The Progesterone Hydroxylase Cytochrome P450 Multicomponent System of *Streptomyces roseochromogenes*: Purification, Characterisation and Regulation

A Thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the University of London

by

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Dedicated to
my parents

ABSTRACT

Streptomyces roseochromogenes, NCIB 10984, hydroxylates exogenous progesterone to 16α hydroxyprogesterone and thereafter in a second phase bioconversion to 2β,16α-dihydroxyprogesterone. Characterisation of this reaction was carried out at the whole cell level. The cellular components responsible for this reaction were also purified to homogeneity. *S. roseochromogenes* contains a cytochrome P450 and two electron transfer proteins, roseoredoxin and roseoredoxin reductase. A reconstituted incubation containing these purified proteins and the natural electron donor, NADH, produced identical hydroxyprogesterone metabolites as intact cells.

In sodium periodate ($NaIO_4$) supported incubations, the initial rate of progesterone hydroxylation was marginally higher than in the natural reconstituted system but the product yield was significantly lower. The yield data showed that the reconstituted natural pathway, supported multiple rounds of hydroxylation in contrast to a likely single round by a minority of P450s in the periodate reaction.

When *S. roseochromogenes* was incubated with exogenous progesterone for 25 h the major metabolite, 16α-hydroxyprogesterone was produced in 3.6 fold excess to the minor metabolite 2β,16α-dihydroxyprogesterone. In a reconstituted system containing highly purified progesterone 16α-hydroxylase cytochrome P450, roseoredoxin and roseoredoxin reductase, both metabolites were produced but in a 10:1 ratio. When *S. roseochromogenes* was pre-incubated with progesterone and the purified components of the hydroxylase system assayed as before, the ratio of 16α-hydroxyprogesterone to 2β,16α-dihydroxyprogesterone produced, decreased to 2.8:1, virtually identical to the ratio in whole cell biotransformations. Reconstitution assays containing all combinations of hydroxylase proteins purified from progesterone pre-incubated and control cells, identified roseoredoxin as solely responsible for the observed changes in *in vitro* metabolite ratios. The fact that the 2.8:1 ratio

was also obtained when *S. roseochromogenes* was exposed to cycloheximide prior to progesterone pre-incubation: pointed to post translation modification of roseoredoxin. Separation of two isoforms by 2-D isoelectric focusing supported this proposition.

A partial 10 amino acid sequence was obtained for both the cytochrome P450 and roseoredoxin for the purpose of probe design for eventual cloning. An amino acid sequence search revealed this P450 to be unique and unlike any other known P450 sequence. These two proteins were also successfully crystallised by hanging drop vapour diffusion trials, giving isomorphous crystals. These crystals will be used for structure determinations pending further growth.

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Abbreviations

ATCC	American Type Culture Collection
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethyldiaminetetraacetic acid
$NaIO_4$	sodium periodate
P450	cytochrome P450
S15	the supernatant from 15000 rpm centrifugation of homogenised cells
SDS	sodium dodecyl sulphate or sodium laureth sulphate
TEMED	N,N,N',N'-tetramethylethyldiamine
YMG	yeast extract+malt extract+glucose, growth medium

Note. Where possible, original chart recordings/ printouts have been included in the figures.

Chapter 1

General Introduction

1.1 Aims of this work

To identify and characterise the nature of the biotransformation of progesterone by *Streptomyces roseochromogenes* and to investigate the nature of the enzyme system responsible for this steroid transformation. Enzyme purification and reconstitution *in vitro* will be approached with a view to elucidating the regulation of such an unusual biotransformation in *Streptomyces* species. Following functional determination, structural analysis of the enzyme system will then be pursued through crystallising and amino acid sequencing. This elucidation will be carried out with the ultimate goal of probe design with potential for progression to the identification of analogous steroid transformation systems in other species, thereby firmly establishing the role of steroid biotransformation in *Streptomyces* within the field of prokaryotic P450 steroid hydroxylating systems. Asymmetric specific activation of unreactive centres of highly saturated molecules such as steroids is a major objective in synthetic chemistry. Development of regimes for the retention of such stereospecific catalysis by non membrane bound enzymes *in vitro*, is therefore important.

1.2 This Thesis

Chapters presented in this work are individually introduced and subsequently discussed, to give a background to the subject matter of each chapter. Following this work, it is now known that the steroid biotransformation by this *Streptomyces* species is a 2β and 16α hydroxylation, mediated by a cytochrome P450 system. Therefore, cytochromes P450 and steroid biotransformation are discussed in chapter 1.

1.3 BIOTRANSFORMATION

The first observation of biotransformation was in 1857 when Louis Pasteur observed the fermentation of lactic acid from sugar by yeast. Biocatalysis and bioconversion are alternative names for biotransformation which is defined as the synthesis of chemical compounds from precursor substrates, not by synthetic chemical processes but by a whole cell or cell-free biological system. The most widely studied whole cell biotransformations are by fungi. For example, the biotransformation of testosterone, a male sex hormone and pregnenolone, a precursor of many steroidal hormones catalysed by the fungus *Botrytis cinerea* (Farooq & Tahara 2000). Testosterone yielded 7,17-dihydroxyandrostan-3-one and pregnenolone yielded 3,11,16-trihydroxypregn-5-en-20-one and 11,16-dihydroxypregn-4-ene-3,20-dione. *Cephalosporium aphidicola* hydroxylates 3, 5-cycloandrostanes to their respective 6-ketones (Bensasson *et al.*, 1999). Testosterone is hydroxylated to 5-dihydrotestosterone by two species of *Penicillium* : *P. chrysogenum* and *P. crustosum* (Cabeza *et al.*, 1999).

Microorganisms from soil samples were screened for hydrocortisone and prednisolone production, the fungus which showed hydrocortisone production was *Cunninghamella blakesleeana* whereas the bacteria which showed prednisolone production ability were *Bacillus sphaericus* (Manosroi *et al.*, 1999). Bacterial biotransformation being of great significance also. Indeed a soil bacterium *Streptomyces roseochromogenes*, is the subject of the work presented here (Berrie *et al.*, 1999).

Obtaining stereospecific biotransformations is of great biotechnological importance. Finding such reactions of recalcitrant centres of large carbon compounds mediated by cytochromes P450 is one biotechnological usage of biotransformation. Cytochromes P450 are of great use because they have wide substrate specificities, many of them have been characterised and their

properties in vitro have been reported for many P450 systems. The *Streptomyces* genus has several examples of cytochrome P450 mediated catalysis which are surveyed in chapter 3 but such mediations are not reported for steroidal biotransformation. *Streptomyces lividans* encodes a highly specific non-P450 esterase fusidic acid enzyme (FusH) that converts the steroid antibiotic, fusidic acid into an inactive derivative (VonDerHaar *et al.*, 1997). As discussed in chapter 3, steroidal bioconversion is documented in *Streptomyces* but not its P450 catalysis. This thesis reports rare steroidal biotransformation, namely 2β and 16α hydroxylation of progesterone.

Streptomyces stereoselective steroid bioconversion is reported for example, at skeletal positions 3α, 20β, 3β and 17β by *S. hydrogenans* (Feller & Traeger, 1985). Stereoselective P450 hydroxylation of steroids at every carbon skeletal position is now reported for fungi. Such a comprehensive library does not yet exist for bacteria and more specifically *streptomyces*.

BIOTRANSFORMATIONS OF STEROIDS BY OTHER STREPTOMYCES SPECIES:

Table 1.1 illustrates the usefulness of a *Streptomyces* P450 library.

The classes of biotransformation reactions catalysed by enzymes in bacteria fall into many categories. For the purposes of this work, we are primarily interested in the oxidation of steroids and more specifically the hydroxylation of the steroid hormone, progesterone. Oxidation is the most important category of microbial transformations. As discussed, it is extremely difficult to achieve the stereospecific targeting of a particular carbon centre within the perhydrocyclopentanophenanthrene ring system of steroids or indeed of the side chain.

Steroid Structure Carbon Position	Streptomyces species	Reference
16α	*S. achromogenes* *S. annulatus* *S. diastaticus* *S. fimicariusaureus* *S. flaveolus* *S. chrysomallus*	Vondrova, O. & Capek, A. (1963) Transformation reactions of steroids as a diagnostic feature in the classification of *Actinomycetes*. Folia Microbiol. 8, 117
16α	*S. aureus* *S. fimicariusaureus* *S. microflavus*	Vondrova, O. & Hanc, O. (1960) 16α hydroxylation of **progesterone** by a strain of *Actinomyces globosus*. Folia Microbiol. 5, 247
16α	*S. coriofaciens*	Ghanem, K. M., Sallam, L. A., Elrefai, A.H. & El-Helow, E. (1987) Egypt J. Microbiol. 22, 27-
16α	*S. griseoluteus*	Vondrova, O. & Capek, A. (1963) Transformation reactions of steroidsas a diagnostic feature in the classification of *Actinomycetes*. Folia Microbiol. 8, 117. [
16α	*S. griseus*	Vondrova, O. & Capek, A. (1963) Transformation reactions of steroids as a diagnostic feature in the classification of *Actinomycetes*. Folia Microbiol. 8, 117 Vondrova, O. & Hanc, O. (1960) 16alpha hydroxylation of progesterone by a strain of *Actinomyces globosus*. Folia Microbiol. 5, 247. [Progesterone]
16α	*S. mediocidicus*	Kita, D. A. et al. (1961) Theintroduction of the 16α-hydroxy group into oestrogens by *Streptomyces*. Nature 190, 627.
16α	*S. lipmanii*	Vondrova, O. & Capek, A. (1963) Transformation reactions of steroidsas a diagnostic feature in the classification of *Actinomycetes*. Folia Microbiol. 8, 117 Vondrova, O. & Hanc, O. (1960) 16α hydroxylation of progesterone by a strain of *Actinomyces globosus*. Folia Microbiol. 5, 247. [Progesterone]
16α	*S. olivaceus*	Vondrova, O. & Capek, A. (1963) Transformation reactions of steroids as a diagnostic feature in the classification of

		Actinomycetes. Folia Microbiol. 8, 117 Vondrova, O. & Hanc, O. (1960) 16α hydroxylation of progesterone by a strain of *Actinomyces globosus*. Folia Microbiol. 5, 247. [Progesterone]
16α	*S. halstedii*	Kita, D. A. et al. (1961) The introduction of the 16α-hydroxy group into oestrogens by *Streptomyces*. Nature 190, 627.
15α	*S. aureus*	Fried, J. (O. Mathieson Chem Corp.) (1956) 7- and 15-Hydroxyprogesterone. US Patent 2,753,290.
12β	*S. diastatochromogenes* *S. owasiensis*	Nazaki, Y., Mayama, M., Akaki, K. & Satoh, D. (1965) Microbial 12β-hydroxylation of digitoxin, a steroidal glycoside. Agric. & Biol. Chem. (Japan) 29, 783. [3β,14-Dihydroxy-5β,14β-card-20(22)-enolide 3β-tridigitoxiside {digitoxin}
11β	*S. fradiae*	Collingsworth, D. R. et al. (1953) A partial microbiological synthesis of hydrocortisone.. J. Biol. Chem. 203, 807
11β	*S. fradiae*	Vondrova, O. et al. (1963) Hydroxylation of the steroid molecule by strain of *Streptomyces fradiae*. Folia Microbiol. 8, 176
11β	*S. fradiae*	Colingsworth, D. R., Brunner, M. P. Haines, W. J. (1952) A partial microbiological synthesis of adrenal cortex hormones. J. Am. Chem. Soc. 74, 2381-2382. [17α,21-Dihydroxyprogesterone {Cortexelone}, 21-Hydroxyprogesterone]
9α	*S. aureofaciens*	Fried, J et al. (1955) The use of microorganisms in the synthesis of steroid hormones and hormone analogues. Rec. Prog. Hormone. Res. 11, 149-177
7β	*S. griseoflavus*	Nazaki, Y., Mayama, M., Akaki, K. & Satoh, D. (1965) Microbial 12β-hydroxylation of digitoxin, a steroidal glycoside. Agric. & Biol. Chem. (Japan)

		29. 783. [3β,14-Dihydroxy-5β,14β-card-20(22)-enolide 3β-tridigitoxiside {digitoxin}]
7β	*S. cellulosae*	Nazaki, Y., Mayama, M., Akaki, K. & Satoh, D. (1965) Microbial 12β-hydroxylation of digitoxin, a steroidal glycoside. Agric. & Biol. Chem. (Japan) 29, 783. [3β,14-Dihydroxy-5β,14β-card-20(22)-enolide 3β-tridigitoxiside (digitoxin)
7β	*S. rimosus*	Nazaki, Y., Mayama, M., Akaki, K. & Satoh, D. (1965) Microbial 12β-hydroxylation of digitoxin, a steroidal glycoside. Agric. & Biol. Chem. (Japan) 29, 783. [3β,14-Dihydroxy-5β,14β-card-20(22)-enolide 3β-tridigitoxiside {digitoxin}
6β	*S. rimosus* *S. diastatochromogenes*	Vondrova, O. & Capek, A. (1963) Transformation reactions of steroids as a diagnostic feature in the classification of *Actinomycetes*. Folia Microbiol. 8, 117
6β	*S. aureofaciens* *S. fradiae*	Fried, J et al. (1955) The use of microorganisms in the synthesis of steroid hormones and hormone analogues. Rec. Prog. Hormone. Res. 11, 149-177
6β	*S. griseocarneus*	Vondrova, O. & Capek, A. (1963) Transformation reactions of steroids as a diagnostic feature in the classification of *Actinomycetes*. Folia Microbiol. 8, 117
4	*S. griseus*	Williamson, J., van Orden, D. & Rosazza, J. P. (1985) Microbiological hydroxylation of oestradiol: formation of 2- and 4-hydroxyestradiol by *Aspergillus alliaceus*. Appl. Environ. Microbiol. 49, 563-567 [17β-Oestradiol]
4	*S. griseus*	Trower, M. K., Sariaslani, F. S. & Kitson, F. G. (1988) Xenobiotic oxidation by cytochrome P450-enriched extracts of *Streptomyces griseus*. Biochem. Biophys. Res. Commun. 157, 1417-1422. [17β-Oestradiol]
3β	*S. violascens*	Kamei, T., Takiguchi, Y., Suzuki, H.,

		Matsuzaki, M. & Nakamura, S. (1978) Purification of 3β-steroid oxidase of *Streptomyces violascens* origin by affinity chromatography on cholesterol. Chem. Pharm. Bull. 26, 2799-2804.
2	*S. griseus*	Williamson, J., van Orden, D. & Rosazza, J. P. (1985) Microbiological hydroxylation of estradiol: formation of 2- and 4-hydroxyestradiol by *Aspergillus alliaceus*. Appl. Environ. Microbiol. 49, 563-567 [17β-Oestradiol, Oestrone]
2	*S. griseus*	2-hydroxylation Trower, M. K., Sariaslani, F. S. & Kitson, F. G. (1988) Xenobiotic oxidation by cytochrome P450-enriched extracts of *Streptomyces griseus*. Biochem. Biophys. Res. Commun. 157, 1417-1422. [17β-Oestradiol]
C17 - 20 side chain cleavage	*S. lavendulae*	Fried, J. et al. (1953) Side-chain degradation, ring D cleavage and dehydrogenation in ring A. J. Am. Chem. Soc. 75, 5764 Fried, J. & Thoma, R. W. (O. Mathieson Chem. Corp.) (1957) Prednisolone. US Patent 2,793,164 Fonken, G. S. & Murray, H. C. (Upjohn Co.) (1961) Microbiological conversion of pregnane-series steroids. US Patent 2,981,659 Peterson, D. H. et al., (1957) Metabolism of progesterone by *Cylindrocarpon radicicola* and *Streptomyces lavendulae*. J. Bacteriol. 74, 684. [Progesterone]

Table 1.1 Steroid skeletal carbon position reactions biotransformed by *Streptomyces* species.

Availability of biotransformation systems is important in terms of the development of new drugs such as anti-inflammatory agents. It was the

discovery that cortisone 21-acetate had anti-inflammatory properties which triggered the field of fungal steroid biotransformation. The advantages of using micro-organism systems of biotransformation is exemplified by cortisone, which would require thirty chemical steps to synthetically form from deoxycholic acid. 11α hydroxylation however is achieved quickly by the fungus *Rhizopus arrhizus* (Peterson & Murray, 1952).

1.4 CYTOCHROMES P450

The advantages of being able to identify cytochrome P450 as responsible for a biotransformation are discussed above.

Cytochromes P450 are a large group of enzymes which are haem proteins (Omura & Sato 1964[1]). They are found in essentially all eukaryotes and in some bacteria. They catalyse a wide range of chemical reactions by acting as monooxygenases when in the presence of O_2 and NAD(P)H and one or more electron-transfer proteins (Guengerich, 1983). Monooxygenase, means that only one of the oxygen atoms of molecular oxygen, is incorporated in the substrate. These enzymes catalyse the oxygenation of an array of hydrophobic carbon compounds. When in the reduced state and bound to carbon monoxide, they show a characteristic absorption peak at 450 nm (Omura & Sato 1964[2]).

1.5 CYTOCHROMES P450 NOMENCLATURE

Cytochromes P450 are a superfamily of haem containing protein that catalyse the metabolism of many lipophilic endogenous substances and exogenous substrates. The nomenclature for the CYP superfamily is based upon divergent evolution of the genes (de Wildt . *et al.*, 1999). CYP genes which share at least 40 % homology are grouped in a family denoted by an Arabic number. For example CYP3. A subfamily, which is indicated by a letter, represents highly

related genes. For example, the designation CYP3A. This is a subfamily of CYP3 genes which are the most abundant liver P450s. Many drugs are mainly metabolised by subfamily CYP3A (Wrighton *et al.*, 1996). The addition of a further arabic numeral designates the individual gene. For example CYP3A4. The CYP isoforms of the CYP3A subfamily have at least 85 % sequence homology.

1.6 SPECTRAL PROPERTIES OF CYTOCHROMES P450

Spectral properties of the P450 molecule vary greatly due to the 'state' of the molecule (Remmer *et al.*, 1966). The state of the molecule varies due to the arrangement of the double bonds of the haem moiety and these are influenced by the axial bonds to the centrally co-ordinately bonded iron ion. Spectral changes come about through changes in the organisation of the d orbital electrons. Iron has five electrons in the d orbital, thus one is unpaired and the iron is therefore low spin. This occurs when there is no substrate bound (Malmström, 1982). When substrate binds, the five electrons are all unpaired thus two electrons occupy the higher energy d orbital. In this case the iron is referred to as high spin (Jefcoate, 1978). The spectral changes induced by binding at the active site are discussed in more detail in chapter 5.

1.7 STRUCTURE OF CYTOCHROMES P450

These enzymes contain protoporphyrin as cofactor. Unlike most other haem proteins, the P450 haem is not covalently bound to the protein. Six co-ordinate covalent bonds can be formed by the iron of haem. Four of the bonds are with the four pyrrole nitrogen atoms of haem and are planar. The fifth bond in P450 always involves a thiolate ion with a cysteine residue in the protein. This attaches the haem to the protein. The sixth bond is not always present but when

present is usually to water (Grasdalen *et al.*, 1976). The fifth and sixth bonds are axial to the plane of the protoporphyrin ring.
Various aspects of P450 structure are surveyed in chapter 8.

1.8 BINDING OF SUBSTRATE TO P450 AND IDENTIFICATION OF AMINO ACID RESIDUES INVOLVED - AN APPLICATION OF P450 MODELLING

Current understanding of substrate binding has arisen through modelling eukaryotic P450 structures on soluble bacterial P450 X-ray structures and their primary sequences, particularly P450 101 (Gunsalus & Wagner 1978). This modelling technique has faults and seems to require many assumptions such as the effect upon catalysis of the hydrophobic domain but for the purposes of substrate binding interactions, has proved very useful (Poulos *et al.*, 1985).
Conformational change induced in a P450 when it binds substrate is associated with a shift of the Soret peak from 420 nm to 390 nm (Omura & Sato 1964[2]), although these figures vary very slightly from one P450 to another. Binding of substrate results in displacement of the iron at the centre of the haem moiety from the plane of the ring towards the thiolate sulphur - Figure 1.8a and Figure 1.8b.

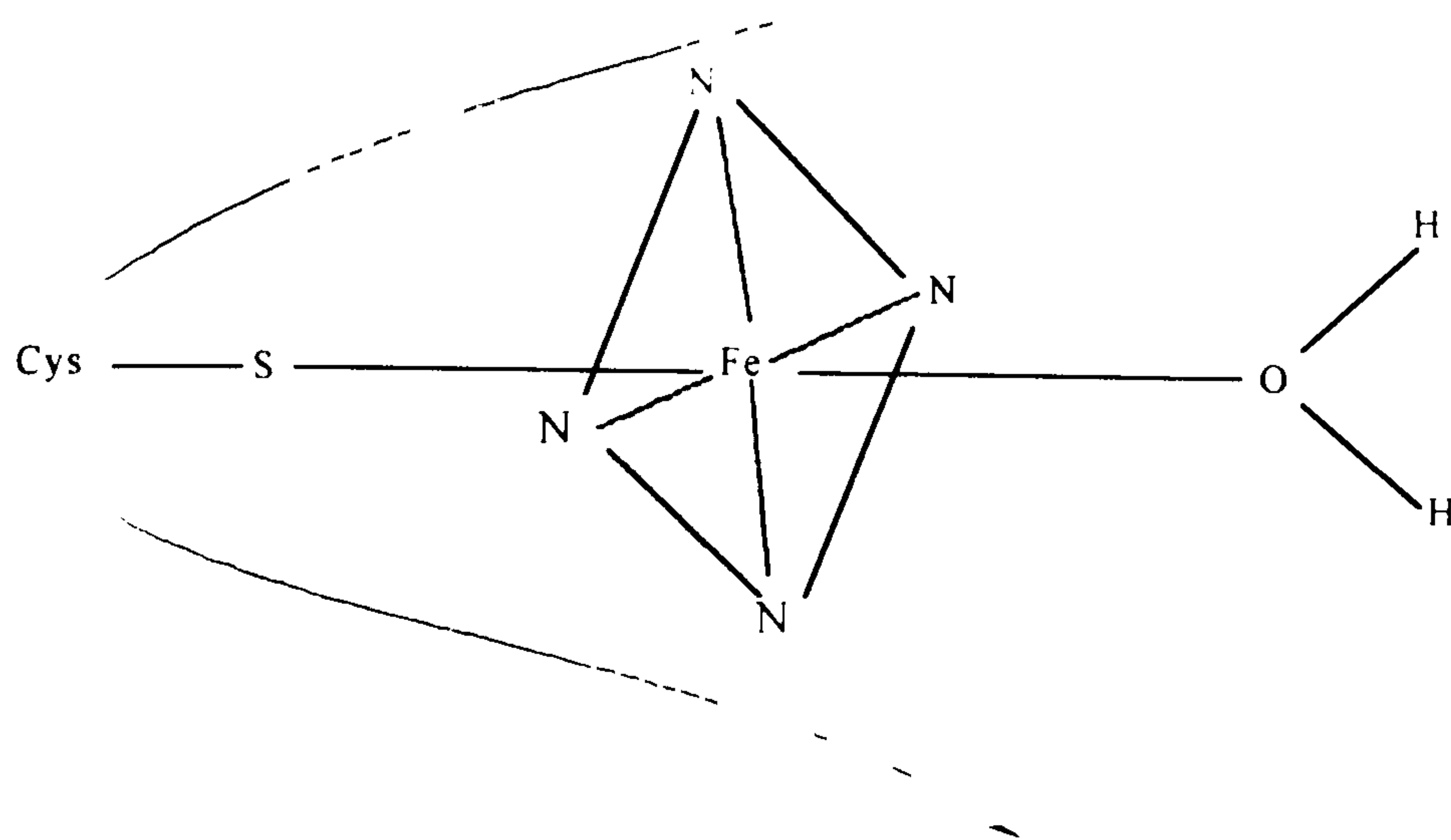


Figure 1.8a Hexacoordinate low spin cytochrome P450 haem binding

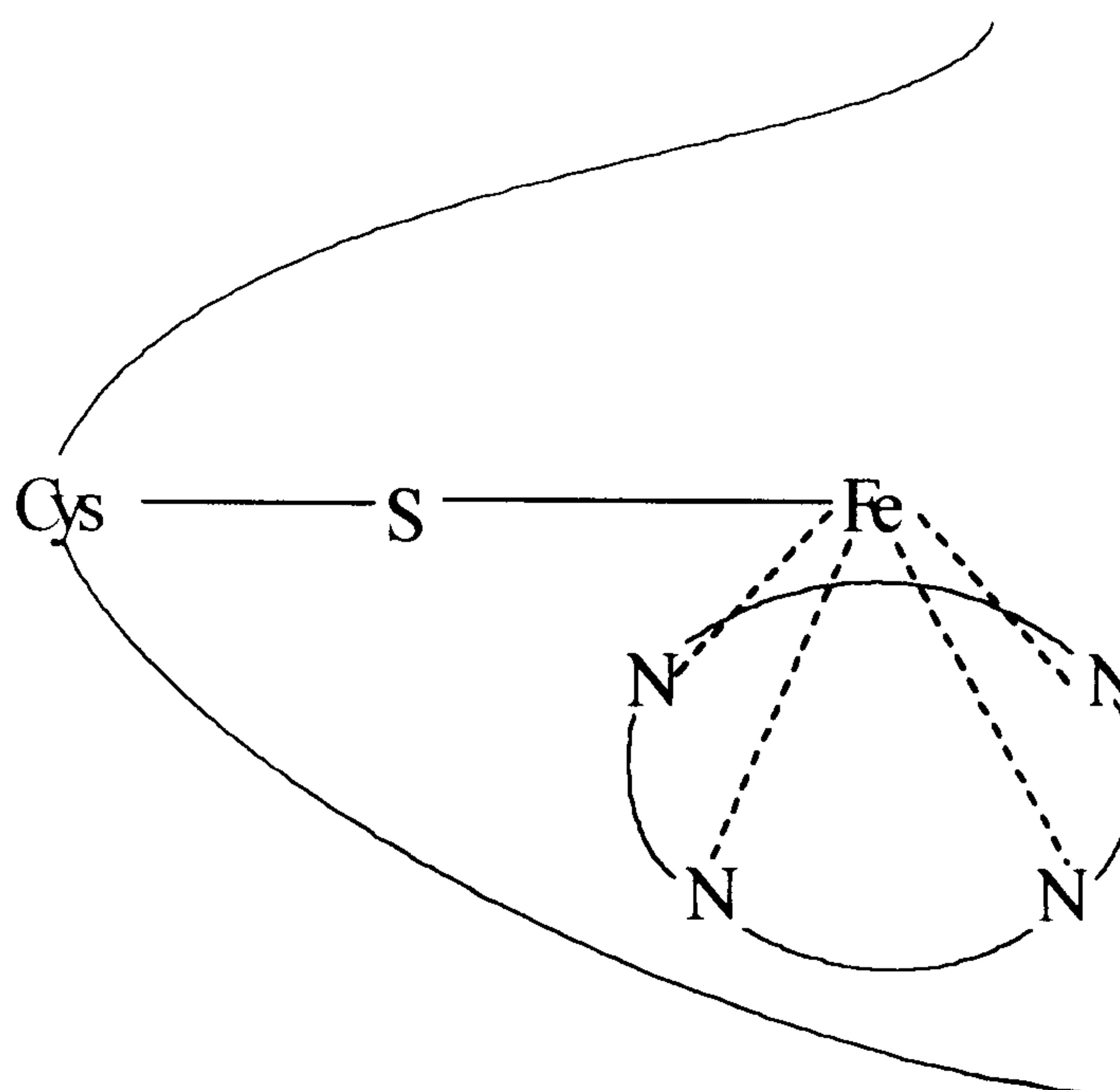


Figure 1.8b Pentacoordinate high spin haem binding site. Orientation of heam does not alter - just the relative position of the iron within the plane of the haem

Obstruction of the sixth ligand, which in most P450s is water, causes a shift of the iron from the plane of the porphyrin toward the fifth ligand (O'Keefe *et al.* 1978). This makes the complex more susceptible to reduction due to the increase in ionic radius of the newly formed high-spin pentacoordinate iron. Other evidence to support this view was presented in 1970 (Tsai *et al.* 1970)

with studies on the binding between P450 from *Pseudomonas putida* and its substrate, camphor. EPR signals of this soluble P450 were observed and the low spin signal decreased upon binding with camphor as the high spin signal increased.

Much evidence for the way substrates bind P450s has been gathered through site directed mutagenesis and to some extent, computer modelling. This work has its roots in the X-ray structure of $P450_{cam}$.

Site directed mutagenesis, selective chemical modification, construction and expression of chimeric cytochromes P450 or computational chemistry can be used to obtain information about substrate binding in this group of enzymes. Chimeric proteins♦ containing sequences of P450 1A1 & 1A2 have been constructed (Pompon 1988) (Pompon & Nicholas 1989). A region of 35 residues (Aligned positions 253-299) has been proposed to play a major role in the control of P450 1A1 activity toward carcinogenic polycyclic aromatic hydrocarbons (Pompon & Nicholas 1989). This region slightly overlaps a peptide sequence from aligned sequences 294-350 that has been suggested to play a role in the binding of fatty acids to P450 2C2 (Imai *et al.*, 1989). Chimeras of P450 2C2 laurate hydroxylase (rabbit liver) & 2C14 testosterone 16α hydroxylase (rat liver), which exhibits 82% amino acid sequence homology but have different substrate selectivities, have been constructed to identify amino acids responsible for substrate binding (Imai & Nakamura 1989).

P450 2A1 & 2A2 display 88% amino acid sequence similarity and exhibit different hydroxylation specificity toward testosterone. Sequence dissimilarities are primarily localised within four discrete regions of the polypeptides that are separated by regions of absolute sequence identity. From the hydroxylation profiles of various chimeras of P450 2A1 & 2A2 it was proposed that substrate specificity is determined by a region positioned between

♦ Also for a novel human-bacterial P450 chimera (Shimoji *et al.* 1998)

alignment positions 365 & 461, which corresponds to the I & J helices of P450 101 (*P. putida* P450 101 model) (Hanioka *et al.*, 1990). In P450 101, the I helix lies directly over the haem and is involved in substrate binding (Poulos *et al.*, 1987). In this region, 13 amino acid differences exist between P450 2A1 & 2A2. One of these differences occurs at alignment position 392 exactly matching the single amino acid substitution G310D by which lanosterol 14-demethylase P450 51 is changed into an inactive enzyme P450 SG1 (Ishida *et al.*, 1988). Mutations of the active site residues F87 and Y96 greatly enhanced the activity of cytochrome P450(cam) (CYP101) from *Pseudomonas putida* for the oxidation of the polycyclic aromatic hydrocarbons phenanthrene, fluoranthene, pyrene and benzo[a]pyrene (Harford Cross *et al.*, 2000), thereby enabling development of computational models of P450 catalysis. Computer modelling has predicted that Glu302 of aromatase P450 19 at alignment position 392 forms part of the active site of this enzyme (Graham-Lorence *et al.*, 1991) and site-directed mutagenesis has confirmed the prediction. Substitution of Glu 302 to Ala, Val or Gln resulted in complete loss of activity, whereas mutagenesis to the functionally similar amino acid Asp resulted in only a 30% decrease in activity. Furthermore, in the same region (Aligned positions 365-461) the tripeptide Ala305-Ala307-Pro308 (Aligned positions 396-398) was found to be important in substrate binding (Graham-Lorence *et al.*, 1991). P450s belonging to the family 4, share a common 16 residue peptide alignment positions 386-401 that is unique among these P450s and has been suggested as being involved in substrate recognition. (Gasser & Philpot 1989). The construction of a functional chimera from approximately 50% bacterial (cytosolic) cytochrome P450cam and 50% mammalian (membrane-bound) cytochrome P450 2C9 has been reported (Shimoji *et al.*, 1998). The protein is soluble and catalyses the oxidation of 4-chlorotoluene using molecular oxygen and reducing equivalents from bacterial putidaredoxin and putidaredoxin reductase. This chimera provides a novel method for addressing structure-function relationships and may prove useful in the design of oxidants for benign

and stereospecific synthesis, as well as catalysts for bioremediation of polluted areas. These results provided the first evidence that bacterial P450 enzymes and mammalian P450 enzymes may share a common tertiary structure (Shimoji *et al.*, 1998). The mechanisms of human ovarian steroidogenesis have also been studied by the formation of a range of chimeric P450s using fragments of the 5'-untranslated region of bovine P450(17 α) and human P450SCC (Simpson *et al.*, 1991).

More recently, the computational molecular dynamics of substrate binding have been modelled upon the P450 of *Bacillus megaterium* (fatty acid hydroxylase). Computer simulations have been used to examine substrate induced conformational change. Cytochrome P450 BM3, of bacterial origin, is one of only five isozymes of the ubiquitous family of over 400 metabolising haemoproteins with a known crystal structure and only one of two with both substrate-free and substrate-bound forms determined. P450 BM3 is of particular interest since it has a similar function and similar substrates as mammalian P450s particularly of the 4A subfamily. Thus, the extent to which the substrate free form of P450 BM3 undergoes conformational change upon binding of a typical fatty acid substrate, palmitoleic acid, has been the subject of recent study (Chang & Loew 1999). Specifically, molecular dynamic's simulations were performed for each of the two substrate-free forms found in the asymmetric X-ray structure and for the two corresponding substrate-bound forms, constructed by docking palmitloeic acid into each of them. Comparisons of the results showed that palmitoleic acid binding had little effect on the conformation of the more closed substrate-free form of P450 BM3. By contrast, in the more open substrate-free form, this same substrate induced a closing of the entrance to the substrate-binding channel (Chang & Loew 1999).

The construction of a three-dimensional model of human CYP2E1 has been reported. It is based on homology with the haemoprotein domain of the unusual bacterial P450, CYP102. Interactive docking of a number of human

CYP2E1 substrates is consistent with their known positions of CYP2E1-mediated metabolism (Lewis *et al.* 2000). Amino acid residues within the putative active site of human CYP2E1, including those associated with the binding of substrates and inhibitors, are shown to correspond with those identified by site-directed mutagenesis experiments conducted on CYP2 family isoforms and they are known to affect substrate metabolism regioselectivity (Lewis *et al.* 2000). Molecular models, generated using molecular replacement or distance geometry methods, can be used to dock substrates and/or inhibitors in the active site to explain various aspects of enzyme function. The majority of modelling research has dealt with enzyme-substrate interactions in the active site. The analysis of these interactions has helped us to better understand the mechanism of P450 catalysis and provided the structural basis for the regio- and stereospecificity of substrate oxidation as well as susceptibility to inhibition or inactivation. The models have been utilised to identify and/or confirm key residues and to interpret rationally experimental data. The alteration in activity in a mutant P450 can be related to changes in enzyme-substrate/inhibitor interactions, such as the removal or appearance of van der Waals overlaps or changes in compound mobility. Homology models can also help to analyse P450-redox partner interactions and identify critical determinants of protein stability. We can expect further development of molecular modelling methods and their increasing contribution into research on P450 function as an integral part of a combined theoretical-experimental approach (Szklarz *et al.*, 2000).

1.9 STEROID BINDING SITES OF CYTOCHROMES P450

Steroid-metabolising cytochromes P450 share a region of high homology which might be involved in the binding of steroids (Mornon *et al.*, 1989). Homologous sequences in human progesterone, mineralocorticoid, oestrogen and androgen receptors have been identified (Picardo-Leonard & Miller 1989). As all these proteins must bind the steroid nucleus, sequence similarities are

expected. The consensus steroid binding site is characterised by a sequence from alignment positions 448-468: L-(P,L)-L-L-(+/-)-X-X-(R,K)-(D,E)-X-L-(R,K)-L-(+/-)-P-V, where L=Leu, (P,L)=Pro or Leu, (+/-) is any changed residue, X is an unchanged amino acid, (R,K) is Arg or Lys, (D,E) is Asp or Glu, P is Pro V is Val.

In P450 2C2, amino acids in the region from aligned positions 137-182 are essential for hydroxylase activity (Imai *et al.*, 1989).

The region from aligned positions 137-182 forms part of the substrate binding domain of P450 2C4 & 2C5 (Kronbach *et al.* 1989). P450 2C5 catalyses progesterone 21-hydroxylation with a low apparent K_m and at rates similar to adrenal 21-hydroxylase P450 21A. P450 2C4 exhibits a 10-fold higher apparent K_m than 2C5 for binding progesterone but has no 21-hydroxylase activity. Constructing hybrids between these two enzymes showed that region 113-118 (aligned positions 168-175) contains the sequence responsible for affinity of P450 2C4 & 2C5 toward progesterone (Kronbach *et al.*, 1989). Only one mutation, V113A (aligned position 168) was required to lower the K_m of P450 2C4 for progesterone hydroxylation (Johnson *et al.*, 1990 and Kronbach *et al.*, 1991[2]). The same mutation in P450 2C1 conferred progesterone 21-hydroxylase activity on this enzyme (Kronbach *et al.*, 1991[1]). Thr-115 & Asn-118 of P450 2C4 (aligned positions 170 & 175) were shown to be located at the surface of the enzyme (Kronbach *et al.*, 1991[2]).

Substrate orientation in 2B1 & 2B2 is determined by Ile-114 & Leu-58 (aligned positions 168 &175) (Aoyama *et al.*, 1989). Both enzymes hydroxylate testosterone in the 16α & 16β positions as well as producing 17-keto and 16β-hydroxy-17 keto derivatives. When Ile-114 and Leu-58 of P450 2B1 & 2B2 were both replaced with Phe, the mutated enzymes did not produce the 16β hydroxylated products of testosterone (Aoyama *et al.*, 1989).

Alignment position 168 in P450 $_{coh}$ and 2A3 (Val-117 & Ala- respectively) is located in the active site (Lindberg & Neigishi 1989). These two enzymes

differ by only 11 amino acid residues in their primary structure but exhibit divergent catalytic activities. Each of these residues has been separately altered by site-directed mutagenesis. The activities of the two enzymes depends critically on the identity of amino acid residues at alignment positions 168, 284 & 468. Substitution of Phe-209 of $P450_{coh}$ (Aligned position 284) by Leu converts the specificity of the enzyme from 7-hydroxylation of coumarin to the steroid hydroxylation of P450 2A3. Enzyme kinetic studies showed that Phe 209 constitutes part of the substrate binding site (Juvonen R.O. *et al.*, 1991). The proposed active-site residue at alignment position 468 forms part of the suggested steroid binding site (positions 448-468). Taking these data into consideration, one amino acid position and two regions are very interesting: alignment position 168, the region from positions 392-398, and the steroid binding site(positions 448-468). Alignment position 168 might match with helix B' of P450 101, which contains the active site residue Tyr-96. Region 392-398 is homologous to a part of the I helix of P450 101 (cam), whereas region 448-468 coincides with helix K and a β3-sheet of P450 101 (cam) containing the active site Val-295 (Ouzounis & Melvin 1991). Although the sequence homology between membrane-bound P450s in the region is high, it is still sufficiently divergent to explain the observed differences in the substrate selectivities. Therefore, residues near these alignment positions in all P450s may constitute part of the active site.

Sequence alignment of human cytochrome P450 3A4 with bacterial enzymes of known structure has provided a basis from which to predict residues involved in substrate oxidation. Substitutions were made at four residues (I301, F304, A305, and T309). In testosterone hydroxylase assays, all of the mutants displayed rates of total product formation similar to wild-type 3A4. However, with progesterone as the substrate, mutants F304A, A305V, and T309A exhibited altered product ratios and/or changes in the rates of product formation (Domanski *et al.*, 1998).

Homology modelling of mammalian steroidogenic cytochromes P450 (CYP) from families CYP11, CYP17, CYP19 and CYP21 based on a novel protein sequence alignment with CYP102 , generated models from the CYP102 crystal structure template (Lewis & Lee Robichaud 1998). These results were consistent with experimental information from site directed mutagenesis studies, steroidal substrate specificity and active site inhibitor studies. Interactive docking studies with both substrates and inhibitors of these enzymes indicated key residue interactions with the putative active site regions of each isoform investigated. This pointed to potential determinants of substrate specificity within these related enzymes (Lewis & Lee Robichaud 1998).
It is important to remember that remote site substitutions may affect substrate binding, the electron transfer process, oxygen binding or the incorporation of the active oxygen species. Also the validity of conclusions drawn on active site residues will depend on the validity of the alignment of a particular P450 and P450 101.

1.10 THE P450 CATALYTIC CYCLE AND THE PEROXIDE SHUNT PATHWAY

All investigations in this report, except for reconstitution of the P450 steroid hydroxylase, in chapters 6 and 7, make use of the peroxide shunt pathway. This is because P450s are multicomponent enzyme systems. Other proteins are required for the transfer of electrons from NAD(P)H to P450. Typically in prokaryotes, which fall into the type II P450 category, require ferredoxin reductase and ferredoxin for this electron transfer to P450.

