FIGURE 17.

The elution profile obtained following the chromatography of an ammonium sulphate fraction on a Blue-B dye-ligand column. The sample chromatographed was from the 50% ammonium sulphate treatment of the eluted fraction from the first calcium phosphate gel addition to fraction "B2", and was applied to a 15 x 2.5 cm diameter Blue-B column. Sample volume was 23 ml with a total number of units* of enzyme of 676.5×10^3. After sample application to the column, approximately 10 ml of 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, was run into the gel bed over the sample. Once the gel bed had run dry, the flow of buffer into the column was turned off for a period of 1 hour. After this period, 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, was allowed to run through the column at a flow rate of approximately 0.9 ml/min. in order to elute the enzyme. The period of equilibration directly after sample application for 1 hour was in order to allow greater resolution of enzyme (which is not bound to the column) from contaminating proteins which may bind only weakly to the dye-matrex, and which could appear in fractions containing the enzyme if they were not exposed to the dye-matrex for periods of time sufficient to enable their interaction with the dye-ligand.

KEY:

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

* Protein concn. (mg/ml).

The fractions between which the eluted enzyme is chromatographically pure are designated by the dashed arrows.

---

*One unit of NADPH-cytochrome *c* reductase catalyzes the oxidation of 1 nmol of NADPH per min.

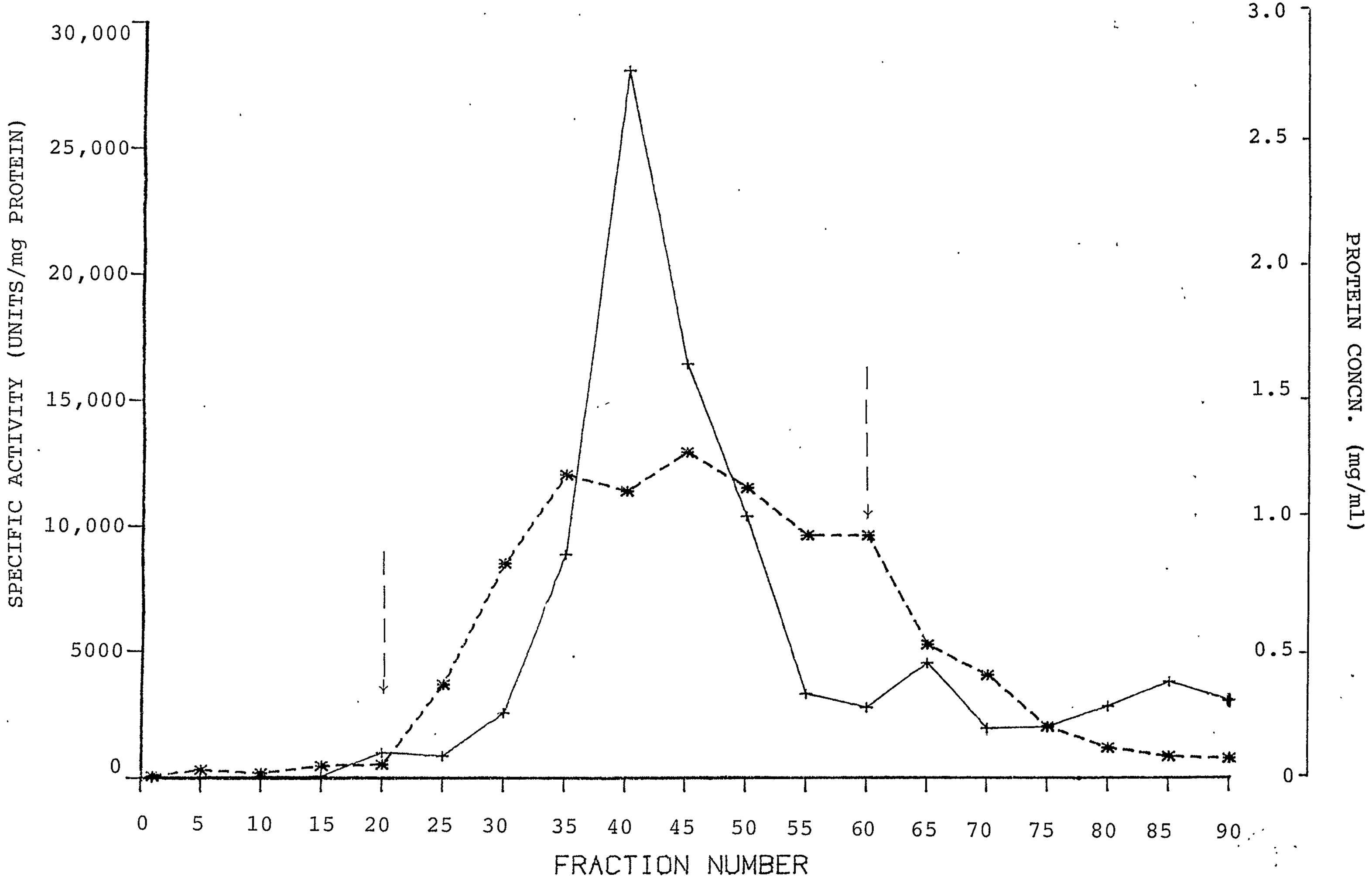

SPECIFIC ACTIVITY (UNITS/mg PROTEIN)
30,000
25,000
20,000
15,000
10,000
5000
0
PROTEIN CONCN. (mg/mL)
3.0
2.5
2.0
1.5
1.0
0.5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
FRACTION NUMBER

FIGURE 18.

The elution profile obtained following the chromatography of an ammonium sulphate fraction on a Blue-B dye-ligand column. The plot represents the same column run as in Fig. 17, but re-plotted in terms of enzyme units* *versus* enzyme specific activity. The sample was from the 50% ammonium sulphate treatment of the eluted fraction from the first calcium phosphate gel addition to fraction "B2", and was applied to a 15 x 2.5 cm diameter Blue-B column and chromatographed as described in Fig. 17. Fractions 20 to 60 were pooled, with a volume of 1.1 ml being recorded per fraction. On the basis of the excellent correlation between total enzyme units and enzyme specific activity, the protein eluting between fractions 20 to 60 was judged to be chromatographically purified NADPH-cytochrome *c* reductase.

KEY:

\+ Total units of enzyme

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

The fractions between which the eluted enzyme is chromatographically pure are designated by the dashed arrows.

---

*One unit of NADPH-cytochrome *c* reductase catalyzes the oxidation of 1 nmol of NADPH per min.

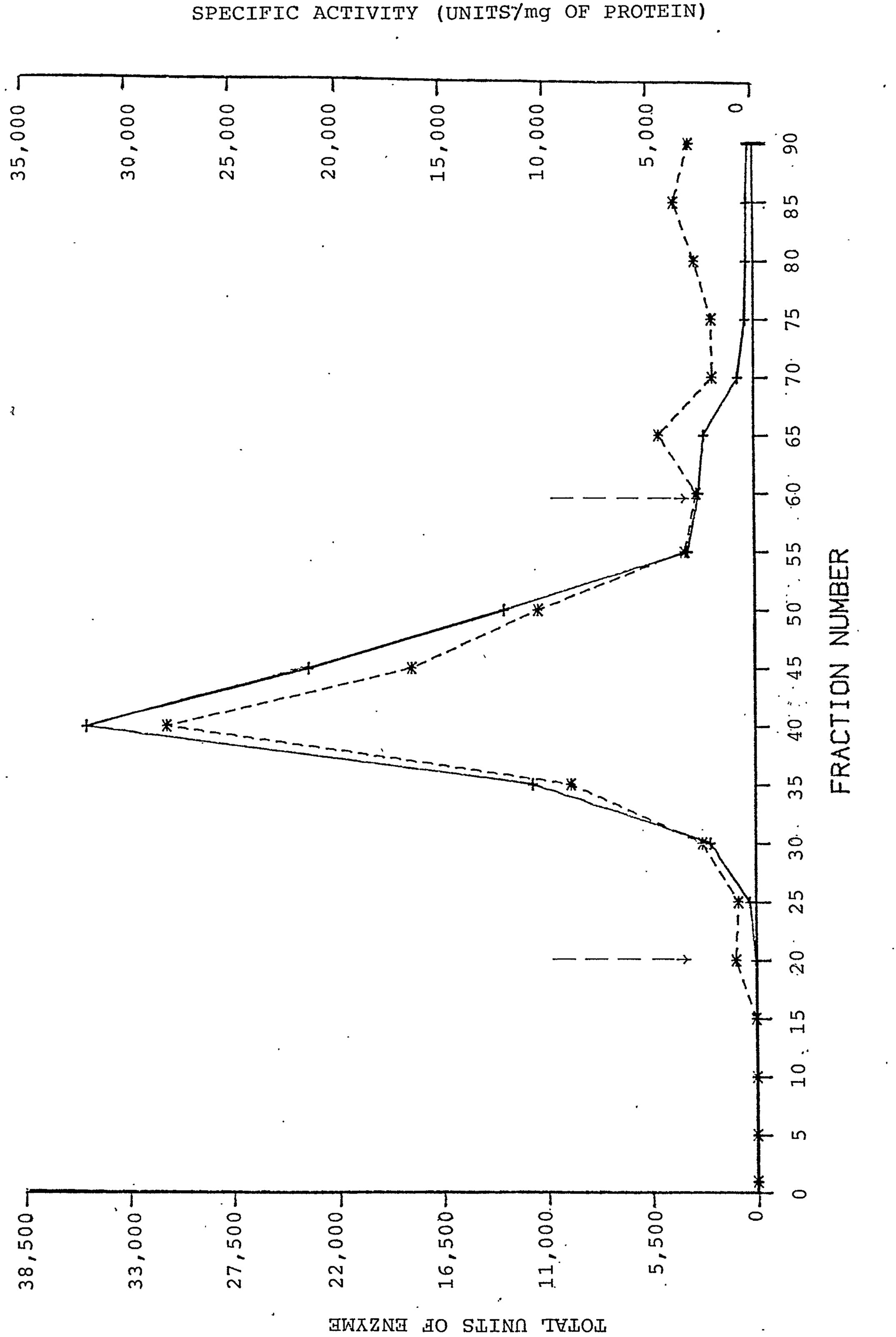

SPECIFIC ACTIVITY (UNITS/mg OF PROTEIN)
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
FRACTION NUMBER
38,500
33,000
27,500
22,000
16,500
11,000
5,500
0
TOTAL UNITS OF ENZYME

TABLE XV: Overall results of the final purification procedure for the second large scale enzyme preparation. The purified enzyme from this preparation was subsequently used in further 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.
*A	600	5.348	3209	(100)	10.0	0.534	(1.0)
§B2	222	6.679	1483	46	6.0	1.169	2.2
§C1	14	69.50	973.2	#30	10.0	6.646	12.4
¶D	44	24.00	106.0	#33	1.2	20.50	38.4

* 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 after the 50% ammonium sulphate treatment of the fraction eluted from the first calcium phosphate gel addition to fraction "B2". This "C1" fraction also represents the pooled samples from the refractionation of fraction "C3" from the first large scale enzyme preparation.

¶ Fraction "D" represents the pooled fractions obtained from the dye-ligand affinity column (*viz.* fractions 20 to 60).

The percentage yield of enzyme quoted here with respect to the crude extract fraction ("A") is based on pooled fractions from this preparation and from the previous large scale enzyme preparation, thus the yield appears higher by 7% (see p.41) than it would be if previous fractions had not been pooled with those from this preparation.

¶ Specific activity is expressed as in Table XI.

into account though, an excellent conservation of total units of enzyme from the crude extract was still able to be achieved. Furthermore, dialysis of the enzyme preparation at high concentrations of protein and enzyme (after elution from the calcium phosphate gel) leads to enzyme denaturation and loss in total units. This observation has been noted earlier in this study, but the reasons for the denaturation occurring on dialysis into low ionic strength phosphate buffers are not completely understood, and further investigation into this phenomenon was not persued.

The pooled fractions from the affinity column were then extensively dialyzed against glass distilled water with FAD omitted in order to make the enzyme preparation as salt free as possible prior to further analysis. During the latter stages of this dialysis, a yellow proteinaceous precipitate was seen to form. Subsequent lyophilization of the dialyzed material yielded approximately 20 mg of yellow powder (around 10 mg of which was still salt material as judged by later analyses). The purified flavoprotein was then stored at -18^{0}C until further needed.

3.2.6 *ELECTROPHORETIC AND SPECTRAL ANALYSES AS CRITERIA FOR ENZYME PURITY.*

In defining the homogeneity of the enzyme preparation and in monitoring the purification process, various electrophoresis procedures were followed (as described in Chapter 2, section 2.5.12). In examining homogeneity, absorption spectra were also performed and served to check for any gross contamination present.

In developing a gel system for giving a good resolution electropherogram of NADPH-cytochrome *c* reductase, many procedures were followed. Unfortunately preliminary experiments with the use of simple 5% acrylamide SDS-slab gels gave poor resolution and unless the protein sample had pretreated by incubation at 100^{0}C for 5 min. with SDS and β-mercaptoethanol, severe protein aggregation would occur in the sample wells. Furthermore, even with the appropriate pretreatment, neither the 5% acrylamide gels nor the 4% to 11% gradient acrylamide slab gels gave adequate and unambiguous resolution of the protein bands to a standard high enough to allow a readily determinable value for the enzyme's subunit molecular weight to be achieved. Poor resolution was similarly a problem during non-denaturing gel electrophoresis in agarose or starch gel media, with aggregation and poor protein

mobility being the main difficulties. Cellulose acetate strip electrophoresis also did not give adequate resolution to the desired standard.

Discontinuous acrylamide gel electrophoresis, however, was able to provide excellent resolution of the individual protein components present in crude extracts through to the purified enzyme preparation.

A separation of the protein components present in some crude extracts utilized in this study is presented in Fig. 19. The densitometer scans of the various lanes of the gels shown in Fig. 19 are given in Fig. 20. From this data it is clear that the strain RL503, which bears the plasmid pRL3, shows a marked increase in the level of NADPH-cytochrome *c* reductase protein over wild-type cells. However this data indicates no further protein bands that can be distinguished in the crude extract of strain RL503 which are not present in wild-type crude extracts and which could correspond to other soluble proteins coded for by pRL3. Later gel electropherograms, (Fig. 21) however did suggest extra protein components existant in strain RL503 crude extracts and further discussion of this observation is given in Chapter 4.

An electrophoretic analysis presenting the soluble protein profiles in crude extracts of partially purified enzyme preparations is given in Fig. 21. Importantly the combined ammonium sulphate and calcium phosphate gel treatments visually, by electrophoresis appear to give a "purified" enzyme preparation (seen in Fig. 21; gel A, lane 7). On re-staining the gel with silver nitrate, however, it is clear that at this stage in the purification procedure the enzyme is at best only partially purified (although the main protein band corresponds to the enzyme). Fig. 22 presents the gel scanning data based on lanes of the gel in Fig. 21. This data indicates the relative content of impurities present at this particular stage in the purification. Furthermore, by relatively quantifying the peak areas obtained by scanning the region of the gel in Fig. 21 where the band corresponding to NADPH-cytochrome *c* reductase appeared in lanes 4 and 5, it was calculated that the areas were in the ratio of 17.6 to 1 for the lanes with crude extracts of plasmid and wild-type *E. coli* strains respectively. The ratio of the corresponding specific activities was determined as 36.6 to 1 which

FIGURE 19.

Discontinuous-SDS acrylamide slab gel electrophoresis of the protein components present in various crude extracts of *E. coli* K-12. The procedure of Weber and Osborn, (1969) as modified by Clark and Switzer, (1977) was used. Samples were run under denaturing conditions in the following manner. 15 µl of sample (crude extract) was added to 5 µl of 10% SDS, 5 µl of absolute β-mercaptoethanol and 5 µl of 10x polyacrylamide tracking dye (bromophenol blue, 1% w/v; sucrose, 50% w/v; xylene cyanol, 1% w/v). The resulting mixture was then spun for 20 s in an Eppendorf Centrifuge. Samples were then heated at 100°C for 5 min. and cooled to room temperature. A 15 µl sample was then removed and applied to the respective sample well. Electrophoresis was at 3.57 V/cm; then once the tracking dye had reached the stacking/resolving gel interface, the voltage was adjusted to 1.79V/cm, and the electrophoresis was ran for another 8 to 12 hours until the marker dye had reached the end of the resolving gel.

Cytochrome *c*, when included in any slab gel electrophoretic run, proved useful as a visible protein marker and could be readily seen as an orange band migrating between the bromophenol blue and xylene cyanol dye fronts during the electrophoresis.

The direction of electrophoresis is downwards.

Lane.	¶Strain from which extract was obtained.	Extract ¶specific activity (units/mg protein).	#Growth conditions.
1	§RL434	2.7	DJ
2	*RR1	77	DJ
3	+RL503	3174	DJ
4	RR1	1.5	Cys
5	§RL421	2.7	DJ

NOTE:

Gel A was stained with coomassie blue.

Gel B was as in gel A but re-stained with silver nitrate.

The protein band corresponding to NADPH-cytochrome *c* reductase is marked by the dashed arrow (the position of the enzyme band in these crude extracts was determined from molecular weight standards which were electrophoresed on the same gel; the approximate molecular weight of the subunit being already known from the work of Siegel and Davis, (1974)).

* Strain RR1 is a wild-type *E. coli* strain (see section 3.3.1,pp.47,48 for further description).

+ Strain RL503 bears the plasmid, pRL3.

§ Strains RL434 and RL421 have deletion and point mutations in *cysJ* respectively.

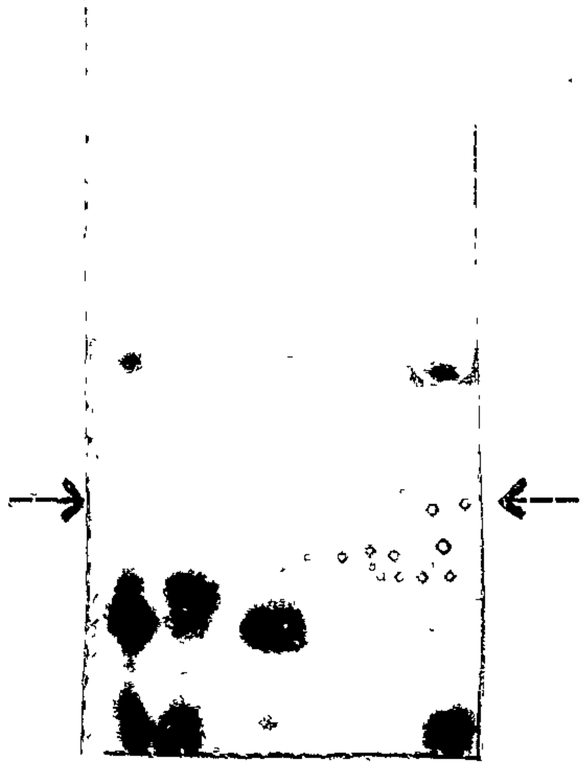
Growth was either in L-djenkolic acid (DJ) for *cys* gene derepression, or in cysteine (Cys) for *cys* gene repression.

¶ Specific activity of NADPH-cytochrome *c* reductase 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.

¶ All extracts prepared for electrophoresis were obtained by sonification of cells of the strains listed.

