THE EFFECT OF NUTRIENT LIMITATION AND R-PLASMIDS ON THE PROPERTIES OF *ESCHERICHIA COLI*

A THESIS PRESENTED BY

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TO THE TWO MEN IN MY LIFE

MY FATHER AND MY HUSBAND

THE EFFECT OF NUTRIENT LIMITATION AND R-PLASMIDS ON THE PROPERTIES OF *Escherichia coli*

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When simple salts media were formulated for the growth of *Escherichia coli* K12 W3110, with (R+) and without (R-) the R-plasmid RP1, it was found that the growth yield of *E. coli* R+ was lower than that of R- for magnesium, potassium and phosphate, although the glucose and nitrogen requirements were the same. In addition, *E. coli* R+ required added ferrous ion at high cell concentrations. RP1 was stable in such media and also during storage on simple salts agar, although it was relatively unstable when stored on nutrient agar.

RP1 lowered the minimum inhibitory concentrations in nutrient broth of cetrimide and chlorhexidine, but did not affect that of phenol. In simple salts media, the sensitivity to disinfectants varied with nutrient-depletion. *E. coli* R+ was more sensitive to chlorhexidine than R-, but magnesium (-Mg) or phosphate (-P)-depleted cells were more resistant than carbon(-C)-depleted ones. *E. coli* R+ (-C) was also more sensitive to cetrimide than R- (-C), but whereas (-P) increased the sensitivity of both cultures, (-Mg) increased the sensitivity of R- and decreased that of R+. R+ and R- (-C) had the same sensitivity to phenols; (-P) or (-Mg) had the same effect as on cetrimide sensitivity.

Although nutrient-depletion and RP1 caused changes in surface charge, these could not be correlated with changes in resistance.

Key Words: *E. coli* R-plasmid media nutrient-depletion disinfectants

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ABBREVIATIONS

E. coli	Escherichia coli
R^+	E. coli carrying the R-plasmid RP1
R^-	E. coli without the R-plasmid RP1
-C	glucose-depleted
-Mg	magnesium-depleted
-P	phosphate-depleted
CM	cytoplasmic membrane
OM	outer membrane
MLP	murein-lipoprotein
PS	polysaccharide
P	peptide bond
PL	phospholipid
LPS	lipopolysaccharide
KDO	2-keto-3-deoxyoctanoic acid
NA	nutrient agar
ON	over night
CDM	chemically defined media
NB	nutrient broth
OD	optical density
DM	Davis Mingioli medium
MIC	minimum inhibitory concentration
M	molar solution
MOPS	3-(N-Morpholino)propanesulphonic acid
conc	concentration
rpm	revolution per minute

Statistical calculations based on Bailey, N.T.J., Statistical Methods In Biology. (1969). The English Universities Press Ltd., London.

ORIGIN AND SCOPE

Much work on the Gram-negative organism, Pseudomonas aeruginosa has been done in this laboratory concerning the effect of nutrient concentration on its growth (Boggis, 1971). Boggis (1971) and Kenward (1975) provided evidence for the changes in the outer membrane of P. aeruginosa associated with changes in the growth environment which are reflected in its sensitivity to antimicrobial agents.

It was of interest to compare the nutrient requirements and devise a simple salts medium for another Gram-negative organism Escherichia coli K12 W3110 and for the same species carrying the R-plasmid, RP1.

The effect of the R-plasmid on the envelope of E. coli was studied indirectly by investigating the effect of nutrient depletion of R^+ and R^- cultures on their sensitivity to three membrane-active disinfectants; chlorhexidine, cetrimide and phenol, as resistance to these is thought to depend on the properties of the cell wall.

1. INTRODUCTION

1.1. CELL ENVELOPE OF Escherichia coli

1.1.1. INTRODUCTION

In this section the cell envelope of Escherichia coli will be dealt with in detail; other Gram-negative bacteria will be mentioned to help elucidate the structure of E. coli.

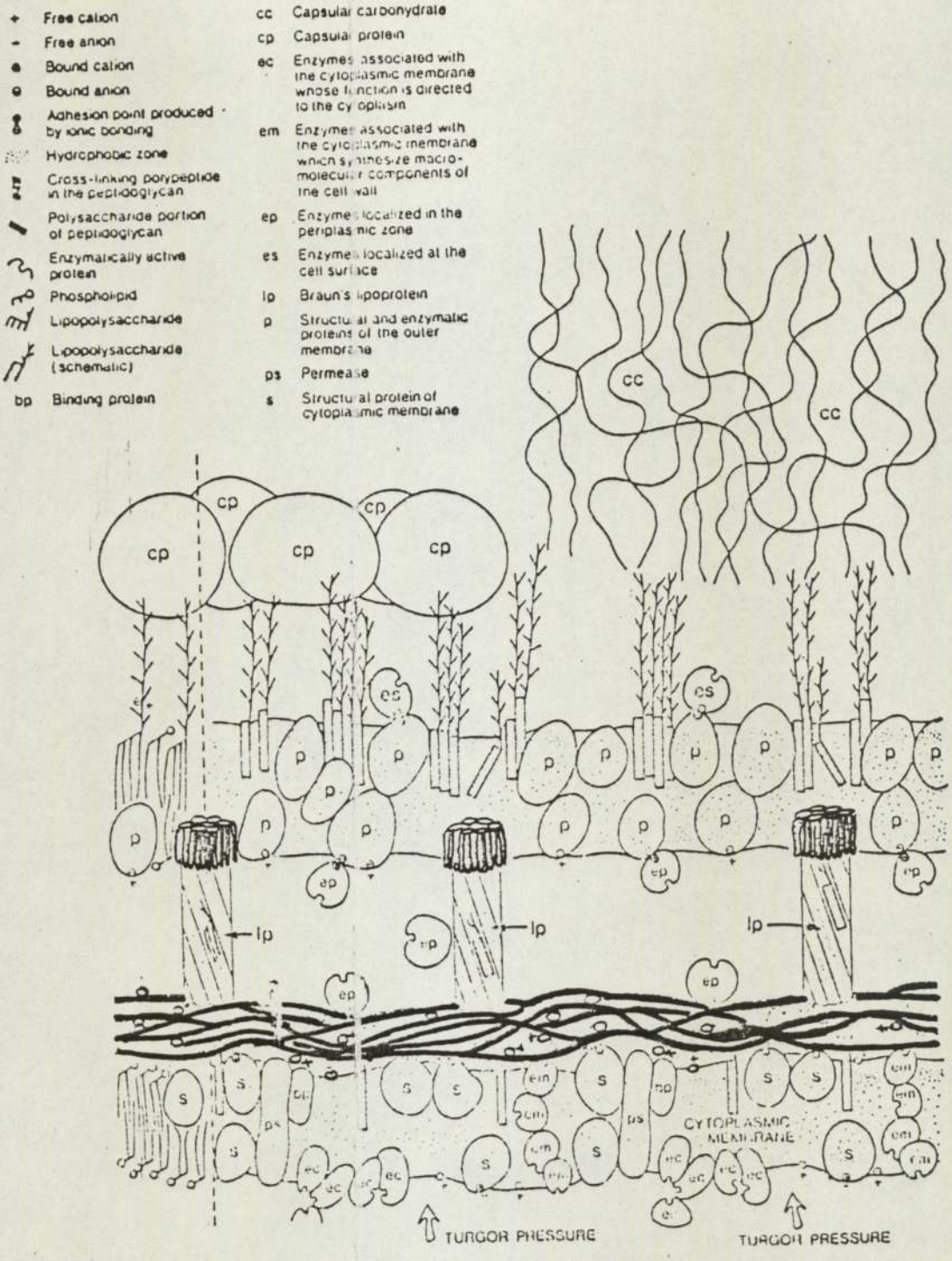
The structure external to the cytoplasm is the cell envelope. It consists of the cytoplasmic membrane (CM), murein-lipoprotein complex, the periplasmic space, the outer membrane (OM) and structures external to the OM such as carbohydrate and slime layers. Fig 1 shows an idealized Gram-negative envelope proposed by Costerton & Cheng (1975).

Many excellent reviews on the Gram-negative cell envelope exist, detailing both the chemistry (Salton, 1964; Martin, 1966; Roger & Perkin, 1968) and the structure and function of the Gram-negative cell envelope (Martin, 1969; Glauert & Thornley, 1969; Reavely & Burge, 1972; Braun, 1973; Leive, 1974; Costerton, Ingram & Cheng, 1974). In addition, Costerton & Cheng (1975) reviewed the cell envelope of Gram-negative and Gram-positive bacteria, concentrating on their role in antibiotic resistance.

1.1.2. THE CYTOPLASMIC MEMBRANE

The CM is located between the cell wall and the cytoplasm. Singer (1972, 1974) gave a useful description of its structure in his liquid crystal model (Figs 2a and 2b). In this model the phospholipids form a liquid hydrophobic layer. The dominant phospholipid in marine Pseudomonad ATCC 198 55 was found to be phosphatidylethanolamine with less diphosphatidylglycerol (Martin & Macleod, 1971).

Fig 1: The Gram-negative cell envelope (Costerton & Cheng, 1975).



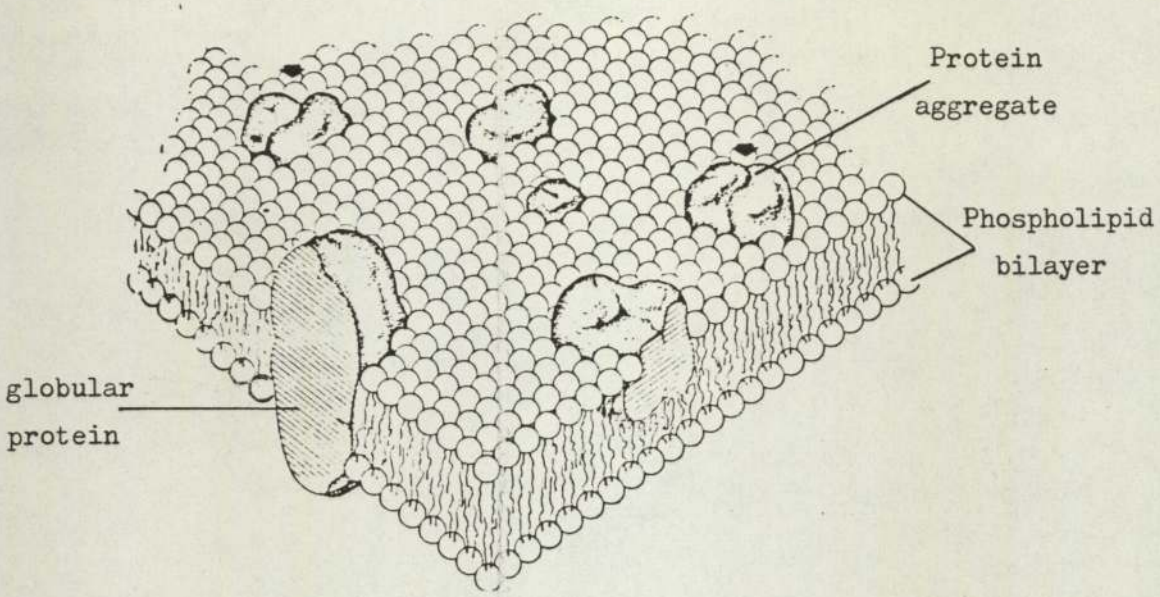


Fig 2a: A schematic representation of the three-dimensional organization of the cytoplasmic membrane (Singer, 1972).

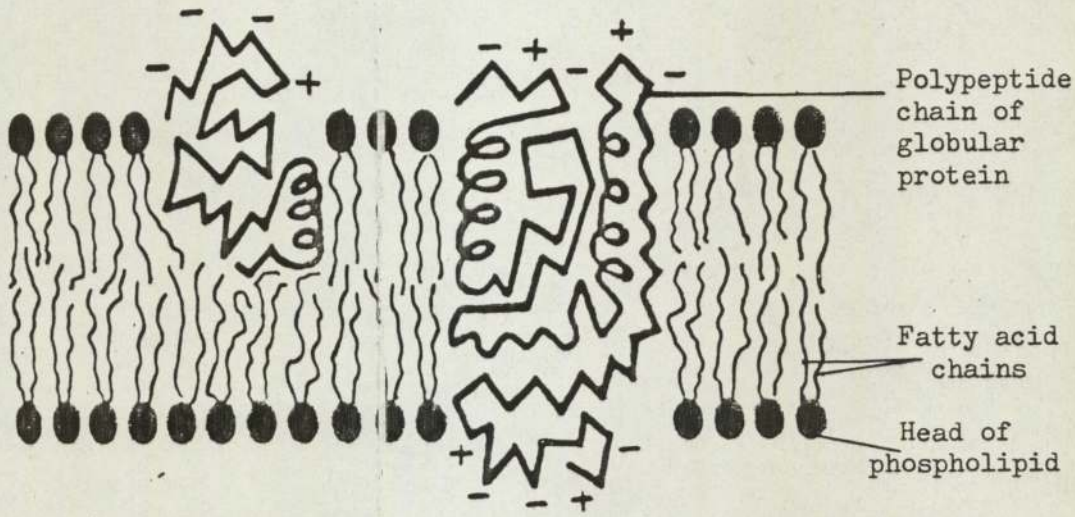


Fig 2b: A diagramatic representation of a cross section of the cytoplasmic membrane (Singer, 1972).

The globular proteins are partially embedded in the phospholipid matrix and partially extend from it (Fig 2b). These proteins have amphipathic structure i.e. their ionic and highly polar groups are largely located on the membrane surface in contact with the aqueous medium, while their non-polar residuesare in the membrane interior (Singer, 1972). Some of these proteins are the permeases which are concerned with the active transport of molecules through the phospholipid layer (Kaback & Hong, 1971). Schnaitman (1970), using E. coli J-5, concluded that the cytoplasmic membrane contains about one-third of the envelope protein and one-half of the phospholipid.

Due to the elastic nature of the cytoplasmic membrane, osmotic forces cause it to form vesicles against the peptidoglycan layer (Costerton & Thompson, 1972). However, in plasmolysed E. coli, the cell wall and cytoplasmic membrane remain in close contact at various points due to turgor pressure of the cytoplasm which is about $3 - 5\ kg\ cm^{-2}$ (Bayer, 1968).

A close relationship exists between the cytoplasmic membrane and cell wall since enzymes located within the cytoplasmic membrane are responsible for polymerizing phospholipid (Bell, Mavis, Osborn & Vagelos, 1971), lipopolysaccharide (Osborn, Gander & Parisi, 1972) and mucopeptide. The mucopeptide strands remain attached to the enzyme sites as they are integrated into the wall (Rogers, 1970).

1.1.3. THE MUREIN-LIPOPROTEIN COMPLEX

The murein-lipoprotein complex is the innermost layer of the cell wall whose function is to maintain cell rigidity. Forsberg, Costerton & Macleod (1970) found that only cells bound by peptidoglycan maintain their shape because treatment with lysozyme caused the loss

of muramic acid and diaminopimelic acid, and the cells were thus converted to spheres. Isolated "murein sacculi" retain the shape of the cells from which they were derived (Forsberg, Costerton & Macleod, 1972). Burman, Nordstrom & Bloom (1972) and Tseng & Bryan (1974) concluded that in addition to the structural function it may possibly act as a barrier to the passage of certain drugs. E. coli K12 and other Gram-negatives showed increased sensitivity to cholate after treatment with penicillins, due to the effect of penicillin which causes a reduction in the degree of cross-linking of the murein. This is due to the sensitivity of the enzyme, which provides end groups for the synthesis of new polysaccharide chains. This makes the murein more flexible and alters the arrangement of the lipoprotein. Thus the barrier function was altered.

The chemical composition of the murein layer is alternating N-acetyl glucosamine and N-acetyl muramic acid, about 12 - 13 A^{o} apart and linked together by 1,4-glycosidic bonds. The polysaccharide chains are cross-linked by peptide side chains. Fig 3 shows the Braun (1973) model of the murein net and the chemical composition of one lipoprotein molecule which is a major protein of E. coli, localized in the periplasmic space. One end of it is covalently linked to the murein net (Braun & Hantke, 1975).

The lipoprotein molecules are 100 - 120 A^{o} apart from each other along the polysaccharide chain (Fig 4) (Braun, 1973).

Braun, Rehn & Wolff (1970) found that lysine, through the ϵ-amino group of its C-terminal, links the lipoprotein molecule to the carboxyl group of meso-diaminopimelic acid of murein in E. coli, and that about 250,000 lipoprotein molecules are distributed over a one-layered murein net (Braun, 1973). E. coli is less sensitive to EDTA than P. aeruginosa since lipoprotein comprises a major constituent in

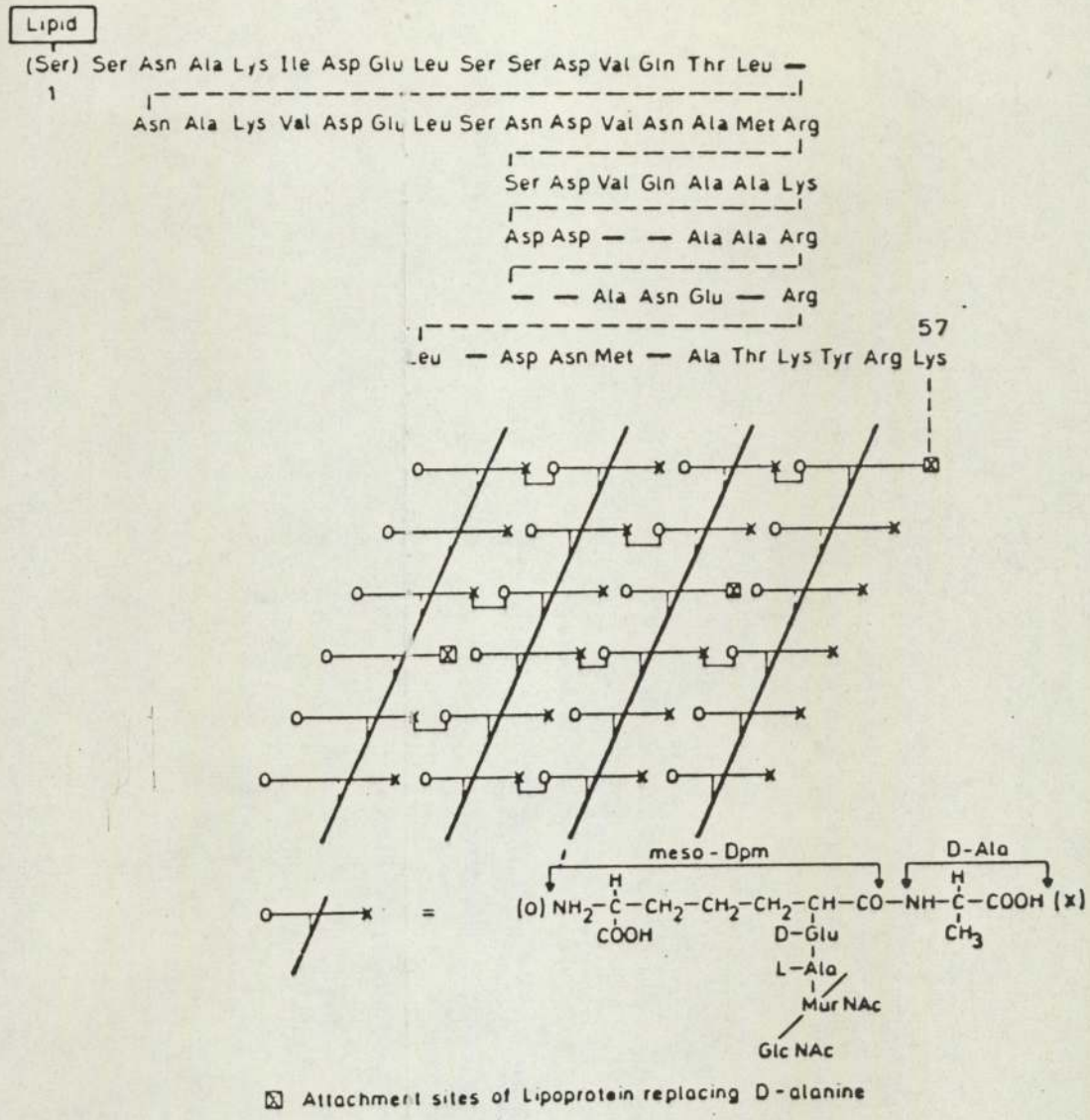


Fig 3: The lipoprotein-murein complex of E. coli (Braun, 1973).

The parallel heavy lines symbolize the polysaccharide chains. They are cross-linked by the T-like peptide side chains which are drawn to allow a long-range covalent fixation of the murein, which is a necessity for E. coli and other Gram-negative bacteria. Since E. coli is cross-linked only to the variable extent of 15 - 30% some links between meso-Dpm (O) and D-Ala (X) were left open. But despite the lack of some cross-linking peptide bonds, the conformation of the peptide side chain is considered to be the same for all. On average, one lipoprotein is covalently linked to every 10 - 12th disaccharide unit of the murein. three attachment sites are indicated in the murein net. The sequence of only one lipoprotein molecule is drawn and presented in a way that emphasizes its repetitive design.

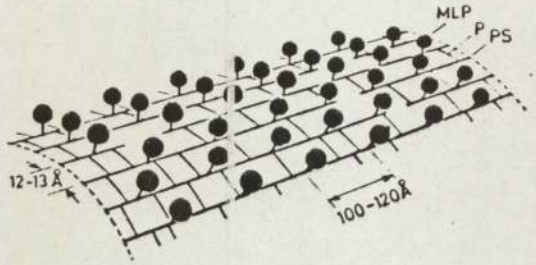


Fig 4: Tentative distribution of the lipoprotein molecules over the murein net (Braun, 1973).

It is proposed that the spacing of the lipoprotein molecules along the polysaccharide chains is 100 - 120 A° and that the polysaccharide chains are 12 - 13 A° apart. The direction in which the polysaccharide chains run in this section of the rigid layer is not known. They could run parallel to the E. coli rod as drawn here but also in any other direction.

MLP - murein-lipoprotein
P - peptide bond
PS - polysaccharide

the rigid layer of the former, while it comprises a minor constituent in the latter (Martin, Heilmann & Preusser, 1972).

1.1.4. THE PERIPLASMIC SPACE

The definition of the periplasmic space is still under discussion. It was orginally described by Mitchell (1961) as an enzyme-containing compartment bounded on the outside by a "molecular sieve" and on the inside by the cytoplasmic membrane. The molecular sieve is equivalent to the outer membrane of Gram-negative bacteria (Forge & Costerton, 1973). If so, the periplasmic space comprises the area within the murein network layer and the open spaces between lipoproteins.

Several enzymes, such as guanosine-5-triphosphatase, are found in this space (Brockman & Heppel, 1968). In addition, 5-nucleotidase, 3-nucleotidase, acid phosphatase, alkaline phosphatase and ribonuclease 1 have been shown to be located in the periplasmic space of E. coli (Cerny & Teuber, 1971) and several other Gram-negative bacteria such as P. aeruginosa (Cheng, Ingram & Costerton, 1970, 1971; Cerny & Teuber, 1972).

The periplasmic space, which is unique to Gram-negative organisms, may also contain antibiotic-degrading enzymes such as the penicillin-degrading enzyme β-lactamase (Richmond, 1975; Brown, 1975).

1.1.5. THE OUTER MEMBRANE

The outer membrane (OM) acts as a distinguishing factor of the cell wall of Gram-negative organisms since no structure comparable to it has been found in Gram-positive bacteria.

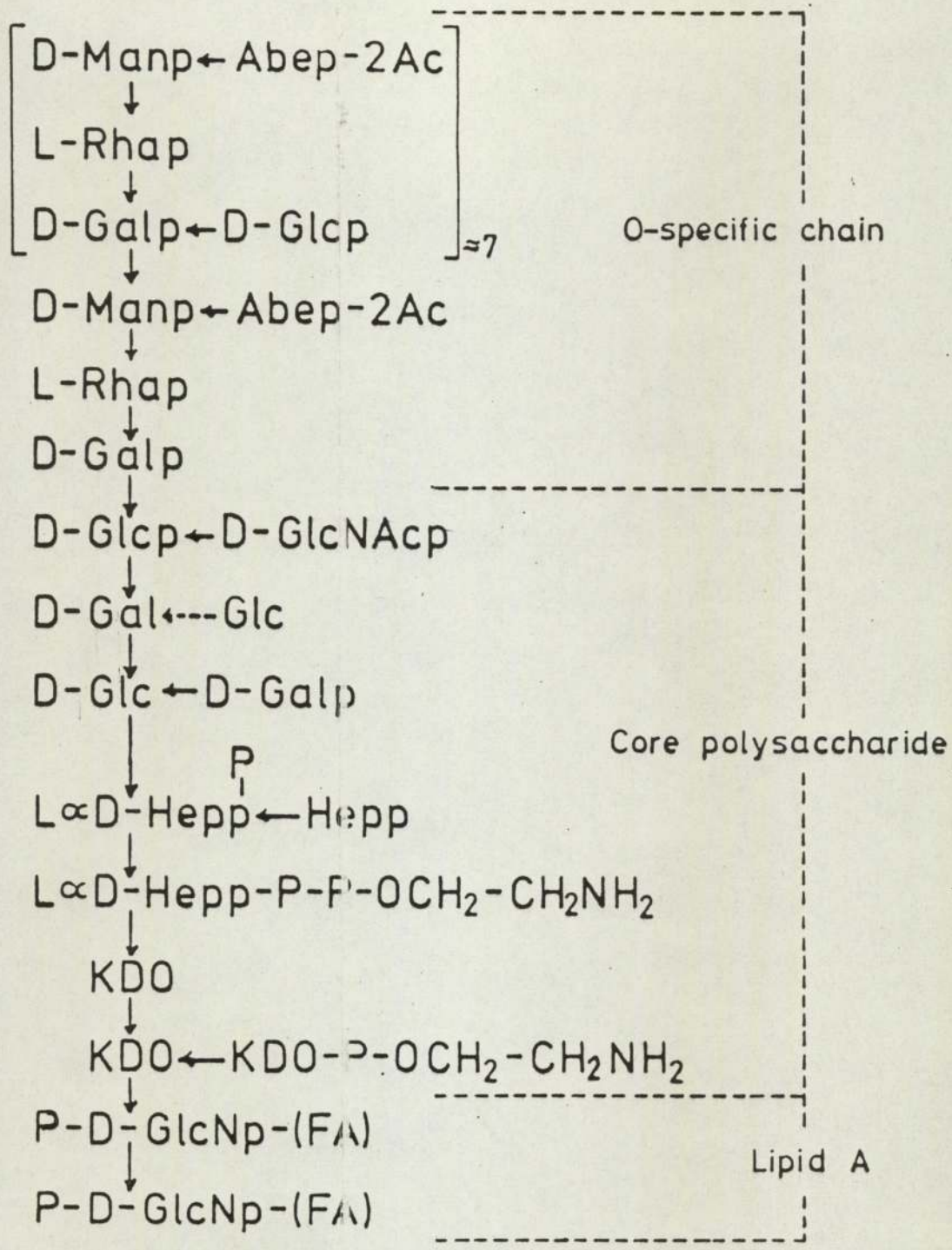
It is composed of phospholipids, hexagonally arranged in a closely packed bilayer containing protein subunits (Costerton et al., 1974). It differs from the cytoplasmic membrane in that it contains considerable amounts of lipopolysaccharide (LPS) constituting theO-antigen and endotoxins of bacteria (Clarke, Gray & Reaveley, 1967 a,b,c). Fig 5 shows the chemical structure of the lipopolysaccharide of Salmonella typhimurium (Luderitz, Galanos, Lehmann & Rietschel, 1974). This structure is similar in all bacteria so far studied, although there is some variation in chemical composition between species (Meadow, 1974). Three regions are recognisable:-

1. The O-antigen-specific side chain.
2. Core polysaccharide, which is a hydrophilic part of the molecule, which protrudes into the surrounding environment (Glauert & Thornley, 1969).
3. Lipid A endotoxic region, which is the hydrophobic core of the outer membrane (Glauert & Thornley, 1969).

Ames, Spudich & Nikaido (1974) reported the presence of multiple major proteins in the outer membrane of S. typhimurium, of molecular weights between 33,000 and 36,000. Kamio & Nikaido (1977) concluded that these proteins are exposed on the outside and located in the outer half of the outer membrane and produce aqueous channels in the lipopolysaccharide-phospholipid bilayer by penetrating through the membrane.

Ames, Spudich & Nikaido (1974) and Smith, Kamio & Nikaido (1975), using S. typhimurium wild type and deep rough mutants (heptose-less or containing 1 or 2 heptose molecules, but no glucose) noted a close relationship between the content of these proteins and LPS; alterations in the LPS of the mutants caused decreases in the level of major protein components per unit surface area and increased the

Fig 5: Structure of S. typhimurium lipopolysaccharide (Luderitz et al., 1974)



Man=Mannose; Abe=Abequose; Rha=Rhamnose; Glc=Glucose; Gal=Galactose; GlcN=Glucosamine; Hep=L-glycero-D-mannose; KDO=2-keto-3-deoxy-octonate; Ac=Acetate; p=Phosphate; P=esterified phosphate; FA=fatty acid

phospholipid content. The protein content may be related to the LPS as they are complexed together.

Fig 6 shows a proposed model of the outer membrane of S. typhimurium (Smith, Kamio & Nikaido, 1975). Note the high protein content in the wild type and Rc mutant, with phospholipid forming the inner layer of the membrane. This lack of exposed phospholipid prevents the passage of hydrophobic molecules into the interior of the membrane (Nikaido, 1976), while in Rd and Re mutants more phospholipid and less protein are found. The exposed phospholipid allows the rapid penetration of hydrophobic molecules (Nikaido, 1976). Nakae & Nikaido (1975) found that isolated OM rather than the peptidoglycan acted as a diffusion barrier in E. coli and S. typhimurium with an exclusion limit of about 900 daltons for oligosaccharides compared with 100,000 daltons of Gram-positive Bacillus megaterium.

Hockstra et al. (1976) noticed that E. coli released a complex containing LPS, phospholipid and proteins into the medium during growth. The fragments had vesicular-shape and were found to be unmodified outer membrane. Braun & Hantke (1975) showed that the E. coli outer membrane contains lipoprotein, which exists in two different forms; the free form and the bound form covalently linked to the peptidoglycan. Inouye et al. (1976) managed to purify the free form and found that the amino acid composition, fatty acid composition and peptides were identical to those of the bound form, with a high α-helical content (87%) compared to that of the bound form.

Garten & Henning (1974), using E. coli K12, found that the protein of OM ghosts can be separated into three major bands, I, II and IV, and one minor band, III. Band IV is a lipoprotein formed of homogeneous peptide chains. Proteins I and II contain no

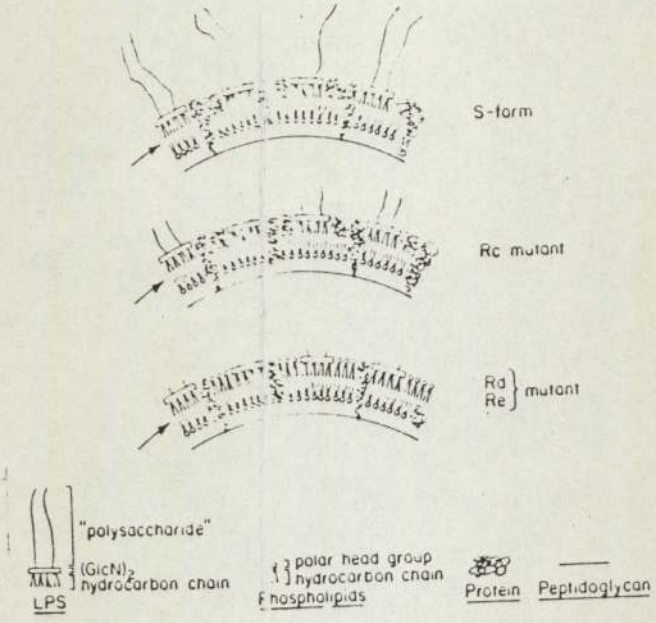


Fig 6: Proposed model for the structure of the outer membrane in S. typhimurium (Smith, Kamio & Nikaido, 1975).

The arrow shows the presumed plane of fracture.

phosphorus, palmitic acid or lipopolysaccharide, but small amounts of glucosamine. They have a similar amino acid composition except for their content of histidine, phenylalanine and proline (Garten & Henning, 1974), which suggests that they may be structural proteins. They appear to act as phage receptors and transport vitamin B12 and iron (Garten, Hendennach & Henning, 1975).

Decad & Nikaido (1976) concluded that the molecular sieve property of the OM of Gram-negatives is due to the presence of water-filled pores, that allow the diffusion of small hydrophilic molecules. However, large molecules which cannot penetrate these pores may be transported by proteins (DiMasi et al., 1973).

Reithmeier & Bragg (1977) concluded that extensive protein-protein interactions through amino groups occur in the OM of E. coli. Verkleij et al. (1977), using E. coli K12, fractured through the OM, found that the inner surface of the outer layer is densely occupied with particles consisting of LPS aggregates stabilized by divalent cations and probably complexed with protein/or phospholipid which appear to resemble those found in P. aeruginosa by Roger, Gilleland & Eagon (1969). Alphen et al. (1977) noted the resistance of E. coli K12 to the action of phospholipases and concluded that phospholipids, which are present in small amounts in the outer leaflet of the OM (as indicated by the smooth areas following freeze fracture), are shielded by LPS and/or proteins, which prevent their degradation in intact cells. Phospholipase-sensitive mutants lack major OM proteins and have an increased phospholipid content.

1.1.6. EXTERNAL FEATURES

Some Gram-negative bacteria have a protein surface layer

external to the LPS layer (Thornley, Glauert & Sleytr, 1974). This protein layer can be removed without affecting the bacterial survival in the laboratory (Murray, 1968). In addition, many have pili protruding from the surface. In E. coli these are long, thin appendages arising just below the cytoplasmic membrane and serve for the transfer of genetic materials and as receptors for RNA and DNA phages (Bradley, 1966, 1973).

Many Gram-negative bacteria have a loosely associated capsule composed of polysaccharide. Bayer & Thurow (1977), using E. coli strain Bi 161/42 (O9:K29(A):H^-) reported that the polysaccharide capsule appeared as a homogeneous layer of 250 - 300 nm in thickness. The purified polysaccharide from the capsule contained filamentous elements, the smallest measuring 250 nm in length and 3 - 6 nm in width, while the slime polysaccharide contained filamentous bundles $>$ 100 nm in length. It is possible that the composition and production of slime varies with cultural conditions as in P. aeruginosa (Brown & Scott-Foster, 1971; Goto, Murakawa & Kuhara, 1973).

1.2. EFFECT OF Mg^{2+} DEPLETION ON THE GRAM-NEGATIVE CELL ENVELOPE

1.2.1. ROLE OF Mg^{2+} IN THE GRAM-NEGATIVE ENVELOPE

Gram-negative bacteria such as E. coli and Sphaerotilus natans contain considerable quantities of divalent metal cations, such as Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} (Rouf, 1964). Lederberg (1956) associated the divalent cations in the envelope with maintaining the stability of the cytoplasmic membrane, since spheroplasts formed from E. coli cells, when incubated in the presence of sucrose (20% w/v) penicillin (10^3 u ml^{-1}) and $MgSO_4$ (0.2% w/v), tend to lyse if Mg^{2+}

is not included. The divalent cations are also responsible for the maintenance of the structural conformation and activity of membrane-bound enzymes (Kundig & Roseman, 1971). They provide cross-linkages between the LPS components (Wilkinson & Galbraith, 1975) and the rest of the OM (Asbell & Eagon, 1966), presumably the phospholipid (Yu & Jordan, 1971; Costerton, Ingram & Cheng, 1974) and the polysaccharide chains of the murein layer (Vincent & Humphrey, 1963).

