NMR STUDIES IN THE BIOSYNTHESIS OF PORPHYRINS

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ABSTRACT

High-field NMR spectroscopy has been applied to the study of two enzymes in the porphyrin pathway and the reactions which they catalyse. Porphobilinogen (PBG) deaminase and uroporphyrinogen III (uro'gen III) cosynthetase are the two enzymes responsible for the formation of the porphyrin macrocycle and the action of these enzymes in the conversion of PBG, [2,11-^{13}C]-PBG and [3,5-^{13}C]-PBG to uro'gen III has been studied by ^{1}H and ^{13}C NMR. The principal intermediate which accumulated was the correspondingly labelled hydroxymethylbilane (HMB). The complete ^{1}H and ^{13}C NMR assignment of PBG is presented, and an analysis made of the effects of pH on the chemical shifts of selected resonances.

The procedures for the generation of enzyme-substrate complexes using [2,11-^{13}C]-PBG and [3,5-^{13}C]-PBG with deaminase, and the methods employed for their purification are described. Use of ^{13}C NMR in an attempt to detect the substrate bound to the enzyme revealed that the linewidths must be inordinately large. The complex was found to disproportionate with time when stored at 25°C. However, enzyme-bound uro'gen I was detected, both in the intact protein, and in the oligopeptides from tryptic digestion and peptide mapping.

The synthesis of [5-^{2}H]-ALA and [5-^{3}H]-ALA and their conversion to the corresponding PBG by the action of partially purified ALA dehydratase are described. The isolation and purification of ALA dehydratase, PBG deaminase (to homogeneity) and uro'gen III cosynthetase from *R. spheroides* are covered.

The first detection of an enzyme-substrate complex by ^{3}H NMR is described, using [^{3}H]-PBG as the substrate and deaminase as the enzyme. The linewidths were found to be extremely large, and dependent upon temperature, giving chemical shifts which allowed tentative conclusions to be made as to the nature of the nucleophilic group on the enzyme to which the substrate binds. The catalytic competence of this complex was also demonstrated, by displacing bound [^{3}H]-PBG with unlabelled PBG. During the resultant formation of [^{3}H]-uro'gen I, a transient low-intensity signal was detected which has been tentatively assigned to the highly reactive exomethylene pyrrolenine species, proposed in several mechanistic schemes for porphyrin biosynthesis. Mechanistic interpretations of these results are also presented.

This thesis is the original composition of the author's work, unless stated otherwise, and has not been submitted for any other degree.

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A special word of thanks is due for Jean Long, without whom the research group would have surely ground to a halt, and who with patience and forbearance transformed my arcane scrawl into no less than a work of art, as the typewritten pages here clearly illustrate.

SYMBOLS AND ABBREVIATIONS

A	acetate side-chain ($-CH_2CO_2H$)
ALA	5-aminolaevulinic acid
ALAD	ALA dehydratase
ALAS	ALA synthetase
AQ	acquisition time
A_x	absorbance at a particular wavelength "x"
C	uro'gen III cosynthetase
CD	circular dichroism
CoA	coenzyme A
CSA	chemical shift anisotropy
D	deuterium
D	PBG deaminase
DCC	dicyclohexylcarbodiimide
DMF	N,N-dimethylformamide
DMSO	dimethylsulphoxide
DOVA	4,5-dioxovalerate
DP	decoupler power
δ_x	chemical shift of nucleus "x" in parts per million (ppm), directly or indirectly referenced to TMS, TSP or p-dioxane
ES	enzyme-substrate complex
ε_x	extinction coefficient of a particular wavelength "x"
FAB-MS	fast atom bombardment mass spectroscopy
FID	free induction decay
FT	Fourier transform
$\gamma_{(x)}$	gyromagnetic ratio (for nucleus "x")
GB	Gaussian broadening

$\hbar$	Planck's constant h, divided by 2Π
HMB	hydroxymethylbilane
HPMD	high performance mixing device
IR	infrared
^{n}J (A-B)	n-bond coupling constant between nuclei A and B
K_i	dissociation constant of enzyme-inhibitor complex (the inhibitor constant)
K_m	concentration of substrate giving half maximal velocity (the Michaelis constant)
KPhos	potassium phosphate buffer
LB	line broadening
λ_{max}	wavelength of absorbance maximum (UV)
MS	mass spectroscopy
M_r	relative molecular mass
NOE	nuclear Overhauser effect
ν_{max}	wavelength of absorbance maxima (IR)
NAD(P)H	β-nicotinamide adenine dinucleotide (phosphate)
NMR	nuclear magnetic resonance (spectroscopy)
NS	number of transients (scans)
NZP	number of points removed from the start of an FID
P	propionate side-chain ($-CH_2CH_2CO_2H$)
PBG	porphobilinogen
pH_{opt}	optimum pH for maximal enzyme activity
pI	isoelectric point
PW	pulse width ("flip angle")
RD	relaxation delay
RF	radio frequency (electromagnetic radiation)
R_f	retention factor (TLC)

SAM	S-adenosylmethionine
SI	size of data table, in data points X 10^3 (K)
SW	spectral width
t	time in minutes
$t_{½}$	half-life in minutes
T	tritium
T	temperature in °C
T_1	spin-lattice relaxation time
T_{1DD}	dipole-dipole spin-lattice relaxation time
T_2	spin-spin relaxation time
τ	time in milliseconds
τ_c	correlation time
TMS	tetramethylsilane (Me_4Si)
Tris	tris(hydroxymethyl)aminomethane
TSP	sodium 3-(trimethylsilyl)-3,3,2,2-tetradeutero-propionate ($Me_3SiCD_2CD_2CO_2Na$)
UV	ultraviolet
V_{max}	velocity of an enzyme reaction when saturated with substrate
χ	magnetic susceptibility

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PART III. EXPERIMENTAL PROCEDURES

For Eilean,

who put up with so much.

PART I

INTRODUCTION

CHAPTER 1

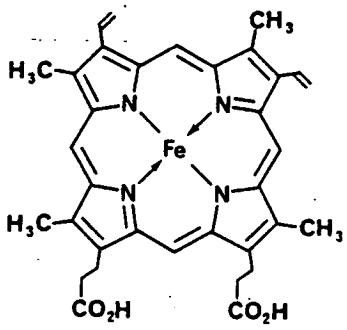
INTRODUCTION

1.1 General Introduction

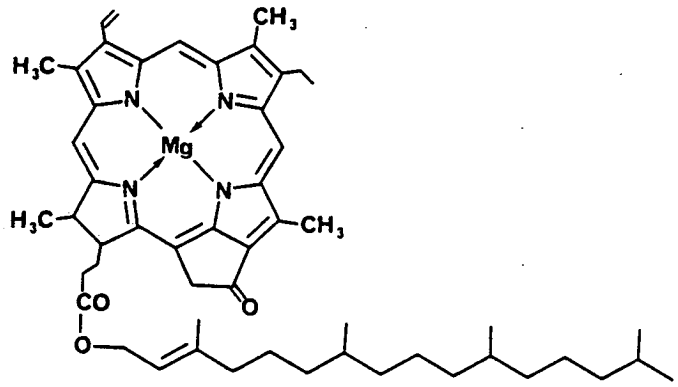
Man and many other forms of life owe their continued existence in part to a family of molecules called porphyrins. They are also known as the life pigments, since in addition to being highly coloured, the porphyrin pigments occupy a rôle of central importance to most living organisms. Furthermore, these pigments are interrelated as tetrapyrroles, and there are many similarities between them. Principally, each contains a metal atom fixed within a macrocyclic nucleus composed of four five-membered heterocyclic rings. Each metal confers upon the macrocycle its own distinctive colour: iron, the deep red colour of haem (1), as found in haemoglobin; magnesium, the green colour of chlorophyll (2); and cobalt, the red-purple colour of Vitamin B_{12} (3).

It is small wonder then, that over the last century scores of chemists, biochemists and biologists alike have tried to unravel the mechanisms by which Nature builds such molecules. As early as 1880 Hoppe-Seyler[1] suggested that haem and chlorophyll were related to one another. During the 1920's and 1930's, a tremendous number of basic studies on the chemistry of haem and chlorophyll were carried out. The work by Willstätter and his colleagues[2] established that haem and chlorophyll were both tetrapyrroles. Fischer and his colleagues[3] refined this work by defining the structure and position of the various side-chains attached to the tetrapyrroles, allowing a clearer distinction to be made between the two compounds. In this

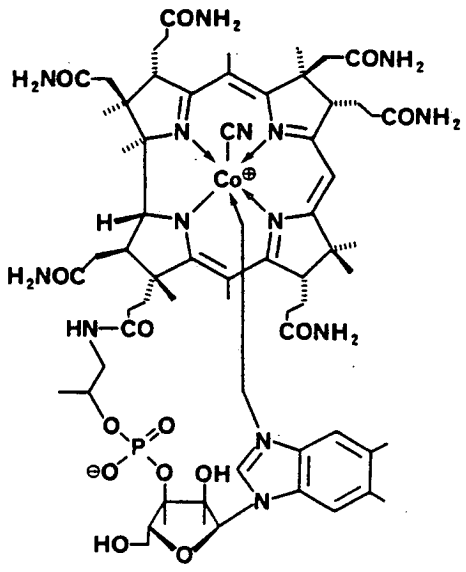
way Fischer established the structure of haem, which he later confirmed by total synthesis in 1929. In contrast, chlorophyll, although its structure was known in 1934, was not synthesised until 1960.[4]



HAEM (1)



CHLOROPHYLL a (2)



VITAMIN B_{12} (3)

It was not until 1948 that Vitamin B_{12} was isolated, and chemical studies on its composition demonstrated that it too was a tetrapyrrole and thus related to haem and chlorophyll. However, its structure proved to be more elusive, since the corrin ring was

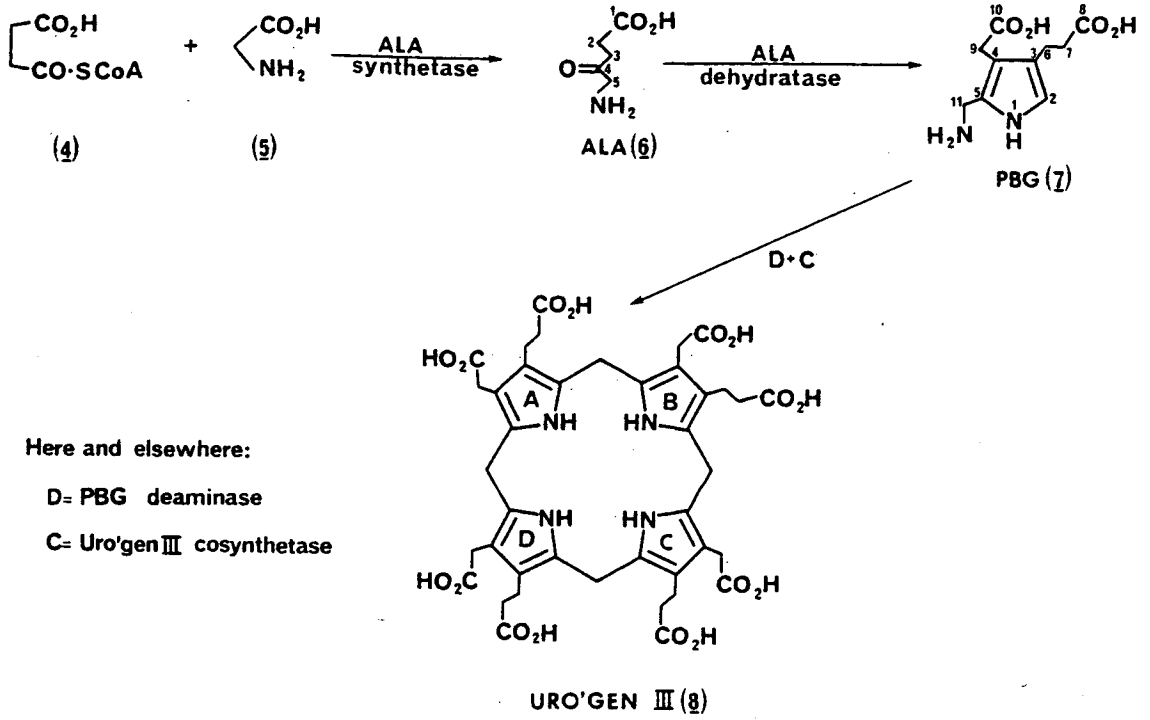
unexpected in that its Rings A and D are directly bonded, in contrast to the porphyrin ring, in which the same attachment is mediated by a methine group. Its structure was elucidated principally by Hodgkin and several other teams of workers in 1955,[5-11] and its total synthesis, accomplished nearly 20 years later by Woodward[12] and Eschenmoser,[13] is undoubtedly one of the most significant landmarks of modern synthetic organic chemistry.

Since a large number of excellent reviews[14-18] exist on the biosynthesis of porphyrins, this chapter will be restricted to the tremendous developments that have taken place over the last 10 years. For this purpose, the next section will highlight the salient features of porphyrin biosynthesis. Subsequent sections will give a more detailed history of each step leading to the porphyrin macrocycle, and a brief look at the metabolic events beyond.

1.2 The Type III Problem Delineated

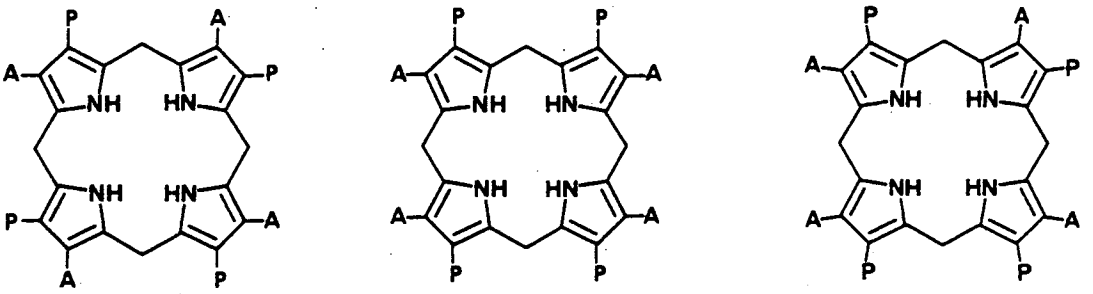
The biosynthesis of porphyrins takes place in many living systems, and occurs by the route shown in Scheme 1. Considering their moderately complex structures, it is astonishing that haem (1), chlorophyll (2) and Vitamin B_{12} (3) have such humble origins as that of glycine (5) and succinyl-coenzyme A (4). However, glycine and succinyl-CoA condense to give δ-aminolaevulinic acid (ALA, 6). Two molecules of ALA further condense to give the monopyrrole porphobilinogen (PBG, 7), which polymerises to give the reduced porphyrin uroporphyrinogen III (uro'gen III, 8).[19]

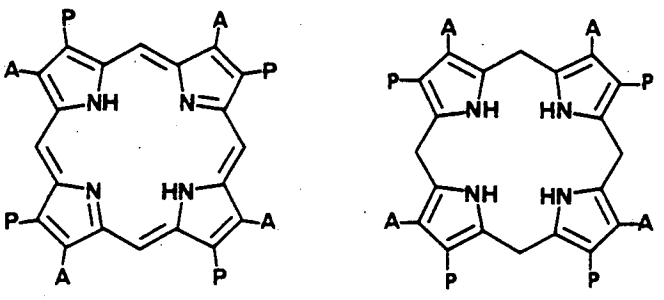
There are four possible isomers of uro'gen, differing in their arrangements of the acetate and propionate side-chains around the



SCHEME 1

periphery of the tetrapyrrole macrocycle, designated types I, II, III, and IV. The same possibilities obviously occur for the uroporphyrins as well (e.g., uroporphyrin I (12)).





Here and elsewhere:

A = $-CH_2CO_2H$

P = $-CH_2CH_2CO_2H$

Prima facie it is a curious thing that the type III isomer predominates in living sytems, the type I isomer occurring in the shells of an oyster *Pinctada vulgaris*,[20] and in the urine of patients suffering from congenital porphyria, while the type II and type IV isomers are not known in any living systems. In the type III isomer, the D ring would appear to have been rearranged so that the propionate side-chains in the C and D rings lie adjacent. Thus it seems as if Nature has evolved in such a way as to assemble the type III porphyrin, when the simplest chemical pathway would lead to the type I isomer. For straightforward head-to-tail polymerisation of PBG (7) might lead one to expect the type I isomer to predominate, and it has even been suggested[21] that Nature forms the unexpected isomer. This, however, is to misstate the enigma. It has been well established[22] that the polymerisation of PBG (7) in acidic conditions gives rise to a statistical mixture whose composition is invariably 50:25:12.5:12.5 types III:IV:II:I. If the implications of the results from prebiotic chemistry[23] are to be believed, along with the discovery of porphyrins on meteorites,[24] then it seems reasonable to suppose that porphyrins occurred early on in the primeval Earth's history. There is evidence that corrins may even have predated the porphyrins.[25] Thus it is not unreasonable to suggest that Nature evolved its biochemical architecture to optimise the synthesis of the statistically most favoured isomer, since it was already the most abundant. This point will become all the more apparent when the individual enzymes in the metabolic pathway are examined in detail later in the chapter.

The problem of how it is that the type III porphyrin predominates both *in vivo* and *in vitro* has puzzled Man for the last 35 years, and

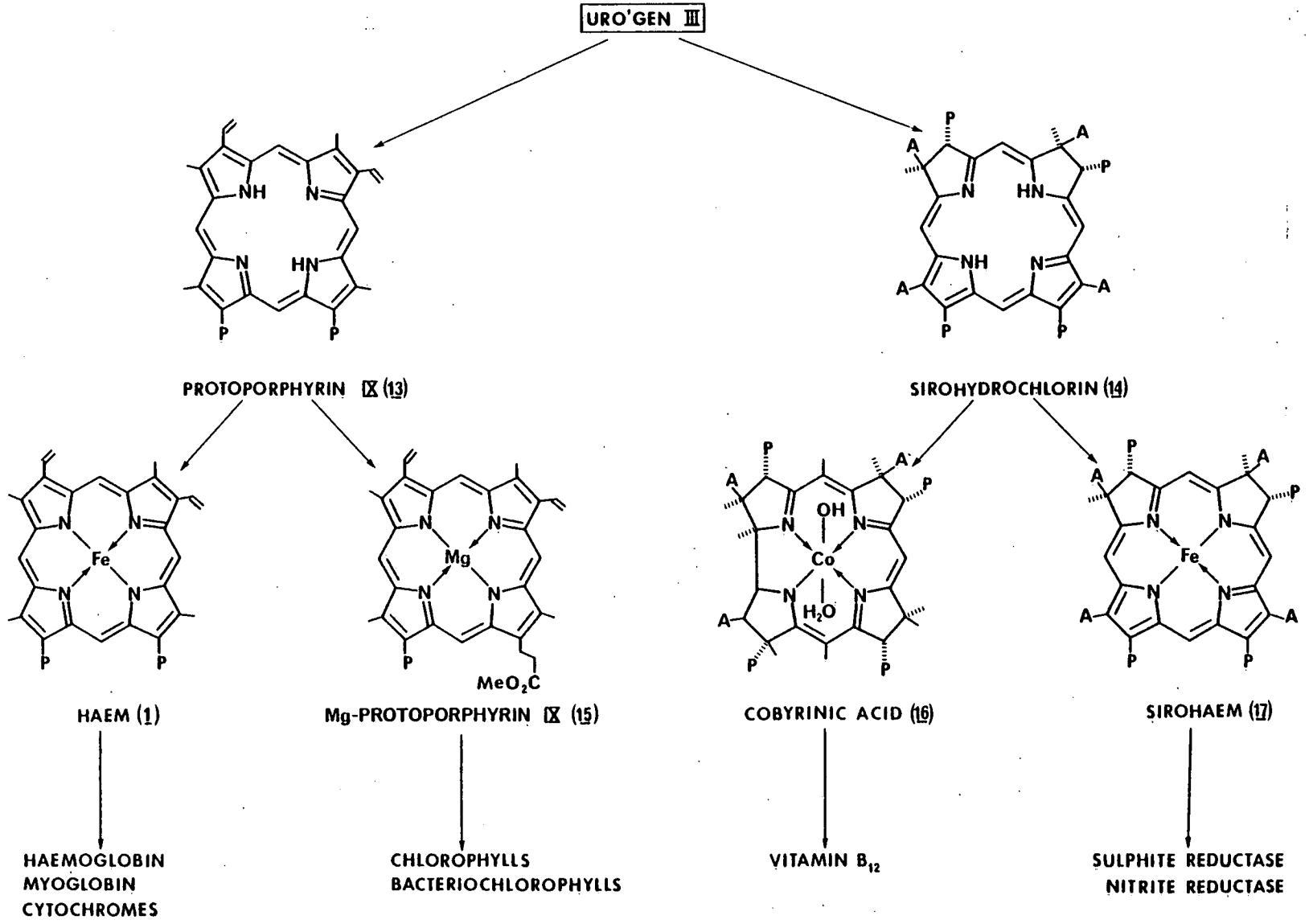
is known as the Type III Problem. It encompasses not only the intriguing mechanisms by which the type III isomer is formed both chemically and enzymically by rearrangement of the D ring, but also how the tetrapyrrole nucleus is built up from the monopyrrole PBG. To fully realise the importance of the Type III Problem, we have to be aware of the significance of uro'gen III (Scheme 2).

A large proportion of the animal kingdom relies upon the haem of haemoglobin to transport oxygen around the body, and this oxygen is produced as a result of the actions of chlorophyll on the aerial carbon dioxide produced by animals, in the process of photosynthesis — a process necessary for the plant's continued existence. This mutual ecological interdependence becomes all the more fascinating when we realise that both chlorophyll and haem have as their common biosynthetic precursor uro'gen III, as indeed do the bacteriochlorophylls, the cytochromes, the sulphite and nitrite reductases, and Vitamin B_{12}.

Thus the understanding of the biosynthesis of uro'gen III is central to our understanding of the biosynthesis of a wide range of essential molecules. Furthermore, that understanding can only come from a detailed study of the Type III Problem, and it is studies directed towards this goal that form the basis of this thesis.

1.3 The Use of NMR in Biological Studies

With the advent of Fourier-transform (FT) nuclear magnetic resonance (NMR) spectroscopy, its use and applicability to the study of biomolecules has gained in recent years. In the field of porphyrin metabolism it is used quite extensively, and since it is also the technique of choice for much of the work presented in Part II, it is appropriate to discuss some of its capabilities.


URO'GEN III
PROTOPORPHYRIN IX (13)
SIROHYDROCHLORIN (14)
HAEM (1)
Mg-PROTOPORPHYRIN IX (15)
COBYRINIC ACID (16)
SIROHAEM (17)
HAEMOGLOBIN
MYOGLOBIN
CYTOCHROMES
CHLOROPHYLLS
BACTERIOCHLOROPHYLLS
VITAMIN B_{12}
SULPHITE REDUCTASE
NITRITE REDUCTASE

SCHEME 2

The basic theory of NMR is covered by a number of excellent texts[26-30] and also its use in biological research.[31-35] This section will be concerned with the use of NMR to study biological macromolecules with special reference to the nuclei ^{1}H and ^{13}C. Other nuclei used in biological NMR are listed in Table 1.1, along with some of their NMR properties. A more detailed summary of NMR relaxation is given in Appendix 1.

Some of the types of biomolecules of interest here which are amenable to study by NMR are proteins, enzyme-inhibitor complexes, and much more recently, enzyme-substrate complexes. Much of the early work in the study of proteins by ^{13}C NMR was performed by Allerhand,[36] who found that the resolution of the carbon resonances is greatly affected by the freedom of rotation of the carbons under observation. The backbone carbons of proteins tend to exhibit broad resonances, whereas relatively free side-chain carbons yield sharp lines. This also explains why denatured proteins show much sharper lines, due to the gains in degree of freedom. The increased resolution obtained with denatured protein is also due to the elimination of chemical shift non-equivalence caused by folding of the polypeptide chain. Sharp resonance lines in protein spectra also arise from non-protonated carbons, due to the inherently longer T_2 values. Increasing the molecular weight of the protein also results in an increase in linewidth, since the protein tumbles more slowly and thus has a longer correlation time τ_c.

In many protein studies, high resolution spectra may be obtained at moderately low field strengths, and indeed only sp^3 carbons benefit from higher field strengths. This can be explained by the contribution of CSA to the relaxation mechanism for non-sp^3 carbons

TABLE 1.1. NMR properties of biologically relevant nuclei.[a]

Isotope	Spin $I/\hbar$	Natural Abundance N/%	Magnetic Moment[b] μ/μ_N	Gyromagnetic Ratio $\gamma/10^7$ rad T^{-1} s^{-1}	Quadrupole Moment $Q/10^{-28}$ m^2	NMR Frequency[c] /MHz	Relative Receptivity[d]
^1H	1/2	99.985	4.8371	26.7510	-	300.133 (500.135)	1.000
^2H	1	0.015	1.215	4.1064	2.73×10^{-3}	46.073 (76.775)	1.45×10^{-6}
^3H	1/2	$<10^{-16}$ (radioactive)	5.1594	28.5335	-	320.121 (533.294)	1.21×10^{-16}
^{13}C	1/2	1.108	1.2162	6.7263	-	75.473 (125.767)	1.76×10^{-4}
^{15}N	1/2	0.37	-0.4901	-2.7107	-	30.416 (50.684)	3.85×10^{-6}
^{17}O	5/2	0.037	-2.2398	-3.6266	-2.60×10^{-2}	40.687 (67.801)	1.08×10^{-5}
^{19}F	1/2	100	4.5506	25.1665	-	282.407 (470.56)	0.8328
^{23}Na	3/2	100	2.8610	7.0760	0.12	79.388 (132.290)	9.25×10^{-2}
^{31}P	1/2	100	1.9581	10.829	-	121.513 (202.490)	0.0663

[a]Data taken from Ref. 28; [b]Data listed are the maximum observable values; [c]Frequency given for a 7.0497 T instrument, with values for a 11.7470 T instrument in parentheses; [d]The receptivity is the sensitivity of a given nucleus expressed relative to protons.

at field strengths >4 T.[37] By the same token, however, the contribution of dipolar relaxation is low for non-protonated carbons at high field strengths, and extremely significant for protonated carbons.

The assignment of specific resonances in a high resolution spectrum of a protein is not an easy task, particularly as much overlapping can occur, and the chemical shifts will reflect also the local environment of the particular residue. Synthesis of model peptides provide a useful way of assigning specific resonances, and Table 1.2 shows the chemical shifts for all the amino acid carbons in model peptides.

Other methods for probing the structure of proteins by NMR includes covalent modification using ^{13}C-enriched reagents. In this way Nigen and co-workers[38,39] found multiple carboxymethylation of myoglobin and mono-carboxymethylation of ribonuclease A when these enzymes are reacted with [2-^{13}C]-bromoacetate. By comparison with model compounds, the multiple resonances observed with the myoglobins can be assigned to carboxymethylation of histidine, lysine and terminal amino groups. Many other examples of this technique of modifying proteins with ^{13}C-enriched molecules can be found in the literature,[40-44] but perhaps a more subtle approach has been the use of inhibitors, which are capable of binding to the active site of the enzyme, thereby providing an active site probe.

Rich and co-workers[45] analysed the ^{13}C NMR of an adduct between pepsin and a pepstatin analogue. When a carboxyl enriched ketone analogue of pepstatin was used, it was found to bind to pepsin, giving rise to a new signal consistent with the formation of a tetrahedral adduct. Similarly, recent work in this laboratory[46,47] has made use of ^{13}C-enriched inhibitors to study the active sites of

TABLE 1.2. ^{13}C chemical shifts of the common amino acid residues in neutral D_2O.[a]

Amino Acid Residue	C=O	αC	βC	γC	δC	εC	ζC
Gly	172.7	43.5					
Ala	175.8	50.8	17.7				
Val	174.9	60.7	30.8	19.3 18.5			
Ile	174.8	59.6	36.9	25.4 15.7	11.3		
Leu	175.9	53.6	40.5	25.2	23.1 21.6		
Ser	172.6	56.6	62.3				
Thr	172.7	60.2	68.3	20.0			
Pro trans	175.2	61.6	30.6	25.5	48.2		
Pro cis		61.3	33.1	23.2	48.8		
Asp	174.2	52.7	39.8	178.4			
Glu	174.8	54.9	28.9	34.6	182.8		
Lys	174.7	54.4	27.5	23.1	31.8	40.5	
Arg	175.0	54.6	28.8	25.7	41.7		157.6
Asn	173.1	51.5	37.7	175.6			
Gln	174.0	54.1	28.1	32.2	179.0		
Met	175.0	53.9	31.0	30.7		15.0	
Cys	175.7	57.9	26.0				
					Ring carbon atoms		
Trp	176.7	56.7	27.4		C2,C5	126.0,122.9	
					C3	108.4	
					C4,C6	120.3,119.3	
					C7	112.8	
					C8	137.3	
					C9	127.5	

TABLE 1.2. continued

Amino Acid Residue	C=O	αC	βC	γC	δC	εC	ζC
					Ring carbon atoms		
Phe	176.0	57.4	37.0		C1	136.2	
					C2,C6	130.3	
					C3,C5	130.3	
					C4	156.0	
Tyr	176.0	57.4	37.0		C1	128.0	
					C2,C6	130.0	
					C3,C5	117.0	
					C4	156.0	
His	172.6	53.7	28.0		C2	135.2	
					C4	118.7	
					C5	130.3	

[a]Data taken from Ref. 31, p. 175, and chemical shifts are expressed in ppm relative to TMS, for linear oligopeptides of the type H-Gly-Gly-X-Gly-Gly-OH or TFA-Gly-X-L-Ala-OCH_3, where X is the amino acid given in the table.

trypsin and papain. Interestingly, it was found that while quaternary carbons at 7 T gave a linewidth of *ca.* 10 Hz, mono-protonated carbons show a linewidth of *ca.* 50 Hz, and methylene carbons around 100 Hz. This is obviously due to the effect of protons. Recently this has been circumvented[48] by replacing the protons with deuterium atoms. This decreases the dipolar relaxation effects, and the scalar coupling only amounts to some 30 Hz for a -CD and 45 Hz for a $-CD_2$.

Even more difficult than the study of enzyme-inhibitor complexes, is the study of enzyme-substrate complexes. Two general techniques may be employed here: (i) for multisubstrate enzymes, incubate with only one substrate enriched with ^{13}C; (ii) for monosubstrate enzymes, "freeze out" the adduct by use of low-temperature, or cryoenzymological techniques. Later in this thesis we shall see examples of technique (i) as applied to one of the enzymes in the porphyrin pathway. Technique (ii) is one of the most difficult techniques, and it has been used in this laboratory[49] to characterise the acyl intermediate formed by papain and [carboxyl-^{13}C]-N-benzoylimidazole.

The use of ^{1}H NMR to study biomolecules is in a sense more difficult than ^{13}C NMR. While ^{1}H NMR is considerably more sensitive, this is a disadvantage with proteins, which possess large numbers of protons falling in the range 0-12 ppm, as opposed to ^{13}C where the range is 0-300 ppm. No selective enrichment can be made, and assignment of the resonances is often very difficult. Perhaps the greatest value of ^{1}H NMR in the study of proteins has been its use in the study of the pH dependence of selected residues. More recently, the application of NOE-difference spectroscopy and 2-dimensional NMR techniques has enabled workers to determine valuable information about the secondary and tertiary structure of proteins.[50,51]

The ability to study proteins in detail by NMR provides a powerful method for probing the subtleties of enzyme-reaction mechanisms. This is particularly important for the porphyrin pathway, in which many of the enzyme reactions have complex mechanisms which are little understood. Such investigations will be outlined in more detail in Part II.

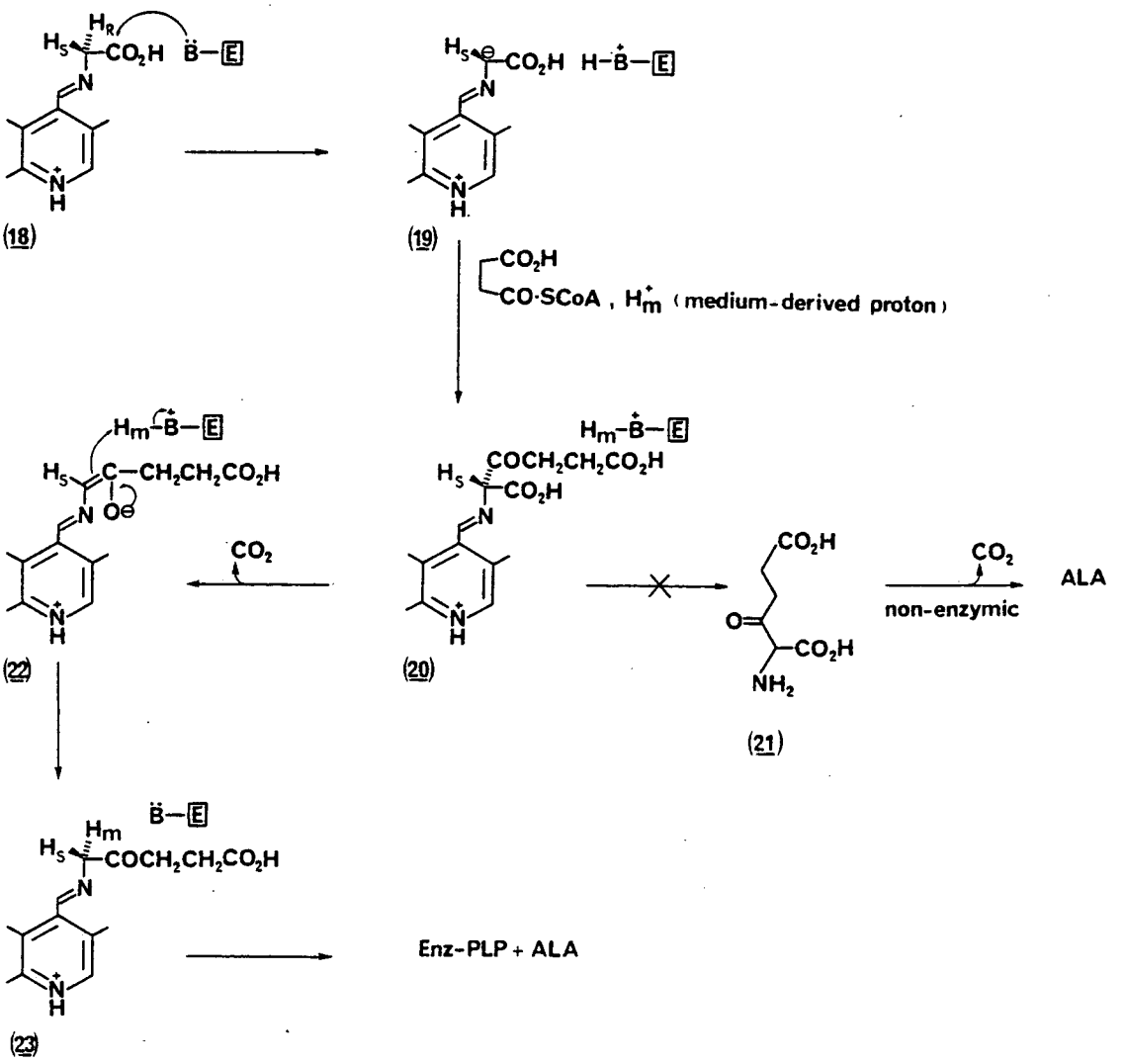
1.4 Porphyrin Biosynthesis

The pathway depicted in Scheme 1 shows the results of some classic studies in porphyrin biosynthesis. The nature of some of the enzymic intermediates involved in haem biosynthesis was inferred from labelling studies by Shemin and co-workers around 1950.[19] Similarly, Granick[52] found that chlorophyll biosynthesis followed such a route in the early stages, using mutants of *Chlorella*. Shemin[53] subsequently demonstrated the formation of corrins from the same initial building blocks.

1.4.1 The Biosynthesis of ALA

(i) Biosynthesis in animals and bacteria.

As mentioned in Section 1.2, ALA (6) in animals and bacteria is formed from the condensation of glycine with succinyl-CoA by the action of ALA synthetase. The mechanism of this enzyme has largely been established by Akhtar and his colleagues,[54-56] and is shown in Scheme 3. By a series of elegant chiral labelling studies, Akhtar showed that the first step in the enzymic reaction is the binding of glycine to the cofactor pyridoxal phosphate, via a Schiff's base (18). Removal of the *pro-R* hydrogen of glycine

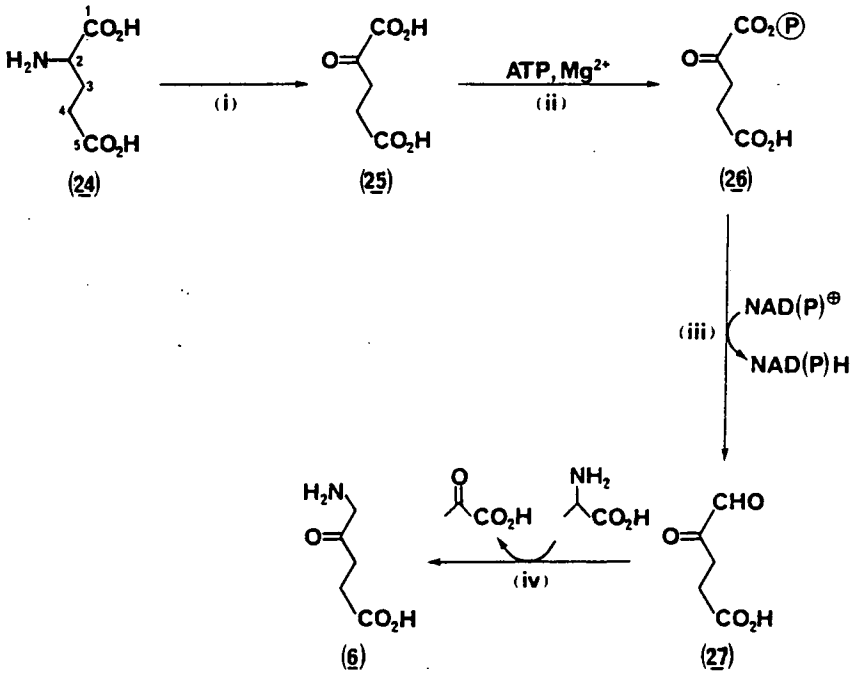


SCHEME 3

forms (19) which condenses with succinyl-CoA to give the intermediate (20). This species was shown to decarboxylate, giving (22) and not 1-amino-2-oxoadipate (21), the latter being able to decarboxylate non-enzymically to give ALA. Using [^{3}H]-glycine Akhtar proved, by determining the chirality of the ALA produced, that decarboxylation must take place on the enzyme surface. The species (22) thus generated becomes subsequently protonated, incorporating a proton from the medium and yielding ALA via (23).

(ii) Biosynthesis in plants.

The biosynthesis of ALA in plants occurs by an altogether different route from that of animals and bacteria. This pathway is often known as the C_5 pathway, since when Beale and his co-workers first proposed it,[57] glutamate was found to be elaborated into ALA. The details of the mechanism for the sequence of enzyme-reactions which convert glutamate into ALA are not fully understood, and there is still considerable uncertainty between the postulated mechanisms.[58] Perhaps one of the most viable mechanisms is shown in Scheme 4.



SCHEME 4. (i) Glutamate transaminase; (ii) A phosphorylase; (iii) A reductase; (iv) ALA transaminase.

Whether or not the enzymes act in a multi-enzyme complex has been the subject of recent speculation.[58,59] However, the essence of the mechanism includes transamination of glutamate (24) to give 2-oxoglutarate (25) which after phosphorylation by an Mg^{2+}-dependent

enzyme, reduction by an NADPH-dependent enzyme gives 4,5-dioxovalerate (DOVA, 27). The conversion of DOVA (27) into ALA is catalysed by a well-characterised[59,60] enzyme, 4,5-dioxovalerate:alanine transaminase (sometimes called DOVA or ALA transaminase) in a classic transaminase reaction.

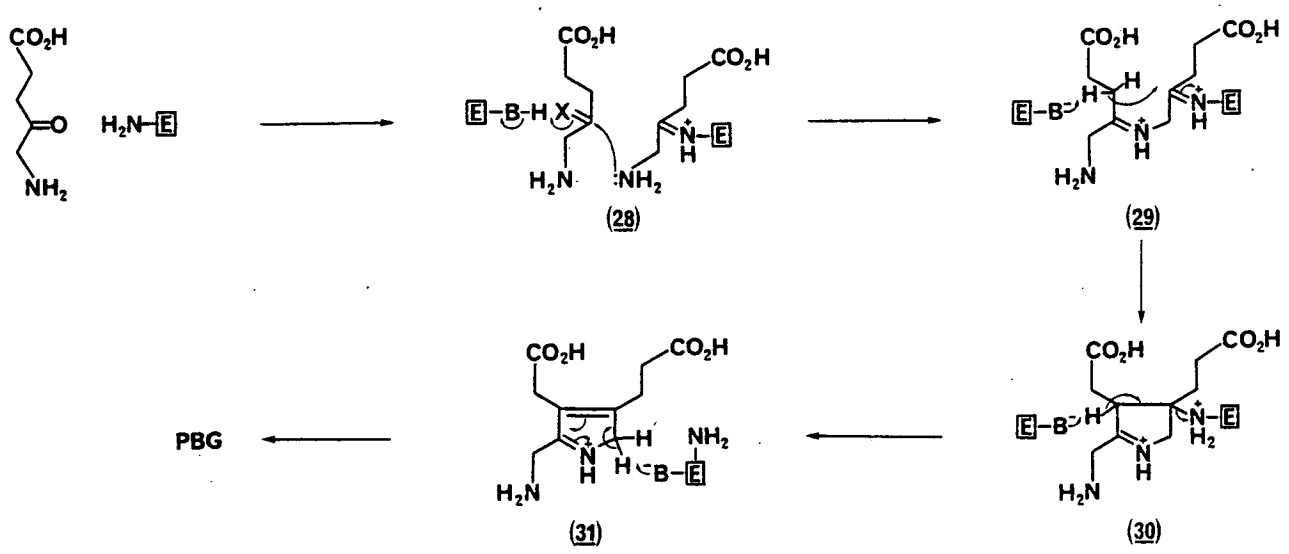
Many of the enzymes in the C_5 pathway have also been detected in animals and bacteria. While the picture for ALA biosynthesis in higher plants seems fairly clear, the extent to which this pathway operates in animals and bacteria is as yet uncertain. Further work is needed in order to establish the contributions from both pathways.

1.4.2 The Biosynthesis of PBG

The transformation of ALA (6) into PBG (7) is mediated by the action of the enzyme ALA dehydratase (sometimes called PBG synthetase). The enzyme catalyses a typical Knorr reaction between two molecules of ALA, involving aldol condensation, dehydration and Schiff's base formation.

Studies by Shemin and co-workers[61-63] established that the substrate molecule becomes covalently attached to the enzyme via a Schiff's base with the ε-NH_2 of a lysine residue. Further stereochemical investigations by Abboud and Akhtar[64] confirmed that the *pro-R* hydrogen originally present at C-5 of ALA is lost stereospecifically during PBG formation. These results were consistent with the hypothetical mechanism put forward by Shemin.[63] Later investigations by Jordan and Seehra[65,66] revised the original idea that the first molecule of ALA to bind to the enzyme gives rise to the half of the molecule with the acetate side-chain. Using both

[^{14}C]- and later [^{13}C]-ALA in single-turnover experiments, Jordan showed that the first ALA molecule to bind gives rise to the half of PBG with the propionate side-chain, as shown in Scheme 5. The



SCHEME 5

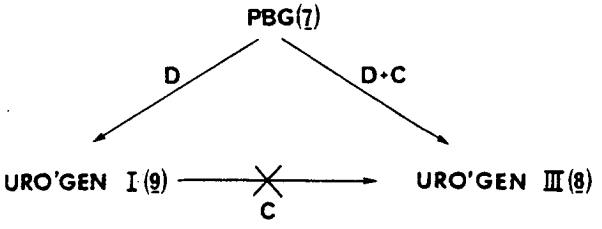
single-turnover technique involves incubating the enzyme initially with a small quantity of labelled ALA, and then following through with a large excess of unlabelled substrate.

The mechanism outlined in Scheme 5 shows the speculations of Jordan[66] which are consistent with the experimental data. The exact details for the mechanism currently await the application of such techniques as ^{13}C NMR and X-ray spectroscopy.

1.4.3 The Biosynthesis of Uro'gen III

Of all the problems in porphyrin biosynthesis, it is the biosynthesis of uro'gen III that has attracted the most attention. Since it is also the focal point for the studies to be described in Part II, it will be examined in some detail.

The pioneering studies of Bogorad, Granick and Rimington[67,68] established the cooperative effort of two proteins, differing in their stability to heat, in the transformation of PBG (7) into uro'gen III (8). The heat-stable enzyme is known as PBG-deaminase (also called by a variety of other names, including uroporphyrinogen I synthase, HMB synthase, and also simply "deaminase", to which it will be referred hereafter.)* The thermally labile enzyme, which was found to be completely inactivated when heated at 55°C for 30 minutes, is known as uroporphyrinogen III cosynthetase (hereafter referred to as "cosynthetase"). It was found that deaminase catalyses the condensation of four molecules of PBG in the formation of one molecule of uro'gen I and four molecules of ammonia. Cosynthetase, however, shows no catalytic activity when incubated with either PBG (7) or uro'gen I (9) (see Scheme 6). This adds a new dimension



SCHEME 6

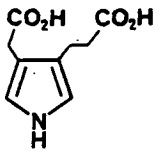
to the Type III Problem as delineated in section 1.2, for here are two enzymes which when acting together give the biologically useful

---

*The IUB has recently recommended the name "HMB synthase", but as will be revealed by the work presented here, this name no better represents the enzyme's function than does uroporphyrinogen I synthase. However, the name PBG deaminase is non-committal as to the nature of the product of the enzyme, and is therefore the name employed throughout this thesis.

isomer, and when acting separately, give rise to the biologically useless isomer.

Deaminase was first isolated from spinach leaves in 1955.[69,70] Since then it has been purified from a number of sources, and some of its properties are summarised in Table 1.3. From such data, it is generally accepted that deaminase is a monomer with $M_r \simeq 37{,}000$. The values for the Michaelis constant (K_m) show that the enzyme has a high affinity for PBG. The enzymes from all sources show strong non-competitive inhibition by sulphydryl reagents such as p-chloromercuric benzoate, mercuric chloride, and N-ethyl maleimide. These effects can be reversed by dialysis with thiol reagents such as 0.1 M cysteine, dithiothreitol or β-mercaptoethanol. Competitive inhibition has been demonstrated using analogues of PBG, such as opsopyrroledicarboxylic acid (32) ($K_i \simeq 0.28$ mM) and iso-porphobilinogen (33) ($K_i \simeq 0.51$ mM).[80-83] When other pyrrole analogues were used, it was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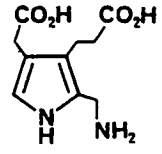
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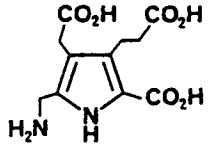
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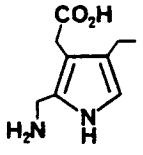
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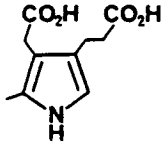
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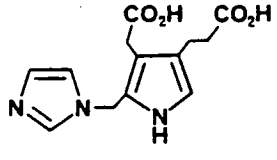
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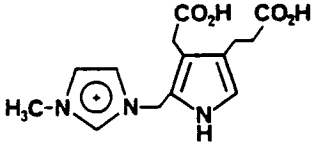
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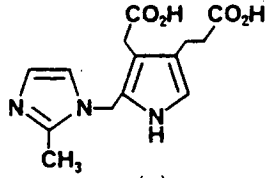

(39)

TABLE 1.3. Properties of PBG deaminase (EC 4.3.1.8).

Source of Enzyme	M_r/daltons	pH_{opt}	pI	K_m(PBG)/M	Max. Spec. Activity[a] /Umg^{-1}	Ref.
R. spheroides	35-36,000	7.8-8.0	4.46	4×10^{-5} (pH 7.8-8.0)	27	71
	36-39,000	7.6 (Phos)	-	$1.3\text{-}2 \times 10^{-5}$ (pH 7.6)	128	72
Bovine liver	-	7.4	-	5×10^{-6}	0.01	73
Wheat germ	38-40,000[g]	8.2 (Phos)	4.2-4.5[g]	5×10^{-5}	1.5[b]	74
Spinach leaves	38-40,000	8.1[c]	4.2-4.5	7.2×10^{-5} (pH 8.2)	0.8[b]	75,76
Euglena gracilis	39-43,000	7.0-8.1[d]	-	7×10^{-5} or[e] 1.95×10^{-4}[e]	6.0, 28[f]	77
Human erythrocytes	20-30,000	7.4 (Phos) 8.2 (Tris)	-	1.3×10^{-4} (pH 7.4)	16	78
	37,000	8.0 (Tris)	6.2	6×10^{-6} (pH 8.0)	2.34	79

[a]1U = 1 μmol uroporphyrin produced per hour at 37°C. Note that 1u is also used, which is defined as 1 nmol uroporphyrin produced per hour. [b]1U = 1 μmol PBG consumed per hour - see Ref. 76; [c]From Ref. 70; [d]From Ref. 17, p. 77; [e]From Ref. 125; [f]From Ref. 136; [g]From Ref. 75.

found that PBG α-carboxylic acid (34) had no effect,[84] both the ethyl analogue of PBG (35) and the acidic pyrrole (36) acted as competitive inhibitors, and the dimethyl ester of (36) showed no inhibitory effect.[78] Finally, PBG-imidazole (37) and its two methylated derivatives (38) and (39) showed no effect.[85] Such results clearly point to the involvement of one or both carboxyl groups in binding,[78] and some degree of tolerance to the nucleophilic group attached to C-11.

The only reported amino acid analysis of deaminase is shown in Table 1.4, for the human enzyme. To date, no sequencing information on the enzyme has been published.

Cosynthetase was first isolated from wheat germ in 1958.[70] It proved more difficult to purify, in particular to separate it from deaminase. This fact, coupled with the lack of a simple, accurate assay method until recently, has resulted in scant information on the enzyme. Furthermore, as we shall see, many of the studies of this enzyme have employed a deaminase-cosynthetase mixture, which is why our mechanistic investigation will be unable to focus on each enzyme in turn.

Some of the properties of cosynthetase are summarised in Table 1.5. Early assay methods for the enzyme relied upon separating the uroporphyrin I and III isomers. Complete and reproducible separation has only recently been achieved using HPLC methods, either of the derivatised coproporphyrins,[91,92] the uroporphyrin octamethyl esters,[93-95] or the uroporphyrin free acids.[96,97] Much more recently, a rapid assay method has been reported which does not involve separating type I and III isomers.[98]

TABLE 1.4. Amino acid composition of human deaminase.[a]

Amino Acid	Mol/37,000 g of Protein	Nearest Integer/ 37,000 g of Protein
Aspartic acid	36.4	36
Theonine	18.0	18
Serine	20.7	21
Glutamic acid	40.1	40
Proline	12.0	12
Glycine	29.9	30
Alanine	22.9	23
Valine	17.0	17
Half cystine	2.4	2
Methionine	0.1	0
Isoleucine	15.0	15
Leucine	39.1	39
Tyrosine	1.0	1
Phenylalanine	7.8	8
Lysine	16.7	17
Histidine	3.4	3
Arginine	10.2	10
Tryptophan	0.0	0

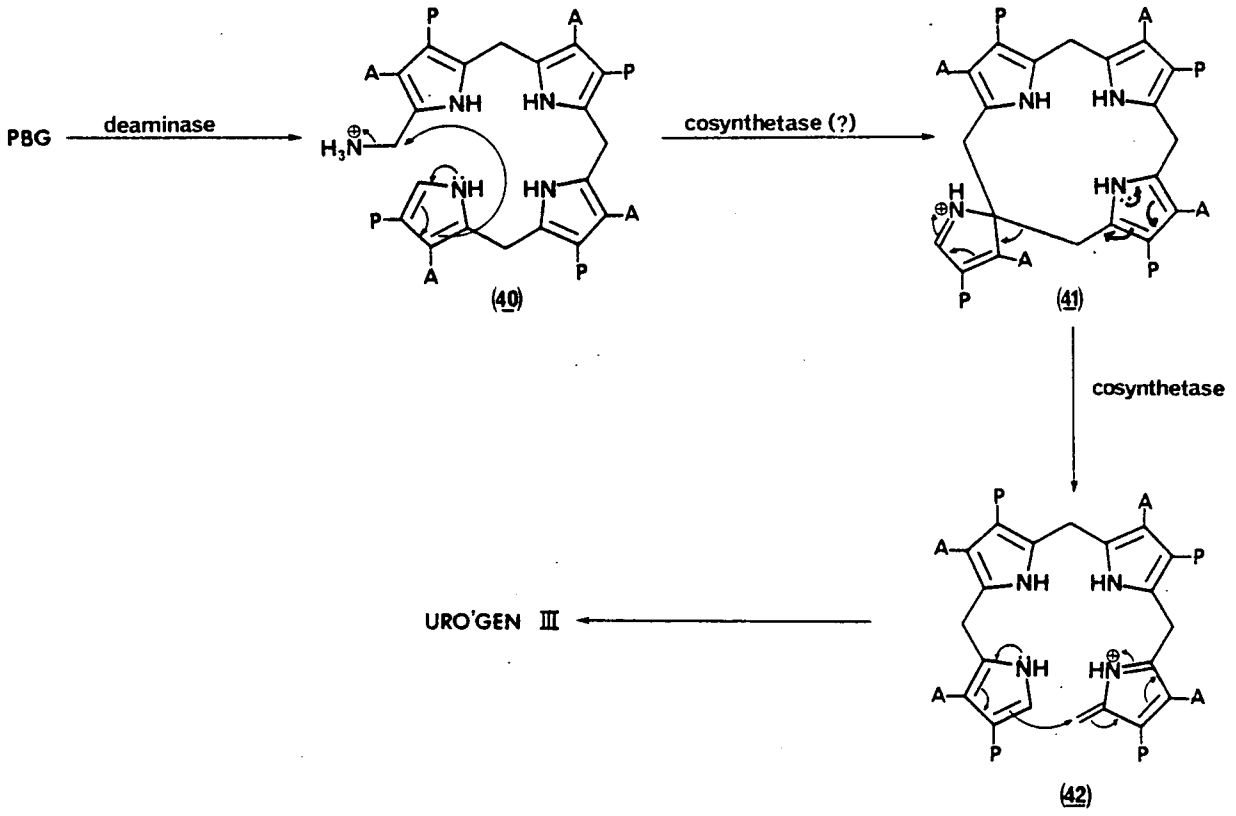
[a]Data taken from Ref. 79.

TABLE 1.5. Properties of uro'gen III cosynthetase (EC 4.2.1.75).

Source of Enzyme	M_r/daltons	pH_{opt}	pI	K_m(HMB)/M	Max. Spec. Activity[a] /Umg^{-1}	Ref.
Wheat germ	60-65,000	-	6.1-6.7	-	-	75
	-	8.2	-	-	-	86
Spinach leaves	-	-	-	-	-	76
Mouse spleen	33-60,000	8.5	-	-	-	87
Human erythrocytes	-	-	-	-	-	88
R. spheroides	30,000 (15,000 subunit)	7.8 (Phos or Tris)	4.6-4.8	-	82	89
E. gracilis	-	-	-	11.3×10^{-6}	-	90

[a] 1U = 1 μmol of uro'gen III per hour.

Initial views about the action of deaminase and cosynthetase favoured an association between the two proteins,[75,78,99] with cosynthetase even operating as a "specifier protein". This stems from the observation that the rate of consumption of PBG by deaminase is increased by added cosynthetase at low substrate concentrations.[87] Apart from one report,[100] one of the striking features of uro'gen I or III biosynthesis was the absence of formation of any detectable free intermediates. For at least a decade no experimental answers were forthcoming and this generated a flow of speculative thinking. Some 25 different hypothetical mechanisms as to the nature of the rearrangement were proposed during this period.[101] Work over the last ten years has served to eliminate a large majority of these, and now only variations on one mechanism, put forward by Mathewson and Corwin[102] in 1960, remain favoured. In this mechanism (see Scheme 7) a linear tetrapyrrole is assembled and then intramolecular

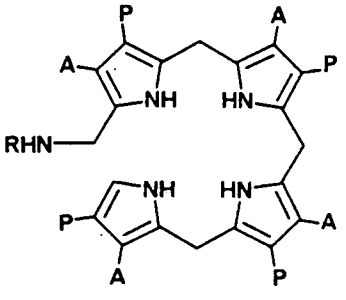


SCHEME 7. Mathewson and Corwin mechanism for uro'gen III formation.

rearrangement of one PBG unit gives rise to uro'gen III via a "spiro" intermediate (41).

While the Mathewson and Corwin mechanism is favoured now, up until the early 1970's almost all the proposed mechanisms seemed tenable. Studies of the Type III Problem then focused on two main questions: (i) What is the timing of the rearrangement? (ii) What is the nature of the rearrangement? The discovery that the action of deaminase on PBG in the presence of ammonium ions or hydroxylamine led to the accumulation of oligopyrroles,[99,103] stimulated considerable interest in the first question. Interest settled upon the possible intermediacy of di- or tri-pyrroles in porphyrin biosynthesis, and this gave rise to intense synthetic effort in three groups in Buenos Aires,[104,105] Cambridge,[17,106] and Yale.[107] For some time results were inconclusive due to anomalous incorporations and non-enzymic reactions of these species. The reader is directed to reviews of this period of research for further information.[16,17]

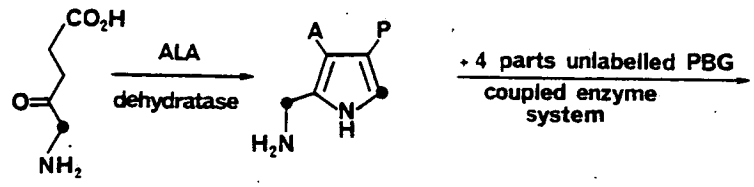
Since the timing of the rearrangement could take place in theory at any level between monopyrrole and tetrapyrrole, the search for free intermediates continued. The possibility for the intermediacy of tetrapyrroles, or bilanes as they are also known, was recognised quite early on by Radmer and Bogorad[108] in 1972, when they isolated a substance whose structure they inferred to be that of aminomethylbilane (40).



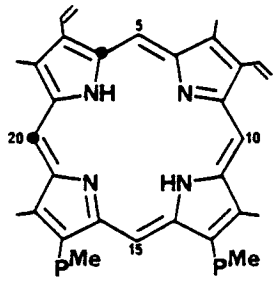
(40) R = H
(43) R = HO
(44) R = MeO

Further credence to this observation came a year later, when Davies and Neuberger[71] reported the detection of polypyrroles which accumulated when the enzyme and PBG were incubated in the presence of amines of the type RNH_2, where R=H, HO, or MeO. These were identified as tetrapyrroles by a variety of techniques, and it was found that aminomethylbilane (40) released ammonia on non-enzymic cyclisation to give uro'gen I. Likewise, the other two tetrapyrroles (43, 44) released the corresponding bases on cyclisation. Furthermore, the lack of accumulation of any oligopyrroles other than bilanes seemed strong evidence in favour of the latter's intermediacy in porphyrin biosynthesis.

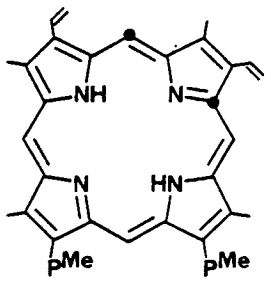
In the same year as the observations of Davies and Neuberger, Battersby and his colleagues[109,110] turned their attention to the second question, what is the nature of the rearrangement? By elegant use of ^{13}C NMR, Battersby showed that the porphyrin D-ring is rearranged intramolecularly. He did this by incubating [2,11-^{13}C]-PBG (45) with deaminase-cosynthetase in a coupled enzyme system from avian erythrocytes and bovine mitochondria, which produces protoporphyrin IX (47-50), analysed as its ester. The [2,11-^{13}C]-PBG was available from [5-^{13}C]-ALA (46) by the action of ALA dehydratase,[111] and the crucial step was to dilute this PBG five-fold with unlabelled PBG prior to conversion to protoporphyrin IX. ^{13}C NMR analysis in the presence of praseodymium shift reagent revealed three doublets with ^3J = 5.5 Hz and one with ^1J = 72 Hz. The latter was assigned to C-15 (50) on the basis of rational synthesis of ^{13}C-labelled protoporphyrins,[112-114] and thus establishing that the D ring is intramolecularly rearranged (see Scheme 8).



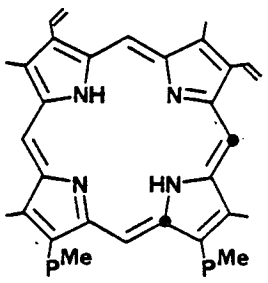
[5-^{13}C] ALA (45) [2,11-^{13}C] PBG (46)



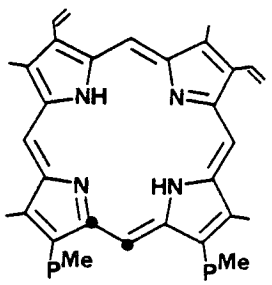
(47) ^{3}J = 5·5 Hz



(48)



(49)

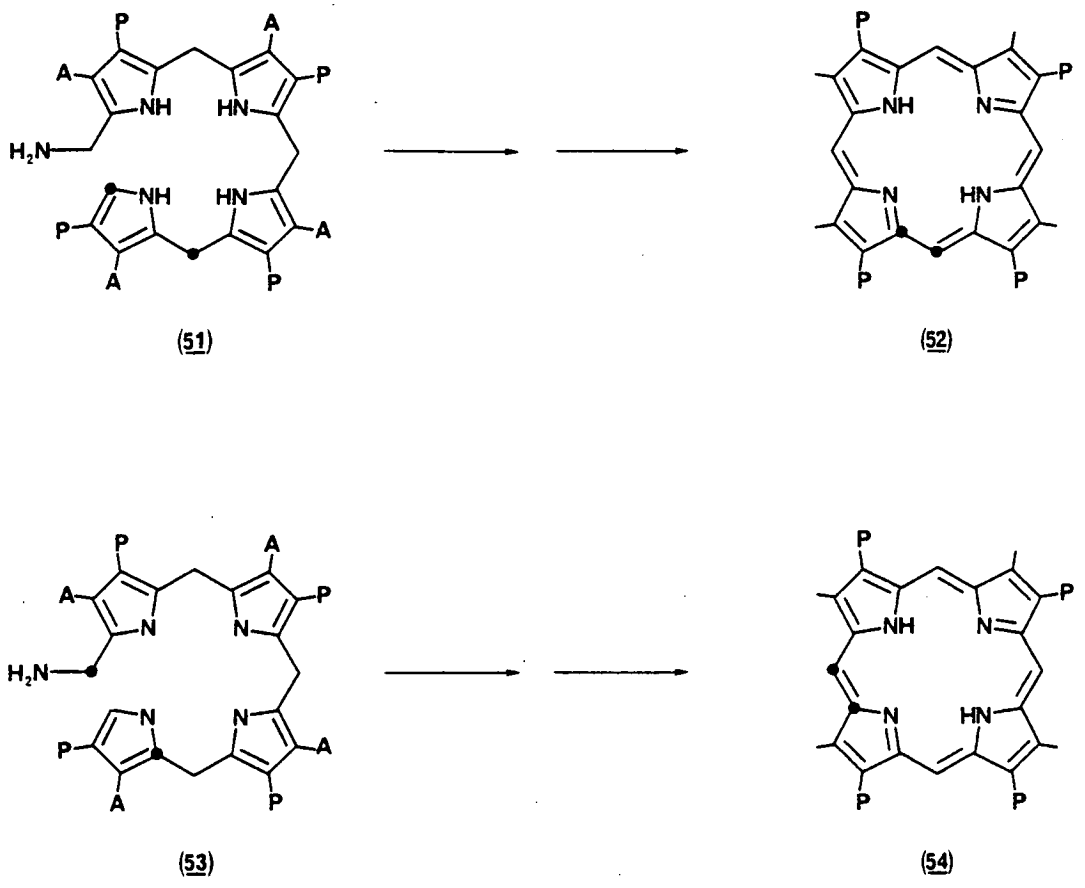


(50) ^{1}J = 72 Hz

SCHEME 8

Although the timing of the rearrangement appeared to be at the tetrapyrrole level, the first test of this came in 1975 by Müller *et al.*[115] in Stuttgart. He found that incubating [4-^{14}C]-ALA with a cell-free extract from *Clostridium tetanomorphum* in the presence of aminomethylbilane (40) resulted in significant radioisotopic dilution over the blank run without bilane, when the product cobyrinic acid (16) was isolated. Shortly afterwards the Cambridge group confirmed unequivocally using ^{13}C NMR that the rearrangement takes

place after head-to-tail combination of four PBG units. They synthesised aminomethylbilane[116,117] bearing two different ^{13}C-labelling patterns (51, 53), incubated each with deaminase-cosynthetase from *Euglena gracilis*, and analysed the isolated coproporphyrin III esters (52, 54) by ^{13}C NMR.[118,119] Use of praseodymium shift reagents again enabled a distinction between the two products. Coproporphyrin (52) showed a doublet (^1J = 72 Hz) which was strongly affected by shift reagent, indicating[110] that it corresponds to C-15, whereas coproporphyrin (54) also showed a doublet (^1J = 72 Hz) but this was virtually unaffected by shift reagent (see Scheme 9). Confirmation



SCHEME 9

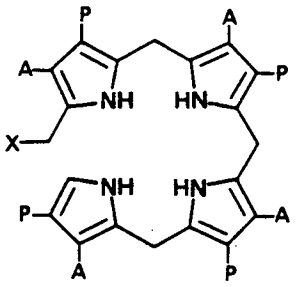
of these NMR observations had already been provided by total synthesis of the protoporphyrins.[110]

These results, especially using the labelling pattern depicted in (54), proved beyond doubt that intramolecular rearrangement takes place at the tetrapyrrole exactly as in Mathewson and Corwin's hypothesis. Further studies by the Cambridge group in 1978,[120,121] using synthetic isomeric aminomethylbilanes bearing one or more rings "inverted", showed that the unrearranged bilane (40) served as the best substrate for the *Euglena* enzyme system, in contrast to a report from Frydman *et al.*,[122] where such bilanes did not serve as substrates at all. Such results led Battersby to conclude[120] that the aminomethylbilane (40) was the enzymic intermediate between PBG and uro'gen III.

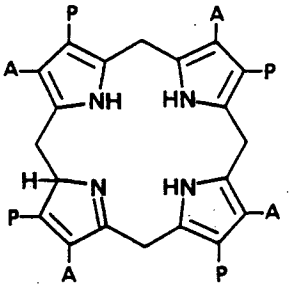
Also in 1978, Scott and co-workers[123] in Texas presented ^{13}C NMR evidence for the direct observation of a transient free intermediate, which they called pre-uroporphyrinogen or pre-uro'gen. They incubated [11-^{13}C]-PBG with highly purified deaminase from *R. spheroides* in the NMR tube, and watched the formation of uro'gen I. Having characterised the spectra of PBG and uro'gen I, when the enzymic reaction was allowed to proceed until 47% of the PBG had been consumed (11 minutes), a set of new signals appeared at 21.85-22.15 ppm and 54.78 ppm. As the reaction proceeded to completion these signals disappeared, and the meso carbon atoms of uro'gen I appeared at 21.63 ppm. When purified cosynthetase was added, none of these signals were observed and the uro'gen III signal increased proportionally.

These results were rationalised in terms of five possible structures for the intermediate (40, 41, 55-57). Since uro'gen III was formed at the expense of pre-uro'gen, it presumably served as a substrate for cosynthetase and suggested that it was formed from a

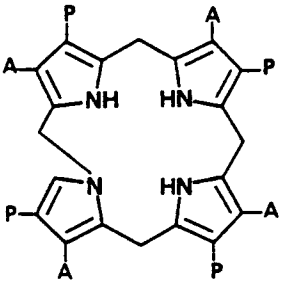
tetrapyrrole. The signals were thus consistent with (55) as a possible structure, especially since acid or base treatment of



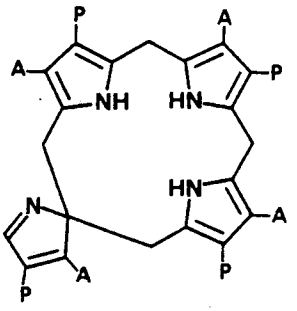
X = OH, HYDROXYMETHYLBILANE (55)
X = NH_2 (40)



URO'GEN I TAUTOMER (56)



"N-ALKYL" (57)

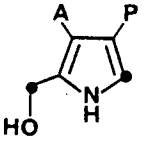


"SPIRO" (41)

pre-uro'gen gave only uro'gen I. The aminomethylbilane (40) was discounted on the basis of chemical shift, since a $^{13}CH_2NH_2$ in such a system would be expected to give a signal at 34.6 ppm. Of the four structures (41, 55-57), note that the uro'gen I tautomer (56) might be expected to arise from chemical cyclisation of a tetrapyrrole prior to uro'gen I formation. However, the uro'gen I tautomer (56) was ruled out when [2,11-^{13}C]-PBG served as a substrate, since no ^{13}C-^{13}C coupling was detected at 54.78 ppm, as required for structure (56). No distinction could be made between the remaining structures, (41), (55) and (57), due to the instability of pre-uro'gen ($t_{\frac{1}{2}}$ = 4 minutes at 37°C, pH 8).

Further evidence that pre-uro'gen acted as a substrate for cosynthetase was provided by the same group,[124] when deaminase-free solutions of pre-uro'gen (obtained by filtration through a membrane) were incubated with cosynthetase and the products analysed by HPLC as their porphyrin esters. Thus Scott and co-workers were able to show that the enzymes deaminase and cosynthetase act *in tandem* and not in association with one another.

These observations stimulated a period of intense research by both the Texas group and the Cambridge group. The first rejoinder from Cambridge reported[125] the observation that there was a clear lag in the formation of uro'gen I from aminomethylbilane (40). The lag was even more evident when consumption of PBG by deaminase, and production of uro'gen I, was determined. No such lag was observed when deaminase-cosynthetase was used. To study the nature of the intermediate that must account for the lag, the Cambridge group generated the intermediate from [11-^{13}C]-PBG, adjusted the pH to >12, and analysed the species by ^{13}C NMR. They found signals at 24.46 ppm and 57.21 ppm, which probably arose from the same species as the Texas group were studying, taking into account shift differences due to referencing. The same signals were also obtained without pH adjustment, but were much shorter-lived ($t_{\frac{1}{2}}$ = 4 minutes at 37°C, pH 8.25). These results were interpreted as evidence for the hydroxymethylbilane (55), on the basis of ^{13}C NMR analysis of hydroxymethylpyrrole. The hydroxy analogue (58) of



(58)

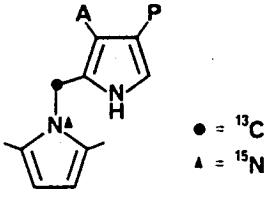
[2,11-^{13}C]-PBG was found to give a signal corresponding to HO-$\underline{CH}_2$-pyrrole at 57.27 ppm (pH >12), and furthermore found to act as a good substrate for deaminase-cosynthetase. ~~Like~~ As with the Texas group, addition of deaminase-cosynthetase to the intermediate produced enhanced quantities of uro'gen III, which led Battersby to support the view that the two enzymes associate in some way rather than acting independently.

In order to validate the earlier work with aminomethylbilane, the Cambridge group also showed that incubation of [11-^{13}C]-PBG with deaminase in the presence of $^{15}NH_4^+$ resulted in the formation of the "trapped" intermediate $^{15}NH_2$-$^{13}CH_2$-bilane, which exhibited a doublet at 38.3 ppm (^1J (^{13}C-^{15}N) = 3.68 Hz). Non-enzymic runs with synthetic [aminomethyl-^{13}C]-bilane and $^{15}NH_4^+$ showed no indication of nucleophilic displacement of the amino group.

