THE SURVIVAL OF BACTERIA CONTAINING R-PLASMIDS

A thesis submitted by

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TO MY PARENTS, WIFE, CHILDREN AND FAMILY

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The survival of bacteria containing R- plasmids

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The quantitative minimal requirements of Escherichia coli K12 W3110, with (R+) and without (R-) the R- plasmid RP1 were determined in anaerobic batch culture using a chemically defined medium (CDM). Under these condition the R+ strain had a greater requirement for K^+ than the R-, but a smaller requirement for SO_4^{2-} with other requirements being similar. In general, the nutritional requirements under anaerobic conditions for both strains were quantitatively greater than those under aerobic conditions.

The ability of the R- and R+ strains to survive in competition with each other, at $D \simeq 0.1\ h^{-1}$ was examined in the chemostat. When R- cells were added to R+ cultures (in a ratio of 1:10), the R- strain took over under glucose (G-lim) and phosphate (P-lim) limited conditions. In the reverse experiments, the level of the R+ strain fluctuated about the original inoculum level. The R+ strain did not disappear completely in these mixed culture experiments. There was evidence that its persistence was possibly due to preferential attachment to the vessel walls. In K^+-limited conditions (K^+-lim), the R+ strain took over at low K^+ concentrations, but not when the K^+ concentration was increased. Under the same condition as used for competition experiments, the apparent K_S values of G-lim and P-lim R- cultures were lower than those of R+ ones. With K^+-lim cells. R+ cultures had lower K_S values than R- at low K^+ concentrations, but similar values at higher concentrations. However, in all limitations the apparent K_S value increased as the concentration of the growth limiting nutrient in the inflowing medium increased. In K^+-lim experiments R- and R+ small colony variants were detected on NA plates. These had similar biochemical properties to those of the parent cultures. The experimental conditions did not affect the stability of the plasmid.

The effect of various growth environments on the cell envelope was investigated. Aerobic cultures had higher 2-keto-3 deoxyoctonic acid contents, with wider variation between depletions and R- and R+ strains, than had the anaerobic cultures. A protein with an apparent M_r of 34,000 present under most growth conditions was absent from the R- strain grown aerobically (K^+-lim) and all iron-depleted cultures.

The R- plasmid decreased the resistance of E. coli to drying, but had no effect on the sensitivity of cells to Tween 80 or to sodium deoxycholate. Sensitivity to the latter was increased by anaerobiosis. Sensitivity to NaCl depended upon the depleting nutrient.

The relevance of anaerobic competition and survival under adverse conditions to persistence of R- plasmids in the wild is discussed.

Key words: Escherichia coli; R- plasmid; nutrient limitation; cell envelope; survival.

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Origin and Scope of the work

A considerable amount of work has been done concerning the aerobic growth and survival of micro-organisms in normal and adverse conditions. However, little is known about the effect of R- plasmids on the persistance of the host bacteria in the gut and in the environment which constitutes a major problem in relation to antibiotic therapy. Factors affecting this will be analysed, both in batch and chemostat cultures.

SYMBOLS AND ABBREVIATIONS

AMPS	Ammonium persulphate
ATP	Adenosintriphosphate
CDM	Chemically defined medium
CFA	Colonization factor antigens
CM	Cytoplasmic membrane
D	Dilution rate
D_c	Critical dilution rate
DDW	Double distilled water
DOC	Deoxycholate
DPG	Diphosphatidylglycerol
ECA	Enterobacterial common antigen
EDTA	Ethylene diamine tetra acetic acid
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
f	Flow rate
FA	Fatty acid
g	Grame
G-lim	Glucose-limited
h^{-1}	Per hour
Inc	Incompatibility
K^+	Potassium
KDO	2-keto-3-deoxyoctonic acid
K^+-lim	Potassium-limited
K_s	Saturation constant
L	Litre
L_n	Logarithms to the base e (natural logarithms)
log	Logarithms to the base 10
LPS	Lipopolysaccharide

LT	Heat-labile
µg	Microgramme
µl	Microlitre
µ	Micron
mg	Milligramme
ml	Millilitre
mM	Millimole
MIC	Minimum Inhibitory Concentration
Min	Minutes
M	Moles per litre
M_r	Molecular weight
MOPS	3-N-morpholinopropane sulphonic acid
μ	Growth rate
μ_m	Maximum growth rate
nm	Nanometre
NA	Nutrient agar
NB	Nutrient broth
OD_{470}	Optical density read at 470nm wavelength
OM	Outer membrane
OMP	Outer membrane protein
ON	Overnight
P	Protein
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PAL	Peptidoglycan-associated lipoproteins
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PG	Phosphatidyl glycerol
PL	Phospholipid

P-lim	Phosphate-limited
R-	Without plasmid
R+	With plasmid
REL	Readily extractable lipid
R-plasmids	Resistance-plasmids
RP1	Plasmid RP1 confers resistance to ampicillin, carbenicillin, kanamycin, neomycin and tetracycline
rpm	Revolutions per minute
S	Sulphur
SAM	S-adenosylmethionine
SCV^-	Small colony variant without R-plasmid RP1
SCV^+	Small colony variant with R-plasmid RP1
SDS	Sodium dodecyl sulphate
S_R	Concentration of limiting-nutrient in medium reservoir.
ST	Heat-stable
TEMED	$NNNN^1$ tetramethylethylene diamine
Tris	2-amino-2-(hydroxymethyl)-propane-1.3-diol.
VT	Vero toxin
WT	Wild type

1. INTRODUCTION

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1.1. Importance of R-Plasmid-Carrying Bacteria in Diseases

1.1.1. Introduction

Most clinically significant antibiotic resistance is determined by genes located on extrachromosomal DNA elements called plasmids (Falkow, 1975; Broda, 1979; Hardy, 1981). Different species of bacteria carry characteristic types of plasmids, some of which can mediate their own transfer by conjugation. This has contributed to the rapid spread of antibiotic resistance in large numbers of bacteria.

There has been much discussion about the threat to health posed by resistant bacteria such as coliform bacteria in the gut, on the one hand, and the part played by antibiotics in maintaining this reservoir of resistant organisms on the other hand (Smith and Armour, 1966; Richmond, 1972). There is much evidence that treatment of man and animals with many different antibiotics (particularly ampicillin and tetracycline) selects effectively for a predominantly resistant gut coliforms (Smith, 1967), but even the flora of those not receiving antibiotics usually contains a few resistant organisms (Datta, 1969; Lincoln et al, 1970). Resistant bacteria may even be found in individuals who have never been given antibiotics (Mare, 1968; Gardner et al, 1969). Lacey (1975) stated that the use of antibiotics has undoubtedly increased the number of plasmid-positive cells; he suggested that future chemotherapeutic strategy against Staph. aureus should be aimed at reducing the incidence of plasmid carriage in this organism, not only to retain the usefulness of a particular antibiotic, but to remove the organisms most valuable evolutionary weapon.

Antibiotic resistance is also very common in gut coliforms and it is likely that every one well, or ill, carries some R+ coliforms. Datta (1969) examined faecal specimens collected before admission from patients awaiting non-urgent surgery. Resistant strains were isolated from 52% of preadmission samples;

up to 60% of these were carrying R- factors. Faecal samples from healthy infants, who had not attended a hospital or received antibiotics, were collected and examined for the presence of antibiotic resistant coliforms. R- factor-containing strains were detected in 81% of the cases examined (Moorhouse, 1969). Linton et al (1972) examined the faecal coliforms in healthy adults and children in Bristol. None of these individuals had any history of antibiotic treatment or hospital attendance. About 73% of the children and 49% of adults carried resistant strains, and about half of these carried R- plasmids.

Very important from the clinical stand point is the frequency with which R- plasmids are passed from non-pathogenic to pathogenic bacteria. Such passage takes place most often in the intestinal tract (Smith, 1969; Smith, 1977; Richmond and Petrocheilou, 1978; Gyles et al, 1978; Datta et al, 1981). One such transfer may result in a sudden acquisition of resistance to one or more commonly used antibacterial agents. The spread of R- plasmids in the intestinal tract is by successive transfer. Although the frequency of transfer of R- plasmid is low (Wiedemann, 1972), antimicrobial agents exert a powerful selection force (Howe et al, 1976).

1.1.2. Prevalence of antibiotic-resistant bacteria causing epidemic diseases

The transfer of drug resistance by R- plasmids is of considerable clinical importance. An increasing number of infections due to resistant organisms are being reported from many areas in the world. During 1969 and 1970, a pandemic of bacillary dysentery caused by Shigella dysenteriae, occurred in Mexico and Central America. Many communities in seven countries had outbreaks of unusually severe dysentery with high morbidity and mortality. Over 500,000 clinical cases were reported in the countries involved. The hospital case-fatality rates were as high as 35% (Balows, 1977). In Guatemala alone, 12,500 people died in one year (Gangarosa et al, 1972). This pandemic

strain Shigella dysenteriae was resistant to chloramphenicol, tetracycline, streptomycin and sulfadiazine. In 1972 an extensive epidemic of typhoid fever swept through Mexico and eventually into the U.S., infecting 100,000 people and killing 14,000 (Eskridge, 1978). Clearly, the resultant disease was more severe, more protracted, with a higher rate of complications than is usually seen with typhoid fever (Gonzalez-Cortes et al, 1973; Olarte and Galindo, 1973). The epidemic Salmonella typhi had a resistance pattern identical to that of the pandemic strain of Shigella dysentriae. It was demonstrated by several investigators (Farrar and Eidson, 1971; Gangarosa et al, 1972) that the multiple drug resistance in both organisms was due to the presence of a transferable R- plasmid. The occurrence of these two epidemics, within a short period of time, in the same geographical region, and due to different organisms with identical, transferable antibiotic resistance patterns, led to the speculation that the same R- plasmid may have increased the virulence of these two enteric pathogens (Gangarosa et al, 1972). Several workers (Thorne and Farrar, 1973; Datta and Olarte, 1974) compared the genetic properties of R- plasmids isolated from strains of S. dysenteriae implicated in the 1969-1970 epidemic in Central America with those found in selected Salmonella typhi strains isolated in the 1972 Mexican outbreak. It was immediately apparent that these plasmids were significantly different. Subsequent compatibility tests revealed that the R- plasmid from the two epidemics belonged to different compatibility groups. Thus, Datta and Olarte (1974) found that 17 strains of Salmonella typhi originating in the Mexican epidemic carried R- pasmids of compatibility group H, where as all of the Shigella dysenteriae isolates examined harboured incompatibility group O plasmids. Clearly, therefore, the plasmids implicated in these geographically related epidemics of typhoid fever and bacillary dysentery were genetically distinct (Thorne and Farrar, 1973).

An example, in which R- plasmids caused a drastic alteration in chemotherapeutic strategy was the emergence of plasmid-borne ampicillin,

chloramphenicol and kanamycin resistance in Haemophilus influenzae (Dang et al, 1975; Elwell et al, 1975; van Klingeren et al, 1977). H. influenzae type b is responsible for a number of serious infections of children, particularly meningitis and epiglottitis. In a 1978 study of bacterial meningitis in the United States, it was reported that 18% of H. influenzae isolates, causing meningitis or bacteremia, were resistant to ampicillin due to an R- plasmid, compared to a 5% incidence in a similar survey in 1976-1977 (Center for Disease Control, 1979).

During 1976, strains of Neisseria gonorrhoeae harbouring B-lactamase-producing plasmids were detected (Elwell et al, 1977) and since their recognition, ampicillin-resistant gonococcal strains have been isolated in more than 15 countries; most cases have been epidemiologically linked with either the Far East or West Africa. Resistant isolates from each source harbour R- plasmids that are highly related yet physically distinguishable (Elwell et al, 1977; Roberts et al, 1977). The two gonococcal R- plasmids share significant (70-90%) DNA sequence homology with an ampicillin-resistant R- plasmid (RSF0885) isolated in 1974 from H. influenzae (Elwell et al, 1975; Duncan et al, 1978). These plasmid-containing strains of N. gonorrhoeae have been shown to be unstable in the absence of selective antibiotic pressure (Roberts et al, 1977). About 30% of all recent N. gonorrhoeae isolates in the Philippines and 16% in the Republic of Singapore were found to contain B-lactamase-producing plasmids (Center for Disease Control, 1979). One of the factors thought to contribute to the high prevalence of R+ strains of N. gonorrhoeae in these two particular areas is the widespread prophylactic use of oral penicillins, especially by prostitutes.

In addition, the discovery of R plasmids in Bacteroides that can be transfered to E. coli (Mancini and Behme, 1977; Guiney and Davis, 1978; Saunders, 1978) has potentially serious implications. The enormously large

population of obligate anaerobes in the colon constitutes an extensive pool of resistance plasmids capable of infecting more conventional pathogens such as Salmonella and Shigella. Moreover, the emergence of transferable drug resistance in Bacteroides fragilis will undoubtedly complicate the successful treatment of infections caused by this important anaerobic pathogen.

Another example of plasmid-mediated antibiotic resistance is that of trimethoprim. Gruinberg and Show (1976) reported that between the year 1971 and 1975 little change was found in the level of trimethoprim resistance among urinary tract isolates. A further survey during 1975 and 1977 (Amyes et al, 1978) disclosed a slight but significant increase in the incidence of that of trimethoprim resistance with, however, a very sharp increase in the proportion of this resistance attributable to transferable R- plasmids. To provide information and establish a basis for observing any future changes in the incidence and types of trimethoprim resistance encountered clinically, Towner et al (1980) carried out two six-month surveys of enterobacteria isolated from clinical specimens during 1978 and 1979. The study revealed that there was no overall increase in the incidence of trimethoprim resistance, but the proportion of resistance attributable to transferable R- plasmids almost trebled. These reported differences in the apparent prevalence and importance of R- plasmids may well be a consequence of the properties of individual plasmids present in the particular environment sampled.

Rudy and Murray (1984) stated that in 1980, as part of their study on the prophylaxis of travellers' diarrhoea in Mexico, students from the United States received a two-week course of oral trimethoprim, trimethoprim plus sulfamethoxazole or placebo. Stools from these students were tested before, during and after therapy, and the results revealed the development of high-level resistance to trimethoprim in most enteric strains isolated from antibiotic-treated students. This study was continued by Murray et al (1982) who found that coliform counts were not appreciably reduced by antibiotic

therapy. E. coli represented 96% of the resistant isolates. To eludicate the origin of resistance, mating studies were conducted (Murray and Rensimer, 1983) with a recipient strain of E. coli and trimethoprim-resistant faecal isolates of E. coli. 41 of the 100 resistant strains tested had transferable type resistance and 23 of the 41 transconjugants were resistant to trimethoprim, ampicillin and streptomycin and contained a plasmid with a molecular mass of 35 mega dalton. A recent investigation (Rudy and Murray, 1984) demonstrated that at least 60% of transferable trimethoprim-resistance plasmids studied were identical. The authors concluded that these results may explain in part the surprising degree of resistance encountered in trimethoprim consuming individuals in that region. This in turn shows the crucial role of the selective pressure of antibiotics on the evolution and persistence of R- plasmid carrying bacteria.

Plasmids have also been used to identify and characterize epidemic strains of a number of different bacteria that have been involved in outbreaks of both nosocomial and community-acquired disease (Courtney et al, 1980; Markowitz et al, 1983; Nolte et al, 1984; Ling and Chau, 1984). For example, plasmid analysis was used in the investigation of several outbreaks of nosocomial Legionnaires' disease. Brown et al (1982) were the first to study plasmids as epidemiologic markers in the investigation of nosocomial Legionnaires' disease. These authors found that a single 80-megadaltons plasmid was present in a majority (61%) of the environmental isolates that they had obtained. However, no plasmids were found in isolates obtained from 23 patients with Legionnaires' disease, which led the authors, who also used a serological subtyping scheme, to suggest that the most common plasmid-free environmental isolate was the infecting strain. Recently, a small outbreak of nosocomial Legionnaires' disease has been investigated (Nolte et al, 1984), in which plasmid finger printing of clinical and environmental strains were used in an attempt to identify the source of the infecting strain. Results of this analysis showed that the only source of the infecting strain was the hot-water tanks.

In conclusion, the probelm of bacterial drug resistance became apparent soon after the introduction of antibacterial agents. Although chromosomal drug-resistance can give rise to clinical problems, most of the current difficulties caused by multiple resistant bacteria arise because the strains contain R- plasmids. The greatest problems arise when they occur in bacteria which cause major epidemics such as Salmonella typhi and Shigella dysenteriae or in strains which cause infections in hospitals e.g. enterobacteria and P. aeruginosa.

In fact, the major reason for the rapid increase in R+ bacteria which usually accompanies antibiotic therapy appears to be the selection of pre-existent R+ strains, rather than the rapid spread of R- plasmids to previously sensitive strains which the patient is harbouring.

In order to control the prevalence of antibiotic-resistant bacteria, the WHO working group (1983) on antibiotic resistance has recommended the following measures.

(a)- Surveillance of bacterial resistance at both national and international level using standardized method of antibiotic susceptibility testing in order to provide data to the health authorities, doctors and pharmaceutical companies. This data would provide a rational basis for usage and the future development of antibiotics.

(b)- Control of antibiotic use in hospitals. The use of antibiotics should be based on precise clinical diagnosis of the nature of the infection and directed against specific pathogens identified by culture or inferred from the site of the infection. The antibiotic(s) should be chosen in accordance with the results of sensitivity testing.

This WHO memorandum also sets out guide lines for the national and international surveillance of resistance in human pathogens and resistance determinants in the general population. It also highlights the essential elements of the strategy for control of antibiotic usage in hospitals such as the establishment of appropriate hospital antibiotic policies elaboration of general strategies and the monitoring of antibiotic use.

1.2. General Characteristics of Escherichia coli

Enterobacteriaceae is one of the largest distinctive families among the Gram-negative non-photosynthetic true bacteria. What distinguishes them from all other gram-negative rods with similar properties is their facultatively anaerobic nature; they are capable of using the fermentation of carbohydrates as a means of anaerobic growth, but possess a respiratory electron transport system which enables them to grow aerobically at the expense of a wide range of oxidizable organic substrates.

The classical representative of this group is E. coli, an intestinal commensal of mammals and birds that can survive only a short period of time outside the host (Larson, 1984). The characteristics of Escherichia that distinguish it from other enterobacteria are: most of its strains are usually motile, form gas from glucose, ferment lactose, produce indole, give a positive methyl-red reaction and a negative Vosges-Proskauer reaction, do not utilize citrate as sole source of carbon, grow in KCN, hydrolyse urea, liquefy gelatin and produce H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated and gluconate is not oxidized. Most strains decarboxylate lysine (Cowan, 1974). Some strains differ from the typical ones in one or two of these characteristics, e.g. motility, gas formation, lactose fermentation or non-utilization of citrate, but are nevertheless accepted as E. coli (Sojka, 1965).

Studies of the antigenic structure of E. coli revealed over 160 different O antigens, 50 H antigens (Holmes and Gross, 1984; Larson, 1984) and 90 K antigens (Larson, 1984). The latter antigens have been divided into three classes according to the effect of heat on the agglutinability, antigenicity and antibody-binding power of bacterial strains that carry them. So far about 30 L antigens are known to be heat labile (when heated at 100^{o} for 1h); about 26 A antigens, which are polysaccharide capsular material, are heat stable and can produce a Quellung reaction; and over 25 B antigens are heat labile like the L antigens, but with the heated antigen (for 2h at 121^{o}) still capable of absorbing antibody (Larson, 1984). Strains possessing K antigens have increased virulence properties that include increased resistance to phagocytosis and antimicrobial agents. The synthesis of some K antigens is probably plasmid-mediated.

E. coli strains predominate among the aerobic gram-negative commensal organisms present in the healthy gut. Serological studies have shown that the type present are not only numerous at any one examination but that over a period of time the types fluctuate: some types persist over relatively long periods of time, where as others are quite transient (Wallick and Stuart, 1943; Sears et al, 1950; Mason, 1980; Mason and Richardson, 1981).

1.2.1. Epidemiology and pathogenicity

E. coli is a part of the normal human gastrointestinal flora and does not usually cause disease. It can, however, cause pyogenic infections if introduced into other body sites. Extra intestinal disease can occur, especially in the urinary tract, as a result of hematogenous, lymphatic or direct spread. It is the principle cause of about 20% of nosocomial infections (Larson, 1984) of which two-thirds arise in the urinary tract in the form of cystitis, pyelitis and pyelonephritis. E. coli is also the most

frequently isolated organism from the urinary tract infections that are not related to instrumentation or hospitalization. The organism also causes sepsis in operation wounds and abscesses in a variety of organs, and neonatal meningitis and septicaemia. Most of the extra-intestinal disease is caused by a few - 10 or less of the 160 serotypes (Larson, 1984), and none of these are enterotoxigenic strains.

The use of serotyping showed that epidemics of infantile enteritis were caused by strains of E. coli belonging to particular O serogroups, members of which became known as enteropathogenic E. coli (EPEC). Most outbreaks take place in infants under 18 months, though many cases are in children up to 5 years old and some infections cause diarrhoea in adults. During the 1950s many epidemics due to EPEC were reported among babies in hospitals and nurseries in Europe and North America (Rogers, 1951; Rogers and Koegler; Wright and Roden, 1953). Further serious outbreaks occurred in Great Britain and Ireland in the 1960s and early 1970s. In late 1967 there was an epidemic in several hospitals in Teeside in which the mortality rate was high (Lancet 1968): two E. coli strains were responsible, O 119H6 and O128H2. A year or so later a similar outbreak due to E. coli O114H2 occurred in hospitals in Manchester areas (Jacobs et al, 1970). In late 1970 and early 1971 outbreaks due to E. coli O142H6 occurred in several hospitals in the Glasgow area (Love et al, 1972; Kennedy et al, 1973). The same strain was responsible for an outbreak in a Dublin hospital (Hone et al, 1973). Not all strains of the entero-pathogenic serotypes are able to cause gastroenteritis. McDonald and Charter (1956) showed that intestinal colonization of infants with strains of O-groups 26, 111, 125 and 125 caused no illness. The particular strains that cause gastroenteritis are distinguished from others in the same serotypes found in symptomless excreters, which do not form enterotoxin (Taylor et al, 1961; Smith and Halls, 1967; Sack et al, 1971). The power to produce enterotoxin can be acquired by a non-toxigenic strain by the receipt of a plasmid transmitted by conjugation from a toxigenic strain (Smith and Halls, 1968).

Although EPEC enteritis now appears to be of relatively little importance in temperate areas with good standards of hygiene, it is still common in tropical countries (Majya et al, 1977) and in communities in which hygiene is poor (Gurwith and Williams, 1977). The epidemiology of EPEC enteritis in tropical countries differs in some respects from that in Europe and North America (Rowe, 1979). Schroeder et al (1968) reported a water-borne outbreak due to E. coli 0111 that affected adults attending a conference centre in the United States; and two food-borne outbreaks have been reported in Great Britain (Vernon, 1969; Report 1974).

E. coli may become pathogenic in the intestinal tract by two mechanisms (Larson, 1984): enterotoxin production or enteroinvasiveness. Each enterotoxigenic E.coli (ETEC) strain may produce one or both of two enterotoxins, a heat-labile (LT) and a heat-stable (ST) toxin. To be fully virulent, ETEC must also possess surface pili, which aid in colonization of the bowel. These colonization factors related to the pili (CF1 and 11) as well as enterotoxigenicity and antibiotic resistance are plasmid-mediated and can be transferred between strains. ST producing E. coli can only be identified with an infant mouse assay, but LT can be detected in the faeces with tissue culture and immunoabsorbant assays. The enterotoxin binds to receptor sites in intestinal cells and interferes with cellular metabolism, causing diarrhoea and altered fluid and electrolyte balance. Hemolysins and colicins, also plasmid-mediated, are produced by some strains. Colicins bind to receptors on surfaces of other sensitive E. coli or related enteric strains and disrupt the cytoplasmic membrane. Most enteric strains do not produce ST or LT and are non-invasive, but a few strains of serogroups 0114 (Burnham et al, 1976) and 0128 (Reis et al, 1979; Ryder et al, 1979) have been shown by the methods of the infant mouse and tissue culture tests to be enterotoxigenic. Several workers have reported that other EPEC strains cause the accumulation of fluid in ligated loops of rabbit ileum (Taylor et al, 1958; Smith and Gyles, 1970). Klipstein et al

(1978) identified strains of EPEC from outbreaks of infantile enteritis that were not ST or LT producers. On further study the authors found that extracts of the isolated strains caused a net efflux of water in the perfused rat gut. This suggested that EPEC produced an enterotoxin that was not detected by the standard tests for ST and LT.

Konowalchuk et al (1977) showed that culture filtrates of certain strains of E. coli had a cytotoxic effect on monolayers of Vero cells; this contrasted with the 'cytotonic' effect of LT on cells in tissue culture. The cytotoxin (Vero toxin or VT) differs from LT and ST in that it has no action on the Y1 adrenal or Chinese hamster ovarian cell lines commonly used to detect ST. 25 VT-producing strains among 253 EPEC strains of 110 groups were isolated from infants with diarrhoea in the United Kingdom (Scotland et al, 1980), they included 20 members of serotype 026: H11, two of serotype 026: H and two of 0128: H2. Johnson et al (1983) and Riley et al (1983) reported that 0157: H7 VT-forming strains of E. coli were responsible for the outbreaks of haemorrhagic colitis in the United States and Canada. VT forming strains have also been isolated from sporadic cases of haemolytic-uraemic syndrome in Canada (Karmali et al, 1983).

It is clear that there is considerable diversity among the strains previously designated EPEC. A few are enterotoxigenic and can be regarded as ETEC. Some produce Vero cytotoxin and some are strongly adhesive to intestinal epithelial cells.

1.3. Origin and Evolution of Antibiotic-Resistance Plasmids (R- plasmids)

The original discovery of R-plasmids was made in Japan following a rise in the incidence of antibiotic resistance of *Shigella* in that country. They were first isolated in a strain of *Shigella flexneri* 2b (no. 222) by

Nakaya et al (1960) and termed 222 by Watanabe and Fukasawa (1960). Since the discovery of these plasmids, a high proportion of antibiotic resistance in Enterobacteriaceae and Pseudomonads has been shown to be mediated by plasmids.

The origin of R- plasmids is not known. They were completely unknown before the use of antibiotics, but not all R- plasmids have evolved recently, that is since the use of antibiotics on a large scale. R- plasmids specifying resistance to tetracycline and streptomycin have been found in strains of E. coli which were preserved by freeze-drying in 1946, before the clinical use of these antibiotics (Smith, 1967).

R- plasmid-mediated antibiotic resistance determinants may have evolved in species of bacteria other than the pathogens and commensals, in which resistance is often encountered to-day (Falkow, 1975). For example, bacteria in the genus Streptomyces have mechanisms to protect their potentially susceptible targets from the antibiotics which they produce. These genes may have been transferred to other bacteria as plasmid- or transposon-encoded drug resistance or both. Also, there is evidence that soil bacteria commonly harbour antibiotic-resistance plasmids (Falkow, 1975; Bingham et al, 1979; Docherty et al, 1981; Polak and Novick, 1982; Show, 1983), presumably to help them combat antibiotics released in their environment by competitors. These genes could have been transferred to clinically important bacteria either directly or via a number of intermediate hosts and plasmid vectors.

Classic R- plasmids are large plasmids with two functionally distinct parts, one is the resistance transfer factor (RTF), which contains genes for autonomous replication and for conjugation. The other part is smaller and carries resistance determinants (R- d), it varies widely in size and in its content of genes for drug resistance (R genes) (Davies and Smith, 1978).

There is both epidemiological (Farrar et al, 1972; Gruenberg and Show, 1976) and experimental (Guinee, 1965; Salzman and Lydia, 1968; Smith, 1969; and Anderson et al, 1973) evidence suggesting that one source of R- plasmids for pathogens is R+ coliforms, especially R+ E. coli strains, which can be recovered in significant numbers from domestic sewage and its receiving waters (Grabow and Prozesky, 1973; Linton et al, 1974; Fontaine and Hoadley, 1976). There has been concern that the use of sewage-polluted waters for recreation is a significant route for dissemination of R- plasmids from excretors back into the general population.

Other sources of R- plasmids are fomites and carriers. Lowbury and Babb (1972) isolated R- plasmid containing P. aeruginosa and Pr. mirabilis from the hospital environment. Skin, nasal and even intestinal carriers of Staphylococci containing R- plasmids were reported by Lacey (1975).

R- plasmids specifying resistance to new drugs can be detected shortly after their introduction to medicine. R- plasmids coding for resistance to trimethoprim and to gentamicin were first detected in 1972, about three years after the introduction of these drugs. Sometimes a longer lag phase may occur before the resistant strains appear; neomycin-resistant strains of Staph. aureus emerged only after nine years of neomycin use and gentamicin-resistant strains after a similar period (Shanson, 1980). Ampicillin has been one of the main antibiotics used against H. influenzae since 1960. In 1970 the first report of ampicillin resistance appeared (Gunn et al, 1974), a resistance which was later shown to be due to production of beta-lactamase (Williams et al, 1974). The percentage of beta-lactamase producing strains has continued to increase from 1.5% in 1977 to 6.5% in 1981 (Howard et al, 1978; Philpott-Howard and Williams, 1982).

1.3.1. Origin and properties of R- plasmid RP1

The use of carbenicillin (at the Burns Unit; Birmingham Accident Hospital) for the treatment of infected burns (which often have mixed infections with P. aeruginosa and various species of Enterobacteriaceae, including Pr. mirabilis, E. coli and K. aerogenes) led to the rapid emergence, by selection, of highly resistant carbenicillinase-producing strains of P. aeruginosa. These strains rapidly displaced the sensitive ones (Lowbury et al, 1969). They were found to possess an R- plasmid (now called RP1) determining linked resistance to tetracycline, kanamycin, carbenicillin, ampicillin and cephaloridine which was transferable between strains of Enterobacteriaceae and P. aeruginosa in vitro (Fullbrook et al, 1970) and in experimental burns of mice (Roe et al, 1971).

A survey reported by Lowbury and Babb (1972) showed little contamination of the hospital (Birmingham Accident Hospital Burns Unit) environment with Enterobacteriaceae or P. aeruginosa carrying RP1; however, at the same time, Proteus species, Providencia, Klebsiella and E. coli carrying this factor were quite commonly isolated from burns, as well as some strains of RP1-containing P. aeruginosa. This means that the presence of this factor was apparently confined to the burns wards and dependent on selection by therapeutic use of carbenicillin. When carbenicillin treatment was stopped for several months, the carbenicillin-resistant organisms disappeared.

The multiple drug-resistance specified by RP1 was accompanied by the acquisition of an extra chromosomal piece of covalently closed circular DNA of molecular weight about 4×10^7 dalton and of buoyant density $1.719/cm^3$ (60% guanine plus cytosine) (Grinsted et al, 1972).

Plasmids RP1 and RK2 are examples of plasmid clones that were identified in the Birmingham Accident Hospital. These plasmids have a wide host-range (Roe et al, 1971). They can be transferred by conjugation to a wide range

of gram-negative bacteria (Olsen and Shipley, 1973). There are usually one or two copies of any one of these plasmids per host cell (Hardy, 1981).

Plasmids can be classified by their incompatibility relations. When a collection of plasmids is tested, in pairs, each against all, they fall into incompatibility groups. This means of classification has been applied for example, to plasmids in E. coli (Datta, 1975) and in Pseudomonads (Jacoby, 1977). Plasmids RP1 and RK2 fall into incompatibility groups P (Inc P) (Hardy, 1981) and have similar conjugation systems (Datta, 1975, Gorai et al, 1979).

RP1 conjugative pili are involved in conjugation and genetic transfer and also act as receptors for different phages (Wilson and Dick, 1983). These pili are located at the cell pole and it is thought that they pull the phage to the cell surface and then retract and disappear into the cell (Bradley, 1974). The Inc P pili are morphologically rigid, having a thickness of 8nm and during the conjugation process lead to aggregation of the cells (Bradley, 1980).

1.4. Structure and Composition of the Gram-Negative Bacterial Cell Envelope

The bacterial cell envelope is a complex structure existing between the cytoplasm and the surrounding environment; its main function is to regulate the uptake of essential nutrients and to protect the cell. The cell envelope of gram-negative bacteria consists of two cell membranes separated by a periplasmic space and a single layer of peptidoglycan (Fig. 1). It is capable of going through dramatic compositional changes in response to environmental conditions (Inouye, 1979; Nikaido and Nakae, 1979; Lugtenberg and van Alphen, 1983).

The outer membrane (OM) (Fig. 2) which consists of lipopolysaccharide (LPS), phospholipid (PL) and protein (P) is covalently linked to the peptidoglycan via a lipoprotein. It protects the cell against harmful compounds like bile salts and enzymes, and also prevents proteins leaking from the periplasmic space into the medium (Di Rienzo et al, 1978). The periplasmic proteins usually have either catabolic functions or nutrient binding functions, an essential step in some active transport systems (Dills et al, 1980).

The cytoplasmic membrane (CM) contains PL, P, all known active transport systems and electron transport systems and many of the cell envelope enzymes. It acts as an anchor for DNA at least during replication and plays a major role in active transport (Rosen, 1978).

Surrounding the cytoplasmic membrane is the peptidoglycan layer which is a rigid cross-linked polymer responsible for the resistance of the cell to osmotic lysis and is partly responsible for retaining the rod shape of gram-negative bacteria (Henning, 1975).

The OM and CM are interconnected by the so-called "zones of adhesion"; about 200-400 of these locations are present per cell covering about 5% of the membrane surface (Bayer, 1979).

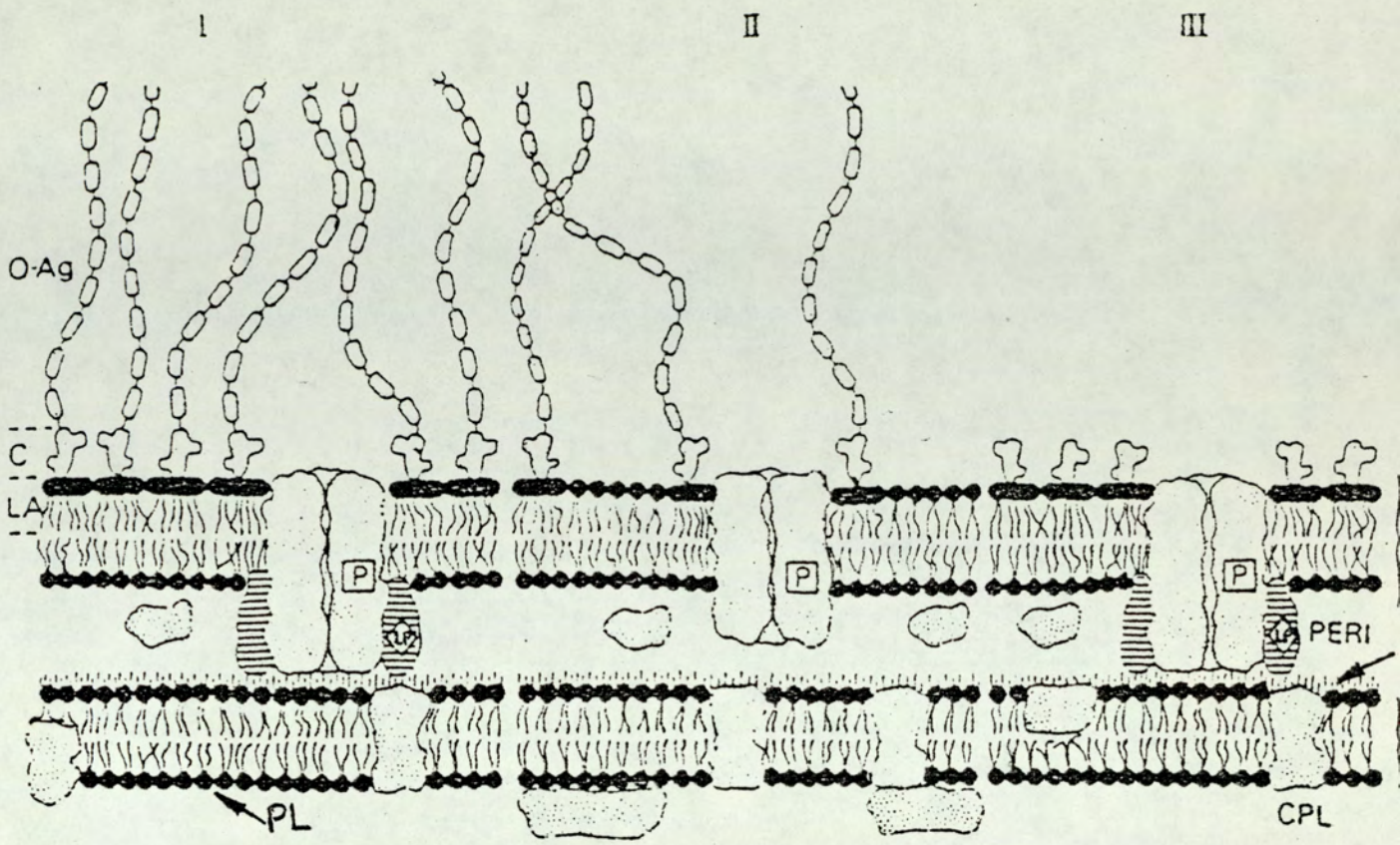


Fig. 1. Model of the cell envelope of gram-negative bacteria.

CPL	Cytoplasm
CM	Cytoplasmic membrane
PG	Peptidoglycan
OM	Outer membrane
PERI	Periplasmic space
LPS	Lipopolysaccharide (LA, C and O-Ag)
LA	Lipid A
C	Core
O-Ag	O-antigen
PL	Phospholipid
LP	Lipoprotein
P	Porin protein

(From Lugtenberg, 1981).

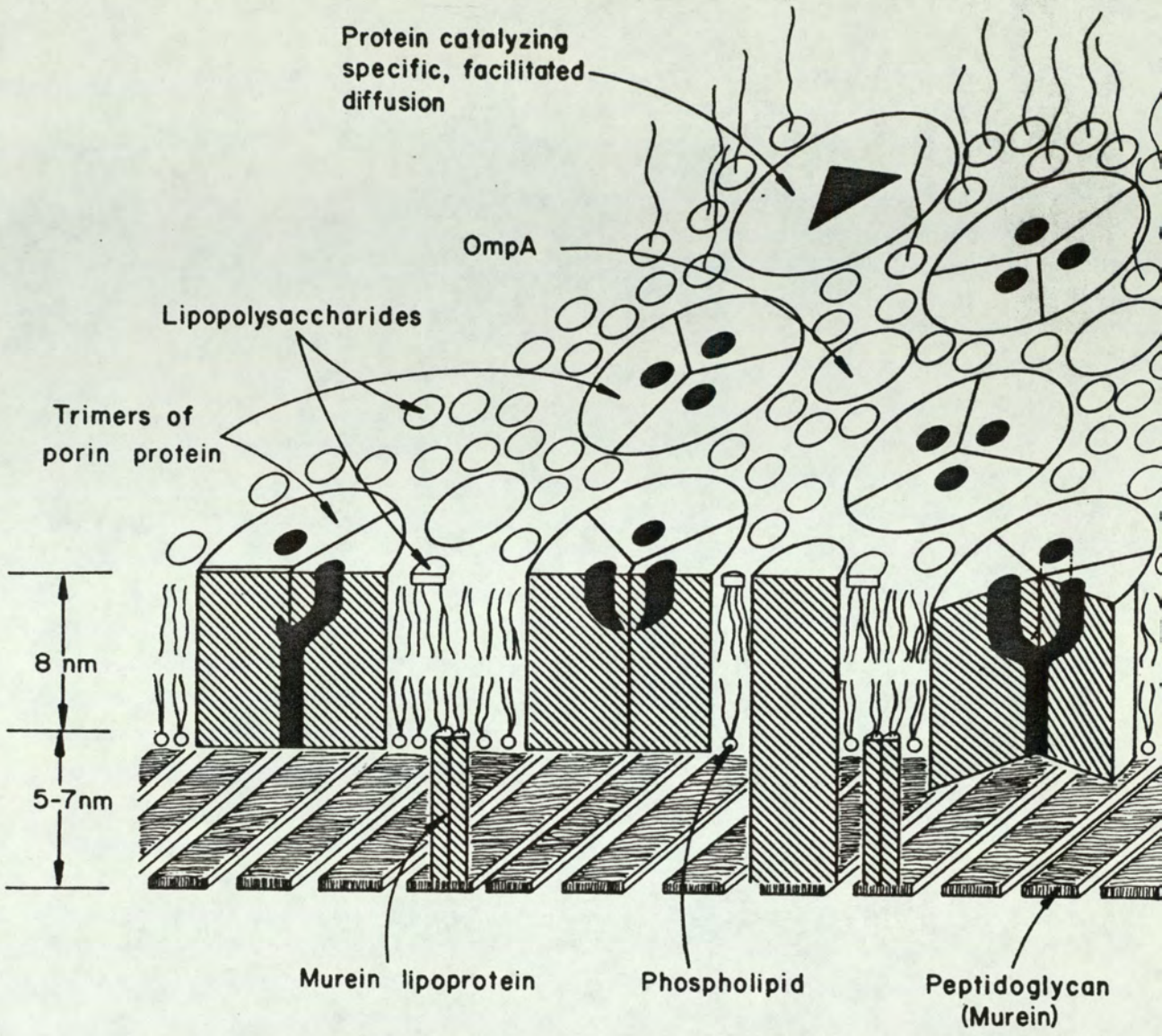


Fig. 2. Schematic model of the E. coli and S. typhimurium outer membrane[a]

(a) Note that some features (such as the length of the saccharide chain of LPS) are not drawn to scale. The specific channel is drawn as a monomer for simplicity and Lam B protein is known to exist as a trimer (Nikaido and Vaara, 1985).

Besides the three main layers mentioned above, several structures or organelles may be present. Sometimes the outer membrane is covered by a layer of capsular material, usually consisting of polysaccharides (Troy, 1979), or by a so called "additional layer", consisting of a regular pattern of subunits, usually protein in nature (Sleytr, 1978). These layers can be involved in protection and adhesion. When present, appendices like flagella (responsible for motility) and pili or fimbriae (involved in adhesion) are anchored in the cell envelope (Lugtenberg and van Alphen, 1983).

1.4.1. Composition, structure and properties of the outer membrane of gram-negative bacteria.

The thickness of the OM of E. coli as determined by electron microscopy was found to be 7.5nm (Glauert and Thornley, 1969). The major components of the OM are LPS, PL and protein (9-12% of the total cellular protein). Cations and the enterobacterial common antigen (ECA) which are common to almost all the Enterobacteriaceae, are minor constituent (Mayer and Schmidt, 1979; Wicken and Knox, 1980). ECA forms 0.2% of the cellular dry weight (Lugtenberg and van Alphen, 1983).

In the following sections specific components of the OM are described. It must be emphasized that both its quantitative and qualitative compositions depend greatly on the growth conditions.

1.4.1.1. Lipopolysaccharide

LPS is also known as endotoxin because it is toxic and is firmly bound to the OM of gram-negative bacteria. It is a unique component which is located exclusively in the outer leaflet of the OM. LPS is an amphipathic molecule consisting of three different regions (Fig. 1), namely: the "O" specific polysaccharide (antigen) side chain, the R specific polysaccharide core which is a hydrophilic part of the molecule that protrudes into the surrounding environment and lipid A, which is the hydrophobic core of the outer membrane (Glauert and Thornley, 1969; Wicken and Knox, 1980; Westphal et al, 1983). In E. coli, lipid A consists of a glucosamine dimer substituted by fatty acyl groups of which B-hydroxymyristic acid, which is specific for LPS, forms a major fraction. The core consists of an inner and outer region. The inner region contains mainly sugars of which heptose (L-glycero-D-manno-heptose) and 3-Keto-2 deoxyoctonate (KDO) are practically specific for LPS. The outer region contains a number of more common sugars like glucose, galactose and

N-acetyl-D-glucosamine (van Alphen et al 1978; Lugtenberg, 1981). KDO links the core polysaccharide with lipid A via an acid labile bond (Wicken and Knox, 1980). The negatively charged phosphate and KDO residues and the positively charged amino residues are assumed to play important roles in the intramolecular interactions (Alphen et al, 1978). The O-antigen consists of up to about thirty repeating units, each containing 3-6 sugar residues. Enterobacterial O-antigens can be homopolysaccharides or heteropolysaccharide with or without short chain branches (Westphal, et al, 1983). It has been shown by SDS-PAGE of LPS preparations that in most smooth E. coli strains, part of the LPS core is not substituted with an O-specific chain and LPS preparations from a given smooth strain are heterogenous with respect to the length of the chains. Thus, the chain length of the O-antigen, even in a culture of one strain is very diverse (Palva and Makala, 1980; Goldman and Leive, 1980) and provides the opportunity for subtle variations in the molecular make up at different locations on the surface of one bacterium (Munford et al, 1980; Tsai and Frasch, 1982). The huge diversity noticed in the building of the repeating unit of the O-antigen within one species, is the basis for O-serotyping, an immunological method used to identify substrains of one species in great detail (Orskov et al, 1977). The LPS of various laboratory "rough" strains like K12, B and C, lacks the O-antigen (Fig. 1 structure III; Lugtenberg, 1981).

1.4.1.2. Phospholipids

The phospholipids of the Enterobacteriaceae are situated in the cell envelope and generally resemble those of E. coli (Cronan, 1979). Phospholipids are mainly composed of three species: phosphatidylethanolamine (PE) which is by far the most abundant species of the PL in the OM (85%), large amounts of phosphatidylglycerol (PG) and diphosphatidylglycerol (cardiolipin). Qualitatively, phospholipids of the OM and CM are similar but the OM is enriched with PE when compared with the CM using conventional culture conditions

(Osborn et al, 1972; Lugtenberg and Peters, 1976; Lugtenberg and van Alphen, 1983). Table 1 illustrates the structure of the major membrane PL and fatty acids of E. coli.

1.4.1.3. Proteins

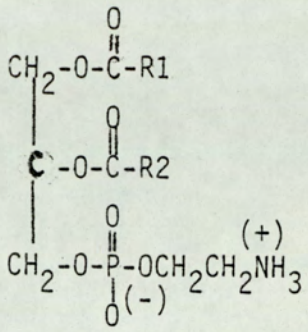
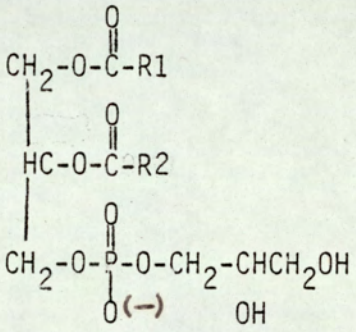
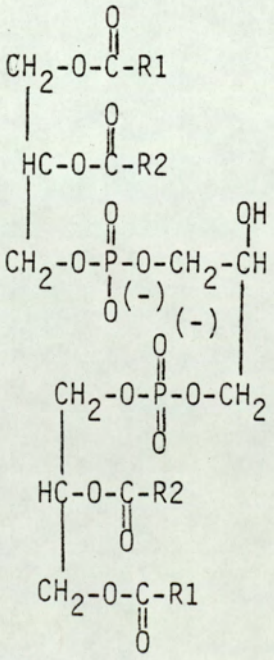
The outer membrane of E. coli contains only a few proteins when compared to the cytoplasmic membrane (Inouye, 1975). As the OM is very poor in enzymatic activity (Osborn et al, 1972), the indentification of the protein components is mainly dependent on separation of proteins in bands using SDS-PAGE which gives high resolution (Lugtenberg et al, 1975; Pugsley and Schnaitman, 1978).

Membrane proteins are either structural, conferring rigidity on the membrane, or functional, allowing it to have considerable enzymatic activity. Another function of OM proteins is to form pores. The degree of association between protein and membrane lipids varies markedly and allows two groups of proteins, peripheral (extrinsic) and integral (intrinsic), to be recognised (Singer, 1971, 1974). The molecular weight of a protein can be estimated by SDS-PAGE, however, as a proven or supposed single amino acid substitution can result in a severe alteration of electrophoretic mobility (Noel et al, 1979; Pugsley et al, 1980), the apparent molecular weight value should be interpreted with care.

The OM of E. coli K12 is considered as typical in that it possesses three major classes of proteins namely: the porin proteins, OMP A protein and lipoprotein (the arrangement of these proteins in the OM is schematically shown in Fig. 2 (Nikaido and Vaara, 1985). The term "major protein" is often relative as in several cases the growth conditions affect the amount of a given protein and a minor protein may become a major protein when it is fully induced

Table 1

Major membrane phospholipids and fatty acids of *E. coli*

Phospholipids	Structure
Phosphatidylethanolamine (PE)	
Phosphatidylglycerol (PG)	
Diphosphatidylglycerol (DPG)	

Fatty acids	
Palmitic acid (C16)	$CH_3(CH_2)_{14}COOH$
Palmitoleic acid (C16)	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$
Cis-Vaccenic acid (C18)	$CH_3(CH_2)_5CH{=}CH(CH_2)_9COOH$

(Lugtenberg and van Alphen, 1983). For example, when E. coli cells are grown under iron starvation conditions the proteins involved in the uptake of ferric chelator complexes become predominant protein species (McIntosh and Earhart, 1976; Pugsley and Reeves, 1976). Under various growth conditions, the total amount of major OMP per unit of OM surface area is constant (Hasumi et al, 1978). This observation shows that a regulation mechanism is operative for major proteins and provides a practical measure for the cell surface area (Lugtenberg and van Alphen, 1983).

1.4.1.3.1. Peptidoglycan-associated pore proteins

Several OM proteins are designated as 'peptidoglycan associated' proteins, these proteins are characterized by their strong but non-covalent bonding with the peptidoglycan fraction (Lugtenberg and van Alphen, 1983). Porin proteins are tightly but non-covalently linked to peptidoglycan and have a high beta structure content (Inouye, 1979). Electron micrographs of negatively stained porin protein peptidoglycan complexes reveal that the porin protein molecules are arranged as a hexagonal lattice layer with a 7.7nm repeat (Rosenbusch, 1974; Steven et al, 1977). There are approximately 1.5×10^5 molecules of porin protein per cell and the hexagonal layer covers about 60% of the outer surface of the peptidoglycan layer (Steven et al, 1977).

Porin proteins are arranged as trimers in the OM. Each trimer contains three separate functional channels i.e. one per porin, which are 1.5 → 2.0nm in diameter (Schindler and Rosenbusch, 1978; Tokunaga et al, 1979a). These porins exist in either the open or closed form and are in equilibrium with each other.

Proteins are named according to their structural genes (Reeves, 1979). Thus, a protein previously known as 11*, d, 3a, B, Tol G protein or 010 is now called Omp A protein as gene omp A is the structural gene for this protein (Lugtenberg and van Alphen, 1983).

E.coli K12 contains 2 peptidoglycan-associated proteins (Lugtenberg et al, 1976; Schmitges and Hennings, 1976; Hasegawn et al, 1976), known as Omp F protein and Omp C protein with apparent molecular weights of 37,000 and 36,000 dalton respectively. These two proteins are immunologically related to each other as well as with Pho E protein (Overbeeke et al, 1980), an inducible pore protein in those strains (Overbeeke and Lugtenberg, 1980). The latter was found to be specific for phosphate and phosphate-containing compounds (Korteland et al, 1982). Other strains of E. coli sometimes produce additional porins e.g. protein K which is found among the encapsulated strains (Paakkanen et al, 1979; Sutcliffe et al, 1983; Whitfield et al, 1983). So far peptidoglycan-associated proteins have been detected in all strains of Enterobacteriaceae tested (Lugtenberg et al, 1977; Nixdorff et al, 1977) and these proteins cross react with E. coli proteins (Overbeeke and Lugtenberg, 1980; Hofstra and Dankert, 1979; Hofstra et al, 1980). The family of peptidoglycan-associated proteins is even larger if one takes into consideration that not all proteins are constitutively present but some are produced under certain growth conditions only (Overbeeke and Lugtenberg, 1980; Hancock and Carey, 1980), by plasmid (Achtman et al, 1977; Iyer, 1979; Moll et al, 1980) or by a (pro) phage (Schmitges and Henning, 1976; Pugsley and Schaitman, 1978). Rosenbusch (1974) showed that in E. coli B, the Omp F porin was a single polypeptide containing 336 amino acids and apparently lacking in any non-protein moiety. None of the porins purified so far are particularly hydrophobic in their amino acid composition (Rosenbusch, 1974; Garten and Henning, 1974) and they are known to carry a negative charge (Schmitges and Henning, 1976). It has also been found that both Omp C and Omp F proteins have very high contents of B structure (Rosenbusch, 1974; Nakamura and Mizushima, 1976) which is in contrast to many other "intrinsic" membrane proteins (cytoplasmic and outer membranes) which have a high α-helix content. These OM proteins show considerable alteration in mobility on SDS polyacrylamide gels after heating of the proteins in SDS. Nakamura and Mizushima (1976) showed that this was due to gross

conformational changes in the proteins which altered the B structure to α-helix. The modification of protein after heating in SDS is characteristic of porins; so too is their association with the underlying peptidoglycan. This was demonstrated by treatment of the cell envelope at a temperature below 70°, which solubilized all the OM components other than the lipoprotein and the peptidoglycan-associated proteins (the porins).

The characteristics of individual general diffusion pore proteins of E. coli and S. typhimurium are summarized in Table 2. It has been suggested that Omp C and Omp F protein of E. coli K-12 are products of the same structural gene (Schmitges and Henning, 1976; Bassford et al, 1977); this, together with the fact that these proteins are hardly or not at all separated in many gel systems (Schaitman, 1973; Garten and Henning, 1974) has led to the misunderstanding that E. coli K-12 contains one general pore protein. It is clear now that these constitutive pore proteins of E. coli K-12 are coded for by distinct but structurally related structural genes (Lugtenberg and van Alphen, 1983). The polypeptides are similar but differ in several properties (Lugtenberg et al, 1976; van Alphen et al, 1978; Ichihara and Mizushima, 1978).

The impermeability of the enterobacterial OM to bile salts and its extremely high permeability for nutrients and other solutes with a M_r up to about 700 daltons (Nikaido, 1979), was explained by Nakae and Nikaido (1975). Decad and Nikaido (1976) developed the concept of water-filled transmembrane pores and, by incorporating porin proteins into artificial LPS-PL bilayers, they illustrated that these artificial vesicles had the same molecular sieving properties as the intact OM. Their results showed that the function of the pore proteins is to form passive diffusion pores; this property made them called "porins" (Nikaido, 1979). In E. coli they have been shown to be homologous and heterologous combinations of Omp C and Omp F (Ichihara and

Table 2

Characteristics of some *E. coli* and *S. typhimurium* peptidoglycan-associated general diffusions pore proteins*

Protein species	OmpF protein (*E. coli* strains K-12 and B)	OmpC protein (*E.coli* K-12)
M_r	37,205	36,000
Number of copies/cell	Up to 10^5	Up to 10^5
(Part of) receptor for phage/bacteriocin	TuIa, T2, TP1, K20, TP2, TP5, colA.	TuIb;T4 Me1; PA-2 SS1; TP2, TP5, TP6
Structural gene	ompF. min 20.7	ompC. min 47.1
Isoelectric point (pH)	5.9-6.2	n.d.
Oligomeric form	Trimer	Trimer
Pore diameter (nm)	1.4	1.3
Further characteristics	Gene ompF has been cloned; it hybridizes with the phoE gene; synthesis of OmpF protein is repressed by high osmolarity; synthesis positively controlled by cAMP; 70% amino acid sequence homology with phoE protein.	For effect of osmolarity see under OmpF protein. Smaller effective diameter than OmpC pore.

(From Lugtenberg and van Alphen, 1983)

* Association with peptidoglycan is meant in the operational sence only. The group of proteins listed in this table has in common an antigenic relation with OmpF protein and/or OmpC protein of *E. coli* K-12.

Mizushima, 1979) which are large enough to span the thickness of the OM (Tokunuga et al, 1979b).

The diffusion rate of solutes is estimated by the difference in concentration between both sides of the membrane and by other factors such as solute size, hydrophobicity and charge. The largest molecules of oligosaccharides which can diffuse through a pore channel of Enterobacteriaceae are those with M_r of < 600 daltons (Nakae and Nikaido, 1975; Decad and Nikaido, 1976). This size limit corresponds to a pore diameter of 1nm (Nikaido, 1979). Pore diameters have also been calculated from conductivity measurements through black lipid films and found to be of 0.9 - 1.4nm in diameter (Nikaido, 1979; Schindler and Rosenbusch, 1978; Benz and Hancock, 1981).

1.4.1.3.2. Omp A protein

There are about 10^5 copies of Omp A protein per E. coli cell. Its complete amino acid sequence consists of 325 residues with a M_r of 35,129 (Chen et al, 1980). Of the lysine residues 6-24% are present as allysine (α-amino adipic acid semialdehyde) as a result of an enzymatic post-transcriptional modification process (Diedrich and Schnaitman, 1978; Mirelman and Siegel, 1979). The Omp A protein is rich in B structure (van Golde et al, 1973; Nakamura and Mizushima, 1976) and is heat modifiable i.e. its apparent M_r on SDS polyacrylamide gels is higher after denaturation in SDS by heating (30,000) than in its non-denatured form (28,000). A protein cross-reactive with Omp A protein was detected in strains of E. coli (Overbeeke and Lugtenberg, 1980; Hofstra and Dankert, 1980) and of other Enterobacteriaceae (Hofestra and Dankert, 1980; Beher et al, 1980). The non-heat modified form of Omp A protein is due to the high content of β structure and excessive binding of SDS in the absence of heating in SDS (Heller, 1978; Chen et al, 1980). Omp A protein does not bind strongly to peptidoglycan, however, some Omp A is covalently

linked to the diaminopimelic acid residues of the peptidoglycan layer in staionary phase E. coli cells (Diedrich and Schnaitman, 1978). Omp A protein with Braun's lipoprotein is involved in maintaining both the structural integrity of the OM as well as the rod shape of the cell. It also plays a role in F pilus-mediated conjugation apparently by stabilizing mating aggregates (Lugtenberg and van Alphen, 1983).

1.4.1.3.3. Lipoproteins

The lipoprotein (or Braun's lipoprotein) found in E. coli and species of Salmonella and Serratia, is a small, well characterised protein (Braun and Rehn, 1969). The lipoprotein from E. coli (M_r 7200) contains 58 amino acid residues and is covalently linked to the underlying peptidoglycan via the amino group of its C-terminal lysine residue. At its N-terminal end, the cystein carries an amide-linked fatty acid as well as a diglyceride moiety. Also in contrast to the heat modifiable proteins, the lipoprotein is rich in α-helical regions (Braun, 1975).

The lipoprotein is by far the most abundant protein of the cell (Braun, 1975). In E. coli it is present in approximately 7×10^5 copies per cell (Nikaido and Nakae, 1979). About two thirds of the total amount is present in a free form, the remaining third being covalently bound to the peptidoglycan (Inouye et al, 1972). Both free and bound forms of similar lipoproteins have been reported to exist in Salmonella and Serratia species, but in Proteus mirabilis, only the bound form of such a lipoprotein has been detected (Braun et al, 1970; Halegoua et al, 1974) and this is only present in relatively small quantities (Gmeiner et al, 1978). Both forms of the lipoprotein have been purified and sequenced (Braun and Bosch, 1972 a and b) and the free form has been crystalized (Inouye et al, 1976; De Martini et al, 1976).

The lipoprotein is not essential for the survival of the cell. However, it may play a role in stabilization of the OM (Hirota et al, 1977; Suzuki et al, 1978). When both the lipoprotein and Omp A protein are missing, cells are unable to grow in the rod form and require high concentrations of Mg^{2+} or Ca^{2+} for growth (Sonntag et al, 1978). Moreover a substantial increase in blebbing was observed in the double mutant and the peptidoglycan layer was no longer connected with the OM (Sonntag et al, 1978) suggesting a role of the proteins in the determination and maintenance of the rod shape, in the stabilization of the OM structure and in anchoring the OM to the peptidoglycan. Recent experiments carried out by Wensink and Witholt (1981) are additional evidence for the latter function. They showed that OM vesicles released by growing E. coli cells contain only a small amount of free lipoprotein, hardly any bound lipoprotein and reduced amounts of Omp A protein. The vesicles also contained reduced amounts of protein V, a protein assumed to be identical to a newly discovered lipoprotein (Wensink and Witholt, 1981).

Another class of lipoprotein, peptidoglycan-associated lipoproteins (PAL), which occur closely but non-covalently associated with peptidoglycan, has recently been found in some gram-negative bacteria like E. coli (Ichihara et al, 1981), P. mirabilis (Mizuno, 1979) and P. aeruginosa (Mizuno, 1979; Mizuno and Kageyama, 1979). They are not immunologically cross-reactive with Braun's lipoprotein (Mizuno, 1981). Several new lipoproteins, which are immunologically different from both PAL and Braun's lipoprotein, have been found in E. coli (Ichihara et al, 1981). Four of these lipoproteins were located in the OM and two in the CM. Thus the minimum total number of biochemically different lipoprotein species in E. coli is nine (Ichihara et al, 1981).

1.4.1.3.4. Other outer membrane proteins

The characteristics of E. coli pore proteins which are not antigenically related to the family of peptidoglycan-associated general diffusion pore

proteins have been summarized by Lugtenberg and van Alphen (1983) and are shown in Table 3.

The receptor of phage T6 and colicin K, is an OM protein of M_r 26,000 (Alderman, 1979; Manning and Reeves, 1978). It has been purified and identified by Manning and Reeves (1978). Biologically active T6 receptor invariably contains LPS (Manning and Reeves, 1978) and *in vivo* experiments suggest that core sugars play a role in a later step of the T6 infection process (Krieger-Brauer and Braun, 1980). It is involved in facilitating the diffusion of all nucleosides and deoxynucleosides except cytidine and deoxycytidine (Hantke, 1976; Krieger-Brauer and Braun, 1980).

The bacteriophage lambda receptor protein of M_r 47,392 (Clement and Hofnung, 1981) is induced in the presence of maltose and is an essential component for the lambda phage (Konisky, 1979). It is involved in the uptake of maltose and maltodextrins (Wandersman *et al*, 1979) at low substrate concentrations (0.001 - 1.0mμ) (Szmeleman and Hofnung, 1975). Once induced it becomes a major OM protein comparable in size with the pore protein (Braun and Krieger-Brauer, 1977). The bacteriophage lambda receptor protein is peptidoglycan-associated and appears to facilitate the diffusion of other nutrients (Braun and Krieger-Brauer, 1977; Nakae and Ishii, 1980). A similar protein which cross reacts with *E. coli* lambda protein has been identified in *Salmonella* (Palva, 1979).

Vitamin B12, M_r 1,327, is too large to move through the general diffusion pores and requires a specific OM protein (M_r 60,000) to facilitate its translocation across the OM. This protein is also the receptor for phage BF23 and the E. colicins. The cell can be guarded against phage and colicin killing effects by the binding of vitamin B12 to the receptor protein (Konisky, 1979).

The outer membranesreceptor for ferric enterobactin protein with a M_r of 81,000 is converted to a M_r 74,000 protein by an OM protease, which has

Table 3

Characteristic of E. coli K-12 pore proteins not antigenically related to peptidoglycan-associated general diffusion pore proteins

Protein	Conditions for optimal expression	M_r	(Part of)receptor for phage/colicin	Structural gene	Proposed function	Further characteristics
Phage T6 receptor	Co-regulated with nucleotide transport	26,000	T6, Col K	tsx.min9.2	Uptake nucleosides and deoxynucleosides	Synthesis catabolite repressible; increased amounts synthesized in cytR and deoR mutants; up to about 4×10^4 copies can be present per cell.
Phage λ receptor	Presence of maltose	47,392	λ;K10;TP1;TP5; SS1	lamB.min91.0	Uptake maltodextrins	Synthesis is catabolite repressible; up to 10^5 copies per cell can be present; pore diameter 1.5nm. Synthesized in reduced amounts in perA mutants and in heptose-less LPS mutants.
ButB protein	Vitamin B12 limitation	60,000	BF23, E.colicins. Col A	btuB.min89.0	Uptake vitamin B12	Up to 200-300 copies per cell.
Cir protein	Fe^{3+} limitation	74,000	Col I, Col V	cir.min 44	Uptake complexed Fe^{3+}?	Synthesis of Cir, FepA and 83K
FhuA protein	Fe^{3+} limitation	78,000	T1,T5,Ø80,Col M	fnuA.min3.4	Uptake Fe^{3+} ferrichrome	proteins is reduced in perA mutants.
Fec protein	Presence of citrate	80,500	-	fec. min 7	Uptake Fe^{3+}-citrate	
FepA protein	Fe^{3+} limitation	81,000	Col B, Col D	fep. min 13	Uptake Fe^{3+} enterochelin	
83K	Fe^{3+} limitation	83,000	-	Unknown	Uptake complexed Fe^{3+}?	

(From Lugtenberg and van Alphen, 1983)

chemical and physical properties ascribed to protein a (Fiss et al, 1979). In growth under iron-limited conditions, gram-negative bacteria de-repress the synthesis of several OM proteins which in E. coli and S. typhimurium have M_r between 74,000 and 83,000 (Griffiths, 1983). In vivo, during an infection with E. coli, these proteins are present in amounts similar or greater than the so called OM proteins (Griffiths, 1983). They are involved in the uptake and release of iron from the chelators (Griffiths, 1983).

As yet, several other OM proteins with unidentified functions have also been reported to exist in E. coli. Examples include protein a, M_r 40,000 (Lugtenberg et al, 1975), protein III, M_r 17,000 (Henning et al, 1973). LPS-binding protein, M_r 15,000, (Geyer et al, 1979), proteins induced by sulphate limitation, M_r 15,000 and 19,000 (Lugtenberg and van Alphen, 1983) and several phage and plasmid-coded OM proteins (Lugtenberg and van Alphen, 1983).

1.4.1.4. Enzymes in the outer membrane

Unlike the CM, the OM is poor in enzymatic activities and the first enzyme found in the OM of E. coli was the phospholipase A1 (White et al, 1971; Bell et al, 1971; Osborn et al, 1972). It has a M_r of 28,000 (Scandella and Hornberg, 1971; Nishijima et al, 1977). Other OM enzymes which have since been discovered are lysophospholipase, lysophosphatidic acid phosphatase and UDP-glucose hydrolase (Osborn and Munson, 1974). Presently it is not known how many enzymes are responsible for the different biological activities in the OM (Lugtenberg and van Alphen, 1983), but two of them have recently been purified. The casein-hydrolyzing enzyme, designated as protease IV, M_r 23,500, (Regnier, 1981 a and b) and leader (or signal) peptidase with an apparent M_r of 39,000 (Zwinzinski and Wickner, 1980). The latter enzyme has been found to exist in equal abundance in CM and OM of E. coli (Zwinzinski et al, 1981).

1.4.1.5. Molecular structure and organisation of the outer membrane

When the OM of gram-negative bacteria is considered as a whole, it has been found that in S. typhimurium there are approximately 1.5×10^6 molecules of PL per $1\mu m^2$ of the OM and 1.9×10^5 molecules of LPS in the same area (Smith et al, 1975). This latter figure represents the number of LPS molecules, which are each assumed to contain three monomeric units. Nikaido and Nakae (1979) suggested that the OM-outer layer of the WT gram-negative bacteria consisted of 41% LPS and 59% protein and the OM-inner layer, 58% PL and 42% protein. It has more recently been reported that the OM-outer layer consisted of 68% LPS and 32% protein with the inner layer containing 46% PL, 50% protein and 4% lipoprotein (Lugtenberg and van Alphen, 1983). The discrepancies in results were attributed to differences in calculated surface area occupied by LPS, the LPS:P:PL ratios and in the strains and growth conditions used (Lugtenberg and van Alphen, 1983). It is therefore important to realise the crucial effect of growth conditions on the composition of the OM, which render it difficult to make any meaningful generalisations concerning the overall composition of the OM. It is known from studies utilizing ferritin-labelled antibodies (Muhlradt and Golecki, 1975) and exogenous galactose oxidase (Funahara and Nikaido, 1980) directed against the O-antigen of LPS that all the LPS is to be found located on the outer leaflet of the OM. A substantial proportion of the LPS molecules form tight complexes with the OM proteins, the in vitro biological activity of which, is often dependent on the presence of the LPS (Lugtenterg and van Alphen, 1983). On the other hand, the PL is present only on the inner leaflet of the OM and hence, they are inaccessible to detergents and bile salts. Mutants with defective LPS, in which the O-chain (the polysaccharide moiety of LPS) is reduced ("rough" and "deep rough" mutants), and pore-protein deficient mutants are sensitive to these and other hydrophobic agents. They possess increased amounts of PL which are possibly located in the outer leaflet of the OM (Wilkinson et al, 1972; Tamaki and Matsuhashi, 1973;

Smith et al, 1975; Kamio and Nikaido, 1976). Also in these mutants much of the protein is not normally incorporated into the OM, possibly because the defective LPS is unable to interact with the proteins (Koplow and Goldfine, 1974; Ames et al; 1974). Electron-spin resonance studies have provided additional evidence showing that for both OMs and model bilayers, PL and LPS are segregated into separate domains with the vast majority of PL molecules localized in the inner layer of the OM (Nikaido et al, 1977; Takeuchi and Nikaido, 1981).

