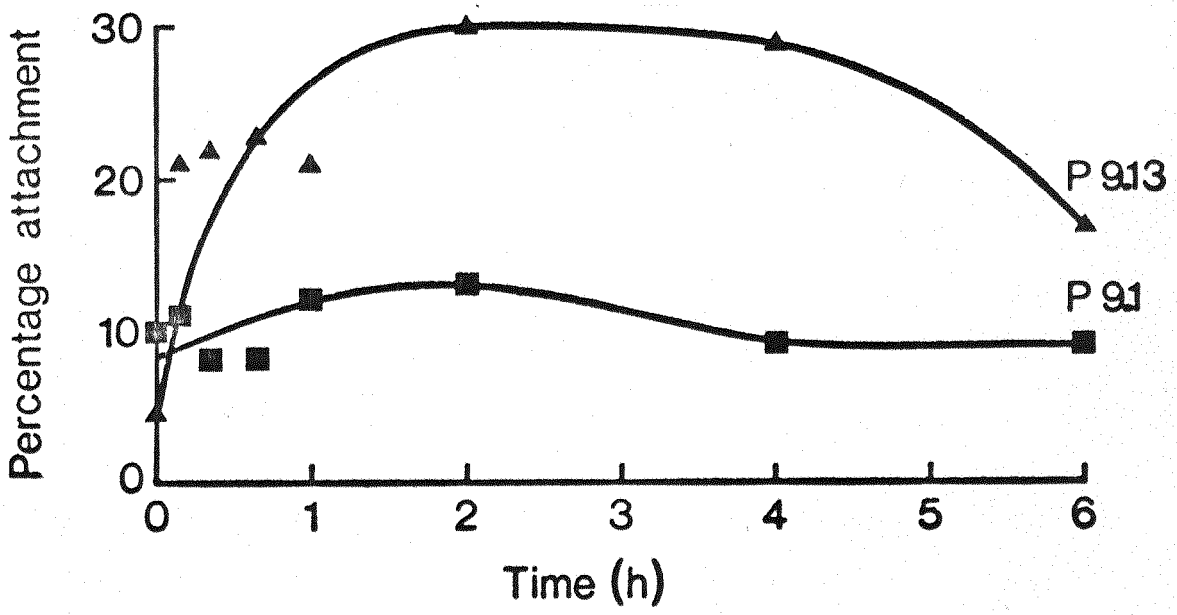


Figure 11 Time course of the attachment of outer membrane vesicles of variants to buccal epithelial cells

Student's 't' test was performed on the data and showed the difference in percentage attachment of vesicles of P9-1 and P9-13 to buccal cells was significant with a confidence limit of less than 0.1%.

Attachment of outer membrane vesicles of variants to substituted agarose gels Experiments were performed to determine the relative hydrophobicity and surface charge characteristics at different pH values (pH 4 to 8.5) of the outer membrane vesicles.

Results (Fig. 12) showed that vesicles of P9-1 and P9-13 were relatively hydrophobic in that they attached most avidly to alkyl-substituted gels having chain lengths C_6, C_8 and C_{10} of a range C_0 to C_{10}; and that variant P9-13 was more hydrophobic than variant P9-1.

Attachment to DEAE gels (Fig. 13) showed vesicles of variant P9-1 to have an attachment advantage over those of P9-13 between pH 5 and 8, but outside this range the situation was reversed.

Attempted inhibition by sugars of the attachment of outer membrane vesicles to buccal cells Experiments were performed with a number of sugars (Table 7) selected because of their occurrence on mammalian cell surfaces, in order to screen for inhibition of attachment of outer membrane vesicles to buccal cells.

Results (Table 7) showed no significant inhibition of attachment of vesicles to buccal cells.

Periodate oxidation of sugar groups on the surface of outer membrane vesicles Sugar groups on the surface of outer membrane vesicles were selectively destroyed by periodate oxidation in order to further investigate the possible role of such groups in attachment to buccal cells.

Vesicles (9 to 13 µg protein) were suspended in attachment buffer pH 7 without bovine serum albumin, containing 10mM sodium periodate and incubated for 30 min at 4°C. An equal volume (80 µl) of 10mM sodium borohydride solution in the same buffer was then added and the mixture was incubated for a further 30 min at 4°C. Controls were included in which the outer membrane vesicles were only subjected to 5mM sodium borohydride.

Results (Table 8) showed no significant change in attachment of periodate treated outer membrane vesicles to buccal cells.

