BIOCHEMICAL ANALYSIS OF THE RECENT PLASMID-ENCODED TRIMETHOPRIM-RESISTANT DIHYDROFOLATE REDUCTASES IN GRAM-NEGATIVE BACTERIA

by

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Abstract

The most important mechanism of trimethoprim resistance is the plasmid-encoded production of an additional trimethoprim resistant dihydrofolate reductase. The epidemiology of plasmid-mediated resistance to trimethoprim has been studied by biochemical typing of the enzymes responsible. In recent years several new trimethoprim resistant dihydrofolate reductases have been identified. The type III enzyme, considered the rarest of the plasmid-mediated dihydrofolate reductases was isolated once in New Zealand in 1979 and never subsequently detected. However the biochemical properties of a trimethoprim resistant dihydrofolate reductase isolated in Nottingham were examined as DNA gene probing had suggested that the enzyme was a type III and the biochemical properties confirmed this.

The enzymes responsible for trimethoprim resistance in two outbreaks of Shigella in the United States were examined. Detailed biochemical analysis suggested that the two enzymes were different from each other but similar to the type III. Therefore the three enzymes were subsequently renamed types IIIa, IIIb and IIIc. The properties of the type IIIb enzyme were very similar to the original type IIIa; however, sequence analysis of the N-terminal of this protein showed that it was quite distinct from the type IIIa.

The type IV dihydrofolate reductase was isolated in South India in 1984 and is the only inducible plasmid-mediated dihydrofolate reductase. Examination of the induction process suggested that the resistance mechanism of the enzyme was similar to chromosomal hyperproduction where resistance is achieved not because

of the insensitivity of the dihydrofolate reductase, but because it is produced in such an amount that it "swamps" the inhibiting trimethoprim. Purification and sequence analysis of the type IV dihydrofolate reductase revealed that the enzyme was similar to the chromosomal dihydrofolate reductase and that it was complexed with NS1 an *Escherichia coli* DNA binding protein.

The biochemical properties of the type V dihydrofolate reductase, which was isolated in Sri Lanka in 1985, were similar to those of the type I enzyme, with the exception that the type V has an unusually low molecular mass when measured on Sephadex. On native polyacrylamide gel electrophoresis however the two enzymes co-migrate, this biochemical similarity suggests the two enzymes are closely related.

The efficiency of plasmid-mediated resistance to trimethoprim has compromised the use of this drug in many parts of the world, from biochemical studies it is clear that plasmid-mediated enzymes continue to evolve.

"It is a capital mistake to theorize before one has data."

Sir Arthur Conan Doyle

in "Scandal in Bohemia"

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Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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Abbreviations

ad	adenine
bp	base pairs
b.d.	bis die
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DM	Davis-Mingioli
DNA	deoxyribonucleic acid
DSTA	diagnostic sensitivity test agar
dTMP	Thymidylic acid
dUMP	Uridylic acid
EDTA	ethylenediaminetetraacetic acid
FPLC	fast protein liquid chromatography
FUdR	5-fluoro-2´-deoxyuridine
gly	glycine
HPLC	high-performance liquid chromatography
ID_{50}	dose giving 50% inhibition
inc	incompatibility group
IEF	isoelectric focusing
kb	kilobases
met	methionine
MIC	minimum inhibitory concentration
Mr	molecular size
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue
Mtx	methotrexate
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
OD	optical density
PABA	para-amino benzoic acid
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
psi	pounds per square inch
R	resistance
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TD_{50}	time at 45° (minutes) giving 50% inhibition
thy	thymidine
Tn	transposon
Tp	trimethoprim

Amino acids in sequence figures are represented by the standard one or three

letter convention, as described by Stryer, L. (1981), BIOCHEMISTRY, W.H. Freeman and Co.

Accession Numbers

The amino acid sequences reported in this thesis have been registered in the protein database of the National Biomedical Research Foundation (NBRF), Washington DC, USA and have been assigned the following accession numbers.

IIIb - A33005

IV - A33004

Chapter 1

Introduction

1.1 Antimicrobial Chemotherapy

As soon as it was proposed by Koch and Henle (Henle, 1840; Koch, 1884) in the last century that diseases were caused by specific microorganisms, attempts were made to control them with the use of chemicals. The first stage in the development of chemotherapy really involves disinfection and antisepsis; the chemical agents used, chlorine phenol and detergents, being too toxic for systemic use (Lister, 1867a, 1867b). These chemicals were used largely for the sterilisation of surgical equipment and at low concentrations for the treatment of water.

If chemical agents were to be used successfully to treat human infections it was realised that they must exhibit selective toxicity i.e. be toxic to the bacteria but not to the host (Ehrlich, 1913). Ehrlich in 1907 had discovered salvarsan a synthetic arsenic compound effective against *Treponema pallidum* the syphilis pathogen, this had been introduced to clinical medicine in 1910 and therefore the search for further chemicals or magic bullets active against bacteria continued.

This search however appeared fruitless and it was not until 1935 when Domagk discovered the selective antibacterial activity of prontisil (Domagk, 1935) and the active constituent identified as sulphanilamide (Tréfouel *et al.*, 1935) that antibacterial chemotherapy became a practical proposition and the antibacterial activities of sulphonamides were quickly exploited (White and Reeves, 1989).

In the light of the discovery of sulphanilamide the search continued for other novel chemicals with antibacterial activity. This approach however has so far produced relatively few new antibacterials with the exception of trimethoprim, nalidixic acid and very recently the fluorinated 4-quinolones. Arguably the most important result of Domagk's work was that it revived interest in the subject of antimicrobial chemotherapy, and led to a re-evaluation of the work by Fleming, who in 1929 had discovered that microorganisms themselves produce antimicrobial agents or antibiotics (Fleming, 1929).

Fleming had described an antibiotic produced by the fungus *Penicillium notatum*, however little work had been carried out on the compound. Chain *et al.* purified the antibiotic in 1940 and it was not until this point that it became clear that penicillin was a far more effective chemotherapeutic agent than anything that had previously been discovered.

This heralded the golden age of antibiotics as it was realised that effective antibiotics were naturally produced by soil organisms. Rather than the search for novel chemicals which had been going on the switch was made to mass screening of soil organisms for new antibiotics.

With hindsight it is perhaps not surprising that if some organisms were producing

antibiotics others must be producing compounds which overcame the inhibitory effects of the antimicrobials, as the range of different antimicrobials was developed so did the variety of resistance mechanisms (Goldstein *et al.*, 1974). More importantly resistance did not always develop by stepwise mutational events as had been predicted, but could exist on stable extrachromosomal pieces of DNA, known as plasmids, which could be transferred between bacteria (Akiba *et al.*, 1960; Mitsuhashi, 1969). The most common plasmid resistance mechanism is drug inactivation and the best studied system is the β-lactamase hydrolysis of penicillins (Philippon *et al.*, 1989; Medeiros, 1989).

The growing realisation that naturally occurring antibiotics had naturally occurring antagonists renewed interest in the development of completely synthetic antibacterials. It was argued that as there would be no naturally occurring enzymes that could break down these drugs resistance would be less of a problem.

It was against this background that trimethoprim was launched in 1969. A completely synthetic antibacterial agent it offered new hope in the fight against bacterial resistance, but also offered the microbiologist a unique "model" system for studying the development of resistance from day one.

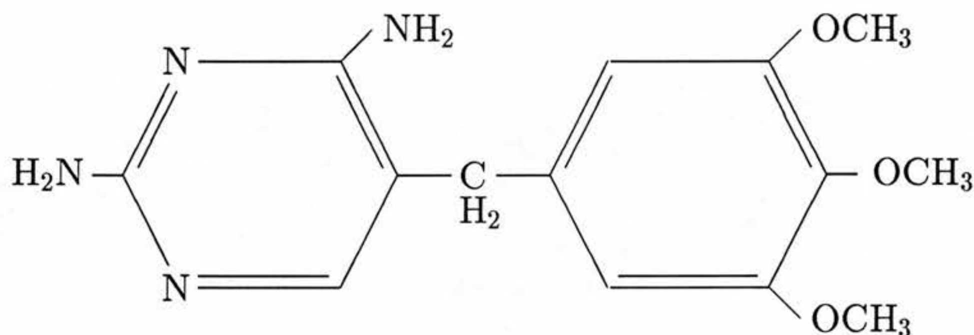
1.2 Trimethoprim

Trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine) (figure 1) is one of a series of compounds first fully described by Roth in 1962. It had been observed that many 5-benzyl-2,4-diaminopyrimidines possessed a high degree of antimalarial activity (Falco *et al.*, 1951a, 1951b) and that this was highest in

compounds which contained a methyl group in the pyrimidine 6 position (Falco *et al.*, 1951a). When the 6-alkyl group was removed antimalarial activity was decreased, however, the antibacterial action of the compound was increased (Roth *et al.*, 1962). A series of 5-benzyl-2,4-diaminopyrimidines were synthesised and tested for antibacterial activity. Trimethoprim was selected for further study on the basis of the magnitude and breadth of its antibacterial activities. The first clinical use of trimethoprim was in the treatment of a *Proteus* septicemia in combination with polymixin and sulphonamides in 1962 (Noall *et al.*, 1962). Apparent synergy between trimethoprim and sulphonamide meant that trimethoprim when it was launched in 1969 was marketed as a sulphonamide potentiator, and only available in combination with sulphamethoxazole (Bushby and Hitchings, 1968; Darrell *et al.*, 1968; Bushby, 1969, 1973). It was claimed that the combination had several advantages, sequential blockade in the same pathway would confer clinical advantage, the combination of the two drugs was synergistic and the combination would delay the emergence of resistance (Hitchings, 1973). In order to understand the rationale for this and the subsequent resistance mechanism, it is necessary to examine the pathway of folate metabolism and the action of trimethoprim.

Both sulphamethoxazole and trimethoprim act on the pathway producing tetrahydrofolate an essential co-factor in the biosynthesis of several amino acids, purines and pyrimidines (Huennekens and Osborn, 1959; Blakely, 1969). The biochemical pathway for folate biosynthesis was described by Brown in 1971 (figure 2). The enzyme dihydropteroate synthetase catalyses the condensation of P-amino benzoic acid and dihydropteridine to form dihydropteroate. Dihydrofolate syn-

Figure 1. The structure of trimethoprim.

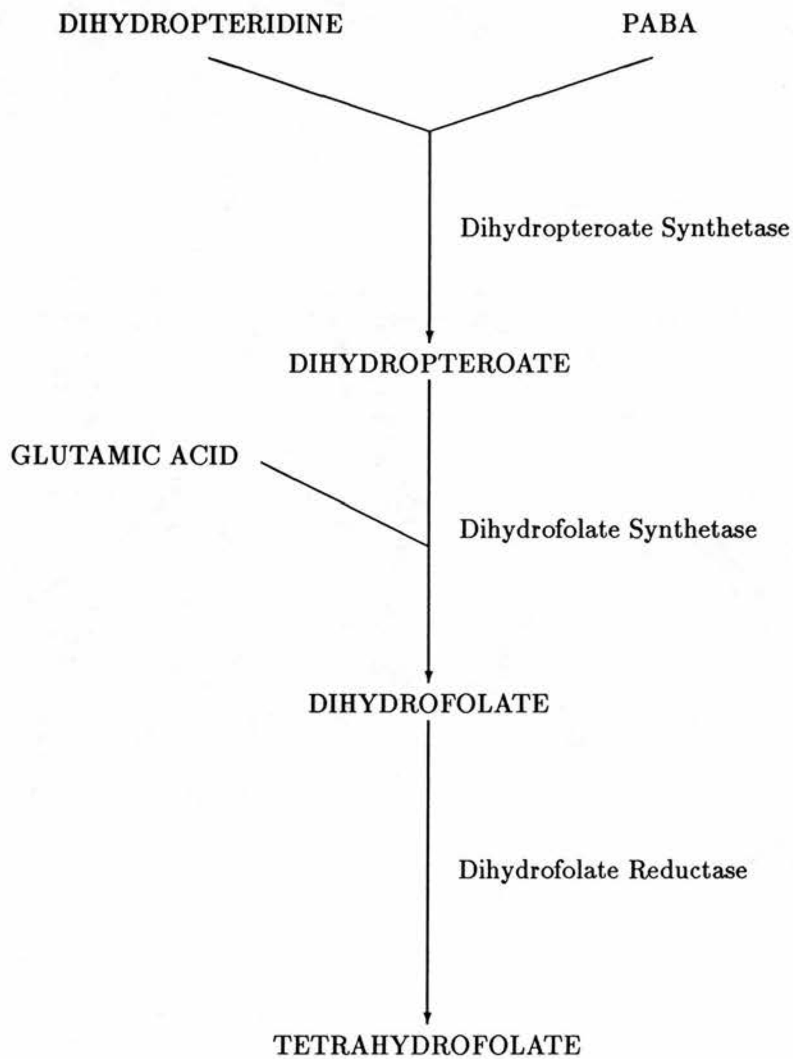


TRIMETHOPRIM

thetase then catalyses the addition of one or more glutamic acid residues to form dihydrofolate. This is then reduced by the action of dihydrofolate reductase to form tetrahydrofolate.

Sulphamethoxazole and trimethoprim both act as competitive inhibitors of enzymes in this pathway, sulphamethoxazole inhibits dihydropteroate synthetase (Brown, 1962), whilst trimethoprim acts by inhibiting dihydrofolate reductase (Burchall and Hitchings, 1965). The basis for their selective toxicity is different however. The dihydropteroate synthetase reaction inhibited by sulphur compounds does not occur in mammalian cells which utilise preformed folates. Although the reduction of dihydrofolate to tetrahydrofolate catalysed by dihydrofolate reductase does occur in both microbial and eukaryotic cells trimethoprim is a much more potent inhibitor of microbial dihydrofolate reductase than of the mammalian enzyme (Burchall, 1969). Although trimethoprim has been known to be selective because it binds less readily to mammalian dihydrofolate reduc-

Figure 2. The biosynthetic pathway of tetrahydrofolate employed by bacteria.



tase than bacterial dihydrofolate reductase, the exact method of this specificity has only recently been elucidated. X-ray crystallography studies demonstrating that trimethoprim can fit well into the substrate binding site of *Escherichia coli* dihydrofolate reductase but not the mammalian enzyme (Matthews *et al.*, 1985b).

1.2.1 Trimethoprim in Combination

As with any new antibacterial agent, when trimethoprim was launched several claims were made about its efficacy and long term prospects. In the case of trimethoprim however the most contentious issue revolved not about the drug itself but the decision to use it in combination with sulphamethoxazole and whether or not the advantages proposed for the combination were valid.

In terms of the first two claims (namely that the synergism of the 2 drugs caused by sequential blockade in the same pathway conferred clinical advantage) while there is little doubt that a synergistic effect can be observed *in vitro* (Bushby and Hitchings, 1968; Darrell *et al.*, 1968; Bushby, 1969), the reason for this synergism has been questioned, Poe (1976) arguing that it was not because trimethoprim and sulphonamide inhibit reactions in the same pathway but owing to the fact that sulphonamides may also bind to bacterial dihydrofolate reductase.

The situation *in vivo* is much less clear cut, and synergy *in vivo* has never been adequately demonstrated although the two drugs appear synergistic in experimental infections in mice (Bohni, 1969; Grunberg, 1973). It can be argued that if Poe's theory of blockade at the same site was correct it would be difficult to imagine how potentiation would occur *in vivo*. Trimethoprim is such a potent an-

tibacterial in its own right that synergy would only occur if it was in subinhibitory levels and the sulphonamide was present in adequate amounts, an unlikely scenario (Lacey, 1979). Studies comparing the clinical efficacy of trimethoprim alone and in combination with sulphamethoxazole have shown no difference between the two (Lacey *et al.*, 1980; Mabeck and Vejlsgaard, 1980). Furthermore models of urinary tract infection have demonstrated that the presence of sulphamethoxazole contributed nothing to the dominant activity of trimethoprim (Greenwood and O'Grady, 1976; Greenwood, 1979).

Since 1972 in Finland, and 1979 in the United Kingdom trimethoprim has been available as a single agent (Lacey, 1982). The monotherapy appears to be as successful as the combination in the treatment of urinary and respiratory tract infections (Brumfitt and Pursell, 1972; Kasanen *et al.*, 1978; Lacey *et al.* 1980; Brumfitt *et al.* 1985). The use of trimethoprim alone also has the advantage of fewer side effects which were associated with sulphamethoxazole (Lacey *et al.*, 1980).

One area in which trimethoprim and sulphamethoxazole do appear to be synergistic *in vivo* is in the case of *Pneumocystis carinii* pneumonia. *Pneumocystis carinii* is the most common infection in patients with acquired immuno-deficiency syndrome and the combination is an effective treatment (Fischl *et al.*, 1988; Shafer *et al.*, 1989). Because of the high incidence of side effects associated with sulphamethoxazole trimethoprim has also been used in combination with dapsone to treat pneumonia caused by *Pneumocystis carinii* (Leoung *et al.*, 1986).

The question of whether the combination prevented the emergence of resistance is

much harder to assess. Studies *in vitro* showed that exposure of laboratory bacteria to trimethoprim alone produced a significantly larger increase in resistance amongst *Escherichia coli* than exposure to the combination (Darrell *et al.*, 1968). The relevance to this *in vivo* however appears doubtful and a number of studies have found no difference in the emergence of resistant organisms in groups treated with trimethoprim alone or in combination with sulphamethoxazole. Lacey *et al.* in 1980 demonstrated no difference in cure rates and emergence of resistance in a randomised double blind trial of 279 patients suffering from respiratory and urinary tract infections. A later study specifically looking at respiratory tract infection also failed to find any difference in the emergence of resistance between trimethoprim and cotrimoxazole (Amyes *et al.*, 1986a). Other studies have also suggested the combination did not delay resistance (Kasanen and Sundquist, 1982; Brumfitt *et al.*, 1983) and that the rise in resistance after the introduction of trimethoprim alone was caused by increased use of the drug (Huovinen *et al.*, 1986).

It is now generally accepted that the use of the combination did not delay the emergence of resistance nor did it give an improved clinical response (Lacey, 1982) and trimethoprim monotherapy has now been used successfully for a number of years (Turnridge, 1988). Levels of resistance are similar in countries where trimethoprim is predominantly administered alone and in those which use the combination (Kasanen and Sundquist, 1982). However the argument is by no means over (Maskell and Pead, 1989).

1.3 Epidemiology of Trimethoprim Resistance

Resistance to trimethoprim was detected shortly after its introduction in the United Kingdom in 1969 (Lacey *et al.*, 1972; Grüneberg, 1976) and France in 1971 (Acar and Goldstein, 1982), indeed some strains of *Klebsiella* isolated before the introduction of trimethoprim have been found to be trimethoprim resistant (Hamilton-Miller and Grey, 1975). The most important resistance mechanism has been plasmid-mediated, following the first report of transferable resistance in 1972 (Fleming *et al.*, 1972). Initially it was difficult to gain accurate figures on trimethoprim resistance because wide variations existed between hospitals in the same area (Huovinen *et al.*, 1982; Amyes, 1986), and inappropriate testing methods were often employed (Amyes and Smith, 1974a). However a detailed picture of trimethoprim resistance worldwide has now been built up (Huovinen, 1987; Turnridge, 1988; Elwell and Fling, 1989). One clear pattern that has emerged is that there is now a distinct difference between resistance levels in developed and Third World countries (Murray *et al.*, 1982; Farrar, 1985). Considering first the developed countries:

1.3.1 United Kingdom

Trimethoprim resistance levels (MIC > 8 or 10 mg/L) in United Kingdom urinary isolates have generally remained around 10 - 15%. Brumfitt *et al.* (1983) reported 13% of hospital isolates resistant in 1981, this is similar to a 12 year follow-up survey at the Whitington hospital which showed that the percentage of trimethoprim resistant isolates remained fairly constant around 9.8% - 13% (Chirnside *et*

al., 1985). Resistance levels in general practice have generally been lower than in hospital, with the proportion of resistant strains ranging from around 5% to 11% (Brumfitt *et al.*, 1983; Grüneberg, 1984; Amyes, 1987). There have been higher incidences of resistance in the United Kingdom. In one Edinburgh long stay hospital, 64% of urinary bacteria were found to be trimethoprim resistant (MIC $>$ 10 mg/L) (Young and Amyes, 1983). This appears to have been an isolated example as the resistance in urinary bacteria in the Edinburgh general hospital population over a three year period was only 16.5% (Amyes *et al.*, 1986b). In common with other countries trimethoprim resistance is a particular problem in geriatric hospitals (Bendall *et al.*, 1989).

Although overall resistance to trimethoprim has remained fairly constant there have been changes in the nature of resistance. In some areas the percentage of resistant strains that can transfer resistance has increased (Chirnside *et al.*, 1985) as has the proportion of high level resistant strains (Brumfitt *et al.*, 1983). Whilst in others there has been a shift from plasmid to chromosomal carriage of high level resistance genes (Towner *et al.*, 1982; Amyes *et al.*, 1986b; Kraft *et al.*, 1986).

1.3.2 Finland and Sweden

Finland was the first country in the world to register clinical use of trimethoprim alone for urinary tract infection in 1973, and consequently the levels of trimethoprim resistance have been monitored closely. In hospital strains, resistance to trimethoprim-sulphamethoxazole rose from 8% in 1971 to 35% in 1983 (Huovinen *et al.*, 1986). Two distinct increases in trimethoprim resistance were observed;

the first after the wider use of trimethoprim-sulphamethoxazole, the second resulting from the dramatic increase in the consumption of trimethoprim alone in 1977. Most importantly resistance has been shown to be primarily related to the total use of trimethoprim, regardless of whether it is in combination or alone (Huovinen *et al.*, 1986). As in the United Kingdom, wide variations in resistance levels have been reported between hospitals (Huovinen *et al.*, 1982, 1983). It was realised that it was important to define hospital type in these studies as resistance was considerably lower in an oncology hospital (22%) than a geriatric hospital (71%) despite approximately the same trimethoprim consumption in each (Dornbusch and Toivanen, 1981). In outpatients trimethoprim resistance has increased from 3% to 19% in the ten years 1978 - 1988 (Heikkilä *et al.*, 1990). Although trimethoprim alone represented 25% of all drug usage for urinary tract infection, the proportion of trimethoprim resistant strains in Finland was the same as in countries where only the combination was used (Kasanen and Sundquist, 1982). Much higher levels of trimethoprim resistance have been found in Finland than Sweden 31-49% as opposed to 1.6-3.6% (Dornbusch and Toivanen, 1981; Dornbusch and Hagelberg, 1983). This probably reflects much higher usage of trimethoprim in Finland at this time.

However, usage in Sweden has increased and in 1984 3% of the population of Jamtland had received trimethoprim. Among older age groups, one third received trimethoprim in a six year period (Sköld *et al.*, 1986). One result of this increased usage was that one third of patients with resistant organisms had never been treated with trimethoprim and Rydberg and Cederberg (1986) demonstrated that trimethoprim resistance was capable of spreading among families of outpatients

being treated with trimethoprim.

1.3.3 United States

Levels of trimethoprim resistance in the United States have remained relatively low with 3 - 8% of *Escherichia coli* being resistant (Murray *et al.*, 1985).

1.3.4 France

Resistance to trimethoprim in hospitals in Paris was 17.9% as far back as 1972 and this had risen to 24.8% in 1979 (Acar and Goldstein, 1982). By 1984 resistance had only increased slightly to 25.5%. However the proportion of strains with high level resistance increased markedly from 40.2% of strains in 1972 to 95.4% in 1984 (Goldstein *et al.*, 1986b). From 1983 to 1988 resistance decreased in all groups of isolates (Goldstein and Acar, 1990).

1.3.5 Developing Countries

Generally speaking much higher levels of trimethoprim resistance are found here than in the developed countries. The introduction of trimethoprim-sulphamethoxazole into these countries has largely replaced ampicillin because of its longer shelf life, low cost and b.d. dosage (Farrar, 1985). In India in 1984, 64% of urinary bacteria have been reported to be resistant to trimethoprim (Young *et al.*, 1986a). In contrast in Sri Lanka, a trimethoprim resistance level of only 3.9% has been reported but this included sampling normal healthy members of the population (Sundström *et al.*, 1987). This is an important point as it is becoming apparent

that carriage of resistant organisms in the normal commensal population may be an important factor contributing to the high level of resistance found in the Third World. Strains capable of transferring low level trimethoprim resistance have been isolated from apparently healthy students in Nigeria (Lamikanra *et al.*, 1989) and in the Sudan 76% of children have been shown to harbour commensal gut flora with high level trimethoprim resistance (Shears *et al.*, 1988).

In hospitalised patients in Nigeria 63.3% resistance has been reported for Gram-negative bacilli causing urinary tract infection (Lamikanra and Ndep, 1989). Trimethoprim resistance in Tanzania was lower than this, 36% of the enterobacteria and 27% of the urinary bacteria being resistant (Amyes, 1986). In South Africa 48.5% of enterobacteria have been found to be trimethoprim resistant (Wylie and Koornhof, 1989). High incidences have been found in Chile where 44% of the *Escherichia coli* in a paediatric hospital in Santiago were resistant (Murray *et al.*, 1985) and 34% resistance to trimethoprim was found in enterobacteria from a range of hospitals (Urbina *et al.*, 1989). In Bangkok 40% resistance has been reported in a general teaching hospital (Murray *et al.*, 1985).

Perhaps the major difference between developed and underdeveloped countries is seen with resistance among *Shigella* spp.. Resistance among Shigella was initially very rare (Gordon *et al.*, 1975; Nelson *et al.*, 1976; Barada and Guerrant, 1980; Macaden and Bhat, 1985); however, increasing use of trimethoprim as the drug of choice for Shigellosis (Salter, 1982) and for travellers' diarrhoea (Ericsson *et al.*, 1990), has meant that trimethoprim resistant Shigella are now a major problem in many parts of the world (Elwell and Fling, 1989).

The emergence of trimethoprim-sulphamethoxazole resistance in Shigella was first reported by Bannatyne *et al.* (1980). It was found that 3% of Shigella strains isolated in Ontario, Canada in 1978 were resistant to cotrimoxazole (Tonin and Grant, 1987). Since then alarming increases in resistance to trimethoprim have been reported for many countries. In Bangladesh resistance has risen from 17% in 1982 to 64% in 1984 (Shahid *et al.*, 1985). In another study in Bangladesh, within one family, resistance rose from 5% to 83% between 1979 and 1983, in this case inadequate dosing with cotrimoxazole before admission may have accounted for the high resistance, and in general this is a common problem in the Third World (Zaman *et al.*, 1983). In Vellore, South India, where *Shigella* spp. have been the commonest etiological agent of gastroenteritis for many years 84% of *Shigella flexneri* and 88% of *Shigella shigae* have been found to be resistant to trimethoprim. Of the resistant strains, 84% had MICs > 1000 mg/L (Jesudason *et al.*, 1989). Large numbers of Shigella which could freely transfer trimethoprim resistance have been isolated in Korea (Chun *et al.*, 1981), and cotrimoxazole resistant Shigella emerged in Brazil in 1983 when it was discovered that 3 out of 4 *Shigella flexneri* isolates could transfer trimethoprim-sulphamethoxazole resistance to *Escherichia coli* K12 (Tiemens *et al.*, 1984). In common with many developing countries trimethoprim-sulphamethoxazole is freely available in Brazil and is a constituent in many antidiarrhoeal medicines. In Thailand resistance among *Shigella* spp. has risen from 3% in 1982 to 29% in 1986 in one paediatric hospital (Chatkaeomorakot *et al.*, 1987). Although mainly a problem of the Third World one European country, which has a serious problem with cotrimoxazole resistant Shigella, is Spain with up to 96.9% resistant by 1982 (Lopez-Brea *et al.*,

1983).

One unusual feature of trimethoprim resistance in Shigella is that resistance may be disseminated by the epidemic spread of one or two bacterial clones. Studies on an epidemic of *Shigella dysenteriae* in central Africa suggested that a single clone was responsible and that the plasmid content changed in response to antibiotic selective pressure (Frost *et al.*, 1982, 1985). A fifteen year study of *Shigella sonnei* in Bulgaria has shown that resistance was associated with the spread of two distinct bacterial clones (Bratoeva and John, 1989).

This is not always the case though as reports from Spain have shown a high prevalence of non epidemic *Shigella sonnei* resistant to cotrimoxazole (Palenque *et al.*, 1983). The most likely reason for this is that Shigella in this area acquire resistance from faecal flora in which R-plasmids are abundant.

Trimethoprim resistant *Shigella* spp. have also increased in the United Kingdom from 1.3% in 1979 to 16.8% in 1983 (Gross *et al.*, 1984). This was an alarming increase; however, it should be borne in mind that 61% of cases were from patients recently returned from abroad or who had been in contact with recent travellers. Interestingly, there have been occasional reports of trimethoprim resistant Shigella among specific groups, in countries where Shigella resistance is not normally a problem. One such example was a rapid rise in trimethoprim resistance on a Navajo reservation in the United States (Griffin *et al.*, 1989), Shigella infections are relatively uncommon in the United States but are a recognised problem on Indian reservations (Blaser *et al.*, 1983).

1.3.6 Trimethoprim Resistance in Salmonella and Staphylococcus

The two other areas which have been of great interest in the epidemiology of trimethoprim resistance have been the development of resistance in Salmonella and in the Gram-positive bacteria, in particular, Staphylococci. These two areas will be considered in turn:

There has been a lot of interest in antibiotic resistance in Salmonella, as it is one area in which there has been a clear spread of genes between animal and man. Initially trimethoprim resistance was rare, the first example was in 1975 when there was a *Salmonella typhimurium* outbreak in cattle (Richards *et al.*, 1978) and in 1976 there were four such outbreaks. However, overall plasmid determined resistance in strains of Salmonella 1976 to 1977 remained low at only 0.3% (Richards *et al.*, 1978).

In 1978 the incidence of resistance began to increase and by 1981 was 6.7% (Ward *et al.*, 1982). The source of the resistance genes was bovine and associated with specific phage-types. Multiresistant *Salmonella typhimurium* of phage-types 204 and 193 appeared in 1977 and spread epidemically in cattle. By 1979 there were 290 cases of these resistant phage-types causing infections in humans (Threlfall *et al.*, 1980). A new phage-type 204C appeared in 1979 and was associated with trimethoprim resistance (Threlfall *et al.*, 1980). By 1985 59% of *Salmonella typhimurium* from cattle and 4% of strains from humans were of phage-type 204C (Threlfall *et al.*, 1986). Almost all trimethoprim resistance in *Salmonella typhimurium* and the majority in other serotypes was plasmid-encoded (Threlfall

et al., 1983). Trimethoprim resistant phage-types of *Salmonella tymphimurium* have spread from Britain to the continent by the export of infected calves (Rowe *et al.*, 1979).

The pattern of trimethoprim resistance in *Salmonella typhi* has been watched closely as it was quickly realised that trimethoprim-sulphamethoxazole was an effective treatment for typhoid (Akinugbe *et al.*, 1968; Pugsley *et al.*, 1969) and trimethoprim was regarded as the drug of choice in chloramphenicol resistant *Salmonella typhi* (Butler *et al.*, 1977, 1982). Resistance has been slower to develop than in *Salmonella typhimurium* and only three cases were documented between 1970 and 1982 (Ward *et al.*, 1982). It was first reported in 1981 when a patient with enteric fever acquired resistance to cotrimoxazole during therapy (Datta *et al.*, 1981). Studies in India have shown *Salmonella typhi* to be highly sensitive to cotrimoxazole (Paramasivan *et al.*, 1977). However a study in Peru in 1981 to 1983 has shown 14% of *Salmonella typhi* to be resistant to trimethoprim (Taylor *et al.*, 1985) and in Mexico 16% resistance has been found (Solórzano *et al.*, 1987).

Considering Gram-positive organisms: trimethoprim has been advocated for use in Staphylococcal infections of the respiratory tract (Garrod, 1969) and for cases of Staphylococcal osteomyelitis caused by penicillin resistant organisms (Craven *et al.*, 1970). Trimethoprim has also been used successfully in combination with rifampicin to treat life threatening Staphylococcal infections (Grüneberg *et al.*, 1984) where it was recognised to have great potential in patients with penicillin hypersensitivity.

In the early 1970s resistance to trimethoprim or cotrimoxazole was relatively low,

1.6% among hospital isolates although 18.5% were resistant to sulphamethoxazole (Nakhla, 1972). Lewis and Lacey (1973) found that the increase in multiresistant strains of *Staphylococcus aureus* was low at around 1.0%, moreover this resistance appeared intrinsic and there was no evidence of it being plasmid-mediated. At this time, methicillin resistant *Staphylococcus aureus* from Europe and the United Kingdom were uniformly sensitive to trimethoprim-sulphamethoxazole (Seligman 1973). However by 1977 cotrimoxazole resistance among hospital *Staphylococcus aureus* in one London hospital had risen to 12.6% (Chattopadhyay, 1977), the reason for this sharp rise was that cotrimoxazole usage had, by this time, risen so much that it was second only to ampicillin. By 1981 the level of resistance among hospital isolates of *Staphylococcus aureus* was only 6% (Brumfitt *et al.*, 1983). There are very few reports of the incidence of trimethoprim resistance in the rest of the world but in the United States the level of trimethoprim resistance in *Staphylococcus aureus* is around 10% (Archer *et al.*, 1986) while in coagulase negative species is around 42% (Galetto *et al.*, 1987).

Trimethoprim resistance has also been found in other Staphylococcal species in the United Kingdom, 38.7% of *Staphylococcus epidermidis* from infected urines in hospital in London and 11.5% in general practice have been found to be resistant to trimethoprim (Brumfitt *et al.*, 1983). Resistance in *Staphylococcus saprophyticus* was lower 0% in hospital and 1.7% from general practice, overall resistance in Gram-positives was 15.2% in hospital and 3.8% in general practice (Brumfitt *et al.*, 1983).

These results are in agreement with those of Richardson (1983) who reported 30% of clinically significant *Staphylococcus epidermidis* from a wide range of hospitals

from the United Kingdom and abroad resistant to trimethoprim whilst *Staphylococcus saprophyticus* was uniformly sensitive. Interestingly the proportion of resistance in *Staphylococcus epidermidis* was 12.6% in strains responsible for the normal skin flora in schoolchildren. The incidence of trimethoprim resistance among methicillin resistant *Staphylococcus aureus* has increased markedly from the 0% reported in 1973 (Seligman, 1973) to 69% from a more recent worldwide selection of strains (Maple *et al.*, 1989). A further survey of *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates from university hospitals throughout Europe found 26% *Staphylococcus aureus* and 77% of *Staphylococcus epidermidis* resistant to trimethoprim. As well as the higher incidence of resistance among *Staphylococcus epidermidis* these strains were more frequently connected to methicillin resistance (Burdeska and Then, 1990).

1.4 Mechanisms of Resistance

A number of mechanisms conferring resistance to trimethoprim have been reported. There are four well characterised resistance mechanisms 1) Thymineless strains, 2) Impermeability, 3) Alterations in the chromosomal dihydrofolate reductase, 4) Plasmid-encoded production of an additional trimethoprim insensitive dihydrofolate reductase.

The clinical significance and occurrence of these is very different and each will be discussed:

1.4.1 Thymineless Strains

Thymineless mutants lack the enzyme thymidylate synthetase required for the synthesis of thymidine and utilise exogenous thymine or thymidine (Barner and Cohen, 1959). Provided thymine or thymidine is present, these mutants are highly resistant to trimethoprim (Amyes and Smith, 1975). Indeed trimethoprim can be used to select for thymineless mutants *in vitro* by growing bacteria in the presence of both trimethoprim and thymine (Stacey and Simson, 1965; Bertino and Stacey, 1966). As levels of thymine are very low in urine, blood and body tissue it was felt that the emergence of thymineless mutants was unlikely *in vivo* (Maskell *et al.*, 1978). However, the emergence of trimethoprim resistant thymineless mutants associated with cotrimoxazole therapy has been reported on a number of occasions (Barker *et al.*, 1972; Maskell *et al.*, 1976; King *et al.*, 1983). In urinary tract infection, a thymine like compound has been detected in sufficient concentration to support the growth of thymine requiring organisms (Maskell *et al.*, 1978). In one study between 1972 and 1979 thymineless bacteria accounted for 1.5% of trimethoprim resistant strains isolated per year (Acar and Goldstein, 1982).

One area where thymineless strains have an important role in determining trimethoprim resistance is cystic fibrosis, where trimethoprim-sulphamethoxazole is widely used prophylactically to suppress Staphylococcal infection (Marks, 1989). The levels of resistance have consequently been studied closely and in one study 21% of patients with *Staphylococcus aureus* contained thymine dependent *Staphylococcus aureus* as their predominant Staphylococcal isolate. All had received

trimethoprim-sulphamethoxazole prophylactically for an average of 30.9 months (Gillagan *et al.*, 1987). In the case of cystic fibrosis thymine dependent organisms can survive because the bronchial secretions in these patients contain many deteriorating cells which act as a source of thymine. The importance of thymine dependent *Staphylococcus aureus* in cystic fibrosis may be greater than realised as the organisms cannot grow on the media routinely used for the isolation of *Staphylococcus aureus* (Gillagan *et al.*, 1987).

1.4.2 Impermeability

Impermeability is often cited as a mechanism of resistance to antibiotics and there are two distinct areas to be considered. Firstly organisms which are intrinsically resistant to trimethoprim because of a natural generalised permeability barrier and secondly those that undergo changes in permeability as a response to antibiotic challenge.

Impermeability as a mechanism of intrinsic resistance is thought to be the reason why *Pseudomonas aeruginosa* is resistant to trimethoprim (Werner and Goeth, 1984). As the dihydrofolate reductase from *Pseudomonas aeruginosa* is sensitive to trimethoprim (Burchall, 1973) it was argued that resistance must result from the impermeability of the outer membrane although this view has been challenged (Scudamore and Goldner, 1982). Lack of permeability is thought to account for the resistance of *Pseudomonas cepacia* to a range of antibiotics including trimethoprim; however, it may not be the only resistance mechanism as an altered dihydrofolate reductase with decreased sensitivity for trimethoprim has been isolated from resistant *Pseudomonas cepacia* (Parr, *et al.* 1987; Burns

et al. 1989).

Acquired impermeability giving resistance appears to be common; however this is often concluded without any positive evidence and rather results from failure to detect any other resistance mechanism (Hamilton-Miller, 1979; Burchall *et al.*, 1982). There have been some cases though where impermeability is clearly important in determining resistance. Amyes and Smith (1977) reported resistance to trimethoprim caused by the presence of plasmid R388s, which promoted the host strain to become mucoid. Simultaneous resistance to trimethoprim, chloramphenicol and nalidixic acid has been reported in *Klebsiella pneumoniae* (Smith, 1976). This pattern of resistance has also been reported among clinical isolates of *Klebsiella*, *Enterobacter* and *Serratia* spp. (Acar and Goldstein, 1982). These mutants can be selected *in vitro* at high frequency with the use of only one of the antibiotics (Gutmann *et al.*, 1985). In these strains binding of chloramphenicol to ribosomes was normal and dihydrofolate reductase levels were the same in the mutant and wild type. The resistant mutants showed a lower uptake of glucose and chloramphenicol and examination of the outer membrane revealed decreased levels of a 40 kilodalton protein in the mutants (Gutmann *et al.*, 1985). In this case the sole resistance mechanism appears to be decreased permeability but the overall significance of impermeability in the epidemiology of trimethoprim resistance has not been clear.

1.4.3 Alterations in Chromosomal Dihydrofolate Reductase

Trimethoprim resistance resulting from alterations in the chromosomal dihydrofolate reductase can be manifested by a) increased production of the enzyme, thereby swamping the inhibiting trimethoprim, or b) by changes in the structure of the enzyme giving increased resistance to trimethoprim inhibition (Grey *et al.*, 1979; Smith and Calvo, 1982).

Considering strains resistant by mechanism a), increased levels of dihydrofolate reductase have been reported in *Streptococcus pneumoniae* (McCuen and Sirotnak, 1974) and in *Escherichia coli* (Sheldon and Brenner, 1976; Smith and Calvo, 1979). Baccanari *et al.* (1975) reported elevated levels of dihydrofolate reductase from *Escherichia coli* RT 500 which was resistant to 500 mg/L trimethoprim. This strain produced a 300 fold increase in enzyme activity over the wild type and 10 fold more than a corresponding strain resistant to 128 mg/L trimethoprim. Increased synthesis of chromosomal dihydrofolate reductase appears not to result from gene amplification but from increased efficiences of RNA polymerase and ribosome sites (Flensburg and Sköld, 1987). A recent clinical isolate of *Escherichia coli* resistant to > 1000 mg/L expresses chromosomal dihydrofolate reductase 200 times more than the sensitive *Escherichia coli* K12. Interestingly the dihydrofolate reductase activity increased when the strain was cultivated in the presence of trimethoprim and this induction was dependent on drug concentration (Tennhammar-Ekman *et al.*, 1986). Overproduction of the chromosomal enzyme may be an important mechanism of resistance in *Haemophilus influenzae* (Degroot *et al.*, 1988).

Alterations in the structural gene resulting in a dihydrofolate reductase with decreased affinity for trimethoprim (mechanism b) have been detected in a number of *Escherichia coli* strains (Sheldon, 1977; Baccanari *et al.*, 1981; Smith and Calvo, 1982) and recently in *Pseudomonas cepacia* (Burns *et al.*, 1989). These altered dihydrofolate reductases are usually not the sole resistance mechanism but are often more highly expressed than the wild-type enzyme (Baccanari *et al.*, 1981; Flensburg and Sköld, 1987). Indeed changes in the structural gene may not only increase resistance to trimethoprim but affect the level of expression simultaneously (Sheldon, 1977).

Although alterations in chromosomal dihydrofolate reductases can give high level resistance to trimethoprim , their occurrence is not often clinically significant and are much less common than plasmid-mediated resistance (Steen and Sköld, 1985).

1.4.4 Plasmid-Mediated Resistance

By far the most important mechanism of trimethoprim resistance has been the plasmid-encoded production of an additional trimethoprim resistant dihydrofolate reductase enabling the blocked chromosomal enzyme to be bypassed (Amyes and Smith, 1974b; Sköld and Widh, 1974). Plasmid-encoded resistance to trimethoprim was first reported by Fleming *et al.* (1972) and is now widespread. The trimethoprim resistant dihydrofolate reductases found in Gram-negative bacteria have been divided into a number of classes based on their biochemical profiles (Huovinen, 1987; Amyes, 1989).

Table 1. Biochemical properties of the plasmid-mediated dihydrofolate reductases.

Enzyme	Tp ID_{50}* μM	Mtx ID_{50} μM	TD_{50} mins	DHF K_m μM	Tp K_i μM	Size daltons
Ia	57	4.4	0.5	5.6	7.4	35000
Ib	32	2.8	1.2	11	41	24500
IIa	70000	1100	> 12	4.6	6100	35000
IIb	80000	750	> 12	8.3	150	35000
IIc	20000	1000	> 12	4.2	400	34000
III	2.0	—	—	0.4	0.019	16900
IV	0.2	0.02	> 12	37	0.063	46700
V	10	—	> 5	—	—	—
VI	200	7.25	0.4	31.25	75	10000
VII	30	3.0	1.5	20.0	7.0	11500
S1	50	0.002	> 12	10.8	11.6	19700

* See list of abbreviations p5

Initially resistance was dominated by the spread of two genes encoding dihydrofolate reductase types I and II, the type I was particularly common probably because the gene was located on a transposon (Barth *et al.*, 1976). However more recently a number of new enzymes have been identified and now seven main types have been distinguished in Gram-negative bacteria, and one type in Staphylococci (table 1).

Type I dihydrofolate reductase

The early plasmid-mediated dihydrofolate reductases fell into two types, both of which confer high level trimethoprim resistance (Pattishall *et al.*, 1977). The type I enzyme encoded by plasmid R483 has a molecular mass of 35,000 and could

be separated from the host enzyme on the basis of gel-filtration. The enzyme is produced to a level about 10-times that of the chromosomal dihydrofolate reductase. The dose required for 50% inhibition (ID_{50}) of the type I enzyme for trimethoprim and methotrexate was 57 μM and 4.4 μM respectively and these figures are several thousand fold higher than the chromosomal enzyme (Pattishall *et al.*, 1977). The type I enzyme is markedly heat sensitive losing half its activity after 30 seconds at 45°C.

The type I dihydrofolate reductase consists of a dimer with a subunit size of 18,000 (Fling and Elwell, 1980; Novak *et al.*, 1983). It has been purified and its N-terminal amino acid sequence determined (Novak *et al.*, 1983). However, because of its lability, the complete sequence could only be elucidated by nucleotide sequencing (Fling and Richards, 1983). The sequence of the type I shows no homology with the type II enzymes but significant homology with the *Escherichia coli* chromosomal enzyme.

The gene coding for the type I dihydrofolate reductase resides on a transposon Tn7 (Barth *et al.*, 1976) and this has proved to be an important factor in determining the spread of the type I enzyme. The type I enzyme is ubiquitous and has proved to be the most commonly detected plasmid-mediated dihydrofolate reductase (Pulkkinen *et al.*, 1984; Steen and Sköld, 1985; Huovinen *et al.*, 1986; Delgado and Otera, 1988). Indeed it has been shown that a rapid rise in trimethoprim resistance in one area can be attributed to the appearance of Tn7 as opposed to other resistance genes (Huovinen *et al.*, 1985).

Although originally plasmid-mediated the type I dihydrofolate reductase has been

found with increasing frequency on the bacterial chromosome (Towner *et al.*, 1978, 1980; Towner, 1981; Fling *et al.*, 1982; Steen and Sköld, 1985; Amyes *et al.*, 1986b). In one study of trimethoprim resistant *Escherichia coli* from hospital and community, the proportion of strains which had chromosomal carriage of Tn7 rose from 38 to 70% (Kraft *et al.*, 1986). It is not clear as yet if this movement of Tn7 from plasmid to chromosome will lead to changes in the incidence of resistance. The type I enzyme has been found integrated into the chromosome of *Vibrio cholerae* on the transposon Tn1527 which is closely related to Tn7 (Goldstein *et al.*, 1986a) and has also been found in the same clinical strains as the type II dihydrofolate reductase (Fling *et al.*, 1982; Burchall *et al.*, 1982; Papadopoulou *et al.*, 1986).

A second transposon mediated type I like dihydrofolate reductase has been characterised (Young and Amyes, 1985). This enzyme encoded by Tn4132 has very similar properties to the type I enzyme but a lower molecular mass of 24,500. It is probably closely related to the type I and has been named the type Ib, and the type I renamed type Ia (Young and Amyes, 1985).

Type II Dihydrofolate Reductase

The type II enzyme encoded by plasmid R67 has a much lower specific activity than the type I. It is produced in amounts the same or less than the chromosomal enzyme (Pattishall *et al.*, 1977). However it is able to confer high level resistance as the enzyme is practically insensitive to trimethoprim and methotrexate (ID_{50} Tp 70,000 μM ID_{50} Mtx 1100 μM). The molecular mass of the type II enzyme

is 36,000 when measured by gel filtration and polyacrylamide gel electrophoresis (Smith *et al.*, 1979). The enzyme was thought to consist of four subunits each 9000 in size (Fling and Elwell, 1980) and amino acid sequencing of the purified enzyme suggested that all the subunits were identical (Smith *et al.*, 1979). However more recently X-ray crystallography studies have revealed that, in common with the type I enzyme, the type II may be a dimeric molecule (Matthews *et al.*, 1986), and it is likely therefore that the four subunits interact as dimers. Complete amino acid sequencing of the type II enzyme from R67 showed that it had no homology with any of the other dihydrofolate reductases (Stone and Smith, 1979). This sequence has been confirmed by the nucleotide sequence of the type II gene from R67 (Brisson and Hohn, 1984).

The type II enzymes have now been split into three classes all of which are biochemically similar but are distinguishable by sequence analysis (Stone and Smith, 1979; Zolg and Hänggi, 1981; Flensburg and Steen, 1986). The original type II enzyme encoded by plasmid R67 is termed the IIa and the IIb and IIc are encoded by plasmids R388 and R751 respectively. Comparison of the amino acid composition of the three enzymes has shown that they are almost identical. There are only 11 (out of 78) amino acid differences between the enzymes encoded by plasmids R751 and R388 and only 17 differences when either enzyme is compared with the R67 enzyme (Flensburg and Steen, 1986).

The epidemiology of the type II enzyme has been studied extensively, it is widespread though not as common as the type I, despite the fact that the IIc is found on a transposon (Fling and Elwell, 1980). The incidence of the type II dihydrofolate reductase genes has been studied with radiolabelled DNA probes and the propor-

tion of trimethoprim resistant strains carrying type II genes has been found to be 26% in a worldwide selection of clinical bacteria (Fling *et al.*, 1982) and 60% in Chile (Murray *et al.*, 1985). In one hospital in the United States trimethoprim resistance was shown to be the result of the spread of a single conjugative plasmid, with a wide host range, which contained the type II dihydrofolate reductase gene (Mayer *et al.*, 1985). This situation has not been seen in Europe. The type II enzyme has recently been identified in *Vibrio cholerae* (Tabtieng *et al.*, 1989). Unlike the type I enzyme there is no evidence as yet that a transposon carrying the type II dihydrofolate reductase gene has migrated into the bacterial chromosome.

Type III Dihydrofolate Reductase

The type III enzyme was reported in a single bacterial species *Salmonella typhimurium*, and was detected because of its lack of hybridisation with radiolabelled DNA probes for the type I and II enzymes (Fling *et al.*, 1982). The type III dihydrofolate reductase differs markedly from the types I and II. It has a lower molecular mass 16,900, is monomeric and only confers low level resistance (MIC Tp 64 mg/L) (Joyner *et al.*, 1984). The type III enzyme is much more sensitive to trimethoprim inhibition than either of the other two enzymes (ID_{50} Tp 2 μM, K_i 19 nM). However, it is able to confer resistance as it has a very strong affinity for the substrate dihydrofolate (K_m 0.4 μM). Amino acid sequencing of the first 20 amino acids of the protein showed it to have 50% homology with the *Escherichia coli* chromosomal enzyme (Joyner *et al.*, 1984).

The complete DNA sequence for the type III gene has recently been elucidated (Fling *et al.*, 1988) and shows it to be much more closely related to Gram-negative bacterial chromosomal dihydrofolate reductases than to the other plasmid-mediated enzymes. The type III enzyme shows 51% homology with the *Escherichia coli* and 44% homology with the *Neisseria gonorrhoeae* chromosomal enzymes (Fling *et al.*, 1988).

The type III enzyme was found originally on a broad host range plasmid (incQ) but appears not to have spread. However, as the enzyme only confers low level resistance, this apparent rarity may actually be attributed to a lack of detection as isolates with low level trimethoprim resistance are not normally screened for transferability of resistance. Towner and Pinn (1981) detected a transferable plasmid conferring only a moderate level of resistance to trimethoprim and stressed the need in all plasmid surveys to test all trimethoprim resistant isolates, regardless of their MIC, for their ability to transfer.

Type IV Dihydrofolate Reductase

The type IV enzyme was identified during a survey of antibiotic resistance in South India in 1984 (Young *et al.*, 1986b). Subsequent biochemical analysis revealed the type IV was unusual in a number of ways 1) It is an inducible enzyme which is rare for plasmid-encoded resistance enzymes in Gram-negative bacteria, 2) It has a large molecular mass (46,700) and 3) It is only partially resistant to trimethoprim (ID_{50} Tp 0.2 μM) (Young and Amyes, 1986a). Furthermore, the expression of the enzyme depends on the presence of methionine adenine and

glycine in the media (Young *et al.*, 1986b).

The Type V Dihydrofolate Reductase

This novel enzyme was identified in Sri Lanka and was detected because of a lack of hybridisation with DNA probes for the types I and II enzymes. This evidence along with a partial biochemical profile suggested that it may be a novel enzyme (Sundström *et al.*, 1987).

Type VI Dihydrofolate Reductase

A high-level trimethoprim resistant *Proteus mirabilis* strain isolated in South Africa contained a 79 kb plasmid (pUK 672) which encoded trimethoprim resistance. The dihydrofolate reductase produced was biochemically different from any of the other plasmid-mediated enzymes and was designated type VI (Wylie *et al.*, 1988). The type VI enzyme is the smallest of the plasmid-mediated dihydrofolate reductases with a molecular mass of 10,000, the enzyme is highly resistant to inhibition by trimethoprim and methotrexate (ID_{50} Tp 200 μM ID_{50} Mtx 7.25 μM). The type VI enzyme is very heat labile losing half of its activity after 24 seconds at 45°C (Wylie *et al.*, 1988).

Type VII Dihydrofolate Reductase

Plasmid pUN835 from an *Escherichia coli* responsible for an outbreak of porcine diarrhoea contained a dihydrofolate reductase gene which did not probe with di-

hydrofolate reductase genes I - V (Amyes *et al.*, 1989). Biochemical characterisation has shown this enzyme to have similar properties to the type Ia dihydrofolate reductase. However it has a much lower molecular mass of 11,500 (Amyes *et al.*, 1989).

S1 Dihydrofolate Reductase

Until 1983, trimethoprim resistance in Gram-positive organisms was not plasmid-mediated. However, in 1983 a trimethoprim resistance gene was identified on plasmid pSK1 from multi-resistant *Staphylococcus aureus* from Australia (Lyon *et al.*, 1986). The plasmid confers high level trimethoprim resistance and encodes a dihydrofolate reductase which is unlike any of the enzymes found in Gram-negative bacteria and it has been designated S1 (Young *et al.*, 1987). The S1 enzyme is produced in large amounts, has a molecular mass of 19,700, is monomeric and is resistant to trimethoprim (ID_{50} 50 μM) but sensitive to methotrexate (ID_{50} 0.002 μM) (Young *et al.*, 1987). A plasmid-mediated dihydrofolate reductase conferring trimethoprim resistance has also been isolated in the United States (Coughter *et al.*, 1987) but the enzyme has also been shown to be type S1 (Tait and Amyes, 1989). The S1 dihydrofolate reductase has been detected on both the chromosome and on plasmids and the position of the gene appears to affect the level of resistance conferred (Galetto *et al.*, 1987).

The gene encoding the S1 dihydrofolate reductase has now been sequenced (Rouch *et al.*, 1989) and shows most homology with the *Staphylococcus aureus* chromosomal enzyme (Hartman *et al.*, 1988; Amyes and Tait, 1990). At present it appears

that the S1 dominates trimethoprim resistance in staphylococci, whether this remains the situation has yet to be seen.

The efficiency of plasmid-mediated resistance to trimethoprim has compromised the use of this drug in many parts of the world. Recently there has been a rapid increase in the number of plasmid-mediated dihydrofolate reductases. This increase in numbers may not be as rapid as it appears but partly due to more sophisticated techniques for detection and distinguishing between enzyme types. Biotin labelled DNA probing has proved a useful tool for the screening of isolates (Carter *et al.*, 1987; Towner *et al.*, 1988; Towner, 1990). Probing has shown that whilst the type I and II enzymes are still common a significant proportion of resistance is caused by other enzyme types. Chatkaeomorakot *et al.* (1987) for instance have shown that 58% of enterotoxigenic *Escherichia coli* did not contain genes for the types I, II or III dihydrofolate reductases. It is clear that plasmid-mediated resistance to trimethoprim is still evolving.

1.5 Other Antifolate Drugs

There are a number of other drugs which act on the pathway of folate metabolism. These are not all antibacterial, methotrexate is an inhibitor of mammalian dihydrofolate reductase and is an important anticancer treatment and pyrimethamine has marked antimalarial activity (Burchall, 1979; Sandler and Smith, 1989).

In terms of antibacterial antifolates a number of trimethoprim-like compounds have been developed. It was realised that minor alterations in the benzyl moiety of trimethoprim could lead to impressive biological changes and this led to the de-

velopment of 2,4-diamino-5-(3,5-dimethoxy-4-methoxyethoxy-benzyl)-pyrimidine better known as tetroxoprim (Liebenow and Prikryl, 1979). Like trimethoprim this compound strongly inhibits prokaryotic dihydrofolate reductase but only weakly inhibits the eukaryotic enzyme (Aschhoff and Vergin, 1979). A combination of tetroxoprim and sulphadiazine has been shown to be synergistic *in vitro* (Grimm, 1979; Wiedemann, 1979) and has been used successfully clinically in the treatment of urinary and respiratory tract infections (Ferber and Ahrens, 1979; Pust *et al.*, 1979). However tetroxoprim is not resistant to the enzymes conferring resistance to trimethoprim and therefore offers no real advantage over trimethoprim (Bywater *et al.*, 1979).

Lipophilic analogues of trimethoprim bearing 3,5-dialkyl-4-hydroxy substituents in the benzyne ring have been developed which are much more active *in vitro* against *Neisseria gonorrhoeae* than is trimethoprim (Roth *et al.*, 1988). However *in vivo* studies showed that these drugs were extensively metabolised and their development was discontinued (Roth *et al.*, 1988). Similarly 3,4-dimethoxy-5-alkenyl, -5-alkyl and -5-alkoxy analogues of trimethoprim which had high *in vitro* activity against anaerobic organisms had a short half life and were disappointing *in vivo* (Roth *et al.*, 1989). However one of a series of 2,4-diamino-5-[(1,2-dihydro-6-quinolyl)methyl] pyrimidines containing *gem*-dimethyl or fluoromethyl substituents at the 2- position of the dihydroquinoline ring, (2,4-diamino-5-[[1,2-dihydro-2,4-dimethyl-3-fluoro-2-(fluoromethyl)-8-methoxy- 6(1H)quinolyl]methyl]-pyrimidine), has outstanding activity against Gram-positive organisms. The compound has as broad a spectrum of antibacterial activity as trimethoprim and the results of *in vivo* tests are awaited (Johnson *et al.*, 1989).

Chapter 2

Materials and Methods

2.1 Bacterial Strains

The bacterial strains used were as follows:

Escherichia coli HB101 (pUN972) transformant containing the type IIIa dihydrofolate reductase gene (located on a 0.85 kb *Pst*1 fragment) cloned into pUC18, from Kevin Towner.

Escherichia coli C600 (pBH600), transconjugant containing the original clinical plasmid responsible for trimethoprim resistance in a *Shigella sonnei* outbreak in a nursing home in Tennessee, from Neil Barg.

Escherichia coli JM101 (pBH7001a) an *E. coli* clone consisting of a 1.8 kb *Pst*1 fragment encoding the trimethoprim resistance gene from an outbreak of *Shigella sonnei* in a National Park cloned into pUC9, from Neil Barg.

Escherichia coli JM83 (pFE1242), strain containing a recombinant plasmid formed by inserting partial *Taq*1 fragments encoding the type IIIa dihydrofolate reduc-

tase from pAZ1 cloned into pUC9, from Lynn Elwell.

Escherichia coli $J62_{-2}$ (pUK1123), an *E. coli* transconjugant containing the original clinical trimethoprim R-plasmid specifying the type IV dihydrofolate reductase, from Hilary Young.

Escherichia coli C600 (pUK1150), an *E. coli* clone containing plasmid pBR322 with a 2.6 kb *Hind*III fragment of pUK1123 encoding the type IV dihydrofolate reductase, from Hilary Young.

Escherichia coli C600 (pUK1152), an *E. coli* clone containing a 4.1 kb *EcoR*1 fragment of pUK1123 containing the type IV dihydrofolate reductase gene ligated into pACYC184, from Hilary Young.

Escherichia coli C600 (pUK1140), an *E. coli* clone containing a 4.1 kb *Eco*R1 fragment of pUK1123 containing the type IV dihydrofolate reductase gene inserted into pBR322, from Hilary Young.

Escherichia coli C600 (pUK1148), contains the recombinant plasmid formed by inserting a 1.7 kb *Cla*1 fragment of pUK1123 encoding the type IV dihydrofolate reductase into pBR322, from Hilary Young.

Escherichia coli HB101 (pLK09) transconjugant containing the recombinant plasmid pLK09. This contained a 1.25 kb insert which included the type V dihydrofolate reductase from plasmid pLM020, from Ola Sköld.

Escherichia coli HB101 (pUK2000) an *E. coli* clone containing plasmid pUC18 with a 0.48 kb *Hpa*1 fragment encoding the type V dihydrofolate reductase, from Ola Sköld.

Escherichia coli C600 (pFE872) an *E. coli* clone containing plasmid pBR322 with a 1.6 kb *Taq*1 fragment encoding the type Ia dihydrofolate reductase, from Lynn Elwell.

Escherichia coli J62$_{-2}$
Escherichia coli J53
Escherichia coli K12 met$^-$
Escherichia coli C met$^-$
} all from Sebastian Amyes.

2.2 Media

2.2.1 Complex Media

The complex media used were Nutrient Broth No. 2 (CM67), Diagnostic Sensitivity Test Agar (CM261), Isosensitest Broth (CM473) and Columbia agar base (CM331B), (Oxoid, Basingstoke, Hants).

2.2.2 Minimal Media

Double strength minimal salts medium (DM) was prepared as described by Davis and Mingioli (1950) (table 2). Single strength minimal medium DM base was prepared by diluting double strength DM base with an equal volume of distilled water prior to autoclaving. For the preparation of diluents, single strength DM base was distributed in 9.9 ml and 4.5 ml aliquots which were autoclaved at 15 psi for 15 minutes. Supplement solutions: for incorporation into minimal medium are shown in table 3.

Table 2. Preparation of double strength Davis Mingioli basal medium (DM)

K_2HPO_4	14.0g
KH_2PO_4	6.0g
Tri-Sodium Citrate.$2H_2O$	0.9g
$MgSO_4.7H_2O$	0.2g
$(NH_4)_2SO_4$	2.0g

These ingredients were dissolved in a litre of distilled water in the order given. 50 ml quantities were distributed and autoclaved at 15 psi for 15 minutes.

Table 3. Supplement solutions.