1.2.2. EFFECT OF Mg^{2+} DEFICIENCY ON THE CELL ENVELOPE

Nutrient depletion has a major effect on the properties and chemical composition of the cell wall of P. aeruginosa (Boggis, 1971; Kenward, 1975).

Mg^{2+} deficiency in E. coli results in the formation of filaments and changes in the permeability of membranes, as indicated by it staining less intensely with methylene blue (Brock, 1962).

Fill & Branton (1969), using electron microscopy, noted that Mg^{2+} starved E. coli have increased membranes and infolding of the cytoplasmic membrane near the ends of the cells. This increase in membranes can be correlated with the results of Gunter, Richter & Schmalbeck (1975) concerning the changes in phospholipid composition of E. coli grown in conditions of Mg^{2+} deficiency. When E. coli was grown in Mg^{2+} depleted batch culture the total cellular phospholipid increased corresponding to an increase in membranes. They also found an increase in total phospholipid following Mg^{2+} starvation. When expressed relative to total extracted phospholipids they found more cardiolipin and less phosphatidylglycerol and phosphatidylethanolamine. However, Gilleland et al. (1974) found that Mg^{2+} depleted P. aeruginosa envelope contains less phosphorus, which contradicts the findings of

Gunter et al.. Possibly this is because Gunter et al. used whole cells, or because an estimate of total phosphorus in the envelope would include the phosphate present in the LPS. Gilleland et al. also found more carbohydrate, 2-keto-3-deoxyoctaroic acid (KDO) and protein in Mg^{2+} depleted cell envelopes than in cell envelopes from Mg^{2+} sufficient cells, associated with an increase in the number of spherical units embedded in the inner surface of the OM under conditions of Mg^{2+} depletion. The units were released from Mg^{2+} sufficient cells by EDTA and were found to be a protein-LPS complex (Roger, Gilleland & Eagon, 1969). In Mg^{2+} deficient cells the units appeared crowded and disorganized and were not removed by EDTA (Gilleland, 1977). The resistance to EDTA caused by Mg^{2+} depletion (Brown & Melling, 1969 a,b) could be explained by this increase in the protein-LPS content of the cell wall. The importance of the LPS in resistance was confirmed by Sanderson, MacAlister, Costerton & Cheng (1974), who found that a S. typhimurium mutant having incomplete LPS was less resistant to antibiotics that act on intracellular targets than the wild type.

Tempest & Ellwood (1969) found, using Aerobacter aerogenes, that the size of bacterial cells and their cell wall chemistry varied with growth rate; increasing the growth rate of Mg^{2+} limited cells decreased the cell wall KDO and heptose contained in the LPS layer (the opposite was found with C-limited cells). This change correlates with changes in the reactivity or content of the O-antigen associated with the LPS layer. Ellwood & Tempest (1972) reviewed the effect of environment on bacterial cell walls of various species. They noted that with Klebsiella aerogenes the bacterial cell wall content, expressed as a percentage of the total cell weight, decreased with increasing growth rate.

Kenward (1975) studied the lipid composition of the

P. aeruginosa cell envelope under Mg^{2+} adequate and Mg^{2+} depleted conditions. He found that after Mg^{2+} starvation significant changes in the cell wall phospholipid composition occured. The phospholipid composition of the wall was related to the presence or absence of Mg^{2+}, but bore no relationship to the resistance of the cell to EDTA or polymyxin. Mg^{2+} depleted cells with added calcium or manganese were sensitive to these agents and had the same phospholipid pattern as Mg^{2+} depleted cells with no added cations but which were resistant to EDTA and polymyxin.

Although many workers have related resistance to antimicrobial agents to the phospholipid content of the cell envelope, finding a decrease in resistance with increasing phospholipid (Brown & Watkins, 1970; Pechey, Yau & James, 1974; Feingold, Hsu-Chen & Sud, 1974), Kenward (1975) concluded that phospholipid composition could not be related to resistance in this instance and that other factors such as the cation content of the cell wall must be taken into consideration.

In conclusion, one of the major effects of Mg^{2+} depletion is a change in the chemical composition of the cell envelope. Mg^{2+} depleted cell envelopes appear to have less phosphorus and less phospholipid, while more LPS and protein appear to be present. These changes may explain changes in permeability and response to antibacterial agents.

1.3. THE EFFECT OF R-PLASMIDS ON THE GRAM-NEGATIVE CELL ENVELOPE

1.3.1. INTRODUCTION

R-plasmids were first discovered in Japan in 1950 by Watanabe and his coworkers while they were investigating the rapid transmission

of multiple drug resistance between E. coli and Shigella. The origin of R-plasmids is not known. That they were completely unknown before antibiotics were introduced has been disproved by the study carried out by Smith (1967). Using E. coli species preserved since the pre-antibiotic era, he discovered that they also carried R-plasmids.

Lowbury et al. (1969), Sykes & Richmond (1970) and Grinsted et al.(1972) described the R-plasmid RP1 which is a group of genes conferring resistance to tetracycline, neomycin, kanamycin and a wide range of β-lactam antibiotics, including carbenicillin. It originated in a strain of P. aeruginosa and was freely transmissible between that and Proteus mirabilis and E. coli. The acquisition of this multiple drug resistance was accompanied by acquisition of an extrachromosomal piece of covalently closed circular DNA of molecular weight about 4×10^7 daltons.

1.3.2. THE EFFECT OF R-PLASMIDS ON SURFACE PROPERTIES

Many plasmids confer antibiotic resistance by coding for antibiotic-destroying enzymes. However, in some cases, a structural alteration of the bacterial cell envelope contributes to the resistance, and there is increasing evidence that plasmids code for a variety of changes in specific components of the bacterial cell envelope (Holloway, 1975).

Curtis & Richmond (1974) concluded that intrinsic resistance gene (or genes) of the plasmid RP1 specify the synthesis of material in the surface layer (thus modifying the envelope) of E. coli which impedes the access of penicillin to its target. They suggest that in Gram-negative bacteria the greatest protective effect of β-lactamase is obtained when the enzyme is stituated behind a

barrier which provides a slow feed of substrate (penicillin) to the enzyme. RP1 provides genes for both the enzyme and some degree of enhancement of the barrier, achieving higher levels of penicillin resistance than plasmids that code for β-lactamase only.

Richmond & Curtis (1975) studied the effect of β-lactamase-less mutants of RP1 on the penicillin resistance of env A mutants of E. coli K12, which are altered in their surface properties, and have a lowered resistance to penicillins. The presence of RP1 amp 1 increased the MIC value of env A cells and therefore it probably carries a gene (or genes) that can repair the env A lesion, suggesting that the R-plasmid determinants are closely linked to those that enhance the intrinsic resistance of normally occuring strains. Sensitivity to lysozyme as well as to actinomycin D, naladixic acid and rifampicin is part of the phenotype of another mutant of E. coli, AS19. Sensitivity to lysozyme indicates changes in the surface of the cell since lysozyme can not normally penetrate to the murein of Gram-negative bacteria. However, insertion of RP1 amp 1, affords some protection against these antibiotics and lysozyme due to the introduction of a barrier between the sensitive murein layer in the wall and the outside of the cell. Results with a further mutant amp 1 irp 1, which has also lost the gene specifying intrinsic resistance, confirm that RP1 alters the surface layers of bacteria to make access by antibiotics to their targets more difficult.

Plasmid-mediated intrinsic resistance to penicillin is not provided by all R-plasmids since R1 plasmid showed no such properties.

Levy, McMurry & Palmer (1974) have shown that a minicell system (cells containing no nuclear DNA, but only R-plasmid DNA) can be used to detect a new inner membrane protein associated with R-plasmid-mediated tetracycline resistance in E. coli. R-plasmids

may also cause protein to be incorporated into the outer membrane of bacteria. Beard & Connolly (1975) noted that the outer membrane of E. coli K12, carrying a derepressed R-plasmid, produced many pili which were absent in strains carrying the repressed R-plasmid. Analysis of the strain with the derepressed R-plasmid showed that it had, as a constituent of its cell envelope, a protein of molecular weight approximately 12,500 which was absent from the strain with a repressed R-plasmid.

Kenward & Brown (1976) studied the influence of the R-plasmid RP1 on the composition of walls isolated from P. aeruginosa. Walls from $RP1^+$ cells contained less Mg^{2+}, Ca^{2+}, KDO and diphosphatidylglycerol, while the content of diaminopimelic acid, lysyl-phosphatidylglycerol and phosphatidylethanolamine was higher than in the cells not containing the R-plasmid. When extracted lipids were examined, it was found that $RP1^-$ cells lost most of their Mg^{2+}, Ca^{2+} and phosphorus, whereas no significant loss occured during the extraction of the $RP1^+$ walls, suggesting that divalent cations were more firmly bound, and that they play a role in resistance of $RP1^+$ strain to EDTA.

It is well known that Gram-negative cells are less susceptible to attack by antibiotics than Gram-positive cells due to the presence of LPS in the envelope which provides an effective permeability barrier. Scott et al. (1974), using E. coli, found that the presence of an R-plasmid rendered it more susceptible to attack by rifampicin derivitives. These antibiotics have side chains with free carboxyl groups and in 'normal' cells do not penetrate easily into the LPS barrier. Since the R-plasmid did not render the envelope non-specifically leaky (as indicated by the loss of resistance to rifampicin, but not to penicillin) it is possible that some structural component of the LPS wall was being coded for by the presence of the

R-plasmid.

Hesslewood & Smith (1974) studied envelope alterations produced by the presence of an R-plasmid in Proteus mirabilis. It was found that the ability of P. mirabilis to swarm was increased by the presence of one R-plasmid, but decreased by the presence of a different one. When both R-plasmids were introduced into the same cell, it was the first one introduced that was phenotypically expressed.

The level of extracellular proteolytic enzyme liberated by the cells was increased in those cells bearing the R-plasmid which inhibited swarming, and decreased in cells bearing the R-plasmid which enhanced swarming. It is therefore possible that the R-plasmid was producing an alteration in the cell envelope rather than coding for enzyme formation. This was confirmed when it was noticed that cells bearing the R-plasmid coding for decreased swarming were more susceptible to attack by sodium deoxycholate (Hesslewood, 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.

It was also suggested that the mucopeptide layer, in association with the lipoprotein binding it to the outer membrane, might be affected. Increased mucopeptide bonding is associated with decreased proteolytic activity and increased swarming. However, no proof of either mechanism was put forward.

In conclusion, relatively little work has been performed on the actual changes in the envelope by the R-plasmid, presumably because it is only in the last few years that techniques for separating and studying Gram-negative cell envelopes have become available.

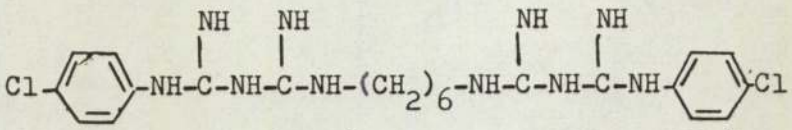
All the available evidence suggests that R-plasmids may produce alterations at two or three sites in the envelope, coding for

the synthesis of protein which is incorporated into the cytoplasmic membrane, and producing alterations in the LPS and other lipid components of the cell wall. There is also a suggestion that in some species they may affect the structure of the mucopeptide layer.

1.4. MODE OF ACTION OF SURFACE ACTIVE ANTIBACTERIAL AGENTS

1.4.1. MODE OF ACTION OF CHLORHEXIDINE

The cationic surfactant, chlorhexidine, is a white, odourless



Chlorhexidine : 1,6-di-(4-chlorophenyldiguanido)hexane

base. Due to its cationic properties, it is incompatible with anionic surfactants and other anionic compounds. Its bactericidal activity is related to inoculum size and increases with increasing alkalinity of the medium (Longworth, 1971). Chlorhexidine is rapidly adsorded by bacterial cells, causing cytological changes including permeability changes and changes in the cells optical properties.

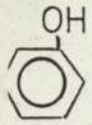
Hugo & Longworth (1964 a) noted that the amount of chlorhexidine adsorbed on the cell surface of E. coli depended on the concentration of chlorhexidine, the cell density and on the pH of the suspending medium. The amount of chlorhexidine bound increases with increase in the pH of the medium, presumably due to an increase in the degree of ionisation of groups on the bacterial surface rather than a change in the ionisation of the chlorhexidine molecule. It reacts with the negatively charged groups on the bacterial surface causing a reduction in charge and therefore in electrophoretic mobility (Hugo & Longworth, 1966).

After adsorption, what follows depends on the chlorhexidine concentration; at low concentrations of chlorhexidine a rapid loss of cytoplasmic constituents occurs (Hugo & Longworth, 1964 a; Ray & Wiseman, 1964, 1965). Hugo & Longworth (1965), using electron microscopy, noted the formation of ghosts devoid of cytoplasmic constituents. A progressive increase in the amount of drug adsorbed is noted with an increase in chlorhexidine concentration. However, a decrease in the rate of loss of cytoplasmic constituents occurs, surface protuberances appear and the cytoplasm changes to a coagulated appearance. These changes are accompanied by an increase in bactericidal activity (Hugo & Longworth, 1964 a). This work has been confirmed by Davis *et al.* (1968) and Davis & Field (1969). In addition, chlorhexidine stimulates dehydrogenase activity at low concentrations and at higher concentrations inhibits it (Hugo & Longworth, 1966). Thus, the action of chlorhexidine on *E. coli* is thought to be as follows:-

1) The primary action is adsorption of chlorhexidine on the surface of the cell. There is little evidence about the effects on the outer membrane.
2) Disorientation of the cytoplasmic membrane lipoprotein prevents it from acting as an osmotic barrier.
3) The subsequent events depend upon the concentration of the drug present; low concentrations only cause leakage of cytoplasmic constituents. Higher concentrations are bactericidal and coagulate cytoplasmic constituents.

1.4.2. MODE OF ACTION OF PHENOL

Phenol is inactive in the unionized form. Phenols are more active therefore at acid pH values (Hamilton, 1971).



Phenol

The primary mode of action of phenol is membrane damage and the consequent leakage of cellular constituents. Bean & Das (1966) represented the uptake of several phenols by absorption isotherms. The uptake of dilute solutions suggested partitioning between the cell and the aqueous solution, but at higher concentrations a change in the uptake pattern suggested protein precipitation. Judis (1966) noted that protoplasts of Micrococcus lysodeikticus bound less of each of the phenol derivatives than the whole cells, suggesting that cell walls do bind a portion of the phenol derivatives, and that the mucopeptide complex is capable of associating with these compounds.

A bacteriostatic concentration of phenol immediately inhibits the synthesis of protein (Pullman & Reynolds, 1965). Judis (1966) found that serum or serum albumin has the ability to decrease the association of phenol derivatives with bacterial cells, possibly by two mechanisms; the protein competes with the bacterial cells for the phenol derivatives or else the proteins coat the bacterial cell making the surface of the bacteria unavailable for penetration by phenol. Binding of phenol to bacterial protein could account for the antibacterial action, as this binding could lead to disruption of the integrity of the bacterial membrane and inactivation of enzymes (Starr & Judis, 1968).

Ribonucleic acid and DNA synthesis is also inhibited by phenol in exponentially growing cells. However, respiration and the synthesis of adenosine triphosphate continue for some little time (Pullman & Reynolds, 1965).

1.4.3. MODE OF ACTION OF CETRIMIDE

The quaternary ammonium disinfectant, cetrimide (cetyl trimethyl ammonium bromide) has the following formula:-

$$C_{16}H_{23}(CH_3)_3N^+ Br^-$$

The antimicrobial activities of quaternaries are decreased by the presence of organic matter due to the adsorption of quaternaries on to proteins. They are more active at alkaline pH values (Hamilton, 1971).

The mode of action of cetrimide is similar to that of chlorhexidine (Davis, Bentley & Field, 1968). Salt & Wiseman (1968) studied the uptake of cetrimide by Escherichia coli and suggested that it occurs in two distinct phases; the primary phase is characterized initially by an increase in the proportion of cetrimide adsorbed with increase in the added concentration, followed at higher concentrations by progressive decreases in the proportion adsorbed. The molecules in the primary monolayer have their polar groups adjacent to the cell so that the new surface presented to the solution is hydrophobic allowing the adsorption of a second layer with their polar groups distal to the cell. The surface formed would be positively charged and therefore further uptake of cetrimide is not possible. Cetrimide then penetrates into the cell possibly into the hydrophobic lipid layer of the cell membranes.

After exposure of various species of bacteria to a bactericidal amount of cetrimide below that required for 99.99% killing, the leakage of 260 nm-absorbing material in 5 min is proportional to the percentage of cells killed. However, when cell suspensions are treated with sufficient cetrimide to kill 99.99% of the cells within 5 min, the

rapidity with which cellular constituents appear in the suspending fluid varies from one species to another (Salton, 1951).

2. MATERIALS

2.1. BACTERIA

2.1.1. Properties:

Escherichia coli K12 W3110 was kindly provided by Dr. P. A. Meacock (Department of Genetics University of Leicester). This strain is "wild-type" K12 cured of sex factor F and bacteriophage λ (Bachmann, 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, specifying resistance to tetracycline, ampicillin, kanamycin, neomycin cephaloridine and carbenicillin (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 transferred from there to *E. coli* K12 met^- and from the latter to *E. coli* K12 W3110.

Throughout this work, *E. coli* K12 W3110 is designated R^- and *E. coli* K12 W3110(RP1) is designated R^+.

2.1.2. Maintenance of stock cultures:

The R^- culture was streaked on to nutrient agar (NA) and MacConkey agar plates (to check for contamination), incubated at 37° overnight (ON) and one of the resultant colonies on NA transferred to a NA slope. After ON incubation at 37°, slopes were stored at 4°. Fresh agar slopes were prepared every three months.

The R^+ strain was maintained in the same way, except that tetracycline (25 μg ml^{-1}) was added to the NA plates to make sure that only colonies containing the R-plasmid were used to inoculate slopes.

The organisms were also stored on chemically defined medium

(CDM) slopes. Isolated colonies from the agar plates were subcultured into nutrient broth (NB), incubated at 37° ON and drops serially subcultured twice into CDM. The final CDM culture was transferred to a CDM slope and incubated at 37° ON. Fresh CDM slopes were prepared at four week intervals. The stability of the R-plasmid was checked and it was found to be very stable within this period (see results p37).

2.2. CHEMICALS

Water: all water was deionized and then distilled in a glass still.

Chemicals used in preparing CDM were AnalaR grade and were obtained from British Drug Houses Chemicals Ltd., Poole, Dorset, England, Fisons Scientific Apparatus Ltd., Bishop Meadow Road, Loughborough, Leicestershire LE11 ORG, England, or Hopkins & Williams Ltd., Chadwell Heath, Essex, England.

L-Methionine: British Drug Houses Chemicals Ltd..

MOPS, (3-(N-Morpholino)propanesulphonic acid) $C_7H_{15}NO_4S$: British Drug Houses Chemicals Ltd..

Polymyxin B sulphate B.P.: Wellcome Brand, kindly donated by Burroughs Wellcome & Co., London, England.

Multodisks: Oxoid Ltd., Southwark Bridge Road, London SE1 9HF, England.

Ampicillin (Penbritin regd.): Beecham Research Laboratories, Great West Road, Brentford, Middlesex TW8 9BD, England.

Kanamycin: Winthrop Laboratories, Surbiton-Upon-Thames, Surrey, England.

Tetracycline: Lederle Laboratories Division, London, England, as achromycin tetracycline hydrochloride.

Chlorhexidine gluconate 5% w/v (Hibitane): Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, England.

Phenol: Fisons Analytical Reagent, Fisons Scientific Apparatus Ltd..

Cetrimide: British Drug Houses Chemicals Ltd..

Hydrochloric acid: AnalaR, British Drug Houses Chemicals Ltd..

2.3. MEDIA

2.3.1. Complex media for stock cultures and for viable counts

Nutrient Broth (NB): Oxoid nutrient broth, code CM1.

Nutrient Agar (NA): Oxoid nutrient agar, code CM3.

MacConkey Agar: Oxoid MacConkey agar, code CM7.

All the above mentioned media were obtained from Oxoid Ltd.. The media were prepared as instructed by the manufacturers.

Lab M Agar: (used for CDM slopes), code MC2, London Analytical & Bacteriological Media Ltd., 50 Mark Lane, London EC3R 7QJ, England.

2.3.2. Media used for transferring R-plasmid

Double strength Davis Mingioli base (Davis & Mingioli, 1950) was distributed in 50 ml volumes and sterilized. The volume was made up to 60 ml by adding aseptically glucose (final conc. 2%), ampicillin (final conc 10 $\mu g\ ml^{-1}$), and, where necessary, methionine (final conc. 50 $\mu g\ ml^{-1}$). This media was mixed with 40 ml double strength Lab M agar and approximately 20 ml poured into petri dishes.

2.3.3. Chemically defined media

A simple salts medium was prepared based on the usual requirements for E. coli (CDM A, Table 1, p24). This was used for the orginal CDM slopes and for initial studies on nutritional requirements. As the requirement for each component was defined, the basic medium used for further studies was modified at each step (Tables 2 & 3, p25&26).

Table 1: Chemically defined media. Concentration (M) of nutrients which support growth to required optical density

Nutrient \ CDM	A	B	C	D	E	F	G
Glucose	5×10^{-3}	2.6×10^{-2}	2.8×10^{-2}	5×10^{-3}	5.6×10^{-3}	7.6×10^{-3}	8.5×10^{-3}
Mg^{2+}	1×10^{-4}	6.6×10^{-5}	2.8×10^{-4}	2.7×10^{-5}	5.25×10^{-5}	4.15×10^{-5}	7.8×10^{-5}
PO_4^{3-}	1×10^{-1}	1×10^{-3}	6×10^{-3}	3.4×10^{-4}	1.13×10^{-3}	5.2×10^{-4}	1.7×10^{-5}
K^+	5×10^{-5}	3.7×10^{-4}	5×10^{-4}	7.3×10^{-5}	1.02×10^{-4}	1.12×10^{-4}	1.62×10^{-4}
NH_4^+	1×10^{-3}	2.12×10^{-2}	2.12×10^{-2}	4.2×10^{-3}	4.2×10^{-3}	6.7×10^{-3}	6.7×10^{-3}
SO_4^{2-}	1.1×10^{-3}	3.1×10^{-4}	1.25×10^{-4}	6.2×10^{-5}	4.5×10^{-5}	9.8×10^{-5}	7.2×10^{-5}
Fe^{2+}	-	1.8×10^{-6}	3.6×10^{-6}	-	6×10^{-7}	-	1.1×10^{-6}
MOPS	-	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}
Na^+	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}

Key to Table 1 on page 24a

Key to Table 1

CDM	Culture	Theoretical OD* obtainable	Use and experiments
A	R^-	not known	Initial slope and experiments
B	R^-	10	Slope and checking buffer
C	R^+	10	Slopes
D	R^-	2	Growth rate, antibacterial agents, R-plasmid stability
E	R^+	2	Growth rate, antibacterial agents, R-plasmid stability
F	R^-	3	Potentiometric titration
G	R^+	3	Potentiometric titration

*For media D, E, F and G this refers to the basal medium

For depletion the media were modified as follows:-

Nutrient	Medium concentration (M)			
	D	E	F	G
- C (glucose) (OD)	0.5×10^{-3} (0.2)	0.5×10^{-3} (0.2)	5.1×10^{-3} (2.0)	5.7×10^{-3} (2.0)
- Mg (Mg^{2+}) (OD)	0.05×10^{-5} (0.1)	0.25×10^{-5} (0.1)	1.25×10^{-5} (1.0)	2.45×10^{-5} (1.0)
- P (PO_4^{3-}) (OD)	0.1×10^{-4} (0.1)	0.5×10^{-4} (0.1)	1.6×10^{-4} (1.0)	5.6×10^{-4} (1.0)

Table 2: Chemically defined media used to determine concentrations (M) required of each nutrient for the growth of the R^- culture

Limiting constituent / Salt	Glucose	Mg^{2+}	NH_4^+	SO_4^{2-}	PO_4^{3-}	Na^+	K^+	Fe^{2+}	Trace elements
$(NH_4)_2SO_4$	5×10^{-3}	5×10^{-3}	-	-	2.12×10^{-2}	2.12×10^{-2}	2.12×10^{-2}	2.12×10^{-2}	2.12×10^{-2}
NH_4Cl	-	-	*	2.12×10^{-2}	-	-	-	-	-
$(NH_4)_2HPO_4$] $NH_4H_2PO_4$]	1×10^{-3}	1×10^{-3}	-	1×10^{-3}	*	1×10^{-3}	1×10^{-3}	1×10^{-3}	1×10^{-3}
Na_2HPO_4] KH_2PO_4]			1×10^{-3}	-	-	-	-	-	-
NaCl	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	-	5×10^{-5}	5×10^{-5}	5×10^{-5}
KCl	3.4×10^{-4}	3.4×10^{-4}	3.4×10^{-4}	3.4×10^{-4}	3.4×10^{-4}	3.4×10^{-4}	*	3.7×10^{-4}	3.7×10^{-4}
Na_2SO_4	-	-	3.5×10^{-4}	*	-	*	-	-	-
$(NH_4)_2FeSO_4.7H_2O$	1.8×10^{-6}	1.8×10^{-6}	1.8×10^{-6}	1.8×10^{-6}	1.8×10^{-6}	1.8×10^{-6}	1.8×10^{-6}	*	-
$MgSO_4.7H_2O$	6×10^{-5}	*	-	-	6.61×10^{-5}	6.6×10^{-5}	6×10^{-5}	6×10^{-5}	6×10^{-5}
$MgCl_2.6H_2O$	-	-	6.61×10^{-5}	6.6×10^{-5}	-	-	-	-	-
MOPS	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}
D(+)Glucose	*	2.6×10^{-2}	2.6×10^{-2}	2.6×10^{-2}	2.6×10^{-2}	2.6×10^{-2}	2.6×10^{-2}	2.6×10^{-2}	2.6×10^{-2}
Trace elements	-	-	-	-	-	-	-	-	*

- salt not used, * nutrient under study

Table 3: Chemically defined media used to determine concentrations (M) required of each nutrient for the growth of the R^+ culture

Salt \ Limiting constituent	Glucose	Mg^{2+}	NH_4^+	SO_4^{2-}	PO_4^{3-}	Na^+	K^+	Fe^{2+}	Trace elements
$(NH_4)_2SO_4$	2.1×10^{-2}	2.1×10^{-2}	-	-	2.12×10^{-2}	2.12×10^{-2}	2.12×10^{-2}	2.12×10^{-2}	2.12×10^{-2}
NH_4Cl	-	-	*	2.12×10^{-2}	-	-	-	-	-
$(NH_4)_2HPO_4$] $NH_4H_2PO_4$	1×10^{-3}	1×10^{-3}	-	6×10^{-3}	*	6×10^{-3}	6×10^{-3}	6×10^{-3}	6×10^{-3}
Na_2HPO_4] KH_2PO_4	-	-	2×10^{-3}	-	-	-	-	-	-
NaCl	5×10^{-5}	5×10^{-5}	-	5×10^{-5}	5×10^{-5}	-	5×10^{-5}	5×10^{-5}	5×10^{-5}
KCl	3.4×10^{-4}	3.4×10^{-4}	-	3.4×10^{-4}	3.7×10^{-4}	3.7×10^{-4}	*	3.7×10^{-4}	3.7×10^{-4}
Na_2SO_4	-	-	3.5×10^{-4}	*	-	*	-	-	-
$(NH_4)_2FeSO_4.7H_2O$	1.8×10^{-6}	1.8×10^{-6}	1.8×10^{-6}	3.6×10^{-6}	3.6×10^{-6}	3.6×10^{-6}	3.6×10^{-6}	*	-
$MgSO_4.7H_2O$	6×10^{-5}	*	-	-	2.8×10^{-4}	2.8×10^{-4}	2.8×10^{-4}	2.8×10^{-4}	2.8×10^{-4}
$MgCl_2.6H_2O$	-	-	2.5×10^{-4}	2.8×10^{-4}	-	-	-	-	-
MOPS	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}	2.5×10^{-2}
D(+)Glucose	*	3.235×10^{-2}	2.6×10^{-3}	3×10^{-2}	3×10^{-2}	3×10^{-2}	3×10^{-2}	3×10^{-2}	3×10^{-2}
Trace elements	-	-	-	-	-	-	-	-	*

- salt not used, * nutrient under study

On the basis of these results, CDM, in which each ingredient was theoretically able to support growth of the R^- or R^+ organisms to a required optical density (OD), were devised (Table 1).

2.3.4. Sterilization of media and antibacterial agents

All media were sterilized by autoclaving at 121° for 20 min. Chemically defined media constituents were also sterilized by autoclaving (phosphate buffer was prefiltered for clarification), except glucose and MOPS which were sterilized by filtration.

Chemically defined media were prepared by mixing ingredients, making up to the appropriate volume and refiltering before use.

Antibiotics and disinfectants were usually sterilized by filtration. When the disinfectants were tested in NA they were added to the melted agar and then sterilized by autoclaving.

2.4. EQUIPMENT

Spectrophotometer: Unicam S.P.600 Pye-Unicam Instruments Ltd., York Street, Cambridge, England, was used with matched glass cuvettes.

pH meter: measurements of pH were carried out using either Pye model 290 pH meter (Pye-Unicam Instruments Ltd.) or EIL 7030 pH meter, together with 28000chart recorder, Bryans Southern Instrument, Mitcham, England.

Water bath: Grant shaker bath S50 type, Grant Instruments, Cambridge Ltd., Barrington, Cambridge.

Centrifuge: International Equipment Company, 300 Second Avenue, Needham Heights, Massachusetts, U.S.A..

Automatic M.L.A. pipettes: 0.25 ml and 0.1 ml sizes and tips (sterilized by autoclaving), Frost Instruments Ltd., Wokingham, Berkshire RG11 1BZ.

Millipore membrane filtration apparatus: Millipore, Heron House, Wembley, Middlesex, England.

Membranes sartorius: membrane filter GmbH, D-3400 Göttingen, West Germany. Pore size 0.2 µm for sterilization, 0.45 µm for clarification.

Whirlimixer: Fisons Scientific Apparatus Ltd..

Glassware: Corning Glass Ltd., (Pyrex) Laboratory Division, Wearglass Works, Sunderland, England.

2.4.1. Preparation of membranes used in millipore filters

Membranes were boiled in three changes of distilled water prior to sterilization by autoclaving. This procedure was followed to remove wetting agents and other chemicals which might affect OD measurements (Brown, Farwell & Rosenbluth, 1969).

2.4.2. Preparation of glassware for use with CDM

Glassware was rinsed in tap water and totally immersed in 5% v/v Decon 90 (Decon Laboratories Ltd., Ellen Street, Portslande, Brighton BN4 1EQ), ON at room temperature. Decon-treated glassware was rinsed once in distilled water, once in 1% v/v hydrochloric acid, then six times in distilled water and twice in deionized distilled water.

After rinsing, the glassware was dried at 60°, covered with aluminium foil and sterilized by dry heat (160° for 3 hr).

3. EXPERIMENTAL METHODS

3.1. GROWTH OF BACTERIA IN CDM

Growth experiments were done using as inoculum a culture grown in CDM B or C (see Table 1, p24) which had been washed three times in a medium lacking the nutrient under study and resuspended at OD 1.0. 0.25 ml of this suspension was inoculated into 24.75 ml of the appropriate medium (see Tables 2 & 3, p25&26) in 100 ml conical flasks.

Flasks were incubated at 37° in a water bath with a shaking rate of 120 throws per min. The media were inoculated approximately 1 hr prior to the first OD readings. Samples for the estimation of OD were removed at 30 min intervals at the beginning of incubation and near the end of exponential growth, but at 60 min intervals during most of the logarithmic phase. They were diluted as necessary. Undiluted samples were returned to the flasks to prevent excessive reduction of culture volume, but samples (0.5 ml) which had to be diluted were subsequently discarded.

3.2. MEASUREMENT OF GROWTH

3.2.1. Choice of wavelength

The turbidity of a culture is determined either by measuring the intensity of refracted light emerging from a culture or by measuring the reduction in intensity of the undeviated light emerging from the culture.

Certain basic conditions should be considered when selecting a suitable wave-length for the measurement of the turbidity of a bacterial suspension. Ideally the absorption by cell pigments, media constituents and metabolic products should be minimal, and the wave-length should be that which allows maximum sensitivity to changes in OD.

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 to OD changes.

E. coli is a non-pigmented organism. Handley, Quesnel & Sturgiss (1974) found that the lowest convenient wavelength to satisfy the above conditions was 420 nm, which was therefore used throughout this study.

3.2.2. Measurement of OD

The turbidity or OD of a particular bacterial suspension at low concentration is related to the reduction in intensity of incident light by the Beer-Lambert Law which can be expressed as

$$OD \propto \log \left(\frac{I_o}{I}\right)$$

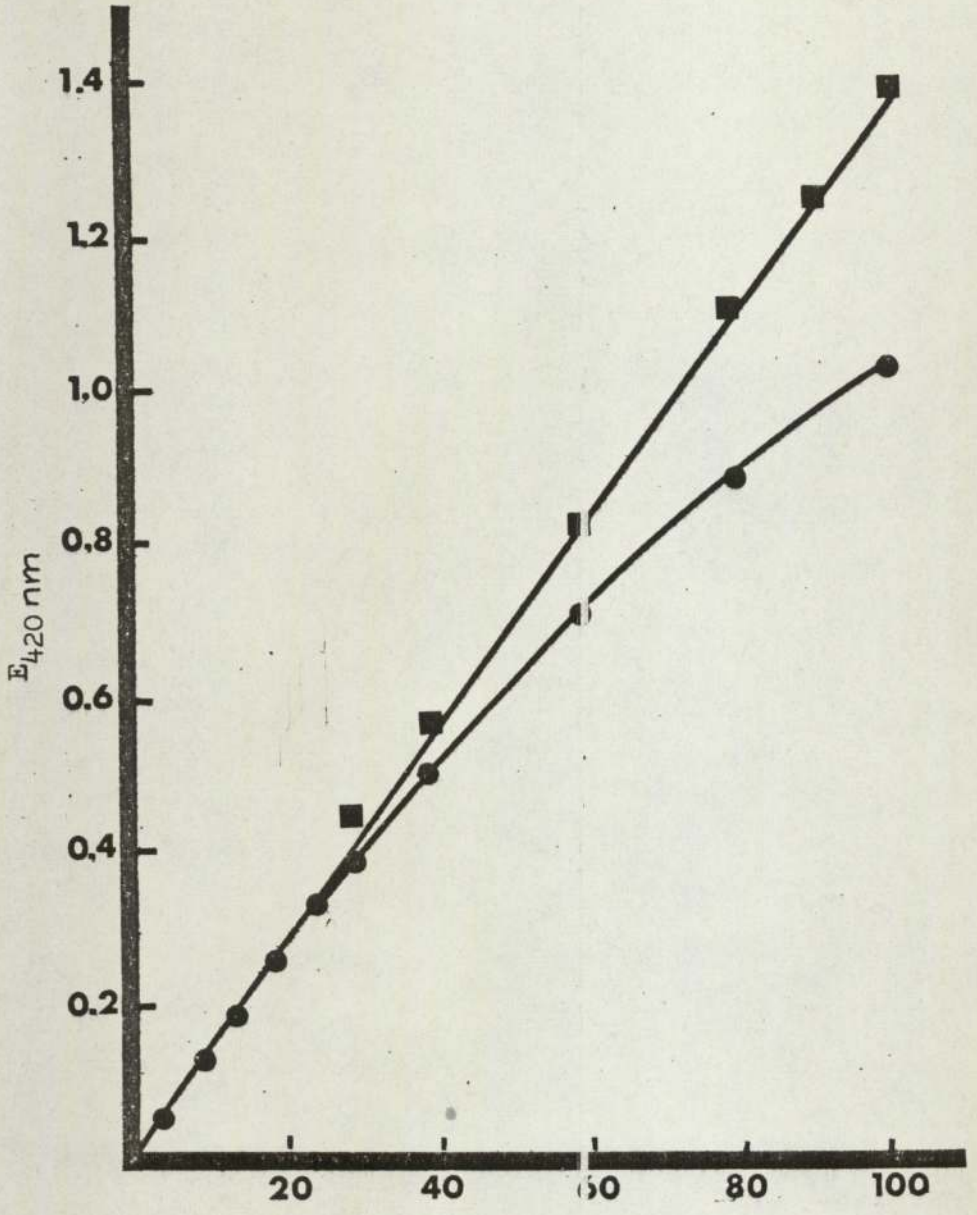
where I_o = intensity of incident light
I = intensity of emerging light

provided that the light path is a constant length. However, at high concentrations of bacteria, this relation does not hold because of secondary scattering of the light (Meynell & Meynell, 1970). It was therefore necessary to determine the OD at which the Beer-Lambert Law was no longer obeyed.

