THE MODES OF SYNTHESIS OF CELL ENVELOPE COMPONENTS IN ESCHERICHIA COLI

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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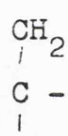
CONTENTS

CHAPTER 1. INTRODUCTION

In this introduction I shall attempt to describe our present understanding of how the surface layers of the bacterium Escherichia coli grow and how the cells divide. In the first section the biosynthesis of the various components of the cell envelope will be described, including the occurrence of a common mechanism, a "lipid cycle", involved in the synthesis of both peptidoglycan and lipopolysaccharide. The structural relationships of the different constituents will then be discussed, together with a description of experiments which lead us to believe that most of the cell envelope components are inserted into the growing envelope at a very few sites on the cell surface, rather than by a process of random intercalation of new material. The next section will contain a discussion of our knowledge of the regulatory processes acting to ensure that at each cell division, two daughter cells are produced which are, in all important aspects, identical with the original bacterium. Finally, the evidence relating to the growth of cell envelope components in bacteria will be discussed in the context of the aims of the present study.

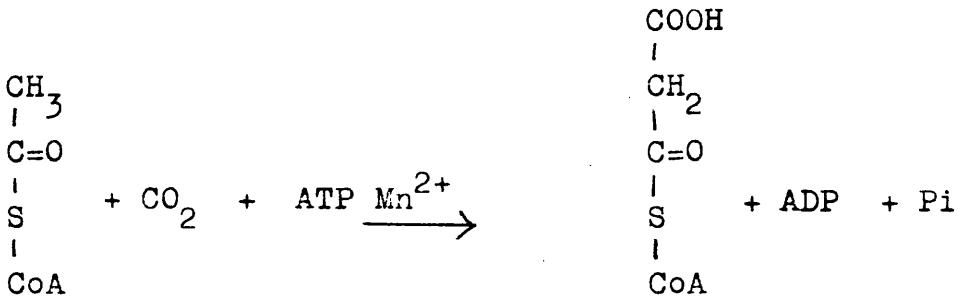
TABLE 1. FATTY ACIDS OF *E.COLI*. (CRONAN AND VAGELOS, 1972)

TYPE	STRUCTURE	SYSTEMATIC NAME	TRIVIAL NAME
Saturated	$CH_3-(CH_2)x$ -COOH	Dodecanoic (x=10)	Lauric acid
		Tetradecanoic (x=12)	Myristic acid
		Hexadecanoic (x=14)	Palmitic acid
		Octadecanoic (x=16)	Stearic acid
Unsaturated	$CH_3-(CH_2)_5-$  x-COOH	*cis*-9-Hexadecenoic acid (x=7)	Palmitoleic acid
		cis-11-Octadecenoic acid (x=9)	*cis*-Vaccenic acid
Cyclopropane	$CH_3-(CH_2)_5-$  x-COOH	*cis*-9,10-Methylene hexa-decanoic acid (x=7)	none
		cis-11,12,Methylene octadecanoic acid (x=9)	Lactobacillic acid
Hydroxy	$CH_3-(CH_2)x$ $-CHOH-CH_2-$ COOH	D(-)-3-Hydroxy tetradecanoic acid	β-Hydroxy-myristic acid

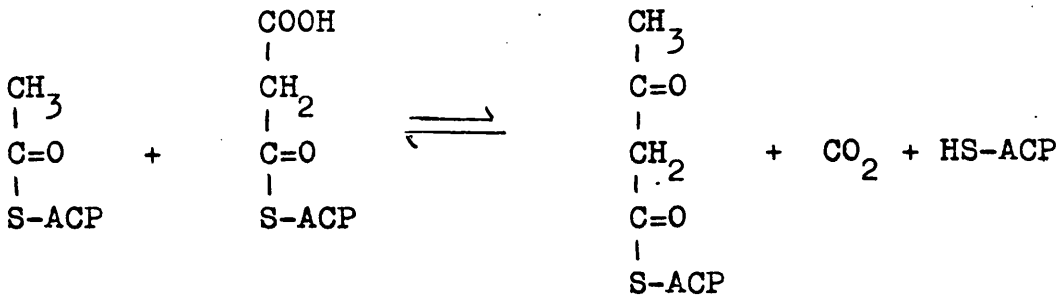
1.Ia. BIOSYNTHESIS OF LIPIDS IN E.COLI

The fatty acids occurring in E.coli are shown in Table 1. The saturated acids are mostly palmitic and myristic with trace amounts of lauric and stearic acids. The identification of both saturated and unsaturated acids is based on their gas chromatographic behaviour. This technique coupled with infra red spectroscopy has shown that the unsaturated fatty acids are all cis monoenes. Cis-vaccenic acid has been shown to be the sole octadecenoic acid (Cronan, 1967) present in E.coli which indicates that oleic acid is not synthesised by this species. This is corroborated by the discovery that lactobacillic acid is the sole C_{19} cyclopropane acid in this organism, since cyclopropane fatty acids are synthesised from the corresponding unsaturated fatty acids (Chalk & Kodicek, 1961). Hydroxymyristic acid is characteristic of the Lipid A moiety of lipopolysaccharide in this organism (Osborn, 1969).

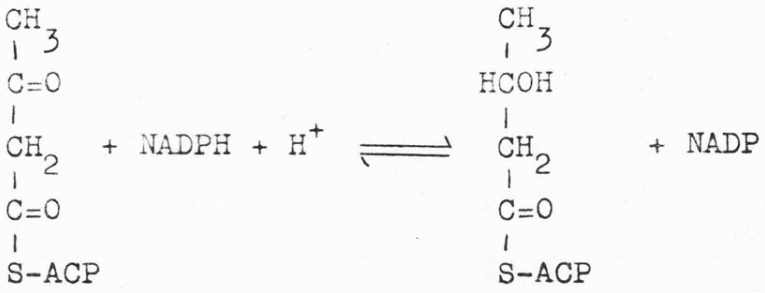
The enzymatic aspects of fatty acid synthesis have been reviewed by Volpe and Vagelos (1973). The first step is the production of malonyl CoA from acetyl CoA by acetyl CoA carboxylase. This involves the fixation of carbon dioxide by the action of the coenzyme biotin.



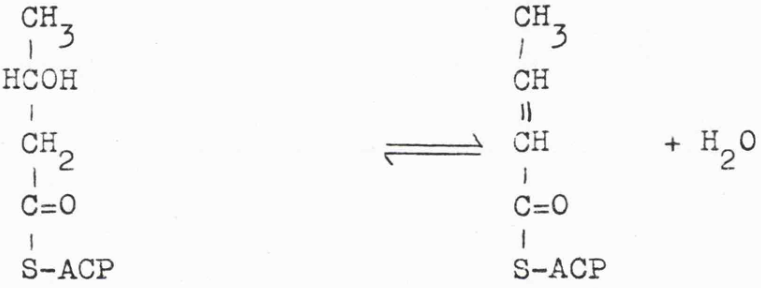
This reaction is important, since it is the use of malonyl CoA as a source of C_2 fragments, which provides the thermodynamic drive for the whole synthetic reaction. The synthesis of the fatty acids from these two initial compounds, acetyl CoA and malonyl CoA, is catalysed by an enzyme complex, fatty acid synthetase. This contains several enzymes which perform a cycle of reactions, each time adding a C_2 fragment to the growing fatty acid chain. The central component of the complex is the acyl carrier protein (ACP) which contains a prosthetic group of similar structure to CoA, and to which all the intermediates are linked by thioester bonds. First of all, two enzymes catalyse the transfer of the acetyl or malonyl group from CoA to ACP. Both reactions seem to occur by a two step process, with an enzyme-acetyl (malonyl) intermediate. This is followed by the condensing reaction catalysed by β-ketoacyl-ACP synthetase.



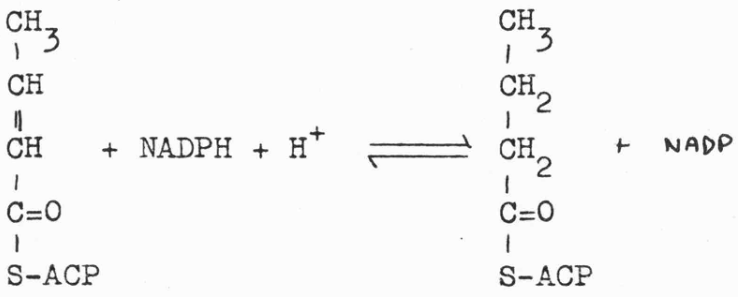
The product then undergoes the first reduction catalysed by β-ketoacyl-ACP reductase to yield the D(-)-β-hydroxyacyl-ACP derivative.



This is then dehydrated to form a trans -2-enoyl-ACP thioester by the action of β-hydroxyacyl-ACP dehydrase.

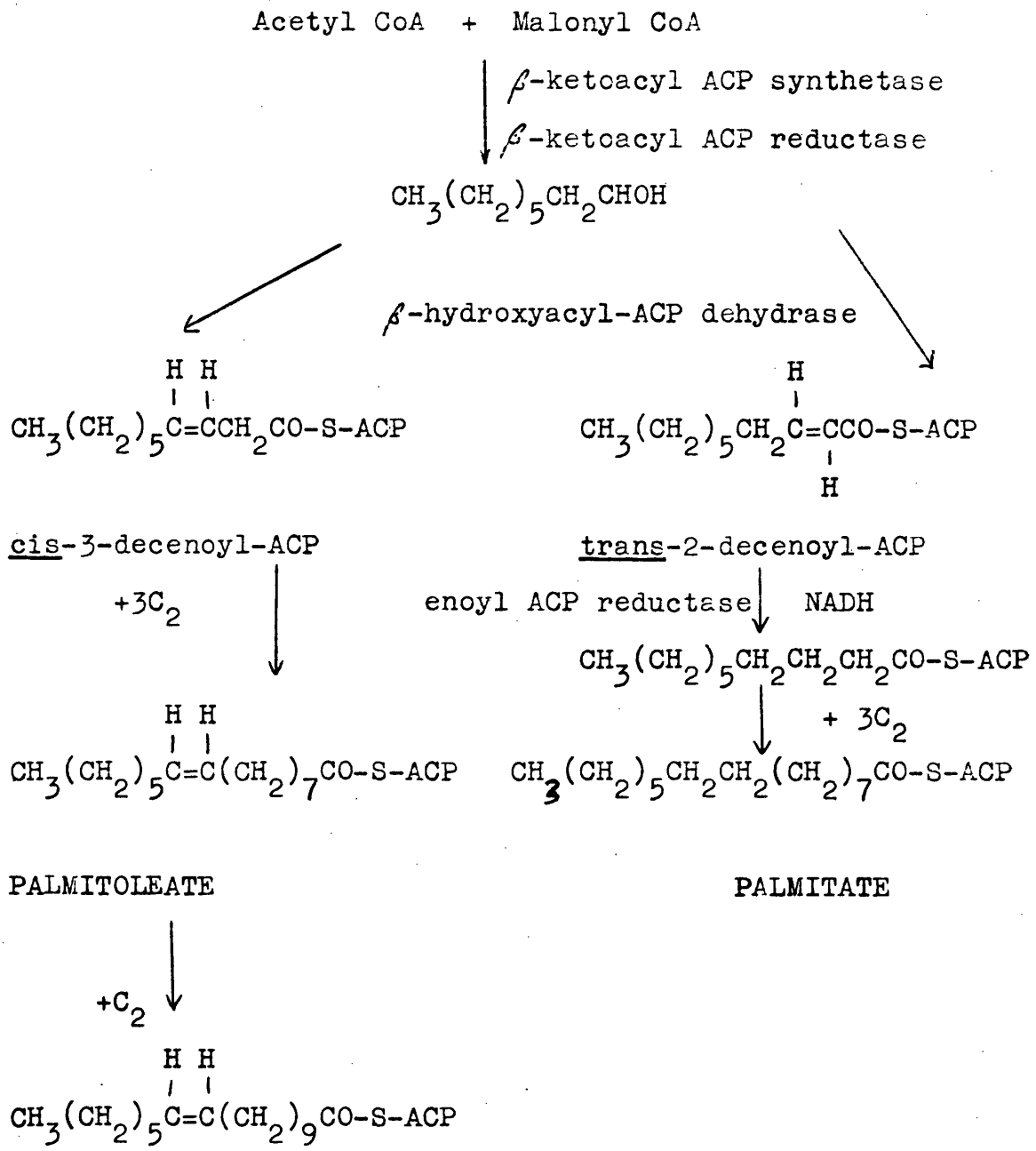


Finally another dehydration catalysed by enoyl-ACP reductase completes the first cycle.



The cycle then repeats with another C_2 fragment being added from malonyl-ACP. Two questions may be posed of this scheme of reactions: does it provide for the synthesis of all the fatty acids, and what determines the length of the individual fatty acids produced? There are at least three different β-hydroxyacyl-ACP dehydrase activities in E.coli. One is specific for chain lengths from C_2-C_8 (Mizugaki et al. 1968). The

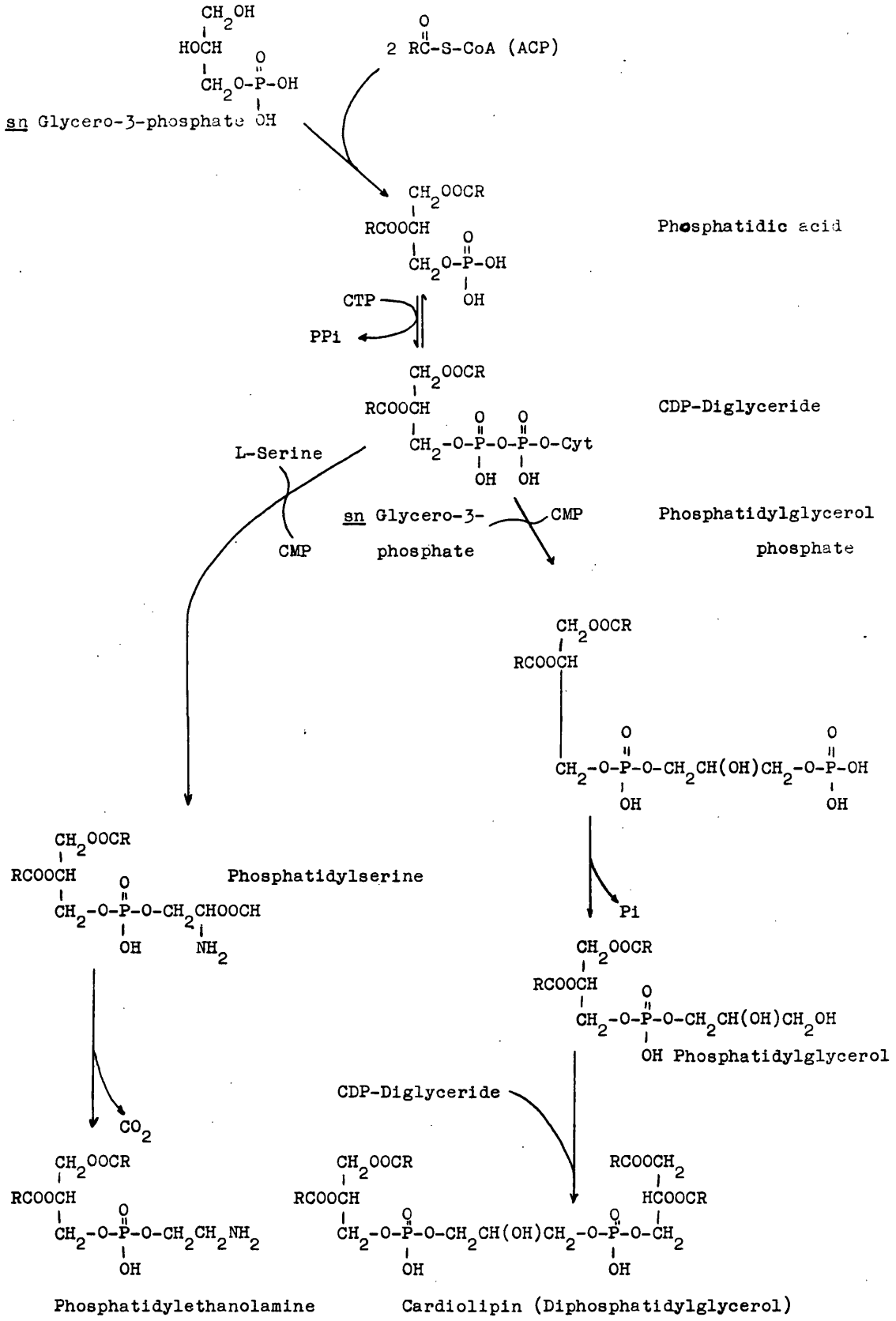
FIGURE 1. PATHWAY OF FATTY ACID SYNTHESIS SHOWING THE BRANCH POINT BETWEEN SATURATED AND UNSATURATED FATTY ACIDS. (FROM CRONAN & VAGELOS, 1972).


Acetyl CoA + Malonyl CoA
β-ketoacyl ACP synthetase
β-ketoacyl ACP reductase
CH3(CH2)5CH2CHOH
β-hydroxyacyl-ACP dehydrase
CH3(CH2)5C=CCH2CO-S-ACP
CH3(CH2)5CH2C=CCO-S-ACP
cis-3-decenoyl-ACP
trans-2-decenoyl-ACP
+3C2
enoyl ACP reductase
NADH
CH3(CH2)5CH2CH2CH2CO-S-ACP
+ 3C2
CH3(CH2)5C=C(CH2)7CO-S-ACP
CH3(CH2)5CH2CH2(CH2)7CO-S-ACP
PALMITOLEATE
PALMITATE
+C2
CH3(CH2)5C=C(CH2)9CO-S-ACP
cis VACCENATE

second catalyses the reaction with any chain length, but is least active with the C_{10} substrate (Birge and Vagelos, 1972). Both enzymes yield trans-2-enoyl-ACP thioesters. The third enzyme is specific for C_{10} substrates and yields two products (Bloch, 1969). One is the trans-2-enoyl-ACP ester and the other is the cis-3-enoyl-ACP ester. As shown in Fig. 1 this latter product is not reduced but forms the precursor of the unsaturated fatty acids. Support for this conclusion comes from the isolation of mutants, deficient in this C_{10} specific enzyme, which are unable to synthesise unsaturated fatty acids (Silbert & Vagelos, 1967). The determination of the chain length appears to be a function of the specificity of the fatty acid synthetase enzymes (Law & Snyder, 1972) although a specific palmityl-ACP thioesterase has been described by Barnes & Wakil (1968). The functions of this enzyme in vivo is unclear, since the ACP derivatives of the fatty acids, as described below, are incorporated directly into phospholipid. The cyclopropane acids are synthesised by methylation of unsaturated fatty acid side chains of phospholipids using S-adenosyl methionine as a methyl donor (Cronan, 1968). Synthesis of lipid A will be dealt with in section 1.Ic.

The mechanism of synthesis of phospholipids in E.coli is shown in Fig. 2. (For a review of the evidence for the occurrence in E.coli of only the species shown, see Cronan & Vagelos, 1972). Radioactive precursor studies have indicated that sn glycero-3-phosphate is the precursor of phospholipids and this was confirmed by the isolation of a mutant requiring this substance or

FIGURE 2. PATHWAYS OF PHOSPHOLIPID SYNTHESIS IN E.COLI (FROM CRONAN & VAGELOS, 1972).


sn Glycero-3-phosphate
2 RC-S-CoA (ACP)
Phosphatidic acid
CTP
PPi
CDP-Diglyceride
L-Serine
CMP
sn Glycero-3-phosphate
CMP
Phosphatidylglycerol phosphate
Phosphatidylserine
Pi
Phosphatidylglycerol
CO_2
CDP-Diglyceride
Phosphatidylethanolamine
Cardiolipin (Diphosphatidylglycerol)

glycerol for growth (Kito, Lubin & Pizer, 1969; Hsu & Fox, 1970). Cronan, Ray & Vagelos (1970) isolated a mutant unable to synthesise 1-acyl-sn-glycero-3-phosphate at 37°C whilst Hechemy & Goldfine (1971) isolated a similar thermolabile mutant able to synthesise this compound, but unable to perform the second acylation necessary to form phosphatidic acid. It therefore seems that two distinct enzyme activities are necessary for the formation of phosphatidic acid. Ray, Cronan, Mavis & Vagelos (1970) have shown that this reaction proceeds with great specificity : palmitic acid is virtually confined to incorporation in the 1-position, whilst the unsaturated fatty acids are primarily attached to the 2-position. The substrates for this reaction in vivo are not known. Both CoA and ACP thioesters are active substrates in vitro but experiments of Overath, Pauli & Schairer (1969), which show that acyl CoA synthetase is required for the incorporation of exogenous fatty acids, indicate that perhaps ACP esters may be substrates when newly synthesised fatty acids are incorporated into phospholipids, whilst CoA esters are substrates for renewal of phospholipid acyl chains with pre-existing fatty acids. CDP diglyceride has not been isolated from E.coli, but an enzyme system is present for its synthesis (Carter, 1968) and for its utilization in the synthesis of phosphatidyl serine and phosphatidyl glycerol (Chang & Kennedy, 1967a,b). Similarly, phosphatidyl-glycerol phosphate has not been isolated, but enzymes are present for both its synthesis and dephosphorylation (Chang & Kennedy, 1967b). The mechanism of synthesis of cardiolipin is not

so clear. The reaction shown in Fig. 2 has been demonstrated by Stanacev, Chang & Kennedy (1967), but an alternative, involving the condensation of two molecules of phosphatidyl-glycerol, with the release of free glycerol, has been proposed by Rampini *et al.* (1970). The enzyme system for the synthesis of phosphatidylethanolamine has been demonstrated by Kanfer & Kennedy (1964).

All the enzymes involved in phospholipid synthesis have been isolated from the particulate fraction of bacteria, and van den Bosch *et al.* (1970) have demonstrated an association between fatty acid synthetase and the cell surface of *E.coli*, by radioactive labelling of the acyl carrier protein, followed by electron microscopy and autoradiography. Analysis of grain distribution showed that the acyl carrier protein is located in or near the inner surface of the cytoplasmic membrane. It therefore seems likely that fatty acid biosynthesis and phospholipid synthesis proceed in association with the cytoplasmic membrane, and that phospholipids are inserted directly into the membrane. However, the basis of the metabolic regulation of lipid synthesis in *E.coli* is not clear. The fact that lipid composition remains fairly constant in various growth media indicates that regulation is not at the level of small molecule precursors. It has been suggested by Volpe & Vagelos (1973) that the turnover of the ACP prosthetic group may play a role in this respect. Merlie & Pizer (1973) have obtained evidence that amino acid starvation in *rel*$^+$ strains but not in *rel*$^-$ strains

causes reduced phospholipid synthesis, and that ppGpp irreversibly inhibits the synthesis of phosphatidic acid *in vitro*. However, the mechanisms that control the qualitative composition of the lipids in the cell envelope are not known.

1.Ib. BIOSYNTHESIS OF PEPTIDOGLYCAN

The peptidoglycan of *E.coli* and of most bacteria appears to be composed of β-1,4-linked pyranoside chains, consisting of N-acetylglucosamine and N-acetylmuramic acid residues arranged alternately. The N-acetylmuramic acid molecules are substituted in the completed peptidoglycan with a tetrapeptide of alternating D- and L- amino acid residues. These peptide moieties are linked in turn, through peptide bonds, to their counterparts on other glycan chains, thus forming a complex network of interconnected polysaccharide chains. The structure of *E.coli* peptidoglycan is shown in Fig. 3 (Ghuysen & Shockman, 1973). In *E.coli* the tetrapeptide structures are linked directly, typical of species belonging to chemotype I (Schleifer & Kandler, 1972). The length of individual peptidoglycan chains has been estimated in *E.coli* and is found to be approximately 100 amino sugar residues (Henning, Rehn, Braun, Höhn & Schwarz, 1972) in agreement with results for other organisms studied, where the glycan chain length varies from 10-50 disaccharide units (Braun & Hankte, 1974). The degree of polymerisation is such that *E.coli* peptidoglycan is

FIGURE 3. SCHEMATIC DIAGRAM OF PEPTIDOGLYCAN STRUCTURE (GHUYSEN & SHOCKMAN, 1973).

a) Structure of the glycan chains

M : N-acetylmuramic acid

G : N-acetylglucosamine

b) Structure of the tetrapeptide unit

c) Complete structure

Ala : alanine Glu : glutamate

DAP : meso diaminopimelate

$\longrightarrow$: α peptide linkage

$\xrightarrow{\gamma}$: γ peptide linkage

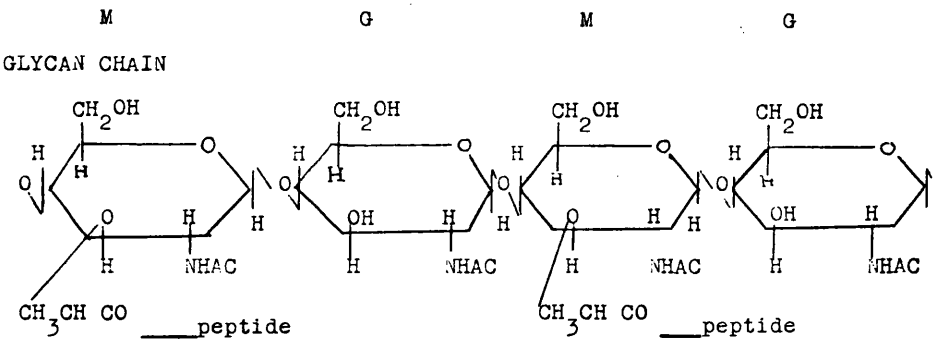
$\longrightarrow$: site of action of

(1) N-acetylmuramidase

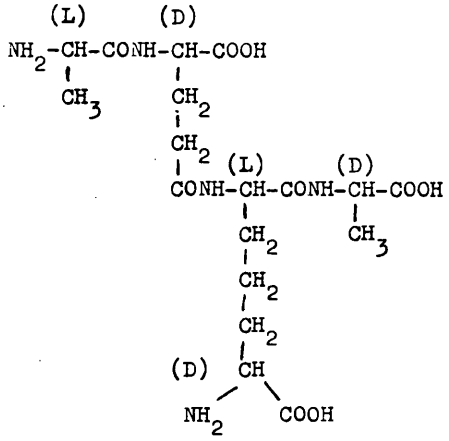
(2) N-acetylglucosamidase

(3) N-acetylmuramyl-L-alanine amidase

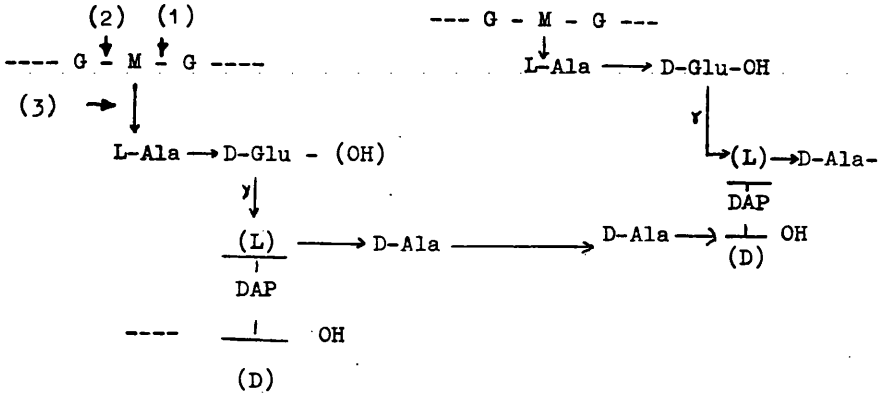
a



b TETRAPEPTIDE



C COMPLETE STRUCTURE



peptide linkage

described as "loose", with only some 50% of the peptide units being cross linked (van Heijenoort et al. 1969).

Figure 4 shows a scheme for the synthesis of peptidoglycan (taken from Ghuysen & Shockman, 1973, which is a comprehensive review of the subject). As can be seen, it is possible to divide the process into three distinct phases which occur in the cytoplasm, the cytoplasmic membrane, and in the cell wall respectively. The first reaction 3 is directly analogous to the production of UDP-glucose in the synthesis of glycogen, and after reaction 4, the precursor is then destined to become peptidoglycan. There follows the stepwise addition of the amino acid residues of the peptide unit and the enzymes involved (6,7,8,9 Fig. 4) all depend on ATP and Mg^{2+} or Mn^{2+}. It is interesting to note that unlike the normal process of peptide bond formation in protein synthesis, this reaction sequence is dependent upon the specificity of the enzyme catalysing the reaction itself.

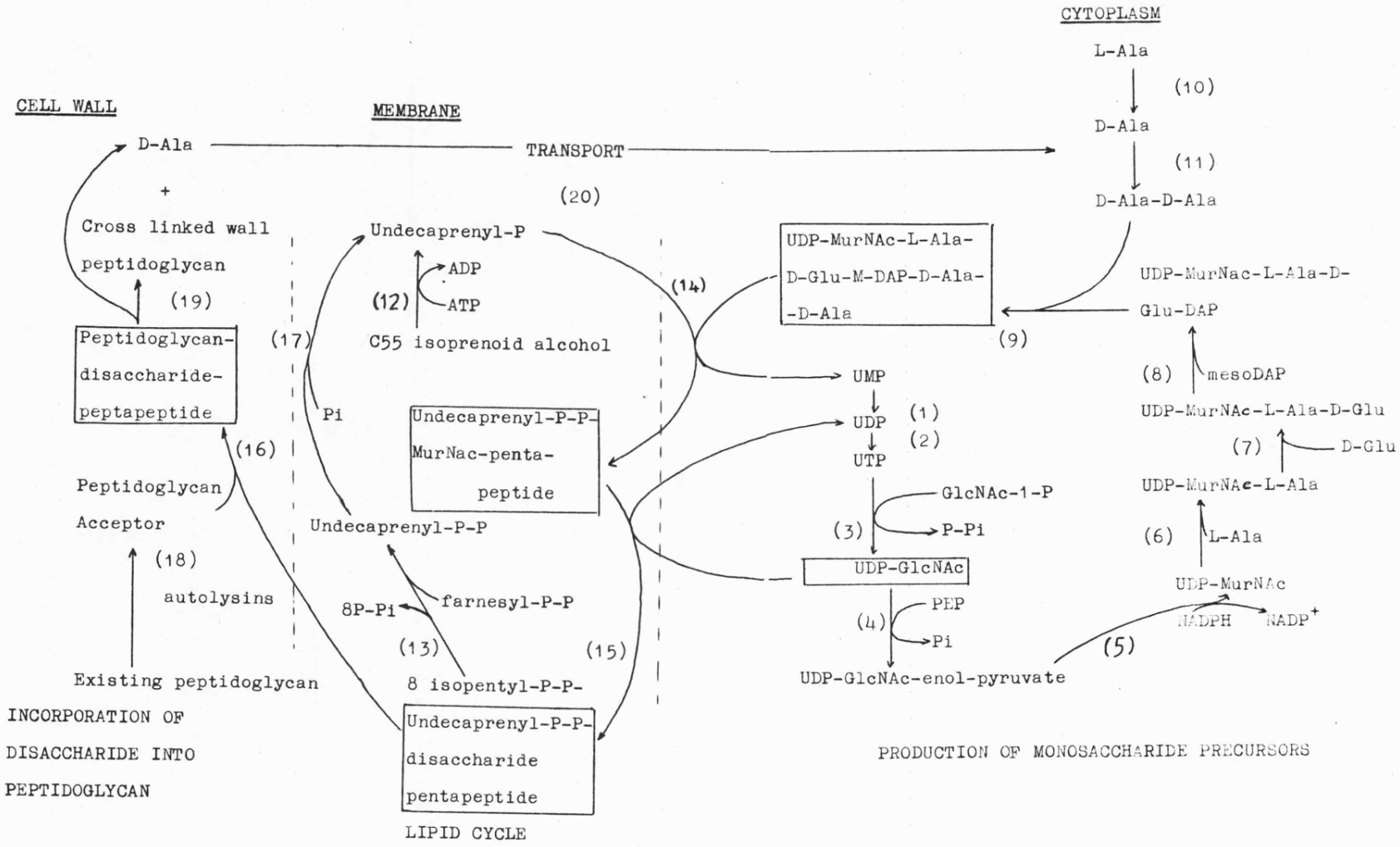
The "lipid cycle" which is localised in the membrane is responsible for the addition of soluble precursors in the cytoplasm to pre-existing peptidoglycan in the cell wall. This aspect of peptidoglycan synthesis appears to be of general occurrence, though it has been most extensively studied in Staphylococcus aureus. The lipid carrier itself is a C_{55} isoprenoid alcohol, made up of eleven isoprene units. Wright et al. (1967) have shown that the peptidoglycan carrier isolated from S.aureus will act in the biosynthesis of lipopolysaccharide in Salmonella, and competition for the carrier occurs in vitro between

FIGURE 4. SYNTHESIS OF PEPTIDOGLYCAN SHOWING THE COMPARTMENTALISATION OF THE REACTION SEQUENCE BETWEEN DIFFERENT PARTS OF THE CELL.