The foregoing picture became somewhat more complicated when the Texas group reported evidence[126] in favour of the N-alkylated macrocycle (57) for the structure of pre-uro'gen. They made use of ^{13}C and ^{15}N NMR to analyse the pre-uro'gen generated as before using [11-^{13}C, 1-^{15}N]-PBG. The ^{13}C NMR spectrum revealed a doublet at 54.78 ppm (^1J (^{13}C-^{15}N) = 6 Hz) where previously the non-^{15}N species showed a singlet, when run at 0°C after a 3 minute incubation of [11-^{13}C, 1-^{15}N]-PBG with deaminase at 37°C. On heating to 37°C, the signals disappeared with concomitant formation of uro'gen I. When the ^{15}N NMR spectrum was obtained, a resonance at 155.7 ppm assigned to the ring D pyrrole nitrogen appeared as a doublet (^1J (^{15}N-^{13}C) = 6 Hz). Further observations with synthetic aminomethylbilane (40) obtained from Frydman's laboratory, supported Frydman's view[122] that it is not a substrate for cosynthetase, for

it was unaffected by purified cosynthetase. Furthermore, it could be detected in pre-uro'gen preparations, presumably due to trapping of the intermediate with ammonium ions. This was corroborated when an ammonia-consuming enzyme system (glutamate dehydrogenase, α-keto-glutarate, NADH) was added, and no aminomethylbilane formed. It was suggested that the same trapping mechanism could give rise to the hydroxymethylbilane also.

The Cambridge group rapidly responded[127,128] by reporting the total synthesis of hydroxymethylbilane (55) (hereafter referred to as HMB) and its incorporation into uro'gen III. Furthermore, its chemical, spectroscopic and quantitative enzymic properties were identical to the HMB generated enzymically. It served as a substrate for deaminase-free cosynthetase in an identical manner to the enzymically generated HMB. Further evidence against the involvement of an N-alkyl intermediate was presented when a model compound (59)



(59)

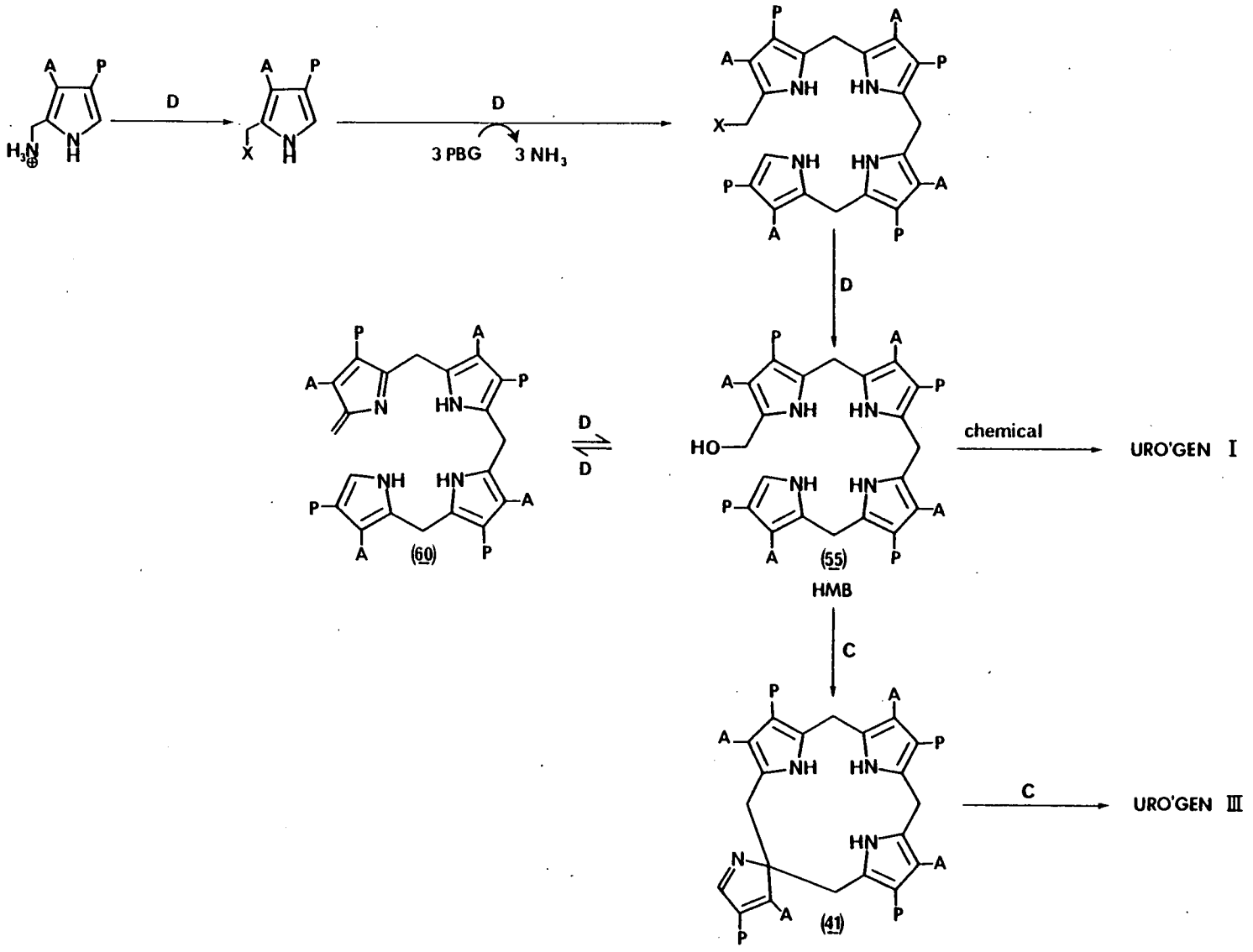
was synthesised, and its ^{13}C NMR spectrum at pH 8.0 showed a doublet at 42.00 ppm, some 12 ppm to higher field than pre-uro'gen, with a one-bond ^{1}J (^{13}C-^{15}N) of 2.4 ± 0.1 Hz, both at pH 8.25 and >12. From this, the 6 Hz doublet observed in Texas was seen as an artefact.

Further observations by the Texas group[129] helped to clarify the situation, when under conditions of high concentrations of enzyme and substrate, a resonance at 54.78 ppm (37°C, pH 8.2) exhibiting two-bond coupling (^{2}J (^{13}C-^{15}N) = 1.8 ± 1 Hz) was detected. Thus

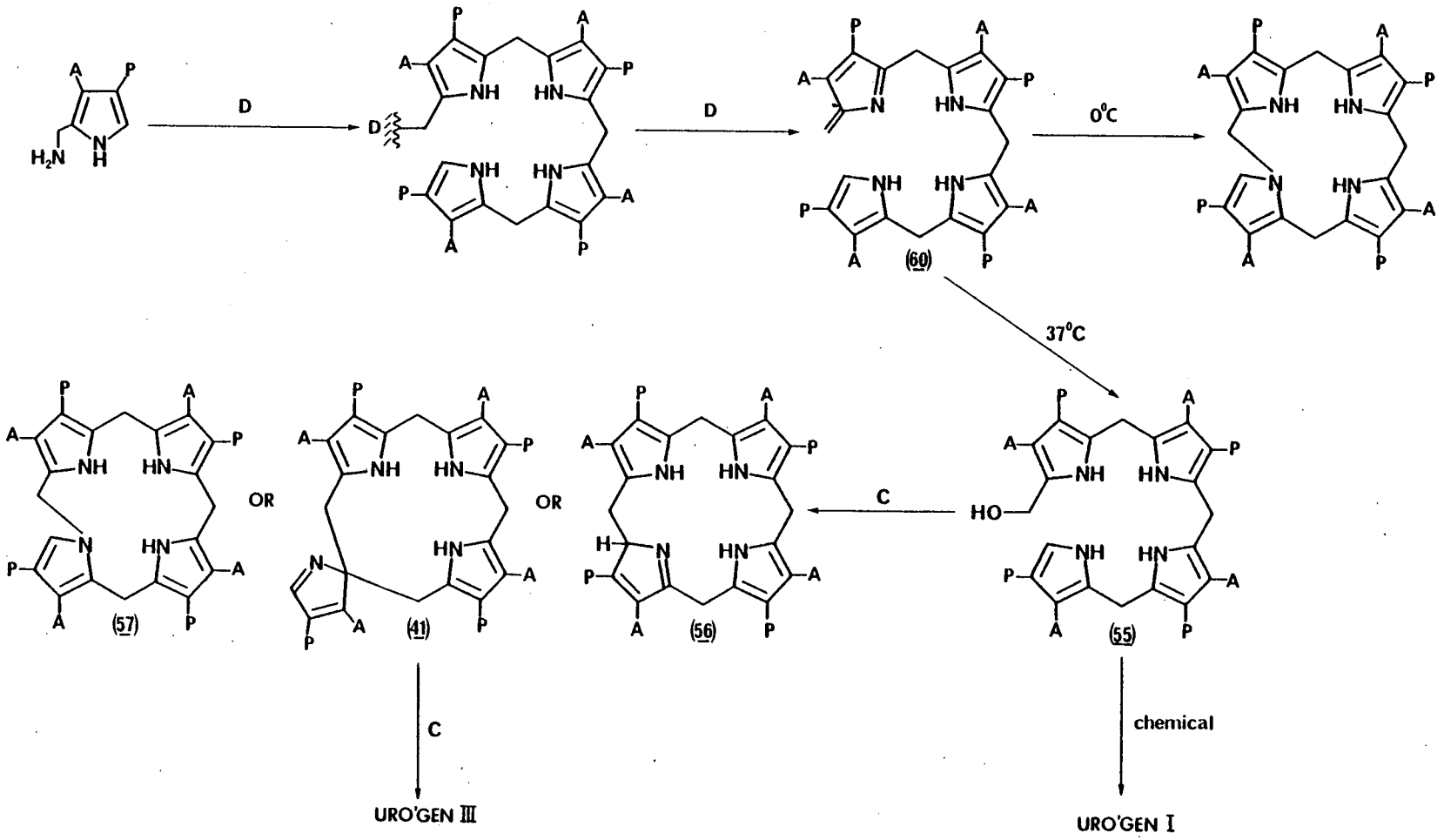
it was clear that at pH 8-12 and 37°C, both groups were handling the same species, namely HMB (55). However, that the 6 Hz doublet may be an artefact was discounted since it had been repeatedly observed with both 4.7 T and 7.0 T spectrometers. The anomalous chemical shift and coupling constant could be explained if it is recognised that the N-alkyl macrocycle (57) at 0°C is conformationally distinct from the model N-alkyl species (59). The reason why the Cambridge group failed to detect the 6 Hz coupling could be explained as a result of their spectra not being recorded at pH 7-8.5 and 0°C. Therefore, the Texas group concluded that HMB represents the nucleophilic trapping of an exomethylene pyrrolenine species with water at 37°C, and it is this latter species that represents the true substrate for cosynthetase.

The Cambridge group then reported[130] their findings at pH 7-8.5 and 0°C. Incubation of [11-^{13}C, 1-^{15}N]-PBG with deaminase at 37°C and pH 8.25 for 7 minutes, cooling to 0°C, and then recording the ^{13}C NMR spectrum at 4°C revealed no signs of any 6 Hz coupling. Again the doublet at 57.1 ppm (^{1}J (^{13}C-^{15}N) = 2.4 Hz) was observed. When hydroxylamine was added to the enzyme-free solution of bilane, and deaminase subsequently added, the hydroxylamino derivative (43) of HMB accumulated, representing the nucleophilically trapped version of a more reactive species, such as an exomethylene pyrrolenine (60, see Scheme 10).

It is worth pausing at this point in 1980 in the chronology of events surrounding the discovery of the free intermediate between PBG and uro'gen III, to note that this is where the work to be described in Part II of this thesis commenced. The two working hypotheses held by both Cambridge[131] and Texas[132] groups in 1980



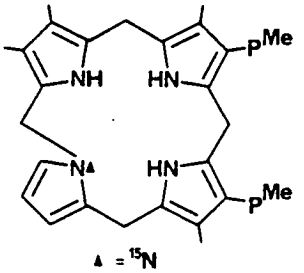
SCHEME 10. Battersby's mechanism for uro'gen III formation (1980).



SCHEME 11. Scott's mechanism for uro'gen III formation (1980).

are summarised in Schemes 10 and 11. In Battersby's mechanism, PBG units are built up into a linear tetrapyrrole on the surface of the enzyme (X group is unknown) which becomes free from the enzyme as HMB (55), a species in equilibrium with the exomethylene pyrrolenine (60). HMB is then the substrate for cosynthetase, in acknowledgement of the fact that the enzymes act *in tandem*, and forms uro'gen III via the spiro species (41) originally proposed by Mathewson and Corwin. In contrast, in Scott's mechanism the species that comes off the surface of the deaminase is the exomethylene pyrrolenine (60), which at 0°C could form the N-alkyl species (57), while at 37°C forms HMB (55) which could go via any one of (57), (41) or (56) to give uro'gen III. Clearly a sustained NMR effort is required if any other intermediates are to be detected, allowing a clearer definition of the mechanism to be made.

Strong evidence against the intermediacy of an N-alkyl structure came late on in the research presented here, in 1983 from Gossauer and Scott,[133] when they reported the total synthesis of a close analogue (61) of the proposed structure (57). Analysis of the



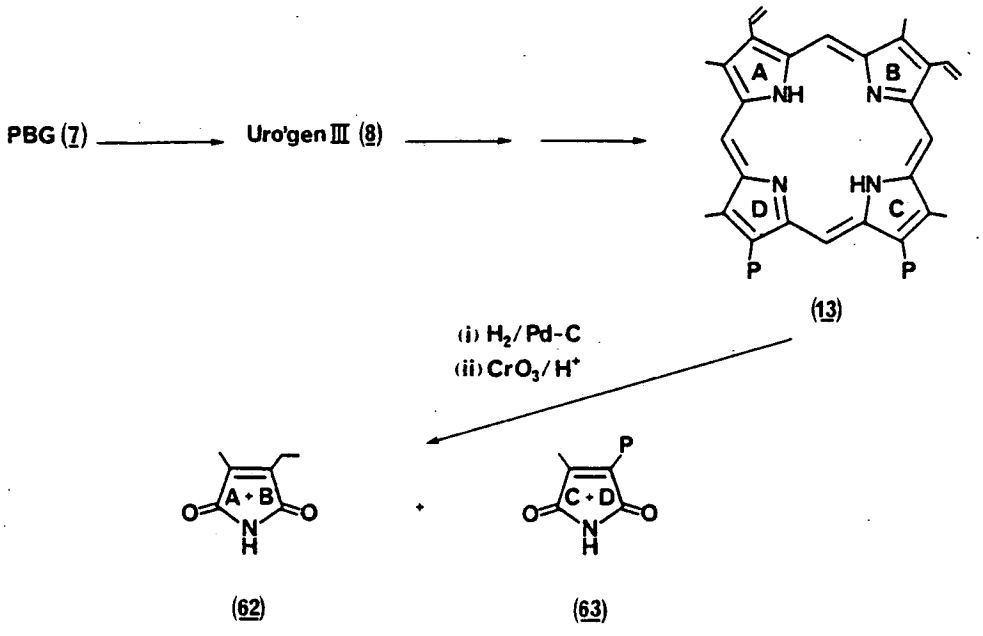
(61)

putative methylene by natural abundance ^{13}C NMR showed a doublet at 41.52 ppm with a one-bond ^{1}J (^{13}C-^{15}N) of 10.7 Hz. This suggests that the 6 Hz coupling may perhaps be an artefact from say, temperature variations in the NMR spectrometer. However, its repeated

observation in a number of spectrometers, and with ^{13}C and ^{15}N, suggests that some effect was observed that is not yet understood.

More detailed studies on deaminase have recently cast light upon the action and mechanism of the enzyme, and this has served to direct the research described in the latter half of Part II. The order of assembly of the four units was investigated independently by Battersby and co-workers[134-136] and Jordan and Seehra.[137,138] Battersby's group employed relatively large amounts of deaminase-cosynthetase to ensure complete loading of the first binding site of the enzymes using unlabelled PBG. Flushing through with [11-^{13}C]-PBG gave uro'gen III which was isolated as its copro-porphyrin III ester for analysis by ^{1}H NMR using europium shift reagents. Integration of the resulting triplets gave an accurate method for quantifying the amount of ^{13}C in the molecule. It was found to be in the order C-20 > C-5 > C-10 > C-15, suggesting clearly a logical order of formation of A, B, C and then D rings.

Jordan's experiments used a similar single-turnover technique in which 1, 2 or 3 molar equivalents of [2,11-^{14}C]-PBG was initially mixed with 1 molar equivalent of deaminase. Excess unlabelled PBG was then used to complete the turnover to uro'gen III, and the product isolated using a coupled enzyme system, as proto-porphyrins IX (13). Chemical degradation of the protoporphyrin IX yielded two different well-characterised maleimides whose specific radioactivity could then be determined (see Scheme 12). The ethyl-methylmaleimide (62) thus obtained arises solely from the A and B rings of the porphyrin, and likewise the haematinic acid (63) arises from the C and D rings. The results proved that the rings were assembled in the order A, B, C and then D.



(62) (63)

SCHEME 12

Following the report from Anderson and Desnick[79] in 1979 of the identification by gel electrophoresis of five catalytically competent forms of human deaminase, representing native enzyme and one, two, three and four bound PBG units, Jordan and Berry[139] investigated the generation of similar enzyme-substrate complexes using [3,5-^{14}C]-PBG and deaminase from *R. spheroides* in a rapid mixing device. Gel-filtration on G-50 Sephadex yielded radioactively labelled protein, which would not result from pre-treatment of the deaminase with opsopyrroledicarboxylic acid (32) or boiling the enzyme. The catalytic competence of the complex was demonstrated by adding excess unlabelled PBG and generating ^{14}C-labelled uro'gen I. The complex was found to be remarkably stable, suggesting a covalent linkage between enzyme and PBG, a fact further corroborated when SDS treatment failed to displace radioactivity. Hydroxylamine, however, displaced radioactivity both in the active and in the SDS-denatured complexes, presumably by nucleophilic displacement.

Jordan and co-workers[140,141] went on to identify the existence of four bands by gel-electrophoresis again representing, as in the case of the complexes of Anderson and Desnick, the native enzyme and enzyme-substrate complexes bearing one, two and three bound PBG units. Interestingly, no complex corresponding to bound tetrapyrrole could be detected. Using this method for the generation of complexes bearing [^{14}C]-PBG, and the rapid-turnover technique described above, Jordan was able to elegantly prove that each band did indeed represent the enzyme-substrate complexes that initially seemed likely.

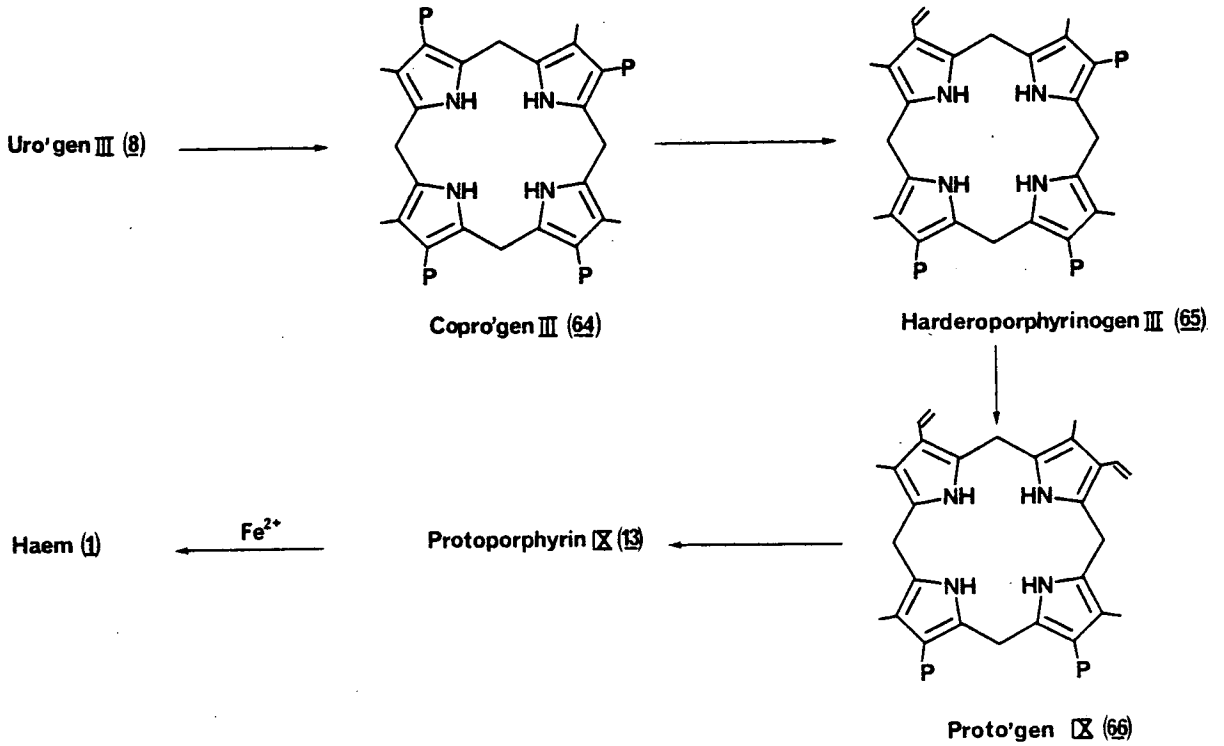
The identification and characterisation of such enzyme-substrate complexes, and the discovery that they are unusually stable clearly suggests that a technique such as NMR could be profitably applied to probe the structure of the active site of this relatively little studied enzyme.

1.4.4 The Later Stages of Porphyrin and Corrin Biosynthesis

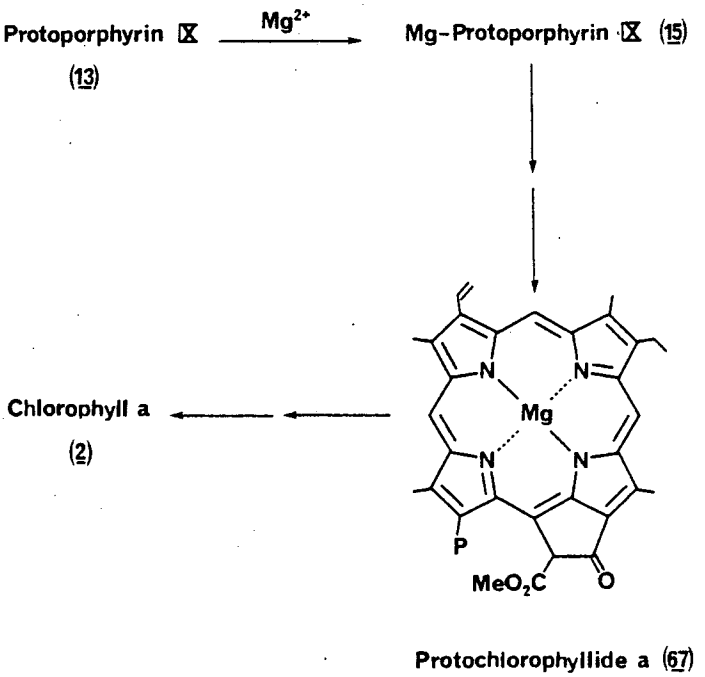
As indicated in Scheme 2, uro'gen III represents the divergence point for porphyrin and corrin biosynthesis. The biosynthesis of haem from uro'gen III is shown in Scheme 13. For further details of each step, the reader is directed to Reference 14 which provides an excellent account of the later stages of porphyrins and corrin biosynthesis.

Chlorophyll branches from the haem pathway at protoporphyrin IX, as shown in Scheme 14.

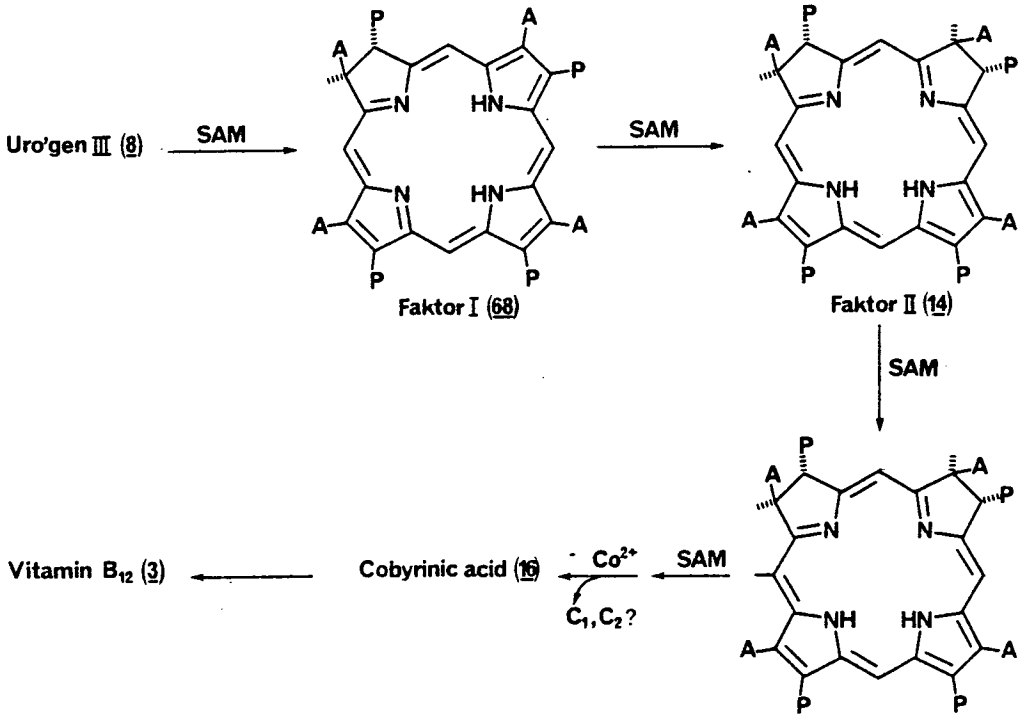
Finally, corrin biosynthesis follows probably the most complex pathway in tetrapyrrole biosynthesis, as shown in Scheme 15. After



SCHEME 13



SCHEME 14



SCHEME 15

it was established[53,99] that ALA, PBG and methionine are involved in the biosynthesis of cobyrinic acid (16), and that cobyrinic acid is elaborated to vitamin B_{12},[142] Scott confirmed[143,144] that uro'gen III is incorporated into vitamin B_{12}, using whole cells of *P. shermanii*. Further work showed that uro'gen III is incorporated without rearrangement.[145]

Parallel studies in three laboratories[146-148] revealed that of the eight methyl groups arranged around the periphery of the corrin macrocycle, seven are derived from methionine, or more properly S-adenosyl methionine or SAM, and one from ALA. Furthermore, Battersby[148-150] and Scott[151] showed that the *pro-R* methyl group at C-12 of B_{12} (3) is derived from methionine. Proof that all the seven methionine-derived methyl groups are transferred intact came simultaneously from the laboratories of Arigoni,[152] Battersby[153] and Scott.[154]

An insight into the sequence of methylation of uro'gen III came with the discovery[155] of sirohydrochlorin (14), the determination of its structure,[156-158] and its successful incorporation into cobyrinic acid.[156-159] This was also confirmed when Müller[160,161] isolated Faktors I (67), II (14) and III (69) from *P. shermanii*, and Faktor II showed all the physical characteristics of (14). It seems likely that the actual biosynthetic intermediate is the reduced dihydrosirohydrochlorin. With the structure of Faktor I rigorously assigned,[161,162] the next step on the pathway to cobyrinic acid came with the determination[156,163] of the structure of Faktor III (69). This was seen obviously to be a trimethylisobacteriochlorin at the outset, but the position of the third methyl group proved difficult to assign.[164-166] The problem now remains, what are the other methylated (from SAM) intermediates on the cobyrinic acid pathway? So far, no light has been shed on this area.

The extrusion of C-20 of Faktor III to give the corrin macrocycle was originally thought[167] to come off as the C_1 unit of formaldehyde. However, there is now strong evidence[168] that it comes off as a C_2 unit, possibly as acetic acid, with the C-20 methyl group still attached. Despite even more recent evidence based on acetic acid trapping experiments,[132,169-171] and also a precedence from Eschenmoser's synthetic studies,[172] there is really little evidence that the liberation of acetic acid is an enzyme-catalysed event. Indeed from Eschenmoser's work, it seems possible that the acetic acid could be lost spontaneously, and this is borne out by the large amounts of acetic acid seen in the blank runs.[132,169-171] Further work in this area is needed to establish the mechanism for corrin formation.

PART II

EXPERIMENTAL

RESULTS AND DISCUSSION

CHAPTER 2

NMR STUDIES OF URO'GEN BIOSYNTHESIS

2.1 Introduction

At the time when this research commenced, three questions within the Type III Problem remained in doubt or unanswered: (i) What is the nature of the intermediate produced by the action of deaminase on PBG, and is it the true substrate for cosynthetase? (ii) How is the linear tetrapyrrole bound and assembled on the surface of deaminase? (iii) What is the mechanism by which cosynthetase catalyses the inversion of the D ring to give uro'gen III? The work described in this chapter makes use of high-field high-resolution NMR instrumentation in an attempt to address questions (i) and (iii). Investigations into question (ii) will be considered in subsequent chapters.

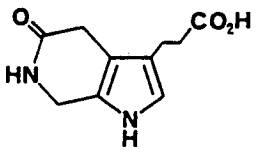
The working hypothesis held in this laboratory (see Scheme 11) did not allow an unequivocal decision to be made between the proposed intermediates in the transformation of PBG into uro'gen I or III. Also, Battersby's mechanism (see Scheme 10) left uncertainty as to the true substrate for cosynthetase — was it HMB (55) or the exomethylene pyrrolenine (60), since they are supposed to be in equilibrium with one another? Accordingly, it was felt that NMR might provide a means for detecting other transient species as the enzymic reactions proceeded.

2.2 ^{1}H NMR Studies

Initial studies attempted to make use of the inherent sensitivity of ^{1}H NMR to detect any unusual species as PBG is converted

to uro'gen I by deaminase. The proposed exomethylene pyrrolenine (60), while known to be highly reactive in model systems,[173] might exhibit a unique olefinic ^1H resonance, even if fleetingly.

Since PBG lactam (70) was readily available in this laboratory (prepared according to literature methods[174] by Dr. M. Kajiwara),



PBG lactam (70)

alkaline hydrolysis provided easy access to large quantities of PBG. Deaminase was purified from *R. spheroides* by methods similar to those described in Chapter 3 omitting the electrophoresis step, by Dr. R. C. Davies.

In a typical experiment, PBG (2 mg) was dissolved in D_2O/Na_2CO_3 and mixed with deaminase (100 U) in the NMR tube at 4°C. Spectra were then recorded at 4°C, with a time-interval of 3 minutes between spectra over the course of 5 hours. Such a 300 MHz ^1H NMR time-course is shown in Figure 2.1. For this experiment the deaminase was dialysed against several changes of 99% D_2O phosphate buffer at pH 8.0, and the residual HOD resonance suppressed by homonuclear irradiation. We can clearly see the initial signals of PBG, assigned in Table 2.1, being lost as uro'gen I formation takes place. Here it was hoped that the exomethylene species (60) might be detectable, but no olefinic resonance was observed. Later literature precedents[175] suggested that such a species would exhibit a chemical shift *ca.* 4.9 ppm, which would be unavoidably obliterated by the HOD signal.

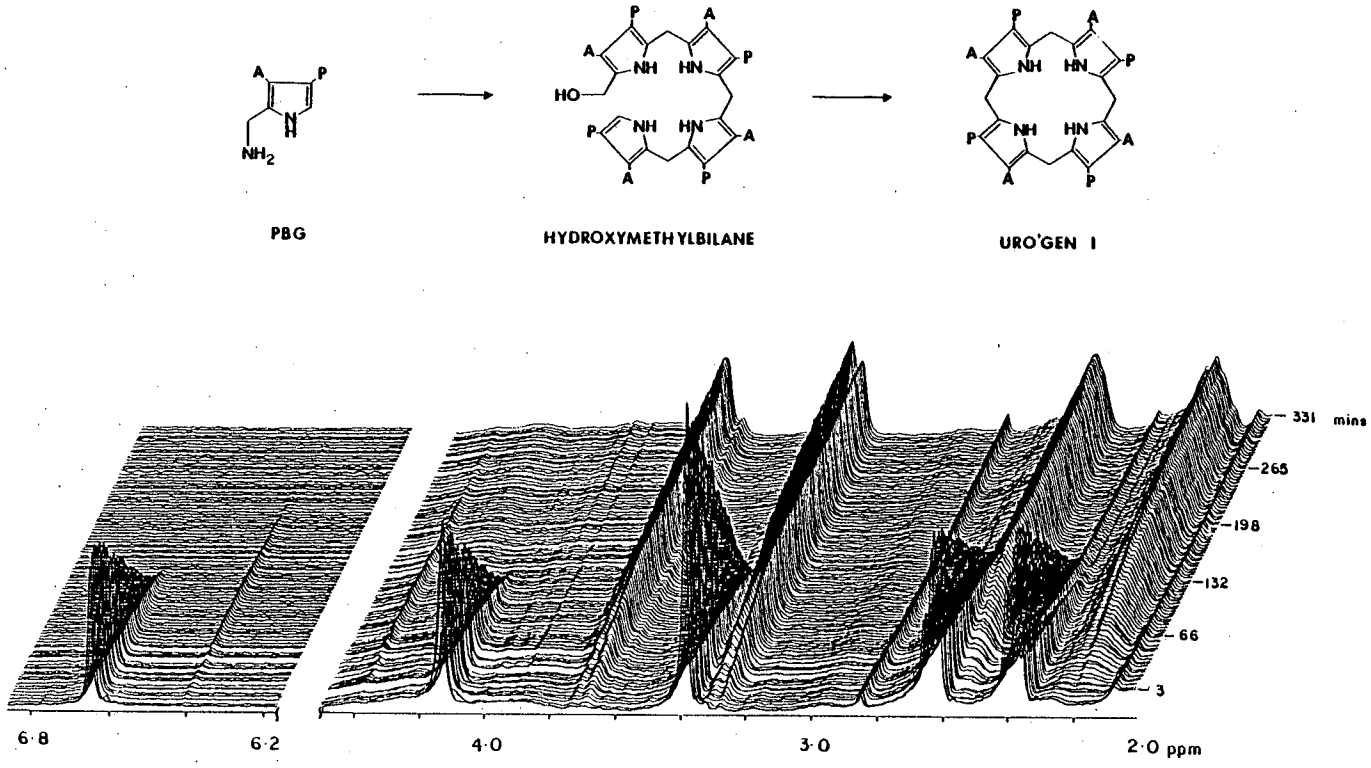
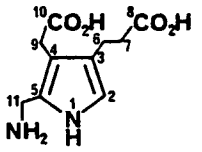


FIGURE 2.1. 300 MHz ^{1}H NMR time-course of PBG and deaminase, pH 8.0, 4°C.

TABLE 2.1. The ^1H NMR assignment of PBG at pH 8.0.

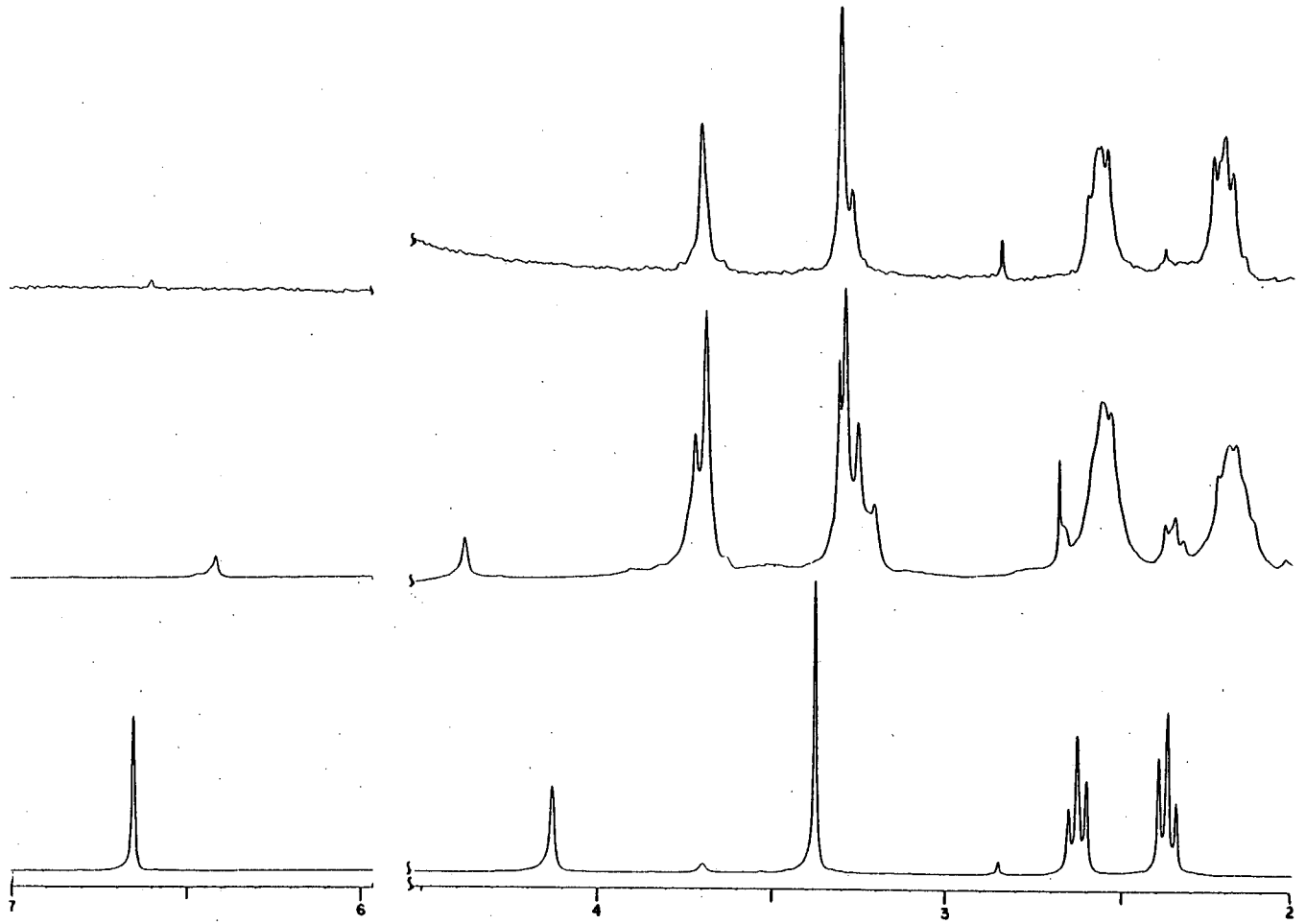


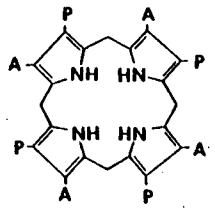
^1H	δ/ppm[a]
C-7	2.41 (2H, t)
C-6	2.67 (2H, t)
C-9	3.41 (2H, s)
C-11	4.16 (2H, s)
C-2	6.69 (1H, s)

[a]Internally referenced to TSP.

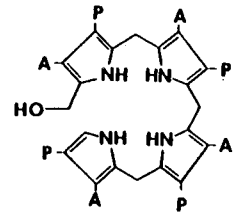
However, additional signals of lower intensity than substrate or product can be seen at 4.4 and 6.4 ppm, corresponding to the HO-$C\underline{H}_2$-pyrrole and pyrrole-$\underline{H}$ of HMB (55), respectively. These assignments were confirmed when compared with a sample of synthetic HMB prepared by Dr. I. Ichinose (see Figure 2.2).

The effect of pH on the chemical shifts of PBG was also examined, since it was felt that there may be some spread of shifts at different pH values, due to the three ionisable groups of PBG. Figure 2.3 shows a 300 MHz NMR study of PBG at a wide range of different pH values. For this experiment, PBG (*ca.* 3 mg) was dissolved in 0.1 M 99% D_2O-phosphate buffer at pH 7.27, and titrated with 0.2 M NaOD in 99% D_2O, measuring the pH directly. The pH values are uncorrected for D_2O, and the sample was referenced using a coaxial insert of 1% TMS in CCl_4. The chemical shifts are also uncorrected for magnetic

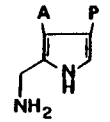




UROGEN I



HYDROXYMETHYLBILANE



PBG

FIGURE 2.2. 300 MHz ^{1}H NMR spectra of PBG, HMB (synthetic) and uro'gen III, pH 8.0, 25°C.

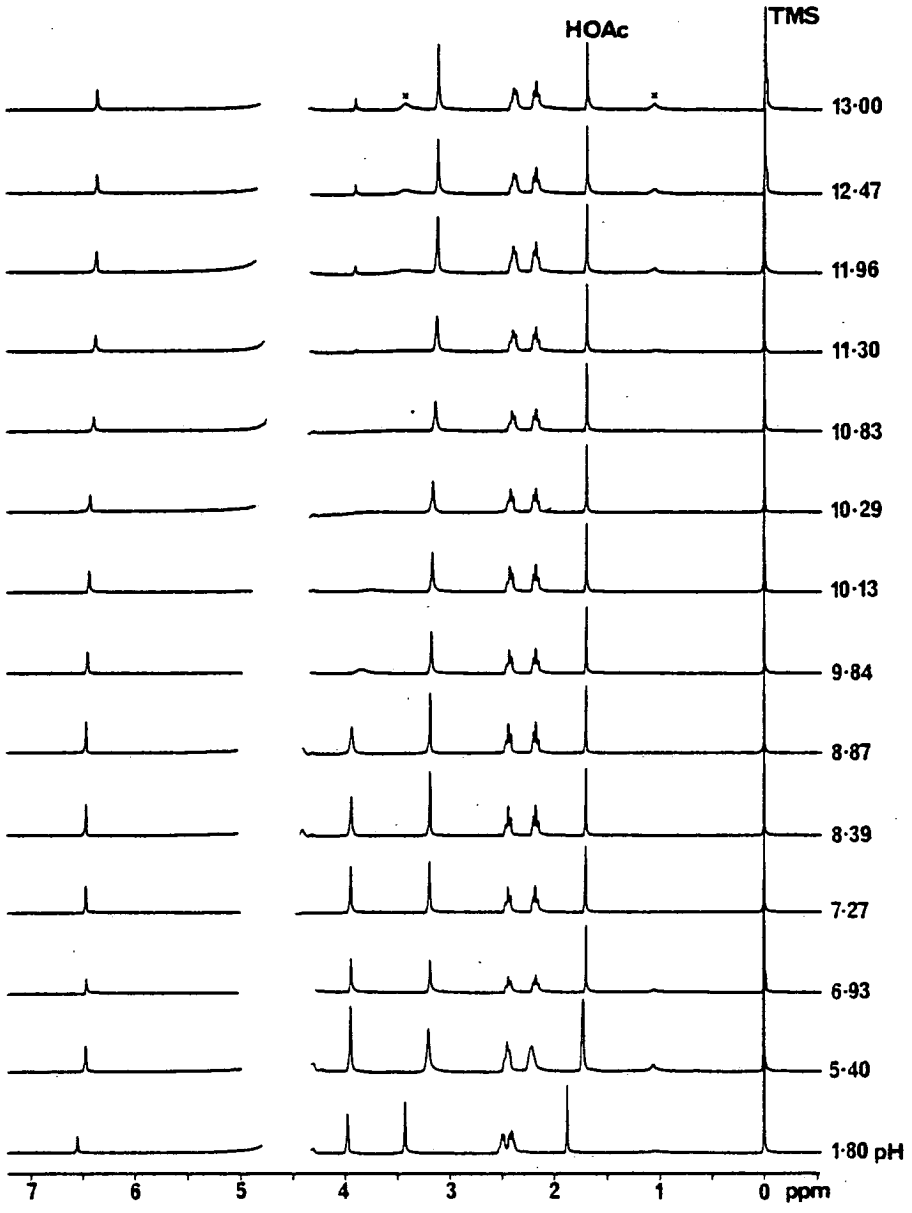


FIGURE 2.3. 300 MHz ^{1}H NMR spectra of PBG at various pH's, 25°C.

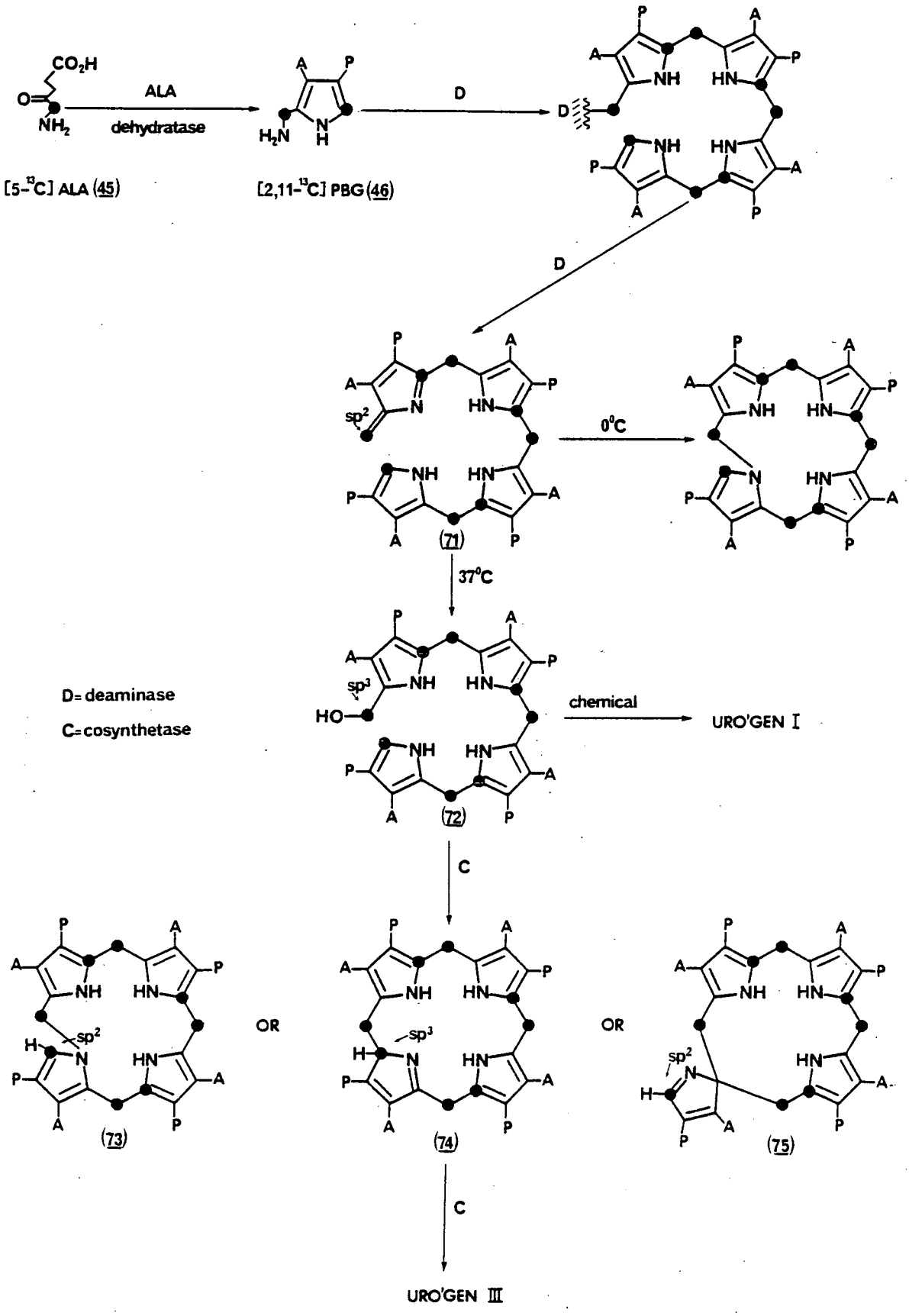
susceptibility differences. As with the other ^{1}H work, the HOD resonance was suppressed by homonuclear irradiation, and the residual line omitted from Figure 2.3 for clarity. The most interesting feature of Figure 2.3 is the severe broadening of the aminomethylene protons near the pK_a of the amino group, which is reported to be 10.1.[176] This is presumably due to a rapid equilibrium between the

protonated and deprotonated aminomethylene species, resulting in an averaged, broadened resonance. As the pH is raised to a point where much of the amino group is deprotonated, the methylene resonance reappears some 0.05 ppm upfield to where it appears at pH 7.27. Additionally, two low intensity peaks at 1.1 and 3.4 ppm appear at both alkaline and acidic pH. These probably represent decomposition products, since by this stage the sample had become highly coloured. Further interpretations of the effect of pH changes on PBG are presented in Section 2.6.

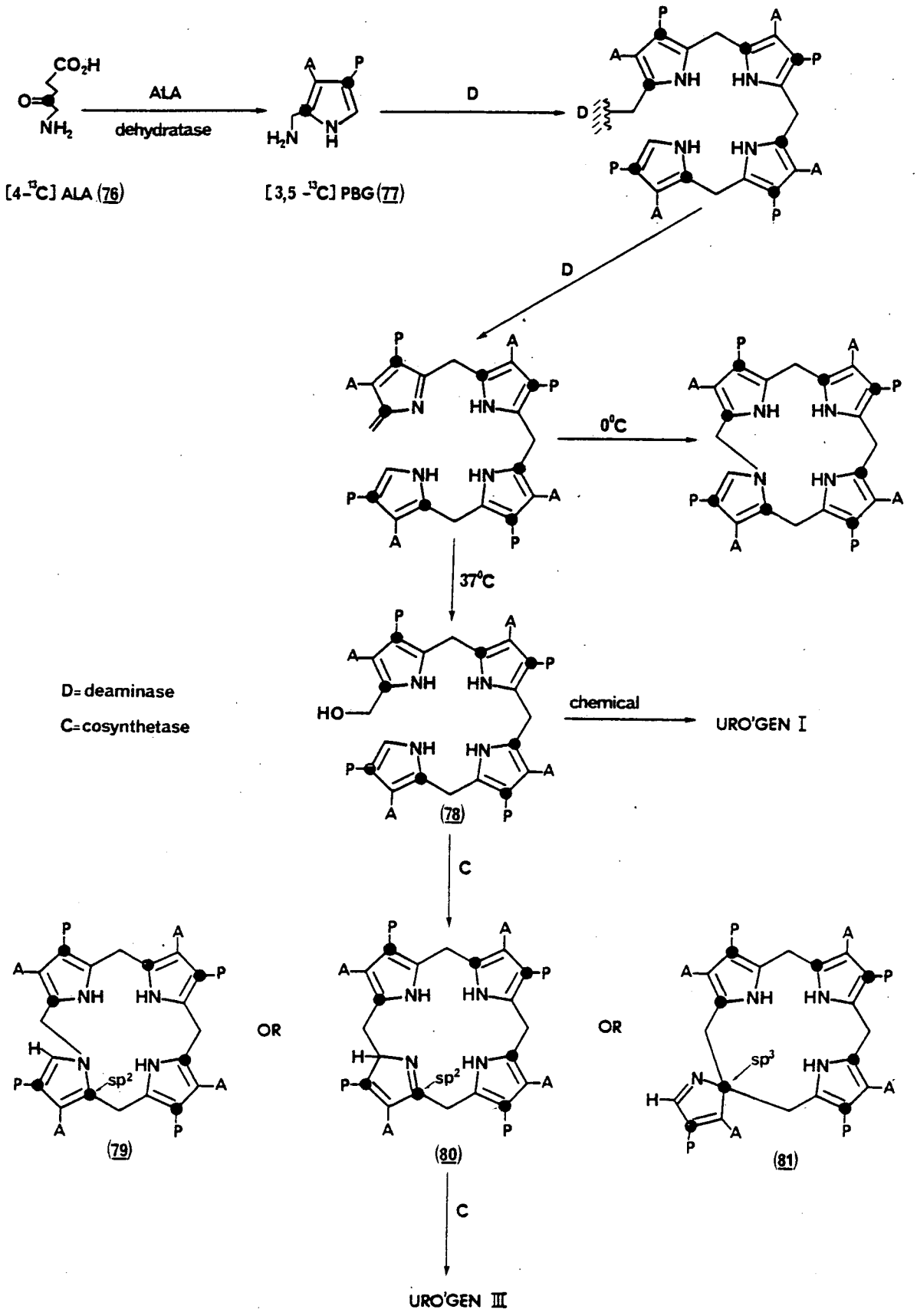
2.3 Synthesis of ^{13}C-Labelled ALA

If we again consider the mechanistic hypothesis held in this laboratory (Scheme 11), clearly the question as to the mechanism of cosynthetase might be approached by appropriate ^{13}C-labelling studies. For example, [5-^{13}C]-ALA (45) would afford a sample of HMB (72) which, on transformation into uro'gen III, could allow a choice to be made between the proposed intermediacy of the uro'gen I tautomer (74) and the other two species (73 and 75), since it has a unique sp^3 carbon (see Scheme 16). Additionally, if the exomethylene species (71) occurred at NMR detectable concentrations, the sp^3 carbon of PBG might be seen changing to an sp^2 carbon.

On the other hand, a distinction between the spiro intermediate (81) and the other two species (79 and 80) could be made using HMB (78) derived from [4-^{13}C]-ALA (76) (see Scheme 17). Thus taken together, these two labelling patterns would allow a distinction to be made between all three proposed intermediates.


ALA
dehydratase
D
[5-13C] ALA (45)
[2,11-13C] PBG (46)
D
0°C
37°C
D= deaminase
C= cosynthetase
chemical
URO'GEN I
C
OR
OR
C
URO'GEN III

SCHEME 16

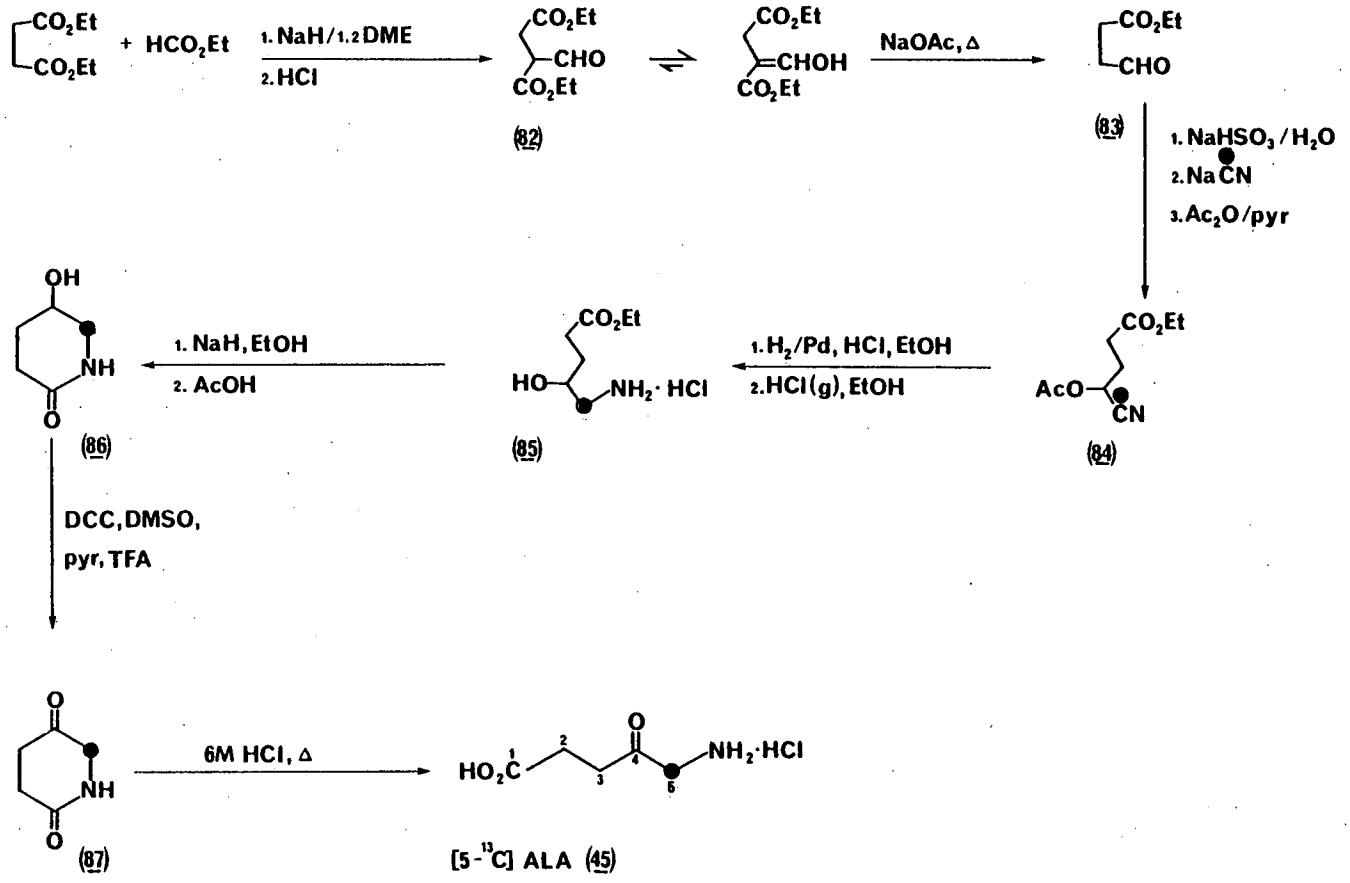

ALA
dehydratase
D
[4-13C] ALA (76)
[3,5 -13C] PBG (77)
0°C
37°C
chemical
URO'GEN I
C
OR
OR
URO'GEN III
D= deaminase
C=cosynthetase

SCHEME 17

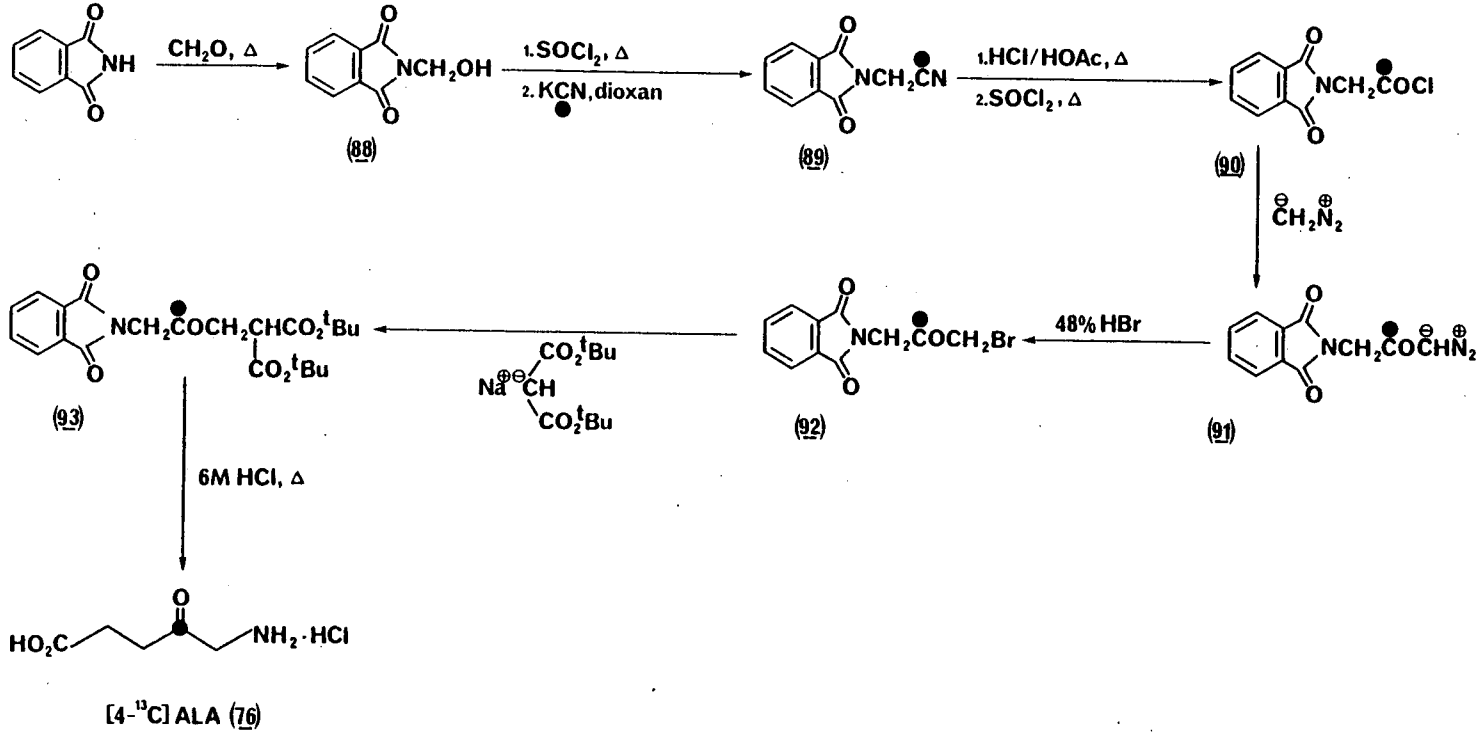
The initial requirement was therefore samples of [5-^{13}C]- and [4-^{13}C]-ALA, which could then be converted into [2,11-^{13}C]- and [3,5-^{13}C]-PBG, respectively, by the action of ALA dehydratase. The method employed for the synthesis of [5-^{13}C]-ALA was essentially that of Battersby *et al.*,[111] with certain modifications (see Scheme 18). The most significant modification occurs in the conversion of the 5-hydroxy-2-piperidone (86) into 5-oxopiperidone (87), in which the order of addition of reagents for the Pfitzner and Moffat oxidation is critical, and the reaction does not work if the published order of addition is followed. Hydrolysis of the 5-oxopiperidone (87) affords ALA hydrochloride, but here care is needed, since refluxing HCl starts to decompose ALA after *ca.* 4 hours. Such decomposition products have been the subject of a recent study.[177]

The method used for the synthesis of [4-^{13}C]-ALA was essentially that of Mitta *et al.*[178] outlined in Scheme 19. The only modification introduced here was the use of refluxing 6 M HCl to hydrolyse the esters, decarboxylate, and hydrolyse the phthalimido group. The recommended mixture of HOAc and HCl was found to yield large amounts of impurities, which could be readily circumvented using 6 M HCl for 5 hours. Again, over-refluxing at this stage led to decomposition of ALA.

With moderately large quantities of [4-^{13}C]- and [5-^{13}C]-ALA in hand, the isolation and purification of ALA dehydratase could be approached as a means to generate [3,5-^{13}C]- and [2,11-^{13}C]-PBG.


CO2Et
CO2Et
+ HCO2Et
1. NaH/1,2 DME
2. HCl
CO2Et
CHO
CO2Et
(82)
CO2Et
CHOH
CO2Et
NaOAc, Δ
CO2Et
CHO
(83)
1. NaHSO3/H2O
2. NaCN
3. Ac2O/pyr
CO2Et
AcO
CN
(84)
1. H2/Pd, HCl, EtOH
2. HCl(g), EtOH
CO2Et
HO
NH2 · HCl
(85)
1. NaH, EtOH
2. AcOH
OH
NH
O
(86)
DCC, DMSO,
pyr, TFA
O
NH
O
(87)
6M HCl, Δ
HO2C
NH2·HCl
[5-13C] ALA (45)

SCHEME 18


CH2O, Δ
1.SOCl2, Δ
2. KCN, dioxan
1.HCl/HOAc, Δ
2.SOCl2, Δ
(88)
(89)
(90)
(91)
(92)
(93)
48% HBr
6M HCl, Δ
[4-13C] ALA (76)

SCHEME 19

2.4 Isolation and Purification of ALA Dehydratase

For most of the preparations of PBG throughout this work, the ALA dehydratase employed was partially purified from *R. spheroides*. In certain special situations, further purification was necessary, but in general the procedure followed the steps in Figure 2.3.

(i) Sonication of *Rhodopseudomonas spheroides*.
(ii) Centrifugation at 35,000 g.
(iii) Centrifugation at >60,000 g.
(iv) Ammonium sulphate precipitation (20-35%).
(v) Centrifugation at 16,000 g.
(vi) Gel-filtration on G-100 Sephadex.
(vii) Concentration on Amicon PM-10 Ultrafilter.
(viii) Chromatography on DEAE-cellulose.
(ix) Concentration on Amicon PM-10 Ultrafilter.

FIGURE 2.3. Isolation and purification of ALA dehydratase.

This procedure is a modification of the methods of Shemin and co-workers.[179] In a typical preparation, 100 g of wet cells were suspended in phosphate buffer (300 cm^3, 0.05 M, pH 7.6) and sonicated. After low- and high-speed centrifugation, and ammonium sulphate precipitation, a crude pellet was obtained (by centrifugation), which was redissolved in Tris buffer (0.01 M, pH 8.0) and applied to a freshly equilibrated G-100 Sephadex column. The gel-filtration step helps to remove contaminating deaminase, as shown in Figure 2.4, the presence of which would seriously lower the yield of PBG in a dehydratase incubation, since some would be converted into uro'gen I.

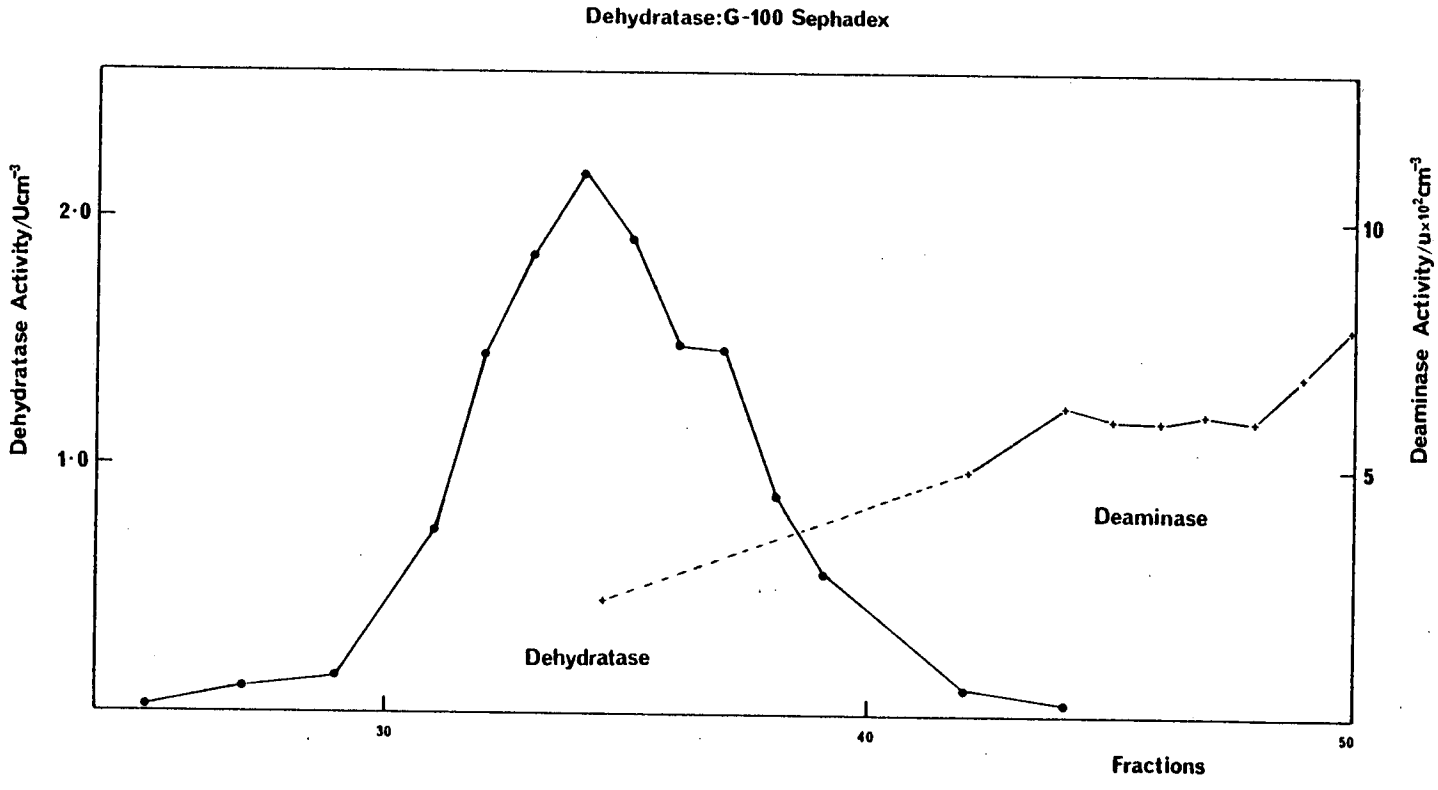


FIGURE 2.4. The purification of ALA dehydratase on G-100 Sephadex.

After concentration by ultrafiltration, the pooled enzyme activity was applied to a freshly equilibrated DEAE-cellulose column, and eluted with a salt gradient of 0.05 M to 0.5 M KCl in Tris buffer (0.01 M, pH 8.0) (see Figure 2.5). The pooled enzyme activity (usually *ca.* 450 U) was used for PBG preparations. The method for assay of enzyme activity was that of Burnham and Lascelles.[180]

2.5 Synthesis of ^{13}C-Labelled PBG

In a typical PBG preparation, partially purified dehydratase (*ca.* 200 U) was pre-incubated at 37°C in the presence of cysteine (0.01 M) and $MgCl_2$ (0.01 M), in nitrogen-degassed phosphate/$NaHCO_3$ buffer (0.1 M, pH 8.0). [^{13}C]-ALA (50 mg) was dissolved in the same buffer, adjusted to pH 8.0 and added to the enzyme. Incubation was continued at 37°C under nitrogen, and the reaction monitored by removing aliquots and assaying for PBG using modified Ehrlich's reagent.[181] The reaction was largely complete in 2 hours, and stopped by addition of $CuSO_4$ solution. The precipitate was removed by centrifugation, and the supernatant containing the PBG lyophilised. The lyophilate was redissolved in water, adjusted to pH 6, and then chromatographed on Dowex 2 acetate. This afforded, after lyophilisation of the acetate buffer, pure PBG acetate salt in *ca.* 60% isolated yield. The acetate salt thus obtained was used for most of the NMR work described here.

