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THE PURIFICATION, GENETICAL AND BIOPHYSICAL
CHARACTERIZATION OF THE CYSJ GENE PROTEIN

FROM ESCHERICHIA COLI K-12

A thesis submitted as part requirement
for the degree of Bachelor of Science (Dent.)
in the University of Sydney

by

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University of Sydney.
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Finally I thank the Academic and Technical staff and my fellow students in the Department, who assisted me in every way possible during this project, and who made my stay here a memorable and very pleasant experience.
ABSTRACT

NADPH-cytochrome c reductase, the product of the *cysJ* gene of *E. coli* K-12, was purified from an overproducing strain of *E. coli* constructed during the project. The purified enzyme was judged to be over 95% homogeneous on the basis of extensive electrophoretic analysis, and the purified preparation was used to obtain a subunit molecular weight estimate of 63,400 Da by SDS-discontinuous acrylamide slab gel electrophoresis. An amino acid composition was also obtained and experiments were carried out in order to define an N-terminal amino acid sequence. Other physical analyses were conducted and an investigation into the specific binding properties of the enzyme to a dye-ligand affinity medium was performed.

The purification scheme, developed *de novo*, utilized affinity chromatography as its central purification feature with the final purity of enzyme being increased approximately 500-fold over wild-type crude extracts.

Implications with respect to the mode of action of the *cysB* protein on the regulation of the *cysB* gene is discussed from the results obtained during this study.
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CHAPTER 1

INTRODUCTION

1.1 CYSTEINE BIOSYNTHESIS IN Escherichia coli K-12 AND Salmonella
typhimurium.

The genes coding for the structural and regulatory products necessary for cysteine biosynthesis in E. coli K-12 and s. typhimurium are grouped in defined areas of the chromosome, such as to constitute a regulon (Smith, 1971). The cysteine biosynthetic pathway in both of these organisms has been well documented (Kredich and Tomkins, 1966; Jones-Mortimer, 1968b) and involves two converging branches, a carbon and a sulphur branch. On the sulphur branch, sulphate is actively transported into the cell and activated via adenosine 5'-phosphosulphate (APS) to form 3'-phosphoadenosine 5'-phosphosulphate (PAPS) which is reduced to sulphite, then further, to sulphide. On the carbon branch, serine is converted to O-acetyl-L-serine (OAS) which reacts with sulphide to form cysteine (Fig. 1). The positions of the cysteine genes on the genetic map of E. coli K-12 are given in Fig. 2. In S. typhimurium the orientation of the cysJINHC gene cluster is inverted with respect to the orientation as read in E. coli (Jones-Mortimer, 1973), and cysB lies at 34 min. (Sanderson and Hartman, 1978) due to an inversion of the genes pyr-cysB-trp with respect to that found in E. coli.

The pathway is subject to feedback inhibition by L-cysteine, which is a specific inhibitor of serine transacetylase (Kredich and Tomkins, 1966; Kredich et al., 1969). Sulphate permease has also been shown to be feed-back inhibited by L-cysteine (Ellis, 1964; Ellis, 1966). In addition, sulphate permease (Dreyfuss, 1964), ATP-sulphurylase and APS-Kinase (Pasternak, 1962; Ellis et al., 1964; Wheldrake and Pasternak, 1965; Kredich, 1971), PAPS reductase and sulphite reductase (Dreyfuss and Monty, 1963a) and O-acetylseryserine sulphhydrylase (Kredich and Tomkins, 1966) have also been demonstrated to be repressed by growth on L-cysteine.

Wheldrake, (1967) demonstrated an inverse relation between the intracellular concentration of cysteine and the specific activity of the sulphate activating enzymes.
FIGURE 1

The cysteine biosynthetic pathway of *E. coli* K-12 and *S. typhimurium*: regulation (Jones-Mortimer, 1968c) and gene-enzyme assignment (Kredich, 1971).

\[\begin{align*}
\text{cysA} & : \text{ sulphate permease} \\
\text{cysB} & : \text{ positive regulatory gene} \\
\text{cysC} & : \text{ adenosine 5'-phosphosulphate kinase} \\
\text{cysD} & : \text{ ATP sulphurylase} \\
\text{cysE} & : \text{ serine transacyetylase} \\
\text{cysH} & : \text{ 3'-phosphoadenosine 5'-phosphosulphate reductase} \\
\text{cysJIG} & : \text{ NADPH-sulphite reductase} \\
\text{cysK} & : \text{ O-acetylséine sulphydrylase A}
\end{align*}\]
**Part A**

**REGULATION:**

\[
\begin{align*}
\text{SO}_4^{2-} &\rightarrow \text{APS} \rightarrow \text{PAPS} \rightarrow \text{SO}_3^{2-} \rightarrow S^2^- \\
\text{cysA} &\rightarrow \text{cysD} \rightarrow \text{cysC} \rightarrow \text{cysH} \rightarrow \text{cysJG} \rightarrow \text{cysK} \\
\text{INDUCTION} &\rightarrow \text{cysB} \rightarrow \text{PROTEIN} \\
\text{SERINE} &\rightarrow \text{OAS} \\
\text{ACETYL CoA} &\rightarrow \text{FEEDBACK INHIBITION}
\end{align*}
\]

**Part B**

**GENE - ENZYME RELATIONSHIPS:**

\[
\begin{align*}
cysE &\rightarrow \text{Serine} \\
cysK &\rightarrow \text{OAS sulphydrylase A} \\
\text{cysA} &\rightarrow \text{SO}_4^{2-} \rightarrow \text{APS} \rightarrow \text{PAPS} \rightarrow \text{SO}_3^{2-} \rightarrow S^2^- \\
\text{Cell} &\rightarrow \text{ATP} \rightarrow \text{APS} \rightarrow \text{PAPS} \rightarrow \text{NADPH} \\
\text{Wall} &\rightarrow \text{sulph- kinase reductase sulphite urylase} \\
&\rightarrow \text{MVH-sulphite reductase} \\
&\rightarrow \text{NADPH-cytochrome c reductase} \\
cysC &\rightarrow \text{cysD} \rightarrow \text{cysH} \rightarrow \text{cysI} \rightarrow \text{cysJ} \\
\end{align*}
\]
FIGURE 2

The positions of the cysteine genes on the linkage map of *E. coli* K-12 (Bachmann and Low, 1980).
E. coli K-12 map, 1980.
Pasternak et al., (1965) showed that the sulphur branch enzymes are not all repressed in a co-ordinate manner, suggesting a mechanism of differential repression whereby the synthesis of the activating enzymes is more sensitive to lower concentrations of cysteine than are the reducing enzymes.

Jones-Mortimer et al., (1968) have noted that OAS is another factor in the pathway's regulation. They have postulated that OAS is an inducer of the pathway, and cysE mutants, which lack the ability to make OAS, can only be induced by the addition of OAS to the medium. By the isolation of cysK mutants in E. coli, strains were found which had both lowered levels of 0-acetyl-L-serine sulphydrylase activity and partially constitutive levels of NADPH-sulphite reductase, due to the high intracellular levels of OAS in those strains. Introduction of a cysE mutation into the strains led to a significant decrease in NADPH-sulphite reductase levels in these double mutants, clearly demonstrating the inductive properties of OAS (Fimmel and Loughlin, 1977a).

Jones-Mortimer et al., (1968) have observed that cysteine and inorganic sulphide prevent induction by exogenously supplied OAS, indicating that repression over-rides induction. Growth of cells on L-cysteine leads to repression, and growth on L-djenkolic acid or glutathione leads to derepression of the enzymes for sulphate assimilation (Dreyfuss, 1964; Karbonowska et al., 1977).

Analysis of the cysB region has indicated that this gene is also involved in cysteine biosynthetic regulation (Dreyfuss and Monty, 1963b; Spencer et al., 1967). In E. coli, cysB mutants are pleiotropically negative (that is, they lack more than one of the enzymes needed for cysteine biosynthesis) and are proven as regulatory gene mutations; hence in order to synthesize the enzymes of sulphate assimilation in E. coli, the presence of OAS, a low intracellular concentration of cysteine and a wild-type allele of the cysB locus are all necessary (Jones-Mortimer, 1968a,b,c.)

The cluster of genes involved in sulphate activation and reduction are grouped together at 59 min. on the E. coli gene map, (Bachmann and Low, 1980); ordering of genes in this cluster being defined by Mizobuchi et al., (1962). The enzymic activities of the products of genes cysC, cysD and cysH were established by Dreyfuss and Monty, (1963b) and of cysI and
cysJ by Siegel and Kamin, (1971). Loughlin, (1975) by assaying enzyme levels in point and deletion mutants with lesions in the cysJ gene of *S. typhimurium*, indicated that the genes cysJ, cysI and cysH form an operon transcribed from cysJ to cysH. Certain mutant strains, deleted through cysIJ and H, had been shown however by Kredich, (1971) to possess ATP sulphurylase and APS kinase activity indicating that genes cysC and cysD are not part of the cysJIH operon. Thus this demonstrated that cysJIH reads as one transcriptional unit with the genes cysC and D not forming part of this unit. Further support for this evidence was obtained by Loughlin, (1976) through the characterization of four nonsense mutants in the cysJ gene of *S. typhimurium*. Preliminary experiments in *E. coli* (R.E.Loughlin, personal communication) have indicated a similar genetic organization to that found in *S. typhimurium*.

The purification, and subsequent physical characterization, have been carried out on a number of the cysteine biosynthetic enzymes to date: sulphate permease (Dreyfuss and Pardee, 1965; Pardee, 1966; Pardee et al., 1966), sulphite reductase (Siegel et al., 1973; Siegel et al., 1982), serine transacetylase (Baecker and Wedding, 1980), O-acetylserine sulphhydrylase (Kredich and Tomkins, 1966; Becker et al., 1969; Floss et al., 1976) and on the multicomponent enzyme complex, cysteine synthetase in *S. typhimurium* (Kredich et al., 1969; Becker and Tomkins, 1969; Cook and Wedding, 1976, 1977, 1978).

1.2 POSITIVE REGULATION BY THE cysB GENE PRODUCT.

Englesberg and Wilcox, (1974) have defined the criteria necessary for designating a system as positively regulated:

1. The isolation of deletion or nonsense mutations within the putative positive regulatory gene, this resulting in a pleiotropic negative phenotype.
2. The exclusion of the regulator gene from the operon(s) that it controls.
3. The isolation and mapping within the regulator gene of pleiotropically constitutive mutants.
4. The demonstration of *trans*-dominance in these constitutive mutants over their pleiotropic negative and inducible alleles.
5. The isolation and characterization of cis-dominant constitutive mutants in the controlled operons as revertants of the pleiotropic negative ones.

Evidence is summarized below which indicates that the cysB gene obeys many of the above criteria and hence is most probably a pure positive regulator of cysteine biosynthesis in E. coli and S. typhimurium.

The regulatory nature of the cysB gene product was noticed by Spencer et al., (1967) in S. typhimurium, whereby mutations that they isolated leading to a constitutive expression of the sulphur branch enzymes were localized in the cysB region of the chromosome. Studies by Jones-Mortimer, (1968c) in E. coli indicated that the wild-type allele, cysB\(^+\), is dominant to the mutant allele in stable and transient merodiploids, and that complementation in vitro was not observed between extracts of cysB mutants and mutants lacking only sulphite reductase. Hence from this data, it was postulated that \(\sigma\)-acetyl-L-serine and the product of the cysB\(^+\) gene were jointly involved in the positive control of the cysteine regulon, resembling in their functions the positive regulation exerted by the combination of arabinose and the araC\(^+\) gene in arabinose utilization (Sheppard and Englesberg, 1966). This postulate was in agreement with the pleiotropic negative phenotype observed in cysB mutants.

Tully and Yudkin, (1975) isolated amber and temperature sensitive mutations in the cysB gene of E. coli, indicating that the gene codes for a protein. Fine-structure mapping and complementation analysis of the E. coli cysB gene (Tully and Yudkin, 1977), demonstrated that the isolated point mutations belonged to a single complementation group, consistent with the results of Cheney and Kredich, (1975) for the cysB gene of S. typhimurium. The pleiotropic negative phenotype of cysB mutants does not appear to be due to negatively complementing subunits either, since an intact cysB\(^+\) gene complements all cysB mutations isolated (Tully and Yudkin, 1977); hence since mutations leading either to loss of expression or to constitutivity are both found in cysB (Cheney and Kredich, 1975) it seems probable that this gene codes for an effector molecule that controls cysteine biosynthesis in a positive fashion (Englesberg and Wilcox, 1974).
Fimmel and Loughlin, (1977b) by DNA-RNA hybridization, used a defective specialized lambda transducing phage carrying part of the cysJIIHC gene cluster (λdcysJIIHD) to detect cysteine specific mRNA synthesized _in vivo_. The results obtained indicated that cysteine biosynthesis is controlled at the level of transcription by the inducer OAS, the _cysB_ protein and cysteine. Jagura _et al._, (1978) used merodiploid strains of the types _FcysB^+/cysB^-_ and _FcysB^+/cysB^C_ in _S. typhimurium_ and studied in these strains the expression of sulphite reductase and _O_-acetylserine sulphydrylase (_cysB^C_ being the constitutive allele of _cysB_). It was found that under conditions of derepression, _cysB^+_ is dominant to _cysB^-_, and that in growth in cysteine, _cysB^C_ is dominant to _cysB^+_. Merodiploids of the type _FcysB^+/cysB^-_, bearing chromosomal point mutations, are derepressed to levels which are either less than, equal to, or greater than those of wild-type. These results predict a multimeric structure for the _cysB_ protein (Mascarenhas and Yudkin, 1980) and the formation in merodiploids of _cysB^-/cysB^+_ hybrid molecules with altered capacities for gene activation. Furthermore, the constitutive alleles _cysB1352_ and _cysB2341_, which allow partial to complete derepression of the cysteine biosynthetic enzymes, were isolated in a strain lacking serine-transacetylase and hence lacking wild-type levels of OAS. It is assumed, therefore, that the _cysB^C_ protein has been altered in such a way as to obviate the interaction with OAS to activate cysteine gene expression. Similar mutations in the arabinose regulatory gene _araC_ (Sheppard and Englesberg, 1966; Englesberg _et al._, 1969) have been isolated, but in contrast to the cysteine system, _araC^C_ is recessive to _araC^+_ due to the negative control exerted by the P1 conformation of the _araC_ protein. However, _cysB^C_ being dominant to _cysB^+_, would indicate that _cysB_ is a pure activator of transcription.

