INVESTIGATIONS INTO THE MECHANISM OF PORPHOBILINOGEN DEAMINASE.

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To my wife, Clare.

Abbreviations and Nomenclature

Abbreviations

ALA	5-Aminolaevulinic acid
ATP	Adenosine triphosphate
CoA	Coenzyme A
f.p.l.c.	Fast protein liquid chromatography
GTP	Guanosine triphosphate
h.p.l.c.	High performance liquid chromatography
Hz	Hertz
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
n.m.r. spectroscopy	Nuclear magnetic resonance spectroscopy
PBG	Porphobilinogen
SDS	Sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) amino methane

Nomenclature

In the structures drawn in this thesis both the beta substituents of the pyrrole rings and of the porphyrin macrocycle have, in some cases, been abbreviated to single letters to save space. These abbreviations are as below:

A = $-CH_2COOH$; P = $-CH_2CH_2COOH$; M = $-CH_3$;

V = $-CH_2CH_2$

In the text tetrapyrromethanes are abbreviated by writing the beta substituents in order reading from either the NH_2 or OH functionality.

Enzyme-intermediate complexes are abbreviated to ES (enzyme with one substrate molecule bound), ES_2 (enzyme with two substrates bound) and ES_3 (enzyme with three substrate molecules bound). Since the substrate has been deaminated on binding to the enzyme it is not strictly the substrate; however, the notation S has been adopted for simplicity.

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Abstract.

Porphobilinogen deaminase has been purified and crystallized from an over-producing recombinant strain of Escherichia coli harbouring a hemC containing plasmid which has permitted the isolation of milligram quantities of the enzyme. Determination of the molecular weight of the enzyme by SDS gel electrophoresis (35,000) and gel filtration (32,500) agrees with the gene derived molecular weight of 33,857. The enzyme has a K_m of 19μM, an isoelectric point of 4.5 and an N terminal sequence NH_2-MLDNVLRIAT.

The enzyme is shown to contain a novel dipyrromethane cofactor which is linked covalently to the protein. The structure of the cofactor is proposed on the basis of its reaction with Ehrlich's reagent and from its chemical properties. Specific labelling of the dipyrromethane cofactor by growth of the E. coli in the presence of 5-amino[5-^{14}C]laevulinic acid demonstrated that the cofactor is not subject to catalytic turnover. The structure of the cofactor was further confirmed as a dipyrromethane made up of two linked pyrrole rings by [^{13}C] n.m.r. studies after the deaminase was specifically labelled with [^{13}C] by growth of the bacteria on 5-amino[5-^{13}C]laevulinic acid. The chemical shift data indicate that one of the pyrrole rings of the cofactor is covalently attached to the deaminase enzyme through a cysteine residue. Evidence from protein chemistry studies confirm that cysteine-242 is the covalent binding site for the cofactor.

The formation of the dipyrromethane cofactor of E. coli was shown to depend on the presence of 5-aminolaevulinic acid. A hemA$^-$ mutant formed inactive porphobilinogen deaminase when grown in the absence of 5-aminolaevulinic acid since this strain was unable to biosynthesise the dipyrromethane cofactor. The mutant formed normal levels of deaminase, however, when grown in the presence of 5-aminolaevulinic acid.

Porphobilinogen, the substrate, interacts with the free α-position of the dipyrromethane cofactor to give three stable enzyme-intermediate complexes, ES, ES_2 and ES_3. Experiments with regiospecifically labelled intermediate complexes have shown that, in the absence of further substrate molecules, the complexes are interconvertible by the exchange of the terminal pyrrole ring of the complex. The formation of enzyme-intermediate complexes is accompanied by the exposure of a cysteine residue suggesting that substantial conformational changes occur on binding substrate. Experiments with the α-substituted substrate analogue, α-bromoporhobilinogen, have provided further evidence that the cofactor is responsible for the covalent binding of the substrate at the catalytic site. Based on these findings it has been possible, for the first time, to construct a mechanistic scheme for the deaminase reaction involving a single active site which is able to catalyse the addition or removal of either NH_3 or H_2O. The role of the cofactor as both a primer and as a means for regulating the number of substrates bound in each catalytic cycle is discussed.

Chapter 1.

The biosynthesis of the tetrapyrrole macrocycle.

1.1 Introduction.

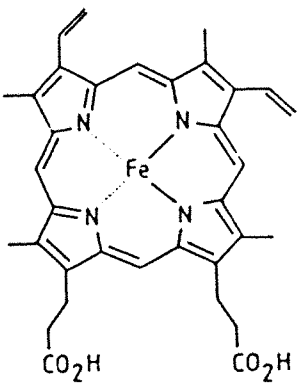
Tetrapyrroles make up a range of compounds that are ubiquitous among living systems and play an essential role in a wide variety of different biochemical processes (Figure 1). Haem, for instance, when it is attached to globin forms haemoglobin and is responsible for the ligating and carriage of oxygen, carbon dioxide and hydrogen ions (Perutz, 1970, 1972). Haem also acts as the prosthetic group in the cytochromes where it is capable of accepting electrons thus making the haem molecule an indispensible part of the electron transport chain and of the cytochrome P_{450} based detoxification reactions (Lemberg and Barrett, 1973). Haem also serves as the catalytic centre of the enzymes catalase and peroxidase. Other modified tetrapyrroles such as chlorophyll, cobalamin (vitamin B_{12}), sirohaem and factor F_{430} are involved in the light trapping processes of photosynthesis (Rabinowitch & Govindjee, 1969), enzyme catalysed rearrangements and methylations (Barker, 1972; Akthar & Wilton, 1970, 1972; Weissbach & Taylor, 1970), sulphite and nitrite reductases (Siegel et al, 1973; Murphy et al, 1973, 1974; Scott et al, 1979) and as the cofactor for the enzyme methyl CoM reductase which is involved in the final steps of the reduction of carbon dioxide in methanogenic bacteria (Thauer, 1982) respectively.

The appearance of the tetrapyrrole macrocycle in evolutionary terms, therefore, is of great significance as it conferred on biological systems not only the possibility for aerobic life but also the ability to utilise solar energy. The biosynthesis of these important natural products has been of great scientific interest as the pathway has led to a more thorough understanding of the processes of control, regulation and economy of effort that nature has always chosen to employ in the construction of any biological component.

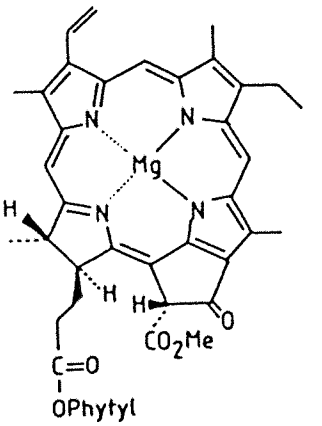
The biosynthesis of all compounds which have a tetrapyrrole nucleus appear to share the same pathway up to the formation of the first macrocyclic intermediate, uroporphyrinogen III (Scheme 1). The first committed step towards the biosynthesis of tetrapyrroles is the formation of 5-aminolaevulinic acid. This amazingly stable aminoketone is dimerized in a condensation reaction to form the pyrrolic building block porphobilinogen. Deamination and tetrapolymerisation of porphobilinogen leads to the formation of preuroporphyrinogen, which after ring rearrangment and cyclisation, gives rise to uroporphyrinogen III. This point in the pathway sees the first divergence in the biosynthetic pathways of the tetrapyrroles with the synthesis of vitamin B_{12} following a different route to that of haem and chlorophyll.

FIGURE 1

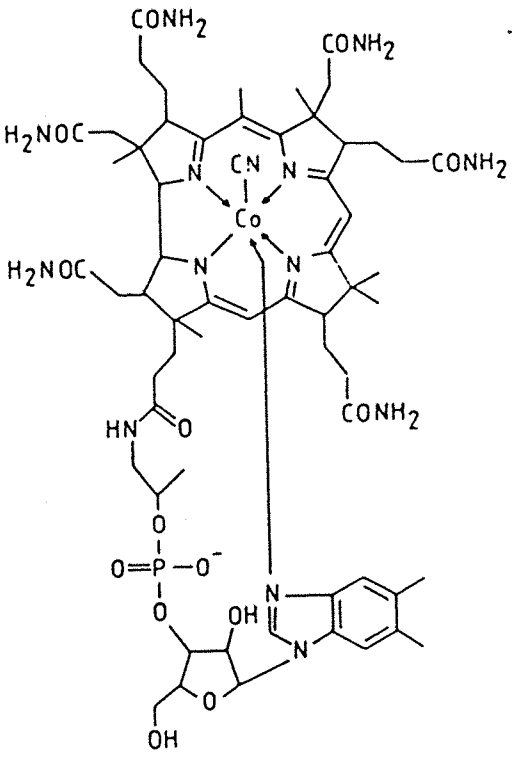
The structures of some biologically important tetrapyrroles.



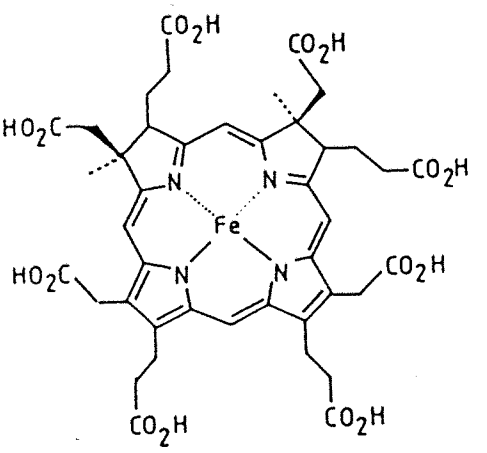
haem



chlorophyll



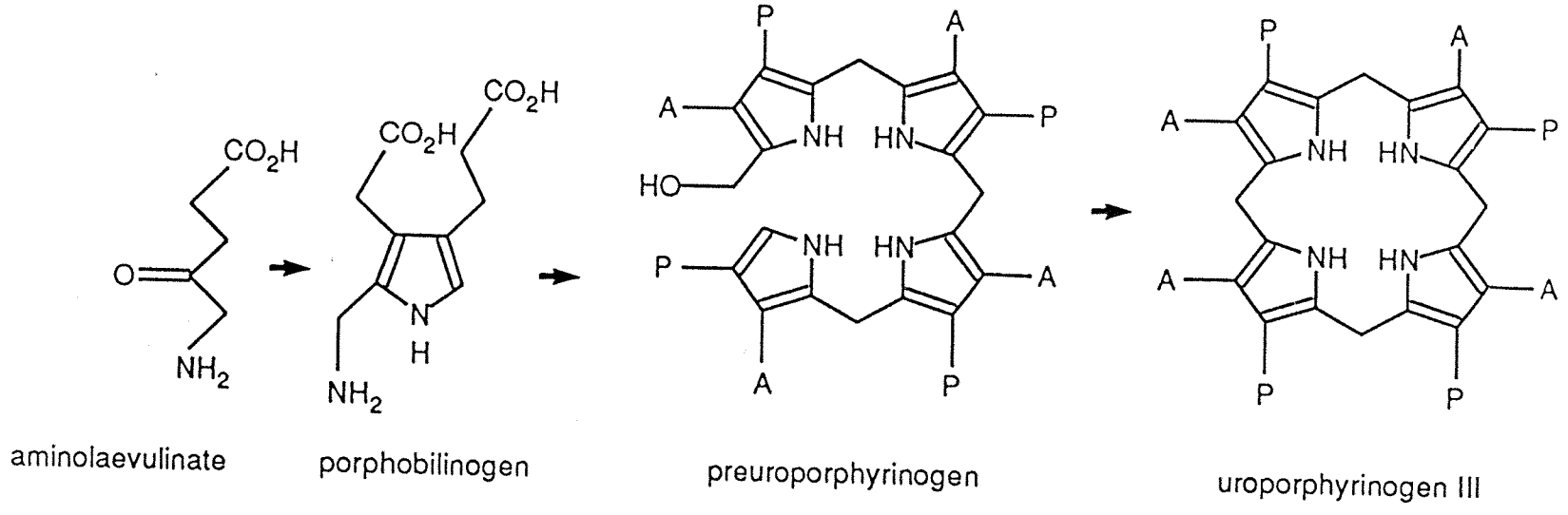
vitamin B_{12}



sirohaem

SCHEME 1

The biosynthesis of uroporphyrinogen III from 5-aminolaevulinic acid.



This common biosynthetic pathway for the construction of all tetrapyrroles has been the subject of extensive scientific research over the past forty years and has been very well documented in several reviews (Akhtar & Jordan, 1978; Battersby & M^{c}Donald, 1976; Dolphin, 1979, 1982; Leeper, 1985 a and b, 1987). This chapter, however, reviews the results of this interest over the last ten years and incorporates some of the more recent findings which have been made in this area especially by the application of the modern techniques of molecular biology. The individual reactions of each stage of the pathway are discussed separately as follows.

1.2 The biosynthesis of 5-aminolaevulinic acid.

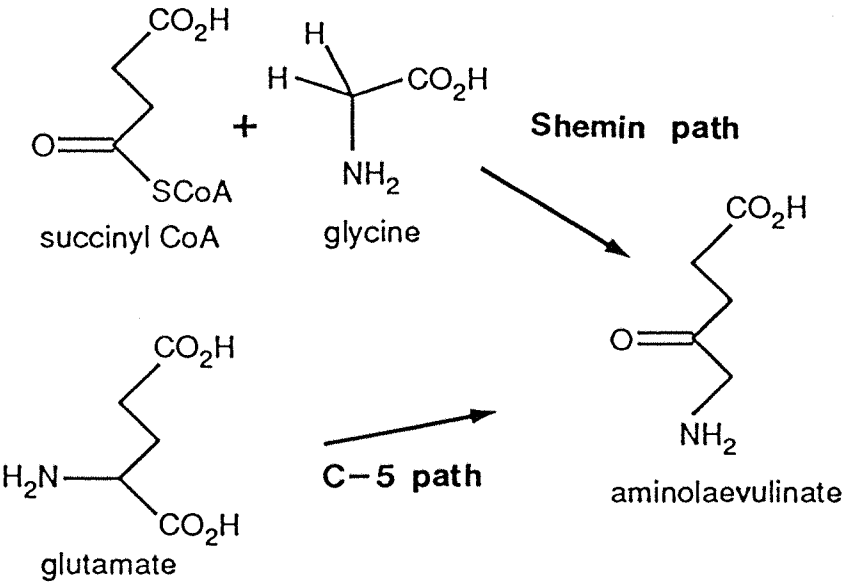
Since the synthesis of 5-aminolaevulinic acid represents the first committed step towards the biosynthesis of tetrapyrroles this initial reaction in the biosynthetic pathway has been of particular interest as it is the regulatory step and acts as the flux control in the synthesis of the final tetrapyrrole product (Granick, 1966; Lascelles, 1968; Keng & Guarente, 1987). The involvement of 5-aminolaevulinic acid as a tetrapyrrole precursor was deduced from the classical experiments of David Shemin (Shemin & Rittenberg, 1945; Shemin & Kumin, 1952; Shemin & Russell, 1953) who demonstrated in animals and some bacterial systems, that 5-aminolaevulinic acid was incorporated into the main nucleus of haem in a similar fashion to the uptake of glycine and succinate. The discovery of an enzyme capable of synthesising 5-aminolaevulinic acid from glycine and succinyl-CoA merely corroborated this hypothesis (Gibson et al, 1958; Kikuchi et al, 1958) (Scheme 2). This route to the formation of 5-aminolaevulinic acid was found not to occur in higher plants or bacteria with an incomplete citric acid cycle. In 1973 Beale and Castlefranco reported that both glutamate and α-ketoglutarate were incorporated intact into the nucleus of chlorophyll and the idea of a straight 5-carbon (C-5) pathway for the formation of 5-aminolaevulinic acid was born (Scheme 2). Since these two routes for the formation of 5-aminolaevulinic acid are so radically different they will be dealt with independently.

1.3 The synthesis of 5-aminolaevulinic acid from glycine and succinyl-CoA.

The pioneering work of Shemin and co-workers (Shemin & Rittenberg, 1945; Shemin & Wittenberg, 1951; Shemin & Russell, 1953; Shemin et al, 1955) established the labelling pattern of haem synthesised from glycine and succinyl-CoA and also established that the same pattern could be obtained from specifically labelled 5-aminolaevulinic acid. The enzyme responsible for the formation of 5-aminolaevulinic acid was discovered simultaneously by two independant groups of workers (Gibson et al, 1958; Kikuchi et al, 1958) who prepared cell free extracts capable of condensing glycine and succinyl-CoA into 5-aminolaevulinic acid. Since that time 5-aminolaevulinic acid

SCHEME 2

The two biosynthetic routes to the formation of 5-aminolaevulinic acid.



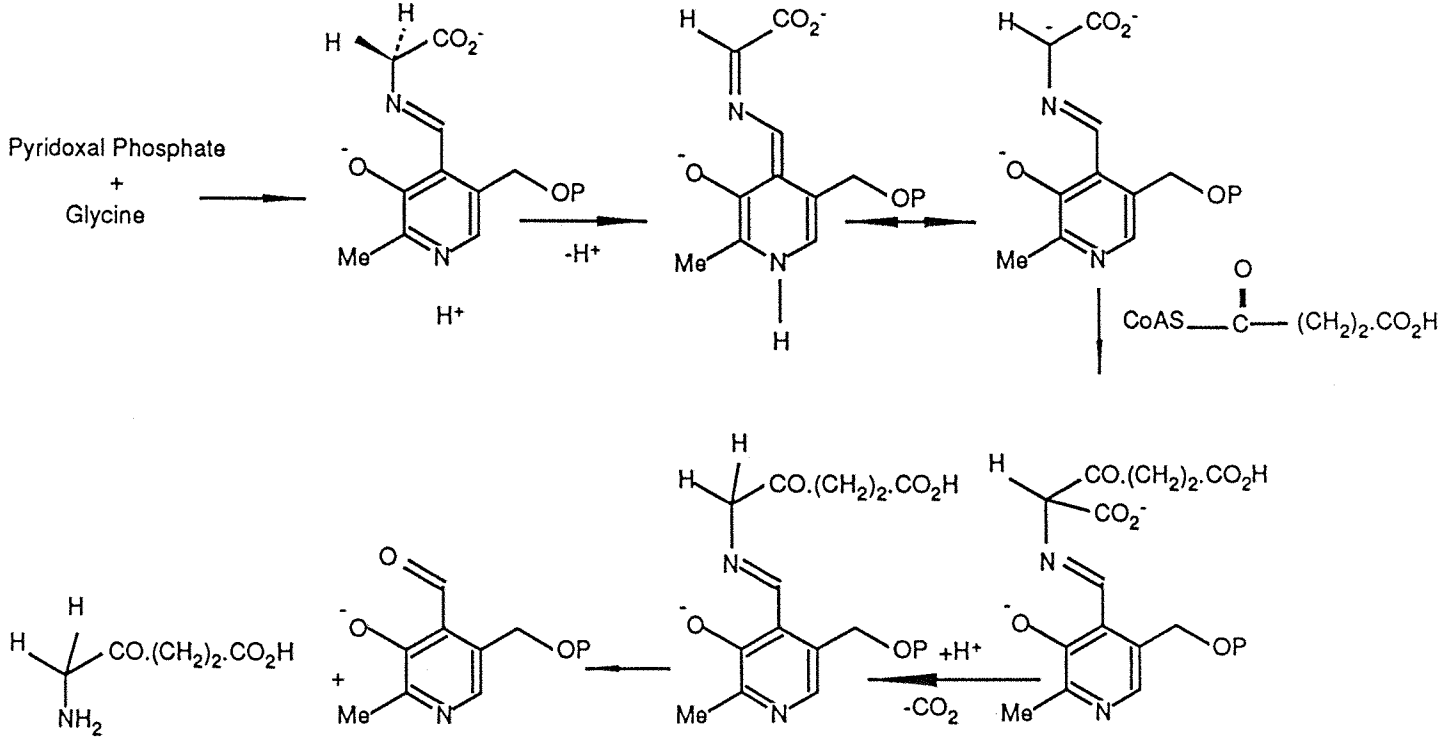
synthase has been purified from many sources including Rhodobacter sphaeroides (Davies & Neuberger, 1979), Euglena gracilis (Dzelzkalns et al, 1982), rat liver (Ohashi & Kikuchi, 1979), chicken embryo liver (Srivastava et al, 1983) and yeast (Volland & Felix, 1984) and this has given rise to a substantial amount of information regarding the properties of the enzyme. In eukaryotic systems the enzyme is found in the mitochondria reflecting the requirement for succinyl-CoA. The purified enzymes appear to consist of homodimers with subunit molecular weights ranging from 40,000 to 70,000 Daltons. Kinetic studies with the enzyme have shown that succinyl-CoA binds much more tightly to the enzyme with a K_m of around 5μM compared to glycine which has a K_m of around 1mM. Glycine, however, shows a greater substrate specificity with aminomalonate being the only glycine analogue to show any substantial degree of inhibition although even this compound does not act as a substrate (Matthew & Neuberger, 1963). Succinyl-CoA shows a lower substrate specificity than glycine, several Coenzyme A esters capable of acting as poor substrates in the enzyme reaction, leading to the formation of the corresponding aminoketone products (Gibson et al, 1955; Jordan & Shemin, 1972).

The enzyme from all sources is ubiquitous in its need for pyridoxal phosphate which acts as a cofactor and is essential for catalytic activity (Gibson et al, 1958; Gibson, 1958). Spectroscopic data of the holoenzyme (enzyme with cofactor) indicates that the absorbance of the enzyme-cofactor complex varies with pH. At either acidic or basic pH values the complex absorbs strongly at 415 nm indicating the presence of a Schiff base, whereas at neutral values the complex absorbs strongly at 330 nm (Fanica-Gaignier & Clement-Metral, 1973). Since the enzyme is active at neutral pH values this suggests that the cofactor is attached to the enzyme through a carbinolamine type bond. This is further substantiated by the fact that the cofactor can be easily removed from the enzyme by dialysis which indicates a weak interaction between the enzyme and cofactor. Furthermore it has been shown that, at neutral pH, the addition of sodium borohydride has little effect on the enzyme activity.

The earlier work of Shemin (Radin et al, 1950) had demonstrated that the C-1 position of glycine was lost during the formation of 5-aminolaevulinate. Subsequent work by Zaman et al (1973) has shown that the pro R hydrogen of glycine was specifically removed on conversion of [2RS-$^3H_2$;2-^{14}C]glycine into 5-aminolaevulinic acid by aminolaevulinic acid synthase. In the absence of succinyl-CoA the enzyme does not catalyse the decarboxylation of glycine but does catalyse the exclusive exchange of the pro-R hydrogen of glycine (Jordan & Laghai, 1976). It is this deprotonation step that generates an activated anionic species capable of reacting with succinyl-CoA which is then followed by the decarboxylation as shown in scheme 3. This scheme was shown to be correct after the absolute stereochemistry of the reaction was determined by analysis of the C-5 position of 5-aminolaevulinate synthesised by the enzyme from

SCHEME 3

The mechanism and stereochemistry of the ALA synthase reaction.



2RS[$^3H_2$]glycine (or 2S[^{3}H]glycine) (Abboud et al, 1974). This demonstrated that the pro-S hydrogen was derived from glycine and the pro-R hydrogen was derived from the medium. Thus the overall reaction appears to go through one inversion and one retention of configuration (Akhtar et al, 1976). The interest in the steric course of enzyme catalysed reactions is not always apparent to the reader but in the absence of the tertiary structure of the protein it is the only means of obtaining an idea of the topography of the active site of the enzyme.

The enzyme appears to show some form of feedback inhibition as low concentrations of haem inhibit the enzyme which suggests that the enzyme is an important regulator of tetrapyrrole biosynthesis (Granick, 1966; Lascelles, 1968). There is also evidence that haem inhibits at the level of transcription (Keng et al, 1987).

The use of recombinant DNA technology has led to the identification, isolation and cloning of the hemA gene which encodes for the 5-aminolaevulinic acid synthase protein. The complete nucleotide sequence for the gene from chick embryo (Borthwick et al, 1985), yeast (Keng et al, 1986), Rhizobium (Robertson-M^{c}Clung et al, 1987) and human (Bowden et al, 1987) have all been published and show remarkable homology. These sequences also reveal that the initial protein, which is synthesized in the cytosol, is a precursor form of the mitochondrial enzyme which loses an N-terminal portion when it is transported.

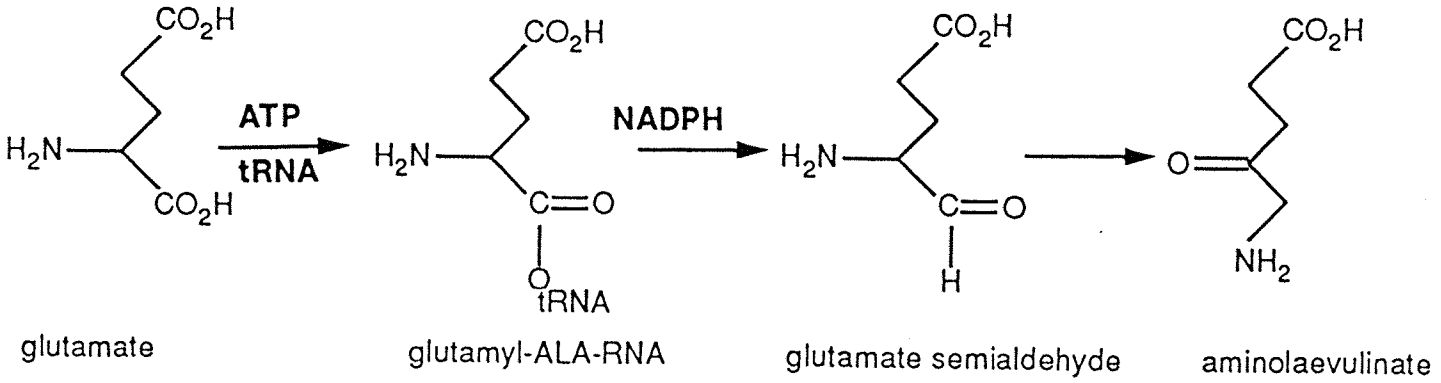
The nature of the enzyme groups responsible for the catalysis have not been identified. A lysine group has been shown to be involved in the stabilization of the bound pyridoxal phosphate cofactor (Nandi, 1978 a) and a thiol group has been implicated in the mechanism of the yeast enzyme (Volland & Felix, 1984) but this lack of information is a reflection of the very small quantities of the purified enzyme that have so far been obtained. Undoubtedly with the application of molecular cloning and the use of over-expression systems it will not be long before more detailed accounts of essential amino acids in the enzyme mechanism will be given.

1.4 The synthesis of 5-aminolaevulinic acid from glutamate.

Although the presence of 5-aminolaevulinic acid synthase had been detected in animals and some bacterial sources, in higher plants there appeared to be a complete absence of the classical Shemin pathway for the synthesis of 5-aminolaevulinic acid. It was not until 1973 that Beale and Castlefranco demonstrated that the 5 carbon atoms of 5-aminolaevulinic acid were derived directly from the intact carbon skeleton of glutamic acid. Since that time it has become apparent that the C-5 pathway is, in fact, more common than the Shemin path and its presence has been demonstrated in higher plants (Beale & Castlefranco, 1974), algae (Weinstein & Beale, 1983) and methanogenic bacteria (Friedmann et al, 1987). This raises the issue as to why nature appears to require

SCHEME 4

The C-5 biosynthetic pathway of 5-aminolaevulinic acid according to Kannangara et al (1984).



two distinct routes for the formation of 5-aminolaevulinic acid.

The answer to this apparent anomaly relates to the correlation between economy and availability. The classical 5-aminolaevulinic acid synthase enzyme does not require the input of any energy equivalents as it uses the high energy intermediate succinyl-CoA, which can be readily obtained from the citric acid cycle. This sole energy source is all that is required for the formation of the tetrapyrrole macrocycle in the Shemin pathway. However, this means that the enzyme has to reside in a cellular compartment where succinyl-CoA is readily available which, in the case of eukaryotes, is in the mitochondria. Thus in organisms where there is no citric acid cycle or in organisms which require a high level of 5-aminolaevulinic acid outside the mitochondria, for instance in greening chloroplasts during chlorophyll synthesis, there has to be an alternative route for the synthesis of 5-aminolaevulinic acid. The only problem with the C-5 pathway is that it is energetically unfavourable and, as will be described in more detail below, requires four ATP equivalents in the form of one ATP molecule itself and one nicotinamide reducing equivalent, to convert glutamate into 5-aminolaevulinic acid (Kannangara et al, 1984).

Although the C-5 pathway was initially discovered by Beale and Castlefranco the elucidation of the mechanism by which this process takes place was achieved by Kannangara and co-workers (1984). In a series of experiments spanning from 1978 to 1986 they were able to dissect the individual enzyme steps responsible for the transformation of glutamate into 5-aminolaevulinic acid and to set a precedent for the involvement of a transfer RNA in a reaction other than protein synthesis (Scheme 4).

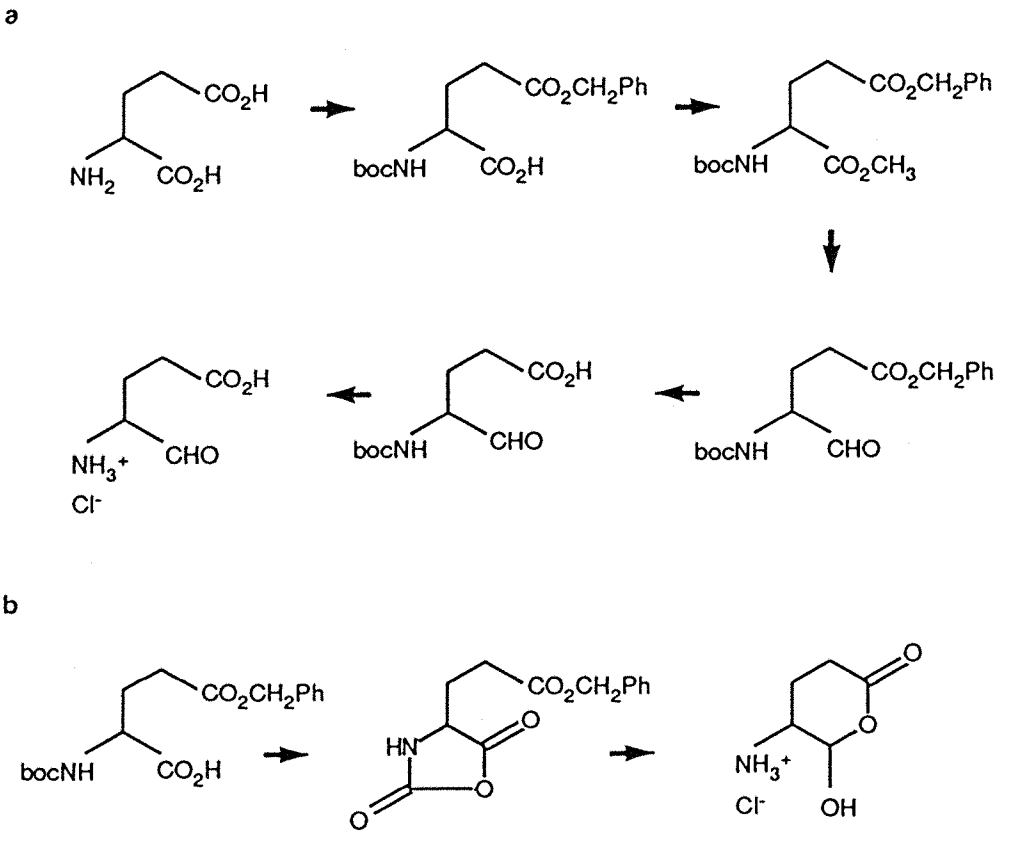
The initial breakthrough for these workers was the isolation of the enzyme system responsible for the conversion of glutamate into 5-aminolaevulinic acid from greening barley chloroplasts. They were able to show that this process required ATP or GTP and a reducing agent in the form of NADH or NADPH. The requirement for one energy equivalent and one reducing equivalent suggested that the glutamate was first activated to a high energy phosphate intermediate followed by some form of reduction and transamination. In an attempt to synthesise this high energy intermediate, which was initially thought to be glutamate-1-phosphate, they accidentally made an intermediate which acted as a substrate and which they thought to be glutamate-1-semialdehyde. This compound, which was synthesised from the acid chloride of protected glutamate, could be added to their assay system to yield 5-aminolaevulinic acid (Kannangara & Gough, 1978; Houen et al, 1984). Fractionation of their enzyme system by serial affinity chromatography gave rise to three fractions which converted glutamate to 5-aminolaevulinic acid only when combined together. One of these fractions was able to convert the synthetic glutamate-1-semialdehyde into 5-aminolaevulinic acid on its own. When the other two fractions were combined together in the presence of glutamate, ATP

SCHEME 5

The synthesis of glutamate semialdehyde as described by Jordan et al (1988, a)

a) open chain synthesis

b) cyclic synthesis



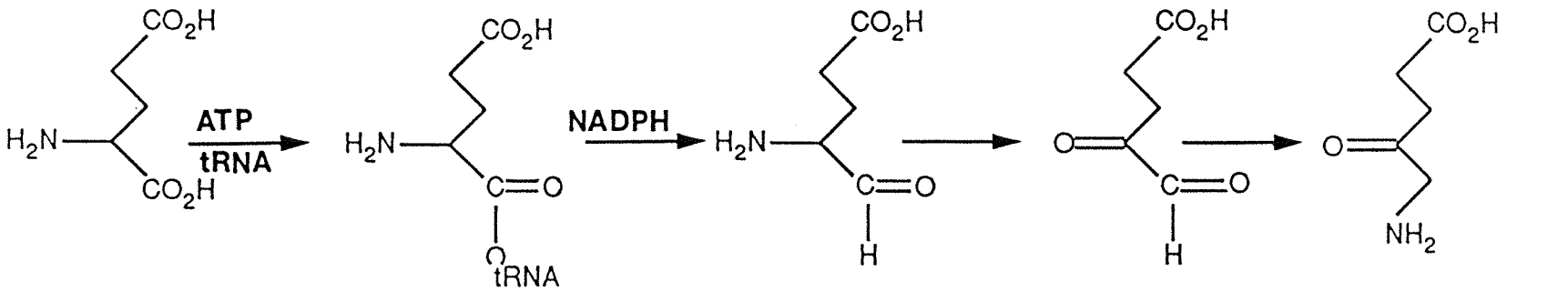
and NADPH the product formed was not 5-aminolaevulinic acid but glutamate-1-semialdehyde. Spectroscopic analysis of one of these two fractions revealed that the fraction had a spectrum characteristic of nucleic acids. The involvement of a nucleic acid species in this fraction was further confirmed when it was demonstrated that ribonuclease was capable of inactivating this fraction in the reconstituted assay system (Kannangara et al, 1984). Isolation and purification of this RNA species by h.p.l.c. and polyacrylamide gel electrophoresis followed by sequencing revealed that the RNA was in fact a chloroplast encoded glutamate transfer RNA (Schön et al, 1986). Investigations into the final affinity column fraction showed that it contained the enzymes capable of binding the tRNA to glutamate, by a process that required either ATP or GTP, and of releasing the glutamate-1-semialdehyde product from the tRNA complex by another process that required NADPH. Thus a route for the conversion of glutamate into 5-aminolaevulinic acid was devised and is shown in scheme 4. Glutamate is first activated by a tRNA synthase in an ATP dependent step which forms an amino-acyl bond between glutamate and a specific glutamate tRNA in an analogous reaction to tRNA activation in protein synthesis. The specificity of this particular glutamate tRNA ensures that the tRNA-glutamate complex in this case becomes destined for conversion into 5-aminolaevulinic acid. The tRNA-glutamate complex is next converted into glutamate-1-semialdehyde by a multi-enzyme process that involves release from the tRNA followed by dehydrogenation. It is at this stage in the mechanism that the NADPH reducing agent is required and it is also the least characterised of all the steps in the pathway. The final reaction in this transformation is the transamination of glutamate-1-semialdehyde into 5-aminolaevulinic acid by the enzyme glutamate-1-semialdehyde aminotransferase. This process is strongly inhibited by gabaculline and aminoxyacetate (Weinstein & Beale, 1985), well known pyridoxal phosphate antagonists. The nature of this inhibition suggests that no amino donor is required and that the enzyme exists in the pyridoxamine phosphate form (Hoober et al, 1988).

The idea of such a reactive species as a free amino aldehyde being involved in this pathway has been questioned recently by Jordan and co-workers (1988 a). Having synthesised glutamate-1-semialdehyde by a different route (Scheme 5) to Houen et al (1984) they found that the free aldehyde species was predictably very unstable and did not give rise to 5-aminolaevulinic acid formation in enzyme extracts from greening barley. By following the synthesis of the glutamate-1-semialdehyde reported by Houen et al (1984) and Kannangara and Gough (1978) they came to the conclusion that the compound made in this manner had a cyclic structure (see scheme 5). The cyclic structure of the material could explain the remarkable stability of the compound and could also still fit in with the requirement for a tRNA species in the pathway.

Evidence for a completely different intermediate in the biosynthesis of 5-

SCHEME 6

Proposed reaction sequence to the formation of 5-aminolaevulinic acid with the participation of dioxovaleric acid as suggested by Breu and Dörnemann (1988).



aminolaevulinic acid has also been submitted which involves dioxovaleric acid (Dörnemann & Senger, 1980). In this case the workers claim that in certain algae species dioxovaleric acid is transaminated into 5-aminolaevulinic acid and that glutamate-1-semialdehyde and dioxovaleric acid can be interconverted by another enzymic process (see scheme 6) (Breu & Dörnemann, 1988). However, the evidence from higher plant systems does not support this view.

1.5 The biosynthesis of porphobilinogen.

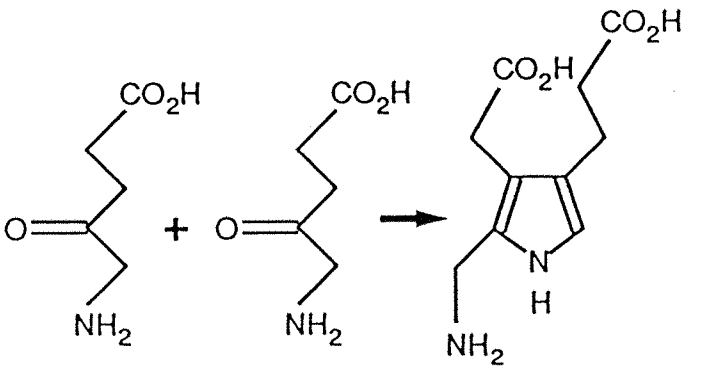
Porphobilinogen is biosynthesised by the catalytic dimerisation of two molecules of 5-aminolaevulinic acid by the enzyme 5-aminolaevulinic acid dehydratase (Scheme 7a). The enzyme has been purified from a wide variety of sources including human erythrocytes (Anderson & Desnick, 1979), bovine liver (Gibson et al, 1955), R. sphaeroides (Nandi et al, 1968) and spinach (Liegdens et al, 1983) although the best characterised proteins are those isolated from R. sphaeroides and bovine liver. Depending on the source of the enzyme, there are reported to be differences relating to pH optima, metal requirement and state of aggregation. Generally the enzyme isolated from animal sources has a low pH optima of between 6 and 7 with a requirement for a zinc ion, which can be removed by the chelating agent EDTA, and has a molecular weight of 280,000 Daltons made up of eight identical subunits each of molecular weight 35,000 Daltons. The enzyme isolated from bacterial sources has a higher pH optima of around 8.0 to 8.5 with a requirement for a monovalent cation and no inhibition by EDTA. The specific activity of the bacterial enzyme is about five times higher than that reported for the mammalian enzyme but it is not clear as to whether the enzyme exists as a hexamer or as an octamer.

All the 5-aminolaevulinic acid dehydratases thus studied are inhibited by sodium borohydride only in the presence of 5-aminolaevulinic acid or the competitive inhibitor laevulinic acid, suggesting the involvement of a Schiff base in the mechanism (Shemin, 1976). Laevulinic acid will, in fact, act as a substrate for the R. sphaeroides enzyme but only in the presence of 5-aminolaevulinic acid and gives rise to the methylpyrrole, shown in scheme 7b, with the laevulinic acid molecule giving rise to the acetic acid half of the final pyrrole product (Nandi & Shemin, 1968). On the basis of this result it was suggested that the first substrate molecule to bind to the enzyme bound at the A (A for acetate) site and formed a Schiff base while the second substrate molecule occupied the P (P for propionate) site. However, the converse was actually found to occur by investigating the problem with single turnover experiments. By exposure of the enzyme to one equivalent of labelled substrate or less followed by the addition of an excess of unlabelled substrate Jordan and Seehra (1980) were able to label specifically only one side of the PBG molecule. The position of the label in the product was determined

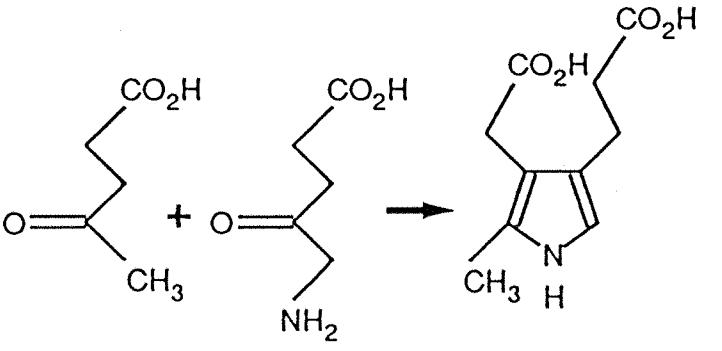
SCHEME 7

Reaction of ALA dehydratase with; a) 5-aminolaevulinic acid **b**) 5-aminolaevulinic acid and laevulinic acid.

a



b



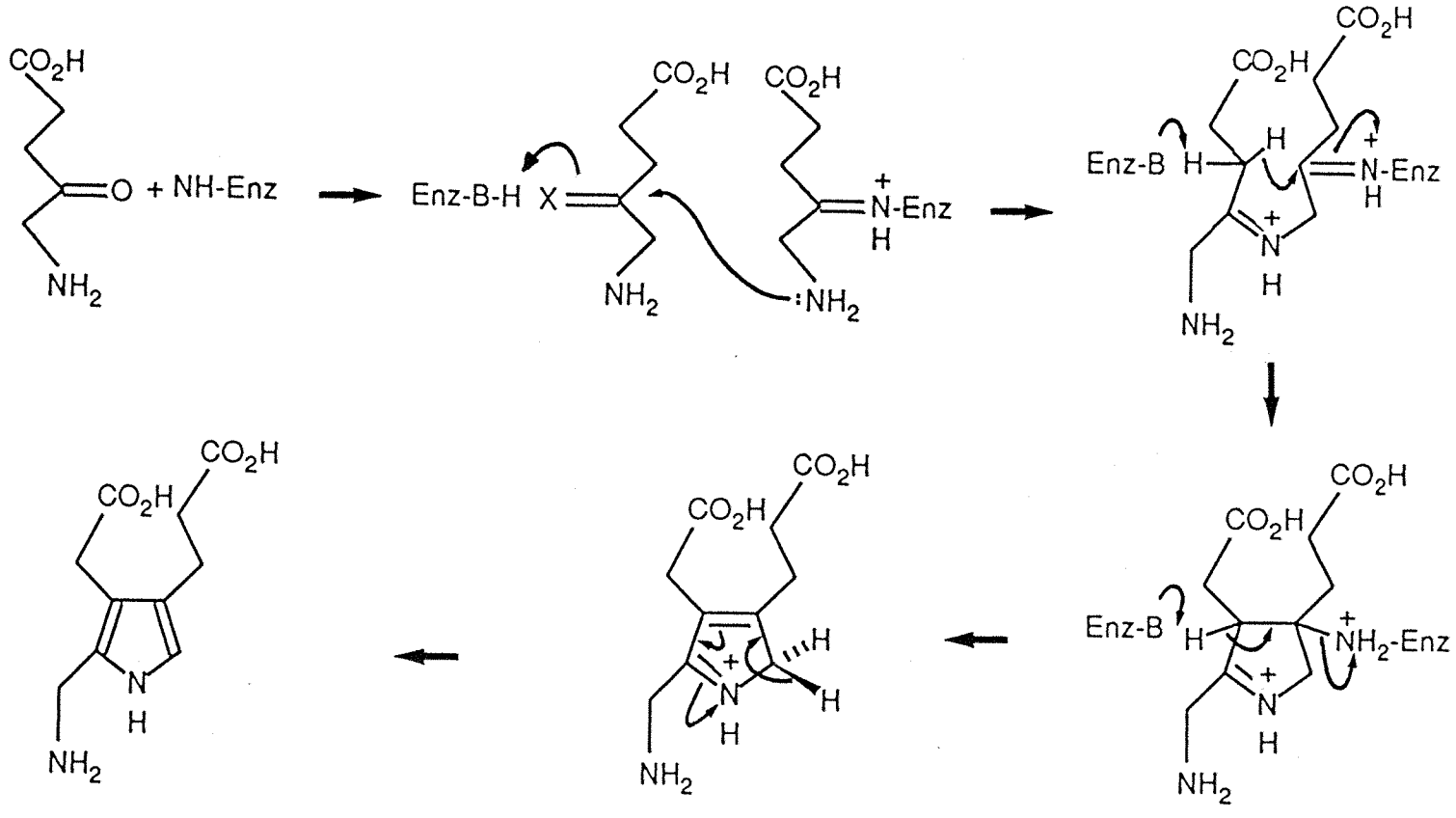
separately by n.m.r. and by chemical degradation to give the unequivocal result that the first molecule of 5-aminolaevulinic acid to bind to the enzyme does so by forming a Schiff base at the P site. This development gave rise to a new enzyme mechanism shown in scheme 8. In this mechanism it can be seen that two molecules of 5-aminolaevulinic acid are condensed in a Knorr type synthesis. The first 5-aminolaevulinic acid molecule forms a Schiff base onto which the enol form of the second molecule of 5-aminolaevulinic acid can react. A dehydration step followed by an enzyme catalysed deprotonation results in the formation of porphobilinogen. The removal of the proton at the C-2 position was shown to be specific when porphobilinogen formed from 5RS-amino-[$^3H_2$]laevulinic acid retained only 50% of the original tritium whereas 5S-amino-[^{3}H]laevulinic acid retained all its original label. These observations demonstrated that it was the pro R hydrogen of the C-2 position that was removed (Chaudry & Jordan, 1976).

The role of the metal ions is still far from clear. The mammalian enzyme is capable of binding up to eight zinc or cadmium ions per octamer but only requires four for full catalytic activity (Bevan et al, 1980) thus suggesting that some form of half site reactivity may be in force. This idea gathers further support from the fact that the dimer form of the enzyme is the lowest aggregation state that remains active (Batlle et al, 1978). The use of n.m.r. to probe the binding of ^{113}Cd to the enzyme yielded a single resonance at k=79ppm (Sommer & Beyersmann, 1984). Interestingly this signal was not affected by the addition of substrate to the enzyme thereby suggesting that the substrate molecule does not interact directly with the bound metal ion. Similar conclusions were reached by other workers who were able to remove the zinc ion by alkylation of an essential SH group and found that the inactive protein was still able to form a Schiff base with the substrate (Jaffe et al, 1984). The nature of the environment surrounding the bound zinc ion in the bovine liver enzyme was studied with the use of extended X-ray-absorption fine structure and suggested that the metal ion was ligated to the protein through three sulphur atoms (Hasnain et al, 1985). Together all this information suggests that the metal ion is not involved in the binding of the first substrate molecule but is probably involved in the ability of the enzyme to accept the second molecule.

The apparent disparity between the bacterial and mammalian enzymes with respect to the metal requirement is also something of a mystery. The cloning and sequencing of the human (Wetmur et al, 1986) and rat (Bishop et al, 1986) cDNA has allowed a direct comparison of the two predicted primary protein sequences and it shows that there is a considerable amount of homology between the structures. From this data it has been possible to identify a putative metal binding site. It will be very interesting to see, when the bacterial hemB gene is sequenced, if it too contains a potential metal binding site. The bacterial enzyme may bind a metal ion so tightly that it cannot be readily removed by EDTA.

SCHEME 8

The mechanism for the biosynthesis of porphobilinogen according to Jordan and Seehra (1980).



The enzymic group responsible for the binding of the first substrate molecule had been identified as a lysine since after borohydride inactivation of the enzyme, in the presence of radioactive substrate followed by protein hydrolysis, a radioactive species corresponding to a lysine derivative could be detected after chromatography (Nandi, 1978 b). With the use of the same approach but by chemically cleaving the protein with cyanogen bromide, Jordan and Gibbs (1986) were able to purify and sequence active site peptides from the bovine and human enzymes. The two sequences were, as expected for an active site region, very similar. The amino acid sequence of the human dehydratase active site also aided and abetted Desnick and co-workers (Wetmur et al, 1986) in the cloning and sequencing of the cDNA for the human enzyme. All the predicted protein sequences from the various cloned enzymes show a very high degree of conservation around this active site lysine.

Apart from the active site lysine no other essential amino acid groups have, as yet, been identified in the catalytic mechanism. It is well known that the enzyme is remarkably sensitive to oxygen and thiol reagents. This sensitivity is related to the presence of two highly reactive sulphydryl groups which, under oxidizing conditions, are able to form an S-S bond. These two sulphydryl groups are apparently involved in the binding of the zinc ion. The role of these groups and any other important amino acid residues will probably not be absolutely known until the fine tertiary structure of the enzyme has been resolved by X-ray crystallography.

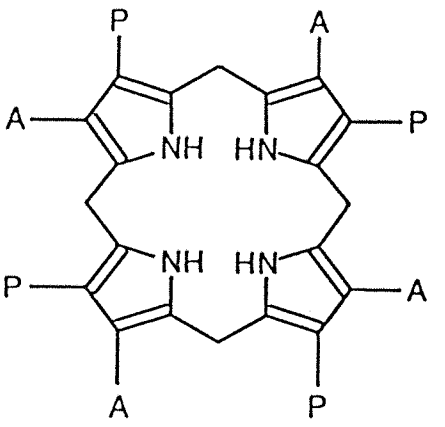
1.6 The biosynthesis of uroporphyrinogen III.

It is only within the last ten years that a full understanding of the process by which four molecules of porphobilinogen are converted into uroporphyrinogen III has been gained. It had been demonstrated back as early as 1958 (Bogorad, 1958 a,b,c) that the enzymic formation of uroporphyrinogen III required the presence of two enzymes, porphobilinogen deaminase and uroporphrinogen cosynthase, the latter one of which was extremely heat sensitive. Thus addition of porphobilinogen to a heat treated spinach extract resulted in the formation of uroporphyrinogen I whereas incubation of the non-heat treated sample with porphobilinogen gave rise to uroporphyrinogen III. Out of the four possible isomers of uroporphyrinogen, it is only the type III isomer, the isomer with ring d inverted, that is physiologically important (Figure 2). Although uroporphyrinogen I was formed by the deaminase, like porphobilinogen it did not act as a substrate for the heat sensitive cosynthase enzyme. It therefore became obvious that uroporphyrinogen I was not a true physiological intermediate but arose as a result of a chemical transformation of the true product of the first enzymic reaction. This led to an extensive investigation to find the true product of this first reaction. The heat stable enzyme, porphobilinogen deaminase, was shown to form uroporphyrinogen I from four molecules of porphobilinogen with the release of four molecules of ammonia (Bogorad, 1958 a, b; Frydman & Frydman, 1970), whereas the heat sensitive enzyme, the cosynthase, seemed to modify the reaction of the deaminase and steer the reaction towards the construction of the type III isomer (Scheme 9).

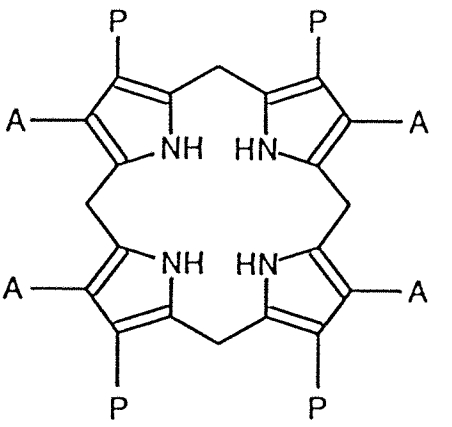
The product of porphobilinogen deaminase, up until 1978, was thought most likely to be the linear unrearranged aminomethylbilane (Figure 3) as this molecule was converted into uroporphyrinogen I by the action of the deaminase and in the presence of added cosynthase it gave rise to uroporphyrinogen III (Battersby et al, 1978). However, this compound was, in fact, a much poorer substrate for the deaminase than porphobilinogen and was not a substrate for the cosynthase alone. The discovery of the product of the deaminase catalysed reaction was finally achieved by Burton et al in 1979 (a). They found that by incubating an excess of [11-^{13}C] labelled porphobilinogen with deaminase in an n.m.r. tube they could obtain not only the expected resonances at around 23 ppm for the meso-methylene carbon atoms of a polypyrrole but also a much smaller signal at 57 ppm. The integration of the peaks was in the ratio of 3:1 showing that one of the carbon atoms in this tetrapyrrole was in a different environment to the other three. This compound was also very unstable with a half life of four minutes giving rise to uroporphyrinogen I exclusively. More importantly this compound acted as an excellent substrate for the cosynthase, and was rapidly converted into the uroporphyrinogen III isomer (Jordan et al, 1979). Essentially the same results were obtained by Battersby et al

FIGURE 2

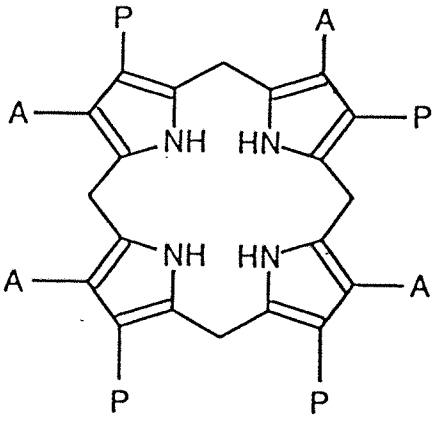
The four isomers of uroporphyrinogen.



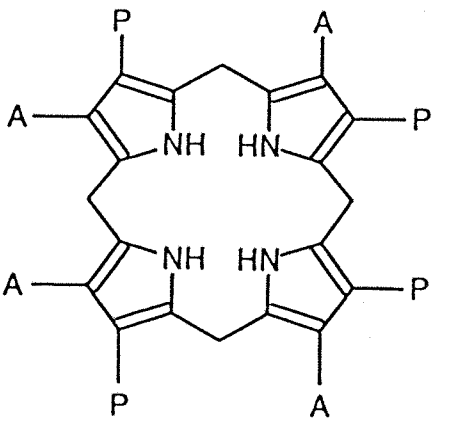
uroporphyrinogen I



uroporphyrinogen II



uroporphyrinogen III



uroporphyrinogen IV

SCHEME 9

The reaction of porphobilinogen with porphobilinogen deaminase in the absence and presence of cosynthase as observed by Bogorad (1958 a and b).