Two series of dilutions of a dense stationary culture of E. coli K12 W3110 were prepared following one washing, using CDM without glucose for one set and sterile distilled water for the other. Dilutions were mixed using a Whirlimixer. The OD of the suspensions were measured.

The relationship between OD and cell concentration obeyed the Beer-Lambert Law up to an E_{420} of 0.25 for both diluents (Figs7 &8).

Fig 7 : Relation between real and observed optical density of E. coli

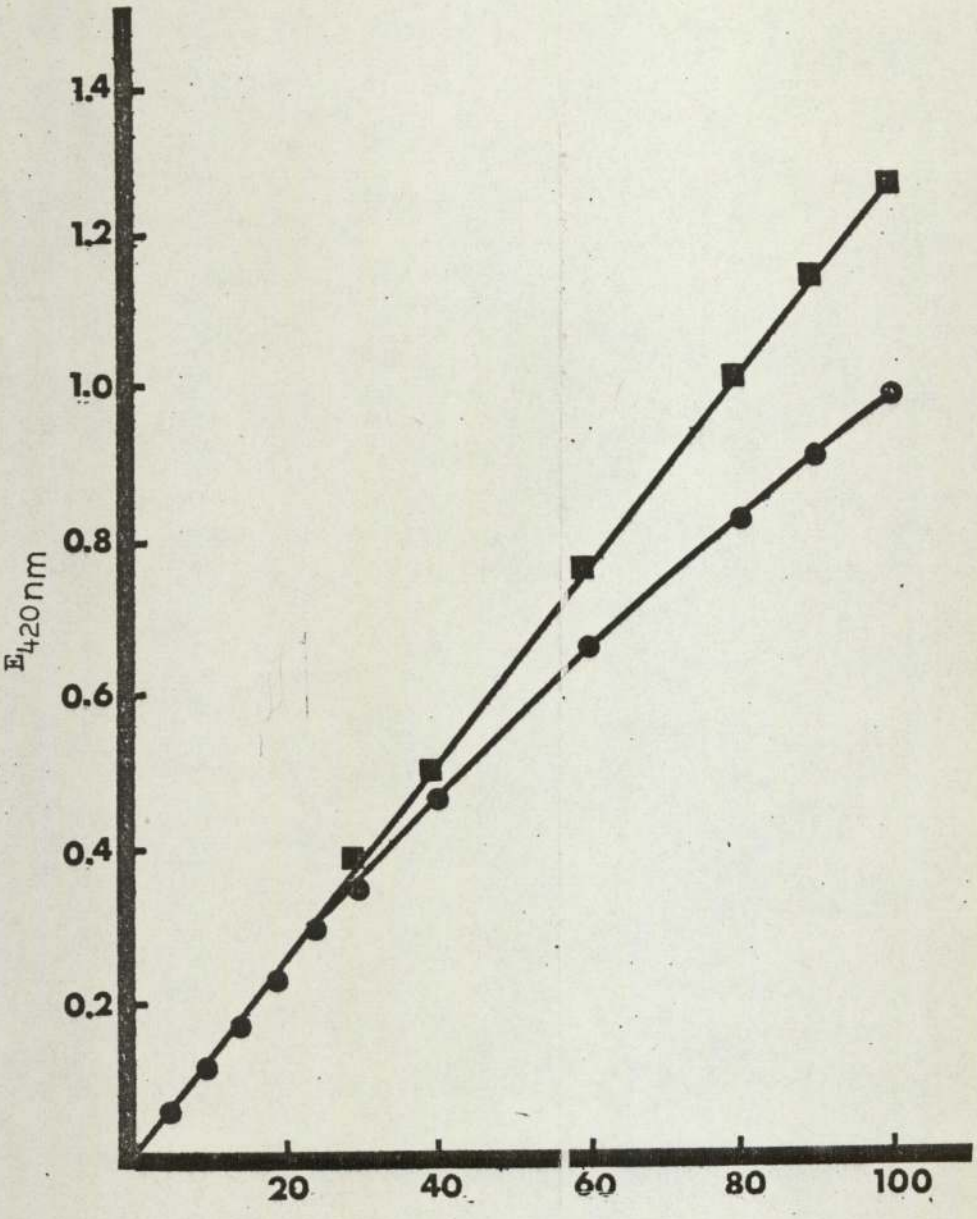


KEY

● Observed E_{420} of undiluted suspension - mean of duplicates

■ Corrected E_{420} of diluted suspension - mean of duplicates
CDM as diluent

Fig8 : Relation between real and observed optical density of E. coli



KEY

● Observed E_{420} of undiluted suspension - mean of duplicates

■ Corrected E_{420} of diluted suspensions - mean of duplicates
water as diluent

Above an E_{420} of 0.25 the OD increased less than expected in proportion to the increase in cell concentration. When the suspensions with OD greater than 0.2 were diluted with the same diluents, linearity was restored. Therefore, in all experiments, suspensions with an OD likely to be greater than 0.2 were diluted 10-fold in distilled water before measurement.

Koch (1961) reported that this reduction in the amount of light scattering in dense suspensions could be due to secondary light scattering redirecting the refracted light toward the photocell in the spectrophotometer. An alternative explanation (Kenward, 1975) is that the cells closest to the incident light prevent the contribution to light scattering of those cells furthest from the light source by "shielding" them.

3.3. PREPARATION OF NUTRIENT DEPLETED CULTURES

A CDM was chosen so that cultures stopped growing exponentially at an appropriate OD (see Table 4, p32).

Glucose limited cultures were harvested at once, while the others were incubated until the OD reached that of the glucose-limited cultures. Depleted cultures were centrifuged at 30° and resuspended to the same OD in a fresh sample of appropriate CDM (see Table 4, p32) which had been prewarmed to 37°. They were then equilibrated for one hr at 37° except for the cultures used for potentiometric titration.

3.4. COLONY COUNTS

Serial dilutions of the cultures (10^{-1}, 10^{-3}, 10^{-5}) were

Table 4: Preparation of depleted cultures

Experiment	Limiting nutrient	OD at end of exponential growth	OD at end of incubation	Resuspending medium
Stability of R-plasmid and Resistance to antibacterial agents	glucose	0.2	0.2	CDM minus glucose
	Mg^{2+}	0.1	0.2	CDM minus glucose & Mg^{2+}
	PO_4^{3-}	0.1	0.2	CDM minus glucose & PO_4^{3-}
	SO_4^{2-}	0.1	0.2	CDM minus glucose & SO_4^{2-}
	K^+	0.1	0.2	CDM minus glucose & K^+
	NH_4^+	0.1	0.2	CDM minus glucose & NH_4^+
Potentiometric titration	glucose	3.0	3.0	Water
	Mg^{2+}	1.0	3.0	
	PO_4^{3-}	1.0	3.0	

prepared using NB. From the final dilution, 0.1 ml was inoculated on to a set of plates and 0.25 ml on another set. The inoculum was spread over the agar surface using a sterile spreader, and then left for 30 min to dry. Plates were incubated at 37° for 24 hr and then the colonies were counted, if possible using plates in which the numbers of colonies ranged between 200 - 300.

3.5. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF ANTIBACTERIAL AGENTS

Graded quantities of stock antibiotic or disinfectant solutions were added to eight test tubes in triplicate, containing 2.5 ml sterile NB (double strength) and the volume made up to 4.9 ml with sterile distilled water if required. Four control tubes containing only NB

and water were prepared. All tubes except one of the controls were inoculated with 0.1 ml culture (final conc approx 10^5 cell), incubated at 37° and examined for growth (turbidity) after 24, 48 and 72 hr.

Samples from tubes showing growth were Gram-stained and examined microscopically to check for contamination. Where appropriate, a loopful of culture from each tube showing growth was streaked on antibiotic agar plates to check for the presence of the R-plasmid.

3.6. TRANSFER OF THE R-PLASMID

R-plasmid RP1 was transferred from *E. coli* J6-2-2(RP1) to *E. coli* K12 W3110 via *E. coli* K12 met$^-$.

E. coli J6-2-2(RP1) and *E. coli* K12 met$^-$(RP1) will be referred to as donors, while E. coli K12 met$^-$ and *E. coli* K12 W3110 as recipients.

Overnight NB cultures of donor and recipient were mixed (1 : 10 respectively) in MacCartney bottles and incubated at 37° for 3 hr. 5 ml Davis Mingioli (DM) medium (see section 2.3.2.) was added and the contents centrifuged for 20 min at 100 rpm using an M.S.E. bench centrifuge. The pellets were resuspended in DM medium.

Serial dilutions up to 10^6-fold were prepared. From each of these dilutions 0.25 ml was inoculated on to the appropriate DM agar plates (Table 5, p34). Plates were incubated at 37° for 48 hr, and compared with controls inoculated with single cultures treated in the same manner.

3.7. STABILITY OF THE R-PLASMID

3.7.1. Resistance conferred by the R-plasmid

Since the R-plasmid RP1 confers resistance to six antibiotics,

Table 5: Selective media used in transferring R-plasmid

Transfer	Donor	Recipient	Selective medium
1	J6-2-2(RP1)	K12 met$^-$	DM + methionine (50 μg ml^{-1}) & ampicillin (1 μg ml^{-1}) (lack of other amino acids eliminates J6-2-2(RP1))
2	K12 met$^-$(RP1)	K12 W3110	DM + ampicillin (1μg ml^{-1}) (lack of methionine eliminates K12 met (RP1))

ampicillin, tetracycline, neomycin, kanamycin, cephaloridine and carbenicillin, the transfer of all determinants was confirmed using "multodisks" (Table 6).

Table 6: Confirmation of complete R-plasmid transfer using multodisks

Antibiotic	Strain of E. coli				
	J6-2-2(RP1)	K12 met$^-$	K12 met^{-1}(RP1)	K12 W3110 K12	W3110(RP1)
Ampicillin (2 μg ml^{-1})	R	S	R	S	R
Neomycin (10 μg ml^{-1})	R	S	R	S	R
Kanamycin (5 μg ml^{-1})	R	S	R	S	R
Tetracycline (10 μg ml^{-1})	R	S	R	S	R
Cephaloridine (5 μg ml^{-1})	R	S	R	S	R
Carbenicillin (100 μg ml^{-1})	R	S	R	S	R

KEY: R resistant i.e. no zone of inhibition

S sensitive i.e. zone of inhibition greater than 2 mm from edge of paper

Nutrient agar plates were flooded with diluted cultures (Garrod, Lambert & O'Grady, 1973, Antibiotics & Chemotherapy, 4th ed., 1973, Churchill Livingstone, Edinburgh & London), the excess removed and the plates allowed to stand for 10 min before adding the multodisks.

Ampicillin, tetracycline and kanamycin were used to study the stability of RP1. The MIC of these antibiotics against the R^- and R^+ strains are shown in Table 7.

Table 7: The MIC of kanamycin, tetracycline and ampicillin against R^- and R^+ cultures

Strain	MIC ($\mu g\ ml^{-1}$)		
	Kanamycin	Tetracycline	Ampicillin
R^-	1 - 2	1 - 2	2 - 4
R^+	>64	32 - 64	>64

3.7.2. The effect of storage on the stability of the R-plasmid

E. coli R^+ was stored on both NA and on CDM slopes at 4°. The stability of the R-plasmid when stored on these slopes was checked by preparing inoculum cultures from the slopes (see section 2.3.3., p23) and using them to grow glucose depleted cultures (OD 0.2).

These cultures were incubated for a further 2 hr and counted on NA plates and NA plates containing kanamycin (20 $\mu g\ ml^{-1}$), tetracycline (10 $\mu g\ ml^{-1}$) or ampicillin (20 $\mu g\ ml^{-1}$).

3.7.3. The effect of nutrient depletion on the stability of the R-plasmid

Glucose (-C), magnesium (-Mg), phosphate (-P), sulphate (-S), potassium (-K) and nitrogen (-N) depleted cultures were prepared

(see section 3.3, p 31) from R^+ cultures stored on CDM slopes for 12 and 16 weeks and counted on NA plates and NA plates containing antibiotics (as in section 3.7.2., p35).

3.8. POTENTIOMETRIC TITRATION OF R^+ AND R^- CELLS

Depleted suspensions were prepared (section 3.3., p31), the OD adjusted to 1.0, and 15 ml transferred to a water-jacketed vessel maintained at 37°. The pH was measured, and then 0.1 ml of hydrochloric acid (pH 2.99) added and the change in the pH of the suspension monitored. This process was repeated until the pH was less than 3. The amount of H^+ bound was calculated from the difference between the H^+ added and the equilibrium pH.

3.9. ACTION OF ANTIBACTERIAL AGENTS ON DEPLETED CULTURES

3.9.1. Measurement of lysis

14.9 ml of a depleted culture (see section 3.3, p 31) was transferred to a 25 ml conical flask at 37° and polymyxin (0.1 ml) was added. Optical density readings were taken 5 min after adding polymyxin and at regular intervals up to 60 min.

3.9.2. Action of disinfectants

Disinfectant-agar plates were prepared from NA autoclaved with the appropriate concentration of disinfectant, and dried for 1 hr at 37°. Depleted cultures were inoculated on to these plates and the counts compared with counts on NA without added disinfectant.

4. RESULTS

4.1. STABILITY OF THE R-PLASMID

4.1.1. Stability on storage

Cultures counted on plates containing the three different antibiotics showed no significant difference at the 95% level (χ^2 test). The results have therefore been plotted as the means of these counts (Fig9) from which it is obvious that when E. coli R^+ was stored on CDM slopes, the R-plasmid was relatively stable compared with its stability when stored on NA plates.

4.1.2. Effect of nutrient depletion on stability

The effect of nutrient depletion on the stability of the R-plasmid was determined on two different occasions, using CDM slopes as inoculum. The results are summarized in Table 8 (p38).

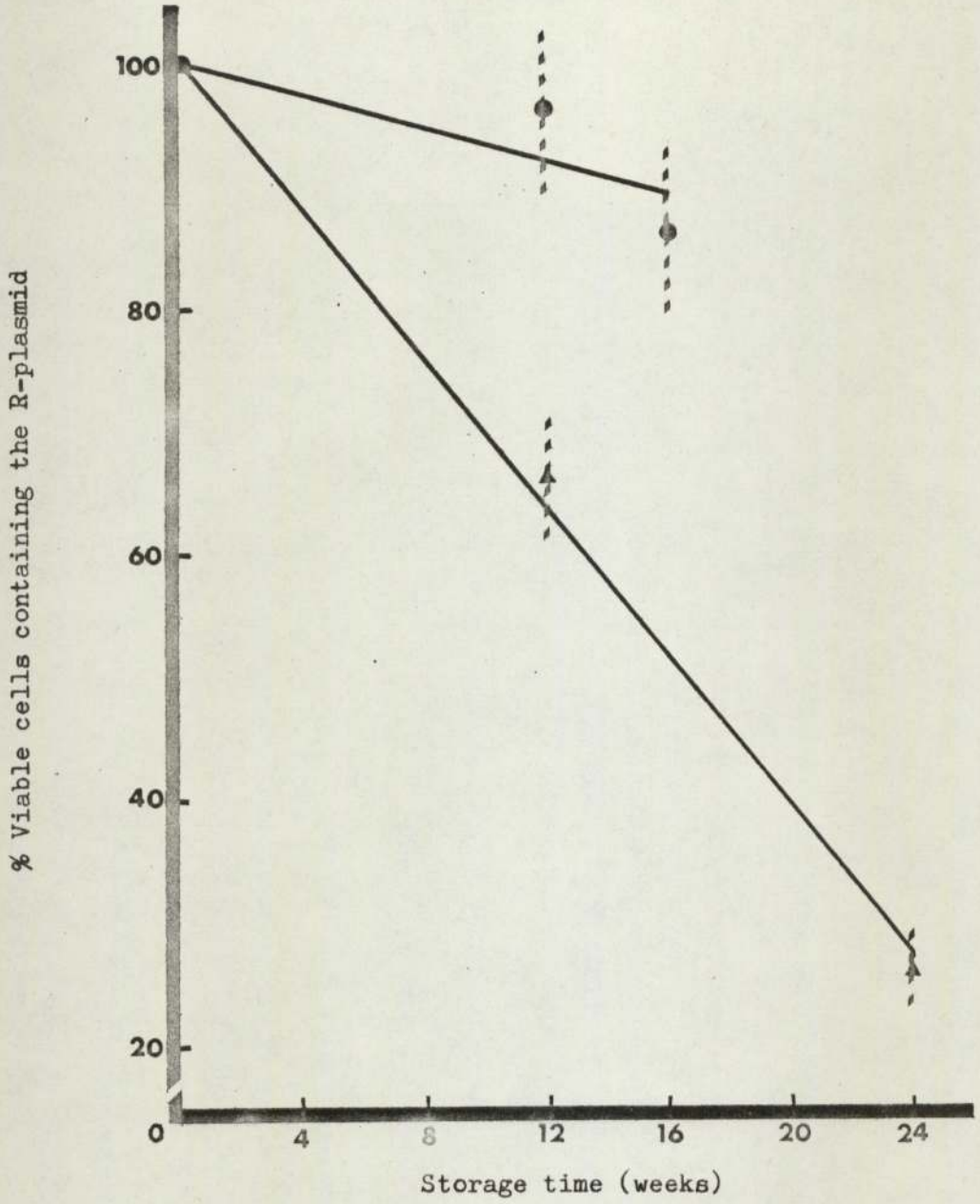
It is clear from Table 8 that there is no significant difference in the % colonies resistant to ampicillin, kanamycin and tetracycline and that the various depletions have no effect on the stability of the R-plasmid.

4.2. DESIGN OF QUANTITATIVELY DEFINED SIMPLE SALTS MEDIA FOR E. coli

The ingredients of CDM suitable for the growth of E. coli are well known (Davis et al., 1973). However, quantitative studies, in which yield is related to nutrient concentration, have not been reported. Such data is needed to design a balanced medium, with no ingredient in gross excess, and to design media which will allow cultures to be depleted of any one ingredient.

For each ingredient studied, the basal medium has to be appropriately adjusted (Tables 2 & 3, p25&26). As far as possible,

Fig 9 : The effect of storage on CDM and NA slopes on the stability of the R-plasmid



KEY

● R^+ culture stored on CDM slope. The results are the mean of three separate tests using agar plates containing Tetracycline (10 μg ml^{-1}), Kanamycin (20 μg ml^{-1}) or Ampicillin (20 μg ml^{-1})

▲ R^+ culture stored on NA slope. The results are the mean of three separate tests using agar plates containing Tetracycline (10 μg ml^{-1}), Kanamycin (20 μg ml^{-1}) or Ampicillin (20 μg ml^{-1})

Confidence limits P = 0.05

Table 8: The effect of nutrient depletion on the % cells carrying the R-plasmid

A. After storage for 12 weeks

Depletion	% Colonies resistant to			$\chi^2_{(2)}$	P
	Ampicillin (20 μg ml^{-1})	Kanamycin (20 μg ml^{-1})	Tetracycline (10 μg ml^{-1})		
- C	97	97	94	0.0625	>0.05
- P	95	98	86	0.84	>0.05
- K	82	98	79	2.42	>0.05
$\chi^2_{(2)}$	1.45	0.0068	1.305		
P	>0.05	>0.05	>0.05		

B. After storage for 16 weeks

Depletion	% Colonies resistant to			$\chi^2_{(2)}$	P
	Ampicillin (20 μg ml^{-1})	Kanamycin (20 μg ml^{-1})	Tetracycline (10 μg ml^{-1})		
- C	88	78.3	93.2	1.33	>0.05
- Mg	92	81	95	1.21	>0.05
- P	99	82	96	1.64	>0.05
- K	72	88	77	1.72	>0.05
- S	97	84	92	0.91	>0.05
- N	92	84	95	0.628	>0.05
$\chi^2_{(5)}$	5.066	0.649	2.871		
P	>0.05	>0.05	>0.05		

the concentration of the ingredients are sufficient to allow a theoretical OD of 10. However, until all ingredients have been studied, there may not be sufficient data to allow this.

4.2.1. Choice of buffer

Most text books recommend pH 7.0 to be used, and early experiments indicated that this would be satisfactory, so it was used throughout. An initial experiment was done using ammonium phosphate adjusted to different pHs as the sole buffer. The results are summarized in Table 9.

Table 9: Effect of pH on growth of E. coli K12 W3110

Initial pH	Final OD reached	Final pH
4.6	0.37	-
5.0	0.35	-
5.5	0.36	3.5
5.9	0.50	3.8
6.4	0.56	5.3
6.5	0.44	-
6.8	0.70	3.9
7.0	0.80 , 0.62	3.8
7.3	0.92	4.0
7.37	0.68	-
7.6	0.76	-

Buffer: 10^{-3}M ammonium phosphate

From the table it is clear that growth ceased at a very low OD, although the basal medium concentration was subsequently found to allow a theoretical OD of 10. The pH after growth was checked; it was very acid and presumably prevented further growth. These results suggest that this concentration of ammonium phosphate has insufficient buffering power to neutralise the acid formed during growth. A higher

concentration of phosphate (0.2M) was used but it was found to be toxic.

Accordingly an organic acid "biological" buffer, MOPS, was used. To check for any possible toxicity that MOPS might have, cultures to which different concentrations of MOPS had been added were compared with a culture to which no MOPS had been added but in which the ammonium phosphate concentration had been raised to 0.1M. From Fig10 it can be seen that cultures with up to 0.5M MOPS had the same doubling time and the same growth yield as one without any MOPS. 0.025M MOPS was therefore used throughout this work, to allow a margin of safety.

4.2.2. Glucose requirement

Both the R^- and R^+ cultures depleted of glucose show characteristic growth curves (Fig11 &12). Growth ceases sharply as glucose becomes limiting.

A linear relationship exists between the maximum OD and glucose concentration (Fig13) in the medium up to about OD 3.0. Later work (Table 10, compare with Table 2) showed that the cultures were unable to grow to higher ODs, because of lack of NH_4^+ and, for the R^+ culture, lack of Mg^{2+}. The requirement for carbon does not differ significantly for the two strains.

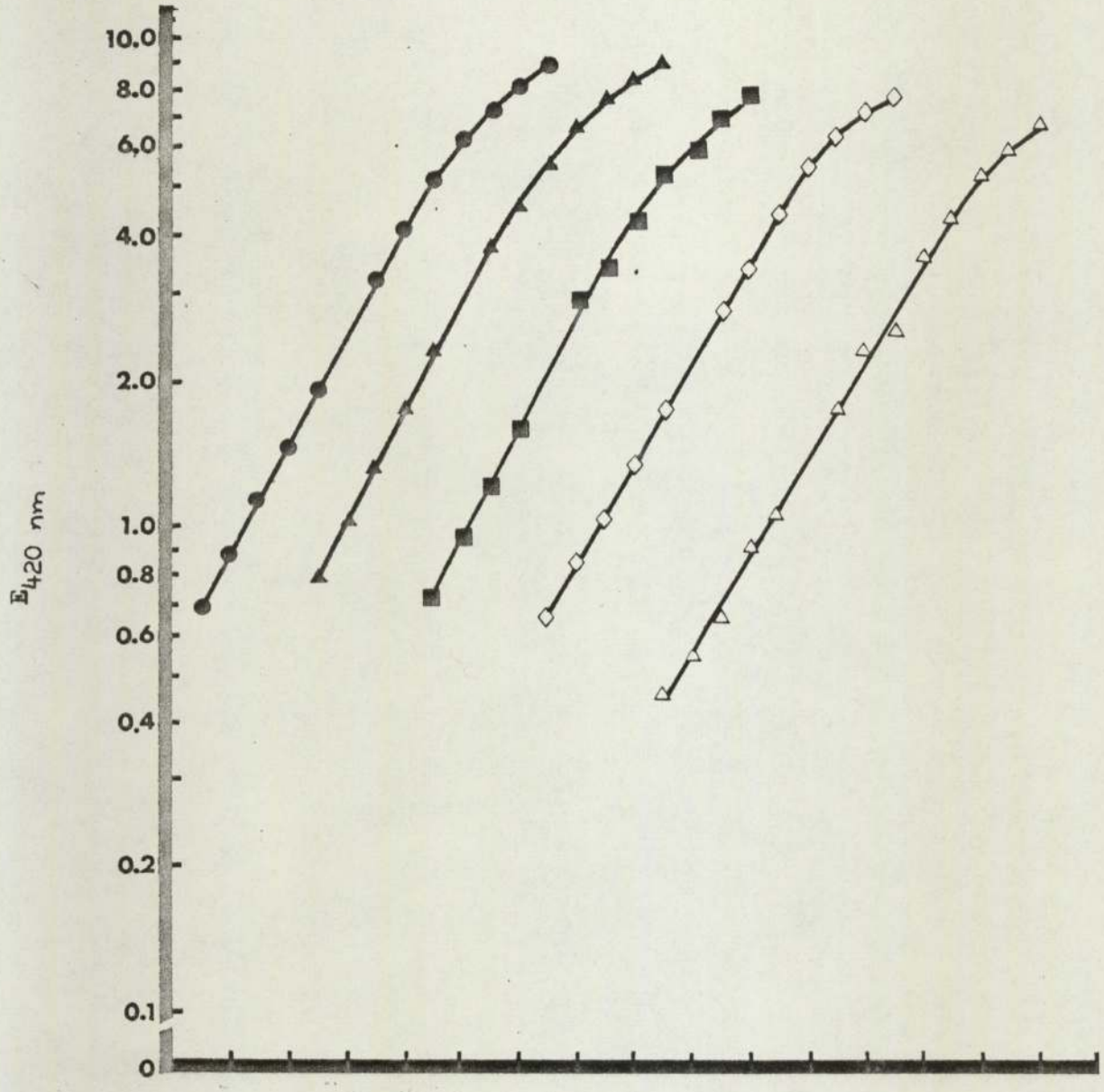
4.2.3. Magnesium requirement

Figs14&15 showsthe growth curves for a range of magnesium concentrations added to the medium, from 6×10^{-6} to 10^{-4}M.

It is clear that, unlike glucose depleted cultures, the growth of - Mg cultures, both R^- and R^+, does not cease sharply, but progressively slows as the Mg^{2+} becomes limiting.

The OD at which growth ceases to be exponential was taken as the onset of limitation and designated as $OD_{(onset)}$. It was

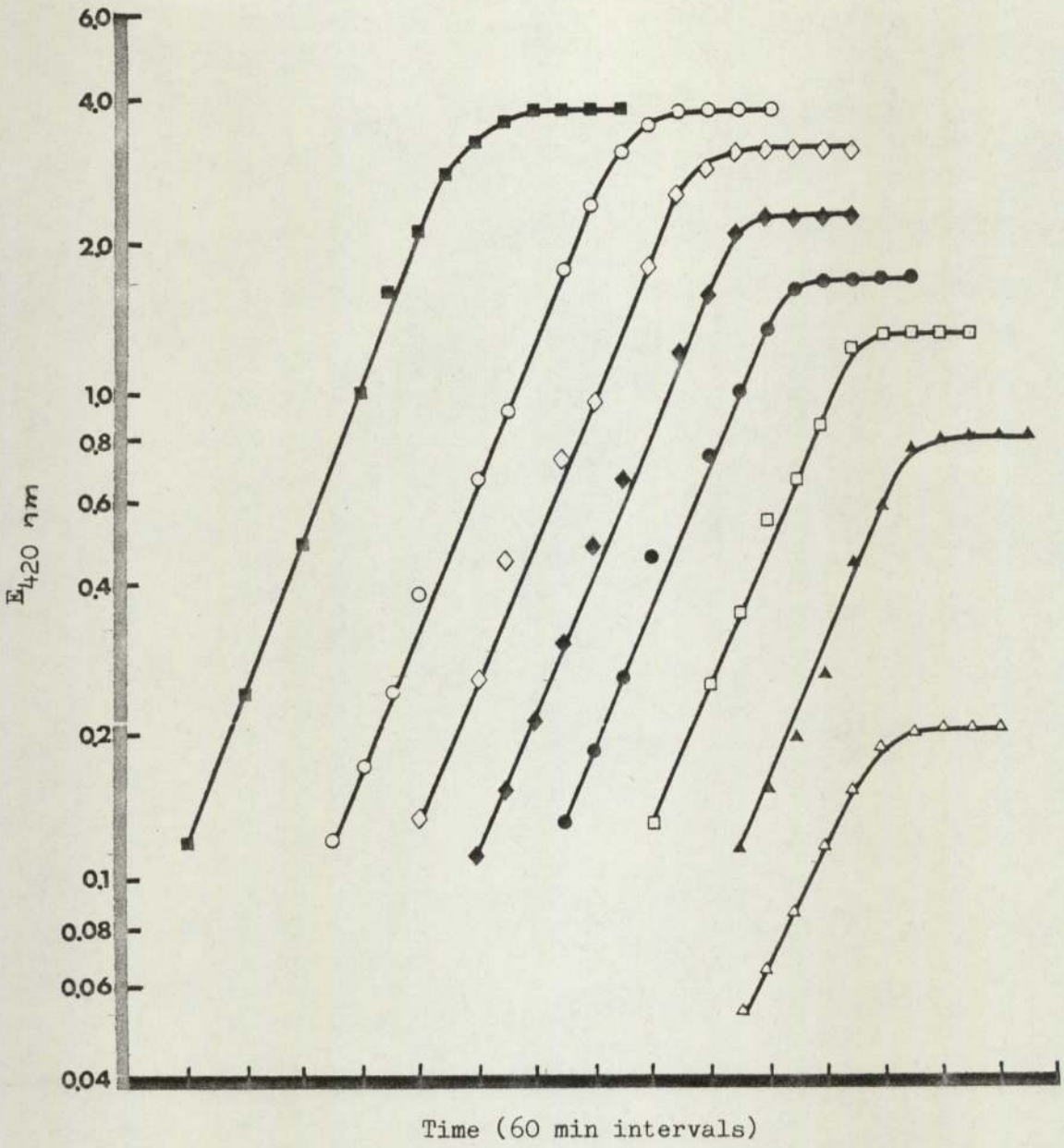
Fig10 : Effect of the concentration of MOPS on doubling time and pH



KEY

MOPS Concentration (M)	Change in pH Initial	Final	Doubling Time (min)
● No added MOPS	7.2	7.3	81
▲ 0.025	7.0	7.3	81
■ 0.05	7.0	7.4	80
◇ 0.075	7.0	7.3	90
△ 0.1	7.0	6.5	90

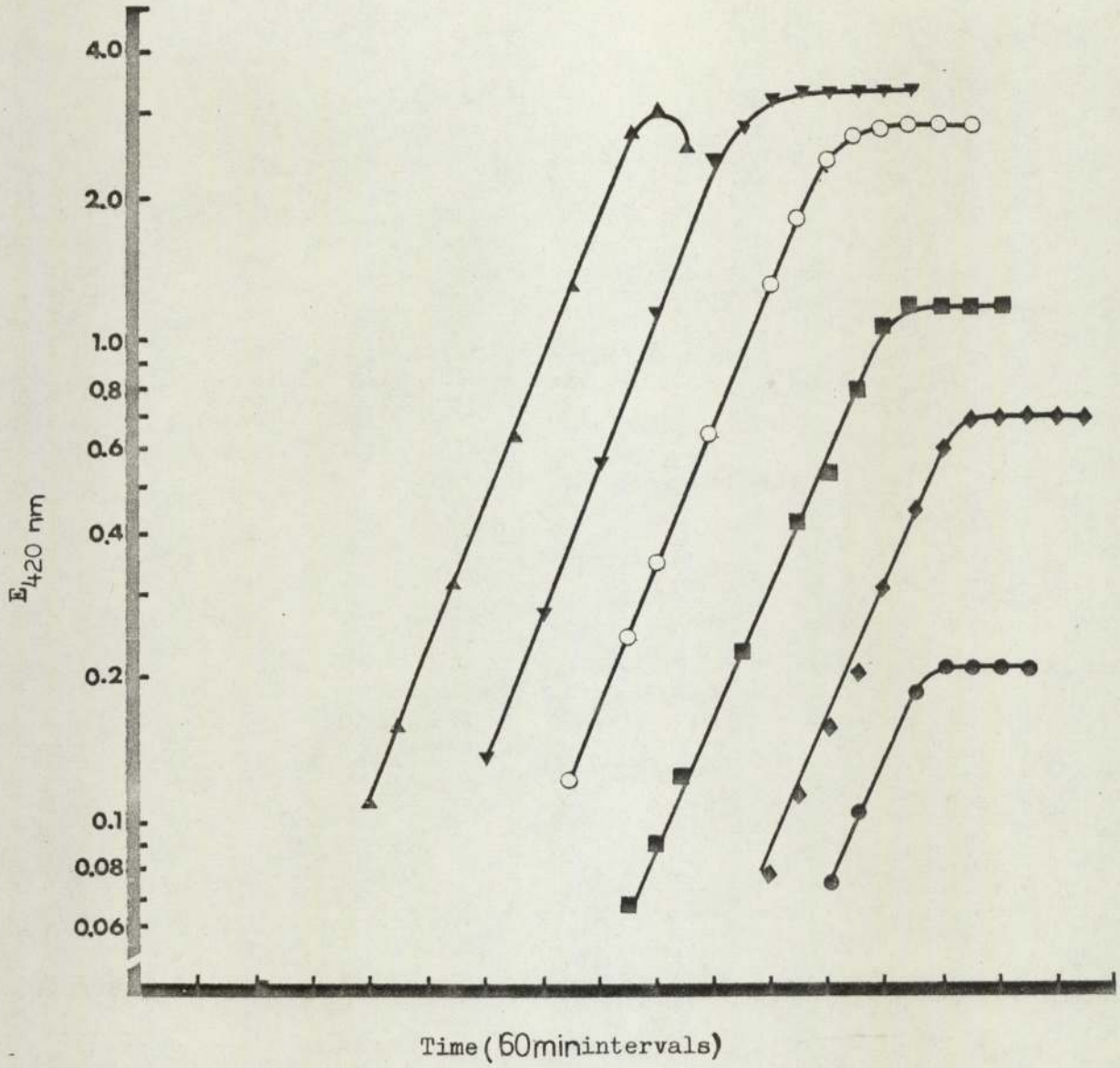
Fig 11 : Effect of glucose concentration on the growth of E. coli R^-



KEY

Glucose concentration ($M \times 10^{-3}$)			
△	0.5	◇	8.0
▲	2.0	○	12.0
□	3.0	■	13.0
●	4.0		
◆	6.0		

