RESISTANCE TO FUSIDIC ACID IN ESCHERICHIA COLI MEDIATED BY THE TYPE I VARIANT OF CHLORAMPHENICOL ACETYLTRANSFERASE

by

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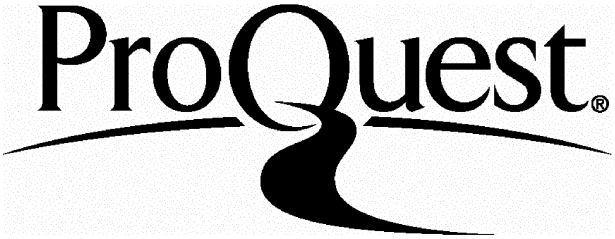

DEDICATION

To Jo and my parents

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ABSTRACT

Plasmid-encoded fusidic acid resistance in Escherichia coli is mediated by a common variant of chloramphenicol acetyltransferase (EC 2.3.1.28), an enzyme which is an effector of chloramphenicol resistance. Resistance to chloramphenicol is a consequence of acetylation of the antibiotic catalysed by the enzyme and the failure of the 3-acetoxy product to bind to bacterial ribosomes. Cell-free coupled transcription and translation studies are in agreement with genetic studies which indicated that the entire structural gene for the type I chloramphenicol acetyltransferase is necessary for the fusidic acid resistance phenotype. The mechanism of resistance does not involve covalent modification of the antibiotic. The other naturally-occurring enterobacterial chloramphenicol acetyltransferase variants (types II and III) do not cause fusidic acid resistance. Steady-state kinetic studies with the type I enzyme have shown that the binding of fusidic acid is competitive with respect to chloramphenicol. The inhibition of in vitro polypeptide chain elongation which is observed in the presence of fusidic acid is relieved by addition of purified chloramphenicol acetyltransferase and equilibrium dialysis experiments with tritiated fusidate have defined the stoichiometry and apparent affinity of fusidate for the type I enzyme. Further binding studies with fusidate analogues, including bile salts, have shown some of the structural constraints on the steroidal skeleton of the ligand which are necessary for binding to the enzyme. Determinations of antibiotic resistance levels and estimates of intracellular chloramphenicol acetyltransferase concentrations support the data from in vitro experiments to give a coherent mechanism for fusidic acid resistance based on reversible binding of the antibiotic to the enzyme.

ABBREVIATIONS

Standard abbreviations used are those recommended by The Biochemical Society in "Policy of the Journal and Instructions to Authors" Biochem. J. (1981) 193, 1 - 27. Some of these and the non-standard abbreviations employed are listed below. The genetic nomenclature is based on Demerec et al. (1966) as modified by Backmann and Low (1980).

A_{260}	Absorbance at wavelength 260 nm.
Acetyl CoA	Acetyl-S-coenzyme A
Amp^R/Amp^S	Resistance/Sensitivity to the sodium salt of D [-]-α- aminobenzyl penicillin
ATP	Adenosine 5'-triphosphate
Bisacrylamide	N, N'-methylene-bis-acrylamide
bp	DNA base pair
BSA	Bovine serum albumin, fraction V
cAMP	Adenosine 3' : 5' - cyclic monophosphate
CAT	Chloramphenicol acetyltransferase
CAT_I	" " type I
CAT_{II}	" " type II
CAT_{III}	" " type III
cat	The gene for chloramphenicol acetyltransferase type I
Cephalosporin P1	6β-acetoxy-7β-hydroxy-11-deoxyfusidic acid
Chloramphenicol	D(-) threo-1-(p-nitrophenyl)-2-dichloroacetamido-1, 3-propanediol
Chloramphenicol base	D(-) threo-1-(p-nitrophenyl)-2-amino-1, 3-propanediol
Cm or Cml	Chloramphenicol

Cm-base	Chloramphenicol base
Cml^R/Cml^S	Resistance/Sensitivity to chloramphenicol
CoA	Coenzyme A-SH
cpm	Counts per minute
CTP	Cytidine 5'-triphosphate
CRP	cAMP receptor protein
dATP	2-deoxyadenosine 5'-triphosphate
dCTP	2-deoxycytidine 5'-triphosphate
DEPC	Diethylpyrocarbonate
dGTP	2-deoxyguanosine 5'-triphosphate
DNAase	Deoxyribonuclease
DTNB	5, 5'-dithiobis (2-nitrobenzoic acid)
DTT	DL - dithiothreitol
EDTA	Ethylenediaminetetraacetic acid, disodium salt
EF-G	Elongation factor G
EF-Ts	Elongation factor Ts
EF-Tu	Elongation factor Tu
Fus^R/Fus^S	Resistance/Sensitivity to sodium fusidate
Fusidate	Sodium salt of fusidic acid unless otherwise implied
Fusidic acid	3α, 11α-dihydroxy-16β-acetoxyfusida-17 (20) - [16,21-cis], 24-dien-21-oic acid
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
GTPase	GTP hydrolysing activity
Helvolic acid	1-dehydro-3-keto-6β-acetoxy-7-oxo-11-desoxyfusidic acid

$I.C_{50}$	The concentration of antibiotic required for 50% inhibition of bacterial colony formation
IgG	Immunoglobulin G
Kan^R/Kan^S	Resistance/Sensitivity to the sulphate of kanamycin
Kb	Kilo base pairs of DNA
MOPS	Morpholinopropanesulphonic acid
M_r	Relative molecular mass (dimensionless)
mRNA	Messenger ribonucleic acid
MW	Molecular weight (daltons)
Nal^R/Nal^S	Resistance/Sensitivity to the sodium salt of nalidixic acid
Pi	Inorganic phosphate (orthophosphate)
psi	Pounds per square inch
RNAase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
Str^R/Str^S	Resistance/Sensitivity to the sulphate of streptomycin
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tet^R/Tet^S	Resistance/Sensitivty to tetracycline
Tris	Tris (hydroxymethyl) aminomethane
tRNA	Transfer ribonucleic acid
TTP	Thymidine 5'-triphosphate (2-deoxyribose sugar moiety)
UV	Ultraviolet radiation
UTP	Uridine 5'-triphosphate (ribose sugar moiety)

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CHAPTER ONE

INTRODUCTION

1.1 Discovery of chloramphenicol

The isolation of chloramphenicol was first described by Ehrlich and co-workers (1947). When first discovered the antibiotic was produced by *Streptomyces venezuelae* a soil Actinomycete. Subsequently it has been found to be produced by a number of Actinomycetes (reviewed by Shaw, 1983). Production of chloramphenicol in *S. venezuelae* has been reported to be under plasmid control (Akagawa *et al.*, 1975).

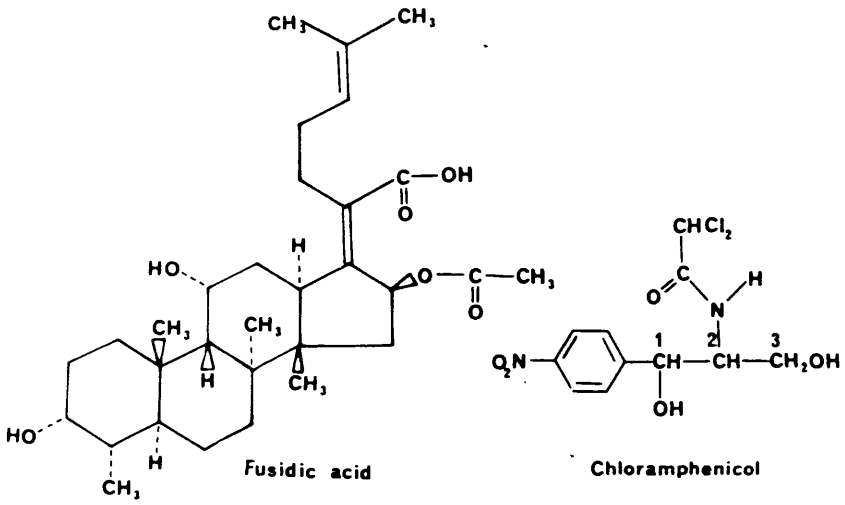
1.2 Structure of chloramphenicol

Chloramphenicol was shown to have the simple structure 1-(*p* nitrophenyl)-2-dichloroacetamido-1,3-propanediol, but of the four possible isomers only the D(-) *threo* stereoisomer is antibacterially active (Fig. 1; Maxwell and Nickel, 1954).

Chloramphenicol is sparingly soluble in aqueous solutions the maximum concentration at biological temperatures being of the order of 5mM (1.62 mg/ml). The antibiotic is more soluble in organic solvents such as ethanol and effectively aqueous solutions of chloramphenicol for bacteriological work can easily be prepared by dilution of an ethanolic solution.

Due to its simple structure the antibiotic is made commercially by chemical synthesis (reviewed by Ehrlich, 1982).

Figure 1.



The structures of fusidic acid and chloramphenicol.

1.3 Antibacterial spectrum of chloramphenicol

Chloramphenicol is a broad spectrum bacteriostatic antibiotic active against most of the commonly encountered laboratory and clinically isolated species of bacteria. Low level tolerance is, nevertheless, observed in at least some Bacillus and Pseudomonas species (reviewed by Shaw, 1983).

1.4 Clinical usage of chloramphenicol

Due to its early discovery and availability chloramphenicol rapidly came into wide oral and intravenous use (principally marketed as "Chloromycetin" by Parke-Davis Ltd.). Occurrences of bone marrow toxicity and adverse reactions in new born children has, however, led to its use being restricted to certain life-threatening infections (reviewed by Ehrlich, 1982 and reviewed by Shaw, 1983). These side effects are probably, at least in part, related to the action of chloramphenicol on mitochondrial metabolism (see paragraph 1.10).

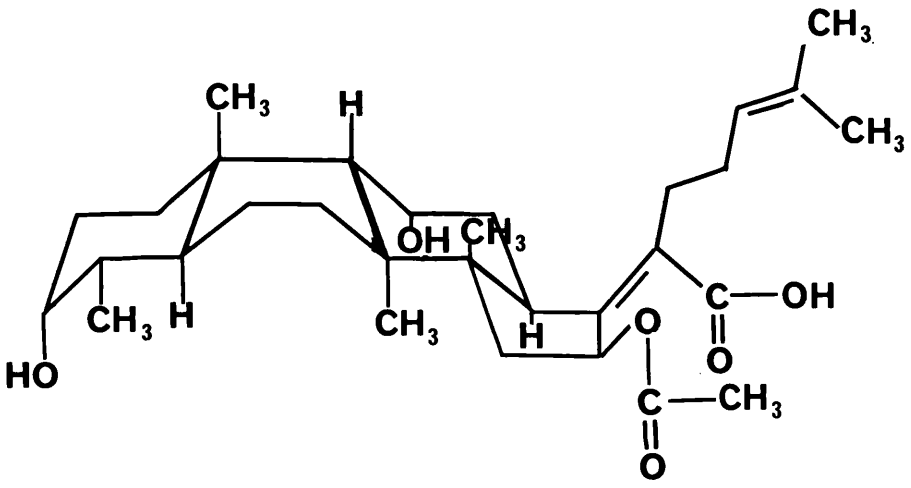
1.5 Discovery of fusidic acid

The isolation of fusidic acid was first described by Godtfredsen and his colleagues (1962a). The antibiotic was extracted from the fermentation broth of a strain of Fusidium coccineum, a fungus originally discovered in a sample of monkey dung collected in Minomo, Settu (Tubaki, 1954).

1.6 Structure of fusidic acid

Spectroscopic and chemical studies (Godtfredsen and Vangedal, 1962; Arigoni et al., 1963, 1964; Godtfredsen et al., 1965) have shown that fusidic acid is the tetracyclic triterpenoic acid depicted in Fig. 1. (3α, 11α-dihydroxy-16β-acetoxyfusida-

Figure 2.



Conformation of fusidic acid

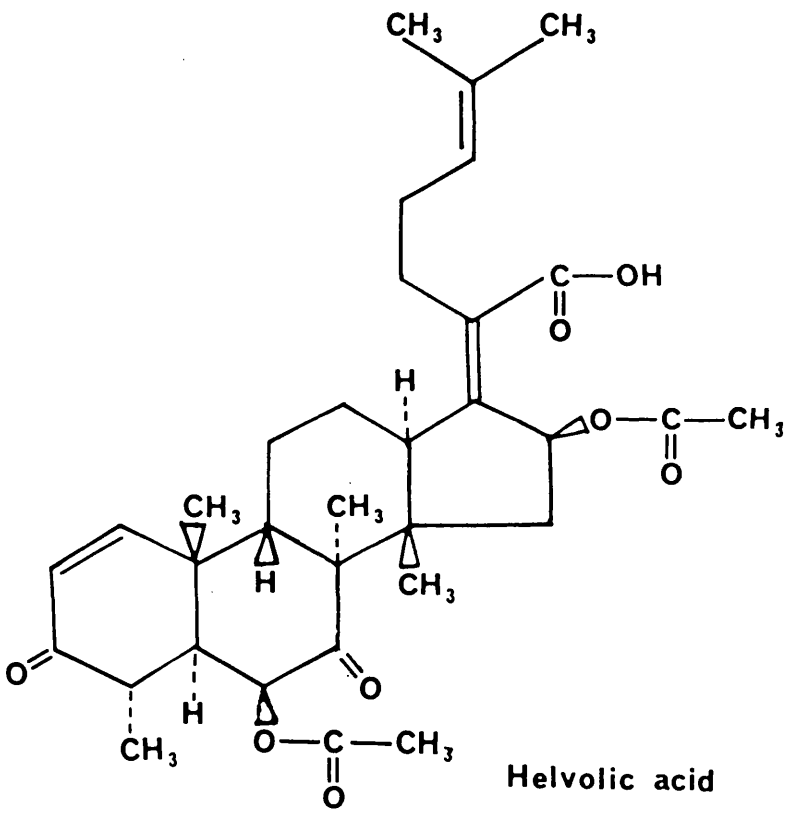
17(20)-[16,21-*cis*], 24-dien-21-oic acid). This structure was subsequently confirmed by the X-ray crystallographic analysis of the 3-*p*-bromobenzoate salt of fusidic acid methyl ester (Cooper and Hodgkin, 1968). The antibiotic has the characteristic cyclopentenoperhydrophenanthrene skeleton of a steroid but with an unusual stereochemistry which causes it to be forced into a "boat" conformation (Fig. 2).

Fusidic acid is only sparingly soluble in aqueous solutions. Accordingly the freely soluble sodium salt was usually used in preference to the free acid in the experiments described in this study. References to fusidic acid in the text will thus be synonymous with sodium fusidate unless otherwise stressed.

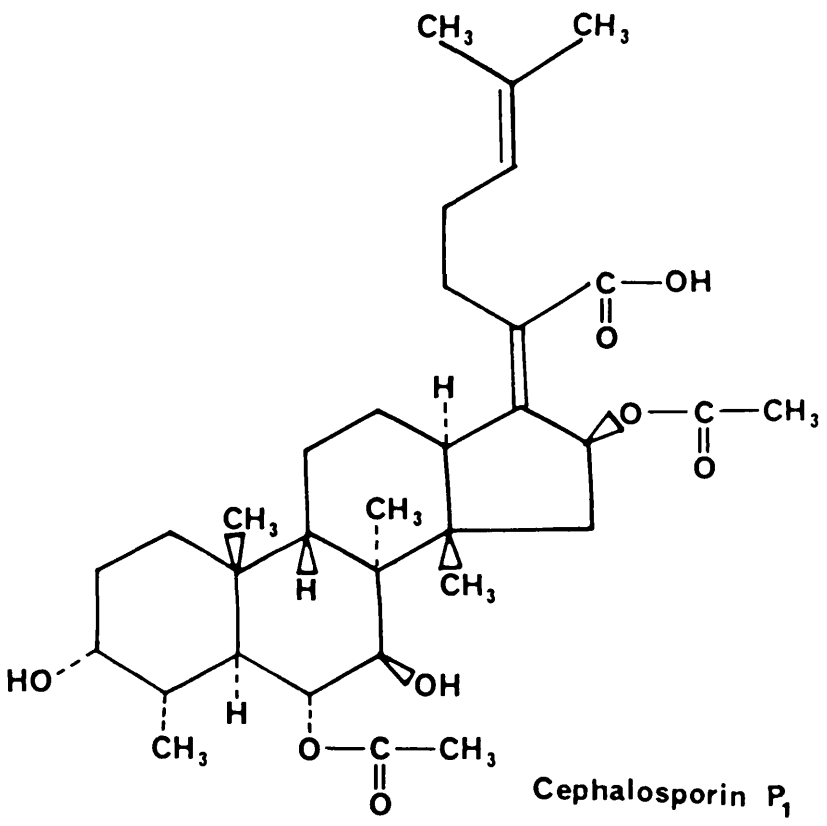
1.7 Antibacterial spectrum of fusidic acid

Fusidic acid is the most antibacterially potent member of a small group of related bacteriocidal compounds of fungal origin that also includes helvolic acid and cephalosporin P1 (Fig. 3). (N.B. The terminology here is confusing since cephalosporin P1 is not chemically related to the β-lactam type cephalosporins which inhibit peptidoglycan synthesis). The group have qualitatively similar, though not identical, antimicrobial spectra (von Daehne *et al.*, 1979).

Broadly speaking, most Gram-positive bacteria are sensitive to these antibiotics ($I.C_{50}$<0.5μg/ml≅1.0μM) whereas Gram-negative bacteria, especially coliform bacilli, are tolerant to high concentrations ($I.C_{50}$>500μg/ml≅1.0mM) (von Daehne *et al.*, 1979). Most eukaryotic organisms are insensitive to fusidane



Helvolic acid



Cephalosporin P_1

Figure 3.

The structures of helvolic acid and cephalosporin P1.

antibiotics (von Daehne et al., 1979).

1.8 Clinical usage of fusidic acid

Fusidic acid is used principally as an antistaphylococcal agent. The sodium salt is generally employed in a non-aqueous topical preparation (the acetoxyl group at position 16, Fig. 10, being too unstable in water for pharmacological requirements) and is marketed as "Fucidin" by Leo Pharmaceuticals Ltd. (Godtfredsen et al., 1962b, von Daehne et al., 1979). Sodium fusidate is also administered orally and the diethanolamine salt of the antibiotic can be given by intravenous infusion (reviewed by Anderson, 1980).

Fusidic acid is metabolised by the liver and the bulk of these metabolites are excreted via the bile (Godtfredsen and Vangedal, 1966).

1.9 The elongation stage of protein synthesis

Protein synthesis is a complex process consisting of three main stages. It is not relevant to this study to reproduce these in fine detail. Each has been the subject of a review article. The stages are initiation (Grunberg - Manago and Gros, 1977), elongation (Bermek, 1978) and termination (Caskey, 1977).

A résumé of the elongation stage in prokaryotes is, however, included here as it lends clarity to the two subsequent paragraphs (1.10 and 1.11).

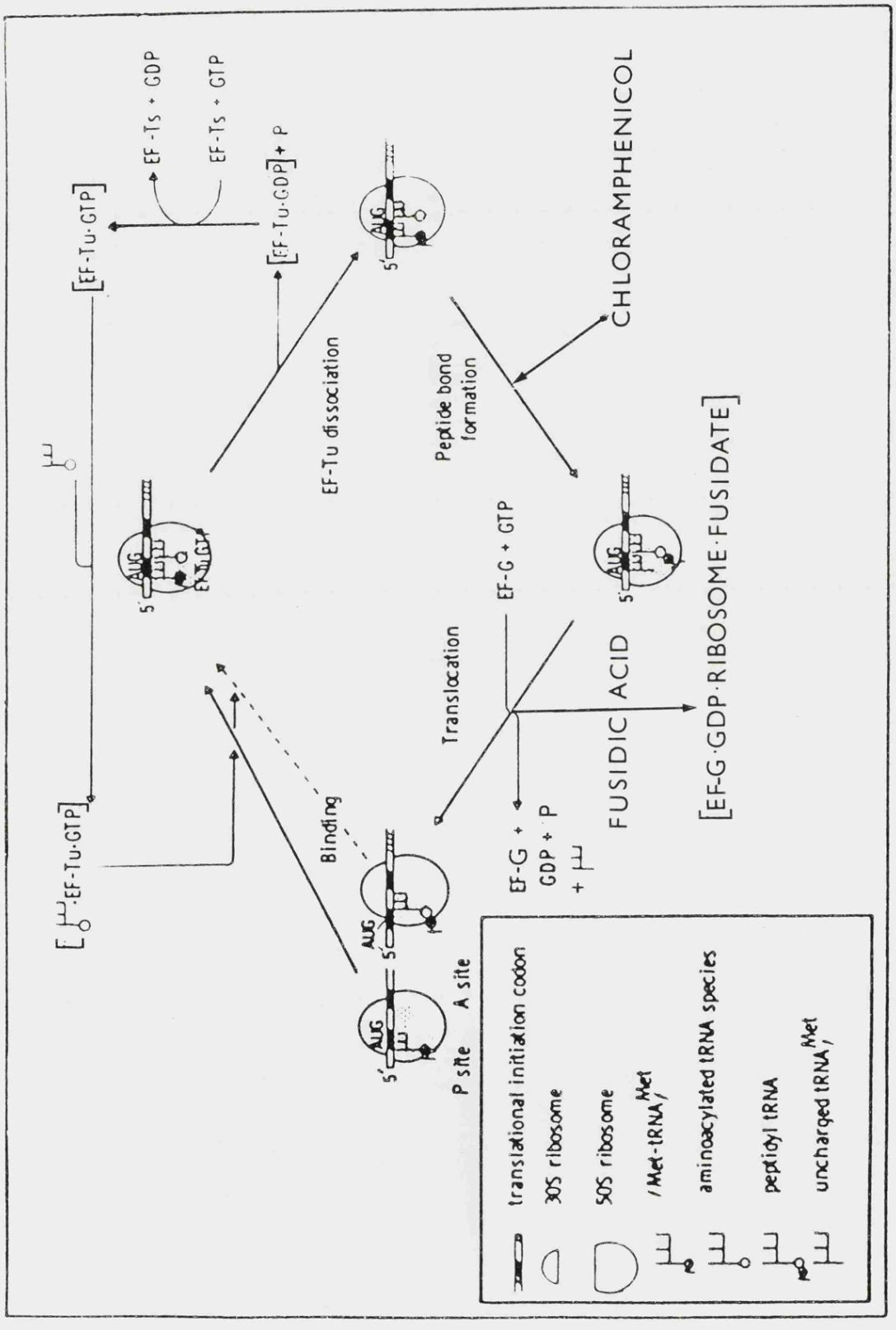
Protein synthesis on prokaryotic (70S) ribosomes requires, in addition to soluble protein catalysts and an energy source, two species of RNA. Messenger RNA (mRNA) encodes the order of the amino-acid residues of the polypeptide in the form of discrete blocks of three nucleotides termed codons. The other RNA species, the transfer RNAs (tRNAs) act as amino-acid carriers being esterified to them by specific aminoacyl tRNA synthetase enzymes. Each tRNA molecule has a specific three nucleotide anticodon domain complementary to a single mRNA codon.

Two tRNA binding sites on the 70S ribosome have been inferred from the use of specific inhibitors of protein synthesis. These are the aminoacyl-tRNA (or A) site and the peptidyl-tRNA (or P) site.

Initiation, the stage that precedes elongation, results in the presence of a specialised charged tRNA species (N-formylmethionyl-tRNA) in the ribosomal P site.

Elongation begins with the binding of a second charged tRNA to the ribosomal A site (see Fig. 4). This tRNA carries the amino-acid which will become the second residue in the polypeptide. The charged tRNA enters the A site as a ternary complex formed by interaction with a binary complex which is, itself, a product of GTP and one of the three soluble protein catalysts of elongation, EF-Tu. The nucleotide bases of the codon and anticodon hydrogen bond and subsequent hydrolysis of the GTP allows EF-Tu to and GDP to dissociate as a binary complex. (The [EF-Tu : GTP] complex is then regenerated by the joint

Figure 4.



The elongation stage of protein synthesis.
(adapted from Glass, 1982).

action of GTP and another elongation factor, EF-Ts).

The next step, catalysed by the large ribosomal subunit, is the formation of the peptide bond (often termed peptidyl transfer). The peptidyl transferase region of the 50S ribosomal subunit contains the ribosomal proteins L2, L27, L6, L16 and L24 (Cundliffe, 1981). The carboxyl ester linkage between the N-formylmethionine and its cognate tRNA is cleaved and the N-formylmethionine then condenses onto the N-terminus of the amino-acyl tRNA that is present in the A site forming a peptide bond. The net result of this transfer step is a dipeptidyl-tRNA species in the A site and an uncharged initiator tRNA in the P site.

Binding of the third elongation factor EF-G (M_r 80,000) and a molecule of GTP to the ribosome then occurs. This promotes displacement of the now uncharged tRNA that is present in the P site by the newly-formed dipeptidyl-tRNA, a process that requires the hydrolysis of GTP to GDP and is linked to the translocation of the ribosome one codon along the mRNA in the 5' to 3' direction. EF-G is then released from the ribosome for re-use in further translocation reactions.

EF-G synthesis in E.coli is linked to ribosomal protein synthesis and the factor can comprise more than 2% of the soluble protein of bacteria in exponential growth. It is probably present in at least as many copies as there are ribosomes. Although GTP is required in order to allow EF-G to bind to ribosomes, its breakdown is not needed for this purpose since it can be replaced in the binding reaction by its analogue GMP-PCP. (This analogue is a potent inhibitor of GTPase reactions). Translocation cannot take place after the substitution, however, which implies

that hydrolysis of GTP is required for movement of the ribosome along the mRNA (reviewed by Lewin, 1974).

Further elongation of the peptide chain occurs by essentially repeating the above events. At the beginning of each cycle the growing polypeptide is contained within the P site as peptidyl-tRNA while charged tRNAs enter into the A site. Peptidyl transfer takes place and the ribosome then translocates to position the new peptidyl-tRNA in the P site and expose a new codon in the A site.

The process continues (using two GTP molecules per peptide bond as indicated) until the A site contains an in phase nonsense codon for which there is no tRNA species.

The antibiotic puromycin acts in elongation as an amino-acyl tRNA analogue. It binds to the ribosome at the A site of the peptidyl transferase centre and accepts the peptide chain from the charged tRNA in the ribosomal P site.

The peptidyl-puromycin so formed diffuses from the ribosome and elongation of the nascent polypeptide is thus prematurely terminated. The puromycin reaction provides a useful model system for studying peptide bond formation on ribosomes. As discussed below direct inhibition of the puromycin reaction represents a direct inhibition of the peptidyl transferase centre and the reaction can help to elucidate the mode of action of certain antibiotics.

1.10 Mode of action of chloramphenicol

Chloramphenicol inhibits prokaryotic protein synthesis both *in vivo* and *in vitro* (reviewed by Cundliffe, 1981). It does not inhibit cell-free protein synthesis based on cytoplasmic extracts derived from mammalian cells, plant cells, yeasts and a protozoan. Chloramphenicol will, nevertheless, inhibit protein synthesis in both intact mitochondria and chlorplasts and also in their extracts.

The antibiotic acts by blocking protein chain elongation by inhibiting peptide bond formation (paragraph 1.9; Fig. 4). This was demonstrated by the drug inhibiting the ribosome-mediated production of polyphe-puromycin, polylysylpuromycin, fmet-puromycin and further demonstrated by its blocking effect on the release of nascent peptides from bacterial ribosomes by puromycin. As stated above (paragraph 1.9) this represents a direct inhibition of the peptidyl transferase centre.

Nevertheless in systems producing polypeptides an apparent inhibition of the puromycin reaction could be the indirect result of the blocking of the translocation step since nascent peptide linked to tRNA would be located in the ribosomal A site. Hence it was important to demonstrate the effect of chloramphenicol on the isolated peptidyl transferase reaction using a different approach. The so-called "fragment reaction" was employed for this purpose. In this system oligonucleotides bearing N-substituted amino-acids which bind to the ribosome at the P site of the peptidyl transferase centre were reacted with puromycin in the presence of washed 50S ribosomal subunits and ethanol. Chloramphenicol was shown not to inhibit the binding of acetyl-leucyl-pentanucleotide to 50S subunits

and also since acetyltenamine that was bound in the absence of drug was readily released by puromycin it was suggested that the drug does not act by masking the ribosomal P site close to the transferase centre. However, in contrast, the antibiotic acted to inhibit binding of unsubstituted aminoacyl-pentanucleotides (which bind to the ribosome at the A site of the peptidyl transferase & therefore do not donate peptides to puromycin). It had earlier been shown that chloramphenicol does not significantly reduce binding of aminoacyl-tRNAs to the ribosomal A site. Hence it is now believed that chloramphenicol prevents the recognition by the transferase domain of its accepting substrate in the A site. Models for the molecular mechanism of this inhibition based on chloramphenicol having an analogous structure to a variety of possible substrates have been proposed but these models fail to be compelling.

Chloramphenicol has been demonstrated to bind to the ribosomes of a number of prokaryotes but not to ribosomes isolated from yeast, protozoa, rat liver or pea seedlings. The binding of chloramphenicol to the E.coli ribosome has been reviewed by Cundliffe (1981). Binding occurs on the large ribosomal subunit (50S) requires magnesium and potassium ions and is readily reversed by washing. Only the D(-) threo stereoisomer (paragraph 1.2) binds and at bacteriostatic concentrations one molecule of chloramphenicol binds per 70S ribosome (Kdiss = 2×10^{-6}M) although a second molecule can bind elsewhere with a lower affinity at higher antibiotic

concentrations (Kdiss = 2 x 10^{-4}M). The sites of these ribosome-chloramphenicol interactions have been investigated by the use of ribosome reconstitution experiments and affinity labelling. The crucial protein in the reconstruction of the high-affinity chloramphenicol bindings site on the 50S ribosomal subunit and the primary target for electrophilic affinity analogues is the large ribosomal subunit protein L16. This protein has been identified as one of those present in the peptidyl transferase centre (paragraph 1.9).

1.11 Mode of action of fusidic acid

Fusidic acid inhibits *in vivo* and *in vitro* protein synthesis in both prokaryotic and eukaryotic organisms. *Escherichia coli* spheroplasts and cell-free extracts are far more sensitive to the antibiotic than whole cells indicating that the intrinsic tolerance of *E. coli* is due to the lack of cell wall permeability (Harvey *et al.*, 1966).

Additionally the fusidic acid producing fungus (paragraph 1.5) seems to exclude the antibiotic from its mycelium rather than tolerate its intracellular presence (Godtfredsen, 1967).

Tanaka and co-workers (1968) observed that in the absence of both tRNA and mRNA the "uncoupled" hydrolysis of GTP could be catalysed solely by purified bacterial ribosomes and EF-G. Furthermore, this GTPase activity could be inhibited by fusidic acid. It was thought, therefore, that fusidic acid was a specific inhibitor of the "coupled" hydrolysis of GTP that is required for normal translocation (paragraph 1.9, Fig. 4). The antibiotic was also shown to inhibit the equivalent "uncoupled" GTPase activity of eukaryotic ribosomes and EF-2 (Malkin and Lipmann, 1969).

Bodley and colleagues (1969) found that in addition to inhibiting GTP hydrolysis, fusidic acid enhanced the recovery of GDP in [ribosome: EF-G:GDP] complexes. These stabilised complexes were later shown to consist of equimolar amounts of the four components, ribosome, EF-G, GDP and fusidate (Bodley, *et al.*, 1970a, Okura *et al.*, 1970). The apparent conflict of

fusidate action was elegantly resolved by varying the relative concentrations of the components in the "uncoupled" GTPase reaction. (Bodley et al., 1970b). When ribosomes and EF-G were present in molar excess and the GTP level was low the rate and extent of GTP hydrolysis was unaffected by fusidic acid. When ribosome and GTP were in excess but EF-G was limited, fusidic acid inhibited hydrolysis but an initial burst of GTPase activity remained. The quantity of hydrolysis was equivalent to the molar amount of EF-G. From this Bodley et al., (1970b) proposed that fusidic acid does not inhibit GTP hydrolysis per se but by stabilising the intermediary [ribosome: EF-G:GDP] complex prevents further rounds of elongation and concomitant GTP hydrolysis. The implication is, therefore, that fusidic acid will allow one round of translocation to occur before inhibition of protein synthesis is established.

This interpretation was supported by the work of Cundliffe (1972) who observed that ribosomes blocked by high concentrations of fusidic acid could still donate nascent peptides to the amino-acyl tRNA analogue puromycin (paragraph 1.9). This shows that when fusidic acid has stabilised the intermediary complex the nascent peptide is, in fact, already in the P site; hence translocation has occurred. Accordingly it was suggested that fusidic acid inhibits elongation, not by inhibiting GTP hydrolysis or translocation, but by impeding access to the ribosomal A site by its action at what has been termed the "single ribosomal GTPase" site (reviewed by Cundliffe, 1981). Briefly, the hypothesis is that the elongation factors EF-Tu and EF-G, which are required for amino-acyl tRNA binding and translocation

respectively (paragraph 1.9), compete in a mutually exclusive manner at a single binding domain in the 50S ribosomal subunit. Binding of either factor constitutes a GTPase centre. GTP hydrolysis at this centre can be used during the binding of amino-acyl tRNA (when the EF-Tu complex is present; paragraph 1.9) or for the displacement of uncharged tRNA in the P site by dipeptidyl tRNA (when the EF-G complex is present; paragraph 1.9). The blocking role for fusidic acid at the single ribosomal GTPase site was subsequently demonstrated in vitro. GTP, purified ribosomes and EF-G that are stabilised by fusidic acid prevent the binding of the [EF-Tu:GTP:Amino-acyl tRNA] complex to the ribosome (Richman and Bodley, 1972).

1.12 Chromosomal resistance to chloramphenicol

Three genetic loci on the E. coli K12 chromosome have been identified as being associated with low level chloramphenicol resistance ($I.C_{50}$<15μg/ml, ≅50μM). These are cml A, omp F (cml B), and cml C and map at 18, 21 and 72 minutes respectively.

The cml A mutation is associated with decreased uptake of the antibiotic and maps at a significant distance from any of the ribosomal subunit genes (reviewed by Backmann and Low, 1980).

The omp F locus is in the structural gene for the outer membrane protein 1a and mutations at omp F are associated not only with decreased chloramphenicol uptake but also with tetracycline resistance, colicin A resistance and bacteriophage Tu Ib resistance. (Reviewed by Backmann et al., 1976; Backmann and Low, 1980; Backmann, 1983).

The cml C mutation was obtained by localised mutagenesis of the major ribosomal subunit gene-cluster at 72 minutes but has not been assigned to a specific protein (Baughman and Fahnestock, 1979). The locus rpl P which codes for the ribosomal protein L16 (see paragraph 1.10) lies in the same dense 72 minute gene cluster. It can be speculated, therefore, that the cml C and rpl P loci may share identity.

A fourth chromosomal mutation, amp B (Nordström et al., 1968) providing increased resistance to ampicillin and chloramphenicol, may equate to one of the above genes already mapped.

High level, chromosomally-mediated, chloramphenicol resistance (>100μg/ml; ≅600μM) has been reported in an uncharacterised E. coli strain (Smith and Worrel, 1953) and in E. coli B (Sompolinsky et al., 1968). In the former case the resistance mechanism was via slow reduction of the nitro group (Fig. 1) whereas with E. coli B the mechanism involved inactivation of the antibiotic by acylation. Additional experiments to investigate and genetically map these mutations have not been performed.

1.13 Chromosomal resistance to fusidic acid

Fusidic acid resistance in E. coli K12 to an antibiotic concentration in excess of the natural tolerance has been described. Two types of mutation have been mapped fus A and fus B at 73 and 14 minutes respectively (reviewed by Backmann and Low, 1980).

The most commonly isolated mutation, fus A, results in a modified elongation factor EF-G (paragraph 1.11). The "uncoupled" ribosome - dependent hydrolysis of GTP catalysed by this factor is less sensitive to fusidic acid due to decreased stabilisation of the [ribosome: EF-G:GDP] complex (Okura et al., 1970, 1971).

The mutation fus B is a temperature-sensitive pleiotropic mutation which also effects RNA synthesis, trimethoprim sensitivity and several other characteristics (Isaksson and Takata, 1978).