Ostrowski and Hulanicka, (1979) isolated a mutation, _cys-2332_, in the promoter region of the _cysJII_ operon, present in a _cysB_ deletion strain. This strain gave a constitutive expression of the _cysJII_ genes, hence indicating that this particular mutation has decoupled the transcriptional initiation process from the presence of _cysB_ and OAS, rendering the operon refractory to cysteine inhibition. The presence of sulphite reductase (encoded by _cysJ, cysI_ and _cysG_) activity in a _cysB-,cys2332_
double mutant indicated that *cysG* was not controlled by *cysB*. This demonstrated that along with *cysE* (Becker and Tomkins, 1969; Kredich, 1971), *cysG* is independent of OAS induction in *E. coli* and *S. typhimurium*, thus these two genes are not part of the regulon that is formed by the other genes involved in cysteine biosynthesis. The presence of sulphite reductase in this double mutant strain also eliminates a model whereby the *cysB* protein converts *O*-acetylserine to a true inducer which in turn inactivates a repressor produced by an unknown regulatory gene, as one would expect to find mutants lacking repressor and such mutants would show constitutivity for all cysteine genes controlled by *cysB*, whereas the *cys-2332* mutation affects the transcription of only the *cysJIIH* operon.

By use of the specialized transducing phage λ*cysB*, recombinant phages λ*cysB242* and λ*cysB257* were obtained, (Mascarenhas and Yudkin, 1980) each of which carried an amber mutation. By the comparison of polyacrylamide gel electropherograms of labelled extracts from UV irradiated bacteria that had been infected with a λ*cysB*+ or with a λ*cysB*− amber phage, this led to the identification of a 39,000 dalton polypeptide that was the product of the *cysB* gene; the protein was then purified to near radiochemical homogeneity and was found to be an oligomer with an isoelectric point close to pH 7.0.

Recently Jagura-Burdzy and Hulanicka, (1981) have used gene fusion techniques to investigate the control of the *cysB* gene itself. In this instance the *lacZ* gene was fused to and placed under the control of the promoter for the *cysB* cistron. In fusion strains the *cysB* gene is inactivated by the fusion event, but the *lacZ* gene coding for β-galactosidase activity is expressed. However, on the introduction of a *cysB*+ allele on a plasmid or episome into the fusion strains, the β-galactosidase levels fell. This implies an autoregulation by the *cysB* protein on its own gene. The direction of *cysB* transcription was then determined by having a specialized transducing phage, λ*cysB-lac*+, induced from the parent fusion strain by UV and using this phage to transduce a mutation in the *trp* proximal region of the *cysB* gene to wild-type *cysB*+ at high frequency thus indicating that the direction of transcription is from the *trp* side of the *cysB* gene to the *pyr* side of the gene.

On growing the parent fusion strains and the plasmid or
episome bearing strains on varying sulphur sources, β-galactosidase levels were not observed to vary significantly. These two classes of strains contain a \( \text{cysB}^- \) and \( \text{cysB}^+ \) allele respectively, indicating that unlike the control of the cysteine biosynthetic genes the \( \text{cysB} \) gene does not respond to the intracellular concentration of cysteine. In some of the fusion strains however, it was noted that the \( \text{cysB} \) locus did not appear to be repressed on introduction of the \( \text{cysB}^+ \) allele and it is suggested that a constitutive mutation in the controlling region of the \( \text{cysB} \) gene is involved in making the locus refractory to high intracellular concentrations of the \( \text{cysB} \) protein. It was suggested that these strains could be useful in producing large amounts of \( \text{cysB} \) protein.

Furthermore, Trudinger and Loughlin, (1981) have proposed that the \( \text{cysB} \) protein exists in two conformations, an inactive form which results from cysteine binding, and an active form which is the result of the binding of the inducer, OAS, and as repression over-rides induction, it can be implied that cysteine binds with greater affinity than does OAS to the \( \text{cysB} \) protein.

In this project, it is hoped that when a \( \text{cysB}^+ \) strain carries a hybrid plasmid bearing the \( \text{cysJ} \) gene and its promoter, that the \( \text{cysB} \) protein binding sites present on the hybrid plasmid (to an extent of approximately twenty copies per cell, without chloramphenicol augmentation) will derepress the synthesis of the \( \text{cysB} \) protein by titrating out the protein through a binding reaction onto the promoter sites on the available plasmids. Such an expectation is based on the assumption that the mechanism of action of the \( \text{cysB} \) protein is to bind to DNA at the promoters for the cysteine genes. Thus, by derepressing the autoregulatory system proven to act on the \( \text{cysB} \) gene it should be possible to obtain plasmid mediated \( \text{cysJ} \) enzyme enhancement to levels commensurate to the number of \( \text{cysJ} \) promoter sites present in the cell cytosol.

1.3 THE MALTOSE BIOSYNTHETIC SYSTEM: A FURTHER EXAMPLE OF PURE POSITIVE CONTROL.

Two widely spaced operons are involved in maltose utilization, designated as \( \text{malA} \) and \( \text{malB} \) (at 75 min. and 91 min. on the
E. coli gene map respectively, [Backmann and Low, 1980]). However, fine structure mapping, (Silhavy et al., 1979) has indicated that each of the two proposed operons is composed of two smaller operons, and MalA and MalB are now used to designate these two groups of operons (Fig. 3).

The malT cistron, which lies within MalA, (Débarbouillé and Schwartz, 1979) produces a protein which acts as the positive regulator for both MalA and MalB, evidence for which is given below. A review of the maltose system is given by Birge, (1981).

Silhavy et al., (1976, 1977), by isolating strains of E. coli in which the lacZ gene was fused to any one of the maltose operons, observed mutants which could be isolated and constitutively produce β-galactosidase. Several of these mutants carried a mutation in malT and these mutations, called malTc, are both cis and trans-dominant over their wild-type alleles, and the failure of the malT+ product to repress constitutive expression, resulting from either a malTc mutation (Débarbouillé et al., 1978) or from initiator constitutive mutations, similar to the cys-2332 lesion (Hofnung and Schwartz, 1971) indicates that maltose is regulated in a strictly positive manner by the malT gene product. A similar conclusion has been reached for the D-serine deaminase system of E. coli (Bloom et al., 1975).

A brief summary of genetic regulation in other biochemical pathways is given by Fimmel, (1977).

1.4 PLASMIDS AS CLONING VEHICLES.

Plasmids can be categorized into one of two major types: conjugative or non-conjugative, depending on whether or not they carry a set of genes called the tra genes, that promote bacterial conjugation.

Plasmids can also be categorized on the basis of whether they are maintained as multiple copies per cell (relaxed plasmids) or as a limited number of copies per cell (stringent plasmids). The replication of stringent plasmids is of necessity coupled to chromosome replication, hence their low copy number.

Generally, conjugative plasmids are of a relatively high molecular weight and are present as one to three copies per host cell chromosome (Table I). An exception is the conjugative
FIGURE 3.

The maltose regulon in *E. coli*. The products of cistrons *malF*, *malG* and *malK* have not yet been purified but like the products of *malE* and *lamb*, they are involved in the transport of maltose and maltodextrans. The directions of transcription of the four *mal* operons are indicated by broken arrows (Débarbouillé and Schwartz, 1979).
TABLE I: The properties of some conjugal and non-conjugative plasmids.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (Kbp)</th>
<th>Conjugative</th>
<th>No. of plasmid copies per cell</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>4.4</td>
<td>No</td>
<td>10-15</td>
<td>*Amp&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\text{Tet}&lt;sup&gt;R&lt;/sup&gt;$</td>
</tr>
<tr>
<td>ColE1</td>
<td>7.0</td>
<td>No</td>
<td>10-15</td>
<td>Colicin</td>
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<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>R6K</td>
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<td>F</td>
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<td>Yes</td>
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<td>-</td>
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<td>RI</td>
<td>109.1</td>
<td>Yes</td>
<td>1-3</td>
<td># MDR</td>
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</tbody>
</table>

*Ampicillin resistance.
$\text{Tetracycline resistance.}$
$\text{Streptomycin resistance.}$
# Multiple Drug Resistance.
plasmid R6K which has an estimated size of 41.9 Kbp (Kilo base pairs) and is maintained as a relaxed plasmid.

Plasmid incompatibility is the inability of two different plasmids to co-exist in the same host cell in the absence of selective pressure. Groups of plasmids which are mutually incompatible are considered to belong to the same incompatibility group or class. Currently over twenty-five incompatibility groups have been defined for plasmids present in E. coli. Plasmids belonging to incompatibility class P, for example RP4, are termed promiscuous, for they are capable of promoting their own transfer into a wide range of Gram-negative bacteria. Such promiscuous plasmids thus offer the potential of readily transferring cloned DNA molecules into a wide range of genetic environments.

Reviews of plasmid physiology are given by Falkow, (1975) and by Novick et al., (1976).

Prokaryotic and eukaryotic DNA segments have been introduced into and amplified on bacterial plasmids and viruses, making available for study large quantities of specific genes, gene clusters and regulatory DNA sequences (Hershfield et al., 1974; Kedes et al., 1975; Struhl et al., 1976). In addition, regulation of the foreign DNA sequence may be controlled from the proximal regulatory sequences on the plasmid vector or from within the foreign DNA insert itself. Evidence for the former control of insert genes has been obtained by Selker et al., (1977). By using EcoRI digestion of the specialised transducing phage φ80trpE-A carrying the entire tryptophan operon of S. typhimurium, a fragment was obtained containing intact trpA, trpD and trpC and another separate fragment was obtained carrying an intact trpA gene. The trpA fragment inserted into EcoRI cleaved plasmids, CoIE1 and pCRI, was expressed regardless of its orientation of insertion. Mitomycin C, a compound that induces colicin E1 production in CoIE1 containing bacteria, stimulated tryptophan synthetase α subunit production, (the product of the gene trpA) in cells containing CoIE1-trpA plasmids with the trpA fragment inserted in one orientation but not the other. Hence in the inducible plasmids, trpA can be expressed from the colicin E1 promoter external to the DNA insert.
Whether regulation is effected from within the foreign DNA insert or is dependent on fragment orientation with respect to the vector's own regulatory machinery, use can be made of the phenomenon of escape synthesis (Willard and Echols, 1968), or gene dosage effect, first noticed in strains transduced by a phage carrying a gene whose product was produced in excess of wild-type levels in the phage bearing strains.

Moir and Brammar, (1976) have shown how readily trp transducing phages derived in vivo may be used to persuade E. coli to produce greatly increased levels of the enzymes encoded by the trp operon.

On persual of the gene dosage effect, hybrid plasmids were used by Backman et al., (1976) and by Backman and Ptashne, (1978). These plasmids were constructed so that the promoter for the lac operon had been placed adjacent to the repressor gene of phage λ by in vitro recombination techniques. Levels of repressor thirty-fold higher than single lysogens were obtained in the plasmid bearing strain. This therefore is an example of control of the foreign DNA insert mediated by an externally positioned DNA regulatory system.

Fling et al., (1978) further studied the escape synthesis effect by utilizing the structural gene for a highly trimethoprim resistant dihydrofolate reductase isoenzyme present on the E. coli resistance plasmid, R67. By digesting the plasmid with EcoRI they obtained a 10.9 Kbp fragment. This DNA segment was then inserted, in vitro, into a series of small multi-copy cloning vehicles. The increase in enzyme gene number resulted in a proportionate amplification of trimethoprim resistant dihydrofolate reductase synthesis, so that plasmid copy numbers in the range of eighteen to thirty copies per chromosome corresponded with increased enzyme levels of twenty to thirty fold (Table II). Thus this is an example of the regulatory control of enzyme synthesis exerted from within the foreign DNA insert, as indicated by when the orientation of the insert was reversed in some of the constructed plasmids, the enzyme levels remained high.

Recently, further use of plasmid enhanced gene product levels has been used by Mercereau-Puijalon et al., (1978), Burrell et al., (1979), Goeddel et al., (1979) and Roberts et al., (1979) to obtain quantifiable amounts of certain gene products produced
**TABLE II:** The relationship between plasmid copy number and enzyme levels (Fling et al., 1978).

<table>
<thead>
<tr>
<th>Host strain</th>
<th>Hybrid plasmid</th>
<th>Cloning vehicle</th>
<th>Plasmid copy No.</th>
<th>Amplification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> J5</td>
<td>R67</td>
<td>-</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> HB101</td>
<td>pIE028</td>
<td>pBR313</td>
<td>31.0</td>
<td>28x</td>
</tr>
<tr>
<td><em>E. coli</em> 711</td>
<td>pIE028</td>
<td>pBR313</td>
<td>30.0</td>
<td>19x</td>
</tr>
<tr>
<td><em>E. coli</em> HB101</td>
<td>pIE024</td>
<td>pBR333</td>
<td>24.0</td>
<td>23x</td>
</tr>
<tr>
<td><em>E. coli</em> C600</td>
<td>pFE332</td>
<td>pBR322</td>
<td>18.0</td>
<td>35x</td>
</tr>
</tbody>
</table>

* Measured by the ratio of the dihydrofolate reductase specific activity in the hybrid plasmid strain to that produced in strains bearing plasmid R67.
in exceedingly small quantities in the wild-type strains.

1.5 THE HYBRID PLASMID pRL3.

The genealogy of pRL3 is outlined below.

The specialised transducing phage $\lambda$cy$\text{s}$$\text{J}$ was utilized by Smith, (1981) to construct the hybrid plasmid pRL1 by in vitro recombination (between $\lambda$cy$\text{s}$$\text{J}$ and the multipurpose cloning vehicle pBR322). $\lambda$cy$\text{s}$$\text{J}$ is able to transduce the cy$\text{s}$$\text{J}$ point mutant strain RL144 to Cys$^+$ but was not able to transduce the cy$\text{s}$$\text{J}$ point mutant, JM57, to cysteine prototrophy, and thus pRL1 was presumed to carry only the cy$\text{s}$$\text{J}$ gene and its promoter. From the mapping data presented for pRL1 (Smith, 1981) it was deduced that the known location of the cy$\text{s}$$\text{J}$ gene and its promoter, assumed to be approximately 1.5 Kbp in length, lay within the 8.8 Kbp HindIII-BamHI bacterial derived fragment of pRL1. On subsequent subcloning experiments (M.T. Smith, personal communication) a 5.9 Kbp HindIII-SalI fragment, within the larger 8.8 Kbp fragment, was ligated into pBR322 (likewise digested) and was used to transform RL421. It was observed that this chimeric plasmid gave rise to Cys$^+$ colonies, and hence was presumed to carry a fully functional cy$\text{s}$$\text{J}$ gene and its promoter.