2.6 Complete ^{13}C NMR Assignment of PBG

While the assignment of the ^{13}C resonances for [2,11-^{13}C]-PBG at pH 8.0 is relatively straightforward, C-2 displaying a resonance

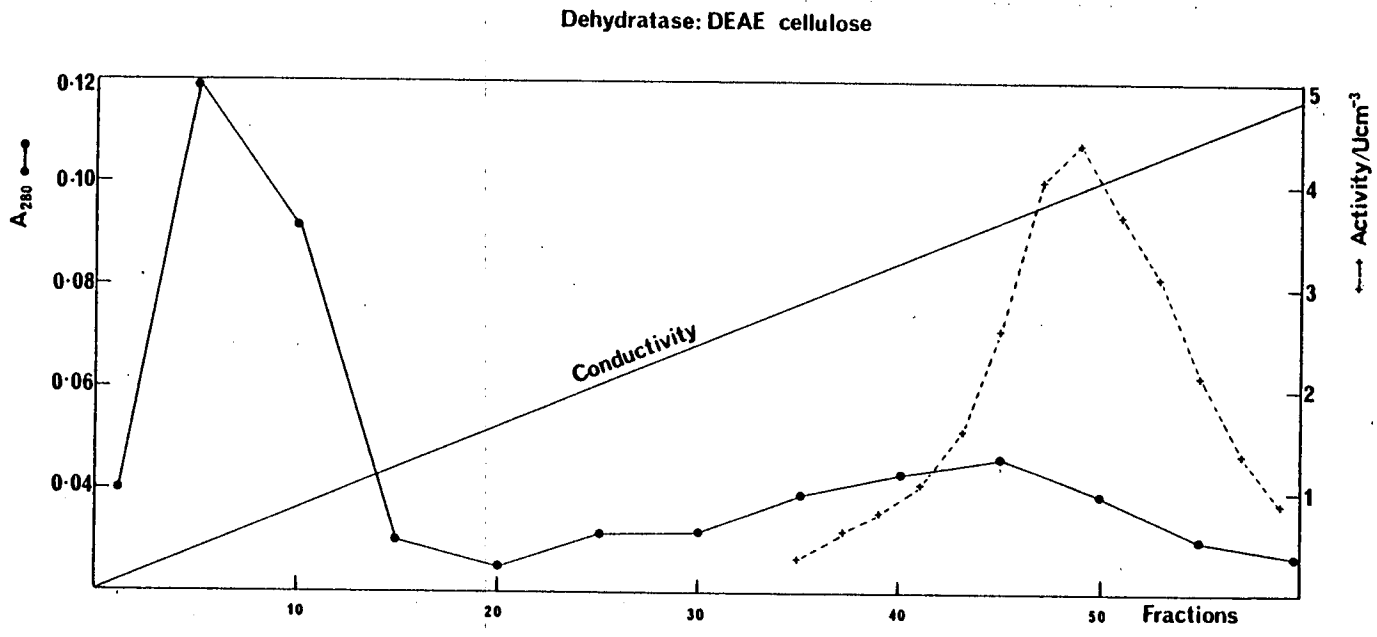
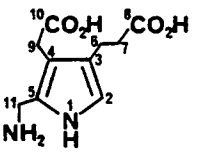


FIGURE 2.5. The purification of ALA dehydratase on DEAE-cellulose.

at 116.1 ppm and C-11 at 34.4 ppm, the assignment of [3,5-^{13}C]-PBG at pH 8.0 is not so clear. As can be seen from Figure 2.7, assignment of the resonances at 120.6 and 122.5 ppm is impossible without recourse to selective ^{1}H-decoupling experiments. When these were performed, as shown in Figure 2.8, the resonance at 120.6 ppm clearly arises from C-5, and the one at 122.5 ppm from C-3.

These selective ^{1}H-decoupling experiments led us to investigate unlabelled PBG, whose complete ^{13}C NMR assignment had not been made. The results of these experiments are shown in Table 2.2.

TABLE 2.2. The ^{13}C NMR assignment of PBG.



^{13}C	δ/ppm[a]
C-6	21.7 (t)
C-9	32.8 (t)
C-11	34.4 (t)
C-7	38.2 (t)
C-2	116.1 (d)
C-4	117.4 (s)
C-5	120.6 (s)[b]
C-3	122.5 (s)
C-8	181.1 (s)
C-10	183.0 (s)

[a]Internally referenced to p-dioxane, δ_C = 66.5 ppm.
[b]Ambiguous assignment confirmed unequivocally by biosynthetic labelling.

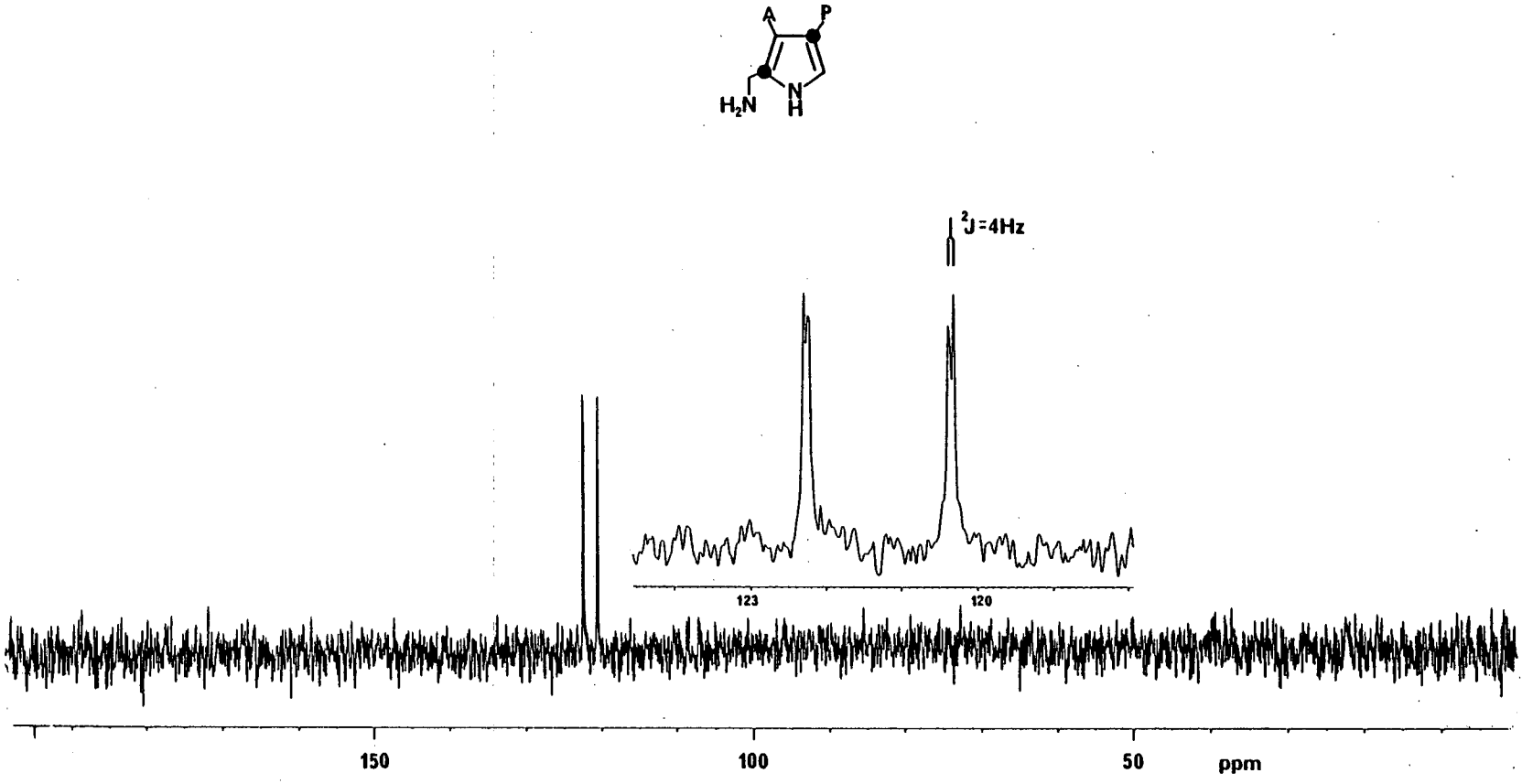


FIGURE 2.7. ^{13}C NMR of [3,5-^{13}C]-PBG, pH 8.0, 25°C.

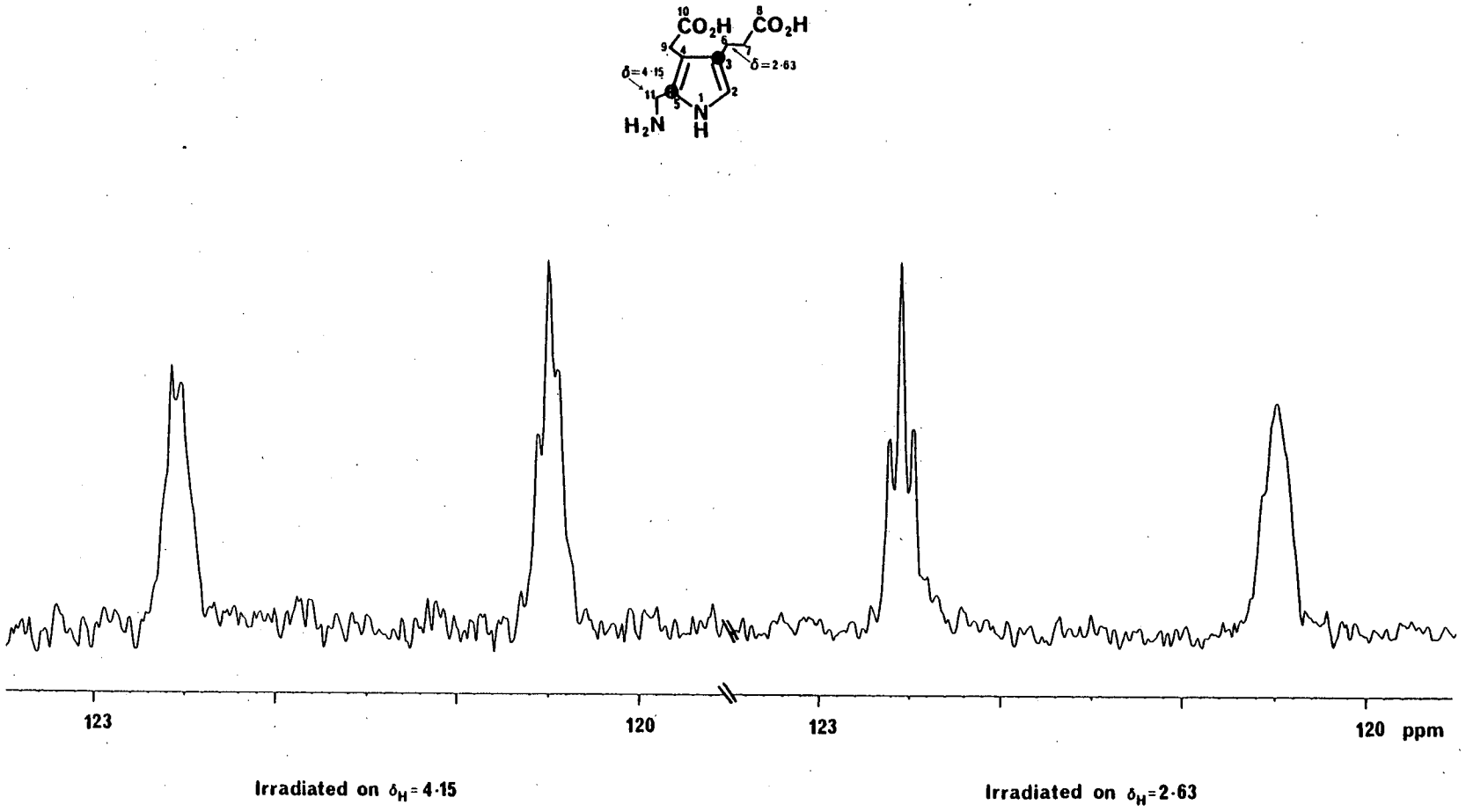


FIGURE 2.8. ^{1}H-decoupling experiments on [3,5-^{13}C]-PBG, pH 8.0, 25°C.

Further investigations on [3,5-^{13}C]-PBG revealed that C-5 is sensitive to the pK_a of the amino group in the side-chain, much as was found for the C-11 protons in Section 2.2. Figure 2.9 shows the effect of varying the pH on [3,5-^{13}C]-PBG. From such a pH titration, we can obtain a value for the pK_a of the amino group (see Figure 2.10) which is somewhat higher than the value of 10.1 mentioned previously. This difference can be attributed to the fact that a given nucleus may not exhibit marked changes in chemical shift at all pK_a values, which can give rise to inaccurate pK_a values.[182] The fact that the carbon β to the amino group is affected more than the α-carbon has been the subject of much theoretical speculation.[182-185] For example, the effect has been observed in lysine, in which the δ-carbon shifts some 4.66 ppm downfield on deprotonation of the ε-NH_2. Shifts were also observed in similar compounds such as ε-aminocaproic acid (4.23 ppm downfield) and hydroxylysine (3.56 ppm downfield).[182] It also occurs in simple linear alkylamines such as pentylamine which exhibits a downfield shift of 5.96 ppm at the β carbon on deprotonation of the amino group.[185] Aromatic amino acids such as phenylglycine show quite a dramatic shift of 7.95 ppm downfield for C-1 of the phenyl ring. Batchelor *et al.*[185] have rationalised the shifts in terms of a uniform linear electric field, the effect of which they call "LEFS". The direction of this field effect depends largely upon the polarisability of the C-X bond under study, which obviously affects the shielding of the carbon atom, and also its orientation. Although this effect can be expected to fall off with distance, and indeed the uniform field effect contributions fall off monotonically with distance from the amino group, the anomalous β-shifts have been rationalised in terms of field gradient effects. The charge density

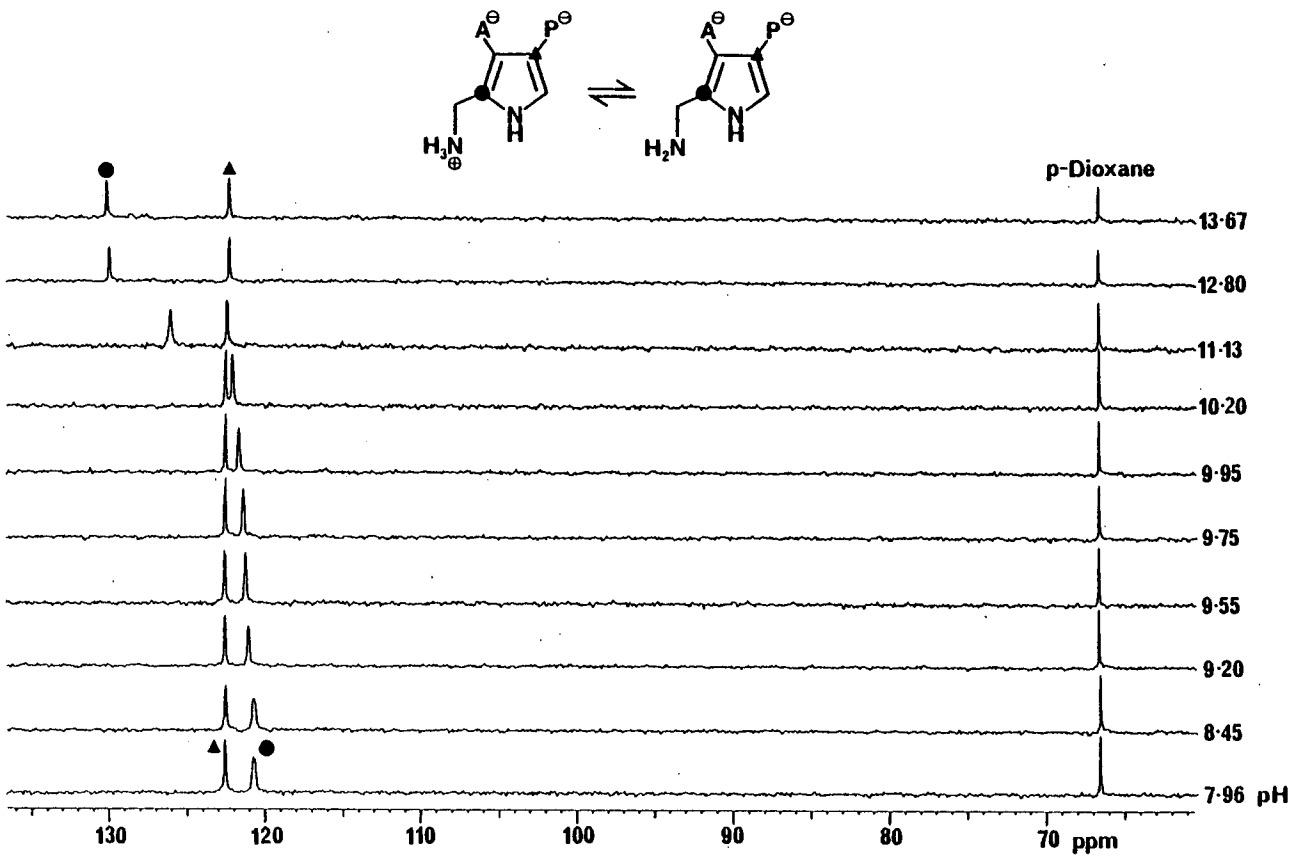


FIGURE 2.9. 75.5 MHz ^{13}C NMR spectra of [3,5-^{13}C]-PBG at various pH's, 22°C.

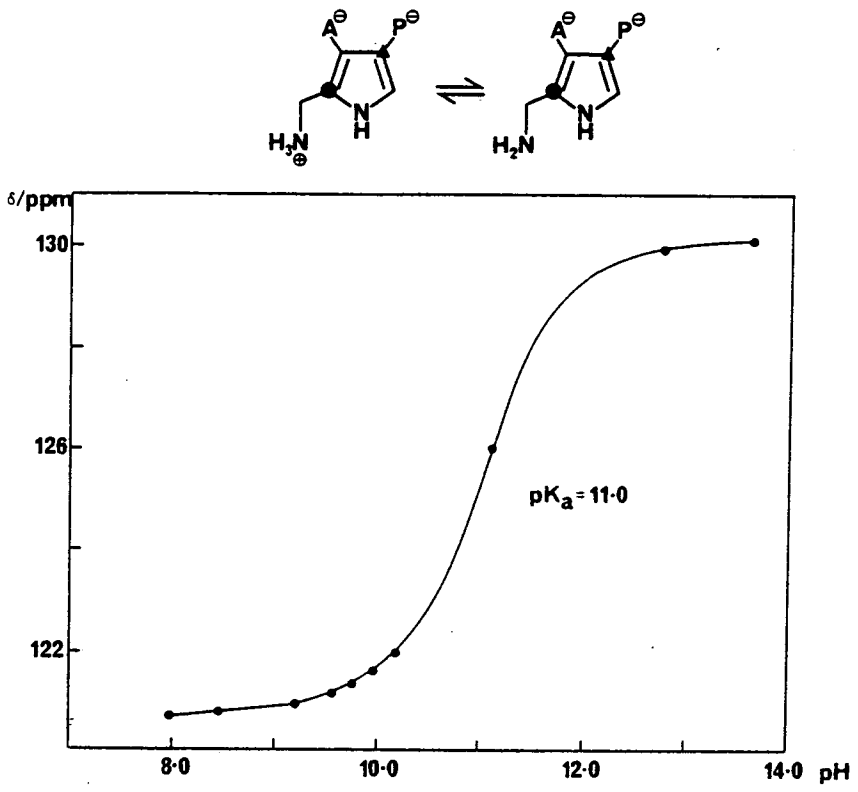
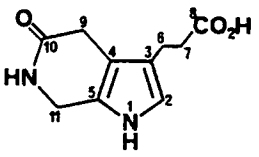


FIGURE 2.10. Titration curve for [3,5-^{13}C]-PBG.

on a carbon atom depends upon the sum of the field gradient and the uniform field effects. Batchelor *et al.* suggest that the field gradient effect dominates in β-shifts of carbons next to amino groups.

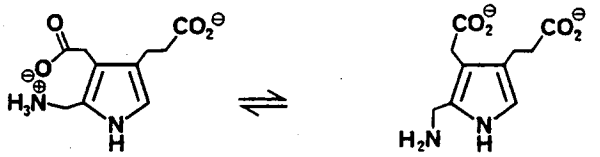
With aromatic amino acids, such as PBG, the bridgehead carbon which is β to the amino group is thought to be affected on deprotonation, due to the orientation of the aromatic ring. However, conformation must also play an important part on this shift. To investigate this possibility, the tentative ^{13}C NMR assignment of PBG lactam (70) was determined (see Table 2.3). The lactam can be regarded as an extreme conformational case of ion pairing between the ionised carboxylate and the protonated amino function. If deprotonation gives rise to large downfield shifts arising also because of conformational changes, then we can imagine that the lactam would exhibit a chemical shift at C-5 upfield from species I. As we can see from

TABLE 2.3. The ^{13}C NMR assignment (tentative) of PBG lactam.



^{13}C	δ/ppm[a]
C-6	21.35
(C-9	28.02)[b]
C-7	38.10
(C-11	40.05)[b]
C-4	109.55
C-2	115.62
C-3, C-5	119.26
C-10	174.30
C-8	182.84

[a]Internally referenced to p-dioxane, δ_C = 66.5 ppm.
[b]These are tentative assignments.



I ⇌ II

Table 2.3, C-5 in the lactam occurs at 119.26 ppm, some 3 ppm upfield from C-5 of PBG. Of course, these observations do not prove that on deprotonation of the amino group, the conformation of PBG in solution is such that the acetate and aminomethyl side-chains are much further apart. In forming a lactam, the orientation of the α- and β-carbon atoms have much more stringent requirements. However, a conformational effect seems likely to be a dominant feature in determining the shift at C-5 due to deprotonation of the amino group in PBG, since even in aromatic systems, a shift as large as 9.4 ppm has not been reported. Furthermore, the C-11 proton resonance is probably so broad in the ^{1}H NMR spectrum of PBG at pH 10.3 (see Figure 2.3) because of a rapid equilibrium between two conformers.

2.7 ^{13}C NMR Studies using [2,11-^{13}C]-PBG

Preliminary investigations were conducted using small quantities of substrate (2 mg) and enzyme (*ca.* 20 U) in a 5 mm NMR tube, observing ^{13}C at 75.5 MHz. In these experiments spectra of HMB (73), uro'gen I and uro'gen III were characterised. Since the transformation of HMB into uro'gen III might involve initial formation of an exomethylene species, [2,11-^{13}C]-PBG was used to generate a sample of HMB by the action of deaminase, and then cosynthetase (purified by Dr. R. C. Davies) and mercaptoethanol were added. In this case no trapped species were observed, and the final spectrum showed natural abundance mercaptoethanol and enriched uro'gen III.

Since no new intermediates were detected in these comparatively small-scale experiments, they were repeated using larger quantities of deaminase in a 10 mm NMR tube. In order to establish optimal NMR

conditions, a sample of [2,11-^{13}C]-PBG (2 mg) was incubated with deaminase (26 U) at 37°C for two hours, freeze-dried and redissolved in nitrogen-degassed D_2O (2 cm^3). This sample of uro'gen I was studied under various decoupler conditions as shown in Figure 2.11, since it was considered to be the best model for whatever intermediates might accumulate.

Gated decoupling is usually employed in situations where the heating effects of decoupling need to be minimised. The decoupler is switched on only during the RF pulse and subsequent acquisition time, and then switched off for a variable time τ. It was found that for uro'gen I, 0.8 watts ^{1}H-decoupling power (12 H in Bruker notation) was the most suitable, and continuous decoupling did not seriously impair signal-to-noise, which would arise from significant heating effects. This was an advantage, since no time would be wasted while the decoupler is switched off, as in the case of gating, during a kinetics time-course in which detectable intermediates may be short-lived. Under these decoupling conditions, the pulse width (or 'flip-angle') was varied in an attempt to optimise the acquisition parameters. Here the object was to acquire the maximum data (i.e., signal-to-noise) in the minimum time.

Using these NMR conditions, uro'gen I was characterised at pH 8.0, 25°C, as shown in Figure 2.12. The spectrum differs slightly from the published one.[186] The meso carbons occur as a doublet centred on 21.81 ppm (^1J (^{13}C-^{13}C) = 50.3 Hz) with the line in the centre of the doublet corresponding to isolated ^{13}C atoms (34%). This arises because the starting ALA was 90% enriched in ^{13}C, resulting in 90% X 90% = 81% enrichment in ^{13}C in PBG. In uro'gen I we are concerned only with two PBG units lying adjacent, and the

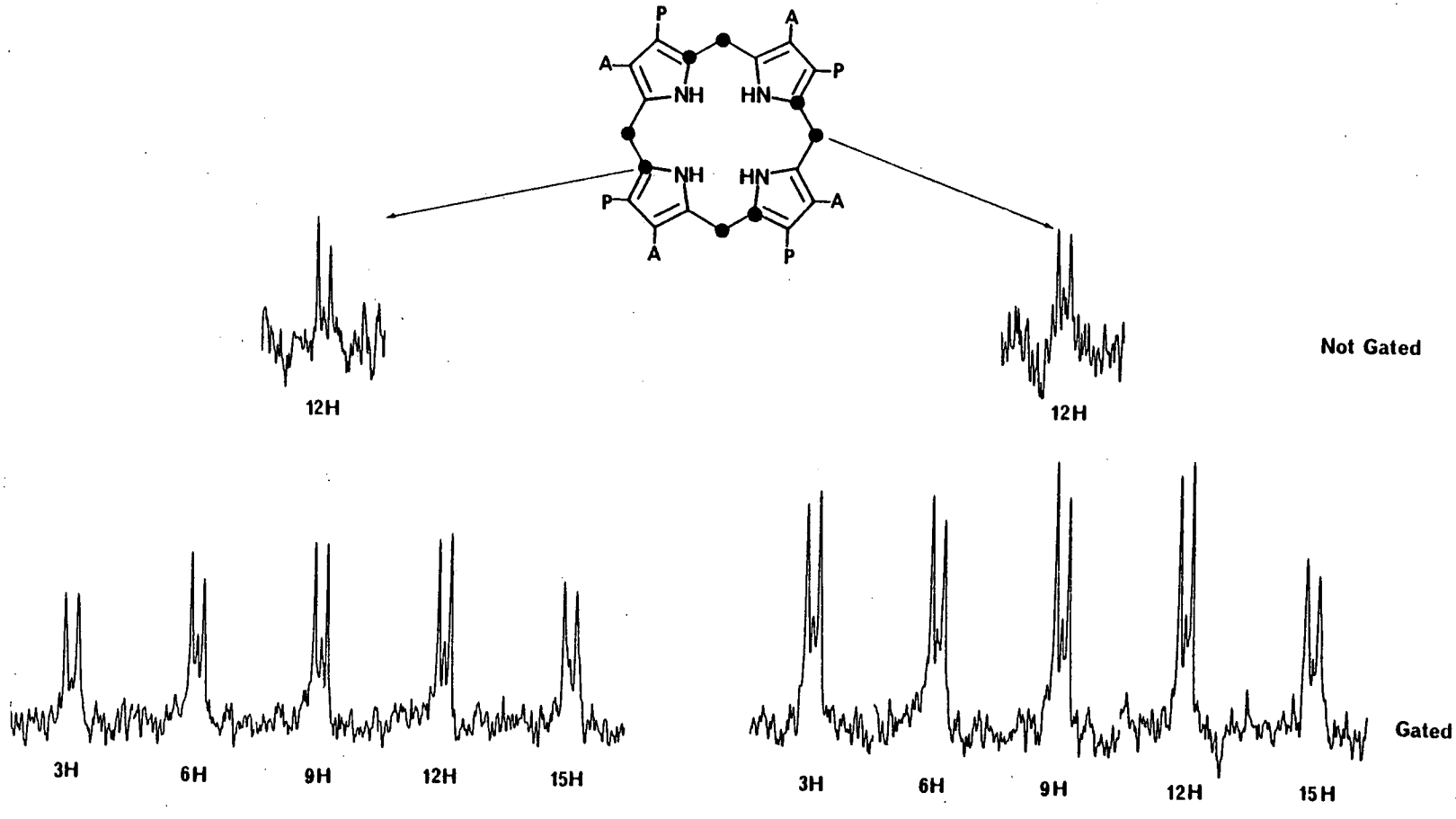


FIGURE 2.11. 75.5 MHz ^{13}C NMR spectra of [2,11-^{13}C]-PBG-derived uro'gen I under various decoupler conditions, with and without gating, pH 8.0, 25°C.

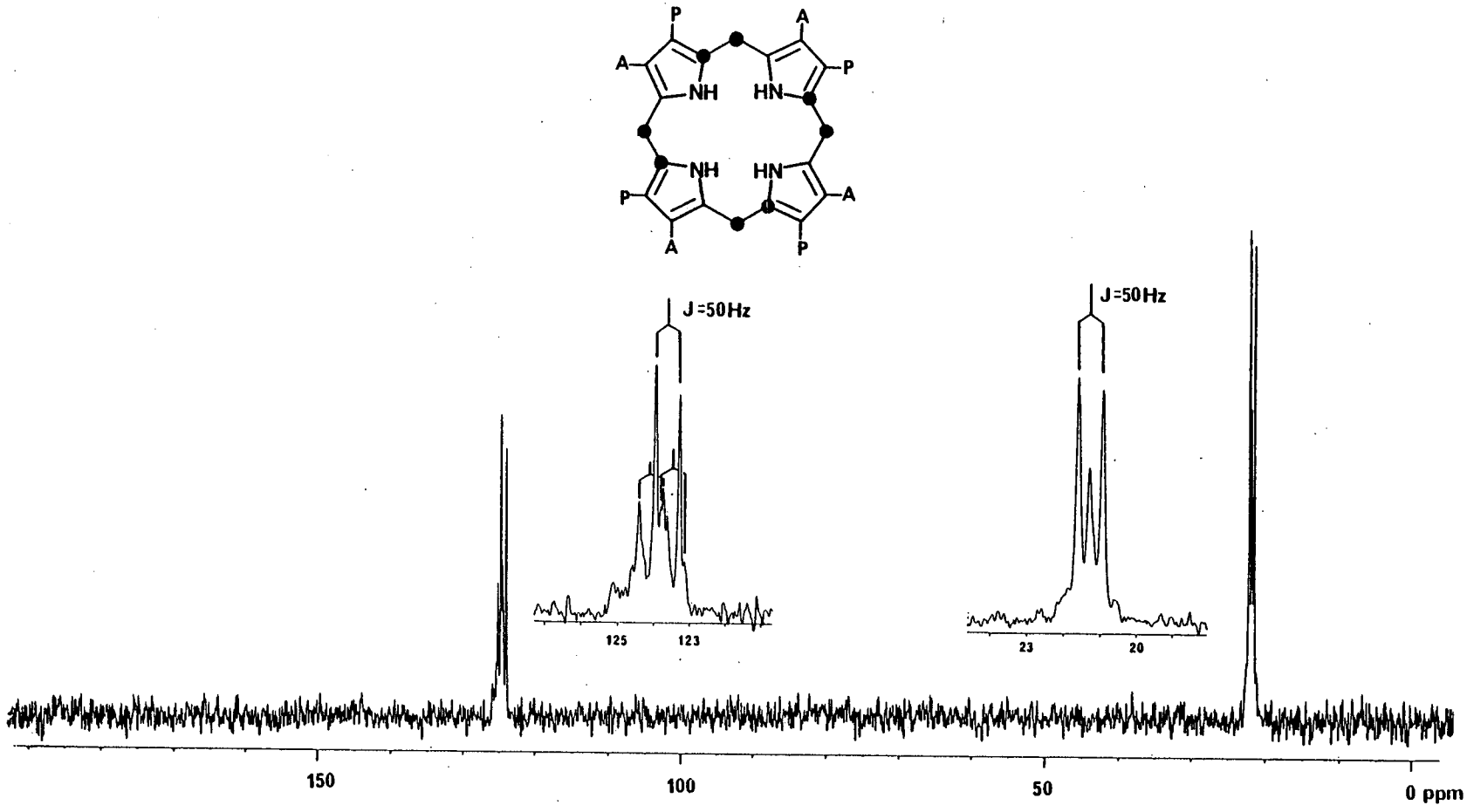


FIGURE 2.12. 75.5 MHz ^{13}C NMR spectrum of [2,11-^{13}C]-PBG-derived uro'gen I, pH 8.0, 25°C.

odds of two ^{13}C atoms being directly bonded in that situation would be 81% X 81% = 65%. The α-pyrrolic carbons show a similar doublet centred on 124.12 ppm (^{1}J (^{13}C-^{13}C) = 50.3 Hz). The other 50 Hz doublets visible in the spectrum arise from a small amount of uro'gen III contaminating the sample. This is due to a minute presence of cosynthetase in the deaminase used to prepare the uro'gen I.

Similarly, uro'gen III was prepared from [2,11-^{13}C]-PBG and analysed by ^{13}C NMR. The spectrum is shown in Figure 2.13, which differs slightly from the published one.[186] However, we can clearly distinguish a doublet centred on 20.68 ppm (^{1}J (^{13}C-^{13}C) = 50.0 Hz) arising from C-5 and C-10, on which is superimposed a triplet (^{1}J (^{13}C-^{13}C) = 50.0 Hz) centred on 20.68 ppm, due to C-15, and a broad singlet from C-20 also at 20.68 ppm. The pattern for the pyrrolic carbons (C-4, C-7, C-14 and C-16) is less easy to interpret. We would expect C-4 and C-9 to give rise to doublets as with uro'gen I, and C-14 and C-16 to appear as a doublet of doublets either completely superimposed or displaced slightly from one another. Such a pattern can be distinguished in Figure 2.14, in which a doublet centred on 123.47 ppm corresponds to C-4 and C-9, and a broad doublet centred on 123.63 ppm corresponds to C-14 and C-16. It should be noted that this interpretation differs from the one presented in the literature,[186] in which a two-bond ^{13}C coupling was suggested to be of the order of 7.5 Hz. In heterocycles such as uro'gen I or III, the two-bond ^{13}C coupling could be expected to be of the order of 4 Hz (see Section 2.8). Such a doublet (^{2}J (^{13}C-^{13}C) ≈ 4 Hz) arising from ^{13}C in C-14 and C-16 but not at C-15 would be expected to occur at very low relative intensity, hardly distinguishable from the noise, since only 11.6% of the

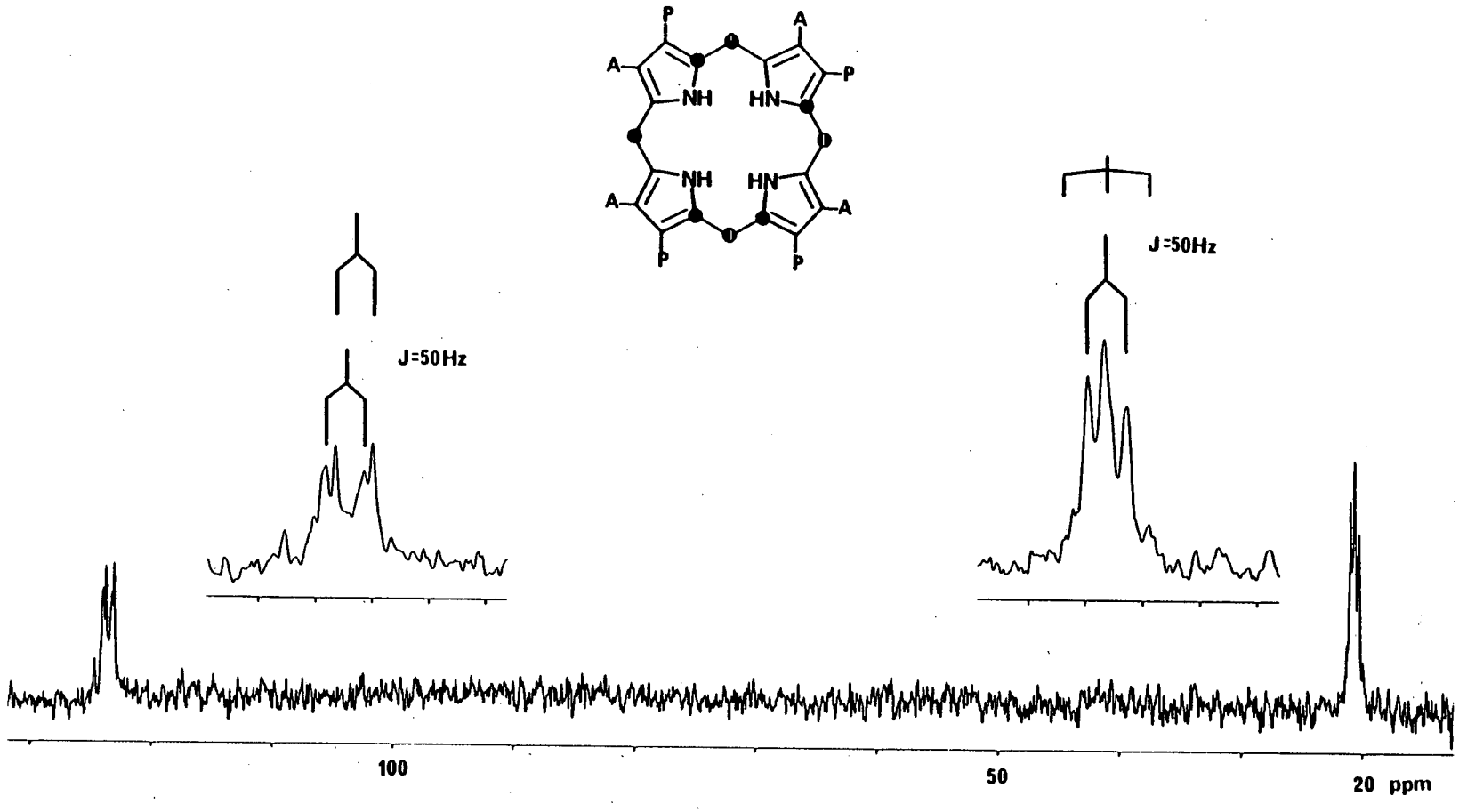


FIGURE 2.13. 75.5 MHz ^{13}C NMR spectrum of [2,11-^{13}C]-PBG-derived uro'gen III, pH 8.0, 25°C.

sample could contain ^{13}C atoms in these positions but *not* at C-15. It is, however, surprising that the published spectrum failed to resolve the doublet arising from C-4, C-9 and the doublet arising from C-14, C-16, since they are separated by some 17.5 Hz.

With the optimum NMR conditions rigorously established, the search for new transient intermediates could begin. In the first instance, [2,11-^{13}C]-PBG (5 mg) in pyrophosphate-D_2O buffer (pH 8.5) was incubated with deaminase (400 U) for 3.5 minutes at 37°C, adjusted to pH 12 with NaOH, and cooled. This was studied in the NMR for 36 minutes at 4°C (see Figure 2.14), and from this spectrum, tentative assignments could be made (see Table 2.4). We can see from Figure 2.14 that some PBG remains, and some uro'gen I has formed, but the predominant species is HMB in which all the carbons can be identified. Interestingly, residual PBG occurs at 34.87 and 113.48 ppm. When [2,11-^{13}C]-PBG was studied by ^{13}C NMR at pH 12, it was found that C-11 occurs at 35.35 ppm as opposed to 34.4 ppm at pH 8.0, and C-2 occurs at 113.64 ppm as opposed to 116.1 ppm at pH 8.0. Also present in the spectrum of HMB is another peak very close to C-11 of PBG at 34.68 ppm, which represents a small amount of aminomethylbilane.

To this sample of HMB, purified cosynthetase (20 U) in phosphate buffer (pH 8.0) was added and sufficient 3 M HCl to bring the pH back to 8.0. Spectra were then recorded at 4°C every 4.5 minutes over 2 hours, and the full time-course is shown in Figure 2.15. Even when blocks of spectra are added together, such as in Figure 2.16, no new intermediates are detectable. Note that the residual peaks which remain throughout the spectra occur at 33.77 and 112.74 ppm, corresponding to aminomethylbilane which is *not* acting as a substrate for cosynthetase.

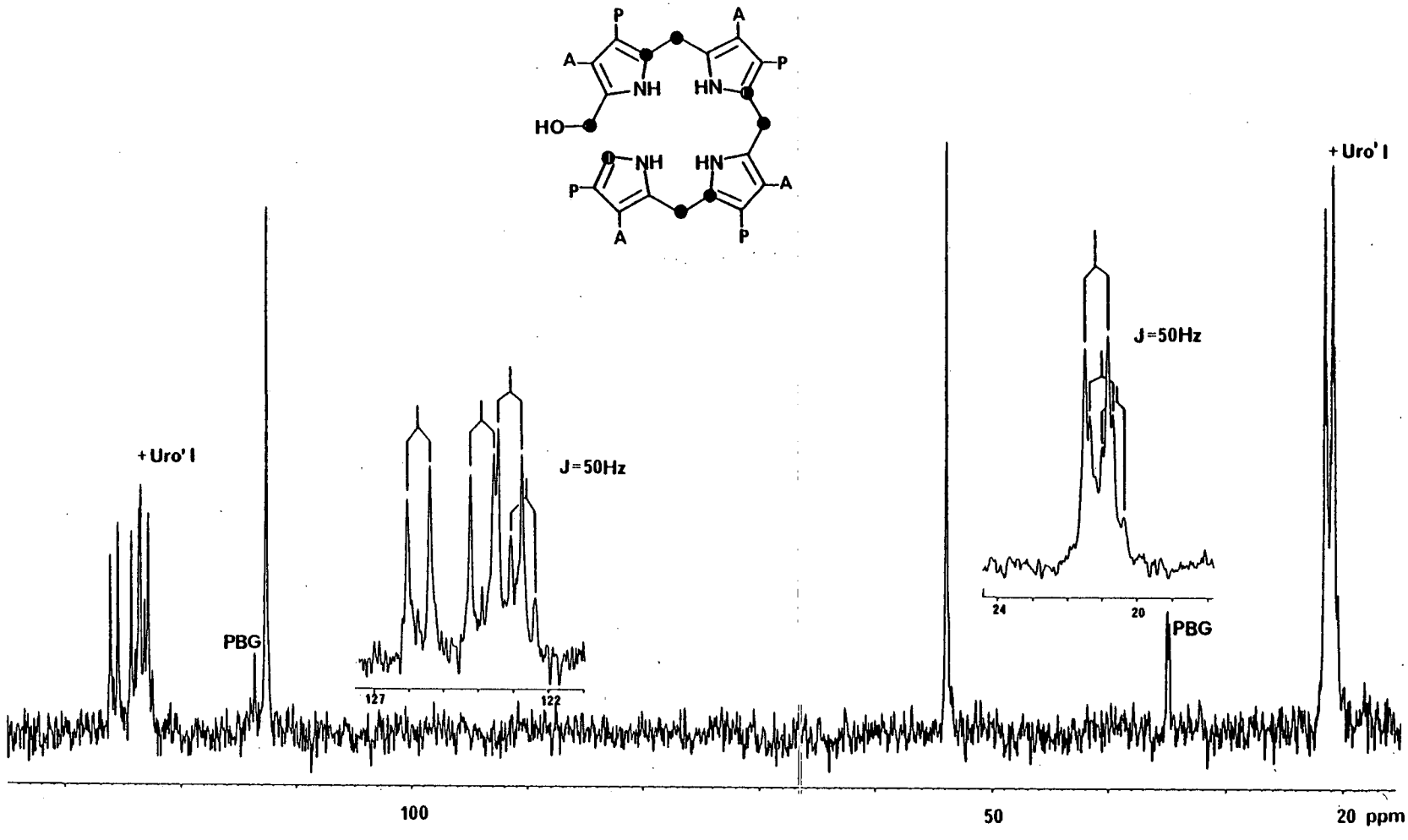
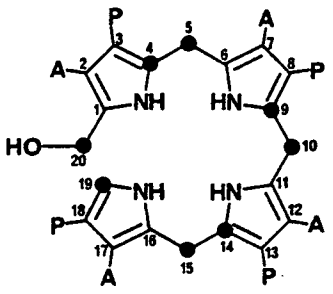


FIGURE 2.14. 75.5 MHz ^{13}C NMR spectrum of [2,11-^{13}C]-PBG-derived HMB at pH 12, 4°C.

TABLE 2.4. The ^{13}C NMR assignment (tentative) of [2,11-^{13}C]-PBG-derived HMB.



^{13}C	δ/ppm[a]
C-15	20.7
C-10	21.1
C-5	21.2
C-20	53.8
C-19	112.5
C-14	123.1 (d, ^{1}J (^{13}C-^{13}C) = 50 Hz)
C-9	123.9 (d, ^{1}J (^{13}C-^{13}C) = 50 Hz)
C-4	125.7 (d, ^{1}J (^{13}C-^{13}C) = 50 Hz)

[a]Indirectly referenced to p-dioxane, δ_C = 66.5 ppm.

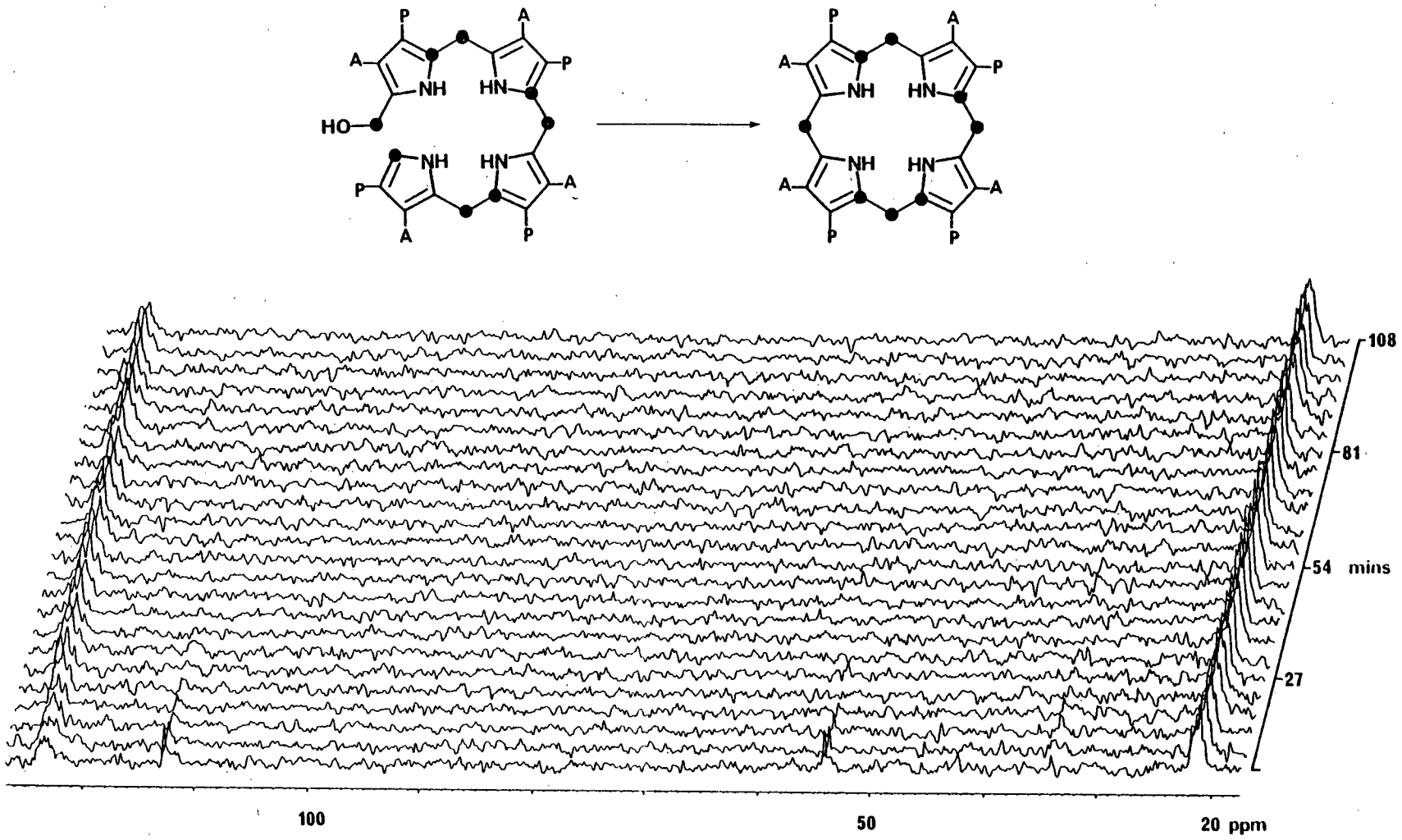


FIGURE 2.15. 75.5 MHz NMR spectra of full time-course of [2,11-^{13}C]-PBG-derived HMB with cosynthetase, pH 8.0, 4°C.

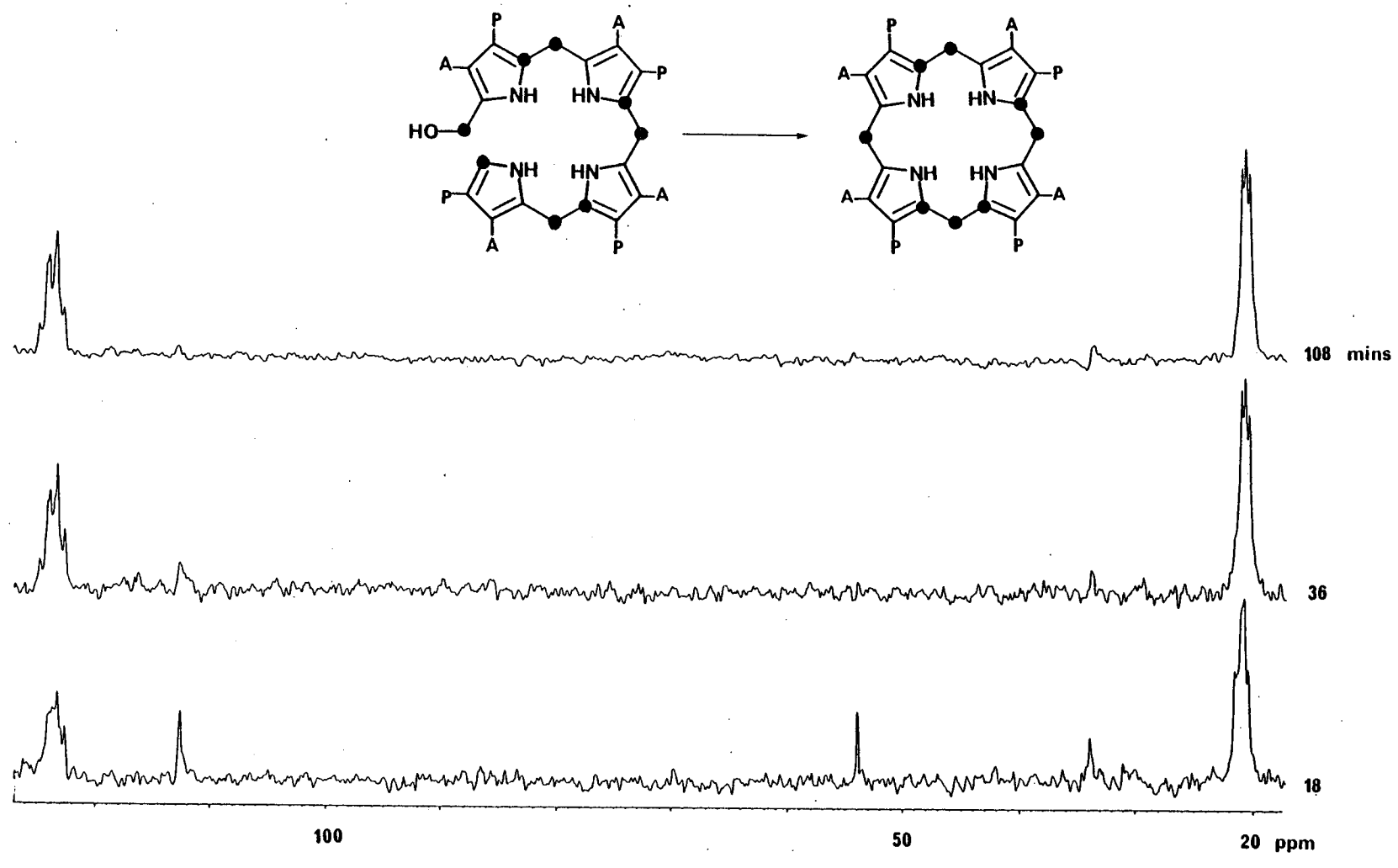


FIGURE 2.16. 75.5 MHz NMR spectra of [2,11-^{13}C]-PBG-derived HMB with cosynthetase, pH 8.0, 4°C.

With the comparative lack of success in the detection of new intermediates using [2,11-^{13}C]-PBG, attention turned to [3,5-^{13}C]-PBG which being quaternary labelled, might afford a greater chance of detecting some transient species.

2.8 ^{13}C NMR Studies using [3,5-^{13}C]-PBG

Preliminary investigations were conducted with [3,5-^{13}C]-PBG (2 mg) in pyrophosphate-D_2O buffer (pH 8.0), in a 5 mm NMR tube. Again optimum decoupling conditions were established, where it was found that 0.8 W (12 H) continuous decoupling gave rise to the most satisfactory spectra. Here the two-bond couplings were readily resolved (^{2}J (^{13}C-^{13}C) = 4 Hz). This same sample was split into two parts, one being used to generate a sample of uro'gen I, and the other being used to make uro'gen III. Both uro'gen samples were characterised at ambient temperature, pH 8.0 over a narrow spectral width (SW = 1,000 Hz), in order to enhance resolution. Figure 2.17 shows the spectrum obtained from uro'gen I. This is clearly composed of a double doublet centred on 117.74 ppm (^{2}J (^{13}C-^{13}C) = 4 Hz, ^{3}J (^{13}C-^{13}C) = 2 Hz) arising from C-3, C-8, C-13 and C-18, and an identical pattern centred on 126.06 ppm arising from C-1, C-6, C-11 and C-16. Incidentally, that the ^{13}C atoms on the acetate side of the pyrroles should give rise to the doublets at 126.06 ppm is by no means obvious, and in fact the clue to this assignment came from the spectrum of uro'gen III.

The spectrum of uro'gen III (see Figure 2.18) is not so easy to interpret, and only assignments to broad families of peaks can be made. We would expect C-3 and C-17 to show two clear doublets

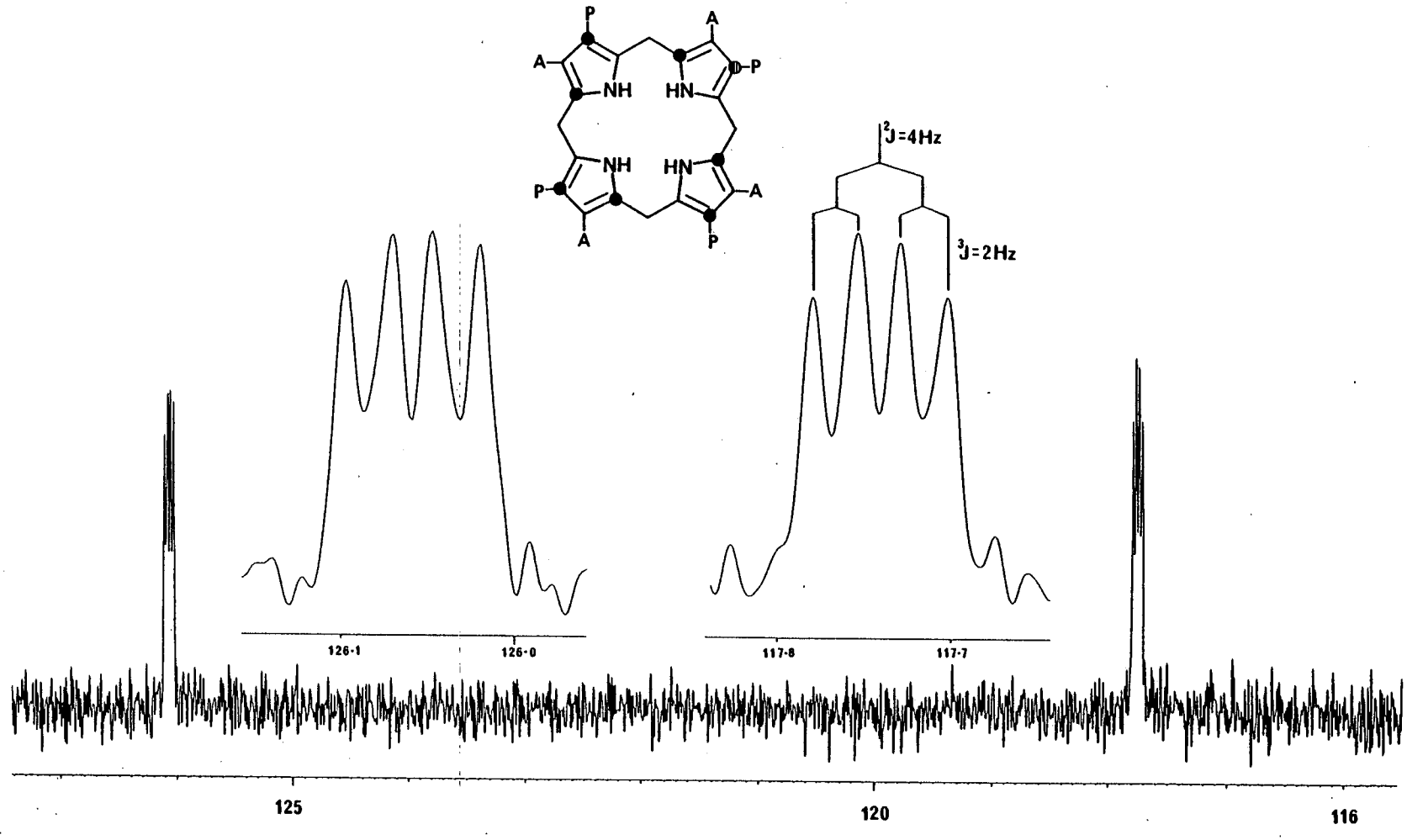


FIGURE 2.17. 75.5 MHz ^{13}C NMR spectrum of [3,5-^{13}C]-PBG-derived uro'gen I, SW = 1,000 Hz, pH 8.0, 25°C.

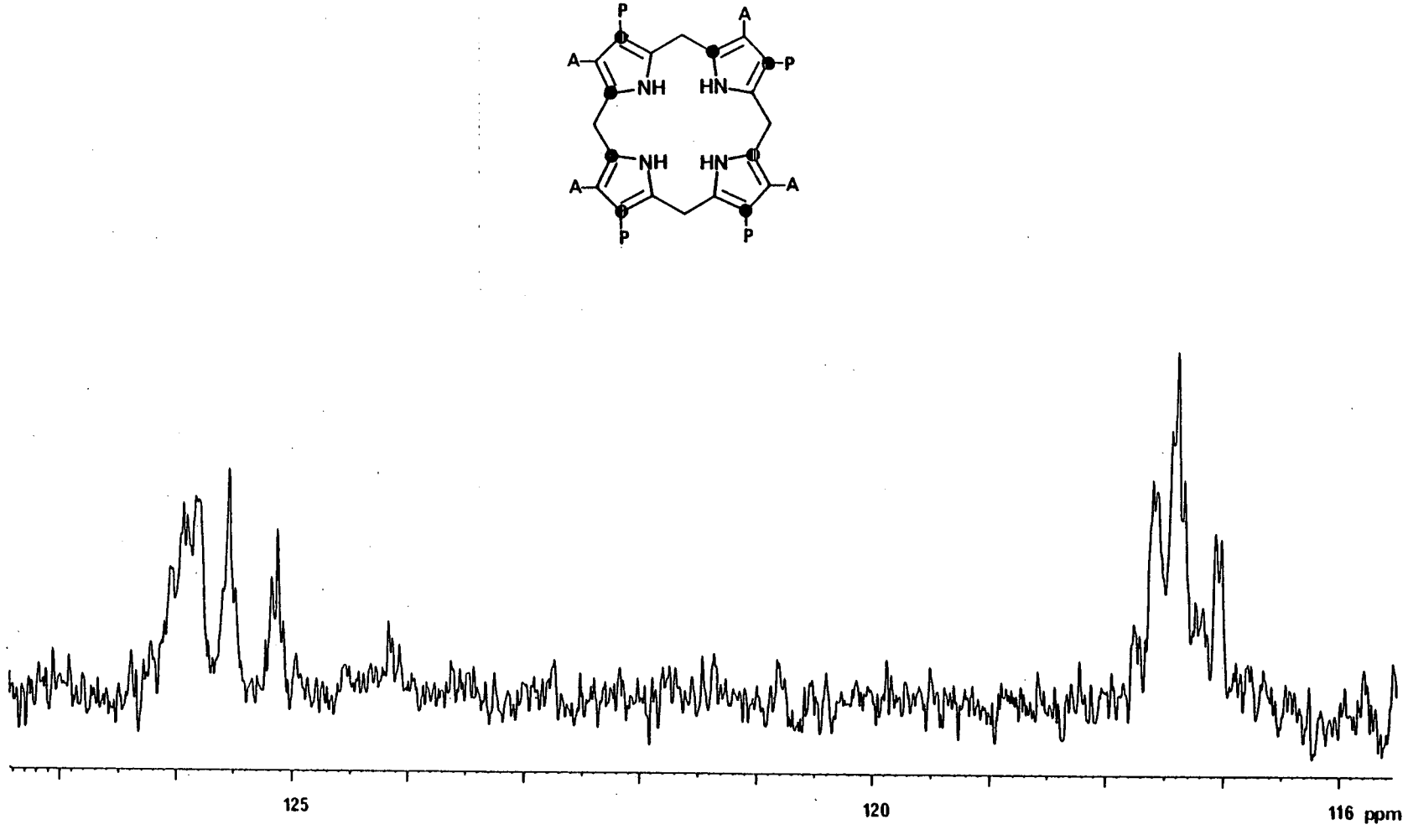


FIGURE 2.17. 75.5 MHz ^{13}C NMR spectrum of [3,5-^{13}C]-PBG-derived uro'gen III, SW = 1,000 Hz, pH 8.0, 25°C.

(^2J (^{13}C-^{13}C) = 4 Hz), one at 117.03 ppm (^2J observed) and the other in the family of peaks centred at 117.37 ppm, probably at 117.34 ppm (^2J observed). For C-3 and C-8, we would expect two doublet of doublets (^2J (^{13}C-^{13}C) = 4 Hz, ^3J (^{13}C-^{13}C) = 2 Hz) which occur at 117.39 ppm (not completely resolved) and at 117.56 ppm (not completely resolved). In the case of the other family of peaks arising from the acetate side of the pyrroles, C-6 and C-11 would be expected to give rise to two doublet of doublets (^2J (^{13}C-^{13}C) = 4 Hz, ^3J (^{13}C-^{13}C) = 2 Hz), which occur centred at 125.90 ppm (not resolved) and 125.81 ppm (not resolved). For C-1 and C-19 we would expect a unique environment giving rise to a known two-bond coupling (^2J (^{13}C-^{13}C) = 4 Hz), and an unknown two-bond coupling. Thus we find an unresolved doublet of doublets at 125.52 ppm and a more revolved double doublet at 125.33 ppm.

With the substrate and two possible products characterised, the best possible NMR conditions were investigated. Uro'gen I in pyrophosphate-D_2O buffer (2.0 cm^3, pH 8.0) in a 10 mm NMR tube was studied at 10°C with 0.8 W (12 H) continuous decoupling. A matrix was constructed varying pulse width and acquisition time against signal-to-noise over a fixed accumulation time of 4.5 minutes. In this way the optimum pulse width and recycle time were selected.

Initially, deaminase (272 U) in pyrophosphate-D_2O buffer (pH 8.5) was studied in the NMR spectrometer for 9 minutes, and no extraneous peaks occurred in the areas of interest. [3,5-^{13}C]-PBG (5 mg) in pyrophosphate buffer was added to the enzyme, incubated at 37°C for 4 minutes before adjusting to pH 12 with NaOH and cooling to 0-5°C. A clearly resolved spectrum of HMB was obtained and is shown in Figure 2.19. Here all eight carbons are distinguishable

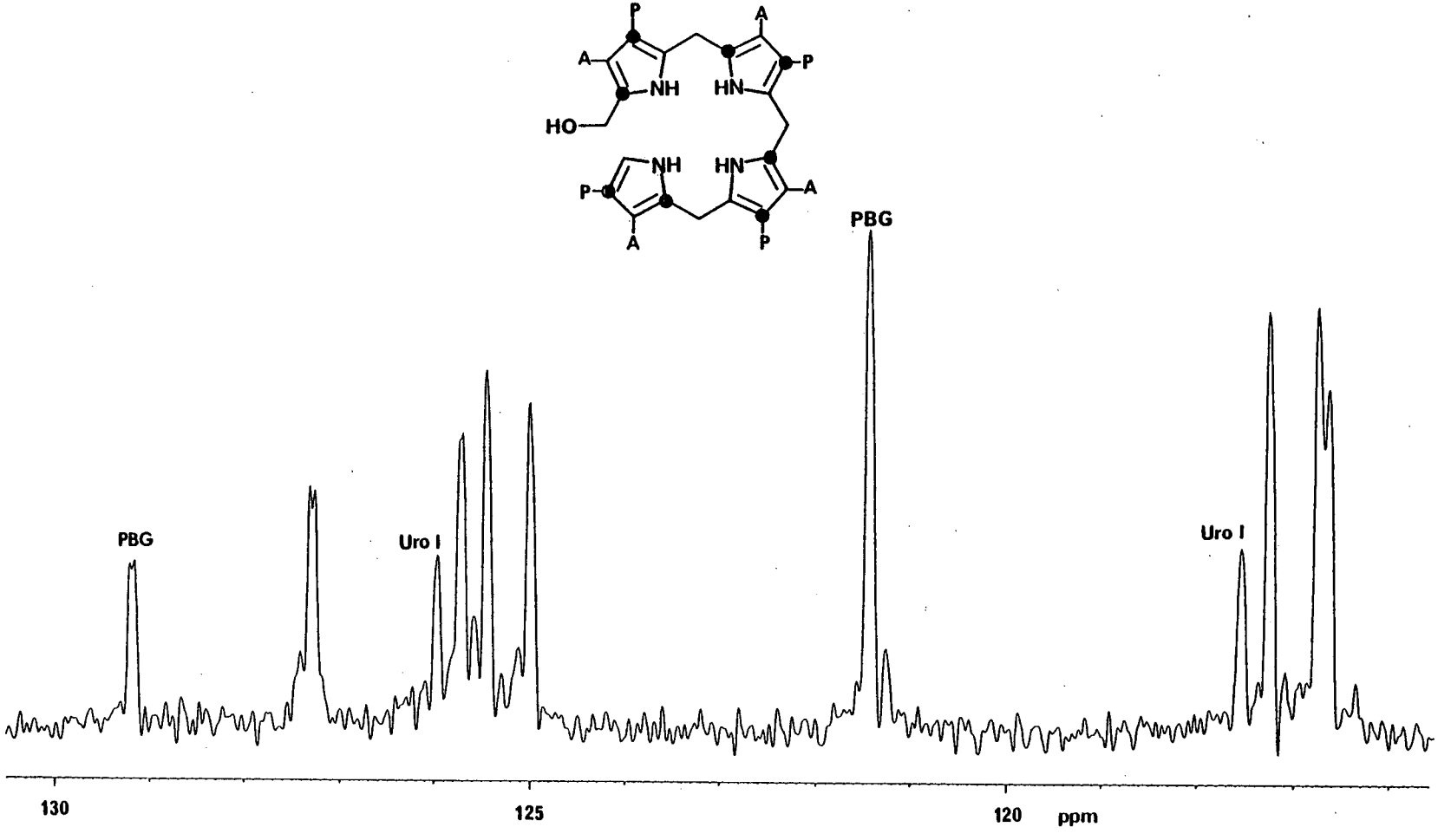
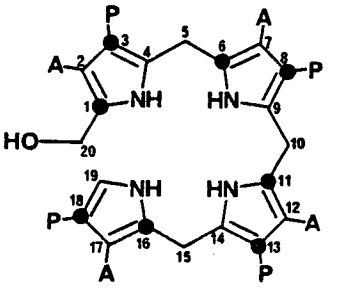


FIGURE 2.19. 75.5 MHz ^{13}C NMR spectrum of [3,5-^{13}C]-PBG-derived HMB, pH 12, 4°C.

and the tentative assignments are given in Table 2.5. Also evident in the spectrum is the presence of uro'gen I at 117.54 and 125.98 ppm, and also residual [3,5-^{13}C]-PBG, with C-3 coinciding with C-18 of HMB at 121.42 ppm and C-5 occurring at 129.16 ppm. This latter chemical shift for C-5 of PBG is clear from the earlier work (Section 2.6) on the pH titration of [3,5-^{13}C]-PBG.

TABLE 2.5. The ^{13}C NMR assignment (tentative) of [3,5-^{13}C]-PBG-derived HMB.



^{13}C	δ/ppm[a]
C-8	116.63
C-13	116.75
C-3	117.25
C-18	121.42
C-16	124.99
C-6	125.44
C-11	125.69
C-1	127.28 (d, ^{2}J (^{13}C-^{13}C) = 4 Hz)

[a]Indirectly referenced to p-dioxane, δ_C = 66.5 ppm.

This sample of HMB was readjusted back to pH 8.0 with HCl after the addition of cosynthetase (30 U) with rapid mixing, and spectra accumulated at 4.5 minute intervals over 3 hours and at *ca.* 4°C. The full time-course is shown in Figure 2.20. Here we can see the loss of HMB and also residual PBG, and concomitant formation of uro'gen III. However inspection of the full spectral width reveals no new sp^3-carbon bearing intermediates or indeed any sp^2 intermediates (see Figure 2.21). In the expansion of the first hour of the reaction (Figure 2.21), no detectable intermediates are revealed. Note that the low-intensity peak which occurs at 21 ppm is also present in the non-enzymic blank (Figure 2.23) and probably represents resonances from the deaminase present.

In order to test the possibility that some intermediates might be present in vanishingly small quantities, a non-enzymic control was performed. HMB derived from [3,5-^{13}C]-PBG was analysed by ^{13}C NMR at pH 8.0, 5°C (see Figure 2.22) and its spectrum is not significantly different from that obtained at pH 12. This was allowed to cyclise chemically as a non-enzymic control (see Figure 2.23). Careful comparison of the series of spectra from the cosynthetase-mediated reaction with the spectra for the cosynthetase-free reaction does not reveal any detectable transient intermediates under these NMR conditions.

Although both the ^{1}H and ^{13}C NMR techniques employed in the prosecution of the research outlined above did not reveal any of the putative intermediates in the mechanistic Scheme 11, they did allow a thorough study of HMB and uro'gens I and III. These studies therefore laid the foundations for much more detailed studies on the enzyme deaminase, where knowledge of all the NMR detectable intermediates and products was absolutely essential.

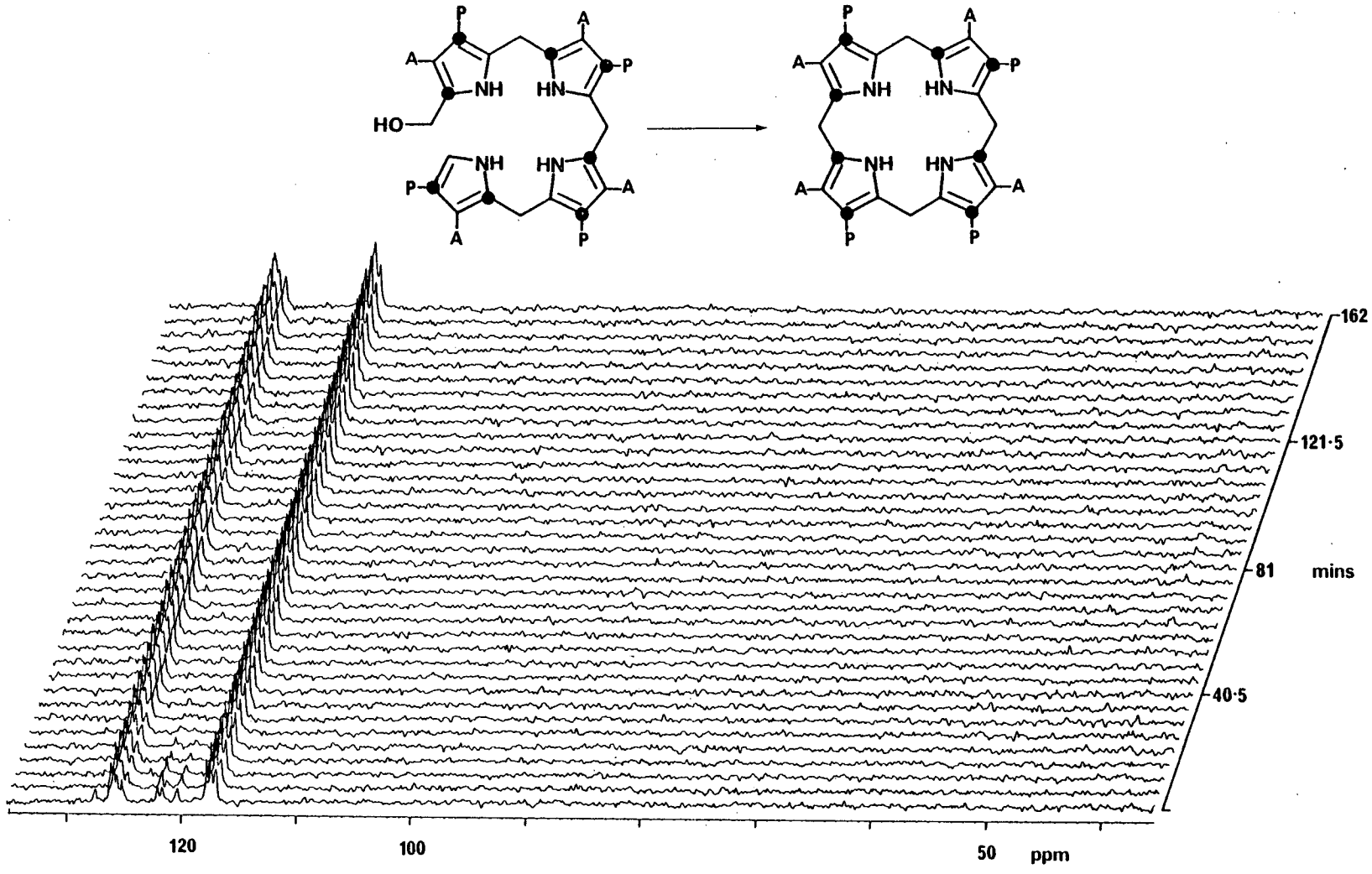


FIGURE 2.20. 75.5 MHz ^{13}C NMR spectra of full time-course of [3,5-^{13}C]-PBG-derived HMB with cosynthetase, pH 8.0, 4°C.

