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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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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 *cysJIHDC* 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*-acetylserine sulphydrylase (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).

cysA: sulphate permease

cysB: positive regulatory gene

cysC: adenosine 5'-phosphosulphate kinase

cysD: ATP sulphurylase

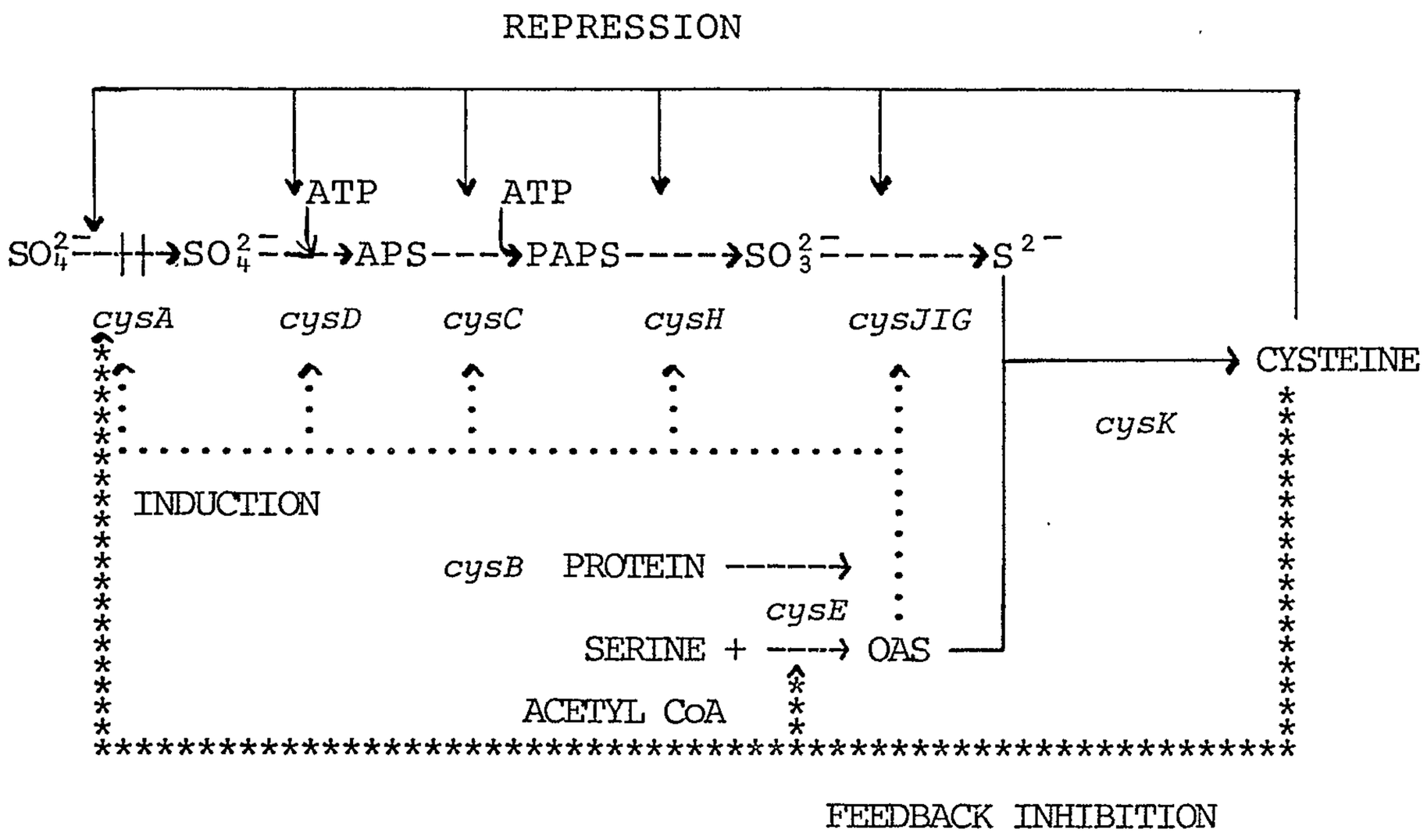
cysE: serine transacetylase

cysH: 3'-phosphoadenosine 5'-phosphosulphate reductase

cysJIG: NADPH-sulphite reductase

cysK: *O*-acetylserine sulphydrylase A

Part A *REGULATION:*



Part B *GENE - ENZYME RELATIONSHIPS:*

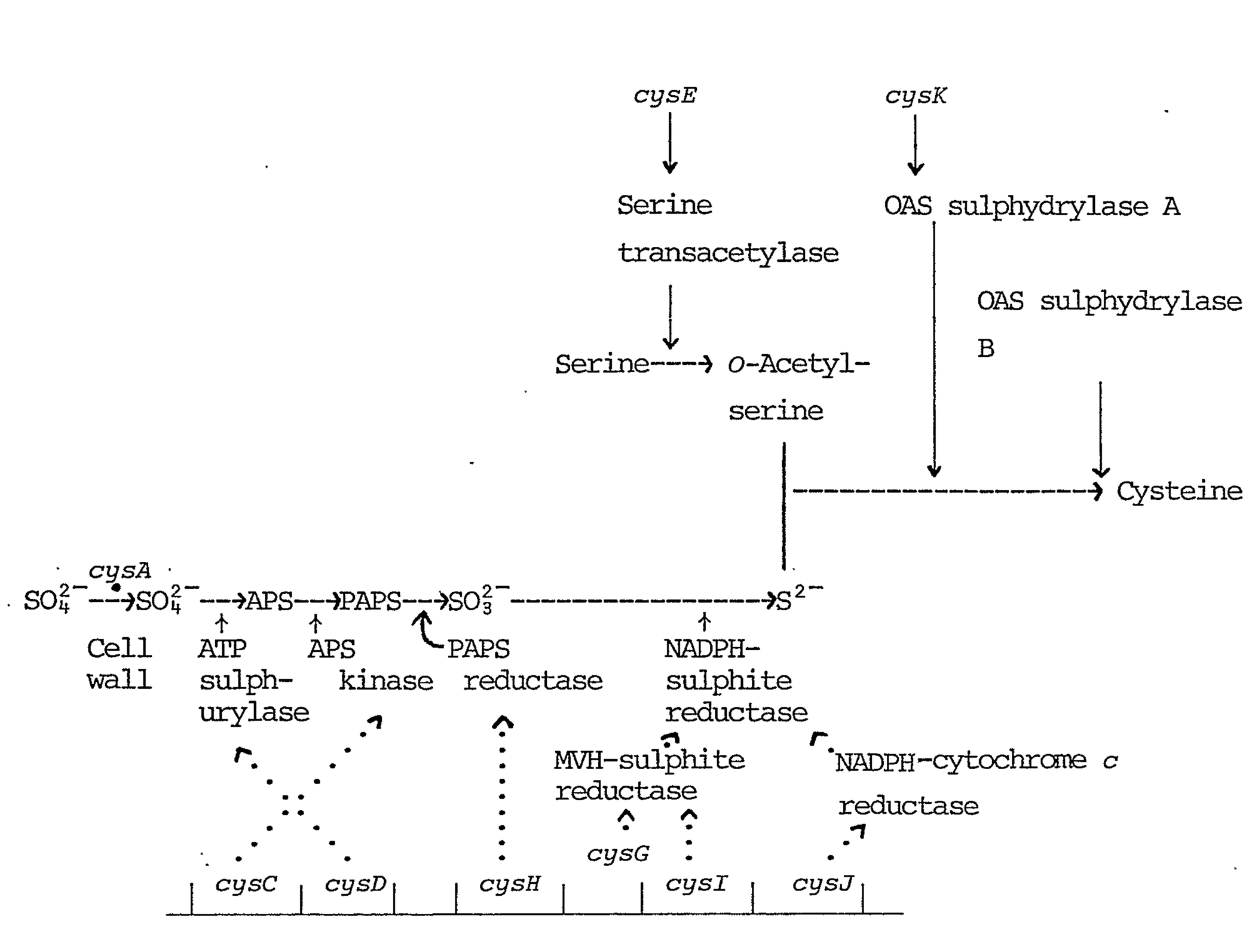
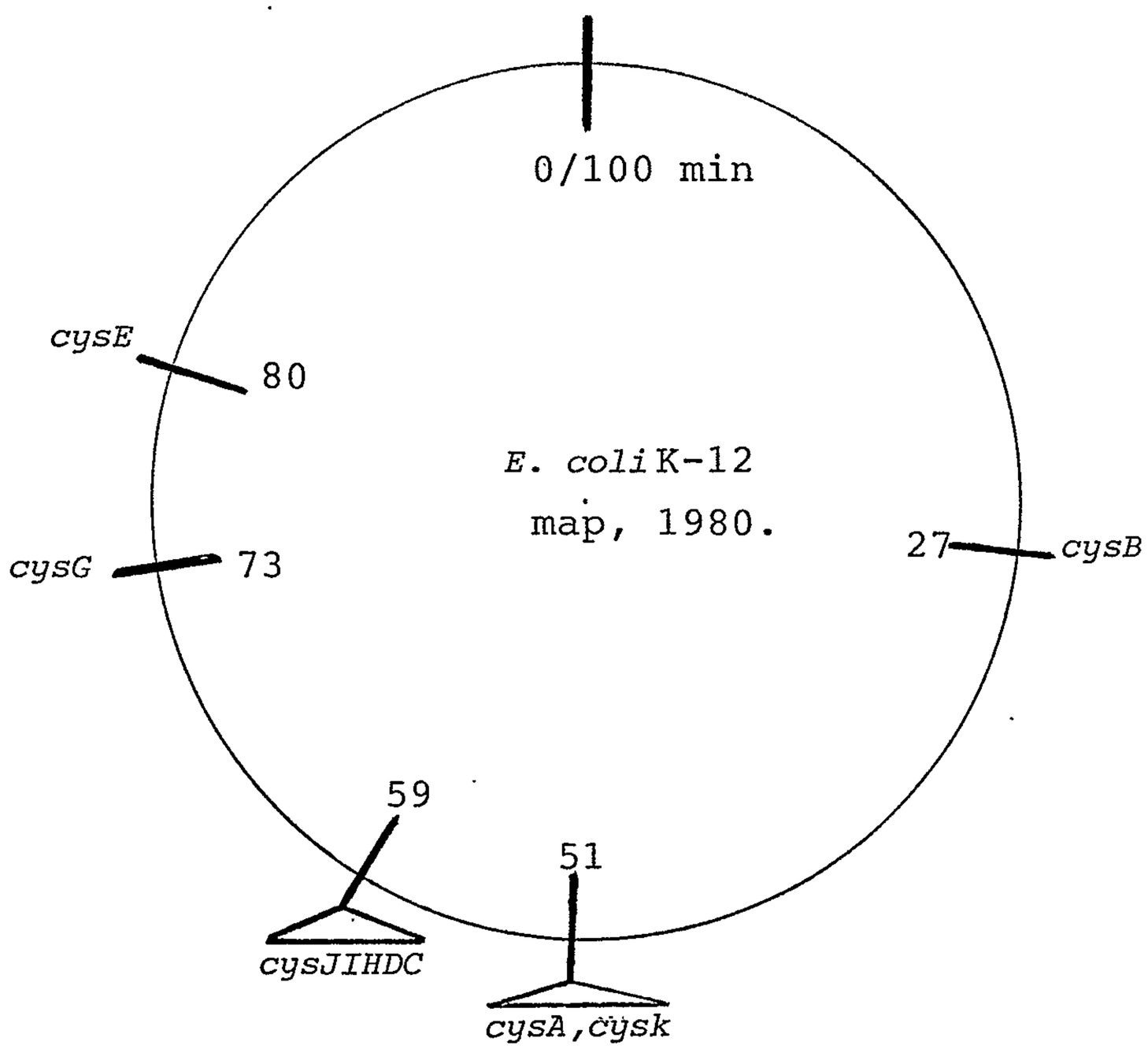


FIGURE 2

The positions of the cysteine genes on the linkage map of *E. coli* K-12 (Bachmann and Low, 1980).