The plasmid so derived was named pRL3 and was on preliminary investigation reported to be of 9.6 Kbp in size (M.T. Smith, personal communication). From this data, $\lambda$cy$\text{s}$$\text{J}$ was characterized as a galactose-type specialised transducing phage (M.T. Smith, personal communication). Thus, pRL3 has been demonstrated to carry 5.9 Kbp of bacterial DNA, comprising the cy$\text{s}$$\text{J}$ gene and approximately 4.4 Kbp of unrelated DNA. pRL3 also carries 3.7 Kbp of pBR322 DNA, which codes for a $\beta$-lactamase function, making transformed cells ampicillin resistant and hence readily selectable (Fig. 4).

Another plasmid which arose out of earlier cloning experiments is designated as pRL2 (Smith, 1981). Transformation experiments indicated that this plasmid bore a complete cy$\text{s}$$\text{J}$ gene and its promoter. It was further characterized as being approximately 10 Kbp in size though no restriction enzyme mapping data is currently available on it.

Importantly, pBR322 and thus pRL3, both carry a CoE1 derived replication origin, which allows for autonomous plasmid replication uncoupled from the host cell chromosome and hence the ability to increase in copy number on chloramphenicol treatment, (Clewell, 1972).
The hybrid plasmid pRL3, showing phenotypic characters and the unique sites for the restriction endonucleases \textit{HindIII} and \textit{SalI} (M.T. Smith, personal communication).

1 Kbp is represented by 4 cm.; the arrow indicates the direction of transcription of the ampicillin resistance determinant.

The question marks indicate that the exact position of the \textit{cysJ} gene and its promoter within the 5.9 Kbp of bacterial DNA carried by pRL3, are not known.
pRL3

9.6 Kbp

The cysJ gene and its promoter
The genealogy of pBR322 has also been fully documented (Bolivar et al., 1977a,b; Covarrubias, 1981), and has been used for cloning both prokaryotic and eukaryotic DNA sequences into various bacterial species.

It was anticipated in this project to further utilize pRL3 transformed into a suitable strain of E. coli to effect an increased synthesis of the cysJ gene product and hence to allow a purification of the cysJ protein from a relatively small initial culture volume.

1.6 NADPH LINKED SULPHITE REDUCTASE OF E. coli AND ITS COMPONENTS.

E. coli and S. typhimurium NADPH-sulphite reductase (hydrogen-sulphide: NADP⁺ oxidoreductase; EC 1.8.1.2) has been purified to homogeneity (Siegel et al., 1973) and extensively analyzed on both physical (Faeder et al., 1974; Murphy et al., 1974; Siegel and Davis, 1974; Siegel et al., 1974; Siegel et al., 1977; McRee and Richardson, 1982; Siegel et al., 1982) and genetical aspects (Siegel and Kamin, 1971; Siegel et al., 1971) have reviewed the correspondence between the data obtained through both the physical and genetical approaches in the characterization of sulphite reductase in E. coli and S. typhimurium.

The enzyme catalyzes the six electron reduction of sulphite to sulphide, and is a soluble complex of molecular weight 670,000. It contains, per molecule, (Siegel et al., 1973) four FAD, four FMN, approximately sixteen non-heme iron (NHI)-acid labile sulphides and three to four molecules of a novel octacarboxylate iron-tetrahydroporphyrin prosthetic group, which has been termed siroheme (Murphy and Siegel, 1973; Murphy et al., 1973). With these prosthetic groups the enzyme is a self-contained miniature electron transport chain somewhat analogous to that found in the membrane bound state of mitochondria and microsomes.

E. coli sulphite reductase can further be described as a complex of two functional components, which may be dissociated from one another by urea treatment of the enzyme, (Siegel and Davis, 1974). These two components comprise a flavoprotein, which contains FMN and FAD, and a hemo-protein, which contains the iron-sulphur and siroheme groups. Neither component by itself possesses NADPH linked sulphite
reductase activity. The flavoprotein component can
catalyze the reduction by NADPH of a variety of artificial
electron acceptors, including cytochrome \( c \) and other
diaphorase-type substrates including 2,6-dichlorophenolin-
dophenol (DCPIP), ferricyanide and menadione. The hemo-
protein component can catalyze the reduction of sulphite to
sulphide with the artificial electron donor reduced methyl
viologen (MVH) or with the \textit{in vivo} electron donor NADPH,
providing that an intact flavoprotein component is present;
furthermore, on mixing the flavoprotein and hemoprotein
components, an NADPH-sulphite reductase activity can be
reconstituted \textit{in vitro} (Siegel and Davis, 1974).

The flavoprotein and hemoprotein are each composed of
a single type of polypeptide chain, termed \( \alpha \) and \( \beta \) respective-
ly, with individual molecular weights of approximately
59,000 and 56,000 (Siegel \textit{et al.}, 1971).

The active flavoprotein has been isolated only in an
octameric form (Siegel and Davis, 1974) designated as \( \alpha_8 \);
the hemoprotein can be isolated however as a monomer, capable
of catalyzing MVH linked sulphite reduction. The sulphite
reductase holoenzyme has the structure \( \alpha_8 \beta_4 \) with one \( \alpha \)
chain per flavin in the complex and four hemoprotein monomers.

Studies with site-specific inhibitors have led to the
hypothesis that the FAD and FMN prosthetic groups of sul-
phite reductase can serve quite different roles in the
electron transfer process (Siegel \textit{et al.}, 1974). Thus the
mercurial \( p \)-chloromercuriphenylysulphonate (\( p \)-CMPS), at low
concentrations, promotes the dissociation of FMN, but not
FAD, from sulphite reductase. Even though the NADPH de-
dependent reductase activities of the enzyme are strongly
inhibited by this treatment the inhibition can be reversed
by the addition of large quantities of FMN to the enzyme.
The FAD moiety remains reducible by NADPH in the mercurial
treated enzyme but it cannot transfer electrons to the heme
prosthetic group unless exogenous FMN is supplied. This
result suggests that FAD might serve as the initial port of
electron entry from NADPH into the enzyme complex and that
FMN served as a transmitter of electrons from FAD to the
heme prosthetic group (or to artificial electron acceptors
such as cytochrome \( c \)). Faeder \textit{et al.}, (1974) demonstrated
that the flavoprotein contained four binding sites for pyridine nucleotide (one per FAD) and that the interaction of the enzyme with pyridine nucleotides, as measured by binding studies, is at the level of the FAD prosthetic group and not the FMN group. Earlier studies (Siegel and Kamin, 1968) however, indicated that 7-8 binding sites for NADPH were available per enzyme molecule (that is, two sites per FAD); these observations being recorded by fluorescence quenching. As the studies by Faeder et al., (1974) were dealing with an FMN-depleted enzyme preparation then although NADPH binding and subsequent flavin reduction were both seen to occur, the absence of the FMN moiety may have precluded the correct in vivo stoichiometry of substrate (NADPH) to enzyme from being recorded. Thus it is assumed in this study that for enzyme preparations which are not likely to be FMN-depleted to any extent, that an NADPH to enzyme binding ratio of 8 to 1 is more tenable (see Chapter 3, pp. 54-55).

A model for the postulated mode of electron flow in sulphite reductase is presented in Fig. 5. Siegel et al., (1971) isolated the flavoprotein component in cysG and cysI single and double mutants of S. typhimurium, and showed that it catalyzed an NADPH linked cytochrome c reductase activity, which was absent in cysJ mutants with corresponding wild-type loci in cysG and cysI. Hence from this data the cysJ gene was implicated in coding for the monomeric α subunit of the flavoprotein, which self-aggregates in the cytosol into α₈ octamers. Studies with these mutants allowed a genetic model to be constructed to explain the gene functions for the cysJ, cysI and cysG loci, all three of which need to be present as their wild-type alleles to allow the formation of a catalytically active sulphite reductase holoenzyme molecule.

Thus by the isolation of these mutants, Siegel et al., (1971) were able to prove the identity of the cysJ gene as the producer of an enzyme that catalyzed the NADPH linked diaphorase reactions, cysI as the producer of the hemoprotein subunit and cysG as an enzyme coding for a transmethylation reaction between
FIGURE 5

Postulated direction of electron potential in NADPH-sulphite reductase (Siegel et al., 1974). Double headed arrows represent a reversed electron flow from MVH to diaphorase acceptors and to NADP⁺. Gene assignments are those of Siegel et al., (1971).

The in vivo direction of electron flow is shown in red.

ABBREVIATIONS:

MVH Reduced Methyl Viologen.

NHI Non Heme Iron.

p-CMPS p-Chloromercuriphénylsulphonate.
uroporphyrinogen III and S-adenosyl methionine to form the siroheme prosthetic group of the hemoprotein subunit; a review is presented by Trudingner and Loughlin (1981).

With the high purity of Siegel's preparation of the sulphite reductase holoenzyme, dissociation of the hemoprotein from the flavoprotein subunit was able to be achieved by DEAE-cellulose chromatography with the flavoprotein effectively binding to the DEAE-cellulose (Siegel and Davis, 1974). However previous work (R.E. Loughlin, personal communication) on reduction and alkylation of a pure preparation of holoenzyme and subsequent separation of the cysJ and cysI polypeptides by DEAE-cellulose chromatography in the presence of urea led to poor yields of pure flavoprotein being recovered due to a strong tendency for the two polypeptides to aggregate (as observed in sedimentation studies). Furthermore, on urea treatment of the holoenzyme to separate its components, a denaturation of the NADPH-cytochrome c reductase activity occurred which prevented the specific activity of the separated flavoprotein from being determined. Notably also, as urea is left in solution it degrades, producing cyanate ions, which can potentially block any available protein's N-terminus leading to a decreased yield of termini available for desired amino acid sequencing purposes by the Edman degradation method. Hence in this study it was intended that a strain of E. coli be used that had a deleted cysI gene in order to prevent problems with trying to separate the cysI encoded protein from the cysJ polypeptide.

The assimilatory sulphite reductases of Allium odorum (Tamura, 1965), Aspergillus nidulans (Yoshimoto et al., 1967), yeast (Yoshimoto and Sato, 1968), spinach (Asada et al., 1969) and Desulfovibrio vulgaris (Lee et al., 1971) have also been highly purified and characterized.

1.7 DYE-LIGAND AFFINITY CHROMATOGRAPHY.

The phenomenon of dye-ligand chromatography was first observed by Haeckel et al., (1968) on purifying yeast pyruvate kinase. It was noticed that the enzyme co-chromatographed on a Sephadex® G-200 gel column with blue dextran, a high molecular weight dextran derivatized with the triazinyl dye Cibacron Blue 3GA. It was subsequently shown that the blue
dye was responsible for the binding and not the dextran. Later use was made of the technique to purify sweet corn R enzyme (Marshall, 1970) and blood coagulation factors (Swart and Hemker, 1970). The dye was named blue-Å and became established as a group selective affinity ligand, either in its original blue dextran form, or when coupled directly to other support matrices. The basis for the group selective affinity nature of the dye in binding dehydrogenases and reductases has been proposed by Thompson et al., (1975a,b), Stellwagen et al., (1975) and Stellwagen (1977). According to the postulated theory, the dye interacts specifically with a particular structural feature of most dehydrogenases and reductases, called the "dinucleotide-fold". This nucleotide binding domain, specific for NAD+(H) or NADP+(H) either as an enzyme substrate or cofactor, consists of an approximately 150 residue polypeptide chain segment arranged in four to six parallel ß pleated strands connected by several α helical strands about the ß sheets. A C-terminus in the ß sheet region and the amino acids surrounding it, form a specific binding site for nucleotides and determine the binding selectivity. This structure is found in a wide variety of nucleotide dependent enzymes, and is well preserved in evolution (Rossman et al., 1974, 1975). Hence the dye-ligand is presumed to bind specifically into the nucleotide dependent site on the protein's surface, effecting purification of the particular nucleotide enzyme by employing a common surface property inherent to these particular enzymes; hence the purification process is said to be group specific for this enzyme class.

Further ligands have recently been developed (as reviewed by Fulton, 1980), and designated: blue-B, green-A, orange-A and red-A.

In each of these matrex gel media, the dye-ligands are coupled to a 5% cross-linked agarose support gel via an ether linkage to the triazine ring of the dye; this coupled material being known as the dye-Sepharose matrex. The formula of the blue-A dye and its classified chemical domains is given in Fig. 6.

Recently some very useful purifications have been obtained with the dye-matrex media which are summarized in
FIGURE 6

The structural components of the blue-A dye ligand: I-sulphonated anthraquinone (chromophore), II-sulphonated benzene (bridge), III-triazine ring (active chlorine shown in agarose binding site), IV-sulphonated benzene (terminal). After Fulton, (1980).
Table III.
Use of the dye-ligand technique is anticipated in this project to be helpful in a purification of NADPH linked cytochrome c reductase by the postulated dye-enzyme interaction on chromatography, with subsequent resolution of pure enzyme from contaminating proteins on enzyme elution from the dye with a specific or non-specific eluent.

1.8 THE AIMS OF THIS STUDY.

The purposes of this study were as follows:
1. To develop a highly efficient cysJ protein producing E. coli K-12 strain;
2. To use this strain to develop a purification scheme to purify to homogeneity the cysJ protein;
3. To investigate the biophysical properties of the pure enzyme;
4. To sequence at least the first ten amino acid residues from the N-terminus of the protein;
5. To use the sequence data to compile a listing of the possible DNA sequences coding for the N-terminal region of the cysJ gene, and
6. To draw conclusions from the anticipated plasmid induced levels of enzyme on the properties of the cysB protein with reference to the cysJ gene's activation and regulation.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Dye-Matrex</th>
<th>Elution Conditions</th>
<th>Yield (%)</th>
<th><em>PF</em></th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase</td>
<td>Baker's yeast</td>
<td>Blue-A</td>
<td>5 mM NAD⁺</td>
<td>55</td>
<td>31</td>
<td>Easter-day and Easter-day, (1974).</td>
</tr>
<tr>
<td>EC 1.1.1.1</td>
<td></td>
<td>(agarose coupled)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>Baker's yeast</td>
<td>Blue-A</td>
<td>10 mM NADPH</td>
<td>60</td>
<td>4460</td>
<td>Easter-day and Easter-day, (1974).</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NADH:Nitrate reductase</td>
<td>Chlorella vulgaris</td>
<td>Blue dextran</td>
<td>0-100 M NADH</td>
<td>63</td>
<td>127</td>
<td>Solomon son, (1975).</td>
</tr>
<tr>
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<td>(agarose coupled)</td>
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<tr>
<td>NAD(P)H: Nitrate reductase</td>
<td>Neurospora crassa</td>
<td>Blue dextran</td>
<td>1 mM NADPH</td>
<td>69</td>
<td>27</td>
<td>Greenbaum et al., (1978)</td>
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<td>(agarose coupled)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The purification factor (PF) quoted is that achieved solely by the dye-ligand affinity chromatography step.
CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS.

