THE ROLE OF BACTERIAL SURFACE POLYMERS IN THE ADHESION OF FRESHWATER BACTERIA TO SOLID SURFACES

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1984

ABSTRACT

In the study of the mechanism of non-specific permanent adhesion of bacteria at the solid-liquid interface a number of alternative approaches have been used. These include, the use of thermodynamic models, to explain observations of biocontact phenomena, the use of disruptive chemicals, enzymes and electron microscopy to investigate the nature of the adhesive bond and finally the biochemical analysis of cell surface components possibly involved in the process. In this study all three approaches were combined in an investigation of the role of cell surface components on the attachment of freshwater organisms to solid surfaces. A thermodynamic relationship was found using attachment assays on a number of freshwater isolates emphasising the influence of water on the adhesive process. Further investigations of phenotypic changes in membrane surface composition of a number of Pseudomonas isolates using continuous culture demonstrated the involvement of polysaccharide in the inhibition of adhesion. A number of adhesion mutants were analysed for genotypic changes in outer membrane proteins, lipopolysaccharides and exopolysaccharides which also confirmed the inhibitory role of polysaccharides. The results demonstrate the role of cell surface characteristics in the adaptability of the organism to micro-environments such as a solid/liquid or air/liquid interface or the aqueous phase.

DECLARATION

This thesis was composed entirely by myself, and, except for the following biochemical analysis, all work was carried out unaided. The biochemical analysis of fermenter eluate was carried out by the analytical section of PHLS, Centre for Applied Microbiology and Research Porton Down, Salisbury.

The work described in Chapter 2 and a portion of the work described in Chapters 3 and 4 have been published in the following Scientific Journals and are included in the appendix.

1) Pringle, J.H. and Fletcher, M. (1983). Applied and Environmental Microbiology 45: 811-816

2) Pringle, J.H. and Fletcher, M. (1983). Journal of General Microbiology 129: 2557-2569.

3) Fletcher, M., Bright, J.J., and Pringle, J.H. (1983). Proceeding of Engineering Foundation Conference, Hersley Pocono Resort, White Haven Pen. pp:33-43.

ACKNOWLEDGEMENTS

I should like to thank Dr. Madilyn Fletcher for her supervision and helpful suggestions throughout the project, and also for reading this manuscript. I also wish to thank Professor Derek Ellwood for his interest and his staff at PHLS, Porton Down for their help and friendship during my stay there, and Dr. Ken Flint for his invaluable support.

I am especially grateful to Lynda Richardson for her help in the Laboratory and Dr. John Bright for ideas and computing knowledge. I am also indebted to many others in the Department particularly Dorothy and Malcolm. I must thank Dr. Bill Keevil and Dr. Phil Marsh for accommodation during my stay at Porton Down, their good humour, wise-cracks and suggestions. Finally I wish to thank my wife and family for long hours of patience, my wife's excellent typing and everyone else whose contribution made this thesis possible.

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LIST OF ABBREVIATIONS

A	absorbance
C	degrees centigrade
cm	centimeter
cpm	counts per minute
D	daltons
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EDTA	ethylenediaminetetra-acetic acid
EP	exopolysaccharide
g	gram
KD	kilodaltons
l	litre
M	molar
m	meter
mA	milliampere
mg	milligram
min(s)	minute(s)
mJ	millijoules
mm	millimeter
mM	millimolar
mN	millinewtons
ng	nanogram
nm	nanometer
OD	optical density
pH	-log H^+ concentration
pK'	-log K'
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
t	time
µCi	microcurie
µg	microgram
ul	microlitre
V	volts
V/V	volume/volume
wt	weight
W/V	weight/volume

CHAPTER I

INTRODUCTION

Any sample of submerged solid surfaces from either the sea or freshwater environments immediately demonstrates the ubiquitous nature of attached micro-organisms from its slimy appearance and touch. This thesis examines this phenomenon of bacterial attachment to surfaces with the aim to understand the mechanism of the initial attachment process of aquatic bacteria. This introduction first examines bacterial adhesion in the natural environment in order to describe the ecological significance of microbial attachment and the reason for studying its mechanism (Section 1.1). Secondly the introduction evaluates a number of alternative physiochemical models of microbial adhesion which have been developed in order to investigate biocontact phenomena and can be applied to this problem (Section 1.2). A thermodynamic description of cell adhesion is examined in some detail, and is shown to be an important theoretical approach. The Third section (Section 1.3) of the introduction evaluates the influence of the substratum, the medium and the bacterial surface on bacteria adhesion. Each of these components provides a separate factor whose influence can be investigated. Fourthly, the role of bacterial cell surfaces is examined including:- (i) which surface structures are available for adhesion mechanisms, (ii) exopolymers and their role in cell aggregation and (iii) the effects of cell physiology on the cell surface and the attachment process (Section 1.4). The final section

(Section 1.5) of the introduction summarises the aim and objectives of this thesis and lists the principal approaches of the experimental work of each subsequent chapter.

Some of the terms used in this thesis should be clarified from the onset. Both attachment and adhesion are used as somewhat general terms to describe the interaction of a bacterium with a surface, a process which may be passive or active depending upon the involvement of cell physiological activity, such as polymer production. Adsorption is used in the physio-chemical sense referring to the concentration of dissolved substances on the surface, whereas sorption is avoided, as this refers to both adsorption and absorption. Surfaces are termed substrata and not substrates, as the former refers to the surface and the latter to nutrients and assimilated material. The term polymer refers to any organic molecule of repeating structure and not purely of microbial origin.

1.1 Bacterial adhesion in the natural environment.

The attachment, or adhesion, of bacteria to solid surfaces has recently been recognised as an important process in their ecology. The maintenance of productive soils (Strotzky, 1980), the formation of communities on submerged surfaces in the aquatic environment (Marshall, 1976) and the virulence of many bacterial pathogens for animals (Walker and Nagy, 1980) and plants (Dazzo, 1980; Lippincott and Lippincott, 1980) all depend, in part, upon the attachment process. Economic implications of attachment have prompted studies of fouling-communities in aquatic environments, such as microbial films on heat exchangers (Meadows and Anderson, 1979), oral communities causing dental caries (Gibbons and Van Houte, 1980) and, more recently, control of immobilisation of bacterial cells for industrial processes (Hollo et al, 1979; Venkatasubramanian and Vieth, 1979).

1.1.1 Biofouling

One of the major nuisances of these primary films is the serious fouling problems which result on submerged man-made structures, such as ships hulls, oil platforms, underwater TV camera lenses, sonar domes and various submerged ocean monitoring equipment. (ZoBell and Allen, 1935; Miller et al, 1948; Corpe, 1970; Meadows and Anderson, 1979).
It is now recognised that the basis of biofouling by algal and crustacean populations is the initial colonisation of these surfaces by a succession of bacteria. The use of

toxic agents to prevent growth of this primary slime film has not provided a complete solution as it is known that micro-organisms such as marine bacteria and diatoms will attach to and grow on all but the most highly toxic surfaces. (Miller et al, 1948; Woods Hole Oceanographic Institution, 1952; O'Neill and Wilcox, 1971). This is probably due to the composition of anti-fouling paints which include a matrix of insoluble polymers that can be used as a carbon source by marine bacteria, contributing to the release of the toxin from the surface (Dolgopolskaya and Gurevich, 1968). Therefore, one major reason for investigating the mechanisms of bacterial adhesion and the parameters which affect microbial attachment to surfaces is to determine an effective means of preventing or delaying microbial fouling.

1.1.2 What is the ecological significance of microbial attachment?

It is this question above all that requires some answers in considering the role of the attached community in the overall processes carried out by the microbial community. What advantages may be gained by adopting this immobile phase will depend upon a number of factors, including the conditions at the solid-liquid interface, nutrient concentration, community diversity and the characteristics of the surface. Therefore no general advantages or disadvantages can be ascribed (Fletcher and Marshall, 1983). However the significance of attachment for nutrient utilisation and

growth of certain heterotrophic bacteria has been investigated, and there is now evidence that growth advantages may be available at the solid surface, especially in habitats where low nutrient concentrations occur. (Henkelekian and Heller, 1940; ZoBell, 1943; Jannasch and Pritchard, 1972; Hendricks, 1974; Goulder, 1977). It is likely that this "surface effect" is due to the concentration of nutrients at the surface. ZoBell, (1943) found that in sea water containing glass beads, wool or tubes to give 2-200 cm^2 glass surface ml^{-1}, 2-27% of the organic material was adsorbed on the glass. However a number of questions remain as to whether all organic material concentrated in this way can provide suitable nutrient for bacterial growth and whether the substratum composition does not also influence this process (Fletcher, 1979; Bright and Fletcher, 1983). On the other hand utilisation of nutrients at surfaces clearly occur in bioreactors or immobilised beds of bacterial cells and can provide an efficient means of biodegration. For example the attachment of micro-organisms to stone surfaces or other man-made trickling filter packing provides an extensive microbial film capable of decomposing organic materials and destroying sewage bacteria in this particular method of waste water treatment (Mack et al, 1975). Therefore a second reason for studying bacterial adhesion is to investigate the ecological implication of bacterial growth at the solid liquid interface. That is to determine

whether such a site is energetically favourable to cell growth and whether the cell requires an active or passive mechanism of attachment. Such studies may indicate that immobilisation of bacterial cells could be engineered in bioreactors for use in enzymatic conversions or production of exoenzymes, polysaccharides and other excreated secondary metabolites. (Gerson and Zajic, 1979).

1.1.3 The activity of bacteria at interfaces

Bacteria require aqueous conditions for growth, but the majority of natural habitats are not homogeneous and compromise a variety of interfaces which provide sites for the attachment of bacteria. In nature there is a wide range of liquid/liquid, gas/liquid and solid/liquid interfaces, many of which support bacterial communities. An obvious example of a liquid/liquid interface is the aqueous medium-oil interface, as most oils give a distinct boundary with water. Utilisation of hydrocarbons by microbes is often accompanied by the production of extracellular surface-active materials, which stimulate growth due to increased emulsification of the hydrocarbon phase. (Erickson and Nakahara, 1975; Gerson and Zajic, 1978). More recently, measurements of the cell-aqueous and cell-hydrocarbon interfacial free energies have been made (see Section 1.2.4) indicating that organisms with a relatively hydrophobic surface combined with the ability to metabolise the hydrocarbon phase have a distinct advantage as they are

attracted to the hydrophobic boundary. Experiments with Acinetobacter calcoaceticus have shown that the surface active compounds as well as the changes in relative hydrophobicity in the cell surface are a response to growth on the hydrocarbon substrates (Neufeld et al., 1980). Thus microbial activity at this interface is quickly adapted to utilisation of hydrocarbon substrates by changes in the outer membranes and extracellular products. Microbial activity at this interface has been suggested as a cause of the rapid degradation of oil spillages (Marshall, 1976).

The gas/liquid or air/water interface is ubiquitously part of the natural environment. The sea-air interface covers approximately 70% of the world's surface. When compared to the subsurface, the surface microlayers of the world's oceans contain an accumulation of potential organic nutrients for heterotrophic bacteria. For example, the concentration of dissolved organic carbon in the subsurface water of the North Atlantic has been estimated as

1.3mg carbon l^{-1} as compared with a mean concentration of 1427 mg carbon l^{-1} (2.9g organic matter l^{-1}) calculated for the 0.1μm surface microlayer (Sieburth, 1975)
These surface microlayers also accumulate bacteria to a concentration between 10 to 1000- fold greater than the subsurface cell numbers. Experiments with axenic cultures of marine bacteria indicate that bacterial interaction takes place at sites in or just beneath the surface lipid

film, which is an important component of the surface microlayers (Kjelleberg et al, 1982).

A further interface influencing microbial activity is the solid-liquid interface, which is of central importance to this study. The solids may be inorganic, living or dead materials, such as clays, sandgrains, sediments, rocks, man-made structures, plants, animals and organic remains of living organisms at different stages of decomposition. The first evidence for bacterial adhesion to surfaces was obtained from glass slides that had been submerged in several aquatic environments (Henrici, 1933; ZoBell and Allen, 1935) and subsequent microscopic examination. These observations have led to extensive studies of the succession of micro-organisms during the initial colonisation of surfaces (Corpe, 1973; Jordon and Staley, 1976; Marshall et al, 1976). Epifluorescence and electron microscopic techniques have been used to overcome the problems resulting from the opacity of solid surfaces found in natural environments and have verified the ubiquitous distribution of micro-organisms within the epiphytic community. (Gerchakov, et al, 1977,1978; Marszalek et al, 1979; Dempsey, 1981).

1.1.4 Succession and Microbial film development

The succession of micro-organisms at the solid-liquid interface can be viewed as an autogenic succession resulting from the resident population altering its own environment. (Alexander, 1971).

Preliminary to any bacterial colonisation, the spontaneous adsorption of macro molecules to any surface immersed in aquatic habitats results in a "conditioning film" which alters the physiochemical properties of the interface and provides a concentration of useful nutrient materials at that surface. (The effects of this conditioning film on bacterial attachment will be an important component of this thesis and will be discussed further in this introduction (see Section 1.3.1). Marshall, (1979) has suggested that this situation favours colonisation of the surface by zymogenous or copiotrophic bacteria.

Zymogenous or copiotrophic (Poindexter, 1981) bacteria are terms describing the nutritional characteristics of chemo-organotrophic bacteria that show a rapid response to high levels of nutrient but grow poorly in low nutrient concentrations. They can be best defined as possessing high maximum growth rates (μ max) and low substrate affinities (high saturation constant k_s). By contrast oligotrophic bacteria are able to grow at low nutrient concentrations and are adapted to infrequently or slowly changing environments where slow growth and efficient uptake systems (low μ max and low k_s) are an advantage. Thus the significance of this distinction between groups of chemo-organotrophic bacteria is reflected in the observed succession of bacteria colonising surfaces in the natural aquatic environment and the changes in available nutrients

at this interface. Therefore a transition from a zymogenous to an oligotrophic bacterial community would be expected as the available nutrients are used and become limiting.

Marshall, (1980) has suggested that microbial growth in aquatic habitats and in the aqueous phase of soils is often severely limited by deficiencies in one or several essential nutrients. This limitation results in metabolic dormancy when bacterial size is reduced as metabolic activity is lowered. Evidence from different habitats indicates that this is a common feature of many bacteria in open oceans, oligotrophic lakes and in soils away from the rhizosphere (Jannasch,1958; Gray and Williams, 1971; Novitsky and Morita, 1976; Casida,1977). Experiments in the chemostat at subsaturating concentrations of the limiting nutrient have shown that typical aquatic organisms (eg. Spirillum, Pseudomonas) become smaller with decreasing growth rates, although the oligotrophic spirillum maintained a higher surface area to volume ratio (s/v) than that of the more zymogenous Pseudomonas species (Hirsch et al,1979). In fact, recent experimentation using colonised glass and formvar coated electron microscope grids supports the view that these small bacterial selectively colonise the surfaces in the first few hours of immersion (Marshall, 1971). Corpe,(1973) noted that Gram-negative organisms predominated over this period and grew readily on the

surfaces in the presence of low nutrient levels (0.005% yeast extract). Thus the bacteria responsible for the initial colonisation of surfaces immersed in low nutrient aquatic environments are thought to be physiologically inactive zymogenous or copiotrophic bacteria and that these organisms rapidly recover their normal size and activity once they adhere to the solid surface and utilise the adsorbed, thus concentrated nutrients.

Following colonisation of a surface by a sequence of different zymogenous bacteria, including: Pseudomonas (50-90%), pigmented Flavobacterium species or non-pigmented non-motile Achromobacter species (10-49%) (Corpe, 1973), appreciable numbers of oligotrophic types begin to appear after 24-72hrs. (Marshall et al,1971; Corpe, 1973). These included Caulobacter, Hyphomicrobium and Saprospira spp. which are better adapted than the copiotrophs to the low nutrient condition prevailing at this stage. Later in the successional sequence, cyanobacteria, diatoms, other unicellular algae and protozoa appear as the biomass, numbers and diversity of attached organisms increases and the succession proceeds. Accompanied with this progression from Gram-negative, rod-shaped, primary colonising bacteria to prosthecate forms is the development of an extensive slime matrix which

increases nonlinearly, whereas attached biomass increases linearly (Bryers and Characklis, 1981).

1.1.5 Attachment Mechanisms

Marshall, (1980) has recognised several different mechanisms of adhesion. These include:-

i) Specific permanent adhesion, found in bacteria attaching to specific sites and involving interaction between complementary molecular structures on the bacterial surface and the attachment site (Jones, 1977).

ii) Non-specific permanent adhesion, where micro-organisms from natural habitats attach to different types of surfaces via a relatively non-specific reaction using specialised attachment organelles, such as appendages, prosthecae, or blebs (Hirsch and Pankratz, 1970; Pertsovskaja et al., 1972; Corpe et al, 1976; Ellen et al, 1978). For example, caulobacters attach by means of a substance produced at a special holdfast region at the end on the prosthecate stalk. (Corpe et al, 1976). Where no such specialised structure is produced adhesion is a result of direct interaction of the bacterial surface polymers and the various surfaces involved (Fletcher, 1980).

iii) Finally temporary adhesion, found, for example, when predatcry gliding bacteria (eg. Saprospira sp.) move over colonised surfaces scavenging film bacteria. The gliding movement of one organism has been shown to take place only when an extracellular glycoprotein slime

which functions as a temporary stefan adhesive is produced by the cells. (Humphrey et al, 1979).

1.2 Physiochemical models of microbial adhesion

One approach to the study of adhesion of micro-organisms to surfaces is to model the long and short range and hydrodynamic forces operating between the surface and the adhering cell in order to determine how they influence cell surfaces contacts. This approach has produced a number of modern sophisticated multi-parameter theories, which have been developed for well-defined systems, providing semi-quantitative descriptions based on simplified assumptions of bacteria as discrete colloidal particles coated with biological macromolecules, such as polysaccharides. These models have been helpful in explaining many biological observations relating to microbial adhesion and the forces acting to form permanent (irreversible) or temporary (reversible) adhesion. However, bacterial cells do not completely conform to these over simplified models in that they are able to adapt to changes in their environment through physiological processes.

1.2.1 DLVO theory: Long-range interactions between charged particles and macroscopic bodies

Long-range forces, i.e. those which are significant separation distances greater than about 2nm, are accurately formulated in the now classical DLVO theory, devised by Derjaguin and Landau, (1941) and Verwey and Overbeck (1948). This theory states that the total

interaction between two objects comprises two terms, one (GA) due to the Van der Waals dispersion forces and the other (GE) due to the overlap of the electrical double layers associated with the charge groups present on the particle and the macroscopic surface. These terms are simply added to determine the free energy of interaction, Gi(h), which is related to the mutual force, fi(h) as a function of particle-surface separation through the relationship

$$fi(h) = -dGi(h)/dh \qquad (1.1)$$

where h is the particle/surface separation

and

$$Gi(h) = GA(h) + GE(h) \qquad (1.2)$$

One approximate form for GA is (Visser, 1976)

$$GA = -\frac{A}{6}\left[\frac{2a(h+a)}{h(h+2a)} - h\left(\frac{h+2a}{h}\right)\right] \qquad (1.3)$$

(a refers to the particle radius)

Where A is the net Hamaker constant for the system, this may be expressed in terms of the individual Hamaker constants of the materials involved by the equation 1.4 (typical values ($x10^{20}$J) are as follows (Visser 1976): water 4.4; oxides 10.6; carbon 47.0. Moreover, the calculation of the Hamaker constant for the various cell constituent requires knowledge of relevant spectroscopic

data, and only limited information is available in the literature for biological materials (Nir, 1977). Values for A can be determined from the ionisation potentials and polarisabilities of atoms comprising the particles and medium of the system. Weiss, (1972) has drawn attention to the difficulties associated with the assignment of numerial values to the Hamaker constant of biological systems.

$$A = (A_1^{\frac{1}{2}} - A_3^{\frac{1}{2}})(A_2^{\frac{1}{2}} - A_3^{\frac{1}{2}}) \qquad (1.4)$$

Where '1' refers to the macroscopic body, '2' to the particle, and '3' to the medium. Normally $A_1 > A_3$ and $A_2 > A_3$ for aqueous systems so the A is positive and GA negative, and the net effect of the Van der Waals forces is attraction. However with teflon, where $A_1 < A_3$, then the net effect of Van der Waals forces is a repulsion. G(E), the electrical double layer interaction, is evaluated from the electical potential at each interface about to overlap, that is ψ_{13} and ψ_{23}. The electro-kinetic or zeta-potential, ζ (mv), is measured by cell electrophoresis and isolectric focusing and has been determined for a number of bacteria (Sherbet, 1978). Typical values for ζ range from 0 to $\pm$ 100mV depending upon the net number of surface charge groups and the bulk electrolyte concentration. Using a

model based on the assumption that, in the case of adhesion, a constant potential is maintained during the cell-surface interaction, then G(E) is given by Equation 1.5. For small values of ψ (<50mV) (Hogg et al, 1966):

$$G_{(E)} = \pi \epsilon \epsilon_o \alpha (\psi_{13}^2 + \psi_{23}^2) \left[\frac{2\psi_{13}\psi_{23}}{\psi_{13}^2 + \psi_{23}^2} \ln \left(\frac{1+\exp(-\kappa h)}{1-\exp(-\kappa h)} \right) + \ln[1-\exp(-2\kappa h)] \right]$$

Where ϵ is the dielectric constant of the medium (78;54 for water at 25°C) and ϵo is the permitivity of free space ($8.85 \times 10^{-12} J^{-1} C^2 m^{-1}$). κ depends on the total ion concentration in the bulk solution, that is

$$\kappa^2 = \frac{e^2}{\epsilon \epsilon_o kT} \sum_i z_i c_i \qquad (1.6)$$

Where e is the electron charge ($1.6 \times 10^{-19} C$) K the Boltzmann constant ($1.38 \times 10^{-23} JK^{-1}$) and T the absolute temperature. Zi is the valency and Ci the concentration of ionic species i. κ^{-1} is an estimation of the thickness of the electrical double layer and takes the following approximate values for a simple 1:1 electrolyte solution: κ^{-1} = 100 when $C_i = 10^{-5}$ mol dm^{-3}, κ^{-1} = 10 when $C_i = 10^{-3}$ mol dm^{-3} and κ^{-1} = 1 when $C_i = 10^{-1}$ mol dm^{-3}).

These models do give some indications of the long range forces operating between the cell and the surface in aqueous media and how the ionic strength of the media and ζ of the surfaces influence contact. As bacterial cells are invariably negatively charged, as are natural surfaces, the forms of G(A), G(E) and G(i) = (G(A) + G(E)) which are most useful in cases where the particle and macroscopic surface have the same negative sign are shown in Figures 1.1 (a b c). The shapes of these curves depend on the values of $a_1 \psi 13$ and $\psi 23$, (or $\zeta 13$ and $\zeta 23$) and κ . Generally at low electrolyte concentration, the G(E) term predominates and there is a large free energy barrier or repulsion force to overcome if a particle is to come into close contact with the surface (Fig 1.1a). At high electrolyte concentration (Fig 1.1c) the free energy barrier is eliminated and there is a strong attraction between the surface and the particle. At intermediate electrolyte concentrations (Fig 1.1b) the free energy barrier is still present but much smaller, in the range 0 to 10KT. This will allow a certain fraction of contacts or particle surface collisions inside the primary minimum and also creates a secondary minimum at separations in the range 3-8nm.

The primary and secondary minima are regions of particle/

Figure 1.1

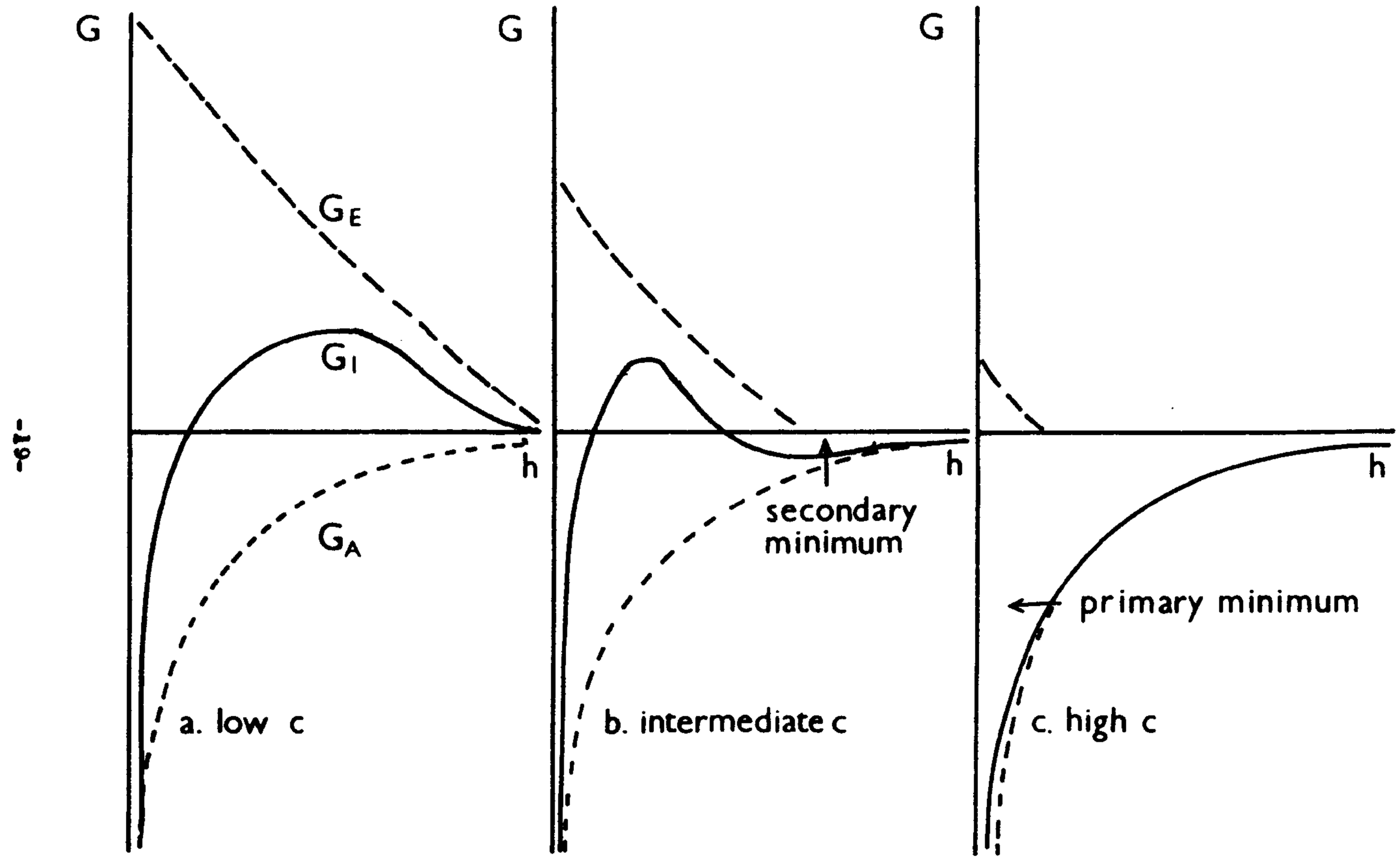

G
G
G
G_E
G_I
G_A
h
h
h
secondary
minimum
primary minimum
a. low c
b. intermediate c
c. high c

Figure 1.1.a,b,c

Interaction free energy (G) versus separation (h)

(h) curves as a function of electrolyte concentration (c)

a) low c (10^{-5}Mol dm^{-3})

b) intermediate c (10^{-3} mol dm^{-3}) and

c) high c (10^{-1}mol dm^{-3}) for a charged particle approaching a macroscopic surface of the same sign. The areas of primary and secondary minima are indicated.

surface separations over which there is a net attraction between the surface and particle. In the primary minimum short range forces predominate where as the secondary minimum relies upon the G(A) and G(E) terms. Because the attraction force of the secondary minimum is generally much shallower (<5KT) than the primary minimum, particles will only reside in this minimum temporarily. This secondary minimum may account for reversible attachment of bacteria which enables a bacterium to remain in close association with the surface without becoming permanently attached (Marshall et al, 1971). However, these theories of colloid stability applied to biological contact phenomena depend on values for a number of parameters being available. Firstly, realistic values for the Hamaker constant of bacterial cell surfaces and solid surfaces are required. The model presented above considers purely dispersion effects in the calculations, and the DLVO theories neglect the induction and orientation effects which are important between polar molecules over short distances (ie.< 1nm). These effects combined with the dispersion interaction make up the Van der Waals energies between molecules. A second approach to long-range Van der Waals interactions between macroscopic bodies is the Lifshitz approach (Lifshitz, 1956) which calculates the equivalent

to the Hamaker constant. Although "A (dispersion Lifshitz)" is a function of h, the separation theory also includes a term "A (orientation and induction)". This theory does not attempt to calculate the sum of the interactions between individual atoms or molecules, but instead the interaction of objects is regarded as occurring through the continuous medium of the fluctuating electro-magnetic field which is always present in the interior of a material medium. The DLVO theory and Lifshitz's theory show good agreement over the long-range interactions, but the Lifshitz model may be more useful at short-ranges, and a combination of the macroscopic DLVO theory and microscopic Lifshitz theory has also been proposed (Nir, 1977).

These models have also been improved by considering formulae to allow for geometry changes to the cell and recently models for cell periphery, where spherical particles possessing multiple layers of varying thicknesses and compositions were considered (Nir, 1977). However, these models are very limited by their need to view the cell surface as a static, rigid structure of constant composition and surface potential. Thus changes in chemical composition of the cell surface which would escape attention in usual double layer Van der Waals descriptions may still dominate the cell contact phenomenon,

particularly if this reaction involves short-range forces of greater magnitude and complexity.

1.2.2 Short-range forces

As mentioned above, short-range forces are particularly important at "close separations" (<0.4nm) and have been emphasised in cell adhesion interactions in a number of publications (Pethica, 1961; Weiss,1967; Rutter and Vincent, 1980; Tadros,1980). They include:-

i) chemical bonds, for example electrostatic, covalent and hydrogen bonds;

ii) dipole interactions, for example dipole-dipole (orientation) (Keesom), dipole-induced dipole (induction) (Debye), induced dipole- induced dipole (dispersion and ion-dipole interactions (London, 1937).

iii) hydrophobic interactions (Kauzman, 1959; Tanford,1979). This last mentioned effect is very misleading as it is associated with the observed tendency of hydrocarbon groups to orientate away from an aqueous phase and is therefore due to the short range forces in i) and ii) influencing water structure and not to a hydrocarbon-water repulsion, due to some unknown force. In actual fact, hydrocarbon-water surface contacts have an attraction for each other and are not repulsive. However, water surfaces and other hydrophilic surfaces have greater attractive forces for each other and therefore create

the impression that certain surfaces are hydrophobic.

The short-range interactions above are therefore of particular importance in aqueous systems and may be repulsive or attractive, depending on the nature of the surface involved. We may view this in terms of the displacement of water molecules from the interface as a micro-organism approaches the microscopic surface. If both surfaces are hydrophilic the short-range interactions are repulsive as both surfaces are sufficiently hydrated to require a net increase in free energy, in order to displace water molecules and spontaneous attractive interactions are always accompanied by a decrease in free energy.

An example of this effect is observed with aqueous dispersions of precipitated silica particles which do not readily coagulate on the addition of electrolytes (Harding, 1971). However, when both surfaces are hydrophobic they attract only weakly the associated surface water molecules which have greater affinity for the bulk solution. Thus water molecule displacement during the approach of hydrophobic surfaces causes a net decrease in their free energy, since there is a net increase in their attraction to each other. (see section 1.2.3 for further details). This is the basis of the "hydrophobic" interaction and suggests that the strongest hydrophobic effects are between hydrocarbon surfaces in

an aqueous medium, followed by hydrophilic-hydrophobic surface interactions with hydrophilic-hydrophobic interactions either weak or repulsive. This "solvophobia" property of water and hydrophobic interactions will be discussed further in sections 1.2.4 - 1.2.5 of the Introduction.

1.2.3 A thermodynamic description of cell adhesion

A useful model for determining the probability of bacterial adhesion in a defined system involves the use of classical thermodynamics. The 2nd law of thermodynamics describes one criterion for an adhesion "reaction" that can occur spontaneously as a negative change in free energy, mediated by changes in enthalpy (decrease) and or entropy (increase). The criterion of spontaneous change, under thermodynamic "irreversible" (non-equilibrium) conditions in a closed system where temperature (T) and pressure (P) are constant, is determined from equation 1.7

$$\Delta S - \frac{\Delta H}{T} > 0 \qquad 1.7$$

Where ΔS is the change in entropy and ΔH the change in enthalpy of the system. These conditions can be determined for such a system using equation 1.8 and quantified as the change in Gibbs free energy (ΔG)

$$\Delta G = \Delta H - T\Delta S \qquad 1.8$$

The condition of spontaneous adhesion would therefore require a net negative change in Gibbs free energy. However, the rate of this process is not determined by this free energy change but by other factors for example the activation energy of the reaction.

This thermodynamic approach can therefore predict whether bacterial adhesion will take place as a spontaneous process provided the change in Gibbs free energy, ΔG, can be evaluated. It would be possible to calculate the ΔG from the DLVO theory or more up to date versions of DLVO theory which consider the long-range forces influencing cell adhesion with the limitations as described in section 1.2 Hall, (1972) has given an equilibrium thermodynamic treatment of a system of interacting colloidal particles of arbitrary geometry and size. It was shown that the properties of a colloidal system can be described completely in terms of the chemical composition, chemical potentials, pressure and temperature. However, in living systems the physiological response of cell-cell and cell-surface interaction can influence the local molecular composition of the cell membranes influencing Van der Waals interactions and cell membrane surface potentials to such an extent that what is required is a non-equilibrium thermodynamic analogue to Halls equilibrium account (Pethica, 1980).

1.2.4 'Wetting' as a description of adhesion

A complementary approach to the colloid stability theory as a means of thermodynamically describing bacterial adhesion is the 'wetting' approach. Again, the change in free energy of the system, which is produced by contact of the surfaces of cell and substratum, gives an indication of the driving forces involved. The free energy change per unit area of contact is related by equation 1.9 to the interfacial free energies of the three interfaces involved.

$$\Delta G^{ADH} = \gamma_{SB} - \gamma_{SL} - \gamma_{BL} \qquad 1.9$$

Where γ_{SL}, γ_{BL} and γ_{SB} are the interfacial free energy of the solid/substratum-liquid, BL bacterium-liquid and SB substratum-bacterium interfaces respectively (Gerson 1980, Good, 1977). Thus in order to determine the ΔG^{ADH} values for all three interfacial free energies are required and are evaluated from equation 1.10 developed from the theory of liquid-liquid interfacial tension by Girifalco and Good,(1953,1954).

$$\gamma_{12} = \gamma_1 + \gamma_2 - 2\phi_{12}\sqrt{\gamma_1\gamma_2} \qquad 1.10$$

Where γ_1 and γ_2 are the free energies of the two interacting surfaces and ϕ_{12}[1] is the interaction parameter

1 Further details of the ϕ_{12} will be discussed below in this Section.

of the two surfaces. Surface free energy, or for liquid surface tension (γ), is described as the energy required to form a new area of surface.

$$\frac{dG}{dA} = \gamma \qquad 1.11$$

Where A = area, γ = surface free energy per unit area and G = Gibbs free energy. It can be thought of as the available energy resulting from surface groups, molecules, or atoms which are able to interact with other surface groups, molecules or atoms which enter into close association i.e. the available bonding energy. The types of interaction are both short and long range forces described in sections 1.2.1 and 1.2.2 and are characteristic of the particular substratum material.

The method of evaluating surface free energy is based upon surface tension measurements of liquids and the measurement of contact angles of liquids on solids. The relationship between a contact angle (θ) of a liquid on a solid and the solid-liquid (γ_{SL}), solid-vapour (γ_{SV}) and liquid-vapour (γ_{LV}) interfacial energies is given by Young's equation:

$$\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos \theta \qquad 1.12$$

Studies of the contact angles of a variety of liquids on clean, low energy polymer surfaces revealed a linear

relationship between cos θ and liquid vapour interfacial energies (γLV) for a homologous series of organic liquids (Zisman, 1964). This empirical result is described by equation 1.13,

$$\cos \theta = 1 + b(\gamma C - \gamma LV) \qquad 1.13$$

Where γC is the critical surface tension of wetting and b is the slope of the relationship between γLV and cos θ.

The intersection of this line with cos θ = 1 occurs at a value of γ_{LV} defined as the critical surface tension for wetting, γc, for that particular series of liquids. Upon first examination the effect of cos θ = 1 on Youngs equation (1.12) is to make, $\gamma SL = \gamma SV - \gamma LV$, and when a liquid spreads over a surface the interfacial free energy approaches zero. Therefore γLV of the liquid approaches γSV of the solid. However the use of this parameter as an estimation of surface free energy has some limitations. Unfortunately plots of cos θ against γLV give different values of γc depending upon the liquid series used for the contact angle measurements. Three different γc's have been described: (i) γC^A obtained using non-polar liquids, e.g. n-alkanes and d_1-n-alkyletes ii) γC^B obtained using polar liquids, e.g. halogenated compounds and esters and iii) γC^C obtained with the use

of hydrogen bonding liquids eg. water, glycerol and formamide (Kitozaki and Hata, 1972). Thus to obtain a γ_C value which is a good approximation of the surface free energy it is necessary to select the series of liquids (i,ii and/or iii) which has the same bonding potential eg. non-polar, polar and hydrogen as the substratum bonding components.

A second parameter that can be used to evaluate surface wettability is the thermodynamic work of adhesion WA. This describes the energy required to separate the two phases at an interface and therefore provides a comparative measure of the interphase bonding between a series of substrata and a single liquid such as water. WA is defined simply as the difference between the surface free energy of the newly formed interface and the sum of the surface free energies of the two interfaces prior to adhesion.

$$WA = \gamma SV + \gamma LV - \gamma SL$$

1.14

WA where water is the liquid phase

$WA(H_2O)$ is probably a more realistic parameter of substratum-wetting in relation to bacterial attachment (as compared to the true surface free energy (γSV)), because the bacterial attachment process takes place at a solid/aqueous interface, where non-polar, polar and

hydrogen bonding may be significant.

Another approach to determine surface free energies and interfacial free energies also makes use of the equation-of-state approach used by Neumann et al,(1974). This is based upon the observation that a plot of $\cos\theta$ against $(\gamma LV)^{-\frac{1}{2}}$ is closer to the empirical relationship than Zisman's extrapolation. (Good, 1977) Therefore, equation 1.13 is better expressed as equation 1.15:

$$\cos\theta = -1 + 2\phi\left[\frac{\gamma SV}{\gamma LV}\right]^{\frac{1}{2}} \qquad 1.15$$

where ϕ is the interaction parameter as defined by

$\phi^2 = \frac{\gamma C}{\gamma SV}$ when $\cos\theta = 1$

Experimentally a strong correlation was found between $\cos\theta$ and γLV when Young's equation of state (1.12) was combined with Good's equation for interfacial free energy (1.10), which incorporates the interaction parameter, (ϕ) as a means of correcting for the differences in bonding potential between the liquid and the solid. If the bonding phases are similar then $\phi = 1$; if the bonding phases are dissimilar $\phi \leqslant 1$. The use of the linear correlation between the interaction parameter and γSL (1.16) enabled the interaction parameter to be used to correct for non-linearities present in plots of $\cos\theta$ against γLV and resulted in the empirical equations 1.17 and 1.18, which enabled values of γSV and γSL to be determined

from easily measured contact angles and liquid surface tensions. (Ward and Neuman,1974, Neuman et al, 1974)

$$\phi = -0.0075\gamma_{SL} + 1.00 \qquad 1.16$$

$$\cos\theta = \frac{\gamma_{LV} + (0.015\gamma_{SV} - 2.00)(\gamma_{SV}\gamma_{LV})^{\frac{1}{2}}}{\gamma_{LV}(0.015(\gamma_{SV}\gamma_{LV})^{\frac{1}{2}} - 1)} \qquad 1.17$$

$$\gamma_{SL} = \frac{((\gamma_{SV})^{\frac{1}{2}} - (\gamma_{LV})^{\frac{1}{2}})^2}{1 - 0.015(\gamma_{SV}\gamma_{LV})^{\frac{1}{2}}} \qquad 1.18$$

Equation 1.17 is cubic with respect to γ_{LV} and some care is required in selecting the correct root. Neumann et al., (1974) have published a fortran program which incorporates selection criteria for the most appropriate value of γ_{SV}. A second program has also been published to calculate the interfacial free energy (γ_{SL}) from values of γ_{IV} and γ_{2V}, where I and 2 can be either liquid or solid, using the equation of state model (Omenyi, 1978). Bedause of the form of equation 1.18, a discontinuity in γ_{SL} may occur for large values of γ_{LV} or γ_{2V}. The computer program therefore calculates γ_{SL} in a manner which is internally consistent with the equation of state approach.

A comparison of ϕ values computed using the equation of state approach above to ϕ evaluated from the molecular (ie. non-thermodynamic) properties of the interface shows good agreement. It has been shown the ϕ_{12} can be

evaluated as

$$\phi_{12} = \phi_{12}(\alpha_1,\alpha_2,\mu_1,\mu_2,I_1,I_2) \qquad 1.19$$

where α is the molecular polarisability, μ is dipole moment and I is ionisation energy (Good, 1977). For polymers α and $\underline{\mu}$ are the polarisability and dipole moment per segment (Good, 1964; Good and Elbing, 1970). An equation has been reported from which ϕ_{12} can be calculated using these parameters (Good and Elbing, 1970). The values of ϕ_{12} range from $\approx$1 for water against organic polymers that form strong hydrogen bonds to 0.6 for water against saturated hydrocarbon polymers. The electrons of non polar aromatic compounds and other weak hydrogen bonding polymers have ϕ_{12} values ranging from 0.7-0.8. These values are difficult to predict using molecular properties of these compounds as the strength of the hydrogen bonds between water and the π electrons of the aromatic ring is not known with any accuracy. However, the range of ϕ_{12} predicted from the equation of state approach fits closely with these values calculated from the molecular properties of the solids and liquids tested.

Returning to the thermodynamic arguments regarding the influence of the free energy of adhesion (ΔG^{Adh}) on

the adhesion of bacterial cells in an aqueous media to solid substrata. Equation 1.9 requires values for γ_{SL}, γ_{BL} and γ_{SB} to be determined before the ΔG^{Adh} can be calculated. However, the equation of state approach has enabled these interfacial free energies to be calculated from theoretical values for γ_{SV}, γ_{BV} and γ_{LV}. Therefore predictions relating ΔG^{Adh} to varying values of γ_{SV} can be made when γ_{BV} and γ_{LV} are held constant. By generating a family of curves γ_{BV} and γ_{LV} variables can also be examined and a thermodynamic model of cell-surface adhesion can be produced. Figures 1.2(a,b,c) show such curves for ΔG^{Adh} as a function of substratum surface free energy, γ_{SV}, for, a) various liquid surface tensions γ_{LV}(mNm^{-1}, mJm^{-2}) and γ_{BV} constant ($70mJm^{-2}$); b) various liquid surface tensions γ_{LV}(mNm^{-1}, mJm^{-2}) and γ_{SV} constant ($60.0mJ\ m^{-2}$); c)various bacterial surface free energies γ_{BV}(mNm^{-1}, mJm^{-2}) and γ_{LV} constant ($73.0mJm^{-2}$). On the basis that spontaneous adhesion of bacterial cells will occur with a negative charge in the free energy of adhesion (ΔG^{Adh}), several predictions regarding the eventual equilibrium partition of cells adhering to the surface can be made:-

i) When $\gamma_{LV}>\gamma_{BV}$, a negative ΔG^{Adh} will result for bacterial adhesion to all surfaces in the range $\gamma_{SV}>\gamma_{LV}$ (fig 1.2.b)

ii) When $\gamma_{LV}=\gamma_{BV}$ a negative ΔG^{Adh} will only result for

Figure 1.2.a,b,c

Change in free energy of adhesion (ΔG^{Adh}) versus substratum surface free energy (γSV) for: a) various liquid surface tensions (γLV) and γBV constant (70 $OmJm^{-2}$); b) various liquid surface tensions (γLV) and γBV constant ($60.0mJm^{-2}$); c) various bacterial surface free energies (γBV) and γLV constant ($73.0mNm^{-1}$). On the basis that spontaneous adhesion of bacterial cells will occur with a negative change in ΔG^{adh} bacterial adhesion would be favoured for the following criterion:-

i) when $\gamma LV > BV$ ΔG^{adh}(-ve) to all surfaces in the range $\gamma SV < \gamma LV$ (fig. 1.2.b)

ii) when $\gamma LV < \gamma BV$ ΔG^{adh}(-ve) for all surfaces in the range $\gamma SV > \gamma LV$ (fig 1.2.a)

iii) when $\gamma LV > \gamma BV$ and $\gamma LV = 72.8mNm^{-1}$ then attachment to low energy surfaces expected

When $\gamma LV < \gamma BV$ and $\gamma BV = 70.0mJm^{-2}$ then attachment to high energy surfaces favoured.

Figure 1.2,a

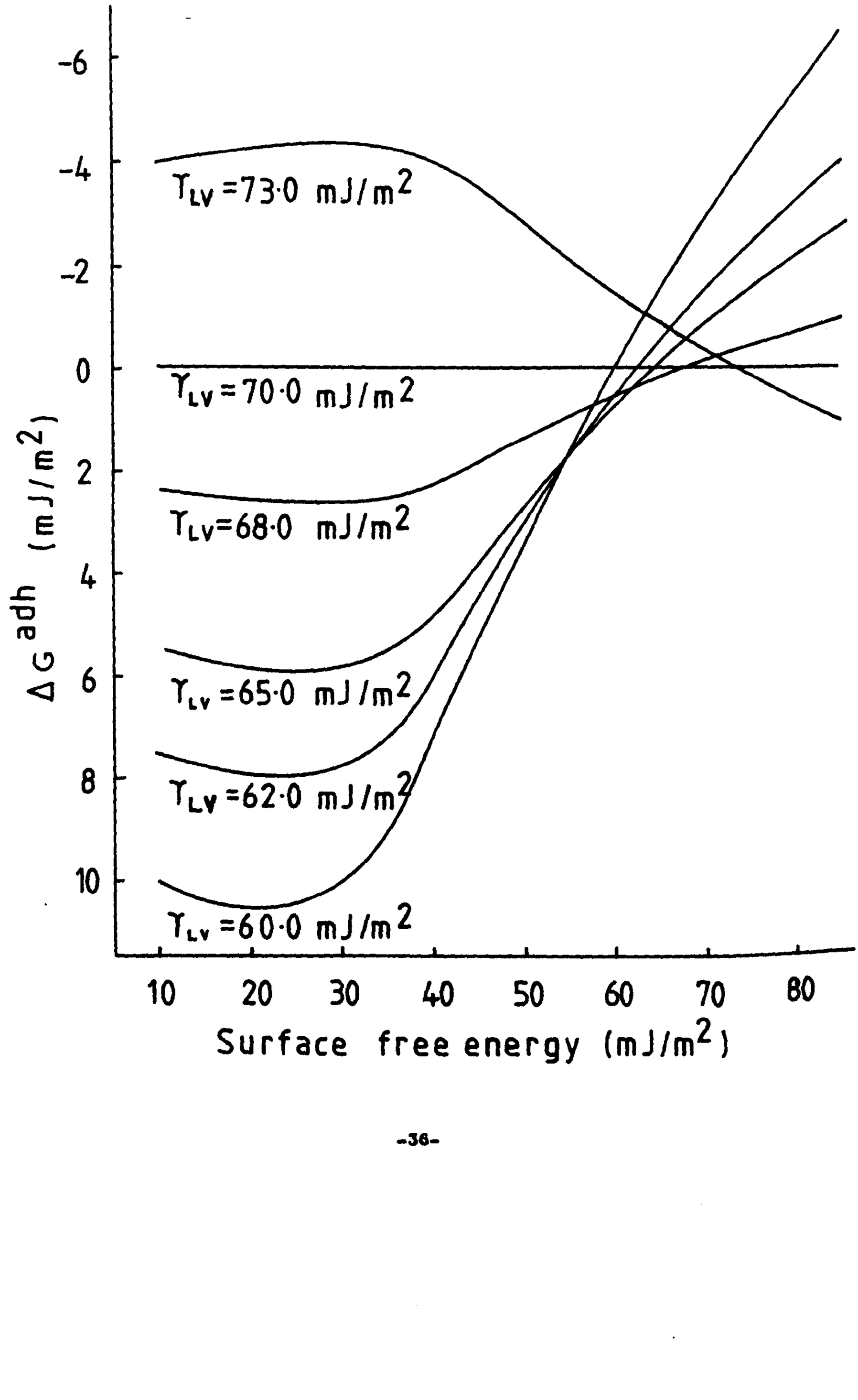

-6
-4
-2
0
2
4
6
8
10
ΔG^{adh} (mJ/m^2)
γ_{LV} = 73·0 mJ/m^2
γ_{LV} = 70·0 mJ/m^2
γ_{LV} = 68·0 mJ/m^2
γ_{LV} = 65·0 mJ/m^2
γ_{LV} = 62·0 mJ/m^2
γ_{LV} = 60·0 mJ/m^2
10 20 30 40 50 60 70 80
Surface free energy (mJ/m^2)

Figure 1.2, b

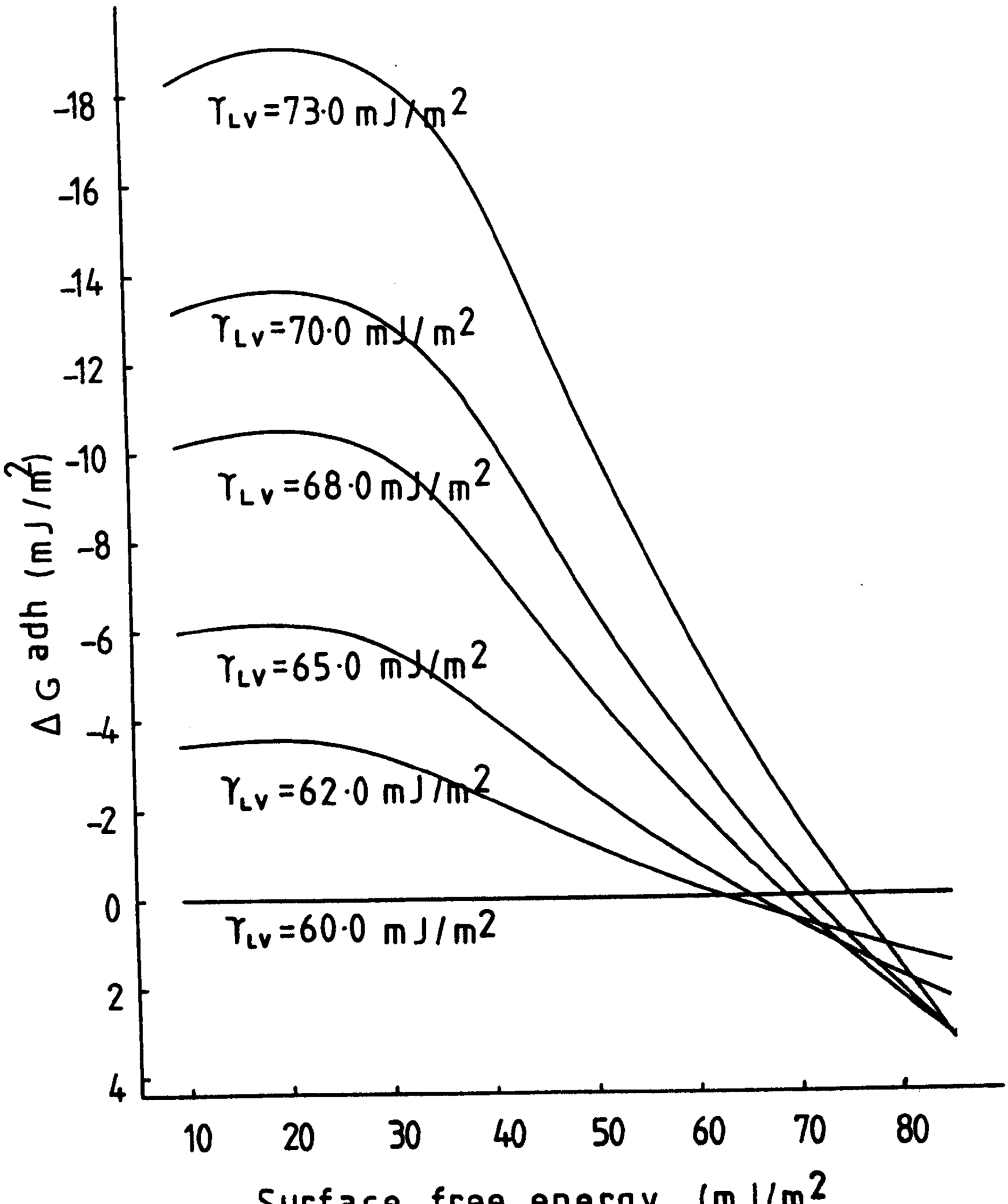
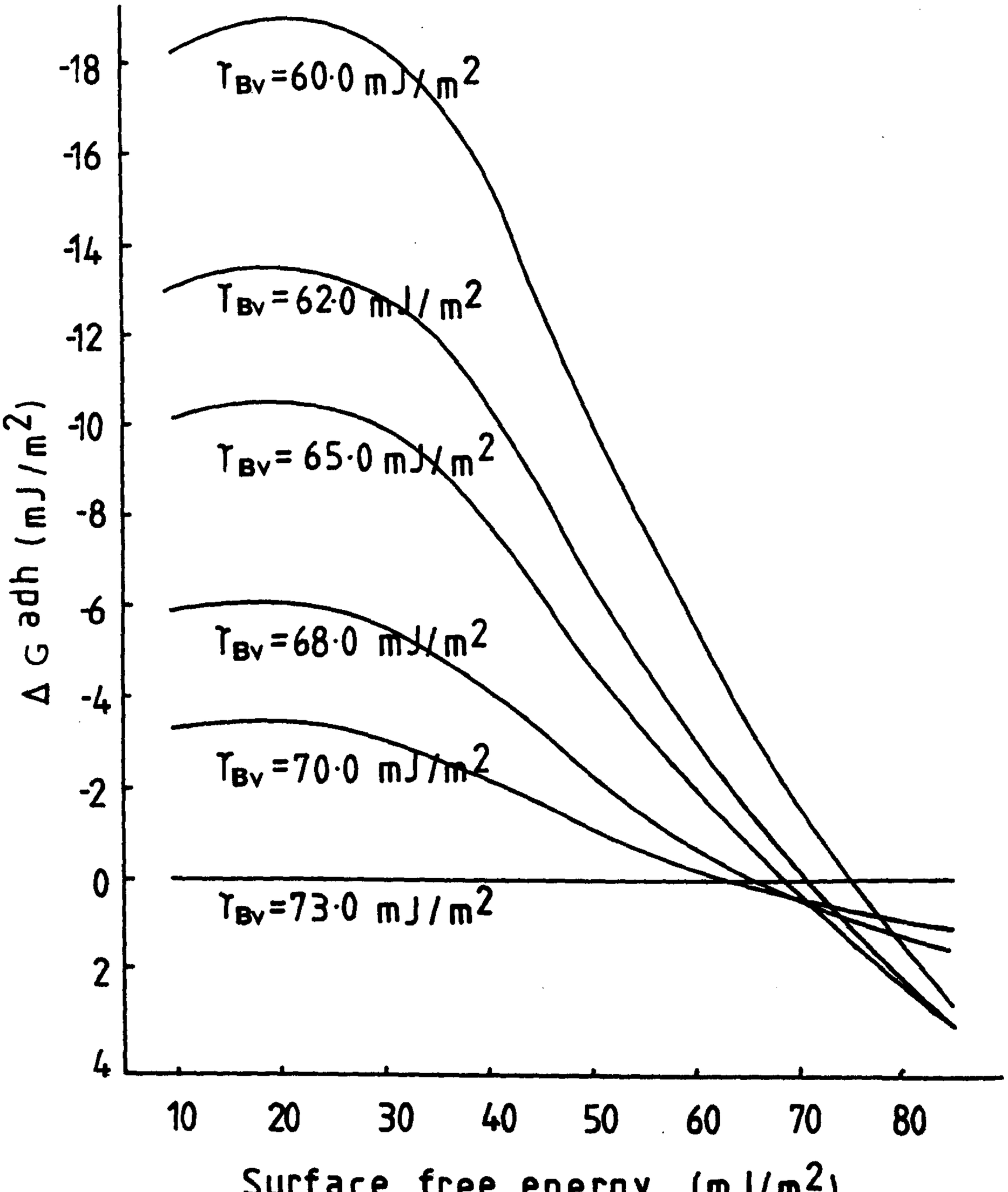

γ_{LV}=73·0 mJ/m^2
γ_{LV}=70·0 mJ/m^2
γ_{LV}=68·0 mJ/m^2
γ_{LV}=65·0 mJ/m^2
γ_{LV}=62·0 mJ/m^2
γ_{LV}=60·0 mJ/m^2
ΔG adh (mJ/m^2)
-18
-16
-14
-12
-10
-8
-6
-4
-2
0
2
4
10
20
30
40
50
60
70
80
Surface free energy (mJ/m^2

Figure1.2,c


-18
-16
-14
-12
-10
-8
-6
-4
-2
0
2
4
ΔG adh (mJ/m2)
γBv=60·0 mJ/m2
γBv=62·0 mJ/m2
γBv=65·0 mJ/m2
γBv=68·0 mJ/m2
γBv=70·0 mJ/m2
γBv=73·0 mJ/m2
10
20
30
40
50
60
70
80
Surface free energy (mJ/m2)

surfaces in the range $\gamma_{SV} > \gamma_{LV}$ (see figure 1.2.a)

iii) When $\gamma_{LV} = \gamma_{BV}$ ΔG^{Adh} will be zero for all surface free energies (figures 1.2 a,b,c)

iv) When $\gamma_{LV} > \gamma_{BV}$ and γ_{LV} = 73 mJm^{-2}(water). ΔG^{Adh} will be increasingly more negative with a reduction in γ_{SV} indicating a maximum level of attachment would be expected to low energy surfaces (Figs. 1.2b,c)

v) When $\gamma_{LV} < \gamma_{BV}$ and γ_{BV} = 70.0 mJm^{-2} then ΔG^{Adh} will be positive for low energy surfaces but reduces in value as γ_{SV} increases, indicating a maximum level of attachment would be expected to high energy surfaces (figure 1.2.a). This model therefore shows how adhesion of bacteria to substrata with a range of surfaces free energies will be strongly influenced by the liquid surface tension LV. Maximum adhesion would be expected to low energy surfaces when γ_{LV} = 72.8 mJm^{-2}(water), whereas reductions in γ_{LV} due to contaminating proteins or surface active organics would reduce this effect. Once γ_{LV} γ_{BV}, the maximum adhesion would switch to high energy surfaces. This phenomenon has been demonstrated when granulocyte adhesion was studied as a function of substrata surface energy (γ_{SV}) for various liquid surface tensions (Zingg et al, 1980). It also may explain the different levels of attachment found with in vitro studies of bacteria from

the same genus and species, as the bacterial surface free energy is also an important parameter. However, several important questions remain:- to what extent does the ΔG^{Adh} influence the level of bacterial attachment, what is the strength of the resulting cell surface contacts; and does this model allow for changes in the cell surface due to extra cellular and extrinsic polymers synthesised by the cell during and after the contact process?

Gerson, (1980) has described an empirical relationship between ΔG^{Adh} and the distribution of cells between the surface and the medium at equilibrium. (Keq). From this point of view the equilibrium under consideration is that between cells free in the medium and cells attached to the solid substratum and is dependent upon ΔG^{Adh} in equation 1.20

$$\log Keq = -\Delta G^{Adh} + B \qquad 1.20$$

where and B are empirical parameters. Keq was determined from the ratio of attached bacterial cells to free unattached cells by direct counting following 24th culture of a number of bacterial species in the presence of commercially available plastics offering a range of surface free energies (γSV). ΔG^{Adh} were determined, using the equation of state approach, from contact angle data collected from the solid substratum and lawns of bacterial

cells, along with the liquid surface tension of the medium. Gerson's results confirm the exponential relationship between a negative ΔG^{Adh} and the equilibrium coefficient of partition of adhering cells to free living cells. The absolute values of $-\Delta G^{Adh}$ reported did not indicate what level of Keq to be expected, as the two species of Staphylococcus investigated showed very little similarity in attached cell numbers, although similar values of $-\Delta G^{Adh}$ were reported for each surface. However, the overall pattern indicated that increased levels of attachment were found to increasingly hydrophobic or low surface energy substrata which would agree with the above model (Gerson and Scheer, 1980).

The strength of bacterial adhesion, on the basis of the thermodynamic arguments, will be dependent upon the types of bonds or forces operating inside the primary minimum of the contacting surfaces (Section 1.2.1). How well matched these forces are depends upon the composition of the interacting substances. Thus cell surface contacts with low energy surfaces may be more frequent, as determined by the $-\Delta G^{Adh}$, yet may be weaker than cell-surface contacts between higher energy surfaces which have greater potential bonding once the repulsion forces have been overcome. Therefore surfaces with greater variety of short range forces including Van der Waals forces, Keesom forces,

Debye forces, London forces and hydrogen bonds will form stronger bonds to the noncovalent phase of a micro-organism which is also held together by these forces.

In adhesion an important "criterion of wetting" describes this relationship as adhesives and adherend solids which have optimal adhesive strength when the γ_{SL} interfacial free energy is a minimum and the contact angle of the adhesive on the adherend approaches zero. However, in an aqueous system, hydrophilic surfaces, which may provide a suitable balance of bonding potential with the bacterial cell surface, are masked with a layer of water in close contact, which will tend to prevent close cell contacts. Hence maximum adhesive strength between a bacterium and substratum will be found when the bacterial surface has greater affinity for the substratum surface than does the water layer.

Thus this approach of "wetting" has been usefully applied to biological systems where qualitative comparisons are required. Unfortunately in characterising the wettability or surface energy of a microbial cell layer the conditions of the Young's equation (equation 1.12) are not met. Like the DLVO theory it cannot account for membrane structure and cell physiology, both of which can respond to the adhesion process. Young's equation requires

conditions of rigidity, homogeneity and insolubility whereas micro-organisms can be deformed and alter shape. They also have complex heterogenous surfaces which release surface active components into the surrounding medium and therefore are difficult to characterise by this approach.

1.2.5 Hydrophobic interactions

The preceeding description of hydrophobic interactions presented above (section 1.2.4) can also be analysed in a thermodynamic framework, and the interactions can be shown to be principally due to Van der Waals interactions. Van Oss et al, (1980) have combined the DLVO theoretical approach with the wettability approach in order to quantify the short range forces acting at separations of 0.2nm between hydrophobic determinants, hydrophilic determinants and both hydrophobic and hydrophilic determinants in water. They used the equation of state approach to evaluate the interfacial free energies, $\gamma_{12}, \gamma_{13}, \gamma_{23}$ (equation 1.18) from $\gamma_{1V}, \gamma_{2V}, \gamma_{3V}$ where typical values may be: γ_{1V} = 34.0 mJm^{-2} (a hydrophobic surface), γ_{2V} = 68.1mJm^{-2} (a hydrophilic bacterial surface) and γ_{3V} = 72.8 mJm^{-2} for water. From equation 1.9 ΔG^{Adh} can be determined for three possible interactions:- ΔG^{Adh} 132, ΔG^{Adh} 131 and ΔG^{Adh} 232, representing hydrophobic - hydrophilic, hydrophobic-hydrophobic and hydrophilic-hydrophilic contacts respectively. However ΔG^{Adh} is related to the Hamaker

coefficient A (Section 1.2.1) by the equation:

$$A = -\Delta G^{Adh}_{12} \, d^{2}$$

1.21

where d is the equilibrium separation distance between two parallel flat (semi infinite) slabs (Hamaker, 1937) Therefore taking d = 2nm for short range separations A values can be determined for each of the aboves interactions:

ie. $A_{131} = +7.25 \times 10^{-20}$ J

$A_{132} = +0.99 \times 10^{-20}$ J

$A_{232} = +0.11 \times 10^{-20}$ J

all of which differ approximately by a decimal order of magnitude. The strongest attraction is the one between two hydrophobic determinants in water (A131). Next in order of magnitude is the attraction between hydrophobic and hydrophilic determinants in water. (A132) which although much smaller still definitely represents a considerable attractive force (Neumam et al, 1979; Van Oss et al, 1979). Finally, the interaction between two hydrophilic determinants in water (A232) is smaller, yet attraction can take place. These energies compare with the strongly attractive interaction between water and the two materials 1 and 2:

(i) A13 = 12;5 $\times 10^{-20}$ J (for the attraction between the hydrophobic material and water;

(ii) A23 = 21.2 $\times 10^{-20}$ J for the attraction between the hydrophilic material and water;

(iii) A33 = 21.9 x 10^{-20}J for the cohesion between water; and

(iv) A12 = 12.7 x 10^{-20}J for the attraction between the hydrophilic material and hydrophobic material following the displacement of water. The above Hamaker constants for the interaction energies of various determinants show how the attraction between the hydrophilic material and the hydrophobic material (A12) is the same order of magnitude as the attraction of the water for the hydrophobic material (A13). Also the attraction of water for the hydrophilic material (A23) is the same order of magnitude as the cohesive forces of water (A33) and is much larger than A12 and A13. Even so when the Hamaker constants are determined for the three phase interaction A131, A132, A232, the hydrophobic determinants show the strongest attraction energies. (A131) followed by hydrophilic-hydrophobic determinants (A132) and two hydrophilic determinants (A132). Van Oss et al,(1980) suggest from this that this hydrophobic effect is due to attraction by van der Waals, forces. Whereas, Kauzman, (1959) and Tanford, (1979) point out that the driving force for this rearrangement must be the displacement of water due to the stronger cohesive attraction of water for itself.

1.3 The influence of the substratum surface, the medium and the bacterial surface on bacterial adhesion.

In the study of bacterial adhesion to solid surfaces, an approach which may be taken is to evaluate the thermodynamic models, presented in the previous section and to determine whether bacterial attachment in a given system can be described or predicted by such models. This rationale has been employed in a number of adhesion studies (Absolom et al, 1979; Dexter, 1979; Fletcher and Loeb, 1979; Gerson and Scheer, 1980) and has been found to be valid over the initial period of adhesion, as long as the bacteria are maintained in stable conditions both physiologically and biochemically. In such experiments it is first necessary to describe the components of the attachment system in physiochemical terms, and thus the bacterial and attachment surfaces and the liquid medium must be examined. The properties of all three components of the attachment process have been found to affect adhesion. However, characterisation of the bacterial surface using this approach has its limitations, as biological systems are chemically complex. Yet, this approach is useful in providing qualitative values for the surface free energy of the bacterial surface which give predictions about the attachment properties of the organism.

1.3.1 The solid surface

The influence of substratum characteristics on the attachment of bacteria from marine and freshwater environments has been extensively studied *in situ* and *in vitro* (Dexter *et al*, 1975; Sechler and Gundersen, 1972; Fletcher and Loeb, 1976; Loeb, 1977; Dexter , 1979; Fletcher and Loeb, 1979; Carson and Allsopp, 1980; Gerson and Scheer, 1980). A number of *in situ* studies on the attachment of bacteria to a range of substrata have shown maximum attachment to the high energy (hydrophilic) surfaces, such as glass, metals, most engineering coatings, nylon, metallic oxides and metals, with increasing exposure (Sechler and Gundersen, 1972; Dexter *et al*, 1975; Dexter, 1979) where as others have found that hydrophobic plastics such as polystyrene and phexiglass become colinised more quickly than glass in the first few hours of submersion (Loeb, 1977; Carson and Allsopp, 1980). In vitro studies on the initial attachment of a marine pseudomonad have shown that these bacteria have a preference for low energy (hydrophobic) surfaces. (Fletcher and Loeb, 1976). In other laboratory experiments many freshwater and marine bacteria, (as well as algae and bryozoa; Loeb, 1977) have shown a similar preference for hydrophobic surfaces and only a few relatively non adhesive strains have attached in slightly higher numbers to hydrophilic

surfaces (Fletcher, J.J. Bright, unpublished data). What allows bacteria to adhere and colonise the hydrophilic surface following immersion in a natural environment may be due to changes in the substratum characteristics that can take place in the first few minutes of exposure. During this period a primary film of organic molecules is adsorbed to the substratum, "conditioning" the underlying surface (Baier, 1970). These surface conditioning films range in thickness from about 10 to 20nm (Baier, 1981) and are therefore difficult to investigate by the routine biochemical analytical methods for determination of constituents. However, a number of more sophisticated, nondestructive methods have been used to measure physio-chemical properties of these surfaces. By using multiple attenuated infra red reflectance spectroscopy on germanium and plastic surfaces dried conditioning films have been found to consist primarily of glycoproteins, proteoglycans or their end-product humic residues (although this technique may over-estimate the contribution of proteinaceous material at the expense of carbohydrate and lipid (Wangersky, 1976). Other non-destructive tests that can be carried out on the dried conditioning films include measurements of optical thickness by ellipsometric techniques of contact potential from vibrating and ionising electrode studies and of crystal structure from Xray diffraction patterns

scanning electron microscopy and determination of critical surface tensions from contact angle measurements (see section 1.2.4). However, these measurements are made on dried films which may bear little relationship to their structure in aqueous solution where they would be hydrated. In an attempt to obtain more meaningful information in relation to bacterial adhesion, Fletcher and Marshall, (1982) have used bubble contact angle measurements. This technique has the advantage that the aqueous phase is still present and thus the conditioned surface will be fully hydrated. Unfortunately, the difficulty in the technique is that the bubble angles obtained in the presence of conditioning molecules are influenced partly by changes in surface free energy and partly by changes in the surface tension of the aqueous phase. Even so, the changes in contact angles reported did reflect differences in the adhesion of Pseudomonas sp. NCMB 2021 in the presence of dilute protein solutions.