GEL A

5 4 3 2 1



GEL B

1 2 3 4 5

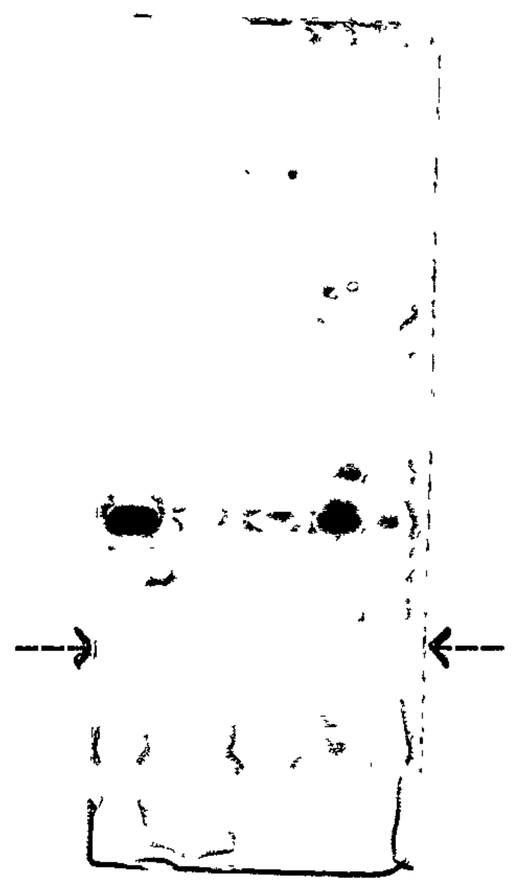
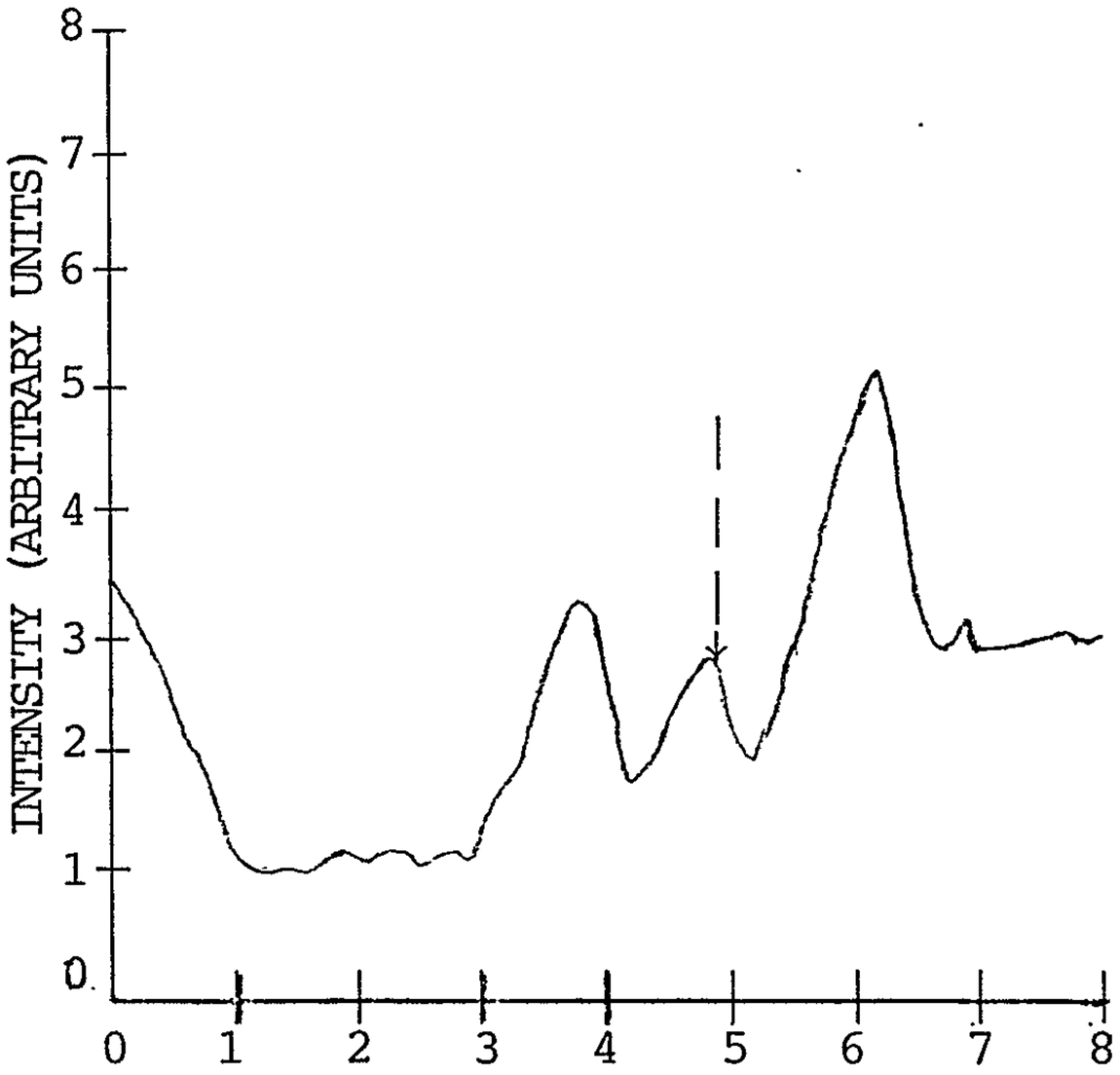


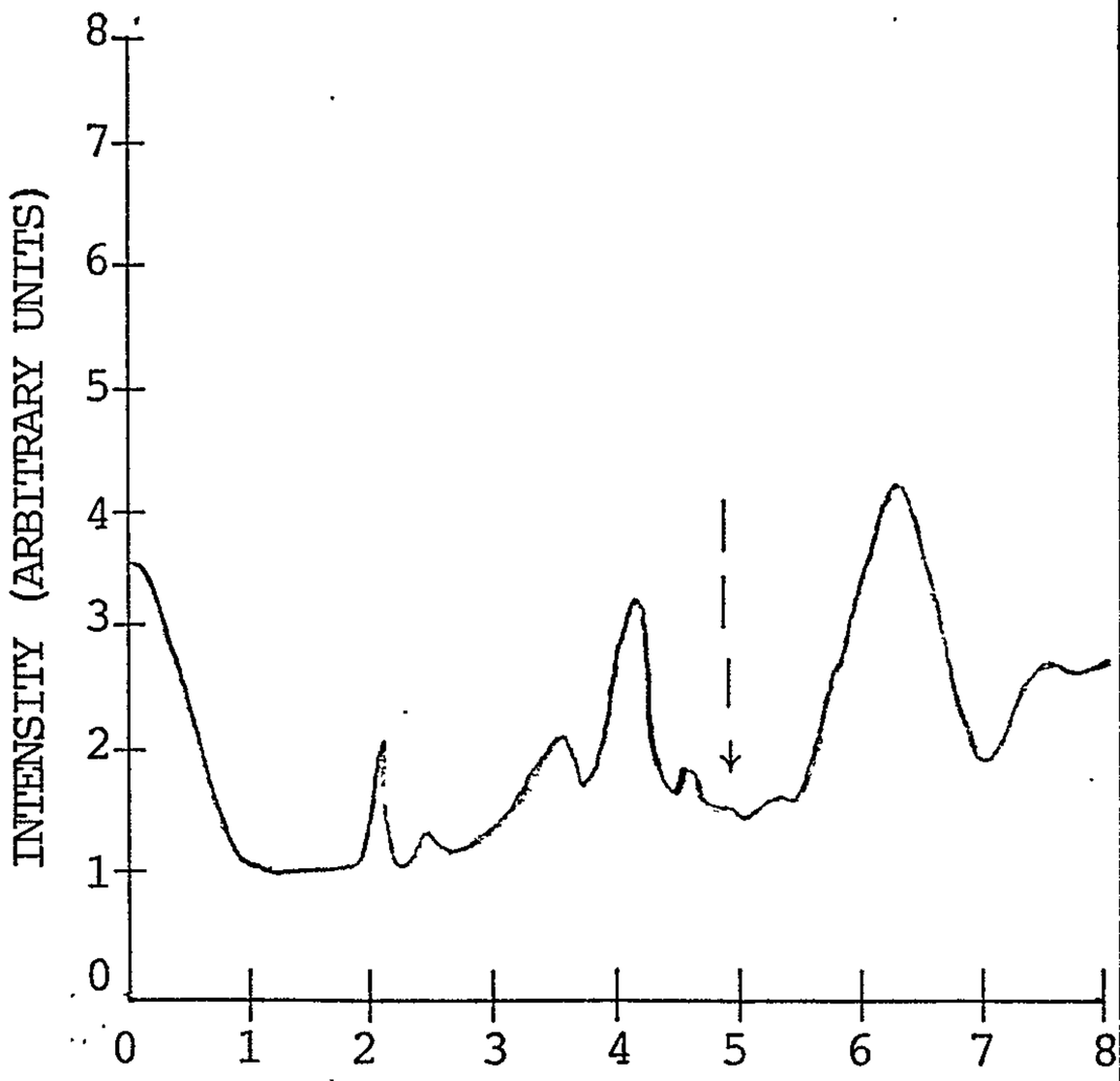
FIGURE 20.

A densitometer analysis of the separation of the proteins present in crude extracts of various *E. coli* K-12 strains. Lanes 3, 4 and 5 of gel A in Fig. 19 were examined by densitometry, with the peak positions corresponding to NADPH-cytochrome *c* reductase marked by dashed arrows. All abscissa and ordinate units are arbitrary and are standardized with respect to each other for each of the scans presented.

LANE 3



LANE 4



LANE 5

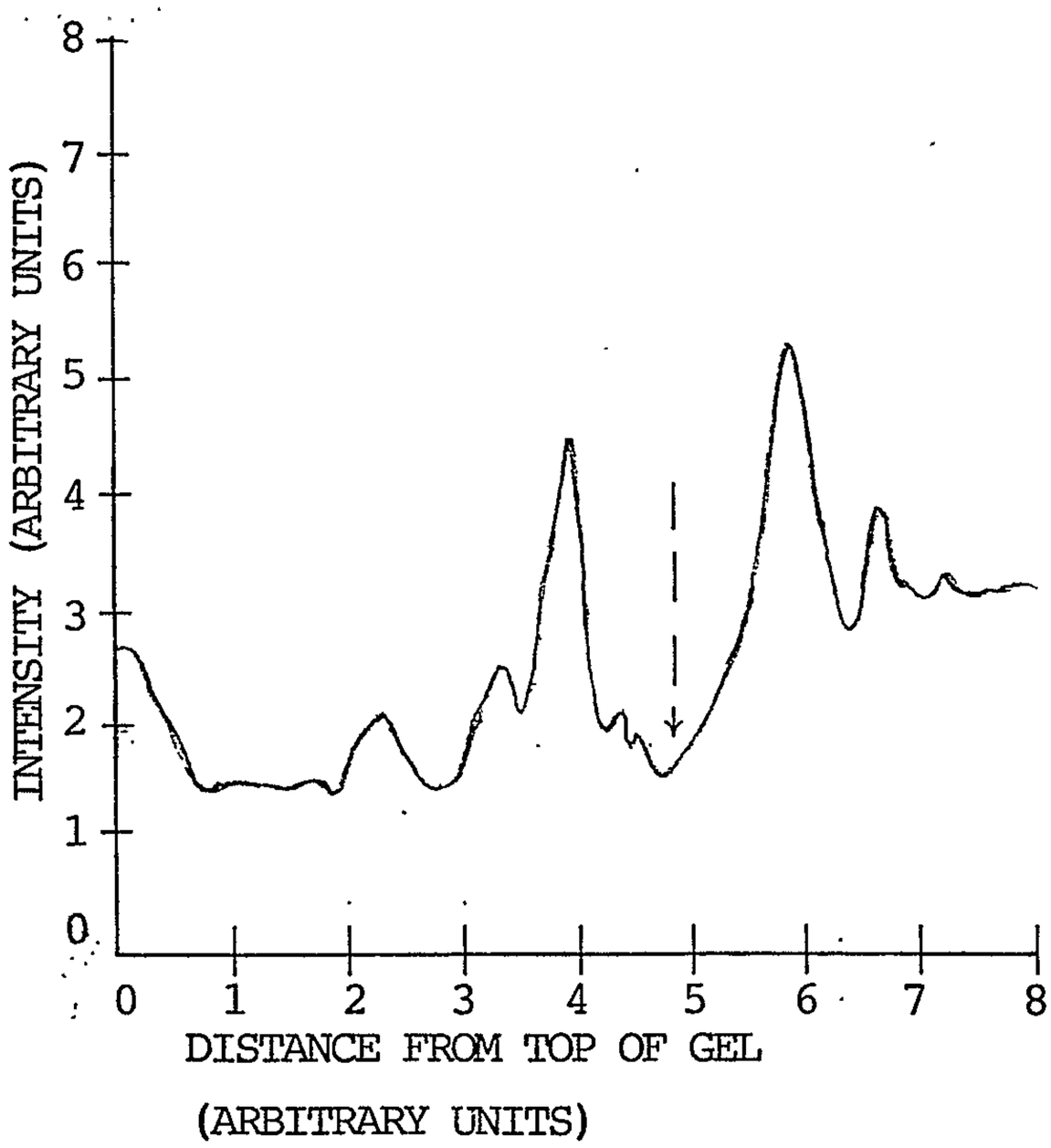


FIGURE 21.

Discontinuous-SDS acrylamide slab gel electrophoresis of crude extracts and partially purified NADPH-cytochrome *c* reductase preparations are presented. Gels were poured and run as described for Fig. 19. Samples were treated and applied to the gel as also described in Fig. 19.

The direction of protein migration is presented here as being in a downwards direction.

Lane.	Strain from which sample was obtained.	Fraction applied.	¶Specific activity of enzyme. (units/mg protein)	#Growth conditions and extract preparation.
1	§RL503	*Eluted fraction.	107	DJ, H
2	RL503	+C1	4173	DJ, H
§3				
4	†RR1	Crude extract.	77	DJ, S
5	¶RL503	Crude extract.	2822	DJ, H
6	RR1	Crude extract.	2.4	Cys,H
7	RL503	+C1	6182	DJ, H
8	RL503	Crude extract.	90	Cys,S
9	†KB9163	Crude extract.	49	DJ, S

NOTE:

Gel A was stained with coomassie blue.
Gel B was as in gel A but re-stained with silver nitrate.
The protein band corresponding to NADPH-cytochrome *c* reductase is marked by the dashed arrow (the position of the enzyme band in these crude extracts and partially purified preparations was determined from molecular weight standards which were electrophoresed on the same gel; the approximate molecular weight of the subunit being already known from the work of Siegel and Davis, (1974)).

* This fraction was eluted from a 10 x 1 cm diameter Blue-B dye-ligand column with the non-specific eluent, KCl, after the gel had been washed with phosphate buffer in the absence of KCl.

+ Two separate preparations of fraction "C1" from the calcium phosphate gel step were electrophoresed here.

§ Strain RL503 bears the plasmid, pRL3; † Strains RR1 and KB9163 are wild-type *E. coli* strains.

Growth was either in L-djenkolic acid (DJ) for *cys* DNA derepression, or in cysteine (Cys) for *cys* DNA repression ; extract preparation was either by use of the Hughe's press (H) or by sonification (S).

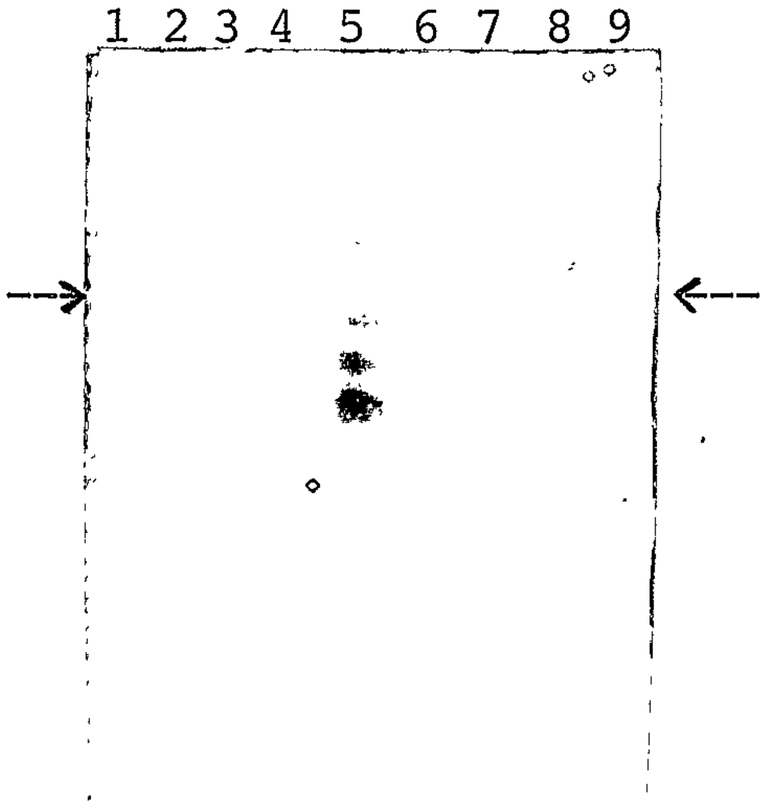
¶ Specific activity is expressed as in Fig. 19.

§ Lane 3 was a *BLANK* lane.

¶ Protein bands present in the pRL3 plasmid,RL503 crude extract not present in wild-type crude extracts are indicated by the crosses.

GEL A

1 2 3 4 5 6 7 8 9



GEL B

9 8 7 6 5 4 3 2 1

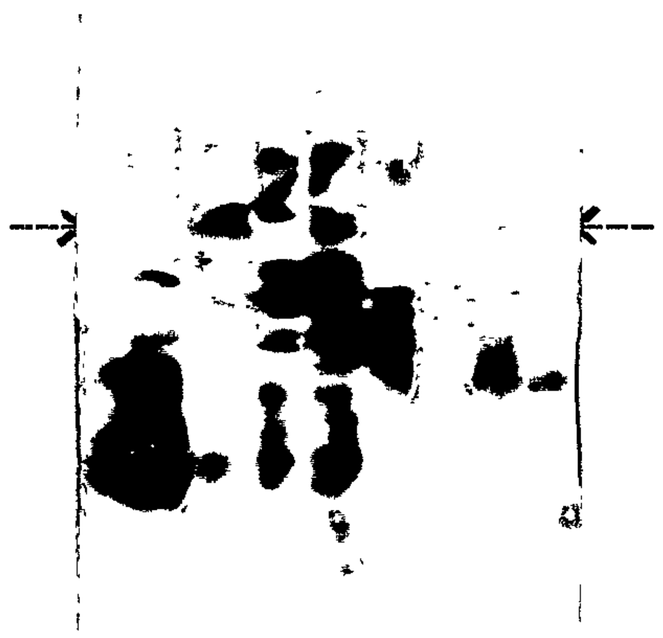
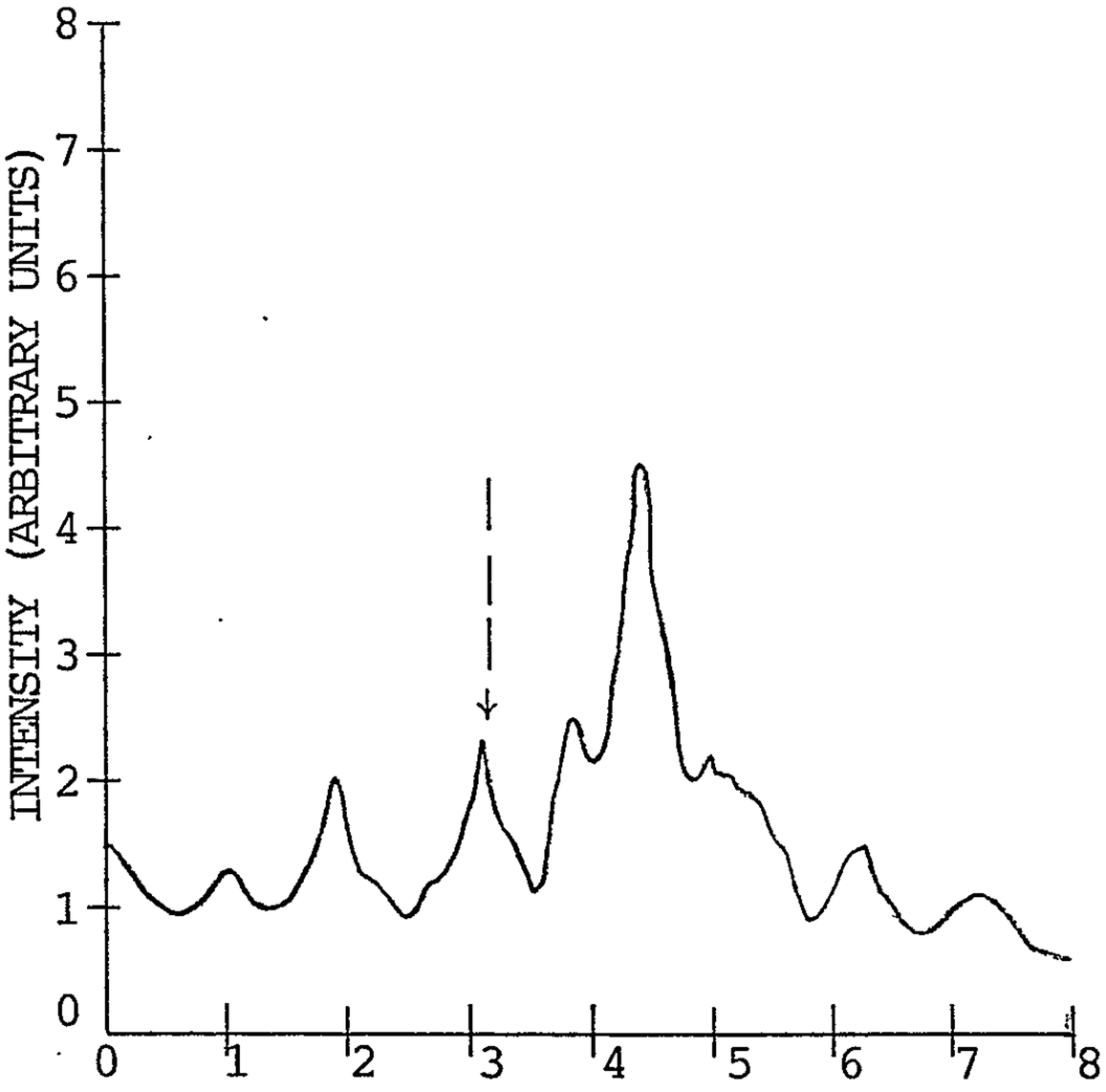


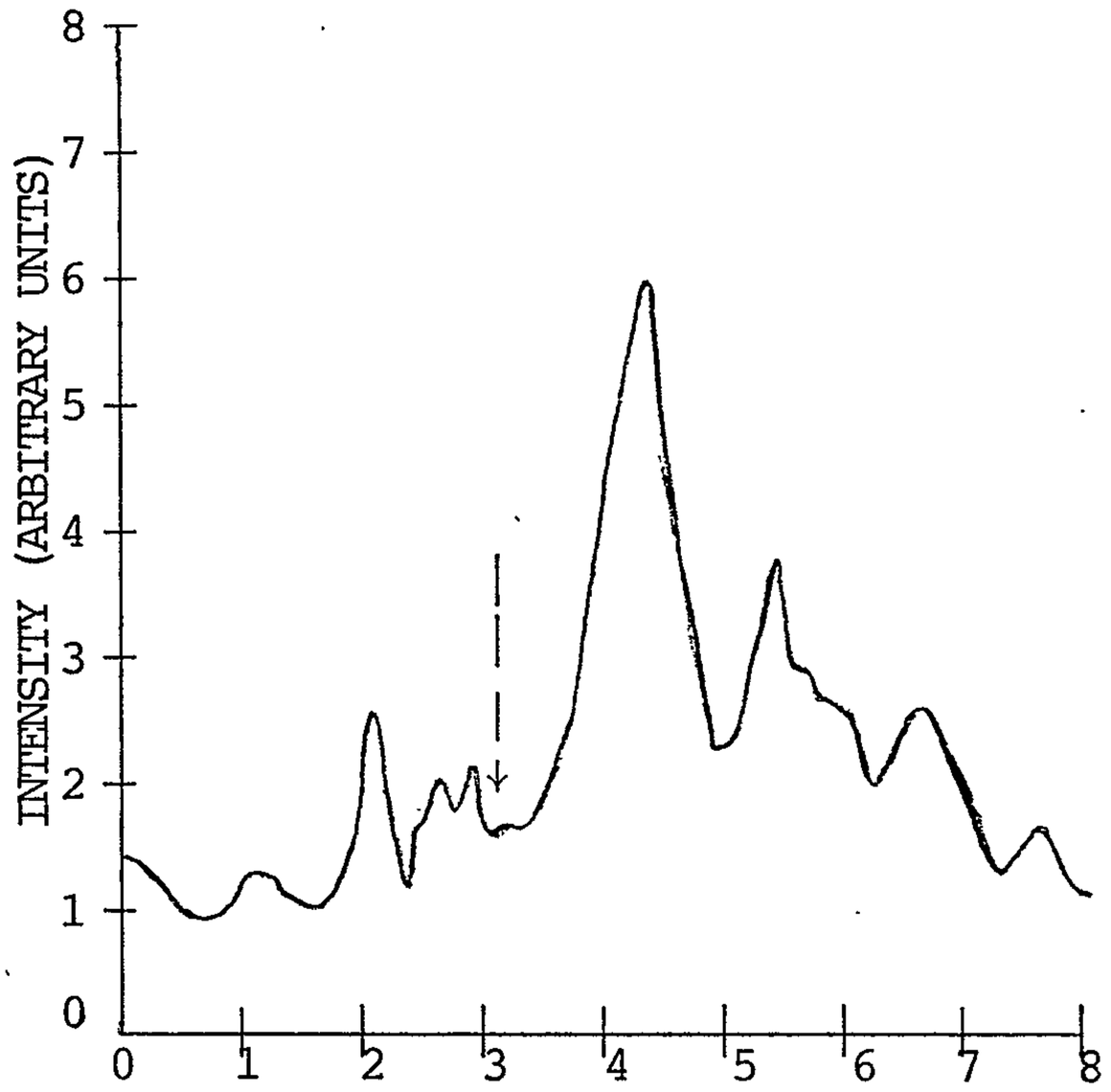
FIGURE 22.