Solution	Source	Strength prepared	Final concentration	Mode of sterilisation
D-glucose	BDH	200mg/ml	2.8mg/ml	Autoclaving
L-proline	BDH	5mg/ml	50μg/ml	Steaming
L-histidine	BDH	5mg/ml	50μg/ml	Steaming
L-tryptophan	BDH	2mg/ml	50μg/ml	Steaming
L-methionine	BDH	5mg/ml	50μg/ml	Filtration
adenine	BDH	5mg/ml	50μg/ml	Steaming
glycine	BDH	5mg/ml	50μg/ml	Steaming
thymidine*	Sigma	5mg/ml	varied	Steaming

Autoclaving was performed for 15 minutes at 15 psi. Steaming was for 30 minutes, filtration was through a Millipore filter with pore size 0.2μm.

*Thymidine solution was also added to complex media as described in the Results.

2.2.3 Preparation of Plates

Complex Media

All laboratory media were made up according to the manufacturer's instructions and were autoclaved at 15 psi for 15 minutes. The plates were poured while the agar was still molten, each containing approximately 15 ml. With antimicrobial drug sensitivity plates, the agar was allowed to cool to 50°C before the appropriate antimicrobial drugs were added and the plates poured. After setting, the plates were dried, inverted at 50°C for 20 - 30 minutes.

Minimal Media

To 50 ml of double strength DM media were added, aseptically, the required amino acid supplements (to give a final concentration of 50 μg/ml), 1.4 ml of 20% D-glucose and the appropriate antimicrobial drugs. Sterile distilled water was added to bring the volume to 60 ml. This mixture was added to 40 ml of molten bacteriological agar No. 1 (L11 Oxoid) and mixed gently before pouring.

2.3 Materials

2.3.1 Chemotherapeutic Drugs

The chemotherapeutic drugs used are listed in table 4. All were supplied sterile and prepared as indicated.

Table 4. Chemotherapeutic drugs.

Chemotherapeutic drug	Method used to dissolve	Supplier
Trimethoprim lactate	H_2O	Wellcome Foundation
Tetroxoprim	lactic acid	Ludwig Heumann & Co.
Pyrimethamine	lactic acid	Wellcome Foundation
Sulphamethoxazole	NaOH	Wellcome Foundation
Aminopterin	NaOH	Sigma
Methotrexate	NaOH	Lederle Laboratories
Streptomycin sulphate	H_2O	Glaxo Ltd.

2.3.2 Reagents

NADPH tetrasodium salt was purchased from the Sigma Chemical Company and was stored desiccated at -20°C. Solutions of NADPH were prepared and used on the same day.

Dihydrofolic acid (DHF) (Sigma) was prepared by dissolving 25 mg of DHF in 0.05M sodium phosphate buffer pH 7.4 containing 0.05M β-mercaptoethanol. This solution was distributed into aliquots which were stored in total darkness at -20°C. No aliquot was refrozen after use. Preparation of DHF for zymographic staining is described later.

5-fluoro-2′-deoxyuridine (FUdR) and 2′-deoxyadenosine were purchased from Sigma and stored at room temperature prior to use. FUdR was added directly to culture media to give the required concentration except when final concentrations below 1.0 μg/ml were required. In these cases the FUdR was diluted in sterile distilled water and the required volume added. 2′-deoxyadenosine was made up

to 5 mg/ml in distilled water and sterilised by steaming for 30 minutes. The required amount was then added to cultures.

EDTA, Folic acid and β-mercaptoethanol were all purchased from Sigma and stored at room temperature.

MTT (Sigma) was stored at +4°C in darkness. A fresh solution of MTT dissolved in distilled water was used for each zymographic stain.

Methotrexate agarose from Sigma was stored in the dark at +4°C until use.

2.3.3 Standard Markers

The standard molecular weight proteins used to calibrate Sephadex gel filtration columns and native polyacrylamide gels are shown in table 5. For SDS polyacrylamide gels a Sigma molecular weight kit (MW-SDS-70L kit) was used. Isoelectric focusing gels were calibrated with an Electran pI calibration kit 4.7 - 10.6 (44270 2G) from BDH.

2.3.4 Buffers

Sodium phosphate buffers were made according to the Data for Biochemical Research, (Oxford University Press). The buffer used routinely for dihydrofolate reductase extraction and purification was 50 mM sodium phosphate buffer pH 7.4 containing 10 mM β-mercaptoethanol and 1 mM EDTA (buffer A).

Table 5. Proteins used as molecular weight markers.

Protein	Molecular mass daltons	Supplier
Bovine serum albumin	66000	Sigma
Ovalbumin	45000	Sigma
Chymotrypsinogen	25500	Sigma
Trypsin inhibitor	20100	Sigma
Cytochrome C	12384	Sigma
A protinin	6500	Sigma
Chain B insulin	3878	Sigma

2.4 Methods

2.4.1 Plasmid Transfer

Donor and recipient strains were grown overnight in 4.5 ml of nutrient broth at 37°C. After overnight growth 0.1 ml of the donor culture and 1 ml of the recipient culture were mixed in 4.5 ml of prewarmed nutrient broth and incubated statically for 18 hours. At the end of this time the mating mixture was vortexed to separate donor and recipient cells. Cells were collected by centrifugation at 4000g for 15 minutes (Heraeus Christ Bactifuge) and resuspended in 5.6 ml basal DM medium and further diluted to 10^{-1}, 10^{-2} and 10^{-4} in DM medium. 0.1 ml of neat culture and each dilution was spread on selective DM plates only allowing growth of a plasmid containing recipient strain. Plates were incubated at 37°C for 48 hours after which time the resultant single colonies were purified on selective plates. Controls consisted of spreading donor and recipient strains on selective plates,

after centrifugation and resuspension in 4.5 ml DM.

2.4.2 Viable Counts

Serial dilutions were made with 1 in 10 and 1 in 100 dilution steps in single strength DM, i.e. 0.5 ml of culture and 4.5 ml diluent or 0.1 ml culture and 9.9 ml of diluent respectively. Suspensions were mixed on a rotomixer (Hook and Tucker Ltd.) and 0.1 ml amounts spread with a sterile glass spreader onto nutrient agar plates. The spread plates were incubated, inverted, at 37°C for 18 hours.

2.4.3 Minimum Inhibitory Concentration (MIC) Determinations on Solid Media

Oxoid No. 2 nutrient broth (4.5 ml amounts) were seeded with an inoculum from a fresh nutrient agar plate and grown overnight at 37°C. A 1 in 10^4 dilution was prepared by serial dilution in DM base and 2 μl of this suspension was spotted onto media containing varying concentrations of the drug with a multipoint inoculator (Denley). All plates were incubated at 37°C for 18 hours with the exception of DM plates which were incubated for 48 hours. Concentrations of drug were usually increased by a factor of two and the MIC was taken as the first concentration permitting no visible growth. A control plate lacking any drug was used in each case.

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2.4.4 Enzyme Preparation

Enzyme was prepared from overnight cultures of bacteria grown in Oxoid Isosensitest broth with vigorous agitation at 37°C. Bacteria were harvested by centrifugation at 6000g for 15 minutes (RC-5B Sorval), washed in Davis Mingioli minimal media and the final pellet resuspended in buffer A. After this point all operations were performed at +4°C. The bacteria were disrupted by sonication (2 x 30s, 8 μm MSE Soniprep) and the cell debris removed by centrifugation (40,000g, 1h).

The resulting crude enzyme preparation could be used to establish how much dihydrofolate reductase the strain was producing (enzyme specific activity), for example in induction studies. However to examine the biochemical properties the crude extract had to be purified further.

Nucleic acids were precipitated by the gradual addition of 0.1 volume 10% streptomycin sulphate. The precipitate was removed by centrifugation at 12000g for 30 minutes in a Sorval RC-5B centrifuge. Dihydrofolate reductase was precipitated from the extract by the addition of ammonium sulphate to 50% and 80%, after each precipitation the pellet was recovered by centrifugation at 12000g for 30 minutes in a Sorval RC-5B centrifuge. The 80% pellet was resuspended in a minimum volume (about 2 ml) of buffer A and then assayed for dihydrofolate reductase activity, resuspended pellets were stored frozen at -20°C until required.

2.4.5 Concentrators

Where protein samples were required to be concentrated to suitable volumes, for running on gel filtration columns, PAGE and IEF gels, Amicon Centriprep 10 and Centricon 10 concentrators were used. These provide fast efficient concentration of small volume macromolecular solutions by ultrafiltration through a low adsorption hydrophobic membrane with a 10,000 dalton cut off. Concentrators were used in accordance with manufacturer's instructions.

2.4.6 Enzyme Assays

Dihydrofolate reductase activity was assayed by the method of Osborn and Huennekens 1958. Dihydrofolate was made up to 1 mM and stored as described in the Materials, NADPH was made up to 1 mM in distilled water and used that day only. The assays were performed in either a Pye Unicam Sp8000, Pye Unicam Sp1800 or Perkin Elmer Lambda 2 spectrophotometer at 37°C. 1 ml quartz cuvettes were used and contained the following reagents:

40 mM sodium phosphate buffer pH 6.0

10 mM B-mercaptoethanol

0.08 mM NADPH

enzyme

distilled water to a volume of 0.95 ml

The blank cuvette contained all these constituents except NADPH. Both cuvettes were placed in the spectrophotometer for four minutes to allow for temperature

equilibration. To start the reaction 0.05 ml of 1 mM dihydrofolate was added to both the blank and test cuvettes, and the decrease in absorbance at 340 nm followed for ten minutes or until the absorbance fell to 0. When a decrease in absorbance occurred before the addition of dihydrofolate, this was taken as dihydrofolate-independent NADPH oxidase activity. This rate was subtracted from the total rate observed in the presence of dihydrofolate, to give the level of dihydrofolate reductase activity.

The decrease in absorbance at 340 nm is caused by reduced absorbancy both by the oxidation of NADPH to NADP and by the reduction of dihydrofolate to tetrahydrofolate. 52% of the combined decrease in absorbancy results from the conversion of dihydrofolate to tetrahydrofolate when both substrates are being consumed stoichiometrically (Mathews *et al.*, 1963), and hence, with this proviso, the rate of enzymic activity was expressed in molar terms of dihydrofolate being converted to tetrahydrofolate. Controls were performed routinely in the absence of enzyme to ensure that the addition of the two substrates together did not result in a change of absorbance.

2.4.7 Protein Estimations

Protein estimations for establishing dihydrofolate reductase specific activity were performed by the method of Waddell (1956). All samples to be tested were suitably diluted in 50 mM sodium phosphate buffer pH 7.4 and the absorbance measured at 215 and 225 nm. The protein concentration could then be calculated with reference to a standard curve which had previously been prepared. This method has been shown to be as precise and more sensitive than the protein

estimation of Lowry *et al.* (1951).

2.4.8 Gel Filtration

Sephadex G50 or G75 fine grade (Pharmacia, Uppsala Sweden) was allowed to swell for 3 hours at 100°C in buffer A, after being allowed to cool any "fines" were carefully decanted and more buffer A added. The Sephadex slurry was poured carefully into an Amicon acrylic column (2 cm^2 x 90 cm) which was maintained at 4°C in an LKB mini cold lab. When the column was fully packed the top was connected and the flow changed to an upward direction with an LKB peristaltic pump. The flow rate was adjusted to around 2 ml per 10 minutes and the column washed continually with buffer A for 48 hours before use.

Samples for separation (1 - 2 ml) were applied to the bottom of the column and eluted with buffer A. Two ml fractions were collected and maintained at 4°C using an LKB Ultrorac fraction collector. Elution was continued until a volume of buffer A, equivalent to the total volume (180 ml) of the column, had passed through. The column was washed for twelve hours between each run with buffer A.

2.4.9 Enzyme Molecular Weight Determinations

Calibration of Sephadex columns was based on the method of Andrews (1964). Five mg of each of the 3 standards chosen were dissolved in buffer A and applied to the Sephadex column. The standard proteins were eluted in buffer A and 2 ml fractions were collected. The position of the protein peaks was determined by

measuring the absorbance of the collected fractions at 280 nm.

2.4.10 Enzyme Kinetics

The biochemical properties of the dihydrofolate reductases being investigated were examined after pooling those fractions, from gel filtration, which showed peak activity. Inhibition of activity by trimethoprim and methotrexate was examined by assaying the enzymes activity in increasing concentrations of the inhibitor. The assays were performed as before with the exception that the required amount of drug was added to both cuvettes and the volume of distilled water altered accordingly.

The temperature sensitivity of the enzymes were examined by incubating the sample in a prewarmed container at 45°C for the requisite time. The sample was then assayed for dihydrofolate reductase activity as normal.

To establish the K_m for the enzymes the activity of each dihydrofolate reductase was assayed under conditions of partial saturation with dihydrofolate and the results analysed by the method of Lineweaver and Burk (1934). The reciprocal of the substrate concentration $1/s$ was plotted against the reciprocal of the rate of enzymic reaction $1/v$. The intercept on the abscissa gives the negative of the reciprocal of the Michaelis Constant (K_m), the K_m value being the substrate concentration at which half the maximal velocity occurs (Dixon and Webb, 1958).

The inhibitor constants (K_i) of the enzymes were calculated by repeating the assays in limiting substrate dihydrofolate but this time in the presence of trimethoprim. Again double reciprocal plots of $1/s$ against $1/v$ were plotted, trimethoprim

inhibition of dihydrofolate reductase is competitive and therefore this double reciprocal plot intercepts the ordinate at the same point as the K_m line. However, the plots in the presence of trimethoprim intercept the abscissa nearer the origin than the lines obtained without inhibition. If the distance to the origin from the intercept is $-\frac{1}{K_p}$ then:

$$K_i = \frac{i}{\frac{K_p}{K_m} - 1}$$

where i is the concentration of the inhibitor (Dixon and Webb, 1958).

2.4.11 Zymography

Bands of dihydrofolate reductase activity were located on polyacrylamide gels by zymographic staining (Tennhammar-Ekman and Sköld, 1979; Broad and Smith, 1982). Immediately after the electrophoretic run, gels were flooded with prewarmed 1.2 mM NADPH in 0.2M sodium phosphate buffer pH 6.0. The gel was then incubated for five minutes at 37°C after which time 0.15 ml of 50 mM sodium phosphate buffer pH 7.4 containing 10 mM dihydrofolate and 50 mM β-mercaptoethanol was added. The gel was incubated for five minutes at 37°C in a dark room, finally 6 mM MTT was added and the gel incubated at 37°C in darkness until bands were visible, typically 5 - 10 minutes. Unspecific reduction of the MTT was identified on control gels stained as described but in the absence of dihydrofolic acid.

2.4.12 Isoelectric Focusing

Isoelectric focusing was performed by a modification of the technique described by Broad and Smith (1982). Dihydrofolate reductase which had been precipitated by 80% ammonium sulphate was diluted 1 in 5 with buffer A containing 0.75 mM NADPH and this was dialysed against buffer A (100 volumes) for 4 hours.

Aliquots of this (10 μl) were applied to the surface of standard polyacrylamide plates containing ampholines capable of producing a gradient from pH 3.5 - 9.5 (LKB products No. 1804-101). Standard pI markers 4.7 - 10.6 (BDH 44270 2G) were also applied to each gel. The samples were focused for six hours on a cooled (9°C) Ultrophor-electrophoresis system (LKB No. 2217) at 500V and 20mA, limited by constant power set at 1W. The gels were stained for dihydrofolate reductase activity as described.

2.4.13 Affinity Chromatography

Affinity chromatography was performed with methotrexate agarose (Sigma) and an Econo-Column (Bio-Rad) (0.375 cm^2 x 20 cm) by a modification of the method described by Kaufmann (1974). Prior to the application of the sample to the methotrexate agarose column, the column was pretreated with the following solutions, 5 ml 1 M K_2HPO_4 containing 0.5g of folic acid, 100 ml 1M K_2HPO_4, and 200 ml 0.1M sodium phosphate buffer pH 5.6 containing 1M NaCl.

The column was then washed with 10 mM sodium phosphate buffer pH 5.6 until the absorbance of the eluate at 280 nm was essentially 0. The column was then

equilibrated with buffer A and the dihydrofolate reductase sample loaded. The column was then washed with buffer A, the protein which did not bind was assayed for dihydrofolate reductase activity to confirm that the enzyme was bound to the column. Washing the column with buffer A was continued until the absorbance at 280 nm was less than 0.01 OD units.

The dihydrofolate reductase activity was eluted by the addition of the following solutions, 2 ml 1M K_2HPO_4, 2 ml 0.5M K_2HPO_4 containing 4 μmoles of dihydrofolate and finally 75 ml of 0.1M K_2HPO_4 containing 1 μM dihydrofolate. Five ml fractions were collected and assayed for dihydrofolate reductase activity. The column was washed in buffer A and stored in the same buffer at +4°C in darkness until further use.

2.4.14 Sequence Analysis

Sequence analysis was performed on an Applied Biosystems 477A instrument, before each analysis the purity of the sample was checked by reverse phase HPLC with an Applied Biosystems 130A microbore separation system. The procedure is as described by Hayes *et al.* (1989).

2.4.15 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out with a Pharmacia Phastsystem (18-1601-01) according to manufacturer's instructions. For SDS polyacrylamide gel electrophoresis PhastGels Gradient 10 - 15% (17-0540-01) and PhastGel SDS Buffer Strips (17-0516-01) were used. Samples to be electrophoresised were di-

luted in loading buffer to give a final concentration of approximately 0.5 $\mu g/\mu l$ and β-mercaptoethanol and SDS added to 5.0% and 2.5% respectively. This was heated at 100°C for 5 minutes and bromophenol blue added to 0.01%.

Samples were loaded onto the gel and electrophoresised at 250V, 10mA and 3.0W for approximately 60VH or until the bromophenol blue dye front reached the anode strip. The gel was then stained with coomassie blue in a Pharmacia Phastsystem development unit (18-1601-01) as described by the manufacturers.

For native electrophoresis PhastGel Homogenous 20 (17-0624-01) and PhastGel Native Buffer strips (17-0517-01) were used. Sample preparation was as before, except no SDS was added and samples were not boiled. The gels were run according to manufacturer's instructions until the tracking dye reached the anodic buffer strip. Native gels were stained either by coomassie blue as above or by zymography as described before.

2.4.16 Fast Protein Liquid Chromatography (FPLC)

FPLC was carried out with a Pharmacia FPLC system consisting of an LCC500 plus controller, UV-M Monitor, FRAC 100 with an HR5/5 Mono Q column. The column was equilibrated with 50 mM Tris-HCl buffer (pH 8.0). Two ml of concentrated dihydrofolate reductase sample (from the pooled peak fractions obtained by gel filtration with a Sephadex G75 column) was added to the column. Separation was achieved by elution with a linearly increasing concentration of sodium chloride (in the above buffer) to a maximum concentration of 1M. Approximately, 34 fractions (1 ml) were collected and each of these assayed for

dihydrofolate reductase activity.

Chapter 3

Results

A. Type IV Dihydrofolate Reductase

3.1 Studies on the Induction Mechanism

The type IV dihydrofolate reductase differs from the other plasmid-mediated enzymes in a number of ways, and perhaps the most important is that it is inducible (Young and Amyes, 1986a). This is very unusual for a plasmid-encoded resistance enzyme in Gram-negative bacteria, however it has been reported for *E. coli* chromosomal dihydrofolate reductase (Tennhammar-Ekman *et al.*, 1986). The only features of the induction mechanism of the type IV enzyme that had been found were that induction could be achieved with increasing trimethoprim concentrations up to 160 mg/L (Young and Amyes, 1986a), and that the increase in specific activity of the dihydrofolate reductase resulted from induction and did not result from the selection of high production mutants (H-K. Young, unpublished results).

The induction mechanism was now studied in more detail to establish how it operated. Induction was studied in *E. coli* J62$_{-2}$ (pUK1123) an *E. coli* transcon-

jugant containing the original clinical plasmid isolated in India in 1984 (Young *et al.*, 1986b). *E. coli* J62$_{-2}$ lacking the plasmid shows no induction of dihydrofolate reductase and dies rapidly when challenged with trimethoprim 10 mg/L in complex media (H.-K. Young, unpublished results). Firstly, the induction of the enzyme under various growth conditions was examined.

3.1.1 Minimum Time for Induction

The induction of the type IV dihydrofolate reductase was investigated by challenging *E. coli* J62$_{-2}$ (pUK1123) with different trimethoprim concentrations for 20 hours and then measuring the enzymes specific activity (Young and Amyes, 1986a). The speed at which the challenged bacteria responded on exposure to trimethoprim should reveal the nature of the induction process. Therefore *E. coli* J62$_{-2}$ (pUK1123) was inoculated directly from a DSTA plate into five flasks containing 250 ml of Oxoid Isosensitest broth containing trimethoprim (40 mg/L). This trimethoprim concentration was chosen for all induction studies as it promoted high levels of enzyme activity without diminishing the recovery of bacteria. The flasks were incubated shaking at 37°C. The contents of each flask were allowed to grow for a set time before the bacteria were harvested and the specific activity in the cleared lysates determined, the results are shown in table 6. Clearly induction was not "switched on" immediately the cells were challenged with trimethoprim but occurred at some time between 5 and 14 hours.

Table 6. Minimum challenge time required for induction of the type IV dihydrofolate reductase in *E. coli* $J62_{-2}$ (pUK1123)

Length of challenge time with Tp 40 mg/L (hours)	Enzyme specific activity nmol DHF reduced/min/mg protein
unchallenged control	5.7
2	2.6
3	7.6
5	3.9
14	24.6
18	23.9

3.1.2 Viable Count and Gram-Stains over 24 hours

There was a considerable time lag between challenge of the bacteria with trimethoprim and the production of elevated levels of dihydrofolate reductase and it was not known what changes were taking place in the bacteria during this time. Presumably if elevated levels of dihydrofolate reductase were not synthesised, to enable the bacteria to overcome trimethoprim challenge, then no growth could occur.

To test this theory the viable count of *E. coli* $J62_{-2}$ (pUK1123) was followed over 24 hours following challenge with 40 mg/L trimethoprim. *E. coli* $J62_{-2}$ (pUK1123) was inoculated straight from a plate into two separate flasks containing 250 ml Isosensitest broth. One flask was challenged immediately with trimethoprim (40 mg/L), the second had no trimethoprim added, the flasks were incubated at 37°C and shaken vigorously for 24 hours. At time 0, and thereafter every 4 hours, a viable count and Gram-stain was performed on the bacteria in

each flask. The results are shown in figures 3 and 4.

Unchallenged *E. coli* J62$_{-2}$ (pUK1123) followed a classic growth curve over the 24 hour period. When bacteria were challenged with trimethoprim, however, a considerable initial drop in viable count was observed. At 8 hours this was reversed and the cells began to grow. By the end of the 24 hour period there was little difference in the viable count of the challenged and unchallenged cultures. Gram-staining carried out in conjunction with the viable counts showed that as the viable count of the challenged cells dropped, cell elongation occurred. After 8 hours, as the viable count increased, the cells shortened and at 24 hours, they were indistinguishable from the unchallenged culture. These results supported the findings of the previous experiment and they suggested that induction probably took place around eight hours after trimethoprim challenge.

3.1.3 Growth before Challenge

The viable count of *E. coli* J62$_{-2}$ (pUK1123) fell after it was challenged with trimethoprim, until induction occurred and reversed the process. It was difficult to establish whether the requirement for induction was that the cells were in a certain growth phase. In the previous experiment challenged bacteria were in lag phase and not actively growing when challenged. To observe the influence of stage in the growth curve, *E. coli* J62$_{-2}$ (pUK1123) was inoculated from a DSTA plate into 250 ml of Isosensitest broth and allowed to grow for various times until challenged with trimethoprim (40 mg/L). After challenge, bacteria were allowed to grow for 20 hours, then harvested and the specific activity in the cleared lysates determined (table 7). After two hours pre-challenged growth, induction no

Figure 3. Viable count of *E. coli* J62$_{-2}$ (pUK1123) over 24 hours in the presence and absence of trimethoprim 40 mg/L.

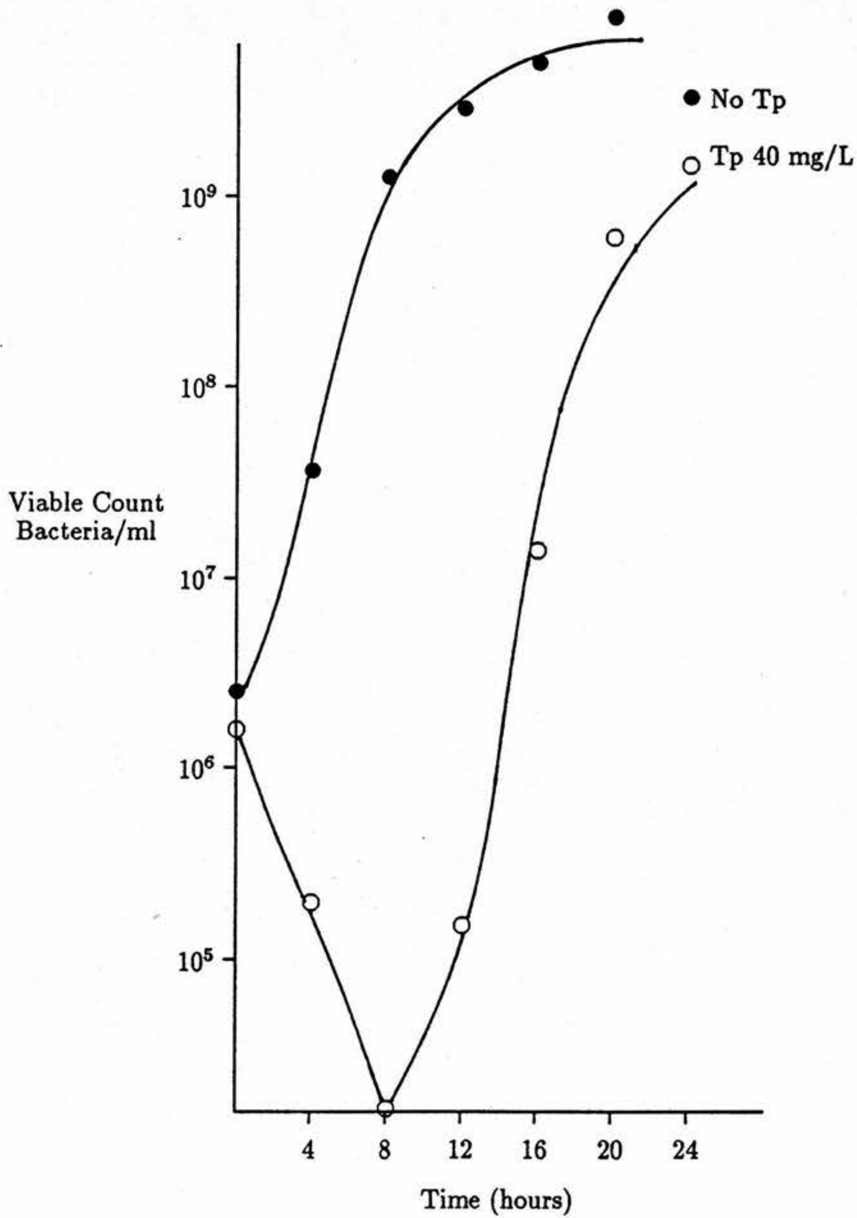


Figure 4. Gram-stains of *E. coli* J62$_{-2}$ (pUK1123) grown over 24 hours in the presence and absence of trimethoprim 40 mg/L

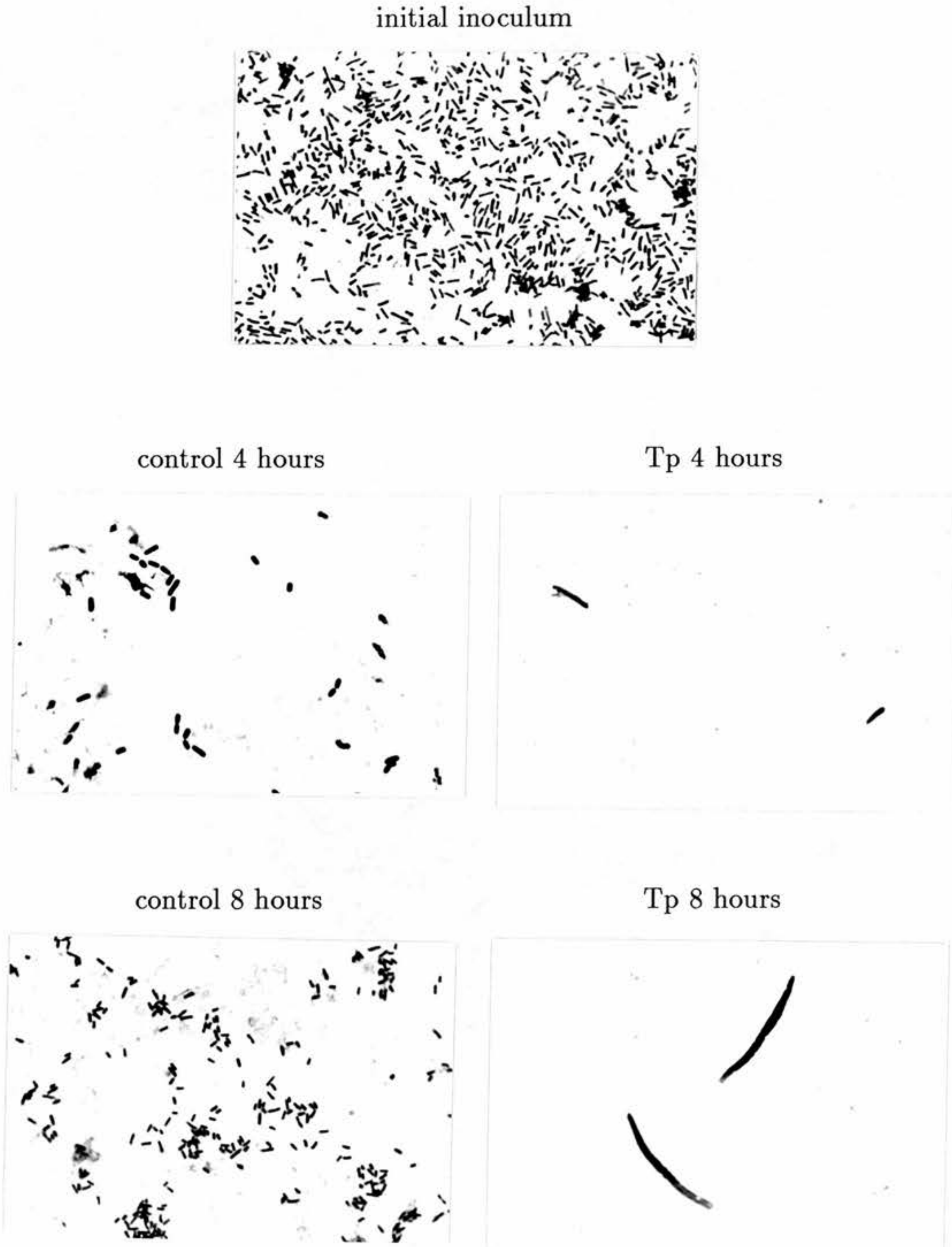


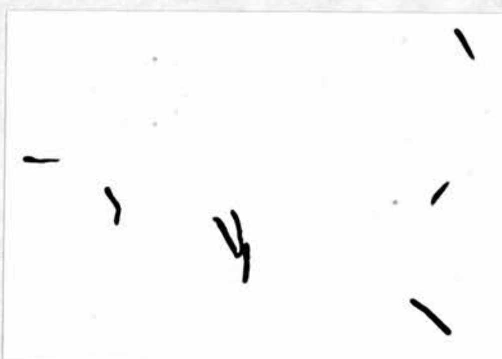
Figure 4 (continued).

control 12 hours

Tp 12 hours

control 16 hours

Tp 16 hours



control 20 hours

Tp 20 hours

Figure 4 (continued).

control 24 hours

Tp 24 hours

longer occurred on addition of trimethoprim even though the bacteria continued to grow. The experiment was repeated employing shorter time intervals in the pre-challenge growth stage (table 8). Clearly a factor was present which affected induction and it appeared at around two hours growth following inoculation. This was probably linked to growth curve.

In order to establish exactly where in the growth curve induction took place *E. coli* J62$_{-2}$ (pUK1123) was inoculated into a series of flasks each containing 250 ml of Isosensitest broth, one flask was challenged immediately with 40 mg/L trimethoprim and the other four at 60, 135, 180 and 240 minutes respectively. A final flask was unchallenged as a control but its growth was followed by viable counts. All flasks were inoculated at the same time and incubated shaking at 37°C, the levels of enzyme production and how this relates to viable count are shown in figure 5.

The results suggest that the bacteria induced dihydrofolate reductase production if challenged in lag phase. However, once the bacteria were in logarithmic phase they were able to grow without inducing dihydrofolate reductase production. This may be explained if bacteria in logarithmic phase are producing something which antagonises trimethoprim. Even more simply there may have been more cells present in logarithmic phase so the amount of trimethoprim capable of acting on any individual cell was less.

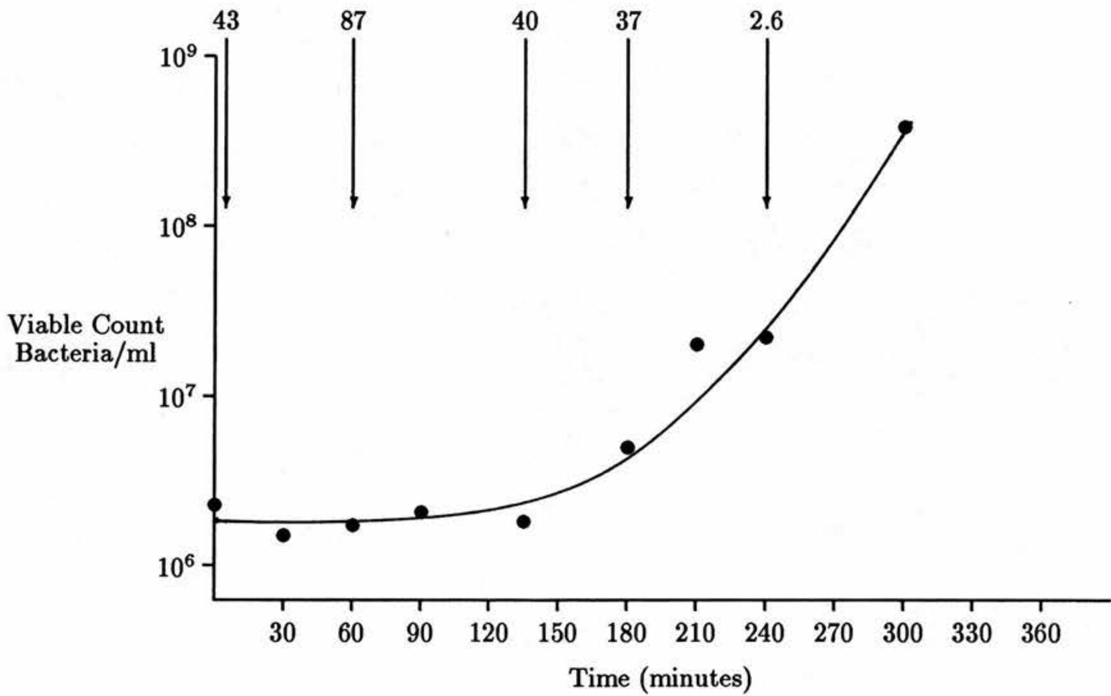
Table 7. The effect of allowing growth before challenge, with Tp (40 mg/L), on induction of the type IV dihydrofolate reductase.

Time between inoculation and challenge with Tp 40 mg/l (hours)	Enzyme specific activity nmol DHF reduced/min/mg protein
0	24.4
2	43.4
3	3.9
4	3.5
5	4.5

Table 8. The effect of allowing growth before challenge, with Tp (40 mg/L), on induction of the type IV dihydrofolate reductase.

Time between inoculation and challenge with Tp 40 mg/l (min)	Enzyme specific activity nmol DHF reduced/min/mg protein
0	31.1
30	68.5
60	44.4
90	52.6
120	3.7

Figure 5. Relationship between growth curve and enzyme induction in *E. coli* J62$_{-2}$ (pUK1123).
Enzyme specific activity (nmol DHF reduced/min/mg protein) when challenged at various points is also shown.



3.1.4 Effect of Method of Inoculation on Induction

The previous results suggested that bacteria, once growing, produced a factor which antagonised the action of trimethoprim. It had been observed that induction was more readily obtained if the challenge flask was inoculated with a loopful of bacteria directly from an agar plate rather than growing up a loopful in a nutrient broth starter culture and employing 1 ml of this as an inoculum. To examine this the two methods of inoculation were compared over a range of trimethoprim concentrations.

For direct inoculation, a loopful of *E. coli* $J62_{-2}$ (pUK1123) was taken from a DSTA plate and inoculated directly into 250 ml of Isosensitest broth containing either 0, 20, 40, 80 or 160 mg/L trimethoprim. The cultures were incubated for 20 hours at 37°C shaking vigorously. Inoculation by nutrient broth starter culture was performed by growing up a loopful of *E. coli* $J62_{-2}$ (pUK1123) in 4.5 ml nutrient broth at 37°C until turbid. One ml of this was used as an inoculum for a series of 250 ml Isosensitest broth cultures identical to those listed above and the flasks were incubated as before. After 20 hours, cells were harvested by centrifugation and disrupted by sonication, the specific activity in the cleared lysates was determined (table 9).

There was a distinct difference in the level of induction between the two groups. Higher enzyme levels were obtained with the inoculum from an agar plate and, furthermore, hyperproduction (specific activity > 20) of dihydrofolate reductase was achieved at a lower trimethoprim concentration, 20 rather than 40 mg/L. To verify this result, a further experiment was performed whereby a single colony

Table 9. The effect of inoculation method on induction of the type IV dihydrofolate reductase in *E. coli* J62$_{-2}$ (pUK1123).

Tp Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein	
	Direct inoculum	Starter culture
0	8.7	13.6
10	4.4	6.3
20	33.8	4.1
40	61.5	26.4
80	34.7	30.3
160	412.3	40.3

Table 10. The effect of inoculation method on enzyme specific activity at a Tp concentration of 20 mg/L.

	Tp Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
Plate inoculum	20	33.6
Starter culture	20	1.7

of *E. coli* J62$_{-2}$ (pUK1123) was selected and split in two. One half was used to inoculate 250 ml of Isosensitest broth containing 20 mg/L trimethoprim and the other half was grown up in 4.5 ml of nutrient broth until turbid and 1 ml of this used to inoculate an identical flask. Both cultures were incubated shaking at 37°C for 20 hours, the dihydrofolate reductase specific activity in each culture was measured. The results (table 10) confirmed that the different inoculation procedures had a pronounced effect on enzyme induction.

The explanation for a lower level of induction when cells were grown in nutrient broth may have been the influence of thymidine. The major difference between nutrient and Isosensitest broth is that the latter lacks thymidine, a compound known to antagonise trimethoprim (Amyes and Smith, 1974a). The carry over of thymidine in the nutrient broth may have been affecting induction levels. Additionally the results of the experiment, measuring the influence of growth before challenge, suggested that once in log phase the bacteria were producing a factor which antagonised trimethoprim, this may have been thymidine. To investigate this hypothesis, a series of induction experiments were carried out in Isosensitest broth in the presence of thymidine.

3.1.5 Effect of Thymidine on Type IV Induction

In the first experiment *E. coli* J62$_{-2}$ (pUK1123) was challenged with trimethoprim (40 mg/L) in the presence of different thymidine concentrations. Bacteria were inoculated directly from a DSTA plate into 250 ml of Isosensitest broth containing trimethoprim (40 mg/L) and thymidine at concentrations of 0, 2, 10, 20 or 50 mg/L. The enzyme specific activities after incubation at 37°C for 20

hours (table 11) demonstrated that the effect of thymidine was marked. A concentration of 2 mg/L was sufficient to allow the bacteria to grow without inducing dihydrofolate reductase production. The experiment was repeated at the lower thymidine concentrations of 2, 0.2 and 0.02 mg/L. The results (table 12) showed that a tenfold decrease in thymidine concentration from 2 mg/L was sufficient to force induction to occur, however, it was not clear if the reverse was true, i.e. at a constant thymidine concentration of 2 mg/L would a tenfold increase in trimethoprim concentration necessitate enzyme induction. To investigate this *E. coli* $J62_{-2}$ (pUK1123) was challenged as before, however in this case, the trimethoprim concentration was raised to 40, 160, 240, 400 mg/L respectively while the thymidine concentration was constant at 2 mg/L. The specific activity of dihydrofolate reductase (table 13) did not rise as the trimethoprim concentration was increased indicating that induction had not occurred. Indeed the specific activity appeared to fall as the trimethoprim concentration increased, at 400 mg/L no enzyme activity was detected although normal growth had occurred.

Because of the importance of thymidine in affecting the induction of the type IV dihydrofolate reductase it was decided to investigate the effect that thymidine has on cell elongation and viable count over 24 hours. The experiment was similar to an earlier experiment in this chapter following the viable count over a 24 hour period. Three flasks containing 250 ml of Isosensitest broth were prepared; the first had no additions, the second contained trimethoprim (40 mg/L) and the third trimethoprim (40 mg/L) plus thymidine (2 mg/L). Each was inoculated with a loopful of *E. coli* $J62_{-2}$ (pUK1123) from a DSTA plate and then incubated at 37°C and shaken vigorously. Viable counts and Gram-stains were performed

Table 11. The effect of thymidine on induction of the type IV dihydrofolate reductase at a Tp concentration of 40 mg/L.

Concentration of thymidine mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
0	33.9
2	2.6
10	2.1
20	1.3
50	1.3

Table 12. The effect of thymidine on induction of the type IV dihydrofolate reductase at a Tp concentration of 40 mg/L.

Concentration of thymidine mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
0	27.4
2	2.3
0.2	17.1
0.02	33.2

Table 13. The effect of thymidine 2 mg/L on induction of the type IV dihydrofolate reductase in the presence of increasing Tp concentrations.

Concentration of Tp mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
40	1.8
160	0.7
240	0.4
400	0.0

on the three cultures at points throughout the 24 hour period.

The results (figure 6) showed that, as before, unchallenged *E. coli* $J62_{-2}$ (pUK1123) followed a classic growth curve. On the other hand the bacteria challenged with trimethoprim (40 mg/L) alone showed a drop in viable count to 8 hours but by 24 hours they had recovered. The bacteria challenged with trimethoprim in the presence of thymidine (2 mg/L) showed no decrease in viable count and grew as the unchallenged culture had done. Similarly when the Gram-stains were examined (figure 7), bacteria challenged with trimethoprim in the presence of thymidine behaved like unchallenged cells and did not elongate. This experiment demonstrated that induction of the type IV enzyme only occurred when the bacteria were under stress. The presence of a given concentration of trimethoprim is not enough.

Figure 6. Viable count of *E. coli* J62$_{-2}$ (pUK1123) over 24 hours, challenged with Tp (40 mg/L) in the presence and absence of thymidine (2 mg/L).

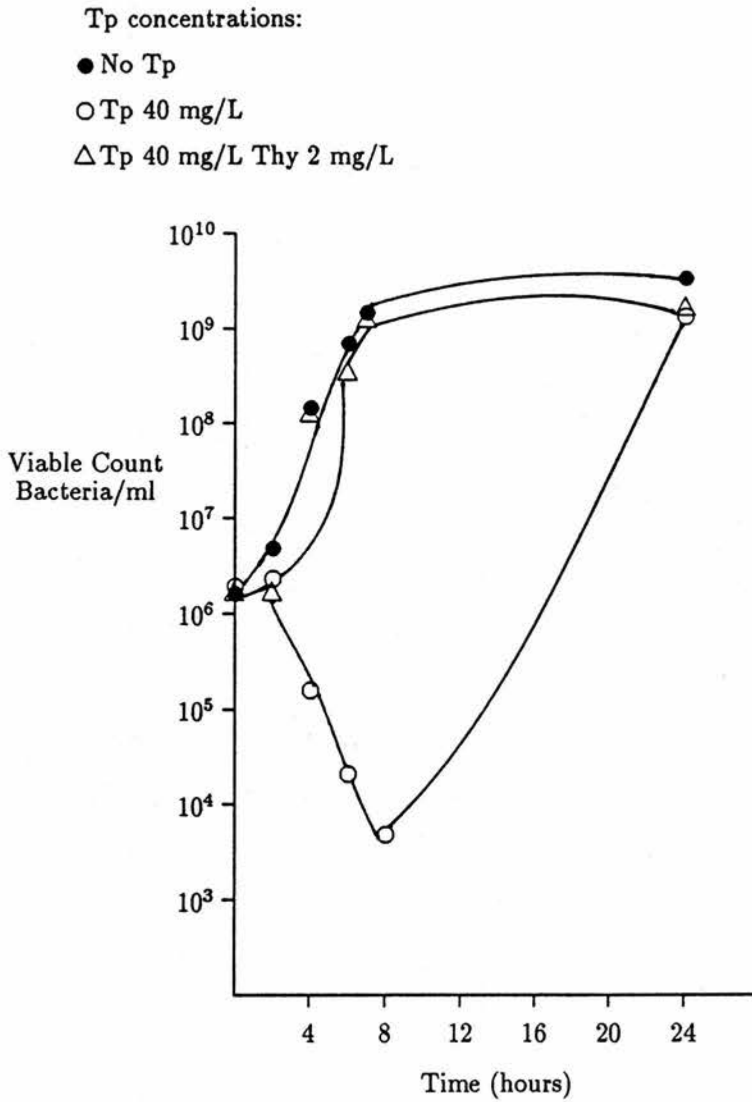


Figure 7. Gram-stains of *E. coli* J62$_{-2}$ (pUK1123) grown over 24 hours with Tp 40 mg/L in the presence and absence of thymidine 2 mg/L.

initial inoculum

control 8 hours

Tp 8 hours

Tp and thymidine 8 hours

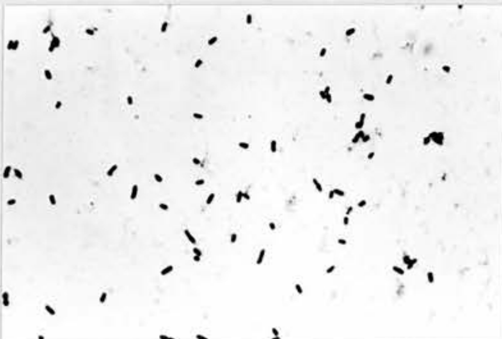
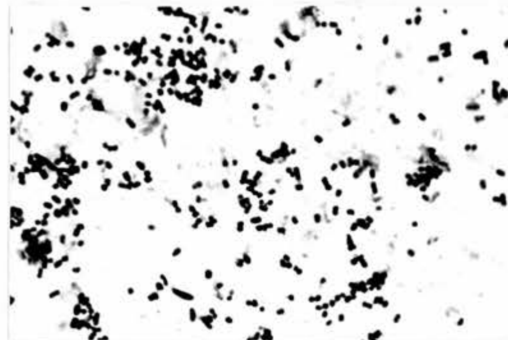


Figure 7 (continued).

control 24 hours

Tp 24 hours



Tp and thymidine 24 hours

3.1.6 Effect of 5-Fluoro-2′-deoxyuridine (FUdR)

Most of the tetrahydrofolic acid formed in *E. coli* by the action of dihydrofolate reductase is used for the synthesis of the thymine moiety of thymidylic acid. As thymidine has such a marked effect on induction, it suggested that the hyper-production of the type IV dihydrofolate reductase may be triggered by thymine starvation. To examine this the effect of FUdR on dihydrofolate reductase levels was examined, FUdR inhibits the enzyme thymidylate synthetase and thus causes thymine starvation.

E. coli J62$_{-2}$ (pUK1123) was grown in Isosensitest broth in the presence of a range of concentrations of FUdR and 2′-deoxyadenosine (20 mg/L) (to prevent the breakdown of the FUdR). Bacteria were inoculated directly from a DSTA plate into 250 ml of Isosensitest broth and grown shaking at 37°C for 20 hours. The FUdR concentrations used are listed in table 14, after 20 hours incubation the specific activities in the cleared lysates were determined. Unexpectedly FUdR did not cause an increase in enzyme production, growth was affected and no pellet was recoverable from the bacteria incubated in the presence of 10 mg/L FUdR. The culture containing 1 mg/L FUdR began to grow shortly before the cells were harvested and in order to check that induction would not have occurred if left longer the experiment was repeated at this concentration but bacteria were not harvested until 30 hours. Results (table 15) showed that again no induction occurred, which suggested that the induction process was triggered by more than simple thymine starvation.

Table 14. The effect of FUdR on levels of dihydrofolate reductase in *E. coli* $J62_{-2}$ (pUK1123).

FUdR Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
0	10.5
0.001	8.9
0.01	10.1
0.1	12.1
1.0	10.6
10	No pellet recoverable

Table 15. The effect of 1.0 mg/L FUdR on levels of dihydrofolate reductase in *E. coli* $J62_{-2}$ (pUK1123) after 30 hours growth.

FUdR Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
0	13.7
1.0	10.1

3.1.7 Effect of Metabolites

The previous experiment suggested that induction of the type IV enzyme was not simply a result of thymine starvation and other factors must be involved. Earlier studies had shown that the MIC of trimethoprim for *E. coli* $J62_{-2}$ (pUK1123) was dependent on the presence of glycine, methionine and adenine in the media (Young *et al.*, 1986b). These three compounds are closely involved in the thymidine pathway (Amyes and Smith, 1974c) and it therefore seemed likely that they would affect induction of the type IV enzyme.

To examine the effect of these three compounds on enzyme production *E. coli* $J62_{-2}$ (pUK1123) was challenged in Davis-Mingioli minimal media containing the auxotrophic requirements for this strain (proline, histidine, tryptophan and glucose). In addition different combinations of methionine, adenine and glycine were added. To compensate for the slower growth rates and the lower bacterial yield in minimal medium, compared with complex media, one litre cultures were used, rather than the 250 ml which had been used previously. As larger volumes were being used a 0.5 ml nutrient broth starter culture was used to initiate growth. Although starter cultures had been shown to give a lower induction in some of the previous experiments, that had been in a total volume of 250 ml. It was felt that the effect of the volume of this starter in one litre would be negligible. The bacteria were grown for 24 hours in the presence of trimethoprim (40 mg/L) and the specific activities of the cleared lysates determined.

The results (table 16) indicated that induction only took place if methionine, adenine and glycine were all present. Interestingly, when any of these were absent,

Table 16. The effect of methionine (met), adenine (ad) and glycine (gly) on the induction of the type IV dihydrofolate reductase in *E. coli* J62$_{-2}$ (pUK1123) when grown in minimal medium.

Supplements* all at a concentration of 50 mg/L			Tp Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein	Growth
	0		0	2.5	+++
	0		40	5.9	+++
met	ad	—	0	2.4	+++
met	ad	—	40	2.2	+++
met	—	gly	0	2.5	+++
met	—	gly	40	0.8	+++
—	ad	gly	0	3.7	+++
—	ad	gly	40	2.3	+++
met	ad	gly	0	3.9	+++
met	ad	gly	40	28.8	+/-

* All media also contained proline, histidine and tryptophan at 50 mg/L and 2.8 mg/ml glucose

normal growth occurred in the presence of trimethoprim. When all three were present little growth had occurred after 24 hours although the bacteria were producing high levels of dihydrofolate reductase. When these bacteria were examined under the microscope they were found to be greatly elongated and when reincubated, for a further 12 hours, growth occurred and the bacteria had returned to their normal size. It appeared, therefore, that what was happening, in the presence of all three supplements, was similar to the situation in complex media but over a longer time period.

The effect of thymidine was examined when all three supplements were present and, as would be expected, it allowed growth over 24 hours in the absence of

elevated levels of dihydrofolate reductase.

The larger volumes required and lower bacterial yield meant that it was impractical to study induction in minimal medium. However the experiment on growth before challenge had suggested that an inoculum effect may be affecting induction levels. It was decided therefore to examine the effect of the inoculum size in Davis-Mingioli medium plus supplements, as this is a more defined system to differentiate between the thymidine effect and any inoculum effect. In the following experiments enzyme specific activities were not determined, cell growth and structure were taken as indications of whether induction had occurred.

A series of *E. coli* J62$_{-2}$ (pUK1123) cultures were grown in 250 ml of Davis-Mingioli medium containing the basic auxotrophic requirements plus methionine, adenine, glycine and trimethoprim (40 mg/L). A control flask which contained no trimethoprim was also included. The bacteria for inoculation into the flasks were grown up in a nutrient broth starter culture until turbid. The inoculum was varied in each flask as follows: In the first set the starter culture was used directly as the inoculum with sizes of 0.5 ml, 1.0 ml and 4.5 ml used for the flasks containing trimethoprim and 0.5 ml for the control. With the second set the nutrient broth starter culture was spun down and the bacteria washed and resuspended in Davis-Mingioli minimal medium. This was used as an inoculum as above. Cultures were incubated shaking at 37°C for 24 hours. To determine if induction was necessary for growth, cell growth and appearance were monitored.

The results (table 17) obtained from the nutrient broth and Davis-Mingioli inocula were identical indicating that there was a definite inoculum effect. With

Table 17. The effect of inoculum size on induction of the type IV dihydrofolate reductase in *E. coli* J62$_{-2}$ (pUK1123) when grown in minimal media.

	Inoculum size (ml)	Tp concentration mg/L	Growth	Viable Count bacteria/ml	Cell appearance
	0.5 (control)	0	+++	2.7×10^9	normal
Nutrient broth	0.5	40	+/-	1.1×10^7	elongated
inoculum	1.0	40	+/-	2.1×10^6	elongated
	4.5	40	+++	5.6×10^8	normal
	0.5 (control)	0	+++	2.5×10^9	normal
Resuspended	0.5	40	+/-	2.7×10^6	elongated
in DM	1.0	40	+/-	4.8×10^7	elongated
	4.5	40	+++	$1.3 \times 10^{9*}$	normal
*Inoculated from	0.5 (control)	0	+++	3.2×10^9	normal
bacteria grown	0.5	40	+/-	8.0×10^5	elongated
in previous	1.0	40	+/-	4.5×10^6	elongated
experiment	4.5	40	+++	7.0×10^7	normal

inoculum sizes of 0.5 ml and 1.0 ml, the presence of trimethoprim (40 mg/L) allowed little growth and the cells were elongated, a response associated with induction. With an inoculum size of 4.5 ml, however, the cells were able to grow normally similar to the unchallenged control. To establish that no genetic change had occurred in the cultures started with the large inoculum, the experiment was repeated with bacteria that had grown from a 4.5 ml starter culture as the inoculum. The same pattern was obtained as before indicating no mutation had occurred (table 17).

Clearly metabolites and thymidine are important in expression of the type IV dihydrofolate reductase but there is also a marked inoculum effect.

3.1.8 Other Antifolate Agents

Trimethoprim is only one of a series of antifolate agents, and their ability to induce the type IV dihydrofolate reductase was examined. Induction experiments were set up with a range of antifolates; tetroxoprim, aminopterin, pyrimethamine, methotrexate and sulphamethoxazole.

The experiments were carried out exactly as with trimethoprim, *E. coli* $J62_{-2}$ (pUK1123) was inoculated directly from a DSTA plate into 250 ml of Isosensitest broth containing different concentrations of the appropriate drug. Cultures were incubated shaking at 37°C and the specific activity measured in the cleared lysates.

The results (table 18) show that there was no induction with any of the antifolates over the range 0 - 160 mg/L, which had produced induction with trimethoprim. It was possible that a higher concentration of these antifolates was needed to cause induction. To study this the MIC of these antifolates for *E. coli* $J62_{-2}$ (pUK1123) was tested and compared to the MIC of trimethoprim (table 19). All the antifolates had higher MICs than trimethoprim suggesting that a higher concentration would be required to induce enzyme production. Only two of the other antifolates tested had MICs of less than 1280 mg/L: tetroxoprim and pyrimethamine, so induction at higher concentrations was confined to these two compounds.

The induction was set up as before and the enzyme levels achieved are shown in table 20. At these concentrations the antifolates were clearly affecting the growth of the bacteria and few cells were recovered from concentrations of 1280 mg/L

Table 18. Induction of the type IV dihydrofolate reductase in *E. coli* $J62_{-2}$ (pUK1123) with antifolates other than trimethoprim.

Antifolate	Drug Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
Tetroxoprim	0	8.1
	20	8.2
	40	5.7
	80	6.9
	160	2.6
Pyrimethamine	0	6.3
	20	1.8
	40	1.6
	80	1.2
	160	1.2
Sulphamethoxazole	0	3.2
	20	2.2
	40	2.9
	80	1.0
	160	4.1
Methotrexate	0	5.6
	20	1.1
	40	0.5
	80	0.3
	160	0.0
Aminopterin	0	6.5
	20	1.8
	40	1.6
	80	1.2
	160	1.2

Table 19. The minimum inhibitory concentrations of various antifolates for *E. coli* $J62_{-2}$ (pUK1123).

Antifolate drug	MIC mg/L	
	$J62_{-2}$ control	$J62_{-2}$ (pUK1123)
Trimethoprim	< 10	< 10
Tetroxoprim	< 10	320
Pyrimethamine	80	320
Sulphamethoxazole	< 10	> 1280
Aminopterin	1280	> 1280
Methotrexate	> 1280	> 1280

from both tetroxoprim and pyrimethamine. Interestingly, induction only occurred with tetroxoprim and not with pyrimethamine.

3.1.9 Induction in Other Strains

Induction had been studied in *E. coli* $J62_{-2}$ but it was not known if this effect was universal as it had not been observed in other strains. Therefore, in order to establish what factors might influence induction, two further bacterial systems were examined. Firstly, as methionine appeared important in determining whether induction occurred, induction was examined in *E. coli* J53, a methionine requiring mutant. Secondly the effect on induction of cloning the type IV gene into another plasmid was investigated.

Table 20. Induction of the type IV dihydrofolate reductase in *E. coli* J62$_{-2}$ (pUK1123) with high level tetroxoprim and pyrimethamine.

Antifolate drug	Concentrations of drug mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
Textroxoprim	0	4.6
	160	3.8
	320	18.5
	640	40.0
	1280	no pellet recoverable
Pyrimethamine	0	5.5
	160	1.6
	320	3.4
	640	0.0
	1280	0.5

Induction in <u>E. coli</u> J53

Plasmid pUK1123 was transferred into *E. coli* J53 from *E. coli* $J62_{-2}$ (pUK1123) by conjugation. Induction in the transconjugant *E. coli* J53 (pUK1123) was studied by a procedure identical to that previously established for *E. coli* $J62_{-2}$ (pUK1123). A colony of *E. coli* J53 (pUK1123), from a DSTA plate, was inoculated into a series of flasks, each containing 250 ml of Isosensitest broth and a different trimethoprim concentration (0 - 160 mg/L). After incubation at 37°C for 20 hours the specific activities in the cleared lysates were determined.

The results (table 21) were different from those obtained for *E. coli* $J62_{-2}$, containing the same plasmid. In *E. coli* J53 production of the type IV enzyme appeared to be derepressed, high levels of activity occurring in the absence of trimethoprim challenge. At a trimethoprim concentration of 160 mg/L the enzyme specific activity was not significantly greater than the basal level of production in the absence of challenge. Furthermore the bacteria were able to grow normally at this concentration unlike *E. coli* $J62_{-2}$ where viability is reduced at a trimethoprim concentration of 160 mg/L.

To investigate if this phenomenon was restricted to *E. coli* J53 or occurred in other methionine deficient strains, plasmid pUK1123 was transferred into two further methionine deficient strains: *E. coli* K12 met^- and *E. coli* C met^-. Before induction studies were performed on these strains the position of the methionine mutation was established in case this had an effect on induction. The methionine mutation was mapped by examining if the strains could utilise homocysteine (figure 8). This revealed that as *E. coli* J53 and K12 could not utilise homocys-

Table 21. Induction of the type IV dihydrofolate reductase encoded by plasmid pUK1123 in *E. coli* J53.

Tp Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
0	32.6
10	8.2
20	6.2
40	10.1
80	42.6
160	35.0

teine, the mutation must be at position A whilst *E. coli* C, which could grow on minimal media containing homocysteine, must have a mutation at point B (figure 8). The MICs of trimethoprim for the three strains were examined on DSTA plates and on minimal media containing glucose proline and methionine (table 22). Interestingly, on complex media, *E. coli* J53 and *E. coli* K12, which had the mutation at point A, had the same MIC while *E. coli* C, which has a mutation at point B, had a higher MIC. On minimal media all had the same MIC 160 mg/L.

Induction studies were performed on *E. coli* C met$^-$ and *E. coli* K12 met$^-$ with trimethoprim over the range 0 - 160 mg/L. The procedure was identical to that used before and the specific activities in the cleared lysates are shown in tables 23 and 24. The results clearly demonstrated that induction did not take place and, like *E. coli* J53, normal growth occurred at a trimethoprim concentration of 160 mg/L. It seems clear from these experiments and the earlier studies that

Figure 8. The interconversion of tetrahydrofolate intermediates and their relationship with purine and protein synthesis (taken from Amyes and Smith, 1974c).