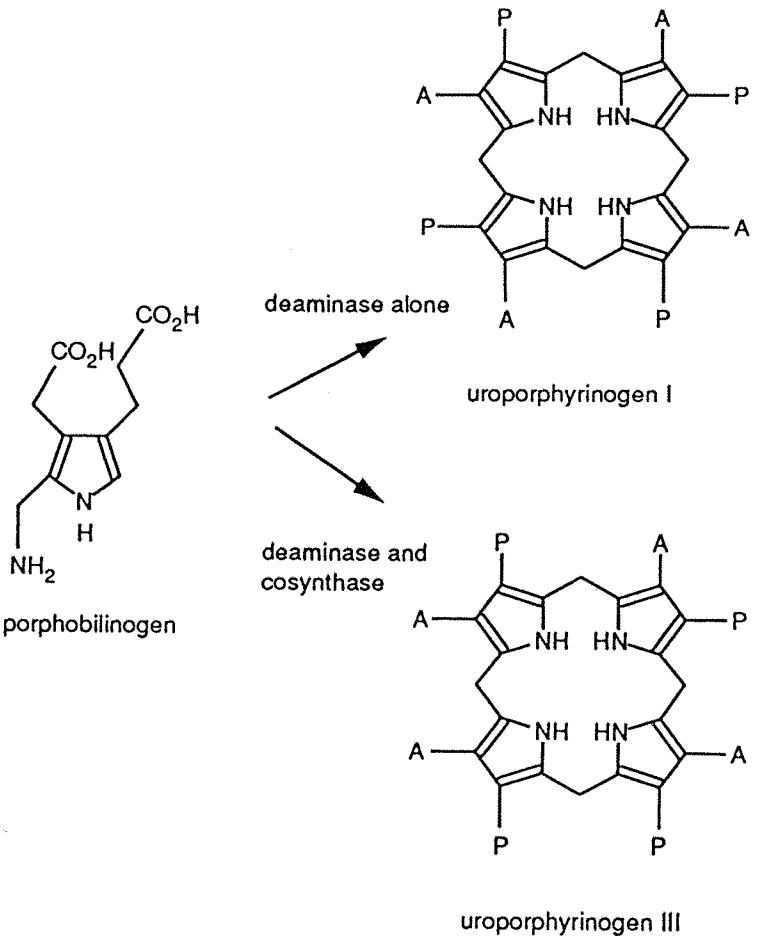
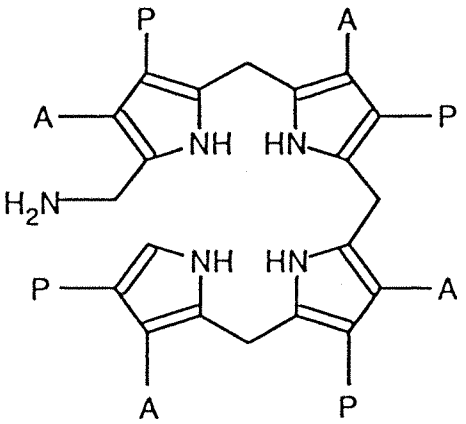


FIGURE 3

The structure of aminomethylbilane.



aminomethylbilane

(1979, a) who subsequently noticed the presence of this transient intermediate. This intermediate was termed preuroporphyrinogen and several proposals as to its structure were made although on the basis of a result obtained by incubating [11-^{13}C, 1-^{15}N]porphobilinogen with the deaminase enzyme, Burton et al (1979, b) suggested that the intermediate may be an N-alkyl porphyrinogen. A different interpretation of the same spectra obtained from the same type of experiment by Battersby et al (1980) led to the proposal of an open chain hydroxymethyl bilane structure for the intermediate (Figure 4). The structure was confirmed after the chemical synthesis of this postulated hydroxymethylbilane intermediate was achieved (Battersby et al, 1979, b) and it was demonstrated that it had identical properties to the intermediate that had been shown by Burton et al (1979, a) to accumulate during the enzyme incubation in the n.m.r. tube.

These experiments thus established, for the first time, the independant roles played by the two enzymes in the biosynthesis of the first macrocyclic precursor of the tetrapyrrole pathway and thereby allowed the mechanisms of the individual reactions to be investigated. The two enzymic steps, the deamination and tetrapolymerisation catalysed by the enzyme porphobilinogen deaminase, and the ring inversion and cyclisation reaction catalysed by the cosynthase, will be dealt with separately from this point onwards.

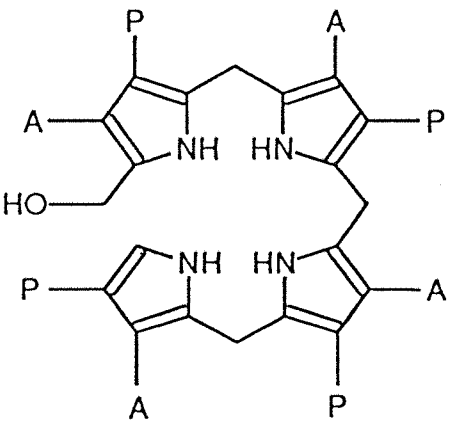
1.7 Formation of preuroporphyrinogen; mechanism of porphobilinogen deaminase.

The enzyme responsible for the deamination and tetrapolymerisation of porphobilinogen is, as has already been stated, termed porphobilinogen deaminase but is also referred to as hydroxymethylbilane synthase. This enzyme has been purified from a number of sources including R. sphaeroides (Jordan & Shemin, 1973), spinach (Higuchi & Bogorad, 1975), human erythrocytes (Anderson & Desnick, 1980), wheat germ (Russell et al, 1984), barley (Warren & Jordan, 1988, a) and E. coli (Hart et al, 1986; Jordan et al, 1988, b). All the enzymes isolated appear to be monomeric proteins with molecular weights of between 35,000 and 44,000 Daltons.

The identification of the product of the reaction catalysed by porphobilinogen deaminase as being a linear unrearranged tetrapyrrole led to speculation that the reaction proceeded in an ordered stepwise manner resulting in the elongation of a polypyrrole chain from the enzyme active site. Evidence for such a reaction had already been submitted when it was found that incubation of the deaminase with porphobilinogen in the presence of either ammonia, hydroxylamine or methoxyamine, compounds which were known to arrest porphyrin production but not porphobilinogen utilisation, resulted in the formation of base substituted pyrroles and polypyrroles (Pluscec & Bogorad, 1970; Davies & Neuberger, 1973). These pyrrole moieties were confirmed as being mono, di, tri and tetrapyrroles after they were separated out by high voltage

FIGURE 4

The structure of hydroxymethylbilane.



preuroporphyrinogen

electrophoresis or by gel filtration. This apparent uncoupling of a previously enzyme bound species was also indicative of a covalent attachment. These results also instigated investigations to determine whether the deaminase could accept polypyrroles as tetrapyrrole precursors. The results of these experiments, however, were disappointing and it was found that the polypyrrole adducts acted more as inhibitors than as substrates.

The order of the tetrapyrrole assembly was next investigated and since the elongation of the tetrapyrrole appeared to proceed in a linear fashion there were only two alternatives that could be considered. Either the pyrrole units were added to the enzyme in the ring sequence abcd or conversely in the order dcba. The answer to the problem was achieved by two independant research groups who both based their methodology on the principal that by reacting the deaminase with a limiting amount of labelled substrate it should be possible to form regio-specifically labelled product. In this way Battersby and co-workers (1979, c) exposed the deaminase to a limiting amount of unlabelled substrate prior to addition of [11-^{13}C] labelled porphobilinogen. The product was transformed into uroporphyrinogen III and esterified prior to analysis by n.m.r. spectroscopy. The result clearly showed that C-20 had the most [^{12}C], followed by C-5, C-10, and C-15 respectively. This demonstrated that the tetrapyrrole assembly proceeded in the order abcd. The same conclusions were reached by Jordan and Seehra (1979) who exposed the deaminase to a limiting quantity of [^{14}C] labelled porphobilinogen prior to the addition of excess unlabelled substrate. The preuroporphyrinogen formed from this incubation was converted enzymically into protoporphyrin and then chemically degraded. Analysis of the degradation products showed that ring a contained the most label with decreasing amounts of radioactivity being found in rings b, c and d.

All these results had suggested that the linear elongation of the final tetrapyrrole product must have been achieved with the enzyme proceeding through stable states with bound mono, di, tri, and tetrapyrroles attached to the active site. Conclusive evidence for such a mechanism was demonstrated by Anderson and Desnick in 1980. They noticed that during their final anion exchange procedure for the purification of the deaminase from human erythrocytes that the deaminase eluted from the column in five peaks. The same five forms of the enzyme could also be separated out by non-denaturing gel electrophoresis and isoelectric focussing. Moreover, when isolated, it was demonstrated in the presence of added substrate that these forms were able to interconvert. When radiolabelled substrate was used the distribution of the radioactivity in these enzyme species was proportionately greater in the enzyme forms with the greater overall negative charge. These results were consistent with the presence of free native porphobilinogen deaminase (E) together with highly stable enzyme-bound intermediate complexes containing either a mono, di, tri or tetrapyrrole (ES, ES_2, ES_3, ES_4). The charge separation of these enzyme intermediates was permitted by the presence of two extra

negative groups with the addition of each pyrrole unit to the enzyme in the form of the two carboxylic acid groups on the pyrrole side chains. Further investigations into these stable enzyme-intermediate complexes with the deaminase from R. sphaeroides by Berry et al (1981) revealed that, in this case, the enzyme only formed three complexes. By using [^{14}C] labelled porphobilinogen these workers isolated the complexes by gel electrophoresis, completed the catalytic turnover by the addition of excess unlabelled porphobilinogen and transformed the labelled preuroporphyrinogen into protoporphyrin. Degradation of the protoporphyrin revealed that the three complexes were enzyme with one, two and three labelled pyrrole units attached. The same three complex forms were also noticed with the enzyme isolated from E. coli (Jordan et al, 1988 b). In both the case of the enzyme from R. sphaeroides and from E. coli there was no sign of a stable enzyme-intermediate form with a bound tetrapyrrole species although the presence of a bound tetrapyrrole has been implicated in the Euglena enzyme (Battersby et al, 1983). The important observation that the complexes were covalently attached to the enzyme was made by Jordan and Berry (1981) when they demonstrated that denaturation of the enzyme-intermediate forms did not result in the release of the bound pyrrole moiety.

This development led to interest in the enzymic group responsible for the binding of the first substrate molecule to the enzyme. On the basis of the difference between the electrophoretic mobility of the E and the ES complex Jordan and Berry (1980) had proposed that the group responsible was not likely to be positively charged. On the basis of various active site directed modifying agents Russell had suggested that the group responsible was a cysteine or an arginine (Russell & Rockwell, 1980; Russell et al, 1984). By first labelling the deaminase with [^{13}C] porphobilinogen and then treating it with proteolytic enzymes Battersby et al (1983) isolated a very small pyrrole containing peptide. N.m.r. analysis of this fragment gave a resonance that suggested the pyrrole was linked to the peptide through a lysine group but in the absence of a [^{12}C] control spectrum this resonance was probably due to [^{13}C] protein natural abundance. These workers also provided further evidence for the involvement of a lysine with inhibition studies using pyridoxal phosphate (Hart et al, 1984). In contrast to the [^{13}C] n.m.r. experiments carried out by the Cambridge group (Battersby et al, 1983), Evans and co-workers (1986) found no resonance that could be attributed to a lysine group. However, by the use of [^{3}H] n.m.r. these workers tentatively assigned one of their resonances to a pyrrole-sulphur link.

The lack of any convincing results in the quest to determine the binding site resulted in several research groups turning to the aid of microbiology and recombinant DNA technology to produce a greater quantity of the protein for study. This strategy had already been employed successfully by Thomas and Jordan (1986) who not only had managed to isolate and sequence the hemC gene encoding porphobilinogen deaminase

but also had produced deaminase high expression systems. This had permitted the isolation of milligram quantities of the enzyme which in turn had facilitated the crystallisation of the protein (Jordan et al, 1988 b). The purification and crystallisation of the deaminase from this recombinant strain of E. coli is described in more detail in chapter 2 of this thesis.

Using enzyme that had been isolated in this way Jordan and Warren (1987) made several important observations that led ultimately to the identity of the group responsible for the substrate binding. They noticed that the native enzyme, enzyme which they had unambiguously shown to have no bound or associated substrate type intermediates, contained an enzyme bound dipyrrole. This conclusion was reached from spectroscopic data of the protein which, under acid conditions, turned pink and, under very strong acid conditions, gave rise to uroporphyrin formation. In a similar fashion crystals of the enzyme grown under mild acid conditions also turned pink. It was interesting to reflect that Berry (1983) had also noticed the presence of a dipyrrollic species during peptide mapping studies of the deaminase isolated from R. sphaeroides, but in this case he had not eliminated the presence of any bound intermediates.

The purified E. coli porphobilinogen deaminase gave a reaction characteristic of a dipyrromethane in the presence of Ehrlich's reagent. More importantly, the ES_2 enzyme-intermediate complex (enzyme with two bound substrate equivalents) exhibited a tetrapyrromethane reaction with Ehrlich's reagent. This simple experiment had demonstrated that the purpose of the dipyrrole was to act as the covalent attachment site for the incoming substrate molecules. Confirmation that the dipyrrole was acting as a cofactor was gained after it was specifically labelled with [^{14}C] by growth of the bacteria in the presence of labelled 5-aminolaevulinic acid. When the purified labelled enzyme was incubated with non labelled substrate it produced non labelled product. The radioactivity could, however, be specifically removed from the protein by treatment with formic acid which gave rise to the formation of labelled uroporphyrin. This Ehrlich's positive group has also been identified by Hart et al (1987) and by using [^{13}C] labelled substrate they had found a resonance which could only be attributed to the first substrate molecule binding to an enzyme bound pyrrole species.

Further work by Warren and Jordan (1988 a) has established that this dipyrromethane cofactor is found in all deaminases. By specifically labelling the dipyrromethane cofactor with [^{13}C] by growth of the bacteria on 5-amino[5-^{13}C]laevulinic acid these workers have not only been able to confirm the structure of the cofactor as a dipyrromethane molecule but also have demonstrated that the cofactor is linked to the enzyme through a cysteine group (Jordan et al, 1988 c). Comparison of the two predicted protein primary structures from the E. coli hemC gene and the human cDNA has revealed that there are only two conserved cysteine groups between the two

sources and the evidence both from protein chemistry and site specific mutagenesis has suggested that the cofactor is attached to the E. coli enzyme at position 242. (This work is described in more detail in chapters 3 and 4).

The presence of a dipyrromethane cofactor at the active site of the deaminase has enormous mechanistic significance and has given rise to a completely new scheme of events. As shown in scheme 10, the tetrapyrrole product is assembled whilst bound covalently to the free α-position of the bound dipyrromethane through the sequence (1)-(2)-(3)-(4). The identification, characterisation and mechanistic implications of this novel cofactor are described in more detail in chapters 3 to 6 of this thesis. With all the information presented in these chapters it has been possible to propose a mechanism for the deaminase that involves the use of two pyrrole binding sites, one catalytic site and a flexible polypyrrole chain. In this respect the mechanism of the deaminase is similar to the mechanism of other enzymes catalysing homopolymerization reactions such as fatty acid synthase where the succesive condensation of each malonyl-CoA molecule occurs at the same active site, with the growing chain attached to a flexible pantotheine chain.

The precise role of the dipyrromethane cofactor and the overall mechanism of the enzyme will undoubtedly be resolved in the near future from the information gained from the X-ray crystallography of the protein.

1.8 Formation of uroporphrinogen III; mechanism of cosynthase.

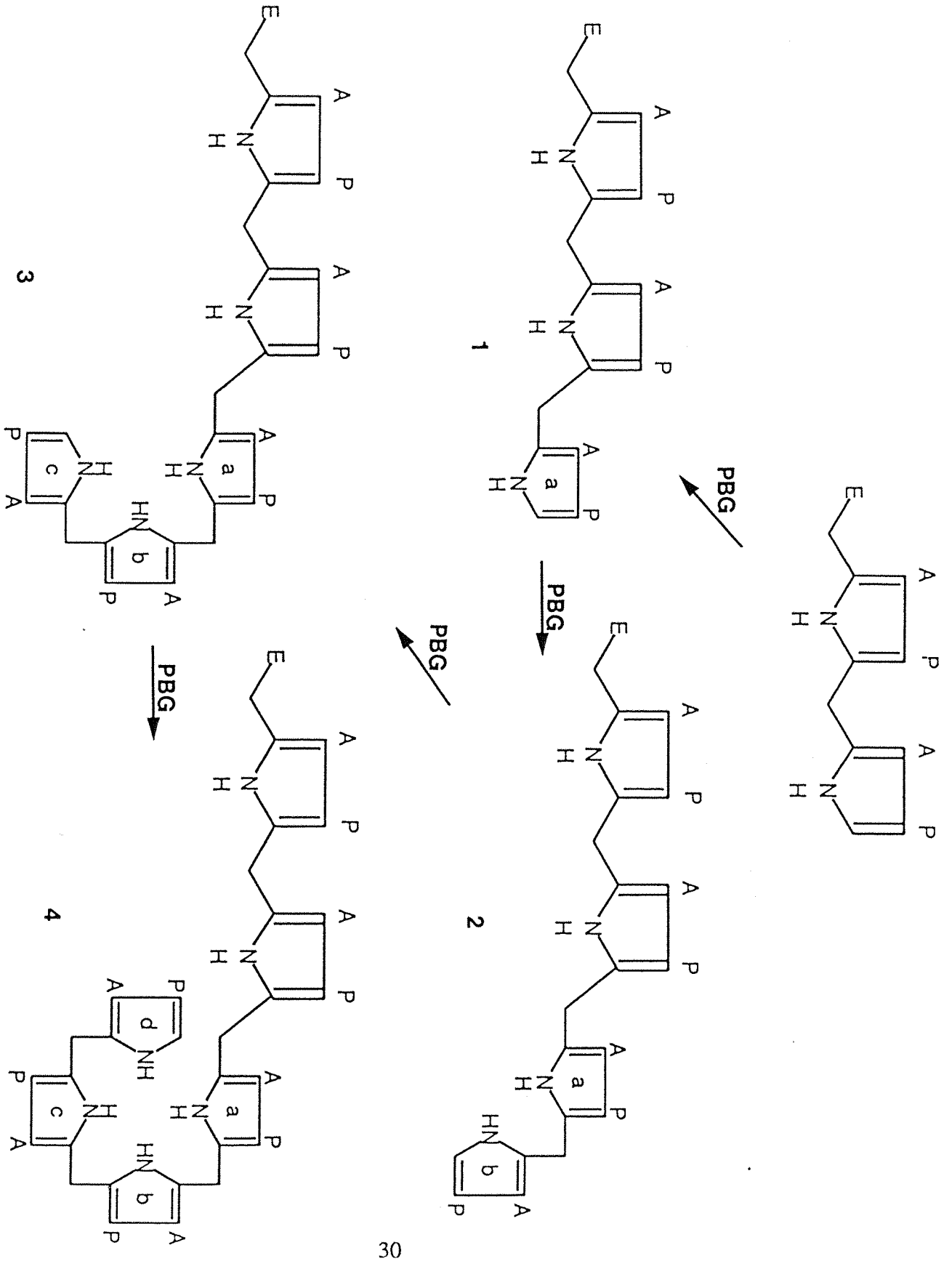
There have been very few experiments carried out on the cosynthase, also referred to as uroporphyrinogen III synthase, due to the difficulties in isolating the enzyme, obtaining the substrate and the lack of a convenient assay system. With the identification of preuroporphyrinogen as the substrate of the enzyme (Jordan et al, 1979) and the development of a rapid assay (Jordan, 1982) there has been an increase in the interest of this amazing enzyme catalysed rearrangement process.

One of the most plausible mechanisms for this enzyme which still agrees with the known facts of the enzyme is shown in scheme 11 and was originally proposed by Mathewson and Corwin (1961). The basis of this proposal revolves around the formation of a spiro intermediate which would then cleave and cyclise to give uroporphrynogen III (Scheme 11). Further evidence for this mechanism has come from studies involving a synthetic spiro-intermediate type analogue (Stark et al, 1985). This analogue was shown to act as a potent inhibitor for the enzyme although it is not transformed by the enzyme (Stark et al, 1986).

Interesting results have come from studies on the substrate specificity of the cosynthase using different synthetic bilanes (Battersby et al, 1979 a; Battersby et al, 1981; Battersby et al, 1982; Diaz et al, 1979; Sburlti et al, 1983)(Table 1). The best

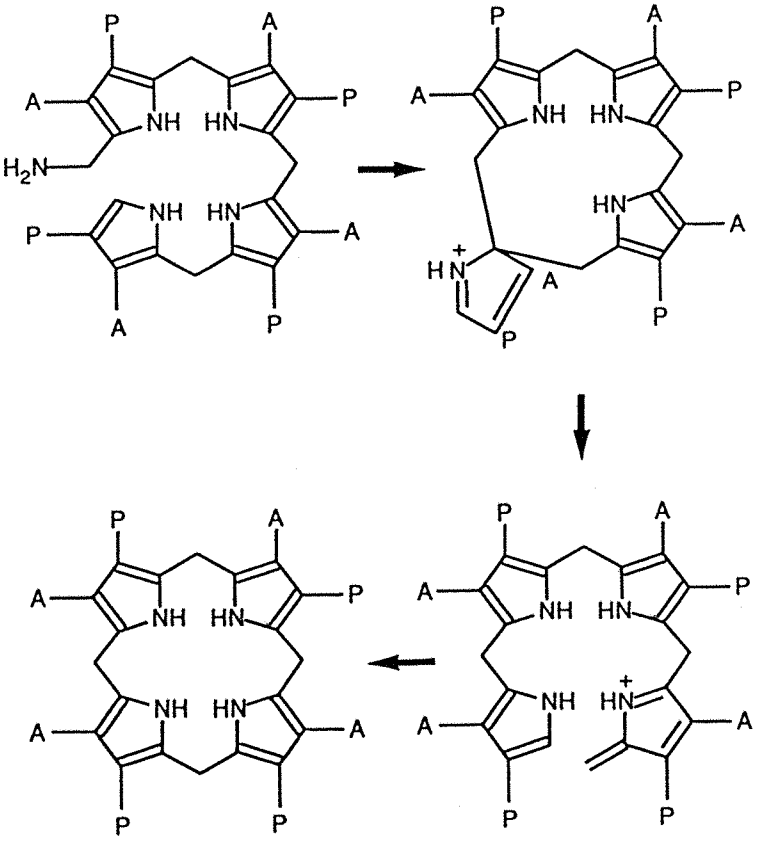
SCHEME 10

Mechanism of porphobilinogen deaminase according to the findings of Jordan and Warren (1987).



SCHEME 11

The Mathewson and Corwin mechanism for the biosynthesis of uroporphyrinogen III.



substrate was, as expected, the unrearranged hydroxymethylbilane which had the side chain order of OH-APAPAPAP. The analogues with inversion of either the a or the b side chain did not act as substrates for the enzyme at all. However, the analogues with inversion of either rings c or d gave rise to rearranged products. Thus the hydroxymethylbilane of the type OH-APAPAPPA, in the presence of cosynthase quite remarkably gives rise to uroporphyrinogen I (see table 1). Since there can be no common intermediate between the type I and the type III bilanes these results suggest that the enzyme recognises and binds the substrate by the presence and orientation of the rings a and b and then carries out a less specific rearrangement and cyclisation process.

The location, isolation and sequencing of the hemD gene has recently been reported by two research groups (Sasarman et al, 1987; Jordan et al, 1987). The gene was found to be immediately adjacent to the hemC locus and under the control of the same promoter. This promoter would also appear to control the transcription of several other unidentified open reading frames which are thought possibly to constitute part of a hem operon.

The purification of the cosynthase has only been reported from three sources with the highly unstable nature of the protein making the isolation of the enzyme extremely difficult. The first reported purification of cosynthase was from Euglena but this was purified only to approximately 70% homogeneity (Hart & Battersby, 1985). The total purification of the enzyme from human erythrocytes (Tsai et al, 1987) represents a remarkable feat in itself as the homogeneous protein was not only purified by a factor of some 50,000 fold but was also partially sequenced. The protein sequence data facilitated the isolation and sequencing of the cDNA. Both the partial purification of the Euglena enzyme and the cosynthase from human erythrocytes relied on a final reverse phase h.p.l.c. stage which, surprisingly, did not lead to total inactivation of the protein. More recently the E. coli enzyme, from a genetically engineered strain of the bacteria, has been purified to homogeneity without the necessity to use a reverse phase column (Jordan & Alwan, 1988). The N-terminus of the E. coli enzyme was found to be in complete agreement with the predicted protein structure. All the purified cosynthase enzymes were found to have molecular weights around 30,000 Daltons and appear to be monomeric.

Little is known about the cosynthase active site and essential amino acids that are required in the catalysed rearrangement reaction. However, with the use of more sensitive and reliable assay conditions and with the use of over-expression systems a more detailed account of the enzymatic machinery will doubtless emerge.

TABLE 1

Comparison of the order of the β-substituents of hyroxymethylbilanes to substrate specificity for the cosynthase.

Order of pyrrole ring β-substituents in ring; a		b		c		d		% enzymic inversion
OH -	A P	A P	A P	A P	A P	A P	A P	100
OH -	A P	P A	A P	A P	A P	A P	A P	0
OH -	A P	A P	P A	A P	A P	A P	A P	95
OH -	A P	A P	A P	A P	A P	P A	A P	45
OH -	P A	P A	P A	P A	P A	A P	A P	0

1.9 The pathway to haem and chlorophyll.

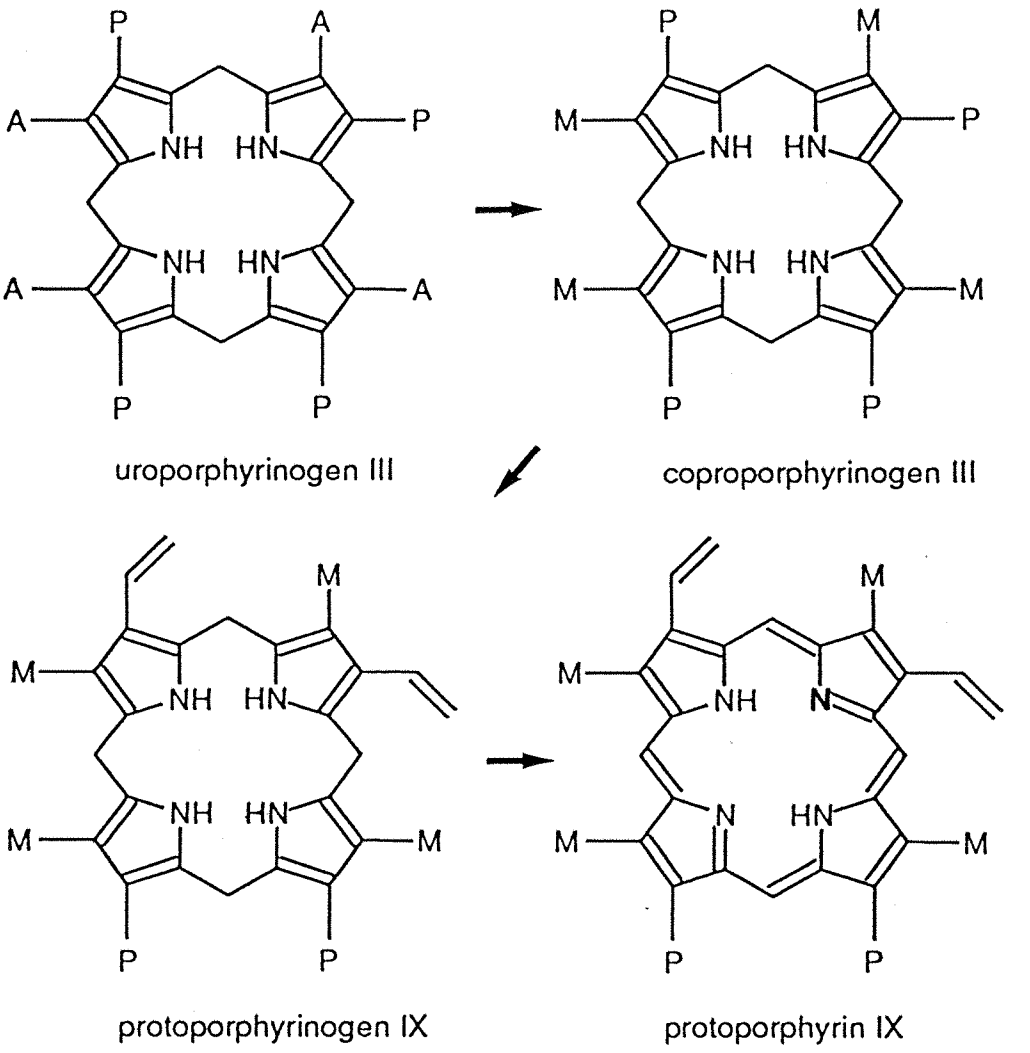
The pathway for the construction of both haem and chlorophyll is the same up to the formation of protoporphyrin IX. This intermediate is formed from uroporphyrinogen III by the action of three enzymes that results not only in an increase of the hydrophobicity of the side chains but also gives rise to an aromatic macrocycle (Scheme 12). Firstly, all four acetate side chains of uroporphyrinogen III are decarboxylated to methyl groups giving rise to coproporphyrinogen III by the enzyme uroporphyrinogen decarboxylase. The sequence of the decarboxylation has been shown to proceed in the ring order dabc (Jackson et al, 1976) and with retention of configuration (Barnard & Akhtar, 1979). Secondly, coproporhyrinogen III is converted into protoporphyrinogen IX by the oxidative decarboxylation of the propionic acid side chains of rings a and b into vinyl groups by the enzyme coproporphyrinogen oxidase. Evidence suggests that the sequence of decarboxylation occurs in the ring order a followed by b (Jackson, 1980) but the overall mechanism of the reaction has not yet been elucidated. Thirdly, the tetrapyrrole nucleus is made aromatic by the action of the enzyme protoporphyrinogen oxidase which removes six hydrogen atoms from protoporphyrinogen IX and converts it into protopoporphyrin IX. The hydrogen moieties are removed from the four meso carbon atom positions and the remaining two hydrogen species are removed from nitrogen atoms. A study of the stereochemistry of the conversion of 11S-[11-$^{3}H_{1}$]porphobilinogen into protoporphyrin IX has shown that only the label at position C-10 is retained (Jones et al, 1979 and 1984). These workers have suggested that three hydrogens are lost as hydride ions from one face of the tetrapyrrole (from C-5, C-15 and C-20) whilst the hydrogen at C-10 is removed from the other side of the macrocycle as a hydrogen ion after tautomerization of the molecule yielding the final product. Insertion of iron into protoporphyrin IX produces haem, whilst magnesium chelation and several more transformations including a methylation and two reductions before the attachment of a phytol chain leads to the construction of chlorophyll a.

1.10 The pathway to vitamin B_{12}, factor F_{430} and sirohaem.

The pathway for the synthesis of these modified tetrapyrroles diverges from the common pathway shared for the biosynthesis of haem and chlorophyll after the construction of uroporphyrinogen III. Although the structure of these compounds have been determined the elucidation of their construction from uroporphyrinogen III is still far from clear. Their synthesis is, however, dependant upon several reduction and methylation reactions and insertion of either cobalt, nickel or iron. There are several comprehensive reviews regarding the synthesis of these tetrapyrroles that give an

SCHEME 12

The pathway from uroporphyrinogen III to protoporphyrin.



accurate account of what is known about their biosynthesis (Dolphin, 1982; Leeper, 1985 a and b; Leeper, 1987). This branch is thought to be the most primitive, in evolutionary terms, of all tetrapyrrole biosynthesis and was probably in operation long before aerobic life began.

CHAPTER 2.

The purification and crystallization of *E. coli* porphobilinogen deaminase.

2.1 Introduction.

Porphobilinogen deaminase is the third enzyme of the porphyrin biosynthetic pathway and in conjunction with the next enzyme of the pathway, the cosynthase, is responsible for the assembly of the tetrapyrrole macrocycle which makes up the nucleus of all haems, chlorophylls and corrins. The enzyme has been purified from a wide variety of sources including Rhodobacter sphaeroides (Jordan and Shemin, 1973), spinach (Higuchi and Bogorad, 1975), human erythrocytes (Anderson and Desnick, 1980) and E. coli (Hart et al, 1986; Jordan et al, 1988 b). Although the enzyme has been obtainable in a purified state since the early 1970's the comparatively small quantities of the protein isolated from these sources had meant that complete characterization of the enzyme was very difficult.

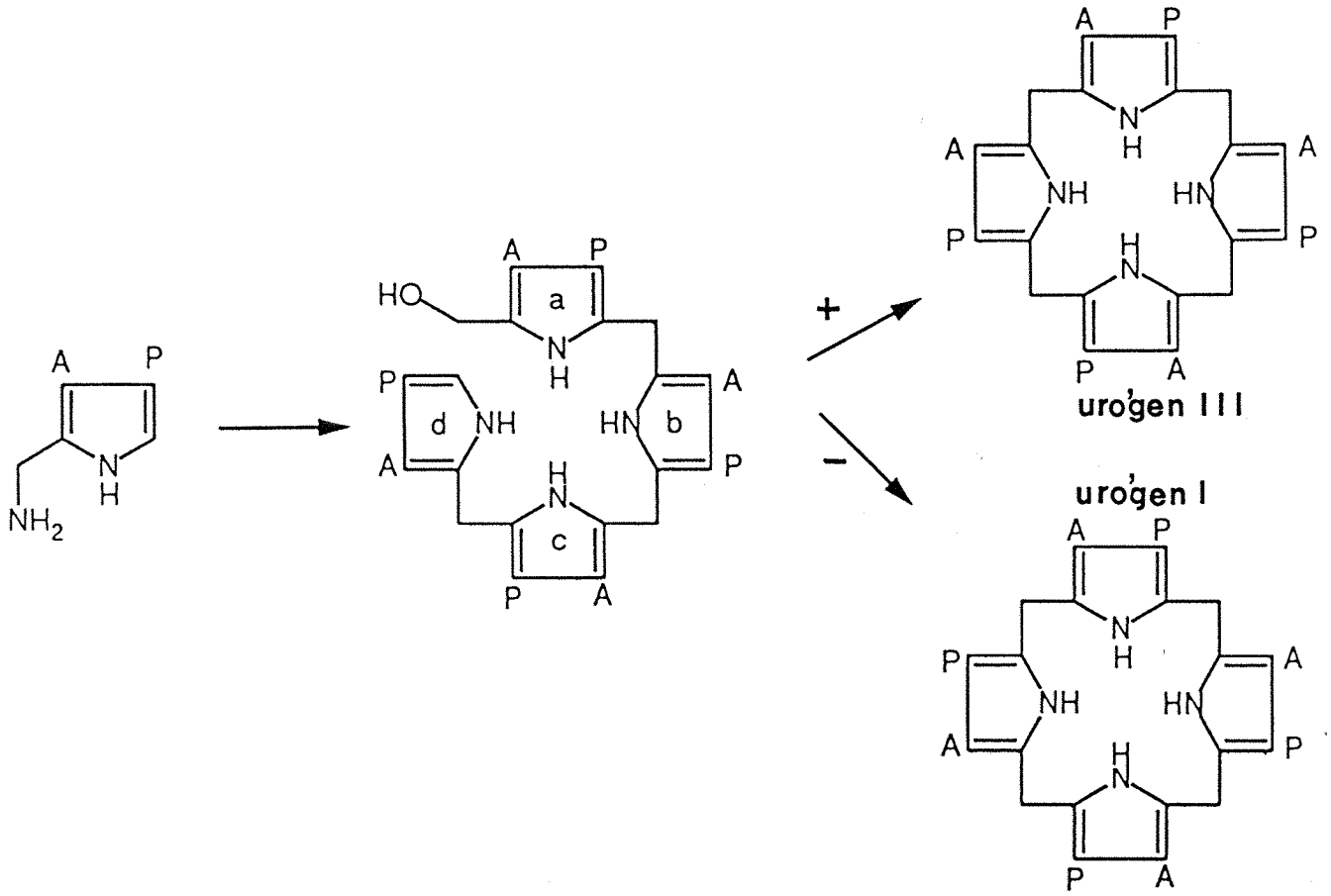
The mechanism of action of porhobilinogen deaminase has been studied for a number of years. The identification of porphobilinogen as a precursor of haem biosynthesis led to the discovery of the enzymes of uroporphyrinogen III synthesis. Classical work in the 1950's by Bogorad established that it was the combined action of two enzymes that was responsible for the synthesis of uroporphyrinogen III (Bogorad, 1958 a, b and c). He showed that heat inactivated one of these two enzymes and that the heat stable enzyme, when incubated alone with PBG, only synthesised the uroporphyrinogen I isomer (Scheme 2.1).

The mechanism by which these two enzymes catalysed the overall reaction was not resolved much further until the discovery of the product of the first enzyme, some twenty years hence. The discovery of the product, a linear hydroxymethylbilane termed preuroporphyrinogen (Burton et al, 1979 a), then paved the way for a more thorough understanding of the enzyme itself. Since that time it has been shown that the deaminase catalyses the synthesis of the linear tetrapyrrole by an ordered, stepwise addition of substrate molecules to the enzyme. The assembly of the ring structure was shown to occur in the sequence a,b,c,d (Jordan and Seehra, 1979; Battersby et al, 1979 c). The ordered attachment of the substrate molecules in the chain elongation has been shown to lead to the formation of enzyme intermediate complexes which are stable enough to be isolated as individual entities (Anderson and Desnick, 1980; Jordan and Berry, 1981).

Identification of the site of attachment of the first pyrrole ring to the enzyme as well as both the number of binding sites and active sites was still far from clear. Evidence for a lysine group being involved in the attachment of the first pyrrole ring had come from [^{13}C] n.m.r. (Battersby et al, 1983) and group specific modification

SCHEME 2.1

The products of incubating porphobilinogen in the presence and absence of cosynthase.



experiments (Hart et al, 1984). Evidence for the involvement of cysteine (Evans et al, 1986; Russell and Rockwell, 1980) and arginine (Russell et al, 1984) was also submitted. However, all these results were unconclusive and lacked any real credance.

The advent of recombinant DNA technology and the application of these techniques to the E. coli porphobilinogen deaminase has resulted in the identification of the gene and the determination of the complete nucleotide sequence (Thomas and Jordan, 1986). Cloning of the gene into the plasmid pBR322 and subsequent transformation of wild type E. coli led to the formation of recombinant strains of E. coli which had elevated levels of the enzyme. One of the above mentioned strains has been shown to overproduce the enzyme by up to nearly one hundred fold. The comparatively large quantities of the protein produced from this strain has provided enough material to permit a thorough investigation into the properties of this remarkable enzyme (Jordan & Warren, 1987; Jordan et al, 1988 a and b; Warren & Jordan, 1988 b).

The main aim, therefore having available a recombinant over-producing strain of E. coli, was to devise a method to purify the enzyme in as high a yield as possible. The large amounts of enzyme obtained could then be used to characterize the protein in full, identify the substrate binding site and allow attempts at crystallization to be made. Some of the work in this chapter has previously been reported (Jordan et al, 1988 b).

2.2 Results and discussion.

2.2.1 Growth and development of bacterial strains harbouring *hem*C plasmids.

The identification, isolation and cloning of the hemC gene gave rise to three recombinant strains of E. coli each of which had elevated levels of porphobilinogen deaminase (Jordan et al, 1985). In order to establish which of these strains would give rise to the highest amount of porphobilinogen deaminase, the level of the enzyme was monitored with time against bacterial cell growth for each of the strains and was compared to the level found in wild type bacteria. The result of this time course is shown in figure 2.1. The level of porphobilinogen deaminase in the three recombinant strains was found to be consistent with that observed by Thomas (1986) in the fact that ST1048 had the highest level of deaminase (about 100 fold increase compared to the wild type species), followed by ST1047 and ST1046 which over-produced the enzyme by about 60 and 30 fold respectively compared to the wild type species. The time course shows that during the exponential phase of growth, porphobilinogen deaminase activity in all four strains increased in parallel with the cell density (Figure 2.1). After some 16-20 hours the cultures showed little further increase in cell density indicating that the bacteria had entered stationary phase. However, the strains harbouring plasmids containing hemC continued to show a steady increase in porphobilinogen deaminase activity over the next 25 hours. Following this period, a brief acceleration in the rate of increase of enzyme activity was seen in ST1048 until a maximum was reached at approximately 55 hours after innoculation. The difference in the levels of the deaminase found between the various strains can be explained by the different sizes of the plasmids. The smaller sized plasmids give rise to higher copy numbers within the cell and this in turn gives rise to a higher level of expression of the particular protein (Table 2.1). Thus pST48 being the smallest of the plasmids was found by Thomas (1986) to have the highest copy number followed by pST47 and pST46 respectively. This copy number to size ratio reflects the activity of the deaminase found in the strains transformed with these particular plasmids. Why the levels of the enzyme take so long to reach their maximum in the cells is unknown, although again it probably reflects the plasmid copy number build up. Obviously to establish this more work is required to investigate the direct correlation between the build up of the deaminase levels and both the amount of plasmid DNA and RNA in the cell.

The conclusion from the monitoring of the cell growth and the enzyme development was that strain ST1048 gave the maximum deaminase levels at a time of 55 hours after innoculation. Thus, for the purification of the enzyme from this source, the

Figure 2.1

Time course of bacterial cell growth and porphobilinogen deaminase activity in strains harbouring plasmids encoding hemC. Cell growth (●-●), porphobilinogen deaminase activity in ST1046 (○-○), in ST1047 (△-△), in ST1048 (□-□) and in HB101/pBR322 (■-■).

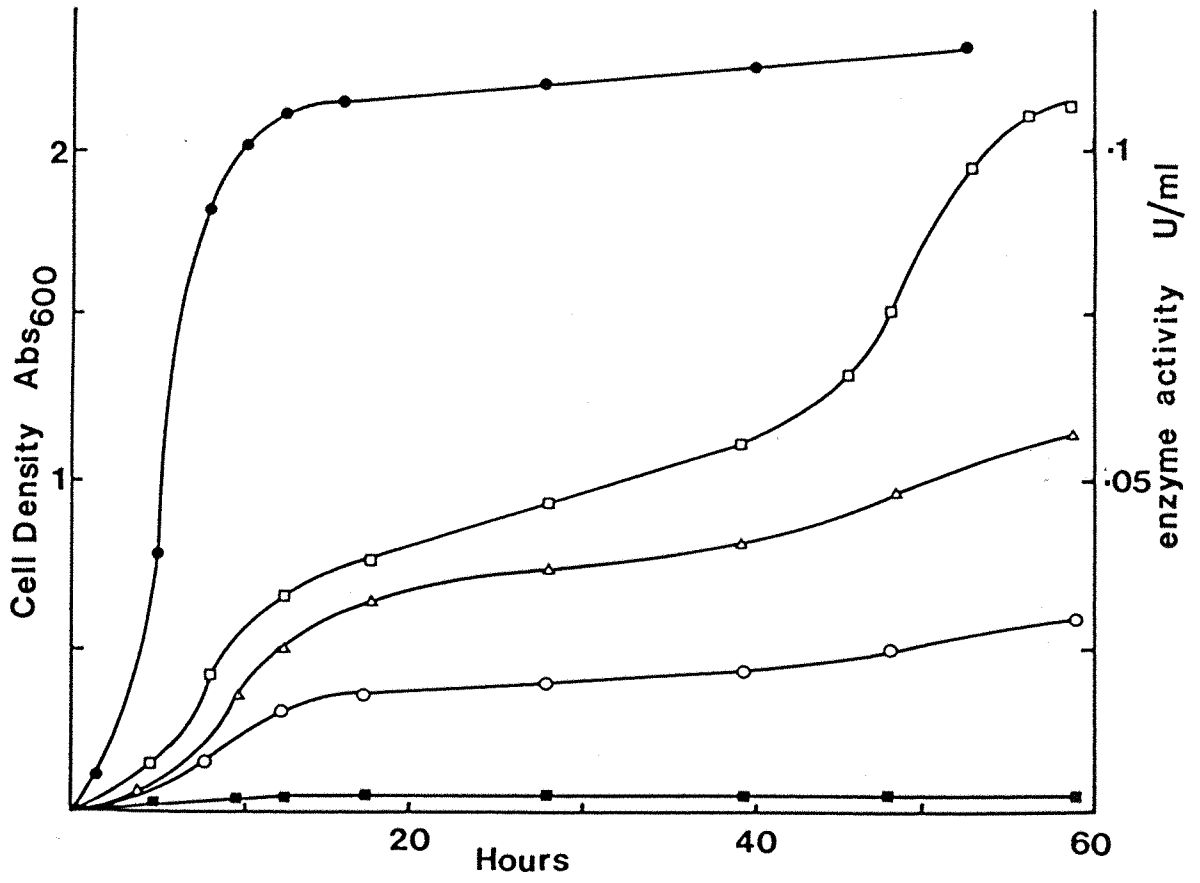


TABLE 2.1

The comparison of the size of hemC containing plasmids to the production of porphobilinogen deaminase. Plasmid size is taken from Thomas (1986).

Bacterial strain	Plasmid size (Kilo-bases)	Deaminase specific activity (μmoles/hr/mg)
Wild type control (HB101/pBR322)	-	0.002
ST1046 (HB101/pST46)	7.24	0.075
ST1047 (HB101/pST47)	5.77	0.121
ST1048 (HB101/pST48)	4.37	0.202

bacteria were harvested after 55 hours.

2.2.2 Purification of *E. coli* porphobilinogen deaminase from ST1048.

The purification of the deaminase from E. coli ST1048 was developed to give as high an overall yield of the protein as possible. This was accomplished by using as few steps as possible in the purification and using procedures which themselves had high yields. The initial strategy was developed from that used to purify the enzyme from R. sphaeroides (Jordan and Shemin, 1973) and from human erythrocytes (Anderson and Desnick,1980). All the steps that were used involved classical techniques that have been adopted for the purification of numerous other proteins, apart from the final f.p.l.c. stage.

The initial sonication step was used to break open the bacteria and to release their cytosolic contents. The conditions used were selected as they gave the highest level of the active enzyme. The use of proteolytic enzyme inhibitors was found to have very little effect on the overall yield of the purified deaminase and were therefore not added to the disruption buffer. Once sonicated the bacteria were immediately heat treated by placing the broken cell extract in a boiling water bath. The extract was maintained at 60°C for 10 minutes. This procedure not only precipitated a large quantity of protein but also inactivated uroporphrinogen III synthase, the next enzyme in the tetrapyrrole pathway. This purification method was used at this stage because it was reasoned that since it conferred a ten fold purification upon the enzyme it may also inactivate some of the proteolytic enzymes present in the extract.

The next stage in the purification was the ion exchange step and this was used largely as a concentration means. Although the deaminase binds tightly to the DEAE cellulose column the low resolution obtained from it only gave a two fold purification at best. However, since the enzyme was eluted from the column in a volume of 200mls this represented a five fold concentration as the supernatant from the heat treatment stage was applied in a volume of 1 litre. In smaller preparations, where the heat treated enzyme can easily be concentrated either by ultrafiltration or by ammonium sulphate precipitation, this stage can be omitted as the final f.p.l.c. ion exchange column procedure gives a much greater resolution. In this larger preparation the halving of the protein content and the much smaller volume obtained makes concentration of the enzyme extract a viable proposition. The concentration of the extract was carried out by ultrafiltration with the use of a PM 10 membrane which allows only proteins of molecular weights of less than 10,000 Daltons to pass through. The enzyme was concentrated to a volume of around 40mls, which was accomplished in a time of about five hours and with only a slight loss of enzyme activity.

The concentrate was applied immediately to the base of a Sephacryl S-200 column which was pumped upwards at a flow rate of 1ml/minute. As the column

developed so the protein extract that had been added could be seen to separate into three yellow coloured bands. The first of these bands to elute was the darkest in colour and was associated with the excluded large molecular weight proteins. The deaminase eluted immediately behind the second of the coloured bands and this proved to be a useful marker for following the migration of the deaminase on the column. The deaminase eluted in a volume of about 200mls.

The enzyme, after the gel filtration stage, when analysed by SDS gel electrophoresis was shown to be the major protein band and to constitute about 20% of the total protein. Although the enzyme at this stage could be applied directly to the f.p.l.c., the loading capacity of the ion exchange column and the rather poor yield of homogeneous protein obtained meant that it was a time consuming and inefficient process. Thus a third column procedure was introduced which differentiated between the proteins on the bases of their hydrophobic properties. The E.coli enzyme was therefore applied to a column of Phenyl Sepharose in the presence of high salt (30% saturated ammonium sulphate (w/v)), conditions under which the enzyme bound quite tightly to the column, and was eluted by decreasing the salt content in the surrounding buffer. It was found that the E.coli deaminase was eluted from the column at around 5% (w/v) ammonium sulphate saturated buffer. This column procedure gave over a 3 fold purification to the enzyme and after dialysis, when the protein was again analysed by SDS gel electrophoresis, the deaminase was found to constitute around 70% of the total protein. This procedure therefore meant that the final f.p.l.c. step could be accomplished in a faster time and with a higher overall yield of homogeneous protein.

The final step in the purification of the deaminase was by the use of a high resolution ion exchange column. The use of such columns in the purifications of proteins has grown exponentially over the last five years and they have proved to be invaluable for the isolation of a number of proteins. The Mono Q HR 5/5 column used in this case was able to separate the deaminase into several broad peaks. Any fractions containing non-homogeneous deaminase were desalted and reapplied to the column at pH 6. This procedure proved to be very useful in "cleaning up" any impure fractions. Another useful method for the purification of crude fractions was to add stoichiometric quantities of the substrate to the enzyme and then to isolate the enzyme substrate complexes which eluted at a higher salt concentration.

The purified enzyme was judged to be homogeneous by electrophoresis on denaturing SDS polyacrylamide gels. A summary of the purification is shown in table 2.2. The purified enzyme had a specific activity of 43μmoles/hour/mg of protein when the protein content was determined by the methods described. When the protein concentration was estimated by measuring the absorption at 280nm and assuming an $A^{1\%}$ value of 10, as used by other workers (Hart et al, 1986), a gross over-estimate of

TABLE 2.2

Purification of porphobilinogen deaminase from 50 litres of E. coli strain ST1048.

PURIFICATION STAGE	VOLUME (mls)	TOTAL PROTEIN (mgs)	TOTAL UNITS (μmoles/hr)	SPECIFIC ACTIVITY (μmoles/hr/mg)	% YIELD
SONICATION	1,000	40,000	6,500	0.14	100
HEAT TREATMENT	1,000	4,200	6,350	1.50	97
ION EXCHANGE DE-52	220	2,000	6,000	3.00	92
SEPHRACRYL S-200	200	260	5,200	20.00	80
PHENYL SEPHAROSE	100	150	4,600	30.00	71
MONO Q F.P.L.C.	40	70	3,000	43.00	46

the specific activity was obtained since the actual $A^{1\%}$ value is 4.7.

The purified deaminase could be stored, after desalting or dialysis against 10mM Tris/HCl buffer pH 8.2, at -20°C for several months without any undue loss of activity.

Other purification procedures were attempted but the protocol described above was found to be the quickest and most reproducible. The only other method that has not been discussed is the precipitation of the protein by ammonium sulphate. As mentioned earlier, this method is useful in smaller preparations of the enzyme in order to concentrate the heat treated extract prior to application to the gel filtration column. Ammonium sulphate precipitation of the enzyme starts at about 45% saturation of the buffer with the ammonium sulphate and the enzyme was usually precipitated in a crude cut between 35-65% saturation. However, once precipitated it was found that centrifugation of the suspension, in order to pellet the precipitate, should not be done at a speed greater than 10,000 g otherwise the enzyme becomes inactivated.

Attempts to prepare an affinity column for the specific purification of the deaminase by attachment of porphobilinogen to a fixed matrix were not successful. Other groups had previously made such columns by linking porphobilinogen to a support via the amino side arm of the pyrrole (Anderson and Desnick, 1980; Williams et al, 1981). These methods had shown a non-specific affinity for the deaminase. The rationale behind the synthesis of this particular affinity column was to try to bind the substrate to the supporting matrix via its free α-position thus leaving the amino side arm free to interact with the enzyme. Although the deaminase bound to the column it again appeared to interact by a non-specific mode and was eluted from the column by a low salt concentration.

2.2.3 Molecular weight determinations.

i) By SDS polyacrylamide gel electrophoresis.

When it was subjected to SDS gel electrophoresis, the enzyme migrated as a single protein band (Figure 2.2, a) with a mobility slightly greater than glyceraldehyde 3-phosphate dehydrogenase (standard molecular weight of 36,000 Daltons). When the mobility of the deaminase was compared to the mobility of the molecular weight standards on a logarithmic plot a subunit molecular weight of 35,000 $\pm$ 3,000 Daltons was obtained (Figure 2.2, b).

ii) By gel filtration through Sephacryl S-200.

The molecular weight of the native enzyme was determined by chromatography through Sephacryl S-200. The enzyme was co-chromatographed with proteins standards of known molecular weight. The deaminase eluted from the column in a single peak of

Figure 2.2

Molecular weight determination of porphobilinogen deaminase using sodium dodecyl sulphate polyacrylamide gel electrophoresis.

a) 10μg of purified porphobilinogen deaminase (right lane); molecular weight standards (left lane).