A densitometer analysis of the separation of the protein components present in various *E. coli* strain crude extracts and in partially purified NADPH-cytochrome *c* reductase preparations. Lanes 2, 4, 5 and 8 of gel A in Fig. 21 were examined by densitometry, with the peak positions corresponding to NADPH-cytochrome *c* reductase marked by dashed arrows. The peaks corresponding to other pRL3 encoded proteins are marked by crosses. All abscissa and ordinate units are arbitrary and are standardized with respect to each other for each of the scans presented.

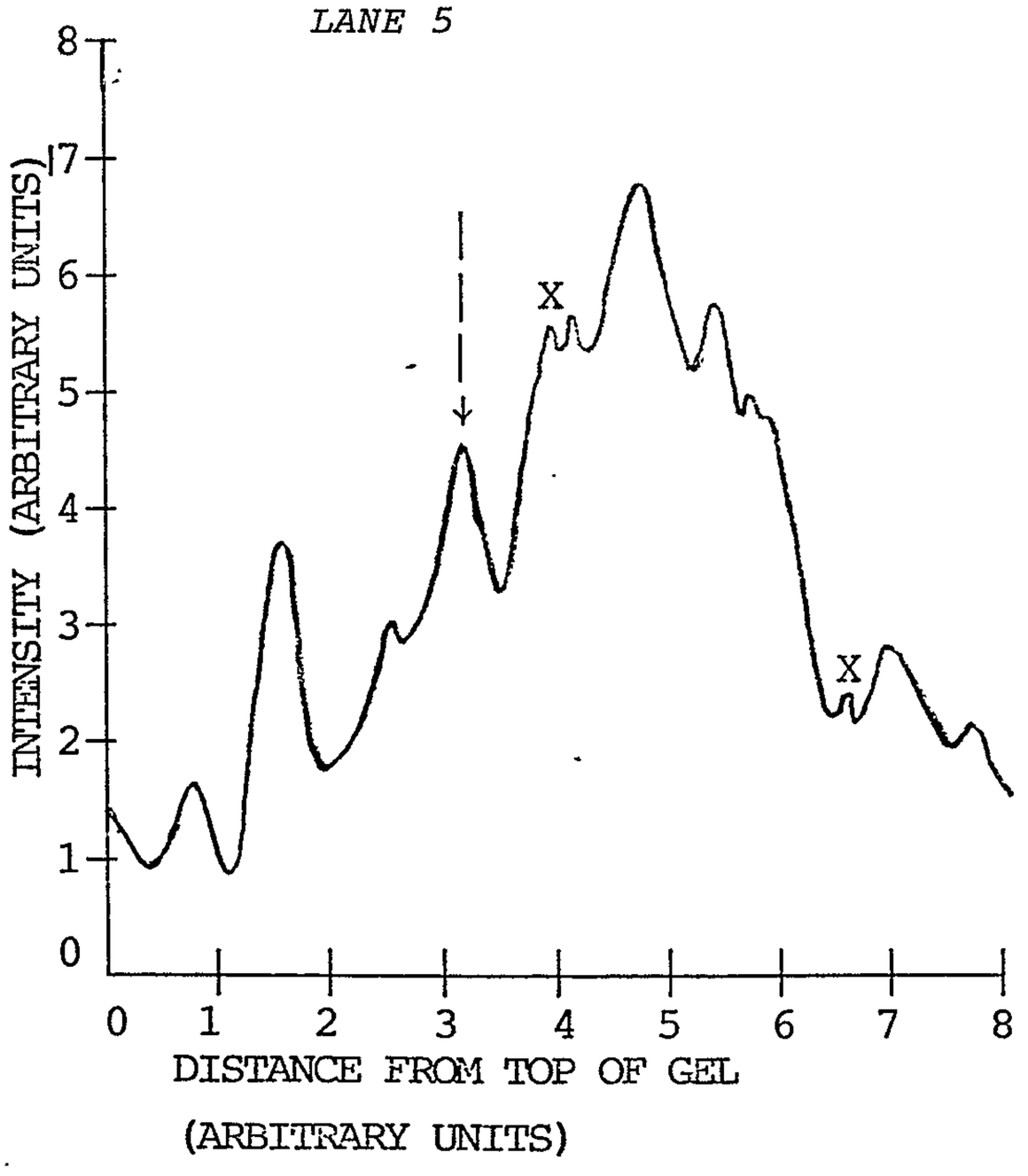
LANE 2



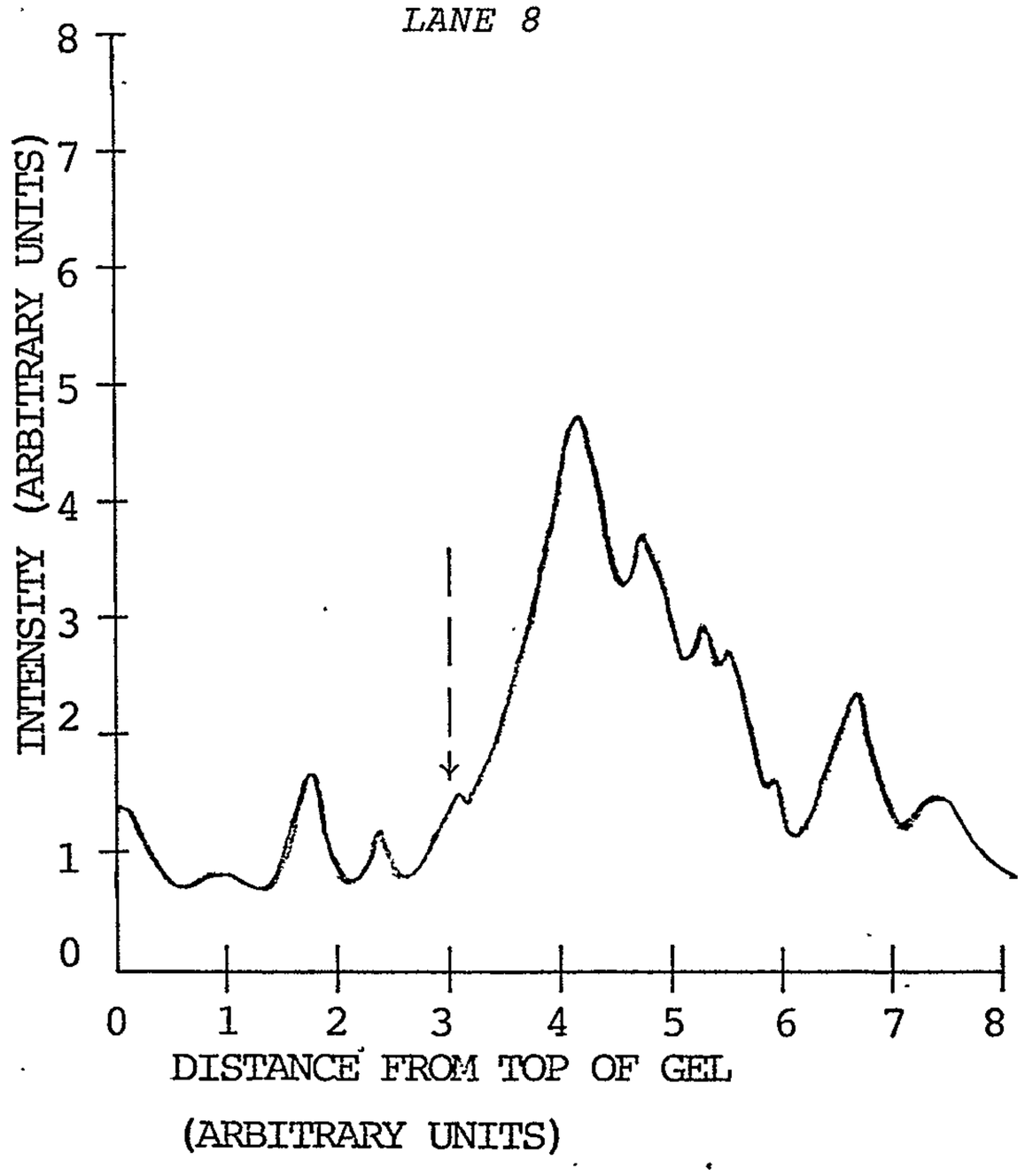
LANE 4



LANE 5



LANE 8



which is in good qualitative agreement with the amount of enzyme seen in plasmid and wild-type crude extracts by electrophoresis.

Lane 1 of Fig. 21 presents the protein profile of a fraction eluted non-specifically from a Blue-B column. Interestingly no band, detectable either by coomassie blue or by silver nitrate staining, could be seen migrating in a position corresponding to NADPH-cytochrome *c* reductase. However, many of the other proteins present in the crude extract of strain RL503 could be observed in this lane, indicating a *selective* removal of the enzyme from these contaminants at the affinity chromatography stage in purification. That the enzyme was not identified by electrophoretic means in the fraction eluted non-specifically from the Blue-B column but that many other extraneous proteins were observed to elute from the column under these conditions, would strongly support the argument that the enzyme was being purified by negative affinity chromatography, that is by the differential removal of contaminants that bind to the dye-ligand. Fig. 23 presents further electropherographic evidence demonstrating the excellent selective purification of the enzyme by the Blue-B chromatography step. Lane 10 of the gel in Fig. 23 represents the electrophoretic protein profile of the calcium phosphate gel eluate which was loaded onto the affinity column and lane 13 represents the protein profile of the fraction appearing in the void volume of the Blue-B column. Clearly then, it is seen that the enzyme has been effectively separated from many protein contaminants present in the calcium phosphate gel fraction by the affinity chromatography step. Fig. 24 (*part A*) presents an electrophoretic analysis in a denaturing gel of the purified enzyme preparation. By this analysis the enzyme appears completely homogeneous after the affinity chromatography stage, as judged by coomassie blue staining. However, on silver nitrate treatment, a number of very minor secondary bands appear along with the primary band corresponding to NADPH-cytochrome *c* reductase. These secondary bands do not resemble closely any dimeric or higher oligomeric subunit form of enzyme appearing on the gels, and could be non-specific proteolytic breakdown products formed on storage of the protein in solution prior to achieving its almost complete purification. That these bands observed by silver nitrate staining corresponded to breakdown products of the enzyme was later strongly supported by the

FIGURE 23.

Tris-urea acrylamide discontinuous-SDS slab gel electrophoresis of crude extracts and partially to completely purified NADPH-cytochrome *c* reductase. The conditions of electrophoresis were those previously described by Jovin *et al.*, (1964). Samples were run under denaturing conditions in the following manner. 10 µl of protein sample was added to 20 µl of 0.05 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, 8 M urea and 0.3 M β-mercaptoethanol, and the resulting mixture was incubated overnight at room temperature in capped tubes. After the incubation period, the tubes were uncapped and 5 µl of 10x polyacrylamide tracking dye (bromophenol blue, 1% w/v; sucrose, 50% w/v; xylene cyanol, 1% w/v) was added to each incubated sample. The mixture was then spun in an Eppendorf Centrifuge for 20 s . 15 µl of the resulting sample was removed and applied to the appropriate electrophoresis well. The electrophoresis was then performed at 10.7 V/cm until the tracking dye had met the stacking/resolving gel interface; the voltage was then adjusted to 7.10 V/cm for electrophoresis within the resolving gel (10 hours duration). Gels were then removed and stained in coomassie blue (poor staining occurring on silver nitrate treatment due to the remaining interfering substances within the gel).

The direction of protein migration is presented here as being in a downwards direction.

Lane.	Strain from which sample was obtained.	Fraction applied.	¶Specific activity of enzyme. (units/mg protein)	#Growth conditions and extract preparation.
1	§RL503	+B1	152	DJ, H
2	†RR1	Crude extract.	50	DJ, H
3	†RL145	Crude extract.	45	DJ, S
4	RL503	B1	152	DJ, H
5	RL503	+B2	1170	DJ, H
§6				
§7				
8	RL503	*C2	1567	DJ, H
§9				
10	RL503	*C1	8356	DJ, H
§11				
12	RL434	Crude extract.	152	DJ, S
13	RL503	¶Purified preparation.	20.5×10^3	DJ, H

NOTE:

The protein band corresponding to NADPH-cytochrome *c* reductase is marked by the dashed arrow (the position of the enzyme band in these crude extracts and *partially* purified enzyme preparations was determined from both, molecular weight standards which were electrophoresed on the same gel and from the position of migration of the single band appearing in the lane carrying the purified enzyme; with the approximate molecular weight of the subunit already known from the work of Siegel and Davis, (1974)).

§RL503 is the plasmid bearing strain; † RR1 and RL145 are wild-type and *cysH*$^-$ *E. coli* strains respectively; + Samples "B1" and "B2" are ammonium sulphate fractions; * Samples "C1" and "C2" are samples from the elution of the first calcium phosphate gel pellet formed from fraction "B2"; ¶ The purified enzyme sample was taken from the pooled 20 to 60 fractions eluted from the Blue-B dye ligand column; #The growth conditions and extract preparation are outlined in a footnote (#) to Fig. 21; §Lanes 6, 7, 9 and 11 are *BLANK* lanes; ¶Specific activity is expressed as in Fig. 19.

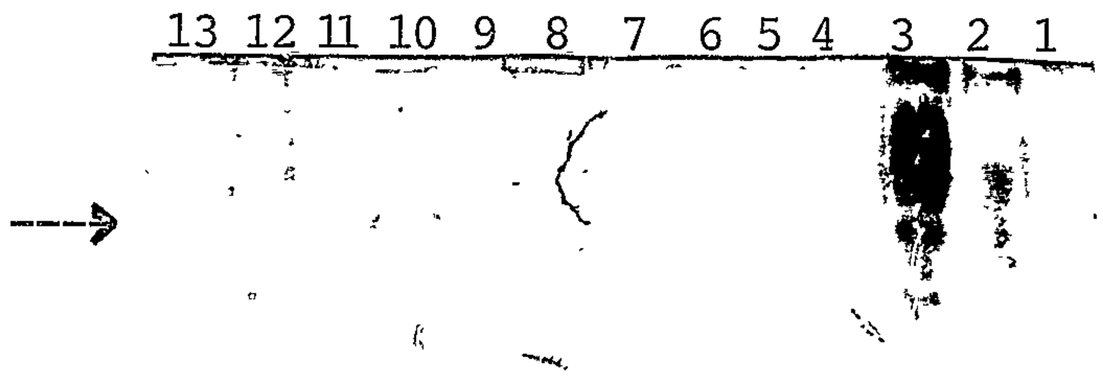

13 12 11 10 9 8 7 6 5 4 3 2 1

FIGURE 24.

PART A.

An electrophoretic analysis of the purified enzyme preparation of NADPH-cytochrome *c* reductase. Samples were run under denaturing conditions after pre-treatment with SDS and β-mercaptoethanol prior to electrophoresis. The exact conditions of sample pre-treatment and electrophoretic methods are presented in Fig. 19, with the gels being of the SDS-discontinuous acrylamide slab gel system of Weber and Osborn, (1969) as modified by Clark and Switzer, (1977).

The direction of protein migration is downwards.

KEY:

GEL A.

A sample of the pooled fractions (*viz*.20 to 60) appearing in the void volume of the final dye-ligand affinity chromatography stage of the second large scale enzyme preparation was electrophoresed. This material has been shown to be chromatographically pure and was considered to be the purest preparation of enzyme obtained during this study.

Staining was with coomassie blue, and the enzyme band visualized by this staining is marked with an arrow. The direct confirmation of the identity of this band as being NADPH-cytochrome *c* reductase was obtained through the use of convenient molecular weight standards run on the same slab gel; the approximate molecular weight of the subunit being already known from the work of Siegel and Davis, (1974).

GEL B.

As for gel A, except that staining was with silver nitrate. The enzyme band is marked with an arrow and "contaminants" appearing on silver nitrate staining of the slab gel are indicated by crosses.

PART B.

A non-denaturing gel electrophoretic analysis of purified NADPH-cytochrome *c* reductase. Use was made of the non-denaturing acrylamide discontinuous *tris*-barbital gel-buffer system of Williams and Reisfeld, (1964) with protein samples being treated in the following fashion. 15 µl of protein sample was combined with 5 µl of 10x polyacrylamide tracking dye (bromophenol blue, 1% w/v; sucrose, 50% w/v; xylene cyanol, 1% w/v). The resulting mixture was then spun for 20 s in an Eppendorf Centrifuge at 4^0C and 15 µl of the solution obtained was applied to the respective sample wells. Electrophoresis was then performed at 7.1 V/cm during both the stacking and resolving periods of the electrophoretic run. Total electrophoresis time was 15 hours. The direction of protein migration is downwards.

KEY:

Lane.	§Strain from which extract was obtained.	Fraction applied.	Extract ¶specific activity of enzyme. (units/mg protein)	#Growth conditions.
1	*RL503	crude extract	535	DJ
2	RL503	pure enzyme	20.5 x 10^3	DJ

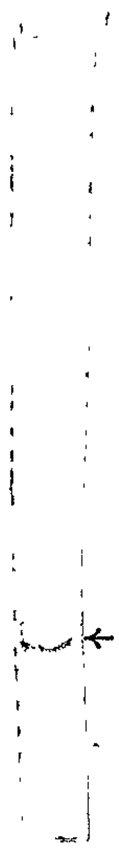
NOTE: Gel C was stained in coomassie blue (with the enzyme's α_8 octameric native state structure, as defined under these non-denaturing gel conditions, being indicated with the arrow), Gel D is as for Gel C, except that staining was with silver nitrate (with the enzyme band under these staining conditions being only barely discerned, as marked with the arrow).

§ All extracts prepared for electrophoresis were obtained from the Hugh's press.* Strain RL503 is the pRL3 plasmid bearing strain.¶ The specific activity of NADPH-cytochrome *c* reductase is expressed as in Fig. 19.

The cell growth conditions are as specified in Fig. 21.

PART A

GEL A



GEL B

PART B

GEL C

GEL D

N-terminal amino acid sequencing analysis of the enzyme from this preparation, with the demonstrating argument presented in the Discussion (Chapter 4). Nevertheless, as it is known that silver nitrate staining is sensitive down to nmol levels of protein (Wray *et al.*, 1981) then it was assumed that the enzyme preparation could not contain significant amounts of these either possible breakdown products or other protein contaminants. Hence it can be said that this preparation of NADPH-cytochrome *c* reductase has an effective percentage purity, as estimated electropherographically, of 95% or greater, and is therefore suitable for subsequent physical analysis.

Fig. 24 *(part B)* presents the results of electrophoresis in a non-denaturing polyacrylamide gel. The enzyme appears pure on coomassie blue staining, but on silver nitrate treatment of the gel, a smear is apparent. When a duplicate gel was run and gel slices prepared and homogenized in 0.3 ml of 0.05 M potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA and 10^{-5} M FAD and assayed for NADPH-cytochrome *c* reductase activity, no activity could be detected in the gel slice corresponding in position to the single band observed on coomassie blue staining. The reason that no activity could be found is that on electrophoresis anomalous local pH changes occur within the gel and alter the stability of the enzyme, leading to its breakdown into dimers, tetramers and so forth, which are not enzymatically active and lead to a smear appearing on silver nitrate staining. Previous workers, (Solomonson *et al.*, 1975) have noted similar breakdown of nitrate reductase from *Chlorella vulgaris* when run in polyacrylamide gels of running pH ~ 8 and the protein bands stained with coomassie blue. Although these workers used gel systems differing in running pH from the systems used in this study (with running pH ~ 7) it can still be infered that the observations with nitrate reductase are similar in nature to those observations with NADPH-cytochrome *c* reductase as far as the denaturation of the enzyme within the gel is concerned, since the phenomenon can be ascribed to unfavourable pH conditions within the acrylamide gel.