Adsorption of macromolecules to a "clean" surface changes the properties of the surface to that of the external molecules of the adsorbed species. Norde and Lyklema, (1978) who have proposed a model of protein adsorption at the solid-liquid interface, suggest that protein adsorption and the affinity of the adsorbed molecule will depend upon the number of positively charged groups which become located at the surface. The affinity of the

protein will be dependent upon a lowering of the negative surface charge of the solid surface or an increase in surface hydrophobicity. Experiments on the adsorption of proteins at the solid/liquid interface are few in number and consist of adsorption isotherms of blood proteins to colloidal particles, plastic substrata and glass, (Van Oss and Singer, 1966; Brash and Lyman, 1969; Mac Ritchie, 1971). The adsorption isotherms of bovine serum albumin (BSA) to hydrophobic and hydrophilic silica particles (Mac Ritchie, 1971) and immuno globulins, human serum albumin, BSA and rabbit haemoglobulin to polystyrene latex particles (Van Oss and Singer, 1966) showed similar isotherms in that:

i) stronger adsorption took place on the hydrophobic silica and latex particles than on hydrophilic silica

ii) adsorption to hydrophobic particles was independent of medium pH

iii) adsorption to hydrophilic silica was very weak at low pH (2.1,3.2) was at a maximum at pH 5.5 (close to the isoelectric point of BSA) and was not detectable at pH 9, probably due to the effect of charge at the particle surface. These results indicate that protein adsorption is mediated by Van der Waal interactions, once surface charge can be overcome, therefore relate to DLVO theory.

The modification of bacterial attachment to the substratum by adsorbed proteins has been demonstrated in a

number of studies. (Meadows, 1971; Fletcher, 1976; Orstavik, 1977). However, the supposition that the surface chemistry of a range of substrata can be completely obscured by the overcoating of these biological macromolecules has not been substantiated (Baier, 1979) and will be examined in this thesis. Baier (1981) suggests that low energy surfaces are more resistant to fouling because of the weaker interaction between the conditioning macromolecules and the solid/liquid interface. Infact the search for "biocompatibility", that is surfaces that do not adsorb macromolecules or cells, has been widely investigated in order to improve the biotolerability of medical implants, with a view to preventing their thrombogenic properties (Andrade,1973), and their resistance to biofouling. A hypothetical surface with surface free energy of zero would be ideal according to the correlation of Lymann et al, (1968) or Baier,(1971). However, blood in contact with air or nitrogen, thought to have such surface properties, would have a high interfacial free energy; therefore it is not surprising that such surfaces do thrombose. Baier, (1971) claims a range of γ_C equal to 20-30 mJm^{-2} may be optimum for biocompatibility which was also supported by Dexter (1979) who found minimal fouling by bacteria on low-energy surfaces from in situ studies. However solid polymers with this range of surface energy are all thrombogenic and do not prevent bacterial

and cell interactions and attachment (Andrade 1977; Fletcher, 1979). Andrade,(1973) has suggested that an ideal surface to be biocompatible, would require an interfacial free energy with blood or water of less than 5 mJm^{-2}. Glass surfaces undoubtedly have higher interfacial free energies with water, as surface charge predominates on this surface and the γ_{SV} of clean glass is 170 mJm^{-2} (Andrade, 1973) Hydrophilic surfaces with high water content and low surface charge, eg. hydrogels, would meet this criterion for biocompatibility. Particularly, if the hydrogel polymer contained a large propertion of water, thus minimising the interfacial free energy with water. Hydrogels used in opthalmology have these properties, and bacterial attachment to these surfaces may indicate to what extent the surfaces are biocompatible, and thus demonstate the inhibition of bacterial attachment via the effects of water at the solid-liquid interface.

The influence of potential surface conditioning proteins, glyco-lipoprotein complexes,glyco-lipids and polysaccharides on <u>in vitro</u> and <u>in situ</u> bacterial attachment is clearly of importance to understanding the attachment process. However well-characterised and clean substrata may be, their apparent characteristics can change rapidly after immersion in an aqueous medium containing bacteria and dissolved macromolecules. <u>In vitro</u> attachment assays can be performed in relatively "clean" conditions with

artificial seawater or freshwater and low organic levels in solution. How quickly and to what extent conditioning films will be established in _in situ_ experiments will vary from one environment to the next, depending upon i) the nature of the macro-molecules present and ii) their concentration in the aqueous environment

1.3.2 The liquid medium

The composition of the liquid medium can also affect attachment, in ways other than the obvious effects on bacterial physiology and cell surface conditioning. In the thermodynamic model thus presented (Section 1.2.3), liquid surface tension (γ_{LV}) is a second parameter, in addition to solid surface energy, influencing bacterial attachment. Changes in γ_{LV} can be provided by surface active macro-molecules such as proteins, detergents or smaller organic molecules, eg. ethanol; alcohol, dimethylsulphoxide (DMSO). _In vitro_ experiments using surface active agents to change γ_{LV} can be used to verify the thermodynamic model. Also induced changes in γ_{LV} provide a means of determining the γ_{BV} without using contact angle methods on bacterial "lawns" as used in other investigations (Van Oss _et al_,1975; Baier, 1979) (See section 1.2.4).

Alternatively, the influence of surface active agents can be viewed in terms of their effects on the intermolecular bonding of macro-molecules and their ability to form both

hydrogen bonds and Van der Waals interactions. Their effects are due in part to changes in the structure of water and the solvation of the hydrogen bonding and hydrophobic groups in aqueous solution. Theoretically surface active agents have been characterised in terms of the types of additives which give rise to hydrophobic solvation as follows:

i) Class I additives (alcohol-like), which are non-polar and poorer hydrogen bond formers than water, will either stabilise or de-stabilise "hydrophobic interations dependent upon temperature and concentration of the additive; alternatively,

ii) Class II additives (urea-like) are highly polar compounds which are thought to be good hydrogen-bonding agents and to act as solubilising agents for hydrophobic interactions over much wider concenttation and temperature ranges. (Brandts, 1969). The use of Class I and II additives over suitable concentration ranges may increase or decrease attachment dependent upon the role of hydrophobic and hydrogen bonding in bacterial adhesion by stabilising or denaturing macromolecules involved in this process.

1.3.3. <u>The bacterial surface</u>

Knowledge of the role of the cell surface structure and surface free energy (γ_{SV}) is required for an adequate description of bacterial adhesion. Investigations in this area can be divided into three basic types: i) thermodynamic, by measuring the surface free energy of the cells using contact angle methods or other alternatives (Van Oss <u>et al</u>, 1975),

ii) chemical and enzymatic treatments which inhibit or disrupt bacterial adhesion and thus indicate the role of specific membrane polymers and exopolymers in attachment (Fletcher; 1980), iii) hydrophobicity measurements of bacterial cells using hydrophobic interaction chromatography (HIC), (Dahlback et al,(1981), bacterial surface binding of radiolabelled dodecanoic acid (Kjelleberg et al, 1980) or the use of biphasic mixtures of dextran and poly(etheylene glycol) to measure bacteria partitioning between phases (Magnusson et al, 1977; Gerson and Akit, 1980; Gerson and Scheer, 1980), iv) and electron microscopic evidence (Fletcher and Floodgate, 1973; Costerton et al, 1978; Latham et al 1978)

1.3.3 i) Contact angle measurements of air-dried bacterial lawns have been used to demonstrate potential pathogenic organisms by comparison of cell surface properties with their susceptibility to phagocytosis (Van Oss et al, 1975) However, their is still some doubt about the significance of bacterial contact angles measurements, as they are made on partially dried and on smooth lawns of cells. Thus the application of Young's equation (1.12) (see section 1.2.4) is at best qualitative as the conditions of rigidity, homogenity and insolubility are not met. Deformation of the cell surface will occur (see Ivanov et al, 1978 for a discussion of contact angles on a deformable solid).

Also cellular components may transfer to the test liquid which would reduce the γ_{LV}, and unless the γ_{LV} value is determined in the experimental situation, then the relationship of Young and the interfacial free energies determined by the equation-of-state would be invalid (Pethica, 1980).

An alternative method of evaluation bacterial surface free energies (γ_{BV}) is based on the thermodynamic model presented in section 1.2.4. By measuring bacterial attachment to a range of substrata with increasing surface free energy (γ_{SV}) and also a range of liquid media surface tensions (γ_{LV}), a value of γ_{LV} can be determined when bacterial attachment is independent of γ_{SV} and $\Delta G^{Adh} = 0$. Under these conditions $\gamma_{LV} = \gamma_{BV}$, as from equation 1.13, for $\Delta G^{Adh} = 0$, $\gamma_{SB} = \gamma_{SL}$ and $\gamma_{BL} = 0$, assuming that the interaction parameter $\phi_{BL} = 1$.

A similar method has been used to determine the surface free energies of platelets and granulocytes independent of contact angle measurements on the cells (Neumann et al,1979, 1980; Absolom et al, 1980). Values of γ_{SV} for these blood cells shows good agreement with values calculated from contact angle measurements, which range from 66.8 mJm^{-2} for human polymorphonuclear lencocytes to 68.3 mJm^{-2} for rat neutrophil (Van Oss et al, 1975). Bacterial surface energies based on contact angle data show a wider range of γ_{BV} from Mycobacterium butyricum at 15.6 mJm^{-2} and Acinetobacter calcoaceticars grown on hydrocarbons 42 mJm^{-2} to Escherichia coli O111 at 67.0 mJm^{-2} and Staphylococcus

aureus - Smith 67.3 mJm^{-2} (Van Oss et al 1975 ;Neufeld at al,1980). Unfortunately the range of organisms tested thus far has been limited to human and animal pathogenic bacteria and organisms involved in hydrocarbon fermentions because of their potential to the field of biochemical engineering in large-scale single-cell protein production (Tannenbaum and Wang, 1975). However, the investigations of medical microbes by Van Oss (1975) do indicate how changes in the outer membrane surface of these organisms (often termed smooth to rough or pathogenic to avirulent) can be reflected in changes to the contact angle, indicating a lowering of the cell surface free energy.

1.3.3 ii) A second method of investigation of the adhesion process has been by chemically or enzymatically treating either i.) free-living or suspended cells and observing any influence on their subsequent attachment to surfaces or ii) attached bacteria to see if the attachment bond is consequently broken down. Chemicals used to denature polysaccharides have included sodium periodate and disodium tetraborate which were found to inhibit or disrupt cell adhesion in attachment studies of marine (Fletcher, 1980) and freshwater bacteria (Marshall, 1972).

This may indicate the involvement of polysaccharides in the attachment process, as periodate ($NaIO_4$,0.02M - 0.2M)

oxidises and cleaves unsubstituted 1:2 glycol groups, while treatment with borate (Na_2BO_4 7 0.1M) causes adjacent hydroxyl groups of sugars to be negatively charged altering the structure of the polysaccharide and the surface of the outer membrane. These chemical treatments are not mild and may also alter the membrane structure, denaturing membrane proteins if not the lipid bilayers. A milder alternative is to use enzymes to disrupt or inhibit attachment, although the presence of the enzyme protein may also influence attachment by conditioning effects (see section 1.3.1). Danielsson et al,(1977) showed that pronase efficiently removed strains of attached marine pseudomonads, whereas trypsin caused their removal to a lesser extent.

1.3.3 iii) A third method of characterising cell surfaces is by measuring the relative hydrophobicity of the cell surface, by one of a number of methods. Cell surface hydrophobicity is a qualitative term which describes the characteristics of the cell which can be measured as the cell surface energy by either i) contact angle measurement ii) attachment where $\gamma_{LV} = \gamma_{BV}$ or iii) measurement of partition coefficients between immiscible liquid phases. This partition coefficient will be related to the free energy of partition (G^{Part}) by equation 1.20

$$\Delta G^{Part} = \gamma_{BL2} - \gamma_{BL1} \qquad (1.22)$$

where BL2 and BL1 equal the interfacial free energy between the bacteria and each of the two liquid phases Gerson and Akit, (1980) have confirmed this model with a number of unrelated organisms using two biphasic mixtures of polyethylene glycol)(MW20,000) and dextran (MW500,000 and 100,000). Interfacial free energies between these mixtures increase with increasing concentration in the poly(etheylene glycol) and dextran solutions yet remain very small compared with solid-liquid interfacial free energies between plastic substrata and water, ie. 66 $\mu N m^{-1}$ ($10^{-3} mJ\ m^{-2}$) for a 6% (W/W) Poly(ethylene glycol) / 8.0(W/W) dextran biphasic mixture compared to 50 mJm^{-2} for a solid hydrocarbon / water interface (Gerson, 1980).

As both surface charge and surface hydrophobicity will influence the Keq (Eq 1.20) of bacterial cells in biphasic mixtures, an alternative method to show the hydrophobic contribution is to increase the interfacial free energy between these mixtures by adding poly(ethylene glycol) or dextran with covalently bound hydrocarbon side chains to ordinary Poly(ethylene glycol) / dextran mixtures For example these hydrophobic ligands of the poly(ethylene glycol) will transfer hydrophobic bacteria from the dextran phase into the poly(ethylene glycol) phase . This has been used by Magnusson et al ,(1977) to compare the surface

properties of smooth and rough Salmonella typhimurium strains and their lipopolysaccharides. Their results indicated that smooth strains of the bacteria do not participate in hydrophobic interactions, while rough strains, lacking the S-specific polysaccharide side chains in the surface lipopolysaccharide were influenced by polymers with covalently linked hydrophobic groups. Both the rough cells and lipopolysaccharide, normally partitioned in the lower dextran phase, could be drawn into the upper poly(ethylene glycol) phase by the addition of Palmitoyl-poly(ethylene glycol). On the other hand, Palmitol-dextran did not influence the partitioning of either rough or smooth cells nor lipopolysaccharide.

A second method of measuring cell hydrophobicity by phase partition is by hydrophobic interaction chromatography (HIC) (Hjerten et al, 1974). This method measures the numbers of cells retained by a hydrophobic column composed of alkyl derivatives of beaded agarose media, which can be regarded as a mosaic of randomly distributed hydrophobic loci in a relatively hydrophilic matrix. The relative hydrophobicity of bacteria isolated from the surface and subsurface water along the Swedish west coast was carried out using this method by Dahlback et al,(1981). They expressed the relative hydrophobicity as the ratio, g/e, between the numbers of cells in the gel (g) and the eluate (e) and found that the enrichment of bacteria at the surface

layers compared to the subsurface showed a positive correlation with hydrophobicity. Similar results have been found in a number of studies of bacterial accumulation at air-water interfaces. (Blanchard and Syzdek, 1978; Hermansson et al, 1979; Kjelleberg and Stenstrom, 1980).

1.3.3 iv) Finally electron/microscopy has been used to visualise the role of bacterial surfaces polymers in solid-liquid bacterial adhesion. A number of studies show bacterial polymers bridging the gap between the bacterial and substratum surfaces (Fletcher and Floodgate, 1973; Costerton et al, 1978; Latham et al, 1978). Histochemical evidence from a study of a marine pseudomonad attached to millipore filter suggested that these connecting fibrils are acidic polysaccharides (Fletcher and Floodgate, 1973) However, the extensive fixation and dehydration steps carried out during the preparation of transmission electron microscopy samples could well account for the apparent simplicity of the cell-surface interaction using this method.

1.4 Bacterial cell surfaces and adhesion

The physiochemical models of cell adhesion presented in sections 1.2.3 and 1.2.4 treat bacterial cells as inaminate colloidal particles but allow predictions of the forces involved and a thermodynamic measure of the change in free energy of adhesion (ΔG^{Adh}) allowing an evaluation of the irreversibility and spontaneity of the process. These models may well be improved and/or modified if the relationship between the cell surface characteristics at the biochemical level and the resulting adhesion were examined. Therefore an alternative approach to determine how bacteria adhere at the solid-liquid interface using a non-specific permanent mechanism would be to examine cell surface components and their role in this form of adhesion. One major problem in this approach is the genetic diversity of the cell surfaces of organisms from even one aquatic environment. Therefore, use can be made of adhesion mutants, in order to determine which cell surface components influence cell adhesion such "sticky" mutants are likely to be enriched for on surfaces when a selective advantage exists for immobile growth. Alternatively "non sticky" mutations may also arise and be enriched to inhibit the cells from becoming attached where growth in the liquid phase is favoured and has a selective advantage.

1.4.1 Description of cell envelope components and their role in adhesion

As the majority of aquatic organisms investigated in this study are Gram-negative bacteria (see section 2.4) attention will be paid primarily to Gram-negative cell enveloped. Electron micrographs of sectioned bacteria show this envelope to be composed of several layers which can be interpreted as two membranes separated by a periplasmic space, which contains a thin, dense layer of peptidoglycan overlying the inner plasma membrane. To the exterior of the peptidoglycan and apparently at the surface of the organism is an outer membrane which has a unit membrane appearance and contains proteins, lipoproteins and protein-lipopolysaccharide (LPS) complexes. In contrast Gram-positive bacteria have a single internal membrane, covered with a wall containing peptidoglycan as its main structural component.

In Gram-negative organisms peptidoglycan is only a minor fraction of the envelope, but in Gram-positive bacteria it may account for as much as 80% of the total weight. Peptidoglycan is a heteropolymer of glycan chains containing alternative residues of muramic acid and glycosamine in β-1,4 linkage. In most bacteria these residues are N-acetylated, with some exceptions. Also, muramic acid residues are substituted with short peptides, consisting

of alternating L and D amino acids which are crosε-linked from one glycan to another to form a macromolecular network of high tensile strength and rigidity. In Gram-negative bacteria the peptide side chains have the basic structure of L Ala-DisoGlu-meso-diaminopimelic acid- D Ala and about 30-50% of these peptides are cross-linked as dimers. It is the reactions of enzymes in the cross-linking of glycan units which gives this molecule its structure and the cell its shape. However, the location of peptidoglycan as a separate layer between the outer and inner membrane of Gram-negative organisms make it unlikely that it can react with macromolecules in the external environment and participate in cell surface interactions.

The outer membrane of several enteric bacteria E coli and various Salmonella species, has been extensively investigated in recent years. This work was initiated by the finding that LPS carries the 'O' antigen specifity of the bacterium. It is responsible for the endotoxic activity of the cell and is a receptor for many bacteriophage. A great number of studies of LPS from Gram-negative bacteria have been made, and numerous reviews dealing with their chemistry, biosynthesis and biological activity have been published (Osborn, 1971; Kadis et al, 1971; Nikardo, 1973; Wilkinson, 1977; Wright and Tipper, 1979).

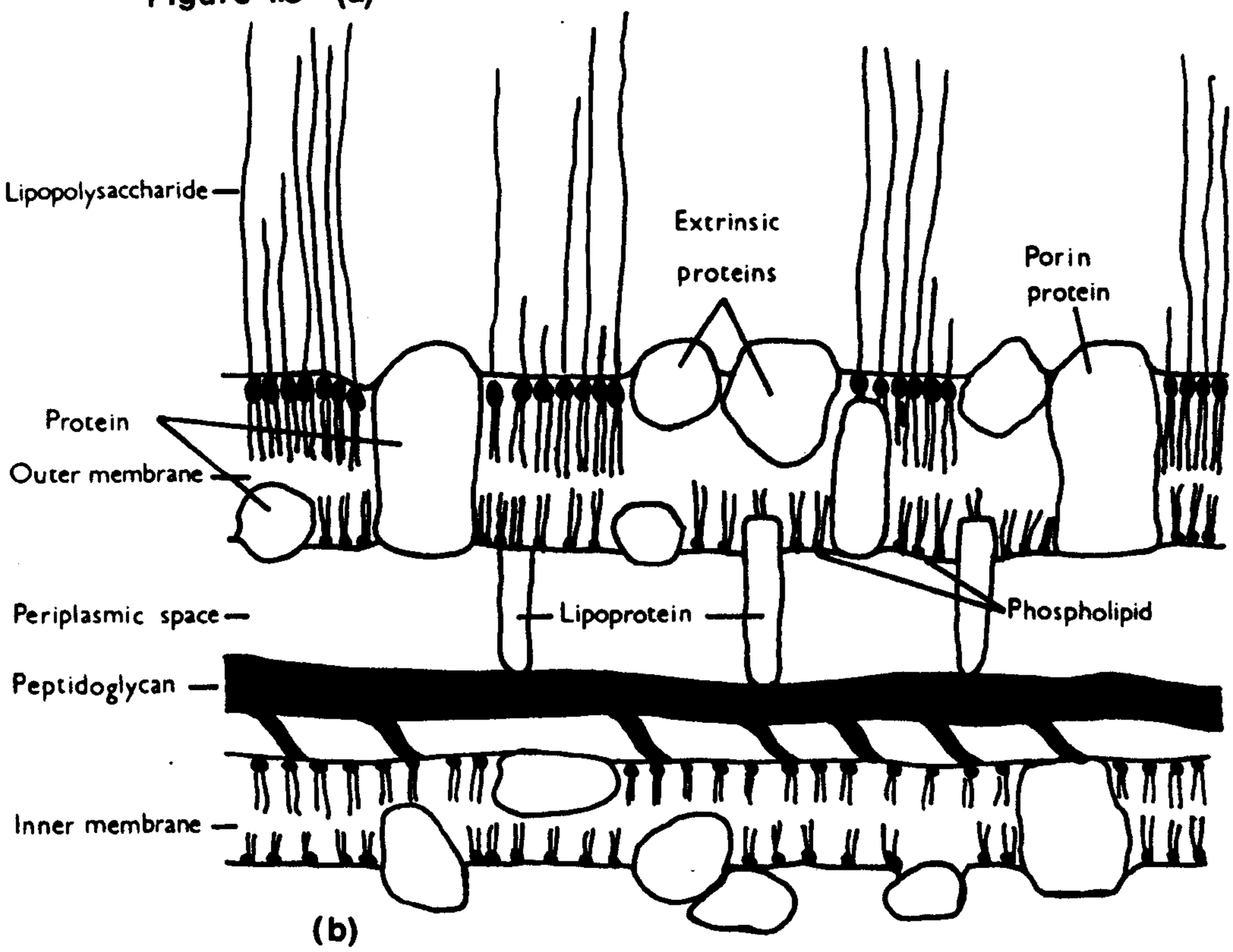
The location of LPS in the outer membrane can be seen in figure 1.3,a and it can be divided into three distinct regions for a classical S-form lipopolysaccharide (fig 1.3b) These regions are covalently linked segments (side-chain, core and Lipid A), each with its distinctive composition, biosynthesis and biological function. The side-chain is the serologically dominant part of the molecule responsible for the O antigenic specifity. It consists of repeating oligosaccharide units, of ten containing rare sugars, and can range in size and complexity. It is absent from the R-form Lipopolysaccharide isolated from R form strains which are mutants in which the genetic defect levels to the biosynthesis of an incomplete core region. The core-region of enteric organisms are conserved parts of the molecule and contain characteristic components, including 3-deoxy-ket-odldonic acid, aldoheptose (both present in the inner core region of Salmonella), glucosamine and 3 hydroxyalkanoic acids (both present in the lipid A region). The keto acid structure is normally identified as a 3-deoxy-D-manno-octulosonic acid ("2-keto-3-deoxyoctonic acid, ie. KDO (figure 1.3.b), while the heptose is typically L glycero-D manno-heptose (Figure 1.3.b). Finally the lipid A structure can be summarised as substituted 6 - 0 - β glucosaminyl glucosame 1, 4 diphosphates with a number of acyl residues ester linked to the available unsubstituted carbons and linked to core oligosaccharides at 3' - position.

Figure 1.3.a,b.

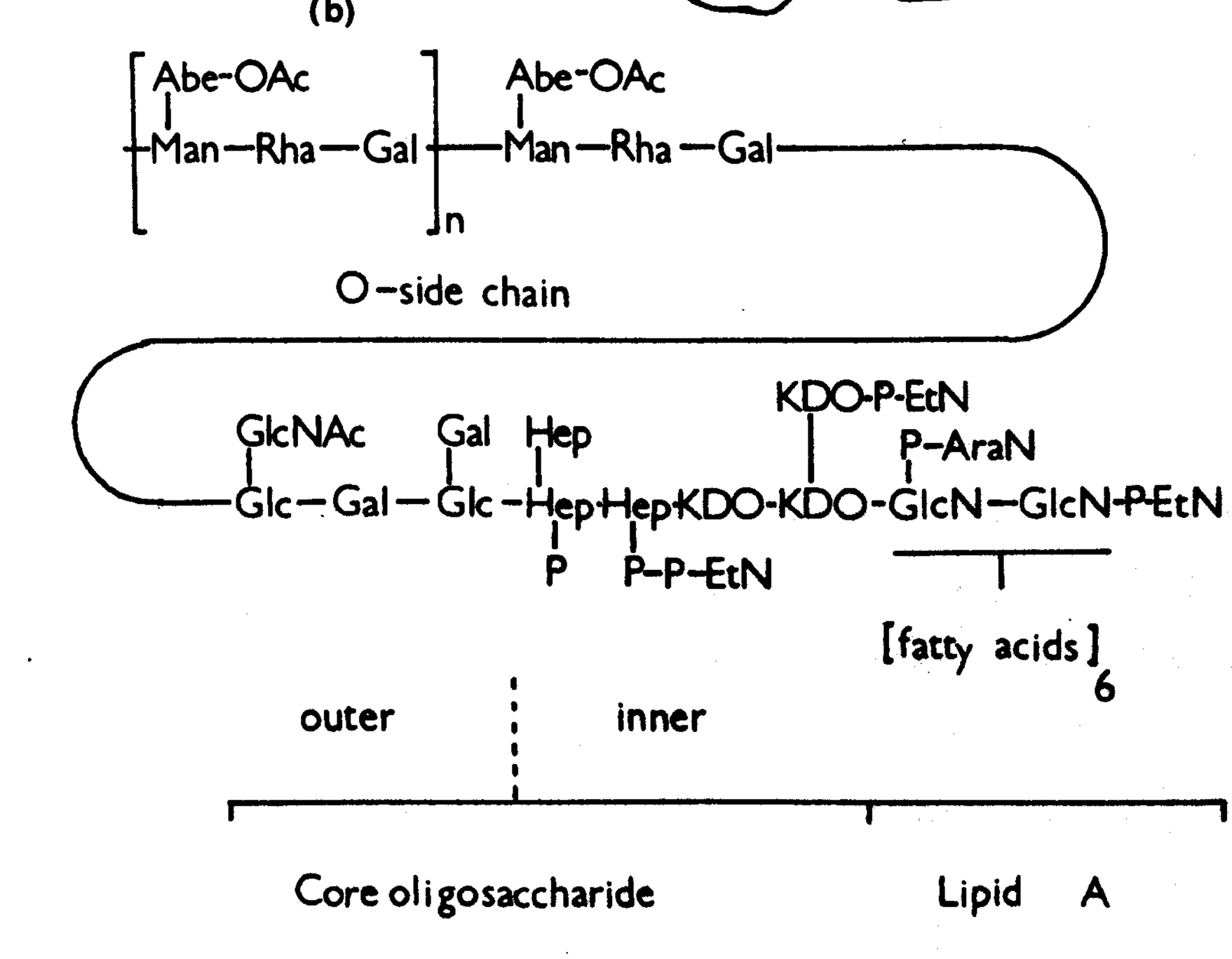
a) Model for the structure of a Gram-negative envelope. Note the varying lipopolysaccharide chain length.

b) Structure of the lipopolysaccharide of Salmonella typhimurium. The molecule is made up of the O-side chain (Abe abequose; Man, D-Mannose; Rha, L Rhamnose; O-Ac, O-acetyl) linked via the core oligosaccharide (Gal, D-Galactose; GlcNAC, N-Acetylglucosamine; Glc, D-Glucose; Hep, L-Glycero-D-mannoheptose; KDO, 3-Deoxy-D-manno-octulosonic acid, EtN, Ethanolamine) to Lipid A (GkN, Glucasamine; AraN, 4-Aminoarabinose). The six fatty acid substituents are present in both amide and ester linkage (After Sutherland, 1977)

Figure 1.3 (a)


Lipopolysaccharide
Extrinsic proteins
Porin protein
Protein
Outer membrane
Periplasmic space
Lipoprotein
Phospholipid
Peptidoglycan
Inner membrane

(b)


Abe-OAc
Man—Rha—Gal
n
O-side chain
GlcNAc
Gal
Hep
KDO-P-EtN
P-AraN
Glc—Gal—Glc—Hep—Hep—KDO—KDO—GlcN—GlcN—P-EtN
P
P-P-EtN
[fatty acids]
6
outer
inner
Core oligosaccharide
Lipid A

With some qualification and refinements, this basic model for (LPS) seems to apply to Salmonella and other enterobacterial genera and is widely applied to the interpretation of the structure and composition of (LPS) from other Gram-negative bacteria (Wilkinson, 1977). Apart from variations in composition, the refinements include i) microheterogeneity in the different regions, ii) the creation of larger molecules in which the basic structural units are cross-linked between the lipid A regions, iii) absence of O specific side-chains from some units in these larger molecules and individual units iv) the existence of side chains differing significantly in length and or composition in the same lipopolysaccharide preparation or v) complexes with outer membrane proteins and other outer membrane components.

From this brief description of LPS it is easy to conclude that changes in the outer or exposed cell surface could well be mediated by either the composition or concentration of LPS molecules. These molecules can extend from the cell surface of smooth Ecoli or S typhimurium up to 150nm from the cell outer membrane (Shands, 1966) and provide effective coverage of more proximal structures on the outer membrane, making such structures inaccessible to antibodies (Smit and Nikardo, 1978) and phages (Lindberg, 1973). Alternatively deep rough LPS mutants of these organisms would appear very

different, as the short or absent carbohydrate chains would leave the cell membrane surfaces exposed to antibodies and phage. For example E coli K12 and B strains are rough mutants and are not wild-type in terms of LPS biosynthesis (Prehm et al,1975, 1976). Their LPS carries only tri-to decasaccharide chains, and estimations of surface coverage by carbohydrate have been made as a maximum of only 8%. (Nikardo and Nakae, 1980). It is therefore very likely that LPS structure and concentration in the outer membrane will greatly influence cell surface interaction of Gram-negative bacteria.

Where cell membrane surfaces are exposed to the environment by a reduced polysaccharide coverage, entrinsic membrane proteins may also be important in cell contact and adhesion. Recently isolation of outer membranes from the cell envelope have been possible by i) equilibrium density-gradient centrifugation (Schnaitman, 1970; Kuplow and Goldfine 1974; Smit et al, 1975) or ii) a method based on selective solubilisation with detergents such as Triton X - 100 in the presence of Mg^{2+} ions (Schnaitman, 1971) or Sarkosyl ionic detergent (Filip et al,1973) which has enabled outer membrane proteins to be distinguished by SDS polyacrylamide get electrophoresis. Electropherograms of outer membrane proteins are usually dominated by only a few prominent bands (Schnaitman, 1970), which have been identified and their properties determined for E.coli and S typhimurium

in a number of studies (Braun et al, 1978; DiRienzo et al, 1978). Of the major proteins in the cell outer membrane Murein lipoprotein exists in the largest numbers of copies ($7x10^5$ copies per cell), two thirds of which is present in a form not covalently linked to the peptidoglycan, which is thought to be involved in the anchorage of the outer membrane to the peptidoglycan (Braun, 1975). A further major group of proteins are the "porins" proteins which form hydrophilic channels and have a relatively non-specific role in the passage of small molecules across the membrane. Also there are heat modifiable proteins some of unknown function, which show different migration properties when heated in SDS prior to electrophoresis (Schnaitman 1973). "Minor proteins" are in general found in a low number of copies per cell, and most have now been shown to be involved in high affinity transport of specific molecules. In addition these proteins serve as receptols for certain bacteriophages and colicins.

Chemical analysis, spin labelling and specific-labelling of whole cells, spheroplasts and isolated membranes with reagents which cannot penetrate the outer membrane have been used to show several features. First LPS is located exclusively in the extrinsic surface of the outer membrane leaflet (Muhlradt and Golecki, 1975; Di Rienzo et al, 1978). Secondly, a large number of the outer membrane proteins have

been shown to be exposed to the external medium by specific-labelling methods (Kamio and Nikaido, 1977). Thus surface proteins are also available for cell surface contacts if sufficiently exposed on the surface of the outer membrane. Phospholipids, the final component of outer membranes, are also asymmetry and predominantly located on the inner or intrinsic surface of the membrane and are therefore of less significance than LPS.

1.4.2 Bacterial surface exopolymers and appendages

The previous section has dealt with the structure of Gram-negative bacterial surfaces in terms of the polymers from which they are made. There are however, other surface exopolymers and appendages which extend from many organisms into the surrounding environment (for a review, see Sokatch, 1979).

1.4.2|) Exopolymers.

The exopolymers of importance to the adhesion of bacteria to solid surface are the capsules and slime layers. freqmently produced by both Gram-positive and Gram-negative bacteria. The distraction between a slime layer, an extra cellular polysaccharide loosely associated with the outer membrane and a capsular polysaccharide, which refers to the polymers visualised round the organism in the light microscope by India ink staining is somewhat ambiguous. The association of these exopolymers with the membrane can be influenced by the growth conditions. Many extracellular

polymers are derived from polymeric components associated with the cell surface during the earlier stages of a growth curve. For example the capsular polysaccharide of Klebsiella aerogenes adheres firmly to the cell surface, and more than 80% of the polymer sediments with the cells (Troy et al, 1971). Alternatively "colominic acid" is an acidic aminopolysaccharide composed of sialic acid (N-acetylneuraminic acid) residues. It can be found in the culture filtrate of Escherichia coli K235 as an oligosaccharide of 10-12 sialyl residues (Troy, 1979) after its release from the membrane associated polysialic acids by acid catalysed hydrolysis. Moreover the release of polymers on the bacterial surface; often described as "sloughing off", may be determined by the micro-environment surrounding the organism, such as pH, the presence of endo-type capsular polysaccharide depolymerases and the structure and composition of the polysaccharide.

Structurally capsules and slime layers are polysaccharides, with the exception of the polyglutamic capsules produced by Bacillus anthracis and certain other members of the genus Bacillus. The polysaccharide structures may be conveniently divided into homopolysaccharides, that is polymers of a single sugar or amino-sugar residue, and heteropolysaccharides, where more than one type of residue is present. Examples of homopolysaccharides are the glucans and levans. Glucans are associated with Gram-positive cocci Leuconostoc and Streptococci and are produced with considerable variation

in degree of polymerisation (DP) and branching. The majority of linkages are (1-6) with branching occurring through (1-3) and (1-4) bond. However, S mutans (cariogenic Streptococci) are known to produce an insoluble glucan (mutan) containing both (1-3) and (1-6) linkages as well as water soluble (1-6) glucans. These organisms have been implicated in the aetiology of dental caries, where the insoluble glucans appear to play a role in adhesion of these organisms to the tooth surface (Gibbons and Van Houte, 1975).

The majority of bacterial exopolysaccharides are heteropolymers often containing uronic acids and/or pyruvyl-ketal groups, plus acetyl groups as common substituants. These polymers are therefore mainly negatively charged but vary in their linkages, DP and branching giving a variety of physical properties. One important physical property contributing towards the adhesive characteristic of these polymers is their rigidity, as determined from their insolubility and the high incidence of either (1-3) or (1-4) linkages in their structure. More detailed accounts covering many aspects of exopolysaccharides in addition to structure occur in Sutherland (1977) and Berkley, et al, 1979).

1.4.2.ii) Fimbriae or Pili

Of the surface appendages the most well studied have been fimbriae or pili, which are known to influence the adhesion of certain bacteria to both mammalian tissue and fungal and

plant surfaces. They were first recognised over twenty years ago by Duguid, et al, (1955) and Brinton, (1959) as filamentous appendages, usually 5 to 25nm in width, but up to 1 to 2 μm in length. They are isolated from Gram-negative and occasionally Gram-positive organisms (Ottow, 1975), by simple agitation followed by high-speed centrifugation and can be analysed by polyacrylamide gel electrophoresis. For example Group 1, subtype 1 fimbriae of E coli consist of a sub-unit protein, pilin, with molecular weight 16,000. Each organism possesses up to several hundred fimbriae pentrichously arranged; their production is determined by the chromosome, and therefore they do not act in conjugation between mating pairs of bacteria as do groups 2 and 5. Fimbriae of this group act as adhesive organelles and can be characterised mainly by their morphology, ability to haem-agglutinate red blood cells, sensitivity or resistance to blocking of adhesion by mannose and other adhesive peculiarities, such as stabilisation of bacterial mating and adhesion to solid surfaces such as glass. Table 1.1 shows the main features of each group of fimbriae and indicates the main groups involved in bacterial adhesion to solid surfaces (from Ottow, 1975)

The mechanism by which fimbriae influence the adhesion of bacteria to a solid surface is thought to be related to their hydrophobic properties. (Brinton, 1965). A chemical

Table 1.1

Classification and properties of Fimbriae or pili (Ottow 1975)

Group	Subtype	Organisms	Adhesive specificity	Other Characteristics
1	1	E coli S.typhimurium (entero bacteria)	fungal plant and animal cells	mannose sensitive haemagglutination peritrichously arranged 7nm dia
	2	Salmonella species	none	mutants of type 1, lack adhesion and haemagglutination
	3	Klebsiella Serratia marcescens	fungal plant cells cellulose fibers glass	mannose resistant lack adhesion to animal cells no haemagglutination Thin 4.8nm dia.
	4	Proteus spp.	fungal plant and animal cells	mannose resistant haemagglutination peritrichously inserted filament very thin 4.0nm dia.
2	-	Most Gram-negative bacteria	bacteria	Sexpili involved in conjugation of bacteria; determined by plasmids
3		Agrobacterium sp other soil bacteria	not known	large dia(40-60nm) and long (3μm); hollow tubes
4		Vibrio	none	Polarly arranged fimbriae, promote bacterial motion
5		Pseudomonas rhodos P. echinoides Agrobacterium Rhizobium lupini	soil bacteria "star formation"	Polarly arranged and conjugation by bacterial aggregation.

analysis of the amino acid composition of subtype 1 fibriae from E. coli (indicated that the Fimbrilin (Pilin)contained at least 163 amino acids, most of them in the L form with a large quantity of hydrocarbon side chains. However, such hydrophobic side chains may be required for the conformational stability of the molecule rather than it being some distinctive property of the filamentous helix of the fimbriae.

1.4.2.iii) Flagella

A second cellular appendage which may be implicated in bacterial adhesion is the bacterial flagellum. Bacterial flagella enable the organism to seek available nutrients as well as avoid harmful or toxic substances by a positive or negative directed motility via the chemotatic response (Adler, 1975, Koshland,1980). Structurally, these appendages can be divided into three parts all of which are composed of one or more proteins. For E.coli the filament is a helical structure, several um long, composed of a single protein, flagellin, molecular weight of the sub-unit being 54,000. .D This is linked to the flagellar hook also made of a single protein of M.W. 42,000. D. Finally the basal structure which anchors the flagella in the cytoplanic membrane is composed of nine polypeptides (Simon et al, 1978). Although these are primarily organelles of locomotion, it is possible that they can act in the adhesion of certain bacteria in two modes.

Firstly, they may provide a mechanism of attachment via interaction with the surface. Meadows, (1971) studied the attachment to glass of three Pseudomonas species, Aeromonas liquifaciances, Escherichia coli, Flavobacterium species and Vibrio anguillarum. From his observations he concluded that bacteria which attached at one end appeared to do so by their polar flagella. (However this evidence is based upon light microscopy of organisms and therefore must be treated with caution.) An alternative mechanism using cell surface components at the poles of this organism may explain these observations. Such a mechanism has been described by Marshall et al,(1971) for Pseudomonas R 3.

A second way that flagella may influence bacterial attachment is by increasing the number of bacterial collisions with the substratum, thus increasing the statistical probability of interaction or overcoming some of the repulsive forces preventing short range contacts (Section 1.2.1). In experiments where the flagella were removed by homogenisation, from cells in an exponentially growing batch culture of a marine Pseudomonas species (NCMB 2021), there was a marked decrease in their subsequent attachment to polystyrene substrata. (Fletcher, 1979). However, it is possible that homegenisation also removes loosely bound surface polymers, also responsible for adhesion but reappearance of motility concomitant with flagella regeneration also coincided with the ability to attach (Fletcher, 1979)

1.4.3 Exopolymers and aggregation

The models presented in sections 1.2 and 1.3 for the adhesion of colloid particles to solid substrata can also be used to predict the aggregation of colloids. This process initially involves the destabilisation of particles followed by the promotion of interparticular collisions facilitating the formation of multiparticle aggregates. Walles, (1968) provided three major categories of aggregation mechanisms: (i) charge effect neutralisation, (ii) polymer bridging and (iii) mutual dehydration, all of which are important to adhesion of bacteria as well as bacterial aggregation. Thus it may be useful to view bacterial adhesion or attachment as a destabilisation of the bacterial surface resulting in interaction with a similarly destabilised substratum. In this way potentially attractive, hydrophobic substrata may be stailised with a conditioning layer of adsorbed macromolecules.

Indeed with some inorganic colloids, such as clays and metal oxides, the surface potential plays a major role in the stability of the suspension. However most micro-organism possess charged hydrophilic surfaces of more complex interactions. Most biological systems receive their charge and stability from ionisation of certain functional groups on the surface and the hydration of both these groups and hydrogen bonding groups. (Harris and Mitchell, 1973). Therefore bacteria or hydrophilic colloids may be stable at zero point charge due to the hydrogen bonding of water molecules to their

highly polar surfaces. Thus two modes of adhesion exist where neutralisation is ineffective: mutual dehydration and polymer bridging. These may both be important in bacterial adhesion at the solid-liquid interface as (i) the exopolymer compositions of the cell surface can be changed to increase surface hydrophobicity allowing dehydration by hydrophobic interaction or (ii) extracellular polymers may act by bridging via complex formation, hydrogen bonding, or proton transfer to enable cell surface-substratum contacts. Since high concentrations of these polymers lead to restabilisation of the cells because of steric effects, optimal concentration of these polymers are required to prevent surface saturation. (Harris and Mitchell, 1973).

In natural systems extracellular polymers may result from lytic products or biological excretions, giving a variety of bacterial polymers that can act as polyelectrolytes bridging cell-cell and cell-substratum contacts. Therefore de-stabilisation of the system by polymer bridging may be a result of passive or active processes. For example in the case of trickling filters there appears to be rather high concentrations of lytic products associated with the biological activity and mass in these systems, implying that lytic products are beneficial to the attachment and aggregation of certain micro-organisms (Harris, 1971). On the other hand, when high carbohydrate wastes are degraded in these systems large accumulations of polysaccharides lead to stable microbial

suspensions allowing few bacteria to adhere (Jones, 1965). Alternatively, cell surface hydrophobicity resulting in mutual dehydration is a passive process which may result from changes in the surface of bacteria due to low nutrient conditions.

In natural aquatic environments, these models of either hydrophilic or hydrophobic colloids may be an oversimplification, and thermodynamic stability will depend upon many variables including the bacterial surface; growth conditions; solid substratum surface, the medium and the type organism. Investigations of bacterial adhesion therefore need to determine the relationship between this stability and bacterial nutritional status in order to establish whether the initial adhesion process is a passive interaction between a destabilised bacterium and or an active response to the solid-liquid interface involving a destabilisation of the surface by an excreted adhesive polymer. The evidence that extensive exopolymer capsules are required for the initial adhesion is limited, and it is likely that secondary exopolymers are produced subsequent to the initial adhesion thus causing firmer associations with the test surface (Fletcher and Floodgate, 1976). These may be due either to a change in the nutritional status of the cell at this interface or to a re-arrangement of the polymer origianlly produced during cell growth prior to the cell-substratum contact. These secondary polymers may be exo-polysaccharides, extrinsic membrane proteins or lytic products from the medium.

1.4.4 Effects of cell physiology on the attachment process

The implication of bacterial polysaccharides in the attachment process has been made by various authors (Corpe, 1970a; Characklis,1973; Geesey et al, 1977; Costerton, 1978, 1981). However few experiments have reproduced the limiting nutrient condition for growth often found in the natural environments. Recently studies of mixed culture enrichments in chemostats under nitrogen and carbon-limitation have been made by Brown et al,(1977) and Wardell and Brown,(1980). These results showed that under nitrogen-limited conditions one dominant species appeared accounting for 70-90% of the population, after a few days at 20°C. This species produced copious amounts of exopolysaccharide, and free surfaces became rapidly coated with polymer. Under carbon-limitation a much greater range of organisms was observed and attachment was more effective, without the appearance of either large amounts of polymer or of specific appendages. The initial non-specific attachment gave rise to the growth of micro-colonies which led to the eventual coverage of the surface. Therefore these results support the view that exopolysaccharides produced in greater than optimum concentrations serve to stabilise bacterial and substratum surfaces.

The effects of cell growth conditions on bacterial wall content and composition has been investigated (Ellwood

and Tempest, 1971). These results for carbon-magnesium- and phosphate-limited growth of Enterobacter aerogenes indicated that the composition of membrane proteins and lipopolysaccharides was greatly influenced by growth rate and limiting nutrient. Therefore, the walls of Gram-negative bacteria vary phenotypically. Thus the ability to attach to surfaces will be dependent upon the growth condition of the organism as well as on its genetically determined outer membrane surface characteristics. This is also the case for exopolysaccharides, as polysaccharide production in some species is concomitant with growth eg. Xanthomonas campestris formation of xantham give throughout fermentation; Moraine and Rogovin, 1973, whereas other organisms produce exopolysaccharides in late exponential phase of growth as in typical of secondary metabolite production. For example polysaccharide production by P. fluorescens (Eagon, 1956), Zoogloea Sp, MP6 (Unz and Farral, 1976) and Pseudomonas sp NClB11264 (Williams and Wimpenny, 1977) occurred in late exponential and stationary phases of growth.

1.5 The aim and objectives of this thesis including the approaches of the experimental work

The aim of the work described in this thesis was to determine a physical and chemical basis for the mechanism of initial attachment of bacteria to solid surfaces, using freshwater bacterial isolates. The experimental work consisted of three principal approaches or objectives:

i) A general study of the physiochemical aspects of the attachment mechanism(s) utilising a thermodynamic approach. The adhesive properties of freshwater isolates were evaluated and related to thermodynamic properties of the adhesive system, with particular emphasis on substratum wettability.

ii) A more detailed examination of the organisms studied in Part (i) to investigate individual components of the adhesive interaction ie. solid surface free energy and liquid surface tension, in an attempt to determine the important factors affecting the thermodynamics of the adhesive interaction.

iii) a. An examination of the surface characteristics of a number of the freshwater isolates in order to examine the importance of environmental factors influencing physiological processes and their effect on the phenotypic expression of adhesive characteristics, these factors included temperature, pH, and nutrient conditions.

iii) b. A study of the importance of genetic components in determining attachment properties. A number of mutants with different attachment properties were obtained and their cell surface components were biochemically analysed in an attempt to identify specific cell surface components which act as adhesives or which prevent adhesion

CHAPTER 2

The Influence of Substratum Wettability on the Attachment of Freshwater Bacteria to Solid Surfaces

2.1 Introduction

In this study, a number of freshwater bacteria were isolated by their attachment to test substrata from a local river. The attachment of each organism to a hydrophobic and hydrophilic surface was evaluated, and further studies evaluated the attachment of selected isolates to a number of substrata with a range of water-wettabilities. The purpose of this work was two-fold: first, to determine whether there was a relationship between bacterial attachment and substratum wettability for a selection of freshwater bacteria, and secondly, to examine this relationship in greater detail in order to characterise the adhesive properties of a number of selected isolates and provide a background for their use in further investigations.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of Organisms

Polytetra-fluorethylene and polystyrene sheets (2.5cm x 7.5cm) and glass microscope slides (see below for suppliers) were suspended just below the surface of fast running water at Baginton Weir, River Sowe, Coventry, England, for 2h (*in situ* pH 8.1 5.5°C). The surfaces were rinsed in sterile distilled water, placed on agar plates, and incubated overnight at 15°C. The substrata were then removed, and the plates were further incubated at the same temperature for 48h. A representative sample of colonies was replated and pure cultures established of several isolates. The medium used for all stages of the isolation was 0.1% (W/V) peptone (Oxoid Ltd. London), 0.07% (W/V) yeast extract (Lab M, London) in distilled water, adjusted to pH 7.4 with 1M NaOH. Agar plates and slopes contained 0.75% (W/V) agar (Lab. M No.2, London). The cultures were maintained on yeast extract peptone agar slopes at 4°C.

2.2.2 Identification Methods

Isolates were preliminarily identified using the scheme outlined for the identification of Gram-negative bacteria, with special referende to the *Pseudomonadaceae* (Shewan *et al*, 1960). Tests included Gram stain, cell morphology, motility, oxidase reaction (Kovacs, 1956), diffusible pigment production (King *et al*; 1954), reaction in O.F (oxidation-fermentation) (Hugh and Leifson, 1953) and sensitivity to 0/129 (2,4 diamino-6, 7-di-ISO-propylpteridine phosphate) (BDH Chemicals Ltd, Poole, England) (Shewan *et al*, 1954)

2.2.3 Culture conditions

Cells were grown in Flasks (250ml) containing 100ml volumes of yeast extract/peptone media (see secton 2.2.1 maintained at 15°C in an orbital shaken incubator at 150 revs/min. The growth of each organism was followed turbidometrically and the cells were harvested in late exponential phase by centrifugation.

2.2.4 Attachment of isolates to hydrophobic and hydrophilic polystyrene substrata

A number of isolates were examined for bacterial attachment to polystyrene as previously described (Fletcher, 1976 , Fletcher, 1977). The cells were washed three times and resuspended with 0.01 M KH_2PO_4 adjusted to pH 7.4 with 1M NaOH. Cell numbers were determined by measuring the suspension absorbance at 540nm, and portions (5ml) of the suspension were transferred to 5cm polystyrene petri dishes (Sterilin, Teddington, Middlesex, England) or tissue culture polystyrene petri dishes (Costar, Cambridge, Mass, USA). These were incubated for 2h at 15°C. The dishes were then washed with phosphate buffer, and attached bacteria were fixed with Bouin fixative and stained with crystal voilet as previously described (Fletcher,1976, 1977). The level of attachment was estimated indirectly by measuring at 590nm (adsorption maximum of crystal violet) the absorption of the stained bacterial film on four randomly selected areas of duplicate dishes.

2.2.5 Attachment of selected isolates to a range of substrata

(i) Substrata The commercially available materials used as substrata included borosilicate glass coverslips (16mm diam., No 1½, Chance Propper Ltd., Warley, England); tissue culture treated polyethylene terephthalate (TC-PET) (Thermanox, Lux Scientific Corporation, Newbury Park, California, USA); polyhexamethylene adipamide (Nylon 6.6) (Polypenco Ltd., Birmingham, England); tissue culture treated polystyrene dishes (TC-PS) (Costar, Cambridge, Mass., USA); polymethylmethacrylate (PMMA) (Perspex, ICI Plastics Division, Welwyn Garden City, England); polyvinylidene fluoride (PVDF) Solvay and Cie Societe Anonyme, Brussels); polyvinyl chloride (PVC) (Cobex, Storey Brothers and Company Ltd., Manningtree, England); polystyrene petri dishes (PS) (Sterilin, Teddington, England); polyethylene high density (PE) (Bexel, Storey Brothers and Company Ltd., Manningtree, England); polytetra-fluoroethylene (PTFE) (Dalau Specialised Plastics Ltd., Clacton-on-Sea, England). Disks of each polymer were prepared using a cork boring tool (16mm diam.) and were cleaned in 2% (V/V) Lipsol detergent (LIP Equipment & Services, Shipley, England) in distilled water, followed by repeated rinsing in distilled water and air-drying. The cleaned substrata were handled only by clean, grease-free forceps. As no attempt was made to ensure the absence of fillers or plasticisers in the plastic substrata, the published contact angles could not be used. Therefore the surface wettability of each substratum

was determined from contact angle measurements using the sessile drop method (Neumann and Good, 1979). Contact angles of quartz-double distilled water on the substrata were measured with a contact-angle goniometer (NRL type, Precision Tool and Instrument, Thornton Heath, England) at 20°C and 44% relative humidity maintained over saturated K_2CO_3. The WA for water was determined for each substratum from the Young Dupré equation (Gerson and Zajic 1979).

$$WA = \gamma_{LV}^{o} (1 + \cos \theta_E) + II$$

Where γ_{LV}^{o} is the surface tension of the liquid in saturated vapour. θE is the equilibrium contact angle and II is the spreading pressure of the adsorbed vapour on the solid. For the purpose of calculation it was assumed that $\theta_A \simeq \theta_E$ (Zisman, 1964) and that the term II was negligible. This is to be expected for systems in which $\theta > 0$ and provided that a low energy solid surface is smooth and molecularly homogeneous (Good, 1977). This is not the case for glass; thus the reported value for glass (Table 2.2) is approximate and represents the work of cohesion for water.

The surface free energy (γSV) for each substratum was determined using the equation-of-state approach as described in section 1.2.4, making use of the Fortran program published by Neumann et al, 1974

ii) Cell suspension preparation and the measurement of bacterial attachment using radiolabelled cells.

The numbers of bacteria attached to the d isks were determined

using a modification of the technique previously described for the measurement of sorption of Streptococcus faecium to glass (Orstavik, 1977) where attached cell numbers were evaluated by liquid scintillation counting of radiolabelled attached cells. Bacterial suspensions were prepared as described in section 2.2.4 and following the washing procedure, the cells were radiolabelled by incubation with ^{14}C-leucine at 1μCi ml^{-1} (Radiochemical Centre, Amersham, England), for 2h at 15^{o}C. The cells were then washed three times and resuspended in 0.01M KH_2PO_4 (adjusted to pH 7.4 with 1MNaOH) to a concentration of 2.5 x 10^9 to 5 x 10^9 bacteria ml^{-1}. The attachment substrata were held in a vertical position in a 25ml universal bottle by a section of silicone tubing of 14mm internal diam. x 20mm external diam. and 10mm length and exposed to the bacteria suspension (6ml) at 15^{o}C for 2h without stirring. Following this attachment period the disks were removed from the universal bottle, after the cell suspension had been removed by flushing with a buffer (100ml) under constant flow conditions. This allowed the transfer of the disks to the scintillation vials with only one passage through a liquid/air interface and was found to be more reproducible than washing the disks by immersion in a number of washing solutions. Before the addition of the liquid scintillation solution the surfaces were air-dried for 1h at 40^{o}C and positioned horizontally in the counting vial.

iii) Liquid scintillation solutions and counting equipment

All surfaces and bacterial suspensions were counted by dissolution in an emulsifier containing liquid scintillator solution of tolulene/Triton x 100 (Octylphenoxy polyethoxy-ethanol) (Sigma, London) (2/1 V/V) with the scintillator 2-(4'-tertiary butylphenyl) - 5 - (4"-biphenylyl-1, 3', 4-Oxadiazole) (butyl PBD) (Fisons Scientific Apparatus, Loughborough, England). Counts were made on a Beckman Instruments Liquid Scintillation system LS-7000, and polyethylene counting vials (Poly Q=11^{TM}, Beckman Instruments Inc, Irvine, California, USA) with open tops were used to facilitate insertion of the disks. The level of quenching was determined using the H number system, a form of external standards monitoring based on the Compton electron spectron generated by an external 137 CS gamma source (Horrocks, 1977)

iv) Calibration of liquid scintillation counts to bacterial cell numbers.

A calibration curve was produced relating liquid scintillation counts to bacterial cell numbers of Pseudomonas fluorescens H2 for each substratum used. The washed radiolabelled cells were resuspended in absolute ethanol and diluted to ten different concentrations between $2.5x10^6$ and $5x10^9$ bacteria ml^{-1}. The cell suspensions (100µl) were then either put in scintillation solution directly in order to measure counts per minute (CPM) per cell or suspensions (100µl) were allowed to spread on the substrata disks, dried, and counted as for

the attached cells (see above). Calibration curves relating CPM.cells^{-1} of cell suspension were therefore produced, and a measure of the loss of counting efficiency was established for each substratum used, by comparison of the calibration curves for each substratum with the calibration curve for free liquid cell suspension. The accuracy of the calibration curve was verified by comparing direct microscopic cell counts of bacteria attached to selected test surfaces with the corresponding calculated cell numbers derived from the calibration curve.

2.3 RESULTS

2.3.1 The attachment of Freshwater bacteria to polystyrene and tissue culture polystyrene substrata.

A quantitative assessment of the attachment of freshwater bacteria to PS and TCPS dishes is given in Table 2.1. The isolates were a representative sample of the organisms plated from the three isolation substrata. Isolates recovered from these surfaces were identified at the generic level where possible and comprised the following:

Pseudomonas 30%; Aeromonas, 26%;
Yellow rods 12%, Acinetobacter 10%;
coryneforms 8%; Alcaligenes 4%;
Flexibacter 4%; Chromobacterium 4% and
Bacillus 2%.

Absorbance values were not transformed to bacterial number for all organisms tested. However, direct counts were made for selected bacteria in order to make some comparisons. The Absorbance (590nm) range of 0.2-0.4 corresponds to attachment density of $2x10^7$ - 10^8 cells per cm^2 and represents high levels of attachment and a large percentage of the available surface covered. The Absorbance (590nm) range 0.05-0.2 corresponds to attachment densities of $5x10^6$ - $3x10^7$ cells per cm^2 and represents moderate attachment, while Absorbance (590nm) range 0.00-0.05 corresponds to attachment range up to $5x10^6$ cells per cm^2 and represents low levels of coverage and attachment.

TABLE 2.1

The attachment of freshwater bacterial isolates to polystyrene and tissue culture polystyrene petri dishes.

Organism No.	Identification	Absorbance 590nm(6n-1) n=8	
		Polystyrene	Tissue culture polystyrene
H2	Pseudomonas fluorescens	0.36 (0.6)	0.017 (.010)
H41	" "	0.33 (0.4)	0.019 (.010)
H15	" "	0.14 (.03)	0.054 (.003)
H45	Pseudomonas aeruginosa	0.33 (.04)	0.162 (.006)
8602	" "	0.30 (.03)	0.190 (.011)
HP	Pseudomonas putida	0.04 (.01)	0.030 (.017)
H40	Pseudomonas sp.	0.33 (.04)	0.172 (.007)
H22	Aeromonas hydrophila	0.004 (.003)	0.004 (.030)
H6	" "	0.09 (.01)	0.090 (.015)
H47	" "	0.13 (.02)	0.13 (.03)
H48	" "	0.18 (.03)	0.13 (.01)
H21	" "	0.24 (.03)	0.22 (.03)
H3	Acinetobacter sp.	0.107 (.020)	0.130 (.014)
H31	Chromobacterium sp.	0.23 (.03)	0.023 (.010)
H4	Flexibacter sp.	0.010 (.008)	0.002 (.002)
H38	" "	0.21 (.01)	0.210 (.006)
H16	Coryneform	0.003 (0.005)	0.000 (-)
H49	Bacillus sp.	0.000 (-)	0.000 (-)

The organisms can be divided into three groups on the basis of their ability to attach to the hydrophobic PS dish as compared to the relatively hydrophilic TC-PS dish.

The first group of organisms, which comprised primarily pseudomonads and was the largest group, attached in high numbers to PS, with significantly lower levels of attachment to the corresponding TC-PS. In the second group, a variety of organisms showed moderate levels of attachment to both substrata. Isolates of the third group showed low levels of attachment, with no preference for either of the substrata that could be detected using this method. Thus no isolate tested showed the ability to attach to TC-PS without being able to attach to PS. No overall generic pattern of attachment was found; nor was there any correlation between the isolation substratum of each organism and its attachment preference. However, a majority of the organisms isolated showed a preference for hydrophobic PS surfaces.

2.3.2 <u>Substratum θ'As and measurement of substratum wettability</u>

The range of substrata, together with the measured advancing contact angles ($\underline{\theta}_A$) Work of Adhesion (WA) for water and Surface Free Energies (γS) are given in Table 2.2. These substrata were chosen as they provide a suitable range of wettabilities and were available commercially in sheet polymer form.

TABLE 2.2

Substrata, water contact angles and work of adhesion

Substrata	Contact angle θ (degrees)	Work adhesion ($\gamma_{LV}o(1+\cos\theta)mJm^{-2}$ [a]	Surface Free Energy γSV (mJm^{-2}) [b]
Borosilicate Glass	0	145.6	72.8
TC-PET	51	119.2	52.5
TC-PS	70	97.7	41.6
Nylon 6.6	66	102.4	44.0
PMMA	74	92.8	39.2
PVDF	76	90.4	38.0
PVC	80	85.5	35.4
PS	90	72.8	29.1
PE	95	66.5	26.0
PTFE	110	48.0	16.7

a $\gamma_{LV}o = 72.8\ mNm^{-1}$

b As determined from the equation of state

2.3.3. Attachment of selected isolates to a range of substrata

The number of bacteria attached to different substrata was evaluated by the calibration of liquid scintillation counts of radiolabelled cells to bacterial cell numbers. The calibration gradients for the range of substrata are shown in Table 2.3 along with the corresponding losses in (^{14}C) efficiency (see Section 2.4.2). The substrata show three levels of reduced efficiency:- 5-7% (PS, TC-PS, PE), 22% (PVC) and 36-42% (PTFE, Glass, TC-PE- NYLON 6.6, PMMA, PVDF). These values were used in conjunction with the calibration curve to calculate attached cell numbers for each test substratum. The attachment profiles of selected isolates for the range of substrata are shown in figures (2.1 - 2.6), which are plots of attachment numbers versus substratum WA and figures (2.7 - 2.12) which are plots of attachment numbers versus substratum γ_{SV}. All six bacterial isolates tested showed a similar relationship between WA or γ_{SV} and attachment numbers. With increasing γ_{SV} or WA the number of attached bacteria increased up to a maximum value, and with further increases in γ_{SV} or WA, the numbers of attached cells decreased. This maximum value occurred within a range of γ_{SV} from 35.4 - 41·6 mJm^{-2} for the six organisms tested. Three isolates Pseudomonas fluorescens H2 (Fig 2.1,2.7), Aeromonas hydrophila H22 (Fig 2.2, 2.8) and chromobacterium sp H31 (Fig 2.3,2.9) showed an attachment maximum at WA = 85.5 mJm^{-2} or γ_{SV} = 35.4 mJm^{-2} (PVC) indicating a greater preference for hydrophobic

TABLE 2.3 Calibration of liquid scintillation counts to bacterial numbers showing β-energy absorption values of the test substrata.

Substratum	Calibration gradient[a] (DPM per 10^6 bacteria)	Ratio of calibration gradient to control gradient R	β energy Absorption (1-R) x 100%
Glass	101.6 ± 2.4 [b]	0.64	36
TC-PET	99.8 ± 7.2	0.63	37
Nylon 6.6	93.4 ± 6.3	0.59	41
PMMA	92.7 ± 8.9	0.58	42
PVDF	91.5 ± 5.3	0.58	42
PTFE	102.9 ± 4.2	0.64	36
PVC	124.7 ± 4.0	0.78	22
PS	150.3 ± 3.0	0.95	5
TC-PS	149.6 ± 3.6	0.94	6
PE	148.6 ± 5.6	0.93	7
Control	159.1 ± 8.6	1.00	0

[a] Calculated slope from regression analysis of DPM against numbers of cells

Correlation coefficients >0.98 for all substrata.

[b] 95% Confidence interval for regression coefficient n = 20.

surfaces than Aeromonas hydrophila H6 (fig 2.4, 2.10), and Pseudomonas fluorescens H15 (fig 2.5,2.11) at WA = 92.8 mJm^{-2} or γSV = 39.2 mJm^{-2} (PMMA) and Acinetobacter sp. H3 (fig 2.6, 2.12) which had an attachment maximum at WA = 97.7 mJm^{-2} or γSV = 41.6 mJm^{-2} (Nylon 6.6).

Figures 2.1-2.6

Relationship between the number of attached bacteria and work of adhesion for the following organisms:-

Figure	Organism	Strain No.
2.1	Pseudomonas fluorescens	H2
2.2	Aeromonas hydrophila	H22
2.3	Chromobacterum sp.	H31
2.4	Aeromonas hydrophila	H6
2.5	Pseudomonas fluorescens	H15
2.6	Acinetobacter sp.	H3

The work of adhesion values were obtained from contact angle measurements of water on a range of substrata. Error bars represent 95% confidence intervals.