It is important to remember that E. coli K12 (a strain of which has been used in the present study) and E. coli B are frequently used in investigations of the OM as rough strains, and are WT in terms of LPS biosynthesis.

Protein occupies 40-75% of the surface area of the OM in the wild type gram-negative bacterial cell. It has been shown that there are some 6.5×10^5 molecules of porin protein per cell (Steven et al, 1977) and about 10^5 molecules of Omp A protein (Lugtenber, 1981). The location of the OM proteins is not fully understood, but in S. typhimurium many of the proteins including all known porins, are incorporated in the outer layer of the OM, and exposed on the cell surface (Kamio and Nikaido, 1977). Neither the bound nor the free form of Braun's lipoprotein appears to be featured on the outer surface of the OM in E. coli and S. typhimurium, which is probably only exposed should there be structural faults in the cell membrane (Nikaido and Nakae, 1979).

Begg and Donachi (1977) proposed a model for the growth of the cell surface of E. coli which involved insertion of newly made porin at the poles of the cell. This in turn would have led to concentration of porin molecules in these two areas. However, both Begg (1978) and Smith and Nikaido (1978) have discovered that the porin protein is distributed evenly throughout the OM. This finding is in agreement with that found by Steven et al (1977) who have

shown that the porin protein of E. coli is arranged in periodic monolayers covering at least 60% of the surface of the peptidoglycan. They reported that although the proteins were strongly attached to the peptidoglycan, yet the periodic structure was maintained in its absence, and that was probably due to strong protein-protein interactions.

Using freeze fracturing electron microscopy methods, van Alphen et al (1978) observed that the various porin proteins complexed with the LPS rather than the lipoprotein. It seems possible that the porin molecules are associated with the LPS (Yu and Mizushima, 1977) and in P. aeruginosa LPS-protein complexes have been clearly identified (Rogers et al, 1969; Gilleland et al, 1973; Stinnet et al, 1973). On the other hand the Omp A protein in E. coli has been found to cross link with the Braun's lipoprotein (Palva and Randall, 1976).

The cell surface of E. coli was studied using freeze etch electron microscopy (Bayer and Leive, 1977; van Alphen et al, 1978). It was revealed that the surface contained numerous randomly-located depressions of about 4.5nm in diameter which were suggested by Bayer and Leive (1977) and van Alphen et al (1978) to be the entrances of the aqueous pores. Freeze-fracture techniques applied to the interior of the OM showed that it could be cleaved into two halves proclaiming a lipid bilayer structure (Smith et al, 1975; Verkleij et al, 1977). The two leaflets clearly differ in that the concave (outer fracture) face is covered with particles having diameters between 4-8nm, whereas the convex (inner fracture) face, contains pits which are presumably complementary to the particles. Particles and pits are assumed to be reflections of interactions between LPS and protein (Gilleland et al, 1974; van Alphen et al, 1978; Nikaido and Nakae, 1979; Osborn and Wu, 1980).

One of the main functions of divalent cations is to maintain the integrity of the outer membrane in gram-negative bacteria. The divalent cations,

magnesium and calcium are necessary for the integrity of the OM, as EDTA treatment of E. coli (Leive, 1965) and P. aeruginosa (Rogers et al, 1969; Roberts et al, 1970) releases predominantly LPS and LPS-protein complexes from the two organisms respectively. These divalent cations presumably act by neutralizing and bonding the LPS anionic groups. However, their effect is not complete in that under a variety of growth conditions a "blebbing" feature has been observed (de Petris, 1967; Smit et al, 1975) which is probably a result of the strong electrostatic repulsion between the components at the OM-outer surface. Braun's lipoprotein serves as an anchor, pulling the OM down to the underlying peptidoglycan layer (Nikaido and Nakae, 1979). E. coli mutants lacking the Braun lipoprotein produce very large "blebs" in the OM which can be reduced by adding magnesium to the medium (Suzuki et al, 1978; Fung et al, 1978). Sonntag et al, 1978 investigated E. coli mutants lacking both the Braun lipoprotein and the Omp A protein. They found that the cell had drastic alterations in the OM. Digestion of the peptidoglycan with lysozyme also causes extensive reorganisation of the OM components (Shands, 1966; Muhlradt and Golecki, 1975). E. coli and S. typhimurium appear to maintain their highly asymmetric OM organisation, at least in part, depending on the lipoprotein-peptidoglycan system and divalent cations. P. aeruginosa has relatively more anionic groups present in its OM (Gray and Wilkinson, 1965) and usually requires high concentrations of magnesium to stabilize the OM and maintain its integrity.

1.4.2. Surface and barrier properties of the outer membrane of gram-negative bacteria

The OM forms a semi-permeable barrier around the gram-negative cell, protecting it from the environment and yet allowing some compounds, both hydrophilic and hydrophobic, to cross the membrane and excluding others, therefore it acts as a molecular sieve.

The OM, particularly the OM proteins, are involved in conjugation. Manning and Achtman (1979) found that Omp A protein was necessary for conjugation. Other proteins are probably involved also, but as yet not much is known about the interaction between OM contents and the required alterations in the cell envelope to enable DNA transfer.

As far as the role lof the OM surface is concerned, the most studied function is that of various OM proteins which act as receptor sites for bacteriophages and colicins (Lugtenberg and van Alphen, 1983). The most familier examples are the λ phage receptor (the lamB gene product); the colicin E and phage BF23 receptor (the protein involved in the specific transport of vitamin B12); the colicin K and phage T6 receptor (the tsx gene product) (Konisky, 1979).

Of the major OM proteins, Omp A protein was found to be the receptor for phage TuII* (Manning et al, 1976), and although both the Omp A protein and LPS are involved, yet the actual protein moiety acts ad the receptor (Datta et al, 1976; Datta et al, 1977). The porin proteins Omp F and Omp C are receptors for phage TuIa and TuIb in E. coli (Datta et al, 1977). The latter two proteins also act as receptors, but, independently of each other for different phages such as TP1 (Omp F) and PA-2 (Omp C).

Certain components in the OM are considered to be partially involved in maintaining the morphology of the cell. Sonntag et al (1978) showed that mutants of E. coli lacking both protein A and lipoprotein became spherical and required higher concentrations of electrolyte (Ca^{2+} and Mg^{2+} in particular) for their optimal growth than was needed for the wild type. However, Henning and Haller (1975) found that mutants of E.coli K12 lacking the porin and Omp A proteins exhibited no morphological difference from the parent strains. Recently, the porins were also found to be important in the maintenance of cell surface structure of E. coli (Nogami and Mizushima, 1983).

1.4.3. Effect of environments on the cell envelope of gram-negative bacteria

The cell envelope structures are complex and interdependent and the protective bacterial envelope can be rendered entirely ineffective by the alteration of any vital component such as the peptidoglycan (Burman *et al*, 1972; Nordstrom and Sykes, 1974). Such alterations can be brought about by both genetic (see section 1.4.4.) and environmental factors.

The structure and composition of the cell envelope is significantly influenced by the environmental conditions (Holme, 1972; Brown, 1975). When placed in environments deficient in an essential nutrient, a growing bacterium changes, not only in its metabolism, but also synthesizes an envelope characteristic of that particular depletion (Brown, 1975, 1977). The envelope composition has been observed to undergo significant changes in response to depletion of cations, anions and glucose as well as changes in growth rate, temperature and osmolarity of the growth medium.

1.4.3.1. Effect of growth medium composition

One of the environmental parameters that commonly influences the properties of microbial cells *in vitro* is the concentration of essential nutrients. The changes in cell envelope structure and composition due to deficiency of different nutrients have been demonstrated by several investigators. For example, Gilleland *et al* (1974) found that the OM of magnesium-depleted cells of *P. aeruginosa* contained more carbohydrate and KDO but less phosphorus; qualitative differences in proteins were also observed. These changes were accompanied by an increased number of highly compact spherical units in the middle layer of OM. The particles were insensitive to EDTA. Similar changes have been observed in *E. coli* (Tsang *et al*, 1976; Verkleij *et al*, 1977) and the particles are thought to consist of a protein-LPS complex (Rogers *et al*, 1969; Verkleij *et al*, 1977). The cell wall of *P. aeruginosa* was markedly influenced

by the lack of magnesium. Under this growth condition amino sugars, total carbohydrate, REL and PL all increased (Kenward et al, 1979). P. aeruginosa cells grown in a magnesium-deficient medium were found to contain decreased magnesium concentration in the envelope which varied in a linear reciprocal relationship with protein H1, (Nicas and Hancock, 1980). They proposed that this protein replaced magnesium at a site on the LPS and rendered the cells resistant to EDTA.

Deficiency of phosphate also affects the PL content of P. fluorescens; Dorrer and Teuber (1977) found that phosphate-depleted cultures synthesize less PG, DPG and PE but produced a positively charged ornithine amide lipid. Another effect of phosphate-depletion is the induction of specific OM protein e in E. coli (Overbeeke and Lugtenberg, 1980). Other inducible proteins resulting from changes in growth medium are those induced in E. coli by sulphate limitation (Lugtenberg and van Alphen, 1983), and under conditions of iron deficiency (Neilands, 1982; Klebba et al; 1982, Griffiths, 1983; Williams, 1984).

The composition of the growth medium in which the organism is grown has been shown to influence the amount of the different OM proteins. The ratio of Omp C to Omp F in E. coli was greatly influenced by glucose concentrations in the medium (Lugtenberg et al, 1976). Cells grown in glucose minimal media had far less Omp C and more Omp F in the OM than in cells grown in nutritionally rich media. The amount of Omp F protein present in glucose-depleted cells tended to be 1.5 to 4 times greater than that of Omp C. Although the amounts of the two porins varied, the change in the total amount of matrix protein was not significant. The authors suggested that the system regulating porin concentrations was stimulated to maintain an approximately constant amount of the porin proteins present in the OM. In addition, because nutrient depletion affects the cell envelope it also influences bacterial resistance to anti-bacterial drugs (Brown, 1977; Turonowsky et al; 1983) and pathogenicity

Costerton et al, 1981; Anwar et al, 1983; Ombaka et al, 1983).

The presence of lipophilic compounds in the growth medium has a significant effect on lipid composition. Salmonella typhimurium exhibited major changes in lipid composition when grown in the presence of either 0.15% sodium deoxycholate or 0.15% sodium benzoate (Tomlins et al, 1982). These lipophilic compounds had directly opposing effects on the lipid profile of the organism, hence, the saturated/unsaturated ratio was markedly elevated in benzoate-grown cells. On the other hand, it was depressed by an even greater margin from the control after growth in the presence of deoxycholate.

The osmolarity of the growth medium is another environmental factor affecting the porin composition of the E. coli outer membrane (van Alphen and Lugtenberg, 1977; Kawaji et al, 1979; Lugtenberg and van Alphen, 1983). It has been found that the amount of Omp F protein decreases dramatically when the organism was grown in media supplemented by high concentrations of NaCl, KCl or sucrose, whereas the Omp C porin concentration increased by almost equal amounts (van Alphen and Lugtenberg, 1977). Similar results were obtained by Kawaji et al (1979), using E. coli as the test organism in a high osmolarity medium (large concentrations of sugars and low M_r dextrans). Under these conditions, expression of Omp C genes was induced whereas that of Omp F was suppressed, leading to a reduction in the concentration of the Omp F protein in the OM, and an increase in Omp C. The surface density of the major OM proteins in S. typhimurium under different growth conditions and growth rates remained almost constant (Alder et al, 1980). Variation in the growth media also had little effect on the surface density of the major OM proteins. It had formerly been suggested (Lugtenberg et al, 1976; Boyd and Holland, 1979) that the exact mechanism regulating incorporation of OM proteins into the envelope was determined by the area available on the cell surface to be occupied by these proteins.

Medium osmolarity also affects the fatty acid content of the OM. When E. coli was grown in a medium of high osmolarity, there was almost complete replacement of unsaturated fatty acids by cyclopropane fatty acids (McGarrity and Armstrong, 1981).

1.4.3.2. Effect of growth temperature

The effect of the growth temperature on the OM and in particular on its lipid contents has been studied. Lipid and phospholipid composition of organisms are affected by the growth temperature (Cronan and Gelmann, 1975), and it has been shown that in E. coli there is an inverse relationship between the amount of unsaturated fatty acids present in the membrane lipids, and the temperature at which the organisms are growing (Raetz, 1978). This is presumably associated with the bacterial requirements to maintain and therefore regulate membrane fluidity. This is in turn linked with the OM proteins, for it has been shown that the lipid fluidity and physical state of the membrane affected the synthesis and structure of the OM proteins (Di Rienzo and Inouye, 1979). The fatty acid composition of the early exponential phase lipids of E. coli was found to depend only very slightly on the growth temperature (McGarrity and Armstrong, 1981). The growth temperature, however, did affect the magnitude of the stationary phase increases in palmatic and cyclopropane fatty acids and the smallest increases were found to be at 20^{o} while the largest were at 42^{o}.

1.4.3.3. Effect of growth rate

Growth rate has also been shown to affect the structure and composition of the cell envelope. Gilbert and Brown (1978b) found that the total PL content of P. aeruginosa cells decreased and the fatty acid content increased with increasing growth rate. The LPS content decreased with increasing growth rate and correlated significantly with drug uptake and sensitivity and it appeared to

determine the degree of penetration of the cell envelope by 3-chlorophenol and 4-chlorophenol.

Decreases in total lipid content with increasing growth rate have been observed for E. coli (Ballesta and Schaechter, 1972). Changes in protein content and certain sugar components of cell walls associated with changes in dilution rates were observed by Collins (1964). He also noticed that faster-growing cells had shorter LPS and underwent partial smooth to rough antigenic variations which were reversed when the dilution rate was decreased.

1.4.4. Effect of R- plasmid carriage on the cell envelope of gram-negative bacteria.

Plasmid-conferred resistance to antimicrobial agents has become the most widespread mechanism of bacterial resistance to antibiotics. This type of resistance is mainly associated with the acquisition of an additional property, usually in the form of an enzyme or enzymes which modify antibiotics, circumvent the target or result in reduced accumulation of the antibiotic (Bryan, 1976, 1979 and 1980). However, in some cases, plasmids induce structural alterations in the envelope of the bacterial cell by coding for a variety of changes in the specific components to which the resistance is attributed.

1.4.4.1. Effect of RP1 plasmid

There is some evidence that penetration of B-lactams can be impaired by R- plasmids (Curtis et al, 1973). This was later confirmed by Curtis and Richmond (1974) who concluded that intrinsic resistance gene(s) of the plasmid RP1 code for the synthesis of material in the OM of E. coli and thus prevents penicillin from gaining access to its target.

Richmond and Curtis (1975) investigated the action of B-lactamase-deficient mutants of RP1 on the penicillin resistance of env A mutants of E. coli which are altered in their surface properties, and have a lowered resistance to penicillins. They found that the MIC of penicillin for env A cells had increased due to the presence of RP1 which probably carried a gene (or genes) that could repair the env A lesion, suggesting that the R-plasmid determinants are closely attached to those that increase the intrinsic resistance of normally occuring strains. Studies with E. coli UB 100S, its envelope mutant DC2 and both strains with the RP1 R- plasmid likewise suggest that RP1 is responsible for changes occurring in the cell envelope that modify susceptibility to some agents (Ahonkhai and Russell, 1979). Transfer of RP1 to P. aeruginosa strains also appears to induce envelope changes in this organism, but all the E. coli and P. aeruginosa strains tested, i.e. with or without R- plasmid RP1, remain sensitive to polymyxin B (Ahonkhai and Russell, 1979).

The cell wall composition of P. aeruginosa is influenced by RP1 with changes in phosphatidylserine and, especially, lysyl phosphatidylglycerol (Kenward et al, 1976) but with reduced cation levels. Walls from R+ cells contained less Mg^{2+} Ca^{2+}, KDO and diphosphatidylglycerol, while they had a higher content of lysyl-phosphatidylglycerol, phosphatidylethanolamine and diamino pimelic acid. When extracted lipids were examined, it was found that plasmid-free cells lost most of their magnesium, clacium and phosphorus, whereas no significant loss occured during the extraction of plasmid-carrying cells. The authors concluded that the divalent cations were more firmly bound, and that they play a role in resistance of RP1-carrying strains to EDTA.

Changes in cell wall structure and composition of P. aeruginosa, associated with the presence of the plasmid RP1, is also indicated by the variation in sensitivity of the cells to EDTA, polymyxin B and cold shock (Kenward et al, 1978. The R+ strain was resistant to the lytic action of EDTA. R- and R+

strains were both sensitive to the lytic action of polymyxin B and the lethal action of cold shock, but the effect was less marked in the R+ cultures. It was also shown by Klemperer et al (1980) that RP1-containing E. coli cells grown in NB were more sensitive to cetrimide and chlorhexidine than the equivalent R- ones. However, the level of sensitivity depended upon whether the R- and R+ strains were depleted of glucose, magnesium or phosphate. When H+ binding capacity was measured, R+ glucose-depleted cultures showed increased binding which was attributed to the presence of the plasmid.

1.4.4.2. Effect of other plasmids

Plasmids may cause proteins to be incorporated into bacterial OM. Beard and Connolly (1975) noted that the OM of E. coli containing a derepressed derivative of R1 plasmid (R1drd19), gave rise to many pili which were absent in strains containing the repressed R- plasmid. Analysis of the derepressed R- plasmid strain revealed the presence of a protein with an apparent M_r of 12,500. This protein was absent from the strain with a repressed R- plasmid.

It is well known that gram-negative cells are less susceptible to antibacterial actions of antibiotics than gram-positive cells, mainly due to LPS in the envelope which provides an effective permeability barrier. The presence of R- plasmids (previously known as R- factors) in E. coli rendered it more susceptible to attack by rifampicin derivatives (Soctti et al, 1974). These antibiotics have side chains with free carboxyl groups that do not penetrate easily into the LPS barrier of "normal" cells. The sensitivity testing carried out showed that R- plasmid containing strains had lost their resistance to rifampicin, but not to penicillin, this observation led to the conclusion that the presence of an R- plasmid did not render the envelope non-specifically leaky and it is possible that some structural component of the LPS wall was being coded for by the plasmid.

In a few cases, the metabolism of LPS (Derylo et al, 1975) and other components of the bacterial cell wall (Hesslewood and Smith, 1974) has been shown to be controlled by plasmids. Hesslewood and Smith (1974) studied envelope alterations caused by the presence of R- plasmids in Pr. mirabilis. They found that the ability of this organism to swarm was enhanced by the presence of the plasmid R-1818, but reduced by the presence of the plasmid R-TEM. However, when both R- plasmids were incorporated into the same cell, it was the one which promoted swarming which was phenotypically expressed. The level of extracellular proteolytic enzyme liberated by the cell was elevated in those cells containing the R-plasmid which enhanced swarming. The possibility that the R- plasmid was producing an alteration in the cell envelope rather than coding for enzyme formation was confirmed when it was observed that cells bearing the R- plasmid coding for decreased swarming were more susceptible to attack by sodium deoxycholate (Hesslewood and Smith, 1974). Since it is known that surface active agents can aggregate the LPS on the surface of gram-negative cells, it is likely that an alteration by the R- plasmid is produced in this region.

Kopecko et al (1980) and Sansonetti et al (1981) reported that a large plasmid, form I, controls the synthesis of the Shigella sonnei form I O-side chains. Antigenic variation in S. sonnei due to form I plasmid loss was associated with changes in the morphology of the colonies. A similar observation was found with S. flexneri (Sansonetti et al, 1982).

Recently, Rosas et al (1983) obtained physico-chemical evidence for the involvement of the plasmid P424 in E. coli envelope alterations. The elimination of this plasmid from E. coli RC424 (wild type) by sodium dodecyl sulphate or ethidium bromide altered its colonial character from smooth to rough (E. coli RC-7) and was associated with an increase in the size of the colonies. The cured bacteria conserved most of the original biochemical properties of the

parent strain. However, some other changes were also observed, such as loss of resistance to chloramphenicol and tetracycline, acquired resistance to ampicillin and the inability to grow in minimal medium and in the presence of detergents. There was a correlation between the loss of resistance to antibiotics and altered morphology of cured bacteria. When E. coli RC424 and cured E. coli RC-7 cells were examined with the transmission electron microscope, the latter showed loss of the two layers corresponding to the OM, increase in peptidoglycan layer thickness and diminished or even absence of a periplasmic space.

In conclusion, all the available evidence suggests that R- plasmids may produce alterations at different sites of bacterial cell envelopes.

1.5. Survival of Bacteria

Introduction

Survival is the avoidance of death threatened by adverse conditions. These conditions can vary widely in their nature and effect on microbial activity. The majority of bacteria can survive, sometimes for a considerable period, in the absence of one or more nutrients and in the presence of one or more adverse conditions, such as starvation, cold, heat, drying and osmotic stress.

1.5.1. Effect of environment on survival of bacteria

The survival characteristics of bacteria depend on the type of organisms and on several other factors, for example, the growth phase from which they are taken (Hegarty and Weeks, 1940; Brown, 1953; Goodlow and Leonard, 1961; Cox, 1966; Cox *et al*, 1971; Dark and Callow, 1973). The death rate of *E. coli* is highest with exponential-phase bacteria and the stationary-phase usually

survive best (Strange and Cox, 1976). The survival in aerosols of continuously-growing E. coli depends less on the growth-limiting substrate in a chemostat than on growth rate; slow growing bacteria survive much better than fast-growing bacteria (Dark and Callow, 1973).

In investigating the mechanisms operative in bacteria that allow them to survive in a dilute environment, Sjogern and Gibson (1981) found that several genera including Escherichia had lower survival rates than Klebsiella when suspended in lake water. They also found that E. coli stressed in distilled water at pH 7.5 had a lower survival rate than at pH 5.5. The higher survival rate of Klebsiella and E. coli at pH 5.5 was attributed to their ability to metabolize internal polymeric components and utilize proton gradients generated by lowering the pH of the environment.

The survival of bacteria during starvation closely correlates with the rate of endogenous metabolism, with slower rates favouring survival (Robertson and Batt, 1973). Druilhet and Sobek (1984) found that S. enteritidis used acid-alcohol soluble material (proteins or peptides) and RNA as endogenous reserves during starvation. E. coli has also been shown to utilize RNA and proteins during starvation (Nath and Koch, 1971).

Starvation has been found to induce the synthesis of anion selective OM protein, transport system for glycerol-3 phosphate which is analogous to the E. coli Pho E porin (Bauer et al, 1985). Thus starvation may modify the nutrient uptake by the organism.

Bacteria employ various survival mechanisms under starvation conditions. Some of the alterations in bacterial surface characteristics during exposure to starvation regime have been studied by Kjelleberg and Hermansson (1984). They found changes in bacterial surface hydrophobicity, charge, and degree of reversible binding to glass surfaces during starvation.

The influence of moisture content on the survival of six faecally-associated bacteria including E. coli present in raw wastewater sludge has been studied by Ward et al (1981). Initially, moisture removal by evaporation enhanced the bacterial survival rate, however, further reduction of moisture content below 50% (by weight) caused a proportional decrease in bacterial numbers. Pr. mirabilis was the most sensitive to the reduction in moisture content and the survival rate was decreased by four orders of magnitude. The effect of ionizing radiation on the same group of bacteria revealed that only S. typhimurium had the highest resistance to radiation. Very similar results were reported by Yeager and Ward (1981).

Drying of micro-organisms without freezing or cooling as a method of preservation has been well studied. Storage after drying in vacuum over P_2O_5 resulted in a good survival of several bacterial species over a period of 4 years (Stamp, 1947). Similar results were obtained by Rhodes and Fisher (1950) with pathogenic and non-pathogenic bacteria.

The survival rate of several bacterial species including Escherichia, when counted immediately after fast and slow drying (20min and 24h respectively) was very similar. However, after prolonged periods of dry storage, the number of viable cells after slow drying was much higher as compared with the rapidly dried cells (Antheunisse and Arkenstèijn-Dijksman, 1979).

Recently, Antheunisse et al (1981) carried out a series of experiments on the survival of micro-organisms after drying and storage. Bacteria suspended in a dextran solution were added to ampoules containing strips of filter paper which were dried without vacuum conditions. The ampoules were sealed and stored in the dark at room temperature. It was found that bacterial cultures of many genera including Escherichia did not show much resistance against dry conditions. Escherichia was not recovered after a storage period

of two years. They concluded that the viability of bacteria after drying and storage on filter paper is less pronounced than that after lyophilization or sealing and storage.

Temple et al (1980) compared the survival rate of E. coli and S. typhimurium in faeces buried in soil under field conditions. The survival pattern of the two enterobacteria was similar at all sites in spite of marked differences in elevation, soil, moisture, exposure and vegetation. However, the overall survival rate of S. typhimurium was higher than E. coli.

Hood and Ness (1982) showed that different organisms respond differently to environmental changes. Survival of V. cholerae varied significantly with incubation temperature. The organism was observed to survive better at 35^{o} than at 25^{o} or 4^{o} in sterile estuarine water and sediments. Furthermore, the organism did not survive in non-sterile sediments at any temperature. In contrast to V. cholerae, E. coli survived better in non-sterile sediments but it did not survive well in sterile estuarine water or sediments. The authors suggested that the survival of V. cholerae was due to the absence of competing organisms and abundance of available nutrients.

Bacteria also survive differently under various environmental temperatures. In a study using four strains of different Proteus species and one of Providencia, the survival rate was determined in two types of sterile soil. At temperature of $18\text{-}20^{o}$ the survival rate was higher than at temperatures of 4^{o} and 37^{o}, it was suggested that the lower survival rate was due to a cold shock at 4^{o} and high metabolic rate at 37^{o} (Papaconstantinou et al, 1981).

1.5.2. Effect of R- plasmid carriage on survival of bacteria

The effect of plasmids on the survival of the host cell has been studied by several workers. Anderson (1973) found that R- plasmid containing E. coli died more rapidly on storage in saline or water and failed to grow as quickly

in broth as corresponding R- plasmid free organisms. Viable counts of R- plasmid RP1 containing E. coli declined faster than of the the R- plasmid-free cultures when each was stored on nutrient agar or simple salts agar slopes (Klemperer et al, 1979).

Kenward et al (1978) showed that the presence of RP1 plasmid in P. aeruginosa decreases the host's sensitivity to lethal action of cold shock. It was suggested that this enhanced survival of R+ cells exposed to cold shock was due to the existence of R- plasmid-coded modifications of the inner and outer membrane. This suggestion was further supported by the inability of 50 to 60% of the R+ cold shock survivors to grow on agar containing, respectively carbenicillin or kanamycin which suggested that these cells may have received non-lethal damage to the outer membrane resulting in the loss of B-lactamase and kanamycin phosphorylase from the periplasmic space.

Dale and Smith (1979) found both E. coli 114 (PH 121) and R- isogenic strains survived extended incubation (150 days at 37^{o}) when grown separately in nutrient broth. However, the authors were not able to demonstrate any difference in the growth characteristics and the survival of the two strains. Recently, Alldrick and Smith (1983), obtained similar results using E. coli containing either the plasmid R46 or its non-self-transmissible derivative in the presence or absence of the isogenic R- parent strain. Neither plasmids conferred any detectable effect on the host's ability to multiply. Similarly under conditions of prolonged incubation neither plasmids conferred a disadvantage on its host when the bacteria were grown in pure culture.

Lacey (1972) found that the presence or absence of individual plasmids in Staphylococci had little effect on their capacity to survive on glass. Similarly, on comparing the survival of strains 649 MR (containing a large number of plasmids) and 649 N (plasmid-free) on glass relative to a standard

strain (no. 6936) at 21^{o}, 30^{o} and 37^{o}, no difference was found in their relative survival (Lacey and Chopra, 1975).

1.6. Growth Requirements

Bacteria, like all other living organisms, require certain nutrients for growth. These nutrients must contain those chemical elements that are constituents of the cellular nutrients and that are necessary for the activity of enzyme and transport systems. In addition, the nutrients must provide the organisms with materials for the production of biologically utilizable energy. These requirements are supplied partially by carbohydrates that are not used exclusively as a source of energy and partially from certain nitrogenous compounds that are built into proteins. Apart from the substances that are synthesized to form the cell mass, there are those nutrients that aid the synthetic processes, such as mineral salts and vitamins. Like man, many micro-organisms are incapable of synthesizing the latter category of essential compounds so that they must be present pre-formed in the basic diet for the proper functioning of the cells.

Almost all members of Enterobacteriaceae grow readily in simple salts media; for E. coli there are a number of widely used chemically-defined media such as that formulated by Davis and Mingioli (1950) and Vogel and Bonner (1956). These media consist essentially of glucose, NH_4^+, Mg^+ and SO_4^{2-} buffered with phosphates. Later, Neidhardt et al (1974) formulated a medium suitable for the growth of S. typhimurium and E. coli. The constitution of this medium was derived from the experimental data of the relation between growth yield and the concentration of several added nutrients for both organisms using a potassium morpholinopropane sulphonate buffer (MOPS). Recently, the specific nutrient requirements for the aerobic growth of E. coli with and without plasmid RP1 were studied by Ismail (1977) which led to the determination of quantitative minimal nutritional requirements for both R- and R+ strains (Klemperer et al, 1979). These consist of the metallic ions (Na^+, K^+, Mg^{2+}, Fe^{2+}), inorganic salts (SO_4^{2-}, PO_4^{3-}, NH_4^+) and glucose as a source of energy. The function of these nutrients is detailed in the discussion under the appropriate nutrient.

1.7. Bacterial Fermentation

1.7.1. Introduction

The term fermentation was first defined by Pasteur. He described fermentations as life in the absence of oxygen. Today fermentation can be defined as those biological processes that do not involve respiratory chains with oxygen or nitrate as electron acceptors (Gottschalk, 1979).

Fermentation is much less efficient than aerobic respiration, consequently, the energy (ATP) yield per molecule of carbohydrate consumed is low (Gottschalk, 1979). In addition the end products of fermentation are not so completely oxidized. Aerobic respiration requires oxygen for oxidative reactions to take place i.e. the oxidative breakdown of carbohydrates is accomplished by a series of dehydrogenations so that oxygen is the final hydrogen acceptor. This involves respiratory chains with high ATP yield. Similarly, fermentation involves a series of dehydrogenations, but in the absence of oxygen, substances other than oxygen accept the hydrogen and are thereby reduced.

The most common fuels for anaerobic fermentation are sugars, particularly D-glucose, but some bacteria can obtain their metabolic energy by anaerobic fermentation of fatty acids, amino acids, purines, pyrimidines depending on the species. In fact, the taxonomic classification of micro-organisms is in part based on their characteristic organic fuels and their fermentation products.

Bacteria carrying out fermentations are either facultative or obligate anaerobes. Facultative anaerobes such as the enterobacteria grow as aerobic

heterotrophs in the presence of oxygen; under anaerobic conditions they carry out a fermentative metabolism.

1.7.2. Fermentative metabolism

Fermentations are usually classified according to the main fermentation end products, such as alcohol, lactate, propionate, butyrate, mixed acids and butanediol fermentations.

Enterobacteria employ the Embden-Meyerhof pathway for hexose breakdown (Wood, 1961). The pathway leading to succinate branches off at phosphenol pyruvate; all other end products are derived from pyruvate. Three enzyme systems act upon pyruvate and the amounts in which the fermentation products are formed depend very much on the activity of these enzyme systems (Gottschalk, 1979). In the mixed acid fermentation large amount of lactate are formed by the action of lactate dehydrogenase, while little is produced in the butanediol fermentation.

1.7.2.1. Mixed acid fermentation

These types of sugar fermentations are carried out by the enterobacteria. The products vary, both qualitatively and quantitatively. These fermentations have one characteristic biochemical feature, which is not encountered in any other bacterial fermentation. This is a special mode of cleavage of the intermediate, pyruvic acid, to yield formic acid:

$$\underset{\text{(pyruvic acid)}}{CH_3COCOOH} + (CoA)SH \rightarrow CH_3COS(CoA) + \underset{\text{(formic acid)}}{HCOOH}$$

Formic acid is, therefore, frequently a major fermentative end product. It does not always accumulate, however, since some of these bacteria possess the enzyme formate hydrogen lyase[4], which splits formic acid to CO_2 and H_2.

$$HCOOH \xrightarrow{4} CO_2 + H_2$$

In such organisms, formic acid is largely replaced as a fermentative end product by equimolecular quantities of H_2 and CO_2.

Organisms belonging to the genera Escherichia (E. coli is the classical representative of enterobacteria and one of the most characteristic members of the normal intestinal flora of man), Salmonella and Shigella ferment sugars to acetic, formic, lactic and succinic acids (Barker, 1956). In addition ethanol, CO_2 and H_2 may be formed. The pathways leading to all these products are summarized in Fig. 3.

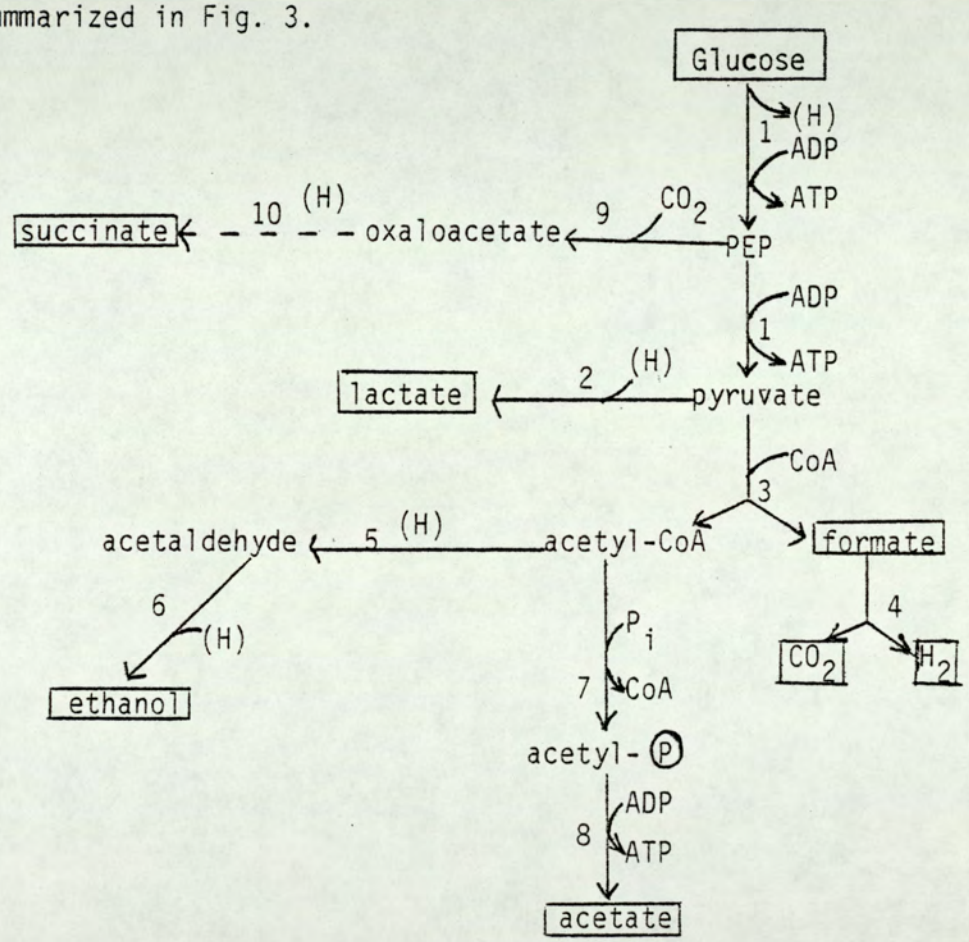


Fig. 3 Pathways of fermentation from glucose (mixed acid fermentations)

1, enzymes of Embden-Meyerhof pathway; 2, lactate dehydrogenase; 3, pyruvate-formate lyase; 4, formate-hydrogen lyase; 5, acetaldehyde dehydrogenase; 6, alcohol dehydrogenase; 7, phosphotransacetylase; 8, acetate kinase; 9, PEP carboxylase; 10, malate dehydrogenase, fumarase, and fumarate reductase; (From Gottschalk, G., 1979).

The most frequent mode of fermentative sugar break down in E. coli is the mixed acid fermentation which yields lactic, acetic acid and succinic acids; formic acid (or CO_2 and H_2) and ethanol. The ratios of the end products may vary considerably, both from strain to strain and in a single strain grown under different environmental conditions e.g. in different growth media (Hernandez and Johnson, 1967). This variability reflects the fact that the end products arise from pyruvic acid through three independent pathways.

1.8. The Continuous Culture of Bacteria

1.8.1. Introduction

In batch cultures, bacteria undergo sequential growth phases. Following inoculation into a nutrient medium, a "lag" phase of variable length is followed by an "exponential" growth phase during which nutrients are consumed and metabolic end products are released into the medium. Growth remains exponential for only a few generations due to exhaustion of essential nutrient(s) A fall in pH and/or dissolved oxygen concentration and accumulation of toxic end products result in the culture entering the "stationary" phase. This growth cycle has been studied in detail by Monod (1942, 1949).

During the exponential growth phase the culture biomass doubles at a constant rate. Thus, if the initial concentration of organisms is X then

$$X = X_o e^{\mu t}$$

$$\log_e X = \log_e X_o + \mu t$$

$$\mu t = \log_e X - \log_e X_o = \log_e \frac{X}{X_o}$$

$$\mu = \frac{1}{t} \cdot \log_e \frac{X}{X_o}$$

When $X = 2X_o$ (i.e. doubles)

$$\mu = \frac{1}{t} \cdot \log_e \frac{2X_o}{X_o} = \frac{1}{t} \cdot \log_e 2$$

But when $X \rightarrow 2X_o$ then t is the doubling time

$$\mu = \frac{\log_e 2}{t_d} \qquad (1)$$

Where t_d is the doubling time, μ is the specific growth-rate constant (Fencl, 1963). The values of μ and td are influenced by the environment, particularly by the concentration of different essential nutrients. The dependence of μ on substrate concentration (S) could be represented (Monod, 1942, 1950) by a Michaelis-Menton type function i.e.

$$\mu = \mu_m \frac{S}{K_s + S} \qquad (2)$$

Where μ_m is the maximum value of μ (i.e. when S is no longer growth limiting). K_s is a saturation constant (numerically equal to the growth limiting substrate concentration at which half of the maximum specific growth rate is reached). In batch culture all nutrients are initially present in excess, hence the exponential growth rate is equal to μ_m.

Monod (1942) showed that there is also a simple relationship between growth and utilization of substrate. This is shown in its simplest form in growth media containing a single organic substrate (for example, glucose, ammonia and salts). Under these conditions the growth rate is a constant fraction, Y, of the substrate utilization rate:

$$\frac{dx}{dt} = -Y \frac{ds}{dt} \qquad (3)$$

Where Y is the yield constant. Thus over any specific period of growth

$$\frac{\text{weight of bacteria formed}}{\text{weight of substrate used}} = Y$$

If the value of the growth constants μ_m, K_s and Y are known equations (1) and (3) provide a complete quantitative description of the "growth cycle" of a batch culture (Monod, 1942). The same constants and equations are equally applicable to the theoretical treatment of continuous culture.

All continuous cultures start as batch cultures and follow the growth cycle described earlier. However, if during exponential growth phase fresh medium is allowed to flow continuously into the culture vessel at a rate equal to that of the outflow, where by the culture grows at a specific growth rate less than μ_m,

then the culture is said to be growing continuously and the culture apparatus is described as a chemostat (Novick and Szilard, 1950b).

1.8.2. Theory of the chemostat

In a chemostat vessel, assuming complete mixing and constant volume, V, medium flows into and out of the vessel at a steady rate, f. The growth rate of bacteria is governed by the dilution rate, D, defined as f/V. D is the number of complete volume changes per unit time and is usually expressed as h^{-1}. Thus the rate at which bacteria are washed out is proportional both to the number remaining, X, and to D

$$-\frac{dX}{dt} = DX \qquad (4)$$

and $\frac{1}{D}$ is the mean residence time of a bacterial cell (Powell, 1965).

In the culture vessel the organisms are growing at a rate expressed by equation (1) and simultaneously being washed out at a rate determined by equation (4). The net rate of increase of concentration of organisms can be predicted from the given balance equation

rate of increase = growth rate - rate of washout

$$\frac{dX}{dt} = \mu X - DX$$

$$= X(\mu - D) \qquad (5)$$

If $\mu > D$, $\frac{dX}{dt}$ is positive and therefore the concentration of the organisms will increase with time, while if $D > \mu$, $\frac{dX}{dt}$ is negative and the concentration of organisms will decrease, eventually to zero. When $\mu=D$, $\frac{dX}{dt} = 0$ and since X is constant, the culture will be in a steady state in which the concentration of organisms does not change with time. Under this condition, the specific growth rate, μ, of the organisms in the chemostat is exactly equal to the dilution rate D.

In the chemostat, the growth limiting substrate is entering at a concentration S_R (reservoir concentration), being mainly consumed by the

organisms and the residue flowing out at a concentration $\bar{S}$; the net rate of change of substrate concentration can be obtained by another balance equation (Herbert et al, 1956).

rate of increase of substrate = rate of input - rate of output - rate of consumption

$$= \text{rate of input} - \text{rate of output} - \frac{\text{growth}}{\text{yield constant}}$$

(From equation 3)

$$\text{therefore } \frac{dS}{dt} = DS_R - DS - \frac{\mu X}{Y} \qquad (6)$$

Substituting μ from equation (2) in equations (5) and (6), it follows that from (5)

$$\frac{dX}{dt} = X \quad \mu_m \quad \frac{S}{K_s + S} - D \qquad (7)$$

and from (6)

$$\frac{dS}{dt} = D\,(S_R - S) - \frac{\mu m X}{Y} \quad \frac{S}{K_s + S} \qquad (8)$$

The last two equations (7 and 8) comprehensively define the behaviour of a continuous culture in which the basic growth relations are given by equations (1), (2) and (3). In a steady state S_R and D are kept constant; if D does not exceed a definite critical value (see Fig. 4), then constant values of $\bar{X}$ (steady state bacterial concentration) and $\bar{S}$ (steady state growth-limiting substrate concentration) will exist for which both $\frac{dX}{dt}$ and $\frac{dS}{dt}$ are zero (Herbert et al, 1956). The steady state values of $\bar{X}$ and $\bar{S}$ can therefore be determined by solving equations (7) and (8):

$$\bar{S} = K_s \quad \frac{D}{\mu_m - D} \qquad (9)$$

$$\bar{X} = Y\,(S_R - \bar{S}) = Y \quad S_R - K_s \quad \frac{D}{\mu_m - D} \qquad (10)$$

The steady state growth limiting substrate concentration, $\bar{S}$, is independent of $\bar{X}$, Y or S_R (equation 9).

From these equations the steady-state concentrations of bacteria and

substrate in the chemostat can be predicted at any value of D and S_R, provided the values of the growth constants μm, K_s and Y are known (Monod, 1950).

In a continuous culture variations of dilution rate lead to changes in the mean generation time and the steady state concentrations of bacteria and substrate (Herbert et al, 1956). The concentration of bacteria has a highest value when the dilution rate is zero (Fig. 4), the chemostat substrate concentration then also being zero. As the dilution rate increases, the substrate concentration increases and the concentration of bacteria decreases, until a value of D, at which the concentration of bacteria becomes zero and the chemostat substrate concentration becomes equal to S_R. Under this condition

$$D_c = \mu m \frac{S_R}{K_s + S_R} \qquad (11)$$

D_c is equal to the highest possible value of μ, which is the value obtained when $\bar{S}$ has its highest possible value S_R (Herbert et al, 1956). When $S_R \gg K_s$, which is true in most cases, then $D_c \simeq \mu_m$. Furthermore from equation (5), at all dilution rates greater than D_c, $\frac{dX}{dt}$ is negative and bacteria will be washed out of the culture vessel faster than they can grow.

The variation with the dilution rate of the steady state concentrations of bacteria and substrate for a number of different values of the inflowing substrate concentration, S_R, is shown in Fig. 5 (Herbert et al, 1956). It can be seen that at a given dilution rate below the critical the concentration of organisms is nearly proportional to S_R, but the concentration of substrate, $\bar{S}$, is independent of S_R i.e. the plot relating D to $\bar{S}$ is the same whatever the value of S_R (Novick and Szilard, 1950).

The curve relating concentration of organisms to dilution rate is seen (Fig. 5) to be displaced vertically as S_R increases, the drop in $\bar{X}$ at high dilution rates being steeper for higher values of S_R. The important facter here is the ratio S_R/K_s (see equation 2). The higher this ratio, the greater

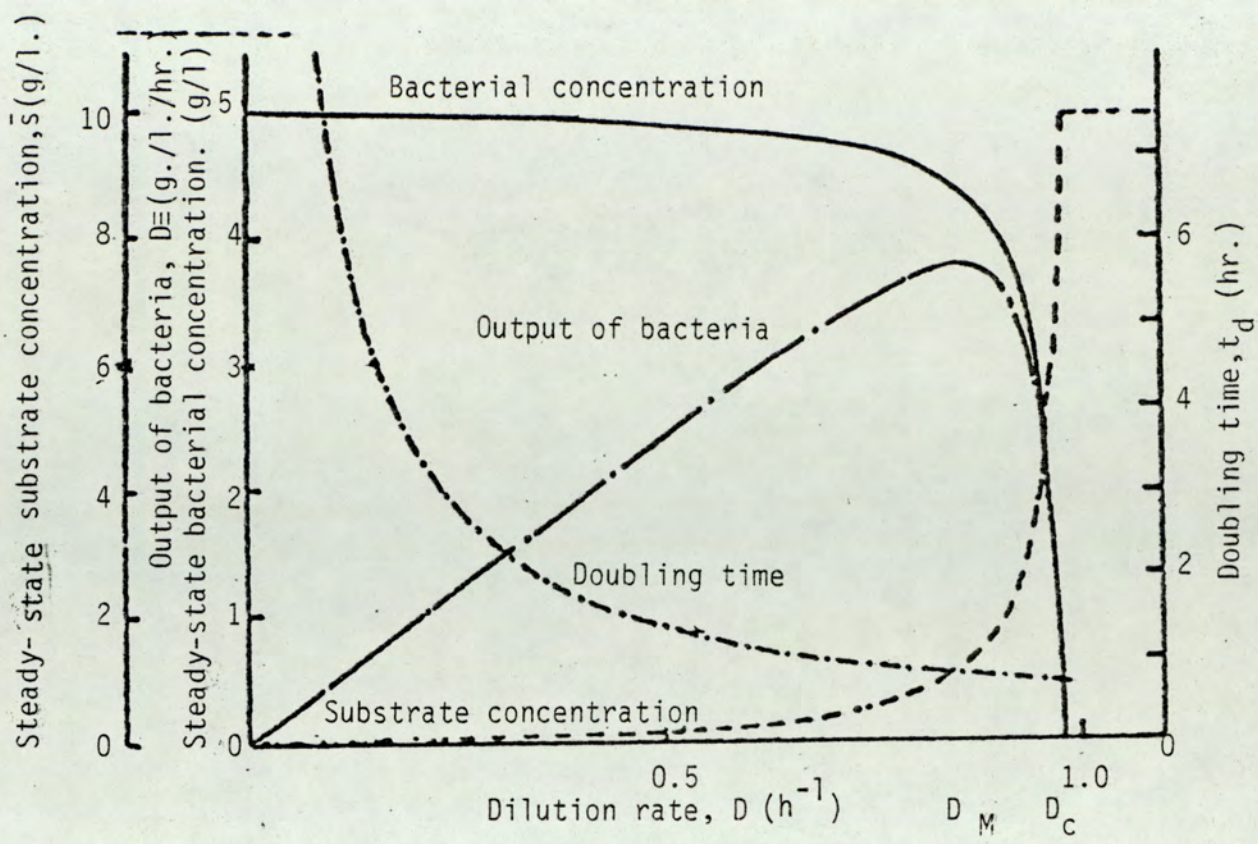


Fig. 4 Steady-state relationships in a continuous culture (theoretical). The steady-state values of substrate concentration, bacterial concentration and output at different dilution rates are calculated from equations (9) and (10) for an organism with the following growth constants: $\mu m = 1.0 hr^{-1}$; $Y = 0.5$ and $K_1 = 0.2$g./l.; and a substrate concentration in the inflowing medium of $S_R = 10$ g./l. (From Herbert et al, 1956)

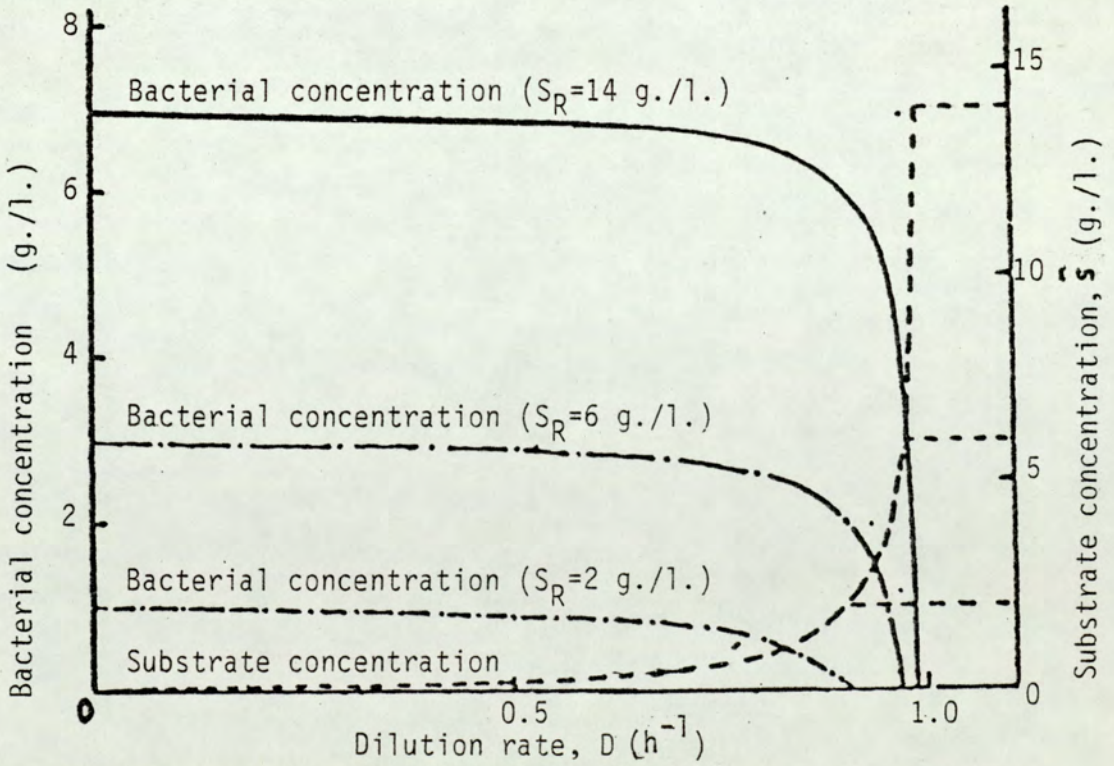


Fig. 5 Effect of varying the concentration of substrate in the inflowing medium (S_R) on steady-state relationships in a continuous culture (theoretical). The curves are calculated from equation (9) and (10) for an organism with $\mu m = 1.0 h^{-1}$, $Y = 0.5$, and $K_s = 0.1$ g./l., for media of three different substrate concentrations. (From Herbert et al, 1956).

the fraction of total substrate that can be consumed without a significant decrease in specific growth rate. Therefore as S_R/K_s is increased the concentration of bacteria is maintained at almost the maximum level up to higher values of D and the critical dilution rate D_c approaches more closely to μ_m (see equation 11).

1.8.3. Theoretical consideration of competition in continuous culture: competition for growth-limiting substrate

Fig. 6 describes the growth characteristics of two organisms A and B with a common growth-limiting substrate. In a steady state culture of organism A, at a given dilution rate the growth-limiting substrate concentration ($\bar{s}$) is maintained at a level characteristic for organism A. If, as in the case of Fig. 6(a), organism B is introduced into a culture of A, the specific growth rate of organism B must be lower than that of organism A at that substrate concentration. This holds for all dilution rates. As a result, organism B is washed out of the culture since it cannot grow at the required rate. In the case of Fig. 6 (b) μ_B would be higher than μ_A at high dilution rates and organism A would be washed out. The reverse would be true at dilution rates below the crossing point of the two curves. Theoretically, co-existence can occur at the crossing point, but it can be shown mathematically that this is essentially an unstable condition (Frederickson, 1977). It is important to recall that $\bar{s}$ is not dependent on the yield of organisms (equation 9, section 1.8.2.) and therefore Y has no influence on the outcome of the competition in the chemostat. One of the crucial requirements for the validity of the arguments as discussed here is that no other interactions occur between organisms A and B (Powell, 1958).

The theoretical models of growth and competition in the chemostat, as based on Monod kinetics, can often account for the behaviour of pure and mixed cultures in spite of the fact that a number of simplifications are made.

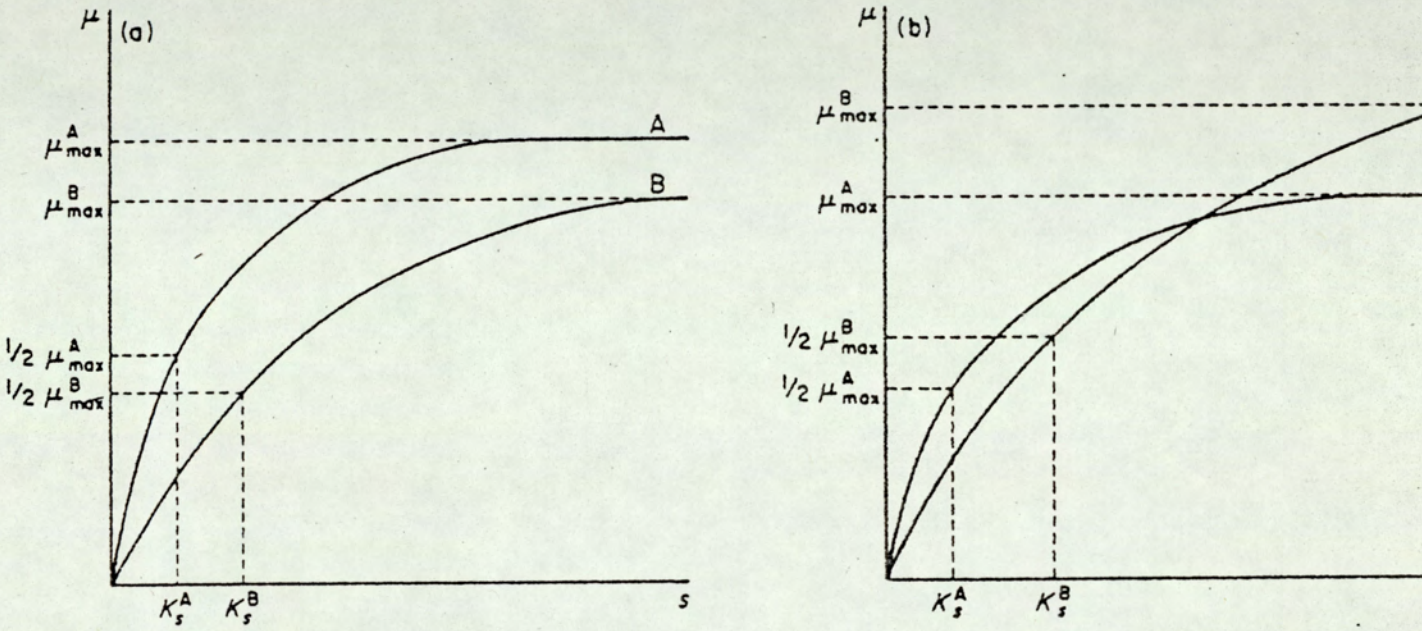


Figure 6 The μ/s relationship for organisms A and B. (a) $K_s^A < K_s^B$ and $\mu^A_{max} > \mu^B_{max}$; (b) $K_s^A < K_s^B$ and $\mu^A_{max} < \mu^B_{max}$ (after Veldkamp 1970).

However, many derivations have been described. First, not all organisms show a typical Monod growth response (Dijkuizen and Harder, 1975). Furthermore, the growth yield is often not constant (Stouthamer, 1979) and may vary with the growth rate due to changes in the cell composition, changes in the efficiency of substrate utilisation or maintenance energy requirement. If in a culture not all the cells are viable, the outcome of the competition may be different for a variety of reasons, the most important being that organisms must grow at a rate higher than the dilution rate in order to maintain themselves in the culture (Pirt, 1975). Another deviation from the idealised behaviour may be due to population effects (Kuenen and Harder, 1982), such as when growth-stimulating or growth-inhibitory substances are excreted, changing the growth characteristics of the organism(s). A special case is the growth of organisms on toxic compounds which become growth inhibitory at relatively low levels. The substrate saturation curve then shows a clear maximum at a low concentration of the substrate and above that declines to zero (Fig. 7).

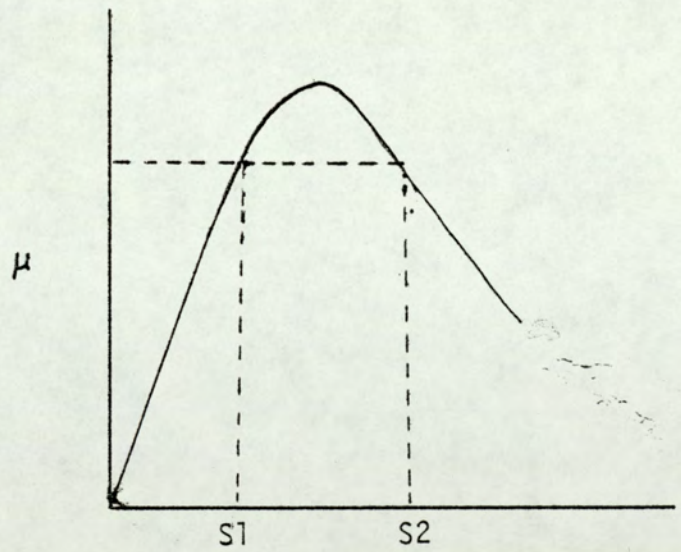


Fig. 7 Relation between the specific growth rate and the concentration of toxic substrate (from Kuenen and Harder, 1982).

Empirical formulae have been proposed to describe the kinetics of growth under these conditions. The Haldane equation is the one most commonly used (Pawlowski and Howell, 1973):

$$\mu = \mu_m \quad \frac{S}{(K_s + S)(1 + S/K_i)}$$

where K_i = the inhibition constant and μ_m = the theoretical maximum specific growth rate.

As pointed out by Veldkamp and Jannasch (1972) and by Harder et al (1977), steady states in the chemostat at submaximal growth rates are theoretically possible at two discrete substrate concentrations, S_1 and S_2 (Fig. 7). Steady states are only possible at a substrate concentration, such as S_1, which is below the concentration allowing maximal growth rate. At concentration S_2 a minor change in the flow rate will lead either to an increase in the concentration of the toxic substrate or to a decrease. In either case the result will be growth inhibition, a subsequent increase of $\bar{S}$, further growth inhibition until eventually the culture washes out. In the former case the substrate concentrations will go down, the growth rate will subsequently increase, lowering even further the substrate concentration. This will eventually lead to the establishment of a new steady state at S_1. Growth at concentration S_2 is possible only by controlling the substrate concentration directly, for example by automatic monitoring, with a substrate-specific electrode.

1.8.4. Bacterial adhesion and growth on the chemostat wall

The importance of surfaces for microbial growth is well recognised. Heukelekian and Heller (1940) showed that the growth of E. coli in glucose-peptone water at glucose concentrations of less that 25 mg l^{-1} was possible only in the presence of glass beads. Jannasch (1958) found that the growth of Bacillus subtilis in dilute media occurred only in the presence of chitin

particles. Furthermore, in a series of studies, Corpe (1970, 1974) showed that bacterial growth in supplemented water was restricted to a glass surface when very low nutrient concentrations were used. Enteric bacteria adsorped to glass surfaces were metabolically more active than organisms in free suspension (Hendricks, 1974). When considered collectively, the above evidence suggests that surfaces have a positive influence on the growth of micro-organisms.

The generally accepted explanation of surface-enhanced growth is that as increased concentrations of molecules occur at an interface, there will be an increased concentration of limiting nutrient at the surface (Marshall, 1976). However, recently Ellwood et al (1982) have proposed an alternative hypothesis based on ideas derived from the chemiosmotic hypothesis of energy conservation (Fig. 8).

The chemostat wall provides a unique environment for the microbial growth. The attachment of bacteria to solid surfaces involves an interaction between the surface and the outer layers of the bacteria. There are three stages in the adhesion of micro-organisms to a surface (Marshall, 1976).

1. Adsorption of the organism on to the surface which is a reversible process.
2. Permanent attachment to the surface, where polymers act as a bridge between the two surfaces.
3. Colonization of the surface by growth of the organism.

The major surface forces operating in adsorption process are (a) London-Van der Waals forces (b) double layer electrostatic interactions, and (c) bridging interactions (Rutter, 1980).

Thus, in general, as bacteria approach a surface, the van der Waals and electrostatic attractive forces can hold cells on to the surface for a short time; during this period cell-surface polymers interact with the surface (polymer bridging). This increases the probability of attachment until enough

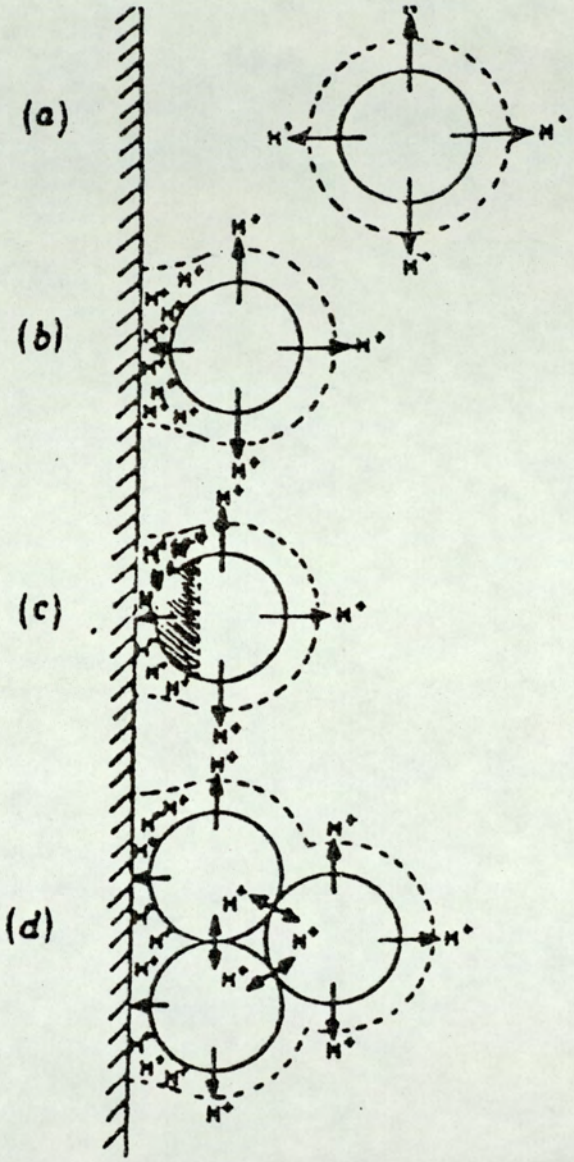


Fig. 8 Postulated chemiostatic interactions of a cell at a surface. The diagram describes (a) a cell in free suspension generating a proton gradient, (b) the interaction of the cell's domain with a surface, establishing a localized higher concentration of extruded protons, (c) localized Δp and ATP synthesis leading to increased metabolic activity and polarity of the cell to drive adhering processes in this region, and (d) the establishment of a microcolony with sharing of proton gradients between members. (After Ellwood et al, 1982).

links or bridges have been formed to hold the cell irreversibly. Further consolidation of attachment would occur by the synthesis of insoluble macromolecules such as mutan produced by S. mutans, that are extruded into the local environment, or by gelation of existing polymers (Morris et al, 1977), or even by precipitation of the polymers (Ash, 1979), perhaps by neutralization with divalent cations (Rutter, 1980). Growth of the attached cells would lead to the production of micro-colonies and eventually to film formation.

Adhesion of bacteria to surfaces is an essential first step in the colonization of microbial habitats, especially in locations where mechanical cleansing mechanisms are operative as in flowing waters and many surfaces in living animals. Adhesive interactions between bacteria and epithelial cells are consequently presumed to be crucial events in the colonization of mucosal surfaces by pathogenic as well as by commensal bacteria (Ofek and Beachey, 1980; Savage, 1980). The presence of specific adhesive mechanisms in different bacterial species illustrate the general importance of such interactions and may account, at least in part, for the specific distribution of organisms in different habitats (Gibbons and van Houte, 1975).

As a bacterium carrying adhesion factors on its surface comes into close contact with a host epithelial cell, it is thought to recognize the corresponding specific receptors located on the epithelial cell surface. The adhesion of bacteria to epithelial surfaces may subsequently lead to irreversible attachment of the bacteria and also the formation of adherent micro-colonies (Kallenius et al, 1980; Marrie et al, 1980). The rate and the affinity of this adhesive process is thought to be solely dependent on the interaction between bacterial adhesion factors and eukaryotic receptors structures (Vosbeck and Mett, 1983).

There is ample evidence that the adhesion of gram-negative bacteria is in most cases mediated by bacterial surface appendages, fimbriae or pili (Duguid

and Old, 1980). Fimbriae consist of protein subunits and are thought to interact in a lectin-like manner with surface carbohydrate structures of eukaryotic cells. They can be distinguished according to the molecular weights of subunits, their amino acid composition and most importantly, the specificity of their binding to certain eukaryotic cells and their antigenicity (Korhonen et al, 1981).

Enteropathogenic E. coli express number of different species-specific adhesion factors that can be distinguished serologically. Thus E. coli strains isolated from pigs with diarrhoea frequently carry adhesion factor K88 (Jones and Rutter, 1972). The genetic information for this adhesion factor as for many others, is usually located on a plasmid. Smith and Linggood(1971) have demonstrated that the expression of both K88 adhesion factor and enterotoxin is required to render an E.coli strain pathogenic.

Similar studies indicate the presence of specific bacterial adhesion factors in human enterotoxigenic E. coli strains. Evans et al (1978) and Evans and Evans (1978) described two adhesion factors, which they called colonization factor antigens (CFA/I and CFA/II) and which have been found in up to 86% of enterotoxigenic clinical isolates in different studies.

1.8.5. Effect of R- plasmid carriage on survival in competition with R- bacteria.

It has often been stated (for example, by Chabbert et al, 1969; Anderson, 1974; Lacey, 1975) that the presence of a plasmid will be disadvantageous to a bacterial cell, under non-selective conditions, because of the energy required, not only for the maintenance of the plasmid, but also for the associated RNA and protein synthesis. Only rarely do organisms carrying R- factors achieve a dominant position in the absence of this selective pressure (Linton et al, 1977).

The ability of a strain of E.coli carrying plasmid PH121 to compete with its R- counterpart in batch culture was investigated by Dale and Smith (1979); they found that when equal volumes of nutrient broth-grown overnight cultures of the two strains were mixed and then diluted 10^7-fold in fresh warm broth, the proportion of plasmid-carrying cells declines rapidly and none was detected after 100 days. The poor performance of R+ cells was attributed to the depletion of metabolizable substrates. At this point the organism would be expected to switch to the use of secondary substrates. However, these can only be metabolized aerobically, so the availability of oxygen rapidly becomes limiting. It is only under these conditions that R+ strain is at serious disadvantages. Similar results were obtained using plasmid R46 or its non-transmissible derivative in E. coli. When the incubation of mixed R-/R+ cultures was prolonged, the possession of either R- plasmid resulted in small but reproducible differences which favoured the R- strain (Alldrick and Smith, 1983).

On the other hand, aerobic chemostat competition studies on E.coli carrying RP1 showed that the R- strain always predominates under phosphate-limited conditions (Melling et al, 1977), with carbon and Mg^{2+} limitations it is the proportions of R+ and R- cells added which determine the outcome. These data indicates the importance of the bacterial environment on the survival of an R+ strain in competition with an R- one. Competition experiments carried out by Wouters et al (1978) showed that E. coli R- cells were able to overgrow the isogenic R+ cells carrying plasmid R6 and a rapid takeover by the R- cells occurred under anaerobic conditions with phosphorus, as well as nitrogen and carbon limitation. The authors did not explain the cause of this take over.

However, Lin et al (1977) showed that lambda lysogens of E. coli have a growth advantage over non-lysogens when grown in glucose-limited aerobic conditions, whereas during anaerobic growth the situation is reversed and the non-lysogens won the competition.

The results of all these competition experiments indicate the importance of the bacterial environment on the survival of the strain with extragenetic elements. Carrying extrachromosomal DNA might be a burden for a bacterium, placing it in an unfavourable competition position during restrictive growth, but the outcome of the competition experiments indicates that this is not always true. In some instances, the extra genes of plasmids might give rise to properties that enable the carrier to survive under certain growth conditions.