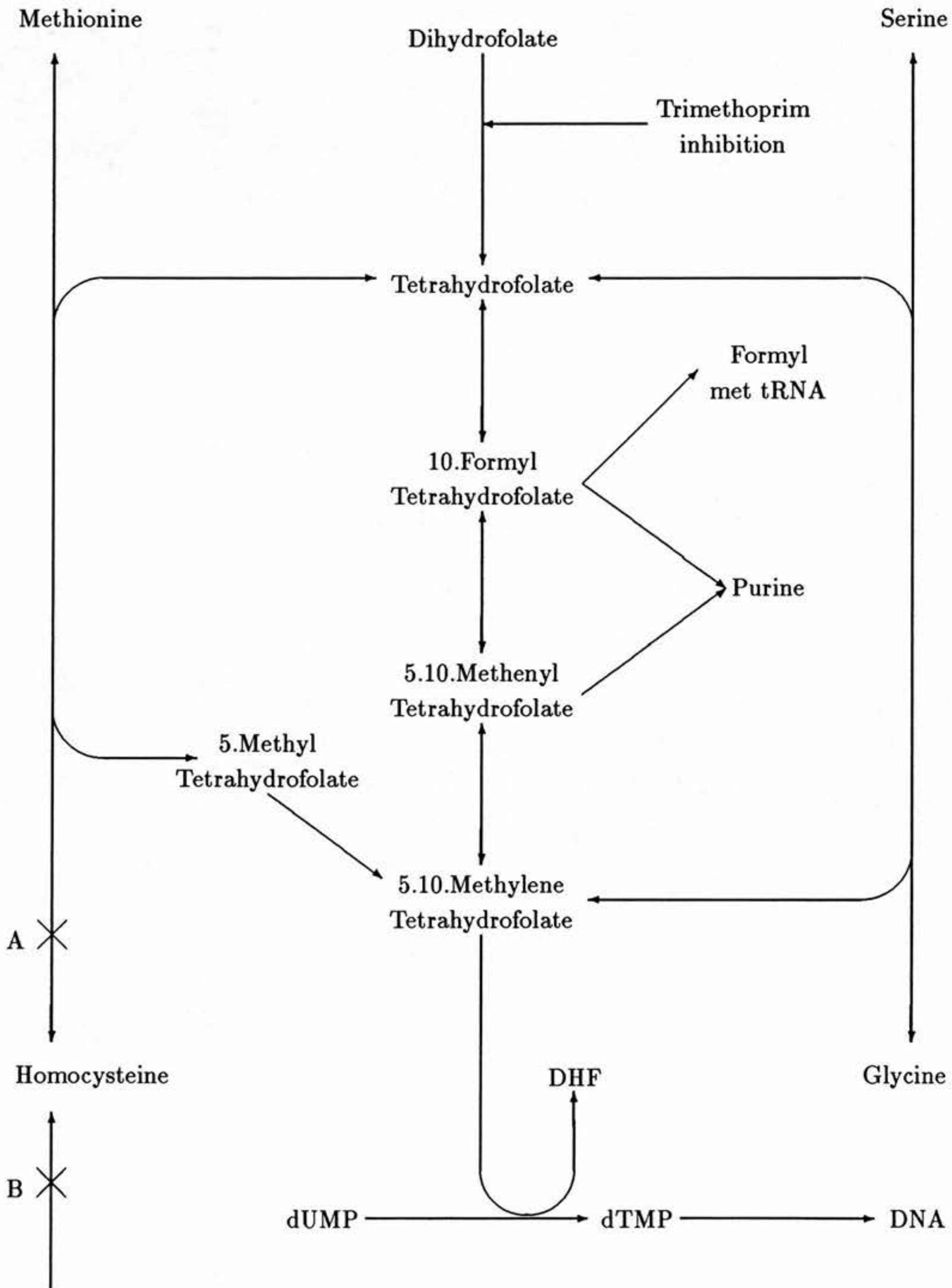


Table 22. MIC's of trimethoprim for *E. coli* methionine deficient strains.

Strain	MIC Tp (mg/L)	
	Minimal Media	Complex Media
E. coli J53 (pUK1123)	160	20
E. coli K12 met$^-$ (pUK1123)	160	20
E. coli C met$^-$ (pUK1123)	160	80
E. coli J62$_{-2}$ (pUK1123)*	160	10
E. coli J62$_{-2}^{*}$	< 5	< 5

* minimal media contained proline, histidine and tryptophan (50 mg/L) and glucose (2.8 mg/ml).

methionine is an important metabolite affecting the induction of the type IV enzyme.

Induction in Clones

The effect on induction of cloning the type IV gene into other plasmids was investigated. The clones used are described in table 25 and figure 9. Induction studies were performed as before except that initially only two trimethoprim concentrations were used: 0 and 40 mg/L. From this study it was apparent that induction was not likely to occur as the unchallenged bacteria were producing high levels of dihydrofolate reductase (table 26). Clone 3, which was producing the least dihydrofolate reductase, was studied further over the full range of trimethoprim concentrations (0 - 160 mg/L) and again no induction took place (table 27).

Table 23. Induction of the type IV dihydrofolate reductase encoded by plasmid pUK1123 in *E. coli* K12 met$^-$.

Tp Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
0	2.2
10	5.0
20	4.4
40	4.4
80	15.7
160	11.2

Table 24. Induction of the type IV dihydrofolate reductase encoded by plasmid pUK1123 in *E. coli* C met$^-$.

Tp Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
0	10.2
10	8.8
20	5.5
40	3.9
80	10.1
160	7.6

Table 25. Type IV dihydrofolate reductase clones.

Clone	
1	*E. coli* C600 (pUK1148) 1.7 kb *Cla*1 fragment of pUK1123 in pBR322
2	*E. coli* C600 (pU1140) 4.1 kb *Eco*R1 fragment of pUK1123 in pBR322
3	*E. coli* C600 (pUK1152) 4.1 kb *Eco*R1 fragment of pUK1123 in pACYC184

Table 26. Induction of the type IV dihydrofolate reductase when cloned.

	Tp Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
Clone 1	0	42.6
	40	33.6
Clone 2	0	40.4
	40	17.6
Clone 3	0	19.9
	40	20.9

Figure 9. Restriction Map of pUK1123.

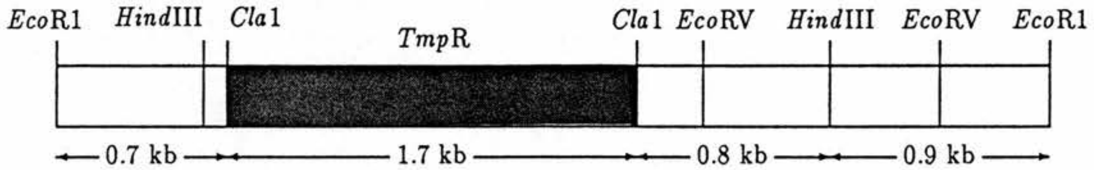


Table 27. Induction of the type IV dihydrofolate reductase encoded by plasmid pUK1152.

Tp Concentration mg/L	Enzyme specific activity nmol DHF reduced/min/mg protein
0	12.0
10	9.7
20	15.2
40	21.4
80	15.9
160	24.0

3.2 N-terminal Sequence and Subunit Structure of the Type IV Dihydrofolate Reductase

Studies on the induction mechanism of the type IV dihydrofolate reductase had suggested that the resistance mechanism was more similar to a chromosomal "swamping" method of resistance, rather than merely the usual bypass mechanism. This fact coupled with the observation that the type IV enzyme is only partially resistant to trimethoprim (ID_{50} 0.2 μM) (Young and Amyes, 1986a) suggest that the type IV could be an intermediate between the chromosomal and highly resistant plasmid enzymes. It was, therefore, important to establish how the type IV enzyme fits into the evolution of plasmid-mediated dihydrofolate reductases.

Sequence analysis represents the ultimate information currently available for the distinction and comparison of plasmid-mediated dihydrofolate reductases. Therefore it was decided to examine the amino acid sequence of the type IV enzyme. Two properties of dihydrofolate reductases make them particularly suitable for sequencing by this method. Firstly, if the enzyme is methotrexate sensitive it should be relatively easy to purify it sufficiently for sequence analysis. Secondly the majority of the active site of all dihydrofolate reductases is situated at the N-terminal end of the protein (Novak *et al.*, 1983; Rouch *et al.*, 1989). Therefore a single run through an automatic amino acid sequencer should provide enough information to deduce the evolutionary relationships.

3.2.1 Purification of the Type IV Dihydrofolate Reductase

The overall purification procedure is outlined in figure 10. The enzyme was purified from 10 litres of *E. coli* $J62_{-2}$ (pUK1123) grown for 20 hours at 37°C in Isosensitest broth containing trimethoprim (40 mg/L). The activity of the enzyme preparation was measured at each stage of the purification procedure. Prior to the gel filtration step 10 ml of the resuspended pellet from ammonium sulphate precipitation contained 41,059 units of dihydrofolate reductase activity. The resuspended pellet was dialysed against buffer A for 4 hours, then concentrated to 2 ml with an Amicon Centriprep 10 Concentrator. The activity in the concentrate was measured and found to contain 40,790 units. The concentrate was then separated on a Sephadex G75 column and 2 ml fractions collected. A clearly defined peak of enzyme activity was identified (figure 11). Fractions 38 - 57 were pooled and the activity reassayed, 24,062 units were present.

The pooled fractions were applied to a methotrexate column. No dihydrofolate reductase activity was detected in the protein which initially passed through the column indicating that the enzyme had been completely bound to the methotrexate agarose. The column was washed continuously with buffer A until the absorbance at 280 nm of the eluate had returned to an OD of less than 0.01, i.e. no further protein was passing through.

The dihydrofolate reductase activity was eluted by the addition of 2 ml 1M K_2HPO_4, 2 ml 0.5M K_2HPO_4 containing 4 μmoles of dihydrofolate followed by 75 ml of 0.1M K_2HPO_4 containing 1μM dihydrofolate (Kaufman, 1974). Five

Figure 10. Purification procedure for the type IV dihydrofolate reductase, the volumes at each stage are also shown.

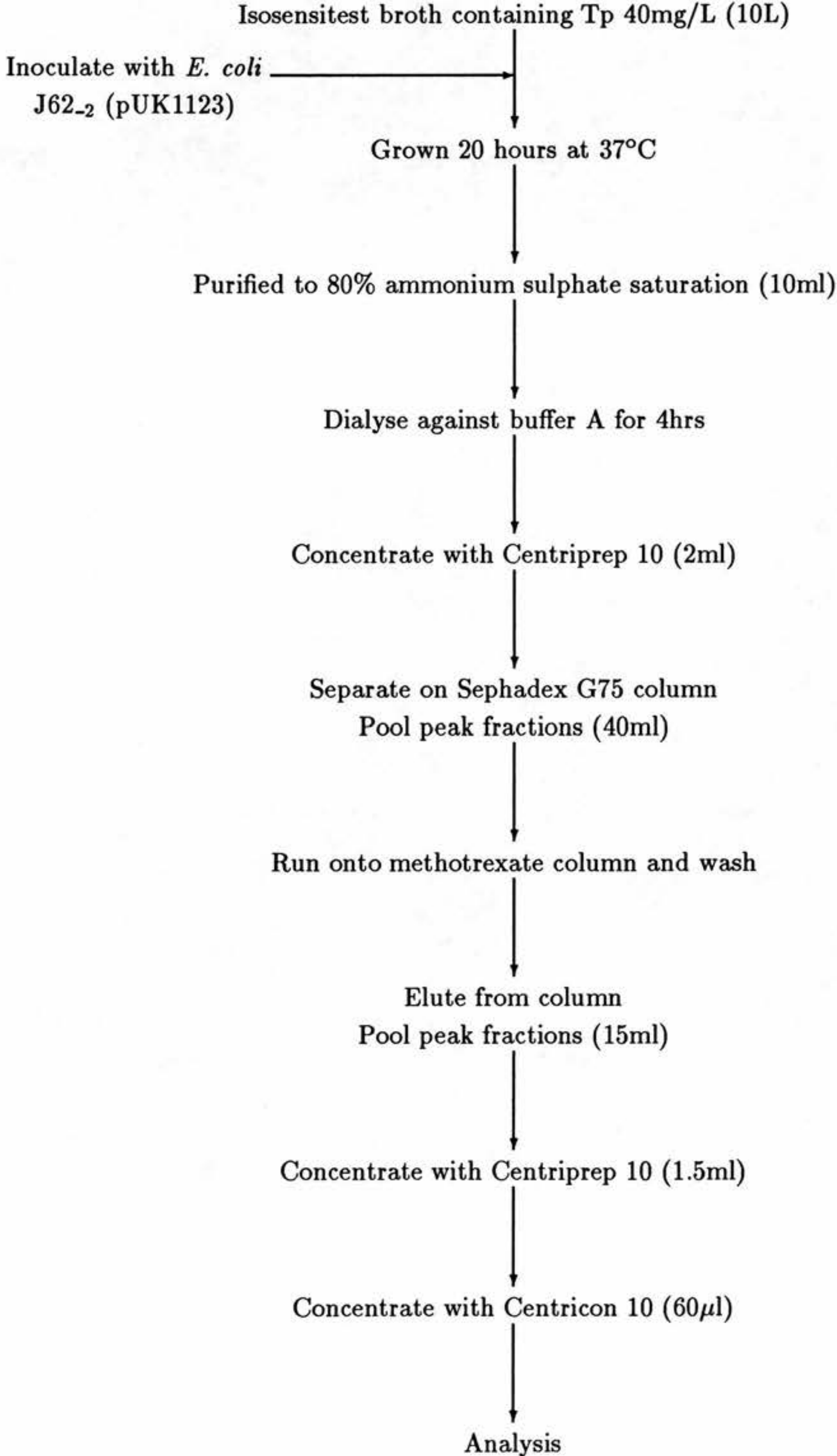
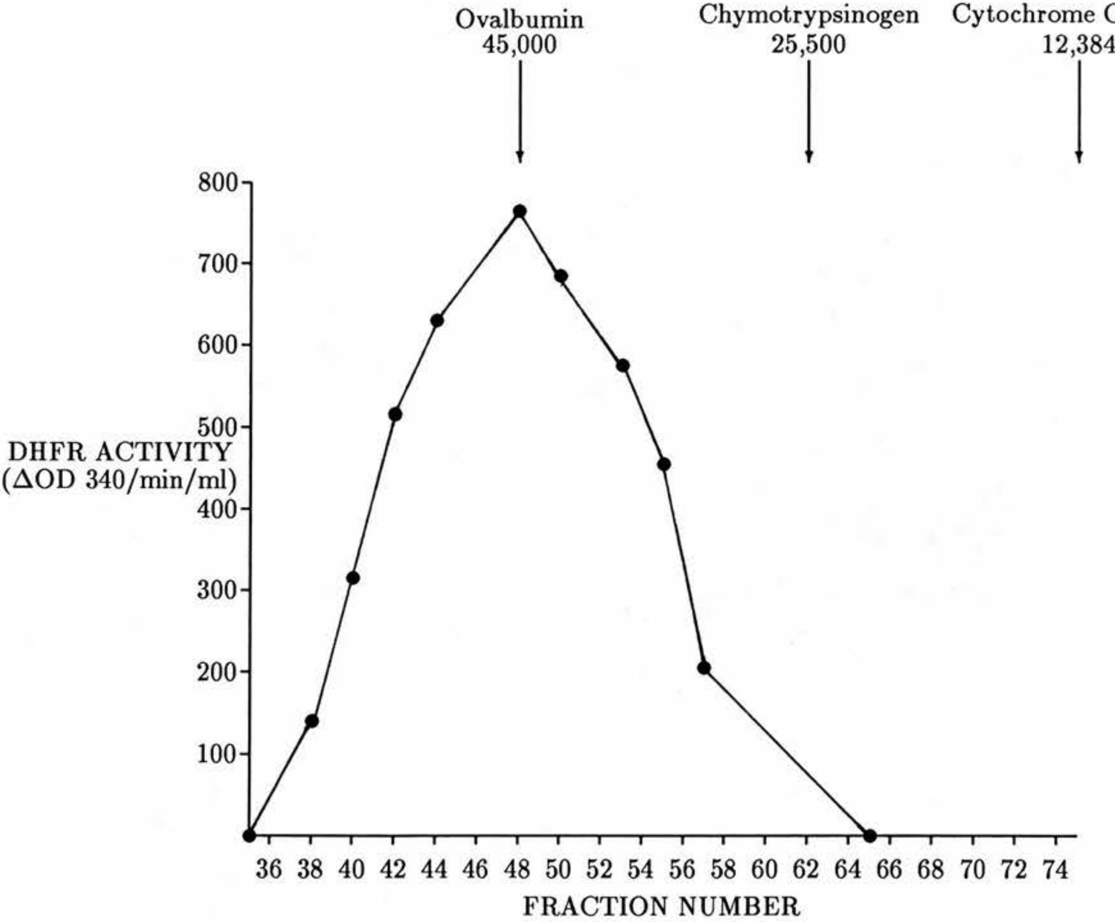


Figure 11. Elution of DHFR activity of *E. coli* J62$_{-2}$ (pUK1123) on Sephadex G75 gel filtration, the elution points of standard markers are also shown. Enzyme had been prepared from a large scale (10L) culture.



ml fractions were collected and assayed for enzyme activity. The three fractions showing peak activity were pooled and contained 14,743 units. This 15 ml sample was concentrated with Amicon Centriprep 10 and then Centricon 10 Concentrators to a final volume of 60μl, which retained 8249 units of enzyme activity.

3.2.2 Native PAGE Analysis of the Purified Enzyme

The efficacy of the purification procedure was checked by applying a 2 μl aliquot of the purified protein to an SDS free homogeneous 20% polyacrylamide gel along with the standard marker proteins. Bovine serum albumin (66,000) ovalbumin (45,000) and trypsin inhibitor (20,100). The gel was run in a Pharmacia Phastsystem until the bromophenol blue dye front had migrated the length of the gel. The gel was then stained with coomassie blue and destained as described in the Materials and Methods (figure 12). This revealed that the enzyme encoded by plasmid (pUK1123) comigrated with ovalbumin (45,000) and produced a single protein band. This not only confirmed that the type IV enzyme had been purified to the native protein but also showed that the protein had a molecular mass of around 45,000. This is virtually identical to the molecular mass of the type IV enzyme determined by gel filtration (Young and Amyes, 1986a).

3.2.3 N-terminal Sequence of the Purified Enzyme

As the purification procedure had apparently removed all contaminating proteins, the native type IV dihydrofolate reductase could be analysed on the automatic amino acid sequencer. As a final check on purity and to establish how much enzyme to load, the purified sample was analysed by HPLC. As expected, a

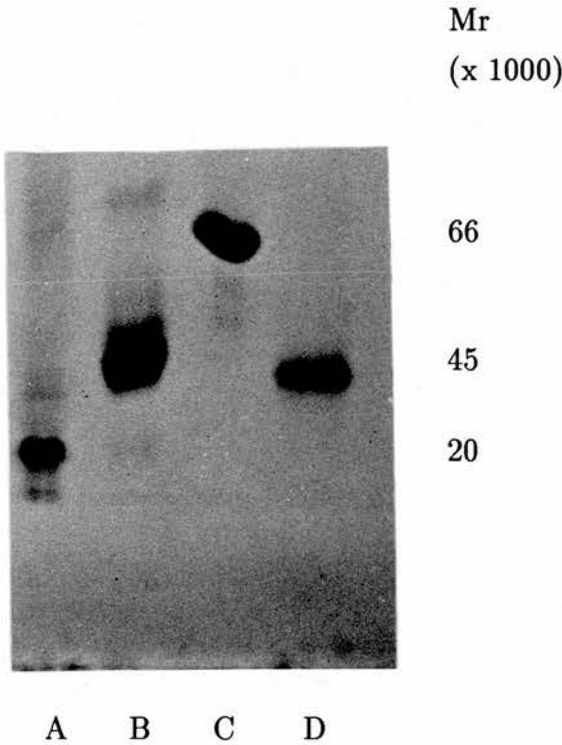
Figure 12. SDS-free polyacrylamide gel electrophoresis of purified dihydrofolate reductase from *E. coli* $J62_{-2}$ (pUK1123). Standard marker proteins are also shown.

Track A: Trypsin inhibitor

Track B: Ovalbumin

Track C: Bovine serum albumin

Track D: Type IV dihydrofolate reductase



single protein peak was obtained, this was applied to an Applied Biosystems 477A sequencer. A clear result was obtained for the first 47 amino acids of the dihydrofolate reductase (figure 13). However a secondary sequence, which was not a dihydrofolate reductase, could also be determined. This secondary sequence was weaker than the primary enzyme and only the first 19 amino acids of this protein could be distinguished. Detailed examination of the GENBANK database revealed that this secondary protein was NS1, an *E. coli* DNA binding protein. The second protein clearly was not a contaminant as a single protein had been found both on Native PAGE and HPLC. This suggests that the native type IV dihydrofolate reductase must have been a complex of these two proteins.

If this was the case then the type IV enzyme which gives a single band on native PAGE should produce subunits of different size when examined under denaturing conditions.

3.2.4 SDS PAGE of the Purified Enzyme

The purified enzyme from pUK1123 was treated with SDS and then applied to a 10-15% SDS containing polyacrylamide gel along with Sigma SDS molecular weight markers (14,000 - 66,000). The gel was run in a Pharmacia Phastsystem and stained with coomassie blue (figure 14).

Unlike the native gel a number of protein bands were present, one major band at around 33,000 and a number of smaller bands. This suggests the existence of subunits of different sizes. The main band is presumed to be the dihydrofolate reductase as the sequence analysis had shown that it was the major protein in the

Figure 13. Amino acid sequence obtained for the purified dihydrofolate reductase from pUK1123

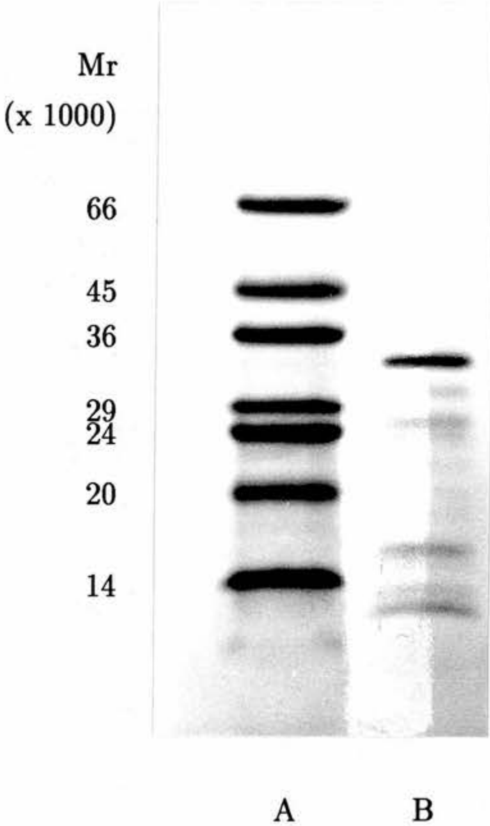
```
IV      Met Ile Arg Met Ile Leu Ala Ile Asn Asn Gln Tyr Phe Ile Gly Lys Asn Asn Thr Leu Met Tyr Arg Leu
NS1     Met Asn Lys Ser Gln ___ Ile Asp Lys Ile Ala Ala Gly Ala Asp ___ Ser Lys Ala
                                         10                                      20

IV      Lys Asp Asp Met Leu Asn Phe Lys Lys Met Thr Gln Asn Asn Ile Val Val Met Gly Arg Lys Thr Phe
                             30                                  40
```

Figure 14. SDS polyacrylamide gel electrophoresis of the purified dihydrofolate reductase from *E. coli* $J62_{-2}$ (pUK1123). Standard marker proteins are also shown.

Track A: Standard marker proteins (14,000 - 66,000)

Track B: Type IV dihydrofolate reductase



sample. The bands of lower molecular weight may represent breakdown products of this and NS1 protein which has a monomeric size of 10,000 but can also exist as a dimer or trimer (Rouvière-Yaniv and Kjeldgaard, 1979). However 33,000 is a large subunit size for a dihydrofolate reductase and to confirm that the enzyme was fully denatured, the gel was repeated but the protein sample was boiled in SDS for up to 30 minutes before being applied to the gel. The result (figure 15) showed that no further denaturation took place and that the true size of the dihydrofolate reductase subunit was 33,000 daltons.

3.2.5 N-terminal Amino Acid Sequence of the Type IV Dihydrofolate Reductase Encoded by pUK1150

The results show that the type IV enzyme was complexed to the NS1 binding protein when encoded by the clinical plasmid pUK1123. However a 2.6 kb *Hind*III fragment from pUK1123 had been cloned into pBR322 to give the hybrid plasmid pUK1150. *E. coli* C600 (pUK1150) produced an active dihydrofolate reductase which was not inducible and had a lower molecular mass than the type IV enzyme encoded by pUK1123. The molecular mass of the pUK1150-derived enzyme is 33,000 when measured by Sephadex gel filtration (figure 16). Preliminary analysis might have suggested that the lower molecular mass of this enzyme resulted from loss of part of the structural gene. However comparison with the results of pUK1123 suggests that the pUK1150 derived enzyme lacks the NS1 binding protein. In order to investigate this further the pUK1150-derived enzyme was purified and the sequence determined. The purification procedure was identical to that for the type IV from pUK1123 and the steps involved and enzyme activity

Figure 15. SDS polyacrylamide gel electrophoresis of the purified type IV dihydrofolate reductase from *E. coli* $J62_{-2}$ (pUK1123) after boiling for 10, 20 and 30 minutes. Standard protein markers are also shown.

Track A: Standard marker proteins (14,000 - 66,000)

Track B: Type IV dihydrofolate reductase boiled for 10 minutes

Track C: Type IV dihydrofolate reductase boiled for 20 minutes

Track D: Type IV dihydrofolate reductase boiled for 30 minutes

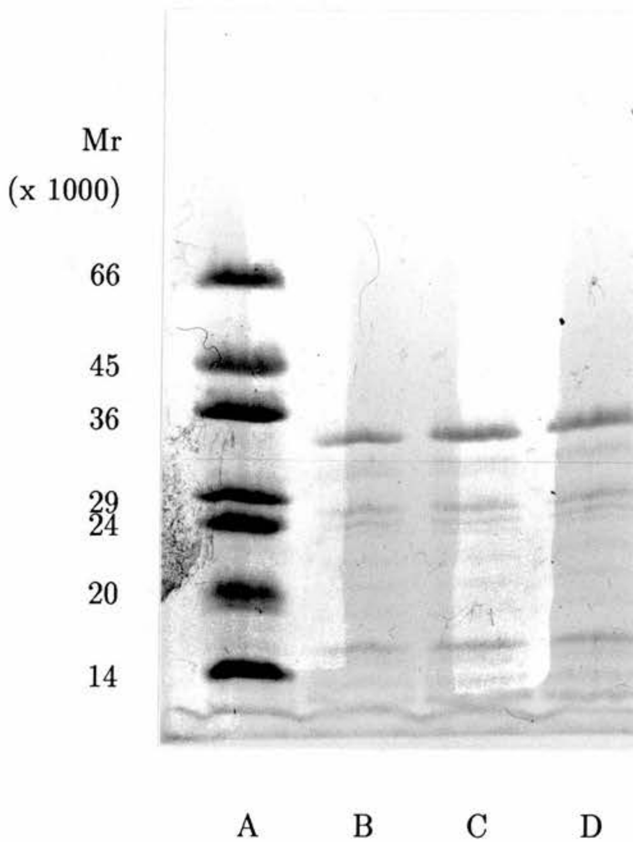


Figure 16. Elution of DHFR activity of *E. coli* C600 (pUK1150) on Sephadex G75 gel filtration.
The elution points of standard markers are also shown.

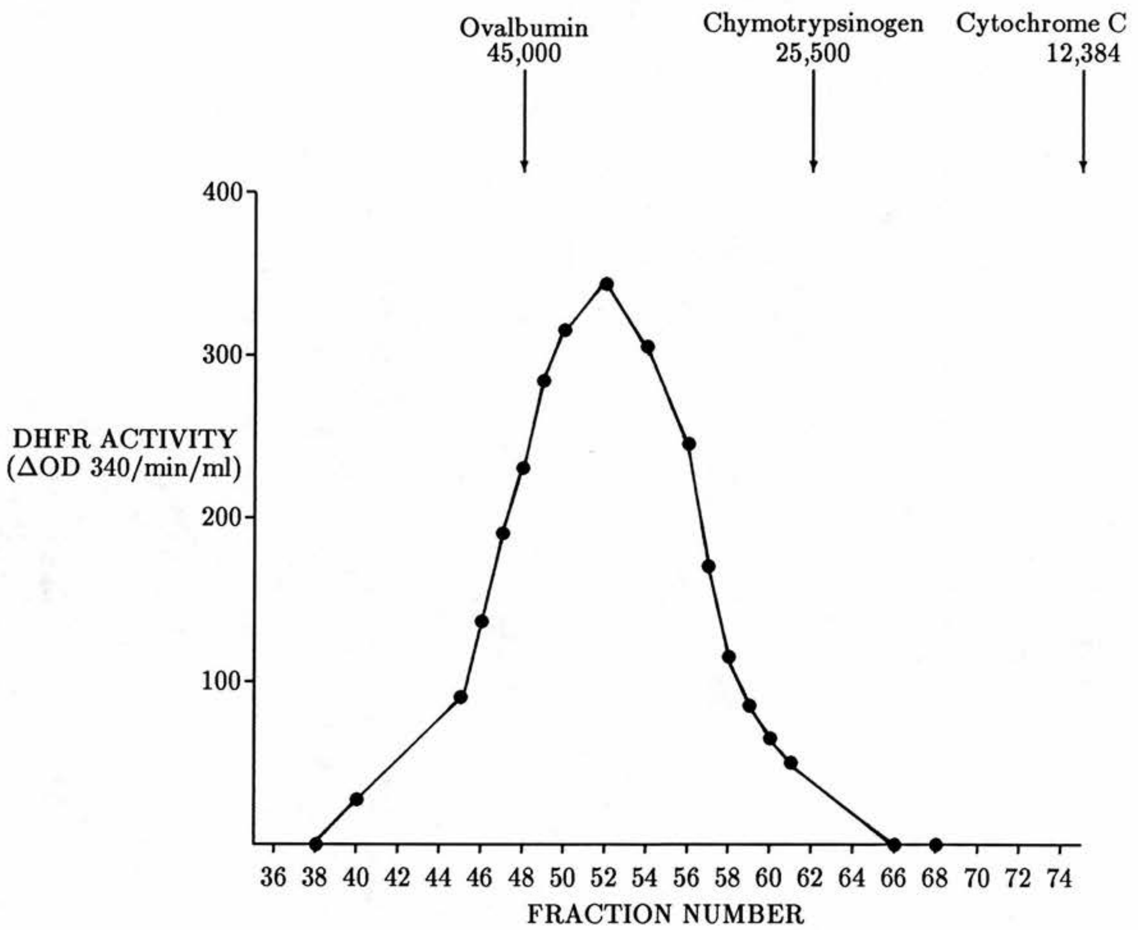


Table 28. Purification of the type IV dihydrofolate reductase encoded by plasmid pUK1150.

Purification stage	Enzyme specific activity nmol DHF reduced/min/mg protein
80% ammonium sulphate precipitate	74,861
2 ml concentrate run onto Sephadex	42,013
pooled from Sephadex	8,722
concentrated from Mtx column	5,316

at each stage are summarised in table 28.

3.2.6 N-terminal Sequence Analysis of the Dihydrofolate Reductase Encoded by Plasmid pUK1150

The purified enzyme was examined on HPLC and a single peak was obtained, it was this peak that was analysed on the Applied Biosystems 477A sequencer. The sequence of the first 49 amino acids was obtained (figure 17) and this was in agreement with that obtained for the type IV encoded by plasmid pUK1123. No evidence of *any* secondary sequence, let alone the NS1 binding protein was obtained, strongly suggesting that the lower molecular mass of the pUK1150 derived enzyme results from the absence of the NS1 DNA binding protein. Comparison of the N-terminal amino acid sequence of the type IV enzyme with those of the other plasmid-mediated enzymes and the *E. coli* chromosomal enzyme, showed that the type IV was most similar to the chromosomal enzyme (figure 17).

Figure 17. Comparison of the N-terminal amino acid sequence of the type IV plasmid-encoded DHFR with the other plasmid-mediated DHFR's and the E.coli chromosomal enzyme.

Identical amino acids are shown by shading. Homologous amino acids (D,E) (R,K,H) (Q,N) (F,Y,W) (I,V,L,M) are underlined. The amino acids involved in the active site are shown by the symbols at the top of the diagram: ▲ binding position of trimethoprim; △ additional binding positions of methotrexate; ■ binding positions of NADPH. Numbering is based on the IV sequence. Gaps have been left to maximise homology.

```
Type IV        M I R M I L A I N N Q Y F I G K N N T L M Y - R L K D D M L N F K K M T Q N N I V V M G R K T F E S
E.coli K-12    M I S L I A A L A V D R V I G M E N A M P W - N L P A D L A W F K R N T L N K P V I M G R H T W E S
Type IIIa    M L I S L I A A L A H N N L I G K D N L I P W - H L P A D L R H F K A V T L G K P V V M G R R T F E S
Type IIIb    T K E A I F A V A E N L A F G L G G G L - P W D T L K D D L Q F F K R L T E G T D D V M G A S T - - -
Type Ia      M K L S L M V A I S K N G V I G N G P D I P W - S A K G E Q L L F K A I T Y N Q W L L V G R K T F E S
Type V       M K V S L M A A K A K N G V I G C G P H I P W - S A K G E Q L L F K A L T Y N Q W L L V G R K T F E S
Type S1      M T L S I I V A H D K Q R V I G Y Q N Q L P W - H L P N D L K H I K Q L T T G N T L V M A R K T F N S
Type IIa     M E R S S N E V S N P V A G N F V F P S N A T F G M G D R V R K K S G A A W Q G Q I V G W Y C T N L T
Type IIb     M G Q S S D E A N A P V A G Q F A L P L S A T F G L G D R V R K K S G A A W Q G Q V V G W Y C T K L T
Type IIc     M D Q H N N G V S T L V A G Q F A L P S H A T F G L G D R V R K K S G A A W Q G Q V V G W Y K T K L T
                                 10                  20                     30                    40
```

3.2.7 SDS PAGE of the pUK1150-derived Dihydrofolate Reductase

The purified enzyme was examined on SDS PAGE and as there was no evidence of the NS1 protein, this enzyme should give a different pattern from the type IV encoded by pUK1123. The enzyme was treated with SDS and then applied to a 10 - 15% SDS containing polyacrylamide gel along with Sigma molecular weight markers (14,000 - 66,000). After electrophoresis the gel was stained with coomassie blue (figure 18). A single band was observed and this corresponded to a molecular mass of approximately 30,000.

The result confirms that unlike the pUK1123 derived type IV the smaller pUK1150 derived type IV is monomeric. The most likely explanation for the lower molecular mass of the pUK1150 derived enzyme is the absence of the NS1 protein.

B. Type III Dihydrofolate Reductases

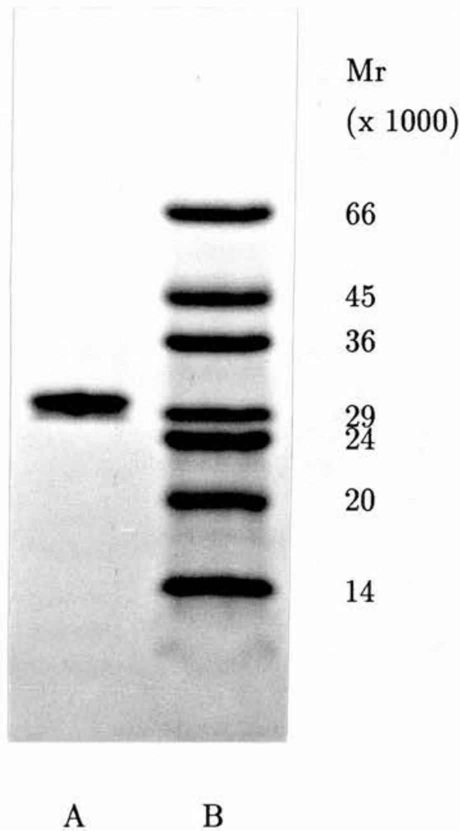
3.3 The Type III Dihydrofolate Reductase in the United Kingdom

The type III dihydrofolate reductase was first identified in New Zealand in 1979 (Joyner *et al.*, 1984). It was isolated from a single strain of *Salmonella typhimurium* and the gene encoding the enzyme was located on plasmid pAZ1. The type III enzyme is unusual amongst plasmid-encoded dihydrofolate reductases for a number of reasons: it confers only low level resistance on its host (MIC 64 mg/L), and is only partially resistant to inhibition by trimethoprim (ID_{50}

Figure 18. SDS polyacrylamide gel electrophoresis of the purified type IV dihydrofolate reductase from *E. coli* C600 (pUK1150). Standard marker proteins are also shown.

Track A: Dihydrofolate reductase encoded by pUK1150

Track B: Standard marker proteins (14,000 - 66,000)



2μM). This latter property is offset because the enzyme is extremely efficient at reducing the substrate as it has a K_m for dihydrofolate of 0.4μM. The type III dihydrofolate reductase has a molecular mass of 16,900 much lower than any of the dihydrofolate reductases that had been isolated before (Joyner *et al.*, 1984). Similarly, unlike the dihydrofolate reductases previously identified, the type III is monomeric.

After the initial isolation of the type III enzyme in 1979, it was never subsequently detected, although it was not clear if this was because the gene was extremely rare or simply resulted from inadequacies in the detection technique resulting from the enzyme's ability to confer only a low level of resistance.

It would seem that the latter is the case as the type III gene has now been detected in *E. coli* isolated from the urine of a hospitalised patient in Nottinghamshire.

3.3.1 Isolation and Probing

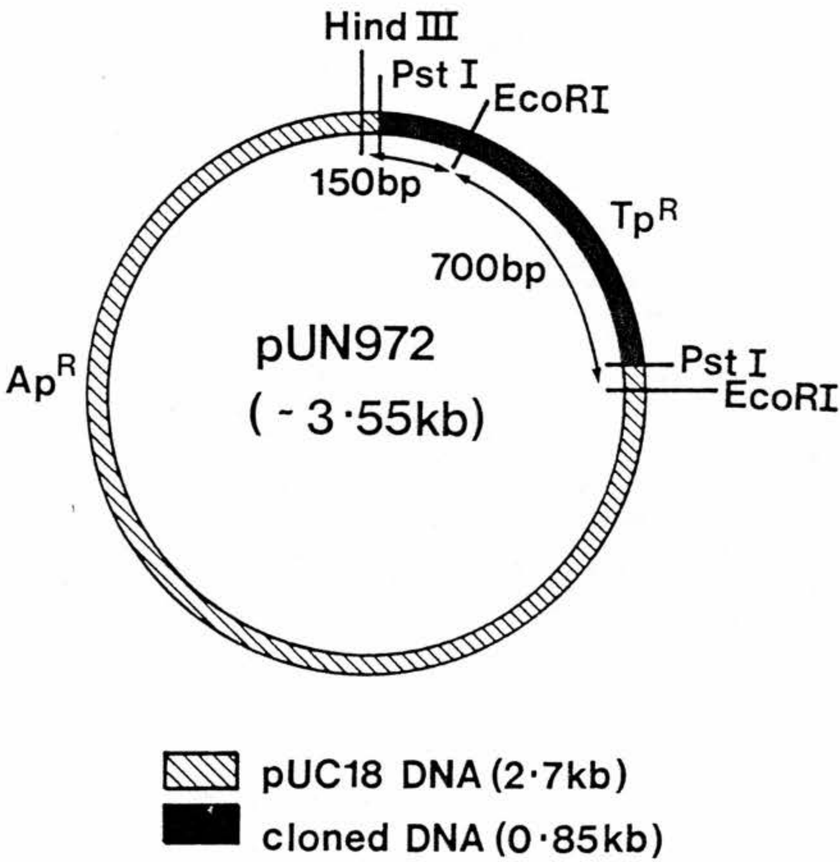
E. coli TM858 was isolated from the urine of a hospitalised patient in Nottinghamshire in 1980. Trimethoprim resistance was not freely transferable and could only be mobilised to *E. coli* J53$_{-2}$ following transposition onto plasmid RP4. The resulting transconjugant *E. coli* J53$_{-2}$ (pUN635 - RP4::3175) was highly resistant to trimethoprim (MIC $>$ 1000 mg/L). Purified plasmid DNA was therefore hybridised with those gene probes known to identify high level trimethoprim resistance genes (i.e. dihydrofolate reductase types I, II and V). However no hybridisation occurred with any of these probes suggesting the presence of a novel dihydrofolate reductase gene.

In collaboration with Dr Kevin Towner in Nottingham a more detailed analysis of the trimethoprim resistance gene and its products was performed. The trimethoprim resistance gene of *E. coli* $J53_{-2}$ (pUN635) was cloned into vector plasmid pUC18. Plasmid pUN635 was digested with the restriction enzyme *Pst*I and a 0.85 kb fragment encoding trimethoprim resistance was ligated into pUC18. The resultant recombinant plasmid pUN972 (figure 19) was introduced by transformation into *E. coli* HB101 and selection made for trimethoprim resistant colonies on DSTA plates containing trimethoprim (50 mg/L). The MIC of trimethoprim for transformants was found to be 64 mg/L, much lower than the original pUN635 containing bacteria.

Plasmid pUN972 was hybridised with all available plasmid dihydrofolate reductase gene probes and positive hybridisation was only obtained with the type III gene probe. Further evidence suggesting that pUN972 carried a type III gene was obtained when a 0.7 kb *Eco*R1-*Pst*I fragment of pUN972, carrying the trimethoprim resistance gene, was used to probe control plasmids encoding previously characterised dihydrofolate reductases. Hybridisation was only detected with the original type III dihydrofolate reductase encoding plasmid pAZ1.

In order to confirm that the pUN972-encoded enzyme was a type III detailed biochemical analysis of the dihydrofolate reductase encoded by pUN972 was performed.

Figure 19. Restriction map of recombinant plasmid pUN972.



3.3.2 Biochemical Properties of the Dihydrofolate Reductase Encoded by pUN972

Molecular Mass

Dihydrofolate reductase was prepared from *E. coli* HB101 (pUN972) which had been grown at 37°C overnight in Isosensitest broth. Enzyme was isolated as described in the Materials and Methods, and 1 ml of the resuspended 80% ammonium sulphate precipitate was applied to a calibrated Sephadex G75 column equilibrated with buffer A and eluted with the same buffer. Two ml fractions were collected every 8.3 minutes and assayed for dihydrofolate reductase activity (figure 20). A single peak of trimethoprim resistant (4μM) dihydrofolate reductase activity was obtained and this corresponded to a molecular mass of 17,000 virtually identical to the mass of the type III enzyme (Mr 16,900). The peak fractions (67 - 72) were pooled for further biochemical analysis.

Inhibition by Trimethoprim

The activity of the partially purified enzyme was assayed in the presence of different trimethoprim concentrations. The activity of the dihydrofolate reductase encoded by pUN972 was directly proportional to the logarithm of the trimethoprim concentration (figure 21) and the enzyme was only partially resistant to trimethoprim with an ID_{50} of 2μM. This is identical to the type III enzyme.

Figure 20. Elution of DHFR activity of *E. coli* HB101 (pUN972) on Sephadex G75 gel filtration.
The elution points of standard marker proteins are also shown.

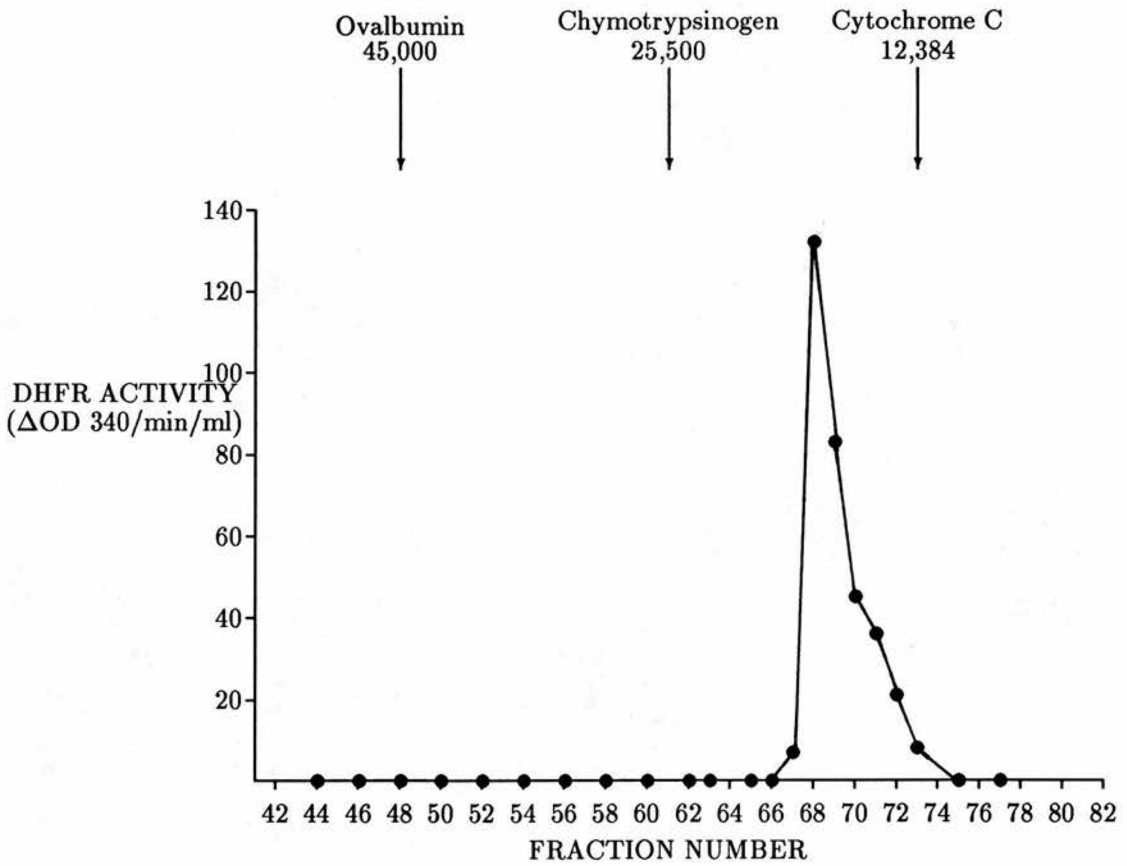
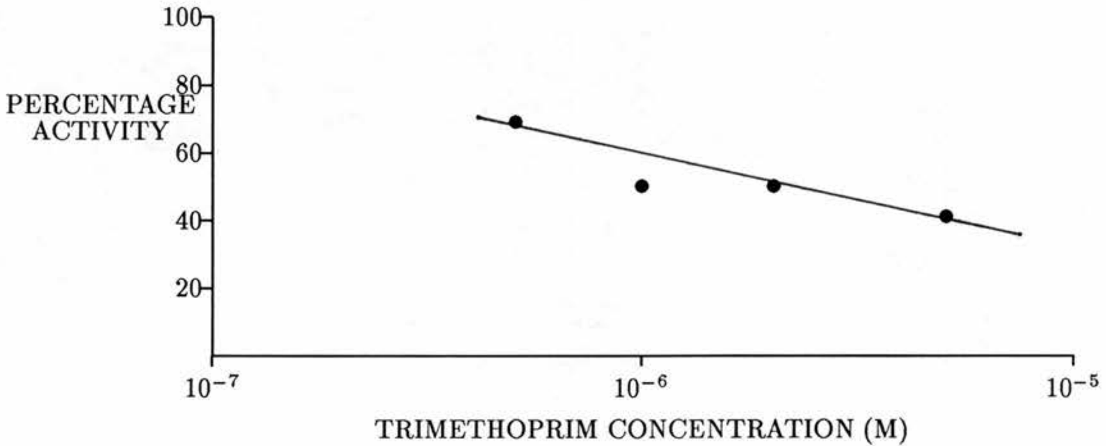


Figure 21. Trimethoprim inhibition of partially purified dihydrofolate reductase from *E. coli* HB101 (pUN972).

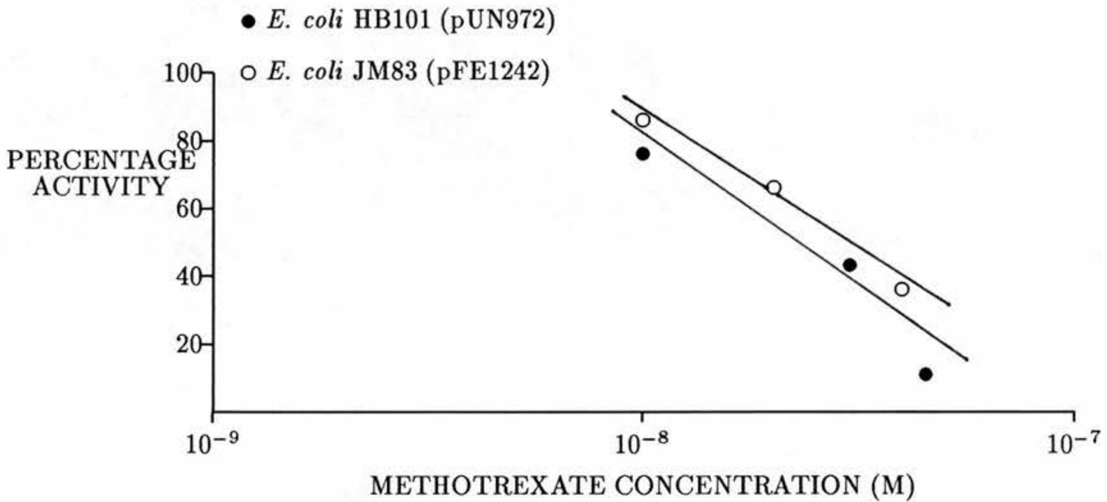


Inhibition by Methotrexate and Heat

The heat and methotrexate sensitivities of the type III dihydrofolate reductase encoded by plasmid pAZ1 were not known. Therefore, in order to compare the type III and pUN972 dihydrofolate reductases, it was necessary to determine these values for the pAZ1 derived enzyme.

Type III dihydrofolate reductase was prepared from *E. coli* K12 JM83 containing the plasmid pFE1242, a recombinant plasmid consisting of a *Taq*I fragment containing the type III trimethoprim resistance gene inserted into the *Acc*I site of pUC9. *E. coli* JM83 (pFE1242) was grown overnight at 37°C in 2 litres of Isosensitest broth and the enzyme prepared in the manner described earlier. After separation by Sephadex gel filtration the active fractions were pooled and the methotrexate and heat sensitivities established and compared with the enzyme

Figure 22. Methotrexate inhibition of partially purified dihydrofolate reductase from *E. coli* HB101 (pUN972) and *E. coli* JM83 (pFE1242).



encoded by pUN972.

Methotrexate Sensitivity

The activities of the dihydrofolate reductases, encoded by pUN972 and pFE1242, were assayed over a range of methotrexate concentrations (figure 22) both enzymes were sensitive to methotrexate ID_{50} $0.02\mu M$ and $0.03\mu M$ respectively.

Heat Stability

The activities of the two enzymes were compared after incubation in prewarmed containers at 45°C for various time intervals. Both enzymes were heat stable maintaining more than 50% of their activity after 12 minutes at 45°C.

Michaelis Menten Kinetics

One of the distinguishing features of the type III enzyme is that it is extremely efficient with a K_m for dihydrofolate of 0.4μM. This enables the type III enzyme to confer a significant degree of resistance despite being only partially resistant to trimethoprim. The efficiency of the dihydrofolate reductase encoded by pUN972 was investigated by assaying its activity under conditions of partial saturation with dihydrofolate and the results analysed by the method of Lineweaver and Burk. The results (figure 23) show that the K_m value for dihydrofolate was 0.4μM, identical to the value previously published for the pAZ1 encoded enzyme. When the assays were repeated in the presence of trimethoprim (5μM), it was found that the maximum velocity remained the same (figure 23) indicating that the drug causes competitive inhibition of the enzyme. The inhibitor constant (K_i) for trimethoprim was 0.04μM, again similar to that reported for the pAZ1 encoded type III dihydrofolate reductase.

The biochemical properties of the enzyme encoded by pUN972 and pAZ1 are indistinguishable (table 29). This confirms the probing results which had suggested that the enzyme was a type III. To confirm that the two enzymes were identical and not merely very closely related isoelectric focusing was used. Isoelectric focusing is extremely useful for detecting differences between proteins as even a difference in a single amino acid can be identified (Amyes and Tait, 1990).

The pI of the type III dihydrofolate reductase encoded by pAZ1 was unknown, therefore, to compare the two enzymes, they were run side-by-side along with a set of pI markers. Dihydrofolate reductase was prepared from *E. coli* HB101

Figure 23. Lineweaver-Burk plot of the partially purified dihydrofolate reductase from *E. coli* HB101 (pUN972).
The reciprocal of the substrate concentration (DHF) (1/S) is plotted against the reciprocal of the dihydrofolate reductase activity (1/V).

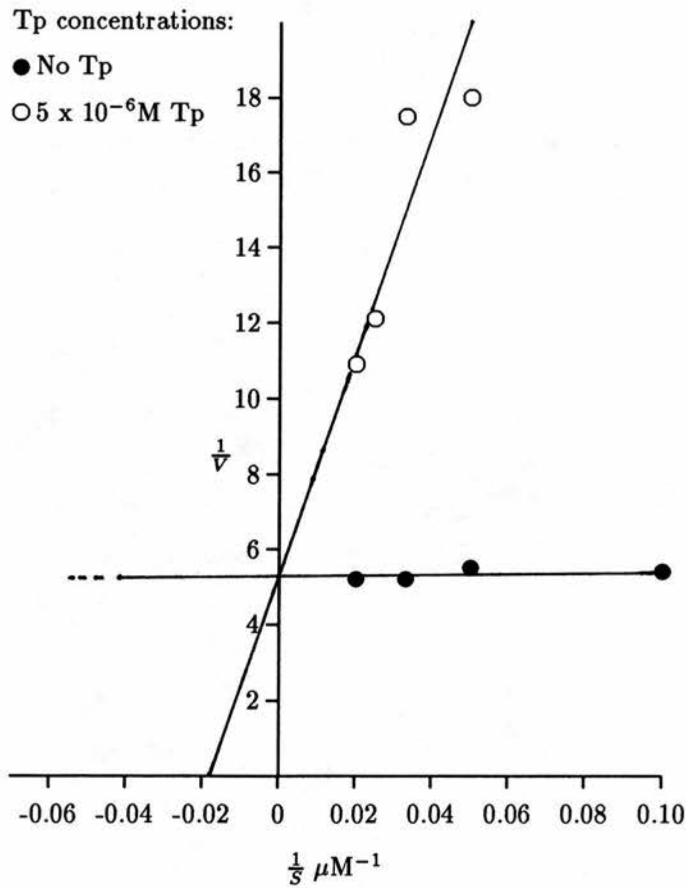


Table 29. Biochemical properties of the type III dihydrofolate reductase and the enzyme encoded by plasmid pUN972.

Enzyme	Tp ID_{50} μM	Mtx ID_{50} μM	TD_{50} mins	DHF K_m μM	Tp K_i μM	Size daltons
Type III	2	0.03*	> 12*	0.4	0.019	16900
pUN972	2	0.02	> 12	0.4	0.04	17000

* Established from *E. coli* pFE1242

(pUN972) and *E. coli* JM83 (pFE1242) as before; prior to isoelectric focusing the resuspended 80% ammonium sulphate precipitate was diluted 1 in 5 in buffer A containing 0.75mM NADPH and dialysed against buffer A (100 volumes) for 4 hours. Ten μl of each sample was then focused and stained as described in the Materials and Methods. The two enzymes co-migrated (figure 24) confirming that they were identical. Analysis of the distance moved by the pI markers showed that each enzyme had a pI of 6.10.

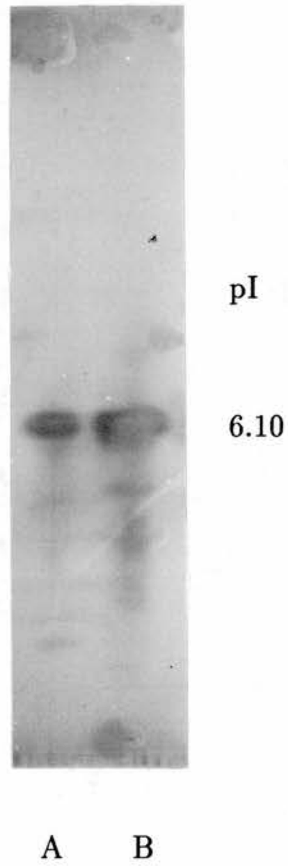
3.4 Low Level Trimethoprim Resistant Dihydrofolate Reductases from Two Outbreaks of Shigella sonnei

The combination of trimethoprim and sulphamethoxazole (cotrimoxazole) is widely used in the treatment of Shigellosis (Salter, 1982). Until recently trimethoprim resistance in Shigella was relatively uncommon (Barada and Guerrant, 1980), however, increased use of trimethoprim-containing therapy in the treatment of Shigellosis has resulted in a large increase in trimethoprim resistance (Elwell

Figure 24. Isoelectric focusing of partially purified dihydrofolate reductase from *E. coli* HB101 (pUN972) and *E. coli* JM83 (pFE1242).

Track A: Dihydrofolate reductase encoded by pUN972

Track B: Dihydrofolate reductase encoded by pFE1242



pI

6.10

A B

and Fling, 1989). Some studies on trimethoprim resistant Shigella have demonstrated the dominance of the type Ia dihydrofolate reductase when the genes were probed with dihydrofolate reductase gene probes (Delgado and Otero, 1988). On the other hand, alternative studies have shown a significant proportion of strains which do not probe with genes coding for either of the commonly occurring type I and type II enzymes (Chatkaeomorakot *et al.*, 1987).

Outbreaks of trimethoprim resistant Shigella have been extremely rare in the United States (Murray, 1986). However two outbreaks of dysentery caused by trimethoprim resistant *Shigella sonnei* have been examined. The first outbreak occurred at a nursing home in East Tennessee in 1985 and involved 60 patients and several medical personnel. The second outbreak was at the Rainbow Family gathering in the Smokey Mountains National Park in North Carolina in 1987, which involved 6000 people (C.D.C., 1987a).

3.4.1 Isolation and Probing of the Trimethoprim Resistance Genes

Isolation and probing of the trimethoprim resistance genes was carried out by Dr Neil Barg, Vanderbilt University, Tennessee. *E. coli* C600 transconjugants were obtained from the *Shigella sonnei* responsible for each outbreak. Transconjugants from the nursing home strains contained only a 97.5 kb plasmid (pBH600) that transferred resistance to chloramphenicol, streptomycin, sulphamethoxazole, tetracycline and trimethoprim. Transconjugants of the National Park strains contained a 90 kb plasmid (pBH700) that transferred antibiotic resistance to ampicillin, streptomycin, sulphamethoxazole and trimethoprim.

Conjugation experiments between strains containing pBH600 or pBH700 yielded transconjugants that contained two plasmids 97.5 kb and 90 kb in size. Since both R-plasmids could co-exist stably, in the same strain without selective pressure, the pBH600 and pBH700 plasmids were of different incompatibility groups. The two plasmids also gave different restriction patterns after cleavage with *Eco*RI, *Hind*III or *Pst*I.

Hybridisation

Southern blots prepared from *Pst*I digests of pBH600 and pBH700 did not show hybridisation with probes for types I, II and III dihydrofolate reductases. The trimethoprim resistance genes were subcloned into plasmid pUC9. Subcloning of plasmid pBH600 produced the hybrid plasmid pBH6001a, the trimethoprim resistance gene residing within a 1600bp *Pst*I fragment. In the case of pBH700, an 1800 bp *Pst*I fragment of the hybrid plasmid, pBH7001a, contained the trimethoprim resistance gene. Probes prepared from these hybridised to the respective parent plasmids and not to plasmids pFE872, pUC4-12 and pFE1242, encoding dihydrofolate reductases types I, II and III respectively. The two probes did not hybridise to each other, *E. coli* chromosomal DNA or lambda phage DNA.

The hybridisation data suggested that there may be two distinct plasmid-encoded trimethoprim resistance genes that were not only different from each other but also distinct from those previously reported. In order to confirm this, detailed biochemical characterisation was carried out on the two enzymes.

The enzyme from the nursing home outbreak was examined in the *E. coli* C600

(pBH600) transconjugant of the original strain as there was sufficient dihydrofolate reductase activity in this strain. However in the case of *E. coli* C600 (pBH700), the transconjugant from the *Shigella sonnei* responsible for the National Park outbreak there was little detectable dihydrofolate reductase activity. In this case the dihydrofolate reductase was recovered from the *E. coli* JM101 strain harbouring plasmid pBH7001a.

3.4.2 Biochemical Properties of the Dihydrofolate Reductase Encoded by Plasmid pBH600

Molecular Mass

Enzyme was prepared from *E. coli* C600 (pBH600) as described in the Materials and Methods and one ml of the resuspended 80% saturation ammonium sulphate precipitate was separated on a Sephadex G50 column. This had been previously calibrated with chymotrypsinogen cytochrome C and the chain B of insulin as molecular mass standard markers. The column was equilibrated with buffer A and the enzyme was eluted in the same buffer. Two ml fractions were collected from the column and these were assayed for dihydrofolate reductase activity. A distinct peak of dihydrofolate reductase activity was obtained at fraction 52 and this corresponds to a molecular mass of 17,000. As this was close to the molecular mass of the chromosomal enzyme the assays repeated in the presence of 4μM trimethoprim and the peak was again found at fraction 52 (figure 25). A molecular mass of 17000 was low but it was very similar to the size reported for the type III enzyme which is 16,900 (Joyner *et al.*, 1984). Fractions 50 to 56 were pooled for further biochemical analysis.

Figure 25. Elution of the DHFR activity of *E. coli* C600 (pBH600) on Sephadex G50 gel filtration.
DHFR activity was assayed in the presence of 4 x 10^{-6}M Tp. The elution points of standard marker proteins are also shown.