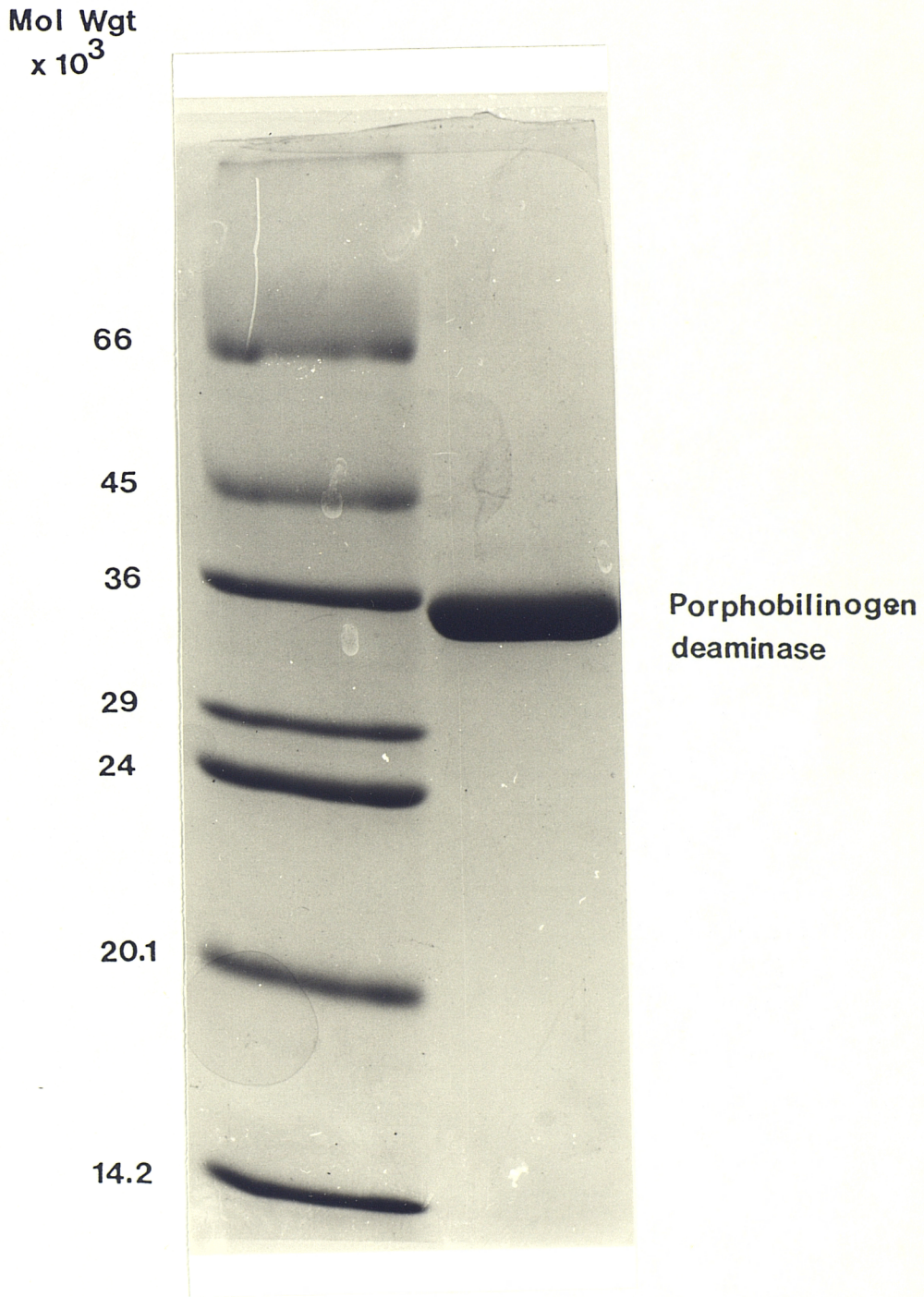
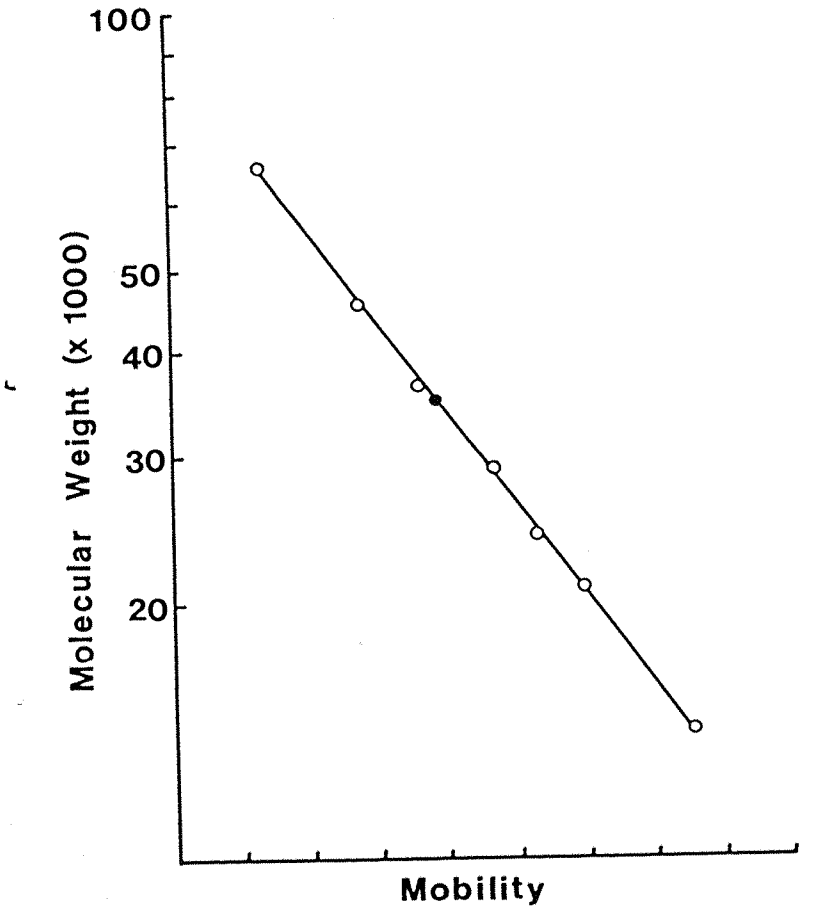


Figure 2.2

Molecular weight determination of porphobilinogen deaminase using sodium dodecyl sulphate polyacrylamide gel electrophoresis.

b) Mobility of E. coli porphobilinogen deaminase compared to the protein standards.



deaminase activity corresponding to a molecular weight of 32,500 ± 2,000 Daltons (Figure 2.3). There was no evidence for higher molecular weight forms under any conditions. The E. coli enzyme thus appears to exist as a monomer as do all other deaminases isolated so far.

2.2.4 Enzyme heterogeneity.

Although the purified deaminase existed as a single protein band after SDS electrophoresis, under non-denaturing conditions 3 major bands could be visualised (Figure 2.4). These bands all exhibited the same deaminase specific activity. When the same sample of purified deaminase was applied to a high resolution anion exchange Mono Q column, using a very slack gradient (0 - 200mM NaCl; 40mls total volume) the protein could be resolved into 5 peaks (Figure 2.5, a). These peaks also all exhibited the same deaminase specific activity. All the isolated peaks from the f.p.l.c. ran as a single band of molecular weight of 35,000 Daltons on denaturing electrophoresis gels (Figure 2.5, b). The fact that the enzyme forms all gave exactly the same molecular weight indicated that the isolated species were not proteolytic degradation products, since to give 5 different forms there would have to be cleavage of at least 5 amino acids from either the N or the C terminus. Such a cleavage would give rise to a difference in molecular weight of at least 500 Daltons between the species which would be noticeable by SDS gel electrophoresis analysis. N-Terminal sequencing of the protein revealed no sign of any heterogeneity. Surprisingly even f.p.l.c. derived peaks appeared to exist in at least two forms when run under non-denaturing conditions (Figure 2.5, c).

It was important to determine unambiguously whether these multiple species were due to enzyme heterogeneity or to the presence of enzyme-intermediate complexes. It is well established that the deaminases isolated from other sources show multiple bands on non-denaturing polyacrylamide gels. These bands have been interpreted as being due to enzyme together with enzyme-intermediate complexes representing different stages of the catalytic cycle "en route" to the tetrapyrrole. Such complexes are susceptible to treatment with hydroxylamine and under these conditions the bound intermediate is released regenerating the free enzyme (Pluscec & Bogorad, 1970; Davies & Neuberger, 1973). The purified E. coli deaminase was therefore treated with 0.2M hydroxylamine and re-analysed by both non-denaturing electrophoresis and f.p.l.c. The hydroxylamine treated enzyme showed no change in mobility indicating that the multiple species were not due to enzyme-intermediate complexes. This was further confirmed by incubating the purified enzyme with [^{14}C]-porphobilinogen to generate the enzyme-intermediate complexes. The resulting enzyme-intermediate complexes were subjected to non-denaturing gel electrophoresis and stained for protein. A duplicate gel was analysed for radioactivity showing that the new bands representing enzyme substrate complexes,

Figure 2.3

Molecular weight determination of native porphobilinogen deaminase by gel filtration on Sephacryl S-200. Enzyme (40 units) and 3mgs of each protein standard were chromatographed on a column (2.5 x 100 cm) of Sephacryl S-200. The molecular weight of the deaminase was calculated from its relative elution volume in comparison to the elution volumes of the molecular weight standards.

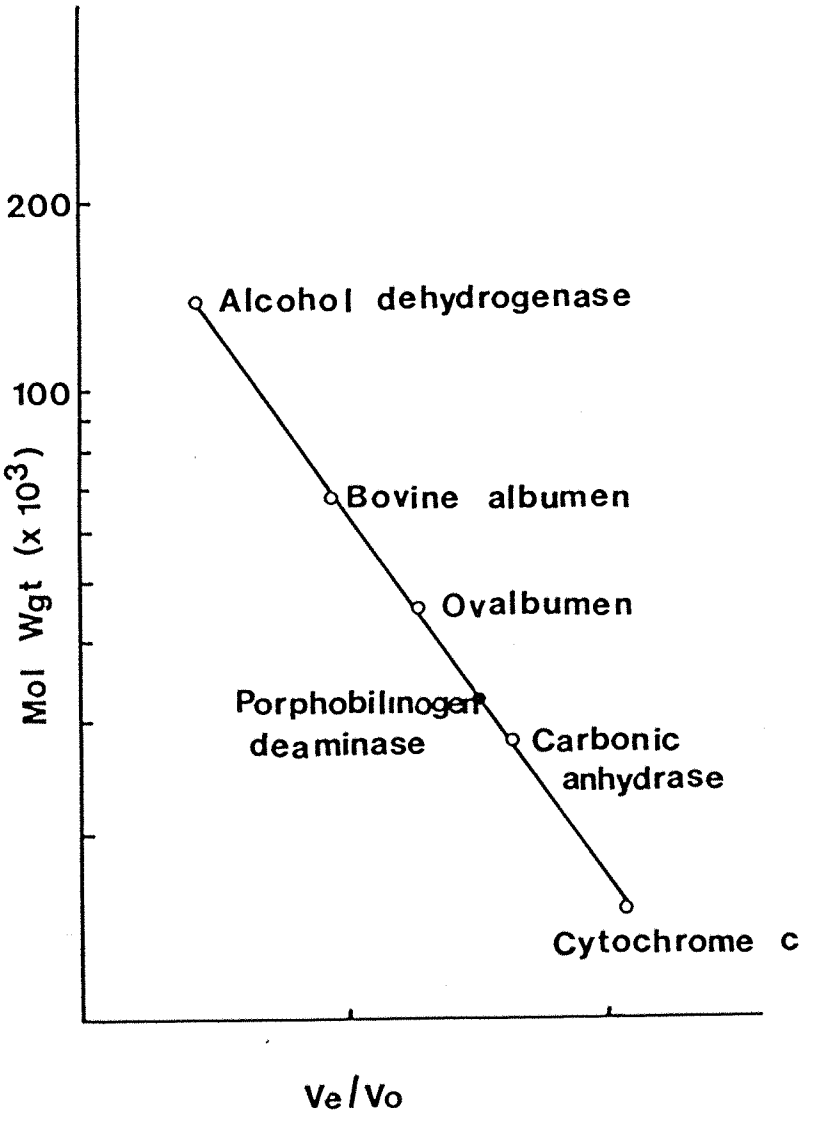


Figure 2.4

Electrophoresis of E. coli porphobilinogen deaminase under non-denaturing conditions. 30μg of the enzyme was subject to electrophoesis as described in the experimental section.

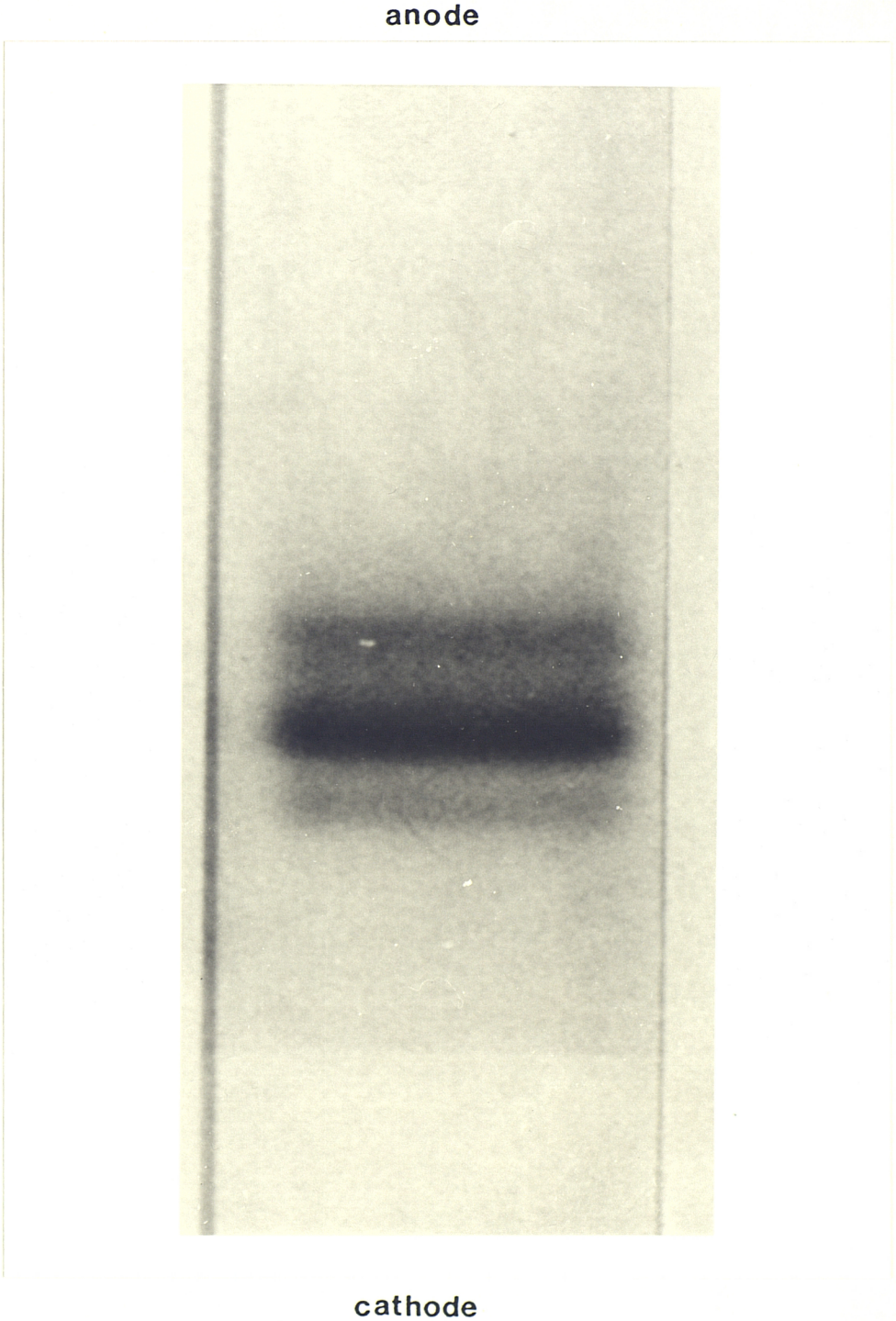


Figure 2.5

Analysis of the different forms of the enzyme by high resolution ion-exchange chromatography and electrophoresis.

a) High resolution ion-exchange chromatography of purified porphobilinogen deaminase using a Mono Q HR 5/5 column. The enzyme eluted in five separate peaks marked A to E, as shown.

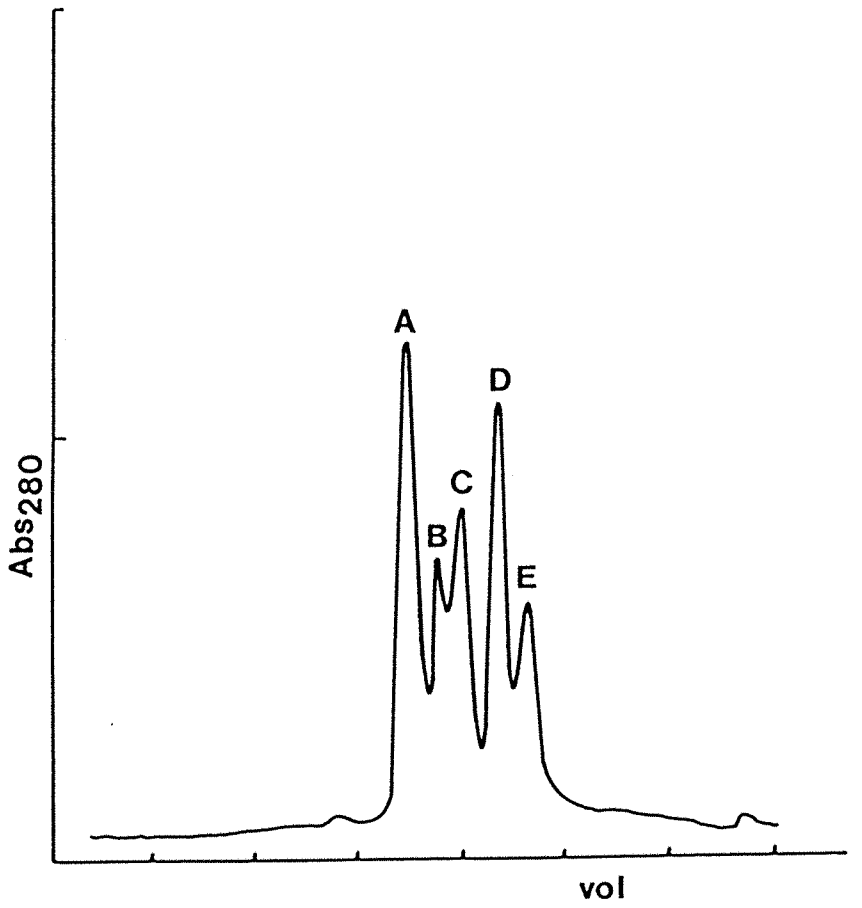


Figure 2.5

Analysis of the different forms of the enzyme by high resolution ion-exchange chromatography and electrophoresis.

b) Sodium dodecyl sulphate polyacrylamide gel electrophoresis of each of the 5 peaks isolated by high resolution ion-exchange chromatography in 2.5a. Lanes 1 and 7 contain molecular weight standards. Lanes 2 to 6 contain 10μg of protein from each peak, A to E, respectively.

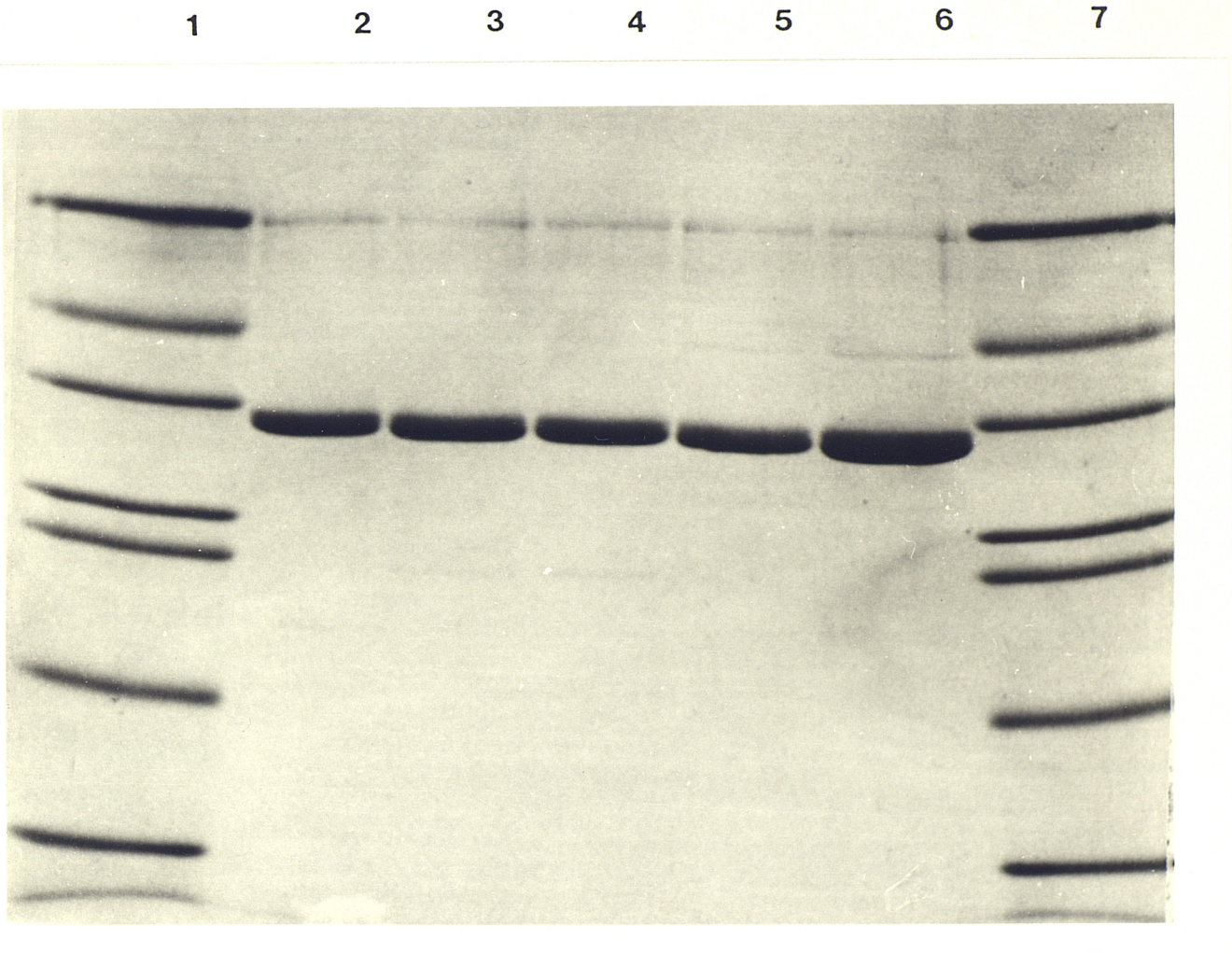
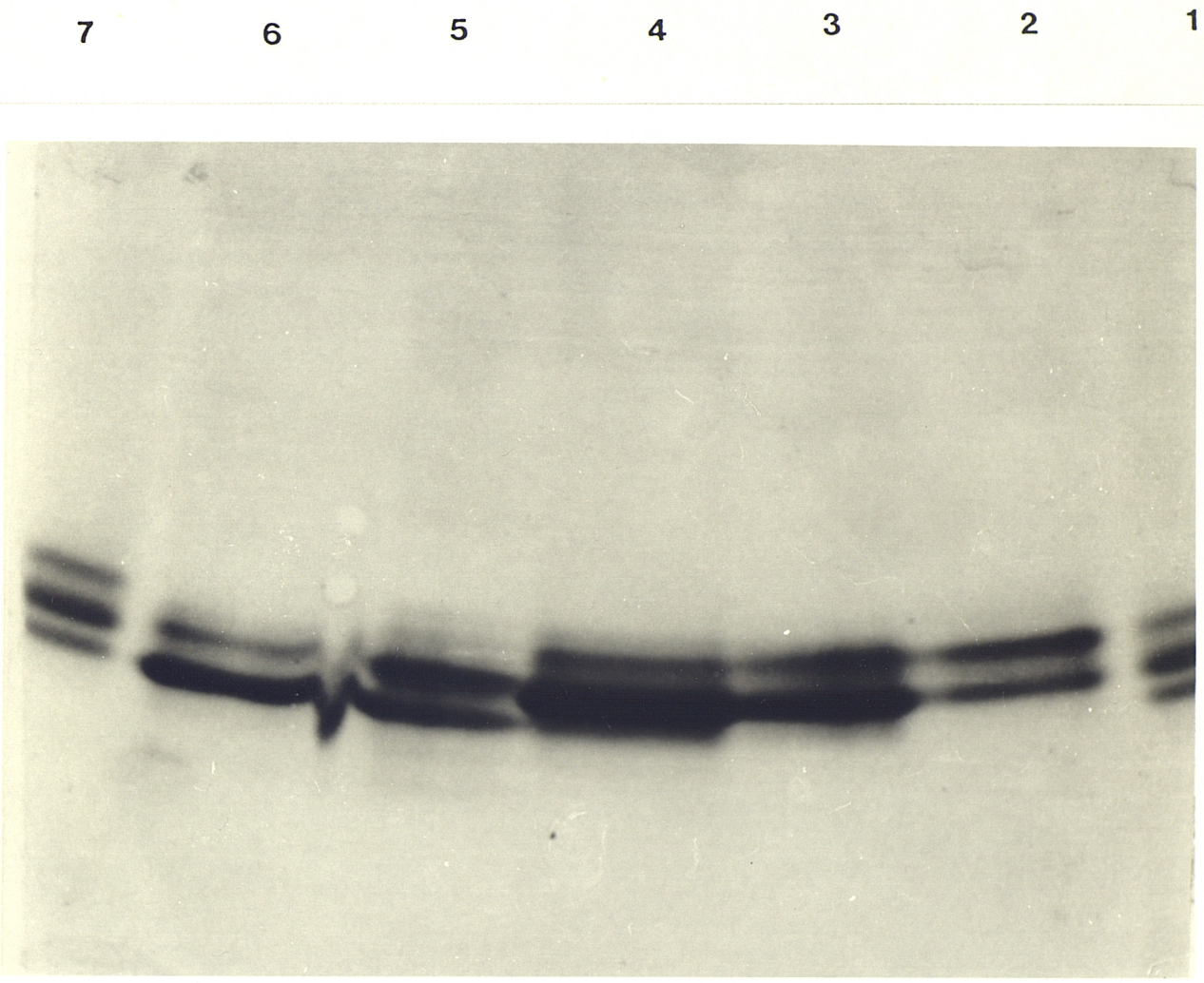


Figure 2.5

Analysis of the different forms of the enzyme by high resolution ion-exchange chromatography and electrophoresis.

c) Non-denaturing polyacrylamide gel electrophoresis of the 5 peaks isolated by high resolution ion-exchange chromatography. Lanes 1 and 7 contain unresolved porphobilinogen deaminase. Lanes 2 to 6 contain 30µg of protein from peaks A to E in 6 (a).



were radioactive. Treatment of these complexes with hydroxylamine resulted in the liberation of the [^{14}C] radioactivity and the regeneration of the original enzyme pattern of 3 bands, all of which were essentially devoid of radioactivity (Figure 2.6). This firmly establishes that the multiple species observed in the purified enzyme are not enzyme-intermediate complexes.

Since all the forms of the enzyme encountered have the same specific activity and the possibility that the multiple species are due to proteolytic degradation products or to enzyme-intermediate complexes, the observed heterogeneity in the purified protein therefore most likely represents slightly different structural forms of the deaminase. This could have arisen by amino acid modification, such as the deamidation of asparagine residues to aspartic acid residues. Whatever the reason for these enzyme forms, it does not seem to affect the ability of each of them to behave as an enzyme with normal activity.

2.2.5 The pH optimum, K_m and isoelectric point.

The effect of pH on the activity of the deaminase was studied over the pH range 6-10 and it was found that the pH optimum was in the broad range between 8.4 and 9.0 (Figure 2.7). The enzyme was inactive below pH 6 and was found to be irreversibly inactivated below pH 4. In unbuffered HCl, however, enzyme activity could be recovered from solutions as low as pH 1 although permanent inactivation occurred if the enzyme was left at this pH for more than two hours. Enzyme activity could also be regained from solutions as high as pH 12 although, again, prolonged incubation under these conditions ultimately led to permanent inactivation. The importance of these pH dependant inactivation studies is discussed in more detail in chapter 3.

The K_m value for the enzyme was determined from a double reciprocal plot after measuring the initial rate of the deaminase reaction with substrate concentrations between 20μM and 200μM (Figure 2.8). The K_m for the E. coli porphobilinogen deaminase was found to be 19 $\pm$ 7μM at its pH optimum. This value is of a similar magnitude to that reported for the E. coli deaminase by Hart et al (1986) and to that found for the enzyme isolated from R. sphaeroides (Jordan and Shemin, 1973).

Flat bed isoelectric focussing of the purified deaminase gave three major bands which ran very close together with an average pI value of 4.5. The three species seen upon isoelectric focussing probably reflect the three species observed after non-denaturing polyacrylamide gel elctrophoresis.

Figure 2.6

Non-denaturing polyacrylamide electrophoresis gel showing the effect of hydroxylamine on [^{14}C]enzyme intermediate complexes derived from purified porphobilinogen deaminase and [^{14}C]porphobilinogen.

Track 1. Protein profile of the [^{14}C]enzyme-intermediate complexes before treatment with 0.2M hydroxylamine.

Track 2. Similar sample to track 1 but following hydroxylamine treatment.

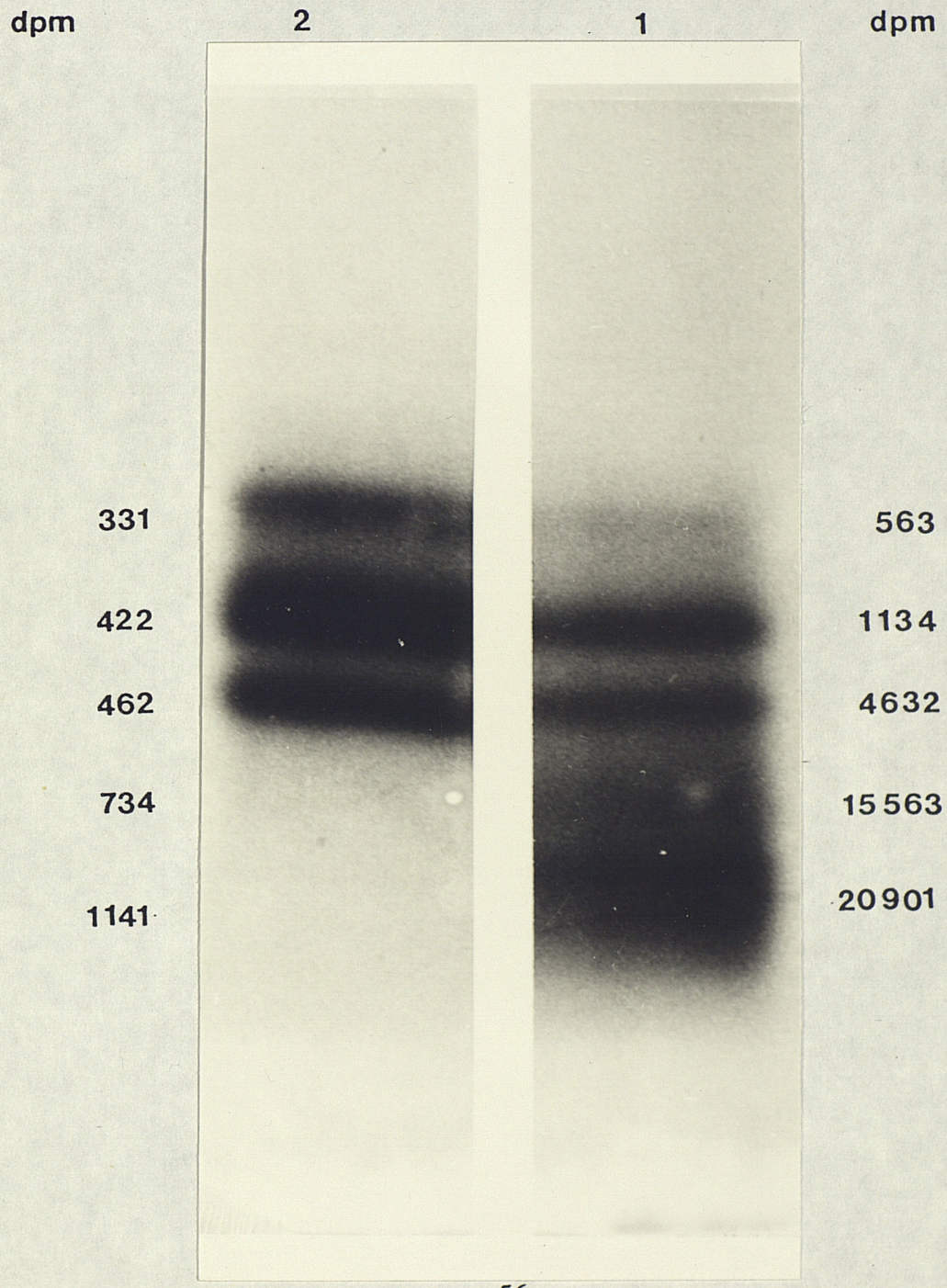


Figure 2.7

Effect of pH on the activity of E. coli porphobilinogen deaminase. Porphobilinogen deaminase activity was determined as described in the experimental section over the pH range 6 to 10 using 0.1M phosphate (○-○), Tris/HCl (□-□) and CHES (△-△) buffers.

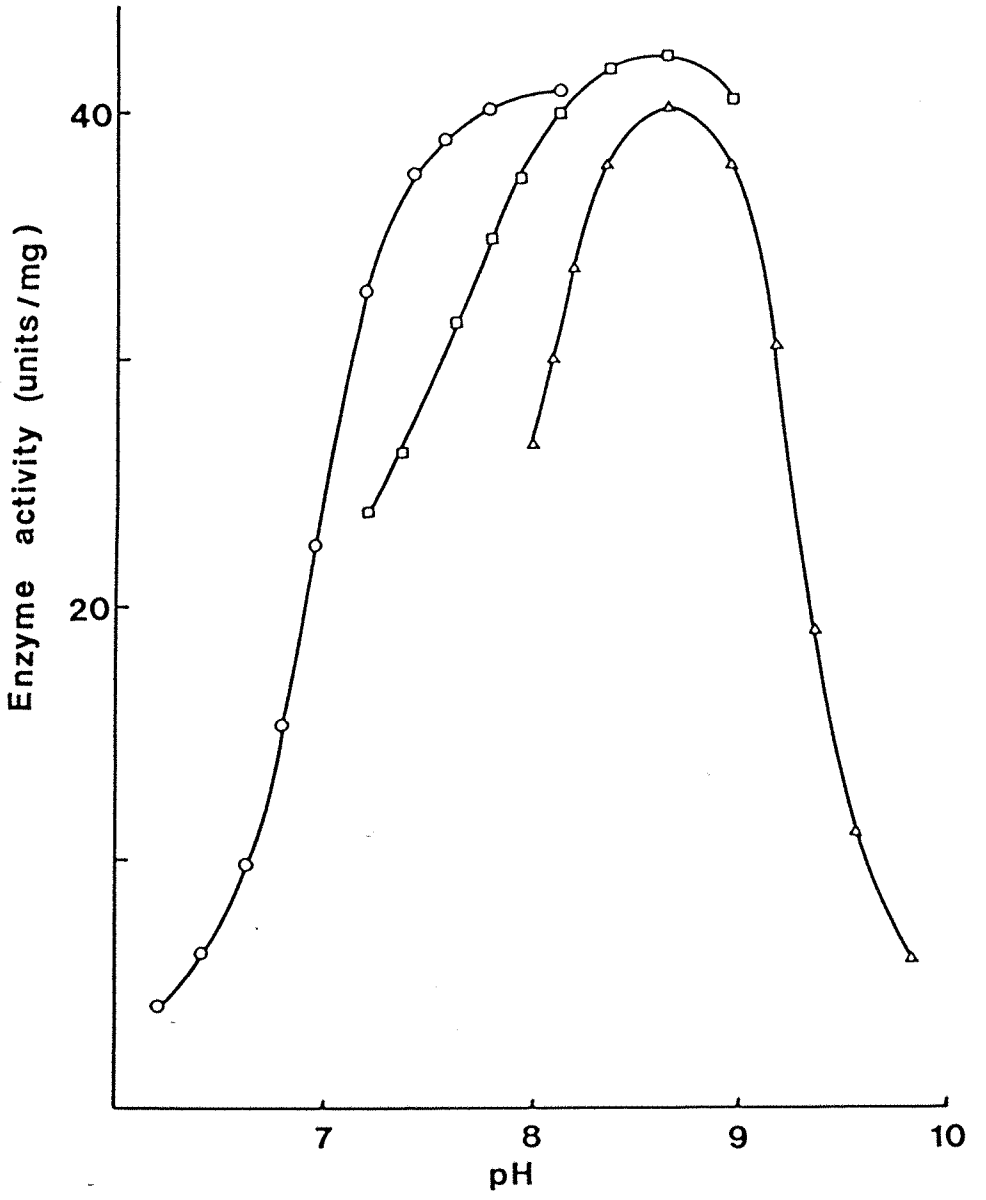
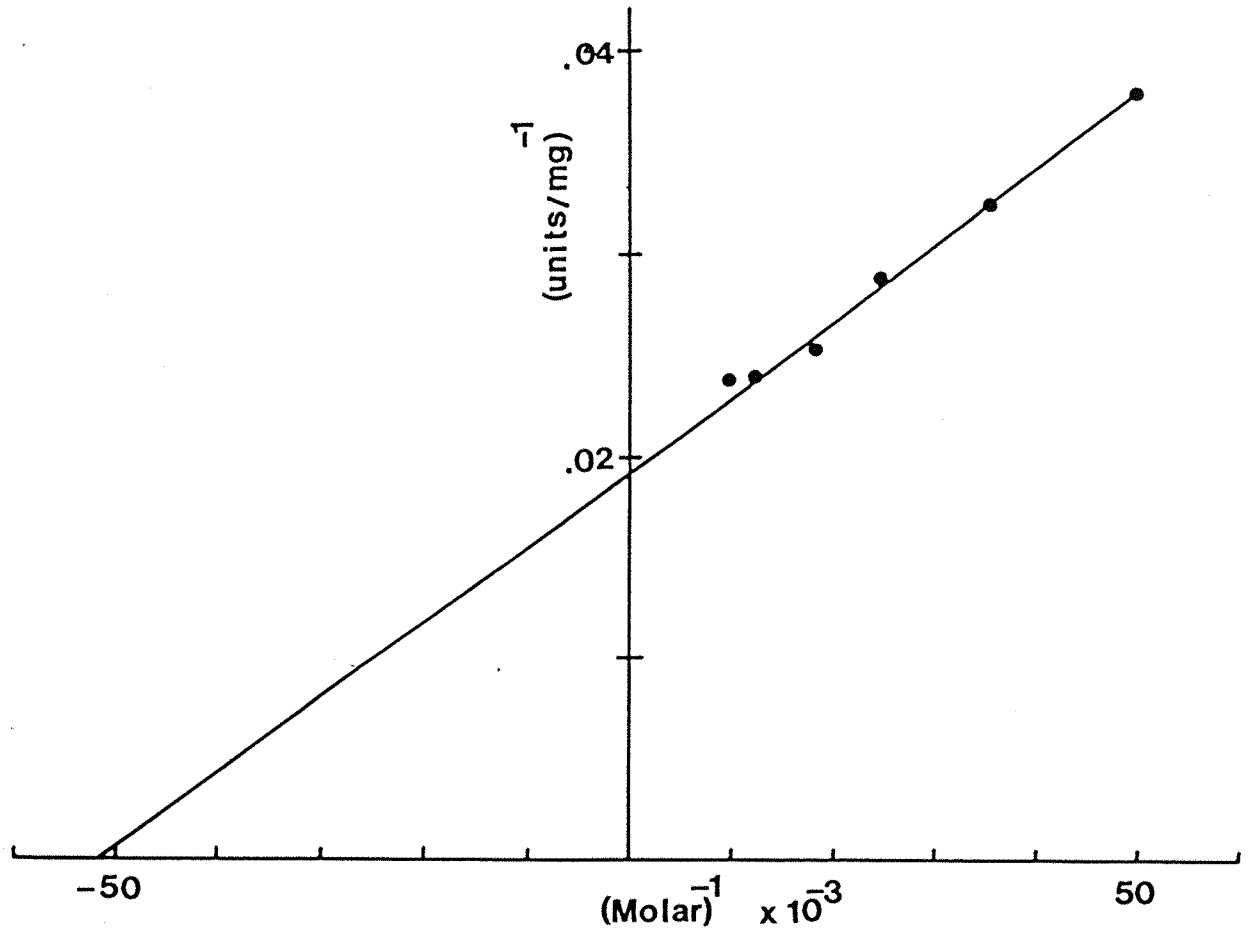


Figure 2.8

Determination of the K_m value of porphobilinogen deaminase with the use of a double reciprocal plot of enzymic rate (μmoles/hr) against substrate concentration (Molar).



2.2.6 N-Terminal analysis

Carboxymethylated deaminase was sequenced in a pulsed liquid sequencer as described in the methods section. The N-terminus was shown to be as follows:

H_2N- M - L - D - N - V - L - R - I - A - T -

This sequence is consistent with that predicted from the complete gene sequence data reported by Thomas and Jordan (1986), but is different to that reported by Hart et al (1986).

2.2.7 Amino acid composition

The amino acid analysis of the purified protein is shown in table 2.3. The composition is in close agreement to that predicted from the full gene sequence. It was interesting to note that the performic acid oxidized protein sample, which was prepared to make a measurement of the number of cysteine residues as cysteic acid, was in fact a pink colour and, when viewed under ultra-violet light was flourescent. This important observation will be dealt with in chapter 3.

2.2.8 Formic acid cleavage.

Analysis of the products of the formic acid cleavage by SDS gel electrophoresis showed two new polypeptides of molecular weights 24,000 Daltons and 11,000 Daltons (Figure 2.9) corresponding to the cleavage at the single aspartic acid (D) - proline (P) link at residues 103-104. This observation lends further support to the protein primary structure predicted from the gene sequence. Treatment of the protein with formic acid also resulted in the formation of uroporphyrin in a similar manner to that formed by perchloric acid oxidation of the protein. Again this aspect will be dealt with in chapter 3.

2.2.9 Crystallization of the *E. coli* porphobilinogen deaminase.

The only technique that can obtain an accurate three dimensional structure of a molecule that has the complex folded nature and size of a protein molecule is X-ray diffraction. Certainly localized information pertaining to the whereabouts of particular reactive groups within a designated area around the active site of enzymes can be obtained from stereochemical analysis of the enzyme reaction, but this information is best interpreted with the protein structure if it is known. X-Ray diffraction analysis of proteins is only obtained from the macromolecule when it is in an ordered crystalline state and it is the preparation of this crystalline state that generally represents the major hurdle in obtaining the required structural information.

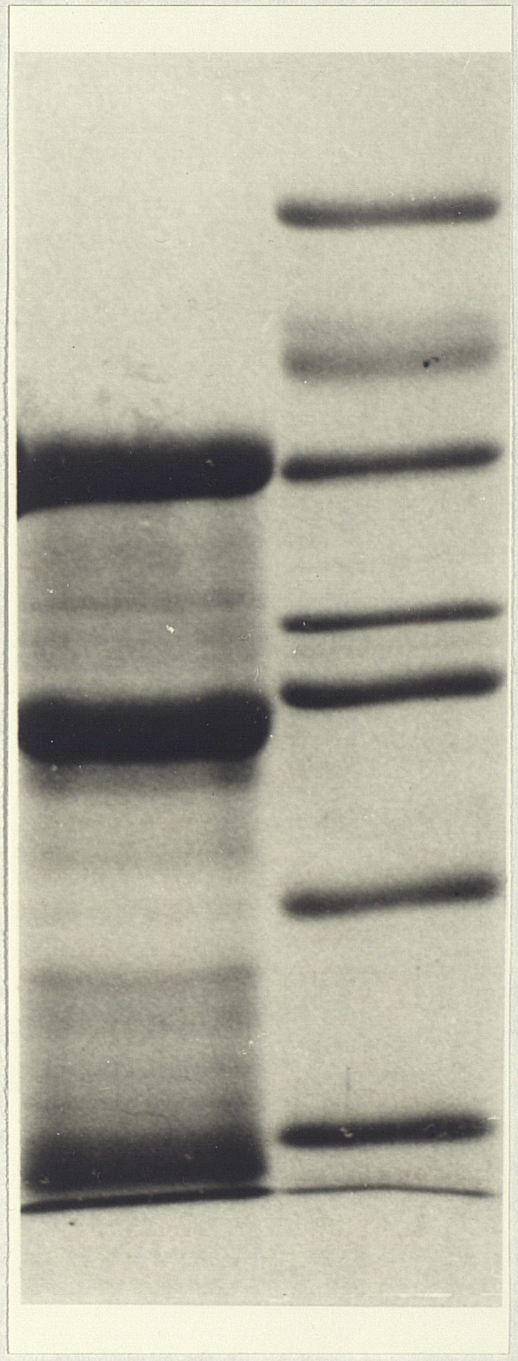
TABLE 2.3

Amino acid composition of purified E. coli porphobilinogen deaminase. Comparison is made to the amino acid composition predicted from the gene sequence.

AMINO ACID	Number of residues observed	Predicted
ASPARTATE/ASPARAGINE	30	30
THREONINE	10	10
SERINE	13	16
GLUTAMATE/GLUTAMINE	27	34
GLYCINE	28	28
ALANINE	30	34
CYSTEINE	3	4
VALINE	20	24
METHIONINE	5	6
ISOLEUCINE	16	19
LEUCINE	40	42
TYROSINE	5	5
HISTIDINE	7	5
LYSINE	8	8
ARGININE	28	27
PHENYLALANINE	4	3
PROLINE	ND	16
TRYPTOPHAN	ND	2

Figure 2.9

Formic acid cleavage of porphobilinogen deaminase. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of 50μg of porphobilinogen deaminase after cleavage with formic acid (left lane). A = uncleaved protein. B = 24,000 Dalton molecular weight fragment. C = 11,000 Dalton molecular weight fragment. Molecular weight standards (right lane).



Protein crystals represent an equilibrium between solid and solute with the equilibrium in favour of the protein coming out of solution. To achieve this equilibrium the protein in solution has to reach a point of supersaturation slowly, whereby it becomes energetically favourable for the protein to enter the solid phase in an ordered arrangement and not to form a mass precipitate. Due to the complicated behaviour of protein molecules in solutions it is very difficult to predict how and when the protein in solution will reach this point of supersaturation. However various techniques have been developed by protein crystallographers which scan over a wide range of possible crystallization conditions and these methods have been well documented (McPherson, 1982; Blundell and Johnson, 1976.). Out of all these methods the vapour diffusion technique is probably the easiest to set up and operate and it was this technique that was employed to crystallize the deaminase.

The conditions under which the E. coli porphobilinogen deaminase was crystallized were chosen initially because the pH was close to the measured pI value of the enzyme and because at this pH, at high concentrations, sodium chloride acted as a good precipitating agent. The protein was initially dialysed against water, lyophilised and then dissolved in 50mM sodium acetate buffer pH 4.8 containg 5% (w/v) NaCl to a final concentration of about 30mg/ml. A 20µl droplet of the protein solution was then placed on a siliconised microscope slide cover which was then inverted over a well in a tissue culture tray that contained 2mls of a 20% (w/v) NaCl solution. This procedure gave rise to diamond-shaped crystals when grown from a protein solution that was more than 95% homogeneous (Figure 2.10, a). It was interesting to note that one of the wells, which was adjusted to pH 4, gave rise to a mixture of pink and yellow crystals (Figure 2.10, b). It was always interesting to note that any precipitates that formed in wells at a pH of less than 4 were a red colour whilst precipitates that formed at pH values higher than 4 turned a deep yellow colour. This observation will be dealt with in more detail in chapter 3.

These diamond-shaped crystals grew over a period of several months and reached a size of about 0.5mm in length. They were, however, wafer-thin and were in the form of platelets. Although these crystals were placed in the path of an X-ray beam the lack of real thickness to the crystals gave rise to only a very faint diffraction picture that was not possible to interpret.

In another prepararion, using deaminase that contained a 20% contaminant, rectangular crystals grew very quickly over a period of about a week (Figure 2.11). The same type of crystals also grew from totally homogeneous protein but took several months to reach the equivalent size. These crystals grew to over 0.5mm in length and more importantly were at least 0.25mm thick. However, when these crystals were viewed under a polarised light source it could be observed that these crystals were made up of

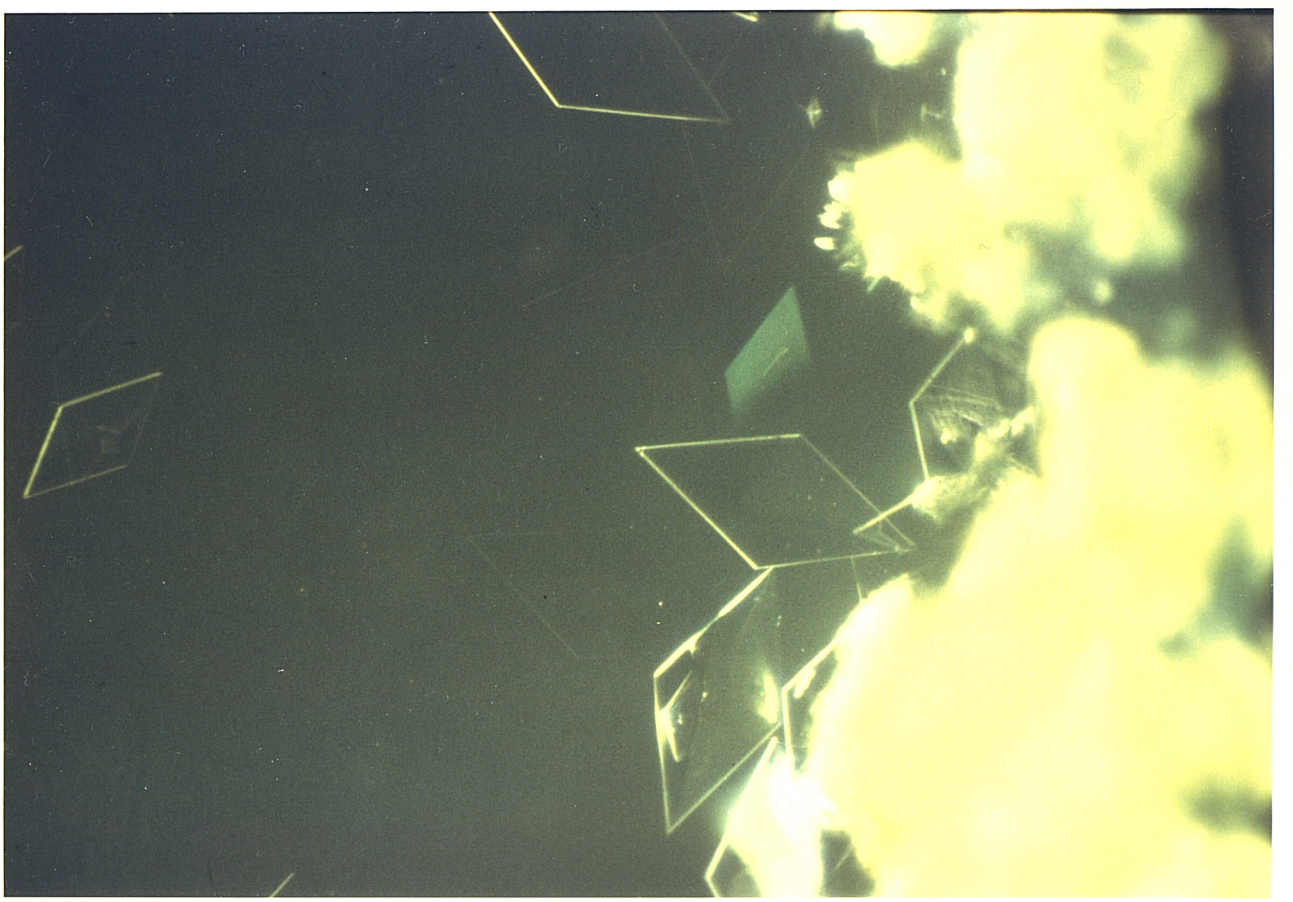
Figure 2.10

Crystals of E.coli porphobilinogen deaminase. The crystals were grown at:-

a) pH 4.8

b) pH 4.5

a



b

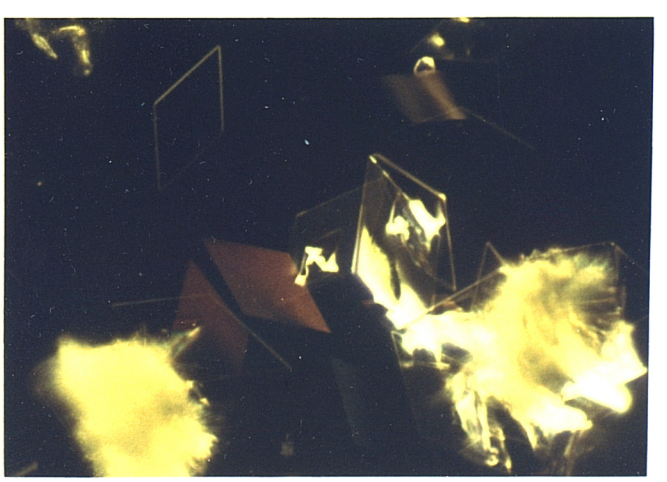


Figure 2.11

Crystals of E.coli porphobilinogen deaminase grown from a different purification batch of enzyme to those in figure 2.10.