Fig. 2.1

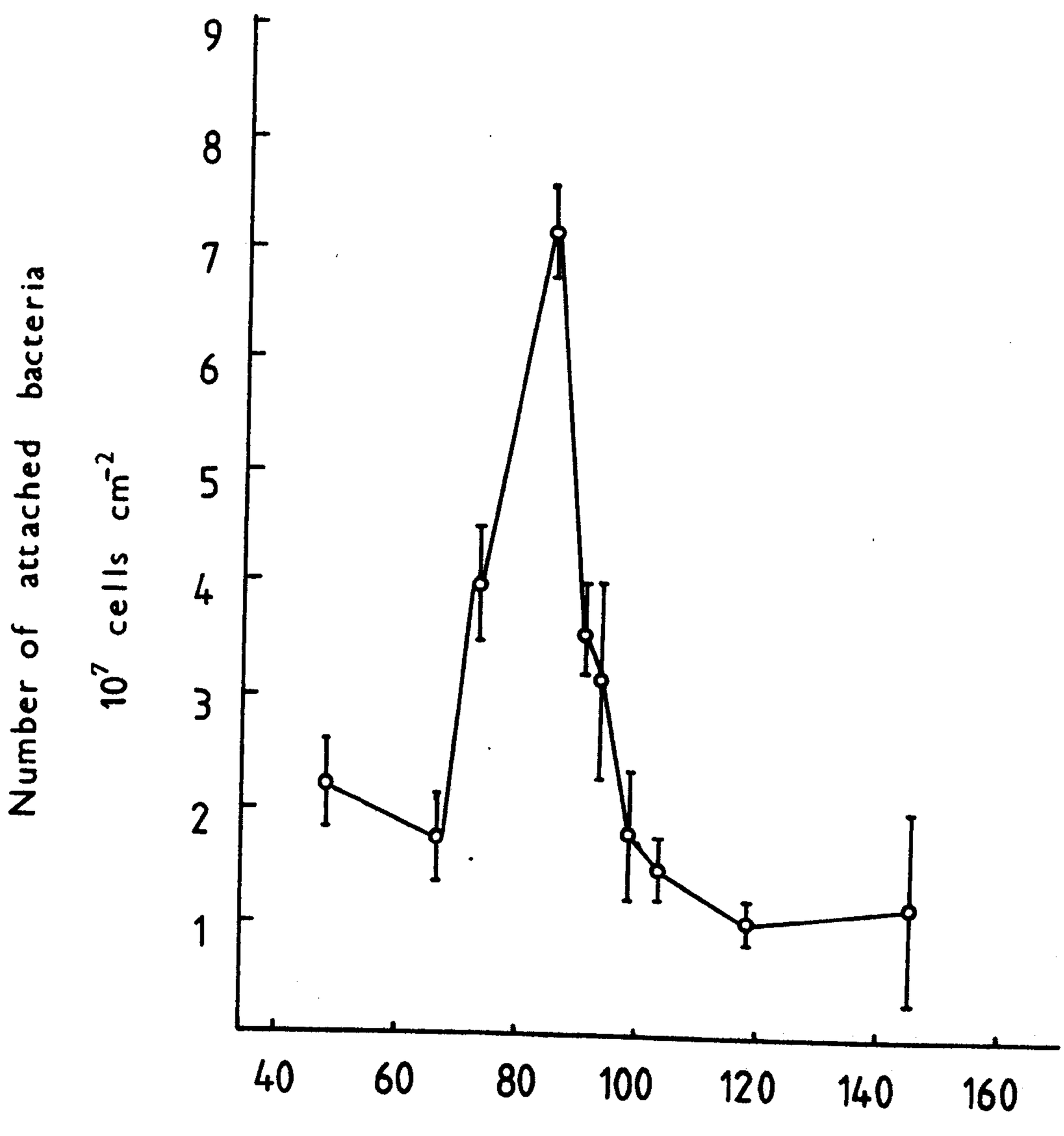

Number of attached bacteria
10^7 cells cm^{-2}
9
8
7
6
5
4
3
2
1
40
60
80
100
120
140
160
Work of adhesion mJm^{-2}

Fig. 2.2

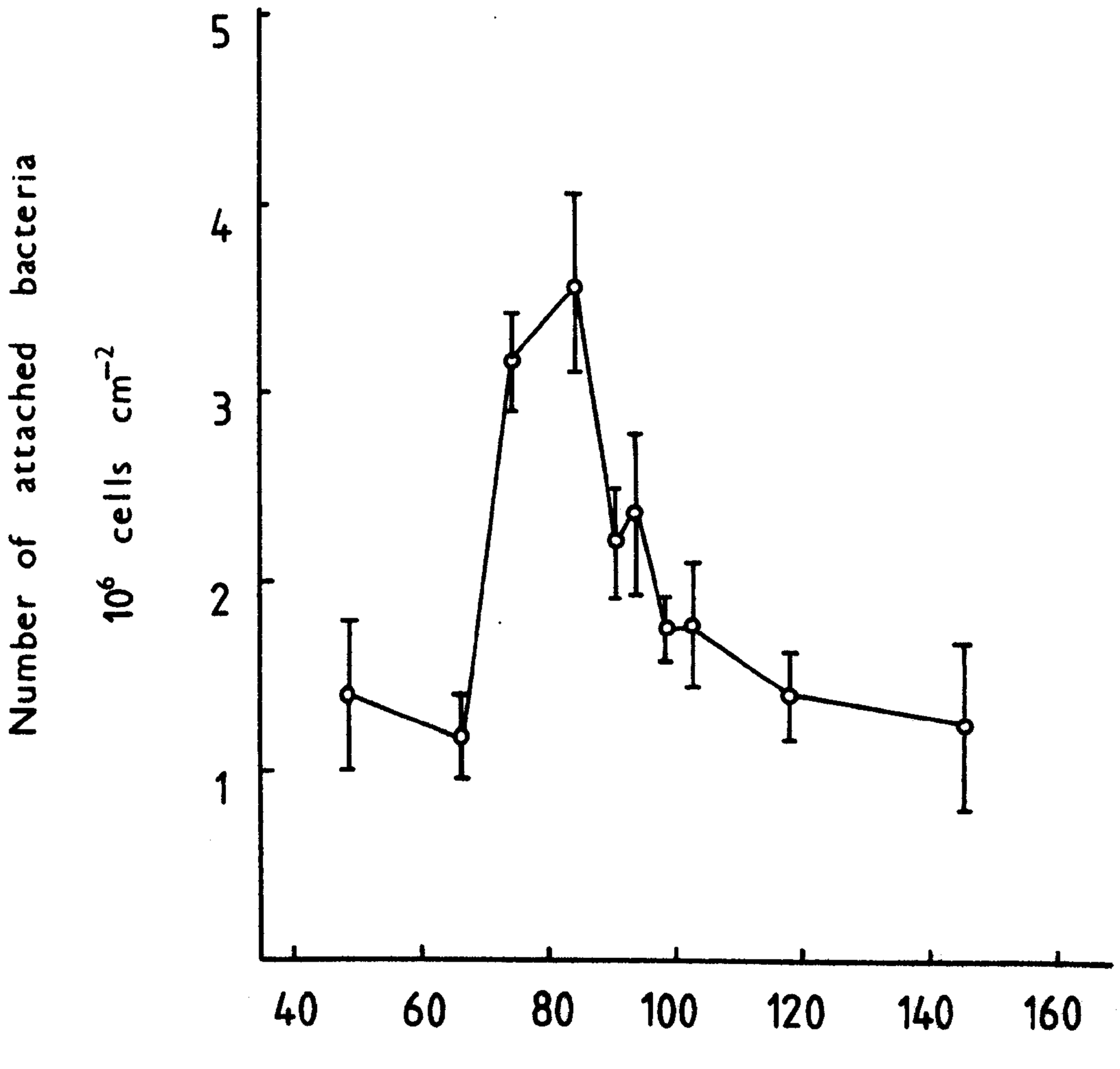

Number of attached bacteria
10^6 cells cm^{-2}
5
4
3
2
1
40
60
80
100
120
140
160
Work of adhesion mJm^{-2}

Fig.23

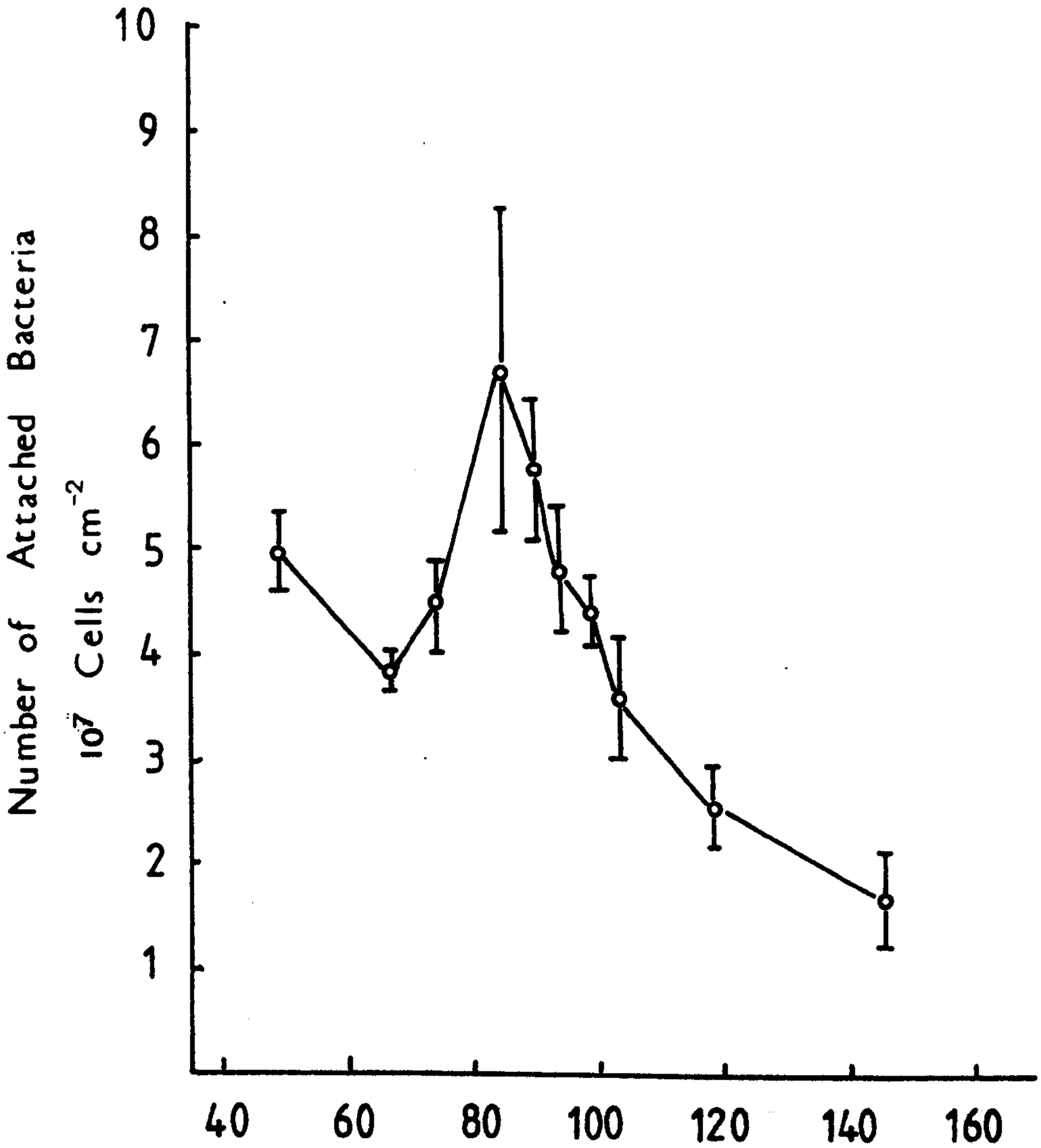

Number of Attached Bacteria
10^7 Cells cm^{-2}
10
9
8
7
6
5
4
3
2
1
40
60
80
100
120
140
160
Work of adhesion mJm^{-2}

Fig.2.4

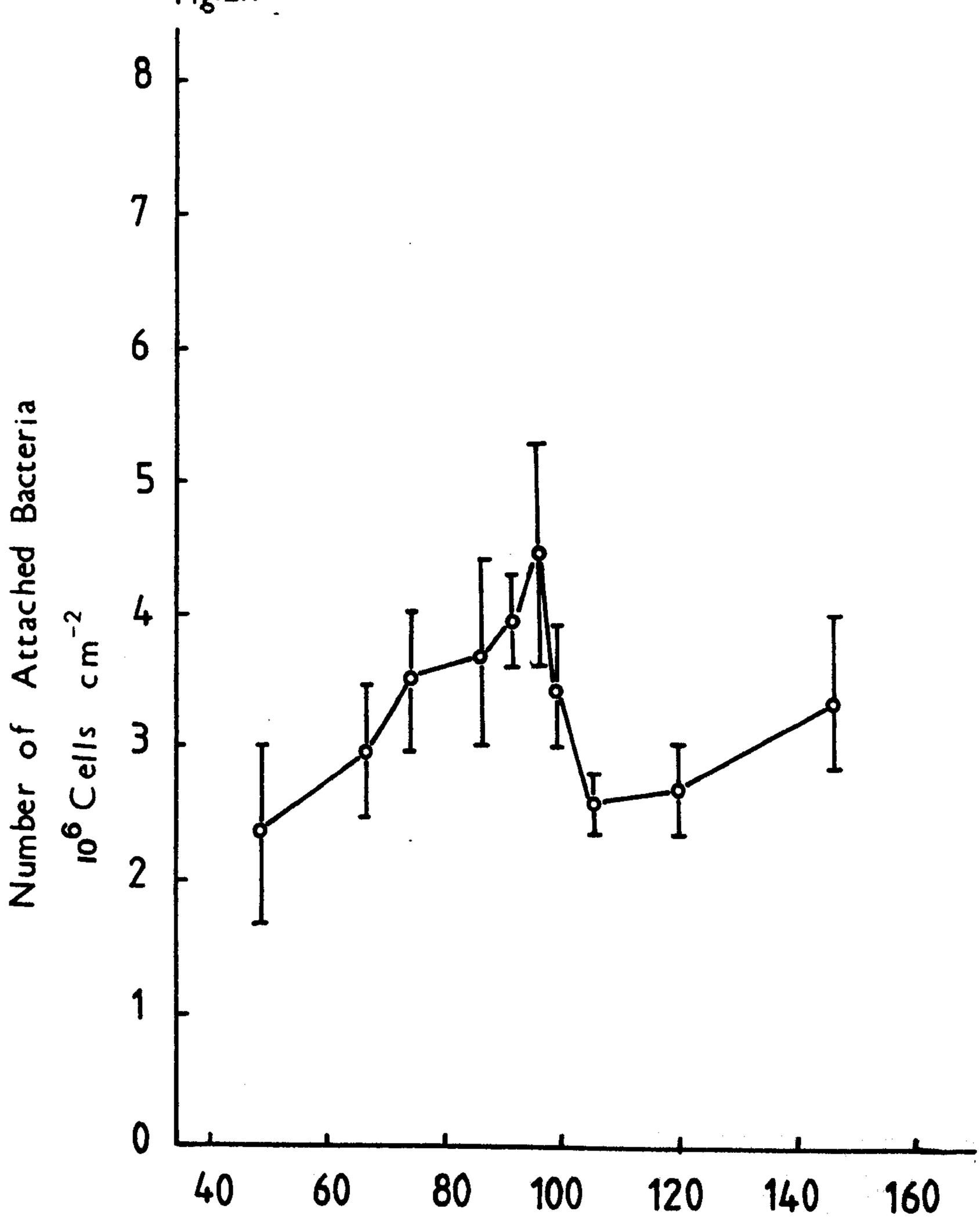

Number of Attached Bacteria
10^6 Cells cm^{-2}
0
1
2
3
4
5
6
7
8
40
60
80
100
120
140
160
Work of adhesion mJm^{-2}

Fig.2.5

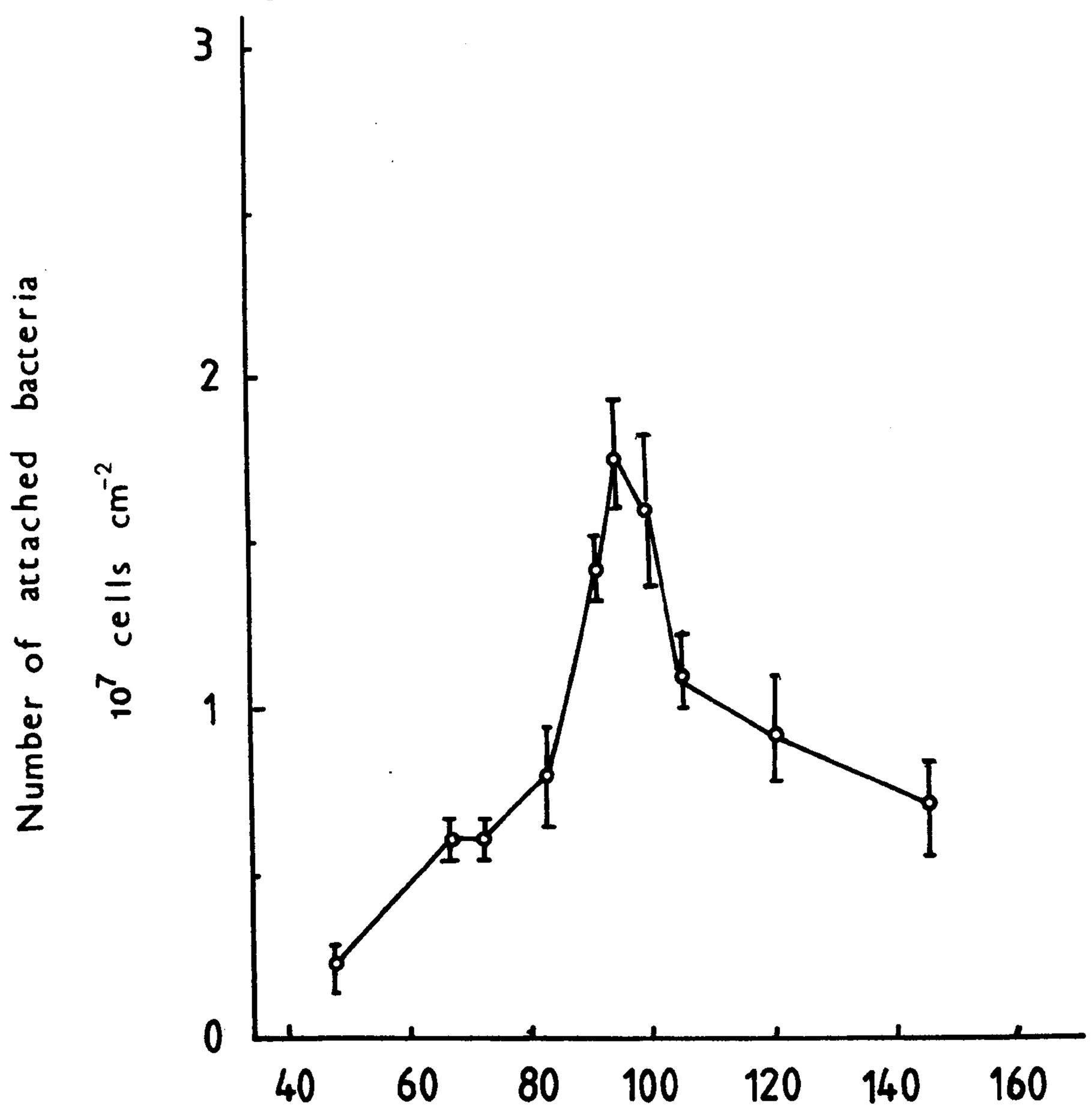
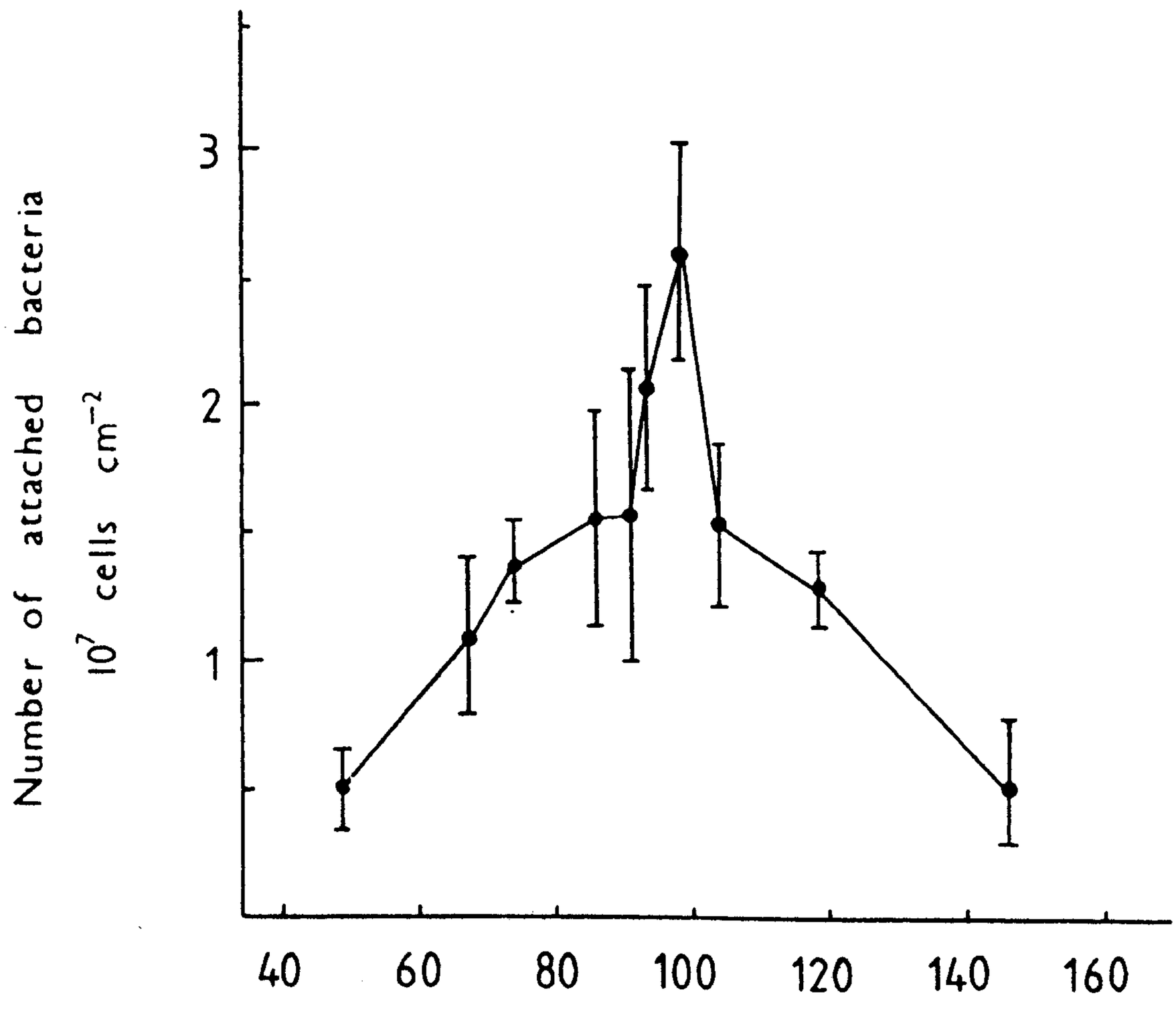

Number of attached bacteria
10^7 cells cm^{-2}
0
1
2
3
40
60
80
100
120
140
160
Work of adhesion mJm^{-2}

Fig. 2.6


Number of attached bacteria
10^7 cells cm^{-2}
3
2
1
40
60
80
100
120
140
160
Work of adhesion mJm^{-2}

Figures 2.7-2.12

Relationship between the number of attached bacteria and surface free energy for the following organisms:

Figure	Organism	Strain No.
2.7	Pseudomonas fluorescens	H2
2.8	Aeromonas hydrophila	H22
2.9	Chromobacterium sp.	H31
2.10	Aeromonas hydrophila	H6
2.11	Pseudomonas fluorescens	H15
2.12	Acinetobacter sp.	H3

The surface free energy values of each substratum was calculated from contact angle data using the computer program published by Neumann et al, (1974). Error bars represent 95% confidence intervals.

Fig.2.7

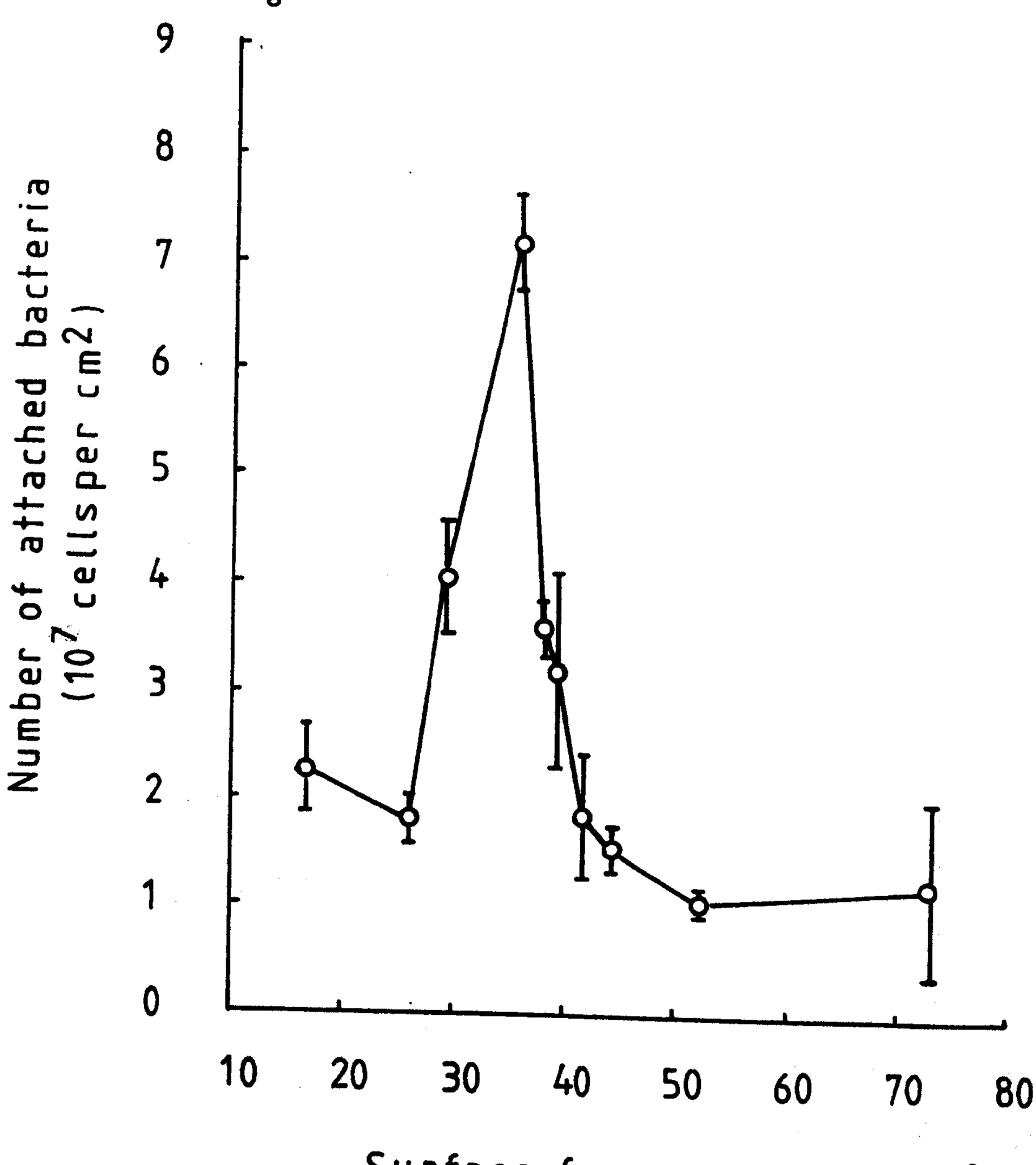

Number of attached bacteria
(10^7 cells per cm^2)
0
1
2
3
4
5
6
7
8
9
10
20
30
40
50
60
70
80
Surface free energy (mJ/m^2)

Fig 2.8

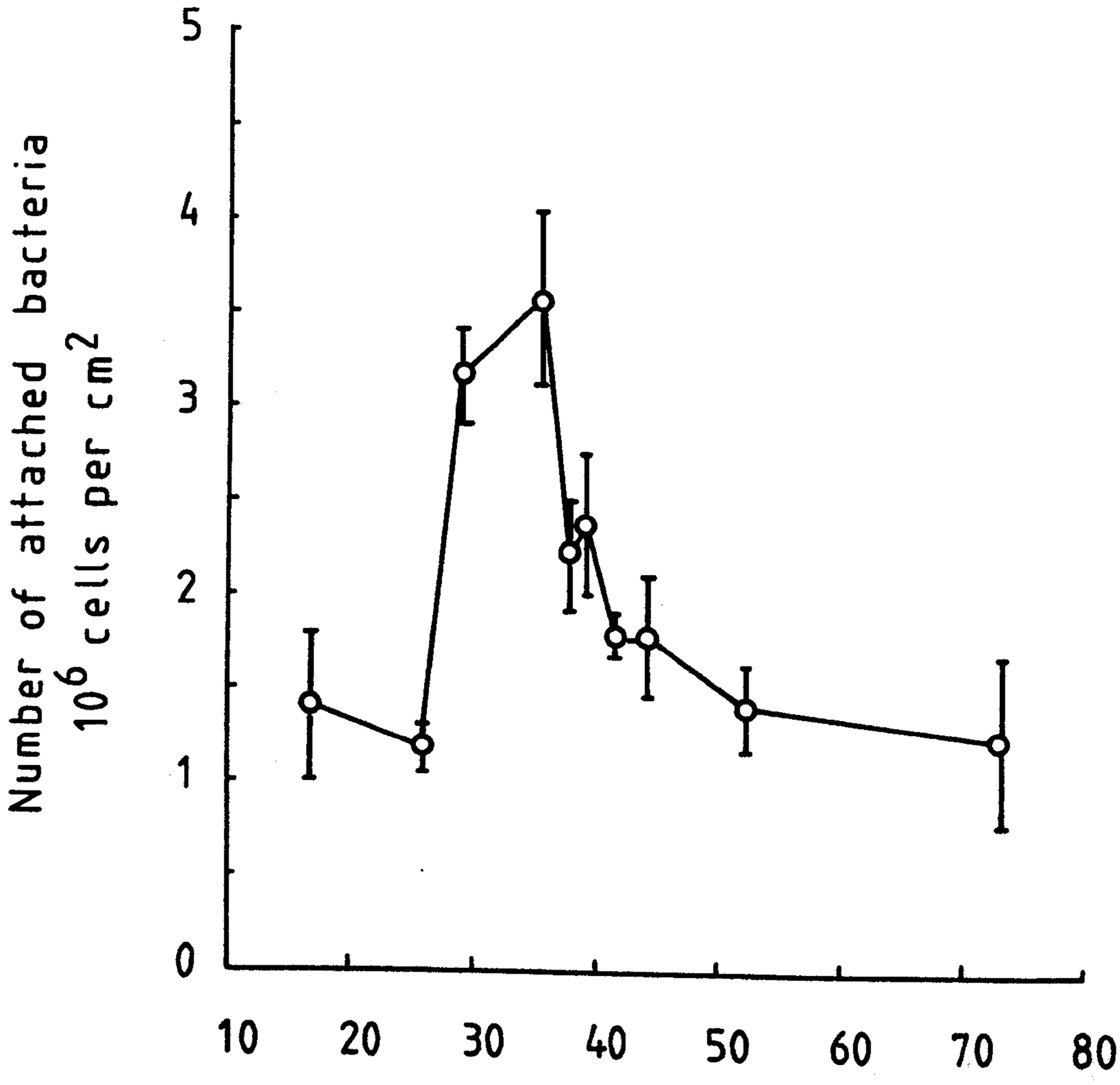

Number of attached bacteria
10^6 cells per cm^2
0
1
2
3
4
5
10
20
30
40
50
60
70
80
Surface free energy (mJ/m^2)

Fig 2.9

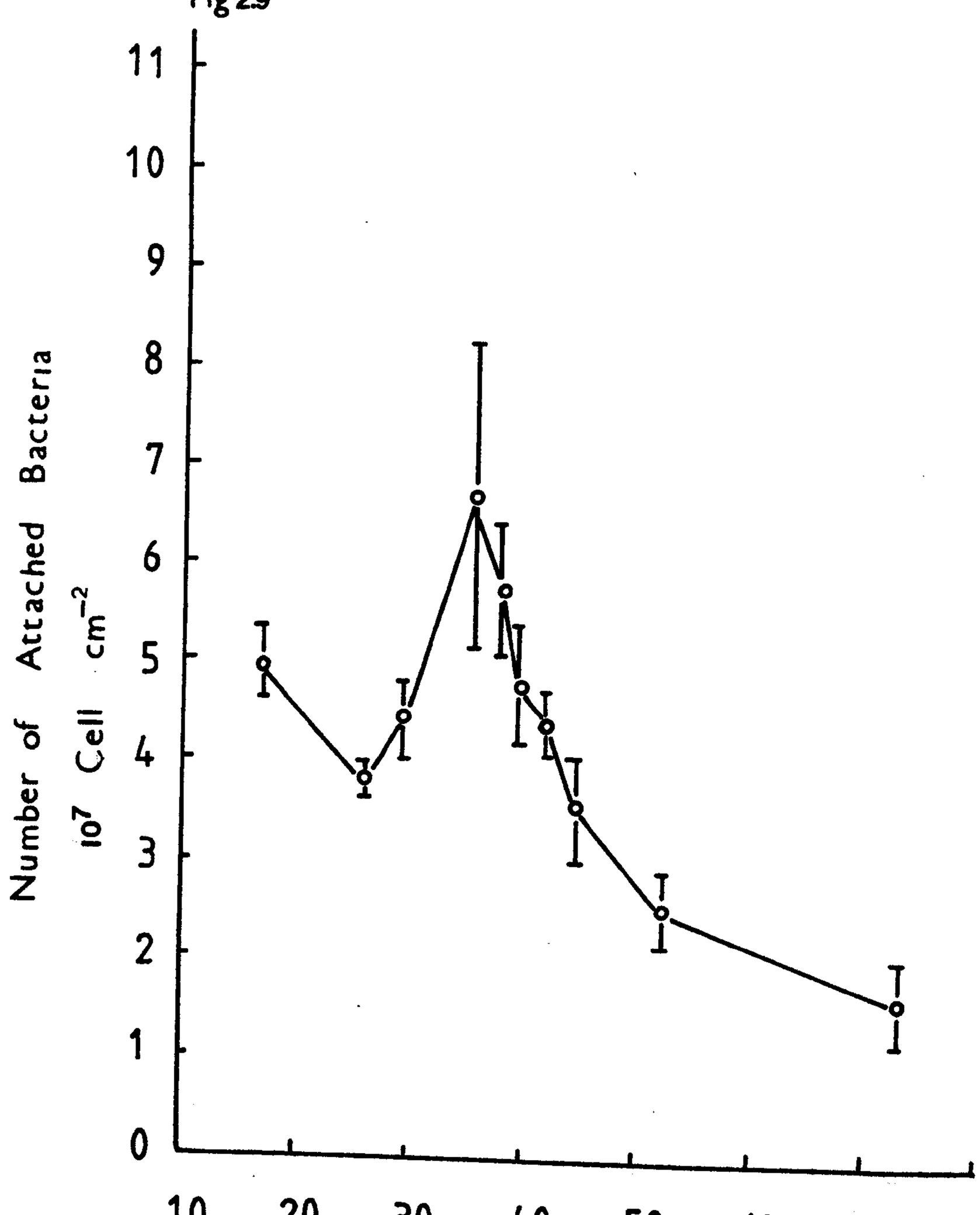

Number of Attached Bacteria
10^7 Cell cm^{-2}
0
1
2
3
4
5
6
7
8
9
10
11
10
20
30
40
50
60
70
80
Surface Free Energy (mJm^{-2})

Fig2.10

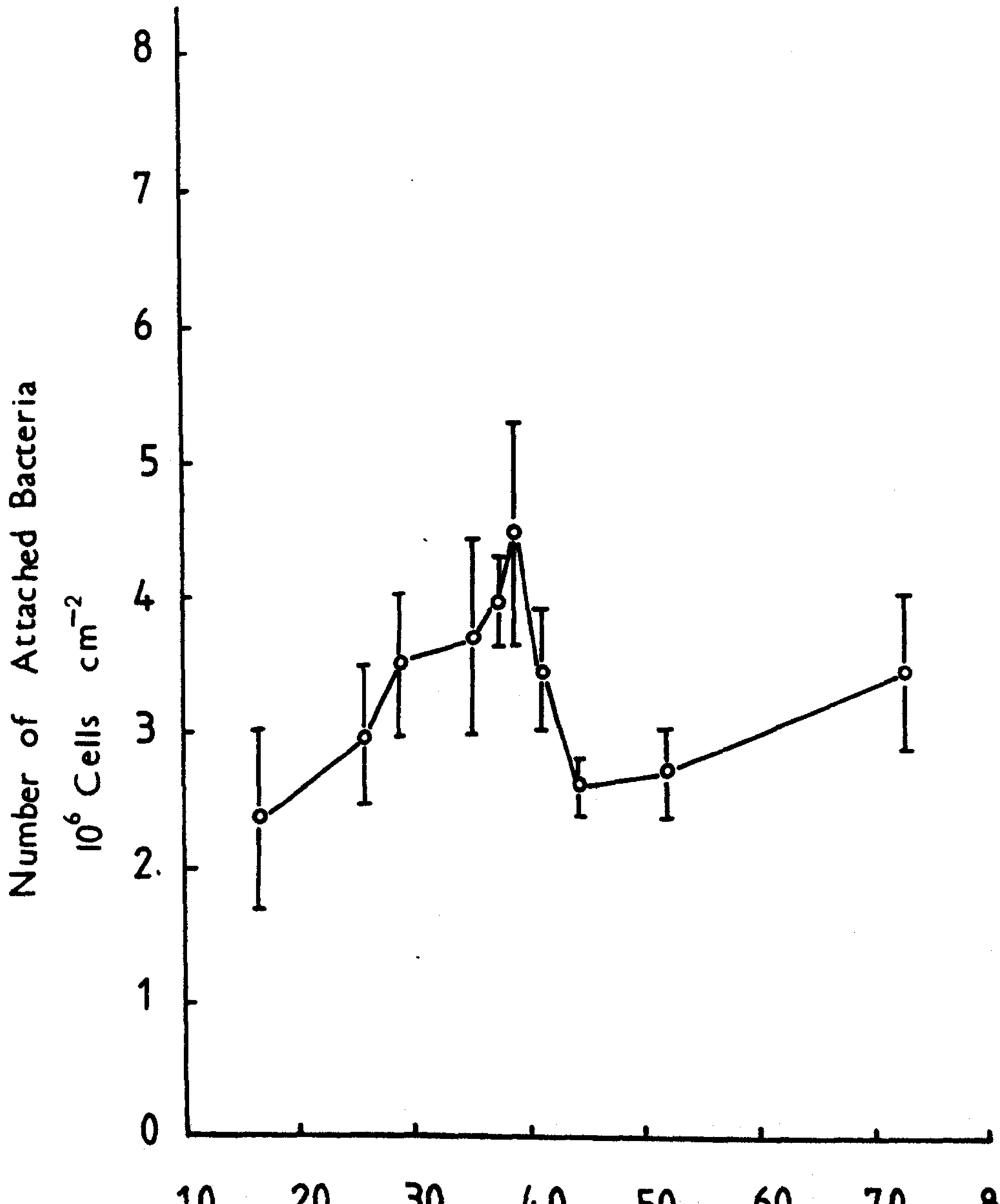

Number of Attached Bacteria
10^6 Cells cm^{-2}
0
1
2
3
4
5
6
7
8
10
20
30
40
50
60
70
80
Surface Free Energy (mJm^{-2})

Fig 2.11

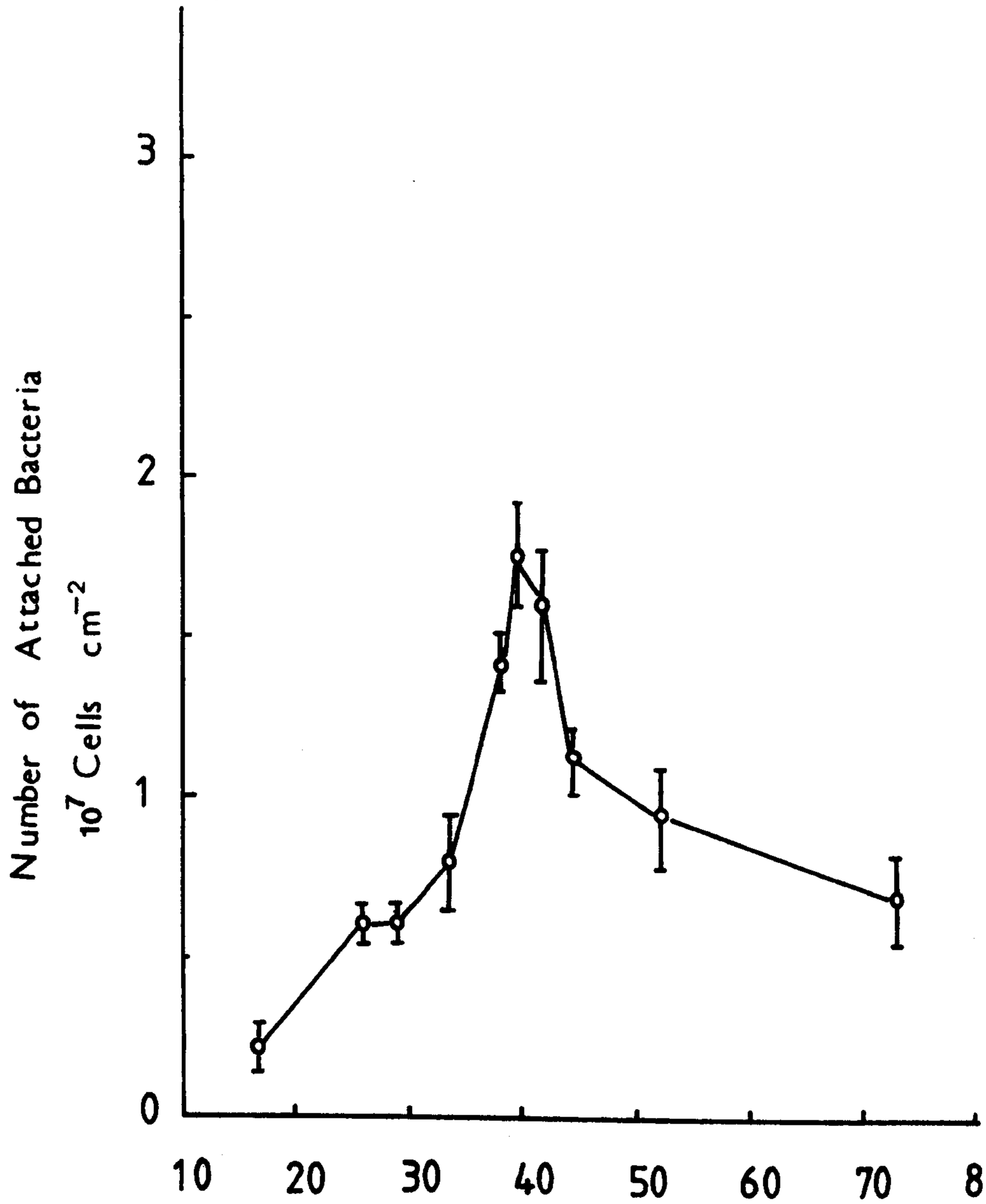
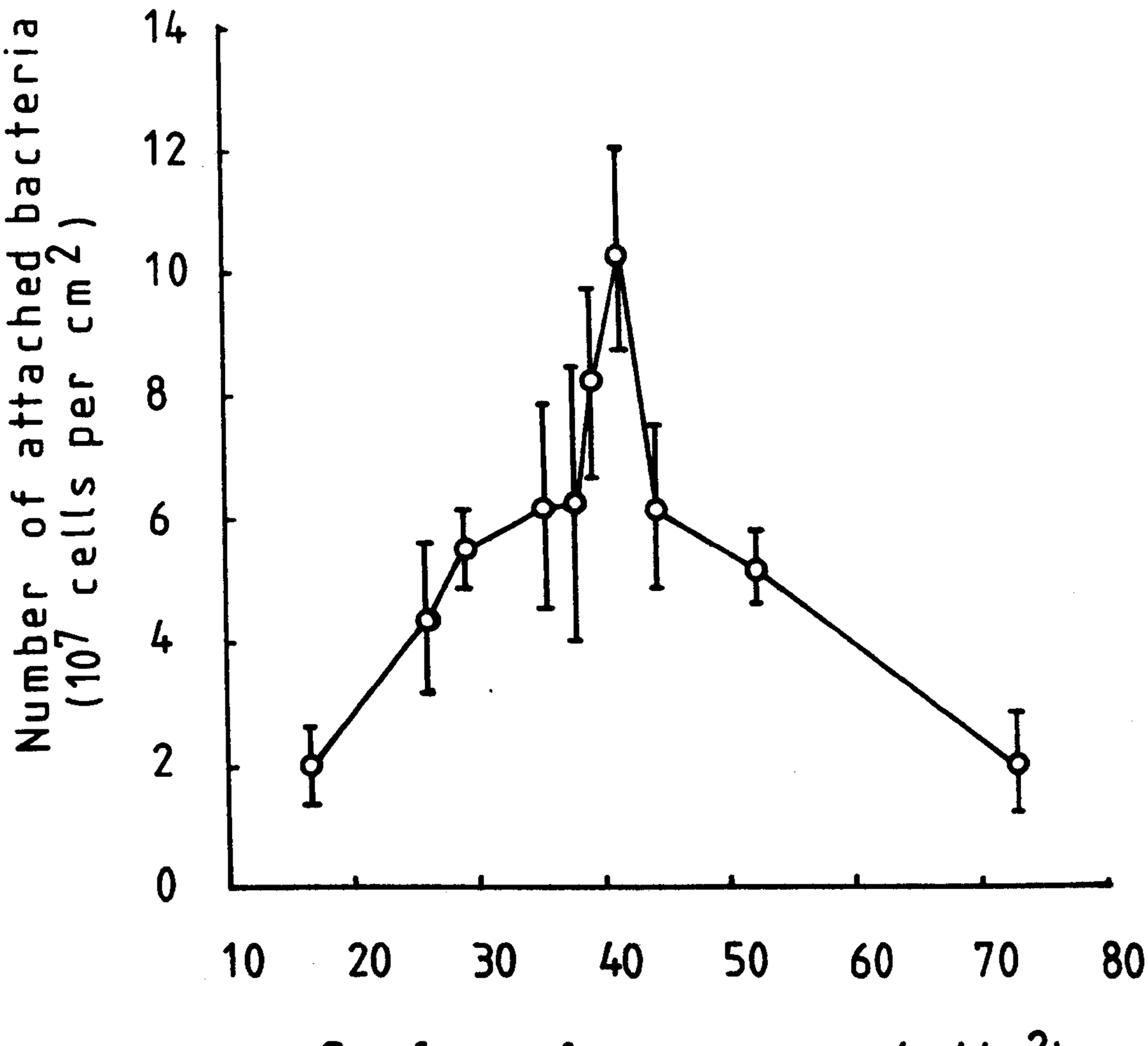

Number of Attached Bacteria
10^7 Cells cm^{-2}
0
1
2
3
10
20
30
40
50
60
70
80
Surface Free Energy (mJm^{-2})

Fig 2.12


Number of attached bacteria
(10^7 cells per cm2)
0
2
4
6
8
10
12
14
10
20
30
40
50
60
70
80
Surface free energy (mJ/m2)

2.4 DISCUSSION

2.4.1 Identification and characterisation of the sample bacterial population

The distribution of organisms recovered from the three isolation substrata and identified at the generic level were characteristic of those zymogenous bacteria found in most aquatic environments. However, the use of standard isolation techniques may prevent the isolation of oligotrophic organisms which are either unable to grow in the high nutrient levels of culture media or are unable to compete with the faster growing zymogenous bacteria. This distribution of bacteria is similar to organisms that dominate aerobic treatment processes (McKinney and Weichlein, 1953; Rogovskaya and Lazareva, 1959; James, 1964; Adamse, 1966; Halls and Board, 1974) and enrichment studies using continuous culture, where liquid and surface population were examined (Larson and Demmick, 1964; Brown et al, 1977; Wardell and Brown, 1980). However, in these continuous culture studies Enterobacteriacae were also prevalent, but were not detected in this investigation probably due to the low temperature (15°C) used throughout the experimentation.

2.4.2 Problems encountered estimating attached cell numbers by indirect radiolabelling

The number of bacteria attached to the different substrata were evaluated by (i) crystal violet dye absorption and

(ii) the calibration of liquid scintillation counts of radiolabelled cells to bacteria cell numbers. The problems of counting radioactive materials on solid supports by liquid scintillation have been previously recorded (Bransome and Gower, 1970). The problems encountered counting on solid supports can be summarised as follows:- (i) heterogeneous counting, where the radioactive sample can be distributed in both the surface film and bulk liquid and counted at different efficiencies; (ii) a type of quenching, called beta absorption, where the surface absorbs β-emissions so they do not produce a measurable excitation of the scintillant, resulting in a decrease in counts from the sample, without any direct measure of the degree of quenching; (iii) geometry effects due to the positioning of the sample on the surface and the position of the surface in the counting vial and (iv) changing counts with time due to effects of the counting solvent on the surface of the sample. Many of these problems can be avoided by methodology. In this instance heterogeneous counting was not found to be a problem, as repeat counts of scintillation vials with the substrata removed, showed only background levels of counts, indicating that very little radioactive sample was eluted from the surface before counting. Geometry effects were minimised by firstly ensuring that the substrata used for calibration controls were evenly coated with labelled cell suspensions.

To prevent water droplets developing on hydrophobic substrata, these bacteria were resuspended in ethanol (50%V/V) which permitted wetting of all test substrata. All dried substrata disks were in the same position in the vials ie. placed horizontally. A changing count rate with time was not experienced with dried substrata disks that remained intact and did not dissolve in the scintillation cocktail. Counts for PS and TC-PS were repeated for several days until stable values were recorded. The counts increased from a loss in efficiency of 22% which was the same as with PVC, and increased within 48hrs to the reported values (see table 2.3). β- absorption, or 'self absorption', could not be eliminated from the counting results by changes in experimental methods. A number of solubilisers were tried in order to elute the radioactive sample from the solid support, however, problems were encountered when solubilising agents reacted chemically with the solid substrata. This method was used however to verify the calibration curve of a number of surfaces. Therefore in order to measure the level of 'β-absorption' accurate calibration curves were produced for each substratum (Table 2.3). The lower levels (5-7, 22%) of β absorption found may be due to the substrata (PS, TC-PS) dissolving in the scintillation solvents or to the scintillation solvents and solutes diffusing into the plastic substrata, causing the surface of the plastic to act as a

plastic scintillator (PE, PVC). Under these conditions the external standards monitoring of the H number system can compensate for any quenching differences. This has been previously shown for high-density polyethylene vials as used in these experiments (Horrock, 1975, Horrock, 1976).

2.4.3 Attachment properties of selected bacterial isolates

An understanding of the attachment properties of bacteria in natural aquatic environments requires detailed information on the substrata, the conditioning components of the aquatic environment and the bacterial populations present. This laboratory study illustrates the importance of substratum properties in the initial stages of attachment of freshwater bacteria to surfaces. The results show that a number of freshwater bacterial strains have an attachment preference for hydrophobic surfaces, even when they were originally isolated on a hydrophilic test surface such as glass. A preference for hydrophobic surfaces has also been found with a marine Pseudomonas sp. (Fletcher and Loeb, 1976) Serratia marcencens, Staphylococcus epidermidis and Staphylococcus aureus (Gerson and Scheer, 1980) in similar laboratory experiment.

Although, the organisms in this investigation showed different levels of attachment, their profiles (fig 2.1-2.12) are similar to curves relating the adhesive strength to the thermodynamic work of adhesion for a plastic adhesive on a

range of plastic surfaces (Dycherhoff and Sell, 1972; Kitazaki and Hata, 1972) Figure 2.13 shows this relationship between the adhesive strength for various lacquers as a function of surface free energy, γ_{SV}, of plastics. The surface free energy of the adhesives (γ_{LV}) are indicated by arrows on the abscissa (taken from Dycherhoff and Sell, 1972). In such a system the change in Gibbs free energy of adhesion, ΔG^{Adh} is related to the interfacial free energies by equation 2.2 and is related to equation 1.9 for the interaction of three interfaces.

$$\Delta G^{adh} = \gamma_{SL} - \gamma_{LV} - \gamma_{SV} \qquad 2.2$$

In this case the γ_{SV} and γ_{LV} are the specific surface free energy and liquid surface tension of the substratum and potential adhesive, respectively. This equation indicates that maximum adhesive strength would be related to a maximum negative charge in free energy of adhesion (ΔG^{adh}) due to a minimum interfacial free energy (γ_{SL}). The γ_{SL} will be a minimum when the interfacial bonding characteristics are similar and $\phi_{SL} = 1$ (see section 1.2.4) Thus maximum adhesive strength would be found when $\gamma_{SV} = \gamma_{LV}$ assuming $\phi_{SV} = 1$. If this is the case then the bacterial surface free energy of the six organisms examined must lie in the range 35.4 - 41.6 mJm^{-2}.

Figure 2.13

Adhesive strength for various lacquers as a function of the surface free energy γ_{SV} of plastic substrata.
The surface free energy of the adhesives are drawn in on the abscissa by means of arrows. This relationship supports the criterion for maximum adhesive strength when $\gamma_{SV} = \gamma_{LV}$ of the adhesive taken from Dyckerhoff and Sell, 1972

Fig. 2.13

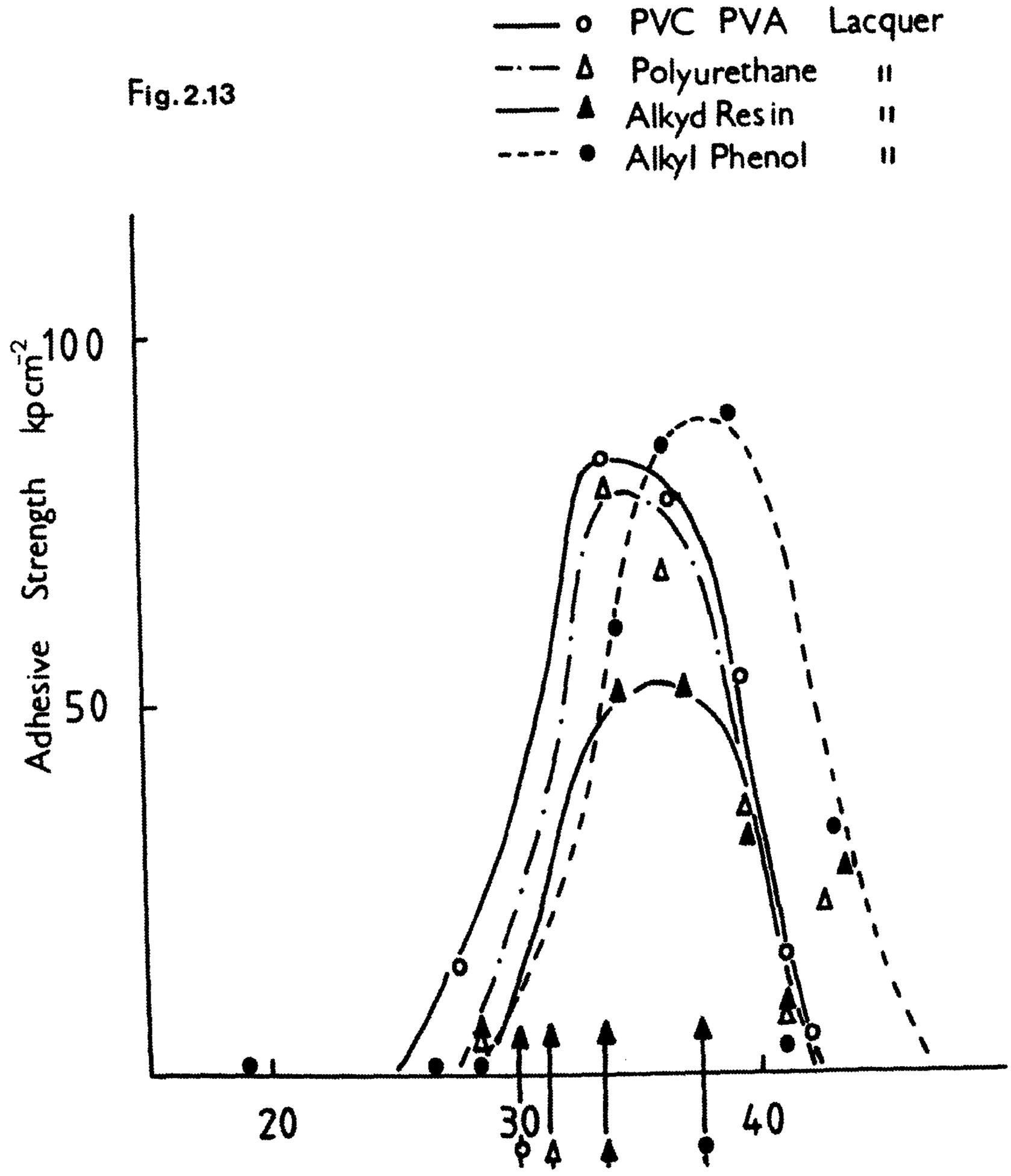

o PVC PVA Lacquer
Δ Polyurethane "
▲ Alkyd Resin "
● Alkyl Phenol "
Adhesive Strength kp cm⁻²
100
50
20
30
40
Surface free energy mJm⁻²

Figure 2.13 cont.

Adhesive	Surface Free Energy	Symbol
PVC PVA Lacquer	30.0 mJm^{-2}	○
Polyurethane Lacquer	31.8 "	△
Alkyd Resin Laquer	34.0 "	▲
Alkyl Phenol Laquer	37.5 "	●

However, equation 2.2 does not include the effects of water on the bacterial surface, substratum interaction, and calculation of ΔG^{adh} must use equation 1.9. The results obtained from the six bacterial isolates show some agreement with the theoretical plots of ΔG^{adh} versus substratum γ SV shown in figures 1.2.b and c, and derived using equation 1.9 and the equation of state approach. An increase in attached numbers was observed with a decrease in γ SV; however the maxima of the bacterial adhesion profiles (figs 2.7 - 2.12) suggest that the profiles could be a result of two opposing trends.

i) ΔG^{adhI} calculated from equation 1.9 and involving the substrata (γSV), the medium (γLV) and the bacterial (γBV)

ii) ΔG^{adhII} calculated from equation 2.2 and involving the substrata γ SV and the bacterial surface adhesive γ BV.

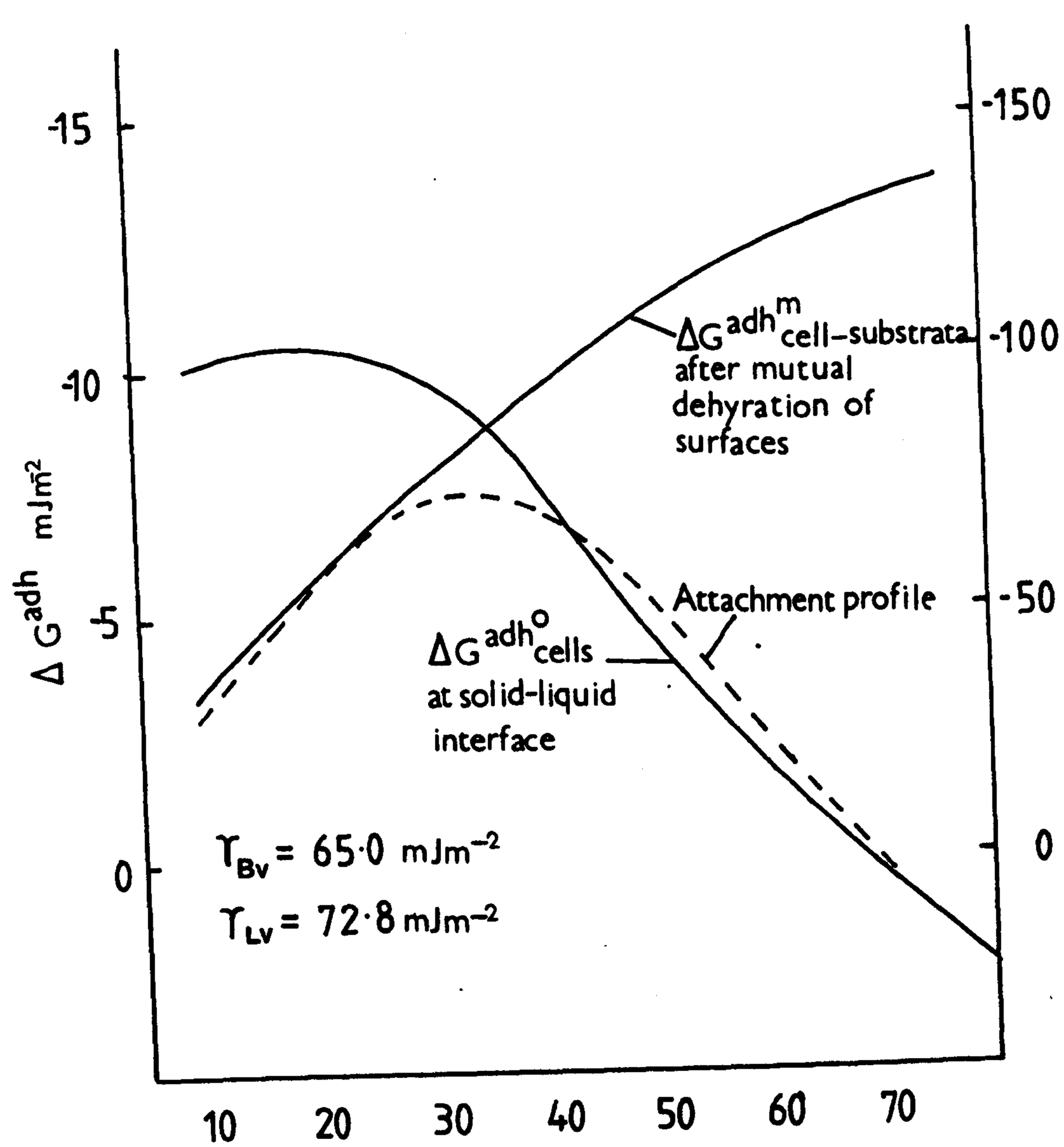
ΔG^{adh^o} gives prediction of the numbers of bacteria attracted to the solid-liquid interface while ΔG^{adh^m} gives predictions of the strength of adhesion when the bacterial and substratum surfaces are mutually dehydrated once in contact. Figure 2.14 shows a theoretical plot of both these curves assuming γ_{BV} = 65.0 mJm^{-2} and γ_{LV} = 72.8 mNm^{-1} for a range of γ SV. ΔG^{adh^o} is increasingly more negative with a decrease in γSV whereas ΔG^{adh^m} shows an increasingly more negative value with an increase in γ SV. A combination of the two curves for ΔG^{adh} would fit the experimental data presented in figures 2.7 - 2.12. Therefore bacteria with hydrophilic

Figure 2.14

Theoretical changes in Gibbs free energy of adhesion (ΔG^{adh}) as a function of the surface free energy (γ_{SV}) of the substrata for bacterial adhesion. ΔG^{adh^o} is the relationship expected for the accumulation of cells at the solid-water interface assuming γ_{BV} = 65.0 mJm^{-2} and γ_{LV}= 72.8 mJm^{-2} as calculated from the equation of state approach and (equation 1.9) ΔG^{adh^m} is the curve of bacterial adhesion after close contact has reduced the hydration of the cell substratum interface. Determined from equation 2.2 assuming that $\gamma_{BV} = \gamma_{LV}$ = 65.0 mJm^{-2} The dotted line labelled attachment profile shows the combined relationship assuming that ΔG^{adh^o} predicts what proportion of the cells accummulate at the interface and ΔG^{adh^m} range - 40 to - 140 mJm^{-2}

Fig 2.14

Fig.2.14



surfaces will tend to be collected by a solid-liquid interface between a low energy substratum and water, which offers a high interfacial free energy available for bonding. Yet the strength of adhesion between the cell and substratum will tend to be greater to higher energy substrata. Hence both bond strength and the level of bacterial accummulation at the substratum surface will influence the attachment numbers.

The general relationship between attached numbers and ΔG_{WA} or γ_{SV} of the substrata was illustrated by all six aquatic bacterial isolates studied. A comparison of the ΔG_{WA} and γ_{SV} maxima for these six isolates is shown in Table 2.4 Spearmans rank correlation test showed no significant difference between three pairs of the maxima from six bacterial strains ($P<0.01$). There was a significant correlation between the maxima in figs 2.1 and 2.2, 2.2 and 2.3, and figs 2.5 and 2.6 ($P<0.01$) were significantly different to the maxima in figs 2.1 and 2.2 ($P<0.05$) (Table 2.5) The position of the maximum attachment was found to be reproducible in repeated experiments and may represent differences in bacterial γ_{BV} if not representing the actual γ_{BV}. (γ_{SV} maxima = γ_{BV} see previous discussion). Bacteria with increasingly hydrophobic surfaces would be expected to form a maximum at lower values of γ_{SV} and the influence of water on this maximum may be less significant as $\phi_{SB} \ll 1$, in this case for high energy substrata.

Table 2.4

A comparison of the substratum WA and γSV maxima for the adhesion of selected bacteria isolates to a range of substrata of varying surface free energy

Organism	Surface	WA[1]	SV[2]
Chromobacterium sp (H31)	PVC	85.5	35.4
Pseudomonas fluorescens (H2)	"	"	"
Aeromonas hydrophila (H22)	"	"	"
Aeromonas hydrophila H6	PMMA	92.8	39.2
Pseudomonas aeruginosa H15	PMMA	92.8	39.2
Acinetobacter sp H3	Nylon 6.6	97.7	41.6

1 WA (mJm^{-2})

2 γSV (mJm^{-2})

Table 2.5

Spearman's rank correlation coefficients (r) for WA and YSV maxima from six bacterial isolates.

	Organism				
Organism	H2	H22	H6	H3	H15
H31	0.90	0.58	0.50	0.29	0.06
H2	-	0.76	0.65	0.25	0.04
H22		-	0.74	0.58	0.45
H6		-	-	0.49	0.55
H3				-	0.85

$$1 - r = \frac{1 - 6\sum d^2}{N(N^2-1)}$$

where d = difference in rank means R^1, from R^2,

and N = number of rank means per isolate

To be significantly the same:-

$r > 0.564$ ($p=0.05$) or 0.746 ($p=0.01$)

To be significantly different:-

$r \leq 0.436$ ($p=0.05$) or 0.254 ($p=0.01$).

2.4.4 Thermodynamic relationships for the adhesion of bacteria in the natural environment

The relationship between attached cell number and substratum γ_{SV} may be valid for the relatively clean conditions of *in vitro* experiments which are carried out in nutrient free media, over a short time scale. Under these conditions the physical aspects of the cell substratum interaction are probably more important than are physiological changes to the cell surface. Also, 'conditioning' of the surface will be due solely to bacterial polymers released from the cell surface. These will depend upon the organism, its culture conditions and rate of production of extracellular and membrane compound under starvation conditions. In studies of bacterial attachment in natural aquatic environments, bacterial adhesion should be strongly influenced by the development of the "conditioning film", and the fact that characteristics of primary colonisers will be influenced by "conditioning" provides the basis of the developing bacterial surface colonisation (Wardell and Brown, 1980, Baier, 1980). Such a conditioning reaction occurs spontaneously only when accompanied by a decrease in the total free energy of the system. Therefore upon conditioning, ie. film adsorption, the original solid-water interface is replaced with a solid-adsorbed organic interface. Such a process would influence the change in free energy of adhesion by i) lowering the liquid surface tension (γ_{LV}) ii) altering the solid surface free energy (γ_{SV}) and iii) influencing the bacterial surface free energy (γ_{BV}).

2.4.5 CONCLUSIONS

i) The organisms isolated from the three test substrata were zymogenous bacteria which are found in most aquatic environments, and the distribution of genera was typical of organisms that can be isolated from enrichment studies of freshwater environments.

ii) A majority of these freshwater bacterial strains showed an attachment preference for hydrophobic surfaces, even when they were originally isolated on a hydrophilic test substratum.

iii) A more detailed investigation of selected organisms showed a similar preference for moderately hydrophobic surfaces and a relationship between attached cell numbers and substratum γ_{SV} or WA. This relationship was characterised by maximum attachment for each organism to a substratum of mid-range of surface energy (35.4 - 41.6 mJm^{-2}). This relationship does not completely support the thermodynamic model presented in section 1.2.4 but demonstrates the important influence of water in any thermodynamic model of bacterial adhesion.

iv) The data illustrates that this selection of freshwater bacteria are spontaneously collected by solid liquid interfaces where the interfacial free energy is high and the short range bonding potential combines polar and dispersion forces. A possible explanation for the attachment profiles is given in that high energy surfaces bind too much water to allow adhesion, but low energy surfaces do not produce cell substratum binding interactions of sufficient strength to withstand

washing during the experimental procedure. Thus, maximum attachment occurs on the intermediate, moderately hydrophobic surfaces.

CHAPTER 3

An Investigation of the Interaction between Bacteria and the Solid/Liquid Interface

3.1 Introduction

In the previous chapter (2) a study of a number of freshwater bacterial isolates, a general relationship was found between the numbers of bacteria adhering to a range of substrata and the wettability of substrata, as determined by contact angle measurement of water. This relationship indicated that although different isolates attached to different extents, ie. in the ranges 10^5 - 10^8 cells cm^{-2}, all strains tested showed a preference for substrata in the middle range of surface free energy, γ_{SV} = 28.8 - 41.2 mJm^{-2}, with lower attached numbers to both lower energy (hydrophobic) and higher energy (hydrophilic) substrata.

The aim of this part of the investigation was therefore to evaluate the thermodynamic model presented in section 1.2.4 in terms of changes in i) the nature of the substratum, due to adsorbed water on conditioning films, ii) the medium, by addition of surface active molecules altering the liquid surface tension, and iii) the bacterial cell surface, by chemical and enzymatic treatments, in order to establish the roles of the substratum, the medium and the bacterial surface in the initial attachment process. This chapter has been divided into three parts dealing with:- i) substrata and substrata conditioning (section 3.2) ii) the medium (section 3.3) and iii) the bacterial surface (section 3.4)

3.1.1 The influence of conditioning films on the adhesion of freshwater bacteria to solid substrata.

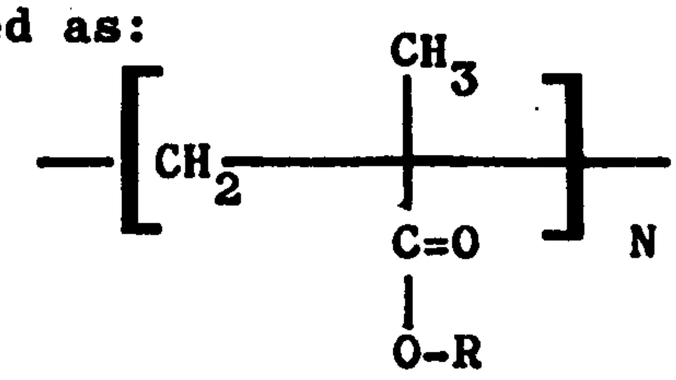
The importance of substratum properties in the initial adhesion of bacteria has clearly been shown from this work and others (Dexter et al, 1975; Loeb,1977; Fletcher and Loeb, 1979; Gerson and Scheer, 1980; Pringle and Fletcher, 1983). As previously described (section 1.3.1), substrata are quickly 'conditioned' following immersion in the natural aquatic environment, and subsequent bacterial attachment must be influenced by such changes to the surface of the substrata. For a surface to be 'biocompatible', reducing this conditioning effect, Andrade, (1973) has suggested that the interfacial free energy between the water and substrata must be less than $5mJm^{-2}$ (see Section 1.3.1). Such interfacial free energies are found between water and hydrophilic surfaces with an adsorbed water layer, due to high water content and low surface charge. Hydrogels used in Opthalmology for contact lenses have these properties and were used in this study to assess the influence of adsorbed water layers on the attachment of bacteria at the solid-liquid interface.

The adsorption of different proteins to surfaces can affect subsequent bacterial attachment (Fletcher, 1976, Meadows, 1971, Orstavik,1977). This investigation examined the effects of other macromolecules including bacterial lipopolysaccharides and dextrans, as well as proteins, on the attachment of freshwater organisms. The influence of conditioning films from natural water samples and bacterial growth media was also determined.

3.1.2 METHODS

3.1.2.1 Attachment of selected isolates to a range of hydrogel substrata

i) Hydrogel substrata The commercially available hydrophilic lens materials used as substrata included SP 55, SP 70, SP 77, and SP 85 (Special Polymers, London, England). These polymers are related to PMMA and are based on acrylic acid esters, particularly the esters of methacrylic acid. The resulting polymer is represented as:



If R is CH_3, the polymer is polymethyl methacrylate (PMMA) and is hydrophobic. If R is CH_2CH_2OH, the polymer is polyhydroxyethyl methacrylate (HEMA) also called Hydron®-S (Levowitz et al,1968). If R is $-CH_2-\underset{OH}{CH}-\underset{OH}{CH_2}$ the polymer is polydihydroxypropylmethacrylate and is used in opthalmology (Refojo and Yasuda, 1965). These polymers are readily cross-linked to form stable three-dimensional networks that form gels in aqueous solution. Contact angles were measured on these surfaces as previously described (section 2.2.5i). They were supplied as lens blanks, 10-12mm in diameter, in a dry state. The lenses were hydrated in sterile distilled water and washed in 2%(V/V) Lipsol solution. After repeated rinsing in sterile distilled water the blank diameters were

measured and compared to the reported expansion factors (published by Special Polymers, London). Attachment to hydrogel blanks was compared to PS, TC-PS and sulphonated polystyrene disks prepared as described in section 3.3.2.2.

ii) <u>Cell suspension preparation and the measurement of bacterial attachment using radiolabelled cells</u> The number of bacteria attached to the hydrogel blanks was determined by a modification of the technique previously described for the measurement of bacterial attachment to substrata disks (see section 2.2.5ii) where attached cells numbers were evaluated by liquid scintillation counting of radio-labelled attached cells. Because of the water content of the expanded hydrogels the surfaces and bacterial suspensions were counted by solubil ation in 80% V/V Lumasolve (Lumac AC Schaesberg, The Netherlands) in distilled water. The hydrogel blank, polystyrene disk, or cell suspension (100µl) were transferred to a 20ml glass counting vial containing 1ml of Lumasolve solution. After 2-4hrs incubation at 50°C 10mls of lipoluma scintillation cocktail (Lumac AC Schaesberg, The Netherlands), was added to each vial. Counts were made on a Beckman instruments liquid scintillation system LS-7000 and the level of quenching was determined using the H number system (see section 2.2.5iii). Calibration curves relating attached cell numbers to scintillation counts were produced using diluted cell suspension for both organisms. These were solubil ised and counted using the above method.

3.1.2.2 Attachment in the presence of dissolved and adsorbed macromolecules The effect of dissolved macromolecules on the attachment to PS and TC-PS petri dishes was tested by adding 100µl of the following stock solutions to 5ml of the bacterial suspension just before addition to the test petri dishes:- bovine serum albumin fraction V (BSA); bovine glycoprotein (BGP); lipopolysaccharide from Escherichia coli serogroup 0111 (LPS); dextran fractions, with average molecular weights 10,000; 40,000; 70,000; 2,000,000 (D10, D40, D70, D2000) (all from Sigma Chemicals Co. St Louis. Mo) These were added at the following concentrations:- BSA 5mgml^{-1}; BGP 5mg/ml^{-1}; LPS 2.5mgml^{-1}; D10 to D2,000 5 mg/ml^{-1} to give final concentration of 0.1 mgml^{-1}. Bacterial suspensions were also prepared in the initial supernatant containing spent growth media (S) and in centrifuged water samples from a number of local natural freshwater sources including the River Sowe (NFS1-6). The effects of adsorbed macromolecules to the attachment substrata were tested by exposing PS and TC-PS petri dishes to 5ml of BSA, BGP, LPS; D10-D2,000 solutions (0.1mgml-0.1M phosphate buffer, pH 7.4), for 2h at 15^{o}C. The S and (NFS1-6) were exposed to the surfaces allowing time for the adsorption of dissolved components for 2h at 15^{o}C. The dishes were then rinsed three times with 0.1M phosphate buffer pH 7.4; this was carried out with utmost care to prevent drying of substrata surfaces. 5ml of bacterial

suspension was added to each dish immediately and attachment was then assayed as previously described in Chapter 2 (section 2.2.4). All substrata were tested in duplicate. Results were expressed both as the absorbance at 590nm of each surface and as the index of attachment (Ia), which is the ratio of the A(590) of the test substratum to that of the control substratum. Thus, values near 1.0 indicate no effect, whereas those significantly below 1.0 indicate an inhibition of attachment.