0/100 min
cysE
80
E. coli K-12
map, 1980.
cysG
73
27
cysB
59
51
cysJIHDC
cysA,cysk

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 *O*-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 *cysJI* 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 sulphydrylase (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 *O*-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 *cysJIHDC* gene cluster (λd*cysJIHD*) 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 on 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 *cysJIH* operon, present in a *cysB* deletion strain. This strain gave a constitutive expression of the *cysJIH* 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 *cysJIH* 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 *cysB*$^-$ and *cysB*$^+$ allele respectively, indicating that unlike the control of the cysteine biosynthetic genes the *cysB* gene does not respond to the intracellular concentration of cysteine. In some of the fusion strains however, it was noted that the *cysB* locus did not appear to be repressed on introduction of the *cysB*$^+$ allele and it is suggested that a constitutive mutation in the controlling region of the *cysB* gene is involved in making the locus refractory to high intracellular concentrations of the *cysB* protein. It was suggested that these strains could be useful in producing large amounts of *cysB* protein.

Furthermore, Trudinger and Loughlin, (1981) have proposed that the *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 *cysB* protein.

In this project, it is hoped that when a *cysB*$^+$ strain carries a hybrid plasmid bearing the *cysJ* gene and its promoter, that the *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 *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 *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 *cysB* gene it should be possible to obtain plasmid mediated *cysJ* enzyme enhancement to levels commensurate to the number of *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 *malA* and *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 $malT^{C}$, 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 $malT^{C}$ 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).

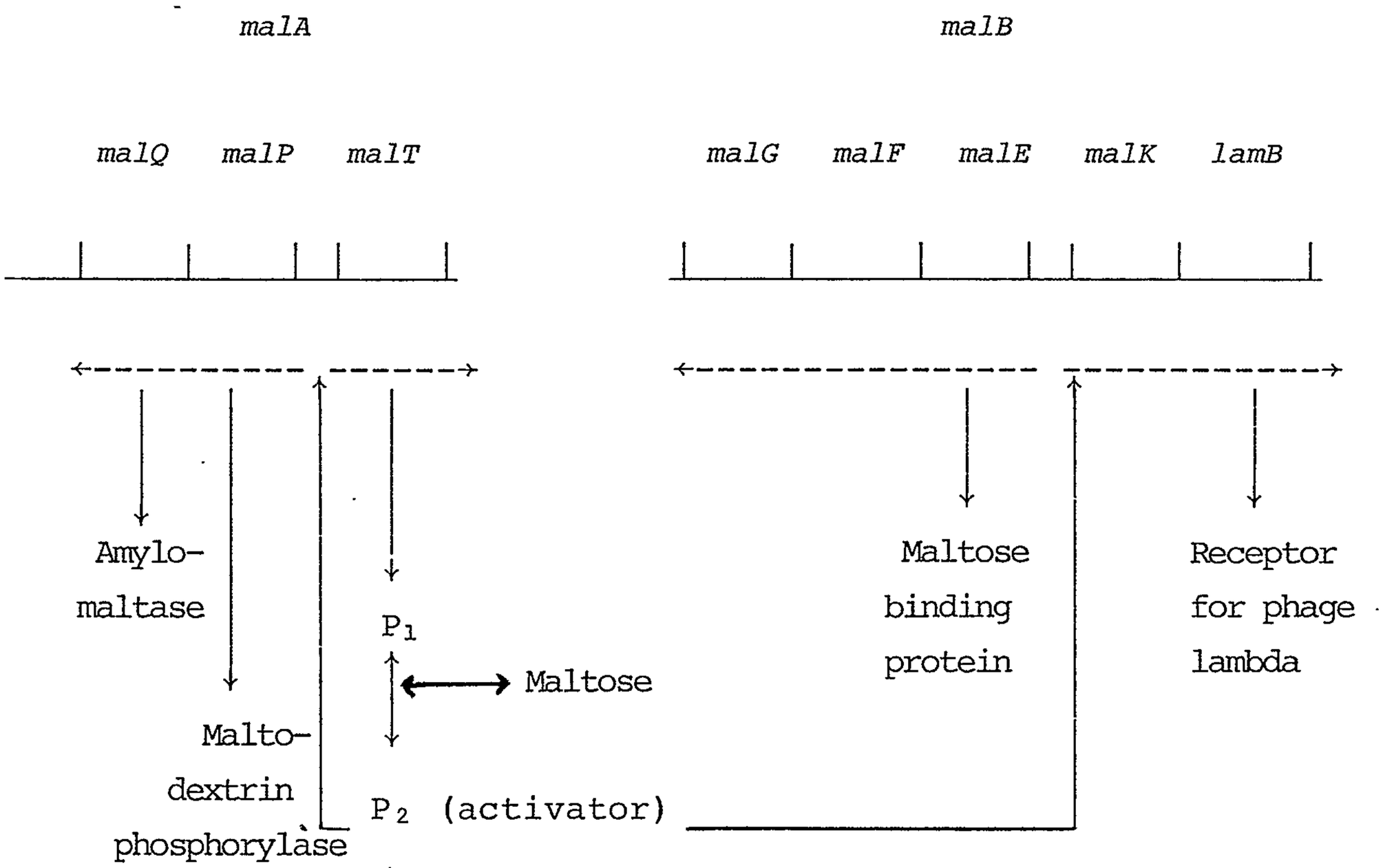

malA
malB
malQ
malP
malT
malG
malF
malE
malK
lamB
Amylo-
maltase
Malto-
dextrin
phosphorylase
P1
Maltose
P2 (activator)
Maltose
binding
protein
Receptor
for phage
lambda

TABLE I: The properties of some conjugative and non-conjugative plasmids.

Plasmid.	Size(Kbp).	Conjugative.	No. of plasmid copies per cell.	Phenotype
pBR322	4.4	No	10-15	*Amp^r §Tet^r
ColE1	7.0	No	10-15	Colicin E1 producer
RSF 1030	9.4	No	20-40	Amp^r ¶Str^r
R6K	41.9	Yes	13-38	Amp^r
F	104.0	Yes	1-2	-
RI	109.1	Yes	1-3	#MDR

*Ampicillin resistance.
§Tetracycline resistance.
¶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. Currenty 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 *Eco*RI digestion of the specialised transducing phage ϕ80*trpE-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 *Eco* RI cleaved plasmids, ColE1 and pCR1, was expressed regardless of its orientation of insertion. Mitomycin C, a compound that induces colicin E1 production in ColE1 containing bacteria, stimulated tryptophan synthetase α subunit prduction, (the product of the gene *trpA*) in cells containing ColE1-*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 *Eco* RI they obtained a 10.9 Kbp fragment. This DNA segment was then inserted, *in vitro*, into a series of small multicopy 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).

Host strain.		Hybrid plasmid.	Cloning vehicle.	Plasmid copy No.	*Amplification factor.
E. coli	J5	R67	-	1.8	-
E. coli	HB101	pIE028	pBR313	31.0	28x
E. coli	711	pIE028	pBR313	30.0	19x
E. coli	HB101	pIE024	pBR333	24.0	23x
E. coli	C600	pFE332	pBR322	18.0	35x

* 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 λ*cysJ* was utilized by Smith, (1981) to construct the hybrid plasmid pRL1 by *in vitro* recombination (between λ*cysJ* and the multipurpose cloning vehicle pBR322). λ*cysJ* is able to transduce the *cysJ* point mutant strain RL144 to Cys^+ but was not able to transduce the *cysI* point mutant, JM57, to cysteine prototrophy, and thus pRL1 was presumed to carry only the *cysJ* gene and its promoter. From the mapping data presented for pRL1 (Smith, 1981) it was deduced that the known location of the *cysJ* gene and its promoter, assumed to be approximately 1.5 Kbp in length, lay within the 8.8 Kbp *Hin*dIII-*Bam* HI bacterial derived fragment of pRL1. On subsequent subcloning experiments (M.T.Smith, personal communication) a 5.9 Kbp *Hin* dIII-*Sal* I 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 *cysJ* 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, λ*cysJ* 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 *cysJ* gene and approximately 4.4 Kbp of unrelated DNA. pRL3 also carries 3.7 Kbp of pBR322 DNA, which codes for a β-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 *cysJ* 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.

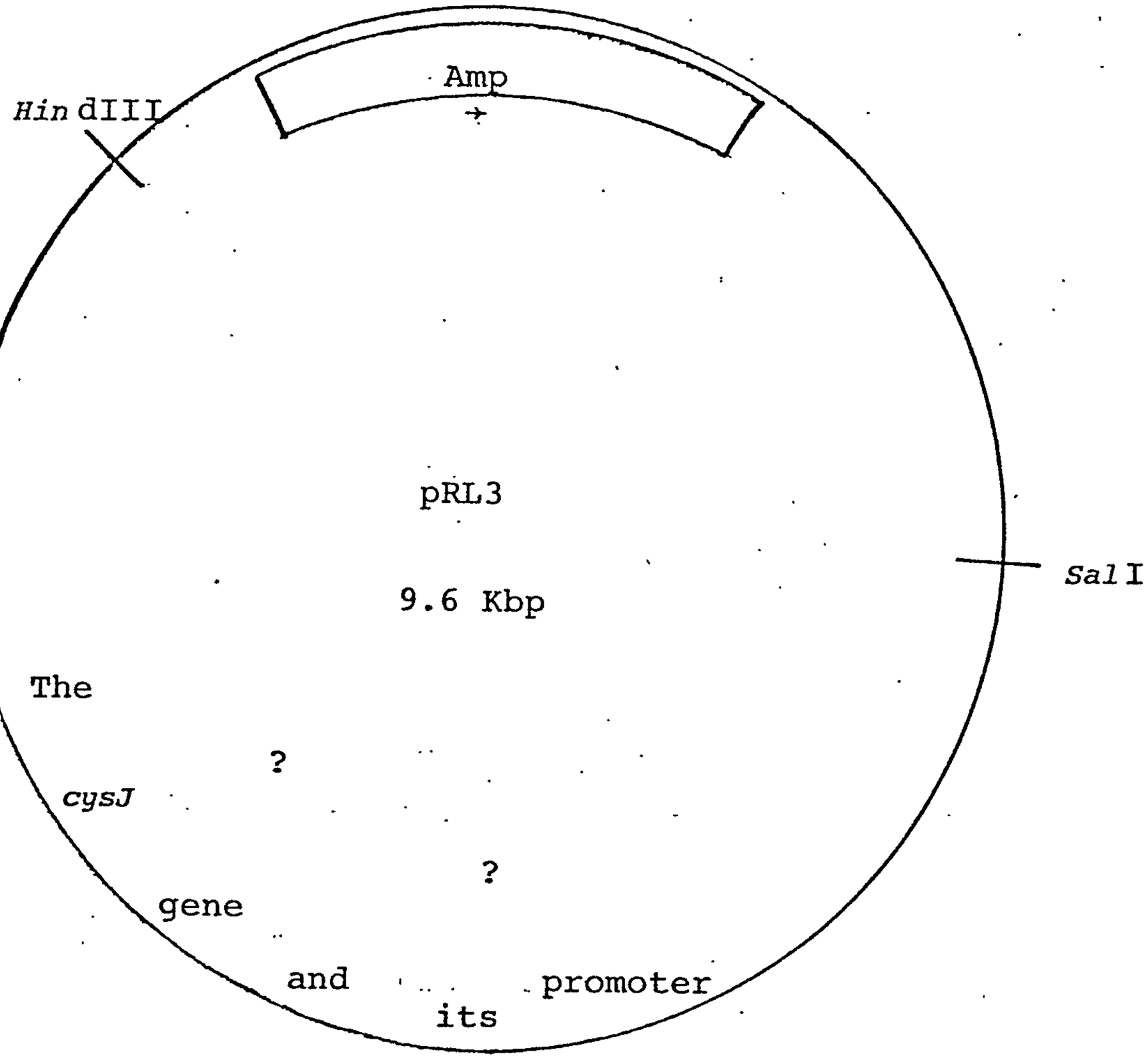
Importantly, pBR322 and thus pRL3, both carry a ColE1 derived replication origin, which allows for autonomous plasmid replication uncoupled from the host cell chromosome and hence the ability to increase in copy number on chloramphenicol treatment, (Clewell, 1972).

FIGURE 4

The hybrid plasmid pRL3, showing phenotypic characters and the unique sites for the restriction endonucleases *Hin*dIII and *Sal*I (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 *cysJ* gene and its promoter within the 5.9 Kbp of bacterial DNA carried by pRL3, are not known.


Amp
HindIII
pRL3
9.6 Kbp
SalI
The
?
cysJ
?
gene
and
promoter
its

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 hemoprotein, 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-dichlorophenolindophenol (DCPIP), ferricyanide and menadione. The hemoprotein component can catalyze the reduction of sulphite to sulphide with the artificial electron donor reduced methyl viologen (MVH) or with the *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 *in vitro* (Siegel and Davis, 1974).