Fig. 25 presents an estimate of the subunit molecular weight of NADPH-cytochrome *c* reductase based on the SDS-slab gel electrophoresis data used in this study. The subunit molecular weight was calculated to be approximately 63,400 Da

FIGURE 25.

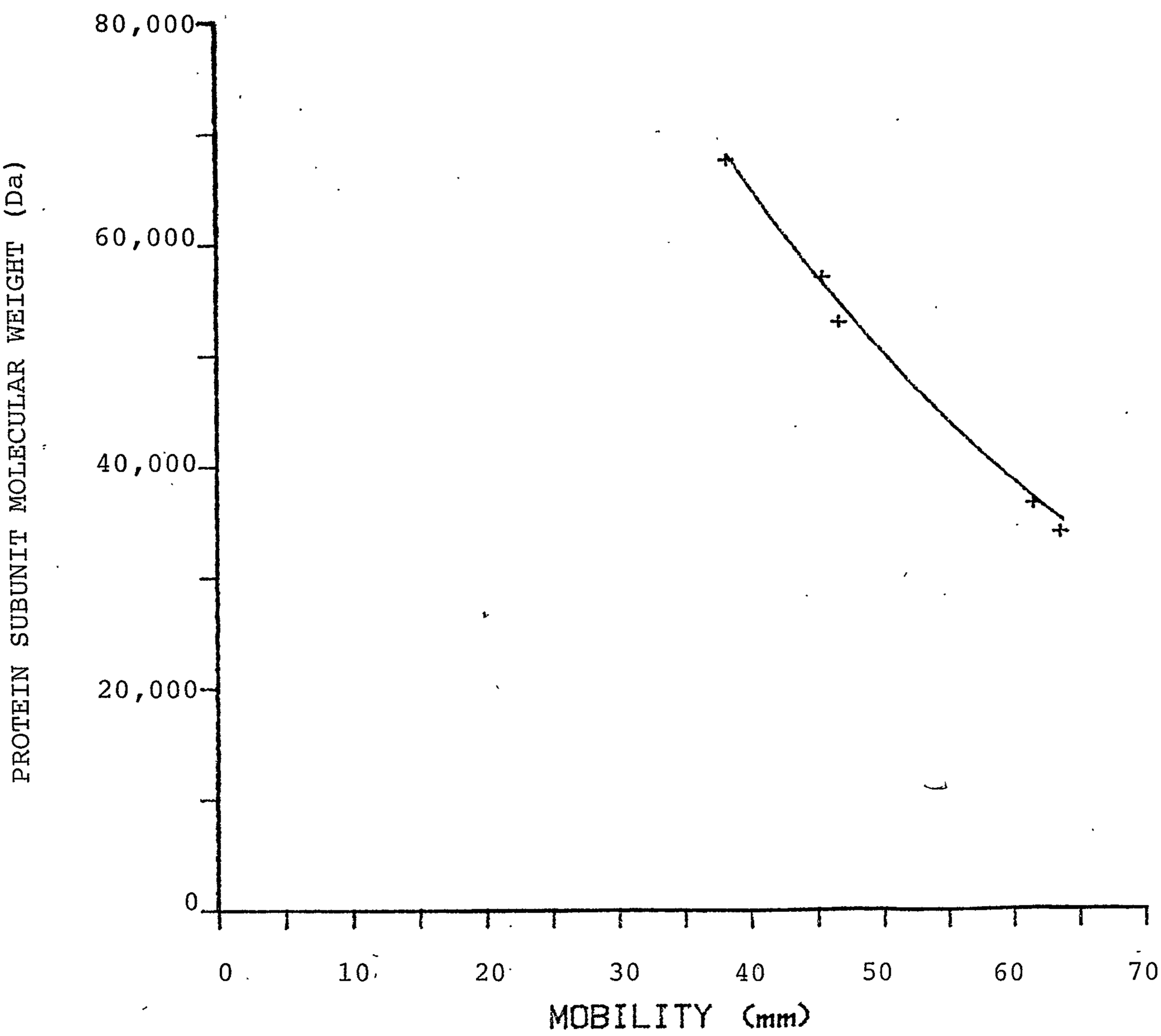
An estimate for the subunit molecular weight of NADPH-cytochrome *c* reductase based on the relationship between protein subunit molecular weight and mobility in an SDS-discontinuous acrylamide slab gel electrophoresis system. Protein electrophoresis was preformed as described in Fig. 19, according to the procedure of Weber and Osborn, (1969) as modified by Clark and Switzer, (1977).

The assumed molecular weights (taken mainly from Weber and Osborn, 1969) of the various proteins used as subunit molecular size standards are listed below.

Protein.	Molecular weight of polypeptide chain (Da).
Glucose 6-phosphate dehydrogenase	67,500
Pyruvate kinase	57,000
Glutamate dehydrogenase	53,000
Lactic dehydrogenase	36,000
Aspartate transcarbamylase, C chain	34,000

All the above proteins exist under native (non-denaturing) conditions as oligomers.

Denatured NADPH-cytochrome *c* reductase migrated with a mobility of 41.0 mm under the electrophoretic conditions utilised.


80,000
60,000
40,000
20,000
0
PROTEIN SUBUNIT MOLECULAR WEIGHT (Da)
0
10
20
30
40
50
60
70
MOBILITY (mm)

which agrees qualitatively with the data of Siegel and Davis, (1974) which predicts a subunit molecular weight estimate of approximately 59,000 Da (based on ultracentrifugation experiments).

Fig. 26 presents the results of tube gel acrylamide discontinuous electrophoreses run with various fractions from the purification procedure including the purified enzyme preparation. These electropherograms simply reinforce previous electrophoresis data in that the enzyme preparation is over 95% pure. Some minor secondary bands were detected though, in the purified enzyme electrophoresis, which could be again breakdown products of the enzyme, but which represent only a small portion of the observed protein migrating in these gels.

On performing tris-borate discontinuous denaturing gel electrophoresis at a pH of 5.77 (running pH 8.64) in the resolving gel, a strong tendency for many proteins to stack and not enter the lower gel was evident. The enzyme appeared to be one of the proteins that failed under these conditions to migrate into the lower gel; however, on increasing the resolving gel pH to 9.81 (running pH 10.01) very few proteins failed to enter the lower gel, with the enzyme penetrating the resolving gel quite effectively. Thus, as all protein samples had been treated as described in Fig. 19, that is under denaturing conditions in the presence of SDS and β-mercaptoethanol, then the observed poor gel penetration can only be due to a precipitation of the protein near its pI value. As later evidence, (section 3.4.1, p. 55) indicated that the enzyme was acidic in nature, then its precipitation at lowered pH (below neutrality) would prohibit good migration into any gel with an unfavourably low enough pH. An accurate determination of the enzyme's pI value was not performed, but qualitative statements arising from both the amino acid analysis and from the discontinuous gel data would suggest a tentative value of between pH 5.5 and 6.5.

In summary, from the electrophoretic analysis, both the ammonium sulphate and calcium phosphate gel steps appear to reduce the total amount of contaminating protein present, without preferentially removing any protein component from the extracts, be it a protein with a high molecular weight subunit structure or a low molecular weight subunit structure. However,

FIGURE 26.

The results of tube acrylamide SDS-discontinuous gel electrophoresis performed with various fractions from the purification procedure including the purified enzyme preparation. Samples were run under denaturing conditions after pre-treatment with SDS and β-mercaptoethanol prior to electrophoresis. The precise conditions of sample pre-treatment are outlined in Fig. 19, with the tube gels being of the SDS-discontinuous acrylamide gel system Weber and Osborn, (1969) as modified by Clark and Switzer, (1977). The electrophoresis was performed at 8.33 V/cm for 3 to 4 hours during *both* the stacking *and* resolving phases of the electrophoresis. All tube gels were stained with coomassie blue (silver nitrate as it was found was quite ineffective in penetrating the 0.5 mm thickness of these tube gels).

The direction of protein migration is downwards.

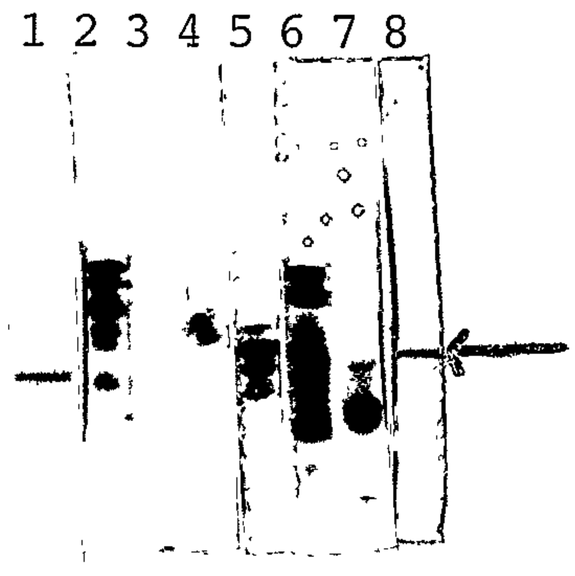
Gel No.	Strain from which sample was obtained.	Fraction applied.	Extract ¶specific activity of enzyme (units/mg protein).	#Growth conditions and extract preparation.
1		+BSA		
2	*RL434	crude extract	2.7	DJ, S
§3				
4		Pyruvate kinase		
5	§RL503	crude extract	3174	DJ, S
6	RL503	¶B2	3146	DJ, H
7	RL503	∇crude extract	2033	DJ, H
8	RL503	Purified enzyme	20.5 x 10^3	DJ, H

NOTE: The protein band corresponding to the subunit molecular form of NADPH-cytochrome *c* reductase is indicated with the arrow, and was determined from the position of migration of the protein band appearing in gel No. 8 (*viz.*the purified enzyme)

* Strain RL434 is the *cysJIH* deletion strain.[§] Strain RL503 is the strain resulting from the transformation of strain RL434 with the plasmid, pRL3.

§ Gel No. 3 was a *blank* with no protein electrophoresed on the gel.

+ The abbreviation used is Bovine Serum Albumin. ¶ Fraction "B2" is from *one* of the initial purification trials, and represents an ammonium sulphate fraction. ∇ This crude extract was from *one* of the initial purification trials. ¶ The specific activity of NADPH-cytochrome *c* reductase is expressed as in Fig. 19. # Growth conditions and extract preparation details are given in Fig. 21.


1 2 3 4 5 6 7 8

on dye-ligand affinity chromatography, the enzyme is seen to be preferentially separated from any interfering proteins present in the eluate fraction from the calcium phosphate gel step. This preferential separation leads to an homogeneous preparation of the enzyme free from any major detectable protein contaminants. In this regard, it is stressed again that the affinity step can be thought of as a *negative* chromatographic separation, in that it demonstrates a *preferential* binding of many contaminating proteins, be it by specific interaction with the dye-ligand or by a non-specific interaction with the agarose support. However the effectiveness of the procedure also relies upon the clear lack of any significant interaction of the enzyme with the dye-matrex and a reason for the poor interaction may be that the size of the native enzyme (molecular weight~460,000) would prevent its entering the agarose beads and binding to the dye-ligand. Previous investigations (see section 3.2.4, p. 35) indicated that some enzyme did in fact bind in a specific fashion to the dye-ligand, but that this percentage of enzyme that did specifically interact with the Blue-B dye would only represent the amount of enzyme that managed to penetrate the agarose beads. Thus it is readily conceivable that the separation effected by the Blue-B column in this instance, was due to the infiltration of the agarose beads by contaminating proteins and their subsequent retention either by specific or non-specific interaction with the dye-ligand and/or the agarose support and by a gel filtration effect created by the nature of the agarose beads, that clearly do not greatly allow penetration by NADPH-cytochrome *c* reductase.

In further monitoring the purification procedure, samples were regularly taken and analyzed by recording their absorption spectra. Fig. 27 presents the data obtained by this method. The spectrum of the purified enzyme shows peaks at 272 nm, 376 to 380 nm and 455 nm, and has a distinct shoulder at 480 nm; the spectrum being that of a typical flavoprotein.

3.3.1 *FURTHER INVESTIGATIONS: GENETICAL ASPECTS*.

The strain, RR1, which has a Cys^+ phenotype, was grown and assayed for NADPH-cytochrome *c* reductase activity. On sonification, a specific activity of 77 units per mg of protein was obtained for growth under conditions of derepression for cysteine biosynthesis, and a specific activity of 1.5 units per

FIGURE 27.

A presentation of the absorption spectra of various fractions sampled during the purification procedure. All samples contained NADPH-cytochrome *c* reductase in its fully oxidized state with the spectra being recorded as described in section 2.5.11 p.24, for all the designated fractions.

KEY:

----- *Fraction "B2" (protein concn.~ 5 mg/ml)

····· §Fraction "C1" (protein concn.~ 0.5 mg/ml)

-·-·- ¶Fractions 20 to 60 pooled from the dye-ligand column (protein concn.~ 1 mg/ml)

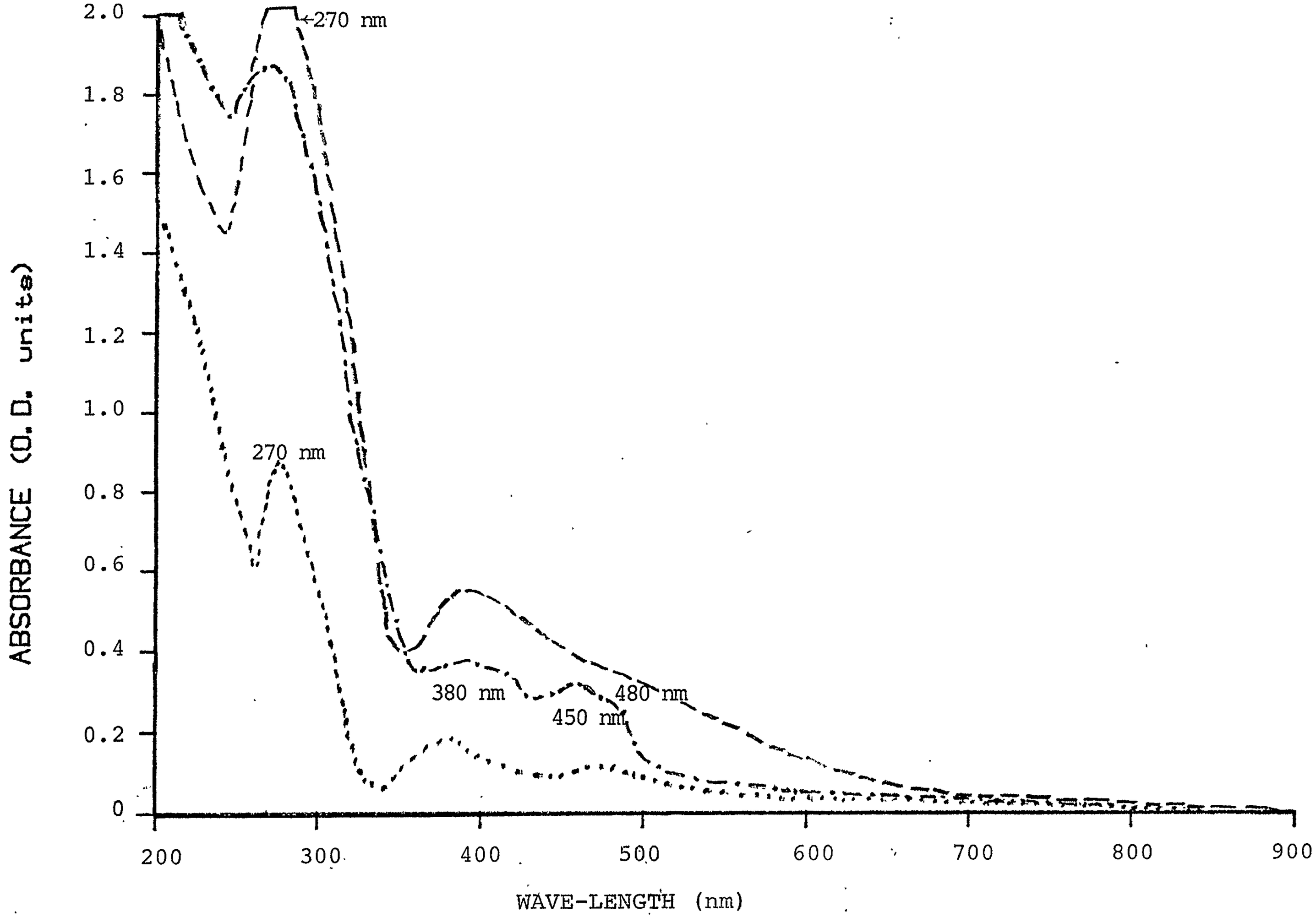
NOTE:

* Fraction "B2" is from the ammonium sulphate treatment of the crude extract fraction from an early enzyme purification trial.

§ Fraction "C1" is the fraction obtained from the elution of the calcium phosphate gel pellet formed on the first addition of calcium phosphate gel to fraction "B2". This particular fraction was from an early enzyme purification trial.

¶ This pooled fraction represents the purified enzyme preparation obtained during this study.

All cuvettes were routinely zeroed at 900 nm before scans were performed starting from this wave-length.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE (O.D. units)
←270 nm
270 nm
380 nm
480 nm
450 nm
200
300
400
500
600
700
800
900
WAVE-LENGTH (nm)

mg of protein was obtained for growth under conditions of repression for the genes involved in cysteine biosynthesis. Extracts of the same strain were also made with the Hughe's press, with a recorded specific activity of NADPH-cytochrome *c* reductase of extracts of cultures grown under conditions of derepression being 49.5 units per mg of protein and 2.4 units per mg of protein for growth under conditions of repression. Notably, the values vary depending on the method used to disrupt the cells, and this variance is possibly due to the concentration of proteases in the Hughe's press extract being higher than in those extracts prepared by sonification, as the protein concentration in the Hughe's press extract was consistently noted to be two fold higher than in the extracts prepared by sonification. Hence from the differences in protein concentration it is conceivable that the Hughe's press extracts contain higher concentrations of proteases that act to breakdown any proteins present in these cell extracts including NADPH-cytochrome *c* reductase. In view of the activity of these proteases it would have been expedient to have included a proteolytic inhibitor into the freshly prepared crude extracts used for protein purification, as more accurate values for the specific activity of the crude extract and all subsequently isolated fractions would have been obtained; furthermore, as presented in the Discussion in Chapter 4, the inclusion of protease inhibitors at an early stage in the enzyme purification could well have prevented the number of non-specific breakdown products of the enzyme being present in the purified enzyme preparation which interfered with the amino acid sequencing procedure to a noticeable extent.

Strain RR1 was then selected as a suitable wild-type control strain in all further enzyme assays as presented below. Table XVI presents the specific activities of the *cysJ* encoded enzyme with the use of various substrate electron acceptors in crude extracts prepared from a variety of *E. coli* strains used in this study. From this data, strains with a deleted *cysJ* gene (strain RL434) or carrying a point mutation in this gene (strain RL421), showed both insignificant levels of NADPH-cytochrome *c* reductase and no NADPH-DCPIP reductase activity. These results are readily understood in view of the catalytic activities displayed by the *cysJ* gene product in its *in vitro*

TABLE XVI: A summary of the specific activities of NADPH-cytochrome *c* reductase with the use of various substrate electron acceptors in crude extracts prepared from a variety of *E. coli* strains. All extracts were obtained from sonified extracts prepared from cells grown under conditions of derepression for cysteine biosynthesis.

Strain.	Relevant genotypic markers borne by:		#NADPH-cytochrome *c* reductase. Specific activity. (units/mg protein)	*NADPH-DCPIP reductase. Specific activity. (units/mg protein)	+NADPH-sulphite reductase. Specific activity. (units/mg protein)	§NADPH-hydroxylamine reductase. Specific activity. (units/mg protein)
	plasmid or phage	*chromosome*				
RL421		*cysJ*	2.7	0.0	0.0	0.0
¶RL421 transduced	λd*cysJIHD*	*cysJ*	48	553.8	4.3	N.D.
RL434		Δ*(cysJIH)*	2.7	0.0	0.0	0.0
¶RL434 transduced	λd*cysJIHD*	Δ*(cysJIH)*	152	361.0	27.2	N.D.
RL502	pRL3 (*cysJ*$^+$)	*cysJ*	1337	1909	1.1	16.1
RL503	pRL3 (*cysJ*$^+$)	Δ*(cysJIH)*	2049	2485	0.0	0.0
§RR1			77	438.5	29.5	313.5

NOTE: ¶ Transduced with phage λd*cysJIHD* from an HFT (high frequency of transduction lysate) of strain RL155. Transductants were selected on the basis of their Cys$^+$ phenotype.

§ Strain RR1 is a wild-type *E. coli* strain used as a control for these assays.

One unit catalyzes the oxidation of 1 nmol of NADPH per min. * One unit catalyzes the reduction of 1 nmol of DCPIP per min. + One unit catalyzes the oxidation of 1 nmol of NADPH per min. § One unit catalyzes the oxidation of 1 nmol of NADPH per min.