Fig 12 : Effect of glucose concentration on the growth of E. coli R^+

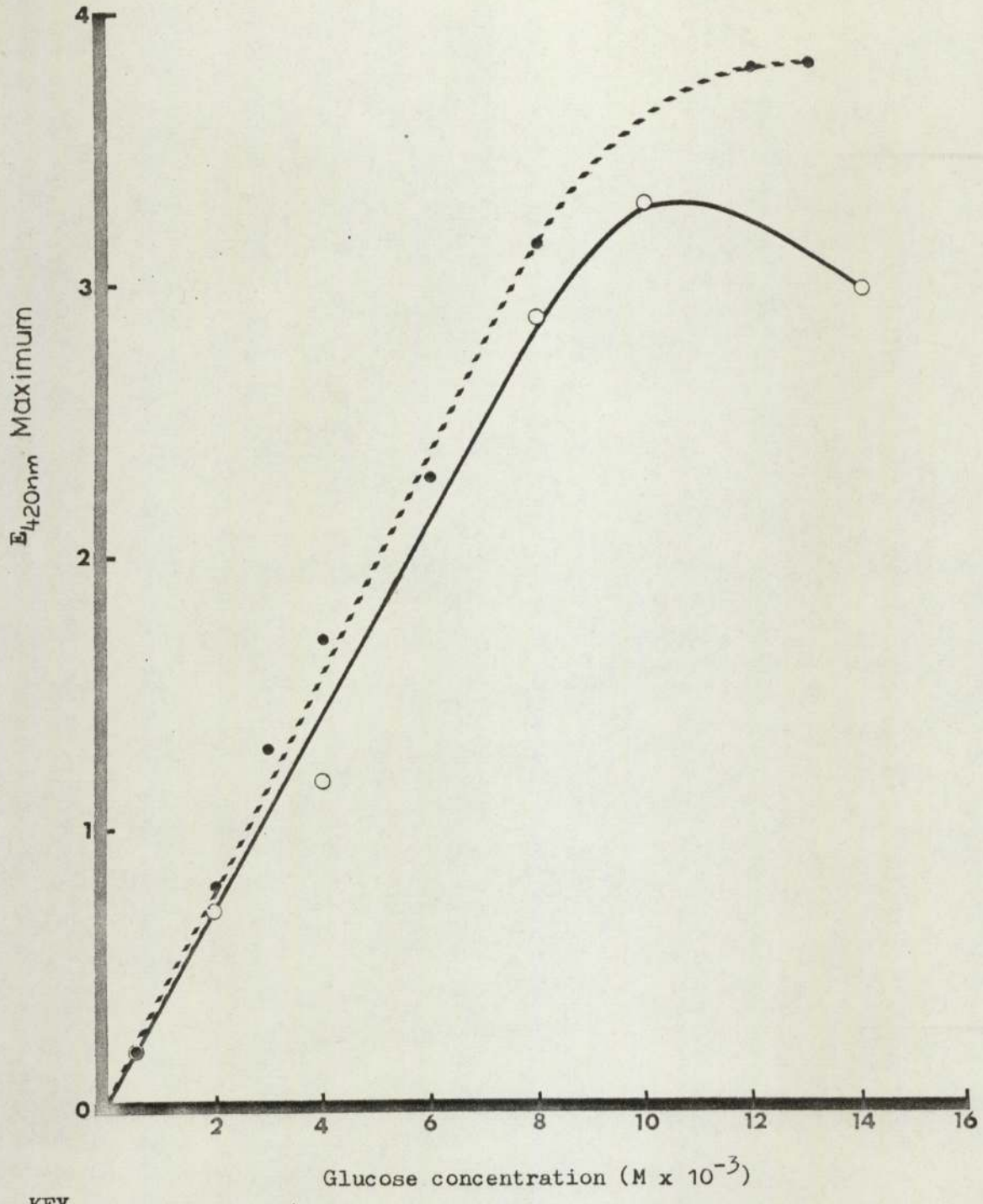


KEY

Glucose concentration ($M \times 10^{-3}$)

● 0.5	▼ 10.0
◆ 2.0	▲ 14.0
■ 4.0	
○ 8.0	

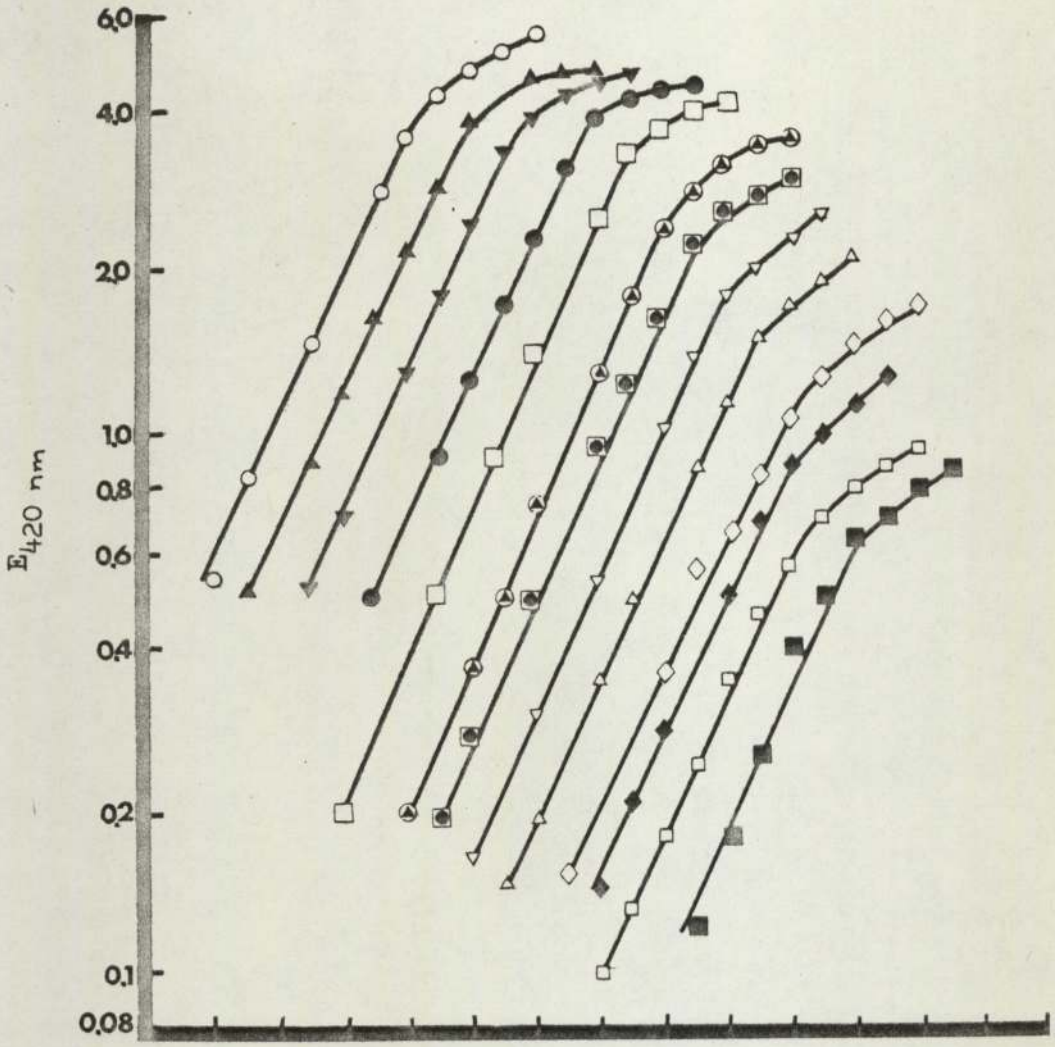
Fig13 : Relation between maximum growth of E. coli R^+ and R^- and added glucose concentration



KEY

Culture	Regression coefficient	P
R^+	0.36	>0.1
R^-	0.39	

Fig 14 : The effect of Mg^{2+} concentration on growth of E. coli R^-

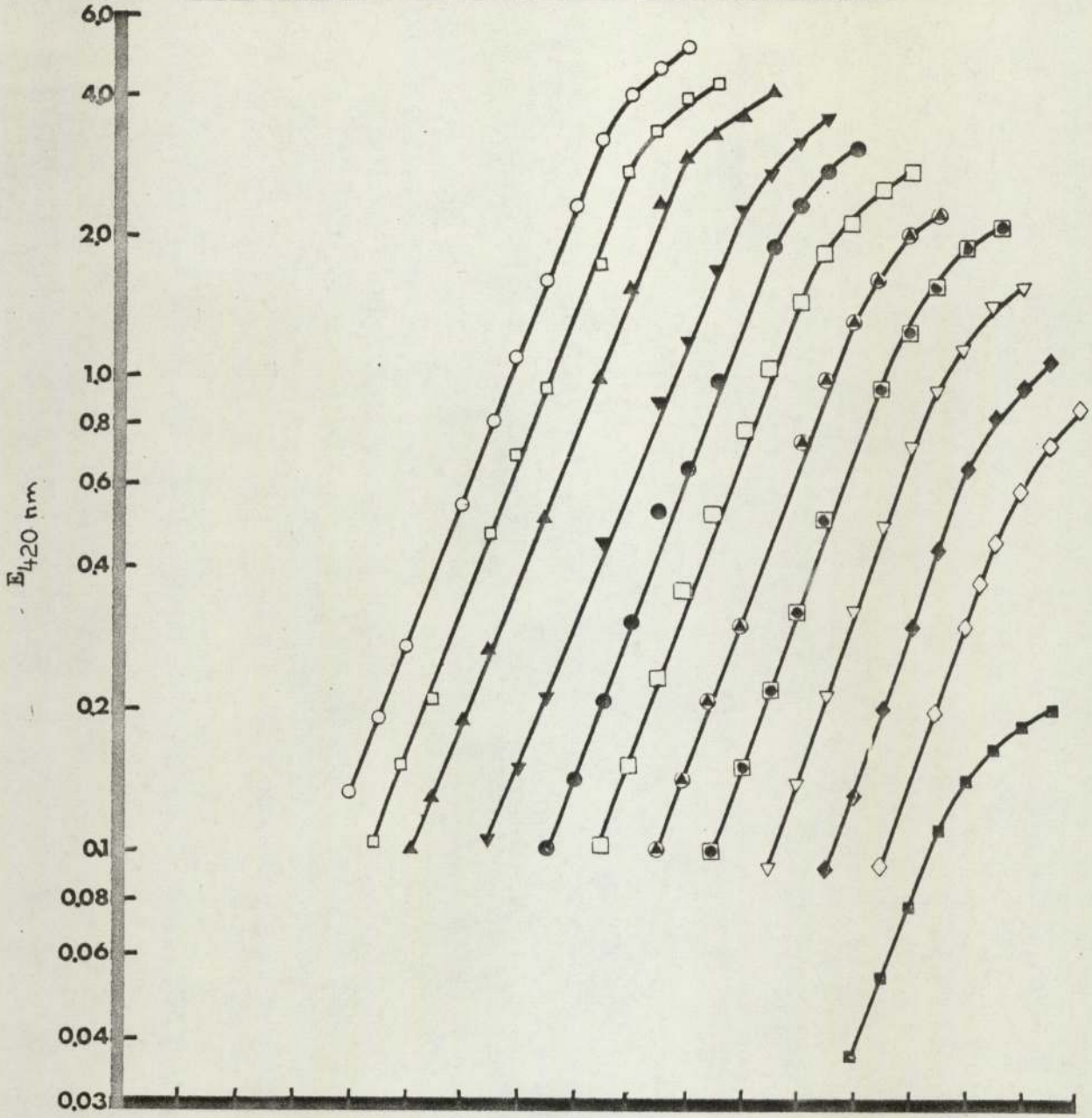


KEY

Added Mg^{2+} concentration ($M \times 10^{-5}$)

■	0.6	⊠	3.0	○	10.0
□	0.8	◬	3.5		
◆	1.0	□	4.5		
◇	1.5	●	5.0		
△	2.0	▼	6.0		
▽	2.5	▲	8.0		

Fig15 : Effect of Mg^{2+} concentration on growth of E. coli R^+



KEY

Added Mg^{2+} concentration ($M \times 10^{-5}$)

	0.4		
◇	1.0	●	5.0
◆	1.5	▼	6.0
▽	2.5	▲	8.0
▣	3.0	□	8.5
◭	3.5	○	10.0
▭	4.5		

plotted against the concentration of Mg^{2+} added to the medium (Fig16).

A linear relationship exists between $OD_{(onset)}$ and Mg^{2+} concentration up to an OD of 3.6 for the R^- culture. For R^+ however, the latter experiment was carried out in a basal medium in which added phosphate was bound to allow a theoretical OD of 2.0.

It is clear from Fig16 that the Mg^{2+} requirement of R^+ cells is about 1.8-fold greater than that of R^- cells, to yield the same cell mass.

The plot of $OD_{(onset)}$ against the concentration of added Mg^{2+} does not pass through the origin. It cuts the abscissa at a concentration equivalent to 1.5×10^{-6}M Mg^{2+} which presumably represents the concentration of Mg^{2+} contaminating the medium, either from the water or from the other chemicals.

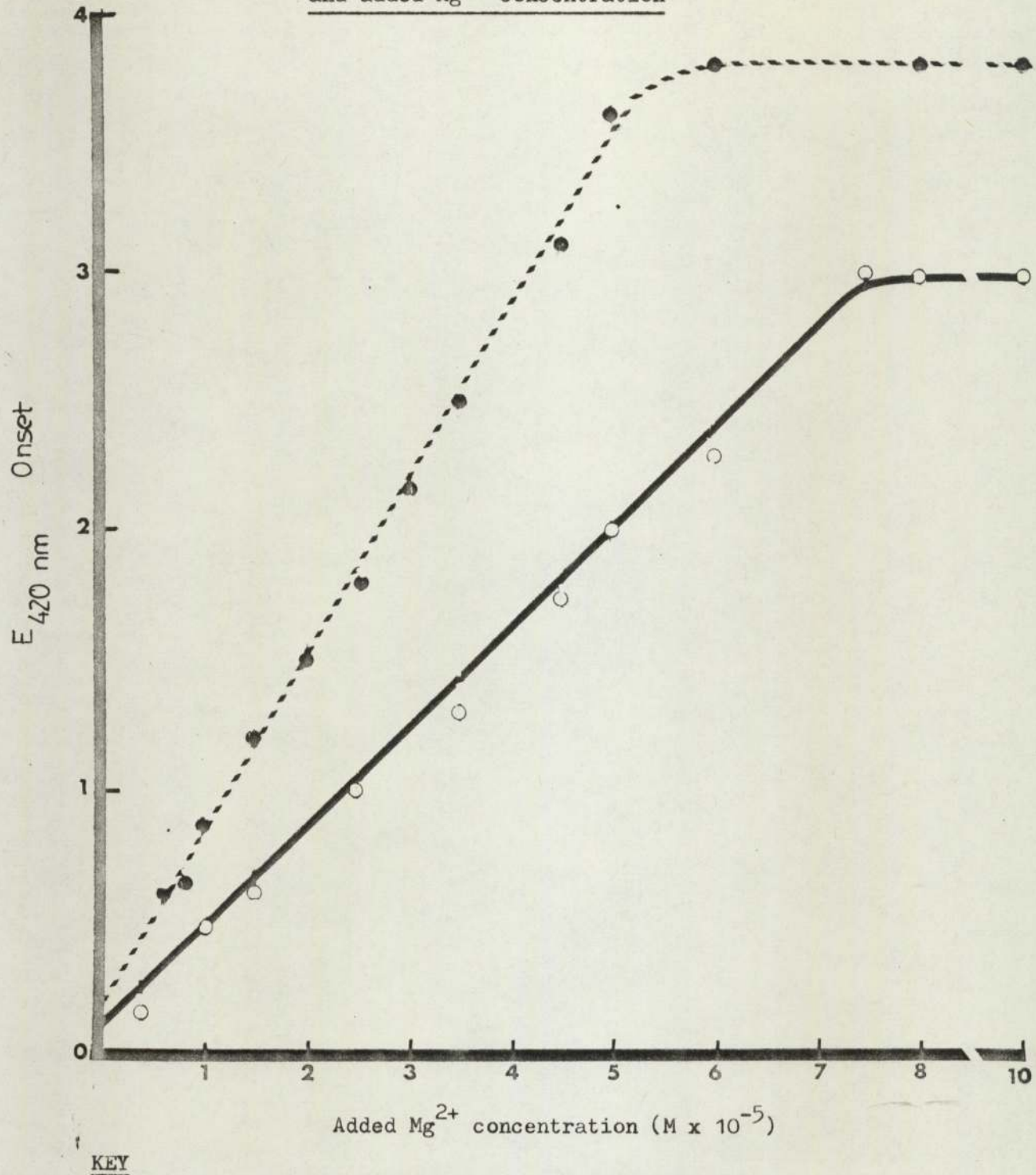
4.2.4. Nitrogen requirement

When NH_4^+ is the limiting component, growth curves for both R^- and R^+ cultures are the same shape as those found for glucose limitation (Figs17 &18).

Fig19 shows the relation between $OD_{(onset)}$ and added NH_4^+ concentration; a linear relationship exists between $OD_{(onset)}$ and NH_4^+ concentration up to OD 3.8 for R^- culture. With the R^+ culture, the relationship appears to be linear up to an OD value of about 2.8. Above this OD the culture was found to be phosphate limited since the concentration of phosphate added was found to allow theoretical OD of 3.0.

No detectable difference in nitrogen requirement was found between the two cultures.

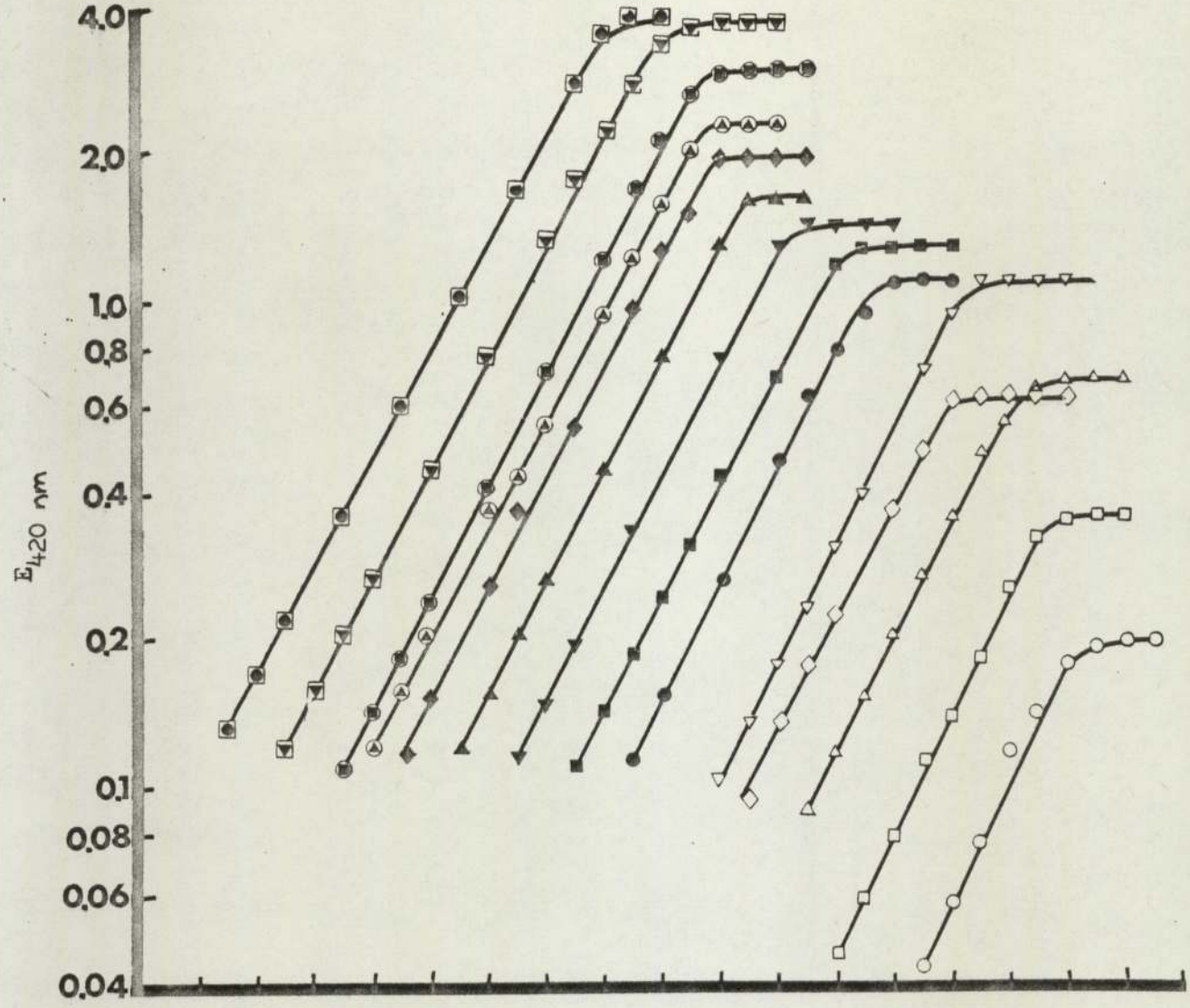
Fig16 : Relation between onset of limitation of E. coli R^- and R^+ and added Mg^{2+} concentration



KEY

Culture	Regression coefficient	P
R^+	0.39	<0.001
R^-	0.67	

Fig17 : Effect of NH_4^+ concentration on the growth of E. coli R^-

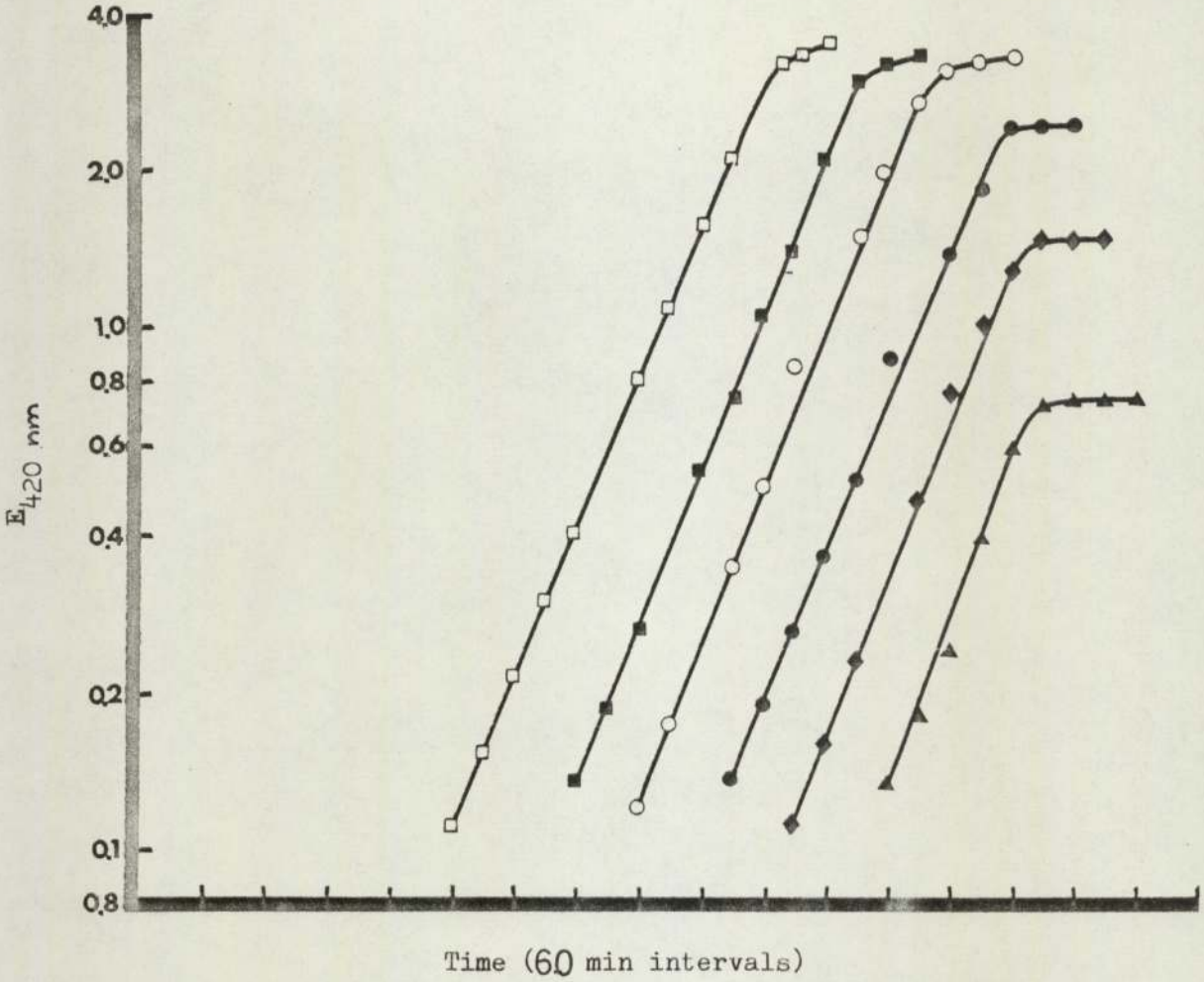


KEY

Added NH_4^+ concentration (M x 10^{-3})

○	0.2	■	2.2	◭	5.0
□	0.6	▼	2.8	◉	6.0
△	0.9	▲	3.2	◪	8.0
◇	1.0	◆	4.0	◙	12.0
▽	1.8				
●	2.0				

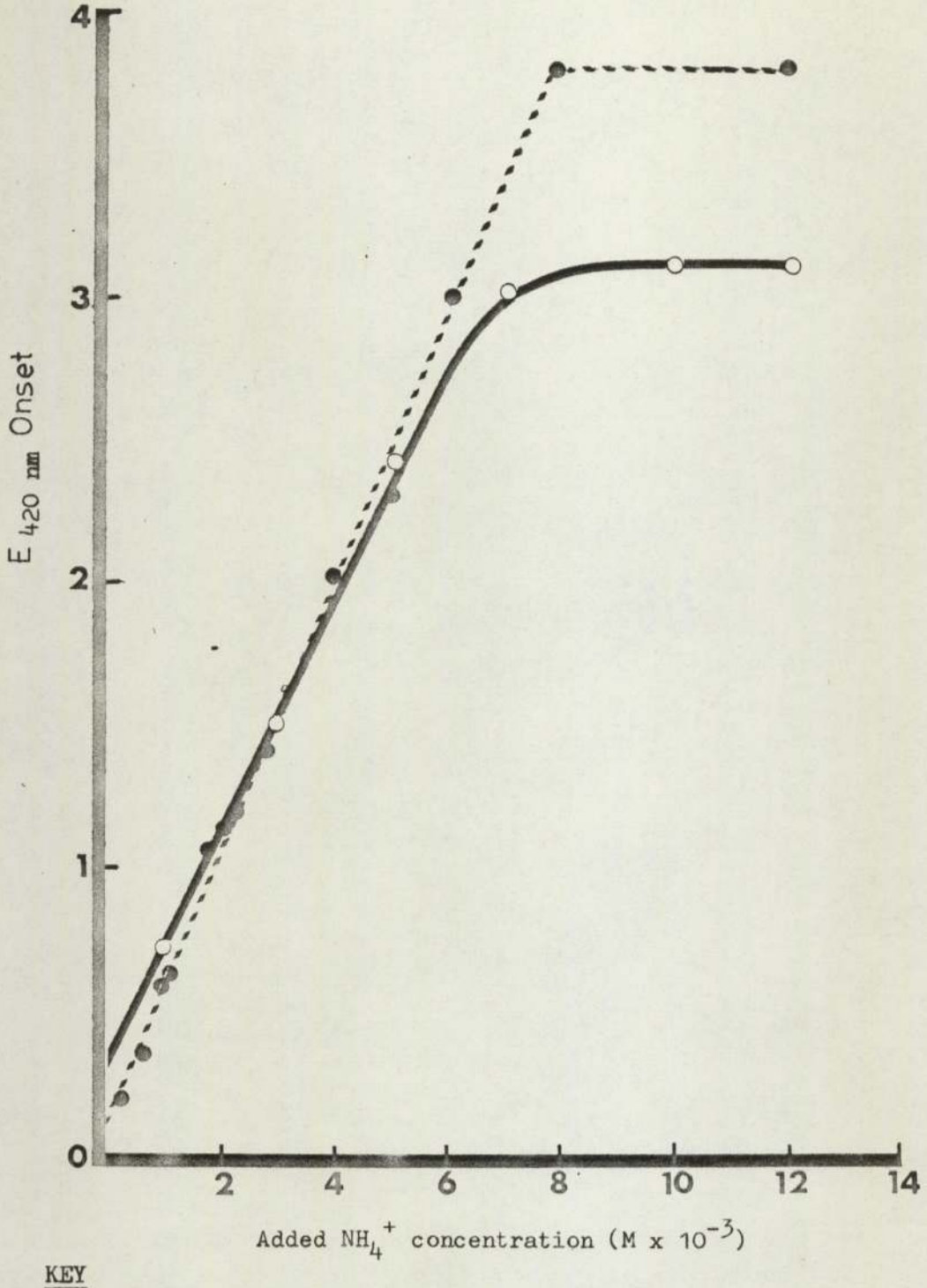
Fig 18 : Effect of NH_4^+ concentration on the growth of E. coli R^+



KEY

Added NH_4^+ concentration ($M \times 10^{-3}$)
▲ 1.0
◆ 3.0
● 5.0
○ 7.0
■ 10.0
□ 12.0

Fig19 : Relation between onset of limitation of E. coli R^- and R^+ cultures and added NH_4^+ concentration



KEY

Culture	Regression coefficient	P
R^+	0.43	
R^-	0.47	> 0.1

4.2.5. Phosphate requirement

Like Mg^{2+} limitation curves, phosphate limitation curves exhibit a slow growth after the onset of limitation (Figs 20&21). The plot of onset of limitation against added phosphate concentration shows a linear relationship up to OD 3.4 for R^-, and up to 2.6 for R^+.

It is clear from Fig 22 that the requirement of R^+ is nearly three-fold more to reach the same OD as that of R^-. The medium contamination, found by extrapolating the plot, was 2×10^{-5}M.

4.2.6. Potassium requirement

Growth curves when potassium limited are similar to those when Mg^{2+} limited, exhibiting slow growth after the onset of limitation (Figs 23&24).

The relationship between the onset of growth and potassium concentration is linear up to an OD of 3.6 for the R^- culture and an OD of 4.5 for the R^+ culture Fig 25.

The requirement of the R^+ for K^+ is more than that of the R^-.

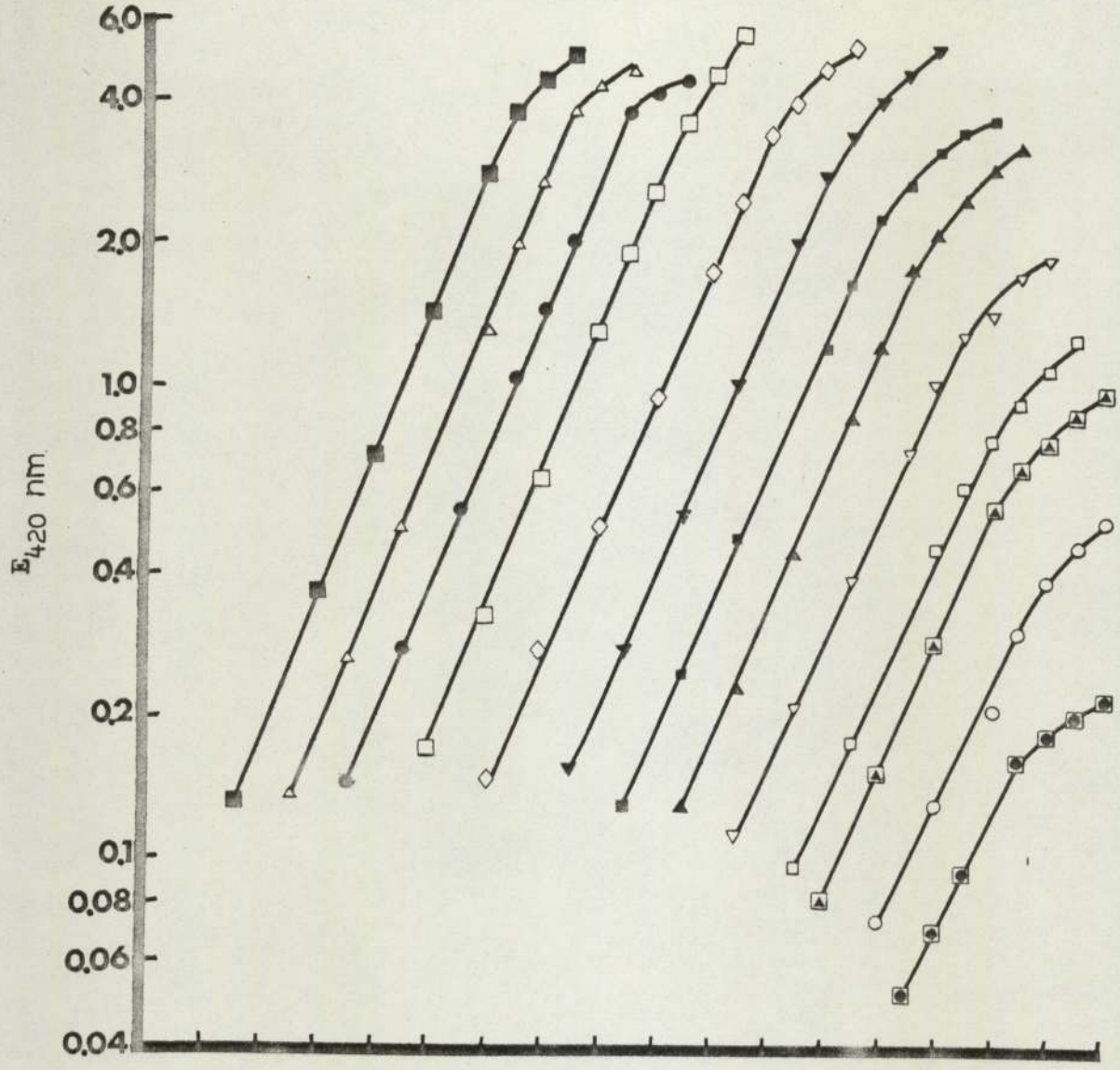
The contamination of the medium, as calculated by extrapolating the plots for the R^- and R^+ cultures on the x-axis, was found to be 0.8×10^{-5}M and 1.2×10^{-5}M respectively.

4.2.7. Sulphate requirement

Growth curves for SO_4^{2-} limitation were also similar to Mg^{2+} limitation curves (Figs 26&27). Linear relationships exist between $OD_{(onset)}$ and SO_4^{2-} concentration for R^- and R^+ up to an OD 3.6 and 4.3 respectively (Fig 28).

Extrapolation of the lines for the R^+ and R^- cultures gave figures of 7×10^{-6}M and 1.1×10^{-5}M respectively, for SO_4^{2-} contamination of the medium.