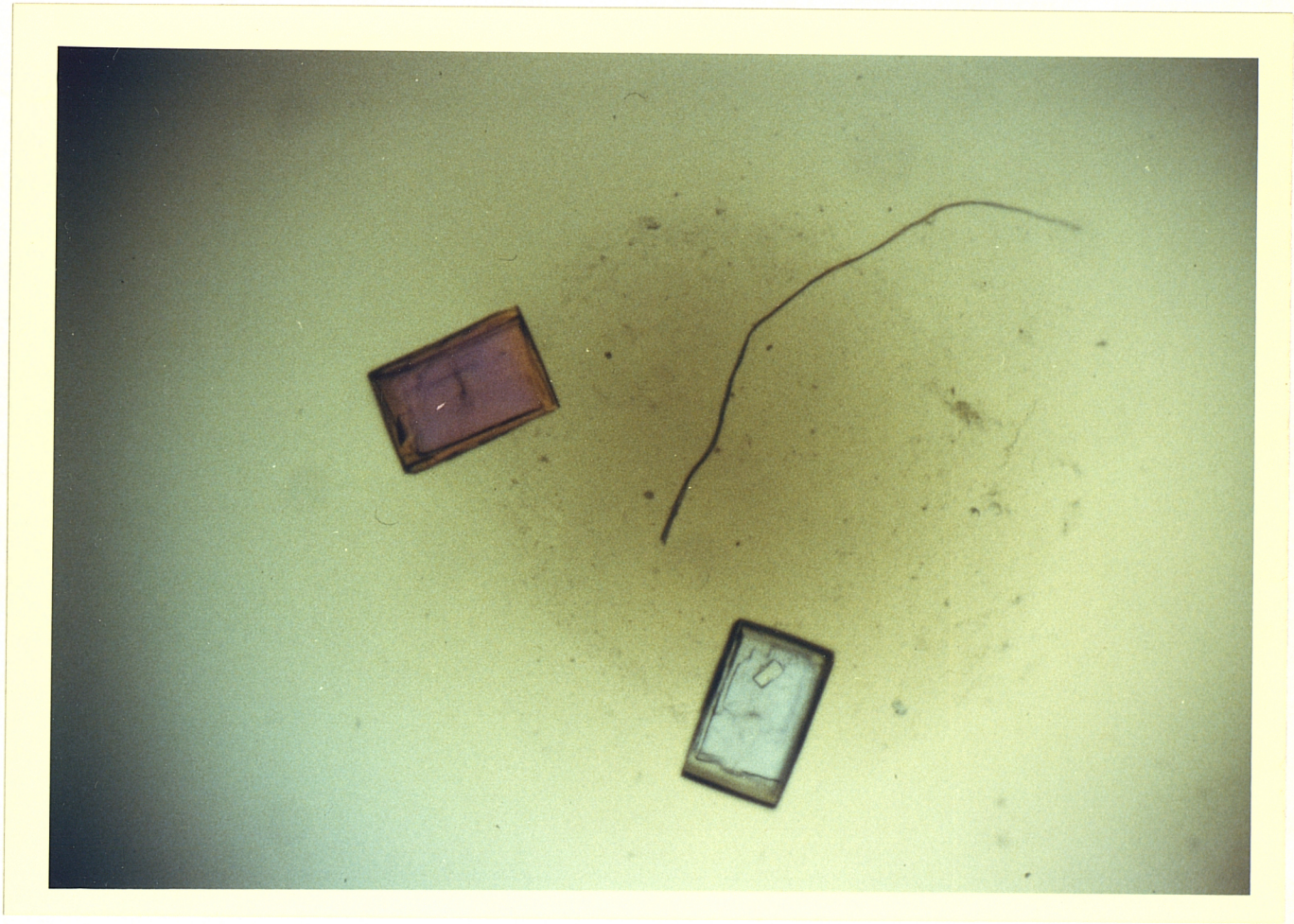
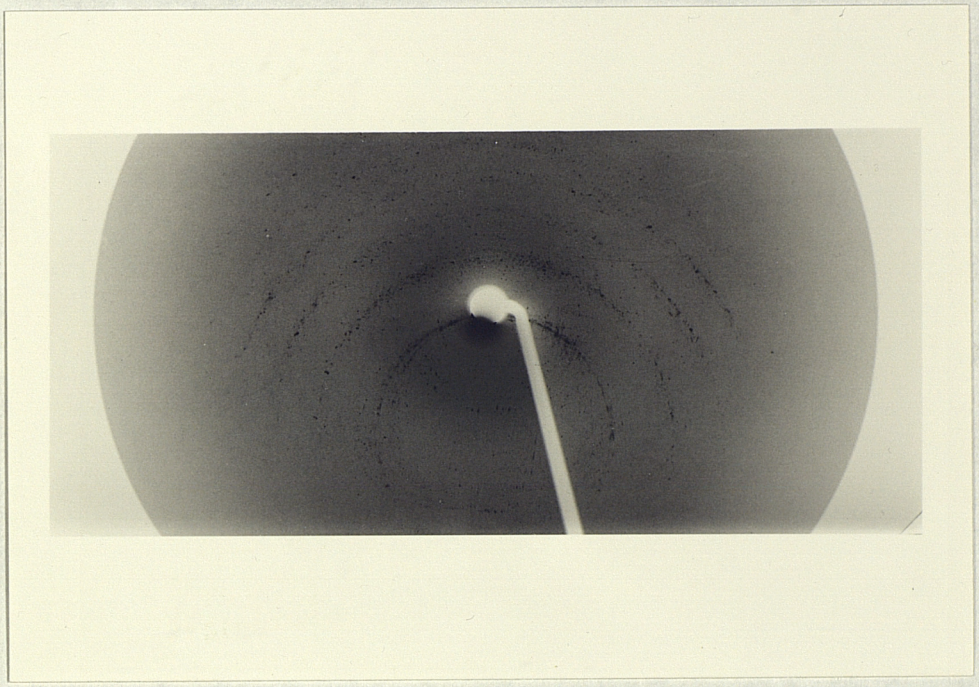
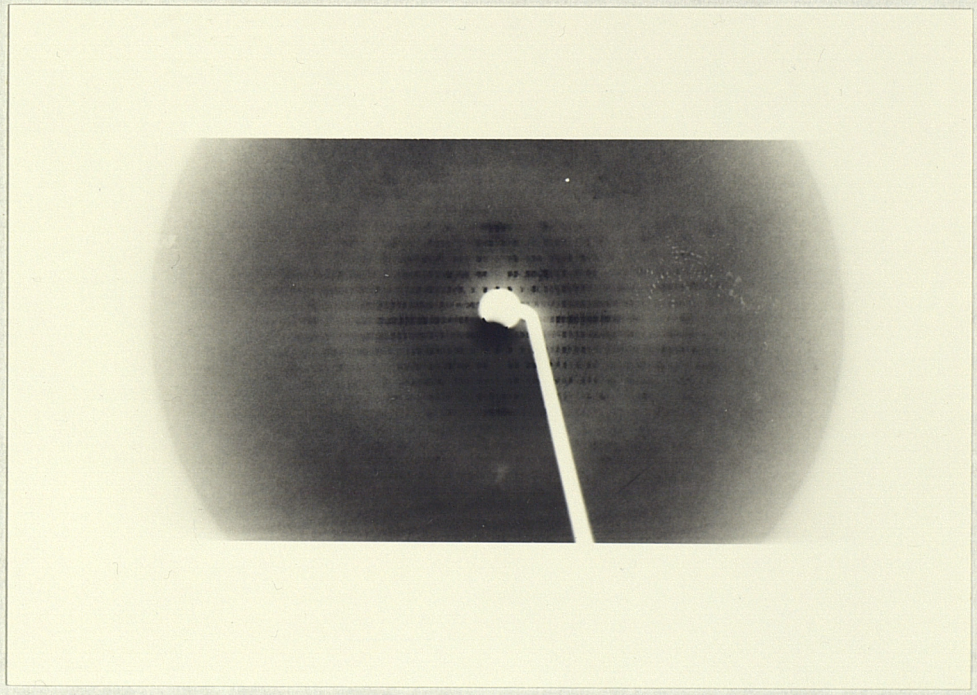


Figure 2.12

X-Ray diffraction pictures of the crystals shown in figure 2.11. The pictures, although showing a centre of symmetry, do not contain single diffraction spots suggesting that the crystals are made up of layers of slightly mis-aligned smaller crystals





layers of crystals in a similar manner to a stack of cards. The X-ray analysis of these crystals confirmed this observation with the diffraction pattern showing multiple aggregates of diffraction spots and several axis of symmetry (Figure 2.12). Again, due to the complex nature of the diffraction pattern, no information regarding the packing arrangement of the protein molecules in the crystal cell could be obtained from this analysis.

Although the deaminase has now crystallized for the first reported time (Jordan et al, 1988 b), to attain any information from X-ray analysis better formed crystals of the enzyme will have to be made. There are many reasons why the crystals did not form properly, possibly due to the microheterogeneity of the protein observed on non-denaturing polyacrylamide electrophoresis gels or possibly due to the high protein concentration of the solution from which the crystals grew, but the fact that crystals grew to a size that was easily manageable is encouraging. It seems quite likely that by manipulation of the conditions stated that it should be possible to grow single crystals especially as it was observed that the rectangular crystals grew from a well formed central core and that the disruptive stacking of the crystal started only after this core grew to a certain size.

Chapter 3.

The identification of a dipyrromethane cofactor at the catalytic site of porphobilinogen deaminase.

3.1 Introduction.

One of the most interesting features of porphobilinogen deaminases is their remarkable ability to form the stable enzyme-intermediate complexes which represent the individual steps of the polymerisation reaction leading to the formation of the tetrapyrrole (Anderson and Desnick, 1980; Jordan and Berry, 1981). The amazing stability of these complexes, which allows them to be isolated, must reflect the chemical linkage between the first pyrrole unit and the enzyme. The form of this linkage has been shown to be of a covalent nature since it was demonstrated that treatment of the enzyme intermediate complex with SDS did not release the bound pyrrole after subsequent gel filtration chromatography (Jordan and Berry, 1981).

The quest to identify the amino acid responsible for the covalent attachment of the first pyrrole ring to the enzyme has been intense over the last decade, although the conclusions from various workers were far from clear and in many cases were contradictory. Lysine had emerged as the most likely candidate on the bases of [^{13}C] n.m.r. studies (Battersby et al, 1983) and pyridoxal phosphate inhibition experiments on the Euglena enzyme (Hart et al, 1984). On the other hand, using a similar n.m.r. strategy as Battersby but with the purified R. sphaeroides enzyme, Evans and co-workers (1986) found no resonances which could be attributed to a pyrrole-lysine linkage but from [^{3}H] n.m.r. studies they did suggest that a cysteine residue may be involved. Using amino acid modifying reagents Russell and co-workers (1984) were able to speculate about the active site amino acids. Although pyridoxal phosphate inhibited the enzyme, these workers found stronger inhibition from arginine-specific reagents with the deaminase isolated from wheat germ and postulated that arginine was responsible for the attachment of the substrate to the enzyme. Evidence for the involvement of a cysteine group had also earlier been presented by these workers on the basis of the enzyme sensitivity towards thiol reagents (Russell and Rockwell, 1980). From electrophorectic data, Berry and Jordan (1981) suggested that the group responsible for the pyrrole attachment would have to be negatively charged rather than positive to explain the difference in mobility of the various ES complexes of the R. sphaeroides enzyme.

From all the observations made in chapter 2 of this thesis it had become clear that the E. coli enzyme had a bound chromophore present, since crystals of the enzyme appeared yellow under neutral conditions yet appeared pink under mildly acidic conditions (Section 2.2.9). Under very strong acid conditions this chromophore gave rise

to porphyrin formation suggesting that it was made up of a pyrrole moiety. The presence of such a chromophore was not unique to the E. coli enzyme as several years earlier Berry (1983) had also noticed a pink coloured compound associated with peptides of the R. sphaeroides enzyme. The pink chromophore suggested the presence of a protonated oxidized dipyrrole but it was not possible during this work to determine whether this was due to an enzyme-bound intermediate. Evans et al (1986) also noticed that the R. sphaeroides enzyme inexplicably yielded uroporphyrin under some circumstances. Our studies with the E. coli deaminase centered on investigating the nature of these observations and was made possible by the availability of milligram quantities of the pure enzyme (Chapter 2). The nature of the chromophore was subsequently identified as a dipyrromethane molecule and at the same time it was demonstrated that this dipyrromethane molecule acted as a cofactor for the enzyme and was responsible for the attachment of the first pyrrole ring, ring a of the final product, to the enzyme (Jordan and Warren, 1987; Warren and Jordan, 1988 c). The discovery, characterisation and mechanism of this novel and unsuspected cofactor is described in this chapter.

3.2 Results and discusson.

3.2.1 Spectral properties of the enzyme.

The purified E. coli porphobilinogen deaminase at high concentrations (10mgs/ml) appears a faint light orange yellow colour and has weak absorption bands at 410nm and 500nm at pH 8 (Figure 3.1, a). It is interesting to note that both crystals of the enzyme and the mother liquor from which the crystals are grown darken to a yellow brown colour over a period of months.

Acidification of the holoenzyme to below a pH value of 4 leads to a dramatic colour change, the solution turning to an intense pink-red colour, which is clearly visible to the naked eye at protein concentrations as low as 0.5mgs/ml. The lowering of the pH to below 4 irreversibly inactivates the enzyme. The absorption spectrum of the deaminase at pH 3.5 is shown in Figure 3.1 (b) and is very different with a large absorption maximum at 490nm.

These spectral recordings are in agreement with the observations made in chapter 2 on the crystals of the enzyme: crystals grown in acid buffer appeared pink in colour, whereas those grown at higher pH were a pale yellow colour. The pink colour is very similar to that given by oxidized protonated dipyrromethanes. These cumulative findings suggested the presence of an enzyme-bound group which when oxidized is protonated to give a pink chromophore under acid conditions but which at neutral pH is almost colourless.

When the holoenzyme was subjected to treatment with acid (50% HCOOH, v/v), over a period of an hour, a strong absorption maximum developed at 405nm characteristic of a porphyrin and the peak at 490nm declined (Figure 3.1, c). Under long wavelength ultraviolet light, this solution exhibited a strong pink fluorescence also indicative of the presence of a porphyrin. The nature of the porphyrin was determined by h.p.l.c. using a reverse phase C18 column as described in the methods section. Comparison with authentic standards established that the porphyrin formed was a mixture of the uroporphyrin isomers I and III in the ratio 80 : 20 (Figure 3.2). The porphyrins formed were not associated with the enzyme protein since after precipitation with trichloroacetic acid (10%, w/v) followed by centrifugation they remained in the supernatant.

It is well established that, under acid conditions, porphyrins are formed from pyrromethanes, first by their polymerization to porphyrinogens which are then rapidly oxidized to their respective porphyrins. These observations are thus consistent with the presence of a dipyrromethane bound to the porphobilinogen deaminase, since liberation of mono-pyrroles from this species would result in the polymerisation to the tetrapyrrole.

Figure 3.1

Spectra of E. coli porphobilinogen deaminase holoenzyme:-

a) at pH 8.0 in Tris/HCl buffer

b) at pH 3.5 in acetate buffer

c) after treatment with formic acid (50%, v/v)

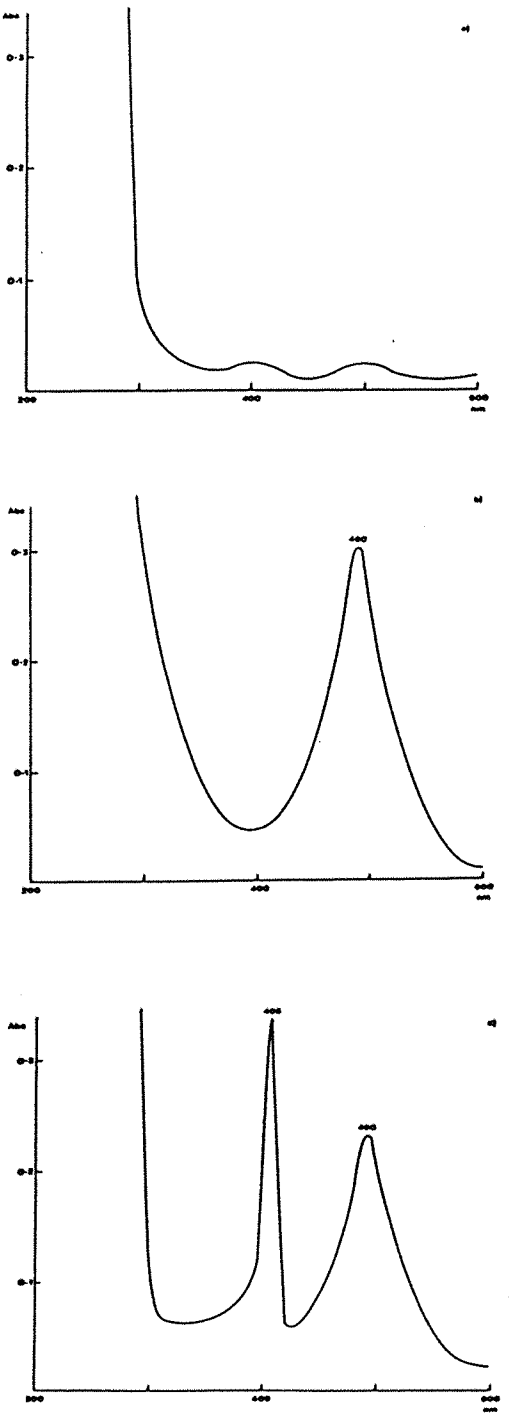
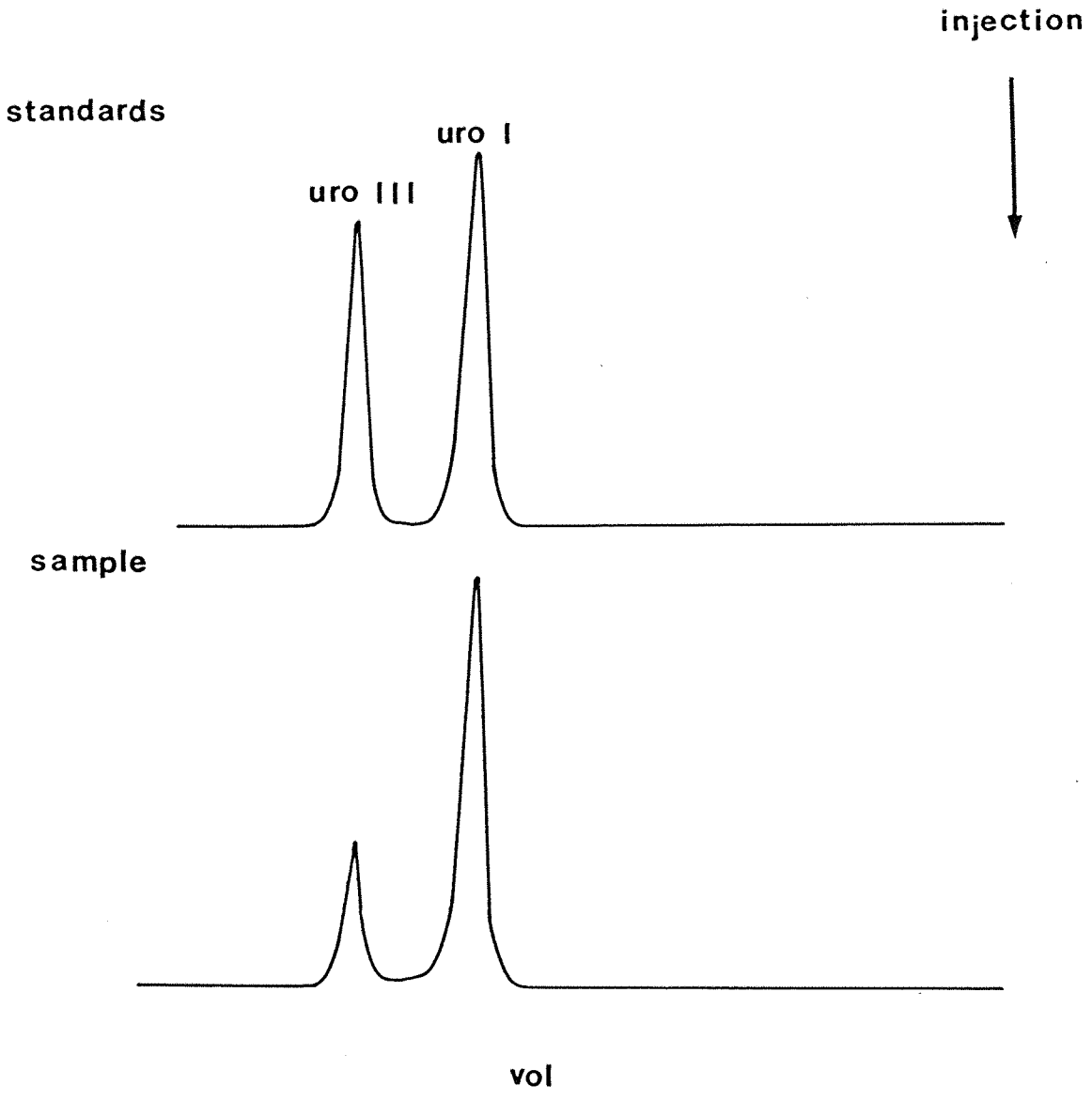


Figure 3.2

Uroporphyrin h.p.l.c. profiles of uroporphyrin I and III standards together with the uroporphyrin sample obtained from formic acid treated porphobilinogen deaminase.



Furthermore, the ability to form uroporphyrin establishes that the dipyrromethane is made up of pyrrole units similar in structure to porphobilinogen.

3.2.3 Stoichiometry of the prosthetic group.

If the enzyme-bound prosthetic group was indeed made up from pyrromethane molecules, then it should be theoretically possible to determine the number of pyrrole rings attached to each protein molecule from the amount of porphyrin formed from a known amount of protein which had been treated with formic acid. However, since the amount of porphyrin formed is dependant upon many unknown factors, treatment of the protein with formic acid does not give an accurate reflection of the stoichiometry. A different strategy was therefore employed where the amount of porphyrin formed from 1mg of holoenzyme was compared to the amount of porphyrin formed from 1mg of either the ES_2 and the ES_3 complex forms of the enzyme. An ES_2 complex form of the enzyme would be expected to give twice the amount of porphyrin compared to the holoenzyme and the ES_3 complex form to give two and a half times the amount. The rationale behind these figures is that if the holoenzyme does contain a dipyrrole moiety, then upon strong acid treatment it should release two pyrrole equivalents which would be able to react together to form porphyrin. On the other hand, the ES_2 form of the enzyme will contain not only the two pyrrole rings of the chromophore but also contain two pyrrole rings derived from the substrate and thus should be able to release four pyrrole equivalents thereby forming twice the amount of porphyrin compared to the holoenzyme. In a similar fashion the ES_3 form of the enzyme will contain three bound substrate derived pyrrole units together with the two pyrrole units of the chromophore giving a total of five units compared to the two units of the holoenzyme and the four units of the ES_2 complex. The actual result is shown in figure 3.3 and shows that the ES_2 complex form gives about twice the amount of porphyrin compared to the holoenzyme and the ES_3 form gives about two and a half times the amount of porphyrin. This result, although not totally conclusive, certainly indicates that there are probably two pyrrole rings bound to each enzyme molecule.

3.2.4 Reaction of the holoenzyme with Ehrlich's reagent.

The likelihood that the enzyme did in fact contain a dipyrromethane prosthetic group suggested that the enzyme should react with Ehrlich's reagent, which is known to give a characteristic colour reaction with such compounds (Pluscec and Bogorad, 1970). The initial reaction of the enzyme with Ehrlich's reagent yielded an intense purple colour over a period of 30 seconds, with an absorption maximum at 566nm. This colour quickly faded over a period of 15 minutes to an orange-yellow colour with an absorption maximum at 495nm (Figure 3.4) in precisely the same way as that given by

Figure 3.3

Comparison of the amount of porphyrin formed by the formic acid treatment of holo-porphobilinogen deaminase to the amount of porphyrin formed by the same treatment of the enzyme-intermediate species with two bound substrate equivalents (ES_2) and the enzyme-intermediate species with three bound equivalents (ES_3).

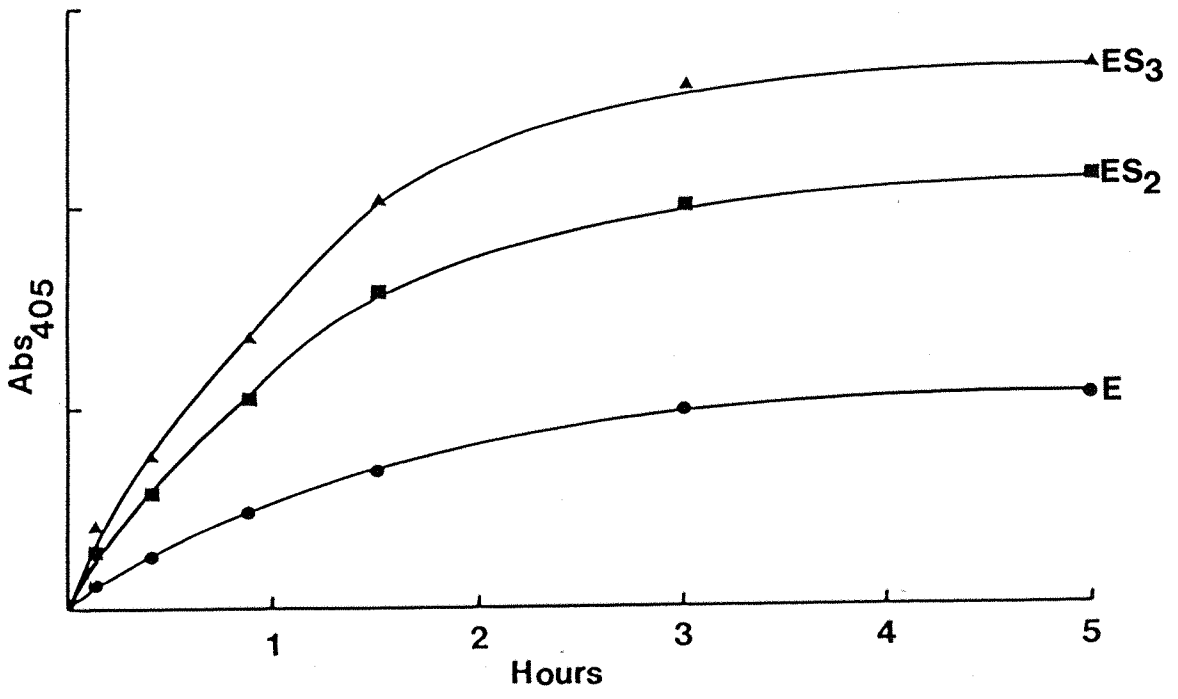
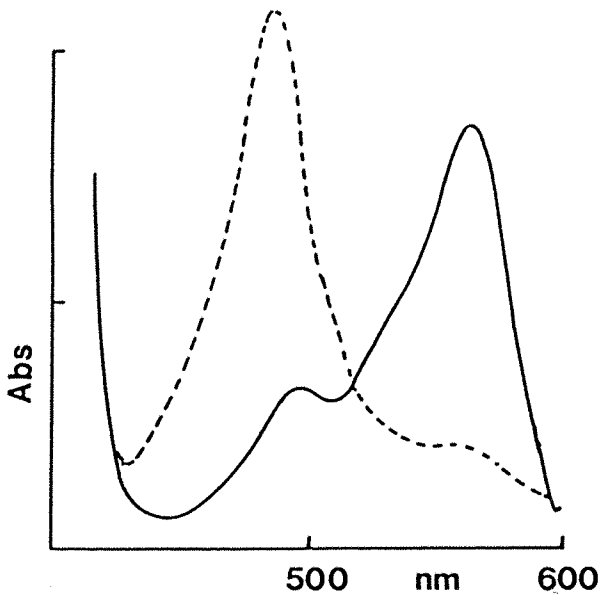


Figure 3.4

Spectra of E. coli porphobilinogen deaminase holoenzyme after reaction with Ehrlich's reagent. Spectra observed after 1 min (———) and 15 min (- - -).



dipyrromethanes. Such spectroscopic behaviour is very strong evidence for the presence of a dipyrromethane (Pluscec and Bogorad, 1970). The reaction with Ehrlich's reagent also demonstrates that the dipyrrole has a free α-position, the significance of which will be discussed below. From the foregoing information it is clear that the dipyrromethane is tightly bound to the enzyme under both weakly basic and weakly acidic conditions. Stronger acid treatment leads to the liberation of a pyrromethane species and its subsequent rapid polymerisation.

The oxidation state of the prosthetic group is likely to be that of a dipyrromethane rather than a dipyrromethene since prior reduction of the holoenzyme with iodine not only inhibited the Ehrlich's reaction but also diminished the yield of porphyrin on treatment with formic acid.

In an attempt to isolate the dipyrromethane, the porphobilinogen deaminase was exhaustively digested with pronase. The digest was then separated by TLC under acidic conditions (butanol/acetic acid/water, 4:1:1, v/v). The oxidised compound migrated as a faint pink band (R_f 0.70) and the remaining reduced species rapidly reacted with Ehrlich's reagent to give an immediate purple colour changing to yellow-orange after 5 minutes. The behaviour of this compound was therefore similar to that of the enzyme bound species.

3.2.5 Mechanistic significance of the dipyrromethane.

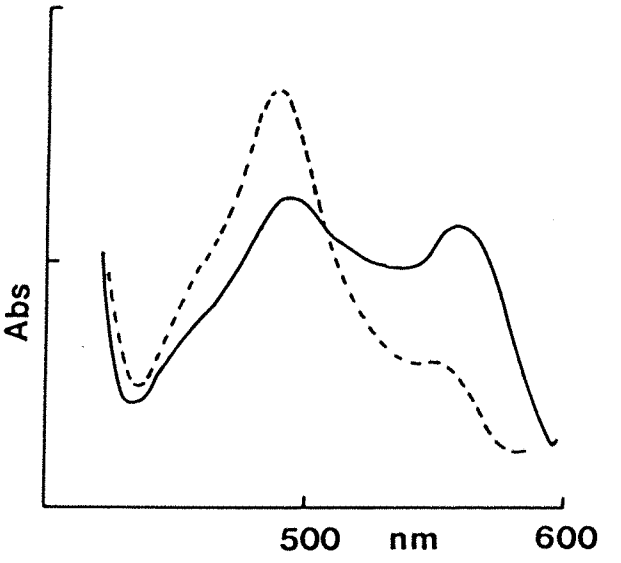
The evidence described above points to the structure of the prosthetic group as being a tightly bound dipyrromethane formed from two molecules of the substrate. In the presence of acid, the enzyme bound dipyrrole is cleaved and the liberated pyrroles are rapidly converted into porphyrin. It could easily be envisaged that this dipyrrole would represent part of the catalytic site of the porphobilinogen deaminase and that the tetrapyrrole product is assembled whilst bound covalently to the free α-position of the bound dipyrromethane.

A crucial test for this hypothesis is that the ES_2 complex should be in the form of a tetrapyrrole structure composed of the dipyrromethane from the holoenzyme linked to the dipyrromethane generated from the binding of two substrate molecules. Accordingly the ES_2 complex was generated and treated with Ehrlich's reagent. As predicted a very rapid colour reaction occurred characteristic of a tetrapyrromethane (Figure 3.5). This is similar to the profile shown for the dipyrromethane except that the absorption transition from 566nm to 495nm occurs about ten times faster (Radmer and Bogorad, 1972) and is powerful evidence for a tetrapyrromethane.

These observations provide further evidence that the native enzyme contains a reactive dipyrromethane cofactor, and more importantly, that this cofactor is involved directly in the sequential binding of the substrate molecules during the catalytic reaction.

Figure 3.5

Spectra of E. coli porphobilinogen deaminase intermediate complex with two bound substrate equivalents (ES_2) after reaction with modified Ehrlich's reagent. Spectra observed after 1 min (———) and 15 min (- - -).



Thus it would appear that the cofactor functions as an anchor for the growing pyrrole chain with the incoming substrate pyrrole units reacting with the free α position of the dipyrromethane molecule. This is shown diagramatically in scheme 3.1.

3.2.6 The synthesis of the dipyrromethane cofactor.

Since the cofactor appears to be made of a strucure of two molecules of its substrate, porphobilinogen, then it was important ot establish whether the cofactor was assembled along the same biosynthetic route as porphobilinogen. This was accomplished by monitoring the specific incorporation of a radiolabelled precursor of porphobililinogen, 5-amino-[5-^{14}C]laevulinic acid, into the enzyme.

In an attempt to label specifically the dipyrromethane cofactor the bacterial culture was grown in the presence of exogenous 5-amino-[5-^{14}C]laevulinic acid. The medium was supplemented with aminolaevulinate because it was known that porphobilinogen was not taken up very well across the cell membranes. In order to ensure that the aminolaevulinic acid was incorporated solely into the deaminase and not into the tetrapyrrole nucleus of cytochromes or into the general amino acid pool, the aminolaevulinic acid was added to the bacterial cultures at a time of 16 hours after innoculation. This time was chosen because the bacteria were entering logarithmic phase and were about to start the maximum rate of production of the deaminase (Section 2.2.1).

By the use of this protocol, 5 litres of bacteria were grown in the presence of 50μCi of 5-amino-[5-^{14}C]laevulinic acid which represented a final concentration of 50μmolar in the medium. After the bacteria were harvested it was found that approximately 7μCi of the [^{14}C] label was taken up by the bacteria. Sonication of the bacterial cells followed by f.p.l.c. analysis of the centrifuged cell extract using a Mono Q column revealed two major radioactive peaks, one of which coincided with the porphobilinogen deaminase and the other which eluted with the porphyrins at the end of the gradient. No other proteins were significantly labelled (Figure 3.6, a). The porphobilinogen deaminase was further purified by the stages previously described and at each stage the radioactivity was found to be **exclusively** associated with the porphobilinogen deaminase enzyme (Figure 3.6, b-d). The resulting homogeneous enzyme (10 mgs) isolated by f.p.l.c. contained 1.5μCi representing an overall yield of 3% of the initial [^{14}C] label used. Based on the enzyme units originally in the extract and the total [^{14}C] label taken up by the bacteria, it was possible to calculate that some 40% of the [^{14}C] radioactivity taken up by the bacteria was associated with the porphobilinogen deaminase dipyrromethane cofactor.

In fact the earlier precautions taken to ensure that non-specific labelling of the deaminase occurred were not really necessary as the bacteria could in fact be incubated with as high a concentration of labelled aminolaeulinic as 0.1M from innoculation time

SCHEME 3.1

The mechanistic significance of the dipyrromethane cofactor in the biosynthesis of preureoporphyrinogen.

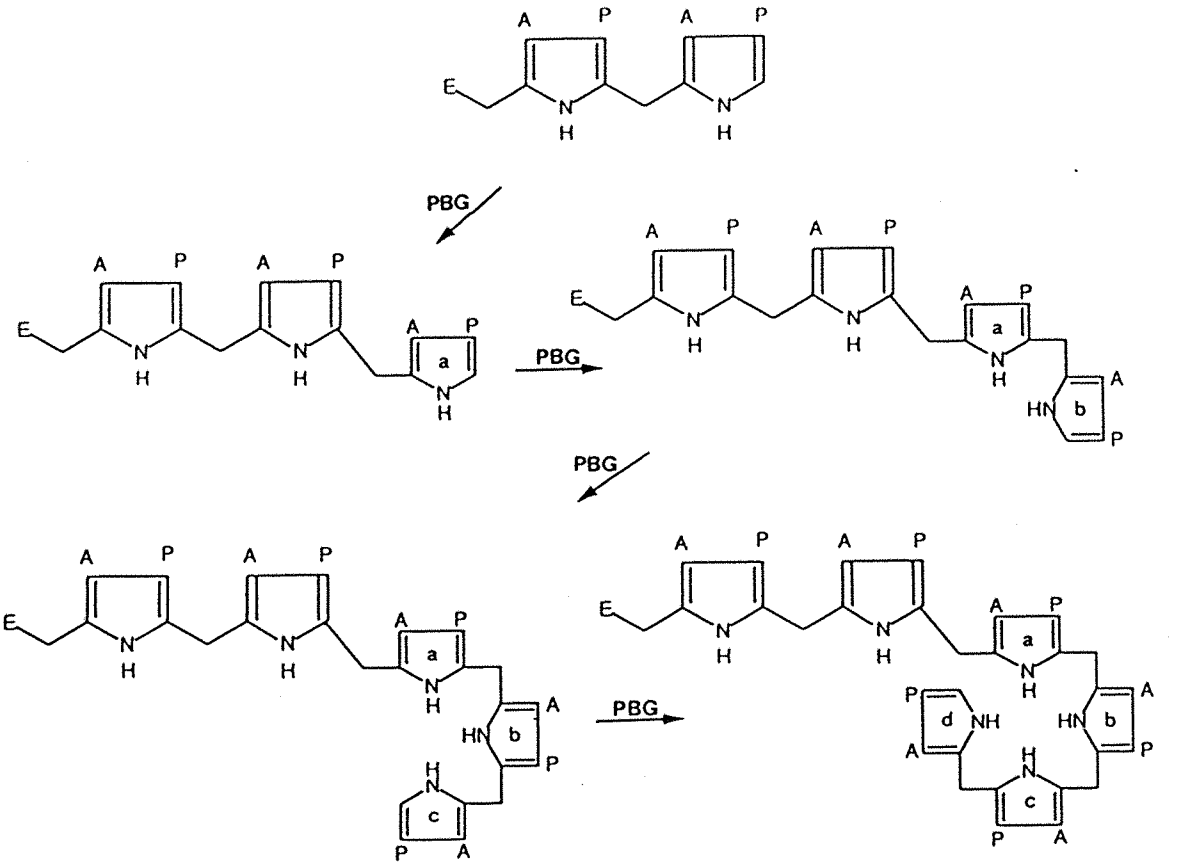


Figure 3.6

Biosynthesis of [^{14}C]porphobilinogen deaminase. E. coli strain ST1048 was grown on medium containing 5-amino[5-^{14}C]laevulinic acid. Cell free extracts were analysed for protein, enzyme activity and radioactivity.

a) F.p.l.c. profile of protein from a cell free extract of sonicated bacteria (DDDD); porphobilinogen deaminase activity (- - -); [^{14}C] radioactivity is shown in a bar chart form.

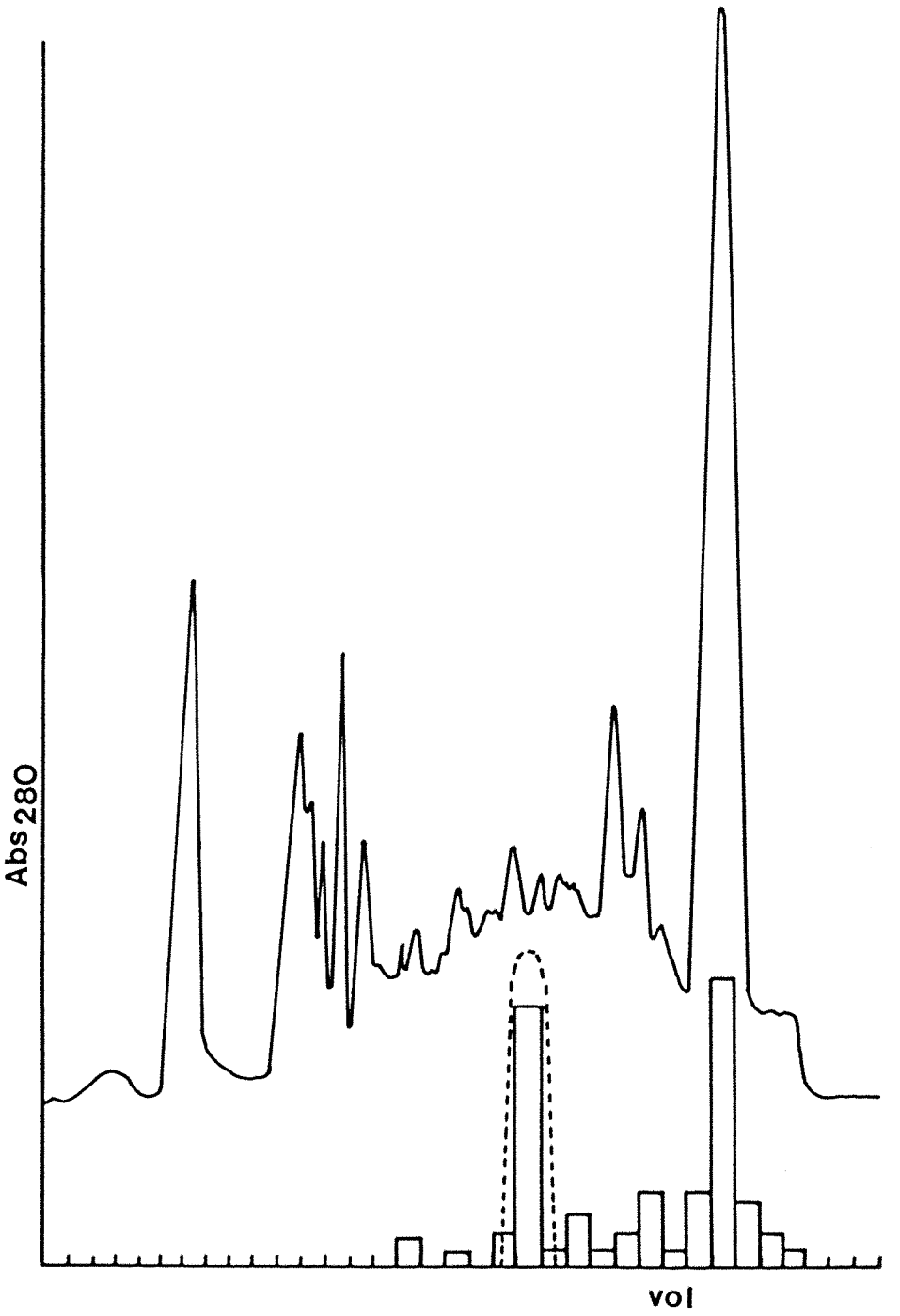


Figure 3.6

Biosynthesis of [^{14}C]porphobilinogen deaminase. E. coli strain ST1048 was grown on medium containing 5-amino[5-^{14}C]laevulinic acid. Cell free extracts were analysed for protein, enzyme activity and radioactivity.

b) F.p.l.c. profile of porphobilinogen deaminase extract after heat treatment and gel filtration (symbols as in a).

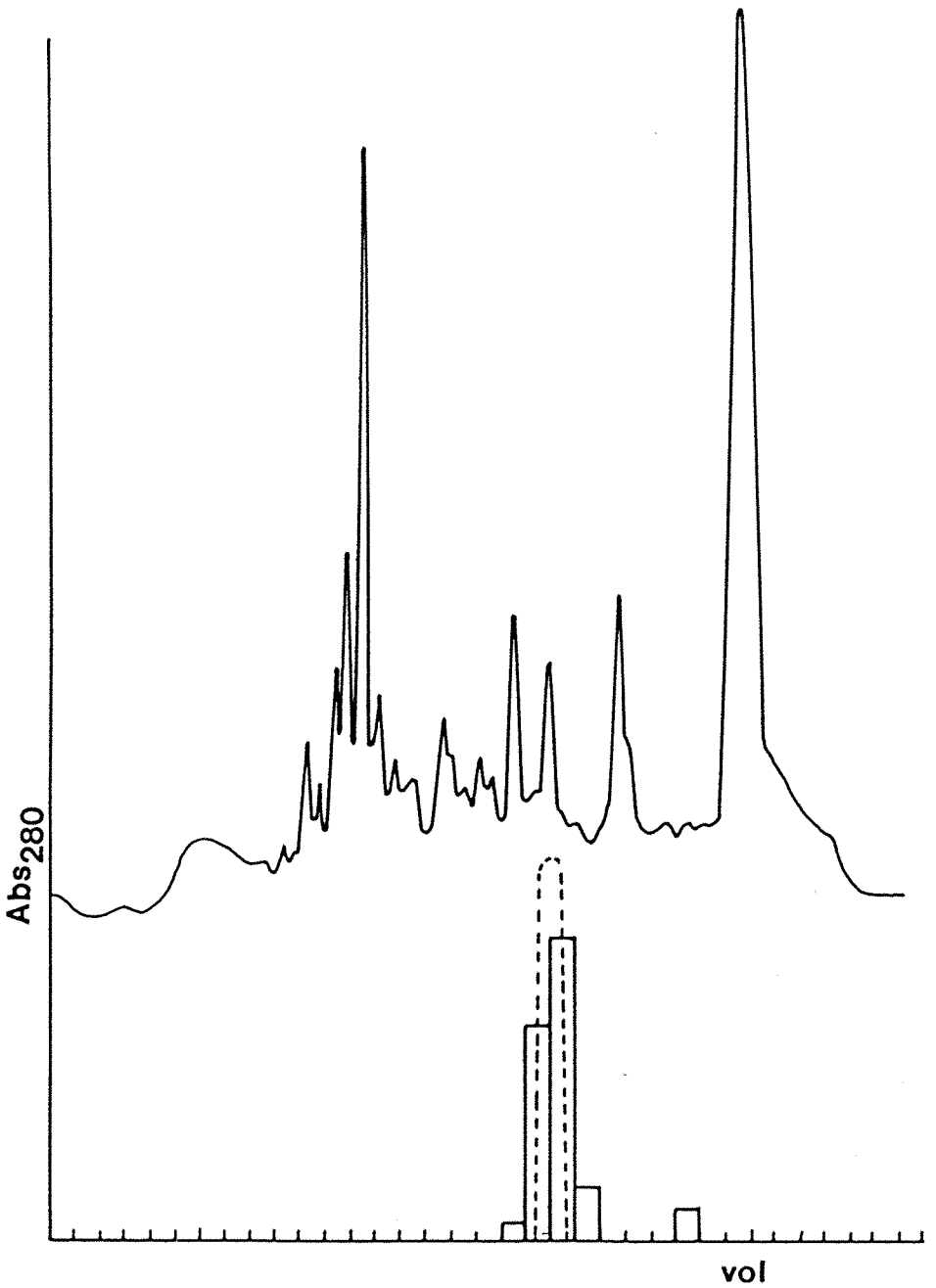


Figure 3.6

Biosynthesis of [^{14}C]porphobilinogen deaminase. E. coli strain ST1048 was grown on medium containing 5-amino[5-^{14}C]laevulinic acid. Cell free extracts were analysed for protein, enzyme activity and radioactivity.

c) F.p.l.c. profile of porphobilinogen deaminase extract after Phenyl Sepharose chromatography (symbols as in a).

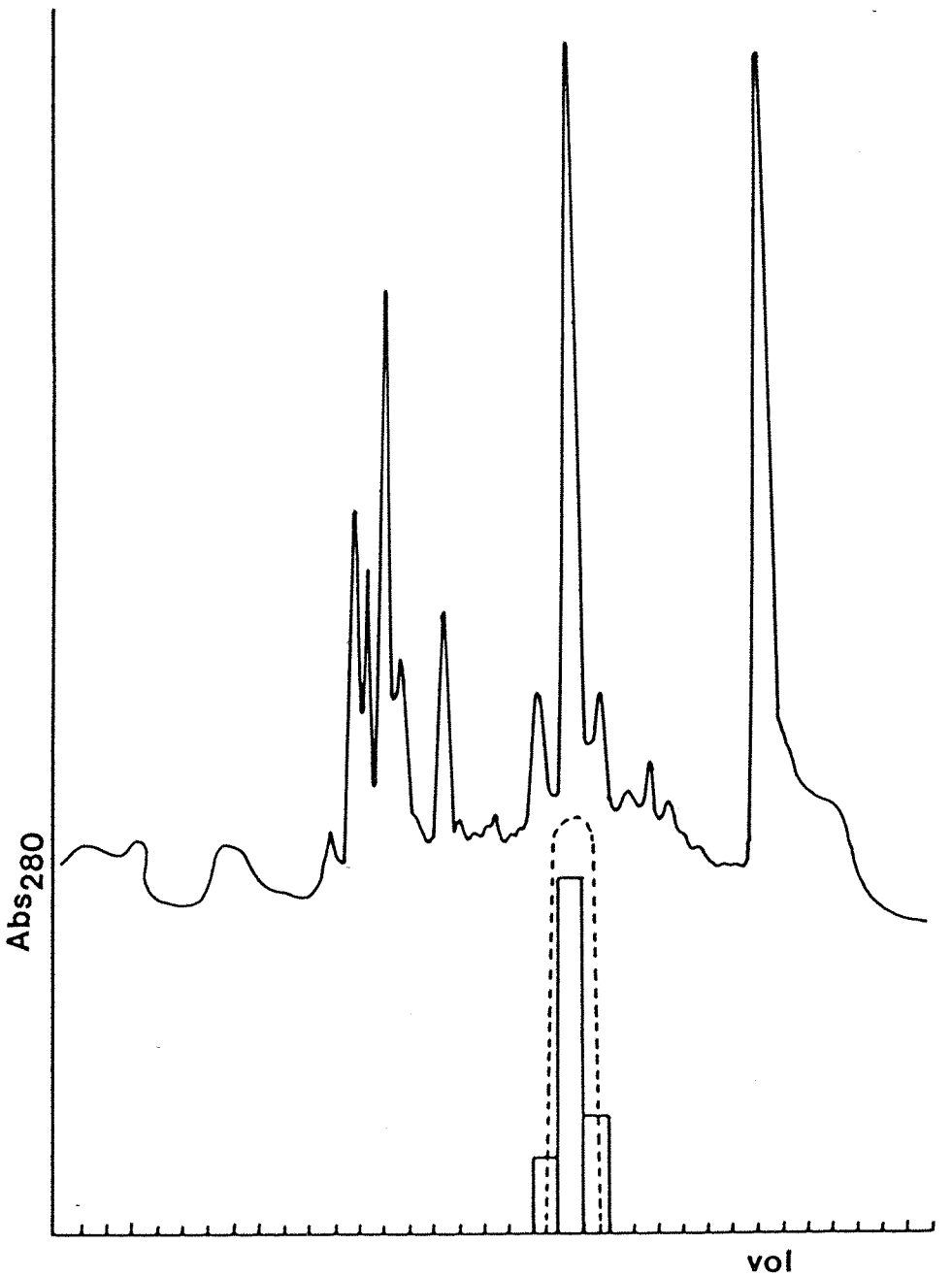
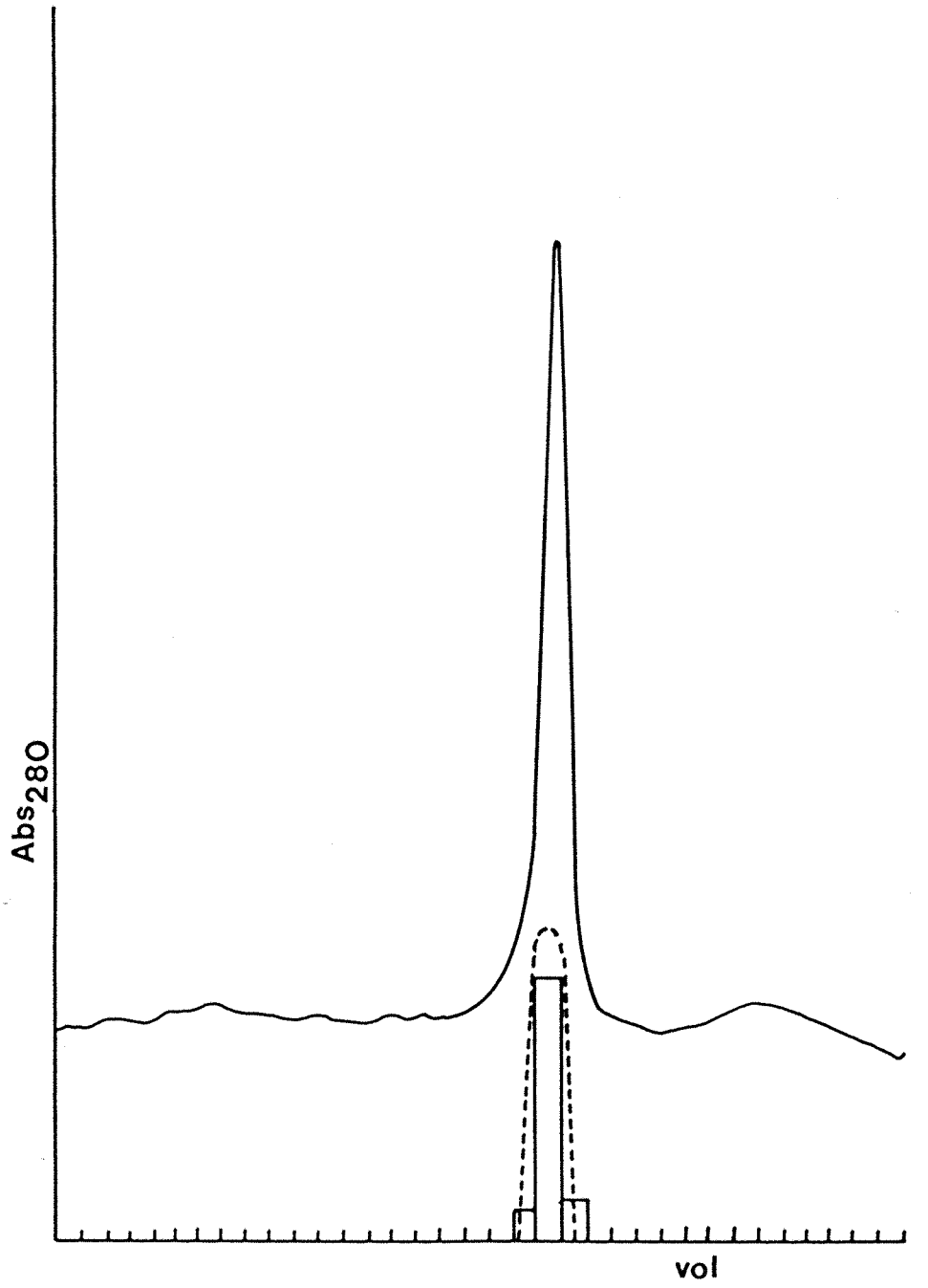


Figure 3.6

Biosynthesis of [^{14}C]porphobilinogen deaminase. E. coli strain ST1048 was grown on medium containing 5-amino[5-^{14}C]laevulinic acid. Cell free extracts were analysed for protein, enzyme activity and radioactivity.

d) F.p.l.c. profile of purified porphobilinogen deaminase (symbols as in a).



onwards and specific labelling of the deaminase still occurred. There was no apparent decomposition of the endogenous aminolaevulinic acid into the general carbon pool as there was no noticeable random labelling of proteins although there was an increase in the level of background radioactivity during the purification of the deaminase in this case which was attributed to the incorporation of labelled tetrapyrroles into hemoproteins.

3.2.7 Confirmation that the dipyrromethane cofactor acts as a true cofactor.

In order to distinguish between the pyrrole rings of the cofactor and those of the bound substrate, two types of separate but related experiments were carried out. First of all the porphobilinogen deaminase labelled in the dipyrromethane cofactor (E*), prepared as described above, was subject to treatment with non-labelled porphobilinogen and the radioactive E*S, E*S_2 and E*S_3 intermediate complexes were isolated by f.p.l.c. as previously described. The results table shows the extent of the radioactivity in each of the complexes. Exposure of any of [^{14}C]-enzyme-intermediate complexes to hydroxylamine, known to release bound substrates from the enzyme (Radmer & Bogorad, 1972; Davies & Neuberger, 1973), led to the regeneration of free active enzyme which, most importantly, contained all the original [^{14}C] radioactivity as judged by the dpm per enzyme unit (Table 3.1). Alternatively the complexes were incubated with 0.5mM porphobilinogen to allow catalytic turnover to take place. Consistent with the above findings, the uroporphyrin was completely unlabelled and the [^{14}C] radioactivity remained in the enzyme-bound cofactor (Table 3.1).

In the second approach, using the converse experiments, the unlabelled native holoenzyme was incubated with [^{14}C]-porphobilinogen (S*) to give the labelled enzyme intermediate complexes ES*, ES_2* and ES_3* which were isolated by f.p.l.c. as above. In this case, incubation of these complexes with hydroxylamine yielded radioactive polypyrroles and enzyme totally devoid of radioactivity (Table 3.1). Alternatively, incubation of these labelled enzyme-intermediate complexes with 0.5mM porphobilinogen liberated all the [^{14}C] radioactivity from the enzyme generating [^{14}C] uroporphyrin I and unlabelled enzyme (Table 3.1).

These experiments firmly establish that the pyrrole rings in the dipyrromethane cofactor and those contributing to the enzyme intermediate-complexes, although structurally related, behave **completely** differently. The dipyrromethane cofactor thus acts as a true cofactor rather than a substrate and is not subject to catalytic turnover. Release of the dipyrromethane cofactor from the [^{14}C] holoenzyme could only be achieved by prolonged treatment with formic acid. This yielded [^{14}C]-uroporphyrin I and III (80:20) and non-radioactive, inactive enzyme protein.

TABLE 3.1

Effect of hydroxylamine and catalytic turnover on [^{14}C]enzyme-intermediate complexes (E^*S) and enzyme-[^{14}C]intermediate complexes (ES^*).