N.D., Not Determined.

reduction of cytochrome *c* and DCPIP, activities which this enzyme does not catalyze *in vivo* due to the absence of these electron acceptors within the *E. coli* cell. When the two strains (RL434 and RL421) are transduced to Cys^+ with the specialized defective lambda transducing phage, λd *cysJIHD* , the enzyme specific activities of NADPH-cytochrome *c* reductase and NADPH-DCPIP reductase are both present to wild-type levels. This indicates that the phage has transduced these strains to $cysJ^+$, thus bringing the *cysJ* gene encoded enzyme levels to wild-type levels. Strains RL502 and RL503 which both carry a plasmid bearing the *cysJ* gene show greatly increased levels of NADPH-cytochrome *c* reductase (as observed previously) and NADPH-DCPIP reductase over wild-type cells. This is again readily understood in terms of the number of copies of the *cysJ* gene in these strains making for an overproduction of the gene product of the *cysJ* gene. When the NADPH-sulphite reductase levels of the strains used were assayed it was observed that only strain RL502 and those strains which had been transduced to Cys^+ had any detectable levels of the enzyme when compared to wild-type cells. This logically follows on from the necessity of having both the *cysJ* and *cysI* and *G* genes all present in the cell before sulphite can be reduced by NADPH, and as strains RL421, RL434 and RL503 lack one or more of these necessary genes then no sulphite reductase can be produced in cells of these strains. However, when strains RL434 and RL421 are transduced to Cys^+, NADPH-sulphite reductase levels can be detected, and although the levels vary in some regards from wild-type, they indicate that these cells are able to reduce sulphite. Furthermore, strain RL502 shows levels of NADPH-sulphite reductase which are significantly lower than wild-type cells. These observations can be explained in terms of the nature of the point mutant present in the *cysJ* gene of the chromosome of this strain. In order that the levels of NADPH-sulphite reductase be so low, the point mutant would have to be exerting polar effects on genes distal from it as seen from the *cysJIH* promoter such that the *cysI* gene would be poorly transcribed and translated. Thus even though there is an abundance of *cysJ* polypeptide in these cells, there is a severely limiting amount of *cysI* polypeptide available, thus leading to greatly reduced levels of NADPH-sulphite reductase as compared to wild-type.

When the levels of NADPH-hydroxylamine.HCl reductase were assayed it was seen that neither strains RL421, RL434 or RL503 had any detectable levels of enzyme. These results could be predicted from the fact that, like sulphite, hydroxylamine.HCl requires the presence of the *cysJ* polypeptide and the *cysI* and *G* polypeptides before its reduction by NADPH can occur *in vitro*. Furthermore, the levels of the enzyme in extracts from strain RL502 show greatly lowered levels of the enzyme as compared to wild-type crude extracts. This assay data supports the results obtained with the use of sulphite as an electron acceptor with regard to the nature of the point mutant present in the *cysJ* gene of this strain.

On mixing crude extracts of the strains RL421 and RL503, a partial reconstitution of NADPH-sulphite reductase activity occurred, the results of which are presented in Table XVII. However from this experiment, only 5% reconstitution of the enzyme could be achieved on mixing the appropriate extracts, as compared to those enzyme levels recorded in wild-type crude extracts. Previous work (R.E.Loughlin, personal communication) using a qualitative assay for MVH linked sulphite reductase, coded by the *cysI* gene in strain JM73 , which bears the same mutation in the *cysJ* gene as does strain RL421, revealed levels of enzyme of only 5% or less as compared to a wild-type strain. This data therefore indicates that the mutation in the *cysJ* gene of strain JM73 could possibly be exerting polar effects on the expression of the genes distal to the lesion as read from the *cysJIH* promoter. This would account for the low levels of MVH linked sulphite reductase present in crude extracts of this strain. Therefore the data in Tables XVI and XVII support each other on the conclusion of the nature of the mutation present in the *cysJ* gene of strain RL421.

Work was then done on assaying the specific activities of NADPH-cytochrome *c* reductase in other strains available. Levels of enzyme in crude extracts of the strain RL145 (which carries a mutation in the *cysH* gene), the strain RL500 (which carries a 17.4 Kbp plasmid bearing the *cysJ* gene and its promoter) and the strain RL501 (which carries an approximately 10 Kbp plasmid bearing the *cysJ* gene and its promoter) were recorded with the ratio of specific activities in the above strains of 1 to 37 to 9 respectively. It appears therefore

TABLE XVII: A partial reconstitution of NADPH-sulphite reductase activity by mixing crude extracts of strains lacking this activity. Strains RL421 and RL503 were grown under conditions of derepression for cysteine biosynthesis and cell extracts were prepared by sonification. Each extract was diluted to a standardized protein concentration of 3.0 mg/ml and then both extracts were mixed and allowed to reconstitute NADPH-sulphite reductase activity in a procedure used unmodified from Siegel *et al.*, (1971). After a period of 20 min. at 0^0C little further reconstitution of enzyme activity was observed to take place.

Crude extracts.	Relevant genotypic markers.	*NADPH-sulphite reductase specific activity. (units/mg protein)	¶Percentage activity of enzyme relative to wild-type (RR1).
RL421	*cysJ,cysI*$^+$	0.0	0.0
RL503	*cysJ*$^+$,Δ*(cysJIH)*	0.0	0.0
RL421 + RL503 (5 min. reconstitution at 0^0C)		1.2	4.1
Rl421 + RL503 (20 min. reconstitution at 0^0)		2.1	7.1

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

¶ The percentage activity of NADPH-sulphite reductase is expressed here with respect to a wild-type strain (RR1) having a specific activity of NADPH-sulphite reductase of 29.5 units/mg protein.

that the specific activities of NADPH-cytochrome *c* reductase present in crude extracts of strains bearing a plasmid carrying the *cysJ* gene and its promoter vary considerably depending on the plasmid involved. In this experiment strain RL145 acts as an effective wild-type strain for the *cysJ* gene with the specific activities of the enzyme produced in the plasmid bearing strains varying four-fold from each other. The data obtained from this experiment indicates that a number of variables are acting to produce the significant differences observed in enzyme activities from these plasmid strains. The possibilities exist that the plasmid copy number has somehow been altered in some of the strains or that perhaps another terminator has mutated upstream from the *cysJ* gene leading to transcriptional read-through and hence leading to the production of fused gene products which would obviate the production of catalytically active NADPH-cytochrome *c* reductase. However many explanations exist, and without further experimentation into the variance of production of NADPH-cytochrome *c* reductase noted here and previously in plasmid bearing strains (see section 3.2.1, pp. 31 and 32), little definite comment can be made on the molecular basis for these recorded differences.

3.3.2 *FURTHER INVESTIGATIONS: ENZYME THERMOLABILITY PROPERTIES.*

NADPH-cytochrome *c* reductase activity decreased rapidly on placing crude extracts containing enzyme at 20^{0}C for periods of up to two weeks, with 0.1% of activity remaining after that length of time. However no loss of activity could be detected on storage of the enzyme at -18^{0}C *or* at 4^{0}C for periods of up to two months.

On heating a crude extract solution from 0^{0}C to over 80^{0}C, various changes in enzyme activity were observed, as plotted in Fig. 28. Notably the activity curve shows a decrease on heating the extract from 0^{0}C to room temperature. This observation, however, was not always reproduced in subsequent experiments, with little or no decrease in activity on heating to room temperature for short periods of time (~ 5 min.). Yet on incubating at room temperature for longer time periods (~ 60 min.) a reproducible decrease in enzyme activity was recorded. However this behaviour is considered to also depend on the protein concentration of the incubation mixture, such that a higher protein concentration leads to a more noticeable decrease in

FIGURE 28.

A temperature dependent activity profile of NADPH-cytochrome *c* reductase. An appropriately diluted enzyme sample in 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, was taken from a crude extract of strain RL503, and was incubated for 5 min. at each of the designated temperatures after having been on ice. Immediately after the incubation period, samples were assayed for NADPH-cytochrome *c* reductase activity at 20^{0}C. At higher temperatures, enzyme activity was lost as indicated independent of any noticeable protein concentration effects within the incubated sample.

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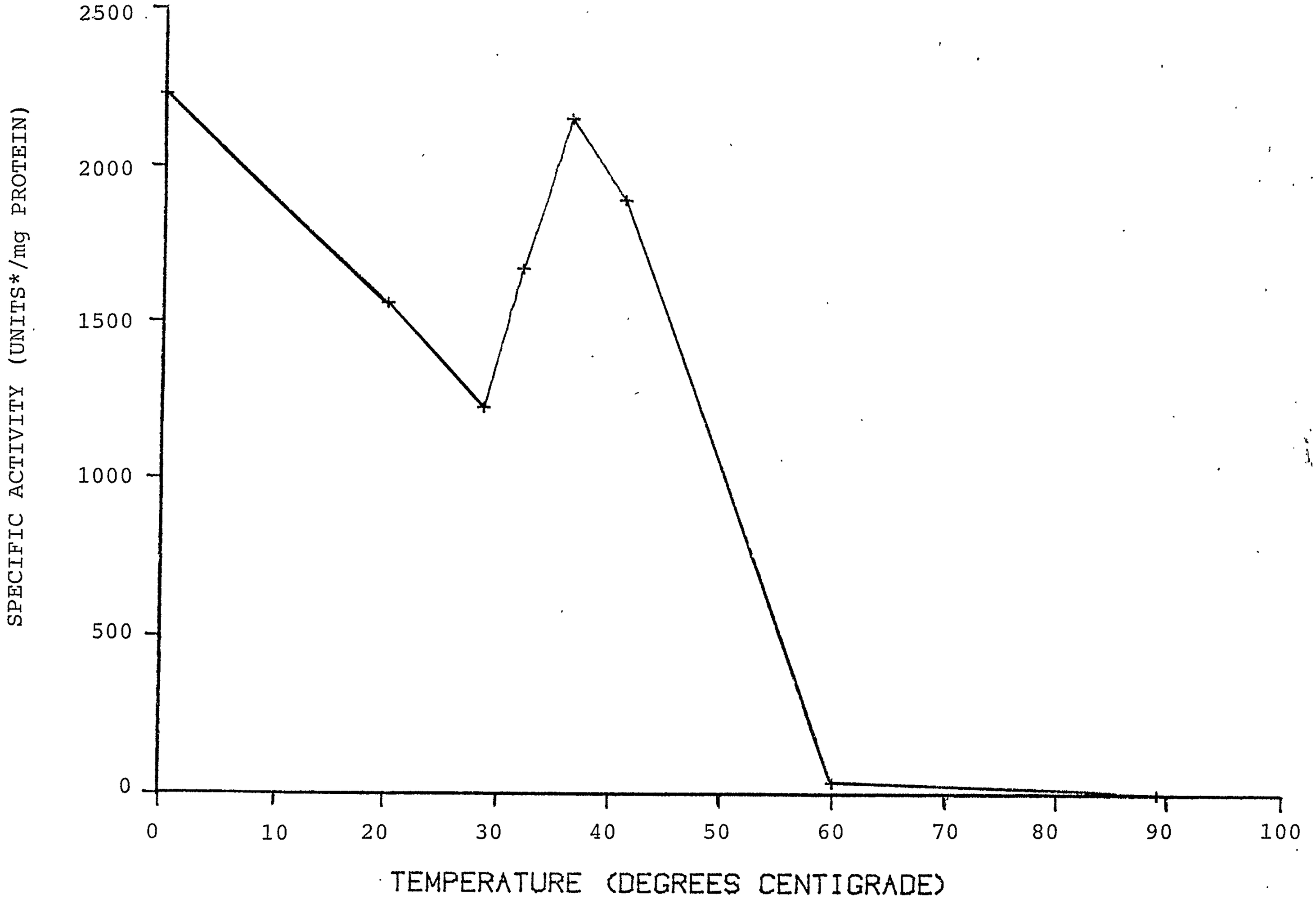
* One unit of NADPH-cytochrome *c* reductase catalyzes the oxidation of 1 nmol of NADPH per min.


SPECIFIC ACTIVITY (UNITS*/mg PROTEIN)
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
80
90
100
TEMPERATURE (DEGREES CENTIGRADE)

in the enzyme's activity on heating to room temperature. Fig. 28 also demonstrates that enzyme activity appears to rise to a peak at a temperature of approximately 36 to 38^{0}C, after which activity rapidly declines. Interestingly, when the incubation mixture was heated at room temperature for a period of time sufficient to note a decrease in enzyme activity, and a duplicate incubation mixture of the same protein concentration and enzyme specific activity was heated to 36^{0}C for the same length of time, no decrease in the enzyme's activity was noted in the 36^{0}C incubated solution when compared to the mixture incubated at room temperature. This observation suggests that the enzyme is maintained in a stable state at body temperature and that its activity displays a cold-lability behaviour at room temperature. Again these results were strongly dependent on the protein concentrations of the incubation mixtures, as determined from preliminary experiments, and as yet no firm relationships between these variables can be drawn. Experiments of a more definite nature were then carried out to investigate the heat-lability behaviour of the enzyme. As was demonstrated in Fig. 28, enzyme activity rapidly falls on heating the incubation mixture to temperatures above 38^{0}C. To furthur investigate this property, an extended incubation at 60^{0}C was performed. The results of this experiment are presented in Fig. 29 and show that at 60^{0}C the enzyme's activity sharply decreases over time, with a slowly decreasing activity function appearing after 5 min. incubation. Furthermore, on cooling the incubation mixture from 60^{0}C to 0^{0}C, a partial renaturation of the enzyme's activity is observed, however further investigations into these and the above phenomena were not performed in the present study.

3.3.3 *FURTHER INVESTIGATIONS: DEPENDENCY OF ENZYME ACTIVITY ON PROSTHETIC GROUPS.*

On dialyzing a pure preparation of enzyme (at approximately 0.1 mg/ml) against 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA but with FAD omitted, a decrease in enzyme activity was observed to occur. The specific activity fell in the order of three- to twenty-fold (the actual decrease being dependent on the initial enzyme concentration prior to dialysis, with the higher the enzyme concentration the more stable the flavoprotein is to this treatment). Furthermore, in an attempt to restore the enzyme's full pre-dialysis activity, the enzyme

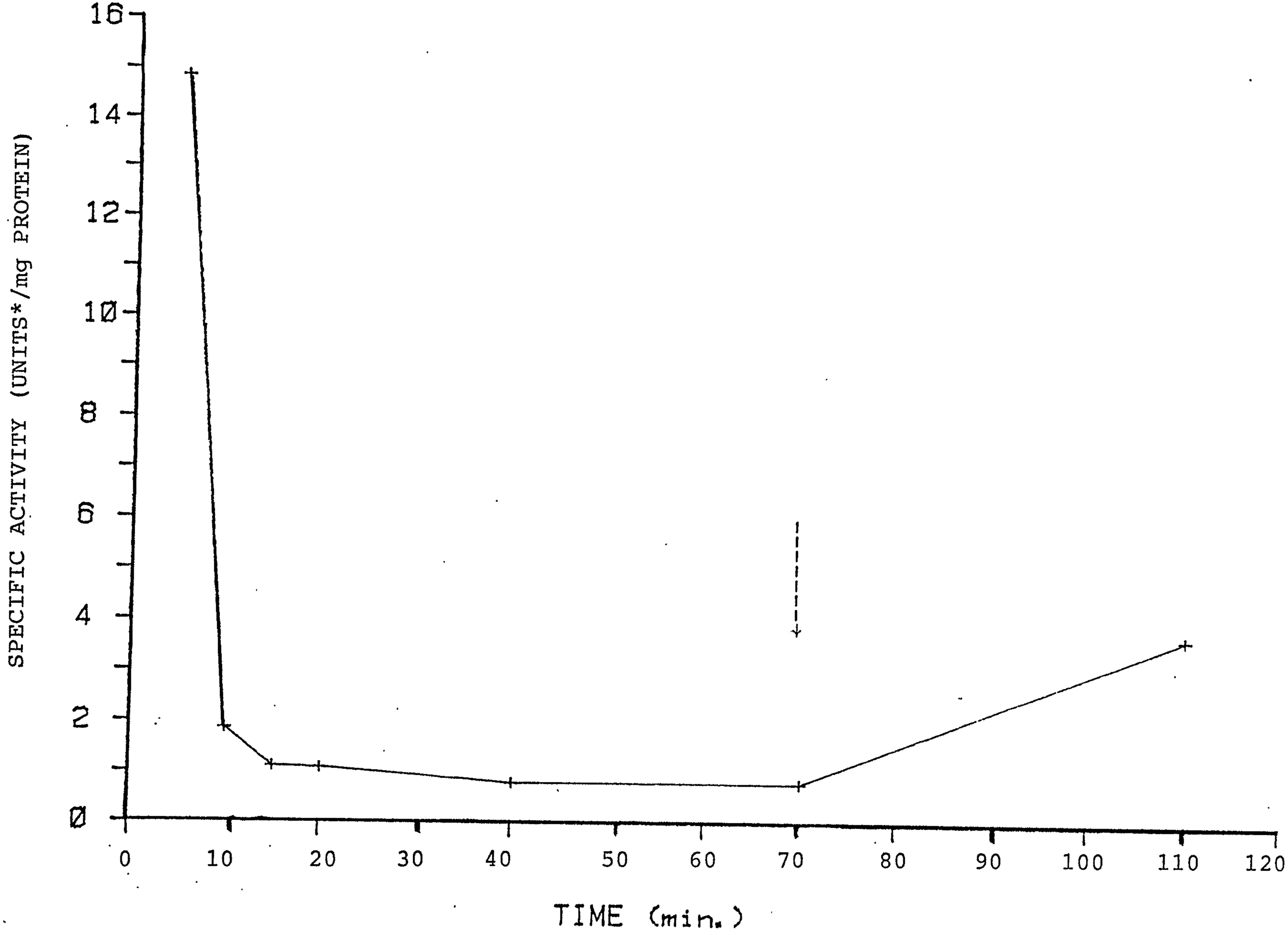
FIGURE 29.

The partial renaturation of NADPH-cytochrome *c* reductase following heat denaturation. The time course assay was performed by removing samples from an undiluted crude extract preparation of strain RL503 incubated at 60^{0}C. Incubation was for the times shown with an approximate extract protein concentration of 10 mg/ml. When samples were removed from the incubation tube they were immediately assayed for NADPH-cytochrome *c* reductase activity. At 70 min. the incubation tube was equilibrated to 0^{0}C and left at this temperature for 40 min. before withdrawing an aliquot for assaying at 20^{0}C. Little variance in enzyme activity was noted within the first time period when the sample had been diluted to 10^{-2}fold or when it had been left undiluted, indicating that protein interaction effects during the incubation of the sample were not very marked during this experiment.

NOTE:

The time at which temperature of the incubation mixture was shifted to 0^{0}C is indicated by the dashed arrow.

* One unit of NADPH-cytochrome *c* reductase catalyzes the oxidation of 1 nmol of NADPH per min.


SPECIFIC ACTIVITY (UNITS*/mg PROTEIN)
16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
70
80
90
100
110
120
TIME (min.)

preparation was re-dialyzed against 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA with *either* FAD *or* FMN present at a final concentration of 10^{-5}M. Notably dialysis against flavin containing buffers resulted in little or no increase in enzyme activity. The results suggest that exogenously present FAD (or FMN) is needed to retain the flavoprotein's activity and that on removal of the externally present flavin, a possible dissociation of the flavin groups bound to the native enzyme occurs leaving the enzyme inactive. Present data would suggest that this dissociation is *not* easily reversable.

On further analysis of more concentrated, pure, enzyme preparations, it was observed from absorption spectra that either a one to one ratio of FAD to FMN groups remained bound to the purified enzyme or that of the flavins bound, FAD was the dominating species with perhaps some residual FMN remaining bound. Unfortunately present spectral techniques could not distinguish the actual ratio of flavins remaining bound to the purified enzyme preparation, however as there is a range of possibilities, that is there may be one FAD bound for every 7 FMN groups, and as every enzyme molecule in the solution may not possess the same ratio of bound flavin, then it would be virtually impossible from absorption spectra *alone* to predict the nature of the flavin groups held by the pure enzyme preparation. However, on the basis of the substantial substrate turnover present in the purified enzyme preparation, it would appear that the ratio of bound flavin groups approached that of a one to one ratio, where both prosthetic groups need to be present in near equimolar amounts in order for the enzyme to carry out efficient catalysis, (Siegel *et al.*, 1974).

3.3.4 *FURTHER INVESTIGATIONS: ENZYME CROSS-LINKING AND FURTHER INHIBITOR STUDIES.*

Attempts to cross-link NADPH-cytochrome *c* reductase with the bifunctional cross-linking reagent dimethlysuberimidate.2HCl which is effective in cross-linking protein subunit structures through the side chains of suitably positioned amino acids such as lysine residues on the protein's surface, were performed. However on incubation with the cross-linking reagent for periods of up to 60 min. no significant decrease in the activity of the enzyme preparation was observed above a simultaneously run control incubation from which cross-linker had been omitted.

In these preliminary observations it is concluded that either cross-linking did occur but that no enzyme activity was lost through the event, or that the enzyme lacked the available groups to be cross-linked.

Cross-linkage experiments have been performed by previous workers (Giri and Ramadoss, 1979) to successfully demonstrate the subunit make-up of the native molecular form of nitrate reductase from *Chlorella vulgaris*. However further investigation into its applicability in the characterization of NADPH-cytochrome *c* reductase is required.

Other experiments were directed at investigating whether various inhibitors or substrates affected the enzyme's activity. To 0.5 ml of an 80% pure enzyme preparation in 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, 0.25 ml of either 2 mM NADPH, 2 mM iodoacetic acid or 2 mM NAD^+ were added in separate tubes. The mixtures were then left on ice for 60 min. and then diluted 10^{-2} fold in 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA, but with FAD omitted; an aliquot was removed from each dilution tube and assayed for NADPH-cytochrome *c* reductase activity. The results indicated that neither of the above incubations lead to any significant inactivation of the enzyme. Implications resulting from the above data are drawn in the Discussion (Chapter 4).