Fig20: Effect of PO_4^{3-} concentration on the growth of E. coli R^-

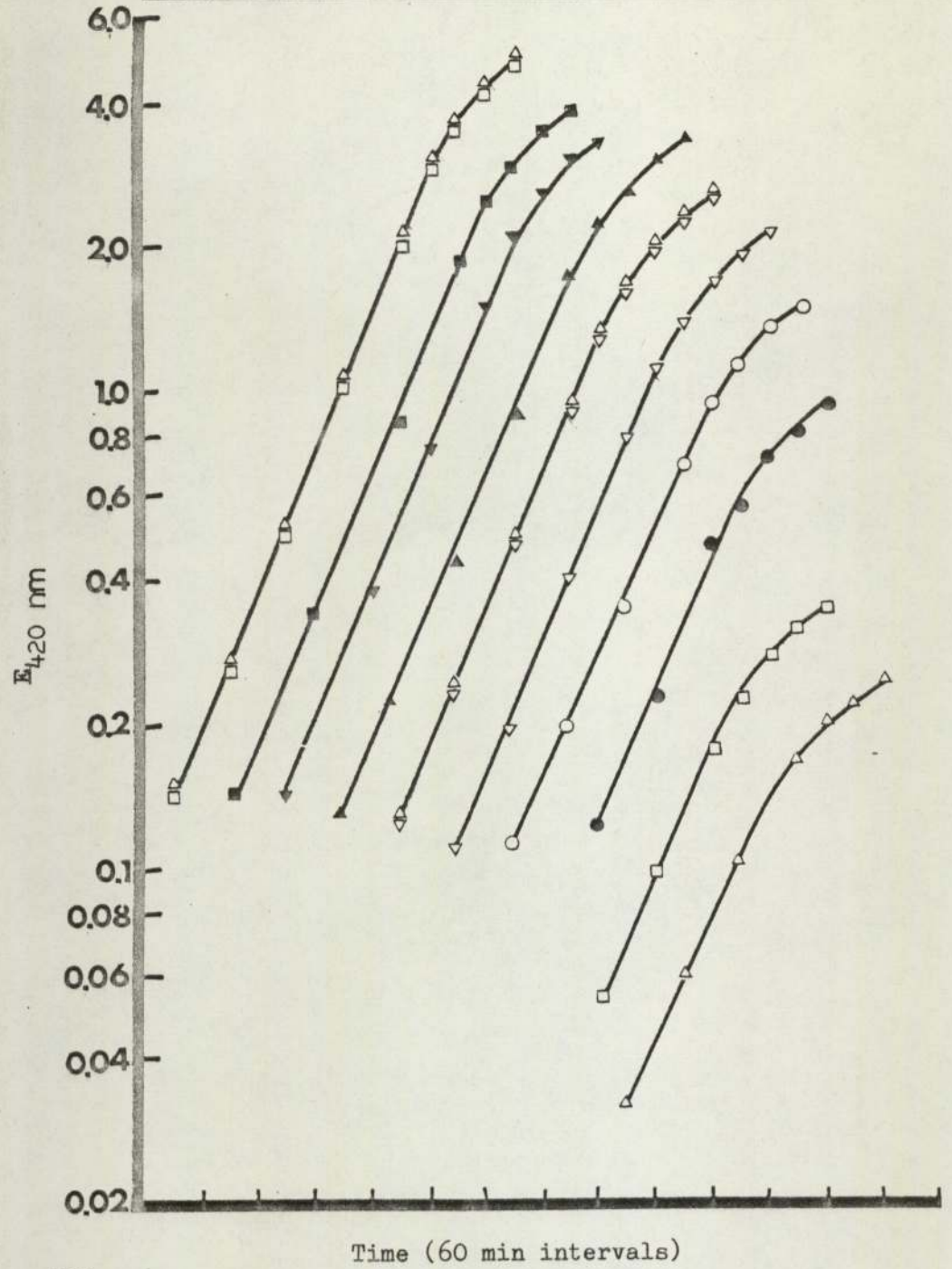


KEY

Added PO_4^{3-} concentration (M x 10^{-4})

◙ 0.2	▲ 3.0	□ 8.0
○ 0.5	■ 4.0	● 10.0
▣ 0.8	▼ 5.0	△ 12.0
□ 1.0	◇ 6.0	■ 20.0
▽ 2.0		

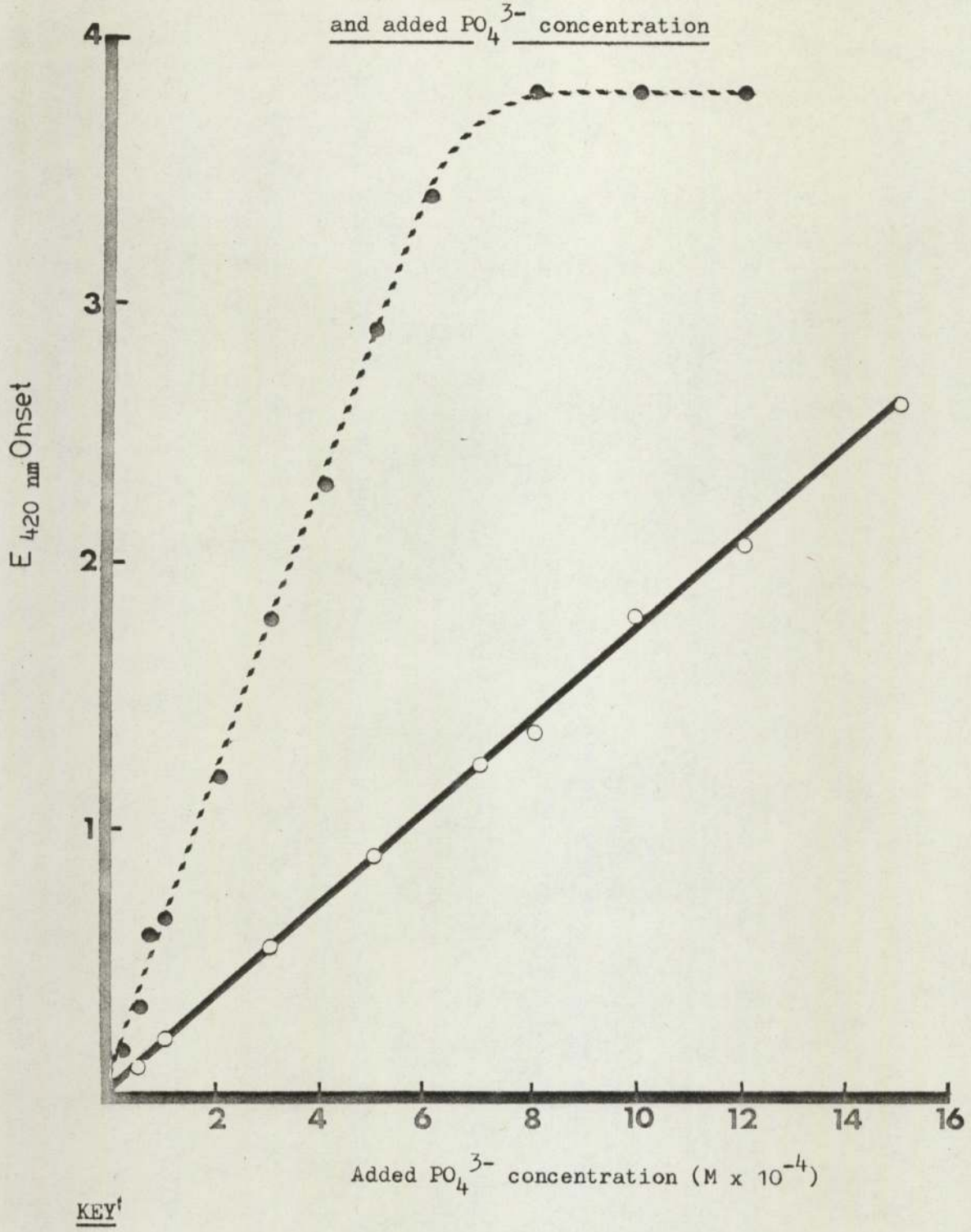
Fig21 : Effect of PO_4^{3-} concentration on the growth of E. coli R^+



KEY

Added PO_4^{3-} concentration ($M \times 10^{-4}$)			
△	0.5	◈	8.0
□	1.0	▲	10.0
●	3.0	▼	12.0
○	5.0	■	15.0
▽	7.0	◈	20.0

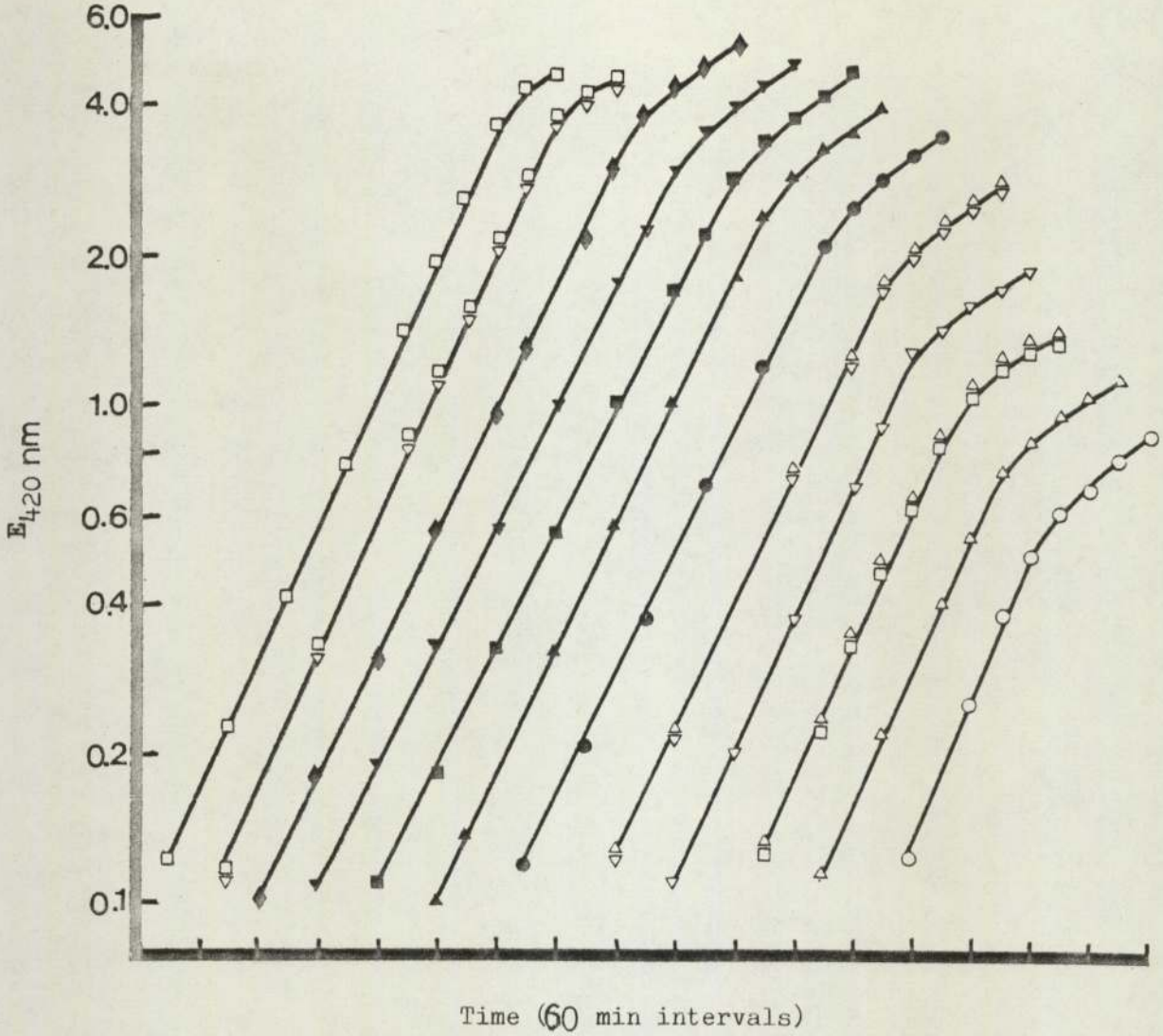
Fig22: Relation between onset of limitation of E. coli R^- and R^+ cultures and added PO_4^{3-} concentration



KEY:

Culture	Regression coefficient	P
R^+	0.173	<0.001
R^-	0.558	

Fig23 : Effect of K^+ concentration on growth of E. coli R^-

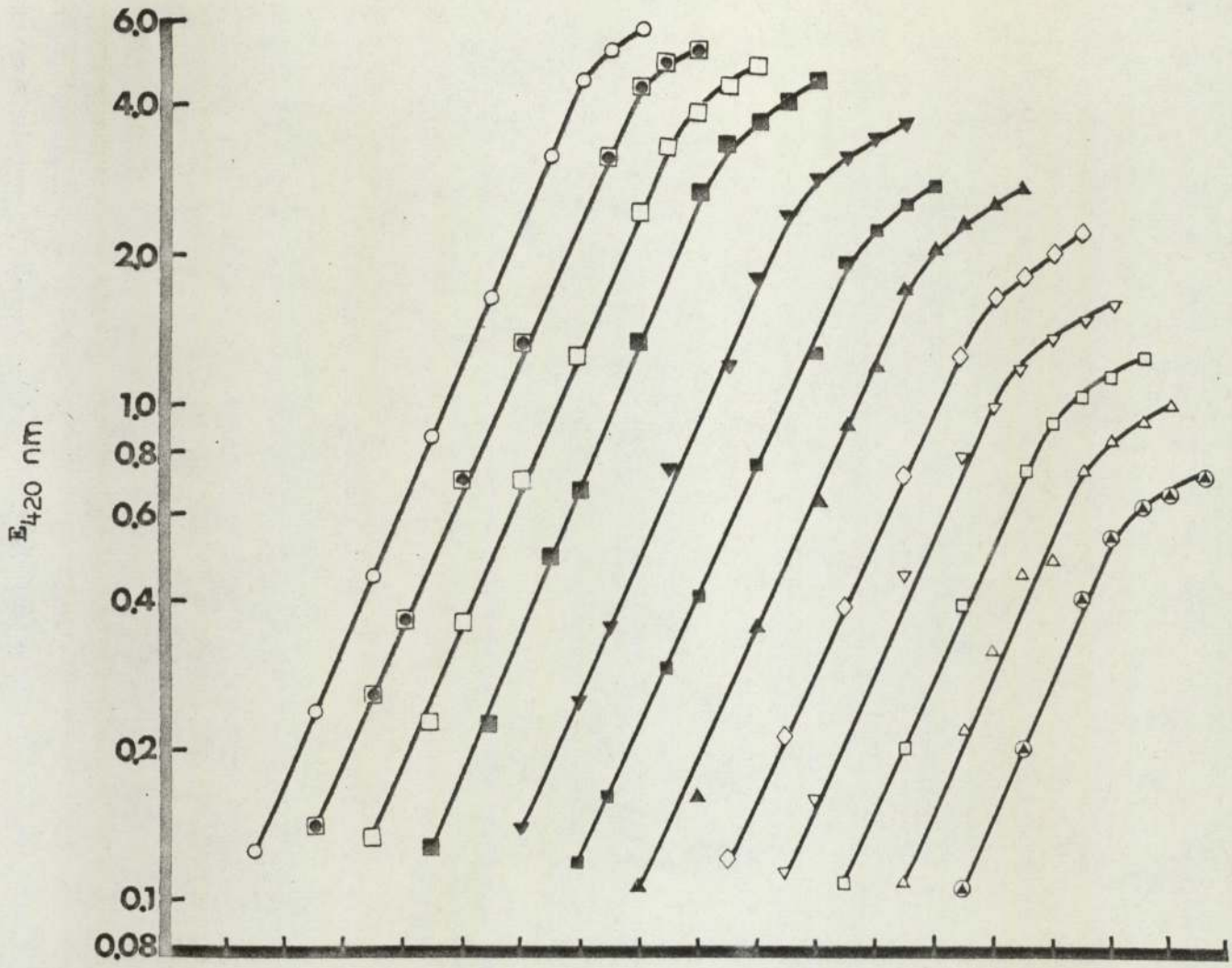


KEY

K^+ concentration ($M \times 10^{-5}$)

○	1.0	▲	9.0
△	2.0	■	10.0
◬	3.0	▼	12.0
▽	5.0	◆	14.0
◈	6.0	⧈	15.0
●	8.0	□	18.0

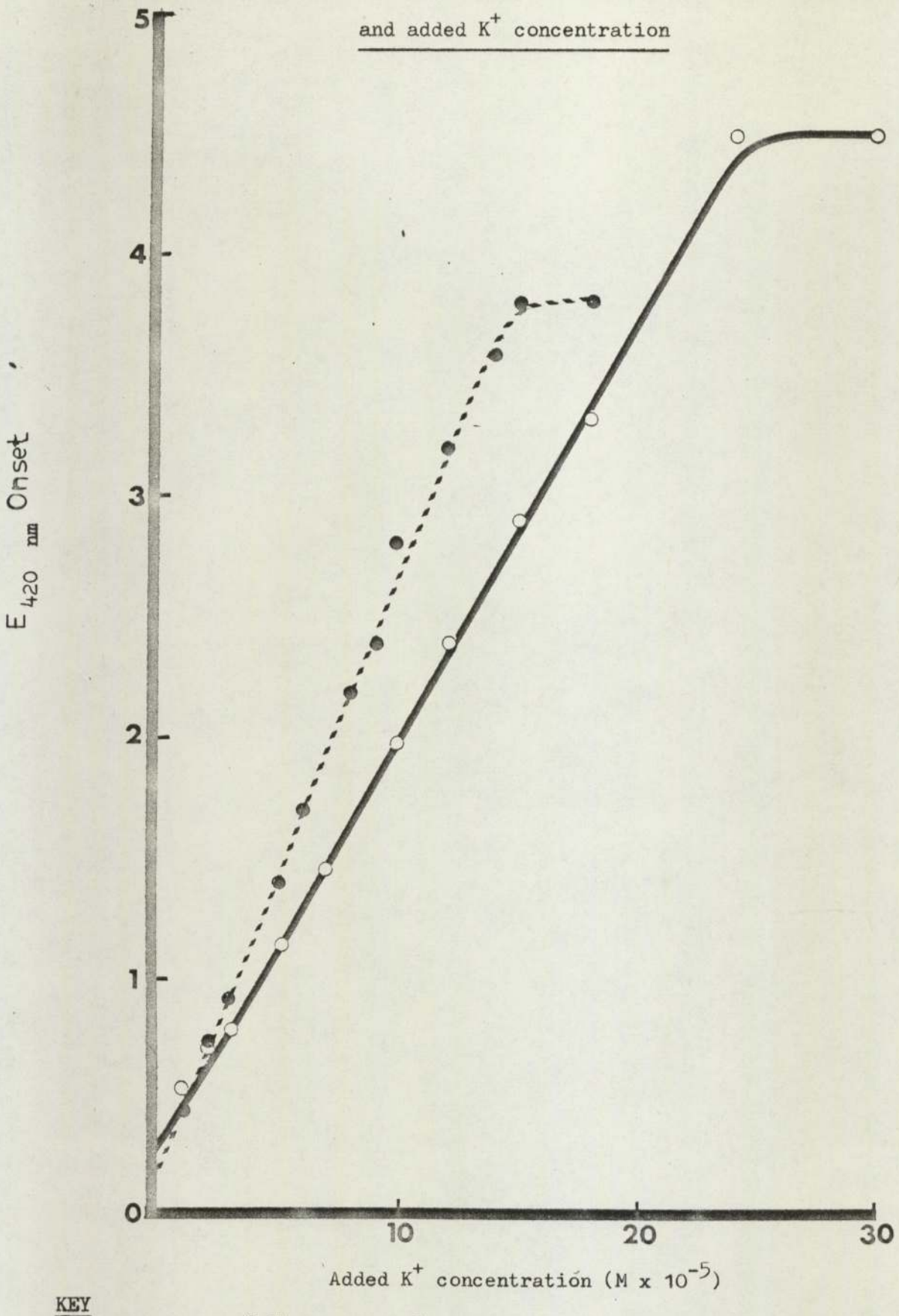
Fig 24 : Effect of K^+ concentration on the growth of E. coli R^+



KEY — K^+ concentration ($M \times 10^{-5}$)

◉	1.0	■	10.0
△	2.0	▼	12.0
□	3.0	■	15.0
▽	5.0	□	18.0
◇	7.0	◙	25.0
▲	9.0	○	30.0

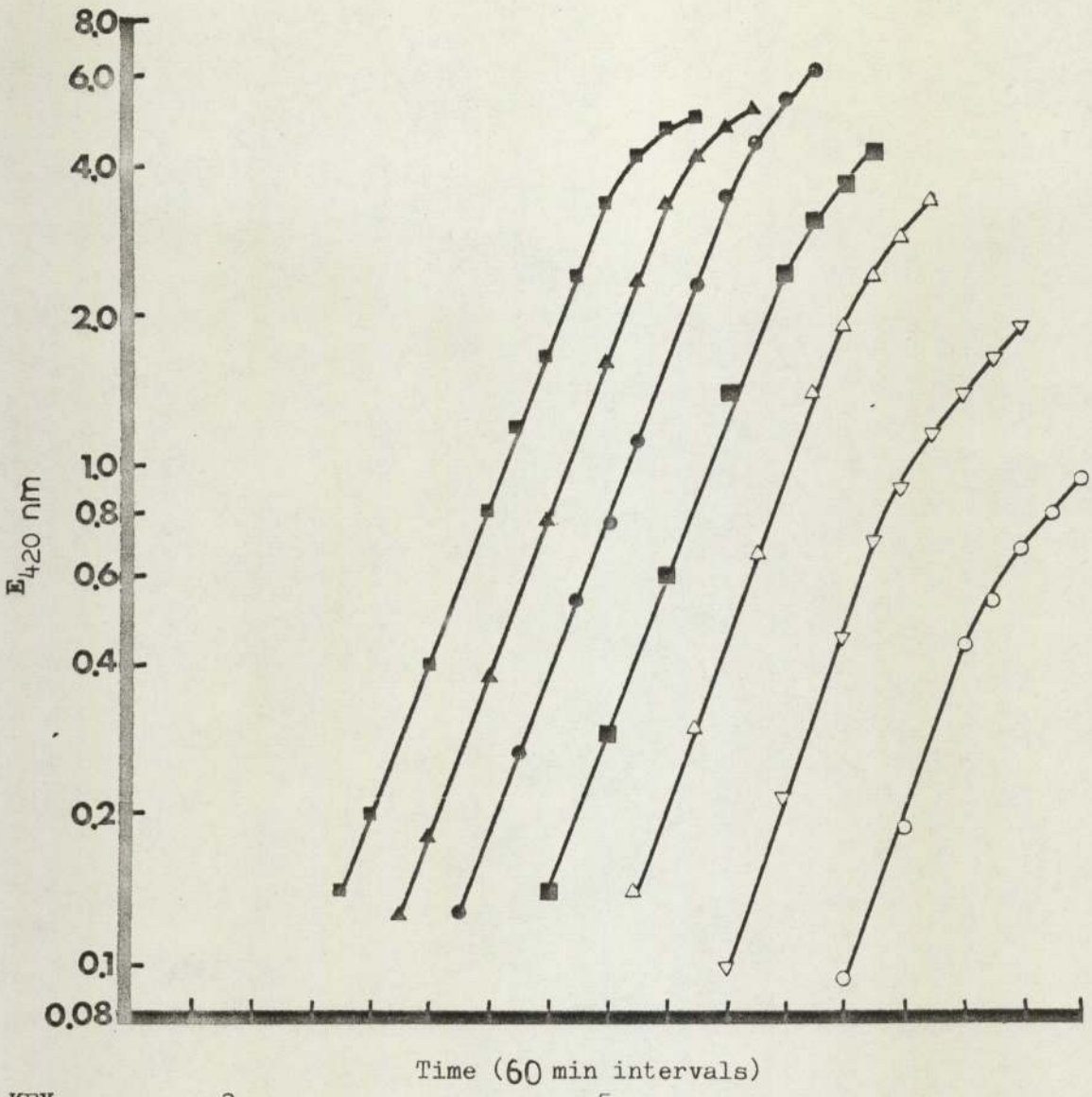
Fig 25 : Relation between onset of limitation of E. coli R^+ and R^- cultures and added K^+ concentration



KEY

Culture	Regression Coefficient	P
R^+	0.172	< 0.001
R^-	0.247	

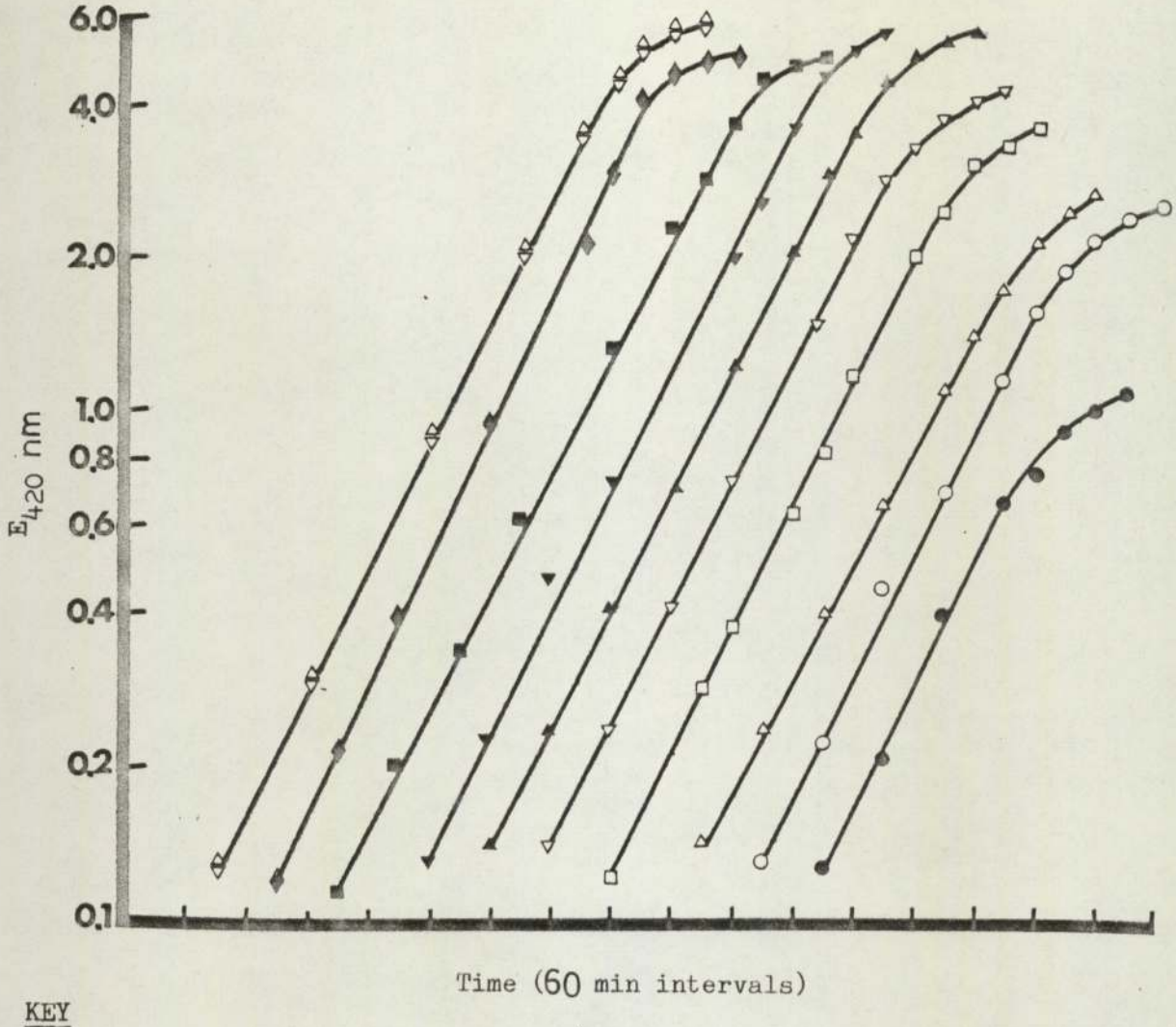
Fig 26 : Effect of SO_4^{2-} concentration on the growth of E. coli R^-



KEY

	SO_4^{2-} concentration (M x 10^{-5})		
○	0.4	▲	15.0
▽	2.0	■	20.0
△	6.0		
■	8.0		
●	12.0		

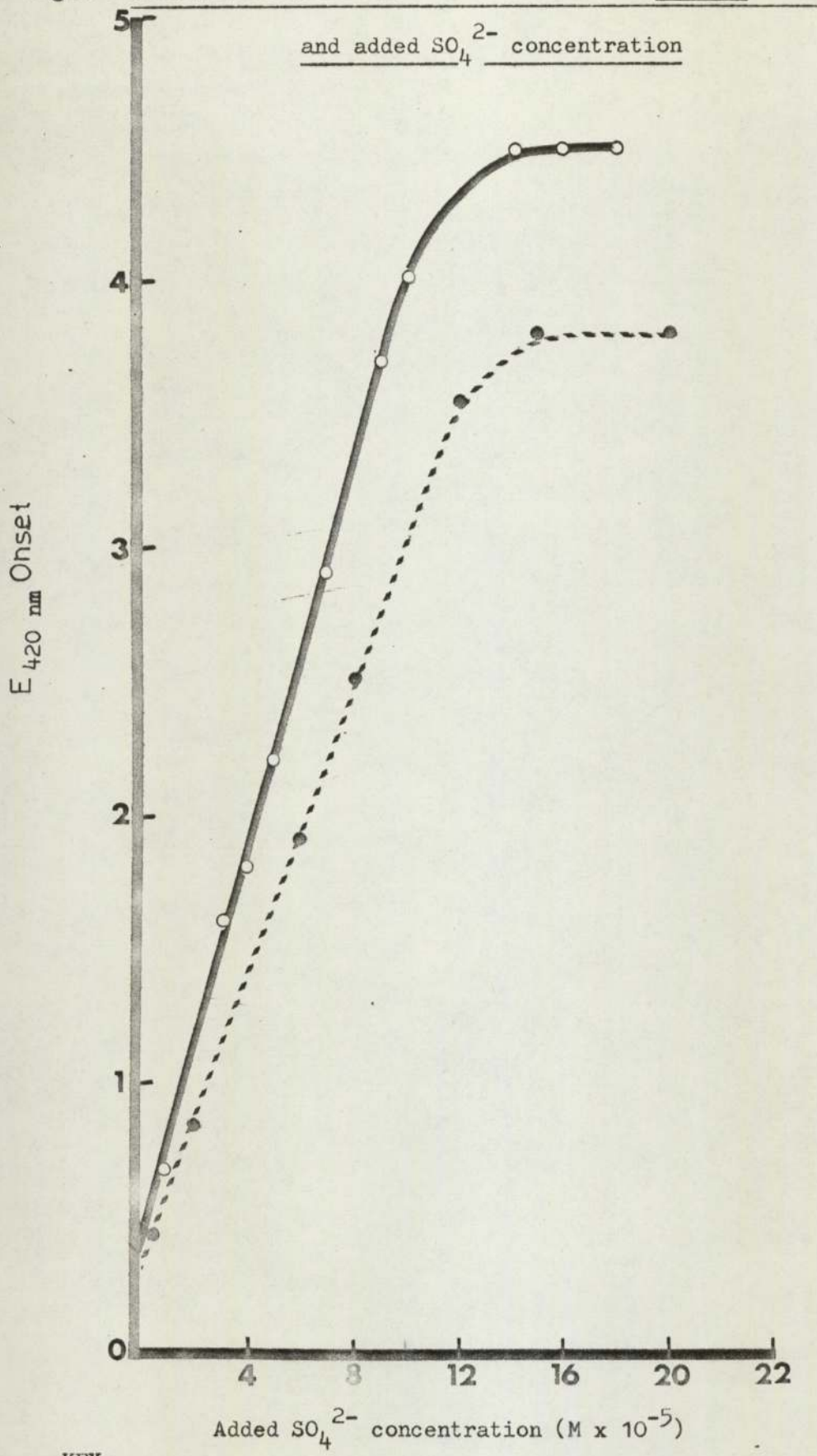
Fig27 : Effect of SO_4^{2-} concentration on the growth of E. coli R^+



KEY

Added SO_4^{2-} concentration ($M \times 10^{-5}$)			
●	1.0	■	14.0
○	3.0	♦	16.0
△	4.0	⟠	18.0
□	5.0		
▽	7.0		
▲	9.0		
▼	10.0		

Fig28 : Relation between onset of limitation of E. coli R^- and R^+ cultures and added SO_4^{2-} concentration



KEY

Culture	Regression coefficient	P
R^+	0.366	< 0.1
R^-	0.271	

4.2.8. Effect of sodium and chloride ions upon growth

The requirement for Na^+ and Cl^- ions of both R^- and R^+ was tested using three media, a control containing no added salt, and two others containing 5×10^{-5}M Na^+ (as Na_2SO_4) and 1×10^{-4}M Cl^- (as NH_4Cl).

Fig29 shows no difference between growth of the test cultures and the controls.

The R^+ culture was tested in a medium allowing growth up to OD 4.5, but the R^- was tested in medium containing glucose in sufficient concentration to allow growth to an OD of 1.0.

4.2.9. The effect of ferrous ion on growth

Fig30 shows the effect of ferrous ion on growth of the R^- culture. It shows that the requirement is satisfied by the amount of ferrous ion present in the medium from other constituents.

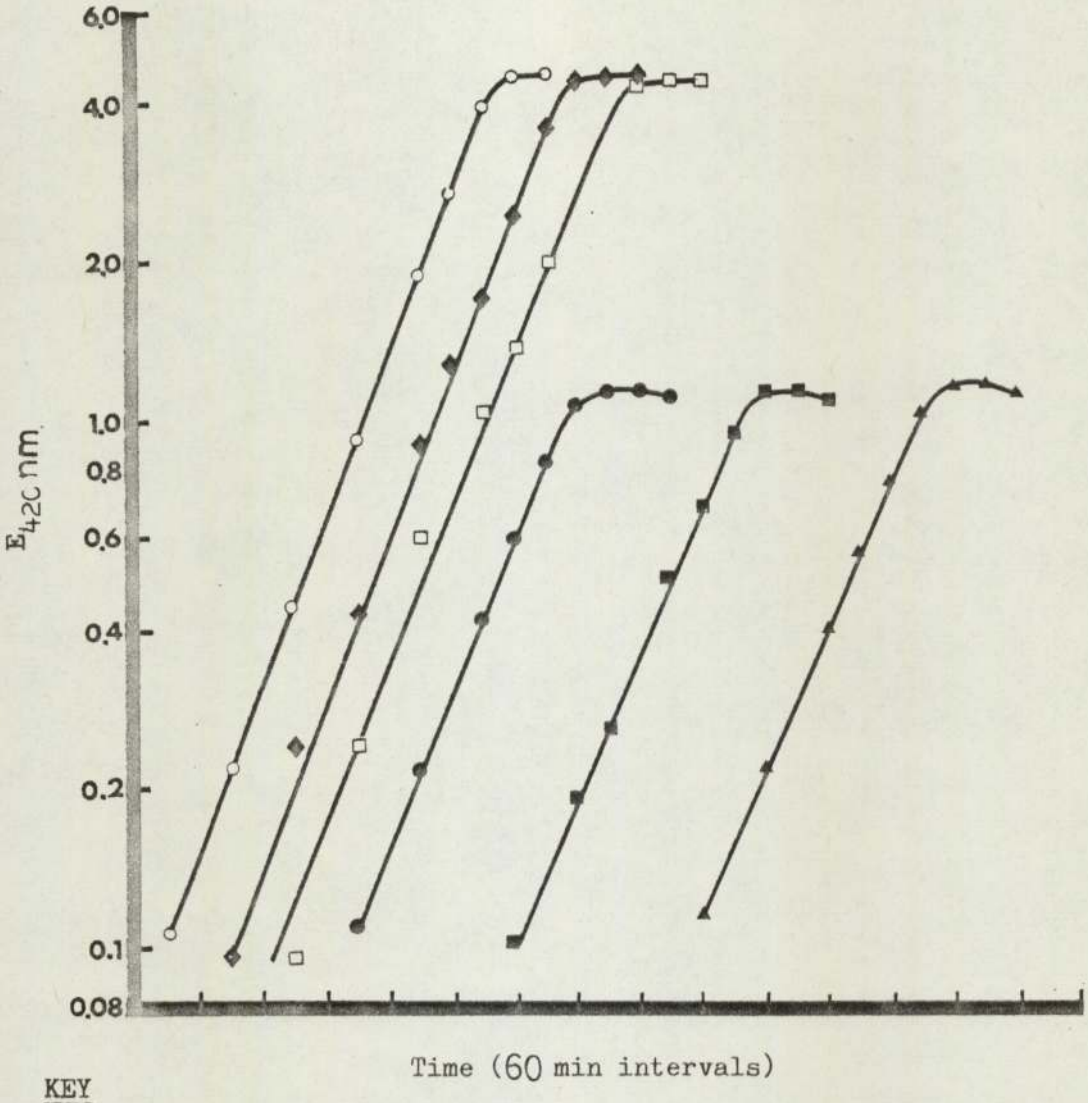
Fig31 shows the effect of different concentrations of ferrous ion on R^+ cells. The growth limitation curves are similar to those of Mg^{2+}. A linear relationship exists between ferrous ion concentration and $OD_{(onset)}$ up to a value of 4.8 (Fig32).