GlcNAc : N-acetylglucosamine

MurNAc : N-acetylmuramic acid

PEP : phosphoenol pyruvate


CYTOPLASM
L-Ala
(10)
D-Ala
(11)
D-Ala-D-Ala
CELL WALL
MEMBRANE
D-Ala
TRANSPORT
+
Cross linked wall
peptidoglycan
(19)
(20)
Undecaprenyl-P
ADP
(12)
ATP
C55 isoprenoid alcohol
(17)
Pi
Peptidoglycan-
disaccharide-
peptapeptide
(16)
Undecaprenyl-P-P-
MurNac-penta-
peptide
Undecaprenyl-P-P
Peptidoglycan
Acceptor
(18)
autolysins
8P-Pi
farnesyl-P-P
(13)
(15)
8 isopentyl-P-P-
Existing peptidoglycan
Undecaprenyl-P-P-
disaccharide
pentapeptide
INCORPORATION OF
DISACCHARIDE INTO
PEPTIDOGLYCAN
LIPID CYCLE
UDP-MurNAc-L-Ala-
D-Glu-M-DAP-D-Ala-
-D-Ala
(14)
(9)
UDP-MurNac-L-Ala-D-
Glu-DAP
UMP
UDP
(1)
(2)
UTP
GlcNAc-1-P
(3)
P-Pi
UDP-GlcNAc
PEP
(4)
Pi
UDP-GlcNAc-enol-pyruvate
(5)
(8)
mesoDAP
UDP-MurNAc-L-Ala-D-Glu
(7)
D-Glu
UDP-MurNAc-L-Ala
(6)
L-Ala
UDP-MurNAc
NADPH
NADP+
PRODUCTION OF MONOSACCHARIDE PRECURSORS

peptidoglycan and lipopolysaccharide precursors (Watkinson, Hussey & Baddiley, 1971). The mechanism of production of the phosphorylated carrier is not known in E.coli. Reaction 12 occurs in S.aureus whilst reactions 13 and 17 are known to occur in Salmonella newington (see Rothfield & Romeo, 1971). The attachment of the peptidoglycan precursor to the lipid carrier, reaction 14, proceeds with the formation of an enzyme-P-N-acetylmuramyl-pentapeptide intermediate. This reaction is inhibited by vancomycin in vitro (Neuhaus, 1972). The reaction results in the transfer of the N-acetylmuramyl pentapeptide and the terminal phosphate of UDP to the carrier. The subsequent transglycosylation, reaction 15, differs in that only N-acetylglucosamine is transferred to form a β-1-4, linked disaccharide unit. The mode of incorporation of the disaccharide unit into pre-existing peptidoglycan is unclear. In E.coli polymerisation of precursors does not occur in the plasma membrane (Braun & Bosch, 1973). It is not even certain whether incorporation is due to transpeptidation, 19, transglycosylation, 16, or both simultaneously (see Braun & Hankte, 1974). The mechanism of transpeptidation has been studied in vitro in particulate preparations and the reaction is inhibited by penicillin (Izaki, Matsuhashi & Strominger, 1968) and involves the release of the terminal D-alanine residue, which is then transported back into the cell, 20, (Fig. 4).

Metabolic regulation of peptidoglycan synthesis seems to operate at several levels. The first is at the alanine branch and involves control of the intracellular

pools of D- and L-alanine, there being separate transport systems for each isomer (Wargel, Shadur & Neuhaus, 1971). Neuhaus et al. (1972) have shown that a high intracellular concentration of alanine represses the synthesis of alanine racemase, 11, and the ratio of D- to L-alanine affects the activity of this enzyme, since the equilibrium constant for the reaction calculated from the Haldane relationship:

$$Keq = \frac{(Km\ \ D\text{-}Ala)\ \ (VmaxL \rightarrow D)}{(Km\ \ L\text{-}Ala)\ \ (VmaxD \rightarrow L)}$$

is approximately unity. Neuhaus and co-workers have also studied D-alanyl-D-alanine synthetase 11 and the results obtained with various inhibitors suggest the presence of multiple product sites on the enzyme which could control the rate of dipeptide formation. Neuhaus & Struve (1965) showed that in Streptococcus faecalis the substrate specificity of the D-alanyl-D-alanine adding enzyme, 9, is complementary to that of the synthetase. This combination of specificities results in the high level of accuracy of peptide synthesis, and also accounts for the growth inhibitory effects of some D amino acids. In this context it is worth pointing out that the mechanism involved in the synthesis of D-glutamate is not clear in E.coli. Exogenous L-glutamate is incorporated into peptidoglycan as D-glutamate, but no racemase activity has been detected (Lugtenberg, Wijsman & van Zaane, 1973).

A second level of control is suggested by the observation of Lugtenberg (see Ghuysen & Shockman, 1973) that UDP-N-acetylmuramyl pentapeptide does not accumulate

in penicillin treated cells, implying that this compound regulates its own synthesis by feedback inhibition. Another obvious possibility for regulation is at the level of incorporation of disaccharide units into peptidoglycan. It is also possible that the genes for the adding enzymes form an operon. The genes for the L-alanine, murC, m-diaminopimelate, murE and D-alanyl-D-alanine, murF, enzymes all map extremely close together at 1-1.5 min. on the E.coli chromosome (Wijsman, 1972).

1.Ic. BIOSYNTHESIS OF LIPOPOLYSACCHARIDE

The lipopolysaccharide of the outer membrane is the most extensively studied element of gram negative cell envelopes. The lipopolysaccharide (LPS) constitutes the major somatic antigen in enteric bacteria and is therefore most important from both an immunochemical and a taxonomic point of view. Not surprisingly the most extensively studied organisms are the various members of the Salmonella group, especially S.typhimurium. However, since this is closely related to E.coli, the general features of LPS synthesis are likely to be the same in both species. Figure 5 shows the structure of S.typhimurium LPS. This is shown to constitute three distinct regions, lipid A, core oligosaccharide and the O-side chain. The KDO_3 (3-deoxy-D-manno-octulosonic acid) lipid A portion of the molecule may well be indispensible to the bacterium, as unconditional mutants defective in the synthesis of this portion of the LPS

FIGURE 5. STRUCTURE OF LIPOPOLYSACCHARIDE SHOWING THE PRINCIPAL REGIONS OF THE MOLECULE (NIKAIDO, 1973)

Abe : abequose Man : mannose

Rha : rhamnose Gal : galactose

Glc : glucose GlcNAc : N-acetylglucosamine

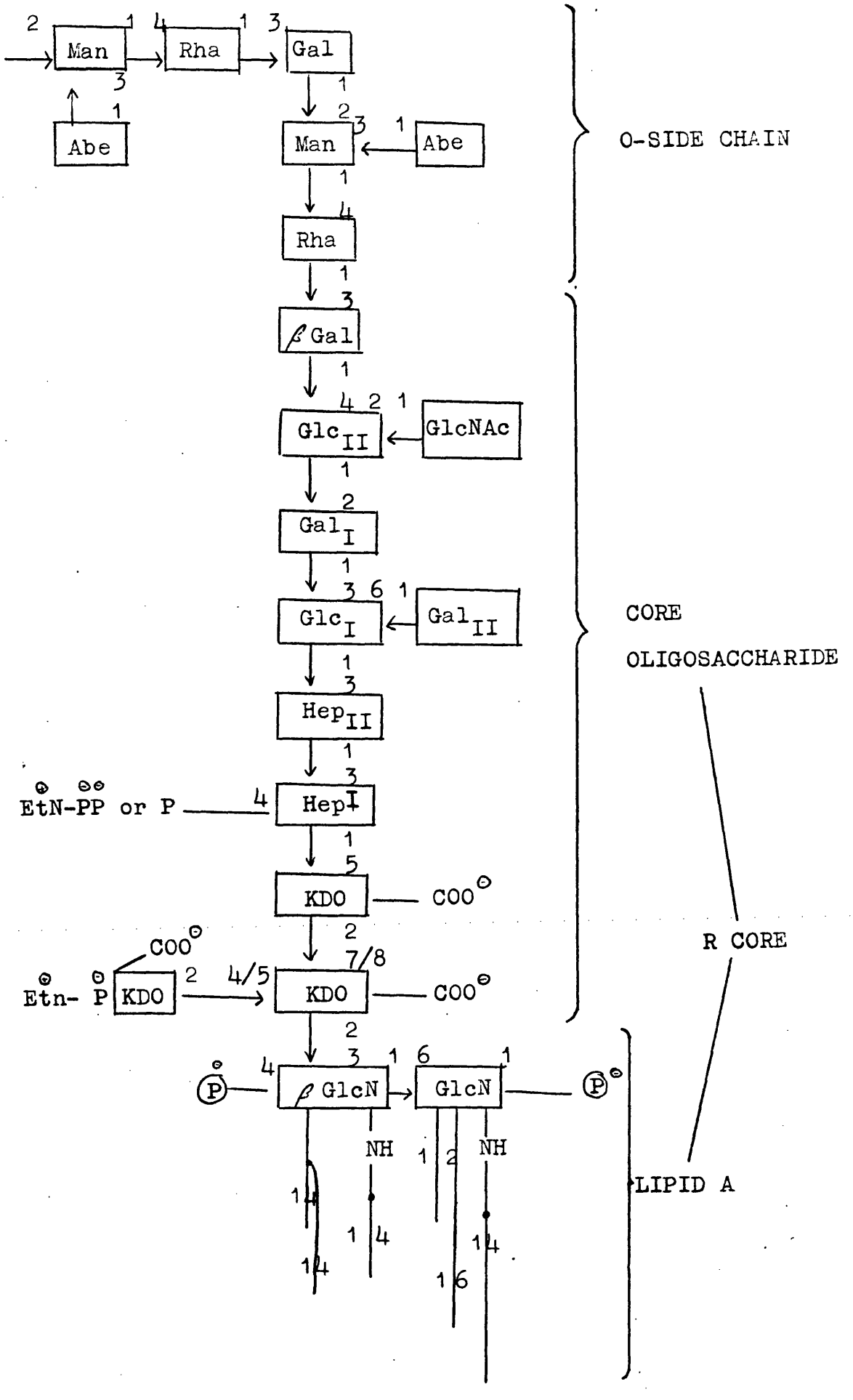
Hep : heptose KDO : 3-deoxy-D-manno-octulosonic acid GlcN : glucosamine

EtN : ethanolamine

$\xrightarrow{1\quad 3}$ type of bond between sugar

$\overset{14}{\text{———}}$: fatty acids showing number of carbon atoms

$\overset{14}{\text{—•—}}$: β-hydroxymyristic acid


Man
Rha
Gal
Abe
Man
Abe
Rha
βGal
GlcII
GlcNAc
GalI
GlcI
GalII
HepII
EtN-PP or P
HepI
KDO
COO⊖
COO⊖
Etn-P
KDO
KDO
COO⊖
P⊖
βGlcN
GlcN
P⊖
NH
NH
O-SIDE CHAIN
CORE
OLIGOSACCHARIDE
R CORE
LIPID A

have never been isolated. In addition to heptose and KDO, phosphate residues are also present in the R core and it is thought that at least some lipid A molecules are cross-linked by phosphodiester bonds (see Nikaido, 1973). It is not certain whether similar bonds also cross-link adjacent core oligosaccharide regions. The lipid A moiety is based on a D-glucosamine skeleton to which various fatty acids, including the characteristic β-hydroxy-myristic acid are attached. The work of Rietschel (see Nikaido, 1973) indicates that all the available sugar groups are esterified to fatty acids, and that the hydroxyl group of the β-hydroxy-myristic acid is also esterified. The appearance of amino acids after acid hydrolysis of LPS has suggested a covalent linkage of LPS to protein, though this has never been conclusively established (Wober & Alaupovic, 1971). The O-side chain consists of repeating subunits of tri - or tetra-saccharides and there is great heterogeneity of chain length in molecules isolated from any one organism. Many laboratory strains e.g. E.coli K12 and B are "rough" mutants, entirely lacking the O antigen. As can be seen from the diagram, there are a large number of ionisable groups in the heptose-KDO region which strongly suggests that cations such as Mg^{2+} may play a very important role in the association of adjacent lipopolysaccharide molecules.

Very little is known about the synthesis of lipid A, but an obvious possibility is that specific transferases transfer each fatty acid to its position in

the glucosamine disaccharide skeleton. Since β-hydroxy-myristic acid is a unique component of lipid A, attempts have been made *in vitro* to demonstrate the transfer of this fatty acid onto partially degraded LPS or lipid A preparations. The only reaction successfully demonstrated so far has been that involving the transfer of this acid from its acyl carrier protein derivative to lysophosphatidyl ethanolamine (Wu & Wu, 1971).

The synthesis of the core oligosaccharide commences with the transfer of KDO from CMP-KDO to lipid A (Heath *et al.* 1966). The mechanism of addition of the subsequent KDO and heptose residues is unclear. The phosphorylation of these latter residues, however, occurs through the action of a specific enzyme, apparently after the addition of the GlcI residue (Mühlradt, 1971). The incorporation of the next two residues, GlcI and GalI were the first reactions studied in cell free systems since mutants unable to form the precursors UDP-glucose and UDP-galactose were available. These mutants produce incomplete LPS which can be then used as a substrate to investigate further reactions. Use of such mutants and cell free systems permitted the following conclusions. Sugars are in fact added sequentially to the growing LPS molecule and not to the free, core oligosaccharide devoid of lipid A. Each step utilises an enzyme specific for the appropriate substrate and nucleotide sugar, (see Nikaido, 1973). A similar reaction sequence has been demonstrated in *E.coli* by Edstrom & Heath (1967). Additional evidence for the occurrence of such a scheme

of reactions comes from the isolation of a series of mutants, each defective in a single step of the sequence. In several studies on the sugar transferase activities Rothfield and various co-workers demonstrated an absolute requirement for phospholipid in each transferase reaction. They first discovered (Rothfield, Osborn & Horecker, 1964) that transferase activity could be released from cell envelopes by prolonged sonication. Using heat inactivated envelope from an appropriate core oligosaccharide mutant as an acceptor, they detected transferase activity in a soluble fraction. However, purified LPS from such a mutant was completely inactive as an acceptor due to extraction of phospholipids (Rothfield & Horecker, 1964). Thus their results indicated that an LPS-phospholipid complex is the acceptor for the transferase reaction. Further studies have indicated that such a complex will bind the transferase enzyme in the presence of Mg^{2+}. On the basis of these and other results Rothfield, Romeo & Hinckley (1972) have proposed a model for the organisation of the components involved in the assembly of core LPS. Lipid A is synthesised within the cytoplasmic membrane, and then the core is built up on it in such a way that the nascent LPS does not leave the membrane. In order to accomplish this, it was suggested that the nascent LPS moves within the plane of the membrane until it meets the correct transferase molecule, when a tight complex is formed. One difficulty in this scheme is that the completed R core must then perform a "flip-flop" transition to the outside of the cytoplasmic membrane where the O-side chain

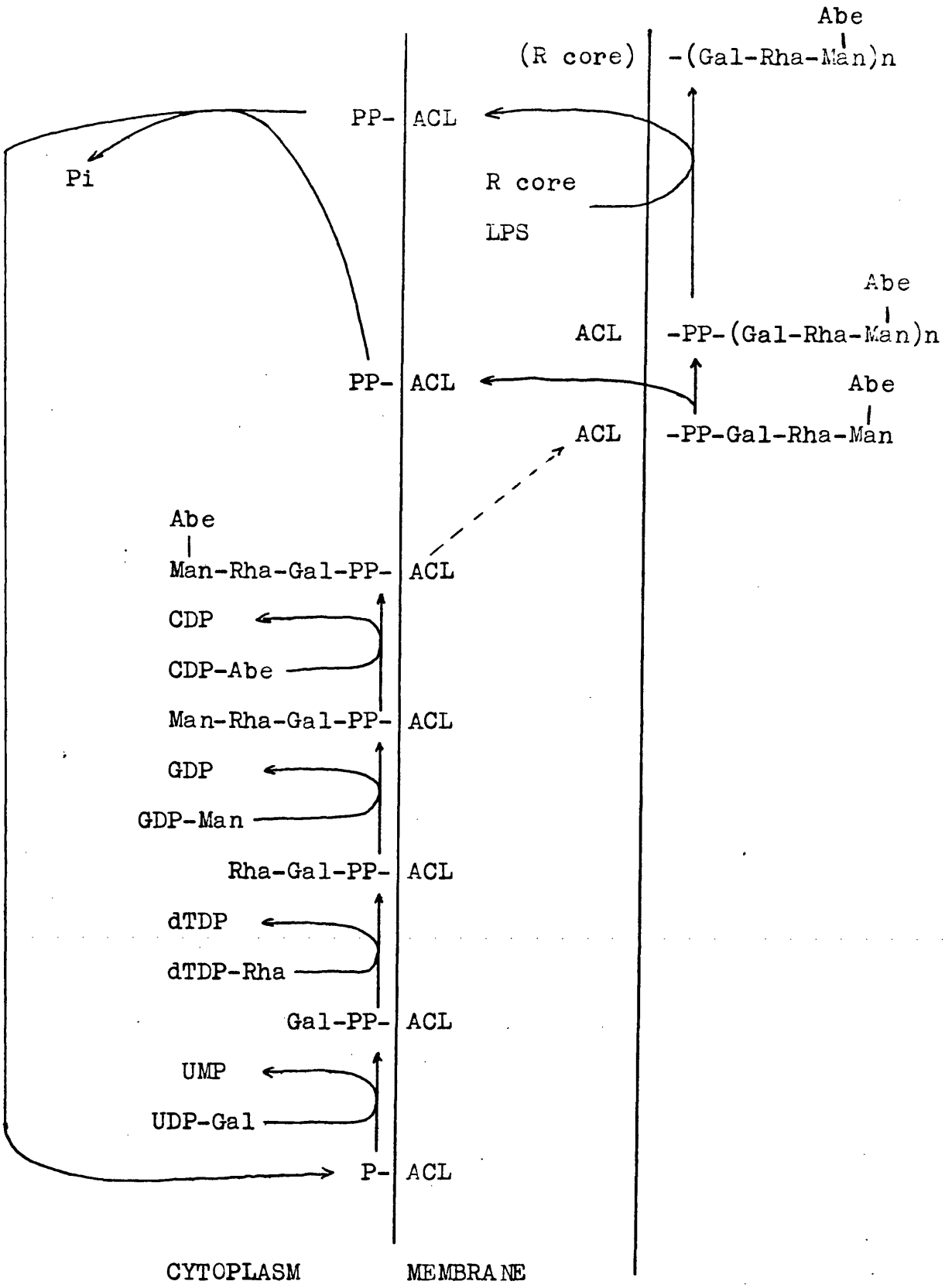
is apparently added to the core.

That the O-side chain consists of oligosaccharide repeating units was indicated by the discovery that the major product of mild acid hydrolysis of LPS was, in the case of S.typhimurium, a tri-saccharide. The investigation of the synthesis of the tri-saccharide in a cell free system confirmed that this was the repeating unit from which the O-side chain is constructed. Thus, using "R core" material obtained from appropriate mutants, it was possible to demonstrate the sequential attachment of O chain sugars, (Zeleznick et al. 1965; Nikaido & Nikaido, 1965). It was thought that a carrier molecule might be involved in this latter process since, although incomplete repeating units could be transferred to the R core in vitro, this did not happen in vivo in mutants defective in the production of complete O-side chain repeating units. Such a carrier was discovered and, as described in Section 1.Ib, appears to be a lipid, identical to the carrier lipid involved in peptidoglycan biosynthesis (Wright, Dankert & Robbins, 1965; Weiner, Higuchi, Saltmarsh-Andrew, Osborn & Horecker, 1965). The subsequent work of Robbins and Osborn revealed the presence of a lipid cycle analogous to that occurring in the synthesis of peptidoglycan, and the results of their work are summarised in Figure 6 (see Nikaido, 1973 for a review of this subject). The observation that in S.anatum exogenous repeating units can be polymerised indicates that the lipid bound intermediates and polymerase molecules are not tightly organised within the

FIGURE 6. DIAGRAMATIC REPRESENTATION OF O-SIDE CHAIN BIOSYNTHESIS SHOWING INTRACELLULAR LOCATION OF REACTIONS (NIKAIDO, 1973)

The abbreviations are the same as Figure 5.

ACL : acyl carrier lipid


Abe
(R core) -(Gal-Rha-Man)n
PP- ACL
Pi
R core
LPS
Abe
ACL -PP-(Gal-Rha-Man)n
PP- ACL
Abe
ACL -PP-Gal-Rha-Man
Abe
Man-Rha-Gal-PP- ACL
CDP
CDP-Abe
Man-Rha-Gal-PP- ACL
GDP
GDP-Man
Rha-Gal-PP- ACL
dTDP
dTDP-Rha
Gal-PP- ACL
UMP
UDP-Gal
P- ACL
CYTOPLASM
MEMBRANE

membrane (Kanegasaki & Wright, 1970). Pulse labelling experiments *in vivo* and *in vitro* have demonstrated conclusively that as in fatty acid synthesis and protein synthesis, polymerisation of O-side chain repeating units is accomplished by transfer of the growing chain onto a monomer unit (Bray & Robbins, 1967; Robbins, Bray, Dankert & Wright, 1967). In view of the fact that the enzymes and carrier involved in O-chain synthesis are membrane bound, it is necessary to ask questions about the detailed topology of the synthetic reactions. The reaction sequence is located in the cytoplasmic membrane since in *rfa* strains, mutants which cannot add O-side chain to the R core, O specific phage will only bind to spheroplasts, i.e. to the outside of the cytoplasmic membrane, (Kent & Osborn, 1968a). Also, enzymes of the lipid cycle have been located in the cytoplasmic membrane (Osborn, Gander, Parisi & Carson, 1972). It seems that since the reactions require water soluble, cytoplasmic sugar nucleotides, the O chain repeating units must be built up on the inner surface of the cytoplasmic membrane. The unit must then move across the membrane, since O side chains are located on the outside surface but not on the inside surface of the cytoplasmic membrane as detected with ferritin-labelled antibodies (Shands, 1966).

The final step in the synthesis of LPS, the joining of the O-side chain to the R core has been demonstrated in a cell free system by Cynkin & Osborn (1968). It is conceivable that repeating units are joined to the R core sequentially rather than as a block, but this seems

unlikely since O-hapten appears to be a precursor of O-side chains in LPS, (Kent & Osborn, 1968b).

1.Id. ENVELOPE PROTEINS

It is not proposed to discuss in detail the mechanism of envelope protein synthesis as there is no conclusive evidence that the process differs from normal protein biosynthesis. Instead, the properties of some envelope proteins and some brief points on their mode of synthesis will be described.

The most thoroughly studied envelope protein in E.coli is a small lipoprotein of molecular weight 7,500 D. This protein is covalently attached to peptidoglycan by linkage to the diaminopimelate residue of the tetrapeptide unit (see Figure 3) thereby displacing the terminal D-alanine residue. The lipoprotein is apparently bound to the outside of the peptidoglycan (Braun, Bosch, Hankte & Haller, 1974) as indicated by the following evidence. Cells can be labelled with diaminopimelate and then treated with lysozyme and EDTA to form spheroplasts. The lysozyme degrades the peptidoglycan giving rise to disaccharide units with the peptide chains still attached, and some of these units still contain bound lipoprotein. Thus the wall label is transferred to the lipoprotein. Subsequent separation of the envelopes by isopycnic centrifugation on sucrose (Osborn, Gander, Parisi & Carson, 1972) demonstrate that 90-95% of the label is associated with the outer membrane. The same result is

obtained using specific antiserum as a probe for the lipoprotein. Braun and his co-workers have demonstrated with whole cells of mutants of E.coli defective in LPS, that the more defective the LPS, the greater is the amount of lipoprotein specific antiserum bound to the cells. Finally, Braun & Bosch (1973) have performed pulse labelling studies which indicate that the lipoprotein is attached to fully polymerised peptidoglycan, rather than to precursors in the cytoplasmic membrane.

It has been calculated (Braun & Hankte, 1974) that there are some 250,000 molecules of the lipoprotein bound per bacterium. This makes the observation of Inouye, Shaw & Shen (1972) that at least two thirds of the total amount of the lipoprotein is present in the outer membrane in an unbound form (i.e. soluble in SDS without prior treatment with lysozyme) somewhat surprising, as 750,000 molecules per cell would make this lipoprotein by far the most abundant protein species in E.coli. Inouye et al. have demonstrated that in pulse chase experiments the SDS-soluble (free) form, appears first and is chased into the SDS-insoluble (bound) form, with an equilibrium being set up between the two forms. The presence of such a large amount of unbound lipoprotein, and some apparently remarkable structural properties of the molecule (see below) have led Inouye (1974) to suggest that the lipoprotein may form polar pores through the outer membrane. The lipoprotein contains covalently bound lipid at the amino terminal cysteine residue (see Braun & Hankte, 1974) and it seems that this could provide the means for

interaction with the outer membrane. Circular dichroism studies reveal that at least 70% of the protein is in an α-helical form and when models are constructed of the protein, the helix has polar residues on one side and hydrophilic residues on the other. Inouye therefore proposes that groups of four or more free molecules could associate with two bound molecules to form a cylindrical array giving rise to a channel or pore which spans the outer membrane.

It was first observed by Hirashima & Inouye (1973), that the messenger RNA for this lipoprotein has an unusually long half-life of about 11.5 mins., as determined by synthesis of the protein in the presence of rifampicin. The lipoprotein lacks histidine and so is virtually the only protein made when a histidine auxotroph is starved of histidine. The synthesis of this protein can therefore simply be followed by measuring the incorporation of amino acid into protein under these conditions. Subsequent studies (Hirashima, Childs & Inouye, 1973; Lee & Inouye, 1974) have demonstrated in fact that the synthesis of the majority of outer membrane proteins is relatively resistant to puromycin and rifampicin, and that the mRNA molecules for these proteins have relatively long half-lives. Differential sensitivity of envelope proteins synthesis to chloramphenicol has been demonstrated by Vambutas & Salton (1970) in Micrococcus lysodeileticus. However, chloramphenicol showed no differential effects in the studies of Inouye et al. Not withstanding this contradiction these studies

remain the only evidence for a separate biosynthetic mechanism for the synthesis of envelope protein in bacteria. In a further investigation of this phenomenon Randall & Hardy (1975) attempted to discover if the ribosomes transcribing these long lived messengers formed a special sub-class. By comparing polysomes from untreated cells with those from rifampicin treated cells (thus enriched for "stable" mRNA) they found that the ribosomes from the treated cells were enriched for protein S1. However, as they describe this is most likely to be an artefact caused by dissociation of S1 from run-off ribosomes in the presence of rifampicin, followed by readsorption onto polysomes. It therefore seems likely that envelope proteins are synthesised on normal ribosomes.

The second most intensively studied protein, or rather group of proteins, are the "major outer membrane" proteins. These are so called because they constitute the major protein bands normally revealed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE). Originally described by Schnaitman (1970) as a single species of molecular weight 42,000 D, subsequent improvements in gel technique have now resolved up to four such proteins (mol. wts. 34-38,000 D) whose relative proportion depends upon both the bacterial strain and the gel system used. The various results obtained have been recently summarised by Lugtenberg *et al* (1975) and by Henning & Haller (1975). One of these proteins (36,500 D) has been purified to homogeneity by

Rosenbusch (1974). The precise relationships between the various major outer membrane proteins observed is not clear, although they do appear to be determined by separate genes (Lugtenberg, personal communication). However, the main interest in these proteins has recently centred on the idea that they are responsible for the determination of cell shape by some sort of self assembly system. If cells are treated with boiling SDS, it is possible to obtain sacculi which are rod shaped entitities composed of peptidoglycan and bound lipoprotein. Alternatively, treatment with Triton X-100, trypsin and lysozyme yields a rod shaped "ghost" (Henning, Rehn & Hohn, 1973). These ghosts appear under the electron microscope to be composed of membrane and contain 25% phospholipid, 25% LPS and 50% protein. The protein is composed of the small lipoprotein, two major outer membrane proteins and one minor component (Henning, Hohn & Sonntag, 1973; Garten & Henning, 1974). If these proteins are responsible for cell shape determination by self assembly, then it should be possible to demonstrate protein interaction over the whole cell surface. Haller & Henning (1974) demonstrated that it was possible to crosslink proteins from whole cells and ghosts into an SDS insoluble form, leaving rod shaped structures after treatment with SDS. Unfortunately not all strains of rod shaped bacteria possess these proteins and Ames, Spudich & Nikaido (1974) have observed that in strains of Salmonella defective in LPS synthesis, these proteins are present in greatly reduced amounts, without any apparently

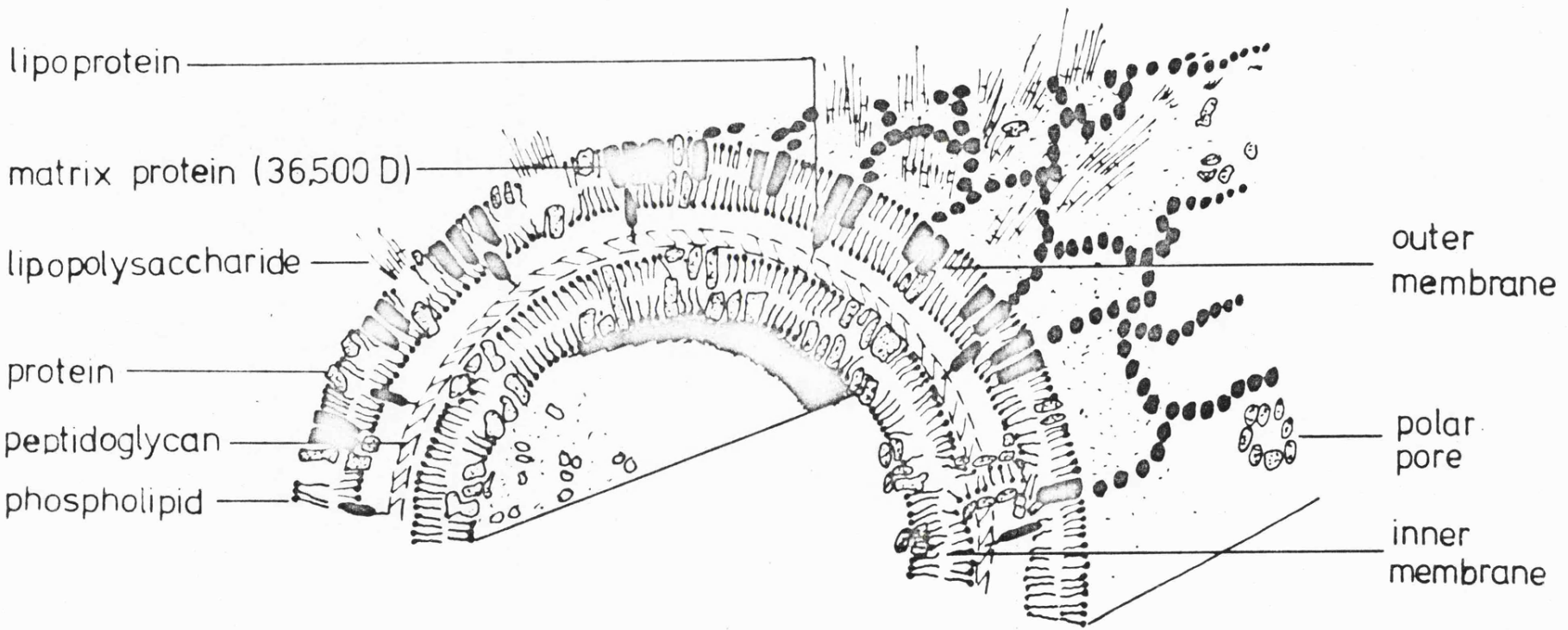
deleterious effects on the organism. Moreover, the crucial evidence forcing the rejection of the notion that these proteins determine cell shape, comes from the isolation by Henning & Haller (1975) of a mutant of E.coli K12 lacking all "major proteins" of the outer membrane.

1.II. STRUCTURE AND GROWTH OF THE CELL ENVELOPE OF E.COLI

The conventional picture of the structure of the cell envelope in gram-negative bacteria is shown in Fig. 7. A rigid layer of peptidoglycan is sandwiched between two membranous structures commonly referred to as the cytoplasmic or inner membrane (IM) and the outer membrane (OM). The aqueous phase between the two membranes is usually referred to as the periplasmic space, and contains many enzymes and transporter proteins, which can be released from cells by osmotic shock (Costerton, Ingram & Cheng, 1974; Bayer, 1974). The peptidoglycan layer as described previously, is seemingly attached to the OM by lipoprotein molecules distributed all over the surface of the peptidoglycan. This explains why treatment of envelopes with proteolytic enzymes releases the OM from the peptidoglycan layer, (de Petris, 1967). It has also been suggested by Rogers (1970) that the cytoplasmic membrane is connected to the peptidoglycan by nascent peptidoglycan. In any event in a living cell hydrostatic pressure would force the IM against the

FIGURE 7. STRUCTURE OF THE E.COLI CELL ENVELOPE (DRAWN BY I.B. HOLLAND) SHOWING THE RELATIONSHIP BETWEEN THE THREE MAIN LAYERS

The position of the 36,500 D protein is not certain


lipoprotein
matrix protein (36,500 D)
lipopolysaccharide
protein
peptidoglycan
phospholipid
outer membrane
polar pore
inner membrane

peptidoglycan. Sites of possible adhesion between the IM and OM have been demonstrated by Bayer (1974) using electron microscopy. The studies of Schnaitman (1970) & Osborn et al. (1972) indicate that in both E.coli and S.typhimurium, the cell envelope is composed of 20% phospholipid, 40% protein and 40% LPS by weight. The outer membrane and peptidoglycan contain 70% of the protein, 50% phospholipid and 90% of the LPS of the whole envelope.