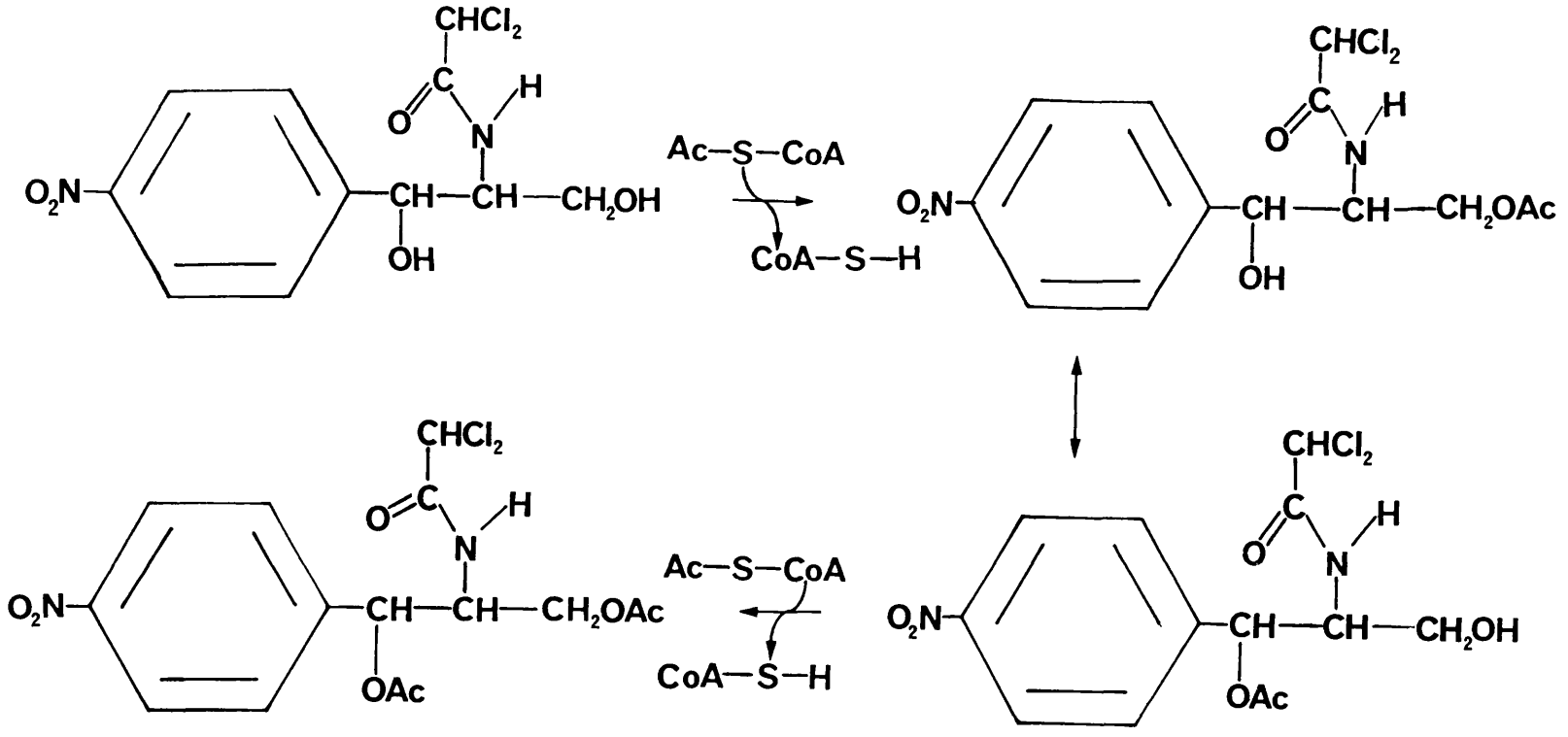


Figure 5.

The acetylation of chloramphenicol by CAT.

1.14 Plasmid-mediated resistance to chloramphenicol

1.14.1 The most prevalent mechanism of chloramphenicol resistance

Plasmid-mediated chloramphenicol resistance in *E. coli* is most commonly mediated by a homotetrameric enzyme which uses acetyl coenzyme A to inactivate the antibiotic by 3-O-acetylation (247 out of 251 clinical isolates of plasmid-mediated chloramphenicol resistance in *E. coli*, Nagai and Mitsuhashi, 1972). The enzyme, chloramphenicol acetyltransferase (CAT, EC 2.3.1.28) has been the subject of a recent extensive review (Shaw, 1983).

The rapid, enzyme catalysed, formation of chloramphenicol-3-acetate is followed by the much slower appearance of chloramphenicol-1, 3-diacetate (Fig. 5). Evidence suggests that a non-enzymic rearrangement of the 3-acetate compound occurs which yields chloramphenicol-1-acetate and that this compound is subsequently re-acetylated at the 3-hydroxy position (Mise and Suzuki, 1968; reviewed by Shaw, 1983). Both monoacetate esters as well as the diacetate ester have been shown to be antibacterially inactive (reviewed by Shaw, 1983).

CAT-mediated chloramphenicol resistance has been shown to act synergistically with chromosomal chloramphenicol resistance mutants (paragraph 1.12; Foster, 1975).

1.14.2 Characterisation of chloramphenicol acetyltransferase

In *E. coli* three naturally-occurring classes of this homotetrameric enzyme have been identified. The variants termed types I, II and III (CAT_I, CAT_{II} and CAT_{III}) were first

characterised on conjugative R-plasmids and the individual enzyme classes each tend to be associated with one or more R-plasmid incompatibility groups. The incompatibility groups, in turn, show considerable homology (Mickel et al., 1977; Sharp et al., 1973) and are associated with a defined host-range of bacterial species (Jacob et al., 1977).

The three CAT variants were originally characterised on the basis of electrophoretic mobility, sensitivity to DTNB (which is a chromogenic coupled substrate in the spectrophotometric CAT assay; paragraph 2.11) and by immunological cross-reaction. Subsequently they have been further characterised by affinity and hydrophobic chromatography, sensitivity to thiol-specific reagents, steady-state kinetic analysis and N-terminal amino-acid sequence. (Gaffney et al., 1978; Zaidenzaig et al., 1979; reviewed by Shaw, 1983).

As additional information is obtained, especially from DNA restriction endonuclease mapping, it is becoming increasingly apparent that the enzyme classes are internally heterogeneous. Nevertheless, at present, the classes are sufficiently homogeneous to warrant their continued usage.

The amino-acid sequence of the CAT_I variant carried on the R-plasmid JR66b has been determined (Shaw et al., 1979) and this has subsequently been confirmed three times by DNA sequence analysis (Fig. 6; paragraph 1.16).

CAT enzymes have been isolated from a large variety of both GRAM negative and GRAM positive bacterial species (reviewed by Shaw, 1983). These enzymes have been studied to varying biochemical genetic extents. The enzymes may be either chromosomally or plasmid-carried and can be either inducibly or constitutively expressed.

Apart from the three E.coli variants studied the most intensively investigated CAT enzymes are those from Staphylococcus aureus. These enzymes are inducibly expressed, carried on small plasmids and immunologically cross-reactive (unlike the E.coli variants). They are not associated with the increased tolerance of S.aureus to fusidic acid. When a type C CAT variant was introduced into E.coli using a "shuttle" plasmid the S.aureus CAT gene was inducibly expressed and conferred low level chloramphenicol resistance to its host. (S. Skinner personal communication). It did not, however, confer fusidic acid resistance to the fusidic acid sensitive E.coli mutant DB10 (paragraph 1.15; this study).

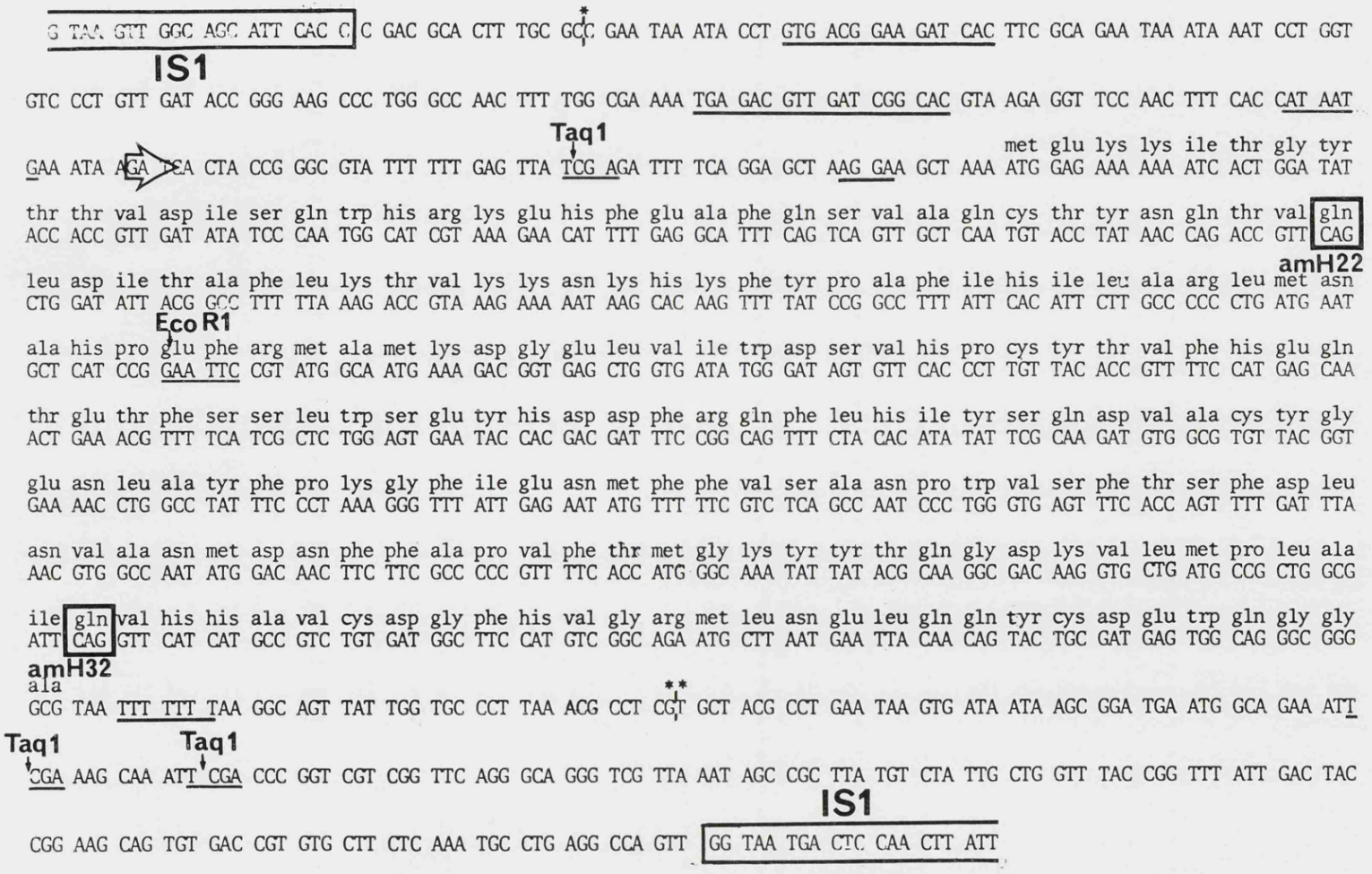


Figure 6.
The DNA sequence of the CAT_I gene
(adapted from Alton and Vapnek, 1979b)

* Start of DNA sequence of pBR325 CAT_I gene (Prentki et al., 1981)

** End of DNA sequence of Tn981 CAT_I gene (Marcoli et al., 1980)

GTG ACG GAA GAT CAC and TGA GAC GTT GAT CGG CAC = cAMP - CRP binding sites)Le Grice & Matzura, 1981.

CAT AATG = promoter, GA start of transcription, AG GA = ribosome binding site) Le Grice et al., 1982.

gln Codon mutated to amber (Völker et al., 1982).

1.14.3 Control of expression of chloramphenicol acetyltransferases

CAT_I is expressed constitutively but this expression, however, is subject to carbon-source mediated catabolite repression. This was first demonstrated *in vivo* by Harwood and Smith (1971) using a CAT_I-encoding R-factor. Subsequently it was confirmed twice *in vitro* (de Crombrugghe *et al.*, 1973; Dottin *et al.*, 1973). In both the latter cases the DNA template was a bacteriophage P1 derivative which carried the CAT_I-encoding transposon Tn9 (Kondo and Mitsuhashi, 1964; N.B. The bacteriophage is now termed P1 CmO. Meyer and Iida, 1979).

For maximal enzyme expression both cAMP (the co-repressor) and the cAMP receptor protein (CRP, the apo-repressor) are required and act together as a positive activator (de Crombrugghe *et al.*, 1973; Le Grice and Matzura, 1981; for review of cAMP/CRP see Adhya and Garges, 1982).

Glucose and some other sugars act as repressing carbon sources so that, unless exogenous cAMP is present, maximal gene expression and hence maximum intracellular CAT_I enzyme levels can only be achieved by using a non-repressing carbon source such as glycerol. The CAT_I structural gene is, unusually, preceded by two cAMP/CRP repressor binding sites but it appears that only the site proximal to the structural gene is required for transcriptional activation (Fig. 6, Le Grice *et al.*, 1982).

Transcription initiates at a guanosine residue 59 bp before the translational start codon (AUG) but the majority of these transcripts terminate after only twelve nucleotides (Fig. 6 Le Grice and Matzura, 1981). The reason for this abortive transcription is not clear and has not been investigated further but may be linked to an attenuation mechanism as observed in bacteriophage lambda and in tryptophan biosynthesis (Yanofsky, 1981).

The expression of both the CAT_{II} and CAT_{III} enzymes appears to be constitutive but has not been investigated in any detail.

1.14.4 Different forms of chloramphenicol resistance in E. coli

A non-CAT, and presumably plasmid-borne, chloramphenicol resistance mechanism based on modification of the antibiotic has been described wherein chloramphenicol is inactivated by reduction of the nitro group to the amino derivative (Fig 1; Molho-Lacroix and Molho, 1952 q.v. paragraph 1.12). A second class that are carried on R-plasmids and encode an inducible mechanism (or group of mechanisms) effect chloramphenicol resistance by preventing the intracellular accumulation of the antibiotic (Nagai and Mitsuhashi, 1972; Gaffney et al., 1981).

1.15 Plasmid-mediated resistance to fusidic acid

To observe the phenotype of plasmid-borne fusidic acid resistance in E. coli it is essential to use a fusidate-sensitive mutant strain as the plasmid host since wild-type E. coli strains are intrinsically tolerant (Datta et al., 1974; paragraph 1.11).

The first study on the occurrence of this resistance was performed by Datta and her colleagues (1974). Among twenty-two naturally-occurring R-plasmids seven were found to confer fusidic acid resistance. Of these, six also encoded chloramphenicol resistance, whilst one R-plasmid, R28, only encoded the fusidic acid resistance phenotype.

1.16 Linkage of chloramphenicol and fusidate resistance

Chloramphenicol and fusidate resistances appear to be commonly found on the same R-plasmid (paragraph 1.15) Genetic mapping studies were performed on two closely related R-plasmids (Mickel et al., 1977) each of which conferred both the fusidic acid and chloramphenicol resistance phenotype.

Both deletion mapping (Dempsey and Willetts, 1976) and restriction endonuclease analysis (Tanaka et al., 1976; Lane and Chandler, 1977; Miki et al., 1978; Timmis et al., 1978a; Blohm and Goebel, 1978) indicated that the chloramphenicol and fusidic acid resistance determinants were closely linked.

The two resistance markers were subsequently shown to be present on transposons related to the small transposon Tn9 (paragraph 1.14.3; Arber et al., 1978). DNA sequence analysis of Tn9 (Alton and Vapnek, 1979b), demonstrated that a 1102 bp sequence flanked by IS1 insertion elements is sufficient to promote and code for both fusidic acid and chloramphenicol resistance (Fig. 6). The only translational reading frame in this nucleotide sequence

capable of encoding a polypeptide of more than sixty-five amino acid residues (including those with possible GUG initiation codons) is that which specifies a protein identical in amino acid sequence to the CAT_I variant determined by Shaw *et al.* (1979).

1.17 Aims of this study

This study was embarked upon to try to find the mechanism behind the phenotype of plasmid-borne fusidic acid resistance in *E.coli* K12. The relationship of this resistance to the gene for CAT_I and to the CAT_I enzyme was also sought. The project was designed to employ three complementary approaches.

Firstly, the genetic approach was to use the available nucleotide sequence information and from it to discern by coupled cell-free transcription and translation experiments the relationship between the CAT_I-encoding 1102 base pair sequence (paragraph 1.16) and fusidic acid resistance. If the CAT_I enzyme was found to be the sole expression product of this region of DNA then it could be concluded that the protein is bifunctional and effects both antibiotic resistances.

The biochemical approach was planned to extend the results of the genetic investigation. Information on the fusidate resistance protein(s) was to be correlated with any detectable changes to the plasmid host cell or to a change in the structure of the fusidic acid molecule.

The third, kinetic, stage was envisaged to confirm and clarify the previous approaches. It was hoped by this to demonstrate the stoichiometry and affinity of any potential antibiotic binding to the resistance protein(s) and by the use of fusidic acid analogues to define the structural constraints on the fusidane skeleton which are required for the resistance mechanism to function. The overall goal was to combine these and other ancillary results both from *in vitro* and *in vivo* experiments so that these would come together to yield a tenable resistance mechanism.

Throughout this study parallel research on the genetic aspects was being performed by a group of researchers headed by Dr. W. Arber in Basle, Switzerland. Soon after I had commenced work the DNA sequence of a smaller variant of transposon Tn9, called Tn981, was published (Marcoli *et al.*, 1980; previously termed Tncam204). This was soon followed by the publication of the DNA sequence of the CAT_I gene from plasmid pBR325 (Prentki *et al.*, 1981). Taken together the three published sequences implied that a 906bp sequence was sufficient to code for both fusidic acid and chloramphenicol resistance (Fig.6).

Two years after my work started the same group proved the genetic hypothesis which my studies were leading towards, namely that CAT_I is a bifunctional protein capable of effecting both chloramphenicol and fusidic acid resistance. My work was based on *in vitro* coupled transcription and translation studies whereas their work was on the isolation of conditional lethal

mutations of the CAT_I gene (Völker *et al.*, 1982)

I was subsequently able to bring my genetic analysis to a close with further experiments *in vitro* using the conditional lethal mutants they had isolated.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Chemicals

2.1.1 Antibiotics

Fusidic acid [11β-^{3}H] diethanolamine salt (47.75 Ci/mol) in ethanol (stored at -80°C) sodium fusidate and its analogues were gifts from Dr. W. von Daehne, Leo Pharmaceutical Products Ltd., Ballerup, Denmark. Cephalosporin P1 and helvolic acid were gifts from Ciba Geigy Pharmaceuticals Division, Horsham, Sussex. Cephalosporin P1 was recrystallized at -20°C from an 80% pure sample to over 99% purity (as determined by thin-layer chromatography) using methanol instead of ethanol in the procedure of Carey et al. (1975). The nonradioactive compounds above were used as the sodium salts when supplied; free acids were converted to their corresponding sodium salts by mild treatment with an equimolar amount of sodium hydroxide (Carey et al., 1975).

Ampicillin (sodium salt), tetracycline, chloramphenicol, kanamycin sulphate, streptomycin sulphate and sodium nalixidate were purchased from the Sigma Chemical Co. Ltd., Poole, Dorset. Spectinomycin as the dihydrochloride pentahydrate was purchased from the Upjohn Company Ltd., Crawley, Sussex.

2.1.2 Other Chemicals

Adenosine 5' [α-^{32}P] triphosphate, triethylammonium salt (400 Ci/mol), Guanosine 5' [γ-^{32}P] triphosphate, triethylammonium salt (27.0 Ci/mol), L-[^{35}S] methionine (1320 Ci/mol) and [5-^{3}H]

Uridine 5'-triphosphate, ammonium salt (20.0 Ci/mol) were purchased from Amersham International plc, Amersham, Bucks.

Complex bacterial growth media, Difco Bacto grade, was purchased from Difco Ltd., Molesey, Surrey.

All other compounds, except those whose source is eluded to in the text, were the best grade available and were purchased from BDH Ltd., Poole, Dorset, Fisons Ltd., Loughborough, Leics. and Sigma Chemical Co. Ltd., Poole, Dorset.

2.2 Bacterial strains

All strains used were *E. coli* K12 derivatives.

E. coli DB10 : F-, *lac* Y1, *mal* A1, *mtl* 2, *xyl* 7, *leu* B6, *thr* 1, *thi* 1, *rps* LA132(Str^R), nal^R, fus^S, *pnp* 7, *rna* 19.
(Datta *et al.*, 1974; supplied by S. Iida).

This strain was used as the sensitive host to score the fusidic acid resistance phenotype conferred by the plasmids used in this study.

N.B. Plasmid-containing strains generally exhibited reduced resistance to sodium nalidixate and streptomycin sulphate (paragraph 5.2.2).

E. coli C600 : F-, *lac* Y1, *leu* B6, *thi* 1, *sup* E44, *ton* A21
(reviewed by Backmann, 1972; supplied by B. Brammar).

This strain was used to observe the phenotype of normal fusidate-tolerant E. coli on antibiotic-containing agar plates. In addition, because growth rates, plasmid yields, and CAT yields were greater for plasmid-containing E. coli C600 strains than when E. coli DB10 contained the same plasmids, it was used as the host when purifying CAT enzymes and plasmid DNA.

E. coli FT1 : F-, pro C32, pur E42, met E70, met B1, lys A23, thi 1, trp E38, str 109 (Str^R). (Taylor and Cronan, 1976; supplied by T. Foster.)

E. coli FT16 and E. coli FT17 : CFA- mutants of E. coli FT1 (Taylor and Cronan, 1976; supplied by T. Foster).

E. coli KHΔ10 : F-, Δ(nad C, ace E, ace F), met B, rel A1, thy A56, tsx 87, pps, azi, ton ? (Langley and Guest, 1978).

E. coli KΔ18 : F-, Δ(nad C, ace E, ace F, lpd) met B, rel Al, thy A56, tsx 87, pps, azi, ton? (Langley and Guest, 1978).

E. coli J53 : F-, pro, met.(Datta et al., 1974)

E. coli J53-1 = E. coli J53, nal^R.(Datta et al., 1974; Gaffney et al., 1978).

E. coli J53-2 = E. coli J53, rif^R.(Datta et al., 1974)

E. coli GM242 : F-, *dam* 3, *rec* A1, *sin* 2, *thr* 1, *leu* B6, *pro* A2, *his* 4, *met* B1, *lac* Y1, *gal* K2, *ara* 14, *tsx* 33, *thi* 1, *deo* B6, *sup* E44, *rps* L260 (Str^R) (obtained from K. Chater).

E. coli ED2149 : F-, Δ(*lac*, *nia*, *gal*, *att*λ, *bio*), $T6^R$. (Dempsey and Willetts, 1976).

2.3 Plasmids

The plasmids used were as listed in Tables 1a and 1b.

2.4 Bacterial growth media

All bacterial growth media was sterilised by autoclaving at 121°C and 1.05kg/cm^2 (=15 psi) for twenty to twenty-five minutes. Liquid media was used within one week of sterilisation and agar plates, stored at 4°C, were used within ten weeks of preparation.

L-broth (Lennox, 1955) per litre : 10g tryptone,
5 g yeast extract,
5 g NaCl,
1 g D-glucose.

2YT broth (Proctor and Rownd, 1982)
per litre : 16g tryptone,
10g yeast extract
5 g NaCl.

Table 1a

R-plasmids and E.coli hosts

R-plasmid	Incompatibility group (Jacob et al.,1977)	E.coli Host (para. 2.2)	Reference
R100 (=NR1=222)	FII	J53	Datta et al. (1974)
EDR51	"	ED2149	Dempsey and Willetts (1976)
EDR104	"	DB10	Dempsey and Willetts (1976) Datta et al. (1974)
R28	"	J53	Datta et al. (1974)
JR70	"	J53-2	Datta et al. (1974)
JR72	"	J53-2	Datta et al. (1974)
R455	FI	J53-1	Datta et al. (1974) Gaffney et al. (1978)

Table 1b. Mini-plasmids and antibiotic resistance levels in E.coli DB10

Plasmid	Antibiotic resistance phenotype in E.coli DB10	CAT enzyme variant (a)	Antibiotic resistances determined by the gradient plate method (para. 2.7) for plasmid-containing E.coli DB10 strains: chloramphenicol (µg/ml)	sodium fusidate (µg/ml)	References
pBR322	Amp , Tet - - -	-	2	5	Sutcliffe (1979)
pBR325	Amp , Tet , Cml , Fus -	I	50	80	Bolivar et al (1978)
pBR325 catam H22	Amp , Tet - - -	-	2	5	Völker et al. (1982)
pBR325 catam H32	Amp , Tet - - -	-	2	5	
pBR328	Amp , Tet , Cml , Fus -	I	25	105	Soberón et al (1980)
pSh141	- Tet , Cml , Fus -	I	45	90	Marcoli et al (1980)
pSh144	- Tet , Cml Fus -	I	45	90	
pKT205	- Tet , Cml - -	II	90	5	(b)
pAH1	- Tet , Cml - -	III	110	5	(c)
pNJ2004	Amp - - - Kan	-	2	5	(d)
pAB02	- - Cml , Fus , Kan	I	40	100	(e)

(a) Gaffney et al. (1978)

(b) pKT205 was constructed by insertion of a PstI restriction fragment from the R-plasmid S-a (Jacob et al., 1977) into the PstI site of the β-lactamase gene of pBR322 (Timmis, 1981).

(c) pAH1 was constructed by insertion of a PstI restriction fragment from the R-plasmid R387 (Jacob et al., 1977) into the PstI site of the β-lactamase gene of pBR322. (A. Hawkins unpublished).

(d) pNJ2004 is an EcoRI-SalI deletion of plasmid pMK2004 (Kahn et al., 1978; Grinter 1984)

(e) pAB02 was constructed by insertion of a PstI restriction fragment from the R-plasmid EDR51 into the PstI site of the β-lactamase gene of pNJ2004 (paragraph 5.2.2.). EDR51 is a deletion derivative of R100 constructed by Dempsey and Willetts (1976).

L-agar as L-broth plus 1.5% (w/v) agar.

2YT agar as 2YT broth plus 1.5% (w/v) agar.

Standard nutrient antibiotic plates : L-agar or latterly 2YT agar was cooled to 60°C and sterile antibiotics at the following final concentrations were added: ampicillin (sodium salt) 50µg/ml, chloramphenicol 80µg/ml and 20µg/ml,tetracycline 10µg/ml, sodium fusidate (MW = 538.8) 40µg/ml, kanamycin sulphate 10µg/ml, streptomycin sulphate 25µg/ml, sodium nalidixate 20µg/ml. Plates were dried open at 42°C for thirty minutes or at 37°C overnight with the lid on prior to use.

M56/2 Minimal medium (Willetts et al., 1969)

0.5M Na_2HPO_4	61.1ml	Solution A (mixed from separate stocks held over trichloromethane) and autoclaved together.
1.0M KH_2PO_4	19.3ml	
10% (w/v) $(NH_4)_2 SO_4$	0.5ml	
0.5% (w/v) $FeCl_2$	0.5ml	
Distilled water	407.1ml	
10% (w/v) $MgSO_4.7H_2O$	1.0ml	Solution B, autoclaved
1% (w/v) $Ca(NO_3)_2$	0.5ml	Solution C, autoclaved

When the solutions were cool (below 65°C) solutions A, B and C were mixed to give 490ml of M56/2 medium.

M56 agar

M56/2 medium at 60°C	490ml
Sterile 4% (w/v) agar at 60°C	500ml
Sterile 20% (w/v) D-glucose	10ml

Minimal selective plates

M56 agar plates were spread just prior to use with sterile additions of 80µl of 0.1% (w/v) thiamine hydrochloride (vitamin B1) and/or 80µl of 1% (w/v) of the appropriate L-amino-acid(s) and co-factor(s). The plates were then dried at 42°C for thirty minutes prior to use.

Low acetate plates

Minimal selective plates as above with sodium acetate as an extra carbon source.

M56/CAT medium

M56/2 Minimal medium	490ml
Sterile 0.4% (v/v) glycerol, 3% (w/v) casamino acids	490ml
Sterile 0.1% (w/v) thiamine hydrochloride	20ml
5mg/ml tetracycline in ethanol	2ml

Crystal violet plates

These were initially prepared from L-agar plus sterile aqueous dye solutions added when the agar was at 60°C to give final concentrations of dye of 2µg/ml and 20µg/ml. After publication of the paper by Proctor and Rownd (1982) 2YT agar plus 2µg/ml crystal violet plates were made by combining all the component

compounds and autoclaving them together as one solution. Dye plates were dried in the same manner as 2YT agar plates before use.

2.5 Sterilisation of additions to growth media

Additions to media and agar were prepared as concentrated stocks. Bile salts, crystal violet and all antibiotics except chloramphenicol, tetracycline and the sodium salts of fusidate analogues were prepared as one hundred fold concentrated solutions.

L-amino acids and co-factors were prepared as 1% (w/v) stock solutions, thiamine hydrochloride as a 0.1% (w/v) stock.

All these aqueous solutions were sterilised individually by passage through sterile (autoclaved) 13mm diameter membrane filters (0.2μm pore size : Sartorius-Membranfilter GmbH, 34 Göttingen, West Germany) which were attached to disposable syringes.

Chloramphenicol (20mg/ml) and tetracycline (5mg/ml) were prepared as stock solutions in ethanol and, after dissolving, were presumed to be sterile.

All additions to growth media were stored at -20°C in small aliquots and removed for use when required. Tetracycline-containing solutions and media were maintained in the dark due to the light-labile nature of the antibiotic. Ampicillin-containing media was unstable and was only deemed to have a

maximum useful life of three weeks when stored at 4°C.

2.6 Maintenance of bacteria strains

Bacterial strains received were inoculated into a 10ml tube containing 1ml of sterile L-broth. The tube was rotated at 20rpm and 37°C overnight. This overnight culture was then streaked onto a 2YT agar plate containing a single, appropriate, antibiotic. For plasmid-containing strains this antibiotic was designed to select for the plasmid. If a multiply resistant plasmid was present the order of choice of the selecting antibiotic was tetracycline, kanamycin, chloramphenicol, fusidic acid, ampicillin, streptomycin, nalidixate. In plasmid-free strains the antibiotic of choice was one which selected for a chromosomal antibiotic resistance marker. Single colonies from these plates were then picked onto a minimal plate which contained suitable additions. After sufficient incubation at 37°C for good growth the minimal plate was replicated to incompletely supplemented minimal plates and/or antibiotic-containing 2YT agar plates. Replica plating (Lederberg and Lederberg, 1952) was performed on a wad of sterile Whatmann 3MM filter paper which was fixed over a replica-plating block, the last replicated plate was always an unsupplemented 2YT agar plate to check that bacterial transfer had occurred onto the previous plates. When these, and the strains constructed in this study,were shown to correspond to their predicted phenotypes glycerol cultures were made for long term storage.

Glycerol cultures were made as follows (after D. Vapnek, unpublished). L-broth was made and aliquoted in 1ml amounts into 2ml screw-topped vials. These were sterilised in batches and stored until required. When a strain had been phenotypically checked out, single colonies were picked from the relevant minimal plate into duplicate L-broth-containing vials. After overnight growth at 37°C 100µl of sterile glycerol and 5µl of dimethylsulphoxide were added to each vial and the mixtures vortexed for fifteen seconds. The vials were then left ten minutes, re-vortexed and frozen at -80°C. The contents of these vials froze solid and when the strain was required for further use it was obtained by scraping a small amount from the frozen surface with a sterile bacterial loop. The duplicate vials were made so that one of them could be repeatedly sampled leaving the other as an uncontamined reserve. The viability of strains maintained in this way remains at a very high level for a great deal of time; strains stored in excess of two years have been found to be both viable and vigorous.

2.7 Determination of antibiotic resistance levels

Resistance levels were estimated by the gradient plate method (Meynell and Meynell, 1970). 50ml of 2YT agar containing an appropriate concentration of antibiotic was set in a wedge in the bottom of a square Petri dish (10 cm x 10 cm). The Petri dish was then returned to the horizontal and the same volume of antibiotic-free 2YT agar was poured in to form an upper wedge. After one hour, when the agar was set and its surface had been dried, bacteria in the early exponential growth phase in 2YT

broth (A_{660} = 0.4) were applied. The application was performed by dipping 0.5cm wide strips of sterile Whatmann 3MM paper into the bacterial suspension, allowing them to drain of excess culture medium, and then laying these momentarily across the surface of the agar. The strips were applied in parallel lines up the concentration gradient which forms by diffusion of the antibiotic out of the lower wedge. Antibiotic resistance was scored after sixteen hours incubation at 37°C by distance of visible growth across the surface of the agar.

2.8 Physical disruption of bacteria

Cell free extracts were prepared from bacteria in the exponential phase of growth (A_{660} = 0.8) using one of the two techniques given below.

2.8.1. Cultures of up to 100ml

Bacterial cells were collected by centrifugation at 17,000g for ten minutes. The pellet of cells was drained (and stored for up to a week at -20°C) and resuspended in cold 50mM Tris/HCl. 5mM chloramphenicol, 0.1mM 2-mercaptoethanol, pH7.8 to give a 15% (wet w/v) suspension.

Cells were disrupted using an MSE 100 Watt ultrasonic disintegrator at full power, adjusted to give an 8μm peak to peak distance. Sonication was carried out using the largest probe that was possible with the tip placed so that it was 3mm below the surface of the suspension prior to disruption. The tube containing the cell suspension was surrounded by ice and sonication consisted of four thirty second bursts each separated

by a thirty second cooling period. Cell debris was removed by centrifugation for thirty minutes at 50,000g and at 4°C.

2.8.2 Cultures of over 100ml

Bacterial cells were harvested as above and resuspended to give a 50% (wet w/v) paste in the above buffer. Cells were disrupted by passage through a pre-cooled Aminco French press (American Instrument Co., U.S.A.) at 700 kg/cm^2 (=10,000 psi), Cell debris was removed as above.

2.9 Protein determinations

Protein determinations were after Lowry et al. (1951).

Three solutions were made up and stored separately at 4°C. Solution A, 10 ml of 2% (w/v) sodium tartrate; Solution B, 10 ml of 1% (w/v) cupric sulphate pentahydrate and Solution C, 1 litre of 0.1M sodium hydroxide, 2% (w/v) sodium carbonate.

A standard curve in the range 50µg/ml to 250µg/ml was made from a 1mg/ml stock solution of bovine serum albumin (BSA) fraction V, stored at -20°C. Each standard had a final volume of 200µl. Three ten-fold serial dilutions of each protein sample were taken each with a final volume of 200µl.

Solutions A, B and C were mixed in the proportions 100µl: 100µl: 10ml respectively. 1ml of the mixture was then added to each protein sample. After ten minutes at room temperature 100µl of a freshly diluted 50% (v/v) aqueous solution of Folin and Ciocalteu's phenol reagent was added and the mixture left

at room temperature for a further thirty minutes (or overnight at 4°C).

Protein concentration was determined as A_{750} in a Pye Unicam SP1800 dual beam spectrophotometer against a protein-free reagent blank. Dilutions for this method were made in distilled water to minimise the errors associated with Tris solutions (Lowry et al., 1951).

2.10 Ouchterlony double diffusion tests

Double diffusion tests were based on the method of Ouchterlony (1949).

PBS2 buffer, pH7.5, was prepared as follows:

NaCl	3.20g
KCl	0.80g
$Na_2HPO_4.7H_2O$	4.60g
KH_2PO_4	0.80g
Distilled water	400ml

A 10cm x 10cm square Petri dish was filled with 20ml of molten 0.70% (w/v) agarose in PBS2 buffer. When set, five arrays of seven 3mm diameter holes were made in the agarose using a cork borer. These holes were arranged in a hexagon with the seventh hole in the centre and such that all seven holes were 7mm apart. The plugs of agarose were removed using a pasteur pipette attached to a vacuum source.

Cell-free extracts (paragraph 2.8.1) to be used as potential antigens were obtained from 20ml cultures growing exponentially (A_{660} = 0.8) in M56/CAT medium (with an antibiotic other than tetracycline substituting if appropriate).

Antigen samples of final volume 10µl calculated to contain approximately 25µg of protein (paragraph 2.9) were applied to the outer wells.

Anti-CAT_I immunoglobulin G (IgG) was a gift from W.V. Shaw and was purified from antisera raised in a goat against CAT_I enzyme isolated from a strain carrying the R-plasmid JR66b (Shaw et al., 1979). A small volume of IgG solution (approximately 50µl) was micro-centrifuged in a 1.5ml Eppendorf tube for two minutes. The supernatant was then removed and diluted twenty fold in PBS2 buffer. 10µl of this diluted IgG solution was applied to the central wells.

The Petri dish was then sealed with tape and incubated at 4°C for forty-eight hours. After incubation the unprecipitated protein was eluted by placing the Petri dish in a small container which held the remainder of the PBS2 buffer. This was shaken overnight on an orbital shaking platform at room temperature and at moderate speed. The Petri dish was then drained and eight 10cm x 10cm squares of Whatmann No. 1 filter paper were applied to the surface of the agarose and a 1kg weight placed on top. After one hour of blotting the filter papers were removed and the Petri dish washed for thirty minutes in excess water on the orbital shaker as above. The agarose was then blotted as before

and the blotted agarose stained with shaking for one hour. The stain was a mixture consisting of 134ml of distilled water, 16ml of glacial acetic acid and 50ml of filtered (as in paragraph 2.5) 0.1% (w/v) Coomassie Brilliant Blue R250 dye in ethanol. The unabsorbed dye was then rinsed off with excess distilled water and the agarose destained, with shaking, in excess 7% (v/v) acetic acid, 5% (v/v) methanol until all of the background dye was removed.