The human gastro intestinal tract is of particular interest as a site for study of the survival and colonisation of R- factor-bearing organisms under natural conditions because it contains a captive population of various organisms that might be involved in R- factor transfer. In vivo Anderson (1974) reported that strains of E. coli carrying R- factors (which mediated resistance to different antibiotics) disappeared from faeces more rapidly than R- factor free strains, when ingested by healthy individuals, who had not been exposed to antibiotics. In contrast, other R+ E. coli persisted in the absence of antibiotics (Hartley and Richmond, 1975). The authors suggested that such persistence seemed to depend more on the nature of the strain than on the plasmid carried.

2. MATERIALS

2. MATERIALS

2.1. Organisms

2.1.1. Properties

Escherichia coli K12 W3110 was kindly provided by Dr. P. A. Meacock (Department of Genetics, University of Leicester).

It is a "wild-type" K12 cured of sex factor F and bacteriophage λ (Bachman, 1972) and is sensitive to ampicillin (25 μg ml^{-1}), streptomycin (25 μg ml^{-1}), tetracycline (25 μg ml^{-1}) and chloramphenicol (50 μg ml^{-1}). (Meacock, personal communication).

The R- plasmid RP1, mediating resistance to β-lactam antibiotics, kanamycin and tetracycline (Lowbury *et al*, 1969; Richmond *et al*, 1972; Grinsted *et al*, 1972) was kindly provided by Dr. S. R. Hesslewood (present address: Department of Physics and Nuclear Medicine, Dudley Road Hospital, Birmingham B18 7QH), in *E. coli* J6-2-2, and transfered from there to *E. coli* K12 W3110 by Ismail (1979).

Throughout this work, *E. coli* K12 W3110 is designated R- and *E. coli* K12 W3110 carrying the plasmid RP1 is designated R+.

2.1.2. Maintenance of stock cultures

The purity of the R- strain was checked by plating on MacConkey and nutrient agar. After overnight incubation at 37^{o} one of the resultant colonies was streaked on a nutrient agar slope, incubated overnight at 37^{o} and stored at room temperature. Fresh agar slopes were prepared fortnightly and strain identity was checked at regular intervals using an API 20E diagnostic kit.

The R+ strain was maintained in the same way, except that tetracycline or kanamycin (15 μg ml^{-1}) was added to the nutrient agar plates to make sure that only R+ colonies were used to inoculate slopes.

2.2. Chemicals

Water: glass double-distilled water was used throughout this work for media and most solutions.

The chemicals used in the preparation of all media were of Analar grade (B.D.H. Chemicals Ltd., Poole, Dorset, England and Fison Scientific Apparatus Ltd., Bishop Meadow Road, Loughborough, Leicestershire LE11 ORG, England) or equivalent.

All chemicals for PAGE were of Analar grade or the highest grade obtainable and were purchased from B.D.H. Chemicals Ltd. (address see above).

2-Keto-3-deoxyoctonic acid (KDO), kanamycin sulphate and sodium N-lauroyl sarcosinate (sarkosyl) were obtained from Sigma Chemical Company, London or Drawer 2, 3500 De Kalb Street, St. Louis, Mo. 63118, U.S.A.

Ampicillin (Penbritin) was obtained in vials containing 500 mg ampicillin as trihydrate (Beecham Research Laboratories, Brentford, Middlesex, TW8, U.K.).

Tetracycline was obtained as achromycin tetracycline hydrochloride from Lederle Laboratories, Fareham Road, Gosport, Hants., England.

Tween 80 (Polysorbate 80) was obtained from Koch-Light Laboratories, London, England.

Sodium deoxycholate (DOC) was obtained from Oxoid Ltd., Basingstoke, Hampshire, U.K.

Reagents for the colorimetric, enzymatic determination of glucose were supplied in kits from Sigma Chemical Company, U.S.A. (address see KDO).

Table 4

Chemically defined media, concentration (mM) of nutrients used in aerobic cultures of *E. coli* R- and R+

CDM / Nutrient	Glucose			Mg^{2+}		PO_4^{3-}	K^+	All nutrients in excess	CDM10		
	A	B(i)	C(i)	D	E(ii)	F	G(iii)	H(iv)		I(R-)	J(R+)
Glucose	*	0.67	8.0	30.0	30.0	30.0	30.0	30.0		25.0	28.0
$MgSO_4$	0.2	0.2	0.2	*	0.01	0.2	0.2	0.2		0.13	0.25
$(NH_4)_2SO_4$	-	-	-	0.2	0.2	-	-	-	-	-	-
KH_2PO_4	0.58	0.58	0.58	0.58	0.58	*	0.06	0.58	$NH_4H_2PO_4$	1.7	5.6
Na_2HPO_4	1.8	1.8	1.8	1.8	1.8	*	3.0	1.8	$(NH_4)_2HPO_4$		
KCl	-	-	-	-	-	0.58	-	-		0.26	0.42
NaCl	-	-	-	-	-	2.4	-	-		0	0
NH_4Cl	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0		16.8	11.9
$(NH_4)_2SO_4.FeSO_4$	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NH_4FeSO_4	0	0.001
MOPS	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0		25.0	25.0

Key: *: Depleted nutrient under study in section 4.1.1. -: Salt not used.

CDM10: For *E. coli* R- and R+ strains formulated by Klemperer *et al* (1979)

(i), (ii) and (iii): Glucose, Mg^{2+} and K^+ depleted CDM respectively;

(iv): CDM, where all nutrients are in excess (in section 4.2).

Table 5

Chemically defined media, concentration (mM) of nutrients used in anaerobic cultures of *E. coli* R- and R+

CDM / Nutrient	I	II	III	IV	V	VI	VI	VIII	IX	X	XI
Glucose	27	18	37	37	37	37	37	37	27	37	37.5
NH_4Cl	15	15	22	44	22	22	22	22	22	15	21.0
$MgSO_4$	0.21	0.21	0.21	0.21	0.42	0.21	0.21	0.21	0.21	0.21	0.42
KH_2PO_4	0.58	0.58	0.58	0.58	0.58	0.58	1.2	1.2	0.58	0.58	0.63
Na_2HPO_4	1.8	1.8	1.8	1.8	1.8	2.42	1.8	3.6	1.80	1.8	4.2
KCl	-	1.0	-	-	-	-	-	-	5.0	1.0	3.57
$(NH_4)_2SO_4.FeSO_4$	0.002	0.0036	0.0012	0.0012	0.0012	0.0012	0.0012	0.0012	0.008	0	0.0021
MOPS	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Strain	R-	R+	R-	R-	R-	R-	R-	R-	R+	R- and R+	R- and R+

Table 6

Key:

* Nutrient under study.

\- Salt not used.

† (Ca^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+} and Mo^{6+})

Table 6

Chemically defined medium used to determine concentration (mM) required of each nutrient for the growth of R- cultures anaerobically

Limiting constituents / Salt	1	2	3	4	5	6	7	Trace elements ($\mu g\ ml^{-1}$) 8	9	10	11	12	13
	Glucose	NH_4^+	Mg^{2+}	PO_4^{3-}	K^+	SO_4^{2-}	Fe^{2+}	Ca^{2+}	Co^{2+}	Zn^{2+}	Mn^{2+}	Mo^{6+}	†
D(+)Glucose	*	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0
NH_4Cl	22.0	*	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
$(NH_4)_2SO_4$	-	-	-	-	-	*	-	-	-	-	-	-	-
$MgSO_4$	0.21	0.21	*	0.21	0.21	-	0.21	0.21	0.21	0.21	0.21	0.21	0.21
MgCl	-	-	-	-	-	0.21	-	-	-	-	-	-	-
Na_2HPO_4	1.8	1.8	1.8	*	2.4	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
NaCl	-	-	-	1.8	-	-	-	-	-	-	-	-	-
KH_2PO_4	0.58	0.58	0.58	*	*	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58
KCl	-	-	-	0.58	-	-	-	-	-	-	-	-	-
$(NH_4)_2SO_4.FeSO_4$	0.0012	0.0012	0.0012	0.0012	0.0012	0.0012	*	0.002	0.002	0.002	0.002	0.002	0.002
MOPS	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
$CaCl_2$	-	-	-	-	-	-	-	0.1	-	-	-	-	0.1
$CoCl_2$	-	-	-	-	-	-	-	-	0.1	-	-	-	0.1
$ZnSO_4$	-	-	-	-	-	-	-	-	-	0.1	-	-	0.1
$MnSO_4$	-	-	-	-	-	-	-	-	-	-	0.1	-	0.1
$(NH_4)_6Mo_7O_{24}$	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1

Table 7

Key:

* Nutrient under study

\- Salt not used.

† (Ca^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+} and Mo^{6+})

Table 7

Chemically defined medium used to determine concentration (mM) required of each nutrient for the growth of R+ cultures anaerobically

Limiting constituents / Salt	1	2	3	4	5	6	7	Trace elements ($\mu g\ ml^{-1}$) 8	9	10	11	12	13
	Glucose	NH_4^+	Mg^{2+}	PO_4^{3-}	K^+	SO_4^{2-}	Fe^{2+}	Ca^{2+}	Co^{2+}	Zn^{2+}	Mn^{2+}	Mo^{6+}	†
D(+) Glucose	*	28.0	28.0	28.0	28.0	28.0	28.0	28.0	28.0	28.0	28.0	28.0	28.0
NH_4Cl	22.0	*	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
$(NH_4)_2SO_4$	-	-	-	-	-	*	0.14	0.14	0.14	0.14	0.14	0.14	0.14
$MgSO_4$	0.21	0.21	*	0.21	0.21	-	0.21	0.21	0.21	0.21	0.21	0.21	0.21
MgCl	-	-	-	-	-	0.21	-	-	-	-	-	-	-
Na_2HPO_4	1.8	1.8	1.8	*	2.4	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
NaCl	-	-	-	1.8	-	-	-	-	-	-	-	-	-
KH_2PO_4	0.58	0.58	0.58	*	*	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58
KCl	1.0	1.0	1.0	0.58	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$(NH_4)_2SO_4.FeSO_4$	0.002	0.002	0.002	0.002	0.002	0.002	*	0.0036	0.0036	0.0036	0.0036	0.0036	0.0036
MOPS	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
$CaCl_2$	-	-	-	-	-	-	-	0.1	-	-	-	-	0.1
$CoCl_2$	-	-	-	-	-	-	-	-	0.1	-	-	-	0.1
$ZnSO_4$	-	-	-	-	-	-	-	-	-	0.1	-	-	0.1
$MnSO_4$	-	-	-	-	-	-	-	-	-	-	0.1	-	0.1
$(NH_4)_6Mo_7O_{24}$	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1

2.3. Media

2.3.1. Complex media for stock cultures, viable counts and biochemical identification

Nutrient broth (Oxoid CM1), nutrient agar (Oxoid CM3) and MacConkey agar (Oxoid CM7) were obtained from Oxoid Ltd., Basingstoke, Hampshire, U.K. The media were prepared as instructed by the manufacturers. API biochemical diagnostic kits were from API Laboratories Products Ltd., Basingstoke, U.K.

2.3.2. Chemically defined liquid media (CDM)

(a) For aerobic growth experiments

E. coli K12 W3110 R- and R+ was initially grown in a simple salts medium based on those formulated by Klemperer *et al* for R- and R+ strains of the same organism (CDMI and J respectively, Table 4) with some alterations in the nutrient concentration to suit the proposed experiments (CDMH, Table 4).

(b) For anaerobic growth experiments

A simple salts medium based on the aerobic requirements for *E. coli* (CDMH Table 4) was used for initial studies on nutritional requirements under anaerobic conditions. Initial R- and R+ inoculum cultures were made in CDMI and J respectively (Table 4).

As the requirement of each nutrient was defined, the basic medium used for further studies was modified stepwise. (Tables 6 and 7). The results obtained from these experiments were used to formulate CDM in which the concentration required of each ingredient was theoretically able to support maximal logarithmic growth of either R- and R+ organisms (CDMI and II respectively, Table 5).

2.3.3. Sterilization of media and antibiotic solutions

All media were sterilized by autoclaving at 121° for 20 min except for sodium deoxycholate (DOC)-containing nutrient agar which was brought to boiling (Oxoid Manual Instruction).

The individual media constituents used for batch cultures were also sterilized by autoclaving except for solutions of glucose, MOPS and Na_2HPO_4 which were sterilized by filtration using membrane filters (pore size 0.22µm). The desired CDM were prepared by mixing aseptically sterile ingredients and when necessary sterile double-distilled water was added to the appropriate volume.

In order to prevent ferrous iron precipitation, 1ml conc. sulphuric acid was added to 1 litre of ammonium ferrous sulphate stock solution prior to sterilization.

All ingredients of chemically defined media used in continuous culture experiments, except glucose, were added to the medium reservoir, dissolved in double distilled water and sterilized by autoclaving. Glucose solutions were sterilized separately and aseptically added to the reservoir.

Membrane filtration was used to sterilize antibiotic solutions which were added aseptically to the melted agar at 56° when NA-antibiotic plates were required.

2.4. Apparatus

Spectrophotometer: Unicam S.P. 600 (Pye-Unicam Instruments Ltd., Cambridge, England).

Spectrophotometric cuvettes: 1cm matched glass cuvettes (Helma Ltd., Westcliff-on-Sea, Essex, U.K.).

pH meter: Pye Model 290 (Pye-Unicam Instruments Ltd., Cambridge, England).

Incubators for batch cultures: Mickle reciprocating thermostatically controlled shaker water bath (Cam Lab Ltd., Nuffield Road, Cambridge, U.K.) or orbital shaking incubator (Gallenkamp Ltd., Loughborough, Leics., U.K.).

Peristaltic pump: MHRE7 flow inducer (Watson Marlow, Falmouth, Cornwall, U.K.).

Flow meter: RS tube complete with float, PTFE stops, fitted polythene ends and calibration chart (Jencons Scientific Ltd., Bedfordshire, U.K.).

Silicon rubber tubing TC 156: (Esco Rubber Ltd., London, U.K.).

Filtration systems: Millipore membrane filtration apparatus (Millipore, Heron House, Wembley, Middlesex, England) were used in conjunction with 47mm diameter membranes, pore size 0.22µm for sterilization and 0.45µm for clarification (Membrane Filter GmbH, D-3400 Gottingen, West Germany) 25mm diameter, 0.22µm membranes (Millipore U.K. Ltd., London and V. A. Howe, London) were used in Swinnex filters (V. A. Howe, London).

Disposable acrodisc 0.2µm pore size (Gelman Sciences, Northampton, U.K.).

Membrane filter preparation: prior to sterilization by autoclaving, membranes were boiled in three changes of distilled water. This procedure was followed to remove wetting agents and other light absorbing chemicals (Brown, Farwell and Rosenbluth, 1969).

Sonicator: Soniprobe type 7530A (Dawe Instruments Ltd., Concord Road, London W3, England).

Centrifuges: MSE Super Minor bench centrifuge for general work and the MSE High Speed 18 (Measuring and Scientific Equipment Ltd., Crawley, Sussex, U.K.).

An I.E.C. M20 (International Equipment Co., Massachusetts, U.S.A.) and a J2 Beckman centrifuge (Beckman Ltd., Bucks., U.K.) were used for harvesting and washing the cultures. An MSE superspeed 50 was used for the preparation of outer membranes.

Whirlmixer: Mixing of bacterial suspension, liquids and solutions was performed using Whirlmixer (Fison Scientific Apparatus Ltd., Loughborough, England).

Microscope: Microscopic counts of bacteria were made using Wild Model M20 Binocular Phase Contrast Microscope (Micro Instruments Oxford Ltd., Oxford, U.K.).

Automatic pipettes and tips: Occasional dilutions, mixing and counts were made using Automatic Medical Laboratory Automation pipettes delivering volumes of 250, 100 and 20µl, and appropriate disposable tips, sterilized by autoclaving (Frost Instruments Ltd., Wokingham, Berkshire RG11 1BZ, U.K.).

Glassware: All glassware was Pyrex Brand (Corning Glass Ltd., Sunderland, U.K.).

Preparation of glassware: Glassware was treated using a standard procedure:

i - Wash with tap water.

ii - 18 to 24h immersion in cold 5% v/v Decon 90 (Decon Labs. Ltd., Ellen Street, Portsland, Brighton, England) or equivalent, followed by rinsing in distilled water.

iii - Immersion in 1% v/v hydrochloric acid for a maximum of 24 h.

iv - Six washes in glass distilled water.

v - Three washes in double distilled water followed by drying at 60^{o} in a thermostatically-controlled drying cabinet. The dried glassware was sterilized either by autoclaving at 121^{o} for 20 min or by dry heat at 160^{o} for at least 3 hours unless otherwise stated.

Oxygen electrode: Measurement of dissolved oxygen in cultures growing under anaerobic conditions were made by using an oxygen electrode (Rank Brothers, Cambridge, England) connected to a calibrated Bryans pen recorder (x y recorder, 26000 A4) (Gallenkamp, Loughborough, U.K.).

Osmometer: Osmolarity was determined by the measurement of freezing point depression using a Knauer Osmometer (Roth Scientific Ltd., Alexander Road, Farnborough, U.K.).

Glass vessel for anaerobic batch cultures (see Fig. 9): made by University Glass Blower, University of Aston in Birmingham.

Chemostats: were made by University Glass Blower, University of Aston in Birmingham as described by Gilbert and Stuart (1977).

Flame photometer: (Pye Unicam Ltd., Cambridge, U.K.).

Haemocytometer: "Improved Neubauer" (Gallenkamp, Loughborough, U.K.).

Colworth Droplette: viewer for viable counts (A. J. Seword, UAC House, London, England).

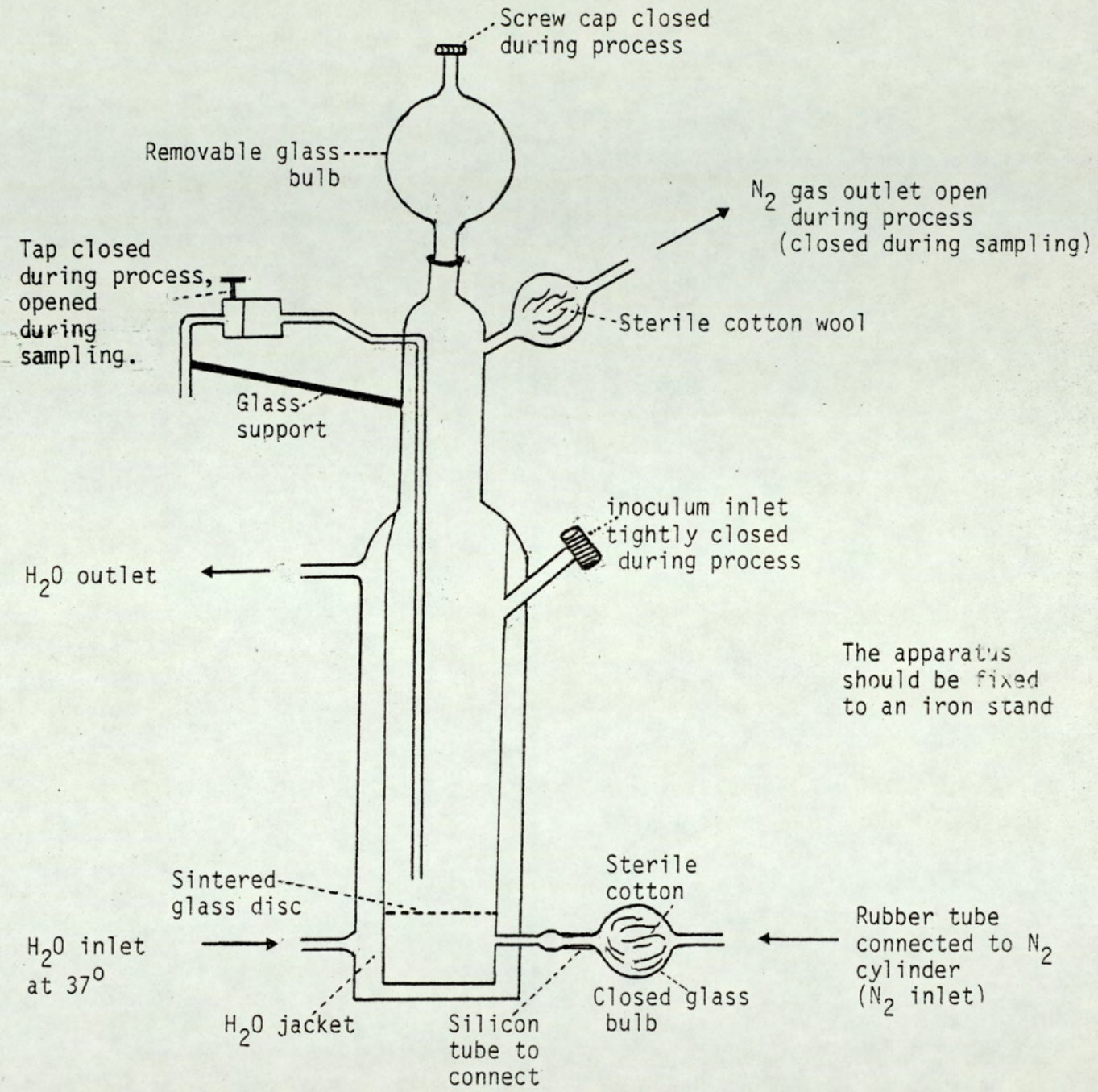
Electrophoresis power supply Model 500 or 200/500 and Agarose Zone Electrophoresis Kit: Model 1415 (Bio Rad Labs., Watford, U.K.).

Magnetic Stirrer: (Gallenkamp, Loughborough, U.K.).

Technicon autoanalyser: (Technicon Instruments Corporation, Tarrytown, New York 10591, U.S.A.).

Figure 9

Diagram of anaerobic (batch culture) glass vessel



3. BASIC EXPERIMENTAL METHODS

3. BASIC EXPERIMENTAL METHODS

3.1. Measurement of bacterial cell concentrations

The methods used to estimate bacterial cell concentrations were two, direct and indirect.

i - Direct method, which involved total or viable counts.

(a) - Total count, that is by direct counting of bacteria when viewed under the microscope (3.2.1.).

(b) - Viable count, that is by adding samples of bacterial suspension to solid culture media and counting the colonies assumed to result from the growth of each living cell in the sample (3.2.2.).

ii - Indirect method, by which the concentration of a bacterial suspension can be measured using a spectrophotometer based on the fact that the turbidity is the result of light scattered by the bacterial suspension. The proportion of incident monochromatic light failing to traverse a bacterial culture unchanged represents the sum of the light absorbed and the light scattered by the organism. Absorption is usually negligible in the absence of pigmentation. Thus by measuring the turbidity or light scattering properties of such suspensions, an estimate of the amount of a cellular material can be made. This method is the most convenient for following the changes in cell concentration during the phases of bacterial growth in liquid cultures.

3.1.1. Theory of measurement of undeviated light

At low bacterial concentrations the intensity of the incident light, Io and that of the transmitted light, I, are related by Beer Lambert's law:

$$I = I_0 . 10^{-\varepsilon l c}$$

ε = extinction coefficient, l = depths of suspension, c= bacterial concentration. By rearrangment of (1) it becomes:

$$\log_{10} I_o/I = \varepsilon.l.c.$$

The value of ε differs for different species (Brown, 1919) and for the same species treated in different ways (Spaun, 1962).

3.1.2. Selection of wavelength for the measurement of turbidity

Certain basic points should be considered when selecting a suitable wavelength for optical density measurements of a bacterial suspension. Ideally, the absorption of light by cell pigments, media constituents and metabolic products should be minimal and the wavelength should be that which permits maximum sensitivity.

Koch (1961) found that the total amount of light scattered is directly proportional to the ratio of cell size to the wavelength of incident light. Accordingly the shorter the wavelength, the more sensitive will be an instrument. 420nm is the lowest convenient wavelength using visible light (Handley, Quesnel and Sturgiss, 1974). However, 470nm was selected for all bacterial growth OD measurements because it was convenient for comparison with other concurrent work.

3.1.3. Measurement of optical density (OD) and its relation to cell concentration

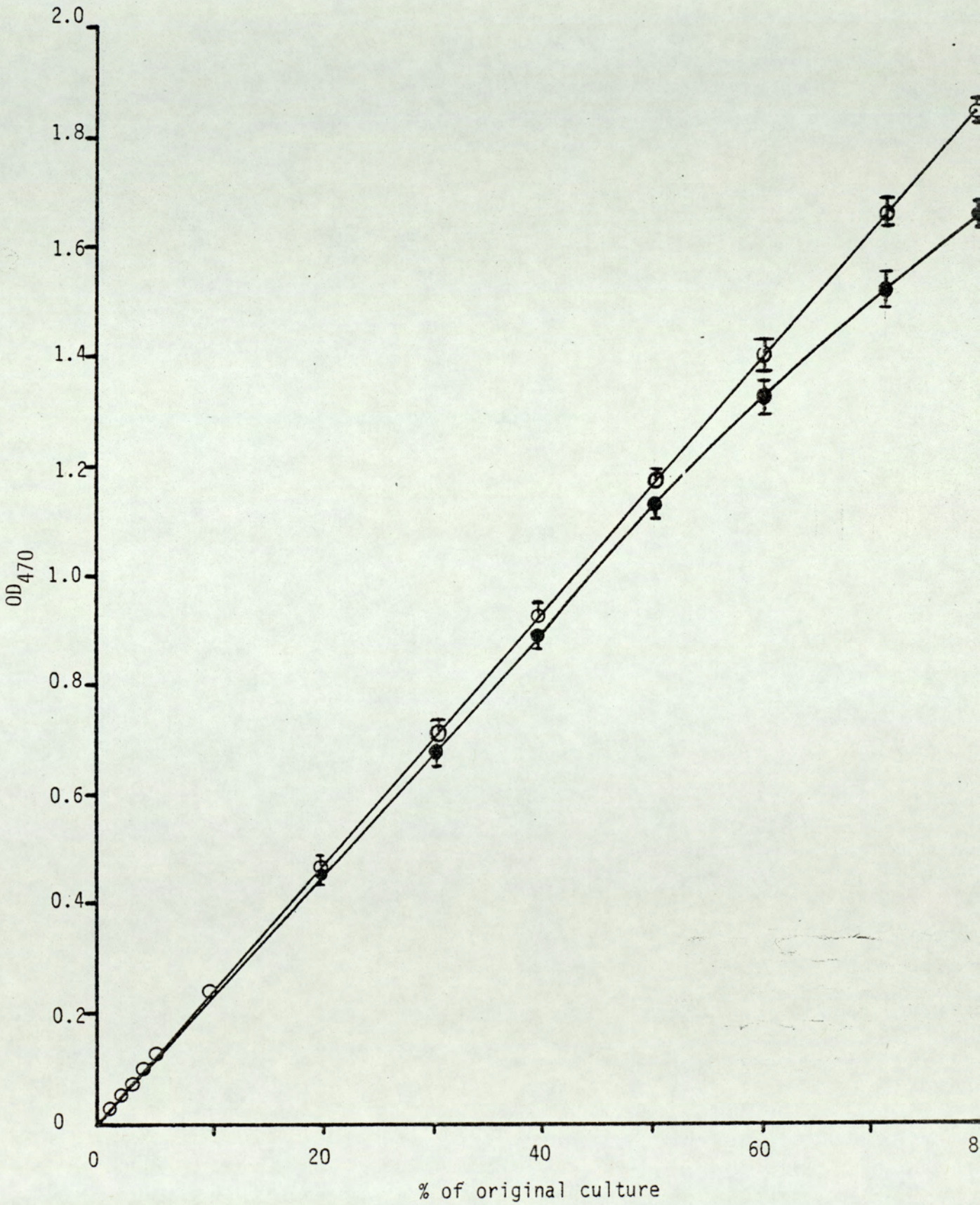
At high cell concentration, the Beer-Lambert relationship does not apply because of the secondary scattering of light (Meynell and Meynell, 1970; Lamanna et al, 1973). The relationship between OD_{470} and E. coli cell concentration was found to obey the Beer-Lambert law up to an OD_{470} of about 0.2 (Fig. 10). A standard curve was therefore made to convert observed OD readings to theoretical ones.

Figure 10

Relation between theoretical and observed optical density for oxygen-depleted *E. coli*

OD_{470} + SD original culture

Diluted sample X dilution
(True OD_{470} + SD)


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD470
0
20
40
60
% of original culture

To determine the relation between OD and cell concentration and also between OD and transmittance, a series of dilutions of a dense stationary phase culture were made in distilled water, in triplicate. The culture had been grown aerobically in the CDM formulated by Klemperer et al (1979), washed once and resuspended in distilled water. OD and transmittance were read. The suspensions were then diluted a second time and the OD again measured (Fig. 10). The principle of the standard curve derivation was based on the Lawrence and Maier method (1977). The graph in Fig. 11 illustrates the relationship between % transmittance and theoretical OD of an E. coli suspension. Using this standard curve, the theoretical OD_{470} of a similar bacterial cell suspension can be read immediately from the observed transmittance.

3.2. Colony counts

3.2.1. Total counts

Total counts were determined using haemocytometer counting chambers having depth 0.1mm (Norris and Powell, 1961; Cook and Land, 1962). Cook and Brown (1965) showed that a chamber of the depth 0.1mm gave less variation in counts than a chamber of 0.02mm depth.

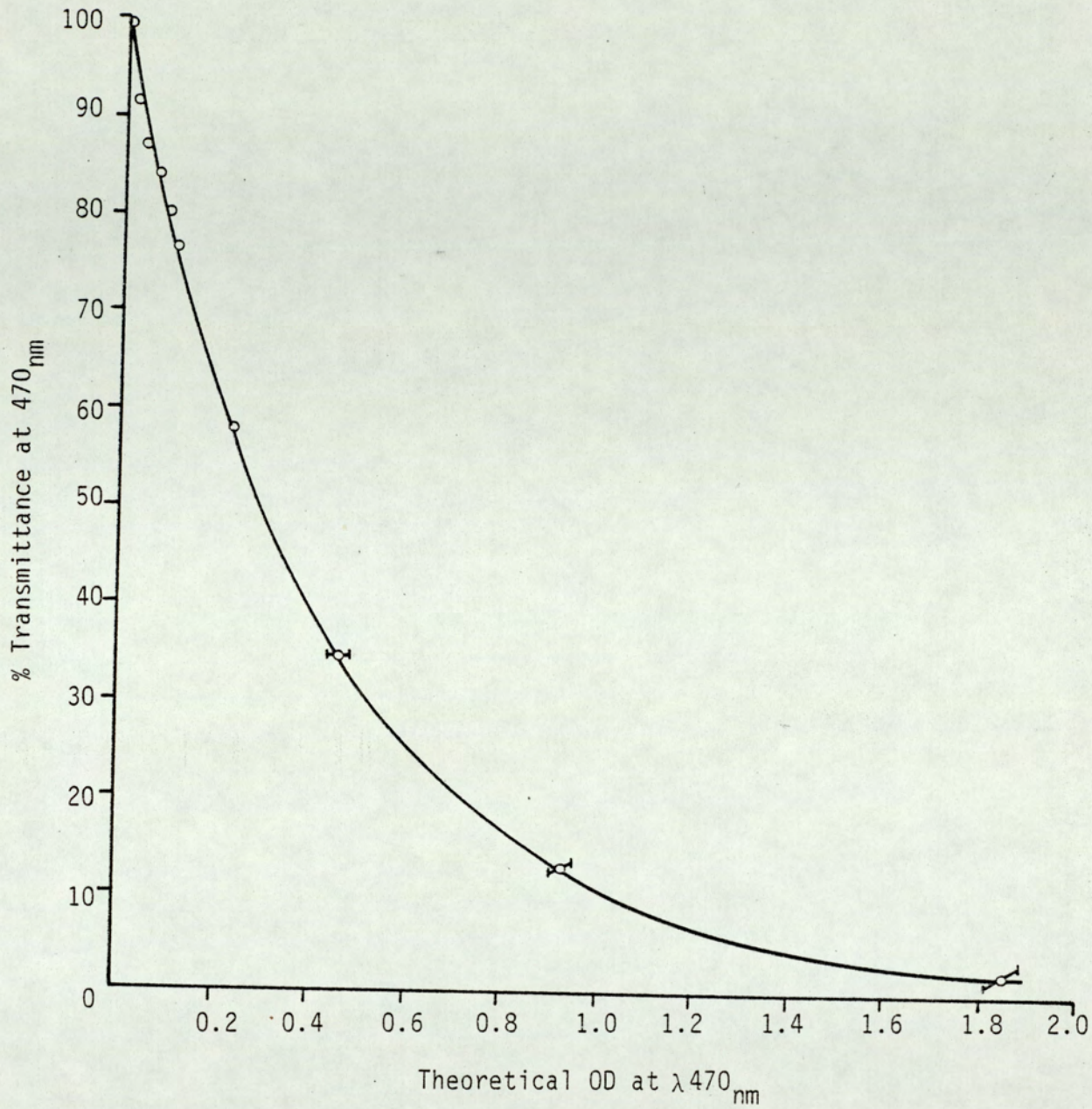
Cell suspensions to which formalin had been added to a final concentration of ca. 1% v/v formaldehyde were diluted in 0.9% w/v NaCl containing 1% v/v formaldehyde so that when counting there were approximately 8-12 immobilized cells per small square of the grid. The diluted suspension was counted immediately or after storage at 4^{o} for less than 48 h, Richards (1965) reported that the storage of Ps. aeruginosa suspension at 2-5^{o} up to 7 days had no effect on the total counts).

After filling, the chamber was allowed to stand at room temperature for 15-20 min in a petri dish containing water-saturated filter paper. The count

Figure 11

Relation between % Transmittance and theoretical OD_{470}

(Standard curve for the conversion of instrumental readings of percent transmittance to theoretical OD_{470})


100
90
80
70
60
50
40
30
20
10
0
% Transmittance at 470nm
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Theoretical OD at λ470nm

was done under phase contrast with a X40 objective and a X10 binocular eyepiece. To check the accuracy of the method the counts from six identical samples were performed. Four counts were made per sample in each of four haemocytometers. Table 9 shows the analysis of variance of the results obtained from Table 8. Subsequent counts were usually performed twice on duplicate samples.

Table 8

Total counts per slide for six replicate counts

Slide / Replicates	Mean counts per small square*				Means
	1	2	3	4	
A	7.63	8.5	6.31	7.5	7.49
B	7.13	8.0	7.88	7.63	7.66
C	7.38	7.5	7.81	8.0	7.67
D	8.13	7.38	7.38	7.19	7.52
E	7.25	7.5	6.94	7.75	7.36
F	7.75	8.19	7.25	7.19	7.6
Total (T)	45.27	47.07	43.57	45.26	-
Means	7.55	7.85	7.26	7.54	-

* Figures under 1, 2, 3 and 4 from A to F represent mean number of cells (X) per 80 small squares in each haemocytometer.

n: number of counts per slide = 6

m: number of slides = 4

nm: total number of observations = 24

1) $\Sigma X^2 = 1372.55$

2) $\frac{\Sigma T^2}{n} = 1368.63$

3) $\frac{(\Sigma X)^2}{n.m} = 1367.61$

Table 9

Analysis of variance of six replicate counts

Source of variation	Sum of squares	Degrees of Freedom	Mean squares	Coefficient of Variation of Means	Variance Ratio (F)
Between counts	(2) - (3) 1.02	m - 1 3	0.34	1.56%	1.74
Within counts	(1) - (2) 3.92	n.m - m 20	0.196	3.19%	

The tabulated values of F for 3/20 degrees of freedom are 4.94 and 3.10 at the 1% and 5% levels respectively. Since the observed value for F (1.74) is less than that of 5% and 1% levels, the variation between counts is not significantly greater than the variation within counts.

3.2.2. Viable counts

Viable counts were made by the surface drop method (Miles and Misra, 1938) using calibrated dropping pipettes which deliver 0.02ml per drop. Cell suspensions were serially diluted in sterile single strength NB. Seven drops of the appropriate dilution were then inoculated on the surface of overdried agar plates. Plates were usually incubated for 18 hours at 37^{o}, 2 days for those containing inhibitors or antibiotics. The viable count was calculated from the mean number of colonies per 0.02ml drop per plate.

To check the reproducibility of viable counts, five samples from a glucose-depleted-stationary phase culture were appropriately diluted (so as to contain roughly 25 colonies per drop) and the colonies counted. The results are shown in Tables 10 and 11, and were subjected to analysis in Tables 12 and 13 respectively.

Table 10

Colony counts per drop for five replicate plates from one sample

(Miles and Misra method)

Count \ Plate	A	B	C	D	E	Mean
1	28	16	20	19	18	20.2
2	18	18	20	17	22	19.0
3	26	23	18	13	17	19.4
4	20	18	22	20	26	21.2
5	19	22	19	21	21	20.4
6	17	20	20	21	22	20.0
7	19	18	18	18	26	19.8
Total (T)	147	135	137	129	152	-
Means	21.0	19.3	19.6	18.4	21.7	-

n = number of counts (drops) Per plate = 7

m = number of plates = 5

n.m = Total number of observations (drops) = 35

X = mean

1) $\Sigma X^2 = 14428$

2) $\frac{\Sigma T^2}{n} = 14049.7$

3) $\frac{(\Sigma X)^2}{n.m} = 14000$

Table 11

Mean colony counts per drop for five replicate experiments

Experiment / Plate	Mean counts per drop (X) 1*	2	3	4	5	Means
A	21.0	22.7	22.4	18.0	22.1	21.24
B	19.3	20.4	21.6	20.6	22.7	20.92
C	19.6	19.3	18.1	22.4	20.3	19.94
D	18.4	22.3	20.7	21.9	21.0	20.86
E	21.7	18.6	23.6	18.3	19.0	20.24
Total (T)	100	103.3	106.4	101.2	105.1	-
Means	20.00	20.66	21.28	20.24	21.02	-

* Numbers are means of drop counts in Table 10.

n = number of replicates per sample = 5

m = number of samples = 5

nm = total number of observations = 25

X = mean

1) $\Sigma X^2 = 10718.5$

2) $\frac{\Sigma T^2}{n} = 10655.9$

3) $\frac{(\Sigma X)^2}{n.m} = 10650.2$

Table 12

Analysis of variance of seven replicate counts from one sample*

Source of Variation	Sum of squares	Degrees of freedom	Mean squares	Coefficient of variation of means	Variance Ratio (F)
Between Counts	(2) - (3) 49.7	m - 1 4	12.43	3.56%	0.986
Within Counts	(1) - (2) 378.3	n.m - m 30	12.61	6.65%	

* Results calculated from data in Table 10.

The variation within counts is not significantly greater than the variation between counts, for the observed value of F (0.986) is less than those tabulated for 4/30 degrees of freedom (2.69 and 4.02 for 5% and 1% respectively).

Table 13

Analysis of variance of five replicate experiments*

Source of Variation In Counts	Sum of squares	Degrees of freedom	Mean squares	Coefficient of variation of means	Variance Ratio (F)
Between plates	(2) - (3) 5.7	m - 1 4	1.43	2.58%	0.457
Within plates	(1) - (2) 62.6	n.m - m 20	3.13	2.57%	

* Results calculated from data in Table 11.

For 4/20 degrees of freedom the tabulated vaolues of (F) are 2.87 at 5% level and 4.43 at 1% level. Therefore the variation in counts within plates is not significantly greater than the variation between plates.

3.3. Dry weight determinations

Plastic centrifuge tubes (50ml) for whole cells or glass centrifuge tubes (10ml) for outer membranes were dried at room temperature to constant weight over phosphorus pentoxide in an evacuated desiccator. 3x36ml samples of the bacterial suspension, in 1% v/v formaldehyde, were pipetted into each tube and centrifuged at 20,000 rpm (8 x 50ml head; J2 centrifuge) at 4^{o} for 30 min. The cell pellet obtained was washed once in 1% v/v formaldehyde-saline solution and once with sterile distilled water before drying to constant weight. For outer membrane dry weight determinations 0.1ml of OM suspension was pipetted into each tube which was then dried to constant weight. Each dry weight was determined at least in duplicate.

3.4. Growth Experiments in CDM

3.4.1. Aerobic growth experiments

These were carried out using as inoculum a culture grown overnight in a simple salts medium (CDMA, D and F, Table 4) in which all nutrients were present in excess except the one under study. The culture was centrifuged at 10,000 rpm for 15 min. at ca. 37^{o}, washed once with sterile pre-warmed distilled water and resuspended to an OD of ca. 2.0 in MOPS solution (50mM, pH 7.4) prewarmed to 37^{o}. 0.25ml of this suspension was added to 24.75ml of the appropriate medium in a 100ml Ehrlenmeyer flask. Each flask was shaken in a water bath at 37^{o}, at a rate of 120 throws per min to provide adequate oxygenation. The media were inoculated approximately one hour prior to the first transmittance reading. Samples for measurement were removed at 30 min intervals during the lag and early stationary phase and at 60 min intervals during the exponential phase. Since all readings were corrected using a standard correction curve (see Fig.11) all samples taken were returned to their original flasks to prevent undue reduction of the culture volumes.

3.4.2. Anaerobic growth experiments

The inoculum was a 24 h culture grown anaerobically in a simple salts medium in which all components except that under investigation were present in excess (Table 6 and 7 for R- and R+ respectively). The 24 h cultures were centrifuged at 10,000 rpm for 15 min at ca. 37°, washed once with sterile prewarmed distilled water and resuspended in prewarmed MOPS solution (50mM, pH 7.6) to the required OD. 0.75ml of these suspensions were added to 74.25ml of the appropriate medium (alternatively 0.5ml to 49.5ml) in an anaerobic glass vessel of 100ml capacity Fig. 9 . For a few hours prior to medium inoculation and throughout the growth experiment oxygen-free N_2 gas was continuously flushed at a rate of ca. 40ml min^{-1} through a sintered disc into the culture, which effected mixing and maintained anaerobiosis. The cultures were enclosed in a water jacket maintained at 37°. Each medium was inoculated approximately 30 min prior to the first transmittance measurement. Samples for measurement were removed at 60 min intervals through the neck of the vessel. While doing this the N_2 exit was clipped forcing nitrogen towards the neck. This process minimized the possibility of contamination and prevented air from gaining access to the culture vessel. All samples taken from the vessel were discarded.

3.4.2.1. Measurement of dissolved oxygen

The oxygen electrode is designed for the measurement of dissolved O_2 in liquid media. The principle of operation is as described by Lessler and Brierly (1969) and its assembly is shown in Fig. 12.

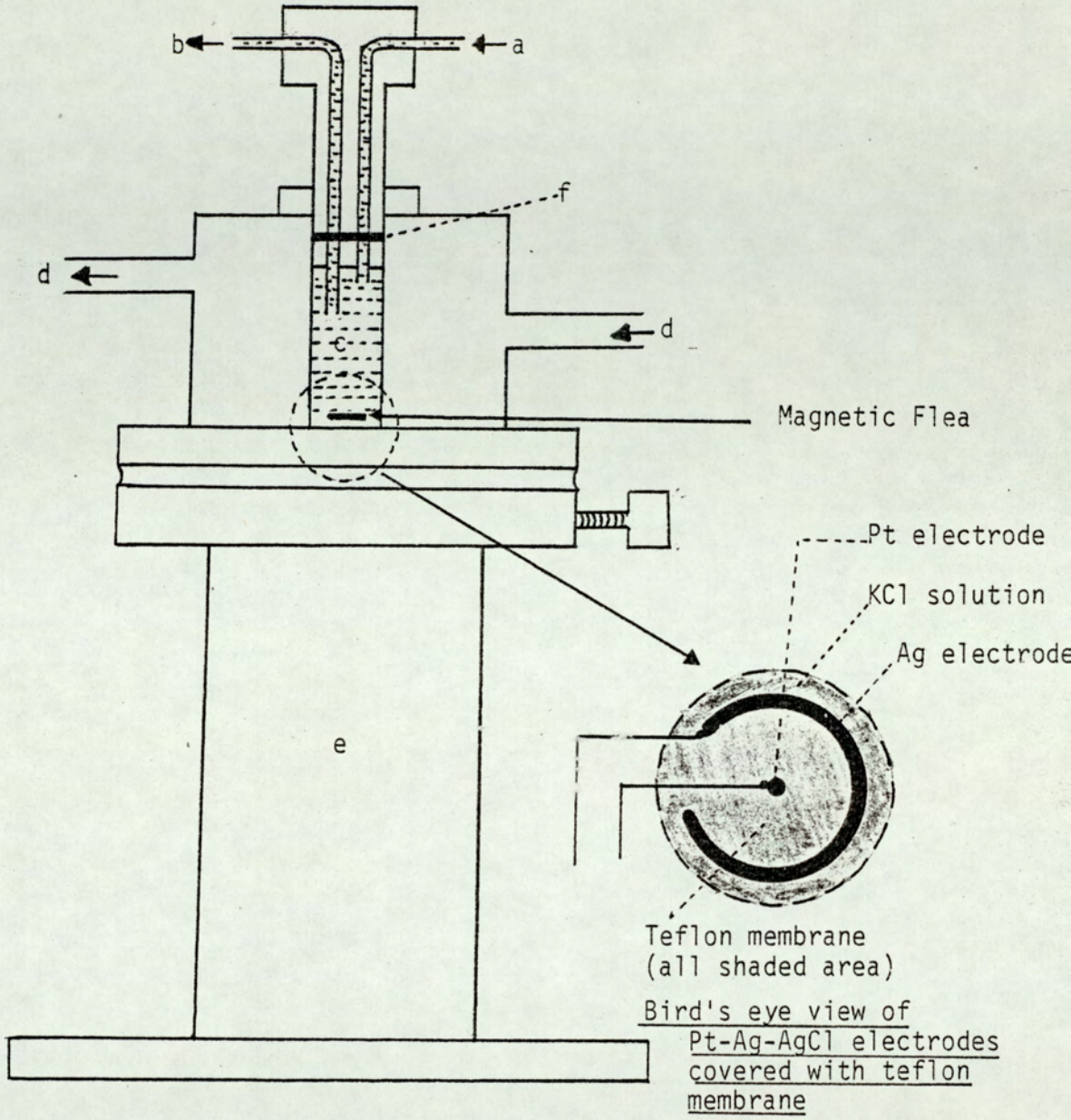
Oxygen diffuses through a thin teflon membrane and is reduced at a platinum surface immediately in contact with the membrane.

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

$$H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O$$

Figure 12

The oxygen electrode assembly



Key:

a) Culture inlet from the culture vessel
b) Culture outlet to the culture vessel
c) Incubation chamber
d) Water jacket
e) Magnetic stirrer
f) Rubber washer (for airtight seal)

The other half cell is also incorporated in the base of the incubation chamber and composed of an Ag-AgCl electrode.

The electrode is maintained at 37^{o} by circulating water through an outer jacket.

To calibrate the electrode, it is switched on and a current of 0.6 volt is applied. Water, saturated with air by stirring in the open air at 37^{o} for 2 h, is then perfused into the incubation chamber and the sensitivity of the recorder adjusted to obtain a maximum deflection. To obtain 0% reading a few crystals of sodium dithionite are added. Similar readings were obtained when chemically defined media were used instead of water.

For the measurement of the amount of dissolved O_2 in the "anaerobic cultures" a sample of CDMIII (Table 5) was saturated with N_2 until near zero % O_2 reading (2-5%) was obtained. This was then compared with the readings for CDM saturated with O_2 and reduced by dithionite. This was done in triplicate and the result used as a standard.

When other CDM were used for different experiments, the % O_2 following saturation with N_2 was measured and compared with the standard. No differences were found. After inoculation of CDM, the percentage saturation was monitored during the lag, exponential and stationary phases of batch culture and also during continuous culture. It was always found to be the same.

3.4.3. Measurement of medium osmolarity

The osmolarity of CDM was determined by a depression of freezing point method (Wallworth and Grant, 1977). Sodium chloride solutions of known osmolarity were used to draw standard calibration curves from which the osmolarity of the CDM could be derived.

3.5. Outer Membrane Protein Profiles

3.5.1. Preparation of outer membranes

At least 100ml bacterial culture OD_{470} 1.0 was required for each preparation. Cultures were centrifuged at 15,000 rpm at 4^{o} for 15 min , washed once with sterile 0.85% saline, resuspended in 10ml distilled water and transferred to a glass beaker partially immersed in an ice-bath. The cells were broken by sonication (5 x 60 sec) and 1.1ml 20% w/v sodium N-lauroyl sarcosinate (sarkosyl) added. Sarkosyl solubilizes the cytoplasmic membrane (CM) but leaves the outer membrane (OM) essentially intact (Filip et al, 1973). After 30 min incubation at room temperature, OM were pelleted by centrifugation at 40,000 rpm (8 x 25ml head, MSE superspeed 50 centrifuge) for 60 min at 4^{o}, washed once in distilled water, resuspended in 0.5ml distilled water and stored at -20^{o} in Bijou bottles.

3.5.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The method of Lugtenberg et al (1975) was used. Gels were composed of a separating gel and a stacking gel; the constituents of each are listed in Table 14.

Table 14

Compositions of separating and stacking gels

	Separating gel	Stacking gel
Stock I	18.75ml	-
Stock II	-	5.0ml
SDS 10% w/v	1.5ml	0.3ml
Tris 1.5M pH 8.8	18.75ml	-
Tris 0.5M pH 8.0	-	7.5ml
Distilled water	20.0ml	16.0ml
TEMED	0.14ml	0.08ml
AMPS 10% w/v	0.2ml	0.1ml

Stock I consisted of 44% w/v acrylamide and 0.8% w/v. N,N'-methylene-bis acrylamide (BIS).
Stock II consisted of 30% w/v acrylamide and 0.8% w/v BIS.
SDS is sodium dodecyl sulphate (Analar grade, specially pure BDH).
TEMED is NNNN' tetramethylethylene diamine and AMPS is ammonium persulphate,

freshly prepared. All solutions except AMPS were stored at 4^{o} for not more than two months. Upon addition of 0.14ml or 0.08ml of TEMED, the polymerisation process was initiated and the gels solidified.

OM protein samples were mixed in an equal volume with the sample buffer (Table 15) and steamed at 100^{o} for 10 min for protein denaturation.

Electrophoresis (using electrode buffer Table 16) was performed at room temperature using a constant current of 40 mA and was ended when the tracking dye had moved approximately 14 cm from the lower border of the stacking gel. Gels were fixed and stained by flooding them with 0.1% w/v Coomassie Brilliant Blue R250 in 50% v/v methanol, 10% v/v glacial acetic acid in water. After overnight soaking in the stain, gels were destained using methanol-glacial acetic acid-distilled water 5% v/v, 10% v.v and 85% v/v respectively.

Table 15

Composition of the sample buffer (pH 6.8)

Ingredients	ml
Tris 0.5M pH 6.8	5.0
SDS 10% v/v	10.0
2-Mercaptoethanol	0.5
Glycerol	5.0
Distilled water	10.0
Bromophenol Blue Solution	0.1

Table 16

Composition of electrode buffer (pH 8.3)

Ingredients	
Tris	6.0g
Glycine	28.8g
SDS 10% w/v	20.0ml
Distilled water to	2000.0ml

3.6. Chemical Assays

3.6.1. Glucose-oxidase-peroxidase method for glucose estimation

To each 0.5ml sample including the blank (0.5ml distilled water) 5.0ml of combined Enzyme-Color Reagent Solution was added and the mixture incubated at 37^{o} for 30 min. The intensity of the colour developed was measured by absorbance at 425nm. A standard calibration curve for D-glucose was determined 4 times and the coefficient of variation found to be ca. 5% (Fig. 13). A standard glucose solution was included in all tests and the absorbance compared. No interference in the test was detected from other medium constituents.

3.6.2. 2-Keto-3 deoxyoctonic acid (KDO) assay

For KDO determinations, a method based on that of Osborn (1963) was used. Samples of KDO standard (0 to 50 $\mu g\ ml^{-1}$), were hydrolysed in 1.25ml 0.05M H_2SO_4 by heating in screw-capped tubes at 100^{o} for 30 min. After cooling, 1.25ml 0.025M sodium periodate in 0.025M H_2SO_4 was added. After 20 min. incubation at 55^{o}, 2.5ml 2% w/v sodium arsenate in 0.5M HCl was pipetted into each tube and thoroughly mixed. Three min later, during which the orange-yellow colour completely disappeared, 2.0ml 3% w/v thiobarbituric acid solution in distilled water was added and the samples placed in a boiling water bath (100^{o}) for 20 min. After cooling to ca. 4^{o} (to minimize evaporation of butanol) 5ml butanol was added, the contents vigorously shaken for about 30 sec. and left to settle to allow butanol aqueous layer separation. Tubes were centrifuged at 10,000 rpm (8 x 12.5 head, I.E.C. centrifuge) for 10 min. The absorbance of each solution at 550nm was recorded. A standard calibration curve is shown in Fig. 14.

To measure KDO in OM, either 0.1ml sample prepared by the sarkosyl method or 0.1ml water-reconstituted sample of dried OM was used. All determinations were performed twice on duplicate samples.

Figure 13

Glucose calibration curve

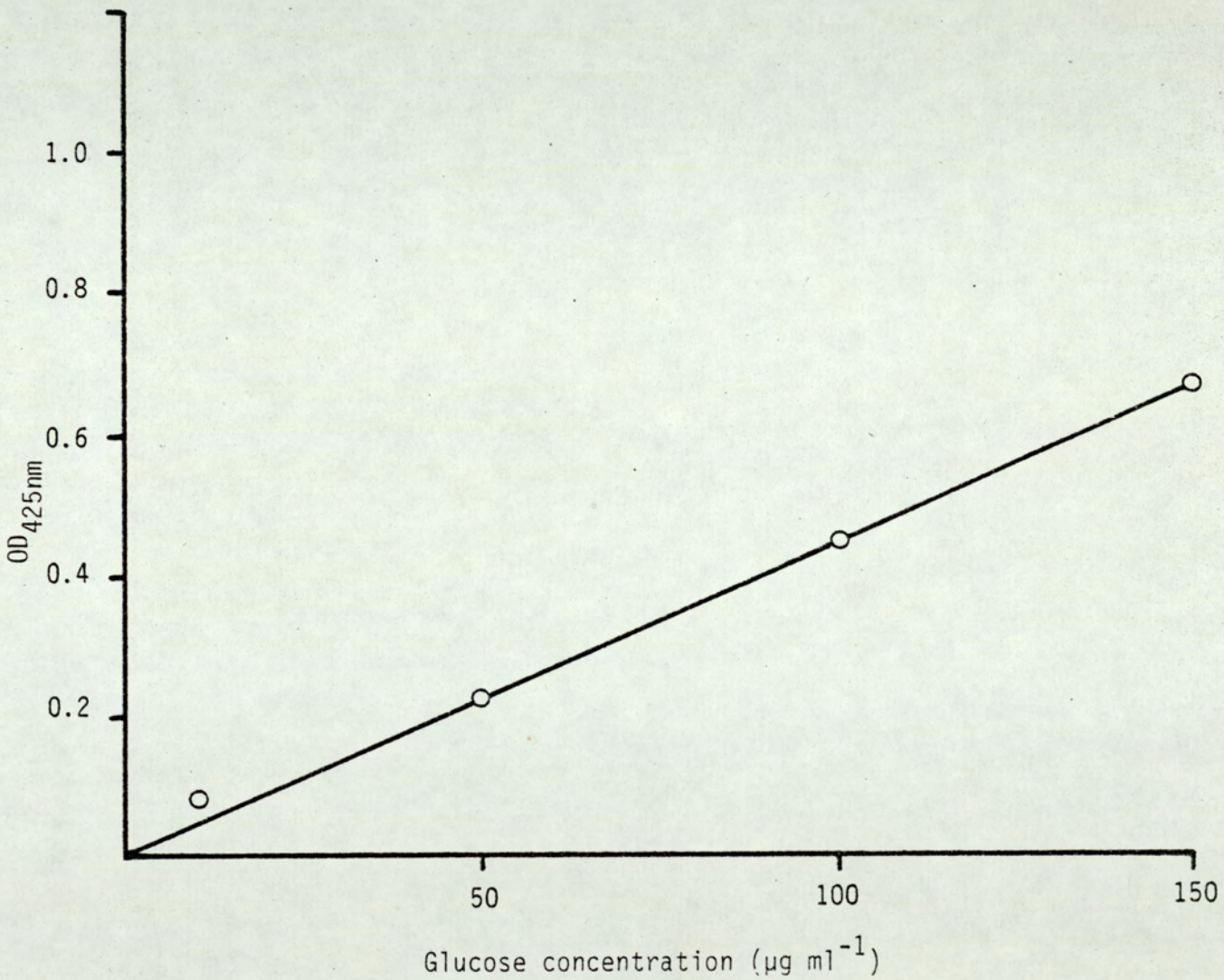
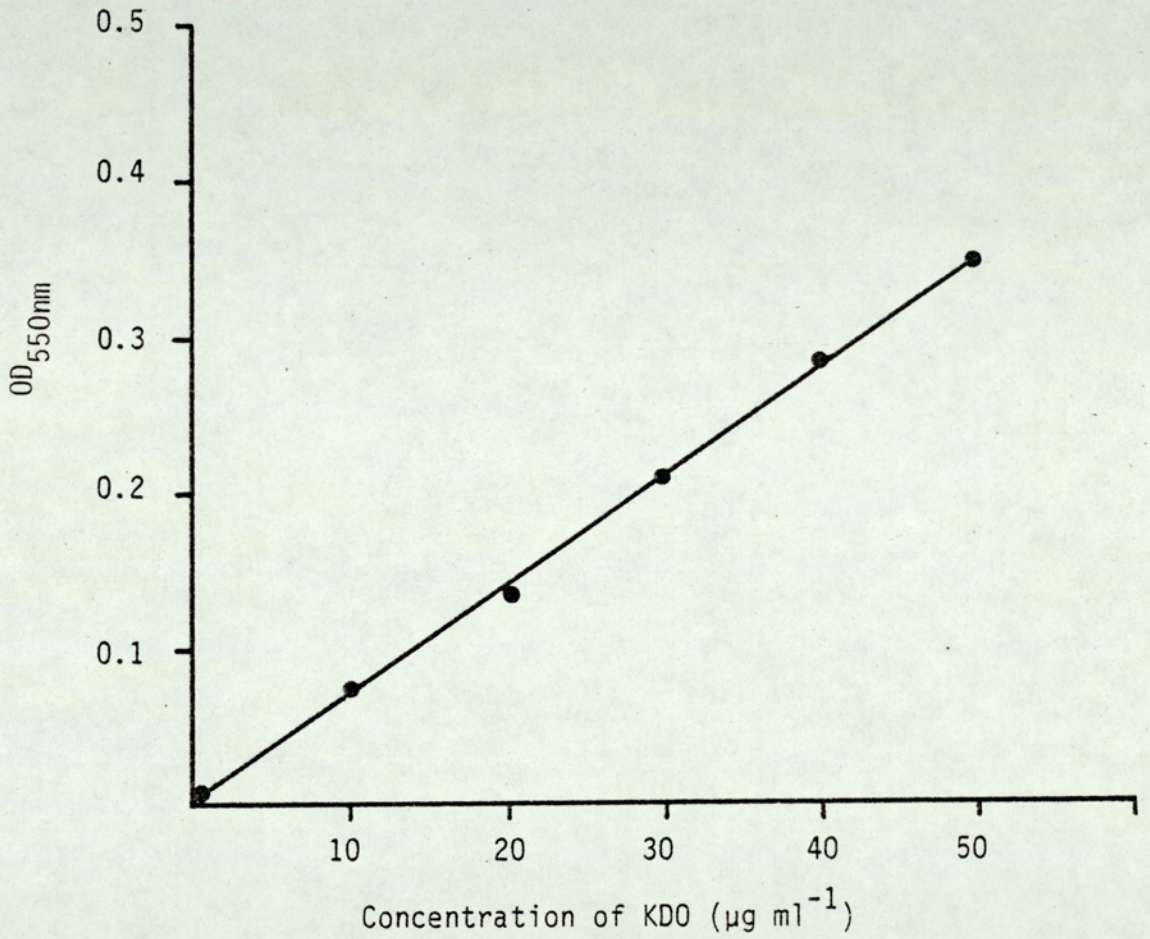


Figure 14

2-keto-3-deoxyoctonic acid (KDO) calibration curve



3.6.3. Determination of total inorganic phosphate

A method based on that of Lundgren (1960) was used. The method was originally developed for the determination of phosphate in detergents and waste water, but is equally applicable to the analysis of physiological fluids.

Condensed phosphates are first converted to orthophosphates by hydrolysis with sulphuric acid. The phosphate concentration is then determined by the reduction of phosphomolybdic acid with ascorbic acid.

Samples of standard phosphate solutions containing 0 to 15 μg ml^{-1} phosphorus (P) were prepared. The % transmittance readings obtained from the autoanalyser were used to construct a standard calibration curve of $\log_{10}$% T against P (Fig.15).

In each experiment, a fresh standard was prepared and checked against the standard curve. Unknowns were assayed in triplicate and the mean values taken.

The reproducibility was assessed by measuring the P content of 7 triplicate samples of a culture filtrate. Table 17 shows the reproducibility of the phosphorus assay.

Table 17

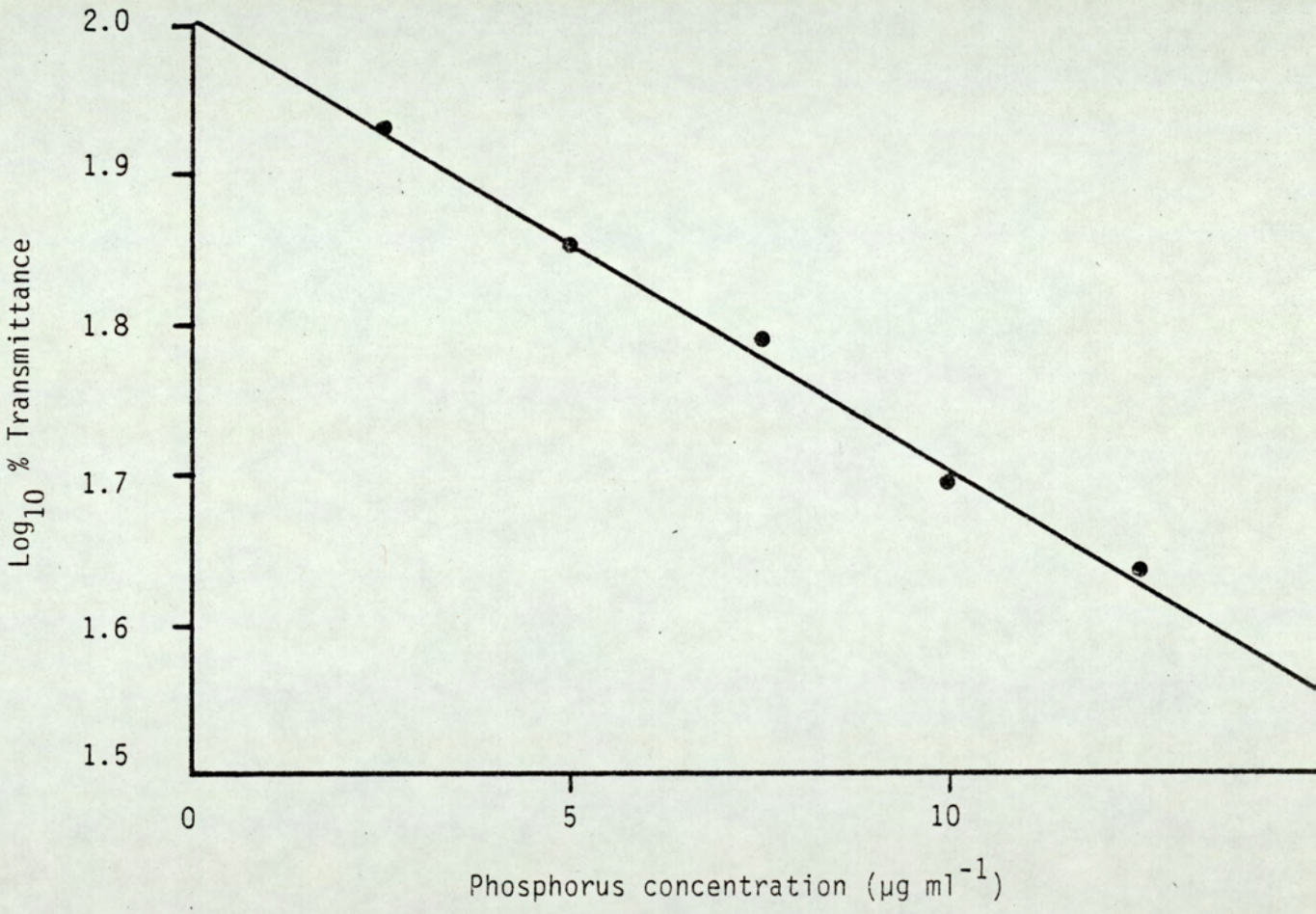
Reproducibility of phosphorus assay

Replicates	Phosphorus (μg ml^{-1})
1	4.165
2	4.165
3	4.491
4	4.491
5	4.213
6	4.213
7	4.120
Mean	4.265
Coefficient of variation	3.69%

Figure 15

Relation between phosphorus (P) concentration and $\log_{10}$% transmittance

(Technicon Analyser)



3.6.4. Assay of potassium

Potassium was assayed by flame photometry. Potassium chloride standard solutions in the range 0 to 0.06 mM were used to prepare the standard calibration curve (Fig. 16).

Standards and unknowns were assayed in triplicate and the mean value taken.

To test the reproducibility of the assay, the K^+ content of 5 replicate culture filtrates was determined. The results are shown in Table 18

Table 18

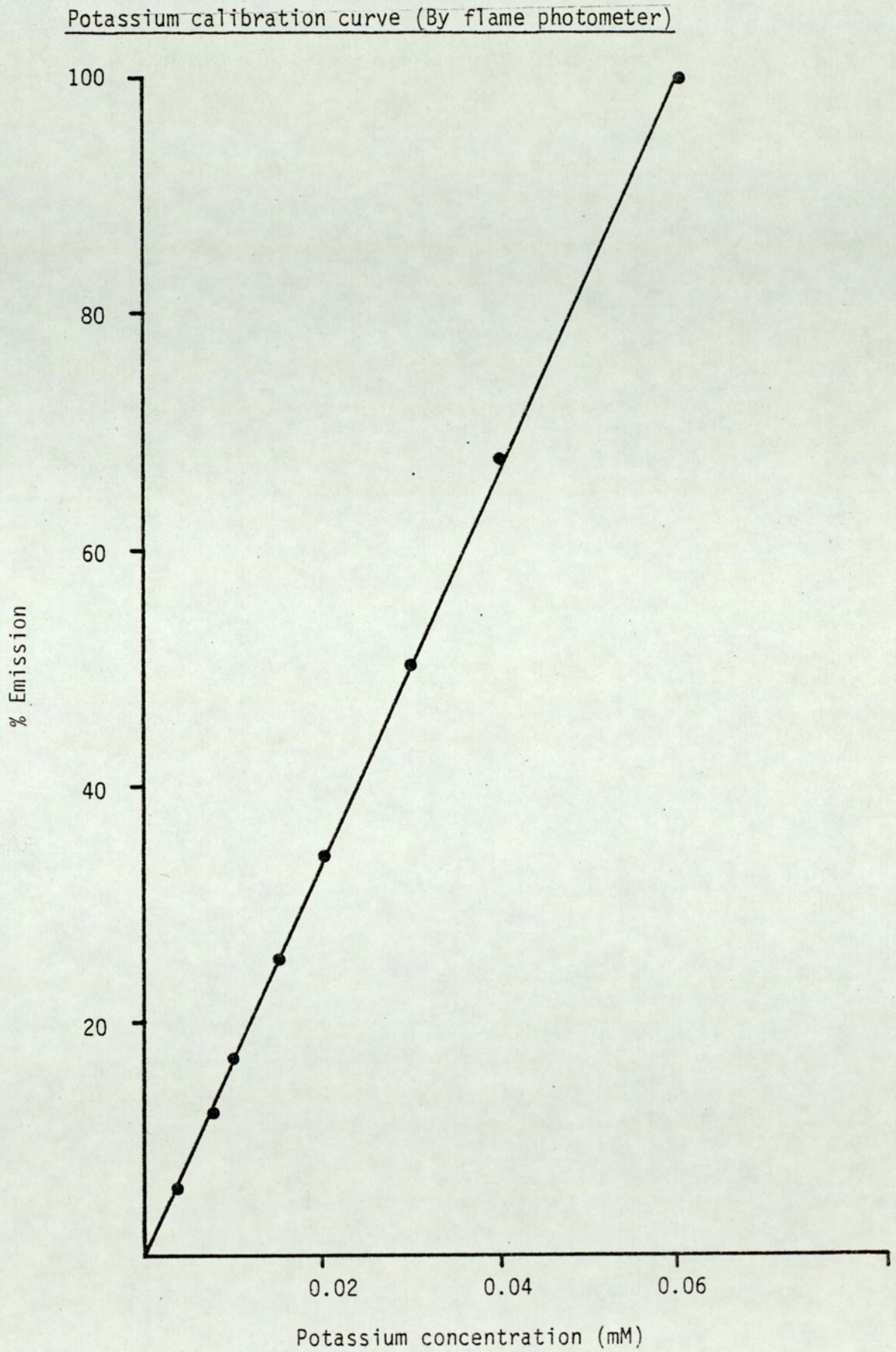
Reproducibility of potassium assay

Replicates	Potassium (mM)
1	0.0067
2	0.0067
3	0.0070
4	0.0073
5	0.0063
Mean	0.0068
Coefficient of variation	5.5%

3.7. Determination of Minimum Inhibitory Concentration (MIC) of Kanamycin Sulphate for E. coli R- and R+ by Plate Dilution Method

Overnight stationary phase cultures in nutrient broth (NB) were appropriately diluted in sterile NB and surface-inoculated by a drop method (Miles and Misra, 1938) on NA plates containing different concentrations of kanamycin SO_4. Plates

Figure 16


Potassium calibration curve (By flame photometer)
100
80
60
40
20
% Emission
0.02
0.04
0.06
Potassium concentration (mM)

were incubated at 37^{o} for 48 hours and viable counts recorded. All determinations were made twice, each in duplicate. The MIC of kanamycin SO_4 for R+ was 24-48 μg ml^{-1} and for R- was 0.4-0.75 μg ml^{-1}.