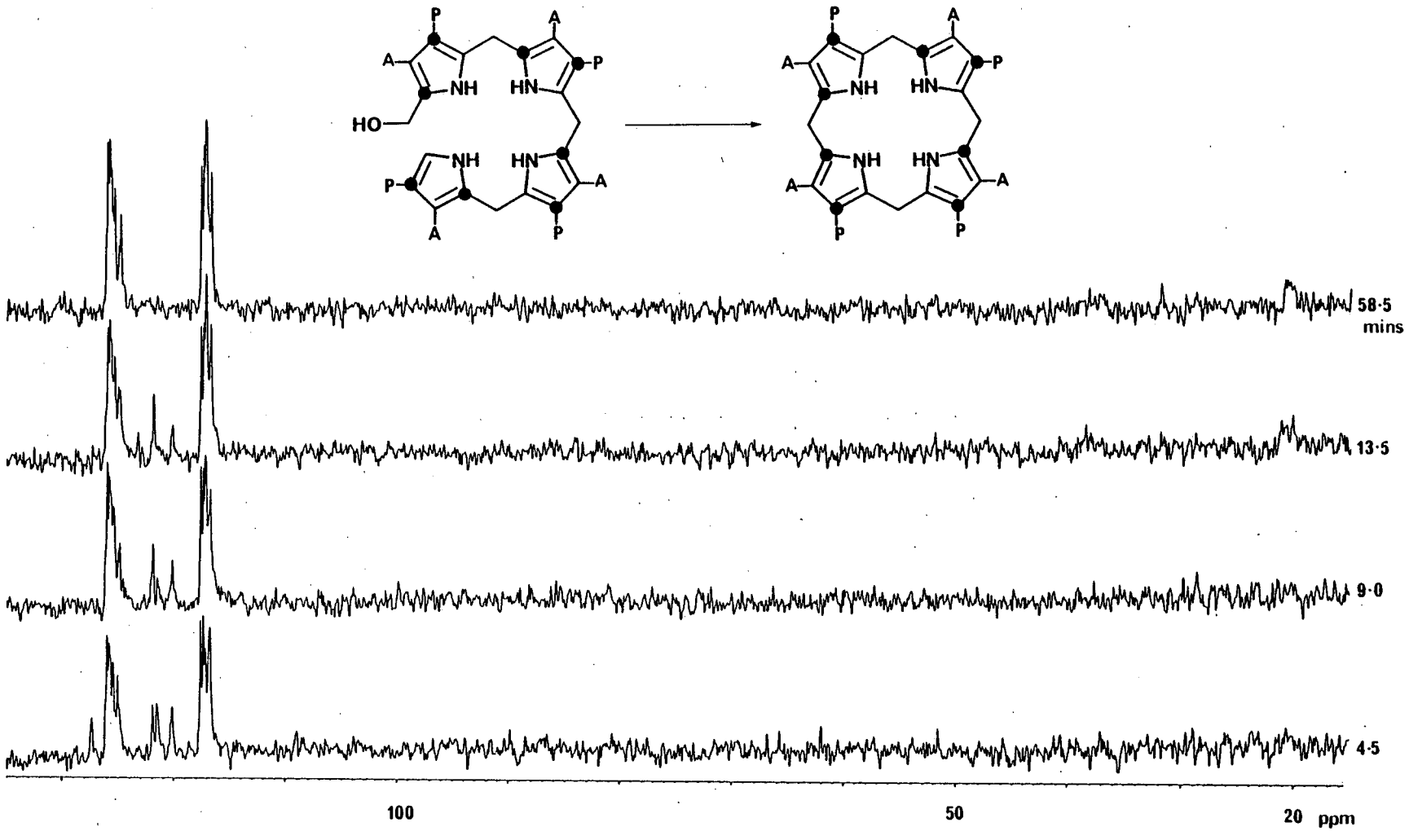


FIGURE 2.21. 75.5 MHz ^{13}C NMR spectra of [3,5-^{13}C]-PBG-derived HMB with cosynthetase, pH 8.0, 4°C.

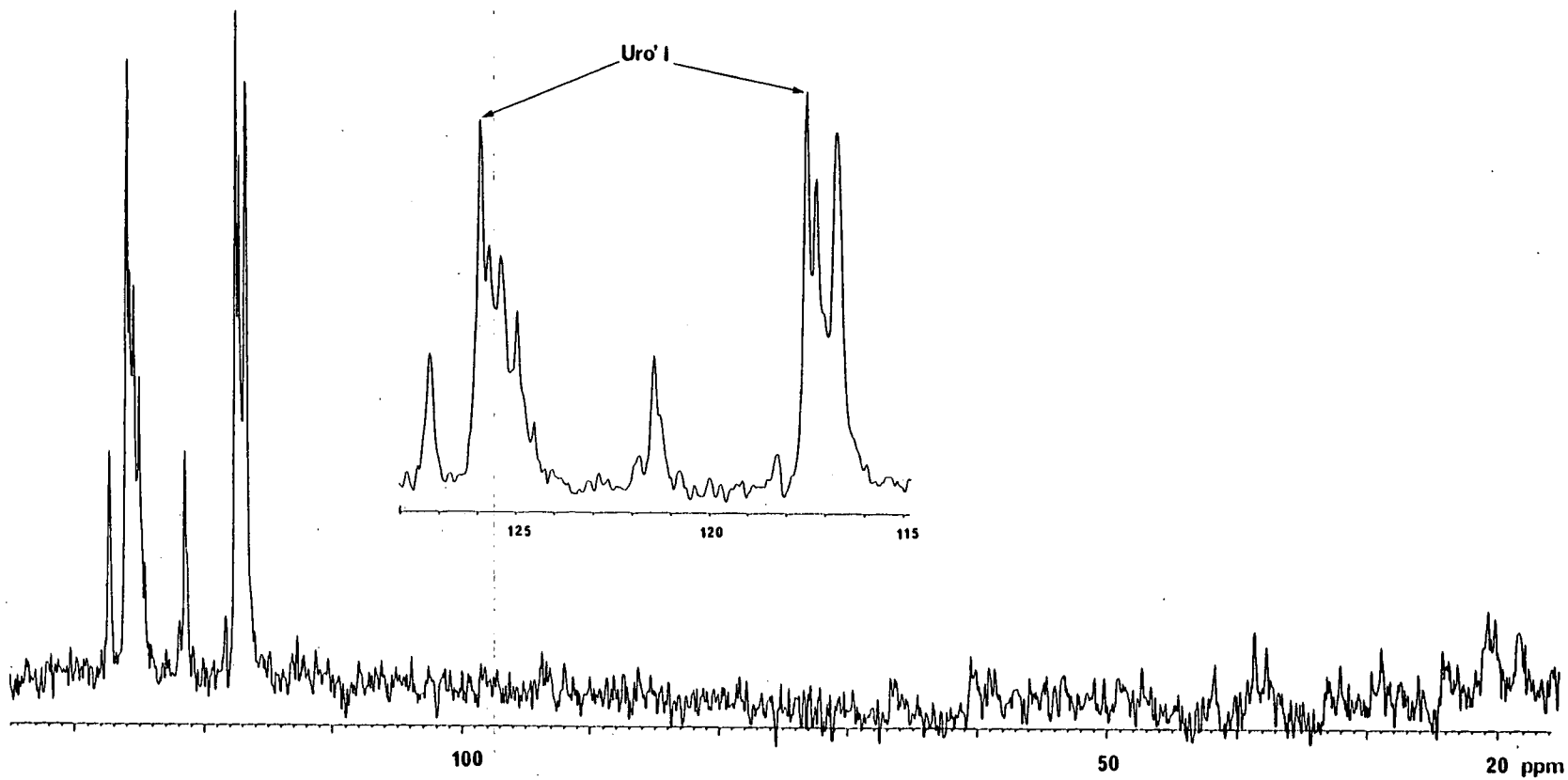


FIGURE 2.22. 75.5 MHz ^{13}C NMR spectrum of [3,5-^{13}C]-PBG-derived HMB, pH 8.0, 5°C.

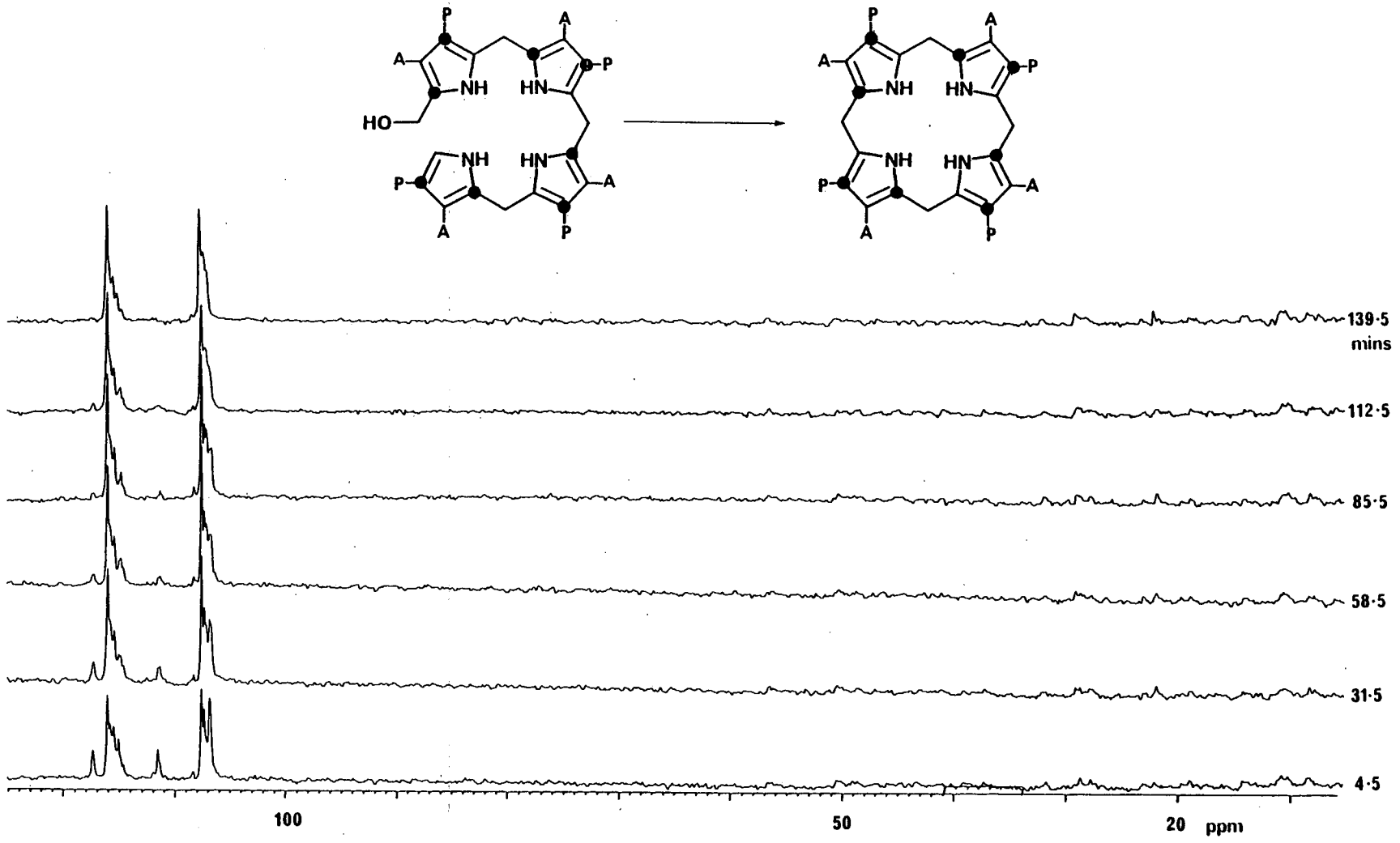


FIGURE 2.23. 75.5 MHz ^{13}C NMR spectra of [3,5-^{13}C]-PBG-derived HMB cyclising chemically (non-enzymic control), pH 8.0, 5°C.

CHAPTER 3

ISOLATION AND NMR STUDIES OF [^{13}C]-PBG-DEAMINASE COVALENT COMPLEXES

3.1 Introduction

Both this chapter and the next are confined to a detailed study of the second question mentioned in Section 2.1, namely how is the linear tetrapyrrole bound and assembled on the surface of deaminase? Following the work by Anderson and Desnick[126] and Jordan and co-workers,[140,141] the possibility of binding ^{13}C-labelled PBG to deaminase and examining the covalent complex by ^{13}C NMR seemed very attractive. Such a complex might furnish vital information about the nature of the active site of deaminase and the way in which the tetrapyrrole is bound, presumably through a suitably nucleophilic group X as shown in Scheme 10.

In the work to be described in this chapter, five major attempts have been made using [2,11-^{13}C]-PBG, [2,11-^{13}C,$^{2}H_3$]-PBG and [3,5-^{13}C]-PBG, and large batches of purified deaminase. Prior to the generation of these complexes, detailed studies had to be performed in order to establish a reliable protocol for their production.

3.2 Preliminary Studies (with Dr. G. Burton)

For the preliminary studies of the enzyme-substrate complex of deaminase and PBG, deaminase was partially purified from *R. spheroides* by the methods outlined in Section 3.3. This deaminase was analysed by flat-bed gel electrophoresis, and conditions optimised for the preparation of the complexes bearing one, two or three PBG molecules. For such experiments a "High Performance Mixing Device" (HPMD) was

constructed, and this was used to mix defined stoicheiometries of PBG and deaminase.

Using [3,5-^{14}C]-PBG, prepared by the action of ALA dehydratase on [4-^{14}C]-ALA, a series of experiments were conducted in which the stoicheiometry of [^{14}C]-PBG and deaminase was varied, and the mixing performed under a variety of conditions. As with Jordan's experiments, four bands could be obtained by gel-electrophoresis on 7.5% polyacrylamide gels when stained either with Coomassie Blue, incubated with PBG and visualised by UV, or when subjected to autoradiography. These bands corresponded to native enzyme and three enzyme-substrate complexes. No band corresponding to bound tetrapyrrole was detected, as Jordan found with the bacterial enzyme. The mobilities of the four bands were determined and for the native enzyme, "mono-PBG", "di-PBG" and "tri-PBG", the mobility/% of tracking dye = 57, 62, 64 and 66%, respectively. This compares favourably with Jordan's reported[141] values of 56, 59, 65 and 70% for the same bands, taking into account differences in gel-operating pH. Varying the stoicheiometry of enzyme and substrate provided the means to optimise the yields, mainly for the complexes bearing one and two substrate molecules. The method for calculating the amount of enzyme made use of Jordan and Shemin's[119] reported specific activity of 128 U mg^{-1} for homogeneous deaminase from *R. spheroides*. Using the $M_r \simeq 36{,}000$ for bacterial deaminase, we can calculate the moles of enzyme from its activity assuming that if the enzyme were completely pure it would have a specific activity of 128 U mg^{-1}.

The data shown in Table 3.1 are typical values obtained from cutting the bands corresponding to complexes bearing one, two and three [^{14}C]-PBG units, dissolving the bands in 30% H_2O_2 and assaying

TABLE 3.1. The effect of [^{14}C]-PBG deaminase stoicheiometries on the resultant enzyme-substrate complexes.

Exptal. Stoich. E:S Ratio[a]	Band E:S Ratio[b]	% Total Bound[c]	% Distribution Radioactivity	% Moles[d]	Exptal. Conditions[e]
1.76:1	1:1	9.3	38.5	56.7	Mixing device[f] at 4°C.
	1:2		53.2	39.2	
	1:3		8.3	4.1	
1:1.26	1:1	29.1	21.1	37.2	Sequential mix at 4°C.
	1:2		55.7	49.1	
	1:3		23.2	13.6	
1:1.88	1:1	25.0	28.0	46.1	HPMD mix at 4°C.
	1:2		52.4	43.2	
	1:3		19.5	10.7	
1:1.88	1:1	26.0	22.8	40.7	HPMD mix at 37°C.
	1:2		55.8	50.0	
	1:3		15.6	9.3	
1:1.88	1:1	30.0	17.7	33.8	Sequential mix at 4°C.
	1:2		55.9	53.3	
	1:3		20.3	12.9	
1:1.88	1:1	32.0	17.5	33.4	Sequential mix at 37°C.
	1:2		55.4	52.9	
	1:3		21.4	13.6	
1:2.63	1:1	23.9	17.4	32.1	Sequential mix at 4°C.
	1:2		55.9	51.5	
	1:3		26.7	16.4	

[a] Calculated using sp. act. of deaminase = 128 U/mg, $M_r \simeq 36{,}000$, and Ehrlich's determination of [^{14}C]-PBG. ($A_{553} = 1.0 \Rightarrow 49.5$ nmol PBG)

[b] Assuming that the bands assigned correspond to the complexes Enz-$(PBG)_n$ where n = 1, 2, 3.

[c] Assayed by radioactive counting.

[d] Normalised, using assumption in (b).

[e] Procedure either employed HPMD or sequential addition with mixing, and then stored at 4°C, or subsequent 37°C incubation for 5 minutes.

[f] An earlier, less-efficient prototype of the HPMD was used here.

the radioactivity by liquid scintillation counting. The error in the values presented is significant, since cutting bands from a gel in which several bands are located close together results in cross-contamination and errors in counting. However, there are general trends from which certain conclusions can be drawn. Since the volumes of enzyme and substrate employed in these experiments were very small (*ca.* 30 μL) using the HPMD did not significantly enhance the yield over sequential mixing. Furthermore, the temperature of mixing did not affect either the yield of bound substrate or indeed the proportions of the three complexes.

The results from these experiments suggested that it would be difficult to generate the "mono-PBG" complex without a significant amount of "di-PBG" complex. While increasing the enzyme:substrate ratio favours the production of the "mono-PBG" complex, the overall amount of substrate bound is lowered. Since the amount of bound substrate was critical for the success of any NMR experiment, this needed to be maximised, while trying to minimise the amount of "di-PBG" complex. In scaling up the mixing to a preparative scale, it is evident that the velocity of the reaction needs to be lowered in order to minimise the formation of product. Thus on a preparative scale, mixing in the HPMD at 4°C seemed to be the best procedure. The optimum enzyme-substrate ratio for preparative procedures was determined using the results of Table 3.1 as a guideline, while acknowledging that the dynamics of mixing on that scale are somewhat different. The preparative techniques are described in Section 3.4

The proteolytic digestion of the complexes was also investigated, and here it was found that while α-chymotrypsin only resulted in partial digestion, pronase was extremely effective. Pronase is an

unusually non-specific proteolytic enzyme which is particularly useful for generating small oligopeptides.[187] These effects were assessed using analytical gel-electrophoresis.

3.3 Isolation and Purification of PBG Deaminase

For most of the preparations of covalent complexes described in this chapter, the deaminase employed was purified from *R. spheroides* to a purity of *ca.* 15% by protein mass. Further purification of the resultant complex afforded samples of highly purified protein (>60% by protein mass and gel-electrophoresis) which were used for much of the experiments described in Chapter 4. The reasoning behind this approach for the purification of the enzyme was as follows. It has been reported[119] that the highly pure enzyme is inherently unstable, whereas formation of the covalent complex confers stability on the protein. It therefore seemed sensible to generate the complex while the enzyme was partially pure, and then highly purify the complex.

The general procedure adopted for the purification of deaminase is shown in Figure 3.1, which includes the steps necessary to obtain enzyme of homogeneous purity. This procedure is a development of the methods of Jordan and Shemin,[119] and Davies and Neuberger.[71] In a typical preparation, 3 kg of wet cells (from 500 L of cell suspension) were processed in eight batches to yield *ca.* 6000 U of deaminase. Thus wet cells (375 g) were suspended in phosphate buffer and sonicated. After low-speed centrifugation to remove the cell debris, the supernatant was heat-treated in small batches and then subjected to high-speed centrifugation. The speed of centrifugation is critical at this point in determining the ultimate purity of the enzyme, forces below 100,000 g being less satisfactory.

(i) Sonication of *Rhodopseudomonas spheroides*.

(ii) Centrifugation at 35,000 g.

(iii) Heat-treatment for 15 minutes at 60°C.

(iv) Centrifugation at 150,000 g.

(v) Ammonium sulphate precipitation (40-55%).

(vi) Centrifugation at 16,000 g.

(vii) Dialysis.

(viii) Chromatography on DEAE-Sephacel.

(ix) Concentration on PM-10 Ultrafilter.

(x) Gel-filtration on G-100 Sephadex.

(xi) Concentration on PM-10 Ultrafilter.

(xii) Heat-treatment for 15 minutes at 60°C.

(xiii) Second gel-filtration on G-100 Sephadex.

(xiv) Concentration on PM-10 Ultrafilter.

(xv) Preparative polyacrylamide gel-electrophoresis.

(xvi) Concentration on PM-10 Ultrafilter.

(xvii) Second preparative gel-electrophoresis.

(xviii) Concentration of PM-10 Ultrafilter.

FIGURE 3.1. Isolation and purification of PBG deaminase.

Crude deaminase was then separated from the high-speed supernatant by fractional ammonium sulphate precipitation, and obtained as a pellet by centrifugation. This was redissolved in Tris buffer and dialysed against two changes of Tris buffer. The dialysate was then applied to a freshly equilibrated DEAE-Sephacel column (see Figure 3.2), and eluted with a salt gradient of 0.05 M KCl to 0.7 M KCl in Tris buffer. DEAE-Sephacel is a commercially available brand of cellulose which is formed into beads and this removes the problems produced by fines which are invariably present in ordinary cellulose. Furthermore, the beaded configuration withstands considerably higher pressures when the column is packed, allowing a denser packing which shows improved chromatographic properties.

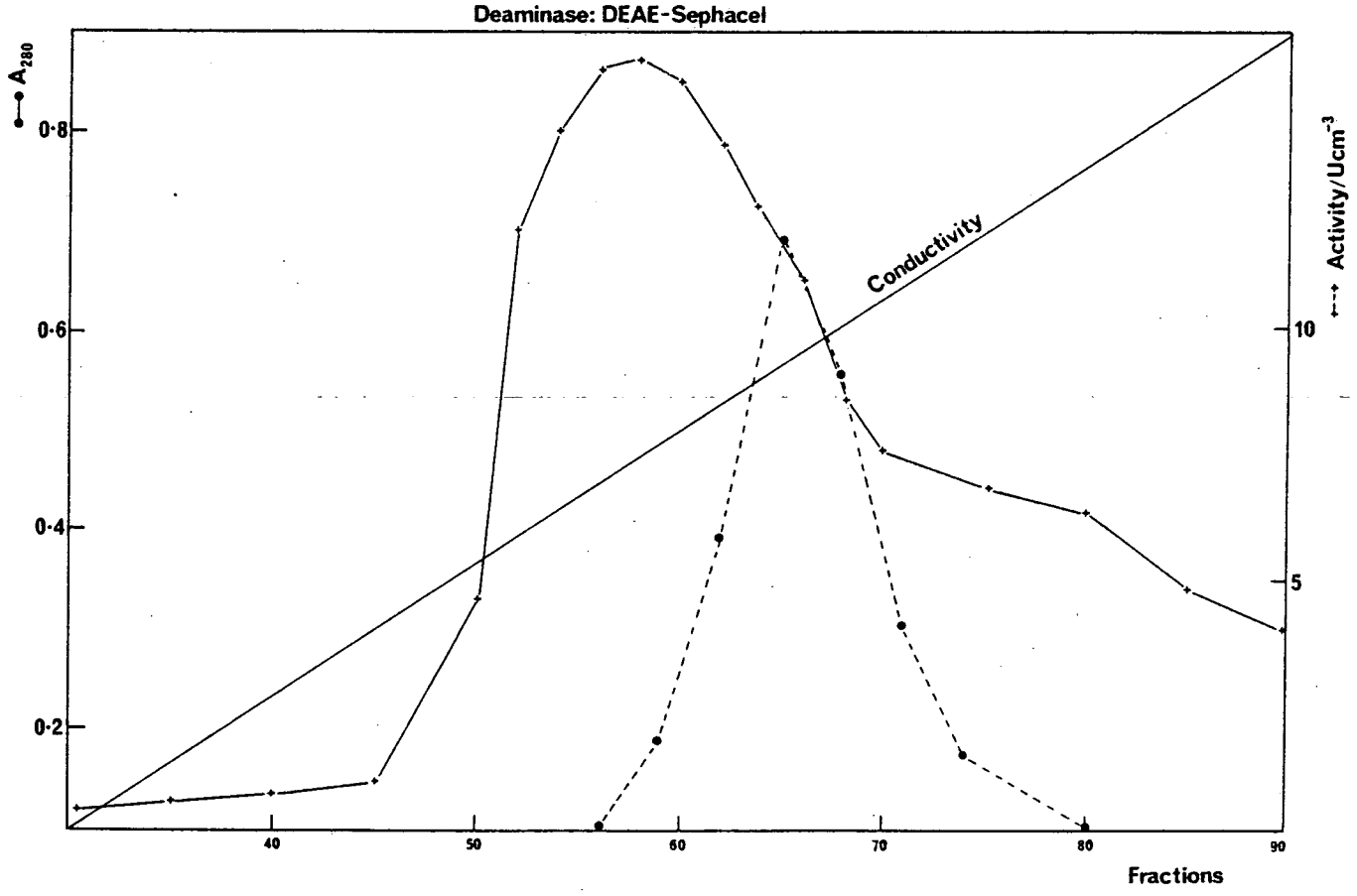


FIGURE 3.2. Purification of deaminase on DEAE-Sephacel.

This cycle was then repeated with another batch of cells and the pooled enzyme from both batches concentrated and applied to a freshly equilibrated G-100 Sephadex column (see Figure 3.3). In

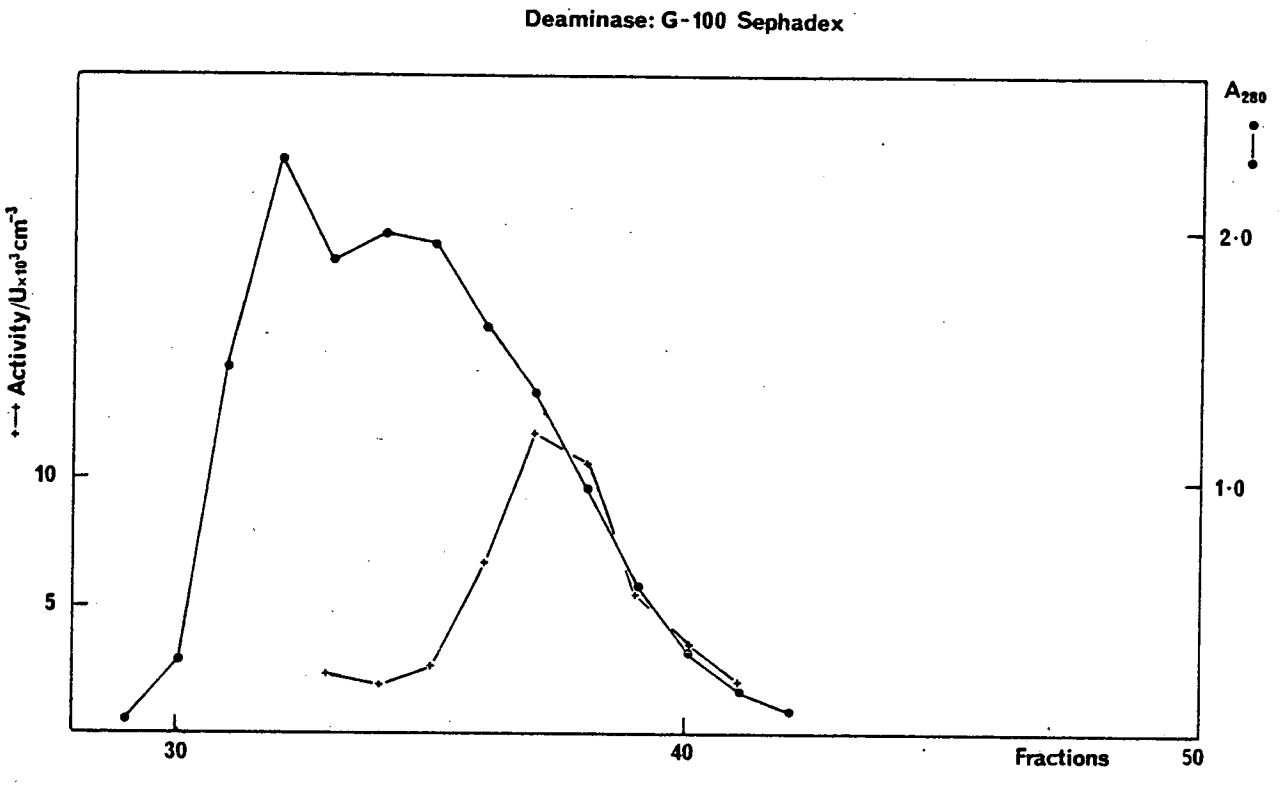


FIGURE 3.3. First purification of deaminase on G-100 Sephadex.

this way the other six batches of cells were processed to furnish in total four samples of enzyme after gel-filtration and concentration. This enzyme activity was then pooled, heat-treated at 60°C for 15 minutes and again applied to a freshly equilibrated G-100 Sephadex column (see Figure 3.4). This step helps to remove a yellow protein which contaminates the deaminase. The two heat-treatment steps served to eliminate the presence of cosynthetase. The pooled enzyme specific activity was typically 12-30 U mg^{-1} (6-12,000 U) at this stage. The further purification steps mentioned in Figure 3.1 will be described in the next section. Routinely the enzyme activity was assayed by comsumption of PBG,[76] using modified Ehrlich's reagent.

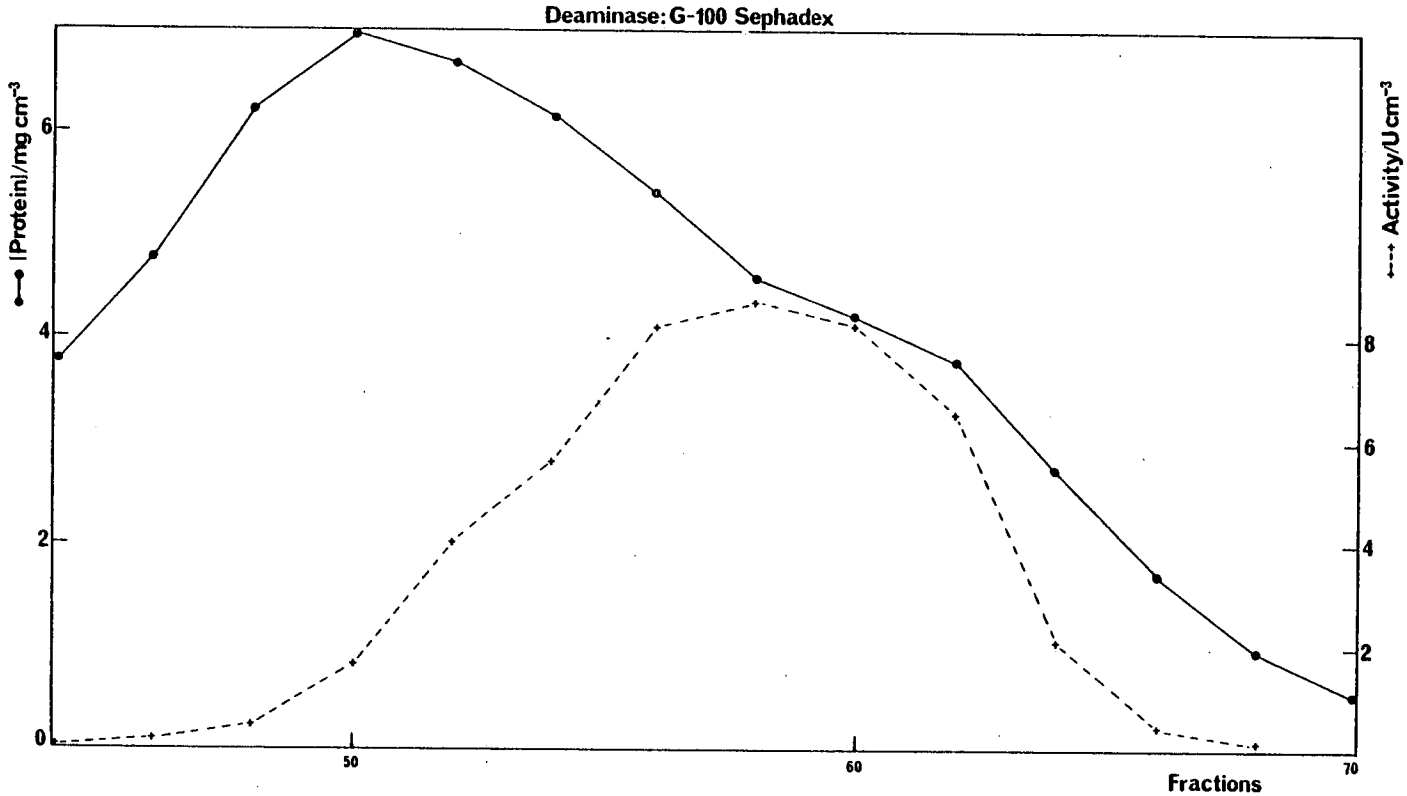


FIGURE 3.4. Second purification of deaminase on G-100 Sephadex.

3.4 Preparation and Purification of the Covalent Complexes

Any NMR experiment designed to study an enzyme-substrate complex must take into account a number of factors which will influence its eventual outcome. The amount of substrate which can be successfully bound is one such factor. Since deaminase is a monomer of $M_r \simeq 36,000$ and a typical preparation yields 6000 U, or 47 mg (1.3 μmol), which could theoretically bind 375 μg of ^{13}C-enriched PBG. This means that the NMR detectable concentration of enriched PBG is equivalent to 37.5 mg at natural abundance (natural abundance of ^{13}C = 1.1%). Thus the substrate, even if we succeed to bind it with a 100% yield, will be present at similar NMR detectable concentrations as the natural abundance protein background. Binding more than one substrate molecule is unlikely to increase the chances of detecting the signals from the substrate, since the labelled resonances are likely to exhibit different chemical shifts. The results of Table 3.1 clearly show that 100% yields are exceedingly difficult to attain, and that the highest yields are achieved when the substrate-to-enzyme ratio is kept high. Accordingly, initial preparations of complex employed an enzyme:substrate ratio of 1 to 3, and were mixed using the HPMD at 4°C, eluting at *ca.* 60 $cm^3\ hr^{-1}$. Minor variations on these conditions were made in each preparation, in an attempt to optimise the yield of "mono-PBG" complex.

In a typical preparation, deaminase (13 μM, 6000 U) and [^{13}C]-PBG (13 μM) and 5 moles % [^{14}C]-PBG were mixed at 4°C using the HPMD, and the resultant solution concentrated by ultrafiltration to a volume of *ca.* 2 cm^3. This was then applied to a preparative polyacrylamide gel (7.5%) and subjected to electrophoresis at pH 8.0

eluting with Tris/Barbital buffer for the upper electrode, and Tris buffer for the lower electrode and sample chamber. Figure 3.5 shows a typical elution profile, detecting protein concentration, enzyme activity and radioactivity. The radioactivity provides an accurate method for assessing the amount of PBG bound, since prior to mixing with deaminase we know the dpm per μmol [^{13}C]-PBG, and thus in the labelled protein the dpm will give the amount of bound PBG. The pooled complex was concentrated by ultrafiltration down to *ca.* 0.45 cm^3 for NMR experiments in a 5 mm tube. The complex thus obtained was usually *ca.* 70% pure and mainly (>90%) corresponded to "mono-PBG" complex (by analytical gel-electrophoresis), bearing approximately 192 μg PBG (51% yield). The dramatic increase in yield of bound PBG over the analytical experiments presumably reflects more effective mixing when using large volumes of enzyme and substrate.

3.5 ^{13}C NMR Studies of [2,11-^{13}C]-PBG-Deaminase Covalent Complexes

The first ^{13}C NMR investigation of the enzyme-substrate complex employed [2,11-^{13}C]-PBG, in which the chemical shift of C-11 would be highly diagnostic of the nature of binding of the substrate. Accordingly, impure complex which had been subjected only to G-50 gel-filtration to remove excess substrate and product was examined. In this case only 50 μg of [^{13}C]-PBG was bound to deaminase (*ca.* 3000 U) and the protein resonance occluded any obvious ^{13}C-enriched sites. After further purification by preparative gel-electrophoresis, the ^{13}C NMR spectrum showed a very small peak at 124.4 ppm (see Figure 3.6), which was found not to be present in electrophoretically

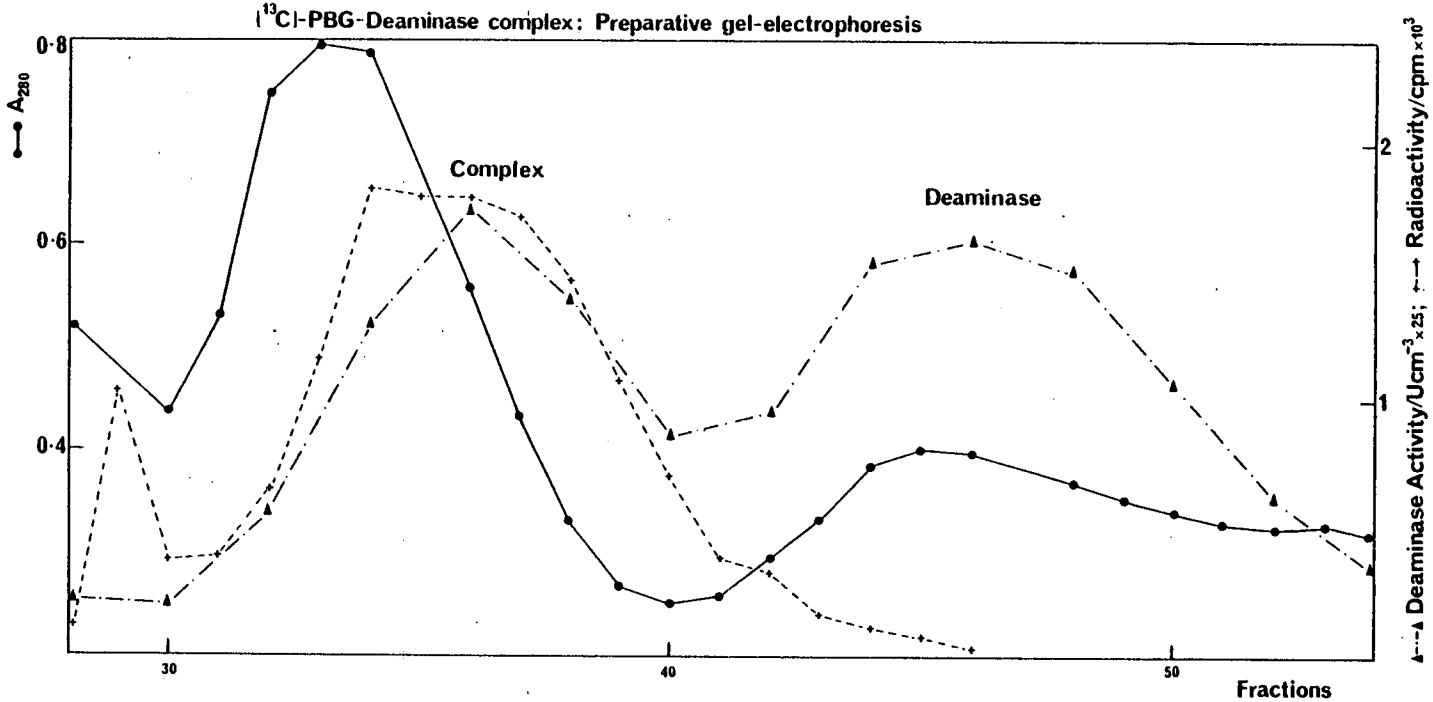


FIGURE 3.5. Purification of PBG-deaminase covalent complex and deaminase by gel-electrophoresis.

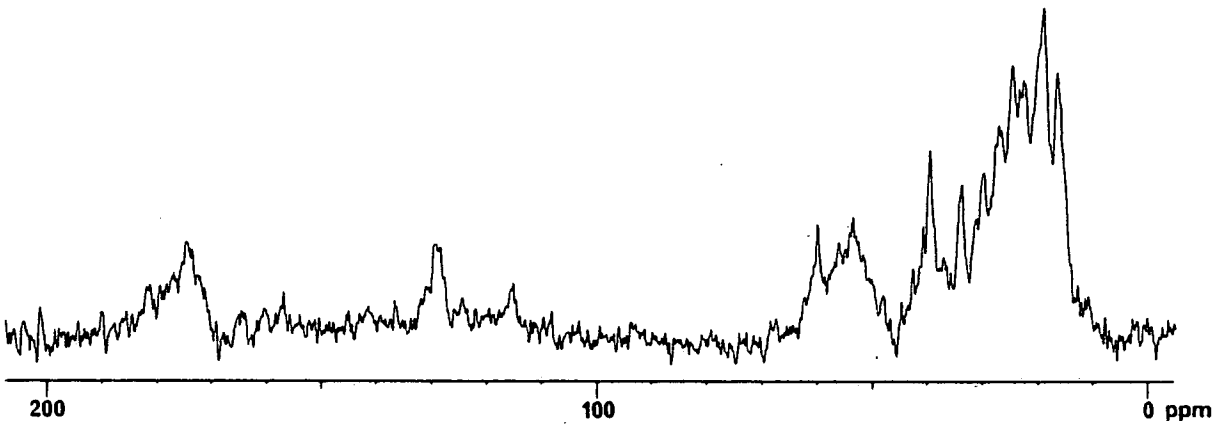


FIGURE 3.6. 75.5 MHz ^{13}C NMR spectrum of [2,11-^{13}C]-PBG-deaminase covalent complex, pH 8.0, 12.5°C.

pure deaminase, also isolated from the preparative electrophoresis (see Figure 3.7). Careful examination of the aliphatic region failed to uncover any significantly enriched peaks. Subtraction techniques also failed to reveal any peaks in that region, due to minor but significant differences between the spectra giving rise to artefacts.

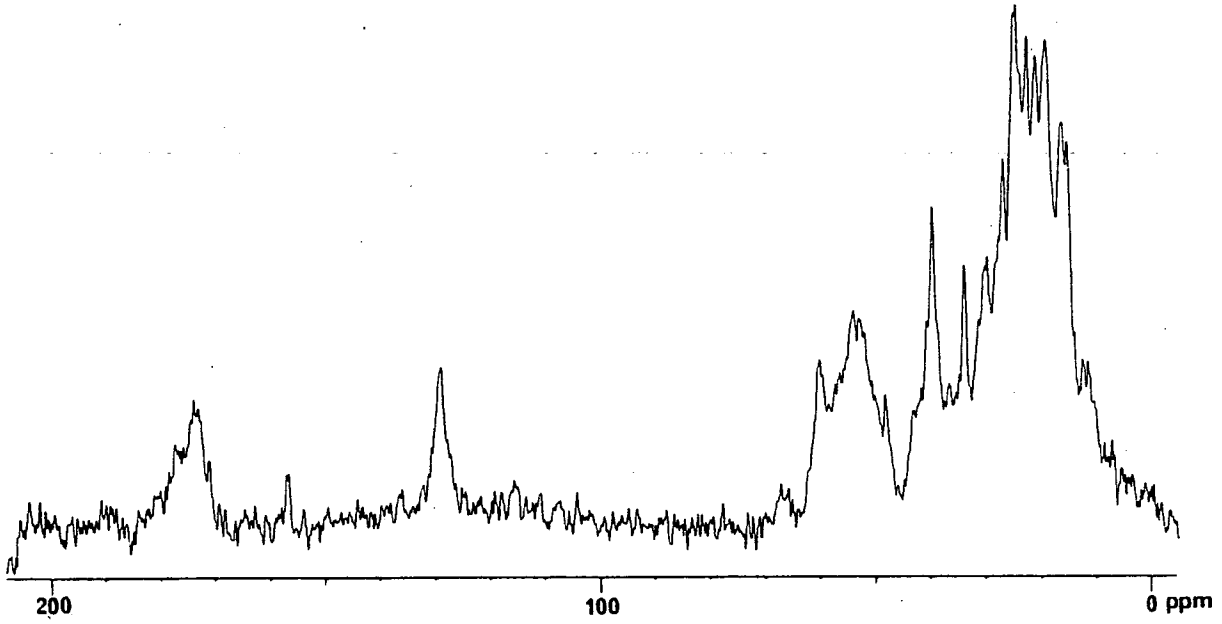


FIGURE 3.7. 75.5 MHz ^{13}C NMR spectrum of electrophoretically pure deaminase, pH 8.0, 12.5°C.

The complex was then incubated with pronase in the NMR tube, using the Carr-Purcell-Meiboom-Gill spin-echo pulse sequence, which optimises narrow lines in the presence of broad ones. After 24 hours, a forest of peaks were observed (see Figure 3.8), corresponding to free oligopeptides. Selected resonances can be assigned to certain amino acid residues using Table 1.2. None, however, could be easily assigned to ^{13}C-enriched PBG. When the digested complex was analysed by ^{13}C NMR using a conventional pulse-sequence, and compared to pure deaminase treated in an identical manner, no distinguishable differences are obvious (see Figure 3.9). Thus no conclusions could be drawn from these spectra, which is in stark contrast to Battersby and co-workers,[188,189] who recently published work along very similar lines in which he obtained a family of peaks at 42-43 ppm, which he assigned to X-$\underline{C}H_2$-pyrrole, and also one at 24.5 ppm, which he assigned to pyrrole-$\underline{C}H_2$-pyrrole. These resulted from oligopeptides from a pronase incubation with the [11-^{13}C]-PBG-deaminase complex. His conclusions are that such results point to the nature of the X group in Scheme 9 being the ε-NH_2 of lysine. It should be noted, however, that Figure 3.9 clearly shows a family of peaks at 41-43 ppm in both the ^{13}C-enriched complex and the pure enzyme. These can be assigned to the α-carbon of glycine, the ε-carbon of lysine, the β-carbon of leucine, and the δ-carbon of arginine, the resonances of which (see Table 1.2) all fall in this region. We chose not to draw conclusions, therefore, from observation of such peaks.

Since the foregoing results were somewhat discouraging, another 3 kg *R. spheroides* was processed to furnish 6000 U of deaminase. This was mixed as usual with [2,11-^{13}C]-PBG + 5% [3,5-^{14}C]-PBG in

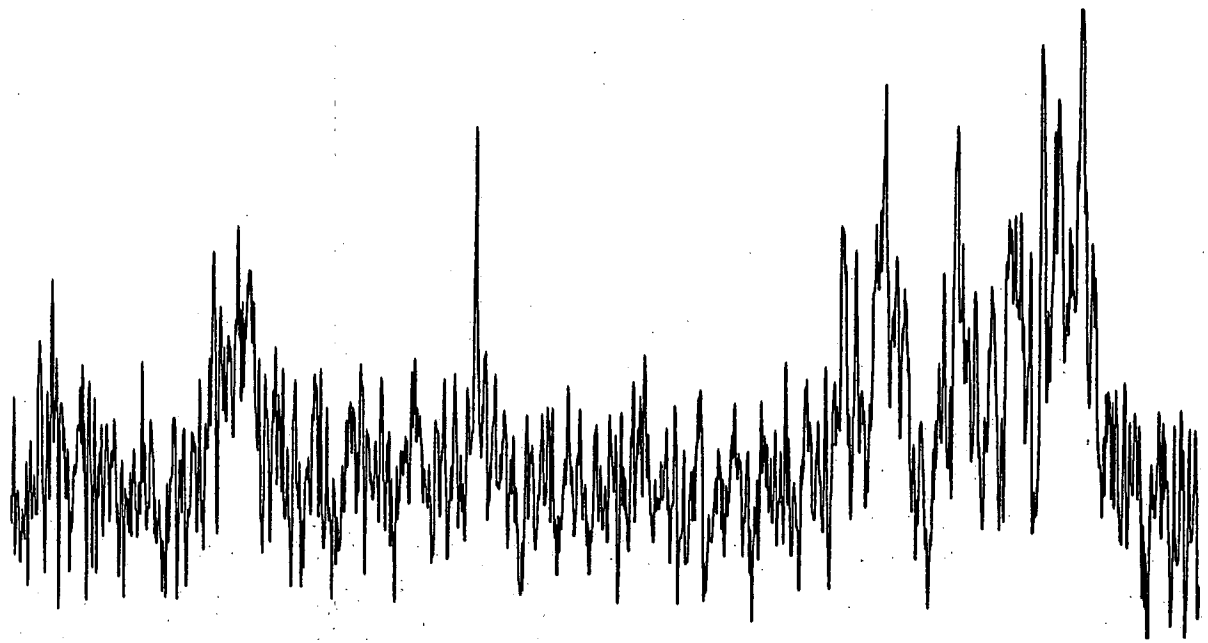


FIGURE 3.8. 75.5 MHz ^{13}C NMR spectra of [2,11-^{13}C]-PBG-deaminase complex + pronase using Carr-Purcell-Meiboom-Gill spin-echo pulse sequence, pH 8.0, 12.5°C.

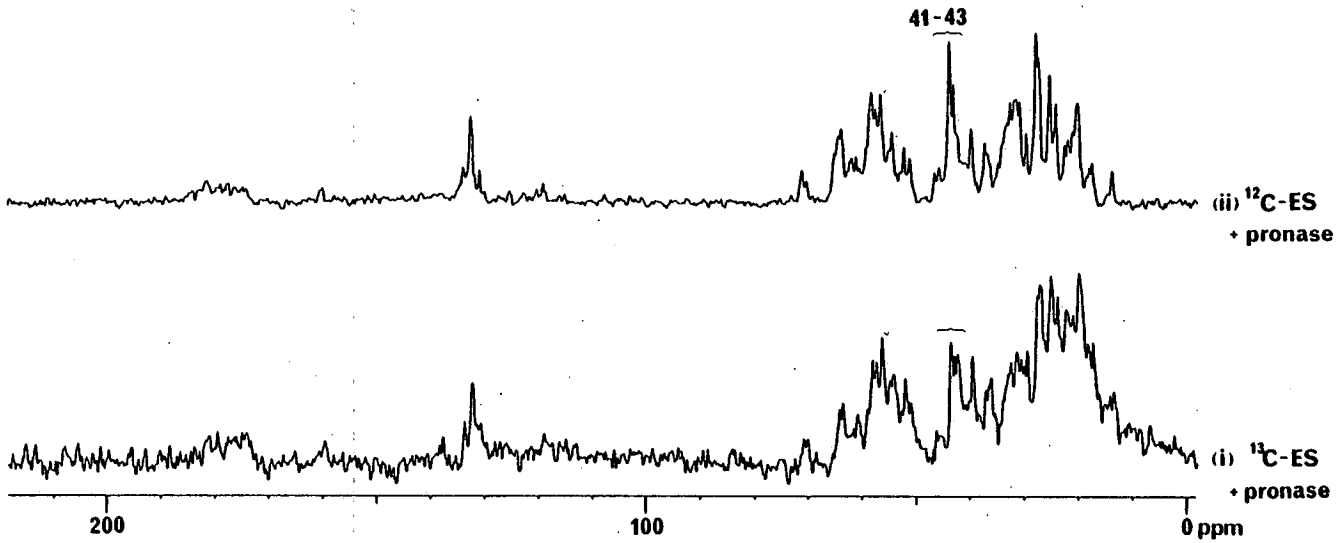


FIGURE 3.9. 75.5 MHz ^{13}C NMR spectra of [2,11-^{13}C]-PBG-deaminase complex (i) and pure deaminase (ii) treated with pronase, pH 8.0, 12.5°C.

our HPMD, and the complex immediately concentrated and subjected to preparative gel-electrophoresis. The radioactive protein corresponding to the complex bearing 192 μg [^{13}C]-PBG, again shown to be "mono-PBG" complex by analytical gel-electrophoresis, was concentrated and examined by ^{13}C NMR. As before, electrophoretically pure deaminase was examined for comparison purposes (see Figure 3.10 (i)). As can be seen from Figure 3.10 (ii), a small extra peak is visible at 123.93 ppm, and again no clear differences in the aliphatic region. When the pure enzyme spectrum was subtracted from the enzyme-substrate spectrum, the spectrum obtained (see Figure 3.10 (iii)) showed clearly the peak at 123.93 ppm, and additionally a peak of similar linewidth at 22.15 ppm. These shifts are consistent with enzyme-bound uro'gen I, which from Section 2.7 exhibits resonances at 124.1 and 21.8 ppm. They would also be consistent with enzyme-bound di- or tri-pyrrole. If the latter were true, then we would expect resonances to occur at *ca.* 112 ppm (see Table 2.4) for the terminal pyrrolic carbon, and at an unknown shift for the methylene directly attached to the enzyme. Figure 3.10 (iii) shows a very low intensity peak at 114.2 ppm, and then two other broad peaks at 44.9 ppm and 31.6 ppm. The poor signal-to-noise does not allow any conclusions to be drawn from these resonances.

In order to obtain a better subtraction, the electrophoretically pure deaminase available from this preparation was examined by ^{13}C NMR, and then mixed with [2,11-^{13}C]-PBG, concentrated directly and examined by ^{13}C NMR. The two spectra obtained are shown in Figure 3.11 (i), (ii). A broad peak at 124.15 ppm is present in the ^{13}C-enriched complex which is not present in the pure enzyme, but otherwise there seems to be little difference. The spectrum is

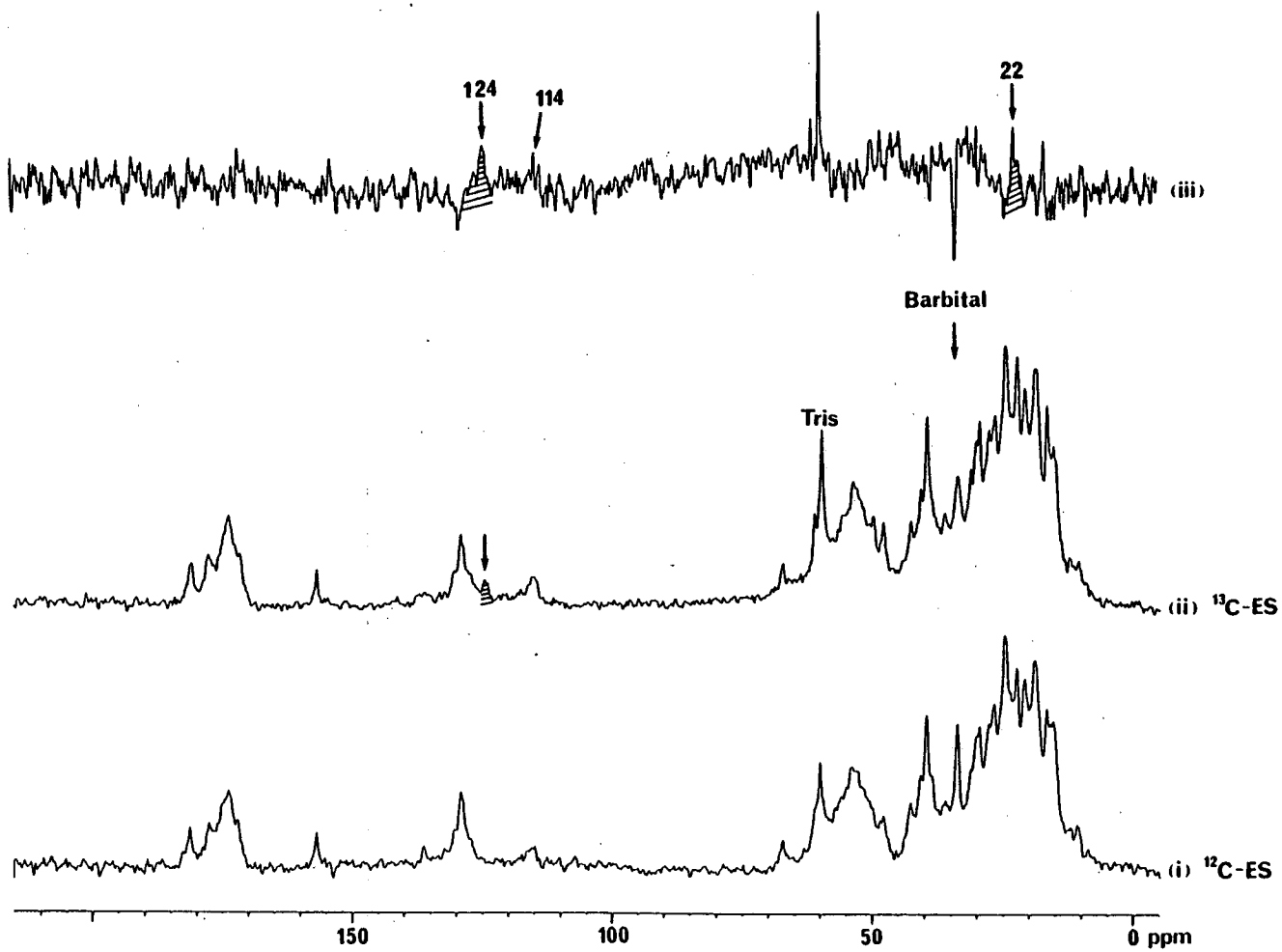


FIGURE 3.10. 75.5 MHz ^{13}C NMR spectra of [2,11-^{13}C]-PBG-deaminase complex (ii), pure deaminase (i), and the subtracted spectrum [(ii) - (i)] spectrum (iii), pH 8.0, 11.0°C.

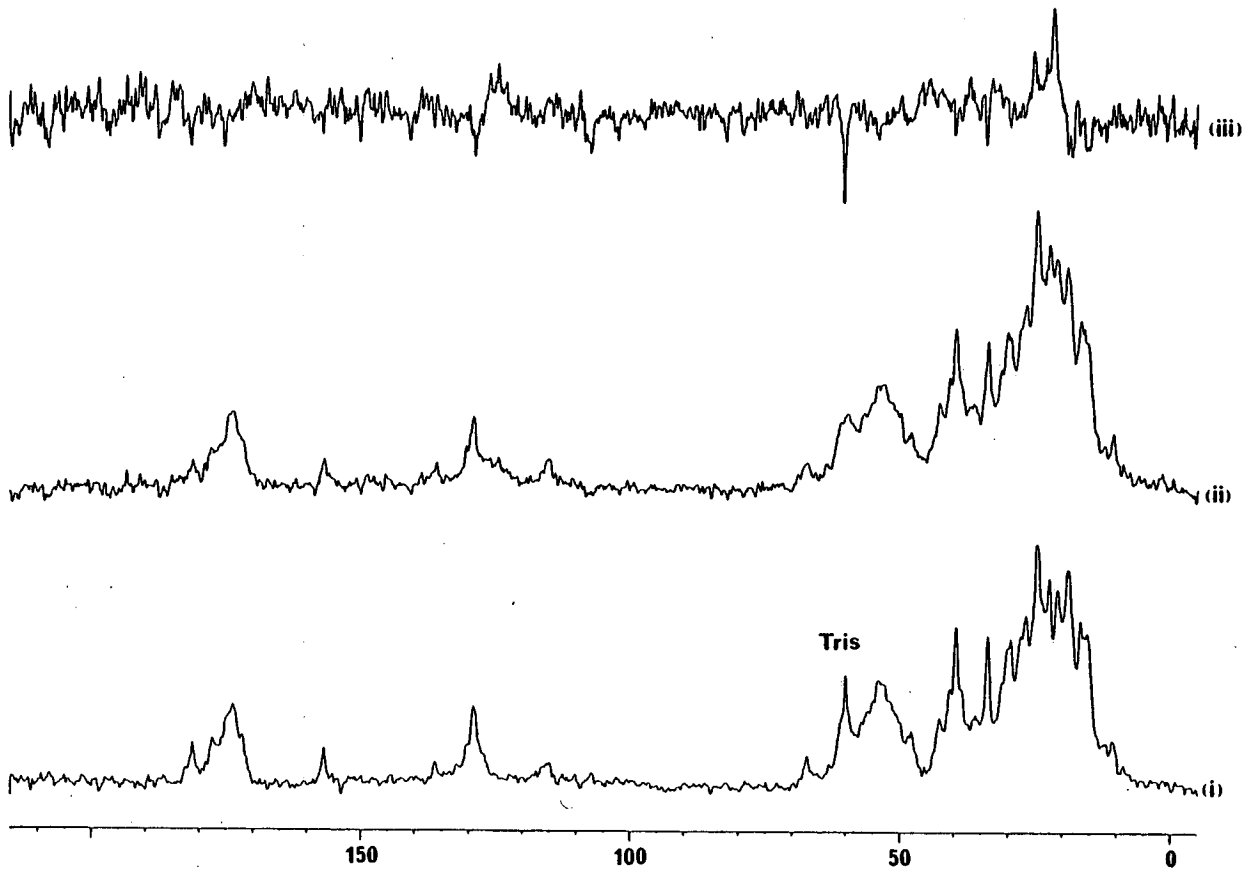


FIGURE 3.11. 75.5 MHz ^{13}C NMR spectra of [2,11-^{13}C]-PBG-deaminase complex (ii), pure deaminase (i), and the subtracted [(ii) - (i)] spectrum (iii), pH 8.0, 11.0°C.

shown in Figure 3.11 (iii). A broad peak centred at *ca.* 124.7 ppm is clearly visible, accompanied by sharp peaks at 21.5 and 25.0 ppm, and three broad peaks at 44.4, 36.9 and 31.9 ppm. These latter peaks could be assigned to residual glycine residues at 44.4 ppm, isoleucine residues at 36.9 ppm and either lysine or residual barbital buffer at 31.9 ppm.

The main batch of complex was subjected to tryptic digestion and the resultant oligopeptides fractionated on G-50 Sephadex (see Figure 3.12). The radioactive peptides (fractions 21-32) corresponding to small molecules (M_r < 2000) were subjected to peptide mapping.[190]

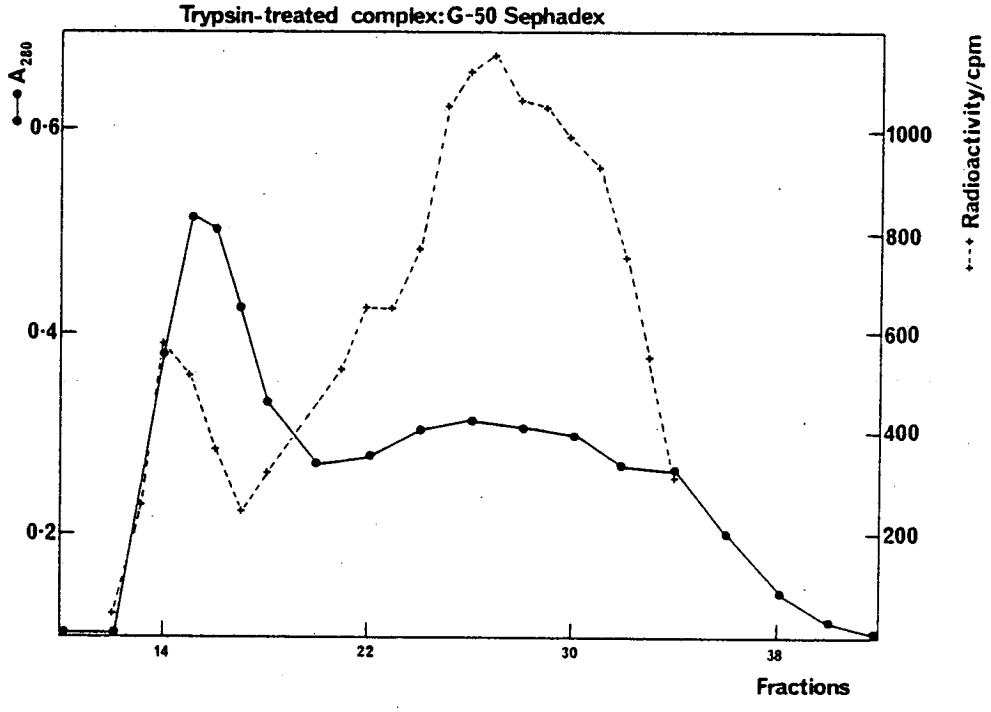


FIGURE 3.12. Purification of trypsin-treated [2,11-^{13}C]-PBG-deaminase complex by gel-filtration on G-50 Sephadex.

In this technique, the oligopeptides are electrophoresed on paper in one dimension and then chromatographed in the descending mode in the second dimension. The peptides were detected using non-destructive fluorescamine, and the map obtained is shown in Figure 3.13. The peptides marked 1, 5 and 6 were eluted off with 0.1 M NH_3 and lyophilised. The larger molecules from gel-filtration (fractions 13-20) were further digested with pronase. In this way seven samples

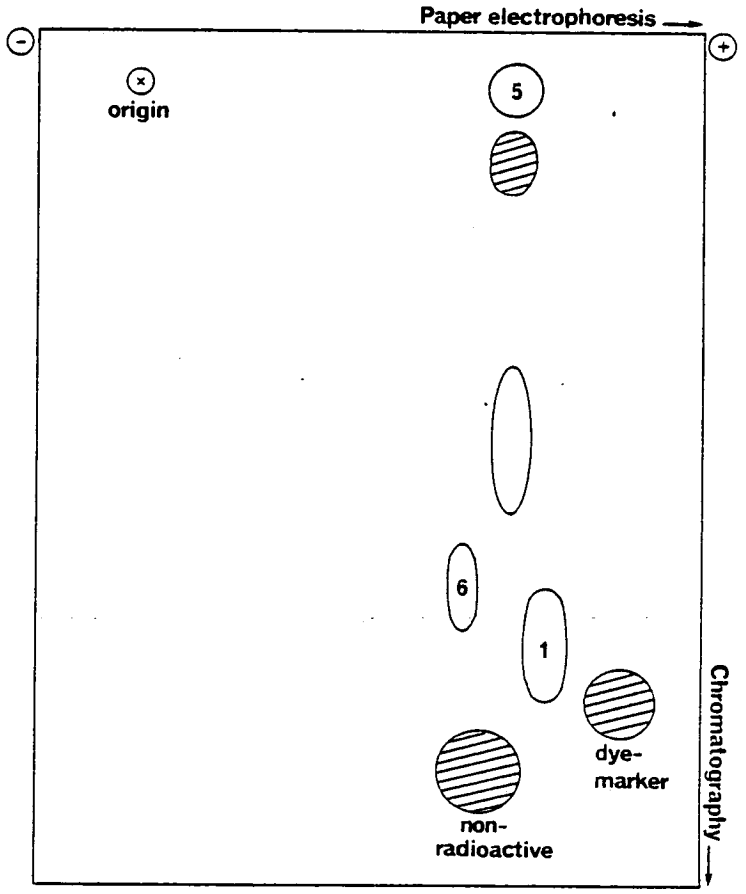


FIGURE 3.13. Peptide map of tryptic peptides from [2,11-^{13}C]-PBG-deaminase complex.

were obtained which were examined by ^{13}C NMR, ^{1}H NMR, UV spectroscopy and mass spectroscopy. For the purposes of further discussion the peptides 1-7 were obtained as follows:

Peptide 1: *ex* map.

Peptide 2: Fractions 21-32 from gel-filtration.

Peptide 3: Fractions 33-41 from gel-filtration.

Peptide 4: Fractions 13-20 from gel-filtration.

Peptide 5: *ex* map.

Peptide 6: *ex* map.

Peptide 7: Fractions 13-20 from gel-filtration after incubation with pronase.

The ^{13}C NMR investigations were difficult with all but two of the peptides (2 and 3) due to prohibitively low quantities (10-50 μg). Figure 3.14 (i) and (ii) show the spectra obtained for peptides 2 and 3. Interpretation of these spectra is extremely difficult, although it is obvious that the substrate is not easily detectable. The ^{1}H NMR spectra displayed resonances much too broad to interpret with any confidence. However, UV spectroscopy revealed that peptide 6 possessed a $\lambda_{max} \simeq 395$ nm, as did peptide 1 to a much lesser extent. This corresponds to peptide-bound porphyrin.

The use of fast-atom bombardment mass spectroscopy (FAB-MS) has recently attracted some attention[191,192] in the sequencing of oligopeptides and proteolytically digested proteins. Since it would also be highly diagnostic for unusual substrate or product molecules attached to the peptide chain, this method was also employed as a means to study the peptides 1, 5 and 6. The results from these endeavours are presented in Chapter 5.

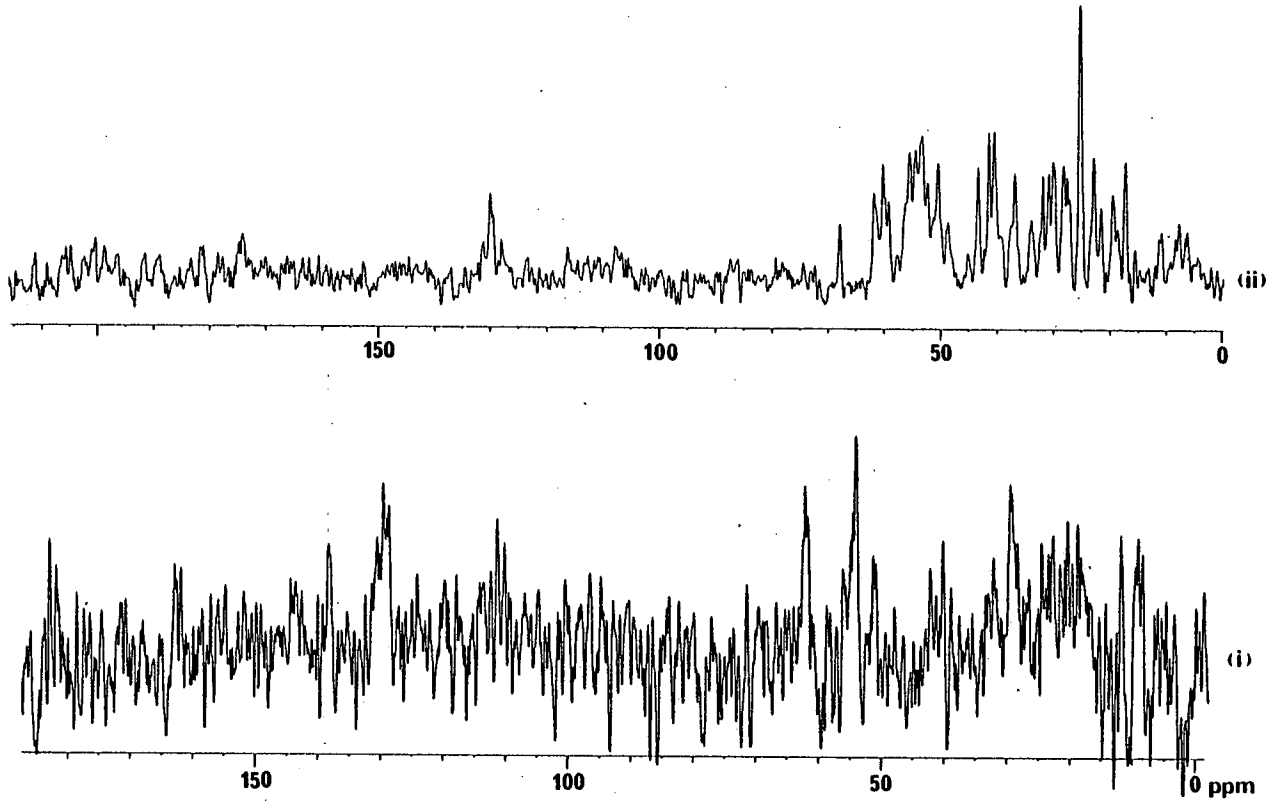


FIGURE 3.14. 75.5 MHz ^{13}C NMR spectra of peptide 2 (i) and peptide 3 (ii).