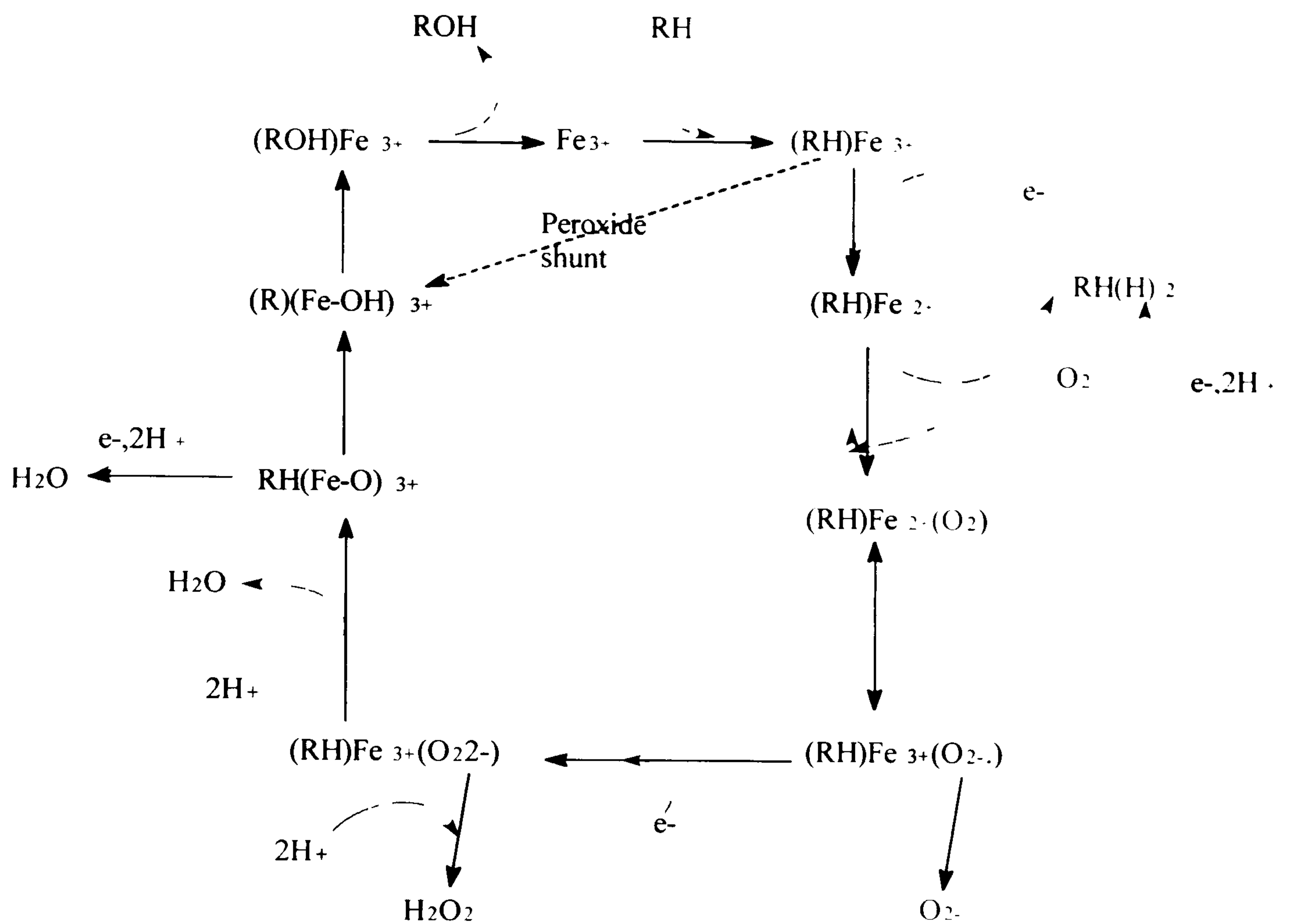


Figure 1.10a The cytochrome P450 catalytic cycle.

These electron transfers are represented by the lower part of the above cycle in Figure 1.10a. In order to eliminate the need for these and to 'short-cut' the cycle, an oxidising agent is added to assays of P450 activity. In these investigations, sodium periodate (Hrycay *et al.* 1975) is used.

The active site of P450 contains iron protoporphyrin IX, bound in part by hydrophobic forces. The fifth ligand is a thiolate ($-S^-$) anion provided by a cysteine residue, a feature that contributes to the unusual spectral and catalytic properties of P450, and the sixth co-ordination position may be occupied by an exchageable H_2O molecule.

Upon reduction of the iron, O_2 (or in competition. CO) can be bound to the sixth position.

The stoichiometry of P450 catalysed reaction is:

$$\mathbf{RH + O_2 + NAD(P)H + H^+ \text{->} ROH + H_2O\ NAD(P)^+} \qquad \textbf{equ. 1.}$$

Where RH is the substrate.

Step 1:

The first step in the reaction cycle is substrate binding. This perturbs the spin state equilibrium of the cytochrome and facilitates uptake of the first electron. This is **step 2**. (Fe^{3+}-RH + e^- $\rightarrow$ Fe^{2+}-RH).

Substrates that undergoes reduction rather than oxygenation, such as epoxides, N-oxides, nitro & azo compounds, and lipid hydroperoxides, accept two electrons in a stepwise fashion to give RH(H_2). To initiate the oxidative reactions, O_2 is bound (**step 3**) to the ferrous P450 with co-ordination to iron *trans* to the thiolate. This intermediate resonance forms are, Fe^{2+} (O_2) -> Fe^{3+} (O_2^-), which are in equilibrium. The substrate remains bound to the P450.

Step 4: transfer of the second electron then occurs, ($Fe^{2+}O_2^{2-}$-RH + e- $\rightarrow$ $Fe^{3+}O_2^{2-}$-RH) This is with the possible involvement in microsomal systems of cytochrome b_5, whose reduction potential is higher than that of P450 reductase (Porter & Coon, 1991).

Step 5 is not well understood but involves splitting of the oxygen-oxygen bond with the uptake of two protons at some stage and the generation of an "activated oxygen", perhaps an iron-oxene (Fe=O)species. and the release of H_2O.

Several resonance forms are possible for the active oxygen intermediate, considering the redox possibilities with the sulphur. iron and oxygen atoms.

Step 6: oxygen insertion into the substrate is believed to involve hydrogen abstraction from the substrate and recombination of the resulting transient

hydroxyl and carbon radicals to give the product. Dissociation of the ROH then restores the P450 to the starting ferric state (**step 7**).

Peroxycompounds (XOOH) may substitute for O_2 and reducing equivalents in a shorten form of the cycle known as the "peroxide shunt". Homolytic cleavage of oxygen occurs with the formation of an iron-bound hydroxyl radical and an alkoxy radical ($XO^{\cdot}$) capable of hydrogen abstraction from the substrate.

It remains to be determined what factors control the regio- and stereoselectivity (see ' substrate binding ' section) of hydroxylation and the identity of the powerful oxidant that is necessary for oxygen insertion into the substrate progesterone without activating groups at or near to the position attacked, when transformed by *S. roseochromogenes*.

1.11 CYTOCHROMES P450 ELECTRON TRANSPORT SYSTEMS

Electrons are derived from either NADH or from NADPH and the reactions can be summarised as :

$$\mathbf{S + NAD(P)H + H^{+} + O_2 \qquad SO + NAD(P)^{+} + H_2O \qquad equ\ 2.}$$

Where S is the substrate.

The P450 molecule is not just an electron transporter like other cytochromes, rather it is responsible for catalysing the monooxygenation of various substrates utilising molecular oxygen and electrons. Electrons therefore need to be transferred to the P450 from NAD(P)H. The haemoprotein itself is not able to react directly with reduced pyridine nucleotides. Other electron transfer proteins are present in most systems for this function. There are two types of P450 electron transfer system, designated Type I

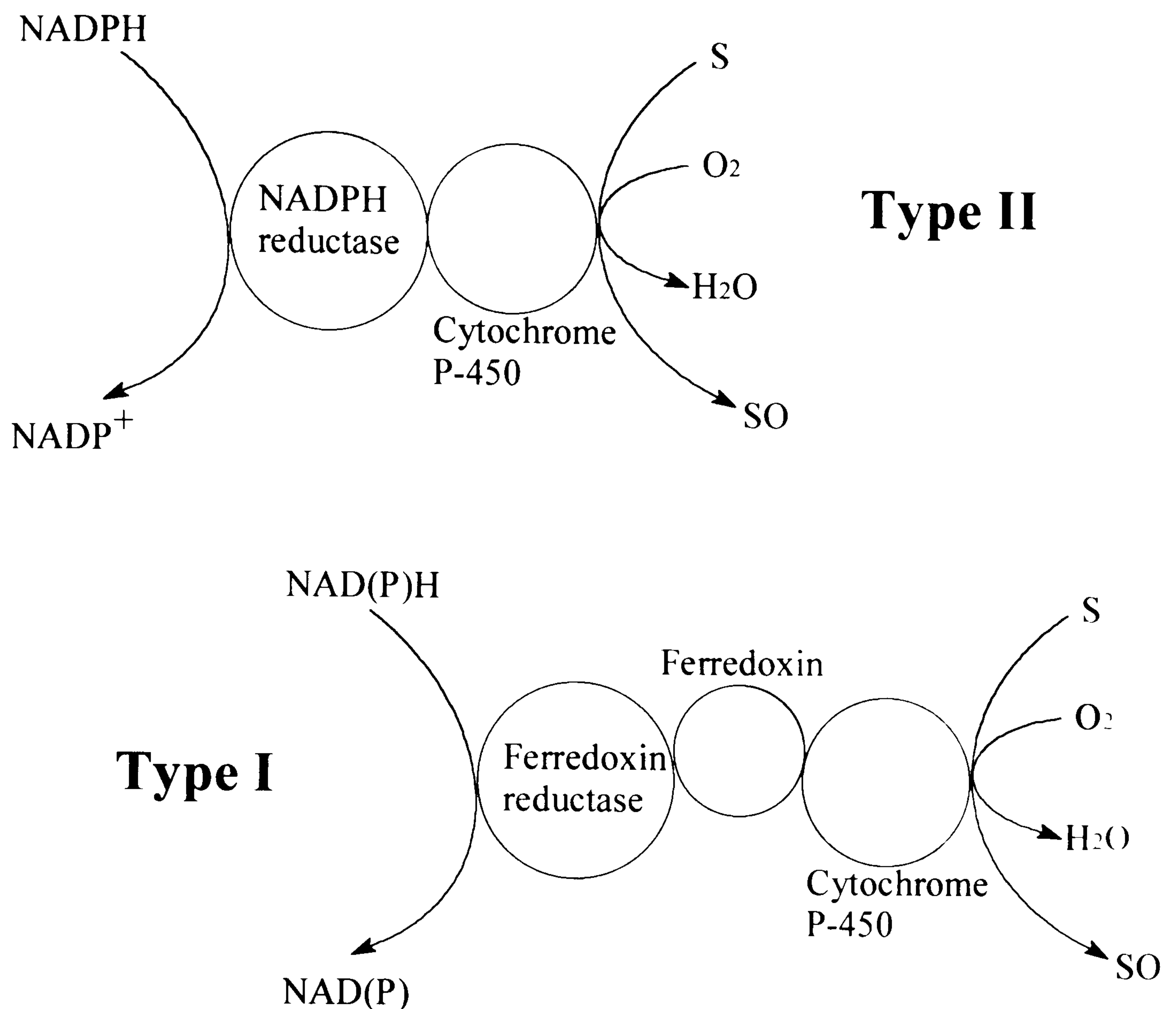


Figure 1.11a Representation of Types Iand II P450 systems. Type II having one extra electron transfer protein requirement than Type I systems.

(generally microsomal) and Type II (generally bacterial and mitochondrial). These are represented in Figure 1.7a. The two types of multicomponent system differ in that, type I occurs in the microsomes of eukaryotic cells. It consists of a FAD and FMN containing reductase and electrons are transferred from NADPH via this to the P450 component. The hydrophobic N - terminal domain of the NADPH-Cytochrome P450 reductase binds the membrane and the P450. The hydrophilic C - terminal domain binds both flavins and electrons

flow from FAD to FMN then to the P450 in two sequential one electron steps. Each reductase can transfer electrons to more than one P450 in mammalian systems (Black & Coon, 1987).

Type II systems occur in mitochondria and bacteria. These systems posses an FAD containing reductase and a small redox iron-sulphur protein, a ferredoxin. All P450 systems identified to date share a common catalytic cycle described above (Koymans *et al.*, 1993).

1.12 THE ROLE OF IRON IN P450 CATALYSIS

A characteristic feature of P450 is the large blue shift in the Soret visible absorption band from *ca* 419nm to *ca* 390nm when substrates bind, indicating a low- to high-spin transition in the Fe atom. In the low spin state the 5d electrons are maximally paired giving a net spin of +1/2, whereas in the high spin state the 5d electrons are maximally unpaired giving a net spin of +5/2 (Fisher & Sligar, 1985).

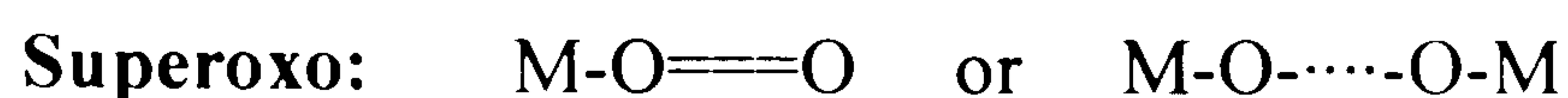
These spin changes and their effect on the spectral properties of P450 reflect changes in the co-ordination environment of the Fe atom with the low-spin spectrum indicative of a strong ligand field axially co-ordinated to the Fe atom on the distal side of the haem. The proximal ligand, the sulphur atom of the cysteine residue, remains attached to the Fe atom in both the low and high spin forms.

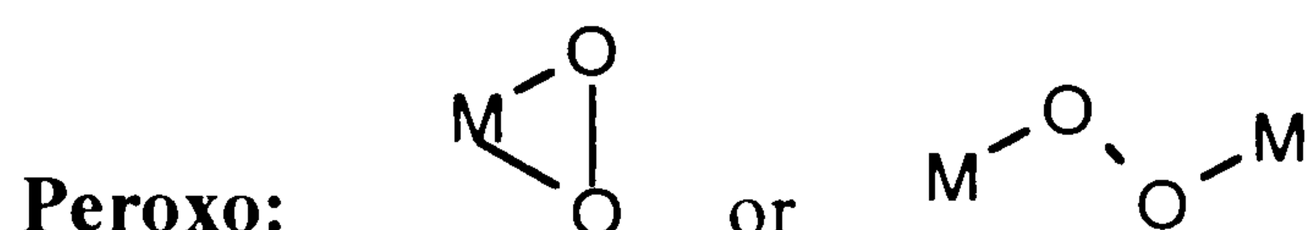
In $P450_{cam}$ the redox potential of the Fe also changes on substrate binding. The potential shifts from *ca* -300mV in the absence of substrate to -173mV when camphor binds, which means that it is thermodynamically more favourable to reduce the iron when substrate is bound. It is the binding of substrate which permits the splicing of oxygen.

The source of electrons required in the catalytic cycle is the iron-sulphur protein putidaredoxin, which possesses a redox potential of *ca* -196mV. Therefore, reduction of substrate-bound enzyme is thermodynamically possible whereas

that of the free enzyme is not, ensuring that reducing equivalents are consumed only in the presence of substrate. The modulation of reduction is achieved entirely by a change in the redox potential and by a change of spin state.

Figure 1.12a Two possible species of covalently bound oxygen. Superoxo or Peroxo forms.



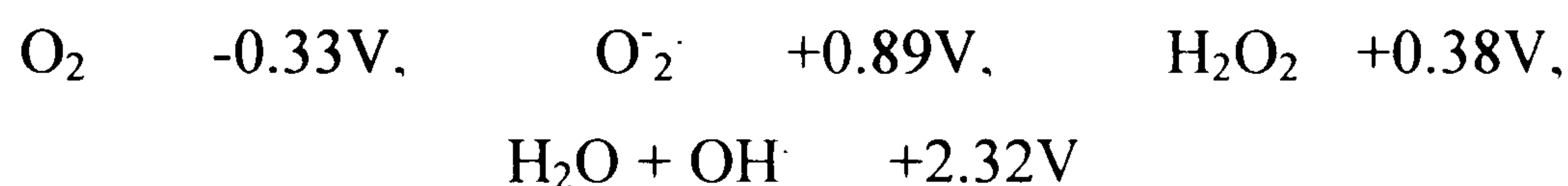


The oxygen in these complexes is not ionic but co-ordinately bound. Thus the oxygen would not exhibit properties of dioxygen ions. Covalently bound oxygen does in many respects resemble either of the two ionic species above in Figure 1.12a, i.e: superoxide & peroxide (Malmström 1982).

The vast majority of oxygenated transition element complexes are diamagnetic but several are paramagnetic. The role of iron in P450 catalysis has also been studied by bacterial P450 modelling as discussed in the previous section and by replacement of the iron centre by other metal ions. For the purposes of modelling, a catalyst has been synthesised comprising a manganese porphyrin carrying four beta-cyclodextrin groups. It catalyses the hydroxylation of substrates of appropriate size carrying tert-butylphenyl groups that can hydrophobically bind into the cyclodextrin cavities (Breslow *et al.*, 1997). In one example as many as 650 catalytic turnovers are seen before the catalyst is oxidatively destroyed and with a rate comparable to that of typical cytochrome P450 enzymes. In another example, a steroid derivative is regio- and stereoselectively hydroxylated at a single unactivated carbon atom but more slowly and with fewer turnovers. The carbon attacked is not the most chemically reactive, and the selectivity is determined by the geometry of the catalyst-substrate complex. Substrates with more flexible binding geometries give more than a single product (Breslow *et al.*, 2000).

1.13 OXYGEN ACTIVATION: A PREREQUISITE FOR ITS INSERTION INTO SUBSTRATE

It is the redox state of the iron centre of the P450 molecule which determines its ability to insert a single atom of oxygen into a substrate. Whether this mechanism is the same in all P450's is unclear and whether the binding of substrate and oxygen are always linked is also unclear. Recent structural studies have suggested that substrate binding sites and distal oxygen binding pockets are not identical (Andersson *et al.*, 1997). The redox chemistry of oxygen underlies the catalytic cycles of peroxidases and oxygenases. Successive 1-electron reductions of dioxygen yield superoxide, hydrogen peroxide, the -OH radical and H_2O with 1-electron potentials (E'_0) in volts as shown below:



The two electron couples linking dioxygen, H_2O_2 and H_2O have redox potentials that are the mean of the corresponding 1-electron values since E must be the same for the different pathways.

The four electron reduction E'_0 ($O_2/2H_2O$) =0.815v, will be mean of the 2-electron values (White & Coon, 1980).

The 1-electron reduction of H_2O_2 has an unremarkable potential E'_0 +0.38v but generates a very potent oxidising agent OH^-. With Fe^{2+} as the reductant this is the Fenton reaction. Depending on how the iron atom is complexed, the OH· radical can remain associated in an iron-oxo complex or be released in the free state. $Fe^{2+} + H_2O_2 \rightarrow [Fe(IV)=O]^{2+} + H_2O$ or $Fe^{3+} + OH\cdot + OH^-$. {Fe(IV)

=Ferryl valence state}.

The catalytic cycles of cytochromes P450 involve Fe(IV) or higher states of iron. Ferryl iron of the Fenton reaction can be almost as reactive as free hydroxyl radical. Peroxidase and cytochromes P450 have succeeded in taming the reactivity of Fe(IV) so that the enzyme does not become inactivated and substrate is modified in a specific way (White & Coon, 1980). These factors are the determinants of the arrangement of electron transfer proteins in P450 systems and also determinants, to some extent of the type of biotransformation which can be achieved.

P450 catalyses the hydroxylation of a wide variety of organic substrates with the concomitant dehydrogenation of NAD(P)H. For the oxygenated P450 species to be formed, a substrate must be bound and the haem-bound iron must be in the reduced Fe^{2+} valence state. The oxygenated species has a spectrum similar to those of oxyhaemoglobin and oxymyoglobin.

The specific interaction of the haem group with the protein residues in the active site determines the ability of the enzyme to hydroxylate bound substrate and the characteristics of a specific reaction. The nature of the fifth ligand in P450 molecules is critical. Originally on the basis of an unusual EPR spectrum, this ligand was thought to be a thiolate anion of a cysteine residue. Subsequent physical, biochemical and modelling studies confirmed the prediction (Malmström 1982). The sixth ligand is thought to be easily exchangeable H_2O., accounting for the low-spin to high-spin transition of P450 on binding of substrate.

The reduction of Fe^{3+}-enzyme is a one electron process, characterised by a half-reduction potential of -326mV. This very low potential can be attributed to the thiolate ligation. It favours the binding of O_2 to the Fe^{2+} form with concomitant transfer of an electron to give $Fe^{3+}.O_2^{\cdot -}$ (superoxide radical). $O_2^{\cdot -}$ is released from the oxyenzyme if no further supply of electrons is available. The resting forms of P450 exist in various states of low-spin high-spin

equilibria (Malmström 1982). An extensive EPR study has shown that the protein can exist in two conformations. One has high-spin Fe^{3+}, which has a higher affinity for substrate (Butler & Hoey 1993).

$(RH)Fe^{2+}(O_2)$ is probably converted to $(RH)Fe^{3+}(O_2^-)$ before receiving the second electron to give $(RH)Fe^{3+}(O_2^{2-})$ ie Fe^{3+} peroxide. The least understood step is the conversion of the peroxide intermediate species to the hydroxylating form, after the breaking of the O=O bonds with the release of one molecule of H_2O.

The Homolytic vs Heterolytic Cleavage Theories

Oxygenation is the feature characteristic of cytochromes P450. The potential for oxygenation of a particular substrate is determined by the ability of a P450 to splice oxygen upon binding to substrate. It is therefore worth considering oxygen insertion at this point.

The Heterolytic Pathway

The peroxide lysis step is the fundamental difference between the oxenoid and quasi-Fenton pathways, the steps after this point being virtually common to the two pathways. Heterolysis of the peroxyacid leaves an oxygen atom (perhaps protonated) coordinated to the iron. The relatively electron-rich iron in the peroxyacid complex could loose a negative charge to carboxylate, which is a good leaving group.

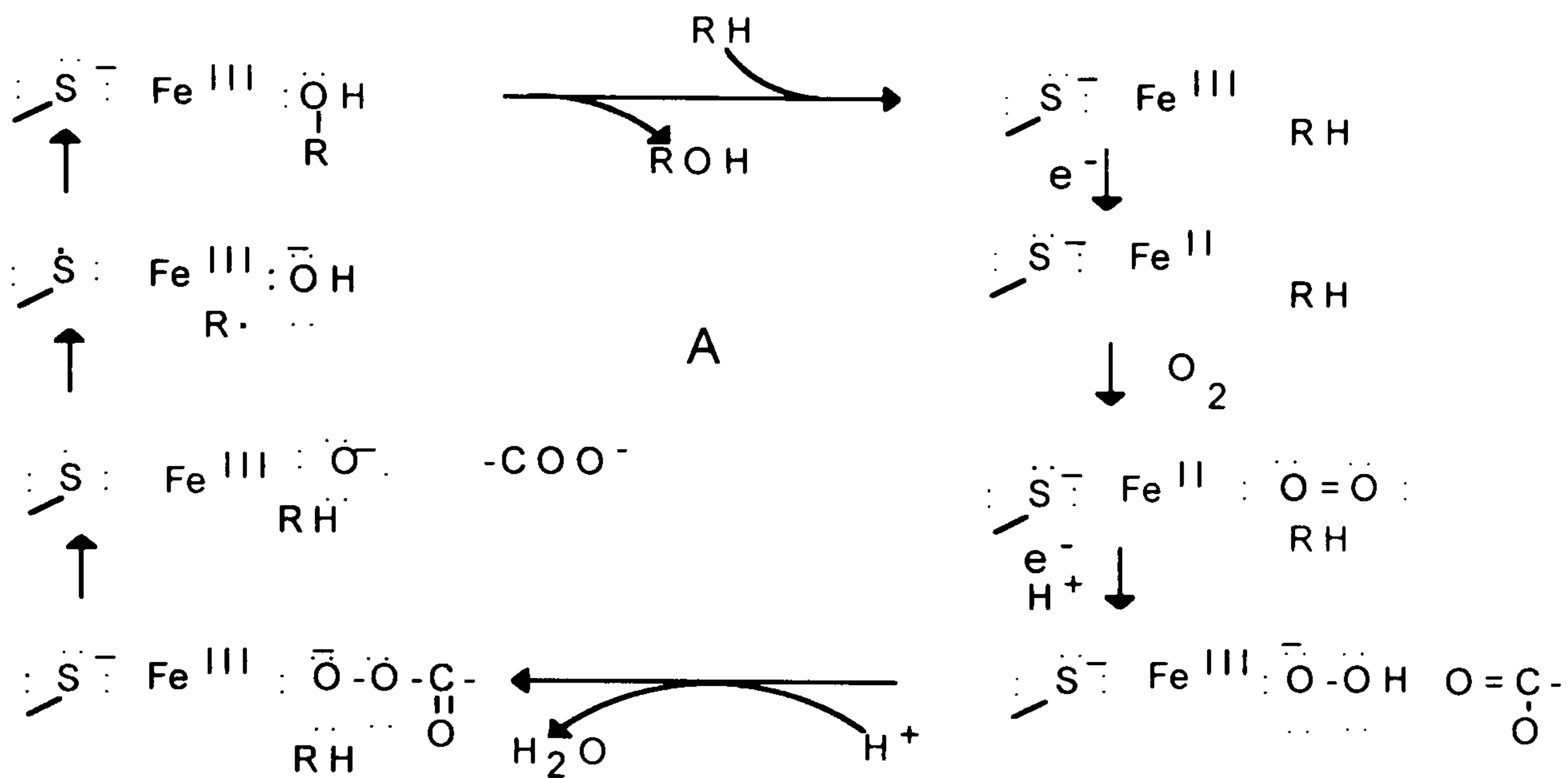


Figure 1.13a. Heterolytic cleavage of the peroxide bond. The oxenoid pathway.

The single iron-bound oxygen atom is the basis for the designation oxenoid pathway.

The Homolytic Pathway

In this pathway, the homolytic peroxide scission resembles the Fenton reaction of ferrous ions with peroxides, hence the descriptor for the pathway, quasi-Fenton.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

In the P450 catalysed reaction the actual one-electron donor-initiating homolysis is thiolate, the iron merely acting as a conduit between the sulphur and the oxygen. The products are the amidyl radical and a thiyl ferric hydroxide complex.

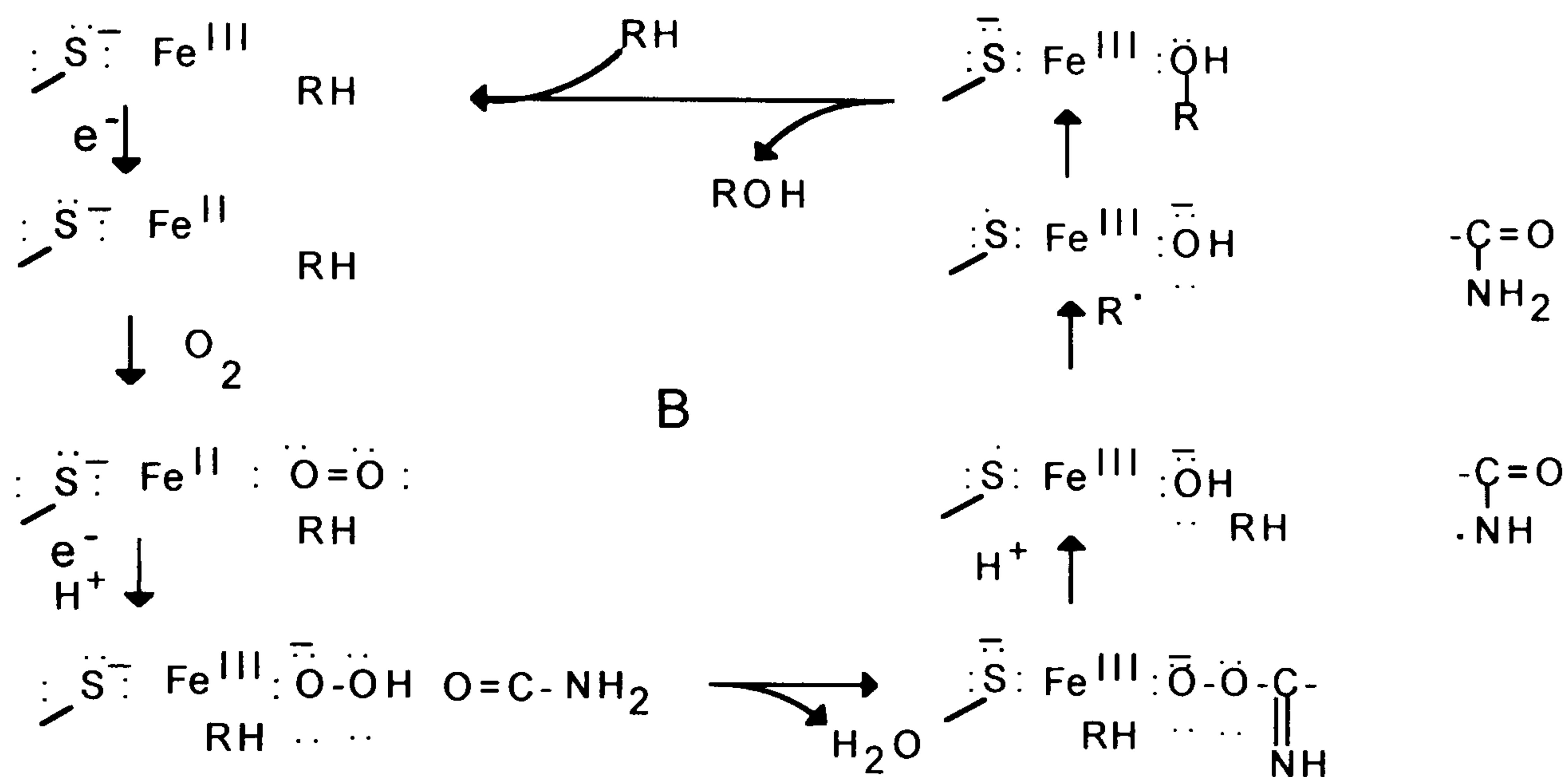


Figure 1.13b. The homolytic cleavage of the peroxide bond. The quasi-Fenton reaction.

Oxygen Insertion into Substrate

It is believed that oxygen insertion into a substrate is a two-step event involving hydrogen abstraction from the substrate and subsequent recombination of the carbon radical and iron-bound hydroxyl radical pair. In the heterolytic pathway, the oxenoid species, $S^{\cdot}Fe^{III}O^{\cdot}$, performs the initial hydrogen abstraction and becomes the hydroxyl radical that virtually immediately recombines with the nascent carbon radical. This process is called oxygen rebound (Groves & McClusky 1976).

1.14 REGULATION OF CYTOCHROME P450 ACTIVITY

The promoters for two inducible herbicide-metabolising cytochrome P450 genes, in *Streptomyces griseolus* have been identified (another *Streptomyces* species being the subject of this work). Transcription from either promoter was shown to be induced by sulfonylurea (chlorimuron ethyl) or phenobarbital. The cytochrome P450-encoding gene (suaC or subC), ferredoxin (suaB or subB) were identified. An inducible, site-specific DNA-binding activity was identified that bound to two similar 8-bp inverted repeat sequences near the sua promoter (suaP). A noninducible DNA-binding activity, distinct from that which bound to suaP, was found that bound to an 11-bp inverted repeat at the sub transcription start point (Patel & Omer, 1992).
A 4-base pair sequence (AAAG) is found to have a regulatory role in the expression of the P450 BM-1 gene in *B. megaterium*. This sequence is found in all eukaryotic and prokaryotic systems which contain barbiturate inducible P450s and is known as a Barbie box. Mutation of the Barbie box lead to the constitutive synthesis of cytochrome P450BM-1 and a 10-fold increase of expression of Bm1P1, a small gene located upstream of the P450BM-1 gene, that encodes a putative regulatory protein (Shaw *et al.*, 2000). Mutation of the P450BM-3 Barbie box significantly increased the expression of both P450BM-3 and Bm3P1 (another small gene located upstream of the P450BM-3 gene that encodes a second putative regulatory protein) in response to pentobarbital induction but left the basal levels unaffected. Bm3R1, a repressor of the P450BM-3 gene, was found to specifically interact with the Barbie box sequences of the *B.megaterium* P450 genes (Liang *et al.*, 1995).

There are numerous other mechanisms of P450 regulation described, such as translational activation as found in the mechanism of P450 progesterone 14α hydroxylase regulation in the fungus *Mucor hiemalis*, where mRNA sequestration is part of the regulatory process. Exposure to substrate releases

sequestered P450 mRNA for translation thereby maintaining a dynamic pool of sequestered mRNA (Antoniou *et al.*, 1994). Another example of the sequestration of mRNA as part of a regulatory P450 mechanism is found in the domestic hen. In the granulosa layer of developing domestic hen follicles the expression of P450 17α-OH mRNA does not directly reflect P450 17α-OH enzyme activity. Moreover, P450 17α-OH mRNA levels dramatically decrease in granulosa cells from preovulatory (compared to developing) follicles. It was proposed that FSH initiates P450 17α-OH enzyme activity to a lesser extent regulates mRNA levels at the time a follicle is recruited into the preovulatory hierarchy and that this action is mediated, at least in part, by the adenyl cyclase/cAMP second messenger pathway. Growth factors such as epidermal growth factor act to prevent premature expression of P450 17α -OH activity in the granulosa layer of follicles that have yet to enter the rapid growth phase of follicle development (Li & Johnson, 1993).

The transcriptional repression of P450 genes is a common regulatory mechanism in mammalian systems such as the steroid 15α hydroxylase of male mice. Steroid 15 α-hydroxylase is a female-specific enzyme in the liver of inbred mouse strain DBA/2J. Assays indicated that the P450 gene is transcriptionally repressed in male mice. BALB/cJ is a variant strain in which the gene is expressed in the males as well as in the females. Genetic crosses between DBA/2J and BALB/cJ indicated that expression of the P450 gene in BALB/cJ males is inherited as a recessive trait and is regulated by a single locus (Aida & Negishi, 1993).

Cell signalling regulation of P450 activity has also been identified as a P450 control mechanism in developmental stages of the bacterium *Myxococcus xanthus* by C signal-dependent promoters of P450 which allow catabolism or anabolism of unusual compounds. These sequences, called C box sequences are cis-acting regulatory elements important for the expression of *M. xanthus*

genes that depend upon intercellular C signalling during development (Fisseha *et al.*, 1999).

A dual control P450 system has been identified in the fungus *Fusarium oxysporum* nitric oxide reductase (P450nor) cytochrome P450 gene, CYP 55. It is regulated in response to oxygen tension by a DNA oxygen binding sensor system. Nitrate and nitrite are the actual inducers of P450nor and its expression is predominantly regulated at the transcriptional level by a system responding to nitrate/nitrite (Tomura *et al.*, 1994).

One of the most widely studied bacterial P450 systems is P450cam from *Pseudomonas putida.* It is often used as a model for mammalian P450 molecules by virtue of being the first P450 crystal structure to be mapped. The problems associated with this are discussed in chapter 5.

There is evidence of autoregulation in the P450 of *Pseudomonas putida.* The regulatory, gene camR on the CAM plasmid of *P. putida* negatively controls expression of the cytochrome P450cam hydroxylase operon (camDCAB) for the camphor degradation pathway and is oriented in a direction opposite to that of the camDCAB operon. The camR gene was found to be autogenously regulated by its own product (Aramaki *et al.*,1993).

The bacteria *Bacillus megaterium* contains a transcriptional repressor Bm3R1. Binding of this to DNA is inhibited by fatty acids. The inactivation of Bm3R1/DNA binding activity results in the activation of transcription of the operon encoding a fatty acid hydroxylase, cytochrome P450 102. This represents a protective mechanism by which *B. megaterium* detoxifies harmful foreign lipids. Polyunsaturated fatty acids (PUFA) activate the transcription of the CYP102 operon. PUFA effects are due to binding directly to Bm3R1. Treatment with the CYP102 inducer, nafenopin, protected cells against PUFA toxicity. Resistance to PUFA toxicity is also observed in a Bm3R1-deficient cells that constitutively expresses CYP102. The resistant phenotype of this Bm3R1 mutant strain is reversed by chemical inactivation of CYP102. Bm3R1

can act as a direct sensor of toxic fatty acids. This was the first evidence of a P450 control system, where fatty acids bind to a prokaryotic transcription factor (Palmer *et al.*, 1998).

B. megaterium also possesses a P450 positive regulatory protein of the barbiturate-inducible P450BM-1 gene (CYP106). BM1P1 and BM1P2 are positive regulatory proteins involved in the expression of the P450BM-1 gene. They act by blocking binding of the repressor protein, Bm3R1 (mentioned above), to the regulatory region of P450BM-1 (He, *et al.*, 1995).

1.15 STEROIDS

Steroids are part of a large group of metabolites called terpenes. Terpenes are based upon a repeating unit known as the isoprene unit, Figure1.15a. Hence, each group of terpenoids are grouped according to the number of repeated units in their basic structure. Therefore each group differs from the next by its members being based around a carbon skeleton five carbons different from the next.

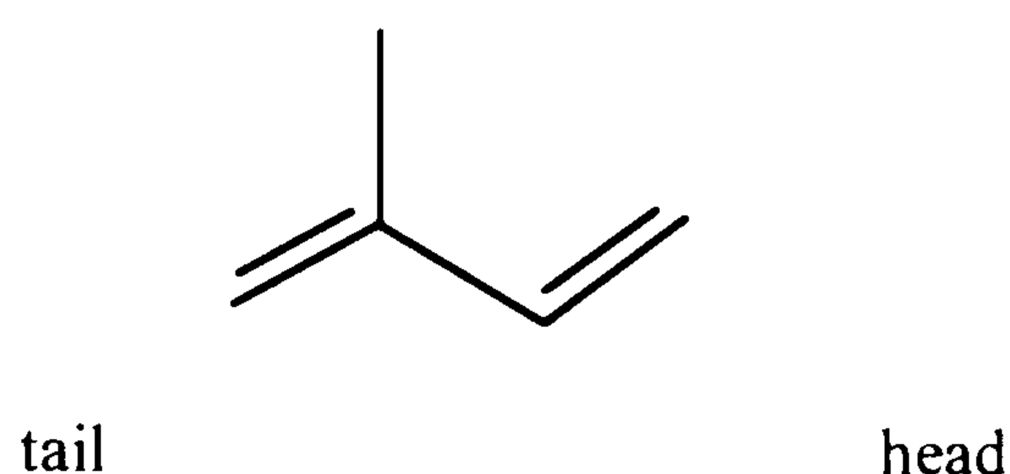


Figure 1.15a The isoprene subunit. 'The steroid building block.'

The classes of terpenes are : C_{10} , monoterpenes; C_{15} , sesquiterpenes; C_{20} , diterpenes; C_{25} , sesterpenes; C_{30} , triterpenes. The steroids are closely related to the triterpenes.

Steroids are polycyclic hydrocarbon compounds which are relatively insoluble in water and soluble in organic solvents. They are important biologically because they form hormones, a vital group of biomolecules which are involved in control mechanisms within multicellular organisms. There are two communication systems within animals : the central nervous system and the blood system. The former communicating on an electrochemical basis and the latter communicating on a chemical basis. The steroid hormones are transported in animals via the blood stream however their release and concentration control is often by the nervous system. So the two communication systems are very much interlinked. The blood system is of course essentially aqueous and although steroids are relatively non-polar they

are transported in the blood as esters (in combination with fatty acids)

Steroids all contain a common hydrophobic nucleus based on phenanthrene reduced to give perhydrophenanthrene i.e: three cyclohexane rings and has a fourth, five membered ring making the perhydrocyclopentanophenanthrene nucleus shown in Figure 1.15b.