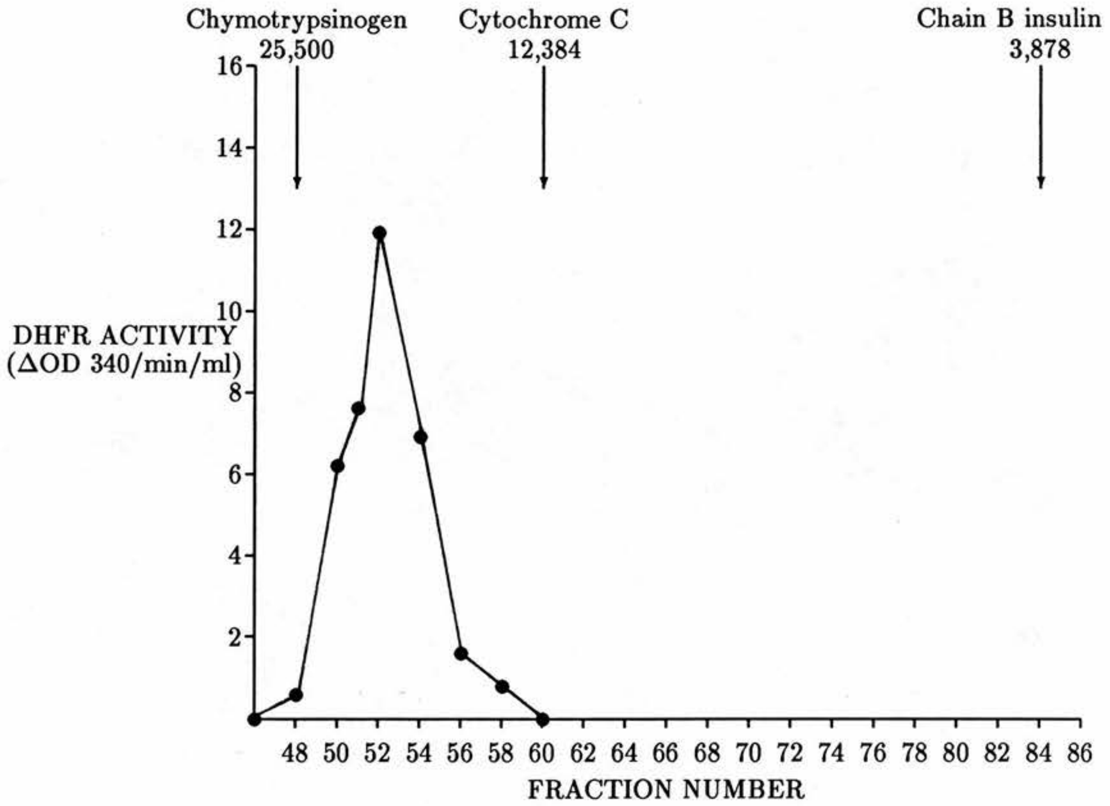
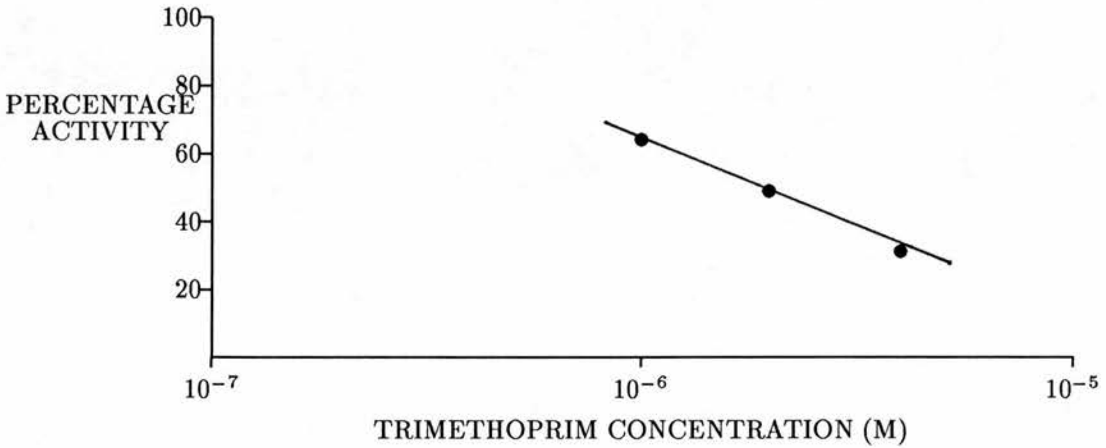


Figure 26. Trimethoprim inhibition of partially purified dihydrofolate reductase from *E. coli* C600 (pBH600).



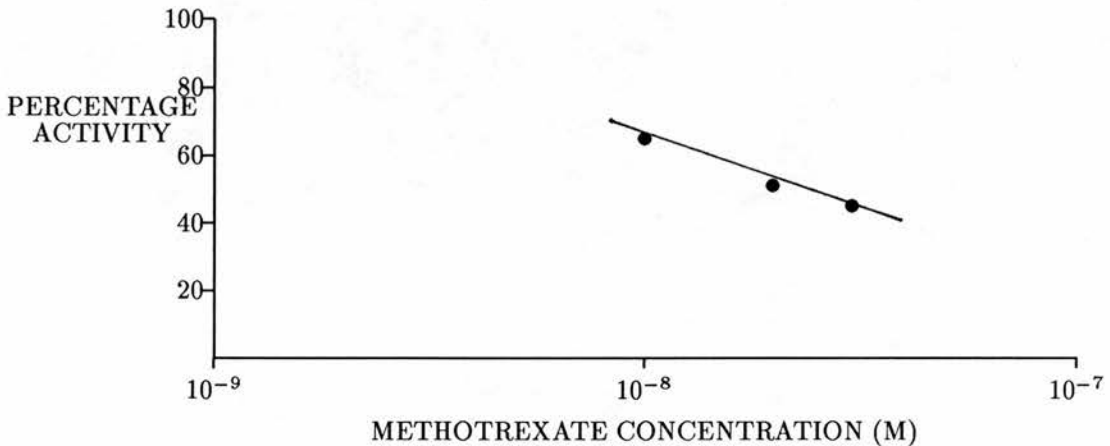
Inhibition by Trimethoprim

The activity of the partially purified enzyme encoded by plasmid pBH600 was assayed in the presence of different concentrations of trimethoprim (figure 26). The enzyme was relatively sensitive to trimethoprim and had an ID_{50} of only $2\mu M$.

Inhibition by Methotrexate

The enzyme encoded by plasmid pBH600 was sensitive to methotrexate. Assaying its activity in different methotrexate concentrations showed that only $0.02\mu M$ was required to reduce the enzymes activity by 50% (figure 27).

Figure 27. Methotrexate inhibition of partially purified dihydrofolate reductase from *E. coli* C600 (pBH600).



Heat Sensitivity

The enzyme derived from the nursing home strain was heat stable maintaining more than 50% of its activity after 12 minutes incubation at 45°C.

Michaelis Menten Kinetics

The activity of the enzyme was assayed in limiting concentrations of the substrate dihydrofolate. The results were analysed by the Lineweaver-Burk plot (figure 28) and this revealed that the enzyme had a relatively high affinity for dihydrofolate with a K_m of 9.52μM. When the assays were repeated in the presence of inhibitory concentrations of trimethoprim, 2 and 5 μM, the enzyme was competitively inhibited by this drug with a mean K_i of 0.40μM.

Figure 28. Lineweaver-Burk plot of the partially purified dihydrofolate reductase from *E. coli* C600 (pBH600).
The reciprocal of the substrate concentration (DHF) (1/S) is plotted against the reciprocal of the dihydrofolate reductase activity (1/V).

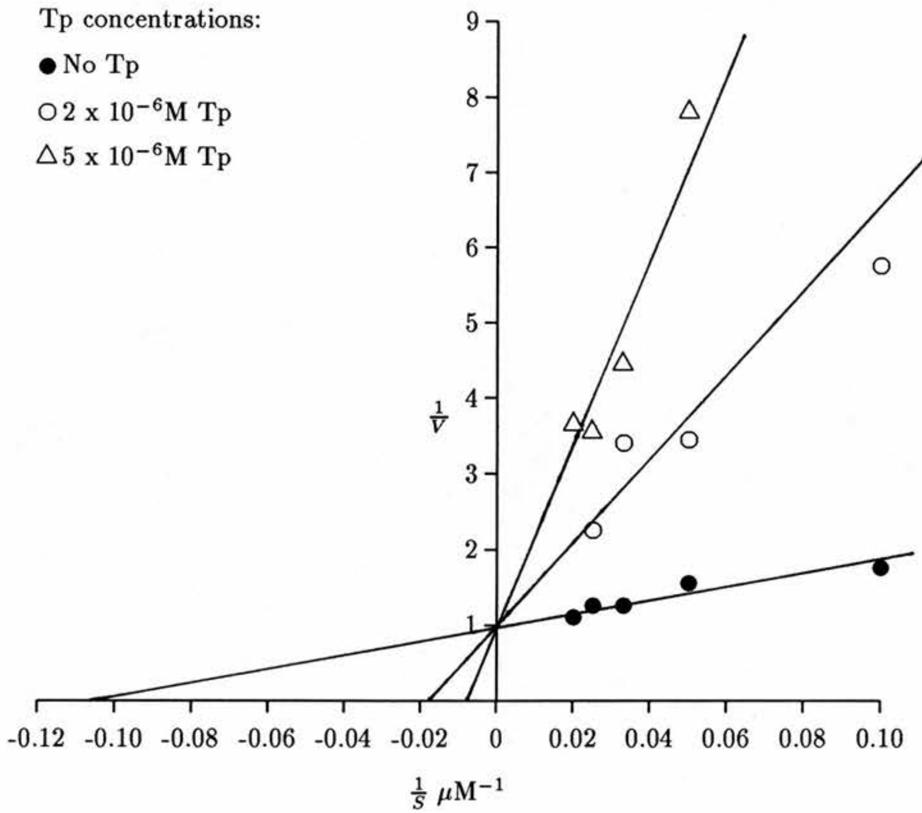


Table 30. Biochemical properties of the type III plasmid-encoded dihydrofolate reductases.

Enzyme Sub-Type	Tp MIC mg/L	Tp ID_{50} μM	Mtx ID_{50} μM	TD_{50} mins	DHF K_m μM	Tp K_i μM	Size daltons	pI
IIIa	64	2.0	0.02	> 12	0.4	0.019	16900	6.10
IIIb	128	2.0	0.02	> 12	9.52	0.40	17000	5.34
IIIc	256	3.0	0.007	8	3.12	0.52	22000	5.65

Examination of the properties of the dihydrofolate reductase from pBH600 showed that it had several properties that were identical to the type III enzyme (table 30) although the enzyme did differ in its Michaelis Menten Kinetics from the type III. The results suggested that this may be a closely related enzyme and for this reason it was named the type IIIb and the original type III renamed the type IIIa.

3.4.3 Biochemical Analysis of the Dihydrofolate Reductase Encoded by Plasmid pBH7001a

Molecular Mass

Dihydrofolate reductase was prepared from *E. coli* JM101 (pBH7001a) as described in the Materials and Methods and separated on a Sephadex G75 column. This had been calibrated with ovalbumin, chymotrypsinogen and cytochrome C as standard molecular mass markers. The enzyme was eluted in buffer A and 2 ml fractions collected, these were assayed for dihydrofolate reductase activity (figure 29). A clearly defined peak was obtained at fraction 62 and this corresponded to a molecular mass of 22,000. Repeating the assays in the presence of

4μM trimethoprim produced a peak at the same point. Peak fractions (58 - 66) were pooled for further biochemical analysis.

Inhibition by Trimethoprim

The activity of the enzyme was measured in the presence of different trimethoprim concentrations (figure 30). This showed that the enzyme was sensitive to trimethoprim with an ID_{50} of 3.0μM.

Inhibition by Methotrexate

The dihydrofolate reductase encoded by plasmid pBH7001a was extremely sensitive to methotrexate. The activity of the enzyme decreased by half in the presence of 0.007μM methotrexate (figure 31).

Heat Sensitivity

The enzyme was only relatively heat stable losing 50% of its activity after 8 minutes at 45°C.

Michaelis Menten Kinetics

The activity of the enzyme was assayed in limiting substrate dihydrofolate concentrations with and without trimethoprim (figure 32). This demonstrated that the enzyme had a relatively high affinity for dihydrofolate (K_m 3.12μM) and was competitively inhibited by trimethoprim (K_i 0.52μM).

Figure 29. Elution of DHFR activity of *E. coli* JM101 (pBH7001a) on Sephadex G75 gel filtration.
Elution points of standard proteins are also shown.

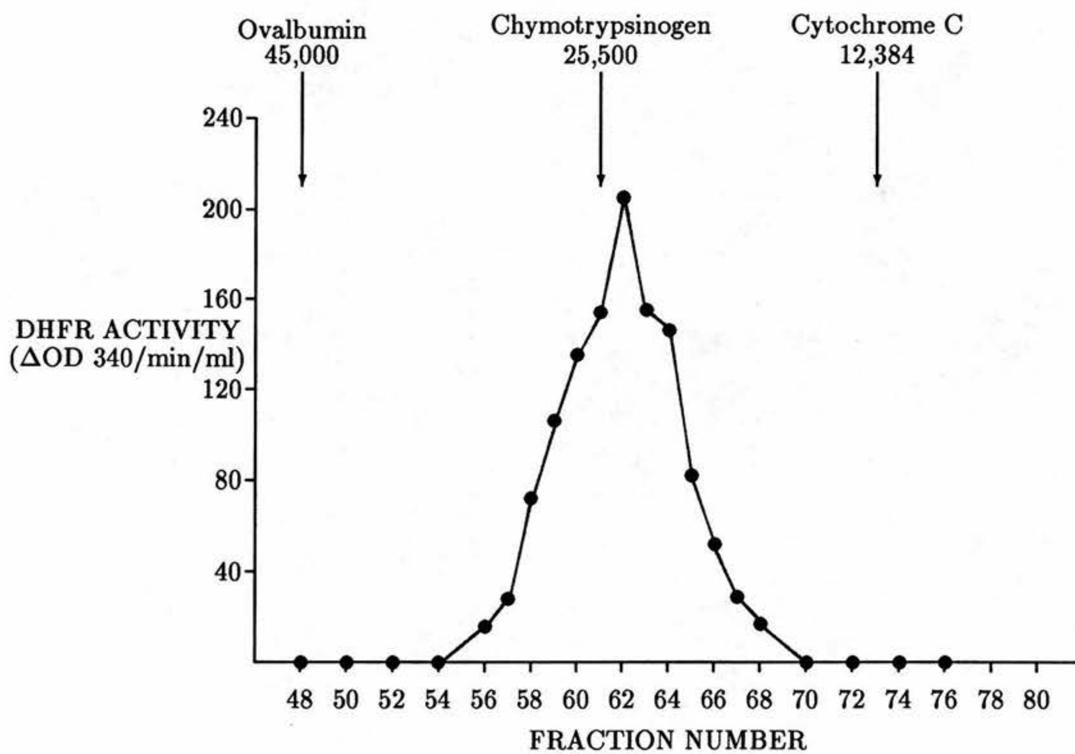


Figure 30. Trimethoprim inhibition of partially purified dihydrofolate reductase from *E. coli* JM101 (pBH7001a).

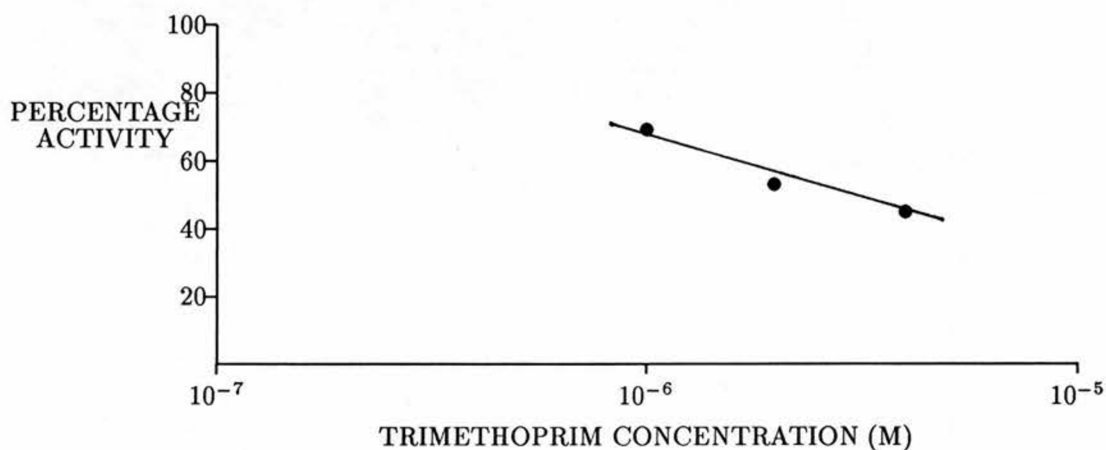


Figure 31. Methotrexate inhibition of partially purified dihydrofolate reductase from *E. coli* JM101 (pBH7001a).

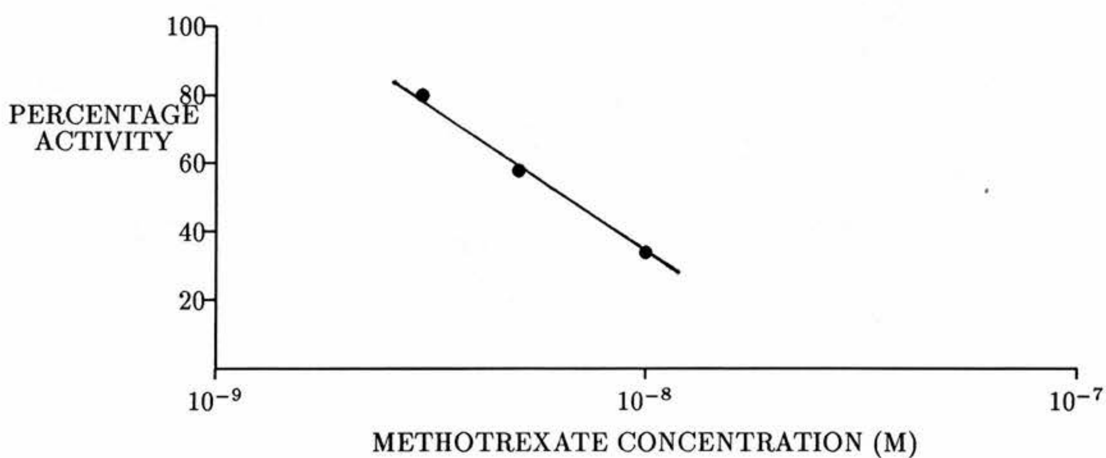
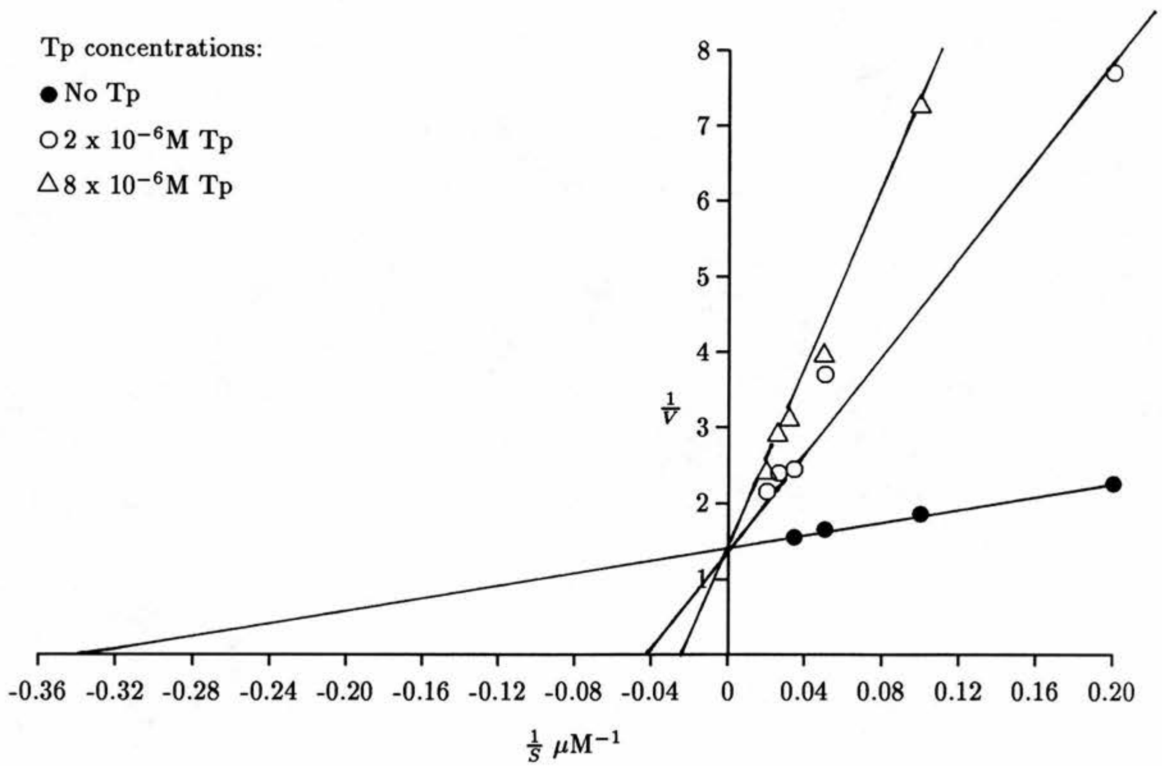


Figure 32. Lineweaver-Burk plot of the partially purified dihydrofolate reductase from *E. coli* JM101 (pBH7001a).
The reciprocal of the substrate concentration (DHF) (1/S) is plotted against the reciprocal of the dihydrofolate reductase activity (1/V).



Biochemically the enzyme encoded by pBH7001a, derived from the strain responsible for the National Park outbreak, was similar to both the IIIa and IIIb enzymes and was, therefore, termed IIIc (table 30). Although all three enzymes show similar antifolate inhibition profiles, the type IIIc appears to be more heat sensitive than the other two. The IIIb and IIIc have similar Michaelis Menten Kinetics to one another and they are significantly different from the type IIIa. On the other hand, the molecular mass of the IIIb is indistinguishable from the IIIa however the IIIc is heavier.

DNA gene probing had suggested that the enzymes from each outbreak were different from each other and that neither was a type IIIa enzyme. The results of the biochemical analysis had demonstrated that while this may be the case, the differences between the enzymes were very small. In order to confirm that these were three distinct enzymes isoelectric focusing was used.

3.4.4 Isoelectric Focusing of the Type IIIa, IIIb and IIIc Dihydrofolate Reductases

Types IIIa, IIIb and IIIc dihydrofolate reductases were prepared from *E. coli* JM83 (pFE1242), *E. coli* C600 (pBH600) and *E. coli* JM101 (pBH70001a) respectively. After enzyme extraction in the normal way the resuspended 80% saturation ammonium sulphate precipitate from each preparation was diluted 1 in 5 in buffer A containing 0.75mM NADPH, and dialysed for four hours against 100 volumes of buffer A. Ten μl of each dialysed sample was subsequently electrophoresed as described in the Materials and Methods. The gel was stained for dihydrofolate reductase activity also as described in the Materials and Methods.

The results (figure 33) showed that the three enzymes produced a distinct and different pattern on an isoelectric focusing gel. Analysis of the distance moved by the pI markers enabled the pI of each dihydrofolate reductase to be established. As before the type IIIa focused clearly and had a pI of 6.10, the IIIb enzyme also gave a defined band and this corresponded to a pI value of 5.34. The IIIc enzyme, however, did not focus as well as the other two enzymes and produced a smeared band at pI 5.65. This proved to be a characteristic of this enzyme and when the gel was repeated with a fresh preparation of type IIIc dihydrofolate reductase the same pattern was obtained.

The results from isoelectric focusing had confirmed that the two enzymes from the Shigella outbreaks were different from each other and that neither was a type IIIa enzyme. There were, however, very strong biochemical similarities between the three, suggesting that they may be closely related in an evolutionary sense. Although isoelectric focusing had demonstrated that the enzymes were different from one another it gave no indication of the degree of difference.

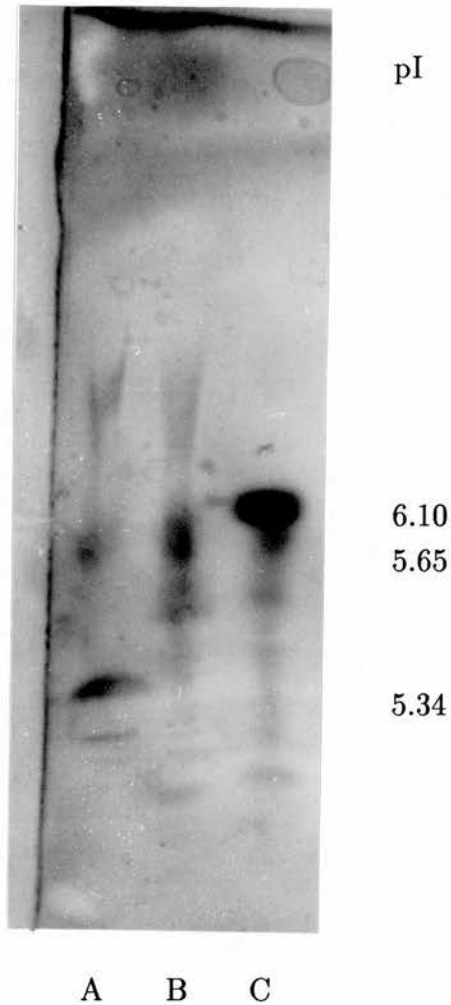
The amino acid sequence of the type IIIa enzyme is known (Fling *et al.*, 1988) therefore if the sequences of the IIIb and IIIc dihydrofolate reductases could be determined this would indicate if these enzymes were closely related. Attempts were therefore made to purify the IIIb and IIIc dihydrofolate reductases in order to establish their amino acid sequences.

Figure 33. Isoelectric focusing of partially purified dihydrofolate reductases from *E. coli* JM83 (pFE1242), *E. coli* C600 (pBH600) and *E. coli* JM101 (pBH7001a).

Track A: Dihydrofolate reductase encoded by pBH600 (type IIIb)

Track B: Dihydrofolate reductase encoded by pBH7001a (type IIIc)

Track C: Dihydrofolate reductase encoded by pFE1242 (type IIIa)

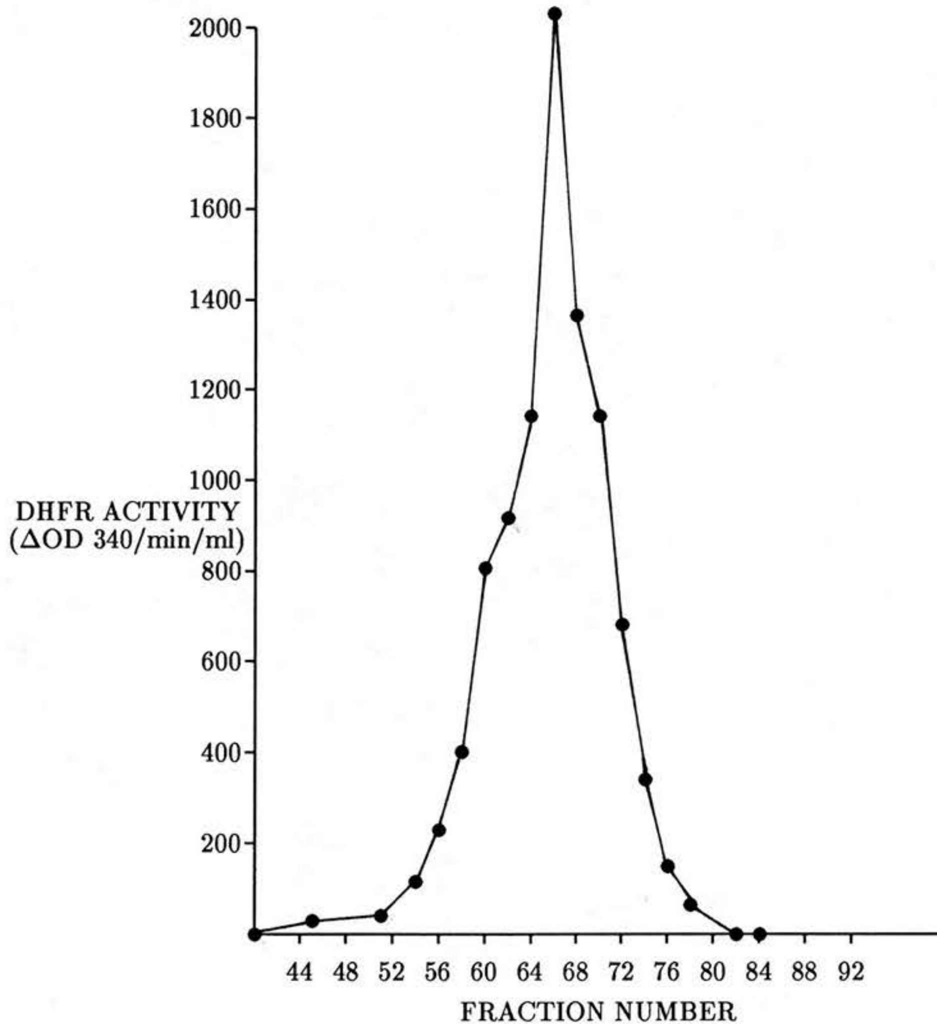


3.4.5 Purification of the type IIIb Dihydrofolate Reductase

Purification was performed as for the type IV dihydrofolate reductase (figure 10). Enzyme was prepared from *E. coli* C600 (pBH600) incubated shaking at 37°C for 20 hours in 10 litres of Oxoid Isosensitest broth. Dihydrofolate reductase was extracted as described in the Materials and Methods and the resuspended 80% ammonium sulphate precipitate was concentrated to 2 ml with an Amicon Centriprep 10 Concentrator. The activity of the enzyme preparation was measured throughout the purification procedure and the 2 ml concentrate contained 78,124 units of dihydrofolate reductase activity. The 2 ml concentrate was separated on a Sephadex G75 column and 2 ml fractions collected. Analysis of these showed that a clearly defined peak of enzyme activity was obtained, (figure 34) fractions 62 - 72 were pooled and the activity reassayed, revealing a total of 28,874 units were present. The pooled fractions were run onto a methotrexate agarose column. No dihydrofolate reductase activity was detected in the protein, which initially passed through the column, indicating that the enzyme had completely bound to the methotrexate agarose.

The column was washed continuously with buffer A until the absorbance, at 280nm, of the eluate had returned to an OD of less than 0.01 i.e. no further protein was passing through. The dihydrofolate reductase activity was eluted by the addition of 2 ml of 1M K_2HPO_4, 2 ml 0.5M K_2HPO_4 containing 4 μmoles of dihydrofolate and 75 ml 0.1M K_2HPO_4 containing 1μM dihydrofolate. Five ml fractions were collected and assayed for enzyme activity, the three fractions showing peak activity were pooled and contained a total of 19,933 units. This

Figure 34. Elution of DHFR activity of *E. coli* C600 (pBH600) on Sephadex G75 gel filtration.
Enzyme had been prepared from a large scale (10L) culture.



15 ml sample was concentrated with Amicon Centriprep 10 and Centricon 10 Concentrators to 30μl, which retained 16,499 units. Before sequence analysis this concentrate was tested for purity by reverse phase HPLC and this revealed the presence of a single protein peak.

3.4.6 N-terminal Sequence Analysis of the type IIIb Dihydrofolate Reductase

Fifteen μl of the purified sample was analysed on an Applied Biosystems 477A automatic sequencer and this gave a clear result for the first 47 amino acids (figure 35). The molecular mass of the enzyme suggested a protein of 162 amino acids, thus approximately 30% of the enzyme had been examined. The sequence obtained starts with a threonyl residue indicating that the initial f-methionyl must have been removed intracellularly. Comparing the sequence with that of the other plasmid-encoded dihydrofolate reductases (type I - V and S1) showed this enzyme was clearly distinct (figure 36). Although biochemically similar to the type IIIa enzyme, the IIIb showed no significantly greater homology with this enzyme than with many of the other plasmid-mediated dihydrofolate reductases.

3.4.7 Subunit Structure of the type IIIb Dihydrofolate Reductase

The subunit structure of the IIIb enzyme was examined by running the purified enzyme on SDS polyacrylamide gel electrophoresis. The type IIIb sample was run on a 10 - 15% Pharmacia Phastsystem SDS gel according to manufacturer's instructions. Sigma molecular weight markers (14,000 - 66,000) were run adjacent

Figure 35. Amino acid sequence of the type IIIb dihydrofolate reductase.

```
Thr Lys Glu Ala Ile Phe Ala Val Ala Glu Asn Leu Ala Phe Gly Leu Gly Gly Gly Leu Pro Trp Asp Thr Leu
                 5                  10                  15                  20                  25

Lys Asp Asp Leu Gln Phe Phe Lys Arg Leu Thr Glu Gly Thr Asp Asp Val Met Gly Ala Ser Thr
                30                  35                  40                  45
```

Figure 36. Comparison of the N-terminal amino acid sequence of the type IIIb plasmid-encoded DHFR with the other plasmid-mediated DHFR's and the E.coli chromosomal enzyme.

Identical amino acids are shown by shading. Homologous amino acids (D,E) (R,K,H) (Q,N) (F,Y,W) (I,V,L,M) are underlined. The amino acids involved in the active site are shown by the symbols at the top of the diagram: ▲ binding position of trimethoprim; △ additional binding positions of methotrexate; ■ binding positions of NADPH. Numbering is based on the IIIb sequence. Gaps have been left to maximise homology.

```
Type IIIb     T K E A I F A V A E N L A F G L G G G L - P W D T L K D D L Q F F K R L T E G T D D V M G A S T
Type IIIa     M L I S L I A A L A H N N L I G K D N L I P W - H L P A D L R H F K A V T L G K P V V M G R R T
Type IV         M I R M I L A I N N Q Y F I G K N N T L M Y - R L K D D M L N F K K M T Q N N I V V M G R K T
E.coli K-12     M I S L I A A L A V D R V I G M E N A M P W - N L P A D L A W F K R N T L N K P V I M G R H T
Type Ia       M K L S L M V A I S K N G V I G N G P D I P W - S A K G E Q L L F K A I T Y N Q W L L V G R K T
Type V        M K V S L M A A K A K N G V I G C G P H I P W - S A K G E Q L L F K A L T Y N Q W L L V G R K T
Type S1       M T L S I I V A H D K Q R V I G Y Q N Q L P W - H L P N D L K H I K Q L T T G N T L V M A R K T
Type IIa      M E R S S N E V S N P V A G N F V F P S N A T F G M G D R V R K K S G A A W Q G Q I V G W Y C T
Type IIb      M G Q S S D E A N A P V A G Q F A L P L S A T F G L G D R V R K K S G A A W Q G Q V V G W Y C T
Type IIc      M D Q H N N G V S T L V A G Q F A L P S H A T F G L G D R V R K K S G A A W Q G Q V V G W Y K T
                                  10                  20                    30                    40
```

to the sample in order to estimate its molecular mass, and the protein bands were visualised by staining with coomassie blue. After electrophoresis on the SDS gel the purified type IIIb enzyme produced a single band of protein when stained (figure 37), this corresponded to a molecular weight of 17,200. As the molecular mass of the type IIIb under conditions, which do not produce denaturation, is 17,000 this result suggests that the type IIIb dihydrofolate reductase must be a monomeric protein.

3.4.8 Purification of the type IIIc Dihydrofolate Reductase by Affinity Chromatography

The type IIIc enzyme was purified from *E. coli* JM101 (pBH7001a) incubated shaking at 37°C for 20 hours in 10 litres of Oxoid Isosensitest broth and enzyme was extracted as before. The resuspended ammonium sulphate precipitate was concentrated to 2 ml with an Amicon Centriprep 10 Concentrator. Separation by Sephadex gel filtration on a G75 column produced a clearly defined peak of enzyme activity which corresponded to a molecular mass of 23,000 (figure 38) fractions 56 to 65 were pooled and this contained a total of 28,874 units.

The pooled fractions were run onto a methotrexate column. In this case considerable dihydrofolate reductase activity was detected in the protein which initially passed through the column indicating that the enzyme had not bound. One possible reason for this was that the methotrexate column had been used several times before and it may have been that it was no longer functioning properly. Therefore a second attempt at purification was carried out with a freshly prepared methotrexate column.

Figure 37. SDS polyacrylamide gel electrophoresis of the purified dihydrofolate reductase from *E. coli* C600 (pBH600). Standard marker proteins are also shown.

Track A: Type IIIb dihydrofolate reductase

Track B: Standard marker proteins (14,000 - 66,000)

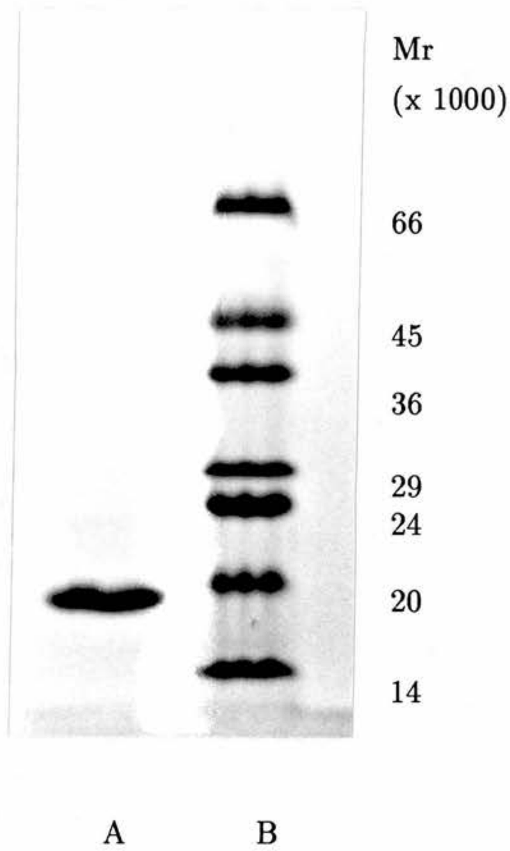
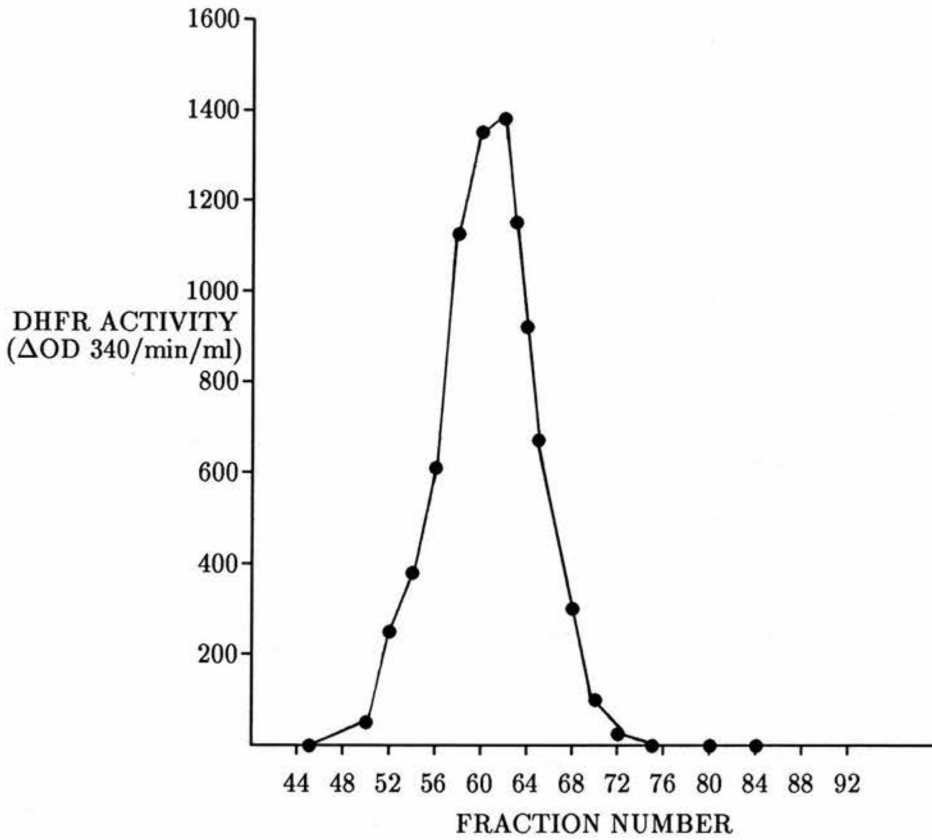


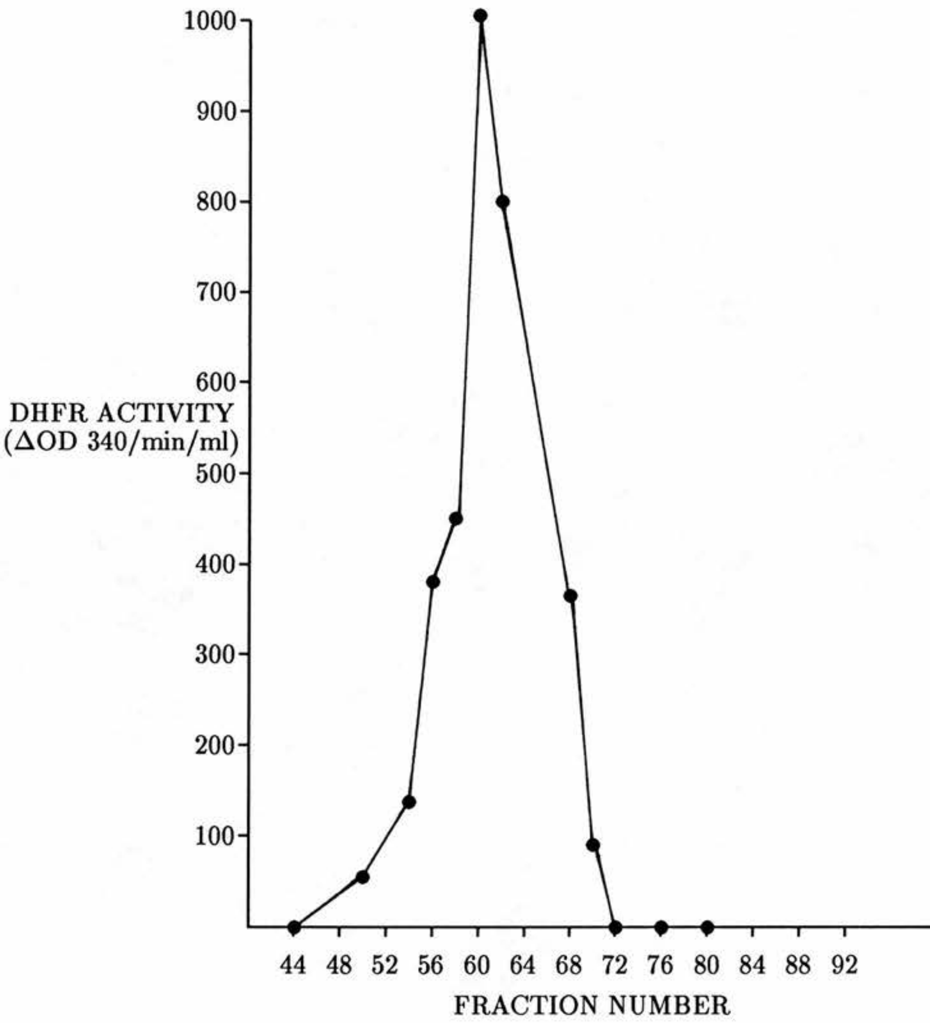
Figure 38. Elution of the DHFR activity of *E. coli* JM101 (pBH7001a) on Sephadex G75 gel filtration.
Enzyme had been prepared from a large scale (10L) culture.



An enzyme extract was prepared as before from *E. coli* JM101 (pBH7001a) and separated by gel filtration (figure 39). Pooled fractions 55 to 68 were applied onto the methotrexate column and the dihydrofolate reductase activity in the eluate which initially passed through was examined. Again considerable dihydrofolate reductase activity was found indicating that the type IIIc enzyme had again not bound to the column, as a fresh column had been used this suggested that the lack of binding must result from a peculiarity of the type IIIc enzyme. The type IIIc enzyme is as methotrexate-sensitive as the type IIIb dihydrofolate reductase (ID_{50} $0.007\mu M$) and it would thus be expected to bind. Dihydrofolate reductase which had passed through the column was assayed in the presence of methotrexate and found to be methotrexate sensitive losing 70% of its activity in the presence of $0.02\mu M$ methotrexate.

Although dihydrofolate reductase was passing through the affinity column some may have been binding and this may be sufficient for sequence analysis. A fresh methotrexate agarose column was prepared and enzyme was extracted from *E. coli* JM101 (pBH7001a) as before. The IIIc dihydrofolate reductase was separated on a Sephadex G75 column and peak fractions pooled. The pooled fractions contained 69,781 units and this was run onto the methotrexate column. The enzyme activity of the protein which had passed through without binding was assayed and this accounted for 53,025 units. The column was washed with buffer A until no more protein came off. Elution of any dihydrofolate reductase that may have bound was attempted by the addition of 2 ml 1M K_2HPO_4, 2 ml 0.5M K_2HPO_4 containing 4 μmoles dihydrofolate then 75 ml of 0.1M K_2HPO_4 containing $1\mu M$ dihydrofolate. Five ml fractions were collected and these were assayed

Figure 39. Elution of DHFR activity of *E. coli* JM101 (pBH7001a) on Sephadex G75 gel filtration.
Enzyme had been prepared from a large scale (10L) culture.



for dihydrofolate reductase activity, a small amount was found in fractions 2 and 3. These two fractions were pooled and concentrated with Amicon Centriprep 10 and Centricon 10 Concentrators to 55 μl. This was assayed for dihydrofolate reductase activity and contained 756 units, which meant that 16,000 units had been lost during the affinity chromotography step. This was probably due to the effect of the methotrexate on the enzyme as it passed through the column as the enzyme is very sensitive to this drug.

The concentrated eluate was analysed by HPLC, and four peaks were obtained. One was significantly larger than the others which were probably breakdown products of this major protein. The large peak was taken for sequence analysis but no sequence could be obtained.

The reason why the IIIc dihydrofolate reductase could not be purified by methotrexate agarose chromotography was not clear although a similar situation has been found with the methotrexate sensitive plasmid-encoded type S1 enzyme from *S. aureus* (Hartman *et al.*, 1988).

3.4.9 FPLC Purification of the Type IIIc Dihydrofolate Reductase

Because of the failure to purify the IIIc enzyme by methotrexate agarose affinity chromotography another method was tried: separation on an anion exchange column, as this can be used to purify dihydrofolate reductase (Iwakura *et al.*, 1983). Enzyme was prepared in the normal way from *E. coli* JM101 (pBH7001a) and separated on a Sephadex G75 column (figure 40), fractions 60 to 68 were

pooled and contained 16,499 units. This was concentrated to 2 ml with an Amicon Centriprep 10 Concentrator and then separated on a Mono Q anion exchange column in a Pharmacia FPLC as described in the Materials and Methods.

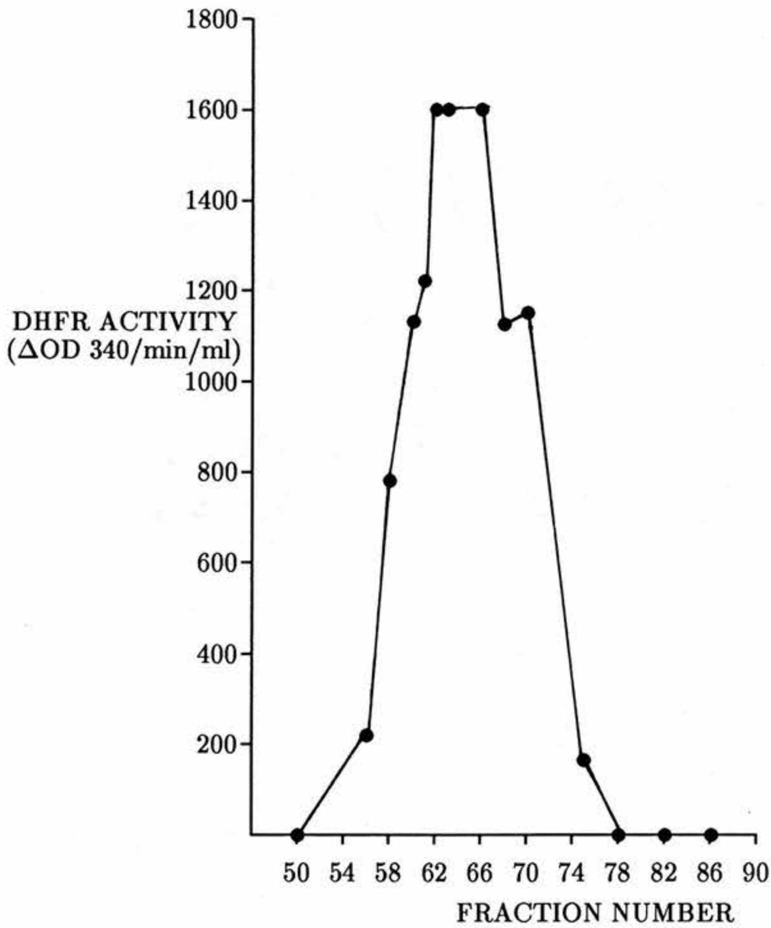
The collected fractions were assayed for dihydrofolate reductase activity but none could be detected. The reason for this may be because the IIIc was unstable in the running buffer used or it may be related to the enzyme's peculiar response on isoelectric focusing.

The inability to purify the IIIc enzyme meant that its sequence could not be determined and compared with the IIIa and IIIb. If the sequence of this enzyme is to be established it will need to be achieved from the DNA base sequence of the type IIIc gene.

C. Type V Dihydrofolate Reductase

The type V plasmid-encoded dihydrofolate reductase was identified during a survey of antibiotic resistance in Sri Lanka (Sundström *et al.*, 1987). The enzyme confers high level resistance on the host bacterium (MIC $>$ 1000 mg/L), and the novel nature of the gene, encoding the type V enzyme, was detected because of its inability to hybridise with gene probes representing dihydrofolate reductase type Ia and II. Partial biochemical characterisation of the enzyme revealed that it behaved differently on an anion exchange column from both the type Ia and II enzymes and it had about a 10-fold lower resistance to trimethoprim than the type Ia (Sundström *et al.*, 1987; Sköld, 1988). The enzyme was subsequently named the type V (Huovinen, 1987).

Figure 40. Elution of DHFR activity of *E. coli* JM101 (pBH7001a) on Sephadex G75 gel filtration.
Enzyme had been prepared from a large scale (10L) culture.



In the original study, trimethoprim resistant strains had been isolated from all over Sri Lanka and it appeared that the type V enzyme was ubiquitous. Unusually the incidence of the type Ia dihydrofolate reductase was very low (Sundström *et al.*, 1987). Before any firm conclusions regarding the origins of the type V enzyme could be made further biochemical characterisation was required.

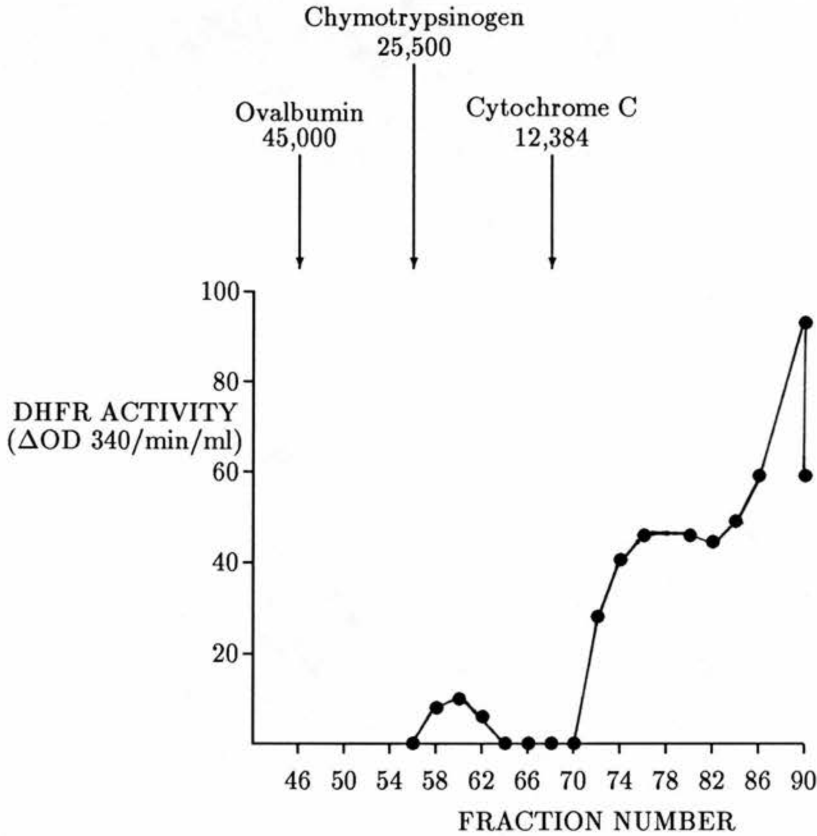
Therefore a complete biochemical profile was performed on the type V dihydrofolate reductase encoded by plasmid pLK09. This recombinant plasmid contained a 1.25 kb insert which included the trimethoprim resistance gene originally from plasmid pLM020 which was transferred from an isolate of *Enterobacter agglomerans* (Sundström *et al.*, 1987).

3.4.10 Biochemical Analysis of the Dihydrofolate Reductase Encoded by Plasmid pLK09

Dihydrofolate reductase was prepared from *E. coli* HB101 (pLK09) and the crude extract was treated with streptomycin sulphate followed by ammonium sulphate as described in the Materials and Methods. One ml of the resuspended pellet from the 80% ammonium sulphate precipitate was applied to a freshly calibrated Sephadex G75 column (90 cm x 2 cm^2).

Two ml fractions were collected and assayed for dihydrofolate reductase activity (figure 41). A small peak of dihydrofolate reductase activity was detected at fraction 60 and a rising level of activity between fractions 72 and 88. The activity at fraction 60 could be inhibited by the addition of 4μM trimethoprim, this fraction corresponds to a molecular mass of 21,000 and clearly represents the

Figure 41. Elution of DHFR activity of *E. coli* HB101 (pLK09) on Sephadex G75 gel filtration.



chromosomal enzyme.

The activity which appeared as a peak at fraction 88 was resistant to trimethoprim at a concentration of 4μM and must result from the plasmid-encoded type V enzyme. Elution from the column at this point suggests the type V enzyme is very small and an accurate molecular mass could not be obtained from a G75 column.

To establish a precise mass for this enzyme, it was prepared from *E. coli* HB101

(pLK09) as before and 1 ml of the resuspended 80% ammonium sulphate precipitate applied to a calibrated Sephadex G50 column (90 cm x 2 cm^2) and 2 ml fractions collected every 13.5 minutes.

Analysis of the collected fractions for dihydrofolate reductase activity revealed a small amount of dihydrofolate reductase at fraction 40 (not shown) which was trimethoprim sensitive and corresponded to the chromosomal enzyme. A major trimethoprim resistant peak of activity was detected at fraction 74 (figure 42) and this corresponds to a molecular mass of 2 - 3000 which is unusually low. Peak fractions were pooled and further biochemical analysis performed.

Inhibition by Trimethoprim

The activity of the type V enzyme was assayed under normal conditions but in the presence of different concentrations of trimethoprim. The results (figure 43) show that the activity was directly proportional to the logarithm of the trimethoprim concentration. The type V enzyme appears moderately resistant with an ID_{50} of $8\mu M$.

Inhibition by Methotrexate

Similarly the activity of the enzyme was assayed in different methotrexate concentrations. Again enzyme activity was proportional to the logarithm of the methotrexate concentration (figure 44). A 50% reduction in enzyme activity was achieved with a methotrexate concentration of $1.5\mu M$.

Figure 42. Elution of DHFR activity of *E. coli* HB101 (pLK09) on Sephadex G50 gel filtration. The elution points of standard marker proteins are also shown.

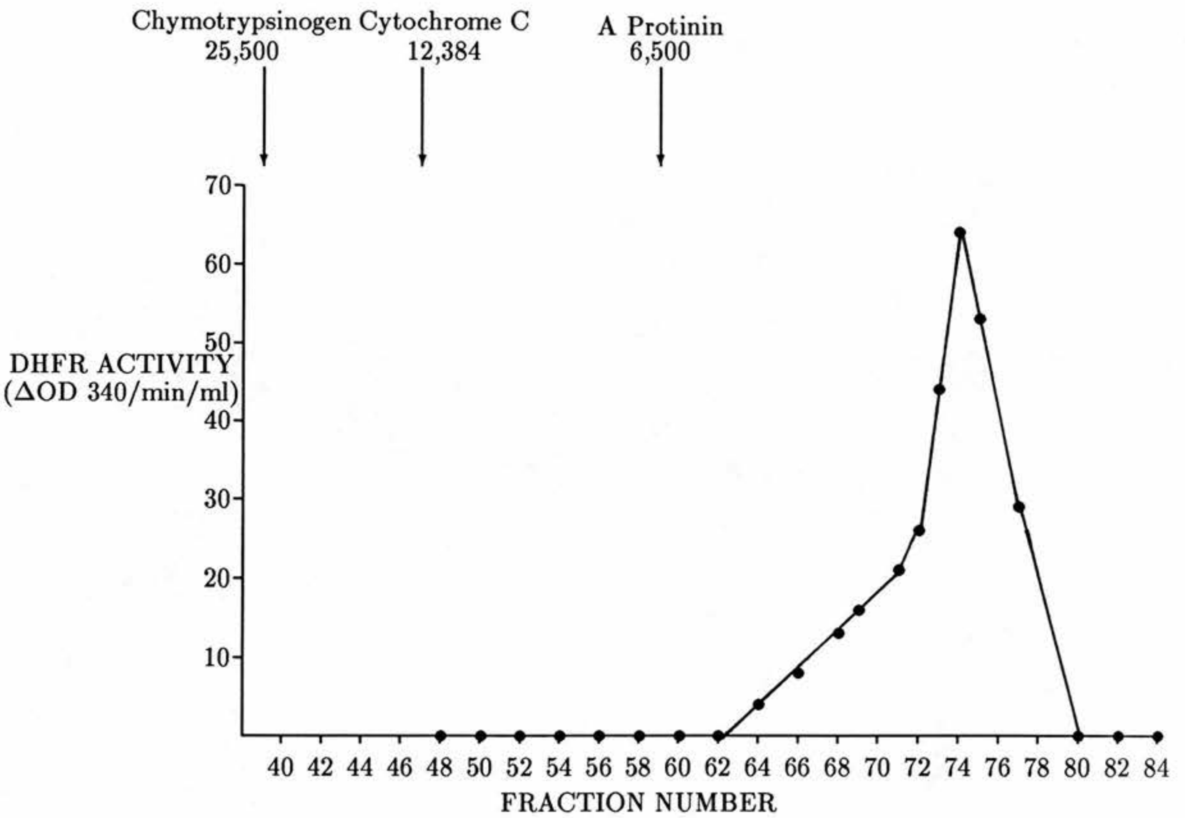


Figure 43. Trimethoprim inhibition of partially purified dihydrofolate reductase from *E. coli* HB101 (pLK09).

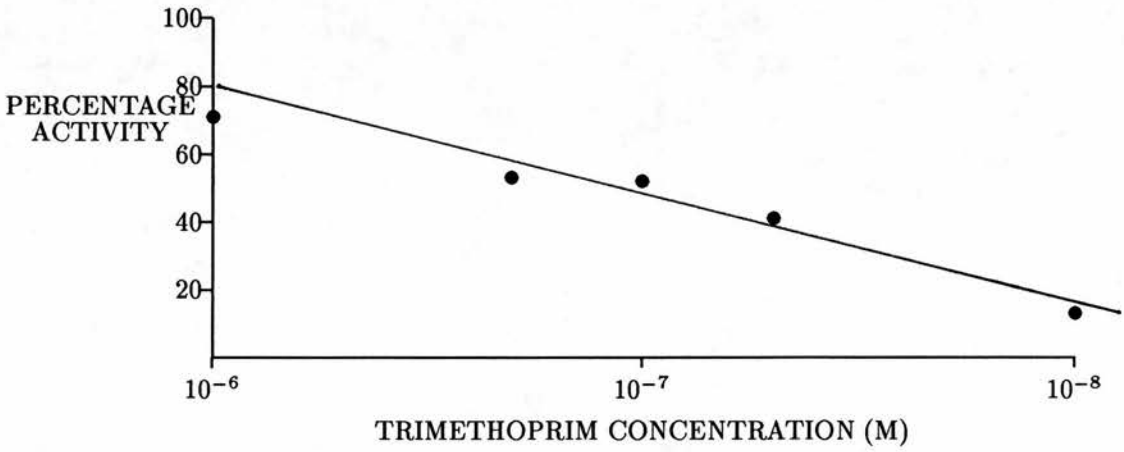
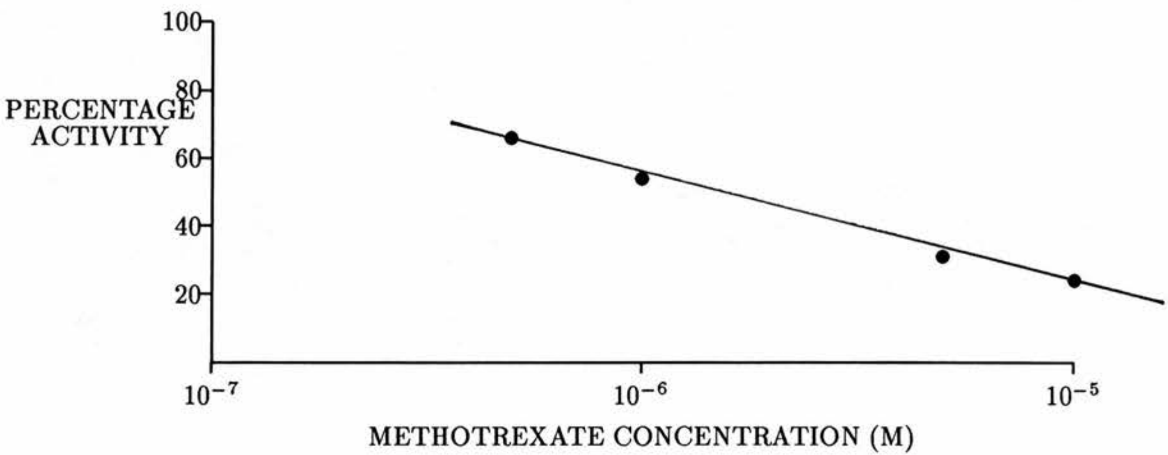


Figure 44. Methotrexate inhibition of partially purified dihydrofolate reductase from *E. coli* HB101 (pLK09).



Heat Stability

The partially purified enzyme was incubated in a prewarmed container at 45°C for various time intervals. The activity of the sample was measured. The type V enzyme was found to be heat sensitive losing half of its activity after 3 minutes at 45°C. This is in contrast to Sundström *et al.* (1987) who reported the enzyme to be heat stable maintaining 80% of its activity after 5 mins at 45°C. The difference may have resulted from the presence of stabilising protein. Therefore the heat stability of the enzyme was compared when partially purified from a Sephadex column and as a crude protein extract. The results (figure 45) demonstrated that the type V enzyme was heat stable only when a significant amount of other proteins were present.