2.11 Preparation of acetyl CoA

100mg of coenzyme A (CoA-SH) was dissolved in 7ml of cold (4°C) distilled water. This was mixed with 1ml of cold 1M potassium bicarbonate. The mixture was then vortexed in a 20ml boiling tube and 16µl of acetic anhydride added to the vortex extremely slowly (over about five minutes). After ten minutes on ice the level of free CoA-SH was estimated in the following way. 10µl of the solution was added to 990µl of a freshly-made 1mM solution of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in 50mM Tris/HCl, 0.1mM EDTA, pH7.8. The A_{412} was then read against a DTNB solution blank in a Pye Unican SP1800 dual beam spectrophotomer. If the acetyl CoA solution gave an absorbance in excess of 0.02 O.D. units further acetic anhydride was added as above.

The concentration of acetyl CoA was determined by combining 0.93ml of the above DTNB solution, 50µl of 5mM chloramphenicol in 50mM Tris/HCl, 0.1mM EDTA, pH7.8, 10µl of concentrated pure CAT enzyme (usually CAT_I) also in the same buffer and 10µl of the unknown acetyl CoA solution. A final A_{412} of 1.36 indicates an initial

acetyl CoA concentration of 10mM. A good yield of acetyl CoA has an A_{412} of 1.8. The acetyl CoA prepared was diluted to 10mM and stored as 50µl and 500µl aliquots at -20°C.

This method results in approximately 10% (w/w) contamination of acetyl CoA with acetyl 2'; 3'-cyclicdephospho CoA. (Commercial supplies of acetyl CoA are rarely above 90% pure also). This compound is a weak competitive inhibitor for pyruvate carboxylase but a substrate for citrate synthase and phosphotransacetylase. It is, therefore, probably a substrate for CAT (Fung et al., 1975, K. Kleanthous personal communication).

2.12 Assay of chloramphenicol acetyltransferase activity

CAT activity was assayed using the spectrophotometric assay of Shaw (1975).

The acetylation of chloramphenicol to chloramphenicol-3-acetate (paragraph 1.14) uses acetyl CoA and generates CoA-SH. The reaction of this reduced CoA with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) yields the mixed disulphide of thionitrobenzoic acid and CoA and a molar equivalent of free 5-thio-2-nitrobenzoate. The latter has a molar extinction coefficient at 412nm of 13,600 litre/mol/cm at pH7.8. (Fig. 7).

The assay was carried out using a Pye Unican SP1800 dual-beam recording spectrophotometer with a temperature controlled cuvette chamber set at 37°C.

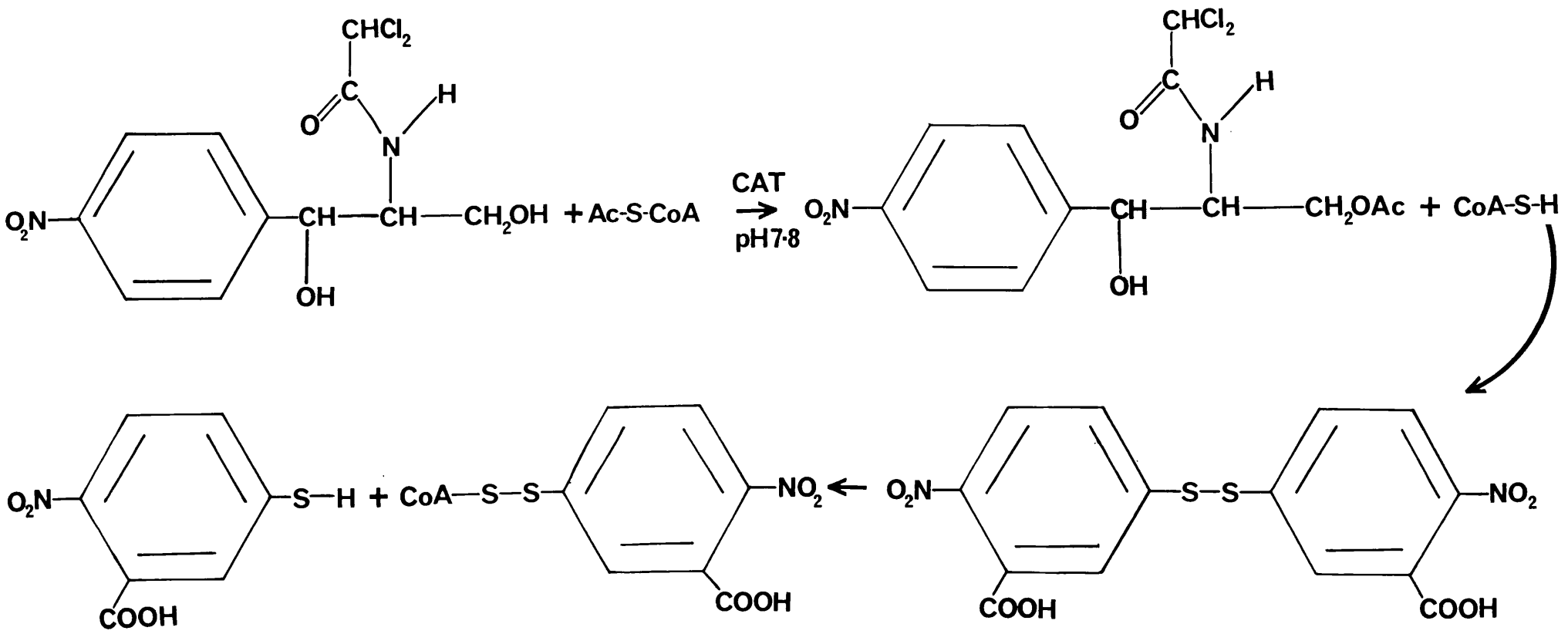


Figure 7.

The basis of the spectrophotometric assay for CAT.

A fresh solution of DTNB was made by dissolving 4mg of DTNB in 9.6ml of 50mM Tris/HCl, 1mM EDTA,pH7.8. Cuvettes containing aliquots of 0.96ml of the DTNB solution were allowed to warm to 37°C in the chamber for a minimum of five minutes and a maximum of fifteen minutes. Then 10µl of 10mM acetyl CoA (paragraph 2.11) was added and allowed to equilibrate for thirty seconds. Next 10µl of enzyme solution was added (if the solution was a crude extract of CAT, the initial rate of acetyl CoA reduction was followed and substracted from the initial rate observed in the presence of chloramphenicol). Finally, 20µl of 5mM chloramphenicol in 50mM Tris/HCl, 1mM EDTA, pH7.8 at 37°C was added and the initial rate of chloramphenicol acetylation was followed for 30 to 60 seconds. The most accurate initial rates were obtained with enzyme dilutions which gave an initial rate of between 0.04 and 0.12 absorbance units per minute and when the chart speed was adjusted to give a trace with a slope of approximately forty-five degrees. The formation of chloramphenicol-1,3-diacetate (paragraph 1.14; Fig. 5) proceeds in the order of one hundred fold slower than the formation of the 3-acetate and is therefore, regarded as having an insignificant effect on this assay (Shaw, 1975). Care must be taken if the enzyme preparation contains mercaptans such as dithiothreitol or 2-mercaptoethanol as these oxidise DTNB and give an immediate increase in A_{412}. This should not, of course, influence the initial rate determination if the absorbance is followed on a chart recorder.

If the observed mean change in A_{412} per minute (subtracting for any background rate in the absence of chloramphenicol) is divided by 13.6 then the result is the number of units of CAT present in to the cuvette (i.e. the number of μ moles of chloramphenicol acetylated per minute at 37°C). The specific activities of the purified CAT variants used in the study were 195, 600 and 570 units per mg for CAT_I, CAT_{II} and CAT_{III} enzymes respectively. The CAT_I and CAT_{III} values being determined from amino-acid analysis (Packman and Shaw, 1981). Whilst the value for CAT_{II} was an estimate based on the units of CAT activity per mg of Lowry determined protein (this study; paragraph 2.9).

Excepting amino-acid analyses all protein determinations are variable with the type of protein assayed (i.e. the BSA standard curve may not equate exactly with the amount of CAT protein). In addition Lowry protein assays are affected by the presence of Tris salts (Lowry et al., 1951). Accordingly, the spectrophotometrically-determined chloramphenicol acetyltransferase activities were deemed more likely to give an accurate quantification of the concentrations of the purified CAT_I and CAT_{III} enzymes and hence were used in preference.

2.13 Purification of chloramphenicol acetyltransferases

The three enzyme variants CAT_I, CAT_{II} and CAT_{III} were purified from separate strains of E. coli C600 carrying the plasmids pBR328, pKT205 and pAH1 respectively. Plasmid pBR328 was used in preference to plasmid pBR325 since it has the same structural gene for CAT_I but its plasmid copy number is elevated 3-fold

(Covarrubias et al., 1981; Table 1b); this plasmid was found to give an approximately 50% increase in enzyme yields. Cells were grown in one to three litres of M56/CAT medium and harvested in the exponential phase of growth (A_{660} = 0.8). M56/CAT medium was used because expression of the gene for CAT_I is sensitive to catabolite repression (paragraph 1.14.3) and CAT_I yields in the absence of glucose or other repressing carbon sources are at least double those obtained in complex media (this study).

Purification of the CAT enzymes used the affinity columns and general methods described by (Zaidenzaig et al., 1979; Packman 1978; Packman and Shaw, 1981a). The affinity resins "K1" and "K2" made by Packman (1978) were each soaked in a solution of 6M guanidine hydrochloride at 4°C for 1 hour. This was to remove any unbound protein that was not eluted during its previous usage. The guanidine hydrochloride was then removed by copious flushing of the resin with distilled water (using a sintered glass funnel, buchner flask and vacuum pump). The water was then flushed out with excess 50mM Tris/HCl, 0.1mM 2-mercaptoethanol, 0.1mM EDTA, pH7.8 (the latter compound was recommended due to its stabilising effect on the CAT_{III} enzyme. K. Kleanthous personal communication). The resin was then allowed to equilibrate in this buffer overnight.

Cells were disrupted (paragraph 2.8.2) and extracts were assayed for chloramphenicol acetyltransferase activity to determine enzyme yields throughout the purification (paragraph 2.12). The freshly-clarified supernatants of the strains that contained CAT_I or CAT_{III} were subjected to a ten minute heat shock. This

was performed in a 60°C water bath by immersion and gentle agitation of a conical flask that held supernatant to one-fifth of its total capacity. The flask was then cooled on ice and the suspension re-centrifuged for thirty minutes at 50,000g and 4°C. Extracts containing CAT_{II} were not similarly treated due to the heat lability of this enzyme.

The crude extracts were then dialysed at 4°C, (to remove intrinsic salt and chloramphenicol) against three changes of five hundred volumes of 50mM Tris/HCl, 0.1mM 2-mercaptoethanol, 0.1mM EDTA, pH7.8, each dialysis lasting at least one hour. After re-assaying the CAT enzyme, 1.0ml of settled, equilibrated, resin was mixed with every 200 units of enzyme. CAT_I was purified using the K1 (low substitution) resin whereas CAT_{II} and CAT_{III} were purified on the K2 (high substitution) resin. Binding of the enzymes to their respective resins was allowed to proceed for ten minutes at 4°C and was monitored by removal of 100µl of the binding mixture. This aliquot was pipetted into a 1.5ml Eppendorf microcentrifuge tube and spun for one minute. Unbound enzyme in the supernatant was assayed and extra equilibrated resin was added to the slurry if further binding was required.

Each bound resin was then poured into a short column and washed with two column volumes of 50mM Tris/HCl, 0.1mM 2-mercapthoethanol, 0.1mM EDTA, pH7.8. The washed resin was then subjected to a second wash of 0.3M NaCl in the same buffer. The resulting column effluent was monitored for protein by its A_{280} against an appropriate NaCl-buffer blank. When the absorbance had

fallen to less than 0.02 units those columns containing CAT_I or CAT_{II} were eluted with buffer containing 0.3M NaCl and 5mM chloramphenicol. The column containing CAT_{III} was similarly treated except that at the point where the A_{280} reached 0.02 units a third wash of several column volumes of 0.6M NaCl-buffer was employed. When the A_{280} had fallen below 0.02 units again this was followed by elution of the enzyme with buffer containing 0.6M NaCl and 5mM chloramphenicol. The elutant was collected in fractions and assayed column fractions of all three CAT variants were stored at -20°C.

Used affinity resin was stored at 4°C in buffer containing 0.02% (w/v) sodium azide.

Each batch of purified enzyme was assayed for homogenity by polyacrylamide gel electrophoresis under denaturing conditions (paragraph 2.14; Figs. 15 and 16).

Before use each stored fraction was dialysed into an excess of buffer to remove salt and chloramphenicol.

21.4 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of proteins was carried under the denaturing conditions of 0.1% (w/v) SDS using a discontinuous buffer system based on that of Laemmli (1970).

15% (w/v) acrylamide/bisacrylamide slab gels (150mm x 140mm x 1.5mm) were prepared as follows.

The lower, separating, gel was poured from a mixture of the following solutions.

(a)	0.75M Tris/HCl, 0.2% (w/v) SDS, pH8.8	13.5ml
(b)	30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide	9.2ml
(c)	distilled water	3.3ml

Solutions (a), (b) and (c) were mixed in a vacuum flask and de-aerated. Next, 950µl of a freshly made solution of 1% (w/v) ammonium persulphate was added, followed by 50µl of TEMED. The resulting mixture was then poured between the glass plates to a level approximately 3cm below the top of the lower plate. It was then overlaid with n-butanol saturated with solution (a) and allowed to set.

The gel was completed by rinsing away the butanol overlay and pouring an upper, stacking, gel. The upper gel had the composition below.

(a)	0.25M Tris/HCl, 0.2% (w/v) SDS, pH6.8	5.00ml
(b)	30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide	0.825ml
(c)	distilled water	3.91ml

After mixing and de-aeration 250µl of freshly made 1% (w/v) ammonium persulphate solution and 15µl of TEMED were added. This was then poured over the lower gel and the gel-slot comb inserted.

Samples (maximum final volume 35µl) containing between 0.02 to 20mg of protein were mixed with 8µl of sample buffer

(0.125M Tris/HCl pH6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 1.4M 2-mercaptoethanol, 0.1% (w/v) Bromophenol Blue), placed in 1.5ml Eppendorf tubes in a boiling water for ten minutes and then cooled on ice prior to loading.

When the acrylamide solution had set and the gel-slot comb had been removed the gel was attached to the vertical electrophoresis equipment. The reservoirs were then filled with running buffer (0.025M Tris/HC1, 0.192M glycine 0.1% (w/v) SDS,pH8.45) and cooled protein samples (see above) were microsyringed into the gel slots. A constant potential difference from cathode to anode was applied at a maximum of 4 Volts/cm (64 Volts) until the dye had passed into the lower gel. The gel could then be subjected to a higher voltage of 8 Volts/cm (128 Volts) and the protein samples were run until the dye reached the bottom of the gel. The total electrophoresis took of the order of five and a half hours. For convenience gels were often run at 3 Volts/cm (42 Volts) overnight.

2.15 Staining of polyacrylamide gels

Polyacrylamide gels were stained in two ways, depending on the amount of protein applied. Those containing 0.2 to 20 µg of protein per gel slot were subjected to Coomassie Blue stain (paragraph 2.15.1), those containing 0.02 to 2µg of protein to silver stain (paragraph 2.15.2). Both staining procedures employed a plastic box to contain the gel and the reagents together with an orbital shaking platform to ensure good circulation and obtain greater sensitivity.

2.15.1 Coomassie Blue stain

This was performed according to Packman and Shaw (1981a). The polyacrylamide gel was soaked for thirty minutes in a solution of 50% (v/v) methanol 10% (v/v) acetic acid, 0.1% (w/v) Coomassie Blue stain R250 (filtered as in paragraph 2.5). The stained gel was then destained in 10% (v/v) acetic acid, 5% (v/v) methanol until the desired amount of unbound dye has been removed.

2.15.2 Silver Stain (based on Merril et al., 1981)

Due to the high sensitivity of this stain gloves were worn throughout the procedure to avoid finger marks. Gels were immersed in 50% (v/v) methanol, 10% (v/v) acetic acid for one hour then transferred to a solution of 5% (v/v) methanol, 10% (v/v) acetic acid overnight. Next day the gels were rinsed in distilled water and then immersed in 10% (v/v) unbuffered glutaraldehyde. The gel was then washed with distilled water for three successive ten minute washes and then soaked in distilled water for two and a half hours.

Silver stain was made thus: 21ml of 0.36% (w/v) sodium hydroxide was added to 4ml of 19.4% (w/v) silver nitrate. The precipitate formed was then dispersed with 2ml of 0.91 specific gravity aqueous ammonia solution. To this was then added 73ml of distilled water. The gel was then immersed in the mixture and was agitated for ten minutes. The gel was then drained, rinsed for two minutes in distilled water and 200ml of 0.0125% (w/v) tri-sodium citrate, 0.2% (v/v) formaldehyde added. When the image had reached the desired intensity (about 30 seconds)

development was stopped with an excess of 1% (v/v) acetic acid. The sensitivity of this method was greatly dependent on the thickness of the polyacrylamide gel and the protocol worked best for thin (0.8mm) gels of 10% or less (w/v) acrylamide/ bisacrylamide.

2.15.3. Drying of stained gels

Stained gels were dryed for autoradiography or storage. The gel was laid on damp Whatmann 3MM paper, covered with cling film and dried with heat for three hours on a gel dryer (Bio-rad) attached to a vacuum pump.

2.16 Mutagenesis

Mutagenesis of bacteria grown in L-broth to exponential phase (A_{660} = 0.8) was carried out using ultra violet (wavelength = 254nm) irradiation with doses of both $100J/m^2$ and $200J/m^2$. Dilutions were taken to determine the percentage of cells killed which, for efficient mutagenesis with this mutagen, is ideally between 95% and 99%.

2.17 Preparation of plasmid DNA

Plasmid DNA was prepared from 1 litre of cells inoculated with 10ml of an overnight culture and grown to exponential phase (A_{660} = 0.8) in L-broth (containing an antibiotic to maintain the plasmid in the cell population).

Two methods were used, the former was a more gentle procedure which was found to be better suited for the isolation of the large R-plasmid DNA molecules.

Mini-plasmid DNA was amplified by the addition of 170µg/ml chloramphenicol overnight (Clewell, 1972), or if the mini-plasmid contained a chloramphenicol resistance gene by the addition of 300µg/ml spectinomycin for the same length of time (Bolivar, 1978).

RNAase was never used due to its deleterious effect on the *in vitro* expression system (paragraph 2.28).

2.17.1 Triton lysis preparation
(adaptation of personal communication) from Dr. B. Ely.

Cells were pelleted at 17,000g for fifteen minutes and resuspended in 24ml of 25% (w/v) sucrose in 50mM Tris/HCl,pH8.0 at room temperature. To this was added lysozyme to a final concentration of 1mg/ml from a fresh 10mg/ml solution prepared in 50mM Tris/HCl,pH8.0. This mixture was kept at room temperature for one minute and then put on ice for five minutes. Next 16.5ml of 0.2M EDTA was gently added and the mixture kept on ice a further five minutes. To this was added 27ml of 2% (v/v) Triton X-100,62.5mM EDTA, 50mM Tris/HCl,pH8.0. After twenty minutes on ice the lysate was vortexed for 8 seconds (three inversions only were used for R-plasmid DNA isolation). This was then centrifuged at 50,000g and at 4°C for thirty minutes (fifteen minutes for R-plasmid DNA).

The supernatant made was then aspirated and ethidium bromide from a 50mg/ml stock solution was added to a final concentration of 0.5mg/ml. This solution was then weighed and 1.12g of caesium chloride was added per gram of supernatant; this gave a relative density of approximately 1.65.

The resulting solution was aliquoted to tubes, paraffin oil added to prevent tube cap erosion, and then each tube was balanced to within 0.1g. The tubes were spun at 120,000g for 48 hours at 20°C. Two dye-bound DNA bands migrated to different positions in the isopynic salt gradient formed. These bands, which are visualised by the fluorescence of ethidium bromide under long wavelength (300nm) ultra-violet light, are the chromosomal (upper) band and the plasmid (lower) band. The plasmid bands of several tubes were removed by syringe and pooled. The pooled bands were then made up to the volume of a single tube with a caesium chloride solution (relative density 1.65) and re-centrifuged under the same, above conditions.

Plasmid DNA was separated from the bound ethidium bromide by extraction with excess propan-2-ol. The caesium chloride was then removed by dialysis (four changes of 1000 volumes of 10mM Tris/HCl, 0.1mM EDTA, pH7.4, each dialysis lasting one hour). Dialysis tubing was prepared according to McPhie (1971).

Dilutions of the plasmid solution were taken and the absorbance at 260nm was measured against an appropriate blank. An A_{260} of 1.0 units was taken to be equivalent to a DNA concentration of 50µg/ml. For more sensitive determinations of DNA concentration as well as purity, a scan of the absorbance of the 220 to 300nm wavelength range was taken. Protein: DNA ratios were estimated using a nomograph constructed by D. Vapnek from the data of Warburg and Christian (1942).

2.17.2. Brij lysis preparation
(adaptation of personal communication from Dr. A.R. Hawkins)

Cells were pelleted at 17,000g for fifteen minutes and resuspended in 40ml of 25% (w/v) sucrose in 0.2M Tris/HCl,pH8.0 at room temperature. To this was added lysozyme to a final concentration of 1mg/ml from a fresh 10mg/ml solution prepared in 0.2M Tris/HCl,pH8.0. This mixture was kept at room temperature for one minute and then put on ice for five minutes. Next 12ml of 0.2M EDTA was gently added and the mixture kept on ice a further five minutes. To this was added 20ml of 1% (w/v) Brij 58 (cetylether), 0.4% (w/v) sodium deoxycholate, 62.5mM EDTA, 50mM Tris/HCl,pH8.0 a few drops at a time with continual swirling of the suspension in order to avoid clumping. After thirty minutes on ice the viscous lysate was centrifuged at 50,000g and at 4°C for thirty minutes. The supernatant made was then aspirated and filtered through an open weave paper towel and collected in a measuring cylinder. To this was added 0.5 volumes of water-equilibrated phenol and 0.5 volumes of trichloromethane: iso-amyl alcohol (24 : 1 v/v). This was mixed by inversion and allowed to stand at room temperature for ten minutes. The mixture was then centrifuged in sealed tubes at 8,000g for five minutes and the aqueous layer removed. To this was added sodium chloride from a 5M stock solution to a final concentration of 0.3M. Next was added 2 volumes of cold (-20°C) 95% (v/v) ethanol, the resulting solution being mixed by inversion. After thirty minutes at -70°C the solution was centrifuged at 17,000g for twenty minutes in sealed centrifuged tubes at the lowest possible temperature setting (-15°C). After centrifugation the supernatant was aspirated and discarded.

The pellet was then drained, dried briefly in a vacuum dessicator and resuspended in 6ml of 0.1M sodium chloride, 10mM EDTA, 50mM Tris/HCl,pH8.0. The resulting DNA suspension was then prepared for isopycnic density gradient centrifugation as outlined in paragraph 2.17.1. With this protocol the pooling and re-centrifugation of plasmid DNA bands was normally omitted as this method yields purer and more concentrated DNA than the Triton lysis preparation.

2.18 Agarose gel electrophoresis

Agarose gels, 0.7 to 2% (w/v) agarose, were prepared by boiling a weighed amount of agarose in the appropriate volume of either of the two electrophoresis buffers given below. Both vertical, and latterly horizontal, agarose gel electrophoresis of DNA was performed. (Maniatis et al., 1982). Vertical agarose gels were run in the same apparatus as polyacrylamide gels (paragraph 2.14) using 3mm spacers and slot combs. The agarose slab was prevented from slipping out from between the plates by a polyacrylamide plug made from 10mls of de-aerated 10% (w/v) cyanogum 41 in agarose gel running buffer plus 40µl of TEMED and 100µl of a fresh solution of 1% (w/v) ammonium persulphate. DNA samples were mixed with an equivalent volume of sample dye (20% (v/v) glycerol, 0.01% (w/v) Bromophenol blue, 5% (w/v) SDS) and applied to the gels.

Two different buffering systems were employed. Tris borate buffer was used for fast-running gels (paragraph 2.18.1) and Tris acetate buffer (paragraph 2.18.2) where the DNA was to be

purified from the gel for further use or when a particularly accurate size determination was called for. Ethidium bromide-stained DNA was visualised under long wavelength (300nm) ultra-violet light.

2.18.1 Tris borate agarose gels

Tris borate buffer,pH8.2 was made in two litre batches at ten times working strength thus:-

Boric acid	110g
Tris	215.6g
EDTA	9.3g
Distilled water	to 2000ml

Agarose gels were made in 1 x Tris borate plus a final concentration of 2µg/ml ethidium bromide and run in 1 x Tris borate buffer without dye. Gels were run at a maximum potential difference of ten volts/cm from cathode to anode.

2.18.2 Tris acetate agarose gels

Tris acetate buffer, pH8.05, was made in two litre batches at ten times working strength thus:-

Sodium acetate	54.4g
Tris	96.8g
EDTA	14.49g
NaCl	21.04g
Distilled water	to 2000ml

The pH was adjusted to 8.05 with glacial acetic acid. Agarose gels made and run in 1 x Tris acetate were stained for 1 hour in the used running buffer supplemented with a final concentration of 2µg/ml ethidium bromide. Gels were run at a maximum potential difference of three volts/cm from cathode to anode.

2.19 Restriction endonuclease digestion

Restriction endonucleases were purchased from Bethesda Research Laboratories, Cambridge and New England Biolabs. DNA digests were performed in 20µl volumes in the suppliers recommended assay buffers. Digests with Pst 1 (EC 3.1.23.31) were performed at 30°C. Those with Taq 1 (EC 3.1.23.39) were performed under paraffin oil at 60°C. Double digests with the enzymes Hind III (EC 3.1.23.21) and EcoR1 (EC 3.1.23.13) were performed by a standard Hind III digestion followed by the addition of 4µl of 500mM Tris/HCl,pH7.5 and the appropriate units of EcoR1 enzyme. Restriction enzymes (except for Taq 1) were inactivatd after digestion by heating at 70°C for five minutes. Taq 1 digests were stopped using an equal volume of water-equilibrated re-distilled phenol followed by extraction with an equal volume of trichloromethane and then ethanol precipitation.

2.20 Estimation of size of DNA fragments

The size of DNA fragments was calculated from agarose gels using the computer algorithm of Schaffer and Sederoff (1981) which is based on a reciprocal plot of mobility vs. size in base pairs. The algorithm requires a set of known standards in order to give a standard error value for the unknown bands. The known standards (paragraphs 2.20.1. and 2.20.3) used are a restriction enzyme digest of plasmid pBR322 (Sutcliffe, 1978) and various digests of bacteriophage λ c1857 S7am (Sanger et al., 1982). Bacteriophage λ DNA was obtained as two separate gifts from Drs. B. Ely and A.R. Hawkins.

Restricted bacteriophage λ DNA was always heated to 70°C for five minutes before use in order to melt the cohesive (cos) ends of the molecule. These can often anneal causing loss of one band and an increase in the size of another and hence can give an erroneous size determination for an unknown restriction fragment.

2.20.1 DNA size fragments from plasmid pBR322 (Sutcliffe, 1978)

Fragment sizes (in base pairs) generated by Taq 1 digestion of plasmid pBR322.

1444
1307
475
368
315
312
141

N.B. It is important to use pBR322 DNA prepared from a dam methylase deficient strain of E. coli (e.g. E. coli GM242; paragraph 2.2) since dam methylation interferes with cleavage at one of the Taq 1 sites. The effect of this methylation is to produce a band of 616 bp that is a combination of the 475 and 141 bp bands (Maniatis et al., 1982; paragraph 3.6.3.).

2.20.2 DNA size fragments from λcI857 S7am (Sanger et al.,1982)

Fragment sizes (in base pairs) generated from bacteriophage λcI857 S7am

Hind III digestion	EcoR1 digestion	Hind III/EcoR1 double digestion
23,131	21,226	21,226
9,418	7,421	5,148
6,557	5,805	4,973
4,361	5,645	4,361
2,322	4,880	4,270
2,028	3,529	2,028
564		1,905
125		1,584
		1,375
		947
		564
		125

2.21 Ligation of DNA molecules

Ligations were performed using T4 DNA ligase (EC 6.5.1.1.). This was either a gift from Dr. B. Ely or was purchased from the Boehringer Corporation Ltd., Lewes, East Sussex. Ligations were carried out in 10µl of the recommended buffer of the latter source (generally with a 3 : 1 molar ratio of insert fragment ends to vector ends) at 16°C for sixteen hours.

2.22 Photography of agarose gels

Photography of ethidium bromide stained agarose gels was carried out using transmitted ultra-violet light (wavelength 300 nm) as described by Maniatis et al. (1982). The film used was either Polaroid Type 57 (ASA 3000 positive film) or Type 55 (ASA 50 positive/negative film). Exposures were made at maximum aperture (f4.5) for between 10 seconds to 5 minutes depending on the film speed, the fluorescence of the gel and the degree of solarization of the transilluminator.

2.23 Transformation of E. coli with plasmid DNA (after Kushner, 1978)

A single colony from a minimal medium selective plate was inoculated into 5 ml of L-broth. The next day 100µl of this overnight culture was inoculated into 10 ml of fresh sterile L-broth in a 100 ml conical flask. When the A_{660} had reached 0.4 units (about three hours) aliquots of 1 ml were withdrawn and pipetted into 1.5 ml Eppendorf microcentrifuge tubes. The tubes were centrifuged for one minute and the supernatant aspirated. The resulting pellet of cells was then gently resuspended (by flicking the tube) in 1 ml of 10mM morpholinopropane sulphonic acid (MOPS), 10mM rubidium chloride (RbCl), pH7.0. This was then re-centrifuged for one minute and the cells gently resuspended in 1 ml of ice-cold 100mM MOPS, 15mM calcium chloride, 10mM RbCl, pH6.5. The tube was held on ice thirty minutes and re-centrifuged for one minute. The pellet of cells was then resuspended in 200µl of the above pH6.5 buffer and undigested or ligated plasmid DNA (0.05 to 0.5 µg) was added in a maximum volume of 20 µl of reaction buffer (larger volumes were ethanol precipitated). This mixture was then held on ice a further thirty minutes and submitted to a heat shock by immersion of the tube in a 55°C water-bath for forty-five seconds. Immediately afterwards, 1 ml of L-broth at 37°C was gently added and the culture incubated, without shaking, for thirty minutes at 37°C. The cells were then pelleted by centrifugation and the pellet resuspended in 110 µl of fresh L-broth. Two one hundred-fold dilutions in L-broth were made of this culture and 100 µl of each of the three serial dilutions was then plated onto selective media. Well over 10^6 transformants were reproducibly obtained per microgram of supercoiled plasmid pBR322 DNA when

E. coli C600 was the transformed strain. However, with *E. coli* DB10 the level of transformation fell to around 10^4 transformants/μg.

2.24 Purification of DNA restriction fragments

DNA restriction fragments were purified from ethidium bromide stained Tris acetate gels (paragraph 2.18.2). The size of bands visualised under ultra-violet light (wavelength 300 nm) was determined (paragraph 2.2) and the appropriate band was cut out of the gel with a scalpel blade. The gel slice was then placed inside a dialysis bag (prepared according to McPhie, 1971) and the bag filled with Tris acetate electrophoresis buffer. DNA was electroeluted from the gel slice on the horizontal gel apparatus as described by Maniatis *et al*. (1982). DNA samples were then extracted with an equal volume of water-saturated *n*-butanol followed by an equal volume of water-saturated re-distilled phenol and then ethanol precipitated.

2.25 Sephadex G50 cleaning procedure for DNA samples

An essentially RNAase-free 1mM Tris acetate, 0.1mM EDTA, 8% (w/v) Sephadex G50 suspension (pH7.5) was prepared as follows.

A 150 ml medical flat bottle was baked at 165°C overnight. This was filled with 100 ml of a 1% (v/v) solution of diethylpyrocarbonate (DEPC) in distilled water. The bottle was then shaken for three hours at room temperature and sterilised in the autoclave. To this was added 12.1 mg of Tris, 3.72 mg of EDTA and 8.0g of Sephadex G50 resin. This was then adjusted to pH7.5 with glacial acetic acid and re-autoclaved.

Glass wool was siliconised by immersion in dimethyldichlorosilane. The excess fluid was drained and the glass wool was then baked at 165°C overnight. A number of 1.5ml Eppendorf tubes were shaken in 1% (v/v) DEPC in distilled water for three hours, drained, autoclaved and dried. The top was then cut off of one of these tubes and placed in the bottom of a 15 ml Coring centrifuge tube. A 1 ml disposable plastic syringe was removed from its sterile packing and plugged with siliconied glass wool. The syringe was then filled with 1 ml of Sephadex G50 suspension and allowed to drain of excess buffer for five minutes. The syringe column was then placed in the 15 ml Coring tube so that the tip of the syringe was inside the Eppendorf tube and the "ears" of the syringe lay across the rim of the Coring tube.

The DNA solution to be cleaned (10 µg covalently-closed plasmid; maximum volume 100 µl) was applied to the top of the column and the whole assembly was then spun at 5,000g for four minutes. The resulting column was dry and the Eppendorf tube contained approximately 520 µl of DNA solution. This was then microcentrifuged at room temperature for three minutes to pellet any resin that has spun through the glass wool. The supernatant was then aspirated and ethanol precipitated (usually in two separate tubes thereby allowing a control and/or spare sample to be simultaneously prepared).

2.26 Nick translation

Nick translation was carried out on a purified restriction fragment that was cleaned by the Sephadex G50 procedure (paragraphs 2.24 and 2.25) and used an adaption of an unpublished

procedure by Dr. A.R. Hawkins.

The nick translation reaction (final volume 100 µl) consisted of 1 µg DNA in 50mM Tris/HCl (pH7.8), 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 50 µg/ml BSA, 25 µM dCTP, 25 µM dGTP, 25 µM TTP and 50 µ Ci 5' (α-^{32}P)dATP (400 Ci/m mole; i.e. final concentration = 1.25 µM). The reaction was started by the addition of 2 units of E. coli DNA polymerase I (Boehringer Corporation, Lewes, East Sussex) and was incubated at 20°C for forty minutes. Nick translation was halted by heating the incubation at 65°C for fifteen minutes.

An 8% (w/v) slurry of swelled Sephadex G100 was prepared by autoclaving a suspension of the resin in 10mM Tris/HCl, 0.1mM EDTA, pH8.0. A 10 ml separation column was then prepared from this slurry in a plastic disposable pipette which was plugged with siliconised baked glass wool (paragraph 2.25).

The nick translated DNA was separated from the unincorporated nucleotide by applying the incubation products to the top of the above G100 column and eluting through with 10mM Tris/HCl, 0.1mM EDTA, pH8.0 buffer. The radioactive nick translated DNA was eluted in the void volume and its progress down the column was monitored with a hand held Geiger-Muller counter. The unincorporated nucleotides were retained by the resin. A small aliquot of the eluted DNA was then counted in Fisofluor 1 scintillation fluid (Fison's Ltd., Loughborough, Leics.) and the cpm/µg of DNA determined.

2.27 Southern blot analysis

DNA:DNA hybridisation analysis (Southern, 1975) was carried out on restriction fragments using a method adapted from an unpublished procedure by Dr. A.R. Hawkins. All manipulations were performed wearing latex gloves.

DNA restriction fragments were electrophoresed on an 0.8% (w/v) agarose gel Tris acetate gel which was stained with ethidium bromide (paragraph 2.18) and photographed (paragraph 2.22). The gel was next soaked in 0.2M sodium hydroxide, 0.6M NaCl for thirty minutes and then immersed in 1.0M Tris/HCl, 1.5M NaCl, pH7.4 for a further thirty minutes. (This procedure makes the DNA single-stranded and promotes efficient transfer of the restriction fragments).

A 3.0M NaCl, 0.3M trisodium citrate solution, pH7.0 was prepared (= 20 x SSC). A piece of nitrocellulose paper (Schierer and Schuell GmbH, West Germany) the size of the agarose gel was then soaked in a one-tenth dilution of this solution (2 x SSC) for fifteen minutes. Twenty large sheets of Whatmann 3MM filter paper were soaked in 20 x SSC and placed in the bottom of a large plastic box. On top of these was placed a sheet of "Saran" wrap with a hole in the centre just smaller than the agarose gel. The gel was then placed over the hole and the soaked nitrocellulose filter was laid on top. The filter was then overlain with two sheets of Whatmann 3MM paper that had been soaked in 2 x SSC. The whole assembly was finally covered with a large wad (> 10cm deep) of paper hand towels and held down by a 1kg weight. The agarose gel was allowed to transfer

its DNA to the nitrocellulose by capillary action at 4°C overnight.

After blotting the nitrocellulose filter was removed from the gel and washed in 2 x SSC for five hours. At this stage both the filter and the agarose gel were checked for DNA transfer under ultra-violet light . The filter was then held on a piece of dry Whatmann 3MM paper with paper clips and baked in an oven at 80°C for four hours.