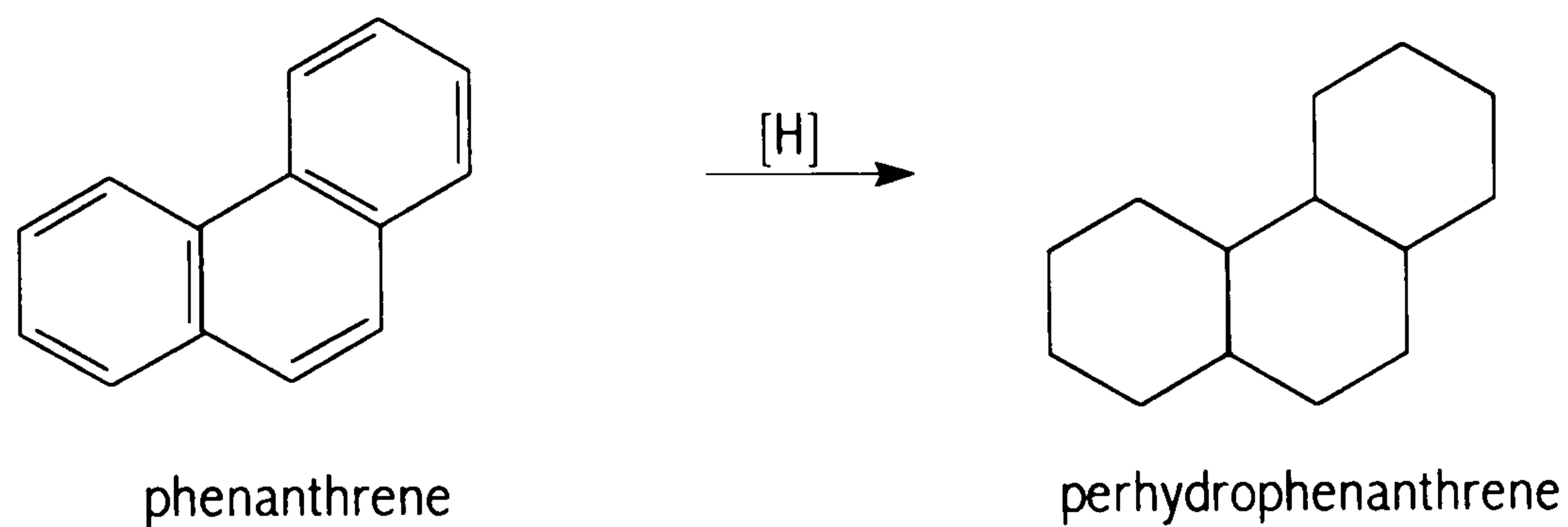


Figure 1.15b. Reduction of phenanthrene forms perhydrophenanthrene.

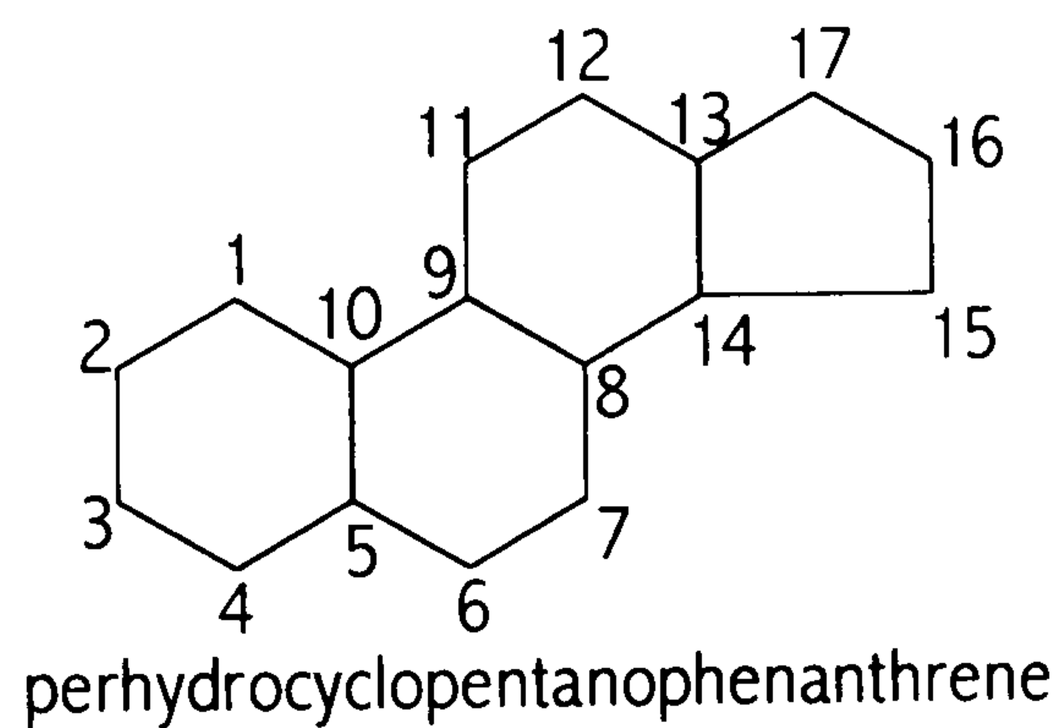


Figure.1.15c. the perhydrocyclopentanophenanthrene ring system - the basic carbon skeleton of steroids, illustrating the numbering convention.

The steroid nucleus is of a planar nature and substituents on the nucleus are designated as 'α - substituents' if orientated away from the observer (behind the plane) and 'β - substituents' if orientated toward the observer. As this is only a projection it is convention to regard any substituent in the same plane as the 18 and 19 methyl groups as β. The 18 and 19 methyl groups are attached to the 13 and 10 carbons respectively. A compound which does not contain this four membered ring cannot be regarded as a steroid. The basic perhydrocyclopentanophenanthrene nucleus can be modified in various ways to produce the parent hydrocarbons of the naturally occurring steroid groups. The source of all steroid hormones is cholesterol which is ingested in food and also synthesised in the liver from leucine or acetate.

1.16 STEROID FUNCTION

Steroids comprise a wide range of hormones such as pregnanes, androstanes, oestranes, glucocorticoids and mineralocorticoids whose functions are chemical communication. Cholesterol is the most important steroid and is a precursor of bile salts which are required for lipid absorption and digestion. Bile salts were the first source of steroids for research in the 1930's. Cholesterol also serves as a precursor of vitamin-D, required in the absorption of calcium.

1.17 BIOSYNTHESIS OF MAMMALIAN STEROID HORMONES

The biosynthetic pathway of steroid hormone synthesis is thought to be the same in all steroid secreting organs such as the ovaries, placenta, testis and adrenals (Hall 1984). All steroid hormones have a common precursor, cholesterol. Steroids are secondary alcohols, but differ from other alcohols in that they are crystalline solids with melting points in the range 100-200° C. Steroids were originally given the name sterols (Gr. *stereos*, solid + -ol).

Cholesterol is a monounsaturated sterol derived from head to tail linkage of isoprene units, Figure 1.15a. Isoprene is not a naturally occurring compound, although the isoprene rule is useful in structure elucidation. The isoprene unit occurs as isopentenyl pyrophosphate and dimethylallyl pyrophosphate, Figures 1.17b and 1.17c respectively,both derived from mevalonic acid, Figure 1.17a.

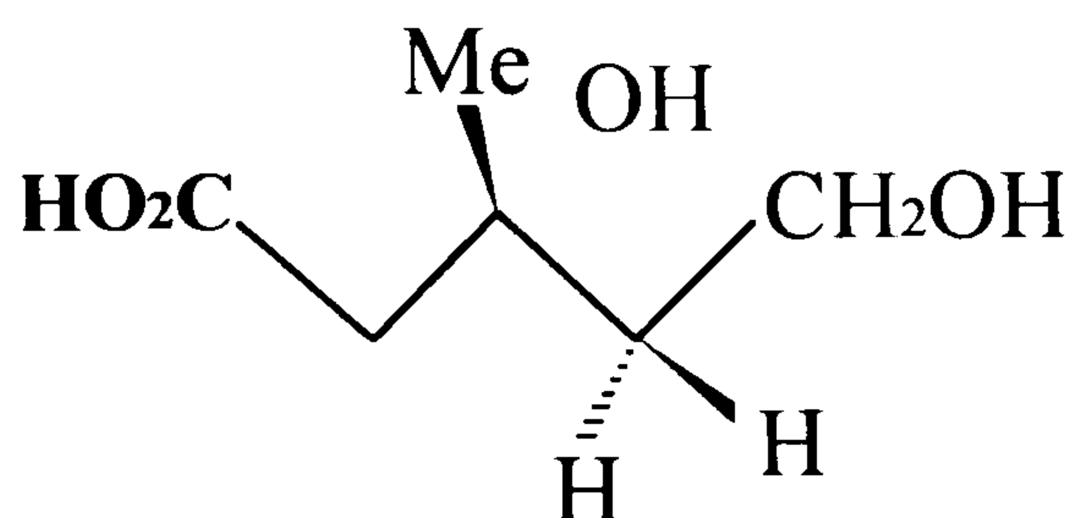


Figure 1.17a. mevalonate

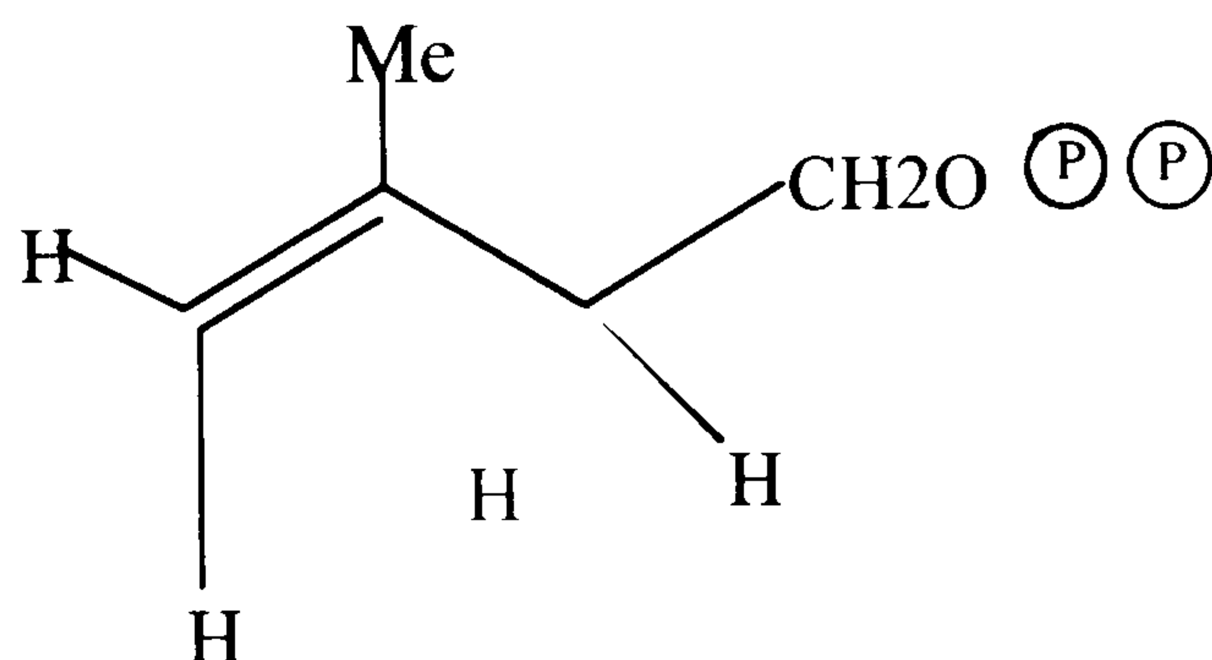


Figure 1.17b Isopentenyl pyrophosphate

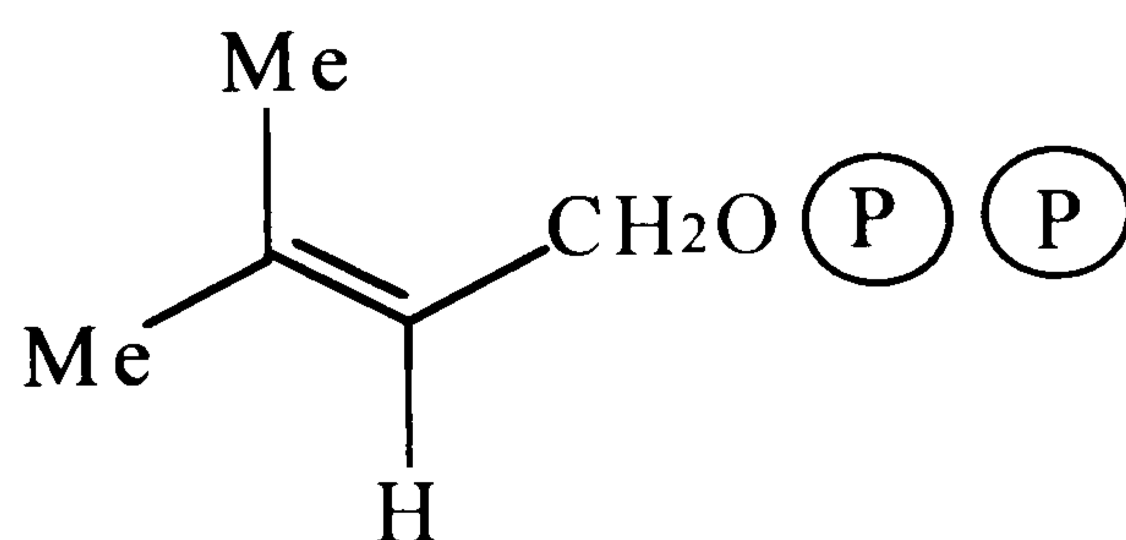


Figure 1.17c. dimethylallyl pyrophosphate

1.18 PROGESTERONE SYNTHESIS AND UTILISATION

Pregnenolone is the major product of the initial side chain cleavage of cholesterol, in the adrenal cortex.

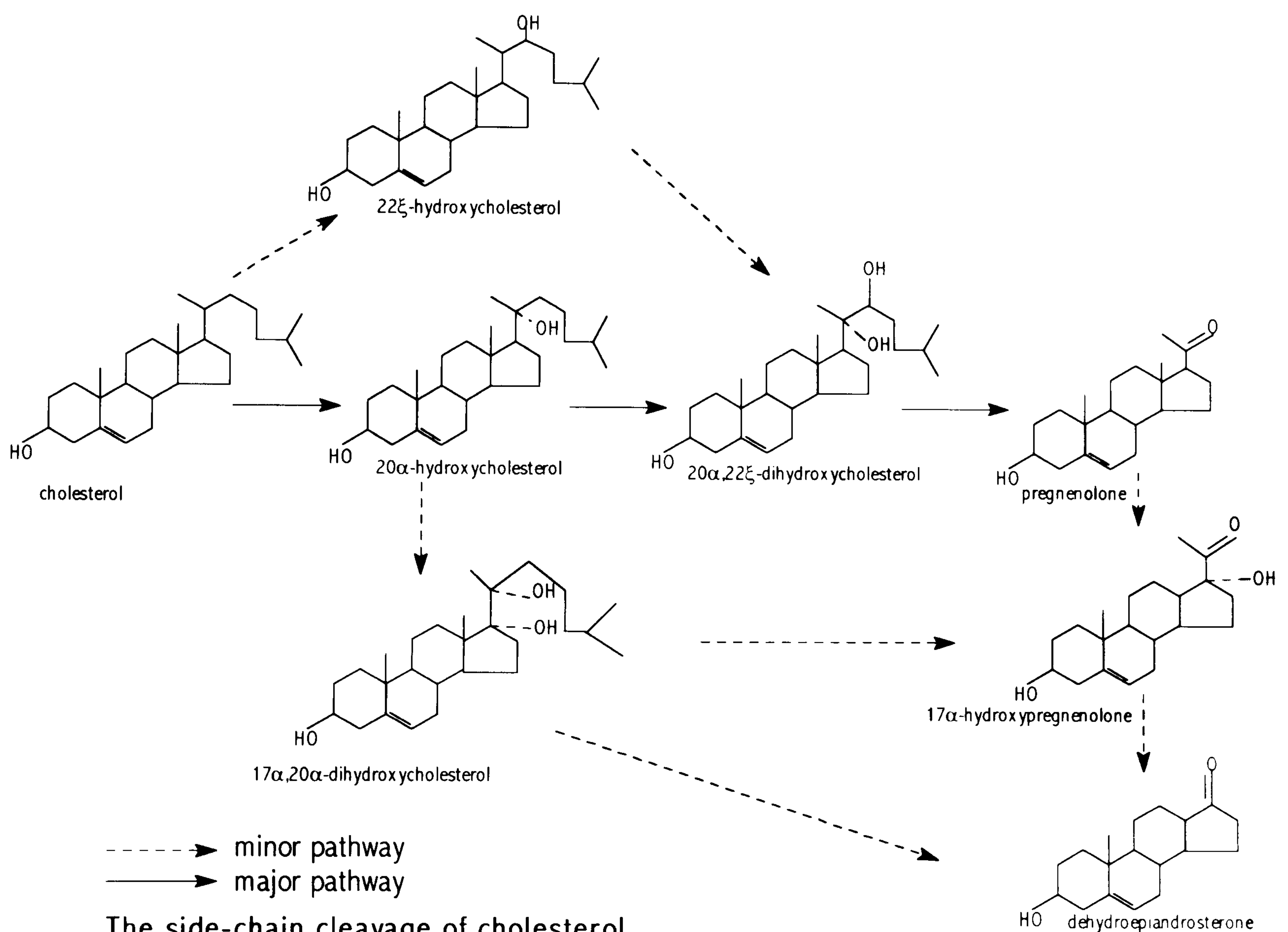


Figure 1.18a. The side chain cleavage of cholesterol. Cholesterol being the source of mamalian steroids. The major and minor metabolic pathways are indicated. The reactions are catalysed by cytochrome P450 isoforms.

Isomerisation of pregnenolone gives rise to progesterone, the steroid hormone of the placenta and corpus luteum and of the maintenance of pregnancy.

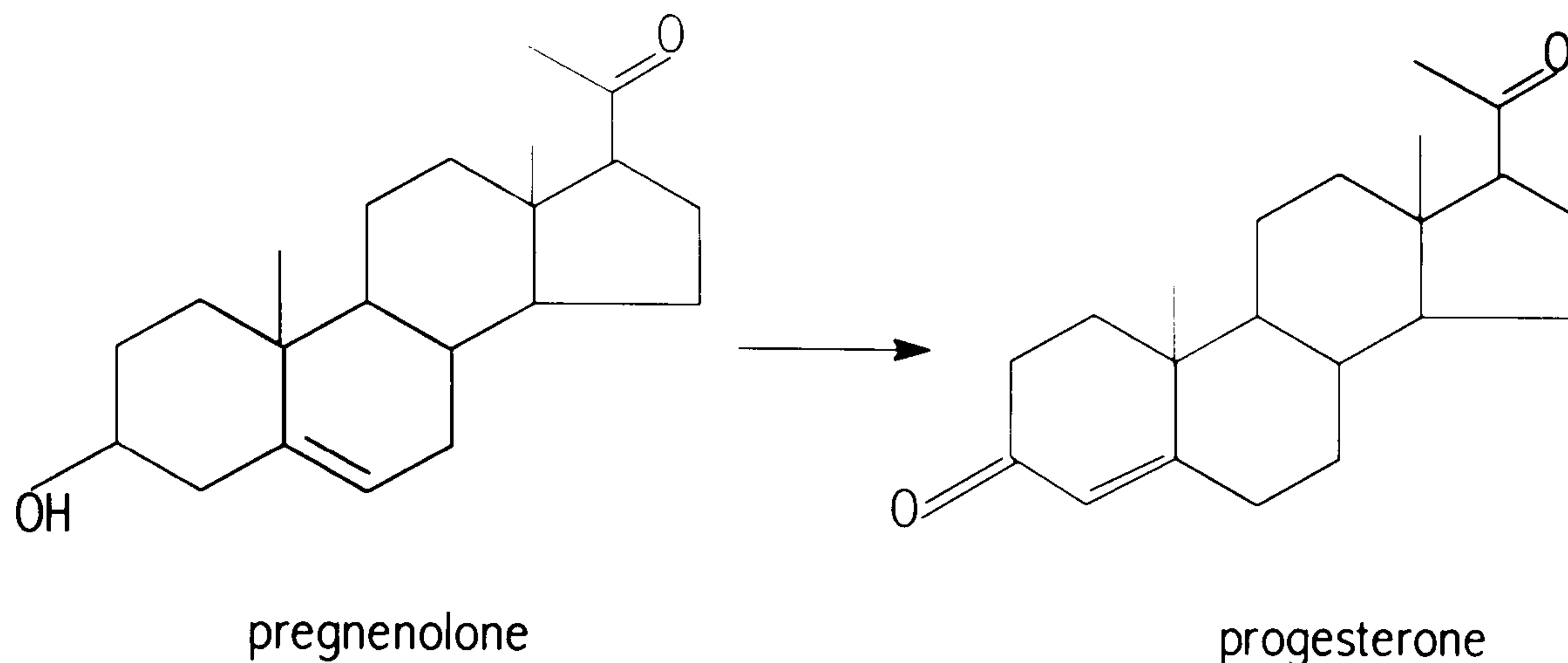


Figure 1.18b. Isomerisation of pregnenolone.

Progesterone contains the Δ^4-3-oxo group of the A ring a characteristic common to all secreted steroid hormones except the oestrogens. Figure 1.18c shows the two fates of progesterone within the adrenal cortex. Three atoms of oxygen are inserted into three distinct positions of the progesterone molecule by enzymes in the microsomes. An atom of oxygen is inserted at the 17α position and at the 21 position. The third oxygen atom is inserted at the 11β position and this is catalysed by a mitochondrial multi P450 enzyme system. 17α,21 & 11β is the preferred sequence for glucocorticoid synthesis but minor variations to this do occur and under normal conditions remain minor pathways unless pathological. For example in congenital adrenal hyperplasia there is a deficiency of 21 hydroxylase. In the zona glomerulosa, progesterone is converted into the mineralocorticoid, aldosterone. First it is hydroxylated at the 21 position by a microsomal hydroxylase system then, at the 11β position by a mitochondrial hydroxylase. Figure 1.18c. shows the pathways in the two histological zones.

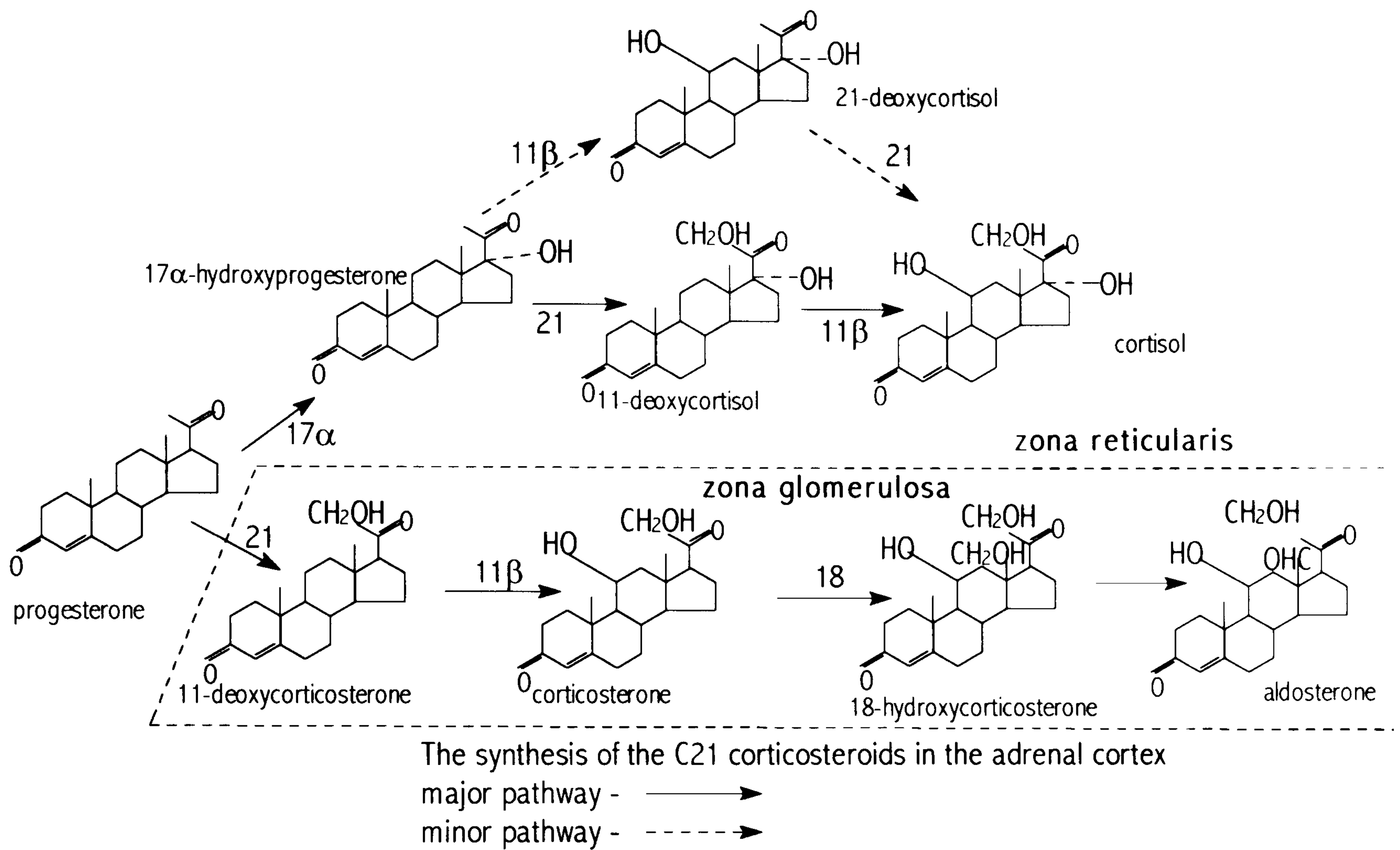


Figure 1.18c The two fates of progesterone within the adrenal cortex. These reactions are catalysed by two CYP3 isoforms (3A4 and 3A5).

Aldosterone, Figure 1.18c, is involved in the maintenance of salt balance and cortisone is required in the process of protein synthesis and inflammation.

1.19 ANDROGEN SYNTHESIS

Androgens are also synthesised from cholesterol cleavage and interestingly are also synthesised in the adrenals from 17α-hydroxyprogesterone and 11-deoxycortisol to yield androstenedione. Testosterone is present in human plasma about ten times more concentrated in men than in women. This is due to the testicular synthesis of testosterone. Figure 1.19d shows adrenal androgen and oestrogen synthesis.

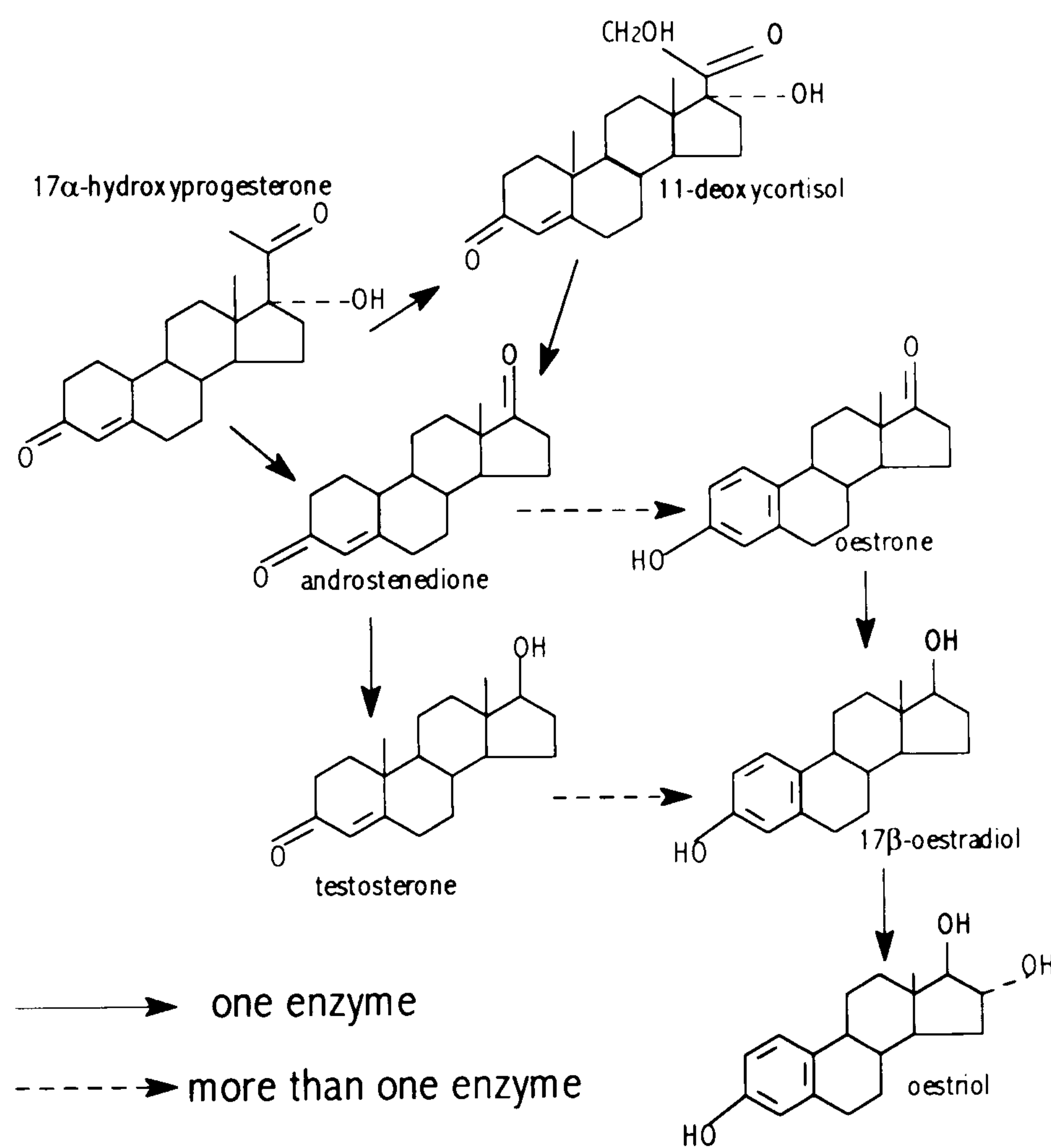


Figure 1.19d. Synthesis of androgens and oestrogens.

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1.20 Overview

The survey above illustrates the huge complexity and number of steroid structures. This section commenced with the example of chemical synthetic pathways to the formation of cortisone which would require thirty separate steps but can simply be achieved by biotransformation. This concept has huge medical implications in terms of P450 modelling techniques for the determination of active site structures of drug and carcinogen metabolising P450s. Indeed 16α hydroxylation of oestrogens is implicated in breast cancer pathology. This is further discussed in chapter 5 but microbial steroid 16α hydroxylation is reported (Younglai & Solomon 1967) as is mammalian 16α hydroxylation (Wong *et al.*, 1989). However the bacterial hydroxylase is not reported to be a P450. The advantage of characterising and purifying bacterial P450s lies in their solubility. Purification and X-ray structural analysis of membrane bound P450s has proved difficult. For example human CYP2E1 which oxygenates ethanol (Bell Parikh & Guengerich, 1999) has been structurally modelled by homology with the CYP102 of *B. megaterium* (Lewis *et al.*, 2000). However a structurally determined eukarotic P450 is reported (Graham & Peterson, 1999). Molecular modelling of steroidogenic cytochromes P450 from families CYP11, CYP17, CYP19 and CYP21 based on the CYP102 crystal structure are also reported (Lewis & Robichaud, 1998). These issues are discussed further in chapter 8. P450 catalysis has many carcinogenic implications, for example P4502B1 from rat liver catalyses the bioconversion of nicotine to cotinine. P4502B1 mRNA showed dose dependent patterns of induction in other tissues expressing P450 2B1, such as the lung. The human homologue of this P450 is P450 2B6. Therefore a useful model of biotransformation is afforded by rat hepatic P450 (Miksys *et al.*, 2000). The development of enzyme systems which are capable of either producing useful compounds for agriculture such as plants expressing antifungal activity and pharmacology, such as the development of antifungal agents is therefore vital.

Industrially applied biocatalysis is also a growing area of P450 application such as production of pravastatin by a *Streptomyces*. expressing P450sca (Matsuoka *et al*., 1989) an important cholesterol-lowering agent. The basis of industrial applications of biocatalysis lies in the availability of sound biotransformation models of reactivity at specific, relatively unreactive centres.

Chapter 2

General Analytical Procedures / Methods

2.1 Whole Cell Biotransformation by and Cell Growth of *Streptomyces roseochromogenes*

2.1.1 Growth and Maintenance of Bacterial Cells on Solid Media

Streptomyces roseochromogenes strain was stored on YMG agar slopes at 4 °C. The slope medium comprised yeast extract 4.0 g/l, malt extract 10.0 g/l, glucose 4.0 g/l and agar 15.0 g/l, in distilled water (Smith *et al.*,1988). Cells were aseptically inoculated onto the slopes inside small screw-cap bottles. Cells were grown in an incubator at 25 °C for 3 days, prior to transfer of the slopes to a refrigerator for storage. Organisms were subcultured every four months.

2.1.2 Growth of Bacterial Cells in Liquid Media

Organisms were grown in 200ml of sterile liquid YMG medium by aseptically inoculating small pieces of the agar slopes into 2.0 l flasks containing submerged coiled wires to aid mixing and aeration. Prior to inoculation of the liquid media, the pH was adjusted to 7.2. The cultures were then incubated in a thermostatically controlled orbital shaker at 25°C and 100 rpm, for 48 hours.

2.1.3 Harvesting of Bacterial Cell Cultures

Following two days of growth, the thick yellow bacterial suspension from each flask was transferred into a 500 ml centrifuge pot. The cells were centrifuged in a Sorvall 5B refrigerated superspeed centrifuge, using a 6 x 500 ml rotor at 8000 rpm and 0-4 °C for 30 min. The supernatant was discarded and the pelleted cells wrapped in aluminium foil and stored at -70 °C until required.

2.1.4 Whole Cell Biotransformation of Progesterone

Progesterone stock was prepared containing 20.0 mg/ml (0.064 M) in absolute ethanol and after a 200 ml cell culture had undergone incubation for 24 h, 1.0 ml of the above stock was added aseptically to flasks. The final progesterone concentration was 0.32 mM. Orbital incubation was then continued as described above, for a further 24 h.

2.1.5 Extraction of Steroidal Products from Cell Culture

After 24h incubation with progesterone, 20.0 ml chloroform was added to the 200 ml culture and mixed by swirling for 1.0 min. The contents of the culture vessel were then poured into a separating funnel and left at 0-4 °C until the chloroform and aqueous layers had separated. The lower chloroform layer was then removed and divided into aliquots of 1.0 ml for storage at 0-4 °C.

2.1.6 Large-Scale Whole Cell Biotransformation of Progesterone for Structural Analysis of Metabolites

The time for maximum transformation of progesterone into metabolites was determined and progesterone transformation was repeated with appropriately scaled up volumes of media and amounts of progesterone.

The metabolites were separated from each other for NMR analysis. Separation was performed on TLC plates as described above except that the steroidal mixture was applied in a long stripe across the lower edge of the TLC plate, rather than as spots. Progress in the separation of the long bands was determined by observing the TLC plates under UV light. The plates were re-run if the separation was insufficient. Bands were cut from the TLC plate and eluted in HPLC-grade methanol overnight. After elution, the pieces of TLC

plate were removed from the methanol and the methanol evaporated. This process was repeated for each metabolite band on the TLC plates, until enough of each had been obtained. The minimum mass of each sample required for ^1H NMR analysis. was 0.5 mg. Purified metabolites were dissolved in $CDCl_3$ for NMR spectroscopy structural analysis, in a Brucker WH 400MHz spectrometer. Structures were assigned by comparing signals of key individual skeletal protons and of substituents e.g. : 4-H, 17α-H, 18H, 19H, 21H and the methine proton of the substituent CHOH functionality thereof, to counterparts in authentic standards and also by comparing the value of the chemical shifts to published signals (Kirk *et al.*, 1990).

2.2 Preparation of Cell Free Extracts and Analysis of Cell Free Transformation by *Streptomyces rosoechromogenes*

2.2.1a Cell Disruption by Homogenisation

Frozen cell pellets were thawed by placing them in sterile phosphate buffer (0.1 M K_2HPO_4 , 0.2 mM EDTA, 1.0 mM DTT, 10 % glycerol, pepstatin 0.7 mg/ml, leupeptin 0.5 mg/l, pH 7.2) ; at 1.5 ml buffer per g of cell pellet (Trower *et al.*, 1989). Once thawed the suspension was transferred to a round bottomed baffled flask and ice-cold acid washed sand was added; 0.5 g per ml of soaking buffer. The flask was then placed over the vertical shaft of a four-blade MSE homogeniser. Cells were homogenised for three minutes (6 x 30 s) with an interval of 1 min between each burst of homogenisation, when the baffled flask was placed on ice to counter the heating effect of the homogenisation.
Sand and cell debris were then removed by centrifugation in a Sorvall 5B superspeed refrigerated centrifuge, using an 8 x 50 ml rotor operated at 0-4 °C and 15000 rpm for 30 min. The supernatant (S15) was decanted from the pellet and retained on ice then re-centrifuged under the same conditions.

2.2.1b Cell Disruption by Sonication

Cell pellets were prepared as above in 2.2.1a. Thawed cells were then placed in a 50 ml beaker in 25 ml aliquots and the shaft of a Dynatech Sonic Dismembrator Model 150 sonicator introduced. Cells were sonicated at a

relative setting of 63 in 30 s bursts before being placed back on ice. The cell debris was then removed by centrifugation as in 2.2.1a.

2.2.2 Transformation of Progesterone in Cell-Free Extracts

Cells were thawed, homogenised and the cell debris removed as described above in section 2.2.1a. To 0.5 ml of the resulting S15 fraction, 3.0 mM progesterone in EtOH was added and 2.0 mM sodium periodate ($NaIO_4$). This incubation mixture was made up to 1.0 ml with incubation buffer: 0.1 M K_2HPO_4 , EDTA 10 mM, DTT 0.5 mM, pH 7.2. Control incubations were also compiled. One control contained 0.5 ml of incubation buffer in place of the 0.5 ml of S15 fraction. A second contained 0.5 ml of S15 which had been boiled for 30 s and cooled to see if spots would appear on TLC analysis.

Progesterone transformation by S15 extracts was optimised by varying concentrations of progesterone, sodium periodate, DTT, EDTA, also varied were pH, temperature and volume of S15 fraction added. Temperature was fixed by incubating assays in a Gallenkamp, orbitally shaking themostatically controlled incubator. pH was adjusted using a Corning pH meter 140. After optimisation, this assay was used in all fractions obtained in purification, to determine the presence of the steroid hydroxylase activity.

In all cases, following the incubation, the steroidal products were extracted from the incubation mixture as described in section 2.5.2.

2.3 Cytochromes P450: Analytical Protocols

2.3.1 Monitoring Haem Absorbance in chromatography Fractions to Locate Cytochrome P450

As discussed in the introduction, the haem moiety of cytochrome P450 is not covalently attached to the protein structure. For this reason haem concentration is not an accurate measure of P450 concentration even if it is approaching homogeneity in any given sample. However, monitoring the 417 nm absorbance of proteins eluted during column chromatography was found to be a very useful guide as to which fraction should be assayed for hydroxylase activity. Of 1.0 ml fractions collected from the columns described above, their absorbances at 280 nm and 417 nm were measured to give an elution profile for total protein and for haem, respectively. The 417 nm absorbance profile proved most useful in locating maximum progesterone hydroxylase activity.

2.3.2 Selective Staining of Haem Proteins in Polyacrylamide Gels (peroxidase activity)

Haem proteins were located in PAGE gels by the hydrogen peroxide - benzidine staining method described by Thomas *et al.*, 1976. Where the haem protein catalysed decomposition of hydrogen peroxide is employed (Thomas *et al.*, 1976). This activity can be linked to a benzidine based substrate to produce a chromophore; the chromophore being indicative of haem protein presence on a polyacrylamide gel.

6.3 mM stock 3,3', 5,5'-tetramethylbenzidine (TMBZ) in methanol and 0.5 M stock sodium acetate, were mixed in a 3 : 7 ratio, to a volume sufficient to soak the gels. The pH was adjusted to 5.0 prior to immersing the gels in this solution

for 2.0 hours in the dark at 4 °C. To this solution H_2O_2 was added to a concentration of 30 mM. A blue stain appeared within an hour.

2.3.3 Obtaining a Dithionite Reduced Carbon Monoxide Difference Spectrum

Two cuvettes, containing either cell extract, partially purified protein from any of the above stages or pure protein in orthophosphate buffer, were placed in a Pye Unicam PU 8800 double beam spectrophotometer and the absorbance range 390 nm - 520 nm (Ruckpaul, 1990) was scanned and a base line set. One cuvette remained in the spectrophotometer while a few granules of sodium dithionite were added to the other cuvette. The contents were mixed by gently inverting five times. Carbon monoxide gas was then bubbled through the dithionite reduced sample for 1.5 min at a rate of one bubble/sec. This cuvette was replaced in the spectrophotometer and the absorbances read from 390 - 520 nm, against the sample blank (Omura & Sato, 1964 [1]).
The amounts of sodium dithionite added were varied and so to were the concentrations of protein sample in the cuvette, until an optimum spectrum was obtained.