3.8. Resistance Conferred by the R- Plasmid RP1

Since R- plasmid RP1 confers resistance to six antibiotics, ampicillin, carbenicillin, cephaloridine, kanamycin, neomycin and tetracycline, the presence of these resistance determinants was confirmed by incorporating ampicillin, kanamycin and tetracycline singly and in all possible combinations in NA. Results of viable counts using the same cultures as in 3.7. are shown in Table 19.

Table 19

Antibiotic resistance of RP1 containing E. coli

Antibiotic (25 μg ml^{-1})	cfu x 10^9 ml^{-1}	
	R-	R+
Ampicillin (A)	0.0	2.8
Kanamycin (K)	0.0	2.9
Tetracycline (T)	0.0	2.7
A + K	0.0	2.6
A + T	0.0	3.0
K + T	0.0	2.7
A + K + T	0.0	2.8
No antibiotics	2.4	3.0

3.9. Survival of *E. coli* R- and R+

3.9.1. Survival in liquid media

Stationary-phase cultures were harvested, centrifuged at 10,000 rpm for 10 min at room temperature and resuspended in sterile warm MOPS solution (50mM, pH 7.4) or in the original menstruum. 3ml aliquots were distributed in clean sterile Bijou bottles and stored in triplicate. Viable counts were done at different time intervals on NA and NA containing 15 $\mu g\ ml^{-1}$ Kanamycin SO_4 plates. Two independent cultures of the R- and R+ strains were used.

To test the effect of the suspending medium, cultures were split into two portions. After centrifugation at 10,000 rpm for 10 min at room temperature the supernatant of one portion was discarded and the pellet resuspended in MOPS solution (pH 7.4) to the original volume. The pellet of the other portion was resuspended in its menstruum. 3ml quantities of each suspension were dispensed in Bijou bottles in triplicate, stored at 25^{o} and viable counts were performed as mentioned above.

3.9.2. Effect of drying at 25^{o} on survival

20 µl samples of stationary-phase cultures of the R- and R+ strains were distributed in a series of sterile specially designed tubes ca. 1cm in length made by cutting Widal test tubes. These were embedded into size 17 perforated bungs, which acted as holders. Just after dispensing, 6 tubes each of R- and R+ were used for zero time viable counts on overdried NA plates or on similar plates containing additional NaCl. After drying samples were reconstituted by adding 0.1ml sterile NB, mixing thoroughly with the same automatic pipette and then appropriately diluting for counting.

In order to ensure complete removal of all adherent dried viable bacteria from the tube walls, tubes were rinsed twice with appropriate quantities of

sterile NB then rubbed with a cotton swab. The first rinse was added to the original dilution while the second rinse and the cotton swab were separately cultured on NA plates. No E. coli colonies were detected on the latter after 2 days incubation at 37°.

3.9.3. Sensitivity of E. coli to sodium chloride, Tween 80 and sodium deoxycholate

Different concentrations of sodium chloride (NaCl), Tween 80 (T80) and their combinations were mixed with dissolved NA and sterilized by autoclaving at 121° for 15 min. NA plates with graded concentrations of sodium deoxycholate (DOC) were prepared according to the Oxoid Manual. Viable counts of R- and R+ cultures were made on these plates, which were then incubated at 37° and inspected for colonial growth and morphology at 1 and 2 days.

3.9.4. Determination of minimum inhibitory concentration (MIC) of NaCl for E. coli R- and R+

Tube dilution method:

Appropriate amounts of NaCl solution were added to sterile glucose-depleted CDM (CDMB, Table 4) or NB (final volume 5ml). 0.1ml of overnight culture in CDMB (ca. 10^6 cfu ml^{-1}) was added to each tube. The inoculated tubes were then incubated at 37° and checked for growth after 24 and 48h. This experiment was done twice with tubes in triplicate. The results are shown in Table 20.

Table 20

Minimum inhibitory concentration of NaCl for E. coli R- and R+

Medium	MIC of NaCl % (w/v)	
	R-	R+
CDM	3 - 3.5	2.5 - 3
NB	> 5	> 5

3.10. The Anaerobic Growth of *E. coli* R- and R+ in a Chemostat

3.10.1. Preparation of media

Using the data obtained from batch culture, the concentration of limiting nutrients was calculated to give OD_{470} of 0.5 to 1.0 (Table 21). Other medium components were in excess at least five times, except Fe^{2+} (2 times) which tends to precipitate.

The weighed quantities of the medium components, except glucose, were dissolved in a small amount of double distilled water in a 20 litre glass reservoir and made up to required volume. The medium pH was adjusted to 7.6 by adding NaOH pellets. The rubber cap was tightly placed in the neck of the reservoir and sealed with aluminium foil and autoclave tape. The separating funnel containing the calculated amount of glucose solution was attached as in the diagram (Fig. 17) and was sterilized by autoclaving at 121^{o} for 2 hours. The glucose solution was transferred aseptically to the medium reservoir while both were still hot. The rubber tubing at junction G was removed and replaced aseptically by a sterile filter and the medium then continuously mixed in the presence of N_2 which was allowed to enter the reservoir at a constant rate of approximately 40 ml $min.^{-1}$.

3.10.2. Calibration of chemostats

The working volume of each chemostat used was measured by pumping water in at ca. 10 ml h^{-1} while oxygen-free N_2 gas was allowed to flow through at a steady rate of 80 ml $min.^{-1}$. After a few hours both the water and gas were cut off and the water in the chemostats was pipetted into a measuring cylinder and the volume recorded. This procedure was repeated a further three times. Table 22 shows the results for all chemostats used. These were subjected to an analysis of variance (Table 23).

Table 21

Glucose-limited (G-lim), Phosphate-limited (P-lim) and Potassium-limited (K^+-lim) media for use in the chemostats

Nutrient	Concentration (mM)		
	G-lim	P-lim	K^+-lim
Glucose	3, 5, 6.2	20.0	20.0
Na_2HPO_4	1.8	0.0	1.8
NaCl	0.0	1.8	0.0
KH_2PO_4	0.6	0.1 or 0.17	0.03 or 0.06
KCl	5.0	5.5	0.0
NH_4Cl	22.2	22.2	22.2
$MgSO_4$	0.2	0.2	0.2
$(NH_4)_2SO_4.FeSO_4$	0.0012	0.002	0.002
MOPS	50.0	50.0	50.0
Medium Osmolarity (mO)	205	225	210

Figure 17

Assembly of medium reservoir

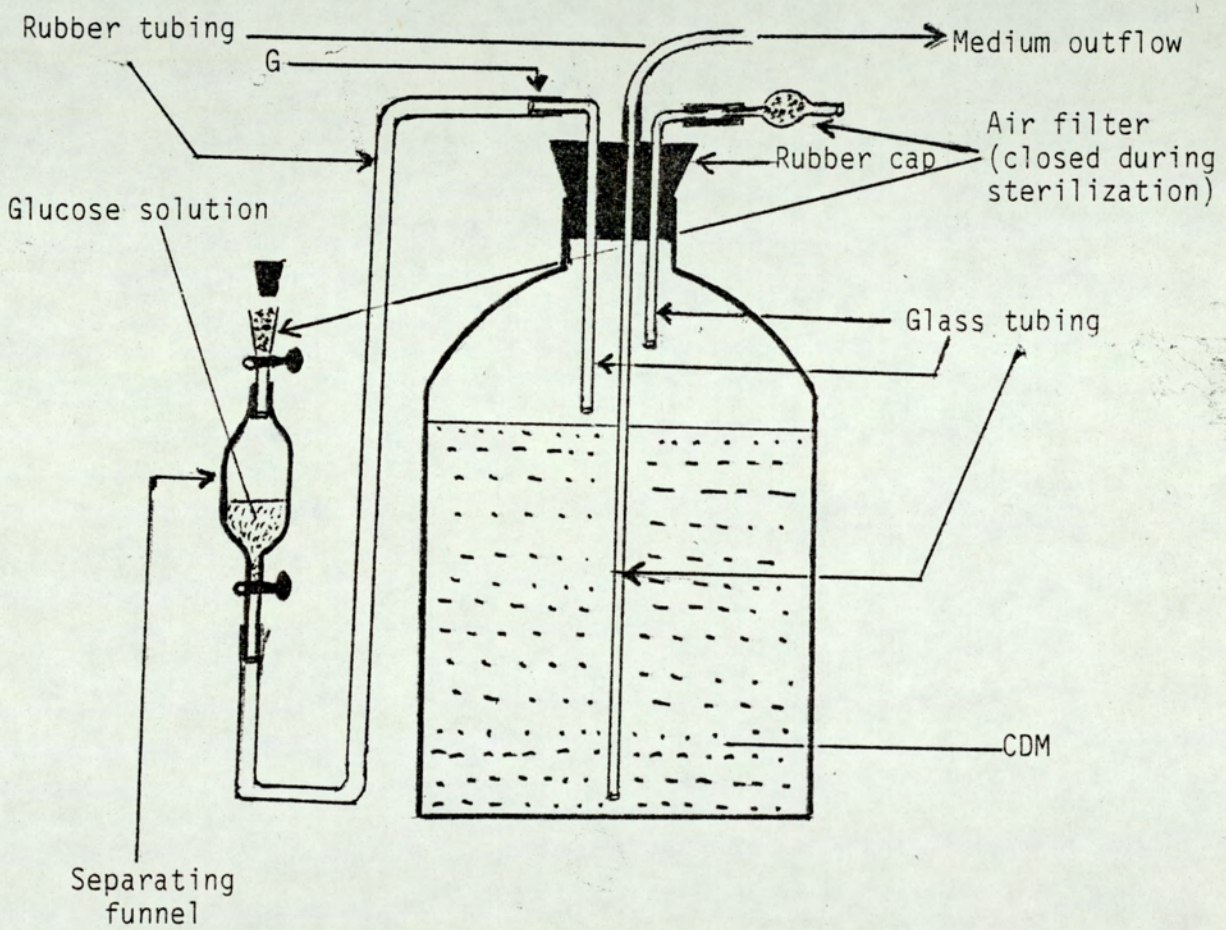


Table 22

Volume of water (ml) in each chemostat for 4 replicate determinations

Volume \ Chemostat No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	48.8	49	50	50	52	50	52	50	48.5	47.8	50.5	50	47	48.5	49
2	49	49	49.5	49	51.5	50.5	51.5	50	48.5	47.5	51	50	47.5	49	48
3	48.5	50	50	49.5	51.5	50	52.5	49.5	48.5	48	51	50.5	48	49	48.5
4	49.5	49.5	50	49.5	52	50	52	50	49	48	50	50	48	49	49
Total (T)	195.5	197.5	199.5	198	207	200.5	207	199.5	194.5	191.3	202.5	100.5	190.5	195.5	194.5

Number of working volume determinations per chemostat (n) = 4

Number of chemostats (m) = 15

Total number of observations (n.m) = 60

X = mean

ΣX^2 = 147584.55 (1)

$\frac{\Sigma T^2}{n}$ = 147475.61 (2)

$\frac{(\Sigma X)^2}{n.m}$ = 147391.44 (3)

Table 23

Analysis of variance of working volume of 15 chemostats on 4 occasions

Source of variance	Sum of squares	Degrees of freedom	Mean squares	Variance ration (F)
Within chemostats	(2) - (3) 84.17	m - 1 14	6.01	2.483
Between chemostats	(1) - (2) 108.94	n.m - m 45	2.42	

The values of F for 14/45 degrees of freedom at the 1% and 5% significance level are 3.9 and 2.62 respectively. Therefore the variation within chemostats is not significantly greater than that between them at 1 and 5% levels. The calculated mean working volume of the chemostats is 49.6 ml which was used for the initial calculation of dilution rate (D).

3.10.3. Use of chemostats

The chemostats were assembled using silicon rubber tubing and sterilized by autoclaving at 121^{o} for 20 minutes. The chemostat jackets were connected in parallel to a water bath equipped with a heater pump adjusted to maintain the culture temperature at 37^{o}. The appropriate sterile medium was taken from

a 20 litre reservoir and added to the culture vessel through a dropper located at the head of the vessel supplied by a Watson Marlow MHRE 7 peristaltic pump with a 10 tube position delta fitting. The pump was able to serve up to 10 chemostats operating at the same dilution rate by inserting fork-like glass tubing in the medium line. All pumps were calibrated and a curve of speed scale versus flow rate (ml h^{-1}) was plotted for each pump. Sterile oxygen-free nitrogen was passed to the base of the chemostat and continuously flushed through the medium at a rate of 80 ml min^{-1} to effect mixing and maintain anaerobiosis. When the medium level reached 3/4 of the chemostat working volume the pump was stopped and the medium was inoculated aseptically with several ml of E. coli from an anaerobic batch culture grown in the same medium. After overnight growth, or when visible growth was observed, the pump was turned on at a low dilution rate for at least 12 h and then readjusted to the required rate. The dilution rate was continuously monitored by pumping water from a water reservoir, using the same inducer pump, and collecting it in a measuring cylinder.

On altering the dilution rate the culture was normally left for 5 medium changes to re-equilibrate.

To check that cultures were limited by the named nutrient as calculated, the concentration of this nutrient was increased by injecting aseptically a few ml of a concentrated solution into the reservoir, using a syringe. A corresponding increase in OD indicated that the original culture was indeed limited by the added nutrient.

The degree of anaerobiosis was measured, as previously discussed in section 3.4.2.1 . It was found that at all dilution rates and media tested the % O_2 saturation did not exceed 5%.

To check for absence of contamination, and that the properties of cultures were stable, samples were removed from the chemostats at intervals and colonial

morphology checked on NA and MacConkey agar plates; samples were occasionally examined microscopically using Gram's stain and checked by routine biochemical tests.

3.10.4. Determination of K_s in single culture chemostats

The apparent K_s for G-lim, P-lim and K^+-lim cultures were determined by measuring the concentration of the limiting nutrient left as soon as the cultures had reached a steady state at a specified D. Approximately 5ml quantities of the appropriate culture were membrane filtered and the filtrates were used for analysis of the limiting nutrient. By applying the following formula (Herbert et al, 1956) one can calculate the K_s of any particular limiting nutrient.

$$\tilde{s} = K_s\left(\frac{D}{\mu m - D}\right)$$

where $\tilde{s}$ is the steady state concentration of the limiting nutrient.

D is the dilution rate.

μm is the maximum growth rate of the organism in batch cultures, calculated from Figures 49, 50 and 51.

3.10.5. Competition experiments

The same media (see section 3.10.1.) and culture conditions (see section 3.10.3.) used in single culture studies were used in the competition experiments. Chemostat cultures of R- and R+ cells were allowed to reach a steady rate of $D \cong 0.1\ h^{-1}$. Approximately 2ml of each chemostat culture was withdrawn and replaced by some of the alternate culture. A few minutes after mixing, and subsequently at definite time intervals, samples were taken for OD_{470} and pH measurements and viable count determinations. The % R+ cells was determined by comparing viable counts on NA containing kanamycin SO_4 (15 $\mu g\ ml^{-1}$) with the counts on NA.

4. RESULTS

4. RESULTS

4.1 Nutritional requirements of *E. coli* R- and R+

Introduction

The ingredients of a chemically defined medium (CDM) suitable for the growth of *E. coli* are well known (Davis *et al*, 1973).

Ismail (1977) investigated the quantitative nutritional requirements of *E. coli* grown aerobically and on the basis of these results, Klemperer *et al* (1979) formulated a chemically defined medium which could be used to prepare a CDM limited in one constituent, that would achieve a required cell concentration in the presence of a controlled excess of non-limiting nutrients.

In the present study the results on depletion of cultures by glucose, phosphate and magnesium were repeated, to check for any variation in the culture and any effect of replacement of $NH_4H_2PO_4/(NH_4)_2HPO_4$ by KH_2PO_4/Na_2HPO_4, NH_4FeSO_4 by $(NH_4)SO_4.FeSO_4.6H_2O$ and 25mM MOPS by 50mM.

4.1.1. Aerobic growth requirements

The R- and R+ cultures - depleted of glucose, magnesium or phosphate (grown in CDMA, D and F respectively, Table 4) show characteristic growth curves (Figures 18, 20 and 22 respectively).

In glucose-depleted cultures, growth ceased sharply as glucose became limiting, in contrast to Mg^{2+} or PO_4^{3-} depleted cultures, where growth did not cease sharply, but progressively slowed as the Mg^{2+} or PO_4^{3-} became limiting. In these experiments, little difference was found in the requirements for R- and R+ (Figures 19, 21 and 23). Table 24 shows the concentrations of added glucose, Mg^{2+} and PO_4^{3-}, calculated to permit exponential growth to an OD_{470} of 1.0 compared with data from Klemperer *et al* (1979).

Figure 18

Glucose-depleted aerobic growth curves of *E. coli* R- and R+

Key

Glucose (mM)	R-	R+
0.56	△	▲
0.67	□	■
2.0	○	●

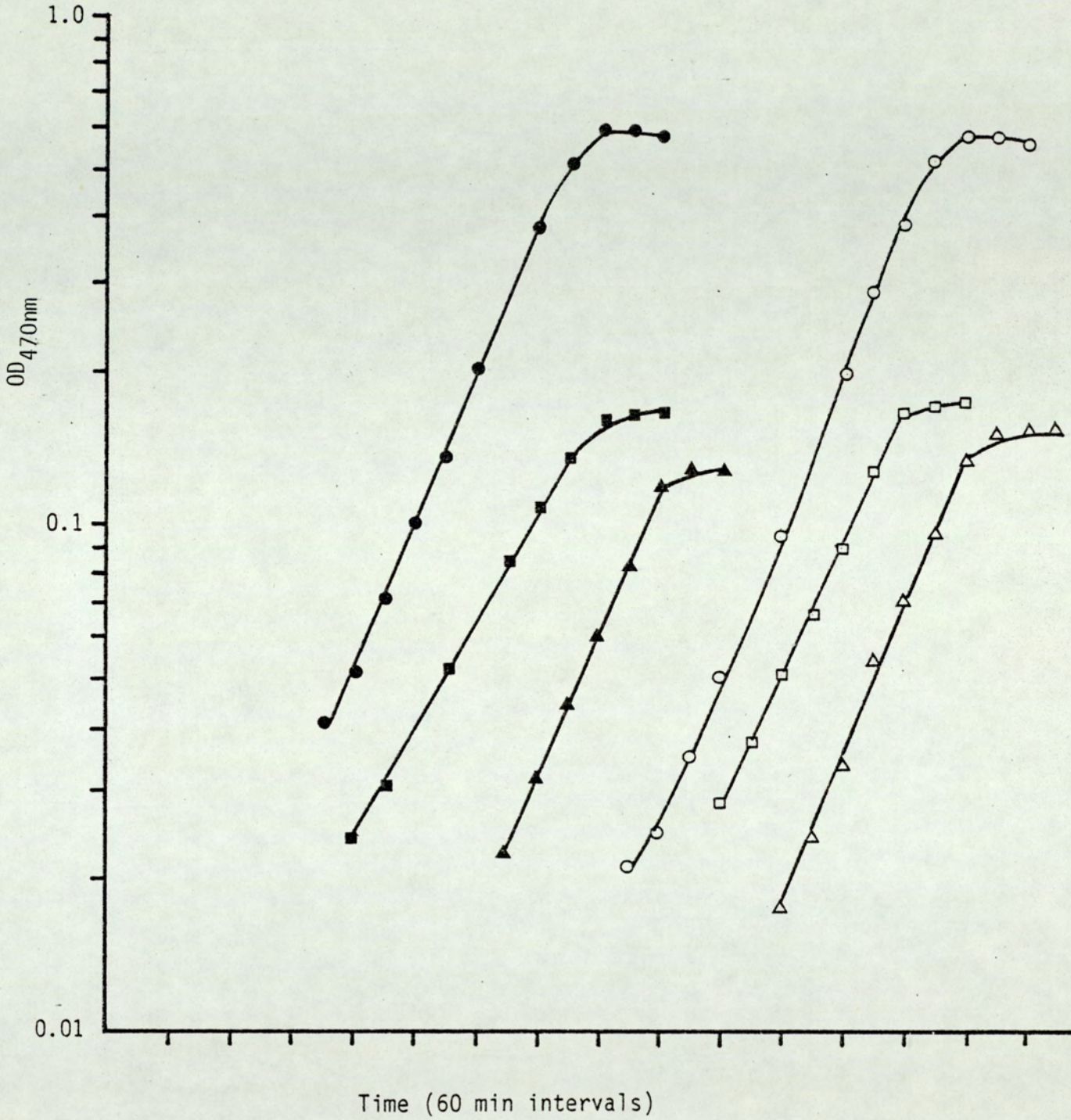

1.0
0.1
0.01
OD_{470nm}
Time (60 min intervals)

Figure 19

Relation between maximum exponential aerobic growth of *E. coli* R- and R+ and added glucose concentration

Key

R- ○

R+ ●

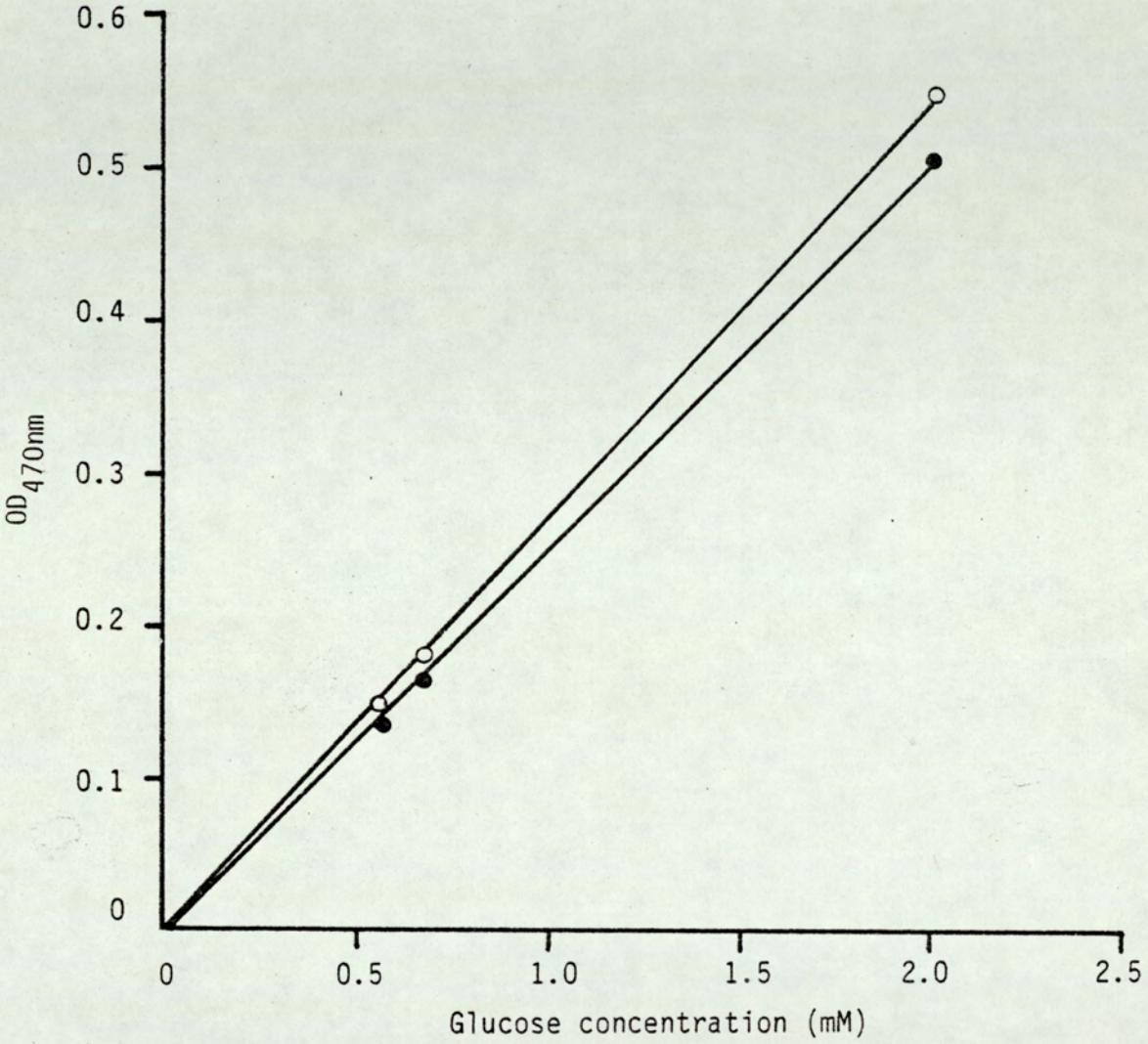

0.6
0.5
0.4
0.3
0.2
0.1
0
OD470nm
0
0.5
1.0
1.5
2.0
2.5
Glucose concentration (mM)

Figure 20

Magnesium-depleted aerobic growth curves of *E. coli* R- and R+

Key

Magnesium (mM)	R-	R+
0.0031	△	—
0.0062	—	▲
0.008	□	■
0.015	○	●

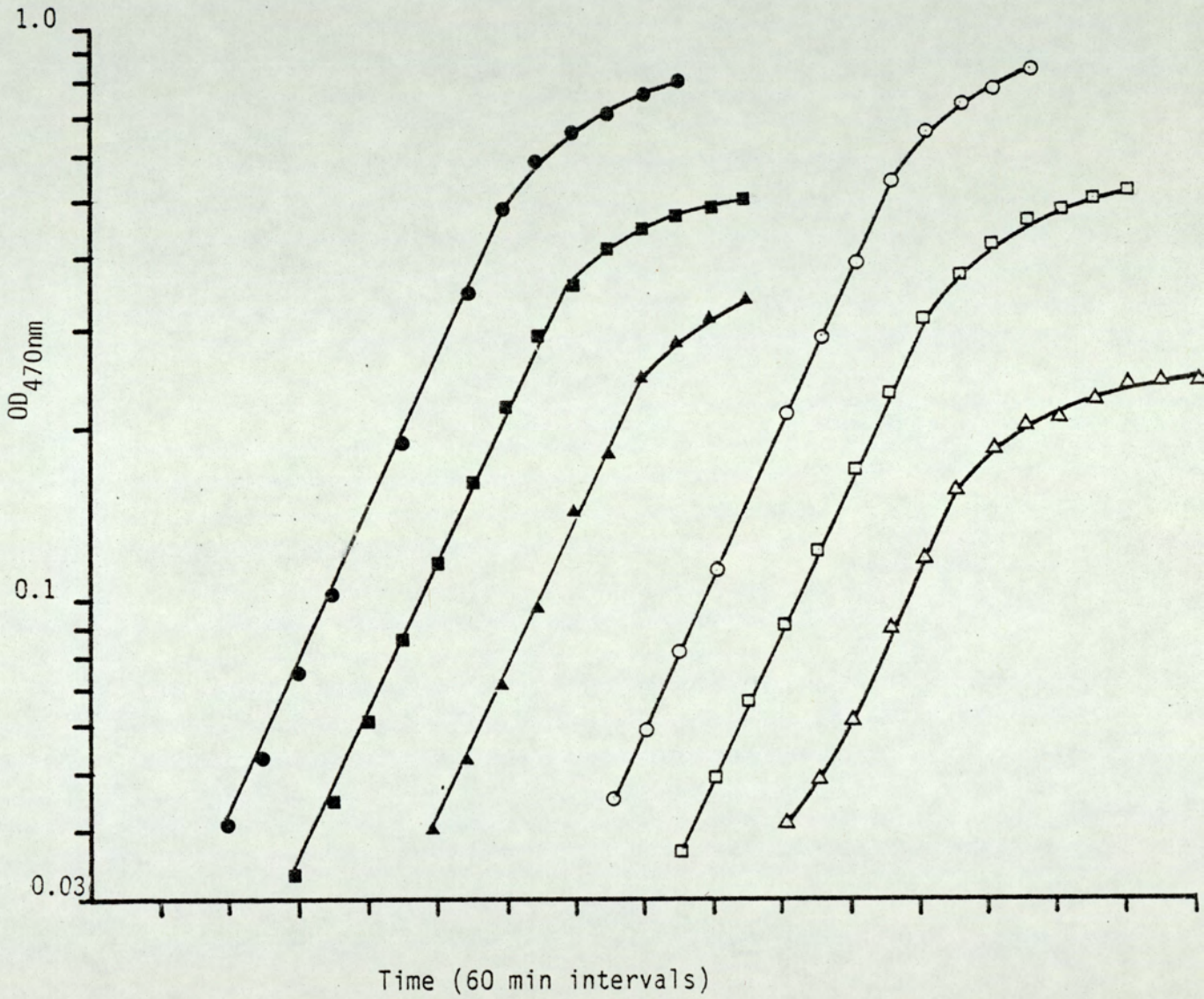

1.0
0.1
0.03
OD_{470nm}
Time (60 min intervals)

Figure 21

Relation between maximum exponential aerobic growth of *E. coli* R- and R+ and added Mg^{2+} concentration

Key

R- 

R+ 

(Each point is the mean of three experiments with SD)

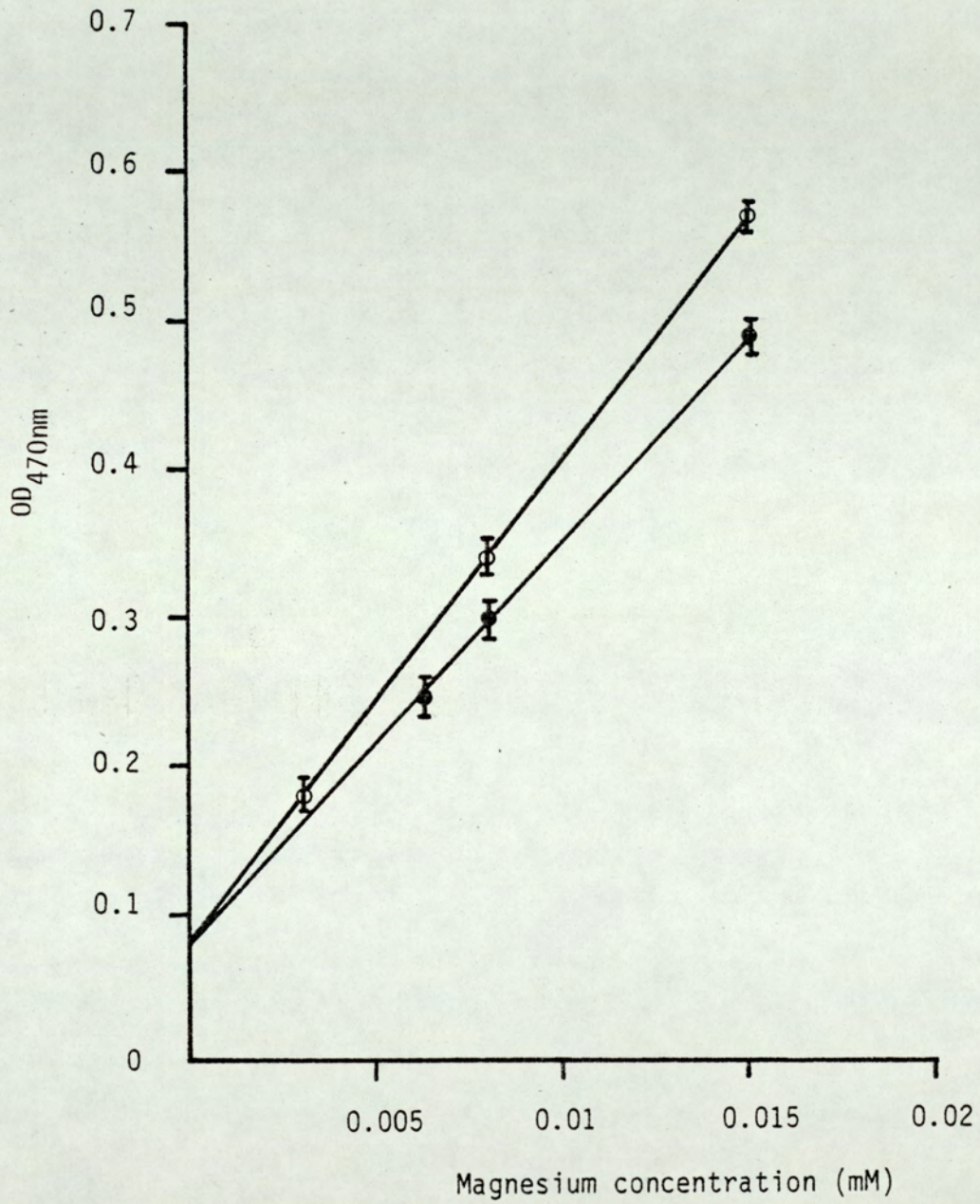

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD_{470nm}
0.005
0.01
0.015
0.02
Magnesium concentration (mM)

Figure 22

Phosphate-depleted aerobic growth curves of *E. coli* R- and R+

Key

Phosphate (mM)	R-	R+
0.48	○	—
0.12	□	■
0.14	—	●
0.3	△	▲

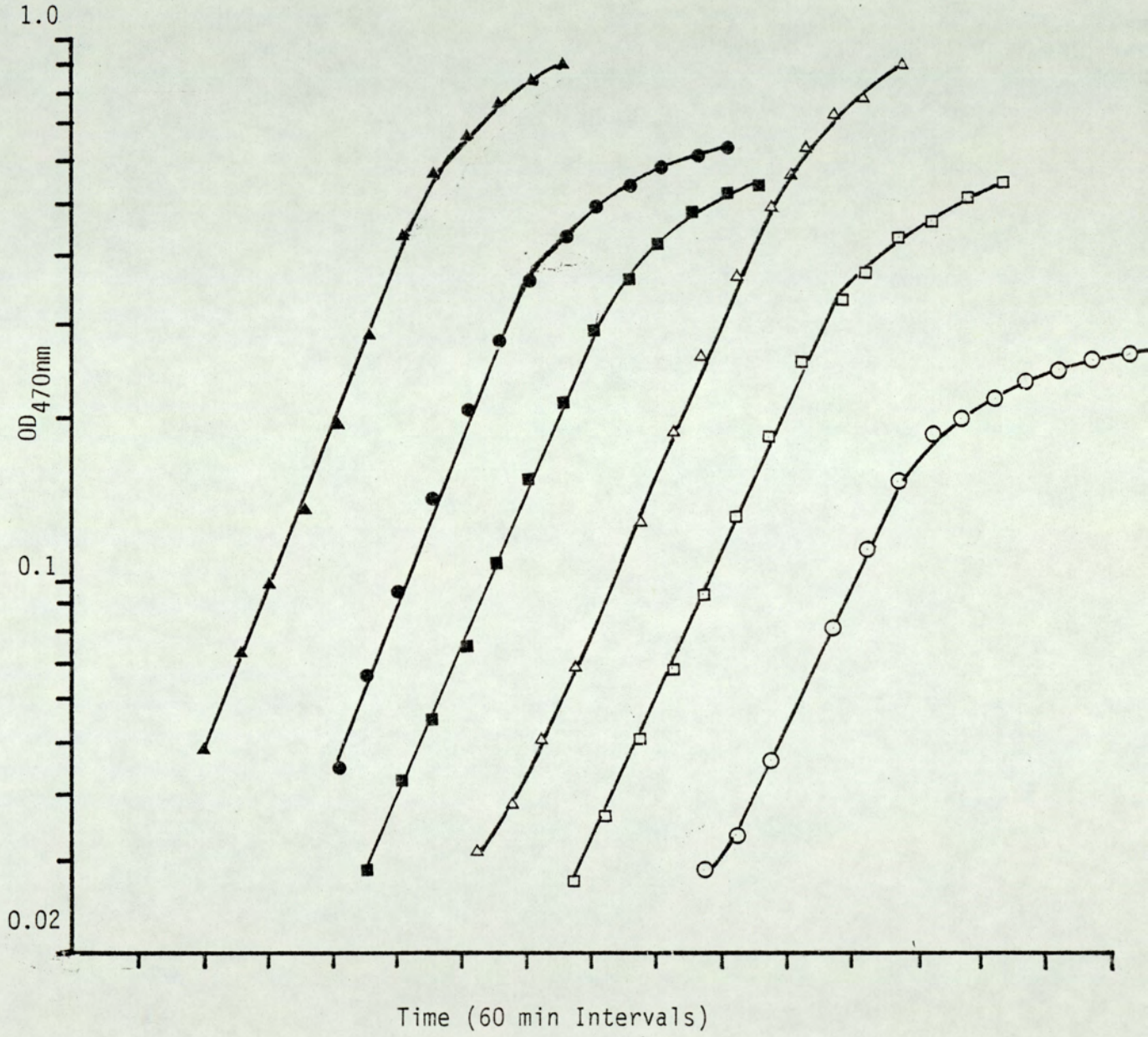

1.0
0.1
0.02
OD_{470nm}
Time (60 min Intervals)

Figure 23

Relation between maximum exponential aerobic growth of *E. coli* R- and R+ and added PO_4^{3-} concentration

Key

R- ○

R+ ●

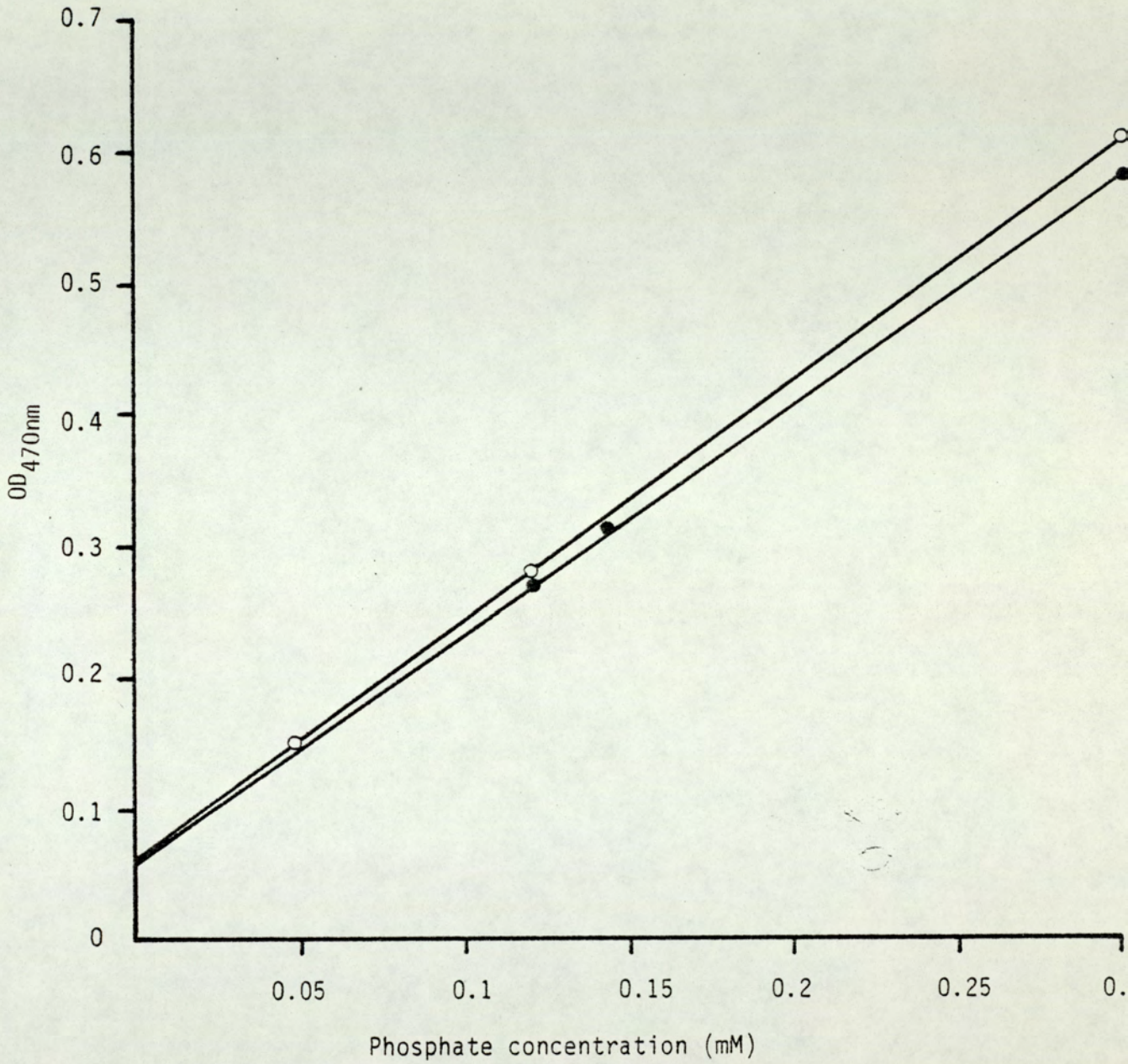

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD_{470nm}
0.05
0.1
0.15
0.2
0.25
Phosphate concentration (mM)

Table 24

Concentrations of added nutrients which permit exponential aerobic growth of *E. coli* R- and R+ to OD 1.0

Depleting Nutrient	Nutrient concentration (mM)			
	R-		R+	
	λ420 (i)	λ470 (ii)	λ420 (i)	λ470 (ii)
Glucose	2.5	3.7	2.8	4.0
Magnesium	0.013	0.026	0.025	0.031
Phosphate	0.17	0.48	0.56	0.51

(i) Klemperer *et al* (1979)

(ii) Calculated from Figures 19, 21 and 23

4.1.2. Anaerobic growth requirements

Introduction

Quantitative data for growth under anaerobic conditions are needed to simulate the conditions in the large intestine. The media used were based on that determined aerobically (CDMH, Table 4) and initially all nutrients were in excess sufficient to allow aerobic exponential growth of R- and R+ cultures to an OD_{470} of at least 10.0. In subsequent experiments, media were adjusted appropriately (Table 6 and 7 respectively). In order to prevent a sharp drop in pH of the growing cultures, the pH of the buffers was increased to pH 7.6. With this system, at the end of the experiments the pH had dropped to between 6.2 - 7.1 depending on the nature of the depleting nutrient.

4.1.2.1. Glucose growth requirement

Figures 24 and 25 show the growth curves of E. coli R- and R+ in CDMI (Table 6 and 7 respectively) containing various concentrations of D(+) glucose as carbon and energy source. At lower concentrations of glucose, abrupt cessation of exponential growth occurred, whilst a more gradual slowing of the growth was observed at higher concentrations.

Figure 26 shows the relationship between OD_{470} and added glucose for a range of glucose concentrations from 0.75mM - 27mM. A linear relationship existed to an OD_{470} of c a. 0.7 and 0.8 for R- and R+ respectively, corresponding to an initial glucose concentration of 5.5mM. Extrapolation of these curves to the X-axis showed zero glucose contamination of the medium.

Figure 24

Effect of glucose concentration on the anaerobic growth of *E. coli* R-

Key

Glucose (mM)	
0.75	△
1.5	⊙
2.25	□
3.0	▽
3.4	⊗
6.7	◇
13.5	◇
20.2	○
26.9	×

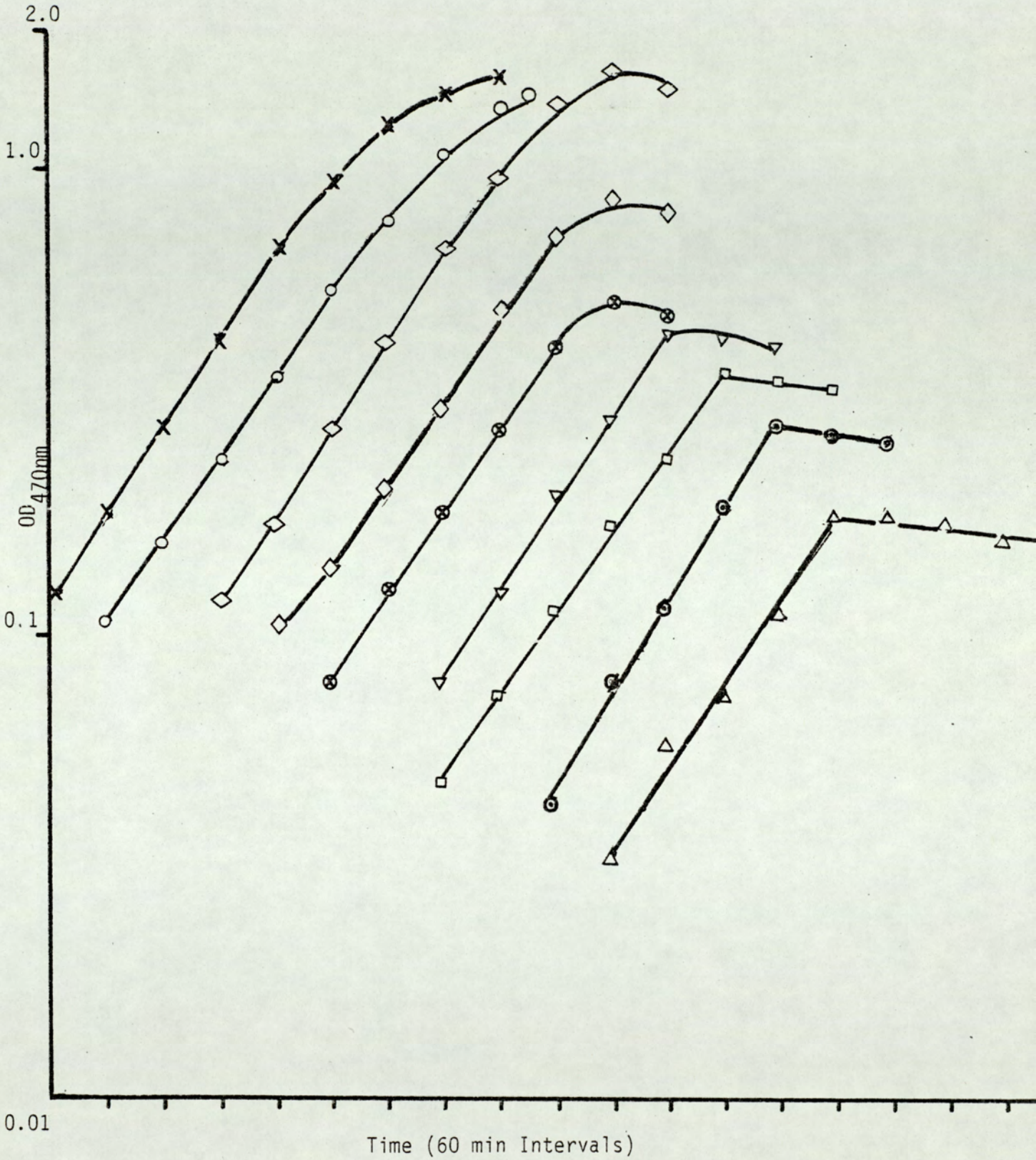

2.0
1.0
0.1
0.01
OD470nm
Time (60 min Intervals)

Figure 25

Effect of glucose concentration on the anaerobic growth of *E. coli* R+

Key

Glucose (mM)	
1.0	●
2.25	⊗
3.0	■
4.5	▲
6.7	◐
11.2	◣
13.5	◧
26.9	■

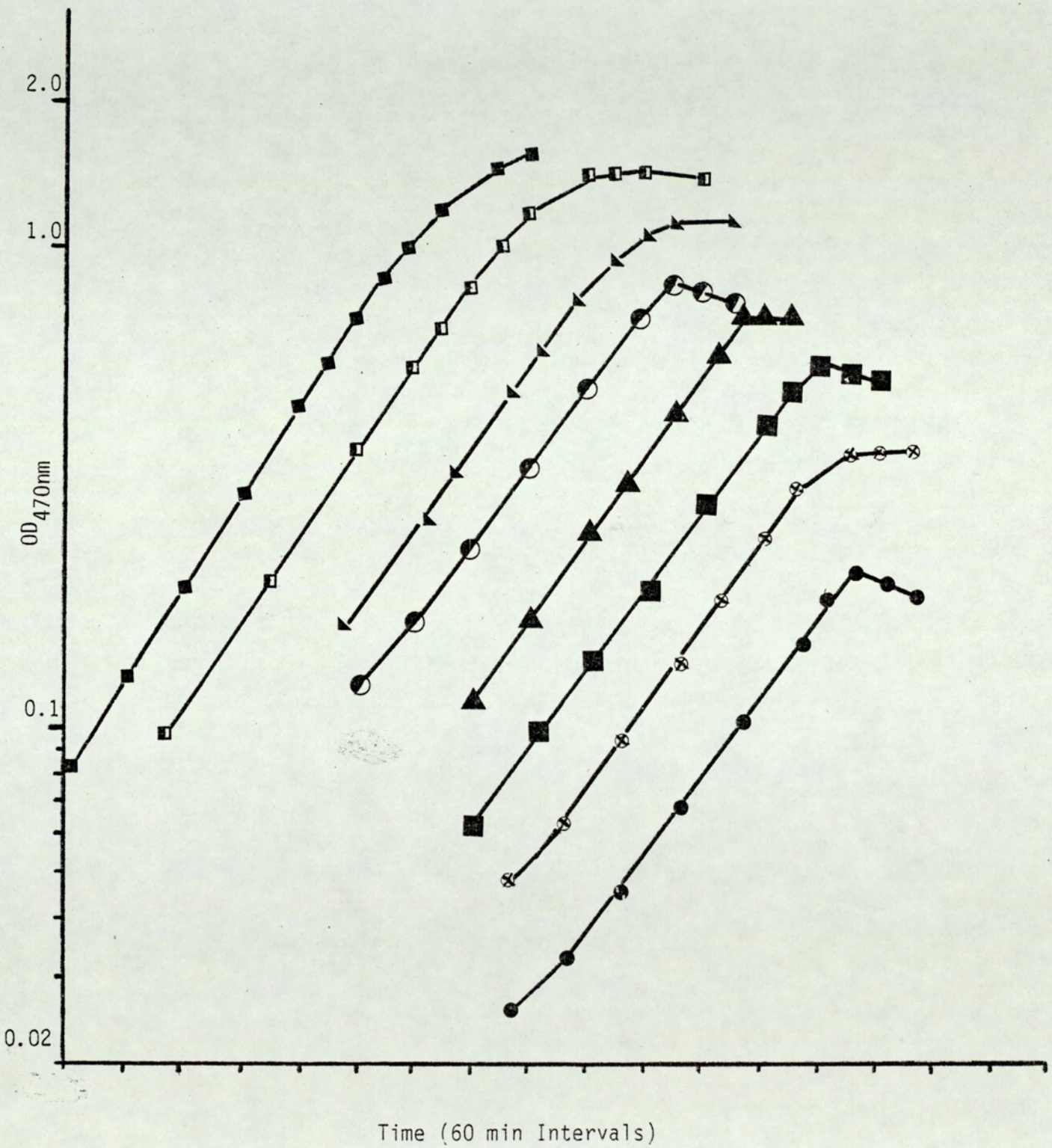

2.0
1.0
0.1
0.02
OD470nm
Time (60 min Intervals)

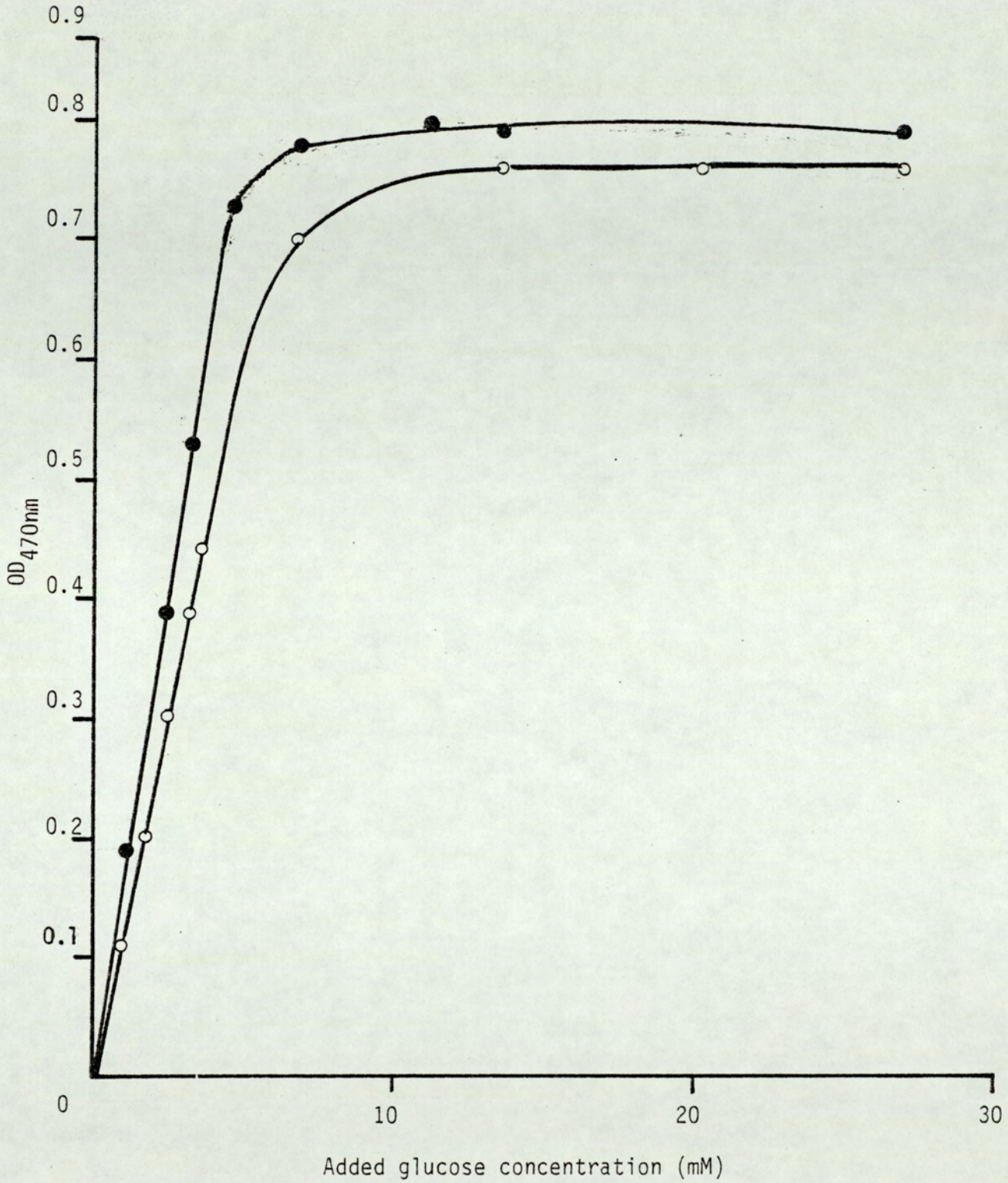
Figure 26

Relation between maximum exponential anaerobic growth of *E. coli* R- and R+ and added glucose concentration

Key

R- ○

R+ ●


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD470nm
10
20
30
Added glucose concentration (mM)

4.1.2.2. Ammonium ion growth requirement

Unlike glucose-depleted cultures, R- and R+ cultures grown in CDM2 (Table 6 and 7 respectively) containing graded concentration of NH_4^+ did not exhibit abrupt cessation of growth; however, as NH_4^+ became restricting, the growth rate decreased abruptly followed by a slow increase in OD_{470} with time (Figures 27 and 28). R+ cells grew significantly slower than R- ones at an NH_4^+ concentration of 0.56mM with doubling time of 4.1 and 1.8h respectively.

The relationship between OD_{470} and added NH_4^+ concentration is shown in Fig. 29 . Linearity was maintained to an OD_{470} max of ca. 0.74 corresponding to an NH_4^+ concentration of 2.3mM.

The medium contamination with NH_4^+ found by extrapolation of the plots, was 0.3mM.

4.1.2.3. Magnesium growth requirement

The growth curves of both R- and R+ cultures obtained when Mg^{2+} was depleted in CDM3 (Table 6 and 7 respectively) were found to be similar in shape to those obtained by NH_4^+-depletion. At Mg^{2+} concentration of 0.01mM the growth rate of the R+ culture was significantly slower than that of the R- one. Figures 30 and 31 show the relationship between OD_{470} and added Mg^{2+}.

The relationship between the OD_{470} at which growth ceased to be exponential and added Mg^{2+} is shown in Fig. 32. Extrapolation of the plots to the X-axis showed Mg^{2+} medium contamination of 0.004mM.

Figure 27

Effect of NH_4^+ concentration on the anaerobic growth of *E. coli* R-

Key

Ammonium (mM)	
0.37	▼
0.56	◉
0.93	■
1.5	▲
2.2	◆
3.3	▽
4.5	○
5.6	◇
22.2	△

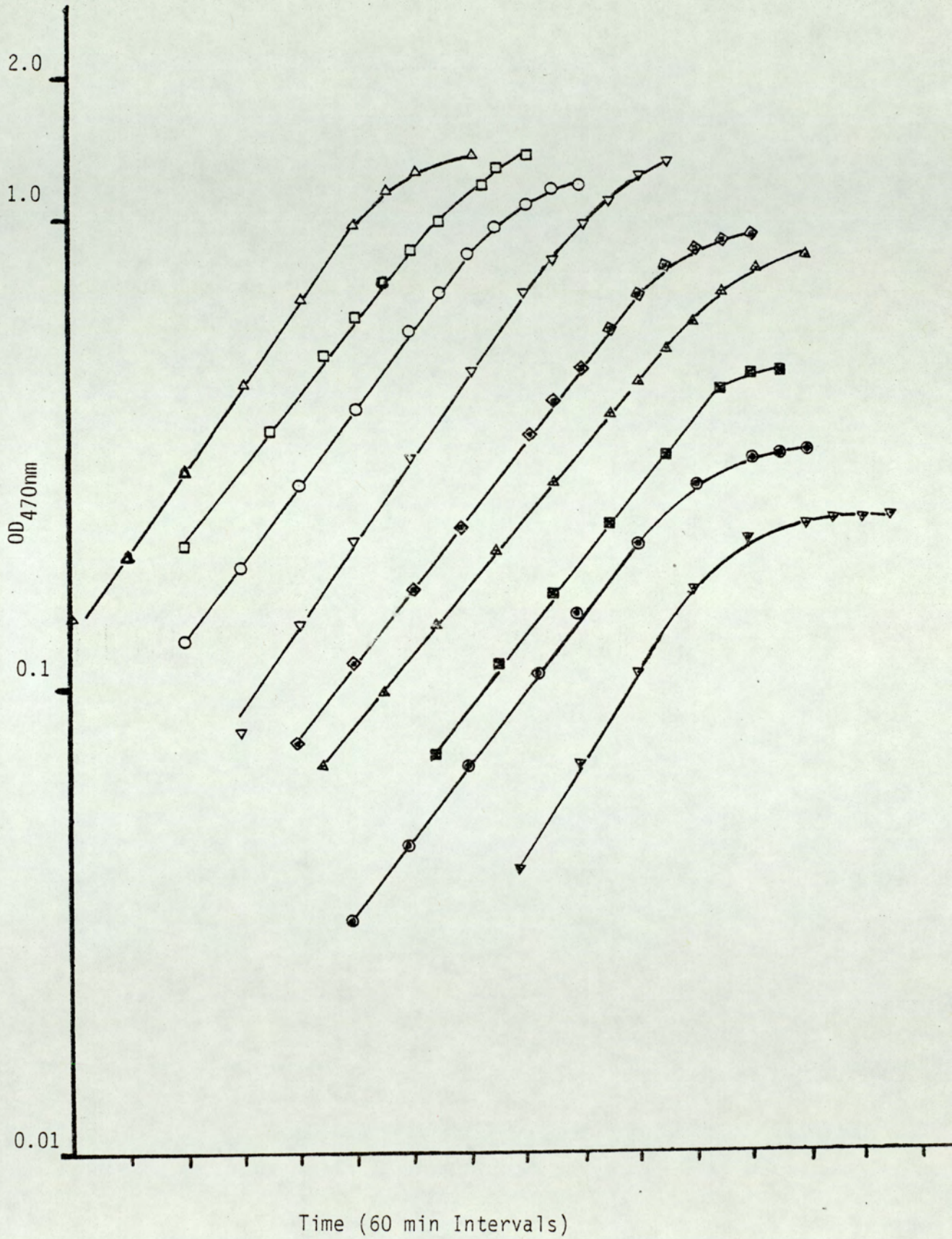

2.0
1.0
0.1
0.01
OD_{470nm}
Time (60 min Intervals)

Figure 28

Effect of NH_4^+ concentration on the anaerobic growth of *E. coli* R+

Key

Ammonium (mM)	
0.56	◆
1.0	▼
1.5	▲
2.0	◢
3.3	■
5.6	●
22.2	✘

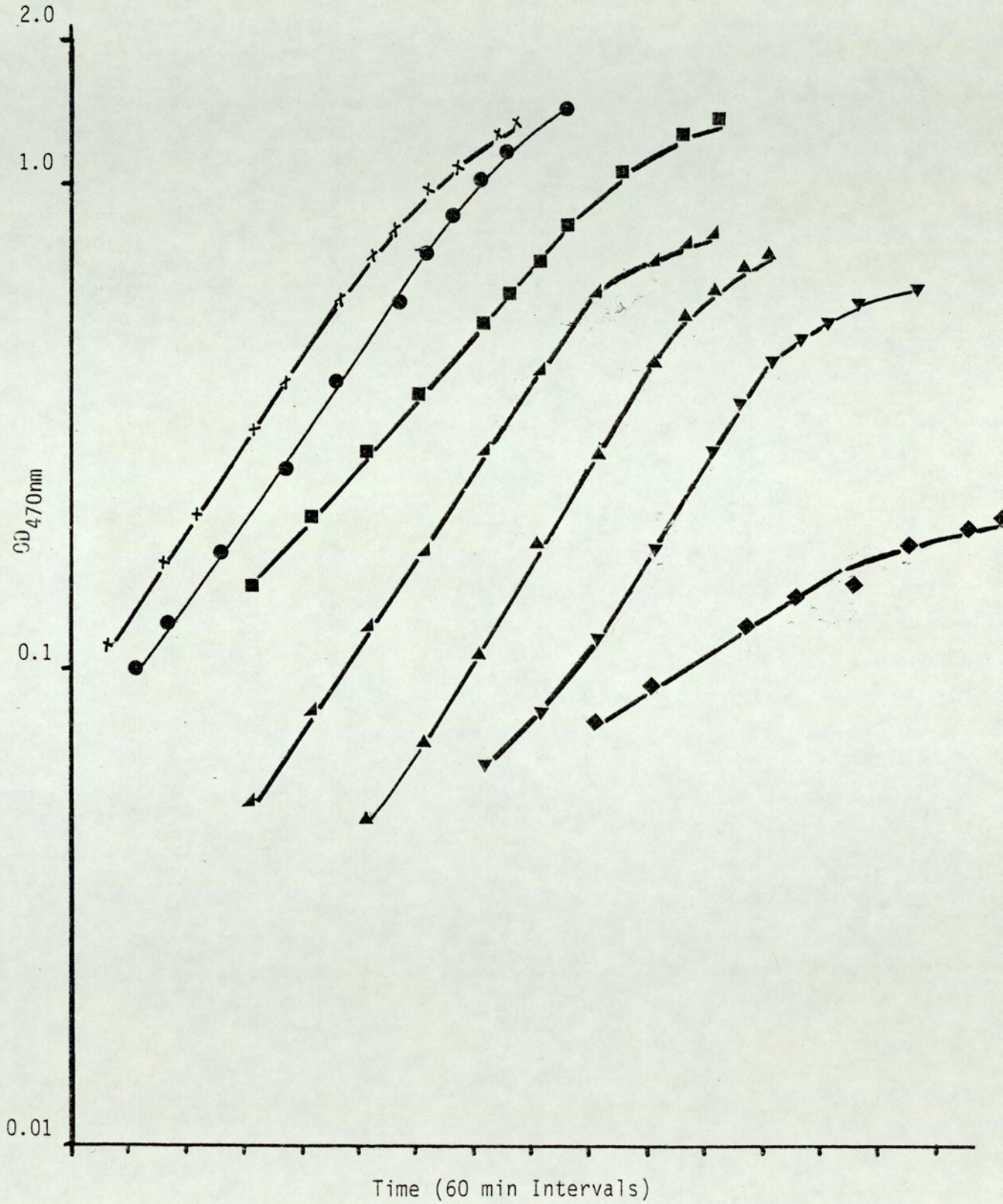

2.0
1.0
0.1
0.01
OD470nm
Time (60 min Intervals)

Figure 29

Relation between maximum exponential anaerobic growth of *E. coli* R- and R+ and added NH_4^+ concentration

Key

R- ○

R+ ●

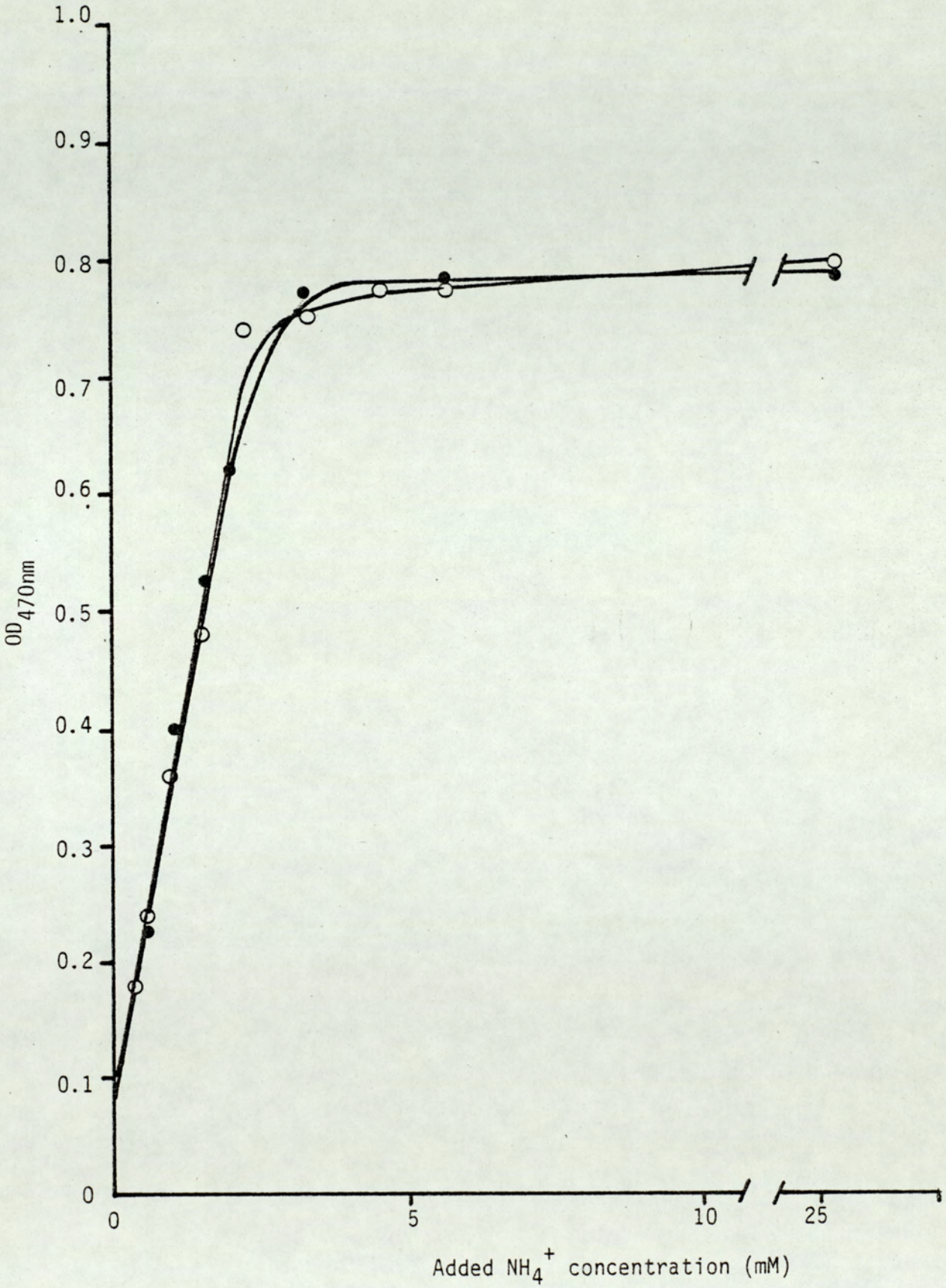

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 470nm
0
5
10
25
Added NH4+ concentration (mM)