The baked filter was then unclipped and incubated at 65°C for five hours in a mixture of 30ml of 10 x Denharts solution (0.2% (w/v) ficoll-400, 0.2% (w/v) bovine serum albumin, 0.2% (w/v) polyvinyl pyrrolidone) plus 30ml of 3 x SSC.

Nick-translated probe DNA, $\geq 10^6$ cpm (paragraph 2.26) was made up to 6ml with distilled water and heated in a boiling water bath for five minutes to convert the DNA to single strands. This was then mixed with 3 ml of 20 x SSC and 1 ml of 10 x Denharts solution to give the hybridisation mix.

The nitrocellulose filter was drained, placed inside a small heat-sealable plastic food pouch and the hybridisation mix (at 65°C) added. The bound DNA and the probe were then allowed to hybridise at 65°C for sixteen hours.

Just prior to ending hybridisation 500 ml of a 0.1 x SSC, 0.005% (w/v) SDS stop solution was prepared and heated to 55°C. The hybridisation bag was then opened (and the mix retained for

potential re-use) and the filter transferred to the stop solution. The stop solution wash was performed at 55°C for thirty minutes and then repeated three times, the final wash being overnight.

The filter was then air dried and placed in a film casette against a sheet of film (Fuji X-ray RX). Autoradiography was carried out at -80°C for ten to one hundred hours depending on the degree of hybridisation and the specific radioactivity of the probe. The film was then processed using Kodak "D-19" developer with May and Baker "Amfix" fixer according to the manufacturer's instructions.

2.28 In vitro coupled transcription and translation

Cell-free coupled transcription and translation studies were carried out using a method based on that of Pratt et al. (1981).

Extracts from E. coli MRE600 (RNAase I^-, prototrophic, group 0, intrinsically fusidate tolerant) and the "low molecular weight mix" (LMM) were gifts from Dr. J. Pratt.

All apparatus was soaked in 1% (v/v) diethylpyrocarbonate (DEPC) overnight and was autoclaved and dried to remove RNAase contamination. All solutions were made from distilled water that had contained 1% (v/v) DEPC for at least twelve hours and which had subsequently been autoclaved twice. Solutions were adjusted to the desired pH by using either an RNAase-free 2M Tris solution or glacial acetic acid. This was because of the inhibiting effect of chloride ions on the in vitro system.

Problems with the level of incorporation of the L-[^{35}S]-methionine into de novo synthesised protein which appeared to depend on the individual preparation of the plasmid DNA were removed by using the DNA purification procedure outlined in paragraph 2.25.

Incubations, in 1.5ml Eppendorf tubes, consisted of 5µg of covalently closed plasmid DNA in 13.5µl of 1mM Tris acetate, pH8.2 together with 7.5µl of LMM, 3.5µl of 0.1M magnesium acetate and 1.5µl of L-[^{35}S]-methionine (1320 Ci/mol, approx. 15µ Ci). This suspension was then vortexed for five seconds and the reaction started by the addition of 5µl of E. coli MRE600 "S30" extract (which was gently mixed by flicking). Incubations were normally performed in a shaking water bath at 37°C.
After thirty minutes the incubation was "chased" by the addition of 5µl of non-radioactive L-methionine (44mg/ml) for five minutes at 37°C.

The incubations were then placed on ice and the amount of radioactive L-methionine incorporated into protein was determined. Samples of 2µl were withdrawn from each tube and spotted onto 2cm diameter Whatmann No. 1 filter discs. The discs were then immersed in boiling 10% (w/v) trichloroacetic acid (TCA) for five minutes (15ml per filter) and washed twice with the same volume of industrial methylated spirits. After drying, along with an untreated control, the filters were counted to toluene scintillant (0.5% (w/v) 2,5-Diphenyloxazole, 0.03% (w/v) 2,2'-p-Phenylene-bis[5-phenyloxazole]). Good incubations were those which retained more than 25% of the initial radioactive counts as TCA-precipitable (i.e. protein-incorporated) counts.

15% (w/v) acrylamide/bisacrylamide gels were prepared (paragraph 2.14) and up to 17.5µl of the incubation mix in an equal volume of sample buffer, could be loaded. For overnight autoradiography (see paragraph 2.27) incubation mix equivalent to 5×10^5 cpm of incorporated counts was applied per track. For lower incorporations, provided that the level of incorporation was five-fold higher than that of the no DNA control incubation, proportionately longer exposures were necessary. Relative molecular mass markers (Sigma MW-SDS-70:- bovine serum albumin, ovalbumin, trypsinogen, β-lactoglobulin, lysozyme) were also loaded. These were identified by Coomassie Blue stain (paragraph 2.15.1) and were marked using ink that was mixed with surplus ^{14}C compounds on the dried gel (paragraph 2.15.3) prior to autoradiography (as in paragraph 2.27).

2.29 Chromatographic investigation of fusidate modification
(after Chopra, 1976 and W. von Daehne personal communication)

Ten, 250ml, conical flasks containing 40ml of 2YT broth were prepared with and without a subinhibitory concentration of sodium fusidate (1µg/ml). Eight of these were inoculated with single colonies of strains of E. coli DB10 and E. coli C600 carrying no plasmid, pBR328, pKT205 or pAH1, the plasmids specifying respectively CAT_I, CAT_{II} and CAT_{III}.

The flasks were shaken for twelve hours at 37°C and sodium fusidate was then added to a final concentration of 40µg/ml. After a further three hours of shaking the total lipids of the culture were extracted using the solvent ratios of Bligh and Dyer (1959). The method was to take 2ml of culture (containing 80µg of fusidate) and to mix this with 2.5ml of trichloromethane

and 5ml of methanol. This was then vortexed for two minutes and 2.5ml of trichloromethane added. After thirty seconds of vortexing 2.5ml of water was added and the mixture vortexed a further thirty seconds. This mixture was then filtered through siliconised glass wool and collected in a small tube which was spun at 17,000g inside a second sealed centrifuge tube for thirty minutes. The alcoholic layer and some of the trichloromethane interface was then aspirated off and 3.125ml of trichloromethane phase (containing 50µg of fusidate) was removed and evaporated to dryness under vacuum. This residue was then resuspended in 5µl of trichloromethane and subjected to thin-layer chromatography. The solvent system used was trichloromethane:cyclohexane:methanol;acetic acid (32:4:1:4, by vol.) and the thin-layer chromatography plates were Merck silica gel 60 F_{254} (20cm x 20cm). The controls of sodium fusidate extracted from water and extracted from the two sterile 2YT medium flasks(with and without the initial 1µg/ml sodium fusidate) and incubated under the same conditions were simultaneously chromatographed. After chromatography the plates were heated at 110°C for ten minutes then submerged in concentrated sulphuric acid for one minute. The detection limit of this method is of the order of 0.25µg of compound. Fusidic acid is observed as a red to purple spot; other fusidate analogues show as red, yellow or brown spots. The sensitivity of this method is such that it can be expected to discern as little as 0.5% modification of the antibiotic.

2.30 Ribosome-dependent GTP hydrolysis (after Bodley et al., 1970a)

Elongation factor G was prepared by Dr. J. Bodley according to the method of Rohrbach et al. (1974) and was a gift from Dr. E. Cundliffe. Ribosomes were prepared by a modification of the method of Cundliffe et al. (1979) and were a gift of Mr. S. Rae.

The reaction mixture (final volume 75µl) contained 15mM Tris/HCl, pH7.8; 10mM magnesium sulphate, 80mM ammonium chloride, 2mM 2-mercaptoethanol, 10 nmol [γ^{32}P]-GTP (approx. 1.5 x 10^6 cpm) and 50pmol elongation factor G. This was pre-warmed to 37°C for ten minutes and the reaction started by the addition of 1pmol (10µl) of ribosome solution. Sodium fusidate and purified CAT were included when required before the addition of the ribosome solution. Three 20µl samples were removed at the end of each incubation (37°C for twenty minutes) and mixed with 20µl of ice-cold 1M perchloric acid in a 1.5ml Eppendorf tube. After ten minutes on ice, 200µl of 5% (w/v) activated charcoal (prepared by the method of Thompson, 1960) was added ~~to~~ and each tube ~~and~~ vortex mixed for thirty seconds to adsorb free ribonucleotides. After three minutes microcentrifugation 100µl of the supernatant containing the hydrolysed radioactive inorganic phosphate was removed for scintillation counting (Fisofluor 1 scintillant; Fisons Ltd., Loughborough, Leics.). A control incubation lacking elongation factor G was included to estimate the extent of background ribosome-independent GTP hydrolysis.

2.31 Equilibrium dialysis

Equilibrium dialysis was performed at 4°C in two separate eight-cell dialysis modules (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Each cell in the apparatus was divided into two 0.5ml chambers by a dialysis membrane with a 12,000 to 14,000 relative molecular mass (M_r) cut off. On one side of the membrane was a fixed concentration of each CAT variant (M_r = approx. 102,500) in 50mM Tris/HCl, pH7.8, 0.1mM 2-mercaptoethanol, 0.1mM EDTA. The opposite chamber contained a variable concentration (0 to 125µM) of the diethanolamine salt of tritiated fusidic acid in the same buffer. Two control places were reserved in each experiment to check (a) that equilibrium had been achieved and (b) the activity of CAT after dialysis.

The modules were then rotated at ten revolutions per minute for ninety hours to allow the highest concentration of fusidate to achieve equilibrium. After dialysis five duplicate samples of 50µl were withdrawn from both sides of each pair of chambers (progressing from lowest to highest fusidate concentration to minimise carry-over errors) and counted in 4ml of Bray's fluid (Fox, 1976). A large volume of this scintillation fluid was used in order to minimise quenching errors. After calculation of the ligand concentrations present after dialysis the resulting data were plotted according to Scatchard (1949). Due to the large amount of data that several of these experiments generate a computer programme was written to save time and increase accuracy (paragraph 2.32).

2.32 Computer programme for equilibrium dialysis data

This computer programme is for the BBC Acorn machine (BBC basic language) and was written with the assistance of Mr. J. Keyte.

The programme does the following:

a) Subtracts a mean environmental background count from each mean tritiated fusidate count and from each tritium standard count.

b) Determines the mean counting efficiency of the scintillation counter against five duplicate tritium standards and prints out the result. (In this case tritiated uridine was used as a surplus supply was available.)

c) Calculates for each cell from the above data the concentration of tritiated fusidate present on both the binding-protein side and on the protein-free side of the dialysis membrane.

d) Plots the concentration data according to Scatchard (1949) and fits a regression line to the resulting set of fourteen plot points. (This appears on the television monitor and can subsequently be transferred to "hard copy" on paper).

e) Finally, the machine prints out the number of tritiated fusidate molecules bound per enzyme monomer (i.e. the stoichiometry of binding) and the strength of this binding (i.e. the equilibrium binding constant, K_d) or in the case of competition equilibrium dialysis experiments the apparent strength of tritiated fusidate binding (i.e. the K_{da} value, see paragraph 6.5).

2.32 Equilibrium dialysis programme

```
 10  DIM COUNTS (29, 4)
 15  DIM X (30)
 16  DIM H (30)
 17  DIM V (30)
 20  DIM MEAN (29)
 25  DIM CORMEAN (29)
 30  FOR I = O TO 29
 35  LET T = O
 40  FOR J = O TO 4
 50  READ COUNTS (I, J)
 60  LET T = T + COUNTS (I, J)
 70  NEXT
 80  LET MEAN (I) = T/5
100  NEXT
110  FOR A = O TO 29
120  CORMEAN (A) = MEAN (A) - MEAN (O)
140  NEXT
150  FOR D = 2 TO 28 STEP 2
160  X (D) = CORMEAN (D) - CORMEAN (D-1)
190  NEXT
195  PRINT "TYPE CONTROL-N"
200  INPUT "AMOUNT OF STANDARD?" STD
210  LET EFF = CORMEAN (29) *100/(STD*2.22*10↑6)
215  PRINT "EFF=" EFF
220  INPUT "VALUE OF F?", F
230  LET L = F*EFF*13805.514
240  INPUT "CAT CONC?", CAT
250  FOR N = 2 TO 28 STEP 2
260  LET H(N) = X(N)*20000/(CAT*L)
270  LET V(N) = X(N)/(CAT*CORMEAN (N))
275  LET @ % = 131850
280  PRINT TAB (2) H(N), TAB (20) V (N)
285  PRINT:
290  NEXT
300  LET SUM X = O
310  LET SUM Y = O
320  LET SUM XY = O
330  LET SUM XX = O
340  FOR N = 2 TO 28 STEP 2
350  SUM X = SUM X + H(N)
360  SUM Y = SUM Y + V(N)
370  SUM XY = SUM XY + (H(N)*V(N))
380  SUM XX = SUM XX + (H(N)*H(N))
390  NEXT N
400  LET b = (SUM XY-((SUM X*SUM Y)/14)/(SUM XX-(SUM X*SUM X)/14)
410  LET a = (SUM Y/14)-(b*(SUM X/14))
420  PRINT "NO. PER MONOMER =" -a/b
430  PRINT "Kd or Kda="  - 1/b
440  YIST = (b * 0.2) + a
450  YSEC = (b * 0.8) + a
460  PRINT YIST, YSEC
470  MODE 5
480  MOVE 200, 860
490  MOVE 200, 160
500  DRAW 200, 860
```

2.32 Equilibrium dialysis programme continued

```
510   MOVE 1200, 160
520   DRAW 200, 160
521   FOR A = 140 TO 159
522   PLOT 69, 700, A
523   PLOT 69, 1200, A
524   NEXT A
525   FOR A = 180 TO 199
526   PLOT 69, A, 260
527   PLOT 69, A, 360
528   PLOT 69, A, 460
529   PLOT 69, A, 560
530   PLOT 69, A, 660
531   PLOT 69, A, 760
532   NEXT A
550   FOR N = 2 TO 28 STEP 2
560   PLOT 69, (200 + (H(N)*500)), (160 + (V(N)*10000))
570   PLOT 69, (200 + (H(N)*500)), (160 + (V(N)*10000))
580   NEXT N
590   PLOT 69, 200, (160 + (a * 10000))
600   PLOT 69, (200 + ((-a/b) * 500)), 160
610   DRAW 200, (160 + (a * 10000))

1000  DATA (5 values of background cpm≡50µl buffer)
1010  DATA (5 values of cpm from the CAT enzyme side
        of membrane, first chamber)
1020  DATA (5 values of cpm from the CAT free side of
        membrane, first chamber)
1030→ 1280 Repeat above for remaining 13 chambers
1290  DATA (5 values of a standard tritium concentration
        in 50µl buffer)
```

2.32 All samples were counted for at least 100,000 counts to minimise statistical counting errors. If this count proved to be an impracticably long time then the sample was counted for ten minutes.

The raw data were typed into the computer programme from line 1000. in their sets of 5 duplicate samples as indicated overleaf. Each value entered was separated by a comma.

On completion of the data entries the command "RUN" was executed and the data were read. The programme then requested the amount of tritium counting standard used in the experiment by diplaying the "AMOUNT OF STANDARD?" prompt. I then fed in the most accurate value possible in microcuries of my tritium standard. This was generally around 0.4μCi and was corrected for radioactive decay. The counting efficiency was then displayed to three decimal places as "EFF="; this was typically around 29%.

The next input prompt was "VALUE OF F?". F stands for fusidate and, in my case, was the specific radioactivity, corrected for decay, of my tritiated fusidate ligand in μCi/mg.

The final prompt each time the programme was run was "CAT CONC?" and here the value entered was the micromolar concentration of CAT monomer present after the ninety hour dialysis. The concentration was determined by the spectrophotometric CAT assay (paragraph 2.12) and was the mean of five estimations (taken from the fusidate-free control chamber). Typically around 7% of CAT activity is lost during the course of the experiment. It was

thought more accurate to use the end-point value of enzyme concentration rather than the initial value, since this is more likely to equate to the concentration of catalytically active enzyme.

The rest of the programme then ran automatically and gave the stoichiometry of the ligand binding ("No. per monomer=") and the affinity or apparent affinity ("Kd or Kda=") depending on whether the experiment performed was a single ligand or a competing ligand dialysis.

The methodology that I used for these experiments may seem somewhat over-arduous but it must be borne in mind that the Scatchard plot (Scatchard 1949) employs some of the same terms on both of its axes. If this intrinsically error prone determination of the equilibrium dissociation constant is then used to determine a subsequent equilibrium constant from a competition dialysis experiment (paragraph 6.5) the errors involved become greatly magnified. It was, therefore, extremely important to minimise sampling, counting and rounding of number errors.

Since the programme was designed to look at fusidate binding the cpm to molarity calculation employed a term which was derived from the molecular weight of the tritiated fusidic acid diethanolamine salt that was used. To use the programme for a different ligand the line listed at 230 must be modified accordingly.

2.33 Synthesis of D(-) threo-1-(p-nitrophenyl)-2-butryamido-1,3-propanediol

D(-) threo-1-(p-nitrophenyl)-2-butryamido-1,3-propanediol was synthesised by a method based on the outline given by Rebstock (1950).

Note similarities to the Croynecin family of antibiotic compounds. Corynecins I→III, Nakano et al. (1976). Corynecin IV = Corynecin I-3-acetate and Corynecin V = Corynecin II-3-acetate (Chemical Abstracts, 1983).

1g of chloramphenicol base (Cm-base. D(-) threo-1-(p-nitrophenyl)-2-amino-1,3, propanediol) was added to 3.0ml of n-butyric anhydride in a 25 ml pear-shaped reaction flask. Anti-bumping granules were added and the mixture refluxed on a steam bath for twenty minutes. The solution was then removed from the steam bath and allowed to cool for ninety minutes in the fume hood. Ice-cold distilled water (50ml) was then added to hydrate the excess anhydride. Thorough mixing was obtained by vortexing the solution for thirty seconds. After one hour on ice the reaction mixture was extracted three times with 50ml of ethyl acetate. The combined extracts were then pooled and decanted into a separating funnel and washed three times with 150ml of 5% (w/v) sodium bicarbonate. This was followed by a further wash with 100ml of distilled water. The ethyl acetate extract was then evaporated to a yellow gum in a rotary evaporator (the evaporation flask being held in a water bath at 40°C and the vacuum being supplied by a water pump). The gum was then dissolved in 100ml of cold (-20°C) acetone and 100ml of cold (4°C) 0.2M sodium hydroxide was added dropwise with thorough mixing. During the whole procedure the flask was held in an ice/salt water bath. (This step serves to remove butryl groups which have been esterified to the Cm-base). The mixture was then left in the ice/salt water bath for one hour and the solution was then neutralised with 2.5M sulphuric acid. Next the solution was returned to the rotary evaporator and partially evaporated to remove the acetone. The aqueous residue was then made acidic (pH3.0) by the addition of further 2.5M sulphuric acid. This was then extracted with two volumes of 200ml of ethyl acetate and the

extracts pooled. The combined extracts were then washed four times with 50ml of 0.5% (not 5%) (w/v) sodium bicarbonate. The extracts were then evaporated to a gum that was subsequently dissolved in 10ml of 1,2-dichloroethane. The product then crystallised out at room temperature. (This process may be "seeded" with crystals from a previous preparation or with a few crystals of Cm-base.) The yield from this preparation is approximately 70%.

To test for purity a few crystals were dissolved in ethyl acetate and applied to a thin layer chromatography plate. (Merck silica gel 60 F_{254}, 20cm x 20cm). The plate was also spotted with control compounds and developed in trichloromethane: methanol (9 : 1, by vol). The 2-butryamido analogue has an Rf (c.0.35) between chloramphenicol (c.0.4) and Cm-base (which remains at the origin). Any esterified compounds produced migrate to an analogous position just behind that of chloramphenicol-1-acetate (Rf c.0.5), chloramphenicol-3-acetate (Rf. c.0.7) and chloramphenicol-1,3-diacetate (Rf. c.0.9) respectively. (The acetates of chloramphenicol may be obtained from the chloramphenicol containing growth medium of CAT - containing strains by extraction with ethyl acetate, Dang-Van et al., 1978; paragraph 1.14.1). Esterified contaminants may be removed by returning to the step where the compound is dissolved in acetone and by following the protocol through to the end. The compound produced by this method co-migrates in the thin layer chromatography system above with a sample of authentic D(-) threo-1-(p-nitrophenyl)-2-butryamido-1,3, propanediol which was obtained from Dr. M. Rebstock. (Parke- Davis Ltd.)

Preliminary infra-red spectral and nuclear magnetic resonance studies (performed in the Department of Chemistry, University of Leicester) are also consistent with this structure.
(N.B. The compound was dissolved in tetrachloromethane for the latter study.)

For a near complete listing of chloramphenicol analogues which have been synthesised chemically and some of the properties of these compounds see Shaw (1983).

RESULTS

CHAPTER THREE

Fusidic acid resistance encoded by R-plasmids

3.1 Fusidate resistance and chloramphenicol acetyltransferase

The study of the fusidic acid resistance phenotypes of naturally-occurring R-plasmids performed by Datta et al. (1974) found that the fusidic acid and chloramphenicol resistance phenotypes are commonly carried on the same plasmid but can be observed separately (paragraph 1.15).

Among the R-plasmids there was a group where both antibiotic resistance phenotypes were present. Of these a number had been earlier investigated to determine if their chloramphenicol resistance was due to chloramphenicol acetyltransferase (CAT; Foster and Shaw, 1973).

It was found that where dual antibiotic resistance was present the plasmid always carried the type I CAT variant (CAT_I) and fusidic acid resistance was never associated with the other two enterobacterial enzyme variants CAT_{II} and CAT_{III}. The latter observations are noteworthy in view of the many close similarities which exist between the three proteins (Gaffney et al., 1978; Zaidenzaig et al., 1979 and reviewed by Shaw, 1983 ; paragraph 1.14.2.).

3.2 Immunological screening for CAT_I

All of the R-plasmids used by Datta et al. (1974) that encoded either fusidate resistance or chloramphenicol resistance or

which gave rise to both resistance phenotypes were obtained. (With the exception of R-plasmid JR73).

Because of the low viability of R-plasmid-carrying strains of E. coli DB10 the R-plasmids were used in the host strains that they were supplied in (Table 1a).

Ouchterlony double diffusion tests (Ouchterlony 1949; paragraph 2.10) were carried out on those strains whose chloramphenicol resistance genotype was unknown.

The double diffusion plate shown (Fig. 8) indicates that the R-plasmids JR70, JR72 (both dually resistant) and R455 (probably sensitive to fusidate - not confirmed; Datta et al., 1974) all make a protein antigenically identical to CAT_I.

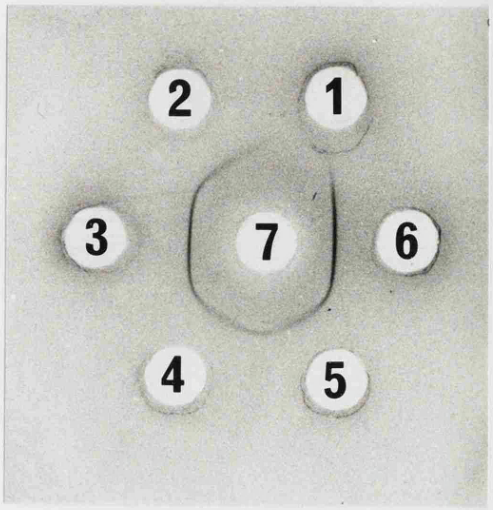
Additionally the plate demonstrates that the fusidate-resistant, chloramphenicol-sensitive R-plasmid R28 make no CAT_I-related proteins.

3.3 The"two genes, one promoter" model

On the basis of the preceding data and the above experiment it appeared that the chloramphenicol resistances encoded by the CAT variants CAT_{II} and CAT_{III} were not associated with fusidate resistance. In contrast the CAT_I enzyme was associated with fusidate resistance but was not the actual effector of the resistance phenotype.

Figure 8.

Ouchterlony test to detect the presence of CAT_I protein



Antigens

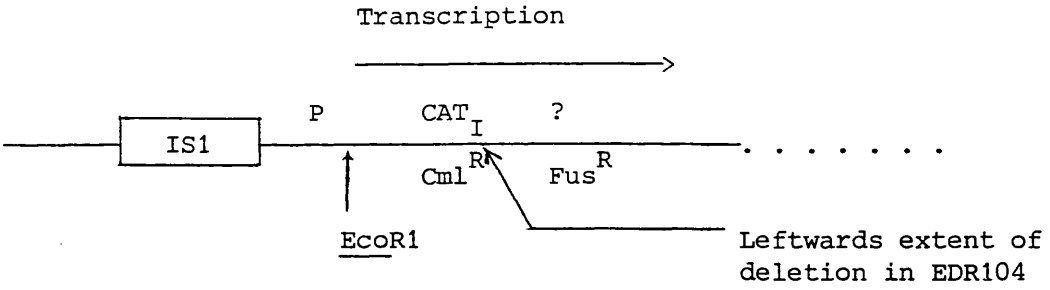
1. R28/E. coli J53
2. JR70/E. coli J53
3. JR72/E. coli J53
4. R455/E. coli J53
5. JR70/E. coli J53
6. JR72/E. coli J53

Antibody

7. IgG versus pure CAT_I protein from JR66b.

This conclusion was supported by the work of Dempsey and Willetts (1976) who reported the isolation from the dually-resistant R-plasmid R100, of a single deletion mutant (EDR104) which had lost fusidate resistance but retained low level chloramphenicol resistance.

Subsequent restriction enzyme mapping of R100 and the related R-plasmid R6-5 and cloning of these fragments (Lane and Chandler, 1966; Miki et al., 1978; Timmis et al., 1978a and b; paragraph 1.16) lead to a modification of the two separate genes model. The refinement proposed was that the structural genes for the chloramphenicol and fusidate resistance effectors were distinct but formed part of a single operon transcribed from a common constitutive promoter, thus:-



The EcoR1 enzyme restriction site was envisaged to cleave the Cml^R-FUS^R operon in or before the structural gene for $Cml^R(CAT_I)$ and result in the absence of expression of the Fus^R structural gene.

3.4 The R-plasmid R28

As stated above (paragraph 3.2) the R-plasmid R28 carries the fusidate resistance phenotype but does not encode chloramphenicol resistance.

A possible explanation of this finding consistent with the above model is that R28 carries a mutant CAT_I structural gene which no longer directs the synthesis of an active enzyme.

Furthermore the mutant CAT_I protein lacks sufficient immunological cross-reactivity to the native CAT_I protein to be detected by the Ouchterlony test (paragraph 3.2; Fig. 8). In addition the defective CAT_I protein fails to effect chloramphenicol resistance but the dual-cistronic mRNA is still synthesised and is still translated to yield the fusidate resistance effector protein.

The rationale is supported by the circumstantial evidence that R28 is in the same incompatibility group (FII; paragraph 1.14.2) as the majority of the CAT_I-encoding plasmids and is, therefore, closely related to them.

3.5 Conflict between DNA sequence and mapping data

The genetic mapping data above suggested two co-transcribed antibiotic resistance genes.

Subsequent DNA sequence analysis of the small fusidate-resistant chloramphenicol-resistant transposon, Tn9 (Alton and Vapnek, 1979b; Fig. 6) pointed to the contradictory conclusion that CAT_I was the effector of both antibiotic resistances. (Cleavage of the DNA at the EcoR1 site causing disruption of the CAT_I structural gene and hence loss of synthesis of the CAT_I enzyme and thereby loss of both antibiotic resistance phenotypes.)

Further DNA sequencing studies (paragraph 1.16) taken together demonstrated that a 906 bp fragment of transposon Tn9 is sufficient to promote and encode both the chloramphenicol and fusidate resistance phenotypes. The only open translational reading frame capable of encoding a polypeptide of more than sixty-five amino acid residues is that which codes for the CAT_I enzyme. A bifunctional role for the CAT_I enzyme as the effector of both antibiotic resistance was hence thought to be highly probable.

3.6 Experiments with R-plasmid R28

The evidence in favour of the "two genes, one promoter" model (paragraph 3.3) was essentially two fold. Firstly with R-plasmid R28 the presence of CAT_I could be dissociated immunologically from fusidate resistance (paragraph 3.2). Secondly, an uncharacterised mutation of R100 appeared to lose fusidate resistance but partially retain chloramphenicol resistance (paragraph 3.3).

Accordingly, experiments were performed to scrutinise the above observations more thoroughly to ascertain if they might be rationalised into a model closer to the "one bifunctional gene product" model suggested by the more compelling DNA sequence data (paragraph 3.4).

The simplest evidence in favour of the two gene model came from the immunological study on the R-plasmid R28 (paragraph 3.2). It was decided, therefore, to subject this R-plasmid to more

thorough examination. The crux of this examination was to determine if the plasmid contained a mutant CAT_I structural gene lacking immunological cross-reactivity as hypothesised in paragraph 3.5, or whether its fusidate resistance phenotype was via a different mechanism.

3.6.1 Chloramphenicol acetylating activity

Overnight cultures of R-plasmid R28/*E. coli* J53 (Table 1a) and the control strain *E. coli* J53 (inoculated from minimal selective plates: paragraph 2.4) were diluted 1 : 100 into 50ml of fresh L-broth in 250ml conical flasks.

Bacteria were grown at 37°C to exponential phase (A_{660} = 0.8) and used to make cell-free extracts (paragraph 2.8.1).

These extracts were then assayed spectrophotometrically for chloramphenicol acetyltransferase activity (paragraph 2.12).

After the appropriate subtractions for chloramphenicol-independent acetylation activity it was shown that neither strain exhibited any spectrophotometrically-observable chloramphenicol acetyltransferase activity. It was concluded from this that R-plasmid R28 does not appear to encode an enzyme with even a low level of chloramphenicol acetylating activity. This finding is, therefore, in agreement with the negative data obtained from the immunological screen for the presence of CAT_I-related proteins (paragraph 3.2).

3.6.2 In vivo mutagenesis

It was reasoned that if R-plasmid R28 were to encode a mutant CAT_I enzyme lacking both immunological (paragraph 3.2) and catalytic activity (paragraph 3.6.1) that this mutation could be relatively small at the DNA level (e.g. a chain termination mutant). Accordingly, the R28-containing strain R28/*E. coli* J53, (Table 1a) was subjected to mutagenesis by ultra-violet irradiation (paragraph 2.16) in order to attempt to back-mutate the CAT_I gene and regain chloramphenicol resistance phenotype. (Ultra-violet irradiation was employed because it is known to mediate all of the possible transitions and transversions as well as causing small frame-shift mutations).

No chloramphenicol-resistant bacterial colonies above the level of resistance afforded by chromosomal mutation (paragraph 1.12) were observed implying that the CAT_I-carrying R-plasmids and R-plasmid R28 are not closely related in their chloramphenicol resistance genotypes.

3.6.3 DNA : DNA hybridisation

In view of the negative data above it was decided to test more rigorously R-plasmid R28 at the DNA level.

R-plasmid DNA was purified (paragraph 2.17.1) from the R28 containing strain (R28/*E. coli* J53; Table 1a), along with control DNAs from strains containing R-plasmids EDR104 (paragraph 3.3) and EDR51 (a deletion isolated by Dempsey and Willetts (1976) which had been deleted for most of the R100 antibiotic resistance genes yet retained both chloramphenicol and fusidate resistance phenotypes).

Each R-plasmid DNA was cleaved with Pst1 endonuclease (paragraph 2.19). This particular restriction enzyme was chosen because the antibiotic resistance genes on several FII incompatibility group R-plasmids have been shown to be grouped together and flanked by IS1 insertion elements (Mickel et al., 1977). The IS1 DNA sequence contains a unique Pst1 cleavage site and thus, as with transposon Tn9, might be expected to generate a small Pst1 fragment containing the entire CAT_I gene or potential CAT_I-related gene (Alton and Vapnek 1979b; paragraph 5.2).

Additional controls of pBR322-based mini-plasmids, pKT205 and pAH1, which carry the CAT_{II} and CAT_{III} genes on short Pst1 restriction fragments (Table 1b) were prepared and digested in the same manner.

As a final control and size marker bacteriophage lambda DNA was digested with the restriction enzymes EcoR1 and Hind III (paragraph 2.20.2).

All of these DNA molecules were subjected to agarose gel electrophoresis and blotted on to nitrocellulose paper (paragraph 2.27).

The resulting blots were then hybridised to a nick translated Taq1 restriction fragment probe derived from pBR325 (paragraph 2.26). The probe fragment, 773 bp, covers the entire structural gene for CAT_I (Fig. 6).

At the stringency temperature used (55°C, paragraph 2.27) fragments known to carry the CAT_I gene were visualised after autoradiography. The level of this detection was more than sufficient to observe restricted R-plasmid DNA fragments which were invisible by the fluorescence of bound ethidium bromide even when this visualisation was coupled to a long photographic exposure (paragraph 5.2.3).

At this comparatively low stringency temperature R-plasmid R28 Pst1 restriction fragments which were clearly visible to the naked eye failed to exhibit any detectable hybridisation.

The conclusion from these results must be that the fusidate resistance phenotype of R-plasmid R28 is not due to a CAT_I enzyme associated mechanism.

(A similar lack of hybridisation was also observed with the controls plasmids which gave rise to CAT_{II} and CAT_{III}-encoding Pst1 fragments.)

N.B. Surprisingly, the largest EcoR1 restriction fragment of bacteriophage lambda DNA was shown to hybridise to the CAT_I-bearing Taq1 restriction fragment probe. Subsequent computer comparisons between the entire sequence of bacteriophage lambda DNA (Sanger et al., 1982) with the whole of the plasmid pBR325 DNA sequence (Prentki et al., 1981) revealed that only the CAT_I gene of the three antibiotic resistance genes of plasmid pBR325 shared any extensive homology with the lambda DNA sequence (i.e. greater than 12 identical nucleotides). This homology is

given below; its biological significance is unclear.

```
                                  phe phe ala pro val phe thr
CAT_I gene sequence (Fig. 6) 5'-TTC TTC GCC CCC GTT TTC ACC-3'
                                  | ||| ||| ||| ||| ||| ||
Lambda DNA sequence          3'-CCG AAGTCGG GGG CAA AAG TGT-5'
(nucleotides 14892 to 14871, Sanger et al., 1982)
```

The empirically-derived formula of Suggs et al. (1981) was used to calculate the maximum temperature for hydrisation of these two sequences. The maximum annealing temperature is predicted as being between 46°C (for the 14-mer) and 58°C (for the 18-mer) under the salt wash conditions used (0.1 x SSC paragraph 2.27). Since the hybridisation wash was at 55°C (paragraph 2.27) the visualisation of the lambda DNA band observed is theoretically entirely feasible.

The above synopsis of the results of the hybridisation performed was from the pooled data of three experiments. The interpretation of the data was initially exacerbated by the use of a probe contaminated with a second DNA fragment. The second fragment was pBR322-related because pBR325 is a derivative of pBR322 (Prentki et al., 1981). This contamination was due to using pBR325 DNA obtained from a dam methylase proficient strain of E. coli. (E. coli C600). The pBR325 DNA was from a plasmid preparation which was amplified by the addition of spectinomycin (paragraph 2.17). The dam methylase enzyme was thus only able to partially methylate this DNA. Although dam methylase and the Taq1 enzyme recognise and act upon different DNA sequences one of the Taq1 sites (TCGA) overlaps with a dam methylase site (G$\overset{*}{A}$TC) in the rare sequence TCG$\overset{*}{A}$TC. (paragraph 2.20.1; Maniatis et al., 1982).

The partial adenine methylation prevented total cleavage of the DNA at this site by Taq1. The nett result being that instead of two pBR322 -derived fragments of 475 bp and 141 bp a small amount of a 616 bp fragment is formed.

When preparing probe DNA by electroeluting (paragraph 2.24) the desired restriction fragment from an agarose gel it was at first thought that the 773 bp Taq1 fragment hearing the CAT_I sequence was well separated from its nearest size fragments (1,307 and 475 bp). Thus the agarose gels were not initially electrophoresised to obtain sufficient separation of the 773 bp and 616 bp bands. Although the 616 bp fragment was not visible by ethidium bromide fluorescence there was enough DNA present to create a mixed probe which recognised the pBR322 moiety of plasmids pBR325, pKT205 and pAH1.

The source of these spurious results, once identified, was removed by the simple expedient of using pBR325 DNA isolated from a dam$^-$ host strain (E. coli GM242, paragraph 2.2) as the source of the CAT_I-bearing Taq1 restriction fragment probe DNA.

CHAPTER FOUR

The nature of CAT_I-associated fusidate resistance

4.1 The mechanism of fusidate resistance

The conclusion from the experiments on the R-plasmid R28 (paragraphs 3.6.1, 3.6.2 and 3.6.3) was that the fusidate resistance genotype of this plasmid was not closely related to that of the more commonly observed fusidate resistance found in association with the CAT_I gene (paragraph 3.1).

It follows, therefore, that the data on R-plasmid R28 cannot be held to conflict with the "single bifunctional CAT_I enzyme" model for chloramphenicol and fusidate resistance which is implicated by the DNA sequence analysis of the CAT_I gene (paragraph 3.4).

4.2 The rationale for abandoning R-plasmid studies

Because of the size and complexity of R-factors (often >60 Kb) the results of experiments performed on mini-plasmids (often < 6 Kb) were thought to be potentially easier to interpret.

It was decided, therefore, for all subsequent experiments to use mini-plasmid containing strains exclusively.