The flavoprotein and hemoprotein are each composed of a single type of polypeptide chain, termed α and β respectively, with individual molecular weights of approximately 59,000 and 56,000 (Siegel *et al.*, 1971).

The active flavoprotein has been isolated only in an octameric form (Siegel and Davis, 1974) designated as α_8; the hemoprotein can be isolated however as a monomer, capable of catalyzing MVH linked sulphite reduction. The sulphite reductase holoenzyme has the structure α_8 β_4 with one α 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 sulphite reductase can serve quite different roles in the electron transfer process (Siegel *et al.*, 1974). Thus the mercurial *p*-chloromercuriphenylsulphonate (*p*-CMPS), at low concentrations, promotes the dissociation of FMN, but not FAD, from sulphite reductase. Even though the NADPH 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 *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 α_8 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 transmethylase 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).

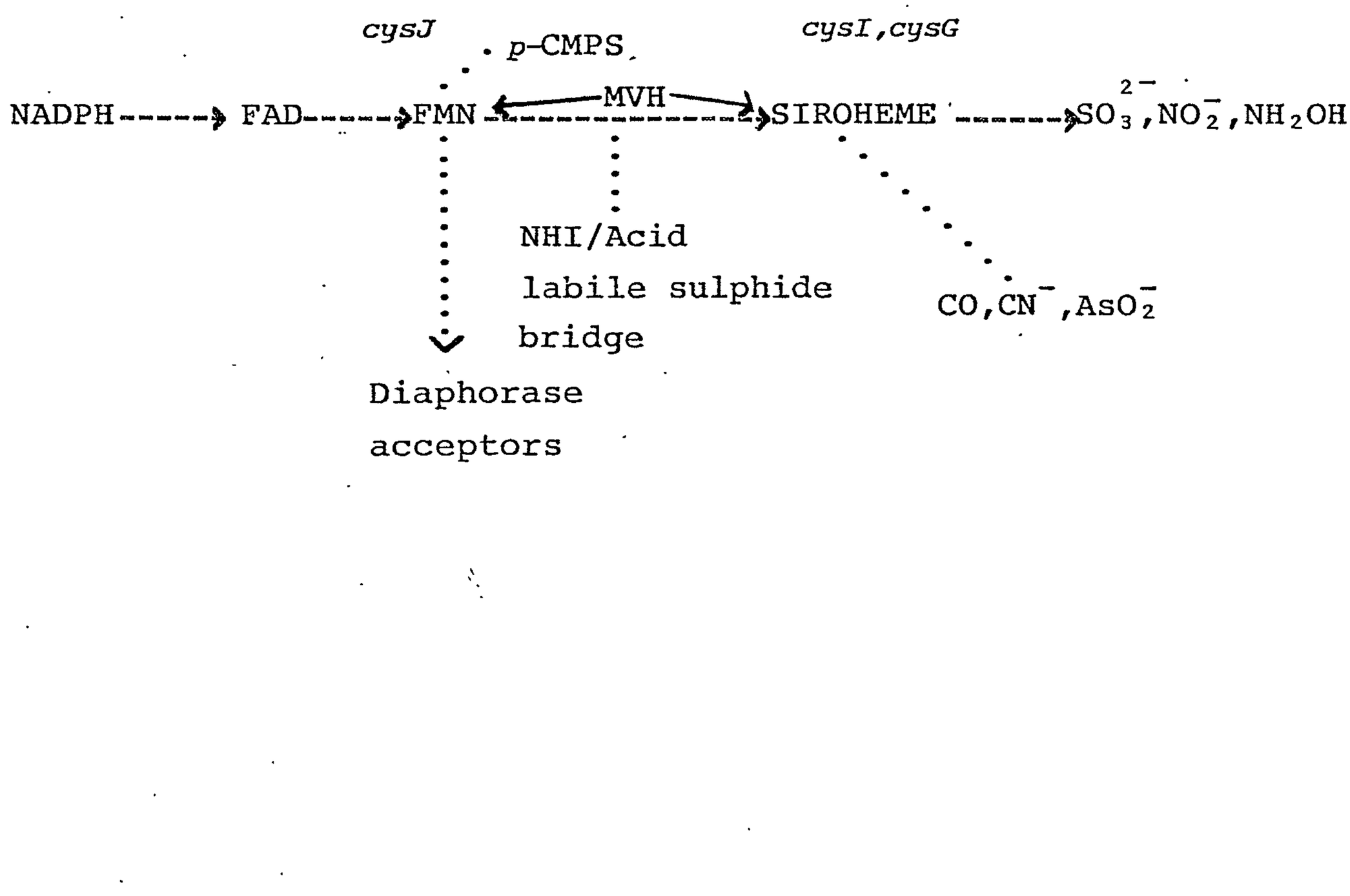
The *in vivo* direction of electron flow is shown in red.

ABBREVIATIONS:

MVH Reduced Methyl Viologen.

NHI Non Heme Iron.

p-CMPS *p*-Chloromercuriphenylsulphonate.


cysJ
p-CMPS
cysI,cysG
NADPH
FAD
FMN
MVH
SIROHEME
SO_3^{2-}, NO_2^-, NH_2OH
NHI/Acid
labile sulphide
bridge
CO, CN^-, AsO_2^-
Diaphorase
acceptors

uroporphyrinogen III and S-adenosyl methionine to form the siroheme prosthetic group of the hemoprotein subunit; a review is presented by Trudinger 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-A 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 β pleated strands connected by several α helical strands about the β sheets. A C-terminus in the β 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).

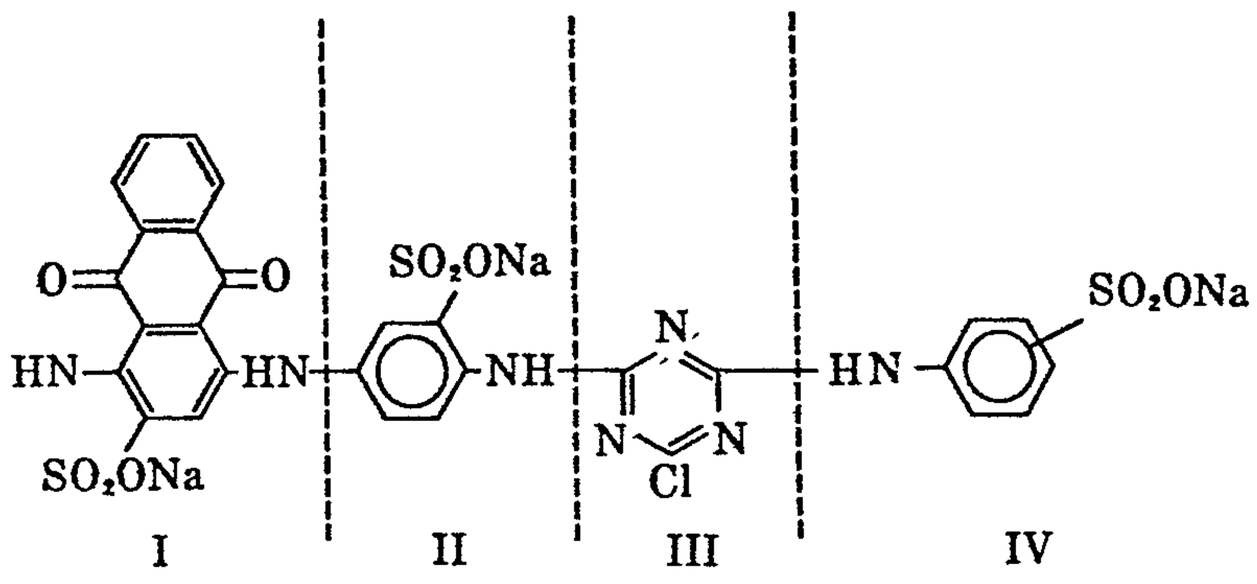

O
O
HN
HN
SO₂ONa
SO₂ONa
NH
N
N
N
Cl
HN
SO₂ONa
I
II
III
IV

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 III: Recent purifications achieved with dye-ligand affinity chromatography.

Enzyme.	Source.	Dye-Matrex used.	Elution conditions.	Yield. (%)	*PF.	Ref.
Alcohol dehydrogenase EC 1.1.1.1	Baker's yeast	Blue-A (agarose coupled)	5 mM NAD^+	55	31	Easterday and Easterday, (1974).
Glucose 6-phosphate dehydrogenase EC 1.1.1.49	Baker's yeast	Blue-A (agarose coupled)	10 mM NADPH	60	4460	Easterday and Easterday, (1974).
NADH:Nitrate reductase EC 1.6.6.1	*Chlorella vulgaris*	Blue dextran (agarose coupled)	0-100 M NADH	63	127	Solomonson, (1975).
NAD(P)H: Nitrate reductase EC 1.6.6.4	*Neurospora crassa*	Blue dextran (agarose coupled)	1 mM NADPH	69	27	Greenbaum *et al.*, (1978)

* 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, lysozyme (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'*-tetramethylethylenediamine 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, *Eco* RI and *Hin* dIII 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 Na_2SO_4 (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 $MgSO_4$ by $MgCl_2$ 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 OF CULTURES.*

Strains of *E. coli* K-12 were stored either in liquid culture (10 ml L broth at approximately 2×10^8 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 -18^0C) and 0.1 ml of which was inoculated into 2 ml of L broth and shaken at 37^0C 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 IV: *ESCHERICHIA COLI* K-12 STRAINS.

Strain.	Genotype.	Source.
χ1776	F^-, *tauA53, dapD8, merA1, supE42,* Δ40 (*gal, attλ, uvrB*), *λminB2, malA25, thyA57, metC65,* Δ 29 (*bioH-asd*), *cycB2, cycA1, hsd1R2,* [pBR322 amp^r, tet^r]	K.D.Brown
KB9163	Hfr, *KL16, nalA900,* Δ(*gal, attλ, bio, uvrB*) *thi*	K.D.Brown
RL145	F^-, *cysH, thr-1, leu-6, trp-1, his-1, argH1, thi-1, xyl-7, ara-13, mtl2, gal-6, lacY1,* λ^-, λ^s *tonA2, str-9, supE* ($SuII^+$)	R.E.Loughlin
RL155	HfrH, Δ*attBB'*, sup^-, Δ(*gal, attλ, bio, uvrB*), str^s, (λd*cysJIHD*), (λ*cI857Sam7*), Δ(*cysJIH*)	R.E.Loughlin
RL421	F^-, *thr-1, leu-6, thi-1, supE44, lacY1, tonA2, cysJ*	*R.E.Loughlin
RL434	F^-, Δ(*cysJIH*), *pro, leu-6, thi-1, lacY,* str^r, r_k^- m_k^-	§R.E.Loughlin
RL500	F^-, *thr-1, leu-6, thi-1, supE44, lacY1, tonA2, cysJ,* [pRL1 amp^r $cysJ^+$]	M.T.Smith
RL501	F^-, *thr-1, leu-6, thi-1, supE44, lacY1, tonA2,* λ^-, *cysJ,* [pRL2 amp^r $cysJ^+$]	M.T.Smith
RL502	F^-, *thr-1, leu-6, thi-1, supE44, lacY1, tonA2, cysJ,* [pRL3 amp^r $cysJ^+$]	M.T.Smith
RL503	F^-, Δ(*cysJIH*), *pro, leu-6 thi-1, lacY,* str^r, r_k^- m_k^- , [pRL3 amp^r $cysJ^+$]	Constructed during this project

TABLE IV: (Cont'd)

Strain.	Genotype.	Source.
RR1	hsdR$^-$, hsdM$^-$, *supE44*, λ^-, *leuB6*, *proA2*, *thi-1*, *trp55*, *ara-14*, *lacY1*, *galK2*, *rpsL20*, *xyl5*	G.Zurawski

NOTE:

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

§ Strain RL434 is derived from *E. coli* strain RR1, 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; λ^s, lambda sensitive; Δ, deletion of area in parenthesis; d*cysJIHD*, defective *cysJIHD* 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^{0}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^{0}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^{0}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^{0}C until good cell density was attained. An HFT (high frequency of transduction) lysate of RL155 containing the phages λ *cI857Sam7* and λd*cysJIHD* 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^{0}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 *Eco* RI and *Hin* dIII and agarose gel electrophoresis followed the procedure outlined

by Smith, (1981) with the appropriate incubation and dilution buffers as specified by Boehringer Mannheim and described by Smith, (1981).