ENZYME SPECIES	[^{14}C] radioactivity in protein after incubation		
	a) alone	b) with NH_2OH	c) with excess PBG
E^*S	3,204	3,174	3,197
ES^*	3,001	5	8

3.2.8 The ubiquitous nature of the dipyrromethane cofactor.

The finding that the E. coli porphobilinogen deaminase contained a dipyrromethane cofactor immediately prompted the question whether this cofactor was a universal active site requirement in all deaminases. To address this issue the deaminases from both a monocotyledon and a dicotyledon plant source were purified. In addition, the deaminase from a mammalian source was also purified so that, in essence, a representative enzyme from each of the major classes of living systems, bacterial, plant and animal, were to be investigated for the presence of the dipyrromethane cofactor. The sensitivity of the E. coli deaminase to Ehrlich's reagent meant that the as little as 30μg of protein could be detected spectroscopically. The deaminases from spinach, barley and human erythrocytes were isolated and purified by the procedures described in the methods section (Table 3.2, a-c). The two plant enzymes purified as single sharp peaks off the final f.p.l.c. Mono Q column procedure. The human enzyme purified as two single sharp peaks off the final f.p.l.c. stage. These two forms of the human enzyme were found to have the same molecular weight and were thought to constitute the holoenzyme and the enzyme with one substrate equivalent bound (ES). The existence of these two major forms of the human enzyme has been well documented in the past (Anderson & Desnick, 1980; Brown et al, 1985). The first of these two forms to elute from the Mono Q column was the enzyme species that was investigated for the presence of the dipyrromethane cofactor as this form of the enzyme was assumed to constitute the holoenzyme. The comparative ease by which these enzymes were purified is a reflection upon the high resolution obtained from the f.p.l.c. Mono Q column. The deaminase from barley chloroplasts was purified to over 75% homogeneity, the spinach enzyme to over 85% and the human enzyme to complete homogeneity (Figure 3.7). This thesis details the first reported purification of porphobilinogen deaminase from barley and also the most homogeneous purification of the spinach enzyme.

When treated with Ehrlich's reagent all the purified deaminases gave positive dipyrromethane colour reactions (Figure 3.8, a-d) establishing that the dipyrromethane cofactor is an essential component of deaminases from all major classes of living systems (Warren & Jordan, 1988 a). This important observation not only indicates the ubiquitous nature of the dipyrromethane cofactor, showing that the presence of the cofactor is not a unique feature of the E.coli enzyme, but also effectively disclaims all the evidence from the past which favoured a direct link between the substrate and the enzyme via an amino acid residue.

TABLE 3.2 (a)

Purification of porphobilinogen deaminase from spinach.

Purification stage	Units (μmoles/hr)	Specific activity (μmoles/hr/mg)
Crude homogenate	35	0.11
Ammonium sulphate precipitation	25	0.55
Heat treatment and centrifugation	23	5.00
Gel filtration Sephracryl S-200	14	11.55
F.p.l.c. Mono Q pH 7.5	8	60.00

TABLE 3.2 (b)

Purification of porphobilinogen deaminase from barley chloroplasts.

Purification stage	Units (μmoles/hr)	Specific activity (μmoles/hr/mg)
Crude chloroplast extract	15	0.50
Heat treatment and centrifugation	11	3.00
F.p.l.c. Mono Q pH 7.5	8	27.00
F.p.l.c. Mono Q pH 6.0	5	55.00

TABLE 3.2 (c)

The purification of porphobilinogen deaminase from human erythrocytes

Purification stage	Units (μmoles/hr)	Specific activity (μmoles/hr/mg)
Cell lysate	45	0.002
Batch ion-exchange DE-52	27	0.095
Gel filtration Sephracryl S-200	25	0.750
Heat treatment and centrifugation	20	1.500
Phenyl Sepharose chromatography	18	15.000
F.p.l.c. Mono Q pH 7.5	16	48.000
F.p.l.c. MonoQ pH 6.0	14	75.000

Figure 3.7

Sodium dodecyl sulphate polyacrylamide gel of porphobilinogen deaminase isolated from E. coli, spinach, barley and human erythrocytes. Lanes 1 and 6 contain molecular weight standards; lanes 2 to 5 contain 10μg of barley, spinach, human and E. coli porphobilinogen deaminase respectively.

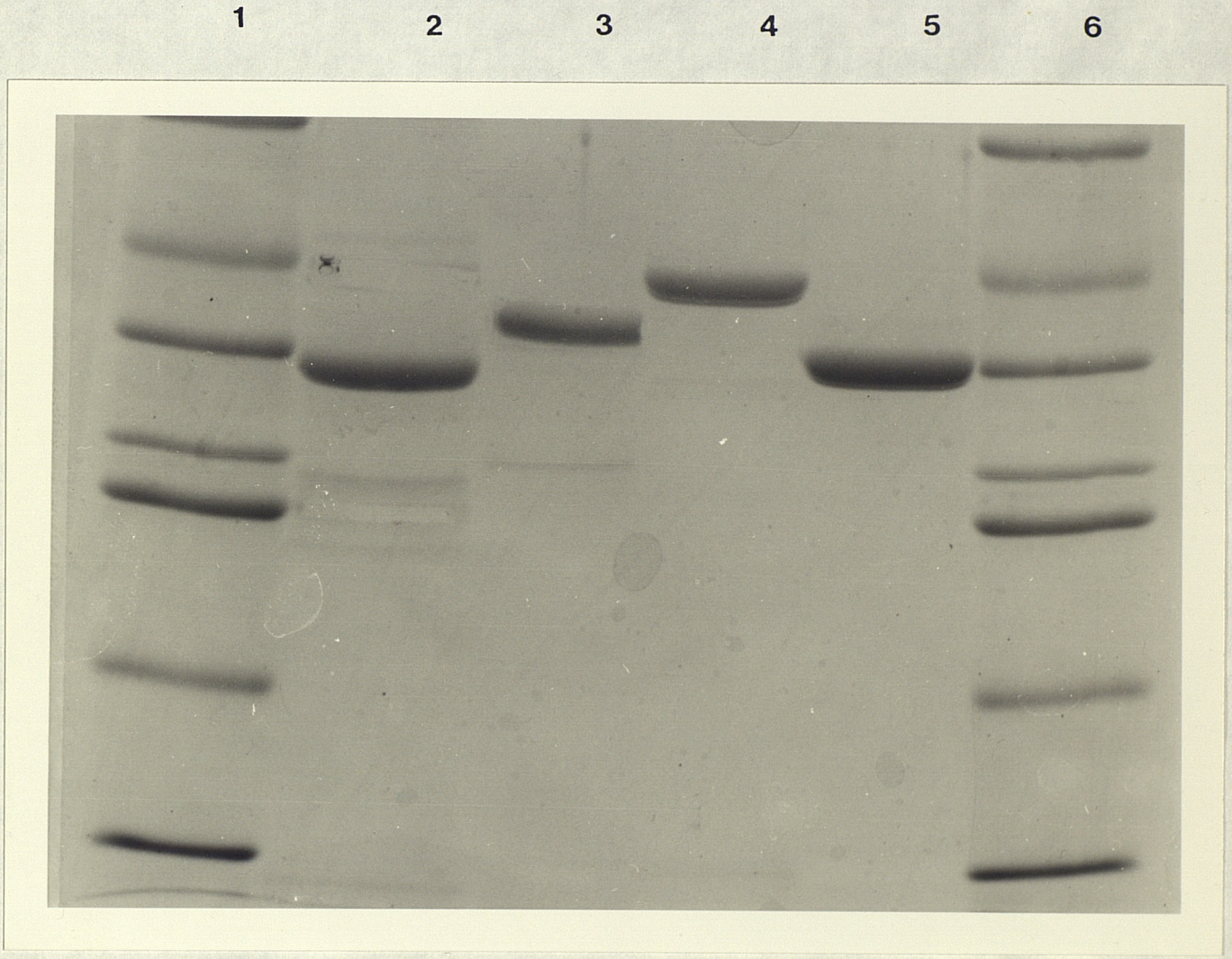


Figure 3.8

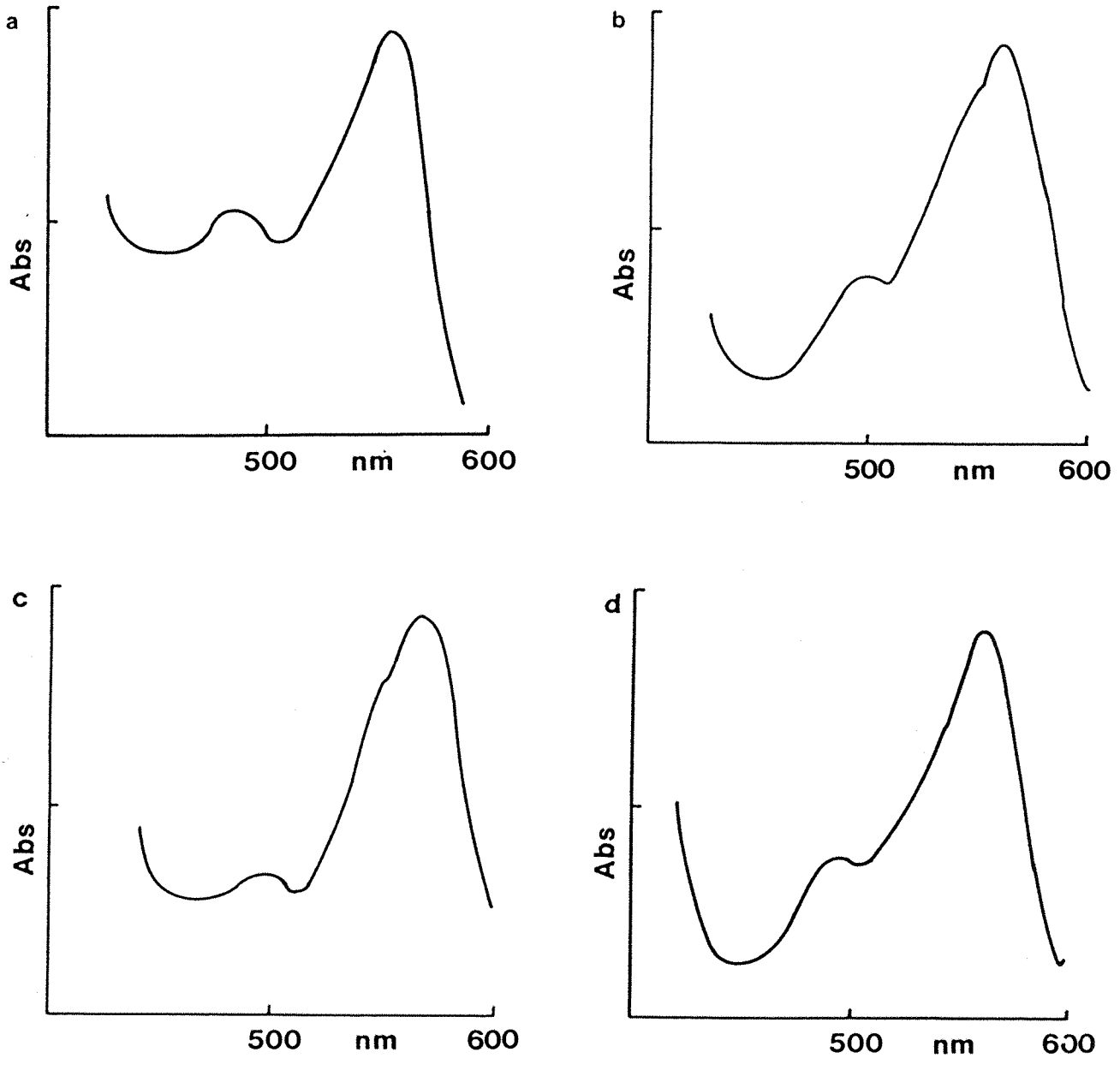
Reaction of Ehrlich's reagent with purified deaminases from different sources. Observed Ehrlich's reaction spectra after 1 min (DDD) with porphobilinogen deaminase isolated from:-

a) E. coli

b) spinach

c) barley

d) human erythrocytes



Chapter 4.

Investigations into the nature of the *E. coli* dipyrromethane cofactor and identification of the amino acid to which it is attached.

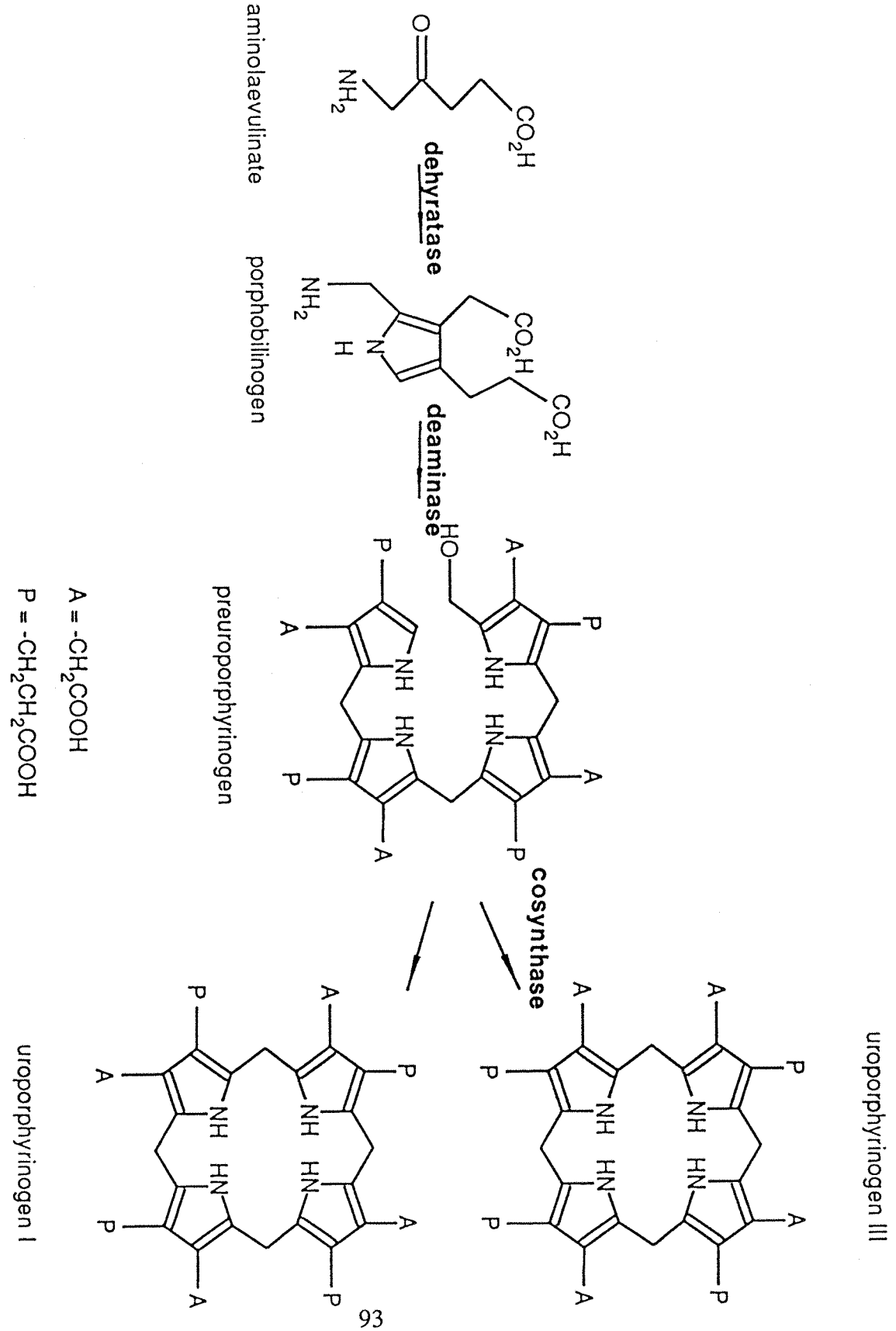
4.1 Introduction.

It is well established that the enzyme porphobilinogen deaminase catalyses the synthesis of the tetrapyrrole by a stepwise mechanism and enzyme-bound intermediate complexes with one, two, three and four pyrrole rings attached covalently to the enzyme active site have been isolated and characterised (Anderson and Desnick, 1980; Jordan and Berry, 1981). The nature of the enzymic group of the E. coli deaminase responsible for the covalent binding of the enzyme-bound intermediates has been shown to be a dipyrromethane molecule which is itself covalently attached to the enzyme (see chapter 3). In summary, this enzyme-bound cofactor reacts with Ehrlich's reagent to give a reaction characteristic of a dipyrromethane (Jordan and Warren, 1987). On binding two molecules of the substrate, the enzyme exhibits an Ehrlich's reaction typical of a tetrapyrromethane, establishing that the cofactor interacts covalently with the substrate (Section 3.2.1). Growth of this E. coli strain in the presence of 5-amino[5-^{14}C]laevulinic acid, the direct precursor for porphobilinogen (Scheme 4.1), has permitted the specific labelling of the cofactor with [^{14}C] radioactivity (Jordan and Warren, 1987; Warren and Jordan, 1988 a and b). Although made up from two porphobilinogen derived units the cofactor, unlike the substrate, is **not** subject to catalytic turnover since the [^{14}C] label remains firmly attached to the deaminase after the enzyme has carried out catalytic turnover (Section 3.2.7).

These results, and others from Chapter 3, provided the experimental evidence to show that the active site of all porphobilinogen deaminases contain the same novel dipyrromethane cofactor, made up by two porphobilinogen molecules linked together (Warren and Jordan, 1988 a). The evidence presented also suggested that the cofactor acts as the anchor point for the substrate molecules during the catalytic construction of the tetrapyrrole. Although this discovery explains the nature of the enzymic group responsible for the attachment of the first pyrrole ring to the enzyme and has eliminated all the previous ideas of various amino acids being responsible for this linkage, it only serves to pose more interesting questions relating to the biosynthesis, insertion and attachment of the cofactor to the enzyme.

SCHEME 4.1

The pathway to uroporphrinogen III from 5-aminolaevulinate.



The work described in this chapter is aimed at trying to answer some of these questions. It provides more information on the biosynthesis of the cofactor (Warren and Jordan, 1988 b and c) and also describes how, with the use of [^{13}C] n.m.r. techniques, the structure of the cofactor has been confirmed. Furthermore the work described establishes that the cofactor is attached to the enzyme through a sulphydryl group of cysteine-242 as has previously been reported (Jordan et al, 1988 c).

4.2 Results and discussion.

4.2.1 Dependance on 5-aminolaevulinic acid for porphobilinogen deaminase activity in a *hem*A⁻ mutant of *E. coli*.

Previous studies using 5-amino[5-^{14}C]levulinic acid, the precursor for porphobilinogen, have shown that this pyrrole is not only the substrate for the enzyme reaction catalysed by porphobilinogen deaminase and is thus the main building block for all tetrapyrroles the but is also the precursor of the dipyrromethane cofactor of the enzyme (Jordan and Warren, 1987; Warren and Jordan, 1988 b and c). In an attempt to investigate this dual role of porphobilinogen further, the effect of a mutation in the hemA gene on the formation of catalytically active porphobilinogen deaminase and the levels of haem were studied in the presence and absence of 5-aminolaevulinic acid. The hemA⁻ mutant will only grow fermentatively under strictly anaerobic conditions since there is little requirement for haem under these conditions. The results in table 4.1 (and illustrated graphically in Figure 4.1) indicate that the level of porphobilinogen deaminase is reduced dramatically and is practically zero in extracts from the hemA⁻ mutant grown in the absence of 5-aminolaevulinic acid. However, when this organism was grown anaerobically, but in the presence of 5-aminolaevulinic acid, the level of the porphobilinogen deaminase was almost identical to that of wild type bacterial strain grown under similar conditions. The small deaminase activity that could be measured in the mutant grown in the absence of 5-aminolaevulinic acid was accounted for by a certain percentage of revertants within the culture. The amount of heme produced in the hemA⁻ mutant when grown on 5-aminolaevulinic acid was also similar to that of wild type E. coli (Table 4.1). It can therefore be concluded that the inability of the hemA⁻ mutant to biosynthesise either 5-aminolaevulinic acid and hence porphobilinogen, prevents the formation of active porphobilinogen deaminase due to the inability of the organism to form the dipyrromethane cofactor.

This conclusion also explains the observations made by other workers who found that not only hemA⁻ mutants but also hemB⁻ mutants failed to produce active porphobilinogen deaminase (Li et al, 1987). This is also as a result of the mutant bacteria being unable to biosynthesise porphobilinogen and hence being unable to form the dipyrromethane cofactor.

In an attempt to activate any preformed "apo" deaminase in the hemA⁻mutant, grown without 5-aminolaevulinic acid, porphobilinogen was added to cell free extracts of the mutant. This resulted in only a partial and extremely variable restoration of porphobilinogen deaminase activity (only up to about 15% of the original activity was recovered at best), even after prolonged incubation with porphobilinogen. It was not possible, in fact, to establish that any "apo" deaminase was present in the extracts from

TABLE 4.1

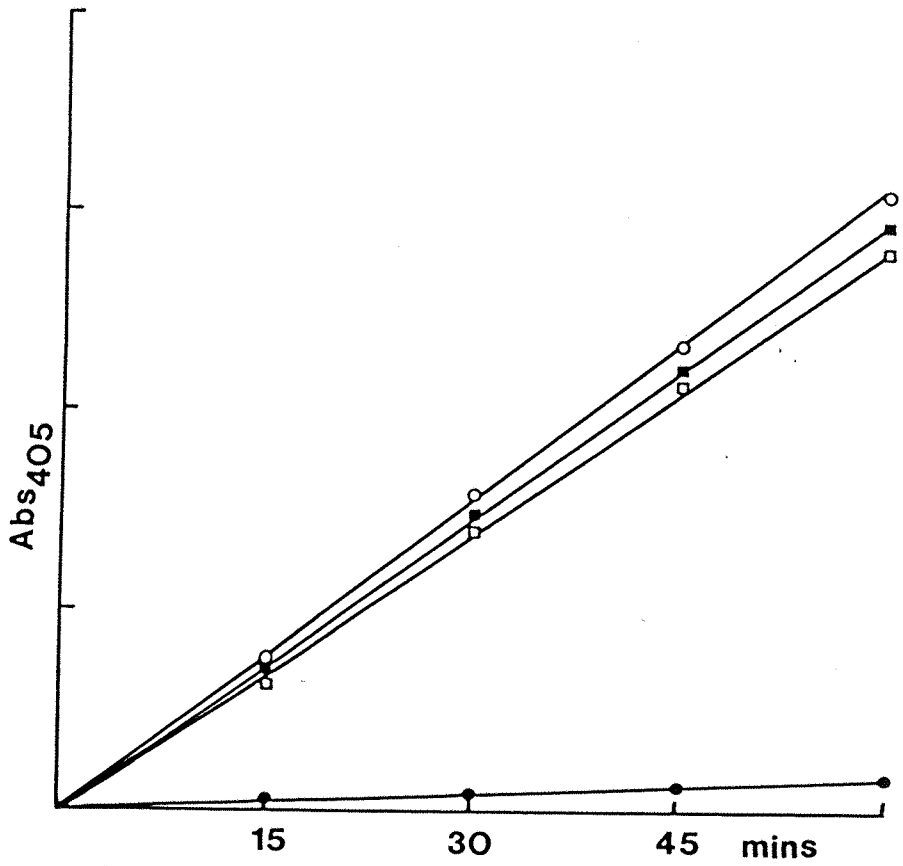
Levels of porphobilinogen deaminase and 5-aminolaevulinic acid dehydratase in E. coli wild type and a hemA⁻ mutant grown in the absence or presence of 5-aminolaevulinic acid.

Bacterial strain and conditions	Deaminase activity (units/mg)	Dehydratase activity (units/mg)
Wild type (A1002) -ALA	15	32
Wild type (A1002) +ALA	14	242
hemA⁻ (A1004a) -ALA	1	350
hemA⁻ (A1004a) +ALA	15	280
hemA⁻/pST48 -ALA	4	-
hemA⁻/pST48 +ALA	125	-

Figure 4.1

Time course for the generation of uroporphyrin I in extracts of wild type and hemA⁻ E. coli. Bacteria were grown anaerobically as described in the experimental section. Cells were harvested after growth in the absence or presence of 5-aminolaevulinic acid. Porphobilinogen deaminase levels in cell free extracts are as follows:-

HemA⁻ mutant in the absence (●——●) or presence (○——○) of 5-aminolaevulinic acid. Wild type in the absence (■——■) or presence of 5-aminolaevulinic acid (□——□).



the hemA⁻ mutant grown in the absence of 5-aminolaevulinic acid because of the very small amounts of the deaminase protein produced in this strain. It was significant, however, that 5-aminolaevulinic dehydratase, the preceding enzyme in the pathway, was present in the hemA⁻ mutant whether 5-aminolaevulinic acid was present or absent (Table 4.1). There did, however, appear to be some control over the level of the dehydratase in the wild type strain which was grown in the presence of 5-aminolaevulinic acid. There would thus appear to be some form of control on the production of the dehydratase in the presence of excess aminolaevulinic acid presumably at the level of transcription. This interesting observation requires further investigation. Since the level of deaminase produced by the wild type E. coli was similar in the presence or absence of added 5-aminolaevulinic acid, it can only be assumed that the "apo" protein was produced but that it was unstable and was rapidly degraded by proteolytic enzymes.

To increase the level of "apo" deaminase the hemA⁻ mutant was transformed with pST48, a plasmid which when present in E. coli HB101 leads to the production of almost 100 times the porphobilinogen deaminase. The transformed strain was also unable to produce active enzyme except when grown on medium supplemented with 5-aminolevulinic acid (Table 4.1). Addition of porphobilinogen to the cell free extracts from the hemA⁻:pST48 strain also failed to substantially activate any "apo" deaminase which was presumed to exist in the extracts. Since both the transformed wild type and the transformed hemA⁻ mutant bacteria had a ten fold increase in the levels of the deaminase compared to the equivalent untransformed strains grown under similar conditions it seemed highly likely that the "apo" deaminase was being produced in the hemA⁻ mutant grown without 5-aminolaevulinate. The only method to determine whether the "apo" deaminase is actually present would be with the use of antibodies. Although it would certainly have been interesting to investigate further the mechanism of how the cofactor is biosynthesised from porphobilinogen and the process by which the cofactor is assembled and attached to the enzyme, the work involved is outside the scope of this thesis. It is worth noting, however, that incubation of extracts presumed to contain "apo" deaminase did on occasion yield up to 15% of active deaminase when incubated for several hours with porphobilinogen suggesting that the "apo" deaminase may be able to catalyse the assembly and insertion of its own cofactor. Speculation on these and related points are covered in chapter 6.

Having transformed the hemA⁻ mutant with a plasmid which could direct a high expression of the deaminase it was theoretically possible to generate deaminase in which the cofactor could be labelled quantitatively if the bacteria were grown in the presence of a labelled precursor such as 5-aminolaevulinic acid. However the transformed hemA⁻ strain, although exhibiting raised levels of the deaminase of a factor of ten fold,

compared to the wild type bacteria, did not produce the levels of deaminase found in the ST1048 strain. This was, most likely, a reflection of the poor growth of the bacteria which produced only about half the cell density of the transformed wild type bacteria. This idea of using a hemA$^-$ mutant transformed with a multicopy plasmid was used to good effect by Scott and co-workers who were able to purify deaminase that had been labelled totally with endogenous [^{13}C] aminolaevulinic acid (Scott, 1988).

The findings with the hemA$^-$ mutant show, for the first time, that the intimate relationship between porphobilinogen deaminase activity and the availability of the heme precursor 5-aminolaevulinic acid is due exclusively to the requirement for the dipyrromethane cofactor at the catalytic centre of the porphobilinogen deaminase enzyme.

4.2.2 The stability of the [^{14}C] labelled dipyrromethane cofactor.

The isolation of homogeneous [^{14}C] labelled deaminase meant that an investigation into the stability of the cofactor could be made readily. From the purification profile it was obvious that the radioactivity was strongly associated with the deaminase protein (Section 3.2.6). Likewise when the deaminase was subjected to non-denaturing polyacrylamide gel electrophoresis the radioactivity was only associated with the protein (Figure 4.2). However, when the deaminase was subject to denaturing SDS polyacrylamide gel electrophoresis only about half of the initial radioactivity remained associated with the protein, the other half being found at the dye front (Figure 4.3). Similar findings were also noticed after formic acid treatment of the labelled enzyme. From precipitation studies it was found that that on initial treatment of the protein with formic acid, about half of the label was released into the supernatant whilst half remained associated with the protein (Figure 4.4). On prolonged incubation with formic acid more of the label was gradually released into the supernatant but even after 30 hours there was still some 25-30% of the initial radioactivity associated with the protein. This residual label was, however, lost from the protein when it was subjected to denaturing SDS electrophoresis. This peculiar behaviour of the [^{14}C]-labelled cofactor probably relates to the oxidation state of the dipyrromethane molecule itself under the various experimental conditions. In its normal active state, when it is bound to the enzyme, the cofactor is reduced and therefore colourless (Scheme 4.2, a). However, with time the dipyrromethane molecule becomes oxidised to give, at neutral pH values, a yellow chromophore (Scheme 4.2, b). This is observed during the crystallisation of the enzyme where the crystals and the mother liquor both turn a deep yellow colour over a period of several months. In a similar manner when the pH of the protein is reduced to below 4, a rapid oxidation and protonation of the cofactor occurs giving rise to a pink colour (Scheme 4.2, c). The state of oxidation of the dipyrromethane molecule reflects its

Figure 4.2

Analysis of the stability of the [^{14}C]-labelled dipyrromethane cofactor by non-denaturing polyacrylamide gel electrophoresis. Porphobilinogen deaminase (30μg), with labelled [^{14}C]-cofactor, was electrophoresed under non-denaturing conditions in duplicate tracts. One of these tracts was stained for protein whilst the other was cut into 2mm slices and analysed for radioactivity.

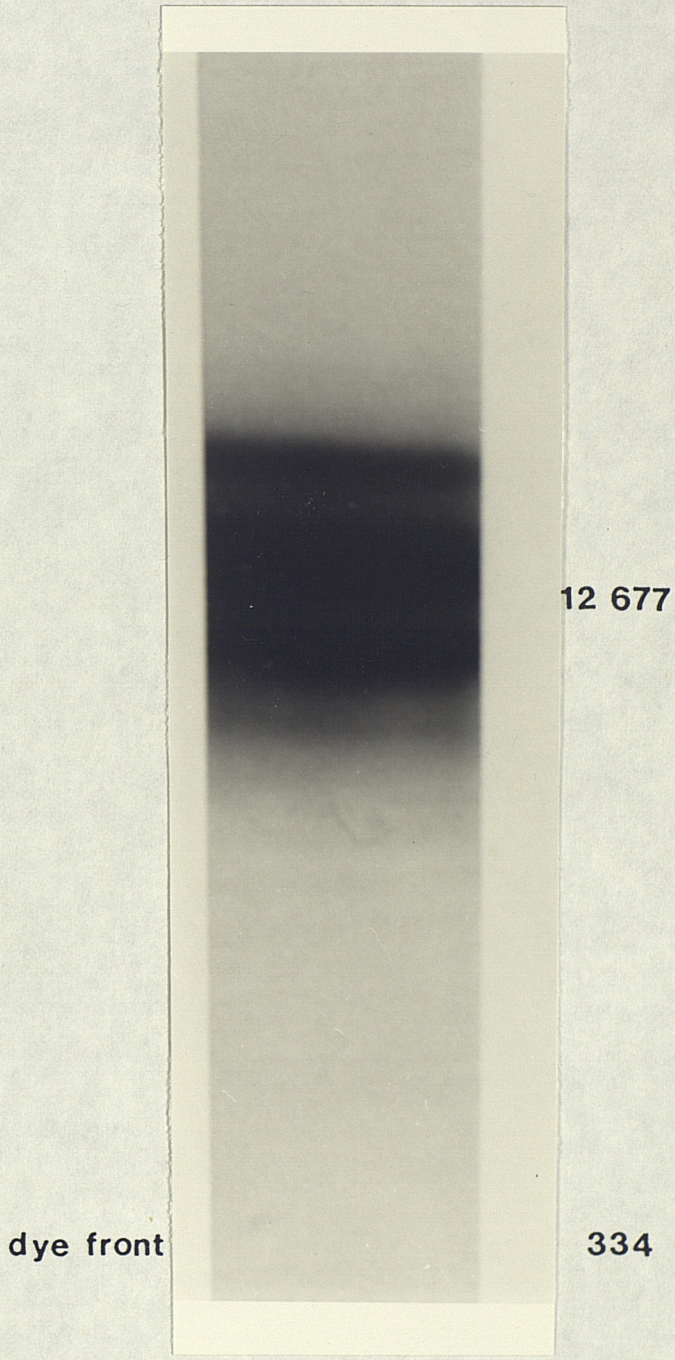
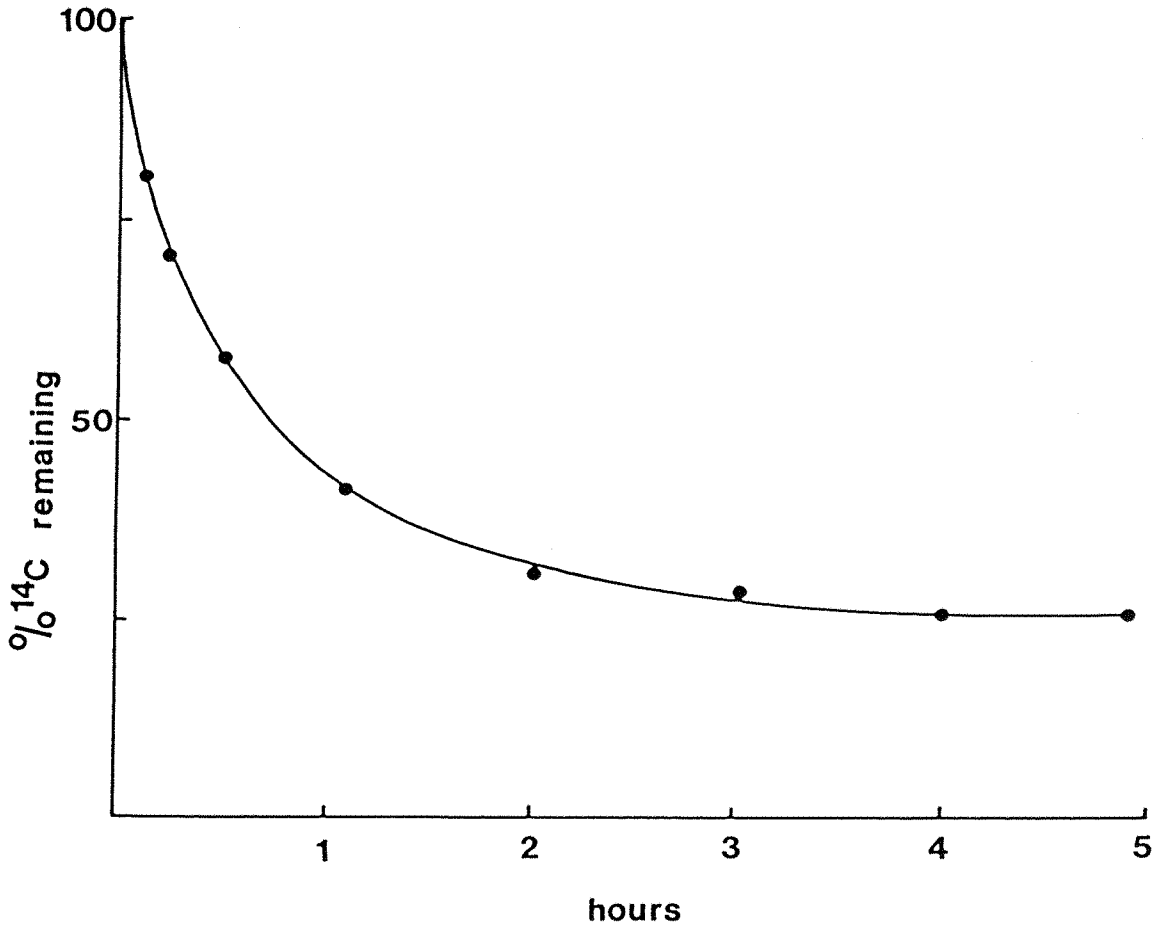


Figure 4.3

Analysis of the stability of the [^{14}C]-labelled dipyrromethane cofactor by denaturing SDS polyacrylamide gel electrophoresis. Porphobilinogen deaminase (20μg), with labelled [^{14}C]-cofactor, was electrophoresed under denaturing conditions in duplicate tracts. One of these tracts was stained for protein whilst the other was cut into 2mm slices and analysed for radioactivity.

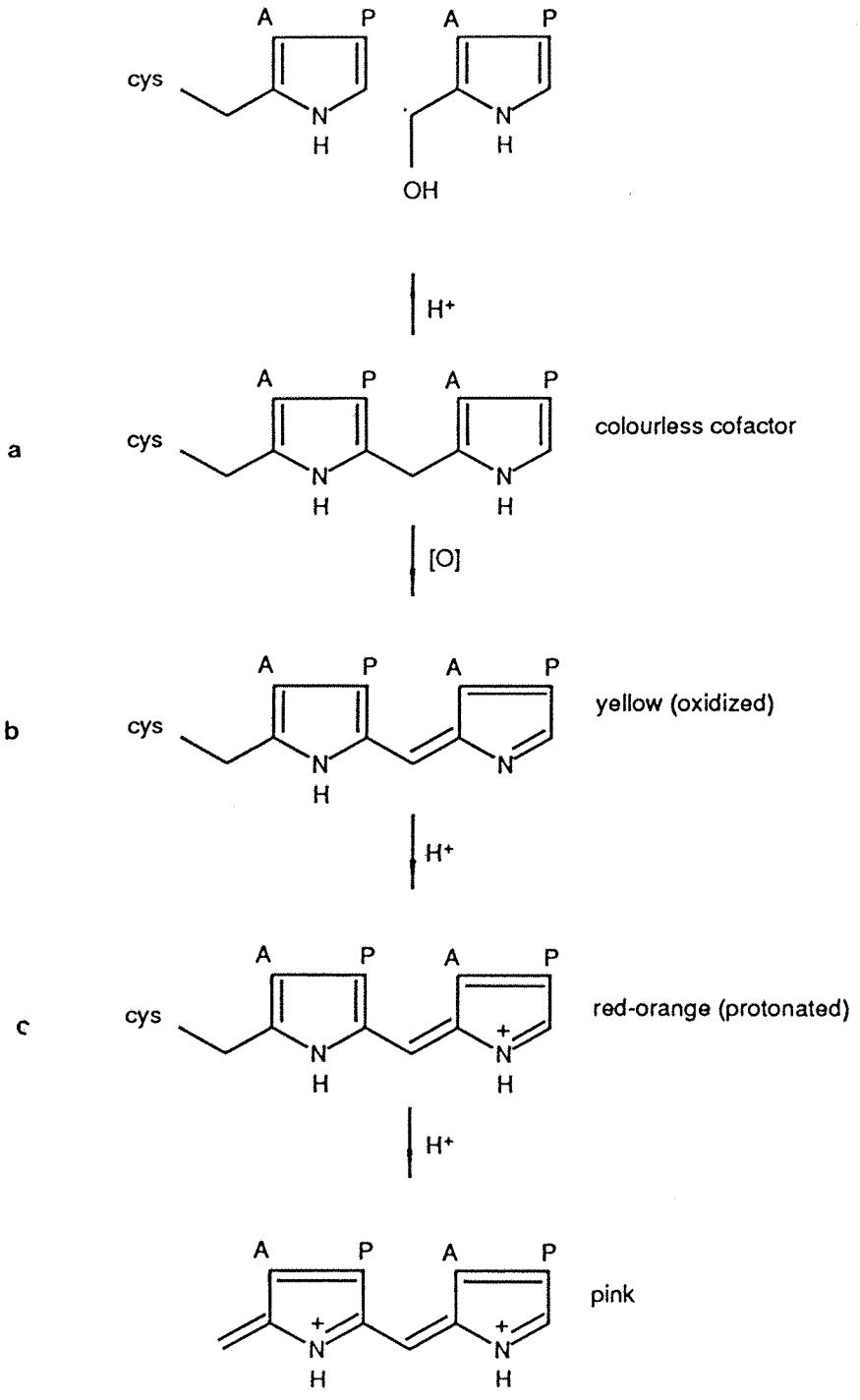
Figure 4.4

Stability of the dipyrromethane cofactor to treatment with formic acid. Porphobilinogen deaminase (1mg; 30nmoles) was treated with aqueous formic acid (50% v/v). At various time intervals a sample of the protein was removed, precipitated with trichloroacetic acid (10%), and centrifuged. Both the supernatant and the precipitated protein pellet were analysed for radioactivity.



SCHEME 4.2

The oxidation states of the dipyrromethane cofactor.



chemical stability. In the reduced active state the dipyrrole can be hydrolysed and broken either at the meso position or at the linkage of the cofactor with the enzyme. However, in the oxidised state the dipyrrole is more stable and cannot be broken by hydrolysis. Thus the oxidation state of the dipyrromethane reflects the stability of the association of the cofactor with the enzyme, and is reflected in the behaviour of the [^{14}C]-labelled protein observed on gels. During non-denaturing electrophoresis the enzyme remains in its native configuration and the cofactor remains associated with the protein. During denaturing electrophoresis the enzyme loses it configuration and, with the presence of β-mercaptoethanol, the cofactor remains in its reduced state thus making it susceptible to cleavage.

4.2.3 Labelling of the dipyrromethane cofactor with [^{13}C] and determination of the structure and attachment site of the cofactor to the enzyme by [^{13}C] n.m.r. spectroscopy.

Since growth of E. coli on 5-amino[5-^{14}C]laevulinic acid resulted in labelling of the dipyrromethane cofactor at the active site of the deaminase it followed that enzyme isolated from E. coli grown in the presence of [^{13}C] precursor would be similarly labelled. The C-5 position of 5-aminolaevulinic acid is incorporated into the C-2 and C-11 positions of porphobilinogen. Incorporation of two molecules of porphobilinogen would thus result in the labelling pattern indicated as shown in scheme 4.3.

Since all the labelled carbon atoms of the dipyrromethane are in unique environments each should exhibit a characteristic chemical shift. The two labelled carbon atoms at the free α-position and the substituted α-position of the pyrrole rings would be expected to show resonances in the aromatic region at around δ=117ppm and δ=124ppm respectively. The meso-carbon atom linking the two rings would be expected to show a resonance at about δ=24ppm and to exhibit a single bond [^{13}C - ^{13}C] coupling to the substituted aromatic carbon at δ=124ppm which, in turn, would be similarly affected. The resonance of the remaining carbon atom would be dependent on the nature of the amino acid to which it is linked.

The enzyme, grown in media supplemented with 5-amino[5-^{13}C]laevulinic acid and tracer 5-amino[5-^{14}C]laevulinic acid, when purified was found to have incorporated 2.4 molecules of labelled ALA per molecule of enzyme representing an incorporation of 60%. The [^{13}C] spectra of the [^{13}C]-labelled deaminase and non-labelled deaminase are shown in Figure 4.5 (a) and (b) respectively. The [^{13}C - ^{12}C] difference spectrum, Figure 4.5 (c), revealed three broad resonances representing labelled carbon atoms from the enzyme-bound cofactor. The aromatic carbons showed signals at δ=116ppm and δ=128.5ppm and the meso-carbon exhibited a signal at δ=24.7ppm. Close inspection revealed that the signal at δ=24.7ppm also had a shoulder at δ= 25.6ppm and that the

SCHEME 4.3

The synthesis of the dipyrromethane cofactor from 5-amino[5-^{13}C]laevulinic acid.

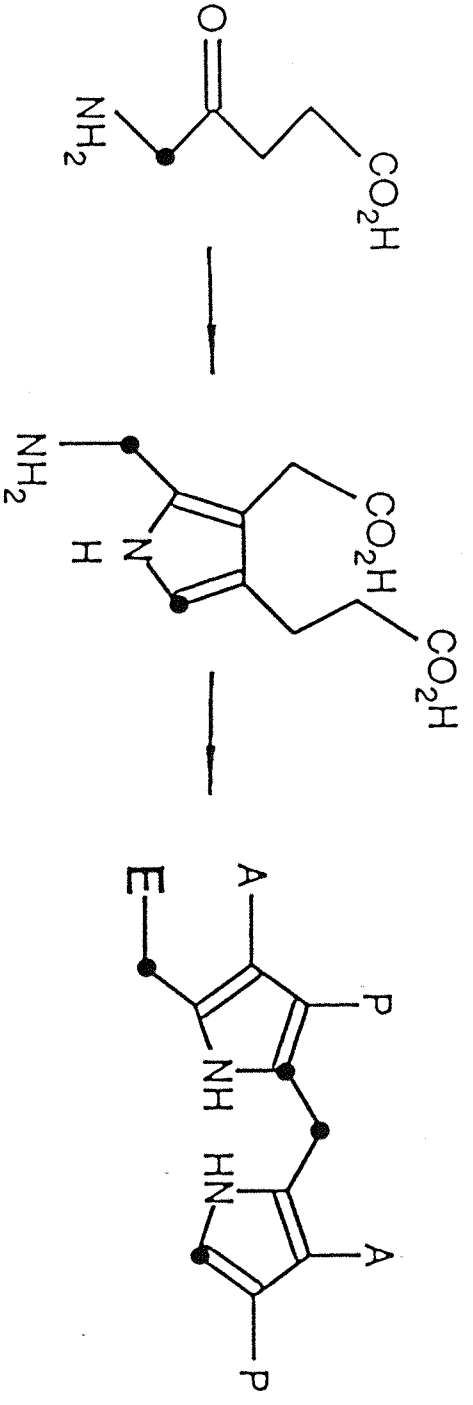


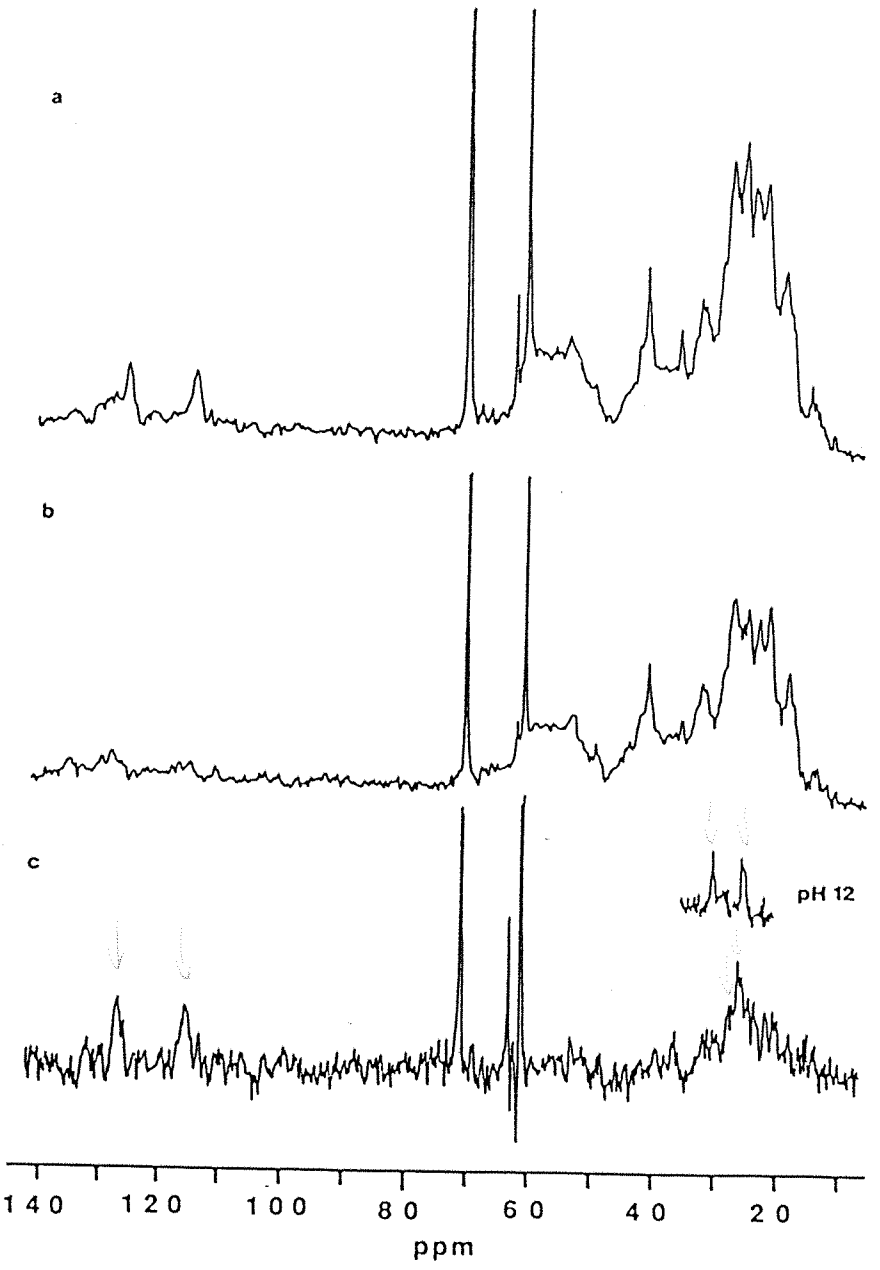
Figure 4.5

[^{13}C] N.m.r. spectra of E. coli porphobilinogen deaminase at pH 8.5.

a) Enzyme isolated from bacteria grown on 5-amino[5-^{13}C]laevulinic acid

b) Non-labelled enzyme

c) Difference spectrum of (a) - (b). Insert shows (c) at pH 12 The sharp signals between 60-70ppm are due to EDTA and residual Tris buffer.



complex integrated to two [^{13}C] atoms suggesting the presence of a possible fourth resonance. In an attempt to visualise the fourth resonance more fully, the labelled enzyme was exposed to pH 12. Under these conditions the deaminase would be expected to unfold. There is no loss of [^{14}C] radioactivity from the enzyme in which the cofactor has been labelled with [^{14}C] after treatment at pH 12 (Table 4.2). Enzyme activity can only be recovered, however, with a rapid adjustment of the pH back to 8.5. Prolonged exposure to pH 12 results in the irreversible inactivaction of the enzyme although the cofactor is not lost from the enzyme. Accordingly, the [^{13}C] n.m.r. spectrum was remeasured at pH 12, as shown in the insert in Figure 4.5 c, revealing the clearly resolved fourth signal centered at δ=29.7ppm. The existence of these four broad resonances, all of which are of similar intensity, is consistent with the strucure of the bound cofactor as shown in scheme 4.3.

The nature of the covalent link between the enzyme and the dipyrromethane may be deduced from the [^{13}C] chemical shift of the fourth resonance exhibited by the enzyme bound cofactor since the [^{13}C] nucleus is extremely sensitive to its environment. For instance, direct bonding of a methylene carbon atom to a lysine ε-nitrogen or to a histidine ring nitrogen would give a chemical shift in the range 40-50ppm, whereas a linkage with the OH group of serine would be reflected by a chemical shift of the order 60-70ppm. The SH group of cysteine would exhibit a chemical shift in the range 25-35ppm (Table 4.3) (Climie, 1979). The chemical shift exhibited by the fourth resonance at pH 8.5 (δ=25.6ppm) is in the range expected for a carbon atom bonded directly to a cysteine residue. At pH 12 the fourth resonance changes to δ=29.7ppm and remains at this value even after the pH was lowered back to 8.5. This movement of the fourth resonance almost certainly reflects a change in the protein structure due to unfolding of the protein. Concurrent with this idea is the observation that the signals at pH 12 become somewhat sharper reflecting a greater disorder in the protein structure. Lowering of the pH back to 8.5, in this case, did not restore enzymatic activity and this was reflected by the fact that the fourth resonance remained at δ=29.7ppm indicating a permanent change in the environment surrounding the cofactor link to the enzyme. Sulphur-porphobilinogen model compounds have been synthesised by Scott and co-workers (1988) and the analogous carbon atom linked to a sulphur atom gives a resonance around δ=29ppm, almost identical to the deaminase labelled with [^{13}C]-cofactor. The lower resonance value of the carbon atom involved in the enzyme-cofactor linkage in the native enzyme in comparison to the model compounds and to the denatured enzyme at pH 12 must represent a pertubation of the environment surronding this carbon atom. The reason for this downfield shift and any mechanistic implications will probably become apparent with the elucidation of the protein crystal structure.

By use of a transformed hemA$^-$ mutant Scott and co-workers, using the

TABLE 4.2

Stability of the dipyrromethane cofactor to alkaline pH with comparison to the catalytic activity of the enzyme.

pH of enzyme solution	Length of time at this pH	% enzymatic activity remaining	% [^{14}C] associated with protein
8.0	2 hours	96	97
8.0	12 hours	91	94
12.0	2 hours	85	96
12.0	12 hours	2	97

TABLE 4.3

Chemical shift values of [^{13}C]-labelled methylene carbon atoms bonded directly to different functional groups. (Taken from Climie, 1979)

Functionality	*C Chemical shift δ
O-*CH_2CH_2CN	65.96
N_{imid}-*CH_2CH_2CN	42.57
N_{amino}-*CH_2CH_2CN	45.10
S-*CH_2CH_2CN	28.27

principle described in section 4.2.1, were able to obtain 90% + enrichment of the cofactor from [^{13}C] labelled 5-aminolaevulinic acid. N.m.r. analysis of the purified protein obtained from this source gave the same, but stronger, signals as described above for the enzyme labelled in our laboratory. Using this highly enriched protein it was also possible to show a [^{13}C - ^{13}C] single-bond coupling between the resonances at δ=128.5ppm and δ=24.7ppm of 54Hz. Further evidence for the involvement of a carbon-sulphur linkage was demonstrated when the fourth resonance at δ=25.6ppm disappeared after the enzyme was treated with Raney nickle (Scott, personal communication).

4.2.4 Identification of the cysteine responsible for the covalent linkage of the cofactor to the deaminase.

Examination of the gene derived primary protein sequence of E. coli porphobilinogen deaminase reveals that four cysteine residues are present (Figure 4.6). Three of these cysteine residues react with thiol reagents such as DTNB under denaturing conditions (Figure 4.7). A comparison of the E. coli and human (Raich et al, 1986) derived protein sequences indicates a remarkable 60% homology between the proteins (Jordan et al, 1988 b). Of the four cysteine groups in the E. coli enzyme only two are conserved between the E. coli and the human deaminases (Figure 4.6). These two conserved cysteine residues are situated at positions 99 and 242 on the E. coli predicted primary sequence and fall either side of the single aspartyl-proline bond at position 103-104 which is cleaved specifically with formic acid (Section 2.2.8). Since the human porphobilinogen deaminase has also been shown to contain a dipyrromethane cofactor (Warren and Jordan, 1988 a) (Section 3.2.8), which must also be bound to a cysteine, one of these two conserved cysteines is likely to be involved with the binding of the cofactor to the E. coli deaminase. In order to determine which cysteine residue is involved a peptide was isolated from the [^{14}C] cofactor-labelled deaminase, after cleavage of the protein by formic acid. Over two thirds of the [^{14}C] label was converted into uroporphyrin but sufficient radioactivity remained bound to permit the isolation of a red coloured peptide containing 20% of the initial [^{14}C] label by gel filtration and reverse phase chromatography. The small formic acid peptide, molecular weight 11,000 Daltons, was found to contain negligible radioactivity. Isolation and purification of the labelled peptide showed it to have a molecular weight of 24,000 Daltons establishing it as the fragment extending from amino acid residue 104 to the C-terminus. Since this [^{14}C] peptide includes cysteine 242 but not cysteine 99 it was concluded that it was the former cysteine which provides the covalent binding site for the dipyrromethane cofactor in the gene derived primary sequence of the E. coli deaminase. The instability of the cysteine-pyrrole bond made further protein chemistry very difficult.