2.3.4 Determination of Cytochrome P450 Concentration in Cell Free Extracts

A P450 sample was placed into one of two cuvettes. Sample buffer was placed into the second cuvette as a blank. The absorbance of the sample was read at 490 nm and at 450 nm. A millimolar extinction coefficient of 91 cm^{-1} mM^{-1} was used to calculate the P450 concentration (Omura & Sato, 1964 [2]) using the equation :

$$[\text{ P450 }] = \frac{A_{450} - A_{490}}{\varepsilon_0}$$

2.3.5 Obtaining *S. roseochromogenes*, Progesterone Hydroxylase Cytochrome P450 Substrate and Inhibitor Binding Spectra

Spectrophotometric analysis of the interaction between the cytochrome P450 progesterone 2β,16α-hydroxylase from *S. roseochromogenes* and substrate, progesterone and inhibitor, ketoconazole (Jefcoate, 1978) was carried out using a Phillips PU 8800 double beam spectrophotometer.

Active fractions from each stage of the purification process were used to obtain the spectra. For each assay 1 ml of fraction was placed in a cuvette in the double beam spectrophotometer and the absorbances of wavelengths from 350 - 510 nm recorded and an absorbance baseline obtained. 0.1 ml of either ketoconazole or progesterone, in varying concentrations in EtOH / 0.1 M phosphate buffer 30:70 v/v was added with varying concentrations of the substrate or inhibitor. Absorbances were recorded across the 350 - 510 nm range to determine the effects of varying concentration. Comparison was also made between spectra obtained from different stages of the P450 purification process (chapter 4).

2.3.6 Inhibition of Catalysis by Ketoconazole

Ketoconazole was added to cell-free incubations of both partially purified and purified progesterone hydroxylase, at concentrations of 0.5 - 8.0 μg / ml. Progesterone was added at 4.0 mM and sodium periodate (Hrycay *et al.* 1975) at 2.25 mM. Incubations were carried out for 3 h before steroidal metabolites were extracted as described in 2.5.2. Methanol dissolved metabolites were observed by TLC and analysed spectrophotometrically, using a Pye Unicam PU 8800 UV/vis spectrophotometer.

2.4 Steroidal Analysis

2.4.1 Cell-Free Assay of Progesterone Transformation

During initial screening and during purification stages, fractions were examined for progesterone hydroxylase activity to ensure that activity had been retained and also to ensure that the specific products of the hydroxylase catalysis were retained. The incubation mixture was prepared as described in section 2.2.2 and products were extracted from this incubation as described in section 2.4.2.

2.4.2 Extraction of Steroidal Products from Small Scale Cell-Free Incubations

Following the incubation described in section 2.5.1, 1.0 ml of chloroform was added to the incubation mixture. The contents of the Bijou bottle were then vortexed and left at 4 °C for 30 min allowing the chloroform and aqueous layers to separate. A glass Pasteur pipette was then introduced gently and the lower chloroform layer withdrawn and transferred into a 2.0 ml Eppendorf microfuge tube. The tube was placed over a thermostatically controlled water bath at 65 °C and evaporated to dryness. The dry steroidal contents of the tube were redissolved in 20 μl methanol prior to TLC analysis (section 2.4.3).

2.4.3 TLC Analysis of Progesterone Transformed in Whole-Cell or Cell-Free Incubations

One of the 1.0 ml steroid samples in chloroform prepared as in section 2.4.2, was placed over a thermostatically controlled water bath at 65 °C and incubated until the chloroform had evaporated. The steroidal products were redissolved

in 20 μl methanol and mixed. Two other tubes were also prepared as controls. One tube contained 20 μl of 20 mg/ml stock progesterone in ethanol. The third contained 20 μl of the cell culture from which the steroidal products had been extracted as described above. These three samples were then spotted in 5.0 μl aliquots onto a Kieselgel 60 F254 fluorescent high performance TLC plate, 1.0 cm from the lower edge and 1.0 cm apart. The sample spots were dried for 1 min under an electric fan. The lower edge of the TLC plate was placed in solvent, ethyl acetate : toluene : petroleum ether, 4:3:3 v/v in a chromatography tank and allowed to develop until the solvent front had risen about 10 cm up the TLC plate. The TLC plate was then removed from the chromatography tank and dried under a heated air flow. The steroidal separation was observed under UV light. Dark spots on a fluorescent green background, afforded by the UV absorbance of the 4-en-3-one chromophore of ring A, indicated the extent of migration and the number of steroidal products.

2.4.4 Quantification of Steroidal Biotransformation Products

Two different procedures were employed to determine the extent of steroid transformation under different conditions and at different stages of the purification process. Yields of progesterone metabolites produced by *S. roseochromogenes* were determined by TLC and HPLC. In the TLC method exactly 5.0 mg of progesterone was added to each incubation and dried transformation products were dissolved in exactly 100 μl HPLC-grade methanol. Equal A_{242} absorbance units of sample contained in *ca* 5 μl were spotted onto fluorescent high-performance Kieselgel 60 F_{254} TLC plates, which were developed in an ethyl acetate/ether/toluene (4:3:3 by volume) solvent system. Steroids were viewed under UV light, by and plates were photographed. Individual spots were scraped from the TLC plate and eluted in HPLC-grade methanol. Dried steroids were re-dissolved in exactly 1.0 ml of

methanol and the UV absorbance at 242 nm was measured. The absolute amount of steroid present was determined by reference to a calibration curve.

Reverse phase high performance liquid chromatography (RP-HPLC) of steroidal metabolites was performed as follows.
The solvent used for RP-HPLC was MilliQ water : methanol, 60 : 40 v/v, which was degassed for 1.0 h prior to priming a Gilson model 303 HPLC pump. Solvent was allowed to flow for 1.0 h through a Whatman Partisil PXS 5/25ODS column with a guard column attached. Solvent was passed through an LDC/ MiltonRoy UV MonitorIII fixed wavelength detector (λ254nm), to detect steroidal products. The UV monitor was connected to a Hewlett Packard Integrator to record the absorbance signals.
The integrator was set to calculate concentration of steroid by proportionality to the area under individual peaks.

2.4.5 Elucidation of the Pathway of 16α-monohydroxy and 2β,16α-dihydroxy progesterone synthesis by *S. roseochromogenes*

To determine whether the 16α-monohydroxylated progesterone metabolite was the direct precursor of the 2β,16α-dihydroxylated product, the incubation was carried out using 1.0 mM [4-^{14}C] hydroxyprogesterone plus 1.0 mM unlabelled progesterone. The extracted products were separated by TLC as described earlier. Spots were cut from the TLC plate and counted, to locate the radiolabelled product. Hydroxylation assays were analysed by HPLC, methanol : water, 60 : 40 v/v as solvent, as described above.

2.5 More General Analytical Procedures

2.5.1 Determination of Protein Concentrations

Protein concentration was determined essentially by the Coomassie blue dye binding method developed by Read & Northcote (Read & Northcote, 1981) and adapted by Dr. Mark Carroll at Queen Mary & Westfield College, Department of Biomedical Sciences.

50 mg Coomassie Blue G250 were dissolved in 50 ml of ethanol and 150 ml of phosphoric acid added with constant stirring followed by distilled water to a final volume between 1 and 2 l, so that the solution was pale brown with a λ_{max} of 470 nm. To a 100 µl sample, 10 µl of 2M NaOH, were added and mixed. 1.0 ml of Bradford reagent prepared above was added and the absorbance read at 595 nm after 2 min against a reagent blank. A standard curve was created using varying concentrations of protein.

The original Coomassie procedure developed by M. Bradford (Bradford, 1976), is only linear up to about 4 µg protein compared to 50 µg protein, by this adaptation.

2.5.2 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis of Proteins

10 % SDS-polyacrylamide gel electrophoresis was carried out in a BioRad Miniprotean II double electrophoresis cell using a modified protocol of Laemlii (1970).

The 10 % resolving gel in 1.0 M tris buffer at pH 8.8 was degassed for 10 min. Then 200 µl ammonium persulphate and 20 µl TEMED were added before pouring the gels. Approximately 50 µl of water was added on top of the resolving gel to aid polymerisation. The 6 % stacking gel in 1.0 M tris, pH 6.8 was degassed and 50 µl ammonium persulphate and 10 µl TEMED were added. After the resolving gel had set, excess water was removed and the stacking gel poured on top.

Protein samples were prepared by adding 15 µl of solubilising buffer, which comprised 20 % bromophenol blue w/v, 12.5 % β-mercaptoethanol v/v, 25 % (Marshall & Williams, 1984) glycerol v/v adjusted to pH 6.8, to 15 µl of fraction and 10 µl of 20 % SDS. Samples were then microfuged before being vortexed, microfuged again and then boiled for 5 min, before being loaded into the gel wells with a syringe.

After running, the plates were prized apart and the gel washed with 10 % TCA (100 ml) for 15 - 20 min, to fix the protein and then washed with 100 ml water for about 5 min. This was followed by a wash with MeOH : water : Acetic acid (45 : 45 : 10) for about 15 min. Gels were then stained in Coomassie blue or silver stained. Gels were washed twice in MeOH / acetic acid solution for 30 min each time. Gels were dried by soaking them in water : acetic acid : glycerol 90:10:2, for 1.0 h and fully dried in a BioRad 200 gel dryer.

2.5.3 Silver Staining of Proteins in Polyacrylamide Gels

The silver staining of gels was carried out essentially according to the method of Rabilloud T. *et al.*, 1988.

2.5.4 Polyacrylamide Gel Isoelectric Focusing (IEF) of Purified Progesterone 2β,16α-Hydroxylase Protein

Polyacrylamide gel solution was prepared as in section 2.5.2, containing an 8 % gel matrix and 2.4 % w/v ampholine (pH 3.5 - 9.5). Gels were run in a BioRad Miniprotean II double electrophoresis cell. Electrode solutions were : anode buffer, 1 M phosphoric acid and cathode buffer, 1 M sodium hydroxide. The gels were prerun at 100 V for 30 min, 150 V for 30 min, then 250 V for 30 min. Samples were then loaded and run at 250 V for 2 h, 300 V for 1.5 h and finally 400 V for 1.5 h.

The gels were then fixed with 10 % TCA and silver stained as described in section 2.5.3.

2.5.5 Two-Dimensional Polyacrylamide Gel Electrophoresis Preparation of IEF gels

IEF gels were prepared essentially by the method of Hochstrasser *et al.*, 1988. 3.0 g Urea were mixed with 0.8 ml stock acrylamide solution (29.2 g acrylamide and 0.8 g bisacrylamide in 100 ml distilled water, filtered through a Millipore filter) , 0.06 g CHAPS , 0.6 ml 10 % w/v Nonidet P40 (in ultra-high purity water). The ampholines used were, 0.1 ml ampholines pH 3 - 10 and 0.5 ml ampholines pH 4 - 8 (Harrington *et al.* 1991). This volume was made up to just under 6.0 ml with ultra-high purity water. The solution was stirred and warmed gently, until all solids were dissolved. The volume was then adjusted to 6.0 ml.

The solution was degassed for 5 min and polymerised by adding 5.0 μl TEMED and 10 μl of freshly prepared 10 % ammonium persulphate solution. Gel rod tubes were filled and overlaid with 100 μl of water saturated with isobutanol. The rod polyacrylamide gels were left to polymerise at room temperature.

Sample buffer was prepared by dissolving 5.5 g urea , 0.1 g CHAPS , 0.2 ml 10 % ampholines (pH 3 - 10) , 1.0 ml NP-40 and 0.1 g DTT in water and then made up to 10.0 ml. This was divided into aliquots and stored at -20 C.
A protein sample of 10 μl was taken and 20μl of sample buffer was added to this. This preparation was vortexed for 10s and incubated at room temperature for 30 min. The sample was then microfuged for 1 min, prior to applying to the top of a rod gel.

Isoelectric focusing was performed in the following manner. Two rod polyacrylamide gels were set up for each protein sample, in a BioRad Model 175 chamber attachment for a BioRad Miniprotean II electrophoresis cell. A syringe with a fine needle was used to apply anolyte to the bottom of each gel to fill any air space. Overlay buffer containing urea and ampholine was added to the top of each gel to expel trapped air. The electrophoresis chamber was placed on a magnetic stirrer and a 2.5 cm stir bar placed inside with 500 ml anolyte (10 mM H_3PO_4). Catholyte (freshly made & degassed 20 mM NaOH) was placed in the upper chamber sufficient to cover the tops of the rod gels. The gels were pre-focused at 200 V for 15 min, 300 V for 15 min and finally 400 V for 15 min. The anolyte and catolyte were discarded and protein samples applied to the tops of each rod gel, followed by overlay buffer. Focusing was carried out at 1000 V for 3 h with continuous magnetic stirring.

After focusing, the rod gels were removed from the tubes by placing in a gel extractor with a syringe attachment containing water. The gels were gently

forced out onto parafilm and 200 μl of Laemlii sample buffer used to stain the length of the gel. After 10 min, the IEF gel was transferred to the top of a 10 % polyacrylamide slab gel in a BioRad Miniprotean II electrophoresis cell, which was prepared as described in section 2.5.2 but which contained a flat upper surface along the top, rather than the sample wells. Also the stacking gel depth was only 5 mm rather than 2 cm. This second dimension gel was run at 200 V until the bromophenol blue band had just migrated off the lower end of the gel. The gels were then fixed as in section 2.5.2 and silver stained as in section 2.5.3.

2.5.6 Non-Denaturing Polyacrylamide gel Electrophoresis

Gels were poured and comprised a 6% stacking gel and 10 % resolving gel as described by Laemlii, 1970. Gels were run in a BioRad Miniprotean II double electrophoresis cell. This SDS-polyacrylamide gel electrophoresis procedure was used to examine the final pure protein samples along with any contaminants, on the basis of charge and size respectively. Non-denaturing gels were prepared in the same way as SDS gels (section 2.5.2) except that SDS was omitted from the sample buffer and β-mercaptoethanol was omitted from the samples. Samples were not heated and the electrode buffer contained no SDS.

2.5.7 Electroblotting of Purified Steroid Hydroxylase for Partial Amino-Acid Sequencing

Following resolution of homogenous P450 by SDS-polyacrylamide gel electrophoresis, the slab gel was soaked in 10 mM CAPS (pH 11) and 10 % methanol v/v (transfer medium) for 30 min. (Matsudaira 1987). A PVDF membrane was soaked in methanol for 15 min. 300 ml of 10 mM CAPS was poured into a Bio-Rad Trans-blot SD semi-dry transfer cell. Three sheets of blotting paper were layered onto the metallic plate in the blotter and the PVDF

membrane placed on top of the blotting paper. The gel was placed onto the membrane and 3 more sheets of blotting paper were layered on top of the gel. Proteins from the gel were electroeluted at 0.5 A for 1 h.

The PVDF membrane was removed and washed for 5 min in distilled water. The membrane was stained with Coomassie blue and stored at -20 °C until sequencing.

2.5.8 Sephadex G-2000 Molecular Sieve Gel-Filtration to Determine the size of Progesterone 2β,16α Hydroxylase P450

20 g of Sephadex G-2000 was weighed and swollen in 0.1 M orthophosphate buffer whilst stirring with a glass rod. The slurry was then autoclaved at 121 °C for 15 min. After cooling the Sephadex was degassed on ice for 1 h and then poured into a glass column to give a bed volume of 70 ml. The column was washed with the buffer as described in section 2.2.2 (running buffer). Active post MIMETIC affinity column fraction (1 ml) was applied to the top of the column along with other proteins of known molecular mass.

The standard proteins run through the column for calibration, were:

cytochrome c M_r 13370, myoglobin M_r 16900, chymotrypsinogen M_r 23240, ovalbumin M_r 43000, phosphorylase a M_r 92000, catalase M_r 247500.

2 ml Fractions were collected using a Pharmacia Frac-100 fraction collector and their elution volume recorded (V_e). A standard curve of molecular mass v elution volume was prepared. 1.0 ml of purified progesterone hydroxylase was then applied to the column and 2.0 ml fractions were collected. When the protein had eluted, V_e was determined and the molecular mass calculated from the calibration curve prepared above. The protein fractions which contained the eluted progesterone were combined and assayed for hydroxylase activity.

2.6 Molecular Biology

2.6.1 Partial Amino Acid Sequence of the Purified P450

Purified protein (7 μg/ml) dissolved in 0.1 M K_2HPO_4 buffer containing 0.5 mM DTT, and 10 mM EDTA and 10 % v/v glycerol was divided into aliquots of 0.1 ml. The protein was partially digested with modified porcine trypsin. N-terminal sequencing of amino acid residues was carried out by acid cleavage of isothiocyanate bound N-terminal residues. Hydrolysed amino acids were identified by HPLC analysis, each amino acid residue being identified by its retention time compared with retention times of authentic amino acid standards. Using searches available on the internet, it was then possible to search for similar protein sequences.

Chapter 3

Whole Cell Biotransformation of Progesterone *by Streptomyces roseochromogenes* and Metabolite Identification by ^{1}H Nuclear Magnetic Resonance Spectroscopy

3.1 INTRODUCTION

This chapter is primarily concerned with an investigation of the effects of culture conditions upon the growth of *S. roseochromogenes* and its ability to biotransform exogenous progesterone. The emphasis here is on the microbiological aspects of the preliminary investigation into progesterone biotransformation by *S. roseochromogenes*.

This chapter also describes the identification of metabolites, by ^{1}H nuclear magnetic resonance spectroscopy (NMR).

3.2 MATERIALS

Streptomyces roseochromogenes strain 10984 was purchased from the National Collection of Industrial and Marine Bacteria (NCIB) Ltd., Aberdeen, Scotland.

Unless otherwise stated, materials used here were of AnalaR quality.

Progesterone was purchased from Sigma Chemicals Co., Poole, Dorset.

For cell growth, yeast extract and malt extract were also purchased from Sigma Chemicals Co.

D(+)-glucose, chloroform, ethyl acetate, HPLC grade methanol and glycerol were purchased from BDH Laboratory Supplies, Poole, Dorset.

Kieselgel 60 F254 TLC plates were purchased from Merck Ltd.,Darmstadt, Germany.

3.3 METHODS

3.3.1 Steroid Transformation *by S. roseochromogenes*

For small-scale analytical experiments, cells were grown at 25 °C for 48 h with continuous shaking (Smith *et.al.*, 1988) (100 rpm in an orbital incubator) in 50 ml YMG in 500 ml conical flasks containing coiled wires to aid aeration and dispersal of biomass. After an initial 24 h of growth,

progesterone (0.25 ml of a 20 mg/ml stock solution in ethanol) was added to each culture flask. After a further 24 h, cells were harvested by centrifugation in an 8 x 50 ml angle rotor spun for 30 min at 8000 rpm and 4°C in a Sorvall 5B centrifuge. The supernatant was decanted and extracted with two equal volumes of chloroform. The organic layers were collected, combined and evaporated to dryness.

To obtain sufficient pure metabolites for structure determination, progesterone transformation incubations were increased fourfold.

3.3.2 Measurement of *S. roseochromogenes* Biomass

Where measurement of cell growth or biomass of *S. roseochromogenes* was required, a 1.0 ml sample of cell culture was removed and diluted x 10 with water and the absorbance measured at 600 nm against a blank of water. The extent of cell growth was then obtained from a biomass calibration curve.

3.3.3 Purification and Structure Determination of Progesterone Metabolites Produced by *S. roseochromogenes*

Metabolites for structure determination were purified from scaled up incubations, as described previously in chapter 2.

3.3.4 Investigation of the Optimal Conditions for Growth of *S. roseochromogenes* Cells

The conditions under which cell cultures were grown was investigated in order to optimise the amount of hydroxylase ultimately recoverable from

harvested biomass. For this purpose two parameters were monitored. The first was the effects of culture conditions upon cell growth and the second was the degree of biotransformation of progesterone per unit biomass produced.

3.3.5 Identification of Progesterone Metabolites Produced by *S. rosechromogenes* strain 10984 by ^{1}H Nuclear Magnetic Resonance Spectroscopy

Metabolites for structure determination were purified as described in chapter 2 from scaled-up incubations. Analyses were by ^{1}H NMR spectroscopy on a Bruker WH400 MHz spectrometer. 16α Hydroxyprogesterone was identified by the "fingerprint" method (Smith *et al.*, 1988), which involved superimposing spectra of authentic monohydroxy steroid standards on the spectrum of putative 16α hydroxyprogesterone. A full range of dihydroxyprogesterone spectra was not available to permit identification of 2β,16α dihydroxyprogesterone by fingerprinting. The structure of this metabolite was assigned by summing the individual shifts of the key characterising signals of the monohydroxylated counterparts. This gave predicted ^{1}H shifts for the unknown dihydroxylated compound. The assignment was confirmed by a full 2-D ^{1}H COSY spectrum analysis (homonuclear correlation spectroscopy).

Spectra presented in this chapter are with reference to Me_4Si as an internal standard. Spectra were obtained at 303 K and the solvent used was $CDCl_3$. Where D was 99.8 %.

3.3.6 Time Course of Progesterone Transformation by *S. roseochromogenes* Strain 10984

In a 25 h incubation of a 200 ml culture, 2.0 ml samples of culture medium were removed aseptically at the time intervals shown in Table 3.4a.

For HPLC analysis, progesterone metabolites were separated in 60% aqueous methanol on an analytical reverse-phase Whatman Partisil PXS 5/25 ODS column. Column effluent was passed through an Pye Unicam PU 4020 UV detector set at 254 nm. Metabolite concentrations were calculated from the areas of the individual peaks eluting from the column measured on a Hewlett Packard Integrator.

3.4 RESULTS

3.4.1 Growth Curve for *S. roseochromogenes*

The profile in Figure 3.4.1a, shows the growth of cells in a 200 ml culture grown in an orbital shaking incubator in a 2 l culture vessel containing a coiled wire wound around the inner circumference of the base of the culture vessel. A 1 ml sample was aseptically extracted every 2 h.

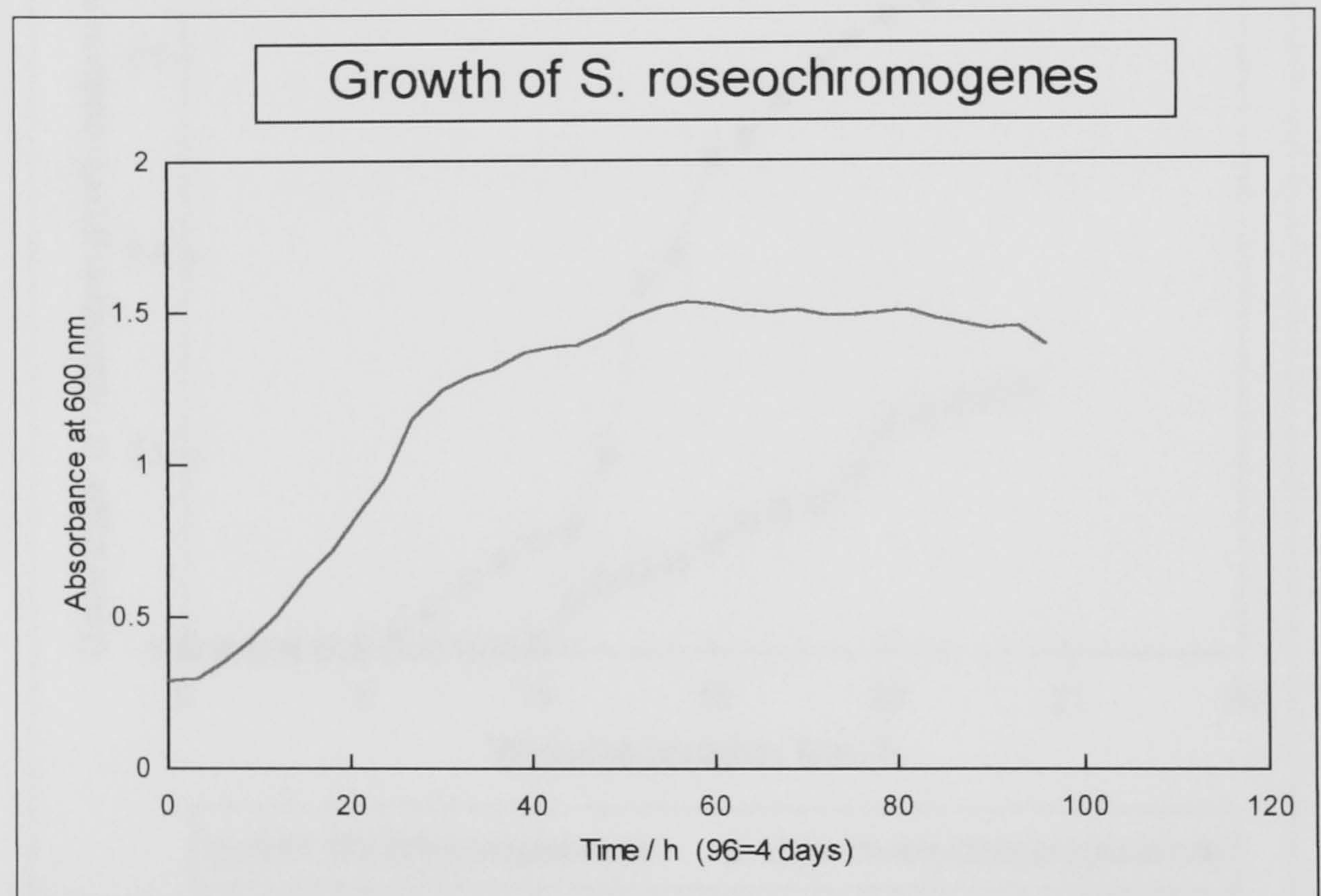


Figure.3.4.1a Growth of *S. roseochromogenes* cells at 25 °C, for the determination of optimal cell incubation time.

The optimum time for late logarithmic growth from the time of inoculation was found to be 30 h. Figure 3.4.1a shows the growth profile in terms of the increase in turbidity of the culture medium with time. A 35 h incubation of 3 l of cell culture in 200 ml aliquots, yielded on average 46 g of cells after harvest.

3.4.2 Quantification of Metabolite Production by Whole Cells of *Streptomyces roseochromogenes*

Cell cultures were incubated with 4.0 mM progesterone for increasing lengths of time, before 20 ml of culture was removed, quenched with chloroform and metabolites extracted and run on TLC plates.

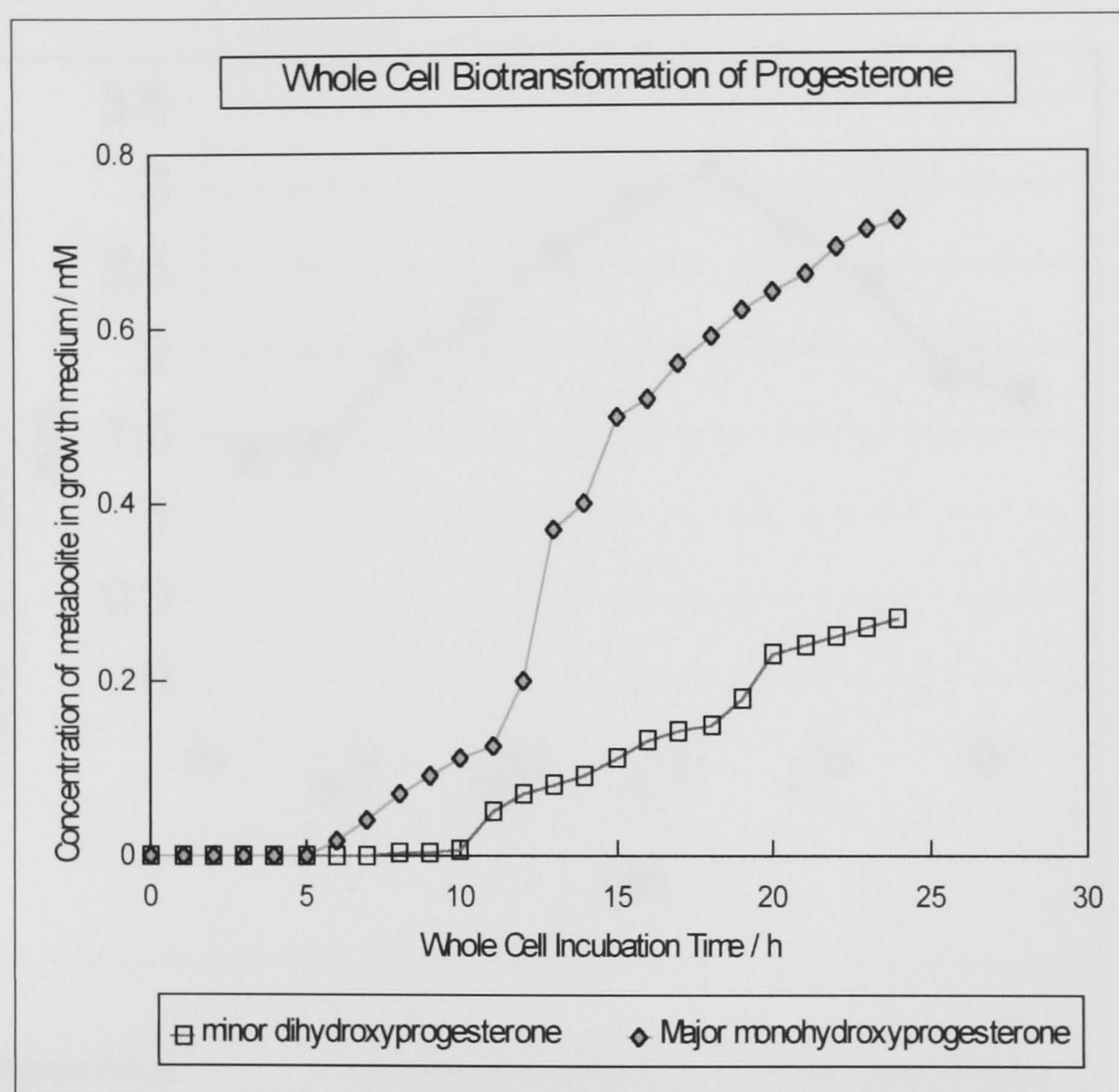


Figure. 3.4.2a *S. roseochromogenes*, whole cell biotransformation of progesterone as determined by TLC elution.

The metabolites were quantified as described earlier. Figure 3.4.2a shows the profile of metabolite production with time. The upper profile shows the production of 16α-hydroxyprogesterone and the lower profile shows the production of 2β,16α-dihydroxyprogesterone.

2β-hydroxyprogesterone was not found to be a product.

3.4.3 The Effect of pH upon Cell Growth of *S. roseochromogenes*

Cells were grown at varying pH values of as indicated in Figure. 3.4.3a, each for 20 h. At 20 h the absorbance of a 10 fold dilution with water was taken. The profile shows that growth was optimal at pH 7.2.

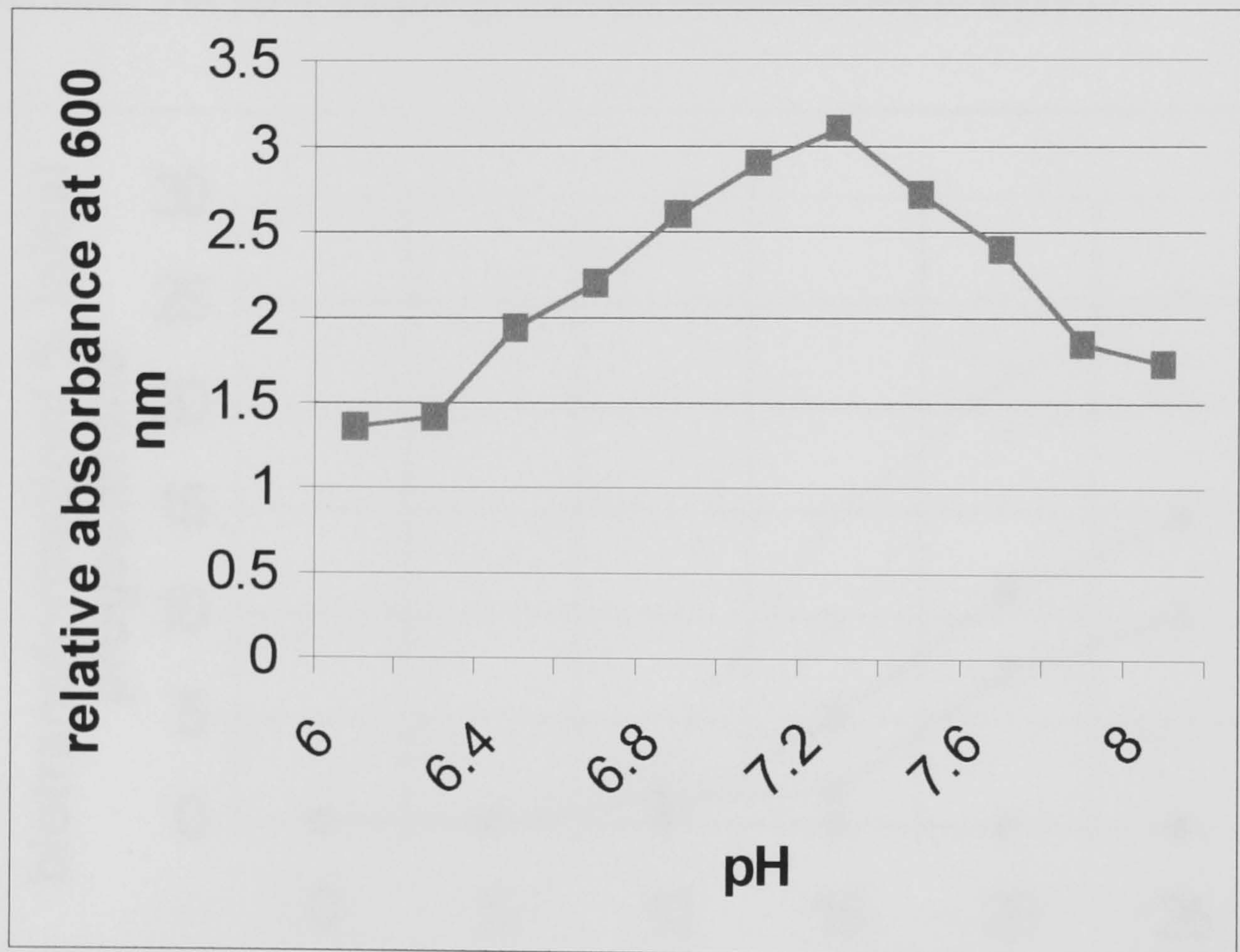


Figure 3.4.3a Cell growth at varying pH in 200 ml culture. For the purpose of identifying the optimum initial pH for cell culture.

3.4.4 The effect of pH on the Transformation of Progesterone by *S. roseochromogenes*

These profiles were obtained by growing cells in the manner described above but the initial pH was different in each case. Although the growth medium was made up at the pH specified, cell growth resulted in pH change over time. The pH of the growth medium therefore refers to initial pH.

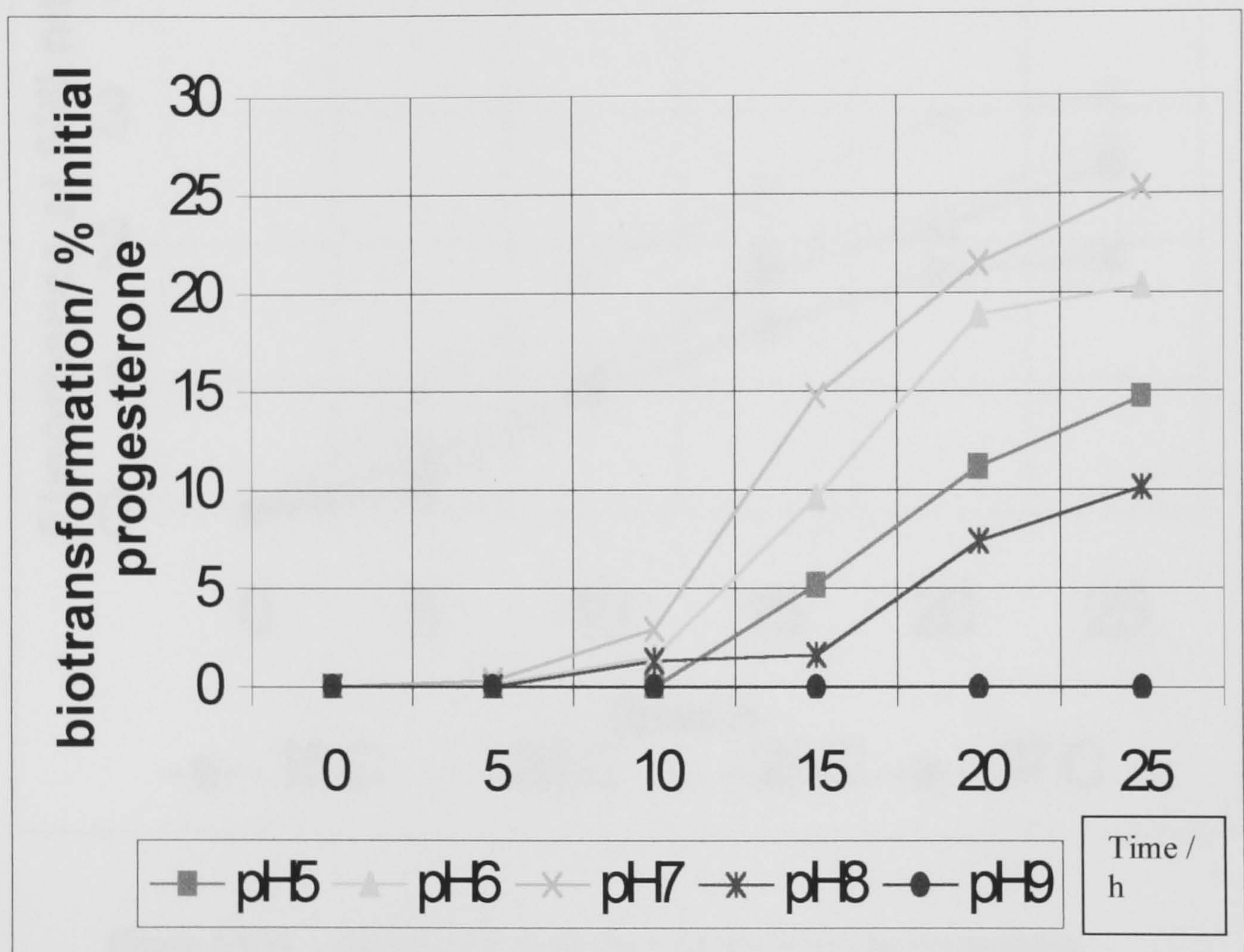


Figure 3.4.4a. Transformation profiles by *S. roseochromogenes* of progesterone with varying pH

The above profiles show a growth medium of initial pH 7 was optimal for maximum progesterone biotransformation by whole cell cultures of *S. roseochromogenes*.

3.4.5 The Effect of Temperature on Cell Growth of *S. roseochromogenes*

Growth at the temperatures indicated in Figure 3.4.5a, was achieved in a thermostatically controlled orbital incubator, except at 37 °C, where the 200 ml culture was incubated in a warm room on an open orbital shaker.

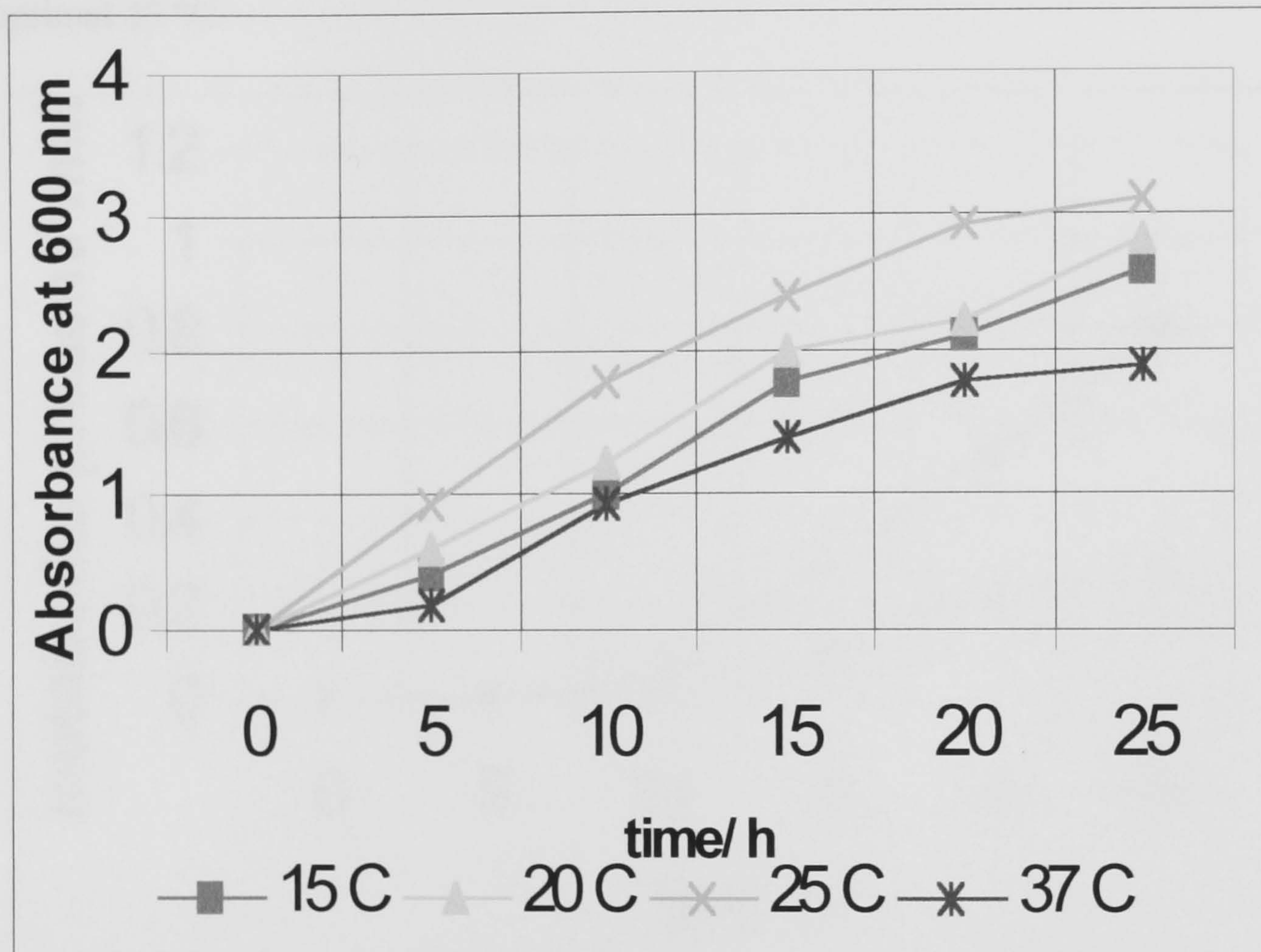


Figure 3.4.5a. Growth of *S. roseochromogenes* at varying temperatures.