The IM is by all criteria a typical membrane, composed of a lipid bilayer containing both integral and peripheral proteins (Singer, 1974). Perhaps the best example of the latter is the Mg^{2+}/Ca^{2+} ATPase whose degree of dissociation from the membrane in vitro is strongly influenced by the concentration of Mg^{2+} in the surrounding medium (Roisin & Kepes, 1972). An important class of IM proteins are the permeases and the availability of convenient procedures for the isolation of fatty acid auxotrophs in E.coli have been particularly useful in studying the effect of lipid composition on, for example, the functioning of the lactose transport system. The lactose transport system is readily inducible and apparently contains a functioning membrane protein. (Though interestingly the presence of the lactose permease has never been demonstrated using envelopes from induced cultures and subjecting them to SDS-PAGE). It has long been known that when grown at different temperatures the fatty acid composition, though not the relative proportions of the phospholipids, of E.coli varies greatly.

As the temperature of growth decreases the proportion of unsaturated fatty acids in the membrane increases. This has two effects : the permeability of the membrane is maintained (see Cronan & Vagelos, 1972), and so, more importantly, is the fluidity of the membrane. Using various fatty acid auxotrophs it is possible to grow bacteria with fairly well defined phospholipid composition. These strains may be used to demonstrate the occurrence of varying phase transitions in membranes as the temperature is decreased and the phospholipids begin to "solidify". Without some sort of control of the fatty acid composition of the membrane, such phase separations could seriously affect the functioning of transport systems in an organism liable to be exposed to different temperatures. That this is so is indicated by the studies of Overath, Hill & Lamnek-Hirsch (1971) which show that the temperature dependence of β-galactoside transport in a fatty acid auxotroph is a function of the fatty acid composition of the cell. It is obviously possible to use this phenomenon as a probe to determine whether the synthesis of functional transport system requires concommitant phospholipid synthesis - a fatty acid auxotroph is grown in the presence of one fatty acid, then the transport system is induced in the presence of another fatty acid, and the temperature dependence of the transport system is determined. The results of Overath et al. (1971), who found that the temperature dependence of lactose transport was a function of the average fatty acid composition, are in direct contradiction to those of

Wilson & Fox (1971), who found that this parameter was a function of the fatty acid present during induction. The results of Wilson & Fox are in agreement with the observation by Hsu & Fox (1970) that in a glycerol auxotroph, the synthesis of a functional lactose transport system requires concommitant synthesis of phospholipid. However, recently Weisberg, Cronan & Nunn (1975) have convincingly demonstrated that the conclusions of Hsu & Fox are unwarrented and that lactose transport can be induced in the absence of phospholipid synthesis. It would therefore seem that the conclusions of Overath et al. are the correct ones.

Another interesting feature of the cytoplasmic membrane is its mode of growth, and here again there is some controversy. The earliest studies of the nature of membrane growth in bacteria were stimulated by the proposal of Jacob, Brenner & Cuzin (1963) that the segregation of daughter chromosomes was achieved by the growth of the membrane between their respective points of attachment to the membrane. Since then other similar models (Clark, 1968b; Sargent, 1974) have been proposed, but all predict that the growth of the membrane, or whatever structure is responsible for separation of daughter chromosomes, should occur in highly conserved, or zonal regions. Numerous approaches have been made to investigate this problem, but all involve either methods of direct visualization of membrane growth, or the isolation of membrane from particular areas of the cell. This latter is possible because of the existence of mini-cells producing strains which bud off small DNA-less

cells from the cell poles. Green & Schaechter, (1972) studied the segregation of phospholipid from parent to daughter cells, and from cells to mini-cells, and concluded that the segregation was random and therefore phospholipids were not inserted into new membrane in conserved regions. A similar conclusion was reached by Lin, Hirota & Jacob (1971), who followed the segregation of old, fully labelled phospholipids, in cells growing in the absence of label, by autoradiography. Tsukagoshi, Fielding & Fox, 1971 used bromostearate as a density label and performed density shifts on synchronously growing cells, and then determined the density of the cell membranes in sucrose gradient. They observed no abrupt change in density after each subsequent generation and concluded that the lipid label was segregating randomly. However, the validity of all these studies is uncertain because the fluidity of the phospholipid in the membranes could easily lead to any label becoming rapidly dispersed over the whole surface even if it was inserted into the membrane only at discrete sites.

Two different approaches to the segregation of membrane proteins have yielded conflicting results. Wilson & Fox (1971) took a mini-cell producing strain induced for β-glucoside transport, freed the culture of mini-cells by banding on sucrose, then induced the culture for β-galactoside transport as well, and determined the ratio of transport activities in whole cells and mini-cells. They concluded that the transport proteins were segregated at random into the mini-cells.

On the other hand, Ryter (1971) studying the growth of new flagella, which are anchored in the inner membrane, in a strain of B.subtilis, temperature sensitive for flagellum production, found that the new flagella appeared in a conserved zone. Kepes & Autissier (1972) also found that induced lactose permease was inherited in a conserved fashion. Therefore it seems possible that the proteins of the inner membrane are inserted and maintained in a conserved state, although the problem of dispersal within the plane of the bilayer is equally great in these experiments as in the experiments on the segregation of phospholipids.

Similar studies on the topology of peptidoglycan synthesis have been performed. Lin, Hirota & Jacob (1971) observing the distribution of ^{3}H-diaminopemelate by autoradiography, among progeny cells growing in the absence of label, concluded that the label was subsequently evenly distributed amongst the progeny. However, Ryter, Hirota & Schwartz (1973) later showed that after very short pulses, the diaminopemelate was incorporated into fairly discrete zones, but on chasing was randomised over the whole surface. This indicates that the peptidoglycan is a dynamic structure presumably undergoing considerable modification during cell growth. In this context it is interesting to note that Schwarz, Asmus & Frank (1969) demonstrated that the site of action of murein hydrolases was restricted to a central zone in the cell. These enzymes have been implicated in the growth of the peptidoglycan layer in many organisms (see Ghuysen &

Shockman, 1973). It therefore seems that insertion of proteins into the cytoplasmic membrane and the growth of the peptidoglycan layer may occur in discrete zones, rather than by intercalation of new material into old. However, the mechanism of insertion of phospholipid is still an unresolved problem.

The outer membrane of gram-negative bacteria is assumed to be a phospholipid bilayer containing, and in association with various proteins. However, a large proportion of the material in this layer of the cell envelope is lipopolysaccharide and this undoubtedly affects radically the properties of this layer. The main function of the OM appears to be that of a "molecular seive" (see Costerton et al. 1974). Nevertheless this is not a completely negative function since a growing number of small ion binding proteins, coupled in some way to specific inner membrane transport systems, are now being identified in the OM. Examples of such systems are λ-maltose, B12, ferrichrome. The structure of the OM under the electron microscope is typical of a membrane and freeze etching studies have revealed a fracture plane in this layer indicating presumably a bilayer of continuous phospholipid and lipid A (Bayer, 1974). The phospholipid composition of this layer in S.typhimurium at least, is significantly different from that of the cytoplasmic membrane : there is a marked increase in the proportion of phosphatidylethanolamine (Osborn et al. 1972). As has already been mentioned above, Inouye has proposed that hydrophilic pores span this membrane through clustering

of the 7,500 D lipoprotein. The "major outer membrane proteins" have until recently been assumed to be intimately associated with the bilayer but Rosenbusch (1974) has reported that at least one of these proteins (B. Lugtenberg) et al. 1975; Mwt. 36,500 D) is specifically associated with the peptidoglycan rather than with the outer membrane. Rosenbusch has further suggested that this protein, possibly in association with other outer membrane polypeptides, may form a highly integrated protein matrix in between the murein and OM layers. Certainly the 36,500 D protein is resistant to trypsin in both whole cells and isolated envelopes (I.B. Holland, personal communication) which indicates that at the very least this protein is buried deep within the outer membrane layer.

The problem of the assembly of the various component of this membrane into an integrated whole is a major one. LPS subunits, previously synthesised in the inner membrane, must presumably bind first to the inner surface of the outer membrane, then "flip" to the outer surface. Yet, as already stated such transitions are very rare if not impossible. Whilst it appears that at least one minor protein species, the bacteriophage λ receptor protein, is incorporated into the outer membrane in the absence of phospholipid synthesis (Randall, 1975a), Randall (1975b) has shown that the same protein is present in decreased amounts in certain LPS deficient mutants. Similarly, observations made by Koplow & Goldfine (1974) with E.coli and Ames et al. (1974) with S.typhimurium, have shown

that in heptose deficient, LPS mutants, there is a significant reduction in the amount of major OM protein in the outer membrane. One possible explanation for this reduction in protein content of the OM for which there is some evidence, however tenuous, is that protein and at least some LPS molecules could be linked through ionic bonds which help to stabilize the membrane. The outer membrane, unlike the cytoplasmic membrane, will remain unfragmented upon treatment with Triton X100 if sufficient Mg^{2+} is present in the surrounding medium (Schnaitman, 1971; De Pamphlis & Adler, 1971). Investigating the release of LPS from E.coli by treatment with EDTA, Levy & Leive (1968) found that a constant fraction of the LPS was resistant to EDTA release. Using a mutant enabling the specific labelling of LPS with galactose, they found that newly synthesised LPS is resistant to EDTA but at later times becomes non-resistant and an equilibrium is set up between the two forms. Nikaido (1973) has suggested that the EDTA resistant fraction might constitute mosaics of LPS-phospholipid whilst the EDTA soluble fraction might be mostly LPS associated with protein, although there is no evidence for this at present.

The possibility that LPS and protein incorporation into the outer membrane could be co-ordinated in some way brings us back to the question of a special synthetic machinery for outer membrane protein. In this connection the studies of Lee & Inouye (1974) may be especially relevant. In addition to demonstrating that outer

membrane proteins have unusually stable mRNA molecules, these workers also demonstrated that these proteins do not apparently accumulate in the cytoplasmic membrane before appearing in the outer membrane. This implies that the bulk, if not all of the newly synthesised outer membrane proteins pass straight into the outer membrane from the cell interior. It is therefore tempting to postulate that they are inserted into the surface envelope at a site of intimate connection between the inner and outer membranes, i.e. at the adhesion sites demonstrated by Bayer. This idea is particularly attractive since there is evidence that LPS is also inserted into the outer membrane at these sites (Mülradt, Menzel, Golecki & Speth, 1973) prior to its rapid dispersal throughout the surface.

Unfortunately there are a large number, perhaps 400, of these sites situated all over the cell surface. In contrast studies on the growth of the cell surface of E.coli, although mainly confined to studies involving phage receptors, appear to show that the outer layer grows from a small number of discrete zones. Thus Ryter, Shuman & Schwartz (1975) observed that pulse induction of the λ receptor (maltose binding protein) resulted in insertion of the receptor into a single although quite wide zone. Similarly Begg & Donachie (1973) using a tsx amber mutant containing a temperature sensitive suppressor, showed that insertion of the T6 phage receptor occurred in a discrete position on the cell surface. A similar result was obtained by Leal & Marcovich (1975) observing zygotes

formed from an Hfr tsx^{s} x F^{-} tsx^{r}. In addition Donachie & Begg (1970) have demonstrated that in cells which are less than a critical length, growth is assymetrical, apparently taking place only from one cell pole. It is therefore hard to reconcile all these observations with the idea that insertion of LPS and protein into the outer membrane is occurring at many sites over the whole cell surface.

1.III. THE CELL CYCLE OF E.COLI

In 1966 Maaløe & Kjeldgaard proposed a most important concept which now constitutes the basis of our understanding of the growth of E.coli. They proposed that whilst the rate of elongation of a macromolecule is constant at a given temperature, independent of the growth rate, the synthesis of the macromolecule may still be regulated by controlling the frequency of its initiation of synthesis. The existence of precise regulatory mechanisms acting upon the synthesis of macromolecules, and indeed cell division itself, is implicit in the early studies of Schaechter, Maaløe & Kjeldgaard (1958) and Kjeldgaard, Maaløe & Schaechter (1958). These workers demonstrated that at different growth rates cells have a characteristic size and macromolecular composition, and that during transitions between these steady states of growth, the pre-shift rates of synthesis of different macromolecules are maintained for different periods of time after the shift, leading to the establishment of different macro-

molecular composition. As early as 1961 Maaløe proposed specifically that the rate of DNA synthesis was controlled by the frequency of initiation of rounds of replication, whilst the time taken to replicate the chromosome remained constant. If it is assumed that to replicate a DNA molecule takes a time comparable to the range of growth rates generally used in batch cultures (0.5-3.0 doublings per hour), such a mechanism would lead to two predictions. At fast growth rates, where the generation time of the culture was shorter than the replication time, dichotomous replication would occur, i.e. a new round of replication would start before the previous round had finished. Conversely at slow growth rates a part of the division cycle would be devoid of ongoing replication. Both these predictions were found to be fulfilled (Maaløe, 1961; Oishi, Yoshikawa & Sueoka, 1964; Pritchard & Lark, 1964, Lark, 1966).

Obviously the question of the control of DNA synthesis can be reformulated : how is the initiation of rounds of replication regulated? Pritchard & Lark (1964) demonstrated that when thymine was added back to a culture of E.coli previously starved of this base, the rate of DNA synthesis was stimulated beyond that observed previous to the period of thymine starvation. They concluded that this stimulation was due to the presence of new replication forks initiated during thymine deprivation. Therefore it seemed possible that the initiation of replication could be controlled in some way by the growth of the cells in the absence of thymine.

The discovery of a technique for producing synchronous cultures of E.coli, or of fractionating populations of cells according to cell age (Helmstetter & Cummings, 1964; Helmstetter, 1967) with minimum disturbance to the cells, permitted more direct measurements of DNA synthesis during the cell cycle. Clark & Maaløe (1967) observed that in glucose grown, synchronous cultures, the rate of DNA synthesis was constant, but then doubled in the middle of the division cycle. Measurement of residual DNA synthesis in the presence of chloramphenicol (to prevent further initiation occurring), showed the observed doubling in rate to be due to a doubling in the number of replication forks, indicating that the rate of synthesis per fork remained constant. They also demonstrated, using two other growth rates, that there was an approximately constant interval between initiation of rounds of replication and cell division, thus providing evidence for a role for the replication of DNA in the control of cell division. Subsequent studies (Helmstetter, 1967; Helmstetter & Cooper, 1968; Cooper & Helmstetter, 1968) have demonstrated that in cultures growing with generation times less than about 70 mins. at 37°C, the time, C, taken for a round of replication is constant at approximately 40 mins. in E.coli B/r. These workers also found that there was a constant period, D, of about 20 mins. between termination of rounds of replication and the subsequent cell division, again implying that cell division is controlled by the replication cycle. Another study implying this connection is that of Donachie, Hobbs

& Masters (1968) who demonstrated that a burst of division occurred approximately 45 mins. after the readdition of thymine to a thymine starved culture.

With this information about C and D, and the data of Schaechter et al. (1958), Donachie (1968) concluded that cell mass at the time of initiation of rounds of DNA replication is remarkably constant. This indicates that rounds of replication are initiated when bacteria reach a critical mass, or, put another way, that at initiation the cell mass per chromosome origin (initiation mass) is constant and independent of growth rate. Various models have been proposed to explain how a cell might titrate its mass (Helmstetter, Cooper, Pierucci & Revelas (1968); Marvin (1968); Pritchard, Barth & Collins (1969); Sompayrac & Maaløe (1973)), but the only biochemical information available on the process of initiation is that it requires RNA and protein synthesis (Lark, 1972; Lark & Renger, 1969; Ward & Glaser, 1969). In this connection it is interesting to note the observation of Schaechter, Williamson, Hood & Koch (1962), that the variation in cell size at the time of division, is smaller than that of cell age at division, the inference being that division as well as DNA replication is regulated by cell size or mass.

Before exploring further the notion that cell division is controlled by the replication of DNA, and by what mechanisms such a control might operate, it is necessary to describe an important discovery of Pritchard & Zaritsky (1970), that the replication time, C, may be

altered under certain conditions, without any effect on the growth rate of the culture. In certain low thymine requiring mutants, the size of the internal thymidine triphosphate pool, is proportional to the concentration of exogenously supplied thymine (see Pritchard, 1974). Pritchard & Zaritsky clearly showed that in such strains the replication velocity, (1/C) is proportional to the external thymine concentration. An independent investigation by Manor, Deutscher & Littauer (1971) demonstrated that under such conditions the step time for the addition of nucleotides to DNA is increased.

Utilizing this phenomenon, Zaritsky & Pritchard (1973) and Meacock & Pritchard (1975) have provided the most convincing experimental data for the nature of the involvement of DNA replication in the regulation of cell division. These experiments involved the study of the kinetics of transitions between different steady state replication velocities, and the effects of such transitions on cell division. If a culture of a low thymine requiring strain of E.coli is growing in a steady state in one thymine concentration, and is transferred to another, there will be a transient change in the rate of arrival of replication forks at the terminus. Therefore if, for example, division is timed from termination of rounds of replication, there will be a delay of D minutes after the shift, before the rate of cell division changes. If division is timed from an earlier event, the delay will be correspondingly longer. In experiments with both E.coli B/r and E.coli 15T$^-$ Pritchard and co-

workers found that after either increasing or decreasing the replication velocity by thymine shift, the same result was always obtained : that division is timed from the termination of rounds of DNA replication. This result is supported by experiments on the effect of inhibiting DNA synthesis on cell division by Clark (1968a,b) and Helmstetter & Pierucci (1968), which also indicate that cell division is timed by termination of rounds of DNA replication.

However, in contrast, the observation that cell division will continue in the absence of DNA replication in B.subtilis (Donachie, Martin & Begg, 1971), and in mutants defective in the initiation of rounds of DNA replication (Hirota, Jacob, Ryter, Buttin & Nakai (1968); Spratt & Rowbury (1971)), has led to the proposal that division is timed from initiation of replication and proceeds as an independent process. On this basis D would be merely the time difference between the two processes, (Donachie & Jones, 1973; Shannon & Rowbury, 1972).

These two alternative models for the timing of division make different predictions about the effect of variation in replication time, C, on the length of the D period. If termination times cell division, D should remain constant and independent of any change in C whilst if initiation is the timing event, D should decrease as C increases. The results obtained by Meacock & Pritchard (1975) demonstrated that the latter is true, but the actual decrease in D was found not to be in proportion to

the change in C. Even so, this result is in direct contradiction to the results obtained from transitions between steady states by these workers. Since, as will be described below, there is nevertheless further compelling evidence that termination does time cell division, it appears that our present notions of the nature of the D period may be too simplistic.

Before considering this additional evidence it is necessary to present the results of experiments designed to yield information on how cells of E.coli grow. Marr, Harvey & Trentini (1966) have observed that at any one growth rate, cells extend only in length, not in width. Studies by Dennis (1971) and Ecker & Kokaisl (1969) indicate that the rates of RNA and protein synthesis increase exponentially during the division cycle, whilst observations of synchronous populations by Kubitschek (1968a,b) and Ward & Glaser (1971) show that cell volume increases linearly, with a doubling in rate during the cycle. Consequently, since cell width remains constant, surface enlargement during the cycle must be linear. However, this linearly increasing cell surface must accommodate an exponential increase in accumulation of cell mass. This situation led Previc (1970) to propose that an increasing internal pressure leads to the observed extension in cell length, and that in order to divide, the bacterium must increase its rate of surface extension to exceed the rate of mass accumulation. Another important observation is that whenever there is a need to accommodate an increase in cell size as a

consequence of increased growth rate or reduced replication velocity, a change in cell width is observed leading to a reduction in the surface area to mass ratio of the cell (Schaechter et al. 1958; Zaritsky & Pritchard, 1973; Meacock, 1975).

In an attempt to explain all these observations Zaritsky & Pritchard (1973) and Pritchard (1974) have proposed that the growth of the cell surface of E.coli, or at least a major component of it, occurs in a linear fashion during the cell cycle with a discrete doubling in rate at some point in the cycle. Further it is proposed that this rate of surface growth is proportional to the growth rate and is linked directly to a late event in the replication cycle. This would explain the reduction in surface area/mass ratio as it has been shown that the effect of an increase in growth rate, or a decrease in the replication velocity, is to reduce the number of copies per unit mass of genes near the terminus relative to genes at the origin (Pritchard, 1974; Chandler & Pritchard, 1975).

There are two alternatives to explain the possible coupling of surface growth to replication. Either the number of (hypothetical) surface growth sites is proportional to the number of chromosome termini - site model - or there is consitutive production of a rate limiting precursor from a gene near the terminus - precursor limitation model. Thus, surface growth continues at a constant rate until the number of termini or the number of genes doubles and then the rate of surface growth doubles, and the excess surface producing capacity will

be used to form a septum. Although the two variants of this model are similar, they lead to different predictions. As we have seen above cell division appears to be timed by termination. The site model postulates that the number of growth sites is proportional to the number of termini, implying that since the rate of extension per site is constant, cell length will be proportional to the number of termini. If, on the other hand the precursor limitation model is true, then the surface area of the cell will be proportional to the number of termini. In fact, measurements of whole cells and isolated sacculi provide good evidence that cell length is proportional to the number of termini per cell, indicating that the site model provides a better explanation of events (Zaritsky & Pritchard, 1973; Meacock, 1975; Pritchard personal communication, Sargent, 1975a in B.subtilis).

It is possible to explain, in terms of the above model, the observation that cell division continues in the absence of DNA synthesis, a fact which may appear to indicate that division is controlled by initiation of rounds of DNA replication (see Pritchard, 1974). Thus most mutants defective in initiation at 42° seem to increase in mass linearly after one generation at high temperature. Since cells under these conditions will have completed rounds of replication, the rate of surface growth should have doubled, and the relative rates of mass and surface accumulation may therefore be maintained at a level similar to that occurring transiently in normally growing cells at the time of septation. Furthermore it is

in fact hard to see how initiation could control the timing of cell division, since there is no change in the number of copies per unit mass of genes at the chromosome origin when either the replication velocity or the growth rate is changed, whilst under these conditions there is a change in cell width. It is therefore very difficult to explain the reduction in the surface area to mass ratio of cells observed under such conditions.

1.IV. AIMS OF THE PRESENT PROJECT

If the ideas developed in the preceding section provide a true explanation for the control of cell division in E.coli, it would seem that at least some components of the cell envelope should be accumulated at a constant rate, with discrete doublings in rate occurring in each cycle.

There have been several previous attempts, to measure the synthesis of envelope components in various species of bacteria, which have yielded conflicting results. Daniels (1969) has reported that in both B.megaterium and E.coli there is a large transient increase in the rate of phospholipid synthesis around the time of cell division. However, both Ohki (1972) with E.coli and Sargent (1973) with B.subtilis demonstrated that the rate of phospholipid synthesis increased continuously throughout the cell cycle. As far as envelope protein is concerned Sargent, (1973; 1975b) has demonstrated that these proteins are accumulated in a

linear fashion throughout the division cycle of B.subtilis, with a doubling in rate approximately coincident with nuclear segregation at two different growth rates. He has also demonstrated that this process is probably controlled by DNA replication, as inhibition of DNA synthesis leads to the continued accumulation of envelope proteins at a constant rate. In contrast to this, Ohki (1972), Shen & Boos (1973) and Ryter, Shuman & Schwartz (1975), have shown that many proteins of the cell envelope in E.coli are synthesised periodically during a discrete but brief period in the cycle. Also, it is possible to deduce from Sargent's (1973) measurement of succinate dehydrogenase activity in synchronised cultures of B.subtilis, that this enzyme is only synthesised periodically. Hoffmann, Messer & Schwartz (1972) have measured the rate of synthesis of peptidoglycan during the cell cycle of E.coli and have concluded that synthesis is linear with doublings in rate occurring a short time prior to division.

Obviously therefore, there are wide discrepancies between the available results, concerning not only the timing of events within the cell cycle, but also whether discontinuous events occur at all. In an attempt to resolve some of these inconsistencies, I have therefore set out to take advantage of the existence of a system for examining the cell cycle of E.coli B/r which results in the minimum disturbance of the growing cultures (Helmstetter & Cummings, 1964; Helmstetter, 1967), and the availability of high resolution SDS-polyacrylamide

gel electrophoresis for the analysis of envelope protein species (Holland & Tuckett, 1972). Furthermore, an extensive analysis of DNA replication and cell division has previously been carried out in the bacterial strain used in these studies (Meacock & Pritchard, 1975) thus greatly facilitating the establishment of possible relationships between the synthesis of envelope components and other events occurring during the cell cycle.

CHAPTER 2

MATERIALS AND METHODS

1. BACTERIAL STRAINS

The strain of E.coli used in these experiments was E.coli B/r LEB16 obtained from P.A. Meacock (Meacock & Pritchard, 1975). It is F^- lacZ str thyA drm, the mutation at the latter locus enabling the strain to grow in low concentrations of thymine, i.e. of less than 20 µg/ml (see Pritchard, 1974). The strain was maintained as frozen cultures in Nutrient broth containing 20% glycerol (Buxton, 1973), and on minimal medium agar plates.

2. CHEMICALS

All chemicals except those in the following list were BDH Analar reagents obtained from Fisons Ltd., Loughborough.

L-proline, L-alanine, thymine, chloramphenicol, phenazine methosulphate, 2,6-dichlorophenol indophenol, reduced nicotinamide, adenine dinucleotide, triethanolamine hydrochloride, oxaloacetic acid, disodium succinate, phosphoenol pyruvate, adenosine triphosphate, dithiothreitol, bovine serum albumin, fibrinogen, ovalbumin (grade V), lactoglobulin and egg white lysozyme were all obtained from Sigma, London.

Lactate dehydrogenase (E.C.1.1.1.27) and pyruvate kinase (E.C.2.7.1.40) were obtained from Boehringer Ltd.

Sarkosyl NL97 was obtained from Geigy U.K. Ltd.

Acrylamide, N,N'-methylene-bis-acrylamide (BIS), and sodium dodecyl sulphate (SDS) were obtained from Bio-Rad Laboratories.

N,N,N',N' - Tetramethylethylenediamine (TEMED) was obtained from Canalco Ltd.

2,5-diphenyloxazole (PPO) and 1,4-BIS-Z-(4-methyl-5-phenyloxazolyl)-benzene(dimethyl POPOP) were obtained from Nuclear Enterprises Ltd.

L-[U-^{14}C] Leucine, L-[U-^{14}C]Histidine, L-[^{35}S] Methionine, [2(n)-^{3}H] Glycerol and [methyl-^{3}H] Thymidine were obtained from the Radiochemical Centre, Amersham.

All chemicals were used as supplied without further purification.

3. MEDIA

The minimal salts medium was that of Helmstetter (1967) and contained per litre of distilled water; 2g NH_4Cl, 6g Na_2HPO_4, 3g KH_2PO_4, 3g NaCl and 0.25g $MgSO_4$ added separately as a sterile 1.0M solution after autoclaving. Proline and alanine were each added at a final concentration of 0.04% w/v as carbon source, and thymine was added at the concentration indicated in each experiment. This growth medium routinely gave generation times of 65 mins. at 37°C for E.coli B/r LEB16. Minimal agar plates were made up with M9 salts containing per litre of distilled water; 6g NaH_2PO_4, 3gm KH_2PO_4, 5g NaCl, 1g NH_4Cl, 11mg $CaCl_2$ and 246mg $MgSO_4.7H_2O$ and solidified with 15g/l

No.3 Oxoid agar. The plates contained 0.04% w/v proline and alanine and the appropriate concentration of thymine.

4. CULTURE CONDITIONS

Appropriate media in conical flasks were inoculated with single colonies from subcultures of E.coli B/r on agar plates and grown at 37°C in a New Brunswick Gyrotory shaking water bath. The ratio between flask volume and culture volume, and the speed of shaking were chosen to ensure that the cultures were adequately aerated.

5. OPTICAL DENSITY

The absorbance (E_{450}) of cultures was determined by withdrawing a 2.0 ml sample and measuring in a Gilford Microsample spectrophotometer at 450nm.

6. TOTAL BACTERIAL COUNTS

The number of bacteria in a culture was determined using a Model B Coulter Counter fitted with a 30 μm orifice. Samples were diluted into an equal volume of 0.9% w/v saline containing 0.8% v/v formaldehyde to fix the cells, and then diluted to an appropriate density for counting in 0.9% saline. Care was taken in selecting the instrument settings to ensure that the smallest cells in the population were counted.

7. ESTIMATION OF PROTEIN CONCENTRATION

The protein content of various envelope preparations was measured by the method of Lowry (Lowry, Roseborough, Farr & Randall, 1951).

8. SYNTHESIS OF PROTEIN

Synthesis of protein in bacterial cultures was measured as the incorporation of L-[U-^{14}C] leucine into ice-cold 5% w/v trichloroacetic acid (TCA) insoluble material. The details of this labelling procedure are indicated in the figure legend for each experiment. The TCA-precipitates were collected onto 27 mm membrane filters (Sartorius 0.45 μm pore size) by suction and washed ten times with 5 ml volumes of ice-cold 5% w/v TCA. Then the filters were dried under an infra red lamp and placed into small vials with constant orientation. The vials were filled with non-aqueous scintillation fluid containing per litre of toluene; 33 mg dimethyl POPOP and 5 g PPO. The small vials were then stoppered and placed in standard Packard vials and the samples counted in a Packard liquid scintillation counter.

9. SYNTHESIS OF PHOSPHOLIPID

The synthesis of phospholipid in bacterial cultures was measured by the incorporation of [2-^{3}H] glycerol (Daniels, 1969) into ice-cold TCA insoluble material as

described in the preceding section. A discussion of this method of labelling phospholipid will be found in Chapter 3.

10. SYNTHESIS OF DNA

Synthesis of DNA was measured as the incorporation of [methyl-^{3}H] thymidine into 5% TCA insoluble material. These samples were processed as described for protein, except that the filters were washed with distilled water at 95°C.

11. ENZYME ASSAYS

The following assays were all performed at 37°C using silica micro-cells in a Pye-Unicam SP1800 recording spectrophotometer. The proportionality between the rate of reaction, measured as the change in extinction per unit time, and the amount of enzyme in each reaction mixture is shown in Figure 8. The rates of reaction were usually constant for at least 10 minutes. All reagents were made up immediately before use and dissolved in the appropriate buffer, except for phenazine methosulphate which was dissolved in distilled water. The "sample" indicated for each assay mixture was usually a bacterial sonicate in 0.01 M sodium phosphate buffer pH7.2. In all cases the figure in brackets shown below refers to the concentration of the solution added to the assay mixture.