4.3 Fusidic acid as an acetyl acceptor

With the hypothesis that the CAT_I enzyme also effects fusidate resistance (paragraph 3.5) and knowing CAT-mediated chloramphenicol resistance to be mediated by acetylation and hence inactivation of the antibiotic (paragraph 1.14.1) it was

reasoned that the mechanism of fusidate resistance might simply be CAT_I-catalysed chemical modification of the fusidic acid molecule. This being despite the apparently large structural dissimilarity of the two drugs (Fig. 1).

Accordingly, the ability of sodium fusidate to act as an acetyl acceptor, in an analogous manner to chloramphenicol, in the spectrophotometric chloramphenicol acetyltransferase assay (paragraph 2.12) was tested.

Cleared cell-free extracts (paragraph 2.8.1) from bacteria grown to exponential phase (A_{660} = 0.6) in 50ml of L-broth plus 10µl/ml tetracycline were prepared.

The bacterial strains used were derivatives of E. coli DB10 carrying the mini-plasmids pBR325, pKT205 and pAH1 (Table 1b) which specify the three enterobacterial CAT enzyme variants CAT_I, CAT_{II} and CAT_{III} respectively (Gaffney et al., 1978).

The crude extracts containing the CAT_{II} and CAT_{III} variants (which are not associated with fusidate resistance, paragraph 3.1) did not, as was expected, acetylate the fusidic acid as evidenced by an absence of an increase in the absorbance at 412 nm. The crude extract containing the CAT_I enzyme also failed to catalyse the acetylation of the antibiotic, indicating that the mechanism of CAT_I-associated fusidate resistance is not via CAT_I-catalysed acetylation of the antibiotic.

4.4 In vivo chemical modification of fusidate

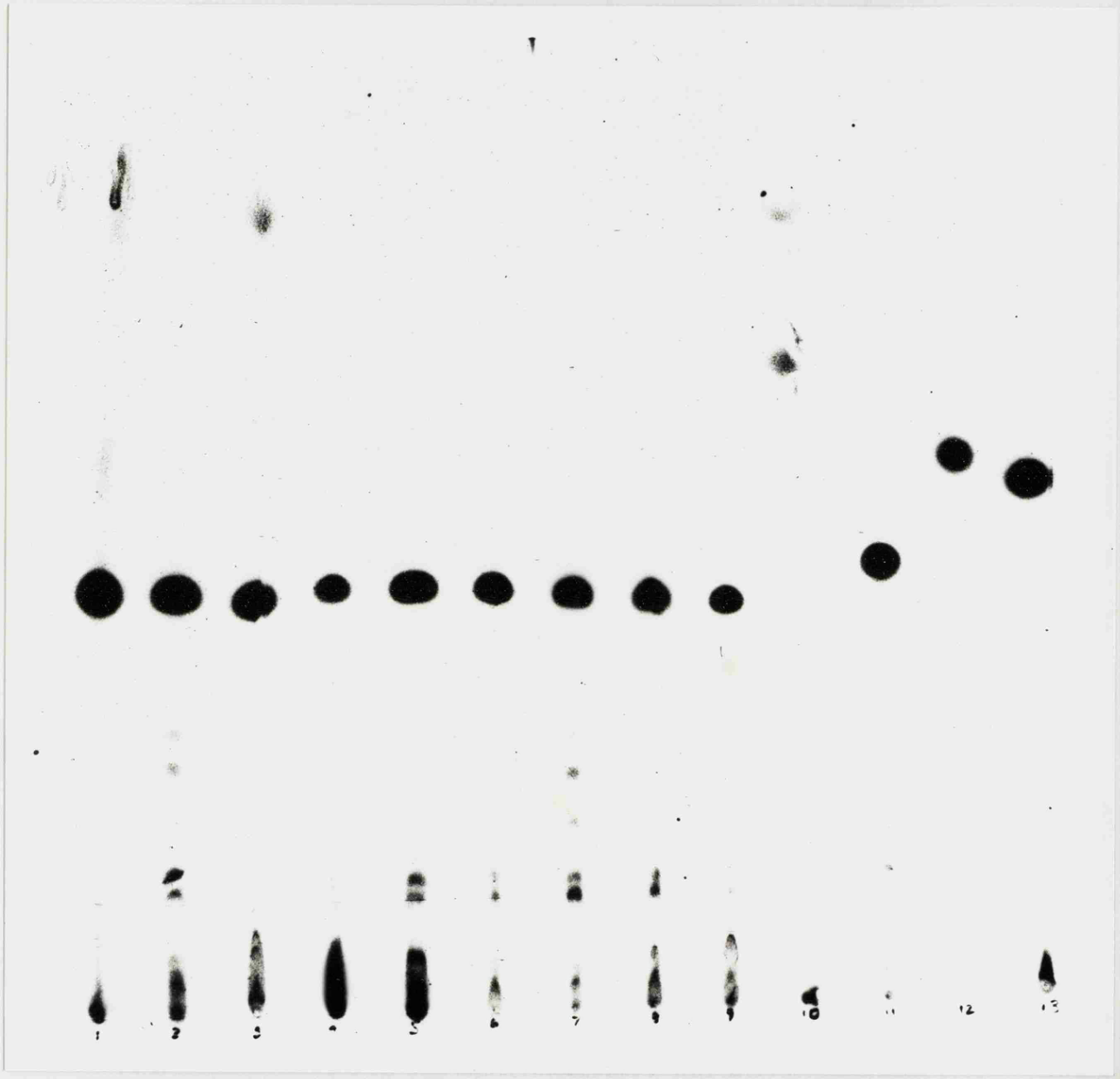
Because of the above negative data obtained for the chemical modification of fusidate *in vitro* it was decided to investigate the possibility of *in vivo* modification as the resistance mechanism. Experiments were performed as described (paragraph 2.29).

No chromatographic evidence of chemical modification of fusidic acid was observed with any of the *E. coli* DB10 and *E. coli* C600 strains employed.

The bacteria grown in flasks containing the initial sub-inhibitory concentration of sodium fusidate (which were included to allow for the possibility that fusidate resistance is inducibly expressed) gave the same chromatographic results as those flasks which lacked an initial low level of fusidate, i.e. that fusidic acid was not modified (Fig. 9).

These findings are in keeping with the results of bioassay studies performed by Werner and Daneck (1981) and by Scannell and Shaw (this laboratory, unpublished experiments). Both groups failed to demonstrate a decrease in the level of anti-staphylococcal growth inhibition caused by pre-exposure of sodium fusidate to *E. coli* DB10 strains which contained R-plasmids (including R-plasmid R28; paragraph 3.4) conferring the fusidic resistance phenotype. The conclusion from this must be that plasmid-mediated fusidate resistance in *E. coli* is not caused by chemical modification of the antibiotic.

Figure 9.



Thin layer chromatography plate to investigate fusidate inactivation.

1. No media, no cells.
2. Media, no cells.
3. *E. coli* DB10.
4. *E. coli* DB10 + pKT205 + [sub.inhib.]
5. *E. coli* DB10 + pKT205
6. *E. coli* DB10 + pAH1 + [sub.inhib.]
7. *E. coli* DB10 + pAH1.
8. *E. coli* DB10 + pBR328 + [sub.inhib.]
9. *E. coli* DB10 + pBR328.
10. VD1577.
11. 3-Epifusidate.
12. 3-Oxofusidate.
13. 11-Oxofusidate.

4.5 Fusidate resistance and phospholipid levels

Werner and Daneck (1981) postulated that E. coli DB10 was fusidate-sensitive because the mutation to sensitivity (see Datta et al., 1974) resulted in reduced levels of cyclopropane fatty acids and phosphatidylethanolamine. They speculated that plasmid borne fusidate resistance might be due to an alteration in the synthesis of these phospholipids which was controlled by plasmid genes.

However, the observation that E. coli DB10 is low in cyclopropane fatty acids may have no immediate or obvious relevance to fusidate sensitivity. The E. coli mutants, FT16 and FT17, isolated by Taylor and Cronan (1976; paragraph 2.2) are defective in the cyclopropane fatty acid synthetase enzyme and ~~have been~~ were shown to exhibit a similar resistance to fusidate (>500 µg/ml) as the fusidate-tolerant E. coli strains C600 and FT1 (the parental strain of FT16 and FT17) as judged by gradient plate analysis (paragraph 2.7; results not shown).

4.6 Resistance studies with fusidic acid analogues

In order to gain further insight into the mechanism of fusidate resistance it was decided to look at the in vivo resistance profiles of a number of E. coli test strains against a selection of close structural analogues of the antibiotic.

A large number of fusidic acid analogues have been synthesised by Leo Pharmaceutical Products and their antibacterial activities have been assayed, principally against Gram positive organisms (von Daehne et al., 1979).

Figure 10.

Structural key to fusidic acid analogues.

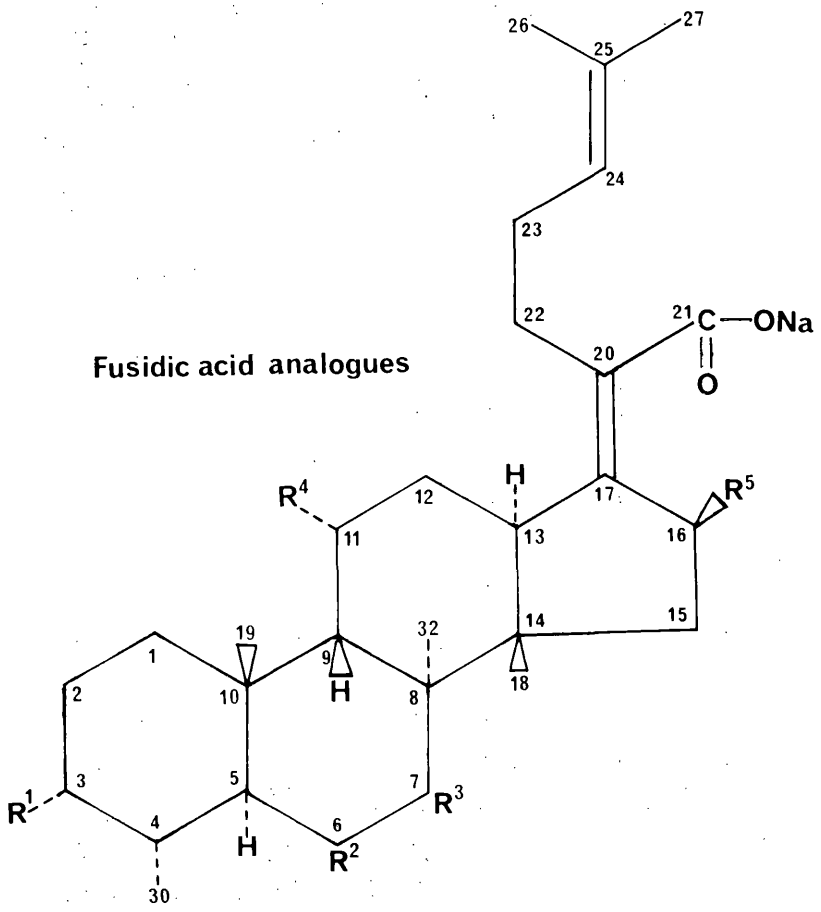


TABLE 2 (see Fig. 10)

FUSIDIC ACID ANALOGUES (von Daehne et al., 1979)

Compound trivial name or designation	1,2 bond	R^1	R^2	R^3	R^4	R^5	13,17 bond	24,25 bond
Sodium Fusidate a.	s	-OH	-H,-H	-H-H	-OH	$-OCOCH_3$	s	d
3-Epifusidate	s	β-OH	"	"	"	"	s	d
3-Oxofusidate	s	=O	"	"	"	"	s	d
3-Bromofusidate	s	-Br	"	"	"	"	s	d
3β-Bromofusidate	s	β-Br	"	"	"	"	s	d
3-Chlorofusidate	s	-Cl	"	"	"	"	s	d
3-Acetylfusidate	s	$-OCOCH_3$	"	"	"	"	s	d
3-Azidofusidate	s	$-N_3$	"	"	"	"	s	d
3β-Mesyl-24,25-dihydrofusidate	s	$\beta-OSO_2CH_3$	"	"	"	"	s	s
7α-Hydroxyfusidate	s	-OH	"	α-OH	"	"	s	d
11-Oxofusidate	s	"	"	-H,H	=O	"	s	d
16-Deacetylfusidate	s	"	"	"	-OH	-OH	s	d
16-Epideacetylfusidate	s	"	"	"	"	α-OH	s	d
16-Deacetylfusidate lactone	s	"	"	"	"	β-lactone	s	d
PR1089 (mixture)	s	"	"	"	"	$\{-SCH_2CH_3$ &$-SCH(CH_3)_2\}$	d	d
PR1246 (mixture) b.	s	"	"	"	"	" $\{$&$-OCHF-CH_2-CH_2F\}$	s	d
PR1249= 16-Isopropylsulphinylfusidate	s	"	"	"	"	$-S(=O)-CH(CH_3)_2$	s	d
VD1303=16-Ethoxyfusidate b.	s	"	"	"	"	$-OCH_2CH_3$	s	d
VD1349 (mixture)	s	"	"	"	"	$\{-OCH_2CHF$ &$-OCH_2CF_3\}$	s	d
VD1545=16-Acetylthio-24,25-dihydrofusidate	s	"	"	"	"	$-SCOCH_3$	s	s
VD1546=16-Benzoylthio-24,25-dihydrofusidate	s	"	"	"	"	$-SCO$	s	s
VD1577=3β-Bromo-16-acetylthio-24,25-dihydrofusidate b.	s	β-Br	"	"	"	$-SCOCH_3$	s	s
24,25-Dihydrofusidate	s	-OH	"	"	"	$-OCOCH_3$	s	s
Helvolic acid sodium salt c.	d	=O	$\beta-OCOCH_3$	=O	-H	"	s	d
Cephalosporin P1 sodium salt c.	s	-OH	$\alpha-OCOCH_3$	β-OH	-H	"	s	d

a. See Fig. 1. b. unstable. c. See Fig. 3. s = single bond. d = double bond.

The steroidal skeleton of fusidic acid is shown in Fig. 10.

The fusidic acid analogues used (Table 2) were employed as their sodium salts when supplied (except compounds VD1349 and VD1546 which were supplied as potassium salts). Analogues supplied as free acids were converted to their sodium salt equivalents by addition of an equimolar quantity of sodium hydroxide. For sparingly soluble compounds it was often necessary to dissolve the compound initially in a minimal volume of methanol and to add this to a large volume of warm (60°C) dilute sodium hydroxide.

For gradient plate analysis (Fig. 11) the 2YT agar solution containing the required antibiotic (paragraph 2.7) was formed by the addition of one volume of four fold concentrated 2YT agar (at 60°C) to three volumes of the above fusidate analogue solution.

The gradient plate analysis (Table 3) revealed that E. coli C600 is intrinsically tolerant to all of the fusidane antibiotics employed. E. coli DB10 however, exhibits a resistance profile qualitatively similar to wild-type Staphylococcus aureus (von Daehne et al., 1979).

The effect of the presence of a number of closely-related mini-plasmids (Table 1b) in E. coli DB10 can also be observed. Mini-plasmids pBR325, pKT205 and pAH1 are all derivatives of plasmid pBR322 and code for the CAT_I, CAT_{II} and CAT_{III} enzyme variants respectively (plasmid pBR322 has no CAT-coding sequence). Plasmid pBR328 is a derivative of pBR325 which exhibits a three-fold increase in copy number (Covarrubias et al., 1981).

Gradient plate analysis of antibiotic resistance.

E. coli C600

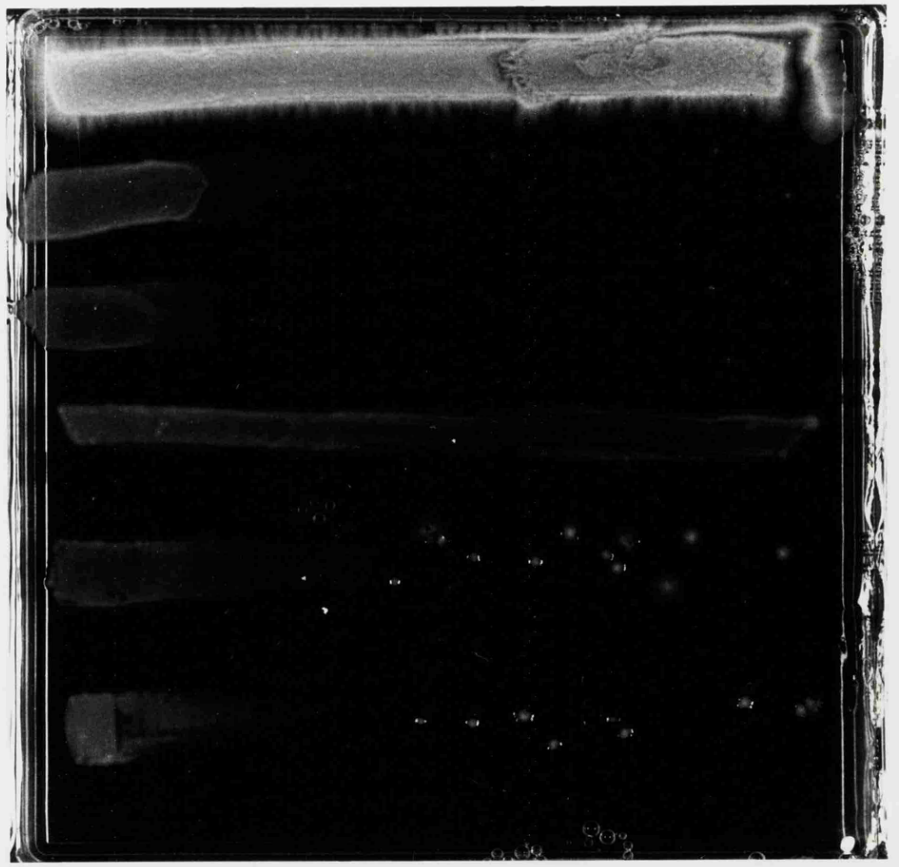
E. coli DB10

E. coli DB10 + pKT205

E. coli DB10 + pBR325

E. coli DB10 + pAH1

E. coli DB10



0 ⟶ 100 µg/ml
24,25 Dihydrofusidate

Figure 11.

Many of the fusidic acid analogues are ineffectual at the concentrations employed even against plasmid-free E. coli DB10. Among the active analogues it can be seen that only the plasmids pBR325 and pBR328 are able to confer resistance. With sodium fusidate itself the increased copy number of pBR328 seems to confer resistance to a higher level that seen with pBR325 (albeit not three-fold higher).

The most striking observation is that the antibacterially active analogues 3-oxofusidate, 7-α-hydroxyfusidate, helvolic acid and cephalosporin P1 are immune to ~~be~~ the resistance mechanism of the CAT_I-gene bearing plasmids. This is in accord with the observations of Völker et al. (1982) who reported that helvolinic acid (6-deactylhelvolic acid) and cephalosporin P1 inhibit E. coli DB10 even when this strain carries pBR325.

The chemical specificity of the resistance mechanism implies firstly that the resistance plasmids do not act simply to restore phospholipid levels (as suggested by Werner and Daneck, 1981; paragraph 4.5) and secondly, if the CAT_I protein causes fusidate resistance, that the CAT_I-antibiotic association is not merely a crude interaction based on the hydrophobic steroidal structure of fusidic acid.

4.6.1 Fusidate resistance and acetylated fusidic acid analogues

The gradient plate analysis (paragraph 2.7; Table 3) also reveals a potential fusidic acid resistance mechanism (paragraph 2.7; Table 3).

Table 3 (see Tables 2 and 5)

Antibiotic Resistance (μg/ml as determined by gradient plate analysis, paragraph 2.7) of test strains against fusidate analogues.

Compound trivial name or designation \ E. coli host strain / plasmid	C600 -	DB10 -	DB10 pBR322	DB10 pBR325	DB10 pBR328	DB10 pKT205	DB10 pAH1
Sodium fusidate	> 500	5	5	80	105	5	5
3-Epifusidate	> 450	> 450	> 450	> 450	> 450	> 450	> 450
3-Oxofusidate	>1000	100	100	100	100	100	100
3-Bromofusidate	>1000	>1000	>1000	>1000	nd	>1000	>1000
3 β-Bromofusidate	> 500	> 500	> 500	> 500	nd	> 500	> 500
3-Chlorofusidate	>1000	>1000	>1000	>1000	nd	>1000	>1000
3-Acetylfusidate	>1000	>1000	>1000	>1000	nd	>1000	>1000
3-Azidofusidate	> 100	> 100	> 100	> 100	nd	> 100	> 100
3β-Mesyl-24,25 dihydrofusidate	> 100	20	20	> 100	nd	20	20
7α-Hydroxyfusidate	> 400	15	15	15	15	15	15
11-Oxofusidate	> 400	100	100	> 400	> 400	100	100
16-Deacetylfusidate	> 500	> 500	> 500	> 500	nd	> 500	> 500
16-Epideacetylfusidate	> 500	> 400	> 400	> 400	nd	> 400	> 400
16-Deacetylfusidate lactone	> 500	> 500	> 500	> 500	nd	> 500	> 500
PR1089	>1000	>1000	>1000	>1000	nd	>1000	>1000
PR1246	> 100	> 100	> 100	> 100	nd	> 100	> 100
PR1249	> 100	30	30	60	nd	30	30
VD1303	> 100	20	20	> 100	nd	20	20
VD1349	> 100	80	80	> 100	nd	80	80
VD1545	> 100	5	5	> 100	nd	5	5
VD1546	> 500	> 500	> 500	> 500	nd	> 500	> 500
VD1577	>1000	>1000	>1000	>1000	nd	>1000	>1000
24,25-Dihydrofusidate	> 100	20	20	> 100	> 100	20	20
Helvolic acid sodium salt	> 150	45	45	45	45	45	45
Cephalosporin P1 sodium salt	> 100	40	40	40	40	40	40

nd = not determined.

The CAT_I enzyme recognises and acetylates the 3-hydroxy group of chloramphenicol as previously outlined (paragraph 1.14.1). From the gradient plate analysis it is clear that acetylation of fusidic acid at position 3 (R^1 in Fig. 10) substantially removes antimicrobial activity. Consequently, if the CAT_I enzyme were able to acetylate fusidic acid *in vivo* at position 3 this would form the basis of a resistance mechanism (despite the *in vitro* experiment, paragraph 4.3, which failed to demonstrate acetylation of fusidate).

The mechanism is only tenable if the relative mobilities of fusidate and 3-acetylfusidate are very similar in the thin layer chromatography system used in the experiment to look for *in vivo* modification of fusidic acid (paragraph 2.29).

When tested, however, it was found that the relative mobilities of fusidate and 3-acetylfusidate were 0.54 and 0.69 respectively. It is clear, therefore, that fusidic acid resistance is not due to 3-O-acetylation of the antibiotic by the CAT_I enzyme.

An alternative resistance mechanism can be envisaged wherein the CAT_I enzyme acetylates fusidic acid at the hydroxyl group at position 11 (R^4 in Fig. 10) since it is known that 11-acetylfusidate exhibits poor antistaphylococcal activity (von Daehne *et al.*, 1979). However, von Daehne and colleagues (1979) state that "due to steric hindrance, the axial hydroxyl group at C11 resists acylation under normal conditions". This steric hindrance, which can clearly be seen in Fig. 2, makes such a mechanism unlikely. A definitive

refution of the hypothesis, however, required some of the 11-acetylfusidate compound, which is no longer available from Leo Laboratories (W. von Daehne personal communication).

CHAPTER FIVE

Molecular cloning and in vitro expression studies

5.1 The models of fusidate resistance

The experiments of Chapter four indicated the specific nature of the fusidate resistance mechanism but did little to resolve the dilemma presented by the conflicting "two genes, one operon" and "single bifunctional protein" models suggested by the preceding data and outlined in paragraphs 3.3 and 3.4.

A potential solution to the problem was that, as established, the CAT_I enzyme effects chloramphenicol resistance but that fusidic acid resistance is mediated by a portion of the CAT_I protein.

This portion might be generated by proteolysis of CAT_I or, alternatively, by premature termination of transcription or translation of the CAT_I mRNA. (The transcription studies of Le Grice and colleagues (paragraph 1.14.3) only extended as far as the EcoR1 site in the CAT_I gene; Fig. 6).

A fourth possibility was that a methionine codon (AUG) within the CAT_I gene mRNA could act as an internal start codon thereby generating a C-terminal fragment of the enzyme.

In view of the more compelling data available from mini-plasmids it was reasoned that by cloning the relevant restriction fragments from several R-plasmids of particular interest a tenable model for fusidate resistance might be gained.

5.2 Molecular cloning of R-factor resistance genes

Of the several R-plasmids already described (Table 1a) the most interesting appeared to be the R100 deletion derivative EDR104 which was isolated by Dempsey and Willetts (1976; paragraph 3.3). This R-plasmid was reported as phenotypically fusidate sensitive and weakly chloramphenicol resistant.

Also of interest were the temperature-sensitive CAT_I enzyme mutants of R100 isolated by Mise and Suzuki (1968) and the point and deletion mutants of the CAT_I gene isolated on the R100-related FII incompatibility group R-plasmids, R1 and R100-1 (paragraph 1.14.2) by Foster and Howe (1973). Neither of these latter two groups of R-plasmids had been tested for their fusidic acid resistance phenotype.

It was initially decided to clone the mutant CAT_I gene from R-plasmid EDR104 (Dempsey and Willetts, 1976) and for this manipulation a suitable restriction endonuclease had to be chosen (see also paragraph 3.6.3). It was known that EcoR1 was unsuitable (Tanaka et al., 1976; Lane and Chandler, 1977) and also that Sal 1 (Miki et al., 1978) and Hind III were probably not ideal (Timmis et al., 1978a). Additionally Bam H1 was probably of little use (Blohm and Goebel, 1978). However, Taylor and Cohen (1979) were able to show that with a derivative of R-plasmid R100 it was possible to clone a Pst1 restriction endonuclease fragment coding for both chloramphenicol and streptomycin resistance phenotypes. This observation was later confirmed by Alton and Vapnek (1979a) with R-plasmid R538-1 (also inc. FII).

Additionally, it was known that both fusidic acid and chloramphenicol resistance could be carried on a small (2.7 Kb) transposon, Tn9 (Alton and Vapnek 1979b). This transposon was first isolated after an *in vivo* recombination event between bacteriophage P1 and the FII inc.R-plasmid R14 (Kondo and Mitsuhashi, 1964). (N.B. The bacteriophage isolated, P1CM, is now termed P1CmO; Meyer and Iida, 1979). Subsequently, Tn9 was shown to comprise of the antibiotic resistance region flanked by direct repeats of insertion element IS1 (MacHattie and Jackowski, 1977) DNA sequence analysis of IS1 revealed that the insertion element contains a single site for the *Pst*1 enzyme (Ohtsubo and Ohtsubo, 1978; Johnsrud, 1979). Because *Pst*1 does not cleave Tn9 between the insertion elements Alton and Vapnek (1979b) were able to clone a small (1.87 Kb) *Pst*1 restriction fragment coding for both chloramphenicol and fusidate resistance into the miniplasmid pBR322 (Table 1b).

From the above restriction enzyme studies and from deletion mapping (Dempsey and Willetts, 1976) it was known that the CAT_I gene of R100 (the parent of EDR104) was close to one end of the resistance-determinant (r-det) region. Furthermore, the r-det region of R100 was shown by heteroduplex analysis to be flanked by IS1 elements (Mickel *et al.*, 1977).

Accordingly, in view of the large amount of evidence above it was decided to attempt to clone the CAT_I gene of EDR104 using the restriction enzyme *Pst*1.

The above Pst1 cloning experiments made use of the unique Pst1 cleavage site in the β-lactamase gene of plasmid pBR322 (Sutcliffe, 1978). Recombinant plasmids not only encoded the cloned R-plasmid resistance genes but also would be demonstrated to have Pst1 fragments inserted into the β-lactamase gene by screening for the loss of the ampicillin resistance phenotype of the vector.

For the EDR104 cloning procedure, however, it was decided to use an alternative vector to pBR322 principally because pBR322 contains a unique EcoR1 restriction site (Sutcliffe, 1978). Experiments were envisaged where the EcoR1 site in the CAT_I structural gene (Fig. 6) would be cleaved in an attempt to separate the chloramphenicol and fusidic acid resistance phenotypes (paragraph 3.3). The presence of an additional EcoR1 site in the recombinant plasmid would cause unnecessary technical complications.

The other, minor, reason why pBR322 was not employed was that it carries a tetracycline resistance gene (Sutcliffe, 1979) and it was not known whether Pst1 cloning from EDR104 might pick up the tetracycline resistance gene of the R-plasmid carried on transposon Tn10 (Dempsey and Willetts, 1976) and hence confuse the recombinant selection process.

A suitable mini-plasmid vector pNJ2004 (Table 1b) was obtained and purified (paragraph 2.17.2). The plasmid carries the β-lactamase gene of pBR322 and the Pst1 cleavage site in this gene is unique to the plasmid. The vector also carries a gene

for kanamycin resistance and additionally lacks tetracycline resistance. Finally, it carries no EcoR1 restriction site.

By using this vector in the cloning procedure E. coli transformants could be selected on the basis of kanamycin resistance and recombinant plasmids could be screened by their ampicillin-sensitive phenotype.

5.2.1 Pst1 restriction endonuclease digestion of R-plasmid EDR51

The objective of the cloning experiments with Pst1 restriction fragments of R-plasmid EDR104 was to isolate a DNA fragment which coded solely for chloramphenicol resistance. Alternatively, if this was not possible, to demonstrate that the entire CAT_I structural gene is required for the fusidate resistance phenotype.

Before embarking on the cloning of the resistance genes of EDR104 it was reasoned that since the laboratory as a whole had little experience in DNA manipulations with E. coli and still less experience with DNA manipulations with R-plasmids, it would be prudent to experiment first on a control R-plasmid.

In the course of their deletion mapping of R100 Dempsey and Willetts (1976) isolated an R-plasmid, EDR51, which has been deleted for the entire r-det region with the exception of the necessary information to code for chloramphenicol and fusidate resistance. This plasmid with its essentially wild-type CAT_I-gene was anticipated to be an excellent control for molecular cloning. The high level chloramphenicol resistance it afforded to its E. coli host (>250µg/ml) was expected to be

a simple phenotype to score amongst the recombinant mini-plasmid population.

R-plasmid EDR51 DNA was purified (paragraph 2.17.1) and cleaved with Pst1 restriction enzyme (paragraph 2.19). On analysis by agarose gel electrophoresis (paragraph 2.18) it was apparent that a large number of bands were generated. Due to this large number of fragments and the low yields of DNA it was found necessary to load the agarose gel with an amount of DNA equivalent to that derived from a one litre culture of bacteria. At first the large number of fragments was thought to be symptomatic of a partial digestion of the DNA, However, subsequent extensive digestion failed to reduce the number of bands (Fig. 12a). Eventually it was found that Pst1 digestion of EDR51 gave thirty fragments ranging from 16.2 Kb to a less than 0.1 Kb with the majority of fragments lying between 1.0 and 3.0 Kb (Fig. 12b). Summation of the fragment lengths gave a value for the size of EDR51 of 66 Kb ± 2 Kb. This value is in close accord with the figure obtained by subtraction of the size of the r-det deletion which formed EDR51 (c. 20 Kb) from the size quoted for the original plasmid R100 of 87.7 Kb (Dempsey and Willetts, 1976).

5.2.2 Cloning of EDR51 Pst1 fragments into plasmid pNJ2004

It was originally decided to isolate recombinant plasmids in E. coli DB10 this being despite the low level of competence obtainable with this host (10^4 transformants per microgram of supercoiled pBR322 DNA). This was in order to permit the fusidic acid resistance phenotype to be scored directly without

Restriction endonuclease digestion of R-plasmid EDR51 DNA

Figure 12a

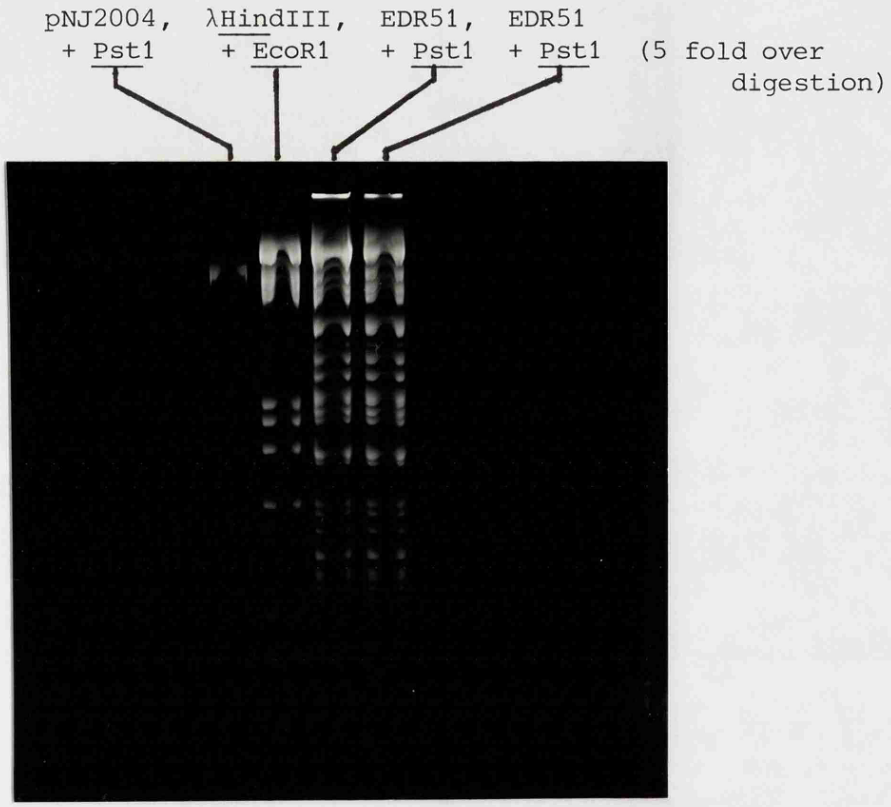
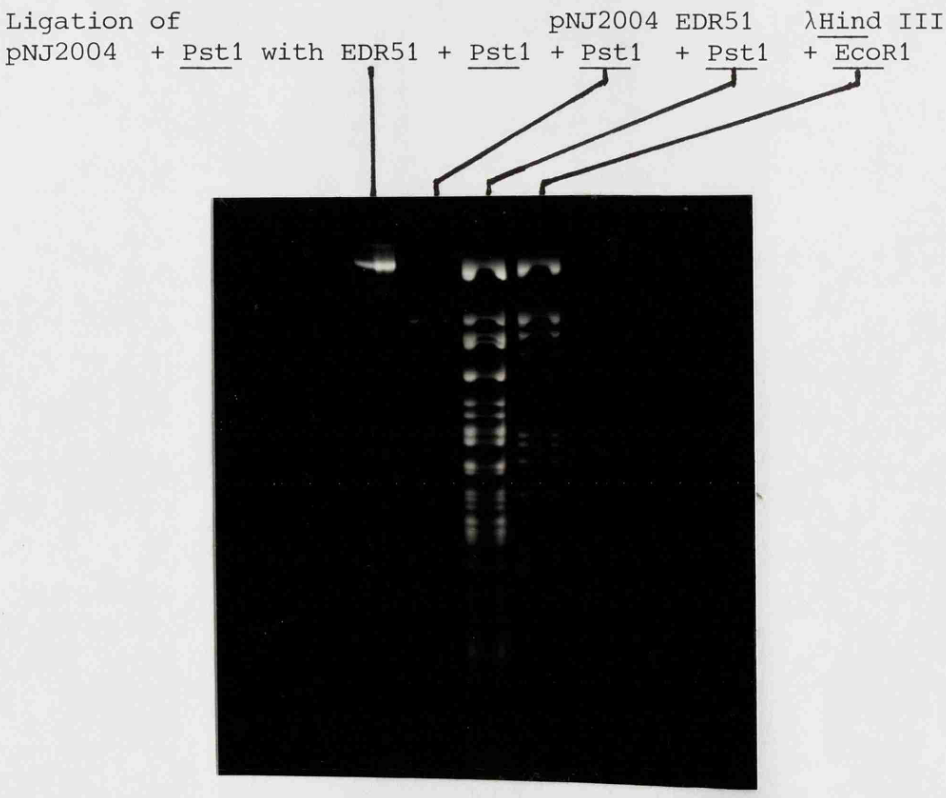


Figure 12b



re-transformation from a fusidate-tolerant host.

Ligation of EDR51 Pst1 fragments into Pst1 cleaved plasmid pNJ2004 was performed at a variety of ratios of fragment-molecule ends to vector-molecule ends ranging from 1 : 1 to 5 : 1 (paragraph 2.21). Ligation was monitored by the increase in size of the DNA fragments as visualised by agarose gel electrophoresis (paragraph 2.18; Fig. 12b). Transformation of competent E. coli DB10 was performed as described (paragraph 2.23).

It was decided, initially, to select directly for the desired recombinant plasmids (i.e. two forms of the plasmid since the Pst1 fragment could be cloned in either orientation).