Agarose Type I (low electroendosmosis), ampicillin (sodium salt), bisacrylamide, calcium phosphate gel (aged), chloramphenicol, cytochrome c (horse heart), dimethylsuberimidate dihydrochloride, L-djenkolic acid, ethidium bromide, FAD, FMN, glucose 6-phosphate dehydrogenase, L-glutamate dehydrogenase, lactate dehydrogenase, lysozime (grade I), NAD$^+$, NADP$^+$, NADPH, pyruvate kinase, riboflavin, ribonuclease A (type 1-A), tetracycline hydrochloride and tris-(hydroxymethyl)-amino-methane (reagent grade) were from Sigma. Acrylamide, bromophenol blue, p-chloromercuribenzoic acid, dichlorophenolindophenol, iodoacetic acid, sodium dodecyl sulphate, $N,N,N',N'$-tetramethylethlenediamine and xylene cyanol were from BDH Chemicals Ltd. Anionic exchange resin beads (AG 501-X8, 20-50 mesh), Bio-gel A-15m (200-400 mesh) agarose and Bio-gel HT (hydroxylapatite) were from Bio-Rad Laboratories. Aspartate transcarbamylase, EcoRI and HindIII were from Boehringer Mannheim. Blue-B gel along with pre-packed 2 ml capacity dye columns containing Blue-A, Blue-B, Green-A, Orange-A, Red-A and unsubstituted 5% cross-linked agarose were from Amicon. Ammonium sulphate (AR grade), EDTA, glycine, sodium dithionite, trichloroacetic acid and urea were from Ajax Chemicals Ltd. Absolute ethanol, hydroxylamine hydrochloride and isopropanol were from May and Baker, Australia Pty. Ltd. Bovine serum albumin was from Armour Pharmaceutical Co. Ltd. England. Coomassie Brilliant Blue was from Michrome, London. DEAE-cellulose (DE-23) was from Whatman. Diethyloxydiformate was from Eastman Kodak Co., Rochester, N.Y. β-Mercaptoethanol was from Fluka. Protamine sulphate was from Mann Research Laboratories. Starch (hydrolysed) was from Connaught Medical Research Laboratories. Tryptone and yeast extract were produced by Difco, Michigan, U.S.A.

All other chemicals used were of the highest grade obtainable and came from various sources.

2.2 MEDIA.

The formulae of nutrient and minimal media are listed in Appendix 1.
2.2.1 **NUTRIENT MEDIA.**

The following media were used where indicated: L broth and L agar.

2.2.2 **MINIMAL MEDIA.**

Davis and Mingioli, (1950) modified salts (Pasternak, 1962) were used for the preparation of sulphate and cysteine selective plates by the method of Lennox, (1955). Supplements for the plates were, glucose (0.2%), biotin (1.0 µg/ml), leucine (40 µg/ml), proline (40 µg/ml), thiamine.HCL (4.0 µg/ml) and either Na₂SO₄ (8.5 mM) or cystine (0.42 mM) for the selection of RL503 transformant clones; proline being replaced by threonine (40 µg/ml) on selection for RL502 transformant clones; other strains were selected on plates containing the supplements dictated by their phenotypic markers. When used, the antibiotics ampicillin and tetracycline were supplied at the concentrations of 100 µg/ml and 20 µg/ml respectively.

Cells used for enzyme assays and enzyme purification were grown in medium E of Vogel and Bonner, (1956) modified by the replacement of MgSO₄ by MgCl₂ and supplemented with glucose (0.2%), L-amino acids (40 µg/ml), thiamine.HCL (4.0 µg/ml) and L-djenkolic acid to 0.15 mM. Ampicillin and chloramphenicol were added to medium E media at the concentrations of 100 µg/ml and 200 µg/ml respectively, where specified.

2.3 **BACTERIAL STRAINS.**

All bacterial strains used were derivatives of *E. coli* K-12, and are listed in Table IV.

2.4 **GENETIC METHODS.**

2.4.1 **GROWTH METHODS.**

Strains of *E. coli* K-12 were stored either in liquid culture (10 ml L broth at approximately 2 x 10⁸ cells per ml) or were stored on L agar plates, for the period of the work. Strains not available in liquid or in plate culture at the commencement of the project were taken from glycerol stocks, (stored at -18°C) and 0.1 ml of which was inoculated into 2 ml of L broth and shaken at 37°C overnight or until good growth was evident. All stock cultures of *E. coli* were checked for their genetic and drug resistance markers by replica plating from a master plate patched from single colonies (Lederberg and Lederberg, 1952) before further work proceeded with the particular strain. Cells from a patch proving to possess
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>χ1776</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, tauA53, dapD8, merA1, supE42, Δ40(gal, attλ, uvrB), λminB2, malA25, thyA57, metC65, Δ29(bioH-asd), cycB2, cycA1, hsdR1R2, [pBR322 amp&lt;sup&gt;+&lt;/sup&gt;, tet&lt;sup&gt;+&lt;/sup&gt;]</td>
<td>K.D.Brown</td>
</tr>
<tr>
<td>KB9163</td>
<td>Hfr, KL16, nalA900, Δ(gal, attλ, bio, uvrB)thi</td>
<td>K.D.Brown</td>
</tr>
<tr>
<td>RL145</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, cysH, thr-1, leu-6, trp-1, his-1, argH1, thi-1, xyl-7, ara-13, metA2, gal-6, lacY1&lt;*/sup&gt;, λ&lt;sup&gt;+&lt;/sup&gt;, λ&lt;sup&gt;−&lt;/sup&gt;, tonA2, str&lt;sup&gt;+&lt;/sup&gt;, supE (SuiI&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>R.E.Loughlin</td>
</tr>
<tr>
<td>RL155</td>
<td>HfrH, ΔattBB', sup&lt;sup&gt;−&lt;/sup&gt;, Δ(gal, attλ, bio, uvrB), str&lt;sup&gt;+&lt;/sup&gt;, (λcysJJH), (λcI857Sam7), Δ(cysJJH)</td>
<td>R.E.Loughlin</td>
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<tr>
<td>RL421</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, thr-1, leu-6, thi-1, supE44, lacY1, tonA2, cysJ</td>
<td>R.E.Loughlin</td>
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<tr>
<td>RL434</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, Δ(cysJJH), pro, leu-6, thi-1, lacY, str&lt;sup&gt;+&lt;/sup&gt;, r&lt;sup&gt;+&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt;</td>
<td>S.R.E.Loughlin</td>
</tr>
<tr>
<td>RL500</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, thr-1, leu-6, thi-1, supE44, lacY1, tonA2, cysJ, [pRL1 amp&lt;sup&gt;+&lt;/sup&gt; cysJ&lt;sup&gt;+&lt;/sup&gt;]</td>
<td>M.T.Smith</td>
</tr>
<tr>
<td>RL501</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, thr-1, leu-6, thi-1, supE44, lacY1, tonA2, λ&lt;sup&gt;−&lt;/sup&gt;, cysJ, [pRL2 amp&lt;sup&gt;+&lt;/sup&gt; cysJ&lt;sup&gt;+&lt;/sup&gt;]</td>
<td>M.T.Smith</td>
</tr>
<tr>
<td>RL502</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, thr-1, leu-6, thi-1, supE44, lacY1, tonA2, cysJ, [pRL3 amp&lt;sup&gt;+&lt;/sup&gt; cysJ&lt;sup&gt;+&lt;/sup&gt;]</td>
<td>M.T.Smith</td>
</tr>
<tr>
<td>RL503</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, Δ(cysJJH), pro, leu-6 thi-1, lacY, str&lt;sup&gt;+&lt;/sup&gt;, r&lt;sup&gt;+&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt;, [pRL3 amp&lt;sup&gt;+&lt;/sup&gt; cysJ&lt;sup&gt;+&lt;/sup&gt;]</td>
<td>Constructed during this project</td>
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**TABLE IV:** (Cont'd)

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<tbody>
<tr>
<td>RRL</td>
<td>hsdR⁻, hsdM⁻, supE44, λ⁻, leuB6, proA2, thi-1, trp55, ara-14, lacY1, galK2, rpsL20, xy15</td>
<td>G. Zurawski</td>
</tr>
</tbody>
</table>

**NOTE:**

* Strain RL421 is a derivative of *E. coli* C600.

$ Strain RL434 is derived from *E. coli* strain RRL, which is a supertransforming strain.

Bacterial genotypic nomenclature is that of Bachmann and Low, (1980) and bacteriophage nomenclature is that of Szybalski and Herskowitz, (1971). Other abbreviations are: λ⁻, absence of prophage; λS, lambda sensitive; Δ, deletion of area in parenthesis; dcysJ1H, defective cysJ1H transducing.
the required markers were then inoculated into 10 ml of L broth and shaken until the correct turbidity was reached, then this liquid culture was used as a working stock culture.

For growth of bacteria, colonies were inoculated into 10 ml of L broth with shaking at 37°C overnight (the first culture period). Minimal medium (50 ml), with the appropriate supplements, was then inoculated with 2.5 ml of the overnight culture and shaken at 37°C for eight hours (the second culture period), then 12.5 ml of this 8 hour culture was inoculated into 250 ml of minimal medium, appropriately supplemented, and shaken overnight at 37°C. Cells were then harvested in the late logarithmic phase of growth.

For all culture growth, the volume of media to capacity of vessel was kept at 0.25 or below, to ensure good aeration.

2.4.2 **PLATE TRANSDUCTION ASSAY.**

Colonies were picked from a master selection plate after their genetic and drug resistance markers had been confirmed. A colony was then inoculated into 10 ml of L broth containing maltose, (0.2%), as sole carbon source in order to induce the lambda receptor on the cell's surface, and ampicillin for drug resistant strains. Growth was overnight at 37°C until good cell density was attained. An HFT (high frequency of transduction) lysate of RL155 containing the phages \( \lambda Ci857 \text{Sam7} \) and \( \lambda dcyS110D \) was streaked across a minimal plate containing maltose (0.2%), the appropriate amino acid supplements and ampicillin for plasmid bearing strains, with sulphate as the sole source of sulphur. The bacteria were then streaked across the lysate and the plate was incubated overnight at 37°C. Resultant colonies were picked and checked for their genetic and drug resistance markers.

2.4.3 **SMALL SCALE PLASMID PREPARATION.**

The plasmid preparation followed the procedure of Davis *et al.*, (1980) or, alternatively, the procedure described by Holmes and Quigley, (1981) was utilized. Plasmid DNA precipitated at the end of the preparation was redissolved in 50 µl of Tris-HCL buffer, pH 8.0, containing RNaseA (as specified by Smith, 1981).

2.4.4 **RESTRICTION ENDONUCLEASE DIGESTION AND AGAROSE GEL ELECTROPHORESIS.**

Restriction endonuclease digestion with *EcoRI* and *HindIII* and agarose gel electrophoresis followed the procedure outlined

The molecular weight of plasmid DNA was estimated from DNA standards run on the gel concurrently with the plasmid unknown. A computer plotted calibration graph is presented in Fig. 7.

Photography was as performed by Smith, (1981).

2.4.5 TRANSFORMATION.

Transformation was done by the method of Cohen et al., (1972) as modified by Smith, (1981). DNA contamination controls, competent cell controls and cell competence test controls were run as specified (Smith, 1981).

Transformants were characterized by having their genetic markers checked and drug resistance phenotype confirmed. Where indicated transformants were isolated, their plasmid DNA prepared, a small sample of which was re-digested, the remaining DNA being retransformed into the parent E. coli K-12 strain; drug resistance and enzyme levels were monitored as an indication of successful transformation and effective plasmid gene expression.

2.4.6 NADPH-SULPHITE REDUCTASE RECONSTITUTION ASSAY.

Crude extracts of strains lacking either the cysJ or cysI polypeptide were mixed in order to reconstitute full NADPH dependent sulphite reductase activity. The reconstitution was performed in vitro according to the procedure of Siegel et al., (1971).

2.5 BIOCHEMICAL AND PHYSICAL METHODS.

2.5.1 PREPARATION OF THE CELL FREE EXTRACT.

Cells were disrupted using a Branson Model B-12 sonifier fitted with a microtip. Cell debris was removed by centrifugation at 28,000 x g for 40 min. and the supernatants (crude extracts) were used for enzyme and protein assays. In large scale work, for protein purification, disruption of bacteria and preparation of the cell free extract was done in a Hughe's press at 6,000 p.s.i. to disrupt the frozen cell pellet; debris was then removed by centrifugation at 28,000 x g for 40 min.

2.5.2 NADPH-CYTOCHROME C REDUCTASE PURIFICATION.

All work leading to the protein purification and the methods used are described in Chapter 3.
FIGURE 7

The relationship between the size and the mobility of DNA fragments obtained by the curve fitting of standards of known size determined by the digestion of λ DNA with the restriction endonuclease HindIII.
2.5.3 NADPH-CYTOCHROME C REDUCTASE ASSAY.

The reduction of cytochrome c was followed spectrophotometrically at 20°C by measuring the increase in absorbance of cytochrome c at 550.5 nm with a 0.2 nm slit width in a Varian DMS-80 spectrophotometer with an attached National VP-653B chart recorder. Cuvettes contained (in a final volume of 1.0 ml): 0.05 M potassium phosphate buffer, pH 7.7, 0.2 mM EDTA, 0.2 mM NADPH, 0.023 mM cytochrome c and 0.1 ml of cell free extract appropriately diluted in 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA. Activity was corrected for endogenous NADPH-cytochrome c reductase levels by omitting NADPH in a control assay.

Millimolar extinction coefficients for the fully reduced and oxidized forms of cytochrome c at 550.5 nm were taken as 27.7 mM$^{-1}$ cm$^{-1}$ and 9.0 mM$^{-1}$ cm$^{-1}$ respectively (Siegel et al., 1964).

Specific activity of extracts is expressed as nmoles of NADPH oxidized per min. per mg of protein present.

2.5.4 ASSAY OF DIAPHORASE ACTIVITY.

The reduction of DCPIP was followed spectrophotometrically at 20°C by measuring the decrease in absorbance of DCPIP at 600 nm with a 1.0 nm slit width in a Varian DMS-80 spectrophotometer with an attached National VP-653B chart recorder.

Cuvettes contained (in a final volume of 1.0 ml): 0.05 M potassium phosphate buffer, pH 7.7, 0.2 mM EDTA, 0.005 mM FAD, 0.04 mM DCPIP, 0.2 mM NADPH and 0.1 ml of cell free extract appropriately diluted in 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA. A millimolar extinction coefficient for the oxidized form of DCPIP at 600 nm was taken as 16.1 mM$^{-1}$ cm$^{-1}$ and on reduction the dye is completely bleached (Siegel et al., 1971).