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*.

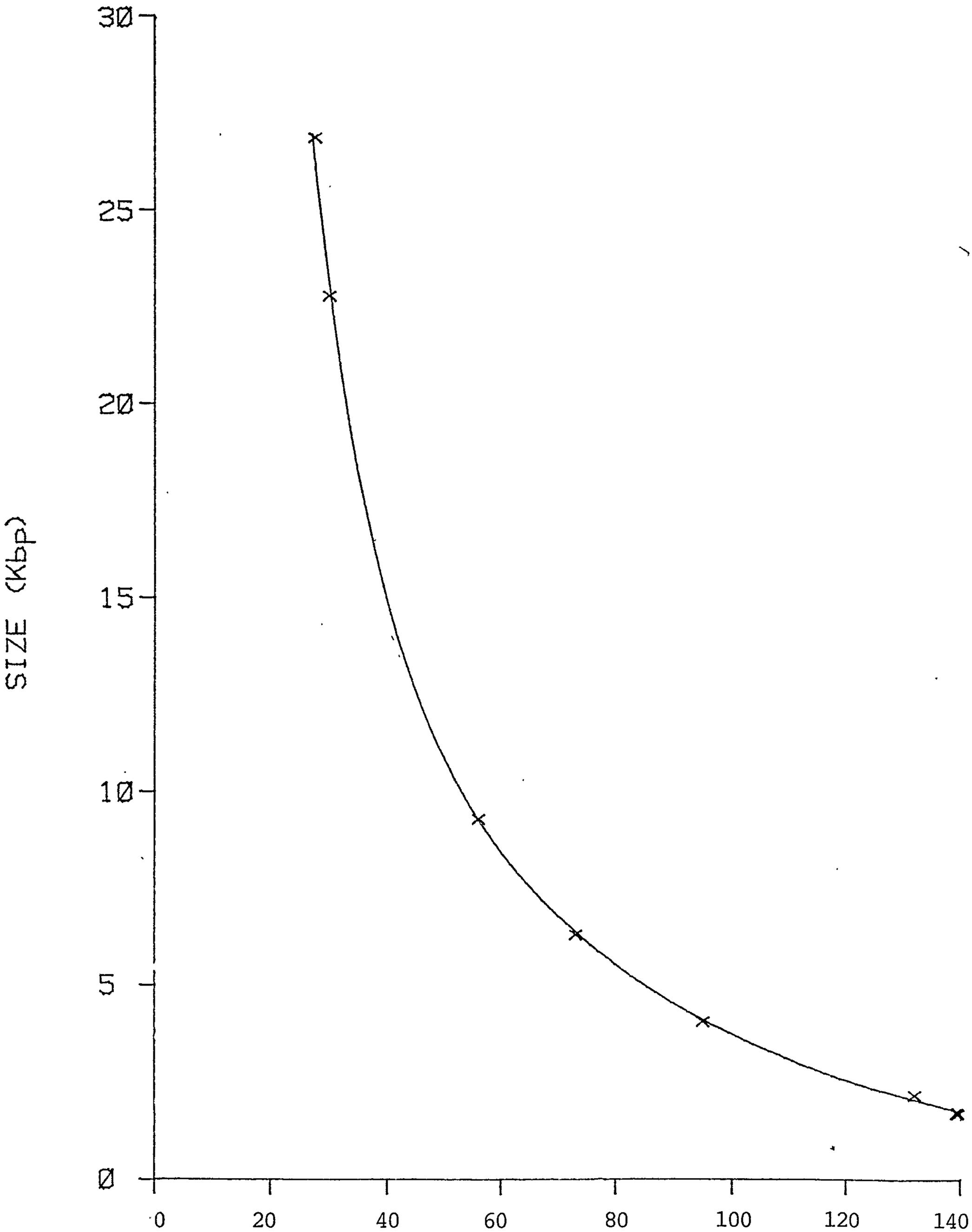
Cells were disrupted using a Branson Model B-12 sonifier fitted with a microtip. Cell debris was removed by centrifugation at 28,000 x *g* for 40 min. and the supernatants (crude extracts) were used for enzyme and protein assays. In large scale work, for protein purification, disruption of bacteria and 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 *Hin*dIII.


30
25
20
15
10
5
0
SIZE (Kbp)
0
20
40
60
80
100
120
140
MOBILITY (mm)

2.5.3 *NADPH-CYTOCHROME C REDUCTASE ASSAY.*

The reduction of cytochrome *c* was followed spectrophotometrically at 20^{0}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^{0}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^{0}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_2SO_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_2SO_3 in a control run on each sample.

Specific activity is expressed as nmoles 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_2CO_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 regeneration were made up from recrystallized urea after removal of the gross excess of cyanate ions by anion exchange chromatography 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 bifunctional reagent dimethylsuberimidate 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 4^0C. The initiation of the reaction was by the addition of solid dimethylsuberimidate 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 spectrophotometer 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 potassium phosphate buffer, pH 7.7, 1 mM EDTA and 10^{-5}M 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

*Sodium Dodecyl Sulphate

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 ran at 4^0C with FAD (10^{-5} M) throughout the gel and in the buffer compartments. Non-denaturing agarose (0.7%) gels were ran 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 4^0C 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 4^0C 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^{0}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^{0}C and then at 20^0 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 $\log_{10}$ 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^{0}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 refering 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 restriction endonuclease analysis; Fig. 8 shows the digestion of pRL3 with the enzymes *Eco*RI and *Hin*dIII. 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. Furthermore by use of the strain RL421 as a recipient in transformation 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 *cysJIH* deletion strain, RL434. Strain RL434 was thus rendered Amp^r but remained Cys^- due to the chromosomal absence of the *cysI* and *cysH* 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

Lane	DNA	Conditions
1	λ	*EcoRI* digestion, complete; *ends cohered.
§2	pRL3	*EcoRI* digestion.
3	λ	As in 1
§4	pRL3	As in 2
5	λ	As in 1

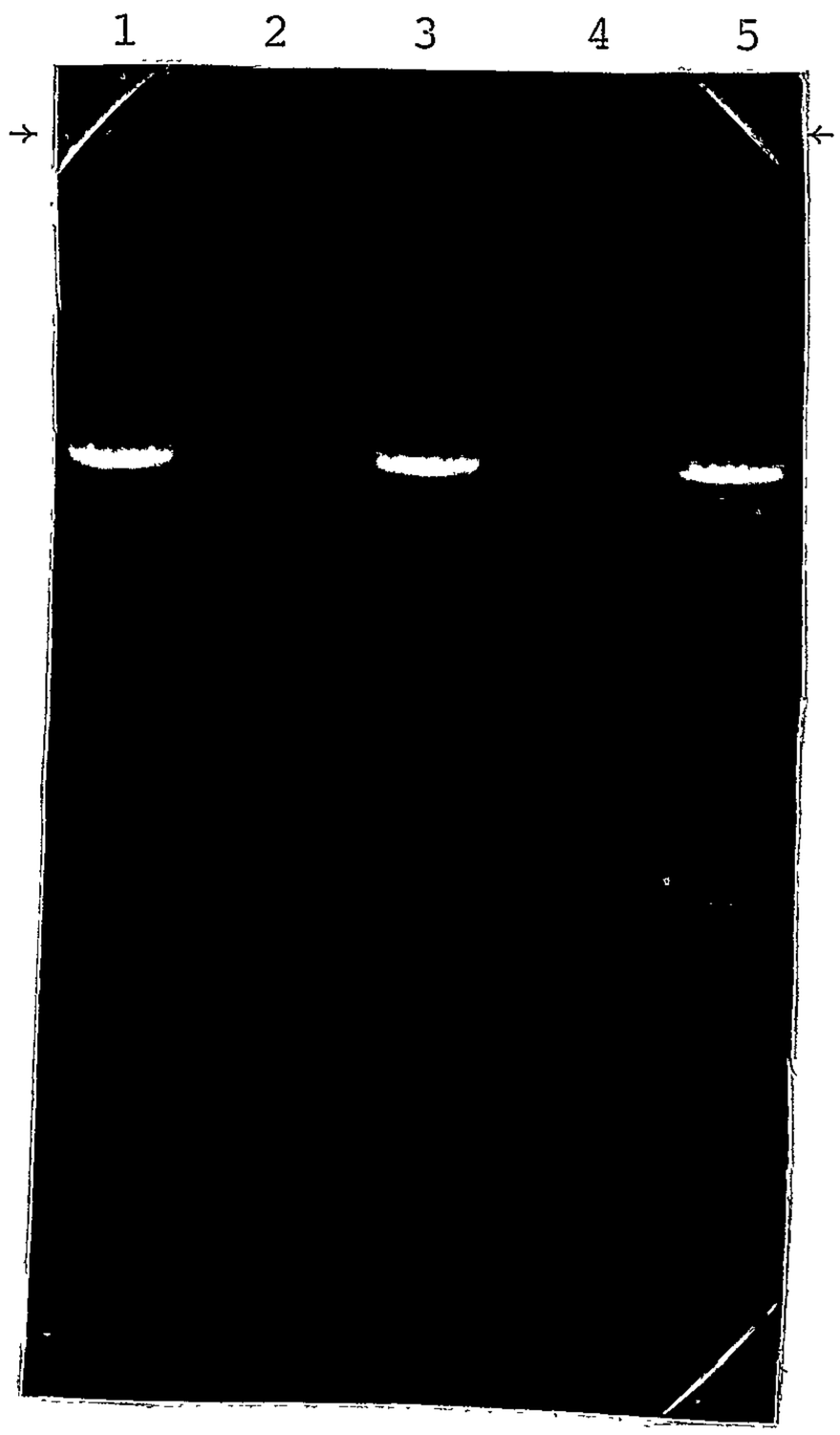
Gel B

Lane	DNA	Conditions
§1	pRL3	*Hin*dIII digestion.
2	λ	*Hin*dIII digestion, complete; *ends cohered.
§3	pRL3	As in 1
4	λ	As in 2
§5	pRL3	As in 1

*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.

GEL A:



GEL B:

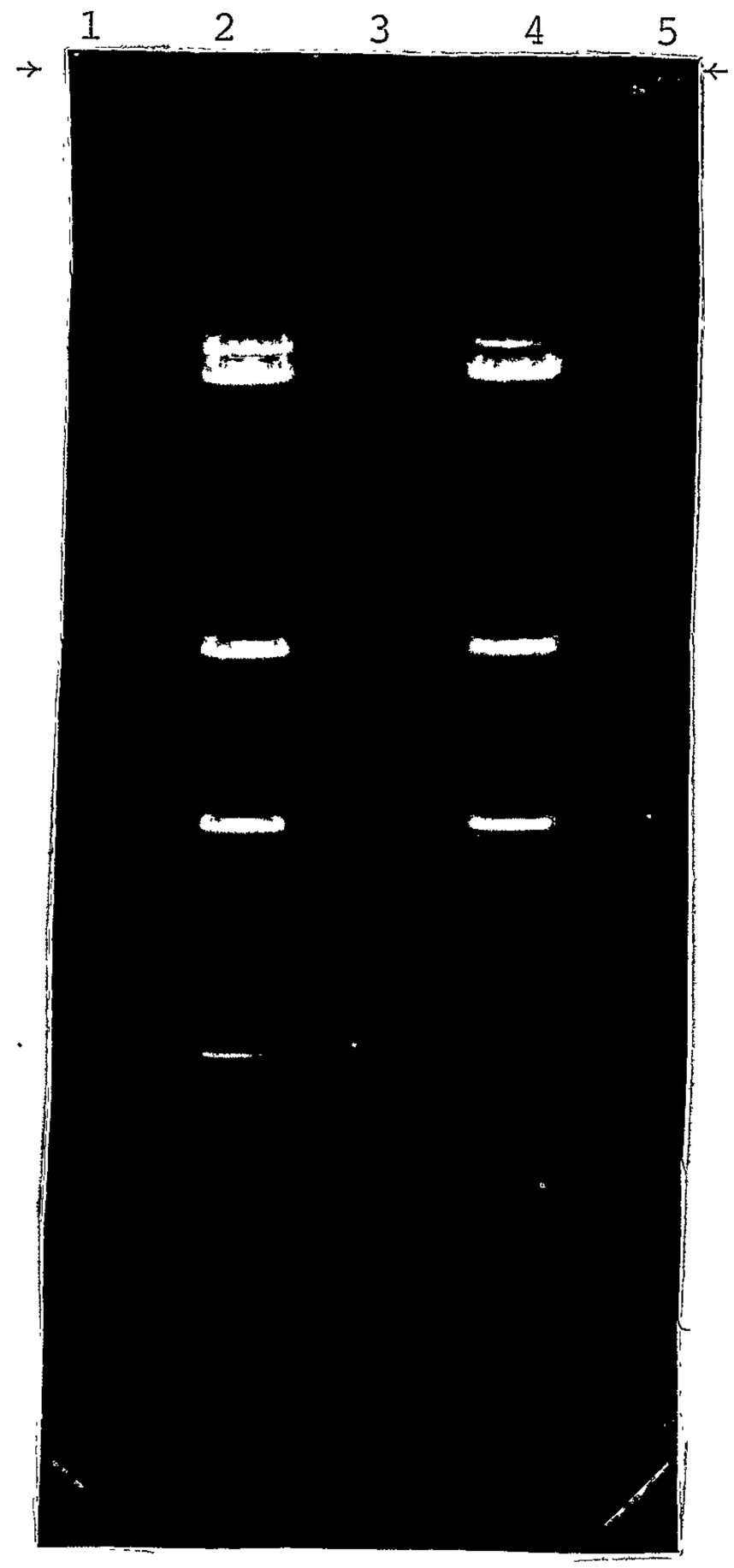


FIGURE 9

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.