Figure 30

Effect of Mg^{2+} concentration of the anaerobic growth of E. coli R-

Key

Magnesium (mM)	
0.008	▽
0.01	◺
0.016	▲
0.021	◘
0.031	◉
0.042	◺
0.083	△
0.104	□
0.208	○

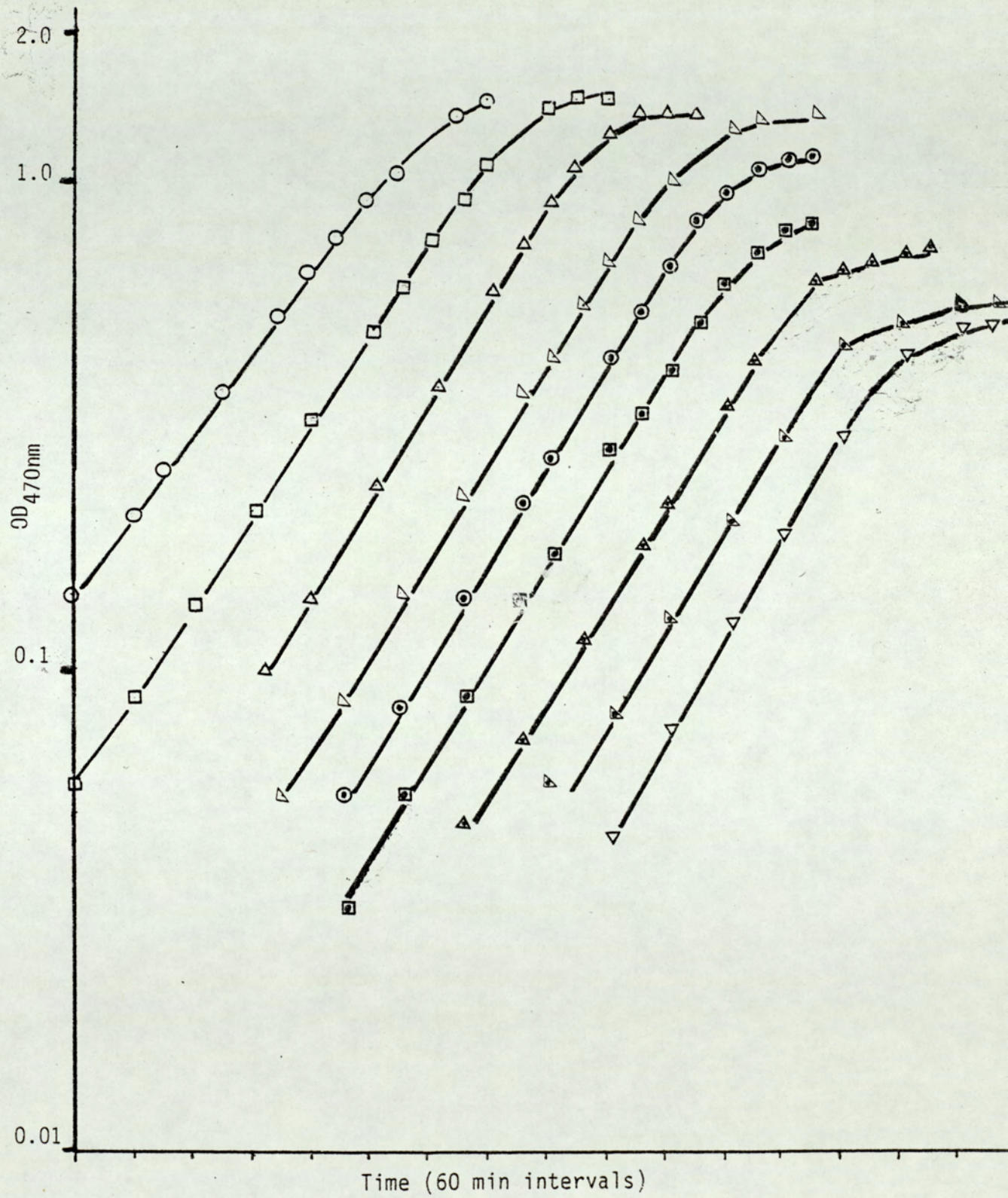

2.0
1.0
0.1
0.01
OD470nm
Time (60 min intervals)

Figure 31

Effect of Mg^{2+} concentration on the anaerobic growth of *E. coli* R+

Key

Magnesium (mM)	
0.01	◆
0.025	▶
0.031	●
0.05	■
0.208	▲

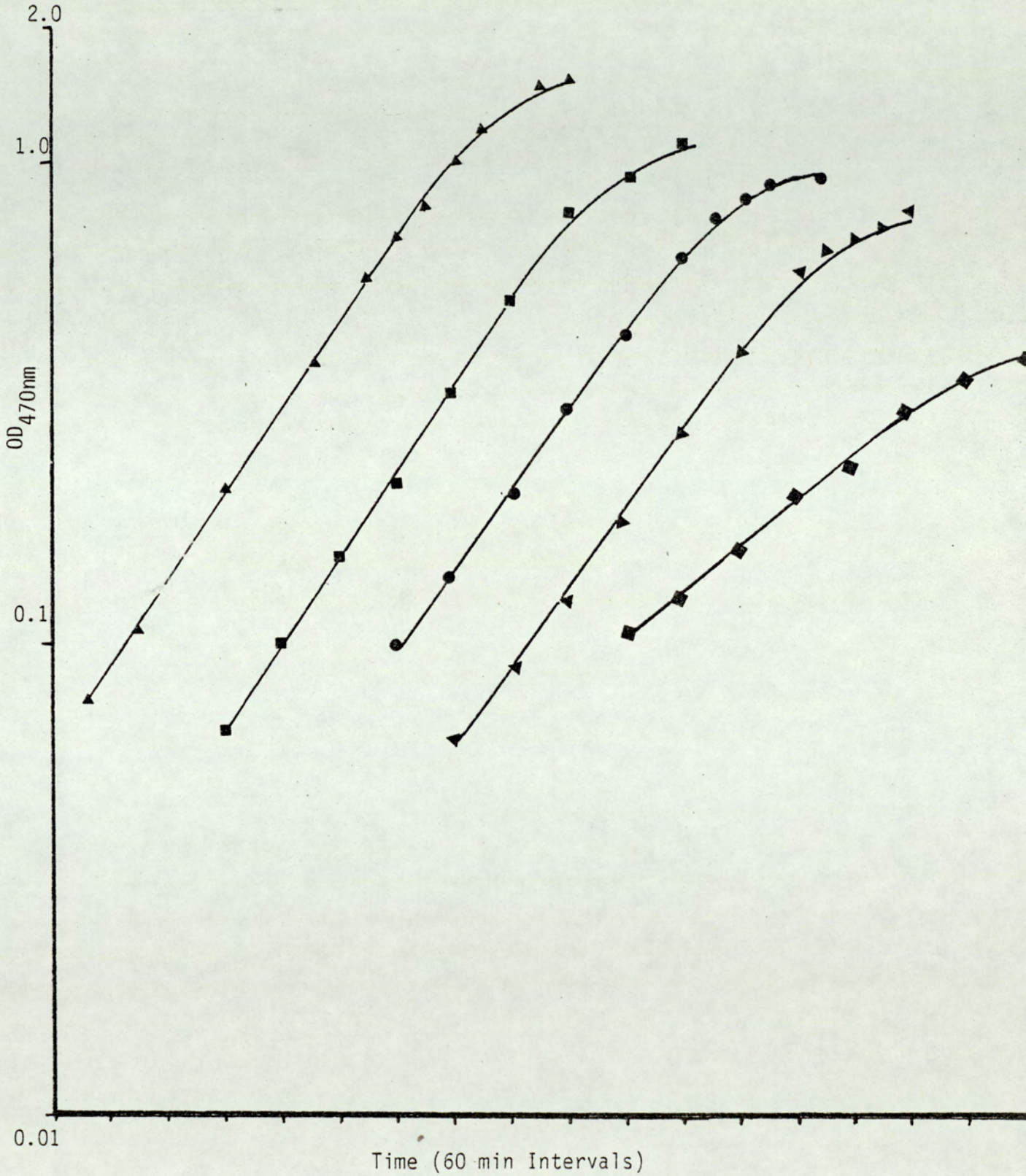

2.0
1.0
0.1
0.01
OD470nm
Time (60 min Intervals)

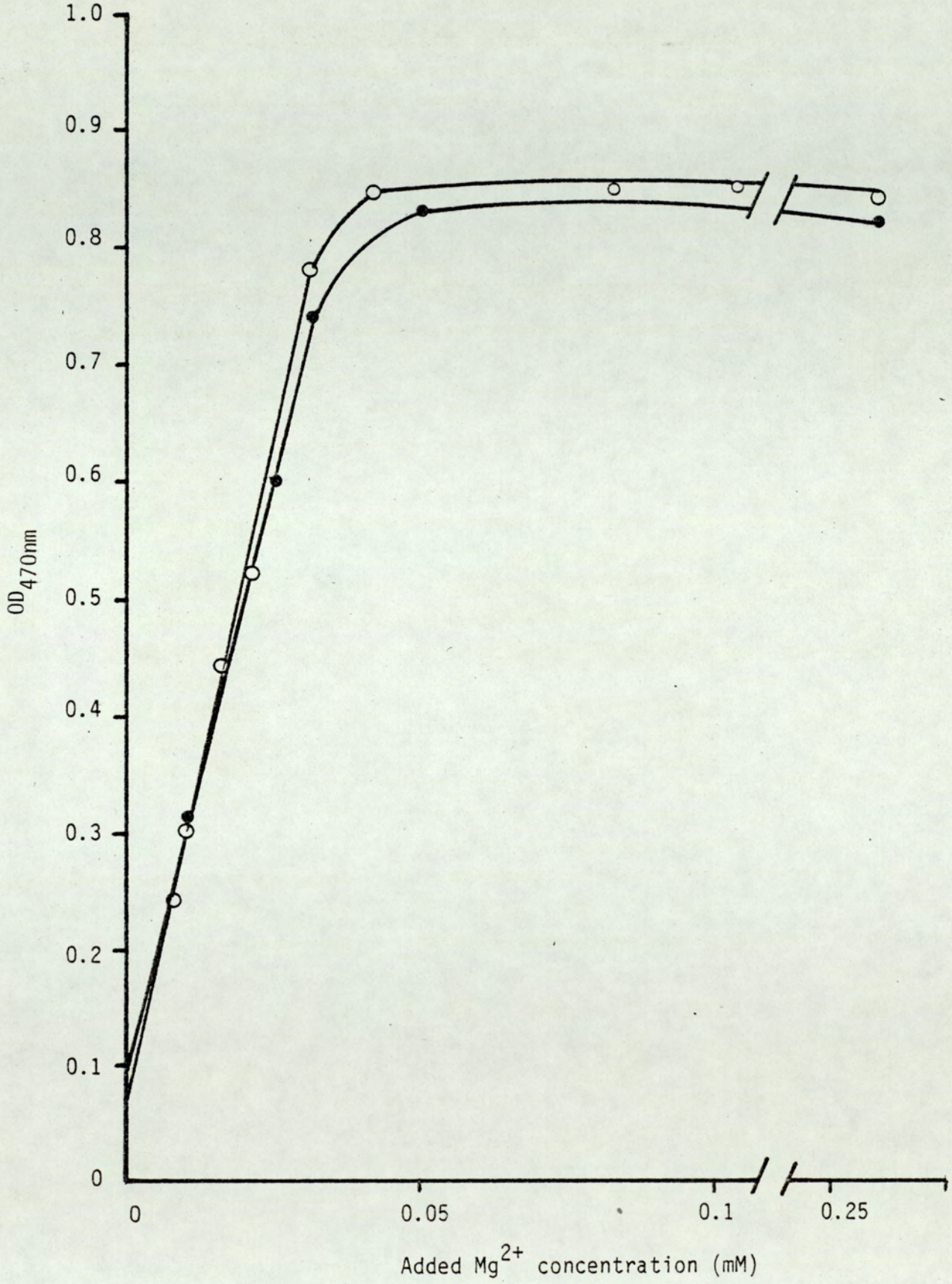
Figure 32

Relation between maximum exponential anaerobic growth of *E. coli* R- and R+ and added Mg^{2+} concentration

Key

R- ○

R+ ●


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD470nm
0
0.05
0.1
0.25
Added Mg2+ concentration (mM)

4.1.2.4. Phosphate growth requirement

Phosphate-depletion growth curves were comparable to those obtained for NH_4^+ depletion. They show a slowing of the growth rate after the onset of depletion. Figures 33 and 34 show the relationship between OD_{470} and added phosphate in CDM4 (Table 6 and 7 for R- and R+ respectively).

The relationship between OD_{470} at which growth ceased to be exponential and added PO_4^{3-} is shown in Fig. 35.

Phosphate contamination of the culture medium was estimated at 0.05mM by extrapolation.

4.1.2.5. Sulphate growth requirement

Figures 36 and 37 show the characteristic growth curves for initial SO_4^{2-} concentrations of 0.0125 to 0.204mM. The shape of R- and R+ growth curves were similar to NH_4^+, Mg^{2+} and PO_4^{3-} depletion curves. In Fig. 38 the graphs show the relationship between maximum exponential growth and added SO_4^{2-} for the R- and R+ strains, using CDM6 (Tables 6 and 7 respectively). The data suggests that there may be a difference in their SO_4^{2-} requirements.

Extrapolation of these graphs to the X-axis gave SO_4^{2-} contamination of 0.015mM in the medium.

4.1.2.6. Potassium growth requirement

The growth curves for K^+-depleted R- and R+ cultures are shown in Figs. 39 and 40 respectively. Following exponential growth the growth rate decreased gradually. Figure 41 shows the relationship between OD_{470} and added K^+ using CDM5 (Table 6, R- and Table 7, R+).

Figure 33

Effect of PO_4^{3-} concentration on the anaerobic growth of *E. coli* R-

Key

Phosphate (mM)	
0.06	▽
0.12	⊙
0.24	◺
0.36	△
0.48	○
0.6	◇
1.19	▬
2.88	▮

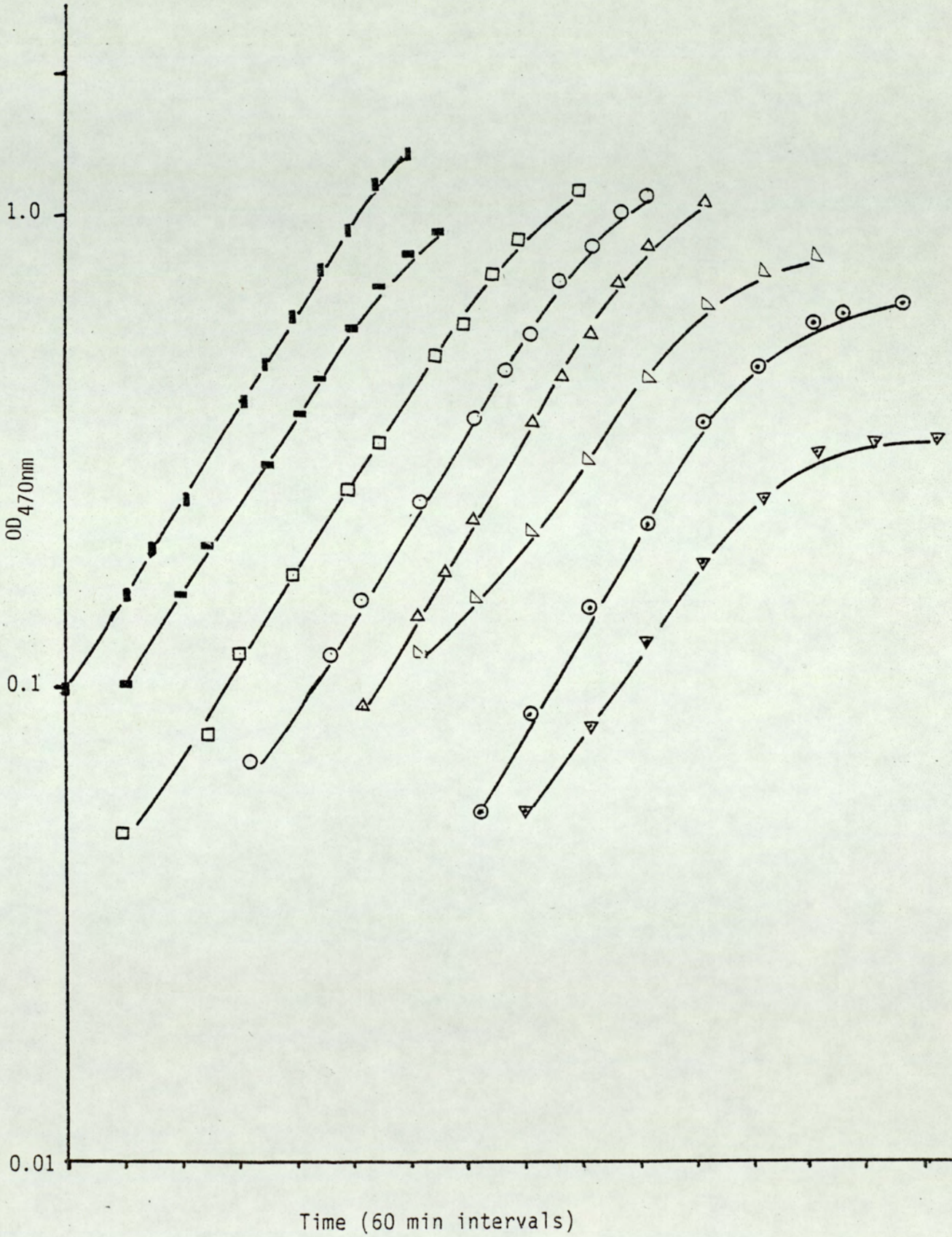

1.0
0.1
0.01
OD470nm
Time (60 min intervals)

Figure 34

Effect of PO_4^{3-} concentration on the anaerobic growth of E. coli R+

Key

Phosphate (mM)	
0.1	X
0.18	◣
0.2	■
0.3	▼
0.5	▬
0.6	●
2.38	▮

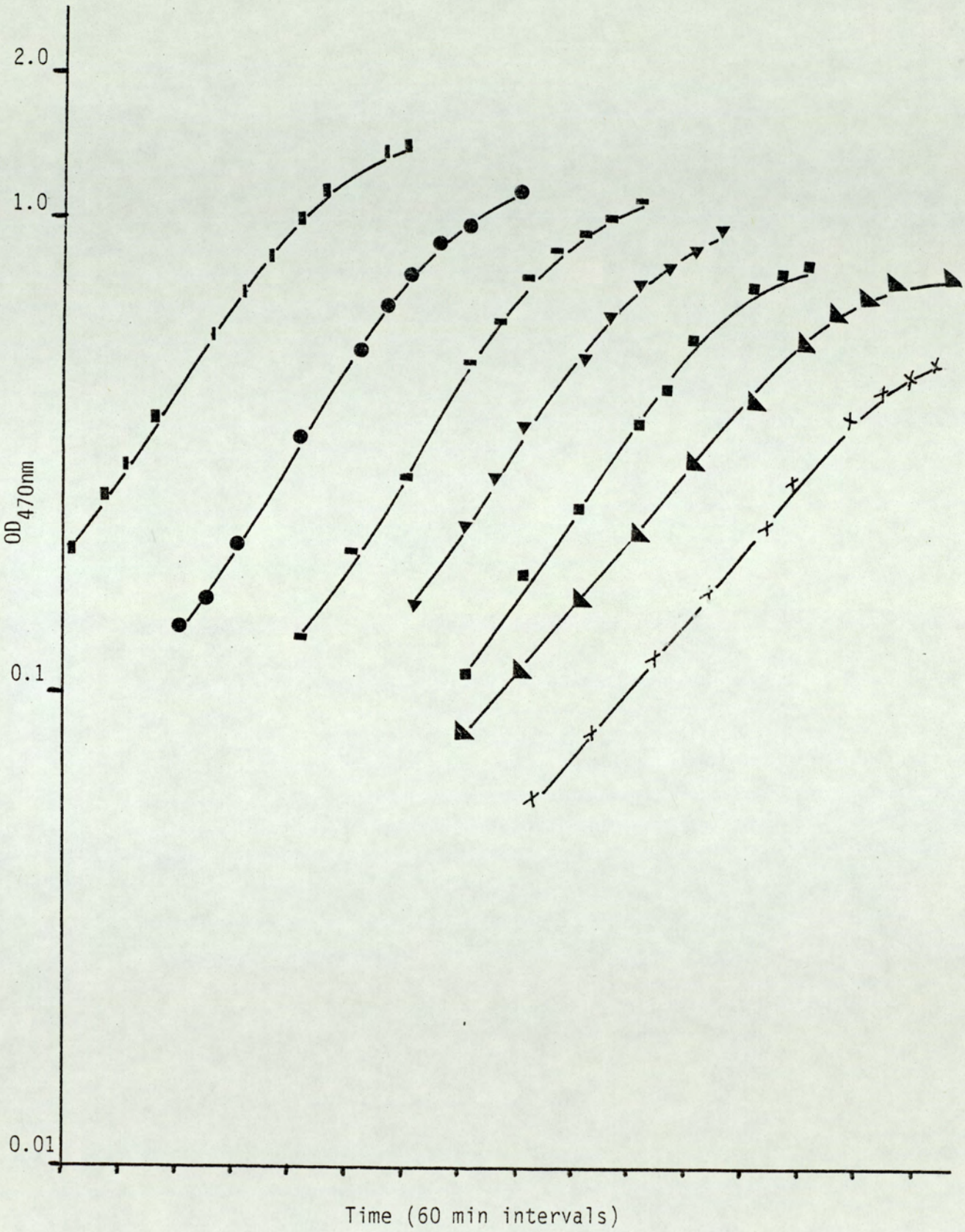

2.0
1.0
0.1
0.01
OD470nm
Time (60 min intervals)

Figure 35

Relation between maximum exponential anaerobic growth of *E. coli* R- and R+ and added PO_4^{3-} concentration

Key

R- ⊙

R+ ●

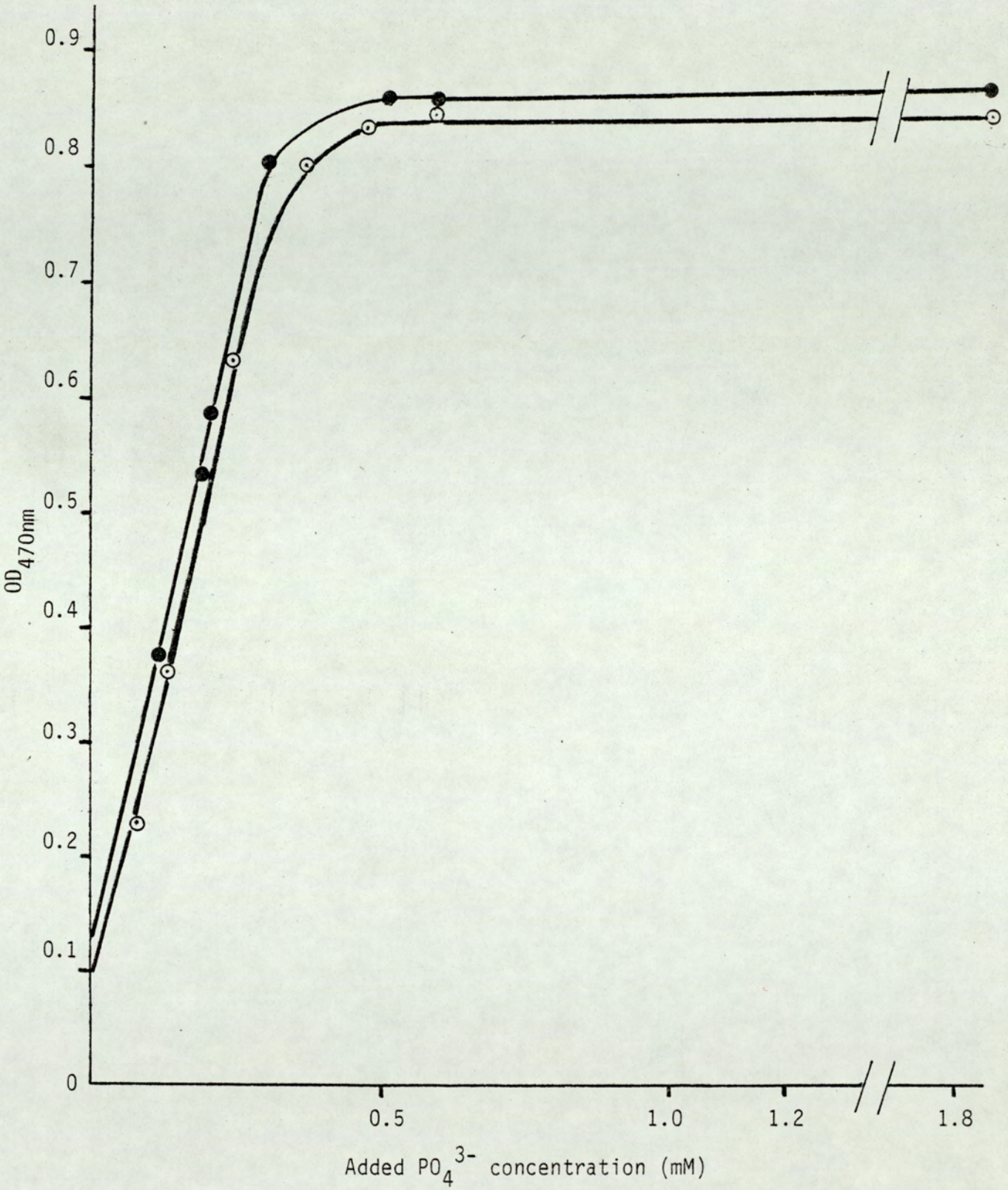

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD470nm
0.5
1.0
1.2
1.8
Added PO4 3- concentration (mM)

Figure 36

Effect of SO_4^{2-} concentration on the anaerobic growth of *E. coli* R-

Key

Sulphate (mM)	
0.0125	○
0.023	□
0.044	×
0.088	◇
0.063	◎
0.204	△

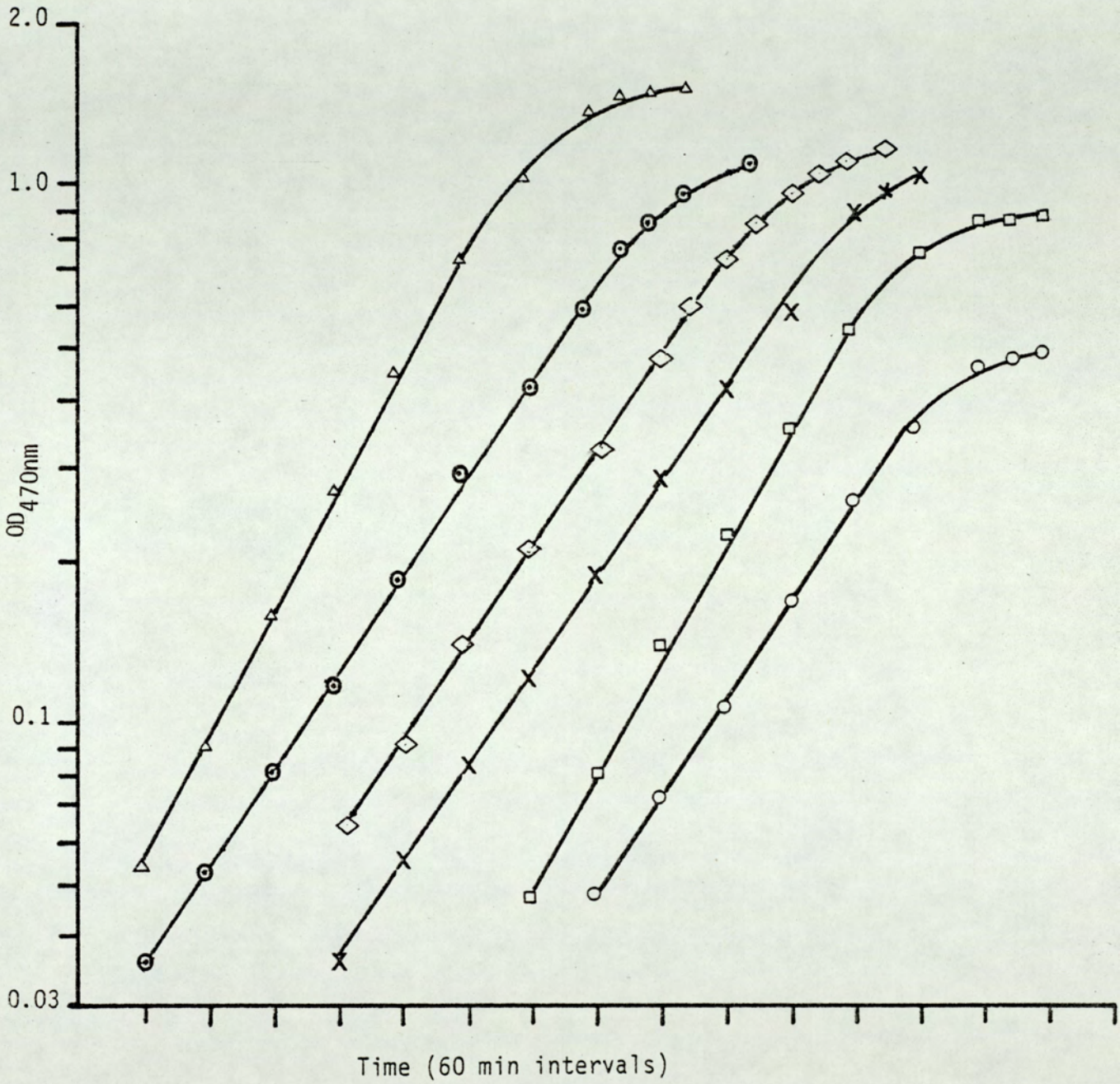

2.0
1.0
0.1
0.03
OD470nm
Time (60 min intervals)

Figure 37

Effect of SO_4^{2-} concentration on the anaerobic growth of *E. coli* R+

Key

Sulphate (mM)	
0.0125	×
0.02	●
0.025	■
0.05	▬
0.1	▮
0.204	▲

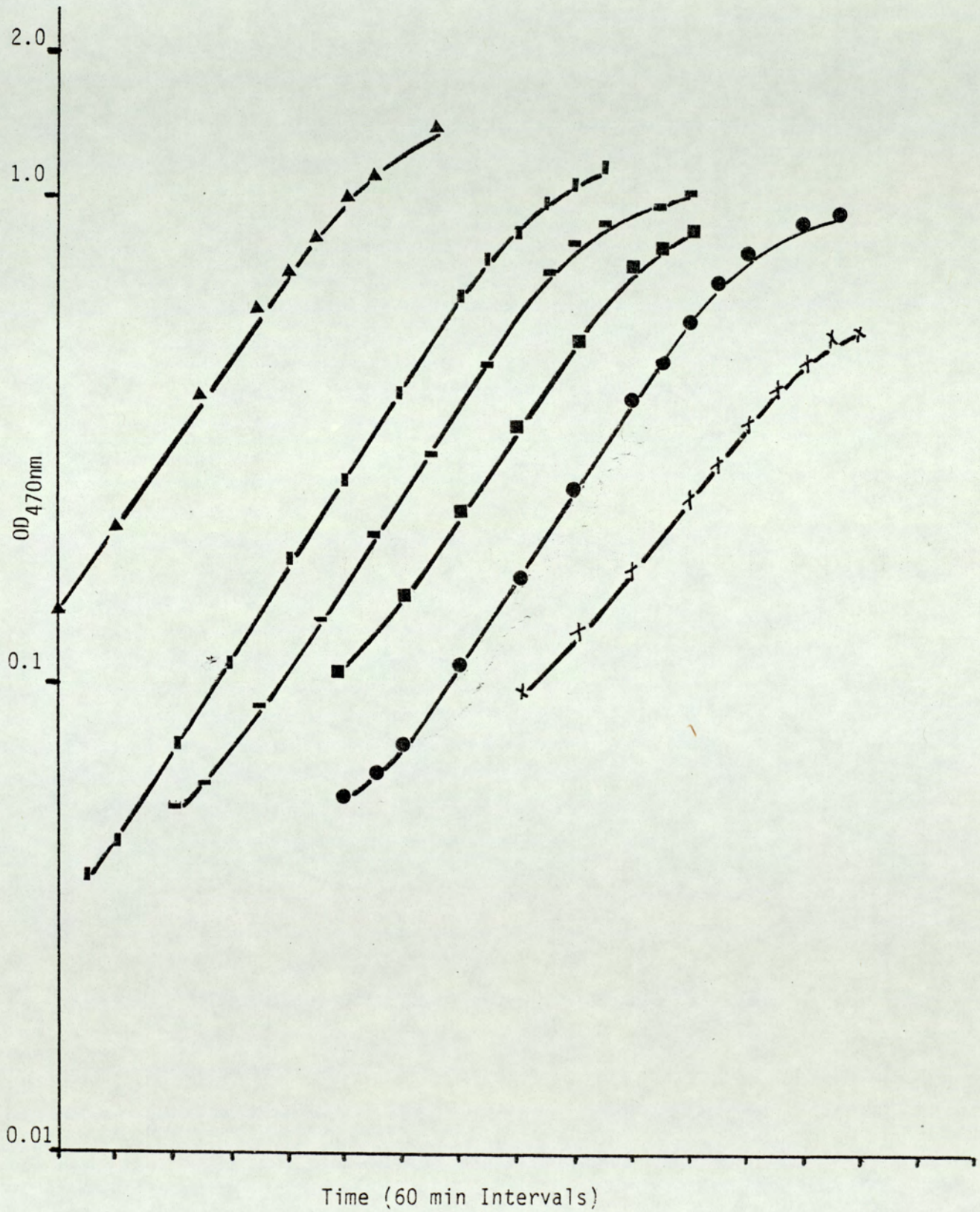

2.0
1.0
0.1
0.01
OD470nm
Time (60 min Intervals)

Figure 38

Relation between maximum exponential anaerobic growth of *E. coli* R- and R+ and added SO_4^{2-} concentration

Key

R- ○

R+ ●

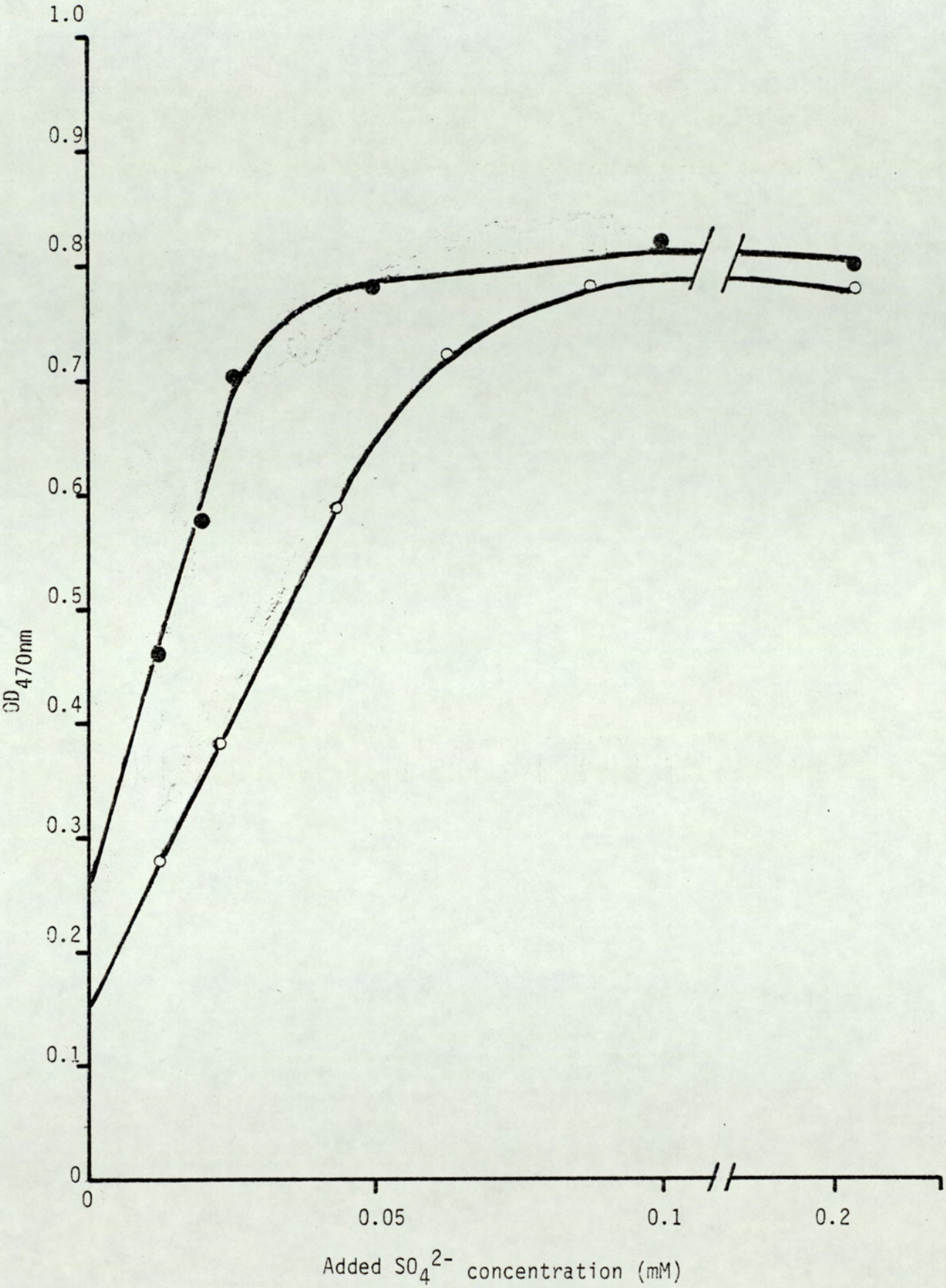

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD470nm
0
0.05
0.1
0.2
Added SO4 2- concentration (mM)

Figure 39

Effect of K^+ concentration on the anaerobic growth of E. coli R-

Key

Potassium (mM)	
0.007	⊠
0.014	✖
0.022	▽
0.029	◤
0.058	◭
0.115	◨
0.173	●
0.23	▲
0.34	◺
0.46	△
0.72	□
0.806	○

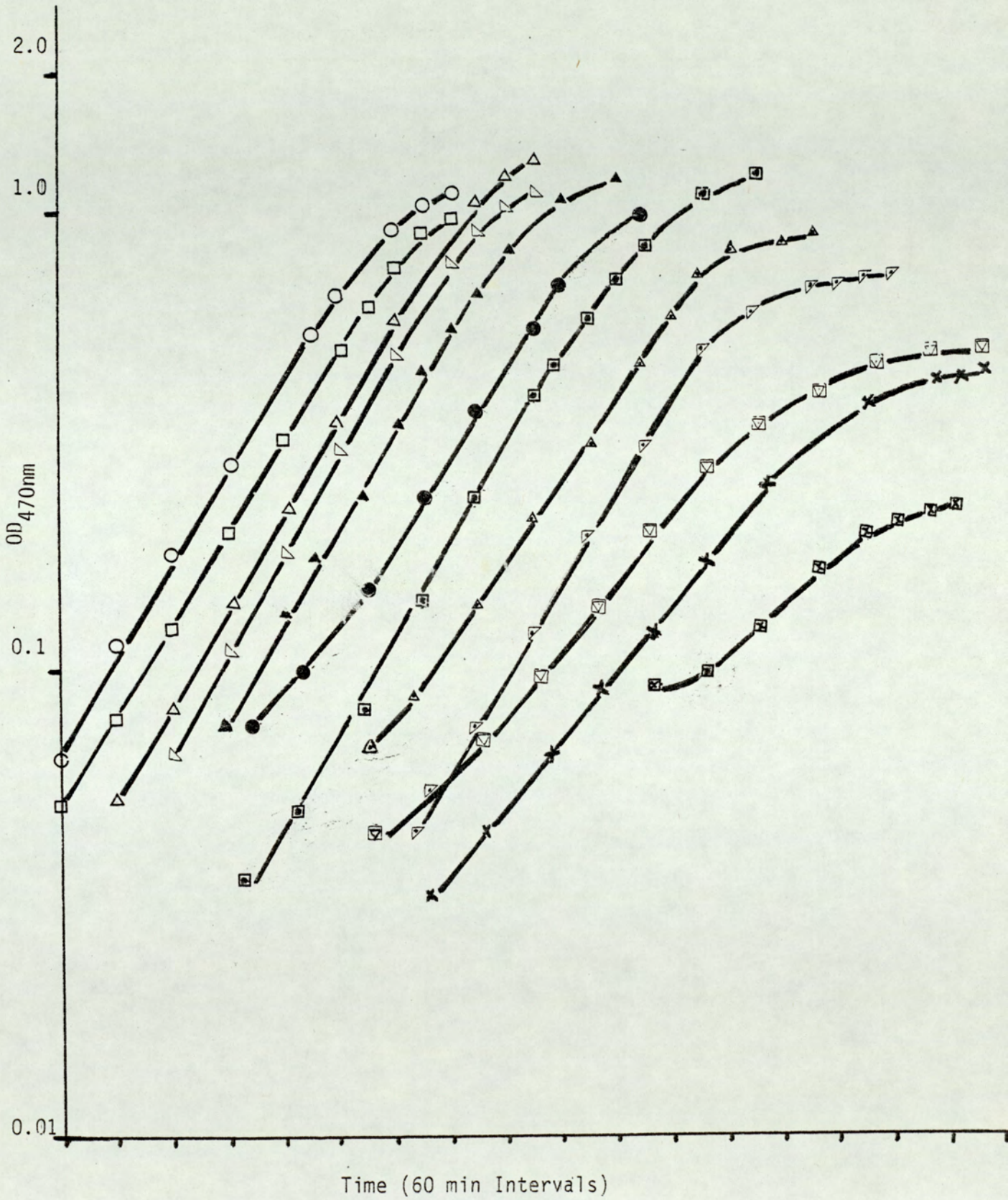

2.0
1.0
0.1
0.01
OD_{470nm}
Time (60 min Intervals)

Figure 40

Effect of K^+ concentration on the anaerobic growth of E. coli R+

Key

Potassium (mM)	
0.014	◉
0.055	⊠
0.13	✖
0.173	◣
0.22	■
0.35	▬
0.4	▮
0.46	◙
0.58	●
0.6	◮
0.72	▲

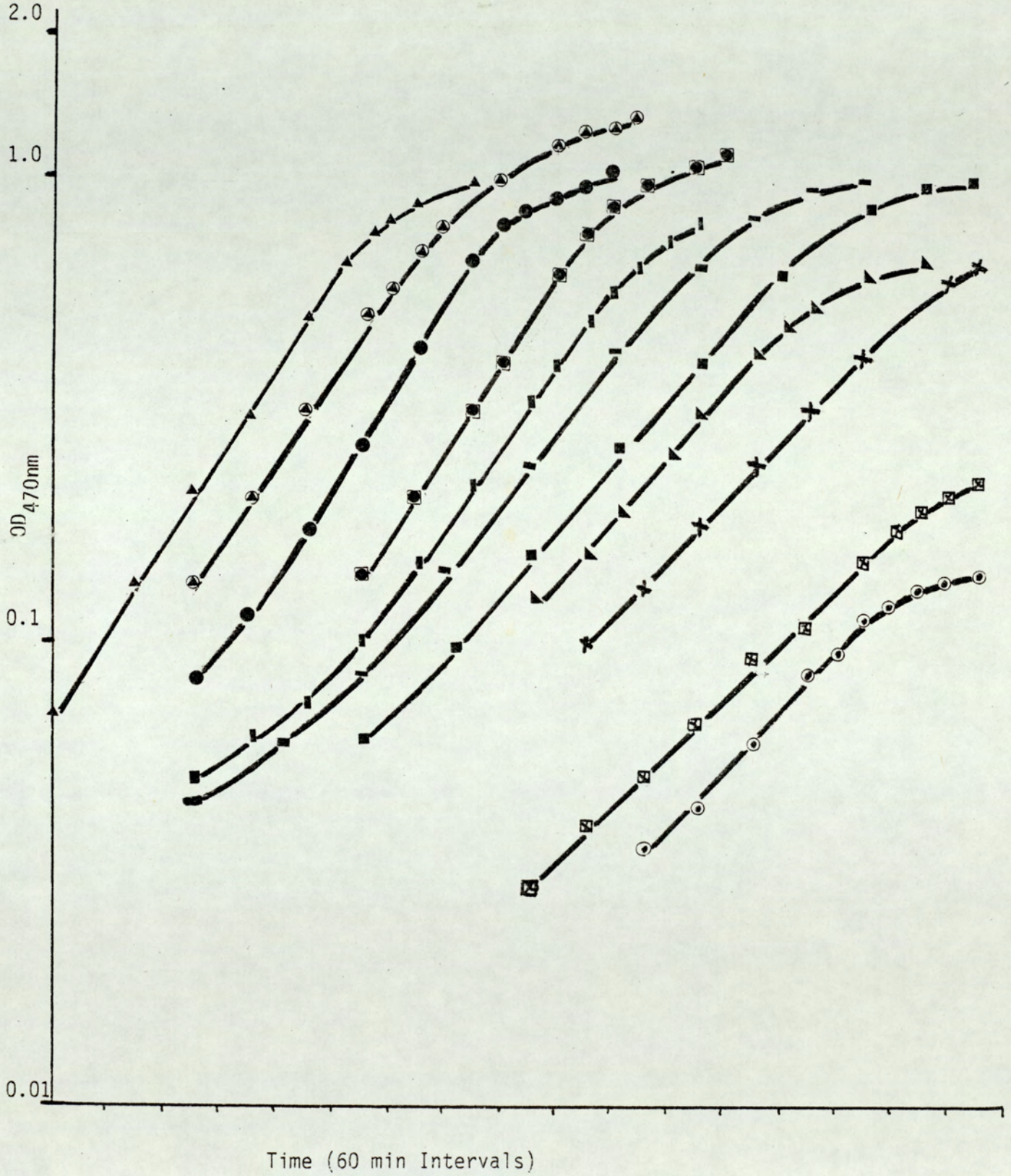

2.0
1.0
0.1
0.01
OD470nm
Time (60 min Intervals)

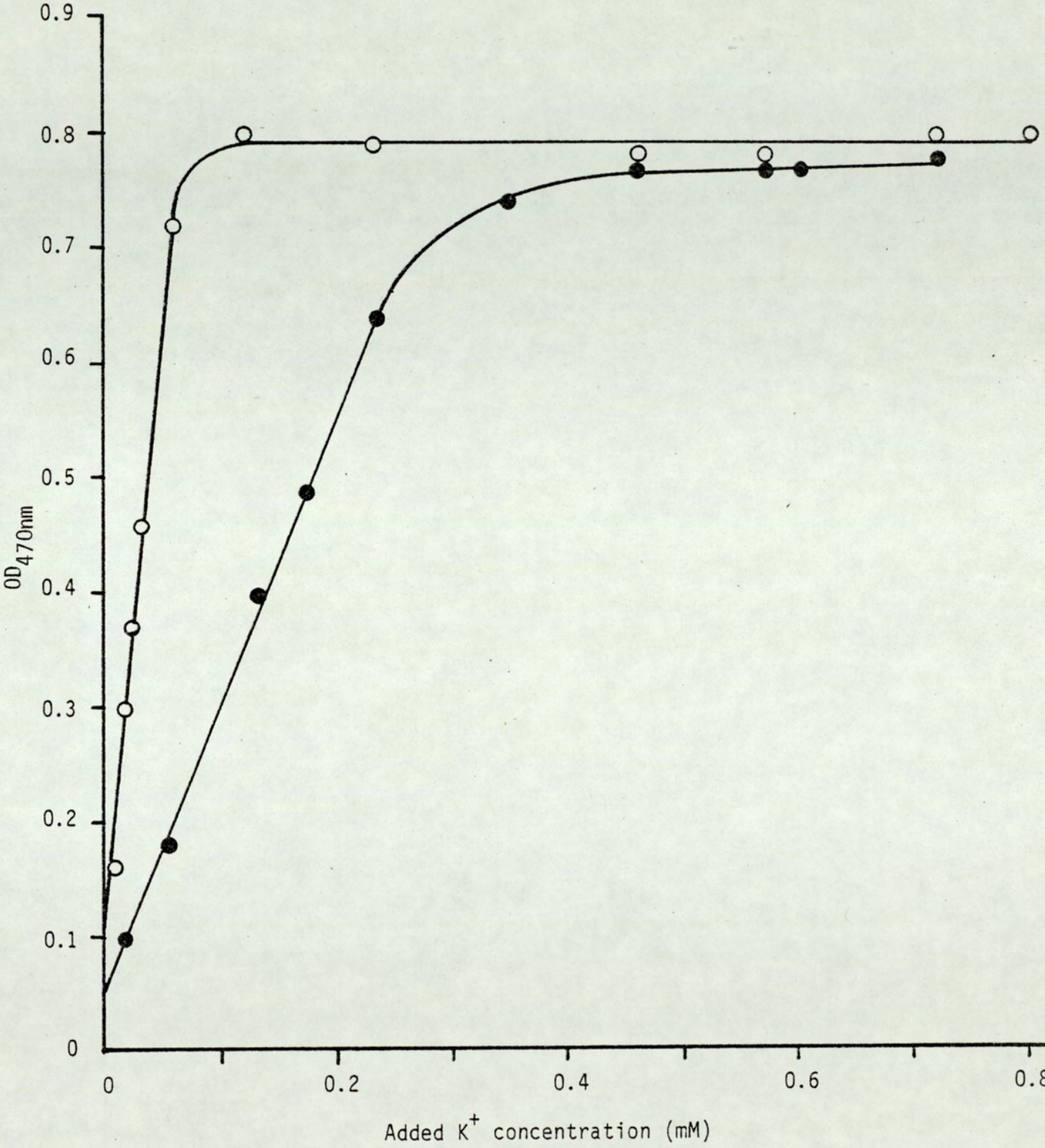
Figure 41

Relation between maximum exponential anaerobic growth of *E. coli* R- and R+ and added K^+ concentration

Key

R- ○

R+ ●


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD_{470nm}
0
0.2
0.4
0.6
0.8
Added K^+ concentration (mM)

Attempts were made to grow the R+ strain in the same concentration of K^+ used for R- exponential growth curves. At less than 0.58mM K^+, the lag phase of R+ cultures was extended to 12 hours or more; however, the maximum exponential growth was comparable to that obtained for R- cultures.

The contamination of the medium, as calculated by extrapolating the plots of OD_{470} max. curves to the X-axis was found to be about 0.015mM.

4.1.2.7. Iron growth requirement

CDMX (Table 5), without added Fe^{2+}, supported growth to an OD_{470} of approximately 0.5 and 0.4 for R- and R+ respectively.

Figures 42 and 43 show the effect of varying the concentration of added Fe^{2+} in CDM7 (Tables 6 and 7 for R- and R+ respectively) on the growth of E. coli R- and R+ cells respectively. The relationship between OD_{470} of maximum exponential growth and added Fe^{2+} is shown in Fig. 44.

Iron contamination of the medium was estimated to be about 0.00045mM by extrapolation.

4.1.2.8. Influence of a group of trace elements on the growth of E. coli R- and R+

Figure 45 shows the growth curves of R- and R+ strains with all ingredients apparently in excess, supplemented by trace elements. When a mixture of each of the following salts: $ZnSO_4.7H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $CaCl_2.2H_2O$, $CoCl.6H_2O$ and $MnSO_4.4H_2O$ ($0.1\mu gml^{-1}$ calculated for Zn, Mo, Ca, Co and Mn respectively) was added (CDM13, Tables 6 and 7), the growth of R- and R+ cultures showed no significant difference from the controls. Trace ions present as contaminants in the medium are presumably sufficient for maximum growth.

Figure 42

Effect of Fe^{2+} concentration on the anaerobic growth of E. coli R-

Key

Iron (mM)	
zero	⊡
0.0001	⊙
0.0002	◿
0.0006	□
0.0012	△
0.0024	○

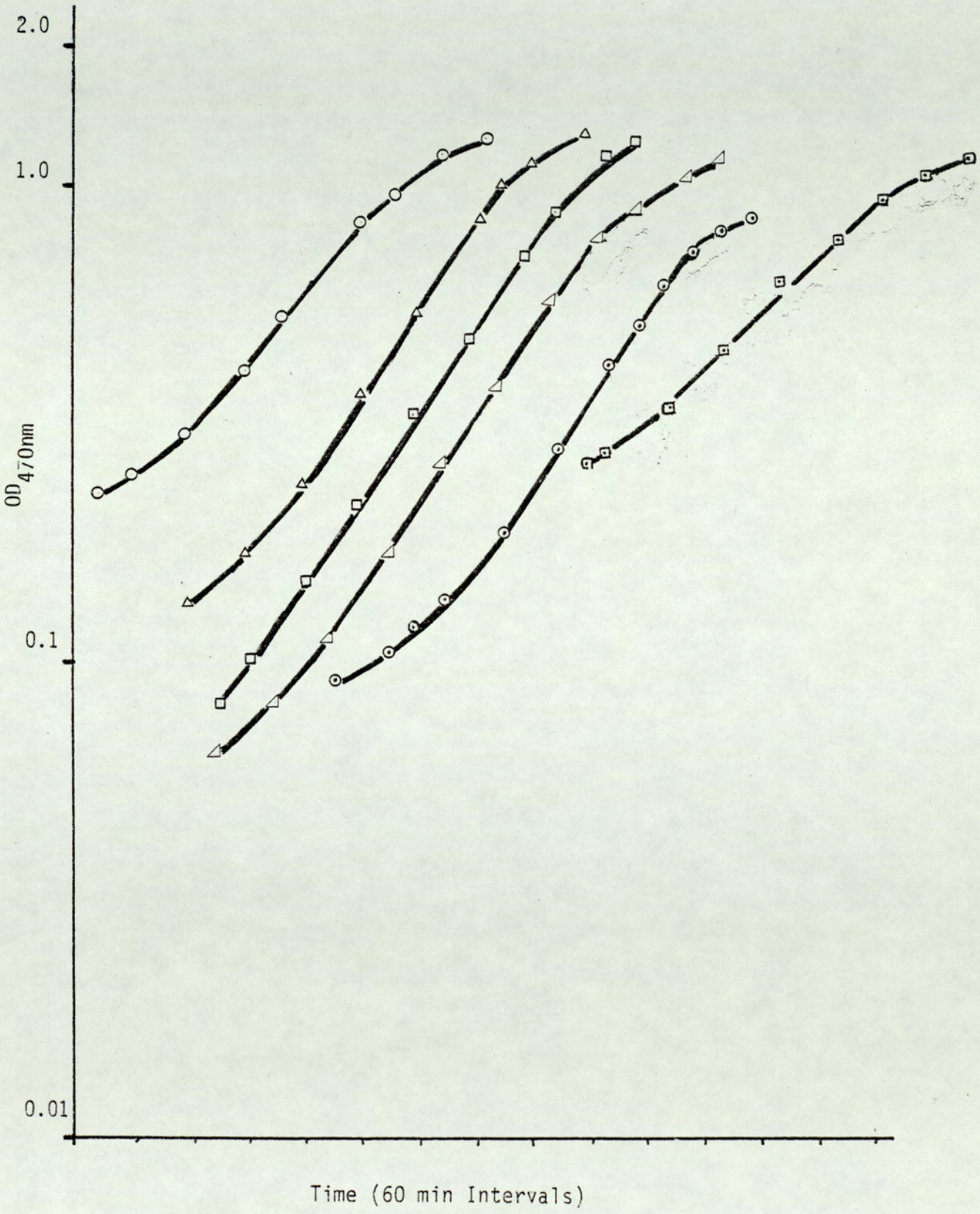

2.0
1.0
0.1
0.01
OD470nm
Time (60 min Intervals)

Figure 43

Effect of Fe^{2+} concentration on the anaerobic growth of *E. coli* R+

Key

Iron (mM)	
Zero	●
0.0002	▼
0.0004	◉
0.0012	■
0.0024	▲

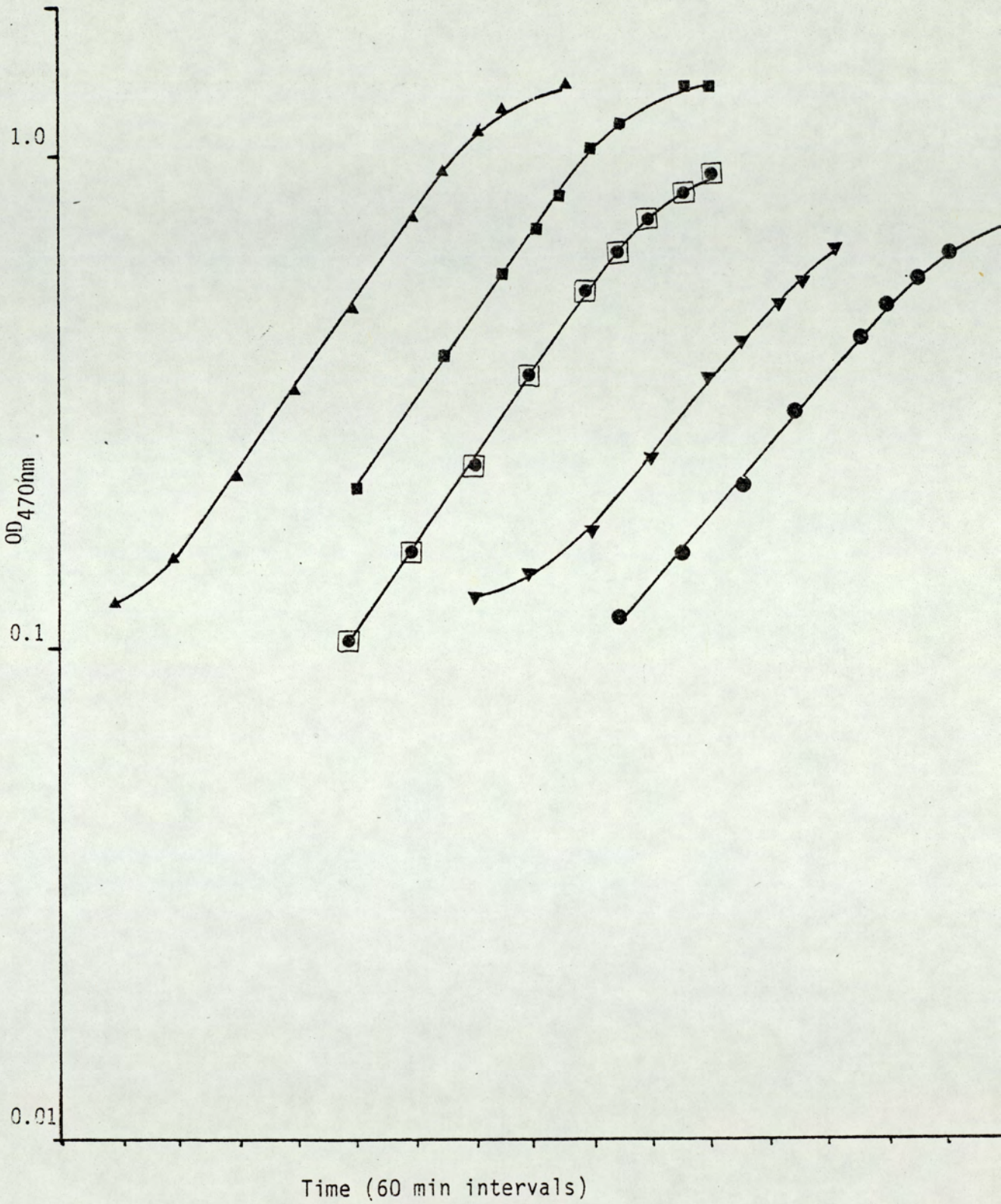

1.0
0.1
0.01
OD_{470nm}
Time (60 min intervals)

Figure 44

Relation between maximum exponential anaerobic growth of *E. coli* R- and R+ and added Fe^{2+} concentration

Key

R- ○

R+ ●

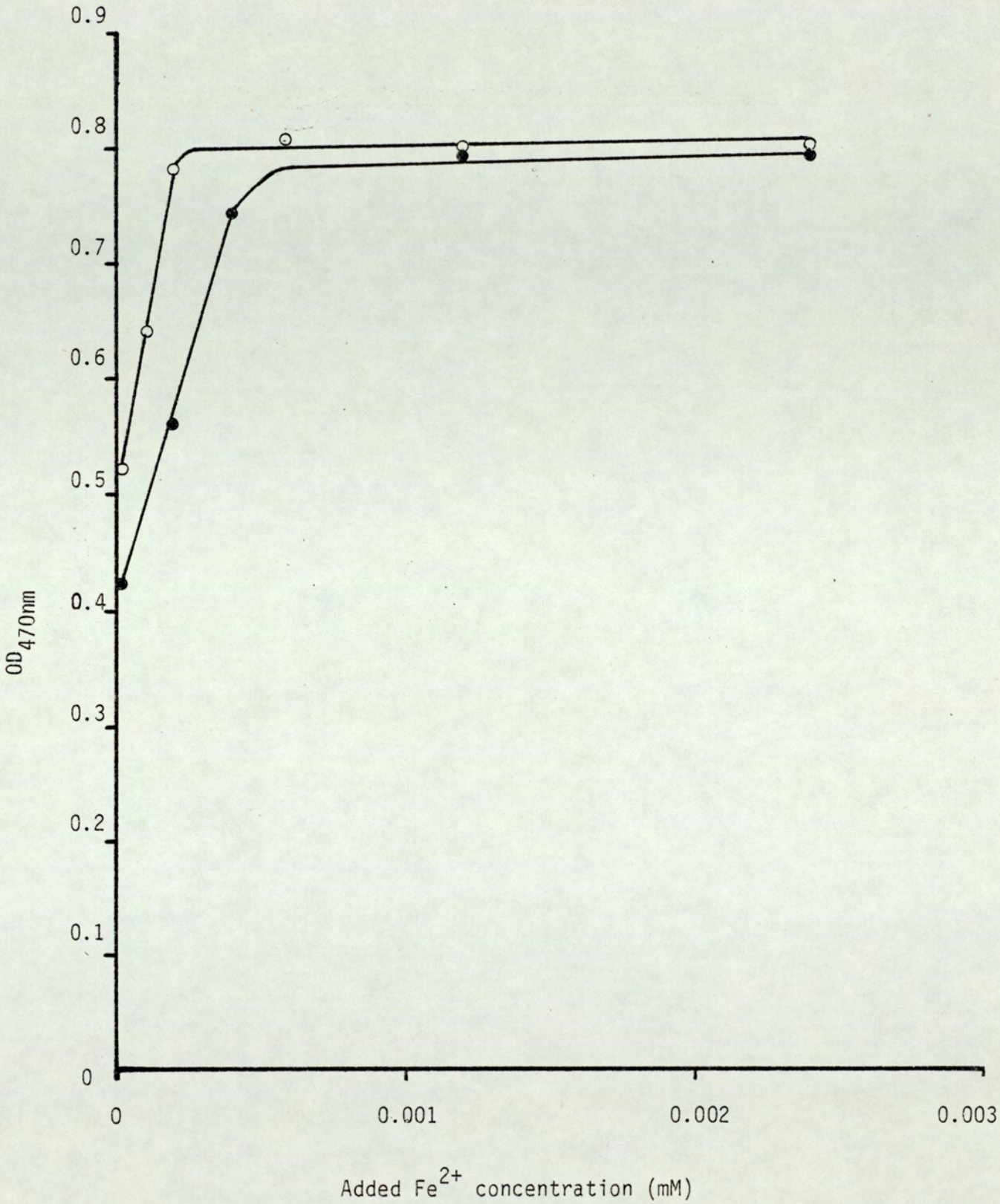
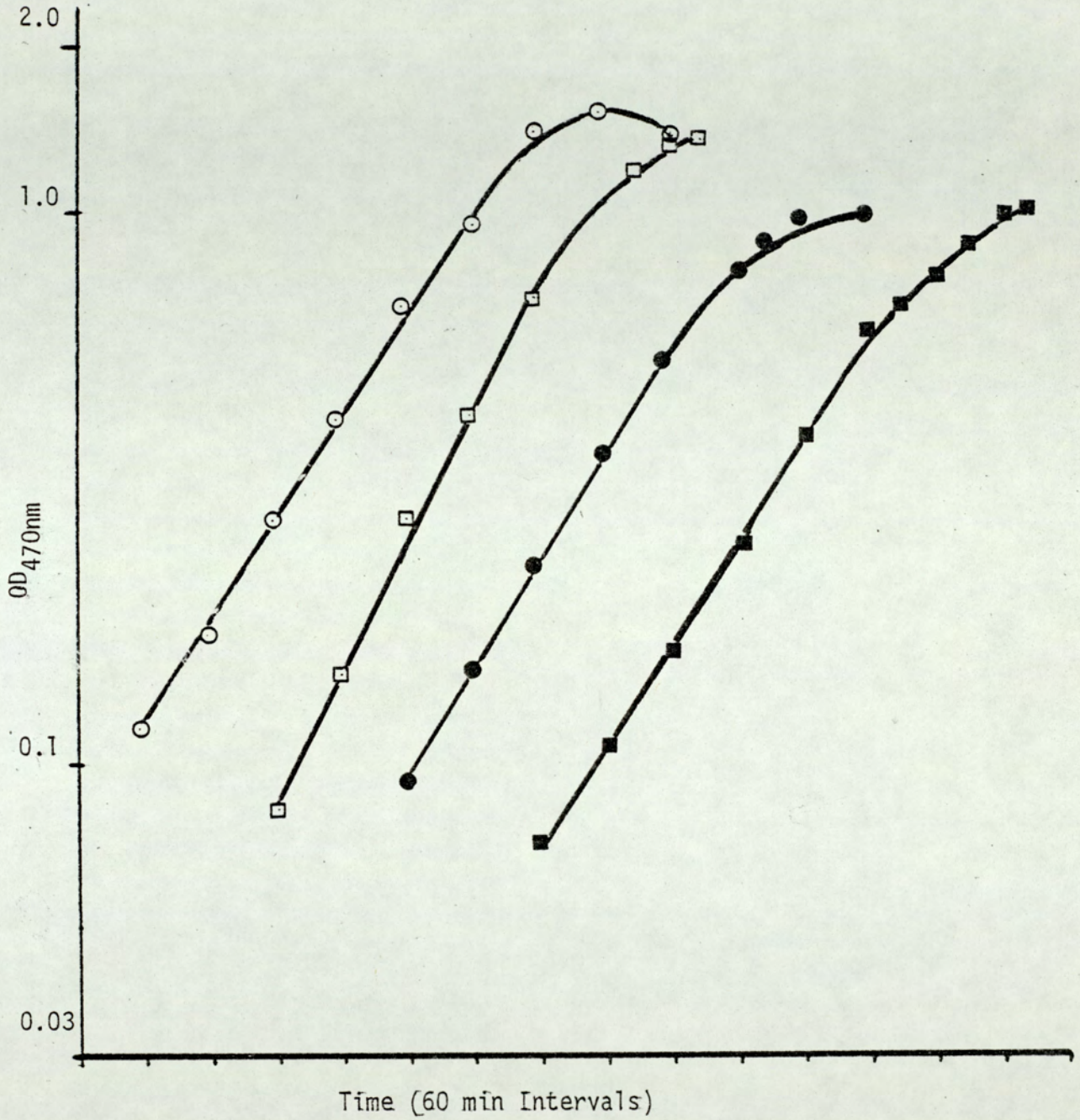

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD470nm
0
0.001
0.002
0.003
Added Fe2+ concentration (mM)

Figure 45

Influence of a group of trace elements on the anaerobic growth of *E. coli* R- and R+

Key

Added trace elements	R-	R+
(Ca, Co, Mn, Mo and Zn; each 0.1µg ml^{-1})	⊙	●
None	⊡	■


2.0
1.0
0.1
0.03
OD470nm
Time (60 min Intervals)

The effect of the individual trace elements on R- and R+ strains are shown in Figs. 46 and 47 respectively. No difference in the growth of the R- cultures was observed when Co, Mn or Mo salts was added (CDM9, 10 and 11 respectively; Table 6). However, addition of $CaCl_2$ or $ZnSO_4$ (Table 6, CDM8 and 10 respectively) resulted in a lowering of the growth rate. In the R+ cultures Ca and Mn appeared to decrease maximum OD_{470}, and Mo, Zn and Co were found to be even more toxic.