3.3.5 *FURTHER INVESTIGATIONS: ENZYME SPECTRAL ANALYSES.*

The pure, fully oxidized enzyme has absorption maxima at 272, 376 to 380, and 455 nm with a strong shoulder at 480 nm, (see Fig. 27). On the addition of successive samples of NADPH to the above preparation, the peak at 455 nm and the shoulder at 480 nm decrease in amplitude, and on further addition of NADPH to the enzyme in solution, a broad flavin semiquinone absorbance occurs over the range of 560 to 640 nm (Fig. 30, No.2). Furthermore, on titrating the enzyme with NADPH, an end point is reached in the decrease in absorbancy of the 455 nm peak and in the 480 nm shoulder (Fig. 31). From this data the ratio of the number of moles of NADPH bound per mole of enzyme is determinable in theory. The calculations indicated that the of the number of moles of NADPH bound per mole of flavoprotein was in the ratio of 200 to 1, a result which was in disagreement from that obtained by previous workers (Siegel and Kamin, 1968) that had indicated from fluorescence quenching data a ratio

FIGURE 30.

Spectral analyses performed on NADPH-cytochrome *c* reductase. By the use of a 0.6 mg/ml enzyme solution of approximately 95% purity, the following spectral scans were performed:

1. Fully oxidized enzyme;
2. Fully oxidized enzyme with 100 µl of 2 mM NADPH added;
3. As in 2., but with 50 µl of 0.23 mM cytochrome *c* added;
4. As in 3., but after 30 min. at 20°C before re-scanning;
5. Fully oxidized enzyme mixed with some crystals of $Na_2S_2O_4$.

All spectral scans were performed at zero time from mixing, unless otherwise stated.

All cuvettes were routinely zeroed at 900 nm before scans were performed starting from this wave-length.

KEY:

-·-·-· ≡ 1. as above

------ ≡ 2. as above

····· ≡ 3. as above

——— ≡ 4. as above

x x x x ≡ 5. as above

NOTE: The peak appearing at ~ 550 to 560 nm is due to the reduction of the added cytochrome *c* within the cuvette containing the enzyme.

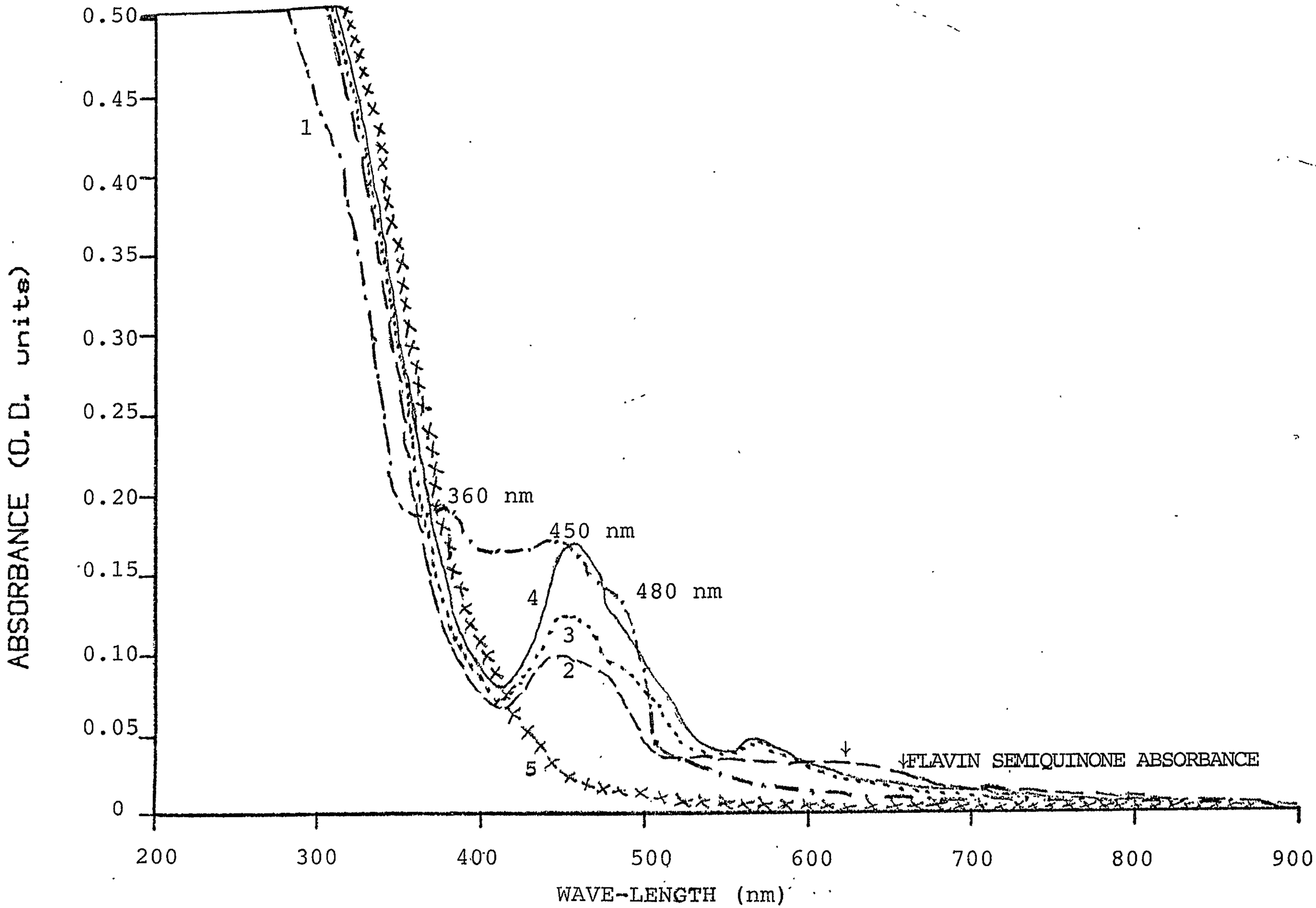
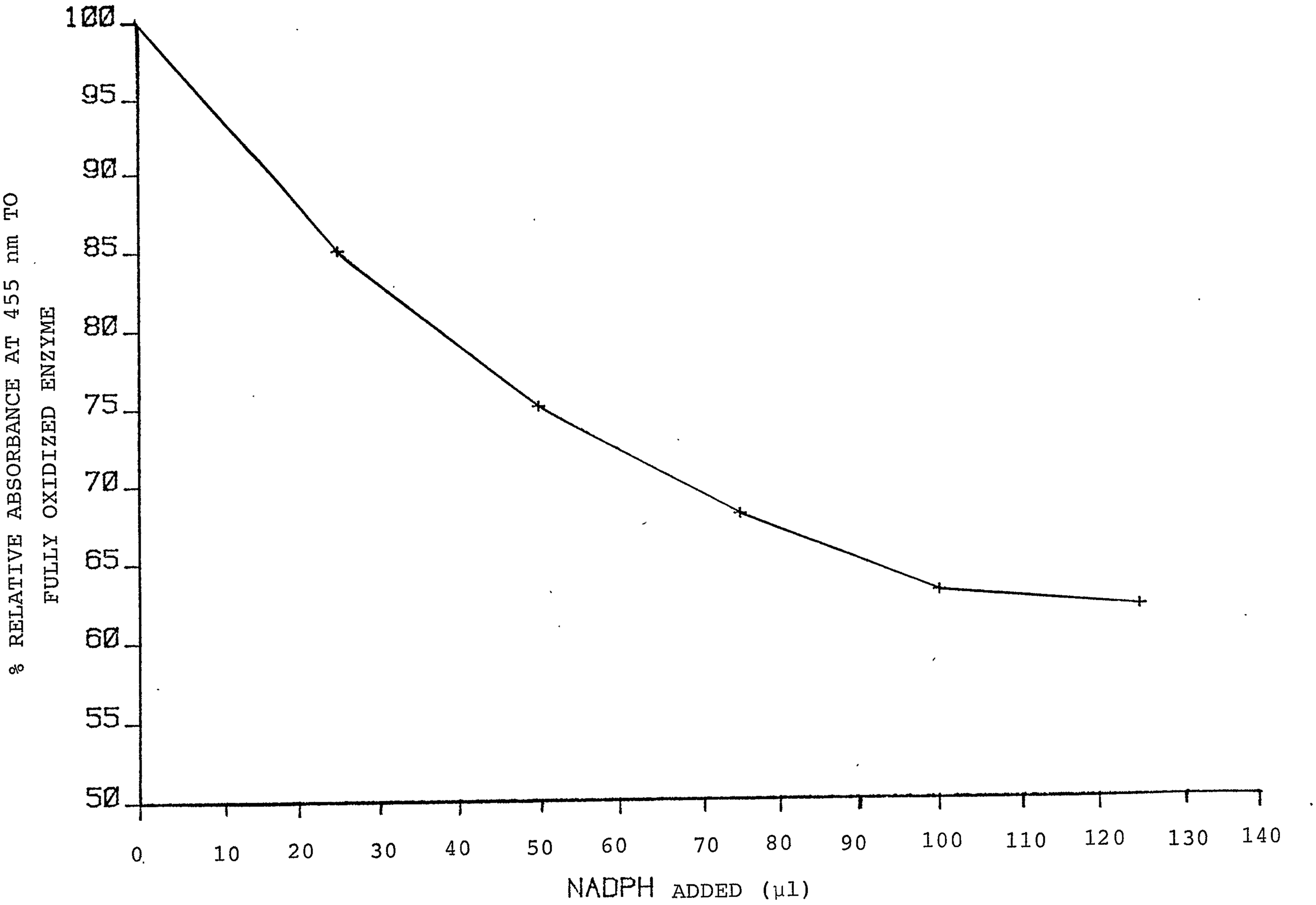

ABSORBANCE (O.D. units)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1
360 nm
450 nm
480 nm
4
3
2
5
FLAVIN SEMIQUINONE ABSORBANCE
200
300
400
500
600
700
800
900
WAVE-LENGTH (nm)

FIGURE 31.

A partial reductive titration analysis of NADPH-cytochrome *c* reductase. To a sample of purified enzyme at 0.6 mg/ml, increasing quantities of 2 mM NADPH were added and mixed. All recordings were performed at zero time after mixing, with an end-point assumed to have been reached on the addition of 125 µl of 2 mM NADPH to the fully oxidized enzyme preparation. No further reduction of the 455 nm peak height was observed on the addition of larger quantities of reducing reagent.


% RELATIVE ABSORBANCE AT 455 nm TO
FULLY OXIDIZED ENZYME
100
95
90
85
80
75
70
65
60
55
50
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
NADPH ADDED (µl)

of 8 to 1 was likely. The discrepancy arises due to the aerobic conditions used in this experiment to record the reduced flavin spectra, as flavin which is enzyme bound would be rapidly re-oxidized by the oxygen present in the solution once it had received electrons from NADPH. Therefore far larger quantities of added pyridine nucleotide are needed to effect, at best, a partial reduction of the enzyme's flavin groups, thus ideally these experiments should be repeated under anaerobic conditions in order to accurately determine the number of moles of NADPH that bind to one mole of native enzyme.

On the addition of cytochrome *c* to the NADPH partially reduced enzyme, a gradual change in peak heights was noted, (see Fig. 30, Nos.3 and 4). Over a period of 30 min. a total recovery of the 455 nm peak and 480 nm shoulder intensities to the values recorded for the fully oxidized enzyme was observed, clearly indicating the substrate dependent re-oxidation of the flavin prosthetic groups of the enzyme.

Crystals of sodium dithionite, when added to the fully oxidized enzyme in solution, lead to bleaching of the 455 nm peak and the 480 nm shoulder (Fig. 30, No.5), with no formation of any intermediary flavin redox states apparent (such as flavin semiquinone).

3.4 THE DEFINING OF AN N-TERMINAL AMINO ACID SEQUENCE OF NADPH-CYTOCHROME *c* REDUCTASE.

3.4.1 *AMINO ACID ANALYSIS OF NADPH-CYTOCHROME c REDUCTASE.*

In order to re-confirm the purity of the enzyme preparation prior to N-terminal amino acid sequencing, two independent determinations of the amino acid composition of the enzyme were performed. The results are presented in Table XVIII. As is very clear from the obtained data, the amino acid composition of the protein purified in the course of this work was, within error, identical to that achieved by previous workers (Siegel and Davis, 1974). Also on the basis of the reproducibility of the results, it can be stated that the preparation represents a highly purified enzyme that appears to be suitable for N-terminal sequencing analysis.

From amino acid analysis "B" presented in Table XVIII an apparent specific volume for NADPH-cytochrome *c* reductase of 0.732 ml/g of protein was calculated according to the method described by Schachman, (1957). A hydration value was found

TABLE XVIII: The results of an amino acid analysis of NADPH-cytochrome *c* reductase. The data presents the results of a previous analysis along with the results of two independent analyses performed on the enzyme preparation obtained during this study.

AMINO ACID:	+ANALYSIS (A):		+ANALYSIS (B):		+ANALYSIS (C):
	% amino acid concn. (nmol/ml).	¶No. Residues per subunit.	% amino acid concn. (nmol/ml).	§No. Residues per subunit.	% amino acid concn. (nmol/ml).
LYS	4.85	26	5.05	23 25	4.81
HIS	1.50	8	1.60	7 8	1.40
*ASN	N.D.	N.D.	}13.96	11 11	N.D.
*GLN	N.D.	N.D.		11 11	N.D.
ARG	5.98	32	5.40	25 27	5.23
§CYS	<1.0	3	<1.0	0.32 0.33	<1.0
ASP	9.91	53	9.40	43 46	8.98
THR	5.23	28	5.20	24 26	4.81
SER	4.67	25	4.95	23 24	4.60
GLU	12.90	69	12.40	56 61	11.86
PRO	5.42	29	5.85	27 29	4.90
GLY	6.92	37	7.45	34 36	7.80
ALA	11.40	61	12.65	58 62	12.43
VAL	7.48	40	7.85	36 38	7.64
¶MET	0.37	5	1.70	8 8	1.59
ILE	3.74	20	3.60	17 18	3.79
LEU	10.65	57	10.20	47 50	10.44
TYR	2.43	13	2.70	13 13	2.78
PHE	3.74	20	3.70	17 18	3.76
TRP	1.69	9	N.D.	N.D. N.D.	N.D.
		Σ535		Σ480 Σ511	

NOTE: +Analysis "A" was performed on a separate preparation of the enzyme by Siegel and Davis, (1974). ¶The number of amino acid residues per subunit was estimated here with the use of a subunit molecular weight value of 58,600 Da. +Analysis "B" was performed in this laboratory, with the enzyme preparation achieved during this study; § For the calculation of the number of residues per subunit, use was made of the subunit molecular weight estimate of Siegel and Davis, (1974) of 58,600 Da for the estimates given in the left column, and of 63,400 Da (this study) for those of the right hand column. +Analysis "C" was performed on this preparation by F. Morgan as an independent estimate.*For analysis "B" ASN and GLN were calculated on the assumption of equimolar amounts of these two amino acids per subunit.§CYS content was estimated in analysis "A" after performic acid oxidation, no acid oxidation was performed in analyses "B" or "C". ¶More extensive hydrolyses are needed for analyses "B" and "C" to accurately determine MET content.

to be 0.428 g of H_2O/g of protein, which was estimated from the amino acid composition according to the procedure of Kunz, (1971). Furthermore, the amino acid analysis data indicates that the ratio of acidic to basic amino acids is approximately 1.8, suggesting that the enzyme is acidic in nature.

The results in Table XVIII indicate that the enzyme subunit molecule is composed of approximately 500 amino acid residues, and from this a size for the *cysJ* gene which codes for NADPH-cytochrome *c* reductase can be predicted to be approximately 1.5 Kbp of DNA.

3.4.2 *THE N-TERMINAL AMINO ACID SEQUENCING ANALYSIS OF NADPH-CYTOCHROME C REDUCTASE.*

As was determined from amino acid analysis "B" in Table XVIII, the number of amino acid residues calculated on the basis of a weighed and hydrolysed enzyme sample, per subunit of NADPH-cytochrome *c* reductase, was in the case of every amino acid (except methionine) lower than previously published data. This systematic inconsistency between the amino acid analysis data indicates that of the weighed sample of protein analyzed there was a certain percentage of this material which was not proteinaceous in nature, that is, residual salt matter that was not removed even by the extensive dialysis prior to lyophilizing the preparation. The exact extent of salt contamination was not realised until preliminary experiments commenced on the N-terminal amino acid sequencing of the protein. In these experiments it was decided to apply the protein sample into the spinning cup in solution form after precisely determining the protein concentration by spectral analysis. Thus the procedure was to place 1.0 mg of the freeze-dried protein into 1.0 ml of glass distilled water (pH ~ 6) and to dissolve the enzyme. However, even after vortexing the mixture for 3 to 4 min. very little protein *appeared* to have gone into solution. This would seem logical in view of the acidic nature of the protein which would lead to its precipitation rather than dissolution at the pH of the mixture. Despite the apparent lack of dissolved protein, an absorption spectrum was performed on the supernatant fraction obtained after spinning the above mixture for 10 min. in an Eppendorf Centrifuge. The spectrum demonstrated peaks at 270 nm and at 450 to 460 nm, with a shoulder appearing at 370 to 380 nm. Using

the peak intensity at 270 nm as a guide, the protein concentration was estimated to be 0.5 mg/ml. This result appeared surprising in that such a relatively large proportion of the protein had in fact dissolved. When a sample of this supernatant was then placed into the Beckman Sequencer and an overnight degradation of 10 residues was programmed, the subsequent analysis of the amino acid derivatives indicated that the N-terminus was blocked, that is, no sequence read-out was ascertained from this initial experiment. However, when a pre-weighed sample of the freeze-dried enzyme was subjected to amino acid analysis and the results analyzed in terms of the amount of protein present in the sample it appeared that at best only 50% by weight of the lyophilized material was in fact protein (see Appendix 3). Thus from this it can be infered that the amount of protein observed to have gone into solution by the spectral analyses was in fact not protein but residual FAD added during the purification procedure and which went into solution producing the observed spectrum (FAD having absorption maxima at wave-lengths very close to the flavoprotein). In addition, when an amino acid analysis was also performed on the supernatant obtained from the initial attempts at dissolving the protein in water, it appeared that very little protein had in fact gone into solution and it was thus calculated that the amount of enzyme placed into the cup was only in the order of picomole amounts, levels which would not be detected by the systems used for analyzing the sequenced amino acid residues, (which are only sensitive down to nanomole amounts).

Thus to obtain an amino acid sequence the protein was placed into the cup as a suspension. The procedure was to place 493 µg of freeze-dried enzyme as a suspension in glass distilled water *directly* into the spinning cup. It should be noted though that although this amount of *weighed* material was placed into the cup, only AT BEST would there be 273 µg of actual protein loaded into the Sequencer, a fact that must be remembered when quantification of the results is attempted. Thus on the assumption that 273 µg of enzyme was placed into the Sequencer this would represent 4.31 nmol of free N-termini available for sequencing if no partial blocking had occurred on purification or if there were no natural blocking effects (the assumption of the amount of N-termini available was

calculated on the basis of the molecular weight estimate for the subunit form of NADPH-cytochrome *c* reductase of 63,400 Da; see section 3.2.6, pp. 45 and 46).

The N-terminal amino acid sequencing results obtained from this experiment are presented in Fig. 32.

When quantification of the results obtained from the N-terminal sequencing analysis was performed, the data presented in Table XIX (*part A*) was obtained. Ideally quantification of the number of amino acid residues released in each cycle of the sequencing run is performed residue by residue, commencing with the first amino acid. However the exact quantification of the first amino acid derivative in this case was not possible since the first residue, threonine, is known to degrade under the conditions of the sequencing experiment into its β-dehydro derivative. This derivative unfortunately does not react quantitatively in the colorimetric reaction used to detect the various amino acid derivatives as they are eluted from the HPLC column during the analysis procedure for the derivatives. Thus in order for some quantification to be attained, the recovery of glutamine (residue No. 3) and the recoveries of valine (residue No.4) and alanine (residue No. 8) were determined (as presented in Table XIX, *part A*).

In order to investigate the precise oligomeric structure of the native form of the enzyme more accurate quantification of the number of removed N-terminal amino acid residues needs to be performed. However despite the lack of accuracy and precision the following argument can be proposed.

On the assumption that 4.31 nmol (or quite possibly even less) of free N-termini were available for sequencing, then the results presented in Table XIX (*part A*) must logically fall within the available range of termini that *could* be sequenced. The listing of the number of N-termini available for sequencing under the experimental conditions employed above for the enzyme existing in various polymeric native states from 1 to 10 is given in Table XIX (*part* B) . On consideration therefore of the available numbers of N-termini and of the recovery trends (notably valine at position 4, with a recovery of 3.57 nmol) then as it is well understood that the recovery of any sequenced amino acid is rarely, if ever, 100% (F. Morgan, personal communication) and that the highest recoveries represent, at best,

FIGURE 32.

The N-terminal amino acid sequence of NADPH-cytochrome *c* reductase. Twenty-six residues were able to be almost completely identified from the protein's N-terminus.

The question mark indicates that the residue at this position in the protein's primary structure is doubtful due to the fact that the resolution of the peaks appearing from the HPLC detector was not unequivocal in the region of *TRP* derivative elution. The same resolution problems are presented by the residue at position "20", where either glutamic acid or glutamine may exist. Despite these queries though, it is considered that the sequence is correct, and that it represents accurately the N-terminal region of NADPH-cytochrome *c* reductase.

RESIDUE:

THR-THR-GLN-VAL-PRO-PRO-SER-ALA-LEU-PRO-LEU-ASN-TRP(?)-GLU-GLN-LEU-ALA-ARG-LEU-GLX-ALA-ALA-THR-THR-ASP-LEU-*

No. FROM
N-TERMINUS:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

TABLE XIX: Quantification of the recovery of suitable amino acids from *(part A)* the sequencing procedure. The recoveries of glutamine (residue No. 3), valine (residue No. 4) and alanine (residue No. 8) were estimated. The results are presented below.