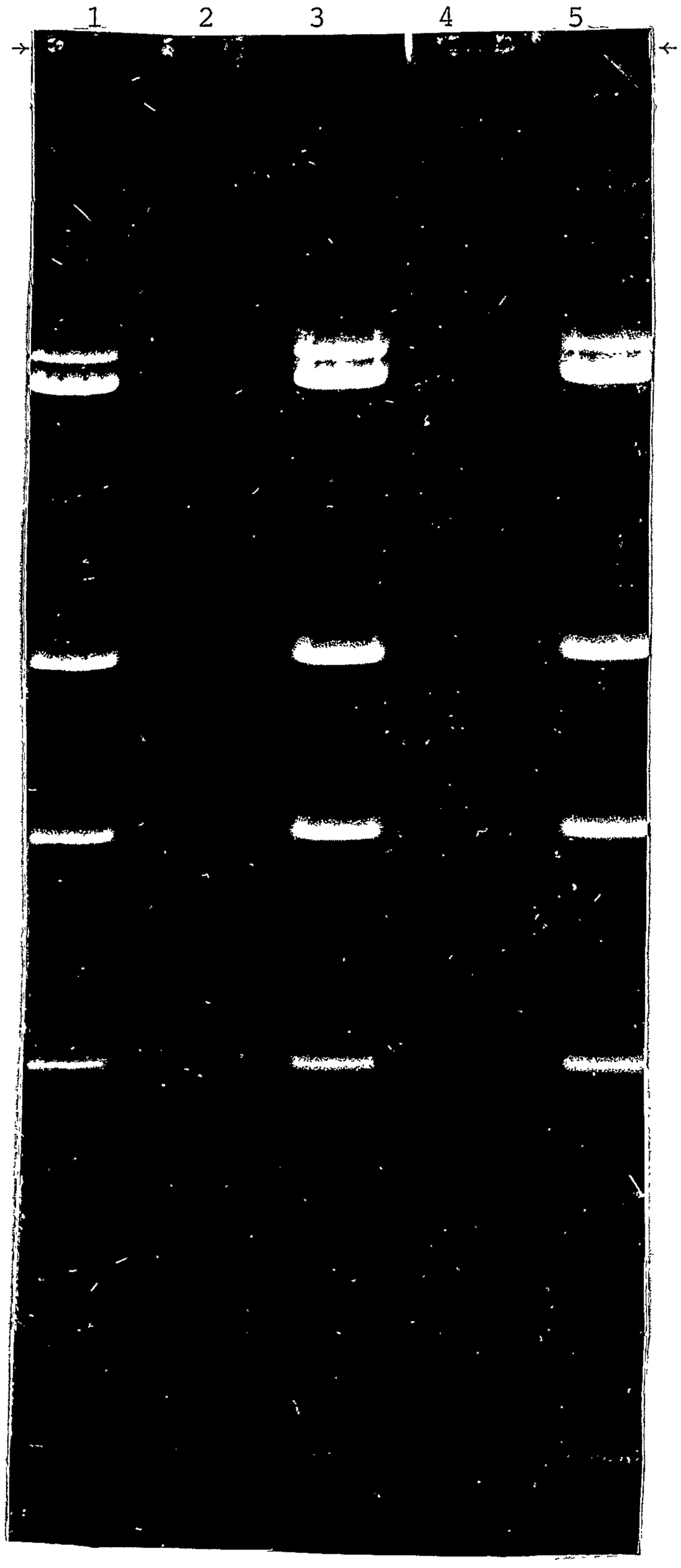
The size of pRL3 was calculated to be 9.54 Kbp from the *Hin*dIII restriction data presented in this Figure, and from the DNA fragment-mobility relationship given in Fig. 7.

LEGEND

Lane	DNA	Conditions
1	λ	*Hin*dIII digestion, complete;§ends cohered.
*2	pRL3	*Hin*dIII digestion.
3	λ	As in 1
*4	pRL3	As in 2
5	λ	As in 1

*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.


1
2
3
4
5

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

TABLE V: The effect of growth conditions on NADPH-cytochrome *c* reductase levels in extracts of a pRL3 plasmid bearing strain *versus* a wild-type *E. coli* strain.

All cell extracts were prepared by sonification.

Strain.	*Growth Conditions.	§Specific Activity.	Extract protein concn. (mg/ml).	Ratio of Specific Activity to wild-type.
WILD-TYPE:				
KB9163	DJ	93.6	14.8	(1.0)
PLASMID BEARING:				
RL503	DJ	3170	8.4	34.0
RL503	Cys	90.0	9.3	0.96
RL503	DJ,CAP	3590	1.2	38.0
RL503	Cys,CAP	9.5	7.8	0.10

*Abbreviations are as follows: DJ, growth in L-djenkolic acid for *cys* gene DNA derepression; Cys, growth in L-cysteine for *cys* gene DNA repression; CAP, chloramphenicol pulse treatment during early exponential culture growth phase for plasmid DNA amplification.

§The specific activity of NADPH-cytochrome *c* reductase is expressed as nmoles of NADPH oxidized per min. per mg of protein present in the extract.

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 *cysJI* 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^{0}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^{0}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^{0}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^{0}C) with the pH adjusted to 7.7 with NH_4OH, 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 resuspended 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 resuspended 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

TABLE VI: Ammonium sulphate fractionation of strain RL503 crude extracts.

Fraction.	Vol. (ml).	Enzyme concn. (units/ml x10³)	Total enzyme (units x10³)	Yield of enzyme(%).	Protein concn. (mg/ml).	¶Specific Activity.	Purification factor.
*A	20.0	108.1	216.3	(100)	16	676	(1.0)
§B1	4.0	3.208	12.8	5.9	5.8	553	<1
§B2	7.4	160.9	119.0	55	11.3	1424	2.1
§B3	5.7	0.802	4.572	2.1	11.6	69	<1
§B4	30.0	0.008	0.241	0.11	0.83	9.7	<1

*Fraction "A" represents the crude extract.

§Fractions "B1" to "B4" represent various ammonium sulphate treated samples derived from the crude extract (see text, pp.30 and 31).

¶The specific activity of the enzyme is expressed as units per mg of protein present in the extract; one unit of enzyme catalyzing the oxidation of 1 nmol of NADPH per min.

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 matrex (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.

TABLE VII: The pattern of enzyme fractionation with calcium phosphate gel from fraction B2. See pp. 32 and 33 for details.

Fraction.	Vol. (ml).	Enzyme concn. (units/ml x 10^3)	Total enzyme (units x 10^3)	Yield of enzyme (%).	Protein concn. (mg/ml).	¶Specific Activity.	Purification factor.
*B2	7.4	16.09	119.0	(100)	11.3	1424	(1.0)
§C1	5.8	10.90	63.2	51.7	1.6	6812	4.78
§C2	5.8	1.069	6.149	5.17	0.67	1596	1.21
§C3	22	0.526	11.6	9.72	1.6	329	<1.0
§C4	2.9	1.604	4.620	3.88	1.3	1337	<1.0
§C5	2.9	0.0	0.0	0.0	0.0	0.0	0
§C6	28	0.0	0.0	0.0	1.8	0.0	0
§C7	13	0.134	1.737	1.46	2.0	67	<1.0
§C8	13	0.0	0.0	0.0	0.2	0.0	0
§C9	13	0.0	0.0	0.0	0.13	0.0	0
§C10	54	0.0	0.0	0.0	0.20	0.0	0
§C11	4.3	0.0	0.0	0.0	0.97	0.0	0
§C12	4.3	0.0	0.0	0.0	0.13	0.0	0
§C13	63	0.0	0.0	0.0	0.40	0.0	0

* Fraction "B2" represents the second ammonium sulphate fraction.

§ Fractions "C1" and "C2" represent the first calcium phosphate gel addition eluates, "C3" being the remaining supernatant; "C4" and "C5" represent the second gel eluates, "C6" being the remaining supernatant; "C7", "C8" and "C9" represent the third gel eluates, "C10" being the remaining supernatant; "C11" and "C12" represent the fourth gel eluates, "C13" represents the remaining supernatant.

¶ The specific activity of the enzyme is expressed as units per mg of protein present in the extract; one unit of enzyme catalyzing the oxidation of 1 nmol of NADPH per min.

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

TABLE VIII: Results from a trial purification of a calcium phosphate gel fraction with the dye-ligand media.

Column.	Fraction.	Vol. (ml).	Enzyme concn. (units/ml).	Total enzyme (units).	Yield of enzyme (%).	Protein concn. (mg/ml).	¶Specific Activity. ($x10^3$)	Purification factor.
	*C1	0.5	3476	1738	(100)	0.83	4.173	(1.0)
CONTROL	§D1	1.0	0.0	0.0	0.0	0.08	0.0	0
	§D2	2.0	1015	2031	117	0.20	5.077	1.22
	§D3	2.0	0.0	0.0	0.0	0.03	0.0	0
BLUE-A	D1	1.0	0.0	0.0	0.0	0.03	0.0	0
	D2	2.0	830	1660	96	0.12	6.915	1.66
	D3	2.0	44	88	5.1	0.08	0.547	<1.0
BLUE-B	D1	1.0	0.0	0.0	0.0	0.00	0.0	0
	D2	2.0	2246	4492	258	0.03	74.9	17.9
	D3	2.0	24	48	2.8	0.08	0.29	<1.0
GREEN-A	D1	1.0	0.0	0.0	0.0	0.03	0.0	0
	D2	2.0	69	138	8.0	0.08	0.856	<1.0
	D3	2.0	695	1390	80	0.13	5.348	1.28
ORANGE-A	D1	1.0	0.0	0.0	0.0	0.04	0.0	0
	D2	2.0	553	1106	64	0.06	9.219	2.21
	D3	2.0	0.0	0.0	0.0	0.25	0.0	0
RED-A	D1	1.0	0.0	0.0	0.0	0.12	0.0	0
	D2	2.0	177	354	20	0.11	1.608	<1.0
	D3	2.0	681	1362	78	0.11	6.189	1.48

* Fraction "C1" presented here is the first calcium phosphate gel eluate from another small scale enzyme preparation whose crude extract, "A", had a specific activity of 2822 units per mg of protein present. The calcium phosphate gel eluate was divided into 0.5 ml samples which were separately applied to each of the dye-columns.

§ Fractions "D1", "D2" and "D3" represent certain fractions collected from the columns; see section 3.2.3. pp.33 and 34, for further details.

¶ The specific activity of the enzyme is expressed as units per mg of protein present in the extract; one unit of enzyme catalyzing the oxidation of 1 nmol of NADPH per min.

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 (C1) 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.