The data represented in Figure 3.4.5a show that the optimum growth temperature was 25 °C. The highest temperature of 37 °C, was least conducive to growth, as this resulted in the lowest increase in culture turbidity; lower than cultures grown at the coolest temperature of 15 °C.

3.4.6 The Effect of Temperature on the Transformation of Progesterone by *S. roseochromogenes*

The comparison of progesterone biotransformation at 10 °C and 25 °C shown in Figure 3.4.6a, illustrates a lesser extent of hydroxylation of progesterone into both metabolites at 10 °C, a temperature less than the optimal 25 °C.

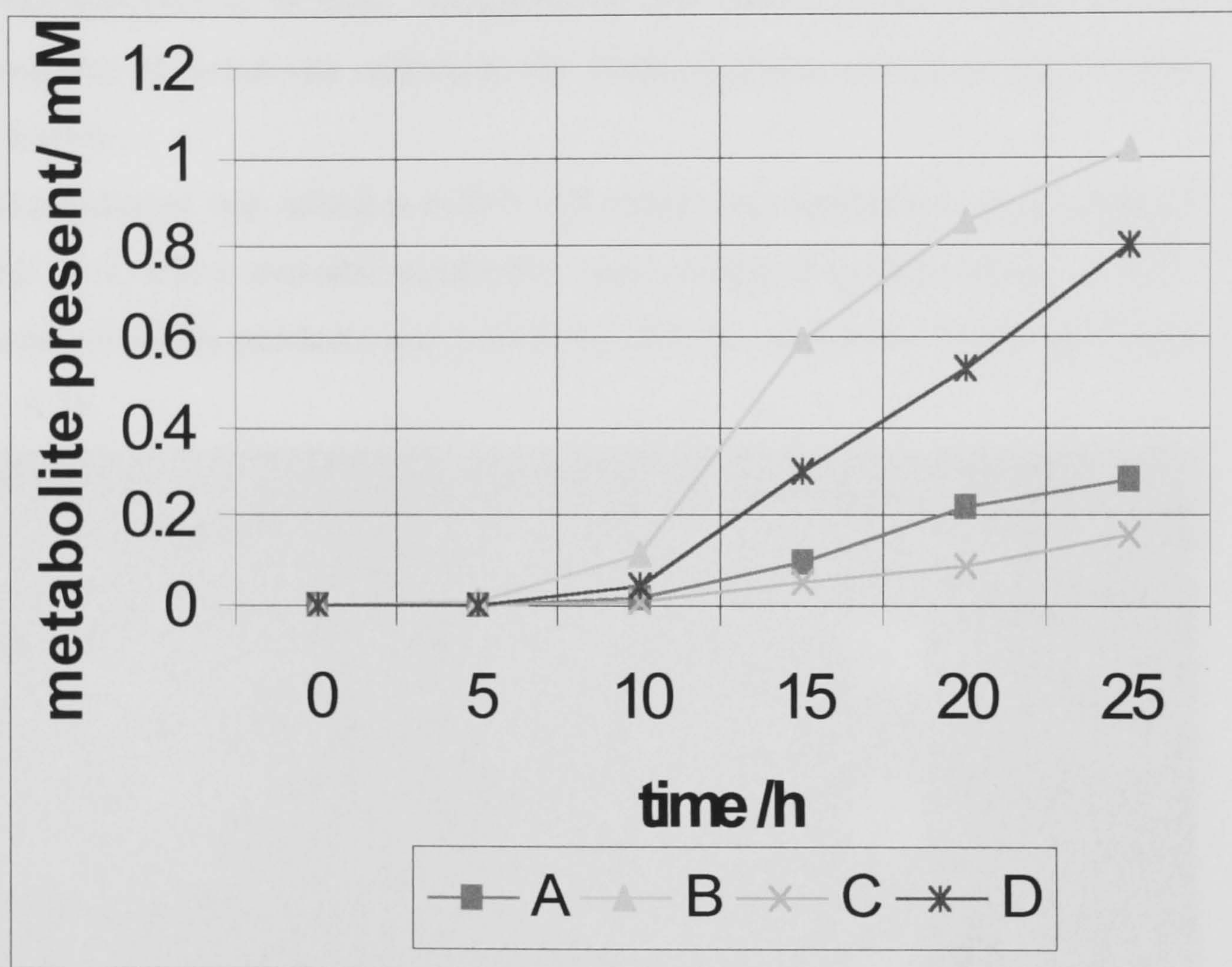


Figure 3.4.6a Progesterone biotransformation by *S. roseochromogenes*.

A minor metabolite production at 25 °C

B major metabolite production at 25 °C

C minor metabolite production at 10 °C

D major metabolite production at 10 °C

Progesterone was maximally transformed into both the major and minor metabolite at the higher temperature of 25° C. Although the profiles are similar at 10° C, the amount of each progesterone metabolite produced was decreased.

3.4.7 Incubation of *S. roseochromogenes* Cells with Progesterone

When progesterone was added to cell cultures, a very similar growth profile was observed to that shown in figure 3.4.1a. Progesterone was added to a concentration of 0.1, 0.2, 0.3, 0.4 & 0.5 mM to observe the effect on growth parameters. Progesterone did not alter the growth rate nor the biomass recovered, and optimum incubation time remained at 35 h for maximal growth of cells. Progesterone was added to cell cultures for the purpose of metabolite collection for NMR analysis, described later in this chapter.

Progesterone was added to a 10 h cell culture and incubation was continued for 25 h before steroidal metabolites were extracted in chloroform. A TLC analysis of the products was performed and the results are shown in Figure 3.4.7a.

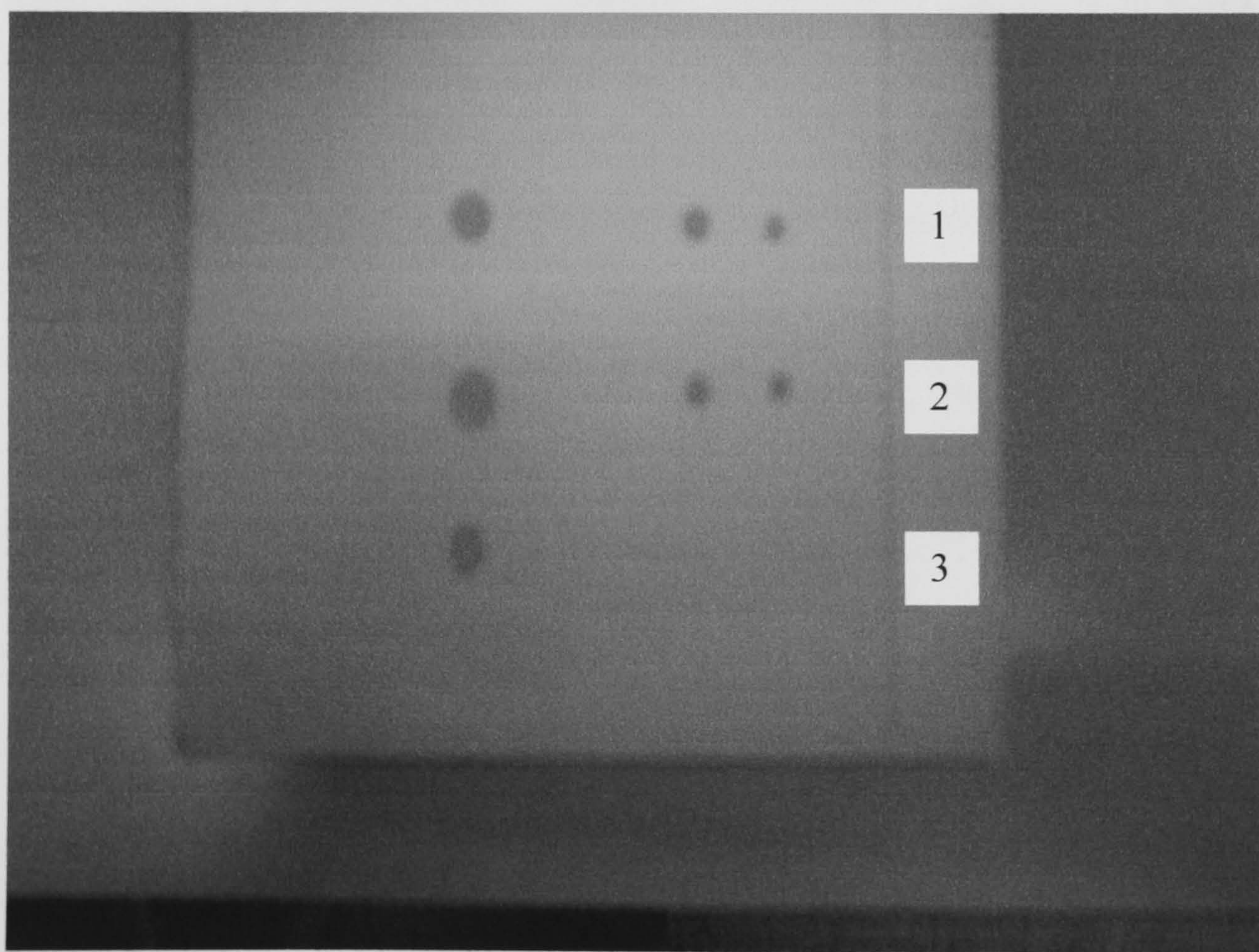


Figure 3.4.7a Photograph under u.v. light showing the two metabolites, as determined by NMR. Migration is from left to right.

Lane 1: Right spot- 2β,16α dihydroxyprogesterone,
Middle spot- 16α hydroxyprogesterone
Left spot- untransformed progesterone

Lane 2: Same as lane 1.

Lane 3: Progesterone alone.

3.4.8 Identification of Progesterone Metabolites Produced by *S. rosechromogenes* Strain 10984 by ^{1}H Nuclear Magnetic Resonance Spectroscopy

Two metabolites were purified from a 25 h progesterone transformation incubation. These metabolites were identified by ^{1}H NMR spectroscopy as 2β,16α-dihydroxyprogesterone and 16α-monohydroxyprogesterone.

The NMR identifying features of 16α-hydroxyprogesterone

The 17α-H signal, a doublet, (Fig. NMR 3 and Fig. NMR 4), is split only by 16β-H, contrasting with the triplet pattern observed in progesterone (Fig. NMR 1 and NMR 2) and all its derivatives hydroxylated at skeletal sites other than C-16 (Smith *et al.*, 1989[2]). The 18-H_3 singlet is virtually unaffected by 16α-OH. The distinctive 16β-H multiplet is at lower field (δ 4.86), Table NMR 1, than the CHOH proton for any other hydroxylated progesterone as a result of the proximity of 16β-H to the 20-oxo group.

	Progesterone (untransformed)	16α OHP	2β OHP	2β,16α DOHP Predicted shift	2β,16α DOHP Predicted location
Proton					
1β	2.04	2.03 (-0.01)	1.56 (-0.48)	-0.49	1.55
2α CHOH	2.35	2.35 (0.00)	4.20 (1.85)	1.85	4.20
16β CHOH	2.19	4.86 (2.67)	2.19 (0.00)	2.67	4.86
4	5.73	5.75 (0.02)	5.82 (0.09)	0.11	5.84
17α	2.54	2.54 (0.00)	2.54 (0.00)	0.00	2.54
18	0.67	0.68 (0.01)	0.67 (0.00)	0.01	0.68
19	1.20	1.19 (-0.01)	1.18 (-0.02)	-0.03	1.17
21	2.13	2.18 (0.05)	2.13 (0.00)	0.05	2.18

Table NMR 1. Chemical shifts for protons [δ(ppm from Me_4Si)]. Where the figures in parentheses are shifts relative to the corresponding proton in progesterone.

Figure NMR 1. The progesterone NMR spectrum from 0 - 2.7 ppm illustrating four characteristic signals: 17α-H at 2.54 ppm, 21-H at 2.13 ppm, 19-H at 1.20 ppm and 18-H at 0.67 ppm. The fifth major characteristic signal for progesterone (4-H) can be seen in Figure NMR2.

Figure NMR 2. The progesterone NMR spectrum from 0 - 12.5 ppm showing all five characteristic signals as follows: 4-H at 5.73 ppm (signal 5), 17α-H at 2.54 ppm (signal 4), 21-H at 2.13 ppm (signal 3), 19-H at 1.20 ppm (signal 2) and 18-H at 0.67 ppm (signal 1).

Figure NMR 3. The NMR spectrum of the major progesterone metabolite produced by *S. roseochromogenes*, identified as 16α hydroxyprogesterone, 0 - 2.7 ppm. As in Table NMR 1, four of the, 16α hydroxyprogesterone shifts, from progesterone (in parentheses) are shown as follows:

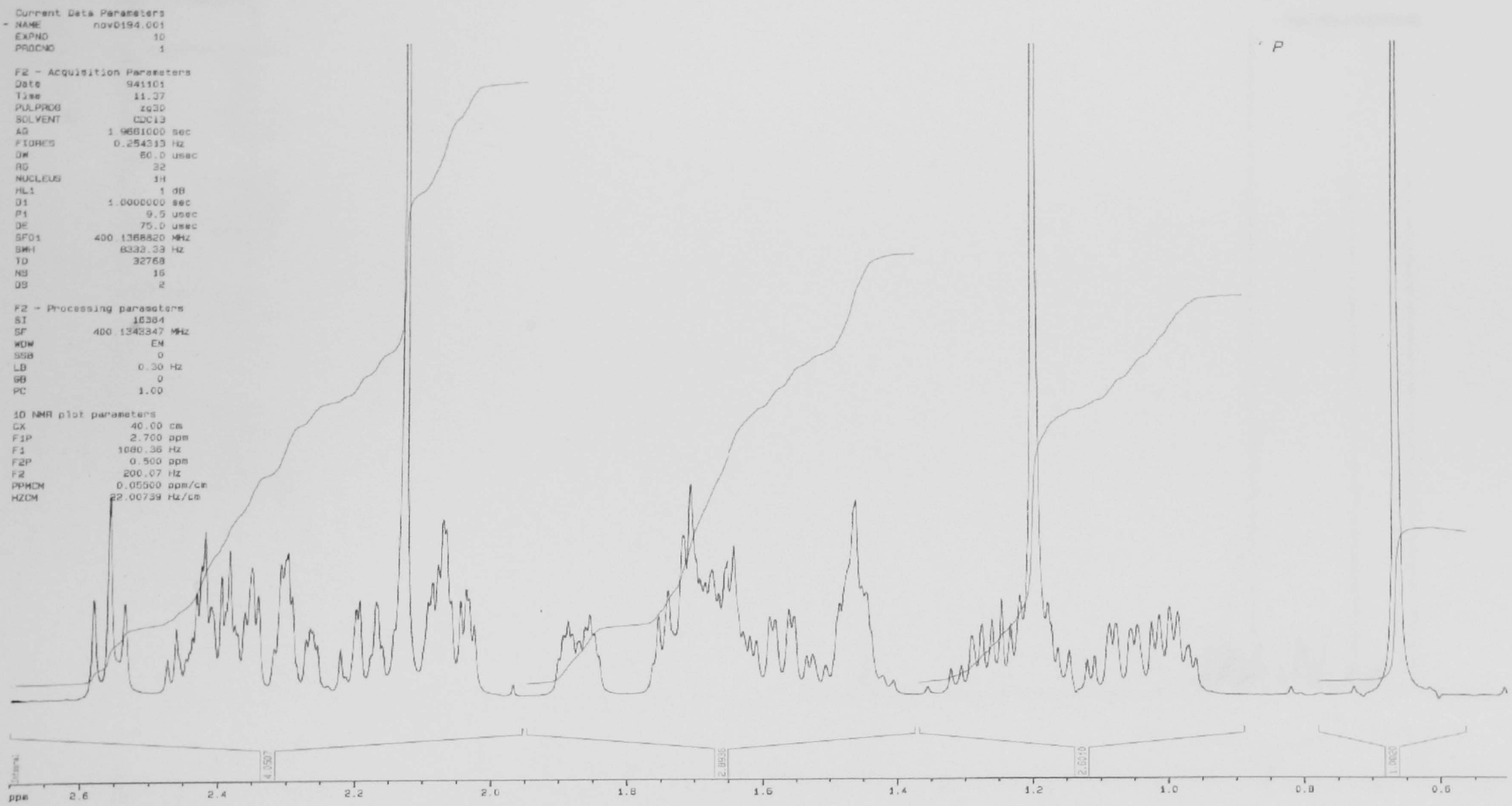
17α-H , 2.54 ppm (0.00)
21-H , 2.18 ppm (0.05)
19-H , 1.19 ppm (-0.01)
18-H , 0.68 ppm (0.01)

The 4-H signal can be observed in Figure NMR 4.

Figure NMR 4. The NMR spectrum of the major progesterone metabolite produced by *S. roseochromogenes*, identified as 16α hydroxyprogesterone. As in Table NMR 1, the 16α hydroxyprogesterone shifts, from progesterone (in parentheses) are shown as follows:

4-H , 5.75 ppm (0.02)
17α-H , 2.54 ppm (0.00)
21-H , 2.18 ppm (0.05)
19-H , 1.19 ppm (-0.01)
18-H , 0.68 ppm (0.01)

Figure NMR 1. NMR spectrum of progesterone



PROGESTERONE

```
Current Data Parameters
NAME           nov0194.001
EXPNO                   10
PROCNO                   1

F2 - Acquisition Parameters
Date                941101
Time                 11.37
PULPROG               zg30
SOLVENT              CDCl3
AQ            1.9661000 sec
FIDRES         0.254313 Hz
DW               60.0 usec
RG                      32
NUCLEUS                 1H
HL1                   1 dB
D1            1.0000000 sec
P1                9.5 usec
DE               75.0 usec
SF01       400.1368820 MHz
SWH            8333.33 Hz
TD                   32768
NS                      16
DS                       2

F2 - Processing parameters
SI                   16384
SF         400.1343347 MHz
WDW                     EM
SSB                      0
LB                 0.30 Hz
GB                       0
PC                    1.00

1D NMR plot parameters
CX                40.00 cm
F1P             12.600 ppm
F1             5041.69 Hz
F2P             -0.400 ppm
F2             -160.05 Hz
PPMCM          0.32500 ppm/cm
HZCM         130.04366 Hz/cm
```

ppm 12 11 10 9 8 7 6 5 4 3 2 1 0

Figure NMR 2. NMR spectrum of progesterone

Current Data Parameters
NAME nov0194.002
EXPNO 10
PROCNO 1

F2 - Acquisition Parameters
Date 941101
Time 11.50
PULPROG zg30
SOLVENT CDCl3
AQ 1.9661000 sec
FIDRES 0.254313 Hz
DW 60.0 usec
RG 1024
NUCLEUS 1H
HL1 1 dB
D1 1.0000000 sec
P1 9.5 usec
DE 75.0 usec
SFO1 400.1368820 MHz
SWH 8333.33 Hz
TD 32768
NS 16
DS 2

F2 - Processing parameters
SI 16384
SF 400.1343902 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

1D NMR plot parameters
CX 40.00 cm
F1P 2.700 ppm
F1 1080.36 Hz
F2P 0.500 ppm
F2 200.07 Hz
PPMCM 0.05500 ppm/cm
HZCM 22.00739 Hz/cm

16α HP

ppm 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6

Figure NMR 3. NMR spectrum of 16α hydroxyprogesterone

```
Current Data Parameters
NAME            nov0194.002
EXPNO                    10
PROCNO                    1

F2 - Acquisition Parameters
Date                 941101
Time                  11.50
PULPROG                zg30
SOLVENT               CDCl3
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FIDRES         0.254313 Hz
DW                60.0 usec
RG                     1024
NUCLEUS                  1H
HL1                    1 dB
D1            1.0000000 sec
P1                 9.5 usec
DE                75.0 usec
SFD1         400.1368820 MHz
SWH             8333.33 Hz
TD                    32768
NS                       16
DS                        2

F2 - Processing parameters
SI                    16384
SF           400.1343902 MHz
WDW                      EM
SSB                       0
LB                  0.30 Hz
GB                        0
PC                     1.00

1D NMR plot parameters
CX                 40.00 cm
F1P              12.600 ppm
F1              5041.69 Hz
F2P              -0.400 ppm
F2              -160.05 Hz
PPMCM       0.32500 ppm/cm
HZCM      130.04369 Hz/cm
```

76526.1

DMF

CDCl3

MeOH

DMF

0.989 1.590 1.739 2.004 3.643 3.157 2.887 83.093 0.909

ppm 12 11 10 9 8 7 6 5 4 3 2 1 0

Figure NMR 4. NMR spectrum of 16α hydroxyprogesterone

NMR data for 2β,16α-dihydroxyprogesterone (* s, singlet; m, multiplet)

The ^{1}H NMR spectrum of 2β,16α-dihydroxyprogesterone has not been previously published. Therefore, this compound was identified by matching the measured values for the chemical shifts of key identifying signals of the unknown metabolite to theoretical values calculated for 2β,16α-dihydroxyprogesterone (Table NMR 1). The calculations involved summing the published values of the key identifying protons in 2β- and 16α-monohydroxyprogesterone (Kirk *et al.*, 1990). 2D COSY analysis confirmed the structure. The features of structural significance of 2β,16α-dihydroxyprogesterone are summarised as follows.

The unknown compound retained the fundamental signals of the progesterone skeleton, i.e. 4-H (s*, δ 5.84,), 18-H_3 (s, δ 0.68), 19-H_3 (s, δ 1.17), 21-H_3 (s, δ 2.18) and displayed the features of dihydroxylation with two clearly separated mid-field multiplets at δ 4.86 and δ 4.2 attributable to methine protons of secondary hydroxylated carbon atoms, the 2α and 16β protons as predicted. See Table NMR 1. This transformation was confirmed by the following spectral data obtained from Figures NMR 5 and NMR 6. The characteristic triplet of 17α-H in progesterone was coincident with a predicted doublet (δ 2.54) consequent on splitting by the 16β-H. 16α Hydroxylation was confirmed by the strong correlation of the 17-H in the COSY with the distinctive mid-field multiplet (2.69 ppm) at δ 4.88 extant at lower field than any other CHOH proton of a hydroxyprogesterone as a result of the proximity of the 16β-H to the 20-oxo group (as mentioned above for 16α hydroxyprogesterone). Correlation of COSY cross peaks of the mid-field multiplet CHOH signal at δ 4.20 with 1α-H at δ 2.49, which was significantly shifted to low field (0.77 ppm) relative to progesterone, and to 1β-H (δ 1.56) and unusually shifted to high field (-0.48 ppm) relative to progesterone, confirmed 2β hydroxylation.

Figure NMR 5. The NMR spectrum of the minor progesterone metabolite produced by *S. roseochromogenes*, identified as 2β,16α-dihydroxyprogesterone. As in Table NMR 1, the 2β,16α dihydroxyprogesterone predicted shifts from progesterone (in parentheses) are shown and are the same as recorded in the NMR spectrum:

17α-H , 2.54 ppm (0.00)

21-H , 2.18 ppm (0.05)

19-H , 1.17 ppm (-0.03)

18-H , 0.68 ppm (0.01)

The 4-H predicted signal is observed in Figure NMR 6.

Figure NMR 6. The NMR spectrum of the minor progesterone metabolite produced by *S. roseochromogenes*, identified as 2β,16α-dihydroxyprogesterone. As in Table NMR 1, the 2β,16α dihydroxyprogesterone predicted shifts from progesterone (in parentheses) are shown and are the same as recorded in the NMR spectrum:

4-H , 5.84 ppm (0.11)

17α-H , 2.54 ppm (0.00)

21-H , 2.18 ppm (0.05)

19-H , 1.17 ppm (-0.03)

18-H , 0.68 ppm (0.01)

Figure NMR 6 also shows the 2α, 4.2 ppm predicted signal and the 16β, 4.86 ppm predicted signal

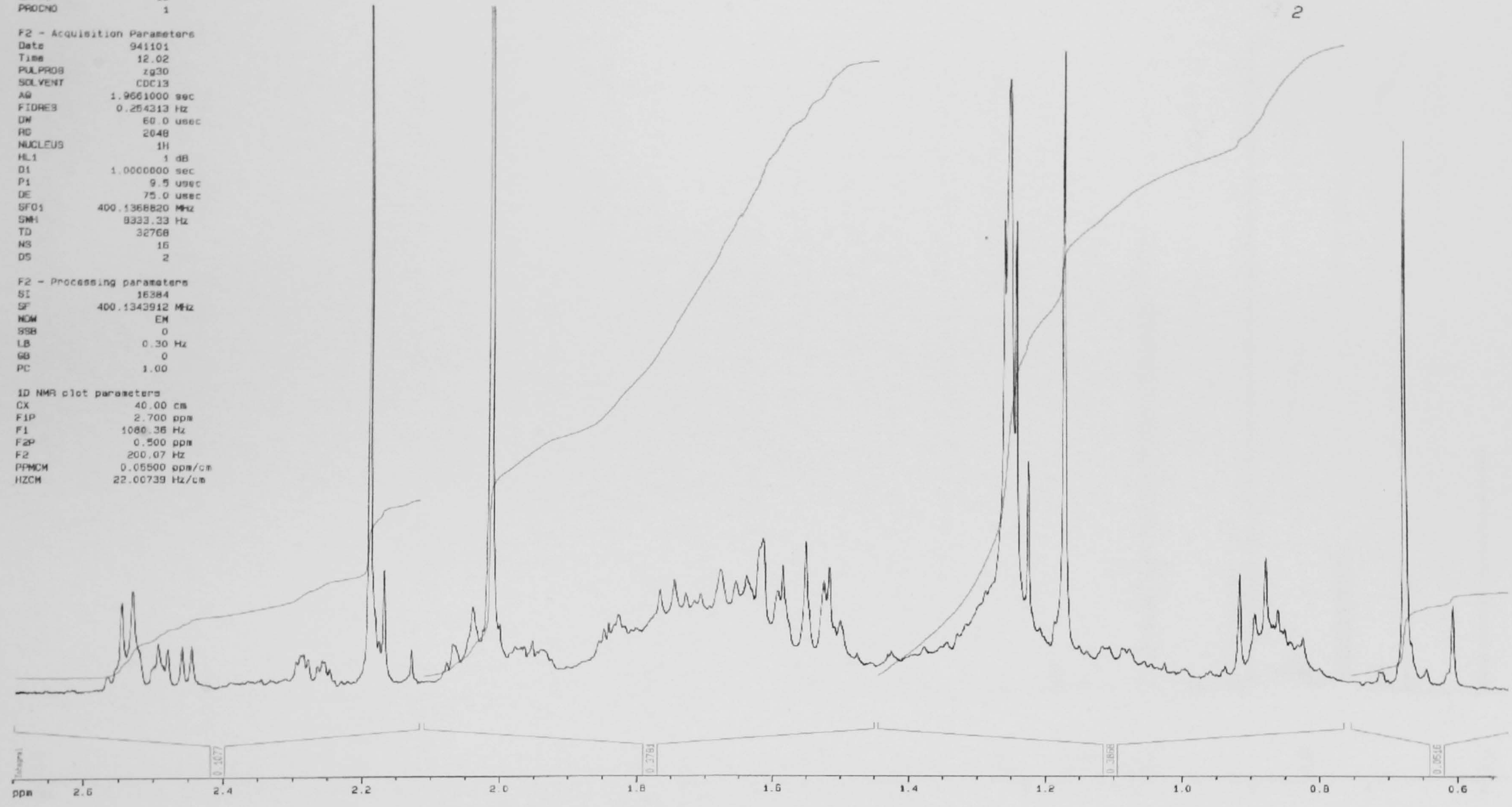


Figure NMR 5. NMR spectrum of 2β,16α dihydroxyprogesterone.

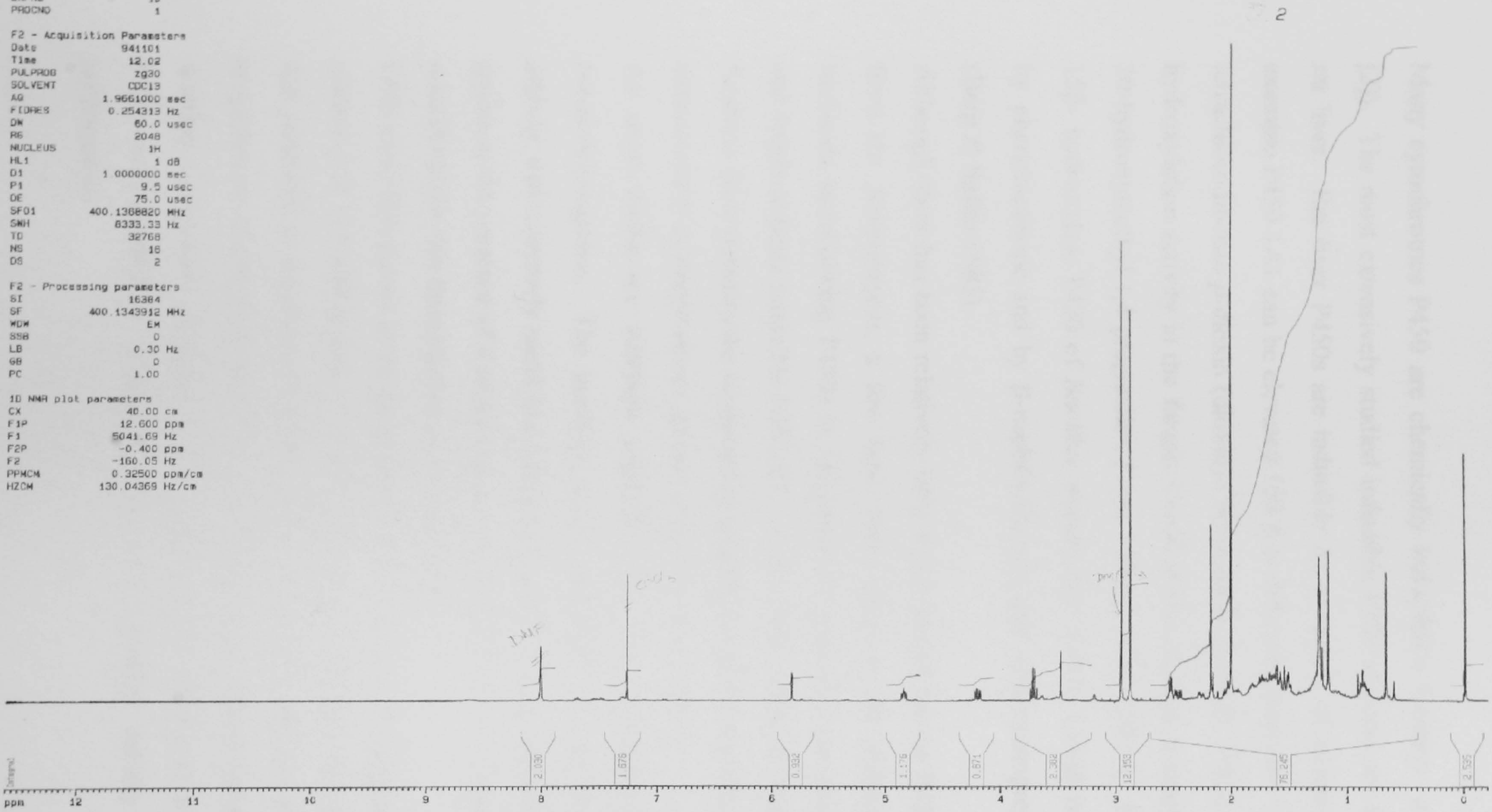


Figure NMR 6. NMR spectrum of 2β,16α dihydroxyprogesterone

3.4.9 Amplification of Progesterone Hydroxylase Concentration in *Streptomyces roseochromogenes* Whole Cells

Many cytochromes P450 are chemically inducible (Conney *et al.* 1973 [2]). The most extensively studied inducible P450 systems are those from rat liver. Rat liver P450s are inducible by a variety of compounds. For example P450 1A1 can be elevated 100 fold following exposure to 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (dioxin) (Waxman & Azaroff, 1992). The 11β hydroxylation activity in the fungus *Cochliobolus lunatus* is amplifiable by 20-hydroxymethyl 1,4-pregnadien-3-one (Jänig *et al.* 1992). The steroid 15β- hydroxylase P450 of *Bacillus megaterium* ATCC 13368 is inducible by phenobarbitone and by β-naphthoflavone and 16α-cyanopregnenolone (Berg & Rafter 1981).

Although there has been relatively little work carried out on P450 systems from the *Streptomyces*, a few have been found to be inducible. The herbicide metabolising P450s in *Streptomyces griseolus* are phenobarbital and sulphonylurea inducible (O'Keefe *et al.* 1988 , Patel & Omer 1992). Soybean flour contains the isoflavanoid genistein, which amplifies the P450 concentration in *Streptomyces griseus* (Trower *et al.* 1989).

As some P450s are substrate inducible this was investigated with *S. roseochromogenes.* The increase in the progesterone 16α hydroxylase activity was extremely small and ranged from a 2 to 6 % increase, for cells grown in the presence of 4 mM progesterone for 35 h at 37 °C and therefore considered for this investigation to be negligible.

Cells were also grown in the presence of soybean flour and separately in the presence of 0-4 mM genistein, to find if a similar response was detected to that observed in *S.griseus* (Trower, 1989). Genistein did not amplify the progesterone 16α-hydroxylase P450 concentration in *S roseochromogenes.*

Another compound, coumarin was screened for its ability to amplify the P450 concentration. Coumarin has a p-coumaryl moiety like the isoflavanoid,

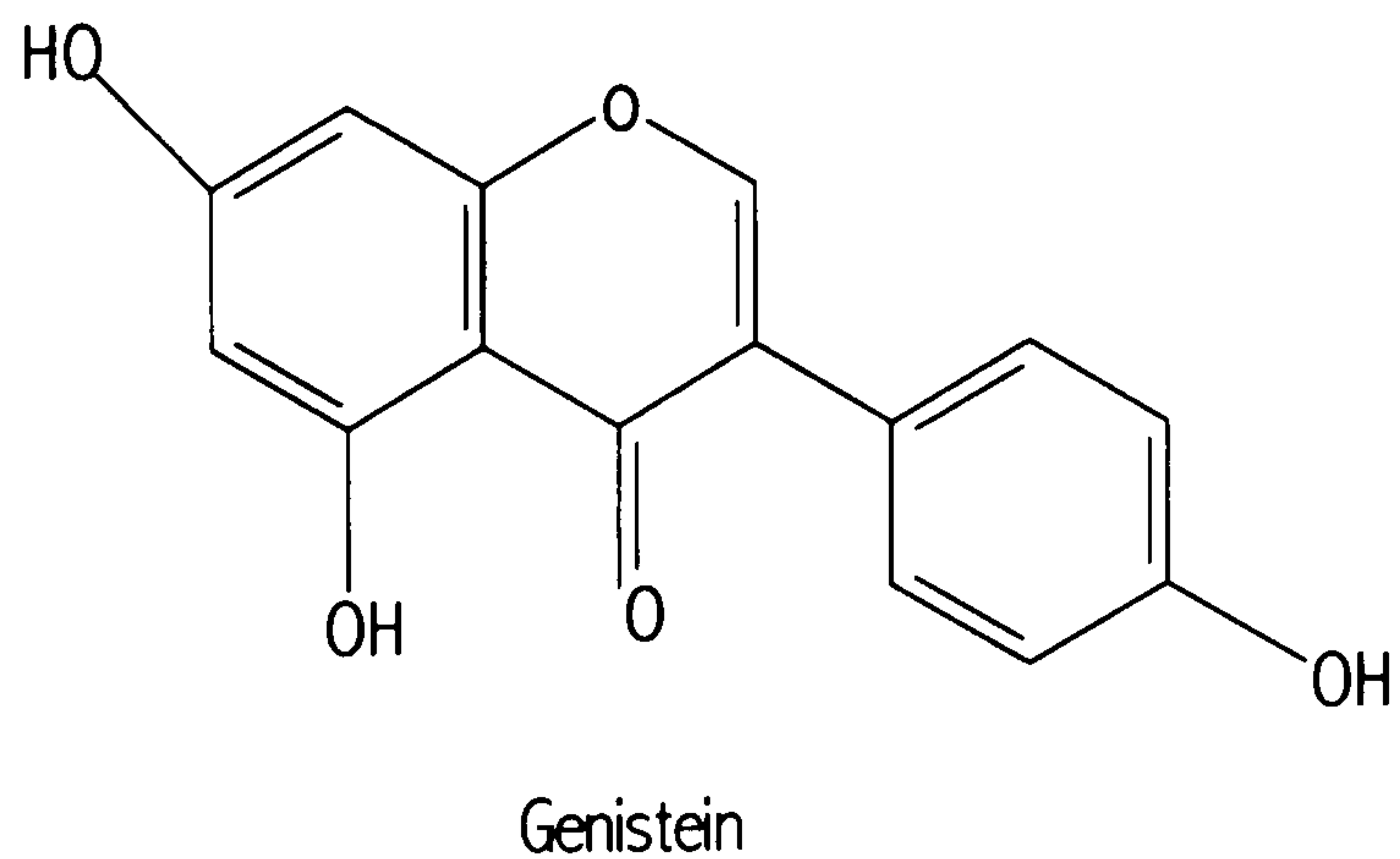


Figure 3.4.A Genistein, an isoflavanoid found to induce P450 concentration in *S. griseus* but not in *S. roseochromogenes*.

genistein. However, coumarin is a smaller molecule because it lacks an extra phenyl group and a hydroxyl group which are present in genistein. Coumarin did amplify the P450 concentration in cells; the extent of which was found to be concentration dependent. Figures 3.4.9a-e, show the optimum coumarin concentration in the growth medium was 1.5 mM.

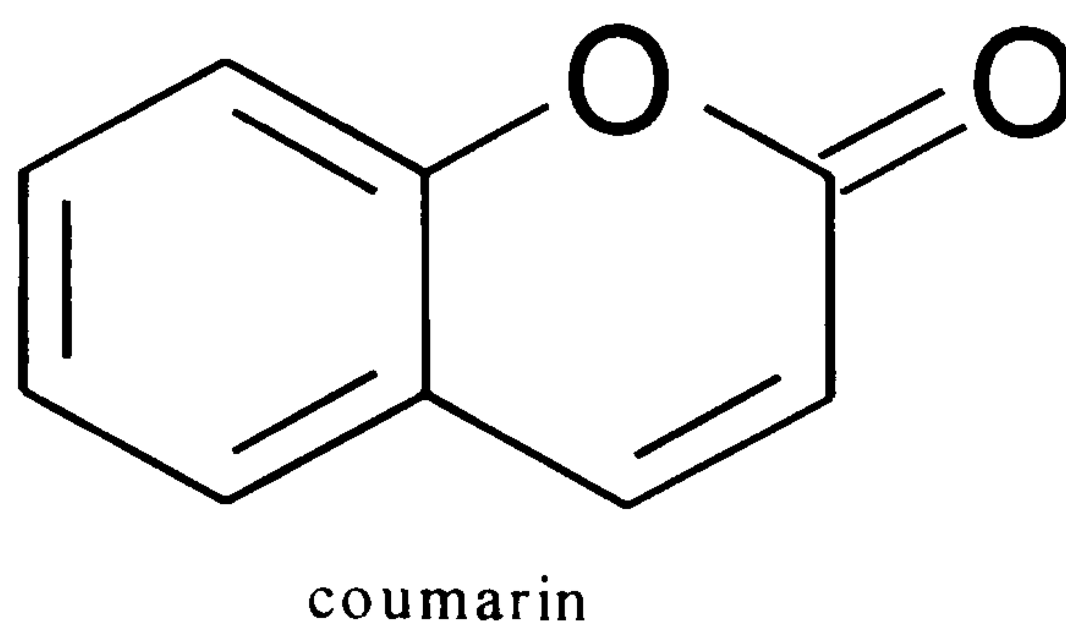


Figure 3.4.B Coumarin, found to augment 16α hydroxylase P-450 concentration in S. roseochromogenes cells.

