THE PHYSIOLOGICAL ACTIVITY OF ATTACHED BACTERIA

by

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CONTENTS

Page.

Acknowledgements viii.

Declaration ix.

List of Abbreviations x.

List of Figures xii.

List of Tables xiv.

Summary xv.

1. General Introduction 1.

1.1. INTRODUCTION 1.

1.2. OBSERVATIONS ON THE INFLUENCE OF SURFACES ON MICROBIAL ACTIVITY 2.

1.2.1. Bacterial numbers 3.

1.2.2. Substrate utilisation 4.

1.2.3. Respiration 4.

1.2.4. Metabolic products 5.

1.3. REASONS WHY THE ACTIVITY OF ATTACHED CELLS MAY DIFFER FROM THAT OF FREE-LIVING CELLS 6.

1.3.1. Species selection 6.

1.3.2. Microenvironmental effects 7.

1.3.2.1. Microenvironmental effects due to surface charge 7.

1.3.2.2. Microenvironmental effects not due to surface charge 11.

1.3.2.3. The influence of molecular adsorption on attached microbial activity 13.

1.3.2.4. The influence of solid surfaces on the uptake and loss of molecules and ions by surface-associated microorganisms 15.

1.4. PURPOSE OF THE STUDY 16.

1.5. CHOICE OF TECHNIQUES 16.

2. Growth and Attachment Characteristics of Marine Bacteria 18.

2.1. INTRODUCTION 18.

Page.

2.1.1. The means by which bacteria attach 18.

2.1.2. The stages of attachment 19.

2.1.3. The attachment process 19.

2.1.4. The influence of environmental conditions on attachment 22.

2.1.5. The influence of physiological activity in attachment 23.

2.1.6. Aims 24.

2.2. MATERIALS AND METHODS 24.

2.2.1. Organisms and growth conditions 24.

2.2.2. Attachment of bacteria to substrata 24.

2.3. RESULTS 25.

2.3.1. Growth characteristics 25.

2.3.2. The influence of time on attachment 25.

2.3.3. The influence of the substratum on attachment 25.

3. Substratum Characteristics 32.

3.1. INTRODUCTION 32.

3.1.1. Evaluation of substratum characteristics 32.

3.1.2. Aims 34.

3.2. MATERIALS AND METHODS 34.

3.2.1. Substrata 34.

3.2.2. Contact angle measurements 35.

3.3. RESULTS 36.

3.3.1. Substratum contact angles 36.

3.3.2. The relationship between θ_B and leucine concentration 37.

3.4. DISCUSSION 37.

3.4.1. Contact angle hysteresis 37.

3.4.2. The relationship between θ_B and θ_R 39.

3.4.3. The influence of leucine on θ_B 40.

3.4.4. Choice of substrata for use in further studies 40.

3.4.5. Summary 41.

Page.

4. Microautoradiographic Evaluation of Substrate Assimilation 43.

4.1. INTRODUCTION 43.

4.1.1. Aims 45.

4.2. MATERIALS AND METHODS 45.

4.2.1. Organism and growth conditions 45.

4.2.1.1. Bacteria incubated with ^{3}H-amino acid mixture or ^{3}H-glucose 45.

4.2.1.2. Bacteria incubated with individual ^{3}H-amino acids 45.

4.2.2. Attachment of bacteria to substrata 46.

4.2.2.1. Bacteria incubated with ^{3}H-amino acid mixture or ^{3}H-glucose 46.

4.2.2.2. Bacteria incubated with individual ^{3}H-amino acids 46.

4.2.3. Incubation of bacteria with ^{3}H-labelled substrates 46.

4.2.3.1. Incubation with ^{3}H-amino mixture and ^{3}H-glucose 46.

4.2.3.2. Incubation of bacteria with individual ^{3}H-amino acids after attachment 47.

4.2.3.3. Incubation of bacteria with ^{3}H-leucine before attachment 47.

4.2.4. Mounting samples onto microscope slides 48.

4.2.5. Microautoradiography 48.

4.2.6. Epifluorescent microscopy 49.

4.3. RESULTS 49.

4.3.1. The relationship between time and bacterial assimilation of ^{3}H-amino acids and ^{3}H-glucose 49.

4.3.2. The relationship between leucine concentration and assimilation by bacteria incubated in static and agitated incubation solutions 50.

4.3.3. The relationship between substratum contact angle and bacterial assimilation of leucine 51.

4.3.4. The relationship between substratum contact angle and bacterial assimilation of different amino acids 52.

Page.

4.3.5. The relationship between bacterial assimilation of leucine and subsequent attachment 52.

4.3.6. The relationship between substratum contact angle and attachment 53.

4.4. DISCUSSION 53.

4.4.1. Assimilation by attached bacteria 55.

4.4.2. Summary 59.

5. An Evaluation of Electron Transport System Activity 60.

5.1. INTRODUCTION 60.

5.1.1. Aims 61.

5.2. MATERIALS AND METHODS 61.

5.2.1. Organism and growth conditions 61.

5.2.2. Attachment of bacteria to substrata 61.

5.2.2.1. Bacteria incubated with INT-ASW solutions 61.

5.2.2.2. Bacteria incubated with INT-ASW leucine solutions 62.

5.2.3. Incubation with INT 62.

5.2.3.1. Incubation of attached bacteria with INT-ASW solutions 62.

5.2.3.2. Incubation of attached bacteria with INT-ASW leucine solutions 62.

5.2.3.3. Incubation of bacteria with INT-ASW leucine solution before attachment 63.

5.2.4. Microscopy 63.

5.3. RESULTS 63.

5.3.1. The relationship between INT concentration and ETS activity of bacteria attached from nutrient-rich and nutrient-poor cell suspensions 63.

5.3.2. The relationship between substratum contact angle and proportion of bacteria demonstrating ETS activity 64.

Page.

5.3.3. The relationship between ETS activity of bacteria and subsequent attachment 65.

5.3.4. The relationship between substratum contact angle and attachment of bacteria incubated with INT 65.

5.4. DISCUSSION

5.4.1. Summary 68.

6. Assimilation and Respiration of ^{14}C-Amino Acids 70.

6.1. INTRODUCTION 70.

6.1.1. Utilisation of organic substrates by attached and free-living microbial populations 72.

6.1.2. Aims 75.

6.2. MATERIALS AND METHODS 75.

6.2.1. Organism and growth conditions 75.

6.2.2. Attachment of bacteria to substrata 75.

6.2.3. Incubation of attached and free-living bacteria with ^{14}C-amino acids 75.

6.2.4. Evaluation of respiration and assimilation 76.

6.2.5. Evaluation of cell numbers 77.

6.2.6. Calculation of uptake kinetics 78.

6.3. RESULTS 79.

6.3.1. The relationship between assimilation or respiration of leucine by surface-associated and free-living cells and substratum contact angles 79.

6.3.2. The relationship between CAQ's of bacteria incubated with leucine and substratum contact angles 79.

6.3.3. The relationship between uptake kinetics of leucine and substratum contact angle 80.

6.3.4. The relationship between velocity of bacterial assimilation or respiration of leucine, glycine, glutamate and arginine and substratum contact angle 80.

Page.

6.4. DISCUSSION 81.

6.4.1. Summary 86.

7. General Discussion 87.

7.1. COMPARISON OF RESULTS FROM THE ACTIVITY STUDIES 87.

7.2. SOME IMPLICATIONS OF THE RESULTS OF THIS INVESTIGATION 88.

7.3. CONCLUSIONS 89.

REFERENCES 92.

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J. J. Bright and M. Fletcher
Applied and Environmental Microbiölogy, 1983, 45, 818-825.

Amino acid assimilation and respiration by attached and free-living populations of a marine *Pseudomonas* sp.
J. J. Bright and M. Fletcher
Microbial Ecology, 1983, 9, 215-226.

ABBREVIATIONS

ASW	:	artificial sea water
CAQ	:	Carbon assimilation quotient
DLVO	:	Derjaguin and Landau, Verwey and Overbeek colloid stability theory
E_h	:	redox potential
ETS	:	electron transport system
G	:	glass treated with ethanol containing HCl
GD	:	glass treated by sonication in detergent
GH	:	glass treated by sonication in water
GU	:	untreated glass
INT	:	2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride
K	:	a value similar to the Michaelis constant equivalent to the substrate concentration when the velocity of uptake is half the maximum rate.
M	:	mica
MAR	:	Microautoradiography
N	:	nylon
NCMB	:	National Collection of Marine Bacteria
P	:	probability
PE	:	polyethylene
PMMA	:	polymethylmethacrylate
PP	:	polypropylene
PS	:	polystyrene
PST	:	tissue culture treated polystyrene
PT	:	platinum
PTFE	:	polytetrafluorethylene
PVC	:	polyvinyl chloride
PVDF	:	polyvinylidene fluoride

r : regression coefficient

rf : roughness factor

S.E.M. : standard error of the mean

SW : natural aged sea water

T : polyethylene terephthalate

Vmax : maximum velocity of reaction

γ_{BL} : bacteria/liquid interfacial tension

γ_{BS} : bacteria/solid interfacial tension

γ_{BV} : bacterium surface tension

γ_{C} : critical surface tension

γ_{LV} : liquid/vapour surface tension

γ_{S} : substratum surface tension

γ_{SL} : solid/liquid interfacial tension

γ_{SV} : solid/vapour surface tension

ΔF : free energy of attachment

θ : contact angle

θ_A : advancing water contact angle

θ_B : bubble contact angle

θ_E : equilibrium contact angle

θ_R : receding water contact angle

θ_W : water contact angle

FIGURES

		Following page.
2.1.	The relationship between the total interaction energy and the separation distance of two approaching surfaces.	19.
2.2.	The relationship between attachment of bacteria to G and to PS and time.	25.
3.1.	Diagramatic representation of advancing, receding and bubble contact angles.	35.
3.2.	The relationship between advancing, receding and equilibrium contact angles and bubble contact angle.	36.
4.1.	The relationship between assimilation of amino acids and glucose by attached and detached bacteria and their associated backgrounds and time.	49.
4.2.	The relationship between assimilation of leucine by attached, detached and free-living bacteria from static and agitated solutions and leucine concentration.	50.
4.3.	A microautoradiograph of attached and free-living bacteria	50.
4.4.	The relationship between assimilation of leucine by bacteria and substratum contact angle	51.
4.5.	The relationship between background silver grains on control substrata incubated with ^{3}H-leucine and substratum contact angle.	51.
4.6.	The relationship between assimilation of amino acids by attached and detached bacteria and substratum contact angle.	52.
4.7.	The relationship between background silver grains on control substrata incubated with ^{3}H-amino acids and substratum contact angle.	52.
4.8.	The relationship between the proportion of ^{3}H-leucine labelled bacteria that attached or remained unattached and time.	52.

Following page.

4.9. The relationship between numbers of attached, or percent detached, bacteria and substratum contact angle. 53.

5.1. Photomicrograph of bacteria attached to G demonstrating ETS activity 60.

5.2. The relationship between the proportion of bacteria attached to and detached from G, demonstrating ETS activity and INT concentration. 64.

5.3. The relationship between the proportion of attached and detached bacteria, demonstrating ETS activity and substratum contact angle. 64.

5.4. The relationship between the proportion of bacteria demonstrating ETS activity that attached or remaining unattached and time. 65.

5.5. The relationship between numbers of attached bacteria or percent detached bacteria and substratum contact angle. 65.

6.1. The relationship between velocity of leucine assimilation by surface-associated bacteria and substratum contact angle.. 79.

6.2. The relationship between respiration of leucine by surface-associated bacteria and substratum contact angle. 79.

6.3. The relationship between total uptake of leucine by surface-associated bacteria and substratum contact angle. 79.

6.4. A modified Lineweaver-Burke plot of leucine uptake kinetics by surface-associated and free-living bacteria. 80.

6.5. The relationship between carbon assimilation quotients of surface-associated bacteria and substratum contact angle. 80.

6.6. The relationship between amino acid assimilation by attached and detached bacteria, amino acid respiration by surface associated bacteria and substratum contact angle. 80.

6.7. The relationship between the carbon assimilation quotients of four amino acids by surface associated bacteria and substratum contact angle. 81.

TABLES

		Following page.
2.1.	Source and morphological characteristics of the bacteria.	25.
2.2.	Growth characteristics of the bacteria.	25.
3.1.	Substratum advancing, receding, bubble and equilibrium water contact angles.	36.
3.2.	Substratum ASW contact angles.	36.
3.3.	Relationship between leucine concentration and substratum ASW bubble contact angle.	37.
6.1.	Data derived from uptake kinetics.	80.

SUMMARY

Assimilation and respiration by a marine *Pseudomonas* sp. was evaluated to determine whether the activity of bacteria attached to solid surfaces (substrata) differed from that of free-living bacteria. Bacteria were allowed to attach to glass and plastic substrata with a range of wettabilities, evaluated by measuring water contact angles. Amino acid assimilation was determined by microautoradiography and liquid scintillation counting, and respiratory activity was determined from $^{14}CO_2$ evolution and electron transport system (ETS) activity evaluated by tetrazolium staining. The uptake kinetics of leucine demonstrated that free-living cells had a smaller half-saturation constant than any of the surface-associated populations but a somewhat greater maximum velocity of uptake than the cells associated with all but the most hydrophobic substratum. Pre-attachment incubation of free-living bacteria with ^{3}H-leucine resulted in the subsequent attachment to all the substrata of approximately a four fold higher percentage of labelled bacteria as compared with populations that remained unattached. When incubated with amino acids at a concentration of 10μg C l^{-1} after attachment, the proportion of attached bacteria that assimilated amino acids and the rate of assimilation per cell, was greater than, or similar to, that of free-living bacteria. However, the proportion of attached bacteria that demonstrated ETS activity and the rate of $^{14}CO_2$ respired per surface-associated cell, was less than, or similar to, that of free-living bacteria. There was little relationship between substratum wettability and the activity of attached bacteria at low substrate concentrations ($\lesssim$ 10μg C l^{-1}) but in the presence of higher leucine concentrations the proportion of attached bacteria that assimilated leucine and demonstrated ETS activity increased with substratum hydrophilicity. The relative activities of attached and free-living bacteria depended upon the substrate, its concentration and the substratum properties. However, the efficiency of substrate utilisation by attached bacteria was generally greater than that for free-living bacteria.

1. General Introduction

1.1. INTRODUCTION

The microbial populations of some natural aquatic environments are largely composed of attached microorganisms (Zobell, 1972; Costerton and Geesey, 1979). This is particularly true in water systems with high concentrations of particulate matter (Kirchman and Mitchell, 1982). Attached (periphytic) microorganisms may consequently make an appreciable contribution to the total microbial processes in a given body of water. Moreover, if, as is generally accepted, (Mitchell, 1974; Marshall, 1976) attached microorganisms are metabolically more active than free-living cells, then the contribution made to these processes by the periphytic population would be proportionately greater than expected by comparison of the sizes of the two population.

The physiological activity of attached microorganisms also has important consequences for man as these organisms can initiate fouling of ships hulls,oil drilling platforms, piplines and heat exchanger equipment (Zobell and Allen, 1935; Miller *et al.*, 1948; Schweisfurth and Mertes, 1962; Corpe, 1973; Baier 1981; Characklis, 1981). The activity of microbial films can, however, be put to good use, as in waste water treatment (Marshall, 1978) and in man-made immobilised cell systems used in biotechnological processes to produce organic compounds such as pharmaceuticals (Abbott, 1976; Chibata and Tosa, 1977; Venkatasubramanian and Vieth, 1979). A better understanding of the factors that influence the activity of attached microorganisms may enable us to evaluate and predict the contribution made by these organisms to the total microbial processes of an environment and may allow us to enhance or restrict, attached microbial growth as required.

Observations from as early as 1901 (Whipple, 1901) have indicated that solid surfaces (substrata) can influence the physiological activity of aquatic microorganisms. Numerous studies (Whipple, 1901; Lloyd, 1937; Zobell, 1937, 1943; Zobell and Anderson, 1936) demonstrated that microbial growth was increased when the ratio of solid surface area to water volume was increased by enclosure in smaller vessels, or by adding glass beads. Zobell (1943) suggested that the influence of solid surfaces (surface effect) was due to greater efficiency of use of extracellular enzymes in utilisation of macromolecular material. However, Heukelekian and Heller (1940) demonstrated beneficial surface effects in the presence of small nutrient molecules not requiring extracellular enzymic breakdown. Observations that the stimulatory influence of substrata on microbial activity only occurred at low nutrient (substrate) concentrations (Heukelekian and Heller, 1940; Jannasch, 1958) have helped to support the suggestion that the beneficial effect of surfaces is due to concentration of nutrients (adsorption) at the solid/liquid interface. This explanation for the surface effect is often stated in microbiology textbooks and is generally accepted (Harvey, 1969; Marshall, 1976; Mitchell, 1974). However, there is no direct evidence to support the assumption that the surface effect is a result of nutrient adsorption. It is, therefore, important to try to obtain some direct evidence to determine the nature of the influence of surfaces on microbial activity before this possible erroneous assumption is further perpetuated.

This chapter describes in detail observations of the influence of surfaces on microbial activity, the mechanisms by which substrata may influence microbial activity and the reasons for choosing the techniques used in this investigation.

1.2. OBSERVATIONS OF THE INFLUENCE OF SURFACES ON MICROBIAL ACTIVITY

Studies have shown that solid surfaces can increase, or decrease, bacterial activity as determined by changes in bacterial numbers, rate of substrate utilisation, respiration or accumulation of metabolic products.

1.2.1. Bacterial numbers

Observations suggest that by increasing the solid/liquid interfacial area of a volume of low nutrient water with solid materials not susceptible to microbial breakdown, the maximum number of microorganisms that the water can support is increased. Whipple (1901) observed that bacteria multiplied faster in small containers than larger ones and suggested this was due to the availability of oxygen. However, Zobell and Anderson (1936) and Lloyd (1937), obtained similar results after eliminating effects due to oxygen availability. All these studies demonstrated a positive correlation between the solid surface area to volume ratio and number of cells that the water supported. However, storage of natural waters in bottles does not always result in an increase in bacterial numbers. Enclosure of natural lake water samples in glass bottles for up to twenty hours had no significant effect on cell numbers in one study (Czeczuga, 1960), and in another investigation there was a negative relationship between the surface to volume ratio of flasks containing eutrophic lake water and the number of bacteria observed in flasks after containment for six hours (Godlewska-Lipowa, 1969).

Increasing the solid surface area of flasks by the addition of glass beads was also shown to increase bacterial numbers, but only at low substrate concentrations (Heukelekian and Heller, 1940). The addition of some solid materials other than glass, to increase the solid/liquid interface area, have also been shown to increase bacterial growth. For example, the presence of talc particles allowed growth of *Escherichia coli* in distilled water, which would not normally support growth (Bigger and Nelson, 1941).

These observations of a general increase in microbial numbers in a volume of low nutrient water with an increase in solid/liquid interfacial area suggest that solid surfaces may somehow increase the substrate available for microbial growth. Surfaces could exert this influence directly by transforming substances unavailable for microbial breakdown into substances susceptible to microbial utilisation (section 1.3.2.3.), or indirectly by increasing the efficiency of substrate utilisation by the microbial population (sections 1.3.2.3. and 1.3.2.4.).

1.2.2. Substrate utilisation

In contrast to the generally stimulatory influence of an increase in solid surface area on cell numbers, surfaces have generally been shown to have an adverse effect on microbial utilisation of substrates. For example, cells of *Micrococcus luteus* absorbed onto an ion exchange resin oxidised glucose and succinate more slowly than cells freely suspended in solution (Hattori and Hattori, 1963), and cells of *Escherichia coli* adsorbed onto a similar resin oxidised glucose, succinate, lactose, xylose, fumarate, and alanine more slowly than cells freely suspended in solution (Hattori and Furusaka, 1959a). A reduction in glucose utilisation was also observed when *Escherichia coli* and *Azotobacter chroococcum* were cultured in the presence of quartz powder (Tschapek and Giambiage, 1956).

The efficiency of substrate utilisation, as determined by growth yield, was found in one study to be much higher for adsorbed cells than for free-living cells (Hattori and Hattori, 1981). If attached cells were generally more efficient substrate utilisers than free-living cells, the apparently contradictory observations described above of an increase in solid surface area generally increasing cell numbers and decreasing the rate of substrate utilisation, may be a result of the larger attached population making slower, more efficient use of the available substrate.

1.2.3. Respiration

Efficient use of substrate by heterotrophic microorganisms may result from the minimum proportion of substrate carbon being respired as carbon dioxide. However, any resulting increase in biomass would result in an increase in the total level of respiration. Observations have been made of both stimulatory and inhibitory surface effects on microbial respiration. A number of solid materials have been shown to increase the respiration of microorganisms. Oxygen consumption by freshwater bacteria attached to glass was greater than that by cells in suspension (Hendricks, 1974), and oxygen uptake by freshwater bacteria in the presence of low peptone concentrations was increased by chitin and silt particles (Jannasch and Pritchard, 1972). Glass beads increased oxygen uptake by *Bacillus megaterium* (Ou and Alexander, 1974) and increased carbon dioxide production by a mixed microbial population and by *Bacillus subtilis* (Parr and Norman, 1964). Oxygen consumption and carbon dioxide production by soil microbiota were accelerated by the presence of the clay montmorillonite (Kunc and Stotzky, 1974, 1977).

Clay particles smaller than bacteria can, however, decrease oxygen consumption by bacteria (Lahav and Keynan, 1962), as can adsorption to cation exchange resin (Hattori and Hattori, 1963). This may be a result of the decrease in surface of the cell through which substrate and oxygen are taken up. Carbon dioxide production was also depressed when soil particles were added to bacteria (Chudiakov, 1926; Peele, 1936) and fungi were adsorbed onto glass (Parr and Norman, 1964; Navarro and Durand, 1977).

1.2.4. Metabolic products

Microbial activity, as determined by the production of metabolites, has generally been shown to be stimulated or not influenced by solid surfaces depending upon the composition of the substratum. For example, methane production by anaerobic bacteria was increased by asbestos particles but not by sand or glass wool (Breden and Buswell, 1933), and methane production by bacteria on fired clay was observed to be three times that by bacteria on PVC, or glass (Murray and van den Berg, 1981). Lactic acid production by some streptococci was increased by adsorption onto hydroxyapatite (Berry and Henry, 1977), and adsorption of *Saccharomyces carlsbergensis* onto glass beads increased the yield of ethanol from glucose (Navarro and Durand, 1977).

These observations indicate that surfaces do not always enhance microbial activity. The precise influence of surfaces on microbial activity may depend upon the type of activity determined, as well as the species present, the substratum composition and the form and concentration of the substrate.

1.3. REASONS WHY THE ACTIVITY OF ATTACHED CELLS MAY DIFFER FROM THAT OF FREE-LIVING CELLS.

There are two types of mechanism that may cause the activities of attached and free-living cells to differ. Firstly, as a result of the selective nature of the attachment process, the species composition of the attached and free-living populations may differ giving rise to different activities as a result of the different physiological capabilities, or microbial interactions of the two populations. Secondly, the environment in the vicinity of attached cells may be more, or less, favourable for microbial activity than that of free-living cells, as a result of the influence of the physico-chemical properties of the solid surface.

1.3.1. Species selection

As physiological activity may be required for attachment of micro-organisms (Fletcher, 1980b), selective attachment of the more active cells from within the free-living population of a single species may occur, thereby giving rise to an attached population with a greater potential activity per cell than the free-living population. Moreover, as attachment of cells from a mixed free-living microbial population is selective with regard to species (Zobell, 1943; Corpe, 1973; Zvyagintsev, 1973; Marshall, 1976), the activities of the attached and free-living populations may differ as a result of differences in species composition. The activity of an attached population may also change with time as a result of the rate of microbial colonisation, which can be influenced by the substratum characteristics (Loeb, 1977; Dexter, 1979), or as a consequence of changes in species composition that occur during succession in a microbial film (Marshall, 1976; Gerchakov, *et al.*, 1977, Marszalek *et al.*, 1979, Dempsey, 1981). The diversity, as well as the biomass and numbers of attached cells increases as succession progresses (Jordan and Staley, 1976). The species composition of the attached population will, therefore, depend initially on the attachment characteristics of the free-living population, the substratum characteristics and the time of exposure of the substratum to the aquatic environment. Thus, there will be spatial and species composition differences in the attached and free-living populations that may give rise to different activities, as a result of the physiological capabilities of the two populations, or the nature of the microbial interactions within the two populations. It is likely that within the attached and free-living populations of a natural body of water there will be differences in competition for nutrients (Jannasch and Pritchard, 1972), mutualism, interdependence, antagonism (Zobell, 1972), predator prey relations (Bitton and Mitchell, 1974; Roper and Marshall, 1974; Bobbie *et al.*, 1978; Ratman *et al.*, 1982) and co-metabolism (Slater, 1978).

1.3.2. Microenvironmental effects

The activity of aquatic microorganisms is largely dependent upon the concentration of various nutrients, ions, exoenzymes and inhibitors in their immediate environment. The charge or physical properties of a solid surface may modify the concentration or availability of these ions and molecules at the solid/liquid interface, as compared with that of the bulk liquid phase, thereby giving rise to different activities of surface-associated and free-living microbial populations.

1.3.2.1. Microenvironmental effects produced by surface charge

The surface charge, or surface potential, may affect

(i) the concentration of ions or charged molecules,

(ii) pH or

(iii) the redox potential (E_h).

(i) Concentration of ions and charged molecules. The distribution of an ion in a solution can be described by the Maxwell-Boltzmann relationship, whereby anions are concentrated and cations repelled at places of positive potential and cations concentrated and anions repelled at places of negative potential, so that

$$[C]_s = [C]_b \exp (z e \psi / k T) \quad (1.1)$$

where [C] = the concentration of an ion at a point where the potential is ψ,

s = concentration at the surface,

b = concentration in the bulk,

z = valence of the ion,

e = electronic charge,

k = Boltzmann constant, and

T = absolute temperature.

Low molecular weight charged substrates may behave in a similar manner to that described for ions (Goldstein *et al.*, 1964). It follows that if the surface and the substrate are of opposite charge, the substrate will be more concentrated at the surface than in bulk solution. In this situation, where the adsorbed species is the substrate for an enzyme reaction, the maximum velocity of reaction (V_{max}) of an enzyme at the surface will be reached at a lower bulk substrate concentration than that of an enzyme in bulk solution. The apparent Michaelis constant (K_m) for the enzyme reaction at the surface ($K_{m\,s}$) will, therefore, be lower than the K_m for the reaction in bulk solution ($K_{m\,b}$.) For surfaces and substrates of opposite charge the reverse will be true and $[C]_s < [C]_b$ and $K_{m\,s} > K_{m\,b}$. Insertion of equation (1.1) into the Michaelis-Menten equation (1.2) gives an equation for the velocity of an enzyme-catalysed reaction at a surface (1.3):

$$V = \frac{V_{max}\, S}{K_m + S} \quad (1.2)$$

$$V_s = \frac{V_{max} (S)_b \exp (z\ e\ \psi/k\ T)}{K_m + (S)_b \exp (z\ e\ \psi/k\ T)} \qquad (1.3)$$

where V = velocity of an enzyme reaction in bulk solution,

V_s = velocity of an enzyme reaction at the surface,

V_{max} = maximum velocity of reaction,

K_m = Michaelis constant, and

S = substrate concentration.

The apparent Michaelis constant of an enzyme reaction can be derived from equation (1.3) to give equation (1.4) (Goldman *et al.*,1971).

$$K_{m\ s} = K_m \exp (-z\ e\ \psi/k\ T) \qquad (1.4)$$

In agreement with the above described phenomenon; Goldstein *et al.*, (1964), Hornby *et al.*, (1966), Wharton *et al.*, (1968a,b) and Goldstein (1970) found the $K_{m\ s}$ of polyanionic enzyme derivatives, reacting with positively charged substrates, were lower than that of native enzymes. The influence of these substrate, enzyme charge interactions on $K_{m\ s}$ decreases as the ionic strength of the solution increases (Goldstein *et al.*, 1964; Wharton *et al.*,1968b). These charge interactions will, therefore, have less influence in sea water systems than in fresh water systems.

(ii) Surface pH. Hydrogen ions can greatly affect microbial activity, and, as with other ions, their concentration at the solid/liquid interface will be influenced by the surface potential. According to Hartly and Roe (1940), the surface potential can be approximated by the electrokinetic or zeta potential(ζ), so that the surface pH can be described by modifying equation (1.1) to give equation (1.5).

$$[H^+]_s = [H^+]_b \exp (-e\ \zeta/k\ T) \qquad (1.5)$$

$$\text{At } 25°C : pH_s = pH_b + \zeta/60 \qquad (1.6)$$

The pH activity curves for enzymes or bacteria associated with charged surfaces, may be displaced by as much as a pH unit, or more as compared with similar activity curves obtained in bulk solution. For example, the pH activity profile of the polyanionic derivative of several proteolytic enzymes acting on their substrates was shifted towards a more alkaline value by 1-2.5 pH units as compared to the native enzymes. Polycationic derivatives of the same enzymes exhibited a similar shift to a more acid pH. (Goldstein *et al.*, 1964; Goldstein and Katchalski, 1968; Goldstein, 1970). The adsorption of bacteria onto anion exchange resin displaced the activity pH profile to the alkaline side by about one pH unit (Hattori and Furusaka, 1959b, 1960, 1961; Hattori and Hattori, 1963; Hattori *et al.*, 1972).

Soils and clays can also influence bacterial activity as a result of their interactions with hydrogen ions. McLaren and Skujins (1963) demonstrated that the pH response curve for nitrification by nitrifying bacteria in the presence of soil particles was 0.5 unit higher than in liquid culture. These workers suggested that this was due to the pH at the site of nitrification, at the negatively charged soil surface, being lower than the pH determined in the liquid phase. Clays, with high cation exchange capacities, may also influence microbial activity by acting as 'buffers' by adsorbing hydrogen ions produced during microbial growth, thereby maintaining a suitable pH for sustained growth (Stotzky, 1966; Kunc and Stotzky, 1977).

(iii) Surface redox potential. The E_h, or electron activity, at a solid surface may be influenced by the oxidative activity of the associated microbial population. If a thick microbial film develops on a surface the oxidative activity of the film may give rise to changes in the species composition of the film and/or changes in facultative aerobic microorganisms from aerobic to anaerobic metabolism. The E_h at a charged surface may also differ from that in the bulk aqueous phase as a result of differences in concentration of oxidising or reducing agents produced by interactions of these agents with the substratum (Hattori and Hattori, 1976).

1.3.2.2. Microenvironmental effects not due to surface charge

The concentration and/or availability of molecules at the solid/ liquid interface may be influenced by their

(i) adsorption,

(ii) entrapment in extracellular polymeric substances or

(iii) modified rates of diffusion.

(i) Adsorption. Although surface potential may play a part in adsorption, other factors not related to surface potential, such as solute/solvent interactions, are important in determining adsorption at the solid/liquid interface. The extent of adsorption of a substance at a gas/liquid interface can be described by the Gibbs adsorption equation (Shaw, 1970). For dilute solutions of concentration (C), the excess concentration of solute per cm_2 of surface, as compared with that in bulk solution (S), is given by equation (1.7).

$$S = \frac{-C}{RT} \cdot \frac{d\gamma}{dc} \qquad (1.7)$$

where $d\gamma/dc$ = ratio of increase of surface tension of the solution with the concentration of solute,

R = gas constant, and

T = absolute temperature.

According to the relationship described by equation (1.7), any solute which causes the surface tension of the solvent to decrease, so that $d\gamma/dc$ is negative, will have a higher concentration at the surface than in bulk solution, since S will be positive. Conversely, if the solute raises the surface tension its concentrations will be lower at the gas/liquid interface than in bulk solution.

The above described principles of solute adsorption at the gas/ liquid interface also apply to adsorption at the solid/liquid interface; however, when a solute is at the solid/liquid interface it will be influenced by the characteristics of the solid as well as the liquid. Polar solute molecules will tend to adsorb onto polar substrata strongly, and non-polar substrata weakly, and vice versa (Shaw, 1970). Moreover, the properties of the substratum to which a molecule adsorbs may make it more, or less, resistant to microbial degradation and in the case of enzymes, may enhance, or decrease, their activity (section 1.3.2.3. *ii*).

(ii) Entrapment in extracellular polymeric substances. Attached microorganisms produce large quantities of extracellular polymeric material which may be involved in attachment. This extracellular material may also entrap molecules and ions that affect the activity of associated attached microorganisms (Costerton, *et al.*, 1978; Geesey, 1982). The large sheath surrounding cyanobacteria is believed to aid in concentrating nutrients from the surrounding medium (Lange, 1976), and some iron oxidising bacteria are thought to trap ferrous ions in their extracellular polymer (Ridgeway *et al.*, 1981). Extracellular enzymes may also be entrapped in the polymer produced by attached microorganisms, thereby, benefitting these organisms by reducing loss of enzymes by diffusion (Zobell, 1972; Tonn and Gander, 1979).

(iii) Diffusion. The activities of microorganisms in microbial films exceeding ~70μm in thickness have been found to be restricted by nutrient diffusion (Kornegay and Andrews, 1968; Saunders and Bazin, 1973). However, the growth of a monolayer of very active attached cells may also be limited by nutrient diffusion (Hattori and Hattori, 1981). In this case the rate of diffusion may be limited as a result of decreased convection near the solid surface. The zone of liquid adjacent to a solid surface with little convection is known as the stationary, unstirred, or Nernst diffusion layer. Depending on the rate of agitation, stationary layers in the order of 10-100μm are invarably found at solid/liquid interfaces (Helfferich, 1962). If the rate of consumption of substrate by attached cells exceeds the rate of diffusion to the surface, a diffusion gradient will be established across the stationary layer and the apparent saturation constant for attached cells will exceed that

for free-living cells (Hattori and Hattori, 1981). The rate of diffusion of substrates through the stationary layer can also limit the rate of reaction of immobilised enzymes producing higher apparent K_m values for these enzymes as compared to native enzymes (Hornby *et al.*, 1968).

1.3.2.3. The influence of molecular adsorption on attached microbial activity.

The concentration of substances at the solid/liquid interface may, or may not, affect attached microbial activity. This will depend upon

(i) the type of molecule adsorbed and

(ii) the influence of adsorption of a molecule on its configuration, which will in turn influence its interaction with surface associated microorganisms.

(i) Type of molecule adsorbed. Molecules that are likely to influence the activity of attached microorganisms include nutrients, extracellular enzymes and inhibitors.

(a) Nutrients. The effect of nutrient adsorption on microbial activity depends upon the bulk nutrient concentration and the influence of adsorption on the availability of the nutrient for microbial utilisation. Heukelekian and Heller, (1940) and Jannasch (1958) observed that in the presence of low nutrient concentrations, surfaces had a beneficial effect on microbial growth, whereas in the presence of high nutrient concentrations, surfaces had little effect. However, the energy of binding of an adsorbed nutrient to a surface, may prevent its utilisation by microorganisms (Stotzky, 1980b). For example, adsorption of aspartate and cysteine onto clays prevented their utilisation by microorganisms even though these amino acids were readily utilised when not adsorbed (Stotzky, 1980b). Furthermore, as well as possible binding energy effects, the availability of large nutrient molecules for microbial breakdown may also be altered by adsorption induced molecular conformational changes (section 1.3.2.3.*ii*).