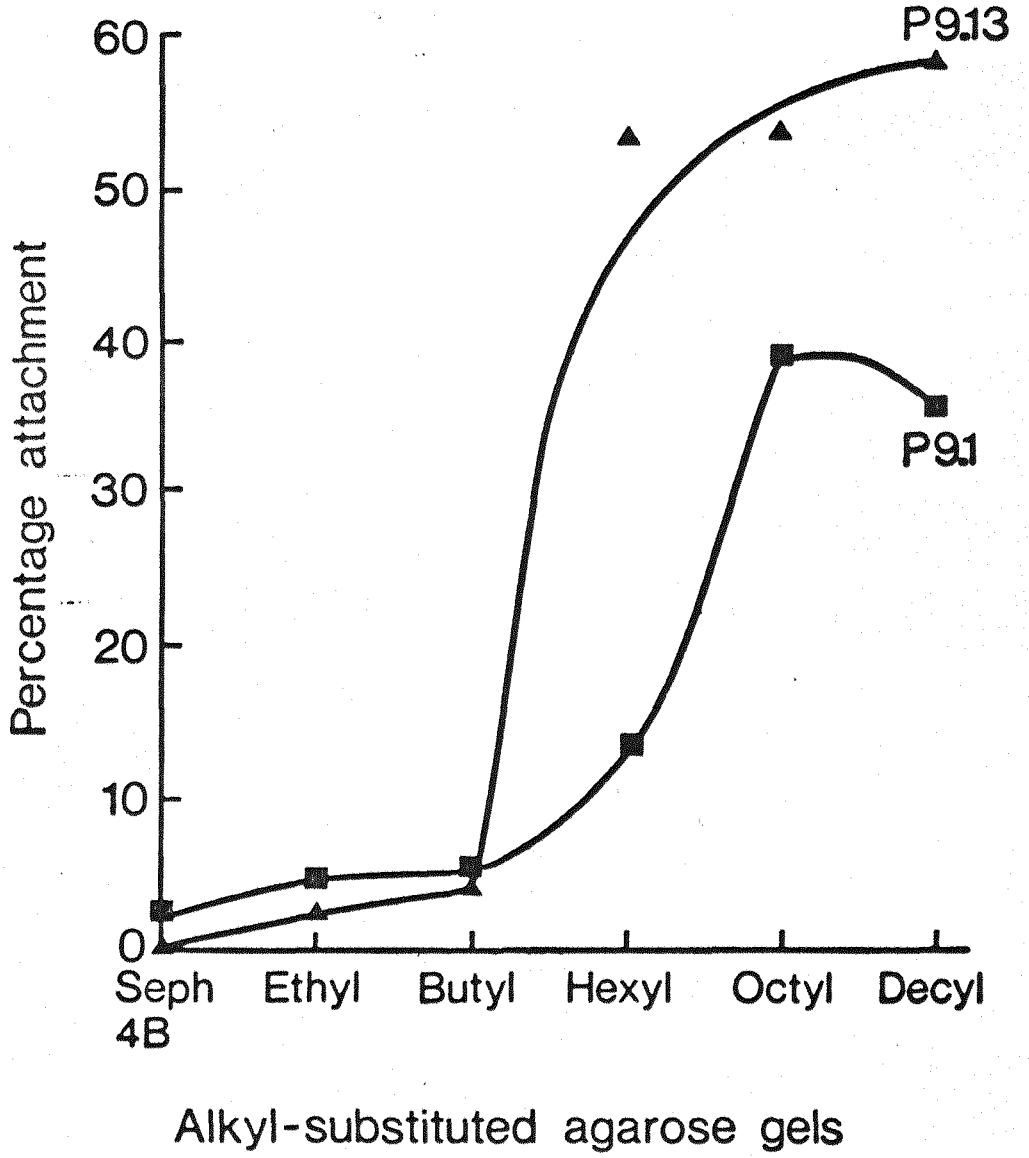


Figure 12 Attachment of outer membrane vesicles of variants to alkyl-substituted agarose gels

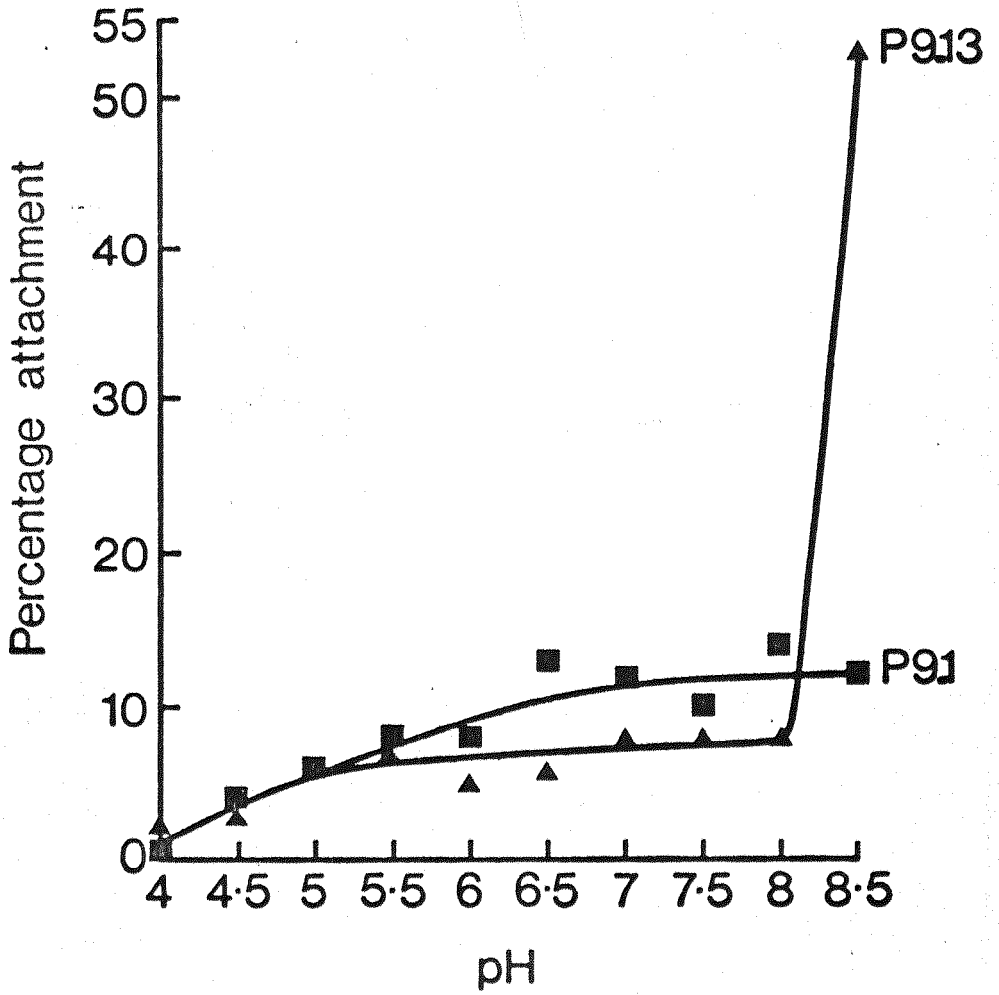


Figure 13 Attachment of outer membrane vesicles of variants to DEAE gel

Table 7

Inhibition of attachment of variants P9-1 and P9-13 to buccal cells by various sugars

Sugars (all 0.1M)	Percentage attachment	
	P9-1	P9-13
N-acetyl-D-glucosamine	37	133
N-acetyl-D-galactosamine	33	105
-L(-)fucose	51	120
-met-D-mannoside	51	110
1-O-met- -D-galactopyranoside	47	109
1-O-met- -D-galactopyranoside	41	126
Glucose	39	96
Mannan	39	78
N-acetyl neuraminic acid	51	104
Sugarless controls	36	100

(Percentages are expressed such that the P9-13 sugarless control = 100%)

Table 8

Periodate treatments of outer membrane vesicles of variants P9-1 and P9-13

	Periodate treated outer membrane vesicles	Sodium borohydride treated outer membrane vesicle controls	Untreated outer membrane vesicle controls
P9-1 percentage attachment	40	38	30
P9-13 percentage attachment	95	110	100

(Percentages are expressed such that the P9-13 untreated control = 100%)

Glycosidase treatments of outer membrane vesicles Sugar groups on the surface of outer membrane vesicles were selectively removed by glycosidase treatment.

Vesicles (19 μg to 29 μg protein) were suspended in attachment buffer pH 5.5 containing mixed exoglycosidases to the final concentrations shown in Table 9; then incubated for 1 h at 37°C and finally centrifuged at 100,000 g for 1 h. The pellet was suspended in attachment buffer pH 7 to a protein concentration of 1.74 μg 200^{-1}. Reducing sugar analysis was performed on the supernatant solutions.