Evidently the ^{13}C-substrate, being protonated at the sites of enrichment, might be expected[193] to possess linewidths of the order of 100-200 Hz, which given the expected intensity of substrate relative to protein background would be very difficult to detect. Furthermore, the previous studies hinted at the presence of broad resonances hidden in the protein background, suggesting that the problem could be solved with more effective subtraction procedures. To achieve this, it was decided to split a large batch of enzyme (9000 U) into two parts, incubating one part with ^{13}C-substrate, and the other with natural abundance substrate. Direct concentration would then furnish two identical samples which could then be examined in a 10 mm NMR tube.

The two complexes thus obtained were initially subjected to a systematic investigation of various parameters, in order to optimise conditions for the subtraction. The T_1's of selected protein resonances were measured, in order to optimise the flip angle and pulse interval. Furthermore, the effect of pH, temperature, ionic strength and protein concentrations were investigated. Of these parameters, it was found that temperature had the most dramatic effect, with accumulations at 25°C being the optimum. When the ^{12}C complex was subtracted from the ^{13}C-enriched complex at pH 8.0 and 7.0 run under optimum NMR conditions, the spectra shown in Figure 3.15 were obtained. In the subtracted spectrum at pH 7.0, there is a broad resonance centred at 130 ppm which probably corresponds to protein arising from slight inaccuracies in the subtraction. The other peak at 27 ppm probably has similar origins. The signal-to-noise in the subtracted spectra at pH 8.0 prohibit any specific assignments. It should be pointed out at this stage that unless

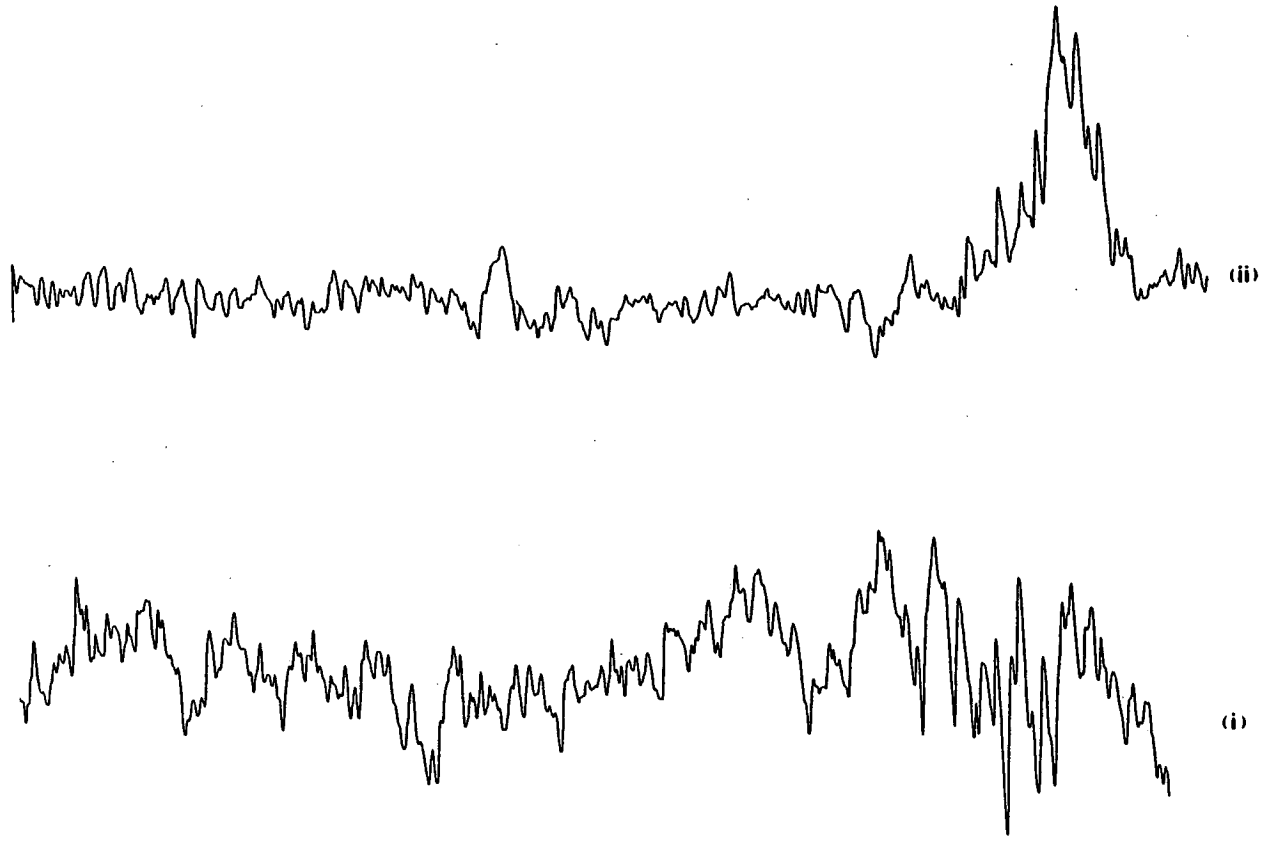


FIGURE 3.15. 75.5 MHz ^{13}C NMR spectra of [^{12}C]-PBG-deaminase complex subtracted from [2,11-^{13}C]-PBG-deaminase complex, different pH's, 11°C.

the substrate was present with excellent signal-to-noise, it is exceedingly difficult to obtain accurate subtractions, since minor variations in temperature, pH or ionic strength can seriously impair the accuracy of this technique.

Accordingly, it was felt that mild acid denaturation of both complexes might yield more information as to the binding of the substrate. Figure 3.16 shows the effect of acid denaturation on both complexes. While the lines sharpen up considerably, there still remains little difference between the two complexes. There is an extra peak at 30.0 ppm, but no such corresponding peak in the aromatic region. It was therefore concluded that further attempts to detect ^{13}C-enriched PBG attached to deaminase must focus on alternative ways to reduce linewidth and increase sensitivity.

3.6 ^{13}C NMR Studies of [2,11-^{13}C,$^{2}H_3$]-PBG-Deaminase Covalent Complex

The foregoing results pointed very strongly to unusually large linewidths for the enzyme-bound substrate. Any other peaks detected, seemed to arise from uro'gen I either bound or associated with the enzyme. Thus any attempts which might assist in decreasing linewidth would increase the chances of detecting the substrate. One method for achieving this is to increase the temperature, but from the previous section, this did not render the linewidth sufficiently narrow to make detection easy. Accordingly, electrophoretically pure deaminase was mixed as usual in our HPMD with [2,11-^{13}C,$^{2}H_3$]-PBG, which was prepared by methods covered in Chapter 4. Here the aim was to remove ^{13}C-^{1}H dipolar relaxation, thereby rendering the carbon more like a quaternary carbon having a linewidth some 2-3 times

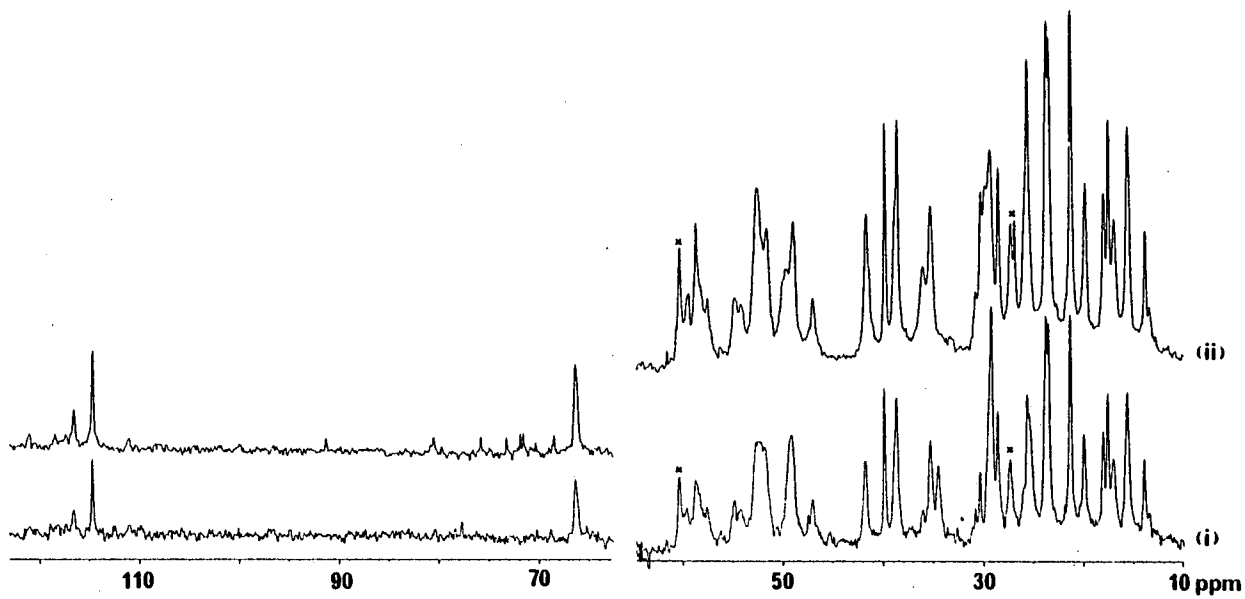


FIGURE 3.16. 75.5 MHz ^{13}C NMR spectra of mild acid-denaturated (pH 2) [^{13}C]-complex (i) and [^{12}C]-complex (ii), pH 7.0, 8 M urea, 25°C.

narrower. Scalar quadrupolar coupling only amounts to *ca.* 45 Hz for a methylene, as mentioned in Section 1.3, so in theory the gains are significant. Furthermore, the experiment can be performed with no decoupling, thereby reducing the protein background due to coupling and thus broadening, and also loss of NOE.

The freshly generated complex was subjected to preparative gel-electrophoresis to remove any uro'gen I which might be associating with the protein, and after concentration, examined in a 5 mm tube at 125.8 MHz. Despite the increased sensitivity of the 11.75 T NMR instrument, accumulations for long periods of time were necessary in order to achieve acceptable signal-to-noise. Figure 3.17 shows the initial spectrum obtained. Here peaks in the region of interest occur at 123.70, 110.89, 92.39 and 88.23 ppm. In the early part of the data accumulation for Figure 3.17 (i), the signals at 92.39 and 88.23 ppm were not visible, suggesting that they are formed slowly. These can be explained as the product of oxidation of uro'gen I which is present in the sample. The ^{13}C chemical shifts of uroporphyrin I from such ^{13}C-labelled PBG are 76 and 98 ppm, which are very similar. The resonance at 123.70 ppm further corroborates this theory, leaving the signal at 110.9 ppm attributable to bound substrate. Unfortunately, however, no such corresponding signal is visible in the aliphatic region. Comparison with a sample of [^{12}C]-complex did not reveal any unique aliphatic resonances (see Figure 3.17 (ii)).

The conclusions to be drawn from these experiments are conflicting and complex. On the one hand, analytical gel-electrophoresis strongly suggests the presence of a "mono-PBG" bound to deaminase, and we are confident that our "mono-PBG" is the same used by Jordan

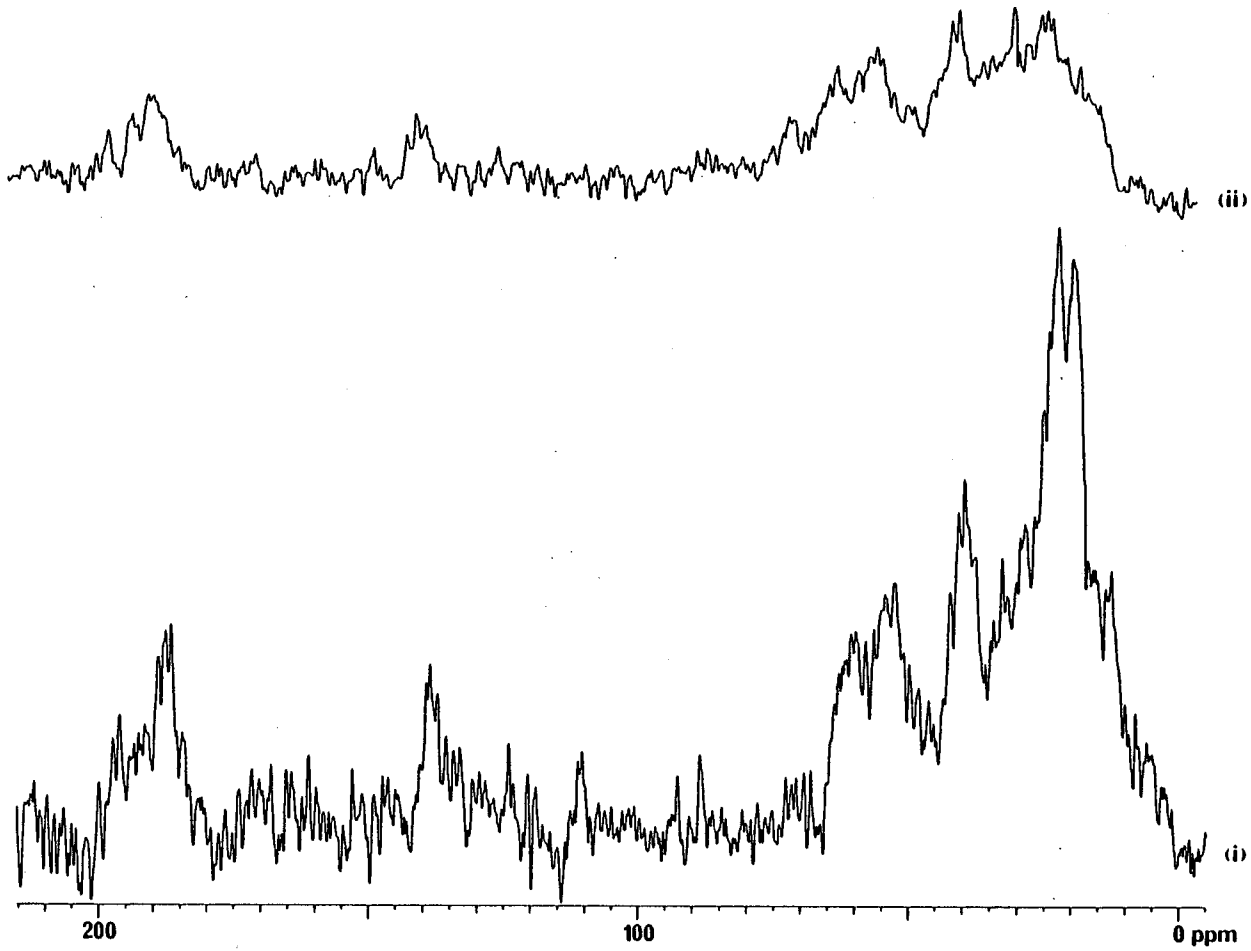


FIGURE 3.17. ^{1}H-coupled 125.8 MHz ^{13}C NMR spectrum of [2,11-^{13}C,$^{2}H_3$]-PBG-deaminase complex (i) and [^{12}C]-PBG-deaminase complex (ii), pH 8.0, 25°C.

in his experiments.[188] However, the NMR results suggest the presence of enzyme-bound uro'gen I. The origins of this uro'gen I may simply be residual product very strongly associating with the protein, or perhaps even more likely, is the disproportionation of the complex at that temperature and concentration. At the point at which bound tetrapyrrole forms, then uro'gen I formation can be expected. More studies on this will be presented in the next chapter.

3.7 ^{13}C NMR Studies of [3,5-^{13}C]-PBG-Deaminase Covalent Complex

An alternative method for circumventing the problems of linewidth associated with protonated carbons is to make use of substrate with quaternary labelled carbons. This is true for [3,5-^{13}C]-PBG, for which the enzyme-bound species might be expected to exhibit linewidths of the order of 50-100 Hz. Furthermore, the substrate resonances should fall in a window region in which no protein resonances occur. Finally, another nice feature of this substrate is that it can be used to test Battersby's tentative assertion[189] that the group X in Scheme 9 is the ε-NH_2 of lysine. This is based on the earlier observation (see Section 2.6) that the chemical shift of C-5 is sensitive to the pK_a of the amino group. Thus, titration of the enzyme-bound species should test the validity of Battersby's assertions, even if the attachment of substrate is made *via* a secondary amine.

For the preparation of the [3,5-^{13}C]-PBG-deaminase complex, the mixing of [3,5-^{13}C]-PBG and deaminase (8000 U, 20 U mg^{-1}) was performed in the presence of 25 mM lysine. Here the idea was to provide a large excess of a small charged molecule which might form

an ion-pair with any uro'gen I produced, without nucleophilically trapping any tetrapyrrole as is the case with ammonia. After gel-electrophoresis, the purified complex, bearing 450 μg PBG, was analysed by ^{13}C NMR at pH 7.6 and 3.7°C. Figure 3.18 shows the

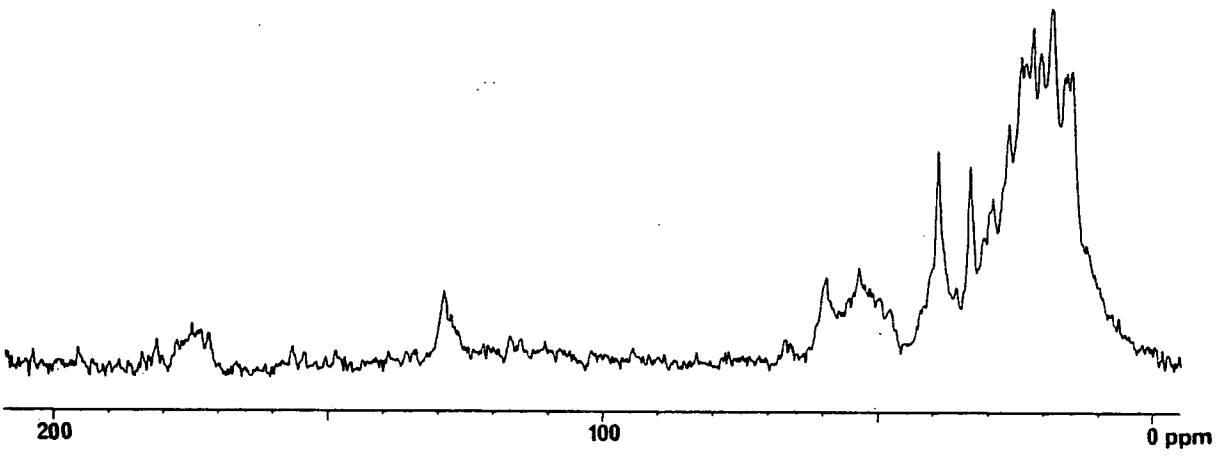


FIGURE 3.18. 75.5 MHz ^{13}C NMR spectrum of [3,5-^{13}C]-PBG-deaminase complex, pH 7.6, 3.7°C.

spectrum accumulated without decoupling but with full NOE. The same sample was then examined over a narrow sprectral width, as was a sample of [^{12}C]-PBG-deaminase complex (see Figure 3.19). The two shaded peaks at 119.87 and 121.45 ppm are assignable to bound substrate. When both samples were re-run at 21.6°C the spectra showed peaks at 120.00 and 121.90 ppm (see Figure 3.20). If we follow the fate of these two resonances with pH (see Figure 3.21), it can be seen that the resonance at 120.00 ppm shifts almost negligibly before it becomes too broad to identify, at pH 10.0. Interestingly, the peak at 121.90 ppm remains relatively sharp and easily assignable to C-3 of PBG. The pH was not adjusted further than pH 10.2 for fear of denaturing the enzyme. When the solution was readjusted to pH 8.0 and re-analysed, the spectrum obtained (Figure 3.20) was very similar

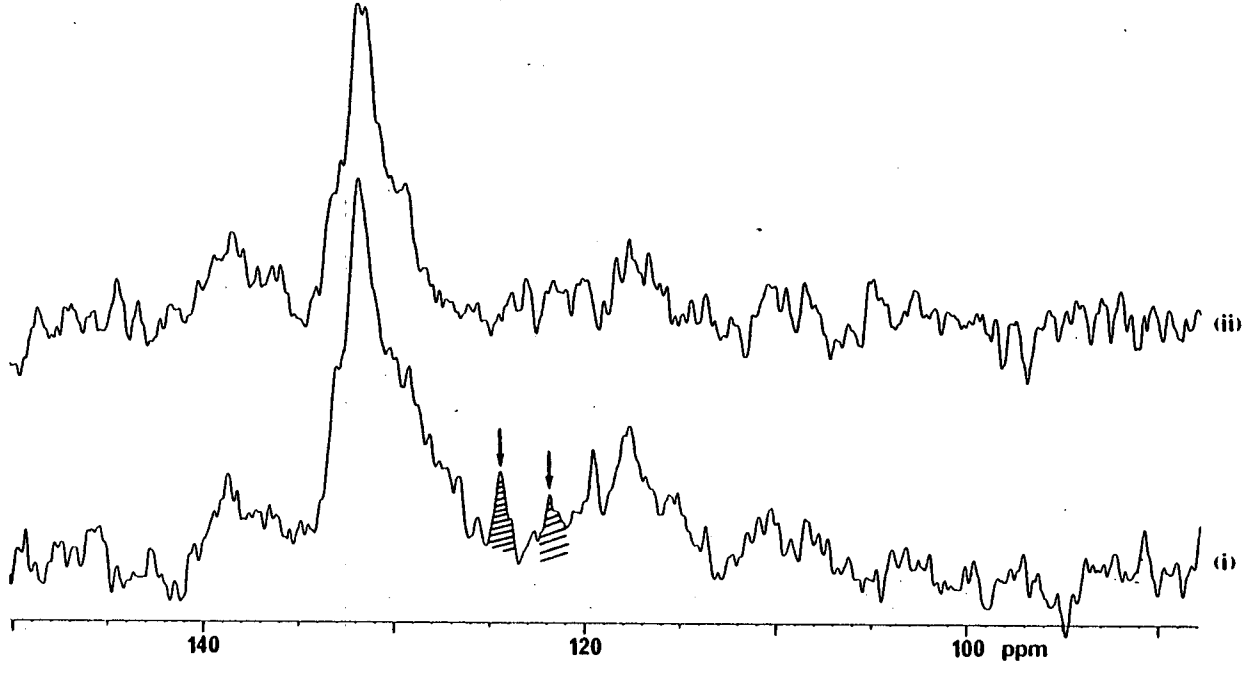


FIGURE 3.19. 75.5 MHz ^{13}C NMR spectra of [3,5-^{13}C]-PBG-deaminase complex (i) and [^{12}C]-complex (ii), pH 7.6, 3.7°C, over a narrow spectral width.

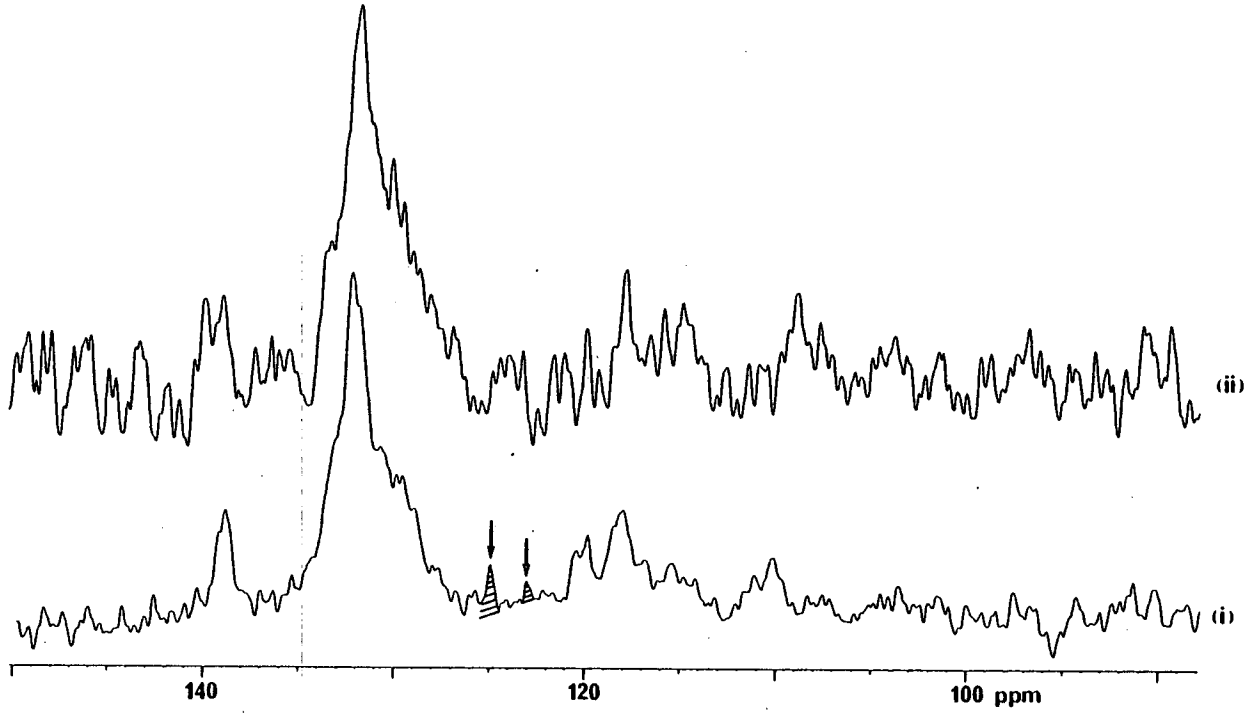


FIGURE 3.20. 75.5 MHz ^{13}C NMR spectra of [3,5-^{13}C]-PBG-deaminase complex (i) and [^{12}C]-complex (ii), pH 7.6, 21.6°C.

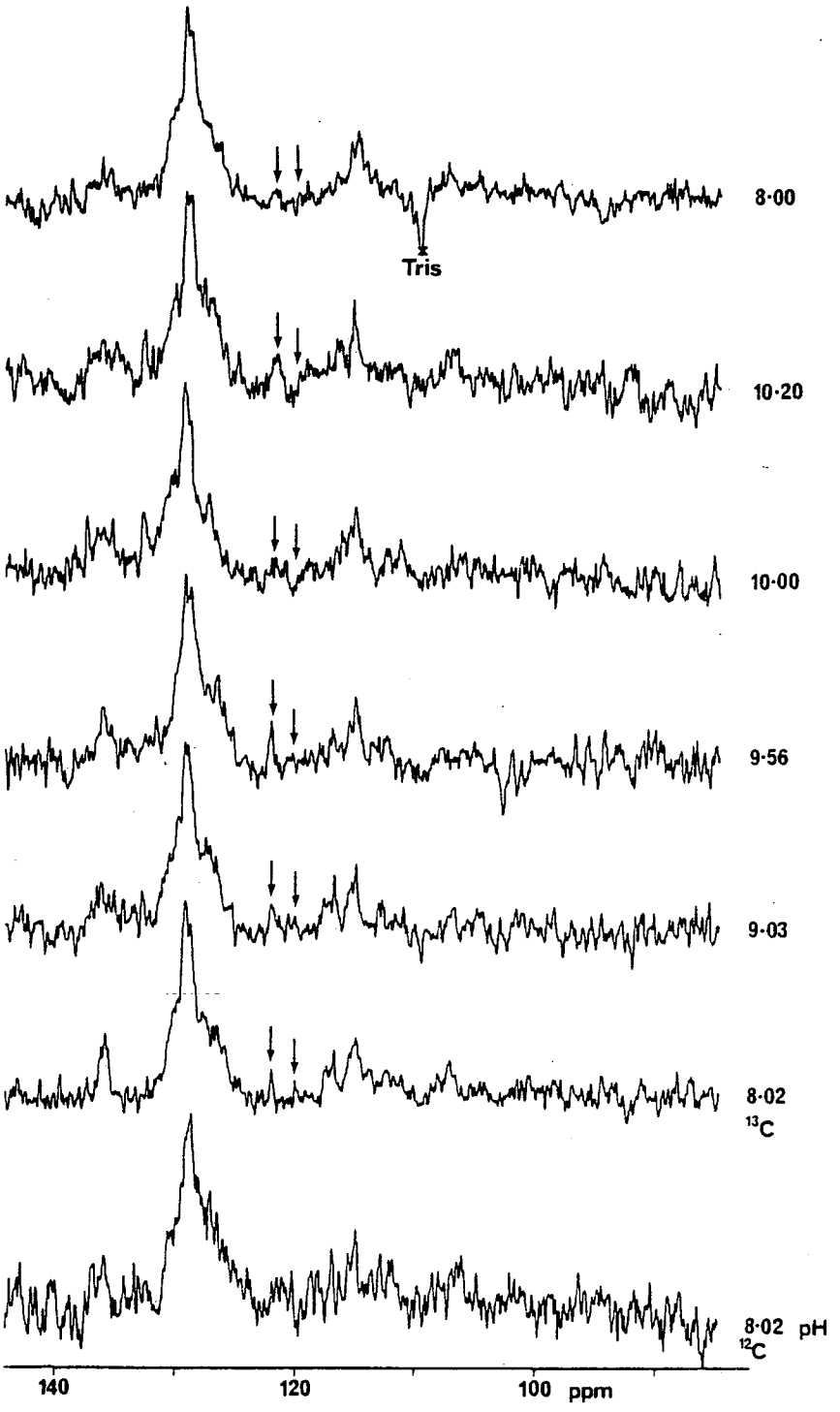


FIGURE 3.21. 75.5 MHz ^{13}C NMR spectra at [3,5-^{13}C]-PBG-deaminase complex and [^{12}C]-complex, various pH's, 21.6°C.

to that obtained before the titration, although most of the resonances were somewhat broader. This latter effect is probably as a result of the high final ionic strength.

This complex was then treated with unlabelled PBG (774 μg). The spectrum obtained is shown in Figure 3.22 (i). When the uro'gen I region was resolution enhanced, the spectrum showed a double doublet centred at 117.6 ppm (^{2}J (^{13}C-^{1}H) = 53 Hz, ^{3}J (^{13}C-^{1}H) = 8.5 Hz) and a doublet centred at 126.0 ppm (^{2}J (^{13}C-^{1}H) = 46 Hz). Since the data was accumulated without ^{1}H-decoupling, the double doublet at 117.6 ppm arises from ^{13}C-^{1}H coupling at C-3, C-8, C-13 and C-18 from the propionate side-chain methylenes, the first methylene giving rise to two-bond coupling and the second methylene giving rise to three-bond coupling. Similarly, the doublet at 126.0 ppm arises from two-bond coupling to the meso protons. Incidentally, this coupled spectrum confirms the assignments made in Section 2.8 for uro'gen I derived from [3,5-^{13}C]-PBG.

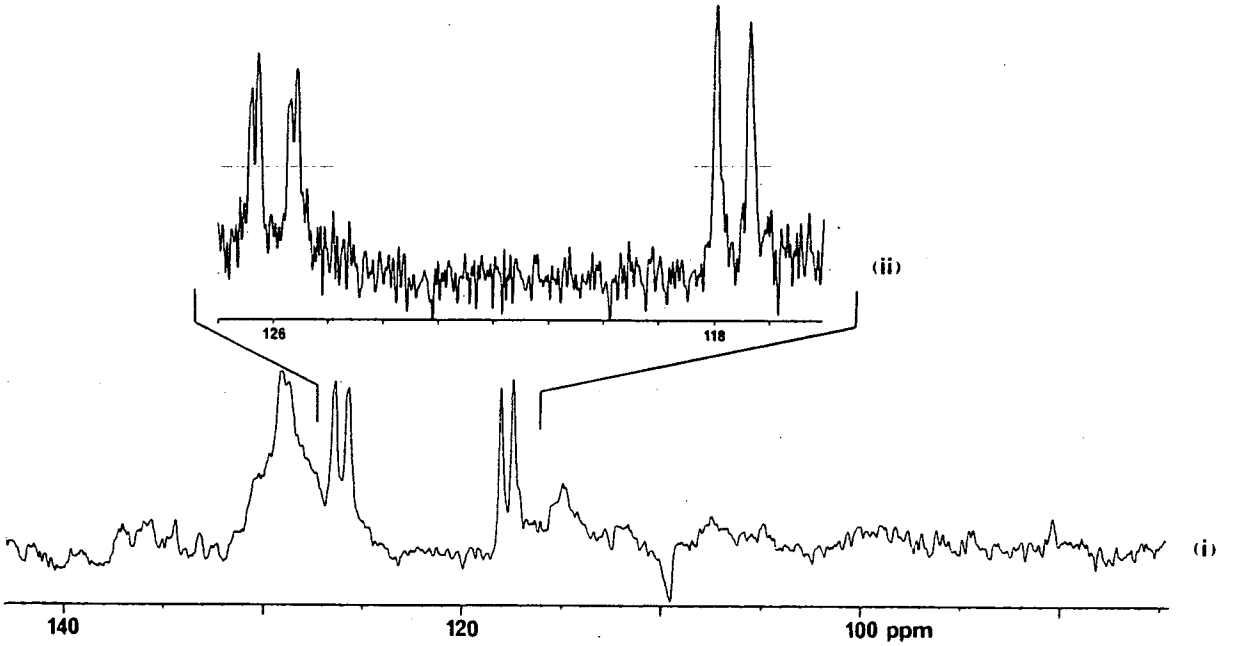


Figure 3.22. 75.5 MHz ^{13}C NMR spectra of [3,5-^{13}C]-PBG-deaminase treated with unlabelled PBG, pH 8.0, 21.7°C, (i). Resolution enhanced, (ii).

CHAPTER 4

NMR STUDIES OF [^{3}H]-PBG-DEAMINASE COVALENT COMPLEXES

4.1 Introduction

The problems encountered in the work described in the previous chapter, in the attempt to study the ^{13}C-enriched PBG-deaminase covalent complexes, had at their roots two basic causes: (i) the linewidths of the covalently attached substrate must be prohibitively large by ^{13}C NMR, and (ii) the protein resonances seriously hamper substrate detection for most ^{13}C-labelling patterns which would furnish useful information.

We therefore set about examining other possible NMR nuclei which have a low natural abundance and a high sensitivity, and which might furnish the information sought. One such nucleus is tritium.

4.2 The Use of ^{3}H NMR in the Study of Enzyme-Substrate Complexes

If we consider the data given in Table 1.1, it is clear that ^{3}H is eminently suited to use in biological NMR. It has a natural abundance of $<10^{-16}$, and an NMR sensitivity at full enrichment some 20% greater than ^{1}H. Indeed, it is the most sensitive NMR nucleus known. This advantage is offset by its radioactivity, but then of all the radionuclides tritium is the least harmful. It is a "soft" β-emitter (0.018 MeV) and has a half-life of 12.3 years. While requiring special handling precautions, the mean range in air travelled by the β-particles is 4.5 mm, and at most levels employed for ^{3}H NMR these are stopped by the dead layers of skin, or the glass walls of the apparatus being used.

As far as NMR instrumentation is concerned, the use of ^3H NMR requires certain additional items in order to equip an ordinary FT instrument adequately. The first of these is a probe suitably adapted to tune the main coil up to the ^3H NMR frequency and have a subsidiary decoupler coil that can be tuned to the ^1H NMR frequency. Also the instrument needs to be equipped with a pulse amplifier and pre-amplifier tuned for the ^3H NMR frequency, and high-quality filters for both the ^3H channel and the ^1H-decoupler channel, capable of selectively filtering either ^3H or ^1H frequencies where appropriate. This is particularly important because the NMR frequencies are so close together that breakthrough of one channel into the other can be a serious problem.

The first reported ^3H NMR spectrum was recorded by Tiers *et al.*[194] on a tritiated ethyl benzene sample of very high radioactivity (10 Ci) at 42.5 MHz. For tritiated water, a satisfactory one-scan spectrum was obtained at a level of 1 Ci cm^{-3} (0.038%).[195] The advent of FT NMR has reduced this limit to 0.5-5 mCi per site of enrichment. A survey of 26 monotritiated substrates showed the relationship

$$\delta_T = \delta_H$$

to be valid to a first approximation.[196]

A number of referencing procedures have been devised which avoid the use of an internal tritiated standard.[196,197,200] One way is to obtain the ^1H NMR spectrum of the sample containing a standard (TMS or TSP), and the absolute resonance frequency of the standard is then multiplied by $\gamma_T/\gamma_H = 1.0666$, to provide a ghost reference frequency for the ^3H spectrum. In another technique, which is employed throughout the work described in this chapter, the ^1H NMR spectrum of the

sample containing a standard is obtained, and the ^{1}H resonances referenced in the normal way. The ^{3}H NMR spectrum is then obtained, and using $\delta_H = \delta_T$, a sample resonance which is remote from the standard and bears both ^{3}H and ^{1}H is used as a reference. The validity of these techniques was confirmed using synthetic monotritiated TMS.[197]

A secondary isotopic shift of 0.021 ppm exists when going from CH_2T to CHT_2.[196] The coupling constant ^{1}J (^{13}C-^{3}H) would be expected to be given by

$$^{1}J\ (^{13}C\text{-}^{3}H) = 1.0666\ ^{1}J\ (^{13}C\text{-}^{1}H)$$

in which the constant (1.0666) again reflects the differences in γ between the two nuclei (γ_T/γ_H). Deviations of ±0.2 Hz have been found, except for chloroform for which a -1.52 Hz discrepancy exists.[198] These deviations are not due to differences in the accuracy of coupling constant measurements, since the linewidths of ^{3}H NMR signals are comparably narrow to those of ^{1}H signals.

When broad-band ^{1}H-decoupling is employed, a ^{3}H-{^{1}H} NOE would be predicted, and after some controversy,[199,200] the existence and value of this NOE has been demonstrated.[201] In theory the maximum NOE is 47% ($\gamma_H/2\gamma_T$) which is quite small when compared to ^{13}C for example, in which the maximum ^{13}C-{^{1}H} NOE is 199%. Since there are usually no significant differential NOE's in a particular sample, the errors in integrating the intensities of ^{1}H-decoupled ^{3}H signals are generally similar to ^{1}H NMR (±2-5%).

While a number of applications of ^{3}H NMR exist for small molecules of biological interest,[202-208] only one example of the study of a biological macromolecule has been reported,[209] in which

the interaction of [6-^{3}H]benzo[a]pyrene and yeast cytochrome P-450 was examined, and a very poor quality ^{3}H spectrum obtained. Thus the work described here represents the first application of ^{3}H NMR to the study of an enzyme-substrate complex. With the appropriate equipment installed by Dr. P. E. Fagerness, we were able to plan a series of ^{3}H NMR experiments designed to solve the problems posed above by ^{13}C NMR. As an initial requirement, it was necessary to be able to synthesise [^{3}H]-ALA of unusually high specific activity in order to yield [^{3}H]-PBG which when bound to deaminase would be sufficiently tritiated (i.e. 0.5-5 mCi per site) to allow detection by ^{3}H NMR with moderate ease. For this purpose, preliminary studies focused on the synthesis of [^{2}H]-PBG as a means of evaluating the synthetic methodology.

4.3 Synthesis of [^{2}H]-ALA and [^{2}H]-PBG

Following a report[210] that the protons at C-5 of ALA are exchangeable at pH 8, a detailed study of the kinetics of deuteration at C-5 and C-3 was conducted using ^{1}H NMR. The kinetic profile obtained is shown in Figure 4.1. From this it is clear that >90% deuteration at C-5 can be achieved with only 15% deuteration at C-3. Since commercially available [^{3}H]-ALA, which is of low specific activity, is invariably 60% tritiated at C-5 and 40% at C-3, this method appeared to provide the means for a significant improvement.

As a test, ALA (1 g) was dissolved in D_2O (50 cm^{3}) and adjusted to pH 8.0 with solid anhydrous sodium carbonate. It was then incubated in a sealed vessel for exactly 225 minutes before adjusting the pH to 1.5 with 20% DCl/D_2O. The mixture was then lyophilised,

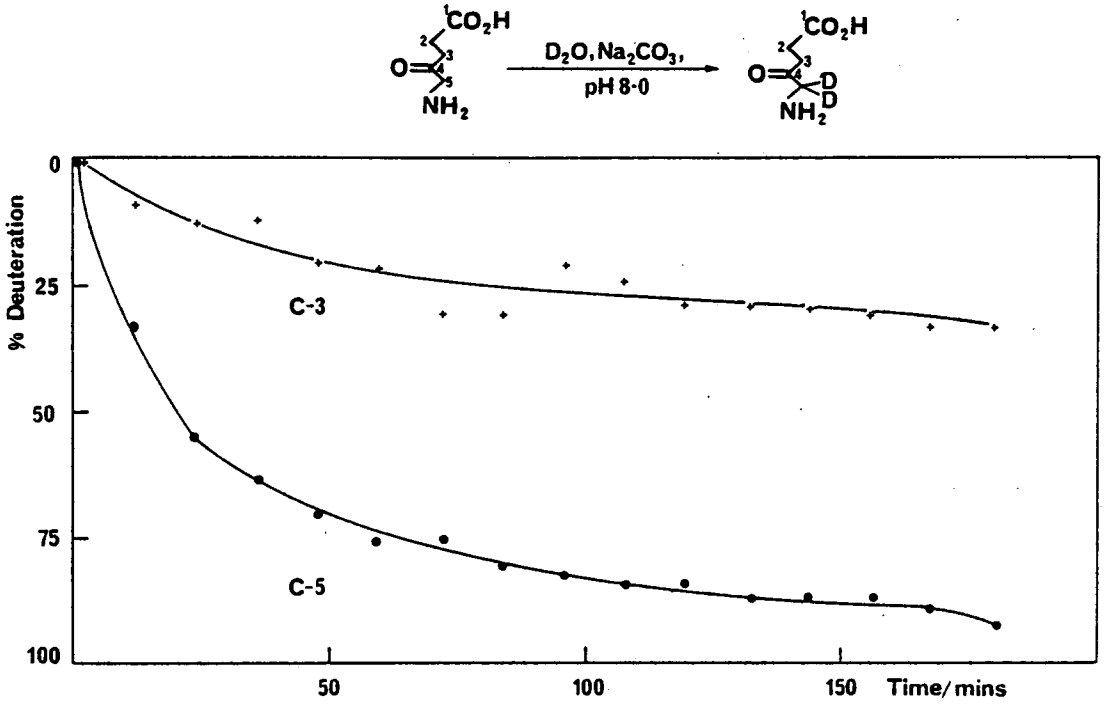


FIGURE 4.1. The deuteration of ALA.

and on recrystallisation the pure [5-^{2}H]-ALA (495 mg, 49% yield) was found to have 90% ^{2}H at C-5 and 15% at C-3 by ^{1}H and ^{2}H NMR and FAB-MS.

Attempts to generate [^{2}H]-PBG from this sample of ALA using ALA dehydratase initially failed for a number of reasons. It was mainly due to the incubation being performed for 2 hours at pH 8.0. At this pH and over this time period, the extent of exchange of deuterium out of the [^{2}H]-ALA is significant. If the amount of enzyme had been increased dramatically and the pH adjusted nearer to 7, then the exchange could be minimised.

Accordingly, the activity of partially purified ALA dehydratase from *R. spheroides* was examined at various pH values in the standard incubation buffer (see Figure 4.2). Clearly at pH 7.0 dehydratase

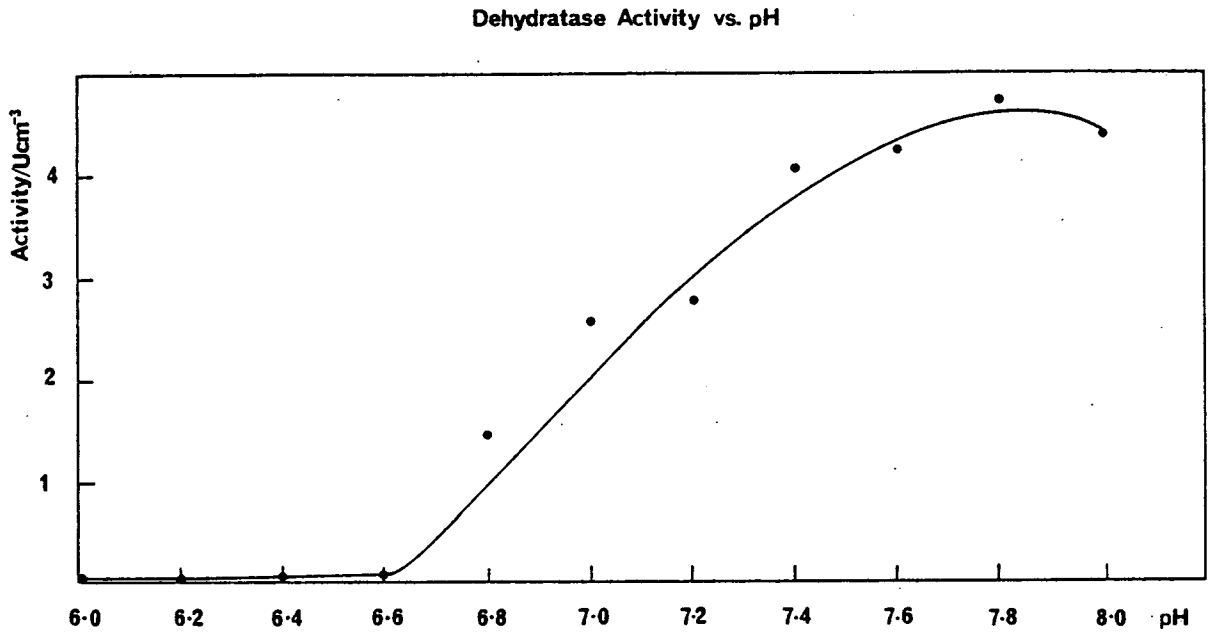


FIGURE 4.2. Dehydratase activity versus pH.

retains 44% of its activity at pH 8.0. Thus when the incubation was conducted at pH 7.0 with a large excess of enzyme, the deuterium incorporation was satisfactory as shown in Table 4.1, although the isolated chemical yield was only 29%, which perhaps reflects an isotope effect on the enzyme activity. Interestingly, the incorporation of deuterium into C-2 was greater than quantitative, probably due to the exchangeability of this proton with the D_2O in which the sample was dissolved for the ^1H NMR determinations. Furthermore, the incorporation at C-11 was some 70% lower than expected, suggesting that the deuterium had been lost from C-5 of ALA prior to PBG formation.

Having established an experimental protocol applicable to the synthesis of [^3H]-PBG, conditions were sought which would be technically simpler. The first criterion here was the tritiated equivalent of D_2O. The deuteration of ALA was performed at

TABLE 4.1 Deuterium incorporation of [2,11-^{2}H]-PBG from [5-^{2}H]-ALA.[a]

Substrate		% D (experimental)	% D (theoretical)	% overall incorporation (ALA → PBG)
ALA	C-3	19	-	-
	C-5	82	-	-
PBG	C-2	56	41	137
	C-11	22	82	27
	C-6	23	19	100

[a]Data calculated from integrals of 300 MHz ^{1}H NMR spectra normalised to resonances not subject to deuteration, i.e. C-2 of ALA and C-9 of PBG.

relatively high dilution, since at pH 8.0 over the course of 2.75 hours, appreciable decomposition of ALA takes place.[177] Negotiations with commercially available custom tritiation services revealed that while 100% T_2O was available, the maximum quantity which could be handled was 100 Ci, which has a volume of 31 µL. Furthermore, adjustment to pH 8.0 must be performed with a volatile base, if problems of removing the labile tritium were not to be encountered.

A series of experiments were performed dissolving ALA·HCl (150 mg) in D_2O (309 µL) and titrating to pH 8.0 with triethylamine. When the necessary amount of triethylamine was established, the experiment was conducted on the same scale as planned for the radiochemical exchange, one in D_2O and one in H_2O aiming for 10% deuteration. Incubation was carried out for exactly 10 minutes (calculated using Figure 4.1) before adjustment with 0.1 M HCl and lyophilisation. Analysis of the 300 MHz ^{1}H NMR spectra, integrating the

methylene at C-5 relative to the one at C-2, revealed that *ca.* 15% deuteration had been achieved.

4.4 Synthesis of [^{3}H]-ALA and [^{3}H]-PBG

In order to gain some knowledge of the isotope-effect in substituting tritium for deuterium and to test the methods worked out above, a sample of [^{13}C]-ALA was sent for custom tritiation by New England Nuclear (Boston, Mass., U.S.A.). The sample of [^{13}C,^{3}H]-PBG subsequently obtained was used in one of the experiments described in Section 3.5, and as well as being radioactive and thus providing an internal means of detecting the complexes produced, the sample provided a ready method for testing the lability of tritium at all the various stages of complex purification.

Therefore [5-^{13}C]-ALA (15 mg, 90 μmol) was sent to New England Nuclear, who performed the custom tritiation as follows. The ALA was dissolved in 100 Ci 100% T_2O and triethylamine added with stirring. This was continued for exactly 10 minutes before adding excess 0.1 M HCl, and removing the labile tritium by cycles of lyophilisation and further addition of 0.1 M HCl. The crude [^{3}H]-ALA was shipped directly in 0.1 M HCl, and converted to PBG as soon as possible after its arrival.

For the incubation with ALA dehydratase, the enzyme (1500 U assayed at pH 7.0) was preincubated at 37°C in phosphate buffer, $MgCl_2$ and cysteine. The [^{3}H]-ALA solution (286 mCi, 3.2 Ci mmol^{-1}) was adjusted to pH 7.0 with NaOH and added to the enzyme. After incubating for exactly 3.5 minutes, the reaction was stopped with $CuSO_4$ and the [^{3}H,^{13}C]-PBG purified by the procedures outlined in

Chapter 2. This yielded pure [^{3}H,^{13}C]-PBG (3.6 mg, 4.7 mCi, 3.5 Ci mmol^{-1}) in 28% chemical yield, as was found in the case with deuterium. If the [^{3}H]-ALA sample had not significantly decomposed, then the PBG produced should have a specific activity of 4.0 Ci mmol^{-1}, which implies a 13% loss of tritium on PBG formation.

One third of this sample (14.6 mCi) was analysed by ^{1}H and ^{3}H NMR, and the spectra obtained are shown in Figure 4.3. The ^{1}H spectrum (Figure 4.3 (i)) clearly shows the 90% ^{13}C-enrichment at C-2 (^{1}J (^{1}H-^{13}C) = 183.5 Hz) and C-11 (^{1}J (^{1}H-^{13}C) = 144.2 Hz), and some ^{3}H-^{1}H coupling at C-6. When the ^{1}H-decoupled ^{3}H NMR spectrum was recorded, it again showed (Figure 4.3 (ii)) the ^{13}C-^{3}H coupling at C-2 (^{1}J (^{3}H-^{13}C) = 195.3 Hz) and C-11 (^{1}J (^{3}H-^{13}C) = 152.1 Hz).

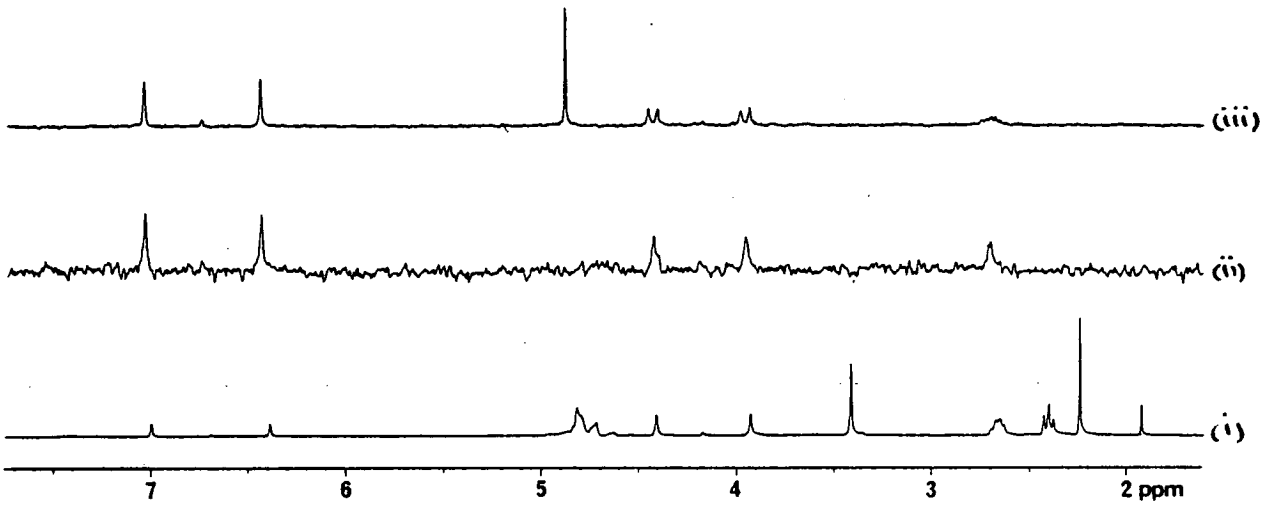


FIGURE 4.3. (i) 300 MHz ^{1}H NMR spectrum of [2,6,11-$^{3}H_{5}$], [2,11-^{13}C]-PBG·HOAc (1.2 mg, 14.6 mCi).

(ii) 320 MHz ^{1}H-decoupled ^{3}H NMR spectrum of sample (i), 2 hours after solvation.

(iii) 320 MHz ^{1}H-coupled ^{3}H NMR spectrum of sample (i), 24 hours later.

Note that from the relationship given in Section 4.2 we would have expected the latter coupling constants to have been 195.7 and 153.8 Hz, which is fairly good agreement. Additionally, the integrals gave a quantitative measure of the ^{3}H distribution, which was 46% at C-2, 35% at C-11 and 19% at C-6. When the ^{1}H-coupled ^{3}H NMR spectrum was recorded, considerable exchange of ^{3}H from C-2 had taken place, giving rise to the HOT resonance at 4.85 ppm. The resonance at 4.14 ppm (C-11) clearly shows ^{3}H-^{1}H coupling (^{1}J (^{3}H-^{1}H) = 15.2 Hz).

When this sample of PBG (10.3 mCi, 852 μg) was used to generate a [^{13}C]-complex, which was later denatured for more detailed ^{13}C NMR studies (see Section 3.7), it was found that 1.57 mCi (15.2% bound) was bound to deaminase (4500 U, 1 μmol). This low incorporation may well reflect appreciable loss of tritium at C-2 of PBG. These results therefore pointed to the necessity for PBG with a high specific activity as is theoretically possible. Furthermore, the incorporation of ^{3}H into ALA was 64% less than the theoretical incorporation (791 mCi, 8.85 Ci mmol^{-1}), implying a k_T/k_D = 0.36. From this a better estimate of the time necessary to ensure complete tritiation was possible.

The first time ALA (15 mg) was tritiated at a high level, the procedure was exactly as with the [^{13}C]-ALA, except that the exchange was allowed to incubate for 360 minutes. This is twice the time necessary for deuterium, with an extra 30 minutes, which is not quite as long as the time predicted from the k_T/k_D obtained above (i.e. 460 minutes) owing to the inconvenience for New England Nuclear. After removal of the labile tritium, the product was dissolved in 0.1 M HCl and shipped in dry-ice. The crude ALA (3.122 Ci,

34.8 Ci $mmol^{-1}$) was incubated with ALA dehydratase exactly as before. This yielded impure [^{3}H]-PBG which, after a second purification on Dowex 2 acetate, furnished [^{3}H]-PBG (250 μg, 144 mCi, 165.9 Ci $mmol^{-1}$) found to be relatively pure by ^{3}H NMR (see Figure 4.4). This dramatic increase in specific activity reflects the fact that the starting ALA was far from pure. This arises from exposing ALA to somewhat extreme conditions. Storage of ALA at pH 8.0 for 6 hours probably resulted in significant chemical decomposition, producing the sorts of impurities identified by Franck *et al.*[177] Furthermore, exposure of ALA to 100 Ci for the same period of time gives rise to considerable autoradiolysis.

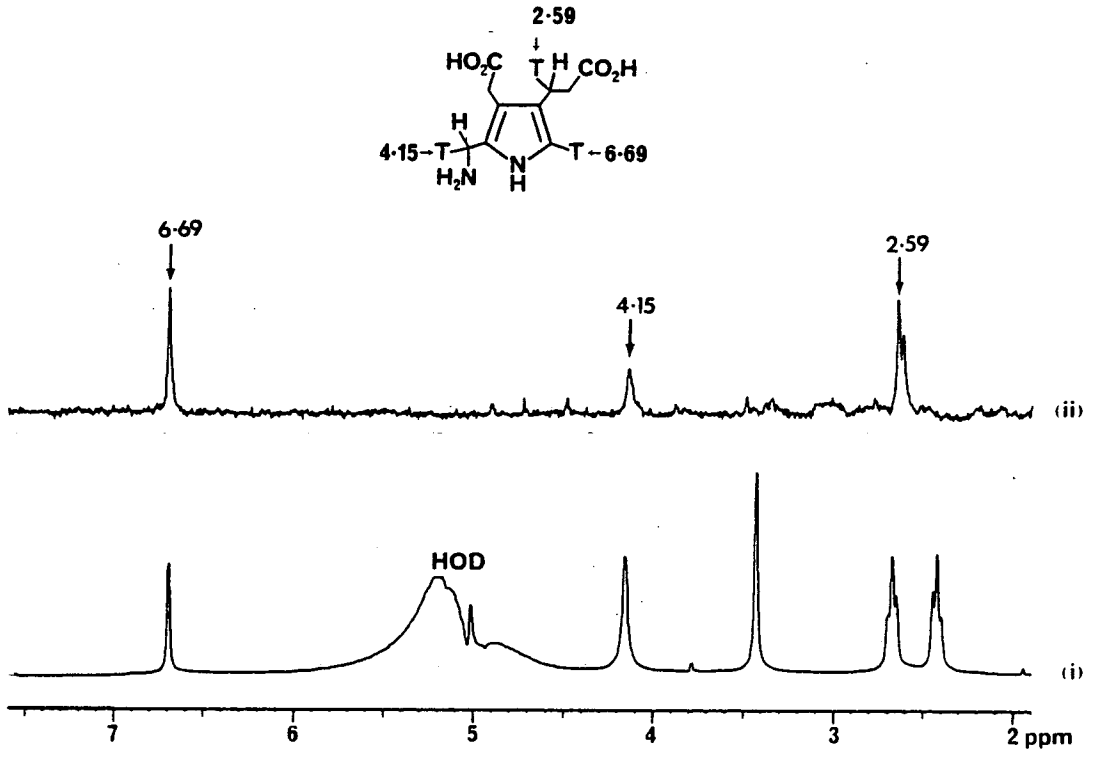


FIGURE 4.4. (i) 300 MHz ^{1}H NMR spectrum of unlabelled PBG (for comparison.
(ii) 320 MHz ^{1}H-decoupled ^{3}H NMR spectrum of [2,6,11-$^3H_5$]-PBG.

The ^{1}H-decoupled ^{3}H NMR spectrum of pure [^{3}H]-PBG (Figure 4.4 (ii)) shows some interesting features. Firstly the resonance due to the propionate methylene would be expected to exhibit a singlet, but in fact shows an unsymmetrical doublet. This is due to the main signal arising from a T-C-H methylene (at 2.59 ppm) and additionally the lower intensity signal arising from a T-C-T methylene (at 2.56 ppm, 0.03 ppm, 9.4 Hz upfield from -TCH). This assignment was confirmed when the ^{1}H-coupled spectrum revealed that the isotope-shifted species was largely unaltered by geminal ^{1}H-coupling. Another feature of the spectrum is the intensity of the ^{3}H signal from the C-11 methylene at 4.16 ppm, reflecting a lower incorporation of tritium into that position. This is consistent with the deuterium results already mentioned, and suggested two possible causes. Firstly, the mechanism by which PBG is formed from ALA is usually thought (see Section 1.4.2) not to involve the aminomethyl side-chain. However, it is conceivable that the enzyme stereospecifically removes a tritium from that position. Secondly, ALA transaminase, the enzyme responsible for ALA biosynthesis in plants (see Section 1.4.1), is known[56,60] to co-chromatograph to some extent with ALA dehydratase. It seems likely that this enzyme is similar to ALA synthetase, both of which are pyridoxal phosphate dependent, and the latter enzyme is known[211] to stereospecifically exchange the *pro-R* hydrogen at C-5 of ALA. Thus treatment of ALA dehydratase with lysine should remove the pyridoxal phosphate, and selectively inactivate the transaminase. In order to assess the effect of lysine on ALA dehydratase activity, some highly purified enzyme was assayed in the presence of lysine and found to be virtually unaffected.

In another preparation of [^{3}H]-PBG, the effect of lysine on the incorporation of tritium into C-11 of PBG was examined. ALA dehydratase was partially purified to the stage outlined in Chapter 2, and then adjusted to 25 mM in lysine and further purified by gel-filtration on G-100 Sephadex. When this was used to synthesise [^{3}H]-PBG from [^{3}H]-ALA (2.438 Ci, 27.3 Ci mmol^{-1}), the product ~~out~~-obtained ~~lined~~ (264 μg, 122 mCi, 132 Ci mmol^{-1}) exhibited only *ca.* 20% more ^{3}H at C-11 over the previous preparation, when assayed by integration of the ^{3}H NMR spectrum.

4.5 ^{3}H NMR Studies of [2,6,11-$^3H_5$]-PBG-Deaminase Covalent Complexes

On the first occasion that [^{3}H]-PBG-deaminase covalent complex was generated, partially purified deaminase (6500 U, 1.6 times excess) was used, and the resultant complex purified by gel-electrophoresis. The pure complex, as usual mainly "mono-PBG", was concentrated and examined by ^{3}H NMR at 10.8°C. In the early accumulations, there was a hint of a signal at *ca.* 6.3 ± 0.2 ppm rapidly exchanging or reacting away. However, the predominant peak was tritiated water (HOT), presumably arising from exchange of the residual aromatic tritium. After approximately 20,000 scans, two peaks were visible, one at 2.4 ± 0.2 ppm corresponding to the propionate side-chain, and one at 3.5 ± 0.2 ppm corresponding to the tritium at C-11 (see Figure 4.5 (i)). Longer accumulations are shown in Figures 4.5 (ii) and (iii), with the aromatic resonance barely visible.

The same sample was examined with a longer recycle time, and the spectrum is shown in Figure 4.6 (i). The two resonances from

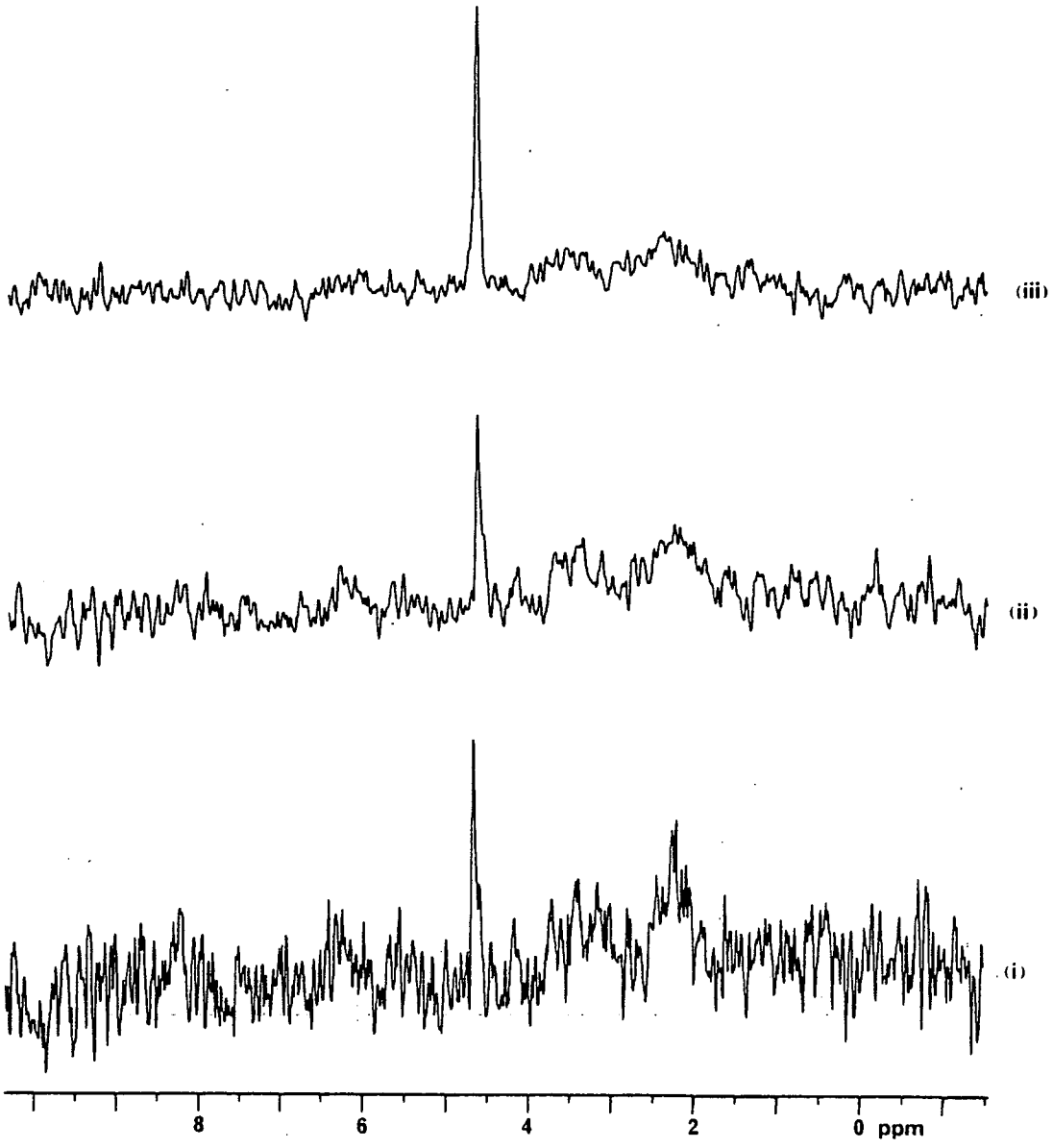


FIGURE 4.5. 320 MHz ^{3}H NMR spectra of [^{3}H]-PBG-deaminase complexes (i) with NS = 22,604; (ii) NS = 34,313; (iii) NS = 43,200, at 10.8°C and pH 8.0.

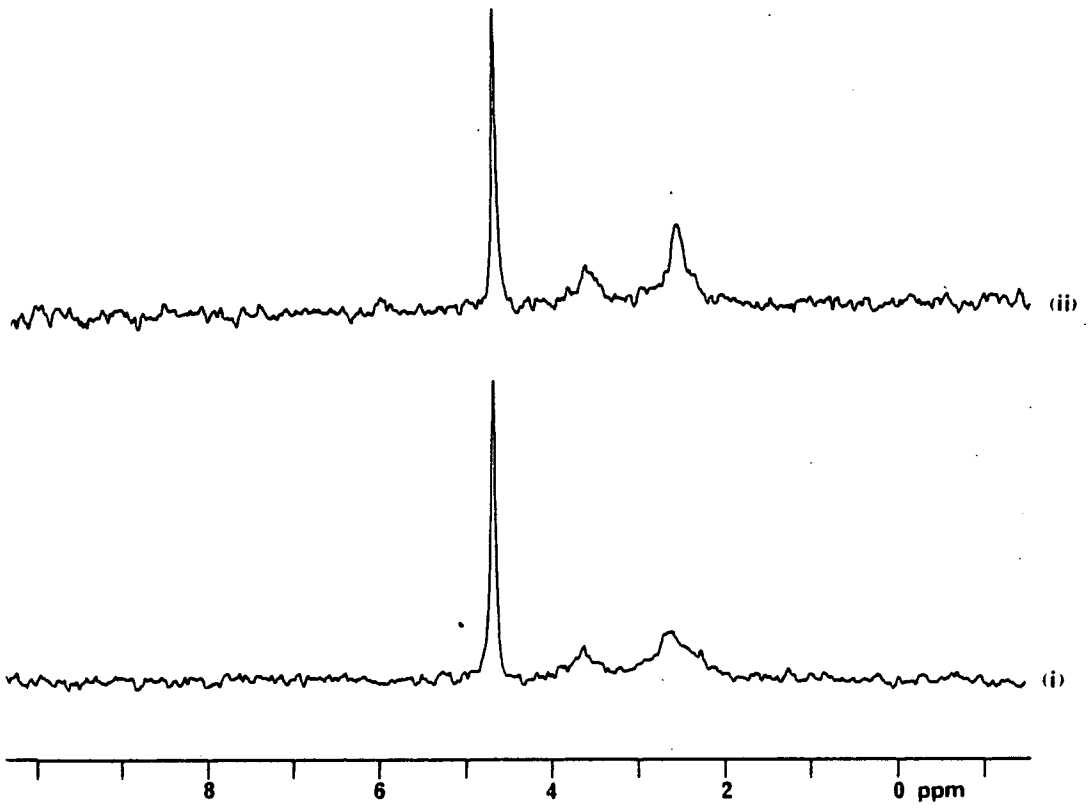


FIGURE 4.6. 320 MHz NMR spectra of [^{3}H]-PBG-deaminase complex at (i) 10.8°C and (ii) 30°C, and pH 8.0.

the complex appeared at 2.6 and 3.6 ppm, and in both cases the linewidths were approximately 150 Hz. When this sample was re-equilibrated to *ca.* 30°C, the linewidths improved to 75 Hz (see Figure 4.6 (ii)). Apart from considerations of linewidth being indicative of our detection of an enzyme-bound species, T_1 measurements, which were of the order of 0.08 s, seemed to be highly suggestive also. The sample was therefore split into two parts and one half treated with unlabelled PBG in the NMR. No reaction seemed to take place and after two further additions of PBG, only 20% of the expected porphyrin formation had taken place when assayed spectrophotometrically. When the protein was assayed for enzyme activity, however, it showed full activity, suggesting a concentration effect. This accords with

Battersby's finding[189] that deaminase is inhibited at high enzyme concentrations such as we encounter in the NMR tube.

As a result of these findings, a portion of the PBG-treated protein was diluted four times, incubated for 10 minutes at 37°C and re-examined by NMR (see Figure 4.7 (ii)). When compared with the protein prior to treatment with PBG (Figure 4.7 (i)) the lines became narrower at 2.6 ppm (propionate side-chains of uro'gen I) and 3.6 ppm (methylene bridgeheads of uro'gen I). Note that no significant chemical shift differences could be detected, suggesting that the species originally observed might be enzyme-bound uro'gen I, or some oligopyrrole gradually forming uro'gen I. When an oxidising agent such as iodine was added to the PBG-treated complex, an extra peak appeared at 4.3 ppm with concomitant loss of the resonance at 3.6 ppm (see Figure 4.7 (iii)). This corresponds to the propionate side-chain of uroporphyrin I. When a portion of the complex which had not been treated with PBG was examined (Figure 4.8 (i)), characteristic resonances of uroporphyrin I at 4.3 ppm (propionate side-chains) and 10.1 ppm (meso-positions — exchangeable) appeared. Interestingly, while the porphyrin peaks occurred at the expense of the peak at 3.6 ppm, the peak at 2.6 ppm remained. Furthermore, re-examination of PBG-treated complex which had not been exposed to iodine revealed the same pattern of resonances (see Figure 4.8 (iii)). Treatment of the PBG and I_2-treated complex with hydroxylamine hydrochloride failed to displace the resonance at 2.6 ppm. Finally, treatment of the untreated complex (as in Figure 4.8 (i)) was examined in 8 M urea, in addition to the porphyrin resonances at 10.2 and 4.4 ppm, the resonance at 2.6 ppm was unaffected, and a new resonance appeared at 5.8 ppm (see Figure 4.9).