(b) Extracellular enzymes. Zobell (1943) observed surface effects beneficial to microbial growth in solutions enriched with sodium caseinate, lignoprotein and emulsified chitin but not with glycerol, glucose and lactate. As the substrates that stimulated growth all require exoenzymic breakdown, it was suggested that the beneficial surface influence was due to the solid substratum retarding the diffusion of exoenzymes and hydrolysates away from attached cells. However, adsorption of enzymes may result in conformational changes or steric restrictions that can alter enzymic activity (section 1.3.2.3.*ii*).

(c) Inhibitors. Adsorption of inhibitors at the solid/liquid interface will have an adverse effect on the activity of attached microbial population unless the process of adsorption inactivates the inhibitors. Removal of toxic substances from the aqueous phase by adsorption may, however, allow growth of bacteria in bulk solution, as was found by Pollock, (1947) with *Hemophilus pertussis* and by Gorelick *et al.*, (1951) with *Brucella*. Harwood and Pirt (1972) attributed the increased growth rate and cell density of *Methylococcus capsulatus*, that accompanied an increase in surface to volume ratio, to adsorption of a growth inhibitor by the glass.

(ii) The influence of substrate adsorption on its utilisation by surface-associated microorganisms. The process of adsorption may transform potentially usable organic substances into more, or less, easily assimilated forms, thereby influencing microbial activity. Kriss (1963), has suggested that the observed increase in numbers of heterotrophic microorganisms in sea water stored in glass containers was due to transformation at the solid/liquid interface of recalcitrant organic substances into assimilable substances. The transformation of these so called 'humic substances' was thought to be a result of adsorption denaturing, or altering the whole humic complex, (or at least the protein component) into a form more susceptible to breakdown by microbial enzymes. Adsorption onto clays

may also make protein molecules more susceptible to microbial breakdown (Stotzky, 1972). If the adsorbed protein is an enzyme then its activity may be increased as a result of it being favourably brought together with its substrate (Stotzky, 1972). However, adsorption may decrease enzymic activity by bringing about unfavourable conformational changes of the enzyme molecule, or steric restrictions on the enzymes availability to high molecular weight substrates (Danielli, 1937; Goldman *et al.*, 1971).

1.3.2.4. The influence of a solid surface on the uptake and loss of molecules and ions by surface-associated microorganisms.

Surfaces may influence the activity of attached microorganisms by directly affecting the cells uptake, or loss, of molecules or ions. Zobell, (1972) suggested that solid surfaces may neutralise the zeta potential, or other electrostatic conditions on the cell wall of attached microorganisms, thereby influencing the uptake and loss of substances by the cell. More recently, Ellwood *et al.*, (1982) speculated that surface associated microorganisms have an energetic advantage over free-living cells as a result of more efficient use of protons in the chemiosmotic processes of the cell. As already described (section 1.3.2.1.*ii*), protons can be concentrated at the solid/liquid interface as a result of charge effects, or by entrapment in extracellular polymers. This could allow more efficient use of protons lost from cells during respiration or ATP utilisation, and taken up during ATP synthesis. In this way, the electrical potential across the cell membrane may be economically maintained to drive processes such as uptake of substrates and loss of metabolites against the concentration gradient.

The uptake of substrates by attached microorganisms may not, however, always be enhanced by solid surfaces. Attachment may restrict the uptake of substrates and oxygen by attached microorganisms as a result of reducing the surface area of the cell which is exposed to the bulk aqueous phase (Hattori and Hattori, 1963).

1.4. PURPOSE OF THE STUDY

Initially this study set out to answer two main questions:

1. Does the activity of attached bacteria differ from that of free-living bacteria?

2. Is the activity of attached bacteria affected by the composition of the underlying substratum ?

In the course of trying to answer these, further questions arose and these were consequently included in the study.

3. Does the activity of bacteria that are initially attached but which become detached differ from that of bacteria that remain attached?

4. When a free-living population is exposed to a surface and some cells attach, do these attached cells represent a sub-population with a different activity to those which remain free-living?

5. Do substratum/substrate charge interactions influence substrate utilisation by attached bacteria?

6. Does the efficiency of substrate utilisation by bacteria differ from that of free-living bacteria?

1.5. CHOICE OF TECHNIQUE

To answer the above questions, techniques had to be used that could clearly distinguish between the activities of attached and free-living bacteria. This is difficult to do for two reasons: firstly, because of the small microenvironment that attached bacteria occupy, and secondly, because of the exchange of cells in the two populations. Also as the numbers of attached bacteria were comparatively small the techniques had to be sensitive. Microautoradiography, tetrazolium staining and ^{14}C labelled substrate assimilation and respiration were found to be suitable on both these counts. ATP assays, using the luciferin-luciferase bioluminescence system and a Lumac® Cell Tester, Model 1030, and oxygen uptake measurements using a Clark oxygen electrode were tried and found not to be sensitive enough techniques

To avoid differences in activities of attached and free-living populations arising from species selection, or interspecies interactions (section 1.3.1.) these studies were carried out using pure cultures. Small substrate molecules were used in the activity studies. This prevented possible complications in the interpretation of results that may have arisen with the use of large substrate molecules due to adsorption altering the availability of the substrate for bacterial utilisation (section 1.3.2.3.*ii*) or effects due to substratum interactions with extracellular enzymes (section 1.3.2.3.*i*(b)).

2. Growth and Attachment Characteristics of Marine Bacteria.

2.1 INTRODUCTION

It is probable that the majority of aquatic bacteria are capable of attachment to solid surfaces. Zobell (1943) found that out of 96 strains of marine bacteria tested, 51% attached to glass, 30% of which attached in large numbers. Similarly, a study of 137 strains of bacteria from 7 genera revealed that 69% of the strains, including representatives from each of the genera, attached to glass and 28% of the strains attached at greater than 10^6 cells/cm^2 (Zvyagintsev, 1973).
The attachment properties of species within each genus varied considerably as can different strains of the same species (Hartley *et al.*, 1978). Attachment may, however, depend to some extent on bacterial morphology. Marshall *et al.*, (1971b) observed that small rods attached in higher numbers than large rods, curved rods, coccoidal,spiral or stalked bacteria.

2.1.1. The means by which bacteria attach

Some aquatic bacteria have special structures thought to be involved in attachment. These structures include fimbriae (pili), fibrils, holdfasts (often produced on the end of a stalk) and other appendages (Ellen *et al.*, 1978; Pertovskaya *et al.*, 1972). The surface components involved in microbial attachment to surfaces have recently been reviewed by Corpe (1980). The majority of aquatic bacteria, however, appear to attach by means of extracellular polymeric adhesives (Fletcher, 1979a), the composition of which probably largely determines the ability of these organisms to attach. Extracellular adhesives are probably produced by cell surface polymerases (Costerton *et al.*, 1978) or, as may be the case with *Flexibacter* species, by goblet-shaped structures within the cell wall (Ridgeway and Lewin, 1973). Adhesives are often largely polysaccarides containing acidic groups (Corpe, 1970; Fletcher and Floodgate, 1973, 1976; Geesey *et al.*, 1977). However 50-80% of the dry weight of extracellular polymer from *Pseudomonas* species NCMB 2021 was shown to consist of protein (Fletcher, 1980a). Pre-attachment treatment of bacteria with inhibitors of protein synthesis (Marshall, 1972; Fletcher, 1980b) and post-attachment treatment with protein digesting enzymes (Danielsson *et al.*, 1977; Wood, 1980; Fletcher and Marshall, 1982a), have suggested that a protein component of the extracellular polyer may be involved in adhesion.

2.1.2. Stages of attachment

Zobell (1943) suggested that attachment mediated by extracellular adhesive polymers could be divided into two phases. Observations by Marshall *et al.*,(1971a) confirmed this and defined these phases as 'reversible sorption', the initial stage when the bacterium is weakly held at the substratum so that it can exhibit Brownian motion and can be removed by washing, and 'irreversible sorption', a time-dependent phase in which the bacterium does not exhibit Brownian motion and cannot be removed by washing. It was also suggested that irreversible attachment was mediated by polymer bridging. Other observations have indicated that firm adhesive can occur spontaneously (Disalvo, 1973; Fletcher, 1980a). After firm attachment there is usually a third phase when growth division of attached cells occurs (Ellwood *et al.*, 1982).

2.1.3. The attachment process

The Derjaguin and Landau and the Verwey and Overbeck (DLVO) colloid stability theory (Marshall, 1976) has been employed as a model system to attachment of bacteria to surfaces. In this model, as a bacterium approaches a surface it comes under the influence of attractive van der Waals forces and electrostatic repulsion forces produced by the cationic clouds that surround surfaces which are generally negatively charged. The balance of these forces are such that net attraction occurs $< \approx 1$ nm from the substratum surface, the primary minimum, and at 5-10 nm away from the substratum, the secondary minimum. These two distances of attraction are separated by an area of repulsion, or primary maximum (Figure 2.1). It has been suggested (Marshall *et al.*, 1971a; Rutter,

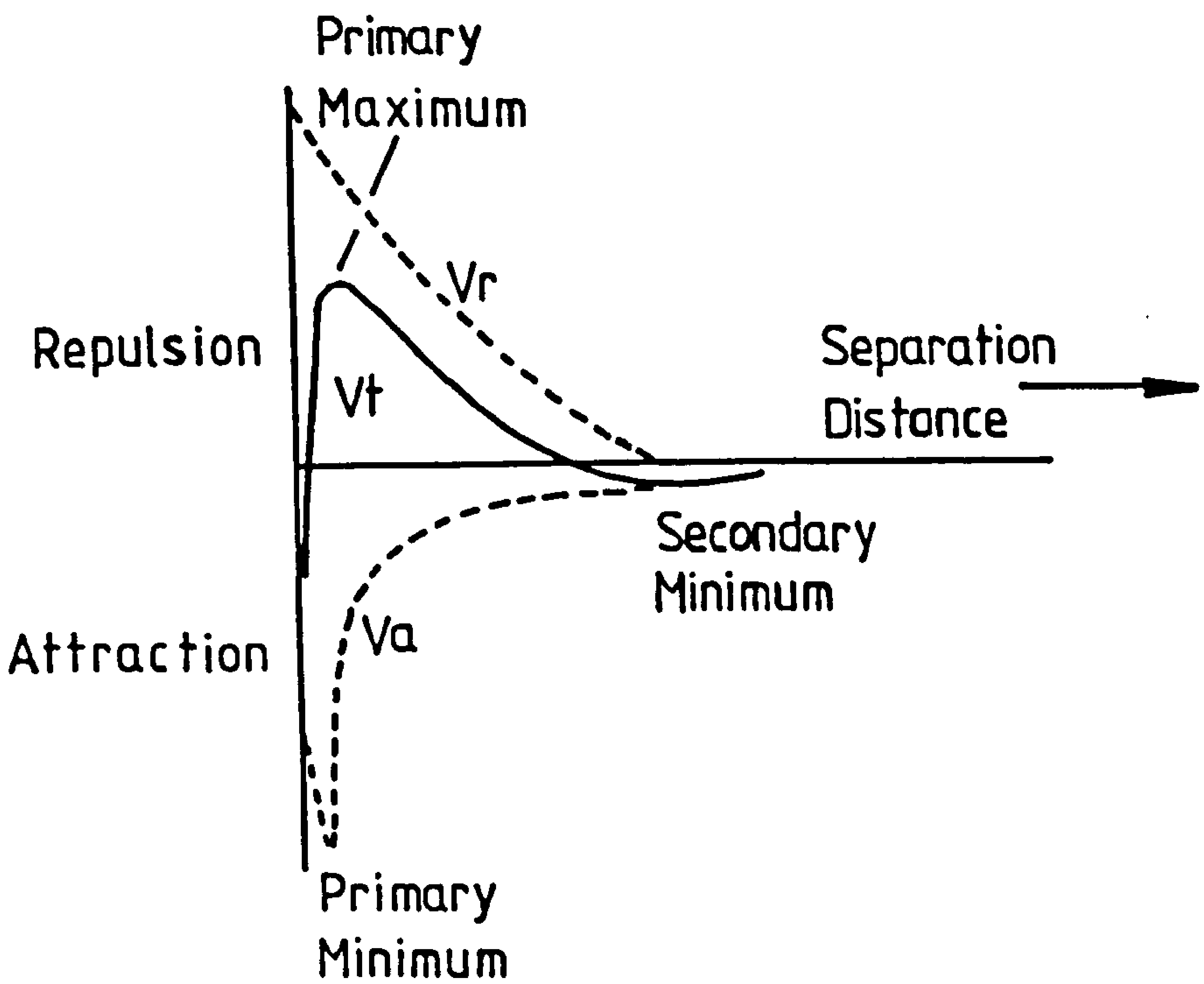


FIGURE 2.1.

The relationship between the total interaction energy Vt and the separation distance of two approaching surfaces. The total interaction energy Vt is obtained by the summation of an attraction curve Va and a repulsion curve Vr. The primary maximum is an area of repulsion that separates two areas of attraction the primary minimum and secondary minimum.

1980), that reversibly adsorbed bacteria are held in the secondary minimum and are prevented from making physical contact with the substratum by the primary maximum. With time the primary maximum is bridged by polymer so that the bacterium becomes irreversibly attached. This physico-chemical DLVO approach to microbial attachment has recently been reviewed by Lips and Jessup (1979), Dolowy (1980), Gingell and Vince (1980), Rutter (1980) and Rutter and Vincent (1980).

The DLVO theory has been developed and expanded so that it presently involves complex calculations of ionic double layer interactions van der Waals forces and entropic (or steric) contributions to the free energy of approach of two surfaces. Although it is not possible to measure the individual components that make up the DLVO theory, it is possible to empirically evaluate the thermodynamic parameters i.e. surface free energy of the bacterium, substratum and liquid components, that are involved in the attachment process. This empirical thermodynamic approach has been compared with the DLVO approach by Pethica (1980) and Tadros (1980).

Surface free energy is a measure of the unsatisfied bonding potential of a surface in a system and comprises van der Waals dispersion forces and electrostatic and polar interactions (Andrade, 1973). The surface tensions (equivalent to surface free energy) of bacteria (γ_{BV}) and solid substrata (γ_{SV}) can be evaluated from contact angle measurements of liquid(s), of known surface tension(s); on bacterial films (van Oss *et al.*, 1975, Gerson and Zajic, 1979; Baier, 1980; Neufeld *et al.*, 1980), or solid surfaces (Zisman, 1964; Newmann, 1974; Newmann *et al.*, 1974), respectively. The surface tension of liquids (γ_{LV}) can be measured directly using standard techniques, e.g. capillary rise, Wilhelmy plate, and platinum ring methods (Shaw, 1970). The values of individual surface tensions γ_{BV}, γ_{LV} and γ_{SV}, so obtained, can be used to calculate the interfacial tensions of bacteria/solid (γ_{BS}), bacteria/liquid (γ_{BL}) and solid/liquid (γ_{SL}) interfaces (Newmann *et al.*, 1974). In general the greater the difference between two surface tensions the greater their interfacial tensions. By incorporation of these interfacial tensions into a simple energy balance equation (1) the free energy of attachment (Δ_F) can be calculated

$$\Delta_F = \gamma_{BS} - \gamma_{BL} - \gamma_{SL} \qquad (1)$$

Attachment is favoured when Δ_F is negative. Clearly from a thermodynamic point of view, in an aquatic environment with a fairly constant γ_{LV}, Δ_F will depend on γ_{BV} and γ_{SV}. Calculations of Δ_F for cell attachment to substrata with a wide range of γ_{SV} reveals that if $\gamma_{BV} < \gamma_{LV}$, Δ_F increases with increasing γ_{SV}, that is, attachment decreases with increasing γ_{SV}, but if $\gamma_{BV} > \gamma_{LV}$ the converse is true, (Newmann *et al.*, 1975). When $\gamma_{BV} = \gamma_{LV}$ Δ_F becomes zero and attachment is independent of the value of γ_{SV}, $\gamma_{BL} = 0$ and $\gamma_{BS} = \gamma_{SL}$ (Newmann *et al.*, 1979).

Both the DLVO and surface free energy approaches may give a fairly accurate physico-chemical account of the non-specific, long-range forces involved in attachment or deposition of cells onto substrata. However, both these approaches have limitations in particular with regard to accounting for short-range forces which are large and specific. These short-range forces occur at the points of contact of the cell and substratum surface and largely govern adhesion, or the forces needed to separate adherents. Adhesion will therefore, be influenced by molecular surface protrusions and specific molecular interactions of the cell and substratum surfaces. Furthermore, these models cannot account for changes in adhesion that may occur with time as a result of changes in physiological activity of the cell.

2.1.4. The influence of environmental conditions on attachment

The attachment characteristics of any particular strain of bacteria depend to some extent on the environmental conditions. For example, the composition of the growth medium both in batch (Marshall *et al.*, 1971a) and continuous culture (Brown *et al.*, 1977) can affect attachment. This may be due to a qualitative or quantitative influence on extracellular polymer production (Ellwood *et al.*, 1982). Attachment can also be influenced in batch cultures by the bacterial growth phase, (Zobell, 1943; Zvyagintsev, 1973; Zvyagintsev *et al.*, 1977; Fletcher, 1977) and in continuous culture by the specific growth (dilution) rate (Ellwood *et al.*, 1974; Rutter and Leech, 1980).

Substratum characteristics can influence the number of cells that attach and the strength of adhesion (Gerson and Zajic, 1979). Attachment has been shown to be quantitatively affected by the substratum surface charge (Fletcher and Loeb, 1979) and surface free energy (Dexter *et al.*, 1975; Dexter, 1979; Fletcher and Loeb, 1979; Gerson and Scheer, 1980; Pringle and Fletcher, 1983). The influence of substratum surface free energy on quantitative attachment is, at least in part, due to the contribution made by the substratum to the adhesive strength between the cell and the substratum (Disalvo and Danials, 1975; Fowler and McKay, 1980; Gingell and Vince, 1980). However, the substratum characteristics, and hence the quantitative attachment of bacteria to substrata, may be modified as a result of adsorption of conditioning films (Goupil *et al.*, 1973; Loeb and Neihof, 1975; Baier, 1980). These films rapidly form by adsorption of macromolecular organic material that may originate as excretory products from living organisms (including those that subsequently attach) or as decompositional products from dead microorganisms (Fletcher and Marshall, 1982b).

Other environmental factors that can influence attachment include electrolyte concentration (Marshall *et al.*, 1971a; Ørstavik, 1977; Fletcher, 1979a), pH (Takakuma *et al.*, 1979; Wood, 1980) and temperature

(Fletcher, 1977; Ørstavik, 1977). As these factors can affect both the physico-chemical aspects of attachment and the physiological activities of the organisms involved in attachment, it is difficult to distinguish the nature of the influence of these environmental factors on the attachment process.

2.1.5. The influence of physiological activity on attachment

The general physiological state of bacteria appears to influence attachment as indicated by a decrease in numbers of attached bacteria (Zvyagintsev, 1973; Fletcher, 1977) and strength of adhesion (Zvyagintsev *et al.*, 1971, 1977) with increase in culture age. This may be due to changes in the type, or quantity of polymer produced (Fletcher, 1980a), or production of polymer degrading exoenzymes (Zvyagintsev *et al.*, 1977).

Physiological activity in the form of motility may also contribute to the attachment characteristics of some organisms. Natural loss, or removal of flagella has been found to parallel a decrease in number of attached cells (Fletcher, 1979a), although this is not always the case (Wood, 1979). The presence of flagella may facilitate attachment via motility by increasing the number of collisions with the substratum, or by producing sufficient force to overcome electrostatic repulsion forces (Fletcher, 1980a). Motility may also influence attachment by allowing positive, or negative, chemotactic responses to attractants, or repellents, that may accumulate at the solid/liquid interface (Adler, 1975; Marshall, 1976).

In this investigation eight strains of marine bacteria, that represent some of the principal genera in the sea (Wood, 1965), were selected. Growth characteristics were assessed from batch growth curves, and attachment characteristics were assessed by evaluating the effects of time, substratum properties, fixation with formaldehyde and growth medium composition on the numbers of bacteria that attached.

2.1.6. Aims

The objective of studying the growth and attachment characteristics of marine bacteria were:

1. To compare the attachment characteristics of a range of strains of bacteria with different morphological and growth characteristics.

2. To determine the influence of physiological characteristics and growth factors on attachment.

2.2. MATERIALS AND METHODS

2.2.1. Organisms and growth conditions

Eight species of marine bacteria were obtained from the National Collection of Marine Bacteria (N.C.M.B.), (Torry Research Station, Aberdeen). The organisms and N.C.M.B. strain numbers were *Corynebacterium erythrogenes* 5, *Micrococcus* sp. 13, *Bacillus filicolonicus* 445, *Bacillus epiphytus* 1045, *Vibrio fisheri* 1281, *Bacillus pacificus* 1862, *Flavobacterium uliginosum* 1863, and *Pseudomonas* sp. 2021. These bacteria were each cultured at 15°C in an orbital incubator (150 rpm) in 100 ml of media comprising 0.1% (w/v) peptone (Oxoid Ltd., London) and 0.07% yeast extract powder (Lab M, London) in aged sea water (SW), pH 8.1. In addition, two of the organisms *B. pacificus* and *Pseudomonas* sp. were capable of growing on glucose and were cultured as above but in 100 ml of media comprising 0.5 g l^{-1} glucose, 0.076 g l^{-1} NH_4Cl and KH_2PO_4 in (SW) pH 8.1. The bacteria were grown to late stationary phase, harvested by centrifugation (10K, 4°C) and resuspended in SW to $\cong 10^7$ cells ml SW^{-1}.

2.2.2. Attachment of bacteria to substrata

10 ml portions of the $\cong 10^7$ cells ml SW^{-1} cell suspension were allowed to attach for 2, 4 and 24h at 15°C to polystyrene petri dishes (PS) (50 mm diameter, Sterilin Ltd., Teddington) and glass coverslips (G) (16 mm diameter, No. 1½, Chance Propper Ltd., Warley), attached to the bottom of each petri dish with double sided adhesive tape. After the period allowed for attachment the dishes were washed with filter sterile SW to remove residual suspended cells and loosely attached organisms.

Attached bacteria were fixed with Bouin's fixative and stained with crystal violet. Replicate samples of each organism were treated as above but were fixed by the addition of 0.5 ml formaldehyde to the 10 ml cell suspension before allowing attachment for 24h.

Attachment was estimated by microscopic counting. The number of bacteria in 50 or 100 100 μm^2-area were counted for duplicate samples using a Zeiss Standard 18 Microscope fitted with x100 oil objective and x10 eye piece with bright field illumination.

2.3. RESULTS

2.3.1. Growth characteristics

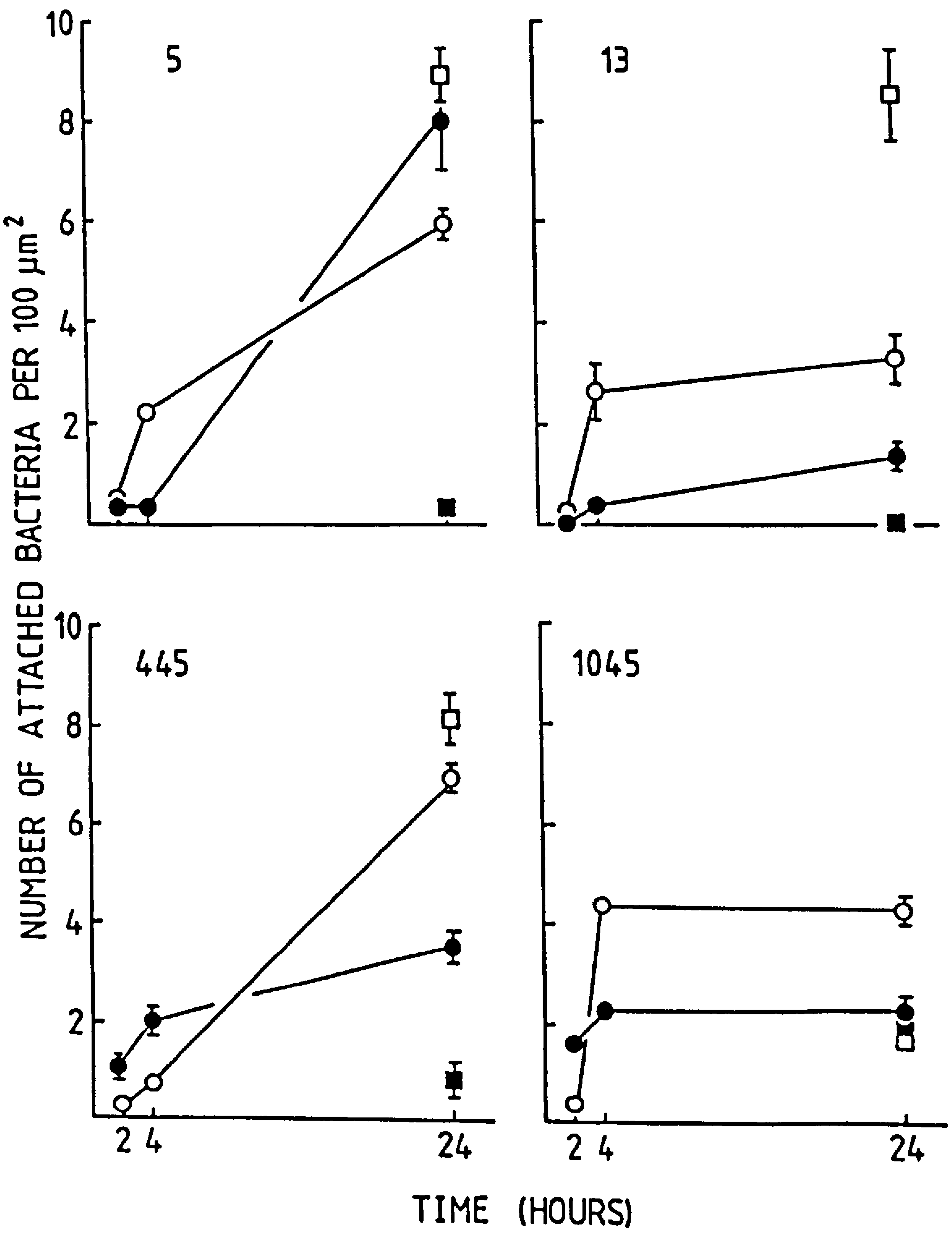
Most of the bacteria were Gram-positive, motile rods (Table 2.1). The growth characteristics of all differed but were somewhat similar for the three *Bacillus* sp., all exhibiting comparatively long lag phases and specific growth rates of approximately $0.1h^{-1}$ (Table 2.2). The growth characteristics did not appear to greatly influence attachment. For example, there was no relationship between attachment and specific growth rate, motility, Gram stain or morphology.

2.3.2. The influence of time on attachment

The rate of attachment over 24h was either linear (e.g. strains 1862 and 2021; Figure 2.2.1), or decreased with time in the form of a saturation curve (e.g. strains 13, 1045 and 1281; Figures 2.2.1. and 2.2.2.). The rate of attachment depended upon the time allowed for attachment, the organism and the substratum. However, even when attachment increased to reach a maximum after 4h (e.g. strain 1045; Figure 2.2.1.) the bacteria covered less than 10% of the substratum surface area.

2.3.3. The influence of the substratum on attachment

The eight strains of bacteria showed no general preference for attachment to G or PS. After 2h bacteria either attached to G and PS to a similar extent (strains 5, 13, 1863 and 2021; Figures 2.2.1., 2.2.2. and 2.2.3.) or attached in higher numbers to PS (strains 445,



<u>FIGURE 2.2.1.</u>

The relationship between time and attachment of bacteria to G(○) and PS(●). Attachment of bacteria to G(□) and PS(■) after fixation with formaldehyde. Error bars represent ± standard error of the mean. The bacteria were *Corynebacterium erythrogenes* 5, *Micrococcus* sp. 13, *Bacillus filicolonicus* 445, and *Bacillus epiphytus* 1045.

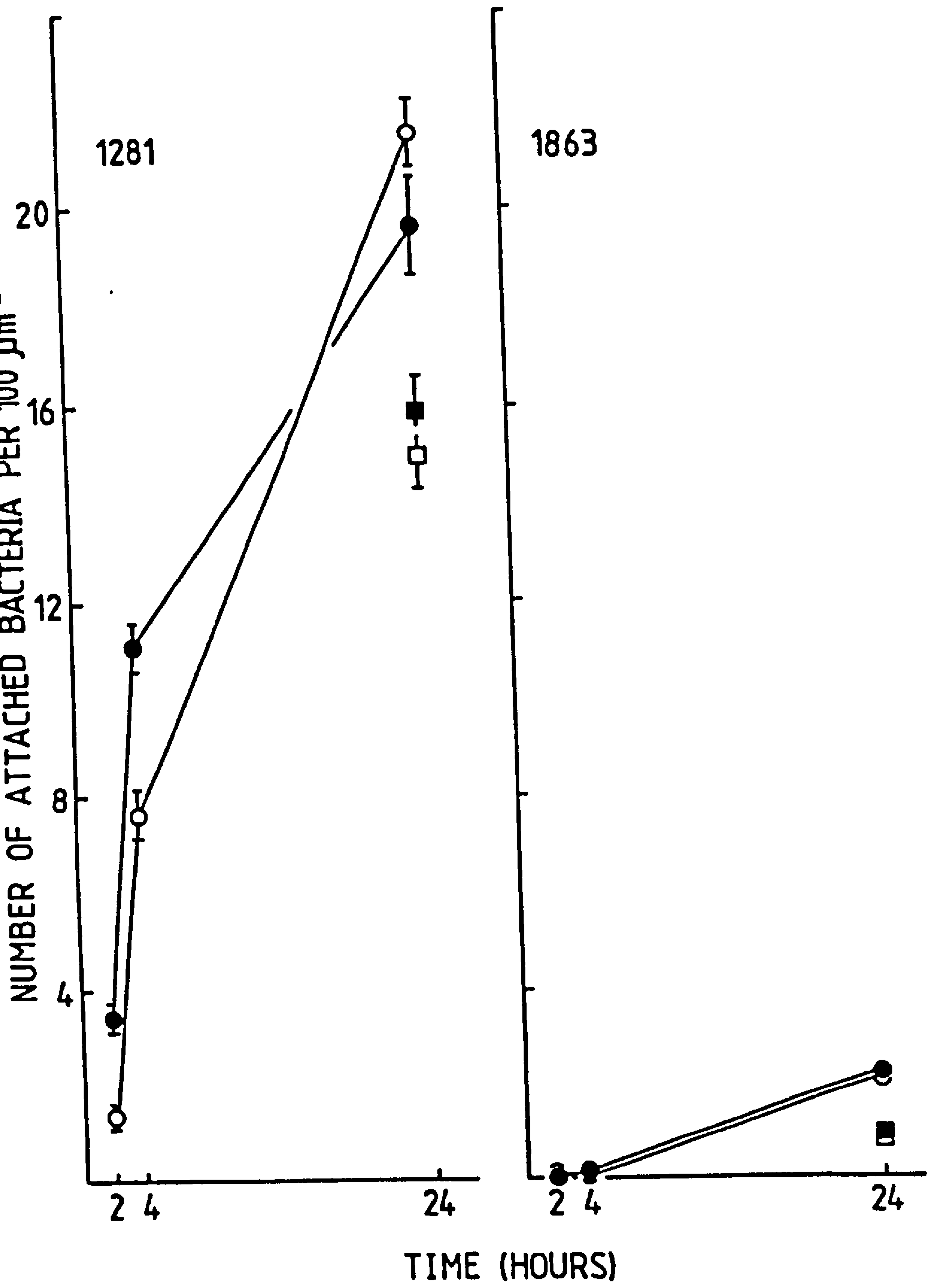


FIGURE 2.2.2.

Attachment of *Vibrio fisheri* 1281 and *Flavobacterium uliginosum* 1863.

Symbols as in Figure 2.2.1.

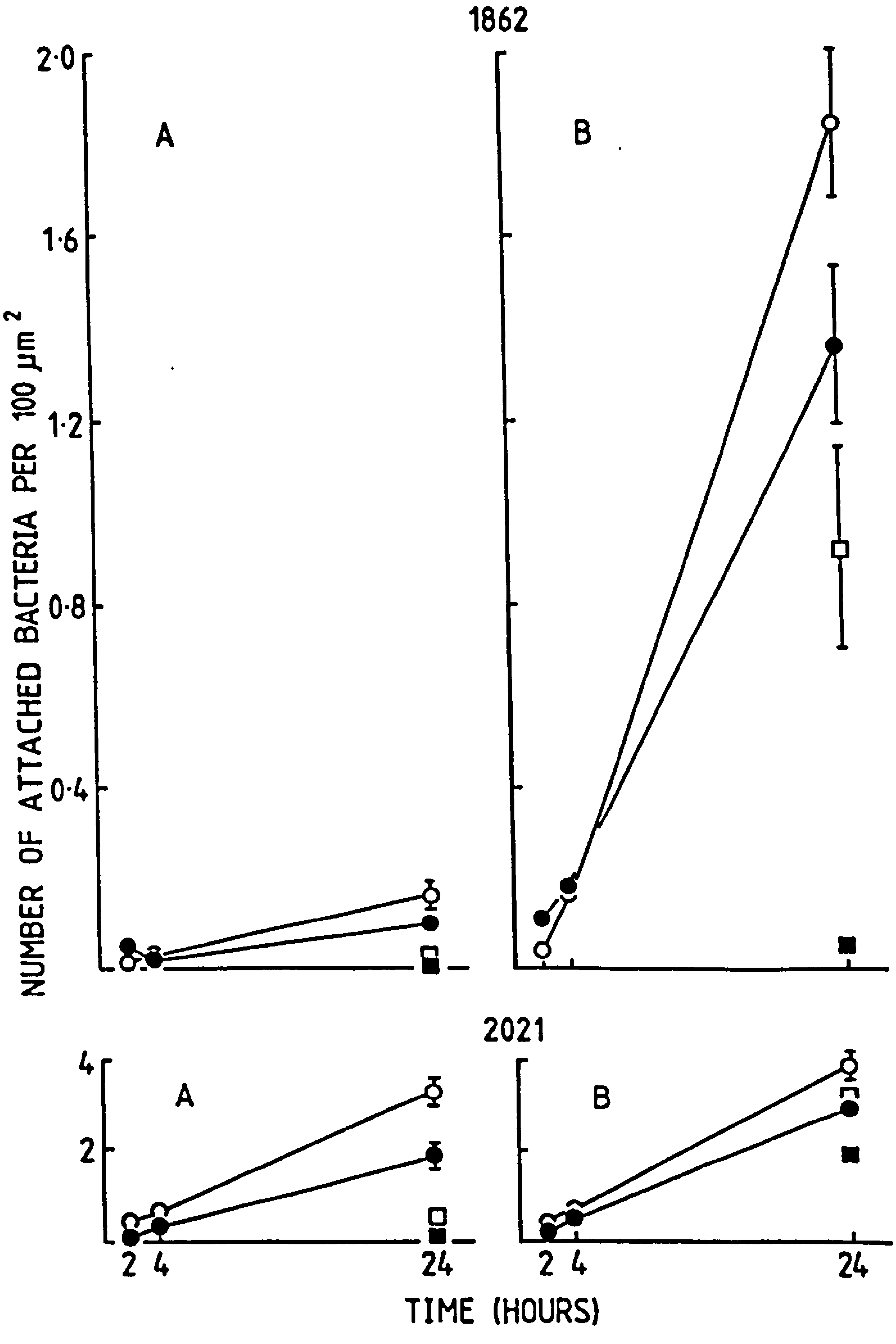


FIGURE 2.2.3.