Endogenous activity was corrected for by running a control with NADPH omitted from the assay system. Specific activity is expressed as nmoles of DCPIP reduced per min. per mg of protein present.

2.5.5 ASSAY OF NADPH-SULPHITE REDUCTASE ACTIVITY.

The reduction of sulphite was followed spectrophotometrically at 20°C by measuring the decrease in absorbance of NADPH at 340 nm with a 1.0 nm slit width in a Varian DMS-80 spectrophotometer with an attached National VP-653B chart recorder.
Cuvettes contained (in a final volume of 1.0 ml): 0.05 M potassium phosphate buffer, pH 7.7, 0.2 mM EDTA, 0.6 mM Na$_2$SO$_3$, 0.005 mM FAD, 0.2 mM NADPH and 0.1 ml of appropriately diluted cell free extract in 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA. A millimolar extinction coefficient for the reduced form of NADPH was taken as 6.22 mM$^{-1}$ cm$^{-1}$ at 340 nm, bleaching occurring at this wave-length on oxidation to NADP$^+$ (Siegel et al., 1971). Endogenous activity was corrected for by omitting Na$_2$SO$_3$ in a control run on each sample.

Specific activity is expressed as nmols of NADPH oxidized per min. per mg of protein present.

2.5.6 ASSAY OF NADPH LINKED HYDROXYLAMINE REDUCTASE ACTIVITY.

The reduction of hydroxylamine was followed spectrophotometrically at 20°C by measuring the decrease in absorbance of NADPH at 340 nm with a 1.0 nm slit width in a Varian DMS-80 spectrophotometer with an attached National VP-653B chart recorder.

Cuvettes contained (in a final volume of 1.0 ml): 0.1 M potassium phosphate buffer, pH 7.7, 0.3 mM EDTA, 10 mM hydroxylamine.HCl, 0.001 mM FAD, 0.2 mM NADPH, 0.1 ml of appropriately diluted cell free extract in 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA.

Endogenous activity was corrected for by omitting hydroxylamine.HCl in a control assay. Use of extinction coefficients is as described in 2.5.5. Specific activity is expressed as in 2.5.5.

2.5.7 ESTIMATION OF PROTEIN CONCENTRATION.

Protein concentration was determined by the method of Lowry et al., (1951) with bovine serum albumin as a protein standard in the range 0 to 250 µg/ml. Interfering substances in some samples arising due to a high phosphate molarity were removed by centrifugation at 28,000 x g for 20 min.

When quick, relative protein determinations were required, the absorbance at 280 nm on a Shimadzu UV-visible 120-02 spectrophotometer against a blank containing 0.05 M potassium phosphate buffer, pH 7.7, 1 mM EDTA and 10$^{-5}$ M FAD, was recorded.

2.5.8 DIALYSIS.

All dialysis was carried out in boiled, Na$_2$CO$_3$ (0.25 w/v%),
pretreated 1 inch diameter dialysis tubing against buffer or
glass distilled water as specified, in a volume ratio of sample
to buffer not exceeding 0.05 for the specified time interval.
2.5.9 CHROMATOGRAPHY.

Bio-gel A-15m, DEAE-cellulose and hydroxylapatite were
used untreated, all fines being decanted before column packing.
Dye matrex Blue-B affinity gel was treated with 0.5 M NaOH and
8 M urea before use to wash away any uncoupled ligand which
forms on storage. All urea solutions used in dye column re-
generation were made up from recrystallized urea after removal
of the gross excess of cyanate ions by anion exchange chroma-
tography on an anionic exchange resin.

The conditions used in each column run are specified when
described in the Results, (Chapter 3).

Fractions were collected by a Gradipore fraction collector
and flow was assisted, when specified, by a Gilson Minipuls-
2 pump set at the column flow rate as described.
2.5.10 IN VITRO SUBUNIT CROSS-LINKING OF NADPH-CYTOCHROME C REDUCTASE.

NADPH-cytochrome c reductase was treated with the bifun-
tional reagent dimethylsulphimidate in a procedure similar to
that employed for nitrate reductase (Giri and Ramadoss, 1979).
The reaction was carried out in 0.1 M potassium phosphate buffer,
ph 7.7, containing 1 mM EDTA, at 4°C. The initiation of the
reaction was by the addition of solid dimethylsulphimidate
at a final concentration of cross-linker of 4 mg/ml to a
purified enzyme preparation with a protein concentration of
0.160 mg/ml in 1.0 ml total volume.

Enzyme activity was followed in a time course assay by
withdrawing aliquots from the reaction mixture and measuring
activity by the standard assay procedure (2.5.3).
2.5.11 ABSORPTION SPECTRA.

Absorption spectra of the purified fully oxidized enzyme
were recorded in a Varian Superscan 3 recording spectrophotomo-
meter at a slit width of 1.0 nm, and in a 10 mm light path.
Spectra were recorded against a blank containing 0.05 M potas-
sium phosphate buffer, pH 7.7, 1 mM EDTA and 10⁻⁵M FAD, with
a total volume of 1.0 ml per cuvette.
2.5.12 PROTEIN ELECTROPHORESIS, GEL STAINING, SCANNING DENSITOMETRY AND
PHOTOGRAPHY.

Analytical tris-glycine acrylamide slab*SDS-discontinuous
gel electrophoresis was performed essentially as described by Weber and Osborn, (1969) as modified by Clark and Switzer, (1977). Tris-borate analytical acrylamide slab SDS-discontinuous gel electrophoresis was as described by Neville, (1971). Analytical urea polyacrylamide discontinuous gel electrophoresis was performed as described by Jovin et al., (1964) with all urea having been treated as described in 2.5.9. Electrophoresis in a 5% acrylamide SDS-slab gel not employing a discontinuous buffer system was as performed by Giri and Ramadoss, (1979), with 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, throughout the gel and in the buffer compartments; these slab gels were routinely pre-electrophoresed at 100 V and 20 mA per gel for 1 hour before sample loading. Gradient polyacrylamide SDS slab gel electrophoresis was as performed by Mascarenhas, (1978) with the modification that the gel concentration range was from 4% to 11% and electrophoresis was carried out to equilibrium at 30 V and 2.5 mA per gel for 16 hours. Non-denaturing analytical polyacrylamide discontinuous slab gel electrophoresis was as described by Williams and Reisfeld, (1964) and was run at 4°C with FAD (10⁻⁵ M) throughout the gel and in the buffer compartments. Non-denaturing agarose (0.7%) gels were run as for the DNA agarose gels (Smith, 1981), but with 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA and 10⁻⁵ M FAD, throughout. The protein agarose gels were performed at 4°C at 15 V and 40 mA for 36 hours. Horizontal non-denaturing starch gel electrophoresis was as according to Smithies, (1955) and was run at 4°C with the buffer conditions as for the non-denaturing agarose gels. The starch gels were electrophoresed at 25 V and 60 mA. Sample treatment for all non-denaturing gels was as performed for the discontinuous gel system described by Williams and Reisfeld, (1964) and will be described in the Results, (Chapter 3).

All polyacrylamide slab gels were poured and run in a Bio-Rad dual slab gel electrophoresis apparatus of gel dimensions 14 x 16 x 0.3 cm. Tube gels of dimensions 6 x 0.5 cm were poured essentially as described by Weber and Osborn, (1969) as modified by Clark and Switzer, (1977) and run under denaturing conditions in a tube gel apparatus. The specific conditions of electrophoresis and treatment of samples are described with the individual gel electropherograms in Chapter Three. Stacking
gels, when used for the discontinuous polyacrylamide gels, were effectively photopolymerized either for 10 min. at 5 cm from a Mineralight UVS-12 source, or for 1 hour under a bright fluorescent light.

Cellulose acetate strip electrophoresis was performed in 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, with approximately 50 to 150 μg samples of protein run at 25 V and 1.8 mA for 15 hours. Samples were treated as for the non-denaturing gel systems, but electrophoresis was run at 20°C.

Staining of gels was as according to Weber and Osborn, (1969) for coomassie blue with the modifications that all staining was done for 1 hour at 42°C and then at 20°C for 12 hours before destaining, and mobility was not corrected for gel swelling; alternatively, tube gels were stained and destained by the trichloroacetic acid method (Solomonson et al., 1975). Slab gels were also stained with silver nitrate according to the procedure of Wray et al., (1981). Scanning densitometry was carried out on a Transidyne 2955 scanning densitometer with an attached National VP-653B chart recorder, at an incident beam wave-length of 590 to 600 nm and with horizontal and vertical apertures of 5 mm and 0.4 to 0.5 mm respectively. Relative quantification of band intensity was obtained by the cut-and-weigh method from the recorder scan.

Gel photographs were obtained by photography of the gels using transmitted light with a Canon camera adjusted to the appropriate aperture setting and with an exposure time of 0.02 s. Kodak plus-X pan (type PX135-36) film was used for negatives which were developed according to the manufacturer's instructions, and prints were made as for the DNA agarose gels (Smith, 1981) on Ilford photographic paper.

Protein subunit molecular weight was calculated from fitting molecular weight standards to a log₁₀ molecular weight versus mobility curve on regression analysis as calculated by computer programme; unknowns were then determined from the plot.

2.5.13 AMINO ACID ANALYSIS.

Amino acid analysis was conducted on a freeze-dried hydrolysed enzyme sample diluted one in ten on a Joel model JLC-6AH amino acid analyzer. Hydrolysis was at 110°C for 24 hours in 6 N HCl; the analysis being done in duplicate with standard amino acids being supplied as external markers. Peak height
data were integrated on an Interdata computer system and corrected to standard amino acid peak areas with the concentrations of the hydrolysed amino acids printed out as nmol/ml of diluted hydrolysate.

2.5.14 PROTEIN SEQUENATION.

All techniques referring to the sequenation procedure are presented in Appendix 2.
CHAPTER 3

RESULTS

3.1 CONSTRUCTION OF AN NADPH-CYTOCHROME C REDUCTASE OVER-
PRODUCING STRAIN OF E. COLI K-12.

In order to construct an NADPH-cytochrome c reductase
overproducing strain of E. coli K-12, use was made of the gene
dosage effect (see Introduction, p.10). Firstly, the plasmid
pRL3 was prepared from the strain RL502 and subjected to restric-
tion endonuclease analysis; Fig. 8 shows the digestion of pRL3
with the enzymes EcoRI and HindIII. pRL3 was then transformed
into strain RL421, which is phenotypically Cys^- and Amp^S, in
order to make this strain both Cys^+ and Amp^R. This experiment
confirmed the earlier data of Smith, (1981) with regard to the
genotypic markers carried by the plasmid pRL3. However, as
strain RL421 is a revertible strain, with Cys^+ revertants being
produced (albeit at a low frequency), then isolated clones
which are both Cys^+ and Amp^R could arise through a reversion
event occurring on the host cell's chromosome and through
transformation by a plasmid which carries no cys DNA but which
does still carry the ampicillin resistance determinant. Furth-
ermore by use of the strain RL421 as a recipient in transforma-
tion it is possible that Cys^+, Amp^R clones could also arise
through recombination between plasmid and host cell chromosome
in the vicinity of the mutated cysJ gene borne by the host cell.
Although both reversion and recombination are unlikely with
the use of strain RL421 as a transformation recipient it was
considered that these possibilities in addition to previously
described problems with the use of a strain carrying a wild-
type allele of the cysI gene (see Introduction, p.15) that a
strain with a deleted cysJ and cysI genes would be more suitable
as a recipient for transformation with the plasmid pRL3. Thus
pRL3 was re-isolated from cultures of the above transformants
of strain RL421 and used to transform the cysJ^I^I^I^I deletion strain,
RL434. Strain RL434 was thus rendered Amp^R but remained Cys^- due to the chromosomal absence of the cysI and cysII genes in
this deletion strain. Transformants of strain RL434 were then
subjected to plasmid purification and restriction endonuclease
analysis; pRL3 being identified and its size noted as being
9.54 Kbp (Fig. 9). The transformant clone of strain RL434
FIGURE 8

Agarose gel electrophoresis of DNA fragments obtained by restriction endonuclease digestion of the plasmid pRL3 isolated from a culture of strain RL502. Lambda DNA was used as a fragment size standard.

The direction of DNA fragment migration is in a downward direction with the sample wells indicated by arrows.

Electrophoresis was performed at 5 V/cm for 10 min. directly after the samples had been placed into their respective wells. The voltage gradient was then reduced to 1.8 V/cm until the bromophenol blue (tracking dye) was approximately 1-2 cm from the end of the gel. Gels were stained with ethidium bromide as according to Smith, (1981).

**LEGEND**

**Gel A**

<table>
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<tr>
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<th>Conditions</th>
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<td>λ</td>
<td>EcoRI digestion, complete;</td>
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<td></td>
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<td>2</td>
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<tr>
<td>3</td>
<td>λ</td>
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<tr>
<td>4</td>
<td>pRL3</td>
<td>As in 2</td>
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<tr>
<td>5</td>
<td>λ</td>
<td>As in 1</td>
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**Gel B**

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<tr>
<td>2</td>
<td>λ</td>
<td>HindIII digestion, complete;</td>
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<tr>
<td></td>
<td></td>
<td>*ends cohered.</td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>λ</td>
<td>As in 2</td>
</tr>
<tr>
<td>5</td>
<td>pRL3</td>
<td>As in 1</td>
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*The two DNA fragments forming the left and right ends of the phage lambda genome remained non-covalently joined to each other under the conditions used for the electrophoresis.

$All indicated lanes containing digested pRL3 were separate aliquots taken from the same plasmid preparation of strain RL502.
FIGURE 9

Agarose gel electrophoresis of DNA fragments obtained by restriction endonuclease digestion of the plasmid pRL3 isolated from a transformant culture of strain RL434. Lambda DNA was used as a fragment size standard.

The direction of DNA fragment migration is in a downward direction with the sample wells indicated by arrows.

The conditions of electrophoresis are as indicated in Fig. 8.

The size of pRL3 was calculated to be 9.54 Kbp from the HindIII restriction data presented in this Figure, and from the DNA fragment-mobility relationship given in Fig. 7.

**LEGEND**

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<td>pRL3</td>
<td>HindIII digestion.</td>
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<tr>
<td>3</td>
<td>λ</td>
<td>As in 1</td>
</tr>
<tr>
<td>*4</td>
<td>pRL3</td>
<td>As in 2</td>
</tr>
<tr>
<td>5</td>
<td>λ</td>
<td>As in 1</td>
</tr>
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</table>

*All indicated lanes containing digested pRL3 were separate aliquots taken from two individual plasmid preparations of the same transformant culture of strain RL434. $See Fig. 8, footnote (*) for definition.
which was analyzed and found to carry the plasmid pRL3, was designated as strain RL503.