4.1.2.9. Summary of growth requirements

A linear relationship exists between OD_{470} at the end of exponential growth and the concentration of the limiting nutrients, glucose, NH_4^+, Mg^{2+}, PO_4^{3-} SO_4^{2-}, K^+ and Fe^{2+}. Linearity was attained to similar OD_{470} values for all nutrients studied. Thus a chemically defined medium may be prepared which will allow the OD_{470} of a culture to be varied to the required level for any particular nutrient. The depletion curves can also be used to formulate a medium containing a controlled excess of nutrients.

One major problem encountered, was the inability to find a CDM in which the strains used would grow exponentially to more than ca. OD_{470} of 1.0. In experiments shown in Fig. 48 the basal medium (CDMI and II, Table 5) was appropriately adjusted (CDMIII to VIII, Table 5) so as the concentration of the ingredients was sufficient to allow a theoretical OD_{470} of more than 4.0. Nevertheless, no culture grew exponentially to an OD_{470} more than ca. 1.0. In practic the chemostat cultures were used at an OD_{470} of less than 1.0.

Table 25 shows a comparison between concentrations of the depleted nutrients which permit exponential growth aerobically and anaerobically to an OD_{470} of 0.5.

Figure 46

Influence of individual trace elements on the anaerobic growth of E. coli R-

Key

Element (0.1 µg ml^{-1})	
Ca	■
Zn	▲
Mo	⊙
Mn	□
Co	△
No trace element	○

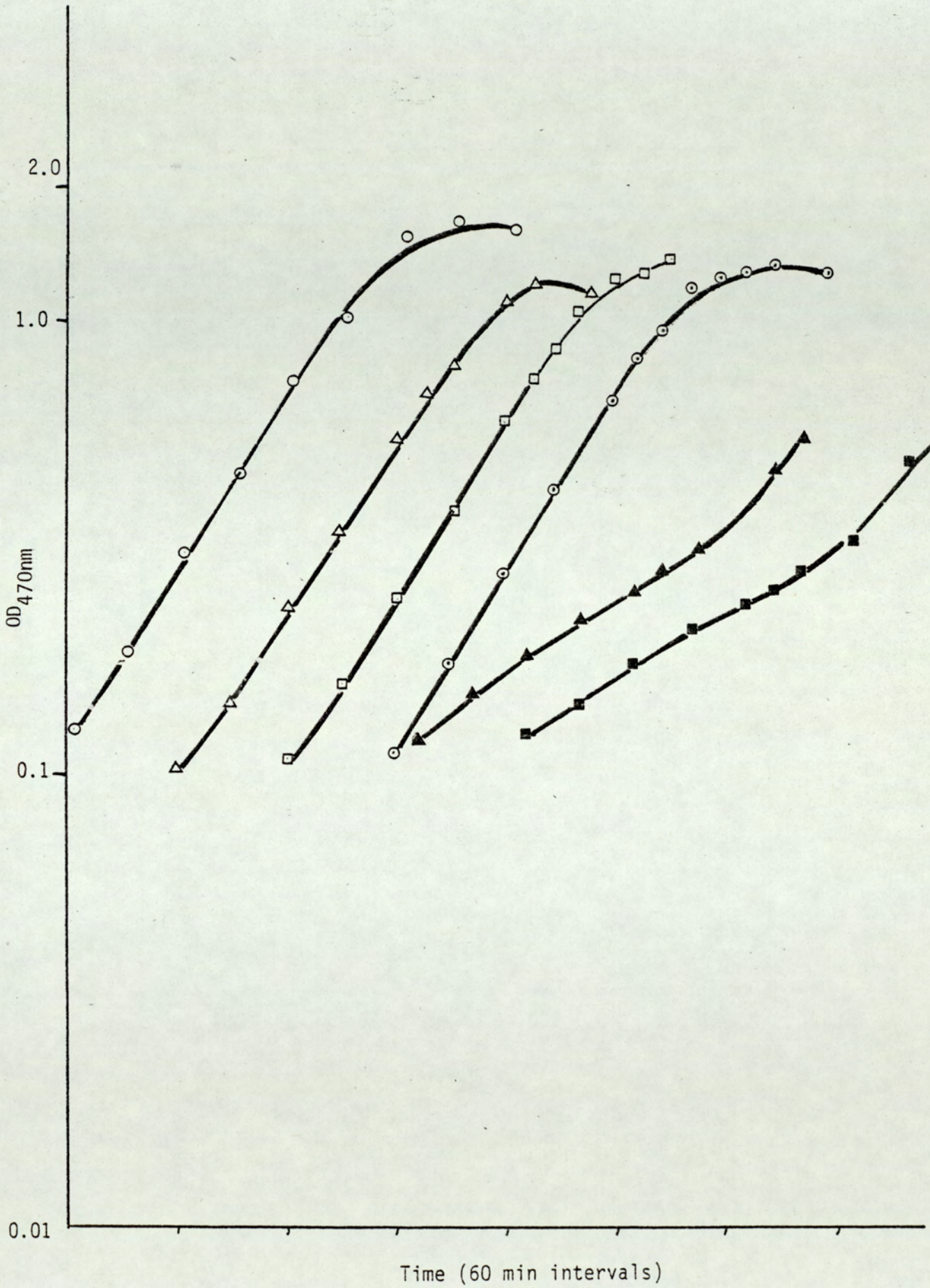

2.0
1.0
0.1
0.01
OD470nm
Time (60 min intervals)

Figure 47

Influence of individual trace elements on the anaerobic growth of *E. coli* R+

Key

Element (0.1 μg ml^{-1})	
Ca	■
Zn	◣
Mo	▼
Mn	●
Co	◘
No trace element	▲

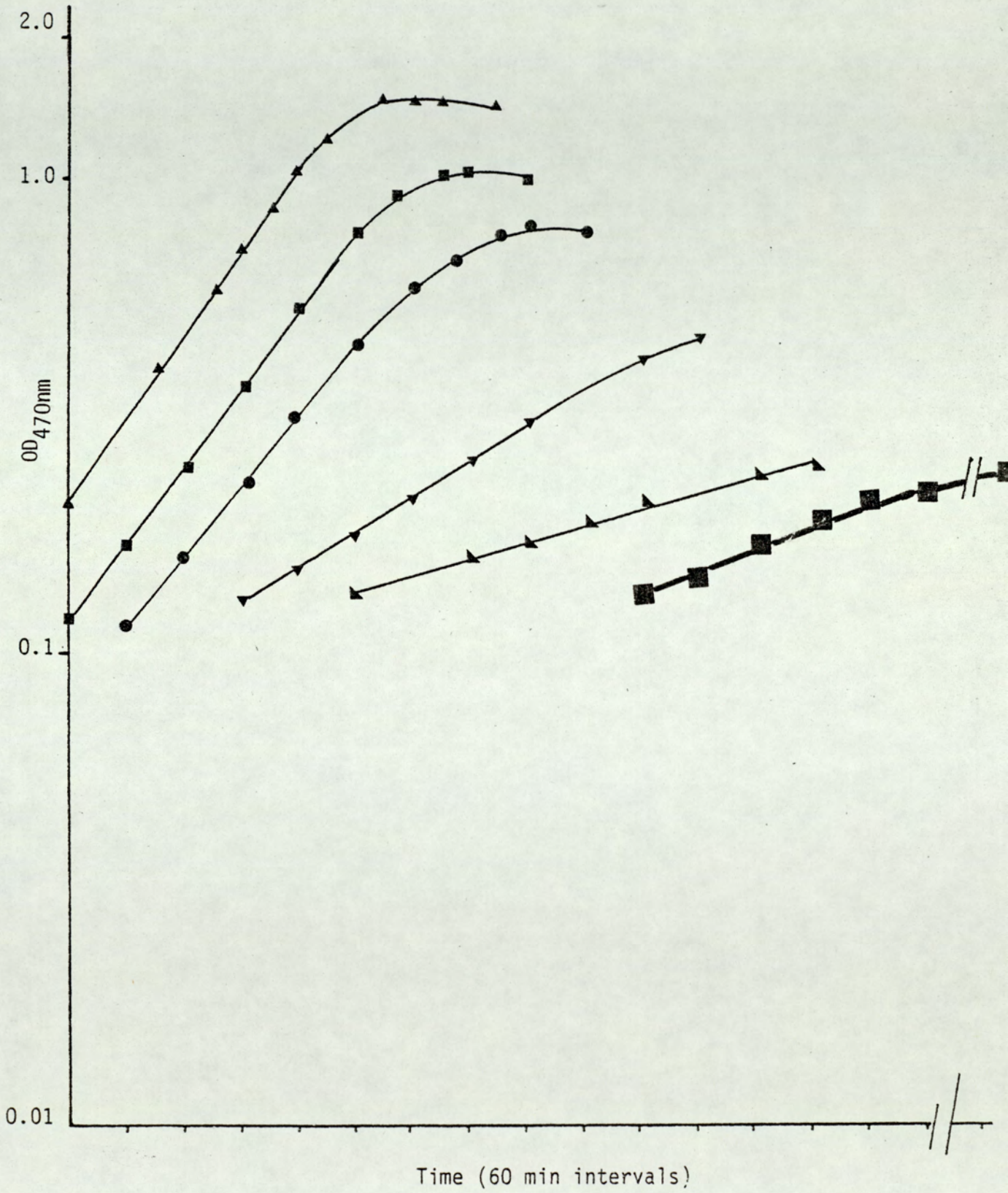

2.0
1.0
0.1
0.01
OD470nm
Time (60 min intervals)

Figure 48

Anaerobic growth curves of *E. coli* R- and R+

Key

R+ R-

◿ Glucose increased to be sufficient for a theoretical OD_{470} of 4.0 (CDM III).

▽ Glucose and NH_4^+ as ◿ (CDM IV).

□ Glucose, Mg^{2+} and SO_4^{2-} as ◿ (CDM V).

○ Glucose and K^+ as ◿ (CDM VII).

△ Glucose and PO_4^{3-} as ◿ (CDM VI).

◉ Glucose, K^+ and PO_4^{3-} as ◿ (CDM VIII).

✖ All nutrients were sufficient for at least a theoretical OD_{470} as ◿ (CDM IX)

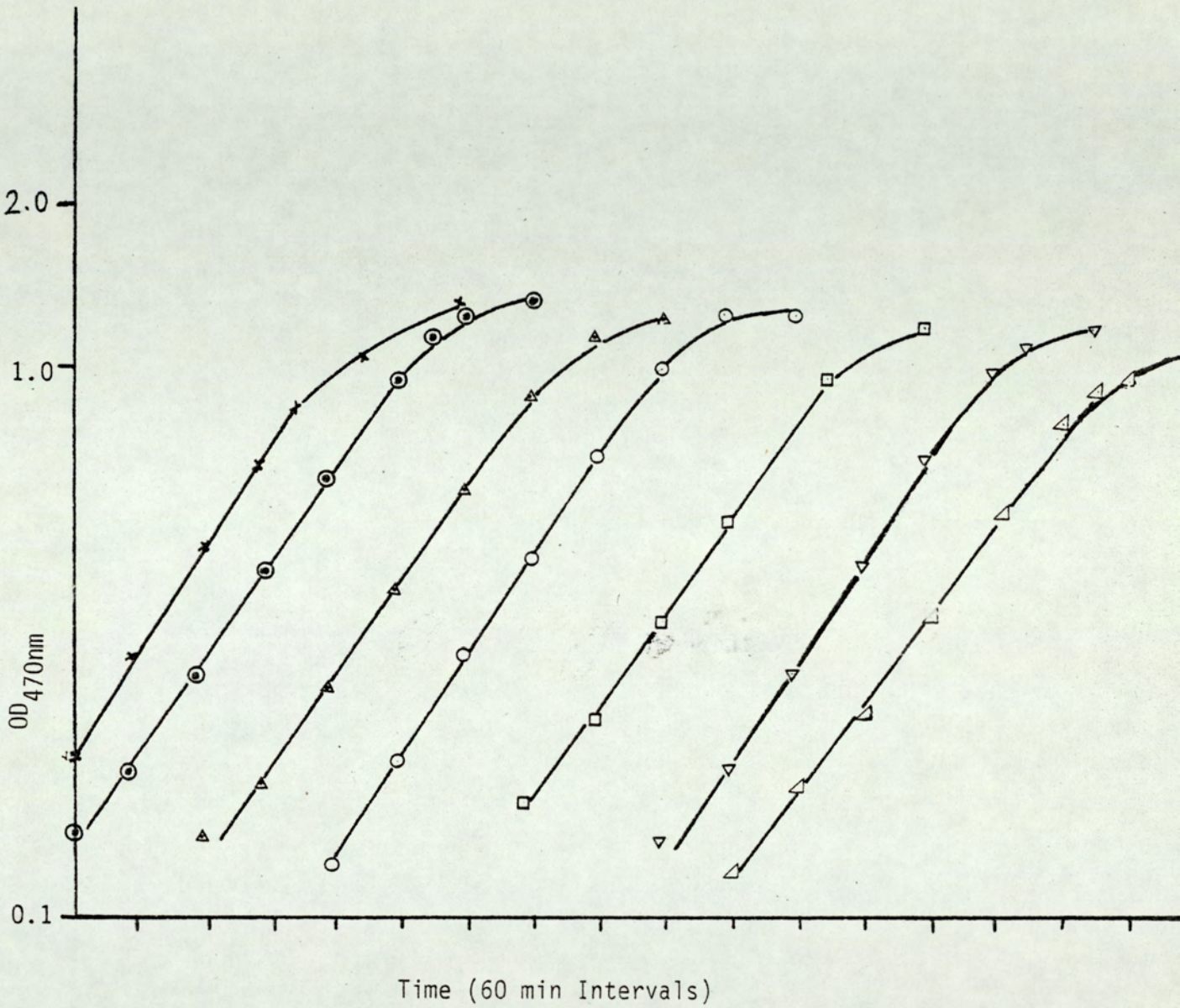

2.0
1.0
0.1
OD470nm
Time (60 min Intervals)

Table 25

Comparison between total[(a)] concentrations of nutrients which permit exponential aerobic and anaerobic growth of E. coli R- and R+ to an OD_{470} 0.5

Nutrients	Nutrient concentrations (mM)			
	Aerobic		Anaerobic	
	R-	R+	R-	R+
Glucose	1.85	2.0	3.9	3.0
NH_4^+	0.975*	0.925*	1.7	1.8
Mg^{2+}	0.015	0.018	0.023	0.025
PO_4^{3-}	0.28	0.29	0.23	0.22
SO_4^{2-}	0.018*	0.014*	0.05	0.03
K^+	0.018*	0.026*	0.05	0.19
Fe^{2+}	0.0*	0.0001*	0.0004	0.0005

(a) Total concentrations calculated from requirements for added ingredient plus estimated medium contamination.

* (Klemperer et al, 1979) using OD measurements at λ 420nm

4.1.2.10. Determination of growth constants from the anaerobic batch cultures of E. coli R- and R+

The growth rate constants (μm) and saturation constants (K_s) for cultures depleted of glucose, PO_4^{3-} or K^+ were calculated from the results previously obtained from batch-culture depletion experiments. For each limiting nutrient, K_s and μm were determined from plots of $\frac{1}{\mu}$ versus $\frac{1}{S}$ were μ is the specific growth rate and S is the corresponding concentration of the depleted nutrient. From Monod's equation

$$\mu = \mu m \frac{S}{K_s + S}$$

as rearranged by Lineweaver and Burke

$$\frac{1}{\mu} = \frac{1}{\mu m} + \frac{K_s}{\mu m} + \frac{1}{S}$$

the slope is $\frac{K_s}{\mu m}$ and the intercept of the line on the Y-axis is $\frac{1}{\mu m}$.

The data for glucose-depleted cultures were calculated from Figures 24 and 25 and plotted in Fig.49 ; for phosphate-depleted cultures from Figures 33 and 34 and plotted in Fig.50 ; and for potassium-depleted cultures from Figures 39 and 40 and plotted in Fig. 51. All plots were calculated regression lines. Results are shown in Table 26 . It can be seen that except for phosphate-depleted R+ cells, two K_s were found for each substrate tested, presumably corresponding to high and low affinity uptake systems. From the appropriate double reciprocal plots the concentrations of the limiting nutrient at the change in K_s value were calculated and are shown in Table 27 .

Figure 49

Double reciprocal plot of *E. coli* R- and R+ anaerobic growth rate versus medium glucose concentration

Key

R- ○

R+ ●

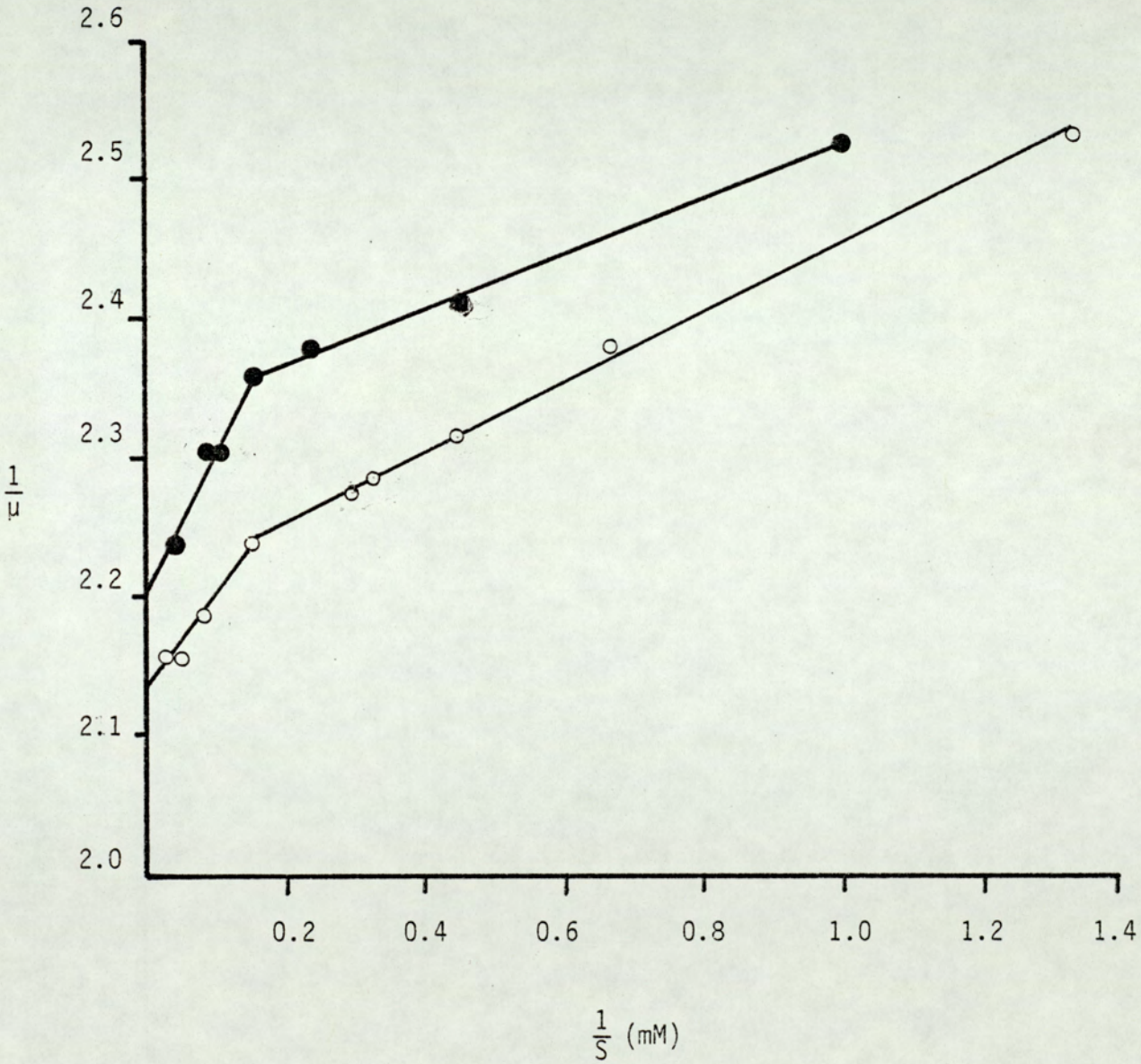

2.6
2.5
2.4
2.3
2.2
2.1
2.0
1/μ
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1/S (mM)

Figure 50

Double reciprocal plot of *E. coli* R- and R+ anaerobic growth rate versus phosphate concentration

Key

R- ○

R+ ●

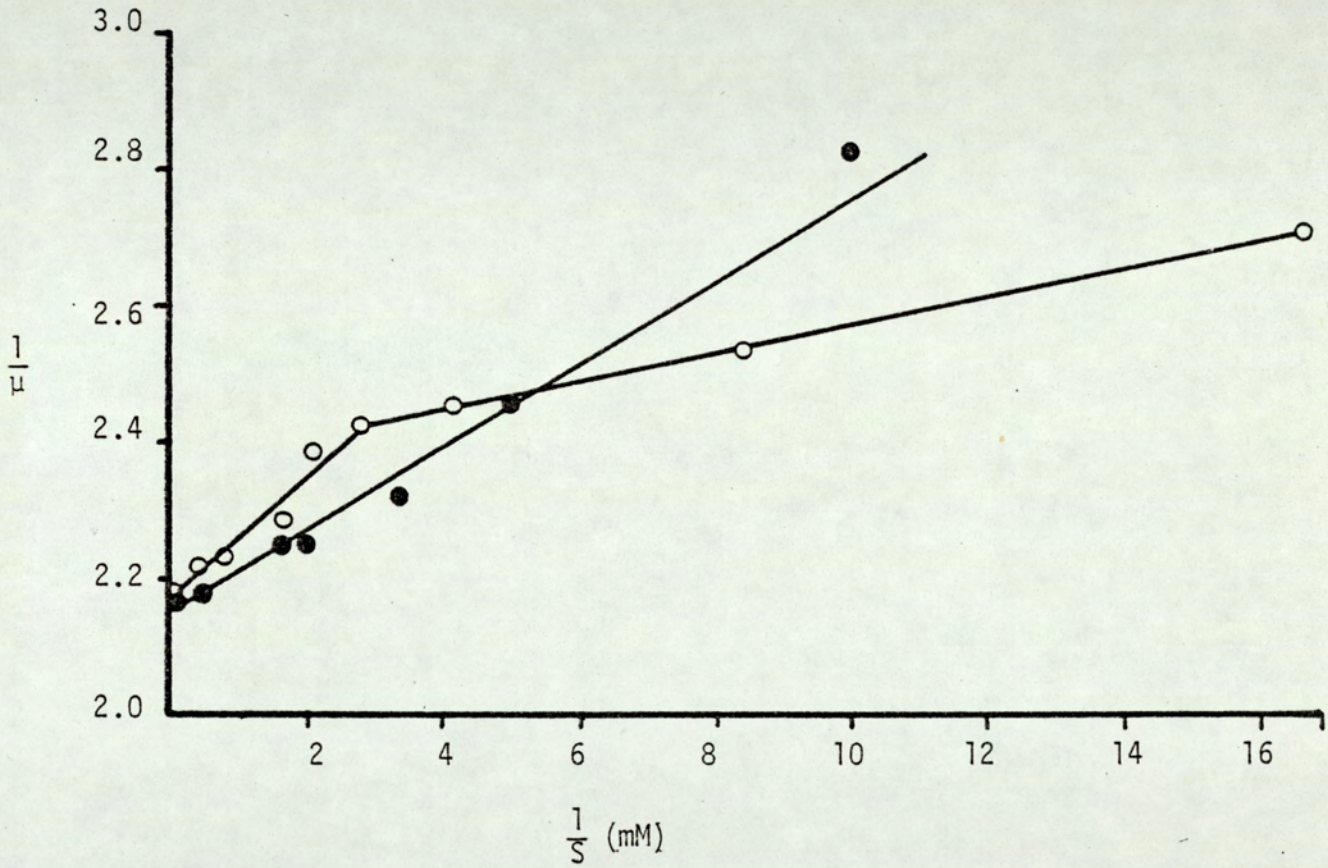

3.0
2.8
2.6
2.4
2.2
2.0
1/μ
2
4
6
8
10
12
14
16
1/S (mM)

Figure 51

Double reciprocal plot of *E. coli* R- and R+ growth rate versus medium potassium concentration

Key

R- ○

R+ ●

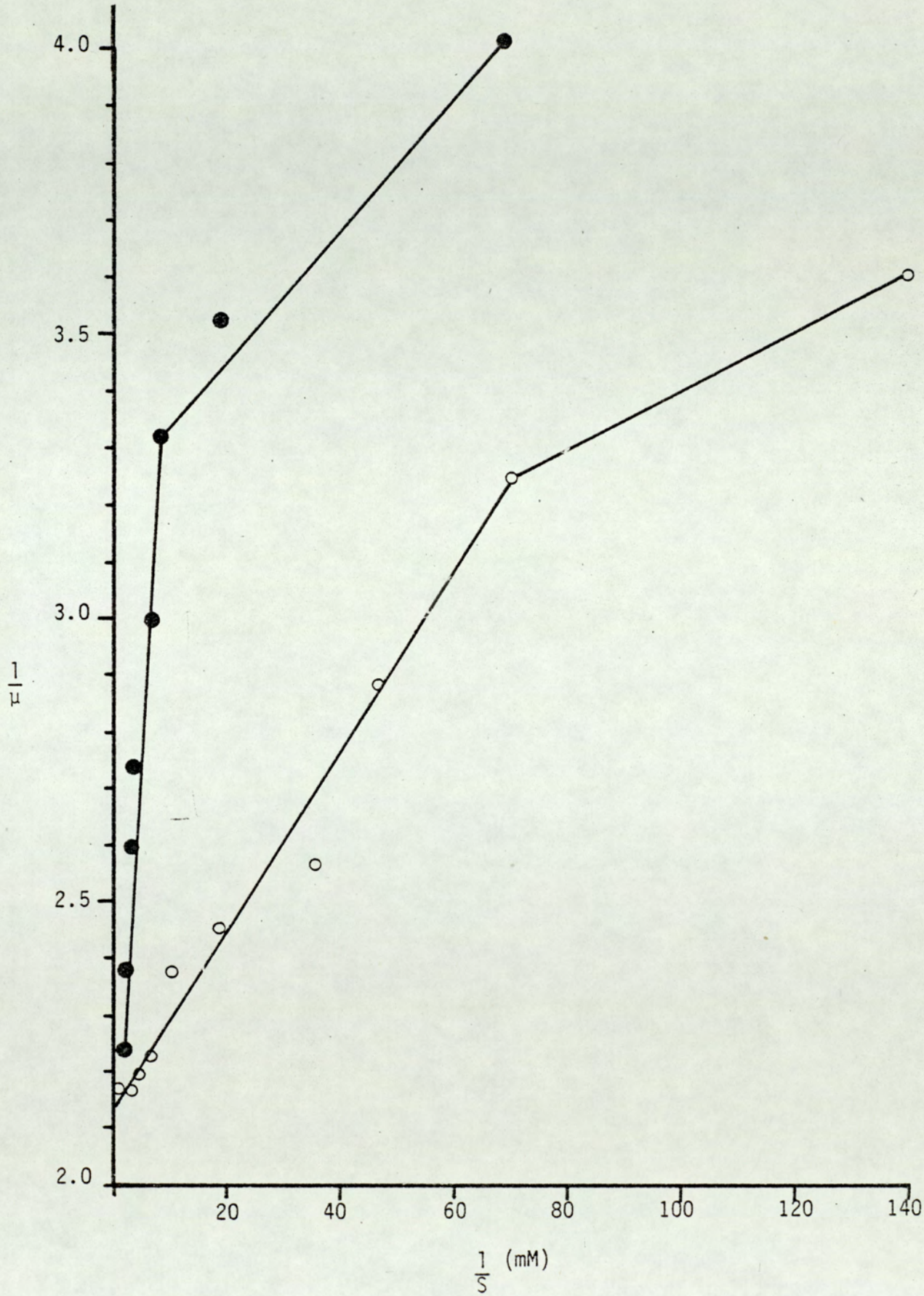

4.0
3.5
3.0
2.5
2.0
1/μ
20
40
60
80
100
120
140
1/S (mM)

Table 26

Growth parameters, $\mu m(h^{-1})$ and K_s (mM), of glucose, phosphate and potassium-depleted anaerobic batch cultures of *E. coli* R- and R+

Depleting nutrient	Growth (i) parameters	R-		R+	
		Substrate concentration		Substrate concentration	
		High	Low	High	Low
Glucose	Slope	0.65	0.25	1.2	0.2
	K_s	0.304	0.114	0.547	0.086
Phosphate	Slope	0.091	0.021	0.059	0.059
	K_s	0.042	0.009	0.027	0.027
Potassium	Slope	0.154	0.005	0.149	0.012
	K_s	0.073	0.002	0.069	0.004

(i) $\mu m = 0.464\ (h^{-1})$

Table 27

Substrate concentrations(mM) at change in K_s (mM) calculated from the double reciprocal plots*

Depleting-nutrient	R-	R+
Glucose	6.67	8.0
Phosphate	0.357	-
Potassium	0.015	0.125

* See Figures 49, 50 and 51.

4.2 Survival studies on aerobic and anaerobic cultures of *E. coli* R- and R+

4.2.1. Survival in liquid medium

4.2.1.1. Effect of storage temperature on survival

It can be seen from Figures 52 and 53 that the survival characteristics of both R- and R+ strains are related to storage temperature. The initial death rate was significantly higher at 37^{o} than at 25^{o} or 4^{o}.

On further storage, there was little change in the viable counts of Mg^{2+} depleted cells (CDME, Table 4) at 4^{o} and 25^{o}; this might have been due to cryptic growth. At 37^{o} the counts of viable cells fell steadily and the death rate of R+ cells appeared to be higher than that of R- ones. On the other hand glucose-depleted cultures, grown in CDMB (Table 4) showed no significant difference in death rate and survival between R- and R+ cells and no cryptic growth was observed.

4.2.1.2. Effect of suspending medium on survival at 25^{o}

Survival curves of R- and R+ cells are shown in Fig. 54 (Cultures were glucose-depleted stationary phase grown in CDMB, Table 4).

MOPS-resuspended R- and R+ cells (pH 7.4) died faster than cells resuspended in their culture supernatant.

There was no significant difference in the survival of R- and R+ strains in either suspending medium.

Figure 52

Effect of storage temperature on survival of Mg^{2+}-depleted stationary phase MOPS resuspended cultures of *E. coli* R- and R+

(mean of two independent cultures each in triplicate, coeff. of variation <10%)

Key

	4^{o}	25^{o}	37^{o}
R-	○	△	□
R+	●	▲	■

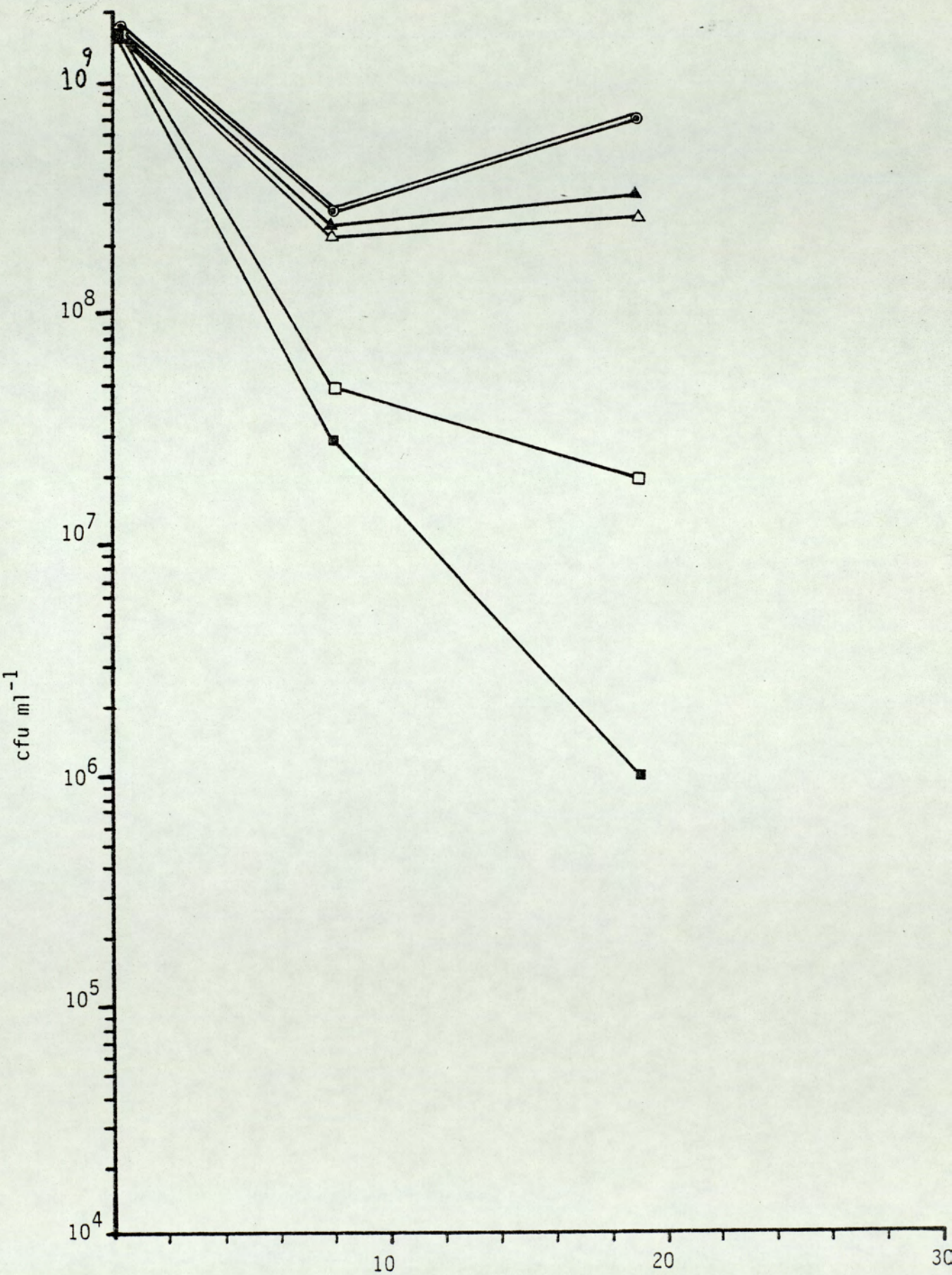

10^9
10^8
10^7
10^6
10^5
10^4
cfu ml^{-1}
10
20
30
Days

Figure 53

Effect of storage temperature on survival of glucose-depleted stationary phase MOPS resuspended cultures of *E. coli* R- and R+

(mean of 2 independent cultures each in triplicate, coeff. of variation < 10%)

Key

	4^{o}	25^{o}	37^{o}
R-	○	△	□
R+	●	▲	■

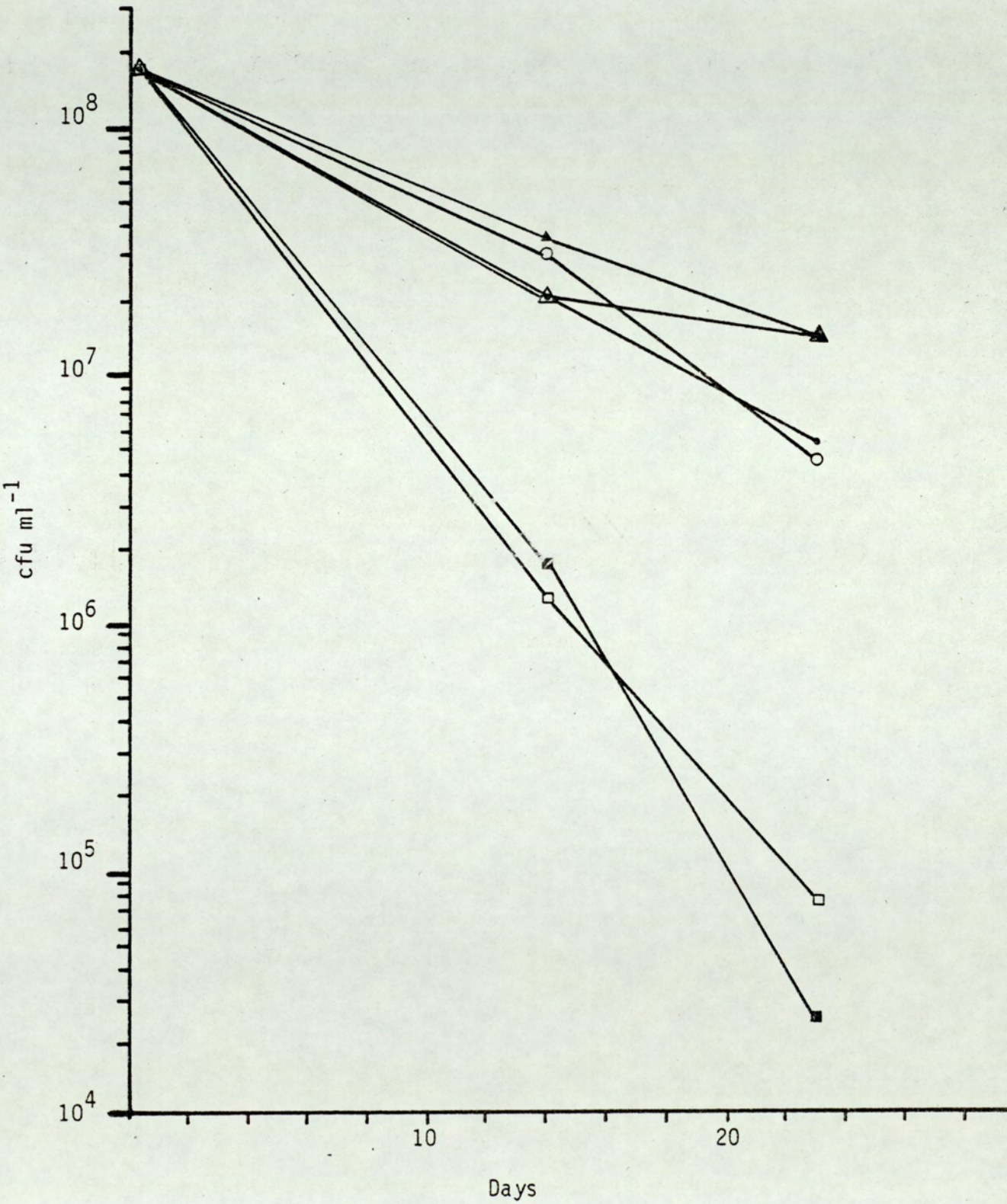

10^8
10^7
10^6
10^5
10^4
cfu ml^{-1}
10
20
Days

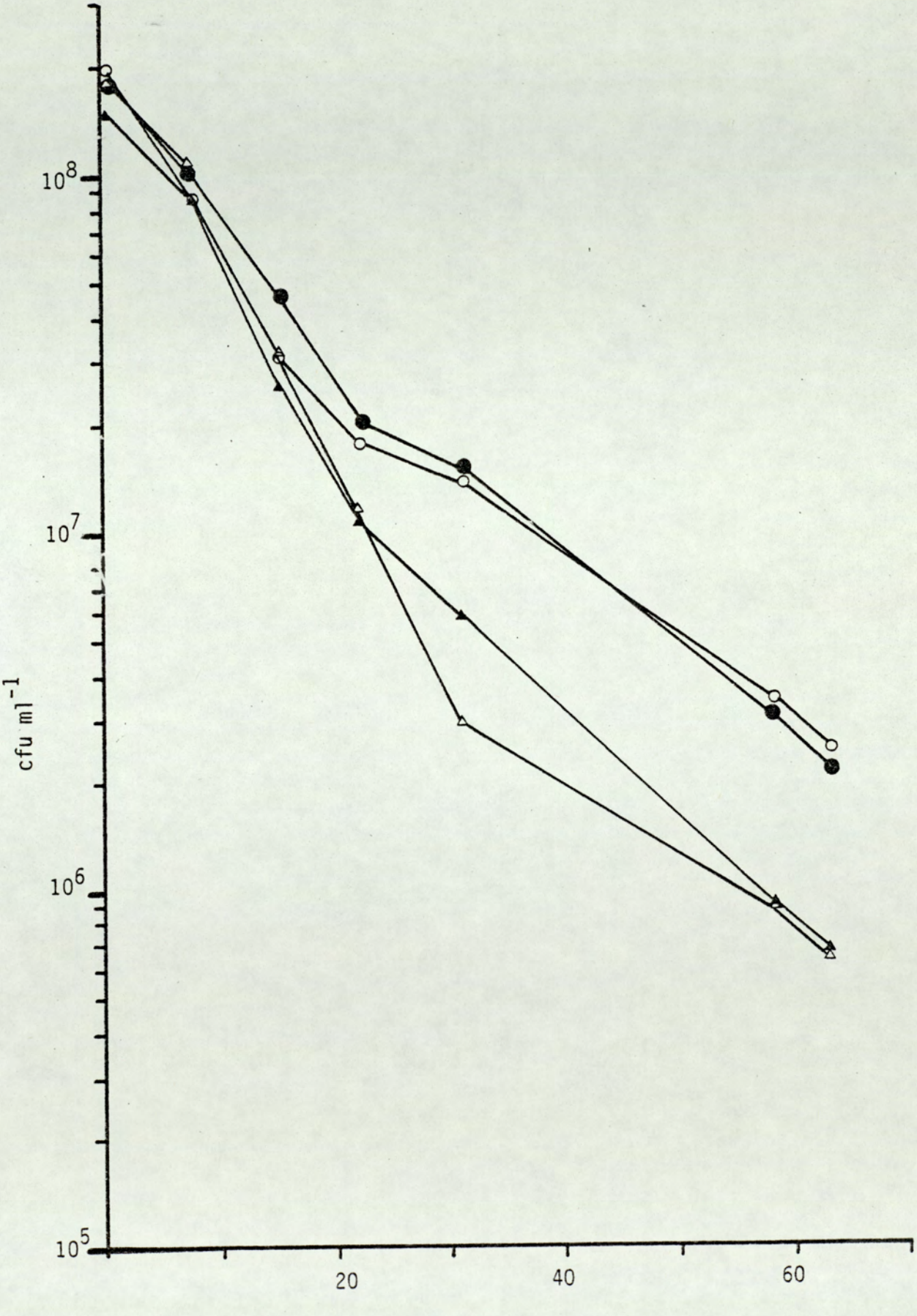
Figure 54

Effect of suspending medium on survival at 25° of glucose-depleted stationary phase cultures of *E. coli* R- and R+

(mean of triplicate determinations of one culture, coeff. of variation < 10%)

Key

	Resuspended in the culture supernatant	Resuspended in MOPS
R^-	○	△
R^+	●	▲


10^8
10^7
10^6
10^5
cfu ml^{-1}
20
40
60
Days

4.2.1.3. Stability of plasmid RP1 in E. coli

4.2.1.3a. Effect of storage temperature on stability of the plasmid

Stationary phase R+cultures depleted of Mg^{2+} or glucose and resuspended in MOPS were stored at different temperature 4^{o}, 25^{o} and 37^{o} (see 4.2.1.1.). Viable counts on NA plates containing 15 µg ml^{-1} kanamycin sulphate showed no significant difference from those performed on NA at all temperatures tested. Figures 55 and 56 represent the mean results of triplicate viable counts of Mg^{2+} and glucose-depleted cultures respectively.

4.2.1.3b. Effect of suspending medium on stability of the plasmid

The stationary phase glucose-depleted culture of R+ cells used in 4.2.1.2. was counted on NA with or without kanamycin sulphate. There was no difference in the count (Fig. 57).

4.2.2. Effect of drying at 25^{o} on survival

After 13 hours, 20 µl samples of stationary phase R- and R+ cells appeared dry, to the naked eye. At that time, over 95% of the cells had died but significantly more R+ than R- (Table 28). (Cultures were glucose-depleted, grown in CDMB, Table 4).

Table 28

Survival of E. coli during drying

Medium	% survival	
	R-	R+
NA	3 ± 0.4	0.5 ± 0.12
NA-NaCl.1.5%	3 ± 0.5	0.3 ± 0.07

On further storage the percentage surviving R- cells fell at approximately the same rate as R+ ones (Fig. 58).

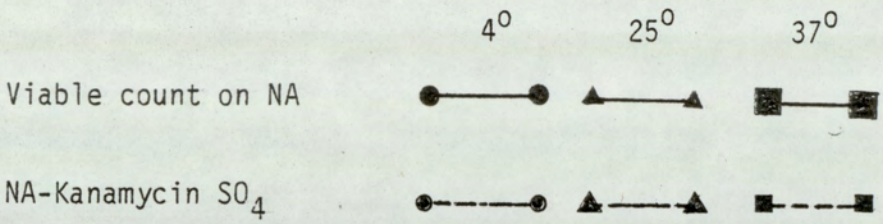
Addition of 1.5% NaCl to the NA on which samples were counted did not reduce the count. This concentration of NaCl was chosen, because it had no detectable effect on the count of cells before drying (see 4.2.3.)

Figure 55

Effect of storage temperature on stability of plasmid (RP1) in Mg^{2+}-depleted cultures of E. coli

(These cultures were the same as those used in Fig. 52)

Key



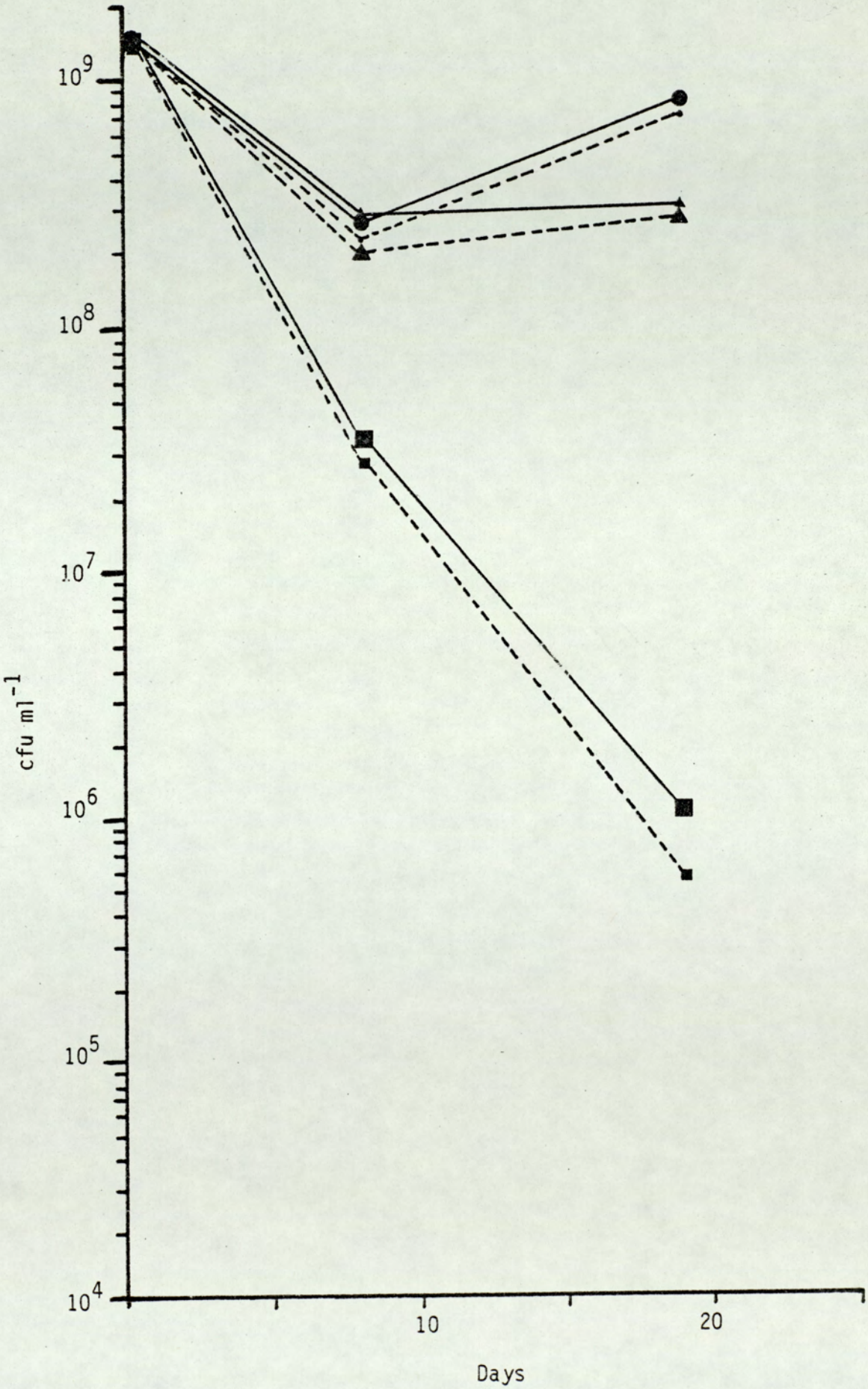
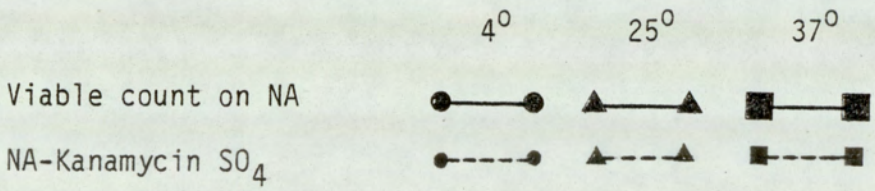

10^9
10^8
10^7
10^6
10^5
10^4
cfu ml^{-1}
10
20
Days

Figure 56

Effect of storage temperature on stability of plasmid(RP1) in glucose-depleted cultures of E. coli

(These cultures were the same as those used in Fig. 53)

Key



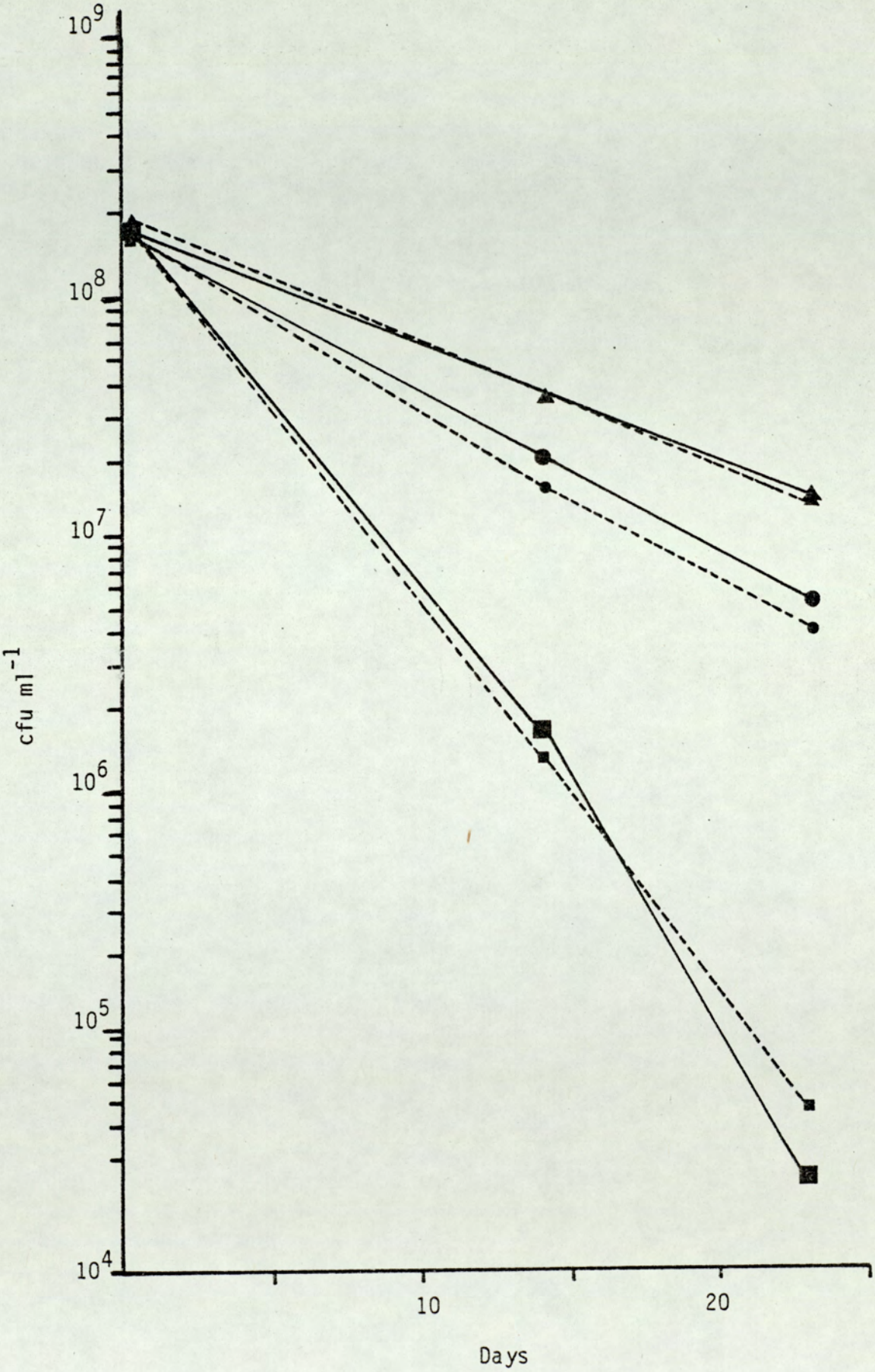
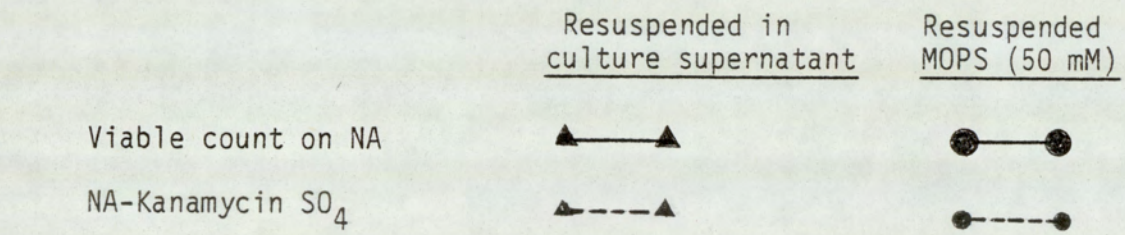

10^9
10^8
10^7
10^6
10^5
10^4
cfu ml^{-1}
10
20
Days

Figure 57

Effect of suspending medium on stability of plasmid (RP1) in *E. coli*

(The cultures were the same as those used in Fig. 54)

Key



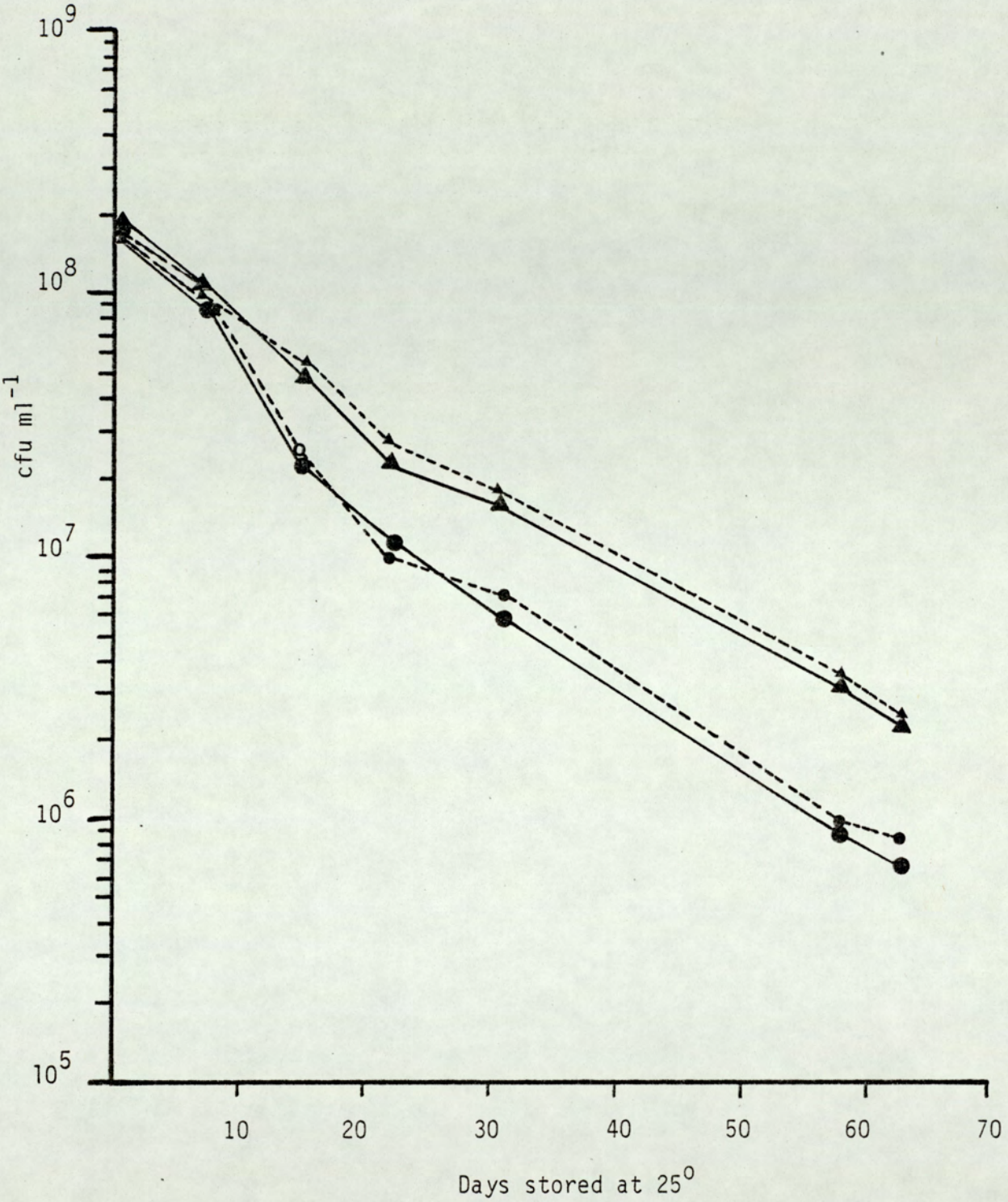
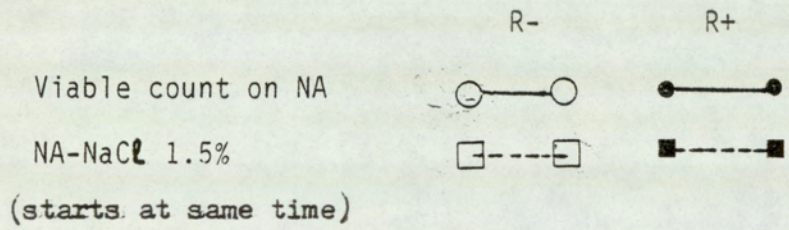
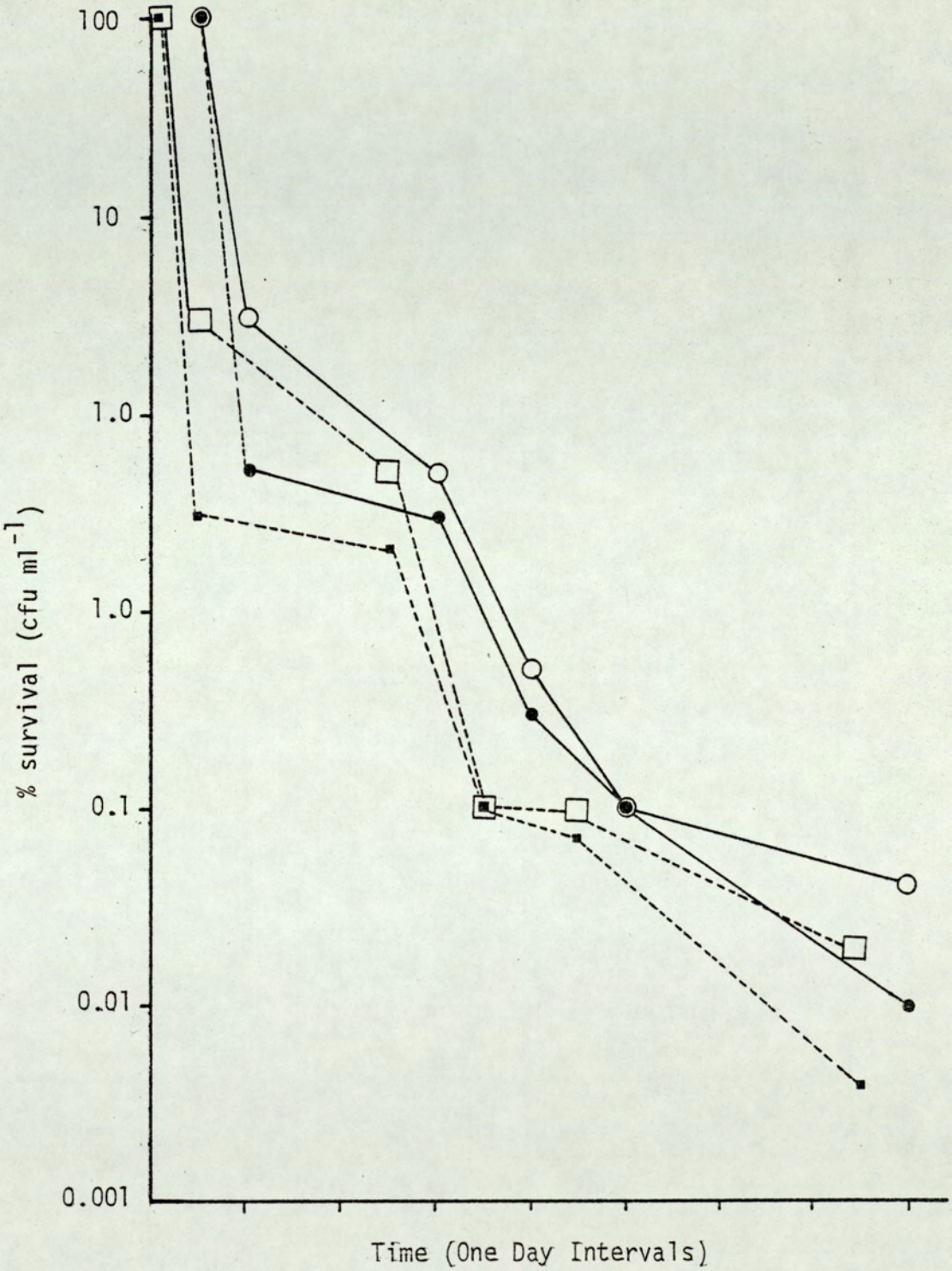

10^9
10^8
10^7
10^6
10^5
cfu ml^{-1}
10
20
30
40
50
60
70
Days stored at 25°

Figure 58

Effect of drying at 25° on survival of glucose-depleted stationary phase cultures of *E. coli* R- and R+

Key




100
10
1.0
1.0
0.1
0.01
0.001
% survival (cfu ml^{-1})
Time (One Day Intervals)

4.2.3. Effect of sodium chloride and Tween 80 on stationary phase cells

4.2.3.1. Effect of NaCl

Cultures depleted overnight were diluted and plated direct on to NA containing different concentrations of NaCl (NA as supplied by the manufacturer contains 0.5% NaCl). The mean values for percentage survival from duplicate experiments are plotted in Fig. 59.

The concentration of NaCl had little effect below 2.5%. At 5.5% NaCl, no colonies were detected. Between these concentrations, the result varied with the depletion and the presence of plasmid RP1.

Glucose-depleted cultures (CDMC, Table 4) were the most resistant and the plasmid had no detectable effect. R- potassium depleted cultures (CDMG, Table 4) were the most sensitive whereas R+ ones were significantly less sensitive. R+ oxygen-depleted cultures (CDMH, Table 4) were apparently more sensitive than R- ones and no R+ colonies were detected at a concentration of NaCl of 4% or more.

Colonial morphology was also affected by the concentration of NaCl. Fig. 60 shows the normal colonial morphology of R- and R+ cells respectively, grown on NA with no additional NaCL. Differences can be detected at 1.5% NaCl. R+ colonies appeared cream-coloured and mucoid, R- colonies were more opaque, smaller and whiter. With 3.5% NaCl, both R+ and R- colonies appeared mucoid and shiny (Fig. 60). The slime was not very viscous and the R+ colonies were almost transparent.

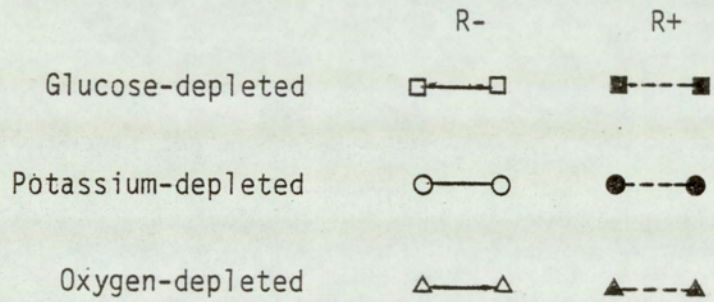
4.2.3.2. Effect of Tween 80

Stationary phase glucose, potassium or oxygen depleted cultures grown in CDMC, G and H respectively, were plated on NA containing different concentrations of Tween 80 (T80).