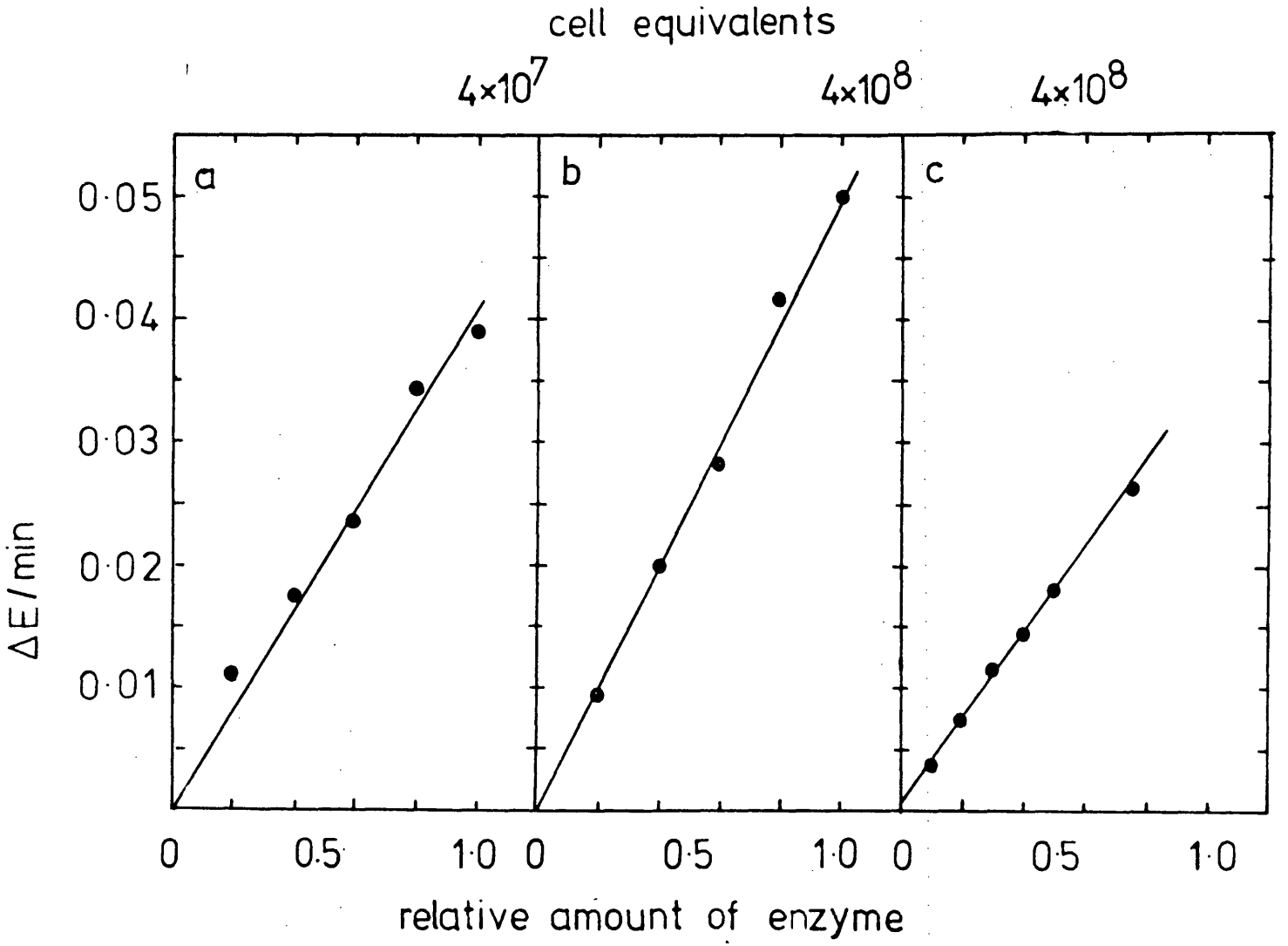
FIGURE 8. PROPORTIONALITY OF RATE OF REACTION WITH AMOUNT OF ENZYME

Cultures of E.coli B/r LEB16 were grown at 37°C in proline/alanine medium supplemented with 20 µg/ml thymine to an E_{450} of 0.25. The cells were harvested, resuspended in 10mM sodium phosphate buffer containing 10mM Mg^{2+} and sonicated as described in section 2.13. The lysates were assayed according to the methods described in section 2.11.

a) Malate dehydrogenase

b) Succinate dehydrogenase

c) Mg^{2+}/Ca^{2+} ATPase


cell equivalents
4×10^7
4×10^8
4×10^8
a
b
c
0·05
0·04
0·03
0·02
0·01
ΔE/min
0
0·5
1·0
0
0·5
1·0
0
0·5
1·0
relative amount of enzyme

Malate dehydrogenase (E.C.1.1.1.37) was assayed according to the method described in the Biochemica Catalogue; Boehringer, 1971. The principle of the reaction is the production of malate from oxaloacetate with the concommitant oxidation of reduced nicotinamide adenine dinucleotide which is observed spectrophotometrically. The assay mixture contained in a total volume of 1.0 ml; 0.1 M potassium phosphate buffer pH7.4, 0.05 ml reduced nicotinamide adenine dinucleotide (NADH) (3mM), 0.05 ml diaminoethane tetraacetic acid (EDTA) (16 mg/ml), 0.01-0.05 ml sample. The reaction was started by the addition of 0.05 ml oxaloacetate (20 mM) and the decrease in extinction at 340 nm was followed.

Succinate dehydrogenase (E.C.1.3.99.1) was assayed according to the method of Arrigoni and Singer (1962). The assay is based on the reduction of phenazine methosulphate by succinate and its dehydrogenase. Reduced phenazine methosulphate is immediately reoxidised by dichlorophenol indophenol and bleaching of the latter dye is estimated spectrophotometrically. The assay mixture, total volume 1.0 ml, contained 0.1 M potassium phosphate pH7.6, 0.05 ml potassium cyanide (0.1M) adjusted to neutral pH with HCl, 0.05 ml dichlorophenol indophenol (1.2mM), 0.05 ml phenazine methosulphate 5 mg/ml, 0.01-0.05 ml sample and 0.05 ml of succinate (0.2M) was added to start the reaction. The decrease in extinction at 600nm was then followed.

ATPase. The membrane bound ATPase described by Roisin & Kepes (1973) was assayed by using a pyruvate

kinase assay (Boehringer, Biochemica Catalogue, 1971) to measure the production of ADP from ATP. The principle of the reaction is as follows:

$$\text{ATP} \xrightarrow{\text{ATPase}} \text{ADP}$$

$$\text{phosphoenol pyruvate} + \text{ADP} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{L-lactate} + \text{NAD}^+$$

The oxidation of NADH is followed spectrophotometrically. The assay mixture contained in 1.0 ml; 0.1M triethanolamine hydrochloride pH7.6, 10 units of lactate dehydrogenase (E.C.1.1.1.27) 10 units pyruvate kinase (E.C.2.7.1.40) both from rabbit muscle, 0.05 ml NADH (3mM), 0.05 ml $MgCl_2$ (40mM), 0.05 ml KCl (2M), 0.05 ml phosphoenol pyruvate (20mM), 0.01-0.05 ml sample and the reaction was started by the addition of 0.05 ml adenosine triphosphate (20mM). The decrease in extinction at 340nm was followed.

12. FRACTIONATION OF BACTERIAL CULTURES INTO POPULATIONS WITH DIFFERENT AGES AND PROCEDURES FOR PULSE LABELLING

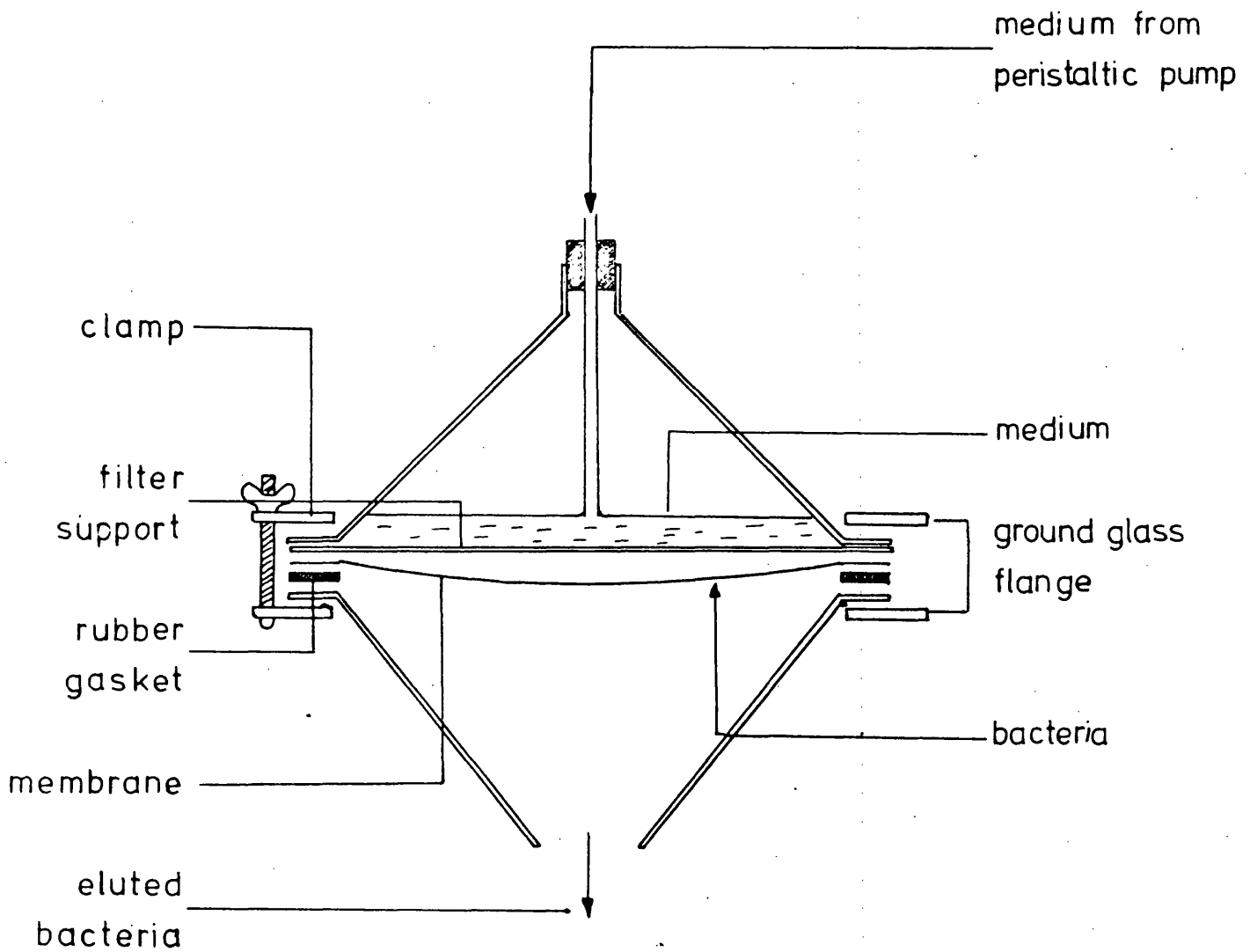
Two separate techniques were employed for age fractionation, both involving similar manipulations. The basic method, described by Helmstetter (1967), allows one to select from a random population of cells a

subpopulation of a particular age. This is achieved by binding an initial culture of bacteria to a Millipore filter and allowing the bacteria to grow and divide. New born cells are then continuously washed off the membrane and can be collected, whilst the parental cells remain attached to the filter. This basic selection may be used in two ways : either the initial culture can be labelled and the fate of the label followed in the progeny cells eluted from the membrane - membrane elution - or the progeny cells can be collected, concentrated and grown as a synchronous culture, which may then be labelled at successive time intervals. The important and fundamental differences between these two methods are discussed in Chapters 4 and 5.

The Helmstetter procedure, which was carried out entirely in a 37°C constant temperature room, will now be described in detail. In both types of experiments a 100 ml exponential culture of E.coli B/r in proline-alanine medium, at a cell density of 10^8 cell/ml (E_{450} = 0.125), was first bound by suction to the surface of a membrane filter (Millipore, grade GS 0.22 um pore size) held in the apparatus (see Fig. 9). The bacteria were then washed with 2 x 100 ml of growth medium, taking care to avoid complete drying out of the filter between washes. Next the filter was inverted by turning over the apparatus and fresh medium was pumped through the filter with a peristaltic pump at maximum rate (22 ml/min). During this time the apparatus was shaken by hand in an effort to remove as many "unbound" bacteria as possible. After 6

FIGURE 9. MEMBRANE ELUTION APPARATUS

The figure is not drawn to scale


medium from
peristaltic pump
clamp
medium
filter
support
ground glass
flange
rubber
gasket
bacteria
membrane
eluted
bacteria

min. the flow rate was reduced to a constant 6 ml/min. The eluate was then collected, either in a series of test tubes (4 or 5 min. periods per tube) for "membrane elution", or into one ice chilled, 1l flask from min. 30 to min. 120 after the start of the elution, in order to collect cells for synchronous cultures. In the latter case, the bacterial suspension was then filtered through an ice-cold membrane filter (Millipore grade HA 0.45 μm pore size) and the cells were concentrated approximately tenfold by resuspending in 50 ml prewarmed medium. The culture was immediately incubated at 37^{o} with shaking, where upon the bacteria grew synchronously for at least two division cycles. The bacterial concentration in the eluate was 2-4 x 10^{6} cells/ml and the initial bacterial concentration of the synchronous culture was therefore approximately tenfold higher.

Pulse labelling for membrane elution. In order to measure rates of synthesis in bacteria by the membrane elution technique, it is only necessary to add isotope briefly to the initial culture and then terminate the pulse by washing the cells on the membrane. Precise details are given for each particular experiment. For measurement of total cellular synthesis, e.g. overall protein synthesis, a sample, generally 5 or 10 ml was removed from each batch of eluate and mixed with an equal volume of ice-cold 10% w/v TCA, and the samples were then processed as described above. When envelope protein synthesis was to be measured, eluate samples (10ml) were immediately chilled and chloramphenicol

(300 µg/ml final concentration) added. Envelopes were subsequently isolated as described in section 13. On some occasions as an alternative to measuring incorporation into whole cells a sample (20ml) of the cleared lysate (Fig. 11) from each sonicated sample was precipitated with 5% w/v TCA in order to estimate the rate of total synthesis. Bacterial densities in the eluate fraction was measured by removing 2.0 ml of culture into an equal volume of formaldehyde/saline solution as previously described.

Pulse labelling in synchronous cultures. In the case of synchronous cultures, pulse labelling was performed by removing 1.0 ml samples from the culture into 1.0 ml fresh prewarmed medium containing isotope. At the end of the labelling period (usually 5 min.) the pulse was terminated. For measurement of total rates of synthesis this was performed by the addition of 2.0 ml ice-cold 10% w/v TCA. The samples were then processed as described above. For measurements of rates of envelope synthesis the pulses were terminated by the addition of chloramphenicol (300 µg/ml final concentration) and either leucine (2 mg/ml final concentration) or glycerol (40 mg/ml final concentration), as appropriate. The control experiment in Fig. 10 indicates that this is an effective method of terminating the incorporation of isotopes into acid precipitable material. Again, in these latter experiments 20 ml samples of the cleared lysate were sometimes removed in order to estimate the total rates of cellular synthesis. Bacterial numbers in the synchronous cultures were estimated by removing 1.0

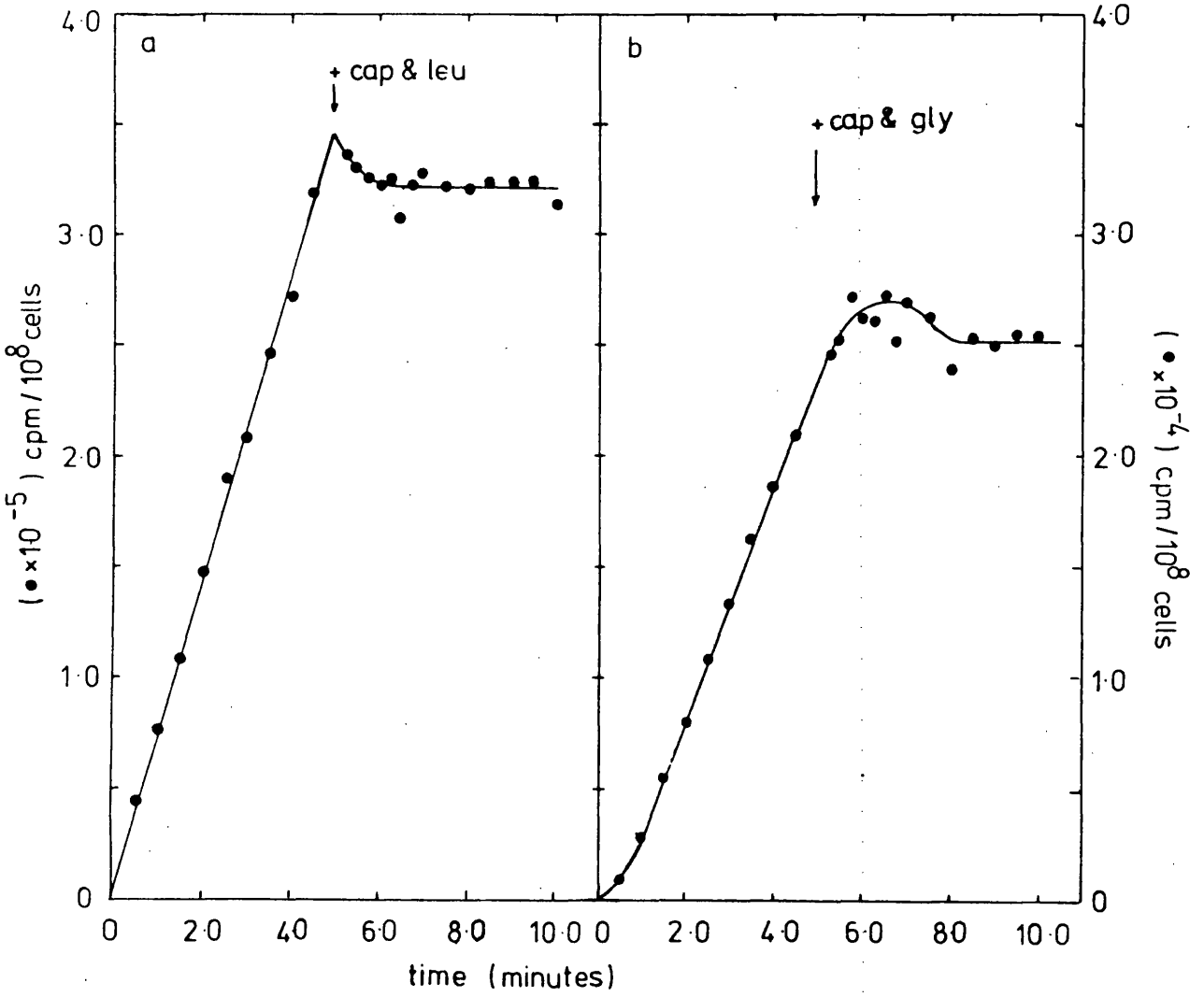
FIGURE 10. INCORPORATION OF $[^{14}C]$ LEUCINE AND 2-$[^{3}H]$ GLYCEROL IN E.COLI B/R LEB 16

Cultures of E.coli B/r LEB16 were grown at 37°C to an E_{450} of 0.25 and 5.0 mls of culture were diluted into 5.0 ml of fresh prewarmed medium containing either 8 µCi $[^{14}C]$ leucine (343 mCi/mmol) or 10 µCi 2-$[^{3}H]$ glycerol (500 mCi/mmol)

a) $[^{14}C]$ leucine : at 30 sec intervals 0.1 ml culture was taken into 1.0 ml 5% w/v ice-cold TCA. After 5 mins. chloramphenicol and leucine were added to a final concentration of 300 µg/ml and 2 mg/ml respectively and sampling into TCA was continued.

The radioactivity in each sample was determined as described.

b) 2-$[^{3}H]$ glycerol : The experiment was performed in an identical fashion except that after 5 mins. chloramphenicol and glycerol were added to a final concentration of 300 µg/ml and 40 mg/ml respectively

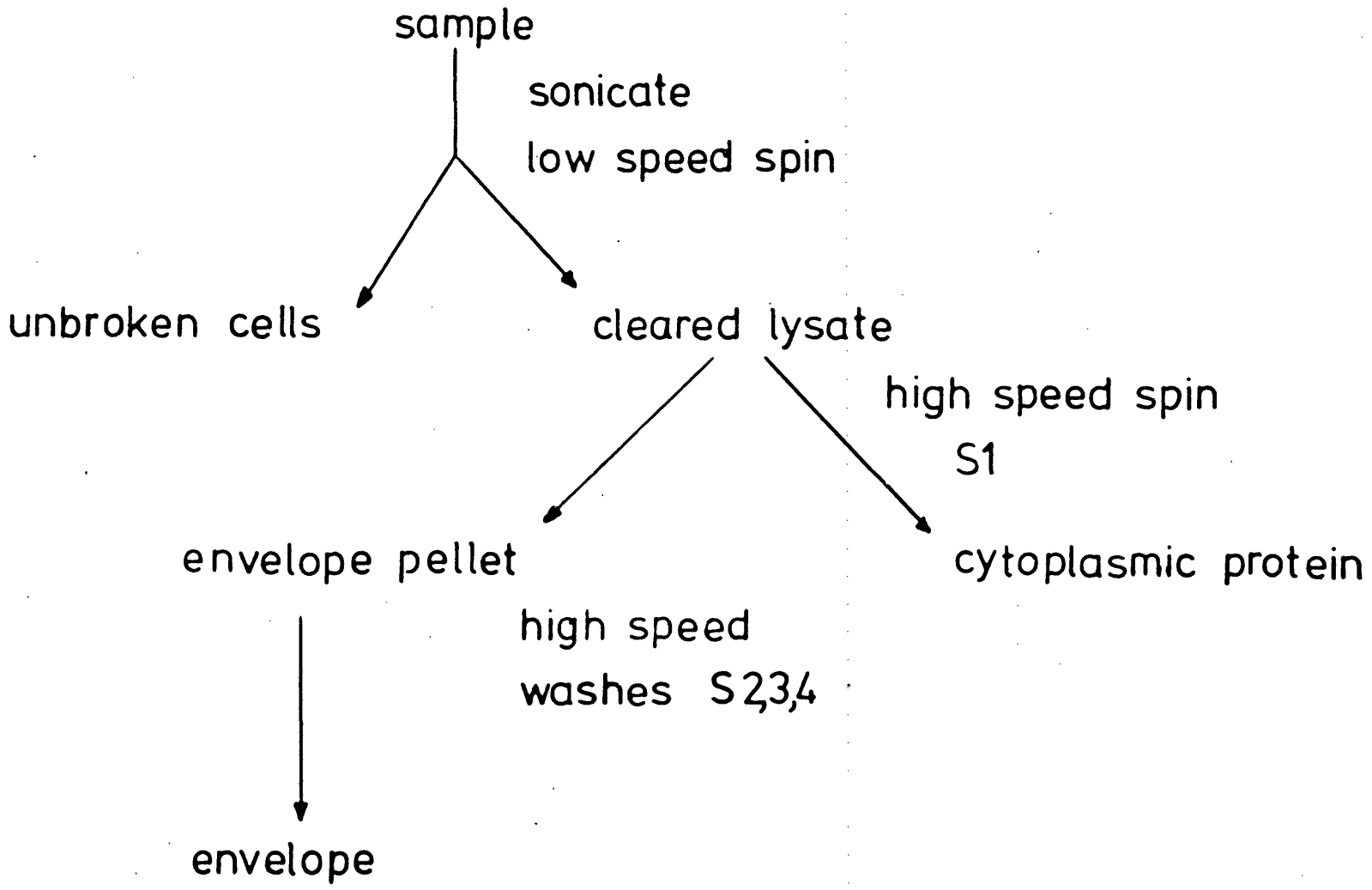

a
b
+ cap & leu
+ cap & gly
4·0
3·0
2·0
1·0
0
0
2·0
4·0
6·0
8·0
10·0
time (minutes)
(• ×10⁻⁵) cpm / 10⁸ cells
(• ×10⁻⁴) cpm / 10⁸ cells

ml of culture into an equal volume of formaldehyde/ saline solution and counting in the Coulter Counter.

13. PREPARATION OF BACTERIAL ENVELOPES

For the preparation of bacterial envelopes, unlabelled carrier cells from an exponential phase culture of E.coli B/r ($E_{450} \sim 0.4$), were added to the radioactive samples to give a total of approximately 10^{10} cells per sample. This represents at least a five hundred fold excess of cold unlabelled cells in the case of samples from both membrane elution fractions and from synchronous culture experiments. Furthermore, in any one experiment all samples contained the same number of carrier cells. Each sample in a test tube (volume at this stage 5-10ml) was next transferred to a 25 ml beaker and the test tube was carefully rinsed with an additional 2.5 ml ice-cold 0.01M sodium phosphate buffer, pH7.2 containing 0.01 M $MgSO_4$. The combined sample was then sonicated for four 30 second intervals with 30 second cooling periods, using the $\frac{3}{4}$" end diameter probe in a 150 watt M.S.E. ultrasonic disintegrator. This and all subsequent operations were carried out at $4^{\circ}C$ and with sonicates or envelope material maintained in the sodium phosphate buffer containing Mg^{2+}. The sample was next transferred to a centrifuge tube and the beaker rinsed with 5.0 ml buffer, which was also added to the centrifuge tube. The samples were centrifuged at 12,000g for 5 mins. at $4^{\circ}C$ in the Sorvall RC-2b centrifuge using an SM-24 rotor. The supernatant

FIGURE 11. SCHEME FOR THE ISOLATION OF ENVELOPES FROM E.COLI


sample
sonicate
low speed spin
unbroken cells
cleared lysate
high speed spin
S1
envelope pellet
cytoplasmic protein
high speed
washes S2,3,4
envelope

which constituted the cleared lysate was kept and any pellet of unbroken cells discarded. The cleared lysates were then centrifuged in the same rotor at 48,000g for 30 mins. at 4°C. The first high speed supernatant, S1. containing most of the cytoplasmic proteins was discarded, the centrifuge tube wiped dry, and the pellets consisting primarily of envelope material were resuspended in a small volume of buffer with the aid of a five second burst of sonication. The volume of each sample was finally made up to 10.0 ml and the samples were recentrifuged at 48,000g for 30 min. at 4°C. The supernatant, S2 was discarded. The pellet was either resuspended in 1.0 ml buffer prior to TCA precipitation or in 0.2 ml of electrophoretic sample buffer prior to gel electrophoresis. The concentration of protein in the latter case was approximately 1.5 μg/μl. The envelopes were stored at -20°C and were stable for many months. The whole envelope isolation procedure is summarised in Fig. 11 and discussed in detail in Chapter 3.

14. SUBFRACTIONATION OF BACTERIAL ENVELOPES ON SUCROSE GRADIENTS

The method of Osborn, Gander, Parisi & Carson (1972) was used. Cells from a 750 ml exponential culture (E_{450} of 0.55) growing in proline-alanine medium were harvested by centrifugation and resuspended in ice-cold 10mM Tris-HCl, pH7.8 containing 0.75M sucrose. The final volume was adjusted to correspond to 7 ml per 100

absorbance units of the original culture. Lysozyme was added to a final concentration of 100 µg/ml and the suspension stirred slowly with a magnetic stirring rod. After 2 mins. two volumes of cold 1.5mM EDTA pH7.5 were added dropwise with slow stirring. Addition of the EDTA took 15 mins. The suspension was then incubated for 15 min. at room temperature. The production of spheroplasts, as monitored by phase contrast microscopy, was approximately 90%. The spheroplast suspension was then subjected to sonication for two 30 sec. periods in batches of 10 ml. The centrifugation procedure described in section 13 was then used to isolate bacterial envelopes from the lysed suspension. The final pellet was resuspended in 1.0 ml 25% w/w sucrose containing 5mM EDTA pH7.5. Gradients were prepared by layering successively 2.1 ml volumes of 50, 45, 40, 35 and 30% w/w sucrose, over a cushion of 1.5 ml 55% w/w sucrose. All these solutions contained 5mM EDTA pH7.5. The envelope material in 1.0 ml 25% w/w sucrose was layered onto the gradients and centrifuged in an SW40 rotor (Beckman L-65B centrifuge) at 35,000 rpm for 16 hours at 4°C. The separated envelope fractions were carefully removed using a syringe fitted with a flat barrelled needle, and after measurement of the refractive index using a refractometor (Bellingham & Stanley Ltd. London), each fraction was diluted with the sodium phosphate buffer used in the preparation of envelopes. The membranes were concentrated by centrifugation in polycarbonate tubes at 48,000 rpm for 2 hrs. at 4°C in a 50Ti rotor using a Beckman L2-50 ultracentrifuge. The

TABLE 2. ELECTROPHORESIS BUFFERS AND GEL SOLUTIONS

A. SEPARATING GEL BUFFER:

0.75 M Tris HCl pH8.8 0.2% w/v SDS

B. STACKING GEL BUFFER:

0.25 M Tris HCl pH6.8 0.2% w/v SDS

C. ACRYLAMIDE SOLUTION:

30% w/v acrylamide 0.8% w/v N;N'-methylene-bis-acrylamide

D. ELECTROPHORESIS BUFFER:

0.025 M Tris 0.192 M Glycine pH8.3 0.1% w/v SDS

E. SAMPLE BUFFER:

0. 25 M Tris HCl pH6.8 20% w/v Glycerol

1% w/v Dithiothreitol 4% SDS

12.5% SEPARATING GEL : 20 ml buffer (A),

16.6 ml acrylamide (C)

1.0 ml ammonium persulphate (15 mg/ml), 0.02 ml TEMED

2.4 ml distilled water

5% STACKING GEL : 5.0 ml buffer (B),

1.6 ml acrylamide (C)

0.5 ml ammonium persulphate (15 mg/ml), 0.01 ml TEMED

2.9 ml distilled water

use of polycarbonate tubes is essential in order to recover at least 50% of each envelope fraction (Nicolaidis & Iyer, personal communication).

15. SUBFRACTIONATION OF ENVELOPES BY SARKOSYL

This method for the isolation of outer membrane material is based on the procedure described by Filip, Fletcher, Wilff & Earhart (1973). Envelopes isolated from sonicated cells by the usual method were resuspended in 10mM sodium phosphate buffer containing 0.5% w/v Sarkosyl NL97 and incubated for 20 mins. at room temperature to solubilise the inner membrane. The outer membrane, which remains largely intact in this detergent, was then pelleted by centrifugation at 48,000 rpm for 2 hrs. at 4°C in the 50Ti rotor. The final pellet was resuspended in the sodium phosphate buffer at a concentration of at least 5 µg protein/µl and stored at -20°C.

16. POLYACRYLAMIDE GEL ELECTROPHORESIS

The basic procedure and buffer system was that of Laemmli (1970). The constitution of the various buffers and gel solutions is given in Table 2. A separating gel concentration of 12.5% w/v with a 5% w/v stacking gel was used routinely. Stock solutions were filtered through Watman No.1 filters to remove dust particles and stored at 4°C in the dark. For the preparation of gels

all solutions were deaerated for several minutes prior to mixing and pouring. The catalyst ammonium persulphate is extremely hygroscopic and variation in polymerisation can occur through the use of different batches containing different amounts of ammonium persulphate. To avoid this, a single large batch of 3 ml aliquots was prepared and stored frozen prior to use for the whole series of electrophoretic analyses. Gels were normally prepared on the day of use, using acid cleaned glassware. It was not found necessary to recrystalise the reagents. Electrophoresis was carried out using a water cooled Bio-Rad slab gel system (Model 220) at a constant voltage of 100V. Up to 40 samples could be analysed at one time on two slab gels 1.5mm thick. Samples containing 1.5 - 4.5 µg protein/ul in the electrophoretic sample buffer were heated at 100°C for five minutes (see Chapter 3) in order to completely disaggregate membrane polypeptides. Then 25 - 50 µl of sample, equivalent to protein from $1x10^9$ - $2x10^9$ cells, was applied to each slot, and 2 µl of 0.25% bromophenol blue was added to every fourth slot along each gel. Bovine serum albumin 65,000 D, fibrinogen 48,000 D and 56,000 D, catalase 56,000 D, malate dehydrogenase 31,000 D, lactoglobulin 18,000 D and lysozyme 14,000 D (all 1 µg/µl in sample buffer) were used as molecular weight standards; 2 µl of each were applied to the gel. The electrophoresis proceeded at a constant voltage of 100V until the bromphenol blue indicator was 0.5 cm from the bottom of the gel (5-6 hrs.). Gels were stained overnight in 300 ml 10% v/v acetic

acid containing 25% v/v isopropyl alcohol and 0.05% w/v Coomassic brilliant blue. Destaining was performed by shaking the gel in 300 ml 10% v/v acetic acid containing 10% v/v isopropyl alcohol for two three hour periods followed by overnight shaking in 10% acetic acid. The gels were then photographed onto 4" x 5" Ilford FP4 film plates using a deep orange filter with background illumination.