Results (Table 9) of reducing sugar analysis showed the evolution of reducing sugars proportional to the cencentration of mixed exoglycosidases used. No significant reduction (Fig. 14) in attachment of mixed exoglycosidase treated outer membrane vesicles to buccal cells was shown even at the highest concentration when 567 μmol reducing sugar was removed. Analysis of treated membranes by SDS-polyacrylamide gel showed no change of mobility for proteins I and IIa.

Periodate oxidation of sugar groups on the surface of buccal cells Sugar groups on the surface of buccal cells were selectively destroyed by periodate oxidation followed by borohydride reduction.

Buccal cells (0.5% packed cell volume) in 0.1M sodium acetate buffer pH 5.5 containing 10mM sodium periodate, were incubated for 30 min at 4°C and centrifuged at 500 g for 5 min. The pellet was washed twice in 0.1M Tris maleate buffer pH 7.4 and the cells were resuspended to a packed cell volume of 0.5% in the same buffer containing 5mM sodium borohydride, incubated for 30 min at 4°C and then centrifuged as above. The pellet was washed twice in attachment buffer pH 7 and the cells were resuspended to a packed cell volume of 5% in the same buffer. Controls were included in which the buccal cells were only subjected to 5mM sodium borohydride.

Results (Table 10) showed a statistically significant reduction in the attachment of P9-13 ($p = 0.02$). A slight reduction was also seen in the attachment of P9-1, but this was largely attributable to borohydride treatment.

Glycosidase treatments of buccal cells Sugar groups on the surface of buccal cells were selectively removed using a mixed glycosidases preparation in order to determine the possible role of such

Table 9

Reducing sugar assays on exoglycosidase treatments supernatants of vesicles

		treatments ($\mu g\ ml^{-1}$ enzyme)		
Variant	Control	50	250	1000
P9-1	26.8	21.6	36	102
P9-13	63	17.6	108	102

(Values are expressed as $\mu g\ ml^{-1}$ of reducing sugar equivalent to a glucose standard)

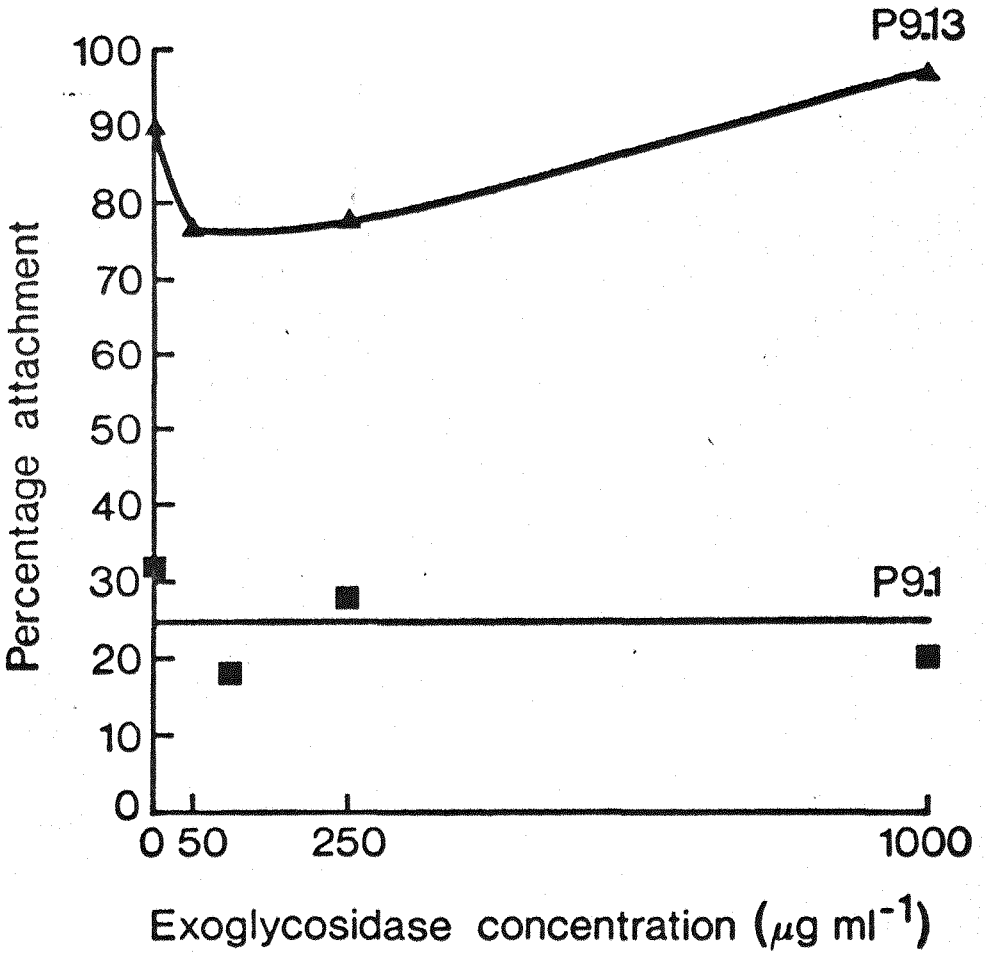


Figure 14 The effect on attachment to buccal epithelial cells of exoglycosidase treatment of outer membrane vesicles of variants

Table 10

Periodate treatments of buccal cells

Variant	Percentage attachment to periodate treated cells	Percentage attachment to sodium borohydride controls	Percentage attachment to untreated buccal cells
P9-1	13	18	32
P9-13	77	100	100

Results are the means of four experiments

(Percentages are expressed such that the P9-13 untreated control = 100%)

groups in the attachment of outer membrane vesicles.

Buccal cells were suspended to a packed cell volume of 5% in attachment buffer pH 5.5 containing neuraminidase and mixed exoglycosidases at the final concentrations shown in Table 11. The suspension was incubated for 1 h at 37°C and centrifuged at 10,000 g for 2 min. The pellet was washed three times in attachment buffer pH 7 and resuspended to a packed cell volume of 5% in the same buffer and used in attachment experiments. Reducing sugar analysis was performed on the supernatant solutions.

Results (Table 11) of the reducing sugar analysis showed increased release of reducing sugars with increasing concentration of glycosidase. A large decrease in attachment (Table 11), proportional to the amount of sugar released was shown with variant P9-13, but this was much less marked with variant P9-1.