Attachment of *Bacillus pacificus* 1862 and *Pseudomonas* sp. 2021 after growth on peptone yeast extract medium (A) and glucose medium (B). N.B. Scale of Y axis for strain 1862 = x10 than for the other strains. Symbols as in Figure 2.2.1.

Table 2.1.

Source and morphological characteristics of the bacteria

NCMB		Isolation Site	Morphology	Motility	Gram Stain
Number	Strain				
5	*Corynebacterium erythrogenes*	U	Coccoid	–	+
13	*Micrococcus* sp.	U	Coccoid	–	+
445	*Bacillus filicolonicus*	Sea Water Marine Mud	Rod	+	+
1045	*Bacillus epiphytus*	Marine Phytoplankton	Rod	+	+
1281	*Vibrio fisheri*	Sea Water Marine Animals	Rod	+	–
1862	*Bacillus pacificus*	Sea Shore Sand	Large Rod	+	+
1863	*Flavobacterium uliginosum*	Marine Bottom Deposits	Rod	–	–
2021	*Pseudomonas* sp.	Sea Water	Rod	+	–

Symbols: U = Unknown

– = non-motile or Gram-negative

\+ = motile or Gram-positive

Table 2.2.

Growth characteristics of the bacteria

NCMB		Length of Lag Phase (h)	μ^1 (h^{-1})	t_d^2 (h)
Number	Strain			
5	*Corynebacterium erythrogenes*	15	0.16	4.45
13	*Micrococcus* sp.	20	0.06	10.91
445	*Bacillus filicolonicus*	30	0.11	6.03
1045	*Bacillus epiphytus*	30	0.13	5.16
1281	*Vibrio fisheri*	5	0.23	3.00
1862	*Bacillus pacificus*	40	0.10	6.93
1863	*Flavobacterium uliginosum*	25	0.06	11.09
2021	*Pseudomonas* sp.	6	0.48	1.45

$^1\mu$ = Specific Growth Rate

^2t_d = Doubling Time

1045 and 1281; Figures 2.2.1. and 2.2.2.) After 4h the preference for attachment to PS was maintained by strains 445 and 1281 (Figures 2.2.1. and 2.2.2.) but reversed for strain 1045 (Figure 2.2.1.) and attachment to G by the other organisms increased over that for PS, (strains 5 and 13; Figure 2.2.1.), or remained similar (strains 1863, 1862 and 2021; Figures 2.2.2. and 2.2.3.).

However after 24h attachment to glass was generally greater than that on PS (strains 13, 445, 1045, 1281, 1862 and 2021; Figures 2.2.1., 2.2.2. and 2.2.3.), although attachment to G and PS by strain 1863 (Figure 2.2.2.) was similar, and for strain 5 (Figure 2.2.1.) attachment was greater on PS.

2.3.4. The influence of formaldehyde fixation on attachment

Pre-attachment fixation decreased attachment to PS by all the strains and attachment to G by all but three strains. The percentage decrease in attachment was greater when the organisms were cultured on a medium containing peptone and yeast extract (Figure 2.2.3.A) than when they were cultured on a medium containing glucose (Figure 2.2.3.B). However, fixation with formaldehyde had no effect on attachment to PS by strain 1045 (Figure 2.2.1.) and increased attachment to G by strains 5, 13 and 445 (Figure 2.2.1.).

2.3.5. The influence of growth medium on subsequent attachment

Attachment to G and PS by strain 1862 cultured on glucose medium (Figure 2.2.3.B) was greater than attachment by this organism cultured on a medium containing peptone and yeast extract (Figure 2.2.3.A), with the exception of attachment to G after 2h. Attachment after 24h to G and PS by strain 2021 was also slightly greater by organisms cultured on the glucose medium. The growth medium did not alter the relative preference of these two strains for attachment to G and PS.

2.4. DISCUSSION

2.4.1. The relevance of attachment characteristics to the physiological activity of attached bacteria

The physiological activity of bacteria may affect attachment and attachment may affect bacterial activity. Therefore, it is important to characterise the attachment properties of bacteria. If activity, or a certain level of activity, is needed for attachment then it would be expected that the active, or most active, organisms of a free-living population would selectively attach. Moreover, the extent to which physiological activity is involved in attachment may depend upon the substratum properties (Fletcher, 1980b), in which case populations of cells with different activities may selectively attach to different substrata.

2.4.2. The influence of time on attachment

Numbers of attached bacteria may increase with time as a consequence of *(i)* the increase in numbers of encounters of bacteria with the substratum and hence opportunity for attachment and *(ii)* time-dependent polymer synthesis stabilising attachment when an organism encounters the substratum.

Pre-attachment fixing of bacteria with formaldehyde would prevent post-attachment polymer production. However, this treatment did not inhibit attachment completely, so polymer production may have increased attachment, although, it was not essential.

Attachment of *Pseudomonas* sp. strain 2021 fits a modified Langmuir adsorption isotherm of molecular adsorption (Fletcher, 1977) whereby the rate of bacterial adsorption is proportional to the culture concentration and the probability of a bacterium coming into contact with a vacant adsorption site. Furthermore, the number of bacteria that are adsorbed is directly proportional to the fraction of surface covered assuming adsorption is limited to a monolayer, which from microscopic examination, appears to be the case. If these criteria apply in this investigation, it would be expected that the rate of increase in numbers of attached bacteria would decline with time, as *(i)* the substratum becomes covered with cells and, *(ii)* to a lesser extent, the numbers of bacteria in solution decreases, assuming no growth occurs. Microscopic examination revealed that there was little, if any, growth during the time allowed for attachment.

The rates of attachment decreased with time for attachment to both G and PS by strains 13, 1045 and 1281 (Figures 2.2.1. and 2.2.2.) and to G and PS by strains 5 and 445 respectively (Figure 2.2.1.). In the case of attachment to both G and PS by strain 1045 and G by strain 13 (Figure 2.2.1.), the rate of attachment decreased to zero after 4h. This suggests that the substrata had become saturated with cells. However, microscopic examination revealed that less than 10% of the substratum surface was covered with bacteria. Similarly, a study of attachment by *Streptococcus sanguis* to glass revealed that saturation coverage was 30% of the total area available (Rutter and Leech, 1980). There are three possible explanations for these observations. *(i)* The substratum became completely covered with associated cells most of which were loosly held and became detached during washing. *(ii)* After four hours in nutrient-free media the bacteria lost their ability to attach. *(iii)* Complete coverage was prevented by mutual repulsion between cells (Rutter and Leech, 1980).

As the distances between attached cells was large, less than 10% of the substratum being covered, mutual repulsion was probably not an important factor in this investigation. Theoretical consideration of the electrostatic repulsion and electrodynamic attraction for a cell interacting with a substratum (Gingell and Vince, 1980) and observations of detachment by red cells (Mohandas *et al.*, 1974) and bacteria (DiSalvo, 1973; Fowler and McKay, 1980) suggest that an increase in substratum hydrophobicity is usually accompanied by a decrease in strength of adhesion. This supports the detachment explanation for the observations in this investigation, as the maximum attachment to G was greater than that for the more hydrophobic PS (e.g. strain 1045, Figure 2.2.1.). As physiological activity may be involved to a greater extent in attachment to hydrophobic surfaces than to hydrophilic surfaces, (Fletcher, 1980b) these observations of the influence of substratum properties on attachment could also be accounted for by bacteria losing their ability to attach with time in nutrient-free media, possibly as a result of loss of physiological activity. This may account for any increase in detachment with time. However, attachment by strains 1862 and 2021 (Figure 2.3.3.) did not decrease over a twenty-four hour period indicating that some species can maintain their ability to attach for more than four hours in nutrient-free media. As there is evidence to suggest that the synthesis of adhesive polymer may be important for attachment of strain 2021 (Fletcher, 1980b), it seems likely that this strain at least was able to maintain the production of its adhesive for twenty-four hours, presumably from endogenous resources.

2.4.3. Influence of formaldehyde fixation on attachment

Pre-attachment treatment of bacteria with formaldehyde may affect attachment by killing the cells thereby stopping physiological processes that may be involved in attachment and/or by modifying the bacteria surface (Sherbet and Lakshmi, 1973). It is difficult to distinguish which of these mechanisms may have influenced attachment. Moreover, previous experiments do not help to clarify the situation as they have not produced consistent results. Killing cells by heat (Ørstavik., 1977) or UV (Meadows, 1971; Ørstavik, 1977; Fletcher, 1980b), does not always influence attachment, although formaldehyde or heat can decrease attachment (Meadows, 1971).

Metabolic inhibitors can also influence attachment. Incubation with KCN, which distrupts electron transport, or the protein-synthesis inhibitors chloramphenicol and puromycin, decreased attachment to PS by strains 5, 13, 1863 and 2021.(Fletcher, 1980b) as did formaldehyde in this investigation (Figures 2.2.1., 2.2.2. and 2.2.3.).

Formaldehyde can influence attachment by blocking cationic amino groups of protein components on the cell surface thereby increasing the negative surface charge. This may increase or decrease attachment depending on the cell surface and substratum composition. Along with other compounds that block cationic amino groups of a protein component involved in the adhesion of *Staphylococcus aureus*, formaldehyde reduced attachment to a cation exchange resin (Wood, 1980). A similar mechanism may have inhibited attachment in this investigation. Strain 2021 has been shown to have a protein component at its cell surface which is probably involved in adhesion (Fletcher, 1980a; Fletcher and Marshall, 1982a). Furthermore, the adhesive polymer of strain 2021 cultured on peptone yeast extract has been shown by electron microscopy to have a different appearance to that produced by cells cultured on glucose (Fletcher and Floodgate, 1973, 1976; Fletcher, 1980a). The results of this study suggest that the surface components of these two types of polymer also differed, possibly in their composition of cationic amino groups. This was supported by the observation that formaldehyde decreased attachment by strain 2021 cultured on peptone yeast extract medium to a greater extent than when cultured on glucose medium (Figure 2.2.3A and B).

It is not clear why formaldehyde increased attachment to G by strains 5, 13 and 445 (Figure 2.2.1.). However, the composition of the adhesive polymer of these organisms was presumably such that the interaction of the polymer surface components with formaldehyde favoured attachment.

2.4.4. Summary

The results of this study of the attachment characteristic of bacteria suggest that:

1. Attachment properties of various strains differ with respect to rate, substratum preference, culture medium and formaldehyde treatment.

2. There was no relationship between attachment properties and general characteristics, e.g. specific growth rate, motility, Gram reaction or morphology.

3. Attachment numbers generally increase with time.

4. Pre-attachment treatment of bacteria with formaldehyde decreased attachment to PS by all the strains and decreased attachment to G by all but three strains.

5. The extent to which formaldehyde treatment influenced attachment was dependent upon the growth medium used to culture the cells.

6. The number of bacteria that attached after twenty-four hours was greatly influenced by the medium on which the bacteria were cultured for strains 1862 but was largely independent of the growth medium composition for strain 2021.

7. The number of bacteria that attached after twenty-four hours was generally greater on G than PS.

3. Substratum Characteristics

3.1. INTRODUCTION

The activity of attached bacteria may be influenced by the properties of the underlying substratum. This is supported by the observations that the activity of bacteria may be stimulated by some substrata but not influenced by other substrata. For example, Bigger and Nelson (1941) found that bacterial growth occurred in distilled water in the presence of 20 out of 75 insoluble inorganic substances. Numerous other studies have indicated that substratum properties may affect the growth and metabolic activities of attached bacteria (section 1.2.).

One of the properties of a substratum that may affect bacterial activity is its charge. The extent to which a clay influences bacterial activity has been found to be generally dependant upon its cation exchange capacity, a measure of the negative charge of clay particles (Stotzky, 1980a). Surface charge and other substratum properties may influence the activity of attached bacteria as a result of affecting the concentration or availability of ions or molecules in the cell's immediate environment or, by directly influencing the cell's uptake and/or metabolic processes (section 1.3.2.). An investigation of the activity of attached bacteria should, therefore, include an evaluation of the substratum properties as this knowledge may lead to a better understanding of the nature of the influence of surfaces on bacterial activity.

3.1.1. Evaluation of substratum characteristics

An important characteristic of a surface that may determine the extent to which it interacts with its immediate environment, whether that be water or bacterium, is its unsatisfied bonding potential. This potential, or surface free energy of a substratum (γ_s) can be evaluated by measurement of contact angles (θ) of liquids on the substratum. If the θ's of a homologous series of liquids, with a range of surface tensions are measured, the surface tension of the liquid that would just wet the solid, so that $\theta = 0$, can be determined (Zisman, 1964). This surface tension should be equal to that of the solid and is known as the critical surface tension (γ_C). However, γ_C may be more or less than γ_S, depending on the compatability of the component interactions of the particular solid and liquid under study (Andrade, 1973). These interactions may include dispersive, polar or metallic intermolecular forces. In an aquatic situation, the extent to which a substratum may be able to interact with its immediate environment, and hence influence attachment and/or subsequent activity of bacteria, may be better reflected by water contact angles (θ_W), than by θ's of other liquids. Indeed, bacterial attachment appears to correlate better with θ_W, or work of adhesion between water and the substratum derived from θ_W, than with γ_C (Pringle and Fletcher, 1973).

Substratum characteristics may quite rapidly change after immersion in natural waters as a consequence of the adsorption of macromolecular organic matter, or conditioning film (Neihof and Loeb, 1972, 1973, 1974; Loeb and Neihof, 1975, 1977). This conditioning will probably modify but not completely obscure the influence of the substratum characteristics on the aqueous phase and attached bacteria (Baier, 1980). Studies *in vitro* have indicated that adsorption of proteins to surfaces can influence attachment (Meadows, 1971; Fletcher, 1976).

To measure the substratum characteristics of a conditioned surface, contact angles have generally been made on dried surfaces (Baier, 1980). Drying, however, produces irreversible conformational changes of the adsorbed molecules. Thus evaluations of the influence of conditioning on surface energy should be made in the aqueous phase. This is possible if contact angles of bubbles on the conditioned surface are measured (Fletcher and Marshall, 1982a).

3.1.2. Aims

The objectives of this study of substratum characteristics were:

1. To characterise a range of substrata by measuring their advancing (θ_A), receding (θ_R) and bubble (θ_B) water contact angles.

2. To determine the relationship between substratum θ_A, θ_R and θ_B.

3. To use the θ_A, θ_R and θ_B determinations to aid selection of suitable materials for use in subsequent experiments designed to evaluate the influence of substratum characteristics on bacterial activity.

4. To determine whether the θ_B of substrata in sea water was influenced by leucine.

3.2. MATERIALS AND METHODS

3.2.1. Substrata

The materials used as substrata were:

African mica (M) (British Mica Co., Ltd., Bedford); glass cover slips (G) (16mm diameter, No. 1½, Chance Propper Ltd., Warley); tissue culture treated polystyrene petri dishes (PST) (Costar, Cambridge, Mass., U.S.A.); polystyrene petri dishes (PS) (Sterilin , Teddington); polyethylene teraphthalate coverslips (T) (15mm diameter, Theramanox, Lux Scientific Corporation, Newbury Park, California, U.S.A.); polyhexamethylene adipamide (N) (Nylon 6.6, Polypenco Ltd., Birmingham); polymethylmethacrylate (PMMA) (Perspex, I.C.I. Plastics Division, Welwyn Garden City); Polyvinylidene fluroide (PVDF) (Solef, Solvay and Cie Société Anonyme, Brussels); polypropylene (PP) (Mass Transfer, Heversham, Cumbria); polyvinyl chloride (PVC) (Cobex, Storey Brothers and Co., Ltd., Manningtree), high density polyethylene (PE) (Bexel, Storey Brothers and Co., Ltd., Manningtree); polytetrafluoroethylene (PTFE) Dalau Specialised Plastics Ltd., Clacton-on-Sea); platinum (PT) (10mm x 10mm electrodes).

Unless otherwise stated, disks (16mm diameter) of the plastic materials were cut with a cork borer and subsequently cleaned by sonication for 1h in filter sterile distilled water. Mica disks (19mm diameter) were cut with a glass cutting tool and cleaved immediately before making θ measurements. θ measurements were made on glass without cleaning (GU) and after treatment by either: *(i)* soaking in ethanol containing 3% (v/v) concentrated HCl for ≅ 48h, followed by rinsing six times in filter sterile distilled water (G), *(ii)* sonication in 2% (v/v) Lipsol detergent (LIP Equipment and Services, Shipley), for 1h (GD), *(iii)* sonication with distilled water for 1h (GH). Platinum was cleaned by flaming until red hot. Polyethylene terephthalate coverslips were supplied sterile and were used without cleaning.

3.2.2. Contact angle measurements

θ_A, were measured immediately after injecting water onto the substrata using a syringe (0.25mm internal diameter needle with the point removed and filed to a 90° bevel) (Figure 3.1.) θ_R were measured after withdrawing water, with the syringe, from the drop used to obtain θ_A (Figure 3.1). θ_A and θ_R were determined for both angles of 10 drops for each of the materials. Filtered (0.1µm porosity filters) distilled water was used for all measurements. In addition θ_A and θ_R (both angles of 5 drops) of filter sterile (0.1µm porosity filter) artificial sea water, (ASW), (Kester *et al.*, 1967) were measured on G, T, PVDF, PE and PTFE. All θ's were measured at ≅ 20°C in a chamber containing saturated K_2CO_3 to maintain the humidity at ≅ 60%. Equilibrium contact angles (θ_E) were calculated from the measured θ_A's and θ_R's as 0.5 (θ_A + θ_R). θ_B, (both angles of 10 bubbles), were determined for all the materials. Bubbles (≅ 2.5mm diameter), were produced by injecting air from a syringe, (with a needle as described above), into a water tight, Perspex (Smith Brothers Asbestos Co., Ltd., Leicester) chamber containing filter sterile (0.1µm porosity filter) ASW. The bubbles floated ≅ 6mm from the point of release to rest against the substratum

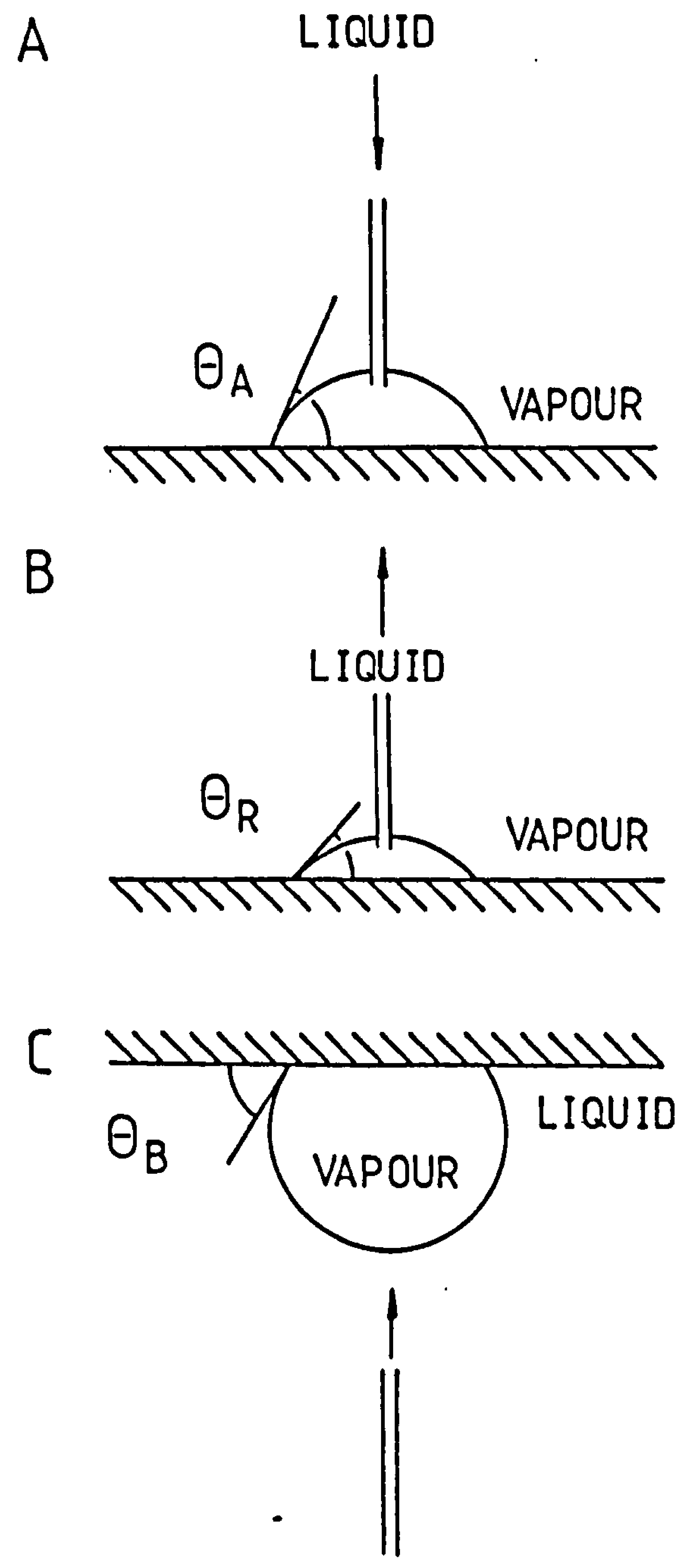


FIGURE 3.1.

Diagramatic representation of (A) θ_A,(B) θ_R and(C) θ_B, determined after injecting liquid onto the substratum θ_A, withdrawing liquid from the substratum(θ_R), or by injecting vapour onto a substratum submerged in liquid(θ_B).

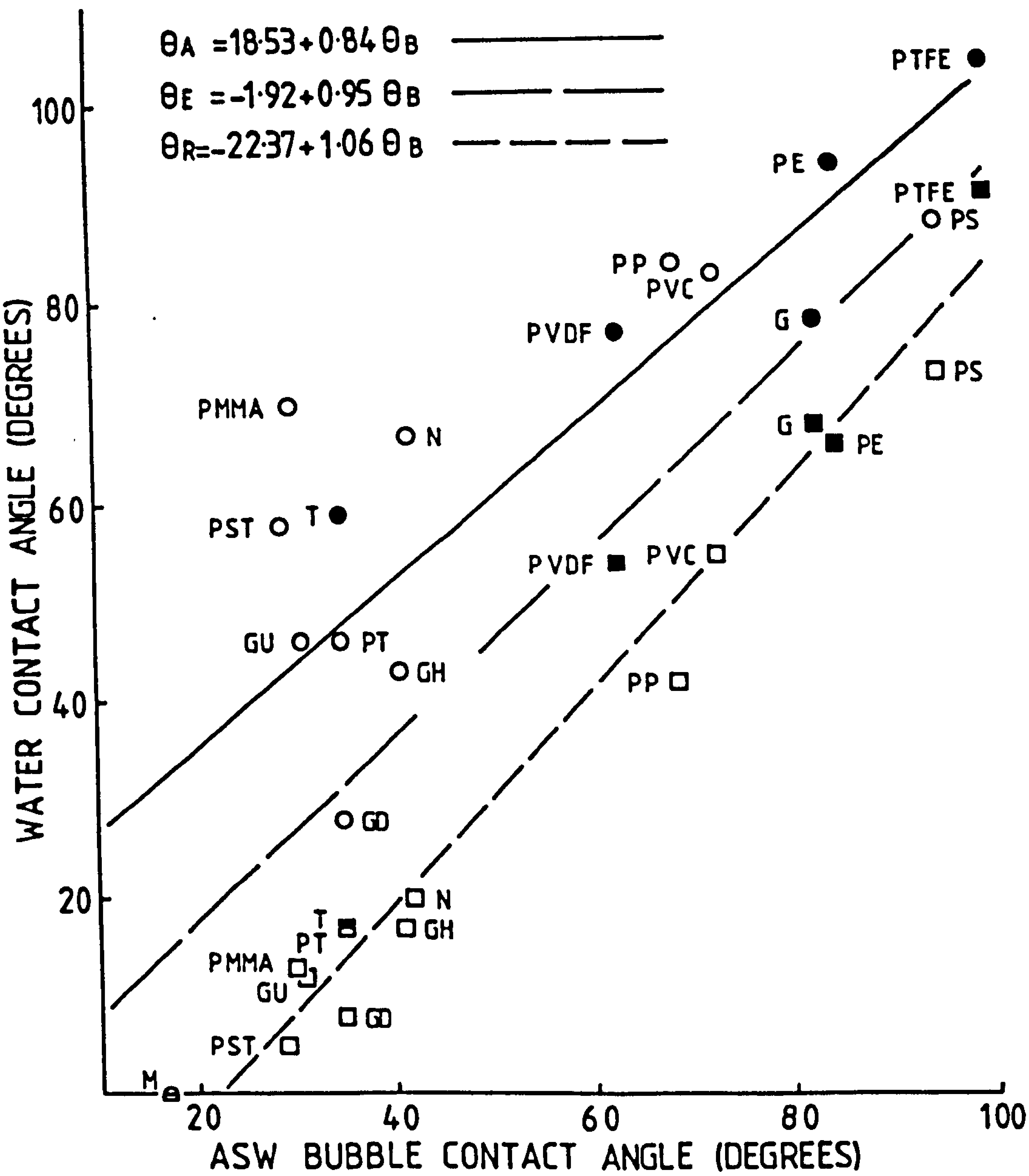


FIGURE 3.2.

The relationship between θ_A (○), or θ_R (□) or θ_E and θ_B. Individual substratum θ_E's, calculated as 0.5 (θ_A + θ_R), not shown. Filled-in symbols are θ's of substrata chosen for subsequent investigations. The equations of the best straight line fit were calculated by regression analysis and are of the form $\theta = a + b\theta_B$, where a equals the values of θ_A, θ_E or θ_R when θ_B equals 0 and b is the slope of the line. The best fit straight lines were θ_A (————), θ_E (—— ——) and θ_R (— — — —).

placed horizontally on a stage at the top of the liquid. (Figure 3.1.) In addition θ_B's on G, T, PE and PTFE were measured in a solution containing 1.0 mg C l^{-1} and 1.0 g C l^{-1} leucine in minimal media (ASW with added NH_4Cl and KH_2PO_4 to produce a carbon, nitrogen, phosphorous ratio of 10:4:1). All θ measurements were made with a Vernier microscope with a goniometer eye piece (Precision Tool and Instruments Co., Ltd., Thornton Heath).

3.3. RESULTS

3.3.1. Substratum contact angles

The θ_A's ranged from 0° for mica, the most hydrophilic surface, to 104° for PTFE, the most hydrophobic surface (Table 3.1.). θ_R's were less than θ_A's and the difference between them increased with the hydrophilicity of the surface (Figure 3.2.). ASW θ_A and θ_R's (Table 3.2.) were similar to those for distilled water (Table 3.1).

Regression analysis of θ_E and θ_B demonstrated that the calculated values of θ_E were similar to θ_B with the regression line passing close to the origin (Figure 3.2.). The equation for the relationship was $\theta_E = 1.92 + 0.95\ \theta_B$. Thus, the value of θ_E when $\theta_B = 0$ was -1.92 and the slope of the line was 0.95. The relationship between θ_B and θ_R was also shown, by regression analysis, to be close to unity, so that $\theta_R = -22.37 + 1.06\ \theta_B$. From this equation it can be calculated that when $\theta_R = 0°$, $\theta_B = 21°$. The difference in values of θ_R and θ_B ($\sim$ 21°) may have been due to buoyancy producing bubble distortion. This was supported by the observation that for mica where $\theta_R = 0$ and θ_B was apparently 17° the bubbles could freely move across the surface suggesting contact of the gas and liquid phases had not taken place. The contribution of bubble distortion to θ_B (approximately 21°) was apparently fairly consistent over the range of θ's measured (Table 3.1., $(\theta_R - \theta_B)$-21°).

Clean glass has θ_A, θ_R and θ_B of 0°. The abnormally high values obtained in this investigation probably reflect the ineffectiveness of the cleaning treatments. Detergent appeared to be the most effective at

Table 3.1.

Substratum advancing, receding, bubble and equilibrium water contact angles

Substratum	Contact angles (degrees)				
	θ_A	θ_R	θ_B	$(\theta_R-\theta_B)^{-21°}$	θ_E[b]
M	0	0	17	4	0
GD	28 (4)[a]	8	35	-6	18
GH	43 (1)	17 (2)	41 (2)	-3	30
GU	46	12 (1)	31 (1)	2	29
G	78 (1)	68 (1)	83	6	73
PST	58	5	29 (1)	-3	32
PS	88	73 (2)	95	-1	81
T	59 (2)	17 (3)	35 (2)	3	38
N	67	20 (1)	42	-1	44
PMMA	70 (2)	13 (2)	30 (1)	4	42
PVDF	77	54 (1)	63 (3)	12	66
PVC	83 (1)	55 (3)	73	3	69
PP	84 (2)	42 (2)	69 (2)	-6	63
PE	94	66 (3)	85 (2)	2	80
PTFE	104 (1)	91	100 (1)	12	98
PT	46 (8)	17 (3)	35 (3)	3	32

[a] Parenthetical values are S.E.M.'s.
S.E.M. of θ's without parenthetical values <1.

[b] Calculated as $0.5\ (\theta_A + \theta_R)$.

Table 3.2.

Substratum ASW contact angles

Substratum	ASW contact angles (degrees)		
	θ_A	θ_R	θ_E[b]
T	62 (1)[a]	11	37
G	75 (1)	66	71
PVDF	79 (1)	49 (2)	64
PE	94 (2)	65 (3)	79
PTFE	106 (2)	90 (1)	98

[a] Parenthetical values are S.E.M.'s.
S.E.M. of θ's without parenthetical values <1.

[b] Calculated as $0.5\ (\theta_A + \theta_R)$

cleaning glass (GD), while the ethanol acid treatment (G) increased the θ's over that of uncleaned glass (GU) (Table 3.1.). These θ's do, however, indicate the extent to which water interacted with the glass surfaces.

3.3.2. The relationship between θ_B and leucine concentration

The θ_B measurements made on substrata in 0g Cl^{-1} leucine (Table 3.3.), differs to some extent from the θ_B for equivalent substrata in Table 3.1., particularly with regard to the most hydrophilic surfaces T and G. This was probably due to variation in different batches of T and G coverslips rather than other experimental variables as the θ_B determinations of the more hyrophobic surfaces PVDF, PE and PTFE were fairly constant. The values in Table 3.3. are, however, comparable with each other as they were made on the same batches of materials. The presence of 0.001 g Cl^{-1} leucine in ASW had little influence on θ_B but 1g Cl^{-1} leucine increased the θ_B (hydrophobicity) of the three most hydrophilic surfaces T, G, and PVDF (Table 3.3.).

3.4. DISCUSSION

3.4.1. Contact angle hysteresis

Liquid contact angles are determined by the solid/vapour (γ_{SV}), solid/liquid (γ_{SL}) and liquid/vapour (γ_{LV}) interfacial energies. This relationship is described by the Young equation:

$$\gamma_{SV} - \gamma_{SL} = \gamma_{LV} \cos \theta_E \qquad (3.1.)$$

where θ_E is the equilibrium contact angle.

In practice θ_E is difficult to determine as contact angles exhibit hysteresis between a maximum θ_A and a minimum θ_R. However, θ_E is probably approximately equal to the mean of θ_A and θ_R (i.e. $0.5\ (\theta_A + \theta_R)$ (Bikerman, 1970). Hysteresis may be caused by surface roughness, heterogenety, or mobility and is, therefore, at a minimum when the solid is smooth, homogeneous, isotropic and rigid (Adamson, 1976). The substrata used in this investigation were rigid so hysteresis was probably due to

Table 3.3.

Relationship between leucine concentration and and substratum ASW bubble contact angle

Substratum	ASW bubble contact angles (degrees) at following leucine concn. ($g\ C\ l^{-1}$)		
	0	0.001	1.0
T	49 (2)[a]	46 (2)	56
G	70 (1)	72 (2)	81
PVDF	63 (3)	—[b]	68 (1)
PE	89	91	88
PTFE	97	100	99 (1)

[a] Parenthetical values are S.E.M.'s. S.E.M. of θ's without parenthetical values <1.

[b] — , Not measured

roughness and or heterogeneity. For a homogeneous surface the apparent or measured contact angle (θ), is related to the true contact angle (θ^1), by the roughness factor (rf) as described in Wenzel's equation:

$$rf = \frac{\cos \theta}{\cos \theta^1} \qquad (3.2.)$$

As surfaces are not perfectly smooth, rf is nearly always greater than one (Zisman, 1964). Thus from equation 3.2. it can be seen that when $\theta^1 < 90°$ then $\theta < \theta^1$ and when $\theta^1 > 90°$ then $\theta > \theta^1$. θ_A's behave in this way but θ_R's behave in the opposite manner. In this investigation θ's were generally $< 90°$; therefore, roughness would have had the effect of producing true θ_A's greater than, and true θ_R's less than, the measured values obtained for θ_A's and θ_R's in this investigation. Thus, if allowance was made for roughness, the difference between the true values for θ_A and θ_R would have been even greater than for the measured values. Surface roughness cannot, therefore, account for the observed hysteresis between θ_A and θ_R.

The substrata used in this investigation, like all other surfaces, were not ideal and hysteresis was probably a consequence of the heterogenetity of the surface. Hysteresis, in this case, may be caused by the liquid being used to make the contact angles being adsorbed onto the high energy areas of the heterogeneous surface. As a result of adsorption, the recession of a drop of water (θ_R) over a solid surface, occurs on a film of water which superficially resembles a high energy surface. However, adsorption may have little influence on θ_A's as an advancing water drop moves over a dry surface. Thus θ_A's are probably essentially determined by the low surface energy areas of a substratum whereas θ_R's are determined by the high surface energy areas which tend to retain liquid during θ_A measurements (Neumann and Good, 1972). Moreover, small patches of high energy sites, in a predominatly low energy surface, will markedly decrease θ_R and leave θ_A relatively unaffected. On such low energy substrata, therefore, θ_A are more characteristic of the greater part of the surface than θ_R. However, the converse also applies, so that for a predominantly high energy surface, θ_R is more representative than θ_A (Blake and Haynes, 1973). The comparatively high θ_A's for the high energy (low θ) surfaces (Figure 3.2.) were, therefore, probably due to low energy patches, possibly in the form of grease contamination. This was supported by the decrease in θ_A's that accompanied increasing thoroughness of cleaning of glass. (Table 3.1.), suggesting cleaning removed contaminating hydrophobic components. The increase in θ_A of glass after ethanol/acid-treatment suggests that ethanol was adsorbed onto the glass surface.