Figure 3.4.9a Transformation in the absence of coumarin

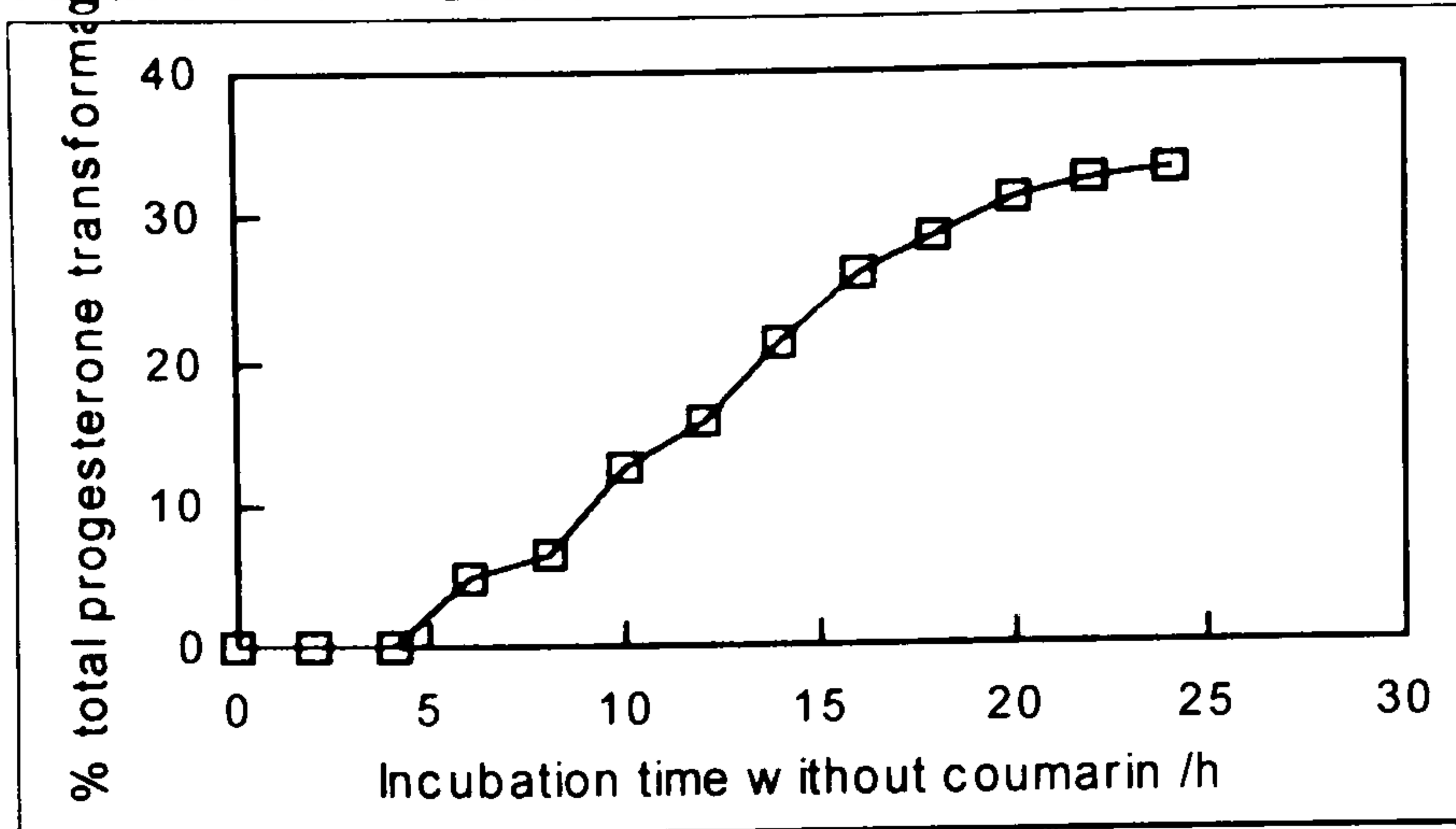


Figure 3.4.9b Transformation in the presence of 0.5 mM coumarin

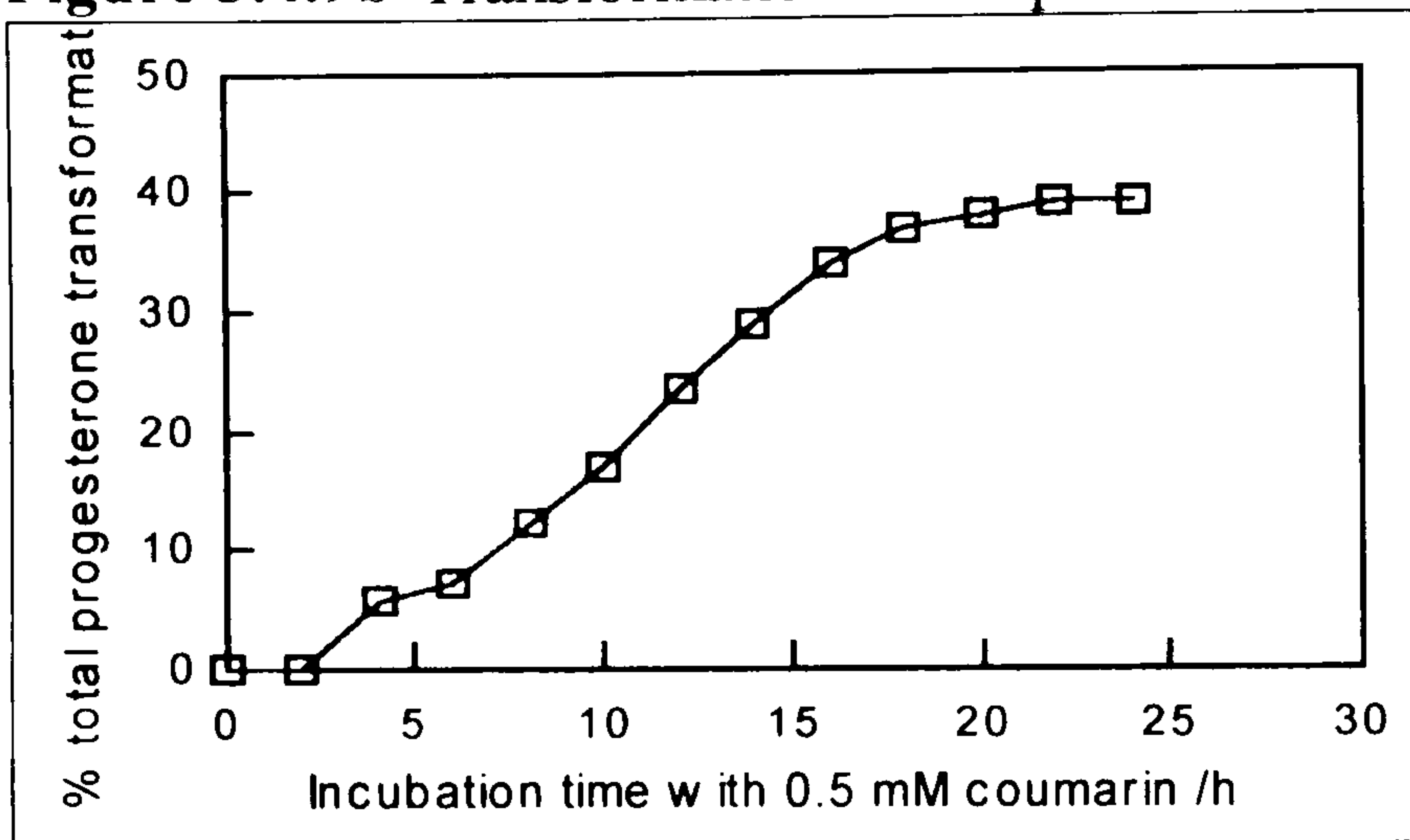


Figure 3.4.9c Transformation in the presence of 1.0 mM coumarin

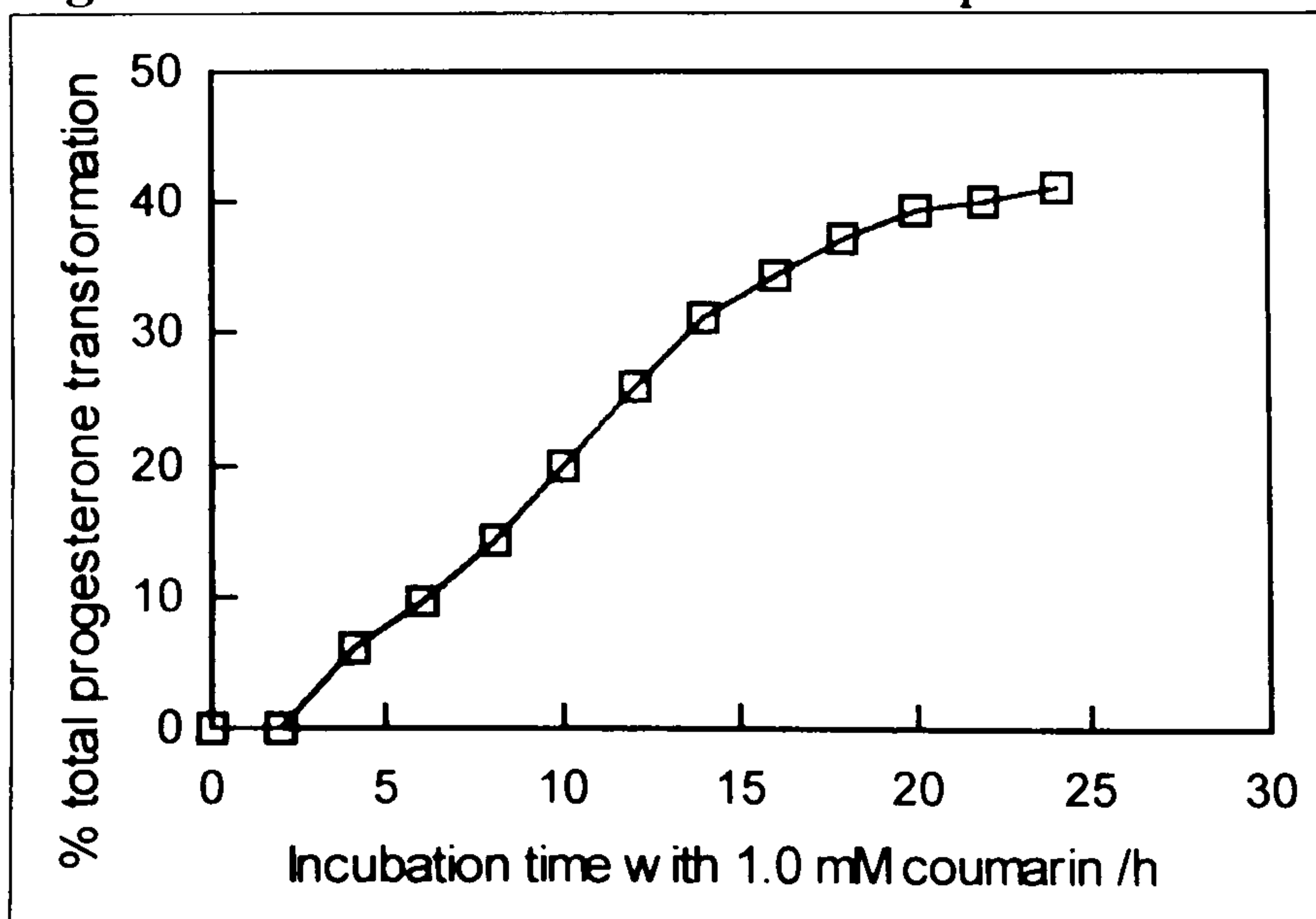


Figure 3.4.9d Transformation in the presence of 1.5 mM coumarin

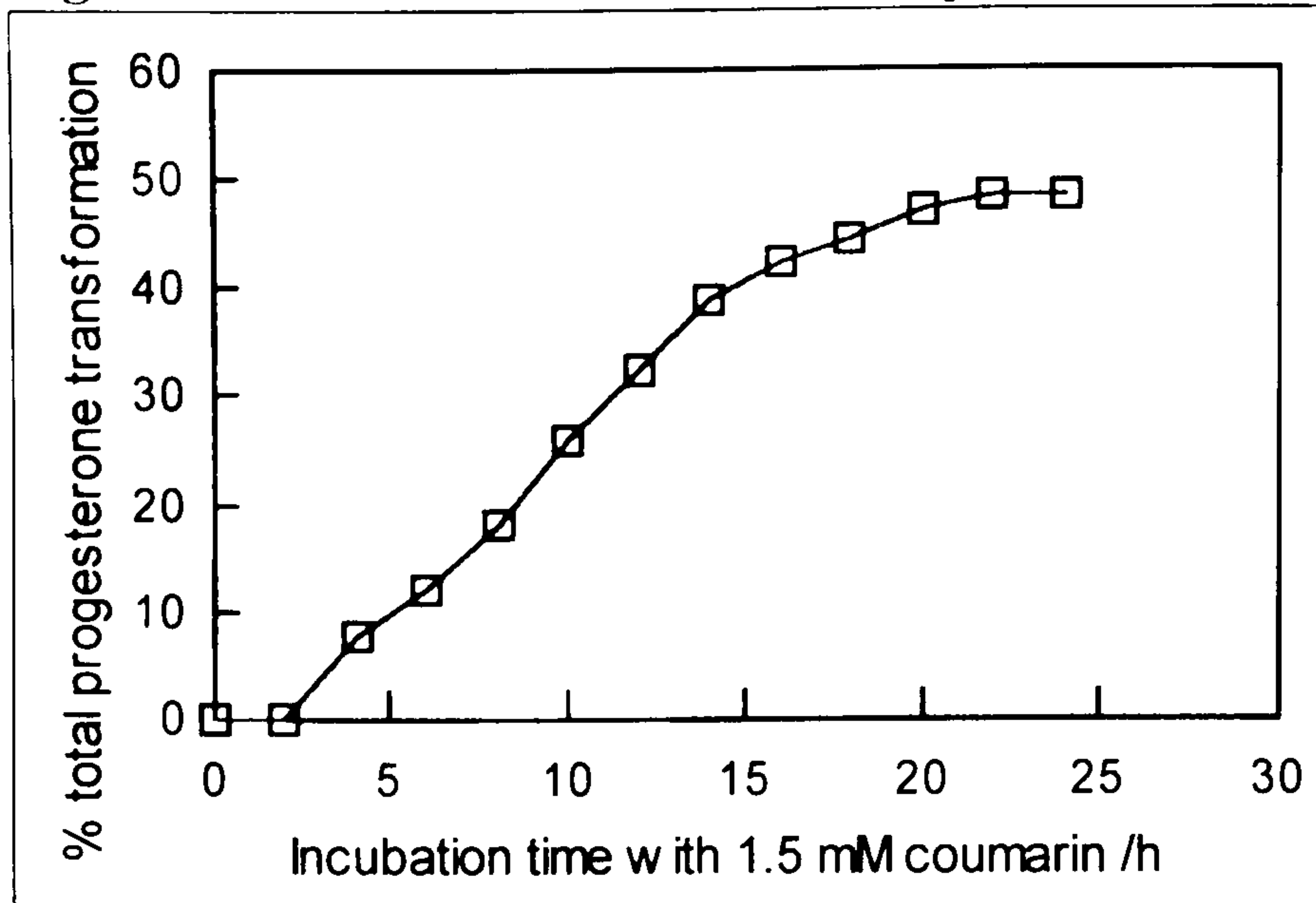
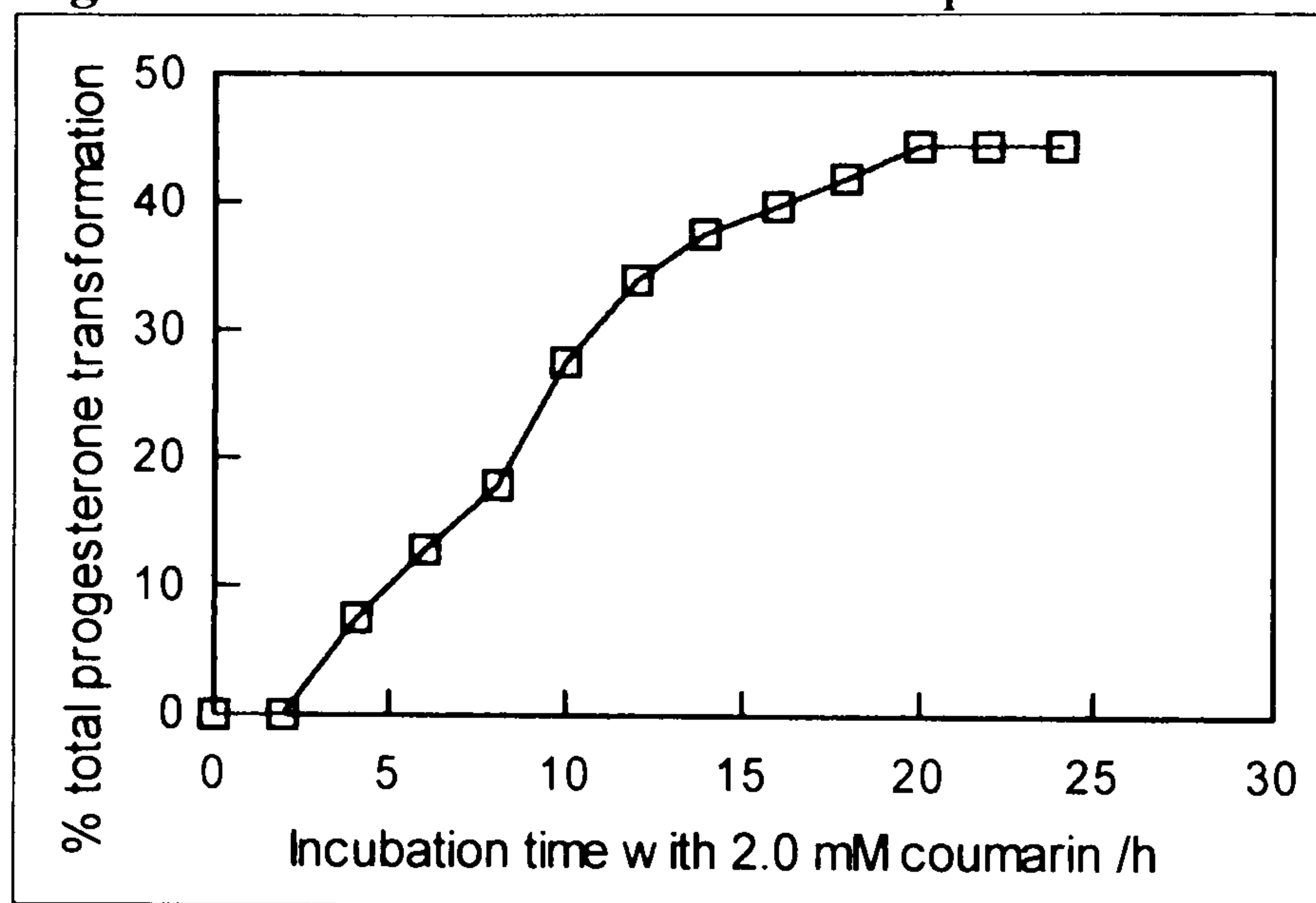


Figure 3.4.9e Transformation in the presence of 2.0 mM coumarin



Figures 3.4.9a-e show the effect coumarin in the growth medium had on the rate and extent of progesterone transformation. Fig.3.4.9a shows that in the absence of coumarin, after 23 h, 32 % of the progesterone was transformed. Fig.3.4.9d shows the optimum concentration of coumarin to be 1.5 mM. After 23 h in the presence of 1.5 mM coumarin, 48 % of the progesterone was transformed. This represents an amplification in hydroxylase activity of 50%. When increased to 2.0 mM, coumarin gave rise to only a 27 % increase in the transformation of progesterone, somewhat lower than that for 2.0 mM coumarin. In the presence of 1.0 mM coumarin it appeared that transformation was not maximally amplified.

3.4.10 Progesterone Transformation Products and Quantification Following Progesterone 16α hydroxylase P450 Amplification

As two metabolites were produced from the transformation of progesterone, the metabolites were quantified to determine whether the production of each was amplified or amplification applied to only one product. Figures 3.4.10a & and 3.4.10b show the results.

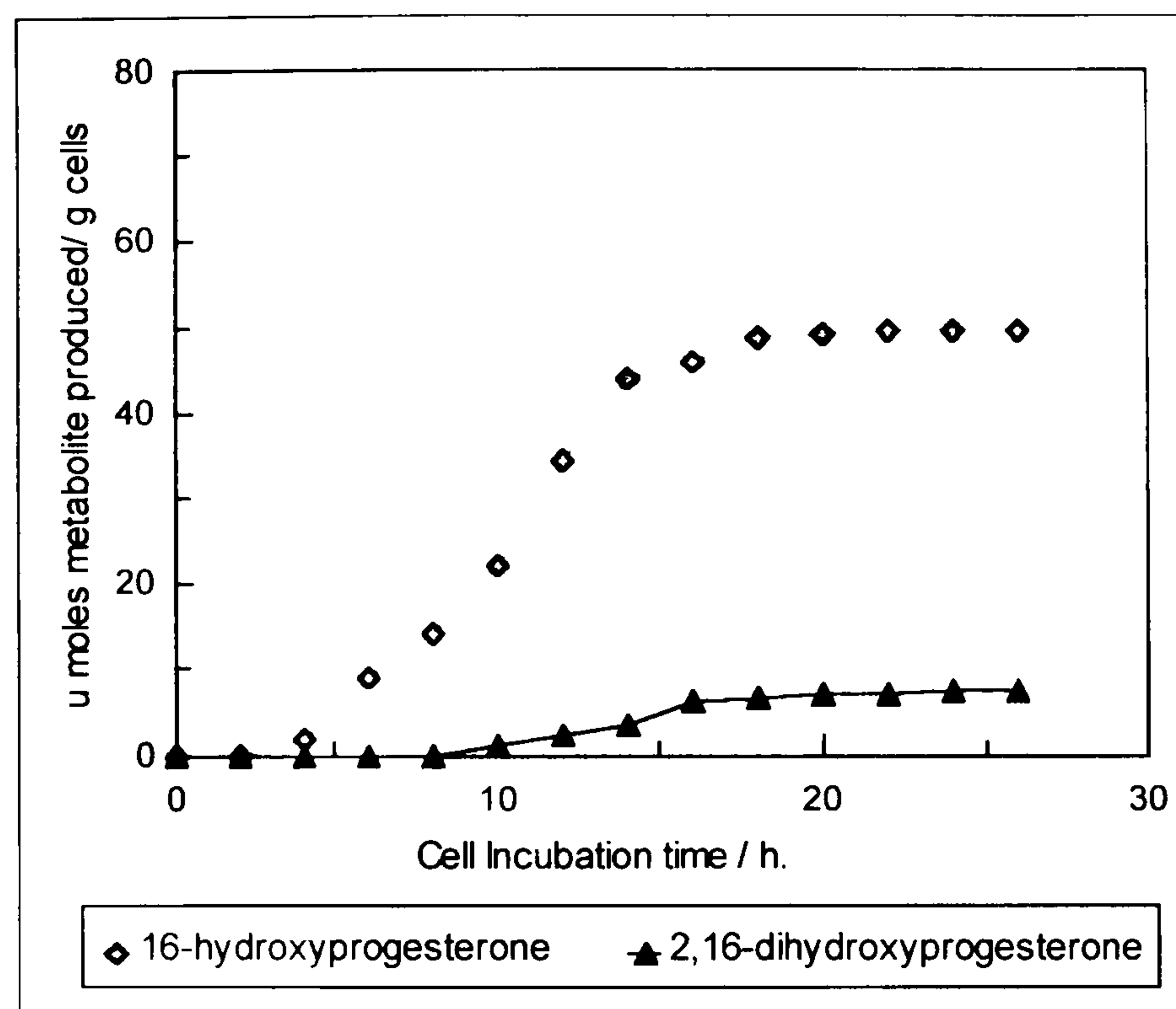


Figure 3.4.10a. Incubation of *S. roseochromogenes* cells with progesterone in the absence of coumarin.

Figure 3.4.10a shows the production of each metabolite in the absence of coumarin. Progesterone is maximally transformed after 20 h. Figure 3.4.10b shows the production of each metabolite when cells were incubated in the presence of 1.5 mM coumarin.

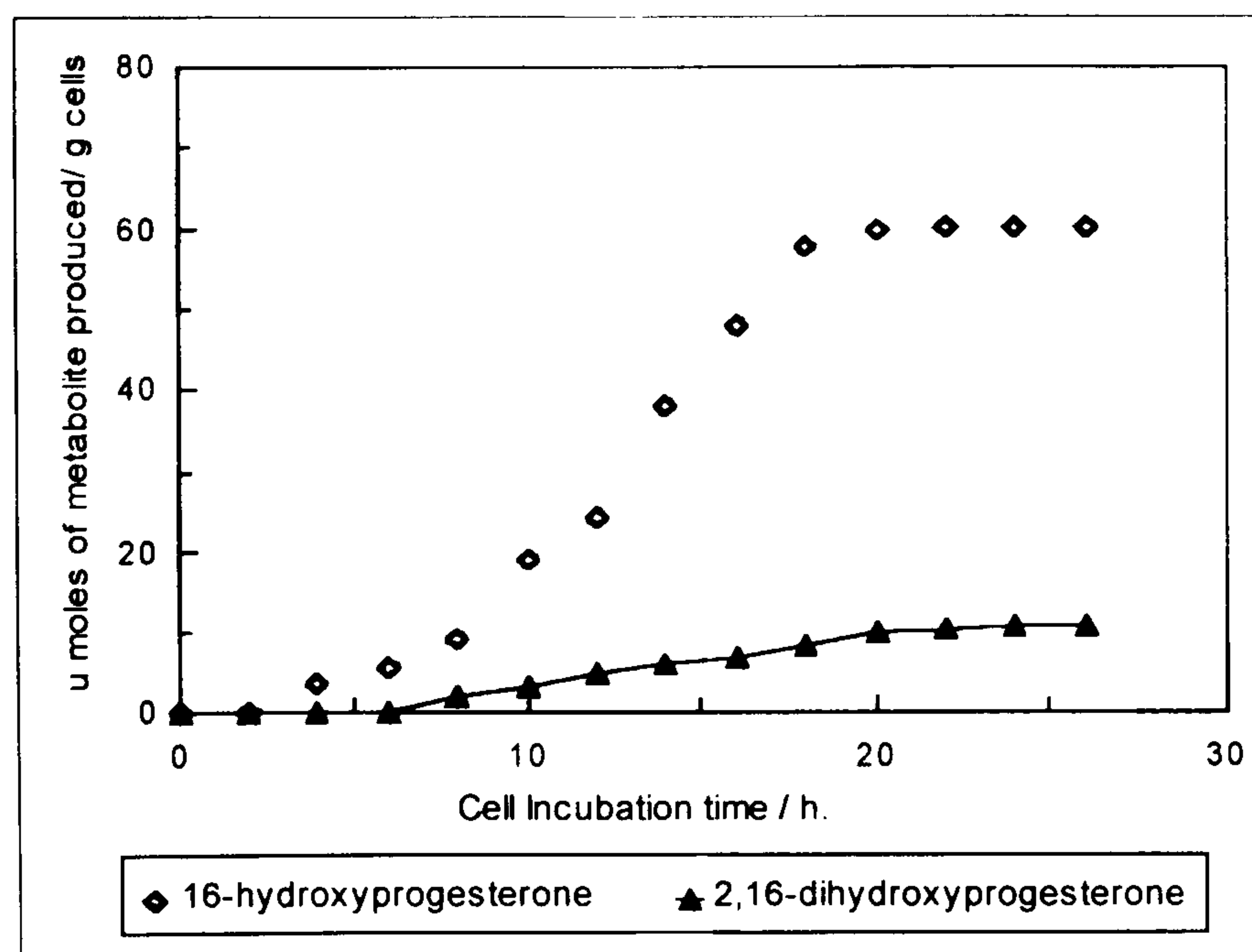


Figure.3.4.10b Incubation of *S. roseochromogenes* cells with progesterone in the presence of coumarin.

Progesterone was maximally transformed after 14 h. In the presence of coumarin, the transformation proceeded up to 48 % which was a 50 % increase in the hydroxylase activity from that shown in Fig.3.4.10a. Maximum transformation occurred sooner in the presence of coumarin and to a greater extent, than in its absence. Amplification of the two hydroxylase activities was approximately equal, i.e : there was not an increase in the proportion of one metabolite over the other. In the presence of coumarin, 2β,16α-dihydroxyprogesterone comprised 14.6 % of the transformation products and 16α hydroxyprogesterone the remaining 85 %. In the absence of coumarin, 2β,16α-dihydroxyprogesterone comprised 15.1 % of the transformation products and 16α- hydroxyprogesterone, the remaining 84 %. This was not considered to be a significant difference. The biomass yields at the end of each incubation were virtually identical.

3.4.11 The Metabolite Production Profile over a 25 h Whole Cell Incubation

16α-Hydroxyprogesterone, the major metabolite of progesterone transformation, first appeared in the culture medium after 6 h of incubation (Table 3.4a). This metabolite was actively produced throughout the entire 25 h incubation but the rate of production significantly slowed after 22 h. A second phase metabolite, 2β,16α-dihydroxyprogesterone, was first detected in the culture medium 2 h after the initial appearance of 16α-hydroxyprogesterone. This compound steadily accumulated during the next 13 h of transformation, but at less than half the rate of synthesis of 16α-hydroxyprogesterone. Production also virtually ceased at 21 h. 2β-Hydroxyprogesterone, the counterpart monohydroxy metabolite to 16α-hydroxyprogesterone, was not observed at any time during the 24 h transformation period. At the end of transformation, 25% of substrate progesterone had been converted into hydroxylated products of which *ca* 72% was 16α-hydroxyprogesterone and *ca* 28% was 2β,16α-dihydroxyprogesterone.

Figure 3.4.11a, shows the HPLC analysis of this 25 h incubation. The legend gives the metabolite identification of each peak. These peaks correspond to the NMR analysis of the metabolites given above.

Time (h)	Total metabolite (% initial progesterone)	2β,16α-DHP (mM)	2β,16α-DHP (% initial progesterone)	2β,16α-DHP (% total metabolites)	16α-HP (mM)	16α-HP (% initial progesterone)	16α-HP (%total metabolites)
1.5	0	0	0	0	0	0	0
6	0.28	0	0	0	0.011	0.275	100
7	0.75	0	0	0	0.03	0.75	100
8	1.53	0.01	0.25	16.3	0.061	1.28	83.66
9	1.95	0.01	0.25	12.8	0.078	1.7	87.18
10	2.90	0.02	0.5	17.2	0.116	2.4	82.76
11	3.75	0.04	1.0	26.7	0.15	2.75	73.33
12	6.6	0.054	1.35	20.5	0.264	5.25	79.55
13	11.05	0.062	1.55	14.0	0.442	9.5	85.97
14	11.8	0.072	1.80	15.3	0.472	10	84.75
15	14.75	0.1	2.50	17.0	0.59	12.25	83.05
16	15.9	0.11	2.75	17.3	0.636	13.15	82.7
17	17	0.13	3.25	19.1	0.68	13.75	80.88
18	19.25	0.17	4.25	22.1	0.77	15	77.92
19	19.85	0.174	4.35	21.9	0.794	15.5	78.09
20	21.50	0.22	5.50	25.6	0.86	16	74.42
21	23.45	0.258	6.45	27.5	0.938	17	72.49
22	24.08	0.263	6.58	27.3	0.963	17.5	72.67
23	24.95	0.278	6.95	27.9	0.998	18	72.14
25	25.28	0.281	7.03	27.8	1.011	18.25	72.19

Table 3.4a. The production of 2β,16α-dihydroxyprogesterone and 16α- hydroxyprogesterone over a 25 h incubation.

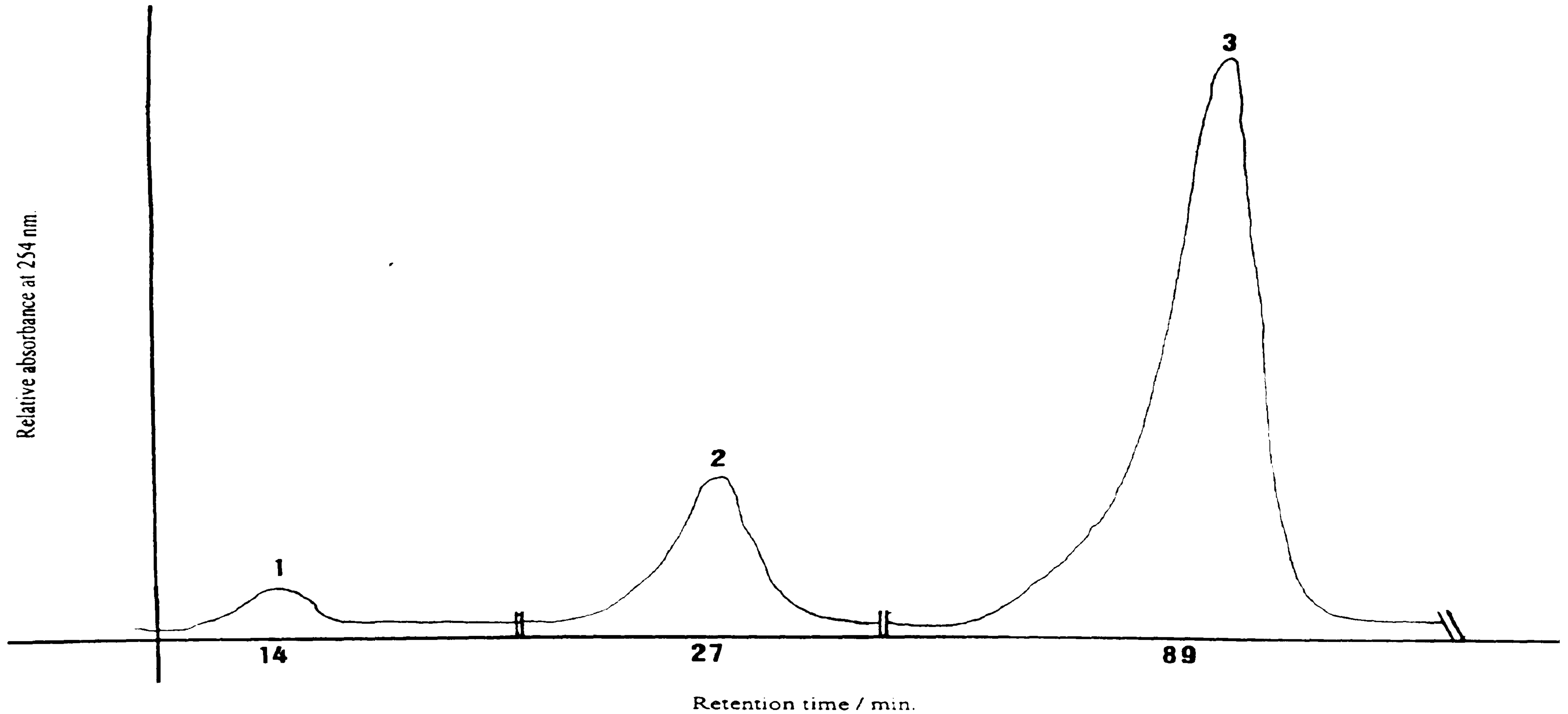


Figure 3.4.11a. HPLC of 25 h progesterone transformation incubation by *S. roseochromogenes*.
Peak 1, 2β,16α-dihydroxyprogesterone (retention time 14 min): Peak 2, 16α-mono- hydroxyprogesterone (retention time 27 min): Peak 3, progestero (retention time 89 min)

3.5 DISCUSSION

The components of the growth medium were varied as were the conditions under which incubation was maintained. After varying the components of the growth medium, a final optimum mixture was obtained. This is outlined in the assay below but the full description of its elucidation is omitted.
In summary, from the results here, the conditions used throughout this work for the growth of *S. roseochromogenes* cells are shown in table 3.5a.

Growth Requirement	**Result**
Yeast Extract in growth medium	4.0 g/l
Malt extract in growth medium	10.0 g/l
Glucose in growth medium	4.0 g/l
pH of growth medium	7.2
Temperature	25°C
Growth time for cells	48 h
Rotation for orbital incubation	100 rpm for 50 ml in 500 ml flasks containing coiled wires.

Table 3.5a Growth conditions for *S. roseochromogenes* cells.

The quantification of progesterone metabolites by TLC elution as described here, was abandoned from this point onward in favour of the far more precise method of HPLC, the description of which is given in chapter 2. HPLC gave much more reproducible results than the TLC elution method. With TLC elution it appeared that total removal of the dihydroxylated metabolite from the TLC plate could not be guaranteed.

Progesterone metabolite identification here, was of importance as this is the first description of the NMR identifying features of 2β,16α-dihydroxyprogesterone.

This chapter described the microbiological aspects of the progesterone biotransformation by *S. roseochromogenes* and provides the basis for investigation into the nature of the hydroxylase.

Steroid biotransformation is well documented in both prokaryotic and eukaryotic organisms such as in the rat (Ferrer *et al.*, 1990), human (Domanski *et al.*, 1998), fungi (Smith *et al.*, 1994) and bacteria Rauschenbach *et al.*, 1993). The whole cell biotransformation of xenobiotics and steroids was investigated in cultured respiratory epithelial cells from rainbow trout (*Oncorhynchus mykiss*) gills (Leguen *et al.*, 2000). Carbon position 16 of testosterone was hydroxylated by trout gill cells, the steroid position of interest in the work presented here. Such biotransformations are associated with cytochromes P450. This is generally not the case in *Streptomyces* species. Steroid biotransformation has been identified in *Streptomyces* (Shirasaka & Tsuruta, 1960) but identification of cytochromes P450 in *Streptomyces* has generally been associated with antibiotic biosynthesis. For example, in *Streptomyces fradiae* the biosynthesis of tylosin is completed by the conversion of the deoxyallosyl moiety of tylosin via bis-O-methylation at 2-OH and 3-OH. Hydroxylation of the polyketide lactone (following its insertion) at C23, is catalysed by the cytochrome P450 enzyme. A ferredoxin is expressed within the same gene cluster as the P450 and other enzymes involved in tylosin biosynthesis (Bate & Cundliffe, 1999). A single cytochrome P450, PicK, is responsible for the hydroxylations of YC-17 and narbomycin that generate methymycin, neomethymycin and picromycin in *Streptomyces venezuelae* (Graziani *et al.*, 1998).

Oligonucleotide primers were applied to amplify DNA fragments of P450 hydroxylase genes. The designed primers were based on several regions of strong similarity to P450 hydroxylases from a variety of *Streptomyces*; in the regions of an oxygen binding site and a haem ligand pocket. These primers were used to amplify DNA fragments from different *Streptomyces* species such as *S. lividans*, *S. parvulus* and *S. coelicolor*. The deduced

amino acid sequences of the isolated fragments revealed significant similarities to known P450 hydroxylases including the product of the suaC or subC genes from *S. griseolus* (Hyun *et al.*, 1998) that is capable of metabolising a number of sulfonylurea herbicides such as chlorimuron ethyl (O'Keefe *et al.*, 1988 and 1993), and to the product of the P450sca2 from *S. carbophilus* which hydroxylates compactin to pravastatin, a tissue-selective inhibitor of 3-hydroxy-3-methylglutamyl-coenzyme A reductase (Matsouka *et al.*, 1989). This method should help researchers in cloning the P450 hydroxylase genes involved in the biosynthesis of useful compounds (Hyun *et al.*, 1998).

In *Streptomyces cinnamonensis* metyrapone (9.0 mM), a P450 inhibitor, caused partial inhibition of the biosynthesis of monensin and coproduction of new metabolites, 26-deoxymonensins A and B by P450 O-demethylation (Pospisil *et al.*, 1994).

Steroid biotransformation has been identified in *Streptomyces hydrogenans*. This is a 20-hydroxysteroid dehydrogenase but not identified as a P450 (Hilhorst *et al.*, 1984).

A variety of soil micro-organisms such as *Pseudomonas, Rhodococcus* and *Streptomyces* species produce cholesterol oxidase (CO), a bifunctional flavoenzyme, which catalyses the oxidation and isomerisation of 3 - hydroxy-5-ene-steroids to 3-keto-4-ene-steroids. Although not a P450 enzyme it is a widely used material in various industrial fields. CO is an important ingredient in most commercially available assay kits for serum cholesterol screening. CO has a broad range of substrate specificity towards various -hydroxy-steroids and has many potential applications as it is stable in the presence of water-immiscible solvents at high concentrations, thus making possible the conversion of water-insoluble substrates (Kitamoto, 1999).