Attachment of trypsin treated outer membrane vesicles of variants to buccal cells Since it had been reported that protein II was very susceptible to protease activity (Swanson, 1978), attachment experiments were performed using outer membrane vesicles which had previously been incubated with trypsin.

Samples (9.48 μg protein 100 μl^{-1}) of outer membrane vesicles suspensions in attachment buffer pH 7 were added to trypsin solution (0.5 μg 840 μl^{-1}) in the same buffer and incubated at 37°C. The enzyme was inactivated by the addition of Soy bean trypsin inhibitor solution (1.0 μg 150 μl^{-1}) in attachment buffer pH 7 at times 0, 5, 10, 20, 30, 40 and 60 min. Controls from which the trypsin was omitted were run in parallel. Samples were removed for attachment experiments (2 x 1.74 μg protein in 200^{-1}) and the remainder was analysed by gradient slab SDS-PAGE and negative-stain transmission electronmicroscopy.

Results (Fig. 15) showed that the attachment of vesicles of P9-1 were unaffected by the trypsin treatment, whilst there was a rapid fall in the attachment of P9-13 vesicles.

Autoradiography (Fig. 16) of trypsin treated ^{125}I-labelled proteins I and IIa analysed on SDS-polyacrylamide gel electrophoresis showed the gradual depletion of both proteins I and IIa. In each the disappearance of protein I was associated with the concomitant appearance of a new peptide of molecular weight 27,500.

Transmission electronmicroscopy showed that the outer membrane

Table 11

Results of glycosidase treatment of buccal cells

Buccal cells treatments	Percentage attachment P9-1 vesicles	Percentage attachment P9-13 vesicles	Reducing sugar released ($\mu g\ ml^{-1}$)
Untreated	31	100	0.1
Neurominidase (0.1 $U.ml^{-1}$)	33	96	0.5
Neurominidase (0.1 $U.ml^{-1}$) and exoglycosidases (1 mg ml^{-1})	20	94	59
Neurominidase (0.1 $U.ml^{-1}$) and exoglycosidases (10 mg ml^{-1})	14	29	213

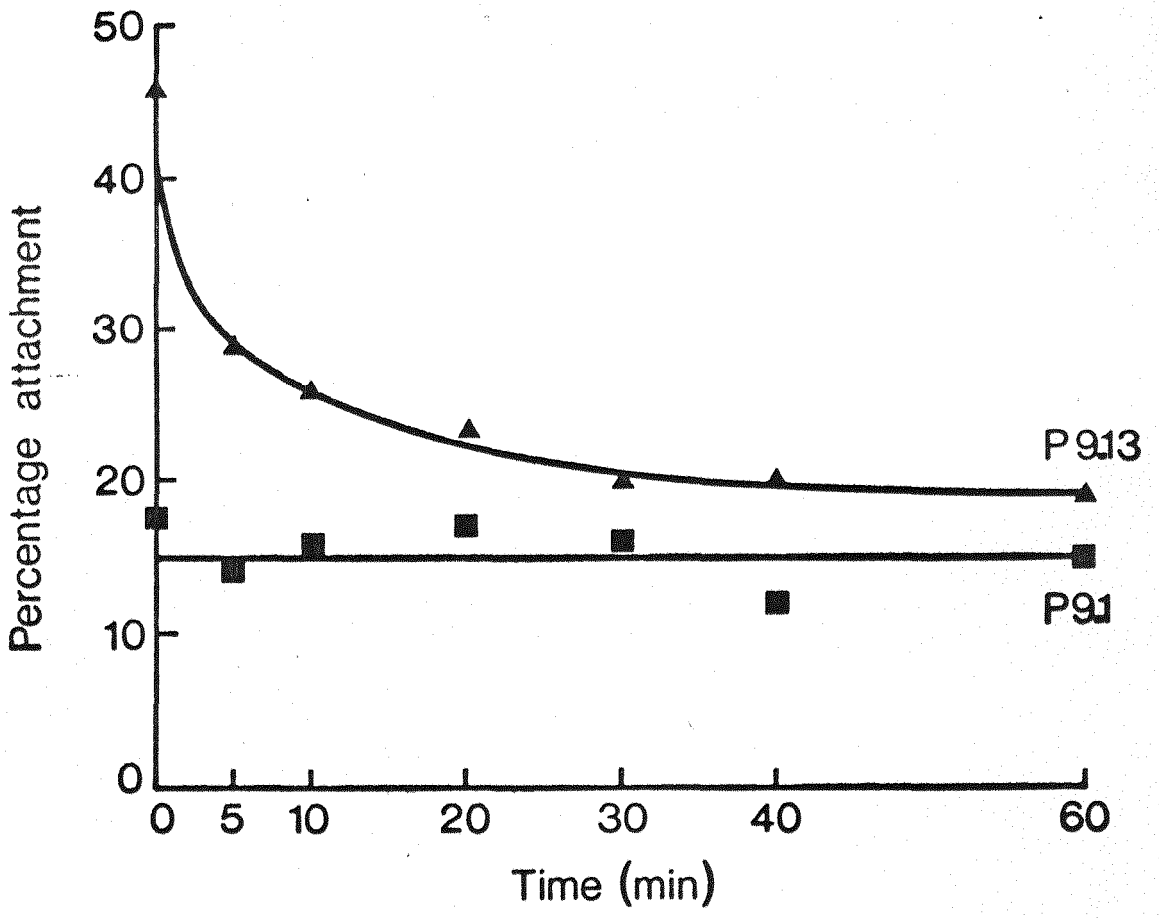


Figure 15 The effect of trypsin treatment of outer membrane vesicles of variants on attachment to buccal epithelial cells