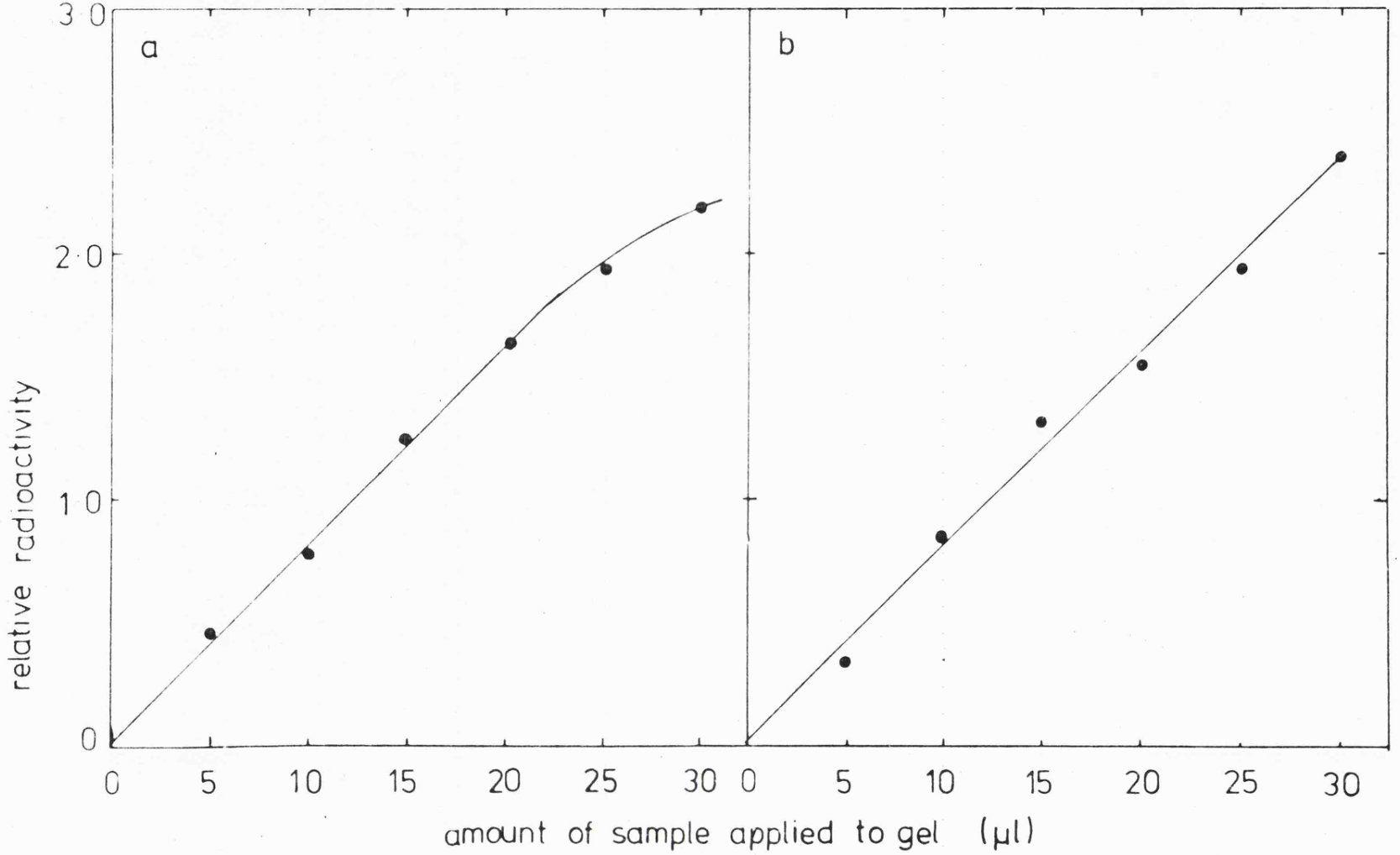
Autoradiography. Radioactive gels were dehydrated by successive washes in dimethyl sulphoxide, followed by impregnation with diphenyl oxazole (PPO) exactly as described by Bonner & Lasky (1974). The gels were then dried onto a sheet of Whatman No.1 filter paper by placing the gel and the filter paper on a supported sheet of porous polythene, wrapping in "Clingfilm" and evacuating this by attachment to a vacuum pump. Heat was supplied evenly over the whole gel surface by judicious placing of two infra red lamps. Drying took approximately 90 mins. The dried gels were then placed in a Kodak X-Ray cassette with a Kodak R.P. Royal X-Omat X-ray film plate and exposed at -70°C as recommended by Bonner & Lasky. As a rough guide, an envelope sample applied to a gel containing 400 cpm ^{14}C-leucine requires approximately ten days exposure in order to obtain a satisfactory autoradiogram. The resulting autoradiograms were developed and together with the negatives of the stained gels were scanned using a Joyce-Loebl microdensitometer. It has recently been reported (Lasky & Mills, 1975) that the response of this system over a wide range of amounts of

FIGURE 12. PROPORTIONALITY OF THE AMOUNT OF RADIOACTIVITY AS DETERMINED BY AUTORADIOGRAPHY WITH THE AMOUNT OF SAMPLE APPLIED TO THE GEL

Different amounts of the same radioactive envelope sample were applied to an acrylamide gel, and following electrophoresis and autoradiography the amount of radioactivity in several bands was determined

a) Band 10 36,500 D protein - 30% total envelope protein

b) Band 14 - 0.7% total envelope protein


a
b
3·0
2·0
1·0
0
relative radioactivity
0
5
10
15
20
25
30
amount of sample applied to gel (µl)

radioactivity is not linear, but, as shown in Fig. 12, the response to the relatively small change in amount of radioactivity for both major and minor protein species occurring in this study, does appear to be linear.

CHAPTER 3. PREPARATION AND CHARACTERISATION OF AN ENVELOPE FRACTION FROM E.COLI

The first problem encountered in any study of a discrete fraction of a cell, is the production of a pure sample of that fraction, free of any contaminating material. The preparation of cell envelopes is made doubly difficult because some components of the envelope i.e. the peripheral membrane proteins, are in effect partitioned between the membranes and the aqueous phase. It therefore becomes necessary to rigorously define the conditions used in the preparation of the envelopes, and to characterise the product as fully as possible. In the first section I shall deal with the preparation and general characterisation of the envelopes and in the second section the detailed analysis of the envelope proteins by SDS-PAGE. Finally I shall present evidence to show that under the conditions employed in this study there is no effect of cell age on the efficiency of isolation of the envelope.

3.I. PREPARATION OF CELL ENVELOPES

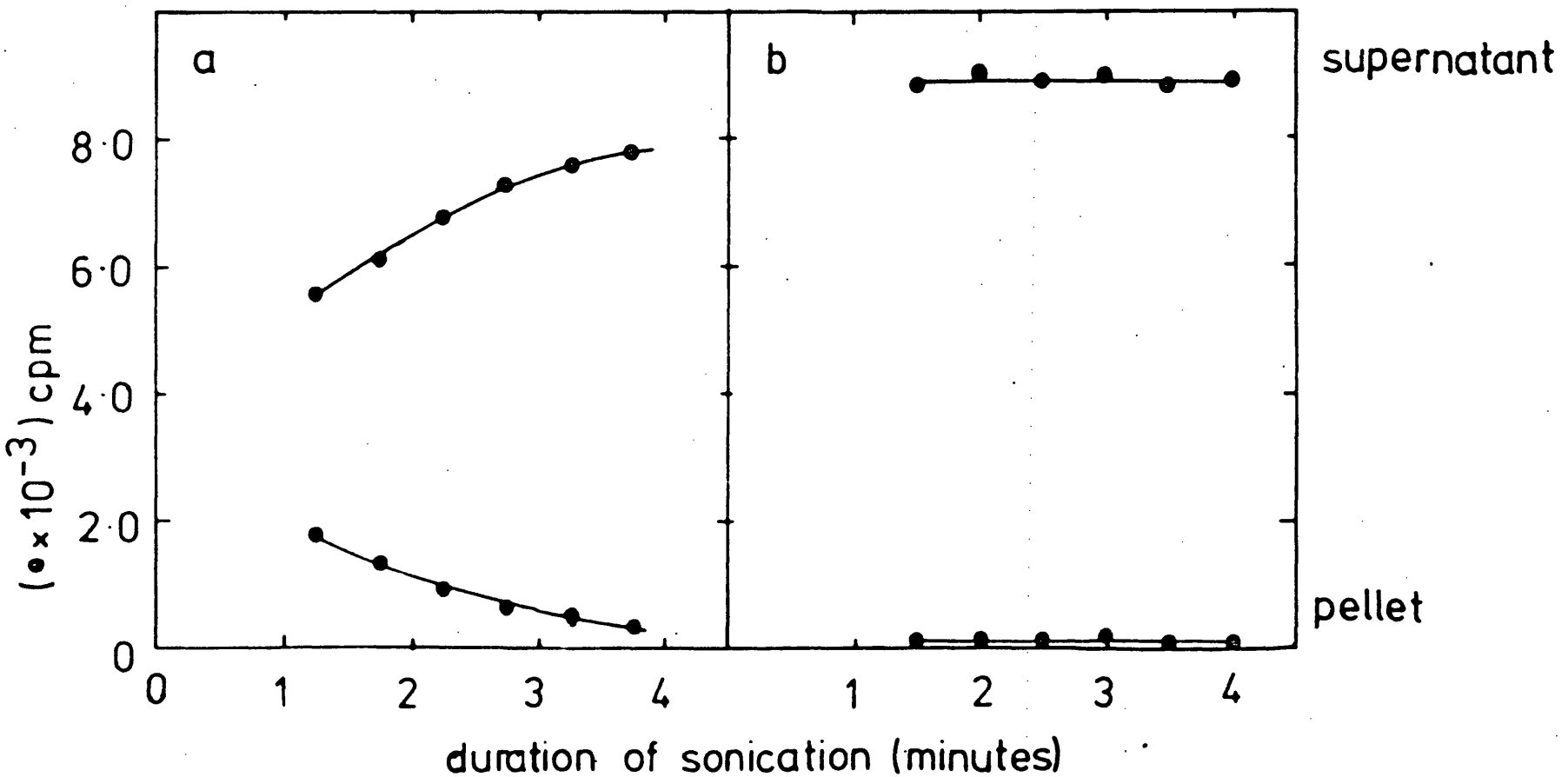
The method adopted in this study for the preparation of envelopes is a modification of the method of Holland & Tuckett (1972) involving differential centrifugation of bacterial sonicates. The basic procedure is outlined in Fig. 11. Since efficient breakage of bacteria is a crucial first step in envelope preparation, experiments

FIGURE 13. EFFICIENCY OF SONICATION

A 20 ml culture of E.coli B/r LEB16 growing at 37°C in proline/alanine medium supplemented with 20 µg/ml thymine was pulse labelled with 0.1 µCi/µg/ml [^{14}C] leucine. After five mins. chloramphenicol and leucine were added to a final concentration of 300 µg/ml and 2 mg/ml respectively, and 4 x 10^{10} cells suspended in 1.0 ml buffer were added

a) 5.0 ml was placed in an 18 ml Sorval centrifuge and sonicated with the exponential probe. At intervals 0.5 mls was removed, diluted with 10 mM sodium phosphate buffer containing 10 mM Mg^{2+} and centrifuged at 6,000 g for 5 mins. The radioactivity in both the pellet and supernatant was determined.

b) The same experiment was repeated except that 5.0 mls was placed in a beaker and sonicated with the $\frac{3}{4}$" end diameter probe


a
b
supernatant
pellet
8·0
6·0
4·0
2·0
0
(•×10⁻³) cpm
0
1
2
3
4
1
2
3
4
duration of sonication (minutes)

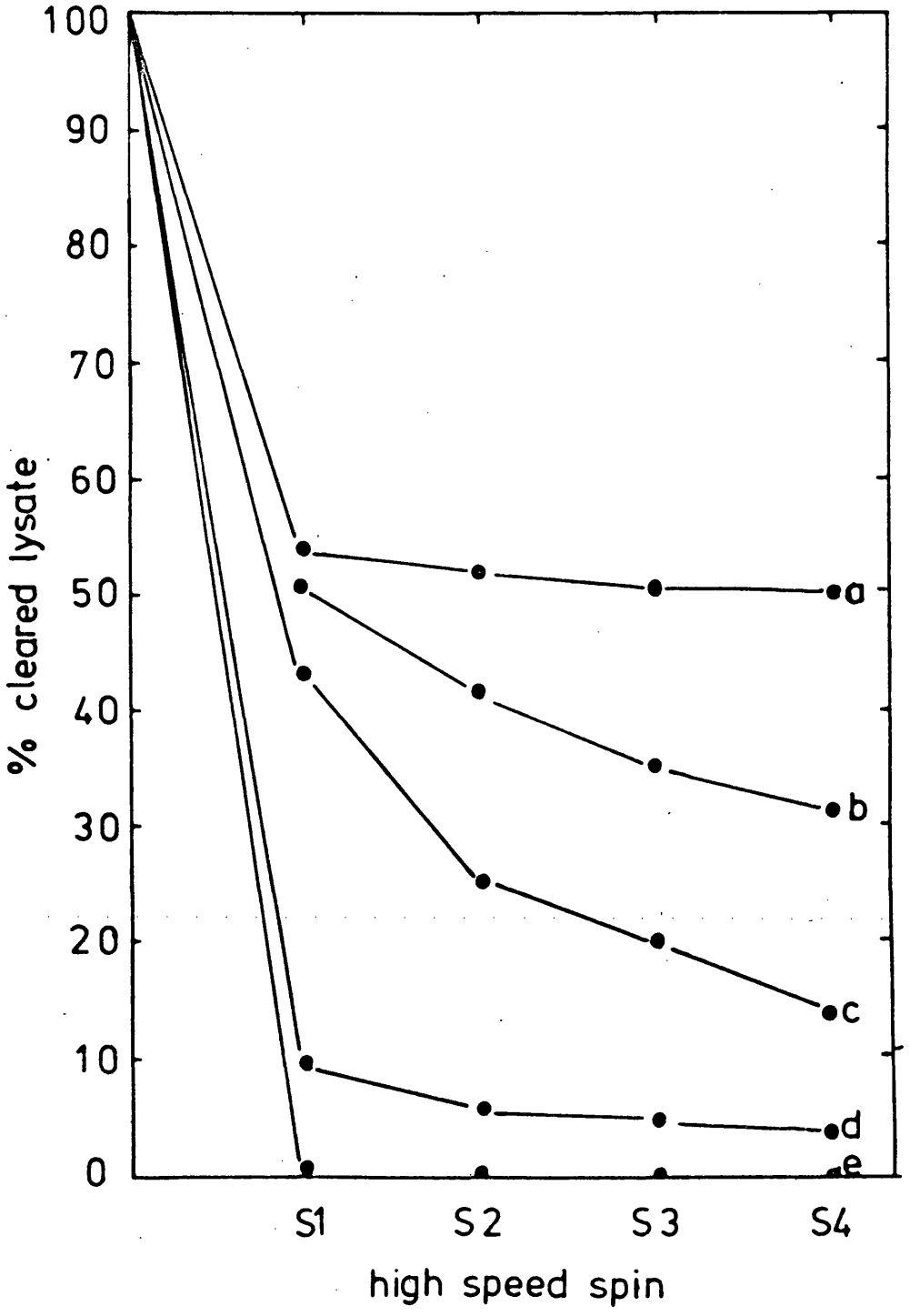
described in Fig. 13, were carried out to test the effect of sonication for different lengths of time on approximately 10^{10} cells under two different sets of conditions. As can be seen, the conditions used in Fig. 13b allow cell breakage with maximum efficiency and these were used throughout this study (see section 3.III). Since envelope proteins are only a relatively minor proportion of the total cell protein it is very important to remove any bacteria remaining unbroken after sonication. Centrifugation at 6,000g for 5 mins. (Fig. 11 - low speed spin) was therefore included in the isolation procedure in order to remove any whole bacteria remaining after sonication. The supernatant from this centrifugation step constituted the "cleared lysate" fraction. This lysate was then recentrifuged at 48,000g for 30 mins. enabling recovery of a crude envelope pellet, which was then subjected to washes by resuspension in buffer, followed by resedimentation at 48,000g. The recovery of material after each high speed centrifugation is presented diagrammatically in Fig. 14. As in the studies of Sargent (1973) malate dehydrogenase and succinate dehydrogenase were used as cytoplasm and membrane markers respectively.

The figure shows that, the isolated envelopes contain approximately 50% of the succinate dehydrogenase activity present in the cleared lysate, implying that 50% of the inner membrane is recovered. In all, 4-6% of the total bacterial protein, estimated as radioactive leucine, methionine or histidine is recovered in the final pellet. Less than 0.5% of the initial malate dehydrogenase activity

FIGURE 14. RECOVERY OF ENVELOPE MATERIAL

The recovery of various components of the cell envelope is presented as a function of the number of washes at 48,000 g. 200 ml cultures of E.coli B/r LEB16 growing at 37°C in proline/alanine medium supplemented with 20 µg/ml thymine were grown to an E_{450} of 0.3. The cultures were then split into 100 ml portions. To follow enzyme recovery samples of all supernatants and pellets produced by the isolation procedure were assayed. For amino acids 5 ml portions were pulse labelled for five mins. with radioactive amino acids ($[^{14}C]$ leucine 8 µCi, 311 mCi/mmol $[^{14}C]$ histidine 8 µCi, 324 mCi/mmol $[^{35}S]$ methionine 16 µCi, 16 µCi/µg) and samples of supernatants and pellets precipitated with TCA and counted. For glycerol 10 µCi 2-$[^{3}H]$ glycerol 500 mCi/mmol was used.

a) succinate dehydrogenase
b) 2-$[^{3}H]$ glycerol
c) Mg^{2+}/Ca^{2+} ATPase
d) amino acids
e) malate dehydrogenase


100
90
80
70
60
50
40
30
20
10
0
% cleared lysate
a
b
c
d
e
S1
S2
S3
S4
high speed spin

is present in the envelopes after the second high speed spin indicating that contamination with cytoplasmic components is very slight. In the first high speed pellet, 50% of the total bacterial phospholipid in the lysate, estimated as 2-[^{3}H] glycerol incorporated into ice-cold TCA insoluble material, is recovered. Also 50% of the Mg^{2+}/Ca^{2+} ATPase is found in this first pellet. However, on subsequent washing of envelope fragments in buffer containing 10mM Mg^{2+} both phospholipid and ATPase are progressively lost. In the case of the enzyme, this is not surprising since the ATPase seems to be a true peripheral membrane protein (Roisin & Kepes, 1973), maximally bound only in the presence of approximately 50mM Mg. The reason for the loss of phospholipid was unexpected and remains unknown. Since repeated washing of the envelopes clearly did result in the loss of peripheral protein as indicated by ATPase and furthermore since contamination by cytoplasmic protein was also minimal after the first wash this more limited procedure was adopted for convenience. Therefore in all subsequent experiments the envelopes were sedimented once at 48,000g and washed once in buffer.

In conclusion, the method described appears to yield some 50% of the total cell envelope, essentially free of cytoplasmic contamination but containing an unspecified, but presumably constant amount, of peripheral protein in addition to integral proteins. Alternative methods of bacterial disruption, e.g. lysis of spheroplasts (see Chapter 2, section 14) would increase this yield, but the

FIGURE 15. GEL ELECTROPHORESIS OF *E.COLI* B/R ENVELOPES

250 ml LEB16 were grown to an E_{450} of 0.4. Envelopes were prepared and the final pellet was resuspended in 0.6 ml electrophoresis buffer E. A second culture was treated similarly except that outer membrane was prepared by treatment with Sarkosyl, and the final pellet resuspended in 0.3 ml buffer E. 5 µl of each were applied to a gel after solubilisation at 100°C along with standard proteins and subjected to electrophoresis

25
20
15
10
7
DYE
band number
DYE-
10 -
15 -
20 -
25 -
sarkosyl pellet-
whole envelope-
molecular weight
-18,000D
-36,500D
-56000D
-76,000D

yields of envelope proteins obtained using spheroplasts were not reproducible from sample to sample. Increased centrifugal force during preparation of envelopes would increase the yield by perhaps 25% (Holland, personal communication) but I shall provide data in the next section which indicate that there is no apparent difference between envelopes isolated at 48,000g and at 100,000g.

3.II. CHARACTERISATION OF THE ENVELOPE PROTEINS OF E.COLI B/R BY SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Polypeptide profiles of E.coli B/r envelopes the appearance of a polyacrylamide gel profile, generated by subjecting total bacterial envelopes to electrophoresis in the presence of SDS, together with the corresponding densitometer scan, is shown in Fig.15. The gel presented in Fig. 15 also displays samples of outer membrane, isolated after removal of the cytoplasmic membrane from envelopes by treatment with 0.5% Sarkosyl, as described in Chapter 2. All samples were solubilised in SDS at 100°C for 5 min. prior to electrophoresis and the gel was stained with Coomassie brilliant blue (Chapter 2, section 16). It was generally found that some 29 distinct bands could be distinguished in the profiles. The RF values and the relative molecular weights corresponding to these bands, computed from the behaviour of standard proteins, is shown in Table 3. The appearance of radioactive profiles may be compared by reference to Figs. 21 and 24. It is of some interest that the membrane proteins of E.coli appear to have relatively low molecular

TABLE 3. ENVELOPE PROTEINS FROM E.COLI B/R AS OBSERVED BY SDS-PAGE

BAND	R.F.	RELATIVE MOLECULAR WEIGHT (IN DALTONS	% TOTAL ENVELOPE PROTEIN	SARKOSYL INSOLUBLE
1	0.99	20,000	4.2	*
2	0.97	21,000	3.1	
3	0.85	26,000	0.5	*
4	0.81	28,000	0.5	*
5	0.80	29,000	0.5	*
6	0.78	30,000	1.3	
7	0.76	31,000	2.9	*
8	0.75	31,500	2.1	
9	0.71	34,000	5.0	*
10	0.68	36,000	29.0	*
11	0.62	40,000	0.5	
12	0.59	42,000	0.5	
13	0.57	44,000	0.5	
14	0.54	46,000	0.7	
15	0.53	47,000	1.8	*
16	0.57	49,000	1.5	
17	0.50	50,000	2.3	
18	0.48	52,000	1.0	*
19	0.46	54,000	0.7	
20	0.44	56,000	2.6	
21	0.43	51,000	0.7	
22	0.40	60,000	1.8	

BAND	R.F.	RELATIVE MOLECULAR WEIGHT (IN DALTONS)	% TOTAL ENVELOPE PROTEIN	SARKOSYL INSOLUBLE
23	0.36	65,000	4.0	
24	0.33	68,000	2.1	
25	0.29	74,000	1.5	*
26	0.27	77,000	1.5	
27	0.26	78,000	2.3	*
28	0.24	82,000	1.5	*
29	0.18	90,000	0.7	

weights since the molecular weight range of the gels is from 18,000 D - 140,000 D and apparently no material is unable to enter the gel. Also shown in Table 3 are the percentages of the total envelope protein in each band. Finally the bands which are thought to correspond to outer membrane polypeptides are specifically indicated. As will be seen in Fig. 35 there is good agreement between the gel profiles of outer membranes prepared by Sarkosyl extraction and by isopycnic centrifugation in sucrose (Osborn et al. 1972).

Sensitivity of the SDS-PAGE system. Fig. 16 shows an empirical curve depicting the minimum number of molecules per bacterium detectable with the acrylamide gel system used in this study. The curve obtained is based on the amount of protein (and known bacterial equivalents) applied to the gel, and the amount of protein in the smallest visible band. The curve can also be used to estimate the number of copies of any envelope protein per cell, if it's proportion of the total envelope protein is known.

Identification of specific polypeptide bands. Methods for identification of polypeptide bands in SDS-gels with known enzyme activities is at a rudimentary stage but it is probable that on a molecular weight basis band 23 is, or at least contains substantial amounts of succinate dehydrogenase (Spencer & Guest (1974)). Bands 9 and 10 correspond to the major outer membrane proteins D and B (Lugtenberg et al. 1975) but it is obvious that in this strain (E.coli B/r) protein B, relative molecular weight

FIGURE 16. MINIMUM AMOUNT OF PROTEIN DETECTABLE BY SDS-PAGE

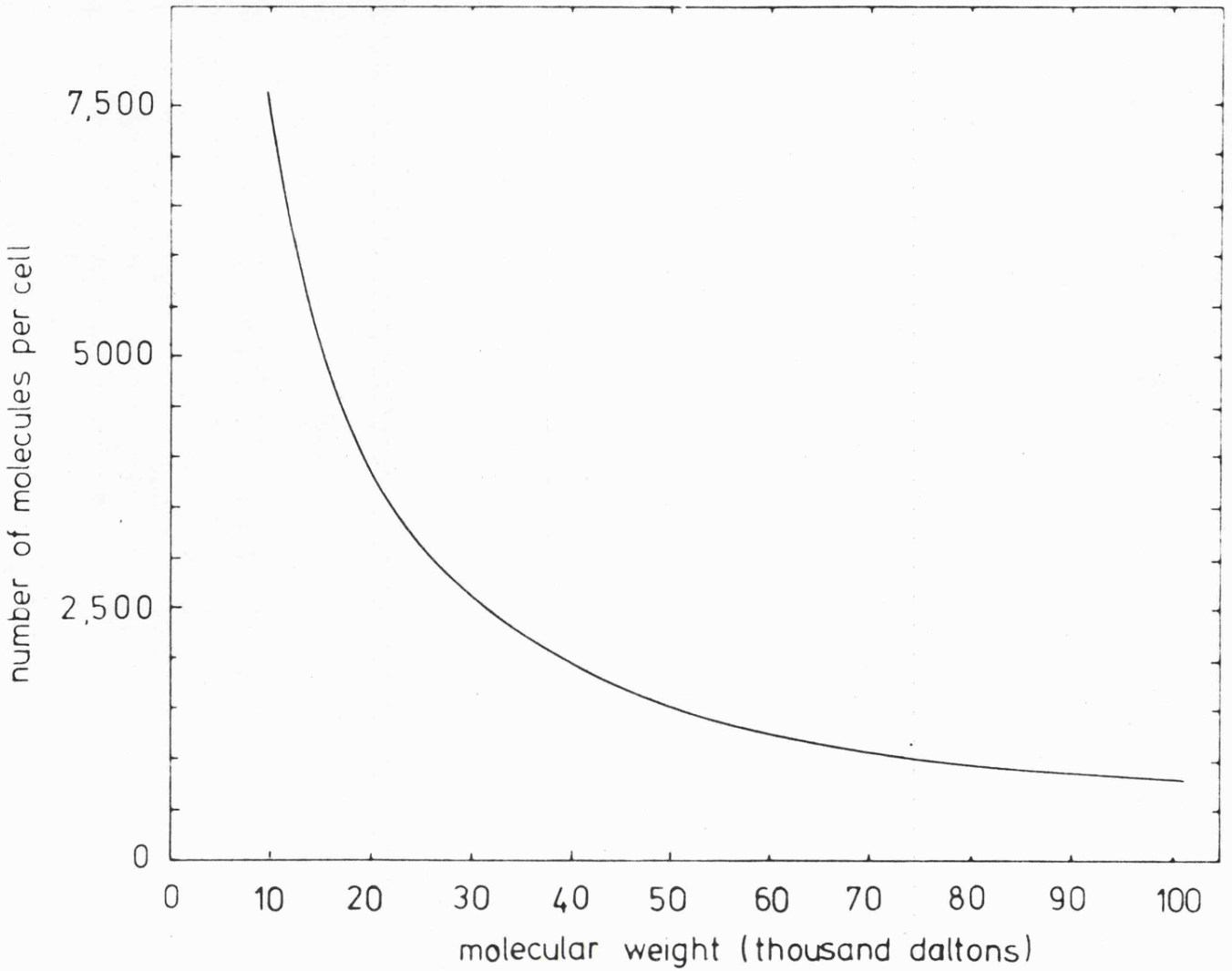

7,500
5000
2,500
0
number of molecules per cell
0
10
20
30
40
50
60
70
80
90
100
molecular weight (thousand daltons)

TABLE 4. EFFECT OF CENTRIFUGAL FORCE ON RECOVERY OF ENVELOPE PROTEINS

BAND NO.	48,000g % TOT ENV PROTEIN	100,000g % TOT ENV PROTEIN	$\frac{48,000g}{100,000g}$
7	2.4	2.5	0.96
8	2.1	2.2	0.95
9	8.6	8.8	1.01
10	30.7	29.6	1.03
11	0.9	1.0	0.90
12, 13	1.9	1.9	1.0
14	0.8	0.8	1.0
15	1.2	1.1	1.09
16	1.1	1.0	1.1
17	2.1	2.1	1.0
18	0.8	0.8	1.0
19	0.7	0.7	1.0
20	2.3	2.2	1.05
21	1.1	1.2	0.91
22	1.0	1.0	1.0
23	2.5	2.2	1.13
24	1.5	1.6	0.93
25	1.2	1.2	1.0
26	0.9	0.7	1.28
27	1.0	1.0	1.0
28	1.1	1.1	1.0
29	0.5	0.6	1.0

36,500 D, is the predominant species.

Effect of centrifugal force during preparation of envelopes and tempeature of solubilisation of samples on the gel profile. Table 4 shows the proportion of protein in each polyacrylamide gel band after analysis of envelope material isolated at either 48,000g or 100,000g and the ratio of the two values. As can be seen there is no significant difference leading to the conclusion that the envelopes isolated at 48,000g are characteristic of the whole envelope, and are not composed of a special sub-fraction. Since sedimentation at the lower gravitational force is very conveniently carried out using a Sorval centrifuge rather than an ultra high speed centrifuge this procedure was adopted for simplicity in all studies.

Fig. 17 shows a gel containing envelopes prepared at 48,000g and 100,000g and solubilised at 70°C and 100°C for 5 min. in SDS prior to electrophoresis. The difference between the 70°C and 100°C samples should be noted. At 70°C there is a great reduction in material in bands 9 and 10 and an accumulation of apparently incompletely solubilised material at a position corresponding to 50-70,000 D. Therefore, as is now general practice, all samples were boiled in sample buffer prior to electrophoresis.

FIGURE 17. EFFECT OF CENTRIFUGAL FORCE ON THE ELECTROPHORETIC PROFILE OF E.COLI ENVELOPES

11 LEB16 was grown to an E_{450} 0.4 and split into four 250 ml portions. Envelopes were prepared from 2 portions at 48,000 g as described in Chapter 2, and from the remaining at 100,000 g in a Beckman L2-65b ultra-centrifuge. Outer membranes were prepared from one portion of envelopes isolated at each speed. Envelopes were resuspended in 0.6 ml electrophoresis buffer E and outer membranes in 0.3 ml buffer, and after solubilisation at both 70°C and 100°C 5 µl samples were applied to the gel and subjected to electrophoresis

band number
DYE
10
15
20
25
sarkosyl pellet
100°
whole envelope
sarkosyl pellet
70°
whole envelope
-100,000g
-48,000g
-100,000g
-48000g
-36500D
-56000D
-76000D
molecular weight

3.III. THE EFFECT OF CELL AGE ON THE PREPARATION OF BACTERIAL ENVELOPES

In the previous two sections I have presented the procedure for the preparation of envelopes and their detailed characterisation, and have concluded that the method yields material which is representative of the whole cell envelope. However, since this procedure is to be applied to a synchronous population of bacterial cells it is important to demonstrate that the efficiency of envelope isolation is not affected in any way by cell age. There is good reason to suppose that this might be a potential problem. Clark (1968a) has demonstrated a differential effect of sonication on bacterial viability in a synchronous culture of E.coli, the bacteria being more susceptible just prior to division. The experiment shown in Fig. 18 in which the release of cytoplasmic protein by sonication is measured as a function of cell age, confirms this observation, but also demonstrates that under appropriate conditions such a differential effect may be avoided. In this experiment bacterial samples from a synchronous culture, pulse labelled with [C^{14}]-leucine, were either sonicated under conditions to produce 80% breakage or completely disrupted, measured in each case by the quantity of labelled debris remaining in the low speed pellet. The sonication conditions used in Fig. 18a and 18b are the same as those used in Figs. 13a and 13b respectively. Following sonication and low speed centrifugation the amount of radioactive protein in

FIGURE 18. EFFECT OF CELL AGE ON EFFICIENCY OF SONICATION

Two synchronous cultures were pulse labelled with 1 µCi [^{14}C] leucine (1 µCi/µg) at intervals. The samples were made up to 10^{10} cells as described in Chapter 2 and sonicated.

a) Sonication in Sorvall centrifuge tube with the exponential probe for 2 mins.

b) Sonication in a beaker with the $\frac{3}{4}$" end diameter probe for 2 mins.

All samples were then spun at 6,000 g in the Sorvall and the radioactivity in the supernatant was determined

(• ×10⁻⁷) cells / ml
(• ×10⁻³) cpm / ml
3·0
5·0
7·0
9·0
6
8
10
20
a
0
20
40
60
80
100
time (minutes)
b
2·0
3·0
4·0
5·0
4
6
8
10
15
(• ×10⁻⁷) cells / ml
(• ×10⁻³) cpm / ml

the clarified lysate was measured. As can be seen in Fig. 18b, where sonication is as efficient as possible, there is a continuous exponential increase in radioactivity, reflecting the continuous increase in the rate of protein synthesis during the cell cycle. However, in Fig. 18a this pattern is not observed. Since sonication is only some 70-80% efficient in this case I interpret this as being due to a differential sensitivity to sonication with cell age, cells being most sensitive (i.e. recovery of released cytoplasmic protein is highest) some forty mins. after birth of the bacteria. Therefore, although there is a differential sensitivity to sonication with cell age, this is not apparent under conditions where sonication is maximally efficient. These conditions are used in all subsequent experiments.

I have assumed that once the cleared lysate is produced there is no further effect of cell age on the isolation of envelopes. Since essentially identical results to those observed with synchronous cultures, are obtained when using the membrane elution technique (Chapter 4), by which bacteria of the same age are always processed, I feel that this assumption is justified.