Michaelis Menten Kinetics

The activity of the type V enzyme was assayed in the presence of decreasing dihydrofolate concentrations in the presence of different trimethoprim concentrations. The results were analysed by the method of Lineweaver and Burk (figure 46). The K_m value for the type V enzyme was $66\mu M$ indicating a moderate affinity for the substrate.

When the enzyme's activity was measured in the presence of 8 and 16 μM trimethoprim the response to decreasing substrate was also first order kinetics and the inverse plot intercepted the ordinate at the same point as the line obtained in the absence of trimethoprim. This indicates that trimethoprim inhibition of the enzyme was competitive. The type V had a mean K_i of $2.5\mu M$.

Figure 45. Heat sensitivity of the dihydrofolate reductase from *E. coli* HB101 (pLK09).

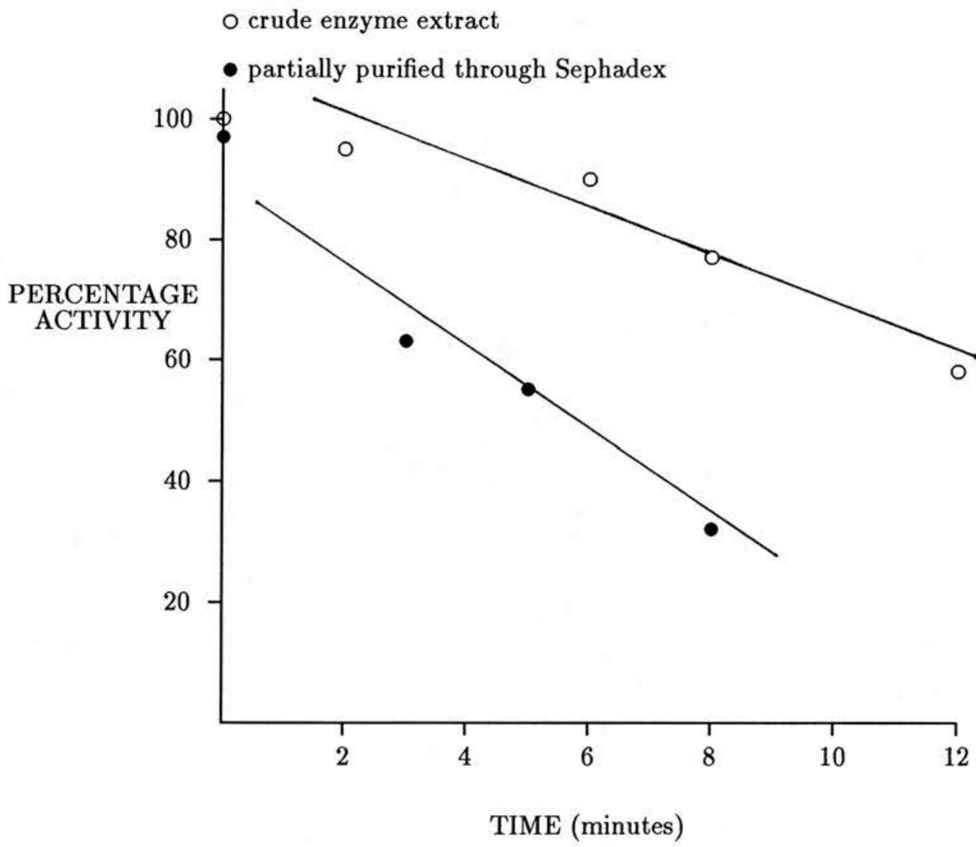


Figure 46. Lineweaver-Burk plot of the partially purified dihydrofolate reductase from *E. coli* HB101 (pLK09).
The reciprocal of the substrate concentration (DHF) (1/S) is plotted against the reciprocal of the dihydrofolate reductase activity (1/V).

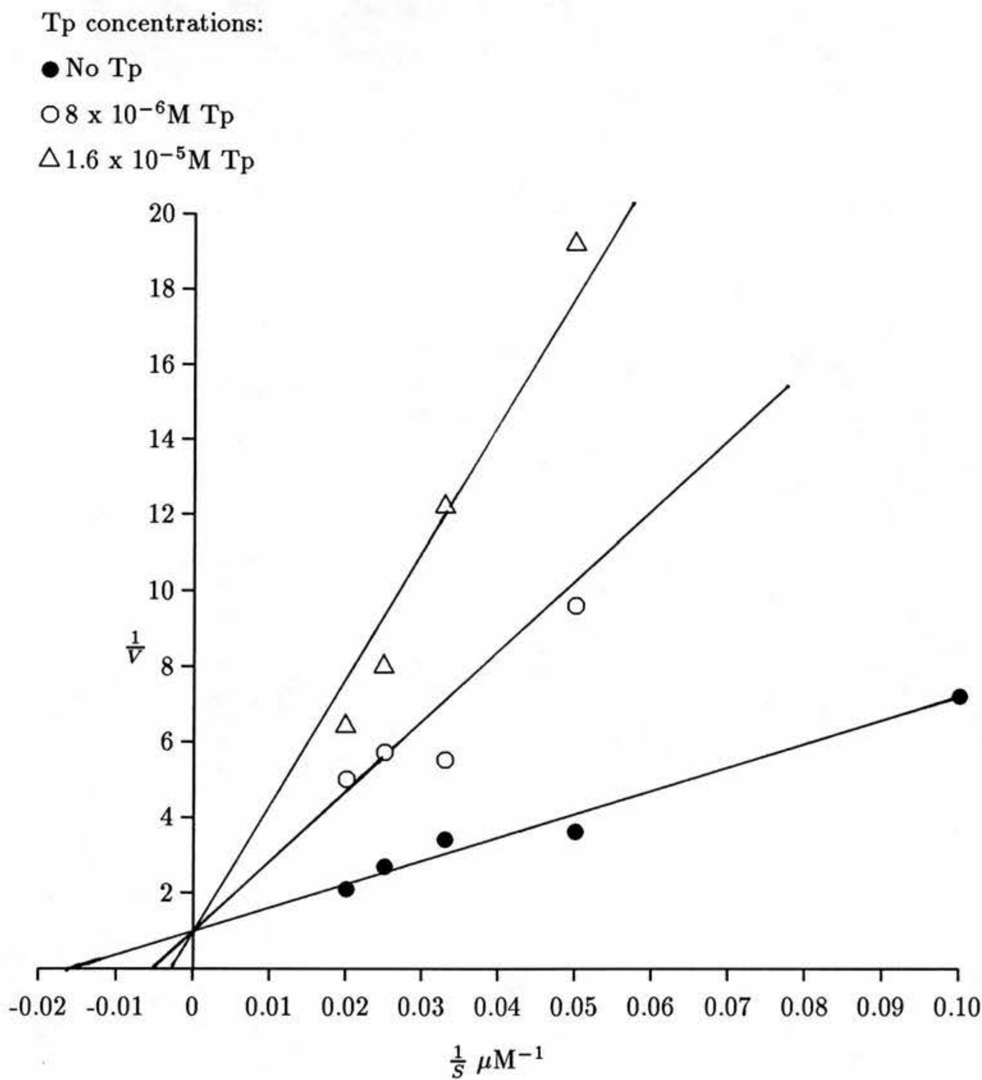


Table 31. Biochemical properties of the type V dihydrofolate reductase from *E. coli* HB101 (pLK09).

Enzyme	Tp ID_{50} μM	Mtx ID_{50} μM	TD_{50} mins	DHF K_m μM	Tp K_i μM	Size daltons
Ia	57	4.4	0.5	5.6	7.4	35000
V	8	1.5	3.0	66	2.5	3000

The results obtained for the type V enzyme were compared with the biochemical profiles of the other plasmid-mediated dihydrofolate reductases. With the exception of the low molecular mass, the type V enzyme appeared most similar to the type Ia dihydrofolate reductase (table 31). The type V enzyme had a very similar K_i, was heat sensitive and there was less than a tenfold difference in inhibition properties between the two.

If the true molecular mass of the enzyme was between 2,000 and 3,000, it was difficult to envisage how the enzyme could remain active. A more likely explanation was that the type V enzyme was, in some way, retarded in its progress through the column thus giving an artificially low molecular mass. A less likely explanation could be that, during the cloning procedure, part of the gene had been cut, resulting in a dihydrofolate reductase of lower molecular mass. However it was difficult to perceive how such a small protein could be active. Therefore in order to confirm the molecular mass and properties, biochemical characterisation was carried out on the dihydrofolate reductase encoded by another type V clone. A 489 bp *Hpa*1 fragment encoding trimethoprim resistance inserted into the *Sma*1

site of pUC18 to give pUK2000 (O. Sköld, personal communication).

3.4.11 Biochemical Properties of the Dihydrofolate Reductase Encoded by Plasmid pUK2000

The type V enzyme was prepared from *E. coli* HB101 (pUK2000) containing this recombinant plasmid and 1 ml of the resuspended pellet from the 80% ammonium sulphate precipitation was separated by Sephadex gel filtration on a G50 column. To obtain an accurate molecular mass it was necessary to have a molecular weight marker smaller than the type V enzyme. Therefore the column was calibrated with chymotrypsinogen and cytochrome C as before, but with the B chain of insulin (Mr=3878) as the low weight marker. A clearly defined peak of trimethoprim resistant dihydrofolate reductase activity was obtained at fraction 80 (figure 47) and this corresponded to a molecular mass of 5,000. The peak fractions (74 to 85) were pooled and used for further biochemical analysis.

Inhibition Properties of Trimethoprim and Methotrexate

The activity of the enzyme was measured with different trimethoprim concentrations and found to be 50% inhibited by a trimethoprim concentration of 20μM. For methotrexate the amount required to reduce the activity by half was 2.3μM (figure 48).

Figure 47. Elution of DHFR activity of *E. coli* HB101 (pUK2000) on Sephadex G50 gel filtration.

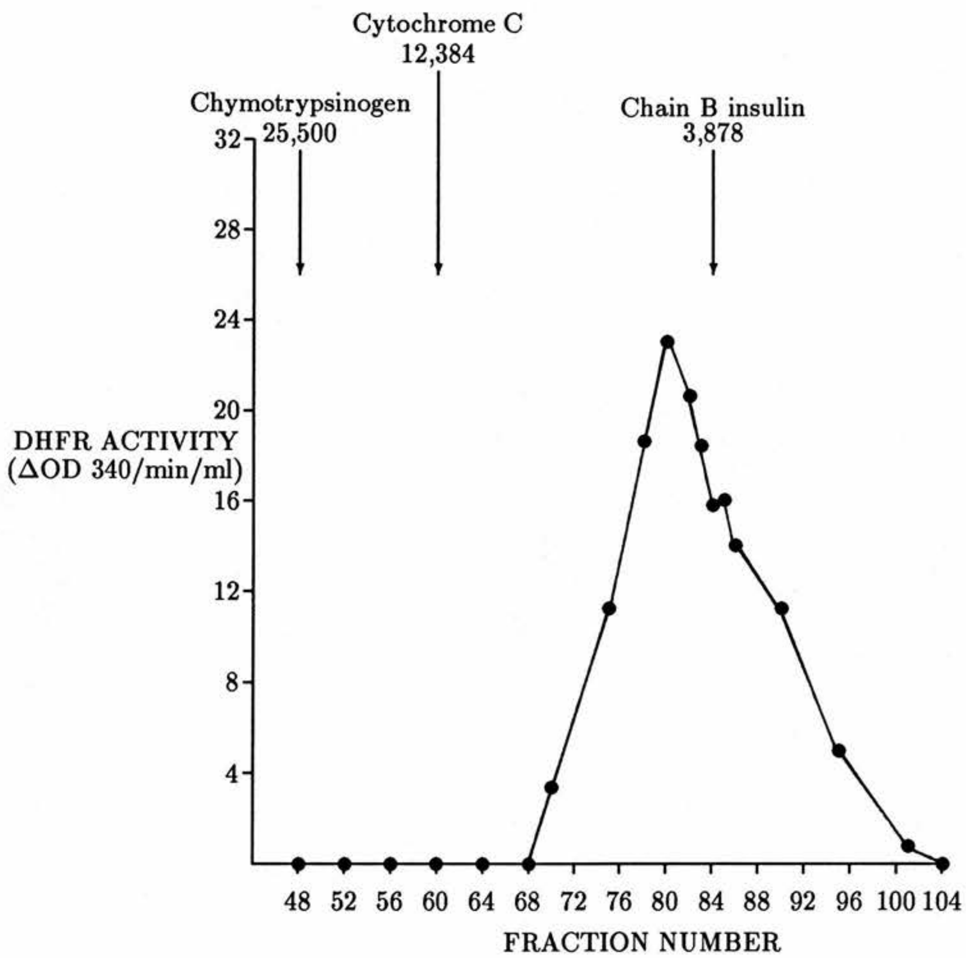
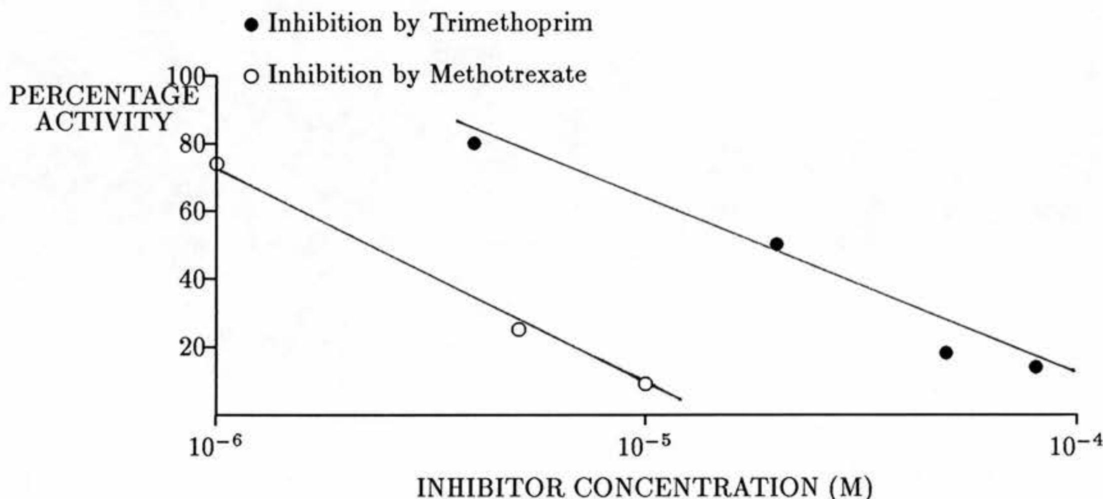


Figure 48. Methotrexate and trimethoprim inhibition of partially purified dihydrofolate reductase from *E. coli* HB101 (pUK2000).



Michaelis Menten Kinetics

The activity of the type V enzyme was assayed under limiting dihydrofolate concentrations in the presence and absence of trimethoprim. The reciprocal of enzyme activity and dihydrofolate concentration were plotted (figure 49). The K_m, for dihydrofolate, of the enzyme was calculated as $15.4\mu M$ and the K_i for trimethoprim was $3.2\mu M$.

The results obtained from the dihydrofolate reductase encoded by pUK2000 were in agreement with those from the pLK09 encoded enzyme and were very similar to the type Ia enzyme except for the apparent low molecular weight (table 32). The results confirmed that the type V must be retained in the column. Assuming the type Ia and type V are closely related it should be possible to alter the type Ia enzyme so that it is retained in the column.

Figure 49. Lineweaver-Burk plot of the partially purified dihydrofolate reductase from *E. coli* HB101 (pUK2000).
The reciprocal of the substrate concentration (DHF) (1/S) is plotted against the reciprocal of the dihydrofolate reductase activity (1/V).

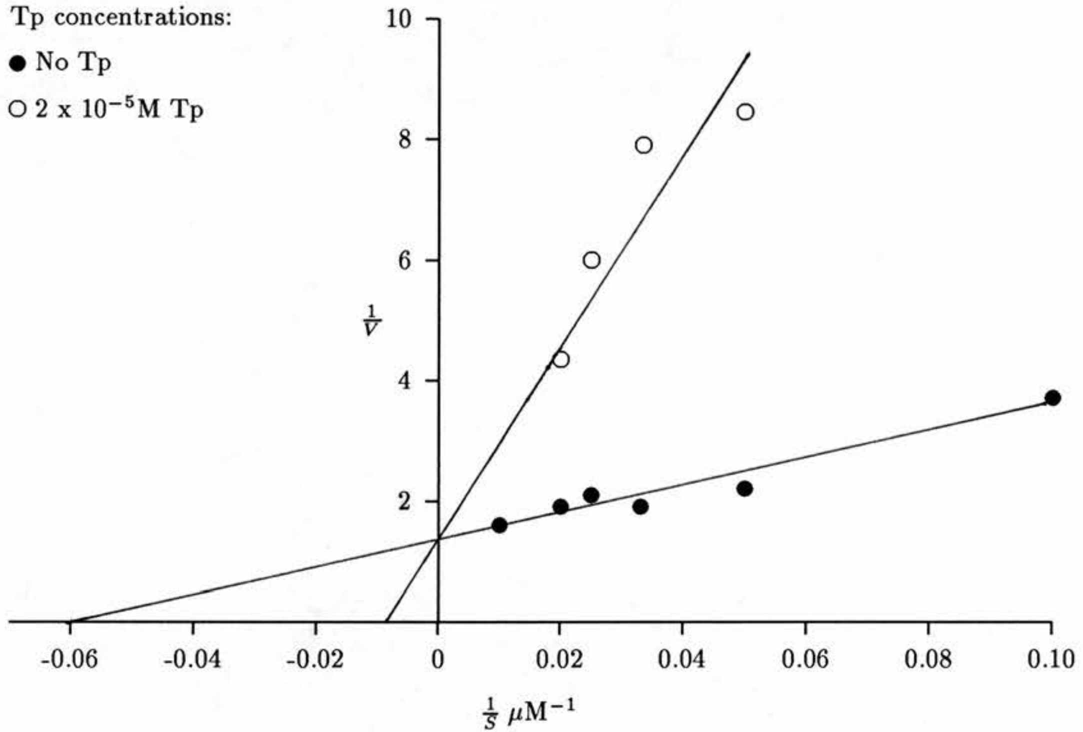
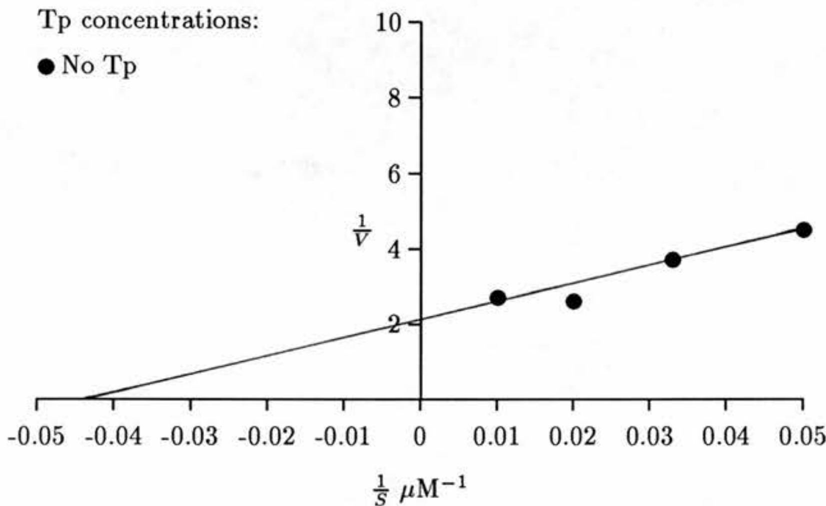


Table 32. Biochemical properties of the type V dihydrofolate reductase from *E. coli* HB101 (pUK2000).

Enzyme	Tp ID_{50} μM	Mtx ID_{50} μM	DHF K_m μM	Tp K_i μM	Size daltons
Ia	57	4.4	5.6	7.4	35000
V (pLK09)	8	1.5	66	2.5	3000
V (pUK2000)	20	2.3	15.4	3.2	5000

Figure 50. Lineweaver-Burk plot of the partially purified dihydrofolate reductase from *E. coli* C600 (pFE872).
The reciprocal of the substrate concentration (DHF) (1/S) is plotted against the reciprocal of the dihydrofolate reductase activity (1/V).



3.4.12 Preparation of the type Ia Dihydrofolate Reductase

A crude type Ia enzyme extract was prepared from *E. coli* C600 (pFE872). The activity of the enzyme was measured in limiting dihydrofolate concentrations (figure 50) and the K_m calculated to be 23μM. A sample of this crude extract was dialysed for four hours against 10mM pH 6.0 sodium phosphate buffer containing 10mM mercaptoethanol in an attempt to disrupt the enzyme, this is a lower pH and ionic strength than the normal buffer and contains no stabilising EDTA.

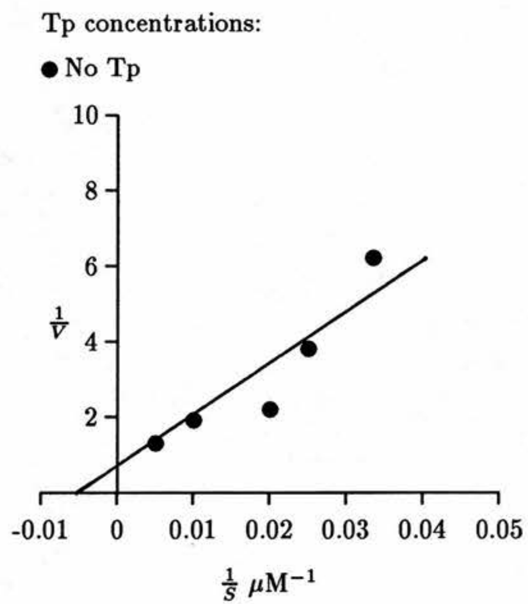
After dialysis the enzyme retained 80% of its previous activity and was still trimethoprim resistant at 4μM. However the K_m of the enzyme was 200μM (figure 51) 10 fold higher than before dialysis. Two ml of the dialysed enzyme was

run on a Sephadex G50 column which had been equilibrated with the same buffer that had been used for dialysis and 1.8 ml fractions were collected every 10 minutes. Activity was assayed from each of the collected fractions but none could be detected. This may have been because the enzyme was too unstable in the absence of EDTA to survive gel filtration. To try and overcome this problem, the experiment was repeated with 1 mM EDTA in the buffers. The same pattern was observed; there was a slight reduction in activity after dialysis but no activity could be detected after 2 ml of the dialysed sample was run through a Sephadex G50 column.

This suggested that after dialysis the type Ia enzyme must be very unstable in low protein concentrations. To examine this the effect of dilution on enzyme stability was investigated. A crude type Ia extract was dialysed against 10mM sodium phosphate pH 6.0 buffer containing 10mM mercaptoethanol and 1mM EDTA for four hours then divided into two aliquots. One of these was diluted 1 in 20 in the same buffer while the other aliquot remained undiluted. The activity in each sample was measured before and after 8 hours at +4°C. The results demonstrated that the dialysed enzyme is unstable when diluted losing 50% of its activity in 8 hours whilst the undiluted sample retained 96% of its activity. This loss of activity occurred after a 1 in 20 dilution. When an extract is run on a Sephadex column it is diluted approximately 1 in 100, as the dialysed extract is evidently unstable when diluted this would explain why no activity was detected after Sephadex gel filtration.

Although the dialysis was affecting the properties of the type Ia enzyme, the dialysed sample was too unstable to run through the Sephadex column. There-

Figure 51. Lineweaver-Burk plot of the partially purified dihydrofolate reductase from *E. coli* C600 (pFE872) after dialysis in 10mM pH 6.0 sodium phosphate buffer containing 10mM mercaptoethanol.
The reciprocal of the substrate concentration (DHF) (1/S) is plotted against the reciprocal of the dihydrofolate reductase activity (1/V).



fore another approach was tried, an undialysed sample of type Ia enzyme was subjected to gentle disruption by freezing and thawing. A 2 ml sample was then loaded onto a Sephadex G50 column equilibrated with buffer A and 1.8 ml fractions collected.

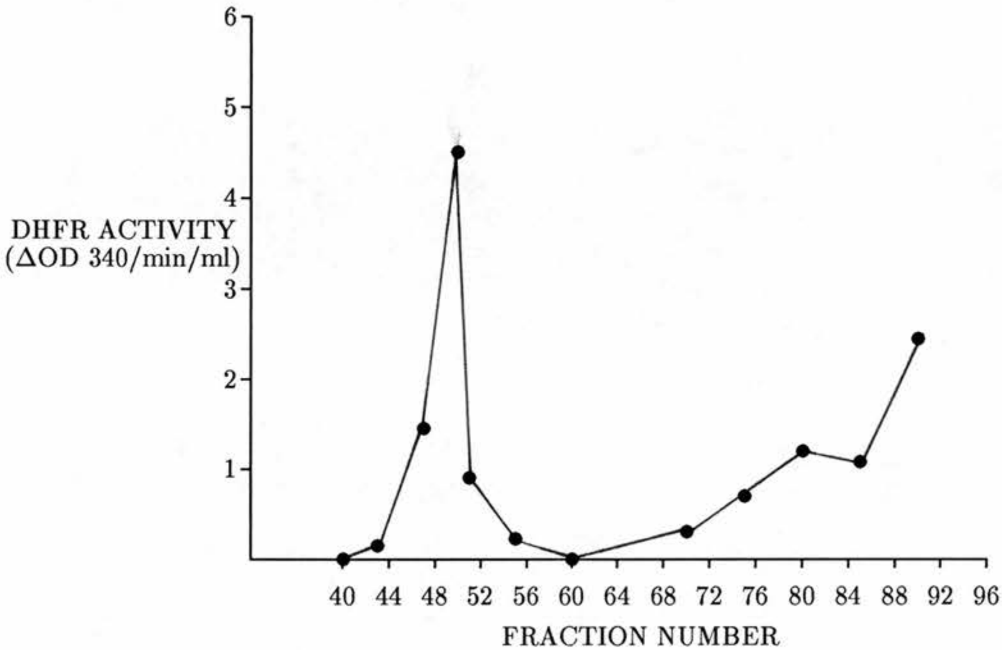
The fractions at the end of the run were assayed for dihydrofolate reductase activity (figure 52). Trimethoprim sensitive chromosomal enzyme was detected in fractions 44 to 50, however low amounts of trimethoprim resistant dihydrofolate reductase were found in fractions 72 to 90. This corresponds to a low molecular mass (less than 5000) and is similar to the type V. The result demonstrated that the type Ia and V behaved in a similar fashion and might be closely related. In addition, it demonstrated that the low molecular mass of the type V from Sephadex was not its true weight. Attempts were therefore made to measure the mass of the type V by a further method.

3.4.13 Estimations of Molecular Mass

Firstly in order to establish that the type V did not have a low molecular mass attempts were made to pass the enzyme through a filter with a molecular weight cut off of 10,000.

A crude type V extract was prepared from *E. coli* HB101 (pUK2000), after establishing that this preparation was active 5 ml were spun in an Amicon Centriprep 10 Concentrator. This has a molecular weight cut off of 10,000 and only proteins of lower mass than this can pass through the filter and are collected in the filtrate collector. After three centrifugations at 4000g the filtrate was assayed for dihy-

Figure 52. Elution of DHFR activity (after disruption by freeze thawing) of *E. coli* C600 (pFE872) on Sephadex G50 gel filtration.



drofolate reductase activity and none was found. The retentate was still active indicating that none of the enzyme had passed through and that the molecular mass of the type V was greater than 10,000.

Native PAGE

The type Ia and type V enzymes have similar biochemical properties except for their molecular mass when they are measured by gel filtration. The previous experiment had confirmed that the molecular mass of the type V, measured by gel filtration, was not its true mass. Therefore the molecular mass of the two enzymes were compared by the alternative method of native PAGE.

The type Ia and type V enzymes were prepared from *E. coli* C600 (pFE872) and

E. coli HB101 (pUK2000) respectively and partially purified with ammonium sulphate. A sample of each was diluted in bromophenol blue and loaded onto a 20% homogeneous SDS-free polyacrylamide gel. The gel was run in a Pharmacia Phastsystem until the bromophenol blue dye front had reached the end of the gel.

As the two enzymes were not purified a specific stain had to be used rather than coomassie blue. The gel was stained for dihydrofolate reductase activity as described in the Materials and Methods (figure 53). The two enzymes co-migrated which indicated that the true mass of the type V enzyme was indeed 35,000 and underlined the inherent similarities between the two enzymes. A sample of the type VII dihydrofolate reductase, which also has similar properties to the type Ia but a lower molecular mass when measured by gel filtration (Amyes *et al.*, 1989) was run alongside. However, unlike the type V dihydrofolate reductase the type VII enzyme genuinely appeared smaller than the type Ia.

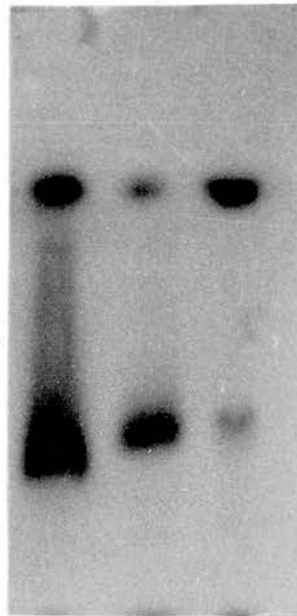
The similarity in biochemical properties between the type Ia and type V dihydrofolate reductases, including their similar molecular masses, suggested these enzymes were closely related. Subsequently the gene encoding the type V dihydrofolate reductase has been sequenced, this confirms the similarity of the two enzymes and the open reading frame predicts an enzyme with subunits of the same molecular mass as the type Ia (Sundström *et al.*, 1988).

Figure 53. SDS free polyacrylamide gel electrophoresis of partially purified dihydrofolate reductases from *E. coli* HB101 (pUK2000), *E. coli* C600 (pFE872) and *E. coli* $J53_{-2}$ (pUN835). Gel was stained by zymography as described in the materials and methods.

Track A: Dihydrofolate reductase encoded by pUN835 (type VII)

Track B: Dihydrofolate reductase encoded by pFE872 (type Ia)

Track C: Dihydrofolate reductase encoded by pUK2000 (type V)



A B C

Chapter 4

Discussion

During the twenty years that trimethoprim has been in clinical use a number of mechanisms of resistance have been characterised (Huovinen, 1987; Elwell and Fling, 1989). Of these the most important has been the plasmid-encoded production of an additional trimethoprim-resistant dihydrofolate reductase (Amyes, 1989). The development and spread of this plasmid-mediated resistance has been studied by biochemical typing of the enzymes responsible, and in recent years, a number of novel enzymes have been identified (Young and Thomson, 1990). This probably reflects not only the continuing evolution of plasmid-mediated resistance, particularly in the Third World, but also reflects more sophisticated methods for detection and distinguishing between enzymes.

The aims of this thesis have been to examine, in more detail, the biochemical properties and induction mechanism of the type IV dihydrofolate reductase and to identify and characterise any novel dihydrofolate reductases which appeared responsible for plasmid-mediated trimethoprim resistance.

4.1 Induction of the Type IV Dihydrofolate Reductase

The type IV enzyme isolated in South India in 1984, is the only plasmid-mediated dihydrofolate reductase which is inducible. Although hyperproduction of the *Escherichia coli* chromosomal dihydrofolate reductase as a resistance mechanism has been well documented (Baccanari *et al.*, 1975; Sheldon and Brenner, 1976; Smith and Calvo, 1979), in only one case has this been shown to be inducible (Tennhammar-Ekman *et al.*, 1986). The type IV dihydrofolate reductase itself confers only partial resistance on its host. Although the MIC in the host strain is dependent on the testing media (Young *et al.*, 1986b) the resistance conferred by the type IV is only moderate even in the optimum medium despite the high levels of type IV dihydrofolate reductase that can be achieved. Furthermore cell viability is greatly reduced at trimethoprim concentrations of 160 mg/L when dihydrofolate reductase production is at its maximum. This is in contrast with the β-lactamases, where inducible enzymes confer high levels of resistance (Livermore, 1987). This contrast suggested that the induction mechanism of the type IV enzyme must be unusual.

Examination of the speed at which *Escherichia coli* J62$_{-2}$, containing the type IV dihydrofolate reductase gene, responded when challenged with trimethoprim, showed that between 5 and 14 hours was necessary before high levels of dihydrofolate reductases were achieved. This contrasts with the induction of the chromosomal enzyme where increased levels of dihydrofolate reductase can be detected within three minutes of challenge with trimethoprim (Tennhammar-Ekman *et al.*,

1986).

It seemed reasonable to presume that the bacteria could not be growing before elevated levels of dihydrofolate reductase were produced, and this was confirmed by following the viable count of challenged and unchallenged bacteria over a 24 hour period. It was observed that the viable count of the challenged cells decreased for 8 hours then increased sharply. This suggested that the induction mechanism was switched on at around 8 hours and the resulting increased production of dihydrofolate reductase allowed the bacteria to overcome the inhibitory effects of the trimethoprim. Gram-staining performed in conjunction with the viable counts revealed that the drop in viable cells was accompanied by cell elongation. This was presumably resulting from the metabolic effects of trimethoprim (Pinney and Smith, 1973; Amyes and Smith, 1974c). However, after this point, the viable count increased and the cells returned to normal.

If *Escherichia coli*, containing the type IV dihydrofolate reductase gene, are allowed to grow before being challenged, no induction of the enzyme occurs. This appeared to be related to the growth curve, in that if bacteria were challenged in lag phase, high levels of enzyme were produced but this did not occur if the bacteria were challenged in logarithmic phase.

The fact that the type IV producing *Escherichia coli* is capable of growing in the presence of inhibitory levels of trimethoprim, without the necessity of enzyme induction, does suggest that the induction occurs only when the bacteria are stressed. It suggests that the resistance mechanism is therefore more akin to hyperproduction than to a classical bypass system.

Thymidine is an important antagonist of trimethoprim (Amyes and Smith, 1975) and its effect on induction is marked. A concentration of 2 mg/L is sufficient to allow growth of bacteria without induction of dihydrofolate reductase. This correlated with the observation that, in the presence of thymidine, the characteristic drop in viable count and cell elongation, seen when bacteria are challenged with trimethoprim, does not occur. This demonstrated that induction was not solely dependent on the presence of a given trimethoprim concentration. Although thymidine had a marked effect on induction, suggesting that the trigger for hyperproduction may be thymidine starvation, inhibition of the enzyme thymidilate synthetase with FUdR caused no increase in enzyme production. Clearly other factors must be involved.

One other area which is certainly important is the presence of metabolites. The MIC of trimethoprim for *Escherichia coli* $J62_{-2}$ (pUK1123) is dependent on the presence of methionine, adenine and glycine in the media (Young *et al.*, 1986b) and these are closely involved in folate metabolism (Amyes and Smith, 1974c) (figure 8). In minimal media, induction of the type IV enzyme is dependent on the presence of all three of these metabolites. In the absence of any one of them, bacteria are able to grow without inducing enzyme production. The importance of these metabolites on induction was further demonstrated by the fact that when plasmid pUK1123 is transferred to methionine-deficient strains induction does not occur. The most likely explanation for this is that, in minimal media containing methionine, glycine and adenine, the presence of trimethoprim prevents the thymidilate synthetase reaction (Amyes and Smith, 1974c). This, bearing in mind the effect of thymidine, may be the trigger for enzyme induction.

If this is the case the reason why FUdR did not cause an increase in dihydrofolate reductase production was not clear. In minimal media there was also a distinct inoculum effect.

Trimethoprim is only one of a series of antifolate agents (Sandler and Smith, 1989) and their ability to induce the type IV dihydrofolate reductase was examined. Using the same concentrations that had been used for trimethoprim no induction was obtained. This might be attributable to *Escherichia coli* J62$_{-2}$ having higher MICs to these drugs than to trimethoprim. Further studies with higher concentrations of tetroxoprim and pyrimethamine showed that induction took place with tetroxoprim. Interestingly, pyrimethamine produced no induction even though it inhibited growth at higher concentrations. It is perhaps not surprising that induction is observed with tetroxoprim as this drug is closely related to trimethoprim and has the same mechanism of action (Aschhoff and Vergin, 1979). The reason why pyrimethamine caused no increase in activity was unclear but is presumably linked to the fact that pyrimethamine is a less efficient inhibitor of bacterial dihydrofolate reductase (Burchall, 1979).

Induction systems in microbial organisms normally act quickly and, once in exponential growth, addition of the inducer rapidly elicits maximum production of the induced enzyme (Wiseman, 1975). It is clear from the results obtained with the type IV dihydrofolate reductase that this is not a classic induction system. High levels of enzyme are not apparent until several hours after challenge and induction does not occur if the bacteria are challenged in logarithmic phase. Furthermore, inducible enzymes are normally located on the bacterial chromosome, this is true not only for metabolic systems for example β galactosidase in *Escherichia coli*

(Monod *et al.*, 1962) but also for enzymes conferring resistance to antimicrobial agents such as the inducible β-lactamases (Sanders, 1989).

As well as being different because of its plasmid location, the type IV induction mechanism also differs markedly from the induction of β-lactamases, where induction is rapid, peak levels of enzyme occurring in 1 - 2 hours (Sanders, 1989), and induction leads to high level resistance (Chamberland, 1989).

Hyperproduction of chromosomal dihydrofolate reductase may be relatively well documented but this is normally the result of stably derepressed mutants, a situation also seen with β-lactamases (Chamberland, 1989; Livermore, 1987), rather than induction. In one case an inducible chromosomal dihydrofolate reductase has been described and this behaves like most inducible enzymes such as the β-lactamases. However the mechanism of induction is not yet understood (Tennhammar-Ekman *et al.*, 1986).

Investigations of the conditions affecting induction of the type IV dihydrofolate reductase revealed that induction of this enzyme appears to be a "last ditch" attempt for the host to survive. Bacteria need to be stressed before induction occurs; and the presence of a given trimethoprim concentration is not enough, suggesting that induction is a response to the metabolic effects of the trimethoprim. Probably the result of stress on the tetrahydrofolate pool although undoubtedly other factors are involved.

Bearing in mind that the type IV enzyme is only partially resistant to trimethoprim (ID_{50} $0.2\mu M$) this suggests that the resistance mechanism appears to be much more similar to a chromosomal swamping type of resistance than a classic

plasmid-mediated bypass.

How important the type IV enzyme will prove to be clinically is a matter for conjecture. The enzyme has been detected only in South India but did appear to be common. It is tempting to speculate that such an unusual resistance mechanism may be the result of conditions prevalent in the Third World. Namely frequent intermittent low dosing with trimethoprim in combination with frequent infections. It is not difficult to imagine how the type IV enzyme could be successful in these conditions especially if located on the same plasmid as other resistance genes. It is also possible that there could be carriage of the type IV gene among the commensal population. Recently bacteria containing transferable low level resistance to trimethoprim have been isolated from healthy members of the population in Nigeria (Lamikanra *et al.*, 1989). The survival of the type IV gene in the West may be hard to visualise but presumably there is the possibility that if the enzyme became continually derepressed high level resistance may be the result. However this may produce a significant drain on the energy resources of the cell.

The similarity of the resistance mechanism with the chromosomal hyperproduction suggests a close evolutionary relationship and this will be discussed later.

4.2 Biochemical Properties of Dihydrofolate Reductases

The plasmid determined dihydrofolate reductases conferring resistance to trimethoprim can be considered in two areas. Those that confer a high level of resistance to

trimethoprim (MIC > 1024 mg/L) and those which confer only a low or moderate level of resistance (MIC $\leq$ 256 mg/L).

In 1979 the first plasmid-mediated dihydrofolate reductase which conferred only a moderate level of resistance to trimethoprim was isolated (Fling *et al.*, 1982). The enzyme (type III) was encoded on plasmid pAZ1, isolated from a strain of *Salmonella typhimurium* from New Zealand, and conferred an MIC of 64 mg/L. Although it subsequently appeared that this enzyme was rare, it was argued that this was only because strains with a low level of trimethoprim resistance were assumed to be non-transferable and not studied in detail (Towner and Pinn, 1981). This view seems to have been correct as a number of plasmid dihydrofolate reductases have now been identified which confer only low level resistance.

The type III dihydrofolate reductase has biochemical characteristics distinctly different from those of the type I and II enzymes (Table 33). Compared with these two enzymes, the type III has a lower molecular mass (16,900) and is only slightly resistant to trimethoprim (ID_{50} 2μM). However despite its susceptibility to trimethoprim and the fact that the enzyme is only produced in small amounts, the type III dihydrofolate reductase can confer a significant level of resistance on its host. It is able to do this because of its high efficiency, having a K_m for dihydrofolate of only 0.4μM (Joyner *et al.*, 1984). The gene encoding the type III enzyme has been sequenced and shows the enzyme to be related to Gram-negative chromosomal enzymes (Fling *et al.*, 1988).

The use of DNA probing has revolutionised the study of plasmid-mediated dihydrofolate reductases (Towner, 1990). The use of such probes has meant that

Table 33. Biochemical properties of the plasmid dihydrofolate reductases.

Enzyme	Tp ID_{50} μM	Mtx ID_{50} μM	TD_{50} mins	DHF K_m μM	Tp K_i μM	Size daltons	MIC mg/L
Ia	57	4.4	0.5	5.6	7.4	35000	> 1000
Ib	32	2.8	1.2	11	41	24500	> 1000
IIa	70000	1100	> 12	4.6	6100	35000	> 1000
IIb	80000	750	> 12	8.3	150	35000	> 1000
IIc	20000	1000	> 12	4.2	400	34000	> 1000
IIIa	2.0	*0.2	* > 12	0.4	0.019	16900	64
IIIb	2.0	0.2	> 12	9.5	0.4	17000	128
IIIc	3.0	0.007	8	3.1	0.5	22000	256
IV	0.2	0.02	> 12	37	0.063	46700	10
V	20	2.3	‡—	15.4	3.2	†5000	> 1000
VI	200	7.25	0.4	31.25	75	10000	> 1000
VII	30	3.0	1.5	20.0	7.0	11500	> 1000
S1	50	0.002	> 12	10.8	11.6	19700	> 1000

* established from *E. coli* pFE1242

† apparent molecular weight by Sephadex gel filtration

‡ dependent on protein concentration

large numbers of trimethoprim resistant strains (including those with only low level resistance) can be screened rapidly and strains containing any potentially novel and interesting dihydrofolate reductases can be identified for more detailed examination (Towner, 1990).

Escherichia coli Tm858, isolated from the urine of a hospitalised patient in Nottinghamshire in 1980, was one such strain. Although this strain and the resultant transconjugant were highly resistant to trimethoprim (MIC $>$ 1024 mg/L), purified plasmid DNA showed no hybridisation with those gene probes known to identify high level trimethoprim resistance genes, i.e. dihydrofolate reductase types I, II and V. However when the trimethoprim resistance gene had been subcloned into pUC18 to give the recombinant plasmid pUN972 and this was introduced into *Escherichia coli* HB101, the MIC was only 64 mg/L. Hybridisation of this plasmid with all available plasmid dihydrofolate reductase probes gave a positive result only with the original type III dihydrofolate reductase encoding plasmid, pAZ1. Biochemically the dihydrofolate reductase encoded by plasmid pUN972 was indistinguishable from the pAZ1 derived enzyme. The molecular weight and Michaelis Menten kinetics of the enzyme encoded by pUN972 were similar to those published for the type III. A direct comparison of the inhibition profiles of the enzymes from plasmids pAZ1 and pUN972 was necessary as the methotrexate and heat sensitivities of the type III from pAZ1 had never been published before, and this revealed that the two enzymes were similar. The final evidence that the two enzymes were indistinguishable was obtained from the isoelectric focusing, refined in this thesis. The two enzymes could not be separated by isoelectric focusing.

It is clear that the enzyme isolated in Nottingham was a type III. One interesting feature of this work is the apparent reduction in the MIC to trimethoprim of the strain carrying the cloned type III enzyme compared to the original isolate. One possible explanation is that a second dihydrofolate reductase gene was present in the original strain and that this masked the presence of the low level type III determinant. As no hybridisation was obtained with probes representing dihydrofolate reductase types I, II and V any high level resistance gene must presumably be specifying a novel enzyme.

The fact that a type III enzyme was present in Nottingham in 1980, barely a year after the initial isolation of this class of enzyme in New Zealand, does suggest that it is much more widespread than was at first realised. The reasons why it may have gone unnoticed are not only that strains with low level resistance were often not studied but, as in this case, the gene may be masked by the presence of another. The ubiquitous type I dihydrofolate reductase has been found in the same isolate as a type II enzyme (Fling *et al.*, 1982; Burchall *et al.*, 1982) so there is no reason why it could not also be present with the type III.

Dihydrofolate reductases conferring low level trimethoprim resistance in two outbreaks of Shigella have also been studied. Trimethoprim resistance in Shigella is now a major problem in many parts of the world, particularly developing countries (Murray *et al.*, 1985; Farrar, 1985; Elwell and Fling, 1989; Dupont, 1989). This is not only because antibiotic usage is higher and less well regulated in these countries, but also because the conditions enabling the epidemic spread of Shigella clones are near perfect. Overcrowded housing, inadequate sewage and water systems and communal cooking facilities all contribute towards the spread

of infection. Trimethoprim resistance in Shigella is not unique to the Third World although, in the West, it does tend to be confined to specific subpopulations; American Indians on reservations, geriatric hospitals and nurseries for example (Blaser *et al.*, 1983; Tauxe *et al.*, 1986; C.D.C., 1987a, 1987b; Griffin *et al.*, 1989).

The two outbreaks of trimethoprim resistant *Shigella sonnei* that have been examined in this thesis both occurred in the United States. One at a nursing home involved 60 people the second at the rainbow family gathering in the Smokey Mountains National Park involved 6000. In the latter case the outbreak was almost certainly the result of the use of communal kitchens, open pit latrines and untreated drinking water (C.D.C., 1987b). Both strains were only moderately resistant MIC 128 mg/L and 256 mg/L respectively and again, initial hybridisation studies demonstrated that the two genes responsible were not of the commonly occurring types and were different from one another. Biochemical characterisations of both enzymes were performed (table 33) and the results suggested that the two enzymes were indeed different from one another but showed similarities to the type III. Consequently, the two enzymes were named IIIb (nursing home) and IIIc (Rainbow Family) and the original type III named IIIa.

In general, the importance of transmissible low level resistance to trimethoprim has not been studied. It is interesting that, like the original type IIIa enzyme, these low level genes have been found in enteric infections. This could be related to the fact that lower levels of trimethoprim are found in the gut during treatment than in the urine. As studies in dogs have shown that after trimethoprim administration much higher levels occur in the urine than in the gut although this does even out over several days (Schwartz and Ziegler, 1969). Although the

type IIIa has been isolated from a urinary tract infection it is important to bear in mind that another high level resistance gene may also have been present.

The discovery of novel trimethoprim resistance genes was not surprising as other studies have demonstrated the occurrence of trimethoprim resistance genes in Shigella which did not hybridise with probes for the commonly occurring enzymes (Chatkaeomorakot *et al.*, 1987). However, in this case this was not followed up by detailed biochemical analysis of the enzymes responsible. The ability of antibiotic resistant clones of Shigella to spread (Frost *et al.*, 1985) presumably means that a novel resistant enzyme can become widespread in a relatively short space of time. It is therefore imperative that the situation in Shigella is monitored closely, not simply for short term treatment strategies, but also to guard against the development and dissemination of further resistant enzymes.

High level resistance to trimethoprim has been dominated by the spread of the type Ia dihydrofolate reductase (Steen and Sköld, 1985). Being located on transposon Tn7 (Barth *et al.*, 1976) enabled this gene to spread efficiently. It is now found integrated in the chromosome as well as on plasmids (Towner *et al.*, 1980; Amyes *et al.*, 1986b) and has been identified outwith the enterobacteria in *Vibrio cholerae* (Young and Amyes, 1986b). However recently a number of other enzymes have been detected which confer high level resistance to trimethoprim.

The first of these, the type V dihydrofolate reductase, was identified during a survey of antibiotic resistance in Sri Lanka in 1985 (Sundström *et al.*, 1987). Probing had been used to screen isolates and, unusually, a very low incidence of type Ia had been found. Examination of the biochemical properties of the type V enzyme

produced an unusual result. Although the majority of the properties of the type V enzyme were like those of the type Ia (table 33) the enzyme had a very low apparent molecular weight when measured on Sephadex gel filtration. Examination of the molecular mass of the type Ia, under different conditions, suggested that the low molecular mass was an artefact. Indeed, this was confirmed by native polyacrylamide gel electrophoresis, which showed that the type V and type Ia enzymes were the same size. These results have subsequently been confirmed by sequence analysis of the type V gene which revealed that it has 75% amino acid homology with the type Ia enzyme and the two subunits have the same predicted molecular mass (Sundström *et al.*, 1988).

There may, in fact, be a number of these high level resistant enzymes which are related to the type Ia. Young and Amyes (1985) characterised a transposon mediated enzyme with similar biochemical properties to the type Ia, interestingly this also had a lower molecular mass. Whether this was a genuine mass or an artefact like the type V is not clear. The recently identified type VII enzyme follows this same pattern (Amyes *et al.*, 1989), although in this case the lower molecular mass does appear genuine as on native polyacrylamide gel electrophoresis the type VII appears smaller than the type Ia. It is also thought that novel type I like enzymes may be responsible for trimethoprim resistance in *Vibrio cholerae* (Ouellette *et al.*, 1988). The reason why some of these enzymes are atypically retarded by Sephadex gel filtration, and appear smaller than they should, is unclear but could be related to a greater number of hydrophobic amino acids.

The type V enzyme originally isolated in Sri Lanka has subsequently been detected in Europe; in Finland (Heikkilä *et al.*, 1990) and in the United Kingdom

(Towner *et al.*, 1990) and it seems likely therefore that this enzyme has been present for some time. Before recent refinements in probing techniques, it would undoubtedly have registered as a type Ia and as probes become more discriminatory it may transpire that there is a vast range of these type I-like enzymes conferring high level resistance. This is certainly suggested by a recent study in Finland which reported 40% of isolates with high level resistance were not due to dihydrofolate reductase type I, II or V (Heikkilä *et al.*, 1990). The finding of the type VII in an animal isolate also suggests these enzymes are widespread (Amyes *et al.*, 1989).

The type II dihydrofolate reductases also confer high level resistance but sequence analysis has revealed these enzymes have a distinctly different origin from the type Ia enzyme (Stone and Smith, 1979; Zolg and Hänggi, 1981; Flensburg and Steen, 1986). Recently a novel dihydrofolate reductase, designated type VI, has been isolated in South Africa. This enzyme is similar in size to a type II subunit and shows high level resistance to trimethoprim and methotrexate like the type II (Wylie *et al.*, 1988). It is not as yet clear if the type II and VI are closely related. A dihydrofolate reductase suspected of being a type II, from probing studies, has been identified in *Vibrio cholerae*. However the enzyme only confers a low level of resistance (MIC 125 mg/L) and biochemical confirmation of this enzyme as a type II is awaited (Tabtieng *et al.*, 1989).

4.3 Sequence Analysis of Plasmid Encoded Dihydrofolate Reductases

The results of sequence analysis represent the ultimate information in the study of the evolution of antibiotic resistance genes. As discussed, plasmid-mediated dihydrofolate reductases have been divided into a number of classes based on their biochemical profiles. But, in addition, as some of the genes responsible have also had their DNA nucleotides or the amino acids of the gene product sequenced, the classification of these enzymes now also takes into account amino acid differences (Stone and Smith, 1979; Zolg and Hänggi, 1981; Fling and Richards, 1983, Flensburg and Steen, 1986; Fling *et al.*, 1988; Sundström *et al.*, 1988; Rouch *et al.*, 1989). The amino acid sequences of the chromosomal dihydrofolate reductases from several bacterial species have also been established (Gleisner *et al.*, 1974; Freisham *et al.*, 1978; Smith and Calvo, 1980; Baccanari *et al.*, 1984; Hartman *et al.*, 1988; Iwakura *et al.*, 1988).

As the number of different enzyme types has increased, sequence analysis has become even more important as some of these enzymes have very similar biochemical properties, for example the types Ia and V. If determination of the enzymes sequence is to become a crucial feature in the distinction of plasmid-encoded dihydrofolate reductases, an alternative to DNA nucleotide sequencing is required since it is too lengthy a process for the routine study of antibiotic resistance. In this thesis rapid sequence analysis was performed using an automatic amino acid sequencer. Two properties make dihydrofolate reductases particularly suitable for this kind of analysis. Firstly most of the active site is situated at the

N-amino terminal end of the protein and therefore a single sequence analysis provides enough information to look at evolutionary relationships (Novak *et al.*, 1983; Rouch *et al.*, 1989). Secondly in many cases the enzyme is easily purified with the use of methotrexate agarose and a native protein, sufficient for sequencing, can be obtained in a few purification steps (Kaufman, 1974).

The known sequences of the plasmid-mediated dihydrofolate reductases are shown in figure 54 along with the sequences of several bacterial chromosomal dihydrofolate reductases. This sequence information can be exploited to evaluate two features, firstly the evolutionary relationships between proteins and secondly the structure-function relationships.

If we first consider the evolutionary relationships: it is obvious when looking at figure 54 that the majority of the enzymes show some degree of homology with each other. The exceptions are the type II group of enzymes, which are clearly different and must have originated from a different source. They show little homology with any other dihydrofolate reductase and their origin remains a mystery. It has been speculated that they have evolved from a Gram-negative chromosomal oxidoreductase (Elwell and Fling, 1989) but this remains unresolved. The recent identification of the type VI dihydrofolate reductase may shed some light on the origins of the type II's but, as yet no sequence analysis on this new enzyme has been performed (Wylie *et al.*, 1988). Likewise the discovery of a type II enzyme which confers only a low level of resistance may provide some answers as to the origin of these enzymes (Tabtieng *et al.*, 1989).

There was a great deal of interest in the sequence of the type Ia dihydrofolate

Figure 54. N-terminal sequence similarities between the plasmid and bacterial chromosomal dihydrofolate reductases. Regions of maximal homology (6 or more) amino acids, identical and homologous, are shown by shading. The amino acids involved in the active site are shown by the symbols at the top of the diagram: ▲ binding position of trimethoprim; △ additional binding positions of methotrexate; ■ binding positions of NADPH. Numbering is based on the Ia sequence. Gaps have been left to maximise homology.

```
Type Ia        M K L S L M V A I S K N G V I G N G P D I P W - S A K G E Q L L F K A I T Y N Q W L L V G R K T
Type IIIa      M L I S L I A A L A H N N L I G K D N L I P W - H L P A D L R H F K A V T L G K P V V M G R R T
Type IIIb      T K E A I F A V A E N L A F - G L G G G L P W D T L K D D L Q F F K R L T E G T D D V M G A S T
Type IV          M I R M I L A I N N Q Y F I G K N N T L M Y - R L K D D M L N F K K M T Q N N I V V M G R K T
Type V         M K V S L M A A K A K N G V I G C G P H I P W - S A K G E Q L L F K A L T Y N Q W L L V G R K T
Type S1        M T L S I I V A H D K Q R V I G Y Q N Q L P W - H L P N D L K H I K Q L T T G N T L V M A R K T
E.coli K-12      M I S L I A A L A V D R V I G M E N A M P W - N L P A D L A W F K R N T L N K P V I M G R H T
S.aureus         T L S I L V A H D L Q R V I G F E N Q L P W - H L P N D L K H V K K L S - - - - - - - - - - -
L.casei            T A F L W A Q N R D G L I G K D G H L P W - H L P D D L H Y F R A Q T V G K I M V V G R R T
S.faecium        M F I S M W A Q D K N G L I G K D G L L P W - R L P N D M R F F R E H T M D K I L V M G R K T
B.subtilis       M I S F I F A M D A N R L I G K D N D L P W - H L P N D L A Y F K K I T S G H S I I M G R K T
N.gon        M L K I T I I A A C A E N L C I G A G N A M P W - H I P E D F A F F K V Y T L G K P V I M G R K T
Type IIa       M E R S S N E V S N P V A G N F V F P S N A T F G M G D R V R K K S G A A W Q G Q I V G W Y C T
Type IIb       M G Q S S D E A N A P V A G Q F A L P L S A T F G L G D R V R K K S G A A W Q G Q V V G W Y C T
Type IIc       M D Q H N N G V S T L V A G Q F A L P S H A T F G L G D R V R K K S G A A W Q G Q V V G W Y K T
                                 10                  20                    30                  40
```

reductase encoded by Tn7, as for many years this was the predominate resistance determinant associated with high level trimethoprim resistance (Steen and Sköld, 1985). The type Ia enzyme shows significant homology with the *Escherichia coli* chromosomal enzyme, 29% direct amino acid matching, and the inclusion of chemically similar residues increases this to 44% (Fling and Richards, 1983). The recently identified type V enzyme, which is biochemically similar to the type Ia is clearly closely related as sequence studies have revealed that the two enzymes show a greater than 75% amino acid homology (Sundström *et al.*, 1988). It seems likely, therefore, that these two genes have a common ancestry and it would be interesting to find out if the type Ib and VII genes show similar homology.

The type IIIa dihydrofolate reductase sequence suggests that this gene originated in *Escherichia coli* or a closely related Gram-negative organism, as the amino acid sequence shows 51% identity with the *Escherichia coli* chromosomal dihydrofolate reductase and 44% with the *Neisseria gonorrhoeae* enzyme (Fling *et al.*, 1988). The type IIIb dihydrofolate reductase isolated in *Shigella sonnei* shares many properties with the type IIIa enzyme, including the fact that it is a monomeric protein. Indeed the type IIIa and IIIb dihydrofolate reductases are only distinguishable by isoelectric focusing. This suggested that the enzymes may be very closely related differing in only one or two amino acids. However, sequence analysis of the N-terminal end of the IIIb has shown that this is not the case. As expected the type IIIb showed no homology with the type II enzymes, but amongst the other plasmid-mediated enzymes the type IIIb showed no greater homology with the type IIIa than any of the other enzymes. Bearing in mind the similarity in biochemical properties between the types IIIa and

IIIb dihydrofolate reductases this is very surprising, suggesting that there must be some evolutionary advantage in the selection of enzymes with this particular biochemical profile.

Induction studies with the type IV dihydrofolate reductase had suggested that the resistance mechanism involving this enzyme was more similar to a chromosomal hyperproduction system than a classic resistant bypass. It was not surprising therefore that sequence analysis of the first 49 amino acids revealed this enzyme to be closely related to the *Escherichia coli* chromosomal dihydrofolate reductase. Indeed 40% direct amino acid matching was found and homology was increased to 58% when homologous amino acids were considered. Sequence analysis of the type IV dihydrofolate reductase also revealed that it was complexed to NS1, an *Escherichia coli* DNA binding protein, and that this contributed to the high molecular mass of this enzyme. The reason why the type IV enzyme is complexed with a DNA binding protein is not clear but may be related to the induction mechanism, since proteins which bind to DNA have been implicated in the control of gene expression (Ptashne, 1986, 1988; Magasanik, 1988).

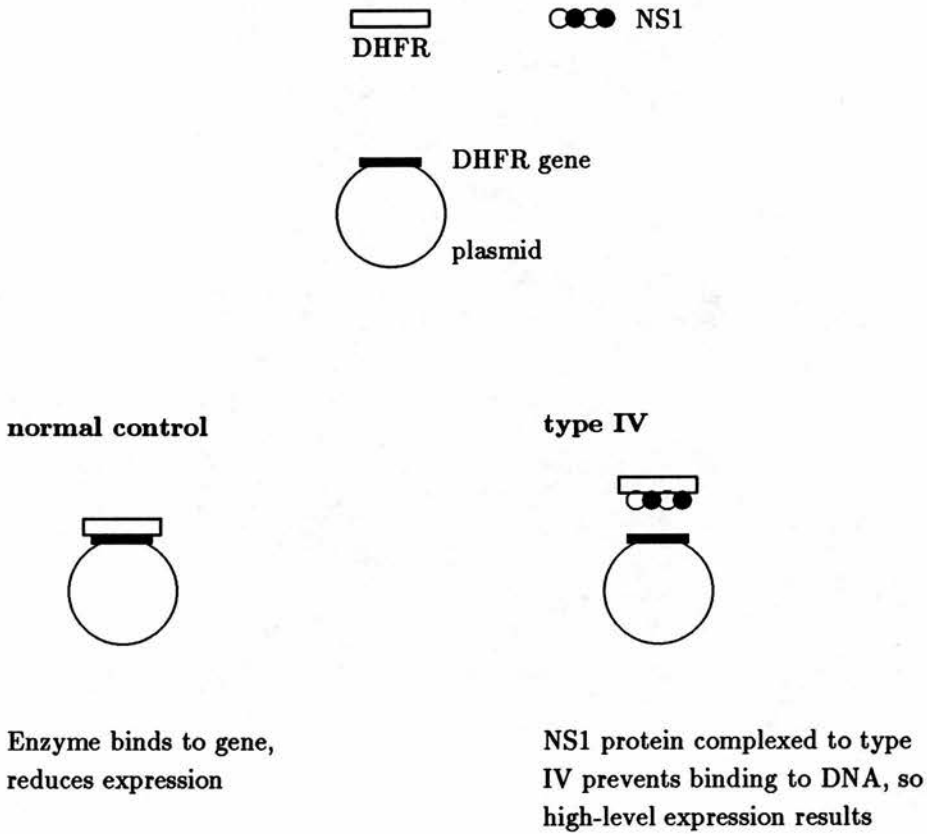
Interestingly the lower molecular mass cloned type IV enzyme, which is non-inducible, is not complexed with NS1. The absence of binding protein in the enzyme from the cloned gene probably results from the lack of NS1 gene in the restriction fragment cloned into pBR322. As this plasmid is a multicopy vector, the necessity for induction of the type IV to produce trimethoprim resistance has been removed, and thus the selection pressure for the NS1 protein gene was not present in the cloning process.