This is conclusive evidence that the cysteine at position 242 in the gene derived

Figure 4.6

Comparison of the predicted protein primary structure between the E. coli gene derived hemC sequence and the human cDNA encoded sequence.

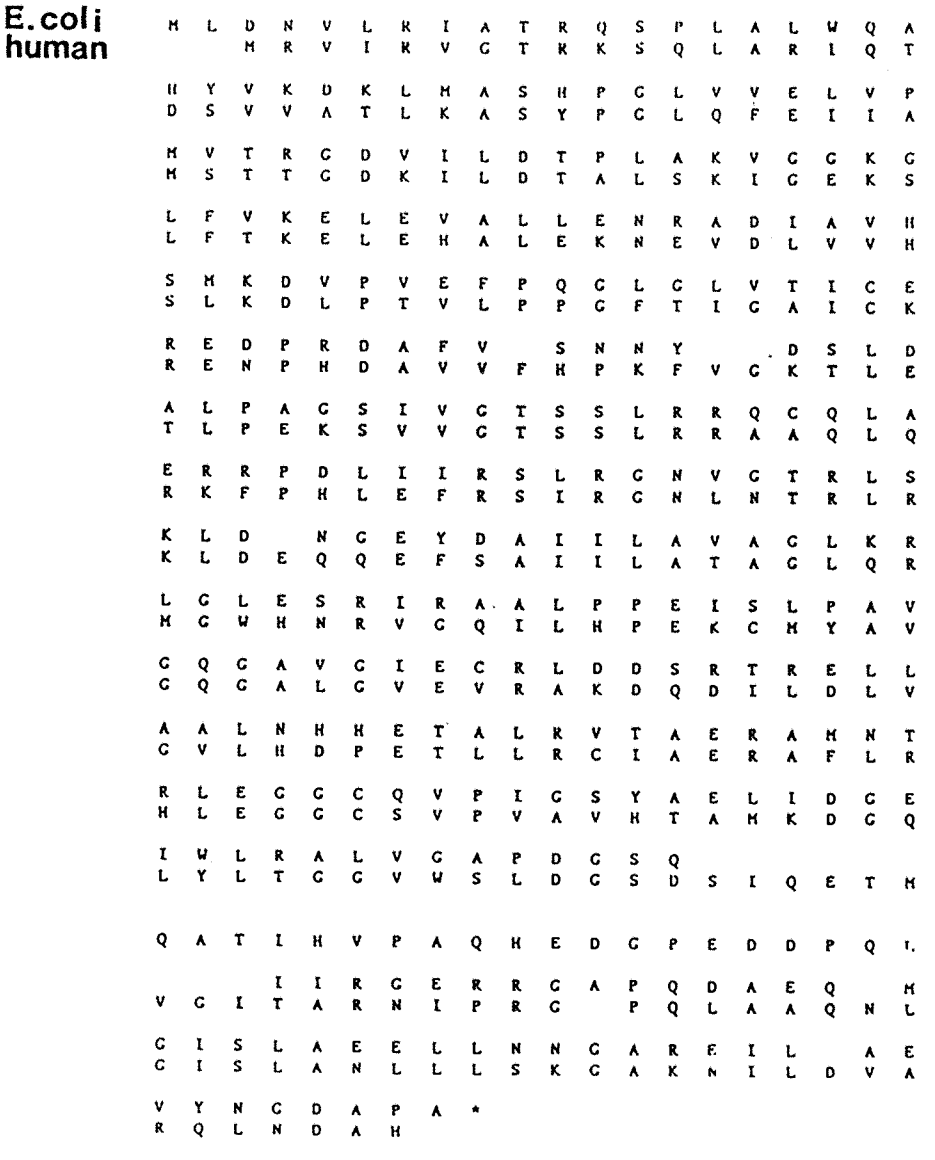
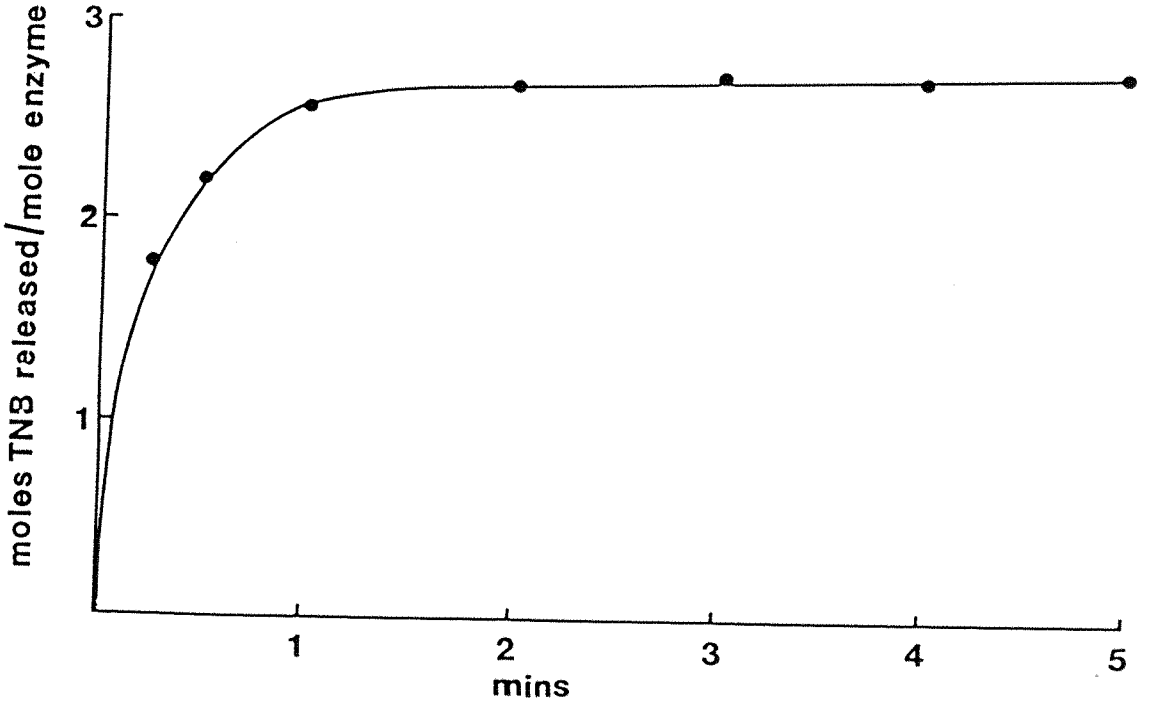


Figure 4.7

Titration of DTNB with denatured E. coli porphobilinogen deaminase to determine the number of enzyme cysteine residues. Porphobilinogen deaminase (1mg; 30nmoles) was denatured with 0.1% SDS and titrated against a solution of 1mM DTNB in 0.1M Tris/HCl buffer pH 7. The absorbance of the liberated thionitrobenzoate was measured at 412nm (ε_{412} = 12,800).



primary structure of the E. coli enzyme is the group responsible for the covalent attachment of the dipyrromethane cofactor to the enzyme. Generation of modified E. coli porphobilinogen deaminases in which either cysteine 99 or cysteine 242 has been substituted by serine using site specific mutagenesis has recently been accomplished and supports this view (Jordan et al, 1988 c). Deaminase containing serine 99 was catalytically active whereas the enzyme containing serine 242 was completely devoid of enzyme activity. The location of the cysteine residue responsible for the cofactor binding in three dimensional terms will be determined with the full elucidation of the crystal structure of the enzyme and this technique may also give an answer as to why the deaminase maintains the cofactor carbon atom which secures the attachment to the enzyme in a pertubated environment.

The use of cysteine to attach covalently the cofactor to the enzyme is not particularly surprising as nature has used this particular group to attach the prosthetic groups of haem to cytochrome c and phycobilin to phytochrome.

Chapter 5.

Studies on Purified *E. coli* Porphobilinogen Deaminase and its Intermediate Complexes.

5.1 Introduction.

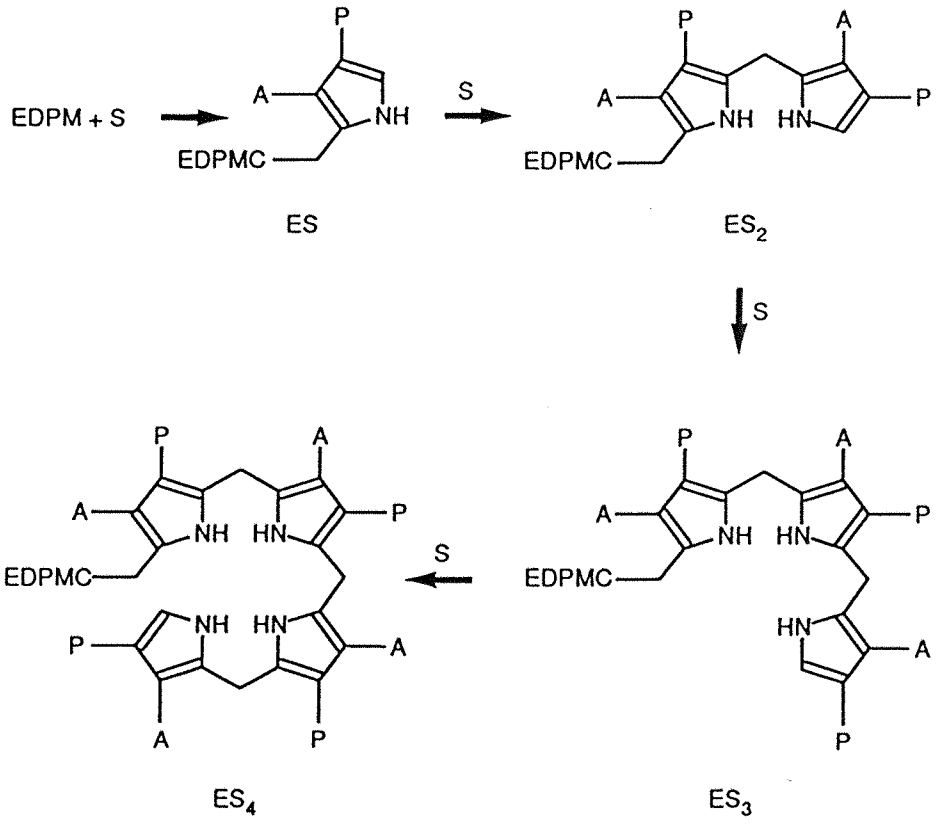
One of the most interesting and remarkable aspects of the mechanism of porphobilinogen deaminase is that it gives rise to the existence of highly stable enzyme-intermedite complexes which are formed as a result of the reaction between the enzyme and its substrate. These enzyme-intermediate complexes have been shown to consist of enzyme with one, two, three and four pyrrole units covalently attached to the enzyme at the active site and thus represent sequential stages in the stepwise synthesis to the final tetrapyrrole product (Anderson and Desnick, 1980; Jordan and Berry, 1981; Jordan et al, 1988 b; Warren and Jordan, 1988 b)(Scheme 5.1).

The involvement of such covalent enzyme bound intermediates had first been suspected in the early 1970's when it had been demonstrated that incubation of the enzyme and its substrate in the presence of nitrogenous bases such as ammonia, hydroxylamine and methoxyamine resulted in the release of base substituted free pyrroles and polypyrroles into the incubation medium (Pluscec and Bogorad, 1970; Davies and Neuberger, 1973). The first evidence suggesting the stability of these enzyme intermediates came from the experiments designed to show the order of ring assembly during the biosynthesis of preuroporphyrinogen. In these experiments the deaminase was first mixed with either a limiting amount of unlabelled or labelled porphobilinogen prior to addition of excess labelled or unlabelled substrate (Battersby et al, 1979 c; Jordan and Seehra, 1979). These experiments led to the formation of regiospecifically labelled porphyrins which were then used to determine the order of ring formation. These experiments proved that there had to be a close and highly stable association between the enzyme and its substrate.

The full extent of this stable association was shown when the individual enzyme intermediate forms were isolated by Anderson and Desnick (1980). They were able to isolate five forms of the human enzyme by an anion exchange procedure and, moreover, they were also able to show that these forms were interconvertible in the presence of substrate. The conclusion from this work was that the human deaminase was able to exist in five forms which represented the free enzyme and enzyme with one, two, three and four pyrroles attached. Using enzyme isolated from R. sphaeroides, Berry et al (1981) demonstrated the presence of three enzyme intermediate complexes. By the use of a procedure that involved regiospecific labelling and some intricate chemistry it was possible to characterise these complex forms as being due to enzyme with one, two and three pyrrole units attached. Both the methods used by these workers involved either

SCHEME 5.1

The stepwise synthesis of the porphobilinogen deaminase enzyme-intermediate complexes.



lengthy chromatographic or polyacrylamide gel electrophoresis procedures that had to be carried out at temperatures no higher than 4°C.

In an attempt to gain a fuller understanding of both the properties of these isolated complexes and of the mechanism of the enzyme itself, especially in the light of the recent discovery that these bound intermediates are anchored to the enzyme dipyrromethane cofactor (Jordan & Warren, 1987), a thorough investigation of the complexes was made. The work in this chapter reports on the nature of the interaction of the substrate porphobilinogen with the dipyrromethane cofactor at the active site of the E. coli porphobilinogen deaminase and also demonstrates that modification of several amino acid groups leads to inactivaction of the enzyme (Warren & Jordan, 1988 b).

5.2 Results and discussion.

5.2.1 Isolation and characterisation of enzyme-intermediate complexes.

Previous studies with deaminases isolated from human erythrocytes and from R. sphaeroides have shown the formation of covalent intermediate complexes on incubation of the native enzyme with the substrate porphobilinogen (Anderson and Desnick, 1980; Jordan and Berry, 1981). These intermediate complexes have, in the past, been isolated either by ion exchange chromatography or by equally lengthy and tedious polyacrylamide gel electrophoresis methods. Under these conditions the complexes are liable to dissociate and it has proved difficult to carry out any detailed investigations.

The availability of f.p.l.c. has permitted the development of a rapid method for the separation of the enzyme intermediate complexes which has allowed a detailed evaluation of their properties to be made. Complete separation of the complexes can be achieved in less than 20 minutes.

When a single peak of purified E. coli porphobilinogen deaminase, isolated by f.p.l.c. (Figure 5.1, a), was rapidly mixed with a half molar equivalent of porphobilinogen and then reapplied to the f.p.l.c. column, two new peaks could be separated (Figure 5.1, b). When a two molar equivalent of substrate was mixed with the enzyme and the resulting mixture was then reapplied to the f.p.l.c. column, a third new peak appeared (Figure 5.1, c). If a ten fold excess of [^{14}C] porphobilinogen was mixed with the enzyme prior to isolation on the f.p.l.c., then a total of four peaks could be visualized corresponding to the original enzyme peak and the three new peaks of the enzyme intermediate complexes (Figure 5.1, d). Determination of the ratios of the enzyme activity, protein content and radioactivity of each individual peak showed that not only did all the peaks have the same specific activity but that the [^{14}C] : protein ratios of peaks 1, 2 and 3 increased with the values of 1.01, 2.10 and 3.19 respectively (Table 5.1). This data suggested that peak 1 was enzyme with a single bound pyrrole unit, peak two contained enzyme with two bound pyrrole units and the final peak 3 represented an enzyme species with three pyrrole units. No evidence was found for a species with four pyrrole units, as was noticed with the human enzyme. This rapid isolation method gave, for the first time, an absolute characterisation to the isolatable enzyme species. These complexes were assigned as ES, ES_2 and ES_3 to differentiate them from the native holoenzyme (E).

5.2.2 Stability of the individual enzyme-intermediate complexes.

The individual complexes, when isolated and stored at 4°C, were found to be remarkably stable. The ES complex, the enzyme intermediate form with one bound pyrrole equivalent, could be stored for up two hours with only a slight decomposition of

Figure 5.1

Isolation and identification of enzyme intemediate complexes. High resolution ion-exchange chromatography of a single peak of porphobilinogen deaminase isolated from a Mono Q column:

a) before treatment with porphobilinogen

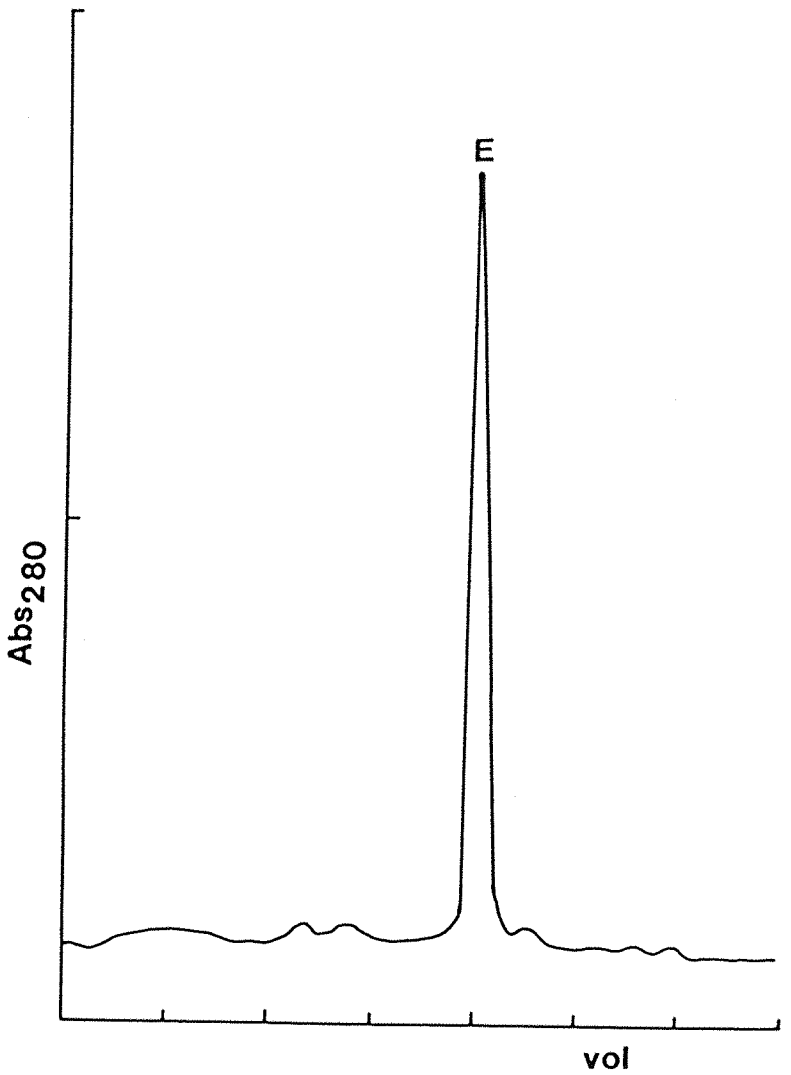


Figure 5.1

Isolation and identification of enzyme intemediate complexes. High resolution ion-exchange chromatography of a single peak of porphobilinogen deaminase isolated from a Mono Q column:

b) after mixing with a half molar equivalent of porphobilinogen

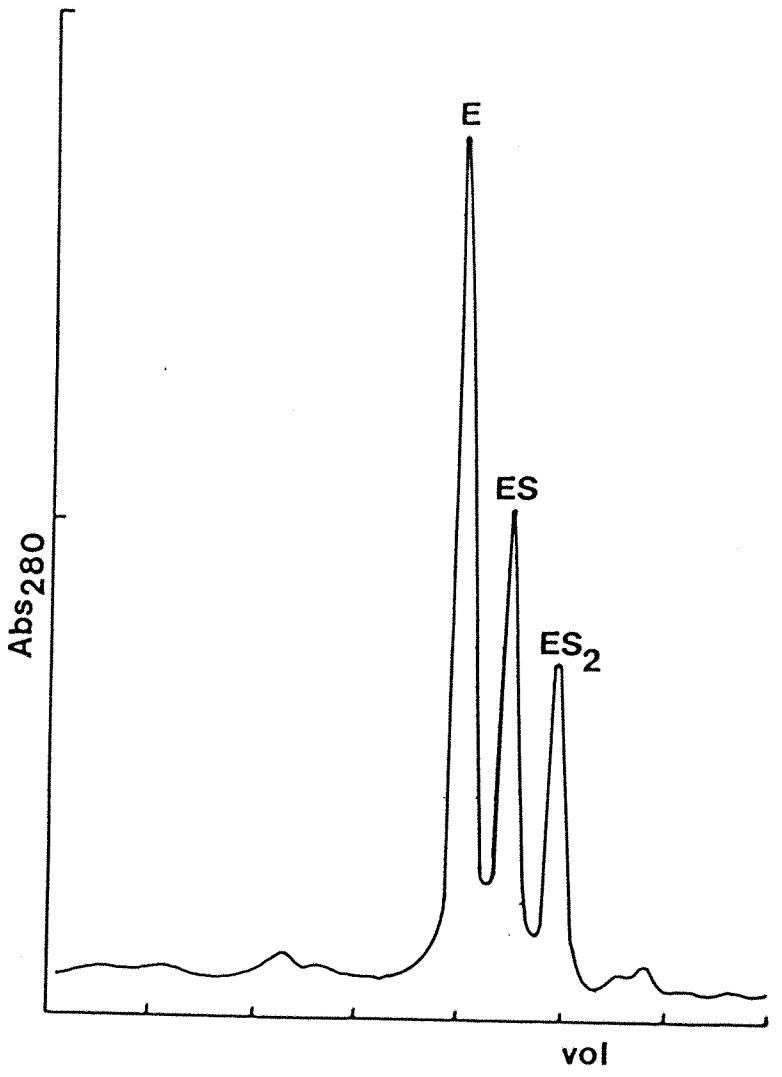


Figure 5.1

Isolation and identification of enzyme intemediate complexes. High resolution ion-exchange chromatography of a single peak of porphobilinogen deaminase isolated from a Mono Q column:

c) after mixing with with two equivalents of porphobilinogen

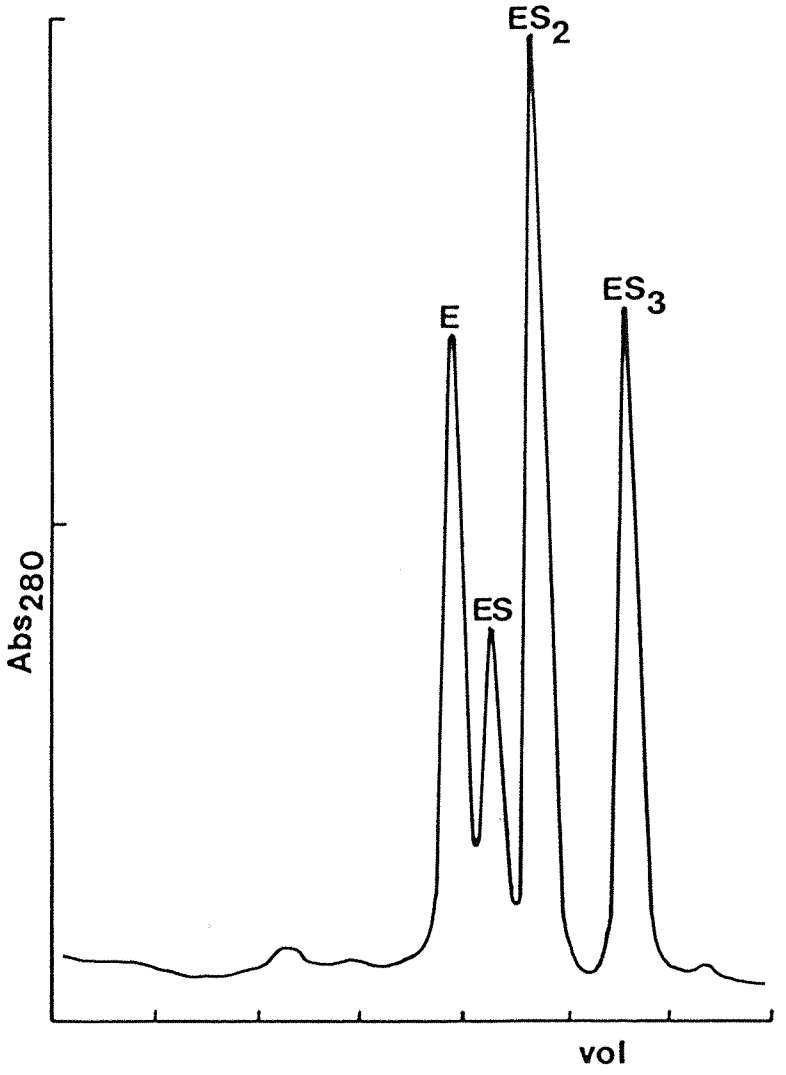


Figure 5.1

Isolation and identification of enzyme intemediate complexes. High resolution ion-exchange chromatography of a single peak of porphobilinogen deaminase isolated from a Mono Q column:

d) after mixing with a ten fold molar excess of porphobilinogen

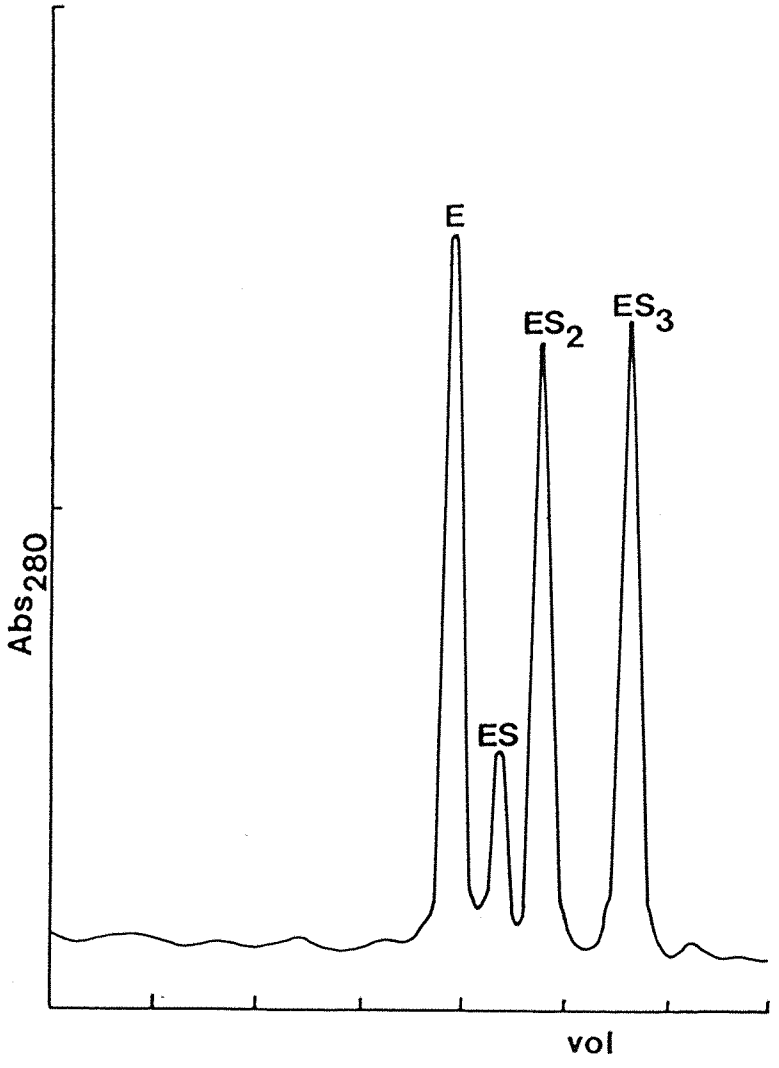


TABLE 5.1

Ratio of substrate (porphobilinogen) : enzyme in isolated peaks obtained from Mono Q high resolution anion exchange chromatography (Figure 5.1) after mixing enzyme with [^{14}C]-porphobilinogen.

Peak	Ratio of substrate:enzyme	Assignment of peak
A	0.14	E
1	1.01	ES
2	2.10	ES_2
3	3.19	ES_3

the major peak into the other enzyme forms (Figure 5.2, a). The ES_2 and ES_3 complex forms were found to be even more stable than the ES form and could be stored for up to 16 hours without any undue decomposition (Figure 5.2, b and c). At higher temperatures the stability of all the complexes is reduced to varying degrees. The ES complex is paricularly labile at 37°C and after 15 minutes at this temperature was rapidly converted into E, ES_2 and ES_3 (Figure 5.3, a). In a similar manner the ES_3 complex was also quite unstable at this temperature being largely converted into the ES_2 form (Figure 5.3, b). The most stable of all the complex forms was ES_2 which remained comparatively unaffected by treatment at 37°C (Figure 5.3, c). Heating all the complex forms to 60°C, however, caused a rapid loss of bound intermediates from all the complexes yielding fully active free enzyme (Figure 5.3, d).

It may be concluded therefore that at 37°C the linkage between the first substrate molecule and the enzyme bound dipyrromethane cofactor is the most labile but that on binding a second substrate molecule a far more stable complex is formed. Addition of a third substrate molecule yields ES_3, a more labile intermediate complex than ES_2, but which is more stable than ES. The greater stability of the ES_2 and ES_3 species is demonstrated by their ability to withstand the conditions of electrophoresis (Figure 5.4). Even though the holoenzyme has been isolated as a single peak from the f.p.l.c. column it still migrates as two bands on non-denaturing polyacrylamide gels. The ES complex form of the enzyme has dissociated into a mixture of all the forms and is not visable on the photograph. The ES_2 and ES_3 complex forms of the enzyme migrate proportionately further towards the anode compared to the holoenzyme.

5.2.3 Release of the bound intermediates from the enzyme complexes.

Previous studies both by Pluscec and Bogorad (1970) and by Davies and Neuberger (1973) established that base substituted pyrroles and polypyrromethanes could be released from the enzyme by treatment with amino compounds such as NH_3, NH_2OH and NH_2-OCH_3. These inhibitory bases have been shown to prevent porphyrin formation yet do not unduly affect the utilisation of the substrate by the enzyme. The released pyrrole moieties are formed by base displacement of the pyrrolic intermediates which are attached to the enzyme complexes. When hydroxylamine was incubated with any of the E. coli porphobilinogen deaminase intermediate complexes free enzyme was regenerated and hydroxylamine adducts of mono, di, tri and tetrapyrroles were released into the solution. These adducts could be separated by high voltage electrophoresis and detected by spraying with Ehrlich's reagent (Pluscec and Bogorad, 1970) (Figure 5.5). When isolated [^{14}C] labelled enzyme-intermediate complexes (ES, ES_2 and ES_3) were treated with hydroxylamine and the released adducts separated out by high voltage electrophoresis it was found, after scanning the chromatogram for radioactivity, that the

Figure 5.2

F.p.l.c. profiles of isolated enzyme intermediate complexes. Each individual complex was isolated and rechromatographed after incubation at 10°C for a number of hours.

a) ES complex after 2 hours at 10°C

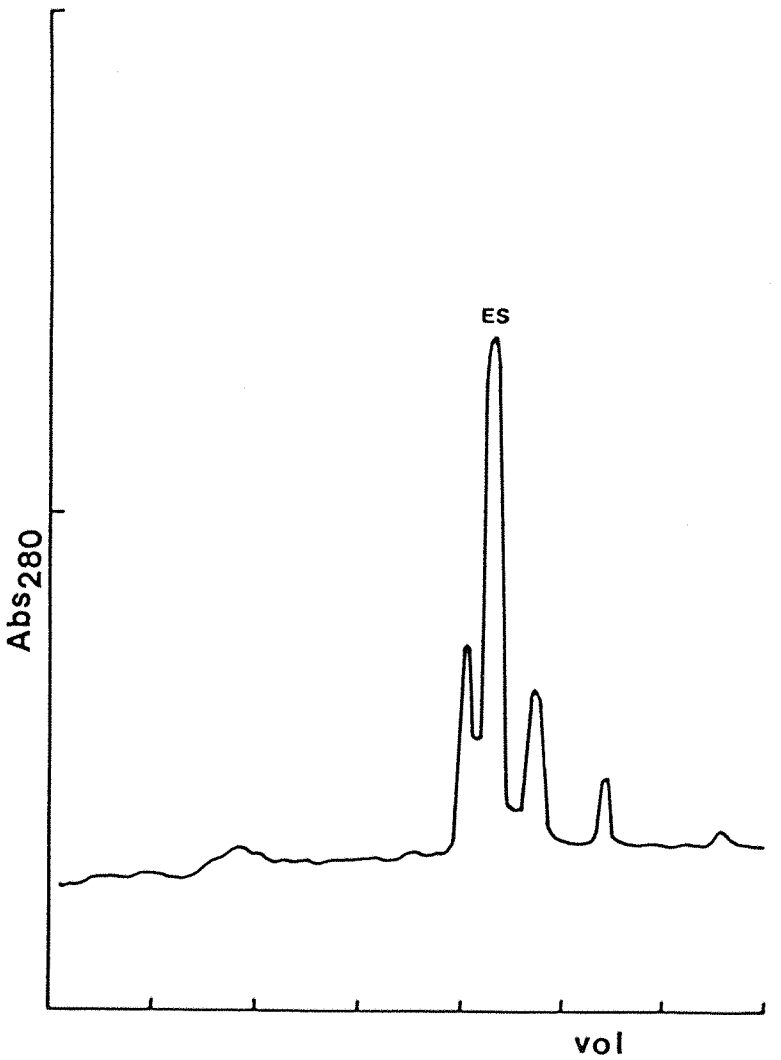


Figure 5.2

F.p.l.c. profiles of isolated enzyme intermediate complexes. Each individual complex was isolated and rechromatographed after incubation at 10°C for a number of hours.

b) ES_2 complex after 16 hours at 10°C

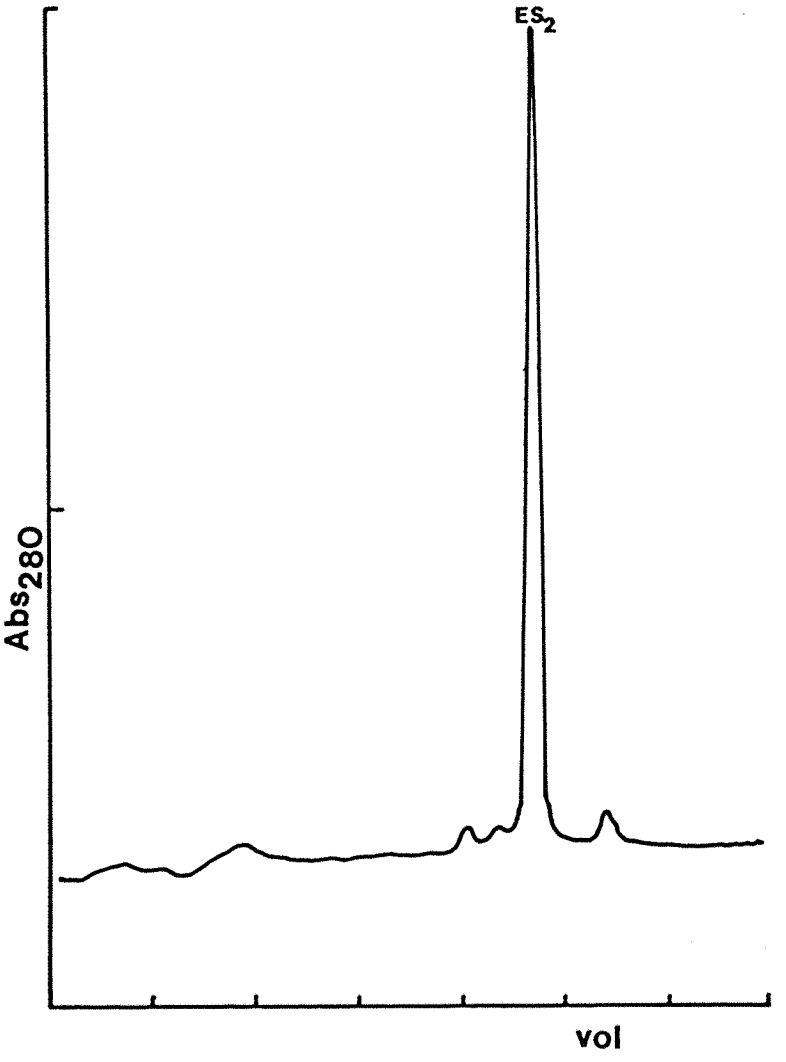


Figure 5.2

F.p.l.c. profiles of isolated enzyme intermediate complexes. Each individual complex was isolated and rechromatographed after incubation at 10°C for a number of hours.

c) ES_3 complex after 16 hours at 10°C

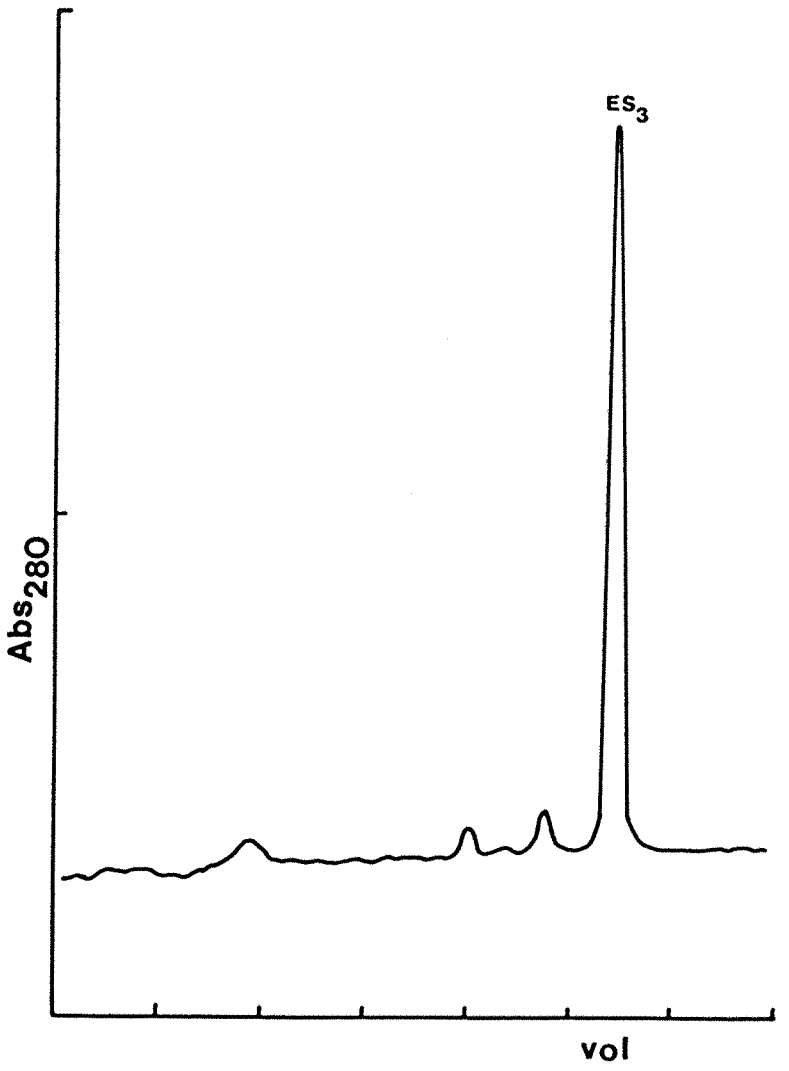


Figure 5.3

Effect of temperature on the stability of the isolated enzyme-intermediate complexes. Each individual enzyme complex was incubated at either 37°C or 60°C prior to reanalysis of the f.p.l.c. profiles.

a) ES complex profile after incubation at 37°C for 15 minutes

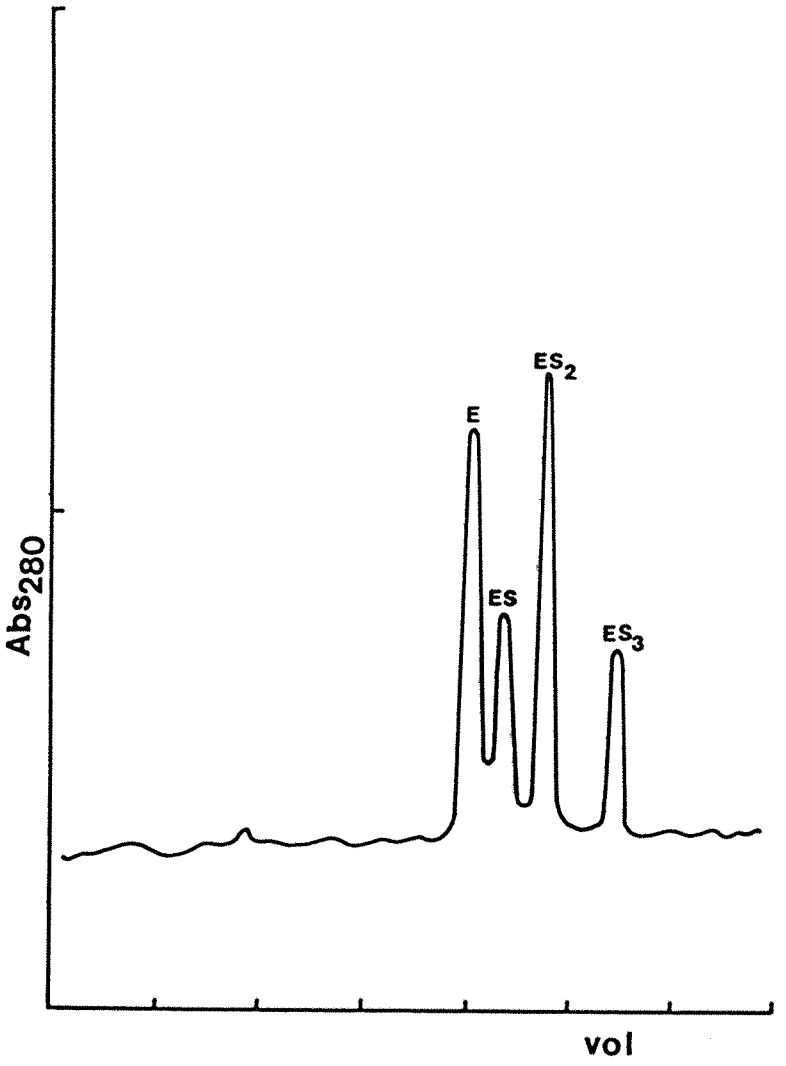


Figure 5.3

Effect of temperature on the stability of the isolated enzyme-intermediate complexes. Each individual enzyme complex was incubated at either 37°C or 60°C prior to reanalysis of the f.p.l.c. profiles.

b) ES_2 complex profile after incubation at 37°C for 15 minutes

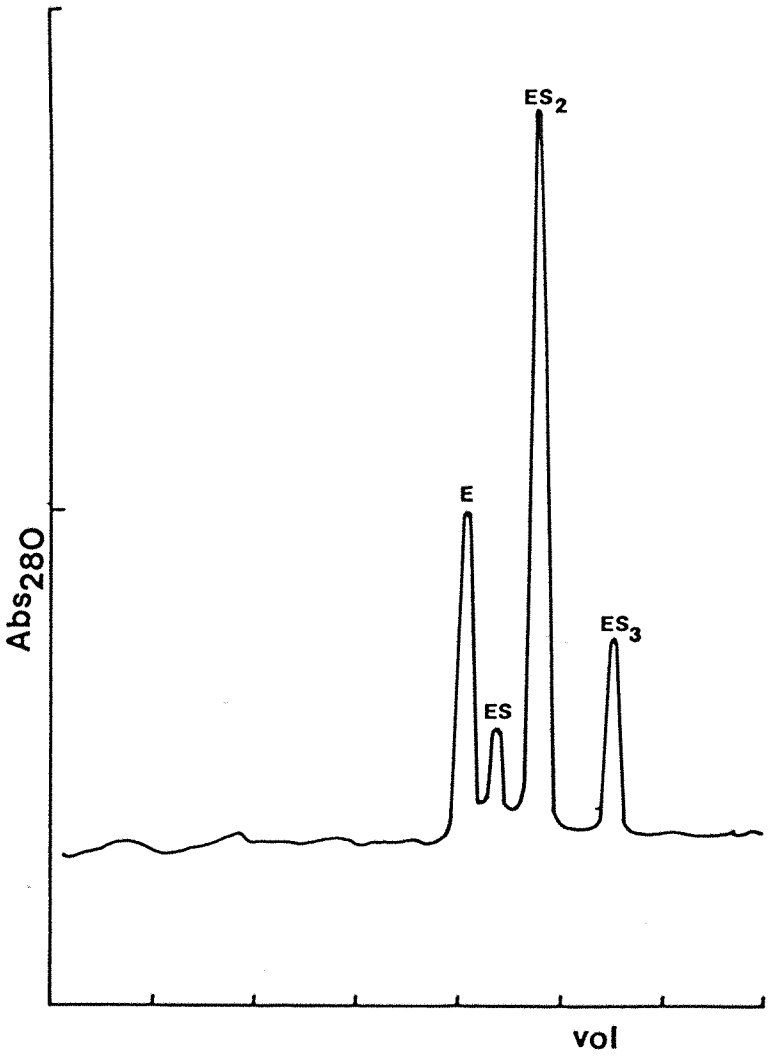


Figure 5.3

Effect of temperature on the stability of the isolated enzyme-intermediate complexes. Each individual enzyme complex was incubated at either 37°C or 60°C prior to reanalysis of the f.p.l.c. profiles.

c) ES_3 complex profile after incubation at 37°C for 15 minutes

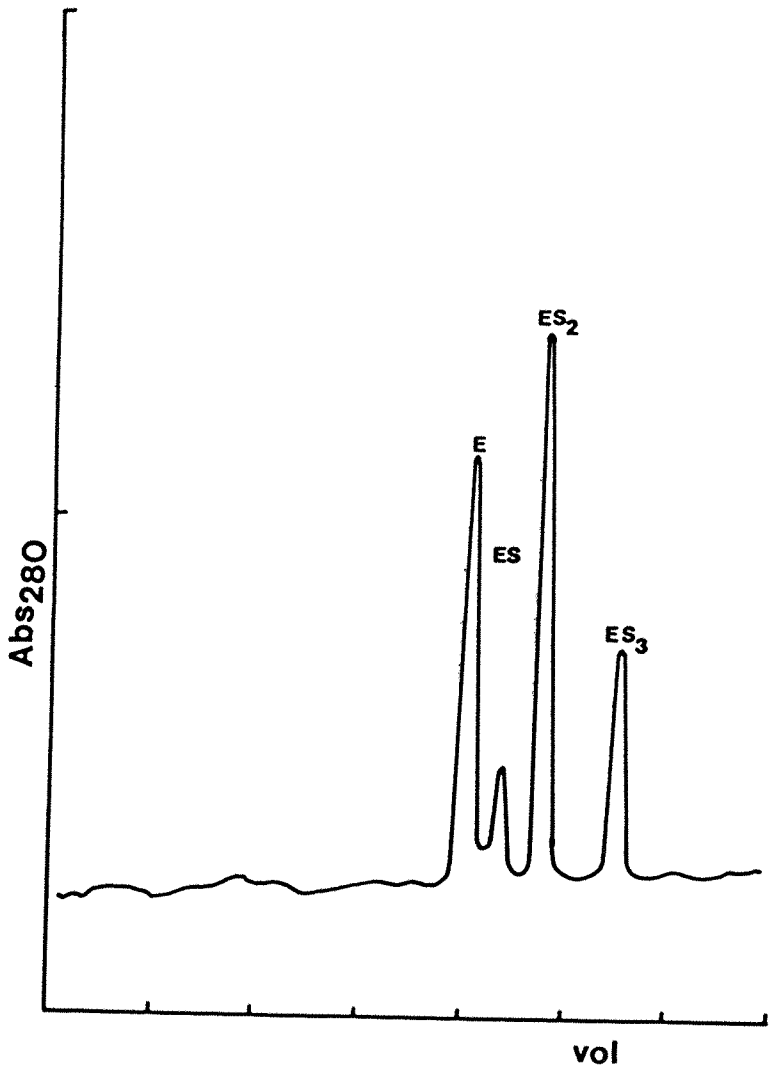


Figure 5.3

Effect of temperature on the stability of the isolated enzyme-intermediate complexes. Each individual enzyme complex was incubated at either 37°C or 60°C prior to reanalysis of the f.p.l.c. profiles.

d) profile of any enzyme-intermediate complex after incubation at 60°C for 15 minutes

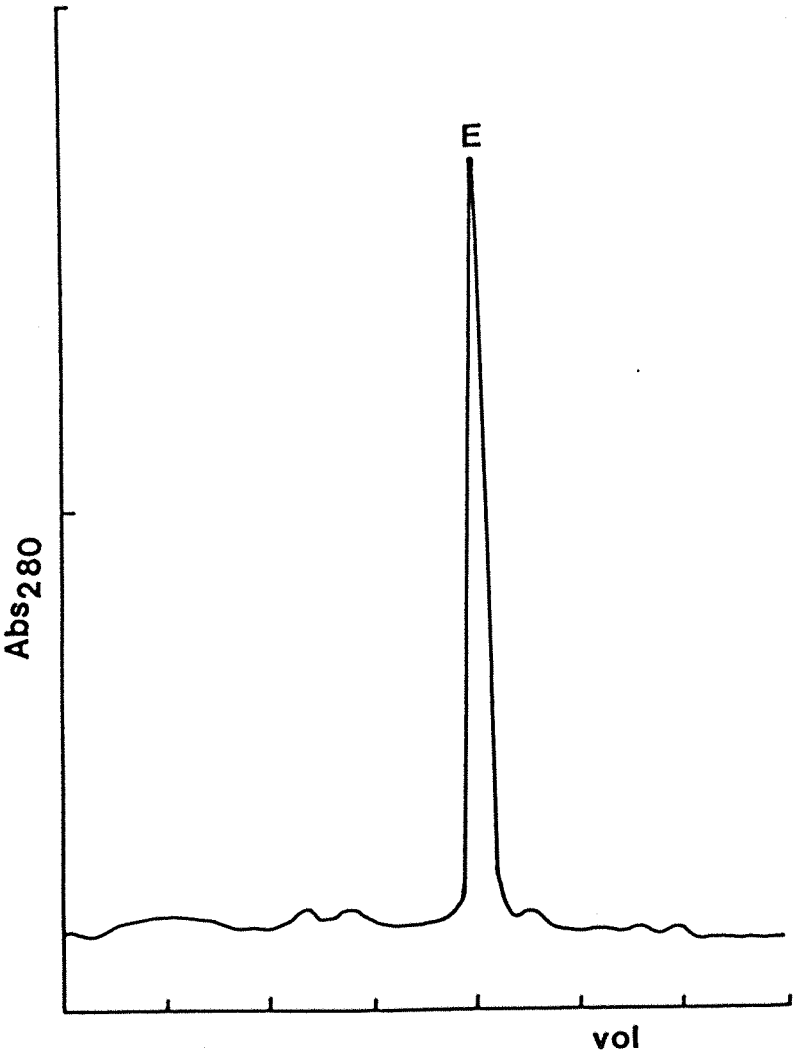


Figure 5.4

Non-denaturing polyacrylamide gel of the isolated enzyme-intermediate complexes. Lanes 1, 2 & 6 contain enzyme (E) alone (10μg); lanes 3 to 5 contain 10μg of ES, ES_2 and ES_3 respectively.

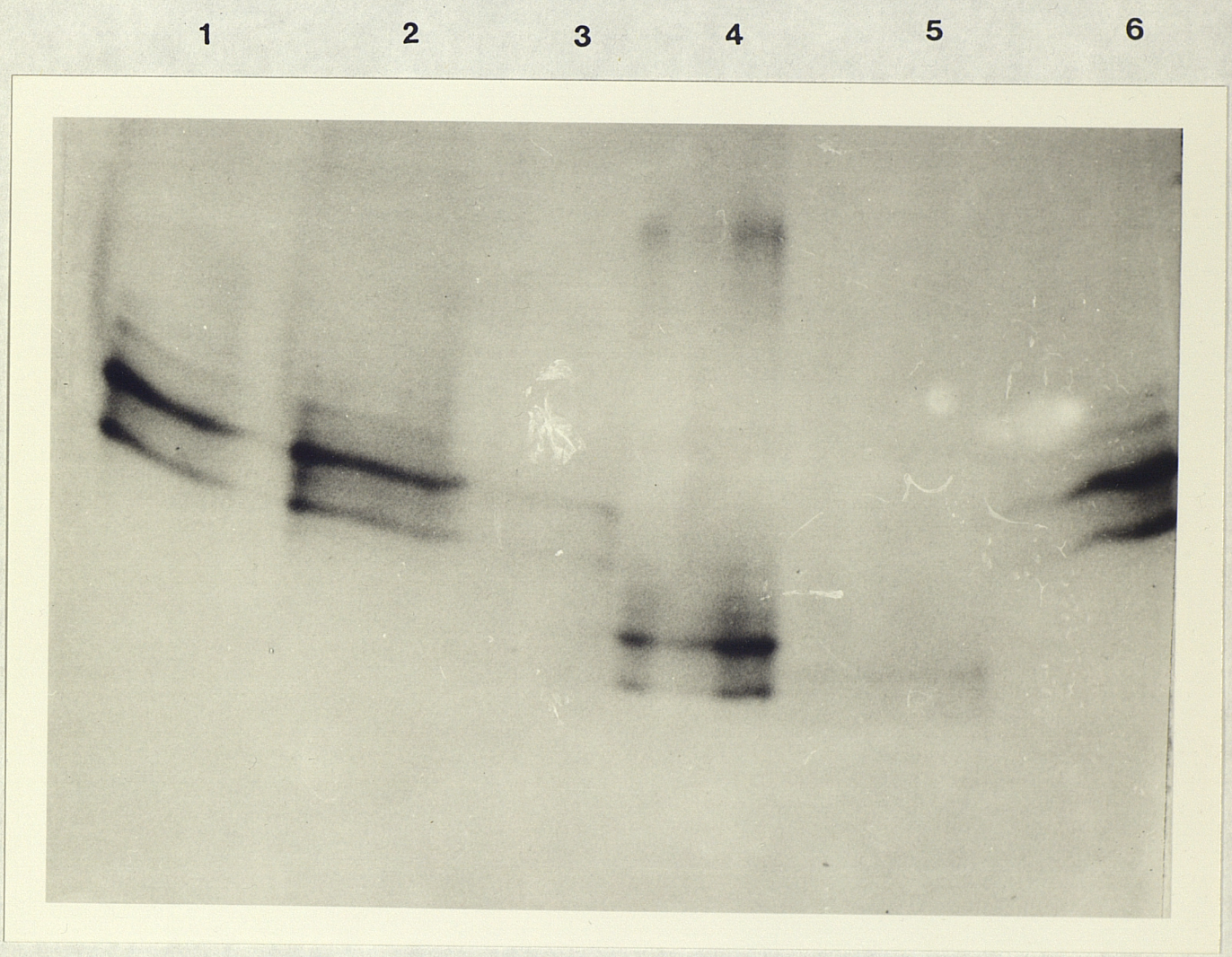
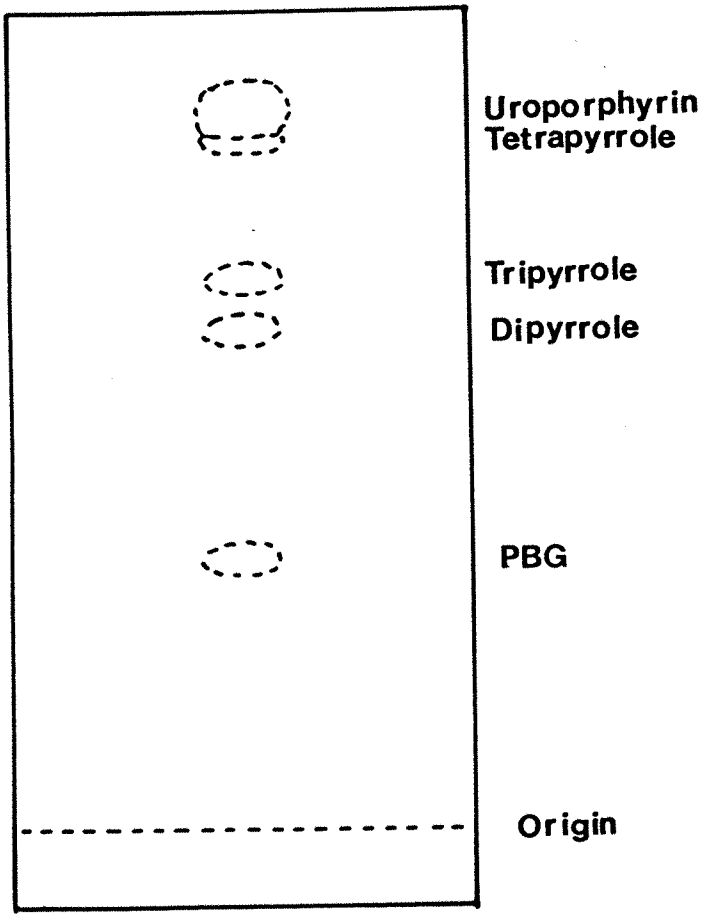


Figure 5.5

Thin layer high voltage electrophoresis of the released enzyme intermediates. Porphobilinogen deaminase (0.5 units) was incubated with 0.2M hydroxylamine in the presence of 100nmoles of substrate for 30 minutes. The base substituted released intermediates were isolated as described in the methods section and identified by spraying the plate with Ehrlich's reagent.



released radiolabelled moiety corresponded to the mono, di and tripyrrole adduct respectively.