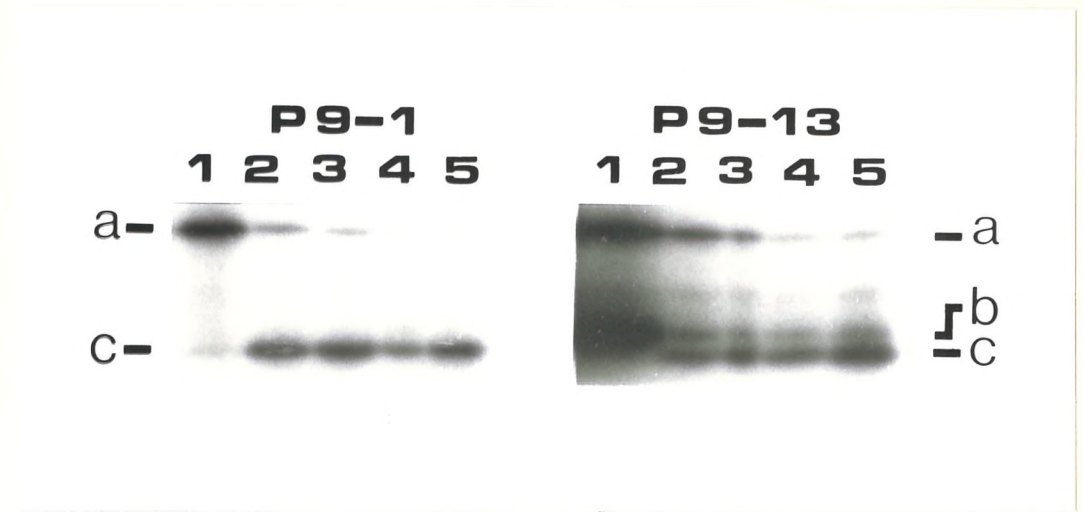


Figure 16 Autoradiography of trypsin treated ^{125}I labelled outer membrane vesicles of variants analysed by SDS-polyacrylamide gel electrophoresis; (a) protein I; (b) protein IIa; (c) "new peptide" (M.W. 27,500) Tracks 1,2,3,4 and 5 correspond with trypsin treatments of 0,20,30,40 and 60 min.

vesicles were still intact after the trypsin treatment (Figs. 17 and 18).

Attempted inhibition by products of trypsin treated buccal cells of the attachment of outer membrane vesicles to buccal cells Inhibition of attachment of vesicles to buccal cells by various sugars, selected because of their occurrence on the surface of mammalian cells, has not been demonstrated. In order to determine if structural integrity of buccal cell surface carbohydrates was essential to attachment, inhibition studies were performed using glycopeptides cleaved from buccal cells with trypsin.

Samples of buccal cells (800 µl of 12% packed cell volume) in attachment buffer, pH 7 were incubated with trypsin (1.2 µg) at 37°C for various time intervals (0, 5, 10, 20, 30, 40 and 60 min). Treatments were terminated by the addition of Soy bean trypsin inhibitor (2.4 µg). Buccal cells were removed by centrifuging the mixture at 10,000 g for 2 min. Attachment experiments were performed in the presence of the trypsin cleavage products.

Results (Fig. 19) show no inhibition of attachment of P9-1 vesicles to buccal cells. The inhibition of attachment of vesicles of P9-13 was statistically significant ($p = 0.05$) in the presence of 416 uM glucose equivalence of buccal cell trypsin products.

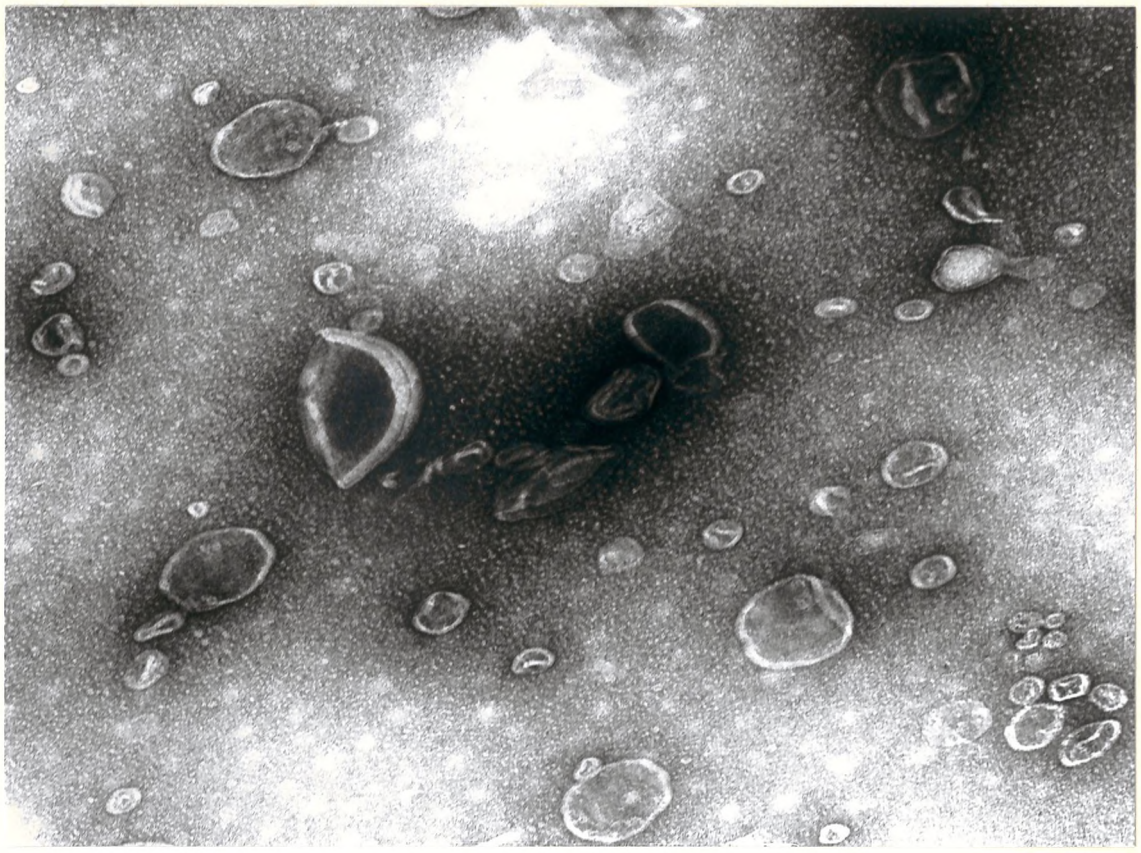


Figure 17 Negatively stained electron-micrograph of untreated outer membrane vesicles of variant P9-13 (x70,000)