For enzyme assays, cultures of strain RL503 were grown under conditions of repression and derepression for the genes involved in cysteine biosynthesis. Cultures were grown in duplicate with one set of cultures grown in the presence of chloramphenicol and the other set grown in the absence of the drug. Chloramphenicol was added to the second growth period of culture growth (see Materials and Methods, p.20) and was subsequently thoroughly washed from the cells by centrifugation (4,000 x g, for 10 min.) and resuspension of the resulting cell pellet in Davis and Mingioli 1x minimal salts; cells were then grown overnight in the absence of chloramphenicol. A wild-type strain, KB9163, was selected and grown under conditions of derepression for the genes involved in cysteine biosynthesis in the absence of chloramphenicol. The enzyme assay data is presented in Table V. From these results strain RL503 is clearly seen as an overproducing strain for the enzyme NADPH-cytochrome c reductase. Growth under conditions of derepression without added chloramphenicol was used for all subsequent cultures utilized for protein purification work. As it was observed that the addition of chloramphenicol to growing cultures of bacteria led to a large decrease in the total protein present in crude extracts prepared from these cells it was reasoned that there was a corresponding decrease in the amount of cysJ encoded enzyme present in these extracts even though the specific activity of the enzyme of interest was unaltered in extracts of cells prepared from cultures grown in the presence of chloramphenicol. Thus as small quantities of purified enzyme were anticipated to be attained it was important to commence purification from cell extracts containing quantities of protein suitable for preparative scale enzyme purification, for this reason chloramphenicol was not employed in any future protein purification work. In addition, each of the cultures of strain RL503 grown in the conditions specified above was subjected to plasmid purification with all the cultures found to contain pRL3 (as determined from agarose gel electrophoresis ran with the appropriate fragment size standards). Furthermore, the strains bearing pRL3 were grown throughout all stages of culturing in the presence of ampicillin in order to prevent
### Spectroscopic Assay of NADPH-Cytochrome c Reductase

DNA amplification

phenocopy pulse treatment during early exponential culture growth phase for plasmid
derepression; Cys' growth in L-lysine for cys gene expression; CAP'-chlamy-
Abbreviations are as follows: D' growth in L-lysinoic acid for cys gene DNA

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Cys', Cap</th>
<th>Cys', Cap</th>
<th>Cys', Cap</th>
<th>Cys', Cap</th>
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<td>5.98</td>
<td>0.38</td>
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<td>3.8,0</td>
<td>1.2</td>
<td>0.90</td>
<td>0.29</td>
<td>3.50</td>
</tr>
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<td>2.0,96</td>
<td>3.9</td>
<td>0.09</td>
<td>0.29</td>
<td>3.50</td>
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<td>14.8</td>
<td>9.36</td>
<td>0.29</td>
<td>3.50</td>
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**Plasmid Bearing**

**WILD-TYPE**

---

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<tbody>
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<td></td>
<td>V. coli strain</td>
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</tr>
<tr>
<td></td>
<td>5.9 X 10^6</td>
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</tbody>
</table>

All cell extracts were prepared by sonication.

B. coli strain

Levels in extracts of a plasmid bearing strain versus a wild-type

**TABLE VI:** The effect of growth conditions on NADPH-cytochrome c reductase
plasmid-free segregants from forming and subsequently dominating the culture. In further experiments it was found to be unnecessary to include ampicillin in either the second or final phases of culture growth, with the drug only being added to the initial overnight culturing period, as NADPH-cytochrome c reductase enzyme levels, reflecting the presence of pRL3, did not vary significantly in the absence of ampicillin as described above. In addition, these initial results show that the plasmid pRL3 undoubtedly bears a fully functional cysJ gene and its promoter, as strain RL503 derived from strain RL434 has a chromosomal deletion extending through the cysJT and H genes and hence cannot revert back to cysteine prototrophy. These assays thus provide conclusive proof of the earlier hypothesis discussed by Smith, (1981) that pRL3 bears a complete copy of the cysJ gene and its promoter.

Genetical implications arising from the above data are presented in the Discussion (Chapter 4).

3.2 PURIFICATION OF NADPH-CYTOCHROME C REDUCTASE.

3.2.1 PURIFICATION TRIALS: CRUDE EXTRACT PREPARATION AND AMMONIUM SULPHATE FRACTIONATION.

Fifty litres of strain RL503 bacterial culture was grown in 250 ml lots at 37°C under conditions of derepression for the genes involved in cysteine biosynthesis. Cultures were harvested by centrifugation at 8,000 x g for 10 min. in a Sorvall RC2B centrifuge, and the cell pellets frozen overnight at -18°C. The cells were then disrupted in a Hughe's press and the weight of the pressate determined. From this point onwards, all operations were performed at 4°C and all specified buffers and reagents added to the cell extract contained 10^-5 M FAD; both procedures being necessary to stabilize NADPH-cytochrome c reductase activity.

To the Hughe's pressate material, 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, was added in the ratio of 2.7 ml of buffer to 1.0 g of pressate, the mixture being thoroughly stirred to a completely homogeneous state. The viscous liquid was then cleared of all cell debris as described in section 2.5.1 (p. 21). The resulting supernatant (crude-extract, designated "A"), was saved and the pellet discarded. To the supernatant a saturated solution of ammonium sulphate, (at 4°C) with the pH adjusted to 7.7 with NH₄OH, was added to
30% saturation over a period of 5 min. with vigorous stirring. The solution was then stirred for another 30 min. then centrifuged at 24,000 x g for 20 min. The pellet obtained was re-suspended in 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA to one fifth the crude extract volume. This fraction is designated as "B1". To the supernatant, saturated ammonium sulphate solution was added to 46% saturation and the solution mixed and centrifuged as above. The pellet was re-suspended also as above but to 0.37 the volume of the crude extract. This fraction was designated as "B2". The remaining supernatant was treated with ammonium sulphate to bring the final saturation to 65% and the solution was mixed and centrifuged as above, the pellet being resuspended also as described above but to 0.29 the volume of the crude extract. This fraction was designated as "B3". The supernatant from the 65% ammonium sulphate treatment was designated as "B4". After a number of trials with small volumes of crude extract, the results presented in Table VI were found to represent the overall trend of enzyme fractionation. Thus the enzyme mainly appears between 30% and 46% saturation, with a doubling of the level of specific activity with respect to the crude extract.

It should be noted that the crude extract levels of enzyme seen in Table VI for strain RL503 are not as high as those presented in Table V. The reason for this large variability is unclear, but it was noted that on various trial procedures that the enzyme levels in the prepared crude extracts were dropping from their initial 30 to 40 fold higher levels than wild-type derepressed cultures to only 10 fold higher levels. However, during these trial preparations subculturing techniques were used and although the cells were still ampicillin resistant and contained plasmid, a mutation, perhaps in the promoter region for the cysJ gene, may have arisen. As another alternative though, it is possible that the very high levels of enzyme present in the pRL3 bearing strain are determined by a very stringent and well balanced control process as dictated by the host’s cysteine regulatory system, thus any minor change in culture conditions may upset dramatically the production of enzyme. Furthermore, it is not unlikely that an abnormal aggregation of enzyme is occurring in the cell’s cytosol leading to deactivation of its activity; yet again this process must be finely
In the extract, one unit of enzyme catalyzing the oxidation of 1 nmol of NADH per min.

The specific activity of the enzyme is expressed as units per mg of protein present.

From the crude extract (see text, pp. 30 and 31).

*Proportion of B to B4 represents various ammonium sulfate-treated samples derived.

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**Table VI:** Ammonium sulfate fractionation of strain R1503 crude extract.

**Proportion of B to B4** represents various ammonium sulfate-treated samples derived.

**From the crude extract (see text, pp. 30 and 31).**

**In the extract, one unit of enzyme catalyzing the oxidation of 1 nmol of NADH per min.**

The specific activity of the enzyme is expressed as units per mg of protein present.

**From the crude extract (see text, pp. 30 and 31).**

**Table VI:** Ammonium sulfate fractionation of strain R1503 crude extract.
balanced within the cell or the levels of enzyme would not fluctuate so dramatically between 10 to 40 fold higher than wild-type. For all future enzyme preparations, cultures were grown on each single occasion from an individual transformant clone of strain RL503, in order to at least avoid any possible variables introduced by subculturing.

In addition to ammonium sulphate fractionation, a fractionation procedure using protamine sulphate as according to Siegel et al., (1973) was attempted. Very low enzyme yields were recovered, however, and the procedure was not pursued any further.

3.2.2 **CALCIUM PHOSPHATE GEL FRACTIONATION.**

The fraction B2 was further fractionated by adsorption onto calcium phosphate gel. The ammonium sulphate fraction was diluted with 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, to a protein concentration of 10 mg/ml. Calcium phosphate gel (after precisely determining the solids content) was then added to a ratio of 0.40 mg of gel per mg of protein present, over a period of 5 min. The resulting suspension was stirred for 30 min. and then centrifuged at 6,000 x g for 10 min. The pellet obtained was eluted by resuspending in 0.4 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, and by leaving the resuspended material to stand for one hour, with the volume of eluting buffer being half the volume of calcium phosphate gel suspension which was added at this step. The resuspended material was then centrifuged as described above, with the supernatant fraction obtained being designated as "C1". To the pellet a second addition of eluting buffer was then added with elution conditions being similar to those already described. The supernatant obtained on subsequent centrifugation was designated as "C2". The final pellet remaining was then discarded.

To the supernatant fraction of the first gel addition (termed "C3") a second amount of calcium phosphate gel suspension was added to bring the total ratio of gel added to 0.6 mg of gel per mg of protein present in "B2". The resulting suspension was stirred and centrifuged as above. The pellet obtained was similarly eluted as above with the successively eluted fractions denoted as "C4" and "C5". To the supernatant fraction of the second gel addition (called "C6") a third lot of calcium phosphate gel suspension was added to bring the total ratio of gel
added to 1.5 mg of gel per mg of protein present in "B2". The suspension was treated as above but the pellet was eluted three times, with the successively eluted fractions being denoted as "C7", "C8" and "C9".

To the supernatant of this third gel addition (called "C10") a fourth calcium phosphate gel addition was made to bring the total ratio of gel added to 1.8 mg of gel per mg of protein present in "B2". This resulting suspension was treated as above and the pellet eluted twice, with successively eluted fractions denoted as "C11" and "C12". The supernatant from the fourth gel addition was kept, and denoted as "C13".

After repeating the procedure a number of times to obtain reproducible results, the pattern of fractionation presented in Table VII emerged.

Hence the enzyme appeared mainly in the first calcium phosphate gel eluates.

3.2.3 DYE-LIGAND AFFINITY CHROMATOGRAPHY: BINDING ASSAYS.

Use was made of 2 ml capacity, dye packed columns, to test for further purification that could be obtained through the use of dye-ligands. Six columns were available, one of which (a control column) contained unsubstituted 5% cross-linked agarose. The five other columns contained, Blue-A, Blue-B, Green-A, Orange-A and Red-A with 5% cross-linked agarose as the support matrix (the respective dye-ligand being directly coupled to the support; see Fig. 6, p.16(a)).

The testing procedure was as follows: firstly, all the columns were regenerated to remove any free ligand with 12 ml of 8 M urea containing 0.5 M NaOH; then a period of equilibration with 12 ml of 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, was carried out for 2 hours, after which 0.5 ml of sample was applied to the column and allowed to drain into the gel bed. After sample application, a further 0.1 ml of 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, was placed onto the column and allowed to drain into the gel bed. Liquid emerging from the column at this stage was collected and designated as fraction "D1". Flow was then stopped and the column was allowed to stand for one hour in order to potentiate an effective protein-dye interaction. After this period the column was washed through with 10 ml of 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, and allowed to drain.
The wash fraction was collected and ultra-filtered to 2.0 ml final volume through an Amicon PM-30 membrane at 40 p.s.i. in an Amicon ultrafiltration cell, model 50, (the resulting fraction being called "D2"). After the column wash step 10 ml of 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA and 1.5 M KCl, was passed through the column in order to elute non-specifically any bound proteins. After application of the eluting buffer the column was allowed to drain. The eluted fraction was concentrated in a manner similar to that described for the wash fraction with the resulting solution being designated as "D3". It was found that in order to achieve an adequate separation of the enzyme from interfering substances at the affinity chromatography step all fractions from the calcium phosphate gel treatment which were intended to be chromatographed were dialyzed against 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, for 12 hours before application to the dye column.

The results that emerged after many trial runs are presented in Table VIII. In this instance the initial culture was grown directly from an isolated transformant clone (subculturing was not used), and the specific activity of the crude extract approached that obtained in the initial experiments (Table V).

The results surprisingly indicate that the enzyme did not bind to the dye columns (only Green-A and Red-A appeared to bind significant amounts of enzyme, but with little increase in purification in either case). Also, proteinaceous material, with no enzyme activity, was observed to pass very rapidly through all the columns, except in the case of Blue-B where the greatest purification of the enzyme was achieved in the wash fraction from this particular column. Notably Blue-B increases the total number of enzyme units from the input fraction by almost 2.5 times, presumably by removing an inhibitor or even highly aggregated forms of the enzyme which might decrease the overall activity in a particular preparation. Furthermore, Orange-A appears to cause a fall in total units, possibly by some denaturation process accelerated by the particular dye- ligand or even perhaps due to a very strong and specific enzyme binding that was not broken even under the strong non-specific eluting conditions used (the fraction of activity appearing in the wash could be due to an overload of the column with enzyme, leading to leakage effects). Of all the columns tested, except
The specific activity of the enzyme is expressed as units per mg of protein present in the extract.

3.2.3. pp. 33 and 34, for further details.

Extractions of fractions 1, 11, and 13, represent certain fractions collected from the columns, see section.

Applied to each of the dye-columns.

Table VIII: Results from a trial purification of a calcium phosphate gel.

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Red-A
Orange-A
Green-A
Blue-A
Control

* Extraction with dye-ligand medium.
for the control column and Orange-A, the Blue-B column was found to bind the least number of enzyme molecules and this combined with the astounding purification obtained in the wash fraction from this column, led to the hypothesis that Blue-B acted to purify the enzyme by both a negative affinity chromatography effect and gel filtration, with the high molecular weight enzyme appearing in the column's void volume. Further studies were then conducted in order to investigate the above results.