3.4.2. The relationship between θ_B and θ_R.

When air bubbles make contact with a solid surface they force the liquid to recede from the surface. Thus, θ_B may be regarded as a form of θ_R, as both are largely determined by the retention of the liquid on the solid. It was not surprising, therefore, that there was a very close correlation between θ_B and θ_R (r 0.985).

Some of the variation between θ_B and θ_R may have been due to differences in the liquid surface tensions (γ_{LV}), which, for ASW was 73.5×10^{-3} N m^{-1} (Riley and Skirrow, 1975) and for distilled water θ_R was 72.7×10^{-3} N m^{-1}. These differences in γ_{LV} would influence θ as can clearly be seen by rearrangement of equation 3.1.:

$$\cos \theta_E = \frac{\gamma_{SV} - \gamma_{SL}}{\gamma_{LV}} \qquad (3.3.)$$

The higher γ_{LV} of ASW would tend to produce a lower cos θ and, therefore, a higher θ. However, in practice the difference in γ_{LV} appeared to be too small to influence the θ's as the ASW and distilled water θ_E were similar (Table 3.1. and 3.2.).

The θ_B's were obviously made under saturated vapour pressure but θ_R were made under a relative humidity of 60%. This has the effect of producing a lower solid surface free energy measurement by θ_B's than by θ_R's (Zisman, 1964). Consequently, for a given substratum, θ_B should be higher than θ_R. This was the case in this investigation so the variation between θ_B and θ_R may have been partly due to the different vapour pressure under which θ_B and θ_R were made. It is difficult to be certain, however, as any differences would be masked by the larger influence of buoyancy causing bubble distortion (section 3.3.1.)

3.4.3. The influence of leucine on θ_B

Leucine may have influenced θ_B by being adsorbed onto the substratum, thereby, affecting the substratum surface energy (γ_{SV}) or by changing the liquid surface energy (γ_{LV}) or both. However, as can be seen from equation 3.3., no matter what the substratum surface energy, and hence γ_{SV} and γ_{SL}, if γ_{LV} changes then so does θ. As the presence of leucine only influenced θ_B's of T, G and PVDF and not PE or PTFE, it appears that θ_B's were not influenced by the effect of leucine on liquid surface tension. Leucine was, therefore, probably adsorbed from the 1 g C l^{-1} solution onto the T, G and PVDF surfaces. This selective adsorption may have been due to the greater unsatisfied bonding potential of these comparatively hydrophilic surfaces. The 1 mg C l^{-1} leucine solution was probably not concentrated enough to allow sufficient leucine adsorption to influence θ_B of the other surfaces.

3.4.4. Choice of substrata for use in further studies

Before this investigation of substratum characteristics, ethanol/acid-treated glass was the only substratum that had been used in this study to determine the influence of surfaces on bacterial activity. It was decided that it was technically feasible to evaluate the influence on bacterial activity of four types of substratum.

This investigation has revealed that the ethanol/acid-treated glass (G) surface used in previous studies was more hydrophobic than uncleaned glass (GU) (Table 3.1.), suggesting ethanol had adsorbed onto the glass. As the extent of ethanol adsorption, and consequently the surface energy of the glass, may have varied with time as a result of desorption in ASW it was decided to only include G in a few subsequent studies for comparative purposes. Four other substrata, each with markedly different surface energies as determined by θ_A, were chosen. These plastic substrata were T, PVDF, PE and PTFE. θ_A's were used in preference to θ_R's or θ_B's to represent the surface energies of these substrata as θ_A's are more characteristic of these predominantly hydrophobic surfaces (section 3.4.1.). The reasons for choosing these substrata were that PTFE was the most hydrophobic surface; T was one of the most hydrophilic, and was convenient to use, as it was supplied in the form of sterile cover slips; PE had been used in a previous investigation of the activity of attached bacteria and was shown to adsorb labelled material released by attached bacteria (Fletcher, 1979b); and PVDF had a θ_A midway between the values of θ_A's for T and PE. These four surfaces had the added advantage that their θ_A's were linearly related (Figure 3.2.), thereby allowing easy detection of any relationship between substratum θ_A and the activity of bacteria attached to these substrata.

3.4.5. Summary

The results of this study of substratum characteristics suggest that:

1. Contact angle hysteresis increased as θ decreased. This was probably caused by substratum heterogenity and in particular as a result of low energy sites on the relatively high energy surfaces.

2. θ_B's were very closely related to θ_R's and were approximately equivalent to θ_R + 21°. The difference between θ_B and θ_R was probably largely due to bubble distortion.

3. θ_B's were similar to θ_E's, calculated as 0.5 (θ_A + θ_R).

4. The salts in ASW had little influence on θ's.

5. A 1 g C l^{-1} solution of leucine increased the θ_B of T, G and PVDF but did not influence the θ_B of PE and PTFE, indicating that leucine was adsorbed onto the most hydrophilic surfaces.

6. Adsorption of leucine from a 0.001 g C l^{-1} solution was not apparent on any of the substrata by θ_B measurements.

4. Microautoradiographic Evaluation of Substrate Assimilation

4.1. INTRODUCTION

The technique of microautoradiography (MAR) can be used to evaluate the growth rate (Brock, 1967), or autotrophic and heterotrophic activities of aquatic microorganisms. MAR works on the principle that radioactive atoms decay emitting radiation which can be detected by a photographic emulsion. This radiation will activate silver halide grains within the emulsion if it is in close proximity to the radioactive source. Upon development the silver halide grains form silver grains that can be observed microscopically. Thus simultaneous microscopic observation of bacteria and silver grains allows an evaluation of bacteria with radioactive material, i.e. bacteria with overlying silver grains.

Much of the literature on the use of MAR in microbial ecology has been reviewed by Hoppe (1977, 1978) and Jones (1979). Several investigations using MAR have evaluated the proportion of actively assimilating microorganisms in the microbial population (Ramsay, 1974; Peroni and Laverello, 1975; Faust and Correll, 1977; Hoppe, 1977; Meyer-Reil, 1978; Fletcher, 1979b). In this type of study, the true proportion of active cells will only be detected if all the cells with the potential for assimilation are labelled. This will occur if the labelled substrate can be assimilated by all the cells in the population and sufficient labelled substrate and time is allowed for all the metabolising cells to accumulate detectable levels of radioactive material. However, the incubation period should not be long enough to allow growth and thus dilution of label amongst progeny. Determinations of the maximum of active cells may, therefore, involve incubation with abnormally high substrate concentrations compared with natural aquatic environments (Hoppe, 1978). Incubation of bacteria with high concentrations of radioactive substrate may also label the most active cells to such an extent that the cells become undetectable upon microscopic examination and indistinguishable from background silver grains. An evaulation of the rates of assimilation by microbial populations can be made after incubation with comparatively low substrate concentrations and short incubation times. In this type of investigation, however, only the most actively assimilating cells of the population will accumulate sufficient radiotracer to become labelled.

To determine the proportion of active cells in a population the numbers of both the total and active cells must be known. Total numbers have been determined in some MAR studies by staining with traditional dyes (Paerl, 1974; Ramsay 1974; Faust and Correll, 1977), but intensely stained cells may not always be clearly distinguishable from silver grains. On the other hand staining with fluorescent antibodies (Fliermans and Schmidt, 1975), or, more conveniently, with acridine orange combined with epifluorescence microscopy, allows clear simultaneous examination of cells and silver grains. This technique has proved suitable for determining the assimilatory activity of attached bacteria (Fletcher, 1979b).

MAR studies have suggested that the properties of an underlying surface whether it be synthetic (Fletcher, 1979b), or a plant leaf (Ramsay, 1974; Ramsay and Fry, 1976) may influence assimilation by attached aquatic bacteria. Evaluations of backgrounds from MAR investigations have also provided evidence of adsorption of labelled material on synthetic substrata (Fletcher, 1979b) and naturally occurring detritus (Paerl, 1974).

The technique of MAR was chosen to examine the assimilatory activities of bacteria as it provides a direct sensitive qualitative evaluation of assimilation by attached and by free-living bacterial populations and may give valuable information on molecular adsorption onto substrata. Tritiated materials were used as they emit low energy beta electrons with a short track length, which produces higher resolution microautoradiographs than higher energy emitters such as ^{32}P, ^{35}S, or ^{14}C. The nuclear track emulsion NTB-2 (Kodak) was specifically chosen as it has a low intrinsic background (Prescott, 1964) and has a high efficiency for tritium compared with other emulsions (Rogers, 1979).

4.1.1. Aims

The objectives of this MAR study were to:

1. Determine whether assimilation by attached bacteria differed from that of detached or free-living bacteria.

2. Determine the relationship between substratum θ_A and assimilation by attached bacteria.

3. Determine the relationship between substrate charge and assimilation by bacteria attached to substrata with a range of θ_A.

4. Determine the relationship between substratum θ_A and adsorption of neutral, acidic and basic amino acids.

5. Determine the relationship between assimilatory activity of bacteria prior to attachment and their subsequent attachment.

6. Determine the relationship between loss of assimilated substrate by attached bacteria and the θ_A of the underlying substratum.

7. Determine the relationship between substratum θ_A, attachment and detachment.

4.2. MATERIALS AND METHODS

4.2.1. Organism and growth conditions

4.2.1.1. Bacteria incubated with ^{3}H-amino acid mixture or ^{3}H-glucose

Pseudomonas sp. NCMB strain 2021 was cultured in 100 ml of medium comprising peptone/yeast extract SW as described in section 2.2.1. Stationary phase cells were harvested by centrifugation (10K, 4°C) and resuspended in SW to ≅ 7 x 10^7 cells ml^{-1}.

4.2.1.2. Bacteria incubated with individual ^{3}H-amino acids.

Pseudomonas sp. NCMB strain 2021 was cultured in 100 ml of peptone/yeast extract medium as described in section 2.2.1. but ASW was substituted for SW. After initial harvesting these cells were washed twice in ASW by resuspension and centrifugation and the final cell suspension was adjusted to ≅ 2 x 10^8 cells ml^{-1}. Growth media were sterilised by autoclaving (122°C/15 min) and sea water used for washing and resuspension was sterilised by filtration (0.2 μm porosity filter).

4.2.2. Attachment of bacteria to substrata

4.2.2.1. Bacteria incubated with ^{3}H-amino acid mixture or ^{3}H-glucose

20 ml portions of cell suspension were added to glass coverslips placed on the bottom of petri dishes. After incubation for 15 mins at 15°C to allow attachment, the glass coverslips with attached cells were washed in SW, to remove loosely attached cells, and transfer to 25 ml universal screw cap bottles containing 5 ml of incubation solution (section 4.2.3.1.).

4.2.2.2. Bacteria incubated with individual ^{3}H-amino acids

7 ml portions of washed cell suspension were allowed to attach, for 2h at 15°C, to G, T, PVDF, PE or PTFE substrata held in an upright position in 25 ml universal screw cap bottles. The substrata were held vertical by placing them in silicone rubber rings cut from tubing (14 mm internal diameter x 20 mm external diameter ≅ 10 mm length). Substrata with attached cells were washed with 100 ml of filter sterile ASW at a flow rate of ≅ 200 ml min^{-1}, to remove residual suspended and loosely attached cells, placed in new silicone rubber rings and transfered to 7 ml of filter sterile incubation solution (section 4.2.3.2.).

4.2.3. Incubation of bacteria with ^{3}H-labelled substrates

4.2.3.1. Incubation with ^{3}H-amino acid mixture and ^{3}H-glucose

To determine the influence of time of incubation with labelled substrate on assimilation, attached bacteria were incubated for ½, 1, 2, 4, and 19h in an orbital shaking (100 rpm) incubator at 15°C in 5 ml SW containing either 1.0 μCi ml^{-1} (≅ 2 μg C l^{-1}) of [^{3}H]amino acid mixture (1.0 μCi ml^{-1}, mean specific activity 31.3 Ci mmol^{-1}), or 10.0 μCi ml^{-1} (≅ 53 μg C l^{-1}) of D-[2-^{3}H] glucose (1.0 mCi ml^{-1}, mean specific activity 35.0 Ci mmol^{-1}) (Radiochemical Centre, Amersham). At the end of the incubation period bacteria were fixed by adding 0.1 ml formalin to the incubation solutions. During incubation some bacteria desorbed and these comprised the detached population as opposed to the attached population that remained on the substrata. The detached cells were collected by filtration and prepared for microscopic examination (section 4.2.4.).

4.2.3.2. Incubation of bacteria with individual ^{3}H-amino acids after attachment

Attached bacteria were incubated for 2h in an orbital shaking (100 rpm) incubator at 15°C in minimal media (section 2.2.1) with the following amino acids. *(i)* 1, 10, 50 or 100 μg C l^{-1} of L-[4,5-^{3}H] leucine (1.0 mCi ml^{-1}, 1.0 Ci $mmol^{-1}$) or *(ii)* 10 μg C l^{-1} of [2-^{3}H] aspartic acid (1 mCi ml^{-1}, 5.1 Ci $mmol^{-1}$), L-[G-^{3}H] glutamic acid (1 mCi ml^{-1}, 3.4 Ci $mmol^{-1}$) (all supplied by Radiochemical Centre, Amersham), L-[G-^{3}H] arginine (1 mCi ml^{-1}, 10 Ci $mmol^{-1}$) or L-[G-^{3}H] lysine 1 mCi ml^{-1}, 10 Ci $mmol^{-1}$) (supplied by ICN, Irvine, Cal. U.S.A.).

To determine the influence of agitation during incubation on assimilation, bacteria attached to T and free-living bacteria were incubated with ^{3}H-leucine medium as above at 15°C for 2h with and without shaking (100 rpm).

To prepare suspensions of free-living bacteria of similar numbers to the attached cell preparations, 0.1 ml portions of the washed cell suspensions were added to 7 ml portions of a corresponding incubation solution and incubated as were the attached populations. Free-living and detached cells were collected by filtration and prepared for microscopic examination (section 4.2.4.)

4.2.3.3. Incubation of bacteria with ^{3}H-leucine before attachment

To determine if the bacteria that attached were truely representative of the free-living population, or whether more, or less, actively assimilating cells selectively attached, bacteria were incubated with ^{3}H-leucine before they were allowed to attach. Bacteria were harvested and washed as described in section 4.2.1.2. but resuspended to a concentration of ≅ 2 x 10^9 cells ml^{-1} in a solution containing 1000 μg C l^{-1} L-[4,5-^{3}H] leucine supplemented with minimal media (section 3.2.2.). The cell suspension was incubated at 15°C for 2h at 150 rpm, washed resuspended to ≅ 2 x 10^8 cells ml^{-1} and allowed to attach for 0.5, 1.0, 1.5, 2.0 or 3.0h.

4.2.4. Mounting samples onto microscopic slides

The attached populations were prepared for microscopic examination by fixing the substrata onto glass microscope slides with double sided adhesive tape. The detached and free-living populations were collected on polycarbonate filters (0.2 μm porosity) (Bio-Rad Labs., Cal. U.S.A.), which had been stained with Irgalan black (Union Color and Chemical, Boston, Mass., U.S.A.) to prevent autofluorescence. The filters were then washed by passing through 2 x 7 ml filter sterile ASW, air-dried and mounted onto slides by dipping the slides in a sterile solution of 1% gelatin in distilled water (45°C) and placing each filter on a wet slide.

4.2.5. Microautoradiography

The method used was based on that developed by Meyer-Reil (1978). Mounted filters and substrata containing the bacteria and corresponding controls (without bacteria but with and without exposure to labelled substrate at the highest concentration used), were coated with NTB-2 nuclear track emulsion (Eastman Kodak Company, New York, U.S.A.) (diluted 1:3 with distilled water) and placed on ice cooled aluminium sheets. When the emulsion had gelled the slides were placed in a light-tight box containing silica gel and kept at -20°C for ≅ 17h, followed by 2h at ≅ 20°C. The emulsion was developed for 30 sec. at ≅ 20°C in Kodak D-19 developer (diluted 1:3 in distilled water), stopped in distilled water for 10 sec, fixed in 30% (w/v) sodium thiosulphate in distilled water for 2 min, washed in running tap water for 15 min, and air-dried.

4.2.6. Epifluorescent microscopy

After development, samples were first soaked for 5 min in citrate buffer (0.2M, pH 6.7), stained for 20 min with acridine orange (B.D.H., Atherstone) (0.02% [w/v] in 0.2 M citrate buffer, pH 6.7), rinsed successively in citrate buffer solutions of pH 6.7, 4.7 and 3.7 and finally rinsed with distilled water. After air-drying specimens were examined, with blue-incident light excitation and bright field illumination, under a Zeiss Standard 18 Microscope, fitted with a x100 oil objective and x10 eye piece, using Cargille Type A immersion oil (McCrone Research Associates Ltd., London). The percentage of bacteria that had assimilated labelled substrate was calculated by relating the total number of cells observed by epifluorescence (in 100 × 100 μm^2 areas, or until 200 bacteria were counted for each slide) to the number of bacteria with overlying silver grains. Silver grains were easily distinguished by adjusting the intensity of the bright field illumination while simultaneously observing bacteria by epifluorescence (Figure 4.3.). Silver grains that did not overlie bacteria were also counted to determine the extent of the background.

4.3. RESULTS

4.3.1. The relationship between time and bacterial assimilation of ^{3}H-amino acids or ^{3}H-glucose

Bacteria attached to G rapidly incorporated ^{3}H-amino acids, so that ≅ 100% were labelled after 4h. This proportion of labelled attached cells was maintained after 19h. Bacteria detached from G incorporated ^{3}H-amino acids more slowly than attached cells, so that only 50% were labelled after 4h, but 100% were labelled after 19h (Figure 4.1A.). The number of background silver grains associated with attached bacteria was greater than that for detached bacteria. Backgrounds associated with attached and detached bacteria increased over the initial 4h incubation period but were little altered after a further 15h incubation period (Figure 4.1C.).

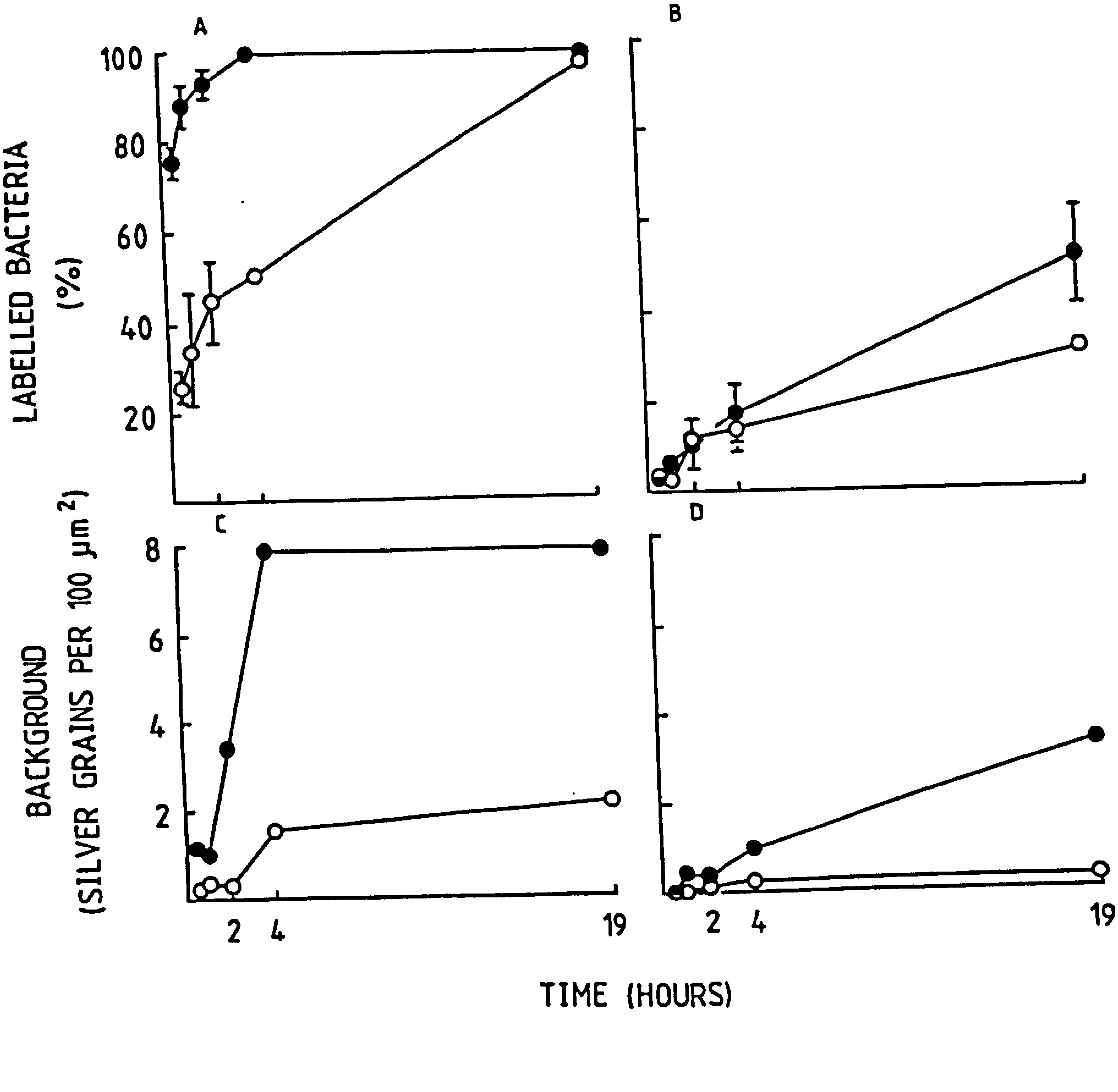


FIGURE 4.1.

The relationship between time and the proportion of bacteria attached to G(●) and detached from G(○), that assimilated ^{3}H-amino acids (A) and ^{3}H-glucose (B). The backgrounds associated with attached (●) and detached (○) bacteria after incubation with ^{3}H-amino acids (C) and ^{3}H-glucose (D). Datum points are means of duplicate samples from one experiment. Error bars are ± S.E.M.

Attached and detached bacteria incorporated ^{3}H-glucose (Figure 4.1B.) more slowly than ^{3}H-amino acids (Figure 4.1A.). When incubated with ^{3}H-glucose there was little difference in the proportion of labelled attached and detached bacteria during the initial 4h incubation period but a larger proportion of attached cells were labelled after 19h (Figure 4.1B.). The backgrounds associated with bacteria incubated with ^{3}H-glucose (Figure 4.1D.) were less than for bacteria incubated with ^{3}H-amino acids (Figure 4.1C.).

Backgrounds associated with attached bacteria incubated with glucose increased with time up to 19h and were greater than that for detached bacteria (Figure 4.1D.).

4.3.2. The relationship between leucine concentration and assimilation by bacteria incubated in static and agitated incubation solutions

Agitation of the incubation solutions enhanced assimilation of ^{3}H-leucine by bacteria attached to T but the extent of this enhancement generally decreased as the substrate concentration increased, so that agitating increased the proportion of labelled attached bacteria when incubated with 1 μg C l^{-1} leucine by 107%, but only increased the proportion of labelled attached bacteria by 16% when incubated with 100 μg C l^{-1} leucine (Figure 4.2.). However, agitating the incubation solution decreased the proportion of detached bacteria that assimilated leucine from 1 and 10 μg C l^{-1} solutions, but increased the proportion of detached bacteria that assimilated leucine from 50 and 100 μg C l^{-1} solutions. Agitation increased the proportion of free-living bacteria that assimilated label when incubated with the highest and lowest concentrations of leucine (1 and 100 μg C l^{-1}) but decreased the proportion of free-living bacteria that assimilated label when incubated with the two intermediate concentrations of leucine (10 and 50 μg C l^{-1}, Figure 4.2.).

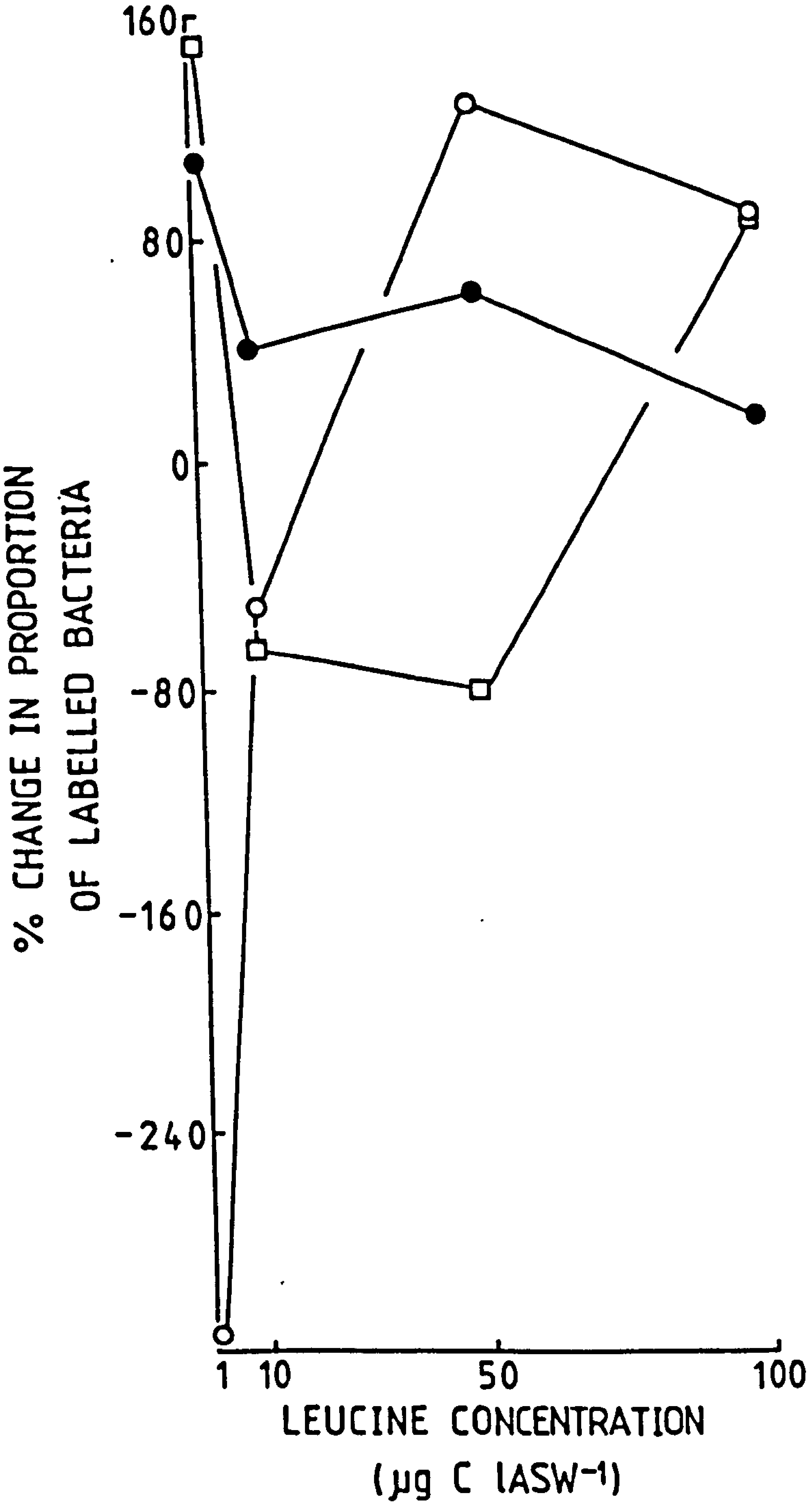


FIGURE 4.2.

The relationship between ^{3}H-leucine concentration and assimilation by bacteria attached to T(●), detached from T(○) and free-living (□), after incubation in static and agitated incubation solutions. The influence of agitation on assimilation by bacteria (vertical axis) was calculated as the proportion of labelled bacteria in samples that were incubated with agitation, divided by the proportion of labelled bacteria in equivalent samples that were incubated without agitation, expressed as percentage. Datum points are means of duplicate samples from one experiment.

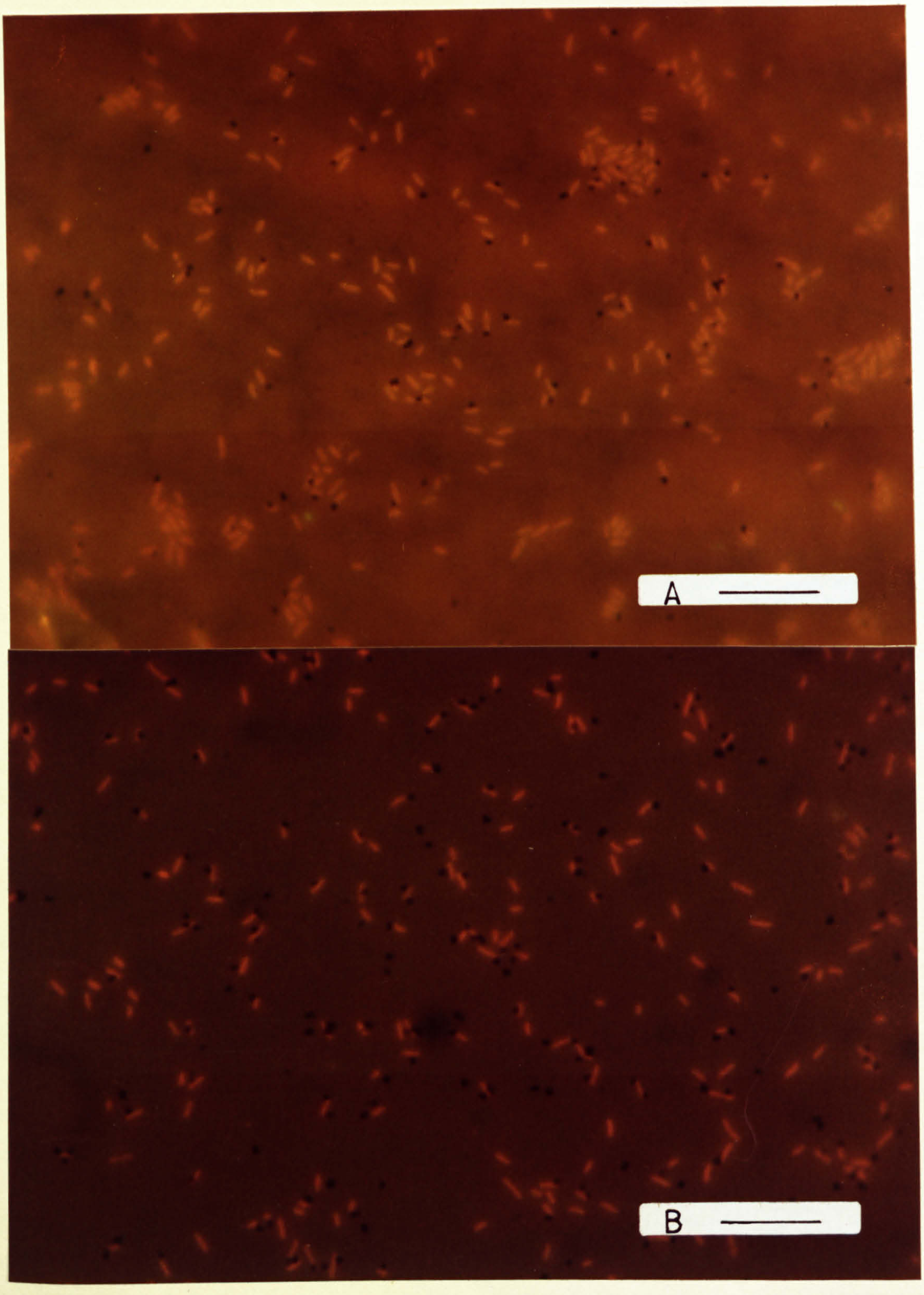
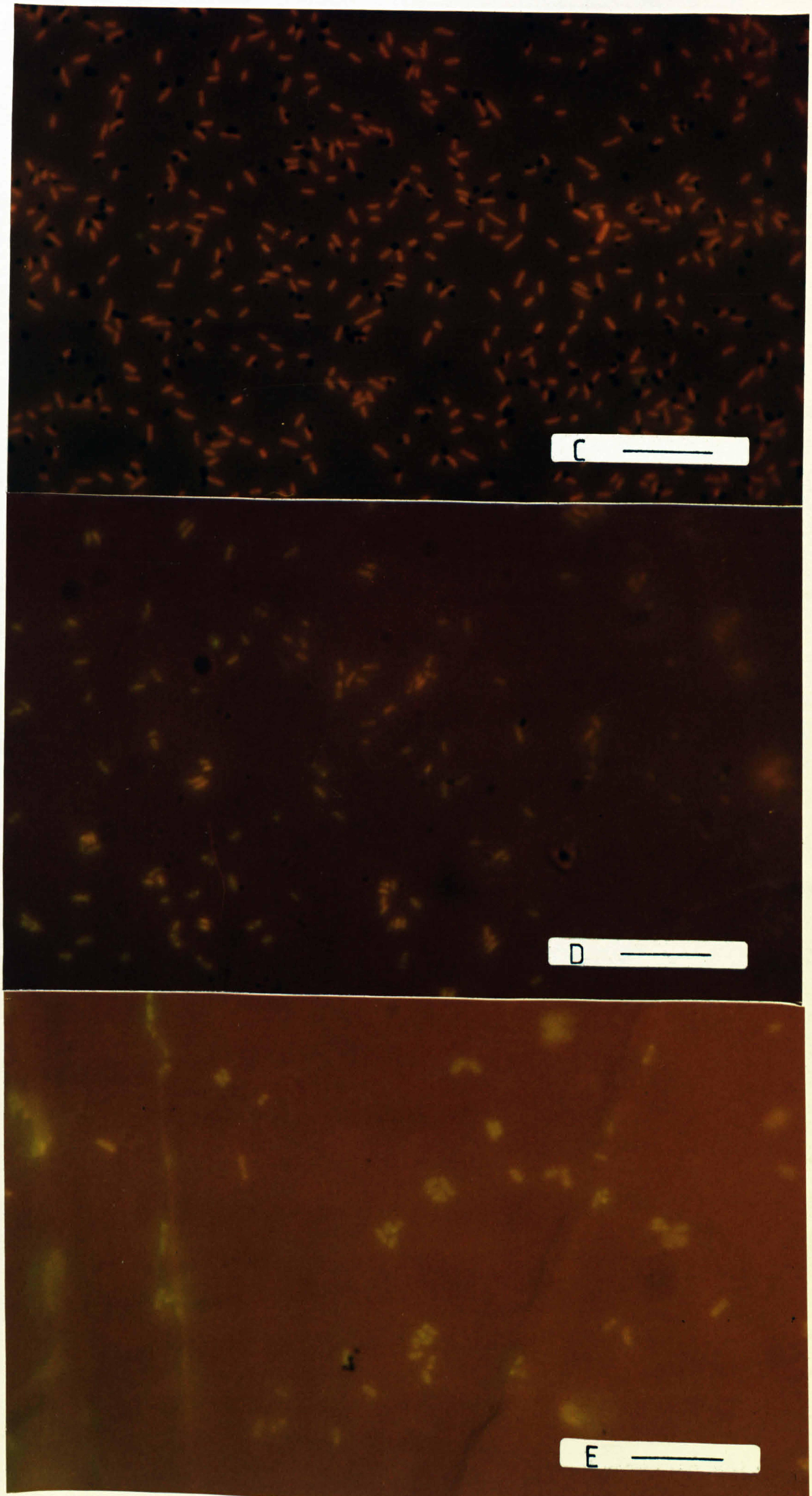


FIGURE 4.3.