AMINO ACID:	RECOVERY (nmol ± 10%):
GLU	2.81
VAL	3.57
ALA	2.81

(part B): The listing of the number of N-termini available for sequencing under the experimental conditions utilised for the enzyme existing in various polymeric native states from 1 to 10. The compilation is based on the assumption that 4.31 nmol of enzyme was placed into the spinning cup and that the enzyme consists of a unique subunit, (termed α).

POLYMERIC STATE:	nmol OF AVAILABLE N-TERMINI:
α_1	0.539
α_2	1.08
α_3	1.62
α_4	2.16
α_5	2.69
α_6	3.23
α_7	3.77
α_8	4.31
α_9	4.85
α_{10}	5.39

a minimum of the optimal recovery that could be obtained, then from an inspection of the data it is proposed that the enzyme exists in its native state as an octameric structure. Furthermore, on the basis of the electropherographic evidence and the sequencing results it is apparent that the enzyme consists of *identical* subunits arranged in an octameric configuration. The only other possibility that exists from the above data is that the enzyme exists in its native state as a heptamer. This possibility however is certainly unlikely intuitively, and is also somewhat unlikely from the obtained data as well, since this conclusion would mean that the percentage recovery of valine was 95%, a value too near to 100% to be really credible (although such recoveries can be obtained, it is unlikely under the conditions used in these experiments).

With any amino acid sequencing experiment the prime concern is the purity of the starting material. During the sequencing cycle there is a strong tendency for non-specific breakdown of the polypeptide chain, a phenomenon that is directly related to the size of the protein being sequenced. These non-specific cleavages arise due to hydrolytic conditions that the protein is exposed to during the degradation cycle. The ideal size of protein to be sequenced by the Edman scheme is in the order of 20,000 Da, a size that is large enough to prevent its premature "wash-out" from the spinning cup yet a size small enough to guard against the occurrence of too many non-specific cleavages along the polypeptide backbone. With a suitable protein of this molecular weight, 70 to 90 N-terminal residues can usually be unequivocally determined, with a good repetitive yield (F. Morgan, personal communication). With regard to this particular enzyme preparation, it was noted that from the very first residue there was a very high non-specific background of amino acids not forming part of the sequence. Although it is not uncommon to find non-specific breakdown products associated with the first residue from the N-terminus (F. Morgan, personal communication) the number of breakdown products observed in the first cycle of the degradation of NADPH-cytochrome *c* reductase would tend to indicate the presence of some contaminating proteins present in the enzyme preparation. The results in tabular form of the nmol of the various amino acid derivatives detected in the first sequencing cycle of the enzyme preparation

are given in Table XX. Interestingly from this data the major contaminating amino acids at the first cycle in the degradation, *viz.* alanine and glycine, appear in amounts almost proportional to there content within the protein as estimated from amino acid analysis. However this trend is not followed by all the amino acids present. Of the 11 amino acid derivatives detected in the first step of the degradation of the protein from the N-terminus, 7 of these were detected, some repetitively, in the first 26 amino acids from the N-terminus. Although these results by themselves do not conclusively indicate the exact nature of the high levels of contaminating amino acid derivatives found, they do indicate that it is most likely that these contaminants are derived from NADPH-cytochrome *c* reductase itself. Furthermore on examination of the percentage recovery of each amino acid derivative from the amount of protein placed into the spinning cup on a nmol basis, it is clear that there is an overall increase in the recovery from the input amount of protein. This would indicate that these additional amino acids encountered in the first degradation are in fact derived from NADPH-cytochrome *c* reductase. However as the precise determination of the recovery of threonine (and also some of the other listed amino acids) depends on the state of their degradation then the above data can not be taken as a conclusive statement on the nature of the observed contamination. As more residues were sequenced from the N-terminus, it became gradually more difficult to discern the primary amino acid sequence from the background levels of contaminating residues. The background became so severe that after residue 26 no further unambiguous sequence read-out could be obtained. This build-up of background "noise" during the latter stages of the degradation was no doubt due to non-specific cleavages occurring along the length of this relatively long polypeptide (~ 500 amino acid residues). However, the high "noise" levels observed during the early stages of the degradation were probably due to a number of causes. These can be thought of in terms of N-terminal "fraying" caused by the action of aminopeptidases during the isolation of the protein, and in terms of breakdown products *viz.* peptides derived from the enzyme and which were co-purified with the native enzyme. These breakdown products could well have arisen from the action of peptidases acting during the course of the

TABLE XX: The results obtained on the first sequencing cycle of the enzyme preparation. The data serves to illustrate the high background "noise" levels of contaminating amino acids detected which gradually grew to unacceptably high levels later on in the sequencing at residue "26", such that the primary sequence was no longer able to be unambiguously identified.

INPUT INTO THE CUP:

4.31 nmol

OUTPUT FROM THE CUP IN THE FIRST SEQUENCING CYCLE:

AMINO ACID:	nmol RECOVERED:	% RECOVERY FROM INPUT:
ASP	0.23	5.34
*THR	4.00	92.8
SER	0.27	6.26
GLX	0.75	17.4
GLY	0.87	20.2
ALA	1.49	34.6
VAL	0.39	9.05
LEU	0.26	6.03
HIS	0.35	8.12
LYS	0.25	5.80
MET	0.46	10.7

NOTE:

* The residue assignment for the first amino acid of NADPH-cytochrome *c* reductase was given to threonine, on the basis of the results presented here.

purification, a consideration that has been previously noted, (see section 3.3.1, p. 48), and therefore the quality of this preparation of NADPH-cytochrome *c* reductase has been lowered by the methods employed during its purification. In order to enable the entire sequence of the protein to be determined, another major purification of the enzyme would be necessary, but with the precautions outlined previously, that is, by the inclusion of peptidase and protease inhibitors at each step in the purification procedure.

With the data obtained on the sequence of N-terminal amino acids for NADPH-cytochrome *c* reductase a listing can be readily compiled for the putative sequence of DNA nucleotides coding for the N-terminus of the enzyme, and thus for the translation commencement region of the *cysJ* gene. This listing for the first 26 residues of the protein is presented in Fig. 33. Out of the 78 nucleotides coding for the first 6 amino acid residues of the enzyme, 44 of these can be predicted with certainty with respect to their position along the DNA sequence of the translation commencement region of the gene. Thus the location of these nucleotides on a DNA sequence could be readily achieved and with this knowledge it would then be feasable to determine, by subsequent mRNA fingerprinting, the precise transcription start site for the *cysJ* gene. From this information the promoter region for the *cysJIH* operon could be ascertained which would then demonstrate whether this particular promoter is typical (in having a structure noted for other promoters under the influence of positive control) or whether it represents a new class of well defined promoter structures which has not yet been investigated.

FIGURE 33.

A listing of the putative sequences of DNA nucleotides coding for the N-terminus of NADPH-cytochrome *c* reductase. The listing is compiled from the first 26 known amino acid residues of this enzyme.

Those nucleotides which are predictable with certainty in their position along the DNA sequence are printed in *italics*.

The question mark indicates that the residue at this particular position in the polypeptide chain is doubtful due to the fact that the resolution of the peaks appearing from the HPLC detector was not unequivocal in the region of *TRP* derivative elution. The same resolution problems are presented by the residue at position "20" where either glutamic acid or glutamine may exist.

For simplicity, the variable third bases present in the second degenerate class of codon triplets representing the amino acids arginine, leucine and serine have been omitted, with these amino acids designated by the first two base pairs of both of their triplet degenerate classes and by the bases in the third position present in the major degenerate class of codon triplets.

Note also that the first base of the codon triplets for glutamic acid *and* for glutamine have both been listed due to the uncertainty of the identity of this particular residue.

AMINO ACID RESIDUE:

1	2	3	4	5	6	7	8	9	10	11	12	13		14	15	16	17	18	19	20		21	22	23	24	25	26
THR-	THR-	GLN-	VAL-	PRO-	PRO-	SER-	ALA-	LEU-	PRO-	LEU-	ASN-	TRP	(?)	-GLU-	GLN-	LEU-	ALA-	ARG-	LEU-	GLX*	-ALA-	ALA-	THR-	THR-	ASP-	LEU-	

DNA CODON ARRANGEMENTS:

T	T		T	T	T		T		T								T					T	T	T	T		
C	C	T	C	C	C	AG T	C	G A G	C	G A G	G			T	T	G A G	C	G C G	G A G	G T T		C	C	C	C	G	G A G
TG	*TG*	*GT*	*CA*	*GG*	*GG*	TC C	*CG*	A A	*GG*	A A	*TT*	*ACC*		*CT*	*GT*	A A	*CG*	T A	A A	C C		*CG*	*CG*	*TG*	*TG*	*CT*	A A
G	G	C	G	G	G		G		G		A			C	C		G					G	G	G	G	A	
A	A		A	A	A		A		A								A					A	A	A	A		

CHAPTER 4.

DISCUSSION.

4.1 GENETICAL ASPECTS.

The gene dosage effect that was observed during this study in strains transformed with the hybrid plasmid, pRL3, can be explained with respect to the regulation of the *cysJ* gene as outlined below.

Before any transcription can commence from the *cysJ* promoter, the *cysB* protein and the inducer, *O*-acetyl-L-serine (OAS), must both be present and the intracellular concentration of cysteine must be at a low level. Under the culture growth conditions used it can be assumed that sufficient OAS is present within the cell and that the cysteine concentration is low within the cell cytosol. However, as expression from the *cysB* gene is controlled by an autoregulatory mechanism and as there are only a few molecules of the *cysB* protein in wild-type cells (Jagura-Burdzy and Hulanicka, 1981) then in order to synthesize increased levels of the *cysJ* protein, the synthesis of the *cysB* protein must be derepressed. Conceivably this could occur by a titration of the *cysB* protein through a binding reaction onto the DNA region of the promoter for *cysJ* on the plasmid, pRL3, leaving little *free cysB* protein within the cytosol to interact back in a repressive manner at the promoter region for the *cysB* gene itself and thus *more cysB* protein would be synthesized within the cell.

When strain RL503 is grown on cysteine, the specific activity of NADPH-cytochrome *c* reductase falls from that recorded when growth was on L-djenkolic acid in approximately the *same* ratio as noted for wild-type cells grown under the same conditions, that ratio being 20 to 25 for cultures grown under derepression as compared to cultures grown under repression for cysteine biosynthesis. Furthermore, it has been noted that the *cysB* locus is itself *refractory* to cysteine repression (Jagura-Burdzy and Hulanicka, 1981), thus the effect of a heightened cysteine concentration within cells must be to bind to the *cysB* protein in such a way as to prevent the *cysB* protein from binding to the available *cysJ* promoters. This inhibition of protein-DNA interaction would serve to bring the *cysB* protein concentration within the cytosol down to wild-type levels on being diluted out on further culture growth, by allowing the now "free" protein to directly autoregulate its own synthesis, as no titration effect of

the protein from the cytosol could occur with cysteine present.

The data in Table V (p.29(a)) presents a summary of the effects of various growth conditions and chloramphenicol supplementation on the specific activity of NADPH-cytochrome *c* reductase from prepared cell extracts. What can not be inferred from the data in Table V and from the discussion above however, is the *maximium* ratio to which the *cysB* gene can be derepressed for its own synthesis over wild-type cells, since enzyme levels can only measure the final stoichiometry of the DNA-regulatory protein interaction, and when either component is present in limiting amounts then the limited component would dictate the amount of enzyme produced and hence assayed for. Thus the level of derepression *inferred* is not necessarily the *possible* level for the *cysB* gene, which could in fact be considerably higher. Furthermore, the kinetics of the protein-nucleic acid association reaction can not be inferred either, from these studies, and in addition, the interaction of the *cysB* protein with other proteins such as RNA-polymerase is a further possibility, but firm conclusions can not yet be drawn on a definition of these crucial factors from studies done to date.

In summary, however, the above data predicts and supports the autoregulation of the *cysB* gene by the *cysB* protein.

The specific activities of NADPH-cytochrome *c* reductase present in crude extracts of strain RL503 were seen to vary considerably from culture to culture, even when subculturing techniques were not employed. The large differences can be ascribed to a combination of events within the cytosol. Firstly, although the *cysJ* gene may be being faithfully transcribed and translated, the enzyme being produced may be anomalously aggregating in the cytosol forming inactive high polymeric forms. This explanation is feasable, yet intuitively it would be anticipated that this phenomenon be consistently observed throughout all prepared crude extracts, that is, the enzyme specific activities of NADPH-cytochrome *c* reductase remain at some fixed level. However under the culturing conditions used the specific activities of the enzyme were observed to constantly fluctuate from crude extract to crude extract and as the cells used for the enzyme purification

were grown from separate individual transformant colonies of strain RL503, then it is most probable that the fluctuations were caused at the genetic level and not by some aggregation of enzyme within the cell's cytosol as an aggregation of enzyme would be expected to be a reproducible event occurring irrespective of the transformant clone used for cell growth. Thus it is presumed that some form of mutation has occurred on some of the plasmids present in the transformants such as to affect transcription and/or translation of the *cysJ* gene carried by these plasmids. This would explain why the enzyme specific activities of NADPH-cytochrome *c* reductase varied so unpredictably and significantly from culture to culture.

The wide range of specific activities of NADPH-cytochrome *c* reductase observed in various *E. coli* strains is given in Table XVI (p.48(a)). Both strains RL502 and RL503 show high levels of enzyme, with their respective parent strains (RL421 and RL434) showing no significant levels of specific activity for the enzyme. On transducing these parent strains then to Cys^+ with the specialized defective transducing phage, λd*cysJIHD*, NADPH-cytochrome *c* reductase levels were seen to increase to approximately wild-type specific activity, indicating that the phage has integrated into the host cell chromosome and that the cell is expressing these introduced genes at a level commensurate to one copy of expressed gene per host cell chromosome.

To reconstitute NADPH-sulphite reductase activity (which is dependent on the *cysJ* and *cysI* genes) from crude extracts of cells lacking either the *cysJ* polypeptide, (RL421) or the *cysI* polypeptide, (RL503) an *in vitro* assay was performed (Table XVII, p.50(a). As the levels of reconstitution were very small it was implied that the point mutant in strain RL421 is polar, hence preventing the effective transcription of the *cysI* gene needed to give wild-type specific activity of NADPH-sulphite reductase on mixing the two crude extracts. Further assays, (Table XVI, p.48(a)) indicated that in crude extracts of strain RL502, NADPH-sulphite reductase and NADPH-hydroxylamine reductase specific activities were significantly lower than in the wild-type strains, (these two enzymes are also dependent on the *cysJ* and *cysI* genes). This further suggests that strain RL421 has a polar mutation in the *cysJ* gene and that although the transformant

strain (RL502) is Cys^+, it still lacks wild-type levels of the *cysI* encoded polypeptide.

Not surprisingly, the plasmid bearing strains show dramatically increased levels of NADPH-DCPIP reductase (which is dependent *only* on the *cysJ* gene, see Table XVI, p. 48(a)) over wild-type crude extracts, however the ratio of NADPH-DCPIP reductase levels between the plasmid bearing and the non-plasmid bearing strains is significantly less than that recorded by assaying NADPH-cytochrome *c* reductase levels in the same strains. Clearly then these assays need to be extensively reviewed before any explanation can be offered with regard to the anomalous variance in ratios observed.

4.2 BIOPHYSICAL ASPECTS.

The binding assays of NADPH-cytochrome *c* reductase to the dye-ligand media revealed that *p*-CMB inhibited enzymatic activity very little under the conditions described, but that it completely blocked any interaction of the enzyme with the dye-chromophore. As other experiments had indicated, the limited binding reaction was in fact a specific event between the NADPH active site on the enzyme and the dye attached to the cross-linked agarose base, thus the inhibition of any interaction when *p*-CMB was added to the enzyme would indicate that *p*-CMB causes a conformational alteration in the protein's structure such as not to greatly interfere with NADPH binding, but to completely abolish some minor structural feature essential to the dye-enzyme interaction.

In other experiments it was found that when the enzyme was incubated with NADPH in either the presence of or absence of FAD then enzyme activity was inhibited in the former case with no inhibition of enzyme activity noted when FAD was omitted from the incubation mixture. This observation is most probably due to the syphoning of reducing power from NADPH through to FAD and thence to dissolved free oxygen in the assay mixture, a reaction which would be running in competition with the reduction of cytochrome *c* in the assay. As activity is not inhibited when FAD is omitted from the incubation then this would support the hypothesis that this phenomenon is due entirely to the redirection of reducing power from NADPH to various electron acceptors.

In summary then, the dye-ligand approach has useful

potential in monitoring any possible steric conformational changes occurring at the enzyme's NADPH active site when the enzyme interacts with various substrates and inhibitors. Yet its full application to further study of this enzyme remains to be performed.

Inhibition of NADPH-cytochrome *c* reductase was not observed *either* with pre-incubation with NAD^+ *or* iodoacetic acid. This indicated that dipyridine nucleotides were not effective in interacting with the NADPH active site of the enzyme and that no sulphydryl groups partake directly in the enzyme's catalytic cycle. (The published data of Siegel and Davis, (1974) indicated that only 3 cysteine residues were present per enzyme subunit, none of which are obviously necessary for enzymatic function based on the incubation data above).

When the enzyme was partially denatured on dialysis into buffer containing no supplemented FAD and then when renaturation was attempted by dialysis back into an FAD containing environment, no significant increase in enzyme activity could be detected. Previous workers (Madyastha and Coscia, 1979) have noted that an irreversible dissociation of FAD occurred from preparations of NADPH-cytochrome *c* (P-450) reductase from *Catharanthus roseus* , leading to a partial denaturation of the enzyme. Clearly then, the partial removal of FAD from NADPH-cytochrome *c* reductase leads to an irreversible denaturation event similar to that noted with other flavoproteins.

4.3 ELECTROPHORETIC ANALYSIS.

On comparing the protein components present in strain RL503 crude extracts to those within the crude extracts of wild-type strains, three new major protein bands were apparent in the plasmid bearing strain. One of these (corresponding to a subunit molecular weight of 63,400 Da) is NADPH-cytochrome *c* reductase, the other two bands (corresponding to subunit molecular weights of 35,200 and 73,500 Da) are other plasmid encoded proteins and are readily detectable by the present gel systems because of their high concentration within the cell (the concentration being proportionate to the number of plasmid copies per cell).

The purified enzyme appears almost homogeneous when

analyzed by silver nitrate staining techniques and it was therefore assumed that the present enzyme preparation was sufficiently pure for sequencing experiments.

4.4 N-TERMINAL AMINO ACID SEQUENCING OF NADPH-CYTOCHROME *c* REDUCTASE.

The data obtained from the sequencing clearly demonstrated the failures of the present purification scheme for NADPH-cytochrome *c* reductase. Firstly, it would have been expedient to have included protease and aminopeptidase inhibitors throughout the purification scheme so as to have avoided the large background "noise" readout from the amino acid derivative detection systems, this "noise" being due to N-terminal fraying and the presence of protein breakdown products. Secondly, a longer period of dialysis would be indicated when trying to remove salt material from the enzyme, as in the present methods it appeared that a great deal of salt material had been carried over with the freeze-dried enzyme which made an analysis of the quantification of the results less definite than it would have otherwise been. Despite these drawbacks though an unambiguous N-terminal amino acid sequence was obtained which demonstrated some features of the protein. From the sequence data it can be said that the N-terminus is neither blocked nor does it still have its N-terminal formyl-methionine group which presumably was split away from the enzyme after translation. The fact that within ten residues from the enzyme's N-terminus there exist three proline residues would suggest that this portion of the protein contains no defined secondary and hence tertiary structure, as proline is characteristically a disrupter of α-helical protein structure. This point concerning the structure of the N-terminal region would seem obvious with respect to its lack of defined structure but it serves to underline the fact that the N-terminus is most likely exposed to the surface of the protein in its native state, or otherwise be buried within the protein in such a way as to be readily available to external solvents when non-covalent forces are broken up within the protein's structure. It is therefore the lack of covalent interactions within the native protein (as seen by the lack of cysteine residues) that the N-terminus is so readily available in this protein for sequencing. However in order to achieve a *full* amino acid sequence for the enzyme it is felt that another

preparation of the enzyme should be obtained as the present enzyme sample has too many breakdown "contaminants" that would severely interfere with cyanogen bromide cleavage techniques used for complete protein sequence determination.

In conclusion then, a preparation of NADPH-cytochrome *c* reductase which was judged to homogeneous on the basis of an electrophoretic analysis and amino acid analysis was used to determine the N-terminal amino acid sequence of the protein. It is felt that this data will be invaluable at future efforts aimed at determining the translation commencement site for the *cysJ* gene and hence from this determine the transcriptional start site for the *cysJ* gene and thus for the *cysJIH* unit of transcription. Once this information is obtained then the promoter region for the *cysJIH* gene cluster can be readily investigated at the molecular level in order to more fully understand the precise manner of regulation of cysteine biosynthesis in *E. coli*.

APPENDIX 1.

FORMULAE OF NUTRIENT AND MINIMAL MEDIA:

L Broth

Per litre: 10 g tryptone
5 g yeast extract
5 g NaCl
Adjusted to pH 7.0 with 1 N NaOH
5 ml 20% glucose (Added after autoclaving).

Routinely supplemented with 0.42 mM cystine.

L Agar

Per litre: 10 g tryptone
5 g yeast extract
5 g NaCl
Adjusted to pH 7.0 with 1 N NaOH
15 g agar
5 ml 20% glucose (Added after autoclaving).

Davis and Mingioli Minimal Medium (modified)

Per litre: 14 g K_2HPO_4
6 g KH_2PO_4
1 g $Na_3citrate.3H_2O$
10 ml 81 mM $MgCl_2$
1.62 g NH_4Cl

Sterile 2x salts are mixed with an equal volume of sterile 2x agar (30g/l), supplements added, and the mixture poured.