In several *streptomyces* examples such as the P450 production of geosmin (1,10-trans-dimethyl-trans-(9)-decalol) produced by *Streptomyces tendae* (Dionigi *et al.*, 1994), P450 monooxygenase activity arises from induction

of a P450 and associated ferredoxin, or of a P450 only, suggesting that some essential electron donor proteins (reductase and ferredoxin) are not co-ordinately regulated with the P450 (O'Keefe & Harder, 1991[1]). The properties of these systems suggest an adaptive strategy whose dual purpose is to maintain a competitive advantage via the production of secondary metabolites, such as in the demethylation of veratrole by cytochrome P450 in *Streptomyces setonii* (Sutherland, 1986) (O'Keefe & Harder, 1991[1]). Hence a potential explanation for the progesterone hydroxylase activity in *Streptomyces roseochromogenes*.

Chapter 4

Cell Free Biotransformation of Progesterone by *Streptomyces roseochromogenes*

4.1 INTRODUCTION

Optimising Conditions for the Examination of the Cell-Free Biotransformation of Progesterone by *S. roseochromogenes*

In order to assess the effectiveness of any procedure used in an enzyme purification process, it is important to be able to assay the activity of the enzyme under optimum conditions. This then provides useful information on the progress of a purification protocol.
The following sections aim to optimise the conditions for progesterone transformation by the 16α hydroxylase from *Streptomyces roseochromogenes*, with the aim of subsequently purifying the protein.

The Progesterone 2β,16α Hydroxylase of *Streptomyces roseochromogenes* may be a Cytochrome P450

The genus *Streptomyces* is a rich source of cytochrome P450 monoxygenase enzymes that are involved in a wide variety of biosynthetic and xenobiotic transformation reactions. In *Streptomyces antibioticus*, this enzyme is responsible for C-8 epoxidation of the lactone ring of the antibiotic oleandomycin (Rodriguez *et.al.*, 1995). The DNA sequence of the *S. antibioticus* P450 is related to the eryF gene of *S. erythraea* (*Saccharopolyspora erythraea*) which codes for a soluble cytochrome P450 (CYP107) that stereospecifically 6-hydroxylates 6-deoxyerythronolide B to erythronolide B during erythromycin A biosynthesis (Shiafiee & Hutchinson 1987). In *S. carbophilus* P450sca hydroxylates compactin to pravastatin, a tissue-selective inhibitor of 3-hydroxy-3-methylglutamyl-coenzyme A reductase (Matsuoka, *et.al.* 1989). The sulphonyl urea herbicide chlorimuron ethyl is metabolised by two inducible *S. griseolus* P450s, P450SU1 (CYP105A1) and P450SU2 (CYP105B1) (O'Keefe *et.al.*, 1988). *S. griseus*

contains a cytochrome P450 that is inducible by the isoflavenoid genistein present in soyaflour (Sariaslani & Kurtz 1986). Extracts of this organism, prepared from soya flour-induced cells and supplemented with spinach ferredoxin and ferredoxin-NADPH reductase are capable of aromatic benzylic, and alicyclic hydroxylation (Trower *et.al.*, 1988). The ChoP gene of *Streptomyces sp.* SA-COO encodes a cholesterol oxidase cytochrome P450 that has a high degree of homology with human and *Pseudomonas* P450s (Horii *et.al.*, 1989).

A plethora of *Streptomyces* species have been widely reported as excellent steroid hydroxylators. C2 and C4 hydroxylation of the phenolic steroid oestradiol is known to be P450 catalysed (Trower *et.al.*, 1988). Skeletal sites transformed in steroids include $\xi 1$, 2β, 6β, 7β, 9α 11α, 11β, 15α and 16α (for examples see footnote[a]) but unlike in the oestradiol bioconversion the nature of these other steroid hydroxylases is unknown.

Mentioned above is the fact that *Streptomyces* species are a rich source of cytochromes P450 and that some *Streptomyces* species are steroid hydroxylators. Despite this, there is no evidence to date reported that any *Streptomyces* P450 is responsible for steroid hydroxylation (except for, Berrie *et al.*, 1999). However, by analogy with steroid hydroxylation in the bacterial species *Bacillus cereus* (Wilson *et.al.* 1966) and *B. megaterium* (Berg *et.al.* 1976) a reasonable assumption is that these hydroxylases are site-selective cytochrome P450 monooxygenases.

4.2 MATERIALS

Materials were obtained from the sources previously described in chapter 3 except that here, epoxy activated Sepharose 6B was purchased from

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[a] Collingsworth D.R. et.al. 1952
Herzog H.L. et.al. 1957
Nazaki Y. et.al. 1965
Smith L.L. et.al. 1961
Fried J. 1956
McAleer W.J. et.al. 1958
Shirasaka M. & Tsuruta M. 1960
Vondrova O. & Capek A. 1963

Pharmacia Biotech Ltd., St. Albans, Herts., U.K.: MIMETIC Blue 1 A6XL affinity resin was obtained from Affinity Chromatography Ltd., Freeport, Ballasala, Isle of Man, U.K. and Cibacron Blue FG3A and NAD-Sepharose affinity gel from Sigma Chemical Co., Poole, Dorset, U.K.

4.3 METHODS

4.3.1 Preparation of Cell-Free Extract

The description of the preparation of cell-free extracts is in chapter 2, along with a description of other methods attempted but not subsequently needed. The treatment and analysis of steroidal metabolites is also discussed in chapter 2.

4.3.2 The CO Binding Difference Spectrum

In order to assay P450 purity at each attempted stage of purification, carbon monoxide binding difference spectra were obtained. Two cuvettes, containing either cell extract from any of the above stages, or pure protein in orthophosphate buffer, were placed in a double beam spectrophotometer and the absorbance range 390 nm - 520 nm (Ruckpaul, 1990) was scanned to set a base line. One cuvette remained in the spectrophotometer and to the second, a few granules of sodium dithionite were added. The cuvette contents were mixed by gently inverting five times. Carbon monoxide gas was then bubbled through the dithionite reduced sample for 1.5 min at a rate of one bubble/sec. This co-treated cuvette was placed back in the spectrophotometer and the absorbances read across the 390 - 520 nm range, against the sample blank (Omura & Sato, 1964 [1]).

The amounts of sodium dithionite added were varied and the concentration of protein sample in the cuvette was varied, until a satisfactory spectrum had been obtained.

4.3.3 Sodium Periodate Dependent Progesterone Hydroxylation by Cytochrome P450

Progesterone was added to a concentration of 4.0 mM to 1.0 ml crude cell-free extract incubations containing 0.5 ml of 0.1 M phosphate buffer. Sodium periodate was added to a concentration of 2.25 mM. Incubations were rotated at 20 rpm on a Gallencamp blood tube vertical rotator, at 25 °C for 10 h prior to steroidal metabolite extraction.

4.3.4 Inhibition of Progesterone 2β,16α-Hydroxylation by Ketoconazole

Ketoconazole was added at 0.5 - 8.0 μg/ml to the cell free incubations described in 4.3.3 above and the steroidal metabolite profiles from each incubation were compared by TLC analysis. This profile comparison was repeated for both partially purified and purified progesterone hydroxylase preparations, also at concentrations of 0.5 - 8.0 μg / ml. Progesterone was added at 4.0 mM and sodium periodate (Hrycay *et al.* 1975) at 2.25 mM. Incubations were carried out for 10 h before steroidal metabolites were extracted as described in chapter 2. Methanol dissolved metabolites were examined by TLC and analysed spectrophotometrically, using a Pye Unicam PU 8800 uv/vis spectrophotometer as described in chapter 2.

4.3.5 Requirement for NADH or NADPH

In the assays described above, $NaIO_4$ removes the requirement for the natural electron donor, NAD(P)H, the electron transfer proteins and molecular

oxygen. To develop the assays with a view to reconstituting the natural P450; the natural electron donor was identified.

All natural cytochrome P450 systems have a requirement for either NADH or NADPH. To the crude cell extracts prepared as described above, NADH or NADPH was added to a concentration of 0 - 0.5 mM. Crude cell-free incubations comprised 0.5 ml cell-free extract made up to 1.0 ml with 100 mM Na phosphate buffer, pH 7.2. To each incubation progesterone was added to a concentration of 0.32 mM (following optimisation of the progesterone requirement). The incubations placed on a vertical rotating platform was rotated for 10 h at 20 rpm prior to steroidal metabolite extraction and examination by TLC as described in chapter 2.

4.4 Purification of the Cytochrome P450 Responsible for Progesterone Transformation in *S. roseochromogenes*

4.4.1 DEAE-Cellulose Ion-Exchange Chromatography

Cell breakage to obtain the S15 fraction, was carried out as described in section 4.3.1. DEAE-52 cation exchange chromatography was carried out as the first stage of purification. CM-32 Anion exchange chromatography was also attempted, but failed to separate the progesterone hydroxylase activity from other proteins as it was not retained on the column. Pre-swollen DE-52 DEAE-cellulose was mixed with orthophosphate buffer (0.1 M K_2HPO_4 , 5 mM EDTA, DTT 0.25 mM, 10 % glycerol, pH 7.2) and degassed under reduced pressure for 1.5 h. The slurry was then poured into a glass column (25 cm x 5 cm) to give a bed volume of 100 ml. The column was thermally equilibrated at 4 °C and washed for 2 h with the column buffer described above. S15 fraction was applied to the column. Once the S15 had permeated the column bed, the column was washed through with 2 x bed volume of column buffer. Proteins were then eluted from the column with 100 ml each of 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM and 350 mM NaCl in the column buffer. Eluted protein fractions were examined by polyacrylamide gel electrophoresis and assayed for progesterone hydroxylase activity, as described in chapter 2.

4.4.2 Preparation of 11α-Progesterone Sepharose 6B Affinity Columns

6.0 g of freeze dried epoxy-activated Sepharose 6B was resuspended in 100 ml of distilled water and gently stirred for 5 min before being transferred to a

sintered glass funnel. The gel was washed for 1 h with 1200 ml of distilled water added in 200 ml aliquots.

After swelling and washing, the gel was coupled with 11α-hydroxyprogesterone (Vertblad 1976) by first resuspending 1.8 mg of this ligand in coupling buffer (100 ml dimethylformamide in 100 ml of 0.1 M potassium phosphate, pH 10). The gel was added to the ligand solution and this slurry was poured into a 1L volumetric flask and mixed by swirling in an orbital incubator at 32 °C for 16 h.

Following this incubation, any excess reactive groups on the Sepharose 6B were blocked (Fox & Hechemy 1978; Kagedal *et al.*, 1978; Simons *et al.*,1977) by washing the gel in the phosphate buffer, then incubating the gel in 1.0 M ethanolamine at 40 °C for 10 h. The coupled gel was then washed with coupling buffer as described above. The slurry was poured into a glass column and the gel washed for 1 h with coupling buffer. The column was then washed alternately in buffers of high and low pH (0.1 M acetate - 0.1 M NaCl, pH 4.0 and 0.1 M borate - 0.5 M NaCl, pH 8.0) (Harvey *et al.*, 1974). Each wash was for 20 min and the cycle repeated thrice.

4.4.3 Steroid Affinity Chromatography

11α-Hydroxyprogesterone was coupled to epoxy-activated Sepharose 6B as described in section 4.4.2. The resulting slurry was poured into a glass column (15 cm height x 1 cm internal diameter) to give a bed volume of 11 ml. The column was washed for 1 h with the column buffer described in section 4.4.1 and equilibrated at 4 °C.

Active post DE-52 fractions were combined then dialysed in 400 ml column buffer and 10 % glycerol for 5 h, twice. The dialysed fraction was assayed for steroid hydroxylase activity as described in chapter 2. Dialysed post DE-52 fraction was then applied to the steroid affinity column and allowed to enter the matrix completely. The column was then washed with column buffer at

pH 7.2 until twice the bed volume had been collected. The fractions were assayed for progesterone hydroxylase activity : a negative result indicated hydroxylase-matrix binding. The column was then washed with buffer containing NaCl in the concentrations used in the DE-52 elution in section 4.4.1. Column fractions were examined by polyacrylamide gel electrophoresis.

When not in use, the column was stored in sodium azide (0.05 M) in 0.1 M phosphate buffer at 4 °C, to prevent bacterial degradation and hydrolysis of the matrix (Fox *et al.* 1978; Landt *et al.* 1978).

4.4.4 MIMETIC Ligand Affinity Chromatography

These column matrices were purchased ready made. Each column resin was bound to a different coloured dye, as specified by the manufacturer, to identify it. When not in use the column resins were stored at 0 - 4 °C, in ethanol / 0.1 M NaCl 25:75 v/v. Column adsorbents used were MIMETIC ligand A6XL: Yellow 2, Blue 1, Blue 2, Red 2 & Red 3. Pilot scale columns were prepared first to establish which resin bound the hydroxylase and provided the most effective protein separation of the dialysed post steroid-affinity active fractions. After identification of the most effective resin, it was used in large scale preparation of progesterone 2β,16α hydroxylase P450. A 15 ml bed volume column was prepared by degassing for 1 h on ice then pouring into a glass column (2 cm x 30 cm). The column was equilibrated and washed with the 0.1 M orthophosphate column buffer at 4 °C. Active post steroid-affinity fractions, which had been combined and dialysed, as in section 4.4.3, were applied to the column immediately followed by two bed volumes of column buffer. Proteins were eluted from the column using column buffer containing 50 mM, 100 mM, 150 mM, 200 mM & 250 mM NaCl. Fractions were assayed for progesterone 2β,16α hydroxylase activity, as in section 4.4.3 then

combined prior to dialysis twice in 400 ml of 0.1 M orthophosphate buffer at 4°C for 3 h.

Column fractions were examined by polyacrylamide gel electrophoresis as described in chapter 2.

4.5 RESULTS

4.5.1 Cell Breakage Techniques for the Disruption of Cells and the Retention of Cell-Free Progesterone 2β,16α Hydroxylase Activity

S. roseochromogenes cells were disrupted by homogenisation and by sonication as described in chapter 2, for the purpose of comparison in order to identify the most effective method of release of progesterone 2β,16α hydroxylase activity from whole cells.

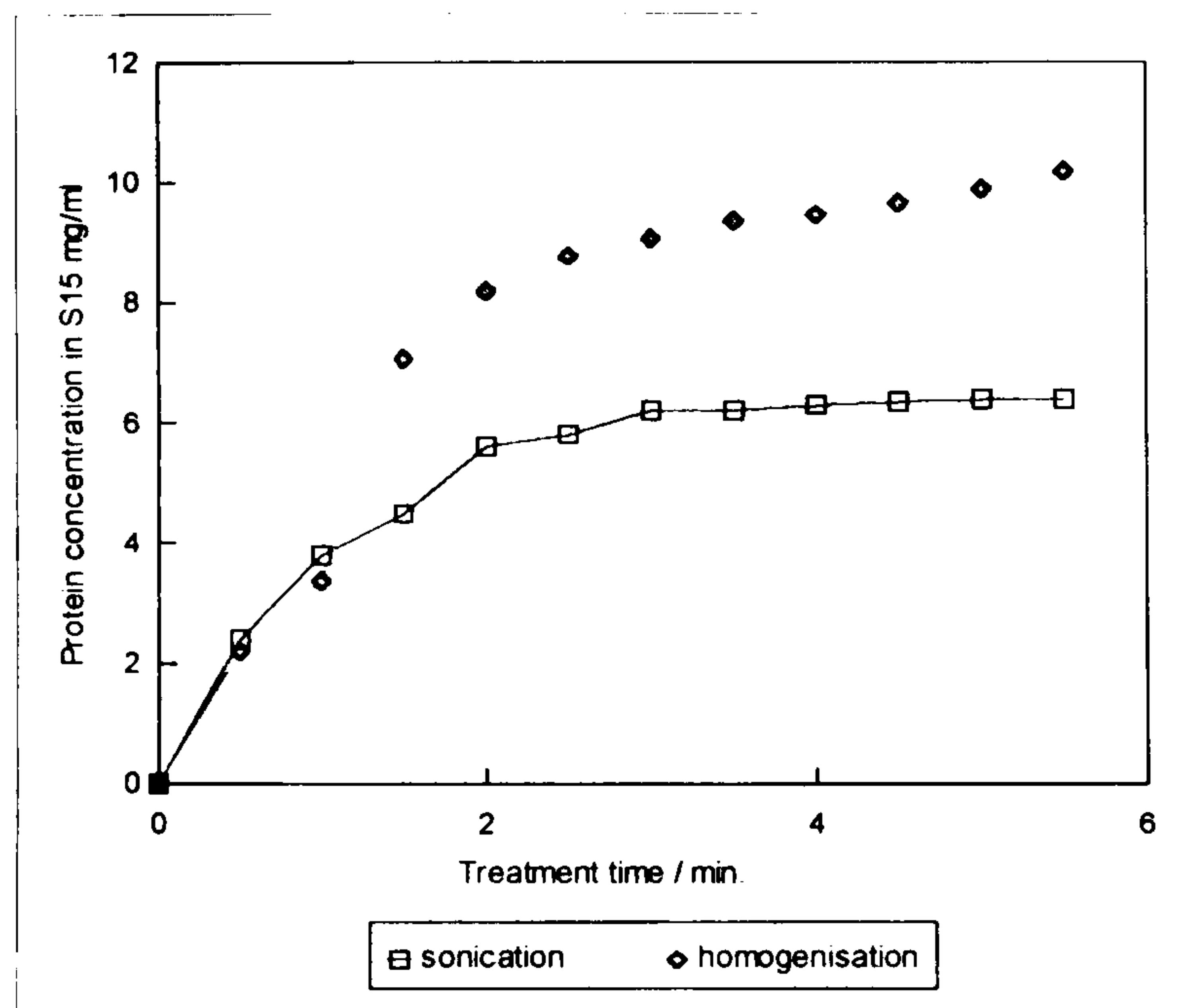

Figure 4.5.1a. Protein concentration of *S.roseochromogenes* obtained by homogenisation and sonication.

The protein concentration obtained by homogenisation using an MSE blender, was greater than that by sonication. The optimum blending time, retaining progesterone 16α hydroxylase activity, was 5.0 min. The results in Figure 4.5.1b, show an optimal homogenisation time of 3 min.

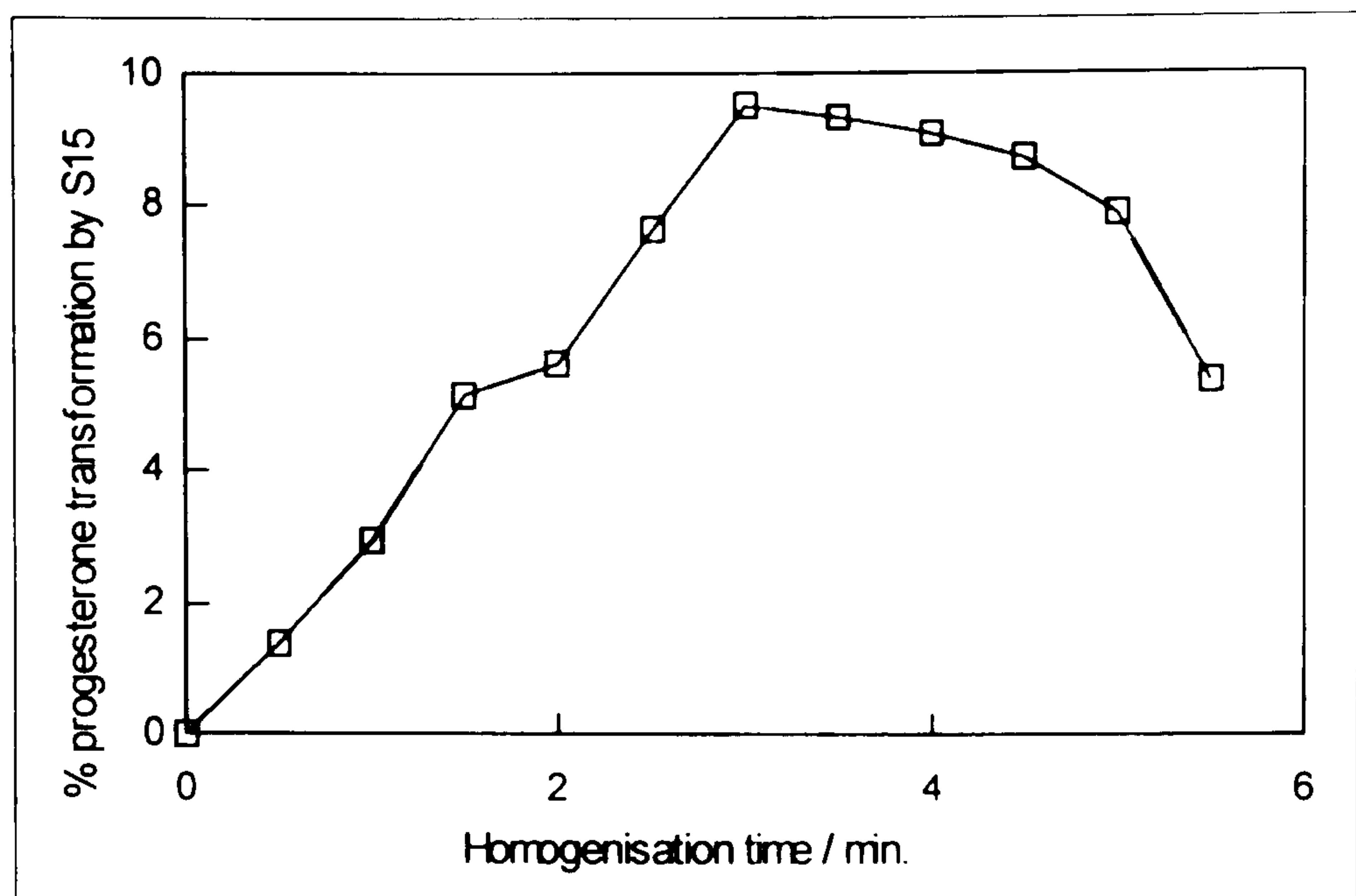

Figure 4.5.1b. The retention of progesterone hydroxylase activity with increasing homogenisation time.

The results in Figure 4.5.1a showed that increasing homogenisation time increased the release of protein, thus resulting in an increasing S15 protein concentration. However, Figure 4.5.1b showed S15 loosing progesterone hydroxylase activity after 3.0 min. homogenisation time. Three minutes was therefore the optimal homogenisation time.

4.5.2 Homogenisation Times and Patterns and the Release of Protein from Whole Cells with the Retention of Hydroxylase Activity 'Striking a Balance'

Homogenising cells continuously for 3 minutes has a warming effect on the ice-cold cell slurry, through friction. Figure 4.5.2a indicates that this may be the case, because cooling at 30 second intervals gave rise to the retention of more of the progesterone 16α hydroxylase activity.

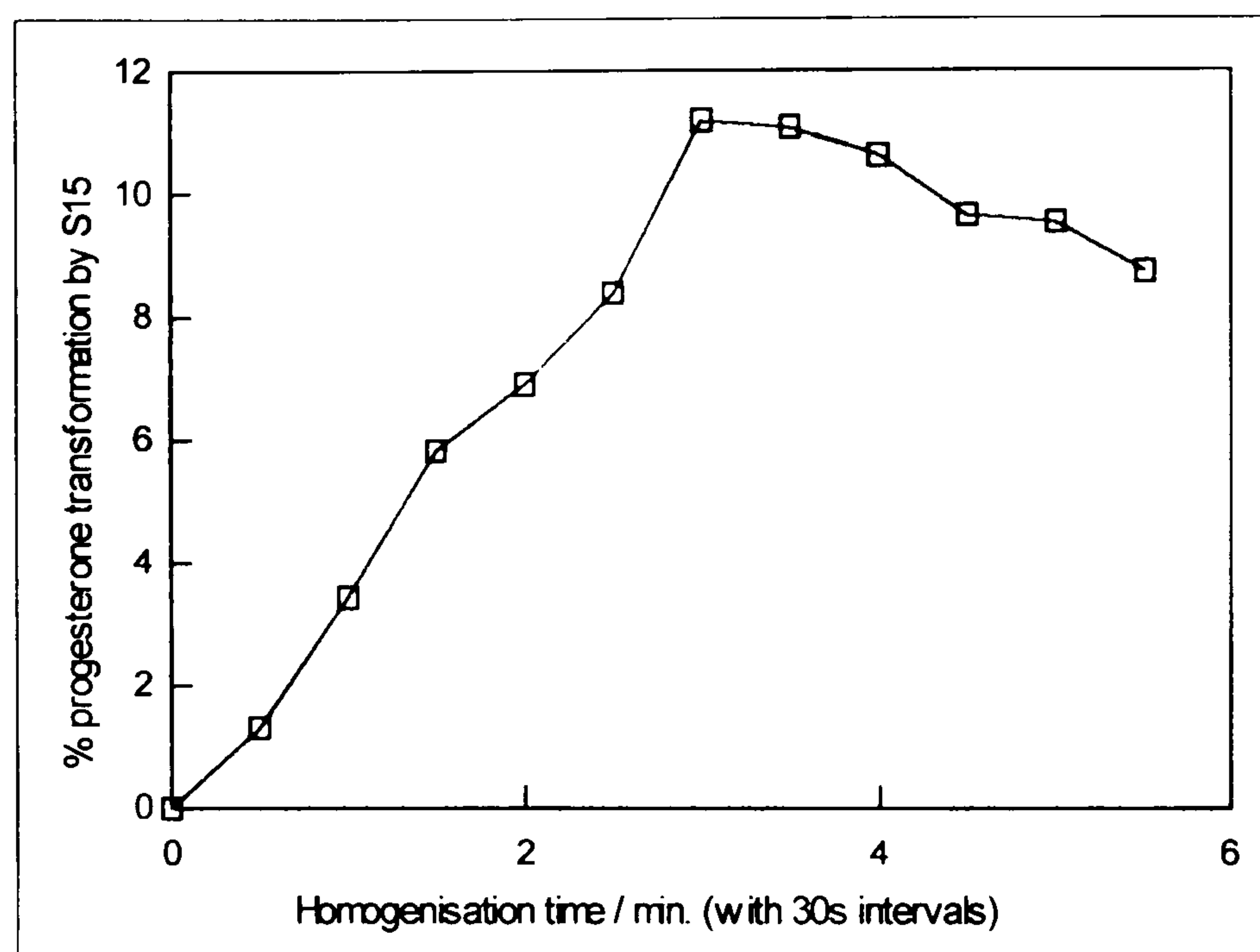

Figure. 4.5.2a. Progesterone hydroxylase activity with 30 second cooling period on ice. Between each 30 second burst of homogenisation.

Six, 30 second bursts of homogenisation with 30 seconds on ice between each burst of homogenisation, provided S15 which was capable of 11.2 % transformation of progesterone when incubated with 3.0 mM progesterone.

4.5.3 The Effect of Protease Inhibitors During Cell Homogenisation

8% Polyacrylamide gels were used to examine the proteins contained in S15. S15 samples were left for increasing periods of time before electrophoresis to reveal the presence of possible proteases. This was established by an increase in the number of bands appearing on an SDS-PAGE slab gel. To prevent degradation, a protease inhibitor, phenylmethylsulphonylfluoride (PMSF) was used during the preparation of cell free extracts (Trower *et al.*, 1989) at a concentration of 7.0 μM from a stock made up in 50:50 water:methanol v/v.

4.5.4 The Effect of Cell Culture Age on Maximum Recovery of Progesterone 16α Hydroxylase Activity

Figure 4.5.4a shows the optimum time for cell harvesting was after 35 h of growth. The figure also shows progesterone transformation by the respective cell free preparations (S15).

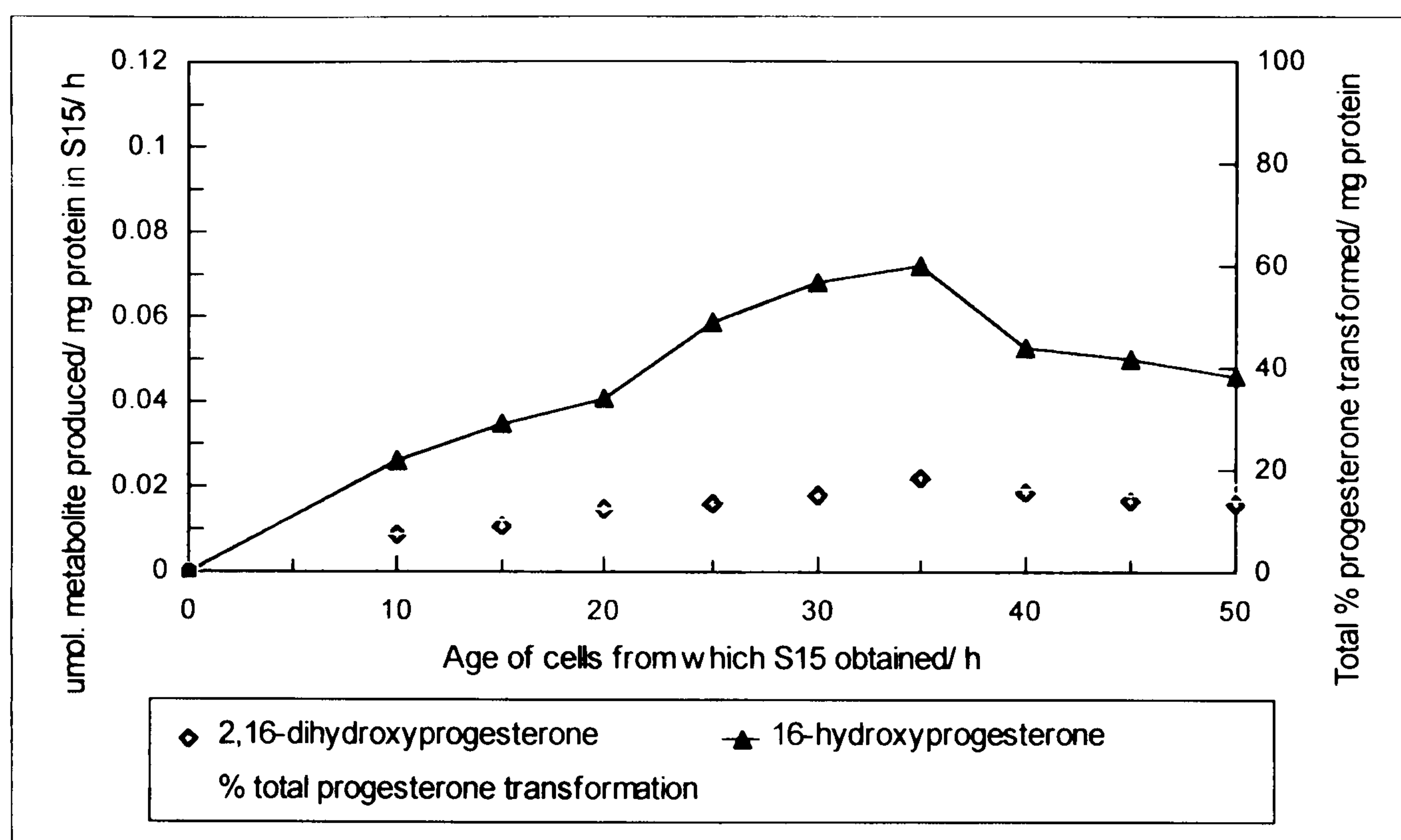

Figure 4.5.4a. The effect of cell culture age on metabolite production by cell-free preparations of the differint aged cultures.

The data in Figure 4.5.4a show a decline in the transformation capability of cell free extracts after 40 h cell growth. Identical results were obtained for both metabolites for all incubation times. Also shown in the figure, is the total percentage transformation of substrate.

4.6 Characterisation of the Progesterone 16α-Hydroxylase System of *S. roseochromogenes*

4.6.1 The Effect of DTT Concentration on Progesterone 16α Hydroxylase Activity in Crude Cell Extracts (S15)

Dithiothreitol (DTT) concentrations were varied as shown in Figure 4.6.1a.

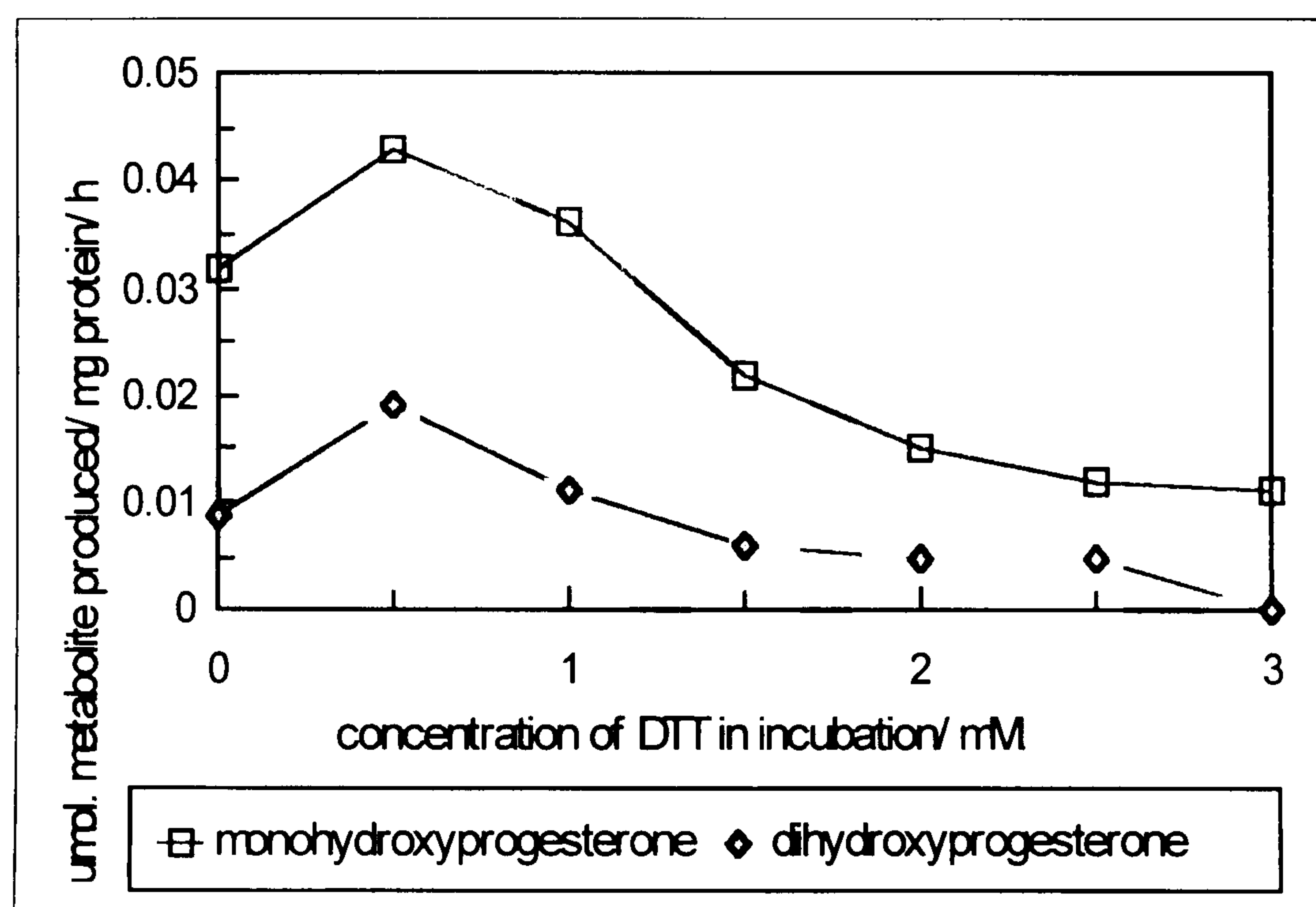

Figure 4.6.1a. Effect of incubation buffer DTT concentration on progesterone transformation by cell free extracts of *S. roseochromogenes*.

When S15 was incubated with 4.0 mM progesterone, 1 mM EDTA at pH 7.2 and 0.5 mM DTT, an increase in the production of the major metabolite of 30% and 100% of the minor metabolite, was observed, compared with incubation in the absence of DTT or the presence of 1.0 mM DTT.

4.6.2 The Effect of EDTA Concentration on Hydroxylase Activity in Crude Cell Extract (S15)

The cell-free incubation conditions described in section 4.6.1 were retained but the concentration of the chelating agent, EDTA was varied from 0 - 30 mM.

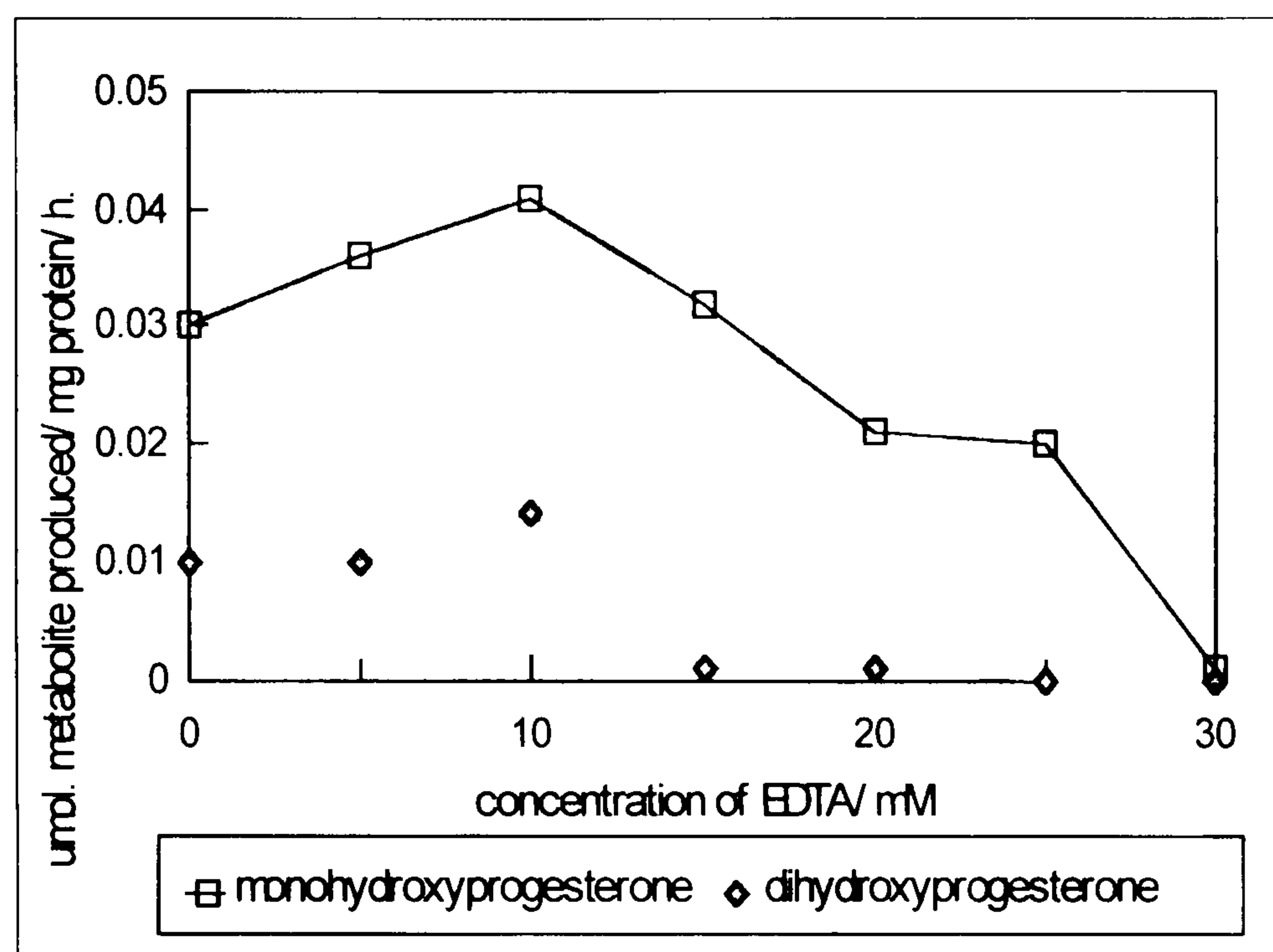

Figure 4.6.2a. The effect of varying EDTA concentration in the incubation buffer on the transformation of progesterone by cell-free extracts.

The data in Figure 4.6.2a show that 10 mM EDTA was the optimum concentration for maximum production of both major and minor metabolite and concentrations of EDTA above 20 mM were inhibitory to progesterone transformation by these cell-free extracts. Above 20 mM EDTA the minor dihydroxylated metabolite was undetectable and at 30 mM EDTA neither metabolite was detectable.

4.6.3 Effect of Glycerol Concentration on the Retention of Progesterone 16α Hydroxylase Activity in Crude Cell Extracts (S15)

Glycerol was added to buffers (Trower *et al.*,1989) in order to stabilise the protein structure of the progesterone 16α hydroxylase, especially during storage at -70°C.