3.2.4 THE MECHANISMS INVOLVED IN DYE-LIGAND PURIFICATION.

Fig. 10 shows the elution profile obtained from a larger scale Blue-B column. The results demonstrate that the enzyme appears in the column's void volume and that a relatively small percentage of enzyme actually is retained by the column and is eluted under non-specific conditions. However, in order to determine whether the enzyme was bound to the column specifically by the dye through the enzyme's NADPH binding site or retained to the column non-specifically by some other means, the enzyme was pre-treated with NADP⁺, NADPH or p-chloromercuribenzoic acid, (p-CMB), prior to chromatography as presented in Fig. 11. Use was made of the Red-A column in this experiment as it has been noted (Table VIII) that a relatively large amount of enzyme does bind to this dye-ligand and hence work with this dye would be readily interpretable in terms of binding specificity. The data in Fig. 11 shows how on pre-treating the enzyme with either NADP⁺, NADPH or p-CMB, no binding of enzyme occurred since there was no delay in the passage of enzyme through the column as compared to an unpre-treated sample. This would suggest therefore that the enzyme-dye interaction is specific as logically, pre-incubation of the enzyme with saturating amounts of NADPH would be expected to block the NADPH sites present on the enzyme's surface and hence interfere with the binding of enzyme to the column. NADP⁺, being a competitive inhibitor of NADPH-cytochrome c reductase, also shows the same pattern of binding inhibition as NADPH, further underlining a specific interaction with the NADPH site on the enzyme during chromatography. Interestingly, p-CMB causes the enzyme also not to bind to the dye-matrex, although the reason for this is not completely understood.

Pre-incubation with either NADPH or NADP⁺, causes a loss in activity of the enzyme of 50% in each case. When the enzyme was pre-incubated with p-CMB, a loss in activity of 10% was
FIGURE 10.

The elution profile obtained from a large scale Blue-B column. A column size of 10 x 1 cm diameter was used for this investigation, with the fraction loaded onto the column being a calcium phosphate gel eluate sample (Cl) of 2.5 ml volume containing 11360 units§ of enzyme at a specific activity of 14030 units per mg of protein present. Notably the peak specific activity appearing from the column was less than that in the input fraction with the total units eluted only representing approximately 15% of the total units present in the input fraction (possibly due to some form of denaturation within the column).

At fraction 60 the inflowing buffer was modified from 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, by the inclusion of KCl to a concentration of 1.5 M, in order to effect the elution of any bound proteins.

A volume of 2.25 ml per fraction was recorded.

KEY:

+ Enzyme specific activity. (units/mg of protein present).
* Protein concn. (mg/ml).

The position at which the inflowing buffer was modified by the addition of KCl is indicated by the dashed arrow.

§ One unit of enzyme catalyzes the oxidation of 1 nmol of NADPH per min.
FIGURE 11.

The elution profile of pre-treated enzyme from a Red-A column. The procedure used was to take 0.5 ml of sample solution containing enzyme and to add to that 0.25 ml of a 2 mM solution of either NADPH, NADP⁺ or p-CMB and to leave the resulting mixture on ice for 1 hour. The pre-incubation mixture was then diluted 50-fold and the 25 ml of diluted solution was applied slowly through the column (at approximately 0.5 ml/min.), in order to prevent premature saturation of the dye-ligand with enzyme. The sample solution contained, before any of the additions listed above, 27 units $^S$ of enzyme activity at a specific activity of 2815 units per mg of protein present (the sample had been previously purified through a 1 x 10 cm Blue-B column). In this case use was made of a 2 ml capacity Red-A column to observe the noted effects on the binding of the enzyme to the dye-ligands.

KEY:

+ p-CMB pre-incubated sample.
* NADPH pre-incubated sample.
X NADP⁺ pre-incubated sample.
† Sample with no pre-incubation (control sample).

$^S$ One unit of enzyme catalyzes the oxidation of 1 nmol of NADPH per min.
% Specific Activity relative to input sample.
recorded. In explanation of these results, it has already been noted that NADP⁺ is a competitive inhibitor of NADPH-cytochrome c reductase activity and that NADPH, while binding to the enzyme during the incubation period could be expected to become oxidized through the FAD present in the buffer to its NADP⁺ oxidation state, thus producing the competitive inhibitor at the enzyme's active site and consequently inhibiting the enzyme. Support for this observation was obtained in later experiments (section 3.3.4 pp.53, 54). Further interpretation of the binding assay experiment is presented in the Discussion (Chapter 4).

In order to further characterize the dye-ligands, a frontal uptake analysis was performed on each of the columns; the data of which is explained in Table IX. Examples of typical profiles are presented in Fig. 12.

The results of Table IX support those earlier obtained (see Table VIII) in that Blue-B appears to bind the least amount of enzyme when compared to the other dye columns. For this reason, and for the purification obtained by Blue-B chromatography, all further purification work involved the use of this particular dye-matrix.

As the experiments with the dye columns indicated, the enzyme when it does bind to the dye-ligand, binds specifically through the NADPH substrate site. By the use of dye-ligands it has been possible to calculate certain substrate dissociation constants such as that for NADPH on the interpretation of the frontal uptake analysis data (Nichol et al., 1974).

Hence by the use of the empirically derived relations used by previous workers, it was calculated that an apparent dissociation constant of 3.2 mM for the binding of the dye-ligand (Red-A) into the NADPH-binding site of the enzyme was observed. However, that this dissociation constant does not represent the actual dissociation constant of NADPH from the enzyme can be seen from the data of Faeder et al., (1974) which gives the dissociation constant of NADPH from the enzyme as being 0.10 mM by the use of equilibrium dialysis experiments. Hence one should be cautioned against the use of data from frontal uptake analysis experiments and using this in the interpretation of various equilibrium constants observed in vitro.

The fact that the dye-ligand has far less affinity for the NADPH-binding site than does NADPH itself, supports the studies
TABLE IX: A frontal uptake analysis of enzyme onto each of the dye-ligand columns. Enzyme samples were loaded onto the columns as described in Fig. 11 (p. 35(b)) for the untreated enzyme control; each dye-ligand column being ran at approximately 0.5 ml/min. The results are expressed as the volume of solution eluted from each column when the total units* of the eluted material is 50% that of the input solution to the column. All values are normalized to those obtained with the unsubstituted agarose control column, and are termed the "relative specific binding coefficient" for that column with respect to the control. From this data, Blue-B appears to bind the least amount of enzyme relative to the control column, the results of which support those earlier presented in Table VIII.

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<th>Column</th>
<th>Relative specific binding coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
</tr>
<tr>
<td>Blue-A</td>
<td>2.99</td>
</tr>
<tr>
<td>Blue-B</td>
<td>1.06</td>
</tr>
<tr>
<td>Green-A</td>
<td>2.71</td>
</tr>
<tr>
<td>Orange-A</td>
<td>2.86</td>
</tr>
<tr>
<td>Red-A</td>
<td>3.71</td>
</tr>
</tbody>
</table>

* One unit of enzyme catalyzes the oxidation of 1 nmol of NADPH per min.

$ All columns were of 2 ml capacity.
FIGURE 12.

Examples of frontal uptake analysis profiles. Samples were applied to the columns as described in Table IX. All columns were of 2 ml capacity.

KEY:

+ Enzyme sample loaded onto a control agarose column.

* Enzyme sample loaded onto a Green-A column.

† Enzyme sample loaded onto a Blue-A column.

The volume of material eluted from the columns when the activity* recorded in the collected fractions represents 50% that recorded in the input sample is indicated by the dashed arrows.

* Activity is expressed in units/mg of protein present, with one unit of enzyme catalyzing the oxidation of 1 nmol of NADPH per min.
% Specific Activity relative to input sample.
with the pre-incubation experiments, where both NADPH and dye-ligand would compete for the NADPH-binding site; NADPH binding far more specifically and securely thus overriding any possible dye-ligand — active site interaction. Implications for conformational steric effects at the binding site for NADPH on the interaction of certain compounds with the enzyme is presented in the Discussion (Chapter 4).

3.2.5 LARGE SCALE ENZYME PREPARATION.

Fifty litres of bacterial culture were grown and harvested to give 75 g wet weight of cells. The purification procedure was run through as described in the previous sections, the only modification being made was that the pellet from the first step in the calcium phosphate gel extraction was resuspended in 0.05 M potassium phosphate buffer, pH 7.7 containing 1 mM EDTA, and centrifuged at 6,000 x g for 10 min. in order to thoroughly wash the pellet preparative to elution with 0.40 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA. The results of the steps up to the affinity chromatography stage are given in Table X. Unfortunately the yield of enzyme in fraction C1 was very low and a second elution of the gel pellet was not performed as little enzyme was expected to be recovered.

Apparently also, a great deal of active enzyme remained in the supernatant fraction (C3). Later attempts to recover this remaining enzyme by fractionation of the supernatant with calcium phosphate gel were successful in being able to recover more enzyme (see Table XI).

At this stage, though, fraction C1 was taken and dialyzed into 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, and the resulting solution was ultra-filtered. However, as severe protein foaming was observed during the ultra-filtration, it was felt that this would lead to considerable risks of enzyme denaturation occurring, hence ultra-filtration was abandoned as a method for reducing the total volume of solution prior to Blue-B gel chromatography. Thus an ammonium sulphate precipitation of the enzyme was performed so as to concentrate the fraction before the dye-ligand step. Ammonium sulphate was added from a saturated solution (at 4°), pH 7.7, to 50% saturation over a period of 5 min. to fraction C1, in order to precipitate many of the proteins present in the fraction. The resulting suspension was stirred for 30 min. and then centrifuged
Specific activity is expressed as in Table VIII (p.314)(a).

Addition of a 5% suspension of calcium phosphate gel to the solution of the first acetone precipitate after the first calcium phosphate gel precipitation, which represents the supernatant sample remaining after the first precipitation of the pellet formed by the addition of the same sample obtained after elution of the pellet formed by the addition of 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM DTT in order to wash the pellet prior to the precipitation was performed. The precipitation was performed on the addition of the first amount of gel to reaction "C", which represents the sample obtained by resuspending the calcium phosphate gel pellet in ammonium sulfate saturation. The amount of the sample, fraction "B", represents the sample of the crude extract, which is the range of 30% to 46%.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>2.059</th>
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<th>110</th>
<th>C3</th>
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</thead>
<tbody>
<tr>
<td>6.89</td>
<td>0.65</td>
<td>96</td>
<td>1.93</td>
<td>6.1</td>
<td>C9</td>
</tr>
<tr>
<td>2.105</td>
<td>0.51</td>
<td>4.29</td>
<td>3.70</td>
<td>67</td>
<td>B9</td>
</tr>
<tr>
<td>1.55</td>
<td>1.01</td>
<td>40</td>
<td>4.21</td>
<td>1.1</td>
<td>A</td>
</tr>
<tr>
<td>2.033</td>
<td>1.97</td>
<td>93.8</td>
<td>40.1</td>
<td>23.4</td>
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</table>

<table>
<thead>
<tr>
<th>Activity</th>
<th>(mg/ml)</th>
<th>Enzyme units/mg</th>
<th>(units/mg) x 103</th>
<th>(units x 103)</th>
<th>Fraction, Vol.</th>
<th>Percent common</th>
<th>Total enzyme yield of protein common</th>
</tr>
</thead>
</table>

The results of the first large scale purification up to the affinity chromatography step are presented below. The data is presented in Table X.
one unit of enzyme catalyzing the oxidation of 1 ml of NADH per min.

The specific activity of the enzyme is expressed as units per mg of protein present in the extract.

Table VII (p. 33)(9)

The designation of reactions is as described in a footnote (§) in Table VII (p. 33)(9).

The reactions from each gel addition have been omitted for clarity. ± Note that the supernatant fraction from the re-extraction of sample "C" are presented. ±

§ Re-extraction of the supernatant by the addition of further amounts of gel. ±

* Fraction "C3" represents the supernatant from the first calcium phosphate gel addition which was:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>0.1&gt;</td>
<td>0.305</td>
<td>2.19</td>
<td>5.2</td>
<td>72.01</td>
<td>0.667</td>
<td>0.08</td>
<td>12.3</td>
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</tr>
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<td>0.1&gt;</td>
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<td>0.667</td>
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<td>2.12</td>
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<tr>
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<td>0.9</td>
<td>72.01</td>
<td>0.667</td>
<td>0.13</td>
<td>3.2</td>
<td>2.12</td>
</tr>
<tr>
<td>0.1&gt;</td>
<td>0.035</td>
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<td>0.7</td>
<td>72.01</td>
<td>0.667</td>
<td>0.13</td>
<td>3.2</td>
<td>2.12</td>
</tr>
<tr>
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<td>2.16</td>
<td>0.7</td>
<td>72.01</td>
<td>0.667</td>
<td>0.13</td>
<td>3.2</td>
<td>2.12</td>
</tr>
<tr>
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<td>2.16</td>
<td>0.7</td>
<td>72.01</td>
<td>0.667</td>
<td>0.13</td>
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<td>2.12</td>
</tr>
<tr>
<td>0.1&gt;</td>
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<td>2.16</td>
<td>0.7</td>
<td>72.01</td>
<td>0.667</td>
<td>0.13</td>
<td>3.2</td>
<td>2.12</td>
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<tr>
<td>0.1&gt;</td>
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<td>2.16</td>
<td>0.7</td>
<td>72.01</td>
<td>0.667</td>
<td>0.13</td>
<td>3.2</td>
<td>2.12</td>
</tr>
<tr>
<td>0.1&gt;</td>
<td>0.092</td>
<td>2.16</td>
<td>0.7</td>
<td>72.01</td>
<td>0.667</td>
<td>0.13</td>
<td>3.2</td>
<td>2.12</td>
</tr>
</tbody>
</table>

I mm NADPH, before the addition of any calcium phosphate gel.

The supernatant fraction to be re-extracted had been eluted from 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mm EDTA.
at 27,000 x g for 1 hour, the pellet obtained being resuspended in 1.30 ml of 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA. The suspension was then dialyzed overnight against the same buffer used to resuspend the above pellet. After dialysis a volume for the fraction of 2.0 ml was recorded. The results of this ammonium sulphate treatment are given in Table XII.

The results indicate that on increasing the protein concentration by reducing the total volume of the fraction, enzyme activity is apparently lost. It was also noted from other experiments that activity is lost on lengthy dialysis of the enzyme (that is, for 24 hours or more) against 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA.

Despite this loss in enzyme activity, the post-dialysis material was used for subsequent Blue-B chromatography. Thus the 2.0 ml of solution was diluted to 4.8 ml in 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, and 1.2 ml of which was applied to a large scale Blue-B column. Fig. 13 presents the elution profile of this column run. Note that a maximum specific activity of 12,300 units per mg of protein present was obtained, which is lower than that of the input dialyzed fraction, indicating that some form of enzyme denaturation had occurred on the Blue-B chromatography step. Despite the drop in enzyme activity and loss of total units, the column was run under the same conditions with successive samples of 1.2 ml being applied to the Blue-B gel and chromatographed. The same elution profile as seen in Fig. 13 was reproduced for each of these successive runs. Fractions 16 to 45 were pooled in each case with the same fractions from each column run and concentrated by ultrafiltration to 6.00 ml total volume (ultrafiltration being indicated here as very little foaming was seen, and hence presumably very little enzyme denaturation was taking place on concentrating the solution).