Bacteria prepared using the combined techniques of microautoradiography and epifluorescence microscopy. Photomicrographs were taken under bright field and UV illumation. Labelled (with overlying grains) and unlabelled bacteria after 2h incubation with 100 μg C l^{-1} ^{3}H-leucine. Free-living bacteria (A) and bacteria attached to T(B), G(C), PE(D) and PTFE(E). Bar = 10 μm.


C
D
E

4.3.3. The relationship between substratum contact angle and bacterial assimilation of leucine

As the substratum θ_A decreased, the proportion of labelled attached bacteria tended to approach, or exceed (leucine concentration 50 µg C l^{-1}), that for the corresponding free-living bacteria (Figures 4.3. and 4.4A.). There was an increase in the significance of the correlation between θ_A and percentage labelled attached bacteria with increase in leucine concentration, so that r was -0.231 (not significant), -0.716 ($P<0.01$), -0.844 and -0.879 ($P<0.001$) for 1, 10, 50 and 100 µg C l^{-1}, respectively. The proportion of labelled detached bacteria (Figure 4.4B.), was generally less than the proportion of labelled attached bacteria (Figure 4.4A.), except for bacteria detached from PTFE, and was similar to, or less than, that for free-living bacteria (Figure 4.4A.).

Examination of control surfaces that had not been exposed to label, indicated that the level of background was very low (<0.1 silver grains per 100 µm²) (Figure 4.5.). Surfaces with, or without, attached bacteria, after exposure to labelled substrate had higher backgrounds than surfaces not exposed to labelled substrate, with the exception of PTFE, which exhibited similar background levels with, or without, exposure to label (Figure 4.5.). When exposed to ^{3}H-leucine, T and PE substrata containing attached cells exhibited higher backgrounds than did those substrata without bacteria. The free-living cell controls, without bacteria, exhibited very low background (<0.1 silver grains per 100 µm²), while the free-living cell samples, containing labelled bacteria, had approximately a two fold higher background level than the controls (Figure 4.5.). Specimens containing labelled detached bacteria had similar background levels to free-living specimens containing labelled bacteria.

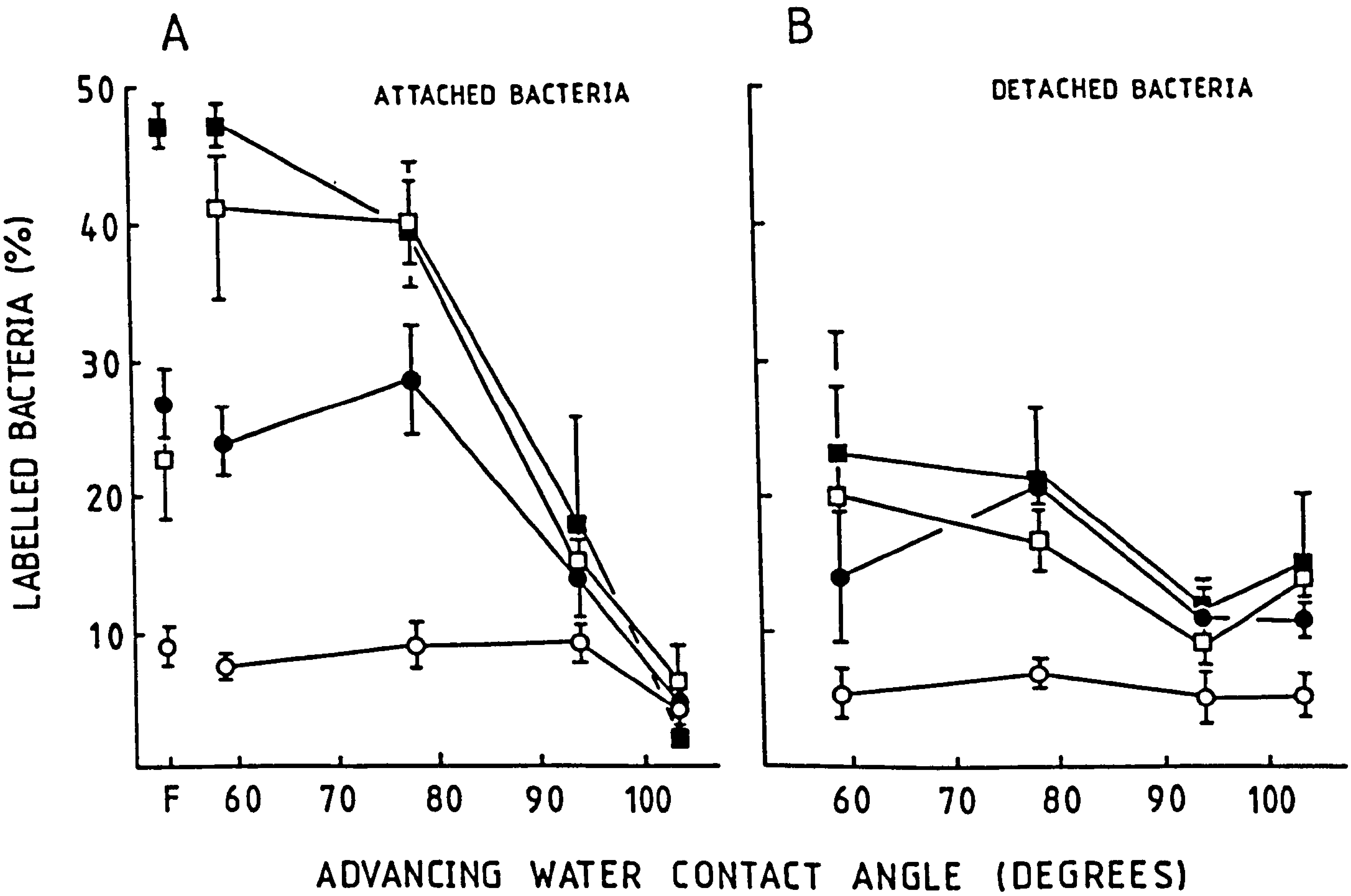


FIGURE 4.4.

The relationship between the proportion of attached (A) or detached (B) bacteria which assimilated ^{3}H-leucine and substratum θ_A. F is the proportion of free-living cells that assimilated ^{3}H-leucine. The ^{3}H-leucine concentrations were 1 (○), 10 (●), 50 (□) and 100 (■) µg $\dot{C}$ l^{-1}. The substrata and θ_A were T 59°, PVDF and G combined 78°, PE 94° and PTFE 104°. Datum points are means of two experiments, each with duplicate samples. Error bars represent ± S.E.M.

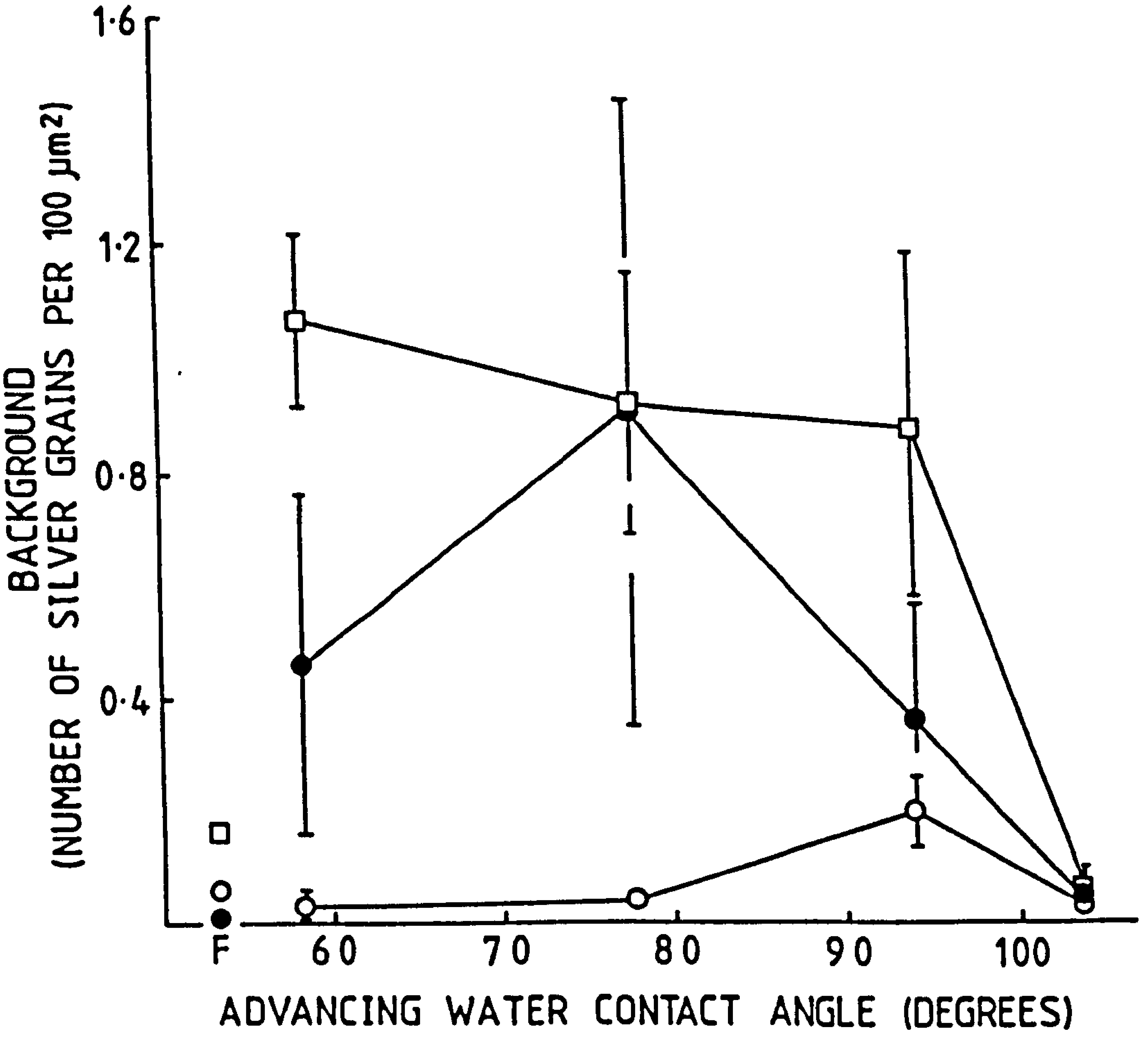


FIGURE 4.5.

The relationship between substratum θ_A and number of background silver grains on control substrata without either bacteria or incubation with ^{3}H-leucine (○), substrata without bacteria but incubated with 100 µg C l^{-1} leucine (●) and substrata with attached bacteria incubated with 100 µg C l^{-1} ^{3}H-leucine (□). F is equivalent free-living bacteria samples. The substrata and θ_A were T 59°, PVDF 77°, G 78°, PE 94° and PTFE 104°. Datum points are means of two experiments, each with duplicate samples. Error bars represent ± S.E.M.

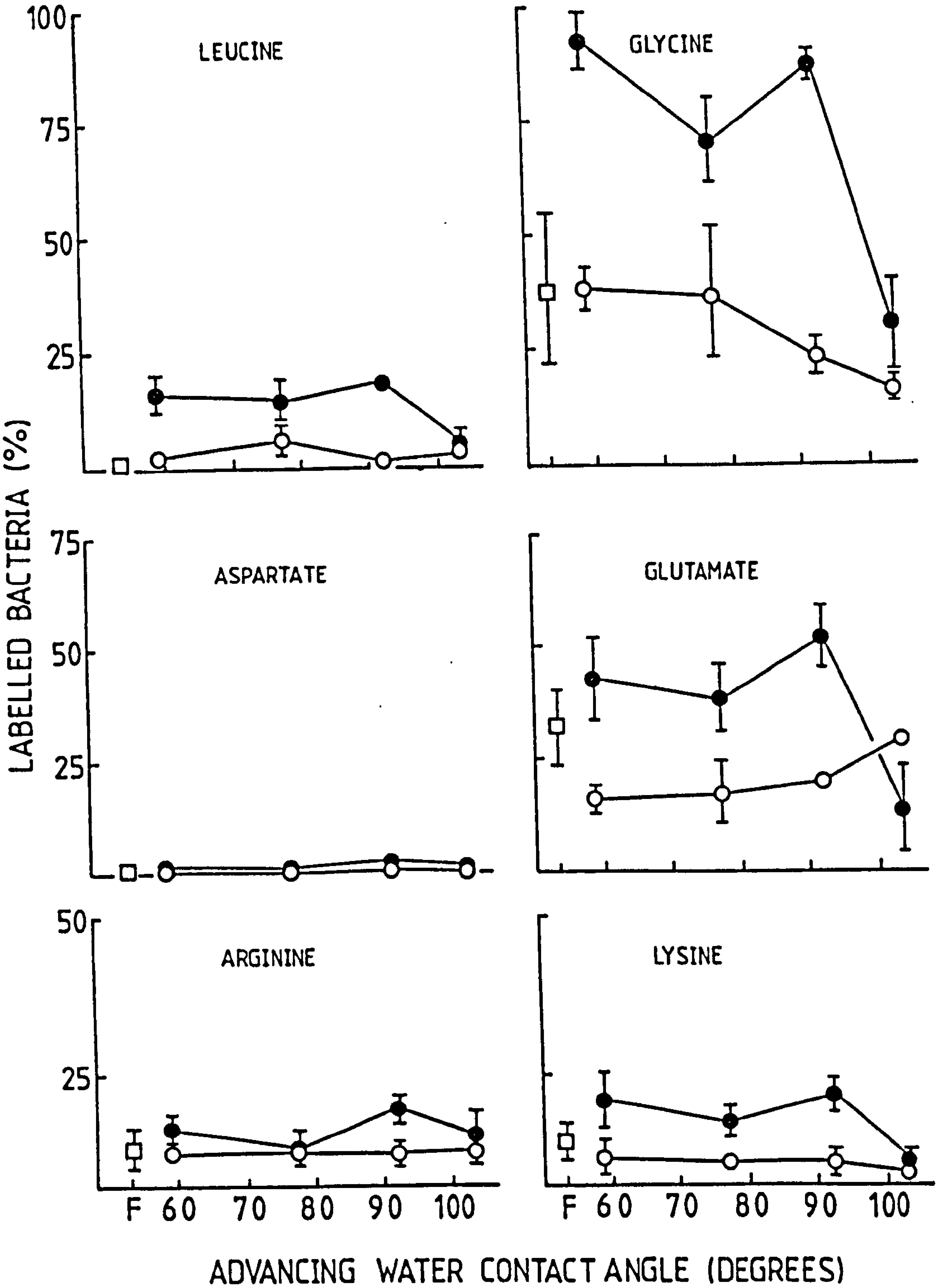


FIGURE 4.6.

The relationship between the proportion of attached (●) and detached (○) bacteria which have assimilated ^{3}H-amino acids and substratum θ_A. F is the proportion of free-living cells that assimilated ^{3}H-amino acids. The amino-acid concentration was 10 μg C l^{-1} and the substrata and θ_A were T 59°, PVDF 77°, PE 94° and PTFE 104°. Datum points are means of two experiments, each with duplicate samples. Error bars represent ± S.E.M.

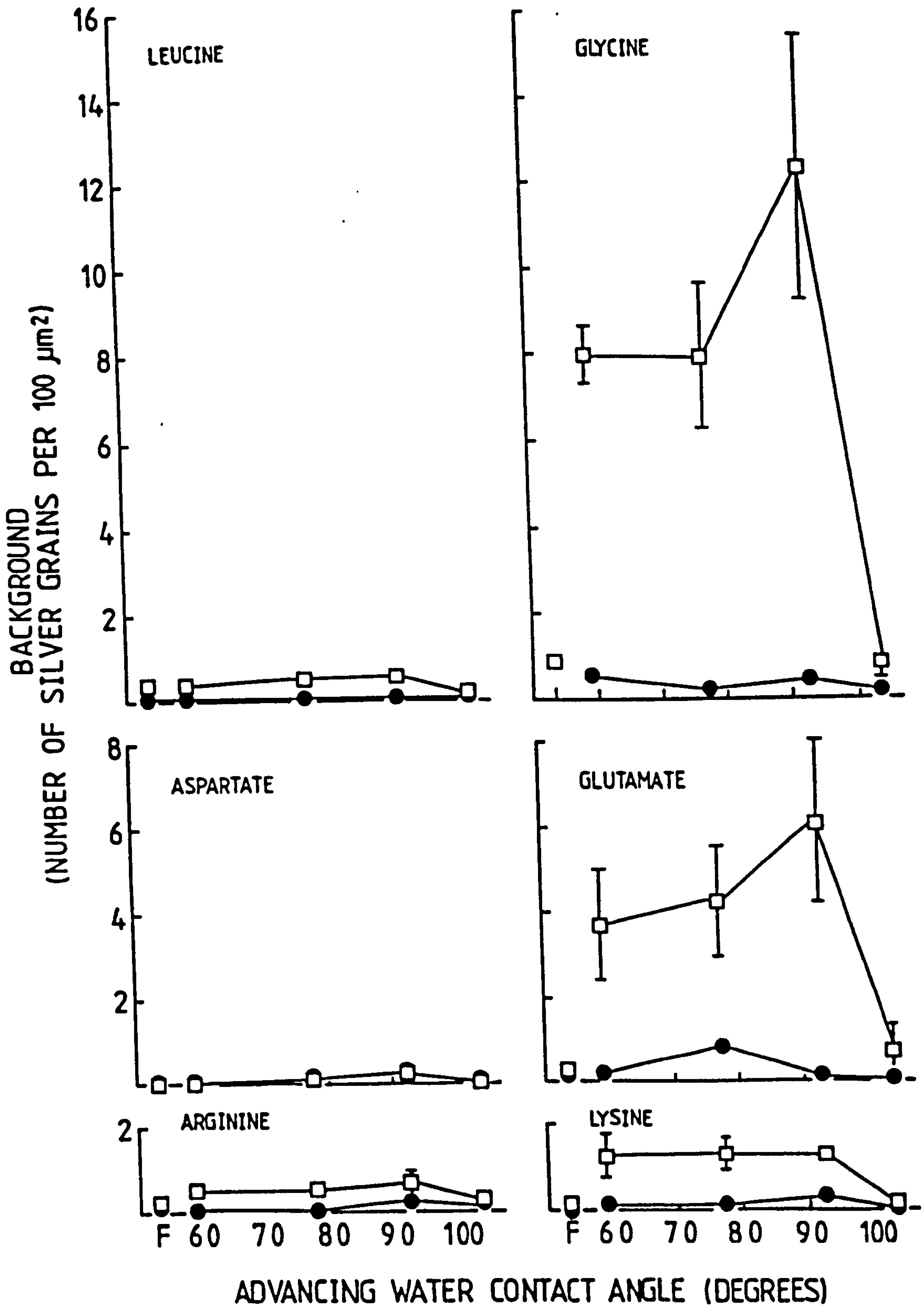


FIGURE 4.7.

The relationship between substratum θ_A and number of background silver grains on substrata without bacteria but incubated with 10 μg C l^{-1} ^{3}H-amino acids (●) and substrata with attached bacteria incubated with 10 μg C l^{-1} ^{3}H-amino acids (□). F is equivalent free-living bacteria samples. The substrata were the same as in Figure 4.6. Datum points are means of two experiments each with duplicate samples. Error bars represent ± S.E.M.

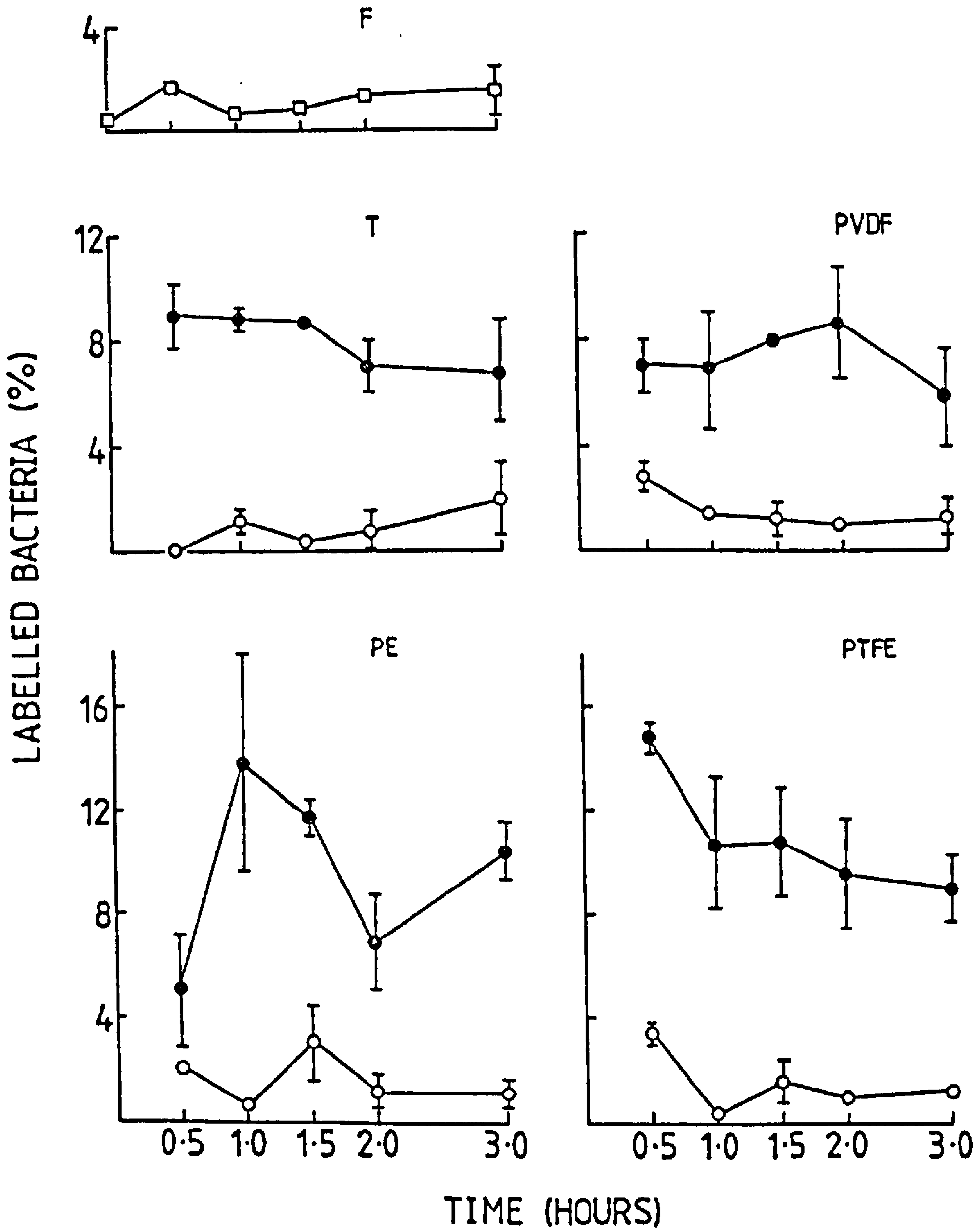


FIGURE 4.8

The relationship between time allowed for attachment and the proportion of bacteria labelled with ^{3}H-leucine prior to exposure to substrata that subsequently attached (●) or remained unattached (○). Free-living bacteria not exposed to a substratum (□). Datum points are means of duplicate samples. Error bars represent ± S.E.M.

4.3.6. The relationship between substratum contact angle and attachment

In all experiments where bacteria were allowed to attach to substrata with a range of θ_A's, the number of attached bacteria increased, with increasing θ_A. For example, this was illustrated by the negative correlation ($r = -0.764$; $P<0.001$) between θ_A and the numbers of cells that remained attached after a 2h attachment period and 2h period of incubation with leucine, and the positive correlation ($r = 0.683$; $p<0.001$) between θ_A and percentage of detached cells (Figure 4.9.). This experiment also demonstrated that there was a strong positive relationship between the percentage of attached cells which assimilated leucine (Figure 4.4A.) and the number of bacteria which remained attached to each of the substrata (Figure 4.9.), so that r was 0.181 (not significant), 0.739 ($P<0.01$), 0.658 ($P<0.01$), 0.747 ($P<0.001$) for 1, 10, 50 and 100 µg C l^{-1} leucine, respectively. This suggests that incubation of bacteria with leucine after attachment may have stabilised or strengthened bacterial adhesion.

4.4. DISCUSSION

The efficiency of NTB-2 emulsion in detecting tritium in a source thickness of 0.5 µm, the approximate thickness of bacteria, is about 16% (Rogers, 1979). MAR may, therefore, underestimate the number of cells that contain isotope in a population. However, given sufficient labelled substrate and incubation time, MAR can be used to determine the proportion of actively assimilating bacteria in a population as demonstrated in Figure 4.1A. If substrate concentrations and incubation times are insufficient to label all the bacteria with the potential for assimilation, then the proportion of bacteria that become labelled over a defined period will reflect the rate of assimilation by that population, as demonstrated in Figure 4.4.

MAR combined with epifluorescence microscopy allows simultaneous observation of the total number of bacteria and the proportion that have

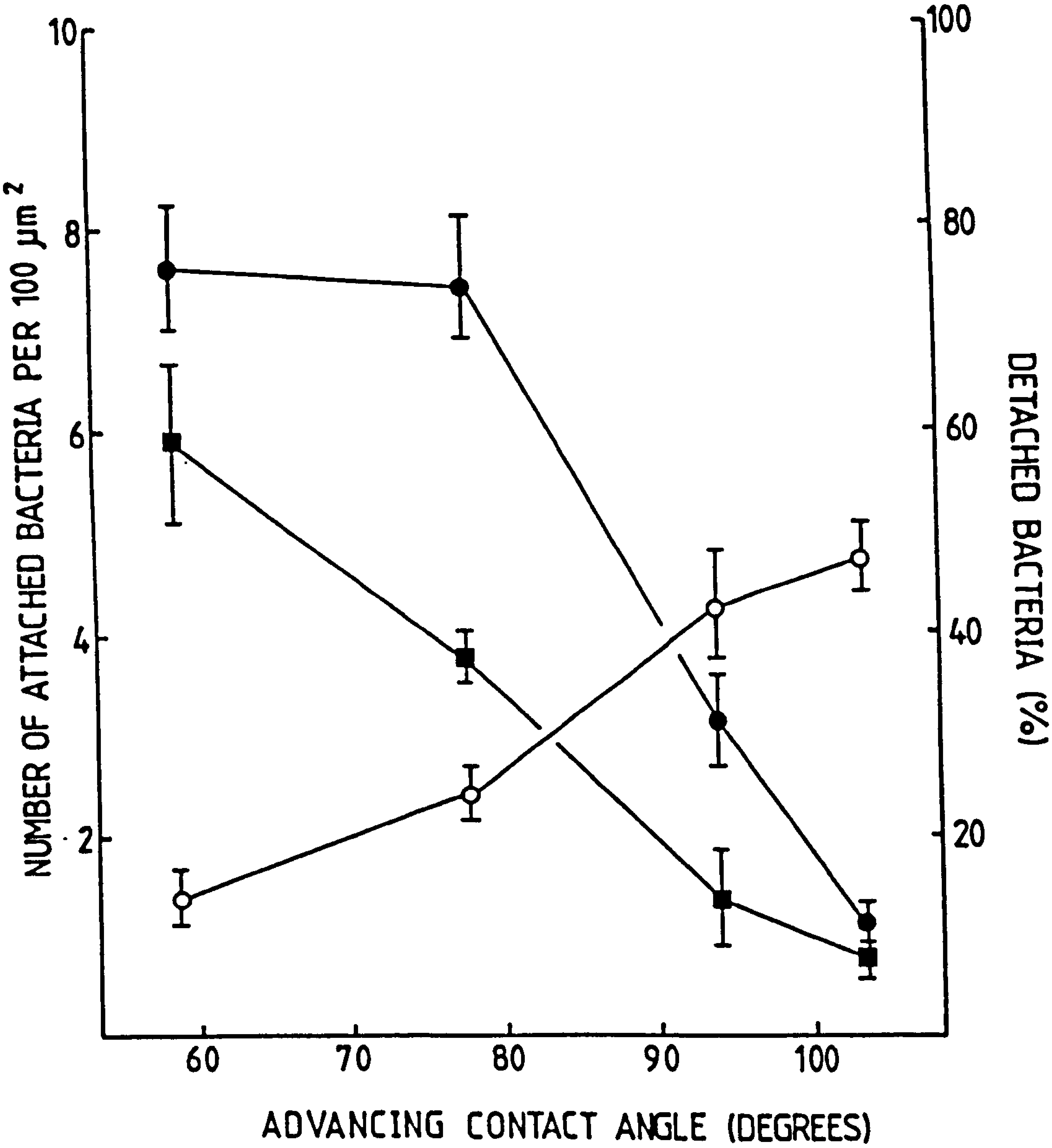


FIGURE 4.9.

The relationship between numbers of attached (●,■) or percent detached bacteria (○) and substratum θ_A. Attached bacteria were those which remained attached after a 2h attachment period followed by 2h incubation with ³H-leucine (●) and those which attached after a 2h incubation period with ³H-leucine followed by 2h attachment period (■). Detached bacteria were those which desorbed from the substrata during the 2h post-attachment incubation with ³H-leucine. The substrata and θ_A were T 59°, PVDF and G combined 78°, PE 94° and PTFE 104°. Datum points for post-attachment incubated samples (○,●) are means of two experiments each with duplicate samples and the datum points for pre-attachment incubated samples (■) are means of duplicate samples from one experiment. Error bars represent ± S.E.M.

accumulated label (Figure 4.3.). Bacteria with overlying silver grains were regarded as having assimilated radioactive substrate and silver grains not associated with bacteria were regarded as background. Background silver grains may be produced by radiation from outside sources unrelated to the radioactive substrate added to the samples, physical formation of a latent image, adsorption of tritiated substrate or metabolised product, or lateral emission of electrons from labelled bacteria. (Rogers, 1979).

In this investigation backgrounds on control surface not exposed to ^{3}H-substrate were very low suggesting that radiation from extraneous sources and physical effects were negligible (Figure 4.5.). However, T, PVDF and G substrata without bacteria but exposed to tritiated leucine had higher backgrounds than the above controls (Figure 4.5.). indicating that leucine was adsorbed by these substrata, which was supported by the influence of leucine on θ_B of the more hydrophilic surfaces (section 3.3.2.). The backgrounds on T and PE substrata incubated with ^{3}H-leucine after attachment of bacteria were higher still (Figure 4.5.) as were the backgrounds on substrata with bacteria labelled prior to attachment (section 4.3.5.), suggesting there was adsorption of labelled metabolites. Labelled bacteria may also increase backgrounds as a result of lateral emission of beta particles. However, if this was the case it would be expected that the free-living bacteria samples, which contained similar or higher proportions of labelled bacteria when incubated with 100 μg C l^{-1} ^{3}H-leucine (Figure 4.4A.), would exhibit similar backgrounds. This was not the case (Figure 4.5.), so it is likely that the higher backgrounds of these attached populations were due to adsorption of labelled metabolites or entrapment of labelled material in bacterial products, e.g. polysaccharides adsorbed on the surface (Geesey, 1982). This may also account for the close relationship between the proportion of labelled attached bacteria and number of background silver grains for samples incubated with

^{3}H-amino acids or ^{3}H-glucose (Figure 4.1.), different concentrations of ^{3}H-leucine (Figure 4.4. and 4.5.) and individual ^{3}H-amino acids (Figure 4.5. and 4.6.).

4.4.1. Assimilation by attached bacteria

Assimilation by attached bacteria was inversely related to θ_A and become more pronounced with increase in leucine concentration (Figure 4.4A.). This suggests that the different activities on the surfaces may have been due to different levels of leucine adsorption and thus concentration on the different substrata. This was supported by the level of the background on the substrata and the influence of different leucine concentrations on substrata θ_B's (section 3.3.2.).

Although assimilation by attached bacteria may have become more pronounced with increase in leucine concentration, as a result of adsorption, it is also possible that the increase in leucine concentration decreased diffusion limited uptake. Adsorption onto a substratum, or assimilation by attached bacteria, sets up a diffusion gradient which may be largely maintained by the relatively stationary liquid layer that is present adjacent to a solid surface (section 1.3.2.2.*(iii)*). This diffusion limitation can be reduced by agitation of the liquid medium. In an experiment where a comparison was made of assimilation by attached bacteria in a range of concentrations of ^{3}H-leucine in agitated and stationary incubation solutions, agitation increased the proportion of labelled attached bacteria, probably by increasing the chance of the substrate coming into contact with the sites of uptake on the bacteria and by reducing the diffusion limitation. This is supported by the general decrease of the influence of agitation with increase in substrate concentration (Figure 4.2.).

If assimilation by attached bacteria in this investigation was influenced by adsorption of amino acids, it might be expected that the charge of the amino acid would influence the comparative assimilatory activities of attached and free-living bacteria, and bacteria attached to substrata with different θ_A. Backgrounds on substrata that were incubated without bacteria in 10 μg C l^{-1} of two neutral (leucine, glycine) acidic (aspartate, glutamate) and basic (arginine, lysine) amino acids, indicated that adsorption was very low and did not appear to be related to substrate charge (Figure 4.7.). The pattern of assimilation of leucine, glycine, glutamate, arginine and lysine by bacteria attached to a range of substrata was also similar (Figure 4.6.), suggesting that weak electrostatic adsorption, not detected by the controls, did not occur. The type of amino acid had little influence on the comparative activities of free-living and attached bacteria, in that assimilation by attached bacteria remained generally higher than that of free-living bacteria, irrespective of substrate charge. Thus the relationship between substratum θ_A and proportion of assimilating bacteria, and the differences in assimilation by attached and free-living bacteria, did not appear to be due to variations in electrostatic adsorption of substrate on the substrata.

Differences in the oxidation rates of amino acids and other small charged substrate molecules by free-living bacteria and bacteria adsorbed onto anion exchange resin, have been attributed to the extent of electrostatic adsorption of the substrate by the resin (Hattori and Furusaka, 1960; Hattori and Hattori, 1963). The electrostatic charge interactions affecting substrate adsorption and the activity of attached bacteria encountered with the substrata used in this investigation are, however, probably more typical of those in aquatic environments than are the charge interactions encountered with anion exchange resins.

The investigation on the influence of a range of amino acid substrates, on attached bacterial activity (Figure 4.6.), did not completely support the results obtained from the experiments with different concentrations of leucine (Figure 4.4.) in that there was not the same clear relationship between θ_A and proportion of labelled attached cells, or backgrounds (Figures 4.5. and 4.6.). This was probably, at least in part, due to the low amino acid concentration (10 μg C l^{-1}) used in the experiment with a range of amino acids, while the relationship between θ_A and assimilation was more apparent at higher substrate concentrations (50, 100 μg C l^{-1}, Figure 4.4A.).