Figure 59

Effect of NaCl on stationary phase cultures of *E. coli* R- and R+

Key



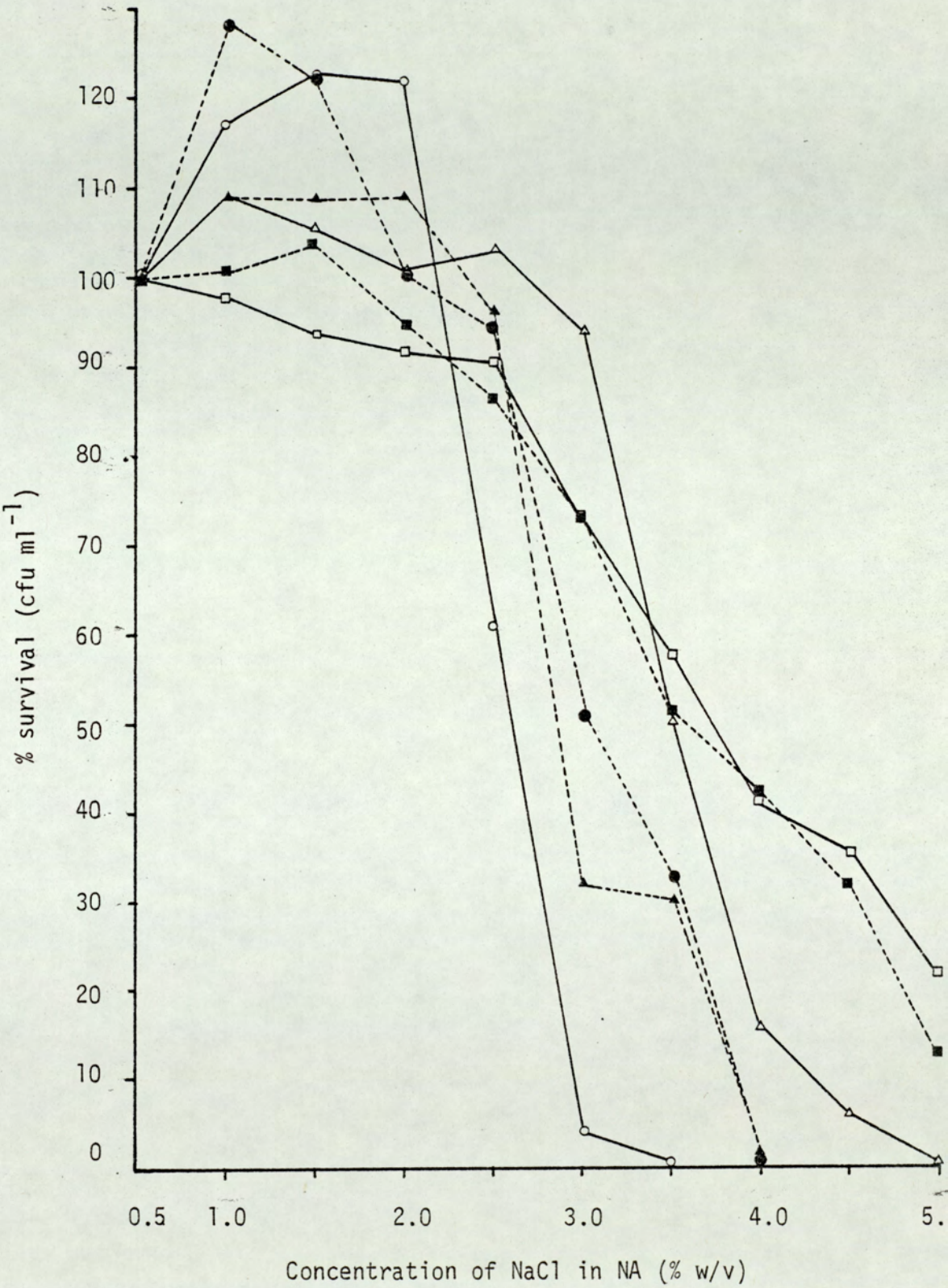

% survival (cfu ml^{-1})
120
110
100
90
80
70
60
50
40
30
20
10
0
0.5
1.0
2.0
3.0
4.0
5.
Concentration of NaCl in NA (% w/v)

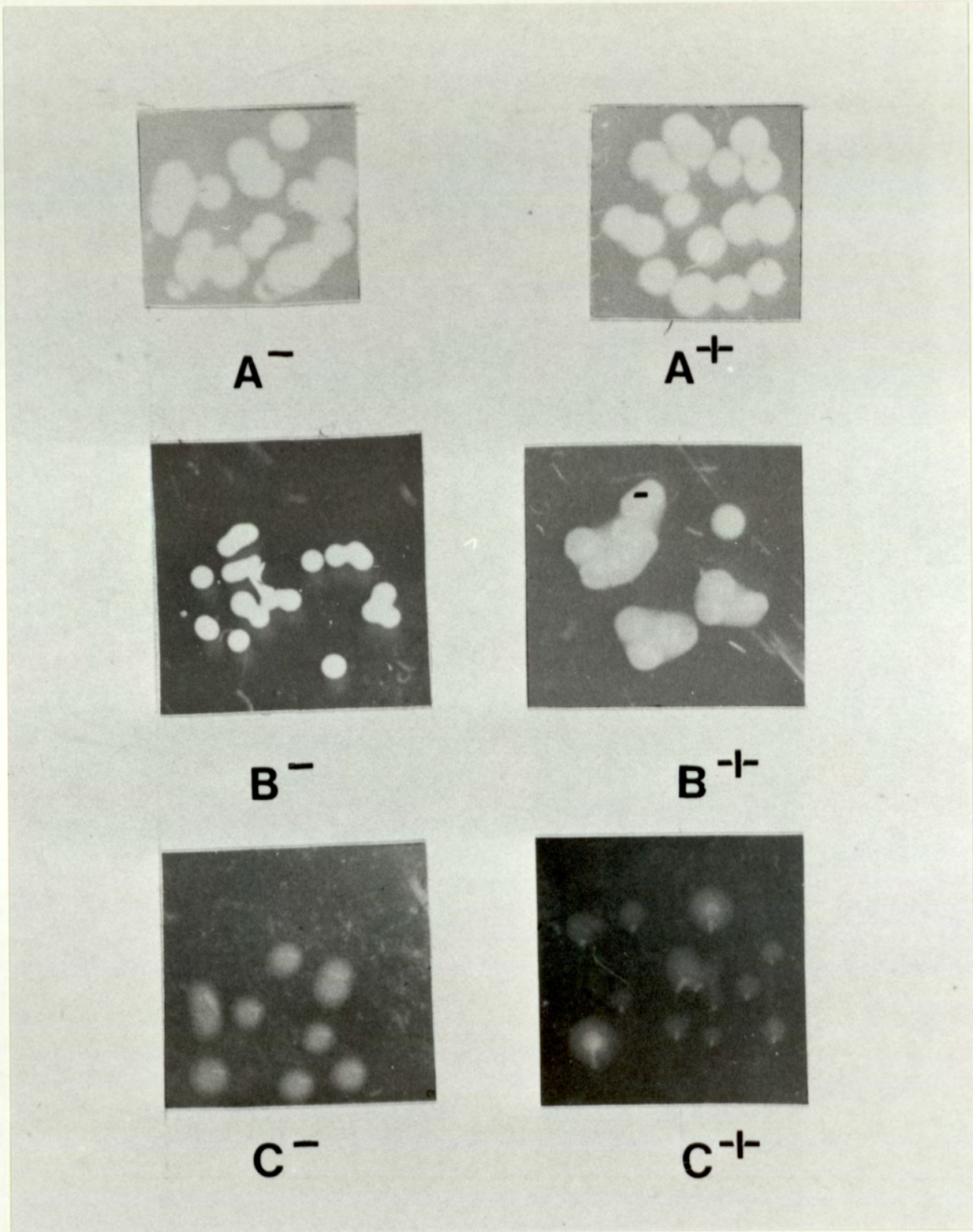
Figure 60

Effect of NaCl on colonial morphology of *E. coli* R- and R+

Key

-	R-
+	R+
A	NA with no added NaCl
B	NA with 1.5% NaCl
C	NA with 3.5% NaCl

All cells were glucose-depleted grown anaerobically in chemostat at $D = 0.1\ h^{-1}$ and incubated aerobically for 48 h on NA plates.


A⁻
A⁻⁺
B⁻
B⁻⁺
C⁻
C⁻⁺

The mean % survival from two experiments was calculated and plotted against concentrations of added T80. From Fig. 61 it can clearly be seen that T80 did not significantly affect the viability of the tested cultures at any of the concentrations used.

Tween 80 had no effect on the colonial morphology of either R- or R+ strains.

4.2.3.3. Effect of combinations of 1.5% w/v NaCl with different concentrations of Tween 80

Figure 62 shows the mean percentage survial in two experiments where cells were plated on NA-1.5% NaCl containing different concentrations of T80. (The cultures used were the same as those described in section 4.2.3.2.; and the counts on NA-1.5% NaCl did not differ from those on NA). There was no significant loss in viability of glucose or oxygen-depleted R- and R+ cultures and of potassium-depleted R+ cultures. Potassium depleted R- cells exhibited a slight but significant fall in % viability.

No significant changes of colonial morphology were detected at any concentration of T80 in the presence of 1.5% NaCl.

4.2.4. Effect of sodium deoxycholate (DOC)

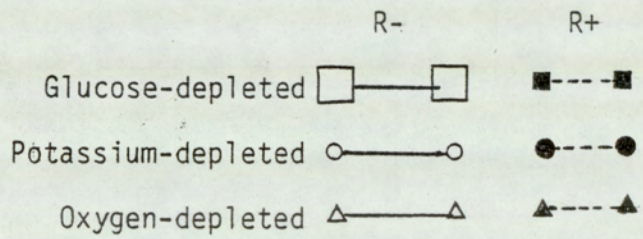
4.2.4.1. Effect of DOC on viability

The action of DOC on R- and R+ cultures of E. coli was tested on chemostat grown cultures (see 3.10.3.) as well as on batch cultures. Results are seen in Fig. 63 . Cells were either from glucose-depleted aerobic stationary-phase cultures grown in CDMC (Table 4) or from glucose-limited anaerobic chemostat cultures grown at D 0.1 h^{-1} in CDMG-lim (Table 21) and plated on NA and NA containing difference concentrations of DOC.

Figure 61

Effect of Tween 80 on stationary phase cultures of E. coli R- and R+

Key



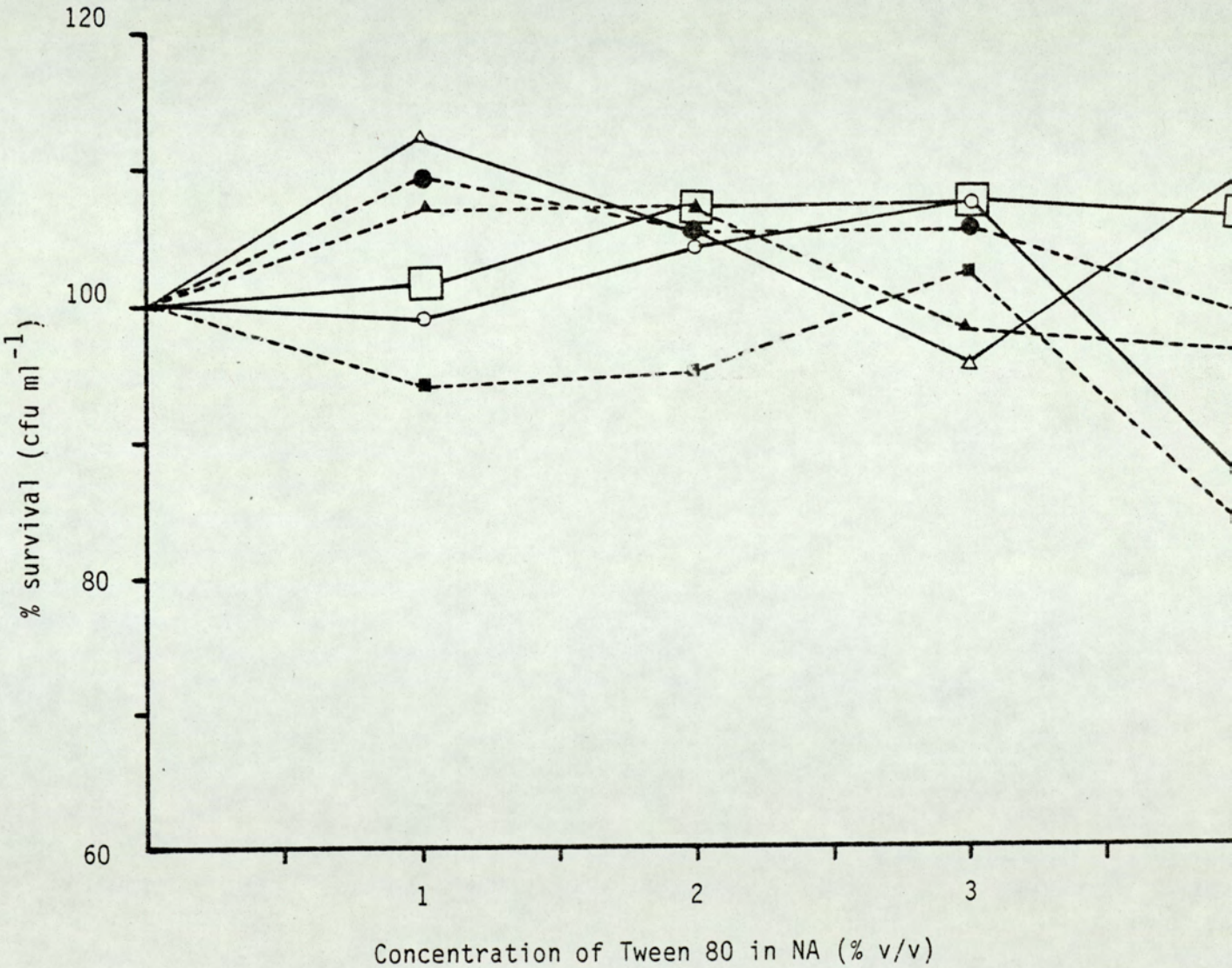
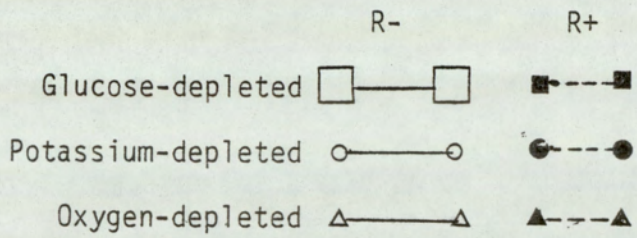

120
100
80
60
% survival (cfu ml^{-1})
1
2
3
Concentration of Tween 80 in NA (% v/v)

Figure 62

Effect of combination of 1.5% w/v NaCl with different concentrations of Tween 80 on stationary-phase cultures of *E. coli* R- and R+

Key



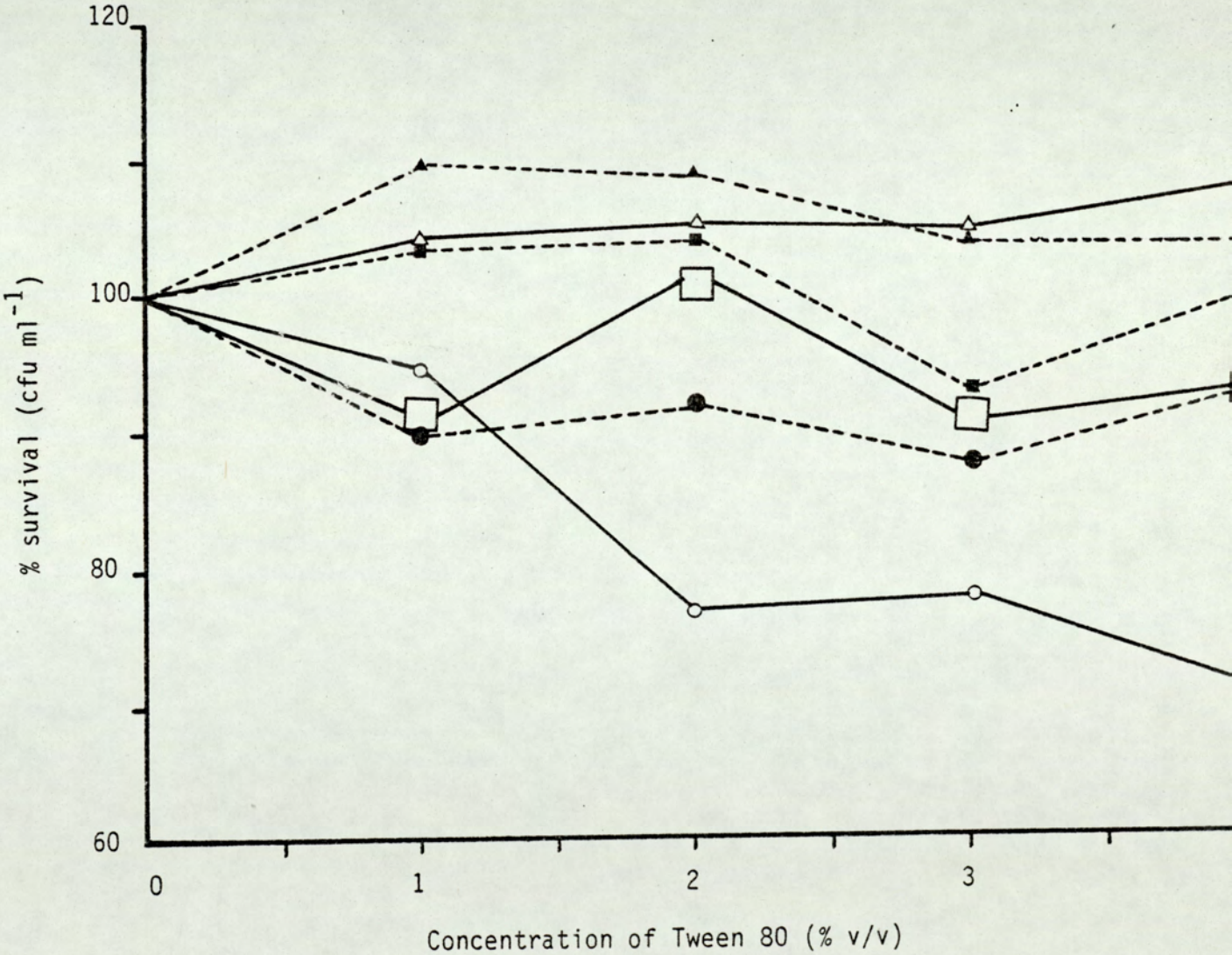
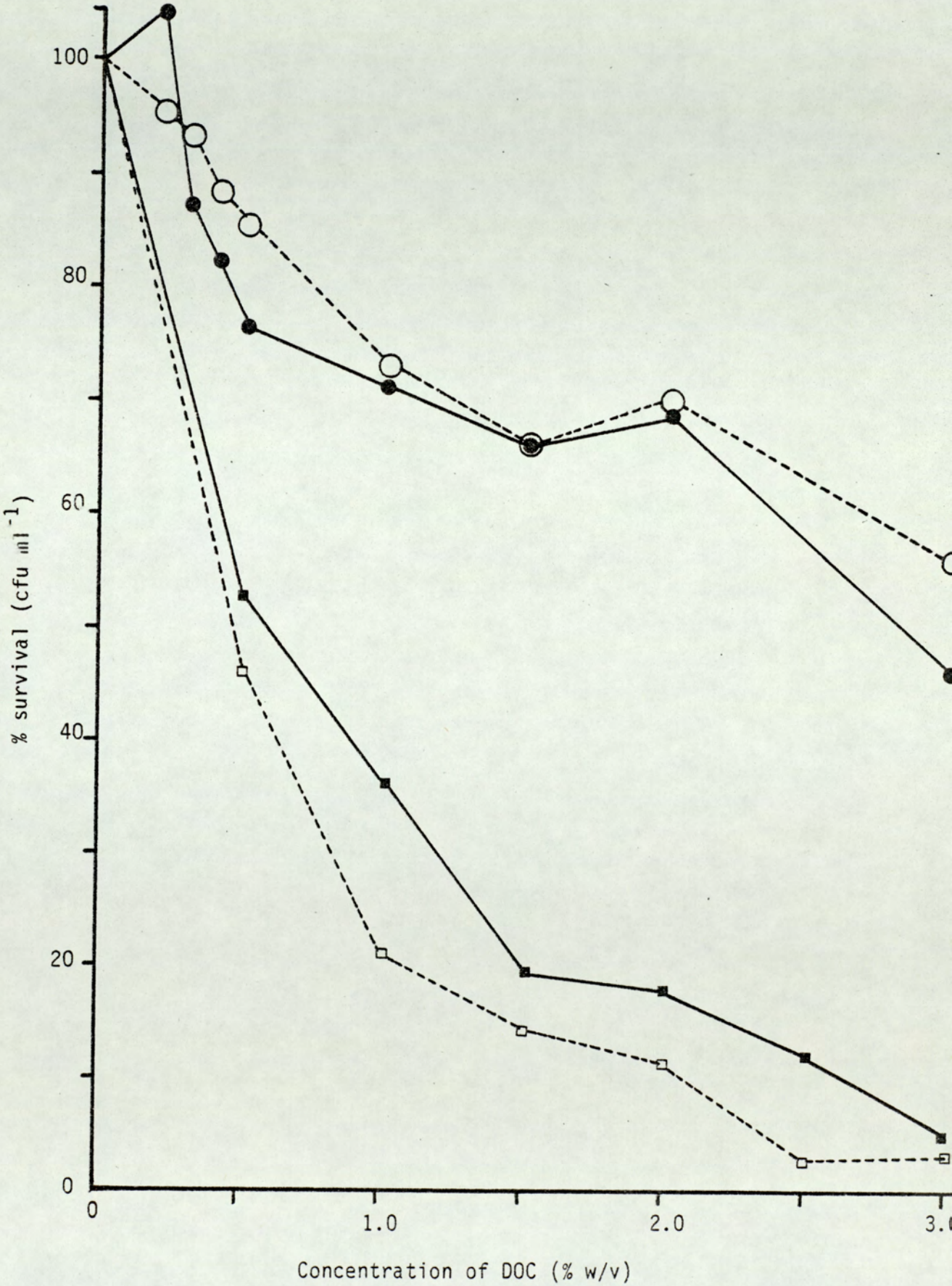

120
100
80
60
% survival (cfu ml^{-1})
0
1
2
3
Concentration of Tween 80 (% v/v)

Figure 63

Effect of sodium deoxycholate on glucose depleted cultures of *E. coli* R- and R+

Key

Culture	R-	R+
Aerobic stationary-phase batch	○---○	●—●
Anaerobic chemostat D = 0.1(h^{-1})	□---□	■—■


100
80
60
40
20
0
% survival (cfu ml^{-1})
0
1.0
2.0
Concentration of DOC (% w/v)

Each point is the mean of two separate experiments. DOC had a significant effect on viability. But no significant difference between R- and R+ strains was observed. The anaerobic chemostat-grown cultures were much more sensitive than the stationary-phase aerobic cultures. Concentrations above 3% could not be tested because of the crystallization and insolubility of DOC.

4.2.4.2. Effect of DOC on colonial morphology

Sodium deoxycholate had no apparent effect on the colonial morphology of glucose-depleted stationary-phase R- and R+ cultures grown under aerobic conditions. However, cells grown anaerobically in the chemostat at D 0.1 h^{-1} showed distinct features when plated on NA containing DOC (Fig. 64). Chemostat grown cells formed colonies which were larger and flatter on NA-DOC than on NA. R+ colonies exhibited concentric rings at DOC concentrations of 1.5% and 3%.

Figure 64

Effect of DOC on colonial morphology of *E. coli* R- and R+ grown anaerobically

-	R-
+	R+
A	NA with no added DOC
B	NA with 0.5% added DOC
C	NA with 1% added DOC
D	NA with 1.5% added DOC
E	NA with 3.0% added DOC

All cells were glucose-limited grown anaerobically in chemostat at D = 0.1 h^{-1} and incubated aerobically for 24 h on NA plates.

A+
B+
C+
D+
E+
A−
B−
C−
D−
E−

4.3. Chemical Analysis of Cell Wall Preparations of *E. coli*

4.3.1. 2-Keto-3-deoxyoctonic acid

2-Keto-3-deoxyoctonic acid (KDO), a marker for lipopolysaccharides (LPS) was assayed in the OM of batch grown stationary-phase cells by the method described in section 3.6.2. The results varied with the nutrient depleted, the presence of the R- plasmid or the conditions of growth (Fig. 65). In every case, cells grown aerobically had more KDO than the same cells grown anaerobically in the same media (CDM XI Table 5 and NB). In some cases, the difference was striking. In glucose, nitrogen and phosphate-depleted R- cells and in potassium-depleted R+ cells, the amount of KDO following aerobic growth was more than double that found after anaerobic growth.

Aerobically grown cells also showed great variation in KDO content with different nutrient depletions depending upon the presence of the R- plasmid. R- strain had more KDO than R+ cells, except following potassium depletion when the R+ cells had three times more KDO than R-.

Anaerobically grown cells showed less variability, with little difference between R- and R+ cells. Glucose, oxygen, magnesium (R- only) and iron-depleted cells (R+ only) had the most KDO.

4.3.2. Outer membrane protein profile

The outer membrane protein (OMP) profiles of *E. coli* R- and R+ as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) are shown in Fig. 66 and 67 . The cultures used were the same as those described in section 4.3.1. The major outer membrane proteins with apparent molecular weights (M_r) between about 30,000 and 40,000 were present in nearly all growth conditions and there appeared to be no major variation in

Figure 65

Concentration of KDO in the OM of batch grown cells of *E. coli* R- and R+

(mean of 2 experiments each in duplicate, OM prepared by sarcosyl method)

Key

Culture characteristics

1 ---------- glucose-depleted

2 ---------- ammonium-depleted

3 ---------- phosphate-depleted

4 ---------- potassium-depleted

5 ---------- magnesium-depleted

6 ---------- sulphate-depleted

7 ---------- iron-depleted

8 ---------- CDM in excess

9 ---------- NB

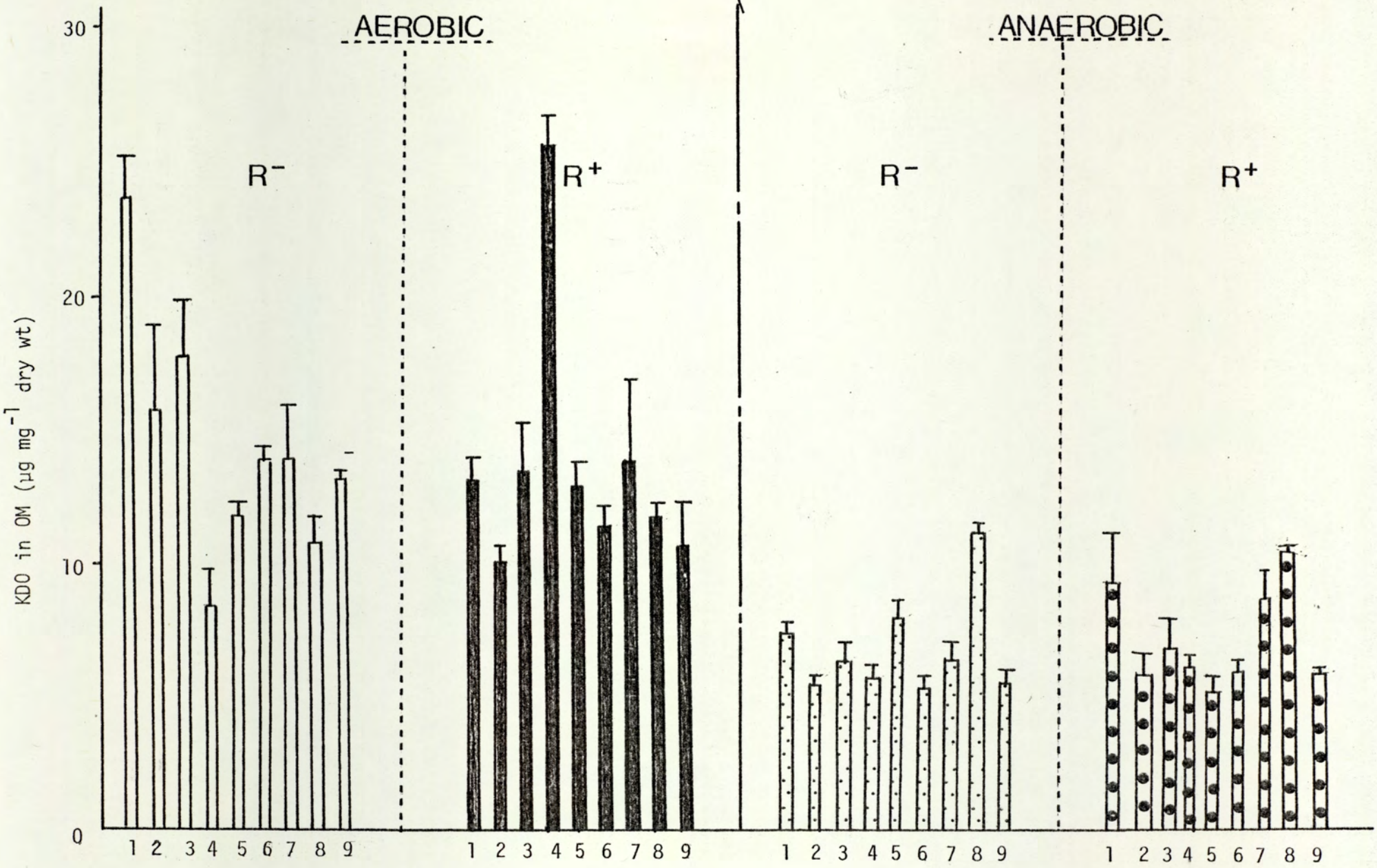

AEROBIC
ANAEROBIC
R⁻
R⁺
R⁻
R⁺
KDO in OM (μg mg⁻¹ dry wt)
30
20
10
0
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9

Figure 66

SDS-PAGE protein profiles of the OM of stationary phase cells of *E. coli* R- and R+ grown aerobically in CDM under different nutrient depletions

Key

R+	R-	Cells
a	b	grown under glucose depletion
c	d	grown under ammonium depletion
e	f	grown under phosphate depletion
g	h	grown under potassium depletion
i	j	grown under magnesium depletion
k	l	grown under sulphate depletion
m	n	grown under iron depletion
o	p	grown in full CDM 10

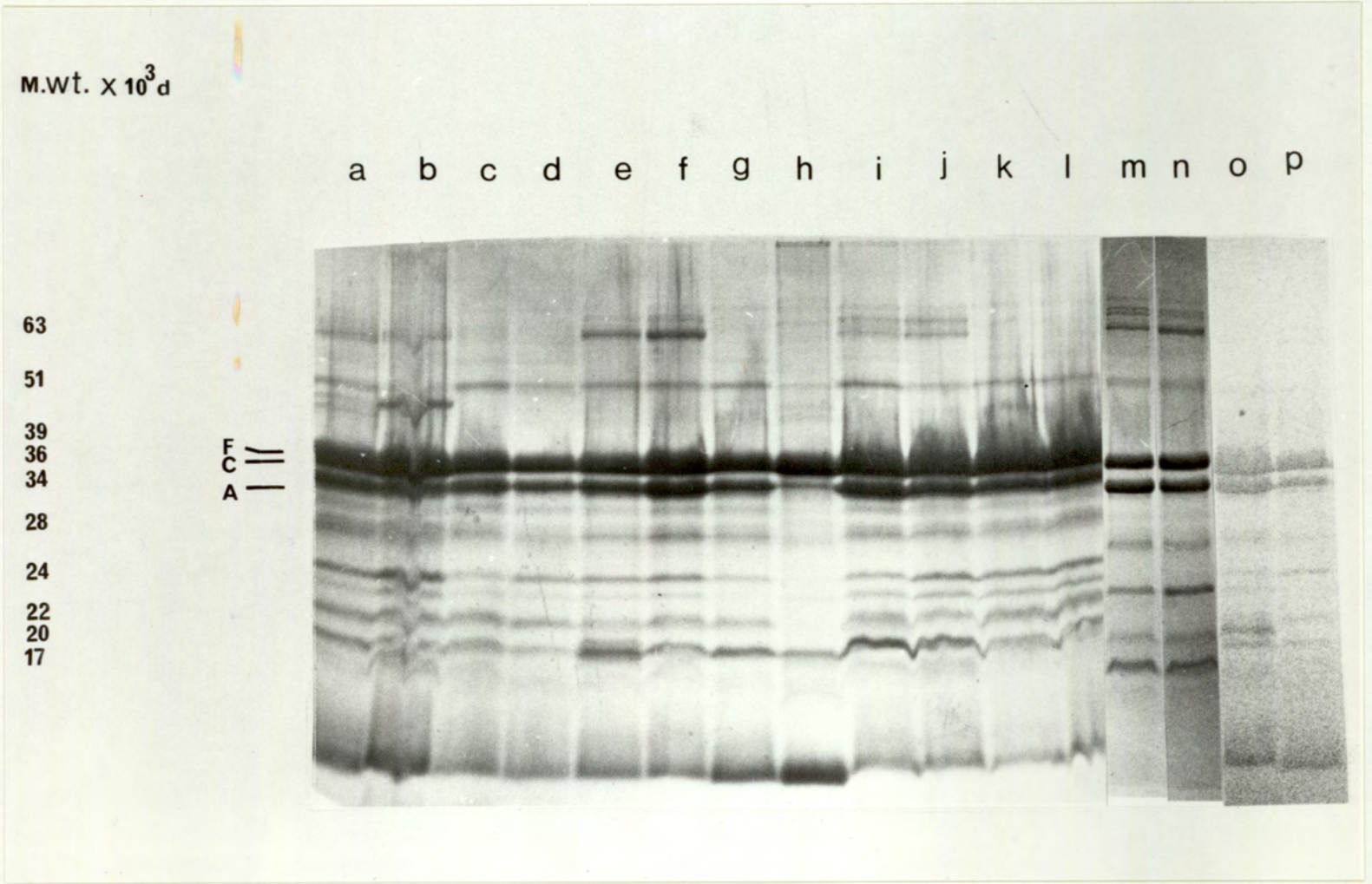
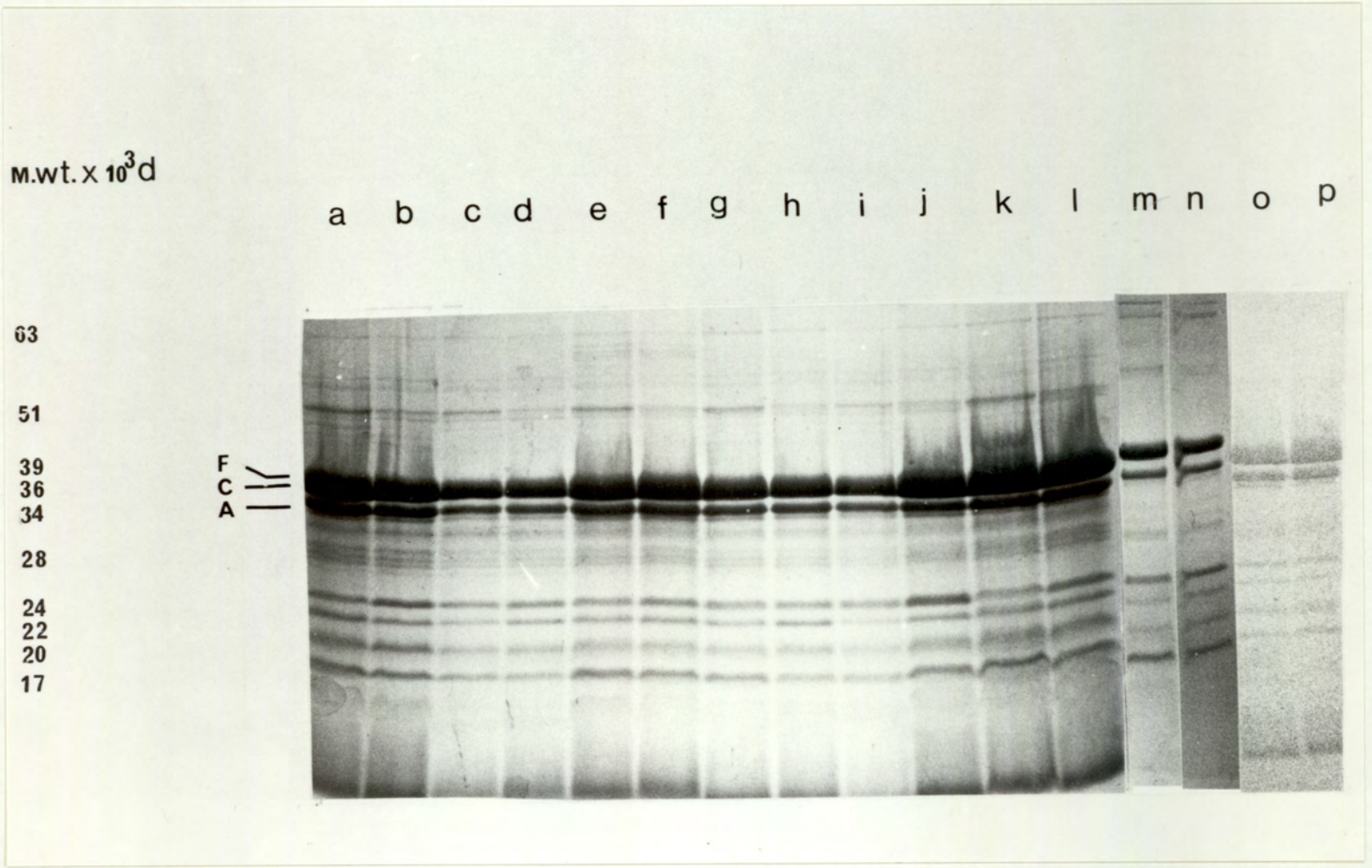

M.wt. X 10³d
a b c d e f g h i j k l m n o p
63
51
39
36
34
28
24
22
20
17
F
C
A

Figure 67

SDS-PAGE protein profiles of the OM of stationary phase cells of *E. coli* R- and R+ grown anaerobically in CDM under different nutrient depletions

Key

R+	R-	Cells
a	b	grown under glucose depletion
c	d	grown under ammonium depletion
e	f	grown under phosphate depletion
g	h	grown under potassium depletion
i	j	grown under magnesium depletion
k	l	grown under sulphate depletion
m	n	grown under iron depletion
o	p	grown in full CDM


M.wt. x 10^3d
a b c d e f g h i j k l m n o p
63
51
39
36
34
28
24
22
20
17
F
C
A

the amount of the OmpF and OmpC (38,800 and 35,600) porin proteins under any conditions. In the R- strain grown aerobically (potassium depleted) and both strains grown aerobically or anaerobically (iron-depleted), the protein with an apparent M_r of 34,000 was not produced. There were also changes in some minor proteins in some cultures. Aerobic, CDM in excess and phosphate-depleted, R+ cultures had an additional protein with an apparent M_r of about 18,500. Aerobic cultures of the R- strain with CDM in excess or depleted in potassium lacked the protein with apparent M_r of about 22,000. In addition the protein with an apparent M_r of about 20,000 was not produced in R- potassium-depleted cultures.

Variations in proteins of high M_r were also found, particularly at a M_r of about 38,800. As expected, a group of proteins in the range of 60,000 - 90,000 were found in iron-depleted cells. These were also expressed in glucose, phosphate and magnesium-depleted aerobic cultures.

4.4. Anaerobic Growth of *E. coli* R- and R+ in Chemostats

4.4.1. The effect of dilution rate on growth parameters

The influence of dilution rate on OD_{470}, total count and viable count of *E. coli* R- and R+ cultures was studied under G-lim and K^+-lim conditions. Mean results from two experiments are shown in Fig. 68 and 69 (G-lim), and 70 (K^+-lim) cultures.

In G-lim cultures an increase in dilution rate was accompanied by a fall in population size, particularly at the higher concentration of 5mM. The optical density did not fall as much as the count. The pH of the cultures fell to 7.4 and 7.1 when glucose concentrations were 3 and 5 mM respectively. These pH values did not vary by more than 0.1 pH unit at the dilution rates tested, over the period of the experiments.

In K^+-lim cultures there were no significant changes in OD or viable count when dilution rates were increased, but, results for R+ cells were slightly higher than those for R- at all dilution rates used. However, up to 35% of the colonies formed when samples were counted on NA were morphologically distinct "small colony variants" (see Section 4.4.2.). The drop in pH values was more than that found in G-lim experiments, 6.9 $\pm$ 0.1 and 6.6 $\pm$ 0.1 pH unit for R- and R+ cultures respectively.

4.4.2. Small colony variants

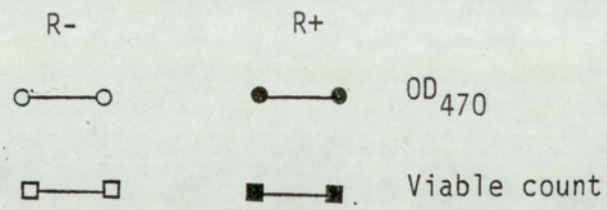
Small colony variants of R- (SCV-) and R+ (SCV+) cells appeared on NA plates, from K^+-limited chemostat cultures. They were found at almost all dilution rates used. The proportion of SCV cells was variable and ranged between 0% and 35% of the parent population (see Fig.70). The SCV were stable when subcultured on NA plates every 48 h for at least three weeks.

Figure 68

Relation between dilution rate and OD_{470} and viable count of anaerobic glucose-limited (3mM) cultures of *E. coli* R- and R+

(Each point is the mean of 3 samples from one chemostat)

Key



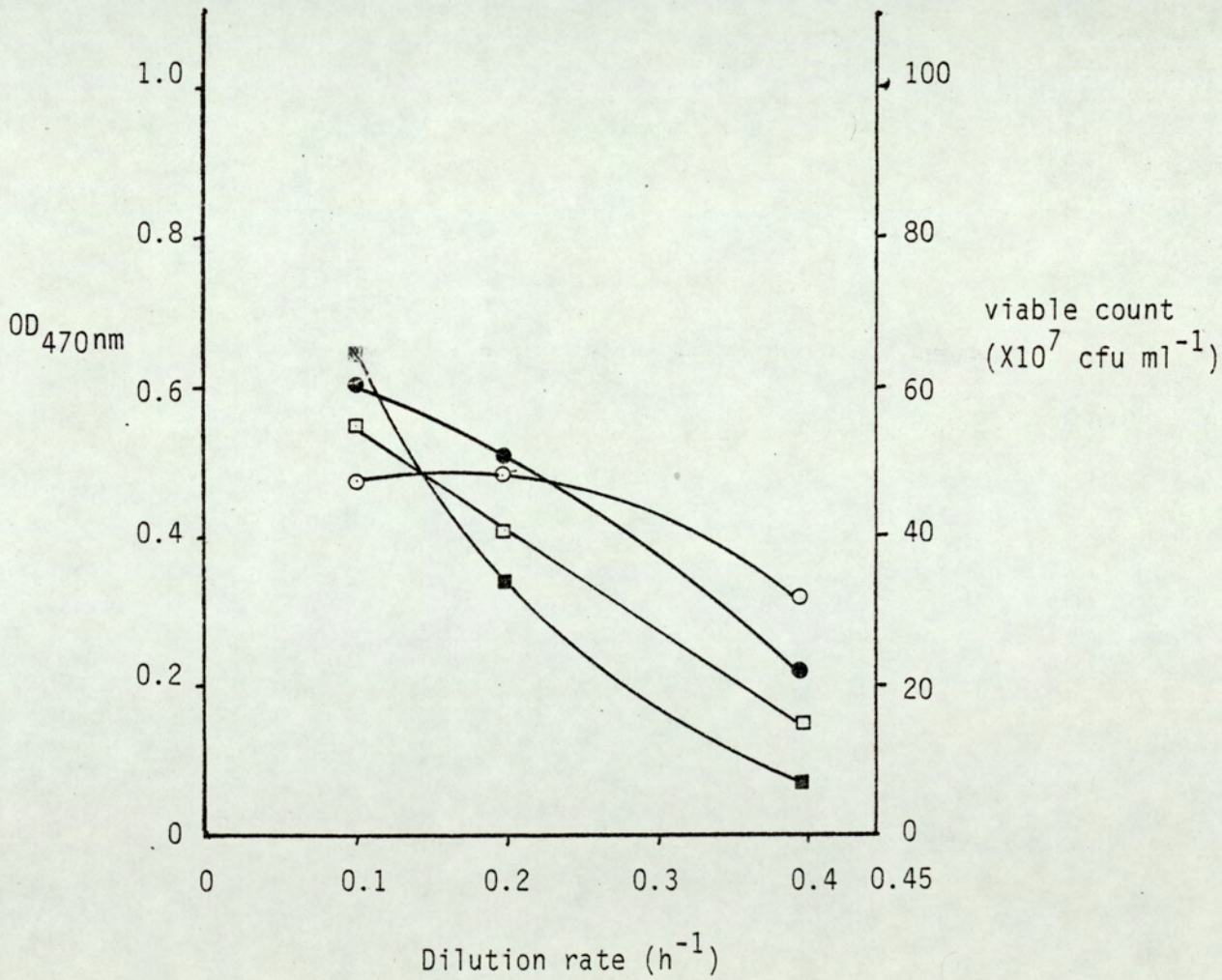
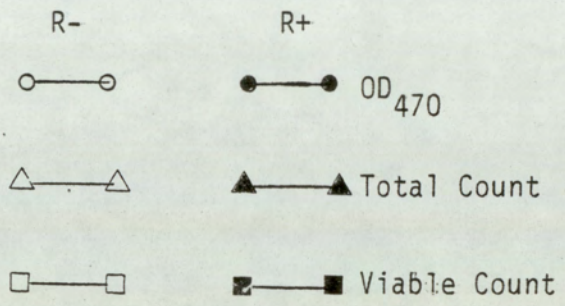

1.0
0.8
0.6
0.4
0.2
0
OD_{470nm}
100
80
60
40
20
0
viable count
($X10^7$ cfu ml^{-1})
0
0.1
0.2
0.3
0.4
0.45
Dilution rate (h^{-1})

Figure 69

Relation between dilution rate and OD_{470}, total and viable count of anaerobic glucose-limited (5mM) cultures of *E. coli* R- and R+

(Each point is the mean of 3 samples from one chemostat)

Key



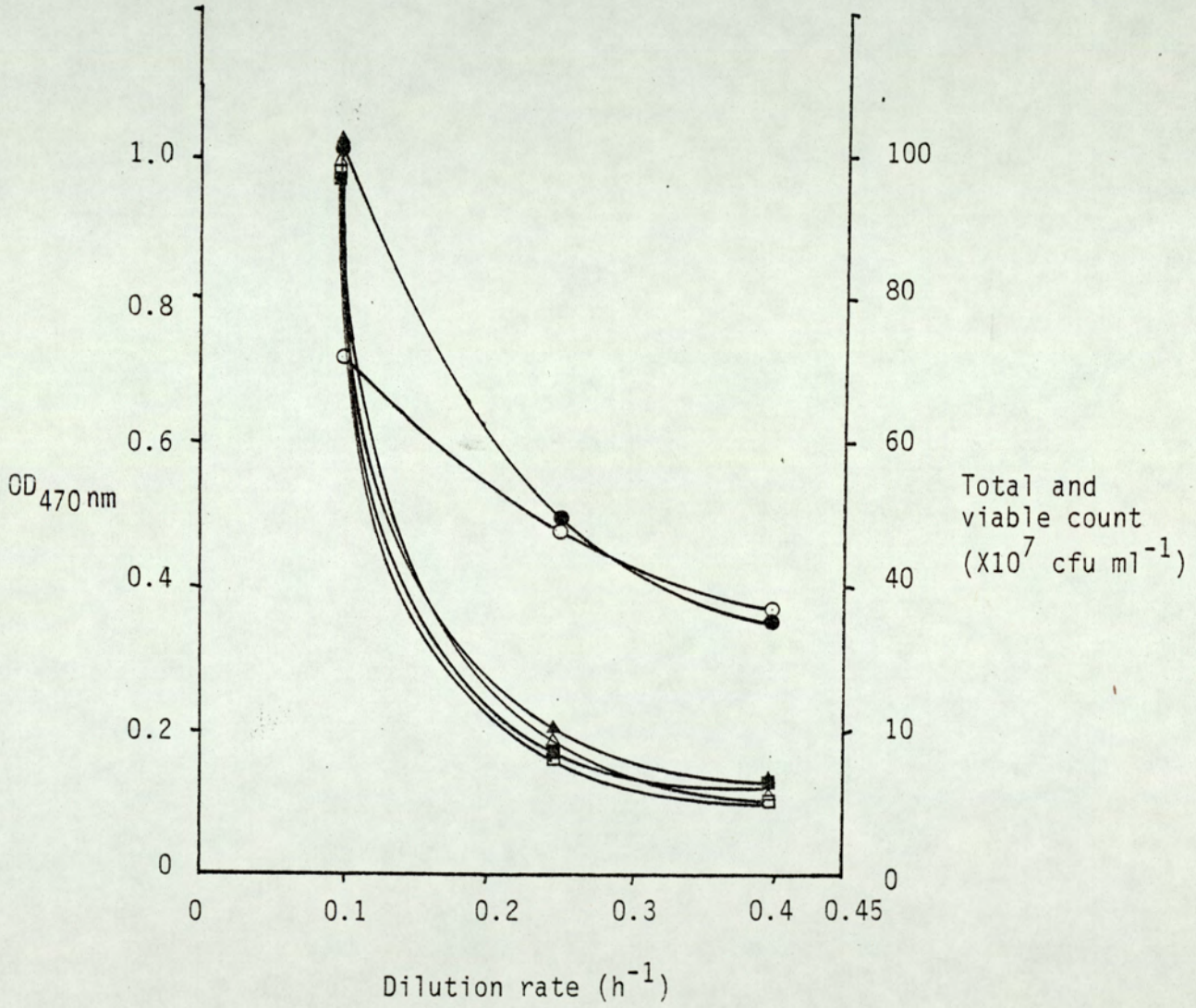
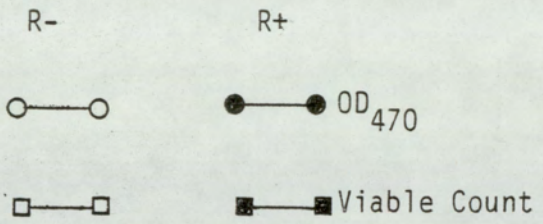

1.0
0.8
0.6
0.4
0.2
0
OD 470 nm
100
80
60
40
10
0
Total and viable count (X10^7 cfu ml^-1)
0
0.1
0.2
0.3
0.4
0.45
Dilution rate (h^-1)

Figure 70

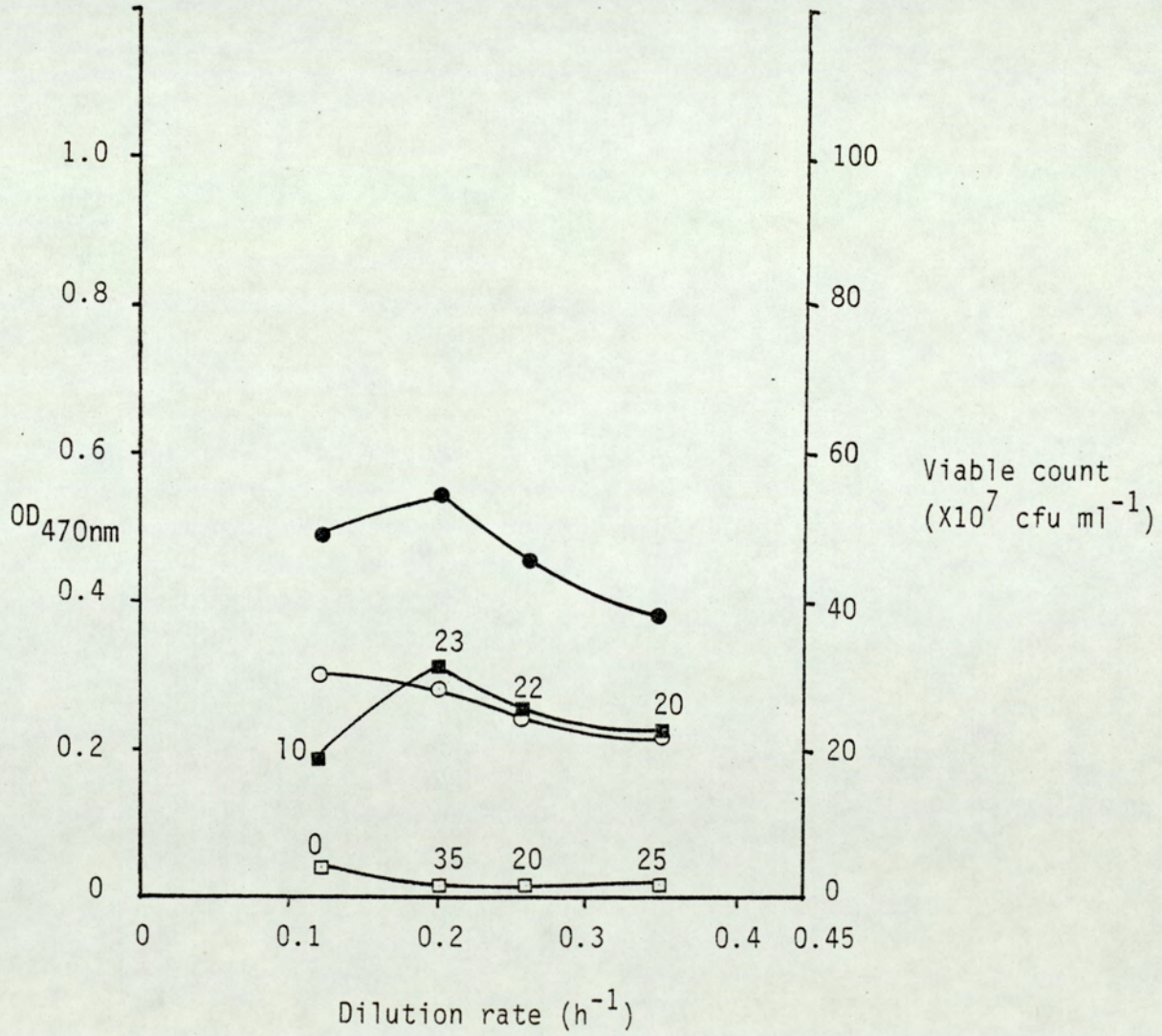
Relation between dilution rate and OD_{470} and viable count of anaerobic K^+-limited (0.06mM) cultures of *E. coli* R- and R+

(Each point is the mean of 3 samples from one chemostat)

Key



N.B. Figures represent % of the count which were small colony variants.


1.0
0.8
0.6
OD470nm
0.4
0.2
0
100
80
60
40
20
0
Viable count
(X10^7 cfu ml^-1)
23
22
20
10
0
35
20
25
0
0.1
0.2
0.3
0.4
0.45
Dilution rate (h^-1)

After 24 h incubation on NA plates, SCV and their parent colonies had a diameter of 1.0mm and 5mm respectively. The methods of Cowan and Steel (1974) and the API 20 E system were used to compare the properties of the SCV- and SCV+ with the original cultures. Only slight differences were found (Table 29).

Table 29

Biochemical tests of R-, SCV-, R+ and SCV+

Test	R-	SCV-	R+	SCV+
Gram's stain	-	-	-	-
Motility	+	+	+	+
Oxidase	-	-	-	-
Catalase	+	+	+	+
Growth on MacConkey	+	+	+	+
API Ortho nitrophenol-galactosidase (ONPG)	+	+	+	+
Arginine dihydrolyse (ADH)	-	-	-	-
Ornithine decarboxylase (ODC)	-	-	-	-
Citrate as C source (CIT)	-	-	-	-
H_2S	-	-	-	-
Urease (URE)	-	-	-	-
Indole (IND)	+	+	+	+
VP test (VP)	-	-	-	-
Gelatin (GEL)	-	-	-	-
Glucose (GLU)	+	+	+	+
Mannitol (MAN)	+	+	+	+
Sorbitol (SOR)	+	+	+	+
Rhamnose (RHA)	+	+	+	+
Sucrose (SAC)	-	-	-	-
Maltose (MEL)	$\pm$	+	+	$\pm$
Amylose (AMY)	-	-	-	-
Arabinose (ARA)	$\pm$	+	+	+

Key: + : Positive - : Negative

$\pm$: Partial fermentation, not increased by further incubation.

SCV+ retained their resistance to kanamycin SO_4 (15 μg ml^{-1} NA). The % of SCV+ on NA-kanamycin SO_4 was equal to that on NA (see Fig. 72).

4.4.3. Stability of plasmid RP1

The stability of plasmid RP1 in E. coli was tested by performing viable counts on NA and NA containing 15 μg ml^{-1} kanamycin SO_4. The plasmid was found to be very stable during the continuous growth period (3-5 weeks), at the selected glucose and potassium concentrations and dilution rates. Typical results are shown in Figures 71 and 72 for G-lim and K^+-lim cultures respectively. In addition, four samples of P-lim R+ cultures at D=0.1 and 0.2 h^{-1} were examined and were found to contain more than 95% cells resistant to kanamycin.

4.4.4. Competition under glucose-limited conditions

As Fig. 73 shows, when the initial count of R+ cells was high, R+ cells were gradually replaced by R- cells. Similar results were obtained at the different glucose concentrations used.

When the initial concentration of R+ cells was low (Fig. 74) the level of R+ cells was maintained at about the same as the inoculum, with some fluctuations. There was no takeover and a number of R+ cells persisted throughout the experiments.

There was no significant changes in the value of the pH, or in the relation between OD_{470} and viable count before and after mixing.

4.4.5. Competition under phosphate-limited conditions

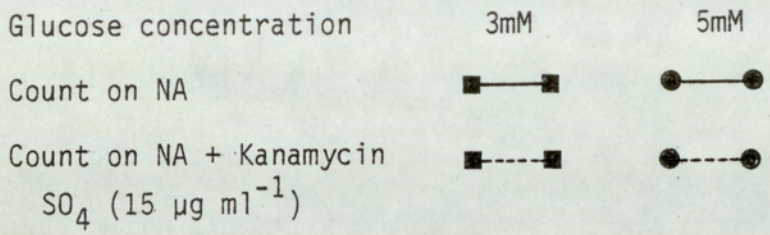
Phosphate-limited single culture chemostats were allowed to reach equilibrium at D=0.1 h^{-1}. About 10% of R+ cells were replaced by R- ones. The R- strain took over from the R+ strain at both concentrations of phosphate

Figure 71

The effect of dilution rate on stability of plasmid RP1 in *E. coli* grown anaerobically in a chemostat under glucose-limited conditions

(Same cultures as in Figure 68 and 69)

Key



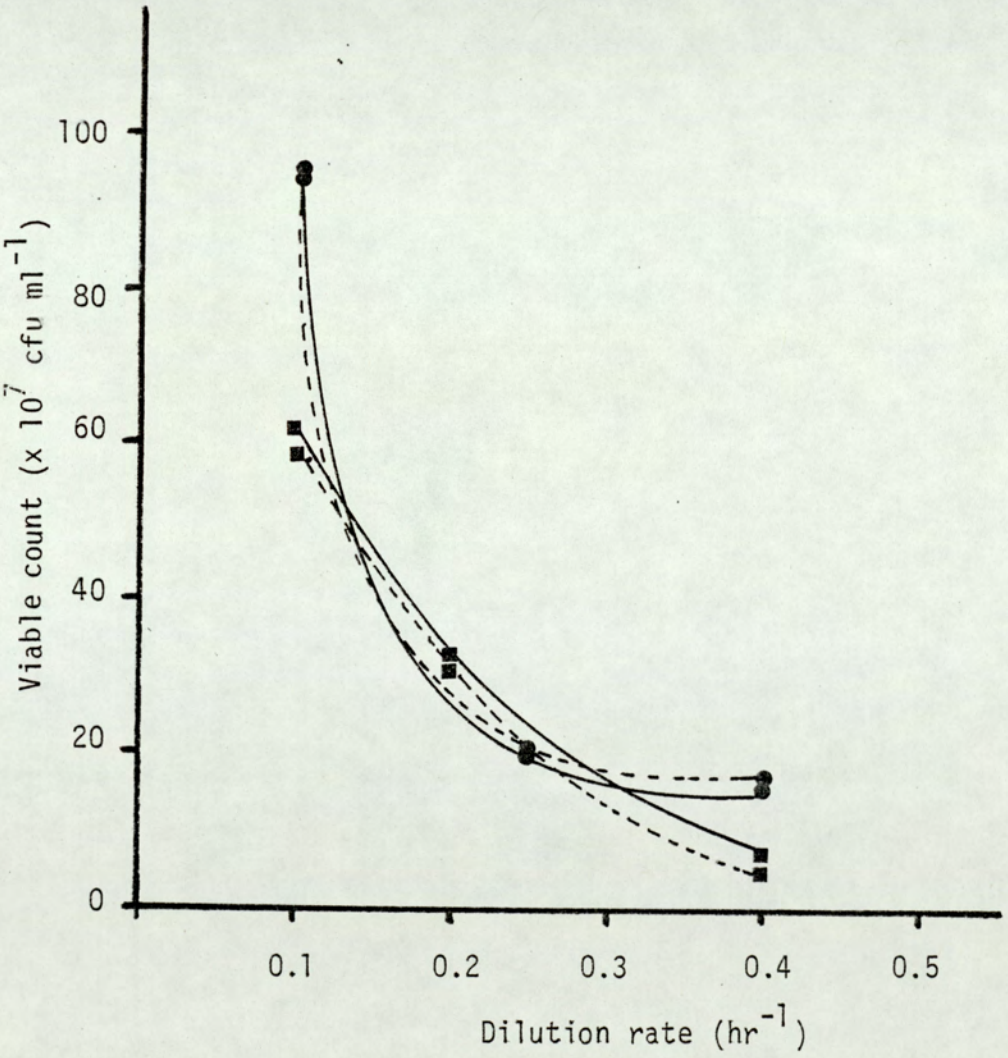
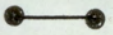

100
80
60
40
20
0
Viable count (x 10^7 cfu ml^{-1})
0.1
0.2
0.3
0.4
0.5
Dilution rate (hr^{-1})

Figure 72

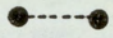
The effect of dilution rate on stability of plasmid RP1 in *E. coli* grown anaerobically in a chemostat under potassium-limited conditions (0.06mM)

(Same cultures as in Fig. 70)

Key



Count on NA



Count on NA + Kanamycin SO_4 (15 µg ml^{-1})

N.B. Figures present over the points are % small colony of the count.

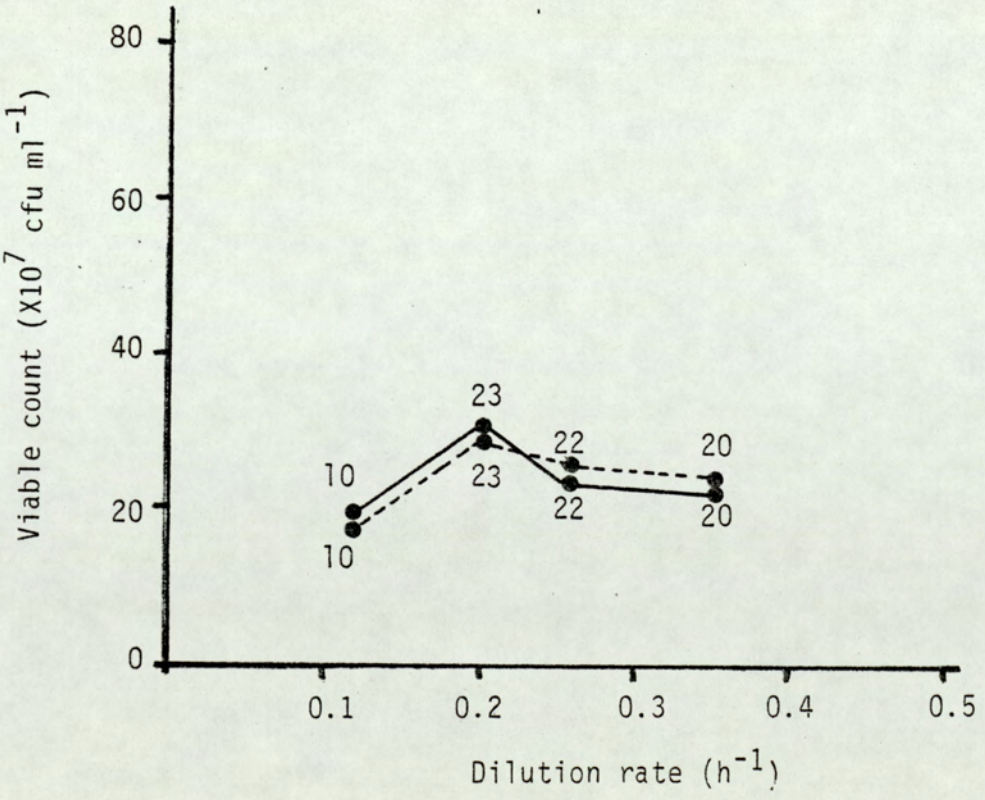

Viable count (X10^7 cfu ml^{-1})
80
60
40
20
0
23
22
20
10
23
22
20
10
0.1
0.2
0.3
0.4
0.5
Dilution rate (h^{-1})

Figure 73

Growth of R+ and R- strains in glucose-limited chemostat cultures

(R- added to R+ cultures)

Key

D (h^{-1})		Added glucose concentration
0.1	■—■	3mM
0.1	●—●	5mM

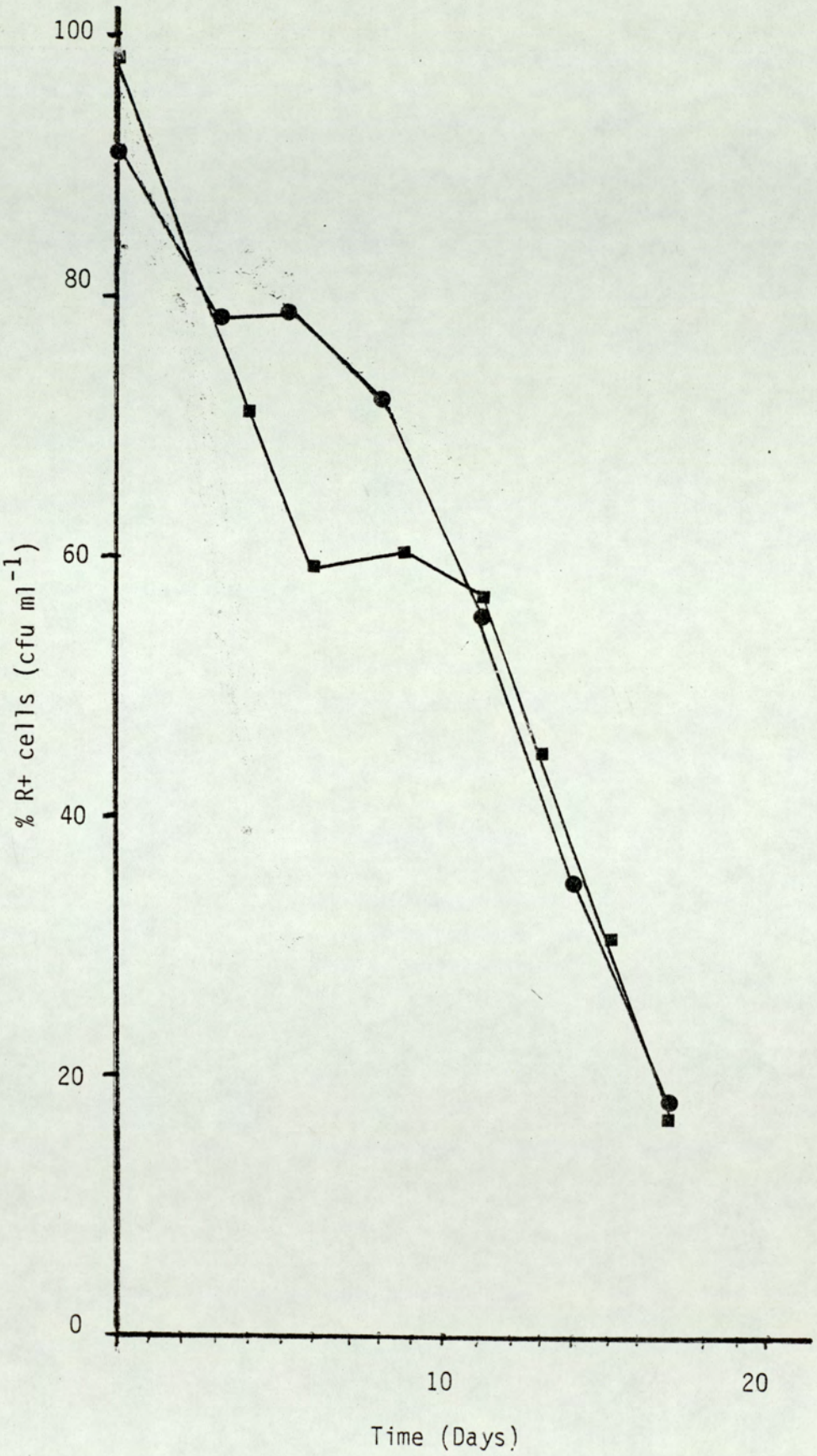

100
80
60
40
20
0
% R+ cells (cfu ml^{-1})
10
20
Time (Days)

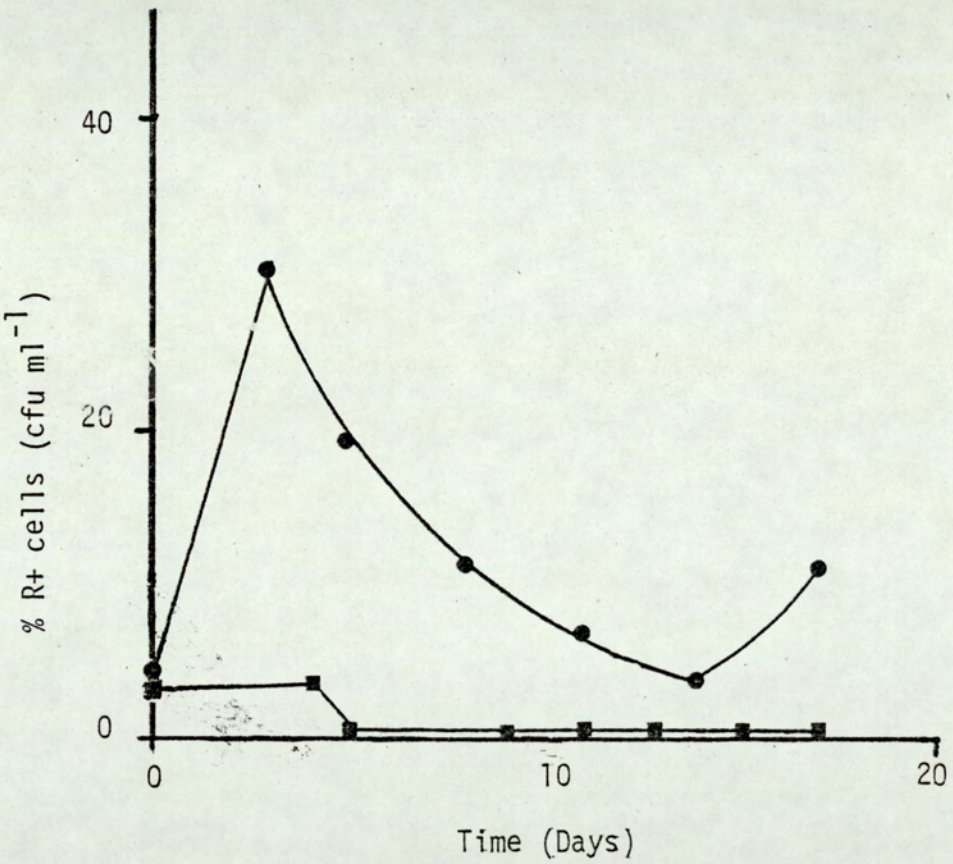
Figure 74

Growth of R+ and R- strains in glucose-limited chemostat cultures

(R+ added to R- cultures)

Key

D (h^{-1})	Added glucose concentration
0.1	3mM
0.1	5mM


40
20
0
% R+ cells (cfu ml^{-1})
0
10
20
Time (Days)

tested; the number of R+ cells declined more rapidly at the higher phosphate concentration (Fig. 75).

Figure 76 shows that when about 10% of a steady state R- population was replaced by its R+ counterpart there was no takeover. The % R+ cells dropped slightly, but the R+ cells did not disappear completely.

As in G-lim competition experiments, similar readings of OD_{470}, pH and viable count were found before and after mixing.