3.2.3 RESULTS

3.2.3.1 The attachment of selected isolates to a range of hydrogel substrata

The numbers of attached bacteria cm^{-2} of hydrogel substrata of increasing expansion ratio are shown in Table 3.1. These values are compared with the attachment to PS, TC-PS and SP petri dishes carried out at the same time. The results indicate that bacterial attachment is lower on hydrogel substrata compared to the solid substrata, PS, TC-PS and SP . There was a decrease in attachment numbers with increasing water content of the hydrogel for both organisms tested. However, the water contact angle for the dry hydrogel surfaces did not produce a consistent relationship when compared to the contact angle of the polystyrene substrata especially when related to bacterial attachment. The sulphonated polystyrene surface had a zero contact angle yet the number of attached organisms was significantly larger than on the hydrogel surfaces, which ranged in contact angle between 11^{o}-34^{o}. Water contact angle measurements on hydrated hydrogel surfaces were not possible.

3.2.3.2 Attachment of bacteria in the presence of dissolved macromolecules

The effects of BSA, BGP, LPS D10-2000, S and NFS1-6 present during the attachment period are shown in Table 3.2. *Pseudomonas fluorescens* (H2) was attached to hydrophobic and hydrophilic Ps substrata for 2h at $15^{o}C$, BSA, LPS, D10-2000 and S inhibited

TABLE 3.1

Attachment of Pseudomonas fluorescens (H2) and Acinetobacter sp. (H3) to a range of hydrogel and polystyrene substrata. The water content and contact angle for water (for dry surface) are given for each substratum. Attachment was for 2h at 15°C.

Organism	Substrata	a Water Content(%w/w)	b Contact angle	c No. attached Bacteria cm^{-2}
Pseudomonas fluorescens H2	SP85	79	11	$6.2\pm1.5\times10^{6}$
	SP77	77	21	$9.0\pm1.0\times10^{6}$
	SP70	70	27	$9.3\pm1.1\times10^{6}$
	SP55	55	34	$1.2\pm0.1\times10^{7}$
	SPS	1	0	$2.1\pm0.13\times10^{7}$
	TC-PS	1	66	$2.9\pm0.12\times10^{7}$
	PS	1	90	$4.3\pm0.4\times10^{7}$
Acinetobacter SP	SP85	79	11	$5.4\pm2.2\times10^{5}$
	SP77	77	21	$6.9\pm3.0\times10^{5}$
	SP70	70	27	$1.9\pm0.4\times10^{6}$
	SP55	55	34	$2.3\pm0.42\times10^{6}$
	SPS	1	0	$1.7\pm0.21\times10^{7}$
	TC-PS	1	66	$6.3\pm0.31\times10^{7}$
	PS	1	90	$6.6\pm0.3\times10^{7}$

a values published by Special Polymers, London

b $(\acute{o}n-1)< 1.5^{o}$ (n=8)

c $(\acute{o}n-1)< 4\times10^{6}$ cells cm^{-2} (n=6)

TABLE 3.2

Attachment to PS and TC-PS Petri Dishes for 2 h at 15°C with macromolecules added to Pseudomonas fluorescens (H2) suspensions. Also attachment using cell suspensions resuspended in culture supernatant (S) or natural freshwater samples (NFS)

(a)

Macromolecule or conditioning solution	A590 of bacteria attached to:		Ia	
	PS	TC-PS	PS	TC-PS
None added	0.164	0.108	1.00	1.00
BSA	0.082	0.053	0.50	0.49
BGP	0.173	0.043	1.05	0.39
LPS	0.039	0.035	0.24	0.32
D10	0.029	0.027	0.18	0.25
D40	0.053	0.029	0.32	0.26
D70	0.046	0.029	0.28	0.26
D2000	0.021	0.024	0.13	0.22
S	0.076	0.075	0.46	0.69
NFS1	0.153	0.104	0.93	0.96
NFS2	0.172	0.109	1.05	1.01
NFS3	0.173	0.105	1.05	0.97
NFS4	0.170	0.099	1.04	0.92
NFS5	0.166	0.103	1.01	0.96
NFS6	0.152	0.107	0.93	1.00

a) $6(n-1) < .01 \quad (n=8)$

attachment to both PS and TC-PS substrata. Whereas, BGP inhibited attachment only to the TC-PS dish surface. Natural freshwater samples NFS 1-6 did not affect significantly the attachment result to either substratum.

3.2.3.3 Influence of macromolecules adsorbed to substrata on subsequent bacterial attachment

BSA, BGP, LPS and conditioning macromolecules except the dextran solutions were found to inhibit bacterial attachment to both PS, and TC-PS substrata (see Table 3.3). However conditioning using natural water samples when allowed to adsorb for 2h at 15^{o}C did not significantly affect subsequent bacterial attachment.

TABLE 3.3

Effects of adsorbed macromolecules from either pure solutions, culture supernatant or natural freshwater samples on subsequent attachment at 15°C for 2 h. (Pseudomonas fluorescens H2)

Adsorbed macromolecule or conditioning solution	(a) A590 of bacteria attached to		Ia	
	PS	TC-PS	PS	TC-PS
None added	0.164	0.108	1.00	1.00
BSA	0.027	0.003	0.16	0.027
BGP	0.041	0.009	0.25	0.083
LPS	0.001	0.000	0	0.0
D10	0.153	0.105	0.93	0.97
D40	0.157	0.110	0.96	1.02
D70	0.183	0.111	1.11	1.03
D2000	0.142	0.113	0.866	1.05
S	0.101	0.053	0.61	0.49
NFS1	0.149	0.102	0.91	0.94
NFS2	0.137	0.100	0.83	0.92
NFS3	0.166	0.096	1.01	0.88
NFS4	0.129	0.099	0.78	0.91
NFS5	0.178	0.098	1.08	0.91
NFS6	0.169	0.104	1.03	0.96

a $(6n-1) < 0.01$ $(n=8)$

3.2.4 DISCUSSION

The adhesion of two bacterial isolates to hydrogel polymers showed a decrease in numbers with increase in the water content of the hydrogel polymer, and reduced attachment levels were found for all the hydrogels when compared with solid polystyrene surfaces. This supports the theory that surfaces of high water content and low surface charge would meet a criterion for 'biocompatibility' as proposed by Andrade, (1973) (section 1.3.1). However, although the SP 85 polymer with 79% water content showed low numbers of attachment, there still remained a significant number of cells in contact with the surface, and undoubtedly these cells would be able to colonise such a surface. Thus even the small interfacial free energy between water and well hydrated surfaces show limited bacterial attachment over the initial colonisation period.

To what extent conditioning takes place on these surfaces is not known. However in a clinical study of soft contact lenses, lenses with a high water content have been shown to have greater deposit formation than those with a low water content (Fowler and Allan Smith, 1983). This would indicate that such surfaces are not biocompatible but serve to concentrate proteins and solutes in the tear fluid when worn. The adsorption of proteins to these hydrophilic surfaces has also been examined (Brash and Lyman, 1969; Baszkin and Lyman, 1980; Holly and Refojo, 1976). Whereas hydrophobic surfaces absorb

serum proteins of a range in molecular weights in an identical fashion, hydrogels absorb low molecular weight protein to a greater extent than high molecular weight proteins. Although both the amount absorbed and the reversibility of adsorption suggest that on hydrophobic surfaces and solid surfaces adsorption is irreversible while adsorption to hydrogels is reversible, the proteins interact less with the gel and sorption may be a pore-penetration process. Baszkin and Lyman, (1980) suggest that the ratio of the work of adhesion of the dispersion forces and the non-dispersion interaction for the solid-liquid interface gives a value (Ia) indicative of the balance of non-dispersive/dispersive forces on the polymer surface and has a similar value to the interaction parameter ϕ_{12}. As this parameter approaches =1, the number of proteins segments attached to the polymer appears to be higher and the protein more strongly bound to the surface. Thus it would appear that maximum protein adsorption occurs when there is a more equal balance of forces between the polymer surface and the high energy liquid or protein solution. Hydrogels and glass may therefore have higher values of Ia $\gg$1 which would give support to this hypothesis (section 1.3.1) as poly(hydroxyethyl methacrylate materials do show low protein adsorption as do very hydrophobic surfaces.

The influence of conditioning films and polymers on bacterial attachment to hydrophobic and hydrophilic substrata was found to be dependent upon the nature of the conditioning

macromolecule and whether attachment took place in the presence of these polymers or after their adsorption to the substrata. The effects of dissolved and substratum adsorbed proteins on bacterial attachment were similar to the observed effects on the attachment of Pseudomonas sp. strain NCMB2021 (Fletcher and Marshall, 1982; Fletcher, 1976), except in this study increased attachment to the PS petri dish in the presence of BGP was not observed. The proteins BSA and BGP inhibited bacterial adhesion through adsorption on the surface (Table 3.3). These adsorbed layers will bind water layers more effectively, than the clean substrata making the surface less suitable for attachment either by modifying the ionic micro-environment of the surface or by effecting the affinity of water for the surface, thus reducing the interfacial free energy of the surface/liquid interface and preventing the spontaneous adsorption of cells. The changes in bonding that will take place with the adsorption of the protein or macromolecule depend upon the surface bonding potential and the role of water and water structure on this process. Conformational changes and denaturation of macromolecules at an interface will influence the uppermost layers exposed to the bacterial surface. This has been investigated only to a limited extent. Fletcher and Marshall, (1980) have shown a relationship between bubble contact angle measurements and macromolecular conditioning at the substratum/liquid interface as a means of evaluating substratum interfacial characteristics and their relevance to bacterial

attachment.

In studies of the adsorption of albumin and γ- globulin to hydrophobic plastic surfaces, using infra red reflection spectroscopy to measure the saturation concentration of protein absorbed as a function of solution concentration at pH 7.4, Brash and Lyman, (1969) showed that these proteins were adsorbed physically but irreversibly as close-packed monolayers in their native state. These conditioning monolayers appeared to be the same when adsorbed to charged surfaces as compared to neutral polymer surfaces. Although, proteins were found to differ in their saturation levels on the range of polymers tested, it was found that on average 100 μg/ml concentrations of protein in solution were required for minimum saturation levels of 1 $\mu g/cm^2$, and Langmuir-type adsorption isotherms were obtained for most systems. This would indicate that high and low energy polymers would be equally conditioned with certain molecules at the same concentration. However it does not explain whether orientation effects of amphoteric molecules may be present.

The presence of conditioning films due to proteins or other macromolecules did not affect attachment when natural water samples were used either to condition the surface prior to attachment or during attachment. These results suggest that conditioning films may not be as quickly developed in freshwater as in seawater or that the dissolved macromolecular concentrations of different aquatic environments may influence the saturation

level of adsorption and surface coverage. Infra red reflection spectroscopy of polyvinylchloride plastic substrata showed rapid (10mins) development of a conditioning film when exposed in Biscayne Bay, Miami Beach, Florida. Yet only a limited amount of conditioning film was present after longer immersion (21hours) in an aquarium. (Goupil et al, 1976). This may reflect the amount of dissolved macromolecules available for conditioning. (Kristoffersen et al, 1982).

Despite their both being largely polysaccharide, the influence of dextrans and LPS on attachment differed. The LPS inhibited attachment to both substrata either in the presence of the cells or when adsorbed to the substrata prior to attachment. On the other hand the range of dextrans showed inhibition only when added to the cells during the attachment period. This indicates that LPS, like the proteins, adsorbed to the substrata and inhibition of attachment was greater when adsorption took place prior to attachment. The dextrans however were not strongly or irreversibly adsorbed to the surface (dextrans were present in elution/washing buffer) and therefore probably had the effect of stabilising bacterial suspensions. Harris,(1971) suggests that these polymers along with the lytic products of cells act by stabilising bacterial suspension preventing either aggregation or sorption to surfaces. This was also the case for supernatant of bacterial cultures (s) which inhibited adhesion either by prior absorption or during attachment. Similar inhibition in attachment was observed for marine pseudomonad NCMB 2021 (Fletcher and Marshall, 1980)

3.2. The effects of changing the liquid surface tension of the attachment media on the attachment of Pseudomonas fluorescens (H2) to polystyrene substrata

3.2.1 Introduction

A second parameter influencing bacterial attachment as indicated by the thermodynamic model presented in section 1.3.1 is due to the surface tension of the medium (γ_{LV}) Changes in γ_{LV} can be produced by surface active macromolecules or smaller organic molecules (section 1.3.2). In these experiments ethanol, dimethylsulphoxide (DMSO) and Urea were used both to induce the liquid surface tension γ_{LV} of the attachment buffer and to influence hydrophobic solvation and hydrogen bonding as class I and class II additives (section 1.3.2)

The results of assays of attachment to polystyrene substrata with increasing γ_{SV} in the presence of varying DMSO concentrations were used to calculate the interfacial free energy, γ_{BV} for this organism in water vapour. The bacterium was grown under nitrogen or carbon-limitation, and γ_{BV} for each growth condition was determined from the γ_{LV} of the particular aqueous DMSO solution in which attachment was independent of γ_{SV}. (see section 1.3.1)

3.3.2 METHODS

3.3.2.1 Organism and culture conditions.

A freshwater isolate of Pseudomonas fluorescens (H2) was used throughout these experiments. The organism was grown in a chemically defined medium containing NH_4Cl (0.2 gl^{-1} for nitrogen limited growth, 2.0gl^{-1} for glucose limited growth), KH_2PO_4(5.55 gl^{-1}) and a mineral salts solution (6ml l^{-1}) containing (gl^{-1}); $MgSO_4.7H_2O$(10), $MnCl_2.4H_2O$ (1.0), $FeSO_4.7H_2O$ (0.4) and $CaCl_2.2H_2O$ (0.1). The carbon source was either glucose or glycerol at (2.0gl^{-1}) and the complete medium was adjusted to to pH 7.0 with 1M KOH (Williams and Wimpenny, 1976). Cultures (100ml) were grown in 1litre Erlenmeyer flasks at 15^{o}C with gyratory incubation (120 rev min^{-1}). Growth was followed turbidometrically, and bacteria were harvested in early stationary phase by centrifugation and resuspended in 0.1M phosphate buffer pH 7.4 to a final concentration of 2.5 - 5 x 10^9 bacteria ml^{-1}, determined from a standard curve relating stationary phase cells to absorbance readings at 540nm.

3.3.2.2 Attachment in the presence of surface active agents.

10ml of bacterial suspensions were mixed with the appropriate volumes of ethanol, (A.R.J. Burrough Ltd), dimethylsulphoxide (Hopkin and Williams) (DMSO) and Urea(A.R., Fisons) to give final concentration of 0.5, 1, 2, 5, 10, and 15% (V/V) for ethanol and DMSO or 0.1, 0.5, 1, 2, 3M for Urea in a final volume of 20ml. Controls were prepared for each experiment by adding distilled water to 10mls of cell suspension to give

a volume of 20mls. 5ml samples of the mixture were immediately placed in a hydrophobic PS petri dish, TC-PS petri dish, and hydrophilic sulphonated polystyrene petri dish (SP). SP were prepared by treatment of PS petri dishes with 10mls concentrated sulphuric acid for 24hrs, after which time they were rinsed repeatedly in sterile distilled water and air-dried. After 2h at 15°C without stirring, bacteria not attached to the dish surface were removed by rinsing in phosphate buffer from a washbottle. The surfaces were then allowed to dry.

3.3.2.3 Measurement of bacterial attachment

The numbers of bacteria attached to polystyrene petri dishes was determined by a modification of the technique previously described for the measurement of bacterial attachment to substrata disks (see section 2.2.5). The cells were radio-labelled prior to attachment experiments and washed three times in sterile phosphate buffer. Following the attachment incubation period and subsequent washing/drying, the petri dishes were broken into small pieces suitable for insertion into a scintillation vial. Care was taken when carrying out this procedure not to disturb the bacterial film. Glass scintillation vials (20ml) containing the broken petri dishes were then filled with 5mls tolulene and were left overnight at room temperature. The vials were then counted following the addition of an emulsifier containing liquid scintillation cocktail of tolulene/Triton X 100 ($\frac{1}{2}$V/V) with the scintillator butyl PBD 12 g l^{-1} to a total volume of 10ml. Counts were

repeated four times over a 24h period. Controls relating bacteria numbers to scintillation counts were prepared by drying ethanol-diluted cell suspensions onto petri dish surfaces and counting using the above procedure. The addition of tolulene to polystyrene petri dishes dissolves the polystyrene completely within 24h, preventing problems in scintillation counting due to the β absorption (see section 2.4) Quenching due to the added polystyrene to the scintillation cocktail was minimal and was determined by H number using a Beckman instruments Liquid Scintillation system LS-7000.

3.3.2.4 Surface tension and contact angle measurements

Liquid surface tension values for the ethanol and DMSO solutions were measured using a Pt, loop and torsion balance (White Elect Inst). Contact angles of double distilled water on the three test substrata were measured as previously described (see section 2.2.5)

3.3.3. RESULTS

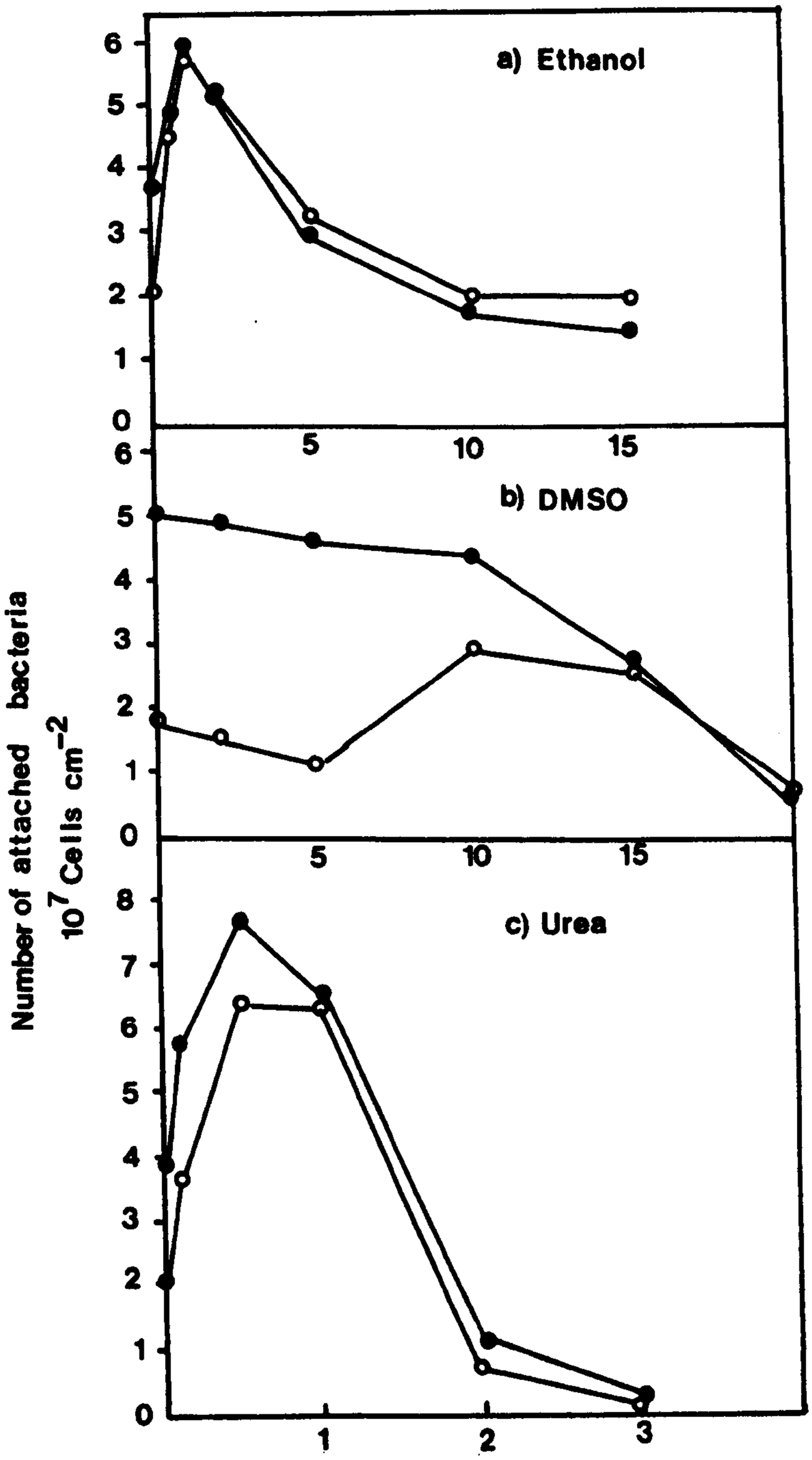
Attachment of bacteria in the presence of surface active agents

The effects of ethanol, DMSO and Urea in relation to concentration on bacterial attachment to both hydrophobic and hydrophilic substrata under carbon limitation are shown in figure 3.1. for *Pseudomonas fluorescens*, . Similar relationships were found between bacterial attachment and the concentration of surface active agent for carbon-and-nitrogen deficient cultures. An increase in attachment up to an attachment maximum was observed with increasing concentration of ethanol and Urea for both growth conditions (Fig. 3.1). Maximum attachment was found on both surfaces at the following concentrations of ethanol and Urea respectively:- 0.17M and 0.5M. At higher concentrations PS and TC-PS show equal levels of attachment as the number of attached bacteria decreased. The results for DMSO (Fig. 3.1) indicate that attachment to the polystyrene substrata decreased slowly with increasing concentration up to 10% (V/V), after which attachment decreased more rapidly. The attachment to TC-PS also decreased slowly for both organisms but then increased to a maximum at 10% (V/V) towards the level obtained on PS, or greater, before decreasing at concentrations greater than 15% (V/V) DMSO. Table 3.4 shows the results of liquid surface tension γ_{LV} measurements for DMSO solutions. The values for γ_{LV} measured agreed well with the reported values for ethanol and DMSO solutions(Dan, 1969). The attachment results of *Pseudomonas fluorescens* H2 from carbon-limited and nitrogen

Figure 3.1

The effects of ethanol (a), DMSO (b), and Urea (c) in relation to concentration on bacterial attachment to both hydrophobic (PS, ●) and hydrophilic (TC-PS, ○) substrata for Pseudomonas fluorescens H2. Similar relationships were found for both carbon and nitrogen limited cultures.

Figure 3.I



Concentration of Surface Active Agent (a)% v/v, (b)% v/v, (c) M.

limited culture for varying DMSO concentrations are shown in figs 3.2 and 3.3. The attachment to PS, TC-PS and sulphonated substrata was used in order to measure the gradient of attachment versus surface free energy for varying concentrations of DMSO. In both experiments the gradient increased with an increase in DMSO concentration or a decrease in γ_{LV} and this relationship was used to calculate γ_{BV} (section 3.3.4)

TABLE 3.4

Liquid surface tension γ_{LV} measurements of DMSO solutions

DMSO concentration %(W/V)	γ_{LV} (mNm^{-1})
0	73.0
2	71.3
5	69.8
10	67.0
15	63.2

Figures 3.2 - 3.3

Relationship between the attachment of Pseudomonas fluorescens H2 and the γ SV of the substrata. Attachment took place in solutions of DMSO in phosphate buffer at 0% (○), 2% (●), 5% (□), 10% (■) and 15% (△) concentrations. Relative attachment numbers were evaluated as dpm of radiolabelled attached cells per $8cm^2$ surface area.

Figure 3.2 Carbon-limited cells.

Figure 3.3 Nitrogen-limited cells.

Fig 3.2

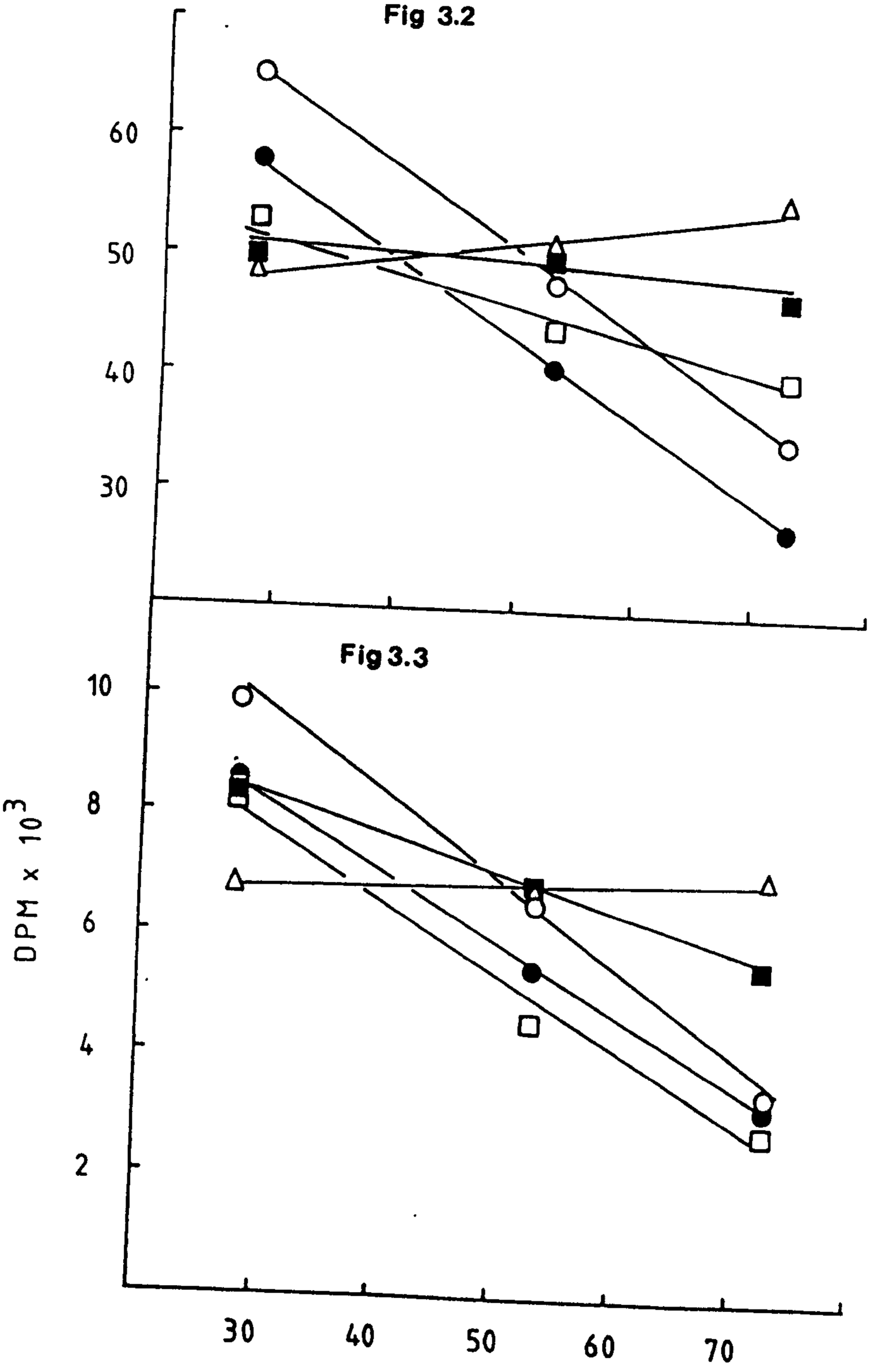


Fig 3.3

3.3.4 DISCUSSION

The liquid medium is important in the attachment process because of the role played by water itself and also because of the presence of dissolved substances which affect adhesion. The influences of dissolved macromolecules on attachment through the modification of surfaces and the formation of conditioning films has been discussed (see section 1.3.1). However solutes can also effect medium properties which influence attachment directly, as liquid surface tension (γLV) is an important influence on the available free energy of adhesion (see Section 1.2.4) (ΔG^{Adh}). It can be difficult to experimentally evaluate this role, however, as surface active agents which alter γLV also tend to be adsorbed at surfaces, thus altering apparent substratum characteristics, and it is not always possible to distinguish between the two effects (Fletcher and Marshall, 1982).

By using the equation-of-state approach to determine interfacial free energies, the theoretical values of ΔG^{Adh} with increasing substratum surface free energy (γSV) at different liquid surface tension (γLV) are shown in figs 1.2 (a,b,c). This thermodynamic model predicts that cell adhesion would increase with an increase in substratum surface free energy γS if γLV was less than the surface free energy of the bacterial cell (γBV). Conversely there would be a decrease in adhesion with increase in γSV if the γLV was greater than γBV. This model has been supported by experiments with

leukocytes and platelets (Neumann et al, 1979; Absolom et al, 1980) In figures 3.4 and 3.5 the results in figures 3.2, 3.3 have been plotted to show the change in slope (attached numbers versus γ_{SV} in Figs 3.2 and 3.3) obtained with different values of γ LV produced by different concentration of DMSO. By plotting the slope of these relationships against γ LV (fig 3.3, 3.5), the intercept of the resultant plot with slope = 0 should give the value of the surface free energy of the bacteria, γ BV. Values of 66.4 and 63.6 mJm^{-2} were obtained for carbon-limited and nitrogen-limited cells, respectively. These values for γBV are similar to others obtained for bacteria (Gerson and Zajic, 1979; Oss, 1979) and have been partly verified by measuring γBV using contact angle measurement of dried cells, although many reservations about this technique still exist (see section 1.3.3). Similar values were also obtained in supplementary experiments using ethanol instead of DMSO. As the physiology of the bacteria may be influenced by the addition of either DMSO or ethanol, respiration measurements were used to check whether the bacterial respiration rate remained unaffected. While this was true for the DMSO concentrations used, the addition of both ethanol and Urea increased the respiration rate of the cells. Similar results were obtained by Fletcher, (1982) in an investigation of the effects on ethanol, propanol and butanol on bacterial respiration of a marine pseudomonad.

Figures 3.4-3.5

Relationship between the slope of attached bacterial number versus γSV(from figs. 3.2 and 3.3) and the γLV of DMSO solutions. The intercept of the resultant plot with slope = 0 gives the value of the surface free energy of the bacteria γBV ie. when $\gamma BV = \gamma LV$.

Figure 3.4 Carbon-limited cells (●)

Figure 3.5 Nitrogen limited cells (○)

Fig 3.4

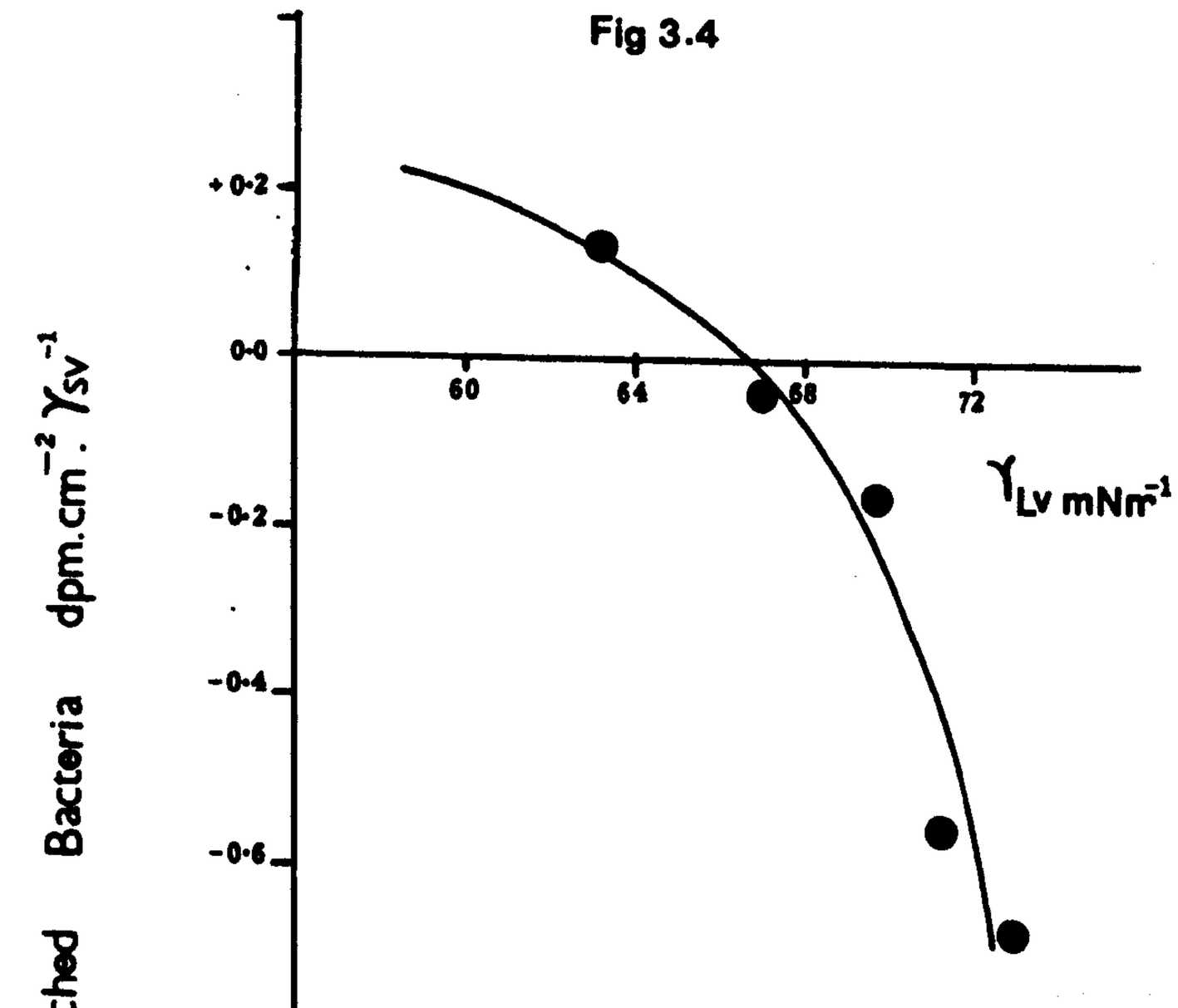
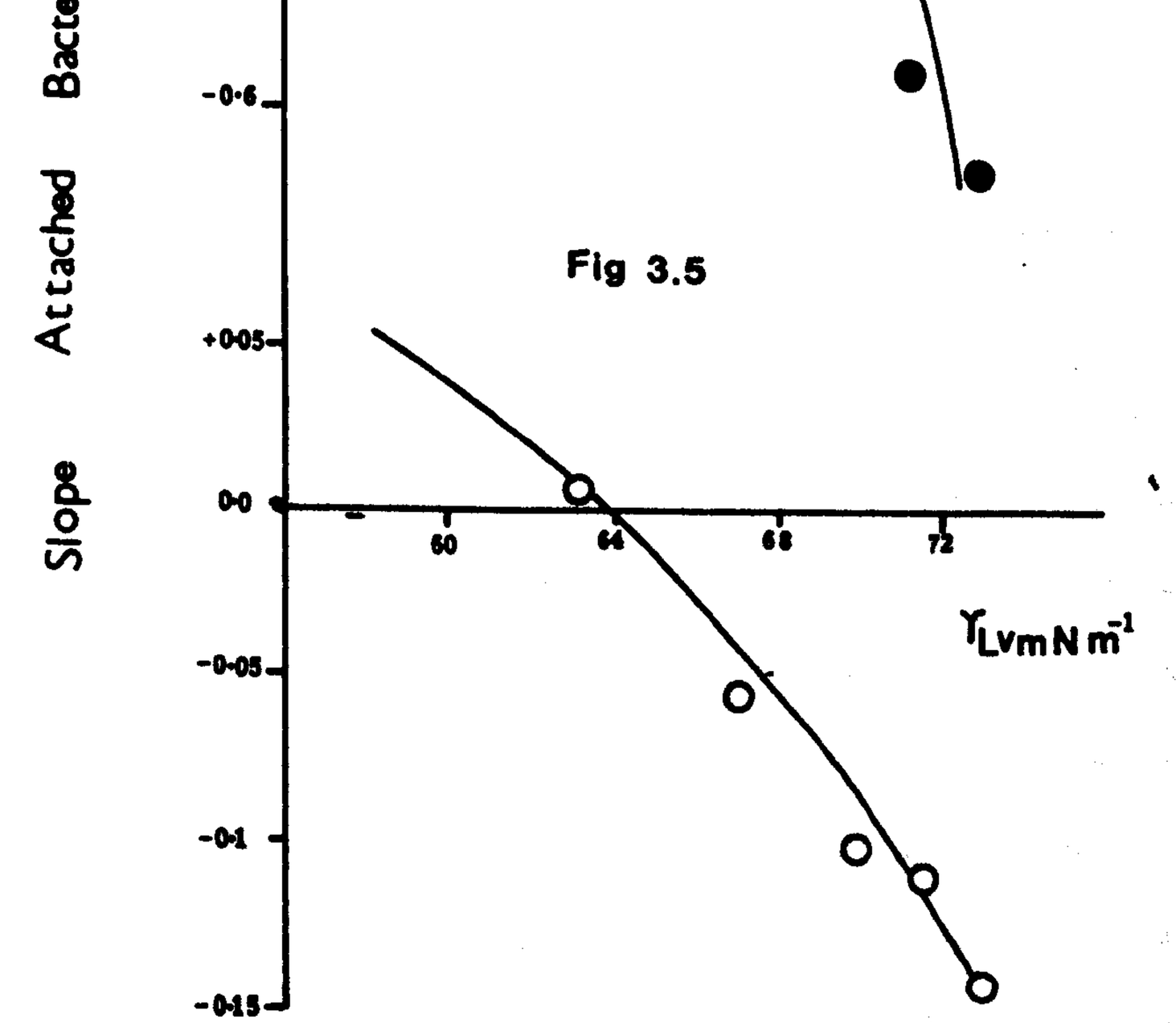

Slope Attached Bacteria dpm.cm^{-2}. γ_{sv}^{-1}
+0·2
0·0
-0·2
-0·4
-0·6
60
64
68
72
γ_{Lv} mNm^{-1}

Fig 3.5


+0·05
0·0
-0·05
-0·1
-0·15
60
64
68
72
γ_{Lv} mN m^{-1}

Thus changes in attachment with these surface active agents may be associated with physiological changes associated with either damage to membranes requiring membrane repair, as was found with Escherichia coli grown in the presence of alcohols (Berger et al, 1980; Ingram, 1976), or effects on the structure of water and the solvation of the hydrogen bonding and hydrophobic bonding groups in solution. The relationship between attachment numbers and concentration of these agents shows similarities with the curves relating the free energy of denaturation of ribonoclease with the composition of ethanol mixtures at 15°C. This relationship indicates that ethanol stabilizes the proteins in the native form, and only at concentrations in excess of 5% V/V will ethanol act as a denaturing agent. This may explain the increased attachment to hydrophobic and hydrophilic substrata at low concentrations of ethanol, as the attachment interaction may require the structural integrity of the cell membrane for adhesion to take place. Once proteins and other macromolecules, which rely on the structure of water for their conformation, are denatured, then weakened bonding between the cell and the surface interface will result (Tanford, 1979). However the data for urea is not consistant with this hypothesis, as urea would behave as a class I additive (section 1.3.2) and should act as a strong solubilising agent for most hydrophobic groups at low concentration and temperature. This may indicate that hydrophobic interaction is not solely responsible for bacterial adhesion and that polar and hydrogen bonds may be significant.

3.4. Studies on the role of the bacterial cell surface on the attachment of freshwater isolates to the solid surface

3.4.1 Introduction

Knowledge of the roles of cell surface structure and free energy (γ_{BV}) is required for a complete ther modynamic description of bacterial adhesion. Investigations in this area have dealt primarily with three experimental variables which can affect adhesions: i) chemical or enzymatic treatments ii) addition of electrolytes and iii) analysis of bacterial surface polymers.

First, chemical and enzymatic treatments which inhibit or disrupt cell adhesion may indicate the possible role of membrane structures or surface exopolymers in attachment. In these experiments treatments were applied to i) the free-cells, to determine their influence on subsequent attachment to substratum, or ii) attached bacteria, to see if the attachment bond was consequently weakened or broken down. These experiments included the use of proteases, hydrophobic liquids, sequestering agents and chemicals that denature polysaccharides.

Second, the effects of cation concentration is an important parameter. These ions dissolved in the attachment media can influence the attachment, indirectly, by influencing cell physiology and membrane permeability (Drapeau and Macleod, 1965), and directly either through their accumulation

at interfaces through the formation of electrical double layer (Shaw, 1970) or by helping to maintain the integrity of the membrane surface through cross linking of acidic groups.

The third variable studied is the influence of the bacterial surface and exopolymers present on the outer membrane on the irreversible attachment of freshwater organism. More detailed examination of these polymers, their concentration, composition, and surface coverage properties is required. Experiments were therefore carried out using a number of strains grown in carbon-or nitrogen-deficient media and on carbon-rich solid media designed to increase surface polysaccharide production. Attachment of these organisms in the presence or absence of their spent growth medium, containing bacterial extracellular polymers, allowed a comparison to be made between exopolymer production and attachment. Further analysis of these polymers was carried out to assess their possible role in attachment and is described in Chapter 4.

In the final subsection of this part (3.4) of the investigation, cell surface hydrophobicity, as determined by hydrophobic interaction chromatography (HIC) was related to the attachment of a number of freshwater strains to hydrophobic polystyrene substrata. This was to determine whether a relationship between attachment to hydrophobic substrata correlates with retention on the HIC gel as a measure of cell surface hydrophobicity (see section 1.3.3)

3.4.2. METHODS

3.4.2.1 The effects of proteases and other membrane modifying agents on attachment

i) organisms and culture conditions Pseudomonas fluorescens (H2) and Acinetobacter sp. were investigated following growth in PYE media under culture conditions that have been previously described (section 2.2.3).

ii) Protease enzyme digestions. Pronase P (Sigma) was either added to the bacterial suspension at the beginning of the 2h attachment period to see if attachment could be prevented (pronase concentration 0.02, 0.2, 2 $mgml^{-1}$ bacterial suspension) or used to detach bacteria after a 2h attachment period where cells were removed by further washing (pronase concentration 0.25 $mgml^{-1}$ bacterial suspension) as a result of enzyme activity. In a third category of experiment bacterial suspensions were pretreated with enzyme for 1h which was then removed by centrifugation of cells and their resuspension in phosphate buffer (pronase concentration 0.25 mg/ml bacterial suspension). Control suspensions either contained proteases denatured by heating at $100^{o}C$ for 20mins or the protease activity was inhibited by adding 0.05ml of phenylmethanesulfonyl fluoride (PMSF) ($7mgml^{-1}$ ethanol) ml^{-1} of bacterial suspension. The presence of ethanol as a solvent for PMSF influenced cell attachment and was therefore included as a control in all assays (.5% V/V ethanol ml^{-} bacterial suspension except for cells only controls. A list of enzyme treatments and controls

is shown in table 3.5a). The numbers of bacteria attached to PS and TC-PS petri dishes for each test assay was measured as previously described (see section 2.2.4) by staining the fixed cells with crystal violet and measuring the absorbance of each dish at four randomly selected areas at 590nm.

iii) <u>The effects of Divalent and Trivalent cations and Ethylenediaminetetraacetic acid (EDTA) on bacterial attachment</u>

Attachment assays to PS petri dishes were carried out in 0.01M ~~HEPES~~ buffer (Sigma), pH 7.0, at 20°C for 2h. The following inorganic salts were pre-incubated with the bacterial suspensions for 1h prior to the attachment assay which was carried out in the presence of the salts:- $CuCl_2$,M + B (AR) $CaCl_2.2H_2O$,M + B (AR); $MgCl_2.6H_2O$,M + B (AR); $MgSO_4.7H_2O$, Fis ons (AR); $LaCl_3.7H_2O$,BDH (SLR); $Al_2(SO_4)_3K_2SO_4.2H_2O$,M + B (SLR)

Similar experiments were carried out in the presence of EDTA but without the pre-incubation in salt solutions. The salts and EDTA were added at the following concentrations: 40mM; 4mM; 0.4mM; 40μM; 4μM and 0.4μM. The relative number of attached bacteria was determined as previously described using crystal violet dye absorption (section 2.2.4). Attachment profiles were also determined for a number of selected organisms in the presence and absence of $MgCl_2$ and $CaCl_2$ (100μM) using the methods described in Chapter 2.

Table 3.5a) Enzyme treatments and controls

Enzyme treatment	Controls
1. Pretreatment of cells with enzyme before attachment assay	i) No enzyme ii) Enzyme + inhibitor (PMSF) iii) Denatured enzyme
2. Attachment of cells in the presence of enzyme	i) No enzyme ii) Enzyme + inhibitor (PMSF) iii) Denatured enzyme
3. Attachment of cells followed by the enzyme treatment to remove attached cells	i) No enzyme ii) Enzyme + inhibitor (PMSF) iii) Denatured enzyme

3.4.2.2 Exopolymer production and bacterial attachment to hydrophobic and hydrophilic substrata

i) Organisms and culture conditions A number of Pseudomonas and Aeromonas strains isolated from the three isolation substrata were used in this study. Each strain was grown on solid and in liquid media for 48h at 15°C. The solid medium contained autoclaved cellophane overlaying nitrogen-deficient medium (Sutherland and Wilkinson, 1965) in agar plates, and the liquid media was either nitrogen deficient or carbon-deficient, chemically defined medium as previously described (section 3.2.2.1)

ii) Attachment Assays. Attachment assays were carried out on harvested cells from solid and liquid media. The cells were harvested from the cellophane overlay using a glass scraper, followed by resuspension in 0.1M phosphate buffer pH 7.4. Attachment assays of washed and unwashed cells were used to provide a comparison of the attachment in the presence and absence of exopolymer. Relative numbers of attached cells were determined using the crystal violet dye absorption method (section 2.2.4)

iii) Exopolymer analysis The exopolymer was isolated from the supernatants from washed cell samples. Volumes (100ml) of the supernatant were dialysed against distilled water at 4°C using dialysis tubing that had been prewashed with 0.01M EDTA/1% (W/V) Na_2CO_3 solution (Williams, 1974) followed by

washing in distilled water until contaminating carbohydrate material was undetectable by the phenol sulphuric acid method (Dubois et al, 1965). Following dialysis(corrections were made for any volume changes during dialysis) the phenol sulphuric acid method to measure the total hexose content of the exopolysaccharide in the supernatant was applied to 1ml of the dialysate. Also total protein was determined by the method of Bradford,(1976). For supernatants where exopolymer was undetectable or at very low concentration by these assay methods, the remaining supernatant was concentrated in an Amicon ultrafiltration system (Amicon Corporation) (YM10 membrane, Diaflo Ultrafiltration membranes). The ultrafiltration membrane had an exclusion limit of 10 KD and was hydrophilic to prevent adsorption of polymers. Assays for total hexoses and protein were repeated on concentrated samples. In samples where exopolysaccharide was present at concentrations above 500$\mu g ml^{-1}$ total hexose, a gravimetric method was used to measure exopolymer (Williams and Wimpenny, 1977). This polymer was isolated by alcohol precipitation dried under reduced pressure and weighed.

iv) Microscopic examination of exopolymer

Microscopic examination of bacterial exopolymers was carried out in order to determine their association with the bacterial cell as capsules or slimes. India ink, crystal violet and Alcian Blue (McKinney,1952) staining was used to demonstrate exopolymers.

3.4.2.3 Hydrophobic Interaction Chromatographs (HIC)

i) Growth conditions and bacterial suspensions for HIC

Several of the isolates examined for bacterial attachment to polystyrene substrata were also screened by HIC tests for their hydrophobic characteristics. Radiolabelled cell suspensions were prepared using the same procedures outlined in section 2.2.5ii) for the preparation of cell suspensions used in the attachment assays. The cells were radiolabelled with either ^{14}C-leucine or ^{3}H-amino acids mixture (1µci ml^{-1}) for 2h., washed three times and resuspended in 0.01M phosphate buffer pH 7.4 to a final concentration of a 10^{9} bacteria ml^{-1}

ii) HIC Hydrophobic interaction chromatography was carried out using a method similar to that described by Dahlback et al,(1981) and Smyth et al, (1978). Hydrophobic derivatives of sepharose (Phenyl sepharose, Octylsepharose, CL-4B Pharmacia Fine Chemicals A B, Sweden) were washed extensively with phosphate buffer to remove fine particles and preservatives. The gel suspensions were allowed to equilibrate at room temperature 20-24^{o}C, and chromatography was performed at the same temperature. Columns comprised short-ended glass Pasteur pipettes (internal diam. 5mm: length 85mm) plugged with a little glass wool and fitted with a plastic syringe barrel reservoir. Gel beds were packed in the pipettes to a height of 30mm (Ca 0.6ml gel bed volume) by gravity feed and washed with phosphate buffer. Radiolabelled bacterial

suspensions (100µl) were allowed to drain into the gel beds and were eluted with phosphate buffer (5mls, flow rate 1-2ml/min). For qualitative results, turbidometrical readings of eluates were compared to an appropriately diluted portion (100µl) of the original cell suspension. For quantitative results 100ul samples of eluates were counted in a dissolution liquid scintillation cocktail as described above 2.2.5,iii). This was compared after an appropriate dilution correction to counts of the original suspension and the degree of adsorption was expressed as the ratio of counts retained by the column (g) divided by counts in the eluates (e), ie g/e.

3.4.3 RESULTS

3.4.3. i) The effects of proteases, cations and EDTA on bacterial attachment

When pronase P was added to the bacterial suspension at the beginning of the attachment period, attachment to both TC-PS and PS substrata was significantly reduced. (Table 3.5) Controls either with added pronase inhibitor (PMSF) or denatured pronase had consistently less effect. However, both reduced bacterial attachment as compared to ethanol treated controls. Pseudomonas fluorescens H2 was affected at all pronase concentrations tested over the range 0.25-0.01 $mgml^{-1}$. The pronase inhibitor PMSF also reduced attachment of H2, particularly to the PS-substratum but not to the same extent as the enzyme treatment. The attachment of the Acinetobacter sp. was affected by enzyme concentrations above 0.02 $mgml^{-1}$ (pronase). However, this organism was not significantly affected by PMSF, and only combinations of high enzyme concentration and PMSF showed any reduction in attachment.

When pronase was used to pretreat the cells prior to the attachment period, a significant reduction in attachment was observed (Table 3.6). Pretreatment with enzyme inhibitor (PMSF) and enzyme or with denatured enzyme had no effect. When bacteria were first allowed to attach and then treated with pronase, the numbers of bacteria remaining, following

TABLE 3.5

Attachment of Pseudomonas fluorescens at 20°C-24°C in the presence of pronase

Enzyme concentration mg ml^{-1}	Treatment					
	Cells only		Inhibitor(PMSF) Ia[a]		Denatured pronase	
	PS	TC-PS	PS	TC-PS	PS	TC-PS
0.0	1.0[b]	1.0[b]	0.46	1.18	1.0	1.0
0.01	0.11	0.37	0.42	0.83	0.93	0.95
0.1	0.09	0.39	0.40	0.87	0.78	0.9
0.25	0.14	0.34	0.28	0.72	0.73	0.9

Attachment of Acinetobacter sp. at 20°C-24°C in the presence of pronase

Enzyme concentration mg ml^{-1}	Treatment					
	Cells only		Inhibitor(PMSF) Ia^{+}		Denatured pronase	
	PS	TC-PS	PS	TC-PS	PS	TC-PS
0.0	1.0[c]	1.0[c]	1.05	0.91	--	--
0.02	0.83	1.3	1.0	1.19	1.05	1.13
0.2	0.18	0.18	0.60	0.64	0.79	0.84
2	0	0	0.30	0.33	0.56	0.73

a) + $(\sigma n-1) \lesssim 0.125$ $(n=8)$

b) 1 A590 = PS 0.178, TC-PS 0.153

c) 2 A590 = PS 0.382, TC-PS 0.280

TABLE 3.6

Effect of pronase on Pseudomonas fluorescens (H2) after pretreatment with enzyme or postattachment treatment and subsequent removal by washing.

Treatment	Ia	
	PS	TC-PS
Control	1.0	1.0
Pretreatment [a]	0.23	0.38
Pretreatment with [a] (PmSF and enzyme)	0.93	0.98
Pretreatment with [a] (denatured enzyme)	1.0	0.95
Post attachment [b]	0.015	0.11
Post attachment [b] (PMSF and enzyme)	0.36	0.45
Post attachment [b] (denatured enzyme)	0.79	0.84

$(\sigma n-1) \leq 0.100 \quad (n=8)$

a) Pretreatment enzyme concentration was as follows:

$0.1 mg\ ml^{-1}$

b) Post attachment enzyme concentration was as follows:

$0.1\ mg\ ml^{-1}$

subsequent washing, was greatly reduced to both substrata and were the lowest values observed for enzyme treated cells. Denatured pronase had no significant influence on post attachment-treated cells, but PMSF and enzyme treatment did reduce attached cell numbers significantly.

3.4.3.ii) The effects of divalent and trivalent cations and EDTA on bacterial attachment

The attachment of Pseudomonas fluorescens H2 to PS - substrata in the presence of varying concentration of divalent and trivalent cations and EDTA is shown in Table 3.7 for the concentration range 0.4μM-40mM. $CuCl_2$ inhibited attachment at concentrations greater than 40μM. Other divalent and trivalent cations increased attachment with increasing concentration to a maximum depending upon the cation, and this was followed by a decrease in attachment with increase in cation concentration at which maximum attachment values were obtained as follows: $CaCl_2$ 4mM; $MgCl_2$ 40μM; $MgSO_4$ 40μM; $LaCl_3$ 0.4-4mM Al_2KSO_4 40μM. At concentrations greater than the maximum attachment concentration bacterial attachment returned to control levels, except for $LaCl_3$ and Al_2KSO_4 showed inhibition. Al_2KSO_4 caused aggregation of the bacteria at 40mM and a significant increase in attachment (Ia=2.6). EDTA caused total inhibition of attachment at concentrations greater than 0.4μM; however, cell lysis was observed at concentrations 40μM and above.

TABLE 3.7

The effects of divalent and trivalent cations and EDTA on bacterial attachment

Cation/EDTA	Concentration Range of Cations and EDTA — Ia [a]					
	0.4μM	4.0μM	40μM	0.4mM	4 mM	40mM
$CuCl_2$	0.97	0.70	0.99	0.27	0.21	0.13
$CaCl_2$	0.96	1.04	1.12	1.25	1.34	1.10
$MgCl_2$	1.04	1.18	1.42	0.98	0.99	0.94
$MgSO_4$	1.02	1.46	1.84	1.18	1.02	0.93
$LaCl_3$	1.00	1.21	1.70	2.22	2.20	1.77
Al_2KSO_4	1.05	1.20	1.46	0.65	0.05	2.6 [b]
EDTA	0.84	0.03	0.02 [c]	0.00 [c]	0.00 [c]	0.00 [c]

a) $(\sigma n-1) \lesssim (0.1)$ (n=16)

b) Cell aggregation observed during attachment assay

c) Cell lysis observed

The attachment profiles of a number of selected organisms and a range of substrata are shown in figures (3.6-3.11) illustrating γSV versus attachment numbers. In every case, the curve for bacterial attachment in the presence of $MgCl_2$ and $CaCl_2$ (100μM) showed an increased level of attachment as compared with the curve for attachment in the absence of these salts. However, the relationship between surface wettability and attachment numbers was maintained as was the position of the attachment maxima.

3.4.3.iii) Exopolymer production and bacterial attachment to hydrophobic and hydrophilic substrata

Cultures (from solid and liquid media) of Pseudomonas and Aeromonas were assayed for attachment to PS and TC-PS and the results for solid and liquid culture are shown in Table 3.8. From this attachment data the pseudomonads could be grouped into three types. i) organisms that attach well from liquid or solid media ie. H2; H15 and H8602, ii) organisms that attach well from liquid culture but poorly from solid media even when washed to remove slime exopolymer, ie. H35 and H40 and iii) an organism that attached poorly from solid and liquid culture ie HP. These findings show some correlation with the analysis of exopolymers released into the growth media or resuspension buffer (Table 3.9). Organisms with reduced attachment had comparatively high values of exopolymer when grown on either solid media (H35, H40) or liquid and solid media (HP) (total hexose content). The

Figures 3.6-3.11

Relationship between the number of attached bacteria and surface free energy in the presence of $MgCl_2$ and $Ca.Cl_2$ (100 μM) (●) compared to attachment in the absence of these salts (○). The following organisms were examined:-

Figure	Organism	Strain No.
3.6	Pseudomonas fluorescens	H2
3.7	Aeromonas hydrophila	H22
3.8	Chromobacterium sp.	H31
3.9	Aeromonas hydrophila	H6
3.10	Pseudomonas fluorescens	H15
3.11	Acinetobacter sp.	H3

The surface free energy of each substratum was calculated from contact angle data using the computer program published by Neumann et al, (1974). Error bars represent 95% confidence intervals.

Fig 3.6

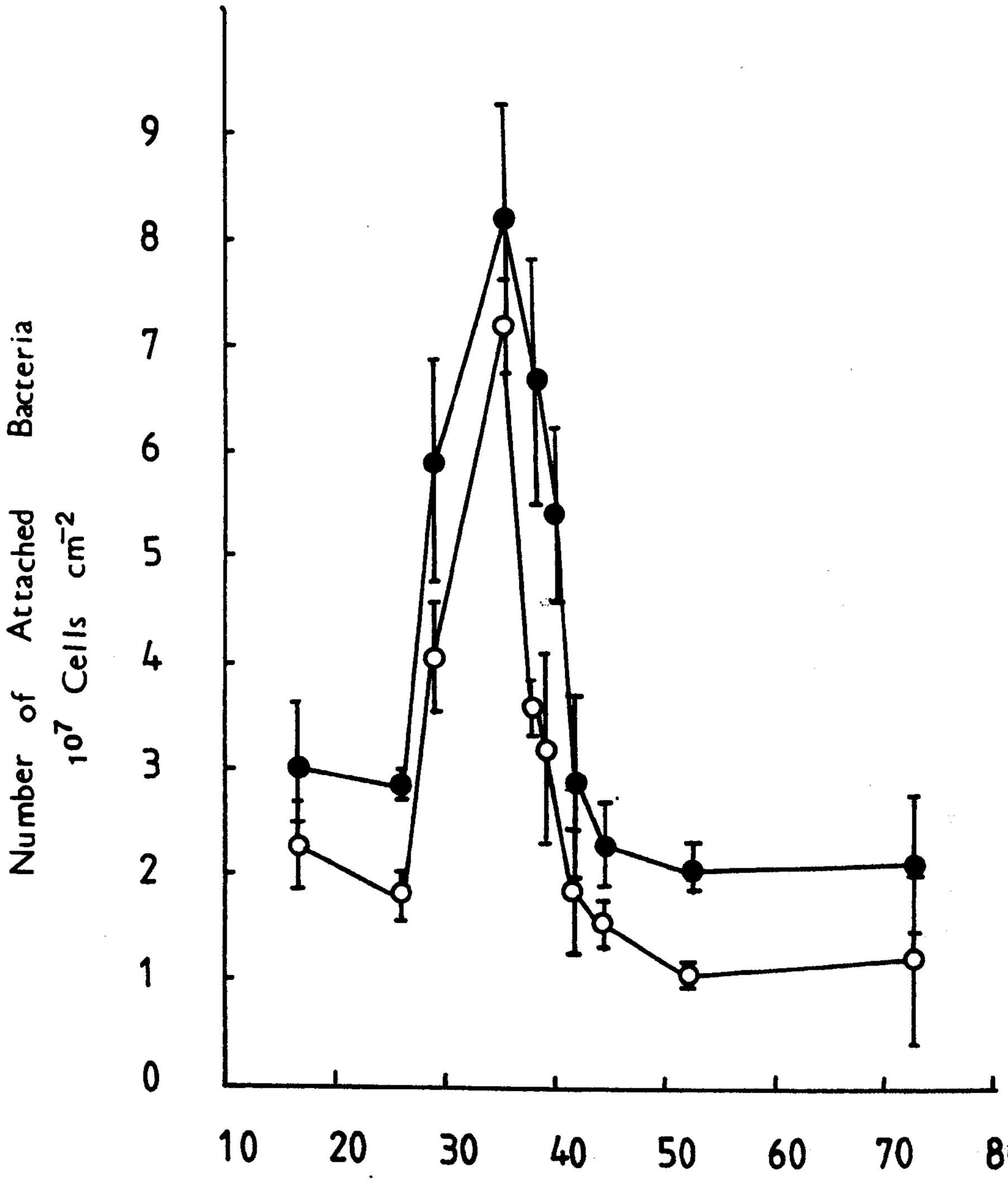

Number of Attached Bacteria
10^7 Cells cm^{-2}
0
1
2
3
4
5
6
7
8
9
10
20
30
40
50
60
70
80
Surface Free Energy m Jm^{-2}

Fig 3.7

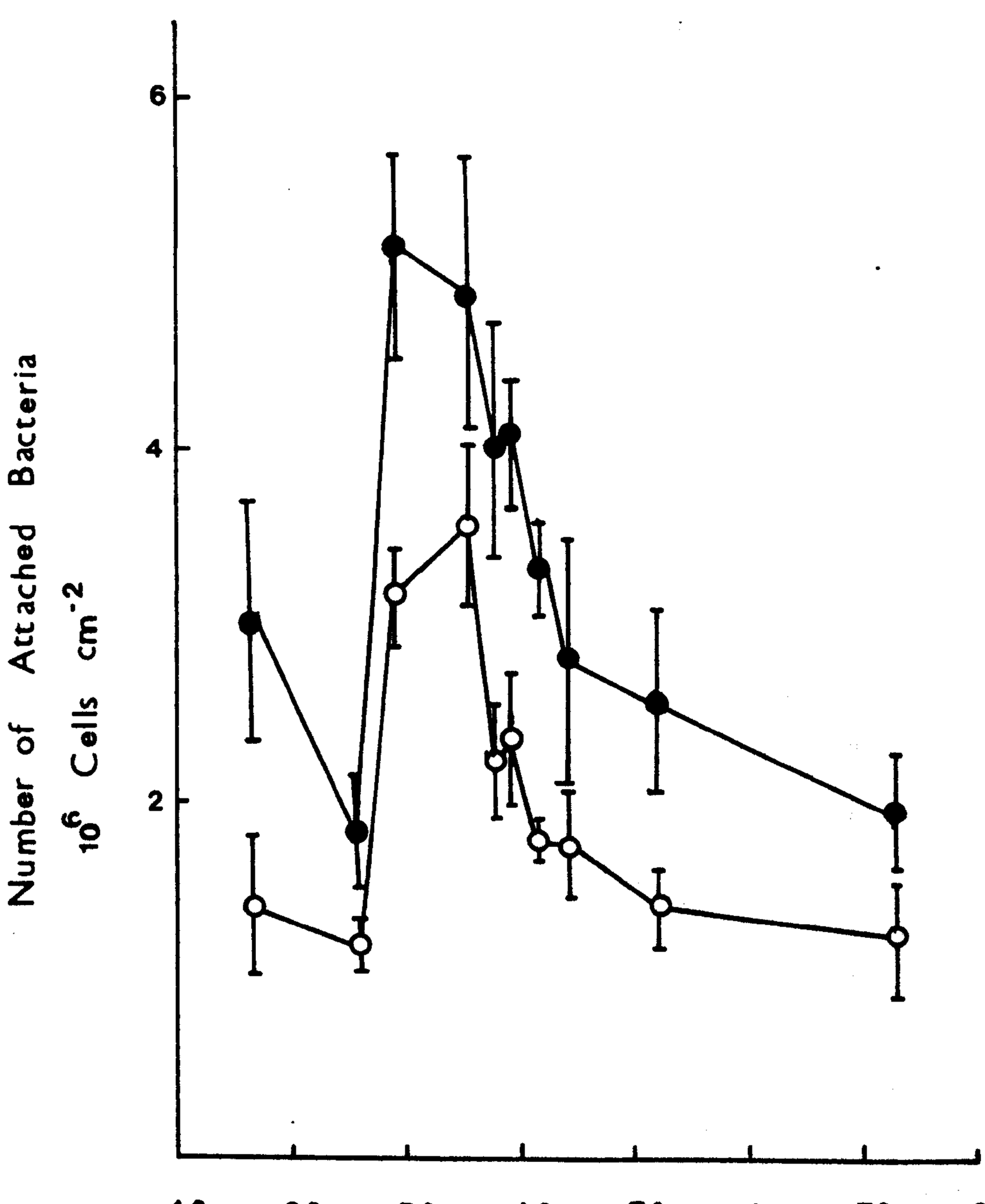

Number of Attached Bacteria
10^6 Cells cm^{-2}
2
4
6
10
20
30
40
50
60
70
80
Surface Free Energy mJm^{-2}

Fig 3.8

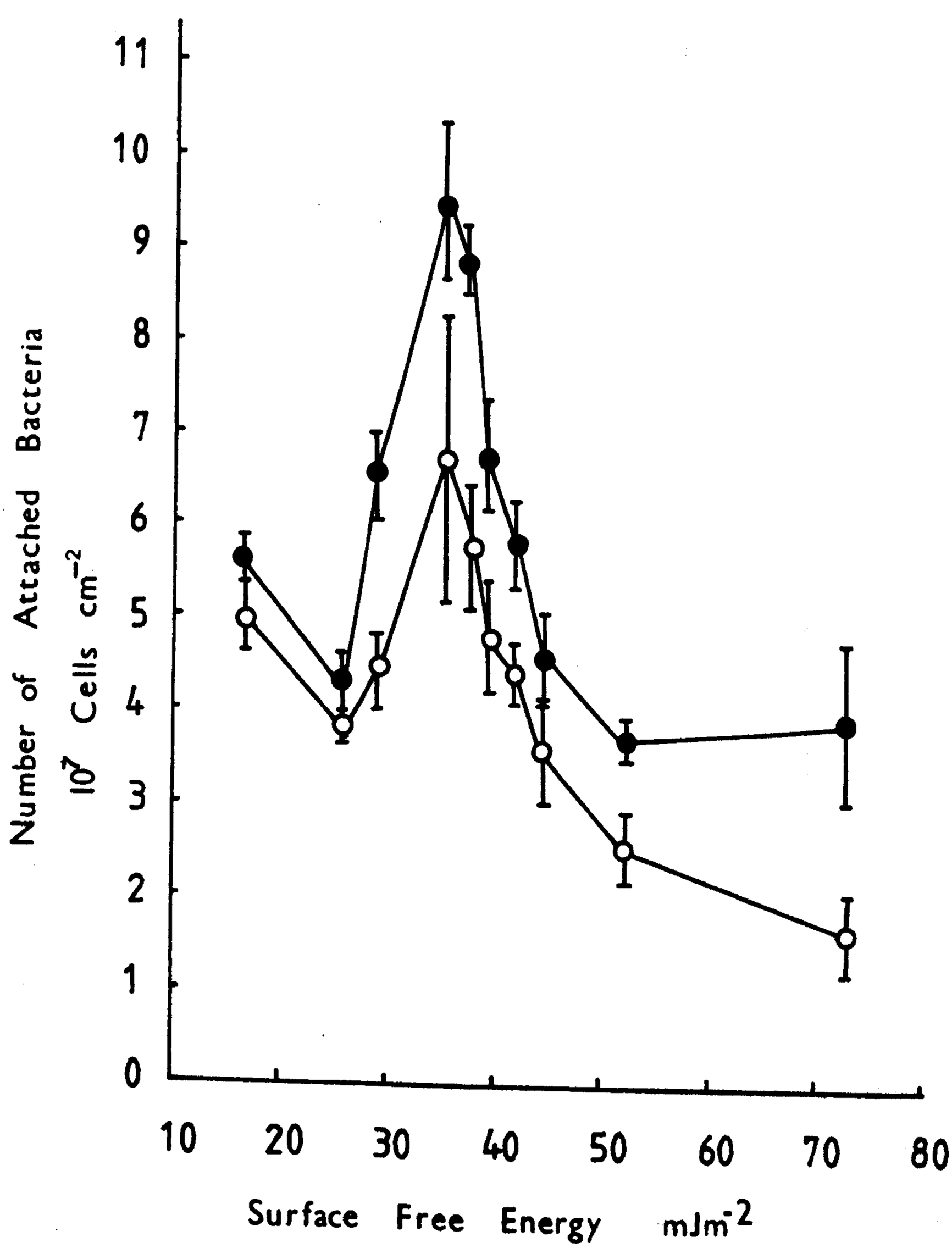

Number of Attached Bacteria
10^7 Cells cm^{-2}
0
1
2
3
4
5
6
7
8
9
10
11
10
20
30
40
50
60
70
80
Surface Free Energy mJm^{-2}