Competent E. coli DB10 was transformed with the ligation mixture. Half of the resulting culture was plated onto chloramphenicol-containing agar (80 µg/ml) and half was spread on fusidic acid plates (40 µg/ml). The concentration of 80 µg/ml chloramphenicol was selected as being mid-way between the level of resistance afforded by the R-plasmid EDR51 (approx. 250 µg/ml; paragraph 5.2.1) and the level of resistance obtainable by chromosomal resistance (15 µg/ml; paragraph 1.12).

Colonies were only observed on the fusidic acid-containing plates. Although, when replicated these colonies proved to be kanamycin resistant and ampicillin sensitive, they failed to exhibit the desired phenotype of chloramphenicol resistance (80 µg/ml) and the E. coli DB10 host phenotypes of nalidixic acid and streptomycin resistance (paragraph 2.2). Consequently,

these colonies were deemed to be spurious and were rejected.

Subsequently it was found, as has also been reported by Werner and Daneck (1981), that E. coli DB10 is more sensitive to a wide range of antibiotics than is wild-type E. coli and that the presence of plasmid frequently acts to increase this sensitivity. This phenomenon is particularly marked for both nalidixic acid and streptomycin resistance and offers an explanation for the loss of these resistances among the transformed E. coli DB10 population.

The cloning procedure was repeated and transformants selected on the basis of kanamycin resistance. Replication of these colonies demonstrated that fifteen percent had lost ampicillin resistance and were, presumably, the products of recombinant plasmids. On extrapolation to the original number of colonies obtained it was calculated that 4.5×10^5 recombinant plasmids had been obtained. Replica plating of these colonies failed to reveal any chloramphenicol resistant clones.

Because only thirty fragments were obtained after digestion of EDR51 DNA with Pst1 (paragraph 5.2.1; Fig. 12b) it was felt that sufficient recombinant plasmids had been screened and that the failure to find a chloramphenicol resistant recombinant plasmid must be because the CAT_I gene of EDR51 contains a Pst1 restriction site and hence was refractory to cloning.

At this time the plasmids pShI41 and pShI44 (Marcoli et al., 1980; Table 1b) became available and were obtained. These are isogenic plasmids based on plasmid pBR322 and carried the CAT_I-coding sequence on a 1.69 Kb Pst1 fragment inserted in opposite orientations into the Pst1 site of the β-lactamase gene. The source of the fragment was the transposon Tn981 (previously termed Tn cam 204) which is related to, but smaller than, Tn9 and which is derived ultimately from R-plasmid R100 (Marcoli et al., 1980).

The plasmids pShI41 and pShI44 along with plasmid pBR325 (Table 1b) were transformed into E. coli DB10 and selected on the basis of tetracycline resistance. These transformants were then tested for their levels of chloramphenicol and fusidate resistance by the gradient plate method (paragraph 2.7). The results (Table 1b) revealed that mini-plasmids carrying the CAT_I-gene are unable to grow on concentrations of chloramphenicol as high as 80 µg/ml.

Once the phenomenon of low level chloramphenicol resistance of CAT_I bearing mini-plasmids had been appreciated it was a simple task to repeat the Pst1 cloning experiment and select transformants on 20 µg/ml chloramphenicol and hence isolate the desired recombinant plasmid pAB02 (Table 1b).

Large numbers of CAT_I genes generated by multiplication of transposons have been reported (Arber et al., 1978; Meyer and Iida, 1979; Chandler et al., 1979). The high levels of chloramphenicol resistance claimed (up to 1 mg/ml) were

attained by gradual increase in the chloramphenicol concentration and with the resulting E. coli taking up to three days to reach the stationary phase of growth. Since the constructs were not transferred to new host strains and since the concentrations and the specific activities of the CAT_I enzymes were not checked the resistance levels need not reflect gene dosage. Chromosomal mutants to chloramphenicol resistance have been shown to act synergistically with CAT_I (Foster, 1975; paragraph 1.14.1) and specific activity mutants of CAT_I could be expected to be found. The slow growth rate of the high level resistance strains may additionally reflect the fact that chloramphenicol is bacteriostatic (paragraph 1.13) and hence a slow turnover of the antibiotic will eventually lead to renewed cell growth. Finally high level chloramphenicol resistance by CAT is probably limited by the size of the acetyl CoA pool (see paragraph 7.3; Uhlin and Nordström 1977 and 1978).

Digestion of plasmid pAB02 DNA with Pst1 endonuclease revealed that it carried a restriction fragment of 1.96 Kb which was identical in size to one of the bands visualised in the Pst1 digestion of R-plasmid EDR51 DNA.

5.2.3 The nature of the mutation which formed EDR104

The route to the cloning of the mutant CAT_I gene of EDR104 (the Dempsey and Willetts (1976) deletion of R100 that was chloramphenicol resistant and fusidate sensitive; paragraph 5.2) was now clear. But as a consequence of preliminary DNA sequence data by S. Iida (personal communication; Marcoli et al., 1980; Iida et al., 1982) and the results of concurrent experiments

described in subsequent paragraphs this was not carried out.

However, in the course of the Southern blotting experiments performed on R-plasmid R28 (paragraph 3.6.3) restriction fragments derived by Pst1 digestion of the DNA of plasmids pAB02, EDR51 and EDR104 were used as controls.

It was observed that the 1.96 Kb fragment in pAB02 and EDR51 hydrises to the nick translated CAT_I gene Taq 1 probe fragment (Fig. 6) and a single band of 1.63 Kb in the EDR104 digest is similarly detected. This means that the deletion of the r-det region observed in EDR104 is larger than the deletion created in the formation of EDR51.

The DNA of the mutant CAT_I gene of EDR104 has now been sequenced by Iida et al.(1982). Its loss of fusidate resistance can be attributed to the removal of seven-amino acid codons from the 3' end of the structural gene and the replacement of this region by DNA of the insertion element IS1. The removal also deletes the protein termination codon and hence the IS1 DNA is transcribed along with the CAT_I structural gene and read as protein coding sequence until an in phase termination codon is reached. The nett result of this mutation is a CAT_I enzyme which is extended by nineteen amino-acid residues, the last twenty six residues being "random" sequence encoded by IS1 DNA. The nature of this mutation is so gross that it does little to assist in the elucidation of the fusidic acid resistance mechanism.

The nature of the IS1-mediated deletion of CAT_I isolated by Dempsey and Willetts (1976)

```
                        Sca1
Native 5' ..... CAG TAC TGC GAT GAG TGG CAG GGC GGG GCG TAA ...  3' (Alton and Vapnek, 1979)  219 Amino-acids
CAT_I           Gln Tyr Cys Asp Glu Trp Gln Gly Gly Ala End
(see Fig. 6)

                                                    IS1 insertion element
                        Sca1                                                                  Tth111I
EDR104 5' ..... CAG TAC TGC GGT AAT GAC TCC AAC TTA TTG ATA GTG TTT TAT GTT CAG ATA ATG CCC GAT GAC TTT GTC ATG CAG CTC CAC CGA TTT TGA .... 3' (Iida et al., 1982)  238 Amino-acids
mutant          Gln Tyr Cys Gly Asn Asp Ser Asn Leu Leu Ile Val Phe Tyr Val Gln Ile Met Pro Asp Asp Phe Val Met Gln Leu His Arg Phe End
CAT_I
```

5.3 Cell-free coupled transcription and translation studies

In vitro transcription and translation experiments were performed in the cell-free system described (paragraph 2.28).

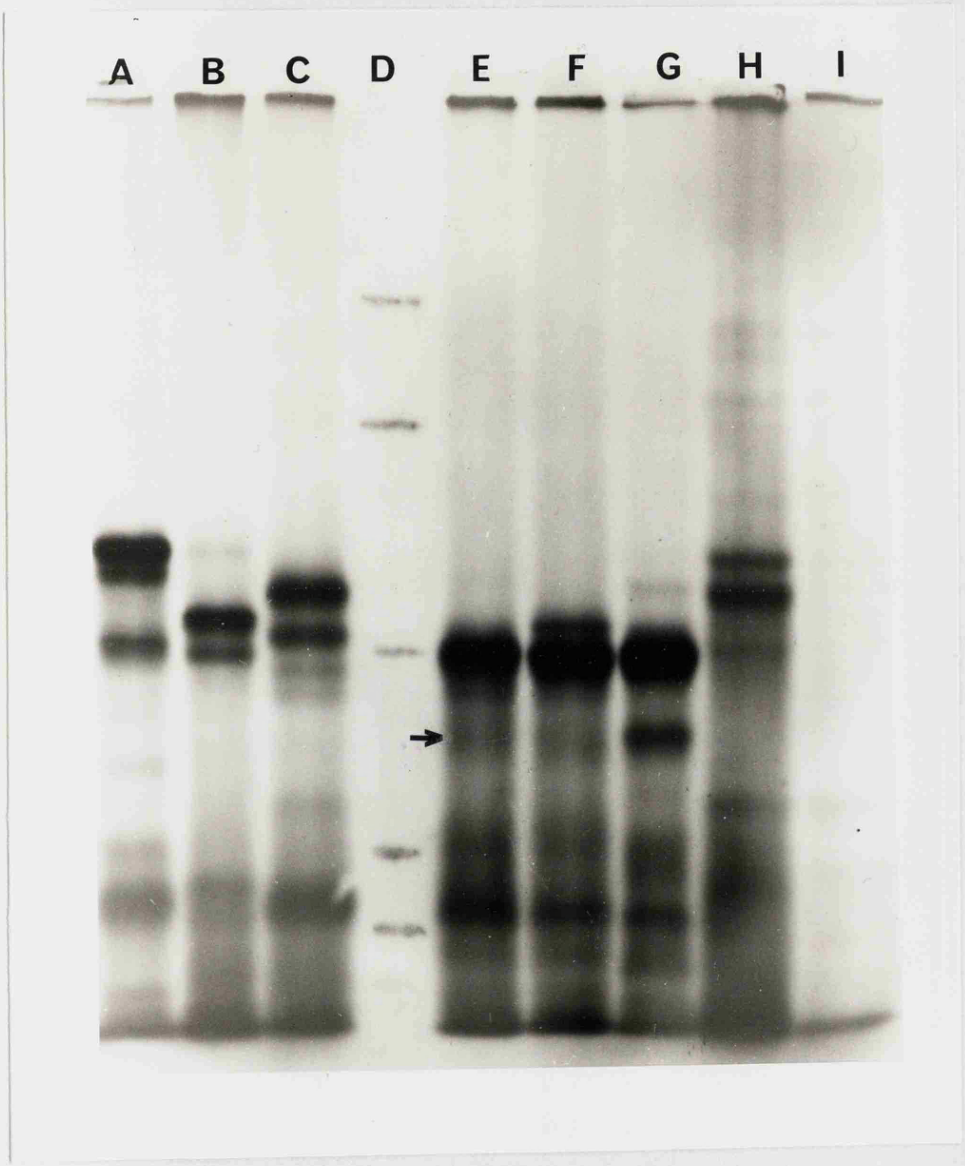
The aim of these studies was to try to observe the number of polypeptides encoded by the CAT_I nucleotide sequence (Fig. 6) so that a model for fusidate resistance could be found which was consistent with the available data (paragraph 5.1). Since neither CAT_{II} nor CAT_{III} are associated with fusidate resistance, (paragraph 3.1) plasmids (as near isogenic as was possible) carrying genes for these variants were used to compare their translation products with those of plasmids specifying CAT_I.

5.3.1 Studies on plasmids bearing the three CAT variants

Track A of the autoradiograph shown in Fig. 13 reveals radioactive protein synthesised in response to the presence of plasmid pBR322 as template DNA. Those of apparent relative molecular mass (M_r) 30,000, 28,000 and 25,000 correspond to the three polypeptides related to β-lactamase which were identified immunologically by Dougan *et al.* (1979) in pBR322 containing minicells (the pre-protein, the active form of the enzyme and a fragment of the β-lactamase respectively. N.B. The 25,000 may indicate disulphide bond formation, Pollitt and Zalkin, 1983) and which were also observed in minicells by Alton and Vapnek (1979a) and Covarrubias *et al.* (1981). Protein bands of apparent M_r 34,000 and of 18,000 which have been reported from minicell studies to be associated with the constitutive tetracycline resistance phenotype (Tait and Boyer, 1978; Alton and Vapnek, 1979a), were not observed. Tracks, B, C and E

Figure 13.

Autoradiograph from in vitro transcription and translation experiments.



Tracks A to C correspond to plasmids pBR322, pKT205 and pAH1 respectively.
Tracks D = M_r standards: 66,000, 48,000, 24,000, 18,400 and 14,300.
Tracks E to H correspond to plasmids pShI41, pShI44, pAB02 and pNJ2004 respectively. Track I is the no DNA control.

in Figure 13 show the translation products of plasmids which were constructed by ligation of appropriate Pst1 restriction fragments into the Pst1 site of the β-lactamase gene of pBR322, the inserted DNA in each case containing the genes for CAT_{II}, CAT_{III} and CAT_{I} respectively (plasmids pKT205, pAH1 and pShI41, Table 1b). Track F reveals the proteins expressed from a plasmid identical with that used in Track E but with the Pst1 restriction fragment specifying CAT_{I} inserted in the opposite orientation (plasmid pShI44) Track H shows the protein bands specified by plasmid pNJ2004. (The apparent M_r 30,000 product corresponds to the major β-lactamase-related protein and the M_r 27,000 band is likely to be the kanamycin resistance protein.) Track G of the autoradiograph reveals the products of plasmid pAB02 prepared by insertion of the Pst1 fragment from EDR51 specifying CAT_{I} into the β-lactamase gene of pNJ2004 (paragraph 5.2.2). Inspection of Tracks B, C, E, F and G reveals that insertion of DNA fragments into the Pst1 site of the β-lactamase gene of both pBR322 and pNJ2004 results in the loss of the β-lactamase-related bands. Tracks E, F and G all strongly express a protein of apparent M_r 24,000 which is the CAT_{I} monomer. (The actual M_r of the CAT_{I} monomer from the amino acid sequence is 25,668 but apparent M_r values in the range 23,500 to 25,000 are commonly observed for CAT_{I} when it is subjected to polyacrylamide gel electrophoresis in the presence of reducing agents and sodium dodecylsulphate Shaw et al., 1979). Tracks B and C both exhibit two major bands; the lower band in each case being equivalent to the apparent M_r of the CAT variant associated with the plasmid. The band observed in Track B of 24,200 correlates with the apparent M_r of the CAT_{II}

monomer; in Track C the 25,500 band corresponds with that expected for the CAT_{III} monomer (Gaffney et al., 1978; see Fig. 15). The most striking feature of this autoradiograph may be seen in Tracks E, F and G which contain the gene products of the only three plasmids which confer fusidate resistance. In addition to the CAT_I band, each track contains a less intense band (M_r 20,000) indicated by the arrow (→). This polypeptide appears irrespective of the orientation of the inserted CAT_I carrying nucleotide sequence (Tracks E and F) and is likely, therefore, to be encoded by and expressed from within the cloned Pst1 restriction fragment. From the DNA sequence (Fig. 6) the largest polypeptide that can be encoded in a different reading frame to that of CAT_I is one of sixty-five amino-acid residues (paragraph 1.16; M_r approximately 7,000). The conclusion from these observations must be, therefore, that the M_r 20,000 polypeptide represents a segment of the CAT_I polypeptide. The possibility that this band was generated by a lack of fidelity of gene expression peculiar to this in vitro system is made unlikely by the observation that a band of M_r 20,000 has been detected by others in the translation products of minicells carrying the CAT_I-coding mini-plasmids pACYC184 (Schröder et al., 1981), and pBR325 (G. Bulnois, personal communication). However, other mini-plasmid studies with different CAT-coding plasmids have failed to observe this minor band (Alton and Vapnek, 1979a; Covarrubias et al., 1981; paragraph 5.2).

5.3.2 Studies on insertion and amber mutations of pBR325

To separate fusidate resistance from the M_r 20,000 product would be to favour the dual role of the CAT_I enzyme as the effector of resistance to both fusidic acid and chloramphenicol. Conversely, to dissociate the fusidate resistance phenotype from the M_r 24,000 band attributed to the CAT_I enzyme monomer would relate fusidate resistance to the presence of the M_r 20,000 polypeptide.

In order to try to resolve this question in an unambiguous manner, three derivatives of plasmid pBR325 were employed which contained DNA fragments of differing lengths inserted into the unique EcoR1 restriction site within the CAT_I structural gene (Fig. 6).

All of the constructs pGB38 (Pratt et al., 1981) p0100I/pBR325 and p0100II/pBR325 (De Vos et al., 1981) were observed to have lost both the chloramphenicol and fusidate resistance phenotypes. Unfortunately they failed to express either the M_r 20,000 or CAT_I enzyme monomer bands in the in vitro system, (data not shown).

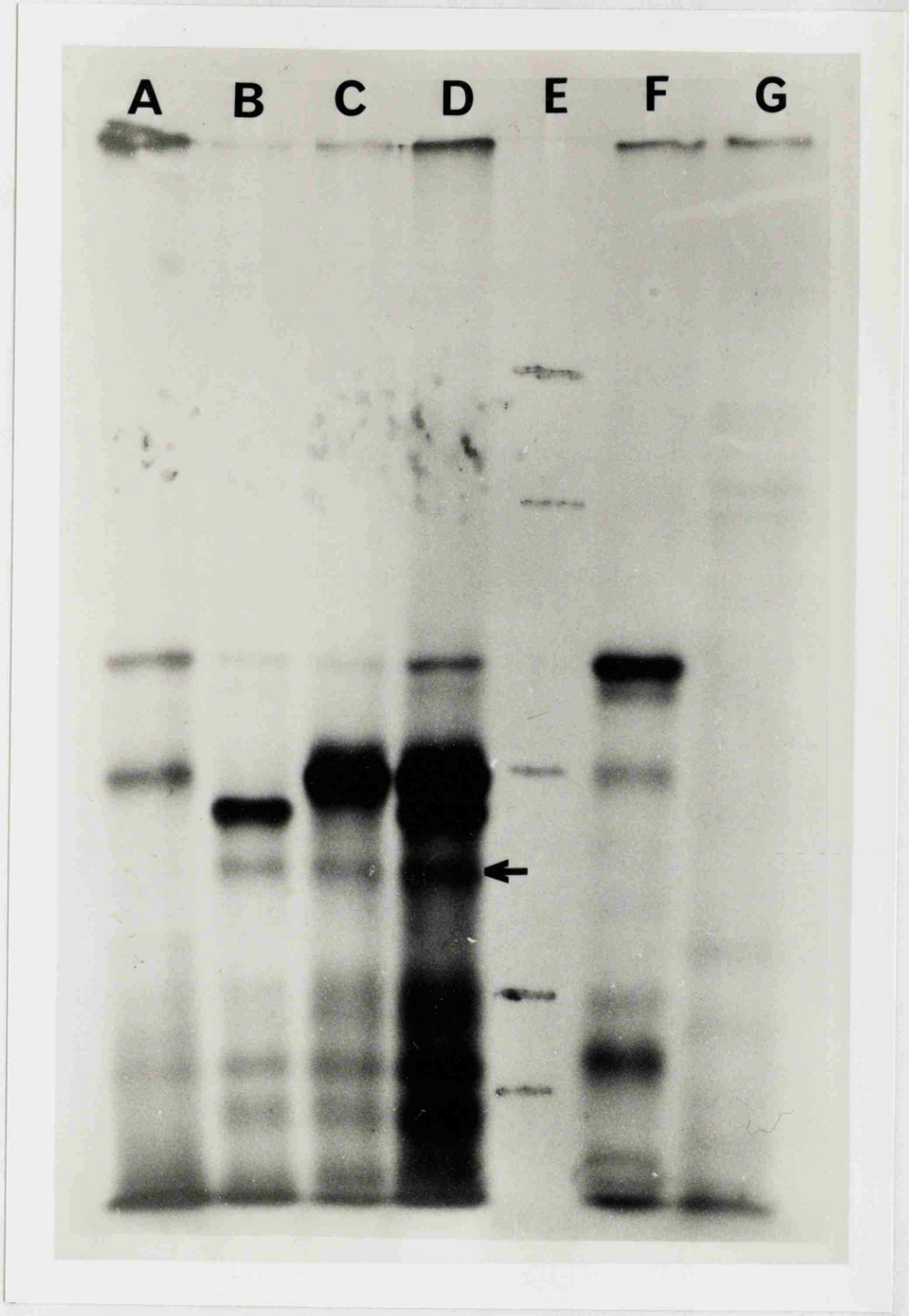
The concomitant presence of fusidate resistance and the M_r 20,000 band was finally dissociated by analysing the protein products directed by two pBR325 derivatives constructed by Völker et al. (1982) in a parallel study.

Völker et al. (1982) demonstrated that amber mutants near the beginning and end of the CAT_I structural gene (Fig. 6) both result in the loss of resistance to chloramphenicol and fusidate. Informational suppression by appropriate suppressor tRNA molecules restores both resistance phenotypes. In addition Völker and colleagues (1982) reported that mutants of the CAT_I enzyme which are temperature sensitive for chloramphenicol resistance (Mise and Suzuki, 1968) are also temperature sensitive for fusidate resistance and simultaneous reversion to temperature-independent resistance was observed. It would appear, therefore, that despite the structural dissimiliarity of the two antibiotics, the resistance phenotype in each case is due to the same gene product (i.e. the CAT_I enzyme; see paragraph 3.4 and 4.3).

Track F of the autoradiograph in Fig. 14 contains polypeptides synthesised in vitro from plasmid pBR322 DNA; Track C shows those expressed from pBR325. The M_r 20,000 band is indicated with an arrow (←). Track A contains the translation products of plasmid pBR325 cat amH22, the derivative carrying a chain terminating amber mutation "early" in the CAT_I coding sequence. No products related to CAT_I are observed either due to the instability of the transcript or of the truncated polypeptide. Track B contains polypeptides expressed by the pBR325 derivative that carries a chain terminating amber mutation "late" in the CAT_I structural gene. The resulting synthesis of a protein of apparent M_r 22,000 corresponds to the exact size of the truncated CAT_I polypeptide that is predicted from the DNA sequence. The polypeptides observed in Track D

Figure 14.

Autoradiograph from *in vitro* transcription and translation experiments.



Tracks A to C correspond to plasmids pBR325 *cat* am H22 (the "early" amber mutation), pBR325, *cat* am H32 (the "late" amber mutation) and pBR325 respectively. Track D is a mixture of the extracts run in B and C. Track E = M_r standards: 66,000, 48,000, 24,000, 18,400 and 14,300. Track F = pBR322. Track G is the no DNA control.

(The difference in intensity of the M_r 30,000 pre-β-lactamase band is due to loading a fixed amount of radioactive protein per track. A consequence of this is that the strong CAT_I promoter makes a great deal of CAT_I and accounts for a large percentage of the total protein applied to the lane. The fraction of the protein that corresponds to the pre-β-lactamase band is thereby decreased.)

of Fig. 14 are a mixture of the translation products which were loaded in Tracks B and C and confirm the observed size difference for the prematurely terminated product. As stated above, both of the amber mutations in the gene for CAT_I result in the simultaneous loss of the chloramphenicol and fusidate resistance phenotypes (Völker et al., 1982). However, the "late" amber mutation (Track B) still expresses the M_r 20,000 band. It can be concluded, therefore that the M_r 20,000 band is not associated with fusidate resistance and that the CAT_I enzyme is sufficient, by itself, to confer both the chloramphenicol and fusidic acid resistances. (Additionally, the M_r 20,000 band must represent an N-terminal fragment of the CAT_I protein.)

Table 4a (paragraphs 2.13 and 6.1)

	Purification Step	Volume (ml)	CAT (U/ml)	Total Units	Protein (mg/ml)	Total Protein (mg)	CAT S.A. (U/mg)	Purification factor	Yield (%)
	Crude extract	40	292.5	11,700	27.45	1098	10.6	1.0	100
	Heat Step	38.5	293.0	11,280	18.44	710	15.9	1.5	96.4
Affinity resin	Tris wash	90.0	0.77	70	-	-	-	-	0.6
	0.3M NaCl/Tris wash	280	0.75	260	-	-	-	-	2.2
	0.3M NaCl/Tris Wash + 5mM Cm	15.5 (five peak fractions pooled)	706.5	10,950	3.62	56.1	195	18.4	93.6

Purification of CAT_I enzyme from pBR328/*E. coli* C600 on "K1" resin

Table 4b (paragraph 2.13 and 6.1)

	Purification Step	Volume	CAT (U/ml)	Total Units	Protein (mg/ml)	Total protein (mg)	CAT S.A. (U/mg)	Purification factor	Yield (%)
	Crude extract	15.0	48.6	729	25.6	384	1.90	1.0	100
Affinity resin	Tris wash	40	0.85	34	-	-	-	-	4.7
	0.3M NaCl/Tris wash	160	1.35	161.4	-	-	-	-	22.1
	0.3M NaCl/Tris wash + 5mM Cm	10.6 (five peak fractions pooled)	50.3	533	0.084	0.89	600	316	73.1

Purification of CAT_{II} enzyme from pKT205/*E. coli* C600 on "K2" resin

Table 4c (paragraphs 2.13 and 6.1)

	Purification Step	Volume	CAT (U/ml)	Total Units	Protein (mg/ml)	Total Protein (mg)	CAT S.A. (U/mg)	Purification factor	Yield (%)
	Crude extract	15.0	218.7	3281	24.3	364.5	9.00	1.0	100
	Heat Step	14.0	239.0	3346	16.6	232.4	14.40	1.6	102
Affinity resin	Tris wash	50	0.90	45.0	-	-	-	-	1.37
	0.3M NaCl/Tris wash	150	1.00	150.0	-	-	-	-	4.57
	0.6M NaCl/Tris wash	200	2.45	490	-	-	-	-	14.9
	0.6M NaCl/Tris wash + 5mM Cm	16.2 (five peak fractions pooled)	153.8	2492	0.27	4.37	570	63.3	76.0

Purification of CAT_{III} enzyme from pAH1/*E. coli* C600 on "K2" resin

CHAPTER SIX

In vitro studies with purified CAT proteins

6.1 Purification of CAT enzymes

Purification of the three enterobacterial CAT variants was performed as described (paragraph 2.13).

Yields and increases in purity of the three enzyme throughout the procedures are set out in Tables 4a, 4b and 4c.

The final purity of CAT_I enzyme was judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (paragraph 2.14) to be homogeneous both by Coomassie Blue stain (paragraph 2.15.1; Fig. 15) and by silver stain (paragraph 2.15.2; Fig. 16).

The final purity of both the CAT_{II} and CAT_{III} variants was judged to be 99% by Coomassie Blue stain but the extra sensitivity of the silver stain procedure indicated a purity of approximately 97.5%.

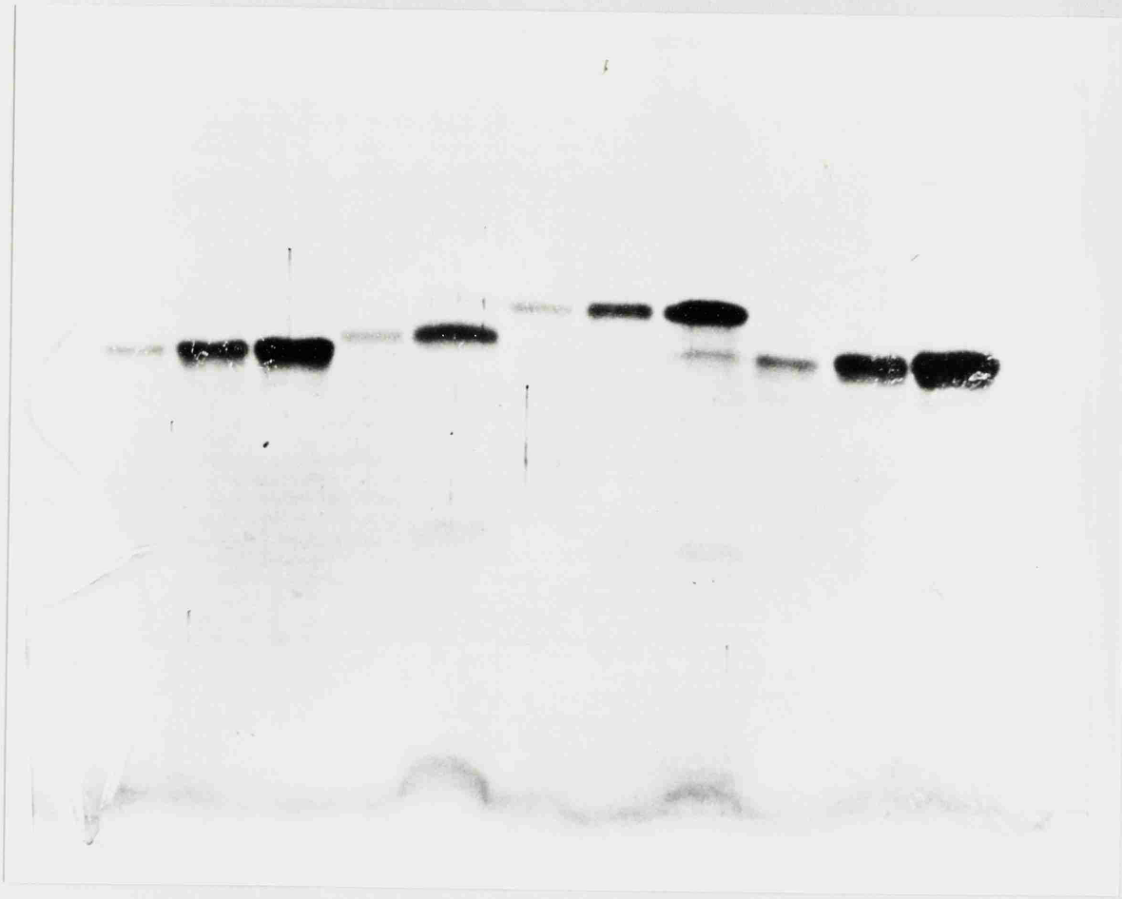
The specific activity of purified CAT_{II} enzyme from strain *E. coli* C600 carrying mini-plasmid pKT205 (Table 1b) was estimated as 600 units/mg protein (paragraphs 2.9 and 2.12; Table 4b).

6.2 Steady-state kinetic analysis of fusidate binding to CAT_I

The steady-state kinetics of the interaction between CAT_I and fusidate was observed by the effect of the presence of fusidate on the rate of acetylation of chloramphenicol in the

Figure 15.

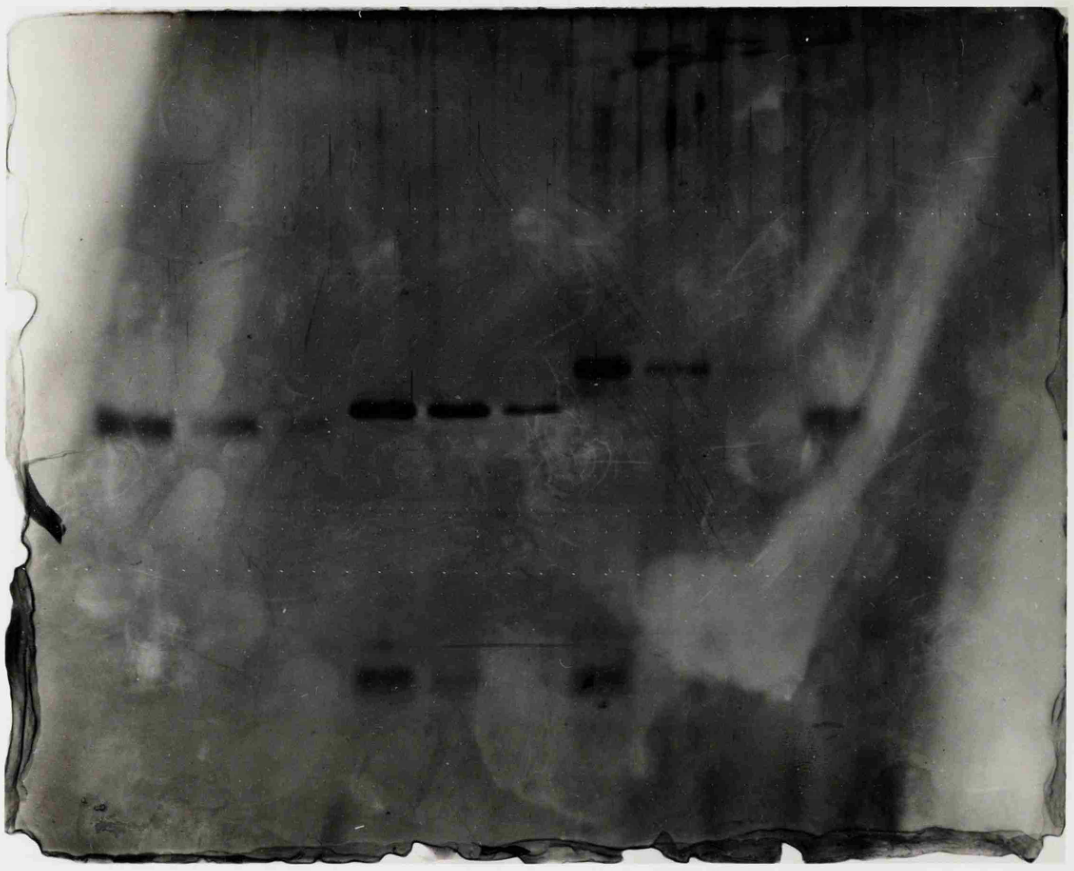
Coomassie Blue stained gel of CAT protein purifications



CAT_I			CAT_{II}		CAT_{III}			CAT_I		
1µg	5µg	20µg	1µg	5µg	1µg	5µg	20µg	1µg	5µg	20µg

Figure 16.