How production of the type IV enzyme is regulated by the DNA binding protein remains unclear but a number of scenarios are possible including gene activation by binding at specific sites (Ptashne, 1986, 1988). Another intriguing possibility may involve the NS1 protein interfering with a dihydrofolate reductase DNA complex. It has been shown that dihydrofolate reductase binds to DNA, and preferentially binds to the DNA that codes for its own production (Gronenborn and Davies, 1981). And it has been speculated that this may play a physiological role *in vivo*, most likely dihydrofolate reductase reduces the level of expression of its own gene (Gronenborn and Davies, 1981). It is possible therefore that because the type IV enzyme is complexed with NS1 it cannot bind to DNA and therefore high expression results (figure 55).

Plasmid-mediated trimethoprim resistance in Staphylococci has only recently become a problem and at the moment one enzyme, the type S1, predominates (Tait and Amyes, 1989; Amyes and Tait, 1990). The gene encoding this enzyme has been sequenced (Rouch *et al.*, 1989). The gene shows a degree of homology with the plasmid-mediated dihydrofolate reductases from Gram-negatives with the exception of the type II's.

The origin of plasmid-mediated antibiotic resistance genes has remained a mystery. In the case of the plasmid-mediated trimethoprim resistant dihydrofolate reductases, this may now be partially resolved. The plasmid-encoded type S1 dihydrofolate reductase in Staphylococci shows closest similarity with the chromosomal dihydrofolate reductase in *Staphylococcus aureus* (Rouch *et al.*, 1989; Hartman *et al.*, 1988; Amyes and Tait, 1990). Similarly, the type IV dihydrofolate reductase shows closest similarity to the *Escherichia coli* chromosomal enzyme

Figure 55. Possible control mechanism for the type IV dihydrofolate reductase.



and, following the Staphylococcal example, appears to be derived directly from it.

Indeed the sequence analysis and biochemical results suggest that the type IV, type Ia, type IIIa, IIIb and V may all have originated from the *Escherichia coli* chromosome. These enzymes could be classified in the same evolutionary path. On the other hand, the type II enzymes, and perhaps time will show the type VI, have followed a completely different evolutionary pathway.

The use of sequence analysis to examine evolutionary relationships among the plasmid-mediated dihydrofolate reductases has provided some answers but inevitably raised new questions. It may eventually raise questions about how in fact these enzymes should be classified. Should enzymes with the same biochemical properties but different sequences be classified in the same subtype or should sequence analysis be the ultimate determinator. This situation is not unique to the dihydrofolate reductases but is currently being debated for the classification of the TEM derived β-lactamases conferring resistance to third generation cephalasporins (Payne, 1990).

The protein sequences can also be used to examine structure-function relationships. The biochemical properties of the plasmid-mediated dihydrofolate reductases are well documented and they vary considerably between the enzyme types. By comparing the sequences with properties it may be possible to correlate changes in sequence with changes in biochemical properties. The residues involved in the active site binding of methotrexate trimethoprim and NADPH have been elucidated (Bolin *et al.*, 1982; Filman *et al.*, 1982; Matthews *et al.*,

1985a) and changes at these points would be particularly interesting. Comparing sequences with biochemical features has enabled the basis of methotrexate sensitivity to be elucidated. All methotrexate sensitive enzymes have an aspartate at residue 28 while in methotrexate resistant proteins a homologous glutamate is present (Fling and Richards, 1983). The type IIIb and IV enzymes are both methotrexate sensitive and each has an aspartate at residue 28.

Alas with trimethoprim binding there seems no such pattern as the residues binding trimethoprim seem highly conserved among the plasmid-mediated enzymes, despite their varying sensitivities to inhibition (figure 54). The only exceptions are the type II enzymes, which are highly resistant to both methotrexate and trimethoprim, which have different residues at the crucial positions. The different sensitivities to trimethoprim in the plasmid-mediated dihydrofolate reductases (other than the type II) may be derived from conformational changes affecting the accessibility of trimethoprim into the active site and this may, of course, involve widely dispersed residues.

However, changes in amino acid sequence leading to resistance have been correlated for isoenzymes of chromosomal dihydrofolate reductases. In one case a single amino acid change from leucine to arginine at position 45 was responsible for a greater resistance to trimethoprim and lower binding of the inhibitor (Baccanari *et al.*, 1981). In another strain a change in the trimethoprim binding site at position 21 from proline to serine lead to increased resistance (Smith and Calvo, 1982). Furthermore use of site directed mutagenesis has revealed the importance of specific hydrophobic residues on ligand binding and catalysis, including phenylalanine at position 31 which is highly conserved among the plasmid-mediated

enzymes (Benkovic *et al.*, 1988).

As mentioned, at present there seems no clear pattern among the plasmid-mediated dihydrofolate reductases accounting for differences in trimethoprim susceptibility. As more information is obtained hopefully such a pattern may emerge and may enable a more rational approach to antifolate drug design. As mentioned in the introduction trimethoprim like compounds continue to be developed and tested for antibacterial activity (Roth *et al.*, 1988, 1989; Johnson *et al.*, 1989). Unfortunately, all studies have, as yet, failed to address themselves as to whether these new compounds can also inhibit the plasmid-encoded trimethoprim resistant enzymes as well as inhibiting the chromosomal dihydrofolate reductase. Unless they can, then there is no real advance over trimethoprim.

4.4 Conclusions

Trimethoprim was the first antimicrobial agent that was specifically designed as a fully synthetic chemotherapeutic agent. It was argued that resistance would be less of a problem as there would be no naturally occurring enzymes which could destroy the drug. While it is true that trimethoprim is not subject to drug inactivation, the bypass mechanism of resistance which evolved is no less efficient. Furthermore the other resistance mechanisms, though of lesser importance, should not be forgotten.

Although resistance to trimethoprim has compromised the use of this drug it is encouraging that overall resistance is low and with carefully monitoring it should remain at this level. However the extensive use of trimethoprim in developing

countries should be watched closely as the high levels and incidences of resistance in pathogenic species is a worrying trend.

The rapid increase in the number of trimethoprim resistant dihydrofolate reductases results from not only the continuing evolution of resistance, but can also partly be attributed to more discriminatory methods of studying these proteins. While questions have been answered many more have been generated and there is still much more to learn.

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C-108 Two Outbreaks of Dysentery Caused by Antibiotic Resistant Shigella sonnei (ARS) Elaborating a Type III Dihydrofolate Reductase (DHFR). N.BARG*, F.HUTSON, S. AMYES, C.THOMSON, M.WHARTON, and W.SCHAFFNER. Vand. Univ. Med. School Nash. TN, Univ. of Edinburgh Med. School Edin. UK., and The Centers for Disease Control, Atlanta, GA.

Bacteria producing a plasmid encoded Type III DHFR enzyme are rare, noted once in a strain of Salmonella typhimurium from New Zealand. ARS isolated from nursing home patients (NH) in 1985 and from campers in a national park (NP) in 1987 were analyzed by plasmid profiles and biochemically. Each one contained the 120 MDa virulence plasmid and 2 (NP) or 3 (NH) small cryptic plasmids. Both strains contained a transferable plasmid (NH 65 MDa, NP 60 MDa) bearing different antibiotic resistance determinants, but both plasmids conferred resistance to trimethoprim (Tp) and sulfamethoxazole. Restriction enzyme cleavage of the two R-plasmids (different incompatibility groups) produced dissimilar patterns. Transformants (NH plasmid DNA) contained the 65 MDa plasmid. The MIC of Tp for both Shigella isolates was 64 μg/ml, the same as strains producing DHFR III. Purified enzyme from C600 transconjugants of each ARS yielded the same biochemical profile as the other, and of a type III DHFR: molecular weight=17 kDa, Tp ID_{50}=2μM, Mtx ID_{50}=.02 μM, DHF K_m=2.8 μM, Tp K_i=0.15 μM, and TD_{50}=12.5 min. We think that both of these ARS elaborate a Type III DHFR and that its gene, being found on 2 different replicons, may have originated from a transposable element.

C-109 Microbial Aetiology of Acute Gastroenteritis among Children in Kuwait. S.K. SETHI* and F.A. KHUFASH. Faculty of Medicine, Kuwait University, Kuwait.

Microbial aetiology of infectious gastroenteritis in 621 hospitalized children was studied during a period of 15 months. A specific microbial diagnosis could be made in 79% of the 621 children. In 5.3% of the 152 controls, one or more enteropathogens could be detected. Rotavirus was the major aetiologic agent present in 278 of 621 (44.7%) patients and 3.3% of controls. Salmonella sp. were the second most important enteropathogens detected in 126 patients (20.3%) - either singly or multiply alongwith other enteropathogens. Campylobacter jejuni and enterotoxigenic Escherichia coli constituted 7.6 and 7.7% respectively while Shigellae and enteropathogenic Escherichia coli were present in 3.0 and 3.4% of the patients. Aeromonas hydrophila was isolated from stools of 7(1.1%) patients. Y.enterocolitica could not be isolated from any of the stool specimens inspite of using both enrichment and selective techniques. It is concluded that rotaviruses and Salmonella sp. account for the major proportion of incidence of gastroenteritis in children in Kuwait. A large number of children with gastroenteritis were infected with more than one enteropathogens. Clinical symptoms and duration of hospital stay of the patients varied according to the associated enteropathogens.

C-110 Survey of Patients with Bloody Diarrhea on the Navajo Indian Reservation using Culture Techniques and DNA Probes for Detecting Bacterial Pathogens. N. STROCKBINE*[1], N. PUHR[1], M. VENKATESAN[2], N. SHAFFER[1], R. TAUXE[1], J. WELLS[1], and I. K. WACHSMUTH[1]. [1] CDC, Atlanta, Georgia and [2] WRAIR, Washington, D.C.

Shigella species have been presumed to be the major cause of bloody diarrhea on the Navajo Indian Reservation. To determine whether other bacterial pathogens are also important, stool swabs from 45 patients with bloody diarrhea were collected and studied for the presence of bacterial pathogens by culture techniques and DNA probes. Colonies were hybridized with the IpaC probe for Shigella/enteroinvasive Escherichia coli (EIEC) and with the SLT-I and SLT-II probes for Shiga-like/Verotoxigenic E. coli (SL/VTEC). Shigella species were recovered from 22 specimens and SL/VTEC was recovered from 1 specimen. Twenty-one of the Shigella isolates were identified by culture and 21 were identified by the IpaC probe. One Shigella was detected by culture that was not identiied by the probe and one was detected by the probe that was missed by culture. No EIEC were identified; Campylobacter species were isolated from four specimens. Recognized or putative bacterial pathogens were recovered from 60% (27/45) of the specimens. These data show that Shigella and, to a lesser extent, Campylobacter are significant causes of bloody diarrhea in this population.

C-111 Etiology of Acute Diarrhea in American Embassy Personnel in Cairo, Egypt. R.L. HABERBERGER, JR.(1), I.A. MIKHAIL[1], J.K. PODGORE[1], C.R. LISSNER[1], L. KEMP[2], D. SPEES[2], J.C. GLENN[1], and J.N. WOODY[1]. [1]U.S. Naval Medical Research Unit No.3, Cairo, Egypt and [2]Health Unit, American Embassy, Cairo, Egypt.

During a two year period, one-hundred and thirty eight American Embassy personnel or their dependents were enrolled in an acute diarrhea study. From the 138 patients we isolated or identified 33 bacterial pathogens, 6 pathogenic parasites including cryptosporidia and 3 cases where we detected rotaviral antigen. We looked at Enterotoxigenic E. coli, Enteroinvasive E. coli and Enteroadherent E. coli during this investigation in approximately thirty percent of the specimens examined. We found Enterotoxigenic E. coli to represent 31% of all pathogens, Shigella sonnei (9.5%), Campylobacter spp (5%), Rotavirus (2%), Shigella boydii (1%), Salmonella Group C_2 (1%), Shigella dysenteriae (1%), Aeromonas hydrophila (1%) and Plesiomonas shigelloides (1%). Susceptibility to twelve antibiotics including cinoxacin, norfloxacin, and furazolidone were performed by the Bauer-Kirby method. All bacterial enteropathogens were universally susceptible to the quinolones, including nalidixic acid. All isolates, with the exception of Salmonella Group C_2 were susceptible to furazolidone. (Supported by NMRDC, Bethesda, MD, Work Unit No. 3M161102BS.AK.311).

C-112 Gastroenteritis Outbreaks in Nursing Homes. F.Y.C. LIN*, C. GROVES, H. WASSERMAN, B.P. LIM, P.POWERS, E.ISRAEL, K.MIDTHUN, K.Y. GREEN, A.Z. KAPIKIAN, Maryland Department of Health and Mental Hygiene, Baltimore, Md. National Institutes of Health, Bethesda Md.

From July 1981 to April 1987, a total of 122 outbreaks of gastroenteritis (GEO) in nursing homes were reported to the Maryland State Health Department. 101 (83%) GEOs occurred between November and March as compared with 21 (17%) between April and October. Salmonella was confirmed as the cause in 8(6.6%) outbreaks. Among them 6 were detected in the warm period (Apr-Oct) while only 2 were confirmed in the cold months (Nov-Mar) ($p < 0.01$). Non-bacterial GEOs in the winter months were characterized by self-limited diarrhea/vomiting of 24-48 hours duration; mean attack rate of 31% for residents, 20% for employees; median duration of outbreak of 7 days. The epidemic curves suggest person-to-person spread as the mode of transmission. 60 stool specimens and 54 paired sera were collected from residents during outbreaks in 7 nursing homes between Dec. 86 and Apr. 87, and were studied for the presence of enteric pathogens, rotavirus, Marin Co. Agent and Norwalk virus. All were negative for Salmonella, Shigella and Campylobacter. Only 1 out of 54 paired sera showed seroresponse to Marin Co. Agent. The role of Norwalk Agents and rotavirus are under current investigation. Our findings indiciate non-bacterial GEO is prevalent in winter months in nursing home residents. Further studies for etiological agents are warranted.

C-113 Diarrheal Illness in a Day Care Center Caused by Enteropathogenic Escherichia coli (EPEC) Serogroup O114. C.A. BOPP*, L.K. GILJAHN, V. HUNDLEY, E.G. SOWERS, T.J. BARRETT, J.G. WELLS, and I.K. WACHSMUTH, Centers for Disease Control, Atlanta, GA and Ohio Department of Health, Columbus, OH.

In July 1987, an outbreak of severe, often chronic, diarrheal illness occurred in a day-care center in Ohio. Twelve of 24 children from 6 weeks to 10 months old were ill. Escherichia coli serotype O114:NM was isolated from 5 of the 12 ill children. The organism adhered to HeLa cells, was positive with the EPEC adherence factor (EAF) probe, and was resistant to ampicillin, carbenicillin, cephalothin, tetracycline, sulfisoxazole and trimethoprim-sulfamethoxazole. It was negative when assayed for heat-labile and heat-stable enterotoxins, invasiveness, Shiga-like toxins I and II, and diffuse adhesiveness. Only serotype O114:H2 has been considered a traditional EPEC within the O114 serogroup, and it was thought to be EAF-negative, or a class II EPEC. On the basis of virulence-associated characteristics, antimicrobial resistance and isolation from infants with chronic diarrhea, we believe that O114:NM should be considered a class I EPEC serotype.

BIOCHEMICAL PROPERTIES OF THE TYPE V PLASMID-ENCODED TRIMETHOPRIM-RESISTANT DIHYDROFOLATE REDUCTASE

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Initially, plasmid-encoded trimethoprim-resistance in clinical enterobacteria resulted from either one of two genes encoding trimethoprim-resistant dihydrofolate reductases (DHFR), type I and type II. However, recently a number of new plasmid-encoded DHFRs have been identified (Huovinen, 1987), distinguishable from one another by their biochemical properties (Amyes, 1986). One of these recently identified enzymes, designated type V, was discovered during a survey of antibiotic resistance among enterobacteria in Sri Lanka (Sundstrom et al, 1987). The enzyme was initially identified on the basis that its gene did not hybridise with a type I or type II DHFR gene probe. However, DNA sequence analysis of the type V DHFR gene predicts an enzyme similar to the type I (Sköld, 1988). To elucidate where the type V fits into the classification of the plasmid-encoded DHFRs, its full biochemical profile has been established.
Enzyme preparation was carried out from 2 litre overnight broth cultures as described by Young & Amyes (1986) and enzyme assays were determined by the method of Amyes & Smith (1974). The molecular mass (Mr) of the enzyme was measured by gel filtration on a Sephadex G-50 column ($2cm^2$ x 90cm) employing chymotrypsinogen, cytochrome c and the B subunit of insulin as standards. Its heat sensitivity was determined at 45°c in a prewarmed container and, after the requisite time, the sample was cooled on ice and the enzyme activity assayed (Young & Amyes, 1986).
A 489 base pair HpaI DNA restriction fragment, containing the whole gene for the type V DHFR, had been cloned into the vector plasmid pUC18 and the enzyme was extracted from *E. coli* containing the resultant hybrid plasmid. The concentration of trimethoprim required for 50% inhibition of the enzyme was 23uM and of methotrexate was 3.5μM. The enzyme had moderate affinity for the substrate dihydrofolate (Km = 15.5μM) and it was competitively inhibited by trimethoprim (Ki = 3.2μM). In the presence of high protein concentrations, the type V enzyme was relatively heat stable, maintaining more than 50% of its activity after 12 minutes at 45°c. When the stablising protein was removed, the enzyme became more heat labile (TD_{50} = 3min). However, the Mr of the type V DHFR was unusually low at 5000.
Although the type V DHFR gene did not hybridise with the type I gene probe, it has a nucleotide sequence similar to the type I gene (Sköld, 1988). Our results show that the inhibition properties of the type V would also classify it as a type I DHFR. However, the most striking feature of the type V was its low Mr, far lower than the type I or any other plasmid-encoded DHFR. These results suggest that the type V enzyme may have resulted from a mutation in the type I DHFR gene, resulting in an enzyme containing little more than the active site. The sequence of the type V predicts an enzyme of 157 amino acids although the Mr suggests an enzyme of only 40 amino acids. It may be that a mutation has inserted a stop codon after 120 base pairs in the open reading frame which prevents further transcription. The similarity of the biochemical properties, of the resultant enzyme, to those of the type I DHFR suggests that a large portion of the protein of the type I enzyme may be unnecessary.

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A-72 Tetracycline Resistance (tet) Gene Expression Increases Aminoglycoside Uptake without Altering Bacterial Transmembrane Potential. T.L. MERLIN*, G.E. DAVIS, and J.K. GRIFFITH. Albuquerque VA Med. Ctr. and Univ. of NM Sch. of Med., Albuquerque, NM.

Extrachromosomal tet genes are common among gram-negative bacteria. Their expression not only confers tetracycline resistance but also enhances susceptibility to commonly-used aminoglycoside antibiotics by increasing their uptake. The mechanism of this increased uptake of aminoglycosides is unknown. Aminoglycoside uptake is usually proportional to the transmembrane potential ($\Delta\Psi$) above a definable threshold. We investigated the relationship between ^{3}H-gentamicin uptake and $\Delta\Psi$ in E. coli (HB101) strains expressing tet at various levels. $\Delta\Psi$ was measured by the distribution of ^{3}H-tetraphenylphosphonium. ^{3}H-gentamicin uptake increased in proportion to the level of tet expression and was blocked by dinitrophenol, which dissipates $\Delta\Psi$. Regardless of the level of tet expression, however, $\Delta\Psi$ was identical in all strains. This indicates that tet expression increases aminoglycoside uptake, not by a mechanism that affects $\Delta\Psi$ directly, but possibly by one which reduces the $\Delta\Psi$ threshold.

A-73 Transfer of the Conjugative 25.2 Mdal Tetracycline Resistance Plasmid from Neisseria gonorrhoeae and Related Species to the Genus Haemophilus. M.C. Roberts*. University of Washington, Seattle. WA.

High-level tetracycline resistance in strains of Neisseria gonorrhoeae, N. meningitidis, Kingella denitrificans, and Eikenella corrodens has been described. The resistance in each species is due to the acquisition of 25.2 Mdal conjugative plasmids that carry the tetracycline resistance determinant TetM. We have shown that these plasmids as well as the B-lactamase plasmids can be transferred by conjugation to a variety of N. meningitidis serogroups and commensal Neisseria species at detectable conjugation frequencies ($> 10^{-9}$). Because these organisms can be found in the same environment as the genus Haemophilus, we determined whether the 25.2 Mdal plasmids could be transferred and maintained in Haemophilus strains. Both the B-lactamase and 25.2 Mdal plasmids could be transferred into Haemophilus influenzae and H. parainfluenzae. This suggests that the 25.2 Mdal plasmid could become established in clinical isolates of Haemophilus and supports the hypothesis that it is very likely that clinical isolates from other genera will at some time acquire the 25.2 Mdal plasmid.

A-74 **Identification of a Large R-Plasmid and Cloning of a Tetracycline Resistance Gene from the Fish Pathogen *Vibrio salmonicida*.** H. SØRUM[1,3]*, M. C. ROBERTS[2], K. FOSSUM[1] and J. H. CROSA[3]. Norw. Col. Vet. Med[1]., Oslo, Norway, Univ. of Washington[2], Seattle, WA, and Oregon Health Sciences Univ.[3], Portland, OR.

Vibrio salmonicida is the causative agent of Hitra disease or cold water vibriosis in farmed Atlantic salmon in Norway. From the late seventies when the disease was first observed, diseased fish have been treated with antibacterial drugs, mainly oxytetracyclin In 1985 the first isolates of the organism that were resistant to tetracycline and the diagnostically used vibriostatic agent O/129 were identified. Fifty six percent of 250 strains isolated from diseased fish along the whole coast of Norway in 1987, were found to be resistant. A large, low copy number plasmid, about 270 kb in size, was isolated from resistant isolates. A tetracycline resistance gene from the plasmid was cloned into the vector pACYC177, mapped and subcloned, resulting in a 1.8 kb fragment containing the gene. The cloned gene hybridized with the class E tetracycline (tetE) determinant described from *Aeromonas hydrophila* and *Escherichia coli* but no corresponding restriction sites have been found. As part of a transcription study the gene was mutated by introducing a stop linker into it. The cloned gene was expressed in *E. coli*, but it has so far not been possible to transfer the large R-plasmid from *V. salmonicida* to *E. coli* or *V. anguillarum by conjugation*. This is the first drug resistance gene isolated from the fish pathogen *V. salmonicida*.

A-75 Analysis of the marA region of Escherichia coli. S. P. COHEN*, H. HAECHLER, and S. B. LEVY. Tufts University School of Medicine, Boston, MA.

Chromosomal multiple antibiotic resistant (Mar) mutants of E. coli require an intact marA region at 34 min on the chromosome (identified by a Tn5 insertion). This region has been isolated and subcloned into a low copy plasmid vector. A fragment of 7.8 kb permits Mar mutants to arise in cells bearing a chromosomal marA deletion. The plasmid clone expresses a single RNA transcript of 1.4 kb whose expression increases at least 50 fold in the resistant mutant. The same RNA species is expressed by chromosomal Mar mutants. A region of 2 kb surrounding the Tn5 insertion has been sequenced, revealing a single transcription unit containing three open reading frames. These were preceded by a strong promoter and two 11 bp complimentary sequences which contain internal dyad symmetry. One is located between the putative -35 and -10 regions and the other just downstream from the putative transcription start site. These sites may serve as binding sites for a transcriptional regulator. The sequence of this promoter region in the opposite direction contains possible overlapping start signals. This organization is strikingly similar to that of the gram negative extrachromosomal tetracycline resistance determinants. Further sequence analysis should identify the change from marA$^+$ to marA.

A-76 **Characterization and Significance of 2 Novel Dihydrofolate Reductases (DHFR) from Trimethoprim (Tp) Resistant *Shigella sonnei*.** N. BARG*, F. HUTSON, L. WHEELER, C. THOMPSON, and S. AYMES. Vanderbilt University Medical School, Nashville, TN and University of Edinburgh, Edinburgh, UK.

Two strains of Tp resistant *S. sonnei* bearing R-plasmids pBH600 and pBH700 elaborated a DHFR. These enzymes had been partially characterized and were most like a Type III DHFR based on $TpID_{50}$ and molecular radius. By agar dilution, each enzyme conferred moderate resistance to Tp with MIC's of 128 ug/ml and 512 ug/ml respectively. Further characterization was provided by DNA hybridization and isoelectric focusing. Subcloning the Tp resistance gene from the R-plasmids indicated that the genes resided on an 1800 bp *PstI* fragment of pBH600 and a 1600 bp *PstI* fragment of pBH700. Probes generated from these fragments did not hybridize to each other or to Types I, II, or III genes on the plasmids pFE872, pUC4-12, and pFE1242. Types I, II, and III probes did not hybridize to pBH600 or pBH700. Isoelectric focusing showed distinct pI's for the enzymes coded for on pBH600 (5.3) and pBH700 (6.5). Neither pI was the same as the Type III enzyme (6.1). Isolates from 8/13 other outbreaks of Tp resistant *S. sonnei* hybridized to the pBH700 derived probe and only 1/13 to the pBH600 derived probe. 2/13 hybridized to the Type I probe and 2/13 to both the Type I and pBH700 derived probes. The genes for the enzymes maintained on pBH600 and pBH700 appear novel. At least the enzyme elaborated by strains bearing pBH700 appears to be widespread among Tp resistant *S. sonnei* isolates in the USA.

A-77 Isolation and Characterization of Dihydrofolate Reductase from a High-level Trimethoprim Resistant Pseudomonas cepacia. J.L. BURNS*, D.M. LIEN, University of Washington, Seattle.

Antibiotic resistant P. cepacia is an important pulmonary pathogen in patients with cystic fibrosis. We examined trimethoprim (Tmp) resistance in 124 CF isolates: 80 (65%) were Tmp^r (MIC≥10 µg/ml) with an MIC_{90} of 200 µg/ ml, 14 had an MIC>500 µg/ml. We selected one of these high-level Tmp^r strains, PC178, in which to characterize the resistance mechanism. There was no evidence of antibiotic inactivation or decreased permeability. Dihydrofolate reductase (DHFR), the target for Tmp activity, was purified from the Tmp^r strain, PC178, and from a Tmp^s strain, PC174, by gel filtration and ion exchange chromatography. The protein of interest was identified on non-denaturing PAGE by DHFR-specific staining. Electrophoretic mobility was similar with estimated enzyme molecular weights of 24K and 23K for PC174 and PC178, respectively. The isoelectric point was identical for both enzymes (pI 6.8) and the K_m (3.1, 4.3 µM) and V_{max} (0.60, 0.76 U/min) for dihydrofolate were similar for the Tmp^s and Tmp^r DHFR. Enzyme specific activity was identical in crude (0.02 U/mg protein) and similar in purified preparations (5.59 vs 10.40 U/mg protein) from PC174 and PC178. However, inhibition studies found a 2-log decrease in susceptibility to Tmp in the Tmp^r enzyme. We conclude that high-level resistance in strain PC178 is due to production of a Tmp^r DHFR.

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The type VII dihydrofolate reductase: a novel plasmid-encoded trimethoprim-resistant enzyme from Gram-negative bacteria isolated in Britain

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Plasmid pUN835 was identified in an *Escherichia coli* strain isolated from an outbreak of porcine diarrhoea on a farm near Nottingham, UK. The trimethoprim resistance gene did not hybridize with any of the gene probes derived from known plasmid-encoded trimethoprim resistance genes. The trimethoprim resistance gene of pUN835 was shown to encode the production of a dihydrofolate reductase which confers high-level resistance on its host. This enzyme was smaller than most plasmid-encoded dihydrofolate reductases (molecular mass = 11,500) and was labile to heat. It had relatively low affinity for the substrate dihydrofolate (K_m = 20 μM) and it was resistant to competitive inhibition by trimethoprim (K_i = 7.0 μM). We classify this novel enzyme as type VII.

Introduction

Three years after the clinical introduction of trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine), bacterial plasmids conferring resistance to this drug were observed in Gram-negative bacteria isolated in the United Kingdom (Fleming, Datta & Grüneberg, 1972). The resistance mechanism encoded by these plasmids involves the synthesis of an additional trimethoprim-resistant dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) which enables the inhibited target of the host bacterium to be by-passed (Amyes & Smith, 1974).

The dihydrofolate reductases encoded by resistance plasmids (R-plasmids) in Gram-negative bacteria were, prior to 1986, divided into three major classes. Of these, the type I enzymes are the most common and consist of two sub-types, a and b. They are heat-labile and have K_i values for trimethoprim of about 20 μM (Pattishall *et al.*, 1977; Amyes & Smith, 1978; Tennhammar-Ekman & Sköld, 1979; Young & Amyes, 1985). The type Ia dihydrofolate reductase (molecular mass = 35,000) is by far the most common of all the plasmid-encoded enzymes, and is encoded by the promiscuous transposon Tn7 (Barth *et al.*, 1976; Fling & Richards, 1983), which has been found in bacteria isolated from many parts of the world. The type Ib enzyme (molecular mass = 24,500), though clearly related to the type Ia, has only been found in the United Kingdom. The type II enzymes consist of four subunits, each with a molecular mass of about 9000 (Smith *et al.*, 1979; Fling & Elwell, 1980). These enzymes are heat

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stable and have K_i values for trimethoprim of about 0.15 mM (Amyes & Smith, 1976; Tennhammar-Ekman & Sköld, 1979). Three different subtypes of these enzymes have been found. The type IIa sub-type has been found in many parts of the world. The third type of enzyme is monomeric (molecular mass = 16,900) and is much more sensitive to trimethoprim (K_i = 19 nM) (Fling, Walton & Elwell, 1982; Joyner *et al.*, 1984). Until recently, the type III dihydrofolate reductase had only been found in a single *Salmonella typhimurium* strain isolated in New Zealand.

Recently, several new trimethoprim-resistant, plasmid-encoded dihydrofolate reductases have been identified. The type IV enzyme was identified in Gram-negative bacteria isolated in South India and differs from the other three types because it is inducible (Young & Amyes, 1986). It is also larger (molecular mass = 46,700), but has a low inhibitor constant for trimethoprim (63 mM). Type V dihydrofolate reductase has been found to be encoded by plasmids isolated in bacteria from Sri Lanka (Sundstrom, Vinayagamoorthy & Sköld, 1987). This enzyme has similar biochemical properties to the type I dihydrofolate reductases, but there are a number of differences in the amino-acid sequence. The recently-reported type VI enzyme (Wylie *et al.*, 1988), on the other hand, is quite different from all those mentioned above. It is small (molecular mass = 10,000) and is more resistant to trimethoprim than any other dihydrofolate reductase, except the type II. Finally, a plasmid-encoded dihydrofolate reductase conferring high-level trimethoprim resistance in a Gram-positive bacterium has been found in *Staphylococcus aureus* isolated in Australia. This enzyme, designated type S1, is unlike those encoded by plasmids in Gram-negative bacteria (Young, Skurray & Amyes, 1987).

In common with the trimethoprim resistance found in Gram-negative bacteria of human origin, plasmid-encoded trimethoprim resistance in bacteria isolated from domesticated farm animals in the United Kingdom has predominantly been mediated by the type Ia dihydrofolate reductase (Wise *et al.*, 1985; Carter, Towner & Slack, 1987; Amyes, 1987). However, an isolate of *Escherichia coli* implicated in an outbreak of porcine diarrhoea on a farm near Nottingham, possessed plasmid DNA that did not hybridize with DNA probes specific for the genes encoding the type I–V dihydrofolate reductases. This paper describes the characterization of this novel plasmid-encoded dihydrofolate reductase.

Materials and methods

Bacterial strains, plasmids and DNA probes

E. coli P20/10/82 (Tp^r) was isolated from an outbreak of porcine diarrhoea in 1982. Plasmid pUN835 was transferred to *E. coli* K12 strain J53-2 in bacterial conjugation experiments as previously described (Wise *et al.*, 1985). Control plasmids encoding previously characterized dihydrofolate reductases were as follows:— R483 (type Ia); pUK163 (type Ib); R67 (type IIa); R388 (type IIb); R751 (type IIc); pAZ1 (type III); pUK1123 (type IV); and pLK09 (type V). All the plasmids were carried by *E. coli* K12 derivatives. Plasmid DNA was isolated as described previously (Carter *et al.*, 1987). DNA probes specific for known dihydrofolate reductases comprised the following:— 499 bp *Hpa*I fragment of pFE872 (type I) (Fling & Richards, 1983); 275 bp *Sau*3A-*Eco*RI fragment of pWZ820 (type II) (Zolg, Hänggi & Zachau, 1978); 822 bp *Eco*RI-*Hind*III fragment of pFE1242 (type III) (M. Fling, personal communication); 1.7 kb *Cla*I fragment of pUK1148 (type IV); 500 bp *Hinc*II fragment of pLK09 (type V)

(Towner, Young & Amyes, 1988). Probes were isolated and labelled with biotin-11-dUTP as described by Towner *et al.* (1988).

Hybridization conditions

Plasmid DNA was isolated on agarose gels and transferred to nitrocellulose filters using a Semidry Electroblotter (Sartorius Ltd.) as described previously (Towner *et al.*, 1988). Routine hybridization conditions were those of Carter *et al.* (1987), with detection of a positive hybridization result by means of a BlueGENE kit (Bethesda Research Laboratories Ltd). The conditions and protocols used were as recommended by the manufacturer. Low stringency hybridization experiments were performed in the same manner, except that only two post-hybridization washes were performed, each in 0.03 M sodium citrate buffer pH 7.0, containing 0.3 M sodium chloride at room temperature for 15 min.

Enzyme preparation and assay

For the determinations of specific activity, dihydrofolate reductase was prepared from 1 l overnight cultures of bacteria grown in Isosensitest Broth (Oxoid Ltd.) with vigorous agitation at 37°C, as detailed by Young & Amyes (1986). The cells were harvested by centrifugation (6000 **g**, 10 min, 4°C) and resuspended in buffer A (50 mM sodium phosphate pH 7.4 containing 10 mM 2-mercaptoethanol and 1 mM EDTA). The bacteria were disrupted by sonication and the cell debris removed by centrifugation (40,000 **g**, 1 h, 4°C). Dihydrofolate reductase activity was assayed at 37°C in 40 mM sodium phosphate buffer pH 6.0, as described previously (Amyes & Smith, 1974). One unit of dihydrofolate reductase activity was defined as the amount of enzyme required to reduce 1 nmol of dihydrofolate per minute. Protein concentrations were estimated by the method of Waddell (1956). For enzyme purification, extracts from 3-l cultures were prepared as above. Ammonium sulphate was added to 50% saturation. After centrifugation (14,000 **g**, 30 min, 4°C), further ammonium sulphate was added to the supernatant to give 80% saturation. After further centrifugation, the pellet was resuspended and dialysed against buffer A. The dialysed sample was applied to a Sephadex G-50 column ($2\ cm^2 \times 100$ cm) that had previously been equilibrated with buffer A and eluted with the same buffer. Molecular mass was determined by Sephadex exclusion chromatography as described previously (Amyes & Smith, 1974). Heat sensitivity, enzyme kinetics and inhibitor studies were investigated as previously described (Amyes & Smith, 1976).

Isoelectric focusing of dihydrofolate reductases

Isoelectric focusing was performed by a modification of the method of Broad & Smith (1982). Aliquots (10 μl) of the dissolved and dialysed pellet obtained from precipitation with 80% saturated ammonium sulphate were applied to the surface of standard polyacrylamide plates containing ampholines capable of producing a gradient from pH 3.5–9.5 (LKB Products No. 1804–101). Standard pI markers were also applied to each gel. The samples were focussed for six hours on a cooled (9°C) Ultrophor electrophoresis system (LKB No. 2217) at 500 V and 20 mA, limited by constant power set at 1 W. The gels were stained for dihydrofolate reductase activity by the method of Broad & Smith (1982).

Results

Isolation and characterization of the trimethoprim resistance plasmid pUN835

E. coli P20/10/82 was isolated from the faeces of a pig in Nottinghamshire in 1982. The strain was highly resistant to trimethoprim (MIC > 1024 mg/l) and the trimethoprim resistance gene freely transfered at a frequency of 5×10^{-5} to the recipient *E. coli* K12 strain J53-2 by conjugation (Wise *et al.*, 1985). Isolation of the plasmid DNA showed that the trimethoprim resistance determinant was carried on a single plasmid (pUN835) of approximately 100 kb. Plasmid pUN835 was shown also to carry resistance genes to kanamycin, ampicillin, streptomycin, sulphonamides, tetracycline and chloramphenicol.

Hybridization of pUN835 with specific gene probes

A previous study (Carter *et al.*, 1987) demonstrated that 82% of trimethoprim resistance plasmids from the Nottingham area carried the gene for dihydrofolate reductase type Ia. Plasmid pUN835 was one of 15 plasmids in the study which failed to hybridize with the type Ia probe. In this present study we confirmed this lack of hybridization, even under low stringency conditions. Plasmid pUN835 was tested therefore, in further hybridization experiments with the available DNA probes for other, previously characterized dihydrofolate reductase types. No hybridization was detected with the probes specific for types II, IV or V enzymes. Hybridization was detected with the type III probe; however, the type III gene has, until recently, been found only in a single *Salm. typhimurium* isolate from New Zealand, and it is known that the type III probe contains a large sequence of DNA external to the dihydrofolate reductase structural gene (Fling *et al.*, 1988). It was decided therefore, to carry out a detailed biochemical analysis of the dihydrofolate reductase encoded by pUN835.

Specific activity of dihydrofolate reductase encoded by pUN835

Cell-free extracts were obtained from *E. coli* (pUN835) cultures grown in the presence and absence of trimethoprim (10 mg/l). When the strain was grown in the absence of the drug, the specific activity of dihydrofolate reductase was 11.5 enzyme units/mg protein and when the strain was grown in the presence of trimethoprim the enzyme preparation had a specific activity of 10.5 enzyme units/mg protein, indicating that the enzyme was not inducible in the presence of the inhibitor. In both cases, the level of enzyme was approximately 10-fold greater than that found in trimethoprim sensitive *E. coli* strains.

Purification of the dihydrofolate reductase encoded by pUN835

Dihydrofolate reductase activity was purified from three litres of culture. After removal of nucleic acids with streptomycin sulphate, the protein was selectively precipitated with ammonium sulphate between 50–80% saturation. The precipitated protein was dialyzed against buffer A and applied to a Sephadex G-50 column. A major dihydrofolate reductase peak was detected (Figure 1). To distinguish the plasmid enzyme from the *E. coli* host dihydrofolate reductase, the fraction assays were repeated in the presence of 4 μM trimethoprim, a concentration shown previously to be sufficient to

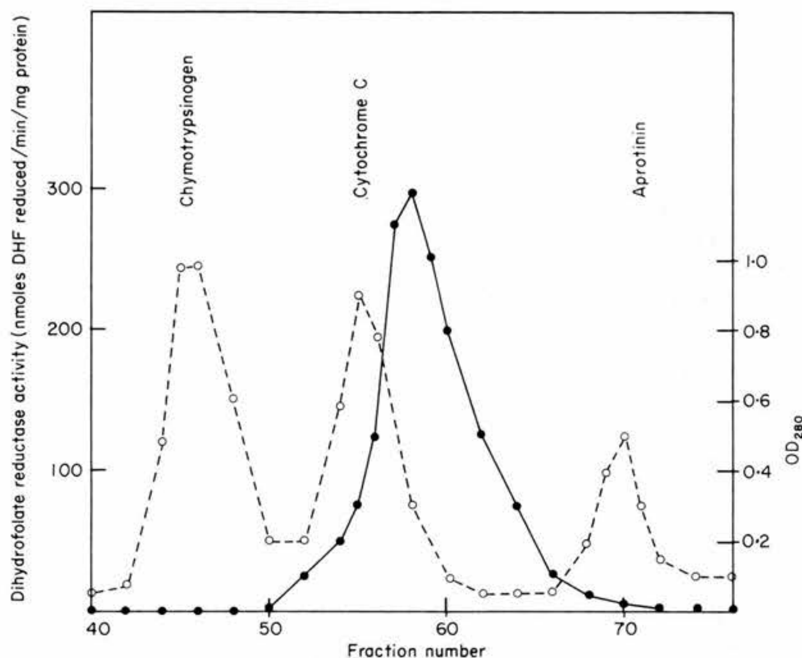

Figure 1. Elution profile of pUN835-encoded enzyme during Sephadex G-50 gel filtration. The dihydrofolate reductase activity obtained after Sephadex exclusion chromatography of a 50–80% ammonium sulphate precipitate from *E. coli* K12 strain J53–2 (pUN835) (●). Elution of standard marker proteins: chymotrypsinogen, molecular mass = 25,000; cytochrome c, molecular mass = 12,384; aprotinin, molecular mass = 6500 (○).

eliminate chromosomal dihydrofolate reductase activity (Amyes & Smith, 1974). The peak value was reduced slightly but the maximum enzyme activity was still found at the same position. When the gel filtration was repeated in the presence of standard proteins, the peak of activity for the plasmid dihydrofolate reductase corresponded to a relative molecular mass of 11,500 (Figure 1).

The peak fraction of the plasmid enzyme (58) had a specific activity of 815.0 enzyme units/mg protein which represented a greater than 50-fold purification of the crude extract. This fraction was used in the further characterization of the enzyme. The plasmid enzyme was heat labile, losing 50% of its activity after 90 sec at 45°C (Table I).

Properties of the dihydrofolate reductase encoded by pUN835

The activity of the pUN835-encoded enzyme was assayed in the presence of increasing concentrations of trimethoprim and methotrexate in order to determine the concentration of each antifolate required to give 50% inhibition (ID_{50}). The ID_{50} of trimethoprim for the plasmid enzyme was 30 μM and of methotrexate was 3 μM. In both cases, the enzyme activity was inversely proportional to the logarithm of the inhibitor concentration (Table I).

The activity of the peak fractions was investigated under conditions of partial saturation with dihydrofolate (DHF) and full saturation with NADPH (50 μM). In control studies, we found that concentrations of NADPH above 10 μM do not limit the

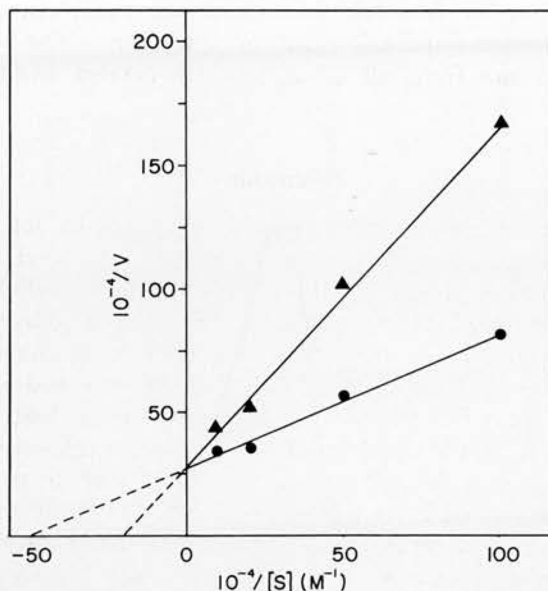

Figure 2. Lineweaver-Burk plot of the partially purified dihydrofolate reductase coded by plasmid pUN835 in the presence (▲) and absence (●) of 10 μM trimethoprim.

activity of this enzyme. The results, analysed by the method of Lineweaver and Burk, showed that the K_m value for dihydrofolate of the plasmid enzyme (Figure 2) was 20 μM. This is similar to the values for most of the other types of dihydrofolate reductases coded by plasmids that occur in Gram-negative bacteria but is about 25-fold greater than the value obtained with the type III enzyme (Amyes, 1986).

When the assays were repeated in the presence of trimethoprim it was found that the V_{max} of the plasmid dihydrofolate reductase remained (Figure 2) identical to that for the uninhibited enzyme indicating that the drug caused competitive inhibition. The inhibitor constant (K_i) for trimethoprim was 7.0 μM, similar to that reported for the type Ia enzyme (Pattishall *et al.*, 1977).

Examination of the enzyme by isoelectric focusing revealed that the dihydrofolate reductase encoded by plasmid pUN835 had an isoelectric point (pI) of 7.1. This is very

Table I. Comparison of dihydrofolate reductases encoded by pUN835 with the type I and V enzymes

Enzyme	Relative molecular mass	Tp[a]ID_{50} (μM)	Mtx ID_{50} (μM)	TD_{50} (min)	DHF K_m (μM)	Tp K_i (μM)
Type VII	11500	30.0	3.0	1.5	20.0	7.0
Type Ia	35000	57.0	5.6	0.5	5.6	7.4
Type Ib	24500	32.0	2.8	1.2	11.0	41.0
Type V	18000[b]	23.0	3.5	3.0	15.5	3.2

[a]Mtx, methotrexate; Tp, trimethoprim; DHF, dihydrofolate; TD_{50}, time taken for the enzyme to lose half its activity at 45°C;
[b]Subunit size.

similar to the pI that we find for the type Ia enzyme (7.1), which has similar biochemical properties but a quite different molecular mass. The pUN835 dihydrofolate reductase is distinct from all other plasmid-encoded enzymes on isoelectric focusing.

Discussion

Plasmid pUN835 encodes the production of a dihydrofolate reductase that is relatively insensitive to inhibition by trimethoprim and confers a high level of resistance to the drug. Failure of pUN835 DNA to show significant hybridization with any of the available gene probes specific for previously characterized dihydrofolate reductase genes identified the enzyme as worthy of detailed biochemical analysis. The properties of this enzyme are distinct from those of the type II, III, IV and VI plasmid-encoded dihydrofolate reductases of Gram-negative bacteria (Amyes, 1986; Young & Amyes, 1986; Wylie *et al.,* 1988) and from those of the type S1 plasmid-encoded dihydrofolate reductase found in staphylococci (Young *et al.,* 1987). However, the pUN835-encoded enzyme does show some similarity to the type Ia, Ib and V dihydrofolate reductases (Amyes, 1986; Thomson & Amyes, 1988). It has similar inhibition profiles with respect to trimethoprim and methotrexate and shares the heat lability of these enzymes. Additionally, the type VII enzyme has a very similar pI to the type Ia enzyme produced by plasmid pFE872, a composite plasmid comprising the type Ia dihydrofolate reductase gene cloned into the vector plasmid pBR322. The pI value of 7.1 is slightly higher than that previously reported for the type Ia enzyme encoded by plasmid R483 (Broad & Smith, 1982). On the other hand, the molecular size of this enzyme is quite different from either the type I or the type V dihydrofolate reductases. In addition, genetic probes made from the type I and type V genes did not hybridize with plasmid pUN835, even under low stringency conditions, thereby demonstrating the lack of DNA homology between the genes coding for these enzymes.

Although pUN835 was isolated from an *E. coli* strain of animal origin, a previous study of the ecology of trimethoprim R-plasmids in the Nottingham area has provided evidence that, although there may only be limited overlap of the bacterial strains from humans and animals, there is a common pool and, therefore, exchange of certain types of R-plasmids carried by these strains (Towner, Wise & Lewis, 1986). It seems almost certain, therefore, that the new type VII dihydrofolate reductase will in the future also be detected in bacteria isolated from the human community.

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PURIFICATION AND RAPID AUTOMATIC AMINO ACID SEQUENCING OF THE TYPE IV DIHYDROFOLATE REDUCTASE

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Resistance to trimethoprim is usually mediated by the plasmid-encoded production of an additional trimethoprim-resistant dihydrofolate reductase (Amyes & Smith, 1974). Thirteen different trimethoprim-resistant dihydrofolate reductases have been identified and distinguished biochemically (Amyes, 1989). Some of the genes responsible for these enzymes have had their DNA nucleotides sequenced. The results of this type of gene sequencing represent the ultimate information in the study of the evolution of genes conferring resistance to antimicrobial agents. However DNA sequencing is a lengthy process for the study of the epidemiology of antibiotic resistance. We report the use of automatic amino acid sequencing to give a rapid partial sequence sufficient to determine evolutionary relationships among trimethoprim resistance genes.

Amongst the plasmid mediated dihydrofolate reductases the type IV is unique as the enzyme is only partially resistant to trimethoprim. In addition, it is the only inducible plasmid-encoded dihydrofolate reductase. In order to see how the type IV enzyme fitted into the evolution of plasmid-mediated dihydrofolate reductases it was essential to determine its amino acid sequence. Two properties make dihydrofolate reductases suitable for rapid amino acid sequencing; firstly the active site is situated at the N-amino terminal of the protein and secondly purification to the native protein is relatively easy with the use of methotrexate agarose affinity chromatography.

Dihydrofolate reductase was prepared from 10L overnight culture of *Escherichia coli* J62-2(pUK1123) in Isosensitest broth, and the bacteria were harvested by centrifugation and disrupted by sonication. The enzyme was precipitated with ammonium sulphate (50-80% saturation) and separated on Sephadex G-75 gel filtration column ($2cm^2$ x 90cm). The fractions showing peak activity were pooled and then applied to a column containing methotrexate agarose ($0.375cm^2$ x 20cm). This column was washed until the absorbance at 280nm was less than 0.01 OD units and all the unbound protein had been removed. The dihydrofolate reductase was then eluted with 2mL of 0.5M K_2HPO_4 containing 4μmoles dihydrofolate and 5mL fractions were collected. The three eluted fractions containing peak activity were pooled and concentrated with Amicon centriprep and centricon concentrators to 60 μL. Reverse phase HPLC analysis showed this preparation to be pure and it was then analysed on an Applied Biosystems 477A protein sequencer.

Sequence analysis gave a clear result for the first fifty amino acids. Comparison of the sequence to that of the other plasmid-mediated dihydrofolate reductases shows the type IV to be clearly distinct. It shows most homology to the *E. coli* resistant chromosomal enzyme with 20 out of the first 50 amino acids showing direct matches. In particular the type IV sequence has a phenylalanine at residue 31, which is the only substitution that the *E. coli* resistant enzyme has compared with the normal sensitive enzyme. The similarity of the type IV dihydrofolate reductase to the *E. coli* resistant enzyme suggests that the induced mechanism of the type IV dihydrofolate reductase is more similar to the hyper-production mechanism of trimethoprim resistance, mediated by the bacterial chromosome, rather than a classic plasmid-mediated resistant by-pass. In this mechanism, sufficient moderately-resistant dihydrofolate reductase is produced which binds all the available trimethoprim and still allows the reduction of dihydrofolate to tetrahydrofolate.

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406/PP20

EMERGENCE OF TEICOPLANIN-RESISTANT COAGULASE-NEGATIVE STAPHYLOCOCCUS.

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EDINBURGEN EX BIBL

Although Staphylococcus haemolyticus and S. epidermidis have been shown to be less sensitive to teicoplanin than S. aureus, few observations with resistant clinical strains have been published. We have recently isolated two strains of coagulase-negative Staphylococcus resistant to teicoplanin, from blood cultures of compromised patients receiving teicoplanin.
The first patient, a 62 year-old woman with lymphoïd leukemia received teicoplanin for the cure of a Streptococcus sanguis II bacteriemia. After 3 days of treatment, fever persisted and blood cultures yielded S. haemolyticus. The second patient, a 64 year-old man with diabetes, developed a central catheter-associated S. aureus bacteriemia. While on teicoplanin therapy, he developed fever and S. epidermidis was isolated from blood cultures. Treatment with IV vancomycin resulted in cure of the infection for both patients. Mics of teicoplanin, by broth dilution, was 32 µg/ml for S. haemolyticus and 16 µg/ml for S. epidermidis. Both strains were fully sensitive to vancomycin (Mics = 1µg/ml). Presence of a teicoplanin inactivating enzyme could not be demonstrated by microbiologic techniques. A decrease of binding affinity of teicoplanin to the cell wall could be shown for S. haemolyticus strain which did not resist to bacteriolysis by lyzozyme after pretreatment by teicoplanin.
Teicoplanin-resistant strains of coagulase-negative Staphylococcus seem to have an increasing clinical importance.

407/PP20

INDUCTION MECHANISM OF THE TYPE IV DIHYDROFOLATE REDUCTASE

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A number of plasmid-encoded dihydrofolate reductases (DHFR) have been reported which confer resistance to trimethoprim (Tp). Induction systems are uncommon in Gram-negative plasmid-encoded resistance genes and only one of the trimethoprim resistance genes is inducible. Little is known about this induction mechanism other than increasing the Tp challenge causes a 600-fold increase in the production of DHFR and that this increase does not result from a genetic change. Further experiments have now been carried out to determine the induction mechanism. DHFR production was not switched on immediately the cells were challenged with Tp. There was a considerable drop in viable count and change in cell morphology before the resistance mechanism was switched on. When E. coli J62-2 (pUK1123) was challenged with Tp induction occurred while the bacteria were in lag phase but not in logarithmic phase. The induction of the type IV DHFR was also very sensitive to the presence of thymidine and this could not be overcome by increasing the Tp concentration. When plasmid pUK1123 was transferred into E. coli J53 and challenged with Tp, no induction occurred and this could be related to the different auxotrophic requirements of this strain. It is known that the type IV DHFR is only moderately resistant to Tp when compared to other plasmid mediated DHFRs. These experiments show that the type IV DHFR is only induced when the organism is under considerable stress. This resistance mechanism is novel for a plasmid mediated enzyme in that it overcomes the inhibition of Tp by producing large quantities of a partially resistant DHFR. It is more similar to the mechanism reported for some strains which have become resistant by over-production of the chromosomal DHFR.

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Identification and cloning of the Type IIIa plasmid-encoded dihydrofolate reductase gene from trimethoprim-resistant gram-negative bacteria isolated in Britain

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Summary. A clinical strain of *Escherichia coli* isolated in Nottinghamshire in 1980 was shown to harbour the type IIIa trimethoprim-resistant dihydrofolate reductase gene, previously identified on only one occasion, in New Zealand in 1979. The gene was identified by hybridisation with an 855-bp type III gene probe and its classification as a type IIIa dihydrofolate reductase was confirmed by detailed biochemical analysis of the enzyme product. The dihydrofolate reductase was identical in size and iso-electric point with the original type IIIa enzyme and shared similar inhibitory and kinetic profiles. The trimethoprim resistance gene was subsequently cloned and the type IIIa dihydrofolate reductase gene was localised to a 700-bp *Eco*RI-*Pst*I fragment. This smaller fragment may prove to be a more specific DNA probe for the future identification of type IIIa dihydrofolate reductase genes.

Introduction

The synthetic antimicrobial agent trimethoprim (Tp) selectively inhibits the bacterial enzyme dihydrofolate reductase (DHFR) (5,6,7,8-tetra-hydrofolate: $NADP^+$ oxidoreductase, EC1.5.1.3) which catalyses the reduction of dihydrofolate to tetrahydrofolate, an essential cofactor involved in the biosynthesis of purines, pyrimidines and amino acids. Resistance to trimethoprim mediated by resistance plasmids (R-plasmids), was first reported by Fleming *et al.*[1] The mechanism of resistance involves the plasmid-encoded synthesis of an additional trimethoprim-resistant dihydrofolate reductase which enables the inhibited host enzyme to be by-passed.[2]

Seven major types of plasmid DHFRs present in gram-negative bacteria have been characterised on the basis of their biochemical properties (see review).[3] They vary in molecular size, heat lability, antifolate sensitivities and Michaelis Menten kinetics. Specific gene probes have now been developed which can identify several of the genes encoding the various enzyme types (see *Materials and methods*).

Strains producing each of the enzyme types I, II, V, VI and VII exhibit a characteristically high level of resistance to trimethoprim—minimum inhibitory concentration (MIC) > 1000 mg/L. In contrast, strains producing the types III and IV plasmid DHFRs are only moderately resistant to trimethoprim (MIC ≤ 64 mg/L). Unlike other plasmid DHFRs, the type III and IV enzymes are only slightly more resistant than the *Escherichia coli* chromosomal DHFR to the inhibitory action of trimethoprim (Ki = 19 nM and 63 nM respectively).[4, 5]

The gene encoding the prototype type III enzyme (now designated type IIIa) was found on a single plasmid (pAZ1) originating in a clinical isolate of *Salmonella typhimurium* in New Zealand in 1979.[6] Two other biochemically similar plasmid-encoded enzymes (types IIIb and IIIc) have recently been reported in *Shigella* strains isolated in the USA.[7] The other moderately resistant DHFR gene, type IV, has been identified on several different plasmids, all of which originated in clinical isolates of bacteria in Southern India.[8]

In this paper we describe the identification of the type IIIa plasmid-encoded DHFR in bacteria isolated in Great Britain. This is the first report of

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the type IIIa trimethoprim resistant DHFR since its original discovery in New Zealand in 1979.

Materials and methods

Bacterial strains and plasmids

E. coli TM858 (Tp^r) was isolated in 1980 from a urine specimen from a hospitalised patient in Nottingham. Trimethoprim resistance was mobilised, in bacterial conjugation experiments, to *E. coli* K12 strain J53-2, following transposition on to the *inc*P group plasmid RP4 as previously described.[9] Control plasmids encoding previously characterised DHFRs were as follows: R483 (type Ia); pUK163 (type Ib); R67 (type IIa); R388 (type IIb); R751 (type IIc); pFE1242 (type IIIa); pUK1123 (type IV); and pLK09 (type V) (see review).[10] All the plasmids were carried by *E. coli* K12 derivatives. Plasmid DNA was isolated as described previously.[11] DNA probes specific for known DHFR genes comprised the following: 499-bp *Hpa*I fragment of pFE872 (type I);[12] 275-bp *Sau*3A-*Eco*RI fragment of pWZ820 (type II);[13] 855-bp *Eco*RI-*Hin*dIII fragment of pFE1242 (type III) (M. E. Fling, personal communication); 1·7-kb *Cla*I fragment of pUK1148 (type IV);[14] 500-bp *Hin*cII fragment of pLK09 (type V).[14] Probes were isolated and labelled with biotin-11-dUTP as described by Towner *et al.*[14] Cloning procedures were as described by Maniatis *et al.*[15]

MIC determination

The MIC of trimethoprim for *E. coli* strains was determined on Diagnostic Sensitivity Test Agar (DSTA; Oxoid) as described previously.[8]

Hybridisation

Plasmid DNA was isolated on agarose gels and transferred to nitrocellulose filters with a Semidry Electroblotter (Sartorius Ltd) as described previously.[14] Hybridisation conditions were those of Carter *et al.*,[11] with detection of a positive hybridisation result by means of a BlueGENE kit (Bethesda Research Laboratories Ltd); the conditions and protocols used were those recommended by the manufacturer.

Enzyme preparation and assay

DHFR was prepared from 2-L overnight cultures of bacteria grown in IsoSensitest Broth (Oxoid) with vigorous agitation at 37°C, as described by Young and Amyes.[5] The cells were harvested by centrifugation (6000 *g*, 10 min, 15°C) and re-suspended in buffer A (50 mM sodium phosphate, pH 7·4, containing 10 mM β-mercaptoethanol and 1 mM EDTA). The bacteria were disrupted by sonication and the cell debris was removed by centrifugation (40 000 *g*, 1 h, 4°C). DHFR activity was assayed at 37°C in 40 mM sodium phosphate buffer, pH 6·0, as described previously.[2] One unit of DHFR activity was defined as the amount of enzyme required to reduce 1 nmol of dihydrofolate/min. Ammonium sulphate was added to 50% saturation. After centrifugation (14 000 *g*, 30 min, 4°C), further ammonium sulphate was added to the supernate to give 80% saturation and, following further centrifugation, the pellet was dialysed against buffer A. It was applied to a Sephadex G-75 column ($2\ cm^2 \times 90$ cm) previously equilibrated with buffer A and eluted with the same buffer. Molecular mass was determined by Sephadex exclusion chromatography as described previously.[2] Heat sensitivity, enzyme kinetics and inhibitor studies were performed as previously described.[16]

Iso-electric focusing of DHFRs

Iso-electric focusing was performed by a modification of the method of Broad and Smith;[17] 10-μl volumes of the re-suspended and dialysed 80% saturation ammonium sulphate pellet were applied to the surface of standard LKB polyacrylamide plates containing ampholines capable of producing a gradient from pH 3·5 to 9·5 (no. 1804–101). Standard pI markers were also applied to each gel. Samples were focused for 6 h on a cooled (9°C) LKB Ultrophor electrophoresis system (no. 2217) at 500 V and 20 mA, limited by constant power at 1 W. The gels were stained for DHFR activity by the method of Broad and Smith.[17]

Results

Identification and cloning of the type IIIa DHFR gene

E. coli TM858 was isolated from the urine of a hospitalised patient in Nottinghamshire in 1980. This strain was highly resistant to trimethoprim (MIC > 1024 mg/L). Trimethoprim resistance was not freely transferable and could be mobilised to *E. coli* K12 strain J53-2 only following transposition on to plasmid RP4. The resulting transconjugant, *E. coli* J53-2 (pUN635 = RP4 : : Tn*3175*), was also highly resistant to trimethoprim (MIC > 1024 mg/L). Therefore, purified plasmid DNA was hybridised with those gene probes known to identify high level trimethoprim resistance genes (i.e., DHFR types I, II and V gene probes). However no hybridisation occurred with any of these probes, which indicated the presence of a novel DHFR gene.

To expedite a more detailed analysis of the trimethoprim resistance gene and its product, the trimethoprim resistance gene of strain J53-2 (pUN635) was cloned into the vector plasmid

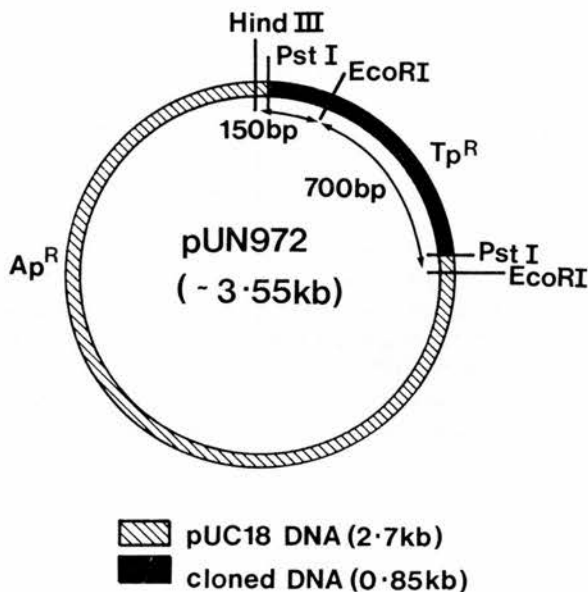

Fig. 1. Restriction map of recombinant plasmid pUN972 carrying the type IIIa DHFR.

pUC18. Plasmid pUN635 was digested with the restriction enzymes *Pst*I and the resulting fragments were ligated into pUC18. The recombinant plasmids were then introduced by transformation into *E. coli* HB101 and selection was made for trimethoprim-resistant colonies on DSTA containing trimethoprim 50 mg/L. A single trimethoprim-resistant clone was obtained which was found to contain an 0·85-kb *Pst*I fragment ligated to pUC18. This recombinant plasmid was designated pUN972 (fig. 1). The MIC of trimethoprim for HB101 (pUN972) was found to be 64 mg/L, much lower than the original pUN635-containing bacteria.