5.2.4 Study of the exchange of the pyrrole rings of the porphobilinogen deaminase-intermediate complexes using regiospecifically labelled complexes.

The aforegoing discussion in section 5.2.3 highlights that the interconversion of the enzyme-intermediate complexes in the absence of added porphobilinogen must involve the release of pyrrole residues from one intermediate followed by their reaction with free enzyme or with another intermediate complex. In order to establish the mechanistic course of these interconversions and to investigate the the nature of this exchange process, ES, ES_2 and ES_3 were prepared regiospecifically labelled at the terminal pyrrole position by titration of the enzyme, ES and ES_2 with limiting [^{14}C]-porphobilinogen (S*) to yield ES*, ESS* and ESSS* respectively. This was carried out at 4°C to prevent any unwanted exchange of label. The labelled complexes thus generated were each independently incubated at 37°C for 15 minutes in the absence of porphobilinogen, the resulting enzyme intermediate complexes were then re-isolated by f.p.l.c. and their radioactivity was determined (Table 5.2). After incubation of ES* at 37°C for 15 minutes the radioactivity was found in all three complexes. The ESSS* terminal pyrrole was also labile but not to the same extent found for ES*. The most stable complex, ESS*, showed far less loss of label as compared to labelled ES* and ESSS*. The conclusion from these experiments is that at 37°C all the complexes are able to release single pyrrole units although the degree of reactivity varies according to the complex. The free pyrrole is then able to participate as a "substrate" in a reaction with other existing enzyme intermediate complexes or with free enzyme, ultimately generating a mixture in which ES_2 predominates followed by ES_3 and ES. Reaction of a liberated pyrrole moiety with ES_3 generates ES_4 which is rapidly released from the enzyme to generate the product, the hydroxymethylbilane, preuroporphyrinogen. This latter reaction pulls the equilibrium to the right leading ultimately to the conversion of all enzyme bound pyrroles to product and the regeneration of free enzyme. This is particularly rapid at 60°C. Since ammonia, the product of the deamination, is not present in the incubation, the back reaction to reform porphobilinogen is not possible, and the pyrrole unit must therefore be liberated by hydration of a reactive enzyme bound species to yield the hydroxy-derivative of porphobilinogen (Scheme 5.2). The hydroxy porphobilinogen has already been shown to act as an alternative substrate (Battersby et al, 1979 a). The exclusive formation of uroporphyrin I suggests that the reactions which occur are all mediated by the catalytic machinery of the deaminase enzyme.

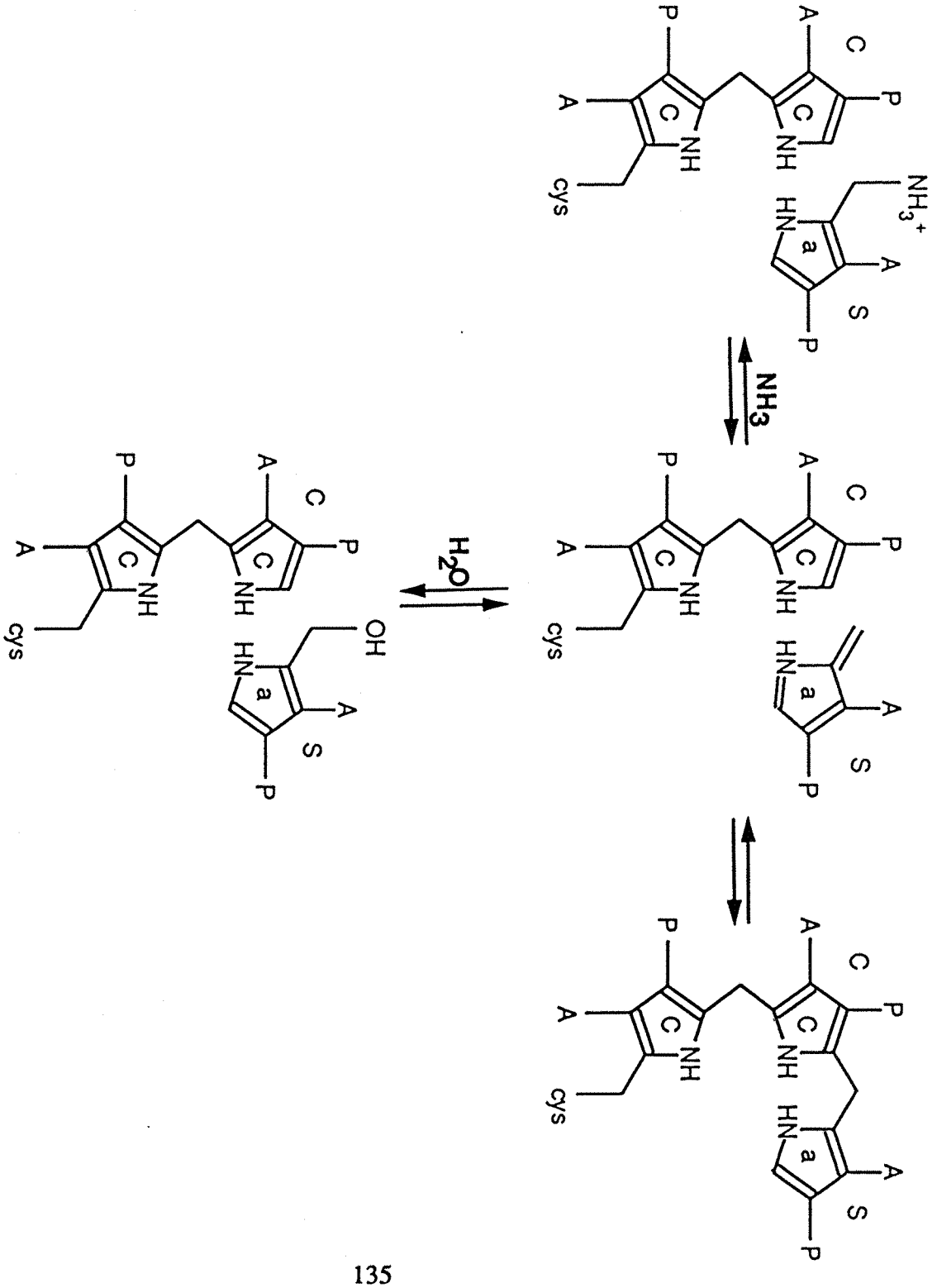
TABLE 5.2

Exchange of the terminal [^{14}C]-substrate in enzyme intermediate complexes. Enzyme intermediate complexes carrying a terminal [^{14}C] "substrate" were prepared as described in the methods section. Complexes ES*, ESS* and ESSS* were individually incubated at 37°C for 15 minutes, after which each was analysed by f.p.l.c. followed by radioactive determination of [^{14}C] label in the resulting complexes. Molar ratios of [^{14}C] : protein were determined for all the complexes (in initial [^{14}C] : protein ratios = 1 : 1).

Intermediate complex	% radioactivity in complexes and product after heating to 37°C				ratio of [^{14}C]:protein after incubation at 37°C		
	ES	ES_2	ES_3	S_4	ES	ES_2	ES_3
ES*	22	40	28	10	1.00	2.07	3.08
ESS*	9	54	22	15	0.40	1.01	1.51
ESSS*	6	31	20	43	0.31	0.42	0.74

SCHEME 5.2

The enzymic conversion of porphobilinogen into hydroxyporphobilinogen.



5.2.5 Susceptibility of enzyme-intermediate complexes to reaction with sulphydryl reagents.

Sulphydryl reagents have been shown to inhibit porphobilinogen deaminase from several sources implying that a cysteine residue may play an important role in the enzyme reaction (Russell et al, 1980; Anderson & Desnick, 1980). N.m.r. studies have also pointed to the involvement of a cysteine in the functioning of the enzyme from R. sphaeroides (Evans et al, 1986).

The native E. coli deaminase is remarkably unreactive to sulphydryl reagents such as p-chloromercuribenzoate, N-ethyl maleimide and 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB) and only high concentrations of these reagents caused enzyme inactivation (Table 5.3). However, in marked contrast, when the reaction of these reagents with enzyme-intermediate complexes was investigated the situation was completely different. For instance, when the ES complex was exposed to N-ethyl maleimide, a time-dependent inactivation of the enzyme was observed. Addition of further substrate residues to give ES_2 and ES_3 resulted in an even more rapid inactivation of the enzyme with N-ethyl maleimide (Figure 5.6). DTNB also showed a greatly increased rate of reaction with the enzyme-intermediate complexes compared to the free enzyme (E) (Figure 5.7).

These observations led to the tentative conclusion that the stepwise addition of substrate molecules causes a conformational change which leads to the exposure of a sulphydryl group previously buried in the native enzyme. Since a considerable conformational change is envisaged during the stepwise addition of substrate molecules to the enzyme-bound cofactor, further study on this cysteine may provide valuable information about the mechanics of the polymerization process. This apparent enhancement of inhibition by mercurial compounds in the presence of substrate was also noticed by Russell and Rockwell (1980) on the deaminase isolated from wheat germ though the reactions in this case were not carried out on individual enzyme-intermediate complexes. They concluded that the apparent enhancement of inhibition by the substrate was either due to a conformational change which exposed a thiol group or that the porphobilinogen had activated the mercurial compound by forming a more reactive reagent. The fact that these workers also noticed the same enhancement of inhibition of the enzyme which had been isolated from a different source is, in itself, indicative that this particular thiol group may be intimately involved in the catalytic mechanism of all porphobilinogen deaminases.

5.2.6 Identification of the reactive cysteine group.

In an attempt to identify the cysteine group responsible for this inhibition an enzyme-intermediate complex mixture was reacted with [^{14}C] n-ethyl maleimide which led to an 80% inactivation of enzymic activity but also resulted in an uptake of 1.3 moles

TABLE 5.3

Effect of inhibitors on native porphobilinogen deaminase and on the enzyme intermediate complex ES_2. Inhibitors were incubated with the enzyme as described in the methods section. Where necessary, the inhibitor was removed by gel filtration prior to assay.

Inhibitor	Concentration	% inhibition	
		E	ES_2
p-Chloromercuribenzoate	5μM	17	35
	10μM	38	93
	100μM	100	100
N-Ethylmaleimide	1mM	11	45
	5mM	47	89
Pyridoxal-5-phosphate	1mM	15	22
	5mM	33	35
	10mM	58	60
Pyridoxal-5-phosphate (after reduction with sodium borohydride)	1mM	77	45
	5mM	81	55
	10mM	83	60
Phenyl glyoxal	1mM	0	0
	5mM	5	7
	10mM	25	23
Butanedione	1mM	0	0
	5mM	1	2
	10mM	5	4

Figure 5.6

Time course to show the effect of N-ethyl maleimide on the inactivation of native porphobilinogen deaminase and on the enzyme-intermediate complexes ES, ES_2 and ES_3. Reaction conditions are as described in the methods section. Native enzyme (o–o); enzyme-intermediate complexes ES (●–●); ES_2 (□–□); ES_3 (■–■)

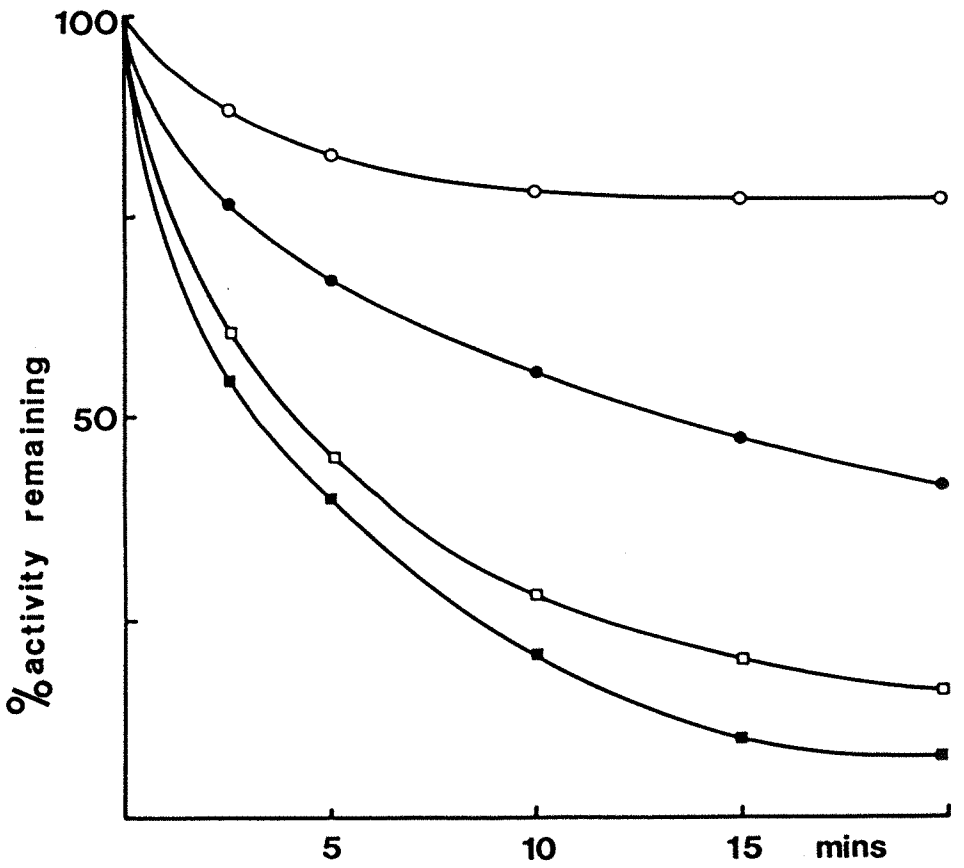
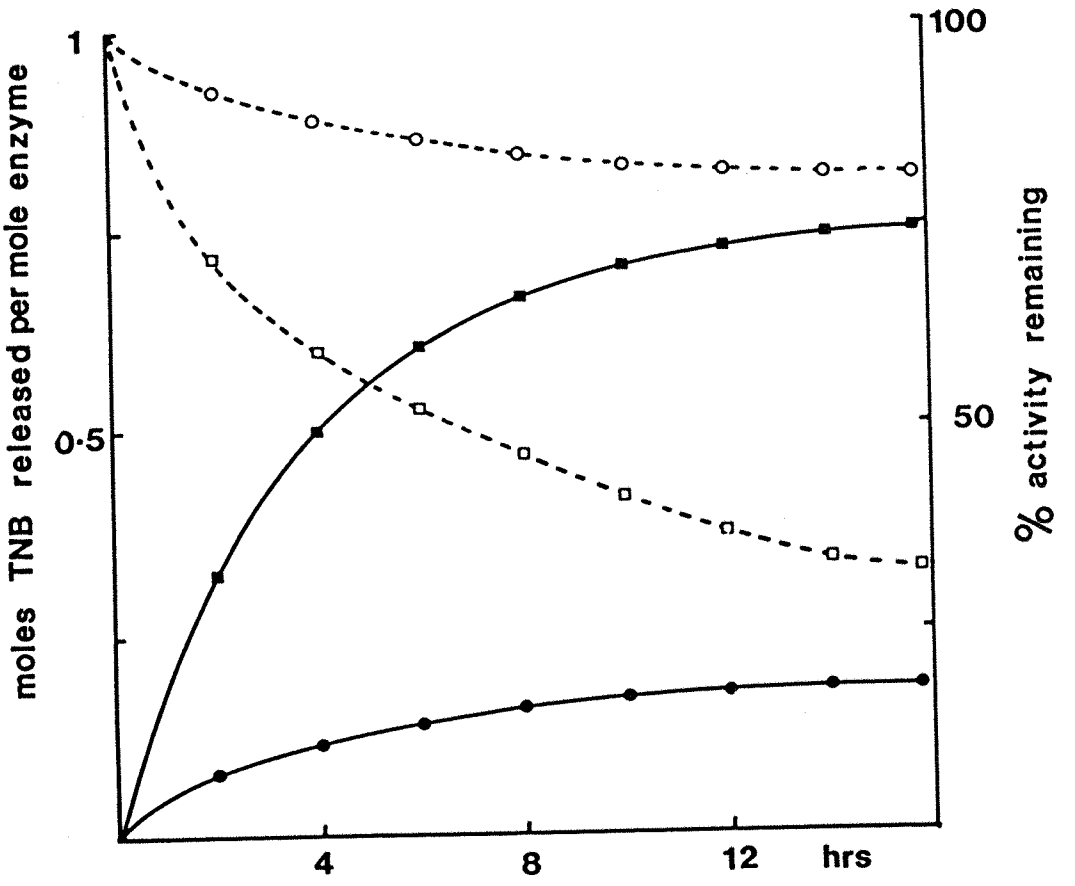


Figure 5.7

Reaction of DTNB with native porphobilinogen deaminase and enzyme-intermediate complex ES_2. Reaction conditions as described in the methods section. Inactivation of E (○---○) and ES_2 (□---□); moles of thionitrobenzoate (TNB) released by E (●——●) and ES_2 (■——■).



of n-ethyl maleimide per mole of protein. The protein was then subject to chemical cleavage of the single aspartic acid (D) / proline (P) linkage by treatment with formic acid as described in section 2.2.8. Analysis of the polypeptide fragments by SDS polyacrylamide gel electrophoresis revealed that two thirds of the counts were associated with the larger, 24,000 Daltons, molecular weight fragment and about one third was associated with the smaller, 11,000 Daltons, fragment (Figure 5.8). This result suggests that there is more than one reactive cysteine within the protein. Since there is only one cysteine group before the aspartyl/proline (position 104/105) linkage then it can be assumed that this group (cysteine 99) is capable of reacting with N-ethyl maleimide. In the larger fragment there are three other cysteine groups although one of these is responsible for cofactor binding (Section 4.2.4). In order to determine which of the cysteine groups, when modified, is responsible for the enhancement of inactivation of the deaminase a more thorough and rigorous investigation is required in the presence of the proper control experiments.

5.2.7 Reaction of *E. coli* porphobilinogen deaminase with pyridoxal-5-phosphate.

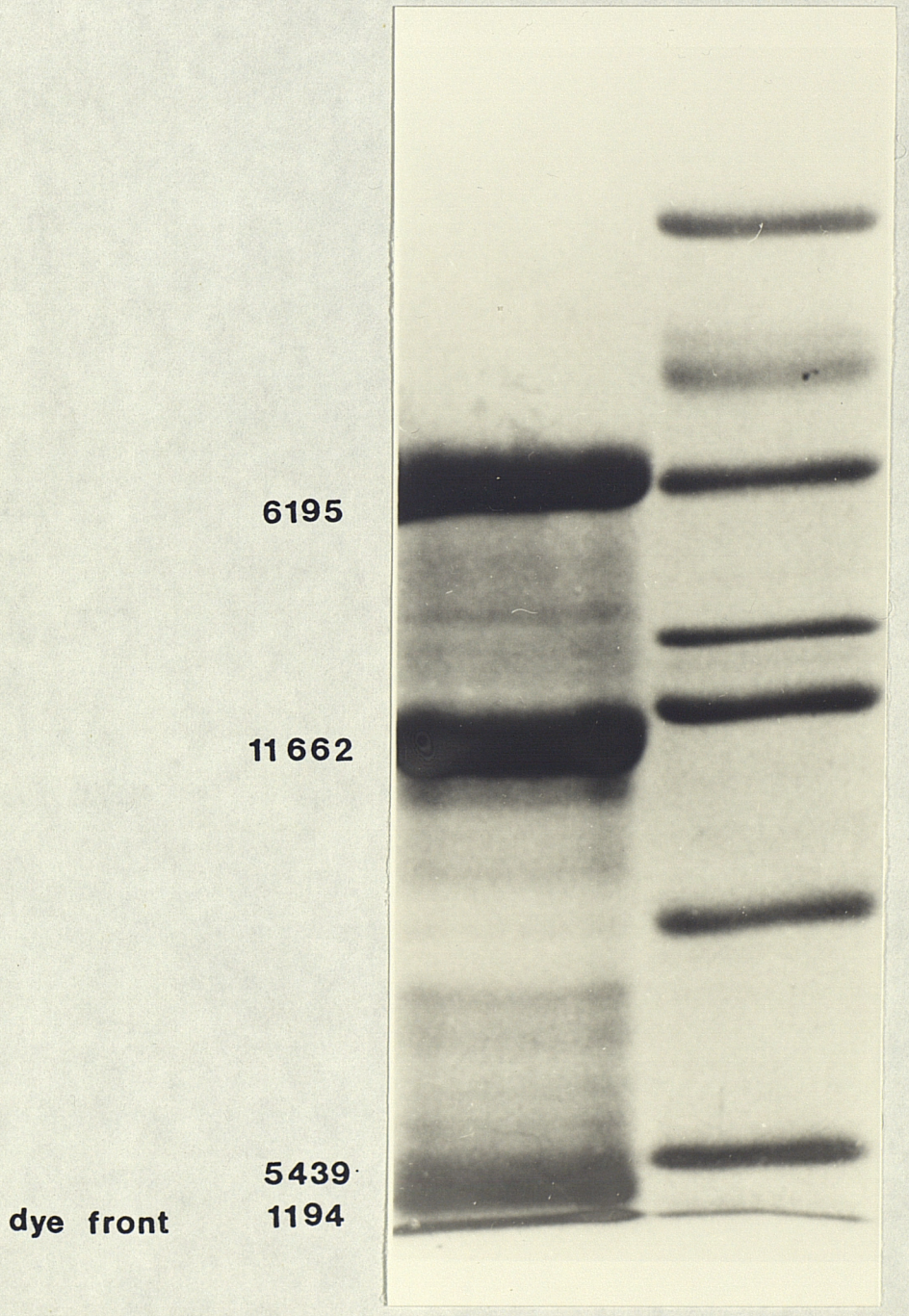
The nature of the active site group which is responsible for the covalent binding of the substrate and intermediates had been a matter of some controversy. From [^{13}C] n.m.r. studies the Euglena enzyme appeared to bind the substrate through a lysine residue. This work had been extended by studies with pyridoxal-5-phosphate which inactivates the deaminase enzyme irreversibly in the presence of sodium borohydride. When porphobilinogen is present, the enzyme is protected from inactivation. Together with the [^{13}C] n.m.r. studies this had been interpreted to indicate the involvement of lysine in a covalent linkage with the substrate.

The discovery that the active site of the porphobilinogen deaminase, not only from E. coli but all other deaminases (Warren and Jordan, 1988 a), contain the dipyrromethane cofactor which is responsible for the binding of the substrate is thus completely different from the conclusions made from the earlier studies on the Euglena enzyme and prompted the investigation of the possible involvement of lysine as an additional catalytically important group.

Accordingly, enzyme was reacted with pyridoxal phosphate in the presence of the substrate porphobilinogen, conditions under which only a marginal loss of enzyme activity occurs (Table 5.3). The enzyme was next reacted with sodium borohydride and the modified enzyme was treated with hydroxylamine to remove the protecting substrate. The enzyme was then reacted, in sequence, with [^{14}C]-pyridoxal-5-phosphate and sodium borohydride as described in the methods. A time dependant loss of activity occured accompanied by incorporation of [^{14}C] label into the deaminase protein. However, the loss of activity did not parallel the incorporation of label into the enzyme

Figure 5.8

Distribution of radioactivity in the peptides after formic acid cleavage of porphobilinogen deaminase that had been reacted with [^{14}C] n-ethyl maleimide. Porphobilinogen deaminase (1mg; 30nmoles) was reacted with a final concentration of 2mM n-ethyl maleimide prior to cleavage of the single aspartic acid / proline linkage by formic acid.



protein as expected for a group involved in the covalent attachment of the substrate (Figure 5.9). In fact incorporation of 1 mole of [^{14}C]-pyridoxal-5-phosphate led to a loss of only 60% of the enzyme activity. Had the modified lysine been involved in a covalent link with the substrate it is unlikely that any enzyme activity would have remained. If a lysine is involved its role is more likely to permit non-covalent interaction between enzyme and substrate. The fact that the substrate has negatively charged carboxyl groups demands that positive sites on the enzyme must be present to allow some form of ionic interaction in the recognition and binding of the substrate.

5.2.8 Identification of the reactive lysine group.

In an attempt to identify the lysine group responsible for this inhibition the labelled deaminase was subject to chemical cleavage with formic acid which breaks the single aspartic acid-proline linkage (position 104-105) in the protein. Analysis of the polypeptide fragments by SDS polyacrylamide gel electrophoresis revealed that the radioactivity was solely associated with the smaller (11,000 Dalton) amino terminal fragment (Figure 5.10). Since six out of the eight lysine residues in the E. coli primary structure are located in the amino-terminal portion of the protein this result was not surprising. The fact that the inhibition with pyridoxal phosphate is found in the deaminases isolated from Euglena and R. sphaeroides (Hart et al, 1984), wheat germ (Russell et al, 1984) and from E. coli (Warren and Jordan, 1988 b), it would seem most probable that the lysine responsible would be conserved between all deaminases. Comparison of the only two known deaminase protein sequences, the E. coli gene derived sequence (Thomas and Jordan, 1986) and the human cDNA derived sequence (Raich et al, 1986), revealed that even in the amino terminal portion of the two predicted structures there are five conserved lysine residues. Further investigations will be required to identify the individual lysine group responsible for the inactivation by pyridoxal phosphate.

5.2.9 Reaction of phenyl glyoxal and butanedione with the *E. coli* porphobilinogen deaminase.

The arginine specific reagents phenyl glyoxal and butanedione have been shown to inhibit the deaminase isolated from wheat germ (Pollack and Russell, 1978; Russell et al, 1984) in a similar manner to the inhibition noticed with pyridoxal-5-phosphate whereby the enzyme could be protected from inactivaction by the presence of substrate. However, when these reagents were incubated with the deaminase isolated from E. coli only a very slight inhibition of enzyme activity could be detected (Table 5.3). This result certainly suggests that there is no readily accessible arginine group in the E. coli deaminase that is involved in the mechanism of the enzyme.

Figure 5.9

The uptake of pyridoxal-5-phosphate and inactivation of the deaminase. Porphobilinogen deaminase (1mg; 30nmoles) was reacted with a final concentration of 2mM pyridoxal phosphate over a period of 5 minutes. At one minute intervals a sample of the reaction was reduced with sodium borohydride and the uptake of [^{14}C] label and enzyme activity were recorded.

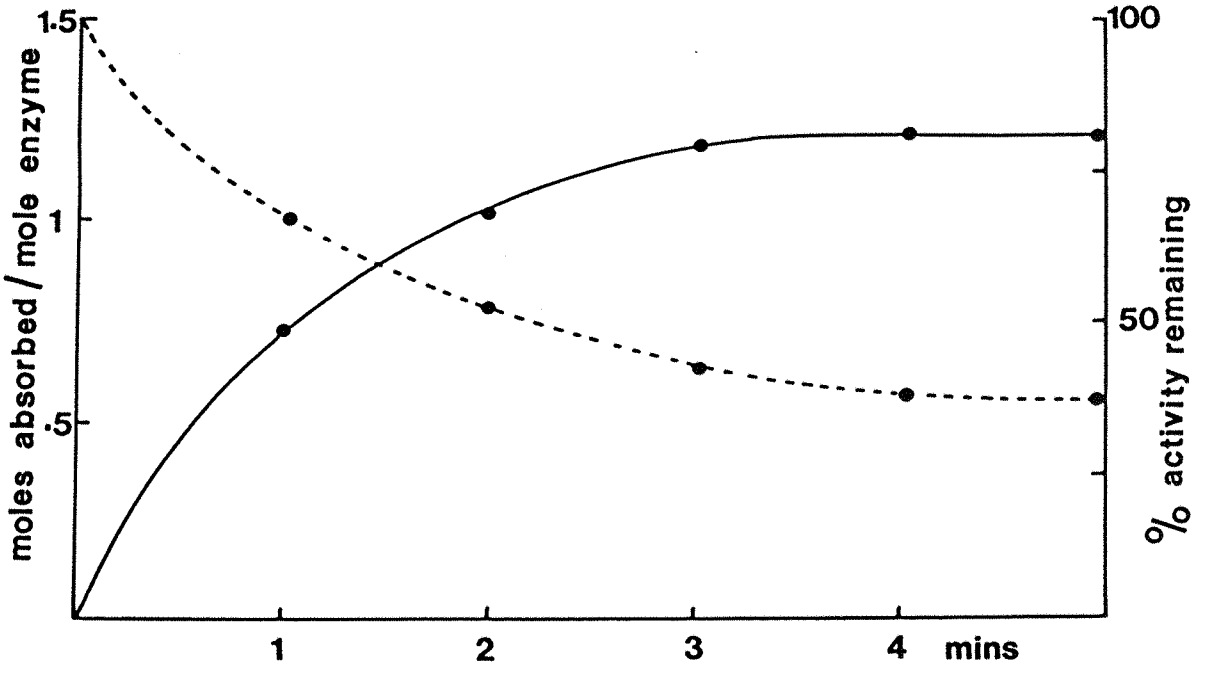
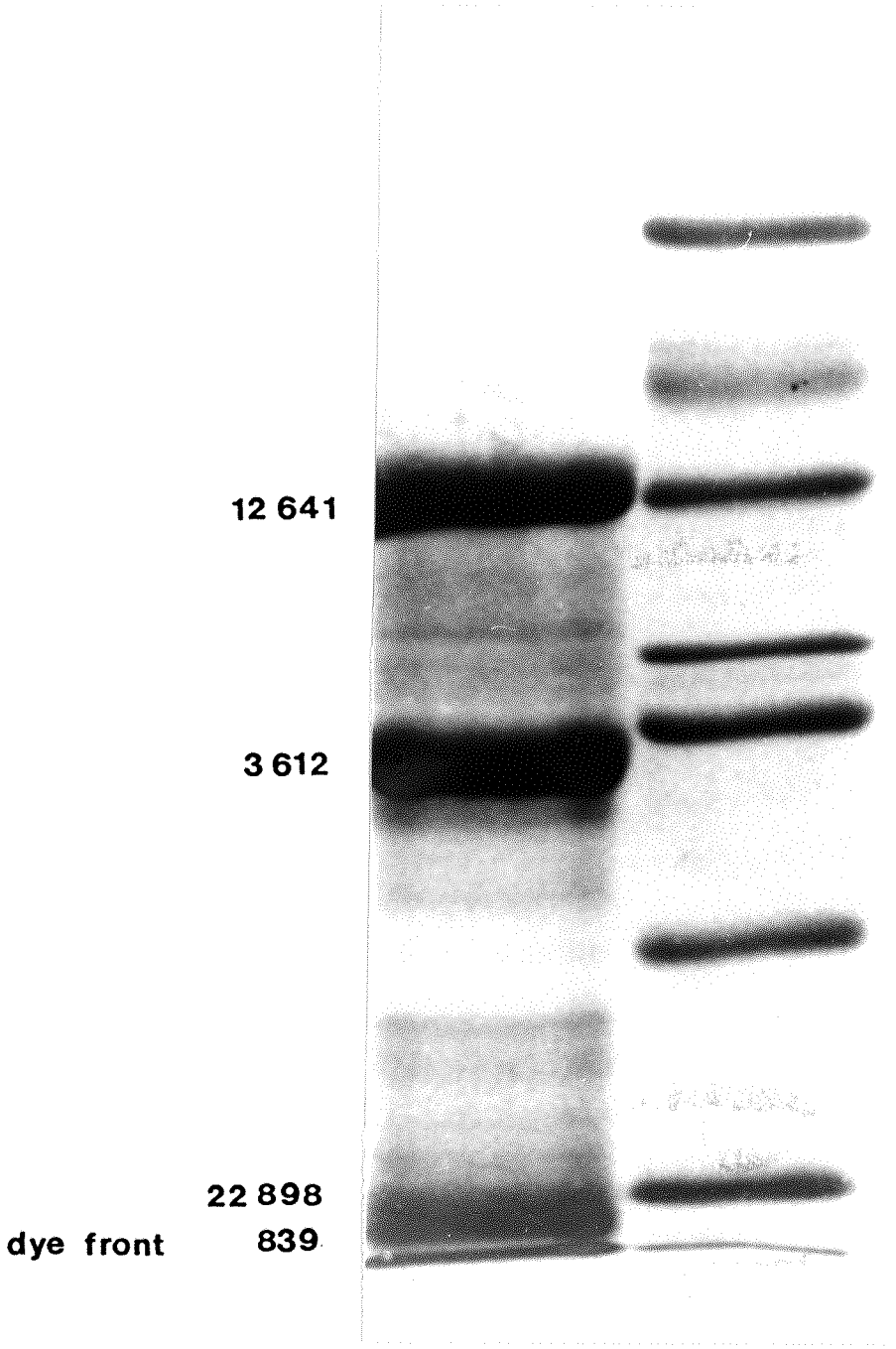


Figure 5.10

Distribution of radioactivity in the peptides after formic acid cleavage of porphobilinogen deaminase that had been reacted with [^{14}C] pyridoxal-5-phosphate and reduced with sodium borohydride.



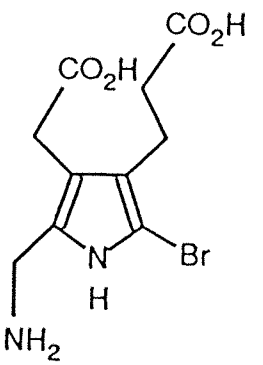
5.2.10 Reaction of 2-bromoporphobilinogen with porphobilinogen deaminase.

The effect of substrate analogues on the activity of porphobilinogen deaminases has been studied extensively as a means to obtain information relating to the mechanism of the enzyme. To this aim workers have synthesized porphobilinogen analogues which differ from the true substrate in the constituents of the acid side chains and in the amino side arm (Wilen et al, 1984). It was found that the analogues did not act as substrates for the enzyme but did, to varying degrees, act as competitive inhibitors. No studies of substrate analogues with a blocked α-position have been reported. Such compounds should act as potent inhibitors of the enzyme since, in the light of the recent developments regarding the action of the dipyrromethane cofactor, the α-position of both the cofactor and the substrate molecule have been implicated in the mechanism of the enzyme itself.

2-Bromoporphobilinogen is closely related to the substrate and differs only in having bromine at the key reactive α-position (Figure 5.11). Bromoporphobilinogen would be expected to bind to the deaminase and form a covalent complex similar to that of the substrate, however the blocked α-position would be expected to prevent the binding of further substrate molecules. As expected, incubation of enzyme with bromoporphobilinogen (25μM) (kindly given to us by Prof. A. I. Scott, Texas A & M University) led to a rapid inactivation of the enzyme within 5 minutes (Table 5.4). The substrate, porphobilinogen, (0.3mM) dramatically reduced the rate of inactivation. This inhibition was hypothesised as being due to the formation of an enzyme-bromoporphobilinogen (EB) complex which, with the presence of a bromine atom at the α-position of the intermediate complex, would be inactive since it would not be able to react with the next incoming substrate molecule. This hypothesis was confirmed when it was found that the deaminase-bromoporphobilinogen complex could be isolated by f.p.l.c. were it chromatographed in a similar but slightly different position to the ES complex (Figure 12, a). Bromoporphobilinogen (B) also reacted with ES and ES_2, yielding ESB and ES_2B as judged by f.p.l.c. analysis (Figure 5.12, b and c). All these complexes were enzymically inactive. Reaction of ES_3 with bromoporphobilinogen completed the turnover yielding bromopreuroporphyrinogen and enzyme, the latter which then reacted with more bromoporphobilinogen to form the enzyme-bromoporphobilinogen complex (Figure 12, d). Bromoporphobilinogen thus appears to act as a suicide inhibitor, which is first recognised by the catalytic site as a substrate and where it is deaminated and then coupled to the enzyme or to an intermediate complex. Once bound however the presence of the bromine atom at the α-position blocks further reaction both with substrate or with additional bromoporphobilinogen residues, leaving the enzyme in an inactivated state. However it is interesting that heating the inactivated

Figure 5.11

Structure of 2-bromoporphobilinogen



Bromoporphobilinogen

TABLE 5.4

Effect of bromoporphobilinogen on the activity of porphobilinogen deaminase.

Inhibitor (pre-incubated for 5 mins with enzyme)	Concentration	% inhibition of enzyme (substrate utilization)
Bromoporphobilinogen	2.5μM	90
Bromoporphobilinogen	25.0μM	96
NH_2OH	0.1M	19
Bromoporphobilinogen + 0.1M NH_2OH	2.5μM	29
Bromoporphobilinogen (with no pre-incubation)	2.5μM	9

Figure 5.12

Reaction of 2-bromoporhobilinogen with porphobilinogen deaminase and enzyme intermediate complexes. F.p.l.c. analysis of enzyme or enzyme- intermediate complexes after reaction with 2-bromoporphobilinogen.

a) Enzyme (E) + bromoporphobilinogen

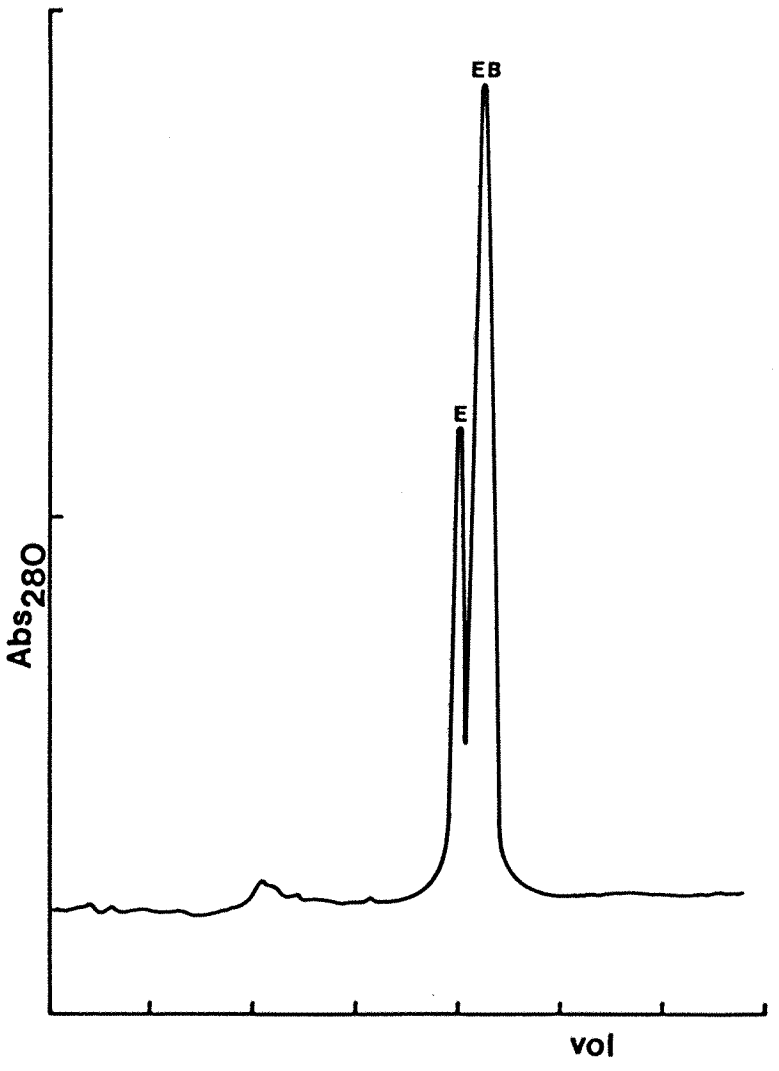


Figure 5.12

b) ES + bromoporphobilinogen

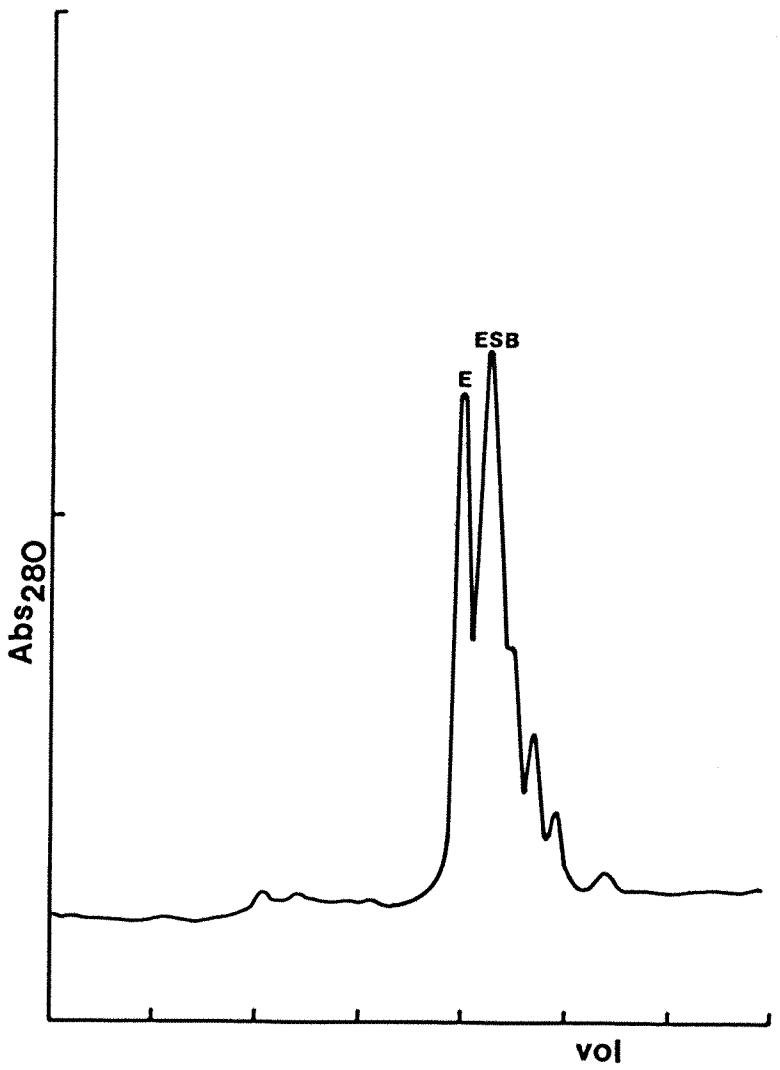


Figure 5.12

c) ES_2 + bromoporphobilinogen

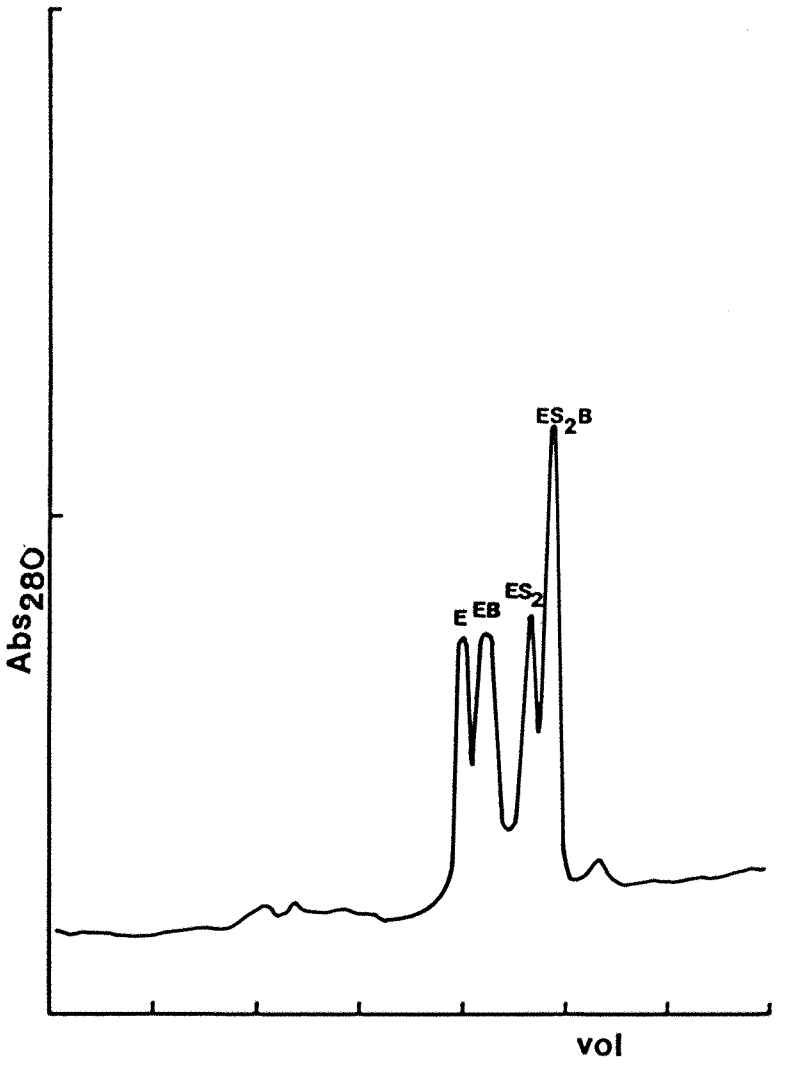
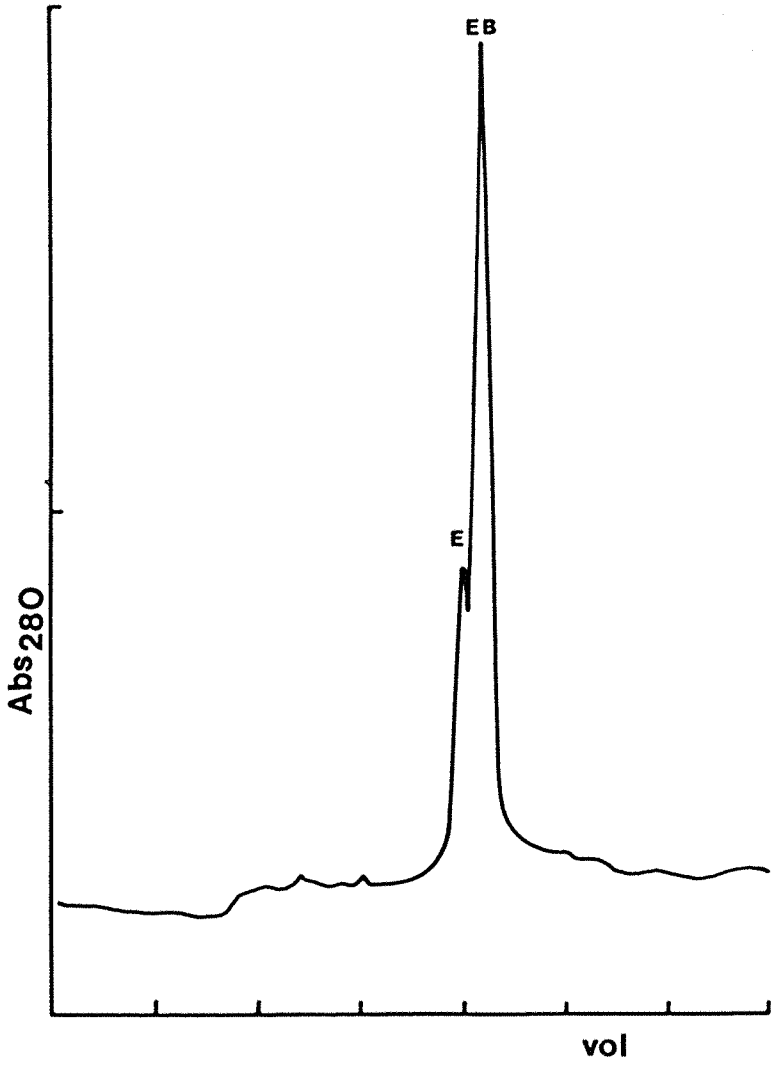


Figure 5.12

d) ES_3 + bromoporphobilinogen



species (EB, ESB, ESSB) or treatment with 0.2M hydroxylamine causes the liberation of the bound bromo-intermediates leading to complete restoration of enzyme activity (Table 5.4). The enzyme, therefore, does not appear to be alkylated or permanently destroyed by the bromoporphobilinogen.

5.2.11 Reaction of the enzyme-bromoporphobilinogen complexes with Ehrlich's reagent.

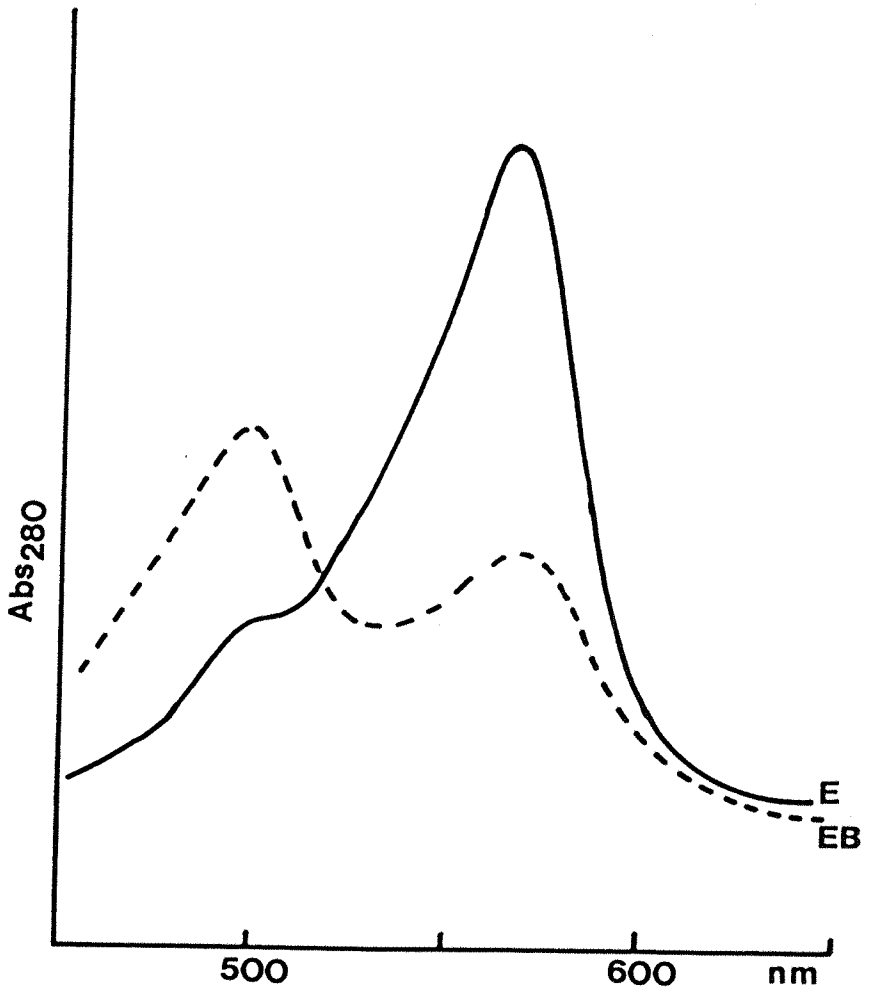
The native porphobilinogen deaminase gives a characteristic Ehrlich's positive reaction due to the resident dipyrromethane cofactor. Since the bromoporphobilinogen reacts with the enzyme by a similar mechanism to the substrate, prior exposure of the enzyme, or enzyme intermediate complexes, to bromoporphobilinogen would be expected to block the reaction with Ehrlich's reagent due to the absence of a free α-position in EB, ESB or ES_2B.

When Ehrlich's reagent was added to enzyme that had been treated with bromoporphobilinogen, the development of the characteristic pink colour was almost completely inhibited. The spectra in Figure 5.13 (a and b) shows the reaction of enzyme with Ehrlich's reagent compared to the reaction of Ehrlich's reagent with the enzyme-bromoporphobilinogen complex. The same result was obtained for ESB and ES_2B where the Ehrlich's reaction was also inhibited.

These observations provide unequivocal evidence that bromoporphobilinogen, an α-substituted substrate analogue, reacts with the enzyme in a similar manner to the substrate but instead of making an active enzyme-intermediate complex it forms a "dead end" inhibitor-enzyme complex. The inhibition of the Ehrlich's reaction with the enzyme-bromoporphobilinogen complex not only demonstrates that the cofactor is directly involved in the sequential binding of the substrate during the catalytic reaction but that the free α-position of the terminal pyrrole in the growing polypyrrole chain is essential for further elongation.

Figure 5.13

Reaction of Ehrlich's reagent with native enzyme (E) and with enzyme-bromoporphobilinogen complex (EB). The reactions and f.p.l.c. conditions are as described in the methods section. The EB complex contained approximately 10% of free enzyme.



CHAPTER 6

Summary and General Discussion.