The purpose of using small samples applied to the column was to avoid a possible saturation effect of the dye-matrex gel with impurities, so that a leakage of contaminants, which would normally bind to the dye-ligand, could be avoided. However, as was evident from this and further experimentation, 50 to 100 ml of dye-matrex gel was able to cope with large quantities of protein, up to 100 mg, before serious saturation problems.
The specific activity is expressed as in Table VII.

The treatment of fraction "CII" obtained after removal of the pellet (fraction "Cu") represents the remaining supernatant fraction from the 20% ammonium sulfate which was formed by the addition of ammonium sulfate to 50% saturation to fraction "Cv".

Table "CII" represents the resuspended ammonium sulfate pellet of sample "Cv", the pellet of fraction "Cv" represents the sample obtained after flotation of the pellet formed by the addition of the crude extract.

<table>
<thead>
<tr>
<th>1.0</th>
<th>0.65</th>
<th>0.014</th>
<th>0.17</th>
<th>0.32</th>
<th>9.16</th>
<th>35.0</th>
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<th>6715</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.29</td>
<td>0.81</td>
<td>4.8</td>
<td>8.70</td>
<td>5</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Fraction "CII" represents the sample obtained after flotation of the pellet formed by the addition of the crude extract.*

**Table XII**: Ammonium sulfate treatment of the extraction obtained by the first addition of calcium phosphate gel to a semi-purified enzyme preparation.
FIGURE 13.

The elution profile from a Blue-B column of an ammonium sulphate precipitated calcium phosphate gel fraction. A large scale Blue-B column was used (25 x 1.4 cm diameter) with the gel equilibrated with 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, before sample application. After the sample was run into the column the flow rate of buffer to the column was turned off for 1 hour in order to allow for the binding of contaminating proteins. When flow rate was resumed fractions of 1.2 ml were collected.

KEY:

+ Enzyme specific activity (units*/mg of protein present).

* Protein concn. (mg/ml).

* One unit of enzyme catalyzes the oxidation of 1 nmol of NADPH per min.
occurred.

From the elution profile presented in Fig. 13, a protein peak can be seen emerging from the column just after the elution of the enzyme. It was presumed therefore that this peak and others eluting after the enzyme represented lower molecular weight contaminating proteins that were effectively separated from the enzyme by Blue-B chromatography. Thus in order to separate from the enzyme any possible higher molecular weight contaminating proteins Bio-gel A-15m gel filtration was performed. However in order to firstly test the applicability of gel filtration, a sample of fraction B2 was applied to a Bio-gel column. The elution profile obtained from the column is given in Fig. 14. From this profile the enzyme peak is seen to elute just in advance of the main protein peak, with some protein peaks appearing ahead of the enzyme. From this data it was reasoned that gel filtration would provide an effective final stage in the purification of the enzyme after Blue-B chromatography by removal of any higher molecular weight proteins not separated from the enzyme by previous steps in the purification procedure.

The ultrafiltered fraction from the Blue-B chromatography step was dialyzed overnight into 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, and applied onto a Bio-gel column. The dialysis into a higher ionic strength phosphate buffer was performed in an attempt to preserve enzyme activity, as low ionic strength buffers have been noted by Naiki, (1965) to severely decrease NADPH-linked sulphite reductase activity in yeast extracts, and supports what has been also observed to occur with NADPH-cytochrome c reductase preparations.

The results from the gel filtration step are presented in Fig. 15. On analysis it was calculated that the total amount of protein eluted from the column appeared to be far higher than the total amount of protein placed onto the column. This discrepancy no doubt arose due to the very small protein concentrations being measured, and at best only 0.15 mg of purified enzyme could be recovered from the column which would be barely sufficient to allow the minimum number of amino acid residues planned to be sequenced (six to ten) to be done and the experiment reproduced. Furthermore, the specific activity of the fractions eluted from the Bio-gel column indicated that
FIGURE 14.

The elution profile of an ammonium sulphate fraction from a Bio-gel column. The sample was applied to a 27 x 0.9 cm diameter Bio-gel A-15m column and eluted with 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, with fractions of 0.35 ml being collected. A sample of 1.0 ml volume at a specific activity* of NADPH-cytochrome c reductase of 3146 units per mg of protein was applied to the gel filtration column.

KEY:

+ Enzyme specific activity (units/mg of protein present).

* Protein concn. (mg/ml).

* The specific activity of NADPH-cytochrome c reductase is defined as the number of nmol of NADPH oxidized per min. per mg of protein.
FIGURE 15.

The elution profile of pooled and concentrated Blue-B chromatography fractions from a Bio-gel column. The sample was applied to a 27 x 0.9 cm diameter Bio-gel A-15m column and eluted with 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, with fractions of 0.39 ml being collected. The applied sample had a recorded specific activity* of 3488 units per mg of protein.

KEY:

+ Enzyme specific activity (units/mg of protein present).

x Protein concn. (mg/ml).

* The specific activity of NADPH-cytochrome c reductase is defined as the number of nmol of NADPH oxidized per min. per mg of protein.
that the samples collected after the gel filtration step were only about 3.5x purer than the crude extract fraction, with an overall decrease in activity from fraction C1 on gel filtration.

In order to examine whether other gel media were more appropriate as a final stage in enzyme purification, both DEAE-cellulose and hydroxylapatite were tested with a fraction eluted from the Bio-gel column. The results are presented in Fig. 16. These preliminary experiments with other gel media indicated that neither of the tested chromatographic media were suitable in further purification of the enzyme since poor adsorptive properties for the enzyme were observed coupled with large losses in enzyme activity on chromatography.

In order to regain enzyme not recovered during the purification procedure in the first calcium phosphate gel addition supernatant, calcium phosphate gel was added to this fraction to give a final gel to total protein ratio of 0.4, 0.6, 1.5 and 1.8 mg of gel added per mg of protein present in the supernatant fraction. The results for the refractionation of the supernatant have been presented in Table XI. Notably the percentage yield of enzyme from the refractionated supernatant indicates that major enzyme denaturation has occurred on calcium phosphate gel treatment of the supernatant fraction. The denaturation may have resulted from the conditions used on gel treatment of the supernatant, as in order to separate the gel from the solution effectively, centrifugation at high speeds was necessary. However, in order to subsequently resuspend the gel pellet, vigorous vortexing of the pellet must be performed so as to render it a suspension. These harsh resuspension conditions could undoubtedly lead to enzyme denaturation and thus give indication to the low recovery of enzyme units on treatment of a sample with calcium phosphate gel. Despite the denaturation effect observed, fractions from the refractionation of the supernatant sample with specific activities above 7,000 units/mg of protein present, were pooled, with the other fractions being discarded.

In order to obtain further quantities of purified enzyme, another large scale purification procedure was performed. Thus a further fifty litres of bacterial culture was grown and harvested to give 146 g wet weight of cells. The cells were
FIGURE 16.

Elution profiles of a Bio-gel fraction from DEAE-cellulose and hydroxylapatite columns. The fraction applied onto each column was a 0.70 ml sample from pooled fractions 110 to 120 from the gel filtration step (see Fig. 15). The samples were dialyzed overnight against 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, and then assayed for total enzyme units before application to columns of 10 x 1 cm diameter. Once the sample had been applied to the column the flow rate of buffer to the column was ceased for 1 hour in order to allow for enzyme binding equilibration to occur. After this period, the flow rate of buffer to the column was resumed at 0.55 ml/min, with a volume of 0.6 ml being recorded per fraction; flow rate being assisted by a Minipuls 2 pump. At fraction 50 the inflowing buffer was changed from 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, to 0.2 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA and 1.5 M KCl, in order to effect the elution of any bound enzyme.

KEY:
+ DEAE-cellulose elution profile.
× Hydroxylapatite elution profile.

The fraction at which the inflowing buffer was changed is marked with the dashed arrow.

* One unit of enzyme activity is defined as that amount of NADPH-cytochrome c reductase that catalyzes the oxidation of 1 nmol of NADPH per min.
then disrupted with the Hughe's press, with the resulting crude extract being treated with ammonium sulphate and calcium phosphate gel as described for the previous large scale enzyme preparation. The first eluted calcium phosphate gel fraction (C1) was then added to those fractions which had been pooled from the refractionation procedure. Protein in the total pooled calcium phosphate gel fractions was then pelleted by the addition of ammonium sulphate to 50% saturation and subsequent centrifugation as described previously. The resulting pellet was resuspended in 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA. Any residual enzyme remaining in the supernatant from the ammonium sulphate treatment was extracted by ultrafiltering the supernatant twice and pooling the finally concentrated solution with the resuspended fraction. The pooled fraction was then dialyzed overnight against 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA. The dialyzed solution was then applied to a large scale Blue-B dye column. A summary of the results obtained for this second large scale enzyme preparation is given in Tables XIII and XIV and in Figs. 17 and 18. The final summary of the purification of sufficient quantities of enzyme to allow for further analysis is presented in Table XV. Notably the final specific activity obtained, of 20,500 units per mg of protein, for the fractions chosen to be pooled from the Blue-B column (viz. fractions 20 to 60), represents a significant increase in the level of enzyme from that obtained in crude extracts of wild-type cultures grown under conditions of derepression for the genes involved in cysteine biosynthesis.

On the basis of the excellent correspondence between specific activity and total units eluted from the Blue-B column, (Fig. 18), the enzyme preparation was considered to be chromatographically pure and Bio-gel A-15m gel filtration was not performed, as previous experiments have indicated that very little further purification can be achieved with this gel filtration step, with only losses of enzyme being obtained.

Due to the addition of the refractionated calcium phosphate gel samples from the first large scale purification procedure to the first eluted calcium phosphate gel fraction (C1) of the second large scale enzyme preparation, the percentage yield of total units of enzyme as referenced to the crude extract of the second preparation appears higher by 7%. Taking this fact
Specific activity is expressed as in Table XI. N.D. 'not determined'.

From the first large scale enzyme preparation (see Table XI, and p.41) samples from the supernatant from the 50% ammonium sulfate treatment of fraction "C1" (II) represent the residual material remaining after ultracentrifugation. The yield of enzyme is referred to as extract ("A") and is not corrected for the addition of aspirin. Fraction "C1" (II) represents the residual material remaining after treatment with 0.05M potassium phosphate buffer pH 7.7, containing 1 mM PMSF, for 2 hours. Fraction "C1" (II) and "C1" (III) after dialysis against 0.05M potassium phosphate buffer, pH 7.7, contain.

Fraction "C1" (II) represents the sample obtained by the second large scale enzyme preparation. Fraction "C1" (II) represents the sample obtained from the first large scale enzyme preparation. Fraction "C1" (II) represents the sample obtained by the first calcium phosphate gel addition to fraction "B2". Fraction "C1" (I) represents the ammonium sulfate fraction obtained in the range of 308 to 468.

Fraction "B2" represents the ammonium sulfate fraction obtained in the range of 308 to 468.

Fraction "A" represents the crude extract.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (%)</th>
<th>Yield (%)</th>
<th>Enzyme Activity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
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<td>C1 (I)</td>
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<td>37.4</td>
<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (II)</td>
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<td>0.12</td>
</tr>
<tr>
<td>C1 (III)</td>
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<td>37.4</td>
<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (IV)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (V)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (VI)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (VII)</td>
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</tr>
<tr>
<td>C1 (VIII)</td>
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<td>37.4</td>
<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (IX)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (X)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XI)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XII)</td>
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<td>37.4</td>
<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XIII)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
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<td>0.12</td>
</tr>
<tr>
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<td>0.12</td>
</tr>
<tr>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XVII)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XVIII)</td>
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<td>37.4</td>
<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XIX)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XX)</td>
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<td>1.13</td>
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</tr>
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<td>1.13</td>
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<tr>
<td>C1 (XXIII)</td>
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<tr>
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<td>C1 (XXVII)</td>
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<td>37.4</td>
<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XXVIII)</td>
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<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XXIX)</td>
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<td>37.4</td>
<td>1.13</td>
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</tr>
<tr>
<td>C1 (XXX)</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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<td>0.12</td>
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<tr>
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<tr>
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<tr>
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<td>1.13</td>
<td>0.12</td>
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<tr>
<td>C1 (XLIII)</td>
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<td>37.4</td>
<td>1.13</td>
<td>0.12</td>
</tr>
<tr>
<td>C1 (XLIV)</td>
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</table>

* Note: The table is truncated for brevity. For a complete table, please refer to the original document or data sheet.
Table XIII. The specific activity is expressed as in Table XI.

The partition factor quoted here is referred to the crude extract fraction (n) from column 5, while the partition factor quoted here is referred to the crude extract fraction (n) from column 5, and the values in column 6 are the calculated partition factor of the gel of the fraction "B" after the Sanger's column of the first and second fraction followed by the addition of the gel of fraction "C".

The assay data from the fractions eluted from the blue-P column was pooled in lots of three. The specific activity of the fraction "C" corresponds to the "C" from Table XI, from which we obtained fraction "C".

The results from the blue-P dye-tyrosine affinity chromatography are:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity (units/mL)</th>
<th>Enzyme (mL/mL)</th>
<th>Protein conc. (mg/mL)</th>
<th>Vol. (mL)</th>
<th>Enzyme conc. (mL/mL)</th>
<th>Total enzyme</th>
<th>Yield (%)</th>
</tr>
</thead>
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<tr>
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<td>N.D.</td>
<td>N.D.</td>
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<td></td>
</tr>
<tr>
<td>(100)</td>
<td>67.65</td>
<td>23</td>
<td>10</td>
<td>2.9</td>
<td>2.9</td>
<td>100</td>
<td>67.65</td>
</tr>
</tbody>
</table>

Note: The best using for physical analysis.

The results of the second large scale enzyme preparation in tabular form. The specific activities from the blue-P dye-tyrosine affinity chromatography are:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity (units/mL)</th>
<th>Enzyme (mL/mL)</th>
<th>Protein conc. (mg/mL)</th>
<th>Vol. (mL)</th>
<th>Enzyme conc. (mL/mL)</th>
<th>Total enzyme</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<td>N.D.</td>
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<td></td>
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</tr>
<tr>
<td>(100)</td>
<td>67.65</td>
<td>23</td>
<td>10</td>
<td>2.9</td>
<td>2.9</td>
<td>100</td>
<td>67.65</td>
</tr>
</tbody>
</table>