4.2.10. The effect of trace elements on growth

Fig30 also shows that when a mixture of 0.1 μg ml^{-1} of each of the following salts : $ZnSO_4.7H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $CaCl_2.2H_2O$, $CoCl_2.6H_2O$ and $MnSO_4.4H_2O$ is added, the growth of R^- culture shows no difference from the control. Trace ions present as contaminants in the media are presumably sufficient for the growth of the R^- culture.

Fig33 shows the effect of trace ions on the growth of R^+ culture. 0.1 μg ml^{-1} or 0.2 μg ml^{-1} resulted in slight increase in growth (from OD 2.0 to 2.7 or 2.5 respectively). However, when the

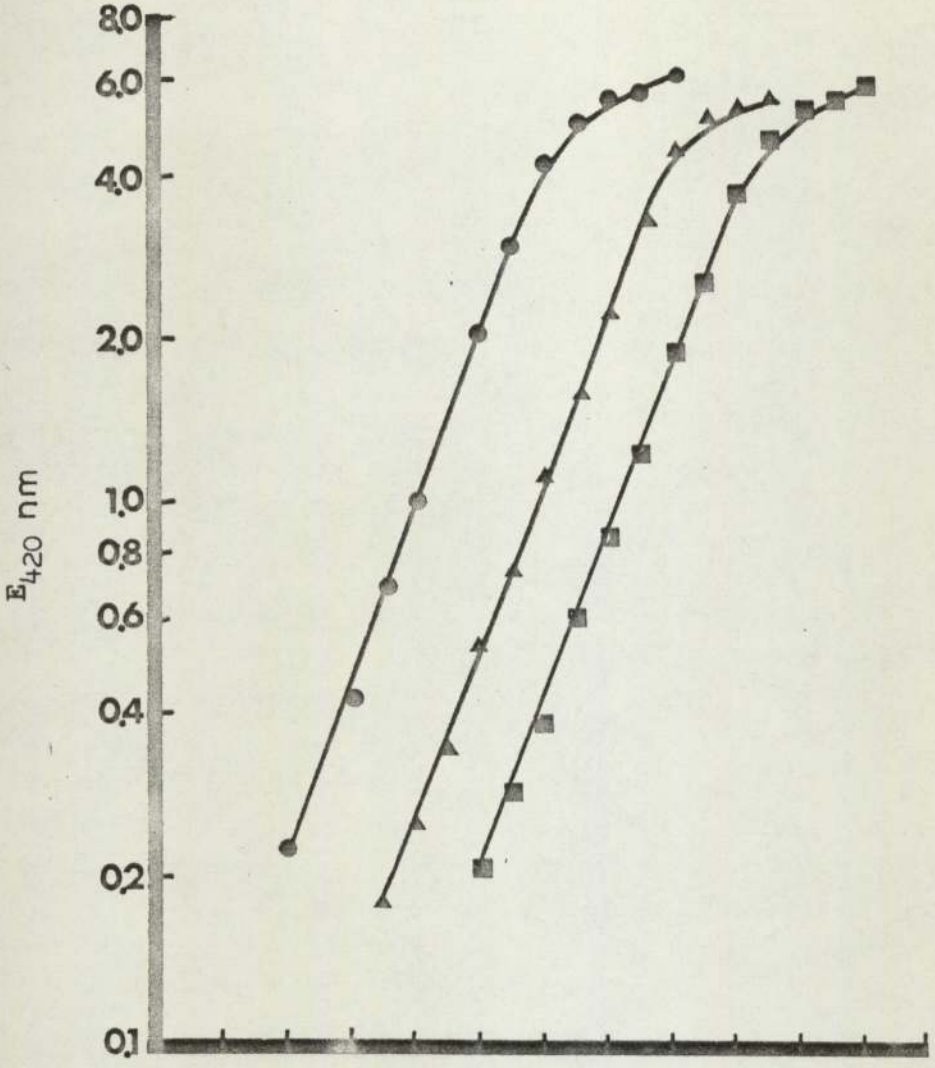
Fig29 : Effect of sodium and chloride ions on the growth of E. coli R^- and R^+



KEY

Added Na^+ and Cl^- concentration	Culture
▲ 1 x 10^{-4}M added Cl^-	
■ 5 x 10^{-5}M added Na^+	R^-
● No added Na^+ and Cl^-	
□ 5 x 10^{-5}M added Na^+	
◆ 1 x 10^{-4}M added Cl^-	R^+
○ No added Na^+ or Cl^-	

Fig30 : Effect of trace elements and iron on the growth of E. coli R⁻



KEY

■ No added trace element or Fe^{2+}

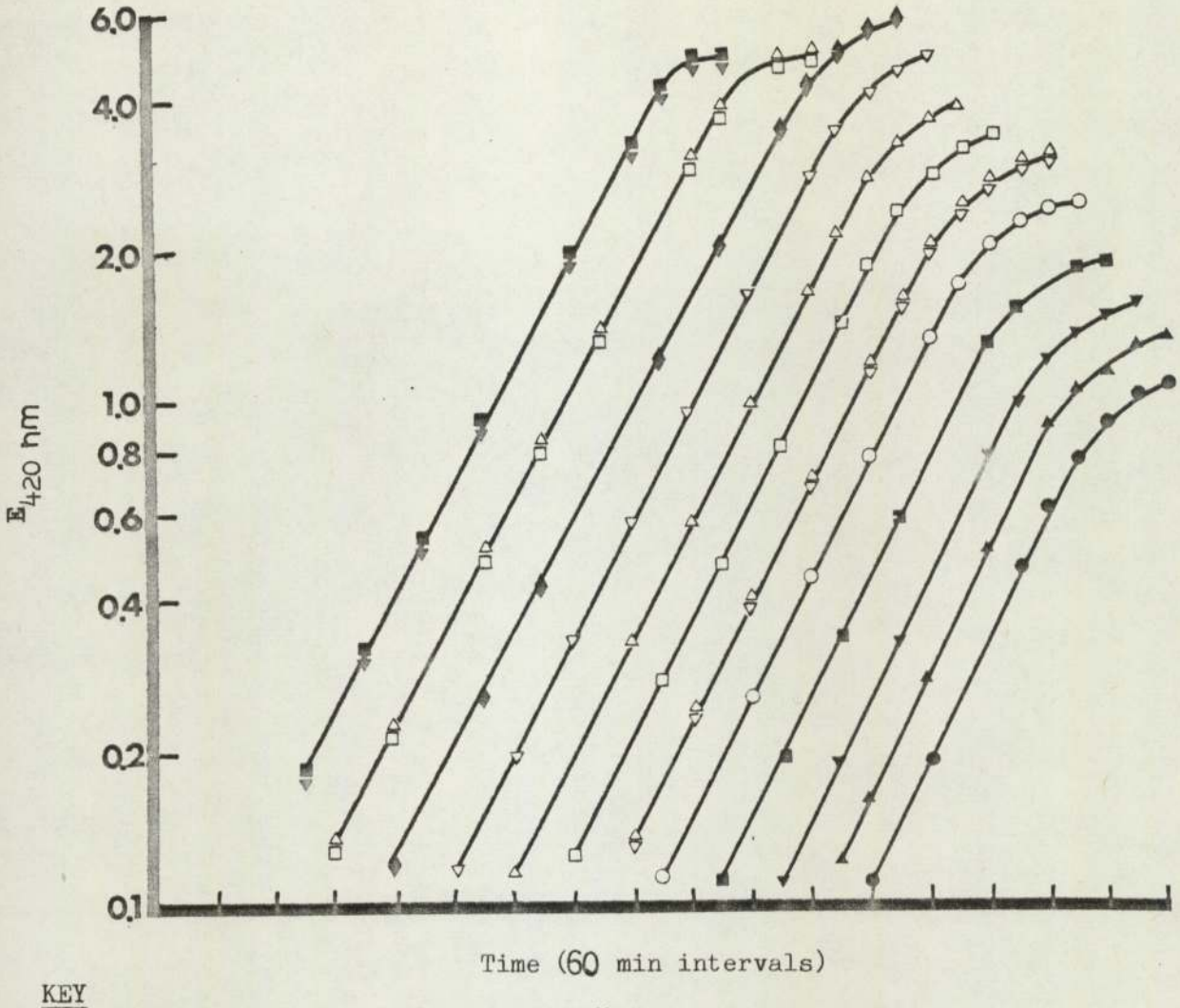
▲ With added Fe^{2+} and trace elements

● With added Fe^{2+}. No added trace elements

Trace elements

$ZnSO_4$	0.1 μg ml^{-1}
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.1 μg ml^{-1}
$CaCl_2.2H_2O$	0.1 μg ml^{-1}
$CoCl_2.6H_2O$	0.1 μg ml^{-1}
$MnSO_4.4H_2O$	0.1 μg ml^{-1}

Fig31 : Effect of Fe^{2+} concentration on the growth of E. coli R^+



KEY

Fe^{2+} concentration (M x 10^{-6})			
●	0	□	0.8
▲	0.05	△	1.0
▼	0.1	▽	1.4
■	0.25	◆	1.8
○	0.5	△	2.0
◈	0.6	▼	3.6

Fig32 : Relation between ferrous ion concentration and onset of growth of *E. coli* R^+ culture

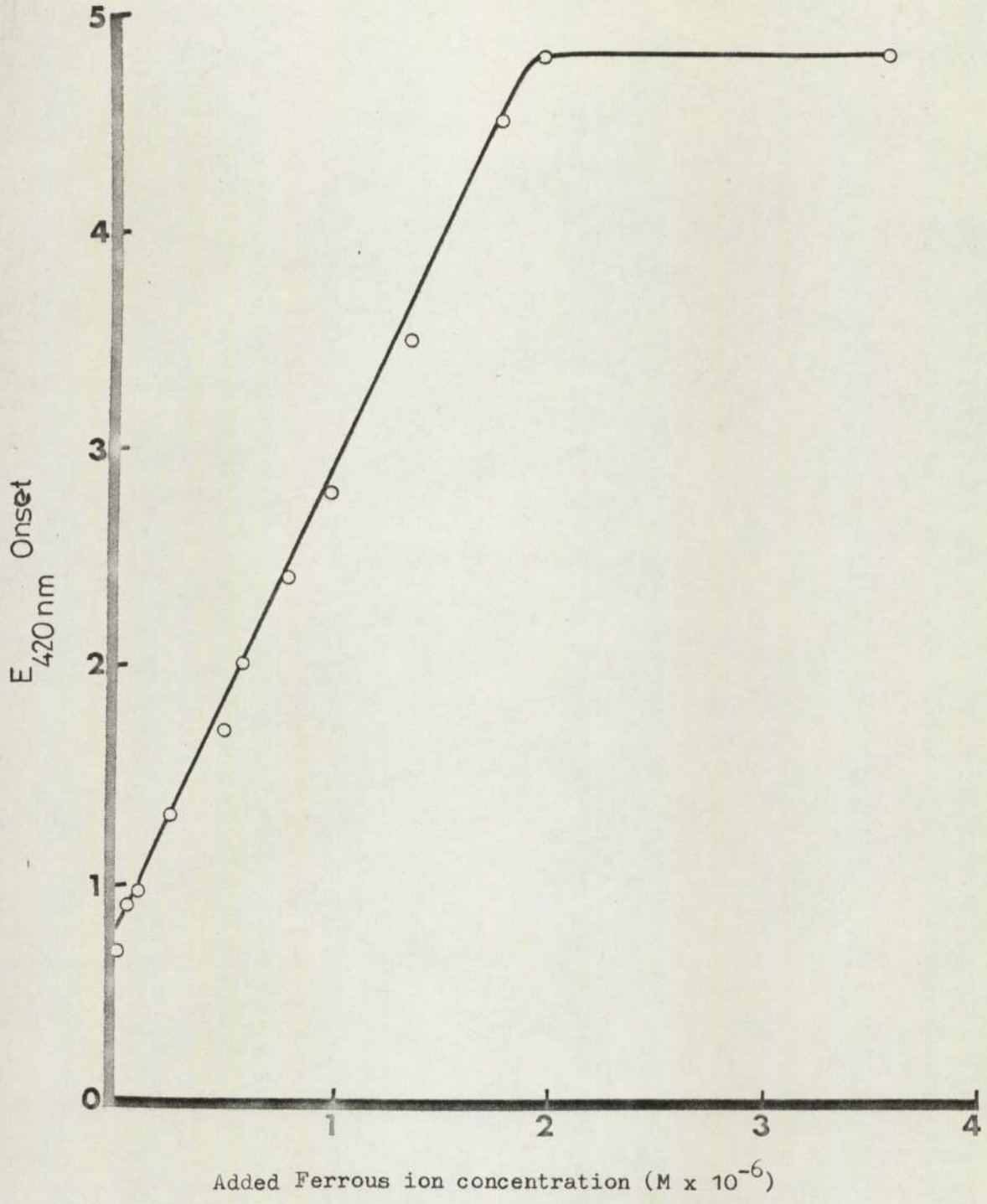
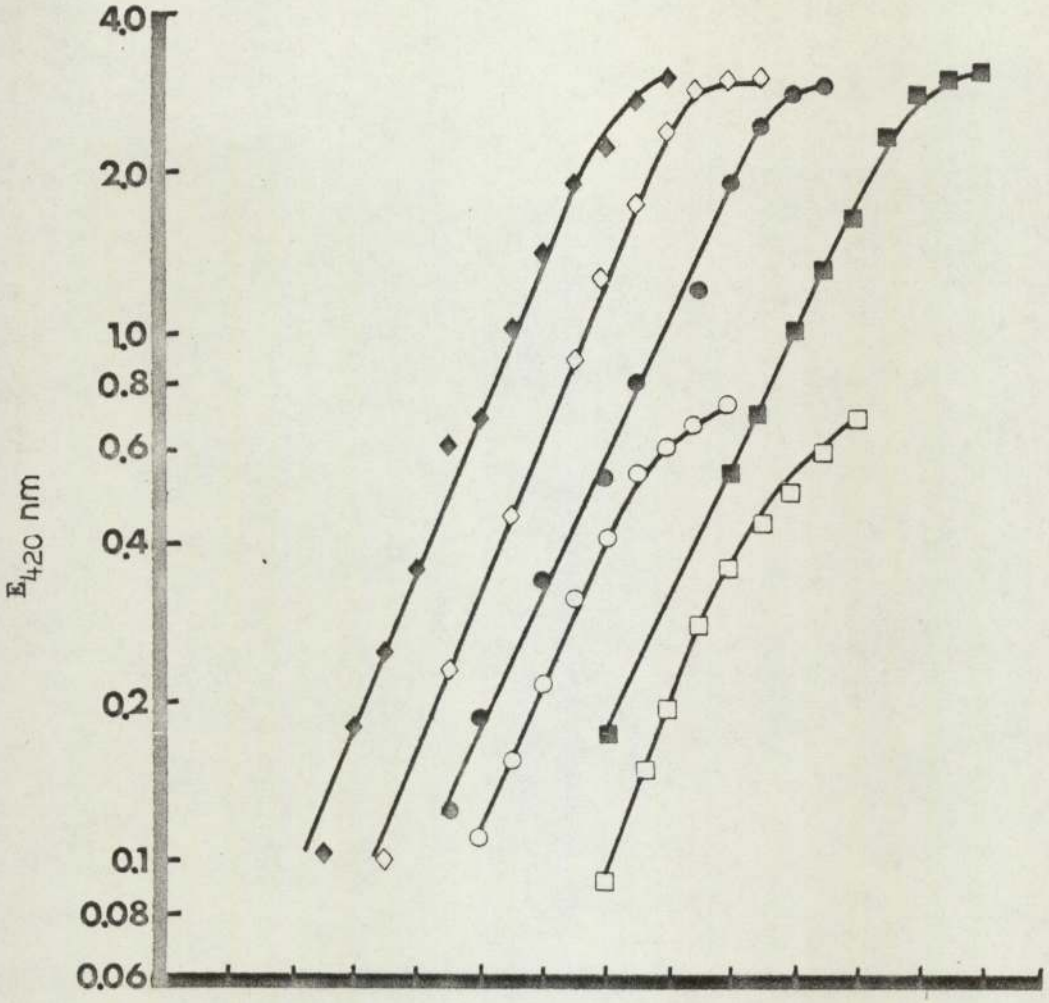


Fig33 : Effect of trace elements and iron on the growth of E. coli R^+



KEY	Fe^{2+} ($Mx10^{-6}$)	Trace elements ($\mu g\ ml^{-1}$)
◆	1.8	0
◇	1.8	0.1
●	1.8	0.2
○	1.8	0.5
■	6.0	0.1
□	6.0	0.5

concentration of trace elements was increased the growth was inhibited at low OD.

Using a high concentration of ferrous ion (6.0×10^{-6}M) with 0.1 μg ml^{-1} or 0.5 μg ml^{-1} of trace elements gave the same result as above.

4.2.11. Summary of media requirements

The results obtained for the medium have been summarized for convenience. Table 10 shows the maximum yield obtainable in this system and the concentration of each nutrient which will theoretically give this yield. Table 11 (see p45) compares the concentration of nutrients required for an arbitrarily chosen OD, and summarizes the different requirements of the R^+ and R^- cells.

Table 10: Theoretical medium to obtain maximum growth in batch culture

	R^-	R^+
OD onset of limitation	3.8	4.8
Nutrient : Concentration (M)		
Glucose	9.6×10^{-3}	1.33×10^{-2}
Mg^{2+}	5.35×10^{-5}	1.21×10^{-4}
NH_4^+	7.9×10^{-3}	1.11×10^{-2}
PO_4^{3-}	6.6×10^{-4}	2.75×10^{-3}
K^+	1.5×10^{-4}	2.64×10^{-4}
SO_4^{2-}	1.3×10^{-4}	1.15×10^{-4}
Fe^{2+}	0	2.0×10^{-6}
Na^+	0	0
Cl^-	0	0

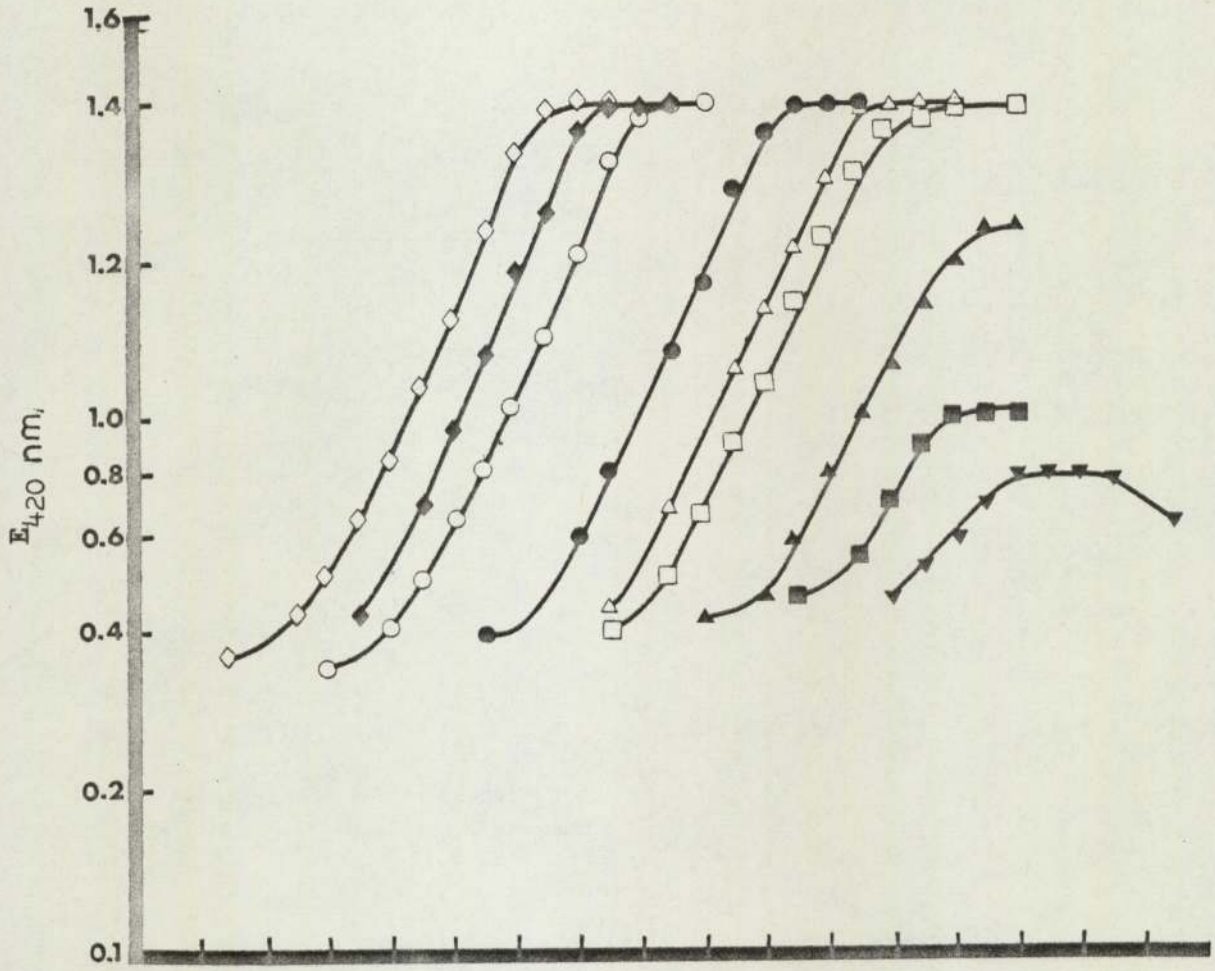
Table 11: Concentration of nutrients added to simple salts media which permits exponential growth of E. coli to an optical density of 1.0 at 420 nm (= dry weight 0.18 mg ml^{-1})

Nutrient	Concentration of added nutrient (mM) (derived from regression line)		P (Comparison of regression coefficient)
	R^-	R^+	
Glucose	2.5	2.8	>0.1
NH_4^+	1.85	1.75	>0.1
Mg^{2+}	0.013	0.025	<0.001
K^+	0.026	0.042	<0.001
Na^+	0	0	-
$HPO_4^{2-}/H_2PO_4^-$	0.17	0.56	<0.001
SO_4^{2-}	0.026	0.019	>0.1
Cl^-	0	0	-
Fe^{2+}	0	0.0001	-

4.2.12. The effect of pH on growth of the R^- culture

All the studies to determine the quantitative nutritional requirements were carried out at pH 7.0 (see p39). Using complete media sufficient to allow a theoretical OD of 10.0, the effect of pH on growth was redetermined (Fig34). Fig35 shows the effect of pH on both yield and doubling time for the R^- culture. A pH higher than 7.0 had no effect on the maximum OD, but did decrease the doubling time slightly, from 68 min at pH 7.0 to 63 min at pH 7.6. The average doubling time throughout this study is about 65 min so it seems that these differences are insignificant.

Fig34 : Effect of pH on the growth of E. coli R⁻

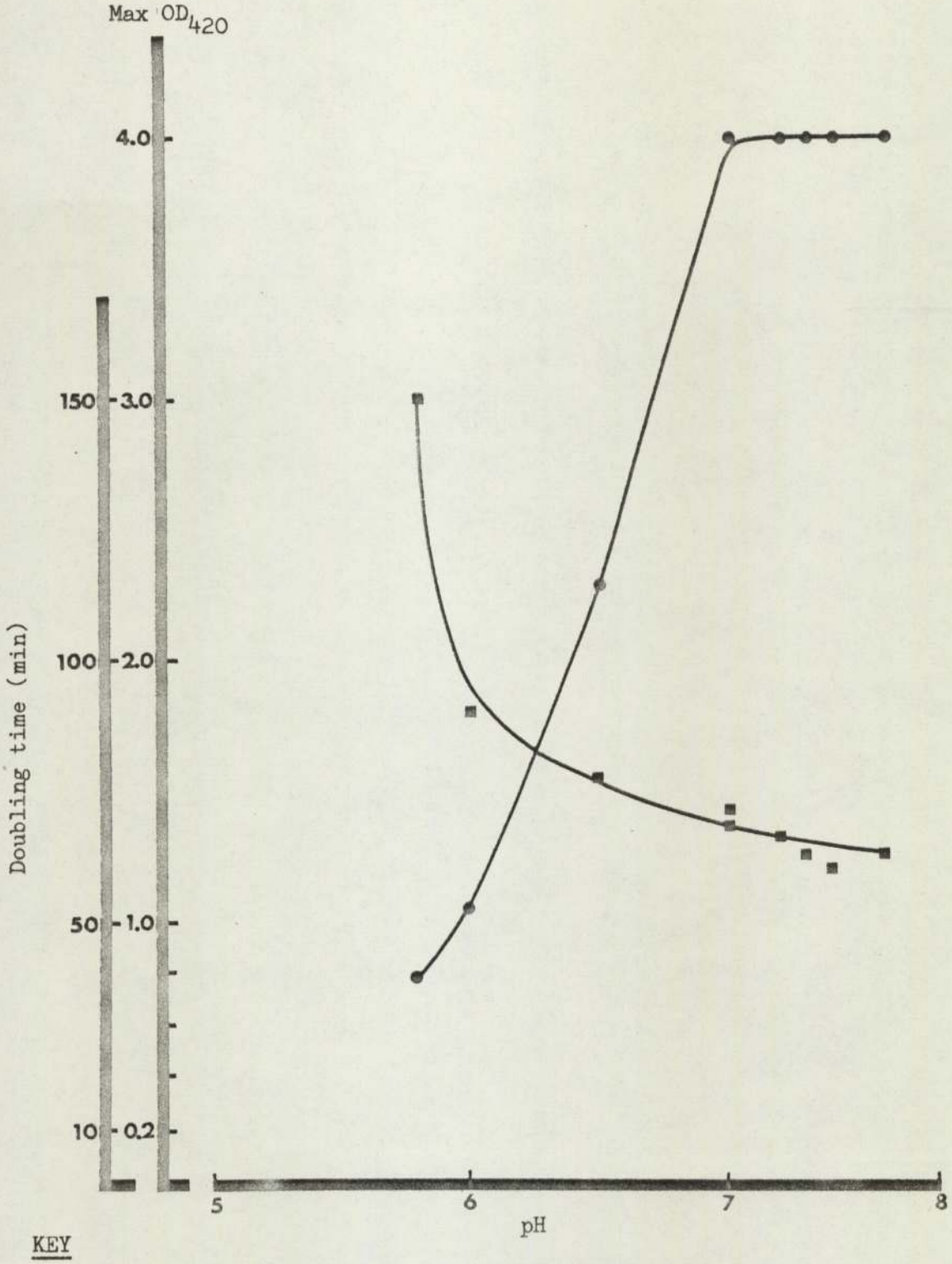


KEY

pH of the culture

▼	5.7	●	7.2
■	6.0	○	7.3
▲	6.5	♦	7.4
□	7.0	◇	7.6
△	7.0		

Fig 35 : Relation between pH, doubling time and maximum optical density of E. coli R^-



4.3. THE EFFECT OF MAGNESIUM AND PHOSPHATE CONCENTRATION ON GROWTH RATE

It was of interest to know whether the difference in the requirement of the R^+ and R^- cultures for Mg^{2+} and PO_4^{3-} was only with respect to the yield, or whether growth rate was also affected. An effect on the growth rate was not observed at the high concentrations used in the limitation studies; lower concentrations of Mg^{2+} and PO_4^{3-} were used to calculate the maximum specific growth rate constant (μ_m) and saturation constant (K_s).

4.3.1. Calculation of growth rate constants and saturation constants for Mg^{2+} and PO_4^{3-}

Figs36 ,37 ,38 and39 show the effect of low concentrations of Mg^{2+} and PO_4^{3-} respectively on the initial growth rate of R^+ and R^- cultures. The basal media was sufficient for a theoretical OD of 2.0.

The saturation constants of R^+ and R^- cultures grown in CDM containing different concentrations of Mg^{2+} and PO_4^{3-} were calculated from Monod's equation (Monod, 1949)

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

where μ = specific growth rate

μ_m = maximum specific growth rate

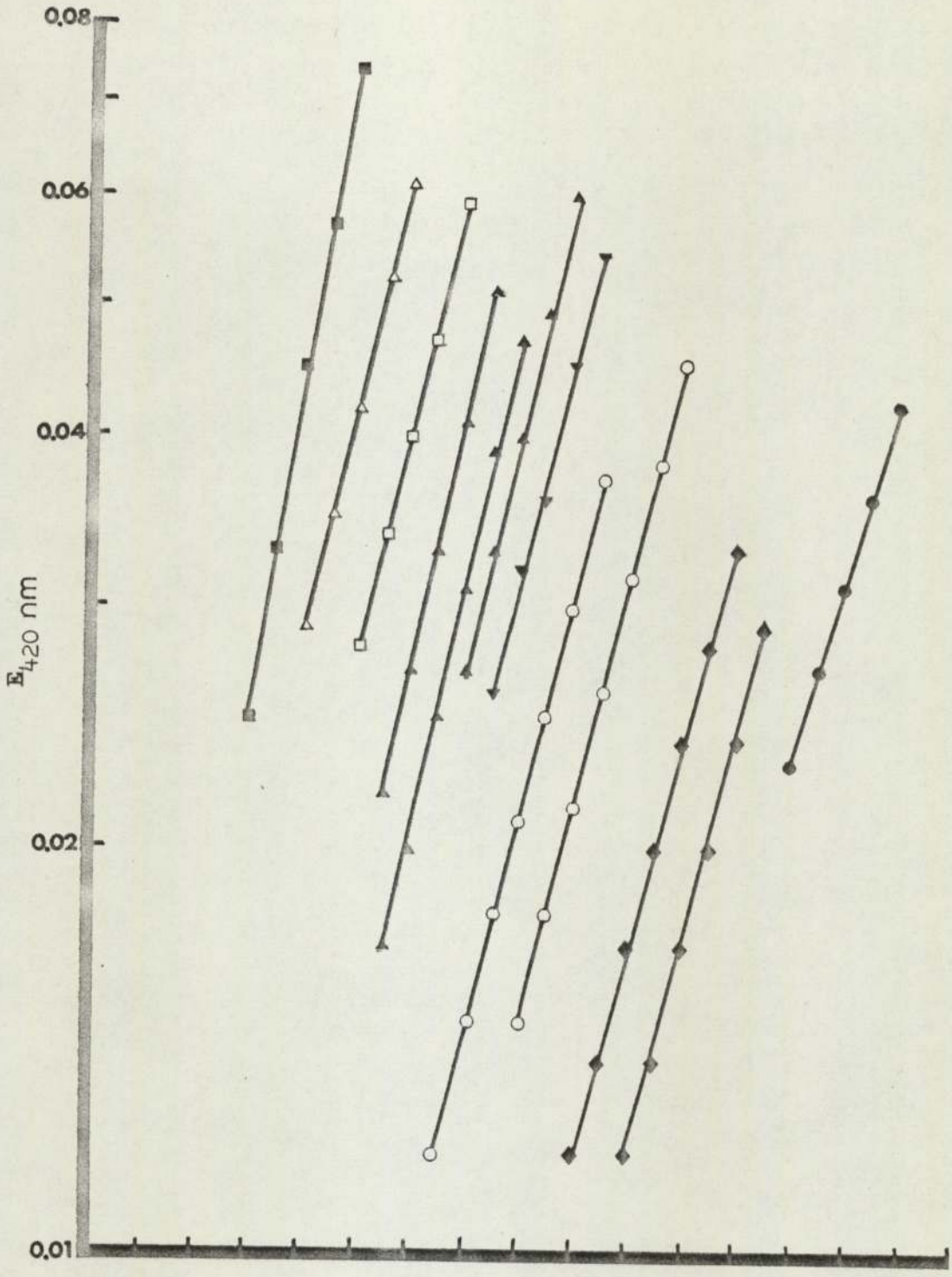
S = nutrient concentration

K_s = saturation constant

A rearrangement of the Monod equation was suggested by Lineweaver & Burke

$$\frac{1}{\mu} = \frac{1}{\mu_m} + \frac{K_s}{\mu_m} \cdot \frac{1}{S}$$

Fig 36: Effect of Mg^{2+} concentration on the growth rate of E. coli R^+

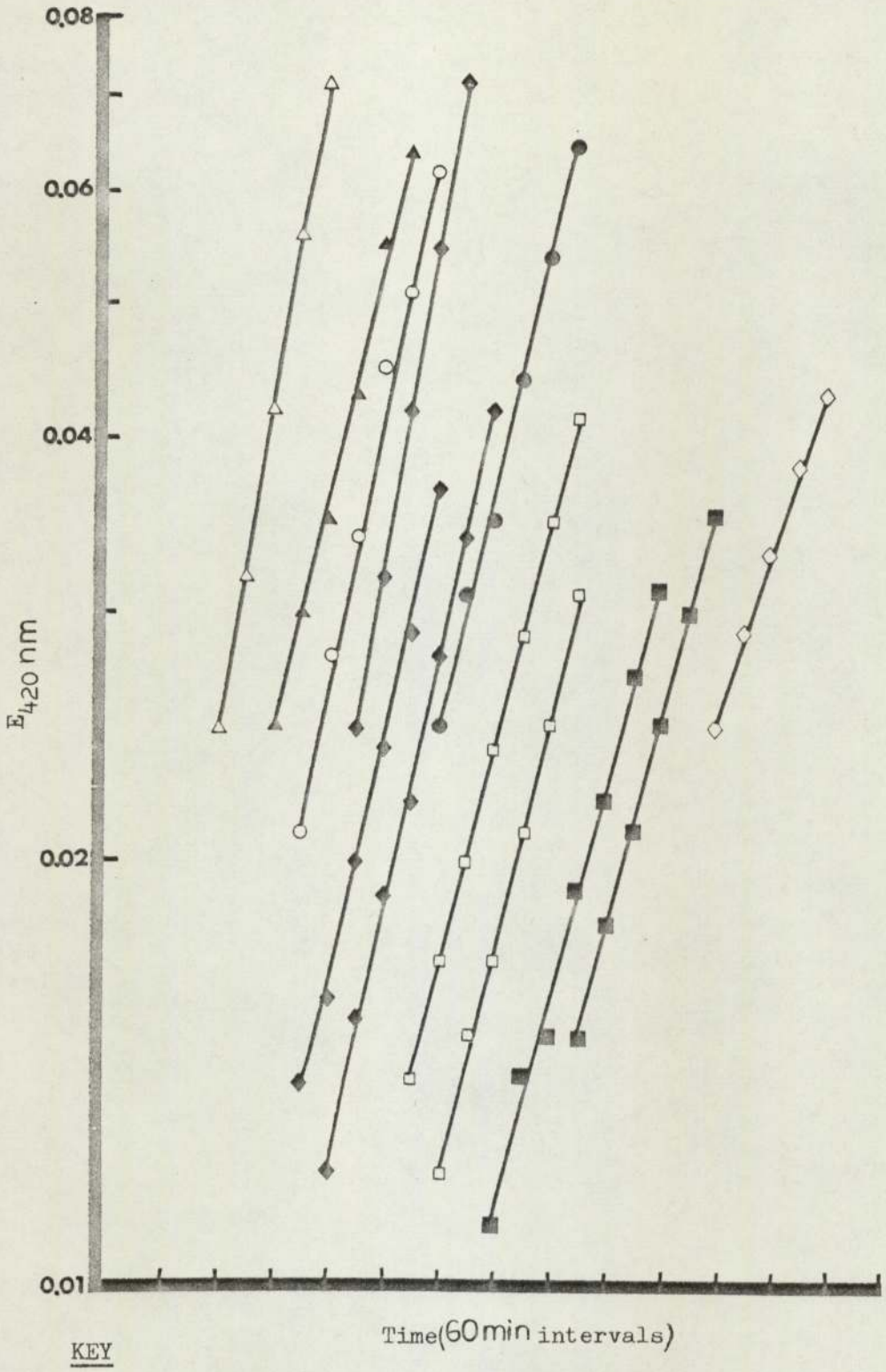


KEY

Mg^{2+} concentration ($M \times 10^{-6}$)