The relationship between the substratum bonding potential (θ_A) and the level of background (Figures 4.5. and 4.7.) and the influence of substrate concentration on uptake by attached bacteria (Figure 4.4.), suggests that the generally higher assimilation by bacteria attached to T, G, PVDF and PE than by detached or free-living bacteria (Figures 4.1., 4.4. and 4.6.), may have been a result of substrate adsorption. This adsorption was probably not due to electrostatic charge interactions (see above).

As differences in the levels of activity of bacteria on the different surfaces could also have been due to preferential attachment of the most actively assimilating bacteria from the free-living population, this possibility was examined. Unattached bacteria were incubated with ^{3}H-leucine and subsequently allowed to attach. This resulted in the labelling of 1-2% of the free-living population but the attachment of a disproportionately high proportion (at least four times greater), of labelled hence presumably more active assimilating bacteria. Although the proportion of labelled bacteria that attached to the different substrata were somewhat similar, the proportion tended to increase with θ_A (means of ≅ 8% on T and PVDF, 9.5% on PE and 11% on PTFE, Figure 4.8.). Assimilatory activity may, therefore, have been more important for attachment to hydrophobic surfaces than to hydrophilic ones, which is supported by other studies (Fletcher, 1980b; Fletcher and Marshall, 1982a).

Despite the apparent selective advantage of potentially higher assimilation by the attached population (Figure 4.8.), this advantage was not always apparent when bacteria were incubated with substrate after attachment to PTFE (Figures 4.4. and 4.6.). This suggests that attachment, particularly when it is to PTFE, decreases assimilation of substrate and/or increases the proportion of label lost possibly by respiration. The proportion of pre-attachment labelled cells that retained label after attachment, decreased with time to a greater extent when attached to PTFE than when attached to the other substrata (Figure 4.8.). The extent of the influence of attachment on assimilation was dependent upon the concentration of substrate as well as the substratum characteristics (Figures 4.4. and 4.6.).

The importance of the physicochemical property of water wettability of a substratum (evaluated by θ_A) to bacterial attachment and assimilatory activity, was supported by the similar values obtained for attachment and assimilation on PVDF and G. These substrata had similar θ_A's, owing to the treatment of G, (see section 3.3.1.) but otherwise have very different characteristics e.g. roughness (observed by light microscopic examination), density and molecular structure.

There was a strong negative correlation between assimilation by attached bacteria or number of attached bacteria, and θ_A, and a positive correlation between proportion of detached bacteria and θ_A. This suggests that assimilatory activity is important for attachment to be maintained. This activity may be involved in production of adhesive polymer to strengthen adhesion of attached bacteria to the substratum.

A previous study on attachment of *Pseudomonas* sp. 2021 to generally more hydrophilic substrata than used in this investigation demonstrated a positive relationship between attachment and substratum θ_A (Fletcher and Loeb, 1979). Nevertheless, there was a similar negative relationship between attachment to the three comparatively hydrophobic surfaces, T, PE and PTFE and θ_A in both studies.
The difference in the attachment characteristics of the marine pseudomonad in the two studies may have been due to differences in the growth phases of the cells used (Fletcher, 1977; Gerson and Akit, 1980), or differences in the extent of cell washing. Washing may remove cell surface components,

residual nutrients, metabolites, or inhibitors thereby influencing attachment by altering the bacteria, or liquid surface tensions (section 2.1.3.) or bacterial activity (Fletcher, 1980b).

4.4.2. Summary

The results of this MAR study demonstrated that:

1. Assimilation of a range of amino acids at a concentration of 10 μg C l^{-1} by attached bacteria, was generally greater than, or similar to, that of free-living bacteria.

2. Neutral, acidic and basic amino acids at a concentration of 10 μg C l^{-1} were not adsorbed onto any of the substrata.

3. Assimilation of leucine by attached bacteria was negatively correlated to θ_A and became more pronounced with increased leucine concentration.

4. ^{3}H-leucine was adsorbed from a 100 μg C l^{-1} solution onto all but the most hydrophobic surface as was labelled material from attached bacteria incubated with this substrate.

5. Assimilation of amino acids by bacteria that detached from T.G. PVBE and PE was generally lower than that of their attached counterparts.

6. Pre-attachment incubation of free-living bacteria with ^{3}H-leucine resulted in the subsequent attachment to all substrata of approximately a four fold higher percentage of labelled bacteria as compared with the population that remained free-living.

7. Attachment was negatively correlated to substratum θ_A.

8. Detachment was positively correlated to substratum θ_A.

5. An Evaluation of Electron Transport System Activity

5.1. INTRODUCTION

For some time tetrazolium salts have been used as electron acceptors in the measurement of microbial dehydrogenase activity. Initially triphenyl tetrazolium chloride was used (Howard, 1972), for which anaerobic incubation was necessary to ensure adequate reduction of the salt to its formazan. Curl and Sandberg, (1961) reported that 2-(*p*-iodophenyl)3(*p*-nitrophenyl)-5 phenyl tetrazolium chloride (INT) successfully competed with oxygen for electrons and therefore could be used under aerobic conditions. Using INT Jones and Simon (1979), obtained a very good correlation between oxygen uptake and electron transport system (ETS) activity of freshwater microorganisms. In this, as in other studies of ETS activity in marine (Packard *et al.*, 1971, 1975; Hobbie *et al.*, 1972; Devol *et al.*, 1976) and freshwater (Zimmerman, 1975) plankton, insoluble coloured formazan, produced as a result of reduction of INT, was extracted with an organic solvent and determined spectrophotometrically. Using this technique samples must contain sufficient respiring cells to produce spectrophotometrically detectable quantities of formazan.

INT staining has recently been used in a similar manner to MAR to microscopically evaluate the activity of microbial populations (Zimmerman *et al.*, 1978). Like MAR, INT staining has the advantage that a qualitative measure of the activity of very small microbial populations can be made. This type of study on ETS activity was first carried out by observing INT-formazan containing bacteria by phase contrast microscopy (Iturriaga and Rheinheimer, 1975). Zimmerman *et al.*, (1978) developed the technique by combining formazan detection with epifluorescence microscopy so that bacteria and INT-formazan spots could be more easily distinguished (Figure 5.1.). This method was shown by Harvey and Young (1980) to be suitable for detecting ETS activity in attached bacteria from a salt marsh estuary. In this study a much greater

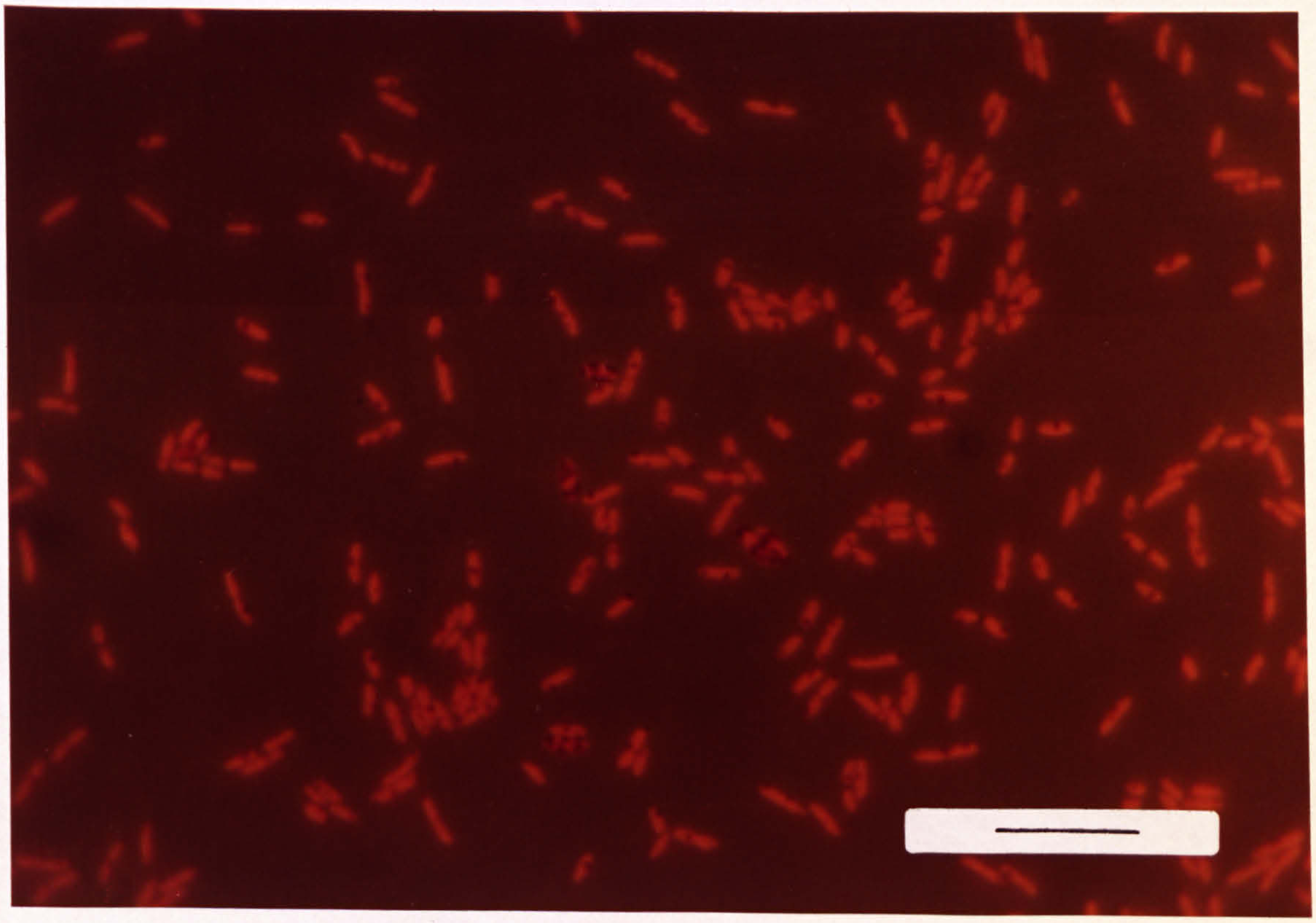


FIGURE 5.1.

Photomicrograph taken under bright field and UV illumination of bacteria attached to G demonstrating ETS activity (with red INT-formazan spots). Bacteria attached during a 1.5h period from a suspension of cells in peptone and yeast extract medium, followed by 20 min incubation with a saturated INT-ASW solution. Bar = 10 μm.

proportion of attached bacteria were found to respire at a detectable level, as compared with free-living cells. INT staining was chosen to examine ETS activity of bacteria, as it is a sensitive technique suitable for qualitatively evaluating respiration by attached and free-living bacterial populations in a manner similar to MAR evaluations of assimilatory activity.

5.1.1. Aims

The objectives of this evaluation of bacterial ETS activity were:

1. Determine whether ETS activity by attached bacteria differed from that of detached or free-living bacteria.

2. Determine the relationship between substratum θ_A and ETS activity of attached bacteria.

3. Determine the relationship between ETS activity of bacteria before attachment and subsequent to attachment.

5.2. MATERIALS AND METHODS

5.2.1. Organism and growth conditions

The marine *Pseudomonas* sp. NCMB strain 2021, was cultured in 100 ml of medium for 40h at 15°C in an orbital incubator (150 rpm). The medium comprised 0.1% (w/v) peptone and 0.07% (w/v) yeast extract in SW, pH 8.1 (section 5.2.1.), or a similar medium but with ASW pH 8.1, substituted for SW (section 5.2.2.2.). The bacteria, which were in stationary phase were harvested by centrifugation (10K 4°C).

5.2.2. Attachment of bacteria to substrata

5.2.2.1. Bacteria incubated with INT-ASW solutions

Harvested bacteria were resuspended in ASW or ASW with 0.1% (w/v) peptone and 0.07% (w/v) yeast extract to ≈ 10^7 cells ml^{-1}. 20 ml portions of the cell suspensions were allowed to attach for 1.5h at 15°C to glass cover slips placed on the bottom of petri dishes. The substrata with attached cells were then washed to remove residual suspended cells, medium and loosely attached cells before transfer, in the same orientation, to the bottom of 25 ml universal bottles containing the incubation solution (section 5.2.3.1.).

5.2.2.2. Bacteria incubated with INT-ASW leucine solutions

Harvested bacteria were washed twice in ASW by resuspension and centrifugation and the final cell suspension was adjusted to ≈ 2 x 10^8 cells ml^{-1}. The cells in 7 ml portions of this suspension were allowed to attach for 2h at 15°C to T, G, PE and PTFE substrata held vertically in silicone rubber rings in 25 ml universal bottles. The substrata were then washed with 100 ml ASW at a flow rate of ≈ 200 ml min^{-1}, to remove residual suspended cells and loosely attached cells, placed in new silicone rubber rings and transferred to 7 ml of filter sterile incubation solution (section 5.2.3.2.).

5.2.3. Incubation with INT

5.2.3.1. Incubation of attached bacteria with INT-ASW solutions

Attached bacteria were incubated for 20 min in an orbital shaking (100 rpm) incubator at 15°C with either 0, 9, 50 or 100% INT-ASW solutions. The 100% INT (BDH, Atherstone) solution was a saturated solution of INT in ASW. At the end of the incubation period the bacteria were fixed with 0.1 ml formalin. Bacteria that detached during incubation were collected by filtration and attached and detached samples were mounted onto microscope slides as described in section 4.2.4. Mounted specimens were stained with acridine orange as described in section 5.2.4.

5.2.3.2. Incubation of attached bacteria with INT-ASW leucine solutions

Bacteria were incubated for 20 min in an orbital shaking (100 rpm) incubator at 15°C with 7 ml portions of a solution containing equal volumes of *(i)* a saturated solution of INT in ASW and *(ii)* a solution of leucine (20, 200, 1000 and 2000 μg C l^{-1}) in ASW giving final leucine concentrations of 10, 100, 500 or 1000 μg C l^{-1}. To prepare suspensions of free-living bacteria of similar numbers to the attached cell preparations, 0.1 ml portions of the washed cell suspension were pipetted into

7 ml of corresponding incubation solutions and incubated as were the attached populations. After the incubation period the bacteria were fixed with 0.1 ml formalin. Free-living and detached cells were collected by filtration and attached, free-living and detached samples were mounted on microscope slides as described in section 4.2.4. Mounted specimens were stained with acridine orange as described in section 5.2.4.

5.2.3.3. Incubation of bacteria with INT-ASW leucine solution before attachment

To determine whether attachment was selective with respect to ETS activity of suspended cells, bacterial suspensions were prepared in the same way as those for pre-attachment incubation with ^{3}H-leucine (section 4.2.3.3.) except that the incubation solution comprised saturated INT with 1000 µg C l^{-1} unlabelled leucine. All incubation and washing solutions were filter sterilised before use.

5.2.4. Microscopy

The mounted samples were stained for 5 min with acidine orange (0.01% [w/v] in 6.6 mM phosphate buffer, pH 6.7) and rinsed with distilled water. After air-drying, the specimens were examined with blue-incident light excitation and bright field illumination under a Zeiss Standard 18 microscope, fitted with x100 oil objective and x10 eye piece. The percentage of bacteria exhibiting ETS activity was calculated by relating the number of bacteria with red INT-formazan spots to the total number of bacteria in 100 x 100 µm² areas, or until 200 bacteria were counted for each slide (Figure 5.1.).

5.3. RESULTS

5.3.1. The relationship between INT concentration and ETS activity of bacteria attached from nutrient-rich and nutrient-poor cell suspensions

ETS activity was demonstrated to occur in a large proportion (95-99%) of the bacteria that attached to G from a cell suspension in peptone yeast extract medium and remained attached during incubation with INT. The bacteria that detached during incubation with INT, after attachment from a peptone yeast extract medium contained a comparatively small proportion of cells with ETS activity ($\sim$ 15%). Similar levels of ETS activity were demonstrated by bacteria that attached from a suspension of bacteria in ASW and remained attached ($\sim$ 5-20%), or detached during incubation with INT ($\sim$ 5-15%) (Figure 5.2.). The INT concentration had little influence on the proportion of bacteria containing INT-formazan spots and so was not limiting the number of bacteria that demonstrated ETS activity.

5.3.2. The relationship between substratum contact angle and proportion of bacteria demonstrating ETS activity

The proportion of bacteria containing INT-formazan spots increased with leucine concentration when cells were attached to T and PE but showed little relationship when attached to G or PTFE, or when free-living (Figure 5.3A.). There was, however, a significant negative correlation between θ_A and percentage of INT-formazan containing attached bacteria at all but the lowest leucine concentration, so that r was -0.44 (not significant), -0.886 (P<0.01), -0.832 (P<0,05) and -0.871 (P<0.01) for 10, 100, 500 and 1000 µg C l^{-1}, respectively. The proportion of bacteria attached to G, PE and PTFE that demonstrated ETS activity was lower than that for free-living bacteria. However, the relationship between the activities of bacteria attached to T and free-living cells depended upon leucine concentration. Detached bacteria generally had a smaller proportion of ETS active cells than did free-living cells. The relative activities of the detached and attached populations depended upon the leucine concentration and substratum. However, the activities of detached cells tended to be higher than their attached counterparts with the exceptions of bacteria that detached from T, during incubation with 100, 500 and 1000 µg C l^{-1} and those bacteria that detached during incubation with 500 µg C l^{-1}, which had lower activities than those which remained attached (Figure

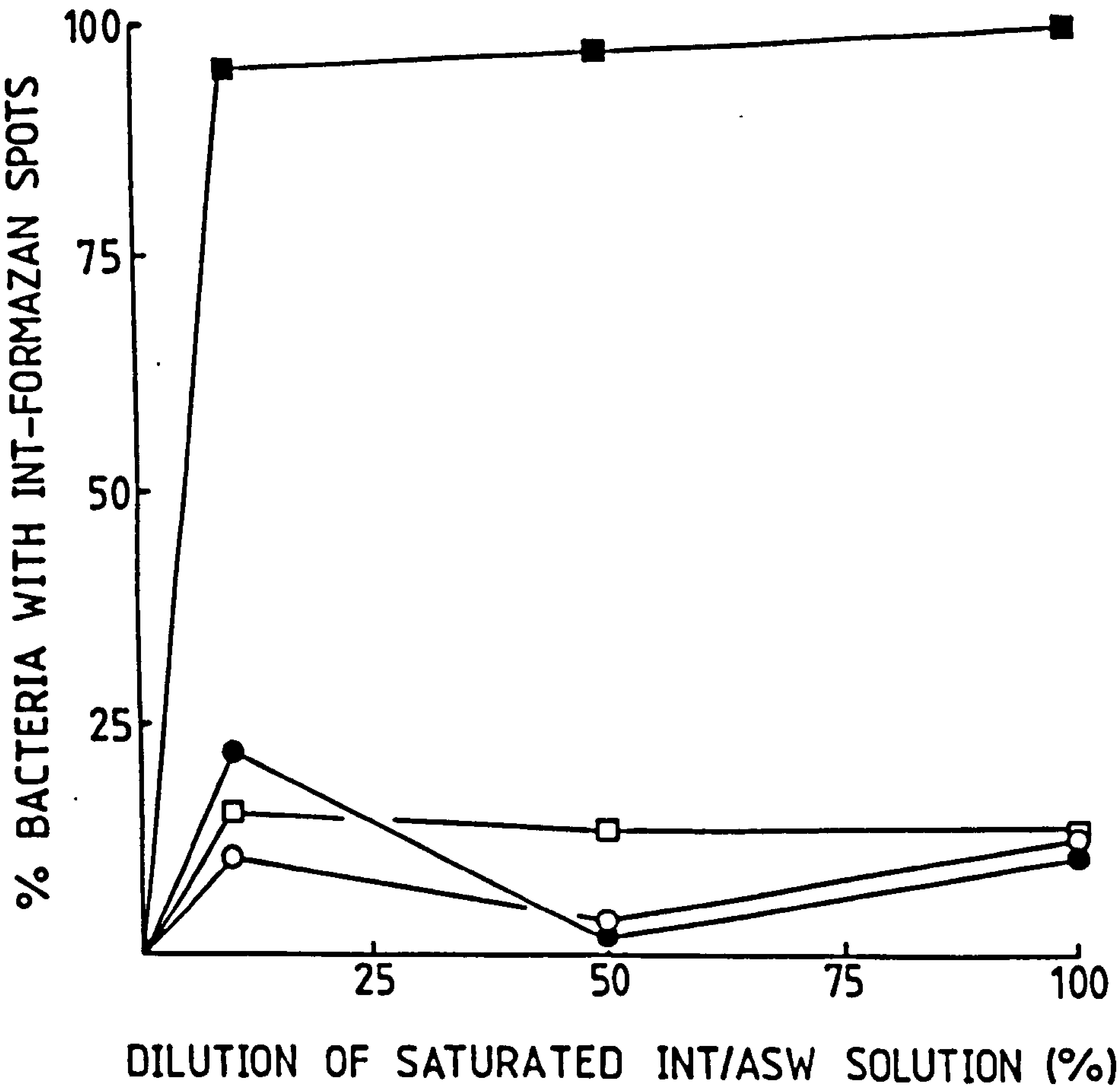


FIGURE 5.2.

The relationship between INT concentration and proportion of attached (●,■) and detached (○,□) bacteria with INT-formazan spots. Bacteria attached to G from cell suspensions in ASW (●, ○) or peptone and yeast extract media (■,□).

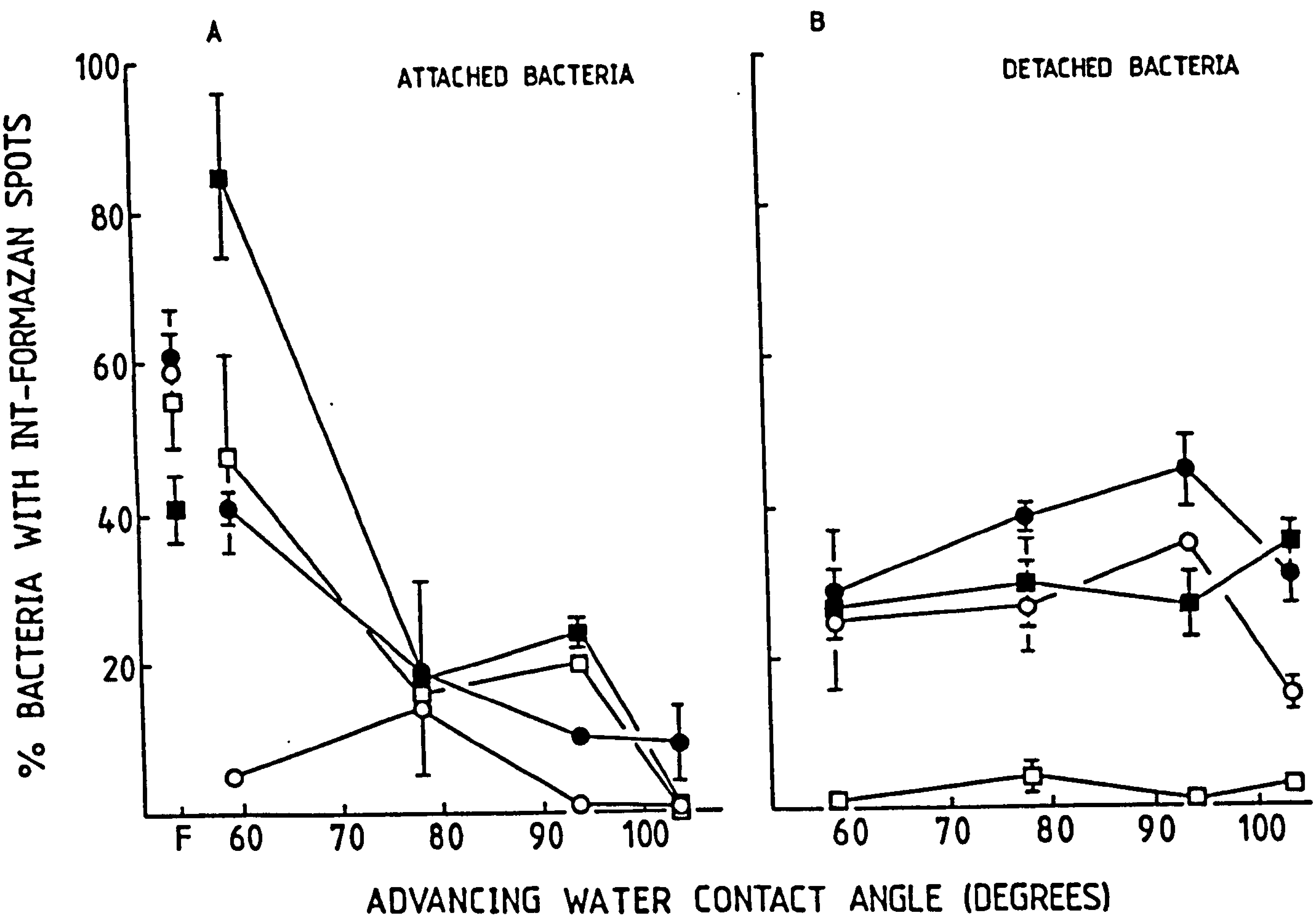


FIGURE 5.3.

The relationship between the proportion of attached (A) or detached (B) bacteria demonstrating ETS activity, indicated by presence of INT-formazan spots, and the θ_A of substrata, after incubation with INT and leucine. F is the proportion of free-living cells with INT-formazan spots. The leucine concentrations were 10 (○), 100 (●), 500 (□) and 1000 (■) µg C l^{-1}. The substrata θ_A were T 59°, G 78°, PE 94° and PTFE 104°. Datum points are means of duplicate samples from one experiment. Error bars represent ± S.E.M.

5.3.).

5.3.3. The relationship between ETS activity of bacteria and subsequent attachment

When bacteria were incubated with INT and leucine before allowing attachment, a disproportionately smaller percentage of bacteria with INT-formazan spots subsequently attached as compared with bacteria that remained unattached (Figure 5.4.). Furthermore, the attached bacteria that exhibited ETS activity had only very faint INT-formazan spots. The proportion of free-living bacteria showing ETS activity was fairly constant over a 3h period. However, the ETS activity of attached cells tended to increase over the initial 1 to 1.5h and for up to 3h either remained at a constant level, with attachment to the more hydrophilic surfaces (T and PVDF), or declined with attachment to the more hydrophobic surfaces (PE and PTFE) (Figure 5.4.).

5.3.4. The relationship between substratum contact angle and attachment of bacteria incubated with INT

Detachment from the substrata, particularly G, was greater after 20 min post-attachment incubation with INT and leucine (Figure 5.5.) than after 2h post-attachment incubation with just leucine (Figure 4.9.). Largely as a result of this difference in detachment, the number of bacteria that remained attached after post-attachment incubation with INT (Figure 5.5.) was lower, particularly for G, than for post-attachment incubation with just leucine (Figure 4.9.). However, attachment remained negatively correlated to θ_A ($r = -0.689$; $P < 0.001$) and detachment remained positively correlated to θ_A ($r = 0.553$; < 0.01). Moreover, although the relationship between attachment and θ_A was somewhat different in this investigation compared with the MAR study, (mainly due to the lower attachment to G), at high leucine concentrations there was still a strong positive correlation between bacterial activity (Figure 5.3A.) and number of bacteria that remained attached to each of the substrata (Figure 5.5.), so that r was 0.049 (not significant),

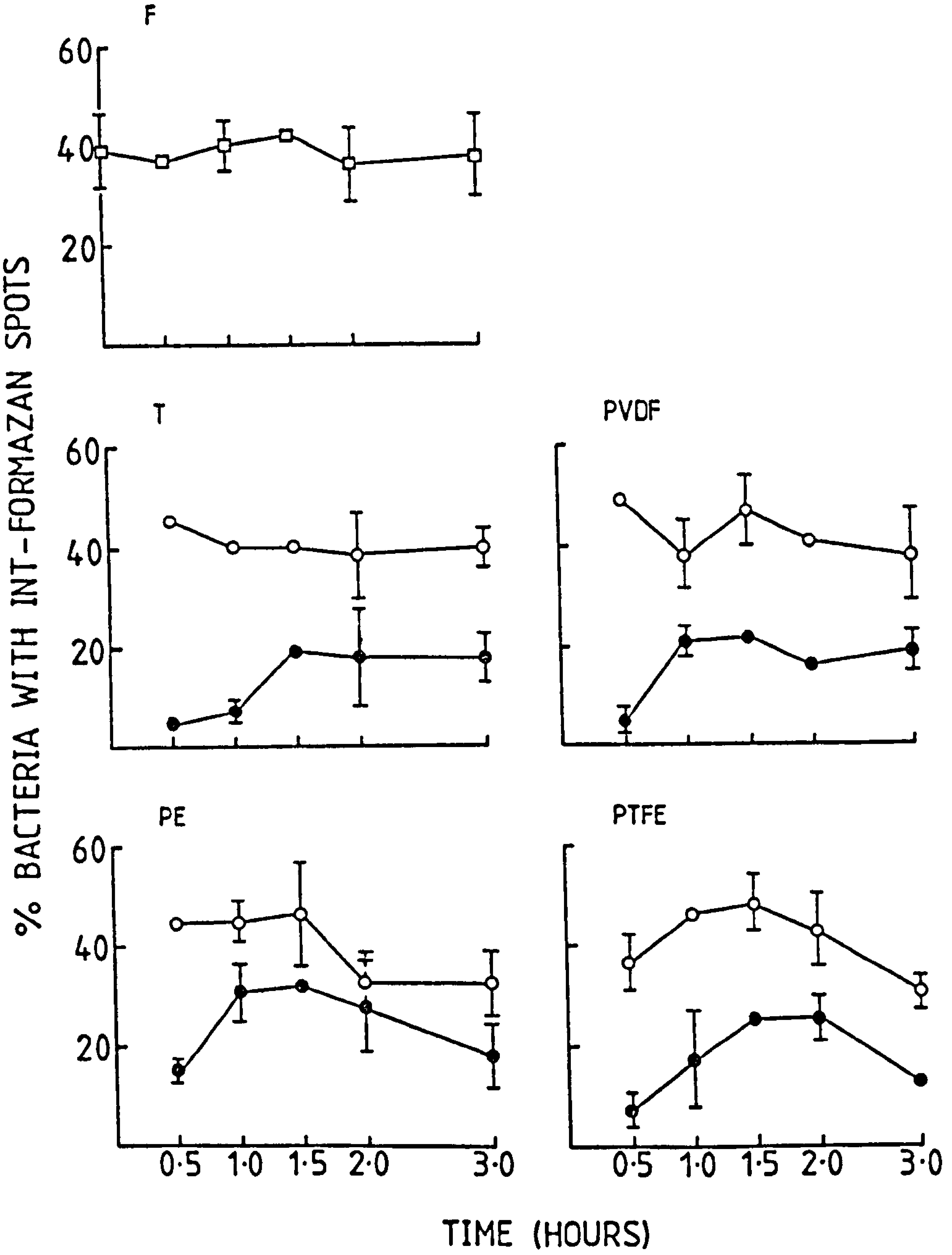


FIGURE 5.4

The relationship between time allowed for attachment and the proportion of bacteria labelled with INT-formazan spots prior to exposure to substrata that subsequently attached (●) or remained unattached (○), or were free-living (□). Datum points are means of duplicate samples from experiment. Error bars represent ± S.E.M.

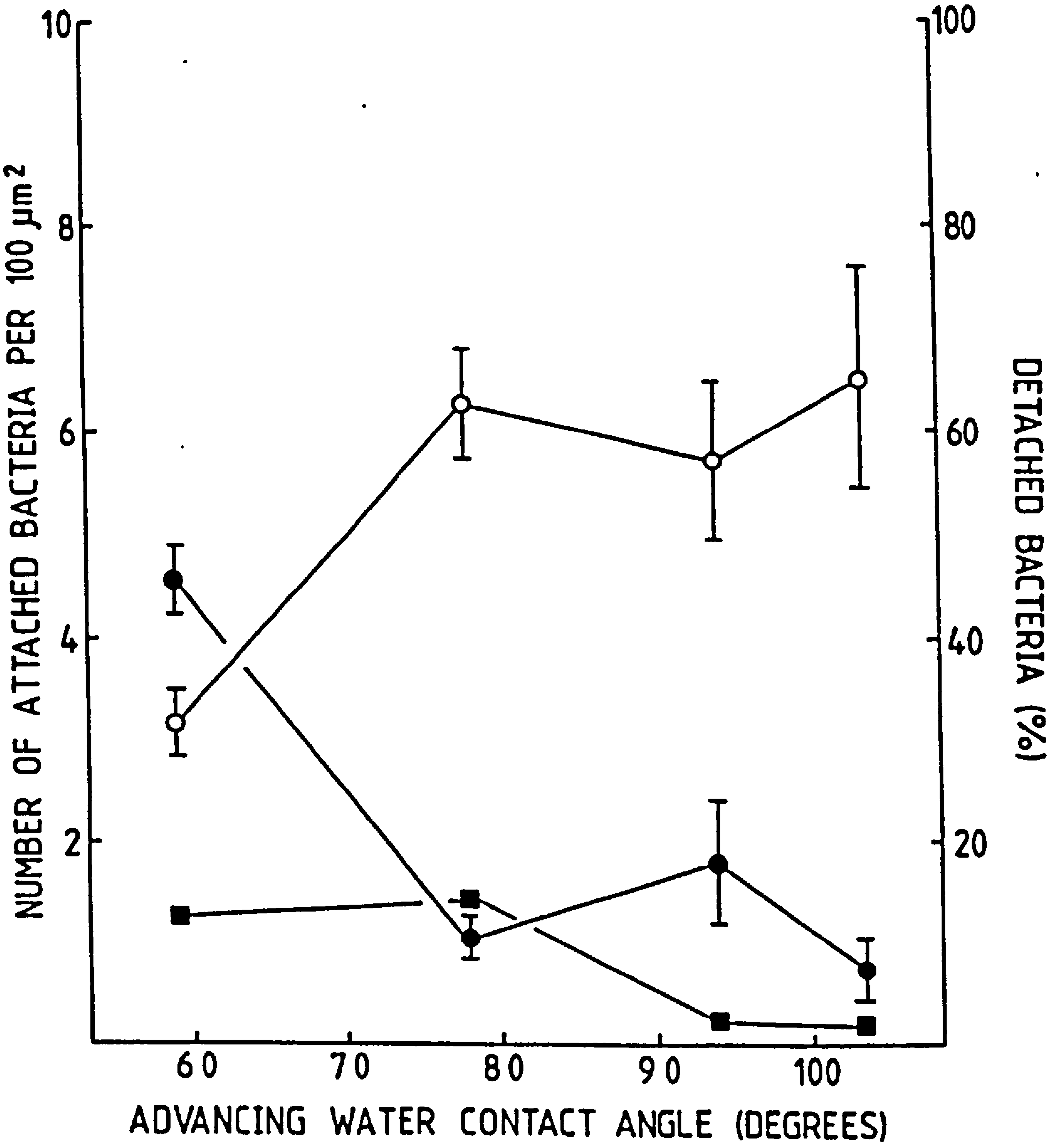


FIGURE 5.5.