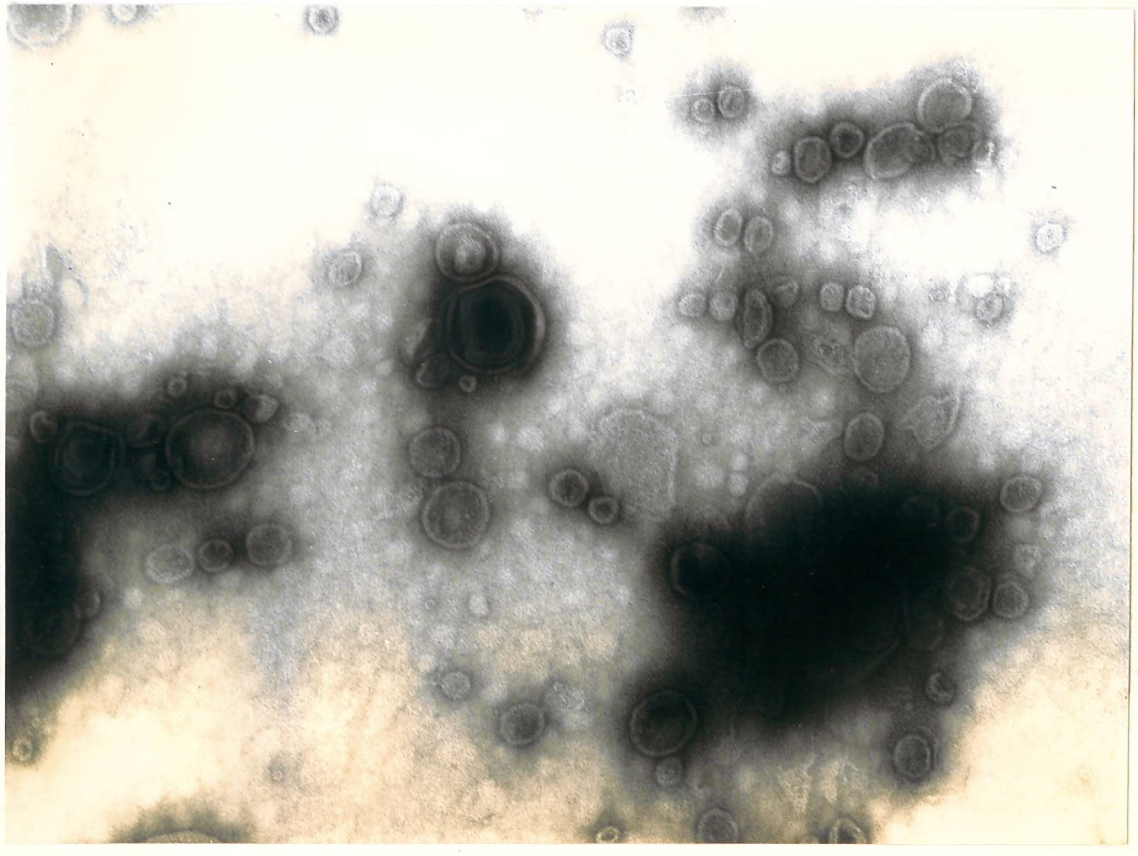


Figure 18 Negatively stained electron-micrograph of outer membrane vesicles of variant P9-13 after 1 h treatment (x70,000)

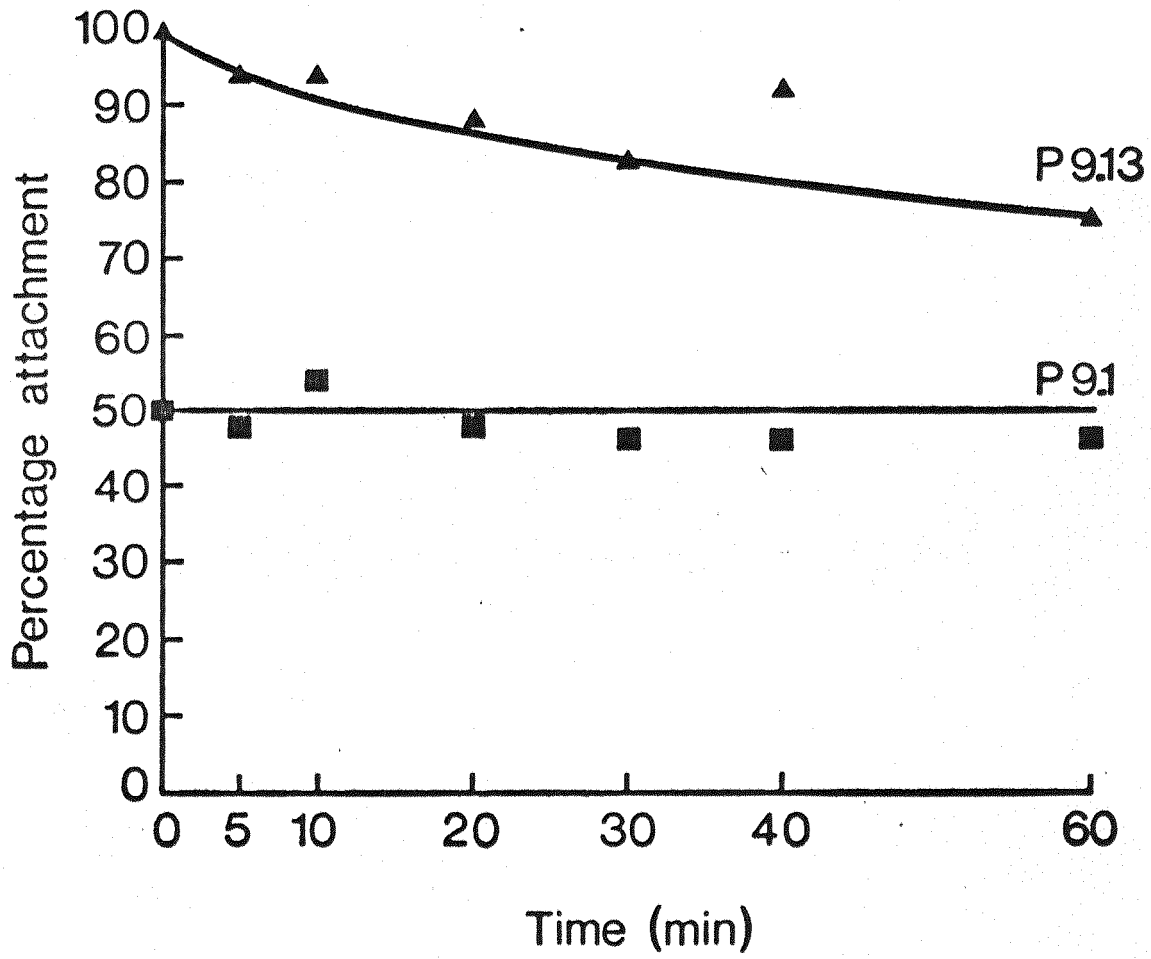


Figure 19 Effects of products of trypsin treatments of buccal epithelial cells for various time intervals, on the attachment of outer membrane vesicles of variants, to untreated buccal epithelial cells

DISCUSSION

Experimental approach Previous studies of the role of gonococcal outer membrane proteins have involved the use of whole organisms (Swanson, 1978; Lambden et al., 1979). This approach is limited by the intolerance of whole cells to extreme chemical treatments or physiological conditions. In order to expand both the range of possible chemical treatments on surface components, and the range of physiological environments, a new approach was attempted using purified membrane proteins.