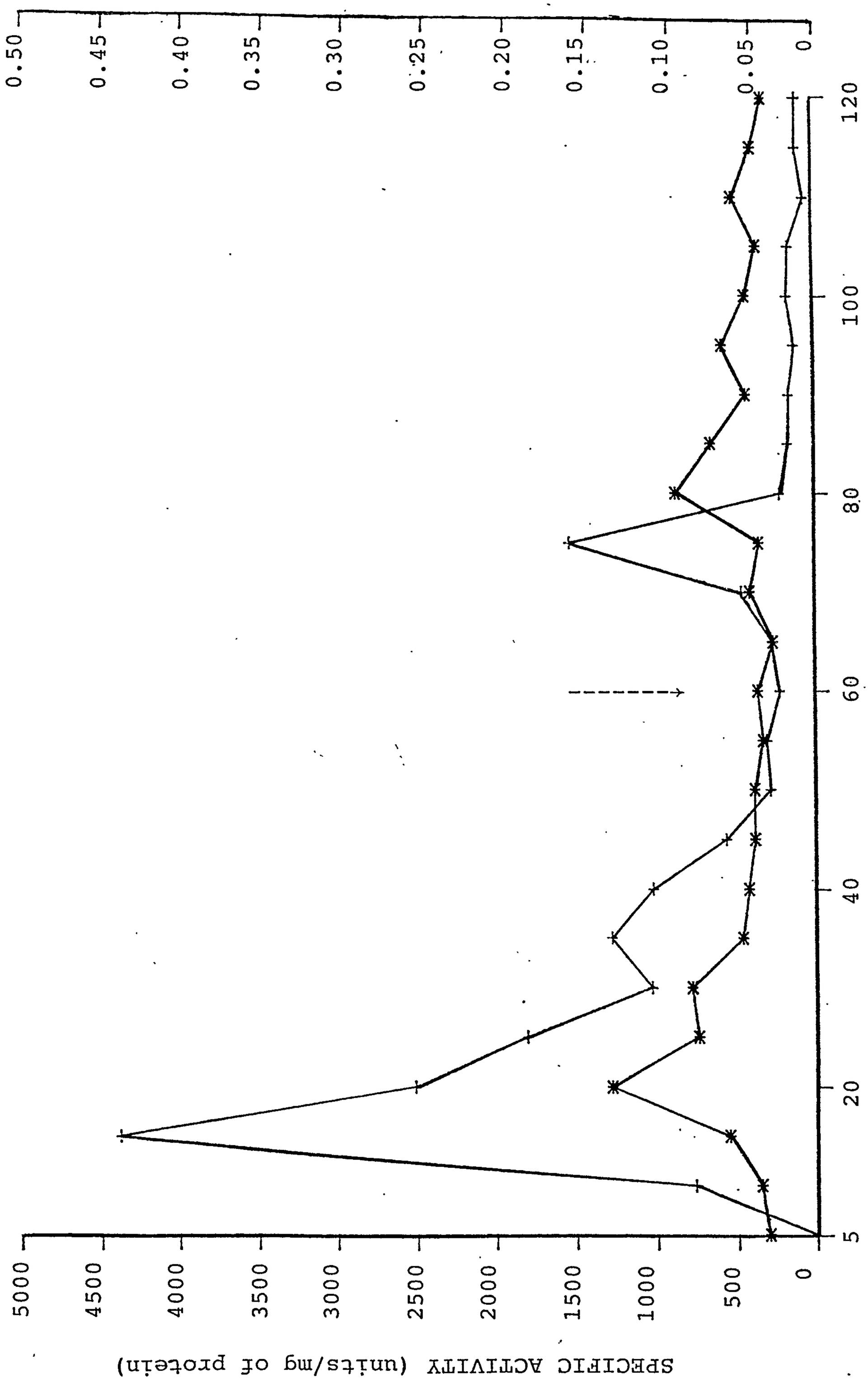

PROTEIN CONCN. (mg/ml)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
120
100
80
60
40
20
5
FRACTION NUMBER
SPECIFIC ACTIVITY (units/mg of protein)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0

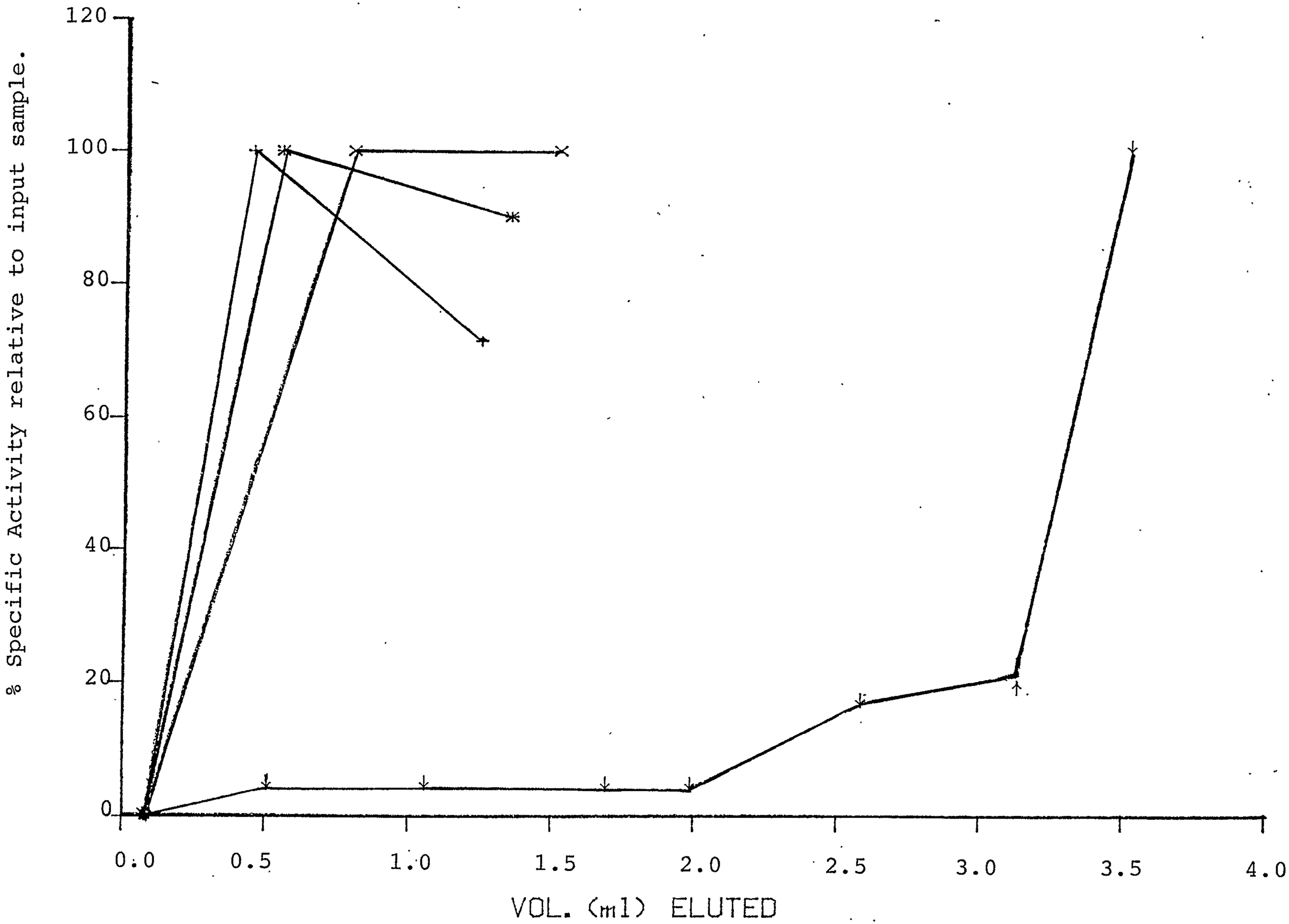
FIGURE 11.

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[§] 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).

[§] One unit of enzyme catalyzes the oxidation of 1 nmol of NADPH per min.


% Specific Activity relative to input sample.
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
VOL. (ml) ELUTED

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-matrex.

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.

§Column.	Relative specific binding coefficient.
Control	1.00
Blue-A	2.99
Blue-B	1.06
Green-A	2.71
Orange-A	2.86
Red-A	3.71

---

* 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.

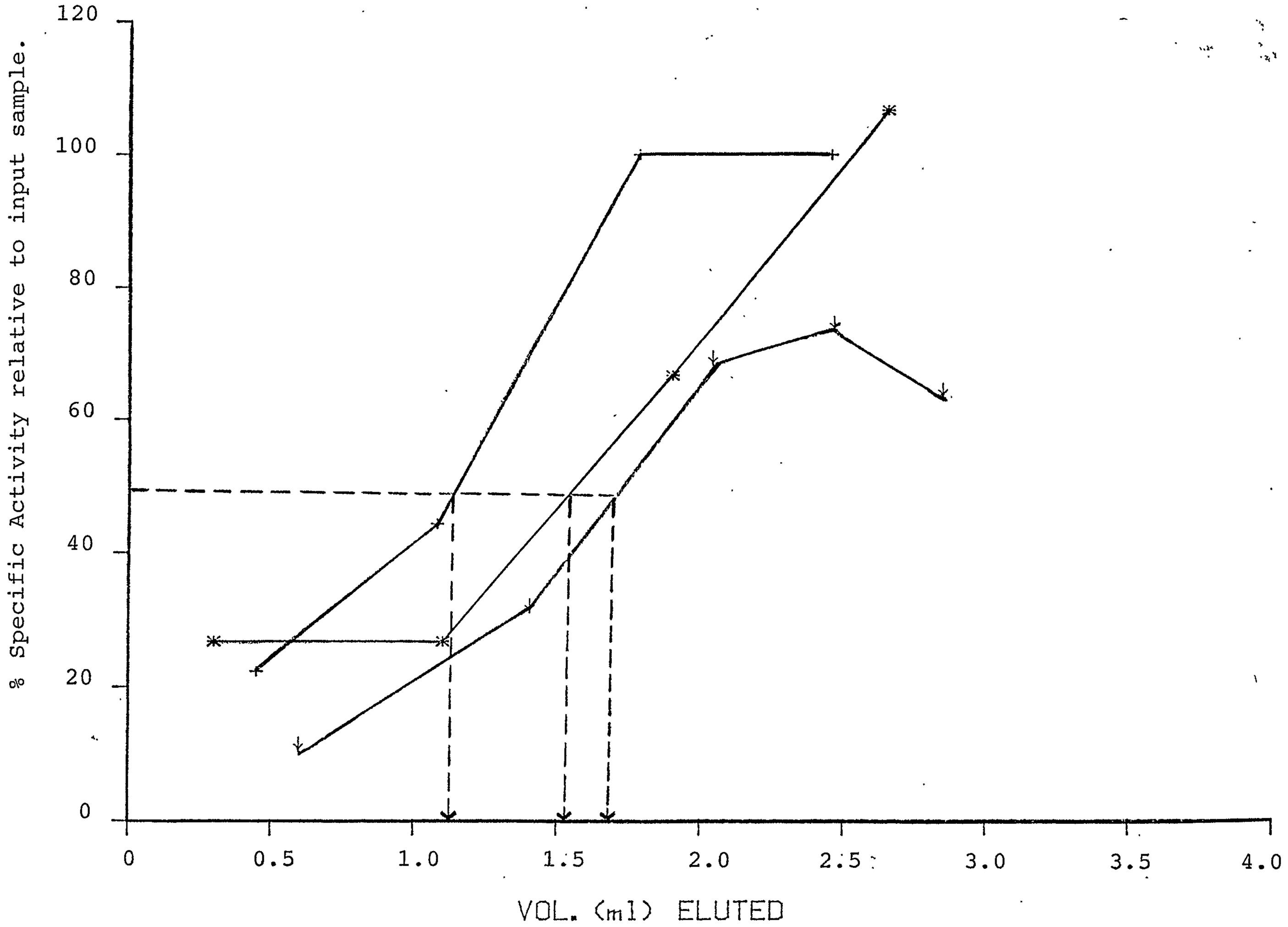
* Enzyme sample loaded onto a Green-A column.

↓ 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.
120
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
VOL. (ml) ELUTED

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 4^0), 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

TABLE X: The results of the first large scale enzyme preparation up to the affinity chromatography step are presented below.

Fraction.	Vol. (ml).	Enzyme concn. (units/ml x10³)	Total enzyme (units x10³)	Yield of enzyme (%).	Protein concn. (mg/ml).	¶Specific Activity. (x10³)	Purification factor.
*A	234	40.1	9385	(100)	19.7	2.033	(1.0)
§B2	87	42.8	3709	40	13.6	3.146	1.55
#C1	40	1.074	42.95	0.45	0.51	2.105	1.04
§C1	20	9.091	183.6	1.96	0.65	14.0	6.89
¶C3	110	18.7	2059	22	2.62	7.144	3.51

* Fraction "A" represents the crude extract.