The relationship between numbers of attached (●,■) or percent detached bacteria (○) and substratum θ_A. Attached bacteria were those which remained attached after a 2h attachment period followed by 20 min incubation with INT and leucine (●) and those which attached after 2h incubation with INT and leucine followed by a 2h attachment period (■). Detached bacteria were those which desorbed from the substrata during the 20 min post-attachment incubation with INT and leucine. The substrata and θ_A's were T 59°, PVDF 77° (pre-attachment incubation with INT), G 78° (post-attachment incubation with INT), PE 94° and PTFE 104°. Datum points are means of duplicate samples from one experiment. Error bars represent ± S.E.M.

0.577 (not significant), 0.807 ($P<0.05$) and 0.910 ($P<0.01$) for 10, 100, 500 and 1000 μg C l^{-1} respectively. Attachment to substrata, particularly T and PVDF, was also reduced after 2h pre-attachment incubation with INT and leucine (Figure 5.5.), compared with attachment after 2h pre-attachment incubation with just leucine (Figure 4.9.), but attachment remained negatively correlated to θ_A ($r = -0.604$; $P<0.001$).

5.4. DISCUSSION

The ETS of respiring bacteria reduces INT to INT-formazan as follows:

$$INT + 2e^- + 2H^+ \rightarrow INT\text{-formazan} + HCl$$

The site of INT-reduction, at least in eucaryotic cells, is probably at, or near, the cytochrome b-ubiquinone complex of the ETS (Kenner and Ahmed, 1975). However, in contrast to mainly diffuse incorporation of red INT-formazan by respiring eucaryotic cells, respiring bacteria accumulate INT-formazan as optically dense, dark red intracellular spots (Figure 5.1.). The size and optical density of the spots corresponds to the intensity of respiration (Zimmerman *et al.*, 1978). Increasing respiratory activity by addition of a substrate in the incubation mixture can increase INT reduction (Curl and Sandberg, 1961, Packard and Healy, 1968). However, cytochemical staining with INT may underestimate respiratory activity within a population, as the respiratory activity of individual cells may be below the level of detection (Zimmerman, *et al.*, 1978).

At least some of the differences in the proportions of cells demonstrating ETS activity in this investigation were due to differences in respiratory intensity of the bacteria in the attached, detached and free-living populations. This is supported by the parallel increase in the proportions of INT-formazan containing cells attached to T and PE with increase in leucine concentration (Figure 5.3.). Also the bacteria that attached to G during incubation with growth medium, were larger, fluoresced bright orange and contained a higher number of spots per cell, than the smaller, faint orange fluorescing bacteria, that attached from a suspension of cells in ASW without added nutrients (Figure 5.1.).

As with assimilatory activity, ETS activity of attached cells was inversely related to θ_A and was generally more pronounced with increase in leucine concentration, suggesting that the substratum influence on bacterial ETS activity was due to different levels of leucine adsorption and metabolism. However, it is not clear why leucine concentration had little influence on the ETS activity of cells attached to G (Figure 5.3A.) as compared with assimilatory activity (Figure 4.4A.). This may have been partly due to the relatively high detachment from G in the presence of INT and leucine (Figure 5.5.) as compared with detachment from G in the presence of just leucine (Figure 4.9.).

In contrast to the generally similar, or higher, assimilatory activity of attached bacteria compared with their detached or free-living counterparts (Figures 4.4. and 4.6.), the ETS activity of attached bacteria, with the exception of bacteria attached to T, was generally lower than that for free-living or detached bacteria (Figure 5.3.). This suggests that the respiratory activity of attached bacteria may have been lower, and the efficiency of substrate utilisation greater, than that for free-living bacteria. This is supported by the $^{14}CO_2$ respiration and carbon assimilation quotient values for these populations (Figures 6.4. and 6.6.). Further details and possible explanations for the difference in efficiency of substrate utilisation are given in sections 6.3. and 6.4.

INT appeared to have an adverse affect on bacterial attachment. This was illustrated by the increased detachment of bacteria after post-attachment incubation with leucine and INT and the reduced attachment after pre-attachment incubation of bacteria with leucine and INT as compared with similar populations of bacteria post-incubated and pre-incubated with leucine alone (Figures 4.9. and 5.5.). As INT reduction probably inhibits some of the cell's ATP synthesis and can inhibit growth

(Jones, 1979), the adverse effect of INT on attachment may have been due to INT's distrupting influence on the metabolic activity of bacteria. Inhibitors of ETS have been shown to decrease attachment of *Pseudomonas* NCMB 2021 (Fletcher, 1980b).

The results of this investigation supports the suggestion that activity favours attachment, as *(i)* there was a close correlation between activity (assimilatory and ETS) of attached bacteria and the number of bacteria that remained attached (Figures 4.4A., 4.9., 5.3A. and 5.5.), and *(ii)* the bacteria that attached had a higher assimilatory activity than those that remained unattached (Figure 4.8.).

5.4.1. Summary

The results of this evaluation of ETS activity of bacteria demonstrated that:

1. The ETS activity of attached bacteria was less than that of free-living bacteria, with the exception of bacteria attached to T, incubated with 500 or 1000 μg C l^{-1} leucine which had similar and higher ETS activities respectively.

2. The ETS activity of detached bacteria was generally lower than that of free-living bacteria, but higher than that of attached bacteria, with the exception of bacteria attached to T and incubated with 100, 500, or 1000 μg C l^{-1} leucine.

3. The percentage of attached bacteria that demonstrated ETS activity when incubated with 100, 500 or 1000 μg C l^{-1} was inversely related to θ_A.

4. The ETS activity of bacteria attached to T and PE increased with leucine concentration but the ETS activity of bacteria attached to G and PTFE or free-living bacteria was largely independent of leucine concentration.

5. After incubation with INT, bacteria that subsequently attached demonstrated lower ETS activity than did free-living bacteria.

6. There was a similar relationship between θ_A and *(i)* the proportion of attached bacteria demonstrating ETS activity when incubated with 500 or 1000 µg C l^{-1}, and *(ii)* numbers of attached bacteria.

7. Incubation with INT decreased subsequent attachment of free-living bacteria and increased detachment by attached bacteria.

8. Attachment and detachment remained, respectively, negatively and positively correlated to θ_A despite the influence of INT on attachment and detachment.

6. Assimilation and Respiration of ^{14}C-Amino Acids

6.1. INTRODUCTION

The heterotrophic activity of aquatic microorganisms has frequently been determined by measuring the uptake of radioactive organic compounds. Parsons and Strickland, (1962) suggested that microbial uptake of labelled substrate in sea water could be described by Michaelis-Menten enzyme kinetics, allowing the calculation of a value similar to the Michaelis constant (K) plus the natural substrate concentration (S_n). The Michaelis constant is also sometimes referred to in bacterial uptake studies as the transport constant (K_t) or half saturation constant. K is equivalent to the substrate concentration when the velocity of uptake is half the maximum rate (Vmax) and is a measure of the affinity of the uptake system for a substrate. Vmax is attained when the uptake sites are continually saturated with substrate. Wright and Hobbie (1965, 1966), developed the kinetic analysis of uptake so that determination of the natural turnover time and theoretical Vmax of organic substrate could be made.

Michaelis-Menten kinetics describes the relationship between the rate of an enzyme-catalysed reaction and substrate concentration. At low substrate concentrations the active sites on the enzyme are not saturated by substrate and the rate of reaction varies with substrate concentration (first-order kinetics). As the substrate concentration increases the sites are covered to a greater extent until the rate is independent of the substrate concentration (zero-order kinetics). When graphically expressed this relationship between the rate of a reaction and the substrate concentration is in the form of a hyperbolic curve which can be mathematically defined by the Michaelis-Menten equation (equation 1.2, section 1.3.2.1.*(i)*).

In applying the Michaelis-Menten model to uptake of substrates by natural, mixed microbial populations a number of assumptions have to be made (Wright, 1973). For example, the "catalytic sites", the transport systems of different microbial cells, are assumed *(i)* to behave as non-allosteric enzymes *(ii)* remain at a constant concentration over the measurement period and *(iii)* not to be induced or inhibited by solute molecules. Despite the lack of justification for making these assumptions, numerous studies have demonstrated that the uptake of a range of amino acids, sugars and organic acids by aquatic bacteria, in coastal water, estuaries, lakes and sediments, can be described by Michaelis-Menten Kinetics (Vaccaro and Jannasch, 1966; Munro and Brock, 1968; Harrison *et al.*, 1971; Robinson *et al.*, 1973; Burnison and Morita, 1974; Wright and Shah, 1975). Some studies have, however, shown low uptake and deviation from this type of kinetics (Vaccaro and Jannasch, 1967; Hamilton and Preslan, 1970; Takahashi and Ichimura, 1971). Uptake by open ocean samples that do not follow Michaelis-Menten kinetics may follow diffusion kinetics (Wright and Burnison, 1979). For example, diffusion-type uptake, probably due to algae, has been reported when uptake is measured at high substrate concentrations (Wright and Hobbie, 1966).

The early determinations of heterotrophic activity using ^{14}C-labelled organic substrates did not account for $^{14}CO_2$ respired and therefore, the values obtained from the kinetic analysis were an underestimate of the total uptake. Assimilation can range from 25 to over 90 percent of total uptake, depending on substrate and microbial population (Wright and Burison, 1979). Williams and Askew (1968) introduced a technqiue whereby respired $^{14}CO_2$ could be captured, allowing calculation of total uptake (assimilation and respiration). Knowledge of the rates of both assimilation and respiration had the added advantage of allowing calculation of the efficiency of substrate utilisation as either the percent of total uptake that is respired (Wright, 1974), or, the 'growth yield', the percent of total uptake that is assimilated (Williams, 1970, 1973).

Among the factors that determine the velocity of uptake of substrates by microorganisms in natural waters, the principal ones are probably temperature, concentration of usable dissolved organic matter and size and physiological state of the microbial population (Wright, 1979). An evaluation of the physiological state of the cells in a microbial population may be obtained by taking into account the number of cells as well as the heterotrophic activity of a microbial population to obtain values of uptake of organic substrates per cell. This type of approach has been made to determine the "specific activities" of microbial cells in estuarine and coastal waters (Wright, 1978, 1979), chemostat grown isolates (Hamilton *et al.*, 1966), and epiphytic bacteria (Fry and Ramsey, 1977), as well as to compare the activities of attached and free-living bacteria (Goulder 1977; Ladd *et al.*,1979; Bent and Goulder, 1981; Bell and Albright, 1982; Kirchman and Mitchell, 1982).

6.1.1. Utilisation of organic substrates by attached and free-living microbial populations

The relative contributions to the total heterotrophic activity in an aquatic system made by the attached and free-living microbial populations will depend upon the physiological activity and size of each of these populations. A number of investigations have shown that uptake of organic substrate per attached cell was greater than that per free-living cell. (Goulder, 1977; Bent and Goulder, 1981; Kirchman and Mitchell, 1982). However, in one study of forty-four diverse aquatic environments, the average turnover time per active free-living cell was lower than that per attached cell (Bell and Albright, 1982). It is possible that such differences in uptake per cell in these studies may, in part, be due to differences in the sizes of the cells. Attached cells have been reported to be larger than unattached cells (Fellows, *et al.*, 1981; Wilson and Stevenson, 1980). In one study the uptake of dissolved adenosine triphosphate by attached cells was one or two orders of magnitude greater than that by free-living bacteria, but this difference could be accounted for by the much larger average cell volume of attached organisms (Hodson *et al.*, 1981).

The size of the attached and free-living microbial populations vary greatly from one body of water to another. In a study of marine, lake, river and creek environments, half the forty-four sites sampled contained a larger attached population and half a larger free-living population (Bell and Albright, 1982). Attached bacteria have been shown to be numerically dominant in some freshwater streams and shallow lakes (Costerton and Geesey, 1979) and some esturine and other aquatic systems with high concentrations of particulate matter (Kirchman and Mitchell, 1982). In midlake and open ocean systems, and in aquatic environments with little particulate matter, free-living bacteria are often numerically dominant (Paerl, 1980).

The contributions by attached and free-living populations to uptake of organic compounds in aquatic systems have been determined by separating the two populations by size-fractionation before, or after, incubation with radioactive substrates. A review of this type of study, made on a variety of inland aquatic ecosystems (Paerl, 1980) showed incorporation of a range of ^{14}C- and ^{3}H-labelled organic substrates by attached and free-living populations can vary dramatically from one aquatic system to another and between different areas or depths within one system. However, incorporation of labelled organic substrates by the >1 μm size fraction, (attached bacteria), in each of the systems was less than 50% of the total. Similarly, a review of published values for assimilation of ^{14}C- and ^{3}H-labelled glucose by microorganisms in marine, estuarine and freshwater ecosystem, indicated that uptake by the >1 μm fraction was less than 50%, with the exception of some studies in freshwater and esturine environments, where assimilation by the >3 μm fraction was greater than 50%, and in one study, reached as much as 99% of total uptake (Kirchman and Mitchell, 1982). In general, it appears that attached bacteria contribute to a greater extent to heterotrophic activity in freshwater systems than in marine systems.

The difference in the relative contributions to the total heterotrophic activity of a system by the attached and free-living populations is probably largely a reflection of the different sizes of the two populations in that system (Bent and Goulder, 1981; Cammen and Walker, 1982). The activity due to the attached population may, however, be larger than suggested by the comparative sizes of the two populations, owing to higher activity per cell of attached microorganisms (Goulder, 1977; Bent and Goulder, 1981; Kirchman and Mitchell, 1982).

The results of the MAR and ETS studies, described in Chapters 4 and 5, suggest that the assimilation by attached bacteria was generally similar, or higher (Figures 4.4. and 4.6.), and respiration lower (Figure 5.3.), than that of the free-living counterparts, indicating that the metabolic efficiency of attached cells may be greater than that of free-living cells. Some studies have shown that attachment can increase the metabolic efficiency of bacteria. For example, the molar growth yield for *Escherichia coli* adsorbed onto an anion exchange resin, was higher than that of equivalent free-living cells (Hattori and Hattori, 1981). In a number of other studies with the same organism and substratum, adsorbed bacteria were shown to exhibit lower oxidations of substrate but higher growth rates (Hattori and Hattori, 1976). However, attachment to a surface that reduces microbial assimilatory and respiratory activity can reduce the metabolic efficiency of attached cells as compared with their free-living counterparts (Gordon *et al.*, 1983).

In this investigation assimilation and respiration of ^{14}C-amino acids by bacteria attached to a range of substrata was measured to determine the effect of attachment on the efficiency of substrate utilisation. The metabolic efficiency was calculated as a carbon assimilation quotient (CAQ), i.e. the amount of carbon assimilated divided by the total carbon uptake, expressed as a percentage.

6.1.2. Aims

The objectives of this evaluation of bacterial assimilation and respiration of amino acids were to:

1. Determine whether assimilation or respiration of ^{14}C-amino acids by attached bacteria differed from that of detached or free-living bacteria.
2. Determine the relationship between substratum θ_A and assimilation and respiration of ^{14}C-amino acids by attached bacteria.
3. Determine whether the CAQ by surface-associated cells differed from that of free-living cells.

6.2. MATERIALS AND METHODS

6.2.1. Organism and growth conditions

The organism was cultured, harvested, washed and resuspended to $\cong 2 \times 10^8$ cells ml^{-1}, as described in section 4.2.1.2.

6.2.2. Attachment of bacteria to substrata

The substrata were T, PVDF, PE and PTFE disks prepared as described in section 3.2.1. Six replicate samples of material were used in each experiment, three each for activity measurements and cell counts. Cells were allowed to attach for 2h at 15° and washed as described in section 4.2.2.2. The six replicate surfaces, with washed attached cells, were then placed in new sterile silicone rubber rings and transferred to 40 ml of incubation solution (section 6.2.3.), contained in a 100 ml conical flask and sealed with an air-tight rubber seal.

6.2.3. Incubation of attached and free-living bacteria with ^{14}C-amino acids

Attached and free-living bacteria were incubated for 2h in an orbital shaking (75 rpm) incubator at 15°C in the appropriate medium (see below). During incubation some of the initially attached bacteria desorbed from the substrata and these were collected by filtration (section 6.2.4.). These comprised the detached population, whereas attached and detached bacteria comprised collectively the surface-associated population. To prepare suspensions of free-living bacteria of similar numbers to the attached populations, 1 ml portions of the washed cell suspension were placed in 40 ml of a corresponding incubation solution and incubated in the same way as the attached populations.

The media comprised minimal media supplemented for the evaluation of uptake kinetics with either 10 μg C l^{-1} of L-[U-^{14}C] leucine (50 μCi ml^{-1}, 340 mCi $mmol^{-1}$) or 10 μg C l^{-1} L[U-^{14}C] leucine with 90, 490 and 990 μg C l^{-1} unlabelled leucine carrier giving total concentrations of 10, 100, 500 and 1000 μg C l^{-1}. The carbon sources used to evaluate assimilation and respiration of different amino acids were 10 μg C l^{-1} each of L[U-^{14}C] leucine (50 μCi ml^{-1}, 340 mCi $mmol^{-1}$), [1-^{14}C] glycine (200 μCi ml^{-1}, 53.4 μCi $mmol^{-1}$), L-[U-^{14}C] glutamic acid (50 μCi ml^{-1}, 282 mCi $mmol^{-1}$) or L-[U-^{14}C] arginine monohydrochloride (50 μCi ml^{-1}, 345 mCi $mmol^{-1}$) (all supplied by Radiochemical Centre, Amersham). All incubation solutions were filter sterilised.

6.2.4. Evaluation of respiration and assimilation

At the end of the incubation period, the attached, detached and free-living bacteria were fixed and $^{14}CO_2$ released by acidification of the incubation solution to pH 1.9 with 0.3 ml 1M H_2SO_4 injected through the rubber seal. $^{14}CO_2$ was absorbed by 2-phenylethylamine (Fisons, Loughborough) which had been injected onto a GF/G glass fibre wick in a well in the centre of the flask immediately before acidification. A further 2h incubation period at 15°C with shaking was allowed for $^{14}CO_2$ absorption. Similar experiments, but without cells and substituting NaH $^{14}CO_3$ for the ^{14}C-substrates, indicated that ≅ 100% of the $^{14}CO_2$ was absorbed by the wicks after 2h. To detect non-biological $^{14}CO_2$ release, ^{14}C-substrate adsorption onto substrata and sterility of solutions, control surfaces without cells were incubated with ^{14}C-substrates as were the surfaces with attached bacteria. Wicks with absorbed $^{14}CO_2$ were placed into vials containing 10 ml Filter-solv TM scintillant (Beckman, High Wycombe).

To measure assimilation of substrates, three of the six replicate attached, detached and free-living samples from each incubated solution were prepared as follows. Surfaces with attached cells were placed into vials containing 1 ml Lumasolve TM (LKB, Croydon) and 200 µl sterile distilled water and incubated for 12h at 50°C to digest the cells before adding 10 ml Lipoluma TM scintillant (LKB) and counting. Attached bacteria were incubated with Lumasolve to remove the cells from the surfaces. This prevented spuriously low scintillation counts caused by the absorption of β-emissions by the various surfaces (Pringle and Fletcher, 1983). To collect free-living and detached cells, respectively, six 5 ml portions from the incubation solutions *(i)* containing free-living cells, or *(ii)* which had contained the substrata, were filtered (polycarbonate filters, 0.2 µm porosity), washed by passing through two 7 ml portions of filter sterile ASW and treated as were the attached cells.

Samples of attached, free-living and detached cells were counted in wide neck polyethylene vials (Beckman Instruments Inc., Irvine, California, U.S.A.) on a Beckman Instruments Liquid Scintillation System LS-7000 to ± 2 sigma counting error, or for 20 min. The counting efficiency of each sample was determined from the H-number, a measure of the shift in the 'Compton edge' produced by sample quenching when exposed to gamma radiation from ^{137}caesium (Horrocks, 1977).

6.2.5. Evaluation of cell numbers

The attached populations were prepared for microscopy by fixing the surfaces (three remaining replicates for each incubation solution) onto glass microscope slides with double-sided adhesive tape. 5 ml portions of suspensions of free-living and detached populations (three replicates from each incubation solution) were filtered onto black polycarbonate filters (0.2 µm porosity), washed by passing through two 7 ml portions of ASW and mounted onto glass slides as described in section 4.2.4. Mounted samples were stained with acridine orange and microscopically examined as described in section 5.2.4. The mean number of cells per 100 μm^2 observed by epifluorescence (in 100x100 μm^2 areas or until 200 bacteria were counted for each slide) was calculated for three replicate samples of attached, detached and free-living cells from each incubation solution. The total number of cells in each incubation solution was calculated for the total substratum surface area (attached cells) or volume (detached and free-living cells).

6.2.6. Calculation of uptake Kinetics

The velocity of assimilation and respiration was calculated as described by Wright and Hobbie (1966), with allowances made for unlabelled substrate carrier, non-biological $^{14}CO_2$ release and ^{14}C-substrate adsorption onto the surfaces. The velocity of assimilation of attached, detached and free-living cells was divided by the number of cells in each population to obtain the mean velocity of assimilation per cell. The total velocity of uptake per cell was calculated by dividing the sum of the velocities of assimilation and respiration by the total number of attached and detached (surface-associated) or free-living cells in the sample. Vmax per cell and K were obtained from a modified Lineweaver-Burke plot of total uptake per cell (y-axis: substrate concentration/uptake velocity, x-axis: substrate concentration) (Wright and Hobbie, 1966). The utilisation of amino acids by surface-associated and free-living cells was compared by calculating a carbon assimilation quotient (CAQ), i.e. the amount of carbon assimilated divided by the total carbon uptake, expressed as a percentage.

6.3. RESULTS

6.3.1. The relationship between bacterial assimilation or respiration of leucine by surface-associated and free-living cells and substratum contact angles

The rates of assimilation by surface-associated cells were generally independent of substratum θ_A, except that assimilation tended to increase somewhat with increase in θ_A at the two highest leucine concentrations (Figure 6.1.). Also at these concentrations the rates of assimilation of surface-associated cells were generally higher than those of free-living cells (Figure 6.1.).

It was impossible to measure respiration of attached and detached cells separately, as CO_2 from both populations was collected on the same wick. Thus all respiration measurements deal with surface-associated (attached and detached) or free-living bacteria. The mean velocity of leucine respiration by surface-associated bacteria did not differ greatly with substratum θ_A (Figure 6.2.). Also respiration of surface-associated bacteria was similar to, or lower than, that of corresponding free-living cells (Figure 6.2.).

The velocity of total leucine uptake (assimilated plus respired substrate) did not vary markedly with substratum θ_A (Figure 6.3.). An exception was cells associated with PTFE and incubated with 500 or 1000 µg C l^{-1} leucine. These generally had higher uptake levels than corresponding bacteria associated with all other surfaces and corresponding free-living cells. The mean total uptake by the other surface-associated populations was similar to that of free-living bacteria (Figure 6.3.)

6.3.2. The relationship between CAQ's of bacteria incubated with leucine and substratum contact angles

With most surfaces, substratum θ_A had little effect on CAQ. However, at 500 or 1000 µg C l^{-1}, the CAQ's for cells associated with PTFE were considerably higher than those for cells associated with the other surfaces (Figure 6.5.). The mean CAQ's of surface-associated bacteria were generally considerably higher than those of free-living cells (Figure 6.5.)

6.3.3. The relationship between uptake kinetics of leucine and substratum contact angle

The total velocity of uptake of leucine per cell over the range 10-500 μg C l^{-1} leucine indicated saturation kinetics, and when transformed to a modified Lineweaver-Burke plot, showed a high degree of linearity (Figure 6.4., Table 6.1.). Bacteria associated with PTFE had K and Vmax values two to four times greater than the equivalent values for the other surface-associated bacteria or free-living cells. Free-living cells had a smaller K than any of the surface-associated populations but a greater Vmax than the cells associated with all but the PTFE substratum.

6.3.4. The relationship between velocity of bacterial assimilation or respiration of leucine, glycine, glutamate and arginine and substratum contact angle

There was no general relationship between amino acid utilisation and substratum θ_A for the four amino acids (Figure 6.6.). The rate of assimilation by attached bacteria was similar to that of detached or free-living cells, except bacteria attached to PTFE (leucine, glutamate, arginine) or PE (glutamate, arginine) had higher assimilation rates. The substratum θ_A had little effect on respiration of surface-associated bacteria, except for cells associated with PE and incubated with glutamate and cells associated with PTFE and incubated with either leucine or glutamate. Respiration of free-living bacteria was when incubated with glycine, higher than, or was similar to that of surface-associated cells. Total uptake of the different amino acids was not greatly affected by substratum θ_A, except with glutamate, and much smaller differences occurred with leucine and arginine. Total uptake of leucine, glutamate and arginine from 10 μg C l^{-1} solutions by free-living cells was higher than that by surface-associated cells with the exception of cells associated with PTFE and incubated with arginine (Figure 6.6.). Free-living and surface-associated cells incubated with glycine had similar total uptake rates.

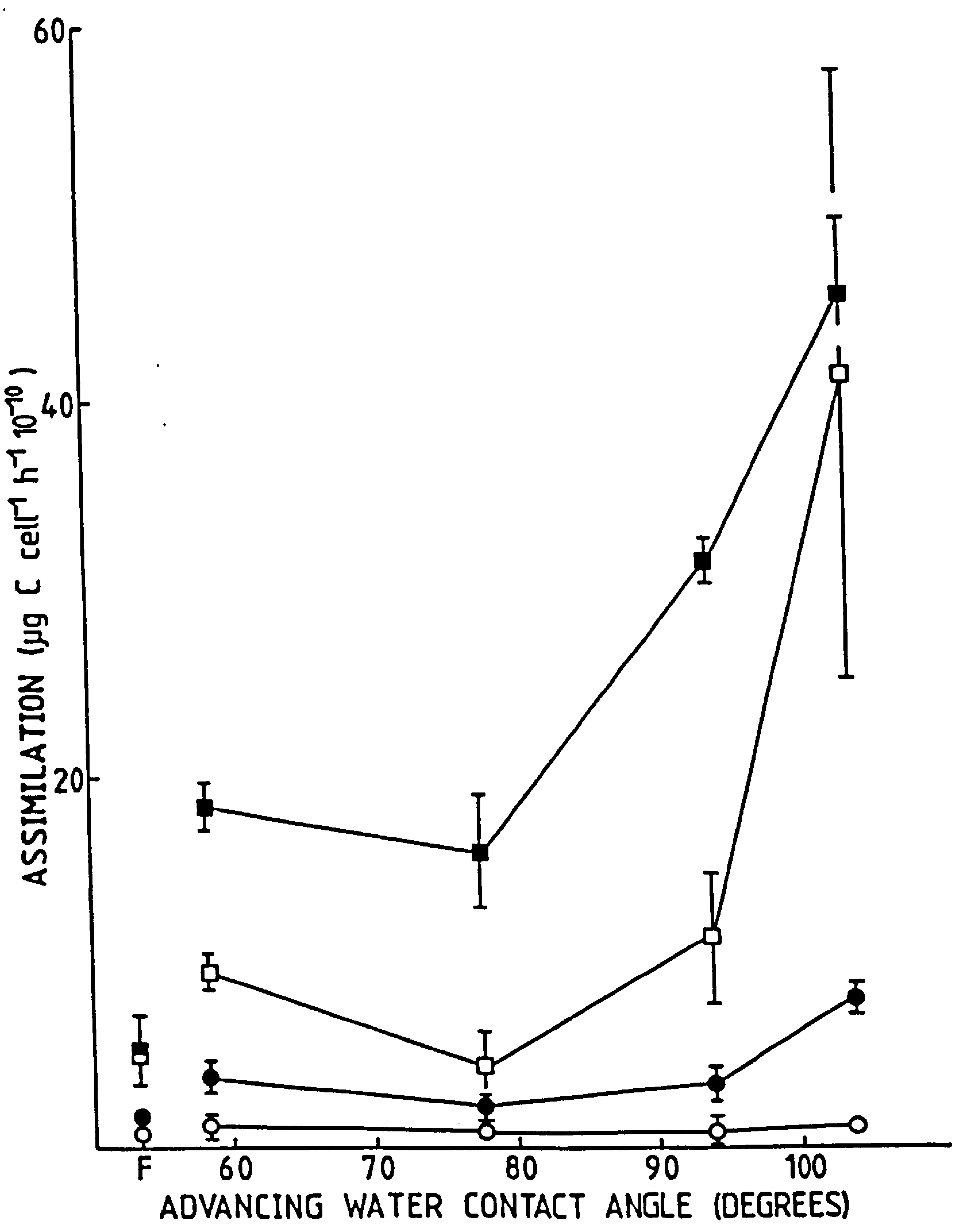


FIGURE 6.1.

The relationship between velocity of leucine assimilation by surface-associated bacteria and substratum θ_A. F is assimilation by free-living bacteria. Leucine concentrations are 10 (○), 100 (●), 500 (□) and 1000 (■) µg C l^{-1}. The substrata and θ_A were T 59°, PVDF 77°, PE 94° and PTFE 104°. Datum points are means of two experiments each with triplicate samples. Error bars represent ± S.E.M.

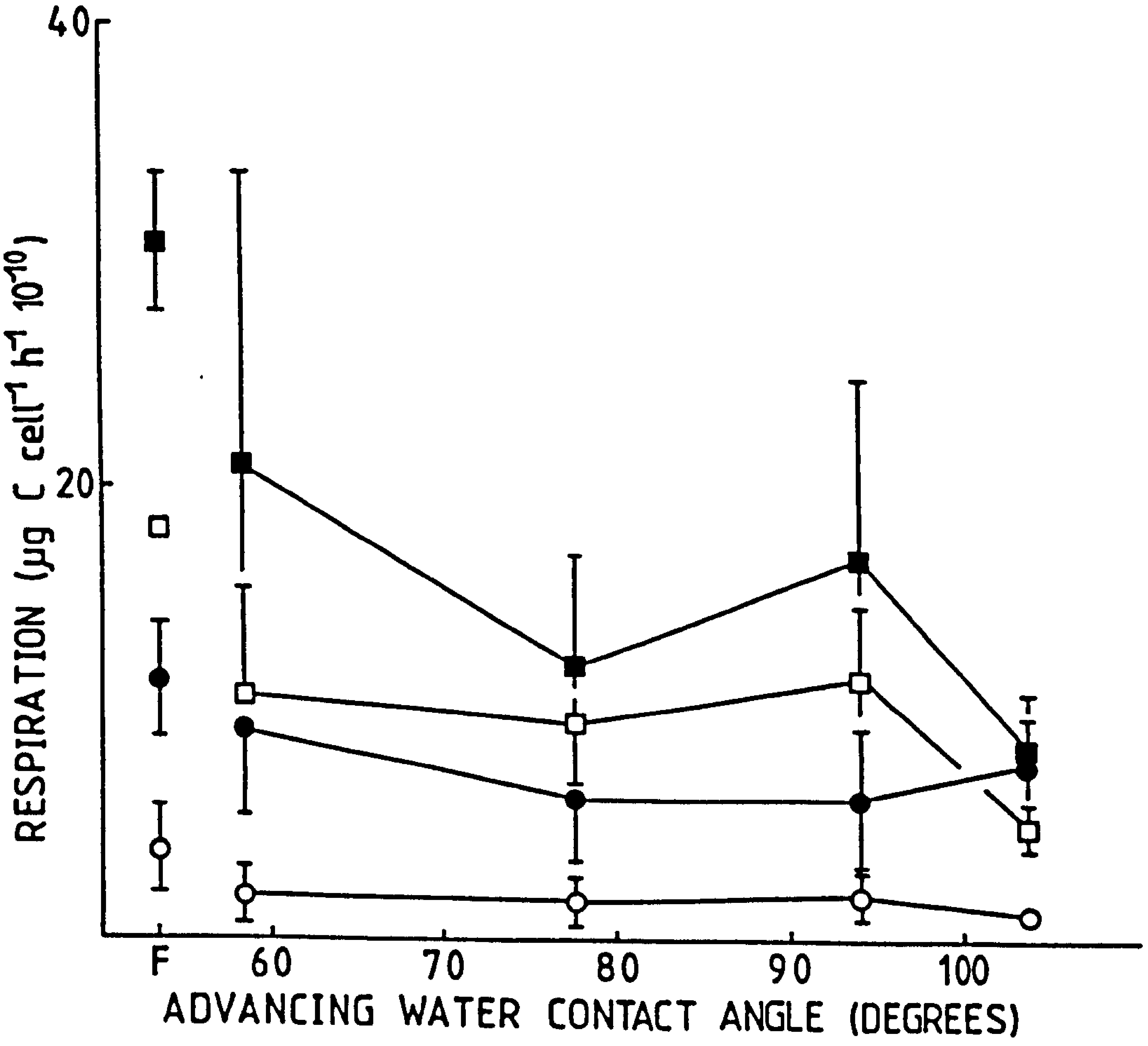


FIGURE 6.2.

The relationship between respiration of leucine by surface-associated bacteria and substratum θ_A. F is respiration by free-living bacteria. Leucine concentrations are 10 (○), 100 (●), 500 (□) and 1000 (■) μg C l^{-1}. The substrata are the same as in Figure 6.1. Datum points are means of two experiments each with triplicate samples. Error bars represent ± S.E.M.

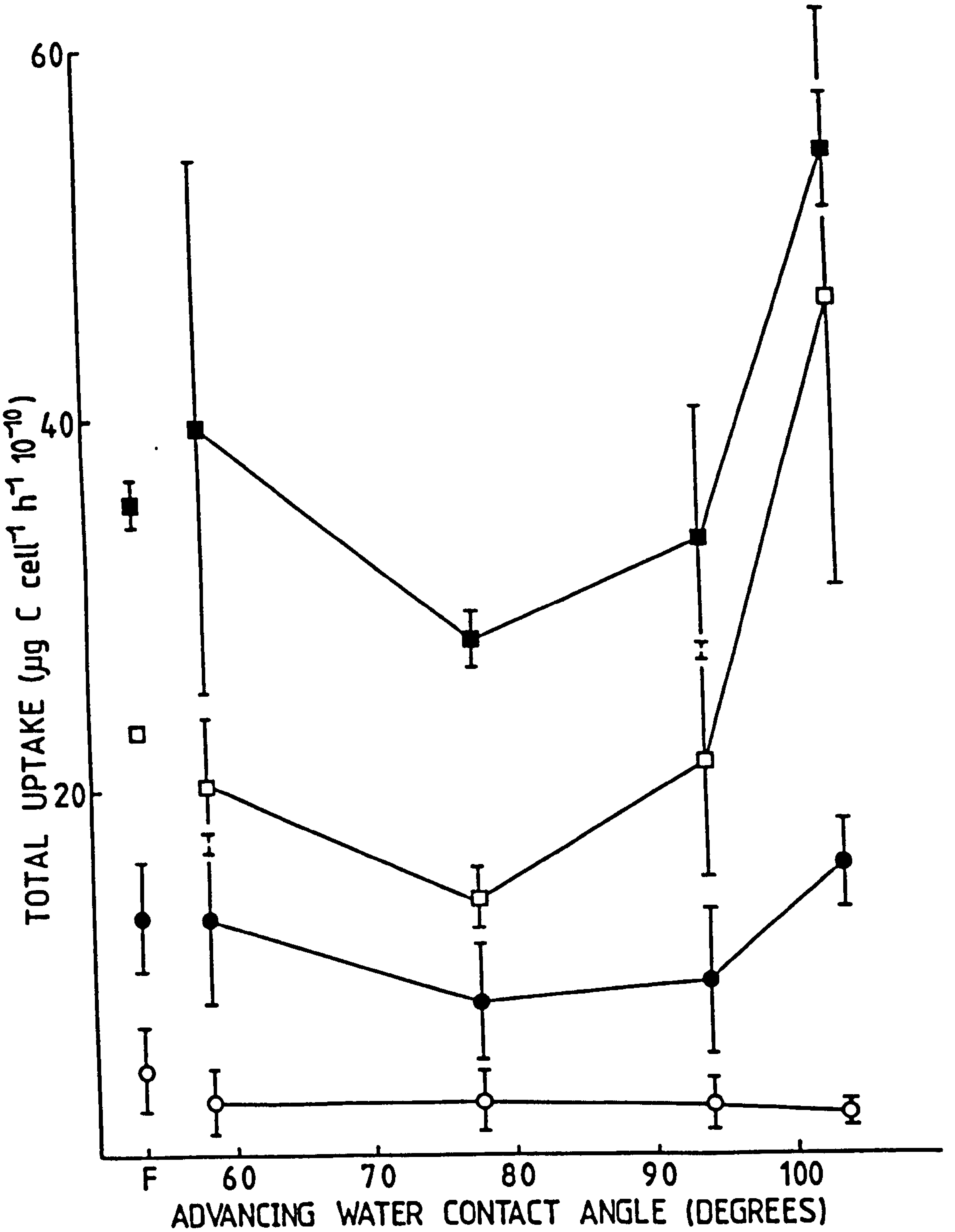


FIGURE 6.3.