In 1983, in the conclusion to his thesis, Alan Berry prophecised that the information required to complete our understanding of the mechanism of porphobilinogen deaminase would result only from the application of the modern techniques of molecular biology and recombinant DNA technology to the problem. The realisation of this foresightedness came to fruition after the hemC gene was isolated, cloned and sequenced by Thomas and Jordan (1986). This cloning work gave rise to the formation of recombinant E. coli porphobilinogen deaminase over-expression systems which produced up to a hundred times the amount of the deaminase compared to that found in the wild type E. coli. In fact the wild type E. coli strain only produced around 30μg of deaminase per litre of cell culture whereas the genetically engineered E. coli strain (ST1048) produced about 3mg of the enzyme per litre of cell culture. With the use of this over-expression system and the knowledge of the gene derived protein primary structure of the deaminase it was hoped that it would be possible to gain a more thorough insight into the mechanism of this remarkable enzyme. Of particular interest would be to determine the number of substrate binding sites, the group responsible for the covalent attachment of the first pyrrole substrate molecule, catalytically important amino acids and an insight as to how the enzyme regulates the size of the polypyrrolic product to a tetramer.

The development of a procedure for the purification of the porphobilinogen deaminase from this over-producing strain of E. coli (chapter 2) therefore represented the first steps towards the complete characterisation of the enzyme. The purification protcol was based on methods that had been used in the past for the purification of deaminases from other sources but also employed the use of an f.p.l.c. system using a high resolution ion-exchange Mono Q column to ensure the complete removal of any impurities. The purified E.coli porphobilinogen deaminase was found to have a native molecular weight of 32,500 Daltons when it was determined by gel filtration and a molecular weight of 35,000 Daltons when the enzyme was subject to denaturing electrophoresis. These values are in close agreement with the predicted, gene derived, molecular weight of 33,857 Daltons for the "apo" deaminase and are in the same range of molecular weights that have been found for all other deaminases so far isolated. The purified E.coli protein did, however, show some form of heterogeneity as it existed in at least five forms that could be separated by high resolution ion exchange chromatography and three forms that could

be separated by non-denaturing gel electrophoresis. These forms were not enzyme-intermediate complexes and since all the species were of the same molecular weight it was unlikely that they represented proteolytic degradation products. The most likely explaination is that these forms contain different modified amino acids within the primary structure caused, possibly by deamidation of asparagine residues. No heterogeneity has been observed with other purified deaminases although the same heterogeneity within the E. coli enzyme has been noticed by Scott and co-workers (Scott, personal communication).

Sequencing of the amino-terminus of the purified protein by a pulsed liquid sequencing method revealed, for the first time, that the amino terminal amino acid was methionine and was therefore in complete agreement with the predicted primary structure data afforded by Thomas and Jordan (1986). Likewise the complete amino acid analysis of the protein as well as the chemical cleavage experiments lended further support to the gene derived data. The physical properties of the protein, the K_m, the pI value and the pH optimum were found to be of a similar order to those found with other deaminases although the specific activity of the enzyme was found to be lower.

The crystallization of the deaminase represents a major achievement in itself as it is only the second enzyme of the porphyrin biosynthetic pathway to be crystallized. Although the crystals that were obtained grew to sizes approaching 1mm in length it was disappointing that the deformities within the crystal structure did not permit the collection of good diffraction data. The fact that crystals were obtained is encouraging and it will probably only take a slight alteration in the crystallization conditions to permit the growth of single well formed crystals.

A direct consequence of both the initial characterisation of the protein and of the crystallization studies was the observation that there was a novel component associated with the protein. This had become obvious during a scan of crystallization conditions when it was noted that deaminase samples left to crystallize below pH 4 turned a red colour. In experiments involving the proteolytic degradation of porphobilinogen deaminase from R. sphaeroides it had also been noticed that a red chromophore appeared (Berry, 1983). It was also noted that samples of the E. coli enzyme, when treated with strong acid, gave rise to the formation of uroporphyrin. The tentative conclusion from these initial observations was that the enzyme contained a bound dipyrrole molecule. A dipyrrole molecule either by itself or when fragmented into individual pyrrole units could give rise to porphyrin formation and could also, when oxidised and protonated, give rise to a red chromophore. As a dipyrrole can be made from two molecules of the substrate, porphobilinogen, it was thought that the presence of a dipyrrole could have arisen from a bound enzyme-intermediate complex. However, this theory had already been dispelled because of the conclusive studies done earlier on the enzyme

heterogeneity which very clearly showed that the purified enzyme contained no bound substrate. At this stage it was argued that if the enzyme contained some pyrrole moiety then the enzyme itself should react with modified Ehrlich's reagent. The reaction of Ehrlich's reagent with mono, di and tetrapyrromethane molecules has been well documented and it had been shown that they all gave different reaction rate profiles and show characteristic spectra (Mauzerall & Granick, 1956; Pluscec & Bogorad, 1970; Radmer & Bogorad, 1972). Reaction of the porphobilinogen deaminase enzyme itself with Ehrlich's reagent was indeed found to give an Ehrlich's positive reaction with a spectrum characteristic of a dipyrromethane. This therefore confirmed the earlier suspicion that the enzyme did, in fact, contain a dipyrromethane molecule and the structure of this compound was further substantiated by n.m.r. studies. The next question therefore related to the function of this dipyrromethane prosthetic group since presumeably there had to be a function for this molecule.

One hypothesis for the function of the dipyrrole was that it could act as the attachment site for the binding of the substrate molecules to the enzyme. This idea was confirmed when it was demonstrated that the enzyme-intermediate complex consisting of the enzyme with two bound substrate molecules (ES_2) exhibited a tetrapyrromethane (bilane) reaction with Ehrlich's reagent. Further evidence for the direct covalent interaction of the substrate with the dipyrromethane cofactor was provided by the experiments with bromoporphobilinogen. This substrate analogue differs only from the substrate in having a bromine atom at the α-position thus making the molecule unreactive towards Ehrlich's reagent. Reaction of bromoporphobilinogen with the deaminase forms an enzyme-bromoporphobilinogen complex which, on reaction with Ehrlich's reagent, no longer gives the typical dipyrromethane reaction. The universal nature of this cofactor was demonstrated when the deaminase from two plant sources and a mammalian source were also shown to contain the dipyrromethane compound.

Further evidence regarding the nature, origin and activity of the cofactor came from labelling experiments with 5-aminolaevulinic acid and investigations with a hemA^- mutant. These experiments confirmed that the porphyrin precursor 5-aminolaevulinic acid is essential for the formation of catalytically active porphobilinogen deaminase. Growth of a hemA^- E. coli mutant in the absence of 5-aminolaevulinic acid resulted in an inactive deaminase whereas addition of 5-aminolaevulinic acid to the growth medium resulted in a normal deaminase level similar to wild type levels. The requirement for 5-aminolaevulinic acid relates directly to its incorporation into porphobilinogen and thence into the dipyrromethane cofactor at the catalytic site of the deaminase. This assertion was confirmed by the specific incorporation of [^{14}C] label into the cofactor when the E. coli was grown on 5-amino[5-^{14}C]laevulinic acid. Subjection of the [^{14}C] labelled enzyme to catalytic turnover with the substrate porphobilinogen, did not result in the loss of any

radioactive label from the enzyme thus confirming that the dipyrromethane was acting as a true cofactor and was not subject to turnover.

Application of this labelling approach to the use of [^{13}C] labelled 5-aminolaevulic acid led to the incorporation of [^{13}C] label into the dipyrromethane cofactor and to the unambiguous determination of the structure of the cofactor by [^{13}C] n.m.r. spectroscopy. The structure of the cofactor was confirmed as a dipyrromethane by this procedure and four resonances, at δ=128.5ppm, δ=117ppm, δ=25.6ppm and δ=24.7ppm, were obtained indicating that the cofactor was made up of two linked pyrrole rings each derived from porphobilinogen. The chemical shift data also indicated that one of the pyrrole rings of the cofactor is covalently linked to the deaminase enzyme through a cysteine residue. Comparison of the predicted primary structure of both the E. coli and the human enzymes reveals that there are only two conserved cysteines between the sequences. Since the human enzyme has been shown to contain the dipyrromethane cofactor as well as the E. coli enzyme then one of these two conserved cysteine residues must be responsible for the attachment of the cofactor. By isolating a formic acid peptide from a sample of [^{14}C] cofactor-labelled deaminase it has been possible to predict that it is cysteine 242 of the E. coli enzyme that is responsible for the cofactor binding.

Studies with purified enzyme intermediate complexes with one, two and three pyrrole units bound covalently to the enzyme have revealed that all three complexes, when incubated in the absence of substrate, are able to participate in enzyme catalysed exchange reactions involving the terminal pyrrole unit. This was demonstrated by the use of regiospecifically labelled enzyme-intermediate complexes in which the terminal pyrrole group of the complex had been labelled with [^{14}C]. The terminal pyrrole unit is removed as the hydroxy-form of the substrate which, as a good substrate itself, can react with any existing intermediate complex. The ultimate result is that the intermediate complexes are transformed into the hydroxymethylbilane product, preuroporphrinogen. From investigations with the enzyme intermediate complexes it can be concluded that the slowest reaction in the absence of porphobilinogen is that in which ES_2 is converted into ES_3. This may be due to a conformational adjustment in the enzyme structure in order to accommodate additional porphobilinogen units. The exposure of a previously unreactive cysteine residue on binding substrate provides preliminary experimental evidence for the occurrence of such a conformational change.

These cumulative results allow us to propose a mechanism for the reaction of the deaminase enzyme. This involves a single active site which is able to catalyse two basic reactions, firstly the deamination of the substrate and the condensation of the deaminated intermediate with either the dipyrromethane cofactor or an enzyme-intermediate complex and secondly, the addition of water to yield a hydroxymethyl product. The latter activity is normally confined to the release of the tetrapyrrole product

but in the absence of substrate any of the enzyme-intermediate complexes can be similarly cleaved albeit at a slow rate. These proposals are summarised in scheme 6.1.

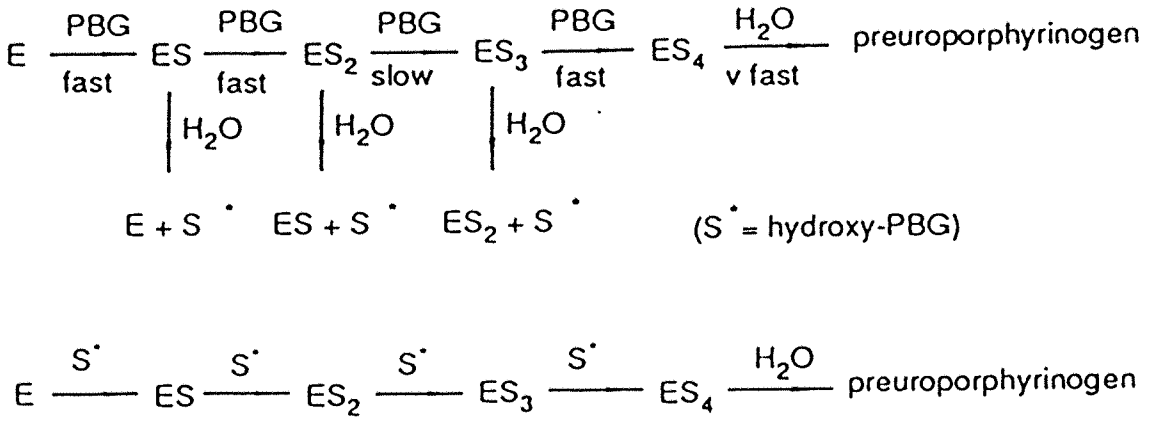
The topography of the catalytic centre may thus be envisaged as having two pyrrole recognition sites, one for the incoming substrate, site S, and the other which can accommodate a pyrrole ring with a free α-position in readiness for the reaction with the substrate at site S. In the native holo-enzyme this site is occupied by the dipyrromethane cofactor and has been designated as site C. Scheme 6.2 outlines the stepwise addition of the four substrates in the assembly of the tetrapyrrole preuroporphyrinogen. The first substrate (ring a) occupies the S site and reacts with the dipyrromethane cofactor which acts as a "primer" for the reaction. The resulting cofactor complex is then translocated at the active site so that the newly bound pyrrole unit (ring a) now occupies the C site. The translocation may occur as a result of the binding of porphobilinogen which may have a higher affinity for the S site than the newly linked pyrrole unit. In the absence of further substrate the pyrrole ring bound in the ES complex remains in the S site and can be released by addition of water. In the presence of substrate the normal deamination occurs at the S site resulting in the formation of ES_2 followed by ES_3 and finally ES_4. At this stage it is envisaged that steric considerations prevent the binding of a fifth substrate and that hydrolytic cleavage, which is normally far slower than the condensation reaction, becomes significant. This results in the liberation of the hydroxymethylbilane product, preuroporphyrinogen. To achieve this the ES_4 complex must relocate at the active site so that ring a is in the S site. This steric effect which prevents any further polymerisation is born out when a space filling model of the tetrapyrrole attached to the two pyrrole rings of the cofactor is made (Figure 6.1). This model shows that if the cofactor is fixed to the protein via an immobile side chain of an amino acid such as cysteine then the flexibility of the dipyrromethane molecule itself allows only enough room for the construction of a tetrapyrrole. The only requirement for the liberation of the tetrapyrrole would be that the a ring of the ES_4 complex occupies the S site. It is well established that the enzyme-bound tetrapyrrole exists in this form since incubation of deaminse with substrate in the presence of ammonia leads to the production of aminomethylbilanes of the type NH_2APAPAPAP (Radmer and Bogorad, 1972). Furthermore, the enzyme is also able to deaminate the aminomethylbilane NH_2APAPAPAP to the hydroxymethyl equivilent. Studies with isomeric bilanes show that the deamination obligatively requires the a ring to be in the AP sequence.

The function of the cofactor is thus twofold, firstly to act as a primer for the reaction and secondly to impose a steric restriction on the number of pyrrole rings which can be incorporated to four. This latter property may be the reason for the fact that the cofactor is permanently linked to the deaminase protein.

There would appear to be subtle differences the in the mechanism of the

SCHEME 6.1

Mechanistic course of events of enzyme-intermediate complexes incubated at 37°C in the absence of further substrate.



SCHEME 6.2

Postulated mechanism for porphobilinogen deaminase using two pyrrole binding sites (S site and C site) and one active site.

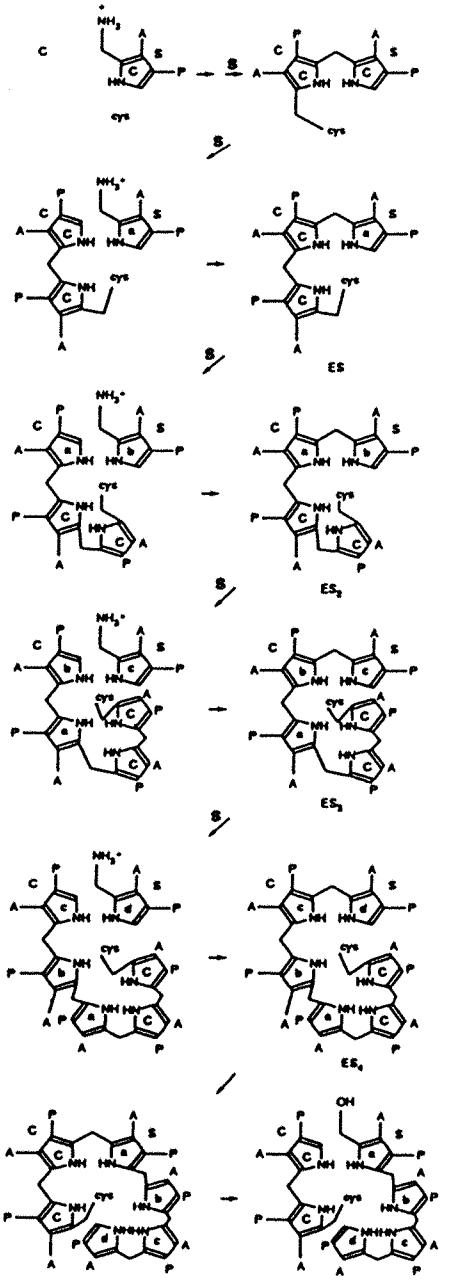
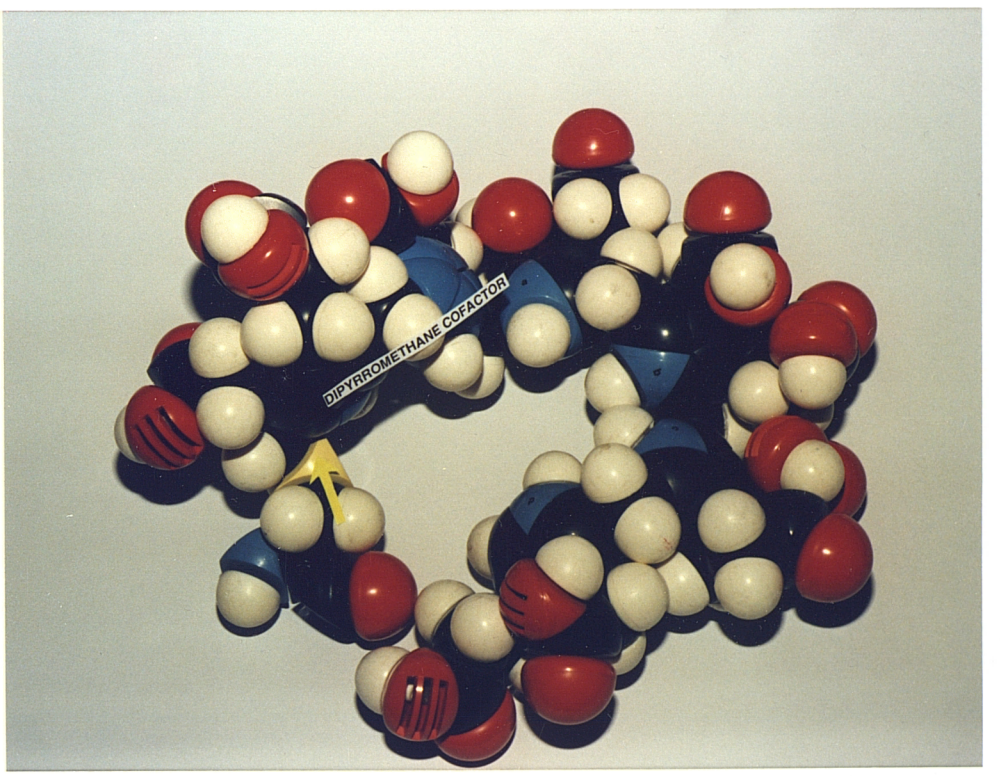


FIGURE 6.1

Space filling model of the dipyrromethane cofactor with four substrate pyrrole rings attached. The second cofactor pyrrole ring is shown in blue and the cofactor itself is shown covalently attached to cysteine-242.



deaminase between the enzyme isolated from eukaryotic sources and from the enzyme isolated from procaryotic sources. Both the enzymes from R. sphaeroides and from E. coli form three stable enzyme intermediate complexes, ES, ES_2 and ES_3 but not a stable ES_4 complex. Out of the complexes formed the ES_2 and the ES_3 appear to be more stable than the ES form. On the other hand, the human enzyme forms all four complexes with the ES form appearing to be the most stable (Anderson & Desnick, 1980). Evidence for an ES_4 complex with the Euglena enzyme has come from indirect studies with preuroporphyrinogen (Battersby et al, 1983). During the isolation of the human enzyme in section 3.2.8 it was found that the human deaminase eluted from the final f.p.l.c. stage in two peaks as had been previously reported for this enzyme (Anderson & Desnick, 1980; Brown et al, 1984). Both these peaks were reacted with Ehrlich's reagent and were found to give slightly different spectra (Figure 6.2, a and b). The first peak gave the normal dipyrromethane reaction as seen with the E. coli enzyme but the second peak gave a spectrum intermediate between the reaction observed for the dipyrromethane reaction and the tetrapyrromethane reaction. This suggested that the second human enzyme form was the ES form and since it had been heat treated during the isolation of the enzyme it therefore represented a much more stable ES form compared to the E. coli enzyme. Thus the eukaryotic enzyme appears to form a very stable complex with the first substrate molecule and also forms a comparatively stable ES_4 complex form which releases the bound tetrapyrrole at a much faster rate in the presence of further substrate molecules (Anderson & Desnick, 1980; Battersby et al, 1983). It is interesting that the molecular weight of the enzymes with these latter properties are about 40,000 Daltons and have an additional protein sequence near the C-terminus. This additional sequence may well act to stabilise the ES_4 complex.

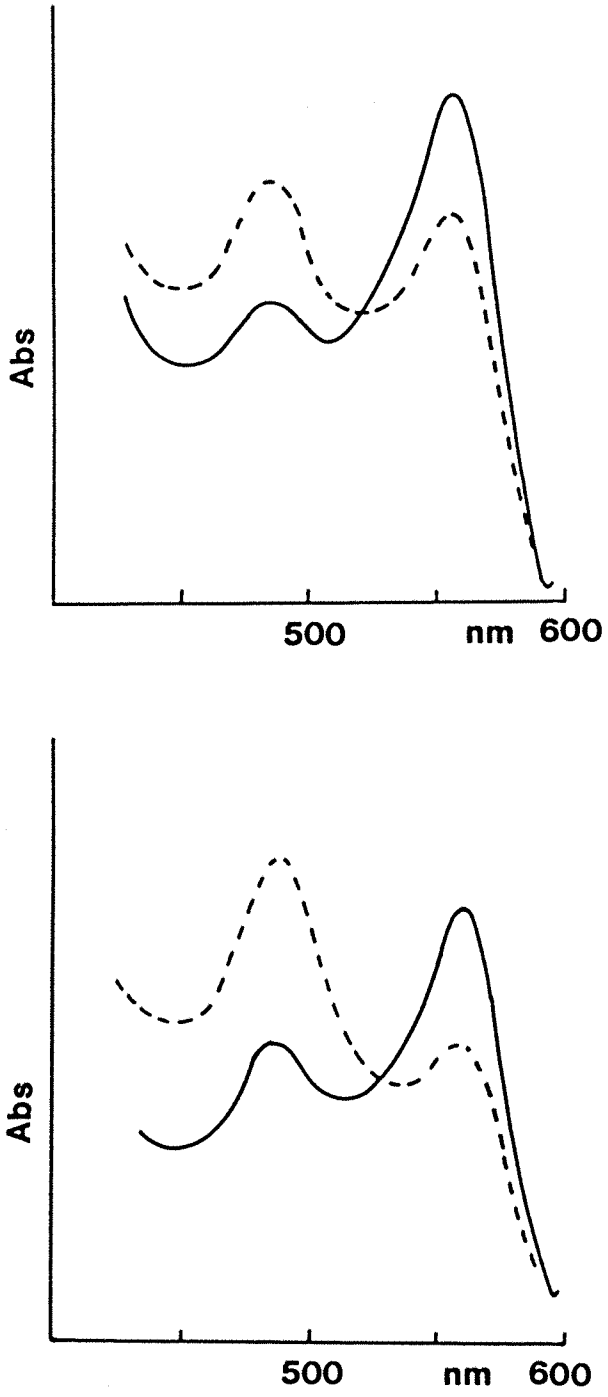
Although no information was gained about the crystal strucure of the deaminase a rough idea of the protein domain structure can be obtained from proteolytic digestion of the native enzyme. In comparison to the proteolysis of the denatured deaminase protein, cleavage of the native deaminase with trypsin is very slow and the sites of cleavage probably represent hinge regions joining different domains. The initial cleavage with trypsin (5% w/w) is very rapid leading to a total inactivation of the enzymic activity within 10 minutes and giving rise to two peptides of molecular weight 28,000 Daltons and 6,000 Daltons. The 28,000 Daltons fragment was still found to have the cofactor attached to it but was not capable of binding any substrate molecules. This first cleavage site was shown, by sequencing, to occur at lysine 64. If the deaminase was left overnight with trypsin then further proteolysis occured giving rise to peptides of molecular weight 17,000 Daltons, 15,000 Daltons and 10,000 Daltons as judged by SDS polyacrylamide gel electrophoresis. These further peptides were shown to arise from cleavage at lysine 158 and arginine 278. Thus it appears that there is one very susceptible region between a

FIGURE 6.2

Ehrlich's reaction with human erythropoeitic deaminase

a) peak 1 after 1 min (———) and five min (----).

b) peak 2 after 1 min (———) and five min (----).



possible N-terminus domain and the rest of the protein and, moreover, this N-terminus domain is essential for normal catalytic activity. Since this N-terminal domain does not contain the cofactor it may contain one of the pyrrole binding sites a suspicion which is further confirmed by the presence of five lysine groups within this small fragment. Three of these lysine groups are found to be conserved between the predicted primary structure of the human and E. coli enzymes. There is a substantial amount of evidence to suggest that a lysine is involved in a non-covalent manner in the enzyme mechanism and it will probably be shown that this reactive lysine group is situated in this N-terminal region.

The results of these cleavage experiments carried out on the native E. coli porphobilnogen deaminase also agree broadly with the computer predicted secondary structure of the enzyme (Figure 6.3).

The work described in this thesis has certainly advanced our knowledge on the mechanism of porphobilinogen deaminase but in attaining the answers to the pertinent mechanistic problems the work has only given rise to more questions relating to the mechanism of biosynthesis and incorporation of the cofactor. In an attempt to answer some of these new questions it has been suggested that the cofactor is required for a dual role, firstly as a primer to attach and anchor the growing polypyrrole chain and secondly to regulate the size of the growing polypyrrole chain so as it does not exceed the size of a bilane. Experimental evidence has shown that the cofactor is biosynthesised from the porphyrin precursors 5-aminolaevulinic acid and porphobilinogen but only preliminary evidence as to how the dipyrrole molecule is made from porphobilinogen or how the cofactor is inserted into the protein was obtained. The evidence would, however, appear to suggest that there is only one logical route leading to the construction and insertion of the dipyrromethane cofactor.

This route would require that the newly biosynthesised "apo" porphobilinogen deaminase protein could react with the first two molecules of porphobilinogen it comes into contact with in the normal catalytic manner giving rise to a dipyrrolic-enzyme bound species. This dipyrrole would be bound covalently to the enzyme through a cysteine group and the stable nature of the interaction would mean that it could not dissociate from the protein. This idea would mean that porphobilinogen deaminase would have three catalytic activities, as a deaminase, as a polymerase and as the cofactor synthase. However, on the experiments carried out on the "apo" deaminase isolated from the hemA$^-$ strain it was found that incubation of the cell extract with either porphobilinogen or with 5-aminolaevulinic acid only gave rise to a small (15%) amount of new deaminase activity.

It could be argued that the cofactor may be inserted by another enzyme. This is unlikely as hemC mutants have not been mapped anywhere other than the hemC gene. However it makes logical sense for the deaminase to synthesise its own cofactor since

FIGURE 6.3

Secondary structure prediction of the *E. coli* porphobilinogen deaminase by the method of Garnier. Symbols used in the semi-graphical representation:
Helical conformation: X Extended conformation: -
Turn conformation: > Coil conformation: *

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          10         20         30         40         50
           ¦          ¦          ¦          ¦          ¦
MLDNVLRIATRQSPLALWQAHYVKDKLMASHPGLVVELVPMVTRGDVILD

XXXXXX------***XXXXXXXXXXXXXXX***X---------------

XXXXXX------***XXXXXXXXXXXXXXX***X---------------

          60         70         80         90         100
           ¦          ¦          ¦          ¦          ¦
TPLAKVGGKGLFVKELEVALLENRADIAVHSMKDVPVEFPQGLGLVTICE

-XXX--X>X-X-XXXXXXXXXXXXXXXXXXX>*-----X>>>*------
-XXX--X>X-X-XXXXXXXXXXXXXXXXXXX>*-----X>>>*------

         110        120        130        140        150
           ¦          ¦          ¦          ¦          ¦
REDPRDAFVSNNYDSLDALPAGSIVGTSSLRRQCQLAERRPDLIIRSLRG

>XXXXXX--->>>>-----X--------*>>------->XX>------>>
>XXXXXX--->>>>-----X--------*>>------->XX>------>>

         160        170        180        190        200
           ¦          ¦          ¦          ¦          ¦
NVGTRLSKLDNGEYDAIILAVAGLKRLGLESRIRAALPPEISLPAVGQGA

>-------*>*XXXXXXXXXXXXXXXXXXX----****------X----
>-------*>*XXXXXXXXXXXXXXXXXXX----****------X----

         210        220        230        240        250
           ¦          ¦          ¦          ¦          ¦
VGIECRLDDSRTRELLAALNHHETALRVTAERAMNTRLEGGCQVPIGSYA

------X>>*XXXXXXXXXXXXXXXXXXXXXXXXXXX>----------
------X>>*XXXXXXXXXXXXXXXXXXXXXXXXXXX>----------

         260        270        280        290        300
           ¦          ¦          ¦          ¦          ¦
ELIDGEIWLRGLVGAPDGSQIIRGERRGAPQDAEQMGISLAEELLNNGAR

--XXXXXX----****>*------>>>****XXXXXXXXXXXXXXXXXXX
--XXXXXX----****>*------>>>****XXXXXXXXXXXXXXXXXXX

         310
          ¦
EILAEVYNGDAPA

X------>>>**XX
X------>>>**XX
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the formation of both the cofactor and product fall in the same generic class of reactions, i.e. deamination and condensation with a nucleophile (either the α-position of a pyrrole or a S^- from cysteine-242.

The next few years will undoubtedly see a foccussing of attention not only on the bioynthesis and insertion of the dipyrromethane cofactor but also on the crystal structure of the deaminase itself. It is, after all, only from the crystal structure of the protein that many of the definative answers to the mechanism will arise.

Chapter 7

Experimental Procedures.

7.1 Materials.

Porphobilinogen was a generous gift from Mr A. Alwan. [3,5,-$^{14}C_2$]Porphobilinogen (11mCi/mmol) was obtained from this department and was prepared as described in (Williams, 1985). 2-Bromoporphobilinogen and 5-amino[5-^{13}C]laevulinic acid were a generous gift from professor A. I. Scott, Texas A & M University, U.S.A. 5-Amino[5-^{14}C]laevulinic acid (49mCi/mmol) was purchased from New England Nuclear. [^{14}C]N-Ethyl maleimide was purchased from Amersham International, Buckinghamshire, England, and also supplied a trial synthesis sample of [4,5-$^{14}C_2$]pyridoxal phosphate (1.93mCi/mmol). Trizma-base, CHES, Coomassie brilliant blue, SDS-7 protein molecular weight standards and antibiotics were obtained from Sigma Chemical Company, Poole, Dorset. Sephacryl S-200, Phenyl Sepharose, PD-10 columns and all f.p.l.c. apparatus were obtained from Pharmacia Fine Chemicals, E. Molesy, Surrey. DE-52 was obtained from Bio-Rad. Tryptone and yeast extract were from Difco Laboratories, Surrey. All other chemicals were supplied by B.D.H., Poole, Dorset, and were of the best grade obtainable.

Spinach was obtained, fresh, from the green grocers and was purchased immediately prior to use. A crude barley chloroplast fraction was a generous gift of Dr C. G. Kannangara of the Carlsberg Laboratory, Copenhagen.

E. coli strains HB101/pBR322, ST1046, ST1047 and ST1048 were obtained from this department and were prepared as described by (Thomas, 1986). All strains were sub-cultured every six months and were stored at 4°C. Bacteria were grown aerobically in one litre batches in two litre flasks of Luria broth supplemented with glucose (1g/l) and ampicillin (30 ug/ml) from 10% innocculae. E. coli strains A1002(mel, ilv, lacI, metE) and A1004a (mel, ilv, lacI, metE, hemA) were a gift from Mr A. Smith, this department and were grown as above but using 2% glucose and under anaerobic conditions.

Plasmid isolation and bacterial transformations were carried out as described in Manniatis (1982).

The development of enzyme activity with bacterial growth was monitored by removing 20ml samples from the cultures. The optical density of the cultures was measured at 600nm to give an indication of the cell growth. The enzyme activity was measured after the cells had been centrifuged at 5,000g for 10 minutes and resuspended in 3mls of buffer and sonicated as described below.

In large scale preparations of the enzyme the bacteria were harvested by

centrifugation in a Sorvall RC 3B refrigerated centrifuge at 5000 r.p.m. for 15 minutes. The cells were then resuspended in a minimal volume of Tris/HCl buffer pH 8.2 and were either stored frozen at -20°C or were used immediately in the purification.

7.2 Preparation of modified Ehrlich's reagent.

Modified Ehrlich's reagent was freshly prepared by dissolving 1g of p-dimethyl amino benzaldehyde in 40mls of glacial acetic acid and 10mls of perchloric acid (60-62%).

7.3 Determination of porphobilinogen deaminase activity.

The enzyme activity was monitored by either one of two methods, namely, either by the disappearance of the substrate (porphobilinogen) or by measurement of the porphyrin formation after cyclisation and oxidation of the product. Each of these methods is described below:

(i) Disappearance of porphobilinogen. The enzyme was incubated in a final volume of 450µl of 0.1M Tris/HCl buffer pH 8.2 at 37°C. The reaction was started by the addition of 100nm of substrate. The assay was monitored by removing aliquots (40µl) at various time intervals and mixing the aliquot with 500µl of modified Ehrlich's reagent and 460µl of water. The amount of porphobilinogen remaining was determined from the optical density measurment recorded at 555nm after 15 minutes ($\varepsilon_{555} = 6.02 \times 10^4 M^{-1}cm^{-1}$)

(ii) Porphyrin formation. Enzyme was incubated in a final volume of 450µl of 0.1M Tris/HCl buffer pH 8.2 at 37°C. The reaction was started by the addition of 100nmoles of porphobilinogen. After a certain time, between 5 and 60 minutes, the reaction was stopped by the addition of 125µl of 5N HCl. This serves not only to precipitate the protein but also causes ring cyclisation of the enzymic product. Oxidation of the porphyrinogen to the porphyrin was enhanced by the addition of 50µl of benzoquinnone (0.1% w/v in methanol). After 20 minutes on ice in the dark, a 100µl aliquot was made up to 1ml with 1M HCl and its absorption at 405nm was recorded against a similar sample which had been stopped at zero time.

7.4 Purification of *E. coli* porphobilinogen deaminase.

Cells being used for the isolation of the enzyme were resuspended in 0.05M Tris/HCl buffer pH 8.2 to a final concentration of 50g of wet weight cells in 500 mls.

7.4.1 Sonication.

The resuspended cells were sonicated in 50ml portions in a M.S.E. ultrasonic disintegrator at an amplitude of between 6-8 microns for a total of 6 minutes in a pulsed fashion of 45 seconds on and off.

7.4.2 Heat treatment.

The extract from the sonicated cells was quickly heated to 60°C in a boiling water bath. The solution was constantly stirred and was maintained at 60°C for 10 minutes. After this time the solution was rapidly cooled to 4°C by immersion in an ice/salt slurry bath. The extract was then centrifuged in an M.S.E. 21 ultracentrifuge at 10,000 rpm for 20 minutes at 4°C (6 x 500ml rotor).

7.4.3 Ion-exchange chromatography.

The supernatant from the heat-treated solution was applied to to a column of DEAE cellulose (10 x 10cm) which had previously been equilibrated in 50mM Tris/HCl buffer pH 8.2. The column was then washed with 1 litre of the same buffer followed by 0.5 litres of 25mM KCl. The enzyme was then eluted from the column by the application of a linear KCl gradient (25-400mM KCl; 1000mls total volume) in 50mM Tris/HCl buffer pH 8.2. Active fractions were pooled and then concentrated in an Amicon ultrafiltration cell fitted with a PM10 membrane.

7.4.4 Gel filtration.

The concentrate, prepared by ultrafiltration (20mls), was applied to the base of a vertical column (75 x 5cm) of Sephacryl S-200 HR, which had been equilibrated in 20mM Tris/HCl buffer, pH 8.2. The column was then pumped upwards at a flow rate of 60mls/hour. Fractions with a high level of porphobilinogen deaminase activity were pooled.

7.4.5 Phenyl Sepharose chromatography.

The pooled active fractions from the gel filtration step were made up to 30% saturation with respect to ammonium sulphate (w/v).The solution was then applied to a column of Phenyl Sepharose (1.5 x 10cm) which had been equilibrated with in 20mM Tris/HCl buffer, pH 8.2, with 30% saturated ammonium sulphate (w/v). The column was then washed consecutively with 100mls of the same buffer, 100mls of buffer with 20% saturated ammonium sulphate (w/v), and finally 100mls of buffer with 15% saturated ammonium sulphate (w/v). The enzyme was eluted with a linear gradient of buffer (total volume 600mls) from 15% to 0% saturation. Fractions (10mls) were collected and those with enzyme activity were again pooled,concentrated and dialysed against 20mM

Tris/HCl buffer pH 8.2. The enzyme elutes at approximately 5% saturated ammonium sulphate.

7.4.6 High resolution ion-exchange chromatography.

The dialysed enzyme solution was applied to a high resolution anion exchange Mono Q HR5/5 column attached to a Pharmacia f.p.l.c. system which had been equilibrated in 20mM Tris/HCl buffer, pH 7.5. The enzyme was eluted using a linear gradient of sodium chloride (0-350mM NaCl,30mls total volume) at a flow rate of 1ml/minute. The absorbance at 280nm was monitored and individual peaks were collected and assayed for porphobilinogen deaminase activity. All active fractions from this stage were analysed by electrophoresis on SDS polyacrylamide gels.

Any fractions that were shown to be impure were rechromatographed to homogeity by high resolution anion exchange chromatography as described above except the procedure was carried out at pH 6.0.

7.5 Purification of porphobilinogen deaminase from spinach.

The spinach enzyme was purified by a modified procedure based on the method described by Higuchi and Bogorad (1975). Valuable assistance was given by a third year project student, Sarah Woodcock, with this purification procedure.

7.5.1 Preparation of crude extract.

0.5Kg of fresh spinach was homogenised in 100g batches with a 100ml of 0.1M Tris/HCl buffer, pH 8.2, in a modified Waring blender for a total of 2 minutes in 30 second pulses with 30 second cooling periods in between. The homogenised solution was passed through a 100μm nylon mesh and was then centrifuged at 5,000rpm for 15 minutes in a Sorvall RC 3B centrifuge to remove any remaining small particulate matter.

7.5.2 Ammonium sulphate precipitation.

The supernant from the crude spinach extract was subject to differential precipitation with ammonium sulphate. The extract was first taken to 30% saturation and the precipitate removed by centrifugation at 20,000 g for 20 minutes. The supernatant was then taken to 60% saturation with ammonium sulphate and the precipitate was collected by centrifugation at 20,000 g for 20 minutes. The deaminase activity was found in this precipitate so the supernatant was discarded.

7.5.3 Heat treatment.

The 30-60% ammonium sulphate precipitate was resuspended in a minimal volume of 0.1M Tris/HCl buffer, pH 8.2, and was heated to 60°C in a hot water bath for 10 minutes after which time it was rapidly cooled to 4°C. The precipitated protein was removed by centrifugation at 20,000 g for 20 minutes and the supernatant was concentrated in an Amicon ultra-filtration unit to around 20mls.

7.5.4 Gel filtration.

The concentrate was applied to the base of a column of Sephacryl S-200 (2.5 x 100cm) which had been previously equilibrated with 20mM Tris/HCl buffer, pH 8.2, and was pumped inversely at a flow rate of 60mls/ hr .. The enzyme eluted from the column as a tight band in a volume of 30mls.

7.5.5 High resolution ion exchange chromatography.

The active fractions from the gel filtration stage were applied directly to a high resolution Mono Q HR 5/5 column attached to a Pharmacia f.p.l.c. system as described in section 7.4.6. The enzyme was found to elute from the column as a single sharp peak well separated from any contaminating proteins. The purity of the enzyme at this stage was such that no further purification was required.

7.6 Purification of porphobilinogen deaminase from barley chloroplasts.

The barley porphobilinogen deaminase was purified from a crude chloroplast fraction that had been prepared as described by Kannangara et al (1984). The deaminase was purified from the fraction by heat treatment of the sample at 60°C as described in section 7.4.2 followed by high resolution ion exchange chromatography on a Mono Q HR 5/5 column both at pH 7.5 and pH 6 as described in section 7.4.6.

7.7 Purification of porphobilinogen deaminase from human erythrocytes.

Porhobilinogen deaminase was purifed from outdated human erythrocytes by a procedure modified slightly from the method described by Anderson and Desnick (1980).

7.7.1 Preparation of crude extract and removal of haemoglobin.

2 Units (1 litre) of outdated human blood was centrifuged at 5,000 rpm in a Sorvall RC 3B centrifuge for 15 minutes. The plasma was decanted and the red cells were washed in isotonic saline (0.9% NaCl w/v). The packed red cells were then

osmotically lysed by the addition of two volume equivalents of distilled water. Unbroken cells and cell debris were removed by centrifugation at 10,000 rpm (18,000 g) for 45 minutes in an MSE 21 centrifuge (6 x 500 ml rotor) and the resultant supernant was made adjusted to 10mM potassium phosphate buffer, pH 6.8. DE-52 cellulose (400mls wet weight volume) was stirred into the supernatant and stirring was continued for a further 30 minutes in order to facilitate a greater binding of the deaminase. After this time the suspension was centrifuged at 5,000 rpm in a Sorvall RC-3B centrifuge for 15 minutes and the supernatant was discarded. The DE-52 resin was then washed twice with 10mM potassium phosphate buffer, pH 6.8, before the deaminase was eluted from the cellulose by washing with buffer containing 100mM KCl.

7.7.2 Heat treatment and gel filtration.

The deaminase containing washing from the previous step was heat treated as described in section 7.4.1 and the precipitated protein was removed by centrifugation. The supernatant was then concentrated in an Amicon ultra-filltration unit to a volume of 20mls. The concentrate was then subject to gel filtration as described in section 7.5.4.

7.7.3 Phenyl Sepharose chromatography.

The active fractions from the gel filtation stage were applied to a column of Phenyl Sepharose (1.5 x 5cm) which had previously been equilibrated in 25mM Tris/HCl buffer, pH 8.0. The column was then washed with three consequetive 50ml volumes of buffer. A red haemoglobin fraction passed unretarded through the column whereas the deaminase was held back by at least two column volumes before it eluted, in a well separated fraction, from the column.

7.7.4 High resolution ion exchange chromatography.

The active fraction from the Phenyl Sepharose column was applied to a MonoQ HR 5/5 column attached to a Pharmacia f.p.l.c. as described in section 7.4.6. The column was run at both pH 7.5 and at pH 6. The deaminase was found to elute from the column as two sharp independant peaks as has been reported before (Anderson & Desnick, 1980; Brown et al, 1985)

7.8 Immobilization of porphobilinogen.

Aminohexyl Sepharose was reacted with a large excess of glutaraldehyde in the presence of carbodiimide in order to generate a matrix with a free aldehyde arm. The gel was washed with 0.5M NaCl to remove the coupling agent and the activated support was then reacted with porphobilinogen at pH 1 for 1 hour. At acid pH values aldehydes are

known to react with the α-position of porphobilinogen. The gel was shown to contain significant quantities of porphobilinogen by following the incorporation of radioactive porphobilinogen into the matrix.

7.9 Crystalization of the *E. coli* porphobilinogen deaminase.

The hanging drop vapour diffusion method (M^{c}Pherson, 1982) was used to prepare microcrystals of the E. coli deaminase. The purified protein was desalted by gel filtration through a PD10 column which had previously been equilibrated with water. The enzyme was then freeze dried prior to resuspension in 50mM sodium acetate buffer, pH 5.0, containing 5% sodium chloride (w/v) to a final concentration of around 30mg/ml. Droplets of size 20µl were suspended from siliconised microscope slide covers over a well in a tissue culture plate which contained 2mls of a 20% sodium chloride solution. The well was made airtight by a layer of grease between the join of the cover slip and the well. The droplet was left for up to 6 months although generally crystals grew within the first 2 months.

7.10 Polyacyrlamide gel electrophoresis.

Polyacrylamide gel electrophoresis under non-denaturing conditions (in the absence of SDS and 2-mercaptoethanol) was carried out by the method of Laemmli and Favre (1973). The coposition of the gel mixes is shown in table 7.1. Enzyme activity on the gel was determined by incubation of the gel for 15 minutes at 37°C with 0.5mM porphobilinogen in 0.1M Tris/HCl buffer, pH 8.2, followed by exposure of the gel to iodine solution (0.01% in .1M HCl) for 5 to 10 minutes. Flouresence due to the formation uroporphyrin was detected using a long wavelength ultra violet lamp. Gels were then stained for protein using Coomassie brilliant blue (0.2% in acetic acid (v/v) and methanol (40%)).

Polyacrylamide gel electrophoresis under denaturing conditions was carried out according to Laemmli (1973). The composition of the gel mixes is shown in table 7.1.

7.11 Molecular weight determinations.

The molecular weight of the deaminase was determined using the Laemmli (1973) gel system which had been calibrated using molecular weight markers. The molecular weight of the native enzyme was determined by chromatography through Sephacryl S-200. The column (2.5 x 100cm) was developed in 0.1M Tris/HCl buffer, pH 8.2, at a flow rate of 15mls/hour. The enzyme was run with standards of known

TABLE 7.1

Composition of solutions for polyacrylamide gels. For non-denaturing polyacrylamide gels SDS and mercaptoethanol are omitted. Gels are run at 30mA constant current.

a) Separating gel

Stock solutions for separating gel	% polyacrylamide gel			
	7.5%	10%	12%	15%
3M Tris/HCl, pH 8.55	6.25	6.25	6.25	6.25
20% SDS	0.25	0.25	0.25	0.25
0.14% Ammonium persulphate	12.50	12.50	12.50	12.50
28% Acrylamide, 0.735% bis-acrylamide	13.40	17.90	21.50	26.75
H_2O	17.60	13.10	9.50	4.25
TEMED	25µl	25µl	25µl	25µl

TABLE 7.1

b) stacking gel

c) sample disruption buffer

Stock solutions for stacking gel	Volume (mls)
28% Acrylamide, 0.735% bis-acrylamide	1.5
1M Tris/HCl, pH 7.0	1.5
0.14% Ammonium persulphate	6.0
H_2O	3.0
TEMED	10µl

Stock solutions	Final concentration	Volume (mls)
20% SDS	2%	1.0
1M Tris/HCl, pH 7.0	0.05M	0.5
Glycerol	6.0%	0.6
Mercaptoethanol	5.0%	0.6
H_2O	-	7.4

molecular weight.

7.12 Radioactivity measurements.

All samples to be measured were made up to a final volume of 1 ml with water to which 9mls of tritoscint, a detergent based scintillant, were added. The samples were then counted in an Intertechnique SL 40 scintillation counter programmed with the appropriate quence correction to convert cpm to dpm.

Radioactive samples from polyacrylamide gels were determined as follows. The gel tracts were sliced into 2mm sections and dissolved with NCS tissue solubiliser (1ml, 9:1 ratio solubilizer:water) for 2 hours at 55°C. The radioactivity was quantitated after 12 hours after the addition of 9mls of tritoscint.

7.13 Formation and purification of enzyme intermediate complexes.

The enzyme intermediate complexes were generated at 4°C by mixing stoichiometric quantities of the enzyme (30nmoles) and substrate (30 - 90nmoles) in a rapid mixing device which consisted of two 1 ml syringes connected by a Y shaped tube. The individual complexes were then isolated and purified by high resolution anion exchange chromatography using a Mono Q HR 5/5 column attached to a Pharmacia f.p.l.c. system which had previously been equilibrated in 20mM Tris/HCl buffer, pH 7.5. The free enzyme and individual complexes were eluted from the column by application of a linear salt gradient (100 - 300mM NaCl; total volume 20mls) at a flow rate of 1ml/min and the isolated peaks were assayed for enzyme activity, protein content and radioactivity.

7.13.1 Formation of enzyme substrate complexes with [^{14}C]-porphobilinogen.

Enzyme (0.1nmole) was mixed with [3,5,-$^{14}C_2$]porphobilinogen (90nm/μCi; 1.5-30nmoles) in 0.1M Tris/HCl buffer, pH 8.2, at 4°C in a rapid mixing device. The enzyme and its substrate complexes were subjected to electrophoresis under non-denaturing conditions and the bands were visualised both by assay of the gel for enzymic activity and staining with Coomassie brilliant blue. Radioactivity was determined by counting 2.5mm gel slices of duplicate gels run under identicle conditions. Alternatively, the enzyme and its substrate intermediates were separated and isolated by high resolution anion exchange chromatography using a Mono Q HR 5/5 as described above.

7.14 Inhibitor studies.

Inhibitor studies were carried out by adding inhibitor to 0.25 units of either enzyme or enzyme-intermediate complex in 0.1M phosphate buffer, pH 8.0, in a final volume of 450µl. After incubation with the inhibitor for 20 minutes at 20°C, the inhibitor was removed by gel filtration and the enzyme activity was determined.

Pyridoxal-5-phosphate inhibition was investigated by incubating either enzyme or enzyme-intermediate complex with pyridoxal-5-phosphate followed by reduction with $NaBH_4$. A freshly prepared solution of 100mM $NaBH_4$ in 0.1M sodium phosphate buffer, pH 8.2, was slowly titrated with the incubation mixture until the solution just became colourless. Duplicate samples of enzyme or enzyme-intermediate complexes were incubated with pyridoxal-5-phosphate but were not reduced with $NaBH_4$.

7.14.1 Reaction with 5,5'-dithiobis(2-nitrobenzoic acid).

About 1mg (30nmoles) of either porphobilinogen deaminase or porphobilinogen deaminase intermediate complexes were reacted with a final concentration of 5mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1M Tris/HCl buffer pH 7. The absorbance of the liberated thionitrobenzoate was measured at 412nm (ε_{412} = 12,800) and was followed over a period of 16 hours at 10°C. The activity of the enzyme in the reaction vial was also recorded simultaneously over the same period of time.

7.15 Separation of released enzymic pyrrolic intermediates.

The pyrrolic components of the deaminase incubation mixtures formed in the presence of the inhibitory bases NH_3, NH_2OH and NH_2OCH_3, were separated from one another by thin layer electrophoresis. Cellulose plates (0.25mm thick, 10 x 20cm) were moistened with 0.05M Veronal buffer, pH 9.2, and were pre-equilibrated for 20 minutes in a potential gradient of 25V/cm. Samples of 20µl of enzyme incubation mixture were applied near the cathode end of the plate and electrophoresis was carried out at 4°C for 2 hours. After electrophoresis the pyrroles and polypyrromethanes were detected by spraying the plate with modified Ehrlich's reagent.

7.16 Carboxymethylation of porphobilinogen deaminase.

Lyophilized deaminase (5mg; 150nmoles) was dissolved in 1ml of 0.1M potassium phosphate buffer, pH 7.0, containing 10mM dithioerythritol (1.5mg/ml), 0.3M $NaHCO_3$ (28mg/ml), 6M guanidine hydrochloride (0.57g/ml), and was incubated for 20 minutes at 37°C. The thiol groups were modified by the addition of 0.1ml of 1M

iodoacetic acid (186mg/ml; adjusted to pH 7 with 1N NaOH) followed by incubation for 5 hours at 37°C under nitrogen. After this time the reaction mixture was thoroughly dialysed against water prior to being freeze dried.

7.17 Performic acid oxidation.

Modification of the cysteine groups to cysteic acid residues was achieved by treatment of the protein with performic acid in the following way. Performic acid was prepared by mixing 1ml of 30% H_2O_2 with 9mls of formic acid at room temperature for two hours prior to cooling to 4°C. The reagent (1ml) was then added to the protein (0.5mg; 15nmoles) and was left at 4°C for 4 hours. After this time an equal volume of water was added and the reaction mixture was then dialysed against water. The modified protein was recovered after the dialysed solution had been freeze dried.

7.18 Amino acid analysis.

A total amino acid analysis was carried out on 6nmole samples of both native and performic acid oxidized protein which allowed a measurment of the cysteine residues as cysteic acid to be made. The amino acid analysis was carried out on a Rank Hilger J120 amino acid analyser. The protein samples were lyophilised in 6M HCl with 0.2% phenol (w/v) at 110°C for 16-20 hours in vacuo. The hydrolysed sample was dried in a vacuum desicator and the amino acids resuspended in 10mM HCl for application to the analyser.

7.19 Isoelectric focussing.

Flat bed isoelectric focussing was performed on an LKB Multiphor apparatus using LKB polyacrlamide gels in the pH range of 3-9 according to the manufacturers instructions.

7.20 Formic acid chemical cleavage of the porphobilinogen deaminase.

Carboxymethylated enzyme (50µg; 1.5nmoles) was dissolved in 300µl of 70% (v/v) aqueous formic acid and was incubated for 36 hours at 37°C. The reaction was terminated by the addition of distilled water (1ml) and the solution was then freeze dried.

7.21 Proteolytic cleavage of porphobilinogen deaminase.

Porphobilinogen deaminase (1mg; 30nmoles)was dissolved in 1ml of ammonium carbonate buffer, pH 8, and was subject to digestion with trypsin (final concentration 5% w/w) or with Streptomyces griseus type XIV protease (pronase) (final concentration 5% w/w) for a period of up to 16 hours at 37°C. The digestion was terminated by freeze drying.

7.22 Separation of peptides.

The peptides were separated initially by gel filtration on a column of Sephadex G-50 (1 x 50cm) and then by reverse phase chromatography using a C-18 reverse phase Pharmacia Pep RPC column attached to a Pharmacia f.p.l.c. system. The system was euilibrated with 0.1% aqueous trifuoroacetic acid and the peptides were eluted by the application of a linear gradient of 0.1% trifluoroacetic acid in acetonitrile to the column.

7.23 N.m.r. spectroscopy.

N.m.r. experiments were conducted in 5mm n.m.r. tubes at 20°C with a Brucker AM-500 n.m.r. spectrometer at an observation frequency of 125.7MHz using a pulse width of 7 μsecs, a repetition time of 1.0 sec and an acquisition time of 0.311 sec. Exponential line broadening of 10Hz was applied to the 16K FID prior to Fourier transform. Samples of enzyme were dissolved in 10mM sodium pyrophosphate buffer (pH 8.5) containing 10% $^{2}H_2O$ at a concentration of 1mM. A sample of non-labelled E. coli porphobilinogen deaminase was used in order to obtain [^{13}C - ^{12}C] difference spectra. This was essential in order to remove resonances due to natural abundance signals from the protein.

7.24 Separation of uroporphyrin isomers.

The nature of the uroporphyrin isomers were determined as described by Wayne et al, 1979. The porphyrins were applied to a reverse phase C-18 column attached to an h.p.l.c. and were separated with an isocratic gradient of sodium phosphate buffer, pH 7.5, containing 4% (v/v) acetonitrile. The column was first run with authentic standards prior to the application of the unknown samples.

7.25 X-Ray crystallography

Crystals were mounted in 1mm diameter Lindemann tubes containing a small resevoir of mother liquor and sealed with wax. Still and precession photographs were recorded with a Nonius precession camera with a crystal to film distance of 100mm. The X-ray source was an Elliott GX20 rotating anode operated at 40KV and 40mA.

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