§ Fraction "B2" represents the ammonium sulphate fraction obtained in the range of 30% to 46% ammonium sulphate saturation.

Fraction "#C1" represents the sample obtained by resuspending the calcium phosphate gel pellet formed on the addition of the first amount of gel to fraction "B2". The resuspension was performed in 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, in order to wash the pellet prior to elution.

§ Fraction "§C1" represents the sample obtained after elution of the pellet formed by the addition of the first amount of calcium phosphate gel to fraction "B2".

¶ Fraction "C3" represents the supernatant sample remaining after the first calcium phosphate gel addition.

¶ Specific activity is expressed as in Table VIII (p.34(a)).

TABLE XI: Fractionation of the supernatant sample from the first calcium phosphate gel addition. The supernatant to be fractionated had been firstly dialyzed overnight into 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, before the addition of any calcium phosphate gel.

Fraction.	Vol. (ml).	Enzyme concn. (units/ml x 10^3)	Total enzyme (units x 10^3)	Yield of enzyme (%).	Protein concn. (mg/ml).	¶Specific Activity. (x10^3)	Purification factor.
*C3	110.0	18.7	2059	(100)	2.62	7.144	(1.0)
§C1	3.0	36.1	109.0	5.30	3.18	11.4	1.60
C2	3.0	14.7	44.41	2.16	2.07	7.104	<1.0
C4	1.5	42.8	64.60	3.14	2.91	14.7	2.06
C5	1.5	9.224	13.93	0.68	1.08	8.541	1.20
C7	6.8	34.8	236.4	11.5	2.75	12.6	1.76
C8	6.8	2.941	20.00	0.97	0.33	9.022	1.26
C9	6.8	0.202	1.372	0.07	0.33	0.604	<1.0
C11	2.3	13.6	30.95	1.51	2.16	6.313	<1.0
C12	2.3	3.476	7.890	0.38	0.75	4.659	<1.0
C13	108	0.667	72.01	3.52	2.19	0.305	<1.0

* Fraction "C3" represents the supernatant from the first calcium phosphate gel addition which was re-fractionated by the addition of further amounts of gel.

§ Fractions from the re-fractionation of sample "C3" are presented. Note that the supernatant fractions from each gel addition have been omitted for clarity. The designation of fractions is as described in a footnote (§) in Table VII (p.33(a)).

¶ The specific activity of the enzyme is expressed as units per mg of protein present in the extract; one unit of enzyme catalyzing the oxidation of 1 nmol of NADPH per min.

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

TABLE XII: Ammonium sulphate treatment of the fraction obtained by the first addition of calcium phosphate gel to a semi-purified enzyme preparation.

Fraction.	Vol. (ml).	Enzyme concn. (units/ml)	Total enzyme (units x10³)	Yield of enzyme (%).	Protein concn. (mg/ml).	¶Specific Activity. (x 10³)	Purification factor.
*C1	20	9091	183.6	(100)	0.65	14.0	(1.0)
§I	1.3	6715	8.730	4.8	8.1	0.829	0.059
§II	35.0	9.16	0.321	0.17	0.014	0.654	0.047

* Fraction "C1" represents the sample obtained after elution of the pellet formed by the addition of the first amount of calcium phosphate gel to fraction "B2" of the initial ammonium sulphate treatment of the crude extract.

§ Fraction "I" represents the resuspended ammonium sulphate pellet of sample "C1", the pellet of which was formed by the addition of ammonium sulphate to 50% saturation to fraction "C1".

§ Fraction "II" represents the remaining supernatant fraction from the 50% ammonium sulphate treatment of fraction "C1", obtained after removal of the pellet (fraction "I").

¶ The specific activity is expressed as in Table VIII (p. 34(a)).

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.

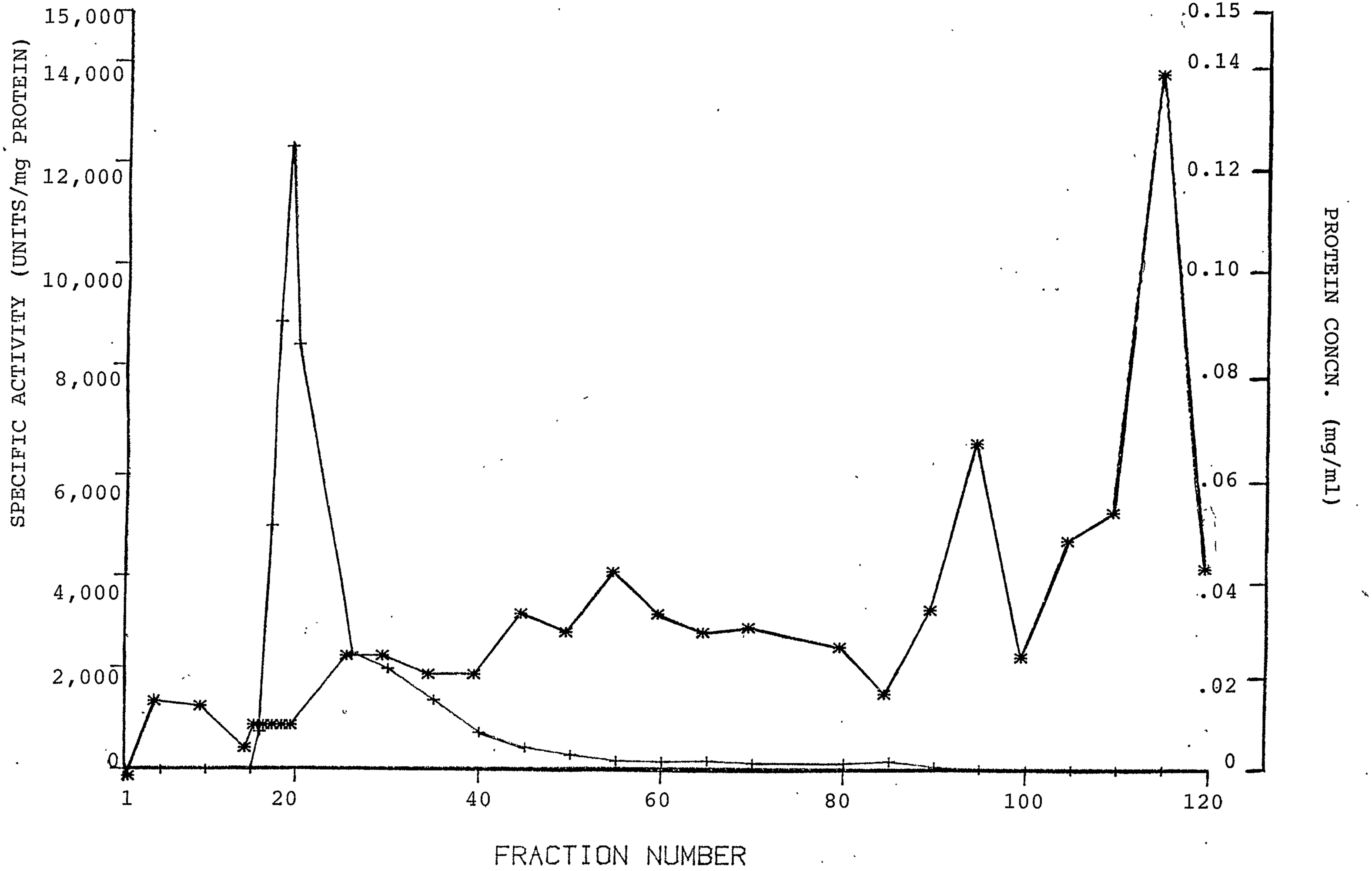
KEY:

\+ 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.


SPECIFIC ACTIVITY (UNITS/mg PROTEIN)
15,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
PROTEIN CONCN. (mg/mL)
0.15
0.14
0.12
0.10
.08
.06
.04
.02
0
1
20
40
60
80
100
120
FRACTION NUMBER

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.

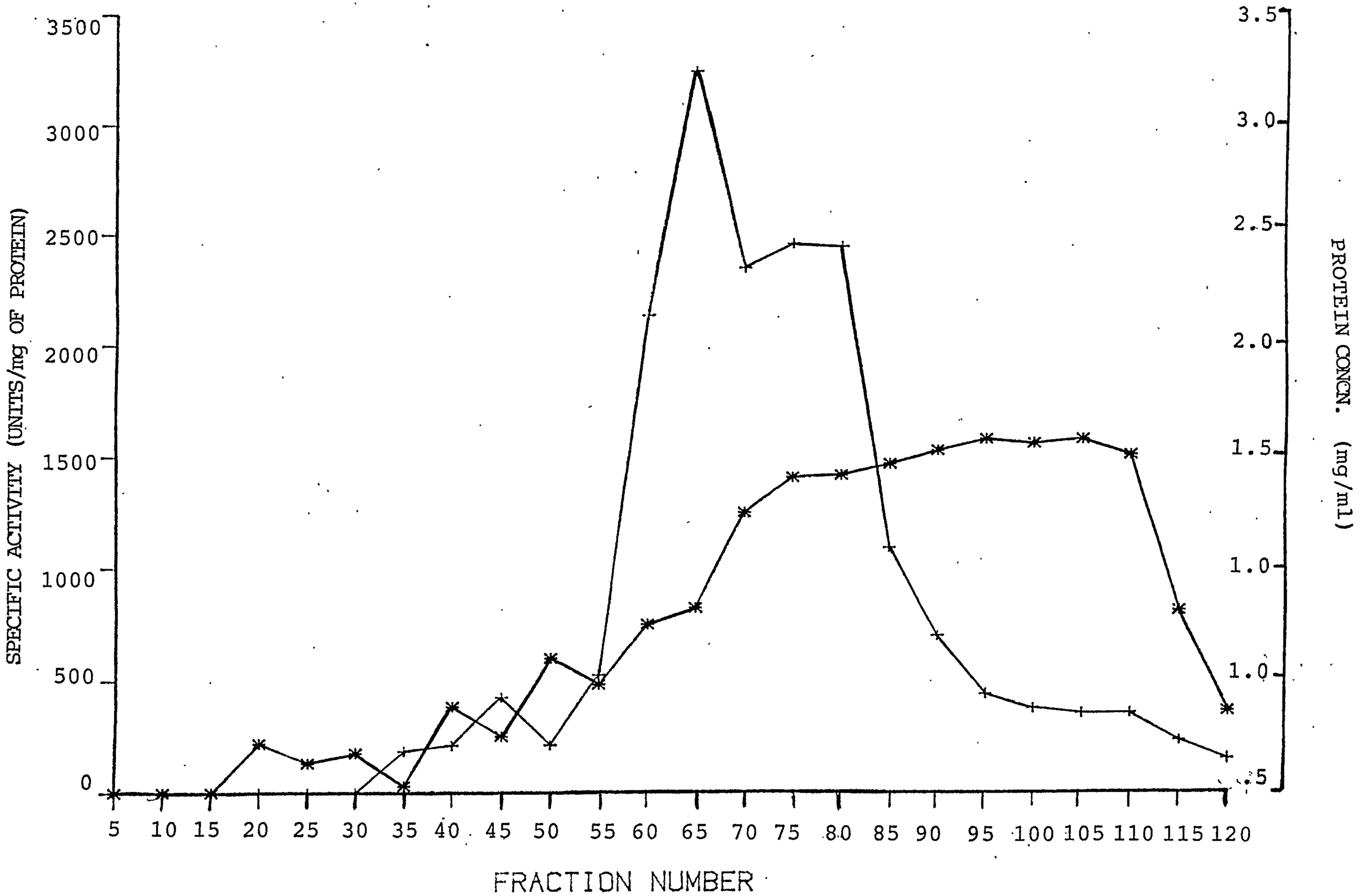

SPECIFIC ACTIVITY (UNITS/mg OF PROTEIN)
3500
3000
2500
2000
1500
1000
500
0
PROTEIN CONCN. (mg/ml)
3.5
3.0
2.5
2.0
1.5
1.0
1.0
.5
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
FRACTION NUMBER

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.