CHAPTER 4. MEASUREMENTS OF SYNTHESIS OF ENVELOPE COMPONENTS BY MEMBRANE ELUTION

The membrane elution technique described in chapter 2 enables the fractionation of a culture of E.coli B/r into portions containing cells of different ages. The rationale and interpretation of these experiments has been described elsewhere in detail (Helmstetter, 1967) and will only be briefly noted here. Firstly, it is known that the distribution of ages within a population of exponentially growing bacteria, is such that there are twice as many young cells as old cells. If, as Helmstetter has described, bacteria are bound to a membrane filter, allowed to grow and eventually one of the progeny of each division washed off the membrane, the expected pattern of appearance of cells in the eluate will be that shown in Fig. 19. Thus the oldest cells in the original population, i.e. those just about to divide, will be the first to divide and to release a progeny cell from the membrane filter. As the experiment proceeds, the progeny of progressively younger cells are eluted. Since there were twice as many younger cells as old cells in the original culture the number of cells appearing in the eluate will increase. After one generation time, the daughters of the oldest cells which remained bound to the membrane after the first division, will divide again and thus the pattern repeats itself. Using this technique it is therefore possible to pulse label the original exponential culture and then, by following the fate of the label in the eluted cells, to construct the pattern

FIGURE 19. THEORETICAL ELUTION PATTERNS

a) Continuous exponential increase in rate of synthesis

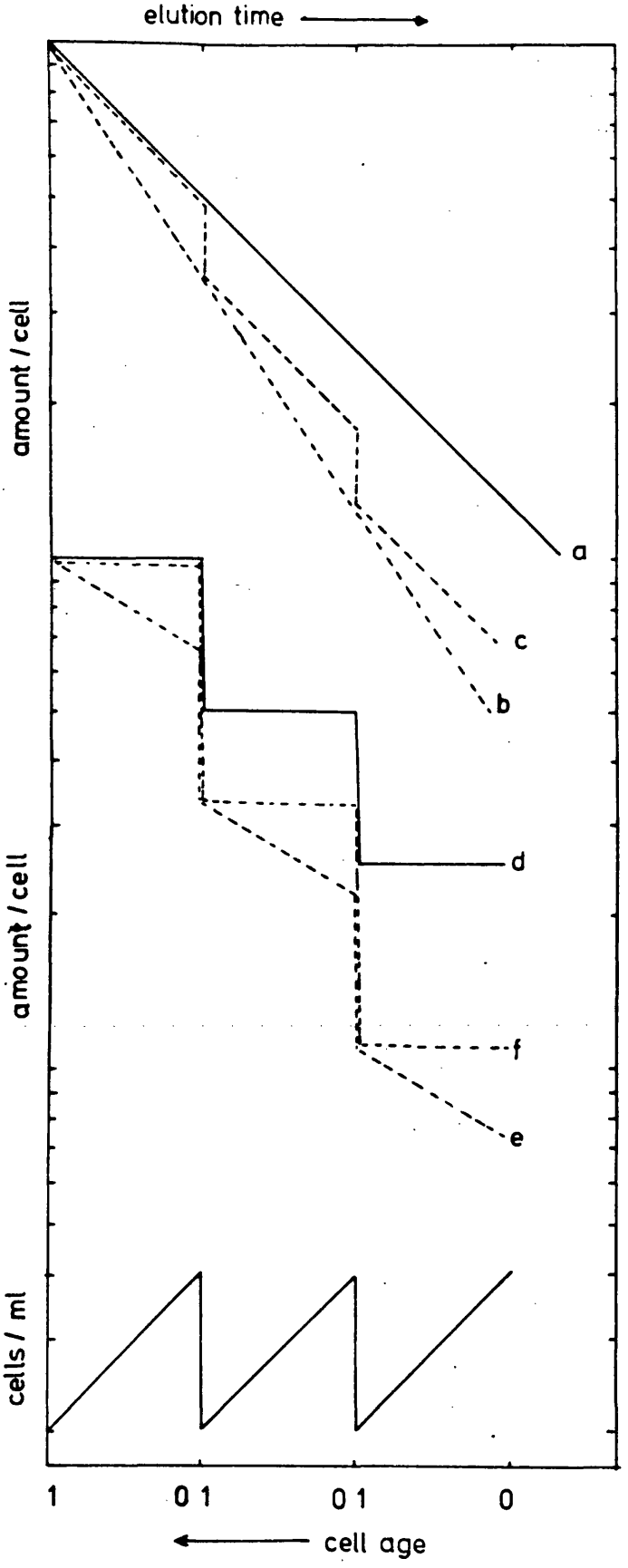
b) Effect of one third turnover per generation on a)

c) As in b) except that turnover occurs at a discrete time

d) Discontinuous linear synthesis with a discrete doubling in rate

e) Effect of one third turnover per generation on d)

f) As in e) except that turnover occurs at a discrete time


elution time
amount / cell
a
c
b
amount / cell
d
f
e
cells / ml
1
0 1
0 1
0
cell age

of synthesis of a macromolecule throughout the cell cycle. Finally, as already indicated, a further most important advantage of this system is that bacteria of identical ages, i.e. newborn cells, constitute all the labelled samples which are subsequently fractionated to produce envelopes. The expected patterns of synthesis of any bacterial component which increases exponentially in rate, or at a constant linear rate with discrete doublings, are shown in Fig. 19. However, it is obvious that this method depends upon there being no appreciable turnover of the component studied. The dashed lines in Fig. 19 show the expected effect of continuous turnover of a component, and turnover which occurs at a discrete time in the cycle.

Turning now to experimental findings, Fig. 20 shows the results obtained after measuring the rate of total protein, phospholipid, and bulk envelope protein synthesis, in E.coli growing in proline/alanine medium. As can be seen, both total protein and phospholipid synthesis appear to increase continuously during the cell cycle in agreement with the results of Dennis & Young (1975) and of Sargent (1973) respectively. Envelope protein synthesis however shows a distinctly different pattern with an increase in rate occurring in the first part of the division cycle. Comparison with the results of Meacock & Pritchard (1975) show that this time is roughly coincident with the middle of rounds of DNA replication in E.coli B/r LEB16, growing in proline/alanine medium.

The stepwise synthesis of envelope protein shown in Fig. 20 is in agreement with the observation of Sargent

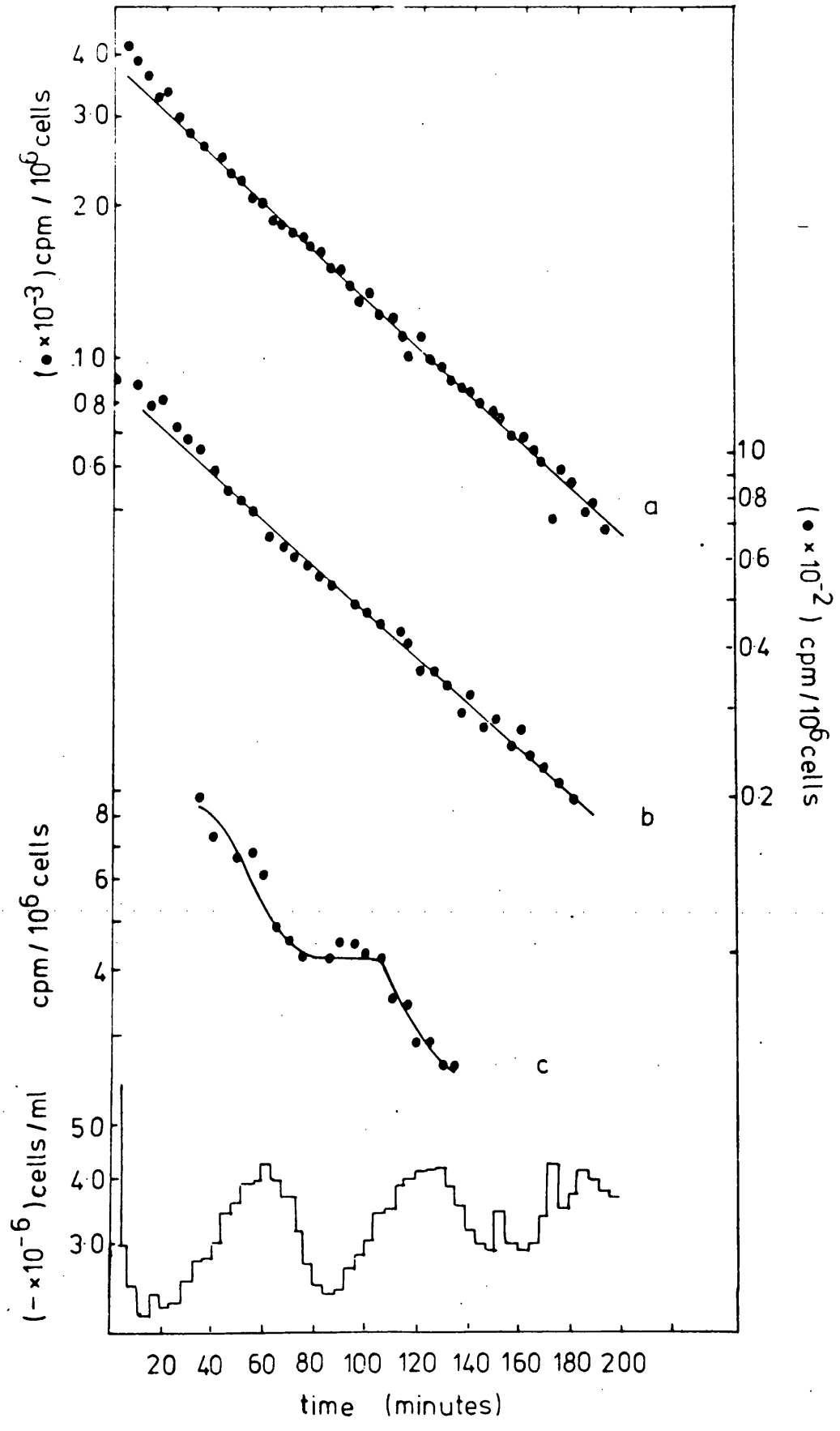
FIGURE 20. RATE OF PROTEIN, PHOSPHOLIPID AND ENVELOPE PROTEIN SYNTHESIS

The initial culture was pulse labelled with 50 μCi $[^{14}C]$ leucine (311 mCi/mmol) or 60 μCi 2$[^{3}H]$ glycerol (1 μCi/μg) for 5 mins. prior to elution. Samples were collected directly into TCA or in the case of envelope proteins, envelopes were first prepared.

a) total protein

b) phospholipid

c) envelope protein

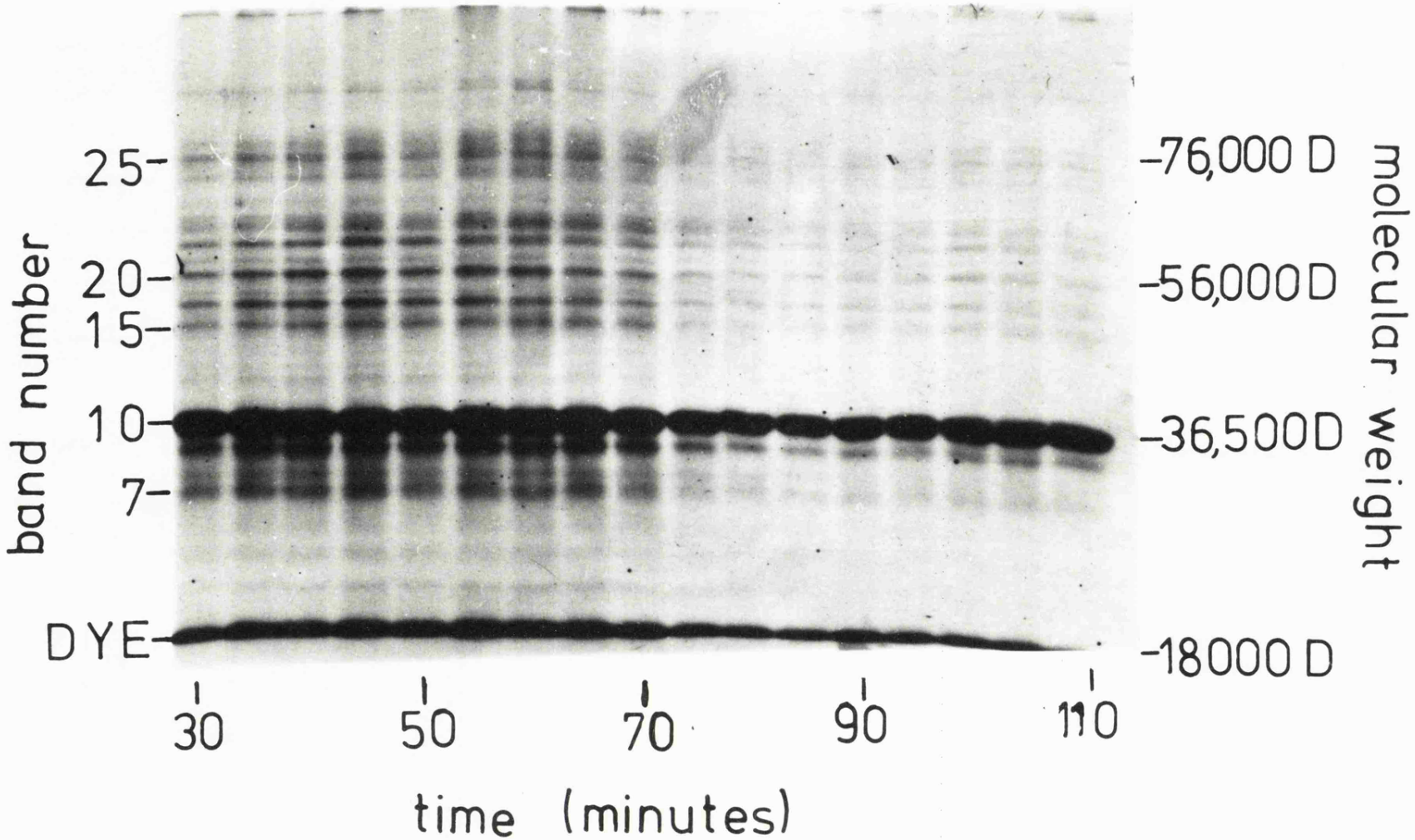

(• ×10⁻³) cpm / 10⁶ cells
4·0
3·0
2·0
1·0
0·8
0·6
a
b
c
(• ×10⁻²) cpm / 10⁶ cells
1·0
0·8
0·6
0·4
0·2
cpm / 10⁶ cells
8
6
4
(– ×10⁻⁶) cells / ml
5·0
4·0
3·0
20 40 60 80 100 120 140 160 180 200
time (minutes)

(1973, 1975b) that envelope protein synthesis in B.subtilis appears to continue throughout the cell cycle at a constant rate, with discrete doublings in rate. From these results it may be concluded that in E.coli B/r envelope protein is also accumulated in a linear fashion and the actual timing of the increase in rate of accumulation, which can only be determined approximately in such experiments, does not coincide with the termination of rounds of replication. This raises the possibility that the synthesis of envelope proteins does not play a role in the timing of cell division. The results also indicate the existence of a regulatory mechanism for envelope protein synthesis distinct from cytoplasmic protein synthesis.

I have referred in the preceding section to the measurement of rates of synthesis of envelope proteins. In fact what has been determined is the combined rate of synthesis and concommitant insertion into the cell envelope. What evidence is there that proteins are not perhaps synthesised at one time in the cell cycle and inserted into the envelope at another? To answer this question, at least in part, the analysis may be refined by taking envelope samples from an experiment as shown in Fig. 20 and subjecting each one to SDS-PAGE followed by autoradiography. Fig. 21 shows an autoradiogram of such a set of samples obtained from an elution experiment. Careful examination of the autoradiogram shows no evidence for the appearance or disappearance of any bands as a function of time. However, it should be noted that samples obtained during the first thirty minutes of

FIGURE 21. AUTORADIOGRAPHIC ANALYSIS OF INDIVIDUAL PROTEIN BAND

Envelopes were isolated from samples taken from an elution experiment following pulse labelling with 50 µCi [^{14}C] leucine (311 mCi/mmol) and resuspended in 0.2 ml electrophoresis buffer E. Following solubilisation at 100°C 25 µl aliquots of each sample were subjected to SDS-PAGE


band number
25
20
15
10
7
DYE
76,000 D
56,000 D
36,500 D
18000 D
molecular weight
30
50
70
90
110
time (minutes)

elution are not analysed as there are still unbound bacteria being washed off the membrane filter during this period. Consequently, maturation of certain proteins might have been missed. On the other hand, attempts to measure directly any post-translational maturation of membrane proteins, using synchronous cultures (see Chapter 6), also proved negative. Consequently I therefore conclude that within the limitation of the method I am measuring the rate of synthesis of envelope proteins.

From the amount of radioactivity in each band of an autoradiogram the rate of synthesis of individual polypeptides may be followed throughout the division cycle. The radioactivity per band is calculated from the area under each peak on a densitometer scan, and then normalised to the amount of protein applied to the gel in each sample. It may be recalled from Chapter 2 that each radioactive bacterial sample was mixed with an excess of unlabelled bacteria to a final total of 10^{10} cells before preparation of envelopes. Therefore, every sample applied to the gel should contain the same amount of protein in, for example, the major 36,500 D band which was used for normalization. The amount of protein in this band was determined by staining the gel with Coomassie brilliant blue, followed by micro-densitometer scanning of photographic negatives of the gels taken prior to autoradiography.

Using the procedure described above to measure the radioactive content of individual bands, it was found that all polypeptides accumulated linearly during the

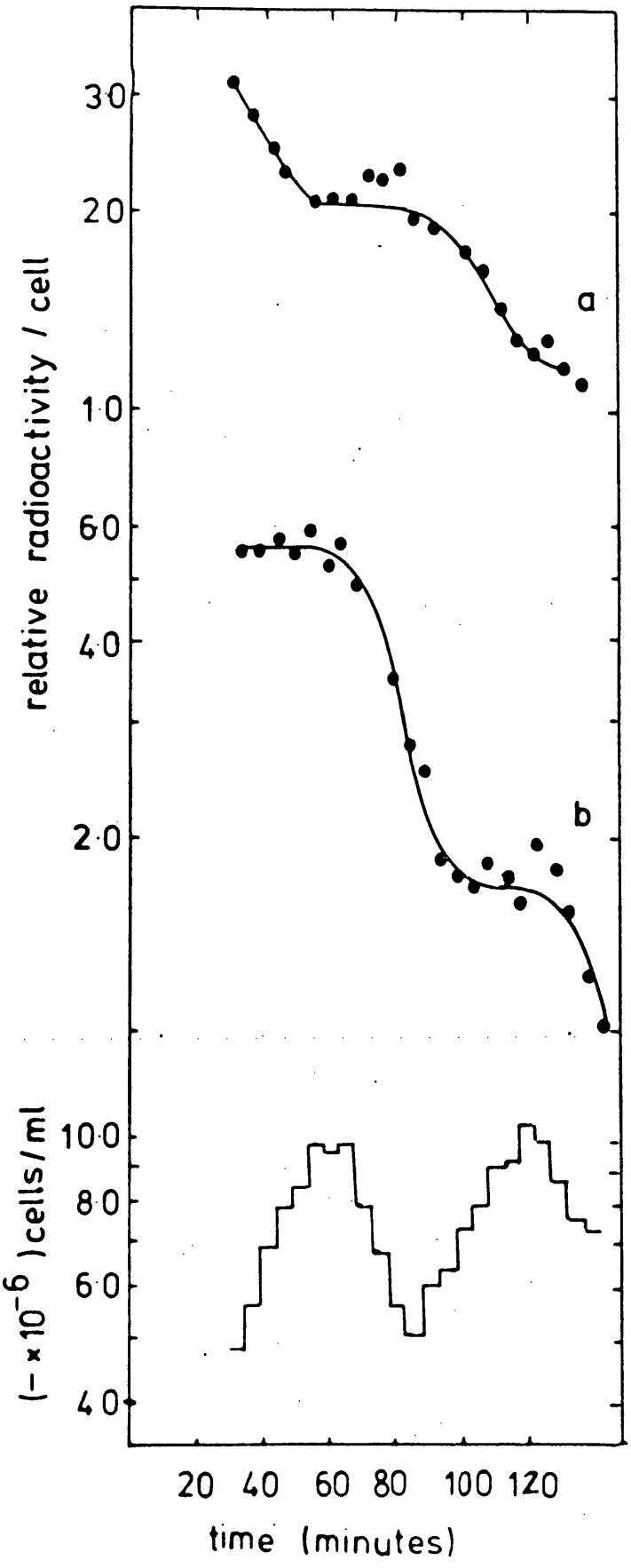
cycle in a fashion similar to that previously observed for envelope protein in bulk. In addition, however, two distinct patterns could be distinguished, each accounting for approximately half of the envelope proteins. One class showed an abrupt doubling in rate of synthesis, whilst the other showed an apparent trebling in rate of synthesis. Fig. 22 shows the results obtained from measurements of one band representative of each of these classes. The apparent difference in timing of the increase in rate of synthesis in the two curves may or may not be significant as it varied considerably from experiment to experiment. The most obvious interpretation of the behaviour of the second class of polypeptides is that some envelope proteins are lost from the envelope, at a discrete time in the cycle, which happens to coincide with the time at which their rate of synthesis doubles (see Fig. 19).

FIGURE 22. RATE OF SYNTHESIS OF INDIVIDUAL PROTEINS

The rates of synthesis of two bands taken from the autoradiogram shown in Figure 21 are presented.

a) 36,500 D protein showing a doubling in rate of synthesis

b) Band 20 showing a trebling in rate of synthesis


30
20
10
60
40
20
relative radioactivity / cell
a
b
100
80
60
40
(- × 10⁻⁶) cells/ml
20 40 60 80 100 120
time (minutes)

CHAPTER 5. MEASUREMENTS OF SYNTHESIS OF ENVELOPE COMPONENTS IN SYNCHRONOUS CULTURES

As described in the previous chapter, the membrane elution technique provides evidence that the accumulation of the bulk and of some individual envelope proteins, occurs at a constant rate, with discrete doublings in rate early in the division cycle, at the growth rate employed. The great advantage of this method is that all the cells which are fractionated are of the same age. There is one disadvantage in that the results obtained may be affected by turnover of the components being studied. I have therefore used the complementary technique of collecting the unlabelled, new-born cells, eluted from a membrane and growing these as a synchronous culture. This method has the disadvantage that the pulse labelling is not performed on an undisturbed, exponentially growing culture. A second potential disadvantage may be ignored, since the experiment described in section 3.III. indicated that under the conditions used there is no effect of cell age on the efficiency of preparation of envelopes from synchronous cultures. The growth of such a culture is shown in Fig. 23. Total protein synthesis increases throughout the cycle as expected and the generation time, calculated from the point where half the bacteria have divided, is equal to the generation time for this strain in batch culture, i.e. 65 minutes. As found by Meacock & Pritchard (1975) under these conditions DNA replication is

FIGURE 23. SYNCHRONOUS CULTURE OF E.COLI B/R

Synchronous cultures of E.coli B/r were pulse labelled for 5' at intervals with 1 µCi of either [^{14}C] leucine (311 mCi/mmol) or methyl -[^{3}H] thymidine (1 µCi/µg)

a) Total protein

b) DNA

c) Cell number

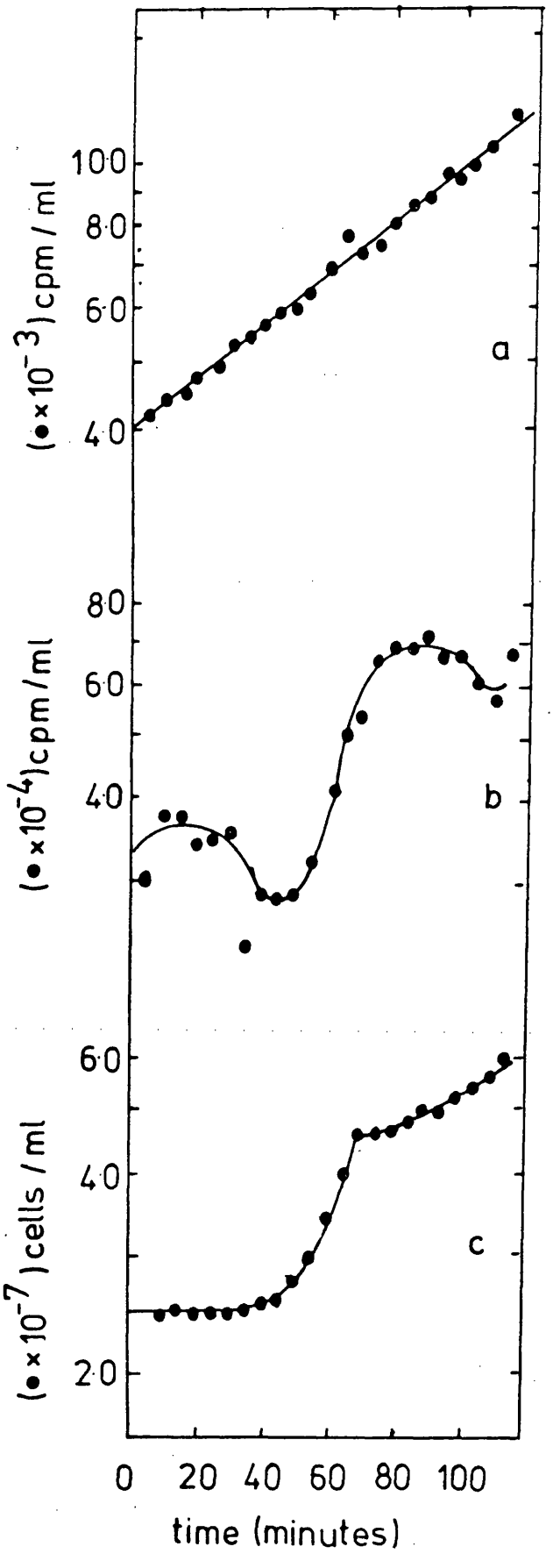
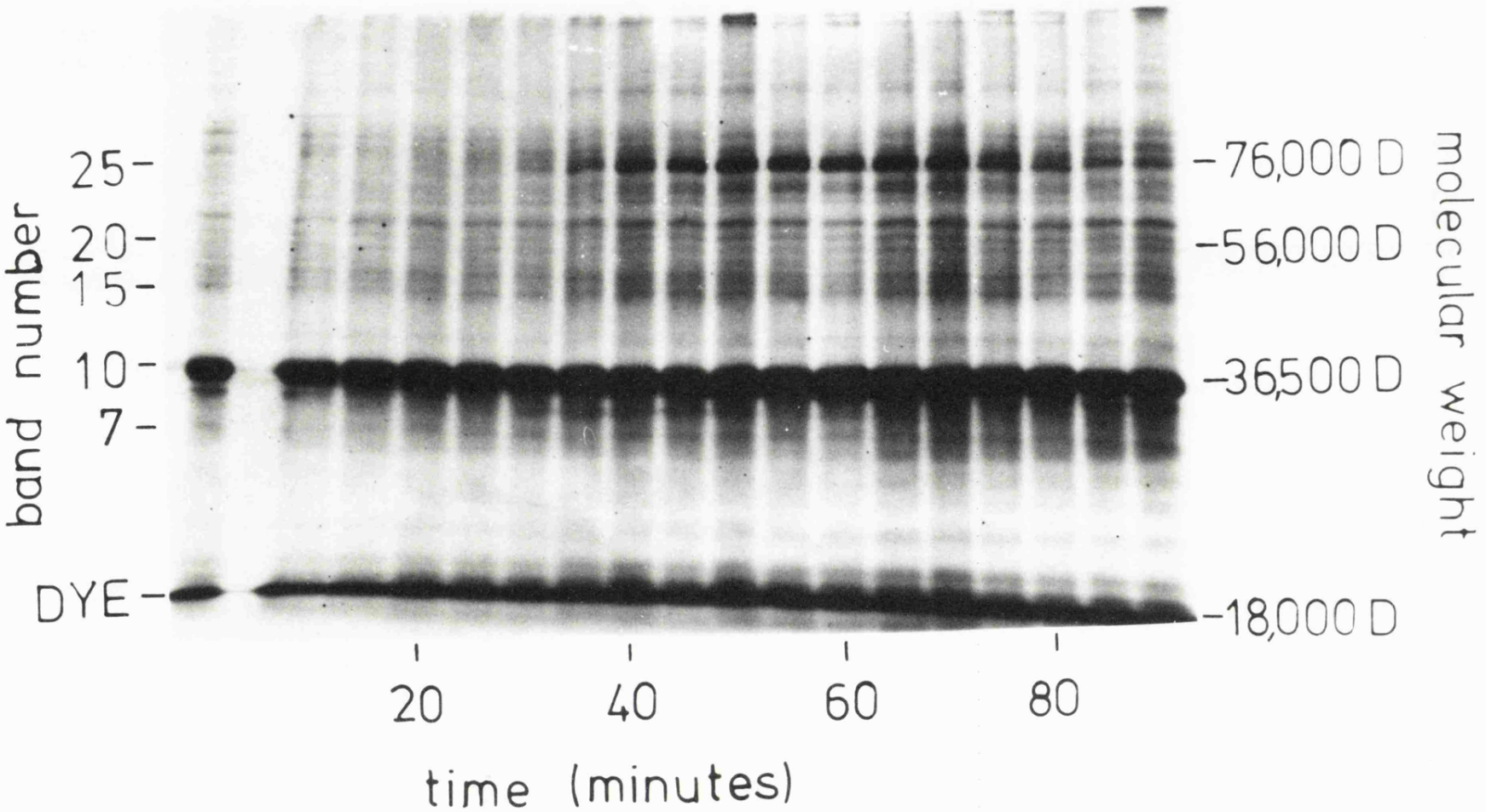

100
80
60
40
(•×10⁻³) cpm / ml
a
80
60
40
(•×10⁻⁴) cpm / ml
b
60
40
20
(•×10⁻⁷) cells / ml
c
0
20
40
60
80
100
time (minutes)

FIGURE 24. AUTORADIOGRAM OF ENVELOPE PROTEINS FROM A SYNCHRONOUS CULTURE

A synchronous culture was pulse labelled for 5 min. at intervals with [^{14}C] leucine. Envelopes were prepared from the sample, resuspended in 0.2 ml electrophoresis buffer E and 25 µl aliquots subjected to electrophoresis


band number
25-
20-
15-
10-
7-
DYE-
-76,000 D
-56,000 D
-36,500 D
-18,000 D
molecular weight
20
40
60
80
time (minutes)

initiated at about the time of cell division, and termination of rounds of replication occurs approximately 20 mins. before division.

Fig. 24 shows an SDS-PAGE autoradiograph of envelope samples taken from a synchronous culture and data obtained from an analysis of two protein bands in this figure is presented in Fig. 25. In addition, in Fig. 25 shows the results of measurements of the synthesis of phospholipid and of bulk envelope proteins in this experiment. As can be seen, phospholipid synthesis is again found to be increasing continuously and exponentially throughout the cycle, whilst bulk envelope protein behaves differently. In fact, bulk envelope protein is accumulated at a constant rate which doubles early in the cycle at a time which corresponds approximately to the middle of rounds of replication (see Fig. 23). This is essentially the same result as that obtained by membrane elution. When the synthesis of the individual proteins as derived from the autoradiograms is determined, it appears that the rate of accumulation of material in each individual protein band also doubles at a discrete time in the cycle. However, no trebling of the apparent rate of synthesis of material in any one band was observed in contrast to the results of experiments utilizing the membrane elution technique. This lends support to the hypothesis that some of the proteins are lost from the membrane at discrete time which possibly coincides with the doubling in rate of synthesis. In this case the membrane elution method will always over estimate the actual rate of synthesis.