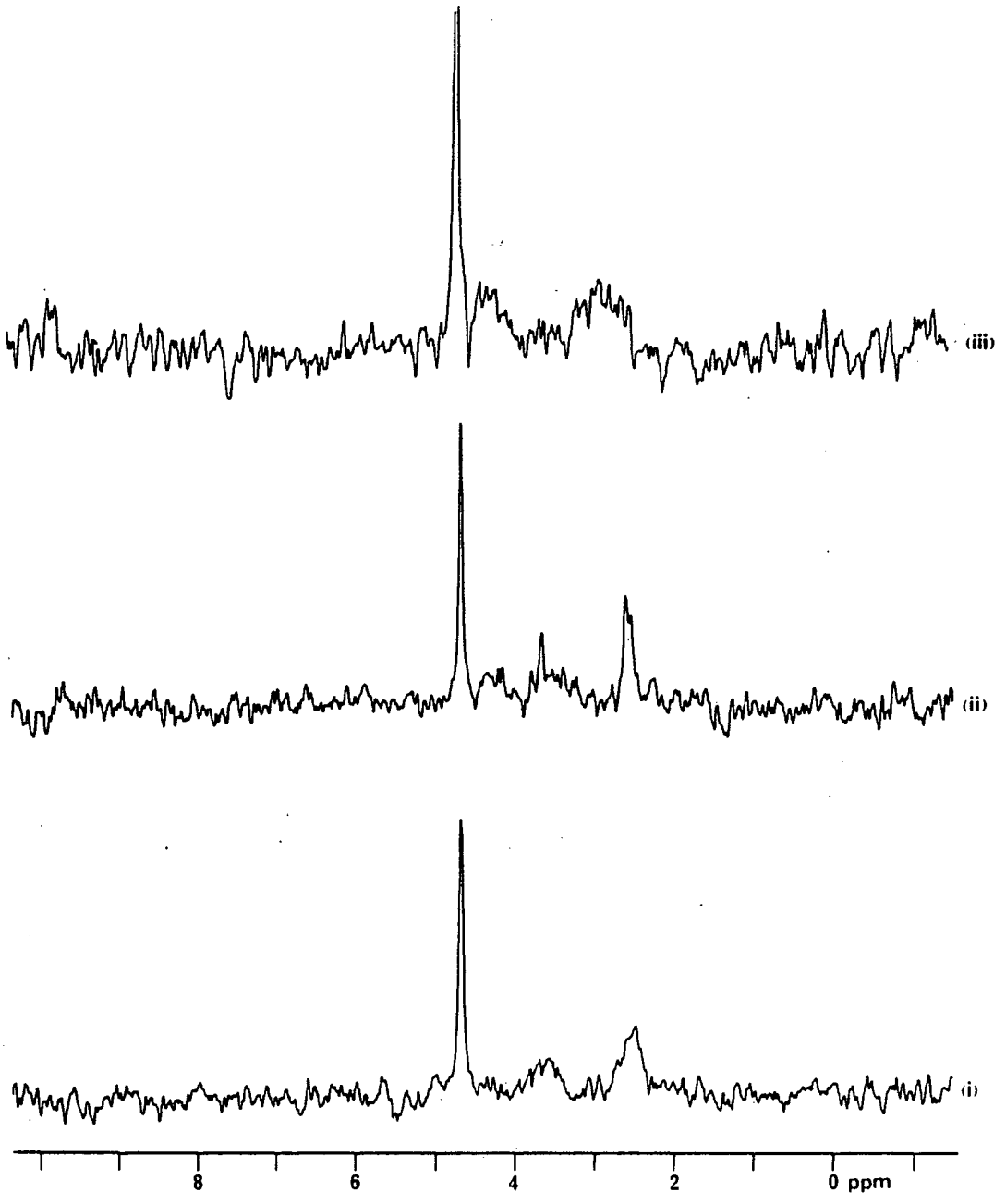


FIGURE 4.7. 320 MHz NMR spectra of [^{3}H]-PBG-deaminase complex (i); with added PBG (ii); with added PBG and I_2 (iii) at 30°C, pH 8.0.

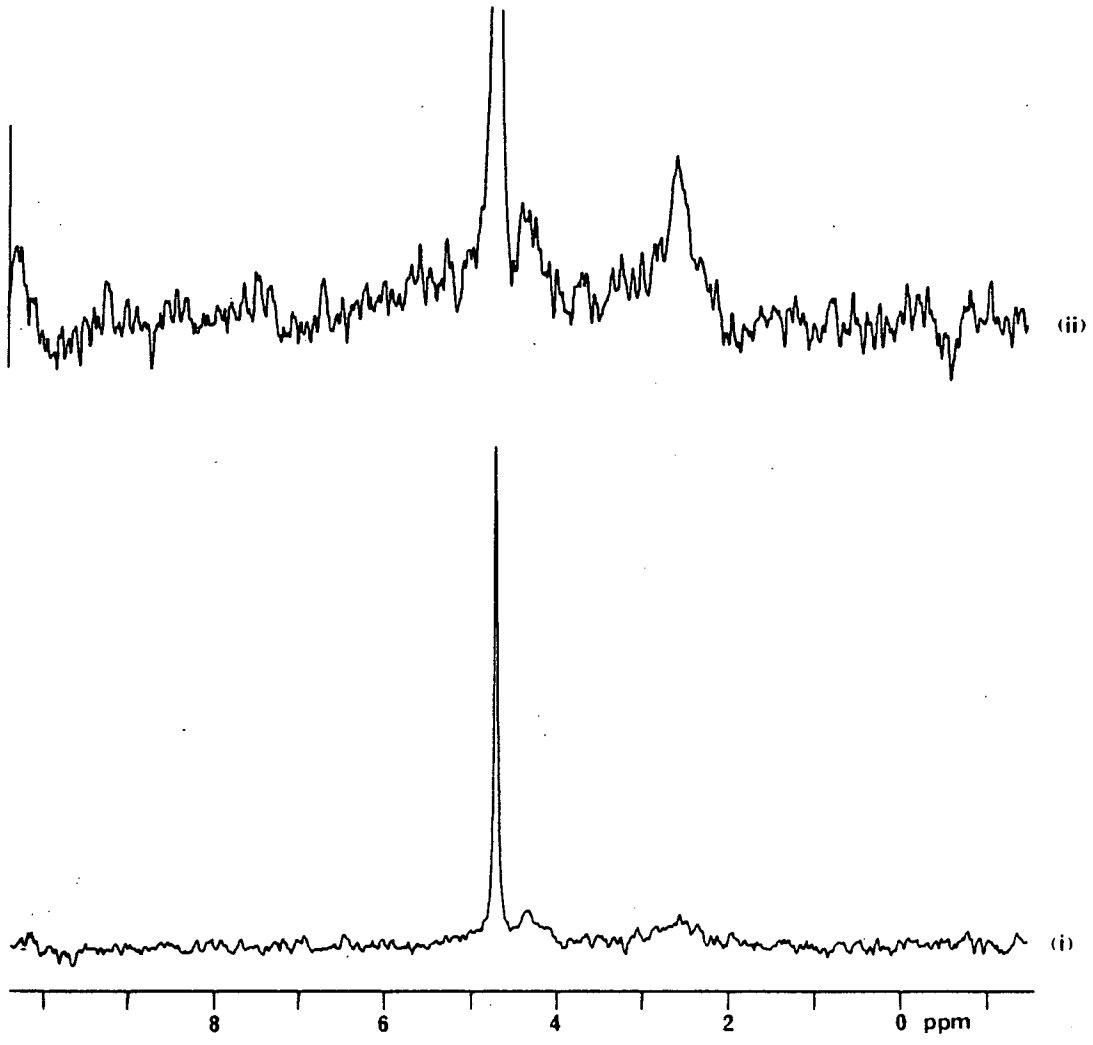


FIGURE 4.8. 320 MHz ^{3}H NMR spectra of [^{3}H]-PBG-deaminase covalent complex after prolonged storage (i) and compared to complex with added PBG after prolonged storage (ii), at 30°C, pH 8.0.

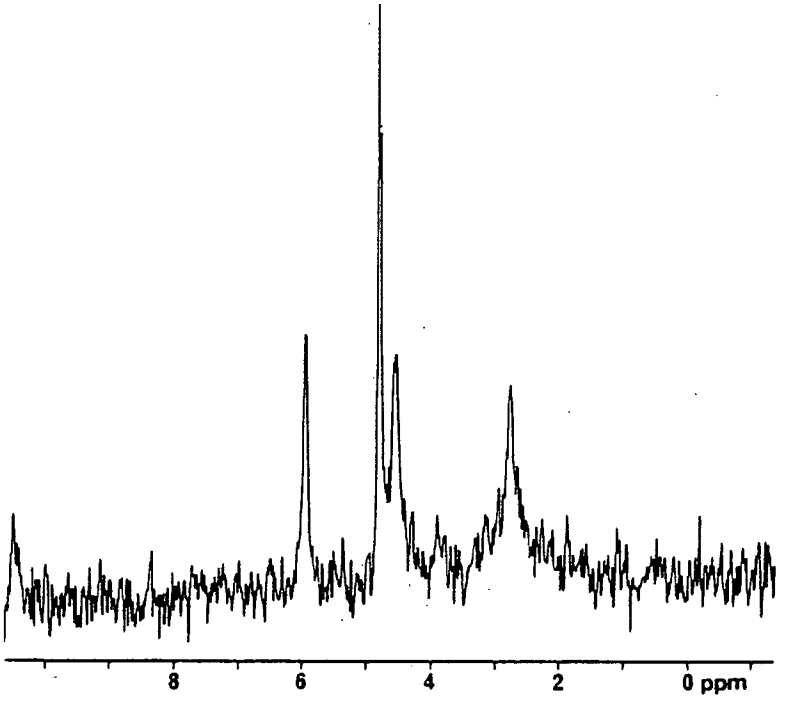


FIGURE 4.9. 320 MHz ^{3}H NMR spectrum of [^{3}H]-PBG-deaminase in 8 M urea at 30°C, pH 8.0.

The interpretation of these results is not straightforward. The indications are that the bound species detected was uro'gen I and not a monopyrrole as first thought. This is corroborated by the detection of porphyrin in the peptides from tryptic digestion of the complex (see Chapter 3). The fact that the complex without the presence of either PBG or I_2 slowly oxidises to give porphyrin further corroborates this theory. However, the fact that analytical gel-electrophoresis initially showed a band well-characterised as the "mono-PBG" complex and then after prolonged storage at 30°C showed a new band corresponding more closely to a tetrapyrrole suggests that the "mono-PBG" complex gradually disproportionates to the bound tetrapyrrole. This tetrapyrrole would appear to cyclise either still covalently attached to the enzyme, or ionically

associated with it presumably through the propionate carboxylate of ring A, and the resultant uro'gen I gradually oxidises to porphyrin. The disappearance of the peak at 3.6 ppm without concomitant loss of the peak at 2.6 ppm, and the "extra" peak at 5.8 ppm after treatment of the complex with urea both remain mysteries to date.

When a repeat of this experiment was planned, certain features of all the previous complex preparations described in this chapter and the preceding one became apparent. The ^{13}C NMR results described in Chapter 3 suggested that while the substrate signals were necessarily of extreme linewidth, disproportionation of the complex to uro'gen I at >25°C took place readily, and the resultant uro'gen I was much more detectable by ^{13}C NMR. Furthermore, this disproportionation seemed to be promoted by the oxidative procedure of preparative gel-electrophoresis. Thus it was clear that a repeat experiment needed to test the disproportionation by analysing the "mono-PBG" complex with time at >25°C, and all the NMR work performed at *ca.* 4°C. The complex should also not be exposed to electrophoresis, but instead electrophoretically pure deaminase used to generate the complex in the presence of 25 mM lysine, to preferentially form an ion-pair between the lysine and the uro'gen I that is produced, rapidly followed by gel-filtration.

Thus electrophoretically pure deaminase (*ca.* 4000 U) was mixed with [2,6,11-$^{3}H_{5}$]-PBG (122 mCi, 27.3 Ci mmol^{-1}) and 25 mM lysine in the HPMD, immediately concentrated, and applied to a freshly equilibrated G-50 Sephadex column. The complex which eluted off was concentrated to *ca.* 0.50 cm^3, and examined by ^{3}H NMR at 5.5°C. A portion of the complex was also examined at 14°C and 23°C (see Figure 4.10 (i)-(iii)). When the spectrum was recorded at 5.5°C,

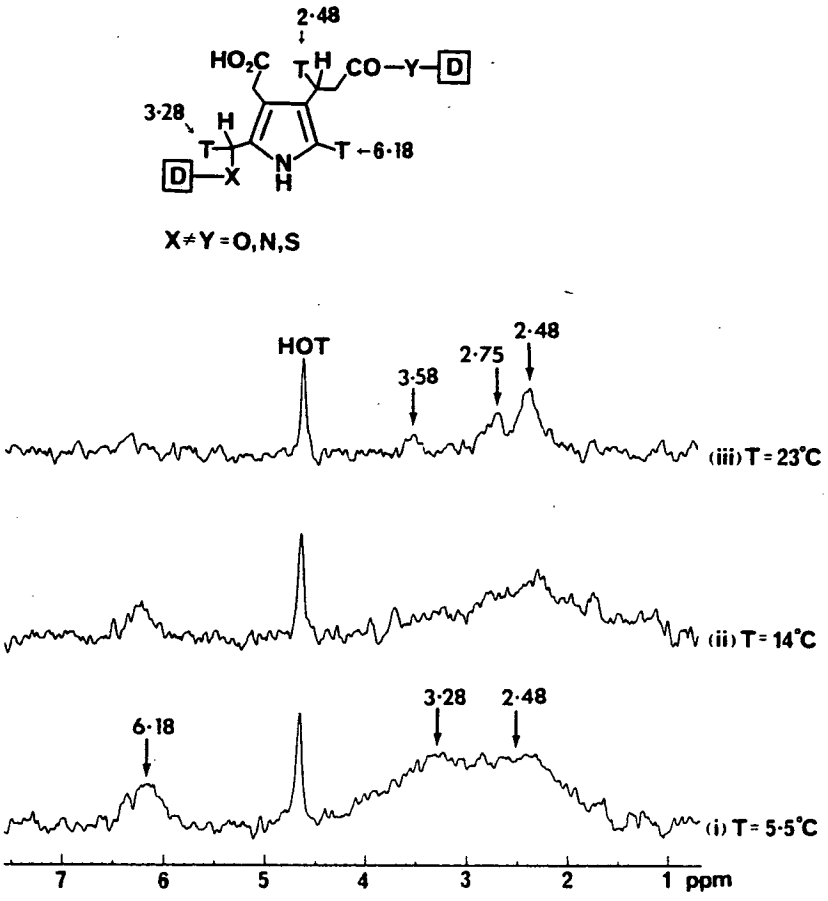


FIGURE 4.10. 320 MHz ^{3}H NMR spectra of [^{3}H]-PBG-deaminase complex at (i) 5.5°C, (ii) 14°C and (iii) 23°C, and pH 8.0.

resonances occur at 6.18 ppm (pyrrole-T), 3.28 ± 0.1 ppm (pyrrole-CT_2-X-Enz) and 2.48 ± 1 ppm (pyrrole-CT_2-CH_2CO_2H). Here it is interesting to note that the aromatic resonance of PBG which normally occurs at 6.69 ppm shifts to 6.17 ppm on binding to the enzyme. On warming to 14°C, the resonance at 3.28 ppm starts to disappear, and a new peak is just visible at 2.75 ppm. At 23°C, the aromatic resonance at 6.17 ppm disappears, and the remaining resonances occur at 3.58 ± 0.05 ppm (20-meso of uro'gen I + pyrrole-CHT-X-Enz), 2.75 ± 0.05 ppm (Enz-pyrrole-$CT_2CH_2CO_2H$) and 2.48 ± 0.05 ppm (uro'gen I propionate side-chain). The latter two signals are considerably

sharper than those obtained at 5.5°C. Also these results can be considered as evidence for the disproportionation of the "mono-PBG" complex to di-, tri- and tetra-pyrroles and subsequently uro'gen I at this temperature (23°C) and protein concentration (*ca.* 1.6 mM). Presumably the signals at 3.58 and 2.75 ppm arise from an oligopyrrole *en route* from bound monopyrrole to uro'gen I. The assignment of the resonances to uro'gen I were confirmed when a sample of enzymatically prepared uro'gen I was examined at 23°C (see Figure 4.12).

To demonstrate the catalytic competence of the complex, unlabelled PBG (950 μg) was added to the portion which had been equilibrated at 23°C, and the formation of uro'gen I studied with time. Uro'gen I showed characteristic resonances at 3.58 ppm (meso positions) and 2.5 ppm (propionate side-chain), exactly as found in ^{1}H NMR studies (see Section 2.2). When this was repeated with the portion which had been stored at 3.5°C, the substrate is clearly connected to uro'gen I (see Figure 4.11) which when warmed to 23°C (see Figure 4.12) exhibited the characteristic resonances found in the ^{1}H NMR studies. Interestingly, however, at 3.5°C the uro'gen I resonances occur at 2.15 and 3.28 ppm, which shift by 0.3 ppm on warming to 23°C. At 3.5°C the uro'gen I is probably still attached or associated with protein, coming free at 23°C. That the shift is not due to temperature is confirmed by consideration of the ^{1}H NMR data for uro'gen I (see Section 2.2) at 4°C.

A noteworthy feature of the spectra in Figure 4.10 is the lack of accumulation of any obvious intermediates such as HMB (55). It seems likely that at this high protein concentration (*ca.* 1.6 mM), intermediates such as HMB, which probably represent the nucleophilic

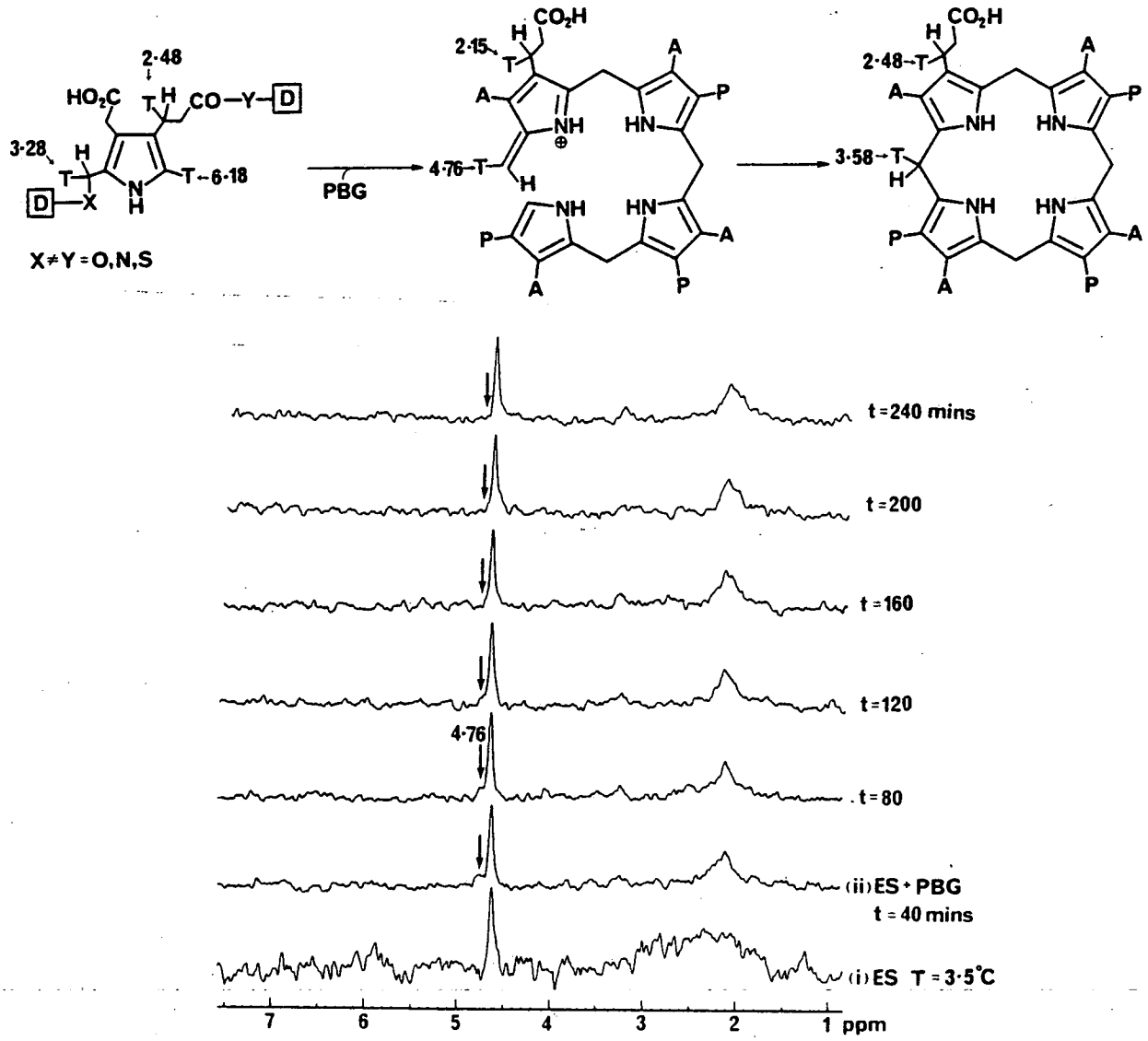


FIGURE 4.11. 320 MHz ^{3}H NMR spectra of [^{3}H]-PBG-deaminase complex + unlabelled PBG (950 μmg) at 3.5°C, pH 8.0.

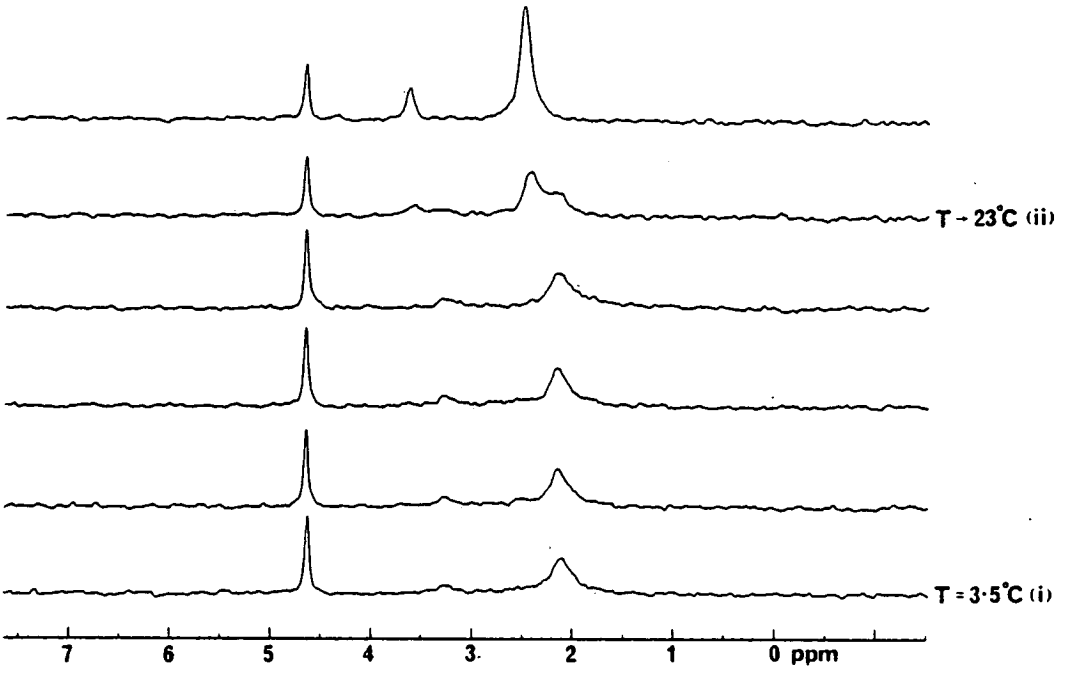


FIGURE 4.12. 320 MHz ^{3}H NMR spectra of [^{3}H]-PBG-deaminase complex + unlabelled PBG at 3.5°C (i) and after warming to 23°C (ii), pH 8.0.

trapping of an exomethylene species (60) with water, never have an opportunity to accumulate. Rather, the highly reactive exomethylene species cyclises directly to uro'gen I. In fact, a transient low-intensity peak appears at 4.76 ppm (Figure 4.11), a chemical shift which is consistent with an exomethylene species. This is perhaps more obvious in Figure 4.13. As mentioned in Section 2.2, model systems exhibit a ^{1}H-resonance at 4.86 ppm.[175]

The unusually large linewidths (*ca.* 150-300 Hz) of the spectra in Figure 4.10 (i) reflect an environment in which the active site of the enzyme is probably buried within the protein. Furthermore, the implications for ^{13}C NMR explain completely the comparative lack of success with that nucleus, since there the linewidths would be substantially greater due to larger T_2 values for the [^{13}C]-complex.

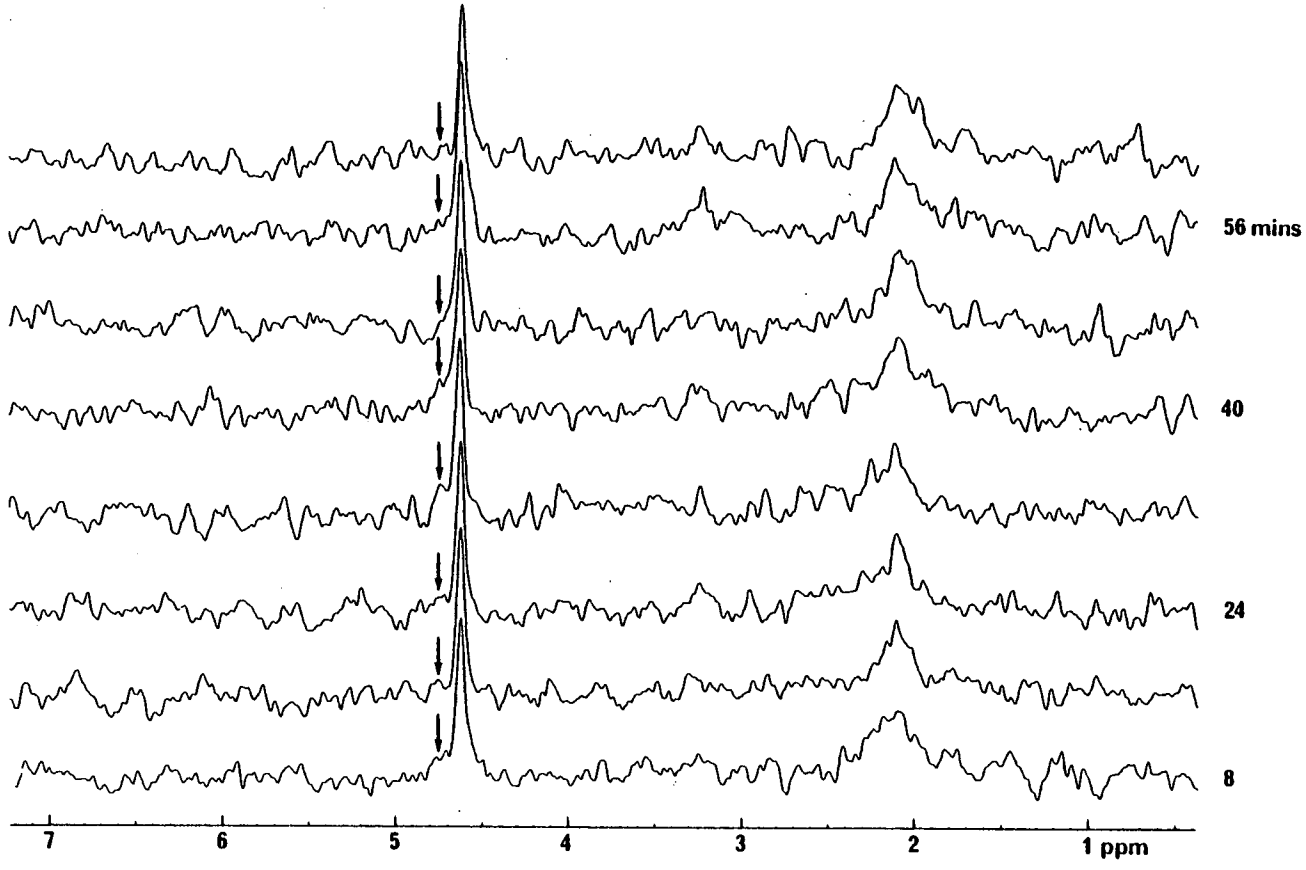


FIGURE 4.13. 320 MHz NMR spectra of [^{3}H]-PBG-deaminase + unlabelled PBG at 3.5°C, pH 8.0.

The propionate side-chain linewidth also suggests that it is either covalently attached or ionically associated with the protein. This is consistent with the reports on the inhibitory effects of PBG analogues (see Section 1.4.3), and also the electrophoretic mobilities of the complexes. Jordan[141] interpreted the increase in the mobility between the "mono-PBG" complex band and the "di-PBG" complex band as evidence for the neutralisation of a negatively charged enzyme group (after loss of $-\overset{+}{N}H_3$), thus providing only a net increase of one negative charge. However, the single net negative charge is much more likely to arise from involvement of one of the pyrrole carboxylates in binding as well as elimination of $-\overset{+}{N}H_3$. The chemical shift of the methylene (C-11) directly attached to the enzyme allows some tentative conclusions to be drawn, and these will be presented in the next chapter.

CHAPTER 5

BIOSYNTHESIS OF PORPHYRINS: CONCLUSIONS AND OUTLOOK

5.1 The Mechanism of Deaminase

The results presented in the previous chapters clearly show that PBG deaminase initially binds one PBG unit, which subsequently condenses with further PBG units to generate ultimately uro'gen I. With the bacterial enzyme, the binding of these PBG units results in three stable covalent enzyme-substrate complexes. At high concentrations and temperatures >25°C, these can gradually disproportionate with time,

$$E \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightleftharpoons ES_3 \rightleftharpoons ES_4$$

in an equilibrium process which lies far to the right under those conditions, and the bound tetrapyrrole gradually forms. This would appear to be inherently unstable and forms uro'gen I via HMB.

The nature of the groups of deaminase involved in the binding of the substrate is difficult to determine in the absence of more detailed information about the protein. Most conventional techniques for the study of binding sites involve the isolation and purification of peptide-bound substrate, but these fail here because the hydrolytic conditions employed result in the polymerisation of the substrate. While proteolytic enzymes can and have been used to obtain oligopeptide fragments, the analysis of these fragments has proved to be extremely difficult, especially since they cannot be further hydrolysed to their constituent amino acids without destroying the

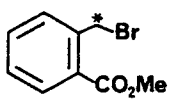
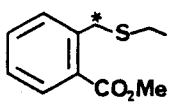
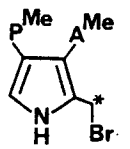
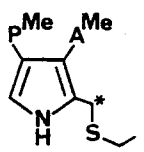
substrate. Unfortunately, to date the analysis of the fragments described in Chapter 3 using FAB-MS is not complete due to technical problems. There are, however, preliminary indications that porphyrin is present in some of the fragments. Thus without crystalline protein, which could be used for a detailed X-ray crystallographic investigation, NMR provides a method *par excellence* for the study of the mechanism of the enzyme.

^{13}C NMR failed to detect enzyme-bound PBG with any confidence, and only detected uro'gen I attached or associated with the protein. ^{3}H NMR, however, provided the means to detect the enzyme-substrate complex and furnished a chemical shift of 3.3 ± 0.1 ppm for the methylene at C-11 of PBG directly attached to the protein. Since PBG can be regarded as a Mannich base, it seems reasonable to expect it to alkylate a group on the enzyme that is probably nucleophilic. Mechanistically it is unlikely to alkylate a histidine or any other aromatic amino acid residue, since it would be extremely difficult to liberate the pyrrole methylene after tetrapyrrole formation had taken place. Similarly, an amide linkage can be ruled out on the basis of its high stability. Much more likely is the involvement of a nucleophilic group on the enzyme, such as the oxygen of serine, the nitrogen of lysine or the sulphur of cysteine. Let us consider models of each of these in turn as a means to rationalise the observed enzyme-substrate chemical shift of 3.3 ± 0.1 ppm A very good model for the serine-O-PBG is the chemical shift of the methylene of HMB (55), which from Section 2.2 exhibits a resonance at 4.4 ppm. Going from a primary alcohol to an ether linkage usually results in a *ca.* 0.2 ppm upfield shift. We can therefore definitely rule out serine oxygen.

For nitrogen, a number of good models exist. PBG itself displays a resonance at 4.2 ppm for the C-11 methylene, and from the work described in Section 2.2, only shifts 0.05 ppm upfield on deprotonation. Aminomethylbilane (40) displays a resonance at 3.92 ppm at pH 12,[115] which implies a chemical shift of 3.97 ppm at pH 8.0. The dipyrrole derivative of PBG exhibits a resonance at 4.05 ppm.[212] Thus, contrary to recent conclusions,[189] the ε-NH_2 of lysine can be ruled out unequivocally.

For sulphur, model compounds were prepared in this laboratory by Dr. N. E. Mackenzie,[213] and the ^1H NMR data is shown in Table 5.1.

TABLE 5.1 ^1H NMR data for models of a sulphur analogue of PBG.[a]

Compound	δ_H (R-$\overset{*}{C}H_2$-X)/ppm
 (94)	4.94 ± 0.05
 (95)	4.10 ± 0.05
 (96)	4.23 ± 0.05
 (97)	3.39 ± 0.1 (calculated)

[a]Data taken from Ref. 213.

The final chemical shift for the model thioether (97) was calculated from (94) - (96) to be 3.39 ± 0.1 ppm, which is in close agreement with the experimentally observed value of 3.30 ± 0.1 ppm.

On the basis of this ^{3}H NMR evidence, combined with the observation that deaminase is strongly inhibited by sulphydryl reagents, an effect which can be reversed by dialysis against thiol reagents (see Section 1.4.3), there is compelling evidence in favour of sulphur for the X group to which the substrate binds, in the form of a cysteine thiol. Although an attempt was made to titrate the enzyme thiol groups using 2,2'-dipyridyldisulphide, none were detected, and it seems likely that if the active thiol were buried in the active site of the enzyme, it would not readily titrate.

Thus the chemical shift obtained from ^{3}H NMR strongly suggests that the nature of the enzymic group to which the substrate binds is a cysteine thiol. There is a *caveat*, however, that some unknown or unusual group could be involved, or that the electronic environment of the active site radically perturbs the bound substrate. The former possibility is unlikely, since from the amino acid analysis of human deaminase (Table 1.4), there are no unusual amino acids, and there is no evidence of a cofactor requirement for the enzyme.

The isolation and purification of deaminase described in Chapter 3 furnished protein *ca.* 70% pure. Therefore, a portion of [^{13}C]-complex which had been incubated with unlabelled PBG was further purified by gel-electrophoresis to provide samples of ultra-pure (>95% pure) deaminase and "mono-PBG" complex. These were sent for amino acid analysis and protein sequencing, but unfortunately the results were not complete at the time of writing.

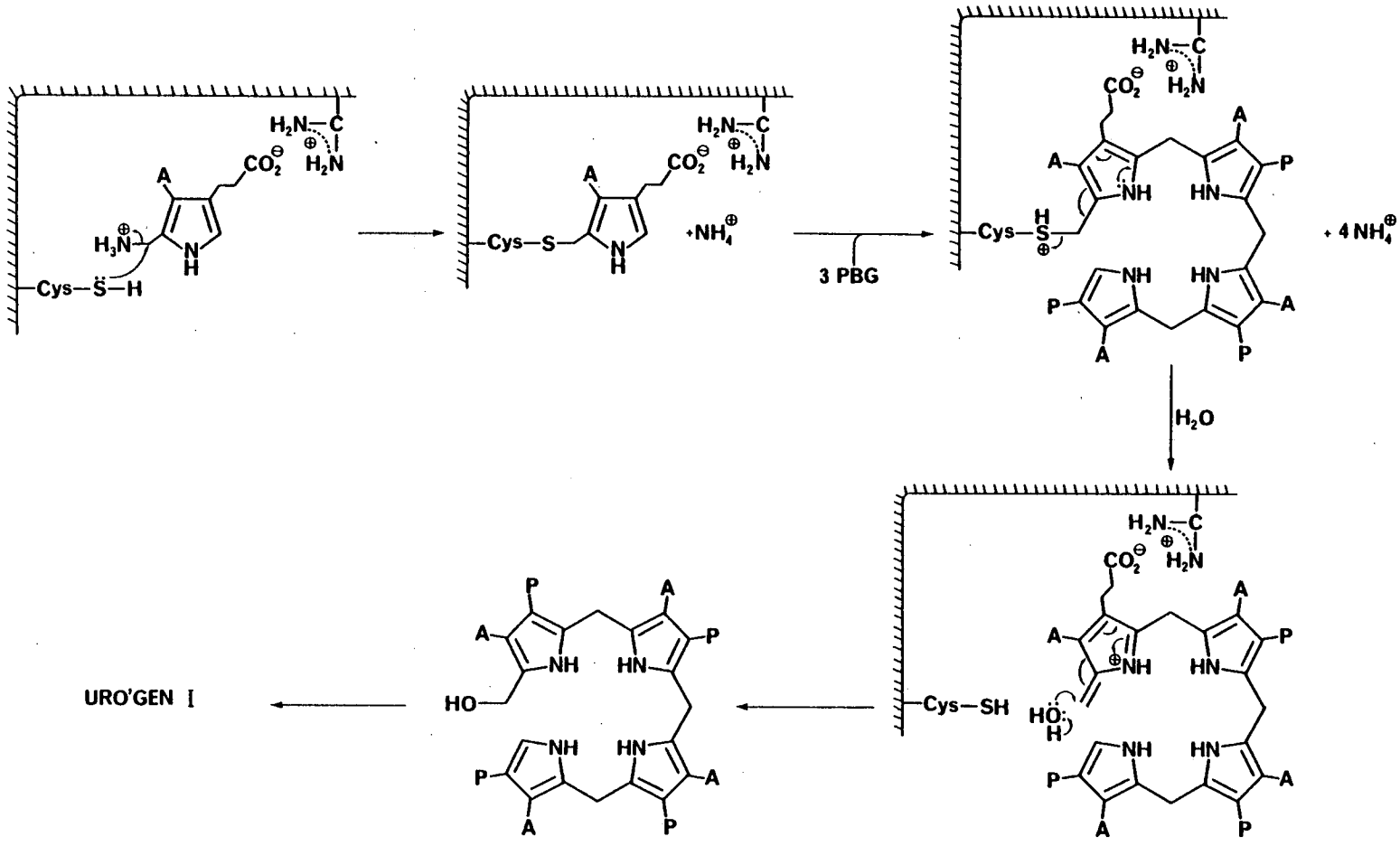
Kinetic studies conducted by Williams *et al.*, using purified deaminase from *Euglena gracilis*[77] and rat spleen[214] have established some interesting features of the deaminase-catalysed reaction. The pH dependence of V_{max} was investigated, and for enzyme from both sources a classic bell-shaped curve was obtained, indicating that two ionisable groups with pK values of 6.1 and 8.9 (*Euglena*) or 7.4 and 8.8 (rat spleen) are important for catalysis. These represent ionisations in the enzyme-substrate (ES) complexes, but without knowledge of the nature of the rate-limiting step in the overall process, or the possibility of equivalent ionisations throughout the series of partial reactions, it is not possible to attribute these pK values to individual reaction steps.

The dependence of K_m with pH was also studied, and found to decrease with decreasing pH. This was interpreted as the absence of the pK in the free enzyme or substrate corresponding to the pK of 6.1 in the *Euglena* enzyme. Thus if the effect is due to ionisation of the substrate part of the ES complex, then the substrate complex must be raised from 3.7 or 4.95[176] for the carboxyl functions to 6.1. If, however, the pK reflects ionisation of the enzyme portion of the ES complex, then the absence of a pK in the measured pH range (pH 6-8.7) may be due to the group having a pK <5.0 in the free enzyme or to the group being buried in the protein and made accessible to the solvent by substrate-induced conformational changes. It was also found that the dependence of V_{max}/K_m with pH showed a single ionisation (pH 8.2) reflecting ionisations in the free enzyme or its three pyrrolic forms.

These results can be further rationalised in the light of the foregoing NMR investigations, which have established that binding

of the first PBG unit takes place through both the C-11 methylene and the C-8 carboxylate (propionate side-chain). The dependence of V_{max} on pH could reflect ionisations in any of the ES complexes, but taking into consideration the dependence of K_m upon pH, it seems likely that the pK of 6.1 arises from a highly perturbed value for the propionate carboxyl of PBG. This unusual value could be explained if the carboxylate function formed an ionic bond with a suitably basic enzyme group, such as the ε-NH_2 of a lysine residue, the guanidino group of an arginine, or the imidazole of a histidine. The other ionisation, as borne out by the dependence of V_{max}/K_m with pH, probably reflects ionisations in the enzyme portion of the ES which is only slightly perturbed (0.7 pH units) by the presence of substrate. If this were true, then the free enzyme pK is closer to typical values for a sulphydryl group (pK 8.3-8.6) than for an ammonium (ε-NH_2) group (pK 9.4-10.6).[215]

Thus a mechanism for deaminase can be proposed based on all the evidence acquired to date, as depicted in Scheme 20. The first PBG unit alkylates a free thiol group with the elimination of one mole equivalent of ammonia, and the propionate forms an ion pair with a lysine or arginine residue,[216] and subsequent condensations with PBG result in the bound tetrapyrrole. At this point the first step in the release of the tetrapyrrole is the regeneration of the free thiol and the formation of an exomethylene double bond. This highly reactive species could be intercepted by endogenous water, or any other suitable nucleophile, as has been shown by numerous trapping experiments.[71,108,188] Either the exomethylene species or its trapped version (HMB) are presumably released into the medium by breaking the propionate ionic bond, for further chemical cyclisation to uro'gen I.



SCHEME 20. Proposed mechanism for PBG deaminase.

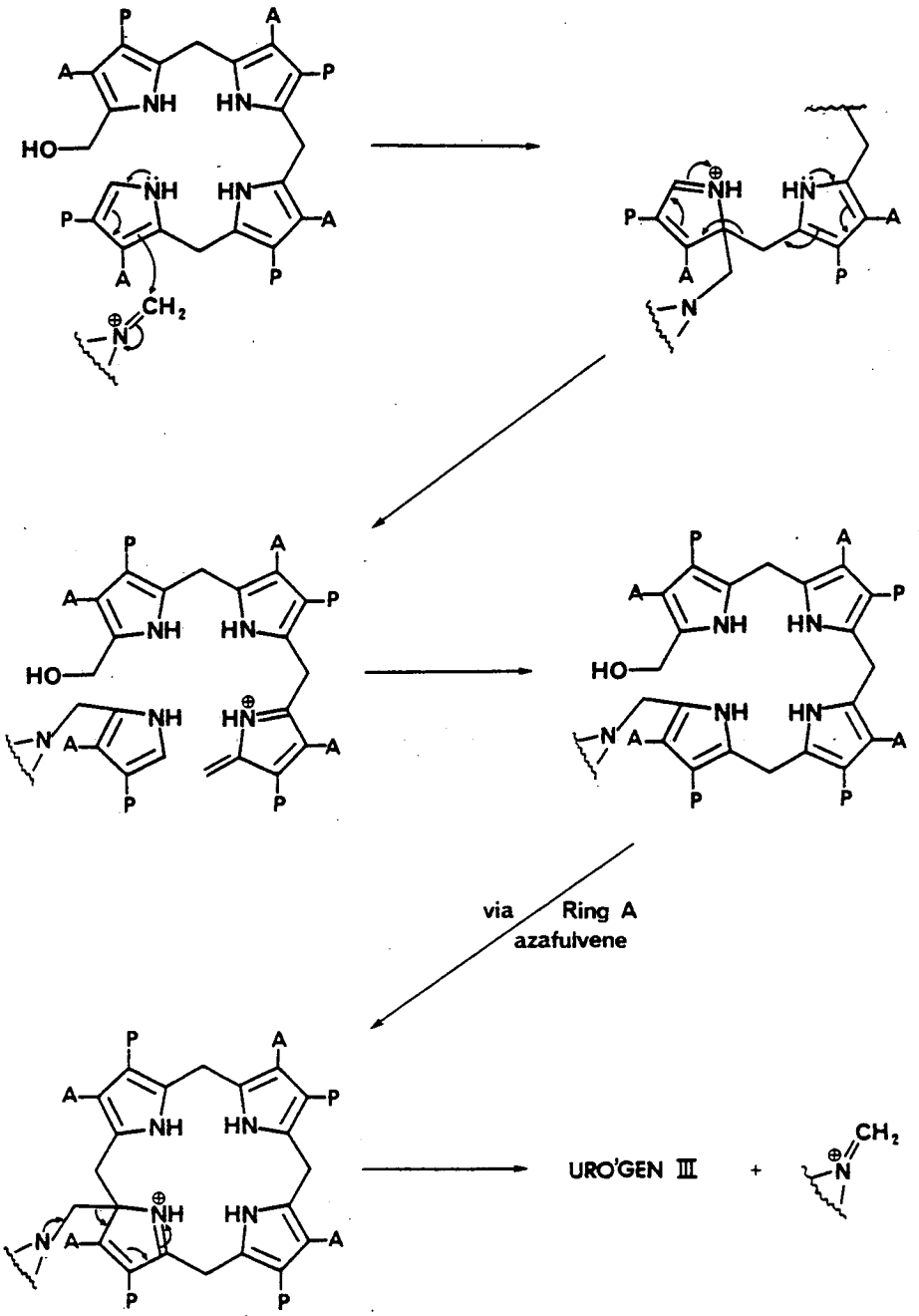
5.2 The Mechanism of Cosynthetase

The results from the experiments in Chapter 2 do not really allow any further conclusions to be drawn as to the mechanism of cosynthetase. However, recent studies by Battersby *et al.*[189] taken together with the studies on deaminase summarised in the previous section, point strongly to the conclusion that HMB (55) is not the true substrate for cosynthetase.

Battersby covalently bound tetrapyrrole from [1-^{3}H]-HMB to deaminase from *E. gracilis*, and treated the complex with cosynthetase. Here it was found that 72% of the bound tetrapyrrole was released and converted to uro'gen III. This contrasts with the 16% of tetrapyrrole released by incubation of the complex at pH 8.0 and 37°C, and >96% released when the complex was incubated with excess PBG.

The experiments described in Chapter 4 established that at high deaminase concentrations, the species released from the enzyme is the very reactive exomethylene pyrrolenine (60). This can be intercepted by water at low protein concentrations, or indeed by cosynthetase if it is present. It has been often suggested[75,78,79] that deaminase and cosynthetase act in a multienzyme complex, and yet it has been shown[124] that when HMB is the substrate, cosynthetase can act completely independently from deaminase. Perhaps an explanation for these disparate viewpoints is that the exomethylene species is stabilised locally by deaminase, and if cosynthetase loosely associates with deaminase, then the exomethylene species immediately binds to cosynthetase for the subsequent ring-inversion step. It is only in the absence of cosynthetase, and at low deaminase concentrations that HMB ever has an opportunity to accumulate. The true substrate for cosynthetase is therefore the exomethylene pyrrolenine.

Unfortunately, none of the studies presented in this thesis shed any light on the intriguing ring-inversion mechanism. While the Mathewson and Corwin mechanism involving the "spiro" intermediate seems a likely candidate (see Scheme 7), alternative mechanisms still continue to be put forward, in the absence of any experimental results. Battersby and co-workers[128] recently proposed the involvement of methylene-tetrahydrofolate in the ring-inversion step (see Scheme 21).



SCHEME 21. Battersby's mechanism for uro'gen III formation (1982).

This is essentially a variation on the mechanisms put forward by Rimington, Johnson and Russell[217-219] for dipyrrole involvement, and an analogous scheme put forward by K. S. Ho.[220] However, until further studies are conducted on cosynthetase, such mechanisms must remain yet more idle speculation.

5.3 Outlook

5.3.1 Deaminase

From the studies outlined for deaminase, the way forward to the investigation of the finer details of the mechanism of the enzyme are clear. While the intermediacy of the exomethylene pyrrolenine species is strongly suggested by what we believe to be its direct observation, and also from the numerous trapping experiments which have been performed, there are two approaches which would establish this unequivocally.

The ^{3}H NMR Approach

The resonance from tritiated water (HOT) casts some doubt on the reality of the resonance assigned to the exomethylene species, owing to the latter's transience and the magnitude of the HOT line partially obliterating the resonance of interest. Thus if the activity of deaminase was examined in a cryosolvent such as DMSO-water, and it were possible to remove the tritiated water completely from [^{3}H]-PBG by repeated cycles of treatment with water and lyophilisation, then there might be a good chance of detecting the exomethylene species directly and more convincingly. The method would involve cooling the [^{3}H]-PBG-deaminase enzyme-substrate complex

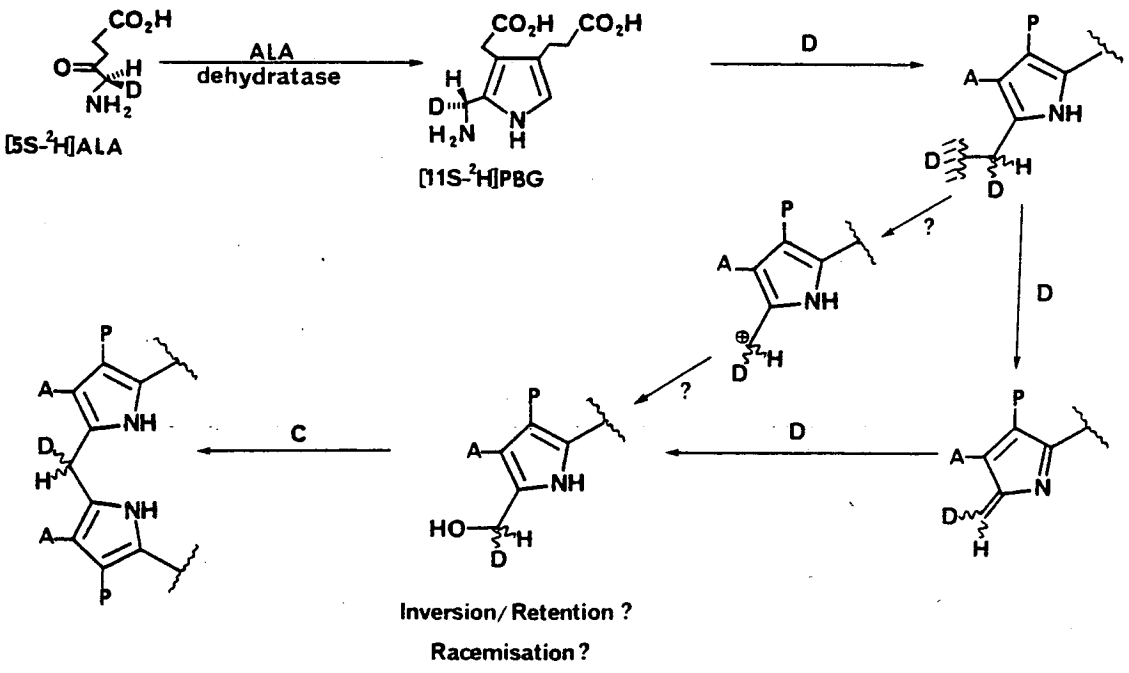
to *ca.* -10°C in the NMR, and treating it with unlabelled PBG. The exomethylene species would then have more time to accumulate and would not be obliterated by the HOT resonance.

The Chiral PBG Approach

If it were possible to follow the stereochemical fate of the methylene directly attached to the enzyme, from PBG through to uro'gen I, the intermediacy of an exomethylene species might be more rigorously tested. For this experiment, large quantities of chiral [11-$^2H_1$]-PBG would be necessary and furthermore a means, hiterto unclear, to determine the stereochemistry of the resultant HMB and uro'gen I, possibly using CD methods by comparison with synthetic material of known stereochemistry. To date, chiral PBG has been prepared only in tracer amounts[55] and in very poor yields from chiral glycine using ALA synthetase. However, with a highly efficient synthesis of [5-^{2}H]-ALA now established (see Section 4.3) there are alternative, much more effective means for producing milligram quantities of chiral PBG. ALA synthetase is known to stereospecifically exchange the protons at C-5 of ALA,[211] and thus incubation of [5-^{2}H]-ALA with this enzyme, and subsequent incubation with ALA dehydratase under the conditions established in Chapter 4, would furnish large amounts of chiral [11S-^{2}H]-PBG. An even easier alternative would be to incubate [5-^{2}H]-ALA with partially purified ALA transaminase from *R. spheroides* and then complete the synthesis of PBG with ALA dehydratase. This approach would be easier because apart from the transaminase having a reportedly higher turnover than ALA synthetase,[221] the purification of ALA dehydratase from *R. spheroides* contains fractions largely distinct from dehydratase

activity that exhibit transaminase activity. Thus two enzymes for the price of one is a distinct advantage. Furthermore, the other enantiomer could be prepared by performing the incubation with unlabelled ALA in a 90% D_2O medium.

The stereochemical course of chiral PBG through to HMB and uro'gen III is shown in Scheme 22. The possible involvement of a carbonium ion could then be ruled out, and if racemisation took place, the intermediacy of the exomethylene species would be clearly indicated. Note that either inversion or retention of configuration could still arise from the exomethylene species being held stereospecifically in the cleft of the enzyme.



SCHEME 22. The stereochemistry of HMB formation.

Further aspects of the precise mechanism of deaminase will become clearer when the sequencing of the protein is complete, hopefully shortly. Additionally, if the gene that codes for

deaminase synthesis could be identified and cloned back into *R. spheroides*, then 80-90% of the bacterial protein mass could be deaminase. Using affinity chromatography with immobilised monoclonal antibodies[222] for deaminase, the crude homogenate from the cloned *R. spheroides* could be purified to give deaminase of >95% purity in one step. With abundant quantities of pure enzyme, the X-ray and solid-state NMR structures could be determined, both with and without the substrate. This will ultimately lead to a deeper understanding of the detailed mechanism for the enzyme.

5.3.2 Cosynthetase

Cosynthetase is a difficult protein to purify in very large quantities, and for NMR investigations to be of any assistance, abundant quantities must be available. Thus the molecular biological techniques outlined for deaminase could also be employed for cosynthetase, and then the ring inversion step studied in much greater detail. For this, cryoenzymological techniques would be necessary, and the enzyme would need initially to be studied in suitable cryosolvents such as DMSO-water. With the most suitable conditions established, a number of approaches would need to be tried.

^{13}C NMR spectroscopy, using [3,5-^{13}C]-PBG-derived HMB as a substrate, might provide the means to detect the spiro intermediate if it is involved, but here again the sp^3 carbon would probably be occluded by the protein background, and possess an extreme linewidth at low temperatures (*ca.* -10°C to -30°C). ^{3}H NMR might again furnish valuable information, especially if a mechanism such as the methylene-tetrahydrofolate one proposed by Battersby were involved.

An alternative to cryoenzymological NMR, which so often suffers from problems of linewidth, would be the use of a "poor" substrate for cosynthetase, such as the D-ring inverted HMB prepared by Battersby *et al.*[90] suitably enriched with ^{13}C, or perhaps a bilane blocked at the free pyrrolic end with a fluorine for example, where ^{19}F NMR could be employed to study the bound tetrapyrrole and the initial stages of ring inversion.

The study of porphyrin biosynthesis over the last decade has been characterised by a lack of detailed knowledge and understanding of the enzymes involved, particularly those responsible for the assembly of the porphyrin macrocycle. The reasons for this are due to the comparatively small quantities of the proteins which are available from the conventional sources, and the absence of non-invasive techniques for investigating these complex enzyme-reaction mechanisms. However, with the advent of cloning techniques, the potential for producing sufficient quantities of both deaminase and cosynthetase for NMR investigations is greatly increased. The manifest virtues of NMR will then be fully exploited in an attempt to unravel the mysteries of these beguiling enzymes.

PART III

EXPERIMENTAL

PROCEDURES

CHAPTER 6

GENERAL EXPERIMENTAL PROCEDURES

Reagents and solvents employed throughout this work were of the highest grade obtainable. Water was doubly-deionised and distilled in glass. Analytical and preparative thin-layer chromatography were carried out on E. Merck GF_{254} silica gel glass-backed plates (0.25, 0.5, 1.0 or 2.0 mm), Brinkmann Machery-Nagel (Düren) silica gel plates, Machery-Nagel neutral alumina plates, or Machery-Nagel DC Fertigplatten cellulose plates (0.1 mm). The solvent systems employed were as follows:

Solvent I: 12:3:5 n-butanol:glacial acetic acid:water.

Solvent II: 80:16:4 benzene:ethyl acetate:methanol.

Solvent III: 1:9 methanol:chloroform.

Column chromatography was carried out on E. Merck Kieselgel 60 Art. 9385 (230-400 mesh) silica gel or E. Merck neutral alumina 90 Activity I Art. 1077 (70-230 mesh) or Brinkmann Avicel microcrystalline cellulose (38 μm particle size).

High-performance liquid chromatography (HPLC) was performed on a Waters Liquid Chromatograph equipped with a Model 6000A solvent delivery system, a Model U6K Universal injector and a Model 440 UV detector operated at 405 nm, or alternatively, a Spectra Physics SP1800 Liquid Chromatograph equipped with an SP8440 UV/vis variable wavelength detector operated at 405 nm, and an SP4100 computing integrator. The columns employed were two μ-Porasil (300 x 3.9 mm I.D., 10 μm particle size) connected in series. The columns were pre-equilibrated for several hours (usually overnight) with n-heptane:glacial acetic acid:acetone:water (600:300:200:1), and

operated using n-heptane:glacial acetic acid:acetone (6:3:2) with a flow-rate of 0.5 cm^3 min^{-1} at a pressure of *ca.* 400 p.s.i. This is essentially the technique of Nordlöv *et al.*,[94] used for the analysis of uroporphyrin isomer ratios in *ca.* 60 minutes per sample.

Melting points were determined using a Kofler-type hot-stage microscope apparatus (Reichert, Austria) and are uncorrected.

Infrared absorption spectra (IR) were recorded as thin films or Nujol mulls using NaCl plates, or in KBr discs using a Perkin-Elmer Model 297 IR spectrophotometer. Abbreviations used to express peak intensities are s — sharp, w — weak.

Ultraviolet/visible absorption spectra (UV) were recorded on a Varian DMS-90 spectrophotometer (Edinburgh), Hitachi Model 100-60, Pye-Unicam SP1800, or Varian Cary 210 spectrophotometers (Texas). For routine determinations, 1 cm pathlength matched quartz cuvettes were used, and for small volumes, 1 cm pathlength quartz micro cuvettes (600 μL capacity) were used. All determinations were carried out at room temperature, except where specified. Absorption maxima (λ_{max}) are reported in nanometers, and absorbances are reported at specific wavelengths (A_x where x is the wavelength in nanometers).

Determinations of pH were made using an Orion Research digital pH meter, Model 601A, with either a standard Orion 10 mm combination electrode, a Beckmann 5 mm combination electrode (for radioactive determinations), or an Ingold 3 mm combination electrode (for 5 mm NMR tubes). Electrodes were standardised in the usual manner with standard buffers from MCB Inc. (E. Merck, U.S.A.) at pH 4.00 and pH 7.00. Corrections for measurements in D_2O have not been made, since the permeability of a deuterated sample through the glass

membrane of the electrode is usually as low as the permeability of a deuterated substrate say, into an enzyme active site. Generally these effects are equal, and there is no gain from making the corrections.

The buffers employed throughout this work were as follows. Potassium phosphate (K Phos) buffer was made up as 0.1 M KH_2PO_4 (monobasic) and adjusted to pH 7.6 with solid KOH. Tris Cl^- buffer was made up as 0.1 M Tris (*ex* Sigma, Trizma reagent grade) and adjusted to pH 8.0 with concentrated HCl. Pyrophosphate buffer was made up as 0.1 M $Na_2P_2O_7$ and adjusted to pH 8.0 with NaOH. Deuterated buffers were prepared in an identical manner using anhydrous salts and D_2O.

Accurate volumes were dispensed using Gilson Pipetman automatic pipettes. Models P-5000 (up to 5 cm^3), P-1000 (up to 1 cm^3), P-200 (up to 0.2 cm^3) and P-20 (up to 0.02 cm^3) pipettes were used in this work, and periodic calibration (using water and an accurate balance) ensured their accuracy.

Weighing was routinely performed on a Mettler Model H10T balance, and accurate masses (to 0.1 mg) were obtained using a Cahn ratio electrobalance.

Bacteria cells were harvested using a New Brunswick Model GLE continuous centrifuge. Sonication of cells was performed using a Heat Systems-Ultrasonics Model W200R Sonicator. Centrifugation at low speeds was carried out using a Dupont Sorvall RC-5B refrigerated centrifuge, using either the SS-34, the GSA or the GS-3 rotors. High-speed centrifugation was performed using a Beckman L3-50 Ultracentrifuge equipped with a diffusion pump and refrigeration, using either a Type 35 rotor, or latterly a 45 Ti rotor. Centrifugation

of small samples at low speeds (<5000 rpm) was performed with a Sorvall GLC-1 Benchtop centrifuge.

All enzyme manipulations were carried out at 4°C in a cold room. Gel-filtration was carried out on either G-10, G-25, G-50 or G-100 Sephadex, purchased from Pharmacia Fine Chemicals, Piscataway, N.Y., U.S.A., or Sigma, St. Louis, MO, U.S.A. DEAE-cellulose and Dowex 2 (Cl^-) were obtained from Sigma, and DEAE-Sephacel from Pharmacia. These were treated before use, as described in the appropriate sections. Columns were pumped using an LKB 2132 Microperpex peristaltic pump under the control of either an LKB Ultrorac 2000 fraction collector or an LKB Superrac 2211 fraction collector. Proteins were detected using either LKB Uvicord S 2138 or Uvicord 8301 UV detectors at fixed wavelengths of 280 nm. Concentration of protein samples was performed using Amicon stirred ultrafiltration cells (Model 8400, 350 cm^3; Model 8050, 50 cm^3 and Model M-3, 3 cm^3), using PM-10 Ultrafilters and operating at 75 p.s.i. of nitrogen pressure.

Analytical gel-electrophoresis was carried out using 7.5% polyacrylamide gels. These were prepared using LKB template and gel-former apparatus (see Chapter 8 for details of gel preparation) and electrophoresis performed on an LKB Multiphor horizontal electrophoresis apparatus and an LKB Model 2103 power supply. The buffers and voltages employed are indicated where appropriate. Preparative gel-electrophoresis was performed using a Buchler Poly-prep 2000 preparative vertical column apparatus, using the buffers and voltages indicated.

Analytical and preparative paper chromatography were performed on a Savant Model FP-22A ethylene glycol/water-cooled flat-bed

apparatus, using the buffers and voltages indicated. Whatman No. 1 or 3 MM paper was used, depending on the scale of the separation.

Determinations of radioactivity were made with a Packard PL Prias Tri-carb liquid scintillation counter interfaced with a Texas Instruments Silent 700 data terminal. Samples were counted in a liquid scintillation cocktail of 5-x cm^3 of Aquasol (New England Nuclear) and x cm^3 of sample, where $x < 0.5$ cm^3. To correct the counts (cpm) to the actual number of disintegrations per minute (dpm), efficiency curves were constructed in the usual fashion, employing both the external standards and channels ratio methods.

Lyophilisation was carried out on a Virtis Freeze-mobile 24 freeze-dryer, and for radioactive samples a special freeze-dryer was constructed. This consisted of a vacuum pump and two large dry ice-acetone traps connected in series with wide-bore rubber tubing, all dedicated to the use of radioactive samples and located in a well-ventilated fume cupboard.

Mass spectroscopic determinations were made using a Kratos MS-50 instrument operated by Dr. D. Russell (Texas). All determinations reported here were made in the fast atom bombardment (FAB) mode, and any special techniques are reported where appropriate.

Routine ^1H and ^{13}C NMR spectra were obtained using a variety of instruments: Varian EM-360, Varian FT-80, ~~deol~~ Varian HA-100, Varian XL-100 (all in Edinburgh) and Varian T-60, Varian EM-390 (Texas). The spectra are reported in the following manner: Chemical shift relative to tetramethylsilane (TMS), (multiplicity, coupling constants in Hz, integrated intensity, assignment). Abbreviations used are those commonly employed: s — singlet; d — doublet; t — triplet; q — quartet; m — multiplet; br — broad; c — complex. For ^1H

spectra recorded in deuterium oxide, sodium 3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionate (TSP) was chosen as an internal reference. For ^{13}C spectra recorded in D_2O, p-dioxane (δ_C = 66.5 ppm) was used.

High-field FT NMR studies were carried out using a Bruker WM-300 wide-bore (Edinburgh) or a Bruker WM-300 wide-bore and a Bruker AM-500 (Texas). All these instruments were equipped with a Bruker Aspect 2000 computer. The WM-300 instrument operated at 7.0497 T and the AM-500 at 11.7470 T. Both instruments used deuterium for locking the field, and in the case of precious biological samples where the presence of an internal reference compound was not advisable, and an external reference capillary impaired already poor homogeneity, the procedure of indirect referencing was employed. In this method, the NMR spectrum of a sample containing internal reference compound is obtained at the identical temperature, pH and ionic strength to the biological sample, using deuterium oxide for the lock signal. The biological sample is subsequently examined using the referencing parameters of the reference sample.

Temperatures in the high-field instruments were maintained with a Bruker VT-1000 variable temperature control unit, using cooled gaseous nitrogen. This nitrogen was cooled either by boil-off from a 220 L stainless steel liquid nitrogen dewar which was transferred to the probe by way of dewared low-temperature transfer lines, or by passing dry nitrogen (obtained by boil-off from a remote liquid nitrogen dewar) through a coiled copper tube immersed in an insulated bath containing isopropanol and stirred using an air-driven aluminium motor and paddle assembly. The isopropanol bath was cooled using a Neslab Cryocool CC-80II immersion cooling element. The temperature

of the bath was monitored using a digital thermocouple, and in general at -50°C the probe could be maintained at *ca.* 2°C, measured using the Bruker Cu-Cu/Ni digital thermocouple which was built into the VT-1000. The sample temperature was then monitored *in situ* in the magnet or just outside using a Luxtron fluoroptic thermometer, which employes a fibre-optic temperature probe. Fine adjustments of the temperature were made using the heating element mounted in the probe with a feedback loop to the VT-1000 control unit.

For biological samples, the superconducting magnet was shimmed for each sample, and the probe tuned to 50 Ω for each sample, using a sweep-generator (Wavetek Model 1002), a signal generator (Wavetek Model 3002), an RF bridge (Texscan RCB-3), and a Tektronix 7854 oscilloscope. For ^{3}H NMR, a Bruker pulse generator, pre-amplifier, and ^{3}H and ^{1}H filters were used. It was also necessary to use an extra filter in the ^{3}H channel to ensure minimal breakthrough of the ^{1}H-decoupler channel into the ^{3}H channel. A Bruker ^{1}H probe was suitably adapted for ^{3}H work by Dr. P. E. Fagerness, and the probe tuned to 50 Ω for both ^{3}H and ^{1}H on the decoupler channel (at least twice), for each sample. The probe also had its thermocouple port blocked below the RF coils, to ensure safety in the event of radioactive sample leakage. In the absence of any feedback mechanism, temperature control was monitored in a non-radioactive sample *in situ*, and the temperature extrapolated for the radioactive sample.

CHAPTER 7

SYNTHETIC PROCEDURES

7.1 Hydrolysis of PBG Lactam

PBG lactam (70, *ex* Dr. M. Kajiwara) was recrystallised from dilute ammonia, by careful addition of glacial acetic acid. This lactam (41 mg) was dissolved in 2 M KOH (0.6 cm^3) and left sealed under N_2 in the dark for 70-75 hours.

To this, sufficient glacial HOAc was added to adjust the pH to 4.5, and the mixture cooled to -10°C for 1½ hours. The PBG monohydrate which separated was collected, washed with ice-cold water, and cold acetone (39 mg, 82% yield).

7.2 Synthesis of [5-^{13}C]-ALA

The procedure outlined below represents a typical synthesis, according to the route in Scheme 18.

Preparation of diethyl formylsuccinate (82)

This preparation was found to be somewhat sensitive to scale, too large a scale sacrificing yields considerably.

Sodium hydride (6.3 g, 0.13 mol of 50% dispersion in oil washed with dry pentane) was suspended in 1,2-dimethoxyethane (40 cm^3, freshly distilled *ex* CaH_2) under nitrogen and at -10°C to -5°C with stirring. To this, diethyl succinate (14.4 cm^3, 0.08 mol freshly distilled *ex* CaH_2) was added slowly over about 10 minutes, maintaining the temperature below 0°C. When the addition was complete, the mixture was allowed to warm gradually to room temperature (*ca.* 20 minutes) in order to allow formation of the diethyl succinate anion.

The mixture was then cooled to *ca.* -20°C while ethyl formate (10.5 cm^3, 0.16 mol freshly distilled *ex* CaH_2) was added *dropwise over one hour*, maintaining the temperature below -10°C. When the addition was complete, the mixture was allowed to warm gradually to room temperature and left stirring overnight.

Water (*ca.* 100 cm^3) was added to the yellow-orange mixture, and extracted with ether (3 times). The aqueous layer was then acidified to pH 3 with 2 M HCl, and re-extracted with ether (3 times). The latter ether extracts were combined, dried ($MgSO_4$) and the solvent removed *in vacuo* to give crude product as a yellow oil (on average 14.2 g, 88% yield).

Preparation of ethyl 4-oxobutyrate (83)

Half of the crude product from the previous step (e.g., 7.1 g, 0.04 mol) was placed in a Carius tube with sodium acetate (0.078 g, 0.95 mmol) and water (15 cm^3). Care was taken to cool the tube to -78°C before sealing. It was then heated for 2 hours at 150°C, and again cooled thoroughly to -78°C before carefully opening. The mixture was decanted out and solid NaCl and water (*ca.* 10 cm^3) were added. The aqueous medium was then extracted with ether (3 times), and the extracts combined, dried ($MgSO_4$) and the solvent removed *in vacuo*.

This whole procedure was performed in duplicate with the other half of the diethyl formylsuccinate, to yield crude product (*ca.* 12.94 g). This was fractionally distilled *in vacuo* through a vigreux column, collecting the fraction distilling at 75°C and 0.1 mm Hg, which was found to be pure product (5.4 g, 59%) by ^1H NMR: δ_H ($CDCl_3$ + TMS) = 9.95 (s, 1 H), 4.22 (q, 2 H), 2.75 (m, 4 H), 1.37 (t, 3 H).

Preparation of ethyl 4-acetoxy-4-[^{13}C-cyano]-butyrate (84)

Ethyl 4-oxobutyrate (83, 4.5 g, 0.035 mol) was added dropwise to a solution of sodium metabisulphite (3.29 g, 0.017 mol) in water (23 cm^3) at 0°C with stirring, and the mixture left stirring at 4°C overnight. It was then cooled again to 0°C and $Na^{13}CN$ (1.77 g, 0.035 mol) in water (10 cm^3) was added dropwise, and the mixture stirred at 0°C for a further hour. The solution was then saturated with NaCl, and extracted with toluene (3 times). The organic layers were combined, dried ($MgSO_4$) and the solvent removed *in vacuo*, to afford crude cyanohydrin (4.2 g).

This was dissolved in pyridine (45 cm^3, freshly distilled *ex* CaH_2) and stirred at room temperature while acetic anhydride (4.13 cm^3, 0.044 mol) was added. Stirring was maintained for 20 hours before 6 drops of water were added, and the solvent removed *in vacuo*. The residue was partitioned between 3 M HCl and ether, and the organic layer washed consecutively with water, saturated $NaHCO_3$, brine and then dried ($MgSO_4$). The solvent was removed *in vacuo* to give crude product (*ca.* 4.7 g, 67% yield). ^{13}C NMR: δ_C = 117.1 (s).