Protein IIa is associated with increased attachment and occurs in the outer membrane of variant P9-13 as the sole representative of the colonial opacity associated proteins (Lambden and Heckels, 1978). Variant P9-13 was therefore excellent starting material for the extraction of protein IIa in a purified form.

Extraction of outer membrane of variant P9-13 with Empigen-BB followed by chromatography on Sephadex G200, produced pure, serologically-reactive proteins I and IIa in detergent solution.

Grant and Hjerten (1977) first used Empigen-BB detergent to obtain native membrane proteins from erythrocytes. It has since been used by Robinson and Manchee (1978) to obtain serologically-reactive membrane proteins from Bordetella pertussis.

Initially an attempt was made to remove the detergent and prepare water soluble micelles of proteins I and IIa in the correct orientation, for use in attachment experiments. However, the method previously used to remove Triton X-100 from various amphiphilic proteins was not successful (Simons et al., 1978).

As an alternative approach, outer membrane vesicles of P9-1 (protein I) and P9-13 (proteins I and IIa) were prepared by lithium acetate extraction of whole organisms and used in attachment experiments.

Attachment experiments Vesicles of outer membranes of variant P9-13 were shown to have, at least, a two-fold attachment advantage over those of variant P9-1, to buccal epithelial cells at physiological pH (Fig. 9). In contrast, the attachment of P9-13 was lower to erythrocytes than P9-1. There was no obvious pH dependence, but the difference of attachment was statistically significant in the physiological pH range (Fig. 10).

Some care is necessary in the interpretation of differences in attachment of variants. It is known that the acquisition of an additional outer membrane protein may result in a simultaneous reduction of one previously present (Heckels, 1978). The insertion of an extra protein in the outer membrane of a variant may cause an unspecified structural alteration resulting in a significant change in attachment properties. Nevertheless these results and previous studies on intact bacteria (Lambden et al., 1979) have shown that the acquisition of protein IIa correlates with an enhancement of virulence related properties.

The contrasting differences of attachment to buccal cells and erythrocytes, imply the presence of a specific receptor for buccal cells on the surface of outer membrane vesicles of variant P9-13; presumably protein IIa.

The efficiency of attachment of P9-13 vesicles to buccal cells was quantified by a plot of Langmuir isotherms of the two variants (Fig. 7) which showed a five fold greater avidity for P9-13. This also showed that the number of available binding sites on the buccal cells is approximately the same for both variants. Since protein IIa is the only component of P9-13 not present in P9-1, an attempt was made to identify any specific binding mechanisms in which it might be involved.

Attempted inhibition of attachment It was assumed that a specific binding mechanism would involve complementary structures on the surfaces of buccal cells and outer membrane vesicles. Obviously competitive inhibition of attachment should result from the presence of one or other of the relevant structural components. Although significant inhibition of attachment to buccal cells by variant vesicles did not result from the presence of any of nine different sugars (Table 1) chosen because of their occurrence on mammalian cell surfaces, this does not necessarily exclude carbohydrates from having a role in attachment. The precise stereochemical configuration of a particular oligosaccharide structure may be essential if it is to function efficiently in a complementary binding system. For example it is known that the interaction between heparin and antithrombin involves a binding site in the heparin molecule composed of up to six disaccharide units arranged in one or more specific sequence(s), and that the carbohydrate unit may not be substituted by any other (Lindahl and Höök, 1978).

The likelihood of the involvement of sugar moieties on the surface of outer membrane was further reduced when it was found that no

significant reduction of attachment followed either the specific periodate oxidation, or glycosidic degradation of carbohydrate structures on the surfaces of variant vesicles. Although substantial quantities of reducing sugars were released by glycosidase treatments of variant vesicles, which increased with increasing amounts of enzyme added, no difference was observed in the mobility of treated proteins by SDS-polyacrylamide gel electrophoresis. This presumably indicates that most of the reducing sugar was released from lipopolysaccharide. If this is the case it would confirm that lipopolysaccharide plays no role in attachment (Trust et al., 1980). The results also suggest that neither proteins I or IIa are significantly glycosylated.

Trypsin treatments Any attachment model which assumes the interaction of complementary structures, has implicit in it the assumption that one of the interacting molecules is a protein (Frazier and Glaser, 1979). Swanson (1978a and b) showed that opacity associated proteins are susceptible to ^{125}I-labelling whilst still inserted in the outer membrane, hence presumably protruding from the surface, and also that, at least, the labelled portion of the molecule may be cleaved off using trypsin. Heckels (personal communication) has shown that the greatest degree of variability in the protein II group lies in the portion of the molecule exposed at the surface.

This study has shown that the removal of a portion of the IIa molecule exposed to trypsin treatment at the surface of the P9-13 vesicles significantly reduces the attachment of those vesicles to buccal cells. Swanson's (1978a) observation that the colonial opacity associated proteins (similar to protein IIa) were more susceptible to trypsin hydrolysis than protein I, was however not confirmed. Nevertheless since the only difference between the membranes is the presence of protein IIa in P9-13 then the lack of any similar reduction in attachment of P9-1 clearly demonstrates the influence of protein IIa on the attachment of P9-13 membranes.

Similar trypsin treatment of buccal cells was not attempted as it had previously been noted that such cells rapidly disintegrate when subjected to trypsin hydrolysis (P.R. Lambden, personal communication). It is possible that buccal cells, being dead, have ruptures in their outer membranes allowing entry to the trypsin and subsequent cellular disintegration.

Assuming that protein IIa has a role as suggested in the specific attachment of variant P9-13 outer membrane vesicles to buccal cells, an attempt was made to identify it's complementary structure on the buccal cell surface. It was initially assumed that such a complementary structure might be a carbohydrate.

The role of carbohydrate structures on the surface of buccal cells in attachment Treatments destructive of carbohydrate structure, and thereby function, similar to those used on variant vesicles, were performed on buccal cells, which were then used in attachment experiments.

Periodate treatment of buccal cells resulted in a statistically significant reduction (13%; $p = 0.02$) in the attachment of vesicles of variant P9-13 to treated cells. An even greater reduction of attachment of P9-13 was obtained with exoglycosidase treated cells, but these are not without certain reservations. A significant reduction in attachment of vesicles of variant P9-13 but not P9-1 to treated buccal cells was seen only at the highest concentration of exoglycosidases (1 unit of neurominadase plus 10 mg ml^{-1} mixed exoglycosidases). The mixed exoglycosidases are in a crude preparation, and at this high concentration contaminating proteases and/or phospholipases may have significant effect; though in one study none were found (S.Morse, personal communication).