4.4.6. Competition under potassium-limited conditions

When approximately 5% of a steady state R+ culture was replaced by its R- counterpart the proportions of R+ and R- cells fluctuated slightly, but overall showed little change at a K^+ concentration of 0.06mM. At 0.03mM the proportion of R- cells dropped rapidly from the initial 5% until none were detectable and remained so throughout (Fig. 77).

The converse experiments were carried out in which about 5% of steady state R- cells were substituted by the R+ strain. The results in Fig. 78 showed that the proportion of R+ fluctuated, but overall there was little change in the ratio of R+ and R-.

The appearance and subsequent fluctuation in the proportions of small colony variants in both mixed chemostat cultures did not apparently affect the competition outcome.

4.4.7. K_s values for G-lim, P-lim and K^+-lim cultures

Accurate values of K_s are difficult to obtain from batch culture experiments, since at low substrate concentrations it is difficult to measure initial growth rate.

K_s values were calculated from the continuous culture experiments by the method described in section 3.10.4. The μm derived from Lineweaver-Burke plots (see Figures 49 , 50 and 51) did not differ significantly. The mean value for μm was therefore used in the calculations.

The results are shown in Table 30 . The K_s value for each limiting nutrient increased as its concentration in the reservoir (S_R) increased. G-lim and P-lim R- cultures had lower K_s values than had their R+ counterparts. With 0.03 mM K^+ in the reservoir the K_s for R- cultures was slightly more than for R+, but at 0.06 mM the K_s was the same.

4.4.8. Persistence of R+ cells in the chemostat

From Figures 74 , 76 and 78 it can be seen that the R+ cells did not disappear completely from mixed cultures at any of the limitations tested. This could have been due to the build up of a solid film of growth. Samples were taken from the vessel wall at the gas/liquid interface, beneath the surface of the liquid on the vessel sides and around the culture outlet. The proportion of R+ cells in these films was greater than expected from counts of the liquid culture, although the numbers varied widely (Table 31).

Figure 75

Growth of R+ and R- strains in phosphate-limited chemostat cultures

(R- added to R+ cultures)

Key

D (h^{-1})	Added phosphate concentration
0.1	0.1 mM
0.1	0.17 mM

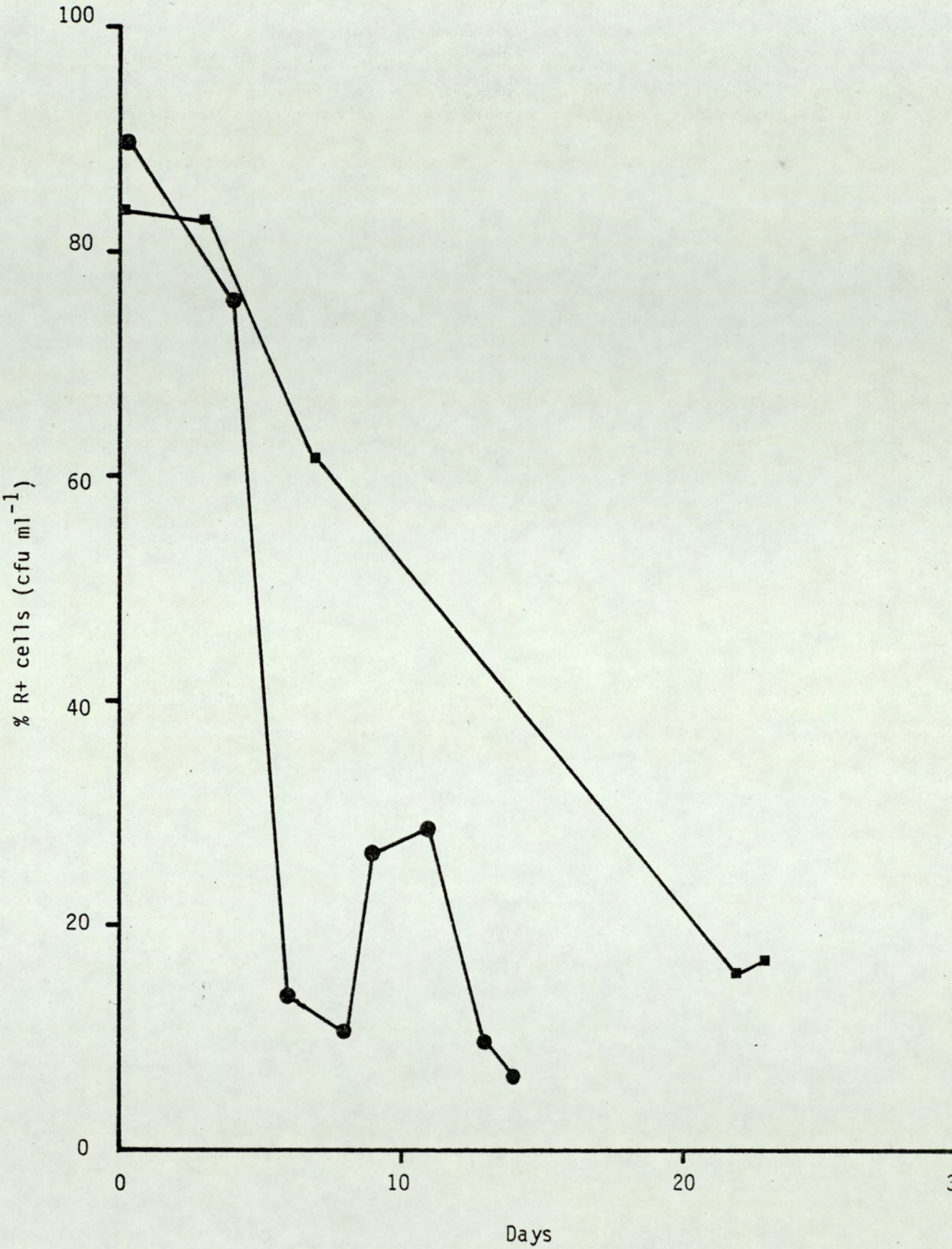

100
80
60
40
20
0
% R+ cells (cfu ml^{-1})
0
10
20
Days

Figure 76

Growth of R+ and R- strains in phosphate-limited chemostat cultures

(R+ added to R- cultures)

Key

D (h^{-1})	Added phosphate concentration
0.1	0.1mM
0.1	0.17mM

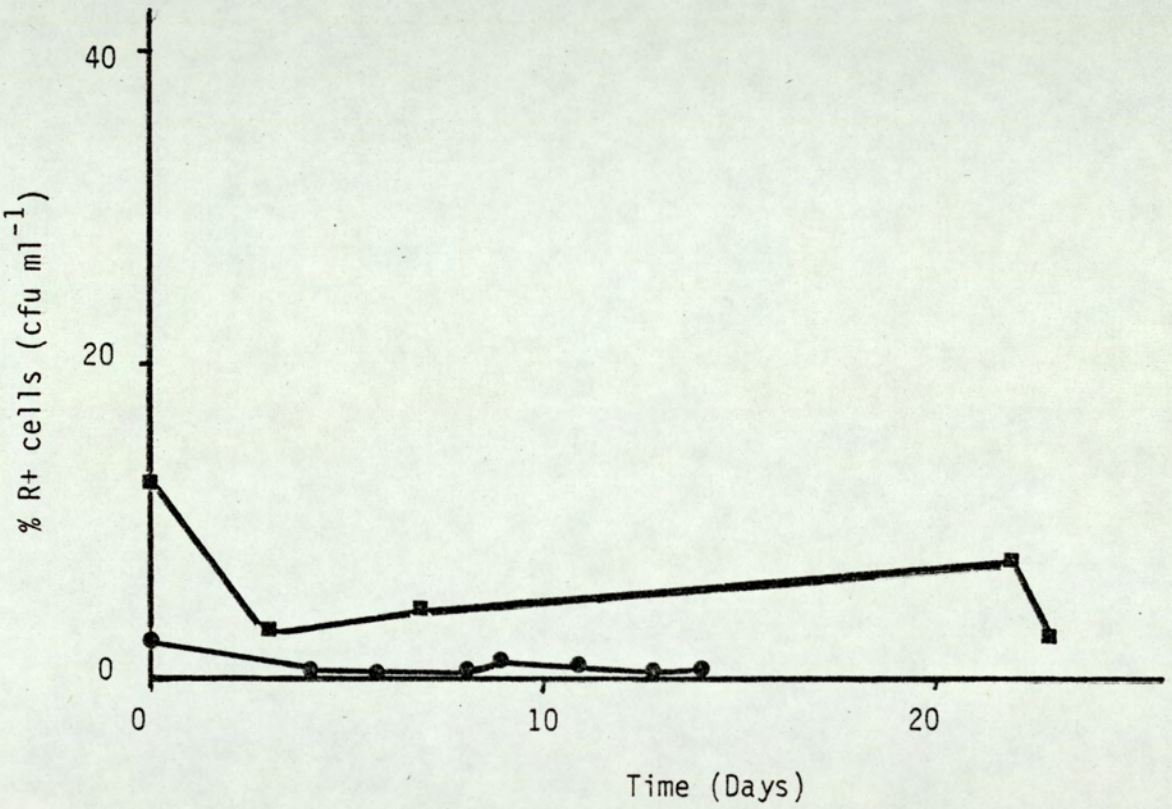

40
20
0
% R+ cells (cfu ml^{-1})
0
10
20
Time (Days)

Figure 77

Growth of R+ adn R- strains in potassium-limited chemostat cultures

(R- added to R+ cultures)

Key

D (h^{-1})		Added potassium concentration
0.1	■—■	0.03mM
0.08	●—●	0.06mM

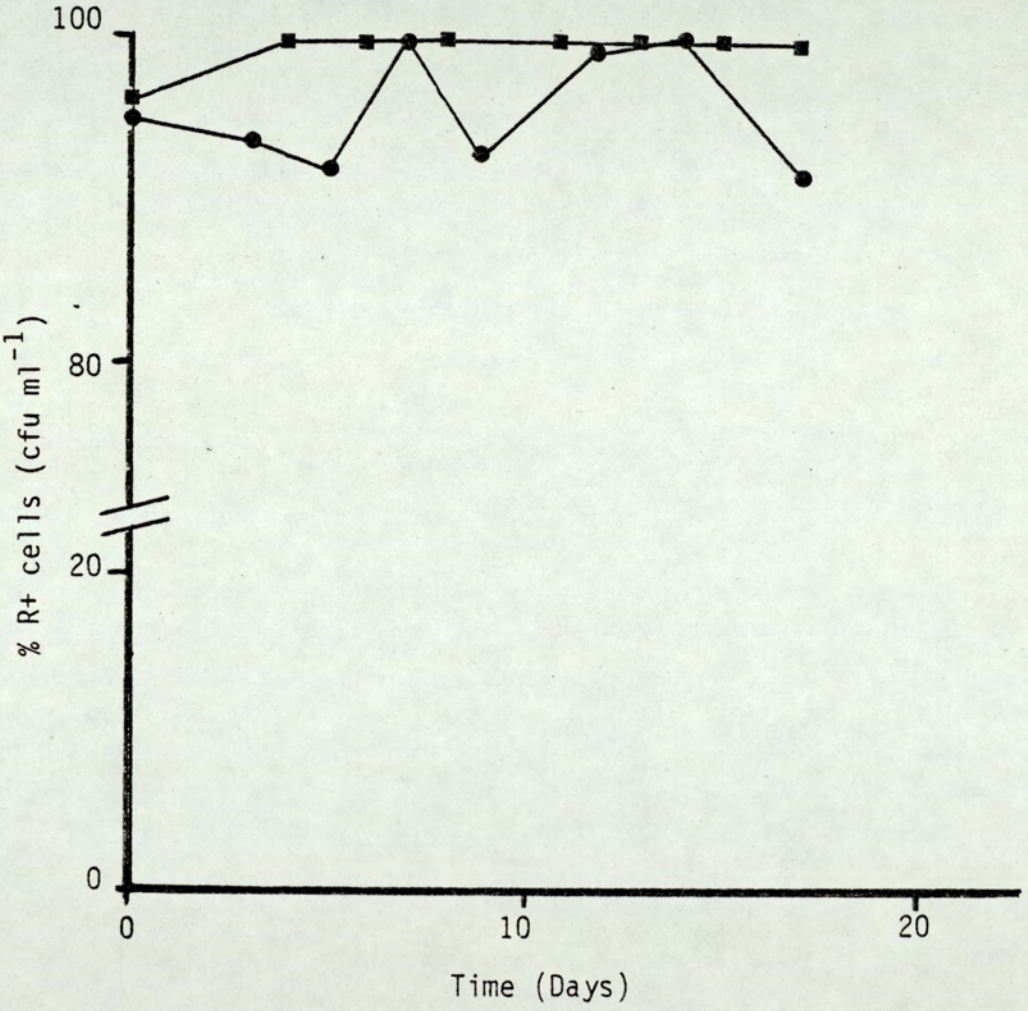

100
80
20
0
% R+ cells (cfu ml^{-1})
0
10
20
Time (Days)

Figure 78

Growth of R+ and R- strains in potassium-limited chemostat cultures

(R+ added to R- cultures)

Key

D (h^{-1})	Added potassium concentration
0.1	0.03mM
0.08	0.06mM

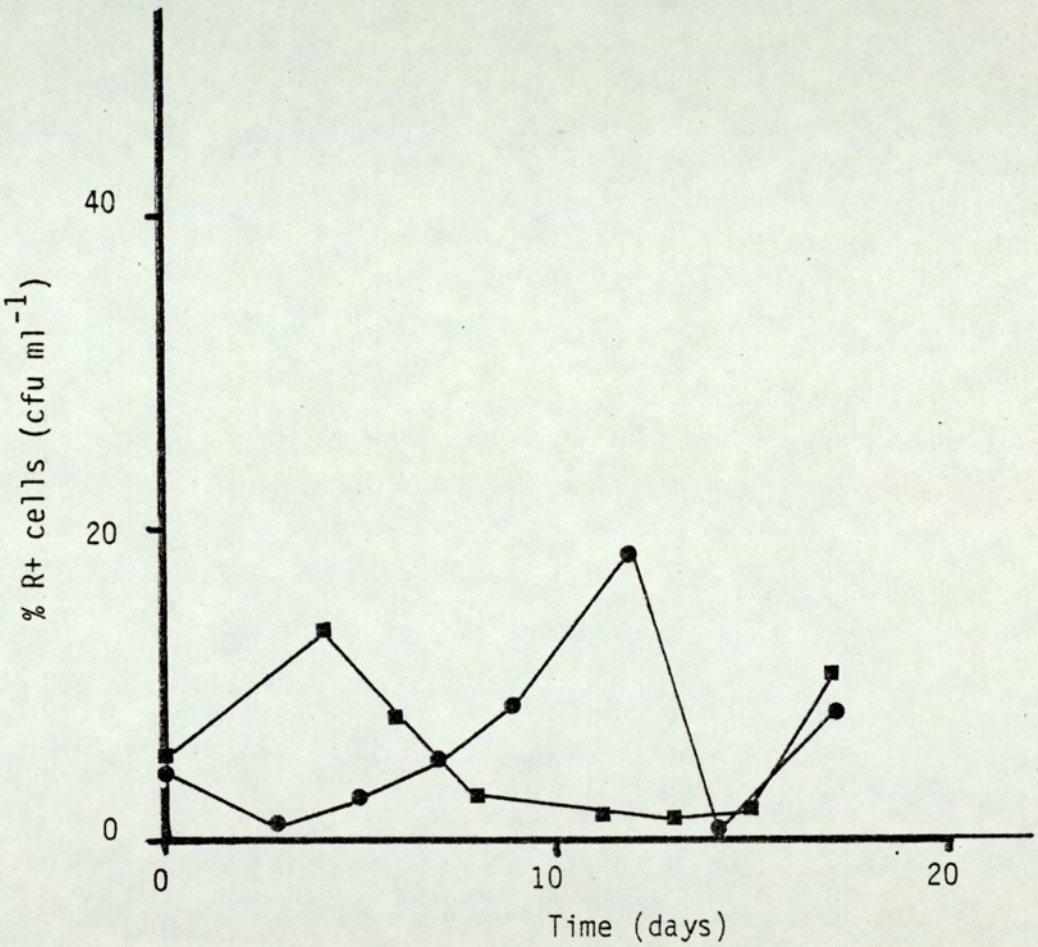

40
20
0
% R+ cells (cfu ml^{-1})
0
10
20
Time (days)

Table 30

Growth parameters used for saturation constant (K_s) calculations of anaerobic chemostat cultures of *E. coli* R- and R+

			R-		R+	
Limiting nutrient	D (h^{-1})	S_R (mM)	$\tilde{s}$ (mM)	Apparent K_s (mM)	$\tilde{s}$ (mM)	Apparent K_s (mM)
Glucose	0.1	3	0.021 ±0.002	0.076	0.025 ±0.005	0.091
	0.085	5	0.053 ±0.005	0.236	0.088 ±0.01	0.392
	0.1	6.2	0.076 ±0.004	0.277	0.114 ±0.01	0.415
Phosphate	0.1	0.1	0.065	0.237	0.074	0.269
	0.1	0.17	0.134	0.488	0.144	0.524
Potassium	0.12	0.03	0.027 ±0.004	0.077	0.024 ±0.003	0.069
	0.08	0.06	0.027 ±0.005	0.13	0.027 ±0.004	0.13

Limiting nutrient: see CDM (Table 21)

μ_m: 0.464 h^{-1}

Table 31

Composition of growth on chemostat walls (i)

Culture	Time (days)	% R+ (cfu ml^{-1}) Liquid (ii) samples	% R+ (cfu ml^{-1}) Wall (iii) samples
G-lim	0	4	-
	17	0.3	24
	0	95	-
	17	7	56
P-lim	0	2	-
	14	0.45	38
	0	90	-
	14	7	43
K^+-lim	0	4	-
	17	5	48
	0	94	-
	17	100	91

(i) See competition experiments section 4.4.4., 4.4.5. and 4.4.6.

(ii) Mean % of 7 counts from one sample.

(iii) Mean of 3 or 4 samples from different positions.

5. DISCUSSION

5. Discussion

5.1. Growth Requirements of *E. coli*

5.1.1. Introduction

The results of this study confirm that *E. coli* R+ as well as R- can be grown anaerobically in a simple salts medium, in batch culture, without the addition of complex growth factors. The maximum exponential growth obtained in the CDM tested was to an OD_{470} of ca. 1.0 although these strains grew exponentially under aerobic condition to an OD_{470} of ca. 4.5 (Ismail, 1977); here, oxygen appeared to be the sole growth limiting factor. Cessation of growth under anaerobic conditions was not due to lack of trace elements; it may have been due to accumulation of toxic end products of metabolism such as ethanol, succinate, lactate, hydrogen and carbon dioxide (Blackwood *et al*, 1956). Tappouni (1984) has shown that addition of fatty acids to digesters markedly decreases survival rate of *Salmonella* sp. The survival of *E. coli* during anaerobic digestion may be limited by fatty acid toxicity.

Furthermore, during catabolism of the glucose, intermediary metabolites and reducing equivalents are produced. Because of the absence of oxygen, the reducing equivalents cannot be transferred to the respiratory chain, so a metabolic bottleneck is created since the carriers of the reducing equivalents, the pyridine and flavine nucleotides, are present in the cell only in low amounts. This accumulation of reduced nucleotides would lead to inhibition of further glucose metabolism therefore depriving the cell of energy. (Neijssel and Tempest, 1979). It is important to know that lack of electron acceptors is probably one of *E. coli* growth-controlling factors in the intestines of both rats and humans (Guiot, 1982).

The shape of the growth curves obtained for R- and R+ cultures were similar to those obtained aerobically for the same organisms (Ismail, 1977), except for curves resulting from lower concentrations of SO_4^{2-}, where sudden cessation of exponential growth occurred. At very low concentrations of NH_4^+

or Mg^{2+} the growth rates of R+ organisms were slower than those obtained at higher concentrations as was expected. However, the growth rates of R- and R+ strains were similar in the CDM tested. The growth rates obtained anaerobically were significantly lower than those found aerobically by Ismail (1977). The maximum growth rates in all media tested were all very similar being approximately 0.42 - 0.48 h^{-1}.

5.1.2. Glucose

Glucose is an essential nutrient for bacterial growth; its role as energy and carbon source has been described in most text books. It has been known for many years that the yield of microbial cells is directly proportional to the amount of the energy source in the medium when this energy source is the growth-limiting factor. In 1960 Bauchop and Eldser concluded from anaerobic growth experiments of Streptococcus faecalis, Saccharomyces cerevisiae and P. lindneri, that the growth yield of an organism is proportional to the amount of adenosine triphosphate (ATP) produced by its catabolic processes. The amount of ATP generated from one molecule of glucose differs with different energy-generating metabolic pathways (Stanier et al, 1979).

When all the nutrients required for the growth of E. coli R- and R+ cultures were present in excess except glucose, growth ceased abruptly as the latter became exhausted (Figures 24 and 25 respectively). However, cultures with high initial glucose concentrations eventually showed a progressive decline in the growth rate (before medium glucose was exhausted, as shown by Clinistix). This indicates that above the concentration of 13.5 (R-) and 11.2mM (R+), glucose may no longer be the depleting nutrient (Fig. 26).

The data obtained in this study showed a significant increase in glucose requirement for R- and R+ cultures grown anaerobically compared with requirements under aerobic conditions (Ismail, 1977). Since only a small fraction of the total energy content of the carbon-substrate can be made available by anaerobic fermentative processes (Teixeira de Mattos and Tempest, 1983), to generate the required amount of energy from glucose by anaerobic metabolism, the organism has to utilize much higher quantities of glucose than by aerobic metabolism. Comparison between aerobic and anaerobic requirements of glucose by E. coli R- and R+ is shown in Table 25. The finding that the requirements of glucose by aerobic and anaerobic R- and R+ cultures are different suggests some

differences in metabolic pathways under aerobic and anaerobic conditions. Furthermore, the higher glucose requirements for both R- and R+ strains under anaerobic conditions may explain the higher K^+ requirements (section 5.1.7.) which is intimately linked to energy turnover.

In some cases, organisms such as K. aerogenes (Tempest and Wouters, 1981) and Ps. aeruginosa (Dawes et al, 1976) possess dual systems for the uptake and metabolism of carbon substrate. A high affinity system is used in the presence of low concentrations of substrate and the low affinity of systems under conditions of carbon excess. Fig. 49 shows the biphasic relationship between the reciprocal of growth rate and the reciprocal of glucose concentration, suggesting a high and low affinity uptake system, depending upon the external glucose concentration. Similar results were obtained for Ps. aeruginosa (Noy, 1982).

5.1.3. Magnesium

Magnesium has many important roles in the bacterial cell, of which stabilization of the ribosomes, acting as a co-factor for a number of enzyme-catalysed reactions, including those involving ATP (Stanier et al, 1979) and those enzymes involved in the synthesis of cell wall components such as fatty acids (Knivett and Cullen, 1967), peptidoglycan (Garret, 1969), lipopolysaccharide (Edstrom and Health, 1967) and phospholipids (White et al, 1971; Hawker and Linton, 1979). There is much evidence that Mg^{2+} is a structural component of the outer membrane of gram-negative bacteria (Costerton et al, 1974) especially in P. aeruginosa (Brown and Melling, 1969a, b; Kenward et al, 1979; Boggis et al, 1979); its interaction with negatively-charged components of LPS and PL is important for cell wall integrity (Wilkinson and Galbraith, 1975). Mg^{2+} is also essential for the integrity of ribosomes and their activity (McCarthy, 1962) in synthesis of ribosomes and RNA (Cohn and Ennis, 1967) and in the control of membrane stability and permeability (Lederberg, 1956; Brock, 1962; Asbella and Eagon, 1966). Tempest et al (1965) found that RNA content, including the ribosomal RNA of Aerobacter aerogenes, was related to the degree of Mg^{2+} limitation. Morgan et al (1966) reported that in aerobically grown Mg^{2+}-depleted E. coli the amount of protein synthesized was proportional to the ribosome content. Under conditions of Mg^{2+} depletion the cell wall of P. aeruginosa is altered rendering the cells more resistant to polymyxin B and EDTA (Brown and Melling, 1969a and b; Boggis et al, 1979) and gentamycin (Nicas and Hancock, I980).

The growth curves of magnesium depleted E. coli R- and R+ differ markedly from those of glucose-depleted ones (Fig. 30 and 31). The growth rate of Mg^{2+}-depleted cultures gradually decreased after exponential growth. Since Mg^{2+} is a component of ribosomes and is involved in protein synthesis it is possible that the decrease in growth rate was due to a fall in ribosomal content and a corresponding decrease in protein synthesis. This hypothesis may be

supported by the findind that in a Mg^{2+}-limited culture of Aerobacter aerogenes, it was found that on increasing the growth rate, the ribosomal RNA as well as magnesium concentration in the cells were increased (Tempest et al, 1965). Also the RNA:Mg^{2+} ratio was found to be constant at several dilution rates. Similar findings were reported using P. putida (Sykes and Tempest, 1965) which give further support to the possibility that the ribosome and RNA content and hence rate of protein synthesis may be controlled by the amount of available magnesium. A linear relationship was shown to exist between OD_{470} onset of limitation and added Mg^{2+} to an OD_{470} ca. 0.8 (Fig. 32). This value is four-fold lower than that obtained by Ismail (1977) for the same strains grown aerobically, presumably because of accumulation of end products as suggested in section 5.1.1.

No significant different in Mg^{2+} requirements was found between R- and R+ strains when grown aerobically or anaerobically; however, both strains required nearly 50% more Mg^{2+} for a given OD_{470} under anaerobic growth conditions. It is possible that the additional Mg^{2+} requirements demanded by E.coli R- and R+ grown under anaerobic conditions may be due to additional protein synthesis, particularly as the NH_4^+ requirements had also increased, see Table 25. It is also possible that this difference in Mg^{2+} requirements may be due to metabolic and structural variations between aerobically and anaerobically grown R- and R+ strains. This hypothesis is supported by the differences in KDO and OMP profile found between aerobically and anaerobically grown cells (see sections 4.3.1. - 4.3.2.).

5.1.4. Phosphate

Phosphorus is normally provided for cells as inorganic phosphate although organic phosphate may be used. It is a component of high energy compounds such as adenosine triphosphate (ATP) in which the energy is stored in the form of phosphoanhydride bonds. It therefore plays an important role in biosynthesis as a component of ATP. Phosphorus is also a constituent of the LPS and PL of gram-negative bacteria. The phosphorus content of bacteria is about 1.5% of the dry weight. However, the content is dependent upon growth rate and temperature varying directly with the former and inversely with the latter (Pirt, 1975). This relationship is due to the stoicheiometry which exists between Mg^{2+}, K^{+}, PO_4^{3-} and RNA (Tempest, 1969). Phosphate-limitation of growth has been shown to result in alterations in bacterial membrane PL, the PL being functionally replaced by neutral lipids and fatty acids (Minnikin and Abdolrahimzadeh, 1974; Minnikin et al, 1974; Gilbert and Brown, 1978a; Noy, 1982), LPS, divalent cations and proteins (Noy, 1982). The synthesis and excretion of a number of phosphate-mobilising hydrolases was also derepressed under phosphate limitation (Tempest and Wouters, 1981).

The growth curves of phosphate-depleted E. coli R- and R+ were comparable in shape to those of magnesium (Fig. 33 and 34 respectively); as phosphate became lacking, there was a progressive decrease in growth rate. This decrease, as in the case of magnesium, may have resulted from reduction in the rate of protein synthesis due to diminished phosphorus concentration (Tempest et al, 1966).

No significant difference was found between the phosphate requirements of R- and R+ cells grown under aerobic or anaerobic conditions (Table 25). This observation may suggest that the components containing PO_4^{3-} are similar. Gilbert and Brown (1978a) found that E. coli R- and R+ strains had equal amounts of PL when grown aerobically under carbon, Mg^{2+} and PO_4^{3-}-depletion. But, PL content of both strains was markedly reduced under PO_4^{3-} depleted conditions.

Fig. 50 showed that only the R- strain grown under anaerobic conditions has a biphasic relation between reciprocal of growth rate and reciprocal of PO_4^{3-} concentration. A similar relationship was also observed in aerobically grown E. coli ML 30 (Shehata and Marr, 1971) and RP1-free E. coli W 3110 (Klemperer et al, 1979). These authors suggested that such relationship indicates the presence of a dual transport system for PO_4^{3-} in the growth medium. In the present study the R+ strain showed only a single phase uptake system. This finding is consisent with that reported by Klemperer et al, (1979).

5.1.5. Nitrogen

Nitrogen, provided as ammonium ions (NH_4^+), is an essential component of proteins, coenzymes and some PL such as phosphatidylethanolamine, purines, pyrimidines, as well as some growth factors (Hawker and Linton, 1979). A variety of inorganic (NH_4^+ salts) and organic (e.g. amino acids) nitrogen sources may be used. Cellular nitrogen forms up to 12% of the bacterial cell dry weight (Pirt, 1975). Under conditions of nitrogen-limitation, the flux of carbon substrate into the cells of E. coli and K. aerogenes may be less tightly controlled. Under this condition the carbon/nitrogen ratio of the media is high and polysaccharide synthesis is favoured. (Tempest and Wouters, 1981).

The exponential growth rate of R- and R+ strains was independent of the initial NH_4^+ concentration except at 0.56 mM when an exponential growth rate of 0.385 h^{-1} for R- (doubling time 1.8 h) and 0.169 h^{-1} for R+ (doubling time 4.1 h) was observed. The R- plasmid had no significant effect on nitrogen requirements when grown under aerobic (Klemperer et al, 1979) or anaerobic conditions (present work); however, the requirements for NH_4^+ were higher for R- and R+ strains when grown anaerobically than aerobically (Table 25). It may be that the additional NH_4^+ requirement for both strains under anaerobic growth conditions was due to extra protein synthesis. This increase in NH_4^+ requirements under anaerobic conditions is parallel to Mg^{2+} demand which is required for protein synthesis.

5.1.6. Sulphate

Sulphur is utilised as inorganic SO_4^{2-} but some organisms require organic S in the form of cysteine or methionine. It represents 0.3-0.4% of the cell dry weight (Pirt, 1975). Bacterial cells require SO_4^{2-} for the synthesis of protein which is initiated by the binding of N-formylmethionyl-t-RNA to the initiation site of mRNA (Gottschalk, 1979). Protein function is dependent on the correct amino acid sequences and its tertiary structure. Disulphide bonds, formed by the oxidation of two cysteine residues, play an important role in the maintenance of their structure. SO_4^{2-} is also required for the synthesis of sulphur-containing amino acids needed for certain functional and structural proteins and serves as ultimate electron acceptor in the anaerobic respiration of strict anaerobes (Stanier et al, 1979).

The iron-sulphur proteins in which iron is bound to cysteine residues of the peptide chain and to sulphide, are electron carriers in the electron transport chain. Coenzyme A contains a terminal sulphydryl group, which is the reaction site of the molecule in biochemical reactions. Coenzyme A is a key intermediate in the metabolism of pyruvate, for example conversion of pyruvate to lipids and oxidation via the tricarboxylic acid cycle.

S-adenosylmethionine (SAM) is a methyl donor in a wide variety of biochemical reactions. The conversion of unsaturated FA to cyclopropane FA (Zalkin et al, 1963) and PE to phosphatidylcholine (Gottschalk, 1979) both involve methylation by SAM. The synthesis of polyamines requires SAM as methyl donor (Hafner et al, 1979). Polyamines have been implicated in ribosomal stabilisation (Turnuck and Birch, 1973) and macromolecular synthesis (Abraham and Pihil, 1981). It has been suggested that under Mg^{2+}-depletion, polyamines may replace Mg^{2+} in its role as a cationic bridge in the OM (Wilkinson, 1975).

Sulphate-limitation results in reduction in non-haem iron proteins with which sulphide is bound in the electron transport chain (Light, 1972). Similar

findings were reported for sulphate depleted E. coli (Poole and Haddock, 1975).

The depletion of SO_4^{2-} in the medium resulted in the gradual reduction in growth rate of R- and R+ strains (Fig. 36 and 37 respectively). Anaerobically, E. coli R- and R+ cells required nearly two fold greater amounts of SO_4^{2-} to give the same population size as aerobically (Table 25). This may be attributed to increase in enzyme and protein synthesis. This suggestion is further supported by the elevated requirements for Mg^{2+} and NH_4^+ which are intimately involved in protein synthesis.

The R- strain under anaerobic growth conditions apparently needs approximately 60% more SO_4^{2-} than the R+ strain to reach the same optical density. This substantial difference in the SO_4^{2-} requirement between the two strains may be partly ascribed to the presence of RP1 plasmid, since there are some structural differences in the outer membrane proteins (see section 5.4.3.).

5.1.7. Potassium

Potassium is a major nutrient for micro-organisms and may be present in bacteria in amounts (g/g organisms) equal to, or even greater than that of phosphorus (Tempest, 1969). Moreover, K^+ is unique among the major nutrients in that it is contained within the cell in an unmodified and largely unbound state, and can be exceedingly mobile (Tempest and Wouters, 1981). It is one of the principle inorganic cations in the cell; it acts as a co-factor for some enzymes (Stanier et al, 1979). Protein synthesis depends on several principal factors of which ribosomal activity is one. K^+ is an essential ion for ribosomal activity; as a result, when K^+ content of bacterial cells is progressively lowered, protein synthesis ceases (Davis et al, 1973). Moreover, there is a correlation between the osmotic tolerance of bacteria and their K^+ content and the maintenance of a relatively constant ionic strength within the cell is of critical physiological importance, because the stability and behaviour of enzymes and other biological macromolecules are strongly dependent on this factor.

The concentration of K^+ present in the cytoplasm is frequently much in excess of that present in the growth medium, and hence under many conditions organisms will need to expend energy in order to concentrate the K^+ within the cell against a sizeable transmembrane K^+ gradient (Tempest and Wouters, 1981). This was demonstrated and quantified, by decreasing the extracellular K^+ concentration in a glucose-limited culture of K. aerogenes from 9 to 0.05mM, a progressive increase in the respiration rate of cells was observed along with a corresponding fall in the yield with respect to both glucose and oxygen (Hueting et al, 1979).

A significant difference in K^+ requirement was found between aerobically (Klemperer et al, 1979) and anaerobically grown cells of R- and R+ (see Table 25). The increase in K^+ demand under anaerobic growth conditions, particularly by the R+ strain may be possibly attributed to synthesis of excess proteins

and additional enzymes to satisfy the requirements of anaerobic metabolism and possibly to maintain a relatively constant ionic strength within the cell. Smith and Neidhardt (1983) using E. coli K12 W 3110 found that the cellular concentrations of 18 polypeptides (including four glycolytic enzymes) was increased by anaerobiosis. Their combined weight fraction during aerobic growth was 81.1, and it increased to 276.8 during anaerobiosis.

5.1.8. Trace elements

In addition to the macro-nutrients discussed previously, microbial growth requires the presence of a range of micro-nutrients or "trace elements". The most important among these are iron, manganese, zinc, cobalt, nickel, copper, aluminium and molybdenum.

The quantitative requirement of microbes for these trace elements generally is so small that enough may be present simply as contaminants of the bulk chemicals to satisfy the growth requirements of a fairly dense population (Tempest and Wouters, 1981). The present work showed no increase in the requirements of trace elements (except iron) for the growth of E. coli anaerobically and their concentrations in the CDM used was presumably sufficient to achieve the required growth.

Iron is essential to cell growth. It is required not only for heme proteins in aerobes but also for certain non-heme enzymes in anaerobes (Davis et al, 1973). Iron is also present in cytochromes and in a number of cofactors, which play an important role as electron carriers (Stanier et al, 1979). The role of iron in biological systems has been the subject of several reviews (Malmstrom, 1970; Coughlan, 1971; Neilands, 1974). Rainnie and Bragg, (1973) have demonstrated that iron deficiency in E. coli may result in an impairment of energy coupling and lower activity of the iron-containing enzymes of the respiratory chain.

As the role of iron associated with oxidative pathways is absent in fermentation processes (Stanier et al, 1979) and heme protein accounts for less than 10% of the total cellular iron (Iorio and Plocke, 1981), the higher requirements of iron for anaerobic growth of E. coli (see Table 25) may be due to increase in cellular demand for iron-proteins and/or iron-containing cofactors.

5.2. Significance of Nutritional Differences Between Aerobic and Anaerobic Environment

Nutritional requirements reflect the metabolism (Hernandez and Johnson, 1967; Boonstra et al, 1978; Tempest and Wouters, 1981; Smith and Neidhardt, 1983) and chemical composition of an organism (Neidhardt, 1963; Tempest and Dicks, 1967; Gilbert and Brown, 1978a and b; Kenward et al, 1979). From the data in Table 25 it is clear that the apparent nutrient requirements of E. coli K12 R- and R+ were greater anaerobically than under aerobic conditions except for PO_4^{3-}. It is unlikely that the higher requirement of most nutrients under anaerobic conditions could be attributed entirely to differences between aerobic and anaerobic metabolism, except for glucose, but, proteins and structural differences in the cell wall may also be involved (see sections 4.3.1. and 4.3.2.).

5.3. The Use of the Chemostat as a Model for the Gut

Some of the characteristics of the gut, such as the temperature, the pH of its contents and their anaerobic nature, can readily be simulated *in vitro*. However, it is difficult to reproduce the same chemical environment in terms of nutrient availability at a realistic dilution rate. Little is known of the rate at which the contents pass through the human gut although it is well reported for ruminants (Howard, 1967). Estimates of bacterial doubling times in the human gut vary from 4-6h (Gorbach, 1978) to 12-24h (Koch, 1971) suggesting dilution rates from 0.04 to 0.14 h^{-1}. The dilution rates calculated from the data of Davenport (1971) and Emonts *et al* (1979) yielded values from 0.01 to 0.32 h^{-1}. The range of dilution rates chosen for competition experiments in the present work was well within this limit at 0.1 - 0.08 h^{-1}, corresponding to doubling times of 6.93 - 8.66 hours. In competition experiments, it is important to set the chemostat at a realistic dilution rate as the inherent growth rate differences of organisms may affect the outcome of competition. Competition results obtained from batch and chemostat culture (Mason and Richardson, 1982) suggested that it is not always the organism with the potentially highest growth rate which secures the dominance *in vivo* (see also section 1.8.5. and Fig. 6(a) and (b)). This could be explained by the low substrate concentrations prevailing in the gut and corresponding low growth rate, the latter being achieved in the chemostat by the use of low dilution rates. Under such conditions selective pressures are advantageous to strains with high substrate affinity for growth-limiting nutrients (Jannasch, 1967; Harder *et al*; 1977).

5.3.1. The effect of dilution rate on growth parameters

In G-lim cultures an increase in dilution rate resulted in a marked reduction in the resident population (see Figures 68 and 69). These curves have characteristics different from those described by Tempest and Dicks (1967) for glycerol-limited A. aerogenes and Noy (1982) for glucose-limited P. aeruginosa both grown under aerobic conditions. These authors found that at low dilution rates the growth yield fell slightly but remained constant at higher dilution rates up to a critical dilution rate. Tempest and Dicks (1967) explained these differences by suggesting that at low dilution rates not all the carbon source added to the culture is utilised in cell structure since a proportion is lost as CO_2 due to oxidation of the carbon source to provide energy for growth and maintenance. The fact that in the present study the cells were grown anaerobically while Tempest and Dicks (1977) and Noy (1982) used aerobic growth conditions may explain the differences in the shape of these curves. In figures 68 and 69 the reduction in the population density was accompanied by an increase in the concentration of residual glucose, with consequent decreased growth. This increase in the concentration of residual glucose with increasing dilution rates may also account for the fall in the cell population.

The effect of glucose-limitation on the cell population is rather different from that of K^+-limitation. With K^+-limitation the optical density and viable count did not change significantly over the range of dilution rates studied (0.1 - 0.4 h^{-1}).

The effect of dilution rate was compared in cultures using 3mM glucose. The pH only dropped to 7.4, so it is unlikely that the accumulation of acidic end products was the cause of the population drop. With K^+-lim cultures in which the pH dropped to 6.9 and 6.6 (with R- and R+ strains), there was no change in population density when the dilution rate was increased from 0.1 to 0.4 h^{-1}. The greater fall in pH with K^+-lim cultures may be explained on the basis that during K^+-limitation when glucose is in excess it is less efficiently metabolised

consequently producing a large amount of acidic end products (Tempest and Wouters, 1981).

An interesting finding for all limitations tested (Table 30) was that, when the limiting substrate concentration in the reservoir was increased the K_s of the corresponding limiting-nutrient increased. Similar findings were reported by Contois (1959) for carbon- and nitrogen-limited cultures of A. aerogenes. A possible explanation for this, is that the concentration of the limiting nutrient is increased, the population density of the culture becomes larger (which is likely to produce environmental changes) and subsequently the amount of toxic end products increase which may inhibit growth processes.

5.3.2. Small colony variants

Under K^+-limitations colonies were isolated which had a completely different morphological appearance from that of R- or R+ wild type. These colonies were designated small colony variants (SCV- and SCV+ for R- and R+ strain respectively) (see section 4.4.2.). The fluctuation in percentage of SCV- or SCV+ was not dependent on dilution rate, but varied randomly (Fig. 70).

SCV could be variants already present in the initial inoculum or induced as a result of phenotypic variation or mutation of the wild type. Plating out of a large numbers of R- cells onto NA without kanamycin SO_4 and R+ cells with and without kanamycin SO_4 (15 μg ml^{-1}) would be expected to select for any SCV that may be present in the population. However when this was done, apparently all the colonies isolated were of wild type appearance. This finding indicates the possible absence of SCV in the stock cultures.

The other two possible mechanisms by which SCV may evolve from the chemostat populations are phenotypic variation and mutation. The behaviour of a bacterial cell is controlled by the characteristics of its genome, which harbours all the information needed to respond to changes in the physico-chemical properties of its environment. None of the microbial species expresses its entire genome under any set of environmental conditions. Cells with similar genotype respond in a similar way to environmental changes by expressing that part of the genome which fits a given set of conditions representing one of its phenotypes for example, phenotypic change occurs at the metabolic level (Konings and Veldkamp, 1980). Hancock (1981) found that P. aeruginosa grown under Mg^{2+}-limited conditions underwent phenotypic change (induction of Omp H1) such that the cells were no longer susceptible to lysis by EDTA, polymyxin and aminoglycosides. But when the cells were grown under Mg^{2+} sufficient conditions the production of Omp H1 was repressed and the cells became sensitive to these agents. In the present study the phenotypic change seems unlikely, since when

the selected small colony variants were transferred and repeatedly subcultured on NA for more than 3 weeks they did not show the morphological properties of the wild type which would be the characteristics of phenotypic response.

The most likely explanation for the appearance of SCV is mutation. Mutation, is an alteration in the nucleotide sequence in the DNA which occurs spontaneously within bacterial populations, and are normally inherited by their progeny. The occurrance of mutants under a given set of conditions has been reported. Lactose-limited chemostat cultures of E. coli undergo takeover by lactose constitutive cells within 100 hours and it has been suggested that this is due to mutation (Dykhuizen and Hartl, 1983). Langford (1984) found that SCV of P. aeruginosa selected from gentamycin containing nutrient broth in chemostat did not revert to the wild type when subcultured 80 times in gentamycin-free nutrient broth. The author concluded that this was not due to phenotypic variation but to spontaneous mutation. Similar situations would seem to apply to the small colony variants found in this study.

5.3.3. Stability of the RP1 plasmid in *E. coli*

During growth of the bacterial host the multiplication of plasmids is independent of that of the chromosome (Engberg and Norström, 1975). They replicate and segregate themselves among daughter cells. A stable plasmid has an inheritability grade of one in the terminology used by Anderson and Lustbader (1975). In this case every daughter bacterium inherits the semi-autonomous plasmid.

The stability of the RP1 plasmid in *E. coli* was examined by growing anaerobically the cells of the R+ strain in the chemostat at different dilution rates under G-lim, P-lim or K^+-lim conditions. The results showed that the plasmid was very stable under all the growth conditions used (Figs. 71 and 72). These results are in parallel to those found by Melling *et al* (1977) for the same strain grown aerobically under conditions of carbon, phosphate or magnesium limitation. A similar observation was also reported by Wouters and Andel (1979) who failed to select for a plasmid-minus population from a parent R6-containing strain of *E. coli* grown aerobically or anaerobically under a number of different growth limitations, including carbon, phosphate and nitrogen limitations. The inherent stability of these plasmids may be attributed to their synchronous replication in the host cell. Some other R-plasmids, on the contrary, are reported to be unstable during growth of the host in chemostat cultures for example, pBR 322 in *E. coli* PC 221 (Jones and Primrose, 1979; Wouters *et al*, 1980) and TP 120 in *E. coli* K12 (Godwin and Slater, 1979). These plasmids were lost during cultivation under various growth conditions.

5.3.4. Mixed culture studies of *E. coli* R- and R+ in the chemostat

The aim of this study was to examine the behaviour of *E. coli* with and without plasmid RP1 under different growth-limiting conditions in the absence of antibiotics. This situation is relevant to the spread of drug-resistant organisms widely, even in the absence of the selective pressure of antibiotics, which led to the evolution of resistant organisms. Such a study is necessary to evaluate the environmental conditions which may influence the maintenance of antibiotic-resistant organisms and plasmid stability. In antibiotic-free media, the plasmid may be at a disadvantage to the host cell and environmental pressures could then discriminate against plasmid-containing organisms. To elucidate this and the mechanisms responsible for competition, mixed culture studies in the chemostats had to be conducted under various environmental conditions.

5.3.5. Competition under glucose-limited conditions

When R- and R+ strains were competing for glucose in G-lim chemostat culture, R- cells consistently achieved dominance (Fig. 73, 74). Since an organism will be selected from a mixture in continuous culture if it is more efficient at utilising the limiting nutrient (Veldkamp and Jannasch, 1972; Harder et al, 1977), the gradual takeover of R+ strain by the R- (Fig. 73), with no differential growth rate advantage of R- over the R+ strain, suggests that the R- strain had a higher affinity for glucose than the R+ strain. This suggestion is further supported by the finding that R- strain had lower K_s values at all dilution rates and glucose concentrations tested (Table 30). It therefore appears likely that the R- cells were more efficient in the uptake or metabolism of glucose than the R+ cells.

The results obtained in this study differ from those obtained for the same strains grown aerobically by Melling et al (1977) in which the proportion of the R- cells gradually fell from the initial 1% to less than 0.01%. It may well be that anaerobic cultures are essentially energy limited (Tempest, 1978), where as aerobically they are carbon-limited (Neijssel, 1976 cited by Tempest, 1978).

5.3.6. Competition under phosphate-limited conditions

Although the apparent phosphate requirements (Table 25) as well as the maximum growth rate (Table 30) of R- and R+ strains were similar, the R- cells gradually displaced the R+ ones in their P-lim cultures (Fig. 75). These results are in accordance with those found aerobically by Melling et al (1977). It seems likely that the R- strain was much more efficient in the uptake or metabolism of phosphate than the R+ strain. This is supported by the observation that lower K_s values were found for the R- than R+ strain (Table 30).

5.3.7. Competition under potassium-limited conditions

The outcome of competition under K^+-lim conditions was somewhat unexpected, particularly as the apparent requirement of R+ batch cultures for K^+ was nearly four-fold higher than that of R-. The efficiency of either strains in scavenging K^+ a-peared to be dependent on the K^+ concentration in the inflowing medium (S_R). When S_R was 0.06 mM there was no takeover whereas at 0.03mM the initial 5% population of R- cells dropped rapidly until none were detected (Fig. 77). Interpretation of these results on the basis of differences in K_s value may explain these observations. In the first case the apparent K_s of R- strain was equal to that of R+, K_s values of both strains were higher than those in the second case (see section 5.3.1. for explanation), which suggests similarity in the efficiency in uptake or metabolism of K^+. In the second case the apparent K_s of R+ strain was less than that of R- (Table 30) which suggests that R+ cells were more efficient in uptake or metabolism of K^+. The emergence of SCV had no obvious effect on the outcome of competition; this conclusion is derived from the finding that the SCV proportions of both strains were nearly constant throughout. However, lack of information on whether or not SCV did exist in batch cultures may lead to uncertainty as to whether the determined apparent K^+ requirements in batch were for a pure culture or a mixture of WT and SCV. The probability of the latter is high for the R+ strain because of the unusually long lag found at low K^+ concentrations.

5.3.8. Persistence of R+ cells in the chemostat

Persistence of the R+ strain even at low levels under G-lim, P-lim and K^+-lim conditions (see Figure 74, 76 and 78 respectively) is of importance particularly in the light of the finding that low levels of R- plasmid containing bacteria occur in some natural environments (Hartley and Richmond, 1975) obviously in the absence of antibiotic selection. Helling et al (1981) reported that plasmid-containing bacteria can survive as a small minority even without direct selection, by attachment to a surface (such as the intestinal lining or the wall of a culture vessel) from which they are not easily released.

Although the work reported here is for anaerobic cultures, nevertheless the data obtained (Table 31) showed that the proportions of attached R+ cells were much greater than those in the liquid cultures. These varied with the nature of the limiting nutrient. Baldini et al (1983) also provided evidence for existence of relationship between plasmid presence and ability to adhere. One of the factors determining the bacterial adhesion is cell surface hydrophobicity (Weiss et al, 1982). It has been shown that E. coli carrying plasmids K88, K99, CFAI and CFII were more hydrophobic (using salting-out techniques) than bacteria without them (Lindahl et al, 1981). Furthermore, Ferreiros and Criado (1984) have reported correlation between the presence of thirty one different R- plasmids including RP4 (RP4 is probably identical with RP1; Hardy, 1981) in three different E. coli strains and variation in hydrophobicity. The variations in hydrophobicity were dependent on the receptor strains and measuring method employed. These observations may explain the variation and high proportion of attached R+ cells found in this study. The variation in the proportion of attached R+ cells with the nature of the limiting nutrient is possibly due to changes in the cell envelope surface characteristics (see section 4.3.1.). Several authors have reported changes in composition and structure and therefore surface associated properties of bacterial cell

envelope with different growth limiting nutrients (Tempest and Ellwood, 1969; Meers and Tempest, 1970; Ellwood and Tempest, 1972; Holme, 1972; Gilleland et al, 1974; Klemperer et al, 1980; Tempest and Wouters, 1981).

Bradley et al (1980) reported that plasmids which determine rigid pili transfer at least 2,000 times faster on solid surfaces than in liquid. As RP1 plasmid determines rigid pili and transfer at a higher rate on solid media than in liquid, it seems likely that the high proportion of R+ cells found on the chemostat wall may be attributed to the transfer of the plasmid from the R+ strain to its R- counterpart. Alternatively or additionally it may be that the difference between R- and R+ strains in the case of attachment to glass is due to the differences in their cell envelopes. Ferreiros and Criado (1984) showed that the inclusion of the RP4 plasmid (which is probably similar to RP1; Hardy, 1981) in different E. coli strains caused alterations in the bacterial surface structures. For example the difference in KDO contents of R- and R+ cells found in the present study (see section 4.3.1.) might explain the difference in the attachment of R- and R+ cells since one of the factors determining the attachment is LPS (Izhar et al, 1982). Furthermore, these strains grown aerobically differ in their sensitivities to disinfectants (Klemperer et al, 1980) and to high concentration of NaCl (see section 4.2.3.1.).

The incomplete disappearence of R+ cells from the mixed liquid culture and the fluctuation in its proportion were possibly due to slow intermittent release of some adherent R+ micro colonies (Costerton and Marrie, 1983).

5.4. Changes in Cell Wall Chemical Composition in Response to Nutrient Depletion and Growth Environment Variation

5.4.1. Introduction

The composition of the bacterial cell wall is not prescribed solely by its genetic content but by environmental factors that circumscribe the expression of the organisms genetic potential (Tempest and Elwood, 1969). The cell wall provides not only a limiting boundary but a surface at which physiologically important molecules must be concentrated and through which they must be translocated into the cytoplasm. Therefore any change in the nature of the environment that produces a constraint to the essential functioning of the cell wall may be expected to effect a modification to its structure so that its functional ability is restored.

5.4.2. Lipopolysaccharide

2-keto-3-deoxyoctonic acid (KDO), a glycosidic component of the LPS of many gram-negative bacteria including E. coli (Ellwood, 1970) has been used as a marker of LPS. KDO gives an indication of the amount of core polysaccharide present on the cell OM. Since other 2-keto-3-deoxy sugar acids (e.g. sialic acid) may react with thiobarbituric acid under the test conditions it is crucial that these acids are either absent from preparations or produce a colour complex with negligible absorbance otherwise falsely high KDO results may occur. For these reasons LPS estimations based on KDO measurements on whole cells were not recommended by Ellwood (1970) and were not used in the present study hence OM KDO contents only were measured.

Various factors such as nutrient limitation and growth rate may affect the KDO content of the cell (Gilbert and Brown, 1978a,b; Dean et al, 1976; Ellwood and Tempest, 1972). For example, phosphate-depleted E. coli K12 (Gilbert and Brown, 1978a,b) and P. aeruginosa (Dean et al, 1976) had lower KDO levels than carbon-depleted cells indicating a reduction in LPS content.

The marked elevated KDO content of E. coli R- and R+ strains under aerobic growth conditions is apparently due to the higher growth rate under aerobic conditions compared with the anaerobically grown cells (see section 4.3.1.). Similar observations have been reported by Tempest and Ellwood (1969). In the present study the KDO content markedly varied with the nutrient-depletion and conditions of growth (Fig. 65) but the significance of this observed difference in cell wall composition is not easily explicable.

The amount of KDO found in the aerobically grown R- strain under all nutrient depletions (except potassium) was higher than the R+ strain. The differences in KDO content and protein profile (to be discussed in the following section) between R- and R+ cell walls suggest that RP1 has coded for a significant change in the OM. This view is supported by the difference

observed in NaCl sensitivity (section 4.2.3.1.) between R- and R+ cells. Kenward et al (1978) reported higher KDO in the cell wall of wild type P. aeruginosa than in RP1 containing strain. This difference in KDO levels together with the variation in PL, diamino pimelic acid and cation content between R- and R+ cell was associated with differences in susceptibility to the lytic action of EDTA and polymyxin as well as the apparent impermeability of R+ cells to tetracycline.

5.4.3. Outer membrane protein profile

SDS-PAGE revealed that both R- and R+ strains of E. coli contain those major outer membrane proteins usually found in other strains of E. coli (Lugtenberg, 1981; Lugtenberg and van Alphen, 1983) in similar relative concentrations. The major outer membrane proteins with apparent molecular weight between 30,000 and 40,000 were present in all growth conditions (Figs. 66, 67) and there appeared to be no significant variation in the amount of the Omp F and Omp C (which were not completely separated). Thus, the two porins Omp F and Omp C are both present, the former being the dominant outer membrane protein. The concentration of Omp F in the outer membrane is greater than that of Omp C particularly when it is grown in minimal media such as the CDM used in the present study. Lugtenberg et al (1976) have reported similar findings, van Alphen and Lugtenberg (1977) suggested that this may be an effect caused by osmolarity. However, in view of the greater efficiency of Omp F as a pore (Nikaido et al, 1980) it is not surprising to find greater concentrations of Omp F than Omp C in the OM of the organism grown in nutritionally poor CDM environment.

Omp A is believe to be pore forming protein (Hantke, 1976; Manning et al, 1977). However, on the basis of the data on porin-deficient mutants (Chen et al, 1980) Omp A protein itself seems unlikely to form pores (Nikaido and Vaara, 1985). This protein was also present in the OM of both R- and R+ E. coli cells under all conditions. These proteins can probably be classified as the major proteins in E. coli which can be identified and compared with proteins found in other strains of E. coli. It is not surprising that these proteins are present in such high concentrations as their functions, both structurally and in terms of permeability of the OM are very important.

The main changes in OM protein pattern under different growth conditions were seen amongst the minor proteins (Fig. 66, 67). Amongst these is the

total absence of a protein with apparent M_r 34,000 under K^+ and Fe^{2+} depletions, possibly this protein requires these elements for expression and is not manufactured when K^+ and Fe^{2+} are in short supply. R+ strain in full CDM and under phosphate-depletion produced an extra protein with apparent M_r 18,500. This might be the result of the presence of RP1 plasmid since the growth conditions were exactly the same for both R- and R+ strains. This may also explain the absence of two proteins of apparent M_r 20,000 and 22,000 from R- strain under potassium depletion. The iron-binding proteins (M_r 60,000-90,000) were present in both R- and R+ strains under glucose, phosphate or magnesium-depletions, however, the relative concentration of these proteins was greater in iron-depleted cultures. The production of high concentration of iron-binding proteins by the organism would compensate for the low level of available iron.

5.5. Effect of Environment on the Survival of *E. coli* R- and R+ Cells Grown Under Aerobic Conditions

5.5.1. Effect of storage temperature

Initially, the drop in viability which occurred in all suspensions was most marked in cells stored at 37^{o} (Figures 52 and 53), the count dropping rapidly for 8 days after which the rate of death of magnesium-depleted cells decreased, particularly R- ones (Fig. 52). Glucose-depleted cells did not change significantly (Fig. 53). Magnesium-depleted cells showed a small increase in count on further storage at 4^{o} and 25^{o}, possibly due to cryptic growth. This phenomenon, so called by Ryan (1959), occurs because dying bacteria leak all sorts of metabolizable small molecules which cannot only protect neighbours from stress, but actually permit their multiplication. The results of these investigations on the effect of storage temperature on the survival rate of *E. coli* are in agreement with the findings of Ryan and Kiritani (1959). They found that the viability of *E. coli* during the stationary phase was decreased by increasing the incubation temperature (0^{o}, 15^{o}, 20^{o}, 25^{o}, 30^{o} and 37^{o}). The survival experiments on fourteen bacterial strains including *E. coli* (Perry and Weinberg, 1973) showed that *E. coli* had higher survival rate at 25^{o} than at 37^{o}, this is also in accord with the findings of the present study.

The effect of temperature on the survival of *E. coli* may be explained on the basis of differences in metabolic rate. Indeed, evidence from several sources suggests that at higher temperatures the rapid metabolism of the endogenous substrates produces energy at a rate greatly in excess of that needed for maintenance, hence, accelerating the death of starved bacteria (Thomas and Batt, 1969a). Alternatively (or even an additional factor), with increasing temperature the fluidity of the cell wall increases and therefore could lead to increased leakage of low molecular weight nutrients from the cell leading to accelerated death. Russell and Harries (1968) found that the

amount of pentoses released from E. coli increases with temperature. For example, the leakage of pentoses from the cells at 37^{o} was 50% more than at 22^{o} or 7^{o}. On the other hand magnesium-depleted cultures (Fig. 52) also showed differences in survival rate between R- and R+ strains at 37^{o}. R+ cells apparently died faster relative to R- cells, an observation which is parallel to that reported by Klemperer et al (1979). This was entirely attributed to the presence of the RP1 plasmid in the host strain. Since aerobically grown R+ cells have greater requirement for Mg^{2+} than R- cells (Klemperer et al, 1979) therefore it is likely that the lower survival rate of R+ cells was due to its higher Mg^{2+} demand, particularly as starvation of bacteria in PO_4^{3-} and saline in the absence of Mg^{2+} leads to a more rapid loss of viability and greater degradation of RNA than comparable organisms starved in its presence as found in K. aerogenes (Tempest and Strange, 1966), Streptococcus lactis (Thomass and Batt, 1968, 1969b) and Zynonomus anaerobia (Daws and Large, 1970). This hypothesis is capable of being tested if the same experiment is repeated in the presence of excess Mg^{2+}.

It is of interest that in vivo Anderson (1974) found that R+ cells of E. coli disappeared from the faeces more rapidly than R- cells. He also suggested that the relative decline in the R- factor populations resulted in impaired vitality of the organisms bearing these plasmids rather than their loss from the cells.

5.5.2. Effect of suspending medium

Figure 54 shows that glucose-starved E. coli R- and R+ cells kept in their original culture medium had a smaller decrease in cell count than those resuspended in MOPS solution (50 mM). These results are in agreement with those of Farwell (1970) who also found that P. aeruginosa cells stored in their culture medium had a lower death rate than when resuspended in saline or water. This can be due to three possible reasons: (a) The culture supernatant still contains excess essential nutrients, except glucose, which could support the living cells. The energy needed to maintain the surviving cells is most likely to be derived from the carbon content of dying cells (Ryan, 1959). (b) MOPS resuspended cells are subjected to a minor osmotic shock during resuspension. Anderson et al (1979) detected sublethal stress in E. coli when this organism was resuspended in various test media. (c) Changing of suspending medium from the culture supernatant to MOPS involved changes in cells surface ions. Gossling (1958) reported that a change of surface ions by changing the medium from phosphate buffer to Ringer led to a high kill of E. coli cells.

5.5.3. Effect of drying

During the process of drying bacteria the hydrogen bound water is removed from the macromolecules of the cell, consequently their configuration is changed e.g. the removal of bound water from DNA results in the formation of thymine dimers and the DNA is no longer capable of proper functioning (Dimmick, 1973). Similarly the biological activities of proteins may be lost and the cytoplasmic membranes are damaged (Strange and Cox, 1976). A further consequences of water loss is that substances may crystallize (Silver, 1965) or achieve toxic concentrations (Bateman et al, 1961; Monk et al, 1957). It has been shown that when bacteria are exposed to drying with concommitant loss of viability, there occurs a damage in the cytoplasmic membrane leading to leakage of cytoplasmic material. For example, Serratia marcescens which had undergone viability losses by drying, released large amounts of ultra violet light-absorbing material (Wagman, 1960). He suggested that this was due to an increase in the cells permeability following membrane damage which was considered by the author as a primary effect of cell drying.

It is obvious from Fig. 58 that during the initial drying process, E. coli R- and R+ died at higher rate than in the period cells appeared to be dried. Similar observation was reported by Fry and Greaves (1951) who found that the highest death-rate of paracolon bacillus (D.201 H) had occured during the early stages of drying. On the other hand, the survival of E. coli also appeared to be affected by the presence of RP1 plasmid. The R+ cells had a lower survival than the R- ones. Overall this result is similar to those of Klemperer et al, (1979), using the same strains stored on NA, Anderson (1973), using the R- plasmid R1 in E. coli stored in saline or water and Calcott et al (1979) using pPL1 plasmid in P. aeruginosa PA01 plated (after exposure to freezing stress) on NaCl or sodium lauryl sulphate supplemented agar medium.

5.5.4. Growth of stationary-phase cells of *E. coli* R- and R+ on nutrient agar containing added NaCl and/or Tween 80

Plasmolysing agents such as NaCl primarily affect the cytoplasm of gram-negative bacteria, causing it to reduce in volume and shrink, drawing away from the outer membrane. The cytoplasmic membrane remains connected with the cytoplasm, and its structure is probably little affected by the stress of plasmolysis. However, the outer membrane is undoubtedly stressed, mainly because it loses much of the structural support supplied by the cytoplasmic membrane. Moreover, plasmolysing agents can be destructive of the integrity of the outer membrane (Bayer, 1968; Decad and Nikaido, 1976). Caulcott (1982) showed that bacitracin and rifampicin, relatively non-destructive agents (because of their M_r being greater than the determined exclusion limit of *E. coli*) to mid-log phase cells of *E. coli* and *P. aeruginosa*, became destructive when these cells were plasmolysed. The author concluded that the consistant reduction in viability of the plasmolysed cells found upon increasing the antibiotics concentration is an indicator of the damage incurred by the outer membrane during plasmolysis.

The presence of RP1 in various gram-negative bacteria has been reported to cause alterations in their cell envelope composition (Shipley and Olsen, 1973; Kenward *et al*, 1978) and variation in the sensitivity to antibiotics (Shipley and Olsen, 1974; Richmond and Curtis, 1975), bacteriophage (Shipley and Olsen, 1973) and disinfectants (Gilbert and Brown,1978a; Ismail *et al*, 1977; Klemperer *et al*, 1980) was also attributed in part to envelope changes mediated by the plasmid.

In the present study the effect of RP1 on the sensitivity of *E. coli* towards NaCl, Tween 80 and DOC was investigated. Fig. 59 showed the effect of different concentrations of NaCl on R- and R+ cells. Glucose-depletion resulted in greater resistance to NaCl above 2.5% than potassium or oxygen-

depletion. Since the nature of a growth-limiting nutrient significantly influences the structure and composition of the cell envelope of gram-negative bacteria (Brown and Melling, 1069; Tempest and Ellwood, 1969; Holme, 1972; Gilleland et al, 1974). Therefore it is possible that the difference in susceptibility to NaCl found between glucose-, potassium- or oxygen-depleted cells may be due to the effect of these depletions on the structure and composition of the cell envelope. On the other hand, variation in sensitivity to NaCl found between R- and R+ strains may also be due to differences in their cell wall composition. The chemical composition of whole cells and cell walls of RP1-carrying P. aeruginosa has been found to be different from that of RP1-free strain in the content of cation, PL, and markers for LPS and peptidoglycan (Kenward et al, 1978). Furthermore, the morphological (Fig. 60) and chemical (Fig. 65) differences found between R- and R+ strains support this hypothesis.

Tween 80 within the range 1-4% was found to have no significant effect on viability of both R- and R+ cells (Fig. 61). Similar observations have been reported by Brown and Winsley (1969, 1971) using P. aeruginosa. They found Tween 80 alone possessed little intrinsic activity against the latter organism. The results obtained upon addition of 1.5% NaCl to the same range of Tween 80 did not differ from those using Tween 80 alone (Fig. 62). Sodium chloride at a concentration of 1.5% had no effect upon viability of either R- or R+ cells when used alone. This concentration was therefore chose to potentiate the action of Tween 80 and possibly reveal any differences in sensitivity to the non-ionic detergent. However the only cells which showed any sensitivity to the combined action of 1.5% NaCl and Tween 80 were potassium-depleted R- cells.

5.5.5. Effect of sodium deoxycholate on viability

DOC, an anionic surface active agent significantly reduced the viability of glucose-depleted cultures of E. coli R- and R+ and the anaerobic chemostat grown cultures were more sensitive than the aerobic batch cultures (Fig. 63). The decrease in the viability of these organisms in the presence of DOC maybe due to its effect on the cell membrane permeability. Humphrey and Cruickshank (1985) found that DOC reduced the viability of Campylobacter jejuni and suggested that DOC affected the cell membrane permeability. Thus the decrease viability may be the consequence of the loss of vital cell constituents including purine and pyrimidine compounds which reflects degradation of cellular RNA (Postgate, 1967).

The anaerobic chemostat grown cultures were more sensitive to DOC than the aerobic stationary-phase cultures (Fig. 63). Fast growing bacteria are usually more sensitive to sublethal and lethal injuries than slow growing ones (Gilbert and Brown, 1978b; Taylor, 1984; Brown and Williams, 1985. Nevertheless, it is difficult to compare two such different growth systems. The markedly different colonial morphology of the chemostat cells (Fig. 64) from those of batch further corroborate this view. DOC causes major changes in lipid composition of the outer membrane of gram-negative organisms, Tomlins et al (1982)found that DOC diminished the viability of S. typhimurium and this was accompanied by decrease saturated/unsaturated membrane lipid ratio. Phosphatidylethanolamines were decreased by 28% whilst phosphatidyl glycerol were increased by 87.5%, DOC also altered the relative distribution of neutral lipids. The colonial morphology of chemostat grown R+ strain was different from the R- in that the former exhibited concentric rings (Fig. 64), this difference is due to the presence of RP1 plasmid. Rosas et al (1983) observed that the removal of plasmid P424 from E. coli RC 424 was associated with drastic changes in the morphology of the colonies. Analysis of the cured strain by scanning and transmission electron microscopy showed important alterations in the size and morphology of the cells.

6. CONCLUDING DISCUSSION

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It would be expected that a variety of different factors may be involved in the survival of R-plasmid containing bacteria, in the absence of antibiotic pressure. Some of the results reported here indicate possible mechanisms.

R+ bacteria disappeared in anaerobic competition experiments, as predicted, but did not disappear completely (see sections 4.4.4, 4.4.5, 4.4.6). The preferential adhesion to glass of R+ bacteria (see section 4.4.8) suggests an important mechanism of survival , assuming that some of the same factors are involved in adherence.

Adherence may involve LPS. It was noted (see section 4.3.1) that iron-limited R+ cells had more KDO, and therefore presumably more LPS, than R-cells. Adherence is also affected by surface charge, electrostatic forces, hydrophobicity, and, in the gut, by the presence of specific receptors (Kallenius *et al*, 1980; Marrie *et al*, 1980; Vosbeck and Mett, 1983). However, no consistent differences in major outer membrane proteins were observed (see section 4.3.2).

Deoxycholate is an antibacterial compound in the gut. However, plasmid RPI did not affect sensitivity to DOC (see section 4.2.4). The osmotic pressure of the intestinal contents must be very high and it was therefore of interest that potassium depleted R+ cells were more resistant to high concentrations of sodium chloride than R- ones, although the reverse was found for oxygen depleted cells (see section 4.2.3.1).

Persistence of plasmid-containing bacteria outside the animal host may also be of medical importance. However, R+ cells actually died faster during drying than R- cells, (see section 4.2.2), and there was no difference in the survival of R+ and R- cells in liquid media (see section 4.2.1).

7. REFERENCES

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