Preparation of 5-hydroxy-[6-^{13}C]-2-piperidone (86)

Crude ethyl 4-acetoxy-4-[^{13}C-cyano]-butyrate (84, 4.7 g, 0.024 mol) and 5% palladium on carbon (4.7 g) in ethanol (140 cm^3) and conc. HCl (9.4 cm^3) was hydrogenated at 20°C and 1 atm. until no further uptake of hydrogen (*ca.* 730 cm^3). The solution was filtered through celite, the solvent removed *in vacuo*, and the residue dissolved in anhydrous ethanol. This was treated with HCl gas until 18 g had been absorbed, and then left at room temperature for 60 hours, after which the solvent was removed *in vacuo*, and then treated with fresh

ethanol and re-evaporated twice. The semi-crystalline residue (85) was dried at room temperature overnight (P_2O_5, vacuum desiccator).

The residue was dissolved in anhydrous ethanol, and to this stirred solution, sodium hydride (1.13 g, 0.045 mol, 50% dispersion in oil) was added in one portion. The mixture was stirred at 20°C for 20 minutes and after neutralisation with glacial acetic acid, was filtered, washing the solid with ethanol. Evaporation of the combined ethanol wastes and chromatography of the residue in a polarity gradient of 1:9 $MeOH/CHCl_3$ → pure methanol on alumina (150 g) afforded pure product (0.9 g) after removal of the solvent *in vacuo*. ^1H NMR: δ_H (D_2O) = 4.22 (m, 1 H), 3.51 and 3.25 (each ddd, 1 H), 2.72-2.24 (m, 2 H) and 2.18-1.15 (m, 2 H); δ_C (D_2O) = 48.4 (C-6); IR: ν_{max} (Nujol) = 3210, 3120 (s) and 1635 cm^{-1}. R_f (Solvent III) = 0.45 (alumina).

Preparation of [5-^{13}C]-5-aminolaevulinic acid hydrochloride (45)

This reaction was found to be rather sensitive to scale, and accordingly has usually been performed on a small scale. Thus the foregoing 5-hydroxy-[6-^{13}C]-2-piperidone (86) was divided into two portions.

5-hydroxy-[6-^{13}C]-2-piperidone (0.45 g, 0.004 mol) was dissolved in dimethyl sulphoxide (6 cm^3, freshly distilled *ex* CaH_2) and trifluoroacetic acid (0.16 cm^3) added. This was left for about 5 minutes under N_2 before dicyclohexylcarbodiimide (2.43 g, 0.012 mol) was added in one portion. Dicyclohexylurea immediately precipitated out and the mixture went a light red/orange colour. This was stirred under N_2 at room temperature for 18 hours before a few drops of water and then ethyl acetate (*ca.* 50 cm^3) were added. The ethyl acetate

layer was separated, dried ($MgSO_4$) and the solvent removed *in vacuo*. At this stage the second portion of starting material similarly processed afforded product which was combined with the first.

The combined residue (87, containing DMSO) was treated with water (47 cm^3) and conc. HCl (84 cm^3) and refluxed for 4 hours. The solvent was then removed *in vacuo*, further water added and the mixture heated with a *minimum* of activated charcoal. Evaporation *in vacuo* again yielded a residue from which the DMSO was finally removed using a high-vacuum rotary evaporator. The resultant oil was redissolved in a small amount of water and applied to a column of Amberlite IR-120 (H^+) ion-exchange resin (bed volume *ca.* 150 cm^3), and eluted with 0.01 M HCl. After elution with *ca.* 300 cm^3 0.01 M HCl, the product was eluted with 1 M HCl (*ca.* 600 cm^3). The solvent was removed by lyophilisation and the residue crystallised from ethanol-ether. Cooling to -20°C was necessary to ensure crystallisation. The product was obtained as cream-coloured crystals (0.819 g, 63% yield). ^1H NMR: δ_H (D_2O) = 4.20 (d, J = 143 Hz, 2 H, $CO{\cdot}C\underline{H}_2{\cdot}N$), 2.98 (2 H, t, $C\underline{H}_2{\cdot}CH_2$), 2.79 (2 H, t, $CH_2{\cdot}C\underline{H}_2$). ^{13}C NMR: δ_C (D_2O) = 48.3 (s, C-5). R_f (Solvent I) = 0.3 (silica, sprayed with ninhydrin; 2% w/v in acetone + 1 cm^3 HOAc).

7.3 Synthesis of [4-^{13}C]-ALA

The procedure described follows the route shown in Scheme 19.

Preparation of N-hydroxymethylphthalimide (88)

Phthalimide (10 g) was heated in a Carius tube at 100°C for 2 hours with formaldehyde (6.3 cm^3, 40% w/v) and water (18.7 cm^3).

The tube was carefully shaken every half an hour to ensure that all the phthalimide dissolved.

On cooling (to -78°C) the sample was removed and white crystalline product appeared. The solvent was removed by evaporation *in vacuo*, and the product recrystallised from 1:1 toluene/EtOH, giving white crystals of pure product (9.6 g, 80% yield, mp 147-9°C [lit.[178] 146-8°C]).

Preparation of N-chloromethylphthalimide

N-hydroxymethylphthalimide (88, 9.6 g, 0.054 mol) was suspended in thionyl chloride (*ca.* 50 cm^3, freshly distilled) and stirred for 2 hours at room temperature. The mixture was then refluxed for 30 minutes, after which the excess thionyl chloride was removed by evaporation *in vacuo*. Complete removal was achieved by azeotroping with dry toluene (twice). The crystalline residue was recrystallised from toluene to afford off-white crystals (9.5 g, 90% yield, mp 134-6°C [lit.[178] 132-4°C]).

Preparation of [1-^{13}C]-phthalylacetonitrile (89)

N-chloromethylphthalimide (3.9 g, 0.02 mol) was dissolved in hot p-dioxane (15 cm^3) and this poured into a solution of $K^{13}CN$ (1.32 g, 0.02 mol) in anhydrous MeOH (*ca.* 30 cm^3). The mixture was allowed to stir overnight at room temperature, filtered to remove KCl (washing the solid with additional warm dioxane), and the solvent removed by evaporation *in vacuo*, giving crude product (5.6 g crude).

Preparation of [1-^{13}C]-phthalylglycine

[1-^{13}C]-phthalylacetonitrile (5.6 g crude) was refluxed with 3:1 acetic acid/conc. HCl (60 cm^3) for one hour, and then evaporated

in vacuo. The residue was dissolved in water, and evaporated *in vacuo* two times. The crystalline product was suspended in ice-water (*ca.* 20 cm^3) and collected, washed with more ice-water, and dried, yielding crude product (*ca.* 4 g).

Preparation of [1-^{13}C]-phthalylglycyl chloride (90)

Crude [1-^{13}C]-phthalylglycine (*ca.* 4 g) was suspended in thionyl chloride (*ca.* 25 cm^3) and heated at 40-50°C for 2 hours, and then refluxed to dissolve all the solid (*ca.* 20 minutes). The excess $SOCl_2$ was removed by evaporation *in vacuo*, complete removal being achieved by azeotroping with toluene (twice), yielding slightly yellow crystalline product (3.3 g, 73% from K^{13}CN, mp 83-4°C [lit.[178] 84-85°C]).

Preparation of 1-diazo-3-phthalimido-[2-^{13}C]-propanone (91)

[1-^{13}C]-phthalylglycyl chloride (3.3 g, 0.015 mol) in anhydrous ether (*ca.* 100 cm^3) was added dropwise to 0.5 M alcohol-free diazomethane in anhydrous ether (98 cm^3, 0.05 mol) maintaining the temperature at -10°C throughout the addition. Almost immediately a flocculent precipitate separated, and the mixture was stored at -20°C overnight. Evaporation of the solvent *in vacuo* yielded crude product as a yellow solid.

Preparation of 1-bromo-3-phthalimido-[2-^{13}C]-propanone (92)

Crude 1-diazo-3-phthalimido-[2-^{13}C]-propanone was suspended in glacial acetic acid (*ca.* 60 cm^3) and 48% HBr (5.0 cm^3) added slowly. The solid dissolved with the evolution of N_2. Stirring was continued for 3 hours after which the solvent was removed *in vacuo*, the remaining HBr being eliminated by successive additions and evaporations of water. The residue was suspended in toluene, filtered, and

evaporated to give crystalline product (2.7 g, 64% from [1-^{13}C]-phthalylglycyl chloride).

Preparation of [4-^{13}C]-5-aminolaevulinic acid hydrochloride (76)

Sodium (0.69 g, 0.03 mol) was suspended in anhydrous ether under N_2 and di-*tert*-butylmalonate (7.0 cm^3, 0.03 mol) was added dropwise with stirring. This was left overnight, after which a white precipitate of a sodium derivative of di-*tert*-butylmalonate formed. This was collected and rapidly suspended in DMF (*ca.* 50 cm^3, freshly distilled *ex* CaH_2), and added to a stirring solution of 1-bromo-3-phthalimido-[2-^{13}C]-propanone (92, 2.7 g, 0.015 mol) in DMF. The mixture was stirred for 24 hours at room temperature, after which the solvent was removed with a high-vacuum rotary evaporator. The reddish semi-crystalline residue (93) was dissolved in dioxane (*ca.* 50 cm^3), saturated with HCl gas at 0°C, and kept at room temperature overnight. After removal of the solvent *in vacuo*, the residue was dissolved in 6 M HCl (*ca.* 60 cm^3) and refluxed for *ca.* 5 hours.

The solvent was removed by lyophilisation, and the residue re-dissolved in water and applied to a column (bed volume *ca.* 150 cm^3) of Amberlite IR-120 (H^+), eluting with 0.05 M HCl (600 cm^3). The product was eluted with 1 M HCl (*ca.* 600 cm^3), and the solvent removed by lyophilisation. The residue from this was crystallised from ethanol-ether, cooling to -20°C overnight. The product was obtained as cream-coloured crystals (1.411 g, 57% yield). ^{13}C NMR: δ_C (D_2O) = 203.9, (s, C-4). R_f (Solvent I) = 0.3 (silica, sprayed with ninhydrin, 2% w/v in acetone + 1 cm^3 HOAc).

7.4 Synthesis of [^{2}H]-ALA

Preparation of 90% D at C-5

ALA hydrochloride (*ex* Sigma, 1.0 g, 0.006 mol) was dissolved in D_2O (50 cm^3, 97.98%, *ex* Cambridge Isotopes, MA, U.S.A.) and the pH adjusted with anhydrous Na_2CO_3 to 8.1 (uncorrected) initially. The solution was left at room temperature sealed in the dark, and approximately every half an hour the pH readjusted to 8.0, for a total of 165 minutes. By this time the mixture had become yellow, and the pH was adjusted to 1.5 with 20% DCl in D_2O (100%, *ex* Aldrich), and the mixture lyophilised. The residue was suspended in absolute ethanol, filtered to remove NaCl, and the solvent removed by evaporation *in vacuo*. The product was then crystallised from ethanol-ether to yield a light brown solid (0.495 g, 49% yield). ^{1}H NMR: δ_H (D_2O) = 4.20 (s, residual ^{1}H at C-5), 2.98 (2 H, br, CH_2CH_2), 2.79 (2 H, t, CH_2 CH_2). ^{2}H NMR: δ_D (H_2O) = 4.2 (s, 2 H, C-5), 2.98 (<2 H, br, CH_2CH_2). R_f (Solvent I) = 0.3 (silica). m/e: MH^+ 170.5 (DCl salt).

Preparation of 10% D at C-5 (Pilot study for [^{3}H]-ALA synthesis)

ALA hydrochloride (15 mg) was dissolved in D_2O (99%, 31 µL) and triethylamine (24.48 µL, distilled *ex* KOH) was added. The mixture was shaken and allowed to stand in the dark for exactly 10 minutes before adding 0.1 M HCl (*ca.* 5 cm^3) and lyophilising.

This was dissolved in D_2O (99%, 0.5 cm^3) for analysis by ^{1}H NMR: δ_H (D_2O) = 4.20 (s, 85% of resonance at 2.79 ppm), 2.98 (2 H, sl. br, t, CH_2CH_2), 2.79 (2 H, t, CH_2 CH_2). R_f (Solvent I) = 0.3 (silica).

Preparation of [5-^{13}C,^{2}H]-ALA

[5-^{13}C]-ALA hydrochloride (15 mg, see Section 7.2) was dissolved in D_2O (1.03 cm^3) and triethylamine (24.48 µL, distilled *ex* KOH) was

added. The mixture was shaken and allowed to stand in the dark for exactly 165 minutes before adjusting to pH 1.5 with 40% DCl in 100% D_2O (Aldrich). Lyophilisation yielded crude product which was used directly for PBG synthesis.

7.5 Synthesis of [^{3}H]-ALA

Preparation of [^{13}C,^{3}H]-ALA (custom preparation by New England Nuclear)

[5-^{13}C]-ALA hydrochloride (15 mg, see Section 7.2) was dissolved in 100% T_2O (100 Ci, 24.48 µL) at room temperature in subdued light. Triethylamine (24.48 µL, freshly distilled *ex* KOH) was added, and the mixture stirred gently for exactly 10 minutes before adding excess 0.1 M HCl (5 cm^3), and immediately lyophilising (in subdued light).

All labile tritium was then removed by cycles of addition of 0.1 M HCl and lyophilisation. The crude product (286 mCi, *ca.* 3.2 Ci mmol^{-1}) was dissolved in 0.1 M HCl (10 cm^3) and shipped directly. It was used for PBG synthesis immediately upon arrival.

Preparation of [3,4-$^3H_4$]-ALA

ALA hydrochloride (15 mg) was treated exactly as above except that stirring was continued for 360 minutes prior to the addition of excess 0.1 M HCl. The crude product (3,122 mCi, 34.8 Ci mmol^{-1}) was dissolved in 0.1 M HCl (5 cm^3) and shipped directly, packed in dry ice.

7.6 Synthesis of [^{13}C]-, [^{14}C]-, [^{2}H]- and [^{3}H]-PBG

Preparation of [^{13}C]- and [^{14}C]-PBG

This preparation was also used for making unlabelled PBG.

A solution of $MgCl_2$ (12.2 mg) and L-cysteine free base (36.3 mg) in buffer (19 cm^3, 0.1 M KH_2PO_4/0.1 M $NaHCO_3$ adjusted to pH 8.0 with KOH) was degassed by bubbling N_2 through it for 15 minutes. Partially purified ALA dehydratase (10 cm^3, 205.5 U, see Chapter 8) was added and the solution pre-incubated at 37°C for *ca.* 5 minutes.

[^{13}C]-ALA hydrochloride (50 mg) dissolved in the same buffer (1.0 cm^3) was added, the pH adjusted to 7.9, N_2 blown over the surface of the solution, and the mixture incubated in a sealed vessel in the dark at 37°C for 90 minutes. The reaction was monitored by removing an aliquot at the start of the incubation, and assaying 10 µL samples every 10 minutes by addition to 3 cm^3 of modified Ehrlich's reagent (see Chapter 8). At the end of the reaction $CuSO_4$ solution (1.3 cm^3, 25 % w/v) was added, and the mixture was cooled prior to centrifugation at 35,000 g (10 minutes). The supernatant was decanted off and lyophilised overnight.

The crude PBG was then dissolved in water (18 cm^3), adjusted to pH 6.0 with HOAc, and applied to a column of Dowex 2 acetate ion-exchange resin (bed volume 35 cm^3, 7 x 2.5 cm). The Dowex 2 acetate was prepared by washing commercial resin (in the chloride form) in a sintered funnel with 1 M NaOH until no more chloride (assayed with 1% w/v $AgNO_3$ solution) eluted (about 3 L for 150 cm^3 resin). It was then washed to neutrality with distilled water, and then with 1 M HOAc (*ca.* 100 cm^3), and again with 1-200 cm^3 distilled water.

After the crude PBG solution was adsorbed onto the resin, the washings from the flask were also applied to the column, and then the column washed with water (75 cm^3), eluting at 40 cm^3 hr^{-1} and collecting 25 cm^3 fractions. The column was then allowed to go just dry and then topped to 1 cm with part of 25 cm^3 of 1 M HOAc. When

this was adsorbed, the rest of the 1 M HOAc was applied, collecting 5 cm^3 fractions. After the 25 cm^3 1 M HOAc was adsorbed, elution was continued with 0.2 M HOAc. Fractions 8-12 contained pure PBG which was obtained as a fluffy white solid after lyophilisation (34 mg, 79%). ^1H NMR (D_2O buffer, pH 8.0): δ_H (PBG) = 6.69 (s, 1 H, C-2), 4.16 (s, 2 H, C-11), 3.41 (s, 2 H, C-9), 2.67 (t, J = 7.8 Hz, 2 H, C-6), 2.41 (t, J = 7.8 Hz, 2 H, C-7).

δ_H ([2,11-^{13}C]-PBG) = 6.69 (d, ^1J (^1H-^{13}C) = 183.5 Hz, 1 H, C-2), 4.16 (d, ^1J (^1H-^{13}C) = 144.2 Hz, 2 H, C-11), 3.41 (s, 2 H, C-9), 2.67 (br, m, 2 H, C-6), 2.41 (t, 2 H, C-7).

δ_H ([3,5-^{13}C]-PBG) = 6.69 (t, ^2J (^1H-^{13}C) = 6.8 Hz, 1 H, C-2), 4.16 (d, ^2J (^1H-^{13}C) = 4.8 Hz, 2 H, C-11), 3.41 (s, 2 H, C-9), 2.67 (q, ^2J (^1H-^{13}C) = 6.8 Hz, 2 H, C-6), 2.41 (q, ^3J (^1H-^{13}C) = 3.3 Hz, 2 H, C-7).

^{13}C NMR data are reported in Chapters 4 and 9.

Preparation of [^2H]-PBG

Partially purified dehydratase (225 U assayed at pH 7.0) dissolved in phosphate buffer (8.1 cm^3, 0.1 M KPhos pH 7.0 + 5 mM β-mercaptoethanol) containing $MgCl_2$ (3.25 mg) and L-cysteine (9.68 mg) was pre-incubated at 37°C for 5 minutes. [^2H]-ALA (10 mg) dissolved in the same buffer (1.0 cm^3) was added and the mixture incubated at 37°C for exactly 10 minutes, before adding $CuSO_4$ solution (430 µL, 25% w/v). After centrifugation at 35,000 g (10 minutes) the supernatant obtained was adjusted to pH 6.0 and applied to a Dowex 2 acetate column (12 cm^3 bed volume, 1.2 x 16 cm). Elution was carried out initially with distilled water, collecting 3 x 8.6 cm^3 fractions. 1 M HOAc (8.57 cm^3) was then applied to the column,

collecting 1.7 cm^3 fractions, before continuing with 0.2 M HOAc. Fractions 10-16 contained PBG which after lyophilisation yielded pure product (2mg, 23% yield). ^1H NMR exactly as unlabelled PBG except integrals different according to the extent of deuteration in the ALA (see Chapter 4).

Preparation of [2,11-^{13}C,^2H]-PBG

Partially purified dehydratase (1160 U assayed at pH 7.0) dissolved in buffer (25 cm^3, 0.05 M KPhos., 99% D_2O, pH 7.0) containing $MgCl_2$ (8.13 mg) and L-cysteine (24.2 mg) was pre-incubated at 37°C for 5 minutes. [^{13}C,^2H]-ALA (crude preparation described above) dissolved in the same buffer (2.0 cm^3, adjusted to pH 7.2) was added, and incubation continued for exactly 4 minutes at 37°C. The reaction was stopped with $CuSO_4$ solution (0.8 cm^3, 25% w/v) and after centrifugation (35,000 g, 10 minutes), the supernatant was adjusted to pH 6.0. Further precipitation took place, so another centrifugation was necessary, before applying to a Dowex 2 acetate column as in the previous preparation. The PBG solution (in fractions 9-15) was lyophilised, affording pure product (3.25 mg, 25% yield). ^1H NMR: δ_H (D_2O) = 6.69 (br, s, <1 H, C-2), 4.16 (br, s, <2 H, C-11), 3.41 (s, 2 H, C-9), 2.67 (br, m, <2 H, C-6), 2.41 (t, 2 H, C-7).

^{13}C NMR: δ_C (D_2O) = 116.1 (s, C-2) and 34.4 (t, C-11).

Preparation of [^{13}C,^3H]-PBG

Partially purified dehydratase (1512 U, assayed at pH 7.0) dissolved in buffer (13.3 cm^3, 0.1 M KPhos, pH 7.0) containing $MgCl_2$ (8.13 mg) and L-cysteine (24.2 mg) was pre-incubated at 37°C for 5 minutes. [^{13}C,^3H]-ALA (286 mCi, *ca.* 3.2 Ci $mmol^{-1}$) dissolved

in 0.1 M HCl (10 cm^3) was adjusted to pH 7.0 with 3.0 M NaOH, measuring the pH directly using a dedicated radioactive pH electrode, and rapidly added to the enzyme. The mixture was incubated at 37°C for exactly 3.5 minutes prior to the addition of $CuSO_4$ solution (0.8 cm^3, 25% w/v). After centrifugation (35,000 g, 10 minutes) the supernatant was decanted off and adjusted to pH 6.0 Since there was further precipitation, another centrifugation step was necessary, and the supernatant applied to a Dowex 2 acetate column, eluting as in the previous preparation. The product eluted off in fractions 11-16, and lyophilisation on a tritium freeze-dryer yielded pure PBG acetate (3.63 mg, 28% yield, 43.76 mCi, 3.5 Ci $mmol^{-1}$). ^3H NMR: δ_T (D_2O) = 6.69 (d, ^1J (^3H-^{13}C) = 195.3 Hz, 1 H, C-2), 4.16 (d, ^1J (^3H-^{13}C) = 152.1 Hz, 2 H, C-11), 3.41 (br, m, 2 H, C-9). ^1H NMR: δ_H (D_2O) = 6.69 (d, ^1J (^1H-^{13}C) = 183.5 Hz, 1 H, C-2), 4.16 (d, ^1J (^1H-^{13}C) = 144.2 Hz, 2 H, C-11), 3.41 (s, 2 H, C-9), 2.67 (br, m, 2 H, C-6), 2.41 (t, 2 H, C-7).

^{13}C NMR: δ_C (D_2O) = 116.1 (s, C-2) and 34.4 (s, C-11).

Preparation of [^3H]-PBG

Partially purified dehydratase (1220 U, assayed at pH 7.0) dissolved in buffer (19.9 cm^3, 0.1 M KPhos, pH 7.0) was pre-incubated for 5 minutes at 37°C. [^3H]-ALA (3,122 mCi, 34.8 Ci $mmol^{-1}$) dissolved in 0.1 M HCl (5 cm^3) was transferred carefully from two vials using a Pasteur pipette into a 10 cm^3 vial containing a small magnetic stirrer-bar, $MgCl_2$ (8.13 mg) and L-cysteine (24.2 mg). The pH was adjusted to pH 7.0, measuring the pH directly, and rapidly added to the enzyme. Incubation at 37°C was carried out for 4.0 minutes in a stoppered flask, whereupon $CuSO_4$ solution (0.8 cm^3, 25% w/v) was

added. After centrifugation (35,000 g, 10 minutes), the supernatant was adjusted to pH 6.0, and applied to a Dowex 2 acetate column and eluted as in the previous preparations. The product eluted in fractions 8-13, and after lyophilisation on a tritium freeze-dryer, yielded impure [^{3}H]-PBG. Since ^{3}H NMR revealed impurities, the sample was applied to another Dowex 2 acetate column (bed volume 5 cm^3, 6 x 1.2 cm), washed with distilled water (3 x 5 cm^3) and then 1 M HOAc (3.57 cm^3), collecting 0.7 cm^3 fractions. Elution was continued at 40 cm^3 hr^{-1} with 0.2 M HOAc and pure PBG eluted in fractions 12-17. Lyophilisation afforded pure product (250 µg, 1.9% yield from unlabelled ALA, 144 mCi, 165.9 Ci mmol^{-1}). ^{3}H NMR: δ_T (D_2O) = 6.69 (s, 1 H, C-2), 4.16 (s, 1-2 H, C-11), 2.59 (s, 2 H, C-6 -CH$\underline{T}$) and 2.56 (s, 2 H, C-6 -C$\underline{T}_2$). ^{1}H NMR: δ_H (D_2O) = 6.69 (br, s, <1 H, C-2), 4.16 (br, s, <2 H, C-11), 3.41 (s, 2 H, C-9), 2.67 (br, m, <2 H, C-6), 2.41 (t, 2 H, C-7).

7.7 Preparation of Porphyrins for HPLC Analysis

The end products from enzymic experiments with deaminase and cosynthetase were generally diluted with buffer (*ca.* 30 cm^3, 0.01 M KPhos, pH 7.5) and oxidised with iodine solution (250 µL, 0.2 N I_2 in 0.2 N KI). The mixture was then lyophilised, and stirred in 5% conc. H_2SO_4/anhydrous MeOH (75 cm^3) for 48 hours. Water (*ca.* 50 cm^3) was then added and the pH brought to neutrality (solid $NaHCO_3$). Extraction with $CHCl_3$ (2 times), and evaporation of the solvent *in vacuo* yielded crude uroporphyrin I/III. This was analysed by TLC (Solvent II) on silica, and generally it was necessary to chromatograph the crude product on a preparative silica plate (2 mm), eluting with Solvent II. Extraction of the silica with $CHCl_3$ and evaporation of

the solvent *in vacuo* then yielded pure uroporphyrin for HPLC isomer analysis.

CHAPTER 8

ENZYMOLOGICAL PROCEDURES

8.1 Growth of *Rhodopseudomonas spheroides* (by S. Miller, K. Mackenzie and C. McCullar)

Rhodopseudomonas spheroides as lyophilised cultures (ATCC 17023) were revived in the medium (10 cm^3) outlined in Table 8.1, which is essentially Lascelles' Medium S.[223] The medium was previously autoclaved in a steriliser at 121°C for 25 minutes at 15 p.s.i. The revived cells were grown anaerobically at *ca.* 30°C under illumination from electric lights, passed through glass jars filled with water for cooling, for 3-4 days or until a healthy-looking culture was obtained. They were then aseptically transferred to agar slants (20 cm^3, 15.0 gL^{-1} agar) and grown in anaerobic jars in the light for 4-5 days. The cultures were used to inoculate 1 L conical flasks containing the above medium (4 slants per flask, flasks completely filled), and the flasks plugged with cotton wool, aluminium foil and sealed with Parafilm. They were grown semi-anaerobically in the light for 3 days, before being aseptically transferred to a 6 L flask containing the same medium (one 1 L flask per 6 L flask, flasks completely filled). These were grown semi-anaerobically in the light turning the flasks one-quarter turn every day, until cells looked green-brown (usually 5 days). It should be noted that the growth times were assessed according to the appearance of the bacteria.

Cells were then harvested using a continuous centrifuge (usually 10 gL^{-1} wet cells), and the cells stored at -20°C until use.

All subsequent manipulations with the cells and enzymes isolated from them were performed at 4°C in a cold room, except where stated otherwise.

TABLE 8.1. Growth medium for *R. spheroides*.

Chemical	Mass/gL^{-1}
Yeast extract	2.0
Sodium glutamate monohydrate	3.8
DL-malic acid	2.7[a]
KH_2PO_4	0.5
K_2HPO_4	0.5
$(NH_4)_2HPO_4$	0.8
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2$	0.04
Nicotinic acid	0.0010
Thiamine HCl	0.0010
Biotin	0.0010
$MnSO_4 \cdot 5H_2O$	0.0012
Ferric citrate	0.00245[b]

[a]Added separately after adjusting to *ca.* pH 7 with NaOH (*ca.* 1.6 g). Final medium adjusted to pH 6.8-7.0 with NaOH.

[b]Dissolved in water prior to addition.

8.2 Isolation and Purification of ALA Dehydratase

Assay

Dehydratase activity was assayed in the following manner. Buffer (1.0 cm^3, 0.1 M KPhos/0.1 M $NaHCO_3$ adjusted to pH 8.0 with KOH), $MgCl_2$ (0.5 cm^3, 0.01 M), L-cysteine (0.25 cm^3, 0.1 M freshly adjusted to pH 7.0 with KOH), water (0.725 cm^3 − x, where x = volume of enzyme solution) and the enzyme (10-50 µL) were preincubated for 5 minutes at 37°C. ALA·HCl (0.025 cm^3, 0.2 M [33.6 mg cm^{-3}] in water) was added, and the incubation continued at 37°C with gentle shaking for 10 minutes. $CuSO_4$ solution (0.1 cm^3, 25% w/v) is then added and the tubes allowed to cool. After centrifugation (*ca.* 2000 g, 5 minutes), 1.5 cm^3 was carefully removed and reacted with 1.5 cm^3 of modified Ehrlich's reagent[181] (0.5 g p-dimethylamino-benzaldehyde in glacial HOAc + 4 cm^3 70% $HClO_4$ and made up to 25 cm^3 with glacial HOAc). After exactly 15 minutes, A_{553} read, and activity calculated using

$$\text{Activity/µmol PBG produced } hr^{-1}\ cm^{-3} = \frac{A_{553} \times 5200}{\text{Vol (µL)} \times t\ \text{(mins)}}.$$

This equation uses ε = 57,700 mol L^{-1} cm^{-1} for [PBG].[180]

Isolation and Purification

In a typical preparation, *R. spheroides* (80-100 g wet cells) were suspended in buffer (300 cm^3, 0.05 M KPhos pH 7.6) and sonicated in 100 cm^3 batches for a total time of 9 minutes in 3 minute bursts with 3 minutes between each burst for cooling. The cell suspension was sonicated in a cooling bath, cooled with circulating ethylene

glycol/water at -10°C. In this way the temperature of the cell suspension could be maintained below +10°C throughout the process.

The homogenate was then centrifuged (35,000 g, 12 minutes) to remove cell debris, and the supernatant carefully decanted. This was then centrifuged at >60,000 g (120 minutes), and the supernatant carefully decanted, taking precautions to avoid the dark-coloured syrupy pigmented protein near the bottom of each centrifuge tube. The supernatant (*ca.* 150 cm^3) was made 10 mM in β-mercaptoethanol, and brought to 20% saturation with ammonium sulphate (15.9 g) with stirring. This was allowed to stir for 30 minutes before centrifugation at 20,000 g (15 minutes). The pellet was discarded and the supernatant brought to 35% saturation with ammonium sulphate (13.2 g). After stirring for 30 minutes, the mixture was centrifuged at 20,000 g (15 minutes), and the pellet containing dehydratase retained. On occasions the supernatant was treated further with ammonium sulphate (19.8 g) to bring it to 55% saturation. After 30 minutes, centrifugation (20,000 g, 15 minutes) yielded a pellet of crude deaminase-cosynthetase which could be used in deaminase preparations (see Section 8.3).

The pellet of dehydratase was dissolved in buffer (*ca.* 20 cm^3, 0.01 M Tris Cl^- pH 8.0) and applied to a freshly equilibrated G-100 Sephadex column (bed volume 1000 cm^3, 31 X 6.5 cm). The G-100 Sephadex was prepared by swelling out *ca.* 58 g of dry beads (40-120 μm particle size) in buffer (2 L, 0.01 M Tris Cl^- pH 8.0) heated on a boiling water bath for 5 hours, stirring occasionally. The column was then packed carefully, and washed with buffer (2 L, 0.01 M Tris Cl^- pH 8.0 + 0.01 M KCl + 5 mM β-mercaptoethanol) with no greater than 20 cm head of pressure.

The G-100 Sephadex column was eluted with 0.01 M Tris Cl^- pH 8.0, 0.01 M KCl, 5 mM β-mercaptoethanol at 30 cm^3 hr^{-1}, collecting 10 cm^3 fractions. Fractions 10-26 were found to contain the active enzyme (*ca.* 770 U) which was pooled and concentrated on a PM-10 Ultrafilter in an Amicon concentrator to a volume of *ca.* 30 cm^3.

The concentrate was applied to a freshly equilibrated DEAE-cellulose column (bed volume *ca.* 500 cm^3, 14 X 6.5 cm). The DEAE-cellulose was prepared by pouring the commercial powder (*ca.* 50 g, 0.88 meq g^{-1}, medium mesh, *ex* Sigma) onto 1 M NaOH (1 L) in a 2 L beaker, and allowing to settle. The excess liquid was decanted off, and the remainder was washed with additional 1 M NaOH (1 L) in a sintered glass funnel, followed with distilled water to neutrality (*ca.* 3 L). The resin was then washed with 1 M HCl (1 L) and again with distilled water to neutrality (*ca.* 2 L). Finally it was washed with buffer (400 cm^3, 0.1 M Tris Cl^- pH 8.0), resuspended in fresh buffer, and left to stand overnight. The column was then packed under *ca.* 2 p.s.i. of nitrogen pressure, and then equilibrated over two nights with (i) 0.01 M Tris Cl^- pH 8.0 buffer (1 L); and (ii) 0.01 M Tris Cl^- pH 8.0 buffer + 5 mM β-mercaptoethanol (2 L).

The DEAE-cellulose column was eluted with a linear salt gradient of 0.05 M KCl (500 cm^3, in 0.01 M Tris Cl^- pH 8.0 + 5 mM β-mercaptoethanol) to 0.5 M KCl (500 cm^3, in 0.01 M Tris Cl^- pH 8.0 + 5 mM β-mercaptoethanol) at 30 cm^3 hr^{-1}, collecting 15 cm^3 fractions. Fractions 38-55 contained the bulk of the enzyme (410 U) which was pooled and concentrated on a PM-10 Ultrafilter to *ca.* 20 cm^3 for use in PBG syntheses.

8.3 Isolation and Purification of PBG Deaminase

Assay

Deaminase activity was routinely assayed by consumption of PBG.[76] A stock solution of PBG (2 mg cm^{-3} dissolved in 0.1 M Tris Cl^- pH 8.0), which could be stored in a freezer for several months, was diluted (75 µL stock PBG + 925 µL 0.1 M Tris Cl^- pH 8.0) and 0.1 cm^3 of this added to 10 µL deaminase which had been allowed to preincubate for 5 minutes at 37°C. Two non-enzymic blanks were also included, and incubation continued at 37°C for 10 minutes. The reaction was then stopped by addition of diluted Ehrlich's reagent (1:0.9 modified Ehrlich's reagent [see Section 8.2]:water). After exactly 15 minutes, A_{553} read, and the activity calculated using the difference between the PBG blank and the enzyme:

$$\text{Activity/µmol PBG consumed } hr^{-1}\ cm^{-3} = 29.7 \times (A_{553}\ (\text{blank}) - A_{553}\ (\text{enzyme}))$$

for 10 µL deaminase and 10 minute incubation. This equation uses ε = 60,606 mol L^{-1} cm^{-1} for [PBG], i.e. when A_{553} = 1.0, [PBG] = 49.5 nmol.

The deaminase activity can also be assayed by production of uroporphyrin I.[76] The assay consitions are exactly the same except that the reaction was stopped by the addition of iodine (2 µL, 0.2 N I_2 in 0.2 N KI) in buffer (0.1 cm^3, KPhos pH 7.5), and making up to 3 cm^3 with buffer (0.1 M Tris Cl^- pH 8.0). After leaving for 30 minutes, a few crystals of $NaHSO_3$ were added, and the A_{395} read (ε = 224,000 mol L^{-1} cm^{-1}). Alternatively, the reaction was stopped by the addition of HCl (2.5 cm^3, 5% w/v), leaving for 30 minutes,

and the A_{405} read (ε = 496,000 mol L^{-1} cm^{-1}). 1 U of activity is defined as forming 1 nmol uroporphyrin hr^{-1}.

Protein concentration was routinely assayed by the method of Warburg and Christian,[224,225] using the relationship

$$[\text{protein}]/\text{mg cm}^{-3} = 1.55\ A_{280} - 0.76\ A_{260}.$$

Isolation and Purification

For this section the purification of a large batch of deaminase suitable for enzyme-substrate NMR investigations is described.

R. spheroides (350-400 g) was suspended in buffer (700-800 cm^3, 0.05 M KPhos pH 7.6) and sonicated in 100 cm^3 batches for a total time of 9 minutes in 3 minute bursts with 3 minutes between each burst for cooling. The temperature was maintained using a cooling bath with circulating ethylene glycol-water at -20°C. In this way the temperature of the cell suspension was kept below +10°C.

The homogenate was then centrifuged at 35,000 g (12 minutes) and the supernatant carefully decanted. This was then heated to 60°C for 15 minutes in 150 cm^3 batches, before cooling in ice and centrifuging at 100-150,000 g (60 minutes). The supernatant (*ca.* 810 cm^3) from this was made 10 mM in β-mercaptoethanol (567 μL) and brought to 40% saturation with ammonium sulphate (183.06 g) with stirring. After 30 minutes, the mixture was centrifuged (20,000 g, 15 minutes) and the pellet containing crude dehydratase retained for large-scale preparations. The supernatant was then brought to 55% saturation in ammonium sulphate (72.09 g) and stirred for 30 minutes. After centrifugation (20,000 g, 15 minutes) the pellet of deaminase was dissolved in 0.01 M Tris Cl^- pH 8.0 buffer (*ca.* 20 cm^3)

and dialysed against two changes of buffer (2 L, 0.01 M Tris Cl^- pH 8.0 + 5 mM β-mercaptoethanol).

The dialysate (*ca.* 50 cm^3) was then applied to a freshly equilibrated DEAE-Sephacel column (500 cm^3 bed volume, 14 X 6.5 cm). The DEAE-Sephacel was prepared in an exactly analogous manner to the DEAE-cellulose as described in Section 8.3. Elution was carried out with a linear salt gradient of 0.05 M KCl (750 cm^3, in 0.01 M Tris Cl^- pH 8.0 + 5 mM β-mercaptoethanol) to 0.6 M KCl (750 cm^3, in 0.01 M Tris Cl^- pH 8.0 + 5 mM β-mercaptoethanol) at 30 cm^3 hr^{-1}, and collecting 15 cm^3 fractions. Typically fractions 61-73 contained active enzyme (*ca.* 1800 U), which was pooled and concentrated on a PM-10 Ultrafilter to *ca.* 30 cm^3.

At this stage the whole process was repeated with another batch of cells, and the pooled enzyme from DEAE-Sephacel chromatography was combined with the first batch, and further concentrated (PM-10) to *ca.* 30 cm^3. This pooled enzyme was applied to a freshly equilibrated G-100 Sephadex column, and eluted with 0.01 M Tris Cl^- pH 8.0 + 5 mM β-mercaptoethanol at 30 cm^3 hr^{-1}, collecting 10 cm^3 fractions. Generally fractions 45-60 contained active enzyme (*ca.* 3200 U) which was pooled and concentrated (PM-10) to *ca.* 15 cm^3.

Another six batches of cells were processed in pairs in an identical manner, furnishing after gel-filtration, four 15 cm^3 samples of the enzyme (total *ca.* 12,000 U) which were pooled and concentrated (PM-10) to *ca.* 20 cm^3. This was then heated at 60°C for 15 minutes to ensure complete inactivation of any residual cosynthetase. No precipitation was observed, so the enzyme was rapidly cooled in ice, and applied to another freshly equilibrated G-100 Sephadex column. Elution was performed with 0.01 M Tris Cl^-

pH 8.0 + 5 mM β-mercaptoethanol at 30 cm^3 hr^{-1}, collecting 10 cm^3 fractions. Fractions 46-62 contained active enzyme and were pooled and concentrated (PM-10) to *ca.* 100 cm^3 (total *ca.* 8000 U, 20 U mg^{-1}).

8.4 Isolation and Purification of Uro'gen III Cosynthetase (by Dr. R. C. Davies)

Assay

Cosynthetase (10 µL, *ca.* 1 U cm^{-3}) and pure deaminase (25 µL, 10 U cm^{-3}) were preincubated together at 37°C for 3 minutes. PBG solution (0.1 cm^3, diluted PBG stock as in deaminase assay) was added, and the reaction stopped after exactly 1 minute by cooling to -78°C in dry-ice/acetone, and then stored in ice. Fresh benzoquinone solution (0.1 cm^3, 1 mg cm^{-3} MeOH) was added as soon as possible, and the mixture left in the dark for 30 minutes. Then saturated $NaHSO_3$ (0.1 cm^3) was added at 0°C for 5 minutes before adding either buffer (3 cm^3, 0.1 M Tris Cl^- pH 8.2) or HCl (2.5 cm^3, 5% v/v). The Soret band was recorded over the range 450-350 nm. For a blank the A_{max} = 0, for deaminase A_{max} ≃ 0.03 and for deaminase + cosynthetase A_{max} ≃ 0.17 at the most. The difference between the deaminase alone and deaminase + cosynthetase gave the activity calculated using ε_{395} (Tris buffer) = 224,000 mol L^{-1} cm^{-1} or ε_{405} (HCl) = 496,000 mol L^{-1} cm^{-1}. 1 U of activity is defined as forming 1 nmol uroporphyrin hr^{-1}, or 1 U forms 1 µmol uroporphyrin hr^{-1}.[98]

Isolation and Purification

R. spheroides (200 g wet cells) were suspended in buffer (400 cm^3, 0.5 M KPhos pH 7.5) and sonicated as for deaminase. After centrifugation at 35,000 g (30 minutes) the supernatant was decanted off and

made 10 mM in β-mercaptoethanol, and brought to 30% saturation with ammonium sulphate. The mixture was then allowed to stir for 30 minutes, and centrifuged (20,000 g, 15 minutes) and the pellet discarded. The supernatant was brought to 55% saturation with ammonium sulphate, stirred for 30 minutes, and centrifuged (20,000 g, 15 minutes). The pellet was dissolved in a minimum volume of buffer (*ca.* 50 cm^3, 0.01 M KPhos pH 7.5) and dialysed overnight against several changes of buffer (0.01 M KPhos pH 7.5 + 1 mM β-mercaptoethanol). The dialysate was then centrifuged at 100-150,000 g for *ca.* 5 hours, and the brown pellet discarded.

The supernatant was applied to a freshly equilibrated hydroxyapatite column (5 X 10 cm) eluting with 0.01 M KPhos pH 6.8 + 1 mM β-mercaptoethanol at 30 cm^3 hr^{-1}, collecting 10 cm^3 fractions. The deaminase present was retained, whereas the cosynthetase was not.

The pooled cosynthetase activity was concentrated (PM-10) to *ca.* 30 cm^3 and applied to a freshly equilibrated DEAE-cellulose column (5 X 12 cm), eluting with a salt gradient of 0.05 M KCl (300 cm^3, in 0.01 M KPhos pH 7.5 + 2 mM β-mercaptoethanol) to 0.4 M (300 cm^3, in 0.01 M KPhos pH 7.5 + 2 mM β-mercaptoethanol) at 30 cm^3 hr^{-1} collecting 10 cm^3 fractions. The cosynthetase activity eluted just before deaminase.

The active cosynthetase fractions were then pooled and concentrated (PM-10) to *ca.* 20 cm^3, and applied to a freshly equilibrated G-100 Sephadex column, eluting with 0.05 M KPhos pH 7.5 + 2 mM β-mercaptoethanol. The active fractions (*ca.* 500 U) were then pooled and concentrated (PM-10) to *ca.* 2 cm^3.

It should be noted that all columns were monitored for both deaminase activity and cosynthetase activity.

8.5 Preliminary Studies on PBG-Deaminase Complexes (with Dr. G. Burton)

For the generation of PBG-deaminase complexes using defined stoicheiometries of PBG and deaminase, either our HPMD (see Section 8.6) or sequential mixing was employed, and the resultant complexes analysed by gel-electrophoresis. The gels were cast using the LKB Multiphor system of gel-former and glass with a rubber gasket, using the following solutions.

Stock Acrylamide

Acrylamide (*ex* Sigma)	22.2 g
N,N'-methylene-bis-acrylamide (*ex* Sigma)	0.6 g
Water	to 100 cm^3

Filtered through a fine sinter to remove solid impurities, and stored at 4°C.

Stock Buffer

Glycine	75 g
Sodium azide	2.5 g
Tris	*ca.* 100 g (to pH 8.9)
Water	to 5 L

7.5% Analytical Polyacrylamide Gel

Stock buffer	33.0 cm^3
Stock acrylamide	22.2 cm^3
Water	7.5 cm^3

These were all mixed and vacuum-degassed for *ca.* 5 minutes before adding rapidly and simultaneously:

Ammonium persulphate (*ex* Sigma) (150 mg/10 cm^3)	3.2 cm^3
N,N,N',N'-tetramethylethylene diamine (TEMED) (*ex* Sigma)	0.1 cm^3

The mixture was then quickly poured into the template, left for 30 minutes to polymerise and then the rubber gasket was removed. The gel was cooled to 4°C for *ca.* 10 minutes and the former removed, yielding a gel (24 X 12 X 0.1 cm) with 9 pockets for samples of volume 14 µL each. The gel was stored at 4°C until use, with a maximum lifetime of 4 days.

Electrophoresis was conducted using electrode buffer consisting of 1:1 stock buffer:water. The gel was pre-electrophoresed for *ca.* 30 minutes in the LKB Multiphor apparatus with cooling fluid at 4°C, at 50 mA (*ca.* 750 V). After the sample was applied with Bromophenol blue tracking dye (1 µL, 0.25% w/v in stock buffer), it was concentrated at 20 mA for 15 minutes. Electrophoresis was then carried out at 40 mA (*ca.* 700 V) for 4-5 hours. The running pH was *ca.* 9.5.

After this time gels were usually developed for protein, staining for 30 minutes in a solution of Coomassie Brilliant Blue R-250 (0.8 g), trichloroacetic acid (200 g) and water (400 cm^3). The gel was then left to destain overnight with destaining solution (600 cm^3 water, 300 cm^3 ethanol and 100 cm^3 glacial acetic acid). Another change of destaining solution the next morning was usually necessary for complete destaining of the gel.

The following solutions were employed for the investigations on substrate-enzyme ratios:

Deaminase (100 U cm^{-3}, 0.01 M KPhos pH 7.6).

PBG (66.7 nmol cm^{-3}, 0.1 M Tris Cl^- pH 8.0).

[3,5-^{14}C]-PBG (60 µCi in 1.0 cm^3 0.1 M Tris Cl^- pH 8.0 and 0.1 cm^3 of this diluted to 1.614 cm^3 0.1 M Tris Cl^- pH 8.0, 37.6 nmol cm^{-3}).

Experiment 1

The above solutions of deaminase and PBG were mixed in equal volumes (30 μL) to give ratios of 1:1, 1:2, 1:3, 1:4 and 1:6 enzyme:substrate, and analysed by gel-electrophoresis together with partially pure deaminase. This revealed a large number of bands corresponding to impurities when stained for protein, but also a strong band corresponding to deaminase (mobility/% of tracking dye = 57) and also an extra broad band (mobility/% of tracking dye = 64) corresponding to the complex. This was confirmed when another gel-electrophoresis was performed using the same complex solutions. This time a strip of the gel was removed for protein staining, and the remainder was incubated in 0.01 M Tris Cl^- pH 8.0 (20 cm^3) containing PBG (640 μg). After 45 minutes at room temperature in the dark, the solution was washed off and the gel treated with iodine in HCl (0.2% w/v I_2 in 1 M HCl). The bands of porphyrin were detected with UV light, and only two for each lane were visible. These corresponded to native enzyme (mobility/% of tracking dye = 55-7) and a broad band corresponding to complexes (mobility/% of tracking dye = 62-4).

Experiment 2

Using the HPMD, the above solutions of deaminase and [3,5-^{14}C]-PBG were used to generate complexes with the enzyme:substrate ratios 1:0.57, 1:1.69, 1:3.38, which were applied to a gel as in Experiment 1. After electrophoresis, the gel was stained for protein, which showed three new bands. The gel was dried out using a vacuum gel-dryer (Bio-Rad) and then subjected to autoradiography using Kodak X-ray film. The exposure was for 6 days at room temperature, and

three bands were detected, as with the protein stain (mobility/% tracker dye for native enzyme = 57%, "mono-PBG" = 62, "di-PBG" = 64, "tri-PBG" = 66). Some bands were cut out and dried (vacuum desiccator) and treated with 0.5 cm^3 30% H_2O_2 at 80°C until completely dissolved (*ca.* 90 minutes). Then Aquasol (4.5 cm^3) was added and the bands assayed by liquid scintillation counting. These results form part of Table 3.1.

Experiments 3 and 4

[^{14}C]-PBG and deaminase were used exactly as in Experiments 1 and 2 to generate complexes either using the HPMD or sequential mixing at either 37°C or 4°C, and the band cut out and assayed as in Experiment 2. The results from these experiments form the major part of Table 3.1.

Experiment 5

The proteolytic digestion of [^{14}C]-complex as generated in Experiment 4 was investigated using α-chymotrypsin (0.74 mg, *ex* Sigma, in 35 μL 0.1 M Tris Cl^- pH 8.0, 1 unit μL^{-1}). α-chymotrypsin (10 units) was incubated with [^{14}C]-complex (68 μL) and also deaminase (25 μL, 1.95 U) at 37°C for 45 minutes. Analysis by gel-electrophoresis revealed that only partial digestion had taken place, and most of the bands present in the native enzyme were present in the digested material.

Experiment 6

Proteolysis of the [^{14}C]-complex (60 μL) and deaminase (40 μL of activity 70 U cm^{-3}) was attempted using Pronase E (5 μL, Type xiv Bacterial protease, 5.8 units mg^{-1} *ex* Sigma, 452 μg in 90.4 μL 0.1 M

Tris Cl^- pH 8.0, 29 units cm^{-3}, 5 $\mu g\ \mu L^{-1}$), incubating at 37°C for 45 minutes. Gel-electrophoresis revealed that an equal distribution of counts occurred in the middle of the gel and in the upper part of the gel, implying that digestion had taken place satisfactorily and a series of [^{14}C]-oligopeptides occurred throughout the gel.

8.6 Preparation and Purification of PBG-Deaminase Complexes

The PBG-deaminase complexes generated for all this work required the construction of a mixing device. This is shown in Figure 8.1.

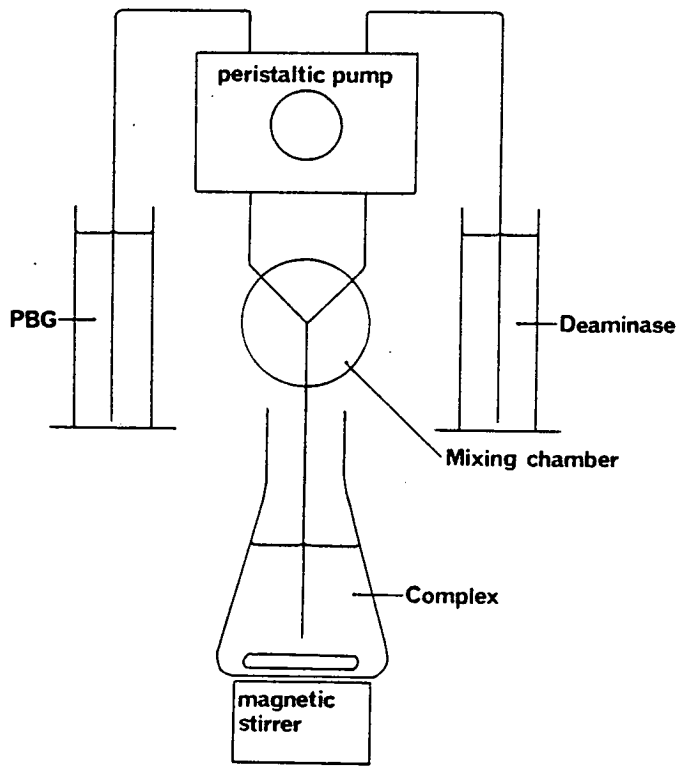


FIGURE 8.1. High Performance Mixing Device ("HPMD").

The mixing chamber was constructed out of a perspex cylinder (3.5 dia X 2 cm) with standard HPLC fittings to three 22 gauge holes meeting at the centre where the mixing takes place. The enzyme and substrate, in identical volumes, were pumped into the mixing chamber by means of a dual-channel peristaltic pump (Gilson Minipuls 2) operating at a flow rate of *ca.* 600 cm^3 hr^{-1}. The complex eluting out of the mixing chamber was passed into a conical flask equipped with a magnetic stirrer.

In a typical preparation, purified deaminase (8000 U, 20 U mg^{-1}, in 50 cm^3 of 0.01 M Tris Cl^- pH 8.0 buffer) and labelled PBG (750 µg [^{13}C]-PBG + 37.5 µg [^{14}C]-PBG, 6789555 dpm µmol^{-1} ^{13}C PBG, in 51 cm^3 of 0.01 M Tris Cl^- pH 8.0 + 5 mM EDTA + [25 mM lysine, optional]) were mixed in the HPMD, and the resultant complex immediately concentrated (PM-10) to *ca.* 2 cm^3.

While the complex was being generated and concentrated, the preparative gel-electrophoresis equipment was assembled. For this the following stock buffers were made:

Stock Tris Buffer

Tris (*ex* Sigma)	41.1 g
Conc. HCl	to pH 7.5
Water	to 2,400 cm^3
	Final pH 7.5

Upper Electrode Buffer

Barbital (*ex* Fisher Scientific)	11.04 g
Tris	2.0 g
Water	to 2 L
	Final pH 7.5

Lower Electrode Buffer and Elution Buffer

Stock Tris buffer:water	1:1

The gel was made using the following solutions:

7.5% Preparative Polyacrylamide Gel

Stock Tris buffer	110 cm^3
Stock acrylamide (see Section 8.4)	74 cm^3
Water	29 cm^3

These were mixed and vacuum-degassed with stirring for 30 minutes prior to the rapid, simultaneous addition of:

Ammonium persulphate (150 mg cm^{-3} water)	7 cm^3
N,N,N',N'-tetramethylethylene diamine (TEMED)	190 µL

After quickly mixing, 210 cm^3 of the solution (10 X 4 cm gel) was poured into the preparative electrophoresis apparatus, equipped with a Buchler gel-supporting nylon screen and a gel-former, and distilled water quickly and carefully layered onto the surface of the gel with a 10 cm^3 plastic syringe and applicator tube. This ensured that the gel polymerised with a flat top surface without a meniscus. The gel was allowed to polymerise at room temperature for 30 minutes before the gel-former was removed, and the apparatus cooled to 4°C in the cold room. The apparatus was connected to a cooling bath circulating ethylene-glycol/water at 0°C. The upper and lower electrode buffers were pumped into the apparatus using peristaltic pumps eluting at 60 cm^3 hr^{-1}, and the waste buffers collected in separate containers to avoid the risk of a short-circuit. Elution buffer was pumped out of the apparatus by a peristaltic pump eluting also at 60 cm^3 hr^{-1} and through a UV detector to the fraction collector.

After all the air-bubbles in all the various chambers had been completely eliminated, pre-electrophoresis was conducted at 65 mA (*ca.* 200 V) for 30 minutes. The sample (2 cm^3) was made 5% w/v in sucrose, and Bromophenol blue tracking dye (10 µL, 0.25% w/v in stock buffer) added. This was very carefully layered onto the surface of the gel using a 10 cm^3 plastic syringe and applicator tube, having previously filled the applicator tube with water so as not to waste valuable complex. Concentration of the sample was carried out at 30 mA (<100 V) for *ca.* 15 minutes, and electrophoresis continued at 50-65 mA (*ca.* 150 V). Initially the elution buffer was collected in bulk (150 cm^3) but after about 6 hours the fraction collector was started, and electrophoresis was continued for approximately another 17 hours, collecting 10 cm^3 fractions. The running pH was *ca.* 7.9.

The fractions were assayed for radioactivity (liquid scintillation counting), protein (A_{280} used as a rough guide, since barbital interferes at this wavelength) and enzyme activity. Typically fractions 38-45 contained complex which was concentrated (PM-10) down to *ca.* 0.4 cm^3 and washed/reconcentrated twice with 0.05 M $P_2O_7^{2-}$ 20% D_2O pH 8.0 buffer. The final sample (0.4 cm^3) was assayed for radioactivity, the total dpm giving the amount [^{13}C]-PBG bound in µmol or µg. This sample was then used in 5 mm NMR studies.

Fractions 46-53 contained electrophoretically pure deaminase (>60% pure, *ca.* 80-120 U mg^{-1}) which was also pooled, concentrated (PM-10) and washed (0.05 M $P_2O_7^{2-}$ 20% D_2O pH 8.0 buffer). This was used either as a blank in a ^{13}C NMR experiment, or for the generation of ultrapure complex. In the latter case, the enzyme and substrate were mixed exactly as before in the mixing device, and after

concentration (PM-10, *ca.* 30 cm^3), applied to a freshly equilibrated G-50 Sephadex column (500 cm^3 bed volume, 19 X 6.5 cm). Elution was performed with 0.025 M $P_2O_7^{2-}$ pH 8.0 buffer + 5 mM β-mercaptoethanol, collecting 10 cm^3 fractions. Fractions 18-28 were combined and concentrated (PM-10) to *ca.* 0.4 cm^3, washing with 0.05 M $P_2O_7^{2-}$ 20% D_2O pH 8.0 buffer, for use in 5 mm NMR experiments.

8.7 Proteolytic Digestion of [^{13}C]-PBG-Deaminase and Purification of Oligopeptides

After examination by ^{13}C NMR, the [^{13}C]-complex (+ 5% [^{14}C]-complex) was lyophilised to give 75 mg of protein. This was dissolved in NH_4HCO_3 buffer (7 cm^3, 0.1 M) and the mixture placed in a test-tube and covered with nitrogen. After preincubation at 37°C for 10 minutes, trypsin (75 µL, 0.76 mg or 1% w/w, *ex* Sigma, TPCK-treated, 10 mg in 1 cm^3 of 10 µM HCl containing 1.1 mg $CaCl_2$) was added, and the mixture incubated at 37°C for 1 hour before further trypsin stock (75 µL) was added. Incubation was continued for a further hour, and the mixture was lyophilised. This was redissolved in buffer (0.05 M NH_4HCO_3) and applied to a freshly equilibrated G-50 Sephadex column (1.8 X 140 cm). Elution was carried out with 0.05 M NH_4HCO_3 buffer at 30 cm^3 hr^{-1}, collecting 3.5 cm^3 fractions. Fractions 21-32 were combined and lyophilised (Peptide 2), as were fractions 33-41 (Peptide 3) and also fractions 13-20 (Peptide 4).

The oligopeptides from fractions 21-32 were redissolved in electrode buffer (*ca.* 200 µL, 1:10:189 pyridine:HOAc:water, pH 3.5) and tracking dye (*ca.* 40 µL, 0.5 mg cm^{-3} DNP-lysine *ex* Sigma and 0.2 mg cm^{-3} xylene cyanole FF *ex* Bio-Rad, both in water) and applied

to a spot 10 cm from one edge and 20 cm from the anode on Whatman's 3 MM paper (46 X 57 cm) pre-wetted with electrode buffer. Flat-bed paper electrophoresis was then carried out in one dimension (100 mA, 2.5 kV) for about 10 hours. The paper was then air-dried, and bisected in two, and the half bearing the peptides subjected to descending paper chromatography in the perpendicular dimension, using n-butanol:HOAc:water:pyridine 15:3:12:10 (by volume) in a pre-equilibrated chromatography tank. Elution took *ca.* 10 hours, and after air-drying, the paper was sprayed with fluorescamine (10 mg *ex* Sigma in 100 cm^3 dry acetone and 1 cm^3 of this diluted to 100 cm^3 with dry acetone + 1 cm^3 dry pyridine). This is a non-destructive reagent which allowed detection of the oligopeptides when viewed under UV light (see Figure 3.15 for map).

The peptides were cut out and cut into small pieces, eluting off the paper with 0.1 M NH_3 (aq). After filtration, the peptide solutions were lyophilised and thus generated Peptides 1, 5 and 6.

Fractions 13-20 (Peptide 4) from G-50 Sephadex gel-filtration were lyophilised, yielding 24 mg of protein after removal of a small amount for further analysis. This was dissolved in buffer (5 cm^3, 0.1 M NH_4HCO_3) and Pronase E solution (300 µL, 2 mg cm^{-3}, 2% w/w in the same buffer) was added. The mixture was incubated at 37°C for 24 hours before lyophilisation to yield Peptide 7.

8.8 Purification of PBG Deaminase for Amino Acid Analysis and Protein Sequencing

The [^{13}C]-complex from Experiment 10 (see Chapter 9, p. 236) after incubation with unlabelled PBG, was subjected to a second preparative

gel-electrophoresis step and described in Section 8.6. This furnished samples of both pure enzyme and "mono-PBG" complex, shown to be >95% pure by analytical gel-electrophoresis. The pooled proteins were desalted by gel-filtration on G-10 Sephadex (bed volume 150 cm^3, 31 X 2.5 cm) eluting with pure water at 100 cm^3 hr^{-1}, and collecting 2 cm^3 fractions. Both the complex and subsequently the pure enzyme eluted in fractions 20-24. These were pooled and lyophilised to furnish pure complex (2.728 mg) and pure enzyme (1.321 mg), assayed spectrophotometrically.

CHAPTER 9

NMR PROCEDURES

The procedures in this chapter cover the ways in which data presented in Part II was (a) acquired and (b) processed. Where this has already been adequately covered, the details have been omitted.

Abbreviations employed are as follows: DP = decoupler power, in Bruker notation 15 H = 0.4 W, 12 H = 0.8 W, 10 H = 1.4 W, 9 H = 1.6 W, 6 H = 3.2 W, 3 H = 6.4 W; SI = size of data table; AQ = acquisition time; PW = pulse width, or "flip angle"; NS = number of scans; RD = relaxation delay; NZP = number of points removed from the start of an FID; LB = line broadening. All data were accumulated with a full spectral width (SW), except where stated otherwise.

9.1 ^{1}H NMR Studies

Studies of PBG and Deaminase (with Dr. R. C. Davies)

For the experiment shown in Figure 2.1, PBG (2 mg) was dissolved in D_2O (99%, 0.1 cm^3) and the pH adjusted to *ca.* 8.0 with a crystal of Na_2CO_3. This was then mixed with deaminase (0.4 cm^3, 100 U, in 0.05 M KPhos pH 7.6 buffer) in the NMR tube at 4°C and rapidly transferred to the NMR. Data was then accumulated at 300 MHz and 4°C, with the low-power (6 L in Bruker notation) irradiation of the HOD line, and with SI = 16K, AQ = 1.64 s, PW = 90° and NS = 4.8. The FID's were accumulated using a kinetics microprogram, with the interval between FID's being 198.88 seconds (3 minutes, 19 seconds). The FID's were then subjected to Fourier transformation. The spectra shown in Figure 2.2 were generated in an identical manner.

Studies on PBG with pH

The pH titration of PBG by ^1H NMR represents a technical problem, since the spectrometer is locked onto the HOD line, which shifts with pH giving rise to changes in the field. Thus PBG (3 mg) was dissolved in buffer (0.1 M KPhos, 99% D_2O, pH 7.27) and referenced to a coaxial capillary insert of 1% (v/v) of TMS in CCl_4. The chemical shifts are not corrected for magnetic susceptibility differences. For a superconducting magnet, the internal chemical shift δ_{int}, can be calculated from the observed chemical shift using

$$\delta_{int} = \delta_{obs} + \frac{4\Pi}{3} (\chi_{ref} - \chi_{sample})$$

where in this case the magnetic susceptibilities $\chi_{ref} = -0.691 \times 10^6$ and $\chi_{sample} = -0.719 \times 10^6$, which gives rise to an effect of 0.117 ppm.

The pH was adjusted using a 0.5 cm^3 syringe mounted in a holder, with a micrometer screw to force the plunger of the syringe in by fractional amounts. The syringe was connected to a teflon tube and in this way very small amounts of 0.2 M NaOD in 99% D_2O could be added, using the teflon tube as a stirrer. Below pH 7.2, 20% DCl in 100% D_2O was judiciously added using a Gilson Pipetman. The pH was measured directly using a 3 mm pH electrode. The pH measurements were not corrected for D_2O.

Data was accumulated with homonuclear irradiation of the HOD line, and with SI = 8K, AQ = 1.139 s, PW = 90°, NS = 16. The FID's were exponentially multiplied with LB = 0.2 Hz before Fourier transformation (see Figure 2.3).

9.2 Complete ^{13}C NMR Assignment of PBG

Preliminary studies on [3,5-^{13}C]-PBG were performed as follows. [3,5-^{13}C]-PBG (2 mg) was dissolved in 0.1 M $P_2O_7^{2-}$, 10% D_2O, pH 8.0 buffer and examined at 75.5 MHz and 25°C, with DP = 3 H gated decoupling, SI = 16K, AQ = 0.541 s, PW = 90°, NS = 1200, and FID was exponentially multiplied with LB = 2 Hz prior to Fourier transformation (see Figure 2.7).

Selective irradiation experiments were made by moving the ^1H-decoupler to the frequency corresponding to the ^1H resonances at C-6 and C-11, at δ_H = 2.63 and δ_H = 4.15. Data was acquired with DP = 3 H gated decoupling, SI = 16K, AQ = 0.541 s, PW ≃ 45°, NS = 4000. The FID's were zero-filled to 32K, exponentially multiplied with LB = 1 Hz and Fourier transformed (see Figure 2.8).

The complete ^{13}C NMR assignment of PBG was performed at 125.8 MHz and 25°C. Unlabelled PBG (20 mg) was dissolved in buffer (453.7 µL, 0.1 M $P_2O_7^{2-}$, 99% D_2O, pH 8.0) to which 3.0 M NaOD in 100% D_2O (46.3 µL) was added. Initially the 500 MHz ^1H NMR spectrum was obtained and an absolute frequency list (expressed later in ppm) drawn up. The coupled ^{13}C NMR spectrum was then obtained: δ_C = 21.7 (t of t, ^1J (^{13}C-^1H) = 127.5 Hz, ^2J (^{13}C-^1H) = 4.0 ± 0.5 Hz), 32.8 (t, ^1J (^{13}C-^1H) = 126.5 Hz), 34.4 (t, ^1J (^{13}C-^1H) = 144.0 Hz), 38.2 (t of t, ^1J (^{13}C-^1H) = 126.8 Hz, ^2J (^{13}C-^1H) = 5.2 Hz), 117.4 (c, m), 120.6 (c, m), 122.5 (c, m), 181.1 (t, ^2J (^{13}C-^1H) = 6.8 Hz), 183.0 (m, ^2J (^{13}C-^1H) = 4.8 Hz, ^3J (^{13}C-^1H) = 4.0 Hz).

Selective off-resonance ^1H-decoupling experiments were then performed, using a very low decoupler power.

Aliphatic Region

(i) Selective decoupling at δ_H = 2.41 ppm.

The triplet at 21.7 ppm collapsed to a singlet, and ^1J decreased for the triplet at 38.2 ppm. All others unaffected.

(ii) Selective decoupling at δ_H = 2.67 ppm.

The triplet at 38.2 ppm collapsed to an unsymmetrical doublet, and J decreased for the triplet at 21.7 ppm. All others unaffected.

(iii) Selective decoupling at δ_H = 3.41 ppm.

For the triplet at 32.8 ppm, ^1J decreased. All others unaffected.

(iv) Selective decoupling at δ_H = 4.16 ppm.

For the triplet at 34.4 ppm, ^1J decreased. All others unaffected.

(v) Selective decoupling at δ_H = 6.69 ppm.

No visible effect.

Aromatic Region

(i) Selective decoupling at δ_H = 2.41 ppm.

Intensity of peak at 117.4 ppm enhanced, and peak at 122.5 ppm sharpened.

(ii) Selective decoupling at δ_H = 2.67 ppm.

Long-range coupling at 116.1 ppm lost to give a clean doublet. The resonance at 122.5 ppm sharpened (showed extra coupling).

(iii) Selective decoupling at δ_H = 3.41 ppm.

Intensity of peak at 117.4 ppm enhanced, and peak at

120.6 ppm showed some fine structure. Peak at 122.5 ppm sharpened considerably.

(iv) Selective decoupling at δ_H = 4.16 ppm.

The resonance at 120.6 ppm sharpened to a quintet, and the peak at 117.4 ppm also sharpened.

(v) Selective decoupling at δ_H = 6.69 ppm.

The resonance at 116.1 ppm split into a triplet with a central singlet representing the decoupled line, and the outer parts of the triplet representing the residual coupling. The resonance at 120.6 ppm was slightly sharper but the others were unaffected.

COOH Region

(i) Selective decoupling at δ_H = 2.41 ppm.

The resonance at 183.0 ppm collapsed to a singlet.

(ii) Selective decoupling at δ_H = 2.67 ppm.

The resonance at 183.0 ppm collapsed to a clean triplet.

(iii) Selective decoupling at δ_H = 3.41 ppm.

The resonance at 181.1 ppm collapsed to a singlet.

(iv) Selective decoupling at δ_H = 4.16 ppm.

No visible effect.

(v) Selective decoupling at δ_H = 6.69 ppm.

The resonance at 183.0 ppm lost its left-hand couplings.

pH Titration of [3,5-^{13}C]-PBG

[3,5-^{13}C]-PBG (2.2 mg) was dissolved in buffer (0.45 cm^3, 0.05 M $P_2O_7^{2-}$, 50% D_2O, pH 8.0) and 3.0 M NaOH (5.2 μL) and p-dioxane

(5.0 μL) added. The pH was measured directly using a 3 mm pH electrode, and the pH adjusted with 1 M β-alanine pH 10.26 buffer. Above pH 10.2, the pH was adjusted by judicious addition of 3.0 M NaOH. Data was accumulated at 75.5 MHz and 23°C with DP = 12 H, SI = 8K, AQ = 0.250 s, PW = 90°, NS = 2712. The FID's were zero-filled to 16K, exponentially multiplied with LB = 2 Hz, and Fourier transformed (see Figure 2.9).

9.3 ^{13}C NMR Studies of Uro'gen Biosynthesis with [2,11-^{13}C]-PBG (with Dr. R. C. Davies)

For these experiments, ^{13}C NMR investigations were conducted at 75.5 MHz, and the deaminase and cosynthetase employed was prepared by Dr. R. C. Davies.

Experiments with [2,11-^{13}C]-PBG in a 5 mm Probe

Experiment 1

[2,11-^{13}C]-PBG (2 mg) in buffer (0.5 cm^3, 0.05 M $P_2O_7^{2-}$, 20% D_2O, pH 8.35) was characterised by ^{13}C NMR at 0°C: δ_C = 34.4 (C-11) and 116.1 (C-2). Deaminase (0.1 cm^3, 176 U cm^{-3}) in the same buffer was added and examined at 0°C. Data was accumulated with DP = 3 H gated decoupling, SI = 16K, AQ = 0.459 s, PW = 90°, NS = 2000. A spectrum of uro'gen I was obtained after exponential multiplication with LB = 3 Hz and Fourier transformation. HPLC analysis of products: 60% I, 40% III.

Experiment 2

[2,11-^{13}C]-PBG (2 mg) was dissolved in buffer (0.1 cm^3, 0.05 M $P_2O_7^{2-}$, 20% D_2O, pH 8.34) and mixed with deaminase (0.3 cm^3, 176 U cm^{-3})

in the same buffer and incubated under N_2 for 4 minutes at 37°C. The reaction was stopped by freezing (ice-salt), warmed to 0°C and cosynthetase (0.1 cm^3, 10 U) added with mixing. The sample was then examined at 1.5°C using a kinetics program to accumulate data every 15 minutes 11 seconds, with DP = 3 H gated decoupling, SI = 16K, AQ = 0.459 s, PW = 90°, NS = 256. Poor spectra of bilane and uro'gen III obtained. HPLC analysis of products: 10% I, 90% III.

Experiment 3

[2,11-^{13}C]-PBG (2 mg) dissolved in buffer (0.1 cm^3, 0.05 M $P_2O_7^{2-}$, 20% D_2O, pH 8.34) was added to deaminase (0.4 cm^3, 176 U cm^{-3}) in the same buffer and incubated under N_2 for 6 minutes at 37°C. The reaction was stopped by freezing (ice-salt), warmed to 1.5°C, and the ^{13}C NMR time-course taken with 15 minutes 11 seconds between accumulations. Data was acquired with DP = 3 H gated decoupling, SI = 16K, AQ = 0.459 s, PW = 90°, NS = 256 and the FID's were exponentially multiplied with LB = 5 Hz prior to Fourier transformation. HMB detected, but short-lived, all cyclising to uro'gen I in *ca.* 30 minutes (see Figure 2.12). HPLC analysis of products: 90% I, 10% III.