●	0.1	▲	0.2 (triplicate)
◆	0.125 (duplicate)	□	0.33
○	0.14 (duplicate)	△	0.5
▼	0.16	■	2.0

Fig 37: Effect of Mg^{2+} concentration on the growth rate of *E. coli* R^-

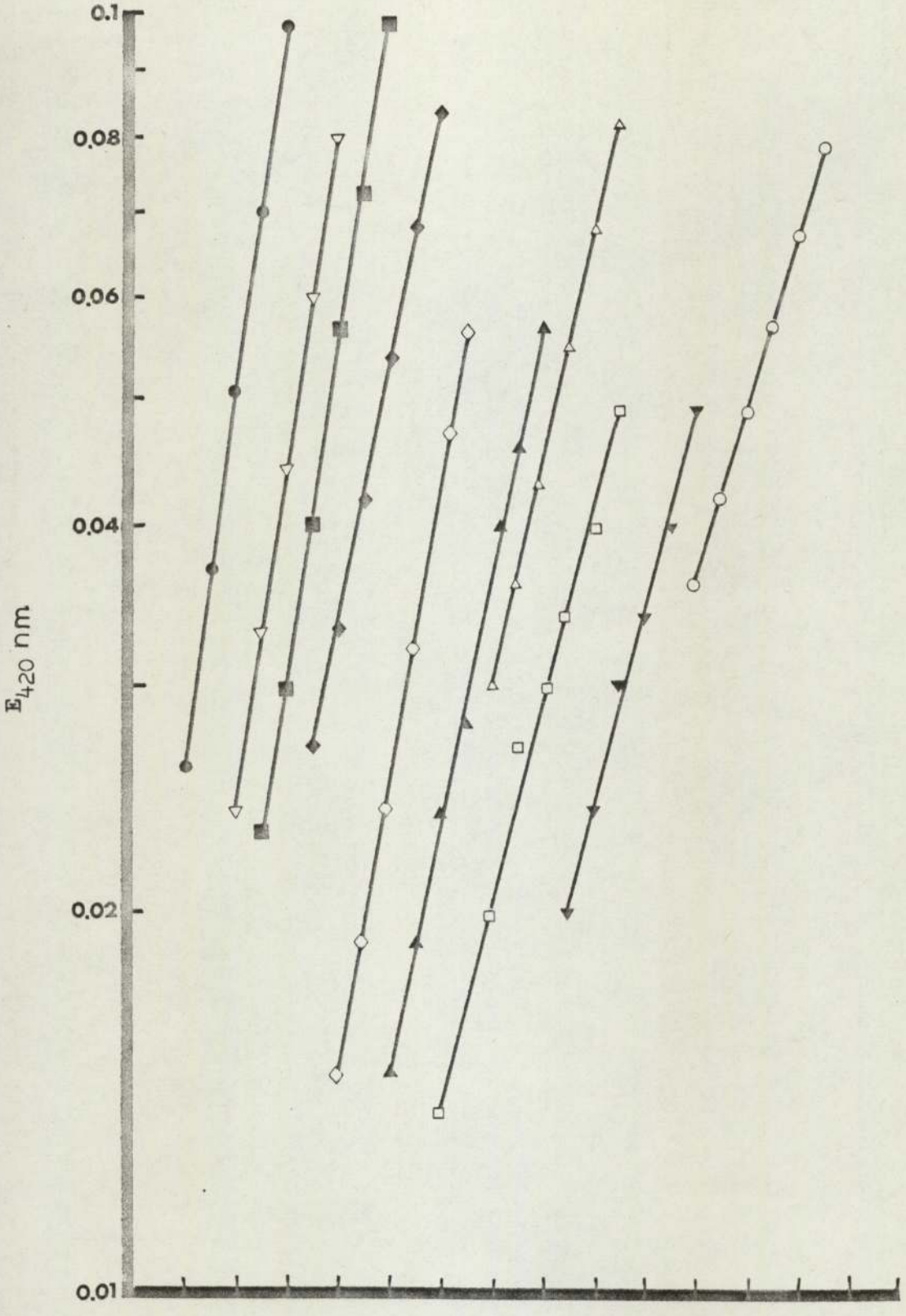


KEY

Mg^{2+} concentration ($M \times 10^{-6}$)

◇	0.1	◆	0.2 (triplicate)
■	0.125 (duplicate)	○	0.33
□	0.14 (duplicate)	▲	0.5
●	0.16	△	2.0

Fig 38: Effect of PO_4^{3-} concentration on the growth rate of E. coli R^+

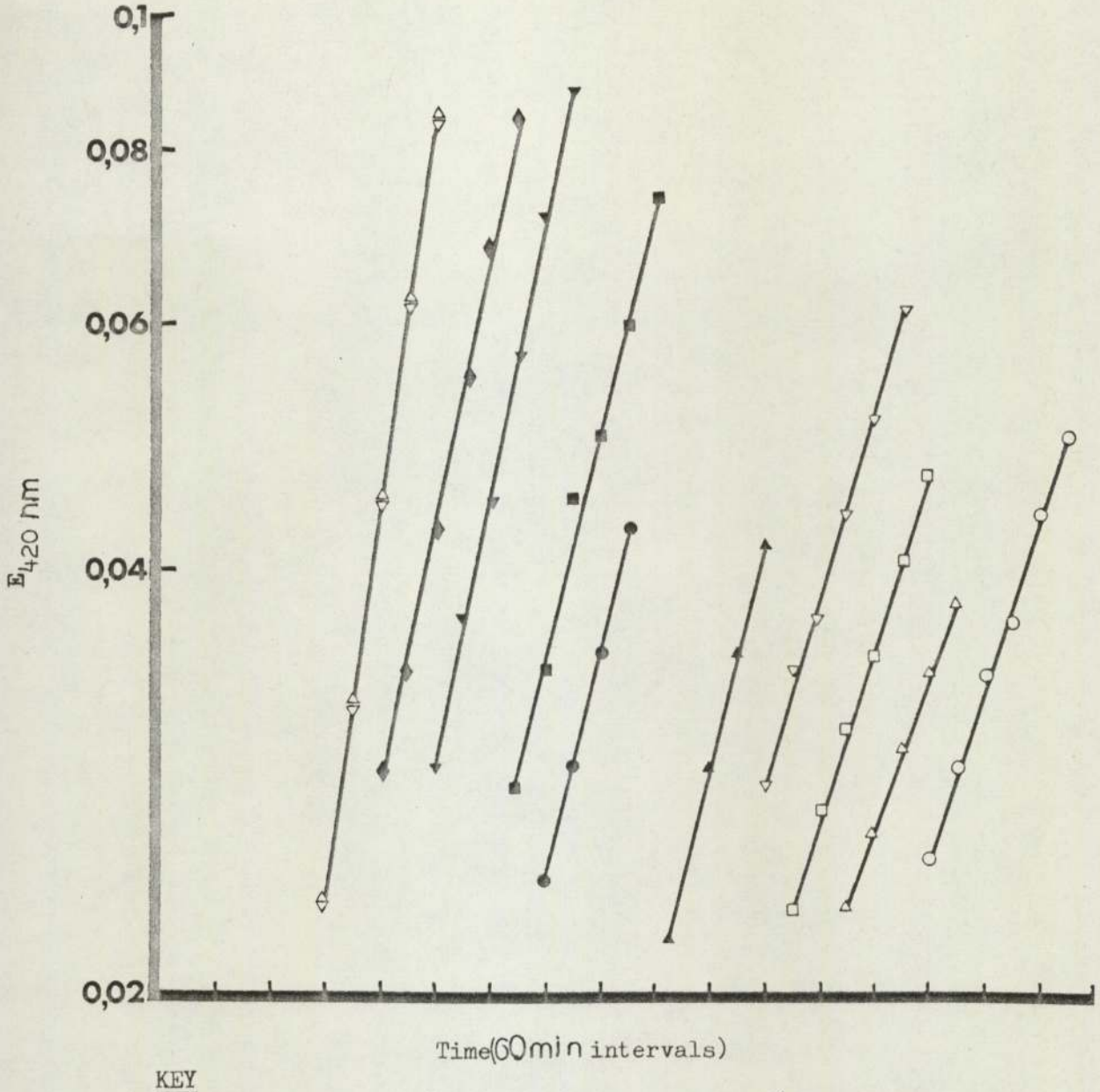


KEY

PO_4^{3-} concentration ($M \times 10^{-6}$)

○ 0.5	△ 1.0	◆ 3.0	● 10.0
▼ 0.6	▲ 1.4	■ 5.0	
□ 0.8	◇ 1.6	▽ 7.0	

Fig39 : Effect of PO_4^{3-} concentration on the growth rate of E. coli R^-



KEY

PO_4^{3-} concentration (M x 10^{-6})

○	0.5	▲	1.4	◆	7.0
△	0.6	●	1.6	◈	10.0
□	0.8	■	3.0		
▽	1.0	▼	5.0		

The data from Figs 36, 37, 38 and 39 were plotted in Figs 40 and 41 as regressions of $\frac{1}{\mu}$ upon $\frac{1}{S}$. In addition, growth rates using relatively high concentrations of Mg^{2+} and PO_4^{3-} in the same basal media, derived from the cultures grown for testing the action of disinfectants Fig 42&43 were also included. The calculated parameters are summarized in Table 12.

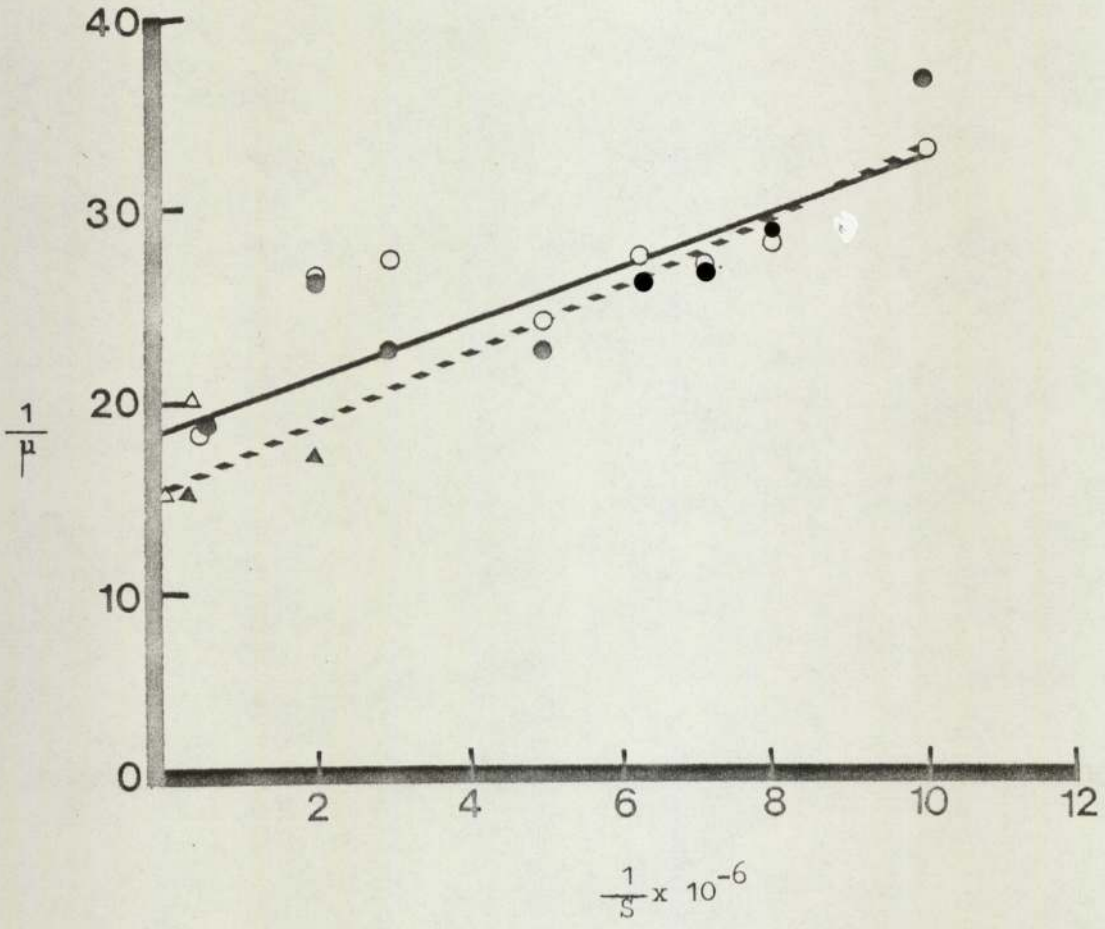
Table 12: Effect of Mg^{2+} and PO_4^{3-} concentration on growth rate and saturation constant

Limiting nutrient	Parameter	R^+	R^-	P
Mg^{2+}	slope $\frac{1}{\mu} : \frac{1}{S}$	0.14	0.18	>0.1
	μ_m	0.54	0.64	
	$K_s(M)^*$	0.09×10^{-6}	0.09×10^{-6}	
PO_4^{3-}	slope $\frac{1}{\mu} : \frac{1}{S}$	0.84	1.12	<0.05
	μ_m	0.64	0.59	
	$K_s(M)$	0.54×10^{-6}	0.73×10^{-6}	

* calculated from combined data from R^+ and R^-

From Figs 40 and 41 and Table 12 it can be seen that the maximum growth rates of R^+ and R^- do not differ significantly. A minimum doubling time of 65 min was confirmed in many experiments, using basal media to allow a theoretical OD of 10 as well as of 2. However, at lower growth rates, there is a significant difference between R^+ and R^- cultures when limited by PO_4^{3-} but not when limited by Mg^{2+}.

Fig40: Double reciprocal plot of growth rate versus Mg^{2+} concentration of E. coli R^- and R^+ culture



KEY

R^+

data from Fig 36

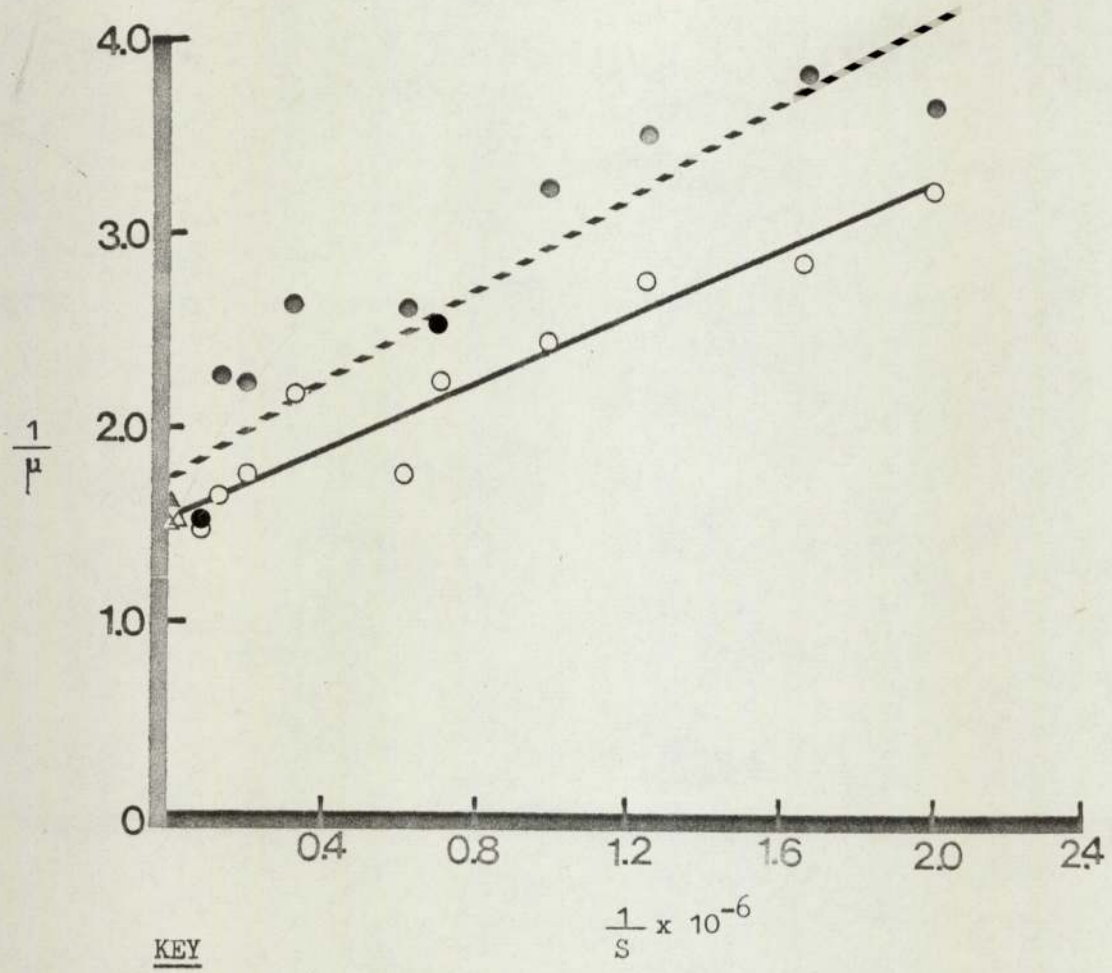
data from disinfectant experiment

R^-

data from Fig37

data from disinfectant experiment

Fig41 : Double reciprocal plot of growth rate versus PO_4^{3-} concentration of E. coli R^- and R^+ cultures



KEY

R^+

data from Fig38

data from disinfectant experiment

R^-

data from Fig39

data from disinfectant experiment

4.4. THE RELATION BETWEEN OPTICAL DENSITY, VIABLE COUNT AND DRY WEIGHT

4.4.1. Relation between optical density and dry weight for exponentially growing cells

Triplicate samples of log phase cells (OD 1.0) were harvested in ice to stop growth, and centrifuged in previously weighed centrifuge tubes. The cells were washed in distilled water, centrifuged and dried over P_2O_5 in a desiccator to a constant weight. The dry weight was found to be 0.18 mg ml^{-1} for both R^+ and R^- (kindly carried out by Dr. Klemperer).

4.4.2. Relation between optical density and viable count for nutrient depleted cells

This was determined for both R^+ and R^- cultures depleted of carbon, magnesium and phosphate, and is shown in Table 13.

Table 13: The effect of nutrient depletion on the relation between viable count and optical density

Depletion	No. of viable cells x 10^{-7} ml^{-1} (OD 0.2)	
	R^+	R^-
C	12.1	19.05
Mg^{2+}	14.0	22.7
PO_4^{3-}	14.2	22.8

4.5 EFFECT OF PHOSPHATE DEPLETION ON LYSIS OF R^+ AND R^- CULTURES BY POLYMYXIN

Table 14 shows that no significant difference was found between carbon and phosphate depleted cells. Considerable day-to-day variation in results led to an investigation into the effect of length of the stationary phase on the lysis of R^- cells, but no significant effects were noted (Table 15).

Table 14: The effect of nutrient depletion on lysis by polymyxin

Polymyxin ($U\ ml^{-1}$)	% Lysis											
	R^-						R^+					
	C-			P-			C-			P-		
20	-	28	-*	36	-	33	-	31	-	34	-	32
40	-	40	22	41	-	41	-	35	21	37	-	36
60	-	-	28	-	-	-	-	-	35	-	-	-

* triplicate experiments

\- experiment not performed

Table 15: The effect of the length of the stationary phase on lysis of carbon depleted R^- cells by polymyxin ($40\ U\ ml^{-1}$)

Stationary phase (hr)	% Lysis
0	44
2	24 46
3	38
5	34 36

4.6. EFFECT OF DISINFECTANTS

4.6.1. The minimum inhibitory concentration of the disinfectants for R^+ and R^-

The result is summarized in Table 16.

Table 16: The minimum inhibitory concentration of the disinfectants

Disinfectant	MIC	
	R^-	R^+
Chlorhexidine ($\mu g\ ml^{-1}$)	5 - 6	0.5
Phenol ($mg\ ml^{-1}$)	2.5	2.5
Cetrimide ($\mu g\ ml^{-1}$)	20 - 25	6 - 8

4.6.2. Effect of nutrient depletion on susceptibility to chlorhexidine

Fig42 shows that when R^- culture is carbon depleted it is more sensitive to chlorhexidine than when phosphate or magnesium depleted. From the confidence limits, it can be seen that there is no significant difference in the sensitivity of the Mg^{2+} and PO_4^{3-} depleted cultures.

The same pattern was found with R^+ cultures, although in all cases they were more sensitive than the equivalent R^- cultures (Fig43).

4.6.3. Effect of nutrient depletion on susceptibility to cetrimide

The effect of cetrimide concentration on % number of colonies formed by the R^- and R^+ culture is shown in Figs44 &45 respectively.

The R^- culture is more sensitive to cetrimide when it is Mg^{2+} or PO_4^{3-} depleted than when it is carbon depleted.

Fig42 : Effect of nutrient depletion on susceptibility of *E. coli* R^- cultures to chlorhexidine

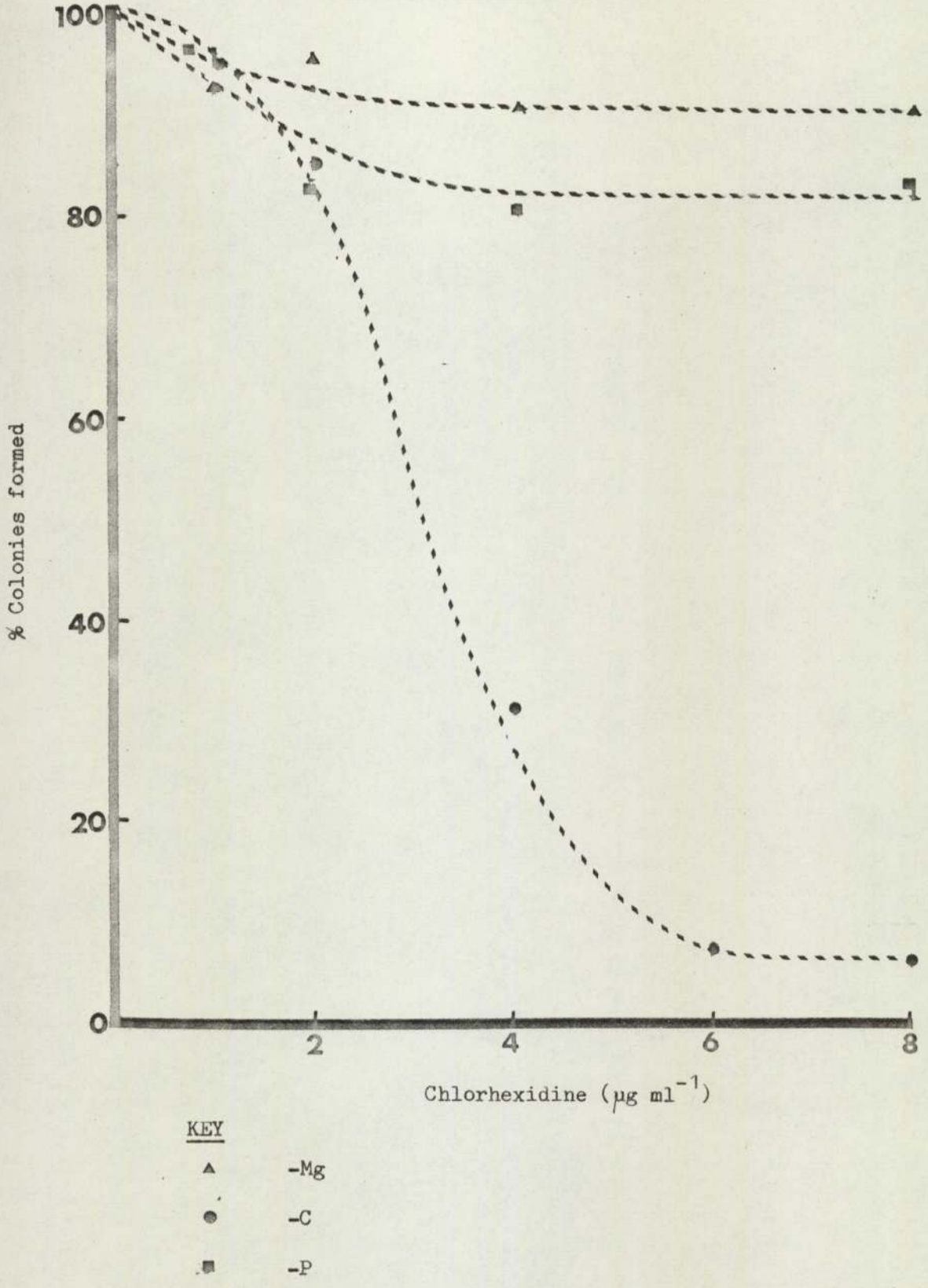


Fig43: Effect of nutrient depletion on susceptibility of *E. coli* R^+ cultures to chlorhexidine

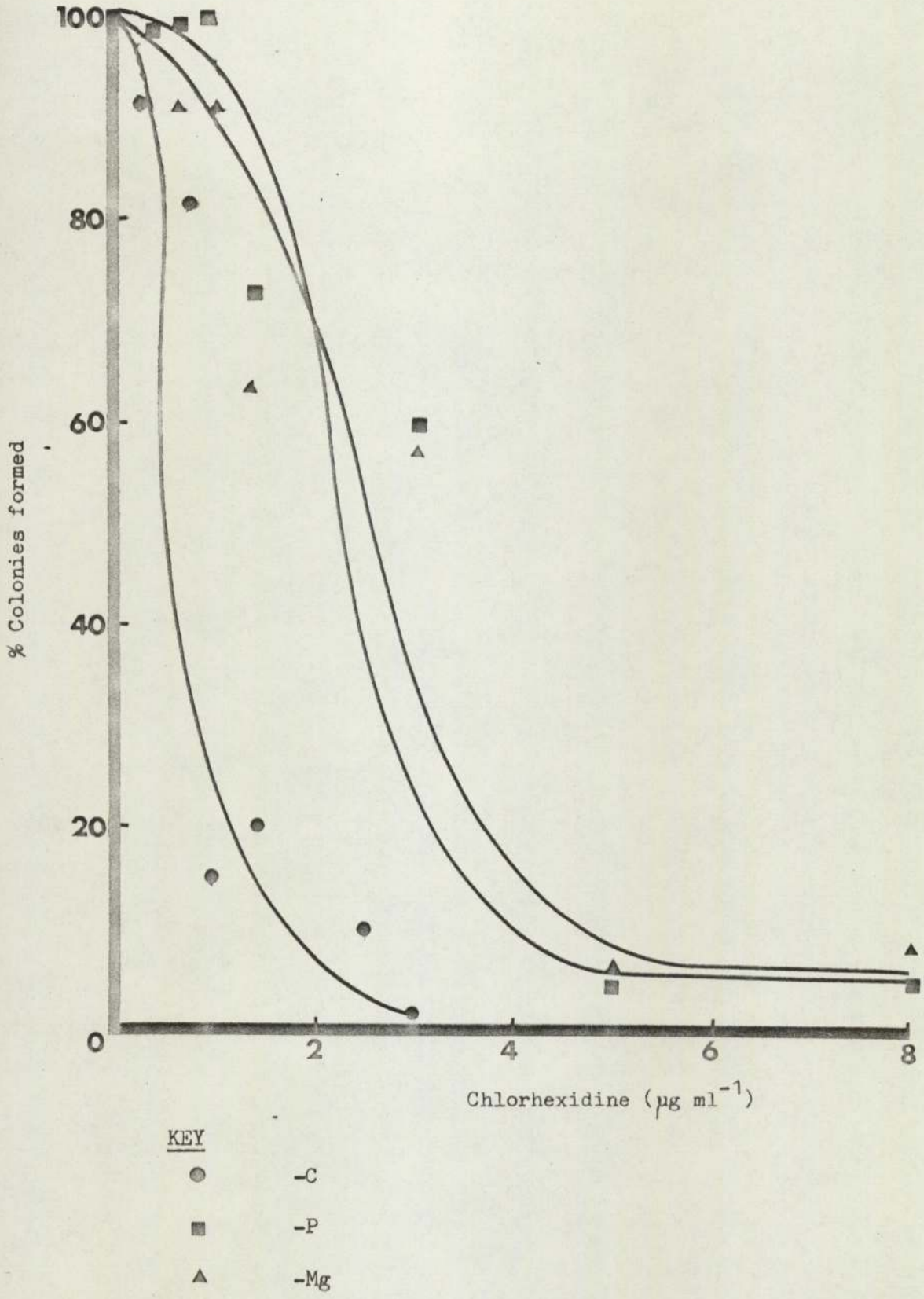


Fig44: Effect of nutrient depletion on susceptibility of E. coli R⁻ cultures to cetrimide

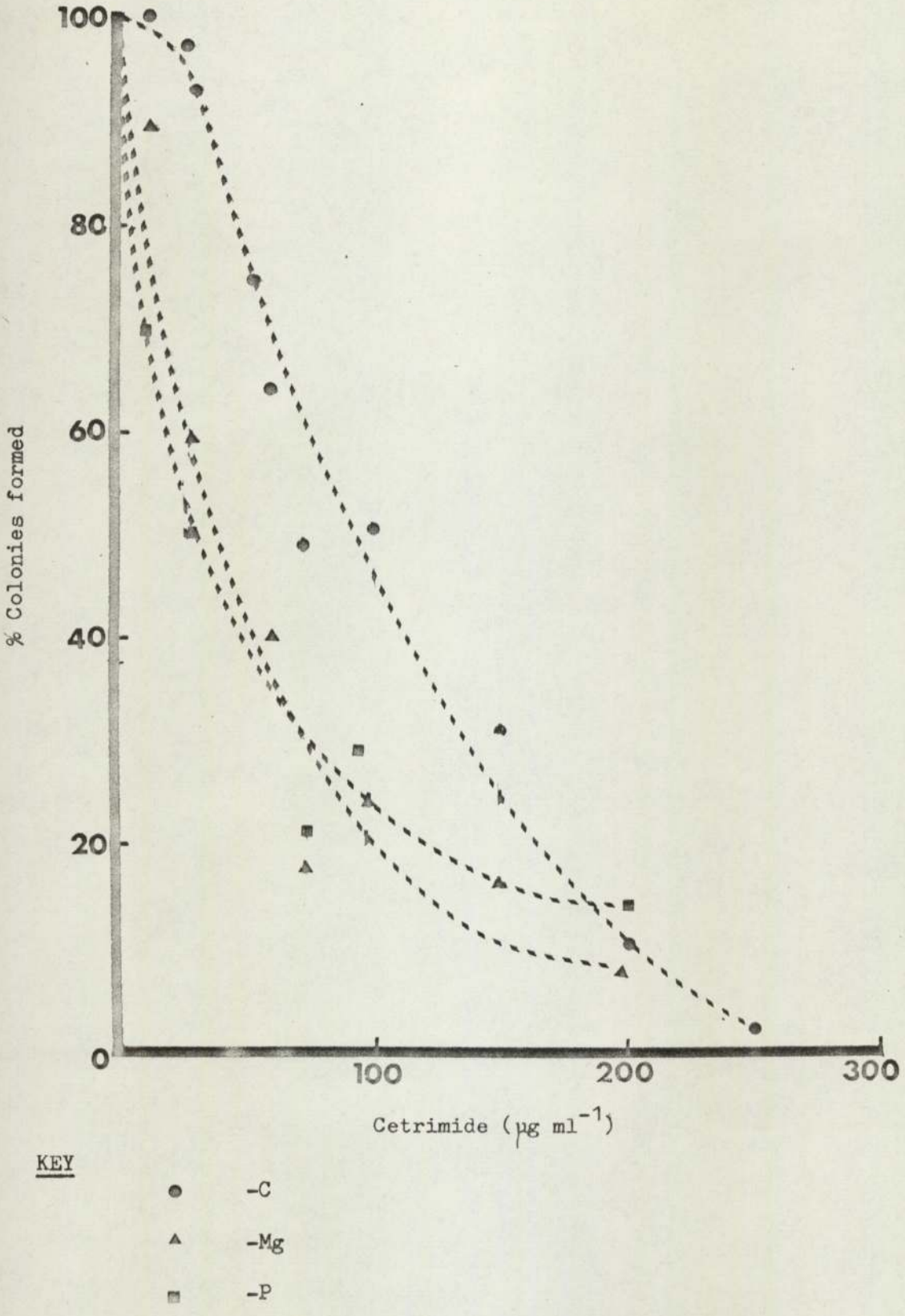
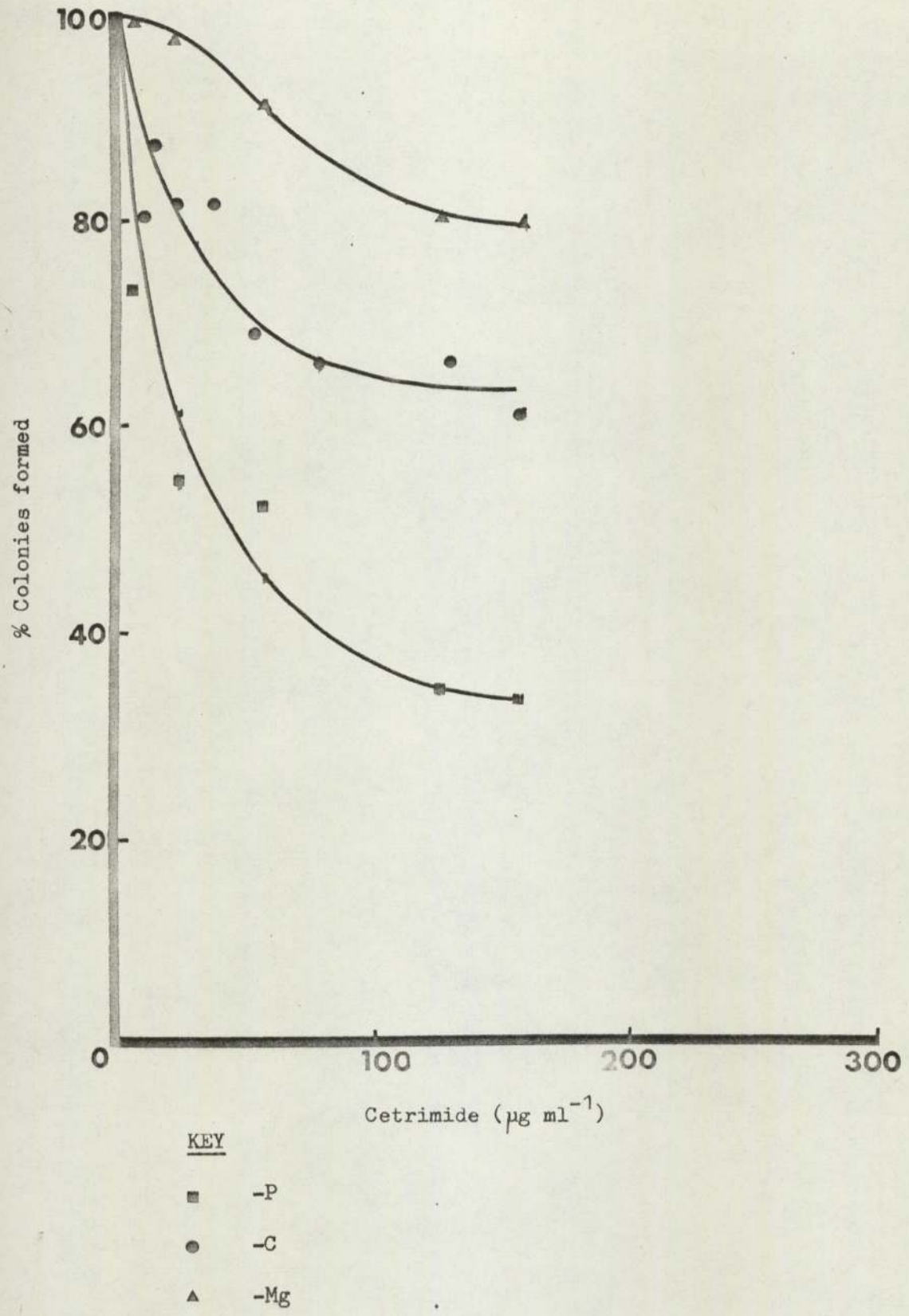


Fig 45 : Effect of nutrient depletion on susceptibility of *E. coli* R^+ cultures to cetrimide



The R^+ culture is also more sensitive when PO_4^{3-} depleted than when it is carbon depleted.