Silver stained gel of CAT protein purifications

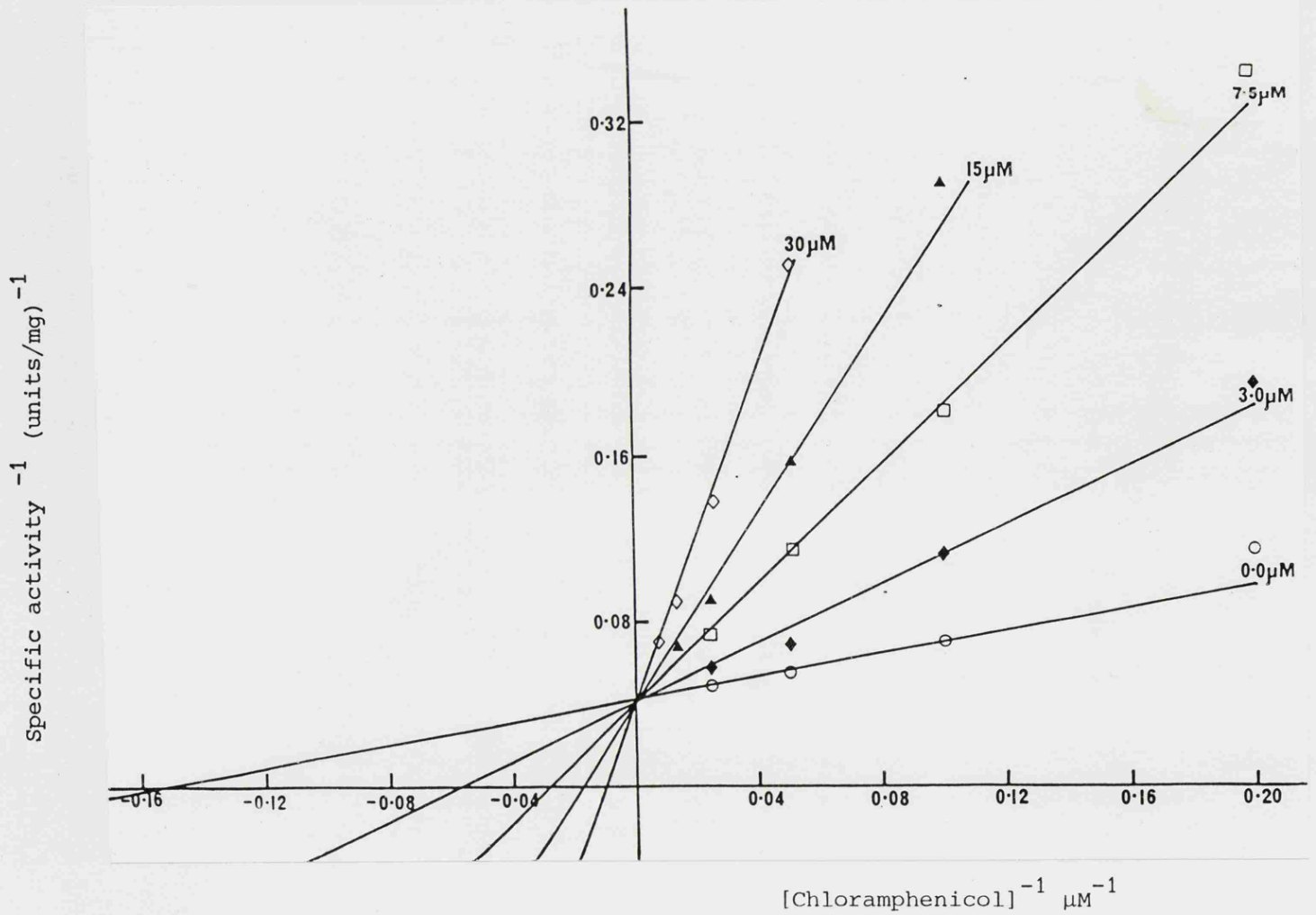


CAT_I			CAT_{II}			CAT_{III}			CAT_I
1µg	0.5µg	0.2µg	1µg	0.5µg	0.2µg	1µg	0.5µg	0.2µg	0.5µg

spectrophotometric assay (Shaw, 1975; paragraph 2.12). An initial concentration of 500 μM acetyl CoA was used for the chloramphenicol inhibition studies and 500 μM chloramphenicol was used with the acetyl CoA inhibition studies. Since the presence of plasmids specifying the enzyme variants CAT_{II} and CAT_{III} have not been associated with fusidate resistance, the effects of fusidate on the rates of chloramphenicol acetylation catalysed by these proteins were examined as controls.
For these studies each of the three CAT variants were used in purified form (paragraph 6.1)

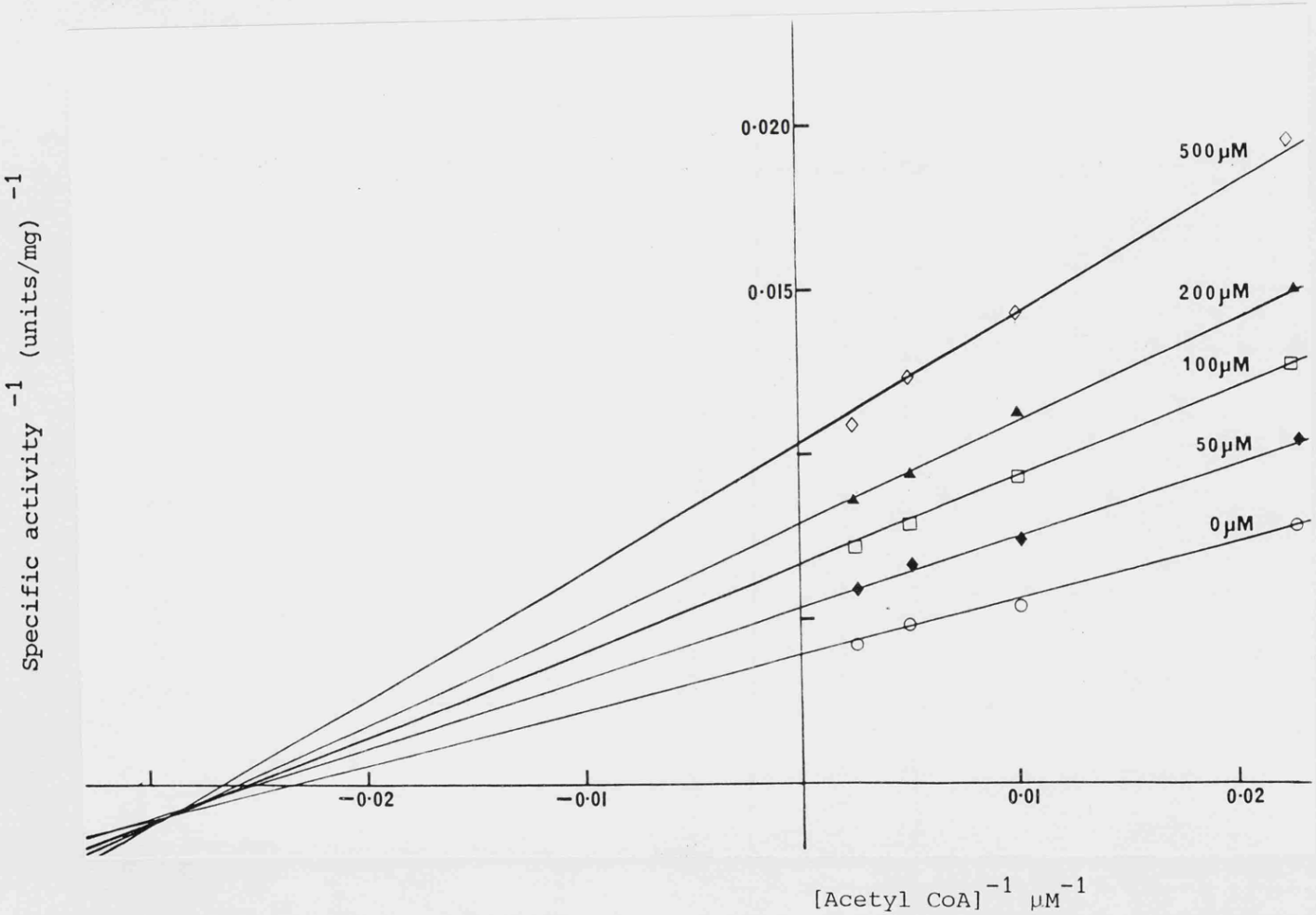
Fusidic acid is not an acetyl acceptor from acetyl CoA in the spectrophotometric assay with any of the three enterobacterial purified CAT variants as had been observed with crude extract (paragraph 4.3). Inhibition of the rate of chloramphenicol acetylation by fusidate was only observed with the type I variant of CAT. The resulting data ~~was~~ were plotted on a double reciprocal plot (Figs. 17a and 17b). The inhibition observed was calculated by the weighted analysis of Cleland (1979) and judged to be competitive with chloramphenicol (Fig. 17a)and mixed uncompetitive-non-competitive with acetyl-CoA (Fig. 17b). The Michaelis constants (K_m) of CAT_I in the absence of fusidate for chloramphenicol and acetyl CoA were 7.2 ± 1.2 μM and 46 ± 5 μM, respectively. The inhibition constant, (K_i), from the secondary plot of the variable chloramphenicol concentration data was 1.7 ± 0.3 μM. Fusidate failed to inhibit the CAT_{II} and CAT_{III} catalysed acetylation of chloramphenicol even when present at a concentration of 500μM.

Figure 17a



Steady-state kinetic plot of the inhibition of chloramphenicol acetylation catalysed by purified CAT$_I$ Sodium fusidate competing with chloramphenicol (acetyl CoA = 500 μM).

Figure 17b.



Steady-state kinetic plot of the inhibition of chloramphenicol acetylation catalysed by purified CAT$_I$ Sodium fusidate competing with acetyl CoA (chloramphenicol = 500 μM).

6.3 Inhibition of in vitro protein synthesis by fusidate

Studies on the inhibition of in vitro protein synthesis by fusidic acid were performed using the cell-free transcription and translation system which was also used to investigate in vitro plasmid-directed protein expression (paragraph 2.28 and 5.3).

The only departures from the method being that half-volume (17.5 µl) incubations were performed to conserve S30 extract and that the de novo synthesised protein was merely quantified as trichloroacetic acid (TCA) precipitable counts and not subjected to sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

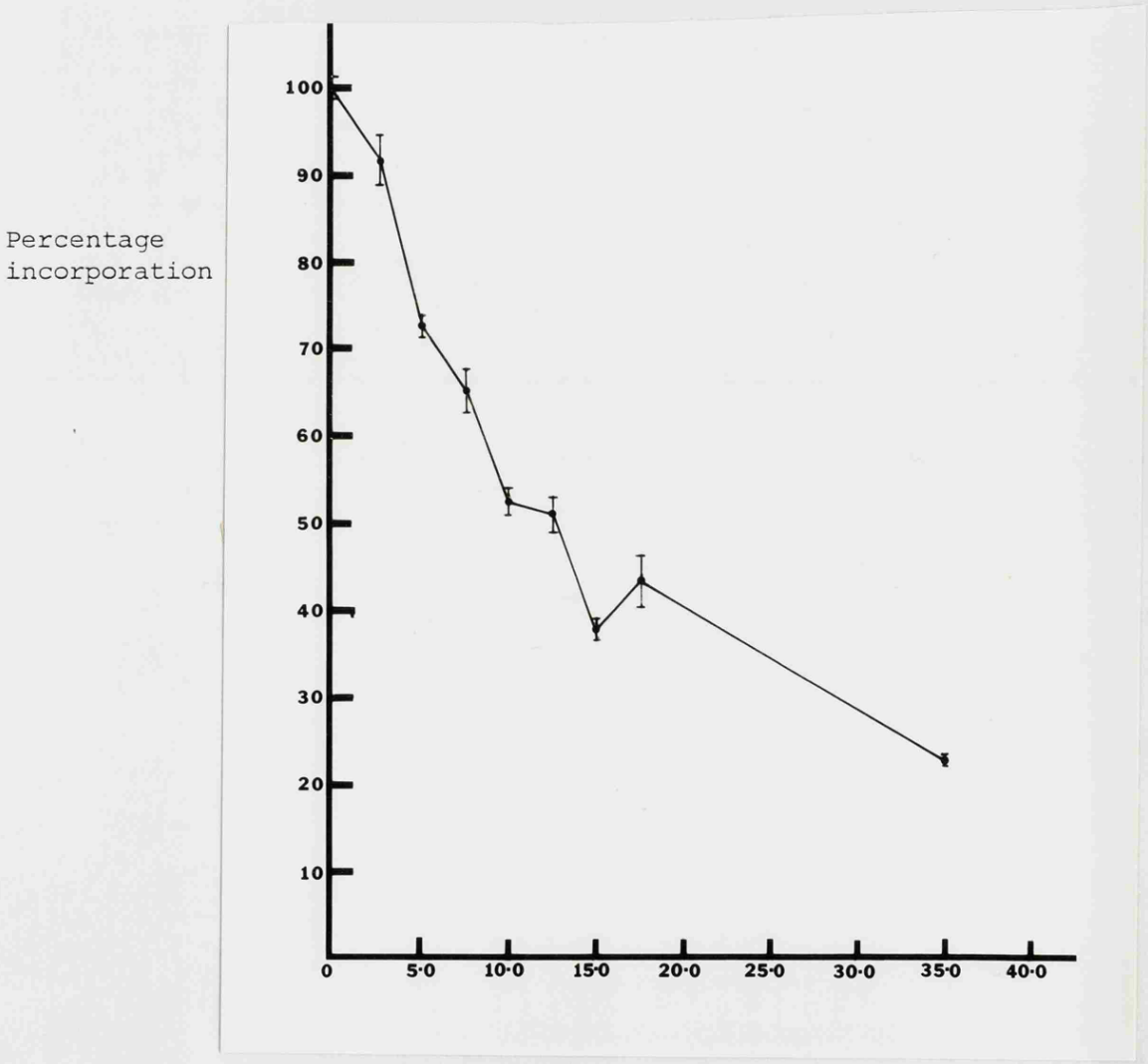
Since it was not necessary to examine the polypeptides expressed the template DNA employed was supercoiled plasmid pBR322 DNA (2.5 µg per incubation). (Any mini-plasmid DNA except those coding for CAT enzymes could, in principle, be used.)

The results of three experiments on the effect of fusidic acid on protein production were combined and are shown in Fig. 18.

From the graph in Fig. 18 it can be seen that fifty percent inhibition of protein synthesis occurred at a final concentration (before the 2.5 µl "cold" methionine chase) of approximately 12 µM sodium fusidate.

Figure 18.

Incorporation of L-[^{35}S] methionine into TCA precipitable protein in the *in vitro* coupled transcription and translation system. Inhibition of incorporation by sodium fusidate.



This figure is in accord with the observations of Harvey et al. (1966) and Tanaka et al. (1968) who reported that E. coli spheroplasts and cell-free extracts are far more sensitive to fusidic acid than the bacteria themselves.

In the experiment of Tanaka et al. (1968) the effect of fusidic acid was studied on a poly U-directed polyphenylalanine synthesising system and approximately sixty-five percent inhibition of protein synthesis was observed at a fusidate concentration of 185 μM. The approximate ten-fold increase in sensitivity of the system employed in this study probably reflects the closer approximation to physiological conditions that were employed.

The purpose of this experiment was to use the system to demonstrate a relief of fusidate-mediated inhibition of protein synthesis brought about by the addition to the incubations of purified CAT_I protein. However, at this time Dr. E. Cundliffe of this Department (Biochemistry) received a gift of purified elongation factor G from Prof. J. Bodley and hence I was able to perform a more defined and more accurate series of experiments free from much of the undesirable complexity of this system (see below).

6.4 Relief of inhibition of in vitro polypeptide chain elongation

The rate of polypeptide chain elongation in vitro was monitored on the basis of the rate of ribosome-dependent GTP hydrolysis (paragraph 1.9). The latter can occur in the absence of both exogenous mRNA and amino-acyl tRNA molecules but is dependent

on the presence of elongation factor G (paragraph 1.11). The maximal rate of GTP hydrolysis under the conditions described in paragraph 2.30 only occurred when fusidate was absent. The mean value of maximal GTP hydrolysis was 101 pmole/min during the twenty minute incubation.

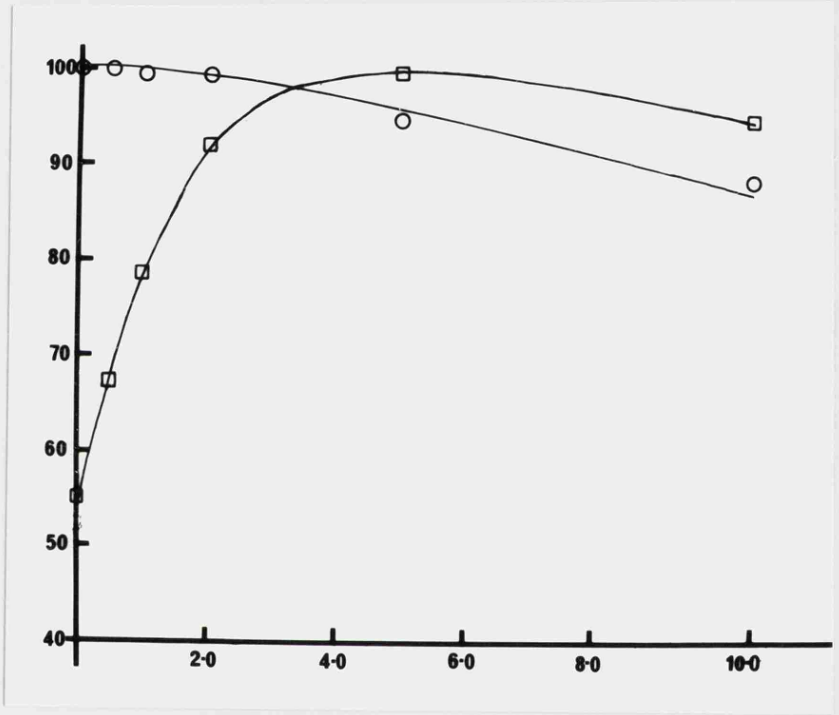
The graph in Fig. 19 shows the effect of the presence of sodium fusidate on polypeptide chain elongation. At a final fusidate concentration of 1.0 µM (0.54 µg/ml) ribosome-dependent GTP hydrolysis is 55% of the maximal rate, an observation in close agreement with the findings of Okura et al., 1970 (q.v. paragraph 6.3). The graph also shows the effect of increasing concentrations of CAT_I on the elongation reaction. Whereas the addition of CAT_I only slightly depresses GTPase activity, it relieves the inhibition caused by the presence of 1.0 µM fusidate. Reversal of the inhibition was judged to be consistent with an equimolar and independent binding of fusidate by each of the four CAT_I subunits. The other enzyme variants, CAT_{II} and CAT_{III}, did not relieve fusidate inhibition even when each present at a final concentration of 10 µM enzyme monomer (results not shown).

6.5 Equilibrium dialysis

In order to further investigate the stoichiometry and avidity of fusidate binding to CAT_I equilibrium dialysis experiments were performed (paragraph 2.31). Enzyme at a monomer concentration of 4.6 µM was dialysed against tritiated fusidic acid and the resulting data were plotted according to Scatchard (1949) (Fig. 20). The equilibrium dissociation constant, K_d, calculated from the slope of this graph was 25.2 µM, and the

Figure 19.

Relief of inhibition of *in vitro* polypeptide chain elongation by type I chloramphenicol acetyltransferase.



-O-O- addition of CAT$_I$ without sodium fusidate.

-□-□- addition of CAT$_I$ with 1μM sodium fusidate.

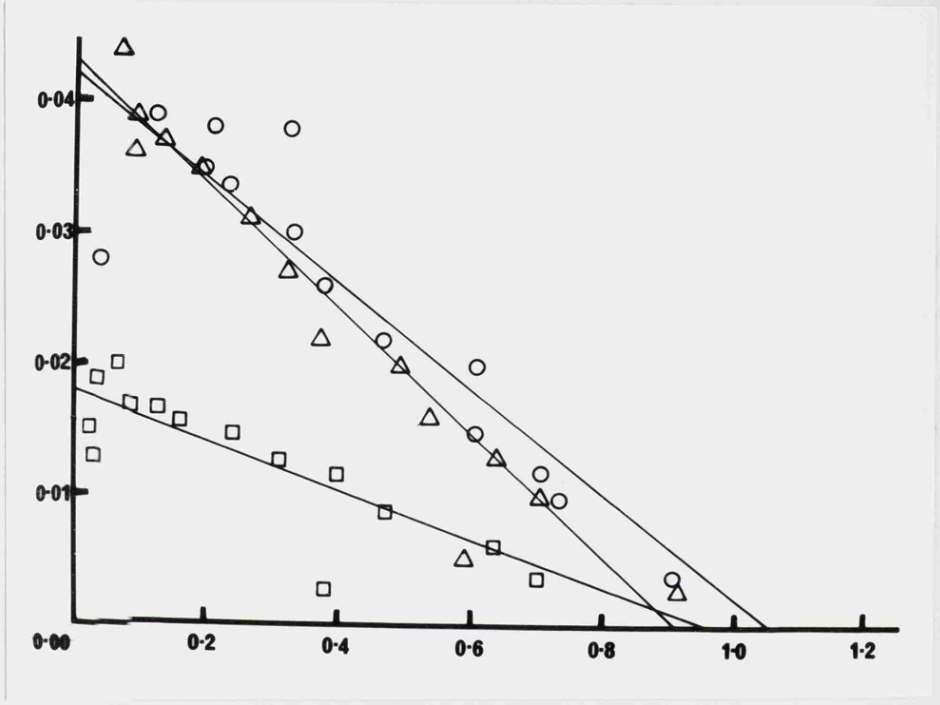
Each point plotted is the mean value of the percentage of maximal GTPase activity from three separate experiments.

value of the intercept on the abscissa (1.06) implies an equimolar binding ratio between CAT_I monomers and fusidate. In addition, the slope of the plot is linear and monophasic indicating that the four binding sites of the CAT_I tetramer bind fusidate independently and non co-operatively (Ferguson-Miller and Koppenol, 1981; q.v. paragraph 6.4). Variation of the fixed concentration of CAT_I in the range 4.0 to 15.0 μM (monomer) did not significantly affect the observed value for the K_d. The enzyme variants CAT_{II} and CAT_{III} were dialysed at fixed monomer concentrations of 1.8 μM and 10.0 μM respectively. Both failed to bind the tritiated fusidate to any detectable extent (results not shown).

During the course of dialysis (90 hrs at 4°C) each of the three CAT variants showed only a small loss of catalytic specific activity (an average of 7 percent with a maximum of 10 percent). Thus, in all determinations of fusidate binding at equilibrium, the protein was in each case predominantly in an enzymatically active conformation. Calculations of the ligand-CAT binding properties were based upon the value observed for the concentration of CAT enzyme at equilibrium as determined in the spectrophotometric assay for chloramphenicol acetylation (paragraph 2.12). Competition binding experiments were carried out to extend the observations that fusidate binding was not merely due to the general hydrophobicity of the antibiotic and was a consequence of more specific interactions (paragraph 4.6). Such experiments were performed as above except that in each case a non-radioactive analogue of fusidate was included at a fixed concentration. The dissociation

Figure 20.

Analysis of the binding of [11β-^{3}H] fusidate to the type I variant of chloramphenicol acetyltransferase by equilibrium dialysis



-○-○-○- [^{3}H] fusidate binding in the absence of other ligands.

-△-△-△- [^{3}H] fusidate binding in the presence of 40μM non-radioactive sodium glycocholate.

-□-□-□- [^{3}H] fusidate binding in the presence of 40μM non-radioactive sodium 24,25-dihydrofusidate.

Extension of the method to other ligands yielded the apparent dissociation constants (K_d) listed in Table 5.

constants of the competing analogues were calculated from the resulting data using the equation of Edsall and Wyman (1958) which can be rearranged thus:-

$$K_{dc} = \frac{[c]}{\frac{K_{da}}{K_d} - 1}$$

where K_d is the dissociation constant of the radioactive ligand at equilibrium (25.2 μM for [^{3}H] fusidate in these experiments).

K_{da} is the apparent dissociation constant in the competition experiment at equilibrium.

K_{dc} is the dissociation constant of the competing ligand at equilibrium.

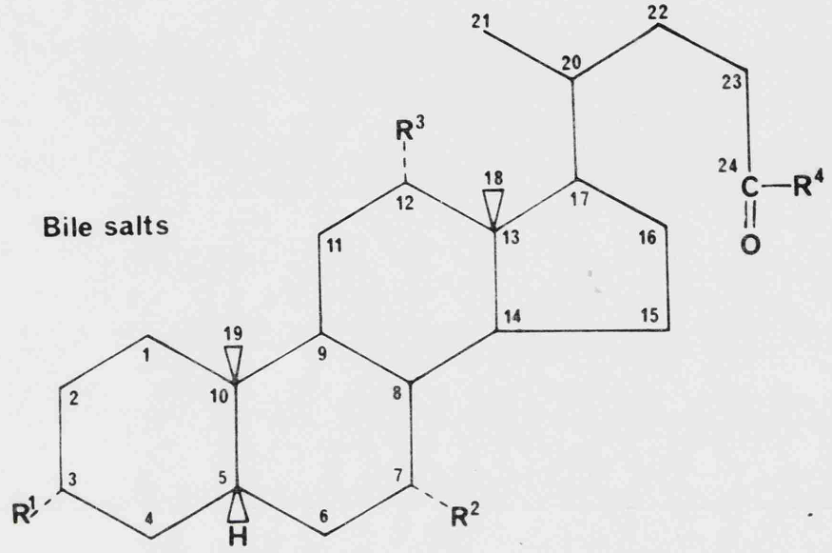
[c] is the final concentration of competing ligand at equilibrium in the absence of binding protein (20 μM for each non-radioactive ligand in these experiments).

To validate the competition dialysis procedure described above, non-radioactive sodium fusidate was employed as the ligand competing against the tritiated diethanolamine salt of fusidate. The K_{dc} value obtained was 31.1 μM as compared with the directly measured K_d of 25.2 μM (Table 5). This small disparity may reflect a low degree of radioautolysis of the tritiated fusidate (D. Sinden personal communication).

Seven fusidate analogues were used in separate competition experiments (Table 5). In addition to compounds having an obvious close structural similarity to fusidate, a study was made of the binding properties of some steroidal bile salts (Fig. 21). Such experiments were prompted by the interesting

Figure 21.

Structural key to bile salts



Trivial name	R^1	R^2	R^3	R^4
Sodium glycocholate	-OH	-OH	-OH	$-NHCH_2CONa$
Sodium taurocholate	-OH	-OH	-OH	$-NH[CH_2]_2SO_3Na$
Sodium glycochenodeoxycholate	-OH	-OH	H	$-NHCH_2CONa$
Sodium taurochenodeoxycholate	-OH	-OH	-H	$-NH[CH_2]_2SO_3Na$
Sodium glycodeoxycholate	-OH	-H	-OH	$-NHCH_2CONa$
Sodium taurodeoxycholate	-OH	-H	-OH	$-NH[CH_2]_2SO_3Na$
Sodium dehydrocholate	=O	=O	=O	-ONa

observations of Proctor and Rownd (1982) that the uptake of crystal violet by strains of E. coli containing the CAT_I enzyme was inhibited by bile salts (also see paragraph 7.1) and the additional knowledge that crystal violet inhibits the CAT_I enzyme competitively with respect to chloramphenicol (Tanaka et al., 1974).

(N.B. The glycine and taurine conjugates of the bile salts cholic acid, deoxycholic acid and chenodeoxycholic acid were used since these three classes of conjugated bile salts constitute the major chemical species in healthy humans. (Sjövall, 1960). The bile salts used are, therefore, the chemical species that wild-type E. coli strains are most likely to encounter in the intestinal environment.)

In addition to being subjected to competition equilibrium dialysis the fusidate analogues and bile salts were tested for their antibacterial activity (Table 5).

Representative plots of two separate competition equilibrium dialysis experiments are shown superimposed on a conventional equilibrium dialysis experiment in Fig. 20.

The results of the experiments presented in this chapter can be summarised as follows. Firstly, kinetic evidence (paragraph 6.2) was given which demonstrated that although fusidic acid was not chemically modified by CAT_I it nevertheless bound to the enzyme very avidly. Furthermore this binding was of a competitive nature with respect to chloramphenicol i.e. a specific domain on the CAT_I enzyme was involved. In addition the kinetics implied that fusidic acid bound to the chloramphenicol binding sites of the CAT_I enzyme in a manner which showed neither negative nor positive cooperativity.

Secondly, as expected, *in vitro* protein synthesis (paragraph 6.3) was inhibited by fusidate and this inhibition could be demonstrated to occur at the elongation step of this process (paragraph 6.4). The inhibition of polypeptide chain elongation moreover could be relieved by the addition of purified CAT_I enzyme and the data again implied that the equimolar binding of CAT_I subunits and fusidate was a process lacking cooperativity.

Thirdly, equilibrium dialysis experiments (paragraph 6.5) extended the above observations and proved the independent and equimolar nature of CAT_I binding by fusidate.

Finally, extension of the equilibrium dialysis technique allowed me to refute the assumption made by both Völker *et al* (1982) and McKell *et al*.(1983; paragraph 8.4) that the CAT_I-fusidate interaction was a crude hydrophobic association primarily based on the steroidal skeleton of fusidate.

Examination of the results of the Steady-state kinetic analysis (paragraph 6.2) and the equilibrium dialysis experiments (paragraph 6.5) show that these techniques do not give comparable values for the strength of the CAT_I-fusidate interaction. The potential reasons for this disharmony are discussed in paragraph 8.2.

The molecular constraints of the interaction which were elucidated from the competition equilibrium dialysis experiments (paragraph 6.5) are also discussed in the same chapter (paragraph 8.1).

Table 5. Antibacterial activity and CAT_I-binding affinities of fusidate analogues.

The minimum inhibitory concentrations (μg/ml) were determined by the gradient plate method (paragraph 2.7). Equilibrium dissociation constants (K_d) were determined using equilibrium dialysis by competition with tritiated fusidate diethanolamine salt (paragraph 6.5; Fig. 20).

Fusidate analogues and bile salts (Fig.10; Table 2; Fig. 21) \ *E. coli* strains (paragraph 2.2)	DB10	DB10	DB10	DB10	DB10	DB10	C600	Equilibrium Dissociation Constant (K_d) with CAT_I enzyme (μM)
Plasmid (Table 1b)	+	+	+	+	+	+	+	
	no plasmid	pBR322	pBR325	pBR328	pKT205	pAH1	no plasmid	
CAT enzyme variant * carried	-	-	CAT_I	CAT_I	CAT_{II}	CAT_{III}	-	
Sodium fusidate	5	5	80	105	5	5	>500	31.1
3-Epifusidate	>450	>450	>450	>450	>450	>450	>450	>500
3-Oxofusidate	100	100	100	100	100	100	>1000	142.3
7α-Hydroxyfusidate	15	15	15	15	15	15	>400	79.5
11-Oxofusidate	100	100	>400	>400	100	100	>400	20.5
24,25-Dihydrofusidate	20	20	>100	>100	20	20	>100	16.8
Helvolic acid	45	45	45	45	45	45	>150	83.0
Cephalosporin P1	40	40	40	40	40	40	>100	>500
Glychocholate	>400	>400	>400	>400	>400	>400	>400	>500
Taurocholate	"	"	"	"	"	"	"	"
Glycochenodeoxycholate	"	"	"	"	"	"	"	"
Taurochenodeoxycholate	"	"	"	"	"	"	"	"
Glycodeoxycholate	"	"	"	"	"	"	"	56.5
Taurodeoxycholate	"	"	"	"	"	"	"	27.6
Dehydrocholate	"	"	"	"	"	"	"	>500

* Gaffney *et al.* (1978), Shaw (1983)

CHAPTER SEVEN

The screening and selection of CAT_I enzyme mutants

7.1 Projected studies on the CAT_I-fusidate interaction

Having determined, as far as available analogues permitted, the molecular constraints placed upon the fusidic acid skeleton for its binding to the CAT_I enzyme (paragraph 6.5; discussed in paragraph 8). I wished to start a preliminary investigation into the complementary facet of the interaction and to look at the constraints present on the amino-acid sequence of the CAT_I protein.

The scope of this study was prompted by the elegance of the amber mutant study performed on the CAT_I structural gene by Völker et al. (1982) and by the amenability of these mutants to analysis in the cell-free coupled transcription and translation system (paragraph 5.3.2).

In their paper Völker et al. (1982) reported that the "late" amber mutation in the CAT_I gene of pBR325 cat am H32 (Fig. 6) could be suppressed using an appropriate tRNA amber suppressor gene so that both chloramphenicol and fusidate resistance phenotypes were regained. However, if this amber mutant, which was originally isolated as a mutation from a glutamine codon (CAG → TAG), was missense suppressed with a tRNA suppressor gene (Sup F) coding for tyrosine the strain regained chloramphenicol resistance but failed to return to fusidate resistance. The reason for this may be that the glutamine (gln) codon suppressed lies at one end of the

chloramphenicol binding site region that is strongly conserved in all CAT proteins studied (reviewed by Shaw, 1983). The amino-acid residue sequence is thus:-

gln val his his ala val cys asp gly arg.

A structure-function investigation of the CAT_I protein was envisaged involving the isolation of mutations in all of the thirty-six amino-acid codons that are mutable to amber in a single step (Fig. 6). These mutants could then be suppressed by a bank of five amber suppressing strains (as employed by Schmitz et al. (1978) and Miller et al. (1979) in their studies on the lac i protein). This suppression would effectively result in thirty-six families of five proteins, each member of the family being identical except at a single amino acid residue (see also Nene and Glass, 1981; Hunter and Glass, 1983 and reviewed by Glass, 1982).

N.B. The use of [^{19}F] 3-fluorotyrosine additionally allows a nuclear spin label to be incorporated into the protein for nuclear magnetic resonance (NMR) studies. This is effected by using a tyr$^-$ supF (Su3) E. coli host cell supplied with [^{19}F] 3-fluorotyrosine. Suppression of amber codons by Sup F causes the incorporation of the spin label into the protein. (Jarema et al., 1981a and b).

The resulting battery of CAT_I protein variants could then be scored at high and low temperatures for their chloramphenicol and fusidic acid resistance phenotypes.

In addition, to these mutants the temperature-sensitive mutants of Mise and Suzuki (1968) and the point mutants of Foster and Howe (1973) could also be investigated (paragraphs 5.2 and 5.3.2). Lastly, the mutagenesis of the CAT_I gene would, of course, generate a great many non-amber point mutants of interest.

The mapping of all of these mutants would be facilitated not only by the known restriction map (Fig. 6) but also by analysis in the in vitro system of the truncated CAT proteins synthesised (paragraph 5.3.2).

As well as a fuller understanding of the CAT_I-fusidate binding constraints the study could also be expected to shed light on the nature of the CAT_I-chloramphenicol and CAT_I-acetyl CoA interactions and to provide data on the residues involved in the CAT_I-subunit interactions (Packman and Shaw 1981a and b).

Finally the work could be correlated with X-ray crystallographic studies on the type I enzyme on which preliminary data have already been gathered (Liddel et al., 1978).

It can be clearly seen that for such a study to be performed well a large number of mutants of the CAT_I gene would need to be screened.

7.2 Screening for catalytically-deficient CAT mutants

As an aid to the isolation of CAT_I amber mutants one could, in principle, use the screen for CAT_I-containing colonies devised by Proctor and Rownd (1982). The method is based upon the competitive binding of crystal violet and other triphenylmethane dyes to the chloramphenicol binding domain of the CAT_I protein (Tanaka et al., 1974). However, this is a screening method and is not applicable to an extension of the CAT_I amber mutant study involving the CAT_{II} and CAT_{III} variants since the dye fails to accumulate in E.coli strains containing these two enzymes (results not shown). Additionally, crystal violet is moderately toxic to wild-type E.coli (and possibly even more toxic to E.coli DB10) and hence the plating efficiency of CAT_I-deficient strains is appreciably less than for CAT_I-containing E.coli strains (Proctor and Rownd, 1982). The method is also unsuitable for the study of the Staphylococcal CAT variants because crystal violet is extremely toxic to many GRAM positive organisms. Transfer of the S.aureus type C variant to an E.coli background (paragraph 1.14.2) does not improve the situation. Although CAT is expressed at a high enough level to confer chloramphenicol resistance the strain fails to accumulate crystal violet (results not shown). Accordingly a more useful positive selection procedure was sought (paragraph 7.3).

An attempt to parallel the work of Proctor and Rownd (1982) based on the authors preliminary communication to W.V. Shaw and using a variety of triphenylmethane dyes was unsuccessful since the method requires very rich complex media plates (paragraph 2.4; Proctor and Rownd, 1982).

7.3 Positive selection for catalytically-deficient CAT mutants

A positive selection procedure which could be used both to detect recombinant DNA plasmids by insertional inactivation of the CAT enzyme (e.g. the cloning of EcoR1 fragments into the EcoR1

site of the CAT_I structural gene, Fig. 6) and which also could be used to isolate point mutants in the CAT gene (paragraph 7.1) was desired.

In personal communications to W.V. Shaw, J.L. Rosner and N. Datta stated that CAT-containing E. coli mutants defective in either ace E or ace F genes were unable to exhibit chloramphenicol resistance on minimal media low in acetate (Shaw, 1983).

Mutants in either the ace E or ace F genes of the pyruvate dehydrogenase complex are unable to synthesise acetyl CoA from pyruvate and need to be supplied with acetate for growth (Langley and Guest 1978; Brown et al., 1977). On low acetate media the acetyl CoA pool is depleted and hence the acetylation of chloramphenicol cannot be sustained. This acetyl CoA drain is, of course, greater than it needs to be merely to provide resistance to chloramphenicol since the chloramphenicol-3-acetate formed re-arranges to the 1-acetate ester and is subsequently re-acetylated to yield chloramphenicol 1,3-diacetate (paragraph 1.14.1; Fig. 5).

N.B. The acetyl CoA drain probably at least in part, accounts for the plateau of chloramphenicol resistance observed by Uhlin and Nordström (1977 and 1978) with R-factor copy number mutants. It may also help to explain the failure to attain high level chloramphenicol resistance when CAT genes are carried on mini-plasmids (paragraph 5.2.2).

If an analogue of chloramphenicol could be found which was devoid of antibacterial activity yet still functioned as an acetyl acceptor it could form the basis of a positive selection procedure for CAT mutants based on the suicide of *ace*$^-$ strains containing catalytically-active CAT enzymes. Furthermore, it would be applicable to all of the CAT enzyme variants and not just the CAT_I variant as the dye based screening is (paragraph 7.2).

By combining data from Shaw (1983) with unpublished correspondence between M. Rebstock and W.V. Shaw I was able to identify a small number of potentially useful chloramphenicol analogues. One of these compounds looked to be not only a useful candidate but also an analogue whose synthesis appeared to be relatively straight-forward.

Synthesis of the compound (D(-) *threo*-1-(*p*-nitrophenyl)-2-butryamido-1,3-propanediol) was performed as described (paragraph 2.33).

As my *ace* mutant strains I chose the *ace* EF deletions *E. coli* KHΔ10 and KΔ18 (Langley and Guest 1978; paragraph 2.2). Deletion mutants were chosen so that they could not revert to give false positives.

Because of extra requirement of *E. coli* KΔ18 for succinate (the *lpd*$^-$ genotype) most of the experiments were performed using *E. coli* KHΔ10 as the plasmid host.

Transformation of E. coli KHΔ10 with plasmids pBR322, pBR325, pBR325 cat am H22 and pBR325 cat am H32 revealed ~~relieved~~ that in the presence of excess acetate (paragraph 2.4) only the strain carrying pBR325 was chloramphenicol resistant. This indicated that E. coli KHΔ10 does not carry an effective suppressor gene (or at least not one able to suppress the two amber mutants used).

Preliminary studies showed that the chloramphenicol analogue was more soluble in water than chloramphenicol and was an extremely good acetyl acceptor in the spectrophotometric assay for chloramphenicol acetylation (paragraph 2.12); indeed with purified CAT_I enzyme it appeared to exhibit a better initial velocity rate than chloramphenicol itself. Additionally, against E. coli KHΔ10 it appeared to be less than three percent as active as chloramphenicol as an antibiotic. Low acetate plates were poured (paragraph 2.4) containing 150 μg/ml of the chloramphenicol analogue (≅0.50 mM) the maximum level tolerated by E. coli KHΔ10. These contained a range of sodium acetate concentrations from 0.2 mM to 2.0 mM. It was expected that since two acetyl CoA molecules are required per molecule of chloramphenicol that acetate concentrations below 1.0 mM would permit the slow growth of the E. coli KHΔ10 strains which lacked CAT (i.e. those containing no plasmid or plasmids pBR322, pBR325 cat am H22 or pBR325 cat am H32; Table 1b). Below 1.0 mM the strains containing CAT (i.e. the pBR325-carrying strain) would not be able to grow.