Vogel and Bonner Minimal Medium E

Per litre of 50x concentrated medium:
670 ml H_2O
8.22 g $MgCl_2.6H_2O$
100 g citric acid.H_2O
500 g K_2HPO_4
175 g $NaNH_4HPO_4.4H_2O$

Final medium: Add 20 ml concentrated (50x) per litre and supplement with glucose and amino acids.

Cystine (Supplement for 1 litre of Medium).

Per litre: 4.2 ml 0.1 M cystine (dissolved in 1 N HCl)
4.2 ml 1 M *tris* base.

APPENDIX 2.

A full treatment of the theory behind the automated Edman degradation procedure is given by Niall, (1977) with an elementary summary of the reactions involved presented in Fig.34. In this project, use was made of the Beckman Model 890C protein sequencer which operates through liquid phase sequencing by means of sample application (either in solution form or in suspension) to a continuously spinning glass cup. The instrument is essentially modular in design, with the various components dedicated to certain functions during the sequencing cycle (Fig. 35, *part A* and *part B*)

The success of any liquid phase sequencing experiment relies on the denaturation of the protein sample applied to the cup with the phenylisothiocyanate (PITC) coupling buffer, Quadrol (*N ,N .,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine), the pK_a of which is slightly less than 9. Quadrol has been noted to be an excellent protein solvent due to the hydrogen bonding properties of the hydroxypropyl groups. It is for its buffering at alkaline pH and protein solubility characteristics that Quadrol is most effective in breaking down the tertiary structure of a protein that has arisen through any non-covalent attractive forces.

Once the protein has been denatured the N-terminus is then sterically available for coupling to PITC and the sequencing cycle can proceed from thereon. The sequencing cycle is not unambiguous however, with many side reactions occurring, the most serious of which is non-specific cleavage of the polypeptide chain, an event which occurs with a higher frequency as the length of the polypeptide chain is increased, optimal conditions for extended cleavage cycles being obtained with proteins of molecular weight 20,000 to 25,000 Da.

The programme format utilised for sequencing proteins on the Model 890C is presented in Table XXI. Before any protein sequencing run however, a 6 hour preliminary mock sequencing period is performed on the synthetic homopolymer, polybrene, (1,5-dimethyl-1,5-diazoundecamethylene polymethobromide, hexarimethane bromide). This procedure is expedient as it serves to coat the inner walls of the cup with a solid support which acts as a "carrier" during the main sequencing run. It is

FIGURE 34.

A simplified diagram of the Edman degradation reaction. The stages are as follows:

I Phenylisothiocyanate (PITC) is covalently coupled to the protein's N-terminus, in alkaline conditions, to form the coupled adduct (the phenylthiocarbamyl protein derivative).

II In anhydrous acid, a nucleophilic attack, by the sulphur derived from PITC, occurs on the carbonyl component of the first amino acid residue, which results in the cleavage of the amino-terminal residue from the polypeptide as an anilinothiazolinone. The remaining protein now is one residue shorter in length (III).

IV-V After extraction of the heterocyclic amino-acid derivative from the residual polypeptide by an organic solvent, it is converted in aqueous acidic conditions *via* the phenylthiocarbamyl form (IV) to the more stable phenylthiohydantoin derivative (V), which is then identified by various chromatographic procedures.

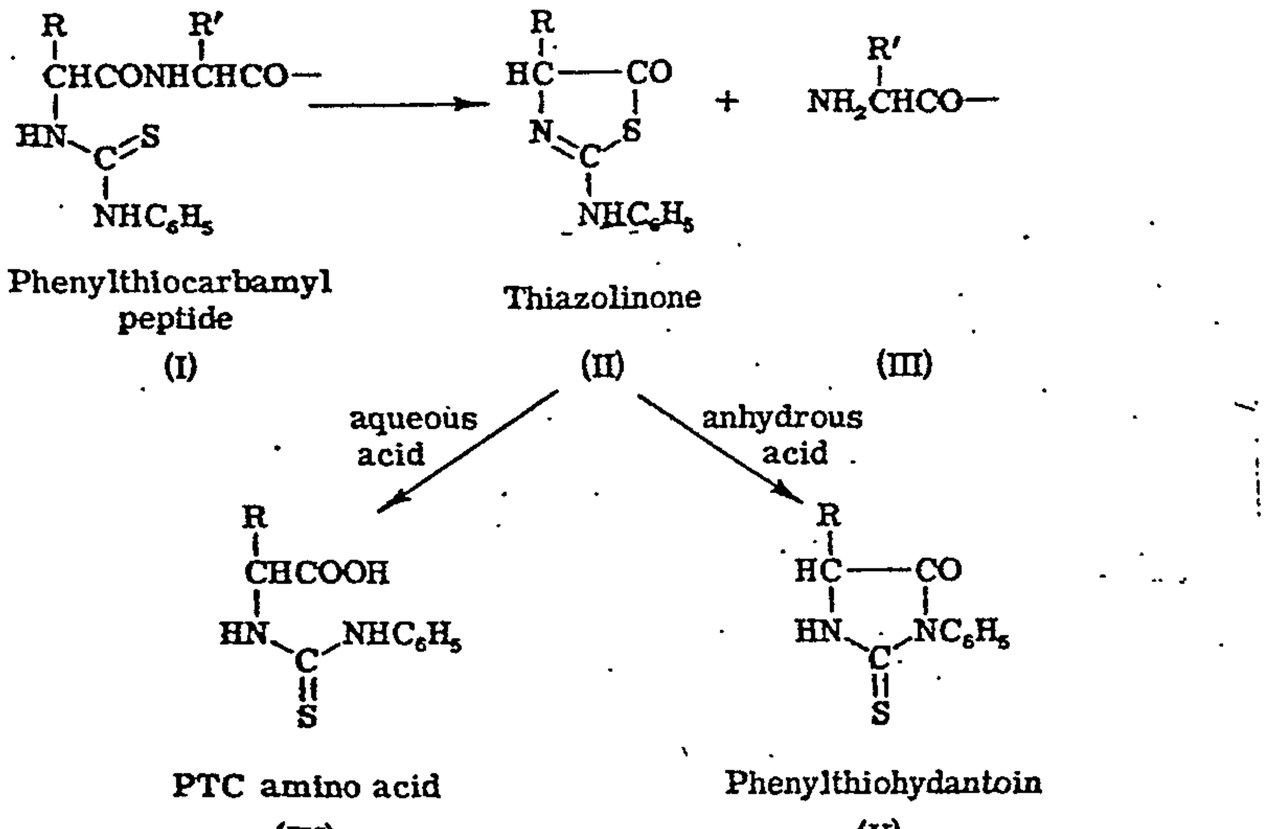

Phenylthiocarbamyl peptide
(I)
Thiazolinone
(II)
(III)
aqueous acid
anhydrous acid
PTC amino acid
(IV)
Phenylthiohydantoin
(V)

FIGURE 35.

PART A:

A schematic modular outline of the design of the Beckman Model 890C sequencer. Only modules I, II and III have so far been automated in the Model 890C.

PART B to Fig. 35 is shown overleaf.

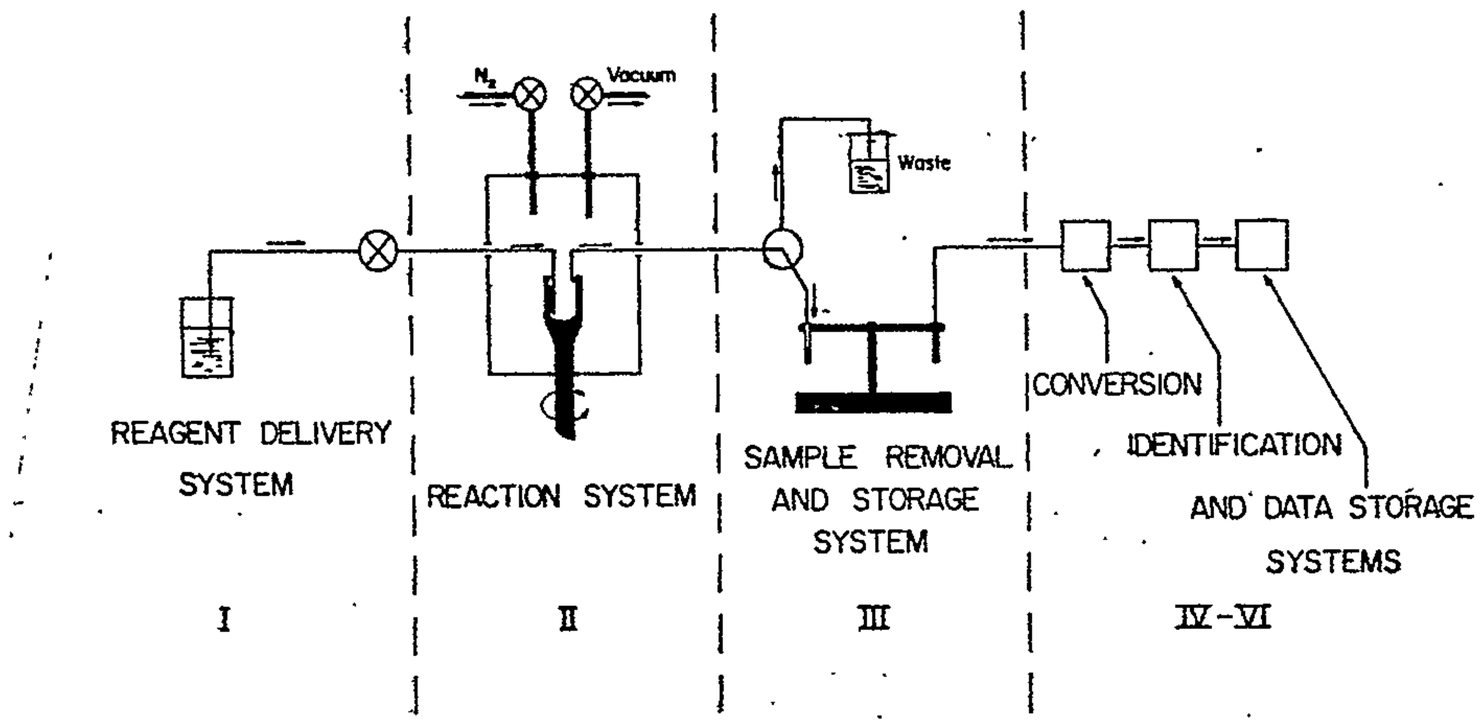

N_2
Vacuum
Waste
REAGENT DELIVERY SYSTEM
REACTION SYSTEM
SAMPLE REMOVAL AND STORAGE SYSTEM
CONVERSION
IDENTIFICATION AND DATA STORAGE SYSTEMS
I
II
III
IV-VI

FIGURE 35. *(cont.,)*

PART B:

A schematic outline in detail of modules I and II of the Beckman Model 890C Sequencer. All operations indicated are automatically controlled through the "basic protein sequencing programme" as given in Table XXI.

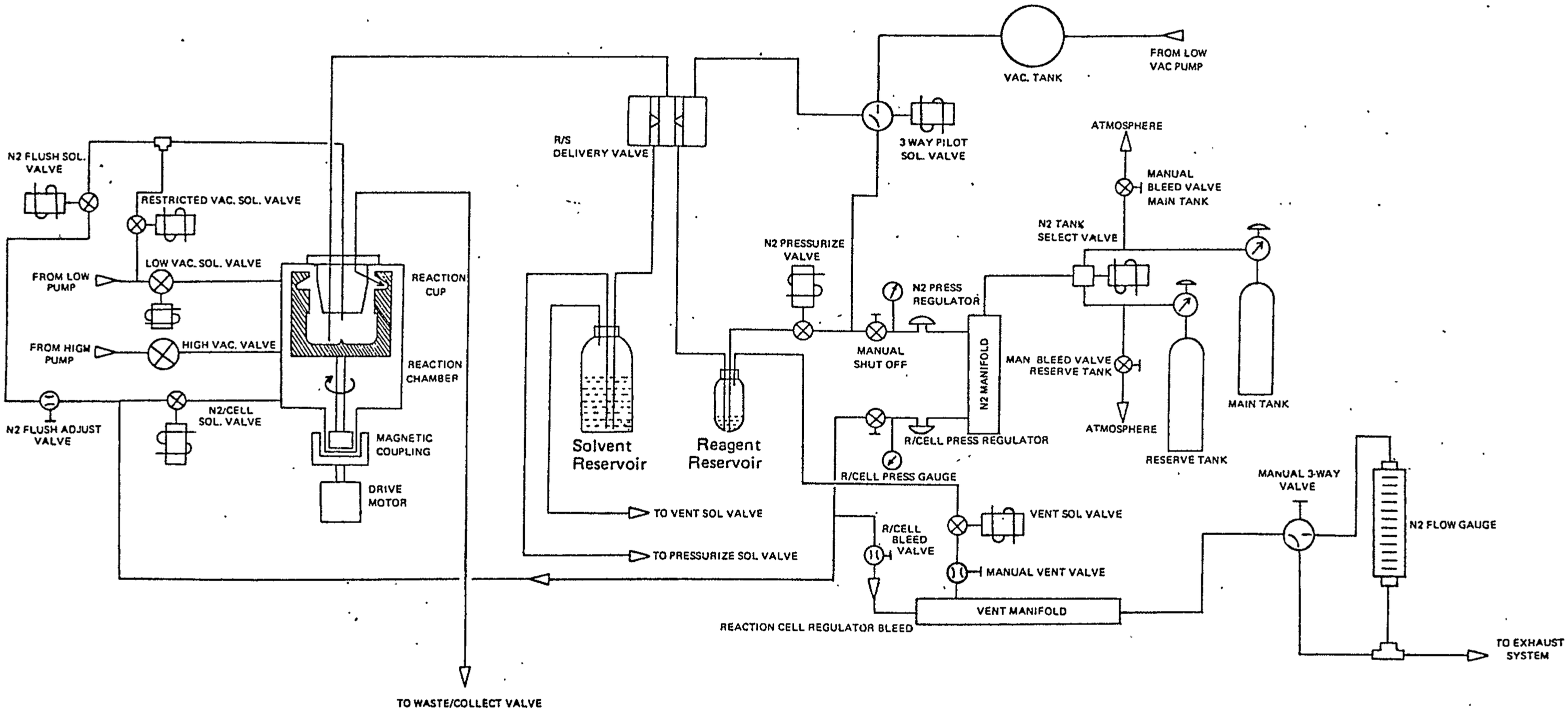

VAC. TANK
FROM LOW VAC PUMP
R/S DELIVERY VALVE
3 WAY PILOT SOL. VALVE
ATMOSPHERE
MANUAL BLEED VALVE MAIN TANK
N2 FLUSH SOL. VALVE
RESTRICTED VAC. SOL. VALVE
N2 TANK SELECT VALVE
N2 PRESSURIZE VALVE
LOW VAC. SOL. VALVE
FROM LOW PUMP
REACTION CUP
N2 PRESS REGULATOR
MANUAL SHUT OFF
N2 MANIFOLD
MAN BLEED VALVE RESERVE TANK
MAIN TANK
FROM HIGH PUMP
HIGH VAC. VALVE
REACTION CHAMBER
ATMOSPHERE
R/CELL PRESS REGULATOR
RESERVE TANK
N2 FLUSH ADJUST VALVE
N2/CELL SOL. VALVE
MAGNETIC COUPLING
Solvent Reservoir
Reagent Reservoir
R/CELL PRESS GAUGE
MANUAL 3-WAY VALVE
N2 FLOW GAUGE
DRIVE MOTOR
TO VENT SOL VALVE
VENT SOL VALVE
R/CELL BLEED VALVE
TO PRESSURIZE SOL VALVE
MANUAL VENT VALVE
VENT MANIFOLD
REACTION CELL REGULATOR BLEED
TO EXHAUST SYSTEM
TO WASTE/COLLECT VALVE

TABLE XXI: The programme utilised to actuate the sequencing cycle in the Beckman Model 890C sequencer.

Programme step:	Programme statements:	Step time (s):
1	Reaction cell pressurize	4
2	R1,R2,S1,S2 nitrogen vent	30
3	R1,R2,S1,S2 pressurize	30
4	Delay	1
5	R1 deliver (waste open)	2
6	Restricted vacuum	40
7	Reaction cell pressurize	4
8	Nitrogen dry	20
9	R2 deliver (waste open)	9
10	Delay	1
11	S2 deliver (waste open)	4
12	Coupling reaction	1230
13	Restricted vacuum	200
14	Low vacuum	100
15	High vacuum and nitrogen flush	300
16	Reaction cell pressurize	4
17	S1 deliver (waste open)	200
18	Post S1 delay (waste open)	4
19	S2 deliver (waste open)	500
20	Post S2 delay (waste open)	60
21	Restricted vacuum	200
22	Low vacuum, R3,S4 vent and FC progress	30
23	High vacuum, nitrogen flush and R3,S4 pressurize	30
24	High vacuum and nitrogen flush	400
25	Reaction cell pressurize	4
26	R3 deliver (waste open)	8
27	Delay	1
28	S3 deliver (waste open)	20
29	Cleavage reaction	120
30	Nitrogen dry	60
31	Restricted vacuum	60
32	Low vacuum	100
33	Reaction cell pressurize	4
34	S4 deliver (FC open)	150
35	Post S4 delay (FC open)	30
36	Restricted vacuum	60
37	Low vacuum	20
38	High vacuum and nitrogen flush	300
39	Return to step 4.	64

Abbreviations are as follows:

R1: 5% PITC in n-heptane
R2: 0.1 M Quadrol in n-propanol/H_2O, 3/4 (v/v)
R3: Anhydrous heptafluorobutyric acid
S1: Benzene
S2: Ethyl acetate
S4: Butyl chloride
FC: Fraction collector

proposed (G. Begg and F. Morgan, personal communication) that the "carrier" adsorbs onto itself any impurities present in the reagents or solvents delivered to the cup and hence this prolongs the extent of any intended degradation (it is also thought that the presence of the solid support guards against peptide washout from the cup on the sequencing of protein cleavage fragments). The polybrene is itself degraded under the conditions used in the sequencing cycle and its effectiveness as a protective agent is lost when the length of the polybrene polymer becomes shorter than that of the intended amino acid segment to be sequenced, be it a peptide or a number of residues from a protein's N-terminus. The preliminary run on the Sequencer thus serves to introduce the support into the cup and also serves to perform any necessary programme adjustments and possible debugging such as to observe that the solvents and reagents are correctly delivered into the cup. After approximately five mock sequencing cycles with the polybrene, the protein sample is then layered into the spinning cup over the carrier layer. The protein is loaded as a suspension or as a solution in a total volume of 400 µl with an approximate protein concentration of 0.5 mg/ml (accurate volumes and concentrations being needed if quantification of the results is desired). Once the sample is applied, the cup is heated to approximately 50^{0}C and a high vacuum is then applied in slow stages to the reaction chamber to dry off the water carried over by the injected sample. The machine is then set to the desired number of cycles and the programme started. The schedule presented in Table XXI is then followed, and repeated until the required number of cycles have been effected. Once the cycles are complete, the split off amino acids (in the anilinothiazolinone form), are removed from the refrigerated fraction collector and are dried down under a stream of nitrogen (to remove the butyl chloride used to extract the amino acid derivatives). After drying, 200 µl of 20% trifluoroacetic acid is added to each sample tube. The tubes are purged with nitrogen, sealed and heated at 80^{0}C for 14 min. then cooled to room temperature. The sample tubes are then unsealed and the trifluoroacetic acid is dried off under a stream of nitrogen, the resulting powder being dissolved in 50 µl of 50% acetonitrile and transferred into an HPLC sample vial. A 5 µl aliquot of the dissolved phenylthiohydantoin-amino acid is then used to

identify the particular amino acid derivative in an HP 1084B high pressure liquid chromatography system. A gradient elution is used, proceeding from 100% 0.01 M sodium acetate buffer, pH 4.5 to 100% acetonitrile. A mixture of external phenylthiohydantoin-amino acid standards are chromatographed before the unknowns in order to calibrate peak positions and areas. Data is printed out as nmoles of unknown amino acid derivative, recovered in each cycle of the degradation, along with the identity of the unknown amino acid residue present at that point in the protein's primary structure.

APPENDIX 3.

In order to estimate the content of protein present in the lyophilized enzyme sample, the following calculation was performed.

From the data given in amino acid analysis "C" (see Table XVIII) the concentration percentage of leucine was taken as 10.44 nmol/ml in a total concentration of amino acids in the hydrolysed sample of 412 nmol/ml; from this data and from the assumption that the subunit molecular weight of the enzyme is 63,400 Da with approximately 64 leucine residues per subunit (the number of leucine residues quoted here was determined from the number of μmoles of leucine per g of protein from the data of Siegel and Davis, 1974; with the actual number of residues being calculated on the basis of the subunit molecular weight estimate of the enzyme from the present study), then the argument below was proposed:

LEU was selected as a representative amino acid and has a nmol/ml % of 10.44. Hence there is $\frac{10.44}{100} \times 412 = 43.0$ nmol/ml of LEU.

Thus 43.0 nmol/ml of LEU represents approximately $\frac{43.0}{64 \times 8}$* nmol/ml of enzyme. Thus from this calculation there are $\left[\frac{43.0}{64 \times 8} \times 8 \times 63,400\right]$ ng of enzyme present. This calculates to be 42.6 μg of protein present in the 85.0 μg of "protein" used for the amino acid analysis. It is felt that the high recorded percentage of non-proteinaceous material remaining in the freeze-dried enzyme sample represents carried over salt which was not effectively removed by the dialysis prior to lyophilization. Despite the high salt content though, a sequencing run was able to be successfully performed on this enzyme preparation by the use of the Beckman Model 890C amino acid sequencer.

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* The subunit structure of the native form of the enzyme was estimated from this present study to be octameric.

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