Fig 3.9

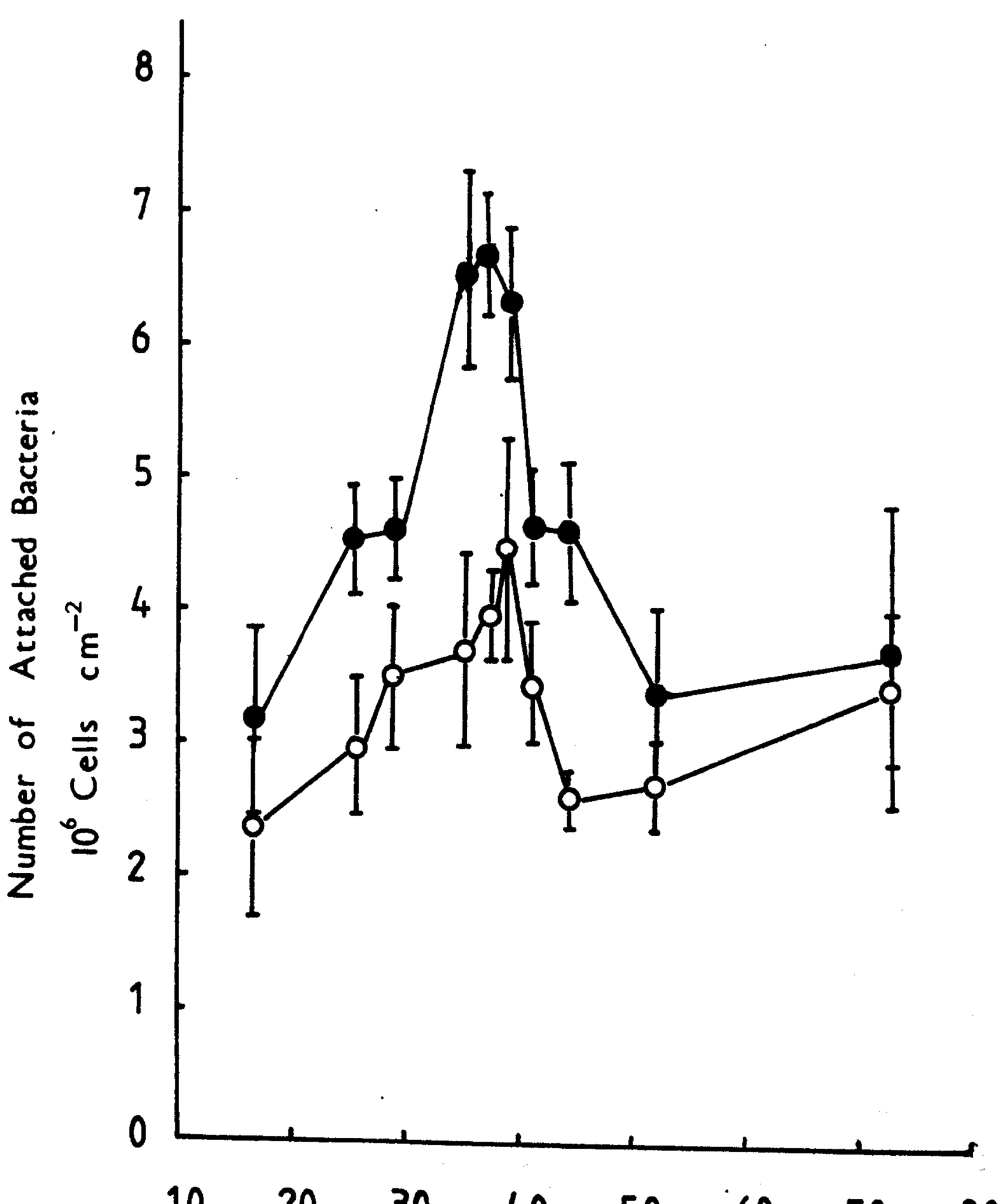

Number of Attached Bacteria
10^6 Cells cm^{-2}
0
1
2
3
4
5
6
7
8
10
20
30
40
50
60
70
80
Surface Free Energy mJm^{-2}

Fig 3.10

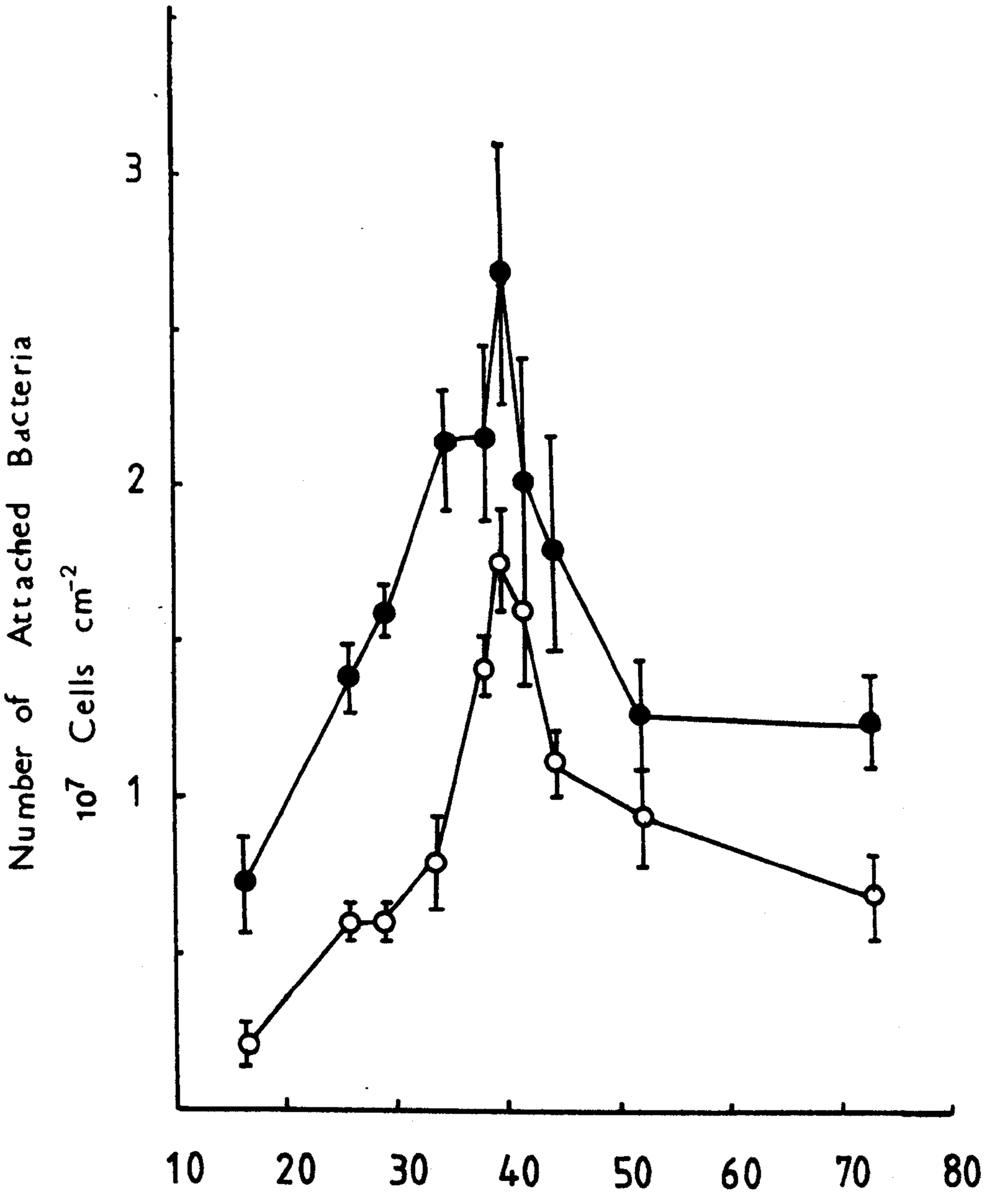

Number of Attached Bacteria
10^7 Cells cm^{-2}
3
2
1
10
20
30
40
50
60
70
80
Surface Free Energy mJm^{-2}

Fig 3.11

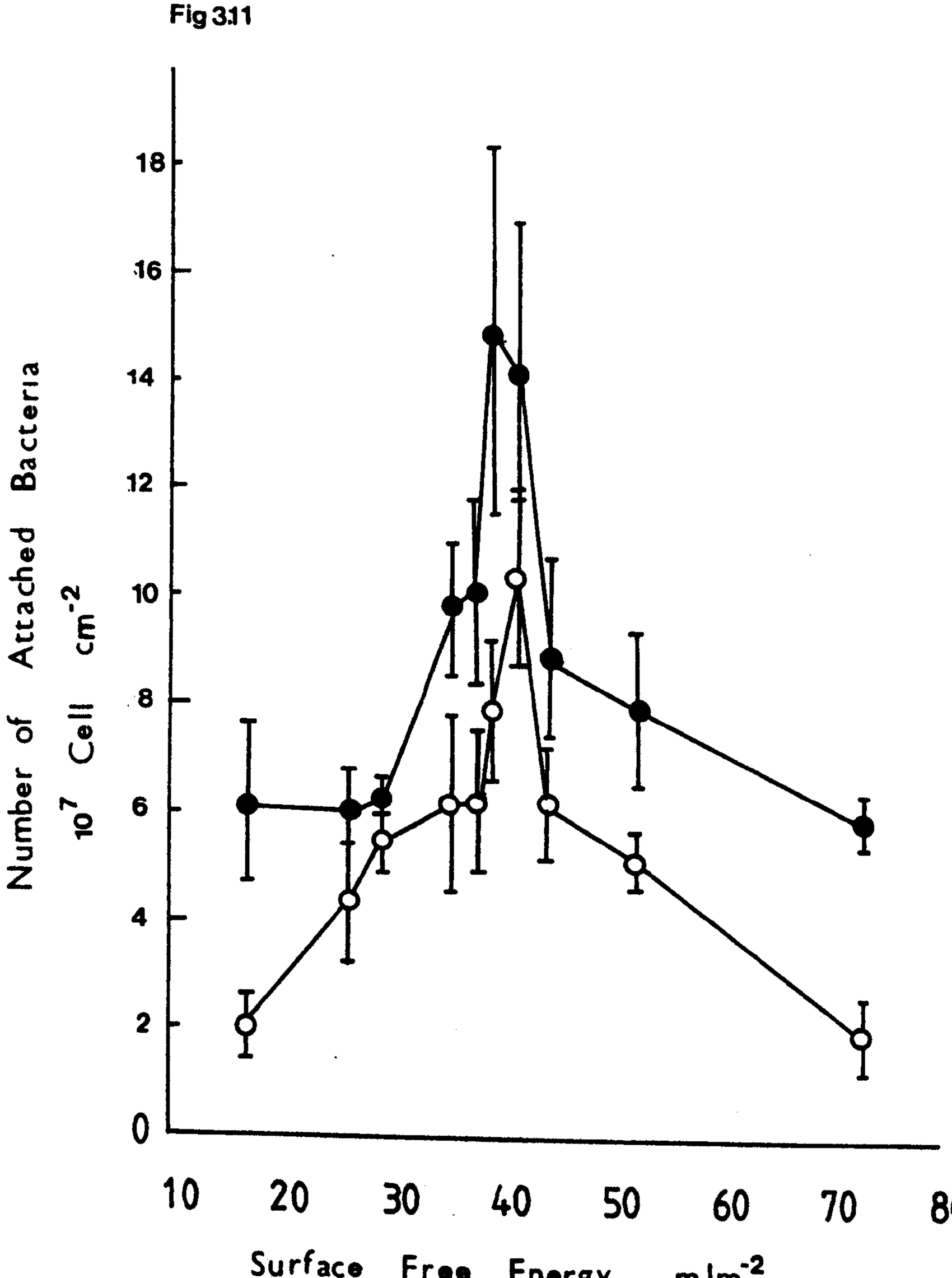

Number of Attached Bacteria
10^7 Cell cm^{-2}
0
2
4
6
8
10
12
14
16
18
10
20
30
40
50
60
70
80
Surface Free Energy mJm^{-2}

TABLE 3.8

Attachment of Pseudomonas and Aeromonas strains from solid and liquid culture in the presence and absence of culture exopolymer.

Organism	Substratum	Attachment ($A590x10^{-3}$)					
		Solid Medium		Nitrogen deficient liquid medium		Carbon deficient liquid medium	
		Buffer[a]	Sup[b]	Buffer	Sup	Buffer	Sup
Pseudomonas							
H2	PS	397	39	625	266	233	84
	TC-PS	276	43	208	265	133	76
H15	PS	236	23	303	183	163	62
	TC-PS	173	37	213	185	124	53
H8602	PS	424	33	558	298	278	74
	TC-PS	301	53	270	301	149	78
H35	PS	39	58	432	267	189	78
	TC-PS	23	76	277	269	123	62
H40	PS	13	37	414	207	279	87
	TC-PS	19	38	200	231	89	32
HP	PS	4	4	36	10	19	10
	TC-PS	3	7	20	12	5	7
Aeromonas							
H22	PS	85	21	19	18	94	12
	TC-PS	43	24	4	26	40	4
H28	PS	22	11	129	23	31	17
	TC-PS	8	3	4	21	6	4
H19	PS	23	13	235	103	28	12
	TC-PS	7	15	105	108	11	3
H37	PS	69	16	123	43	75	18
	TC-PS	32	17	97	52	28	22

a) Attachment of resuspended cells in phosphate buffer

b) Attachment of resuspended cells in culture supernatant

TABLE 3.9

Analysis of Pseudomonas and Aeromonas strains exopolymer from solid and liquid cultures (Total hexoses (TH) or protein (TP) of dialysed exopolymers)

Organism	Analysis	Exopolymer (mg ml^{-1})		
		Solid media	Nitrogen deficient liquid media	Carbon deficient liquid media
Pseudomonas				
H2	TH	0.103	0.014	0.018
	TP	0.182	0.069	0.082
H15	TH	0.052	0.019	0.012
	TP	0.172	0.033	0.042
H8602	TH	0.043	0.019	0.007
	TP	0.166	0.073	0.027
H35	TH	1.277	0.039	0.045
	TP	0.183	0.094	0.088
H40	TH	0.884	0.041	0.053
	TP	0.052	0.039	0.043
HP	TH	0.649	0.564	0.437
	TP	0.173	0.143	0.129
Aeromonas				
H22	TH	0.072	0.023	0.012
	TP	0.183	0.043	0.032
H28	TH	0.088	0.029	0.015
	TP	0.199	0.076	0.072
H19	TH	0.075	0.073	0.016
	TP	0.155	0.099	0.051
H37	TH	0.063	0.029	0.024
	TP	0.142	0.092	0.083

amount of protein in the exopolymer was higher with organisms grown on the solid media than in the nitrogen-or carbon-deficient liquid media(table 3.9.)

All four Aeromonas strains showed lower attachment levels than the Pseudomonas strains with the exception of the pseudomonad (HP). In general increased levels of attachment were found for the nitrogen-deficient culture, except for H22 which showed greater attachment when from the carbon-deficient media. No correlation between exopolymer and attachment level could be made for the Aeromonas strains. However, the presence of exopolymer in the attachment media reduced attachment for most strains examined,Table 3.8 columns (supernatant). Figure 3.12 shows exopolysaccharides stained with alcian blue for the organisms found to produce slime polysaccharide. Although polysaccharide was detected in the supernatant of the resuspension buffer for solid media-grown cultures, a polysaccharide coating was also seen following washing indicating that some polysaccharide was associated with the outer membrane and formed a capsule layer.

3.4.3.iv) Hydrophobic interaction chromatography of bacterial isolates previously examined for attachment to polystyrene substrata

Suspensions of the strains were chromatographed on phenyl and octyl sepharose. Twenty organisms were screened quantitatively to observe the general relationship between hydrophobic

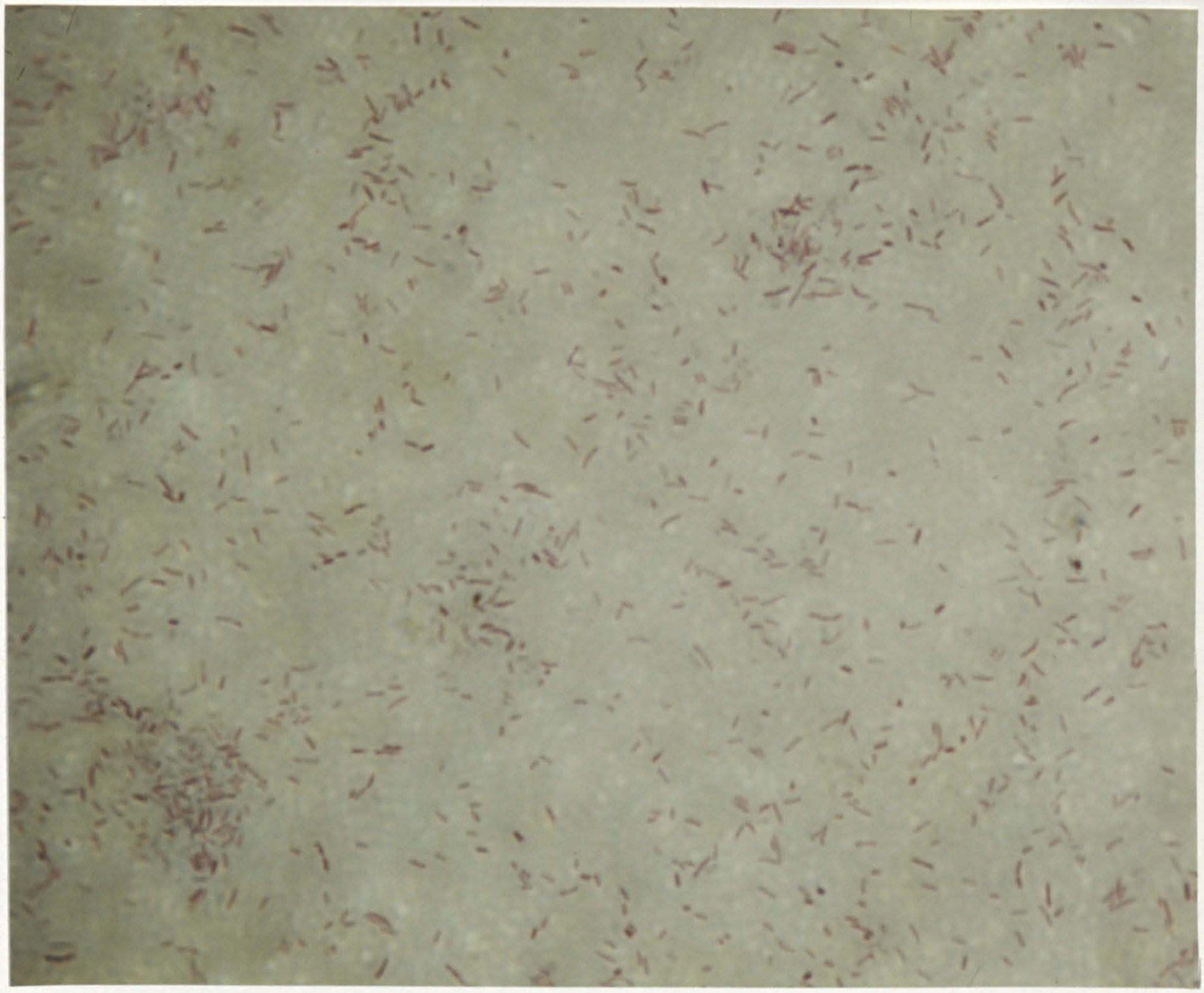
Figure 3.12

Micrographs of Pseudomonas fluorescens H2 and Pseudomonas putida HP grown on Kings B medium at 15°C for 72hours. Micrographs were stained with Alcian Blue for exopolysaccharide and counterstained with Ziehl Neelsen carbol Fuchsin:

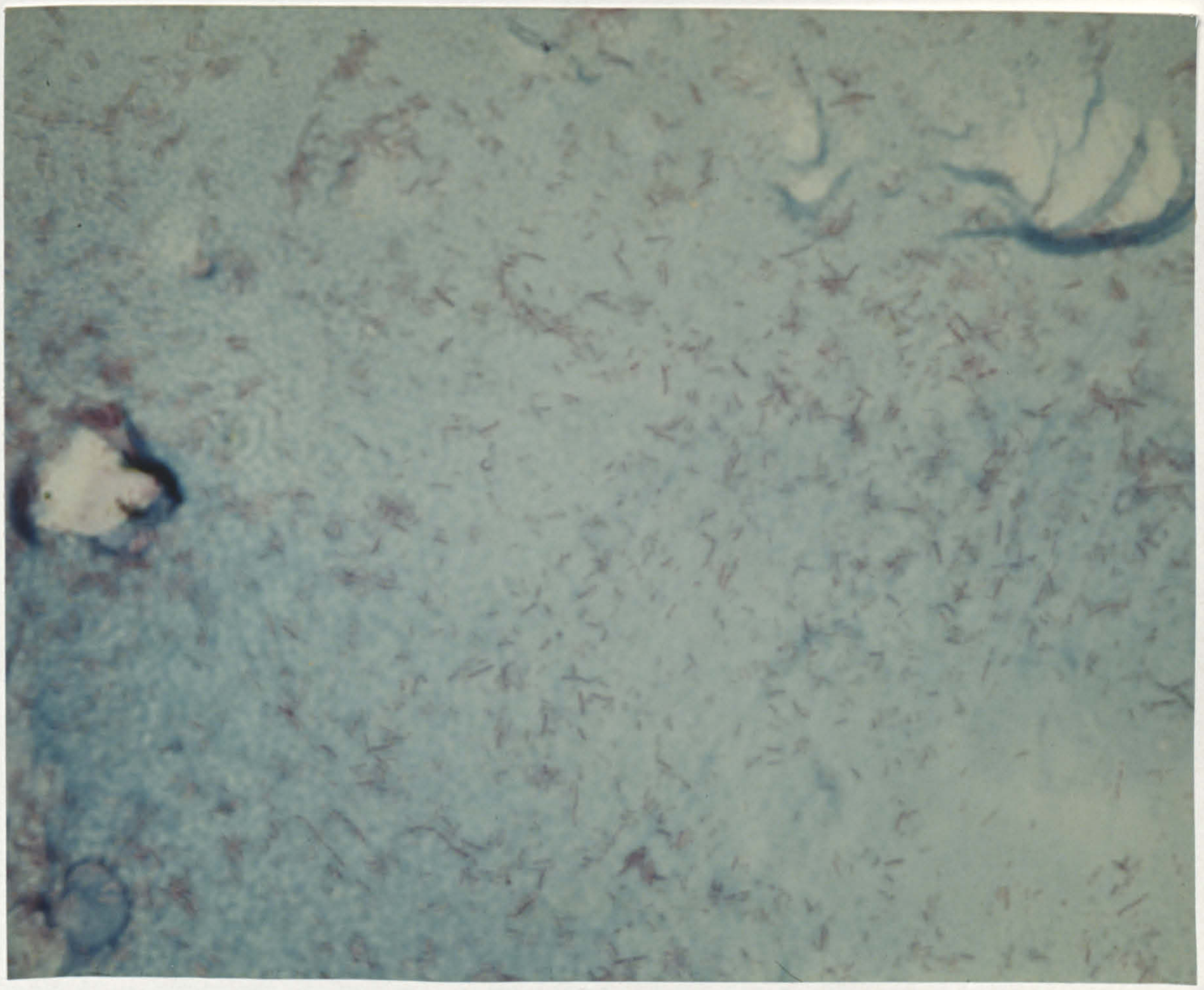
a) Pseudomonas fluorescens H2 showing little exopolysaccharide;

b) Pseudomonas putida HP showing extensive slime polysaccharide staining blue.

a)



b)



interaction chromatography of cells and their attachment to hydrophobic surfaces (Table 3.10). For a more quantitative analysis, radiolabelled cells were chromatographed and the degree of hydrophobicity is expressed as the ratio g/e, representing the radio activity counts for labelled bacteria retained in the hydrophobic gel (g) and those in the eluate (e). The higher the value of g/e, the larger the proportion of cells retained in the gel and thus the greater the tendency to enter into hydrophobic interactions. A value of log g/e of <0 is taken as hydrophilicity of cells (Dahlbeck et al, 1981). Figure 3.13 shows a linear relationship between log g/e versus log attachment for ten organisms. Quantitative values for g/e were determined using radio-labelled cells and attachment to polystyrene was determined using crystal violet staining and absorbance reading at 590nm. Initially, the HIC tests were run on both Octyl and Phenyl gels; however, as similar results were observed for both gels, only the Octyl-sepharose gel was used for this quantitative study.

TABLE 3.10

Hydrophobic Interaction Chromatography of 20 strains previously examined for attachment to polystyrene surfaces

Organism	Attachment to PS[b] A590 ($X10^{-3}$)	A540 ($X10^{-3}$)[a] Phenyl Sepharose	Octyl Separose
H2	364	2	3
H40	332	4	5
H15	337	7	3
H35	329	4	2
H41	332	18	5
H8602	304	9	12
H21	242	33	22
H31	228	39	17
H38	209	72	82
H48	182	68	55
H45	143	18	24
H47	129	69	97
H3	107	93	84
H6	92	114	112
H1	43	112	173
H22	4	213	214
H16	3	293	284
H49	0	243	265
H24	0	229	237

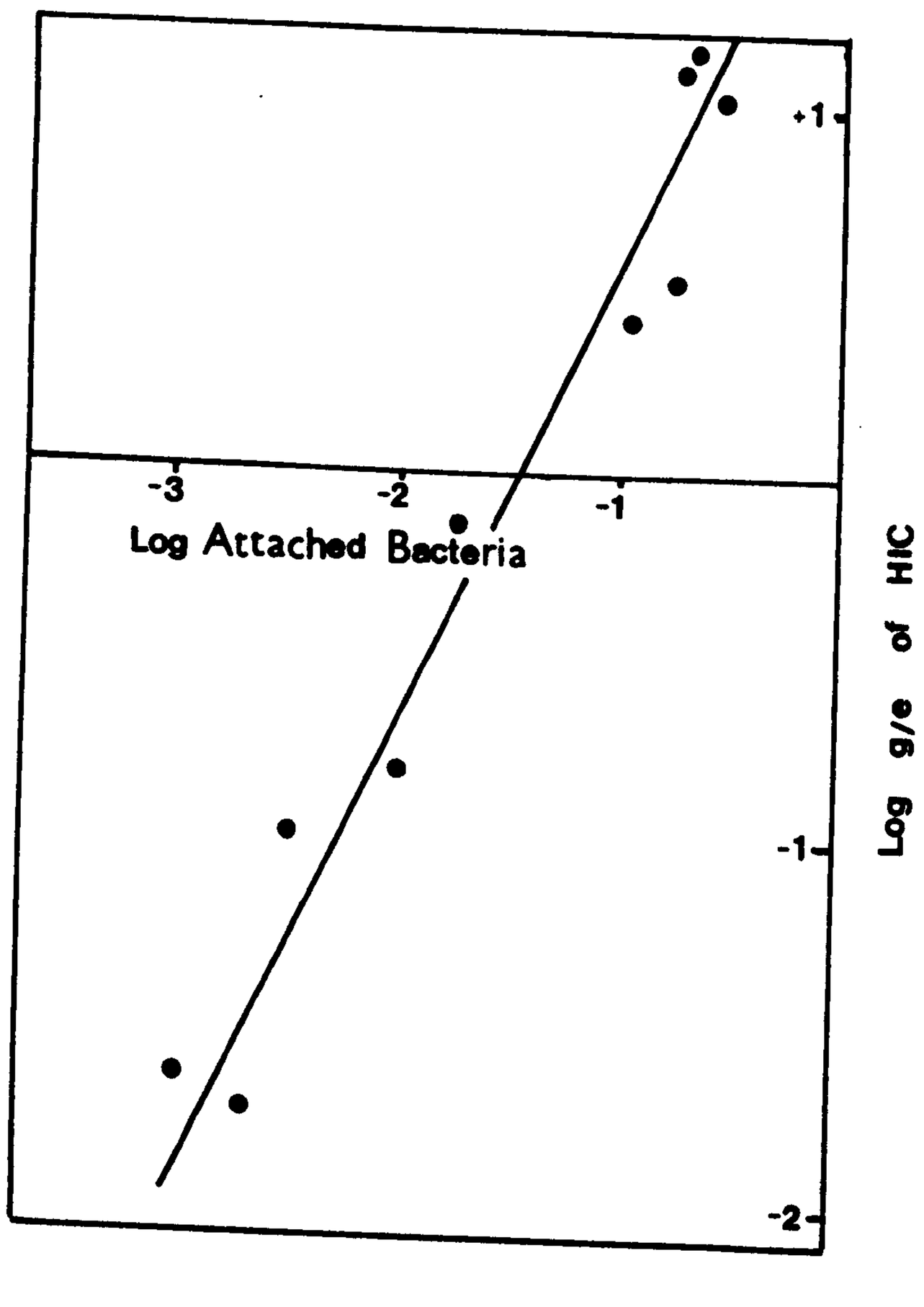
a) 100ul of original cell suspension was adjusted to give a final A540($X10^{-3}$) of 300 ($\pm$10) when diluted in the appropriate elution volume (5mls), A control column containing sepharose Cl-4B was used for each strain examined [A540($X10^{-3}$) of elution buffer was 244 ($\pm$23)c]

b) $\sigma(n-1) \leq 20$, (n=8) c) $\sigma(n-1) \leq 23$ (n=20)

Figure 3.13.

Relationship between log g/e for hydrophobic interaction chromatography and log number of attached bacteria to PS substrata for a range of organisms. Quantitative values for g/e were determined using radiolabelled cells and attachment to polystyrene was determined using crystal violet staining and absorbance reading at 590nm.

Fig. 3.13



3.5 DISCUSSION

The influence of bacterial surface characteristics was first investigated by chemical treatments which were designed to chemically modify the cell surface components. Pronase removed cells attached to both TC-PS and PS petri dishes, and pretreatment with the enzyme also inhibited adhesion. Similar results have been reported for attached marine pseudomonads (Danielsson et al,1977; Fletcher and Marshall, 1982), although Fletcher, 1980 reported that pronase, trypsin and several other enzymes did not remove Pseudomonas sp. NCMB 2021 when it was attached to glass. This work indicated that either extrinsic proteins or exocellular proteins of cells are involved in the adhesion process. Previously bacterial adhesive polymers have usually been considered to be polysaccharide (Costerton et al, 1977; Costerton,1981), and polysaccharide components have been demonstrated by electron microscopy of cells that were part of a bacterial film (Costerton et al, 1977; Fletcher and Floodgate, 1973). However, this indirect evidence may only be true for high energy surfaces, as low energy surfaces show spontaneous adhesion where little time dependence and hence polymer production would be needed for permanent attachment (Fletcher and Marshall,1982). This suggests that the bacterial surface/liquid interface is of major importance to adhesion to hydrophobic substrata. (Fletcher and Floodgate, 1973). Treatment of cells with $NaIO_4$(0.1M) also influenced

attachment to these surfaces as previously reported (Fletcher, 1980) suggesting the cell surface polysaccharide integrity is also important.

The influence of divalent and trivalent cations also supports the view that membranes or exopolymer structure is of importance, as both divalent and trivalent cations increase attachment to PS substrata at concentrations normally found in freshwater aqueous environments (Table 3.7). Higher concentrations caused either inhibition of attachment, which was correlated with bacterial aggregation or with a return to control values which had been determined for cells in cation-deficient buffer (<0.4 μM). At lower concentrations of cations, produced when EDTA was added, almost total inhibition of attachment was recorded. However with the Pseudomonas fluorescens strain H2, cell lysis was achieved at concentrations 4μM EDTA, and is consistent with EDTA induced lysis of other Pseudomonas sp. when treated with sequestering agents. (Shively and Hartsell, 1964; Wilkinson, 1967).

When a number of Pseudomonas and Aeromonas strains were examined for bacterial attachment and the possible associated production of exopolymers, only three of the ten isolates tested produced significant levels of exopolymer above the concentration normally attributed to polysaccharides, membrane proteins and lysed cell products found at low concentration in culture supernatants of Gram-negative bacteria (Rothfield and Pearlman-Kotheniz, 1969). The

production of exopolysaccharide by these strains was correlated with low attachment, and attachment even increased when the bacteria were washed in order to remove any slime layers not directly associated with the cell surface. Aeromonas strains did not produce any exopolymers in significant amounts, yet adhesion of a number of strains was low to both surfaces. This would indicate that outer cell surface composition, as well as exopolymer production, influences the initial adhesion process. Further studies of these polymers and of outer membrane polymers and other membrane components was therefore proposed to investigate the biochemical significance of this result.

One surface characteristic that was found to correlate with adhesion to the PS surface was the HIC properties , ie. increase in cell hydrophobicity (Table 3.10), of the bacterial isolates. This result confirms that HIC can be used to assess surface characteristics related to the adhesion to hydrophobic substrata. It also indicates that bacteria that collect and interact in the surface microlayers at the liquid-air interface, previously shown to have low g/e values (Dahlback et al, 1980) may adhere to the air-liquid interface in a manner similar to their adhesion to solid substrata.

The results so far presented support a model of adhesion from freshwater bacteria through a thermodynamically favourable and spontaneous process producing an irreversible contact through passive mechanism. The role of water in this

interaction is clearly important as hydrated macromolecular conditioning films exclude contact between clean substrata surfaces and bacterial surfaces via stabilisation.

However, this exclusion principle may also be utilised by bacterial cells in situations where the free-swimming state is an advantage. In order to prevent this strong interaction with available substrata cells may produce extensive capsules and slime layers. Alternatively, in the case of organisms that cannot produce such exopolymers, alterations in their outer membrane LPS sufficient to prevent adhesion and to reduce hydrophobic interactions may be maintained. Freshwater bacteria may then utilise an alternative active mechanism requiring polymer excretion when adhering to hydrophilic or conditioned surfaces. This active process is probably involved in a second stage of adhesion where cell-cell contact increases the strength of adhesion of attached cells in the developing film. Therefore, this work has provided a background for further investigations of the exopolymers and outer membranes surfaces of a number of freshwater bacterial isolates in order to determine their role in adhesion. The following sections describe a study designed to answer the question: to what extent can pheno-typic and genotypic changes in the production of these polymers influence adhesion?

3.6 CONCLUSIONS

3.6.1 The influence of adsorbed water and conditioning films at the substratum surface on bacterial attachment

i) The adhesion of two bacterial isolates to hydrogel polymers showed a reduction in attachment numbers with increasing water content. The surfaces were not found to be "biocompatible" as proposed by Andrade, 1973, but significantly limited bacterial attachment over the initial colonisation period compared with PS substrata and charged PS substrata. As these hydrogel polymers have a net neutral surface charge, this inhibition of adhesion can be attributed to the adsorbed water layers and the reduction in interfacial free energy.

ii) Conditioning molecules effects upon attachment were dependent upon the nature of the conditioning macromolecule and whether attachment took place in the presence of the polymers or after their adsorption to the substrata

iii) Proteins and Glycoproteins were inhibitory through adsorption at the substratum interface

iv) The polysaccharide containing macromolecules differed, in that LPS adsorbed to surfaces and inhibited attachment while the dextran adsorption was ineffective in inhibiting bacterial attachment. Dextrans, however, showed stabilising effects when added to the bacterial suspensions prior to the attachment assay

v) Natural water samples were found not to influence attachment by an adsorbed conditioning film. However culture supernatant containing macromolecules released from the cells had inhibitory effects either by adsorption or stabilisation.

vi) These conditioning experiments confirm that conditioning films protect "clean" surfaces from bacterial colonisation and support the hypothesis that initial bacterial colonisation requires spontaneous bacterial attachment prior and during surface conditioning. The resulting bacterial succession at the substrata will be due to changes in this original population.

3.6.2 <u>The influence of liquid surface tension γ_{LV} on bacterial attachment</u>

i) The effects of changes in γ_{LV} of the attachment media on bacterial attachment were dependent upon the organic agent used to change γ_{LV}.

ii) Both ethanol and Urea increase attachment numbers at low concentrations (0.17M ethanol, 0.5M Urea) yet their effects could also be attributable to changes in cell structure through changes in cell physiology (recorded as an increase in respiration rate).

iii) DMSO did not produce this effect and was used to measure γ_{BV} by applying the equation of state model. (Section 1.2.4) and the assumption that $\gamma_{BV} = \gamma_{LV}$ when attachment levels do not change over a range of γ_{SV}. Values for γ_{BV} were 66.4mJm^{-2} and 63.6mJm^{-2} for carbon and nitrogen-limited

cultures respectively, and this is consistent with other methods of evaluating γ_{BV}.

3.6.3 Studies on the role of the bacterial surface on the attachment of freshwater bacteria to solid surface

i) The effects of proteases on attached bacterial cells support the involvement of proteins in the adhesion to hydrophobic substrata (However, proteases may also disrupt membrane integrity)

ii) Increased levels of attachment were observed in the presence of divalent and trivalent cations at concentrations normally found in freshwater aqueous environments. These concentrations increased attachment to a wide γ_{SV} range of substrata.

iii) Exopolymer production by three of the ten isolates tested was accompanied by low attachment levels when compared to washed cells free of polymer. The rest of the strains showed no significant exopolymer production yet varied in attachment abilities. This supports the view that differences in bacterial outer membrane composition influence subsequent attachment

iii) The HIC of a number of freshwater isolates correlated with attachment to PS substrata and can be used to assess surfaces characteristics related to the adhesion of bacteria to hydrophobic substrata.

CHAPTER 4

The influence of phenotypic and genotypic changes in the bacterial cell surface on the attachment of Pseudomonas isolates to test substrata

4.1 Introduction

A detailed study was made of the physiological and bio-chemical conditions which effect the attachment of Pseudomonas isolates in order to determine the mechanism of this irreversible and non-specific adhesion process. To do this the approach which was taken was to assess how phenotypic or genotypic changes in the cell surface structure affect the attachment of these organisms in vitro. This entailed a correlation of changes in the outer cell membrane composition after growth under defined conditions, with accompanying changes in the attachment of the organism, as well as the assessment of the role played by different surface components through the identification and analysis of adhesion mutants.

Four conditions which influence bacterial attachment were initially examined including: time of incubation, with substrata, ie. time allowed for attachment, incubation temperature, media pH and ionic strength. This was followed by an investigation of the effects of growth condition on attachment of Pseudomonas fluorescens H2, prior to its attachment in batch and continuous culture media carbon-

and nitrogen-limitation, this included an analysis of membrane protein and carbohydrate content.

The selection and enrichment of attachment mutants was achieved by incorporation of the "adsubble" process into a continuous culture fermenter or chemostat. The term "adsubble processer" is a contraction of "adsorptive bubble separation processes" and is defined as 'those phenomena and techniques in which dissolved or suspended material is segregated within or removed from a liquid by adsorption or attachment at the surface of rising bubbles' (Lemlich, 1966, 1972; Karger, 1967). For colloidal materials such as a bacterial suspension, if these bubbles form an overflowing or otherwise removable foam facilitating the separation of the material, the process is called foam fractionation and is shown schematically in figure 4.1. Figure 4.2 shows how this principle was incorporated into the continuous culture system. A conventional fermenter design was used, however, by maintaining the liquid level using gas entrainment, an overhead overflow was found to selectively remove foam from the surface of the culture. Under these continuous culture conditions in Carbon or Nitrogen-limited minimal media, the wild-type was found to concentrate in the foam layer and was removed from the fermenter.

The selection pressure that results from this process favoured the enrichment of two major classes of mutants both having cell surface characteristics fundamentally different from the original organism. These were identified by

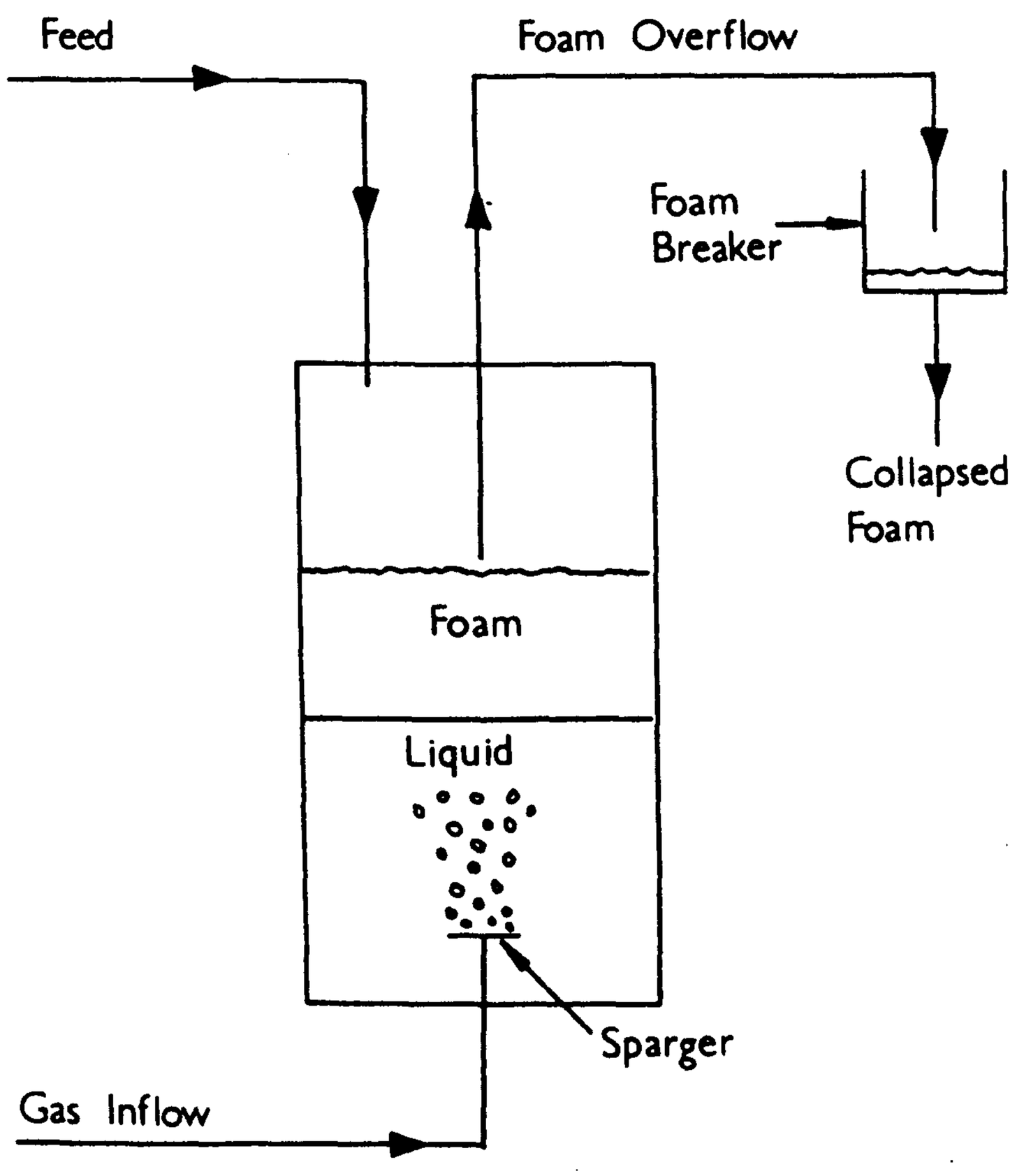


Fig.4.1. A schematic flow diagram for foam fractionation.

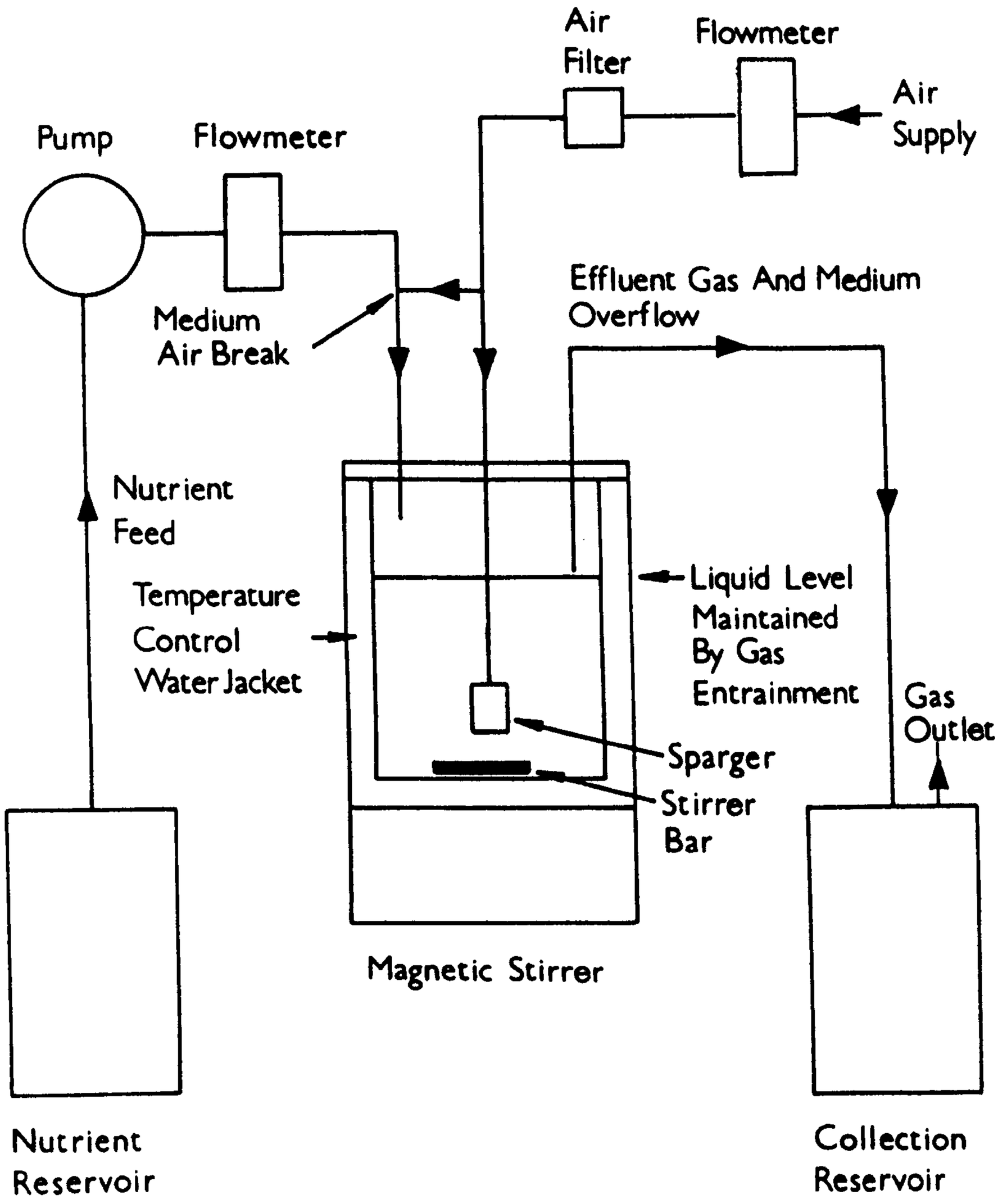


Fig.42. The continuous culture equipment incorporating a foam fractionation system.

stable changes in colony morphology when plated on to either minimal or complex solid media. A number of similar mutants of other Pseudomonas strains were isolated by foam fractionation, or they were identified by colony morphology changes from routine colony platings. This investigation therefore examined these changes in cell surface characteristics in relation to the cell's attachment ability. Extrinsic membrane proteins, lipopolysaccharides (LPS) and exopolysaccharides (EP) of the wild-type and mutants were analysed, following growth of each strain in batch culture. The extrinsic membrane proteins were analysed by radioamidation and radio iodination followed by SDS-PAGE and autoradiography, and component sugar analysis was carried out by GLC of suitable derivatives of the hydrolysate of LPS and EP extracted from each strain. A more detailed examination of the adsorption isotherms of LPS and EP isolated from batch cultures of P. fluorescens H2 wild-type and adhesion mutants was made in order to assess the adsorption characteristics of the surface polymers involved in relation to the attachment of each strain to hydrophobic and hydrophilic polystyrene substrata. Finally, electronmicrographs of these organisms were also prepared in order to determine whether surface appendages such as pili or fimbriae were present.

4.2 METHODS

4.2.1 Identification of test organisms

A number of Pseudomonas isolates were further identified using the identification keys of Hendrie and Shewan, (1979) and King and Phillips, (1978). The tests included: Oxidase (Kovacs, 1956) incubation temperatures of 5°C (up to 3 weeks) and 41°C (in nutrient broth incubated in a water bath)(Haynes and Rhodes, 1962), extracellular hydrolases (gelatin, starch, casein, DNA); single carbon source utilisation performed with simple basal medium of Palleroni and Doudoroff (1972), and including ethanol, glucose, maltose, mannitol; pigment production on Kings A and B medium (King et al, 1954) and antibiotic sensitivity tests determined by a disk (oxoid) method, nutrient agar plates surface-seeded from young (2-3 day) broth culture using the following agents: ampicillin (disk content 25µg), carbenicillin (100 µg), cephaloridine (25µg), chloramphenicol (10µg), colistin (10µg), erythomycin (10µg), gentamicin (10µg), novobiocin (5µg), penicillin (5units), polymyxin B (300 units), streptomycin (10µg), sulphafurazole (500µg) and tetracycline (50µg).

4.2.2 Culture conditions

Pseudomonas isolates and adhesion mutants were cultured in a chemically defined medium as described in section 3.3.2.i. The carbon source was glucose (0.1% W/V) unless otherwise stated and the medium was adjusted to pH 7.0 with 1M NaOH.

i) Batch Cultures were grown in 250ml conical flasks at

15°C in an orbital shaken incubator (150 rev min^{-1}). Growth was followed turbidometrically and cells were harvested in late exponential phase by centrifugation.

ii) <u>Continuous cultures incorporating foam fractionation</u> were grown in a 1 l (Quick-fit, Fisons) chemostat vessel with a working volume of 500ml. This was maintained at 15°C using a temperature controlled water-jacket with a refrigerated cooler (Churchill). Moist air was sparged into the culture vessel at a flow rate of 1 l.min^{-1} and the culture was vortexed using a magnetic motor stirrer (Heidolph Drehzahl, Galenkampf). A constant volume was maintained by gas entrainment (shown in figure 4.2) with an overhead overflow. The nutrient feed was delivered using a peristaltic pump (type 7 MHRE, Watson Marlow)

iii) <u>Continuous cultures avoiding foam fractionation</u> were carried out using a similar chemostat arrangement. However, the volume within the vessel was maintained by a glass tube entering the vessel near the liquid surface and the over-flowing culture liquid removed by means of a peristaltic pump (LKB). The culture was aerated at a rate of 0.2 min^{-1} using a single orifice sparger and an air outlet was included to prevent air pressure in the vessel forcing foam from the surface of the culture. The culture was vortexed using a magnetic motor stirrer (LH Engineering). The bacterial yield was monitored by dry wt. determination of the culture and the supernatant fraction of the fermenter overflow

was analysed for glucose, NH_4^+, Fe^{3+} Mg^{3+} and Ca^{3+} ions.

4.2.3 Quantitative estimation of polysaccharide

Exopolysaccharides (EP) were precipitated, purified and quantified using the methods previously described in section 3.10.2(iii).

4.2.4 Analytical methods

EP concentration of dialysed supernatants was determined by the phenol-sulphuric acid method of Dubois et al, (1976) using glucose or glucuronic acid as standards. Absorbance readings were taken at 488nm for glucose and 482 nm for glucuronic acid. Uronic acid concentrations in the poly-saccharide samples were evaluated by the carbazole method (Bitter and Muir, 1962) using glucuronic acid as a standard. O-acetyl content of uronic acids was determined by the hydroxamic acid reaction (Weissman and Meyer, 1954) with acetylcholine as standard.

Protein concentrations of supernatants and membrane samples were determined by two methods:-

1) Folin phenol reagent (Lowry et al, 1951) and 2) Protein-dye binding using Coomassie Brilliant Blue G-250 (Bradford, 1976)

4.2.5 Preparation of LPS

LPS was extracted from washed cells with aqueous phenol (Luderitz et al., 1965). Approximately 1 g (dry weight) of bacteria was suspended in 50 ml of water and warmed to 65°C. An equal volume of 90% (w/v) aqueous phenol at the

same temperature was added and the mixture stirred vigorously for 5min. The aqueous and phenol phases were separated by centrifugation (3000 g, 30 min, 0°C). Each layer was collected and dialysed for 48h against running tap water to remove phenol. LPS was isolated by ultra centrifugation (100,000 g, 4h), purified by sepharose Cl-4B (Pharmacia) gel filtration (Romanowska, 1970) and dried under reduced pressure over P_2O_5.

4.2.6 Polysaccharide hydrolysis

EP and LPS glycosidic linkages were hydrolysed by four methods, depending upon subsequent chromatography and the nature of the polymer:

i) Uronic acid polymers were hydrolysed for paper chromatography in 88% (V/V) formic acid (5 mg ml^{-1}, 100°C, 5h);

ii) For GLC analysis the uronic acids in the EP were reduced to primary alcohols while they were still part of the EP. Uronic acids as monomers are difficult to quantitatively reduce to the corresponding neutral carbohydrates, even with repeated treatments with $NaBH_4$ (Sjostrom et al., 1974). However, when part of the polymer, the uronic acids can be quantitatively reduced with one treatment with $NaBH_4$ provided the methyl ester is formed first (Smith, 1967). Methyl ester formation, reduction by $NaBH_4$ and hydrolysis in 2N HCl was carried out by the method outlined by Fazio et al, (1982). GLC derivatives were then prepared as for neutral sugars.

iii) Polysaccharides containing neutral sugars and hexosamines were hydrolysed in 1M H_2SO_4 (5 mg.ml^{-1}, 100°C, 16h). Sulphuric acid was then neutralised by the addition of barium carbonate (saturated solution) and the neutral supernatant containing the sugars was dried at 40°C in an evaporator (Evapo-mix, Bucher Instruments, USA).

iv) LPS were hydrolysed in a 40% (W/V) suspension of Dowex 50 w-X8 (H^+) resin (Sigma) in 0.02M HCl (5 mg.ml^{-1}, 100°C, 30h). The hydrolysate was deaminated using the method outlined by Varma & Varma (1976). A freshly prepared solution (0.15 ml) of sodium nitrite (23.3% W/V) was added to 0.5 ml of hydrolysate in a sealed glass tube and subjected to intermittent vortexing at room temperature for 30 mins. Then, the hydrolysate was neutralised in a tandem exchange column containing 5ml Dowex 50 w-X8 (H^+) over 5ml Dowex 1-2X (HCO_3^-). Eluate and washings were collected and fatty acids removed by adding an equal volume of hexane and discarding the upper layer after brief shaking. After this extraction was repeated 2-3 times the aqueous layer was dried in a rotary evaporator (Rotavapor-RE/A, Orme Scientific Ltd) at room temperature.

4.2.7 Paper chromatography

Descending paper chromatography was on Whatman No. 1 paper irrigated in either butan-1-ol/pyridine/water (6:4:3, by vol.) for neutral sugars (Whistler and Conrad, 1954) or in acetone/ethanol/isopropanol/0.005 M borate buffer pH 12

(3:1:1:2, by vol) for uronic acids (Makerjee, 1964; Evans and Linker, 1973). The monosaccharides were detected with aniline phthalate spray reagent (Block et al, 1958).

4.2.8 GLC

GLC of neutral and amino sugars was carried out using either alditol acetate (AA) derivatives (alginate polymers) and peracetylated aldononitrile (PAAN) derivatives.

i) Derivatisation to AA derivatives

Nuetralised hydrolysates were reduced to alditols in $NaBH_4$ (10 mg ml^{-1}) for 1 h. Excess $NaBH_4$ was then destroyed by acidifying the samples with acetic acid, and the samples were dried in an evaporator at 40°C, as for the neutralised supernatants. Methanolic HCl (1% V/V) in three 1 ml portions, was added, and the contents were again dried after each addition to remove the borate ions quantitatively. The residue was then dissolved in acetic anhydride/dry pyridine (1:1 V/V) and heated for 3h at 100°C. The pyridine was extracted with a stream of nitrogen and the residue transferred to chloroform (Sloneker, 1951). G.L.C. analysis was carried out on a Pye series 104 chromatograph model 64 with dual FID detectors, using a column (175 x 0.4 cm) of 3% OV 225 on Chromosorb W(HP) (with N_2 carrier gas at a flow rate of 40 ml min^{-1}) and isothermally at 195°C. The peak areas were computed by a Hewlett Packard 3380 A integrator. Components were identified by their respective retention times in comparison with authentic standards (BDH, Chemicals Ltd.)

ii) Derivatisation to PAAN derivatives

Dried hydrolysates were subjected to successive treatments with (i) 6-7 mg of hydroxylamine hydrochloride in 0.4 ml dry pyridine at 100°C for 4 mins, and ii) 0.5 ml acetic anhydride at 100°C for 10 mins. The solvents were evaporated the PAAN derivatives partitioned against chloroform/water (2:1) by vol and the chloroform layer retained for analysis. PAAN derivatives were analysed using a modification of the GLC conditions described by Linton and Cripps (1978) on a Sigma 3 chromatograph model with single FID detector. Column materials and conditions were the same as for the AA derivatives and components were identified by their respective retention times in comparison with the standard sugars derivatised using the procedure outlined for the hydrolysates.

4.2.9 Isolation of outer membranes

Whole cell membranes were prepared using a method similar to that of Schnaitman,(1970). The calls were harvested from batch cultures by centrifugation and washed 3 times in chilled 0.1 M tris (hydroxymethyl) aminoethane (Tris) buffer pH 7.8, containing 0.1 mM. EDTA. The cells were then re-suspended in the Tris-EDTA buffer (30 ml), and deoxyribonuclease I (1 mg) and ribonuclease A (1 mg) (Sigma) were added to each suspension. The suspensions were cooled to 0°C in ice and passed through a French pressure cell (Aminco) three times at 0.96 - 1.1 x 10^8 Pa, followed by the addition of $MgCl_2$ to a final concentration of 2mM. Unbroken cells were then

removed by centrifugation (5,000 x g, 10 min) and membranes were harvested by further centrifugation (25,000 x g, 1 h) and washed three times in 0.01 M Tris-HCl buffer pH 7.4 by repeated centrifugation (25,000 x g, 1 h).

Outer membranes were separated from the inner membranes by <u>in situ</u> density gradient centrifugation using Percoll density gradient media (Pharmacia Fine Chemicals). An iso-osmotic stock solution of Percoll (SIP) was made by addition of 9 parts (V/V) Percoll to 1 part (V/V) 2.5 M sucrose, and this was further diluted in 0.25 M sucrose (25% V/V SIP) to give a starting density of 1.06 g ml^{-1}. The whole membrane sample (0.5 ml) was then mixed with the Percoll/sucrose media (39.5 ml) and a balance tube containing Density Marker Beads (Pharmacia) was used to monitor the gradient (Kagedal and Pertoft, 1978). The centrifugation was performed in an angle head rotor (34°C, 8 x 50 ml; Beckman centrifuge) for 2 h at 31,000 x g. av. Under these conditions the inner and outer membranes were separated efficiently to either end of the tube and were easily recovered (inner membrane density 1.037 g ml^{-1}, outer membrane density 1.067, 1.081 g ml^{-1}). The membranes were recovered from the Percoll media on a 1.4 x 70 cm column of Sepharose Cl-2B, equilibrated with 0.01 M Tris-HCl pH 7.4 at 4°C, and the void volume fraction was concentrated in an Amicon ultrafiltration system (YM10 membrane).

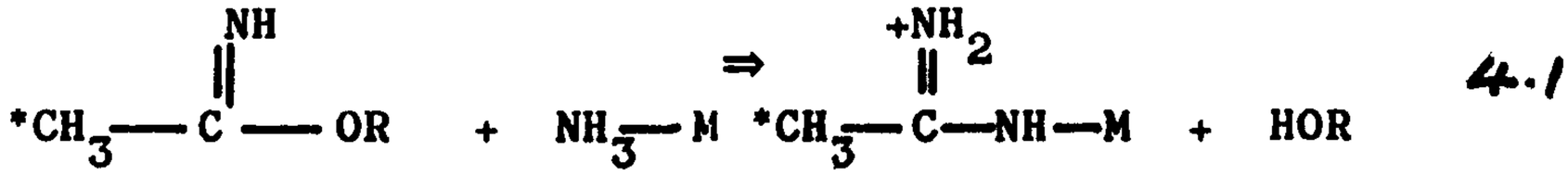
4.2.10 Electrophoresis of outer membrane proteins

Acrylamide-bis acrylamide (BDH Chemicals Ltd) slab gels were prepared using the discontinuous buffer system described by Laemmli (1970). The stacking gel contained 4% (W/V) acrylamide in the presence of 0.125 M Tris-HCl, pH 6.8 and 0.1% SDS. The resolving gel contained 8% /W/V) acrylamide in the presence of 0.375 M Tris-HCl, pH 8.8, and 0.1% SDS. Ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (Sigma) were used as polymerizing agents, and the running buffer (pH 8.3) contained 0.025 M Tris base, 0.192 M glycine and 1.0% (W/V) SDS. The gels were prepared by the method of Ames,(1974) and were run at a constant current of 40mA with the voltage increasing from 80 V to 200 V and the run lasting about 4.5h. When run, the gels were stained by the four staining steps recommended by Fairbanks et al., (1971) using Page blue G83 (Sigma) (0.1%) in propan-2-ol and acetic acid. Finally the gels were dried to filter paper.

4.2.11 The use of radioamidination and radioiodination of membrane proteins in order to demonstrate extrinsic or external proteins of the outer membrane of adhesion mutants.

i) Amidination of intact bacterial cells was carried out using the method previously described by Whiteley and Berg, (1974) for the amidination of outer and inner surfaces of human enthrocyte membranes. ^{14}C Isethionyl acetimidate ($^{14}CIAI$) (Amersham International) is unable to penetrate the membrane and was used to label surface exposed proteins with primary

amines. ^{14}C Ethylacetimidate (^{14}C EAI) Amersham International) can penetrate membranes and was used to label both surface exposed proteins and internal membrane proteins. Equation 4.1 shows the reaction of the imidoesters under mild physiological conditions.



4.1

The methyl group* is radioactively labelled and becomes linked to protein (M). The non-penetrating reagent, IAI ($R=CH_2CH_2SO_3-$) is highly polar and lipid insoluble; the penetrating reagent, EAI ($R = CH_2CH_3$) is relatively non-polar and lipid soluble.

The cells were harvested from batch cultures (100mls) by centrifugation and washed three times in chilled phosphate buffer (pH 8.0 50mM phosphate; Na^+, 175 mM; Cl^- 82mM and K^+3.6 mM) Imidoesters (100µg 45µci) were dissolved in NaOH(0.1M) to give a neutral solution and added immediately to the suspension of cells (10mls) in phosphate buffer. The reactions were run for 30min at 20-25^oC and pH was maintained at 8.0 during this time by titration with 0.01 M HCl. Following the reactions the cells were washed three times in 0.1M Tris buffer, pH 7.8, containing 0.1M EDTA and whole cell membranes prepared as in section 4.2.10.

ii) Iodination of intact bacterial cells was carried out using the method described by Boxer et al, (1974). The cells (1ml suspension) were harvested and washed as in section 2.2.4

and resuspended in 0.1ml in 0.1M phosphate buffer, pH 7.4, in an eppendorf tube. The iodination was carried out in a total volume of 0.4ml containing 10µm KI, 0.4mg of lact-operoxidase ml^{-1}(sigma)London Chemical Co.) and 0.125 mCi of $Na^{125}I$ (carrier-free Amersham International). The reaction was done at room temperature, and H_2O_3 (300nmol total) was added in 20 equal aliquots at 3min intervals. After iodination the cells were washed five times with 0.1M sodium phosphate buffer, pH 7.4, and finally transferred to 0.1M tris pH 7.8 prior to lysis. Whole cell membranes were prepared as in Section 4.2.10.

iii) <u>Electrophoresis autoradiography and fluorography</u>

Radioiodinated or Radioamidinated samples were electrophoresed as described in Section 4.2.10. Prior to electrophoresis the samples were counted by scintillation counting as described in section 2.2.5.iii)in order to assess the level of labelling and allow equal loadings (CPM) of the separate samples. Radioiodiated sample gels were fixed, stained and dried as previously described and exposed to Kodak RP Royal X-omat film (RP/R) at -70°C for 16h. Exposed films were developed for 6mins at 20°C in Kodak DX-80 developer. Radioamidinated sample gels were fixed, equilibrated with dimethylsulphoxide impregnated with 2.5-diphenyloxazole (PPO) by immersion in 4 vol. 20% W/W PPO in dimethylsulphoxide, soaked in water, dried and exposed and developed as above (Bonner and Laskey, 1974)

4.2.12 <u>Isotherms of Adsorption of EP and LPS from adhesion mutants to hydrophobic and hydrophilic polystyrene substrata.</u>

Radiolabelled EP and LPS were isolated and purified from 100ml cultures grown in a chemically defined medium (section 3.2.2.i) containing ^{14}C-glucose (Amersham International) (2.5 μ ci ml^{-1}) as described in sections 4.2.3-4.2.5. The specific activity of each polymer was determined by relating liquid scintillation counts to concentration, as determined by the phenol-sulphuric acid method (section 4.2.4). Adsorption assays were carried out using methods similar to those of Baszkin and Lyman, (1980). Polystyrene, tissue culture treated polystyrene, and sulphonated polystyrene petri dishes were used as substrata (see sections 2.2.4 and 3.6.3). The petri dishes were filled with 0.01M phosphate buffer, pH 7.4 (5mls), containing the required amount of ^{14}C labelled polymer to give a final range of polymer concentrations between 1.0mgml^{-1} and 0.01mgml^{-1}. The adsorption experiment was carried out for 3hrs at 20^{o}C to insure an equilibrium value of polymer adsorption (Baszkin and Lyman, 1980). Each petri dish was then rinsed with 300 ml of phosphate buffer and allowed to dry at room temperature. The concentration of adsorbed polymer was then determined by liquid scintillation counting using the method described in Section 3.6.3.

4.2.13 Electronmicroscopy of negatively stained organisms following culture on solid media and liquid media.

Electron microscopy of a number of isolates was carried out in order to determine whether fimbriae, pili or flagella were important surface characteristics of adhesion. The organisms were grown on solid media or liquid media as previously described, harvested, resuspended in phosphate buffer and transferred to carbon-coated grids. The cells were stained with 2% sodium phosphotungstate acid, pH 7.0 and allowed to dry. Samples were viewed in a Siemers Elmiskop 1A electron microscope and comparisons were made with piliated and fimbriated Escherichia coli.

4.2.14 Attachment Assays

Attachment assays were carried out using the methods described in Section 2.2.4. These were modified in order to study the effects of time, temperature, pH and ionic strength on attachment of Pseudomonas fluorescens H2 as follows:

i) Temperature. Incubation temperatures in the range 4°C to 65°C were used. Prior to incubation the washed cell suspension was diluted 1:9 in buffer previously equilibrated to the appropriate temperature. Following incubation with the substrata unattached cells were removed by washing PS and TC-PS dishes with buffer previously equilibrated to the appropriate temperature.

ii) pH and ionic strength. The effects of pH and ionic strength were determined using a number of hydrogen ion

buffers as described by Good, et al, (1976) covering the range pH 3-12. The ionic strength of these buffers was changed by adding 1M NaCl to give a low ionic strength of 0.01M NaCl and a high ionic strength of 0.5M NaCl. Washed cell suspensions were prepared as previously described (Section 2.2.4) and were diluted 1:9 into the appropriate buffer pH/ionic strength prior to incubation in the PS and TC-PS dishes. Washings were also carried out with the appropriate corresponding buffer.

4.3 RESULTS

4.3.1. Further identification of pseudomonad isolates used in attachment experiments to investigate the role of exopolymers and outermembranes in adhesion

On the basis of the results shown in Table 4.1 all of the isolates examined were identified as fluorescein producing pseudomonads and could be designated *P. aeruginosa*, *P. fluorescens* or *P. putida*. P. aeruginosa was identified by the production of a blue or brown pigment on King's medium A(H45). The *P. fluorescens* isolates (H2, H15, H41) were identified by growth at 4°C but not at 41°C, growth on glucose and mannitol, but not on ethanol or maltose, and ability to hydrolyse of casein (negative) and gelatin (positive). *P putida* (H35, H40) also grew at 4°C but not at 41°C, but failed to attack casein gelatin and mannitol. Strain H2 did show some atypical results of *P. fluorescens* as this strain was found to grow on ethanol and to hydrolyse casein, results typical of *P aeruginosa*. However, it was unable to grow at 41°C and did not produce the pyocyanin pigment on King's medium A. The antibiotic sensitivity of this strain also confirms this identification, as the results are typical of *P. fluorescens* based on the antibiotic sensitivity of these organisms as determined by King and Phillips (1978).

TABLE 4.1

Identification of Pseudomonad isolates

Test	Organism No. H2	H15	H35	H40	H41	H45
Oxidase	+	+	+	+	+	+
Growth at 41°C	-	-	-	-	-	+
Growth at 4°C	+	+	+	+	+	+
gelatin hydrolysis	+	+	+	-	-	-
starch "	-	-	-	-	-	-
casein "	+	-	-	-	-	+
DNA "	-	-	-	-	-	-
Ammonium salt "sugars"						
glucose	+	+	+	+	+	+
ethanol	±	-	+	+	+	+
maltose	-	-	-	-	-	-
mannitol	+	-	-	+	+	+
Pigment production						
Kings A	-	-	-	-	-	+
Kings B	F	F	F	F	F	F
Other pigments	brown pigment	pink pigment	-	-	-	-
Antibiotic sensitivity						
Ampicillin	R	-	-	-	-	-
Carbenicillin	R	R	R	R	R	S
Cephaloridine	R	R	R	R	R	R
Chloramphenicol	R	R	R	R	R	R
Colistin	S	S	S	S	S	S
erythromycin	R	-	-	-	-	-
gentamicin	S	S	S	S	S	S
novobiocin	R	R	R	R	R	R
penicillin	R	-	-	-	-	-
Polymyxin B	S	S	S	S	S	S

4.3.2 An investigation of the attachment of P. fluorescens (H2) following defined growth conditions.

4.3.2.i) The influence of time of attachment on the adhesion of P. fluorescens H2 to polystyrene and tissue culture polystyrene substrata

The attachment of P. fluorescens H2 after growth in defined minimal medium is shown in figure 4.3. Maximum level of attachment was found after 2h (15°C) on both test substrata. Similar curves were determined for the attachment of this organism after growth in complex media or after growth with carbon-or nitrogen-limitation in continuous culture. The initial rate of attachment and the maximum level achieved varied for these growth conditions and was further examined

4.3.2.ii) The influence of temperature of attachment on the adhesion of P. fluorescens H2 to PS and TC-PS substrata

The effects of temperature on attachment of this isolate are shown in figure 4.4. Increasing levels of attachment to both substrata were measured with increasing temperature of incubation (1h) until maximum numbers were recorded in the temperature range 30°C-40°C. Above this temperature range the attachment to both substrata decreased, the rate of decline being greater for the PS than TC-PS, with the result that above 45°C the levels attaching to TC-PS was above PS.