The results of both periodate and exoglycosidase treatments of buccal cells suggest the involvement of a buccal cell surface carbohydrate structure in attachment of variant P9-13 vesicles to buccal cells. To further study this possibility an attempt was made to obtain structurally intact, and therefore functionally significant carbohydrate structures from buccal cell surfaces. Buccal cells were treated with trypsin to release surface glycoproteins, and the products were used in competitive inhibition of attachment experiments. Statistically significant inhibition of attachment of P9-13 but not P9-1 vesicles to buccal cells occurred at the highest concentration of trypsin product used (418 μM of carbohydrate). This suggests that at least part of the advantage conferred by protein IIa is due to the recognition of a specific carbohydrate receptor. The contrasting attachment of variants to buccal cells and erythrocytes suggests that this receptor is present on the surface of buccal epithelial cells, but not erythrocytes. However this does not exclude the possibility that non-specific interactions involving

hydrophobic and electrostatic forces might also contribute to the enhanced attachment of P9-13.

Non-specific interactions Attempts were made to estimate the contribution of non-specific interactions to the difference of attachment of the two variants.

Hydrophobic interactions The alkyl-substituted agarose gels used to estimate the hydrophobicity of outer membrane vesicles of variants had alkyl chain lengths of C_0 to C_{10}. The degree of substitution by alkyl groups was comparable on all gels at a concentration of 0.2M substituent (mol galatose)$^{-1}$ (manufacturer's data). Results showed that variant P9-13 had a significant attachment advantage over variant P9-1 in the chain length range C_6, C_8 and C_{10}. Variant P9-1 showed a peak attachment at C_8, but variant P9-13 had still not reached a conclusive peak at C_{10}.

It has been suggested that the cell surface of variant P9-1 has hydrophobic pockets or regions capable of non-specific binding interactions with the surface of buccal epithelial cells (Trust et al., 1980). Since the presence of protein IIa on the surface of variant P9-13 correlates with increased hydrophobicity and with increased attachment to buccal cells this confirms the importance of non-specific hydrophobic interactions in the attachment of gonococci.

Electrostatic interactions Non-specific electrostatic interactions between bacterial and mammalian cell surfaces greatly influence the efficiency of attachment (Rutter, 1980). The presence of protein IIa in the outer membrane of variant P9-13 might cause changes of cell surface charge which significantly aid it's attachment. The relative charge on the surface of vesicles of the two variants was measured by attachment to diethylaminoethyl agarose gel (DEAE) at various pH (pH 4.5 to 8.5). The interpretation of the results (Fig. 12) is difficult due to the simultaneous titration of groups on the surfaces of both gel and membranes. Nevertheless the results showed that variant P9-13 attached less well to DEAE than variant P9-1 over a significant range (pH 5.5 to 8). Since DEAE gel is positively charged (DEAE: pK 9.5) over the same pH range, variant P9-13 must be less negatively charged than variant P9-1, presumably due to the presence of protein IIa. Thus variant P9-13

will experience a lower repulsive force than variant P9-1 when approaching negatively charged mammalian cell surfaces, so aiding it's initial attachment.

Similar considerations of surface charge may be extended to in vivo situations. The two most favoured sites of gonococcal infection are the urethra and endocervix. Most data suggest a significant pH range for these sites of pH 6 to pH 8, though precise determination is difficult. The range of pH of endocervical mucus during the menstrual cycle has been estimated as pH 6 to pH 8 (Moghissi, 1973; Kroeks and Kremer, 1977). Dahlberg (1978) reported a pH 6.5 to 7 for seminal fluid, and pH 7.5 to 8 for infected seminal fluid. The pH of urine (4.8 to 8) may be irrelevant once initial attachment to the mucosal surface has taken place. An additional factor which may have great importance in vivo, but which has not been investigated is the ionic strength of the environment in which attachment takes place. Nevertheless a reduction in the repulsive force between gonococci and mammalian cell surfaces would increase the probability of the necessary collisions.

Results suggest that protein IIa is a significant factor in the modification of membranes of variant P9-13 affecting non-specific hydrophobic and electrostatic interactions. This in turn may increase the probability of successful specific interactions being made.

Summary Results suggest that protein IIa on the surface of variant P9-13 plays a decisive role in attachment; probably through both specific and non-specific mechanisms. Protein IIa specifically interacts with a complementary carbohydrate structure on the surface of buccal epithelial cells in a lock-key type mechanism. The additional contribution of non-specific hydrophobic and electrostatic interactions to attachment are also dependent on the presence of protein IIa. Membranes of variant P9-13 are both less negatively charged and more hydrophobic than those of variant P9-1. It is not clear what proportion of final attachment is due to non-specific interactions, or whether such interactions are mainly responsible for the reduction of barriers to attachment.

Protein IIa is one of a family of similar proteins which occur in various combinations on the surface of gonococcus strain P9 giving rise to a number of distinct variants. Different variants are associated with a variety of virulence related properties, such as increased attachment to epithelial cells, increased resistance to serum killing.

Other properties, less clearly understood in virulence terms, are associated with some strains, such as an increased attachment to leucocytes and an increased trypsin sensitivity. Obviously the generation of different outer membrane phenotypes allows the gonococcus a flexible response to environmental selection pressures. This presumably is one reason why the gonococcus is such an efficient pathogen. Such phenotypic diversity may seriously limit or prohibit the use of colonial opacity associated proteins in vaccines. However, little information is yet available on serological cross reactions between the different proteins. Antisera to intact outer membranes arenow available, and absorption experiments with P9-1 (containing protein I and lipopolysaccharide) should provide antibodies specific to the protein II group. These could be used to prepare Fab fragments with which to study the effect on attachment of blocking specific proteins and the possible cross-reactivities. The same specific antisera may also be used in immunofluorescent studies, to determine which proteins are expressed on the gonococcal surface in in vivo pathological situations. This information would provide a rational basis for the selection of possible vaccine candidate(s). However protein I, which is consistently present in the outer membranes of all known variants, is the most likely vaccine candidate. Although protein I has been associated with gonococcal serological specificity, and varies between strains, it may still have an important vaccine potential. It is thought, for example, that the gonococci causing pelvic inflammatory disease may be restricted to two serologically specific protein I strains (T.M. Buchanan; personal communication). If this were the case, then vaccines could be prepared to protect those individuals most at risk.

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