When the R^- and R^+ cultures are compared, all the R^+ cultures are more resistant than the corresponding R^- culture.

4.6.4. Effect of nutrient depletion on susceptibility to phenol

Phenol has a high concentration exponent so it is difficult to demonstrate differences in resistance.

When R^+ and R^- cultures were carbon depleted, the effect of phenol concentration on % number of colonies formed was the same for both of them.

However, when the R^- culture was Mg^{2+} depleted it was more sensitive to phenol, and when PO_4^{3-} depleted it was even more sensitive Fig46. The effects of Mg^{2+} and PO_4^{3-} depletion on the resistance of the R^+ culture to phenol were opposite to each other, as was found with cetrimide resistance. In both cases, Mg^{2+} depletion increased resistance and PO_4^{3-} depletion increased sensitivity (Fig47).

4.7. THE EFFECT OF NUTRIENT DEPLETION ON THE UPTAKE OF HYDROGEN IONS BY E. coli

The amount of H^+ taken up at different pHs is shown in Fig48. When carbon depleted, less hydrogen ions are bound by the R^- culture than by the R^+ culture. PO_4^{3-} depleted cultures bound the largest amount of hydrogen ions, and Mg^{2+} depleted cultures the least.

Fig46 : Effect of nutrient depletion on susceptibility of *E. coli* R^- to phenol

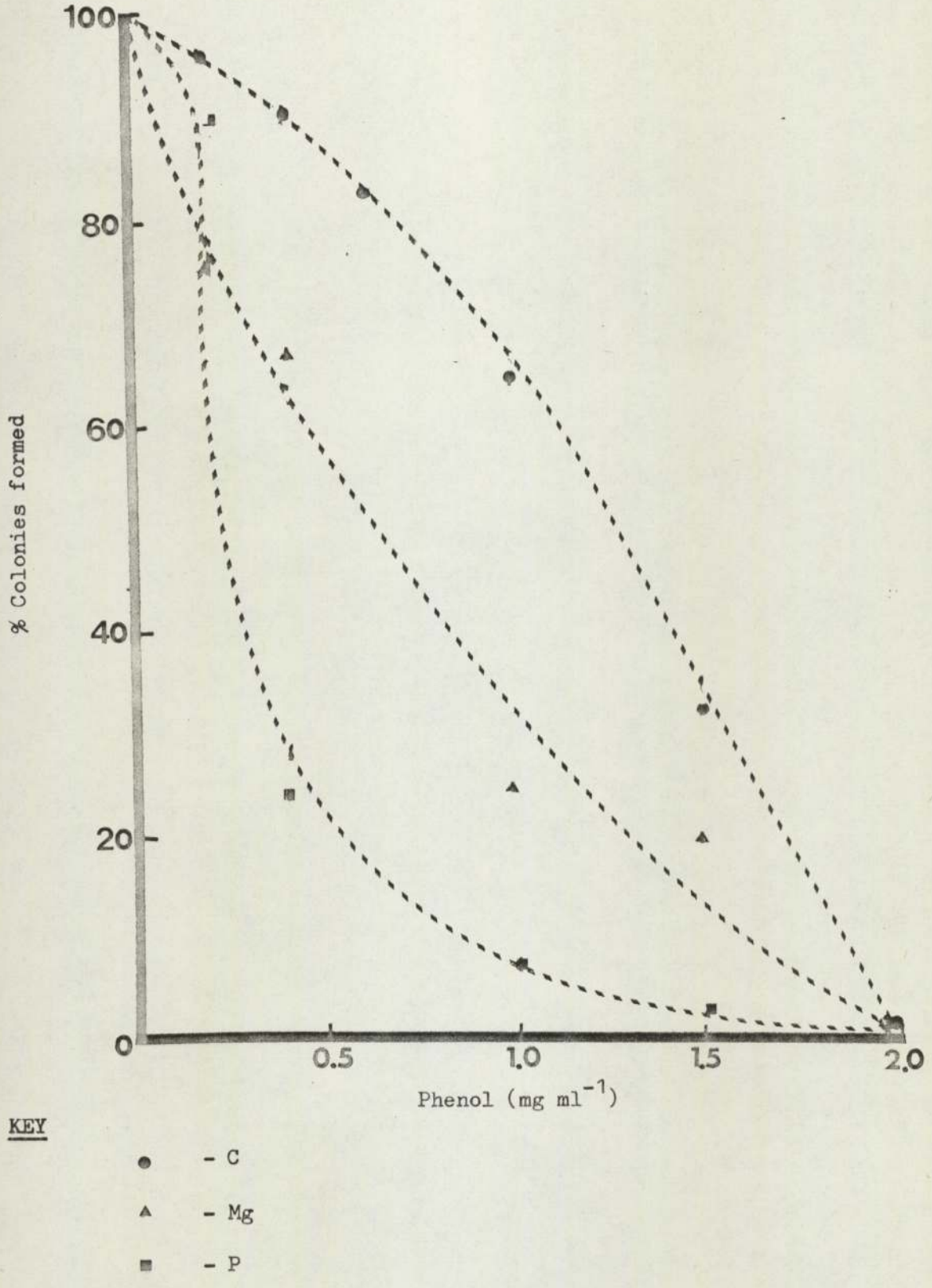


Fig47 : Effect of nutrient depletion on susceptibility of E. coli R^+ to phenol

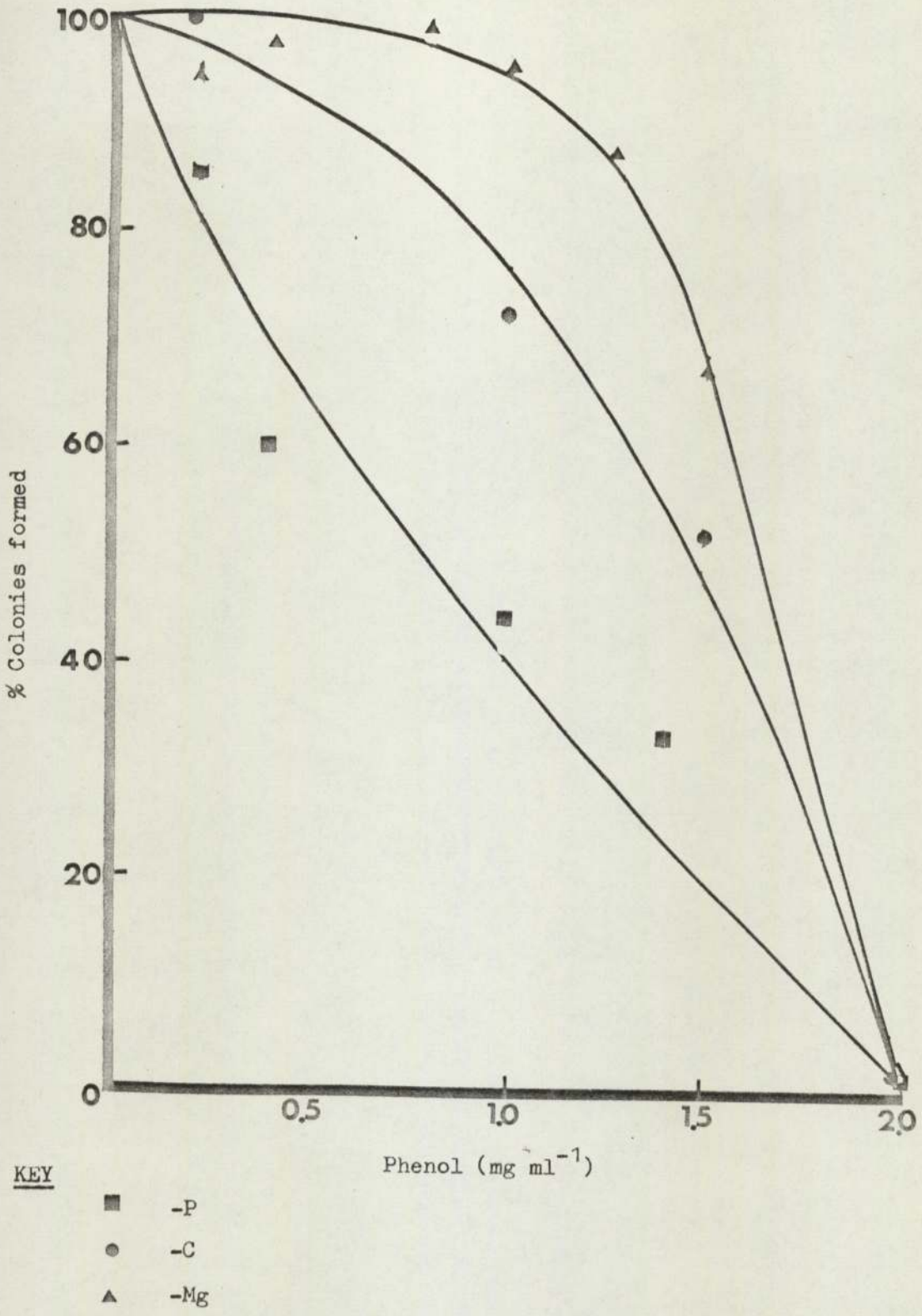
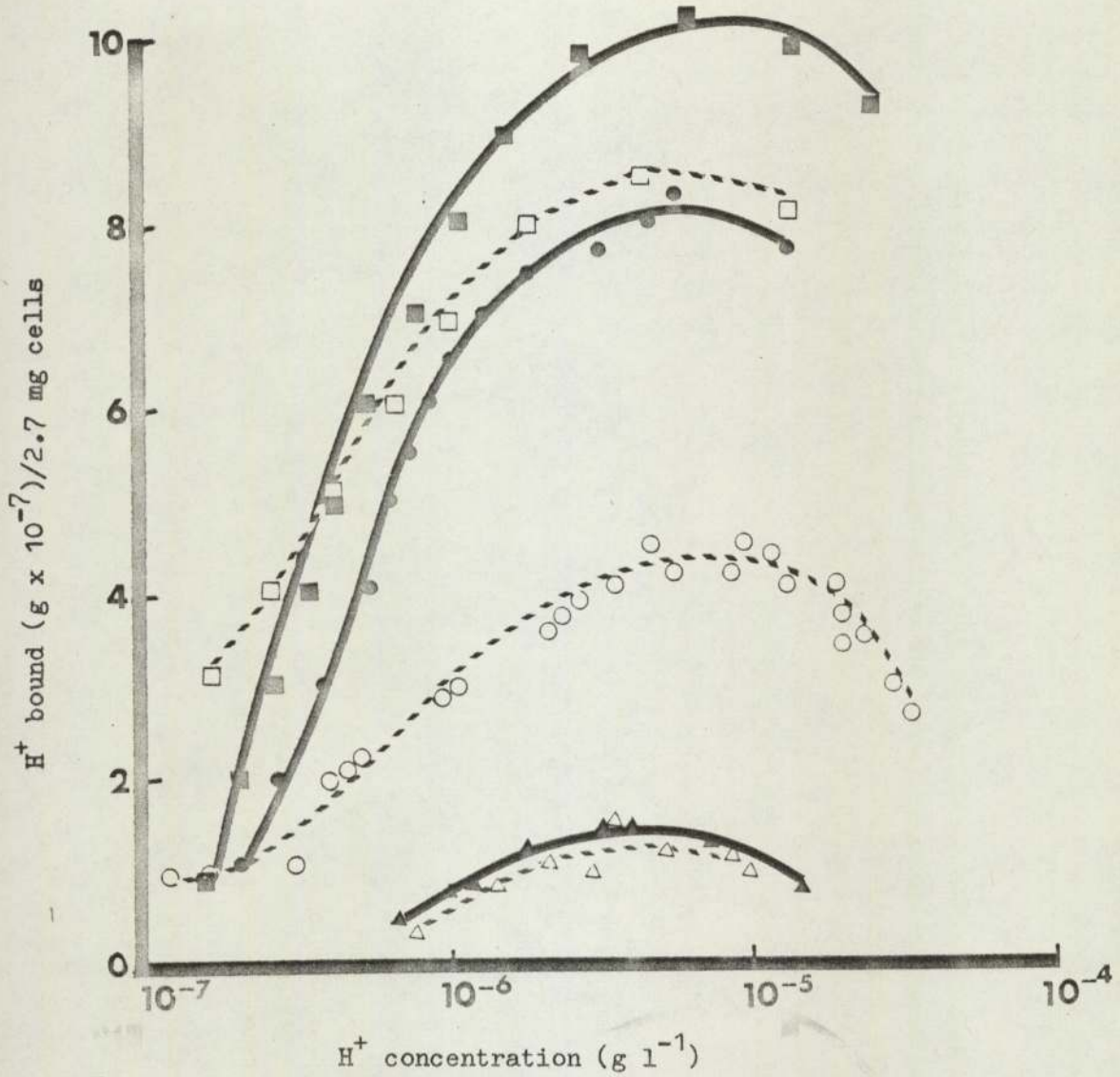


Fig 48: The effect of nutrient depletion on the uptake of hydrogen ions by E. coli R^+ and R^-



KEY

△ -Mg.R^- □ -P.R^-

▲ -Mg.R^+ ■ -P.R^+

○ -C.R^-

● -C.R^+

5.1. NUTRIENT REQUIREMENT OF *Escherichia coli*

5.1.1. Introduction

When growth of a culture is limited by a particular nutrient, the amount of growth which results is directly related to the initial concentration of the nutrient, and will depend upon the way the nutrient is utilized by the cell, and the structure into which it is incorporated.

Table 11 shows that growth yields of R^+ and R^- cells are different for a number of nutrients, which suggests important differences in metabolism and composition. These are discussed in detail below. In addition, the nutrient requirements of *E. coli* R^+ and R^- are compared with those of another Gram-negative species, *Pseudomonas aeruginosa*, for which similar information is available (Boggis, 1971), and for which there are a number of studies of chemical composition, its relation to resistance and the way these vary with nutrient limitation (Brown, 1975).

5.1.2. Glucose

Glucose has a duel role in this medium, serving as a source of carbon and a source of energy.

No significant difference was noted between the glucose requirements of R^+ and R^- cells (Fig 13). However, as carbon represents some 50% of the cell dry weight (Stanier *et al.*, 1977) changes in composition are unlikely to be large enough to be reflected in changes in growth yield. The same glucose requirement therefore suggests that energy-generating metabolic pathways are the same. However, Gilbert & Brown (1977) noted that the percentage of the storage material poly-β-hydroxybutyrate was 10 to 20 times greater in carbon depleted R^+ cells compared with similar R^- cells, representing 5% of the dry weight

of the R^+ cells. Furthermore, differences in iron requirement to be discussed later also point to differences in energy generation.

The growth yield of P. aeruginosa for glucose was found to be lower (Boggis, 1971) than for E. coli. The energy required to synthesize macromolecules probably does not vary much among micro-organisms (Stanier et al., 1977), so the difference in yield can probably be attributed to the relative efficiency of the Embden-Meyerhof pathway of E. coli compared with the Entner-Doudoroff pathway characteristic of Pseudomonads (Davis et al., 1973).

5.1.3. Magnesium

Growth curves of Mg^{2+} depleted cultures (Figs 14 & 15) have a markedly different shape from those of carbon depleted cultures (Figs 11 & 12). There is a gradual decrease in the growth rate after the exponential phase has ceased as can be detected with all cultures limited by compounds required in trace amounts. Presumably this is because the specific permease for the compound is no longer able to maintain saturated intracellular concentrations in very dilute solutions (Stanier et al., 1977).

E. coli R^- had a higher growth yield than E. coli R^+, and that of P.aeruginosa was even lower (Boggis, 1971) (the respective OD obtained using a Mg^{2+} concentration of 10^{-5} M were 0.84, 0.46 and 0.11).

Mg^{2+} has many important roles in the bacterial cell: stabilization of the ribosomes, acting as a cofactor for a number of enzyme-catalyzed reactions, including those involving ATP (Stanier et al., 1977) and those enzymes involved in the synthesis of cell wall components such as fatty acids (Knivett & Cullen, 1967), peptidoglycan (Garrett, 1969), lipopolysaccharide (Edstrom & Heath, 1967) and phospholipids (White et al., 1971 cited by Kenward, 1975). Magnesium has also

been suggested as a structural component of the outer membrane (Costerton, Ingram & Cheng, 1974), especially in P. aeruginosa (Brown & Melling, 1969 a,b).

Tempest, Hunter & Sykes (1965) found that RNA content, including the ribosomal RNA of Aerobacter aerogenes, was related to the degree of Mg^{2+} limitation, and MaCarthy (1962) and Morgan et al. (1966) have shown that protein synthesis in Mg^{2+} depleted E. coli is proportional to the ribosome content. As R-plasmids code for enzyme formation, it might be thought that the additional Mg^{2+} requirement of R^+ cells was due to the additional protein synthesis. However, it is unlikely that the amount of additional protein formed could make a significant difference to the total protein of the cell, and indeed the nitrogen requirements for E. coli R^+ and R^- are the same (Fig19). It seems more likely that large differences in Mg^{2+} requirement are going to be due to structural differences and that these will be found in the cell walls. Such differences may also explain the difference between Mg^{2+} requirement of E. coli and P. aeruginosa, although the higher requirement of the latter for nitrogen (Boggis, 1971) may also indicate increased protein synthesis and an associated increase in Mg^{2+} requirement for this purpose.

5.1.4. Phosphate

Fig22 shows that the growth yield of R^+ cells for phosphate was less than for R^-. Interestingly, the yield of P. aeruginosa (Boggis, 1971) was comparable to that of the R^+ cells. However, phosphate has so many roles in the cell that these comparisons may not be very helpful. Its main structural role is in the membranes and nucleic acids. Gilbert & Brown (1977) found little difference in the phospholipid (PL) content of R^+ and R^- cells. Perhaps the

lipopolysaccharide (LPS) phosphate requirements differed. Brown & Watkins (1970) noted that a polymyxin resistant strain of P. aeruginosa possessed cell walls with different PL content, but not PL composition, compared with walls from a sensitive strain. They also obtained some evidence that LPS differed. Kenward (1976), using P. aeruginosa NCTC 6750, found that RP1 caused increased phosphatidylethanolamine and decreased phosphatidylglycerol. In addition, R^+ cells have the additional DNA of the plasmid. However, this would not affect the phosphate requirement detectably.

5.1.5. Nitrogen and Sulphate

Both nitrogen and sulphate are required for protein synthesis and nitrogen for nucleic acid. The R-plasmid had no effect on nitrogen requirement (Fig 19); although Fig 28 suggests that it may slightly increase the sulphur requirement, there was insufficient data to establish a significant difference. P. aeruginosa was found to require about twice as much nitrogen and sulphur (Boggis, 1971) as E. coli. This could be due to metabolic as well as structural differences.

5.1.6. Potassium

Potassium comprises one of the principal inorganic cations in the cells; it acts as a cofactor for some enzymes (Stanier, 1977) and it is also important for ribosomal function because when the potassium content of bacterial cells is progressively lowered, protein synthesis ceases (Davis et al., 1973).

E. coli R^+ needs 1.5-fold as much K^+ to give the same growth as the R^- culture. It is unlikely that the additional requirement for K^+ by the R^+ culture could be entirely attributed to the additional

enzymes coded for by the R-plasmid, suggesting other differences in metabolism.

5.1.7. Iron

E. coli R^- does not need any added iron to support its growth (Fig30), suggesting that its iron requirement is satisfied by contamination from other nutrients present in the medium. The level of contamination was not estimated chemically, but it is likely to be of the same order of magnitude as that found by extrapolating the graph relating the growth of E. coli R^+ to concentration of added iron back to the x-axis (Fig32), bearing in mind that the basal media used was slightly different. This contamination by iron was found to be about 4×10^{-7} M. A similar high level of contamination was also found by Boggis (1971) using P. aeruginosa.

Fig 30 shows that the R^+ culture requires added iron above an OD of about 0.8, and, from the slope of the graph, it can be seen that the requirement rises steeply in dense cultures. Finch (1976) measured the dissolved oxygen concentration of the medium during the growth of P. aeruginosa and noted that it decreased exponentially during early exponential growth. By an $OD_{470\ nm}$ of about 0.4, the culture was only about 60% saturated with oxygen, and above this OD the rate of oxygen removal progressively slowed. This showed that not only were the cells growing in a progressively oxygen depleted medium but that at high cell concentrations, growth involved utilization of proportionally less oxygen relative to the number of cells present.

The steady decrease in the concentration of available oxygen found by Finch (1976) may be related to the steep rise in iron requirement noted for E. coli R^+ because the main role of iron in the cell is associated with oxidative pathways. Iron is a constituent

of cytochromes which are heme proteins, in which the prosthetic group is composed of a cyclic tetrapyrrole with one atom of iron chelated within the ring. Moss (1952) cited by Smith (1954), using E. coli, found an increase in cytochrome a_2 at low oxygen tension, but it could not be correlated with the changes in respiratory rate. The cytochrome b_1 band disappeared in anaerobically grown cells. Schaeffer (1952) and Waring & Werkman (1944) cited by Smith (1954) also noted quantitative variations in cytochromes in Bacillus cereus with changes in oxygen tension and in Aerobacter indologenes with changes in iron concentration respectively. However, Smith (1954), who examined many species, including E. coli, found no obvious relationship between cytochrome components and the degree of aerobiosis. More recent work has resolved some of the confusion by showing that other medium components affect the cytochromes present in E. coli (Poole & Haddock, 1975) and in addition, iron limitation leads to a decreased synthesis of both cytochromes and the iron-sulphur proteins of the electron-transport chain (Haddock, 1977) leading to an impairment of energy conservation.

Iron is also found in catalase, an enzyme which is found in E. coli; it catalyzes the breakdown of hydrogen peroxide, produced from the oxidation of flavoproteins, to water and oxygen. However, it is not clear why E. coli R^+ might need more catalase when grown under oxygen limitation.

5.1.8. Significance of nutritional differences between R^+ and R^-

Nutritional requirements reflect the metabolism and chemical composition of an organism. The possibility that the difference in the needs of E. coli R^+ and R^- are due at least in part to an effect of RP1 on the cell wall will be considered in the next section. However, the importance of R-plasmids as mediators of resistance and

their wide distribution in the community, including those of sewage, surface water, soil, air and of other environmental sites (World Health Organization, 1976) is becoming even clearer. In Salmonella, the resistance may be acquired in animals or in man in many parts of the world. The chloramphenicol-resistant typhoid outbreak in Mexico and the resistance to chloramphenicol in over 80% of cases of typhoid fever in South Vietnam in 1975 are examples of the conversion of a specific human pathogen to resistance in man as a result of immoderate use of antibiotics (World Health Organization, 1976).

Antibiotic resistance is very common among gut coliforms and it is likely that everyone, well or ill, carries some R^+ coliforms. Datta (1969) cited by Richmond (1972), isolated resistant strains from 52% of patients before admission to hospitals; up to 60% of these were carrying R-factors. Moorhouse (1969) cited by Richmond (1972), isolated 81% strain carrying R-factors from healthy infants in Dublin. In Bristol, faeces from healthy adults and children under five, none of whom were attending hospital or receiving antibiotics, were examined for the presence of antibiotic resistance; 73% of the children and 49% of the adults carried resistant strains and about half the strains carried R-factors (Linton et al., 1972, cited by Richmond, 1972). Richmond (1972) isolated 21% tetracycline-resistant strains from the sewage of Bristol hospitals, and 3% from domestic sewage. This means that the effect of such plasmids on nutrition may have a wider significance.

It seems likely that most bacteria under natural conditions must be nutrient limited (Brown, 1977) and where two species are competing, their nutrient requirements may affect their chance of survival.

Melling, Ellwood & Robinson (1976) examined the effect of the possession of the R-plasmid RP1 upon the ability of E. coli W3110

to survive in mixed culture with isogenic R^- strain under different environmental conditions in a chemostat. They found that the R-plasmid was stable under all growth conditions used, but that in a mixed culture of R^+ and R^- cells, the R^- strain took over under PO_4^{3-} limited conditions, even from a very small inoculum. This occured at dilution rates of 0.3 h^{-1} and 0.1 h^{-1}. They showed that the replacement of R^+ by R^- cells was not due to the formation of a toxic factor by the R^- cells and suggested therefore that the R^- strain is more efficient in the uptake or metabolism of phosphate.

The biggest difference in nutritional requirement of R^+ and R^- cells was found here to be for phosphate (Table 11) and the lower yield of R^+ cells could explain their replacement by R^- cells at high growth rates. However, Kuenen et al. (1975) cited by Veldkamp (1976) noted that when two PO_4^{3-} limited chemostats were inoculated with pond water, a Spirillum species became dominant at a low dilution rate (0.03 h^{-1}), whereas an unidentified rod shaped bacterium dominated the culture at a high dilution rate (0.3 h^{-1}). Further study revealed that the K_s for phosphate of the Spirillum and the rod shaped bacterium was 1.1×10^{-8} M and 6.6×10^{-8} M respectively. Thus, only at high growth rates did differences in yield affect selection. By analogy with these experiments, it might have been expected that at the lower growth rate used by Melling et al. (1976) R^+ cells ($K_s = 0.54 \times 10^{-6}$ M) might have taken over from R^- cells ($K_s = 0.73 \times 10^{-6}$ M). Presumably, the result depends on differences in the yield and growth rate, and the actual dilution rate tested (0.1 h^{-1}) was not enough to alter the balance. It was however, noted by Melling et al. (1976) that the R^+ cells never disappeared entirely. With other limitations (C and Mg^{2+}) Melling et al. (1976) found that it was the proportions of R^+ and R^- cells added which determined the outcome. This is perhaps not

surprising. Although the yield for Mg^{2+} limited cultures was found here to be higher for R^- than R^+ cells, the difference was not as great as for PO_4^{3-} limitation (Table 11). There was no difference in saturation constants (Table 12).

Nutritional differences may play a role in survival in the gut. Smith (1975) found considerable variation in the survival of different mutants of E. coli K12 following ingestion, but that thymine-dependent strains (thy$^-$) in general survived more poorly than thy$^+$ ones. He pointed out that strains with nutritional defects could be useful for potentially dangerous genetic engineering experiments.

Establishment of an infection may also be affected by nutritional requirements. Smith (1976) reported that nutritionally-deficient mutants of Salmonella typhimurium were avirulent unless injected with their required mutants. However, for most bacteria, the tissue and body fluids probably contain sufficient nutrients to support some growth, but nutrient concentrations may affect the rate of growth in vivo and therefore the chances of establishing an infection. Once the tissue is infected, bacterial growth may be controlled by the supply of some particular nutrient which may vary according to the site of infection (Dean et al., 1976; Brown, 1977).

Living cells need iron, and all must form or be supplied with compounds to solubilize and transport the metal. An early host response to bacterial invasion is a reduction in the amount of iron in the blood plasma by halting intestinal assimilation of iron and by increasing liver storage of the metal. However, if the host is hyperferremic, he becomes exceedingly susceptible to even small numbers of invading bacteria (Lankford, 1973 cited by Weinberg, 1974).

The increase in the iron requirement of E. coli due to RP1 may at the same time make the cell less virulent. However, under

conditions of hyperferremia, such differences would disappear.

5.2. EFFECT OF NUTRIENT DEPLETION AND R-PLASMID ON THE CELL WALL

5.2.1. Changes in cell surface

Table 13 shows that although different depletions have very little effect on the cell surface area, the R^+ cells may have a larger surface area than R^- ones since the number of cells are greater in the former and less in the latter in a suspension at a fixed OD. However, the concentration of intracellular constituents also affects the OD of a suspension (Meynell & Meynell, 1970) so some other effect of the R-plasmid may explain this difference.

Measurement of H^+ uptake showed that nutrient depletion has a profound effect on the cell surface, modified slightly by RP1 (Fig 48). There was no change in the isoelectric point, but the amount of hydrogen ion bound varied ($-Mg^{2+} < -C < -PO_4^{3-}$). Hydrogen ions would neutralize phosphate groups, carboxylic groups of amino acids and fatty acids, and ionise the basic groups of amino acids. The biggest change in surface charge was about pH 5 which is near to the pK of many organic acids, suggesting that these compounds of the cell envelope are changing with these depletions. Changes in charge do not correlate with changes in sensitivity to chlorhexidine, cetrimide and phenol (see section 5.2.2.).

Changes in the cell surface may be important in pathogenicity. In recent years several authors have stressed the importance of the interaction between the bacterial surface, serum factors and phagocytic cells (Tagesson & Stendahl, 1973; Hofman & Dlabac, 1974, cited by Finch, 1976). Friedberg & Shilo (1970) cited by Finch (1976) noted that the possession of a complete LPS core is important for resisting

ingestion and for intracellular survival and that the presence of the O-specific side chain contributes further resistance. Finch (1976) found that slow growing ($-Mg^{2+}$) cells were more resistant to killing by phagocytes than (-C) cells and presented evidence that this was due to a difference in resistance to opsonisation and to intracellular killing by cationic proteins.

5.2.2. Changes in resistance to disinfectants

Table 16 shows the effect of the R-plasmid on the MIC of the three disinfectants studied in broth. The presence of the R-plasmid renders the cells more sensitive to chlorhexidine and cetrimide, but with phenol no changes were noted. These results do not support those obtained by Russell(1972) using P.aeruginosa, since he suggested that R-plasmids have no effect on sensitivity to disinfectants. His results have little meaning because he was not comparing the same strains with and without an R-plasmid. However, he did not test the R-plasmid RP1, and not all R-plasmids will necessarily affect the cell envelope.

MIC's show only the effect of an agent on growing cells. A more common situation is where the cells have become stationary from lack of nutrients. In addition to the effect of genetic determinants it is known that cell envelopes show phenotypic variation associated with the availability of nutrients (see p9). Therefore the effects of nutrient depletion and the R-plasmid were studied together and the results are summarized in Table 17.

Resistance to the three disinfectants tested is thought to depend on the properties of the cell envelope (Hugo & Longworth, 1964 a,b). This is confirmed by the changes in resistance demonstrated here. The R-plasmid RP1 is known to affect the cell envelope, as has already been discussed (see section 1.3.2) and it is therefore to be expected that

Table 17: Summary of changes in resistance to disinfectants following nutrient limitation

Disinfectant	*E. coli* R^-			*E. coli* R^+		
	-C	-Mg	-P	-C	-Mg	-P
Cetrimide	.	--	--	+	++	-
Chlorhexidine	.	++	+	---	-	--
Phenol	.	--	---	.	+	--

KEY:- *E. coli* R^- (-C) has been chosen as an arbitary standard with which all cultures are compared.

+,++ cultures more resistant

-,--,--- cultures less resistant

. no changes in resistance

it might affect the MIC's of disinfectants.

The effect of the R-plasmid in (-C) cultures was similar to that on the MIC when they were tested against phenol and chlorhexidine. The resemblance of envelope-mediated-resistance of growing cells to C-limited cells has also been shown for *P. aeruginosa* using polymyxin or EDTA (Brown & Melling, 1969 a,b).

With cetrimide, the result with (-C) cells varied with the cetrimide concentration. However, some differences between growing and stationary cells might be expected, as changes are known to occur in the envelope when cells become stationary (Knivett & Cullen, 1967). Cells depleted of Mg^{2+} or PO_4^{3-} gave a different response from (-C) cells, depending upon the presence or absence of the plasmid and the disinfectant tested.

The effect of depletion on hydrogen ion uptake indicated

changes in the surface but these could not be correlated with changes in resistance.

The effect of Mg^{2+} depletion on the cell wall structure of P. aeruginosa has already been discussed (see p 9), however effects on resistance are likely to vary from one species to another. Mg^{2+} and PO_4^{3-} depletion of Proteus mirabilis causes an increase in resistance to phenol and cetrimide when compared with C-depletion (Klemperer et al., 1977).

Interpretation of these results awaits more information about changes in the composition of the E. coli cell envelope. Using whole cells of E. coli W3110, the only significant effect of RP1 found by Gilbert & Brown (1977) was in the increased levels of β-polyhydroxybutyrate irrespective of the nature of the depleting nutrient. PO_4^{3-} depleted R^+ and R^- had less phospholipid than C-depleted cells, which was compensated for by increases in fatty acid and neutral lipid content. The reduction in phospholipid content of the (-P) culture was accounted for by decreased diphosphatidylglycerol and phosphatidylethanolamine. Also, Kenward (1975), using (-C) P. aeruginosa, found that RP1 caused increased phosphatidylethanolamine and decreased diphosphatidylglycerol in the cell envelope. The importance of phospholipid ratios in relation to permeability of the outer membrane was demonstrated by Boman, Nordstrom & Normark (1974) by measuring the rate of uptake of gentian violet by a number of E. coli K12 mutants.

The increase in positively charged phospholipids might decrease binding of Mg^{2+} ion or binding of cationic surfactants.

Certainly, RP1 affected either the location of Mg^{2+} or the nature of its binding to other components in the wall because it could not be extracted from R^+ cells by organic solvents as readily as from R^- cells (Kenward, 1975).

5.3. CONCLUSION

While it is clear that interpretation of these results must await a detailed study of the changes in composition of the E. coli envelope, a number of conclusions can be drawn. Both the R-plasmid RP1 and nutrient depletion affect the structure of the E. coli envelope and these changes alter its resistance to antibacterial agents.

Depletion of nutrients other than those tested may also cause changes in the envelope and therefore resistance. The amount of some of the proteins in the outer membrane of strains of E. coli are controlled by the iron concentration of the medium (Ichihara & Mizushima, 1977). Sulphate depletion leads to a decrease in endotoxin content in E. coli (Pearson & Ellwood, 1974) and to an increase in resistance to polymyxin of P. aeruginosa (Klemperer, 1976).

Other changes in resistance may occur as a result of changes in growth rate, for example Finch & Brown (1975) showed that the resistance of (-C) P. aeruginosa to EDTA varied with growth rate. Chemostat studies of E. coli R^+ and R^- might yield some interesting results.

Envelope changes may also affect resistance to opsonization and phagocytosis. In addition, pathogenicity may be affected by changes in antigenicity, comparable to changes in P. aeruginosa antigens following Mg^{2+} limitation (Finch, 1976). The increased iron requirement associated with the R-plasmid may actually reduce the virulence of R^+ cells, through 'nutritional immunity' (Weinberg, 1974). Survival of R^+ in competition with the R^- strain will depend on growth yield and growth rates. Colonisation of the gut will depend on relative ability to stick to the mucous membranes and to resist colicins formed by other strains of E. coli (Smith, 1976). Both these

properties depend upon the cell surface. The gut is a poor nutritional environment and its flora is likely to be nutritionally depleted. The different requirements of R^+ and R^- cells will mean that R^+ cells will become depleted of some nutrient before R^- cells.

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