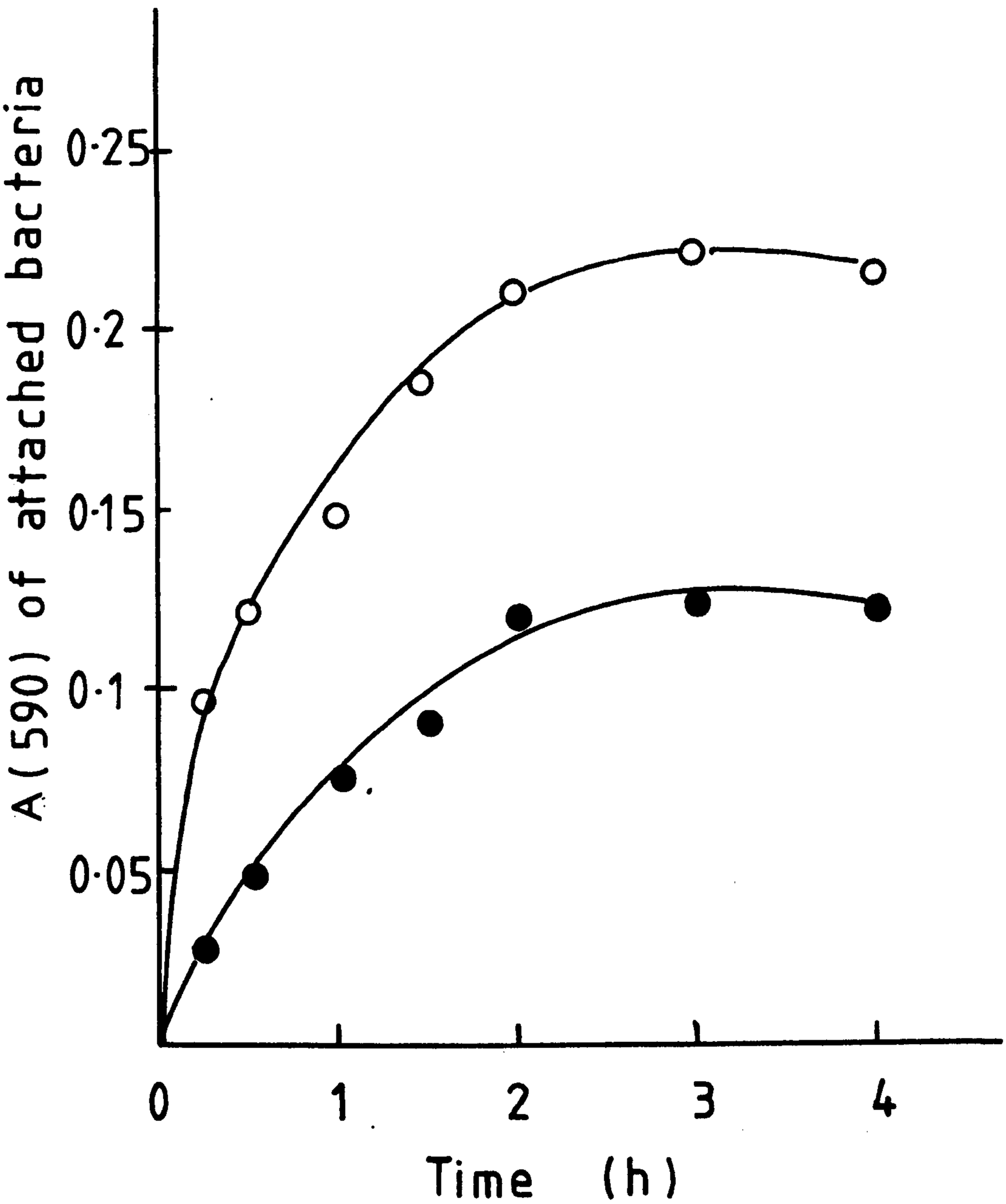


Figure 4.3

Relationship between time of attachment and number of attached bacteria for the adhesion of P. fluorescens H2 to PS (○) and TC-PS (●) substrata. A(590) of crystal violet stained bacteria was used as a measure of attached numbers.

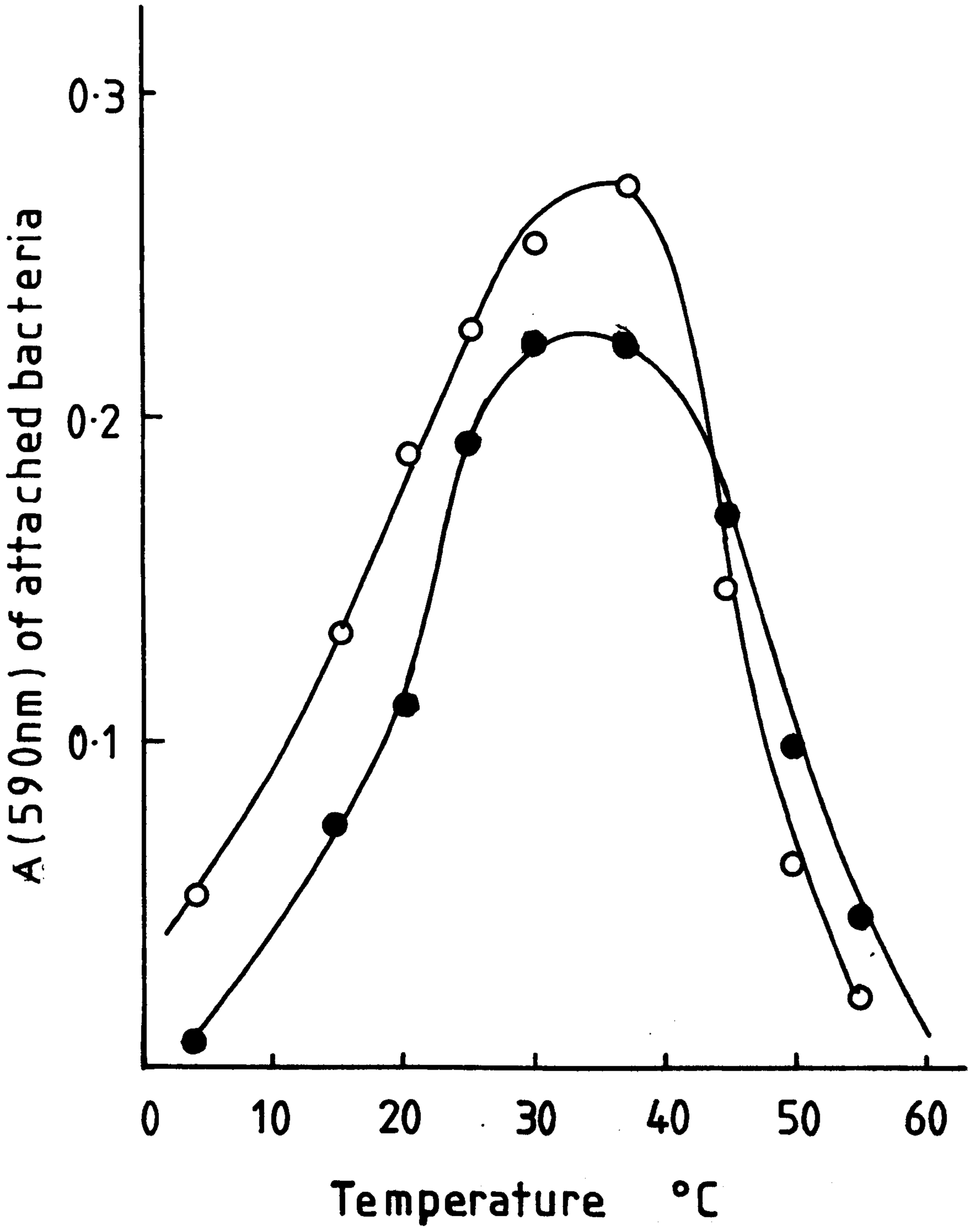


Figure 4.4

Relationship between temperature of attachment and numbers of attached bacteria for the adhesion of P. fluorescens H2 to PS (○) and TC-PS (●) substrata. A (590) of crystal violet stained bacteria was used as a measure of attached numbers.

4.3.2.iii) The effects of media pH and ionic strength on the attachment of P. fluorescens to PS and TC-PS substrata

In order to determine the effects of pH and ionic strength on the attachment of this P. fluorescens (H2) isolate a number of hydrogen ion buffers covering the range pka = 3.71-8.3 were used. Figure 4.5 shows the buffering capacities of this combined 0.01M DMHT buffer over the range pH 3.0-12.0. The effects of this buffer on cell respiration was determined using an oxygen electrode and it was found not to influence the O_2 consumption rate of log phase cells after incubation at 15°C-20°C for 2h compared to similar incubation periods in phosphate buffer. The ionic strength of the buffer was changed by adding 1M NaCl to produce a low ionic strength at 0.01M NaCl and high ionic strength at 0.5M NaCl. The attachment pH profiles at low and high ionic strength are shown in figure 4.6. These profiles show maximum attachment peaks at two pH values, pH 5.5 which was the highest peak in each case and pH 7.5-8.0. The effect of pH was to modulate the level of adhesion and reduce this level significantly at extreme pH values 4.0, and 9.0 and 7.0. The effect of ionic strength was to reduce the level of attachment uniformly over this pH range. Thus both changes in pH and ionic strength did not significantly effect the attachment preference of this organism to the hydrophobic substrata.

Figure 4.5

Titration curve for the combined 0.01M DMHT buffer showing the buffering capacity for the pH range 3.5-11. The effective range for this buffer is pH 4.5-9 over which a constant buffering capacity was achieved.

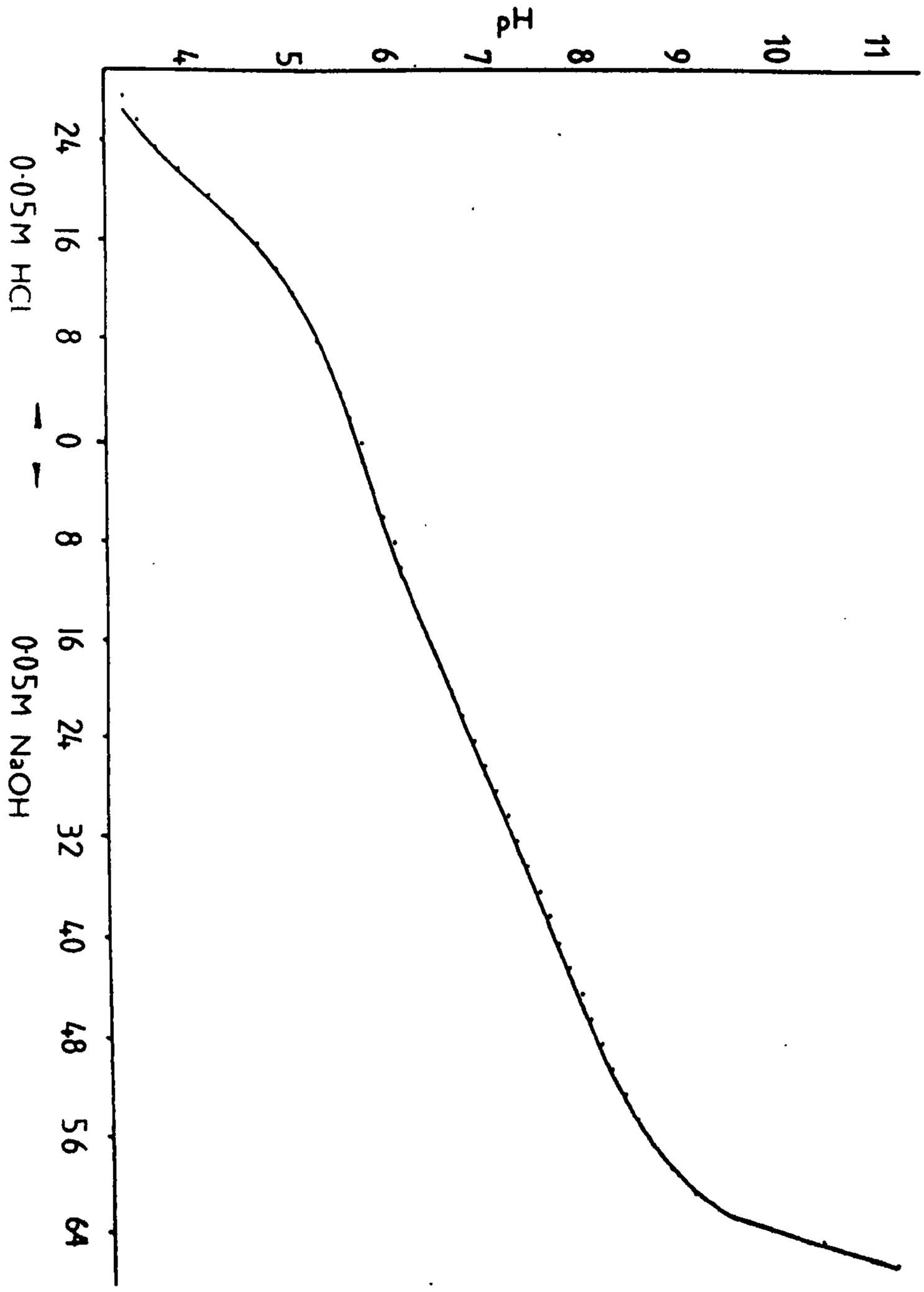
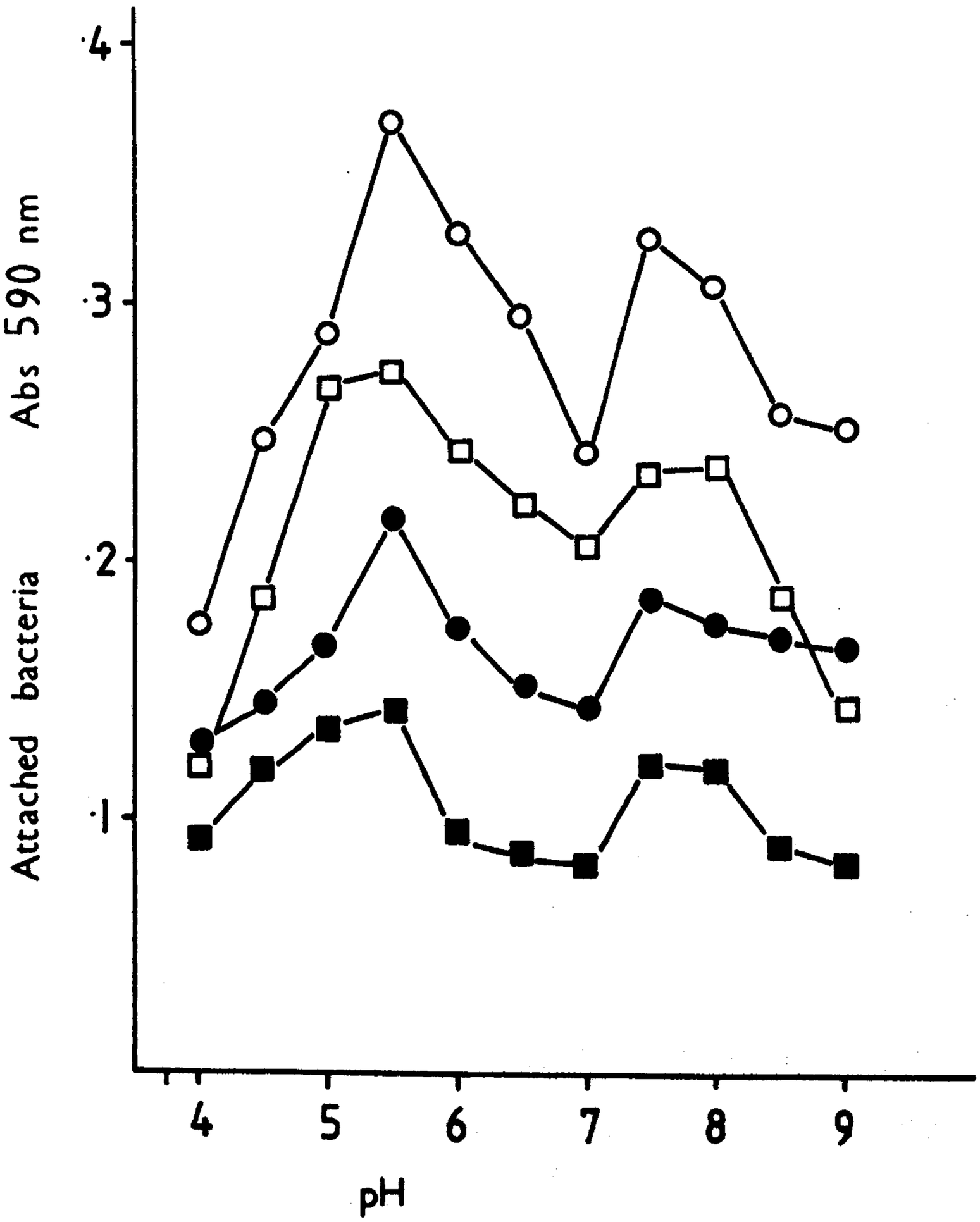


Figure 4.6

Relationship between attachment pH and ionic strength and numbers of attached bacteria for the adhesion of P. fluorescens H2 to hydrophobic and hydrophilic substrata. Attachment took place at the following concentrations of NaCl: PS, 0.01M (○); TC-PS 0.01M (●); PS, 0.5M (□); TC-PS 0.5M (■). A(590) of crystal violet stained bacteria was used as a measure of attached numbers.

Fig 4.6



4.3.2.iv) The adhesion of P. fluorescens H2 following batch culture in chemically defined media varying in concentration of nitrogen salts

The attachment of P fluorescens H2 to PS and TC-PS following batch culture in chemically defined media varying in nitrogen salts concentration is shown in figure 4.7. Stationary phase cells were attached for 1h at 15°C in phosphate buffer and in phosphate buffer containing 0.1% (W/V) glucose. Cells cultured in low NH_4Cl concentration entered stationary phase at a lower cell concentration and attached to both substrata in significantly lower numbers than when cells were cultured in NH_4Cl concentrations $>0.1\ gl^{-1}$. The effects of glucose in the medium of the attachment assay was to significantly increase attachment levels to both substrata at NH_4Cl concentrations $>0.2\ gl^{-1}$ while attachment levels to PS substrata were reduced with the addition of glucose over the NH_4Cl range 0.05-0.2 gl^{-1}. Growth at or above 0.2 gl^{-1} NH_4Cl produced a similar cell concentration at stationary phase and was considered to be carbon limited.

4.3.2.v) The attachment of P. fluorescens (H2) following growth in carbon-nitrogen-and carbon/nitrogen-limited continuous culture

The attachment of P. fluorescens H2 following growth in continuous culture is shown in figure 4.8, 4.9 and 4.10. All three figures show a reduction in attachment levels with

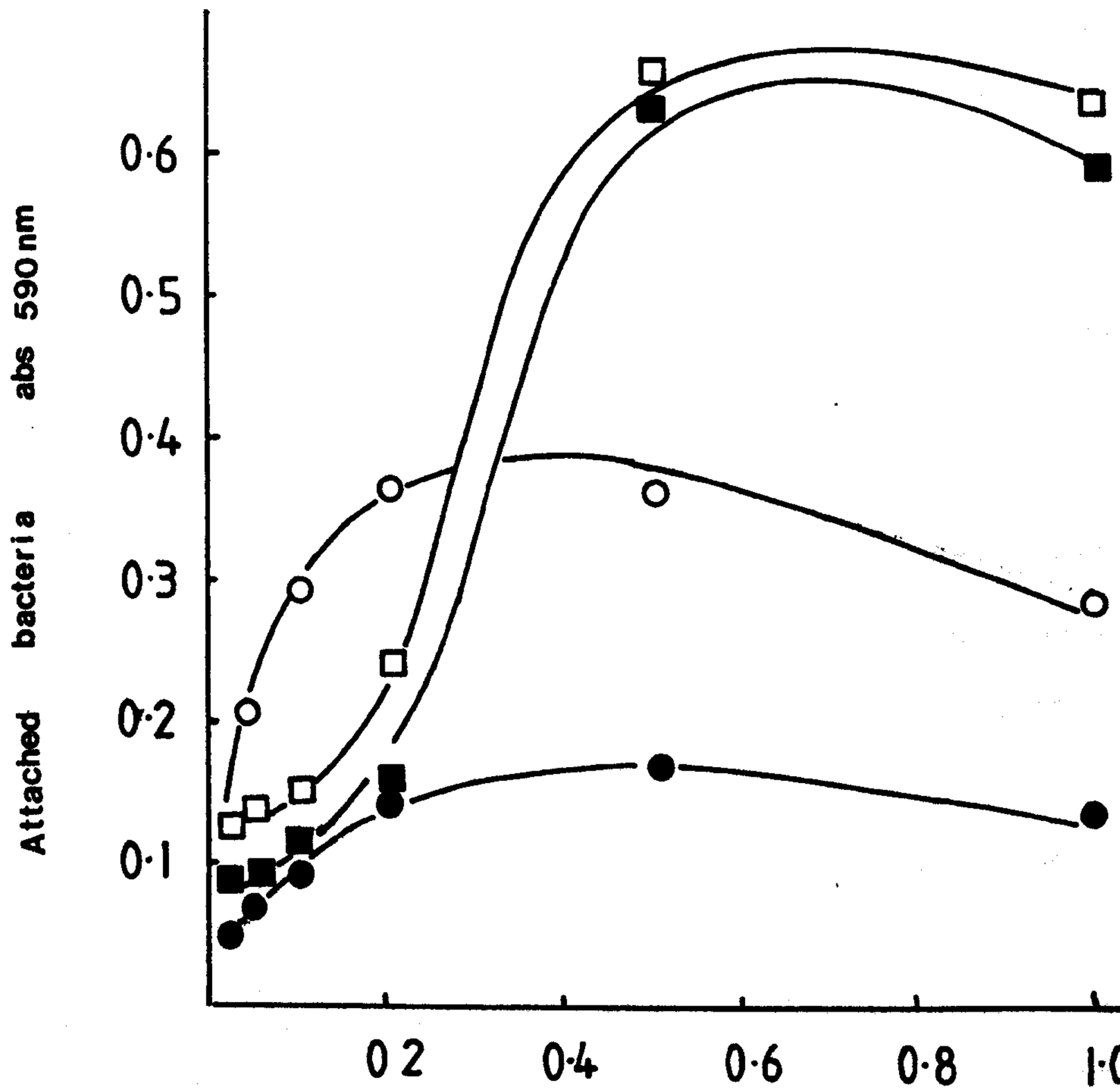
Figure 4.7

Relationship between growth conditions determined by varying the nitrogen salts concentration of a chemically defined media and attached bacteria for the adhesion of P. fluorescens H2. Attachment took place under the following conditions:

Substratum	Medium	Symbol
PS	buffer	○
TC-PS	buffer	●
PS	0.1 gl^{-1} glucose	□
TC-PS	0.1gl^{-1} glucose	■

A (590) of crystal violet stained bacteria was used to measure of attached numbers.

Fig 4.7


Attached bacteria abs 590 nm
0·6
0·5
0·4
0·3
0·2
0·1
0 2
0·4
0·6
0·8
1·0
Concentration of NHCl 4 g.l⁻¹

an increase in dilution rate. Maximum level of attachment were observed for cells under carbon/nitrogen-limitation (4.8) followed by lower levels for carbon-limitation (4.9) and minimum levels for nitrogen-limited (4.10) growth. These continuous culture experiments used the apparatus designed to prevent the enrichment of adhesion mutants as described in section 4.2.2.iii). Table 4.2 shows the influence of dilution rate on the bacterial yield and macromolecular composition of the membrane for the growth limitations given above. For the three conditions of growth limitation investigated, an increase in dilution rate was accompanied by an increase in the yield of bacterial dry wt per gram glucose utilised, and a reduction in the corresponding yield of membrane. It was noted that cell size increased with increasing dilution rate. Table 4.2 also shows changes in protein and carbohydrate composition of the membrane. For all three growth conditions levels of protein decreased with increase in dilution rate, whereas carbohydrate content increased with increase in dilution rate. Maximum levels of carbohydrate were found with nitrogen-limited growth and minimum levels with carbon/nitrogen-limitation. The following media components were analysed throughout the operation of continuous culture experiments in order to determine the true limiting substrate: glucose, NH_3, Ca^{++}, MG^{++}, Fe^{++}

TABLE 4.2

Influence of dilution rate on the bacterial yield and macromolecular composition of the outer membrane during carbon, carbon/nitrogen and nitrogen limited culture of P. fluorescens

	Dilution rate (hr-1)	Yield cells (g. bacterial g. glucose)	Yield(% dry wt) membrane	% of protein and carbohydrate in membrane	
				Protein[a]	Carbohydrate[b]
1) Carbon limited					
	0.2	0.32	17.5	62.0	18.8
	0.1	0.29	19.8	69.0	11.1
	0.066	0.22	23.2	79.0	10.3
	0.033	0.20	25.0	83.0	7.2
2) Carbon/nitrogen limited					
	0.2	0.36	13.2	74	13.3
	0.1	0.28	16.3	69	9.8
	0.66	0.25	20	75	7.2
	0.33	0.22	24	89.0	5.7
3) Nitrogen limited					
	0.2	0.28	22.3	11.0	87.4
	0.1	0.22	27.8	13.0	60.0
	0.66	0.20	33.0	36.0	53.3
	0.33	0.19	39.2	53.0	36.2

a) Protein values were derived by assuming that the proteins have an overall composition similar to bovine serum albumin. Thus the figures may not be accurate but they allow a comparison to be made.

b) The carbohydrate measurments were determined by the phenol-sulphuric acid method (Dubois *et al*, 1976), with glucose as a standard and therefore may not be accurate depending upon the sugar composition being assayed.

Figure 4.8,4.9 and 4.10

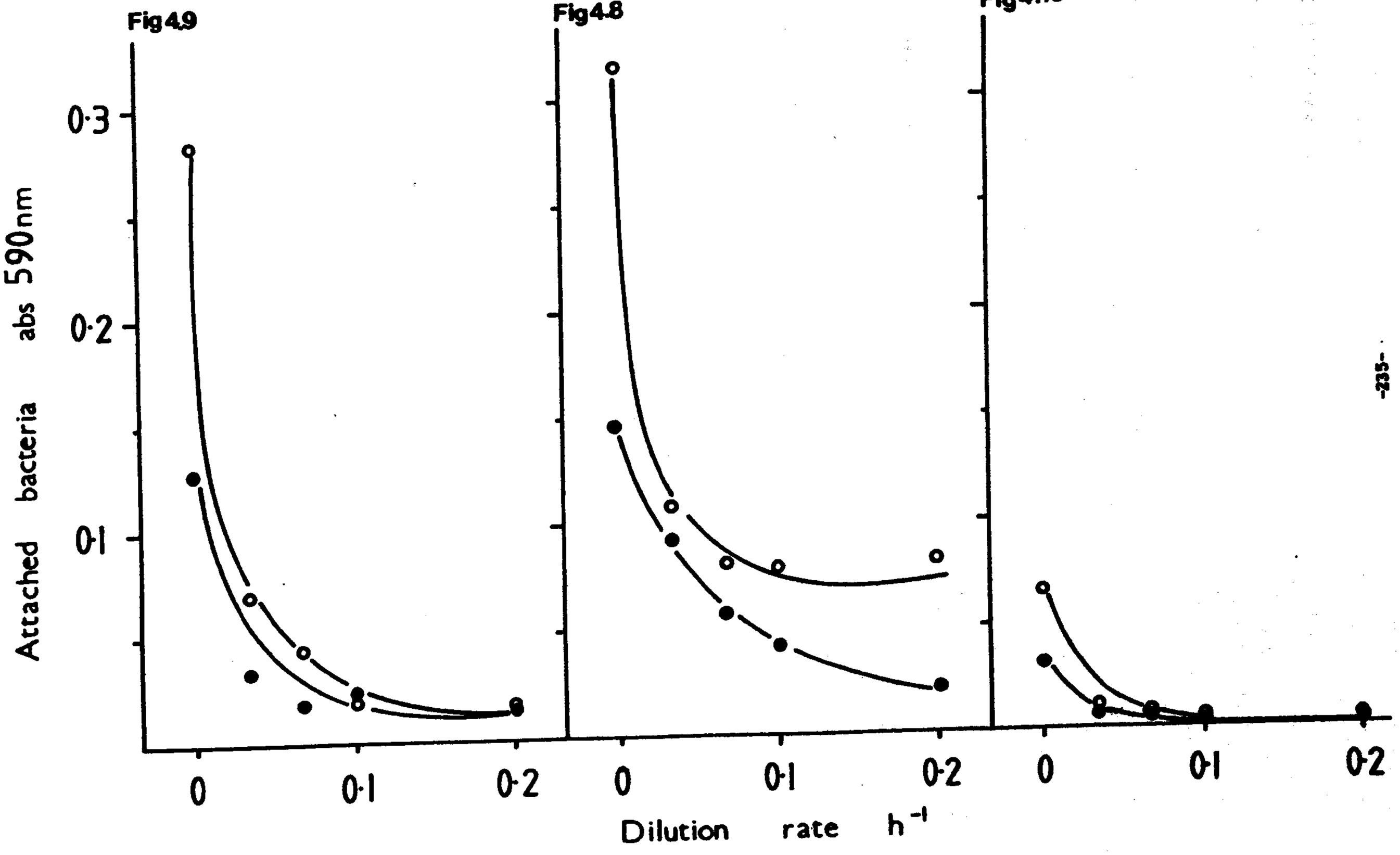
The attachment of P. fluorescens M2 following growth in continuous culture to PS (O) and TC-PS (●) substrata. A (590nm) of crystal violet stained bacteria was used measure attached numbers. The relationship between culture dilution rate and attached numbers was the following growth conditions.

Figure	Growth Conditions
4.8	carbon /nitrogen limited
4.9	carbon limited
4.10	nitrogen limited

Fig 4.9

Fig 4.8

Fig 4.10



4.3.3 An investigation of adhesion mutants of P. fluorescens H2 including an analysis of outer membrane proteins LPS and extracellular polysaccharides.

4.3.3.i) The selection and enrichment of adhesion mutants during the continuous culture of wild-type fluorescens H2

The operation of the continuous culture system was found to select and enrich for two types of adhesion mutants by the foam fractionation of wild-type cells during the initial continuous culture phase (6 days). Figure 4.11 shows the changes in viable counts of wild-type and mucoid mutants during the operation of the continuous culture system, with the dilution rate and exopolysaccharide level detected in the media. It shows the rapid removal of wild-type cells following the initial batch culture and a repeat of this process following the return of cells during a second period of batch culture. Once continuous culture conditions were established the viable counts of both wild-type and mucoid colonies showed a "take over" of the culture by the mucoid mutant and coincided with the production of exopolysaccharide, detectable in the culture medium.

After 17 days culture, the distribution of colony morphologies was determined from samples of the aqueous phase and walls of the fermenter. Table 4.3. shows the viable counts of these samples and the ratios of the mutants to wild-type colonies. Wild-type colonies were just detectable in samples from the aqueous phase, which was dominated by the

Figure 4.11

The change in viable counts of wild-type (●) and a mucoid mutation (●) and in EP concentration (■) during batch and continuous culture of P. fluorescens.

Fig 4.11

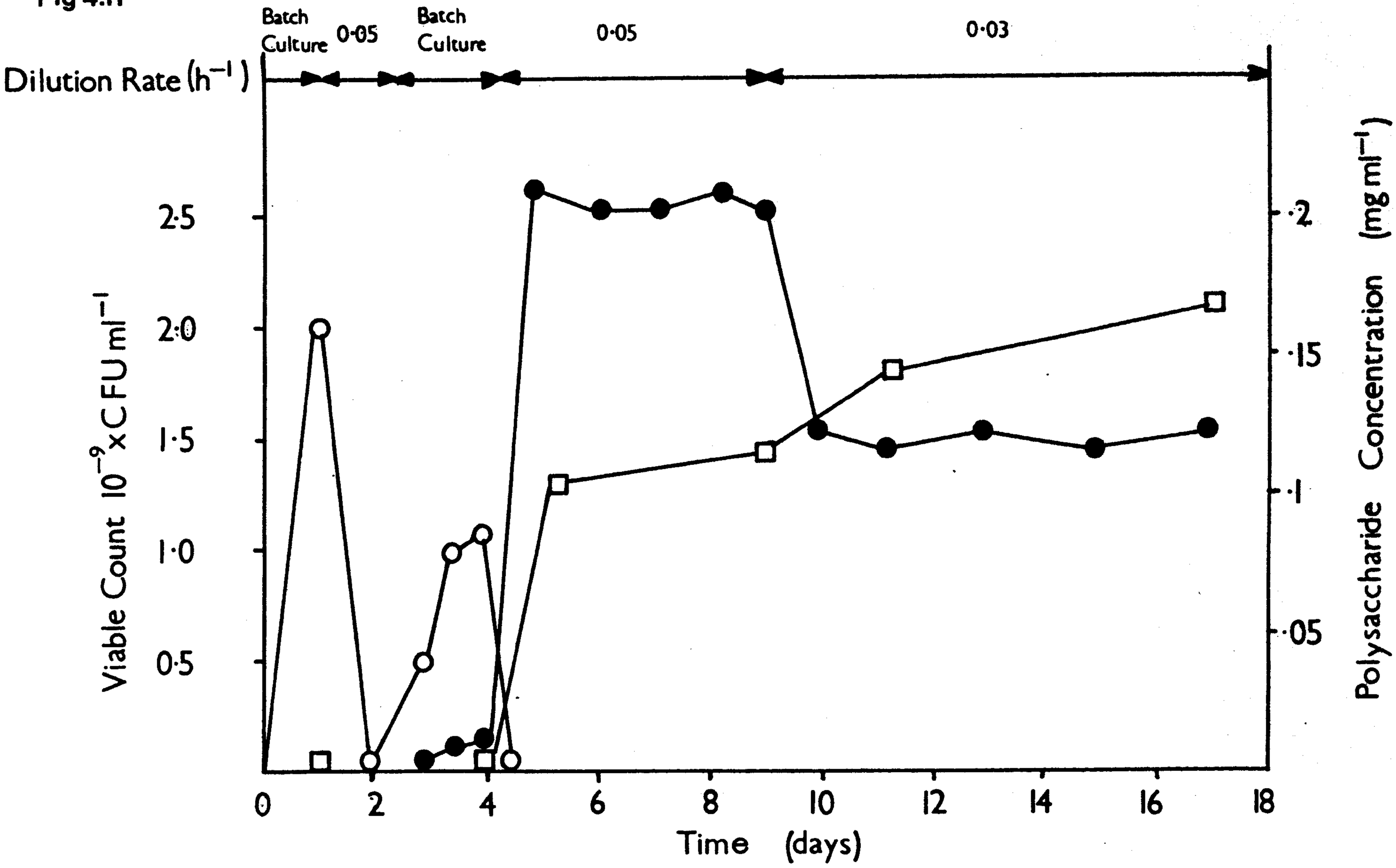

Batch Culture
0·05
Batch Culture
0·05
0·03
Dilution Rate (h^{-1})
Viable Count 10^{-9} x CFU ml^{-1}
2·5
2·0
1·5
1·0
0·5
·2
·15
·1
·05
Polysaccharide Concentration ($mg\,ml^{-1}$)
0
2
4
6
8
10
12
14
16
18
Time (days)

TABLE 4.3

Distribution of the colony morphology sampled from the bulk liquid and fermenter walls after selection and enrichment of attachment mutants.

Colony Morphology	Sample Site [a]	Viable Count CFU ml^{-1}	Ratio of Mutants to wild-type
Wild-type	Bulk liquid	1.0×10^5	1
Mucoid	Bulk liquid	4.8×10^8	4800
Crenated	Bulk liquid	2.0×10^6	20
Wild-type	Fermenter walls	2.3×10^8 [b]	1
Mucoid	Fermenter walls	3.0×10^6 [b]	0.013
Crenated	Fermenter walls	8.6×10^8 [b]	3.7

a Fermenter wall sample sites were below the air-liquid interface

b 1 ml represents a sample area of 10 cm^2

mucoid mutant. However, the crenated morphology was dominant in the wall population with a smaller population of wild-type colonies and only detectable levels of mucoid mutants.

In order to confirm that the changes in colony morphology were due to mutation of the wild-type and not contaminants the sensitivity of a number of colonies of each mutant type to 12 antibiotics was checked and all mutant strains tested showed similar sensitivity patterns to the wild-type. However, although the wild-type was found to be resistant to carbenicillin, enhanced carbenicillin resistance was found for all mucoid strains examined. The crenated mutants studied were all phenotypically similar and no spontaneous reversion to the wild-type condition occurred. However, the mucoid strains showed a high frequency of reversion to the wild-type colony appearance. Figure 4.12 shows the colony morphology of wild-type and the adhesion mutants.

4.3.3.ii) <u>Attachment assays of wild-type and adhesion mutants</u>

In order to confirm that the mutations were affecting the attachment ability of the bacterium, the attachment properties of several strains of each mutant were examined. The attachment assay used hydrophilic and hydrophobic polystyrene petri dishes as test substrata, and attachment was quantitatively assessed by staining the attached bacterial films in crystal violet and measuring the absorbance of this stained film. Table 4.4 shows the attachment results of each mutant type,

TABLE 4.4

The attachment of adhesion mutants of polystyrene and tissue culture polystyrene petri dishes following growth in carbon-limited batch culture.

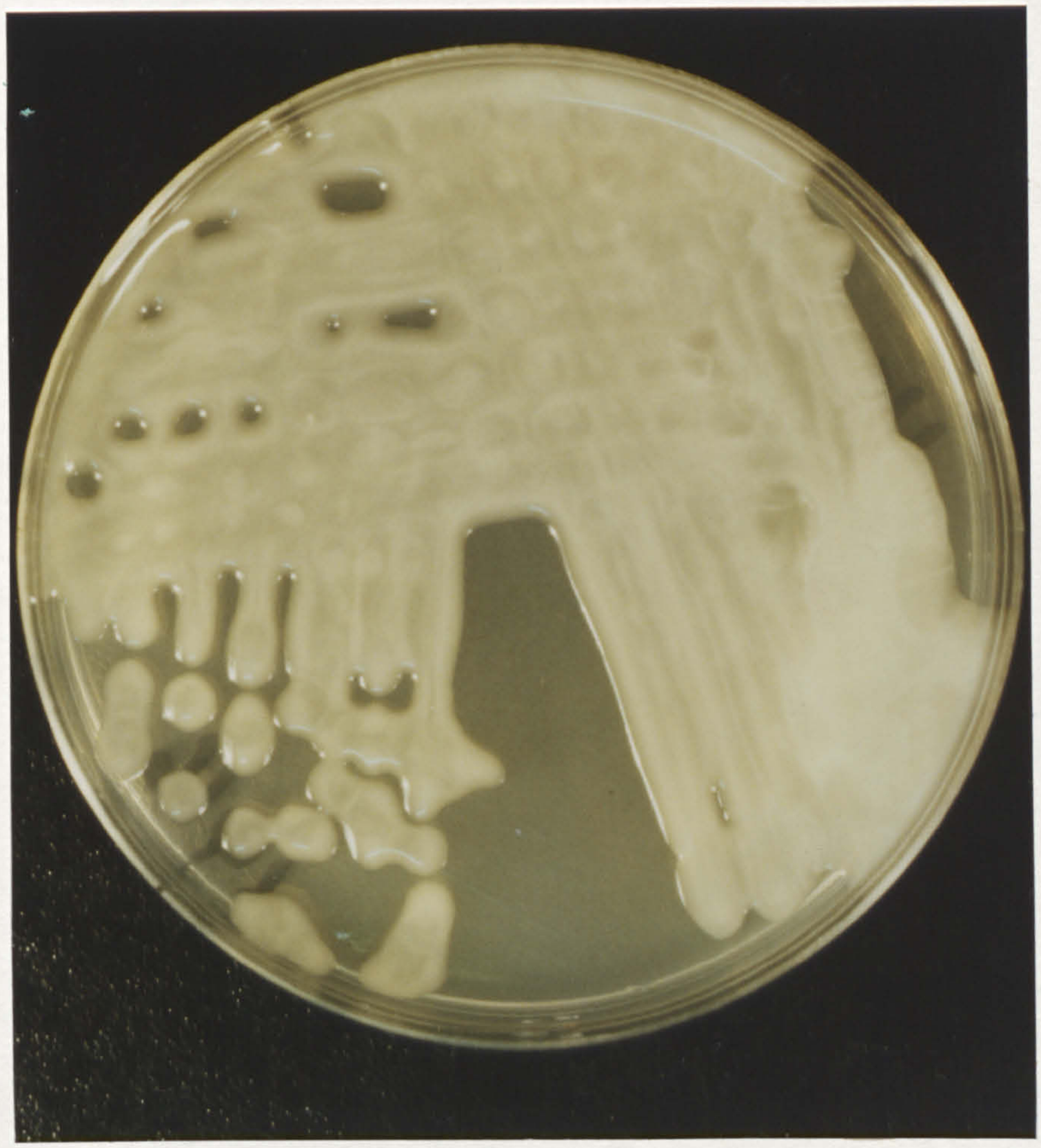
Strain No.	Cell type	Absorbance 590nm (σ_{n-1})n=8	
		Polystyrene	Tissue culture Polystyrene
H2P	Wild-type	0.190 (.022)	0.067 (.007)
H2M1	Mucoid[a]	0.025 (.003)	0.011 (.005)
H2M2	Mucoid[a]	0.051 (.005)	0.018 (.005)
H2S1	Crenated	0.216 (.022)	0.094 (.005)
H2S2	Crenated	0.252 (.034)	0.126 (.007)

a The mucoid mutation is unstable in batch culture, and reversion rate can be high dependent upon growth conditions. Thus cultures may contain significant numbers of cells with wild-type morphology.

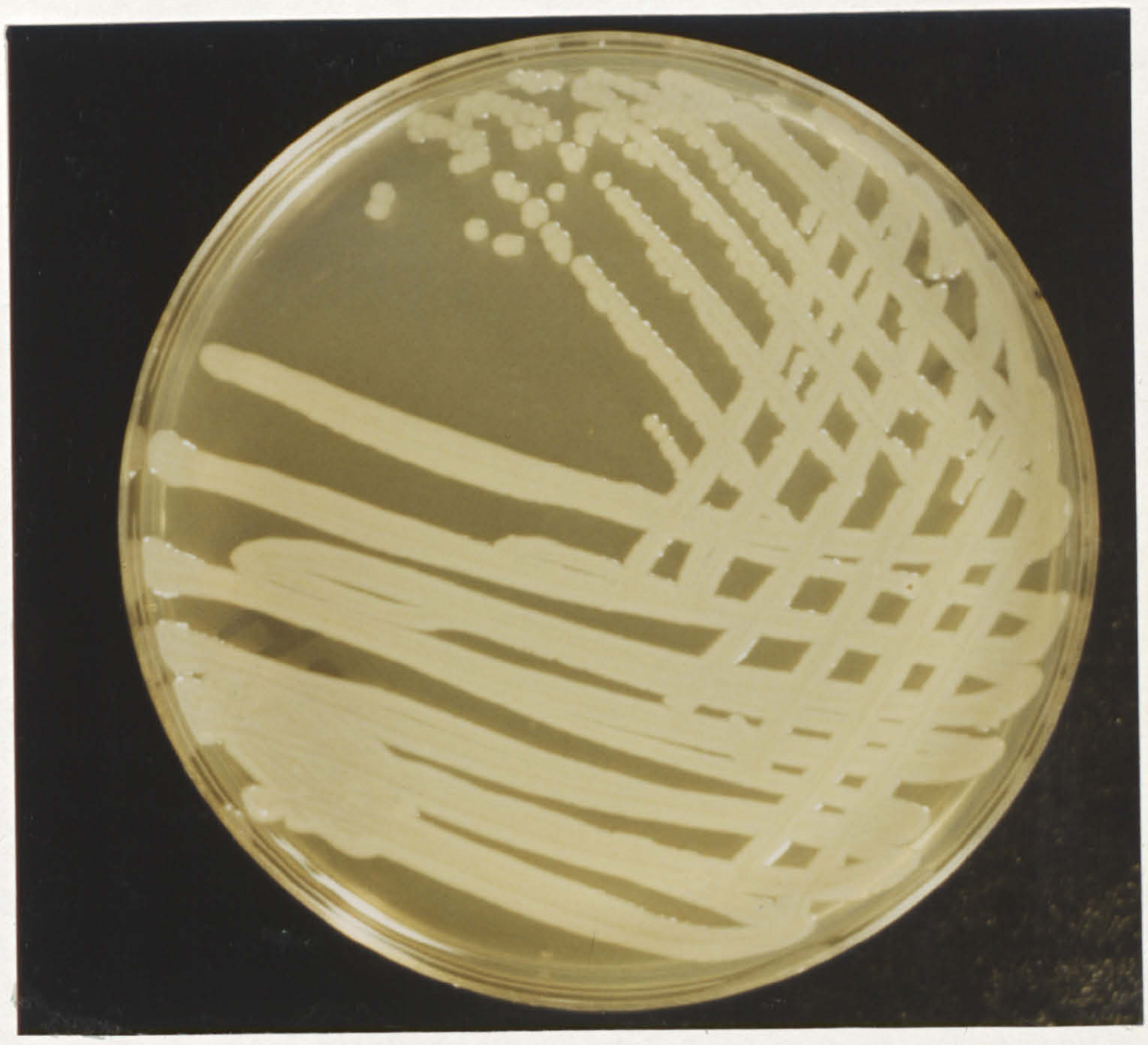
Figure 4.12

Colony appearance of wild-type and adhesion mutant strains of P. fluorescens grown on Kings B medium at 15°C for 72 hours; a) Mucoid mutant; b) wild-type c) crenated mutant.

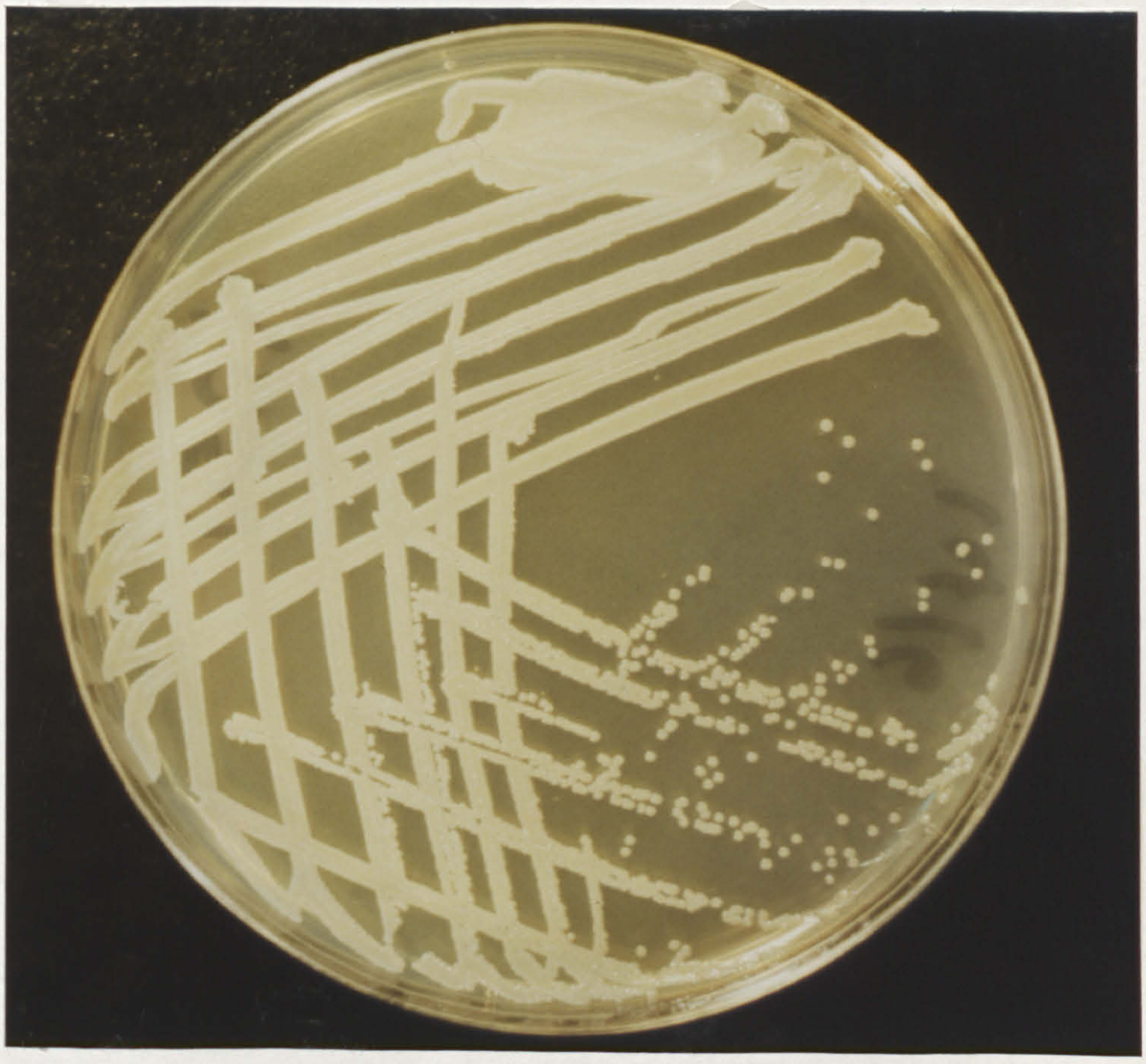
a)



b)



c)



as compared to the wild-type, for both substrata. The results confirm the fermenter findings in that mucoid mutants (H2M1, H2M2) showed lower levels of attached cells than the wild-type, and the crenated mutants (H2S1, H2S2) showed greater levels of attached cells.

4.3.3.iii) Characterisation of the outer cell surface of wild-type cells and adhesion mutants

The membrane of Pseudomonas fluorescens, obtained following disruption with a French pressure cell, were characteristic of Gram-negative cell walls in that they i) pelleted at g values (25,000) that typically sediment cell walls (Nikaido and Nakae, 1979, Schnaitman 1970); ii) contained material which reacted with thiobarbituric acid to produce an absorption spectrum identical with authentic KDO (data not shown), iii) banded in Percol density gradient at a density typical of small membrane vesicles ie. $>1.067, <1.081$ gml^{-1}, iv) contained relatively small amounts of cytoplasmic membrane enzyme activity (<20% NADH oxidase (EC1.6.99.3) as determined by following the decrease in A_{340} described by Darveau et al, (1980) (data not shown). Analysis of outer membrane proteins isolated by equilibrium density gradient centrifugation and separated by SDS-polyacrylamide electrophoresis are shown in figures 4.13 and 4.14. Figure 4.13 shows scans, recorded with a LKB Ultro Scan 2202 laser densitometer, of wild-type H2P2, crenated mutant H2S1 and mucoid mutant H2M2. The major proteins labelled I,II,III, and IV were present in all strains examined. However, when

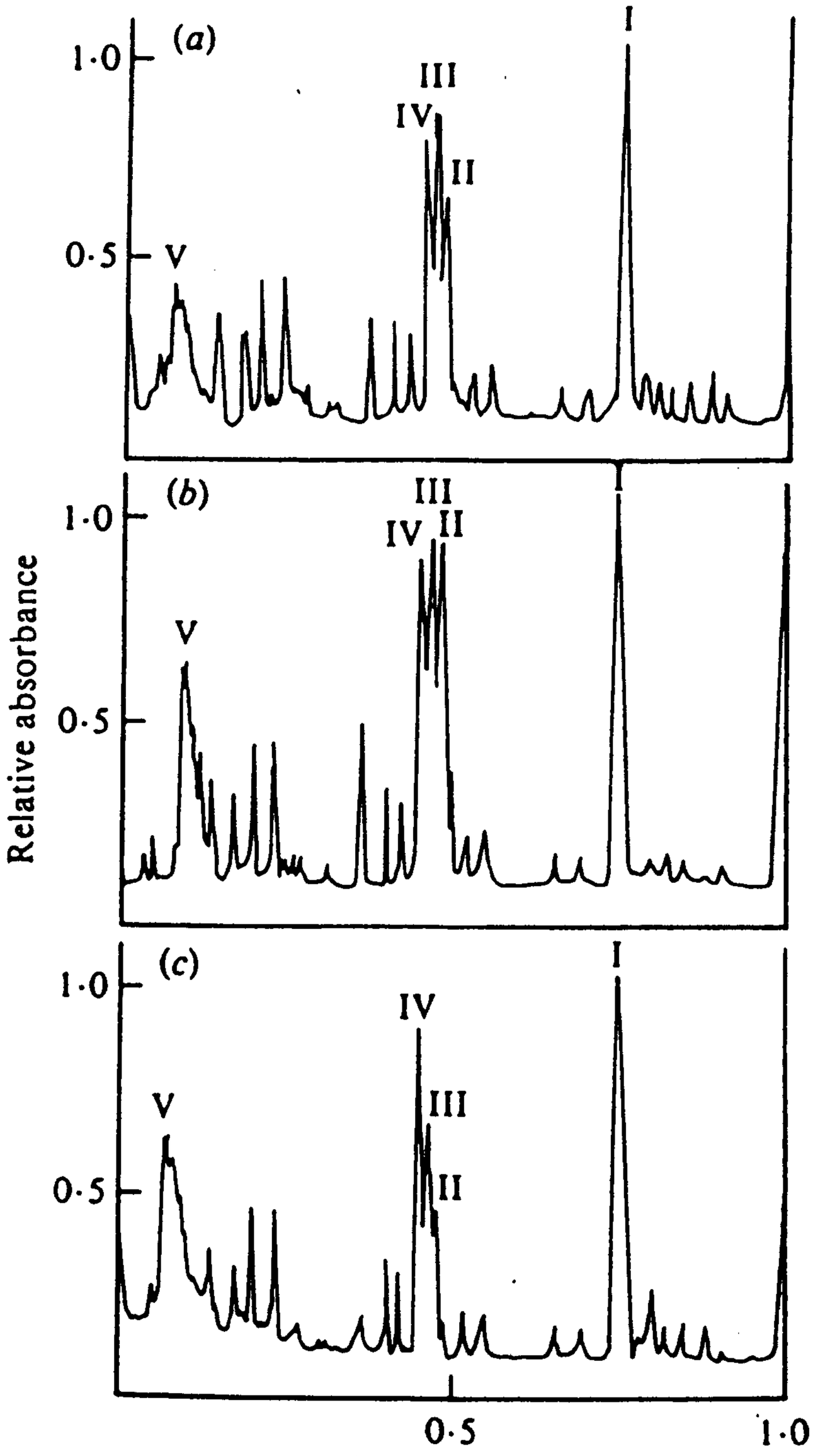
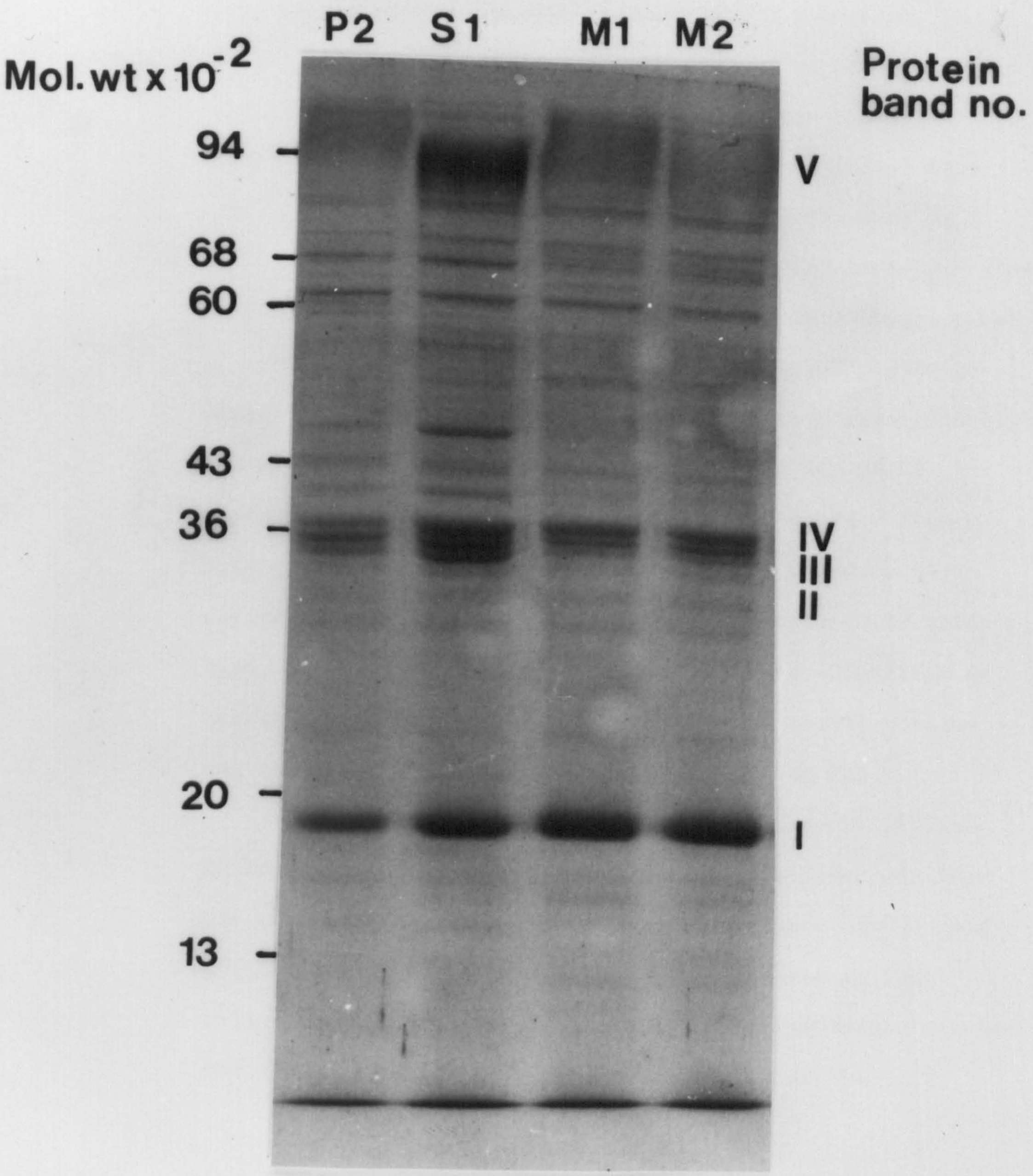


Fig. 4.13 Laser densitometer scan of outer-membrane proteins of (*a*) the wild-type, (*b*) crenated mutant H2S1, and (*c*) mucoid mutant H2M1. The major proteins, labelled I, II, III, IV and V, were present in all strains examined, but their relative proportions differed.

Figure 4.14

Electrophoresis of outer membrane fractions from wild-type (P2), a crenated mutant (S1) and two mucoid mutants (M1, M2).

Figure 4.14


P2
S1
M1
M2
Mol. wt x 10-2
Protein band no.
94
68
60
43
36
20
13
V
IV
III
II
I

outer membrane proteins were prepared from cells grown on ^{14}C amino acids a number of minor bands were identified yielding a minimum of over 30 proteins associated with this fraction. Figure 4.15 shows a fluorogram of SDS-PAGE gel, comparing the proteins present in the wild-type outer membrane and cytoplasmic proteins. The LPS protein complex characteristic of outer membranes was found to have the same mobility as the marker dye bromophenol blue and was demonstrated using PAS staining. Difference in the minor bands of the mutants compared to the wild-type were few. There was some loss of smaller bands from the crenated strains, accompanied by a change in mobility of a diffuse staining region labelled V. The mucoid strain electropherogram was similar to that of the wild-type except for small changes to the concentrations of the minor bands. Figure 4.15 also shows the extracellular proteins found in cell supernatants when concentrated by ultrafiltration of wild-type cell supernatants. These proteins were identified in the supernatants of wild-type crenated and mucoid strains and had the mobility of proteins No 16 and No 31.

The results in Table 4.5 shows the yields of LPS and EP from wild-type and mutant strains. These indicate that the crenated mutants H2S1 and H2S2 had between 40-55% less polysaccharide per cell dry weight as compared to the wild-type and mucoid strains. Also, only the mucoid strains H2M1 and H2M2 produced significant amounts of EP.

Figure 4.15

Fluorogram of SDS-PAGE gel showing outer membrane fraction of wild-type (OM) cytoplasmic proteins (S) and extra cellular proteins (EP) concentrated by ultra-filtration of wild-type cell supernatants.

Figure 4.15

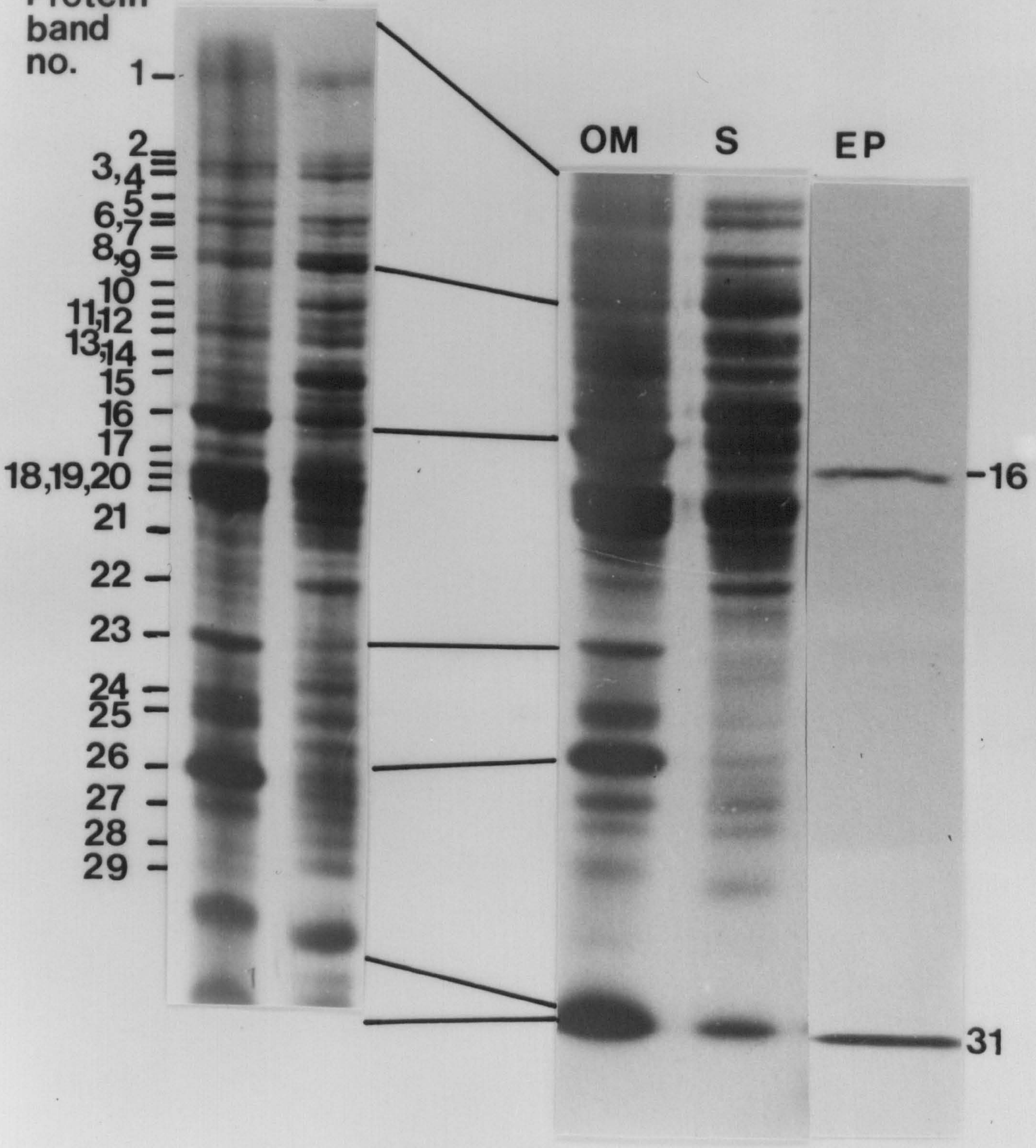

Protein band no.
OM
S
1
2
3,4
5
6,7
8,9
10
11,12
13,14
15
16
17
18,19,20
21
22
23
24
25
26
27
28
29
OM
S
EP
16
31

TABLE 4.5

Lipopolysaccharide (LPS) and exopolysaccharide (EP) yields from Pseudomonas fluorescens strains

Strain	Yield of crude lipopolysaccharide (% wt of dry cells)	Yield of lipid free polysaccharide (% by wt of lipopolysaccharide)	Yield of crude exopoly-saccharide (% of wt of dry cells)
H2P2 (wild-type)	2.87%	17.3%	1.26%
H2M1 (mucoid)	2.39%	19.4%	50.70%
H2M2 (mucoid)	2.26%	20.2%	173.00%
H2S1 (crenated)	1.98%	15.4%	0.87%
H2S2 (crenated)	1.59%	13.8%	0.93%

4.3.3.v) LPS analysis

LPS of each strain was analysed in order to determine whether changes in the mutant characteristics could be attributed to differences in LPS composition. The sugars in the polysaccharide fraction of the LPS were analysed as their respective PAAN derivatives. Amino sugars were deaminated, following resin-catalysed hydrolysis, to anhydroaldoses. Glucosamine, galactosamine, quinosamine and fucosamine are deaminated to 2,5 dideoxy-mannose, 2,5 dideoxy-talose, 2,5,6 trideoxy-mannose and 2,5,6 trideoxy-talose, respectively and these were also analysed as their respective PAAN derivatives. It was assumed that the relative proportions of the PAAN derivatives indicated the respective proportions of underivatised sugar in the original material and that the detector response was the same for each PAAN derivative (Seymour et al, 1979)

The polysaccharide from the wild-type and mutant strains consisted predominantly of glucose (25-30%), ribose (21-24%), dideoxyhexosamines (29-33%), rhamnose (11-15%) and glucosamine (4-6%) (Table 4.6) However, no significant differences were found between mutant types and the wild-type.

Significant amounts of ribose were detected in each strain studied, but this could have been due to extensive contamination of LPS extracts with nucleic acids. Although LPS extracts were purified by gel filtration, no significant

TABLE 4.6 Analysis of lipopolysaccharide from Pseudomonas fluorescens

	Values of each PAAN derivative are expressed as a percentage of the total carbohydrate					
Strain	Glucose	Ribose	Rhamnose	Glucosamine	Galactosamine	Dideoxyhexosamine
H2P2 (wild-type)	25.7	23.6	15.1	4.5	trace	30.9
H2M1 (mucoid)	30.1	20.9	11.9	4.7	trace	32.2
H2S1 (crenated)	30.1	21.9	10.9	4.3	trace	32.7
H2S2 (crenated)	26.4	24.1	13.3	6.5	trace	29.7

amounts of 2-deoxyribose were detected and all strains examined showed consistant amounts of ribose present.

4.3.3.vi) EP analysis

The EP from the wild-type and crenated mutant was difficult to precipitate with ice-cold isopropanol and formed a granular precipitate which could only be removed by centrifugation. The composition of the EP from these strains is shown in Table 4.7 and the major difference is in the amounts of galactose present. When corrected for the presence of galactose, the composition of these EP's was found to be predominantly sugars thought to be specific to the LPS composition (Table 4.6). Thus the EP released by the wild-type and crenated strains may be composed of LPS contaminated with agar which would rise to the galactose. Similar experiments with liquid batch culture confirmed that the galactose component was due to agar contamination, as no galactose was present. (Table 4.7)

Paper chromatography of hydrolysed polymers from mucoid strains revealed a pure uronic acid polymer composed of D-mannuronic acid with variable amounts of its 5-epimer, L-guluronic acid. O-acetyl groups were present on all strains tested at concentrations between 5-10%(W/W) of total EP, as calibrated against the standard. Table 4.7 shows values of mannuronic acid and guluronic acid as mannose and gulose derivatives, as the gulose reduced from

Table 4.7 *Analysis of exopolysaccharide from P. fluorescens strains*

Strain	Values of each derivative are expressed as a percentage of the total carbohydrate						
	Mannose	Glucose/Gulose	Ribose	Rhamnose	Glucosamine	Galactosamine	Dideoxyhexosamine
H2P2 (wild-type)	2·7	27·7	20·3	15·0	3·4	Trace	30·9
H2S1 (crenated)	2·3	30·1	23·0	10·7	4·6	Trace	29·3
H2S2 (crenated)	2·3	30·4	21·6	11·6	3·0	Trace	31·1
H2M1 (mucoid)	56·8*	43·2*	Trace	Trace	—	—	Trace
H2M2 (mucoid)	85·6*	14·4*	Trace	Trace	—	—	Trace

* Alditol acetate derivatives of mannuronic acid and guluronic acid following methyl ester formation and reduction with $NaBH_4$.

guluronic acid has the same retention time to glucose. These values are similar to the carbazole values calculated as per cent of dry weight and based on a glucuronic acid standard.