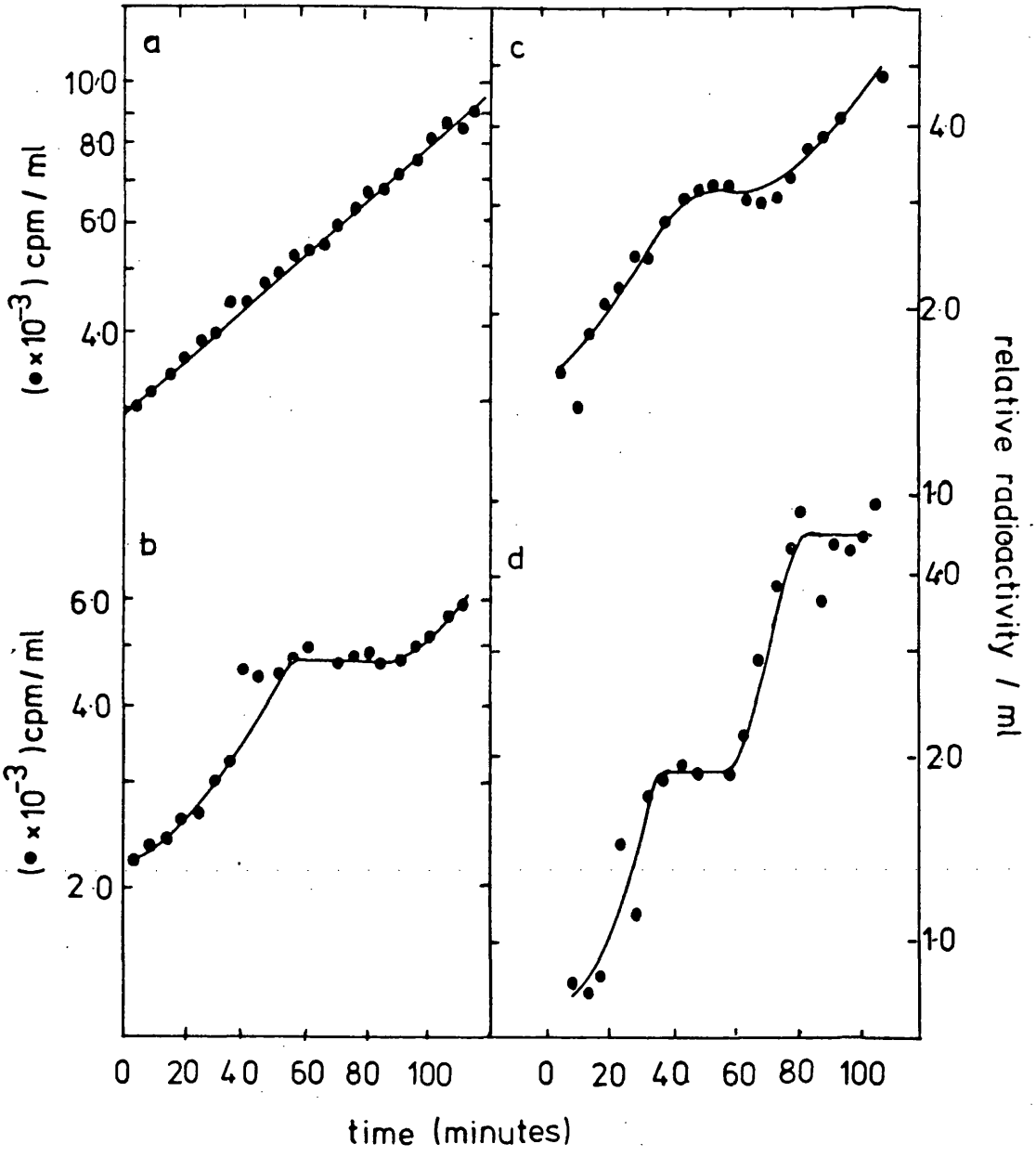
FIGURE 25. RATE OF SYNTHESIS OF ENVELOPE COMPONENTS

a) Phospholipid : the synchronous culture was pulse labelled at intervals for 5 mins. with 1 μCi 2-[^{3}H] glycerol (1 μCi/μg)

b) Envelope protein : the synchronous culture was pulse labelled at intervals for 5 mins. with 1 μCi [^{14}C] leucine (311 mCi/mmol)

c) 36,500 D protein taken from autoradiogram shown in Figure 24

d) Band 20 taken from autoradiogram shown in Figure 24

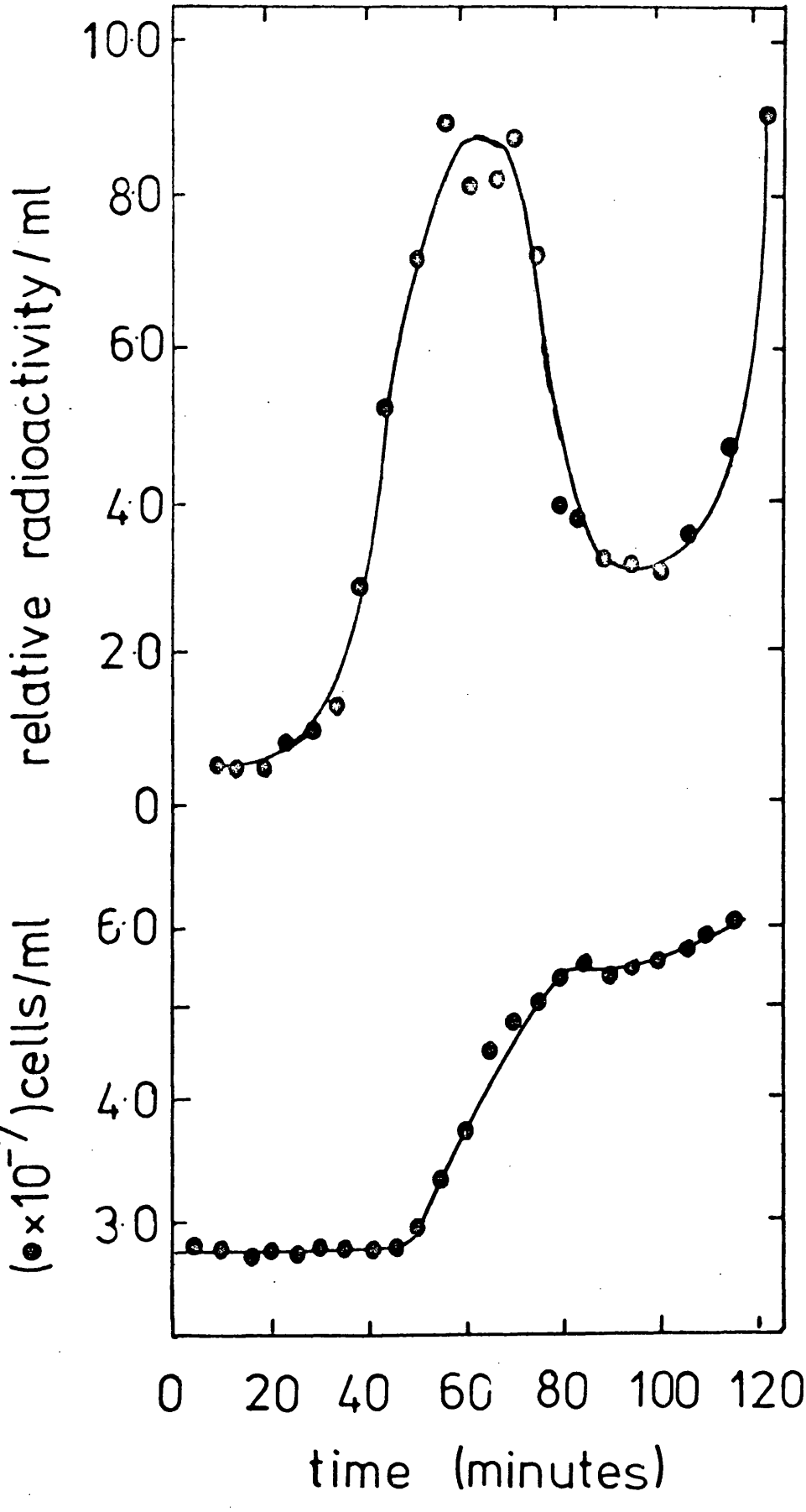

a
b
c
d
10·0
8·0
6·0
4·0
2·0
(• ×10⁻³) cpm / ml
(• ×10⁻³) cpm / ml
4·0
2·0
1·0
4·0
2·0
1·0
relative radioactivity / ml
0 20 40 60 80 100
0 20 40 60 80 100
time (minutes)

Returning to consideration of the results shown in Fig. 24 the most obvious aspect is the appearance of a major new band of 76,000 D at about 40 mins. This band then declines in amount only to reappear again in the second cycle. The timing of the appearance of this band during the cell cycle has been confirmed in several separate experiments and appears to correlate most closely with termination of rounds of replication. This is most clearly shown in Fig. 26. It should be noted that a second apparently "periodic" protein band, just below the 36,500 D band in Fig. 26, is an artefact, as it has only ever been observed once and was coincident with a similar band in the stained profile on this occasion.
The dramatic periodicity of the 76,000 D band does not appear in the stained profile.

There are three possible explanations for the behaviour of the 76,000 D, or periodic protein: either it is made continuously throughout the cycle, but only associates with the envelope at one specific time; or it's synthesis and insertion into the envelope is truly periodic; or that it is only made at one time in the cycle but associates with the envelope at a different time. In relation to the properties of this protein it should be recalled that such a protein is not observed in the membrane elution experiments. This latter fact strongly suggests that the protein may be rapidly lost from the envelope and hence, missed in those earlier experiments. Consequently, attempts were made to elucidate the mode of synthesis insertion and stability

FIGURE 26. RATE OF SYNTHESIS OF PERIODIC PROTEIN

Data taken from the autoradiogram shown in Figure 24


100
80
60
40
20
0
relative radioactivity / ml
6·0
4·0
3·0
(•×10⁻⁷) cells/ml
0
20
40
60
80
100
120
time (minutes)

of this protein in the bacterial envelope by a series of pulse chase experiments and these are described in Chapter 6.

The periodic protein appears in the position occupied by bands 25 and 26 of the stained envelope profile. It is impossible to estimate accurately the molecular multiplicity per cell of this, or any other protein, on the basis of the percentage of leucine incorporated into this polypeptide since the leucine content of the individual bands is not known. However, an approximate estimate would be 10,000 molecules, and from Table 3 it can be seen that bands 25 and 26 each comprise some 1.5% of the total envelope protein on the basis of their affinity for Coomassie brilliant blue. (This affinity may also vary for different proteins, but is assumed to be a fair estimate of the amount of protein present in a band). Therefore, it is possible to estimate from the curve in Fig. 16 that in an exponential culture there are on average 3,000 molecules of polypeptides 25 and 26 per cell. If it is assumed that one of these polypeptides is the periodic protein then obviously, if only a fraction of the cells in an exponential culture at any one time, contain this protein, then these numbers, per cell, will be correspondingly increased. The high molecular multiplicity of this protein indicates that it is more likely to perform a structural rather than a catalytic or regulatory function in the cell. In fact, it is a drawback of the current gel analytical technique, that given the level of detection of polypeptides shown

in Fig. 16, the synthetic pattern of many of the potentially most interesting proteins, present in very few copies per cell, may not be detectable.

I have also measured the activity of succinate dehydrogenase during the cell cycle of E.coli B/r. The result is shown in Fig. 27, and it can be seen that the activity increases continuously throughout the cycle. Assuming that the specific activity of the enzyme remains constant, this finding is not consistent with the mode of periodic synthesis of this enzyme claimed for B.subtilis (Sargent, 1973). A possible explanation for this inconsistency is that under different nutritional conditions quite different modes of synthesis can occur, since in Sargent's study bacteria were grown on glucose as carbon source.

Finally, I wish to conclude that in synchronous cultures of E.coli B/r the rate of synthesis of phospholipid increases continuously throughout the cycle, whilst the rate of envelope protein accumulation is linear with discrete doublings in rate occurring early in the cycle.

FIGURE 27. SUCCINATE DEHYDROGENASE ACTIVITY IN SYNCHRONOUS CULTURES

1.0 ml samples from a synchronous culture were added to 0.2 ml chloramphenicol (500 µg/ml final concentration) and sonicated. 0.5 ml of each sample were assayed for enzyme activity

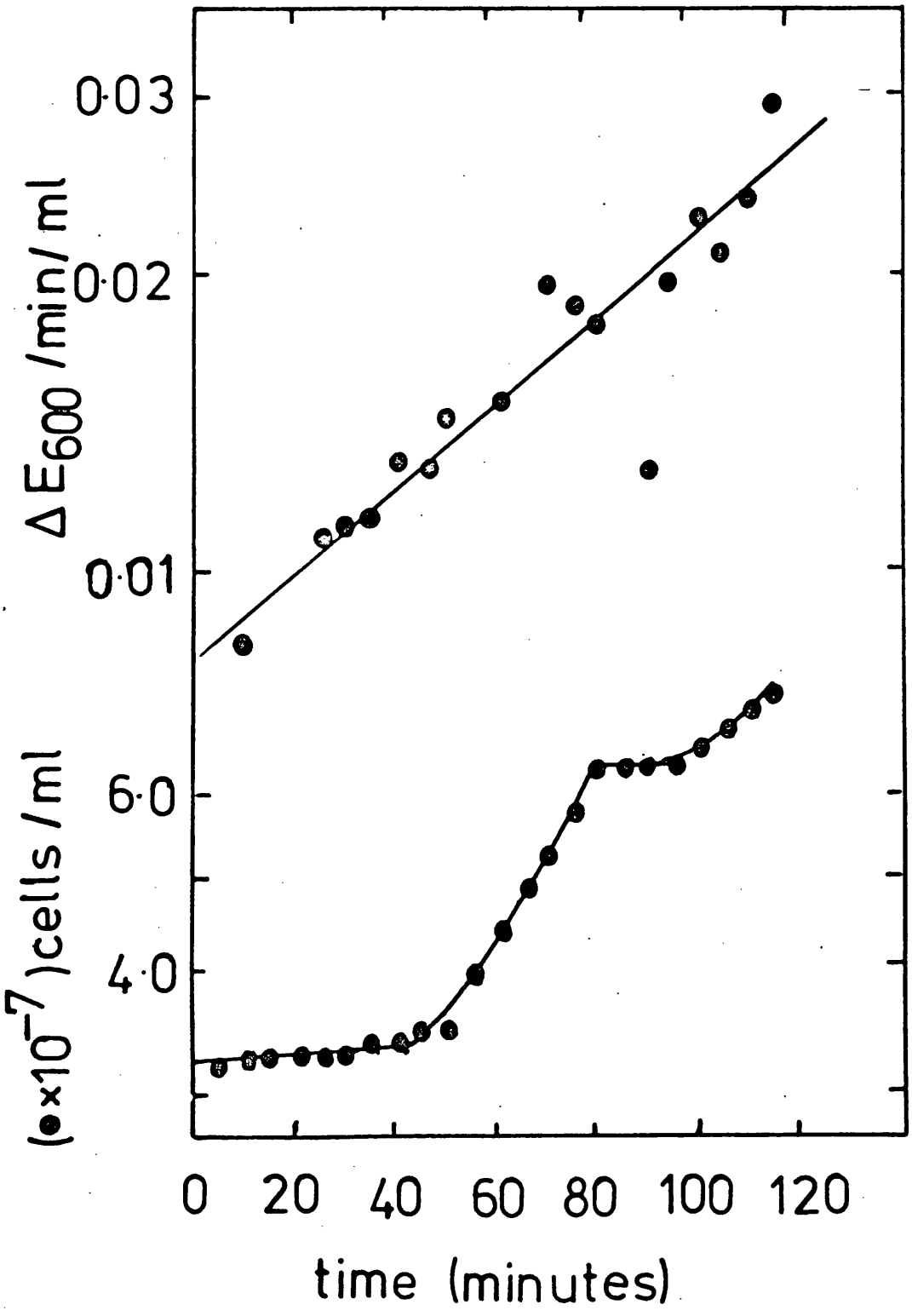
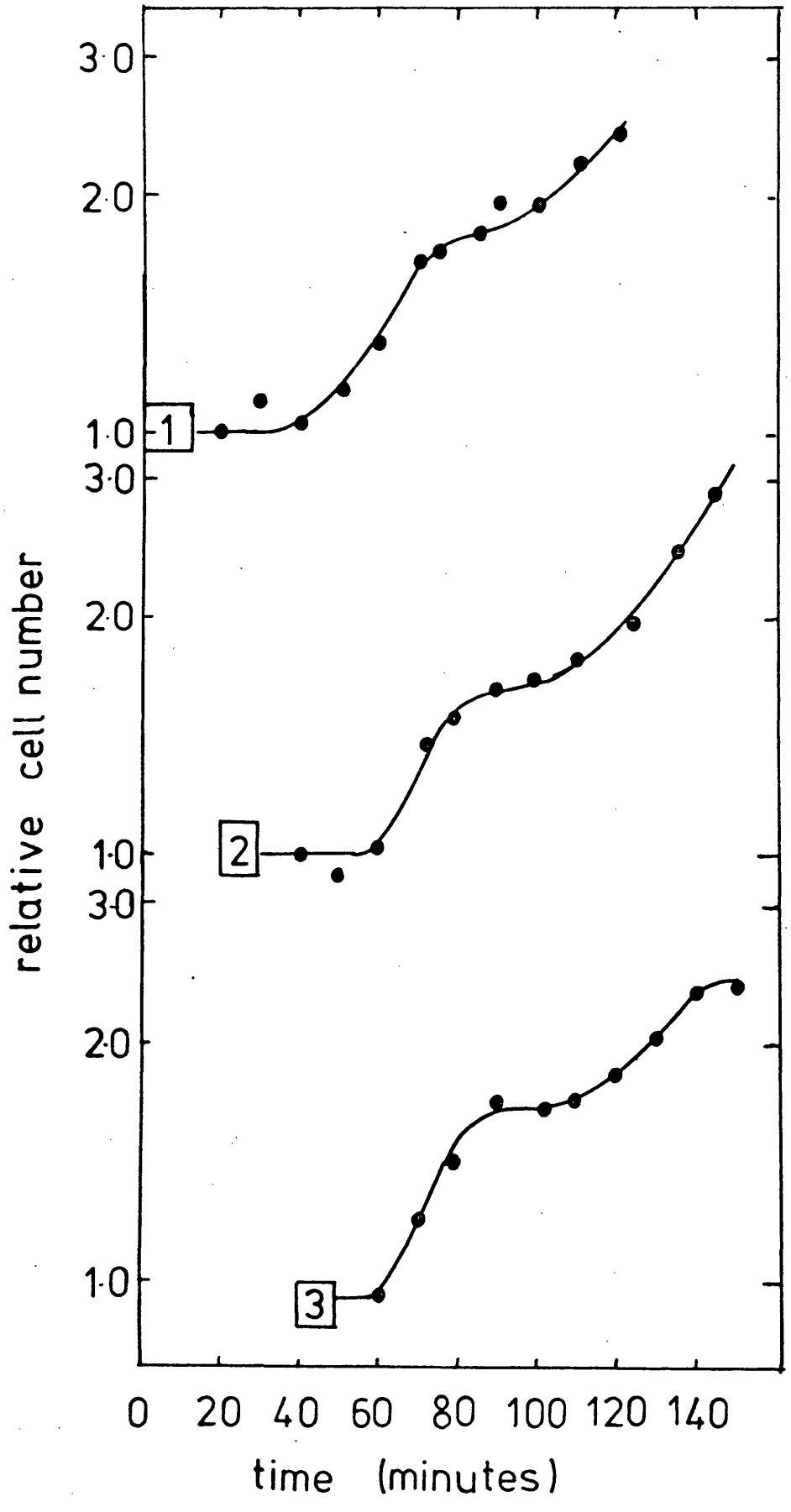

0·03
0·02
0·01
ΔE600 /min/ ml
6·0
4·0
(●×10⁻⁷) cells /ml
0
20
40
60
80
100
120
time (minutes)

FIGURE 28. TIME OF PULSE DURING PULSE-CHASE EXPERIMENTS

The timing of pulse labelling with [^{14}C] leucine and subsequent growth of the synchronous cultures are indicated. Pulse labelling periods were of 10 mins. duration


3·0
2·0
1·0
1
3·0
2·0
1·0
2
3·0
2·0
1·0
3
relative cell number
0 20 40 60 80 100 120 140
time (minutes)

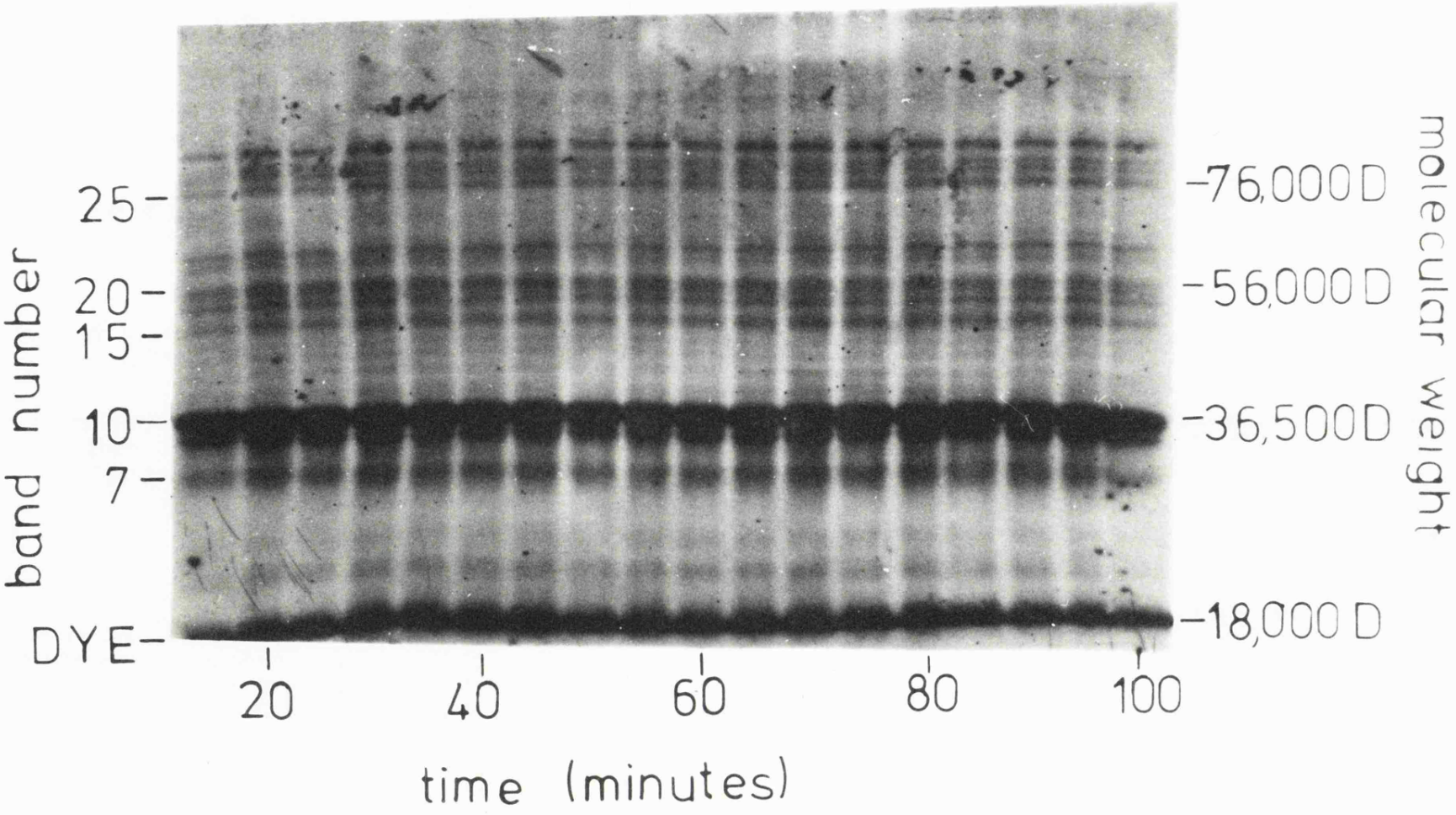
CHAPTER 6. LOSS OF PROTEINS FROM ENVELOPES ISOLATED FROM SYNCHRONOUS CULTURES

In the previous two chapters evidence has been presented which suggests that the accumulation of envelope protein proceeds at a constant rate which doubles at a particular time during the cycle. Moreover, some of the data suggested that at this same time in the cycle there is an efflux of some proteins from the membrane. In addition, a specific protein was observed to appear periodically in the envelope whose proportion indicated that it may also be rapidly dissociated from the membrane at certain times in the cycle. In an attempt to investigate these phenomena further the series of experiments described in Fig. 28 was performed. Synchronously growing E.coli B/r were pulse labelled at different 10 minute periods of the cell cycle, rapidly washed and allowed to continue growth in unlabelled medium. The data in Fig. 28 indicate that the bacteria continue to divide synchronously after such treatment, though in some cases division is slightly delayed. Envelopes were isolated at different times and the radioactivity determined in whole envelopes and in individual protein bands obtained after fractionation by SDS-PAGE.

An autoradiogram of samples taken from a culture labelled just after birth (period 1, Fig. 28) is shown in Fig. 29. It should be noted particularly, that there is no sudden appearance of bands later in the cycle, during growth in unlabelled medium. This indicates that

FIGURE 29. AUTORADIOGRAPH OF ENVELOPE PROTEINS AFTER PULSE LABELLING DURING PERIOD 1

A synchronous culture was labelled for 10 mins. with 50 µCi [^{14}C] leucine (311 mCi/mmol). The culture was filtered (Millipore 47 mm, 0.45 µm pore size) and resuspended in fresh prewarmed medium. At intervals samples were taken and envelopes prepared. These were resuspended in 0.2 ml electrophoresis buffer E and 25 µl aliquots were subjected to electrophoresis


band number
25
20
15
10
7
DYE
20
40
60
80
100
time (minutes)
76,000 D
56,000 D
36,500 D
18,000 D
molecular weight

all the radioactive protein bands observed by SDS-PAGE are composed of material both synthesised and inserted into the envelope at the time of the pulse. The pulse labelling technique used in this study therefore apparently truly measures the rates of synthesis, of envelope proteins and is not merely measuring accumulation of protein within the envelope (see Chapter 4). Since in Fig. 29, there is no appearance of a prominent band at 76,000 D, this indicates that the periodic protein is neither synthesised continuously through the cycle, nor is it synthesised at some time significantly earlier than the time at which it is eventually seen to associate with the envelope. In fact, as shown in Fig. 30, this protein is only labelled when cultures are exposed to radioactivity during period 3 (see Fig. 28).

The behaviour of the bulk and of various individual classes of envelope proteins in a pulse chase experiment, for a culture labelled during period 1 (Fig. 28), are summarised in Fig. 30. The results obtained for the bulk envelope protein show that during growth in unlabelled medium only a small loss of protein from the envelope occurs, perhaps 15% per generation. There is virtually no loss of the class of protein which shows a doubling in rate of synthesis as detected by membrane elution e.g. the major, 36,500 D protein. However, the class of proteins, previously shown to treble in rate of synthesis in membrane elution experiments and which comprise some 50% of the total envelope protein, show a quite high rate of loss from the envelope. This is approximately one

FIGURE 30. LOSS OF PROTEINS FROM ENVELOPES

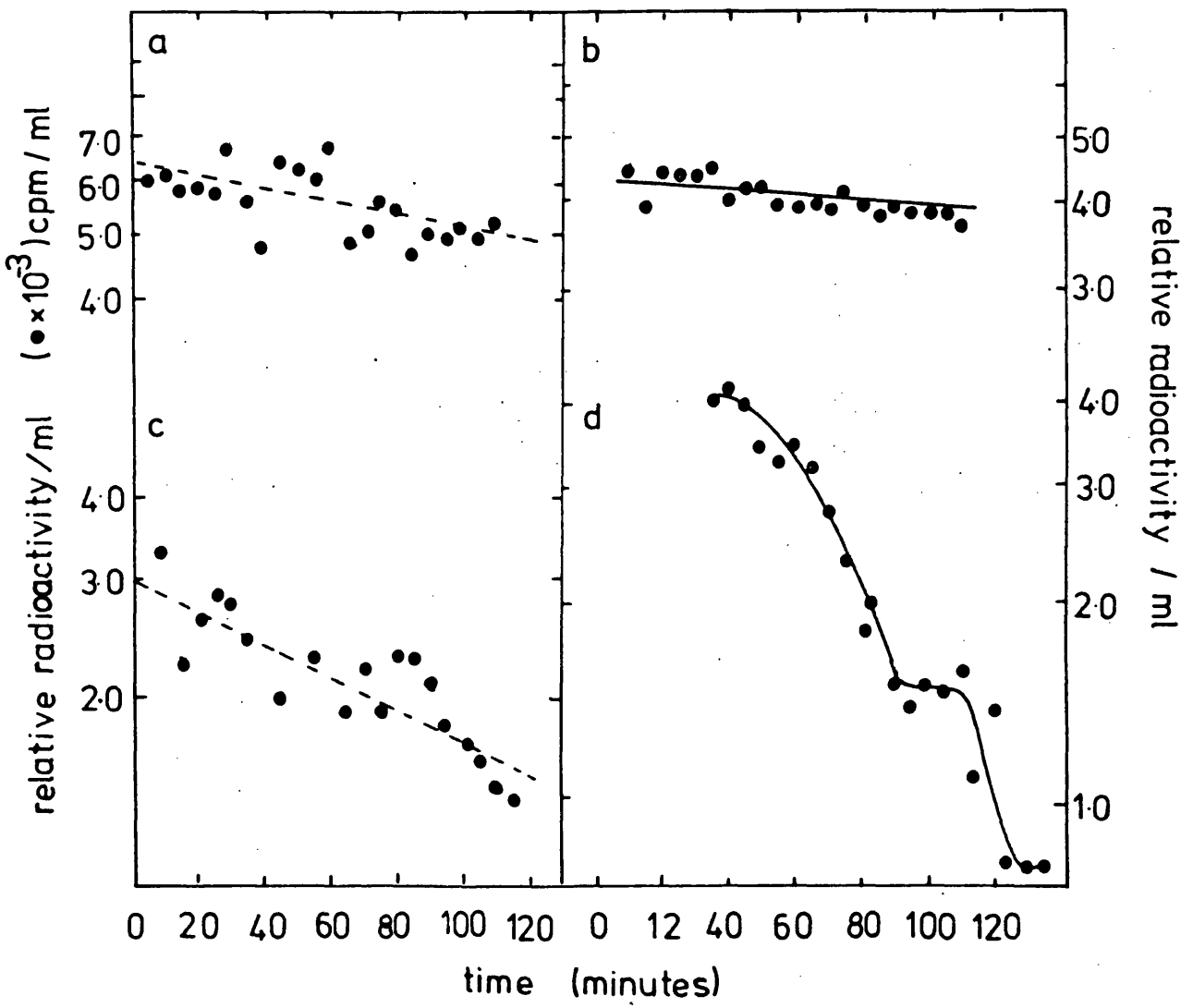
Results are taken from the experiment described in Figure 29.

a) total envelope protein

b) 36,500 D protein

c) Band 20

d) 76,000 D periodic protein


a
b
c
d
7·0
6·0
5·0
4·0
4·0
3·0
2·0
50
40
30
40
30
20
10
relative radioactivity/ml (•×10⁻³)cpm/ml
relative radioactivity / ml
0 20 40 60 80 100 120
0 12 40 60 80 100 120
time (minutes)

third per generation, accounting for most if not all of the loss observed for the bulk envelope protein. If this loss were occurring at a discrete time in the cycle, coincident with the doubling in rate of synthesis of these proteins, this would certainly explain the apparent trebling in rate of synthesis observed in the membrane elution experiments (see Chapter 4 and Fig. 19). Unfortunately, as indicated in the Fig. 30, the data are ambiguous on this point. However, the two observations, high rate of loss from the envelope, and an apparent trebling in rate of synthesis detected by membrane elution, enable the conclusion to be drawn that some proteins are lost from the envelope at a time which approximately coincides with their increase in rate of synthesis.

Data is also presented in Fig. 30 on the loss of the 76,000 D, periodic protein from the envelope. This data was obtained from a culture pulse labelled during period 3 (Fig. 28). As can be seen the loss of this protein from the envelope is rapid and extensive, and this affords a ready explanation for the absence of this protein in gel profiles taken from membrane elution experiments.

I therefore conclude that the synthesis of envelope protein proceeds at a constant rate which doubles at a discrete time during the cell cycle in E.coli B/r. Certain proteins from the cell envelope are lost during the cycle, probably at a discrete time, which at the growth rate employed in these studies, is coincident with

the doubling in rate of synthesis of these proteins. There is also a periodic protein of molecular weight 76,000 D which is synthesised only at a certain time and associates transiently with the cell envelope. The timing of synthesis of this protein is apparently coincident with the time of termination of rounds of DNA replication.