Plasmid pUN972 was hybridised with all available plasmid DHFR gene probes. A positive hybridisation result was obtained only with the type IIIa DHFR gene probe. Further evidence suggesting that pUN972 carried a type IIIa DHFR gene was obtained when a 0·7-kb *Eco*RI-*Pst*I fragment of pUN972 (fig. 1), carrying the trimethoprim resistance gene, was used to probe control plasmids encoding previously characterised DHFRs. Hybridisation was detected only with the original type IIIa DHFR-encoding plasmid, pAZ1.

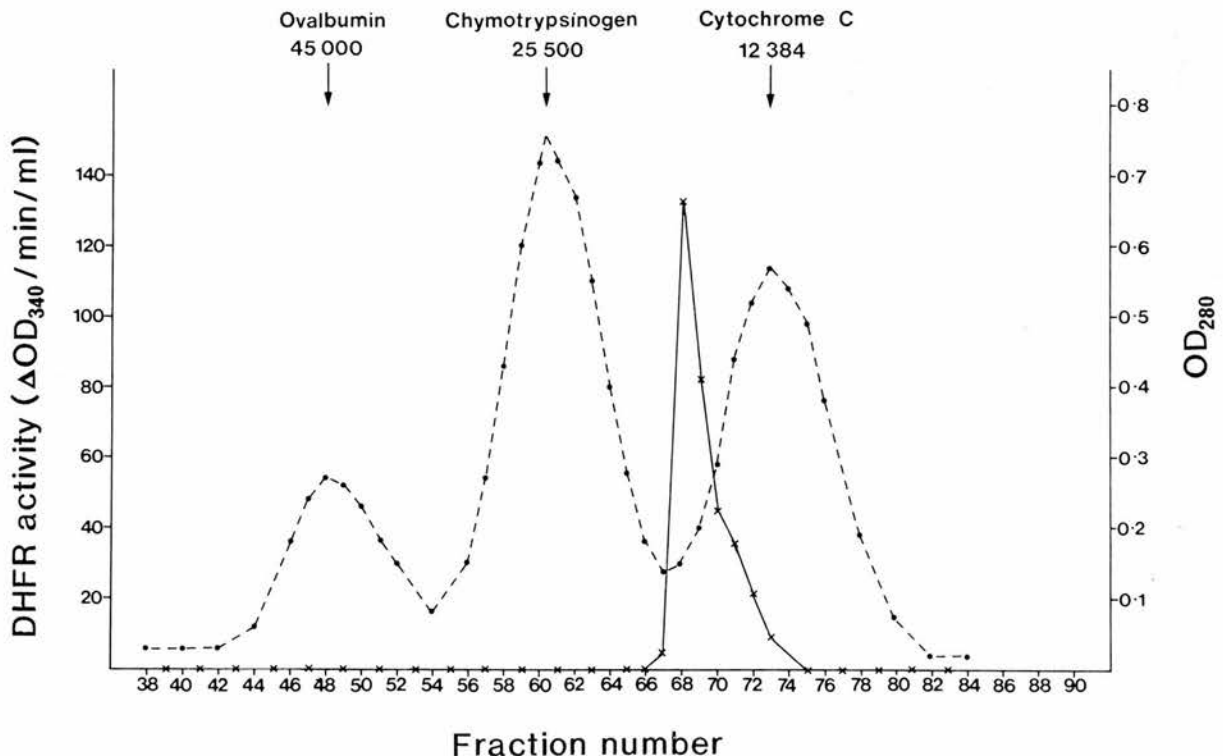

Fig. 2. Sephadex G-75 gel filtration of the type IIIa DHFR. ×——×, DHFR activity obtained after sephadex exclusion chromatography of a 50–80% saturation ammonium sulphate precipitate from *E. coli* HB101 (pUN972). •-----•, Elution of standard marker proteins (OD_{280}).

Purification of the DHFR encoded by pUN972

DHFR activity was purified from 2 L of culture. After removal of nucleic acids with streptomycin sulphate, the protein was selectively precipitated with ammonium sulphate between 50 and 80% saturation. The precipitated protein was dialysed against buffer A and applied to a pre-calibrated Sephadex G-75 column. A major DHFR peak was eluted during gel filtration corresponding to a molecular weight of 17 000 daltons (fig. 2). This is indistinguishable from the 16 900 daltons reported for the original type IIIa enzyme encoded by plasmid pAZ1.

Characterisation of the pUN972 and pAZ1 encoded DHFRs

Inhibitor profiles. To compare the pUN972-encoded enzyme with the original type IIIa enzyme, the DHFR encoded by pAZ1 was also purified and separated by gel filtration of Sephadex G-75. Fractions containing peak DHFR activity were pooled for each enzyme preparation and used for comparative characterisation.

Both enzymes were found to be heat stable, they retained more than 50% activity after being held at 45°C for 12 min. Partially purified DHFR activity was assayed in the presence of increasing concentrations of trimethoprim and methotrexate. Both enzymes were approximately 10 times more resistant to inhibition by trimethoprim than the *E. coli* chromosomal DHFR, losing 50% of their activity in the presence of 4·5 μM trimethoprim. This is also similar to the value previously reported by Joyner *et al.*[4] for the pAZ1-encoded enzyme. Similarly, both enzymes were inhibited by 50% in the presence of 10 times greater concentrations of methotrexate (0·02 μM) than that required to inhibit the *E. coli* chromosomal enzyme.

Michaelis Menten kinetics. The activity of the pUN972 encoded enzyme was investigated in conditions of partial saturation with dihydrofolate and the results were analysed by the method of Lineweaver and Burk (fig. 3). The results (fig. 3)

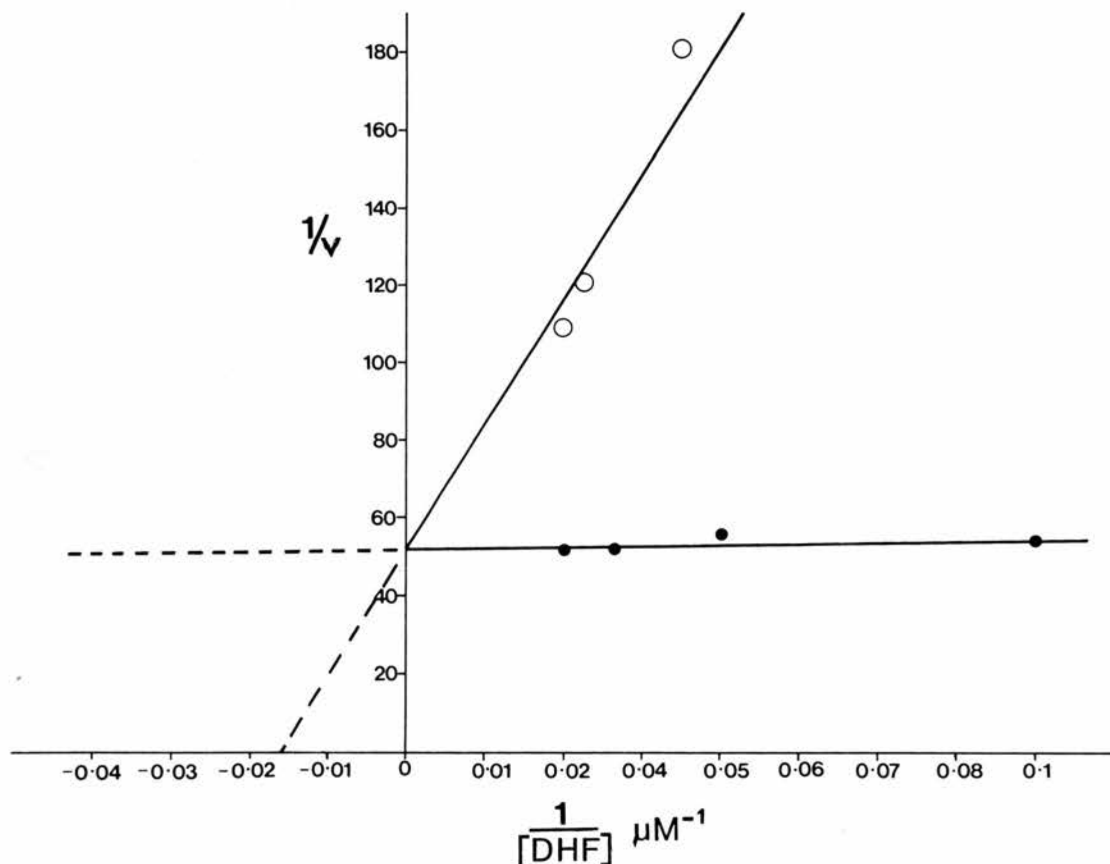

Fig. 3. Lineweaver-Burk plots of the partially purified type IIIa DHFR. The reciprocal of the dihydrofolate concentration (1/[DHF]) is plotted against the reciprocal of the rate of DHFR activity (1/V) in the presence and absence of trimethoprim. •, No trimethoprim; ○, 5 μM trimethoprim.

show that the K*m* value for dihydrofolate was 0·41 μM, a value similar to that previously published for the pAZ1 encoded enzyme. When the assays were repeated in the presence of trimethoprim it was found that the maximum velocity remained the same (fig. 3), indicating that the drug causes competitive inhibition of the enzyme. The inhibitor constant (Ki) for trimethoprim was 0·04 μM, again similar to that reported for the pAZ1-encoded, type IIIa DHFR.

Iso-electric focusing. Final evidence indicating that the pUN972-encoded dihydrofolate reductase was identical to the type IIIa enzyme encoded by pAZ1 was obtained by determination of the iso-electric point of the two enzymes. When run concurrently, both enzymes were found to co-focus with a pI of 6·1, thus confirming the classification of the pUN972-encoded enzyme as a type IIIa DHFR (fig. 4).

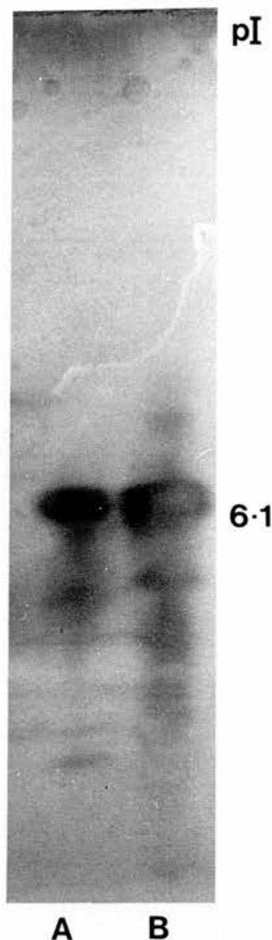

Fig. 4. Iso-electric focusing pattern of the type IIIa DHFRs. Track A, type IIIa enzyme originally obtained from plasmid pAZ1 (New Zealand); track B, type IIIa enzyme obtained from plasmid pUN972 (Nottingham).

Discussion

The original strain of *E. coli* studied in this paper exhibited high level resistance to trimethoprim, characteristic of plasmid DHFR types I, II, V, VI and VII. However, hybridisation studies employing the available DNA probes specific for these DHFR gene types failed to identify a previously characterised DHFR gene. Further genetic analysis, involving cloning of the trimethoprim resistance gene into the vector plasmid pUC18, revealed a DHFR gene conferring only a moderate level of trimethoprim resistance in the recombinant strain. Detailed biochemical analysis of the enzyme product of this gene showed it to be identical with the type IIIa enzyme, encoded by pAZ1, described by Fling *et al.*[6] This is the first report of the type IIIa DHFR gene since its original detection in a clinical isolate in New Zealand.

One interesting feature of this work is the apparent reduction in MIC of trimethoprim for the strain carrying the cloned type IIIa DHFR compared to the original isolate. One possible explanation is that a second DHFR gene, conferring a high level of resistance, was present on the original transposon and that this "masked" the presence of the low level type IIIa determinant; however, other more complicated scenarios involving gene copy numbers or gene regulation are perhaps more feasible. For example, the MIC could vary depending on the orientation of the insert and whether the gene was utilising its own promotors or those of pUC18.

An important consequence of this study has been the production of a new type IIIa DHFR gene probe. The previously available 855-bp DNA probe for the type IIIa gene contains approximately 350 bp extraneous to the structural gene;[18] consequently it has been shown to produce occasional "false positive" hybridisation reactions (K. J. Towner, unpublished results). The gene probe constructed in this study is smaller (700 bp) and may prove, therefore, to be more specific. The elaborate techniques involved in the detection and confirmation of the type IIIa DHFR gene suggest that the apparent low incidence of the type IIIa enzyme may result from a lack of detection rather than a scarcity of the gene. Therefore, the new gene probe constructed in this study may be very useful

in clarifying the epidemiology of what has previously been considered to be an extremely unusual resistance gene.

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N-terminal amino acid sequence of the novel type IIIb trimethoprim-resistant plasmid-encoded dihydrofolate reductase from *Shigella sonnei*

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The type IIIb dihydrofolate reductase, a novel plasmid-encoded enzyme recently identified in *Shigella sonnei*, has been shown to have some similar biochemical properties to the type IIIa dihydrofolate reductase which was first identified in New Zealand in 1979. However, the type IIIb enzyme has a K_i for trimethoprim of 0·4 μM, and a pI of 5·35 (as compared to 19 nM and 6·1 for the type IIIa); both these results suggest that it is a different enzyme from the prototype type IIIa. The type IIIb dihydrofolate reductase was purified by methotrexate agarose affinity chromatography, yielding a pure protein as determined by HPLC. Automatic amino acid analysis of the purified enzyme showed it to be distinct from all other known plasmid-encoded dihydrofolate reductases and quite different from the type IIIa enzyme. The purified enzyme was examined by SDS-PAGE, which revealed that the type IIIb dihydrofolate reductase was a monomeric protein of M_r 17200.

Introduction

Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine] prevents the bacterial NADPH-dependent reduction of dihydrofolate to tetrahydrofolate by competitively inhibiting the enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) (Burchall & Hitchings, 1965). A number of mechanisms of resistance to trimethoprim have been reported, the most important of these being the plasmid-encoded production of an additional trimethoprim-resistant dihydrofolate reductase enabling the trimethoprim blockade of the chromosomal enzyme to be bypassed (Amyes & Smith, 1974). The trimethoprim-resistant dihydrofolate reductases found in Gram-negative bacteria have been divided into a number of classes based principally on their biochemical profiles (for reviews see Amyes, 1989; Huovinen, 1987). In addition, as some of the genes responsible have also had their DNA nucleotides sequenced, the classification of these enzymes now also takes into account amino acid differences (Stone & Smith, 1979; Zolg & Hanggi, 1981; Fling & Richards, 1983; Flensburg & Steen, 1986; Fling *et al.*, 1988; Sundström *et al.*, 1988). Initially resistance was dominated by the spread of two genes encoding dihydrofolate reductases types I and II. The type I was particularly common, probably because the gene responsible was located on a transposon, Tn7 (Barth *et al.*, 1976). However, more recently a number of new enzymes have been identified and now seven main groups have been distinguished in Gram-negative bacteria on the basis of their biochemical properties, DNA/DNA hybridization and amino acid sequences (for a review see Amyes, 1989).

Trimethoprim-sulphamethoxazole is widely used in the treatment of shigellosis (Salter, 1982). Until recently trimethoprim resistance in Shigella was relatively uncommon. However, increased use of trimethoprim in the treatment of shigellosis has resulted in a large increase in trimethoprim resistance. This now represents a major concern in many parts of the world. In Bangladesh the level of trimethoprim resistance rose from 5% in 1979 to 83% in 1983 (Zaman *et al.*, 1983; Shahid *et al.*, 1985) whilst in England and Wales the level of resistance rose from 1·3% to 17% in the same period (Gross *et al.*, 1984). Some studies on trimethoprim-resistant shigella have demonstrated the dominance of the type I dihydrofolate reductase whilst others have shown a significant proportion of strains which do not react with probes for genes encoding for either of the commonly occurring type I and type II enzymes (Chatkaeomorakot *et al.*, 1987). Recently, two novel plasmid-encoded dihydrofolate reduc-

The amino acid sequence reported in this paper has been registered in the protein database of the National Biomedical Research Foundation (NBRF), Washington, DC, USA, and has been assigned the accession number A33005.

tases have been identified from epidemic strains of trimethoprim-resistant *Shigella sonnei* isolated in the USA (Barg *et al.*, 1989). These novel dihydrofolate reductases were identified both by their inability to hybridize with gene probes for dihydrofolate reductases I, II and III and by their biochemical properties. However, in many respects, they appeared biochemically most similar to the type III dihydrofolate reductase. One in particular was very similar; it conferred only a moderate level of trimethoprim-resistance (MIC 128 mg l^{-1}) and its ID_{50} of trimethoprim was 2 μM, which is identical to the value for the type III dihydrofolate reductase (Joyner *et al.*, 1984). The original type III enzyme has been shown to be a monomeric protein of M_r 16900 and the novel enzyme was similarly small. On this basis this enzyme was named the IIIb and the original type III dihydrofolate reductase the type IIIa (Barg *et al.*, 1989). However, the IIIb enzyme did differ in some biochemical properties, most notably in its isoelectric point, from the type IIIa.

To investigate the relationship between these enzymes it was essential to examine the amino acid sequence of this new dihydrofolate reductase. Here we report the use of methotrexate agarose as an affinity column matrix to purify the IIIb dihydrofolate reductase, enabling its N-amino terminal amino acid sequence and subunit structure to be determined.

Methods

Bacterial strains and plasmids. The type IIIb dihydrofolate reductase was prepared for sequence and subunit analysis from *Escherichia coli* C600(pBH600) (Barg *et al.*, 1989).

Enzyme preparation for sequence analysis. Dihydrofolate reductase was prepared from 10 litre overnight cultures of *E. coli* in Oxoid Isosensitest Broth, grown at 37 °C and shaken vigorously (Young & Amyes, 1986). Bacteria were harvested by centrifugation at 6000 *g* for 15 min and resuspended in buffer A (50 mM-sodium phosphate buffer pH 7·4, containing 10 mM-2-mercaptoethanol and 1 mM-EDTA). The bacteria were disrupted by sonication (2 × 30s, 8 μM, MSE Soniprep) and the lysate cleared by centrifugation at 40000 *g* for 1 h at 4 °C. Dihydrofolate reductase activity was assayed at 37 °C in 40 mM-sodium phosphate buffer pH 6 (Amyes & Smith, 1974); the units of activity were nmol dihydrofolate reduced min^{-1}.

Enzyme preparation for biochemical analysis. Enzyme preparation, biochemical analysis and iso-electric focusing were done as described by Amyes *et al.* (1989).

Enzyme purification and sequence analysis. Dihydrofolate reductase activity was precipitated from the crude preparation by the addition of ammonium sulphate from 50 to 80% saturation. The enzyme was resuspended in buffer and separated on a Sephadex G75 gel filtration column (2 cm^2 × 90 cm). Fractions showing peak enzyme activity were pooled and applied to a methotrexate-agarose column (0·375 cm^2 × 20 cm) made up according to the manufacturer's instructions (Sigma). This column was washed with buffer A until the A_{280} was less than 0·01 and all the unbound protein had been removed. The dihydrofolate reductase was then eluted with 2 ml 0·5 M-K_2HPO_4 containing 4 μmol dihydrofolate and washed with 75 ml 0·1 M-K_2HPO_4 containing 1 μM-dihydrofolate (Kaufman, 1974). Fractions of 5 ml were collected. The three eluted fractions showing peak activity were pooled and concentrated with Amicon Centriprep and Centricon concentrators to 30 μl. Prior to sequence analysis the preparation was checked for purity by reverse-phase HPLC analysis. The only protein peak was selected from the reverse-phase HPLC and analysed on an Applied Biosystems 477A protein sequencer (Hayes *et al.*, 1989).

Gel electrophoresis. SDS-PAGE was done on a Pharmacia Phast system according to the manufacturer's instructions. A Sigma molecular weight kit (14000–70000) was used to calibrate the gel.

Results

Biochemical properties of the type IIIb dihydrofolate reductase

The plasmid-borne dihydrofolate reductase gene from the *Shigella sonnei* strain responsible for an outbreak of dysentery at a nursing home in East Tennessee in 1985 was transferred to *E. coli* C600(pBH600). The dihydrofolate reductase from this transconjugant was then examined in detail.

The M_r of the dihydrofolate reductase produced by the transconjugant was 17000 when measured by Sephadex gel filtration. When the measurement was repeated in the presence of 4 μM-trimethoprim to remove any chromosomal dihydrofolate reductase the position of the peak was unchanged. The enzyme was sensitive to the presence of trimethoprim, requiring 2 μM of the drug to give 50% reduction in activity. It was also sensitive to methotrexate, with 0·02 μM-methotrexate reducing the enzyme's activity by half. The enzyme was, however, heat stable, maintaining more than 50% of its activity after 12 min at 45 °C.

The activity of the enzyme was measured at limiting substrate concentrations in the presence and absence of trimethoprim. Analysis by the method of Lineweaver and Burk showed that the type IIIb dihydrofolate reductase had a K_m for dihydrofolate of 9·5 μM and that it was competitively inhibited by trimethoprim with a K_i of 0·4 μM.

Biochemically the enzyme had several properties which were very similar to the type IIIa dihydrofolate reductase isolated in New Zealand in 1979. However, iso-electric focusing of the type IIIb enzyme showed that it focused clearly with a pI of 5·34 which was distinct from that of the New Zealand type IIIa dihydrofolate reductase (Table 1).

Enzyme purification

The activity of the enzyme preparation was measured throughout the purification procedure. Prior to gel filtration the resuspended pellet from ammonium sul-

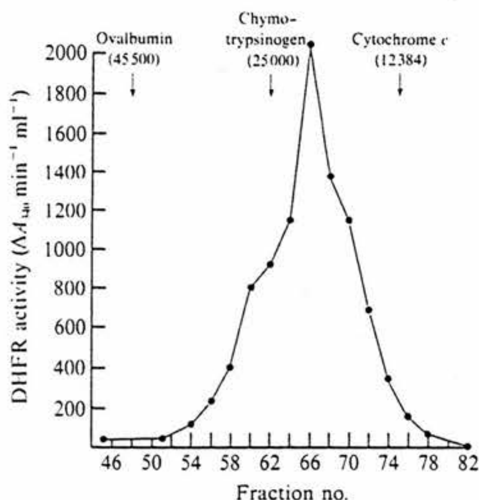

Fig. 1. Elution profile of pBH600-encoded enzyme during Sephadex G-75 gel filtration. ●, Dihydrofolate reductase (DHFR) activity obtained after Sephadex exclusion chromatography of a 50–80% ammonium sulphate precipitate from *E. coli* C600(pBH600). The positions of the elution peaks of the standard marker proteins are indicated by arrows.

phate precipitation contained 78124 units of dihydrofolate reductase activity. Separation on Sephadex gel filtration gave a clearly defined peak of enzyme activity (Fig. 1). Fractions 62–72 were pooled; they contained a total of 28874 units. The pooled fractions were run onto the methotrexate column. No dihydrofolate reductase activity was detected in the protein which initially passed through the column, indicating that the enzyme had been completely bound to the methotrexate agarose. The column was washed continuously until the A_{280} of the eluate had returned to a value of less than 0·01, i.e. no further protein was passing through. The dihydrofolate reductase activity was eluted by the addition of 2 ml 0·5 M-K_2HPO_4 containing 4 µmol dihydrofolate. Fractions of 5 ml were collected and assayed for enzyme activity. The three fractions showing peak activity were pooled and contained a total of 19933 units. This 15 ml sample was concentrated with Amicon Centriprep and Centricon concentrators to 30 µl, which retained 16499 units. Before sequence analysis this sample was tested for purity by reverse-phase HPLC and this revealed the presence of a single protein peak.

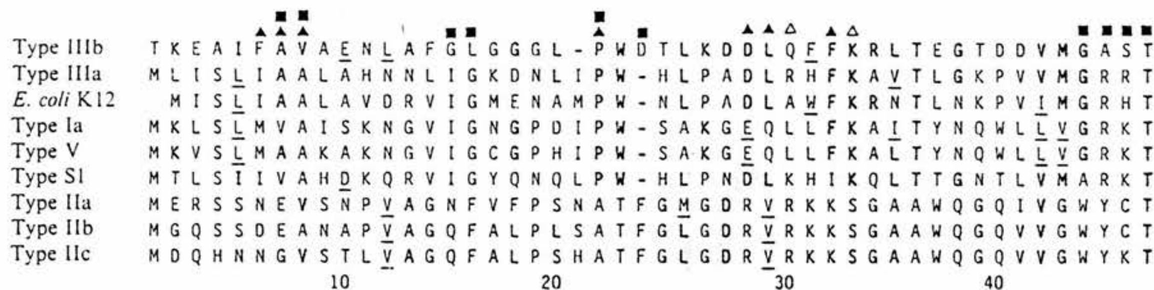

Fig. 2. Determined N-terminal amino acid sequence (47 amino acids) of the type IIIb plasmid-encoded dihydrofolate reductase. Identical amino acids are shown by shading. Homologous amino acids, (D E) (R K H) (Q N) (F Y W) (I V L M), are underlined. The amino acids involved in the active site are shown by the symbols at the top of the diagram: ▲, binding positions of trimethoprim; △, additional binding positions of methotrexate; ■, binding positions of NADPH. Positions are taken from Rouch *et al.* (1989) and numbering is based on the type IIIb sequence.

Table 1. *Comparison of the biochemical properties of the type IIIa and IIIb dihydrofolate reductases*

Enzyme	Tp ID_{50} (µM)	Mtx ID_{50} (µM)	TD_{50} (min)	DHF K_m (µM)	Tp K_i (µM)	M_r	pI	Tp MIC (µg ml^{-1})
IIIa	2·0	0·02	>12	0·4	0·019	16900	6·10	64
IIIb	2·0	0·02	>12	9·5	0·4	17000	5·34	128

Abbreviations: Tp, trimethoprim; Mtx, methotrexate; DHF, dihydrofolate.

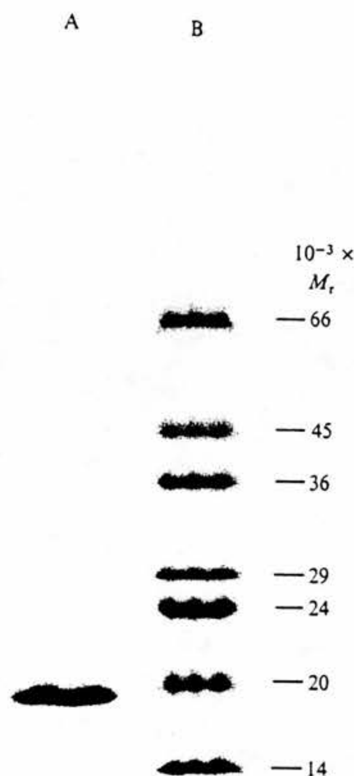

Fig. 3. PAGE of purified dihydrofolate reductase encoded by plasmid pBH600. The bands were visualized by staining with Coomassie blue. A, dihydrofolate reductase from *E. coli* C600(pBH600); B, Sigma standard marker proteins (M_r 14000–70000).

Sequence analysis of the type IIIb dihydrofolate reductase

Fifteen microlitres of the purified sample was analysed on an Applied Biosystems 477A automatic sequencer. This gave a clear sequence for the first 47 amino acids (Fig. 2). The M_r of the enzyme suggests a protein of 162 amino acids, thus approximately 30% of the protein has been examined. The sequence obtained starts with a threonyl residue, indicating that the f-methionyl residue had been removed intracellularly. Comparing the sequence with that of the other known plasmid-encoded dihydrofolate reductases (types I–V and S1) showed this enzyme to be clearly distinct. Although the type IIIb enzyme is biochemically similar to the type IIIa enzyme isolated in New Zealand in 1979, its homology with this enzyme is not significantly greater than that with any of the other plasmid-encoded dihydrofolate reductases.

Subunit structure of the type IIIb dihydrofolate reductase

The subunit structure of the type IIIb dihydrofolate reductase was examined by running the purified enzyme on a Pharmacia Phast system SDS gel. The sample was run on a 10–15% Pharmacia Phast gradient gel according to the manufacturer's instructions. After running on the SDS gel the type IIIb enzyme produced a single band of protein when stained (Fig. 3); this corresponded to an M_r of 17200. As the M_r of the native type IIIb enzyme determined by Sephadex gel filtration is 17000, the enzyme is presumably a monomeric protein.

Discussion

The major groups of plasmid-encoded dihydrofolate reductases have, in the past, been distinguished from one another primarily by their biochemical profiles (Amyes, 1986). Recently, however, the number of different enzyme types has increased (for a review see Amyes, 1989). In some cases the different dihydrofolate reductases have quite similar biochemical properties (e.g. the types I and V) and so it has become necessary to look at the structure of these enzymes in more detail. This has been achieved by the examination of either the DNA nucleotide or amino acid sequences of the enzymes.

If determination of the enzyme's amino acid sequence is to become a crucial feature in the distinction of plasmid-encoded dihydrofolate reductases, an alternative to DNA nucleotide sequencing is required since it is too lengthy a process for the routine study of antibiotic resistance. Therefore a more rapid method is needed and we thus report the use of such an alternative, namely rapid automatic amino acid sequencing to give a partial sequence sufficient to determine evolutionary relationships amongst trimethoprim resistance genes. Two properties make dihydrofolate reductases suitable for rapid automatic amino acid sequencing. Firstly, most of the active site is situated at the N-terminal end of the protein so that a single run through a sequencer provides enough information to evaluate the relationships (Rouch *et al.*, 1989; Novak *et al.*, 1983). Secondly, in many cases the enzyme is easily purified with the use of methotrexate agarose, and a native protein, sufficient for sequencing, can be obtained in a few steps.

Biochemically, the type IIIb dihydrofolate reductase is more similar to the type IIIa enzyme isolated in New Zealand in 1979 than to any other plasmid-encoded dihydrofolate reductase. However, it does differ from the type IIIa enzyme in pI value and in Michaelis–Menten kinetics.

Comparison of the amino acid sequence for the first 47 amino acids with the sequence of the type IIIa plasmid-

encoded dihydrofolate reductase shows the type IIIb enzyme to be clearly distinct (Fig. 2). Only fifteen direct matches are found between the two enzymes; two further amino acids in the type IIIb enzyme were homologous to the amino acids at the same position in the type IIIa. The type IIIb enzyme showed slightly less direct matching with the *E. coli* chromosomal dihydrofolate reductase, with only 13 direct matches and three further homologous amino acids. This in contrast to the IIIa enzyme, which shows a high degree of homology with the *E. coli* K12 chromosomal dihydrofolate reductase. The type IIIb sequence was also compared with the sequences of the other plasmid-encoded dihydrofolate reductases types I, II, V and S1 (Fig. 2). The type IIIb enzyme was distinct from these, showing 10 direct matches with the type Ia and five, six and six direct matches with the types IIa, IIb and IIc respectively. The type IIIb showed 12 direct matches with the type V and 13 with the type S1. It showed 13 direct matches with the type IV enzyme (unpublished results). The sequence data show that although the IIIa and IIIb dihydrofolate reductases are biochemically similar these enzymes are not closely related in an evolutionary sense.

Like the type IIIa enzyme, the type IIIb dihydrofolate reductase is monomeric. It also has a similar M_r. Therefore, there does appear to be some evolutionary advantage in the selection of enzymes with this biochemical profile.

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REVIEW ARTICLE

Trimethoprim resistance; epidemiology and molecular aspects

—a review based upon a Symposium held on 19 April 1989 at the 4th European Congress of Clinical Microbiology, Nice, France

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Introduction. Trimethoprim resistance was first recognised in gram-negative bacteria 20 years ago. Workers in several research centres have studied the epidemiology and molecular aspects of trimethoprim resistance over the intervening years, initially in gram-negative bacteria and more recently in gram-positive bacteria. The introduction of this completely novel synthetic antimicrobial provided a unique opportunity to study the full evolution of bacterial resistance to an antimicrobial drug, without the pre-existing influence of resistance genes selected by other related compounds. Consequently, the model provided by trimethoprim resistance has considerable relevance to our understanding of the evolution of bacterial resistance to antimicrobial agents in general. Recent years have seen exciting and major advances in the use of modern DNA technology to study the epidemiology of trimethoprim resistance. Considerable progress has been made in evaluating the importance of the different resistance mechanisms, especially those carried by resistance plasmids. Nevertheless, in most countries resistance to trimethoprim has only recently reached clinically significant proportions and it remains a widely used and valuable component of the antimicrobial armamentarium. The papers presented in this review are based on a Symposium held at the 4th European Congress of Clinical Microbiology in April 1989. The Symposium provided a forum for the various aspects of trimethoprim resistance to be brought together and resulted in the collation of research material which forms this review.

Evolution and spread of trimethoprim resistance in gram-negative bacteria

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Trimethoprim, in association with sulphonamides, has been used for 20 years in human and veterinary medicine and is still one of the most widely used antibiotics in the world. The reasons for its success are: a broad spectrum of activity, a marked synergic effect on both sulphonamide-susceptible and -resistant strains, excellent clinical results and, particularly important, a very low price. This success has nevertheless one drawback, namely the emergence of bacterial resistance.

Epidemiology of trimethoprim resistance

Comparisons of the incidence of bacterial resistance are difficult because many factors such as methodology, selection and choice of specimens and strains, type of patients and local epidemics greatly influence the percentage of resistant strains. For these reasons, assessment of changes in trimethoprim resistance are really only significant if they come from long-term follow-up studies within a single laboratory.

Resistance to trimethoprim has been carefully monitored for a long period in Finland, the United

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Kingdom and France. In Finland, from 1978 to 1984 resistance to trimethoprim in *Escherichia coli* isolates from outpatients increased slowly (e.g., 5·4% to 9·2% in Turku).[1] A sharper increase has been observed in the last 3 years and is best illustrated by data from the town of Rovanienui, where resistance to trimethoprim was stable at about 5% between 1980 and 1986, but rose dramatically to 14·7% in 1987 and has remained at that level since (P. Huovinen, personal communication). In Paris, resistance to trimethoprim in *E. coli* increased slowly from 1972 to 1979 and then very sharply to 1982 (fig. 1). From 1983 to 1988, resistance to trimethoprim decreased in all groups of isolates. On the other hand, in Nottingham, in a study involving nearly 74 000 bacterial isolates from 1978 to 1988, trimethoprim resistance increased progressively throughout the study period[2] (K. J. Towner, personal communication). In *E. coli*, resistance rose from 2·0% to 15·5% and in *Proteus* spp. from 7·2% to 24·9%. A marked increase in resistance was found between 1979 and 1980 and between 1983 and 1984. Between 1983 and 1984 this sharp increase was found in both hospital strains (*E. coli* from 9·3% to 16·2%, *Proteus* spp. from 11·0% to 22·0%) and community isolates (*E. coli* from 8·5% to 13·7%, *Proteus* spp. from 7·3% to 17·5%).[2] More recently, between 1987 and 1988, another profound increase has been observed in community isolates of *Klebsiella*/*Enterobacter* spp. (15·2% to 24%) and *Proteus* spp. (18·2% to 25·2%) (K. J. Towner, personal communication). In complete contrast, in the USA, the incidence of resistance to trimethoprim has remained very low both in the community as well as in nosocomial strains. The highest incidence amongst hospitalised patients has been 13%.[3]

In developing countries, the incidence of trimethoprim resistance is very disquieting. In Thailand, trimethoprim-resistant bacteria represented 40% of urinary isolates.[4] Similarly, Young *et al.*[5] found that 64% of urinary isolates in India in 1984 were resistant to trimethoprim. In Johannesburg Hospitals (South Africa), 50% of enterobacterial species isolated in 1986–1987 were resistant to trimethoprim.[6] The same problems exist in South America where, in 1987, 25–41% of strains isolated from the community in Santiago de Chile were resistant to trimethoprim.[7]

The importance of antibacterial drug resistance in general, and trimethoprim resistance in particular, becomes acute when considering specific pathogens such as *Shigella* spp., which are responsible for a high rate of morbidity and mortality. Data from several countries indicating an increase in the percentage of trimethoprim-resistant strains among isolates of shigellae are summarised in table I. What are the reasons for this situation?

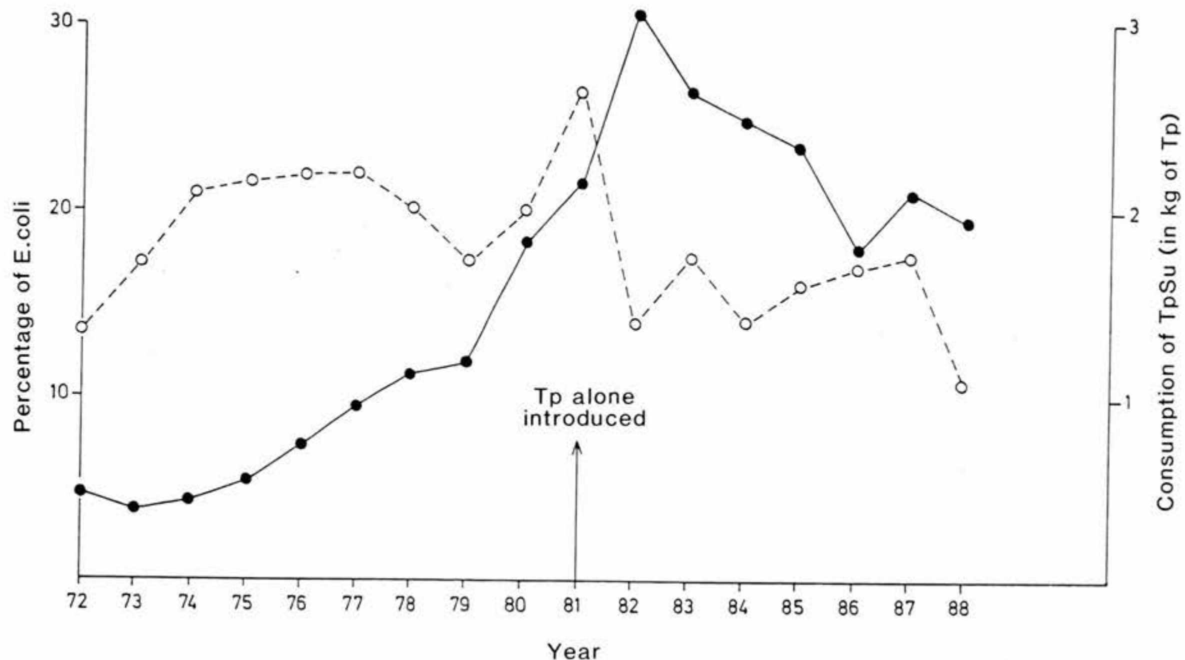

Fig. 1. Proportion of trimethoprim resistance in *E. coli* in the St Joseph Hospital in Paris and its relation to the usage of trimethoprim. ●—● Percentage of *E. coli* resistant to trimethoprim. ○—○ Consumption of trimethoprim.

Table I. Resistance to sulphamethoxazole-trimethoprim among *Shigella* spp.

Country	Year	Tp-R (%)	Total strains tested
Bangladesh	1980	0	1018
	1984	55	890
Mexico	1980	0	55
	1984	6	23
Thailand	1979–1984	35	1033
	1986–1988	61·1	211
Chile	1983	25	85
	1987	37	100
Brazil	1983	23	57

Resistance to trimethoprim, or to any other antibiotic, will spread if three favourable conditions are met: (1) the emergence of stable resistance genes; (2) the presence of resistance vectors such as epidemic strains, plasmids or transposons; (3) continuous or periodic selection pressure.

Emergence of stable trimethoprim resistance genes

Low level resistance to trimethoprim usually results from mutational events such as decreased permeability and quantitative or qualitative modifications of the bacterial target of trimethoprim, dihydrofolate reductase (DHFR).[8] Plasmid-mediated low level resistance has been described, but only on a few occasions (see section by Young and Thomson in this review). Impermeability mutants are particularly interesting because of the cross-resistance to trimethoprim and other antibiotics, especially quinolones, which can act as selectors. This type of mutation has been seen in *Klebsiella, Enterobacter* and *Serratia* and is much more prevalent than it is often assumed to be.[9]

High level resistance to trimethoprim is usually specified by plasmids which encode an additional trimethoprim-resistant DHFR (reviewed by Amyes[10]). Trimethoprim resistance plasmids were first isolated in London in 1971[11] and have quickly spread to all enterobacterial species, *Vibrio cholerae* and even *Acinetobacter* and *Pseudomonas*, although these latter species are intrinsically resistant to trimethoprim. Trimethoprim resistance plasmids have been found in various enterobacteria. This is especially true of *Salmonella* spp., some of which have been implicated in human infections (for review, see Goldstein *et al.*[12]).

The main characteristics of a successful resistance plasmid are to be transferable from one bacterium to another and to encode resistance to several unrelated antibiotics. Sequential studies from different centres have shown fluctuating levels of transferable resistance to trimethoprim among high level resistant strains. For example, in Edinburgh in 1982–83, a sharp decrease (50·7% to 37·2%) in the transferability of high-level trimethoprim resistance genes (normally associated with plasmid-mediated resistance) was observed, even though the incidence of high level resistance had remained constant.[13,14] Similarly at the St Joseph Hospital in Paris, transferable trimethoprim resistance reached a peak during 1980–81 (62·6% of highly resistant strains) then decreased to 45–50% by 1988.

These fluctuations in the transferability of high-level resistance can be explained, at least in part, by the presence of transposable elements encoding resistance to trimethoprim which can jump from one plasmid to another plasmid or on to the bacterial chromosome.[15] Transposon Tn*7*, which encodes resistance to streptomycin, spectinomycin and trimethoprim (DHFR Ia), was the first and the most common of the trimethoprim transposons described.[16] Its presence has been more or less demonstrated or presumed in many bacterial species by hybridisation studies with a probe encoding the DHFR Ia gene.[12] Other transposons, very similar and probably identical to Tn*7*, have been isolated from enterobacteria and *Vibrio cholerae*.[17] A second group of transposons, clearly different from Tn*7*, is represented by Tn*4132*; these encode resistance to trimethoprim alone.[18] Finally, a third group of transposons, represented by Tn*402*, encode a type II DHFR.[19]

Extensive studies of the occurrence of Tn*7* in enterobacteria have been performed in Finland in 1980–81 and in 1983.[20] A general increase in the percentage of enterobacteria harbouring Tn*7* was observed between the two study periods (47·3% in 1980–81 and 56·1% in 1983); however, the percentage of *Klebsiella* strains harbouring Tn*7* was significantly lower than in other enterobacteria during the study periods (12·9% in 1980–81 and 23·5% in 1983). All these fluctuations in the transferability rates can be explained by the movement of Tn*7* into the bacterial chromosome. In a study from Glasgow the percentage of chromosomal Tn*7* rose from 38% to 70% in hospital isolates during two study periods, 1979–80 and 1982.[21] The percentage of chromosomal Tn*7* was also very high in community isolates from Glasgow. Therefore, a general phenomenon can be proposed: after the initial "infection" of a bacterial cell by a plasmid carrying Tn*7*, the transposon will jump and remain on the chromosome even when the initial plasmid is cured spontaneously.

Several other trimethoprim-resistance transposons have been found on transferable plasmids; an epidemic with DHFR type II has been described in Boston.[22] In Paris, we found that 36% of unrelated plasmids isolated during 1981–84 were able to hybridise with a DHFR II specific gene probe. However, there is no evidence yet to suggest that a transposon carrying the type II DHFR gene has migrated into the bacterial chromosome.

Selection pressures

The last factor responsible for the increase in trimethoprim resistance is selection pressure. As seen from fig. 1, the percentage of trimethoprim-resistant *E. coli* did not always follow the consumption of sulphonamide-trimethoprim. After 1982, with the increased use of new cephalosporins and fluoroquinolones, the consumption of sulphonamide-trimethoprim clearly decreased and so did the percentage of trimethoprim-resistant strains. A very interesting study concerning the effects of selection pressure has been done in Nottingham.[2] With the increased use of trimethoprim alone, the percentage of trimethoprim-resistant strains which are susceptible to sulphonamides increased dramatically, especially in *Proteus* spp., such that by 1988, 67% of the *Proteus* strains isolated in the community were susceptible to sulphonamides (K. J. Towner, personal communication).

In several studies, Amyes and other authors demonstrated that ampicillin, which is widely used in many clinical settings, may select bacteria harbouring plasmids encoding joint resistance to ampicillin and trimethoprim.[23,24] Indeed, in our own hospital, between 53 and 87% of the plasmids encode joint resistance to both trimethoprim and ampicillin.

Conclusions

As inferred from epidemiological and genetic studies, resistance to trimethoprim is borne by many different replicons and can be mediated by epidemic strains, plasmids or transposons. These factors, combined with a widespread use in man and animals of trimethoprim, related substances and even unrelated antibiotics, may explain the emergence and spread of trimethoprim resistance in different species and provides a unique epidemiological model of the interaction between genetic elements, antibiotic policy and the resulting emergence, spread and stabilisation of resistance to an individual antibacterial drug.

Trimethoprim resistance in staphylococci

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Plasmid-mediated trimethoprim resistance in gram-negative bacteria emerged within 3 years of the clinical introduction of the drug.[11] The plasmid carriage of trimethoprim resistance undoubtedly contributed to the spread of resistance and the high proportion of resistant gram-negative isolates in certain areas of the world.[5]

Emergence of trimethoprim resistance in staphylococci

In gram-positive bacteria, the story is quite different. Amongst staphylococci, the incidence of trimethoprim resistance is very low, despite the continuous challenge with the drug for 20 years. Indeed, until the advent of methicillin-resistant *Staphylococcus aureus* (MRSA), trimethoprim resistance was virtually unheard of. In MRSA, the first trimethoprim-resistant strains were characterised by a low minimum inhibitory concentration (MIC) ranging from 10 to 250 mg/L.[25–29] This resistance was presumed to be encoded by genes located on the bacterial chromosome as no transfer of the resistance determinants could be achieved. The mechanism of this moderate level of resistance is still unknown, although it has been suggested that it results from an over-production of the bacterial DHFR.[30] This type of chromosomal resistance has been found in both eastern and western Australia, the USA and Europe.[25–29] It is likely that this chromosomal resistance represents an initial stage in the development of high-level resistance to trimethoprim in MRSA.

Plasmid-mediated trimethoprim resistance in staphylococci did not emerge until 1983, when it was found in Australian MRSA isolates.[28,31] They were characterised by their high MIC of trimethoprim (>512 mg/L). At that time, 10% of *S. aureus* strains in the USA were found to be trimethoprim resistant, but this resistance had not then been shown to be plasmid-mediated.[29] Archer *et al.*[29] demonstrated that strains from Virginia and from Pennsylvania were able to transfer their trimethoprim resistance genes and strains from both areas carried trimethoprim resistance plasmids (pG01 and pG05). Although the plasmids from the two

areas were different, they did show common restriction sites which indicated their relatedness to one another.[29]

The trimethoprim resistance plasmids isolated from staphylococci from different parts of Australia were compared and were also shown to have similar restriction patterns to one another, suggesting that they too came from a common source. However, these restriction patterns were, by and large, different from those found in the plasmids obtained in the USA.[30] The one exception was the restriction pattern around the trimethoprim resistance gene, which was common to plasmids from both continents.[30] Thus, although the trimethoprim resistance gene appeared ubiquitous, the plasmids on which it was carried were quite different. In Australian MRSA, 50% of the high-level trimethoprim resistance was mediated by the 28·4-kb plasmid, pSK1. The trimethoprim resistance gene could be located to a 2·75-kb region of plasmid pSK1 by comparing it to spontaneous deletion mutants which had become trimethoprim-sensitive.[32] A 5·1-kb *Eco*RI fragment of pSK1 was cloned into plasmid pACYC184, to form the hybrid plasmid pSK407. This hybrid plasmid was shown to express high-level trimethoprim resistance in *Escherichia coli*. Transposon mutagenesis, with Tn*5*, of the trimethoprim resistance gene showed that it comprised between 0·55 and 0·75 kb, suggesting it could code for a protein of up to 20 Kda.[33]

The type SI DHFR

The pSK1-containing clinical strain encoded a high level of DHFR (Specific Activity = 129·6 nmol dihydrofolate reduced/min/mg of protein). However, staphylococci generally encode much higher levels of chromosomal DHFR than their gram-negative counterparts. The high background enzyme activity made the initial identification of a plasmid-encoded DHFR much more difficult. However, the 5·1-kb *Eco*RI fragment of pSK1, which encoded the trimethoprim resistance gene, had been cloned by Lyon *et al.*[33] into the multi-copy plasmid pACYC184 in *E. coli*. The specific activity of the chromosomal DHFR in this strain was much lower than in staphylococci. Indeed, the DHFR activity encoded by the recombinant plasmid in *E. coli* was more than 20 times higher than that of the same host containing the same plasmid lacking the insert. The plasmid DHFR, which had originally been encoded by pSK1, was partially purified by ammonium sulphate precipitation and gel filtration. The molecular size of the enzyme, when estimated by Sephadex G-75 gel filtration, was 19·7 Kda,[34] which was very close to the 20 Kda predicted by Lyon *et al.*[33] from transposon mutagenesis of the gene. From these results, the enzyme appears to be monomeric.

The pSK1-encoded enzyme is far less susceptible to inhibition by trimethoprim, as 50 μM of the drug is required to inhibit the enzyme by 50% compared with 40 nM for the *S. aureus* chromosomal enzyme. This 1000-fold difference in inhibitory capability is matched by the difference in the MIC of trimethoprim for *S. aureus* strains containing a trimethoprim resistance plasmid and a fully sensitive plasmid-free *S. aureus* strain. Surprisingly, the plasmid-encoded DHFR confers no resistance to the dihydrofolate analogue, methotrexate. All the gram-negative plasmid-encoded DHFRs which confer high-level trimethoprim resistance (MIC > 1000 mg/L) are also resistant to the inhibitory action of methotrexate. Considering the close structural similarity of methotrexate and dihydrofolate, the gram-negative enzymes are remarkable in their ability to distinguish these two compounds by resisting methotrexate, but retaining a high affinity for the substrate dihydrofolate. This makes it virtually impossible to devise a new drug that more closely mimics dihydrofolate than methotrexate but is also selective. However, as the gram-positive enzyme is methotrexate sensitive, the possibility exists to design a new DHFR inhibitor with the inhibitory properties of methotrexate, but the selectivity of trimethoprim, and still be able to overcome the plasmid-encoded enzyme.

The DHFR encoded by pSK1 retained a high affinity for the substrate dihydrofolate, showing a higher binding capability (Km = 10 μM) than the *S. aureus* chromosomal enzyme (Km = 80 μM). Furthermore, although the plasmid enzyme is competitively inhibited by trimethoprim, it has a much higher Ki (11·6 μM) than that of the *S. aureus* chromosomal enzyme (Ki = 6·5 nM). All the properties show that the plasmid-encoded enzyme from Australia was different from the *S. aureus* chromosomal enzyme and from the 12 plasmid-encoded DHFRs found in gram-negative bacteria. We have thus designated this enzyme the type S1.[34]

In the USA, plasmid pG01 was also subsequently shown to encode a DHFR which, by examination of protein production with [^{35}S] methionine in minicells, was estimated to be 18·5 Kda.[35] In this study, the enzyme was less susceptible to inhibition by trimethoprim than the chromosomal DHFR with an ID50 of 7·3 μM. This was around 2000-fold less susceptible than the staphylococcal chromosomal enzyme. These results strongly suggested a

link between the trimethoprim-resistant DHFR encoded by the Australian and the American plasmids. In our hands, the American DHFR had a Mr of 21 000 and very similar kinetic and inhibition properties to the type S1 from Australia.

The *dfr*A gene, encoding the type S1 DHFR, has also been found in coagulase-negative staphylococci isolated in Australia. In particular, when the 0·9-kb *Eco*RI-*Eco*RV restriction fragment of plasmid pSK407 (which consists almost entirely of the trimethoprim resistance gene) was used as a probe, the DNA of four multi-resistant coagulase-negative bacteria hybridised.[32] In three strains (two *S. epidermidis* and one *S. hominis* I), the gene was located on a plasmid. In each case the plasmids were different from each other and from either pSK1 or pG01, the prototype trimethoprim resistance plasmids in *S. aureus*. In the final strain, *S. epidermidis* SK683, no plasmid location for the *dfr*A gene could be found and it was presumed to be located on the bacterial chromosome. The biochemical properties of the DHFRs were all very similar to the type S1 with the exception of the specific activity. In the case of the three plasmid-containing strains, the activity ranged between 27 and 101 nmol dihydrofolate reduced/min/mg of protein, which is high and consistent with the view that the genes are located on small, multi-copy plasmids. On the other hand, the specific activity in *S. epidermidis* SK683 was 7·7 nmol dihydrofolate reduced/min/mg of protein, which is compatible with the location of the gene on the limited copy-number chromosome.

The similarity of the biochemical properties suggest that all the DHFRs are closely-related. However, DHFRs with less than 70% DNA homology have been shown to display identical properties.[10] Conventional DNA-DNA hybridisation studies, performed under stringent conditions, will reveal DNA homologies of greater than 75–85%. Without sequencing the gene, it is impossible to state that two genes or gene products are identical. However, isoelectric focusing is extremely sensitive in its ability to reveal differences of just one amino acid in proteins consisting of 200 residues or more. However, the magnitude of the differences in pI gives no indication of the actual difference between the proteins, merely a demonstration that they are not the same. Conversely, it is rare for two enzymes with different amino acid sequences to have exactly the same isoelectric focusing pattern. The DHFRs encoded by not only the Australian and American *S. aureus* plasmids, but also the Australian and American coagulase-negative staphylococcal plasmids, were examined by isoelectric focusing, employing a modification of the method of Broad and Smith.[36] In every case, the plasmid-encoded DHFR band co-focused at pI 6·54, suggesting that the *dfr*A gene is ubiquitous in staphylococcal strains on both continents (S. Tait *et al.*, unpublished results).

Sequence of the dfrA gene and the type S1 DHFR

The *dfr*A gene in plasmid pSK1 has been ascribed to a 0·75-kb region of the plasmid.[32] This region is flanked by three directly repeated copies of an insertion sequence, IS*257*.[37] Their presence suggests that the *dfr*A gene and the flanking insertion sequences form a composite transposon, Tn*4003*.[30] The ubiquitous nature of the type S1 DHFR and the location of the *dfr*A gene in many different replicons, strongly supports a transposon location of the gene. Furthermore, there is a close similarity of the restriction endonuclease map of Tn*4003* from the Australian plasmid pSK1 with that of the trimethoprim resistance region on the American plasmid pG01, even though the rest of the plasmid is quite different from the American plasmids.[38] This suggests that the American plasmid also has the flanking IS*257* insertion sequence as well as the trimethoprim resistance gene.

DNA sequence analysis has confirmed that the trimethoprim resistance gene is located on a unique composite class I transposon, which is flanked by three rather than the normal two insertion sequences, namely IS*257*.[39] The direct orientation of the three insertion sequences has been shown to confer instability to the carriage of the transposon, by promoting spontaneous deletions of Tn*4003*. Plasmids with these specific deletions have been found both spontaneously within the laboratory and within clinical isolates.

The open reading frame for the *dfr*A gene has been found, by sequence analysis, to be 486 bp, corresponding to a protein of 162 amino acids. The active site of the DHFRs is at the N-terminal, the first 50 amino acids of which are shown in fig. 2. There are some similarities between this sequence and the type Ia plasmid-encoded enzyme in gram-negative bacteria. A closer similarity was noted between the sequence of the gram-positive chromosomal DHFRs from *Lactobacillus casei*, *Streptococcus faecium* and *Bacillus subtilis*.[39–42] However, a recent automated amino acid sequence of the N-terminal of the *S. aureus* trimethoprim-sensitive chromosomal DHFR suggests that the origin of the plasmid-encoded enzyme may lie in the same species in which it was first discovered.[43]

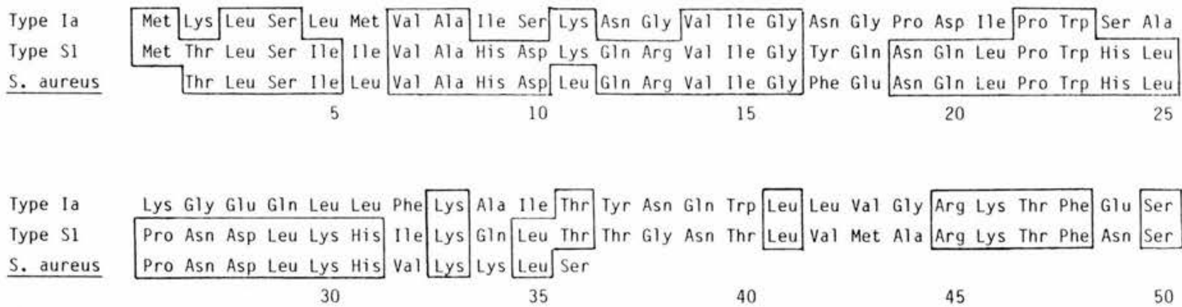

Fig. 2. Comparison of the N-terminal amino acid sequence of the type S1 plasmid-encoded dihydrofolate reductase with the sequence of the *S. aureus* chromosomal enzyme.

Conclusions

Plasmid-encoded trimethoprim resistance in staphylococci appears to be mediated by one gene, *dfr*A, which encodes the type S1 DHFR. The location of this gene on a transposon (Tn*4003*) has ensured that it has spread widely, not only geographically but also within the species of the genus *Staphylococcus*. The spread of the gene encoding the type S1 DHFR by Tn*4003* appears to be very reminiscent of the spread of the type Ia DHFR gene by transposon Tn*7* amongst the plasmids and chromosome of gram-negative bacteria.

Plasmid-encoded trimethoprim-resistant dihydrofolate reductases in gram-negative bacteria

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Plasmid-encoded trimethoprim resistance in gram-negative bacteria was first reported in 1972[11] and was originally associated with extremely high minimum inhibitory concentrations (MICs) of trimethoprim (>1000 mg/L). The mechanism of resistance was later shown to result from the plasmid-mediated production of an additional, trimethoprim-resistant DHFR.[44,45] So far a total of 12 different plasmid-encoded DHFRs belonging to seven major groups (types I–VII) have been identified in gram-negative bacteria (table II).

DHFR types I and II, encoded by plasmids R483 and R388 respectively, were the original plasmid DHFRs described and have now been reclassified as DHFR types Ia and IIb.[10] Both these enzymes are much larger than the chromosomal enzyme with a molecular size of 35 Kda, but differ from each other in their subunit composition—the type Ia enzyme being composed of two identical, inactive sub-units of 18 Kda, whereas the type IIb enzyme is made up of four identical, inactive sub-units of 8·5 Kda.[46] The type II enzymes are also much more heat stable and are, at least, one thousand times more resistant to the inhibitory action of trimethoprim than the type I enzymes.[47,48]

In 1986, a second sub-class of type I DHFR was identified, the type Ib, coded by plasmid pUK163. This enzyme has very similar biochemical properties to the type Ia enzyme encoded by R483, differing only in its molecular size (24·5 Kda). The sub-unit composition of this enzyme has not yet been determined, but it has been postulated that the type Ib DHFR may comprise two partial type Ia sub-units.[18] Both the type Ia and Ib DHFR genes are carried on resistance transposons, Tn*7* and Tn*4132* respectively.

The amino acid sequence of the type Ia DHFR has been determined and compared with the *E. coli* chromosomal DHFR sequence.[49] The two enzymes show 29% identity, increased to 44% when chemically similar residues are included, with the greatest regions of homology being in the amino terminal region which comprises most of the active site of the enzyme.

A number of sub-groups have also been distinguished among the type II DHFRs: the type IIa enzyme, encoded by plasmid R67;[48] the original R388 encoded type IIb enzyme;[46] and the type IIc enzyme coded by plasmid R751.[50] All three DHFR type II enzymes are very similar in their biochemical properties and share extensive (>78%) amino acid sequence homology.[51–53] However, sequence comparisons with other DHFRs reveal them to be quite unlike other plasmid or bacterial DHFRs. Indeed it has been postulated that the type II DHFRs may

Table II. Properties of the plasmid-encoded dihydrofolate reductases

Plasmid type	DHFR sub-type	MIC for Tp (mg/L)	Ki for Tp (μM)	Mr (Kda)	Countries where reported
I	a	>1000	7·4	35·0	All continents
	b	>1000	41	24·4	UK
II	a	>1000	6100	35·0	Europe, America, Asia
	b	>1000	150	35·0	Europe, America, Asia
	c	>1000	400	34·0	UK
III	a	64	0·019	16·9	New Zealand, UK
	b	128	0·15	17·0	USA
	c	512	0·64	22·0	USA
IV		10	0·063	46·7	India
V		>1000	3·2	36·0	Sri Lanka, UK
VI		>1000	162	9·0	South Africa
VII		>1000	7·0	11·5	UK
S1		>1000	11·6	19·7	Australia, USA, UK

have been derived from an oxidoreductase which originally acted on some other substrate.[46, 51]

The type III DHFR, which has now been reclassified as the type IIIa, was first described by Fling *et al.*[54] and is characterised by its low molecular size (16·9 Kda) and unusually low resistance to trimethoprim inhibition.[55] As a result, organisms producing this enzyme exhibit only a moderate level of resistance to trimethoprim (MIC 64 mg/L). Until recently, this enzyme had only been reported on one occasion in a clinical strain of *Salmonella typhimurium* isolated in New Zealand. However, in 1988, C. J. Thomson and colleagues (unpublished observation) identified the type IIIa enzyme in a clinical strain in Nottingham. Recently developed techniques for isoelectric point determination of DHFRs[36] have lead to the identification of two further sub-groups of type III enzymes, types IIIb and IIIc, encoded by plasmids from *Shigella* strains isolated in the USA (N. Barg *et al.*, unpublished observation). These enzymes also confer only a moderate level of trimethoprim resistance on their host, are very similar biochemically to the type IIIa enzyme, but differ markedly in their isoelectric points.