4.3.4 The use of radioamidination and radioiodination of membrane proteins in order to compare extrinsic membrane proteins of the wild-type P fluorescens H2 with adhesion mutants

i) Radioamidination The imidoesters isethionyl acetimidate (IAI) and ethyl acetimidate (EAI) were used to identify membrane proteins. ^{14}C labelled forms of these reagents were used to convert amines to radioactive amidines and IAI was used to label extrinsic or surface proteins, where as, EAI was used to label outer and inner membrane proteins. Table 4.8 shows the relative cpm of ^{14}C labelled membrane and cytoplasmic proteins after radioamidination of intact cells using both IAI and EAI followed by isolation and purification of membranes and cytoplasmic proteins. These results show that very little of the IAI penetrates the cell outer membrane and the values for inner membrane and cell supernatant for IAI incorporation may be an artifact of the purification steps. Figure 4.16 shows a flurogram of a SDS-PAGE gel comparing ^{14}C IAI and ^{14}C EAI labelled cells with ^{14}C amino acids labelled outer membrane proteins and supernatant proteins for wild-type P fluorescens H2. Many of the major protein bands associated with the outer

TABLE 4.8

Radioamidination of Intact wild-type cells using ^{14}C IAI and ^{14}C EAI

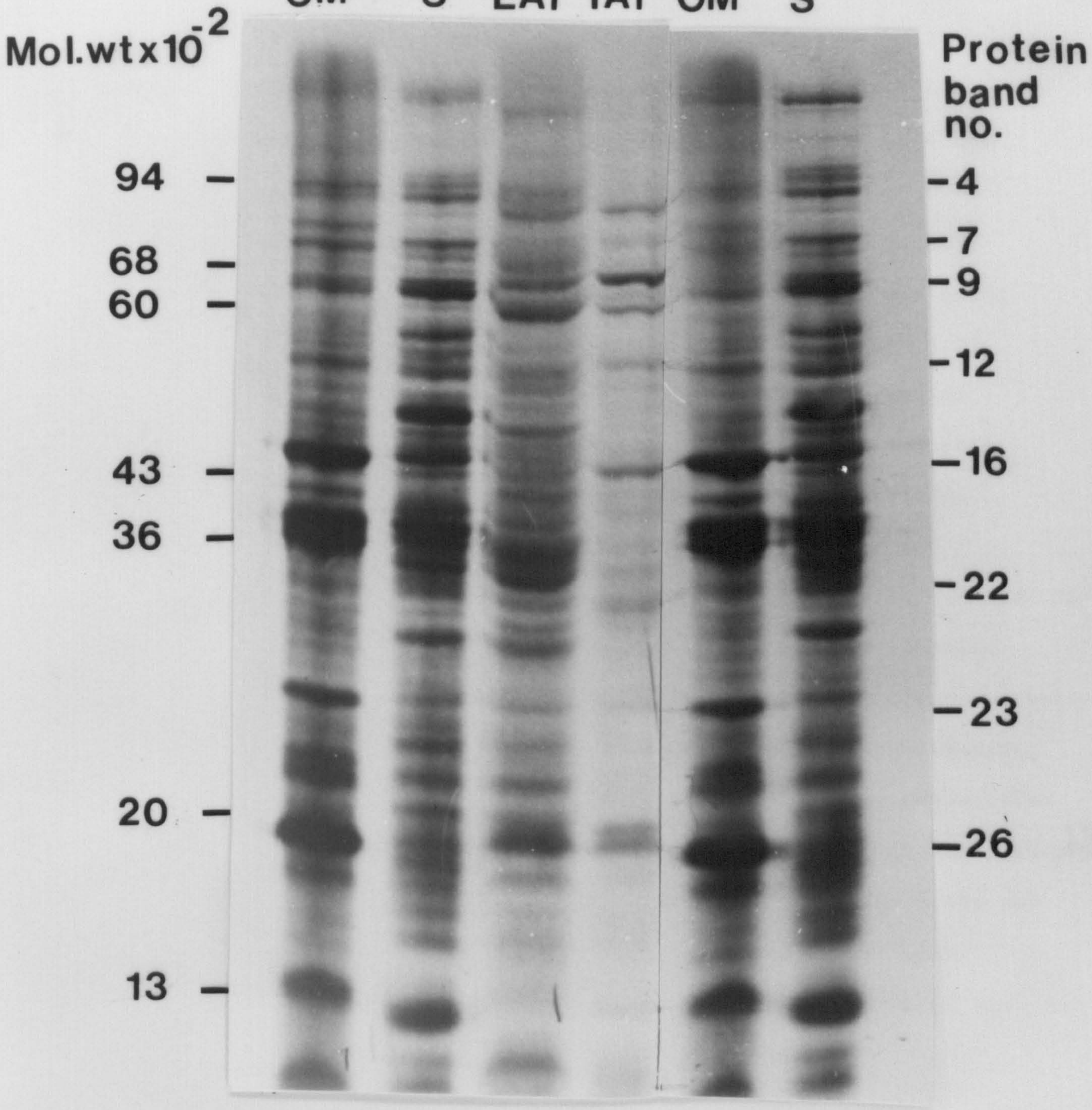
Imidester	Incorporation per sample (cpm)			
	Outer membrane	Inner Membrane	Supernatant	Incorporation
IAI	898000	7500	6300	0.1%*
EAI	958000	1123000	12364000	16.0%*

*cpm values were corrected to dpm using H number relationship with counting efficiency and compared to the total activity used ie. 45µci/sample

Figure 4.16

Fluorogram of SDS-PAGE gel comparing ^{14}C IAI and ^{14}C EAI labelled cells with ^{14}C amino acid labelled outer membrane proteins and cytoplasmic proteins for wild-type P. fluorescens M2

Figure 4.16


OM
S
EAI
IAI
OM
S
Mol.wtx10-2
94
68
60
43
36
20
13
Protein band no.
-4
-7
-9
-12
-16
-22
-23
-26

membrane of this organism were found in the EAI labelled track and were therefore considered extrinsic proteins. Protein bands in the EAI track comprised of the major bands in the outer membrane and cell supernatant tracks combined confirming that EAI was entering the cell. However these compounds were not used further to identify the extrinsic membrane proteins of adhesions mutants as they proved to be too expensive when compared with the radioiodination system.

ii) Radioiodination Comparisons of extrinsic outer membrane proteins of adhesion mutants carried out using ^{125}I radioiodination are shown in figure 4.17 and 4.18. Figure 4.19 gives a comparison of extrinsic proteins labelled by radioiodination and radioamidination along with ^{14}C amino acid labelled outer membrane proteins. Both methods give similar results, and as ^{125}I is inexpensive and does not require fluorography it was used for further analysis of mutant strains. Figure 4.18 shows an autoradiogram of ^{125}I labelled extrinsic membrane proteins of crenated and mucoid mutants compared to the wild-type. No major changes in the outer membrane protein composition was observed for the adhesion mutants. Although the pattern of each mutant showed some changes in the minor bands and the intensity of the major bands S2 track gave poor iodination levels which was repeated on a number of occasions indicating a reduced surface protein composition.

Figure 4.17

Comparison of extrinsic proteins labelled by radio-iodination (^{125}I) and radioamidination (EAI, IAI,) with ^{14}C amino acid labelled outer membrane proteins (OM) and cytoplasmic proteins (S).

Figure 4.17

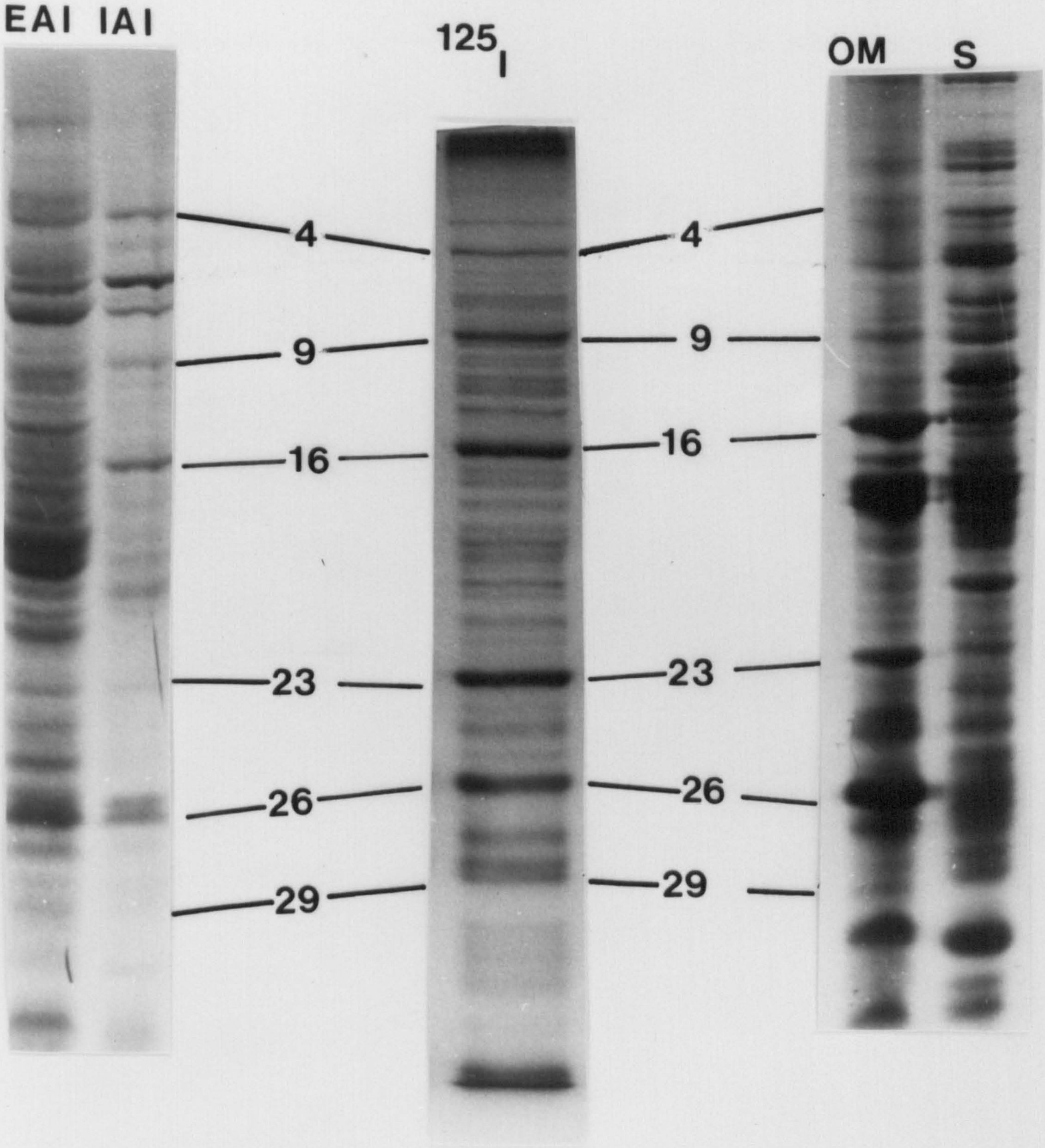


Figure 4.18

Autoradiogram of ^{125}I labelled extrinsic membrane proteins of crenated (S1,S2) and mucoid strains (M1, M2) compared to the wild-type (P). Also included is the page blue stained SDS-PAGE gel showing total cell proteins for each strain.

Figure 4.18

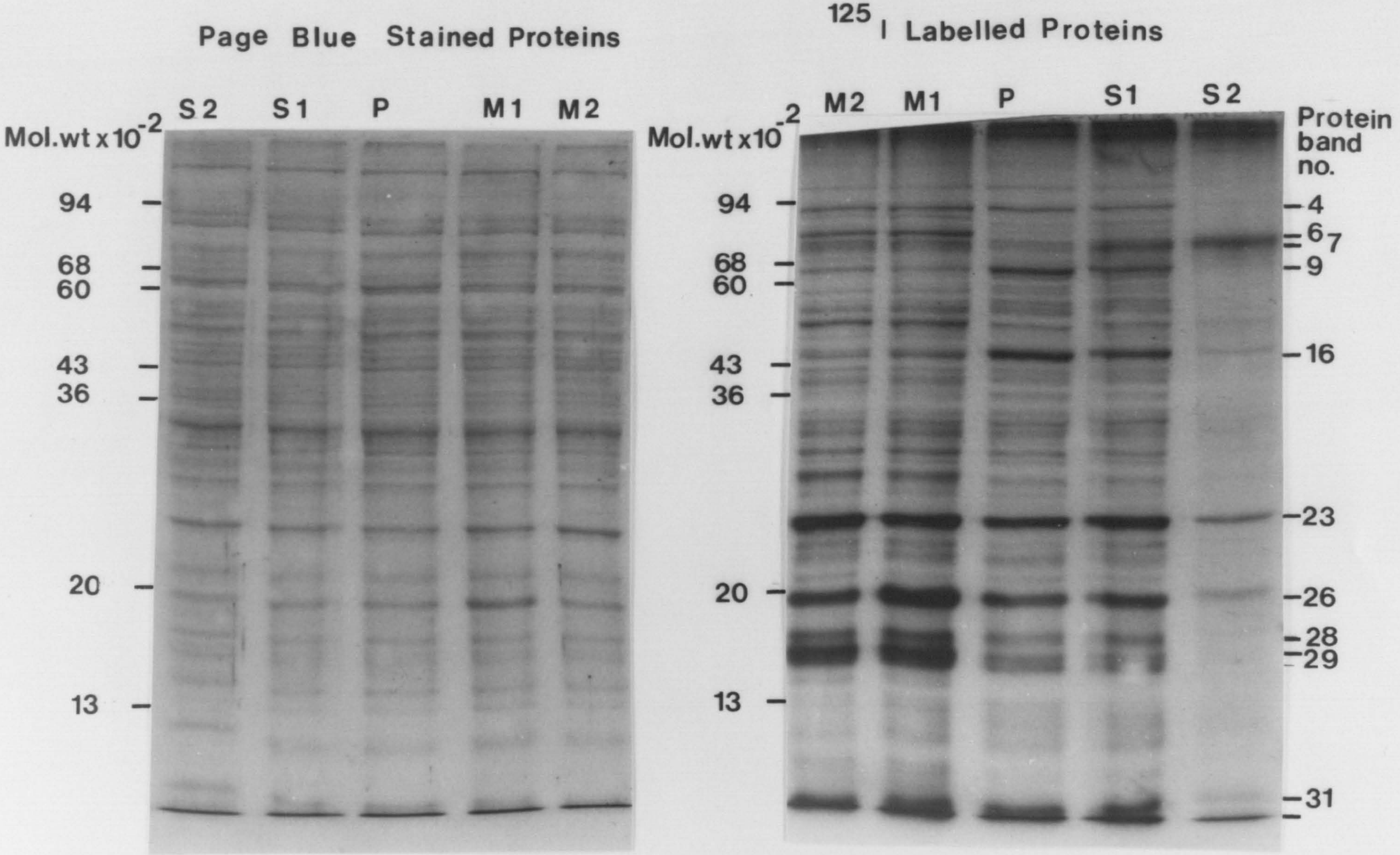
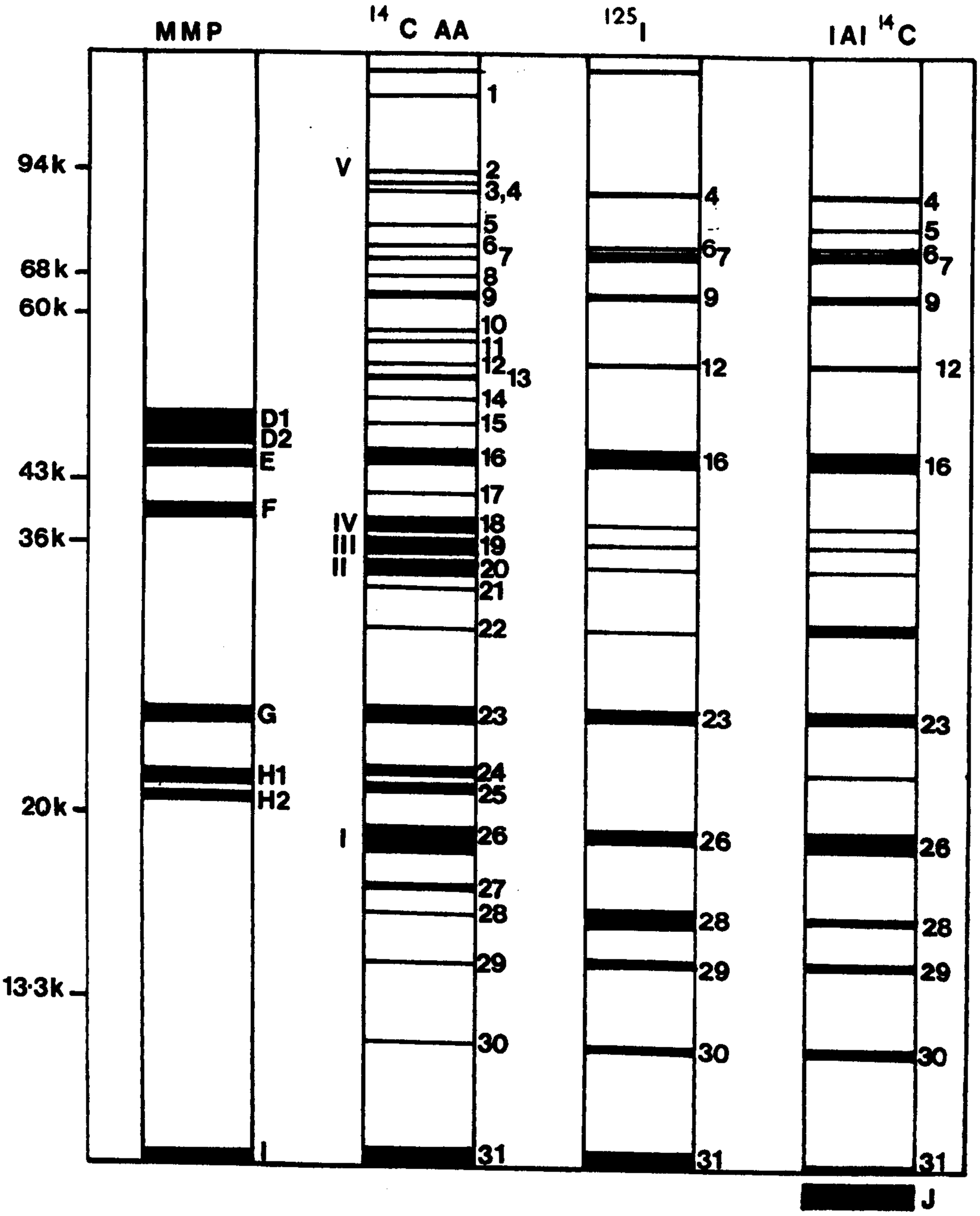

Page Blue Stained Proteins
S2
S1
P
M1
M2
Mol.wt x10-2
94
68
60
43
36
20
13
125I Labelled Proteins
M2
M1
P
S1
S2
Mol.wt x10-2
94
68
60
43
36
20
13
Protein band no.
4
6
7
9
16
23
26
28
29
31

Figure 4.19

Comparison of extrinsic proteins labelled by radio-iodination (^{125}I) and radioamidination (IAI ^{14}C) along with ^{14}C amino acid labelled outer membrane proteins of wild-type P. fluorescens H2. Also included is an outline of the major membrane proteins (MMP) of P. aeruginosa after Hancock and Carey (1979).

Fig 4.19


MMP
^{14}C AA
^{125}I
IAI ^{14}C
94k
68k
60k
43k
36k
20k
13·3k
D1
D2
E
F
G
H1
H2
I
V
IV
III
II
I
1
2
3,4
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J

4.3.5 Characterisation of the outer cell surface of a number of Pseudomonas isolates and their adhesion mutants

After the observed relationship between colony morphology and attachment ability shown by P. fluorescens H2 was recognised, a number of colony mutations of different pseudomonads were tested for attachment ability and a comparison of their surface characteristics was made. Table 4.9 shows a list of these adhesion mutants along with their attachment characteristics. Two types of adhesion mutants were found, ie. either with an increase in attachment ability over that of the wild-type (adh^+) or with a decrease as compared to wild-type attachment ability (adh^-) and both these mutations were found to correlate with changes in colony morphology. Mucoid or smooth colonies became smooth or rough, respectively corresponding to a change from adh^- to adh^+ and rough or smooth colonies became smooth or mucoid, respectively for a change from adh^+ to adh^-.

i) Extrinsic membrane proteins of three pseudomonas isolate (H8602, HP and H40) are shown in figure 4.20. Similar results were obtained for these organisms as for P. fluorescens H2 when extrinsic proteins from wild-type and adhesion mutants were compared using radioiodination. Only a small number of changes were observed in the composition of the minor bands and the intensity of the major bands.

TABLE 4.9

The attachment of adhesion mutants of several Pseudomonas isolates following growth in carbon limited batch culture

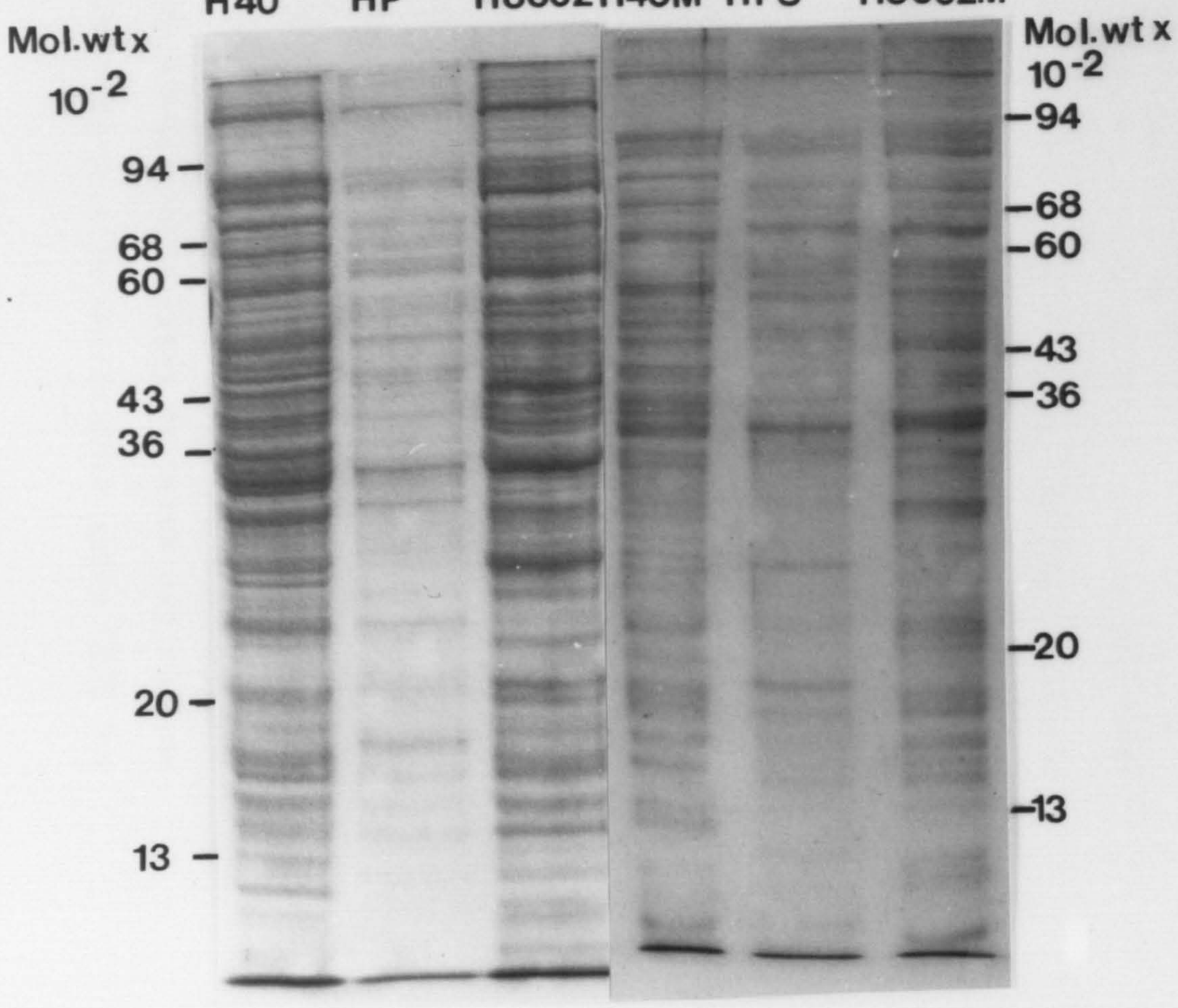
Strain No	A 590 Polystyrene	Tissue culture polystyrene	Colony Morphology
H8602	0.30(0.03)	0.190(0.011)	Smooth
H8602M	0.023(0.005)	0.011(0.004)	Mucoid
HP	0.04(0.01)	0.030(0.017)	Mucoid
HPS	0.31(0.023)	0.170(0.012)	Smooth
H40	0.33(.04)	0.072(0.007)	Smooth
H40M	0.09(.02)	0.043(0.006)	Mucoid
H35	0.203(0.06)	0.075(0.013)	Smooth
H35R	0.356(0.11)	0.275(0.016)	Rough

Figure 4.20

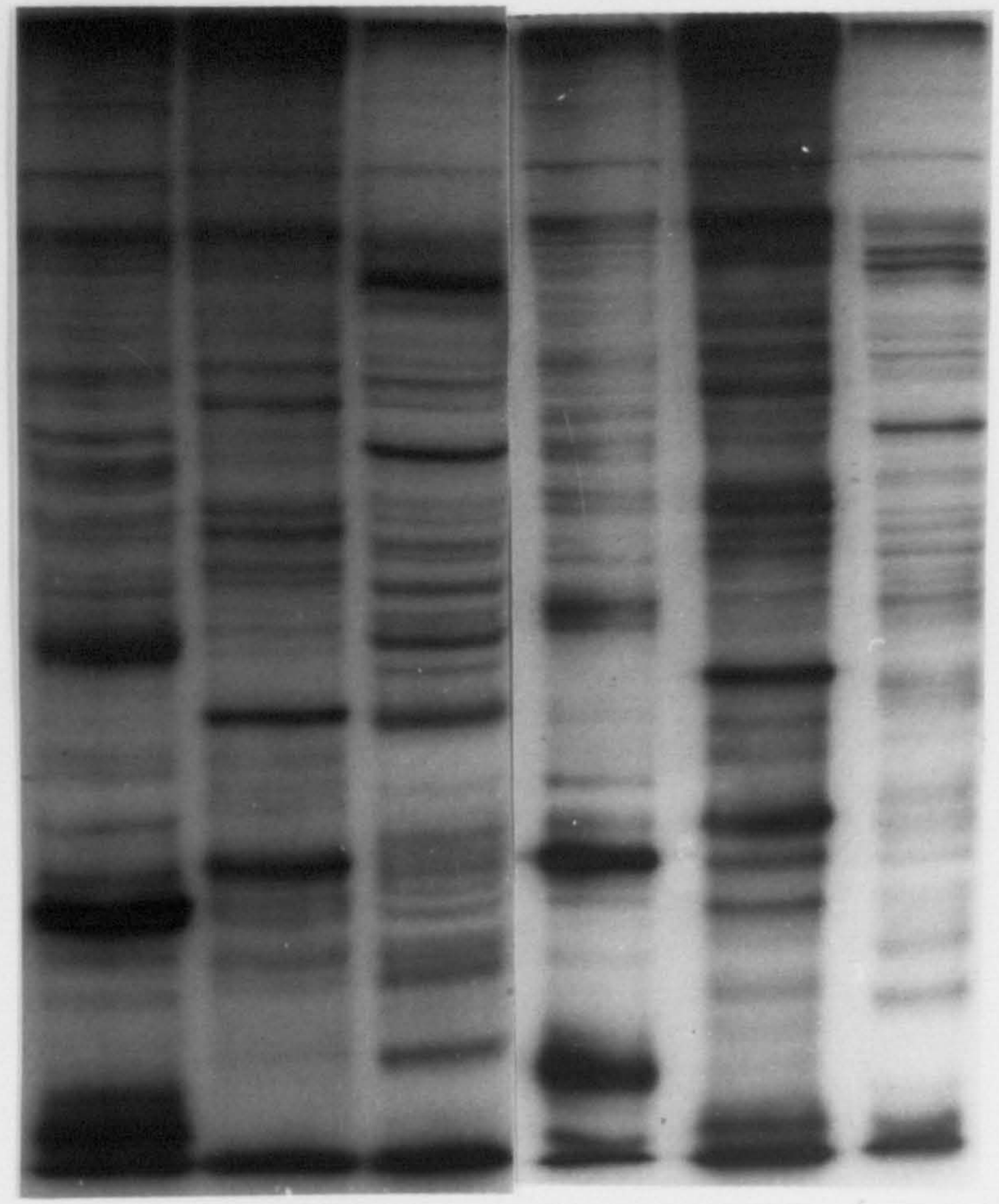
Autoradiogram of ^{125}I labelled extrinsic membrane proteins of H8602, H8602M; HP, HPS and H40, H40M. Also included is the page blue stained SDS-PAGE gel showing total cell proteins for each strain.

Figure 4.20

Page Blue Stained Proteins



^{125}I Labelled Proteins



ii) LPS Sugar composition of wild-type and the adhesion mutants are shown in table 4.10. These were prepared as previously described (4.2.5) and were analysed by GLC of PAAN derivatives. Three strains, H8602 H40 and HP showed similar sugar compositions to their respective adhesion mutants. However, LPS composition of the rough mutant H35R showed a reduction in the relative amounts of rhamnose, glucosamine, galactosamine and dideoxyhexosamines which would indicate a loss of the O-specific chains characteristic of some smooth-rough type mutations in the structure of LPS

iii) EP Composition was examined for these adhesion mutants and compared to the wild-type strains. Table 4.11 shows the sugar composition of EP from wild-type and adhesion mutants. Strains H8602 and H40 showed a change from EP composed predominantly of sugars throught to be specific to the LPS composition in the wild-type to a heteropolysaccharide EP in the mutants. For example a mutant of H8602 produced a uronic acid polymer composed of D-mannuronic acid and L-guluronic acid, and a mutant of H40 produced a glucose/ galactose/rhamnose polymer in the ratio 1:1:2 (H40). Wild-type strain HP also produce a heteropolysaccharide EP composed of glucose and galactose in the ratio 1:1. The ability to produce this polymer in both liquid and solid culture was lost in the mutant HP-S, which produced an

TABLE 4.10 Analysis of lipopolysaccharide from Pseudomonas strains and adhesion mutants

Values of each PAAN derivative are expressed as a percentage of the total carbohydrate

Strain	Glucose	Ribose	Rhamnose	Glucosamine	Galactosamine	Dideoxyhexosamine	Unknowns
H 8602 (wildtype)	22.8	1.3	17.6	18.8	6.7	15.3	18.5
H 8602 (M mucoid)	26.2	1.4	16.3	19.0	8.9	13.4	14.8
H35 (wildtype)	22.2	12.8	18.3	4.0	1.6	14.2	26.9
H35-R (rough)	71.0	8.0	13.3	1.2	trace	3.8	2.7
H40 (wildtype)	20.3	trace	38.4	4.9	6.8	20.2	9.4
H40M (mucoid)	20.7	trace	40.2	5.4	5.8	18.0	9.9
HP (wildtype)	28.8	32.2	18.2	trace	1.5	10.2	9.1
HP-S (smooth)	30.0	30.0	19.0	trace	1.8	9.0	10.2

TABLE 4.11 Analysis of extracellular polymer from Pseudomonas strains and adhesion mutants

Values of each AA derivative are expressed as a percentage of the total carbohydrate

Strain	Glucose	Galactose	Mannose	Ribose	Rhamnose	Unknowns and others
H 8602 (wildtype)	30.6	trace	trace	4.3	20.2	44.9
H 8602 (M mucoid)	42.4 [a]	trace	45.2 [a]	3.3	1.2	7.9
H 40 (wildtype)	20.9	trace	trace	trace	53.2	25.9
H 40 M (mucoid)	28.5	24.5	trace	trace	46.9	trace
HP (wildtype)	46.0	52.0	trace	1.2	trace	0.8
HP-S (smooth)	32.0	trace	trace	11.2	29.0	27.8
H35 [b] (wildtype)	50.5	40.0	trace	4.4	5.1	-

a
Alditol acetates derivatives of mannuronic acid and guluronic acid following methyl ester formation and reduction with $NaBH_4$

b
EP from solid media grown cells

EP composed predominantly of the sugars identified in this strain's LPS. Also shown in Table 4.11 is the composition of EP from strain H35 when grown on solid media. This was also found to be a heteropolysaccharide of glucose and galactose, which may have the ratio 1:1 when allowances are made for the contaminating LPS.

4.3.6 Adsorption of EP and LPS from adhesion mutants and wild-type P fluorescens H2 to hydrophobic and hydrophilic polystyrene substrata.

The adsorption isotherms of EP isolated from wild-type H2P2 crenated mutant HS1 and mucoid mutant H2M2 are shown in figures 4.21, 4.22 and 4.23, respectively. These graphs show the isotherms for three substrata, polystyrene (PS) tissue culture polystyrene (TCPS) and sulphonated polystyrene (SP) and are plots of the amount of polymer adsorbed in $\mu g.cm^{-2}$ against the bulk liquid concentration of each polymer in solution ($mg.ml^{-1}$). These isotherms varied in both concentration range over which the adsorbed polymer was measured and the preference of each polymer for three substrata. The term EP as used here refers to the radio-labelled alcohol precipitated material that was released into the culture medium during growth and therefore EP from the wild-type and crenated culture contains a complex mixture of extracellular products. However the extracellular polysaccharide produced by the mucoid mutant was further purified by gel filtration chromatography and was adsorbed in its pure form. In the case of wild-type EP, both PS and

Figures 4.21-4.24.

Adsorption isotherms of EP isolated from wild-type P. fluorescens H2P2, crenated mutant H261 and mucoid mutant H2M2 are shown in figures 4.21, 4.22 and 4.23, respectively and isotherms for wild-type H2P2, LPS is shown in figure 4.24. The isotherms for three substrata, PS (O), TC-PS (●) and SP (X) plotted for the amount of polymer adsorbed in ug cm^{-2} against the bulk liquid concentration of each polymer in solution (mg ml^{-1}).

Fig 4.21

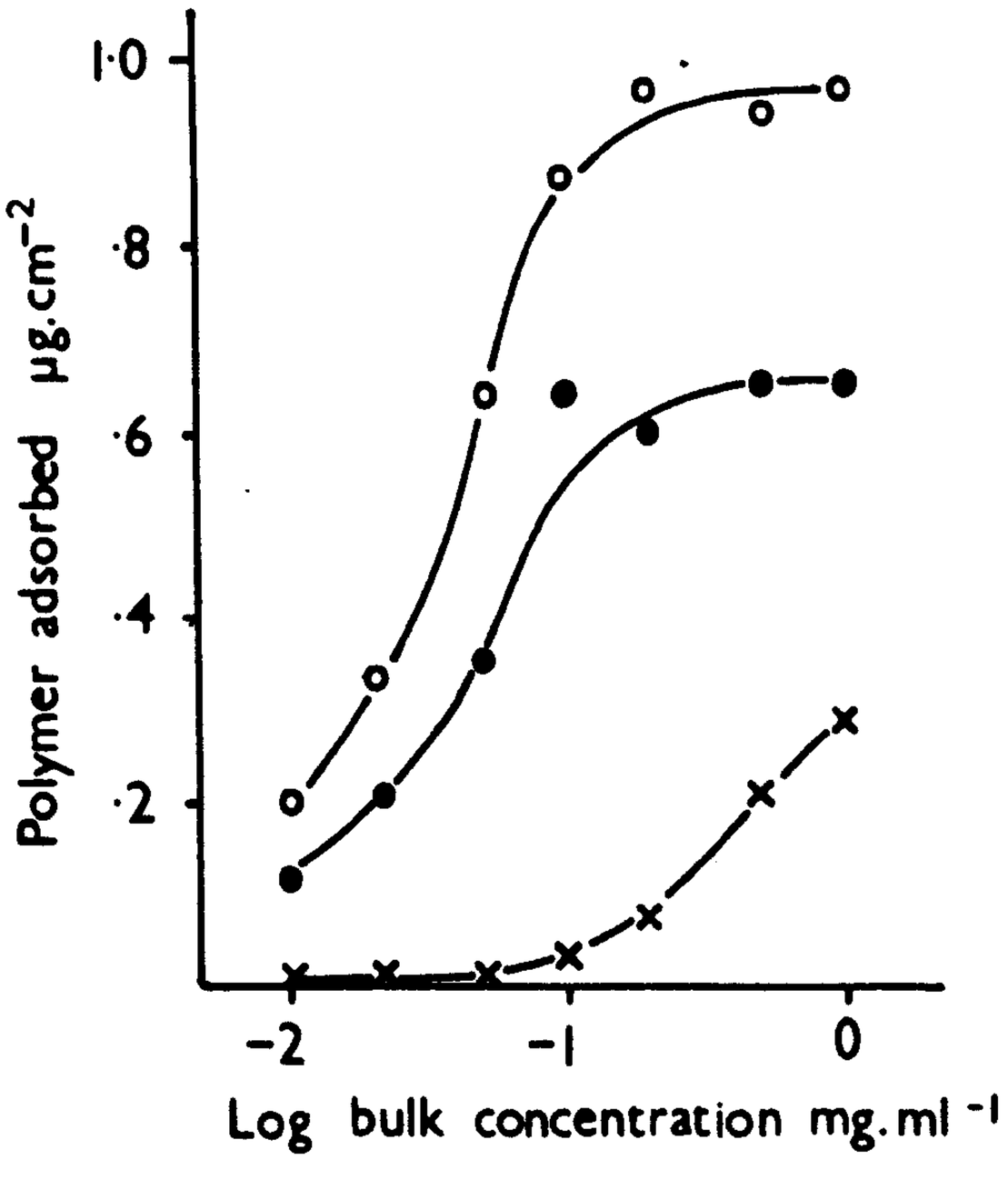


Fig 4.23

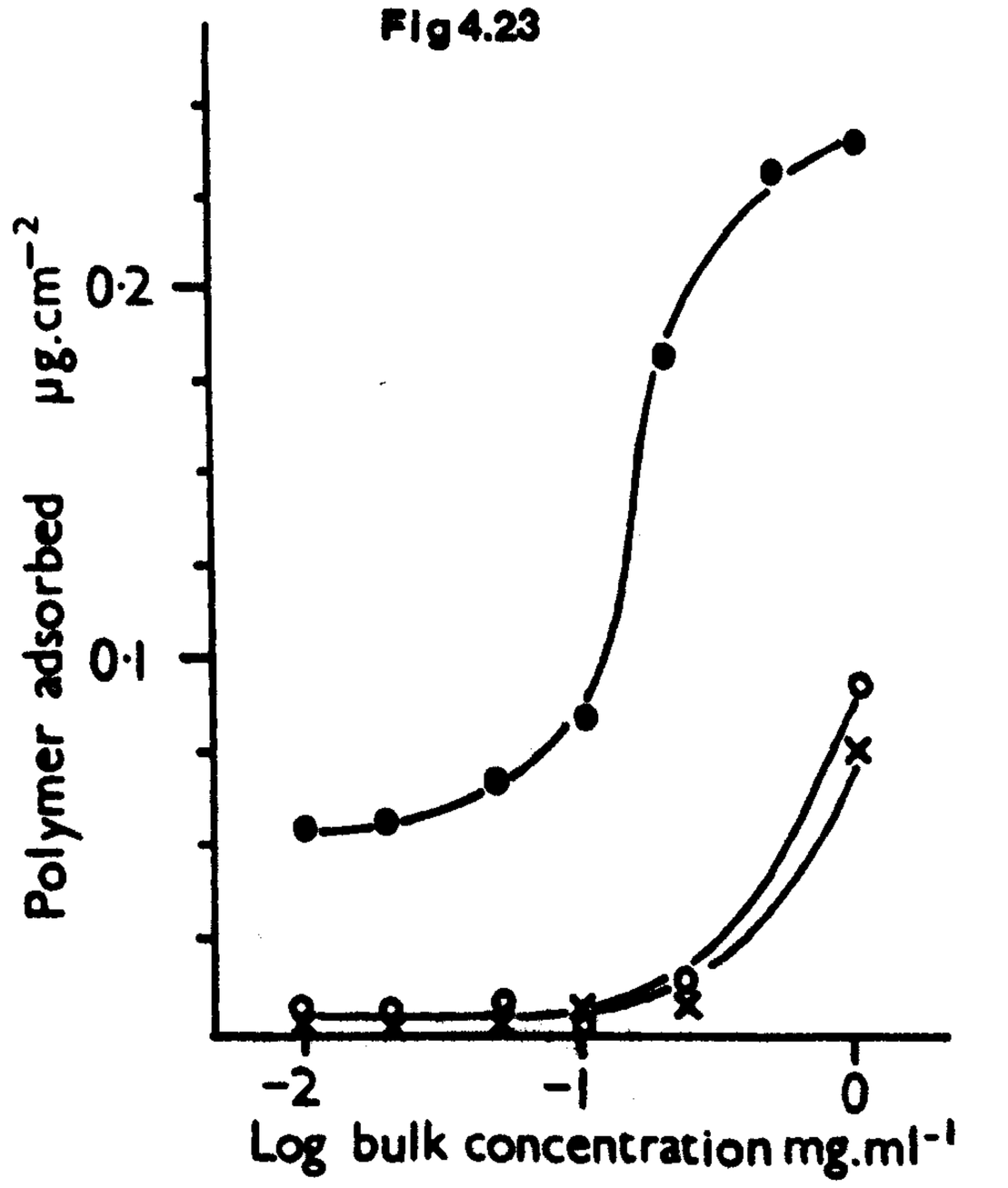


Fig 4.22

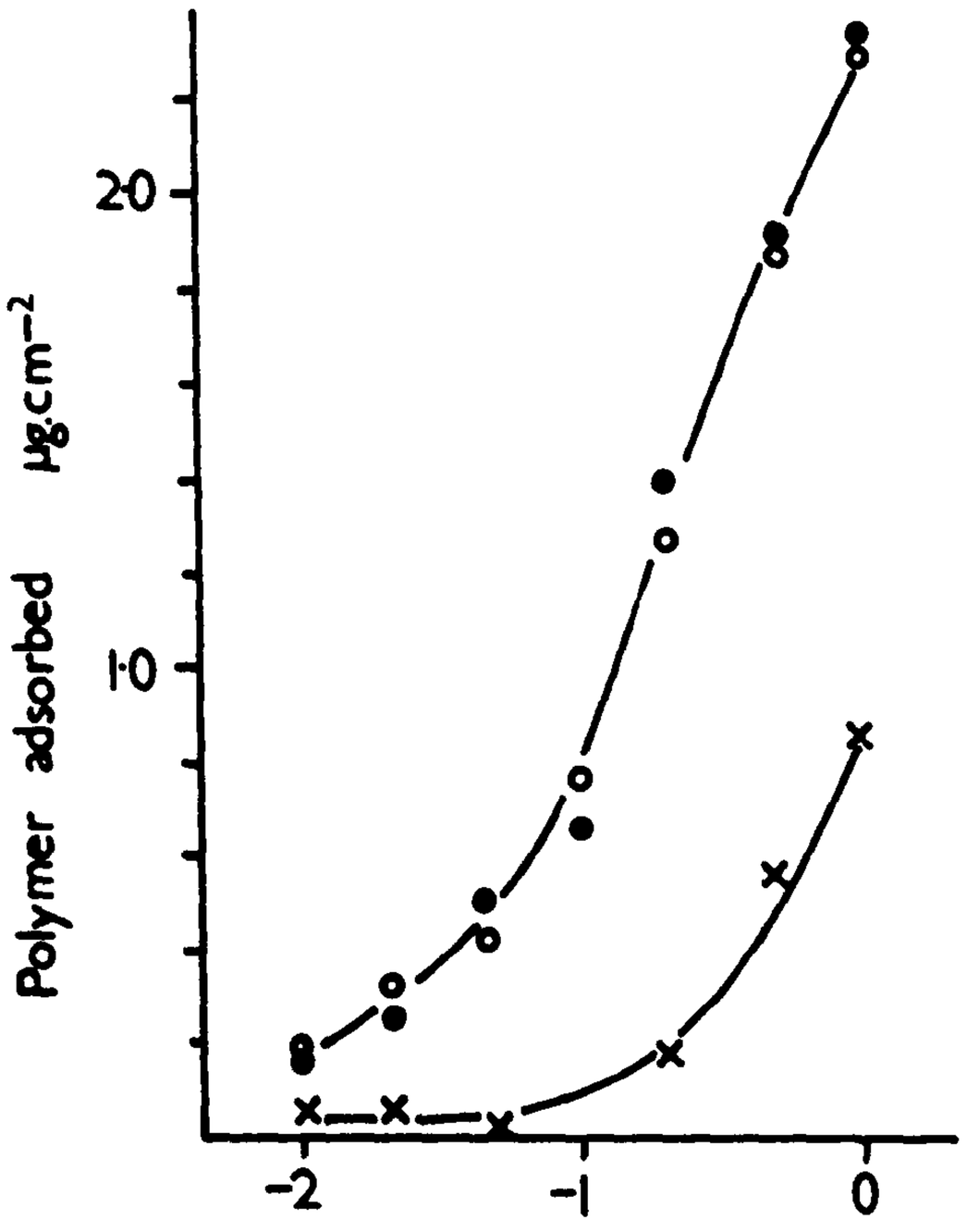
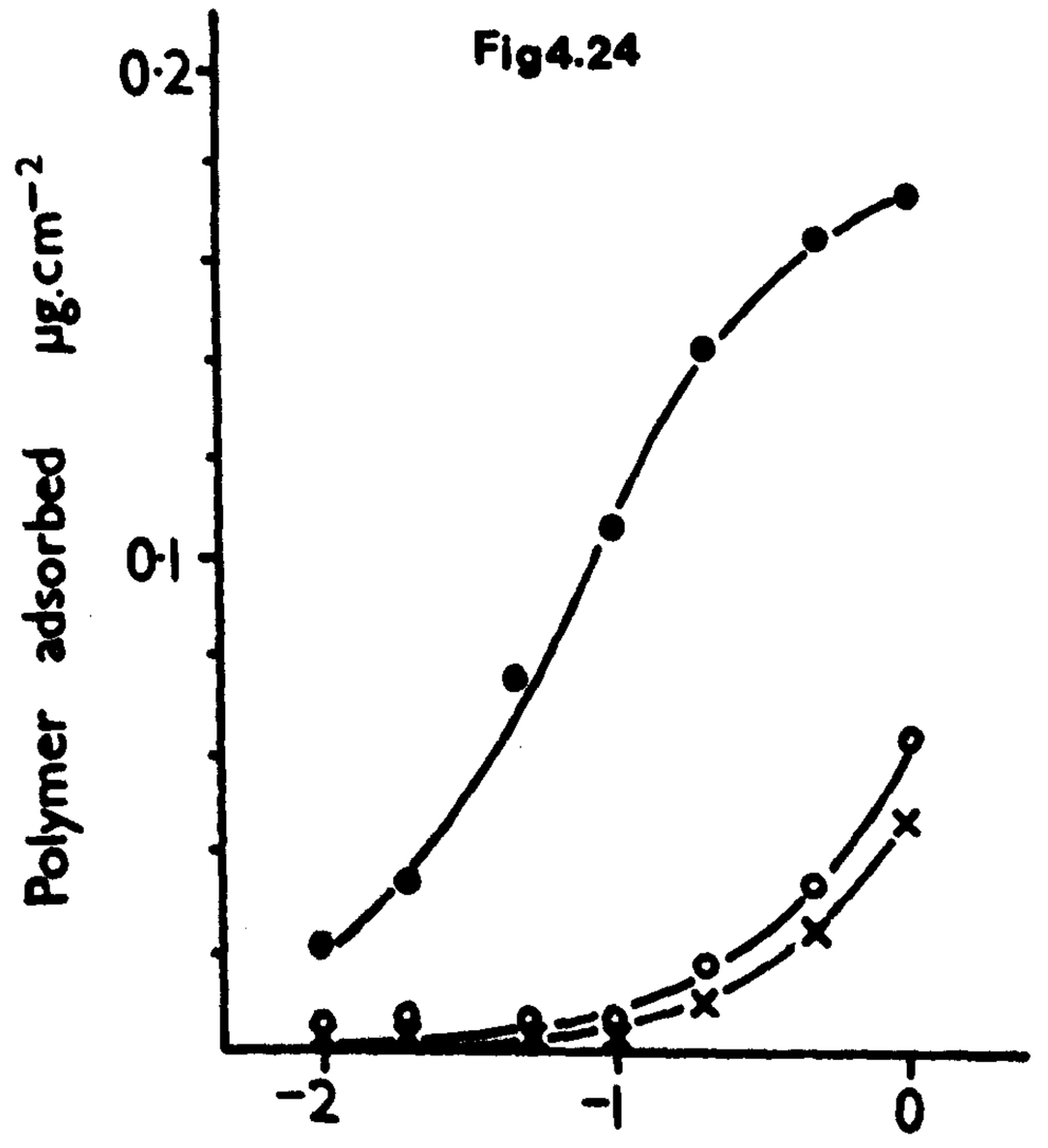


Fig4.24



TCPS isotherms are in the range 0.1-1.0 $\mu g\ cm^{-2}$ with higher levels of adsorption on the PS which levels off at 0.9 $\mu g\ cm^{-2}$ adsorbed polymer at a bulk concentration of 0.1 $mg\ ml^{-1}$ or above. The TC PS isotherm also forms a plateau, at 0.6 $\mu g\ cm^{-2}$, at a bulk concentration of 0.1$mg\ ml^{-1}$ or above. The SP isotherm, on the other hand, was in the range 0.01-0.3 $\mu g\ cm^{-2}$ and did not reach a plateau. The isotherms from the EP isolated from the mucoid mutant (fig. 4.23) were found to span a lower adsorbed polymer concentration range from 0.01 to 0.25 $\mu g\ cm^{-2}$ for all three substrata tested. Increased levels of adsorption were found on the TC PS surface compared to the PS and SP substrata and all three substrata failed to form a plateau within the bulk concentration range examined.

Isotherms from the EP isolated from the crenated mutant (fig. 4.22) were found to span a higher range of adsorbed polymer concentration 0.02-2.5$\mu g\ cm^{-2}$. The TC PS and PS Isotherms were identical within experimental error, while the SP isotherm showed lower levels of adsorption. These isotherms also failed to form a plateau within the bulk concentration range examined.

Isotherms for the wild-type LPS is shown in figure 4.24; these curves are similar to the mucoid EP (fig.4.23) They have the same concentration range for adsorbed polymer and the same preference for the TC-PS substratum.

These experiments were repeated and samples duplicated for each test substratum.

4.3.7 Electron microscopy of negatively stained bacteria following culture on solid and liquid media

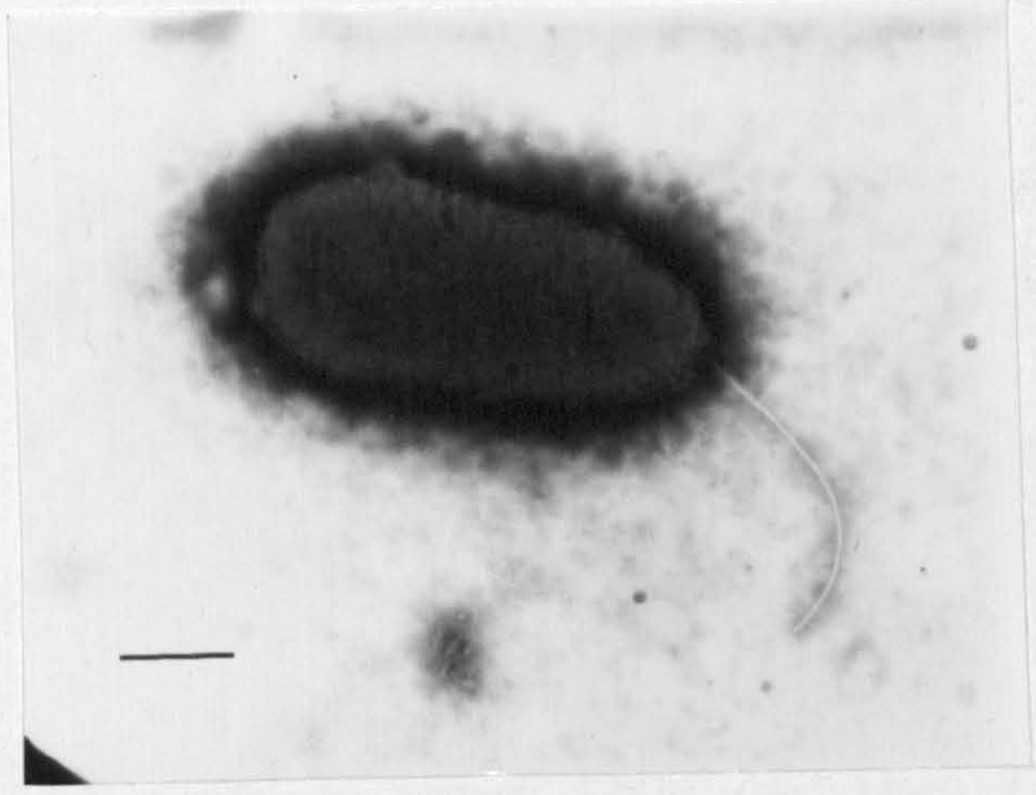
The outer surface structures including flagellum and pili or fimbriae were examined for the wild-type and adhesion mutants by negative staining of cells grown on solid or liquid media. Electronmicroscopy of these Pseudomonas strains previously examined for attachment ability is shown in figures 4.25-30 . Control E coli cells known to possess both pili and fimbriae are shown in figures 4.31-4.32. No pili or fimbriae like structures were visible in the Pseudomonas strains examined, several showed a halo staining zone typical of a bacteria enveloped in a slime or capsular layer (figures 4.28-30), particularly if the cells were grown on solid media. Polar flagella were clearly visible on the majority of cells and the control electronmicrographs show that the staining procedure was good enough to detect pili and fimbriae when present. Figure 4.32 shows E.coli F+ cells with bacteriophage QB in order to demonstrate F pili.

Figures 4.25-4.30

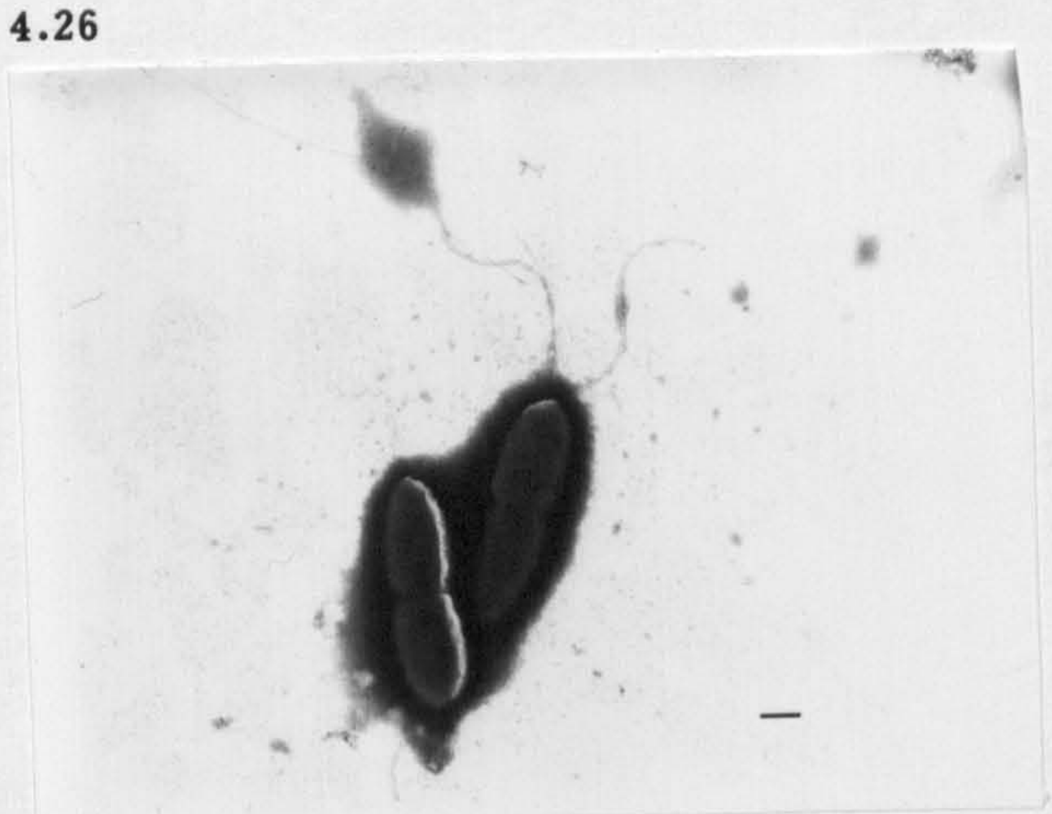
Electronmicrograms of Pseudomonas strains examined for their attachment ability are shown in Figures 25-30. They include the following strains and growth conditions:

Figure	Strain	Growth Conditions
4.25	P. Fluorescens H2	broth
4.26	P. putida H35	"
4.27	P. " H40	"
4.28	P. Fluorescens H2	solid
4.29	P. putida H35	"
4.30	P. " H40	"

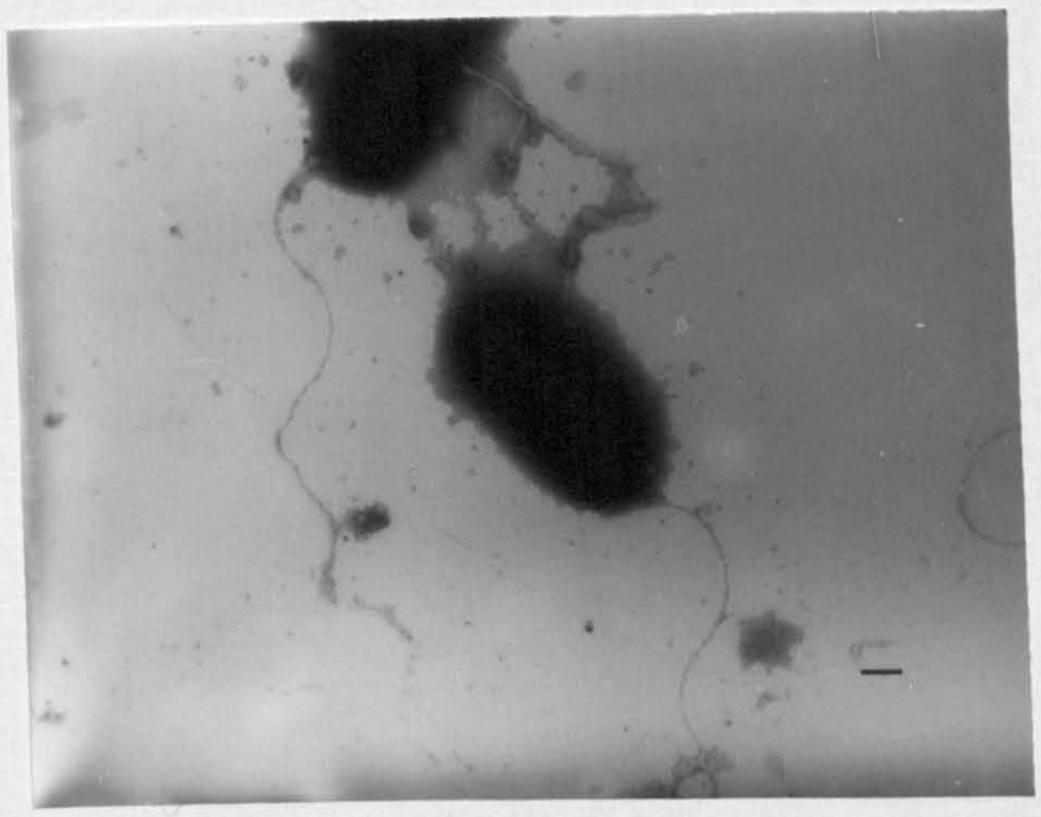
4.25



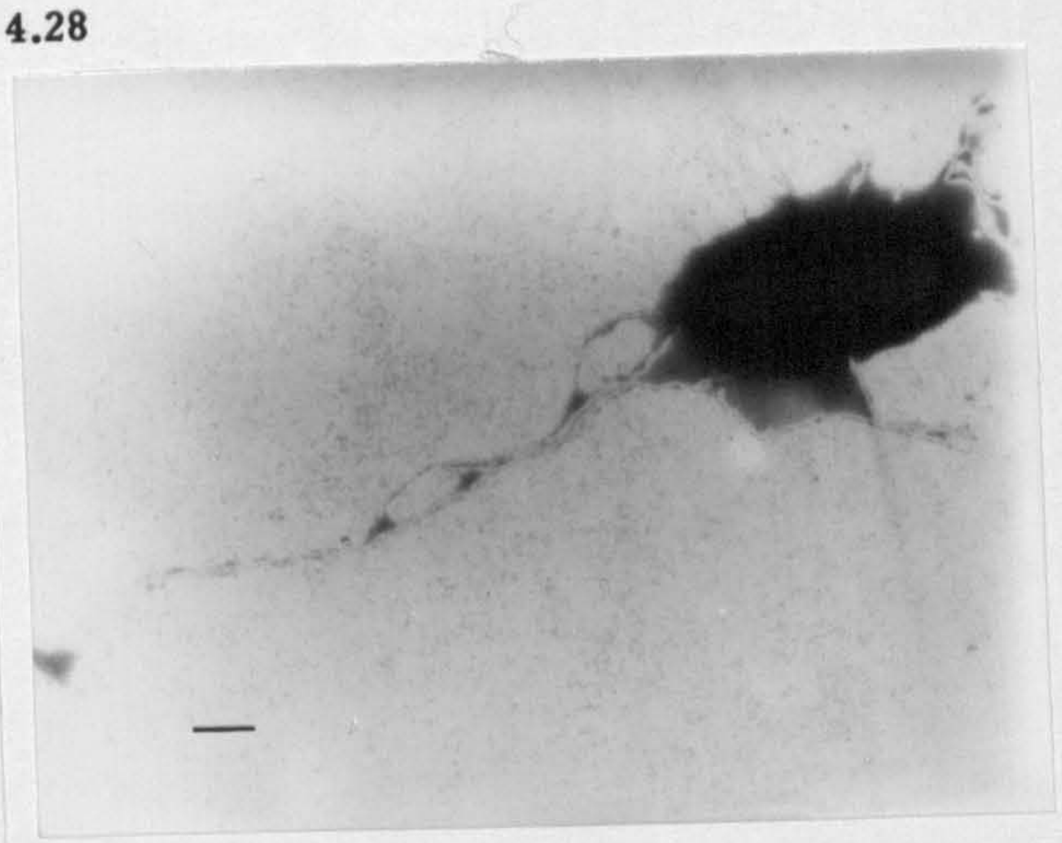
4.26



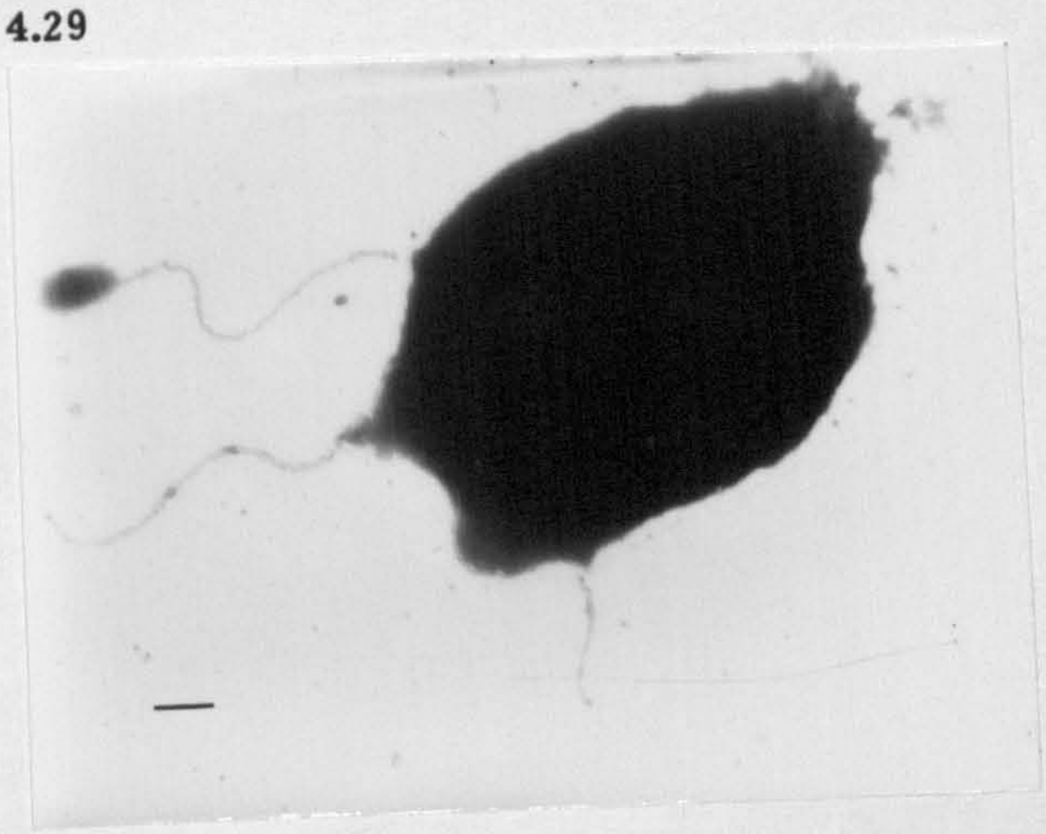
4.27



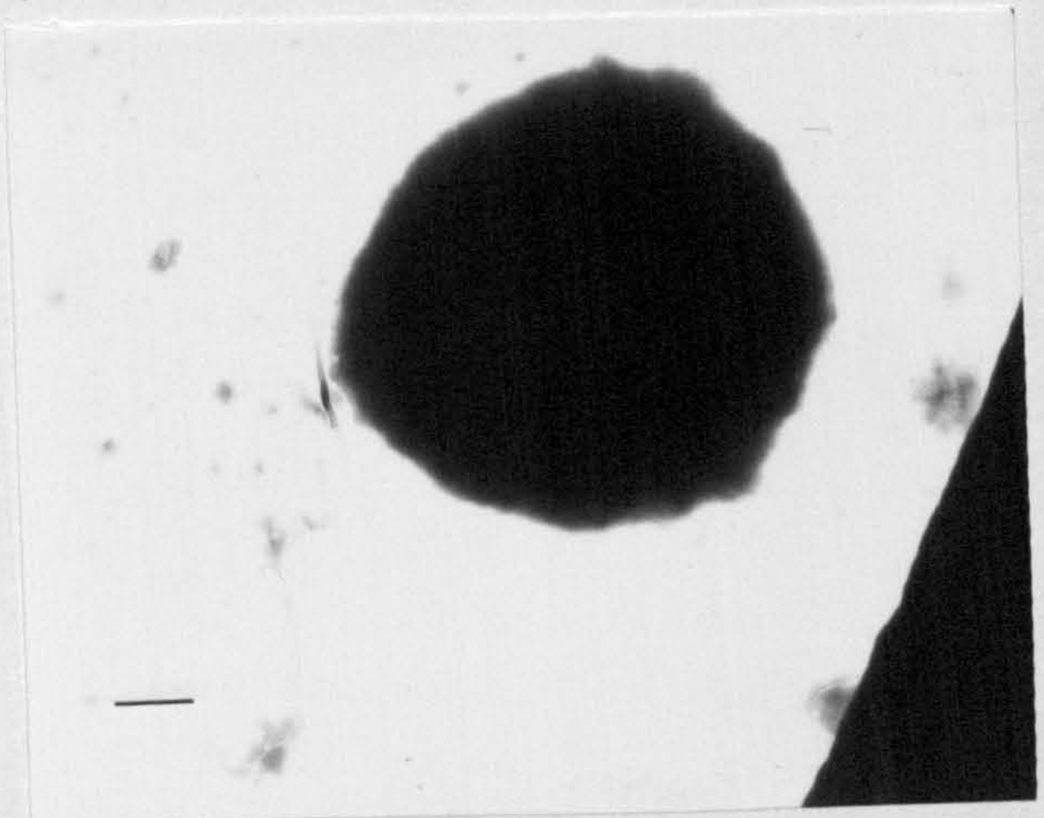
4.28



4.29



4.30

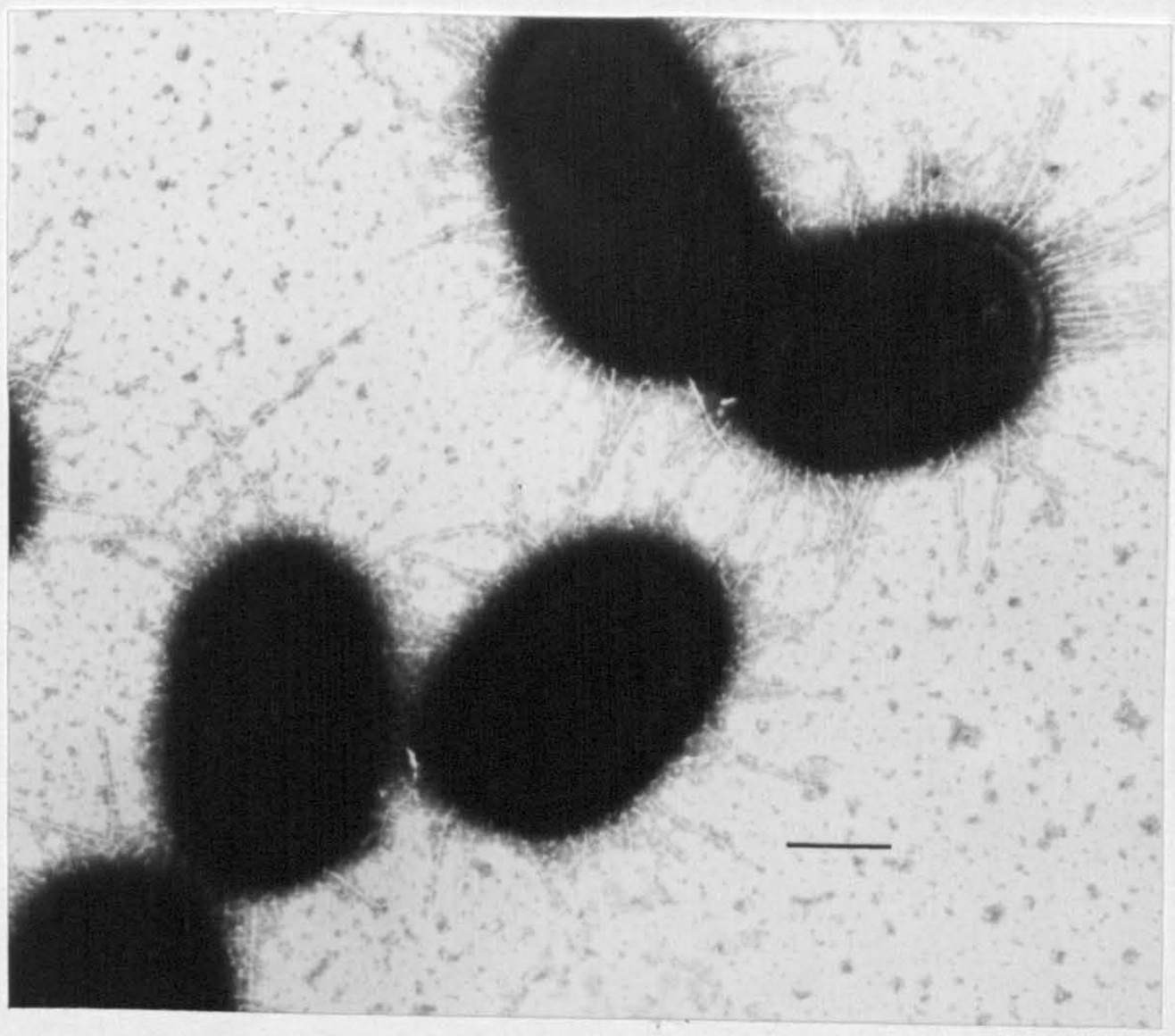


scale bar = 1μm

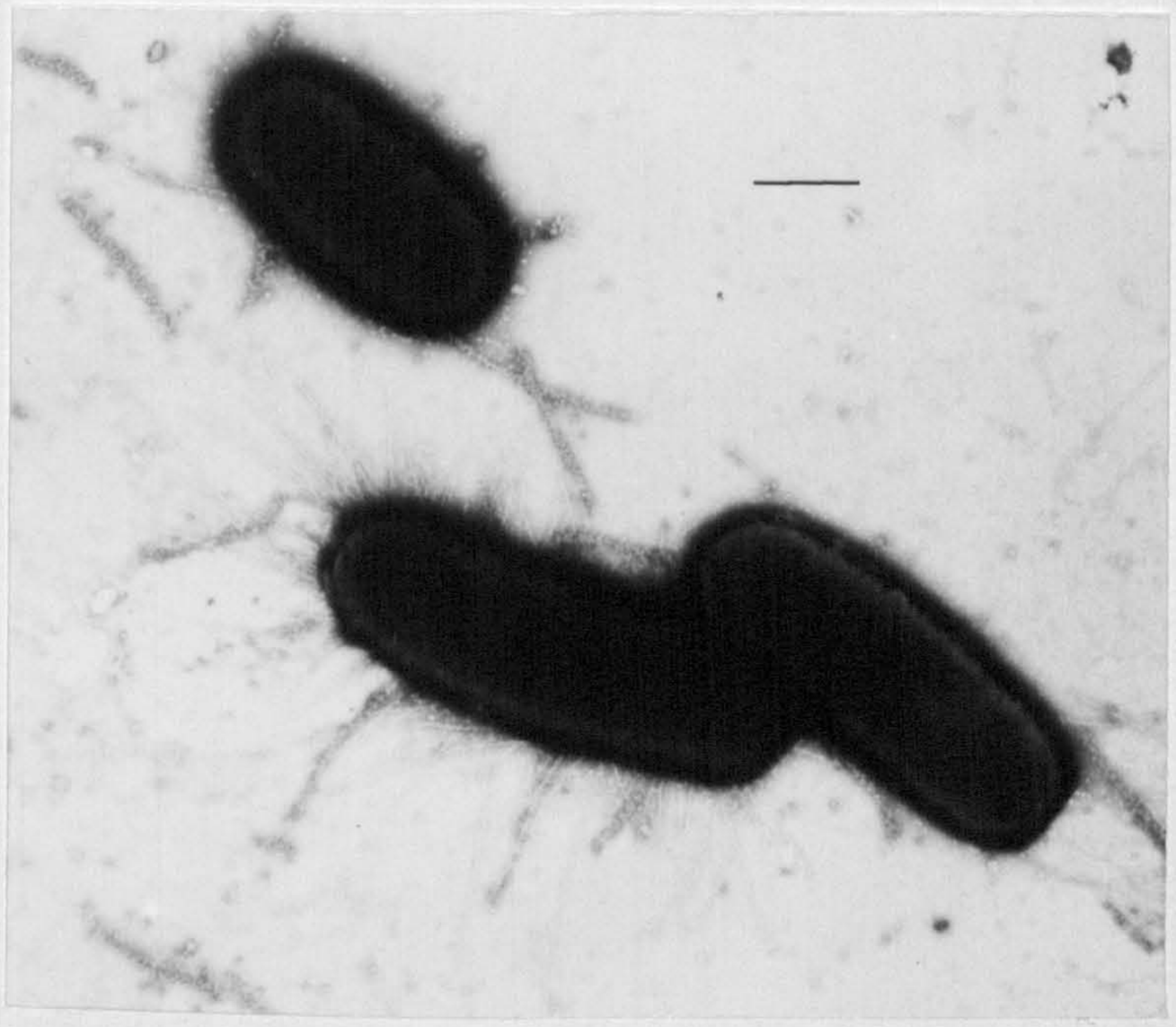
Figures 4.31-4.32

Electronmicrograms of Escherichia coli cells known to possess both pili and fimbriae are shown in figures 4.31 and 4.32 Figure 4.32 shows E. coli F^+ cells with bacteriophage QB in order to demonstrate F. Pili.