The relationship between total uptake (assimilation and respiration) of leucine by surface-associated bacteria and substratum θ_A. F is total uptake by free-living bacteria. Leucine concentrations are 10 (○), 100 (●), 500 (□) and 1000 (■) µg C l^{-1}. The substrata are the same as in Figure 6.1. Datum points are means of two experiments each with triplicate samples. Error bars represent ± S.E.M.

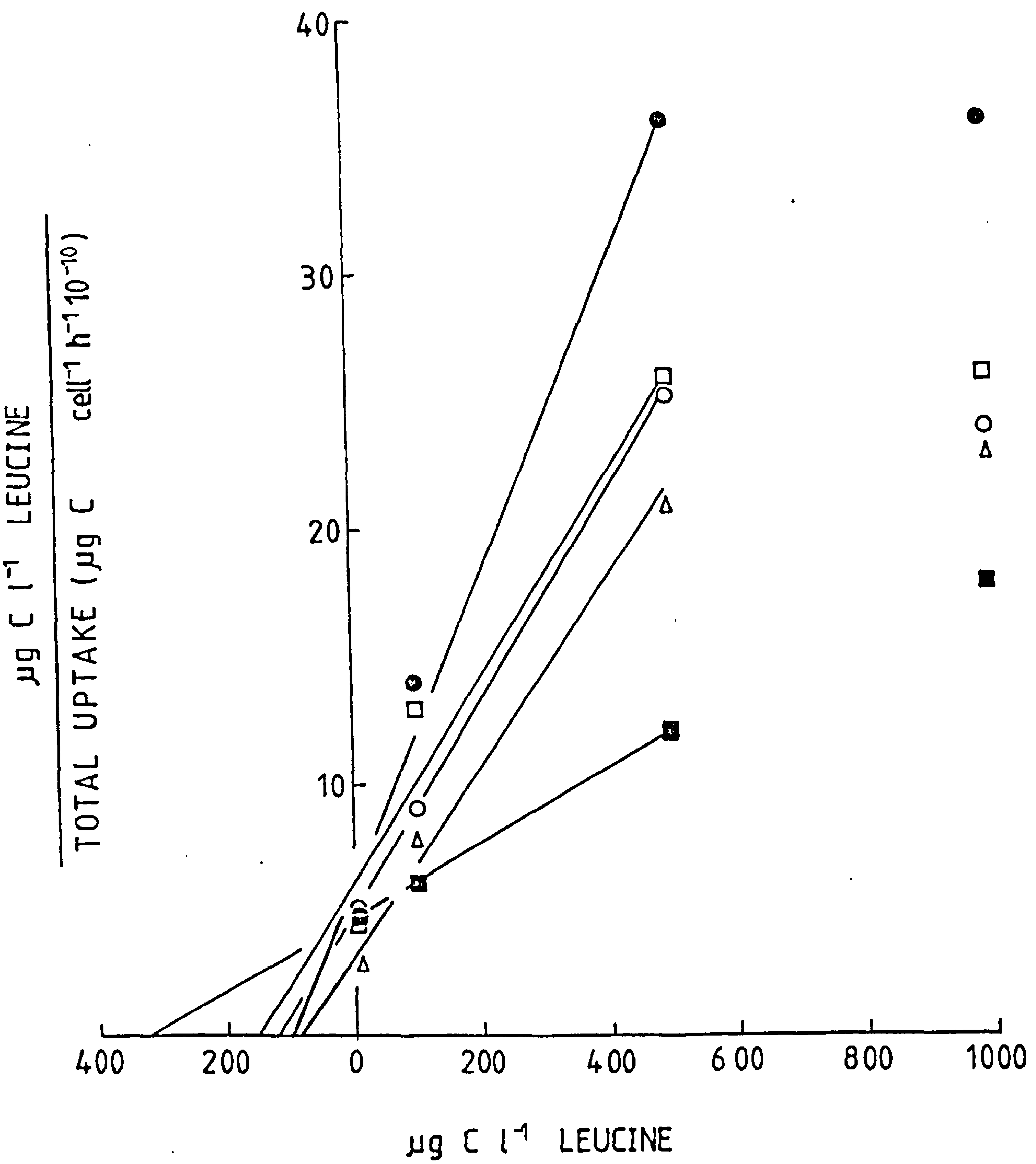


FIGURE 6.4.

A modified Lineweaver-Burk plot of leucine uptake kinetics by bacteria associated with the substrata T(○), PVDF(●), PE(□) or PTFE(■), and free-living bacteria F(Δ). Datum points are means of two experiments each with triplicate samples.

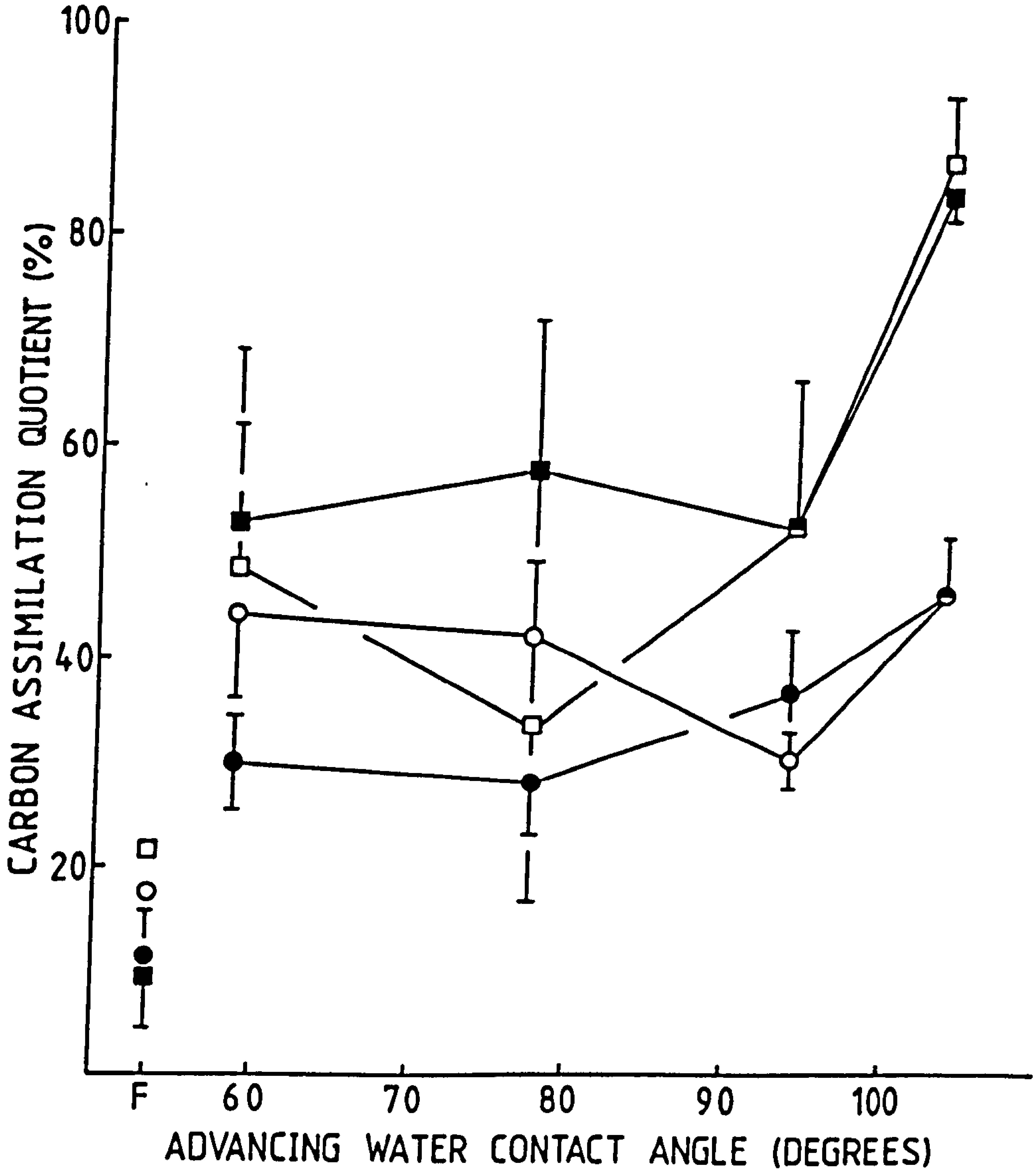


FIGURE 6.5.

The relationship between carbon assimilation quotient (CAQ) of surface-associated bacteria and substratum θ_A. F is CAQ for free-living bacteria. Leucine concentrations are 10 (○), 100 (●), 500 (□) and 1000 (■) µg C l^{-1}. The substrata are the same as in Figure 6.1. Datum points are means of two experiments each with triplicate samples. Error bars represent ± S.E.M.

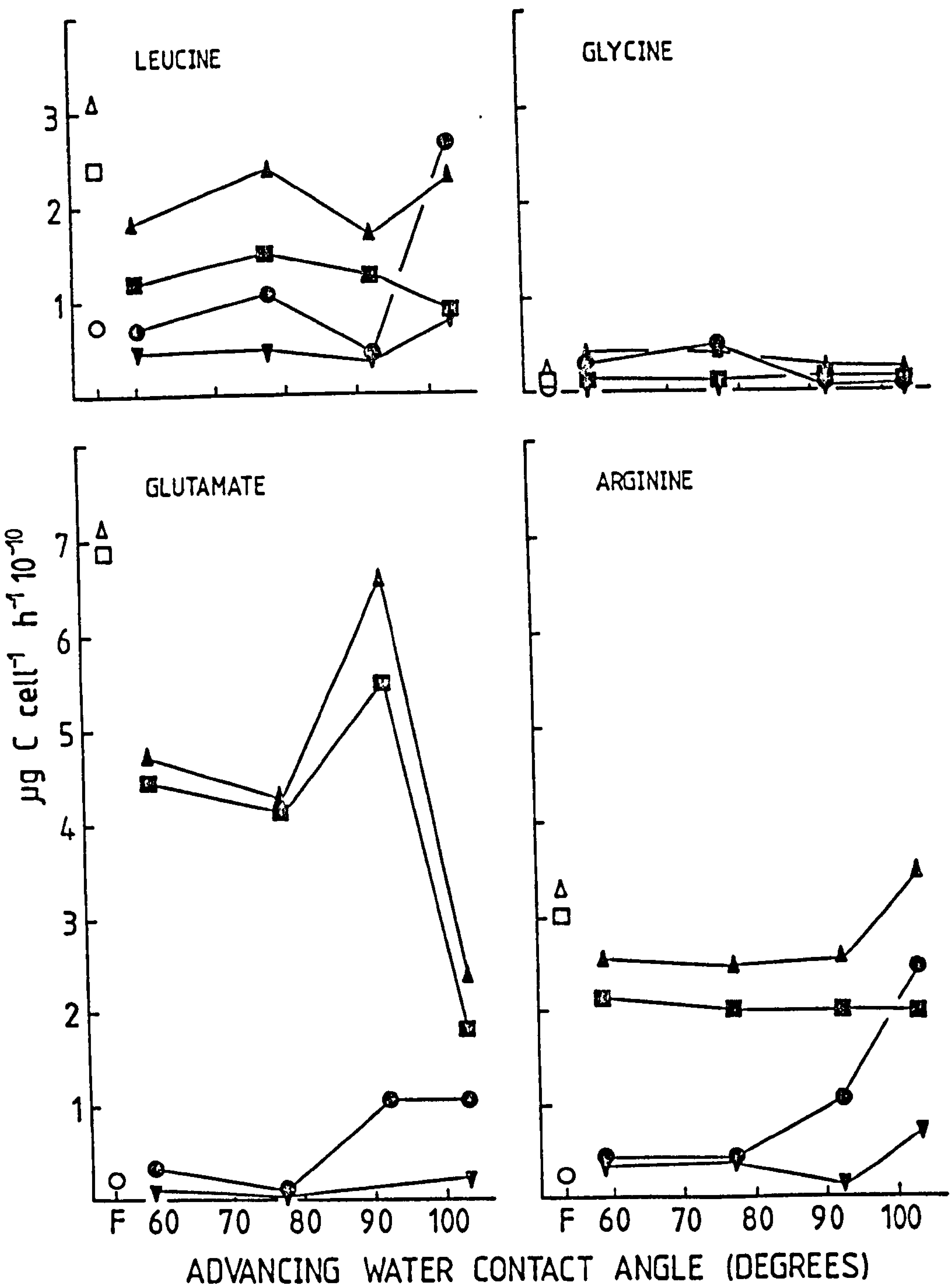


FIGURE 6.6

The relationship between amino acid assimilation by attached (●), and detached (▼) bacteria, amino acid respiration by surface-associated bacteria (■), or total amino acid uptake by surface-associated bacteria (▲) and substratum contact angle. F is assimilation (○), respiration (□) and total uptake (Δ) by free-living bacteria. Bacteria were incubated with 10 μg C ℓ^{-1} of ^{14}C-labelled amino acids. The substrata are the same as in Figure 6.1. Datum points are means of triplicate samples from one experiment.

Table 6.1.

Data derived from uptake kinetics[a]

Population	Surface	K		Vmax $cell^{-1}h^{-1}10^{-10}$		r^b
		$\mu g\ C\ l^{-1}$	μM	μg C	μM	
Surface-associated	T	120	1.67	24	0.33	0.918**
"	PVDF	95	1.32	16	0.22	0.945**
"	PE	155	2.15	25	0.35	0.822*
"	PTFE	307	4.26	66	0.92	0.790
Free-living		86	1.19	27	0.38	0.971**

[a] Uptake Kinetics calculated from modified Lineweaver-Burk plots of total uptake $cell^{-1}$ at 10, 100, and 500 μg C l^{-1} leucine for two experiments.

[b] Correlation coefficient (** $P<0.01$, * $P<0.05$)

The CAQ's of surface-associated bacteria (Figure 6.7.) varied with the substratum θ_A and amino acid and followed a similar pattern to total uptake, except with glycine. The CAQ's for surface-associated cells were greater than, or less often similar to (cells associated with PE and incubated with leucine and cells associated with T or PVDF and incubated with glutamate), that for free-living cells.

6.4. DISCUSSION

In this investigation, assimilation and respiration of ^{14}C-amino acids was measured to determine the effects of substrata on uptake kinetics and efficiency of substrate utilisation. For comparison of the uptake kinetics of surface-associated and free-living cells, the velocity of assimilation, respiration and total uptake was calculated on a per cell basis over the substrate range 10 to 100 µg C l^{-1}. The observations using MAR and demonstrated that given sufficient substrate and time, all cells were capable of amino acid assimilation (Figure 4.1A.), although individual cell rates varied.

The total velocity of uptake per cell for both surface-associated and free-living bacteria, when incubated with 10-500 µg C l^{-1} leucine, showed saturation kinetics as demonstrated by the linearity of the data when transformed to a modified Lineweaver-Burke plot (Table 6.1., Figure 6.4.). However, with the possible exception of PTFE-associated cells, incubation with 1000 µg C l^{-1} leucine produced values for substrate concentration divided by total uptake (y-axis Figure 6.4.) smaller than would be expected from extrapolation of the modified Lineweaver-Burke transformed data obtained at the other substrate concentrations. This suggests that the total uptake per cell from 1000 µg C l^{-1} leucine solutions was greater than that which would be obtained by saturation kinetics. The relatively high uptake by cells incubated with 1000 µg C l^{-1} leucine may have been due to induction of uptake by existing, or new,uptake sites, or diffusion of substrate into these cells.

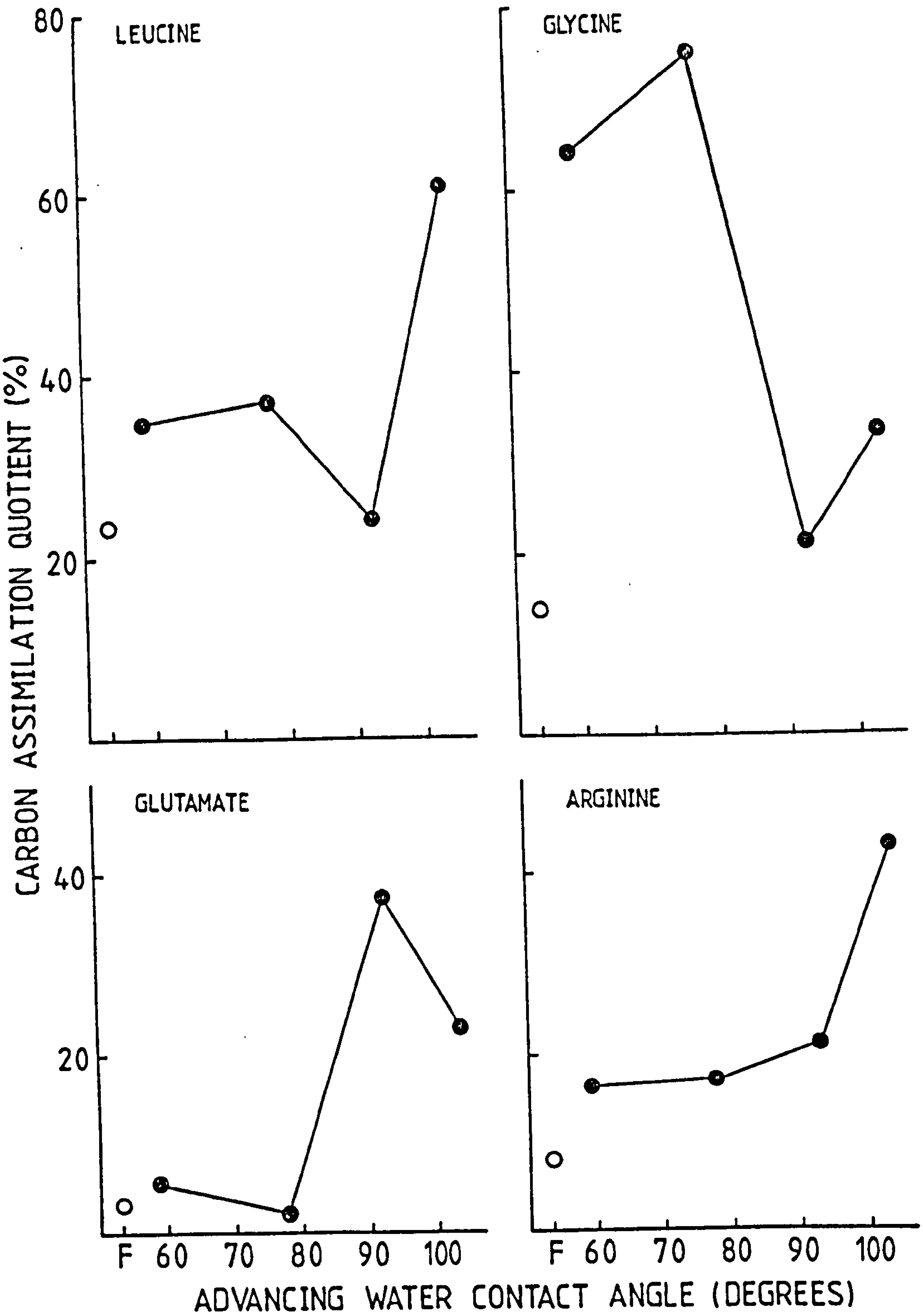


FIGURE 6.7

The relationship between carbon assimilation quotient (CAQ) of four amino acids by surface-associated bacteria and substratum θ_A. F is the CAQ for free-living bacteria. Bacteria were incubated with 10 μg C ℓ^{-1} of ^{14}C- labelled amino acids. The substrata are the same as in Figure 6.1. Datum points are means of triplicate samples from one experiment.

Values for Vmax $cell^{-1}$ and K obtained from the modified Lineweaver-Burke plots demonstrated that kinetic characteristics of substrate uptake by bacteria may be influenced by attachment and by the physiochemical properties of the substratum (Table 6.1.). Surface-associated bacteria had higher K values than free-living cells. These differences may have been real or apparent and not indicative of a real change in the cells affinity for substrate.

Higher apparent K values for surface-associated cells could have been brought about by lower substrate concentration and/or accessibility at the solid/liquid interface as compared with that in the bulk phase (section 1.3.2.1.*i*.). Such lower interfacial substrate accessibility to surface-associated cells could have been caused by *(i)* substrate/substratum interactions (section 1.3.2.3.) or *(ii)* diffusion limitation (section 1.3.2.2.*iii*.). The θ_B and MAR studies indicated that leucine was adsorbed from relatively concentrated leucine solutions (Table 3.3. and Figure 4.5.) onto at least the more hydrophilic substrata. This suggests that the true K value for surface associated cells would actually be greater than could be observed as the substrate concentration in the vicinity of surface-associated cells was greater than in bulk solution. However, adsorption of the substrate may have made it less accessible for uptake by attached cells (section 1.3.2.3.), thereby decreasing the effective concentration of substrate at the substratum surface and increasing the apparent K values of surface-associated cells.

With regard to *(ii)*, the concentration of substrate at the solid/liquid interface may have been lower than in bulk solution as a result of uptake of amino acids by attached cells producing a concentration gradient and diffusion limitation across the 10-100 μm thick stationary layer situated at the solid/liquid interface (section 1.3.2.2.*iii*.). That is, uptake of leucine by surface-associated cells may have been greater than diffusion to them, thereby decreasing the interfacial concentration of leucine and increasing the apparent K of surface-associated cells. Similar diffusion limitation explanations have been proposed for the comparatively high values of apparent K reported for adsorbed bacteria (Hattori and Hattori, 1981) and immobilised enzymes (Goldman *et al.*, 1971). The apparent K values for surface-associated cells may have been lower than the true value of K if the surfaces had adsorbed unknown contaminating substrate (Su), in which case the observed values of K would have been equivalent to K + Su.

The higher K values for surface-associated cells could have reflected a real difference in affinity for substrate if the substratum had influenced the uptake processes of the surface-associated bacteria. This influence could have been brought about by *(i)* the physico-chemical properties of the substratum on substrate transport, or *(ii)* the physical presence of the substratum on the effectiveness of some of the uptake sites of attached bacteria. One mechanism by which the substratum physico-chemical properties might have exerted an influence on active uptake is by affecting transmembrane proton motive force energy-coupled transport (Booth and Hamilton, 1980). The proton motive force is composed of a membrane potential and pH gradient, either of which might be influenced by the properties of an adjacent substratum. Moreover, any substratum effect on the membrane potential may have also altered the permeability of the porins that facilitate movement of small hydrophilic molecules across the membrane (Braun and Hartke, 1981).

The attachment of bacteria to a substratum, no matter what its physico-chemical properties, will decrease exposure of nearly half its surface area to direct exposure to the bulk solution. This may, therefore, reduce the effectiveness of some of the cells uptake sites which might affect V_{max}.

Vmax of bacteria associated with PTFE was considerably higher than that of other surface-associated or free-living cells. The reason for this is not clear, but at 500 and 1000 μg C l^{-1} leucine, PTFE-associated bacteria had comparatively higher assimilation rates with little increase respiration rate. It is possible, therefore, that the properties of PTFE in some way facilitated uptake of leucine without the need to use energy produced by respiration. For example, by enhancing the proton motive force across the cytoplasmic membrane (Konings and Veldkamp, 1980).

Although the total velocity of uptake of leucine by surface-associated and free-living cells was generally somewhat similar for each of the substrate concentrations, i.e. 10, 100, 500 and 1000 μg C l^{-1} (Figure 6.3.), surface-associated bacteria generally assimilated proportionally more (Figure 6.1.) and respired less (Figure 6.2.) than corresponding free-living cells, thus producing considerable differences in CAQ values (Figure 6.5.). Similarly, when incubated with low concentrations (10 μg C l^{-1}) of a range of amino acids, assimilation by attached cells was generally higher than, or similar to, that of free-living cells and respiration of attached cells was lower than, or, similar to free-living cells (Figure 6.6.). The extent of the differences in assimilation and respiration by attached and free-living cells was dependent upon the amino acids and substratum. As a result of these differences in assimilation and respiration, the CAQ's of surface-associated bacteria were also dependent upon substrate and substratum θ_A and were generally higher than the CAQ's of free-living cells (Figure 6.7.). These differences in CAQ may be due to one or more of the following:

(i) Conservation of energy by attached cells due to the substratum acting as a site for accumulation of protons and other positive ions which, according to chemiosmotic theory (Mitchell 1961, 1966), increases the cells energy level by favouring the electrochemical gradient of protons (proton motive force), across the cytoplasmic membrane.

(ii) As yet unexplained differences in the energy requirement for maintenance, biosynthesis or motility.

(iii) Selective attachment of the more metabolically efficient cells.

(iv) Adsorption of radiolabelled metabolites.

(v) Increased incorportion or entrapment of labelled material as a result of extracellular polymer production by attached bacteria.

It is not possible to evaluate the importance of *(i)* or *(ii)*; however, it is plausible that protons and other positive ions either produced by attached bacteria, or originating from the seawater, would associate with the negatively charged substrata thereby energetically benefiting the cell by increasing the electrochemical potential across the cell membrane (section 1.3.2.1.). With regard to *(iii)* the MAR study of amino acid assimilation indicated that the more actively assimilating bacteria of the free-living population preferentially attach (Figure 4.8.), after which there is a further selection of actively assimilating cells as a result of subsequent detachment of bacteria with a lower level of assimilation (Figures 4.4. and 4.6.). With respect to *(iv)*, the MAR study indicated that some metabolites adsorb onto the substrata (Figures 4.5. and 4.7.), but adsorption levels were low and not related to substratum θ_A in the same way as was CAQ in this study. Thus metabolite adsorption was probably not an important factor. Finally concerning *(v)*, extracellular polymeric adhesive is produced by surface-associated bacteria during the second stage of cell adsorption to bridge the bacterium and solid surfaces (Marshall *et al.*, 1971a). This may increase incorporation of labelled material directly, by biosynthesis into polymeric adhesive, or indirectly, by trapping labelled material.

It is likely that more than one of the above described factors contributed to the attached population having higher CAQ's. In sequence the following may have occurred:

(i) Selective attachment of the more actively assimilating cells from the free-living population, followed by further selection by detachment of the less active assimilatory cells.

(ii) Direct and indirect incorporation of labelled material in the extracellular polymer of attached cells.

(iii) Facilitated uptake of substrate as a result of the substratum-enhanced proton motive force across the cytoplasmic membrane.

6.4.1. Summary

The results of this study of assimilation and respiration of ^{14}C-amino acids indicate that:

1. The rates of assimilation by surface-associated cells were generally independent of substratum θ_A and were similar to the rates of free-living cells when incubated with 10 or 100 µg C l^{-1} leucine. When incubated with 500 or 1000 µg C l^{-1} the assimilation rates of surface-associated bacteria increased with increase in θ_A and were greater than for free-living cells.

2. The velocity of leucine respiration by surface-associated cells did not differ greatly with substratum θ_A and was similar to, or lower than, that of corresponding free-living cells.

3. The total velocity of leucine uptake by surface-associated cells did not vary greatly with θ_A and was similar to that of corresponding free-living cells. An exception was cells associated with PTFE and incubated with 500 or 1000 µg C l^{-1} leucine, which generally had higher uptake levels than the other surface-associated cells and free-living cells.

4. Free-living cells had a smaller K than any of the surface-associated populations but a somewhat greater V_{max} than cells associated with all but the PTFE surface.

5. Bacteria associated with PTFE had K and Vmax values two to four times greater than the equivalent values for the other surface-associated bacteria or free-living cells.

6. The CAQ's of surface-associated bacteria were generally considerably higher than for those for free-living cells.

7. General Discussion

7.1. COMPARISON OF RESULTS FROM THE ACTIVITY STUDIES

In general the different methods employed to measure assimilatory and respiratory activity gave fairly consistant results for the relative activities of attached, free-living and detached populations. For example, with both MAR and ^{14}C- uptake studies, when incubated with amino acids at a concentration of 10 µg C l^{-1} the proportion of attached bacteria that assimilated amino acids was greater than, or similar to, that of free-living bacteria, which in turn had greater, or similar, activities than detached bacteria (Figures 4.6. and 6.6.). Similarly, when incubated with 10 µg C l^{-1} leucine both the proportion of attached bacteria that demonstrated ETS activity and the rate of $^{14}CO_2$ respired per surface-associated cell was generally less than that by free-living cells (Figures 5.3. and 6.6.). However, some of the populations of bacteria did appear to demonstrate different activities when examined by different methods, particularly when exposed to comparatively high substrate concentrations. For example, when attached bacteria were incubated with increasing concentrations of leucine the proportion of labelled bacteria on the hydrophilic substrata increased to a greater extent than those on the hydrophobic substrata (Figure 4.4.). However, the rate of assimilation per cell by bacteria on the hydrophobic substrata increased to a greater extent than on the hydrophilic substrata (Figure 6.1.).

It is likely that the differences in the results were due to the different rationales behind the two methods. MAR was essentially a qualitative evaluation of the proportion of bacteria in a population which had reached a detectable level of activity after a given time. In contrast liquid scintillation gave a quantitative measure of assimilation calculated as a rate per cell assuming all cells were active. Some discrepancies in the calculations of rates of assimilation per cell may have arisen from the assumption that all the cells were active although this appeared to be true at least in the presence of very high substrate concentrations (Figure 4.1A.).

It is also possible that some of the differences in assimilatory activity of some of the bacterial populations were due to differences in the isotopes used, their positions in the amino acid molecules and their metabolic rates. However, it is difficult to comprehend how these differences could account for the different responses to increased leucine concentrations by bacteria attached to hydrophilic and hydrophobic substrata.

7.2. SOME IMPLICATIONS OF THE RESULTS OF THIS INVESTIGATION

Observations of the activities of microorganisms from natural aquatic environments often attribute any enhanced activity by attached cells to adsorption of nutrients onto solid surfaces (section 1.1.). Although this investigation has demonstrated that adsorption of an amino acid can occur at least from fairly high concentrations onto moderately hydrophilic substrata (Table 3.3. and Figure 4.5.), it has also demonstrated that attachment of the more active cells of the free-living population and detachment of the less active cells of an attached population, may select an attached population with a higher activity than the free-living population.

Many observations of surface effects have shown that bacterial numbers increase in proportion to the available surface area (section 1.2.1.). However, few explanations have been proposed to account for the increase in the energy source which presumably allows the increase in bacterial numbers. Zobell (1943), suggested that the surface effect was due to more efficient use of extracellular enzymes to break down large adsorbed substrate molecules, and Kriss (1963) proposed that adsorption of large humic substances changes their conformation so that they become susceptable to microbial breakdown. These explanations do not, however, account for surfaces increasing bacterial numbers when small substrate molecules are present (Heukelekian and Heller, 1940). Ellwood *et al.*, (1982) recently speculated that solid surfaces may benefit bacteria energetically by helping to maintain the cells proton gradient, thereby benefiting chemiosmotic process of the cell. However, no direct evidence is available to support this hypothesis. The observations of this investigation provide evidence to support the proposal that surfaces increase the number of bacteria by supporting an attached population of cells that make more efficient use of the available substrate, as measured by CAQ's, than free-living cells. The number of bacteria that a body of water could support therefore increases directly in relationship to the solid surface to volume ratio, because there is a related increase in the size of the attached population, and consequently an increase in the efficiency of substrate utilisation by the total population, and ultimately a proportional increase in the number of bacteria that the substrate can support.

7.3. CONCLUSIONS

The results of this investigation of the physiological activity of attached bacteria suggest that:

1. The assimilatory activity of attached bacteria in the presence of low amino acid concentrations (10 μg C l^{-1}) was generally greater than that of free-living or detached bacteria. This was supported by the observations that:

   a. Attached bacteria had a higher proportion of cells demonstrating amino acid assimilation than did free-living or detached bacteria.

   b. Assimilation rates per attached cell was greater than that for free-living and detached cells.

2. The respiratory activity of attached bacteria in the presence of low amino acid concentrations (10 μg C l^{-1}) was generally lower than that of free-living bacteria or detached bacteria, as demonstrated by:

   a. A lower proportion of attached bacteria demonstrating ETS activity as compared with free-living or detached bacteria.

b. Lower rates of CO_2 evolution per cell for surface-associated bacteria as compared with free-living bacteria.

3. The physiological activity of attached bacteria was apparently more efficient than that of free-living bacteria. This was supported by the observations that:

a. Attached bacteria had a higher proportion of cells demonstrating amino acid assimilation and a lower proportion of cells demonstrating ETS activity than did free-living cells.

b. The ratio of amino acid-carbon assimilation to amino acid-carbon respired was greater for attached bacteria than for free-living bacteria.

4. The activity of attached bacteria may have been influenced by substrate adsorption.

This was supported by the observations that at comparatively high leucine concentrations:

a. The proportion of bacteria that demonstrated assimilatory activity and ETS activity was greater for bacteria on the more hydrophilic substrata.

b. Bubble contact angles for substrata indicated that leucine was adsorbed to a greater extent on hydrophilic substrata.

c. MAR indicated greater adsorption of substrate and metabolites on hydrophilic substrata.

5. Attachment was influenced by bacterial activity.

This was supported by the observations that:

a. The assimilatory activity of free-living bacteria that subsequently attached was greater than that of bacteria that remained free-living.

b. The assimilatory activity of bacteria that subsequently detached was generally lower than that of cells that remained attached.

c. The proportion of bacteria that assimilated substrate was positively correlated to the number of bacteria that attached to that substratum and was negatively correlated to the proportion of bacteria that detached from that substratum.

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