Sequence analysis of the type IIIa enzyme has shown it to share 51% homology with the *E. coli* chromosomal DHFR, suggesting that the type IIIa gene may have originated in either *E. coli* or in a closely related gram-negative organism.[56] Preliminary sequence data on the type IIIb DHFR, however, indicates it to be quite distinct from both the sensitive *E. coli* DHFR and the type IIIa plasmid enzyme (C. J. Thomson and S. G. B. Amyes, unpublished results). Thus, despite the similarities in biochemical properties between the two plasmid enzymes, their origin and evolution appear to have occurred independently of each other.

The type IV plasmid DHFR, identified in a number of different clinical isolates in South India, is larger than other plasmid DHFRs (46·7 Kda), is only 10 times more resistant to the inhibitory action of trimethoprim than the *E. coli* chromosomal DHFR, but is unique in its ability to be induced in the presence of increasing concentrations of trimethoprim.[57] Bacteria producing the type IV DHFR appear almost sensitive to trimethoprim when tested on conventional sensitivity test agar, although in complex broth containing trimethoprim these organisms show a significant level of resistance to the drug, similar to that exhibited by strains producing type III DHFR.[58] The mechanism of resistance conferred by the type IV DHFR differs from other plasmid DHFRs and is believed to occur by a "swamping" mechanism which involves the removal of trimethoprim through binding of the drug to the large number of type IV enzyme molecules induced in its presence. Amino acid sequence data has been obtained for the first 50 residues of the amino terminal region of the type IV DHFR. Comparison with the amino terminal regions of other DHFRs shows that the type IV enzyme shares 40% and 34% homology with the *E. coli* chromosomal enzyme and the type Ia plasmid enzyme respectively (C. J. Thomson and S. G. B. Amyes, unpublished results). This may indicate a common ancestry for the genes encoding these enzymes.

The type V plasmid DHFR was originally identified in clinical isolates from Sri Lanka.[59] This enzyme shows many biochemical similarities to the type Ia plasmid DHFR, although it differs significantly in molecular size, being only 17·5 Kda—a

similar molecular size to the type Ia subunit. The type V DHFR has now been identified in clinical bacteria in Nottingham (K. J. Towner *et al.*, unpublished results). The complete amino acid sequences of the types Ia and V DHFRs have been compared.[60] The two enzymes share 75% homology over their entire amino acid sequence and differ in only six amino acids within the first 50 residues forming the active sites of the enzymes. It seems likely, therefore, that these two enzymes share a common ancestral gene which, in each case, has followed a slightly different evolutionary path.

Finally, two further plasmid-encoded enzymes have recently been identified. The type VI plasmid DHFR has been found in only one clinical strain of *Proteus mirabilis* isolated in South Africa. This enzyme has a low molecular size (10 Kda) and, like the type II enzymes, requires very high concentrations of trimethoprim to inhibit its activity by 50%. These characteristics might indicate a possible relationship with an active type II DHFR subunit. Hybridisation studies, however, appear to indicate that extensive sequence homology does not exist between these two genes.[6] A type VII DHFR has recently been identified in an *E. coli* isolate of animal origin in Nottingham.[61] This enzyme, like the type VI enzyme, is also small and heat labile. However, it has similar inhibitory profiles to the types Ia and V enzymes.

It is evident from the numerous recent reports of new plasmid-encoded trimethoprim-resistant DHFRs that these enzymes are continuing to emerge and evolve in a manner comparable to the β-lactamase enzymes. With advances in molecular and biochemical technologies it is to be expected that novel plasmid-encoded DHFRs will continue to be identified and, hopefully, relationships between, and the origins of, those already distinguished will become clearer.

DNA probes for trimethoprim-resistant dihydrofolate reductases

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As described earlier in this review, seven major types of trimethoprim-resistant DHFRs encoded by plasmids have now been characterised in gram-negative bacteria, with several of these major groups being further divided into related subtypes.[10] Trimethoprim resistance in gram-positive bacteria has been slower to evolve, but at least one distinct trimethoprim-resistant DHFR has been identified in multi-resistant *Staphylococcus aureus*.[34] These enzymes can be distinguished from each other, and from the host chromosomal DHFR, on the basis of several biochemical and biophysical criteria,[18,36,48,55] but such identification is impracticable to perform for large scale epidemiological studies, primarily because of the time required to extract and purify each individual enzyme.

An alternative method of characterisation, which is simple to perform and can potentially be carried out on a large scale in routine microbiology laboratories, involves the use of non-radioactively-labelled DNA probes specific for particular DHFR genes. Carter *et al.*[62] demonstrated that a biotin-labelled DNA probe could be used to rapidly screen a large number of trimethoprim R plasmids for the presence of the DHFR type I gene. This method can be adapted for use with any antibiotic resistance gene for which a suitable DNA probe is available and, consequently, efforts have been made to develop specific DNA probes for each of the known DHFR types. The DNA probes which are currently available for these genes are listed in table III. Information on their specificity is reviewed and updated in the following sections.

DHFR I

The type I DHFR probe consists of a 499-bp *Hpa*I fragment of pFE872, derived originally from R483.[49] This DNA fragment is comprised almost

Table III. DNA probes for specific dihydrofolate reductase genes

DHFR type	Probe
I	499 bp *Hpa*I fragment of pFE872[49]
II	850 bp *Eco*RI fragment of pFE364 (or pFE700)[22] 280 bp *Sau*3A/*Eco*RI fragment of pWZ820[63]
III	855 bp *Eco*RI/*Hin*dIII fragment of pFE1242 (M. E. Fling, personal communication) 700 bp *Pst*I/*Eco*RI fragment of pUN972 (K. J. Towner, unpublished results)
IV	1700 bp *Cla*I fragment of pUK1148[64]
V	500 bp *Hin*cII fragment of pLK09[64]
VI	no probe currently available
VII	300 bp *Eco*RV fragment of pUN1042 (K. J. Towner and G. I. Carter, unpublished results)
SI	500 bp *Eco*RI/*Hin*dIII fragment of pG018[35]

entirely of the structural gene and forms an extremely useful probe for screening purposes. When biotinylated and used in conjunction with the high stringency wash conditions described by Carter *et al.*,[62] hybridisation is detected only with DHFR enzymes belonging to subtypes Ia and Ib. If low stringency wash conditions are used (two post-hybridisation washes only, each for 15 min in $2 \times$ SSC/0·1% SDS), hybridisation can additionally be detected with the type V DHFR gene (K. J. Towner and G. I. Carter, unpublished results). This is not surprising since DNA sequence analysis has shown that there is 68% similarity at the nucleotide level between the type Ia and type V enzymes.[60]

DHFR II

There are at least three subtypes within the DHFR II group, all of which seem to be closely related at the hybridisation level. Fling and colleagues produced a series of probes derived from R67 (subtype IIa), culminating in the use of a 850-bp *Eco*RI fragment from pFE364 or pFE700.[20] This probe (and its relatives) will readily eliminate any resistance genes which do not belong to any of the DHFR II subtypes (i.e., there are no "false-negatives"). Unfortunately, it also includes several hundred bases upstream of the type II structural gene, including a region of homology contained within the widely distributed transposon Tn*21*.[60,65] This probe consequently generates fairly frequent "false-positives" when used to screen trimethoprim R plasmids for the presence of the type II gene. Putative positive hybridisation results should therefore be confirmed either biochemically or by use of alternative probes.

One possible alternative probe for the type II DHFR group is the 280-bp *Sau*3A/*Eco*RI fragment of pWZ820, derived originally from R388 (subtype IIb). Sequencing data shows that the DHFR IIb structural gene is only 234 bp long,[66] of which 153 bp of the 3′-terminal end are included in the probe. When biotinylated and used under high stringency conditions[62] we have observed hybridisation of this probe only with plasmids which have also been subsequently shown by conventional biochemical criteria to belong to one of the DHFR group II subtypes.

DHFR III

The type III structural gene is contained within a 808-bp *Taq*I partial DNA fragment derived from the prototype III DHFR R plasmid pAZ1.[56] This fragment has been inserted into pUC9 to form the recombinant plasmid pFE1242. When used in conjunction with high stringency wash conditions, the 855-bp *Eco*RI/*Hind*III fragment of pFE1242 forms a reliable probe for eliminating resistance genes which do not belong to the type III group (i.e., there are no "false-negatives"). The probe does, however, contain approximately 350 bp external to the structural gene and therefore generates occasional "false-positive" hybridisation results.

The recent recognition of the type III resistance gene in *E. coli* responsible for urinary infection in the UK has enabled an alternative probe to be identified following cloning of the resistance gene into the recombinant plasmid pUN972 (K. J. Towner, unpublished results). A 700-bp *Pst*I/*Eco*RI fragment of pUN972 readily detects genes belonging to the type III group and contains less DNA extraneous to the structural gene than the probe derived from pFE1242. Further evaluation is in progress.

DHFR IV

The gene responsible for the type IV DHFR has been cloned into pBR322 to form the recombinant plasmid pUK1148.[64] The 1·7-kb *Cla*I fragment of pUK1148 forms a useful probe for the type IV gene. This probe also contains DNA sequences external to the type IV structural gene and, therefore, although it was very specific when tested against "control" DHFR plasmids, it can be anticipated that occasional "false-positives" may be generated when it is used to screen trimethoprim R plasmids on an epidemiological scale. To date, however, such "false-positives" have not been observed and the type IV gene has been detected only in bacterial isolates from Southern India.[10]

DHFR V

The type V DHFR gene was cloned into pBR322 by Sundström *et al.*[59] to form the recombinant plasmid pLK09. It was subsequently demonstrated by Towner *et al.*[64] that a 500-bp *Hin*cII fragment of pLK09 could be used successfully as a probe for genes belonging to the type V group. This probe consists almost entirely of the structural gene, but because of the 68% similarity at the nucleotide level between the type Ia and type V enzymes[60] it is important that it is used in conjunction with high stringency wash conditions. When biotinylated and used as a probe under the conditions described by Carter *et al.*[62] we have detected no cross-hybridisation with any of the other recognised DHFR groups.

DHFR VI

A probe is not yet available for this DHFR gene, which has to date only been detected on R plasmids isolated in South Africa.[10] However, no hybridisation has been observed between the plasmid carrying the prototype DHFR VI gene (pUK672) and the probes available for the other DHFR groups (K. J. Towner, unpublished results).

DHFR VII

This new enzyme group has recently been characterised in a trimethoprim-resistant strain of *E. coli* isolated in the UK. It was originally recognised on the basis of the failure of the trimethoprim R plasmid contained within the strain to hybridise with the probes for any of the other known DHFR types.[61] The type VII gene has now been cloned into pUC18, forming the recombinant plasmid pUN1042, and a 300-bp *Eco*RV fragment used as a preliminary probe (K. J. Towner and G. I. Carter, unpublished results). Unfortunately this probe contains only part of the structural gene. The type VII enzyme has very similar biochemical properties to the type I and type V groups,[61] and some cross-hybridisation was indeed detected between the type VII probe and the type V gene (but none of the other groups). Enzymes belonging to the type VII group can, however, be clearly distinguished as neither the type I nor the type V probes show any hybridisation with the type VII gene. Development of a more specific type VII probe is currently in progress.

DHFR S1

The type S1 DHFR is the only trimethoprim-resistant DHFR reported to date from gram-positive bacteria.[10] The gene has been cloned into pBR322 by Coughter *et al.*,[35] who also reported the use of a 500-bp *Eco*RI/*Hin*dIII fragment of the recombinant plasmid pG018 as a probe. This probe should be an intragenic fragment of the structural gene and was shown to fail to hybridise, even under low stringency conditions, with the type I, II or III genes.[35] It has now also been shown that there is a lack of hybridisation between the type S1 gene and the probes capable of recognising the other known DHFR types (K. J. Towner and G. I. Carter, unpublished results).

Conclusions

New groups of trimethoprim-resistant DHFRs are now being reported on a regular basis and it seems that DNA hybridisation procedures offer the best approach for monitoring the evolution and distribution of these important resistance genes on an epidemiological scale. The use of cloning procedures enables DNA probes to be constructed for a particular need. Although requiring a certain amount of specialist expertise, these procedures are relatively inexpensive to carry out. When combined with the development of non-radioactive detection systems for use in hybridisation experiments, it is now certainly feasible for studies on the epidemiology of trimethoprim-resistant DHFR genes to be carried out in most routine microbiology laboratories.

Clinical importance of trimethoprim resistance in staphylococci isolated in Europe

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Among the gram-positive bacteria, staphylococci are the most important pathogens in both community- and hospital-acquired infections.[67,68] The two most important species are *Staphylococcus aureus* and *S. epidermidis* and this communication focuses on these two species only. *S. aureus* is more virulent than *S. epidermidis* and may cause a number of serious infections including wound infections, abscesses, urinary tract infections, endocarditis, pneumonias and bacteraemias with a high mortality.[69,70]

S. epidermidis was, in the past, often regarded as a culture contaminant, but it is now well established that it is a pathogen. Most *S. epidermidis* infections are hospital-acquired and related to the ability of this organism to colonise skin and indwelling foreign devices.[71] The most serious consequences are bacteraemias and, in the USA, coagulase-negative staphylococci are currently the leading cause of nosocomial bacteraemias, with *S. epidermidis* accounting for 60–80% of cases.[72]

Antibiotic therapy

The frequency and severity of staphylococcal infections demands suitable antibiotic therapy. For non-β-lactamase producing staphylococci, penicil-

lin G or V are the drugs of choice. Since nearly all staphylococci nowadays produce β-lactamase, a penicillinase-resistant penicillin such as methicillin or cloxacillin is required, or alternatively, a cephalosporin, imipenem or clavulanate potentiated amoxycillin can be used. These agents, however, are rendered useless once methicillin-resistant staphylococci (MRS) prevail. Since MRS are almost uniquely multi-resistant, i.e., also resistant to gentamicin, erythromycin, tetracycline, and many other antibiotics, few alternatives are left.[68,72,73] Vancomycin is the drug of choice in these instances. Co-trimoxazole and, recently, the fluoroquinolones have proved to be valuable drugs against MRS.[74,75] Resistance to the fluoroquinolones, however, is being acquired at a surprisingly fast rate, especially by MRS.

Resistance to trimethoprim

Trimethoprim resistance in staphylococci has been reported since the early 1980s.[26,32] Trimethoprim resistance genes reside on large plasmids or in the chromosome and code for a novel, trimethoprim-insensitive DHFR.[32,34] In view of the importance of trimethoprim-sulphonamide combinations such as co-trimoxazole in the treatment of infections by MRS, we undertook a study to evaluate the relationship between resistance to trimethoprim, methicillin and sulphonamide, the level of trimethoprim resistance, its mobility and its mechanism.

In this study a total of 269 strains was investigated—196 strains of *S. aureus* and 73 strains of *S. epidermidis*. Only strains exhibiting resistance to one or more of the following antimicrobials were included: trimethoprim (Tp), methicillin (Mt), co-trimoxazole (TpSu) or gentamicin (Gm). All strains came from University Hospitals in Hungary (54 *S. aureus*, 21 *S. epidermidis*), Germany (30 *S. aureus*), Switzerland (43 *S. aureus*, 19 *S. epidermidis*), the UK (30 *S. aureus*) and Spain (39 *S. aureus*, 33 *S. epidermidis*). All strains were identified by growth on mannitol-salt agar, their DNAase and coagulase reaction, the API-Staph system (API SYSTEM S.A., Montalieu Vercieu, France) and the Staphrapid test (Roche). The susceptibility to eight antimicrobials—Tp, sulphamethoxazole (Su), Mt, penicillin (Pc), chloramphenicol (Cm), Gm, erythromycin (Em) and tetracycline (Tc)—was determined on Mueller Hinton Agar (Difco) at 30°C. The breakpoints for resistance were chosen according to the NCCLS recommendations[76] and were- $\geqslant$16 mg/L for Tp, Mt and Gm, and $\geqslant$512 mg/L for Su.

Among the *S. aureus* strains, 117 (60%) were resistant to Mt, 51 (26%) to Tp, 68 (35%) to Su, 85 (43%) to Gm, 186 (95%) to Pc, 54 (28%) to Cm, 118 (60%) to Em and 134 (68%) to Tc; 36 strains (18%) were resistant to MtTp, 48 (24%) to TpSu and 35 (18%) to MtTpSu. Coupled resistance to Mt plus Gm was more frequent; it was present in 66 strains (34%).

Large differences were observed if the results with strains from different sources were analysed separately. Of the 54 *S. aureus* strains from Hungary, 49 were resistant to Mt, 5 to Tp, 15 to Su, 16 to Gm, 4 to MtTp, 5 to TpSu and 4 to MtTpSu. Of the 39 isolates from Spain, 14 were resistant to Mt, 3 to Tp and only 1 to Su. None of these exhibited simultaneous resistance to MtTp or triple resistance to MtTpSu, whereas 14 were resistant to Mt plus Gm. Coupled resistance to MtTp was also rare in the isolates from Switzerland (1 strain), and none was resistant to MtTpSu. The isolates from Germany were generally the most resistant. Of 30 *S. aureus* strains studied, 22 were resistant to Mt, 25 to Tp, 28 to Su, 24 to Gm, 21 to MtTp, 25 to TpSu and 21 to MtTpSu. A rather high proportion of strains resistant to MtTp, TpSu and MtTpSu was also present in the *S. aureus* isolates from the UK (10, 11 and 10 strains, respectively, out of 30).

In contrast to *S. aureus*, the majority of *S. epidermidis* strains were resistant to Tp (56 out of 73, 77%); 27 (37%) were resistant to Mt, 45 (62%) to Su, 48 (66%) to Gm, 73 (100%) to Pc, 25 (34%) to Cm, 44 (60%) to Em and 55 (75%) to Tc. As in *S. aureus*, double resistance to MtTp occurred in 18 strains (25%), to TpSu in 43 (59%) and triple resistance to MtTpSu in 13 (18%). Analysis of the strains of different origin again showed large variation. Of the 21 *S. epidermidis* from Hungary, 6 were resistant to Mt, 17 to Tp, 10 to Su, 4 to MtTp, 10 to TpSu and 4 to MtTpSu, 6 to MtGm. Fifteen of the 19 Swiss isolates were resistant to Tp, 2 to Mt, 11 to Su, 5 to Gm, 2 to MtTp, 11 to TpSu, 1 to MtTpSu and none to MtGm. Twelve of the 33 isolates from Spain were resistant to MtTp, 22 to TpSu and 8 to MtTpSu.

From these data it seems that resistance to trimethoprim occurs more frequently in *S. epidermidis* than in *S. aureus*, and is also more frequently connected to methicillin resistance in *S. epidermidis*. The occurrence of strains resistant to MtTp or MtTpSu varies widely from country to country but is generally low. This underscores the role of co-trimoxazole as a valuable alternative in the treatment of multi-resistant staphylococcal infections.[74]

As regards the mechanism of trimethoprim resistance, the level of resistance was of interest. As shown in fig. 3, the majority of *S. aureus* strains

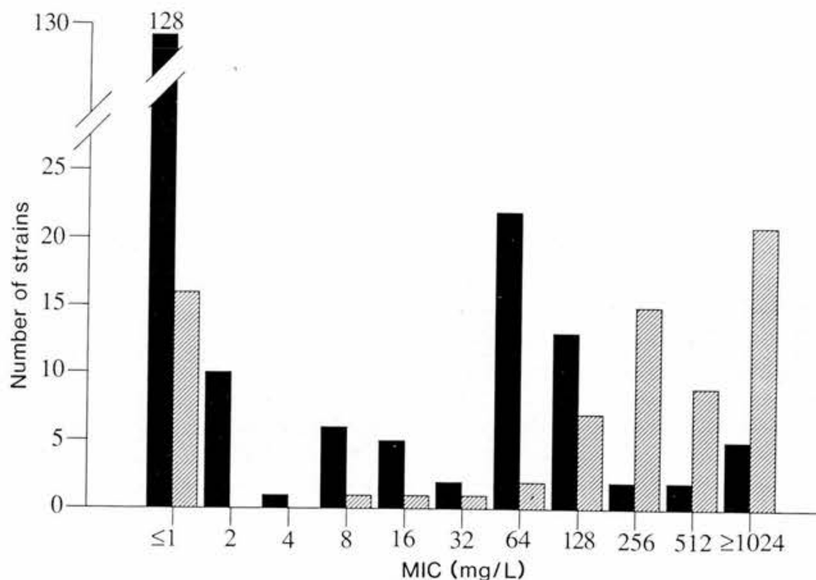

Fig. 3. Distribution of MICs of trimethoprim in *S. aureus* (196 strains; ■) and *S. epidermidis* (73 strains; ▨).

resistant to trimethoprim have MIC values of 64 and 128 mg/L. Only 7 of 196 strains tested exhibited high level resistance with MICs >512 mg/L. In contrast, there is a clear tendency in *S. epidermidis* towards high level trimethoprim resistance, with 30 strains exhibiting MICs of >512 mg/L. These differences in the level of trimethoprim resistance may reflect differences in the mechanism of resistance and in the genetic localisation of the responsible trimethoprim resistance gene.[38]

Transfer of Tp-resistance

Plasmid DNA was isolated from 46 trimethoprim-resistant strains as described by Bennett[77] with slight modifications. Agarose gel electrophoresis showed plasmids in all strains and generally two or more plasmids were present. Protoplast transformation was then carried out with *S. aureus* 113 (NCTC 8325 r^-) following the procedure from Goetz and coworkers[78] with some modifications. No transformants could be obtained after selection on trimethoprim 8 mg/L from the 21 *S. aureus* strains exhibiting MICs for trimethoprim between 32 and 256 mg/L. In the four strains with MICs of 512–1024 mg/L, trimethoprim resistance was transferable. With *S. epidermidis* only two strains with MICs for trimethoprim of 32 and 256 mg/L were tested and trimethoprim resistance was not transferable. In contrast, in 8 of the 19 strains with MICs of 512–1024 mg/L, trimethoprim resistance could be transferred. Details of those strains which showed transferable trimethoprim resistance are shown in table IV. Generally, only one plasmid was transferred to the host, ranging in size from 8·5 to 45 kb. In addition to trimethoprim, resistance to penicillin, cadmium nitrate (Cd), $HgCl_2$ (Hg), sulphonamides or ethidium bromide (EtBr) was often cotransferred. In no case, however, was resistance to gentamicin transferred, in contrast to findings with Australian isolates.[79] Occasionally a small plasmid carrying chloramphenicol (Cm) resistance was simultaneously co-transferred. Overnight growth at 44° in Mueller Hinton broth cured the majority of strains with transferable trimethoprim resistance and loss of trimethoprim resistance always coincided with loss of the large plasmid, whereas the small plasmids (<5 kb) conferring chloramphenicol resistance could not be cured.

Trimethoprim-resistant DHFR

The trimethoprim-resistant clinical isolates exhibited DHFR activities in the crude extract which ranged from 38 to 226 mU/mg of protein, in most cases above 100 mU/mg of protein. This is higher than the basal chromosomal enzyme level, i.e., in *S. aureus* ATCC 25923 34 mU/mg of protein or *S.*

Table IV. Resistance profiles in staphylococci with transferable Tp resistance

Strain	Origin	MIC for Tp (μg/L)	Resistance patterns	
			original isolate	transformant
S. aureus 157/4696	CH	>1024	Tp Su Pc Cm Tc Cd	Tp Pc Cd
S. aureus 853	D	512	Tp Su Mt Pc Em Tc Cd Hg	Tp Pc Cd Hg
S. aureus V2157I	D	1024	Tp Su Mt Gm Pc Cm Em Tc Cd	Tp Pc Cd
S. aureus 1075	CH	>1024	Tp Su Pc Cm Tc Cd	Tp Pc Cd
S. epidermidis 955	CH	>1024	Tp Su Gm Pc Em Tc EtBr	Tp EtBr
S. epidermidis 961	CH	>1024	Tp Su Gm Pc Em EtBr	Tp EtBr
S. epidermidis Hub	CH	>1024	Tp Su Pc Cm	Tp Pc Cm
S. epidermidis H8915	H	>1024	Tp Gm Pc Cm Em Tc EtBr	Tp EtBr
S. epidermidis H15043	H	>1024	Tp Su Gm Pc Em EtBr	Tp Su EtBr
S. epidermidis SP26	SP	1024	Tp Su Pc	Tp
S. epidermidis SP33	SP	1024	Tp Su Mt Gm Pc Em Tc	Tp Pc
S. epidermidis Mur78	SP	>1024	Tp Su Gm Pc Em	Tp Su

CH = Switzerland; D = Germany; H = Hungary; SP = Spain.

aureus 113 (NCTC 8325r⁻) 50 mU/mg of protein. As far as investigated, two enzymes with DHFR activity were found in these strains.[34,43] DHFR activity in the transformants was 1·5–2-fold higher than in the original isolate (with only two exceptions), probably due to the presence of only one plasmid resulting in a higher copy number. The trimethoprim resistance gene from *S. aureus* strain 157/4696 has, meanwhile, been cloned into the pUC18 vector and its nucleotide sequence is presently being determined.

Conclusions

A considerable number of MRS were found also to be resistant to trimethoprim. *S. epidermidis* strains were more generally resistant than *S. aureus*, as observed elsewhere.[38,72] However, the frequency of strains with resistance to both methicillin and trimethoprim varies widely among strains of different origin, but is generally low. Thus, trimethoprim-sulphonamide combinations may often constitute a valuable alternative treatment of infections due to MRS. As observed by others,[32] trimethoprim resistance could be transferred only from strains with a high MIC for trimethoprim. The clear tendency towards high level trimethoprim resistance in *S. epidermidis*, in contrast to *S. aureus*, is in line with previous observations and supports the idea that *S. epidermidis* constitutes a reservoir of resistance genes and that the trimethoprim resistance gene may have evolved in *S. epidermidis*.[38,72]

The dissemination of trimethoprim resistance plasmids in a large isolated community

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Perth, the capital of Western Australia, is a large modern city with a population in excess of one million people. It is, however, nearly 3000 km from the nearest city of similar size and thus is ideal for a study of the appearance and spread of R plasmids in a large isolated community. Trimethoprim, in the form of co-trimoxazole was first introduced into Australia for general clinical use in February 1973. Six years later, records at our hospital showed that there was a low incidence of trimethoprim-resistant enterobacteria. However, 1 year later, in 1980, a marked increase in the number of such isolates was noted.

This paper describes the results of surveys for trimethoprim resistance in human clinical isolates of enterobacteria carried out in 1980–81 and in 1985. In both surveys, isolates were examined for resistance pattern and transferability, together with the size and incompatibility group of any R plasmid conferring trimethoprim resistance. In the first survey, trimethoprim-resistant isolates from pigs were also included although trimethoprim had

Table V. Clinical isolates with trimethoprim resistance

Year	Source	Number of isolates						
		E. coli	*K. pn.*	*K. ox.*	*Ent.*	*Citro.*	*Proteus*	Total
1981	man	33	11	5	3	1	0	53
	pigs	59						59
1984	man	19	24	2	9	1	4	59

K. pn. = *Klebsiella pneumoniae*; *K. ox.* = *Klebsiella oxytoca*; *Ent.* = *Enterobacter* spp.; *Citro.* = *Citrobacter* sp.

never been used for growth promotion or therapeutic purposes in the pig farm. These isolates were part of the normal faecal flora of healthy weaned piglets and were obtained at very low frequencies. Trimethoprim-resistant human clinical and porcine isolates are listed in table V. More than 75% of the human clinical isolates came from urine specimens and *Escherichia coli* and *Klebsiella* spp. accounted for the majority.

A high proportion (86%) of the porcine isolates were capable of transferring trimethoprim resistance, whereas in the human clinical isolates, a lower proportion of transferable resistance was detected (table VI). The incompatibility groups for the transferable trimethoprim plasmids indicate that *Inc*FIV and *Inc*N R plasmids were initially important in the appearance of trimethoprim resistance in both the human and porcine isolates. Four years later, however, *Inc*C R plasmids had virtually replaced *Inc*FIV plasmids in the human isolates.

IncN trimethoprim R plasmids

In the first survey of trimethoprim resistance, the *Inc*N R plasmids ranged in size from 51 to 57 kb. They comprised two categories (SuTpHg resistance and SmSpSuTp resistance) and were transferred from various genera among the human clinical isolates. Representatives of each category had characteristic restriction endonuclease digest patterns and plasmids conferring SmSpSuTp resistance hybridised to the type I DHFR probe used (pFE506) whereas plasmids conferring SuTpHg resistance hybridised to the type II DHFR (pFE420). Both types of IncN plasmids were also detected in isolates from the pigs.

Table VI. Transferable trimethoprim resistance

Year	Number tested	Tra⁺ (%)	Number of isolates in Inc group			
			FIV	N	C	Uc*
1981	59 pig	51 (86)	30	3	†	1
	53 man	19 (36)	11	8	0	0
1985	59 man	35 (59)	1	21	10	3

*Uc = unclassified, i.e., compatible with all plasmids tested.
† — = undetected among the number studied.
Tra^+ = transfer positive.

By 1985 the *Inc*N plasmids had changed. They were larger in size (65–77 kb) and hybridised to the type I DHFR probe. Two thirds conferred resistance to ApTcSmSuTp and were detected in a *K. pneumoniae* strain causing nosocomial infections in the hospital. The remaining six plasmids in this survey came from a variety of hosts including *E. coli, Enterobacter* spp. and a *Citrobacter* sp. They were also larger and had some additional resistance determinants. The *Inc*N R plasmids had successfully maintained themselves in our community over that period.

IncFIV trimethoprim R plasmids

The *Inc*FIV R plamids are of great interest. They had rarely been reported in other parts of the world prior to their isolation by Mee and Nikoletti.[80] They ranged in size from 125 kb to 175 kb (table VII). The plasmids from human isolates exhibited a variety of resistance patterns, only some of which included resistance to Hg, whereas all the plasmids from porcine isolates conferred resistance to Hg. The plasmid collection was divided into six sets, labelled A–F, based mainly on their patterns of resistance.[81] Digestion of the porcine and human *Inc*FIV plasmids, using the restriction enzyme *Eco*RI, showed that plasmids in sets A, B and C shared 13 restriction fragments >1 kb in size amongst all representatives, while three other restriction fragments were common to all but one of the plasmid representatives.

The Cm plasmids from man and pigs (Sets D+

Table VII. Characteristics of the *Inc*FIV R plasmids

Origin	Resistance pattern	Size (kb)	Set
Man	SuTp	155	A
Man	TcSuTp	164	B
Man	TcSmSuTp	170–175	C
Man	TcCmSuTpHg	141–158	D
Pigs	TcCmSuTpHg	147–162	D′
Pigs	SuTpHg	125	E
Pigs	TcSuTpHg	131	F

Su = sulphonamide; Tp = trimethoprim; Tc = tetracycline; Sm = streptomycin; Cm = chloramphenicol; Hg = mercury.

D′) shared 14 fragments in common and another four restriction fragments were present in all but one of the representatives. Between these two groups, however, only three restriction fragments were common—14·6, 8·36 and 4·44 kb. Another four fragments were shared by some members of both groups. The R plasmids from pigs without Cm^r (sets E and F) were almost identical in their restriction fragment patterns. The Tc^r plasmids of set E had one extra restriction fragment of 5·55 kb. These plasmids shared only two of the three common restriction fragments (14·6 and 4·44 kb).

Thus the *Inc*FIV R plasmids with trimethoprim resistance comprised three major groups. They had various biotypes of *E. coli* as the original host, but differed in size, patterns of resistance and restriction endonuclease digest patterns. All, however, hybridised with the type II DHFR probe used and showed no reaction to the type I DHFR probe.[81]

The Tp^r genes from the epidemiologically related *Inc*FIV plasmids were cloned into plasmid pACYC184. The Tp^r gene was present on an *Eco*RI fragment > 20 kb for plasmids of sets A, B and C, a 5·28-kb fragment for sets D and D′, and an 8·81-kb fragment for sets E and F.[82] Despite the size variation, the DHFR gene itself was highly conserved on all plasmids and map differences in the flanking regions provided evidence that the most recent exchange of trimethoprim R plasmids between pigs and man had occurred relatively recently and had originated in the animals. These D and D′ R plasmids had identical *E. coli* hosts, based on biotyping, indicating that the organism, together with its R plasmid, had spread from pigs to man.

Conclusions

The *Inc*FIV trimethoprim R plasmids arose in Perth and exhibited considerable variation in their struggle to establish themselves. Other work has shown that they contain several replication regions and they rearranged their composition, losing and acquiring resistance determinants, during a relatively short time span. The extent of variation within the *Inc*FIV R plasmids is unusual. Most studies of R plasmids conferring resistance to a particular antibiotic and belonging to a particular incompatibility group have shown a considerable degree of structural stability of the plasmid itself for long periods sometimes exceeding a decade. The variation seen in the case of the *Inc*FIV trimethoprim R plasmids may reflect the difficulties of establishing in the local community. Within a few years they had been overtaken by "fitter" *Inc*N and *Inc*C trimethoprim R plasmids. During their stay they produced plasmids of sets A, B and C in man, sets E and F in pigs, and sets D and D′ in both man and pigs. All three groups represent unique lines of evolutionary development and probably arose from a prototype *Inc*FIV plasmid. It is not possible to ascertain whether this original *Inc*FIV plasmid arose in pigs or man, but the data indicate that plasmid exchange between man and animals is occurring relatively frequently.

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Editors' conclusions

The study of trimethoprim resistance has been greatly facilitated by the advent of improved technology. The use of specific DNA probes for the known plasmid-encoded trimethoprim resistance genes has allowed a much more rapid evaluation of the relative importance and dissemination of these different genes. Furthermore, DNA–DNA hybridisation studies have revealed the emergence of a plethora of new plasmid-encoded resistance genes with quite distinct biochemical properties. The advent of rapid automated nucleotide and amino acid sequencing procedures may soon provide us with even more efficient methods of studying possible inter-relationships between these different genes and understanding the evolution of trimethoprim resistance on a wider basis. Further collaboration between molecular biologists and protein biochemists will undoubtedly continue to provide exciting and illuminating advances in the study of trimethoprim resistance in the future.

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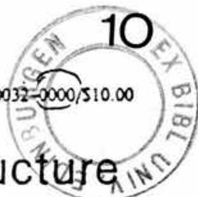

N-terminal amino-acid sequence and subunit structure of the type IV trimethoprim-resistant plasmid-encoded dihydrofolate reductase

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Summary. The type IV plasmid-mediated dihydrofolate reductase (DHFR), from a clinical strain of *Escherichia coli* isolated in South India, was prepared from a transconjugant containing the original clinical plasmid, *E. coli* J62-2 (pUK1123), and from *E. coli* C600 (pUK1150) containing a 2·6-kb *Hin*dIII fragment of pUK1123 cloned into plasmid pBR322. Both preparations were purified by methotrexate affinity chromatography. Automatic amino-acid sequencing of the N-terminal of the purified type IV enzyme from both sources gave an identical sequence which was clearly distinct from other plasmid-mediated trimethoprim-resistant DHFRs. The type IV DHFR showed most homology with the endogenous, chromosomally-encoded *E. coli* enzyme. Amino-acid sequence analysis also showed that the type IV enzyme preparation from *E. coli* J62-2 harbouring the original clinical plasmid, pUK1123, also contained the *E. coli* DNA-binding protein NS1. Analysis by polyacrylamide gel electrophoresis suggested that the type IV enzyme, in its native form, consists of a DHFR of M_r 33 000 coupled to a DNA-binding protein.

Introduction

Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine] prevents the bacterial NADPH-dependent reduction of dihydrofolate to tetrahydrofolate by competitively inhibiting the enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate; $NADP^+$ oxidoreductase, EC 1.5.1.3) (DHFR).[1] Shortly after its introduction in 1968, plasmids which encoded resistance to trimethoprim (T_p) were identified in gram-negative bacteria.[2] The mechanism of resistance was subsequently identified as the production of an additional trimethoprim-resistant DHFR which enabled the blocked endogenous chromosomally-encoded enzyme to be bypassed.[3]

The DHFRs encoded by resistance plasmids (R-plasmids) in gram-negative bacteria have been divided into seven major types based on their biochemical properties.[4] Some of the genes responsible have also been sequenced.[5–10] Initially, plasmid-mediated trimethoprim resistance was dominated by the spread of two genes encoding DHFR types I and II. However, since 1982, a number of new enzymes have been identified, the most unusual of these being the type IV DHFR.

Bacteria harbouring plasmids encoding the type IV plasmid-encoded DHFR were found in South India in 1984.[11] This enzyme confers low-level resistance on the host bacterium so may be a potential intermediate between a sensitive enzyme and the highly-resistant plasmid-mediated DHFR. This enzyme differs from the six other types identified in several ways: (1) it is an inducible enzyme, which is rare for a plasmid-encoded resistance enzyme in gram-negative bacteria; (2) it has a large M_r (46 700); and (3) it is only partially resistant to trimethoprim (ID_{50} $Tp = 0{\cdot}02\ \mu M$).[11] Examination of the induction mechanism of the type IV enzyme and its response to challenge with trimethoprim has suggested that this resistance mechanism is more similar to a resistance mechanism sometimes employed by the bacterial chromosome, namely the production of a mutant enzyme which is moderately resistant to trimethoprim and is hyperproduced, thus swamping the available drug, with sufficient activity remaining to continue the bacterial reduction of dihydrofolate to the active tetrahydrofolate form.[12,13]

It is important to establish how the type IV

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enzyme relates to the other plasmid-mediated DHFRs. We report the use of automatic amino-acid sequencing to give a rapid partial sequence sufficient to determine evolutionary relationships among trimethoprim-resistant DHFRs.

Materials and methods

Bacterial strains and plasmids

The type IV DHFR was prepared for sequence analysis from two bacterial strains: (1) *Escherichia coli* J62-2 (pUK1123), an *E. coli* transconjugant containing the original clinical trimethoprim R-plasmid from a clinical strain of *E. coli* isolated at the Christian Medical College Hospital, Vellore, South India in 1984;[11] (2) *E. coli* C600 (pUK1150), an *E. coli* clone containing plasmid pBR322 with a 2·6-kb *Hin*dIII fragment of pUK1123 encoding the type IV DHFR gene.[14]

Dihydrofolate reductase preparation

DHFR was prepared from 10-L overnight cultures in Iso-Sensitest Broth (Oxoid) grown at 37°C and shaken vigorously.[11] Bacteria were harvested by centrifugation at 6000 *g* for 15 min and resuspended in buffer A (50 mM sodium phosphate buffer, pH 7·4, containing 10 mM 2-mercaptoethanol and 1 mM EDTA). The bacteria were disrupted by sonication (2 × 30 s, 8 μM; MSE Soniprep) and the lysate was cleared by centrifugation at 40 000 *g* for 1 h at 4°C. DHFR activity was assayed at 37°C in 40 mM sodium phosphate buffer, pH 6·0.[3]

Enzyme purification and sequence analysis

DHFR was precipitated from the crude preparation by the addition of ammonium sulphate to 50–80% saturation. The enzyme was resuspended in buffer A and eluted from a Sephadex G-75 gel filtration column ($2\ cm^2 \times 90$ cm). The fractions showing peak activity were pooled and then applied to a column ($0{\cdot}375\ cm^2 \times$ 20 cm) containing methotrexate-agarose. This column was washed with buffer A until the absorbance at 280 nm was less than 0·01 OD units and all the unbound protein had been removed. The DHFR was then eluted with 2 ml of 0·5 M K_2HPO_4, containing 4 μM dihydrofolate, followed by 75 ml of 0·1 M K_2HPO_4 containing 1 μM dihydrofolate;[15] 5-ml fractions were collected. The three eluted fractions showing peak activity were pooled and concentrated, with Amicon Centiprep and Centricon concentrators, to 60 μl. Prior to sequence analysis, each preparation was checked for purity by reverse-phase HPLC analysis. The single peak of protein, determined on the reverse-phase HPLC, was analysed on an Applied Biosystems 477A protein sequencer.[16]

Molecular mass (M_r) determination

Molecular mass determination by Sephadex gel filtration was performed as described earlier.[11] SDS and native polyacrylamide gel electrophoresis (PAGE) were performed on a Pharmacia Phast system according to the manufacturer's instructions. For SDS gels, protein samples were boiled for 3 min in a mixture of SDS and bromophenol blue. For native gels, samples were simply dissolved in a solution of bromophenol blue. Protein bands were visualised by staining with Coomassie Blue. For SDS gels, a Sigma molecular size marker kit (14 000–70 000) was used, and for native gels bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 500) and trypsin inhibitor (M_r 20 100) were employed as standards.

Results

Molecular mass of the type IV DHFR

The type IV DHFR was purified by methotrexate affinity chromatography from *E. coli* J62-2 (pUK1123) and *E. coli* C600 (pUK1150). In both cases, the purified protein was analysed by HPLC and a single protein peak was observed; this was identified as DHFR.

We have shown previously that a partially-purified preparation of the type IV DHFR, encoded by plasmid pUK1123, has a M_r of 46 700 when measured by Sephadex G-75 gel filtration.[11] We now examined the methotrexate-agarose purified enzyme, encoded by plasmid pUK1123, on SDS-free PAGE. On a homogeneous 20% acrylamide gel, the enzyme from plasmid pUK1123 co-migrated with ovalbumin, corresponding to a M_r of 45 500. Staining of the gel with the non-specific protein stain Coomassie Blue (fig. 1) confirmed the high purity obtained by methotrexate-agarose affinity chromatography.

The M_r of the methotrexate-agarose purified enzyme from the cloned DHFR gene in plasmid pUK1150 was determined in a calibrated Sephadex G-75 colum and found to be 33 000 (fig. 2). This lower M_r could have arisen from two causes; (1) the cloning of the type IV gene into plasmid pBR322 may have removed some of the sequence of the gene or it may have put a new stop codon into the sequence allowing the translation of a protein of smaller size; or (2) the protein from the original plasmid pUK1123 may have been composed of two non-identical subunits and only one of the genes had been cloned. If (2) was the case, the original protein would show different M_r values when measured by PAGE with SDS.

SDS-PAGE for M_r determination

The type IV DHFR from the cloned gene in plasmid pUK1150 was treated with SDS and then applied to a 10–15% gradient SDS-containing

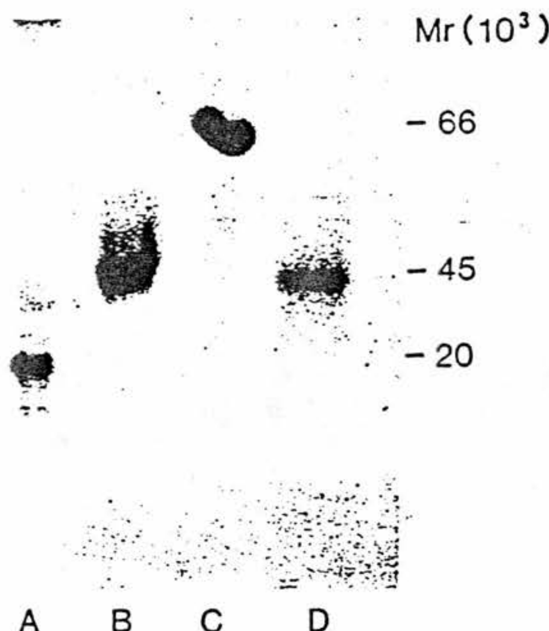

Fig. 1. SDS-free polyacrylamide gel electrophoresis of the purified type IV DHFR from *E. coli* J62-2 (pUK1123). Bands were visualised after staining with Coomassie Blue. **A**, trypsin inhibitor; **B**, ovalbumin; **C**, bovine serum albumin; **D**, type IV DHFR.

polyacrylamide gel. When it was electrophoresed until the dye front reached the end of the gel, it migrated as a single protein band of M_r *c*. 30 000. This result suggests that the enzyme from the cloned gene was a monomeric protein. On the other hand, when the purified DHFR encoded by the clinical plasmid pUK1123 was treated in the same manner, it migrated as one major band and a series of small bands (fig. 3). The major band had a M_r of 33 000. The smallest of the minor bands corresponded to an M_r of *c*. 10 000. The other minor bands were of similar intensity and appeared to have M_r values that were multiples of the smallest band, suggesting that they might be dimeric and trimeric forms of the M_r 10 000 protein. If this was the case, it indicated that the type IV enzyme of M_r *c*. 46 000, found on SDS-free PAGE and gel filtration was, in fact, made up of more than one subunit.

N-terminal amino-acid sequence analysis

HPLC was used to establish that the two enzyme preparations derived from plasmids pUK1123 and pUK1150 were pure proteins. The proteins from the respective HPLC peaks were applied to an Applied Biosystems 477A protein sequencer and a clear result was obtained for the first 50 amino acids of both enzymes (fig. 4). As expected, the DHFR sequences obtained from *E. coli* J62-2 (pUK1123) and *E. coli* C600 (pUK1150) were identical and quite distinct from those trimethoprim-resistant plasmid-mediated DHFRs that have already been sequenced.[5–10] There was, however, one distinct difference between the enzyme preparation derived from the clinical plasmid (pUK1123) and that from the clone. In the former, the presence of a second protein was apparent in the sequence analysis confirming that the native type IV DHFR was composed of non-identical subunits. The first 19 amino acids were distinguishable and comparison with known sequences has identified it as an exact match with the NS1 protein, a DNA-binding protein in *E. coli*.[17] The presence of this second protein in the pUK1123-derived preparation was particularly interesting because this DHFR preparation had appeared as a single protein on HPLC and on SDS-free PAGE. In the preparation derived from the cloned plasmid pUK1150, there was no evidence at all of any second protein. Furthermore, the NS1-binding protein has a very distinctive pattern when measured on SDS-PAGE. It migrates as not only a monomer of M_r 9250, but also as a dimer, trimer and tetramer.[18] This is the pattern that was found with the minor bands of the second protein on SDS-PAGE (fig. 3).

Discussion

Since the introduction of trimethoprim in 1968, several plasmid-mediated DHFRs have been identified that confer resistance to trimethoprim. These enzymes have, in the past, been largely distinguished by their biochemical properties[4] although the amino-acid sequence of many of them is now known.[5–10] Some of the more recently identified enzymes are clearly related to earlier plasmid-mediated DHFRs. For example, amino-acid sequence analysis has revealed that the types Ia and V are closely related.[10] The unique nature of the type IV enzyme, however, suggests that it may not be derived from any of the other plasmid enzymes. Indeed its sensitivity to trimethoprim and the low level resistance it confers would suggest that it is a precursor to the other plasmid-mediated enzymes.

In this investigation, the N-terminal sequence of the type IV enzyme was determined with the use of the Applied Biosystems 477A automatic amino-acid sequencer which allowed rapid sequence analysis. Two properties of the type IV DHFR make it particularly suitable for sequencing by this method; (1) the majority of the active site of all DHFRs is situated at the N-terminal of the protein;[19,20] and (2) purification of the native

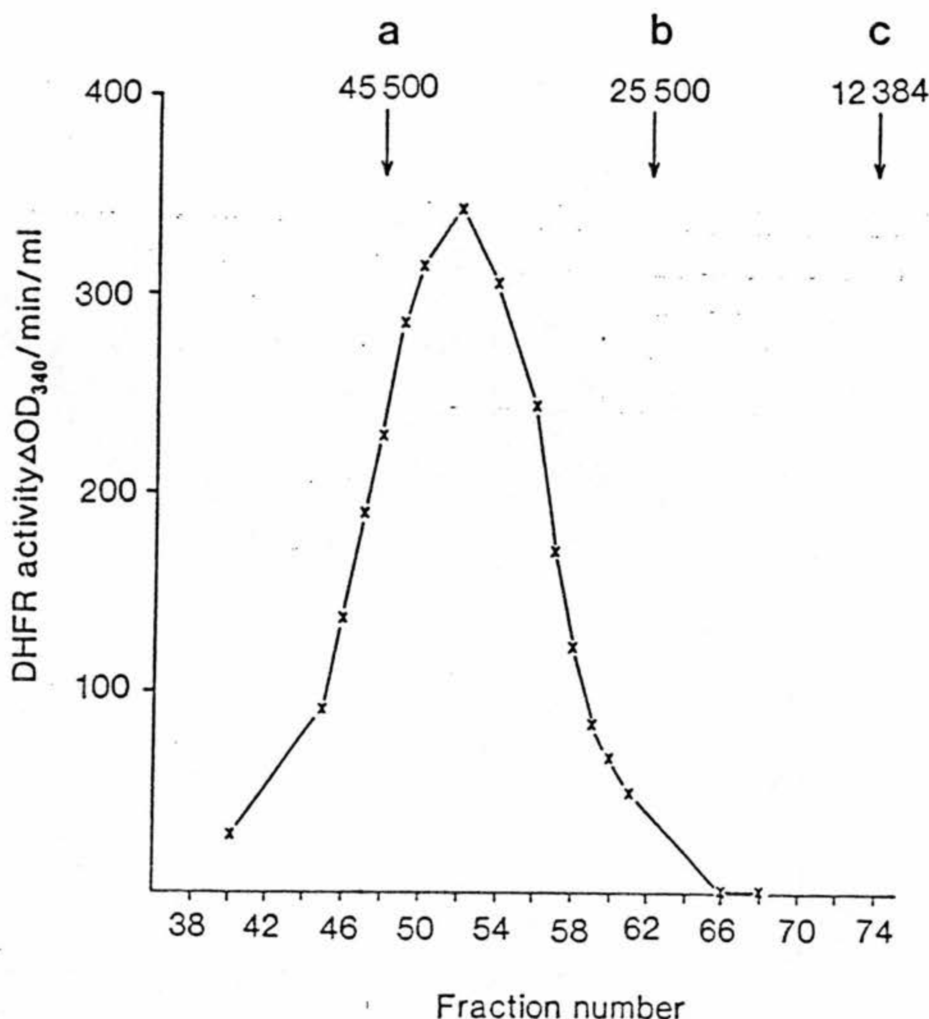

Fig. 2. DHFR activity of an ammonium sulphate (50–80% saturation) precipitate from *E. coli* C600 (pUK1150) analysed by Sephadex G-75 gel filtration. Elution of marker proteins is also shown—a, ovalbumin; b, chymotrypsinogen; c, cytochrome C.

protein is relatively easy with the use of methotrexate-agarose affinity chromatography, a result of the methotrexate sensitivity of the type IV enzyme, a relatively rare property amongst plasmid-encoded trimethoprim-resistant DHFRs.

Comparison of the first 50 amino acids of the type IV sequence with those for the other plasmid-mediated DHFRs sequenced so far (types I–V)[5–10] showed the type IV enzyme to be clearly distinct. However, significant homology occurred with the *E. coli* K12 chromosomally-encoded enzyme. Twenty direct matches were found between the two enzymes and nine further amino acids were homologous with the amino acids at the same position in the chromosomal enzyme.[21] This high degree of similarity between the type IV DHFR and the *E. coli* chromosomally-encoded enzyme is, perhaps, not surprising because the type IV enzyme, like the chromosomally-encoded enzyme, confers only partial resistance to trimethoprim. Furthermore, studies on the induction mechanism of the type IV enzyme have indicated that its mechanism of resistance is more similar to the hyper-production mechanism of resistance mediated by deregulation of the *E. coli* chromosomally-encoded enzyme rather than the classical by-pass mechanism mediated by trimethoprim R-plasmids.[22]

One surprising finding was the presence of the DNA-binding protein NS1 in the purified enzyme preparation from the original clinical plasmid (pUK1123). Analysis of this preparation by HPLC gave a single peak. As a further check on purity the preparation was run on native PAGE and stained with Coomassie Blue, and only a single band was

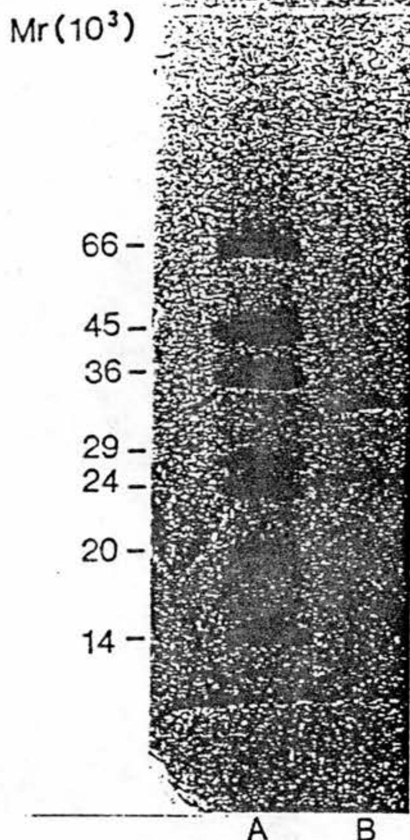

Fig. 3. SDS-PAGE of the purified type IV DHFR from *E. coli* 162-2 (pUK1123). Bands were visualised after staining with Coomassie Blue. A, Sigma mol. wt markers (M_r 14 000–70 000); B, type IV DHFR.

obtained at M_r 45 500 confirming its purity. This corresponds to the M_r of the type IV enzyme when measured by gel filtration. The results indicate that the native type IV enzyme is a complex of the two proteins. SDS-PAGE analysis of this preparation confirmed the existence of subunits of differing size. Purified enzyme from the cloned type IV DHFR from pUK1150 contained no DNA-binding protein. The cloned enzyme has a lower M_r and is monomeric when examined on SDS-PAGE. The most likely explanation for the lower M_r is the absence of the complexed DNA-binding protein.

The reason why the type IV enzyme is complexed with a DNA binding protein is not clear but it may be related to the induction mechanism, since proteins which bind to DNA have been implicated in the control of gene expression.[23,24] The absence of the binding protein in enzyme encoded by the cloned gene probably results from the lack of the NS1 gene in the restriction fragment cloned into pBR322. As pBR322 is a multi-copy vector, the necessity for induction of the type IV enzyme to produce trimethoprim resistance has been removed in the clone,[14] thus the selection pressure for the NS1 protein gene was not present in the cloning process.

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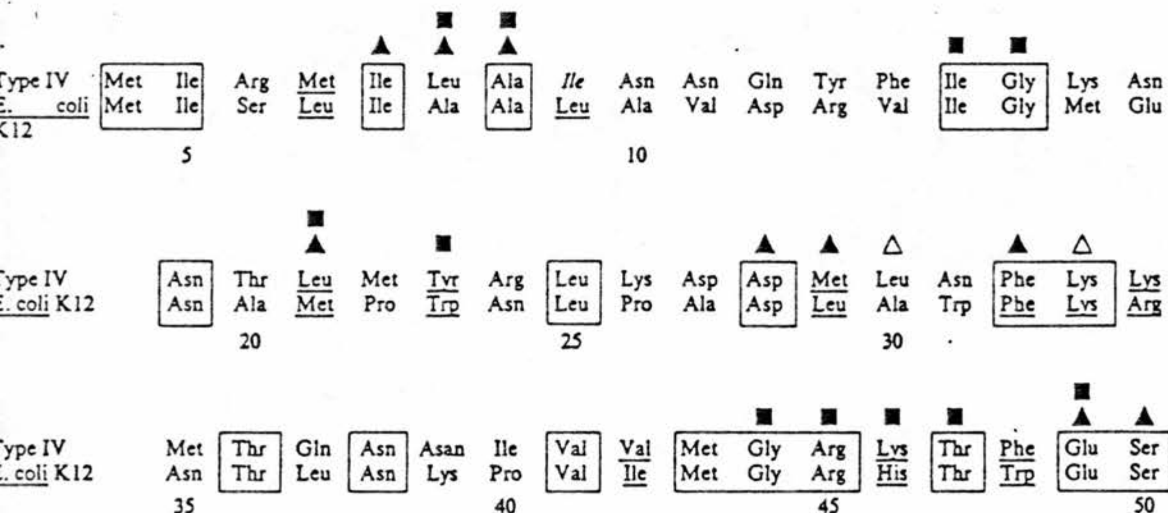

Fig. 4. N-terminal amino-acid sequences of the type IV plasmid-encoded DHFR and the *E. coli* K12 chromosomally-encoded enzyme. The amino acids are identified by standard three-letter abbreviations and are numbered according to the convention of Louch *et al.*[19] Identical amino acids are shown within the line boxes. Homologous amino acids are underlined. The amino acids involved in the active site are shown by the symbols at the top of the diagram: ▲ indicates the binding positions of trimethoprim; △ indicates the additional binding positions of methotrexate; ■ indicates the binding positions of NADPH. Positions taken from Louch *et al.*[19]

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thrombocytes, serum creatinine and liver enzymes were all normal.

During the first two days in hospital the patient experienced profuse diarrhea and abdominal pains. From the day 2, she was given oral norfloxacin, 400 mg twice a day. After two days of treatment the improvement was remarkable and she returned home a few days later in good general condition. The *Shigella sonnei* strain was sensitive in vitro to norfloxacin.

A possible explanation for this rare case of *Shigella sonnei* sepsis might be the absence of the primary filtration of the portal blood through the reticuloendothelial system of the liver, combined with the absence of the splenic clearance function. The double protection afforded by the liver and spleen might be the reason why *Shigella* sepsis is a rare event.

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Detection in the UK of Trimethoprim-Resistant *Escherichia coli* Encoding the Type V Dihydrofolate Reductase

Seven major types of trimethoprim-resistant dihydrofolate reductases (DHFRs) coded for by plasmids in gram-negative bacteria have now been described, with several of these major groups being further divided into related subtypes (1). Of the seven major types, the type V DHFR was originally identified in clinical isolates of enterobacteria from Sri Lanka (2) and has not been reported to occur in Europe. We have previously described a DNA probe suitable for monitoring the spread of plasmids mediating this form of trimethoprim resistance (3). This probe was used in a retrospective study to examine 14 trimethoprim R plasmids originally isolated during the period 1980–82 as part of an investigation into the distribution of the DHFR type I gene in the Nottingham area of the UK (4). The 14 trimethoprim R plasmids selected (Table 1) were those that had previously been found not to show any hybridisation with the DNA probe for the predominant type I gene (4).

Plasmid DNA was isolated on agarose gels and transferred to nitrocellulose filters as described previously (3). The DNA probe for the type V DHFR gene consisted of a *Hinc*II fragment of plasmid pLK09, approximately 0.5 kb in size (3). Probes for other known DHFR genes were those described by Amyes et al. (1). DNA probes were purified, labelled with biotin-11-dUTP (Bethesda Research Laboratories, UK), and then hybridised with the immobilised plasmid DNA (3, 4). Detection of a positive hybridisation result was by means of a Blu-GENE Kit (Bethesda Research Laboratories), using the conditions and protocols recommended by the manufacturer.

Eight of the 14 trimethoprim R plasmids gave a positive hybridisation result with the type V DHFR gene probe (Table 1). These eight plasmids were also found to give negative hybridisation results with the available DNA probes for other DHFR genes (types I, II, III and IV). Cell-free extracts obtained from cultures carrying each of the eight plasmids had DHFR-specific activities varying from 0.39 to 177.4 (Table 1), and it seems that this unusually wide variation may be characteristic of plasmid-encoded type V DHFR enzymes (2). When the properties of the enzyme encoded by pUN663a (chosen because of its high specific activity) were examined in more detail, as described by Amyes et al. (1), they were found to be essentially identical to those of the enzyme encoded by the cloned type V DHFR gene carried by pLK09 (Table 2) and different to those of other previously characterised DHFR enzymes (1).

The type V DHFR shows many biochemical similarities to the original type I DHFR (now termed type Ia). However, it does behave differently from the type Ia when separated on a Sephadex G-50 gel filtration column (5). The type V enzyme is held back in the column and gives an apparent molecular weight of 5,000, possibly due to a greater propensity for hydrophobic amino acids that may retard its progression in Sephadex. This is a characteristic of this enzyme and can be used to distinguish it, although the true molecular weight when measured on native PAGE is approximately the same as the type Ia enzyme. It seems probable, therefore, that these two enzymes may share a common ancestral gene.

Table 1: Plasmids screened for the type V dihydrofolate reductase gene.

Plasmid	Year isolated	Resistance pattern[a]	Hybridisation with type V probe	Dihydrofolate reductase specific activity[b]
pUN108	1979	Tp	-	
pUN166	1980	TpTcKmCmSu	+	0.58
pUN189	1980	TpSm	-	
pUN241	1980	TpTcKmCm	+	2.40
pUN283a	1980	TpSu	+	1.72
pUN365	1981	TpSu	-	
pUN394a	1981	TpSu	+	0.39
pUN433	1981	TpSm	+	10.46
pUN445a	1981	TpSu	+	1.27
pUN494	1981	TpTcKmCmSu	+	1.71
pUN545	1981	TpAp	-	
pUN589	1981	TpSmSuTcKm	-	
pUN663a	1982	TpAp	+	177.40
pUN835	1982	TpKmApSmSuTcCm	-	

[a]Tp: trimethoprim; Tc: tetracycline; Km: kanamycin; Cm: chloramphenicol; Su: sulphonamides; Sm: streptomycin; Ap: ampicillin.
[b]nmol of dihydrofolate reduced/min/mg total protein.

Table 2: Properties of the dihydrofolate reductases encoded by the prototype type V plasmids pLK09 and pUN-663a.

	pLK09	pUN663a
Activity ratio[a]	76	98
Trimethoprim ID50 (μM)	23	20
Methotrexate ID50 (μM)	3.5	2.0
TD50 (min)	3.0	< 2.0
M_r[b] (Da)	5,000	5,000

[a]Activity of enzyme related to the specific activity of the *Escherichia coli* chromosomal enzyme.
[b]Apparent relative molecular weight determined by gel filtration.
ID50: dose of drug to achieve 50 % inhibition of the enzyme.
TD50: time in min at 45 °C to achieve 50 % inhibition of the enzyme.

The fact that the type V enzyme has now been shown to occur in both Sri Lanka and the UK raises the interesting question as to whether evolution of the type V gene has occurred independently in two separate locations, or whether a single evolutionary event has been followed by wide distribution. Our results demonstrate that the evolutionary event distinguishing the type V from the type I DHFR had occurred in the UK at least as early as 1980.

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