4.31



4.32



—— = 1 µm

4.4 DISCUSSION

4.4.1 The effects of physicochemical parameter on the attachment of bacterial cells to test substrata

The first part of this Chapter examined several parameters which influence adhesion of the P.fluorescens H2. The intention was to determine whether an active or passive mechanism was involved in attachment and also to establish whether growth conditions prior to the attachment process influence subsequent attachment.

The relationship between time and the number of pseudomonads attached (figure 4.3) allows two measurements of attachment. First, the initial rate of adhesion can be determined by attachment measurements over 30mins and secondly a measurement of saturation level after 2h. Similar results have been reported for a marine pseudomonad. NCMB 2021 (Fletcher, 1977) and support a physicochemical model of adhesion due to the spontaneous nature of the interaction. The main physiological response on the part of the bacterium influencing attachment is cell motility. Motility increases the chances that a bacterium may encounter a potential attachment surface. The relationship between culture concentration and cell attachment has been previously examined (Fletcher, 1977) with the result that bacterial attachment to test substrata such as polystyrene can be represented as a Langmuir adsorption isotherm. This model describing molecular adsorption from solution onto

a surface also supports the view that bacterial attachment may be controlled to an appreciable extent by nonbiological phenomena.

The effects of temperature on bacterial attachment are shown in figure 4.4. This data may be redrawn in order to determine whether this interaction obeys the relationship originally described by Arrhenius:

$$\log_e v = \frac{-\Delta H}{RT} + C \qquad 4.1$$

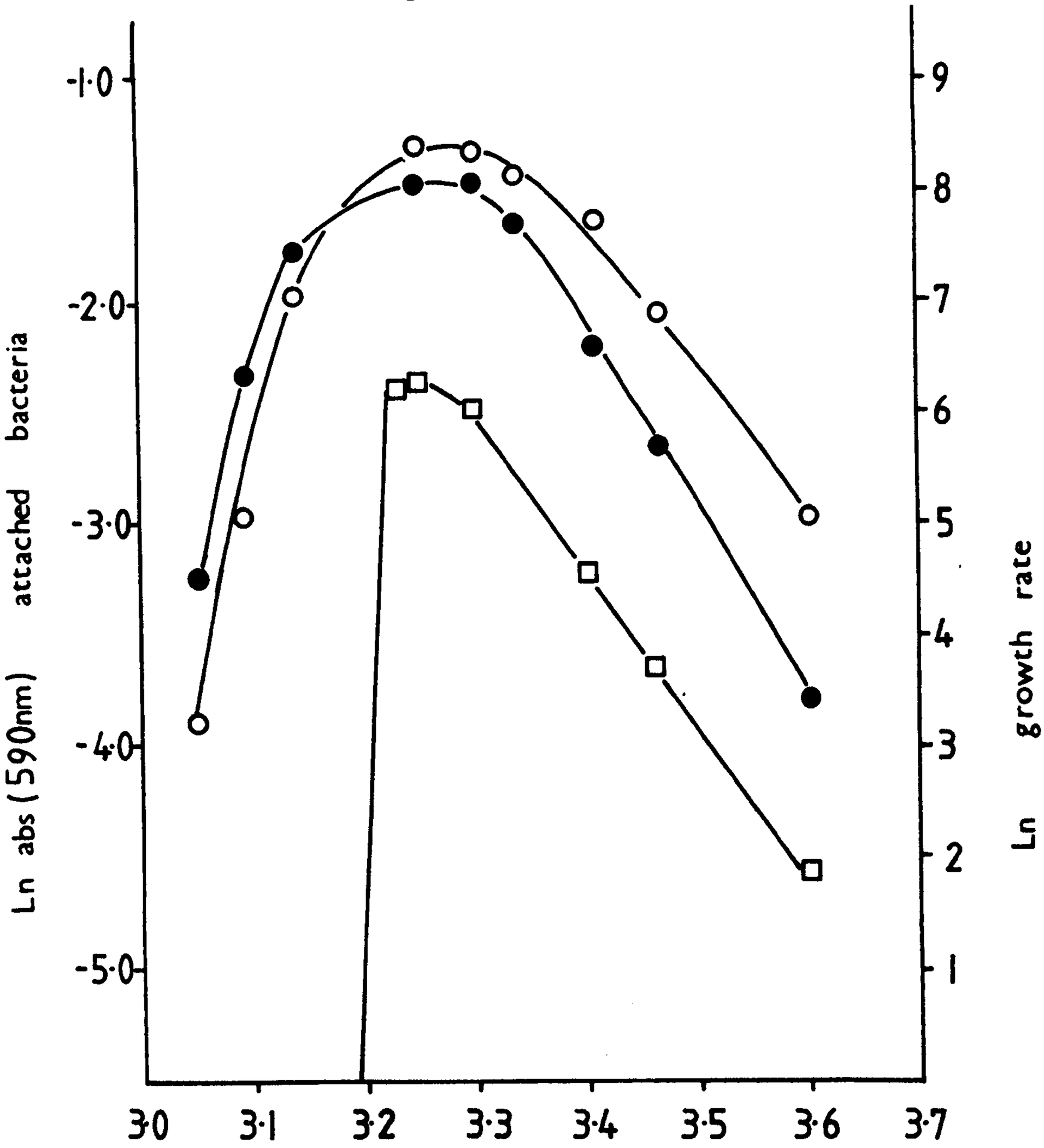
Where v represents the reaction velocity of H ie. the activation energy of the interaction, R the gas constant, and T the temperature in degrees Kelvin. Figure 4.3.3 shows a plot of the rate of adhesion for bacteria attaching to PS and TC-PS substrata as a function of T^{-1}, with a comparable plot of the rate of growth of P. fluorescens H2 also as a function of T^{-1}. Similar curves have been reported for Arrhenius plots of growth rate vs temperature for E coli (Ingraham, 1958). All three curves are linear only over a portion of the temperature range for growth. Although, growth rate falls abruptly at the upper limit of the temperature range, bacterial adhesion falls less abruptly with significant levels well above the maximum temperature for growth. This supports the view that adhesion can take place without time-dependent physiological processes such as polymer secretion or cell respiration. The abrupt fall in growth rate at high temperatures is caused by the thermal

Figure 4.33

The initial Ln rate of attachment of P. fluorescens H2 to PS (○) and TC-PS (●) substrata as a function of T^{-1}, with a comparable plot of the Ln rate of growth of this organism also as a function of T^{-1} (□). In abs (590nm) of crystal violet stained bacteria was used to measure the Ln rate of attachment over 1h.

32

Fig 4.33


-1·0
-2·0
-3·0
-4·0
-5·0
Ln abs (590nm) attached bacteria
9
8
7
6
5
4
3
2
1
Ln growth rate
3·0
3·1
3·2
3·3
3·4
3·5
3·6
3·7
T⁻¹ °Kelvin x 10³

denaturation of proteins in activating cell physiological processes. Adhesion may eventually be inhibited by thermal denaturation of cell membranes and cell surface components.

The decrease in attachment with a lowering of temperature has several explanations involving both cell physiology and physicochemical adsorption. The obvious effect on cell physiology would be to reduce cell growth, often accompanied by an increase in the production of secondary metabolites such as polysaccharides. In this case, however, the cells have been transferred to a low nutrient buffer solution where "shift-down" or starvation conditions would prevail. This would also reduce the growth rate and remove any substrata for polymer synthesis. In studies of "shift-down" growth of E. coli a culture growing in nutrient broth was washed by centrifugation and placed in a synthetic medium where a lower growth rate would be affected. After the shift, cell division and DNA synthesis proceeded for some time, resulting rapidly in small cells. Net protein and RNA synthesis, however, were resumed only gradually and the size and composition characteristic of the new medium were only obtained after three to four hours. (Mandelstam and McQuillen, 1976) Thus the most probable effect of a decrease in temperature is to lengthen the time required by the cell to readjust to its new growth conditions.

These changes in cell physiology must also influence cell motility which has been shown to affect attachment numbers (Fletcher, 1979).

Lower temperatures would also increase the viscosity of the medium and the bacterial surface. At low temperatures "hydrophobic" or Van der Waals forces are weakened while all other types of bonds (except for chemisorption) are strengthened. Therefore adsorption dependent upon weak or short range (Van der Waals) bonding between solute and surface over a number of sites would favour higher temperatures for optimum conditions, (Shaw, 1970).

Two further physical parameters influencing bacterial attachment to solid surfaces are pH and ionic strength of the medium. Theoretically both these factors affect the long range interactions described by the DLVO theory. This model would predict that an increase in bulk electrolyte concentration would reduce the electrical double layers and increase attraction between particle and surface of like charge. The pH would also influence the ζ and the net surface charge. At pH values >pI (the isoelectric point) the cells would bear a net negative charge whereas, at pH values <pI cells would be positively charged. Unfortunately no accurate estimation of the isoelectric point of the test organism could be made as no access to suitable equipment was possible. The isoelectric point, however, of most

Gram-negative bacteria is similar and has been determined for E coli using isoelectric equilibrium experiments to be pI = 5.7 (Sherber and Lakshmi 1973). Calculations based on this value estimate the number of effective negative charges to be 1.32×10^{13} cm^{-2}. By removal of negative or positive charges using chemical modification of the cell surface it is possible to determine the total charges of the cell surface. Values of 1.47×10^{13} cm^{-2} for positive charges and 2.89×10^{13} cm^{-2} for negative charges have been determined for E coli cells treated with ethyleneimine and formaldehyde, respectively. It is interesting to compare these values with the negative charges associated with the substrata. Calculation of the negative surface charge density on the polystyrene dishes using a dye binding method (Marondas and Thomas, 1970) indicated a PS surface charge density (scd) of 6.0×10^{13} cm^{-2} and TC-PS scd of 30.0×10^{13} cm^{-2} (Maroudas, 1975). These charges are probably carboxyl groups from peracid catalysts used in the polymerization and for the tissue culture treated dishes further carboxyl groups are introduced during a corona discharge process (Marondas, 1973). Therefore as both cell surface and substrata are clearly negatively charged, long range repulsion forces would be expected at low ionic strength (10^{-4}M) switching to an attractive force as the double layer is reduced by an increase in ionic strength (10^{-1}M).

Attachment profiles for P. fluorescens H2 at high and intermediate ionic strengths, over a range of media pH values to PS and TC-PS are shown in figure 4.6. Maximum attachment levels to both substrata were obtained at pH 5.0-5.5, which indicates that maximum attachment will take place when the cell net surface charge is minimal ie. close to the cell isolelectric point. A second peak, however, was observed for all pH attachment profiles with maximum attachment levels in the range pH7.5-8.0. This peak cannot be explained in terms of long range forces as both the bacterial surface and substrata will carry a net negative charge over this pH range. Similar pH profiles have been reported for the adsorption of other Gram-negative species to a weakly acidic cation exchange resin, Bio-Rex 70, which is an acrylate based carboxyl resin. (Wood, 1980). Although increased levels of adsorption to these resins were found at pH levels below 5.0, peaks similar in figure 4.6 were found at pH 5.5 and 7.5 for both E. coli and Sal. typhimurium The effect of ionic strength on these pH profiles is opposite to that predicted by the DLVO theory. In fact higher levels of attachment were found at all pH values examined for the intermediate ionic strength (10^{-2}M) when compared to the high ionic strength ($5x10^{-1}$M)(fig. 4.5). These observations again support a hydrophobic mechanism for adhesion, whereby, hydrophobic interactions are weakened by

the addition of NaCl. This may act as a chaotropic ion by virtue of the anionic effect on water structure. (Dandliker and Saussure, 1971) Alternatively NaCl may act on bacterial surface proteins and polysaccharides producing not only the charge of the cell surface but also affecting the conformation of cell wall macro-molecules. (Ou and Marquis, 1970, Wood, 1980). The increased concentration of salt would maintain cell surface solubility and keep the cell in suspension. Changes in pH and ionic strength of the media may also affect cell physiology which could also help to explain these observations.

4.4.2 <u>The effects of cell growth condition both prior and during the attachment assay.</u>

Since the extracellular conditions of the bacterial outer membrane play an important part in the surface attachment of this particular pseudomonad, variations in cell surface macro-molecules, as a result of variations in growth conditions, would be expected to affect adhesion. In this section both batch culture and continuous culture was used to grow the test organism in a defined minimal medium in order to measure the effects of growth variation on attachment ability.

The results of the batch culture experiments gave some indications that greater levels of attachment took place after cells had been grown under carbon limitation, and in particular the transition point from carbon-to

nitrogen-limitation produced maximum attachment levels. When the attachment medium was supplemented with glucose, the effects obtained with the growth conditions in defined medium (section 4.3.2.iv) were exaggerated; nitrogen limited cells showed lower levels of attachment to PS while glucose limited cells showed higher levels of attachment to both PS and TC-PS (figure 4.7). Obviously phenotypic expression of outer surface structures that are responsible for bacterial attachment are influenced by these growth conditions. Great care, however, must be taken in any interpretation of these results as batch cultures are "closed systems" where growth conditions change progressively throughout the culture growth cycle. Therefore this work was repeated in continuous culture in order to obtain steady state conditions for cell growth prior to the attachment assay.

The influence of a "shift-up" in growth conditions by the addition of glucose to the attachment media is also difficult to interpret. The normal effects on cell physiology of such a change in growth conditions is to inhibit DNA replication and stimulate RNA, protein and carbohydrate synthesis. Thus cells grow longer and larger but do not divide until DNA replication recommences. For an Enterobactor aerogenes culture, a "shift up" in dilution rate from 0.004 h^{-1} to 0.24h^{-1} in a single step inhibited cell division and DNA replication for 5h and the

"steady state" concentrations of RNA, DNA and carbohydrate characteristic of E aerogenes growing at a dilution rate of 0.24 h^{-1} were attained 5h, 9h, and 3h, respectively, after setting the dilution rate at 0.24h^{-1} (Tempest et al, 1967). Thus changes in cell size and activity could account for the major changes in attachment observed for carbon-limited cells from stationary phase batch (see fig. 4.6). Such changes would again modify the surface characteristics of the cell (Tempest et al, 1967). The results of continuous culture studies and attachment experiments confirmed that increased attachment ability of this organism resulted from carbon-limited growth at low dilution rates. Addition of glucose or an increase in the dilution rate prior to attachment caused an increase in measured attached cells from both carbon and carbon/ nitrogen-limited culture (data not shown). Nitrogen-limited culture gave minimum attachment and did not show the "shift-up" effect. These results correlate well with the biochemical analysis of cells after steady-state growth under various limitations of carbon and nitrogen (Table4.2). Low attached cell numbers for nitrogen-limited culture were observed for cells with a large proportion of carbohydrate in the membrane. High levels of attachment were recorded for carbon-and carbon/nitrogen-limited culture with a large proportion of protein in the membrane and significant

reduction in carbohydrate. The reduction in attachment with an increase in dilution rate also correlated with a corresponding increase in carbohydrate in the membrane.

The influence of dilution rate with carbon-and nitrogen-limited growth on the bacterial macromolecular composition has been studied with a number of Gram-negative organisms. For example, similar results have been reported for a glycerol-limited culture of Enterobacter aerogenes (Tempest and Ellwood, 1969) whereby the wall KDO and heptose contents increased markedly with growth rate, indicating a general increase in wall lipopolysaccharide content. Not only did the content vary, but the composition and the lipopolysaccharide was also influenced by growth conditions. These phenotypical variations in the membranes of Gram-negative bacteria in response to environmental conditions can thus affect the attachment ability of the organism, used in this study, and further work is required to determine to what extent other Gram-negative and Gram-positive organisms are affected. For this organism, increased levels of polysaccharide associated with the membrane fraction inhibited cell adhesion possibly by an increase in cell surface charge and ionic repulsion or by steric hindrane (Pethica,1961).

4.4.3 Selection and enrichment of adhesion mutants of P fluorescens by foam fractionation

The collection of microbial cells by foam fractionation was first demonstrated by Dognon,(1941) who found that

Mycobacterium tuberculosis cells were easily removed from suspension by foaming, while E.coli Staphlococcus albus and Schizosaccharomyces sp. were concentrated with difficulty. Boyles and Lincoln,(1958) found that the nature of the bacterial surface affected the collection efficiency, and hydrophilic smooth strains of Brucella suis were not concentrated in foam under numerous conditions, whereas hydrophobic rough strains were effectively concentrated.

Previous adhesion studies of the wild-type strain of this organism indicated that the cells are able to enter into hydrophobic interactions with surfaces (Chapter Two, Pringle and Fletcher, 1982) and was confirmed by HIC and by their foam fractionation properties.

In this section the incorporation of foam fractionation into the basic fermenter was found to remove a large proportion of the wild-type cells from the fermenter. Foam fractionation has been reported to concentrate bacterial cells 10^6 fold (Grieves and Wang, 1966) however, total counts of this organism showed 10^3-fold concentration after 2 h operation. This ability to fractionate was also found for Pseudomonas aeruginosa NCIB 8602 which, like P fluorescens H2, produced mucoid and crenated mutants, but no significant fractionation of an Aeromonas hydrophila strain was detected (data not included). Further studies may reveal how common foam fractionation is in natural populations.

The results of continuous culture of wild-type cells was to select two types of mutation. The mucoid exopolysaccharide-producing mutant dominated in the aqueous phase, as it was not removed by the foam fractionation. While the crenated mutant dominated on the fermenter walls (Table 4.3) as it had a greater attachment ability, confirmed by attachment assay (Table 4.4). In studies of wall growth or adhesion in mixed bacterial cultures using chemostats, little attention has been paid to changes in the cell surface characteristics reflected by colony morphology. However, Brown et al., (1977) have reported that differences in attachment levels in a mixed population was related to the presence or absence of a glucose containing polysaccharide. When the chemostat was supplied with glucose limited medium a wide variety of bacterial types was found attached but no surface polymer was apparent. In nitrogen-limited medium the culture contained an abundance of extracellular polymer and little adhesion. In similar enrichment studies, Wardell and Brown, (1980) confirmed that non-specific attachment occurred in C-limited cultures without the appearance of either large amounts of polymer or specific appendages.

Polysaccharide production after the initial stage of attachment is likely to be important in maintaining a biofilm of microbial species, most commonly associated in micro-colonies, flocs, filaments or streamers, films and mats. Thus adsorption isotherms were carried out on surface polymers from the adhesion mutants in order to verify their binding

abilities to the test substrata (4.4.4) and their role in bacterial adhesion. The role of outer membrane proteins is unclear although small differences were detectable in SDS-polyacrylamide gels of outer membrane proteins from the wild-type and adhesion mutants. No significant changes in the major outer membrane bands could be seen. Further studies, however, of extrinsic proteins were made in order to show to what extent these components influence the characteristics of the membrane surface. (see section 4.4.4).

The results of the LPS analysis suggested that the crenated mutants were similar to the crenated (CR) mutants of Klebsiella (Norval and Sutherland, 1969), as in liquid media both mutants autoagglutinated due to reduced lipopolysaccharide content (in this case between 30-55%). These mutants different from Salmonella semi rough (SR) mutants which have polymer with side-chains containing a single oligosaccharide repeating unit (Luderitz et al,1971). The colony morphologies of these mutants are similar, although, these crenated strains lack the ability to form exopolysaccharides at low and high temperatures and are therefore identical to the (CR-O) double mutants. This is to be expected as the wild-type of the pseudomonad unlike the Klebsiella aerogenes, does not produce exopolysaccharide normally. These mutants are thought to be due to lack of sufficient isoprenoid alcohol giving a reduction in the lipopolysaccharide content of the cell to about 30% of normal.

The extent to which changes in the LPS influence the hydrophobicity of the cell surface may be important in cell adhesion to solid surfaces. Magnusson et al.,(1977) demonstrated changes in cell hydrophobicity and charge interaction using an aqueous polymer two phase system to compare the properties of smooth and rough Salmonella typhimurium and their corresponding isolated LPS. The rough Rd mutant of Salmonella typhimurium showed hydrophobic interaction with covalently linked hydrophobic groups in the aqueous polymer system indicating a number of accessible interaction sites at the cell surface. By contrast the smooth (S) bacteria and LPS from the parent strain did not participate in hydrophobic or charge interaction indicating extensive surface hydrophilicity and ionic repulsion. Changes in the LPS content undoubtedly change the coverage of the cell surface by the polysaccharide side chains of LPS. In wild-type strains of E. coli or S. typhimurium, the cell surface is effectively covered by such chains, estimated at 41% of the outer surface area. However, rough mutants reduce this coverage to only 8%, leaving many of the surface proteins and phospholipids exposed (Nikaido and Nakae, 1979).

The composition of this P fluorescens LPS was found to be rich in amino sugars. Previous studies have shown large amounts of 3-amino-3, 6, dideoxy-D-galactose (fucosamine) and 2-amino-2, 6-dideoxyglucose (quinovosamine) present in Pseudomonas strains (Koval and Meadow, 1975; Wilkinson, 1972). The LPS composition of this organism is similar to P. syncyanae as reported by Wilkinson et al,(1973).

The mucoid exopolysaccharide was found to be an alginate composed of D-mannuronic acid and variable amounts of guluronic acid. This mucoid mutant was unstable, and large numbers of reversions to the wild-type morphology occurred, suggesting a chromosomal control mechanism similar to that found with alginate production by P. aeruginosa (Fyfe and Govan, 1980) The change in antibiotic resistance with the mutation to alginate production is also consistant with findings that mucoid mutants of P.aeruginosa and other species of Pseudomonas can be isolated in vitro from wild-type non-mucoid strains by selection for carbenicillin resistance. Therefore changes to the exopoly-saccharide producing mutant in response to environmental changes is rapid due to effective selection and the high frequency of the mutation.

In summary, the results of the attachment assay and the investigation of attachment mutants suggested that polyanionic carbohydrate slimes, produced by many aquatic organisms, may not participate in the initial non-specific attachment to hydrophobic solid/liquid interfaces as previously suggested. (Corpe, 1974; Costerton et al, 1978). The abundant acidic polysaccharide materials described by Corpe (1970) may not be concerned with the primary attachment of the producing cells, but with the development of a subsequent bacterial film, a process requiring time-dependent biosynthesis, cell growth and the formation of cell to cell bridges. On the other hand primary attachment is dependent upon the bacterial surface presented to the substrata and is influenced by the extent

of LPS present. How much LPS coverage may influence this process can be due to both phenotypic and genotypic changes affecting LPS production.

4.4.4 Further studies on the role of exopolymers LPS, extrinsic proteins and extracellular surface appendages on the attachment of several Pseudomonas isolates

The role of extrinsic or surface membrane proteins on the attachment of aquatic organism remains unclear following the investigation of a number of adhesion mutants. Both radio-amidination and radioiodination of extrinsic proteins clearly identified a number of major outer membrane proteins available on the surface for cell-substratum interaction. To identify the role of individual proteins would require the use of specific mutation in these outer membrane proteins. For example an indication of the involvement of protein in the adhesion of a Gram-negative bacterium was provided by comparison of the adhesion of a mutant strain of E.coli deficient in outer membrane protein 1 with that of the parent strain to Bio-Rex 70 (Wood, 1980). The mutant was unable to adhere to this adsorption matrix over the pH range 6.0-7.5, characteristic of the non-specific range of adhesion whereas, the parent strain did adher in the region. This suggests that the presence of a specific protein is necessary for adhesion.

Figure 4.17 shows the differences between the major outer membrane protein of P. aeruginosa with those identified for P.fluorescens (Hancock and Carey, 1979). A number of authors have demonstrated that major outer membrane proteins with

apparent molecular weights ranging from 30,000 to 40,000 are characteristically "heat modifiable" and others are modified by 2mercaptoethanol in the loading buffer. Therefore without a reproduction of the exact solubilisation conditions if is difficult to compare outer membrane proteins in order to determine common function. (Bragg and Hou, 1972; Inouye and Yee, 1973; Ames et al, 1974; Muzushima, 1974; Schnaitman, 1974; Nakamura and Muzushima, 1976; Russel, 1976; Di Rienzo et al, 1978) Two proteins which have a common mobility and are not "heat modifiable" are labelled G and I (Fig. 4.19). The identity of G is unknown, but it is an extrinsic protein (23) whereas I is the lipoprotein, identified by lipid staining, and involved in maintaining the structure of the outer membrane by uniting it to the peptidoglycan and inner membrane. The protein labelled F is head modifiable and is normally bound to LPS. Following heating in SDS-2 mercaptoethanol a number of mobilities are obtained depending upon the conditions used. Thus proteins labelled II III IV or 18,1920 are probably protein F in three states of association with LPS or without LPS (Hancock and Carey, 1979). This protein has been identified as a porin; it may not be located on the outer surface of the membrane and is not a significant extrinsic protein. Proteins 16 and 31(I) (fig. 4.19) have been isolated from growth media after their concentration using ultrafiltration. Both are extrinsic proteins as they are labelled during radioamination and radioiodination and may be important in the adhesion of cells as they are easily released into the medium. The band labelled (16)

(Fig. 4.19) may be two or more proteins which are not resolved by this electrophoretogram. A glucose-binding glycoprotein (GBP) important in glucose chemotaxis has been identified in P. aeruginosa with an average molecular weight of 44,500 which would be expected in this region and would also be easily released into the growth medium (Stinson et al, 1977).

The analysis of adhesion mutants of three Pseudomonas putida strains, HP, H40, H35, and Pseudomonas aeruginosa H8602 revealed that exclusion by surface polysaccharides, either EP or LPS of the outer membrane, reduced attached cell numbers. Therefore, mutations in either the production of EP (H40M, H8602M and HPS) or the composition of the LPS (H35-R) influenced the adhesion of liquid grown cells to both PS and TC-PS substrata. H8602M was isolated from a continuous culture of wild-type cells subjected to foam fractionation and produced a similar alginate polymer to that produced by P.fluorescens H2. H40M produced a heteropolysaccharide of glucose, galactose and rhamnose in carbon-limited batch culture in conditions where wild-type H40 showed little exopolymer production (Table 3.9). HP-S produced no slime EP in liquid culture and on solid media (data not shown) with the effect that mutant cells were able to adhere in greater numbers to both PS and TC-PS substrata.

Finally H35-R showed a change in LPS composition and produced a rough colony form which correlated with an increased level of attachment over wild-type. H35-R, however, did not attach

significantly after growth on solid media where heteropoly-saccharide of glucose and galactose was produced. These results support the observation described in Chapter 3 suggesting an inhibitory role of EP in the primary attachment of bacteria to solid surfaces. Further studies on the role of EP on the eventual stability of a bacteria film is now required to determine the exact function of these polymers following the colonisation of the solid surface.

4.4.5 Adsorption isotherms of EP from wild-type and adhesion mutants

The results of adsorption isotherms of EP from wild-type and adhesion mutants of P. fluorescens H2 can be redrawn as a linear form of the Langmuir equation

$$r/a = KN - Kr \qquad 4.2$$

which yields linear plots of r/a against r, where r is the amount of adsorbed polymer in $\mu g\ cm^{-2}$ of polystyrene; a is the equilibrium solution concentration in $\mu g/ml$, N is the maximum possible adsorbable amount of polymer and k is a constant. The intercept at r/a = 0 yields N, and - K is the slope of relationship. Figures 4.34-4.37 show this relationship for EP and LPS isotherms. The plots are similar to adsorption isotherms of human globulin to polystyrene latex particles (Brash and Lyman, 1967), in that a line of greater slope representing the formation of an adsorbed monolayer was found for each surface and in some cases a second line of lower slope

Figures 4.34-4.37

Adsorption isotherms of EP and LPS isolated from wild-type P. fluorescens H2P2, crenated mutant H251 and mucoid mutant H2M2 redrawn as a linear form of the Langmuir equation (4.2) of r/a against r Where r is the amount of adsorbed polymer in $\mu g\ cm^{-2}$ of PS and a is the equilibrium solution concentration in $\mu g\ ml^{-1}$ The isotherms of three substrata are plotted PS (○), TC-PS (●) and SP (X) for the following polymers:

Figure	Polymer
4.34	wild-type EP
4.35	mucoid EP
4.36	crenated EP
4.37	wild-type LPS

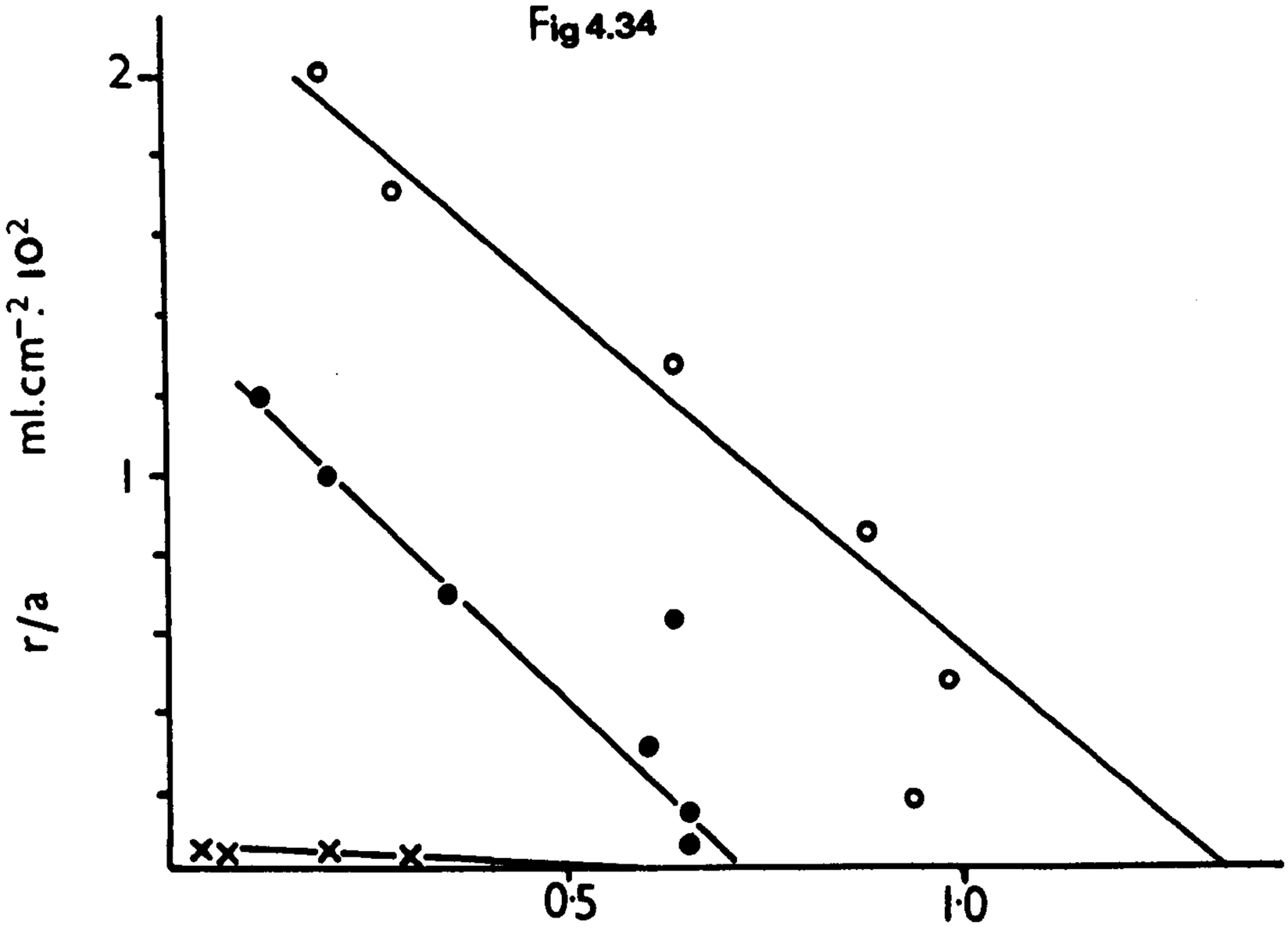


Fig 4.34

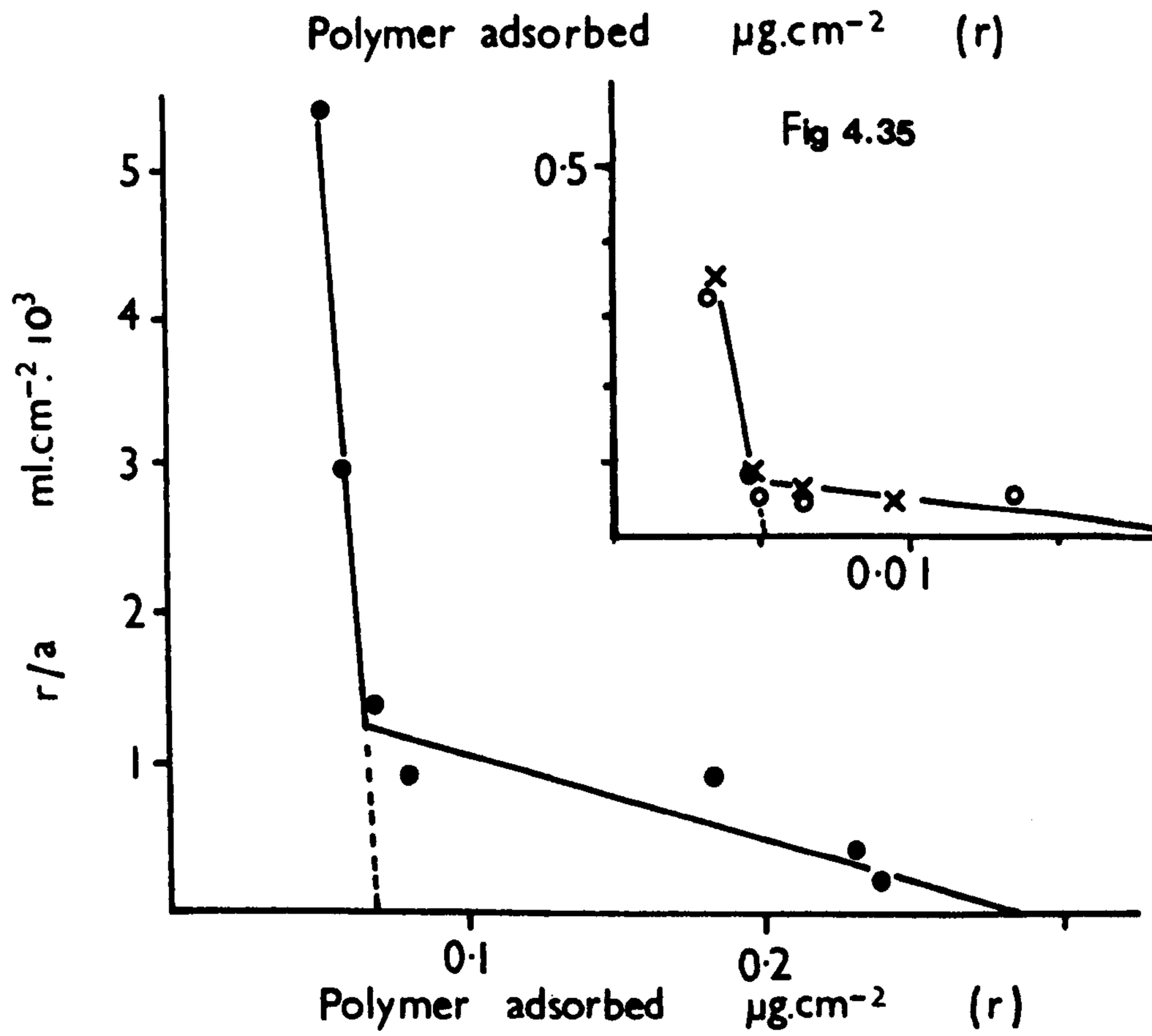


Fig 4.35

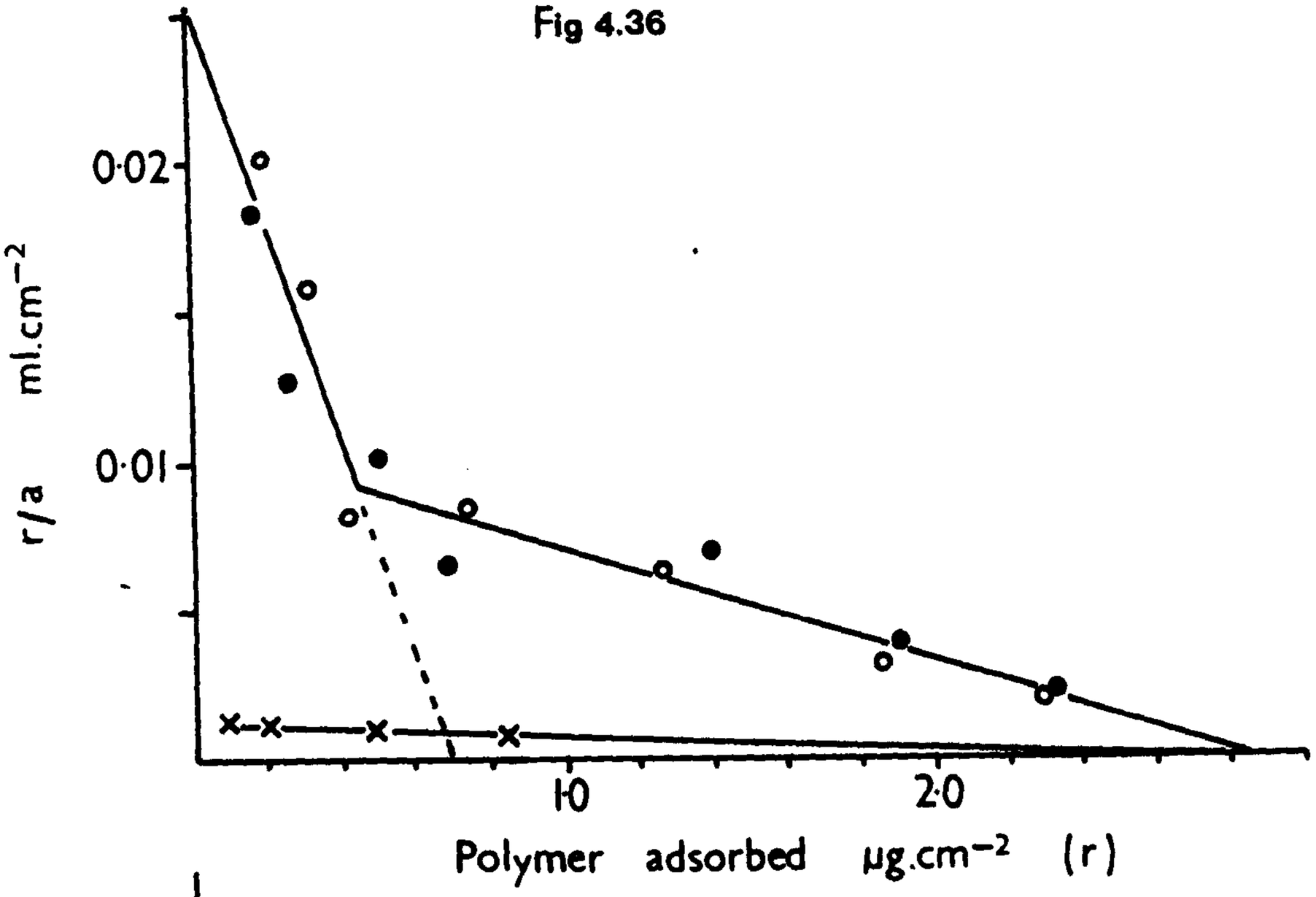


Fig 4.36

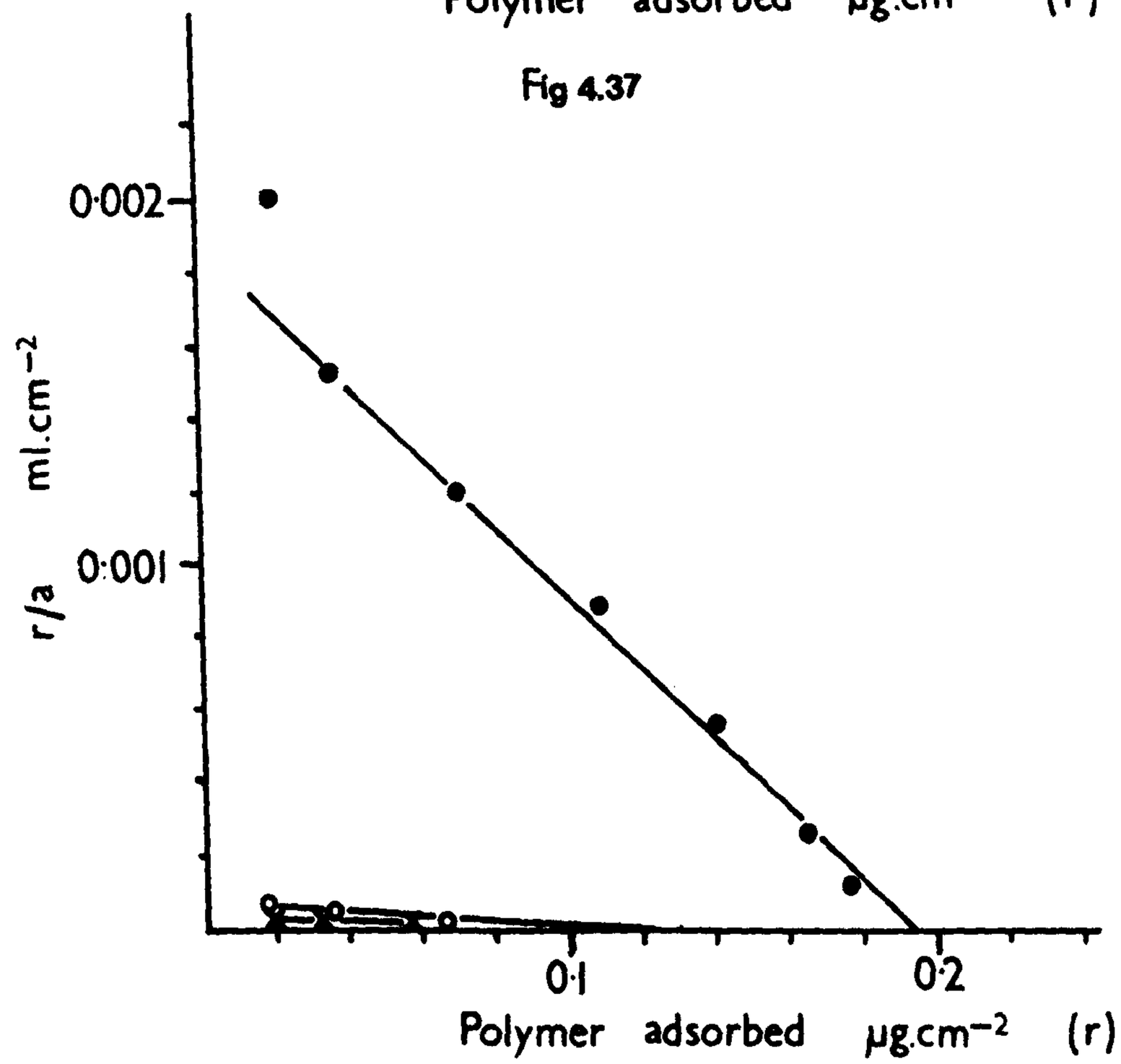


Fig 4.37

was produced representing either further adsorption to the original monolayer or the production of a second layer of adsorbed molecules by rearrangement of initially adsorbed molecules. Table 4.12 shows N values for the various polymer and substratum combinations. Higher N, values were recorded for EP of wild-type and crenated mutants with PS and TC-PS substrata than with SP substrata, indicating that these substrata accomodate more EP per unit area of surface than do surfaces with increased charge (SP). Polymers that contain a greater proportion of larger polysaccharide molecules quickly saturate available sites (Mucoid EP LPS) and therefore show lower N values.

Maximum adsorption of the mucoid polymer was recorded for the TC-PS surface at 0.07 $\mu g\ cm^{-2}$ (figure 4.35). Assuming an average molecular weight of 10^6 daltons, each molecule will occupy a space of 2300 nm^2. It is difficult to determine the size and shape of this linear polysaccharide. The β 1-4 linkages between the monomer units will tend to maintain a linear polymer with a repeating disaccharide unit occupying 6.79 nm^3 (based on the X ray diffraction studies of cellulose fibers) (Bailey, 1965). Thus the area the molecule will occupy on the surface will be 0.86 nm^2 with the strands placed laterally over the surface or 0.81 nm^2 with the strands perpendicular to the surface. The average molecular weight of this molecule determined from gel filtration was 10^6 daltons. However this is misleading, as a large heterogeneity of

TABLE 4.12

Mathematical functions of EP and LPS adsorption derived from the Langmuir adsorption isotherm.

Values for N^a $\mu g.cm^{-2}$

Polymer	Substrata					
	PS		TC-PS		SP	
	N1[b]	N2[c]	N1	N2	N1	N2
H2 wild-type EP	1.28	-	0.72	-	<0.5	-
H2 mucoid EP	0.005	0.02	0.07	0.285	0.005	0.02
H2 crenated EP	0.75	2.81	0.75	2.81	-	2.81
H2 wild-type LPS	0.19	-	0.121	-	0.08	-

a. From the equation (4.2) r/a = KN-Kr when r/a = 0, N = r

b. N1 first value determined for N representing a monolayer formation.

c. N2 further adsorption on the surface when applicable.

molecular weights would be expected. The calculation of area coverage however, will be based on the molecular weight, as the area of the polysaccharide molecule will be directly proportional to the number of repeating units and thus to molecular weight (unlike globular proteins). Assuming a MW of 10^6 daltons, then the estimated area occupied by each molecule agrees well with maximum adsorption coverage, at 2507nm^2 - 2030 nm^2, assuming the orientation of the molecule is lateral to the surface of the substrata.

The adsorption isotherms for the mucoid polymer onto PS or SP substrata were much lower ie. 0.005 µg cm^2 suggesting that few adsorption sites were available on these substrata or that the adsorption bonding was weaker and EP was removed during the washing stage. The level of surface charge on the SP substrata may cause sufficient ionic repulsion to prevent significant levels of adsorption. Alternatively, the PS substrata may form insufficient bonding with the adsorbing EP to prevent the water/air interface from capture of the EP during washing. This water/air interface interaction was not observed for the TC-PS or SP surfaces which were able to remain covered with media during washing. Thus the reduction of bacterial adhesion to these surfaces produced by this mucoid EP may be due to separate mechanism for each substratum. Adhesion to the TC-PS may be inhibited by the adsorption of mucoid EP, preventing cell-surface contacts whereas, adhesion to the PS-substrata may take place only to be lost due to the

weak bonding between the PS surface and water.

The adsorption of EP from the wild-type and crenated strains produded isotherms with N values typical of protein adsorption to similar weakly charged surfaces, ie. a range of 0.5 $\mu g\ cm^{-2}$ - 1.5 $\mu g\ cm^{-2}$ (figures 4.34-4.36). Adsorption to the PS substrata of wild-type EP showed a greater binding capacity than that obtained with TC-PS. (N_1 of PS = 1.75; N_1 of TC-PS = 0.75). However, the crenated EP adsorbed to both TC-PS and PS substrata equally and was able to continue to adsorb following initial monolayer formation at N_1 = 0.75 $\mu g\ cm^{-2}$ to a second adsorption maximum at N_2 = 2.8 $\mu g\ cm^{-2}$. These results indicate that wild-type EP adsorption to both TC-PS and PS substrata inhibit further adsorption when a monolayer of polymer has been adsorbed. However, crenated EP does not inhibit further adsorption, which could be due to the relative concentration of LPS present in the EP.

Finally LPS adsorption (figure 4.37) shows similar results to the adsorption of mucoid EP. Maximum adsorption was to the TC-PS surface at N_1 = 0.195 $\mu g\ cm^{-2}$, with a single saturation value. The polysaccharide component of the LPS may therefore dominate the adsorption characteristics, allowing adsorption to wetted surfaces where charge density would be low enough to prevent ionic repulsion. Both mucoid EP and LPS will have hydrated polysaccharides which would be expected to interact less strongly with the increasingly hydrophobic surfaces. Therefore adsorption of wild-type LPS to the TC-PS from the

wild-type EP may inhibit further protein adsorption, hence the reduction in adsorption concentrations N_1 for TC-PS in figure 4.34. The adsorption of protein from the EP of the crenated strain may dominate this isotherm. In which case it will be affected to a lesser degree by LPS components as was observed for the wild-type EP. Therefore significant concentrations of LPS may enable all three substrata to be covered by a monolayer of LPS as found when attachment to PS and TC-PS was inhibited (section 3.2.3.3).

4.4.6 Electronmicroscopy of test organisms

The electronmicrographs of the Psuedomonas strains used in this study do not establish any clear evidence for the involvement of pili or fimbriae in this initial non-specific adhesion process. This may be due to unsuitable culture conditions to maintain $pili^+$ or $fimb^+$ strains. However, the electronmicroscopy did demonstrate the presence of a hydrated capsule around a number of organisms, which may prevent pili or fimbriae from being seen. Thus their role must remain unsubstantiated as far as this work is concerned and limited to observations of electronmicrographs of bacteria attached in their natural environments.

In conclusion this chapter has demonstrated the effects of phenotypic and genotypic variation in the outer surface polymers on the primary attachment of bacteria to solid surfaces. The expression of surface polysaccharides prior to the cell-surface interaction will prevent adhesion of the cells by affecting the short range hydrophobic interaction characteristic of this form of adhesion. This may be achieved (i) by steric

hinderence following the adsorption of polymer at the surface or ii) by the maintainance of water structure over the outer membrane weakening any short range interactions between cell and surface or iii) by ionic repulsion and long range forces preventing cell surface contacts. These mechanisms depend upon the substratum characteristics, the polymer and the attachment media.

4.5 CONCLUSIONS

This Chapter can be divided into three parts:

i) The influence of physical parameters on the attachment process.

ii) Biochemical analysis of phenotypic changes in cell surface structure, as a result of growth conditions, that influence subsequent bacterial adhesion.

iii) A detailed biochemical analysis of genotypic changes in cell surface structure that modify attachment ability.

4.5.1. Physical factors influencing a passive or active adhesive mechanism

The effects of time of attachment, temperature, pH and ionic strength on the adhesion of *P. fluorescens* H2 supports a passive model of adhesion similar to the adsorption process of the Langmuir adsorption isotherm (Fletcher, 1977). However, although the process may be passive changes in the organisms motility can influence the chances that a bacterium may encounter a potential attachment surface.

The effects of temperature on attachment also supports this model because adhesion was still possible at temperatures above the growth maximum for this organism. Below the growth maximum the adhesion rate of this organism did follow the Arrhenius relationship (4.1) and was significantly reduced at low temperature. The effects of pH and ionic strength did show that ionic interaction were not important in this process as the results for the attachment in varying pH and ionic

strength media were inconsistent with the DLVO theory.

From these limited numbers of observations it is clear that a number of physical and physiological competing effects influence the subsequent adhesion. Any interpretation of the results must decide whether physical parameters influence the adhesive bond or the cell physiology or both.

4.5.2. Phenotypic changes in the cell surface and their influence on subsequent attachment.

The experiments using batch cultures gave some indications that greater levels of attachment takes places after cells had been grown under carbon limitation. This was confirmed by continuous culture experiment which were used in order to avoid problems of growth transitions. A correlation was found between the protein and carbohydrate content of the cell and subsequent adhesion. High levels of attachment were recorded for carbon and carbon/nitrogen limited culture where a large proportion of protein was found in the membrane. Reduction in attachment was found for nitrogen limited culture with a corresponding increase in carbohydrate content of the membrane. Thus increased levels of polysaccharide in the membrane fraction reduced subsequent adhesion.

4.5.3. Genotypic changes in cell surface and their influence on subsequent attachment

Conclusions in this section were based on the identification and biochemical analysis of adhesion mutants. These were initially isolated from a chemostat following enrichment due to foam

fractionation and secondly by examination of colonies showing variation in colony morphology. Mutants showed either increased attachment ability as for the crenated mutants of P. fluorescens H2 or reduced attachment ability as for the mucoid mutants of this strain. Biochemical analysis of the outer membrane proteins, extra-cellular polysaccharides and LPS clearly identify the polysaccharide surface component to be influencial in the adhesive properties of the cell. In general exopolysaccharides and extensive LPS covered membranes showed reduced attachment properties. Attachment potential of the organisms was returned with the loss of production of these polymers. Although no mutants in outer membrane proteins were identified, the role of extrinsic proteins in the initial adhesion process cannot be ignored as the exposure of cell surfaces by the removal of polysaccharide must allow membrane proteins to be made available for cell substratum contact.

Finally, the results of the adsorption isotherms of polymers released into the culture medium clearly demonstrates that the interaction of polysaccharides and proteins at the substratum surface will be influencial in the subsequent bacteria adhesive bond and over production of polysaccharide produces an adsorbed molecular layer of polysaccharide which may inhibit subsequent adsorption and adhesion.

CHAPTER 5

Final Discussion and Conclusions

5.1 Thermodynamic models and their application to the study of bacterial attachment

Thermodynamic models have proved very useful in interpreting and in some case predicting the nature of the interaction of bacterial attachment at the solid-liquid interface. Both the DLVO type of model, a more theoretical approach, and the broad phenomenological descriptions of the surface energy type have enabled some generalisations to be made which have been used to explain observations of biocontact phenomena. For example Van Oss *et al*, (1975) have made use of the surface energy approach in order to grade bacterial contact specificity for phagocytosis.

In the preliminary investigation of this thesis (Chapter 2) a general relationship between attached numbers and the γ_{SV} of the substrata was observed for a number of aquatic isolates. An increase in attached numbers was observed with decreasing γ_{SV} until a maximum, giving a characteristic attachment peak for each organism in the mid-range (γ_{SV}), was reached. This general relationship supports the hypothesis that bacterial cells will spontaneously accumulate at any solid/liquid interface where a high interfacial free energy γ_{SL} is available. The strength of the subsequent adhesion will depend upon i) the available types of bonding at the solid surface ii) the characteristics of the bacterial outer surface and iii) whether time dependent

physiological activity by the bacterium can increase the strength of the interaction.

The accummulation of bacteria/biomolecules at interfaces of high interfacial free energy, can be predicted to some degree by the thermodynamic model described by equation 1.9, relating the change in free energy of adhesion, ΔG^{adh}, to emp irically determined values of γ_{SL} γ_{BL} and γ_{SB}, calculated by the equation of state approach and using theoretical values for γ_{SV} γ_{BV} and γ_{LV} (figures 1.2). This model demonstrates the importance of the water surface tension and the effects of water on hydrophilic and hydrophobic surfaces. Therefore, although hydrophilic surfaces offer stronger bonding potential to the hydrophilic surfaces of bacteria, the adsorbed water layers provide a sufficient thermodynamic barrier as to reduce the number of organism adhering.

In experiments using a controlled surface shear stress, produced by laminar flow, to measure the minimum critical shear required to remove attached and attaching organisms, increased critical shear stress was required with increasing surface free energy to either detach cells or to prevent adhesion (Mohandas et al, 1974; Fowler and Mckay 1980). Therefore surfaces with increasing surface free energy form stronger adhesive contacts with cell surfaces as they provide a greater variety of short range forces, including hydrogen, dipole-dipole and dispersion forces, which will form stronger bonds with the non covalent phase of a micro-organism, which is also held together by these forces.

This model applies principally to "clean" surfaces as used in these in vitro studies. Once surfaces are "conditioned by macromolecules", a reduction in their interfacial free energy (γ SL) will reduce bacterial attachment unless more specific adhesion mechanisms are utilised by the bacterium to form contact with the modified surface.

The thermodynamic approach however does have limitations when applying broad phenomenological descriptions to organisms which have a diversity of biochemical structures, as well as the dynamic ability to change both those structures and the chemical compositions of the environments in which they occur. However, with these limitations in mind it is possible to envisage that the initial colonisation of "clear" or non-conditioned surfaces in natural aquatic environments would be due to the non-specific and spontaneous accumulation of bacteria and macromolecules at the solid-liquid interfaces. This may be particularly important with organisms known to be physiologically dormant in oligotrophic waters ie. Zymogenous bacteria (section 1.1.3)

5.2 The Characteristics of a surface

The thermodynamic arguments of the previous section (5.2) demonstrate that clean surfaces will rapidly change characteristics once exposed to the macromolecular solutes in an aquatic environment. The thermodynamic arguments however, do not assess how quickly this transition will take place or the extent to which different molecular conditioning will influence subsequent bacterial attachment.

Adsorption kinetics and isotherm studies of proteins at the solid-liquid interface have shown that spontaneously adsorbed film development is not instantaneous and is dependant uppn bulk medium concentration (Mac Ritchie,1970) and on the substratum surface characteristics (Brash and Lyman,1969). The results of experiments on the effects of conditioning films of either pure macromolecules or natural polymers obtained from freshwater samples or culture supernatants, on bacterial attachment demonstrates the complexity of the phenomenon:

i) Certain molecules inhibit adhesion purely by surface adsorption and hydration

ii) Other molecules (particularly proteins) condition both solid surface and bacterial cell surface, stabilising them by reversible adsorption and steric hindrance. In natural environments a range of such molecules will be available at different concentrations. Therefore as demonstrated by the natural water sample conditioning (section 3.2.3.3)the levels of organic present in a natural sample will vary considerably from one environment to the next, which will influence conditioning time scales.

Evidence from the results of adhesion of bacteria to hydrogels of increasing water content supports the view that the minimum interfacial energy of adsorbed water layers produces biocompatible surfaces. However, even these surfaces in time become conditioned and do not prevent even low levels

of bacterial attachment. Conditioning films can be viewed as hydrated surfaces. However, this may only be achieved by multilayer configuration, as primary monolayers adsorbed to hydrophobic substrata are known to denature and allow subsequent adsorption of partially unfolded and native protein (Bash and Lyman, 1969). Thus, films may only become bio-incompatible when multiple layers of adsorbed macromolecules are present. This is supported by the observed concentration required to inhibit bacterial attachment (see section 3.1).

5.3 <u>The liquid medium</u>

The liquid medium is important in the attachment process because of the role played by water itself and also because of the presence of dissolved substances which affect adhesion. The influence of dissolved macromolecules on attachment through conditioning of the surface has been discussed (section 5.2). However, small organic solvents such as ethanol and urea influence medium properties by altering water structure and the liquid surface tension.

Their effects on water structure have been examined by studying their effects on micelle and protein denaturation in aqueous solution. For example the addition of ethanol increases the stability of ribonuclease at concentrations up to 5% V/V at 15^{o}C as determined from the free energy of denaturation. (Brandts, 1971.). At concentration greater than 5% (V/V),

ethanol acts as a denaturing agent. The stabilisation effect is thought to be due to ethanol maintaining the apolar groups of the protein internal to the protein structure maintaining the normal or native conformation. With increasing concentrations, solvation effects would allow apolar groups into the aqueous phase, unfolding and denaturing the protein. Thus the effects of ethanol on bacterial attachment at low temperature (15°C), may be similar in that hydrophobic interactions between cell surface components and the substrata are stabilised by the presence of low concentration of ethanol or urea. Higher concentrations will denature and solvate these interactions. Experiments at higher temperatures may confirm this theory as stabilisation only occurs at low temperature.

The effects of solutes can also be explained in terms of liquid surface tension measurements which relate to the ΔG^{adh} by equation 1.13. Using the equation of state approach the interfacial free energies of the bacterium/solid, liquid/solid and the bacterium/liquid can be determined for experimental determined values of γ_{LV} and γ_{SV} and theoretic values for γ_{BV}. This model predicts that cell adhesion would increase with an increase in substratum surface tension if γ_{LV} was less than the surface tension of the cells γ_{BV}. Conversely, there would be a decrease in adhesion with increase in γ_{SV} of the γ_{LV} was greater than γ_{BV}. When $\gamma_{LV} = \gamma_{BV}$, adhesion would be independent of γ_{SV} and allows γ_{BV} to be determined

experimentally. The results obtained for γ BV of Pseudomonas fluorescens using this method and DMSO to decrease γ LV (66.4 and 63.6 mJm^{-2} for carbon and nitrogen limited cells respectively) are similar to values quoted for other bacteria using contact angle methods (Fletcher and Pringle, 1983).

The medium ionic composition is also important in adhesion in organic divalent cations, ionic strength and pH all influence adhesion, probably by changes in cell surface structure and perhaps by changes in cell physiology which also influence cell surface structure and composition. The effects of ionic strength on attachment rule out the predominance of long-range interactions, as described by the DLVO theory in bacterial adhesion to weakly charged surfaces. Increases in ionic strength reduced attachment levels when a reduction in the double layer or of potential produced by an increase in ionic strength theoretically should increase attached cell numbers.

5.4. The bacterial surface

There are two possible interpretations of the mode of bacterial attachment to the solid-liquid interface:

i) The first mode uses the available free-energy of the interface, and the cell adheres despite the apparent lack of designed biological adhesion mechanisms, such as bio-adhesives polymers or anchoring appendages.

ii) A second mode may make use of specific bio-adhesive polymers, especially when the interface may offer a selective advantage for survival but little interfacial free energy to promote adhesion.

Both modes of attachment would have evolved to accommodate the physicochemical properties of interfaces in the aquatic environment and be dependent upon the advantages or disadvantages of an attached existence. Bacteria for which a free-swimming existence or dispersal is an advantage could have a means of avoiding the spontaneous physicochemical attractions of interfaces by an appropriate modification of their cell surface. Therefore it is not surprising that bacterial surface components show such a diverse range of biochemical structure.

In this thesis Zymogenous or copiotrophic bacteria on the basis of the evidence presented probably adopt the first mode of adhesion and possess no specific bio-adhesive polymers. Although electronmicroscopy of attached bacterial cells shows surface polymers which appear to act as adhesives (Fletcher and

Floodgate, 1973; Marshall and Cruickshank, 1973, Costerton et al, 1978), these electronmicrographs were prepared on attached films rather than cells that had just made primary contact with the surface. The growth of bacterial micro-colonies on the substrata after this primary contact would probably in the majority of cases increase the strength of adhesion by cell-cell and cell-substratum contacts of surface extruded macromolecules.

A number of publications have suggested that cell surface polysaccharides are the bacterial adhesives (Corpe, 1973; Fletcher and Floodgate 1973; Costerton et al, 1978). This may be the case under special circumstances where polysaccharides provide a means of cell-cell contact through a more specific polymer bridging model or the production of a water insoluble matrix. (Gibbons and Van Houte, 1975). In fact the role of the majority of polysaccharides may be quite the reverse to bio-adhesives. Adhesion experiments with cells that produce slime or capsular polysaccharides support the view that poly-saccharides provide a hydrated surface layer which is often highly negatively charged, thus protecting the lower surfaces from the physicochemical attraction of many interfaces, stabilising cell-cell contacts and acting as dispersants. Further evidence comes from experiments where proteases are able to remove attached bacteria (Dannielson et al, 1977; Fletcher and Marshall, 1982), showing that short range forces

between surface proteins and the substratum are involved in adhesion.

Various methods are now being evolved to assess the bacterial surface energy, and this general physicochemical parameter correlates well the subsequent attachment properties of the organism, as clearly illustrated by HIC studies and some contact angle data (Gerson, 1981) The reported values for γ BV fall in a broad spectrum from extreme hydrophobicity at 15.6 mJm^{-2} for Mycobacterium butyricum to extreme hydrophilicity at 72 mJm^{-2} for Thiobacillus thiooxidans (Gerson and Zajic, 1979) representing a continum of short range forces from pure dispersion interaction, of so called "hydrophobic bonds",to more polar and electrostatic forces, dependent upon the surface composition. The long-range DLVO theory clearly describes the importance of electrostatic forces as a cell approaches the secondary and primary minima. However, once in close contact it is the effects of adsorbed water and water structure at the solid interface which influences these close range forces and the bonding potential of the organism. To simply say the bonding is hydrophilic or hydrophobic is an inadequate description of the complex range of possibilities available.

In order to determine the role of different surface components in the primary adhesion process, both phenotypic and genotypic changes were examined. This approach has verified

that polysaccharides in the form of LPS or EP exclude bacterial surfaces from surface interactions involving both hydrophobic and hydrophilic adhesion. Cells grown in carbon or carbon/ nitrogen-limitation adhere more readily when the polysaccharide content of the membrane is reduced as growth rate is reduced This finding is consistent with what is known about the metabolic state of zymogenous bacteria that form the bases of the microbial succession of solid surfaces. These cells are physiologically dormant, a situation likely to reduce polysaccharide content of the membrane to a minimum. It was not possible to use the genetic studies to investigate the role of extrinsic membrane proteins, as suitable mutants in outer-membrane proteins were not discovered. However, the mutants that were identified from changes in colony morphology clearly support the exclusion role demonstrated by the variety of polysaccharides analysed. Proteins may also show similar exclusion properties as demonstrated by the Tra-proteins expressed in the transfer genes of R plasmids. These proteins mask specific sites on the cell surface which interact with the pilus (Timis et al, 1979). They also prevent complement fixation and subsequent lysis of the cell by a similar mechanism.

5.5 FUTURE STUDIES

Two areas of study would be envisaged:

i) An investigation of the physiological activity of bacteria in a biofilm and the role played by polysaccharides and other cell products in stabilising of biofilms. The aim would be to identify conditions whereby optimum physiological activity could be produced and maintained in a stable biofilm. Analysis would be made of polysaccharide and protein components of a biofilm isolated from natural aquatic environment by enrichment using a continuous culture system containing a high surface to volume ratio. This approach would also allow assays of physiological activity of the developing biofilm using enzyme assays and analysis of culture eluant.

ii) An investigation of cell aggregation and adhesion control at the genetic level. The aim would be to improve the bio-technology of cell immobilisation and problems associated with downstream processing. The approach would be to study mutations in cell surface components with the view to genetic control of cell aggregation or adhesion by cloning the essential functions on plasmids. This would allow cells surface polymers which control cell aggregation to be manipulated during the trans-formation of organic compounds or the production of cloned gene products of commercial value and cells to be readily collected or removed from these cultures. The results of this thesis indicate that control of LPS production would provide a

sufficiently sensitive system. Alternatively fermenter design can also be important in maintaining selection for a certain genotypes as demonstrated by the principle of foam fractionation and the stable maintenance of alginate production by Pseudomonas fluorescens.

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