CHAPTER 7. THE RELATIONSHIP BETWEEN ENVELOPE PROTEIN SYNTHESIS AND DNA REPLICATION

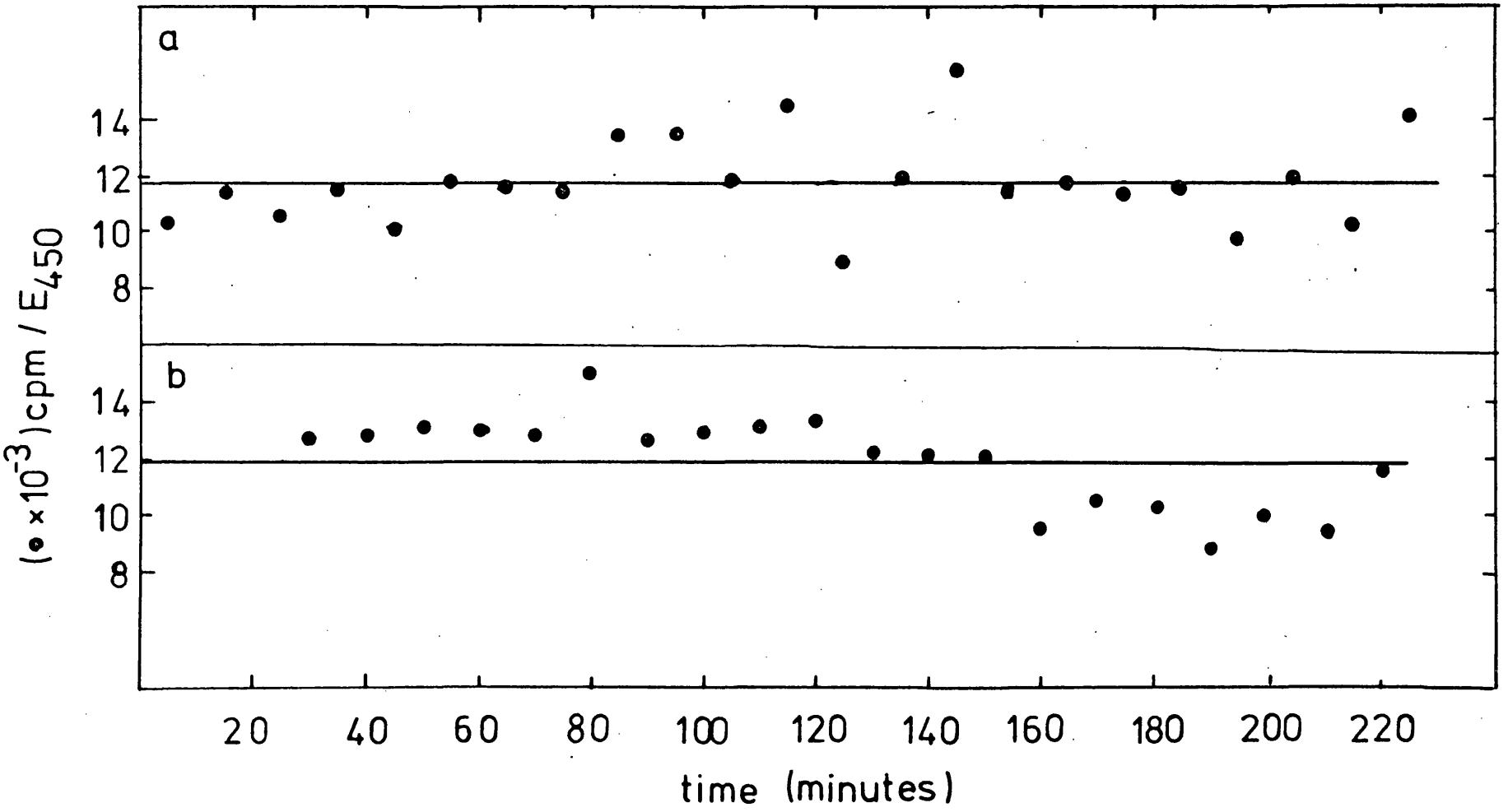
In view of the demonstration of a coupling between cell division and DNA replication, and the formulation of explanations which predict a coupling between surface growth and DNA replication, (see Chapter 1) two experiments were performed in an attempt to demonstrate such a relationship i.e. between envelope protein synthesis and replication in E.coli B/r. Experiments described in chapters 4 and 5 have shown that envelope protein synthesis proceeds at a constant rate which then doubles at about the middle of the DNA replication cycle. One possible explanation for this pattern of synthesis is that a rate limiting element, for example, an RNA polymerase subunit, is synthesised constitutively: i.e. at a constant rate per gene copy. Consequently, when the gene in question is replicated, the rate of synthesis of the gene product will double. Moreover, the amount of gene product will be proportional to the gene concentration (gene copies per unit mass) of its structural gene (Chandler & Pritchard, 1975). Since, by decreasing the replication velocity, it is possible to alter the gene concentration of all genes, apart from those at the chromosome origin, by an amount which depends upon their position on the chromosome, it should be possible to determine the location of such a hypothetical gene on the chromosome. Finally, since it is proposed that the gene product is the rate limiting element in envelope protein synthesis, the location of

FIGURE 31. EFFECT OF THYMINE CONCENTRATION ON ENVELOPE PROTEIN PER MASS RATIO

Two cultures of E.coli B/r LEB16 were grown in the presence of 5 µg/ml [^{14}C] leucine (0.01 µCi/µg) to an E_{450} of 0.1. At intervals samples were taken and chloramphenicol and leucine added (300 µg/ml and 2 mg/ml final concentration respectively) and envelopes were prepared. At the same time the E_{450} of the cultures was measured, and the envelope protein per mass ratio was determined.

a) 20 µg/ml thymine

b) 2 µg/ml thymine


a
b
14
12
10
8
14
12
10
8
(• x10-3) cpm / E450
20
40
60
80
100
120
140
160
180
200
220
time (minutes)

the gene may be determined by measurement of the amount of envelope protein present per unit mass, when the replication velocity is altered at different thymine concentrations (Chandler & Pritchard, 1975).

The results of an experiment designed to test these possibilities is shown in Fig. 31. Cultures of E.coli B/r LEB16, growing in two different concentrations of thymine, were fully labelled with ^{14}C-leucine for several generations. Envelopes were prepared from samples taken at successive intervals of time and the radioactivity in each sample was determined. Meanwhile, total cell mass was determined by measuring the E_{450} for each sample. After successive generation times the cultures were diluted two-fold into fresh prewarmed medium in order to maintain balanced growth at low optical density. The replication times for the two cultures have been determined as 41 mins. and 68 mins. for growth in 20 ug/ml and 2 ug/ml thymine respectively (Meacock & Pritchard, 1975). In the low thymine medium the concentration of genes near the terminus should show a 36% reduction as described by Chandler & Pritchard (1975) and for a gene half way along the chromosome it can be calculated that the concentration should be reduced by about 14% under these conditions.

Reference to Fig. 31 on the contrary shows that essentially the same envelope protein per mass ratio was obtained, irrespective of the replication velocity. This result indicates that either envelope protein synthesis is not directly coupled to the replication of a specific

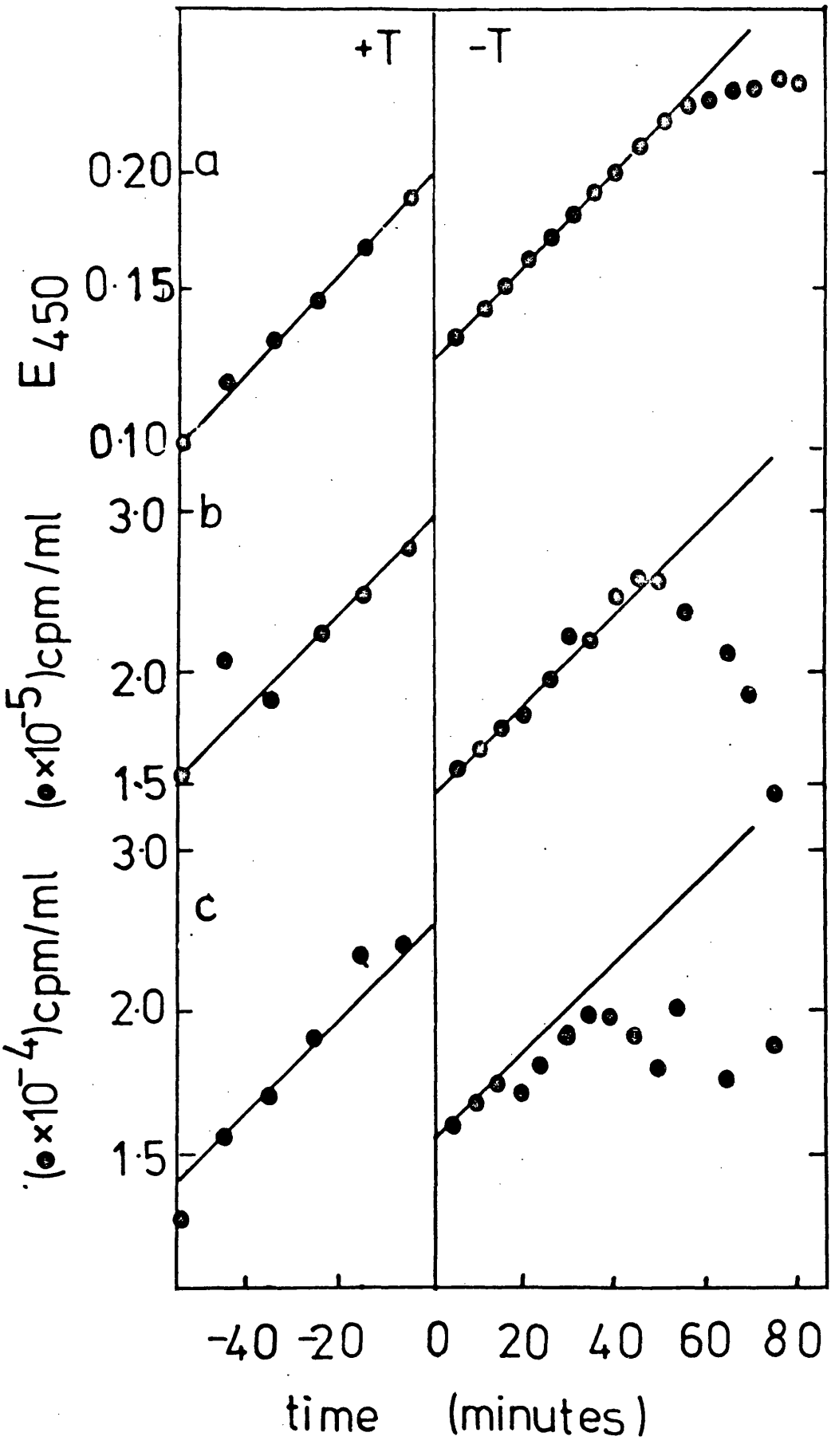
FIGURE 32. EFFECT OF THYMINE STARVATION ON THE RATE OF ENVELOPE PROTEIN SYNTHESIS

A culture of LEB16 was grown to an E_{450} of 0.1. After one generation thymine was removed from the culture. The rate of total and envelope protein synthesis were followed.

a) Extinction at 450 nm

b) Total protein synthesis

c) Envelope protein synthesis

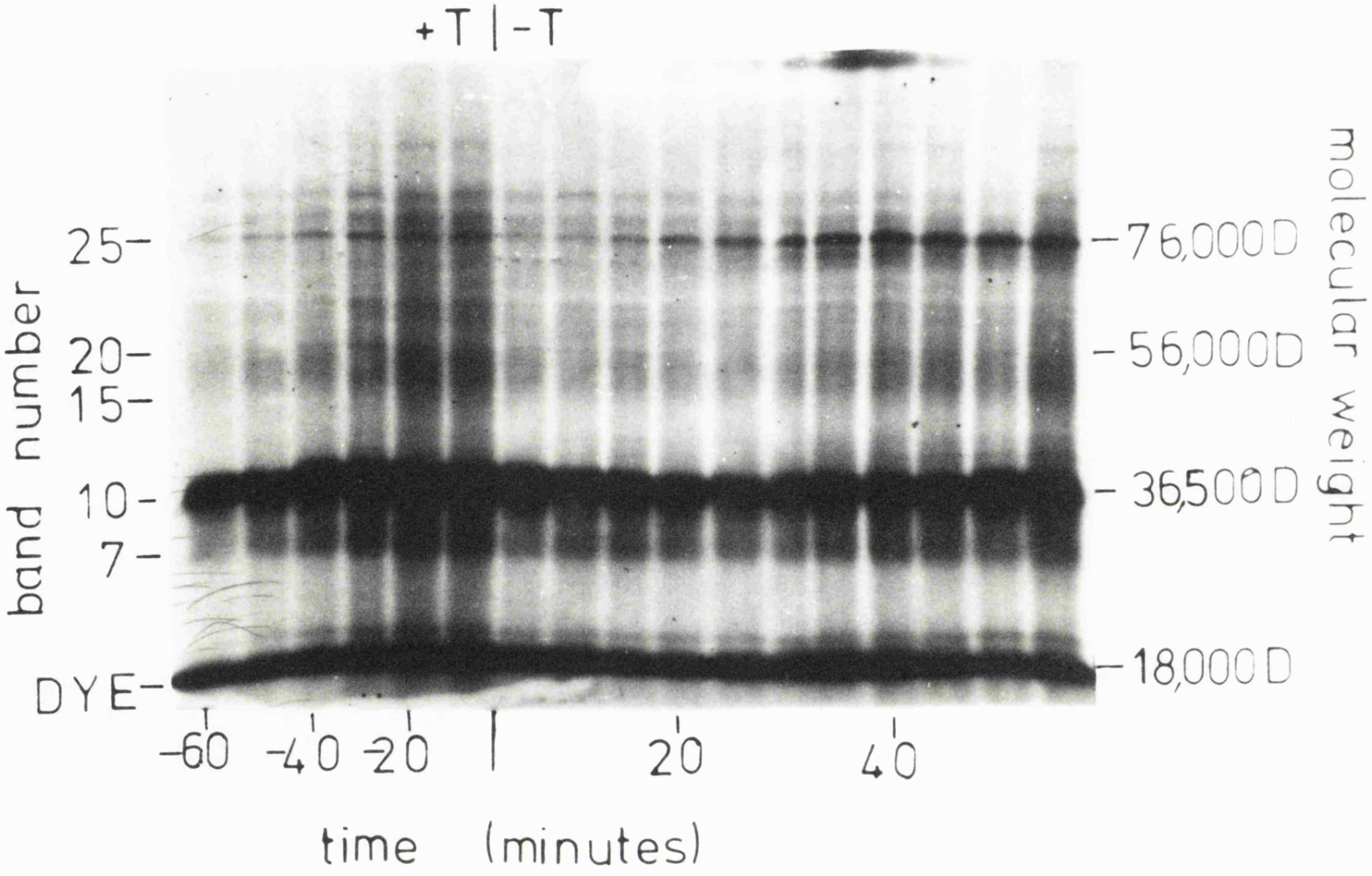

+T
-T
a
b
c
0·20
0·15
0·10
3·0
2·0
1·5
3·0
2·0
1·5
E450
(•×10⁻⁵)cpm/ml
(•×10⁻⁴)cpm/ml
-40
-20
0
20
40
60
80
time (minutes)

gene or that such a gene is too close to the origin for a change in its output to be detected by this technique. This result is however further evidence that, whatever the basis for the linear rate of synthesis of envelope proteins, it is not coupled in a simple way to termination of rounds of DNA replication.

An alternative approach to the problem is to measure the effect of an imposed cessation of DNA synthesis upon envelope protein synthesis. If the rate of envelope protein synthesis is regulated in some manner similar to that discussed above, then after an immediate stop in DNA synthesis, the rate of synthesis of envelope proteins should remain constant. The results of a preliminary experiment to test this hypothesis are presented in Fig. 32. The figure shows that the predicted result is not obtained and in fact the rate of synthesis of envelope protein continues to increase after removal of thymine. This result is also in contrast to that obtained by Sargent (1975b) who showed that the rate of envelope protein synthesis in B.subtilis does remain constant after immediate inhibition of DNA synthesis. This may indicate either that envelope protein synthesis in the two organisms is controlled differently or that the data in Fig. 32 must be considered inconclusive. Individual samples from the experiment shown in Fig. 32 were subjected to SDS-PAGE and autoradiography. The autoradiogram obtained is shown in Fig. 33. Whilst the quality of this gel profile is not sufficiently high to draw meaningful conclusions concerning most individual protein bands, the

FIGURE 33. AUTORADIOGRAPH OF ENVELOPE PROTEINS FOLLOWING THYMINE STARVATION

Envelope samples from the experiment described in Figure 32 were subjected to SDS-PAGE followed by autoradiography.


+T | -T
band number
25-
20-
15-
10-
7-
DYE-
-76,000 D
-56,000 D
-36,500 D
-18,000 D
molecular weight
-60
-40
-20
20
40
time (minutes)

most obvious effect of thymine starvation is that, after a short lag, the synthesis of a protein of 76,000 D molecular weight is specifically induced. As shown in Fig. 34, the rate of synthesis of this polypeptide increases rapidly until after 35 mins. it constitutes 10-15% of the total envelope protein being synthesised at this time. When samples containing this protein are analysed with envelopes containing labelled periodic protein, the molecular weights are indistinguishable, (Fig.35) indicating that the two proteins are probably identical.

In concluding this section it is impossible to say that envelope protein synthesis is regulated by the output of a constitutive gene on the basis of the available evidence. The observation that thymine starvation apparently stimulates the synthesis of the 76,000 D periodic protein is obviously very interesting and will be the subject of further investigation.

FIGURE 34. APPARENT INDUCTION OF PERIODIC PROTEIN SYNTHESIS FOLLOWING THYMINE STARVATION

Data taken from the autoradiogram shown in Figure 32

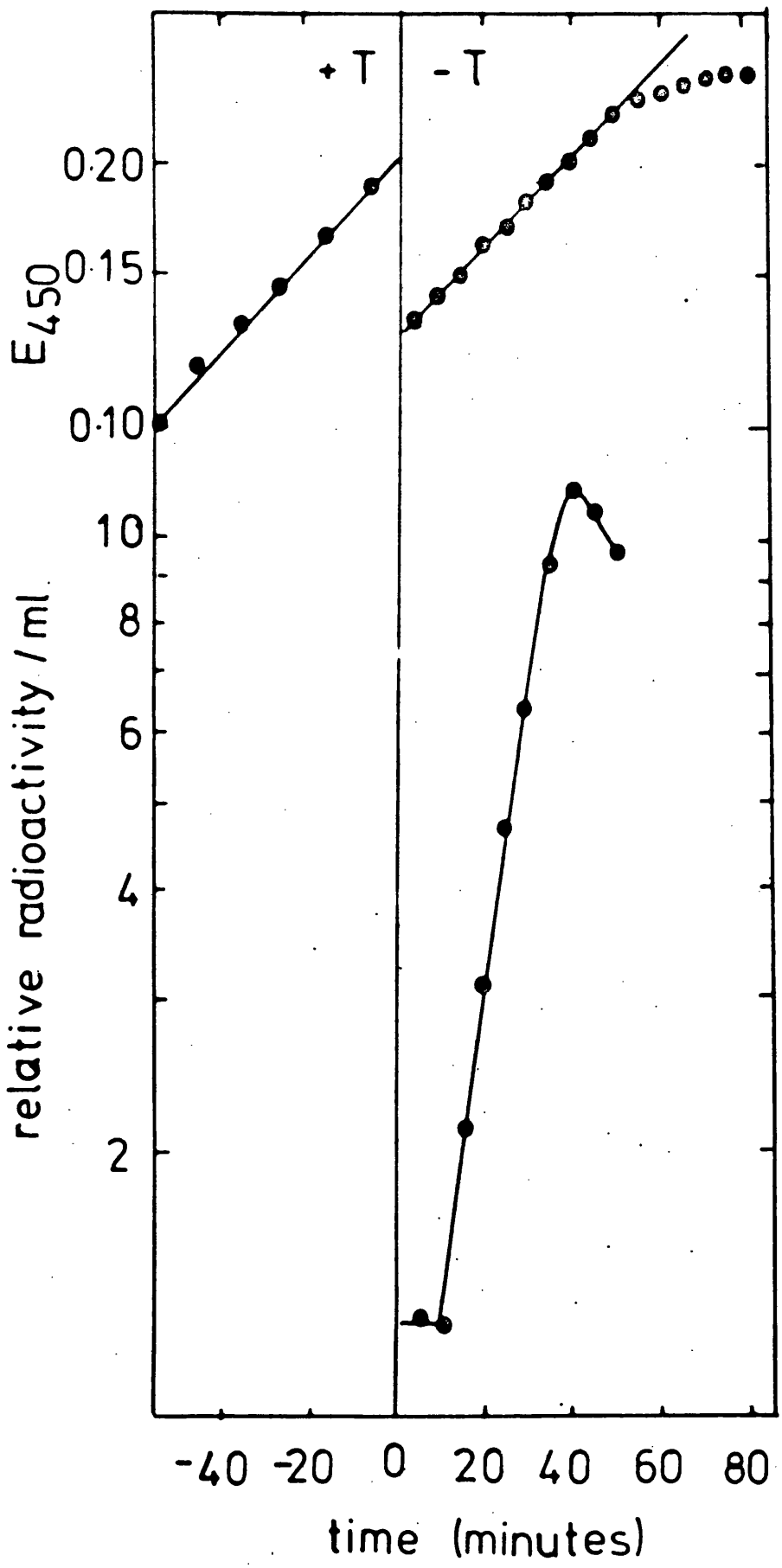

+ T
- T
0·20
0·15
0·10
E_{450}
10
8
6
4
2
relative radioactivity / ml
-40
-20
0
20
40
60
80
time (minutes)

FIGURE 35. ELECTROPHORESIS OF OUTER MEMBRANES PREPARED BY ISOPYCNIC CENTRIFUGATION AND SARKOSYL TREATMENT

a) The gel shows samples from the experiments shown in Figure 32 and from pulse-chase experiments performed after labelling of synchronous cultures during periods 3 and 1 (see Figure 28).

b) Autoradiogram of the gel

1) sample from thymine starvation experiment (Fig. 32)

2) sample from culture pulse labelled during period 1 (Fig. 28)

3) sample from culture pulse labelled during period 3 (Fig. 28)

4) total envelope

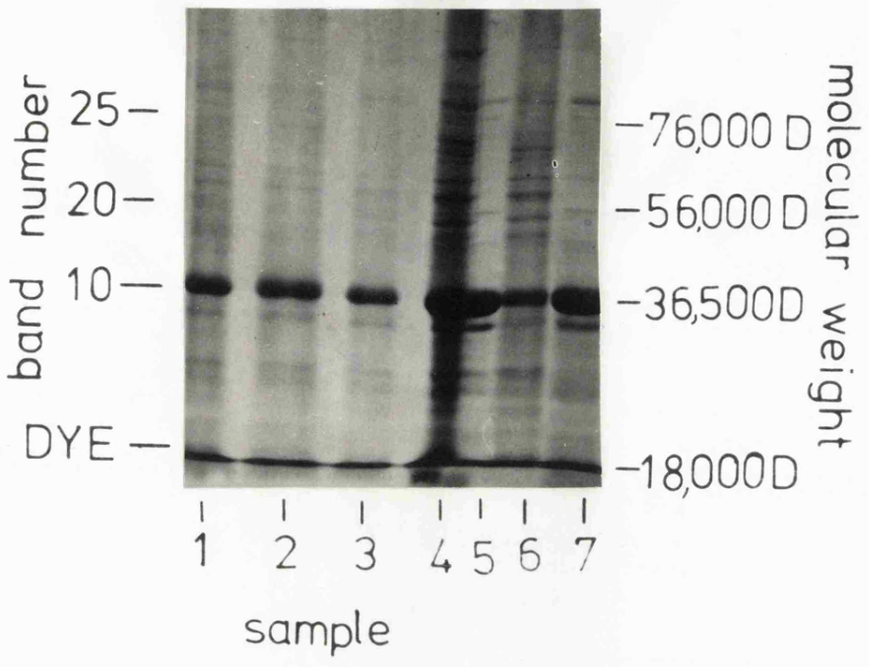
5) Sarkosyl pellet

6) inner membrane (L2)

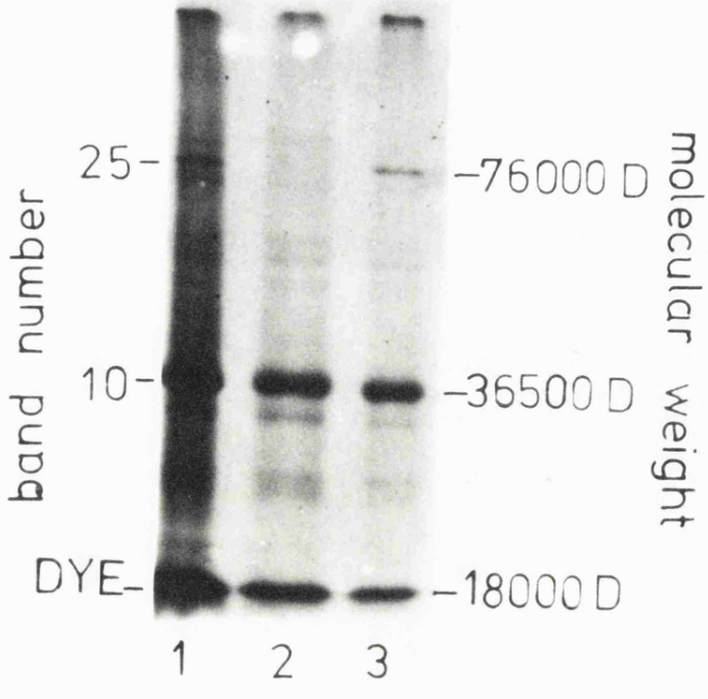
7) outer membrane (H)

6&7 isolated by isopycnic centrifugation in sucrose

a


band number
25
20
10
DYE
76,000 D
56,000 D
36,500D
18,000D
molecular weight
1 2 3 4 5 6 7
sample

b


band number
25
10
DYE
76000 D
36500 D
18000 D
molecular weight
1 2 3

CHAPTER 8. DISCUSSION

As stated in chapter 1, the aim of the present study was to attempt to resolve the confusion, surrounding the pattern of synthesis of envelope components in the bacterial cell cycle, by using improved techniques of age fractionation and analysis. In fulfilling this objective at least the present study has proved successful. However, the elucidation of the regulatory mechanism underlying the observed pattern of synthesis remains a major problem, virtually no progress having been made in this direction.

The first conclusion I wish to draw is that as shown in Fig. 20 and 25, the rate of phospholipid synthesis increases continuously and exponentially throughout the division cycle in E.coli B/r. This is in agreement with the result of Sargent (1973) with B.subtilis but is in disagreement with those obtained by Daniels (1969) with both E.coli and B.megaterium. In addition, current work in the laboratories of Helmstetter and Messer (personal communications) indicate that either continuous, exponential, or stepwise; that is, linear patterns of phospholipid synthesis may be observed depending upon variations in the procedure adopted for measurement of 2-[^{3}H] glycerol incorporation. The basis for this variation is not understood at this time. However, 2-[^{3}H] glycerol is presumed to be a specific label for phospholipid (Daniels, 1969) and experiments carried out in this laboratory (data not shown and A. Boyd

personal communication) and by Sargent (1973) indicate that estimation of 2-[^{3}H] glycerol by precipitation of whole cells with ice-cold 5% w/v TCA provide a true measure of incorporation of precursor into phospholipid. Finally, it should be noted that the experiment performed by Daniels (1969), which involved pulse labelling an exponential culture prior to age fractionation by sedimentation on a sucrose gradient, is especially difficult to interpret, since his data reveal a four to five-fold variation in the size of cells recovered from the gradient. A two-fold variation only would be expected.

The second conclusion I wish to draw is that, as shown in Fig. 20 and 25, the rate of total envelope protein synthesis proceeds at a constant rate which doubles at a discrete time in the division cycle. This observation is in agreement with Sargent's claim that a similar pattern may be observed with B.subtilis (Sargent, 1973) although the degree of synchrony obtained with this latter organism is inferior to that obtained by the methods used in this study. I find no evidence that substantial numbers of individual envelope polypeptides are synthesised only at a certain time in the division cycle as has been reported by Shen & Boos (1973) for periplasmic proteins, Ryter et al. (1975) for the λ receptor protein and by Ohki (1972) for several membrane proteins. It is difficult to draw meaningful conclusions with regard to Ohki's work, since the method employed only synchronised DNA replication and not cell mass. With regard to the studies by Shen & Boos and by Ryter et al. on the levels of specific enzymes or

minor protein species, it should be pointed out that the limit of resolution of the technique employed in this study is strictly limited to the measurement of proteins present in the minimum number of copies per cell shown in Fig. 16. However, I have measured the activity of succinate dehydrogenase (Fig. 27) and find that the amount of this enzyme also increases continuously throughout the division cycle.

The experiments described in Fig. 22 and 30 considered together indicate that at a certain time in the cycle some proteins are lost from the cell envelope. This effect is not apparently confined exclusively to either inner or outer membrane proteins. Such a phenomenon has also been observed by Sargent (1973) in B.subtilis. It is not clear what this loss of protein represents. It may be due to actual turnover of some envelope proteins, or perhaps "remodelling" when new growth sites are inserted into the envelope.

The experiments described in this thesis show that the doubling in rate of synthesis of envelope proteins occurs early in the cycle coincident with the middle of rounds of DNA replication. This result is quite contrary to the notion that termination of rounds of replication is coincident with the observed doubling in rate of synthesis. On the other hand, Sargent (1975b) has claimed that in B.subtilis, the apparent doubling in rate of envelope synthesis is coincident with the termination of rounds of replication, although the data presented are not unambiguous. I should like to propose that the

synthesis of envelope protein is not in fact involved in the timing of cell division in E.coli B/r. If the synthesis of envelope protein is nevertheless linked in some way to the duplication of a particular gene, and there is some evidence that this is so in B.subtilis (Sargent, 1975b), it is a reasonable expectation that the synthesis of envelope protein in E.coli would double at the time observed in this study. This is because measurements of surface area (and hence presumably of at least some envelope components) per unit mass, as a function of replication velocity, indicate that surface area behaves as if it were regulated by the doubling of copies of a gene situated about half way along the chromosome (Meacock, 1975). This follows from the fact that as described in chapter 1, cell length, as opposed to surface area, is apparently regulated by termination of rounds of replication in E.coli (Meacock & Pritchard, 1975; Meacock, 1975). Consequently, if the cell is to maintain a constant composition of envelope protein per unit area, when growing with different replication velocities, it would be expected that the doubling in rate of synthesis should occur in the middle of the replication cycle. That this is the case for a range of replication velocities should now be tested.

The question of whether the observed doubling in rate of envelope protein synthesis, is in fact due to the doubling of the copies of a particular gene as assumed above, has of course not yet been settled. In one test of this hypothesis, the results of the experiment

described in chapter 7, involving the relative enrichment of genes proximal to the origin in bacteria growing in low concentrations of thymine, do not support this view. However, it is questionable if a predicted change of 14% in the envelope protein per mass ratio could have been observed. For example, one difficulty in such measurements may result from a factor evident in Fig. 22 and 25. The data there seem to indicate that the increase in rate of synthesis occurs at different times with respect to the two classes of envelope protein described in chapter 4. In fact, as stated previously, the data are somewhat ambiguous on this point, but if half the envelope protein is doubling in rate of synthesis significantly earlier than halfway through the replication cycle, the predicted change in envelope protein per mass would be considerably reduced.

In a second test of this hypothesis, where further gene duplication was prevented by inhibition of DNA synthesis by thymine starvation, the rate of envelope protein synthesis did not remain constant (Fig. 32). Thus the results did not support the simple hypothesis that the rate of envelope protein synthesis is determined by the number of copies of a particular gene. However, there is some reduction in the rate of envelope protein synthesis although the rate of total protein synthesis did continue to increase after thymine starvation. Whilst no definite conclusions should be drawn from this preliminary experiment, the possibility remains open that factors other than gene duplication can in fact

affect envelope protein synthesis. In addition of course it is possible that a specific class of membrane proteins are directly subject to gene control but their behaviour is being masked by the bulk measurements used here.

Whatever the basis of the regulatory mechanism, the actual process which causes the synthesis of envelope protein but not that of total protein to increase abruptly in rate of synthesis, is unknown. There is some evidence that different factors operate in envelope protein synthesis, as distinct from cytoplasmic protein synthesis in E.coli (Lee & Inouye, 1974), but this is difficult to reconcile with the fact that at least some presumed membrane proteins are translated from polycistronic m.RNA molecules along with cytoplasmic proteins. Notwithstanding this possible objection, one obvious possibility is that envelope protein synthesis is controlled at the level of either transcription or translation, and that the rate limiting element is a cofactor for RNA polymerase or a novel initiation factor for translation. In either case it is possible that there are structural peculiarities in envelope protein m.RNA molecules which would account for the observed stability of some outer membrane protein m.RNA molecules (Lee & Inouye, 1974). Alternatively, the insertion of newly synthesised envelope proteins may only occur at discrete sites in the surface, and that the number of such sites doubles at discrete times during the cycle. If insertion and synthesis are coupled, as seems likely, this would imply the operation of some sort of feedback mechanism which stimulates envelope protein synthesis as the area for insertion is suddenly enlarged.

The presence of a periodic protein of molecular weight 76,000 D in the envelope of E.coli B/r has been established. The appearance of this polypeptide apparently coincides with the termination of rounds of replication although this should now be confirmed over a range of growth rates. This polypeptide is not apparently the same as that reported by Jones & Donachie (1973) to be required for cell division. (This has a molecular weight of 40,000 D, Donachie personal communication). Similarly the 76,000 D protein is not the "X" protein described by Inouye & Pardee (1970) which is also induced by thymine starvation. This protein has a reported molecular weight of 48,000 D. The function of the 76,000 D protein is unknown as yet, but it is interesting that it's synthesis is apparently also stimulated by thymine starvation, indicating that it may be synthesised whenever DNA replication stops. In that case it should not be observed in synchronous cultures where replication occurs throughout the cell cycle.

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SUMMARY

In an attempt to elucidate the patterns of synthesis of some envelope components during the bacterial division cycle, the rate of synthesis of phospholipid and envelope proteins has been determined throughout the cell cycle of E.coli B/r.

To facilitate this study, a convenient method of preparation of envelopes was devised which leads to the recovery of approximately half the membranous material of the bacterial envelope. Further characterisation of this material indicates that it is derived randomly from, and is therefore representative of the whole bacterial envelope. In the first instance the membrane elution technique devised by Helmstetter (1967) was employed, and the following results were obtained. The rate of phospholipid synthesis was found to increase continuously and exponentially throughout the division cycle, whilst the rate of envelope protein synthesis was found to be constant throughout part of the cycle which doubled in rate at a discrete time. Further fractionation of envelope proteins by SDS-PAGE showed that the rate of synthesis of approximately half the envelope proteins increased two-fold, whilst that of the other half increased three-fold. Similar results were obtained using synchronous cultures except that all envelope proteins showed a two-fold increase in rate of synthesis. Pulse-chase experiments performed with synchronous cultures showed that the class of proteins that underwent an apparent three-fold increase

in rate of synthesis were also subsequently lost from the envelope at a greatly increased rate. Combining the results of all three sets of experiments it is concluded that, whilst the rate of synthesis of phospholipid increased continuously throughout the division cycle, the rate of envelope protein synthesis remained constant with discrete doublings in rate of synthesis. Some envelope proteins are lost from the envelope, probably also at a discrete time in the division cycle. The experiments performed with synchronous cultures demonstrated the appearance of a protein synthesised at about the time of termination of rounds of replication, which was only transiently associated with the bacterial envelope. Furthermore, the synthesis of this protein was greatly stimulated by inhibition of DNA synthesis caused by removal of thymine from the growth medium. The role this protein plays in the bacterial cell remains unknown.

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