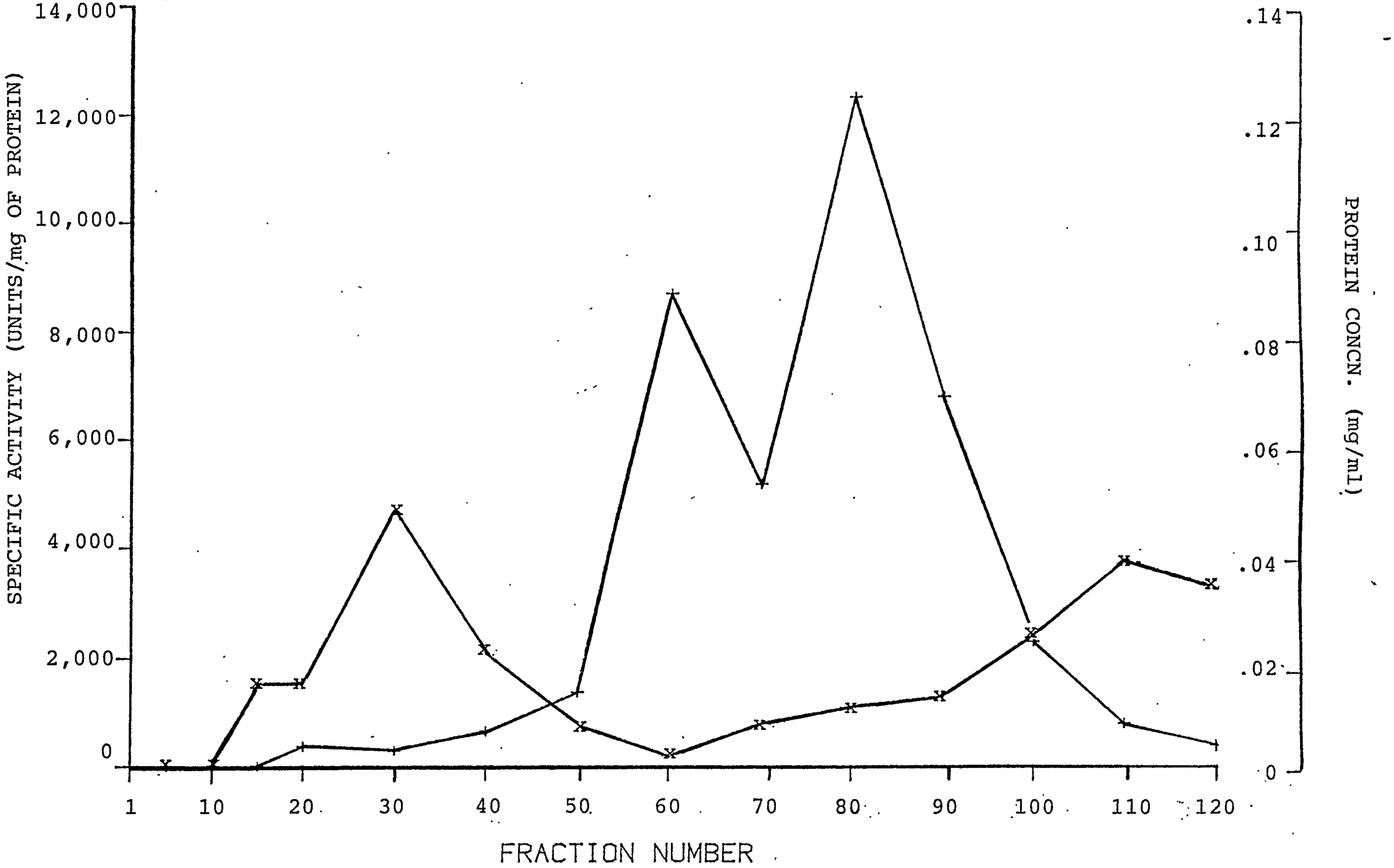
KEY:

+ 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.


SPECIFIC ACTIVITY (UNITS/mg OF PROTEIN)
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
PROTEIN CONCN. (mg/ml)
.14
.12
.10
.08
.06
.04
.02
0
1
10
20
30
40
50
60
70
80
90
100
110
120
FRACTION NUMBER

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.

x Hydroxylapatite elution profile.

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

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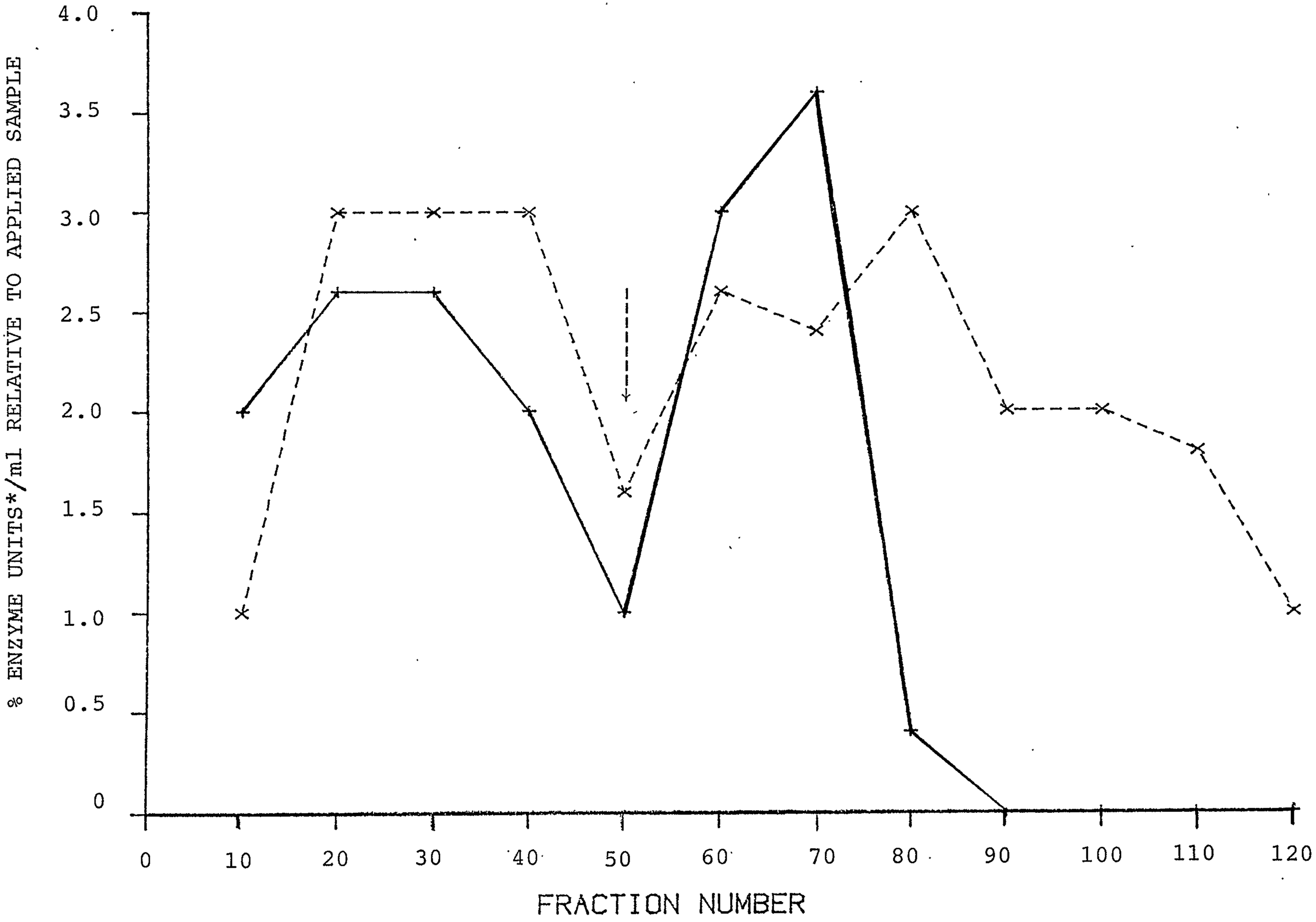
* One unit of enzyme activity is defined as that amount of NADPH-cytochrome *c* reductase that catalyzes the oxidation of 1 nmol of NADPH per min.


% ENZYME UNITS*/ml RELATIVE TO APPLIED SAMPLE
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
70
80
90
100
110
120
FRACTION NUMBER

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 extacts 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

TABLE XIII: Results of the second large scale enzyme preparation up to the dye-ligand affinity chromatography step.

Fraction.	Vol. (ml).	Enzyme concn. (units/ml x10³)	Total enzyme (units x10³)	Yield of enzyme (%).	Protein concn. (mg/ml).	¶Specific Activity.	Purification factor.
*A	600	5.350	3210	(100)	10.0	535	(1.0)
§B2	222	6.680	1483	46	5.71	1170	2.19
C1(I)	24	37.40	898.4	28	4.48	8356	15.6
C1(II)	48	27.70	1332	#42	3.02	9155	17.1
C1(III)	14	69.5	973.3	#30	N.D.	N.D.	N.D
C1(IV)	23	29.4	676.5	#21	N.D.	N.D.	N.D.
C1(V)	80	0.0021	0.168	0.005	N.D.	N.D.	N.D.

* Fraction "A" represents the crude extract.

§ Fraction "B2" represents the ammonium sulphate fraction obtained in the range of 30% to 46% ammonium sulphate saturation.

Fraction "C1(I)" represents the sample obtained through elution of the pellet formed by the first calcium phosphate gel addition to fraction "B2". Fraction "C1(II)" represents sample "C1(I)" pooled with the fractions obtained from the refractionation of the supernatant of the first gel addition to fraction "B2" from the first large scale enzyme preparation. Fraction "C1(III)" represents the sample obtained by the 50% ammonium sulphate treatment of fraction "C1(II)". Fraction "C1(IV)" represents fraction "C1(III)" after dialysis against 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, for 24 hours. Fraction "C1(V)" represents the residual material remaining after ultrafiltration of the supernatant from the 50% ammonium sulphate treatment of fraction "C1(II)".

The yield of enzyme is referenced to crude extract ("A") and is not corrected for the addition of samples from the first large scale enzyme preparation (see Table XV, and p.41).

¶ Specific activity is expressed as in Table XI. N.D., Not Determined.

TABLE XIV: The results from the Blue-B dye-ligand affinity chromatography step of the second large scale enzyme preparation in tabular form. The fractions containing chromatographically purified NADPH-cytochrome *c* reductase (*viz.* fractions 20 to 60) were pooled, dialyzed to remove salt, and lyophilized; the obtained flavoprotein being used for physical analysis.

Fraction.	Vol. (ml).	Enzyme concn. (units/ml x10³)	Total enzyme (units x10³)	Yield of enzyme(%).	Protein concn. (mg/ml).	¶Specific Activity. (x10³)	#Purification factor.
*C1	23	29.4	676.5	(100)	N.D.	N.D.	N.D.
+1 to 5	5.5	0.0	0.0	0.0	0.03	0.0	0.0
6 to 10	5.5	0.0005	0.0029	0.0004	0.02	0.027	<1.0
11 to 15	5.5	0.0027	0.0147	0.0022	0.05	0.055	<1.0
16 to 20	5.5	0.054	0.294	0.044	0.05	0.992	1.9
21 to 25	5.5	0.321	1.765	0.26	0.37	0.873	1.6
26 to 30	5.5	2.19	12.10	1.8	0.85	2.570	4.8
31 to 35	5.5	10.7	58.80	8.7	1.20	8.868	16.6
36 to 40	5.5	32.1	176.5	26.1	1.14	28.1	52.5
41 to 45	5.5	21.4	117.6	17.4	1.30	16.5	30.8
46 to 50	5.5	12.0	66.2	9.8	1.16	10.4	19.4
51 to 55	5.5	3.208	17.6	2.6	0.97	3.321	6.2
56 to 60	5.5	2.674	14.7	2.2	0.97	2.768	5.2
61 to 65	5.5	2.406	13.2	2.0	0.53	4.549	8.5
66 to 70	5.5	0.802	4.412	0.65	0.41	1.971	3.7
71 to 75	5.5	0.415	2.280	0.34	0.21	2.012	3.8
76 to 80	5.5	0.348	1.912	0.28	0.12	2.826	5.3
81 to 85	5.5	0.334	1.838	0.27	0.09	3.798	7.1
86 to 90	5.5	0.241	1.324	0.20	0.08	3.069	5.7

* Fraction "C1" corresponds to fraction "C1(IV)" from Table XIII, which was the sample obtained after the 50% ammonium sulphate treatment and dialysis of the fraction from the elution of the first calcium phosphate gel pellet formed by the addition of the gel to fraction "B2".

+ Each of the fractions eluted from the Blue-B column was pooled in lots of five. The assay data is calculated on the basis of the fractions which had been pooled.

The purification factor quoted here is referenced to the crude extract fraction ("A") from Table XIII. ¶ The specific activity is expressed as in Table XIII.