Experiment 4

[2,11-^{13}C]-PBG (2 mg) dissolved in buffer (0.1 cm^3, 0.05 M $P_2O_7^{2-}$, 20% D_2O, pH 8.34 adjusted to pH 8.7 with 3 M NaOH) was added to deaminase (0.4 cm^3, 176 U cm^{-3}) in the same buffer (pH 8.7), incubated under N_2 for 6 minutes before adjusting to pH 12 with 3 M NaOH and ^{13}C NMR spectra recorded at 0°C. Data was accumulated for *ca.* 4 hours with DP = 3 H gated, SI = 16K, AQ = 0.459 s, PW = 90°, NS = 4500. The FID was zero-filled to 32K, exponentially multiplied

with LB = 3 Hz prior to Fourier transformation. A satisfactory spectrum of HMB was obtained.

Experiment 5

The foregoing HMB solution was adjusted back to pH 8.7 (with 3 M HCl) and to this cosynthetase (0.1 cm^3, 10 U in the same buffer, pH 8.7) and β-mercaptoethanol (5 μL) was added. Data was accumulated at 0°C in a kinetics time-course, with DP = 3 H gated, SI = 16K, AQ = 0.459 s, PW = 90°, NS = 500. The FID's were summed, zero-filled to 32K and exponentially multiplied with LB = 3 Hz prior to Fourier transformation. No detectable intermediates. HPLC analysis of products: 43% I, 57% III.

Experiment 6

[2,11-^{13}C]-PBG (1.5 mg) was dissolved in buffer (0.5 cm^3, 0.05 M $P_2O_7^{2-}$, 90%, pH 8.0) and adjusted to pH 12 (30 μL 3 M NaOH). Data was accumulated with DP = 12 H continuous, SI = 16K, AQ = 0.541 s, PW = 90°, NS = 1000, and the FID was exponentially multiplied with LB = 3 Hz prior to Fourier transformation.

Experiment 7

[2,11-^{13}C]-PBG (2 mg) was dissolved in degassed buffer (2.8 cm^3, 0.025 M KPhos pH 7.6) and deaminase (0.1 cm^3, 10 U) and cosynthetase (0.1 cm^3, 10 U) in the same buffer was added and incubated at 37°C for 2 hours. The mixture was lyophilised and redissolved in 0.5 cm^3 D_2O (99%) and examined at 25°C with DP = 12 H continuous, SI = 16K, AQ = 0.541 s, PW = 30°, NS = 4000, and the FID was zero-filled to 32K, exponentially multiplied with LB = 5 Hz and Fourier transformed to give a satisfactory spectrum of uro'gen III (see Figure 2.13).

Experiments with [2,11-^{13}C]-PBG in a 10 mm Probe

Experiment 8

[2,11-^{13}C]-PBG (2 mg) was dissolved in buffer (0.5 cm^3, 0.05 M $P_2O_7^{2-}$, 99% D_2O, pH 8.0) and diluted with degassed phosphate buffer (2.2 cm^3, 0.025 M KPhos pH 7.6), and incubated with deaminase (0.1 cm^3, 26 U) in the same buffer at 37°C for 2 hours. The mixture was lyophilised, and the residue dissolved in D_2O (2.0 cm^3, 99%). This solution of uro'gen I was studied at 25°C varying DP and PW. Gated decoupling with DP = 15 H, 12 H, 9 H, 6 H and 3 H was used with PW = 90°, and then DP = 12 H continuous with PW = 15°. Data was accumulated with SI = 16K, AQ = 0.541 s, NS = 500, and the FID's were exponentially multiplied with LB = 5 Hz, prior to Fourier transformation (see Figure 2.11). HPLC analysis of products: 89% I, 11% III.

Experiment 9

[2,11-^{13}C]-PBG (5 mg) dissolved in buffer (0.4 cm^3, 0.05 M $P_2O_7^{2-}$, 80% D_2O, pH 8.5 + 10 µL 3.0 M NaOH) was incubated with deaminase (1.6 cm^3, 400 U) in the same buffer for 3.5 minutes at 37°C, before adding 3 M NaOH (25 µL) and cooling (ice-water). Data was accumulated at 4°C for 36 minutes with DP = 12 H continuous, SI = 16K, AQ = 0.541 s, PW = 15°, NS = 5000. The FID was zero-filled to 32K, exponentially multiplied with LB = 3 Hz and Fourier transformed. Satisfactory spectrum of HMB obtained (see Figure 2.14).

Experiment 10

To the foregoing solution of HMB, cosynthetase (0.2 cm^3, 20 U in 0.05 M KPhos pH 8.0 buffer) was added and 3 M HCl (35 µL) added

with rapid mixing to bring the pH to 8.0. The sample was rapidly transferred to the NMR, and a time-course run over 2 hours, collecting data every 4.5 minutes. Data was accumulated with DP = 12 H continuous, SI = 16K, AQ = 0.541 s, PW = 15°, NS = 500 and the FID's exponentially multiplied with LB = 3 Hz prior to Fourier transformation (see Figures 2.15, 2.16). HPLC analysis of products: 15% I, 85% III.

9.4 ^{13}C NMR Studies of Uro'gen Biosynthesis with [3,5-^{13}C]-PBG (with Dr. R. C. Davies)

For these experiments, ^{13}C NMR investigations were conducted at 75.5 MHz, and the deaminase and cosynthetase employed was prepared by Dr. R. C. Davies.

Experiments with [3,5-^{13}C]-PBG in a 5 mm Probe

Experiment 1

[3,5-^{13}C]-PBG (1 mg) in buffer (0.25 cm^3, 0.1 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0) was incubated with deaminase (0.05 cm^3, 10 U and 2.2 cm^3 0.025 M KPhos pH 7.8) for 2 hours at 37°C under N_2 in the dark. This was lyophilised and the residue redissolved in N_2-degassed buffer (0.5 cm^3, 0.05 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0). Data was accumulated with DP = 12 H gated, SW = 1000 Hz, SI = 2K, AQ = 1.024 s, PW = 90°, NS = 27,500 and the FID was zero-filled to 16K prior to Fourier transformation. Fully resolved spectrum of uro'gen I (see Figure 2.17). HPLC analysis of products: 96% I, 4% III.

Experiment 2

[3,5-^{13}C]-PBG (1 mg) in buffer (0.25 cm^3, 0.1 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0) was incubated with deaminase (0.05 cm^3, 10 U and 2.1 cm^3 0.025 M KPhos pH 7.8) and cosynthetase (0.1 cm^3, 10 U) in the same buffer for 2 hours at 37°C under N_2 in the dark. This was lyophilised and redissolved in N_2-degassed buffer (0.5 cm^3, 0.05 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0). Data was accumulated with DP = 12 H continuous, SW = 1000 Hz, SI = 2K, AQ = 1.024 s, PW = 90°, NS = 34,250 and the FID was zero-filled to 16K, exponentially multiplied with LB = 1 Hz prior to Fourier transformation (see Figure 2.18). Partially resolved spectrum of uro'gen III. HPLC analysis of products: 6% I, 94% III.

Experiment 3

[3,5-^{13}C]-PBG (2 mg) in buffer (0.1 cm^3, 0.1 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0, washed into the tube with a further 0.05 cm^3 of the same buffer) was incubated with deaminase (0.4 cm^3, 120 U) in the same buffer at 37°C under N_2 for 4 minutes. 3 M NaOH (10 µL) was added and the solution cooled. Data was accumulated at 3°C with DP = 12 H gated, SW = 1000 Hz, SI = 2K, AQ = 1.024 s, PW = 90°, NS = 6000 and the FID was zero-filled to 16K, and exponentially multiplied with LB = 1 Hz prior to Fourier transformation. Poorly resolved spectrum of HMB obtained. HPLC analysis of products: 95% I, 5% III.

Experiments with [3,5-^{13}C]-PBG in a 10 mm Probe

Experiment 4

The sample from the previous experiment (Experiment 3) was diluted with buffer (2.0 cm^3, 0.1 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0) and

used as a test sample. Data was accumulated at 10°C with 12 H continuous decoupling, and a matrix for varying PW and SI (AQ) was constructed according to signal-to-noise over a fixed accumulation time of 4.5 minutes. The FID's were zero-filled to 16K (where appropriate) and exponentially multiplied with LB = 5 Hz prior to Fourier transformation. Signal-to-noise was measured on the peak of uro'gen I at 117.7 ppm.

	PW 90°	75°	60°	45°
SI/K (AQ)				
4 (0.1362 s)		36.7	45.5	
8 (0.2703 s)	54.7	56.3	32.1	48.2
16 (0.5407 s)	52.4	42.9	56.6	41.7

While these results exhibit the errors in making the signal-to-noise measurement, it was ascertained that PW = 75° and SI = 8K (AQ = 0.270 s) were probably the best conditions for molecules of this sort.

Experiment 5

[3,5-^{13}C]-PBG (5 mg) was dissolved in buffer (0.4 cm^3, 0.05 M $P_2O_7^{2-}$, 90% D_2O, pH 8.5 plus 10 µL 3 M NaOH) and added to deaminase (1.6 cm^3, 337 U in the same buffer) and incubated at 37°C for 3.5 minutes before 3 M NaOH (25 µL) was added. The spectrum obtained of HMB was unsatisfactory, so cosynthetase (78 U, in 0.05 M KPhos pH 8.0) was added and also 3 M HCl (35 µL) with *rapid* mixing to bring to pH 8.1. Data was accumulated at 8°C over 90 minutes collecting every 4.5 minutes, with DP = 12 H continuous, SI = 8K, AQ = 0.270 s, PW = 75°, NS = 1000, and the FID's were zero-filled

to 16K, and exponentially multiplied with LB = 10 Hz prior to Fourier transformation. No new intermediates. HPLC analysis of products: 13% I, 87% III.

Experiment 6

Deaminase (1.6 cm^3, 272 U in 0.05 M $P_2O_7^{2-}$, 80% D_2O, pH 8.5) buffer was analysed for a baseline for 9 minutes at 8°C (NS = 2000) under essentially the same conditions as Experiment 5. No extraneous peaks were detected in the areas of interest. [3,5-^{13}C]-PBG (5 mg) in buffer (0.05 M $P_2O_7^{2-}$, 80% D_2O, pH 8.5) was added to this deaminase solution, and the mixture incubated at 37°C for 4 minutes. The pH was then adjusted to 12 with 3 M NaOH (25 µL) and the mixture cooled to 0-5°C. Data was accumulated at 3-7°C over 36 minutes with DP = 12 H continuous, SI = 8K, AQ = 0.270 s, PW = 75°, NS = 10,000, and the FID was zero-filled to 32K, and subjected to Gaussian multiplication with a Gaussian broadening of 0.350 and LB = -3Hz prior to Fourier transformation (see Figure 2.19). Fully resolved spectrum of HMB obtained.

Experiment 7

To the foregoing solution of HMB, cosynthetase (0.3 cm^3, 30 U in 0.05 M KPhos pH 8.0 buffer) was added and 3 M HCl (35 µL) with *rapid* mixing. Data was accumulated at *ca.* 4°C over 3 hours collecting every 4.5 minutes, with DP = 12 H continuous, SI = 8K, AQ = 0.270 s, PW = 750, NS = 1000, and the FID's were zero-filled to 16K and exponentially multiplied with LB = 5 Hz prior to Fourier transformation. No new intermediates (see Figures 2.20, 2.21). HPLC analysis of products: 21% I, 79% III.

Experiment 8

As a non-enzymic control for Experiment 7, [3,5-^{13}C]-PBG (5 mg) in buffer (0.4 cm^3, 0.05 M $P_2O_7^{2-}$, 80% D_2O, pH 8.5) was incubated with deaminase (368 U, 2.0 cm^3 in the same buffer) for 3 minutes at 37°C before adding 3 M NaOH (25 µL) and cooling. Data was accumulated at 5°C over 90 minutes, with DP = 12 H continuous, SI = 2K, AQ = 1.024 s, SW = 1000 Hz, PW = 90°, NS = 5300 and the FID was zero-filled to 32K and exponentially multiplied with LB = 2 Hz prior to Fourier transformation (see Figure 2.22).

Experiment 9

The foregoing HMB solution was readjusted to pH 8.0 with 3 M HCl (35 µL) and data accumulated for 2 hours every 4.5 minutes at 5°C, with DP = 12 H continuous, SI = 8K, AQ = 0.270 s, PW = 90°, SW = 15,151 Hz, NS = 1000 and the FID's were exponentially multiplied with LB = 5 Hz prior to Fourier transformation (see Figure 2.23). No new intermediates. HPLC analysis of products: 96% I, 4% III.

9.5 ^{13}C NMR Studies of [2,11-^{13}C]-PBG-Deaminase Complexes

Experiments 1-13 were performed in a 5 mm probe at 75.5 MHz, 14-29 in a 10 mm probe at 75.5 MHz, and 30-31 in a 5 mm probe at 125.8 MHz.

Experiment 1

[2,11-^{13}C]-PBG-deaminase complex (50 µg [^{13}C]-PBG bound to 3000 U partially purified deaminase without further purification dissolved in 0.5 cm^3 0.05 M $P_2O_7^{2-}$, 10% D_2O, pH 8.0 buffer) was examined at 12.5°C, with DP = 12 H continuous, SI = 16K, AQ =

0.4925 s, PW = 90°, NS = 51,351 and the FID was exponentially multiplied with LB = 20 Hz prior to Fourier transformation. Protein background clearly visible, but no significantly enriched peaks.

Experiment 2

[2,11-^{13}C]-PBG-deaminase complex (as in Experiment 1) was examined at 12.5°C under different NMR conditions, with DP = 12 H continuous, SI = 32K, AQ = 0.983 s, PW = 72°, NS = 50,000 and the FID was exponentially multiplied with LB = 20 Hz prior to Fourier transformation. While aromatic and carbonyl carbons more clearly visible, no detectable substrate.

Experiment 3

The foregoing complex (0.2 cm^3, in 0.05 M $P_2O_7^{2-}$, 10% D_2O, pH 8.0 buffer), after purification by electrophoresis (see Chapter 8), was studied at 12.5°C, with DP = 12 H continuous, SI = 32K, AQ = 0.983 s, PW = 90°, NS = 154,805, and the first data points removed with NZP = 2. The FID was exponentially multiplied with LB = 20 Hz and Fourier transformed (see Figure 3.6). Substrate signal detected.

Experiment 4

Electrophoretically pure deaminase (0.2 cm^3, in 0.05 M $P_2O_7^{2-}$, 10% D_2O, pH 8.0 buffer) was analysed at 12.5°C with DP = 12 H continuous, SI = 32K, AQ = 0.983 s, PW = 90°, NS = 90,002, and the first data points removed with NZP = 2. The FID was exponentially multiplied with LB = 20 Hz and Fourier transformed (see Figure 3.7).

Experiment 5

To the complex from Experiment 3, Pronase E (1.855 mg, *ex* Sigma, Type xiv) was added directly and studied at 12.5°C over

24 hours. Data was accumulated using the Carr-Purcell-Meiboom-Gill spin-echo pulse sequence with a kinetics time-course, and DP = 12 H continuous, SI = 32K, AQ = 0.983 s, NS = 3600 (X 3). The pulse sequence involves (i) an initial delay of 5 times approx. T_1; (ii) a 90° pulse; (iii) a dephasing delay of 1 msec; (iv) an 180° pulse, phase-shifted 90° relative to the first pulse; (v) a dephasing delay of 1 msec and (vi) a loop to (iii) to make 4 echoes. Three such FID's were gathered, added together, the first points removed with NZP = 2, and the new FID exponentially multiplied with LB = 20 Hz prior to Fourier transformation (see Figure 3.8).

Experiment 6

The previous sample was analysed under standard conditions at 12.5°C using DP = 12 H continuous, SI = 32K, AQ = 0.983 s, PW = 90°, NS = 18,001, and the first points of the FID were removed with NZP = 2. The new FID was exponentially multiplied with LB = 20 Hz and Fourier transformed (see Figure 3.9 (i)).

Experiment 7

Electrophoretically pure deaminase (from Experiment 4) was incubated with Pronase E (0.9788 mg) in the NMR as in Experiment 5 except that the data was accumulated under standard conditions at 12.5°C using DP = 12 H continuous, SI = 32K, PW = 90°, AQ = 0.983 s, NS = 37,533, and the first points of the FID removed with NZP = 2. The new FID was exponentially multiplied with LB = 20 Hz and Fourier transformed (see Figure 3.9 (ii)).

Experiment 8

The sample used in Experiment 6 was diluted with buffer (5.5 cm^3, 0.01 M KPhos pH 7.6), incubated for 22 hours at 37°C and lyophilised. The residue was redissolved in D_2O (99%) and data was accumulated with DP = 12 H continuous, SI = 32K, AQ = 0.983 s, PW = 90°, NS = 17,333 and the first points of the FID removed with NZP = 2 prior to exponential multiplication with LB = 20 Hz and Fourier transformation. The spectrum was too complicated for interpretation due to large number of free amino acids present.

Experiment 9

[2,11-^{13}C]-PBG-deaminase complex (192 µg bound to 6000 U deaminase after gel-electrophoresis in 0.4 cm^3 0.01 M $P_2O_7^{2-}$, 10% D_2O, pH 8.0 buffer) was examined at 11.0°C. Data was accumulated with DP = 12 H continuous, SI = 16K, AQ = 0.492 s, PW = 54°, NS = 79,203 and the first points of the FID were removed with NZP = 2, the new FID zero-filled to 32K, and exponentially multiplied with LB = 20 Hz prior to Fourier transformation. The transformed spectrum was baseline corrected using a spline fit (see Figure 3.10 (ii)). Evidence of bound substrate/product.

Experiment 10

Electrophoretically pure deaminase (*ca.* 800 U, 63 U mg^{-1} in 0.01 M $P_2O_7^{2-}$, 10% D_2O, pH 8.0 buffer) was analysed using DP = 12 H continuous, SI = 16K, AQ = 0.492 s, PW = 54°, NS = 136,803 and the first points of the FID removed with NZP = 2. The new FID was zero-filled to 32K, exponentially multiplied with LB = 20 Hz, and Fourier transformed. The spectrum was baseline corrected using a spline fit (see Figure 3.10 (i)).

Experiment 11

The foregoing enzyme was diluted with buffer (11 cm^3, 0.01 M $P_2O_7^{2-}$ pH 8.0) and used to generate more [^{13}C]-complex. This was concentrated (PM-10) directly to 0.4 cm^3 and examined at 11.0°C. Data was accumulated with DP = 12 H continuous, SI = 16K, AQ = 0.492 s, PW = 54°, NS = 136,802 and the first points of the FID removed with NZP = 2. The new FID was zero-filled to 32K exponentially multiplied with LB = 20 Hz, and Fourier transformed. The spectrum was baseline corrected using a spline fit (see Figure 3.12 (ii)).

Experiment 12

[^{13}C]-oligopeptide (Peptide 2) tryptic digestion of [^{13}C]-complex (from Experiment 9) was examined at 25°C. Data was accumulated with DP = 12 H continuous, SI = 16K, AQ = 0.492 s, PW = 90°, NS = 115,202, and the FID was zero-filled to 32K, exponentially multiplied with LB = 20 Hz and Fourier transformed. The spectrum was baseline corrected using a spline fit (see Figure 3.16).

Experiment 13

Peptide 3 from the tryptic-digested complex was examined at 25°C with DP = 12 H continuous, SI = 16K, AQ = 0.492 s, PW = 90°, NS = 14,400 and the FID was zero-filled to 32K, exponentially multiplied with LB = 20 Hz, and Fourier transformed. The spectrum was baseline corrected using a spline fit (see Figure 3.17).

Experiment 14

[^{12}C]-PBG-deaminase complex (101 µg PBG bound to 4500 U partially purified deaminase without further purification, dissolved in 0.05 M

$P_2O_7^{2-}$, 20% D_2O, pH 8.0 buffer) was examined at 10.5°C. Data was accumulated with DP = 10 H continuous, SI = 8K, AQ = 0.246 s, PW = 90°, NS = 324,500, and the first points of the FID were removed with NZP = 2. The new FID was zero-filled to 16K and exponentially multiplied with LB = 20 Hz prior to Fourier transformation.

Experiment 15

[2,11-^{13}C, ^{3}H]-PBG-deaminase complex (generated identically to the complex in Experiment 14 and dissolved in the same buffer) was examined at 10.5°C. Data was accumulated and processed under identical conditions to Experiment 14. No significant difference detected (see Figure 3.18 (ii)).

Experiment 16

The previous sample was examined under identical conditions except NS = 10,000.

Experiment 17

One quarter of the previous sample was diluted 4 times with buffer (0.0125 M $P_2O_7^{2-}$, 20% D_2O, pH 8.0) and examined under identical conditions to Experiment 16. There was 4 times less signal-to-noise, suggesting that no protein aggregation occurred at high concentrations.

Experiment 18

The previous sample was made 0.05 M and then 0.1 M in KCl and examined under identical conditions. There was a marginal increase in signal-to-noise with increasing ionic strength.

Experiment 19

The previous sample (with 0.1 M KCl) was examined with SI = 16K, AQ = 0.5 s, and then SI = 4K and AQ = 0.1 s. With AQ = 0.5 s, the signal-to-noise was better than both the case with AQ = 0.1 s and also all the previous conditions.

Experiment 20

Progressive saturation was carried out on the previous sample with SI = 4K, AQ = 0.1 s, RD = 0, 0.1, 0.2, 0.4 and 0.9 s, PW = 90°, NS = 10,000. The FID's were zero-filled to 16K, exponentially multiplied with LB = 20 Hz and Fourier transformed. Selected protein resonances at 44, 54 and 130 ppm were used to calculate the following T_1 values: 0.12, 0.23 and 0.52 s, respectively. Using the equation

$$\text{Cos } \alpha_{opt} = e^{-T_R/T_1}$$

and a desired T_R (repetition rate) of 0.25 s, the optimum flip angle α was calculated for each line as 83°, 70° and 52°, respectively.

Experiment 21

The sample from Experiment 14 was adjusted to pH 6.98 (by diluting and re-concentrating on a PM-10 Ultrafilter 3 times with 0.1 M $P_2O_7^{2-}$, 20% D_2O, pH 6.98 buffer) and analysed at 10.5°C. Data was accumulated with DP = 10 H continuous, SI = 8K, AQ = 0.246 s, PW = 90°, NS = 324,502, and the FID was zero-filled to 16K, exponentially multiplied with LB = 20 Hz and Fourier transformed.

Experiment 22

The main sample from Experiment 15, combined with the aliquot removed for Experiments 17-20, was adjusted to pH 7.02 (by diluting and re-concentrating on PM-10 Ultrafilter 3 times with 0.1 M $P_2O_7^{2-}$, 20% D_2O, pH 7.02 buffer) and data was accumulated and processed identically to Experiment 21 (see Figure 3.18 (i)).

Experiment 23

The previous sample was examined with SI = 8K, AQ = 0.246 s, PW = 90°, RD = 0.754 s, NS = 2000 (33 minutes) and the FID zero-filled to 16K, exponentially multiplied with LB = 20 Hz and Fourier transformed.

Experiment 24

The previous sample was examined with SI = 8K, AQ = 0.246 s, RD = 0, PW = 66°, which represents the average of the optimum flip angles calculated in Experiment 20, and NS = 2000 (8 minutes). The FID was processed as before, and the signal-to-noise was found to be comparable. When the sample was examined with NS = 8130 (33 minutes), the signal-to-noise improved significantly.

Experiment 25

The previous sample was re-equilibrated at 30°C and data accumulated as before with SI = 8K, AQ = 0.246 s, RD = 0.754 s, PW = 90°, NS = 2000. The FID was processed as in Experiment 23, and the signal-to-noise dramatically increased. When repeated with PW = 56°, RD = 0, and NS = 8130, signal-to-noise further increased slightly.

Experiment 26

The previous sample was examined under the latter conditions (PW = 56°) with NS = 452,000 and the FID zero-filled to 32K, exponentially multiplied with LB = 20 Hz and Fourier transformed. No detectable substrate/product.

Experiment 27

The sample from Experiment 21 was examined under identical conditions to the previous experiment. Subtractions did not yield any substrate/product.

Experiment 28

The sample from Experiment 26 was treated with 2 drops of 6 M HCl and the precipitated protein collected by centrifugation and redissolved in 8 M urea (2.0 cm^3 + 10% D_2O) and examined at 30°C, with DP = 10 H continuous, SI = 8K, AQ = 0.246 s, PW = 56°, NS = 200,005. The first points of the FID were removed with NZP = 2, and the FID then zero-filled to 32K, exponentially multiplied with LB = 20 Hz and Fourier transformed. The spectrum was baseline corrected using a spline fit (see Figure 3.20 (ii)).

Experiment 29

The sample from Experiment 27 was treated identically as in the previous experiment and examined (NS = 218,130) and processed under identical conditions (see Figure 3.20 (i)).

Experiment 30

[2,11-^{13}C, ^{2}H]-PBG-deaminase complex (5800 U of electrophoretically pure deaminase used, slightly contaminated with tritium, making radioassay of the amount of bound PBG impossible, and further

purified by electrophoresis) dissolved in buffer (0.5 cm^3, 0.1 M $P_2O_7^{2-}$, 90% D_2O, pH 7.0) was examined at 24°C. Data was accumulated with no decoupling, SI = 32K, AQ = 0.590 s, PW = 90° and NS = 264, 812, and the first points of the FID removed with NZP = 2. The new FID was zero-filled to 64K, exponentially multiplied with LB = 50 Hz, Fourier transformed and the spectrum baseline corrected with a spline fit. Substrate signals detected (see Figure 3.21).

Experiment 31

[^{12}C]-PBG-deaminase complex (generated from 6500 U deaminase in a tritium preparation described in Section 9.8) was examined and processed under identical conditions to the previous experiment (see Figure 3.22).

9.6 ^{13}C NMR Studies of [3,5-^{13}C]-PBG-Deaminase Complex

All these experiments were carried out in a 5 mm probe at 75.5 MHz.

Experiment 1

[3,5-^{13}C]-PBG-deaminase complex (450 µg bound to 8000 U partially purified deaminase and purified by electrophoresis) in buffer (0.05 M $P_2O_7^{2-}$, 20% D_2O, pH 8.02) was examined at 3.7°C with DP = 21 H (to maintain NOE's), SI = 8K, AQ = 0.246 s, PW = 90°, SW = 16,666 Hz, NS = 28,800 and the first points of the FID were removed with NZP = 2. The FID was then zero-filled to 32K, exponentially multiplied with LB = 20 Hz and Fourier transformed (see Figure 3.23).

Experiment 2

The previous sample was re-examined under identical conditions over a narrow spectral width, with SI = 10K, SW = 4716 Hz, AQ = 1.077 s. The FID was zero-filled to 16K, and exponentially multiplied with LB = 20 Hz prior to Fourier transformation.

Experiment 3

The previous sample was examined with SI = 2K, AQ = 0.250 s, SW = 4716 Hz, PW = 90°, NS = 115,200 and processed as in Experiment 2 (see Figure 3.24 (i)).

Experiment 4

The previous sample was re-equilibrated to 13.5°C and examined and processed under identical conditions to Experiment 3. Slight improvement in signal-to-noise detected.

Experiment 5

[^{12}C]-PBG-deaminase complex (generated for a tritium study — see Section 9.8) was examined (NS = 132,401) and processed under identical conditions to the previous sample (see Figure 3.24 (ii)).

Experiment 6

The previous sample was re-equilibrated to 21.6°C and examined (NS = 87,083) and processed under identical conditions to Experiment 5 (see Figure 3.25 (ii)).

Experiment 7

The sample from Experiment 4 was examined (NS = 69,707) and processed under identical conditions to the previous experiment (see Figure 3.25 (i)).

Experiment 8

The foregoing sample was adjusted to various pH values with 1 M β-alanine pH 10.26 buffer, using the methods described in Section 9.1. Data was accumulated and processed under identical conditions to those in Experiment 7 (see Figure 3.26).

Experiment 9

To the previous sample at pH 8.0 (adjusted by dilution and re-concentration on a PM-10 Ultrafilter twice with 0.05 M $P_2O_7^{2-}$, 20% D_2O, pH 8.0 buffer) unlabelled PBG (0.7744 mg in 50 µL 0.1 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0 buffer) and a kinetics time-course was started, collecting data every 30 minutes over 6 hours. Data was accumulated under identical conditions (NS = 7200) to Experiment 7, and the FID's were exponentially multiplied with LB = 10 Hz prior to Fourier transformation.

Experiment 10

The end-point of the previous experiment was accumulated overnight under identical conditions (NS = 299,602) to Experiment 9, and the first points of the FID removed with NZP = 2. The new FID was zero-filled to 32K and (i) exponentially multiplied with LB = 10 Hz, prior to Fourier transformation (see Figure 3.27 (i)); and (ii) resolution-enhanced by Gaussian multiplication with a Gaussian broadening of 0.1, and LB = -0.5 Hz, prior to Fourier transformation (see Figure 3.27 (ii)).

9.7 ^{1}H NMR Studies of the Deuteration of ALA

ALA hydrochloride (80 mg *ex* Sigma) was dissolved in D_2O (99.9%) and adjusted to pH 8.0 with solid anhydrous Na_2CO_3. Immediately an aliquot (0.5 cm^3) was removed and examined at 300 MHz and 25°C. Data was accumulated in blocks of 15 minutes over a total of 180 minutes with homonuclear irradiation of HOD line with DP = 346, and SI = 8K, AQ = 1.139 s, PW = 90°, NS = 420, and the FID's were exponentially multiplied with LB = 0.5 Hz prior to Fourier transformation.

The percentage deuteration at C-5 and C-3 was calculated from the peak intensities normalised to the intensity of C-2, which is not susceptible to exchange with deuterium (see Figure 4.1).

9.8 ^{3}H NMR Studies of [2,6,11-$^{3}H_5$]-PBG-Deaminase Complexes

All ^{3}H NMR experiments were performed in a 5 mm probe at 320.2 MHz. The indirect method of referencing was employed. [^{3}H]-PBG containing TSP was examined by tuning the ^{3}H coil to the ^{1}H NMR frequency, and the PBG resonances referenced relative to TSP. The coil was then re-tuned for ^{3}H and the same tritiated resonances were assigned using the relationship $\delta_H = \delta_T$.[196] Since the field was locked onto the HOD line, all subsequent biological samples examined under similar conditions of pH, temperature and ionic strength would be referenced to an accuracy of ±5%.

Experiment 1

[^{3}H]-PBG-deaminase complex (14 mCi bound to 6500 U of partially purified deaminase and further purified by electrophoresis) in buffer

(0.05 M $P_2O_7^{2-}$, 50% D_2O, pH 8.0) was examined at 10.8°C. Data was accumulated with DP = 12 H continuous, SI = 2K, AQ = 0.266 s, PW = 90°, NS = 22,604 and the FID was exponentially multiplied with LB = 6 Hz prior to Fourier transformation (see Figure 4.5 (i)). Data was accumulated for two further periods (NS = 26,500 and 34,313) and processed in the same way (see Figure 4.5 (ii)) and finally for a long run (NS = 43,200) and processed in the same way (see Figure 4.5 (iii)).

Experiment 2

The previous sample was examined at 10.8°C with DP = 12 H continuous, SI = 8K, AQ = 1.065 s, PW = 90°, NS = 42,720 and the FID was exponentially multiplied with LB = 10 Hz prior to Fourier transformation (see Figure 4.6 (i)).

Experiment 3

The previous sample was re-equilibrated to *ca.* 30°C and data accumulated with a kinetics time-course collecting data every hour, with SI = 8K, AQ = 1.065 s, PW = 90°, NS = 3600. The FID was exponentially multiplied with LB = 10 Hz and Fourier transformed (see Figure 4.6 (ii)).

Experiment 4

Progressive saturation was employed to determine the T_1's of the resonances in the previous sample. Data was accumulated with SI = 1K, AQ = 0.133 s, PW = 90°, NS = 3600 and RD = 0, 0.4, 0.9, 1.4 and 1.9 s. The FID's were zero-filled to 8K, exponentially multiplied with LB = 5 Hz and Fourier transformed. The resonances at 2.6 and 3.6 ppm were used to calculate the values for T_1's, both being 0.08 s.

Experiment 5

The previous sample was split into two portions, one being kept at 4°C, and the other examined and processed under the same conditions as in Experiment 3 (see Figure 4.7 (i)).

Experiment 6

To the previous sample, unlabelled PBG (450 µg, in 100 µL 0.05 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0 buffer) was added and data accumulated every 30 minutes over 12 hours, with DP = 12 H continuous, SI = 8K, AQ = 1.065 s, PW = 90°, NS = 1800. The FID's were exponentially multiplied with LB = 10 Hz and Fourier transformed. The linewidths did not seem to have changed significantly, so the T_1's were determined by progressive saturation exactly as in Experiment 4. Again a value of 0.08 s was obtained.

Experiment 7

To the previous sample, further unlabelled PBG (986 µg in 100 µL 0.05 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0 buffer) was added and examined under essentially the same conditions but with PW = 38° and NS = 45,489. The FID was processed as in Experiment 6, and a slight improvement in linewidth had taken place. Porphyrin was assayed spectrophotometrically and this indicated that 20% of the formation expected had taken place.

Experiment 8

An aliquot (0.125 cm^3) of the previous sample was diluted 4 times with buffer (0.375 cm^3, 0.05 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0) and incubated at 37°C for 10 minutes. Data was accumulated with SI = 4K, AQ = 0.532 s, PW = 38°, NS = 136,246 and processed as in Experiment 7

(see Figure 4.7 (ii)). Uro'gen I formation was evident (linewidths decreased) as well as some porphyrin formation (see Figure 4.7 (ii)).

Experiment 9

To the previous sample, iodine (10 μL, 0.2 N I_2 in 0.2 N KI) was added and the sample examined (NS = 268,830) and processed under identical conditions (see Figure 4.7 (iii)). Porphyrin formation clearly took place.

Experiment 10

The other portion of complex from Experiment 5 was re-examined with SI = 8K, AQ = 1.065 s, PW = 90°, NS = 52,387, and the FID exponentially multiplied with LB = 10 Hz prior to Fourier transformation (see Figure 4.8 (i)).

Experiment 11

The other portion of PBG-treated complex from Experiment 8 was re-examined (NS = 83,230) and processed under identical conditions to the previous experiment (see Figure 4.8 (ii)).

Experiment 12

To the sample from Experiment 9, hydroxylamine hydrochloride (50 μL, 0.2 M in 0.2 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0 buffer) was added and data accumulated under identical conditions to Experiment 10, collecting data every hour. Since no changes really took place, the sample was re-run for a longer time (NS = 55,815) and the FID processed as in Experiment 10. No decreases in linewidths of uro'gen I were detected.

Experiment 13

To the sample from Experiment 10, solid urea was added to bring the solution to 8 M, and examined (NS = 79,778) and processed under identical conditions to that of Experiment 10. Considerable sharpening of resonances (see Figure 4.9).

Experiment 14

[^{3}H]-PBG-deaminase complex (16 mCi bound to 4000 U of electrophoretically pure deaminase and purified by gel-filtration on G-50 Sephadex) in buffer (0.4 cm^3, 0.05 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0) was examined over 12 hours collecting data at 30 minute intervals, at 5.5°C. Data was accumulated with DP = 12 H continuous, SI = 8K, AQ = 1.065 s, PW = 90°, NS = 1800 and the FID's added, the first points removed with NZP = 2. The new FID was zero-filled to 16K, exponentially multiplied with LB = 10 Hz, and Fourier transformed. The spectrum was baseline corrected using a spline fit (see Figure 4.10 (i)).

Experiment 15

The previous sample was re-equilibrated to 14°C and examined under identical conditions (7 blocks of NS = 1800), and the FID's added and processed as in the previous experiment (see Figure 4.10 (ii)).

Experiment 16

The previous sample was split into two parts, one part being stored at 4°C, and the other diluted with buffer (200 µL, 0.05 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0 buffer) and re-equilibrated to 23°C. This was examined (9 X NS = 1800) and processed under identical conditions to the previous experiment (see Figure 4.10 (iii)).

Experiment 17

To the previous sample, unlabelled PBG (950 µg) in buffer (50 µL, 0.1 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0) was added and data accumulated as in the previous experiment (at 23°C) except that 30 X NS = 900 (15 minutes) were collected. The FID's were added in fives except for the first one, and processed as in Experiment 14 (see Figure 4.11).

Experiment 18

The remaining portion from Experiment 16 was equilibrated at 3.5°C, and re-examined with SI = 4K, AQ = 0.532 s, PW = 90°, NS = 900 and processed under identical conditions to Experiment 14 (see Figure 4.12 (i)).

Experiment 19

To the previous sample, unlabelled PBG (749 µg) in buffer (50 µL, 0.1 M $P_2O_7^{2-}$, 90% D_2O, pH 8.0) was added and data accumulated in blocks of 7.5 minutes over 225 minutes with DP = 12 H continuous, SI = 4K, AQ = 0.532 s, PW = 90°, NS = 900 and the FID's were added in fives, and the first points removed with NZP = 2. The new FID's were zero-filled to 16K, exponentially multiplied with LB = 10 Hz and Fourier transformed (see Figure 4.12).

Experiment 20

The previous sample was further examined under the same conditions for another 30 X NS = 900, except that after 9 X NS = 900, the temperature was raised to 23°C. The FID's were added in pairs and processed as in the previous experiment (see Figure 4.13).

APPENDIX

APPENDIX 1

Relaxation and Linewidths in Biological NMR

(i) *Spin-lattice relaxation*

This process is due to a net loss of energy of the excited nuclei to the surrounding molecular environment, which can be regarded as an assembly of rapidly fluctuating local magnetic fields. The lost energy is transferred to the lattice as extra rotational or translational energy. The time constant for this process is designated T_1, and it is made up of several components. In large biological molecules, the most dominant features of T_1 relaxation are from dipolar relaxation (T_{1DD}) and chemical shift anisotropy (CSA). In dipolar relaxation, a nucleus such as ^{13}C may be attached to a ^{1}H atom, and here the two spins act as small dipoles located at the centre of the ^{13}C and ^{1}H atoms. Consequently the ^{13}C nucleus experiences a small magnetic field due to the dipolar interaction with the proton, and this depends on the magnitude of the two dipoles (μ_C and μ_H) and the orientation of their line of interaction relative to the applied field. As the molecule tumbles in solution, relaxation can be induced by the local oscillating magnetic field, which for small molecules (M_r <1000) and non-viscous solutions is a function of the correlation time τ_c, and the inverse sixth power of the distance between the two atoms. The correlation time is a measure of how rapidly the molecule undergoes reorientations in solution.

In CSA relaxation, if a non-protonated carbon is placed with its axis perpendicular to the external magnetic field, the shielding

of the carbon by the electrons will be different from that found if the axis is parallel to the field. Rotational motion then produces a fluctuating magnetic field and relaxation may occur by this mechanism. While in many systems, CSA is a negligible relaxation mechanism, at higher magnetic field strengths it becomes increasingly dominant, being the principal relaxation for sp^2 carbons at fields >6 T.

(ii) *Spin-spin relaxation*

In spin-spin relaxation, two nuclear spins in close proximity interchange energy with one another. This can occur because the magnetic field generated by a spinning nucleus has a component perpendicular, or transverse as it is known, to the main field, and thus is ideally set up for inducing transitions in the adjacent nuclear spin. The magnetic field produced by one nucleus will also cause a variation in the field experienced by the other nucleus, and obviously the reverse is true. This process results in spin-spin or transverse relaxation, with an exponential time constant designated T_2. The result is a spreading of the resonance frequency, which leads to a broadening of the resonance lines in the spectrum.

Also dependent on the relaxation mechanisms is the nuclear Overhauser effect (NOE). An NMR spectrum of a nucleus such as ^{13}C obtaied with simultaneous proton irradiation often yields an improvement in signal-to-noise of the carbon resonance in addition to removal of the ^{13}C-^{1}H scalar coupling. This is the NOE phenomenon, and is due to a favourable redistribution of the ^{13}C energy level populations caused by irradiation of nearby protons. Therefore the

NOE depends on the influence of these protons on the relaxation mechanisms. It should be emphasised that while ^{13}C and ^{1}H are the most quoted examples of NOE, any two nuclear spins exhibiting coupling by a relaxation interaction show NOE's, and such nuclei need not necessarily exhibit a scalar coupling J_{AB}.

REFERENCES

REFERENCES

1. F. Hoppe-Seyler, *Z. Physiol. Chem.*, 4, 193 (1880).
2. R. Willstätter, Uber Pflanzenfarbenstoffe, in "Nobelshiftelsen, Stockholm, Les Prix Nobel en 1914-1918", p. 1. P. A. Norstedt, Stockholm (1920).
3. H. Fischer and H. Orth, "Die Chemie des Pyrrols", 2 vols., Akademische Verlags-geschellschaft, Leipzig (1937).
4. R. B. Woodward, *Angew. Chem.*, 72, 651 (1960).
5. E. L. Rickes, N. G. Brink, F. R. Konivszy, T. R. Wood and K. Folkers, *Science*, 107, 396 (1948).
6. E. L. Smith and L. F. J. Parker, *Biochem. J.*, 43, vii (1948).
7. D. E. Wolf, W. H. Jones, J. Valiant and K. Folkers, *J. Amer. Chem. Soc.*, 72, 2820 (1950).
8. J. B. Armitage, J. R. Cannon, A. W. Johnson, L. F. J. Parker, E. L. Smith, W. H. Stafford and A. R. Todd, *J. Chem. Soc.*, 3849 (1953).
9. C. Brink, D. C. Hodgkin, J. Lindsey, J. Pickworth, J. H. Robertson and J. G. White, *Nature*, 174, 1169 (1954).
10. D. C. Hodgkin, J. Pickworth, J. H. Robertson, R. J. Prosen, R. A. Sparks and K. N. Trueblood, *Proc. R. Soc. Lond. A*, 251, 306 (1959).
11. P. G. Lentert and D. C. Hodgkin, *Nature*, 192, 937 (1961).
12. R. B. Woodward, *Pure Appl. Chem.*, 33, 145 (1973).
13. A. Eschenmoser, "23rd Internat. Congress Pure and Applied Chem.", Butterworths, London, Vol. 2, p. 69 (1971).
14. M. Akhtar and P. M. Jordan, in "Comprehensive Organic Chemistry", Ed. W. D. Ollis, Pergamon, London, Vol. 5, pp. 1121-1163 (1978).

15. L. Bogorad, in "The Porphyrins", Ed. D. Dolphin, Academic Press, New York, Vol. VI, pp. 125-178 (1979).

16. D. G. Buckley, *Ann. Rev. Chem. Soc. B*, 74, 392 (1977).

17. A. R. Battersby and E. McDonald, in "Porphyrins and Metalloporphyrins", Ed. K. M. Smith, Elsevier, Amsterdam, pp. 61-122 (1975).

18. B. F. Burnham, in "Metabolic Pathways", Ed. D. Greensberg, Academic Press, New York, Vol. III, pp. 404-539 (1969).

19. D. Shemin, *Harvey Lectures*, 50, 258 (1955) and references therein.

20. A. Comfort, *Science*, 122, 279 (1950).

21. Ref. 17, p. 75.

22. D. Mauzerall, *J. Amer. Chem. Soc.*, 82, 2605 (1960).

23. G. W. Hodgson and C. A. Ponnamperuma, *Proc. Natl. Acad. Sci. U.S.A.*, 59, 22 (1968).

24. G. W. Hodgson and B. L. Baker, *Nature*, 202, 125 (1964).

25. N. H. Georgopapadakou and A. I. Scott, *J. Theor. Biol.*, 69, 381 (1977).

26. M. L. Martin, J. J. Delpuech, G. J. Martin, "Practical NMR Spectroscopy", Heyden, London (1980).

27. T. C. Farrar and E. D. Becker, "Pulse and Fourier Transform NMR: Introduction to Theory and Methods", Academic Press, New York (1971).

28. R. K. Harris and B. E. Mann, "NMR and the Periodic Table", Academic Press, London (1978).

29. J. B. Stothers, "^{13}C NMR Spectroscopy", Academic Press, New York (1972).

30. R. J. Abraham and P. Loftus, "Proton and Carbon-13 NMR Spectroscopy", Heyden, London (1978).

31. K. Wüthrich, "NMR in Biological Research: Peptides and Proteins", North Holland Publishing Co., Amsterdam (1976).

32. R. A. Dwek, "Nuclear Magnetic Resonance (NMR) in Biochemistry", Oxford University Press (1973).

33. T. L. James, "Nuclear Magnetic Resonance in Biochemistry", Academic Press, New York (1975).

34. R. A. Dwek, I. D. Campbell, R. E. Richards and R. J. P. Williams, "NMR in Biology", Academic Press, New York (1977).

35. O. Jardetzky and G. C. K. Roberts, "NMR in Molecular Biology", Academic Press, New York (1981).

36. A. Allerhand, in "Methods in Enzymology", Academic Press, New York, Vol. 61, pp. 458-549 (1979).

37. R. S. Norton, A. O. Clouse, R. Addleman and A. Allerhand, *J. Amer. Chem. Soc.*, 99, 79 (1979).

38. A. M. Nigen, P. Keim, R. C. Marshall, J. S. Morrow and F. R. N. Gurd, *J. Biol. Chem.*, 247, 4100 (1972).

39. A. M. Nigen, P. Keim, R. C. Marshall, J. S. Morrow, R. A. Vigna and F. R. N. Gurd, *J. Biol. Chem.*, 248, 3724 (1973).

40. R. G. Khalifah, *Biochemistry*, 16, 2236 (1977).

41. R. B. Moon, K. Dill and J. H. Richards, *Biochemistry*, 16, 221 (1977).

42. R. B. Moon and J. H. Richards, *J. Amer. Chem. Soc.*, 94, 5093 (1972).

43. N. A. Matwiyoff, P. J. Vergamini, T. E. Needham, C. T. Gregy, J. A. Volpe and W. S. Caughey, *J. Amer. Chem. Soc.*, 95, 4429 (1973).

44. M. G. Choc and W. S. Caughey, *J. Biol. Chem.*, 256, 1831 (1981).

45. D. H. Rich, M. S. Bernatowicz and P. G. Schmidt, *J. Amer. Chem. Soc.*, 104, 3535 (1982).

46. J. P. G. Malthouse, N. E. Mackenzie, A. S. F. Boyd and A. I. Scott, *J. Amer. Chem. Soc.*, 105, 1685 (1983).

47. M. P. Gamcsik, J. P. G. Malthouse, W. U. Primrose, N. E. Mackenzie, A. S. F. Boyd, R. A. Russell and A. I. Scott, *J. Amer. Chem. Soc.*, 105, 6324 (1983).

48. B. D. Bray and E. T. Harper, *J. Amer. Chem. Soc.*, 105, 3731 (1983).

49. J. P. G. Malthouse, M. P. Gamcsik, A. S. F. Boyd, N. E. Mackenzie and A. I. Scott, *J. Amer. Chem. Soc.*, 104, 6811 (1982).

50. G. Wagner and K. Wüthrich, *J. Mol. Biol.*, 155, 347 (1982).

51. K. H. Mayo, P. M. Tyrell and J. H. Prestegard, *Biochemistry*, 22, 4485 (1983).

52. S. Granick, *Ann. Rev. Plant Physiol.*, 2, 115 (1951).

53. D. Shemin and D. C. Bray, *Ann. N. Y. Acad. Sci.*, 112, 615 (1964).

54. Z. Zaman, P. M. Jordan and M. Akhtar, *Biochem. J.*, 135, 257 (1973).

55. M. M. Abboud, P. M. Jordan and M. Akhtar, *J. Chem. Soc. Chem. Comm.*, 643 (1974).

56. M. Akhtar, M. M. Abboud, G. Barnard, P. M. Jordan and Z. Zaman, *Phil. Trans. R. Soc. Lond. B*, 273, 117 (1976).

57. S. I. Beale, *Phil. Trans. R. Soc. Lond. B*, 273, 99 (1976) and references cited therein.

58. R. J. Porra and H.-U. Meisch, *Trends Biochem. Sci.*, 9, 99 (1984).

59. L. Varticousti, J. P. Kushner and B. F. Burnham, *Int. J. Biochem.*, 12, 743 (1980).

60. J. M. Turner and A. Neuberger, in "Methods in Enzymology", Academic Press, New York, Vol. 17A, p. 188 (1970) and references cited therein.

61. R. Schmid and D. Shemin, *J. Amer. Chem. Soc.*, 77, 506 (1955).

62. D. L. Nandi and D. Shemin, *J. Biol. Chem.*, 243, 1231, 1236 (1968).

63. D. Shemin, *Phil. Trans. R. Soc. Lond. B*, 273, 109 (1976).

64. M. M. Abboud and M. Akhtar, *J. Chem. Soc. Chem. Comm.*, 1007 (1976).

65. P. M. Jordan and J. S. Seehra, *J. Chem. Soc. Chem. Comm.*, 240 (1980).

66. P. M. Jordan and J. S. Seehra, *FEBS Letts.*, 114, 283 (1980).

67. L. Bogorad and S. Granick, *Proc. Natl. Acad. Sci. U.S.A.*, 39, 1176 (1953).

68. H. L. Booij and C. Rimington, *Biochem. J.*, 65, 3, 4 (1957).

69. L. Bogorad, *Science*, 121, 878 (1955).

70. L. Bogorad, *J. Biol. Chem.*, 233, 501, 510 (1958).

71. R. C. Davies and A. Neuberger, *Biochem. J.*, 133, 471 (1973).

72. P. M. Jordan and D. Shemin, *J. Biol. Chem.*, 248, 1019 (1973).

73. H. A. Sancovich, A. M. Del C. Batlle and M. Grinstein, *Biochim. Biophys. Acta*, 191, 130 (1969).

74. R. B. Frydman and B. Frydman, *Arch. Biochem. Biophys.*, 136, 193 (1970).

75. M. Higuchi and L. Bogorad, *Ann. N. Y. Acad. Sci.*, 244, 401 (1975).

76. L. Bogorad, in "Methods in Enzymology", Academic Press, New York, Vol. 5, p. 885 (1972).

77. D. C. Williams, G. S. Morgan, E. McDonald and A. R. Battersby, *Biochem. J.*, 193, 301 (1981).

78. R. B. Frydman and G. Feinstein, *Biochim. Biophys. Acta*, 350, 358 (1974).

79. P. M. Anderson and R. J. Desnick, *J. Biol. Chem.*, 255, 1993 (1980).

80. L. Bogorad, *Plant Physiol.*, 32, xli (1975).

81. L. Bogorad, in "Comparative Biochemistry of Photoreactive Systems", Ed. M. B. Allen, Academic Press, New York, p. 227 (1960).

82. A. T. Carpenter and J. J. Scott, *Biochem. J.*, 71, 325 (1959).

83. A. T. Carpenter and J. J. Scott, *Biochim. Biophys. Acta*, 52, 195 (1968).

84. H. Heath and D. S. Hoare, *Biochem. J.*, 72, 14 (1959) and *Ibid.*, 73, 679 (1959).

85. M. Yuan and C. S. Russell, *FEBS Letts.*, 46, 34 (1974).

86. E. Stevens and B. Frydman, *Biochim. Biophys. Acta*, 151, 429 (1968).

87. E. Y. Levin, *Biochemistry*, 7, 3781 (1968) and *Ibid.*, 10, 4669 (1971).

88. E. Stevens, R. B. Frydman and B. Frydman, *Biochim. Biophys. Acta*, 158, 496 (1968).

89. H. Nordlöv, P. M. Jordan, M. M. Schneider, S. Hosozawa and A. I. Scott, unpublished results.

90. A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham and P. S. Pandey, *Angew. Chem. Int. Ed. Engl.*, 20, 293 (1981).

91. A. R. Battersby, D. G. Buckley, G. L. Hodgson, R. E. Markwell and E. McDonald, in "High Pressure Liquid Chromatography in Clinical Chemistry", Eds. P. F. Dixon, C. H. Gray, C. K. Lim and M. S. Stoll, Academic Press, New York, p. 63 (1976).

92. A. R. Battersby, C. J. R. Fookes, K. E. Gustafson-Potter, E. McDonald and G. W. J. Matcham, *J. Chem. Soc. Perkin I*, 2413 (1982).

93. J. C. Bommer, B. F. Burnham, R. E. Carlson and D. Dolphin, *Anal. Biochem.*, 95, 444 (1979).

94. H. Nordlöv, P. M. Jordan, G. Burton and A. I. Scott, *J. Chromatogr.*, 190, 221 (1980).

95. A. H. Jackson, K. R. N. Rao and S. G. Smith, *Biochem. J.*, 203, 515 (1982).

96. A. H. Wayne, R. C. Straight, E. E. Wales and E. Engelert, Jr., *J. High Res. Chrom. & Chrom. Comm.*, 2, 621 (1979).

97. I. C. Walker, M. T. Gilbert and K. Stubbs, *J. Chromatogr.*, 202, 491 (1980).

98. P. M. Jordan, H. Nordlöv, G. Burton and A. I. Scott, *FEBS Letts.*, 115, 269 (1980).

99. L. Bogorad, *Ann. N. Y. Acad. Sci.*, 104, 676 (1963).

100. E. B. C. Llambias and A. M. Del C. Batlle, *FEBS Letts.*, 6, 285 (1970).

101. See Ref. 16 and references cited therein (5 and 10).

102. J. H. Mathewson and A. H. Corwin, *J. Amer. Chem. Soc.*, 83, 135 (1961).

103. J. Plusec and L. Bogorad, *Biochemistry*, 9, 4736 (1970).

104. B. Frydman and R. B. Frydman, *Accounts Chem. Res.*, 8, 201 (1975).

105. B. Frydman, R. B. Frydman, A. Valisinas, E. S. Levy and G. Feinstein, *Phil. Trans. R. Soc. Lond. B*, 273, 137 (1976).

106. A. R. Battersby, D. A. Evans, K. H. Gibson, E. McDonald, L. N. Mander and J. Moron, *J. Chem. Soc. Chem. Comm.*, 768 (1973).

107. A. I. Scott, K. S. Ho, M. Kajiwara and T. Takahashi, *J. Amer. Chem. Soc.*, 98, 1589 (1976).

108. R. Radmer and L. Bogorad, *Biochemistry*, 11, 404 (1972).

109. A. R. Battersby, E. Hunt and E. McDonald, *J. Chem. Soc. Chem. Comm.*, 442 (1973).

110. A. R. Battersby, G. L. Hodgson, E. Hunt, E. McDonald and J. Saunders, *J. Chem. Soc. Perkin I*, 273 (1976).

111. A. R. Battersby, E. Hunt, E. McDonald and J. Moron, *J. Chem. Soc. Perkin I*, 2917 (1973).

112. A. R. Battersby, J. Moron, E. McDonald and J. Feeney, *J. Chem. Soc. Chem. Comm.*, 920 (1972).

113. A. R. Battersby, G. L. Hodgson, M. Ihara, E. McDonald and J. Saunders, *J. Chem. Soc. Chem. Comm.*, 441 (1973).

114. A. R. Battersby, G. L. Hodgson, M. Ihara, E. McDonald and J. Saunders, *J. Chem. Soc. Perkin I*, 2923 (1973).

115. H.-O. Dauner, G. Gunzer, I. Heger and G. Müller, *Hoppe-Seyler's Z. Physiol. Chem.*, 357, 147 (1976).

116. A. R. Battersby, E. McDonald, D. C. Williams and H. K. W. Wurziger, *J. Chem. Soc. Chem. Comm.*, 113 (1977).

117. A. R. Battersby, D. G. Buckley, D. W. Johnson, L. N. Mander, E. McDonald and D. C. Williams, *J. Chem. Soc. Perkin I*, 2779 (1981).

118. A. R. Battersby, C. J. R. Fookes, E. McDonald and M. J. Meegan, *J. Chem. Soc. Chem. Comm.*, 185 (1978).

119. A. R. Battersby, C. J. R. Fookes, M. Meegan, E. McDonald and H. K. W. Wurziger, *J. Chem. Soc. Perkin I*, 2786 (1981).

120. A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham and E. McDonald, *J. Chem. Soc. Chem. Comm.*, 1064 (1978).

121. A. R. Battersby, C. J. R. Fookes, K. E. Gustafson-Potter, E. McDonald and G. W. J. Matcham, *J. Chem. Soc. Perkin I*, 2413 (1982).

122. L. Diaz, R. B. Frydman, A. Valasinas and B. Frydman, *J. Amer. Chem. Soc.*, 101, 2710 (1979).

123. G. Burton, P. E. Fagerness, S. Hosozawa, P. M. Jordan and A. I. Scott, *J. Chem. Soc. Chem. Comm.*, 202 (1979).

124. P. M. Jordan, G. Burton, H. Nordlöv, M. M. Schneider, L. Pryde and A. I. Scott, *J. Chem. Soc. Chem. Comm.*, 204 (1979).

125. A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham, E. McDonald and (in part) K. E. Gustafson-Potter, *J. Chem. Soc. Chem. Comm.*, 316 (1979).

126. G. Burton, H. Nordlöv, S. Hosozawa, H. Matsumoto, P. M. Jordan, P. E. Fagerness, L. M. Pryde and A. I. Scott, *J. Amer. Chem. Soc.*, 101, 3114 (1979).

127. A. R. Battersby, C. J. R. Fookes, K. E. Gustafson-Potter, G. W. J. Matcham and E. McDonald, *J. Chem. Soc. Chem. Comm.*, 1155 (1979).

128. A. R. Battersby, C. J. R. Fookes, K. E. Gustafson-Potter, E. McDonald and G. W. J. Matcham, *J. Chem. Soc. Perkin I*, 2427 (1982).

129. A. I. Scott, G. Burton, P. M. Jordan, H. Matsumoto, P. E. Fagerness and L. M. Pryde, *J. Chem. Soc. Chem. Comm.*, 384 (1980).

130. A. R. Battersby, R. G. Brereton, C. J. R. Fookes, E. McDonald and G. W. J. Matcham, *J. Chem. Soc. Chem. Comm.*, 1124 (1980).

131. A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham and E. McDonald, *Nature*, 285, 17 (1980).

132. A. I. Scott, *Pure & Appl. Chem.*, 53, 1215 (1981).

133. A. Gossauer, W. Neidhart and A. I. Scott, *J. Chem. Soc. Chem. Comm.*, 883 (1983).

134. A. R. Battersby, C. J. R. Fookes, E. McDonald and G. W. J. Matcham, *Bioorg. Chem.*, 8, 451 (1979).

135. A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham and E. McDonald, *J. Chem. Soc. Chem. Comm.*, 539 (1979).

136. A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham, E. McDonald and (in part) R. Hollenstein, *J. Chem. Soc. Perkin I*, 3031 (1983).

137. P. M. Jordan and J. S. Seehra, *FEBS Letts.*, 104, 364 (1979).

138. J. S. Seehra and P. M. Jordan, *J. Amer. Chem. Soc.*, 102, 6841 (1980).

139. P. M. Jordan and A. Berry, *Biochem. J.*, 195, 177 (1981).

140. A. Berry and P. M. Jordan, *Biochem. Soc. Trans.*, 231 (1981).

141. A. Berry, P. M. Jordan and J. S. Seehra, *FEBS Letts.*, 129, 220 (1981).

142. K. Bernhauer, F. Wagner, H. Michna, P. Rapp and H. Vogelmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 349, 1297 (1968).

143. A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara and R. J. Cushley, *J. Amer. Chem. Soc.*, 94, 8269 (1972).

144. A. I. Scott, N. Georgopapadakou, K. S. Ho, S. Klioze, E. Lee, S. L. Lee, G. H. Temme, III, C. A. Townsend and I. M. Armitage, *J. Amer. Chem. Soc.*, 97, 2548 (1975).

145. A. R. Battersby, M. Ihara, E. McDonald, F. Satoh and D. C. Williams, *J. Chem. Soc. Chem. Comm.*, 436 (1975).

146. A. I. Scott, C. A. Townsend, K. Okada, M. Kajiwara, R. J. Cushley and P. J. Whitman, *J. Amer. Chem. Soc.*, 96, 8069 (1974).

147. C. E. Brown, J. J. Katz and D. Shemin, *Proc. Natl. Acad. Sci. U.S.A.*, 68, 4083 (1973).

148. A. R. Battersby, M. Ihara, E. McDonald and J. R. Stephenson, *J. Chem. Soc. Chem. Comm.*, 404, 458 (1974).

149. A. R. Battersby, E. McDonald, R. Hollenstein, M. Ihara, F. Satoh and D. C. Williams, *J. Chem. Soc. Perkin I*, 166 (1977).

150. A. R. Battersby, M. Ihara, E. McDonald, J. R. Redfern and B. J. Golding, *J. Chem. Soc. Perkin I*, 158 (1977).

151. A. I. Scott, C. A. Townsend and R. J. Cushley, *J. Amer. Chem. Soc.*, 95, 5759 (1973).

152. M. Imfeld, C. A. Townsend and D. Arigoni, *J. Chem. Soc. Chem. Comm.*, 541 (1976).

153. A. R. Battersby, R. Hollenstein, E. McDonald and D. C. Williams, *J. Chem. Soc. Chem. Comm.*, 543 (1976).

154. A. I. Scott, M. Kajiwara, T. Takahashi, I. M. Armitage, P. Demou and D. Petrocine, *J. Chem. Soc. Chem. Comm.*, 544 (1976).

155. L. M. Siegel, M. J. Murphy and H. Kamin, *J. Biol. Chem.*, 248, 251 and 2801 (1973).

156. A. R. Battersby, E. McDonald, H. Morris, M. Thompson, D. C. Williams, V. Bykhovsky, N. Zaitseva and V. Bukia, *Tet. Lett.*, 2217 (1977).

157. A. R. Battersby, E. McDonald, K. Jones, J. A. Robinson and H. R. Morris, *Tet. Lett.*, 2213 (1977).

158. A. I. Scott, A. J. Irwin, L. M. Siegel and J. M. Shoolery, *J. Amer. Chem. Soc.*, 100, 316 and 7987 (1978).

159. A. R. Battersby, E. McDonald, M. Thompson and V. Ya Bykhovsky, *J. Chem. Soc. Chem. Comm.*, 150 (1978).

160. R. Deeg, H.-P. Kriemler, K.-H. Bergmann and G. Müller, *Hoppe-Seyler's Z. Physiol. Chem.*, 358, 339 (1977).

161. K.-H. Bergmann, R. Deeg, K. D. Gneuss, H.-P. Kriemler and G. Müller, *Hoppe-Seyler's Z. Physiol. Chem.*, 358, 1315 (1977).

162. A. I. Scott, presented in "3rd Europ. Symp. on Vitamin B_{12} and Intrinsic Factors", Walter de Gruyter, Berlin (1979).

163. A. R. Battersby and E. McDonald, *Bioorg. Chem.*, 7, 161 (1978).

164. G. Müller, K. D. Gneuss, H.-P. Kriemler, A. I. Scott and A. J. Irwin, *J. Amer. Chem. Soc.*, 101, 3655 (1979).

165. G. Müller, K. D. Gneuss, H.-P. Kriemler, A. J. Irwin and A. I. Scott, *Tetrahedron*, 37, 81 (1981).

166. A. R. Battersby, G. W. J. Matcham, E. McDonald, R. Neier, M. Thompson, W.-D. Woggon, V. Ya Bykhovsky and H. R. Morris, *J. Chem. Soc. Chem. Comm.*, 185 (1979).

167. M. Kajiwara, K. S. Ho, H. Klein, A. I. Scott, A. Gossauer, J. Enzel, E. Neumann and H. Zilch, *Bioorg. Chem.*, 6, 397 (1977).

168. A. R. Battersby, N. G. Lewis, R. Neier, G. W. J. Matcham and E. McDonald, *J. Chem. Soc. Chem. Comm.*, 541 (1979).

169. L. Mombelli, C. Nussbaumer, H. Weber, G. Müller and D. Arigoni, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 11 (1981).

170. C. Nussbaumer, M. Imfeld, G. Wörner, G. Müller and D. Arigoni, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 9 (1981).

171. A. R. Battersby, M. J. Bushell, C. Jones, N. G. Lewis and A. Pfenninger, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 13 (1981).

172. V. Rasetti, A. Pfaltz, C. Kratky and A. Eschenmoser, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 16 (1981).

173. H. Falk and K. Grubmayr, *Synthesis*, 614 (1977).

174. J.-H. Furhop and K. M. Smith, in "Porphyrins and Metalloporphyrins", Ed. K. M. Smith, Elsevier, Amsterdam, p. 758 (1975).

175. H. Falk and T. Schlederer, *Monatsh. Chemie*, 112, 501 (1981).

176. S. Granick and L. Bogorad, *J. Amer. Chem. Soc.*, 75, 3610 (1953).

177. B. Franck and H. Stratmann, *Heterocycles*, 15, 919 (1981).

178. A. E. A. Mitta, A. N. Ferramola, H. A. Sancovitch and M. Grinstein, *J. Labelled Compds.*, 3, 20 (1967).

179. D. L. Nandi, K. F. Baker-Cohen and D. Shemin, *J. Biol. Chem.*, 243, 1224 (1968).

180. B. F. Burnham and J. Lascelles, *Biochem. J.*, 87, 462 (1963).

181. D. Mauzerall and S. Granick, *J. Biol. Chem.*, 219, 435 (1956).

182. H. L. Surprenant, J. E. Sarneski, R. R. Key, J. T. Byrd and C. N. Reilley, *J. Magn. Res.*, 40, 231 (1980).

183. W. J. Horsley and H. Sternlicht, *J. Amer. Chem. Soc.*, 90, 3738 (1968).

184. W. Horsley, H. Sternlicht and J. S. Cohen, *J. Amer. Chem. Soc.*, 92, 680 (1970).

185. J. G. Batchelor, J. Feeney and G. C. K. Roberts, *J. Magn. Res.*, 20, 19 (1975).

186. G. Burton, P. E. Fagerness, P. M. Jordan and A. I. Scott, *Tetrahedron*, 36, 2721 (1980).

187. D. G. Smith, in "Methods in Enzymology", Vol. XI, p. 230 (1967) and references cited therein.

188. A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham, E. McDonald and (in part) R. Hollenstein, *J. Chem. Soc. Perkin I*, 3031 (1983).

189. A. R. Battersby, C. J. R. Fookes, G. Hart, G. W. J. Matcham and P. S. Pandey, *J. Chem. Soc. Perkin I*, 3041 (1983).

190. G. Allen, "Sequencing of Proteins and Peptides", Laboratory Techniques in Biochemistry and Molecular Biology Series, Elsevier, *passim* (1981).

191. D. H. Williams, C. V. Bradley, S. Santikarn and G. Bojesen, *Biochem. J.*, 201, 105 (1982).

192. M. Barber, R. S. Bordoli, R. D. Sedgwick, A. N. Tyler and E. T. Whalley, *Biomed. Mass Spectrom.*, 8, 337 (1981).

193. Unpublished observations from this laboratory.

194. G. V. D. Tiers, C. A. Brown, R. A. Jackson and T. N. Lahr, *J. Amer. Chem. Soc.*, 86, 2526 (1964).

195. J. Bloxsidge, J. A. Elvidge, J. R. Jones and E. A. Evans, *Org. Magn. Res.*, 3, 127 (1971).

196. J. M. A. Al-Rawi, J. P. Bloxsidge, C. O'Brien, D. E. Caddy, J. A. Elvidge, J. R. Jones and E. A. Evans, *J. Chem. Soc. Perkin II*, 1635 (1974).

197. J. P. Bloxsidge, J. A. Elvidge, J. R. Jones, R. B. Mane and M. Saljoughian, *Org. Magn. Res.*, 12, 574 (1979).

198. J. M. A. Al-Rawi, J. A. Elvidge, J. R. Jones and E. A. Evans, *J. Chem. Soc. Perkin II*, 449 (1975).

199. J. M. A. Al-Rawi, J. P. Bloxsidge, J. A. Elvidge, J. R. Jones, V. M. A. Chambers and E. A. Evans, *Steroids*, 28, 359 (1976).

200. L. J. Altman and N. Silberman, *Anal. Biochem.*, 79, 302 (1977) and *Steroids*, 29, 557 (1977).

201. J. P. Bloxsidge, J. A. Elvidge, J. R. Jones and R. B. Mane, *J. Chem. Research*, 258 (1977).

202. J. A. Elvidge, J. R. Jones, C. O'Brien, E. A. Evans and H. C. Sheppard, *J. Chem. Soc. Perkin II*, 174 (1974).

203. J. M. A. Al-Rawi, J. A. Elvidge, R. Thomas and B. J. Wright, *J. Chem. Soc. Chem. Comm.*, 1031 (1974)

204. J. M. A. Al-Rawi, J. A. Elvidge, D. K. Jaiswal, J. R. Jones and R. Thomas, *J. Chem. Soc. Chem. Comm.*, 220 (1974).

205. J. M. A. Al-Rawi, J. A. Elvidge, J. R. Jones, V. M. A. Chambers and E. A. Evans, *J. Label. Comp. Radiopharm.*, 12, 295 (1976).

206. J. A. Elvidge, D. K. Jaiswal, J. R. Jones and R. Thomas, *J. Chem. Soc. Perkin I*, 1080 (1977).

207. L. J. Altman, C. Y. Han, A. Bertolino, G. Hardy, D. Laungani, W. Muller, S. Schwartz, D. Shanker, W. H. DeWolf and F. Yang, *J. Amer. Chem. Soc.*, 100, 3235 (1978).

208. V. M. A. Chambers, E. A. Evans, J. A. Elvidge and J. R. Jones, "Tritium nuclear magnetic resonance (tnmr) spectroscopy", Review 19, Amersham Corporation, Illinois, U.S.A., May 1978.

209. S. Libor, J. P. Bloxsidge, J. A. Elvidge, J. R. Jones, L. F. J. Woods and A. Wiseman, *Biochem. Soc. Trans.*, 8, 99 (1980).

210. Craig Townsend, Ph.D. Thesis, Yale University (1974).

211. A. Laghai and P. M. Jordan, *Biochem. Soc. Trans.*, 5, 299 (1977).

212. A. R. Battersby, J. F. Beck and E. McDonald, *J. Chem. Soc. Perkin I*, 162 (1974).

213. Dr. N. E. Mackenzie, unpublished results.

214. D. C. Williams, *Biochem. J.*, 217, 675 (1984).

215. E. J. Cohn and J. T. Edsell, "Proteins, Amino Acids and Peptides", Reinhold Publ. Corp., New York, p. 445 (1943).

216. cf. C. S. Russell, S. Pollack and J. James, *Fed. Proc. U.S.A.*, 43, 1545 (1984).

217. G. H. Cookson and C. Rimington, *Biochem. J.*, 57, 476 (1954).

218. E. Bullock, A. W. Johnson, E. Markham and K. B. Shaw, *J. Chem. Soc.*, 1430 (1958).

219. C. S. Russell, *J. Theor. Biol.*, 47, 145 (1974).

220. K. S. Ho, Ph.D. Thesis, Yale University, p. 99 (1977).

221. L. Varticovski, J. P. Kushner and B. F. Burnham, *J. Biol. Chem.*, 255, 3742 (1980).

222. cf. P. Hérion and A. Boller, *Bioscience Reports*, 3, 373 (1983).

223. J. Lascelles, *Biochem. J.*, 62, 78 (1956).

224. O. Warburg and W. Christian, *Biochem. Z.*, 310, 384 (1941).

225. E. Layne, in "Methods in Enzymology", Vol. III, p. 454 (1957).

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Natural Products – Professor A. I. Scott (5 lectures).

β-Lactam Antibiotics – Glaxo Research (5 lectures).

FT-IR – series of 5 lectures.

Bio-organic Chemistry – series of 5 lectures.

Current Topics in Organic Chemistry – organic staff (10 lectures).

Professor Scott's research group seminars over a period of four years.

Various departmental colloquia and seminars, Monday evening organic seminars over two years.

Various seminars and specialised courses in Texas over two years.