Analysis of the growth of these strains streaked on to the selective plates did not, however, give the result desired. Below 1.0 mM acetate the strain *E. coli* KHΔ10/pBR325 consistently gave a small number of colonies. These colonies were fully resistant to chloramphenicol when acetate was supplied in excess indicating they had not lost the CAT-carrying plasmid.

With this failure the experiment was abandoned but it was later discovered that I had been sent *E. coli* A10 in error. This strain carries the same auxotrophic markers as *E. coli* KHΔ10 (often written for laboratory use "Δ10") but is instead merely a point mutant of the *ace* F gene (Langley and Guest, 1977; 1978). It is altogether possible that the "*E. coli* KHΔ10/pBR325" colonies observed below 1.0 mM acetate are in fact chromosomal revertants to *ace*$^+$. Unfortunately with my research assistantship at an end I was unable to repeat the microbiology. I gather, however, that the work will be taken up by another member of this laboratory.

N.B. For the future of this protocol a higher chemical homologue of the compound could be expected to retain a good deal of acetyl-accepting potential and could be anticipated to be less antibacterially active. Thus a greater concentration of the analogue could be employed in the selection plates and this would thereby make the selection for catalytically-deficient CAT mutants much more clear cut since the analogue would provide a greater CAT-assisted acetyl CoA drain.

CHAPTER EIGHT

DISCUSSION

8.1 The binding of fusidane compounds to CAT_I

The CAT_I enzyme has been shown to bind reversibly to fusidic acid and a number of analogues (paragraphs 6.2 and 6.5; Figs. 17 and 20; Table 5).

By combining the data on binding affinities with the chemical structures (Table 5) of the fusidic acid analogues (Fig. 10; Tables 2 and 5) and bile salts (Fig. 21) used the nature of the CAT_I-fusidane ligand interaction can be assessed. Far from being merely a interaction dependent solely on the hydrophobic properties of the steroidal skeleton it can be seen that the CAT_I enzyme exhibits a strong specific preference for certain functional groups. The preference furthermore is consistent between the two groups of compounds.

In addition to position 3 the only other site tested which fails to tolerate even a small change is position 7. Minor changes in the nature of the groups at positions 4, 5, 9, 11, 12, 13, 14 and 16 are accepted and major changes to the side chain at position 17 are also permitted.

The antibacterial potency of fusidic acid analogues against *E. coli* DB10 has been shown to reflect qualitatively the activities observed against *S.aureus* (von Daehne *et al.*, 1979; Bodley and Godtfredsen, 1972; Table 3). For those compounds

which still function as antibiotics it can be seen that they only act on CAT_I-containing cells if their equilibrium dissociation constants (K_d) are high (Table 5). These compounds thus provide additional circumstantial evidence that, as established, CAT_I is the fusidic acid resistance effector.

8.2 A coherent mechanism for CAT_I mediated fusidate resistance in vivo

For the CAT_I enzyme to represent a plausible resistance mechanism based solely on reversible fusidate binding it ought to satisfy two criteria. Firstly, the concentration of CAT_I protein within the cell must be sufficient to bind and sequester the incoming antibiotic and, secondly, the affinity of fusidate for the enzyme must approximate or exceed that of fusidate for the complex of ribosome GDP and elongation factor G. (The results indicate that there is a 1 : 1 binding stoichiometry between each of the CAT_I subunits and fusidate. paragraph 6.5).

The extent of increase in the observed fusidate resistance of *E. coli* DB10 strains carrying mini-plasmids over the plasmid-free host strain was determined on 2YT agar gradient plates (Tables 1b and 5). For *E. coli* DB10 carrying two of the plasmids specifying CAT_I (pBR325 and pBR328) this increase is 75 and 100 µg/ml respectively, corresponding to an increase in fusidate concentrations of 138 and 185 µM. The specific activity of CAT_I in crude extracts of *E. coli* DB10 carrying pBR325 and of *E. coli* DB10 with pBR328 was determined from cells grown in 2YT broth. Under such conditions CAT_I is present as 2.8% and 3.7% of the total intracellular protein respectively. If 15% of the wet weight of *E. coli* is presumed to be protein

(Lehninger, 1975) this would imply intracellular CAT_I monomer concentrations of 175 and 230 μM respectively.

The apparent affinity of CAT_I for fusidate was determined in two ways which might reasonably be expected to yield similar values. Equilibrium dialysis gave a K_d value of approx. 30 μM (Table 5) whereas steady-state kinetics gave an apparent K_i value 20-fold lower (paragraph 6.2). Although both experiments were performed in near identical buffers, the steady-state kinetic experiments were performed in the presence of near-saturating levels of acetyl CoA at 37°C (500 μM) whereas the equilibrium dialysis in the absence of coenzyme took place at 4°C (paragraph 2.31). The latter conditions being dictated by the known ability of CAT_I to hydrolyse acetyl CoA slowly in the absence of chloramphenicol (Shaw, 1983)

The sequential order of binding of chloramphenicol and acetyl CoA has not been determined for CAT_I but an analysis of the CAT_{III} variant (which does <u>not</u> bind fusidate) by steady-state methods strongly favours a ternary complex mechanism wherein acetyl CoA is the leading substrate (Kleanthous, K and Shaw, W.V. manuscript in preparation). Although the nature or extent of induced changes at the chloramphenicol/fusidate binding site which may attend the prior binding of acetyl CoA are unknown it is possible that the disparity between the K_d and K_i values is accountable to this phenomenon. A less tenable hypothesis is that the binding of fusidate to CAT_I is dramatically temperature-dependent.

If the observed K_i value (1.7 μM) is a more realistic reflection of the situation *in vivo* wherein acetyl CoA is present, then it is worth noting that this value approaches the reported dissociation constants for interactions of the ribosome-GDP-elongation factor G complex with fusidate (0.4 μM observed by Okura *et al.*, 1971) and 24,25-dihydro-fusidate (0.39 μM noted by Willie *et al.*, 1975).

8.3 Possible epidemiological consequences of CAT_I-mediated fusidate resistance

Davis and Smith (1978) postulate and cite examples of a variety of potential mechanisms to account for antibiotic resistance phenotypes. The mechanism proposed by this work is that the CAT_I enzyme acts as an intracellular molecular "sponge". This mechanism is not postulated by Davis and Smith (1978) and appears to constitute a novel resistance process.

The epidemiological significance of this resistance is difficult to gauge since *E. coli* as encountered in the intestinal tract is intrinsically resistant to fusidic acid (paragraph 1.7). However, increased tolerance to bile salts might favour the viability of CAT_I-containing bacteria in the digestive tract. This may to some degree account for the preponderance of CAT_I-containing strains in clinical isolates, over the occurrence of the other two enterobacterial CAT variants. This hypothesised selective advantage could, therefore, enhance the persistence of CAT_I-containing strains in the absence of continued selection for resistance to chloramphenicol.

N.B. The only other organism other than E.coli which has been studied for its chromosomal and plasmid determined fusidate resistance phenotypes is S. aureus. No resistance mechanisms have been identified but it is known that the antibiotic is not modified (Chopra, 1976 and Sinden, 1980).

8.4 Postscript

McKell et al. (1983) have just published the results of a steady-state kinetic analysis of the interaction between fusidate and crude extracts of the CAT_I enzyme. Their results concur with the results of my experiments performed on the purified enzyme and conclude that fusidic acid competes competitively with chloramphenicol. The apparent dissociation constant (K_i) which they obtained is 6.2± 2.8μM in broad agreement to the value of 1.7± 0.3μM which I obtained. However, in the absence of any further experiments McKell et al. (1983) assume that binding occurs via non-specific hydrophobic adsorption.

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Resistance to fusidic acid in *Escherichia coli* mediated by the type I variant of chloramphenicol acetyltransferase

A plasmid-encoded mechanism involving antibiotic binding

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Plasmid-encoded fusidic acid resistance in *Escherichia coli* is mediated by a common variant of chloramphenicol acetyltransferase (EC 2.3.1.28), an enzyme which is an effector of chloramphenicol resistance. Resistance to chloramphenicol is a consequence of acetylation of the antibiotic catalysed by the enzyme and the failure of the 3-acetoxy product to bind to bacterial ribosomes. Cell-free coupled transcription and translation studies are in agreement with genetic studies which indicated that the entire structural gene for the type I chloramphenicol acetyltransferase is necessary for the fusidic acid resistance phenotype. The mechanism of resistance does not involve covalent modification of the antibiotic. The other naturally occurring enterobacterial chloramphenicol acetyltransferase variants (types II and III) do not cause fusidic acid resistance. Steady-state kinetic studies with the type I enzyme have shown that the binding of fusidic acid is competitive with respect to chloramphenicol. The inhibition of polypeptide chain elongation *in vitro* which is observed in the presence of fusidic acid is relieved by addition of purified chloramphenicol acetyltransferase, and equilibrium dialysis experiments with [^{3}H]fusidate and the type I enzyme have defined the stoichiometry and apparent affinity of fusidate for the type I enzyme. Further binding studies with fusidate analogues, including bile salts, have shown some of the structural constraints on the steroidal skeleton of the ligand which are necessary for binding to the enzyme. Determinations of antibiotic resistance levels and estimates of intracellular chloramphenicol acetyltransferase concentrations *in vivo* support the data from experiments *in vitro* to give a coherent mechanism for fusidic acid resistance based on reversible binding of the antibiotic to the enzyme.

Fusidic acid is one of a small group of antibiotics which have a steroidal structure. It inhibits polypeptide chain elongation in prokaryotes by stabilizing the intermediary complex of ribosome with elongation factor G and GDP, thus preventing further rounds of GTP hydrolysis and concomitant elongation of the nascent polypeptide chain (reviewed by Cundliffe, 1981). Chloramphenicol inhibits elongation but at the preceding peptidyl transfer step. Its structure differs in a number of respects from that of fusidic acid.

Most genera of Gram-negative bacteria are intrinsically tolerant to concentrations of fusidic acid that inhibit Gram-positive bacteria (von Daehne *et al.*, 1979). Thus, to observe the phenotype of plasmid-borne fusidic acid resistance in *Escherichia coli*, it is necessary to use a fusidate-sensitive mutant strain as the plasmid host. Datta *et al.* (1974) used the fusidate-sensitive host strain DB10 in the first reported study. Among 22 naturally occurring R-plasmids examined, seven conferred fusidic acid resistance. Six of these plasmids also encoded chloramphenicol resistance, including five which have been shown to specify the type I variant of

Abbreviations used: CAT, chloramphenicol acetyltransferase; CAT_I, CAT_{II}, CAT_{III}, the three naturally occurring enterobacterial enzyme variants; *cat*, gene for CAT; Amp, ampicillin; Tet, tetracycline; Cml, chloramphenicol; Fus, fusidic acid; Kan, kanamycin; Nal, nalidixic acid (all used for antibiotic resistances).

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where the plasmid specified chloramphenicol resistance, spectinomycin at a concentration of 300 μg/ml (Bolivar, 1978).

Determination of antibiotic-resistance levels

Resistance levels were estimated by the gradient plate method (Meynell & Meynell, 1970) using 50 ml of 2YT agar containing an appropriate concentration of antibiotic and set in a wedge in the bottom of a square Petri dish (10 cm × 10 cm). The Petri dish was then returned to the horizontal and the same volume of antibiotic-free 2YT agar was poured in to form an upper wedge. After 1 h when the agar was set and its surface had been dried, bacteria growing exponentially in 2YT broth (A_{660} 0.4) were applied. The application was performed by dipping 0.5 cm wide strips of sterile Whatman 3MM paper into the bacterial suspension, allowing them to drain until free of excess culture medium, and then laying these momentarily across the surface of the agar. The strips were applied in parallel lines up the concentration gradient which was formed by diffusion of the antibiotic out of the lower wedge. Antibiotic resistance was scored after 16 h incubation at 37°C by distance of visible growth across the surface of the agar.

Protocol for investigation of antibiotic inactivation

Eight conical flasks (250 ml) containing 40 ml of 2YT broth were prepared with and without a sub-inhibitory concentration of sodium fusidate (1 μg/ml). Each was inoculated with single colonies of strains of DB10 and C600 carrying no plasmid, pBR328, pKT205 or pAH1, the plasmids specifying respectively the type I, type II, and type III variants of CAT (see Table 1).

After the flasks had been shaken for 12 h at 37°C, sodium fusidate was added to a final concentration of 40 μg/ml. After a further 3 h of shaking, the total lipids of the culture were extracted by the method of Bligh & Dyer (1959). Aliquots of the trichloromethane/lipid mixture calculated to contain 50 μg of antibiotic were evaporated under vacuum and subjected to t.l.c. The solvent system used was trichloromethane/cyclohexane/methanol/acetic acid (32:4:1:4, by vol.) and the t.l.c. plates were Merck silica gel 60 F_{254} (20 cm × 20 cm). Controls of sodium fusidate extracted from water and extracted from sterile 2YT broth (with and without the initial 1 μg of sodium fusidate/ml) and incubated under the same conditions were included. After chromatography the plates were heated at 110°C for 10 min then submerged in concentrated H_2SO_4 for 1 min. The detection limit of this method is of the order of 0.25 μg of compound. Fusidic acid is observed as a red to purple spot (other analogues show as red, yellow or brown spots). The sensitivity of this method is such that it can be expected to discern as little as 0.5% modification of the antibiotic.

Transcription and translation studies

Coupled transcription and translation studies *in vitro* were carried out using the method and cell extracts described by Pratt *et al.* (1981) with 5 μg of plasmid DNA per incubation. M_r standards (Sigma MW-SDS-70) were identified by Coomassie Blue stain (Packman & Shaw, 1981) and marked with radioactive ink prior to autoradiography.

Chloramphenicol acetyltransferase purification

The three enzyme variants, CAT_I, CAT_{II} and CAT_{III}, were purified to homogeneity from separate strains of C600 carrying the plasmids pBR328, pKT205 and pAH1 respectively. Plasmid pBR328 was used in preference to plasmid pBR325 since it has the same gene for CAT_I and because its copy number is elevated (Covarrubias *et al.*, 1981), thus providing a significant increase in enzyme yield. Cells were grown in 1–3 litres of M56/CAT medium and harvested in the exponential phase of growth. M56/CAT medium was used because expression of the gene for CAT_I is sensitive to catabolite repression (Le Grice *et al.*, 1982) and CAT_I yields in the absence of glucose or other repressing carbon sources are at least double those obtained in complex media.

Purification of CAT_I and CAT_{III} was by the procedure of Packman & Shaw (1981) with the following modifications. (*a*) The use of phenylmethanesulphonyl fluoride and deoxyribonuclease was omitted. (*b*) EDTA (0.1 mM) was included in the 'standard buffer' (50 mM-Tris/HCl, pH 7.8, containing 0.1 mM-2-mercaptoethanol) throughout. (*c*) CAT_I was eluted from the low substitution affinity resin with standard buffer containing 0.3 M-NaCl and 5 mM-chloramphenicol. (*d*) CAT_{III} was eluted from the high substitution affinity resin with standard buffer containing 0.6 M-NaCl and 5 mM-chloramphenicol. CAT_{II} was purified without the 10 min heat step because of its heat lability, but the procedure employed the same affinity resin as that used for the CAT_{III} enzyme but with the low salt wash and elution buffers of the CAT_I purification as outlined above. The specific activity of purified CAT_{II} from strain C600 carrying plasmid pKT205 was approx. 600 units/mg of protein.

Chloramphenicol acetyltransferase activity was assayed by the spectrophotometric method of Shaw (1975). One unit is defined as the amount of enzyme which catalyses the production of 1 μmol of product/min at 37°C.

Table 1. *Plasmids used in this study (referenced in text)*

Plasmid	Antibiotic resistance phenotype in DB10	CAT variant*	Antibiotic resistances (μg/ml) determined by the gradient plate method for plasmid-containing DB10 strains	
			Chloramphenicol	Sodium fusidate
pBR322	Amp, Tet – – –	–	2	5
pBR325	Amp, Tet, Cml, Fus –	I	50	80
pBR325 *cat* am H22	Amp, Tet – – –	–	2	5
pBR325 *cat* am H32	Amp, Tet – – –	–	2	5
pBR328	Amp, Tet, Cml, Fus –	I	25	105
pKT205†	– Tet, Cml – –	II	90	5
pAH1‡	– Tet, Cml – –	III	110	5
pNJ2004§	Amp – – – Kan	–	2	5
pABO2‖	– – Cml, Fus, Kan	I	40	100

* Gaffney *et al.* (1978).

† pKT205 was constructed by insertion of a *Pst*I restriction fragment from the R plasmid S-a into the *Pst*I site of the β-lactamase gene of pBR322 (Timmis, 1981).

‡ pAH1 was constructed by insertion of a *Pst*I restriction fragment from the R plasmid R387 into the *Pst*I site of the β-lactamase gene of pBR322.

§ pNJ2004 is an *Eco*RI/*Sal*I deletion of plasmid pMK2004 (Kahn *et al.*, 1978; Grinter, 1978).

‖ pAB02 was constructed by insertion of a *Pst*I restriction fragment from the R plasmid EDR51 into the *Pst*I site of the β-lactamase gene of pNJ2004. EDR51 is a deletion derivative of R100 constructed by Dempsey & Willetts (1976).

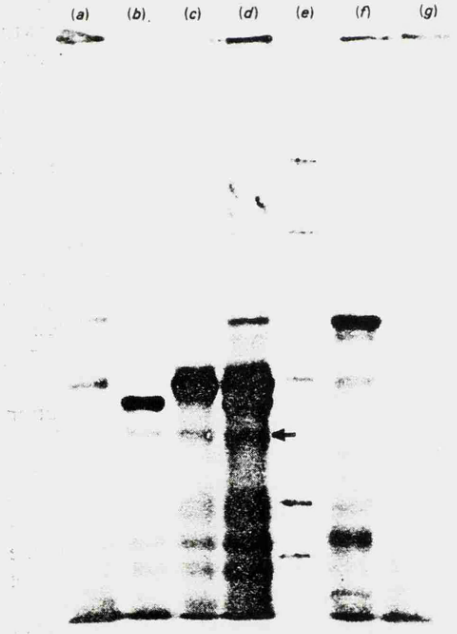


Fig. 1. *Autoradiograph of the results of coupled transcription and translation experiments*
Tracks (*a*)–(*d*) and (*f*) are identified in the text. Track (*e*) contains M_r standards (66000, 48000, 24000, 18400 and 14300). Track (*g*) is the control incubation that has no added template DNA.

tetracycline-resistance phenotype (Tait & Boyer, 1978), were not observed. Track (*c*) shows the polypeptides expressed from pBR325. As well as the pBR322-directed proteins, a protein of apparent M_r 24000 is expressed which is the CAT_I monomer (the actual M_r of the CAT_I monomer from the amino acid sequence is 25668 but a lower value is often obtained with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; Shaw *et al.*, 1979). In addition to these bands, the track contains a less intense band (apparent M_r 20000) indicated by the arrow. The minor band is always co-synthesized with CAT_I, irrespective of orientation, when the CAT_I fragment is inserted into the *Pst*1 restriction site in the β-lactamase gene at pBR322 (results not shown) and must, therefore, represent a segment of the CAT_I polypeptide. (A lack of fidelity of gene expression peculiar to this system is made unlikely by the detection of the same minor band in minicells; Schröder *et al.*, 1981.)

To separate fusidate resistance from the M_r 20000 product would be to demonstrate the dual role of CAT_I as effector of resistance to both fusidate and chloramphenicol. Conversely, to dissociate the fusidate resistance phenotype from the M_r 24000 band attributed to CAT_I would relate fusidate resistance to the presence of the M_r 20000 polypeptide. Such a distinction was achieved by using two derivatives of pBR325 constructed by Völker *et al.* (1982) which carry different chain-

terminating amber mutations within the CAT_I structural gene. Track (*a*) contains the translation products of plasmid pBR325 *cat* am H22, the derivative carrying an amber mutation 'early' in the CAT_I coding sequence. No products related to CAT_I are observed either due to the instability of the transcript or of the truncated polypeptide.

Track (*b*) contains polypeptides expressed by plasmid pBR325 *cat* am H32, the derivative that carries an amber mutation 'late' in the CAT_I structural gene. The resulting synthesis of a protein of apparent M_r 22000 corresponds to the exact size of the truncated CAT_I polypeptide that is predicted from the DNA sequence. The polypeptides observed in track (*d*) are a mixture of the translation products which were loaded in tracks (*b*) and (*c*) and confirm the observed size difference for the prematurely terminated product. Both of the amber mutations in the gene for CAT_I result in the simultaneous loss of the chloramphenicol and fusidate resistance phenotypes. However, the 'late' amber mutation (track *b*) still expresses the M_r 20000 band. It can be concluded, therefore, that the CAT_I enzyme is sufficient, by itself, to confer both the chloramphenicol and fusidic acid resistances.

Steady-state kinetic analysis of fusidate binding to CAT_I

The steady-state kinetics of the interaction between CAT_I and fusidate was observed by the effect of the presence of fusidate on the rate of acetylation of chloramphenicol in the spectrophotometric assay (Shaw, 1975). Since the presence of plasmids specifying the enzyme variants CAT_{II} and CAT_{III} have not been associated with fusidate resistance, the effects of fusidate on the rates of chloramphenicol acetylation catalysed by these proteins were examined as controls. Each of the three CAT variants was purified to homogeneity for such studies and for the experiments described subsequently.

Fusidic acid is not an acetyl acceptor from acetyl-CoA in the spectrophotometric assay with any of the three enterobacterial CAT variants. Inhibition of the rate of chloramphenicol acetylation by fusidate was only observed with the type I variant of CAT. The inhibition observed was judged to be competitive with respect to chloramphenicol and mixed uncompetitive–noncompetitive with acetyl-CoA. [The Michaelis constants (K_m) for chloramphenicol and acetyl-CoA were $7.2 \pm 1.2\,\mu M$ and $46 \pm 5\,\mu M$, respectively, for CAT_I in the absence of fusidate.] The inhibition constant (K_i) from the variable chloramphenicol concentration data was $1.7 \pm 0.3\,\mu M$. Fusidate did not inhibit the CAT_{II}- and CAT_{III}-catalysed acetylation of chloramphenicol even when present at a concentration of $50\,\mu M$ (results not shown).

Relief of inhibition of polypeptide chain elongation in vitro

The rate of polypeptide chain elongation *in vitro* was monitored on the basis of the rate of ribosome-dependent GTP hydrolysis. The latter occurs in the absence of both exogenous mRNA and amino-acyl tRNA molecules but is dependent on the presence of elongation factor G. Maximal rates of GTP hydrolysis under the conditions described were observed only when fusidate was absent. The mean value of maximal GTP hydrolysis was 101 pmol/min during the 20 min incubation. At a final fusidate concentration of $1.0\,\mu M$ ($0.54\,\mu g/ml$), ribosome-dependent GTP hydrolysis is 55% of the maximal rate, an observation in close agreement with the findings of Okura *et al.* 1970. The effect of increasing concentrations of CAT_I on the elongation was measured. Whereas the addition of CAT_I only slightly depresses GTPase activity, it relieves the inhibition caused by the presence of $1.0\,\mu M$-fusidate. Reversal of the inhibition was judged to be consistent with an equimolar and independent binding of fusidate by each of the four CAT_I subunits. The other enzyme variants, CAT_{II} and CAT_{III}, did not relieve fusidate inhibition even when present at a final concentration of $10\,\mu M$ enzyme monomer (results not shown).

Equilibrium dialysis

In order to further investigate the stoichiometry and avidity of fusidate binding to CAT_I equilibrium dialysis experiments were performed. Enzyme at a monomer concentration of $4.6\,\mu M$ was dialysed against [^{3}H]fusidic acid and the resulting data were plotted according to Scatchard (1949) (Fig. 2). The equilibrium dissociation constant, K_d, calculated from the data was $25.2\,\mu M$, and the value of the intercept on the abscissa (1.06) implies an equimolar binding ratio between CAT_I monomers and fusidate. In addition, the slope of the plot was linear, indicating that the four binding sites of the CAT_I tetramer bind fusidate independently and in a non-co-operative fashion. Variation of the fixed concentration of CAT_I in the range $4.0–15.0\,\mu M$ (monomer) did not significantly affect the observed value for K_d. The enzyme variants CAT_{II} and CAT_{III} were studied at fixed monomer concentrations of $1.8\,\mu M$ and $10.0\,\mu M$ respectively. Both fail to bind [^{3}H]fusidate to any detectable extent (results not shown).

During 90 h of dialysis at 4°C each of the three CAT variants had only a small loss in catalytic specific activity (an average of 7% with a maximum of 10%). Thus, in all determinations of fusidate binding at equilibrium, the protein was in each case predominantly in an enzymically active conformation. Calculations of the ligand–CAT binding

properties were based upon the value observed for the concentration of CAT at equilibrium.

Competition binding experiments were carried out to learn whether fusidate binding might be due merely to the general hydrophobicity of the antibiotic or be a consequence of more specific interactions. Such experiments were performed as described above, but in each case a non-radioactive analogue of fusidate was included at a fixed concentration. The dissociation constants of the competing analogues were calculated from the resulting data using the equation of Edsall & Wyman (1958) which can be rearranged thus:

$$K_{dc} = \frac{[c]}{\left(\frac{K_{da}}{K_d}\right) - 1}$$

where K_d is the dissociation constant of the radioactive ligand at equilibrium (25.2 μM for [^{3}H]-fusidate in these experiments). K_{da} is the apparent dissociation constant in the competition experiment at equilibrium. K_{dc} is the dissociation constant of the competing ligand at equilibrium, and [c] is the final concentration of competing ligand at equilibrium in the absence of binding protein (20 μM for each non-radioactive ligand in these experiments).

To validate the competition dialysis procedure as described above, non-radioactive fusidate was employed as the competing ligand. The K_{dc} value obtained was 31.1 μM as compared with a directly measured K_d of 25.2 μM (see Table 2).

Seven fusidate analogues were used in separate competition experiments (Fig. 3). In addition to compounds having an obvious structural similarity

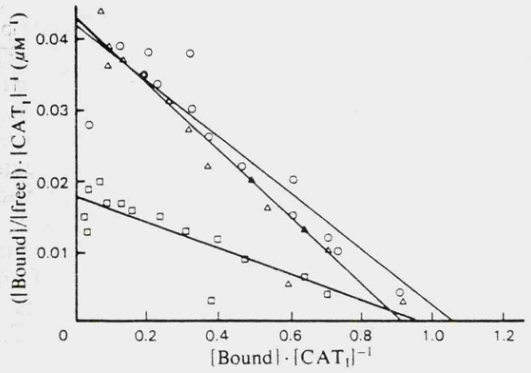
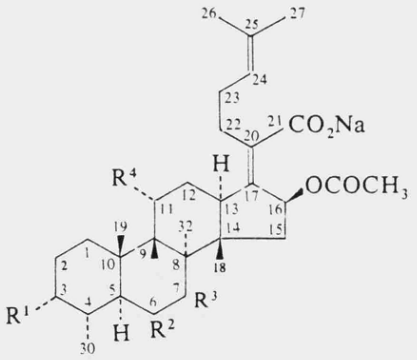


Fig. 2. *Analysis of the binding of [11β-^{3}H]fusidate to the type I variant of chloramphenicol acetyltransferase by equilibrium dialysis*

The experiments were performed as described in the Materials and methods section and were analysed according to Scatchard (1949). ○, [^{3}H]fusidate binding in the absence of other ligands; △, [^{3}H]-fusidate binding in the presence of non-radioactive glycocholate (40 μM); □, [^{3}H]fusidate binding in the presence of 24.25-dihydrofusidate (40 μM). Extension of the method to other ligands yielded the apparent dissociation constants (K_d) listed in Table 2.

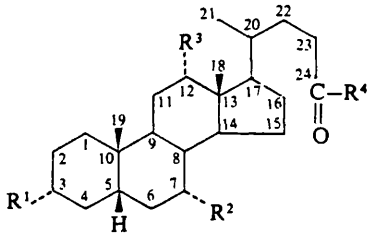


Nature of 1.2 bond	R^1	R^2	R^3	R^4	Nature of 24.25 bond	Trivial name
Single	–OH	–H, –H	–H, –H	–OH	Double	Sodium fusidate
Single	β-OH	–H, –H	–H, –H	–OH	Double	Sodium 3-epifusidate
Single	=O	–H, –H	–H, –H	–OH	Double	Sodium 3-oxofusidate
Single	–OH	–H, –H	α-OH	–OH	Double	Sodium 7 α-hydroxyfusidate
Single	–OH	–H, –H	–H, –H	=O	Double	Sodium 11-oxofusidate
Single	–OH	–H, –H	–H, –H	–OH	Single	Sodium 24.25-dihydrofusidate
Double	=O	β-$OCOCH_3$	=O	–H	Double	Helvolic acid sodium salt
Single	–OH	α-$OCOCH_3$	β-OH	–H	Double	Cephalosporin P1 sodium salt

Fig. 3. *Fusidic acid analogues and isomers used in this study*

Table 2. *Antibacterial activity and CAT_I-binding affinities of sodium salts of fusidic acid analogues used in this study*
The minimum inhibitory concentrations were determined by the gradient plate method. Equilibrium dissociation constants (K_d) were determined using equilibrium dialysis by competition with [^{3}H]fusidate as described in the Materials and methods section and shown in Fig. 2.

Fusidate analogues and bile salts \ Bacterial strains ...	Minimum inhibitory concentration (μg/ml) DB10 + no plasmid	DB10 + pBR322	DB10 + pBR325 (CAT_I)	DB10 + pBR328 (CAT_I)	DB10 + pKT205 (CAT_{II})	DB10 + pAH1 (CAT_{III})	C600 + no plasmid	Equilibrium dissociation constant (K_d) with CAT_I (μM)
Sodium fusidate	5	5	80	105	5	5	>500	31.1
3-Epifusidate	>450	>450	>450	>450	>450	>450	>450	>500
3-Oxofusidate	100	100	100	100	100	100	>1000	142.3
7α-Hydroxyfusidate	15	15	15	15	15	15	>400	79.5
11-Oxofusidate	100	100	>400	>400	100	100	>400	20.5
24,25-Dihydrofusidate	20	20	>100	>100	20	20	>100	16.8
Helvolic acid	45	45	45	45	45	45	>150	83.0
Cephalosporin P1	40	40	40	40	40	40	>100	>500
Glycocholate								>500
Taurocholate								>500
Glycochenodeoxycholate								>500
Taurochenodeoxycholate	>400	>400	>400	>400	>400	>400	>400	>500
Glycodeoxycholate								56.5
Taurodeoxycholate								27.6
Dehydrocholate								>500



R^1	R^2	R^3	R^4	Trivial name
–OH	–OH	–OH	$-NHCH_2CONa$	Sodium glycocholate
–OH	–OH	–OH	$-NH[CH_2]_2SO_3Na$	Sodium taurocholate
–OH	–OH	H	$-NHCH_2CONa$	Sodium glycochenodeoxycholate
–OH	–OH	–H	$-NH[CH_2]_2SO_3Na$	Sodium taurochenodeoxycholate
–OH	–H	–OH	$-NHCH_2CONa$	Sodium glycodeoxycholate
–OH	–H	–OH	$-NH[CH_2]_2SO_3Na$	Sodium taurodeoxycholate
=O	=O	=O	–ONa	Sodium dehydrocholate

Fig. 4. *Bile salts used in this study*

to fusidate, a study was made of the binding properties of some steroidal bile salts (Fig. 4). Such experiments were prompted by observations of Proctor & Rownd (1982) which pointed to an interaction of bile salts with CAT_I.

Fusidate analogues and bile salts were also tested for antibiotic activity against strain DB10 and a number of plasmid-bearing derivatives. The data in Table 2 summarize the results of such experiments as well as the data from competition equilibrium dialysis studies (Fig. 2). As expected, none of the bile salts showed significant antibiotic activity against either a conventional (fusidate-tolerant) strain of *E. coli* (C600) or strain DB10. However, fusidate analogues which inhibited the growth of DB10 and which were observed to be bound avidly by CAT_I (11-oxofusidate and 24,25-dihydrofusidate) were significantly less active *in vivo* against DB10 containing a plasmid specifying CAT_I. An increase in the observed minimum concentration required for inhibition was not observed in the case of DB10 derivatives lacking CAT (pBR322) or in strains with

plasmids specifying CAT_{II} (pKT205) or CAT_{III} (pAH1).

Discussion

Fusidate resistance and phospholipid levels

The absence of any chromatographically detectable change in the fusidate molecule after incubation with CAT_I-containing *E. coli* is in keeping with the findings of bioassay studies performed by Werner & Daneck (1981) and by S. Scannell & W. V. Shaw (unpublished work). Both groups failed to demonstrate a decrease in the level of anti-staphylococcal growth inhibition caused by pre-exposure to fusidate of *E. coli* DB10 strains which contained plasmids conferring fusidate resistance. Werner & Daneck (1981) postulated that DB10 was fusidate-sensitive because of reduced levels of cyclopropane fatty acids and phosphatidylethanolamine. They speculated that plasmid-borne fusidate resistance might be due to an alteration in the synthesis of such phospholipids which in turn was controlled by plasmid genes. However, the observation that DB10 is low in cyclopropane fatty acids may have no immediate or obvious relevance to fusidate sensitivity, since the *E. coli* mutants isolated by Taylor & Cronan (1976) are defective in cyclopropane fatty acid synthetase but are as resistant to fusidate as are wild type *E. coli* strains (results not shown).

Specificity of CAT_I for fusidic acid

It has been reported that CAT_I fails to mediate resistance to the antibiotics helvolinic acid (6-deacetoxyhelvolic acid) and cephalosporin P1 (Völker *et al.*, 1982). Although interesting, these observations fail to give a clear insight as to the nature of the specificity of CAT_I for fusidane compounds, since both analogues differ from fusidic acid at several positions (Fig. 3). The results of the competition equilibrium dialysis experiments described above on several fusidic acid analogues and bile salts (Fig. 4) show the CAT_I binding specificity (Table 2).

In addition to position 3, the only site tested which fails to tolerate even a small change is position 7. Minor changes in the nature of the groups at positions 4, 5, 9, 11, 12, 13, 14 and 16 are accepted and major changes to the side chain at position 17 are also permitted.

A coherent mechanism for CAT_I-mediated fusidate resistance in vivo

The experiments described represent an extension of the genetic proof offered by Völker *et al.* (1982) that the structural gene for CAT_I is required for both the chloramphenicol and fusidate resistance phenotypes. For the CAT_I enzyme to represent a plausible resistance mechanism based solely on reversible fusidate binding it ought to satisfy two criteria. Firstly, the concentration of CAT_I within the cell must be sufficient to bind and sequester the incoming antibiotic and, secondly, the affinity of fusidate for the enzyme must approximate or exceed that of fusidate for the complex of ribosome GDP and elongation factor G. (The results indicate that there is a 1:1 binding stoichiometry between each of the CAT_I subunits and fusidate.)

The extent of increase in the observed fusidate resistance of DB10 strains carrying mini-plasmids over the plasmid-free host strain was determined on 2YT agar gradient plates (Table 1). For DB10 carrying two of the plasmids specifying CAT_I (pBR325 and pBR328) this increase is 75 and 100 μg/ml respectively, corresponding to an increase in fusidate concentrations of 138 and 185 μM. The specific activity of CAT_I in crude extracts of DB10 carrying pBR325 and of DB10 with pBR328 was determined from cells grown in 2YT broth. Under such conditions CAT_I is present as 2.8% and 3.7% of the total intracellular protein respectively. If 15% of the wet weight of *E. coli* is presumed to be protein this would imply intracellular CAT_I monomer concentrations of 175 and 230 μM respectively.

The apparent affinity of CAT_I for fusidate was determined in two ways which might reasonably be expected to yield similar values. Equilibrium dialysis gave a K_d value of approx. 30 μM whereas steady-state kinetics gave an apparent K_i value 20-fold lower. Although both experiments were performed in nearly identical buffers, the steady-state kinetic experiments were performed in the presence of saturating levels of acetyl-CoA at 37°C whereas the equilibrium dialysis in the absence of coenzyme took place at 4°C. The latter conditions were dictated by the known ability of CAT_I to hydrolyse acetyl-CoA slowly in the absence of chloramphenicol (Shaw, 1983). If the observed K_i value (1.7 μM) is a more realistic reflection of the situation *in vivo* wherein acetyl-CoA is present, then it is worth noting that this value approaches that of the reported dissociation constants for interactions of the ribosome–GDP–elongation factor G complex with fusidate (0.4 μM observed by Okura *et al.*, 1971) and 24,25-dihydrofusidate (0.39 μM noted by Willie *et al.*, 1975).

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