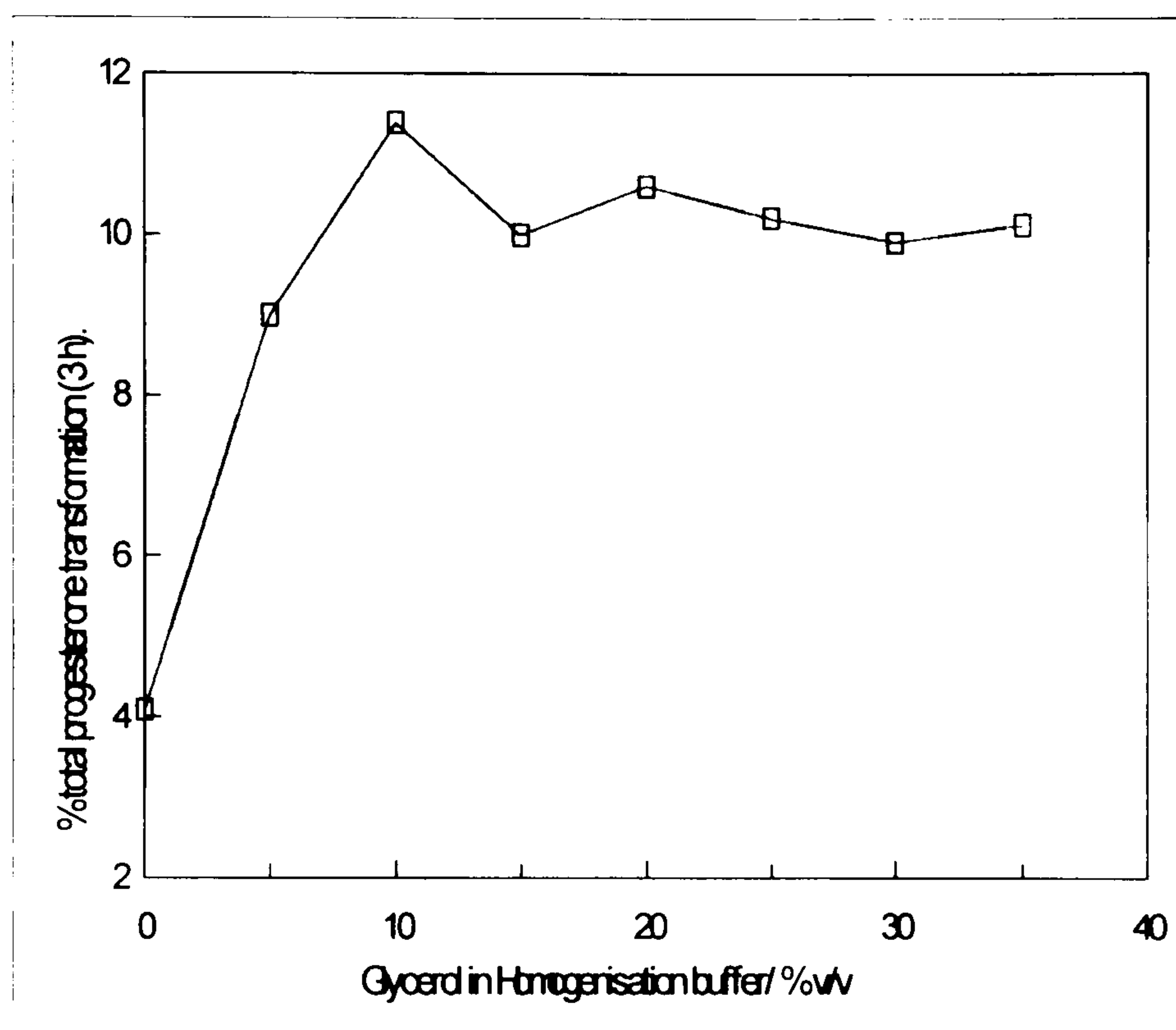

Figure 4.6.3a The effect of increasing glycerol concentration on the total transformation of progesterone by cell-free extracts in a 3h incubation.

The data in Figure 4.6.3a show that 10% glycerol was the optimum buffer proportion to use. Cells were also stored in 10 % glycerol at -70 °C. The presence of glycerol above 10% was of no advantage and increased the viscosity of samples thereby retarding their flow through column chromatography purification columns.

4.6.4 Sodium Periodate ($NaIO_4$) Concentration in Phosphate Buffer for Maximum Progesterone Transformation

Cytochrome P450 systems are multicomponent and require other proteins for transfer of electrons from NAD(P)H to the terminal P450. However, an oxidising agent can be used to obviate the requirement for electrons in a shortened form of the P450 catalytic cycle known as the peroxide shunt pathway, as described in the introduction (chapter 1).

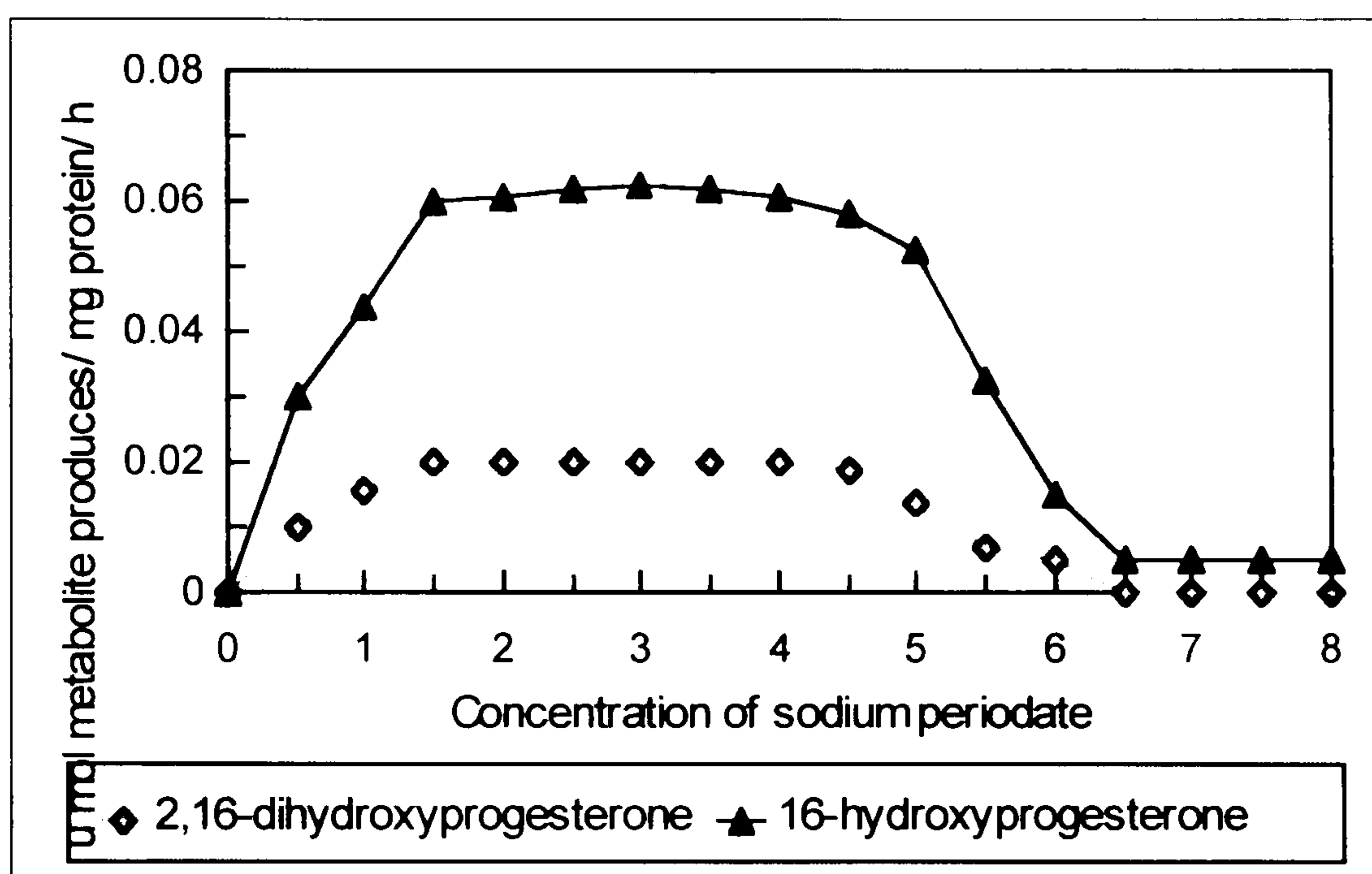

Figure 4.6.4a Effect of varying the concentration of $NaIO_4$ in incubation buffers, on the transformation of progesterone by cell-free extracts.

The data in Figure 4.6.4a show that 2.5 mM sodium periodate was the optimum concentration for the production of both major and minor metabolites in cell-free incubations. Above a concentration of 3.0 mM. sodium periodate appeared to have an inhibitory effect upon the progesterone hydroxylase activity. From 0 - 1.5 mM $NaIO_4$ there was a sharp increase in the rate of progesterone transformation attributable to the increasing concentration of oxidant.

4.6.5 pH Dependence of Cell-Free Transformation of progesterone

S. roseochromogenes cell free extracts were incubated with progesterone, as described above at pH values from 6.4 to 8.6. The effects of this on the biotransformation of progesterone into both major and minor metabolites are shown in the data in Figure 4.6.5a.

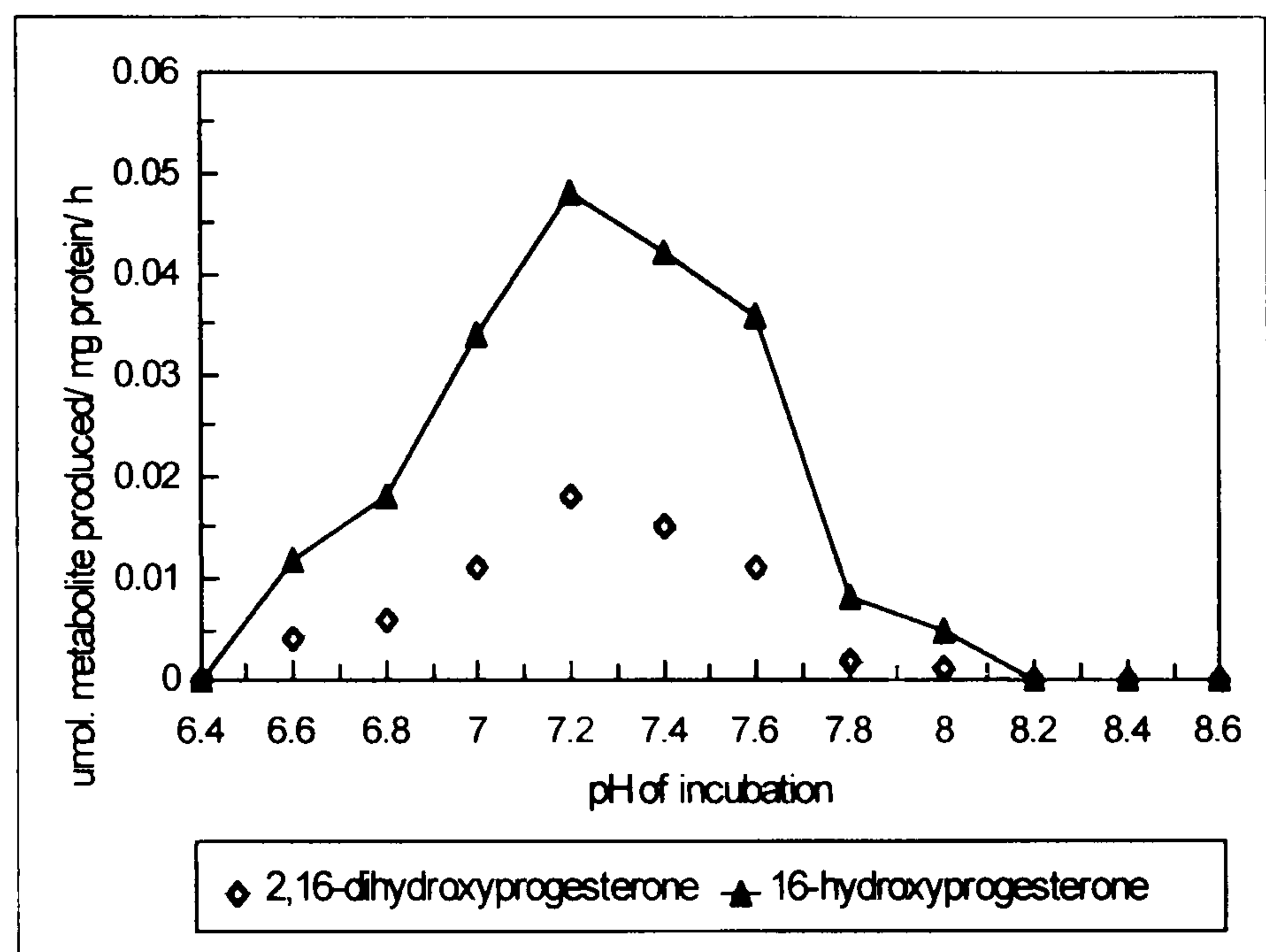

Figure 4.6.5a Effect of cell-free incubation pH on the production of both major and minor metabolites of progesterone transformation.

The data show that a pH of 7.2, was the optimum cell-free pH for progesterone biotransformation assays. This was the same pH as was found to be optimum for the growth of *S. roseochromogenes* whole-cell cultures.

A sharp decrease in the rate of production of the major metabolite was observed above pH 7.6 and a pH above 8.2 totally inhibited the production of both metabolites. A pH below 6.4 was not conducive to progesterone transformation. The data show that over this pH range, the minor metabolite follows a similar production profile as the major metabolite.

4.6.6 Effect of Progesterone Concentration on Hydroxylase Activity

Equal concentrations of protein from cell extracts were incubated with increasing concentrations of progesterone to determine an effect on the hydroxylation of the substrate. The data for progesterone transformation are shown in Figure 4.6.6.a.

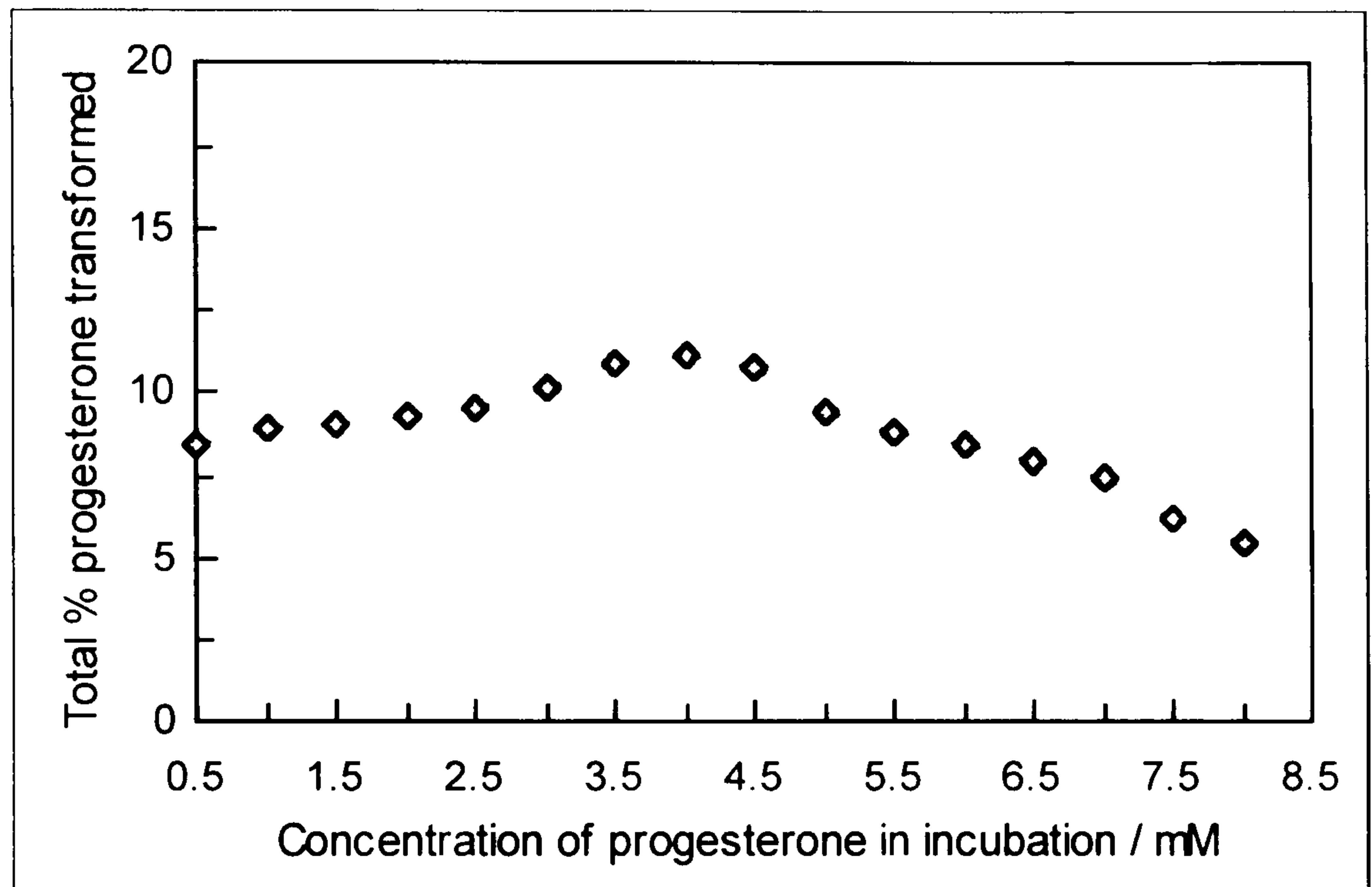

Figure 4.6.6a The effect of progesterone concentration on metabolite production in 10 h cell-free incubations.

Up to 4.0 mM progesterone, the increase in transformation was approximately linear. However, above this concentration, metabolite production was inhibited in that the greater the concentration of progesterone above 4.0 mM, the lower the percentage bioconversion.

4.6.7 Effect of Protein Concentration on the Production of Progesterone Hydroxylase Metabolites

An S15 protein concentration of 6.25 mg/ml yielded the greatest progesterone transformation in cell-free incubations as described above. Increasing the protein concentration above this, may introduce a higher proportion of an

inhibitory factor. Figure 4.6.7a shows the effect of protein concentration on progesterone transformation.

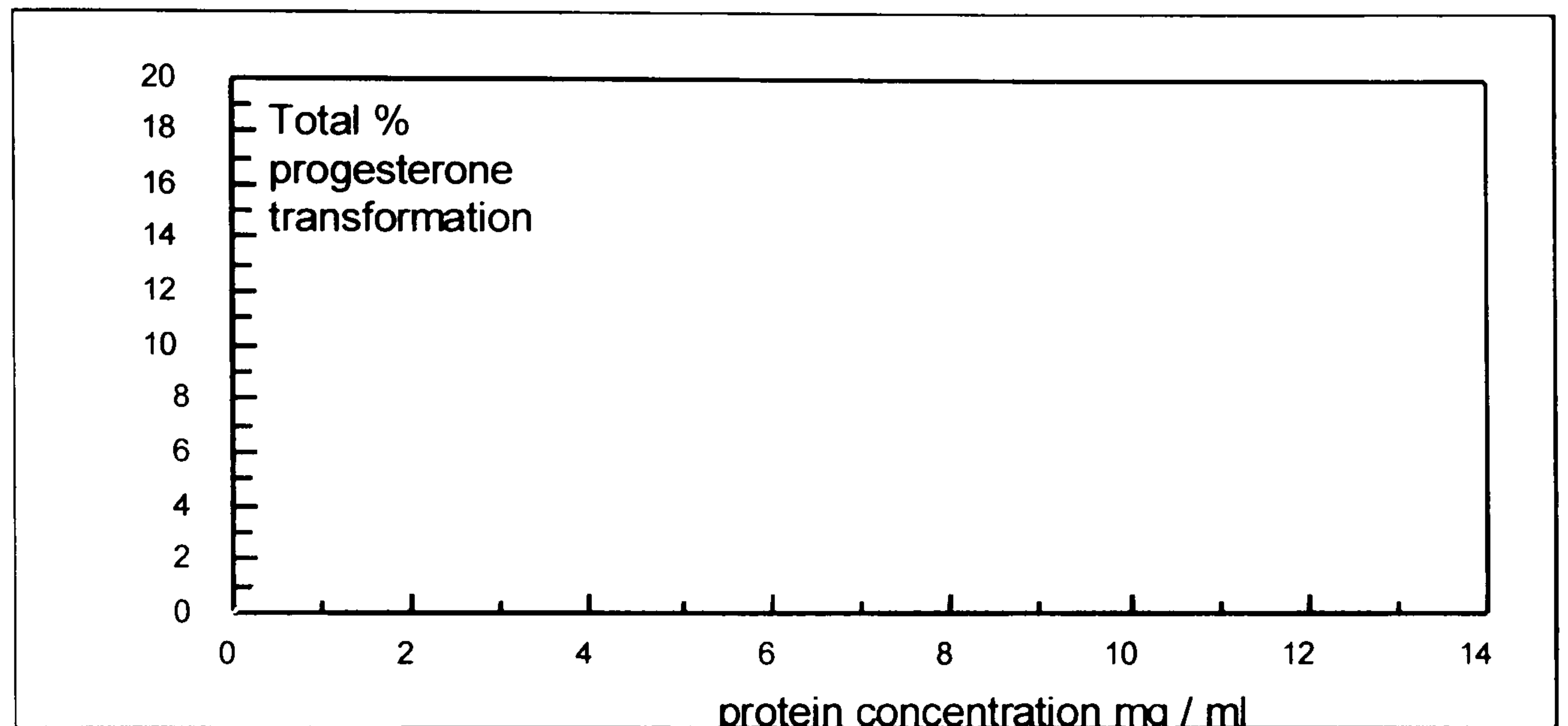

Figure 4.6.7a Effect of increasing the concentration of S15 protein in the cell-free incubation on progesterone transformation.

At this stage, it was not known what proportion of the protein fraction P450 was. Above 6.25 mg/ml of S15, the percentage transformation of progesterone began to fall.

4.6.8 Varying the Incubation Time of Progesterone with Cell Extract (S15)

Cell free assays were incubated for increasing lengths of time, to determine the time required for maximum transformation of the substrate. In Figure 4.6.8a, the data show that a maximum of 12.4 % transformation was observed after 3.0 h under the conditions described above.

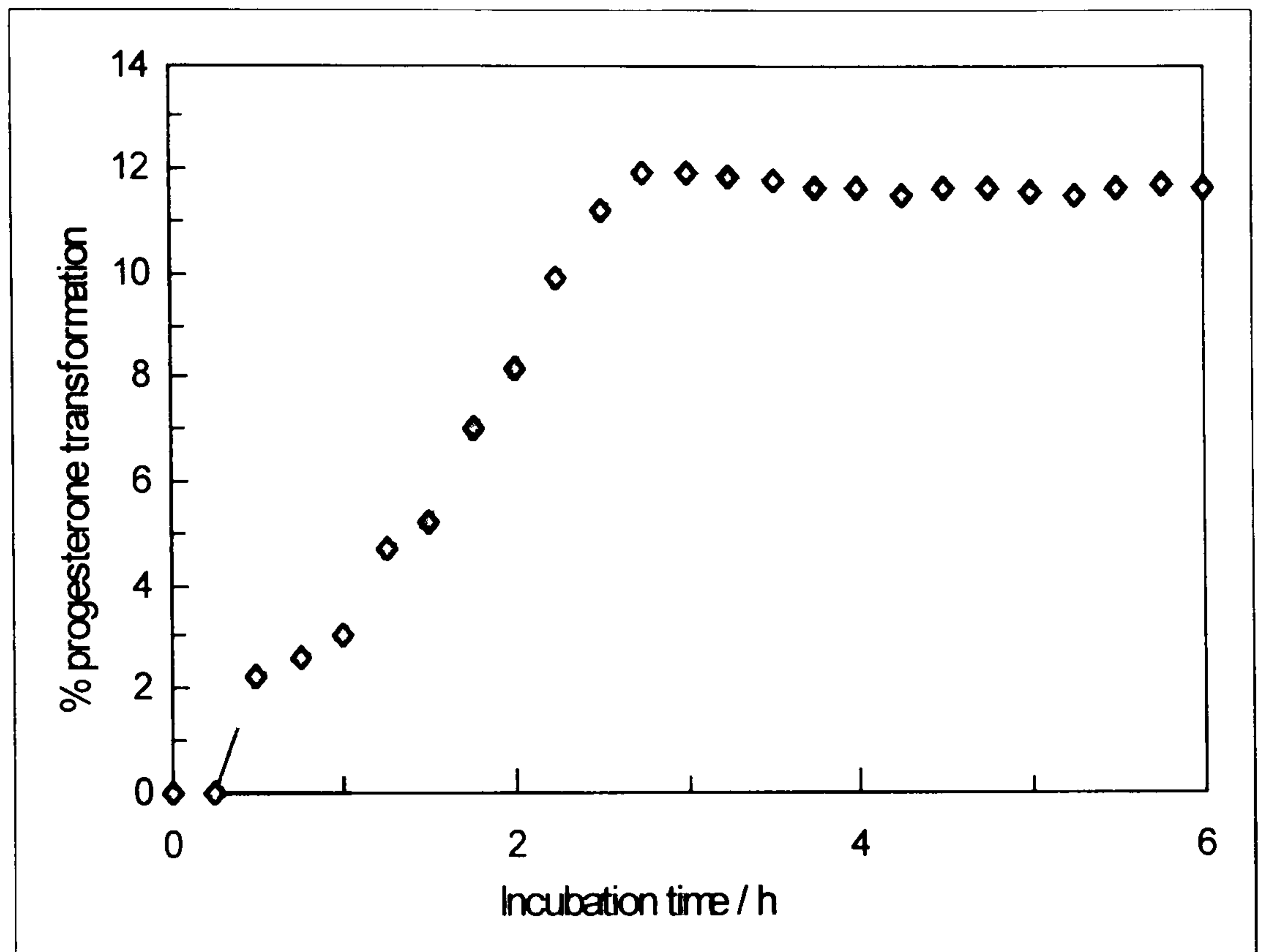

Figure 4.6.8a Increasing incubation times for cell free assays.

Incubation times above 3.0 h did not increase the production of metabolites. Transformation from 0 - 3.0 h was linear.

4.6.9 Inhibition of Progesterone Hydroxylase activity by Ketoconazole

Ketoconazole is a known P450 inhibitor and inhibitor of steroidogenesis. In porcine testicular microsomal P450, ketoconazole inhibits the P450 dependent cleavage of 17α-hydroxyprogesterone at the C17-20 bond (Nagai *et al.*, 1987) in a dose dependent fashion as to in yeast 14α demethylase inhibition by ketoconazole. The study of fungal P450 inhibition by azole antifungal agents has been extensive, for example *Saccharomyces cerevisiae* CYP61, sterol Δ22-desaturase (Kelly *et al.*, 1997[1])

Ketoconazole and other azole P450 inhibitors are used in medicine and industry as antifungal agents, where the development of resistance to such agents is an important consideration at the genetic level. For example the

insertion of the 14α demethylase gene of *Saccharomyces cerevisiae* into azole resistant plasmids thereby forming azole resistant yeasts for industrial fermentation (Kelly *et al.*, 1993 [1]).

Figure 4.6.9a shows the result of the inhibitory effect of ketoconazole on progesterone transformation by S15 at 6.5 mg/ml protein incubated with 4.0 mM progesterone. Incubation was with increasing concentrations of ketoconazole.

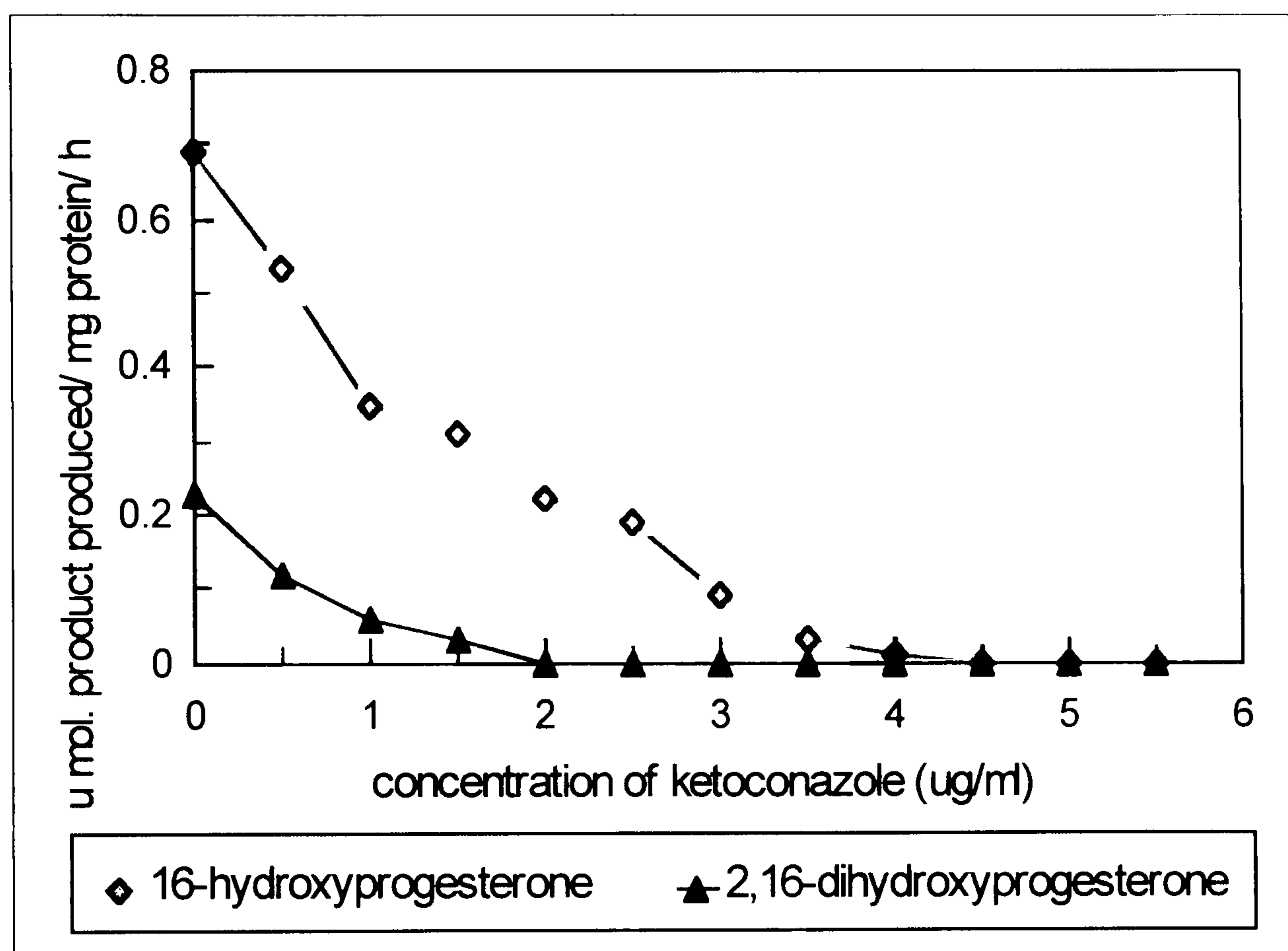

Figure 4.6.9a. The inhibitory effect of ketoconazole on *S. roseochromogenes* progesterone hydroxylase activity.

Figure 4.6.9a shows a dose dependent response to ketoconazole. 2 μg/ml ketoconazole totally inhibited the production of the minor metabolite, 2β,16α dihydroxyprogesterone but 0.22 μmol/mg protein/h of the major metabolite, 16α hydroxyprogesterone, was produced at this concentration. The major metabolite was totally inhibited at a ketoconazole concentration of 4.0 μg/ml.

4.7 Purification of the Progesterone 16α-Hydroxylase Cytochrome P450 in *S. roseochromogenes*

4.7.1 DEAE Cellulose Ion-Exchange Chromatography of S15 Cell-Free Extract

S. roseochromogenes S15 cell free extract was prepared and applied to a DEAE 52 ion exchange column as described in section 4.4.1. The profile of proteins eluting from the column is shown in Figure 4.7.1a.

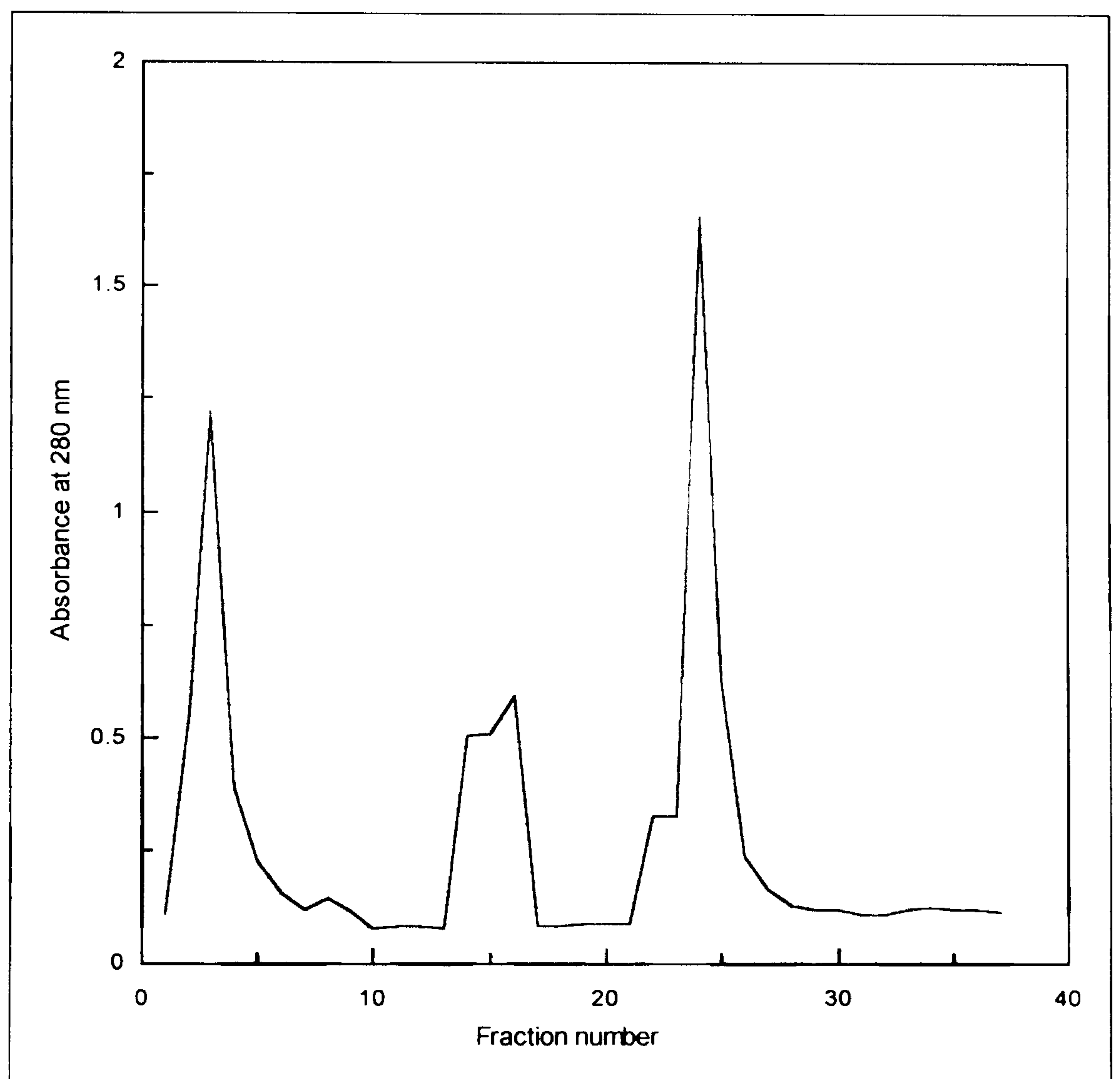

Figure 4.7.1a Elution profile of S15 proteins from the DEAE 52 column.

In the profile in Figure 4.7.1a above, the first peak contained unbound proteins eluting from the column in the wash buffer or 'follow-through'. The second peak is proteins eluted at 100 mM NaCl. and the third peak is proteins which eluted at 200 mM NaCl. The active fractions eluted at 200 mM NaCl.

4.7.2 Dithionite Reduced Carbon Monoxide Difference Spectrum of Post DEAE 52 Column progesterone Hydroxylase Containing Fractions

Post DEAE column fractions, were tested for reduced CO difference spectra indicative of the presence of a cytochrome P450 (Omura & Sato, 1964 [1]).

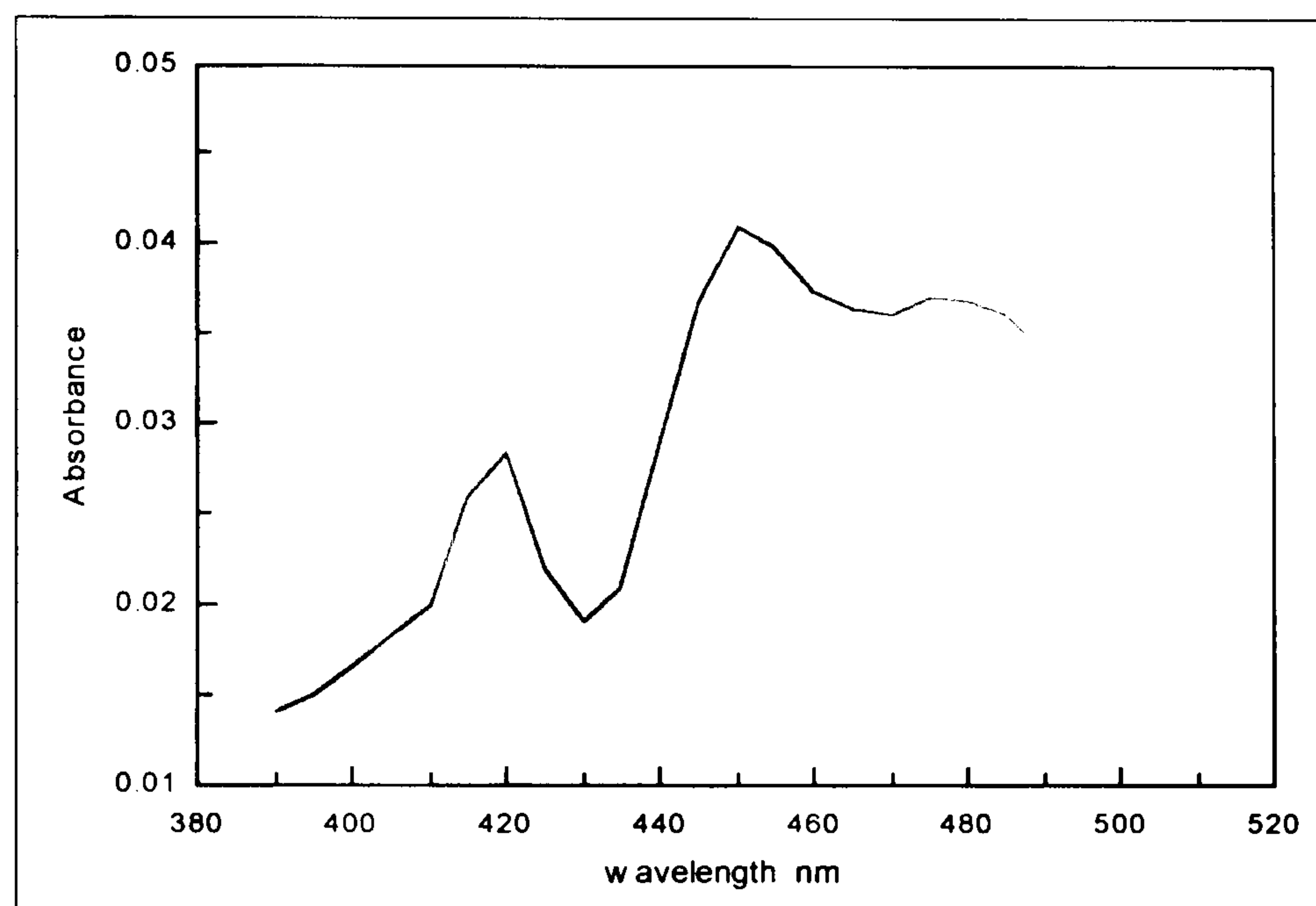

Figure 4.7.2a Dithionite reduced CO difference spectrum for *S. roseochromogenes* progesterone hydroxylase.

The spectrum in Figure 4.7.2a, has a peak at 450 nm indicative of the presence of native cytochrome P450, a trough at 430 nm and a peak at 420 nm corresponding to denatured P450 in the preparation. From this spectrum: a value of A_{450} =0.041 and A_{490} =0.035, were measured. Using a molar extinction coefficient of 91 $cm^{-1}mM^{-1}$ (Omura & Sato, 1964 [2]) these values correspond to a native P450 concentration of 65.9 nM. The total P450 concentration in the fraction must be greater because it contains denatured P450.

4.7.3 Sepharose 6B - 11α-Progesterone Affinity Chromatography of Active Post DEAE 52 Fractions

Fractions from the 200 mM NaCl DEAE 52 eluate which displayed typical reduced CO spectra and progesterone hydroxylase activity were combined and dialysed as described in the methods section prior to application to the sepharose 6B 11αprogesterone affinity column.

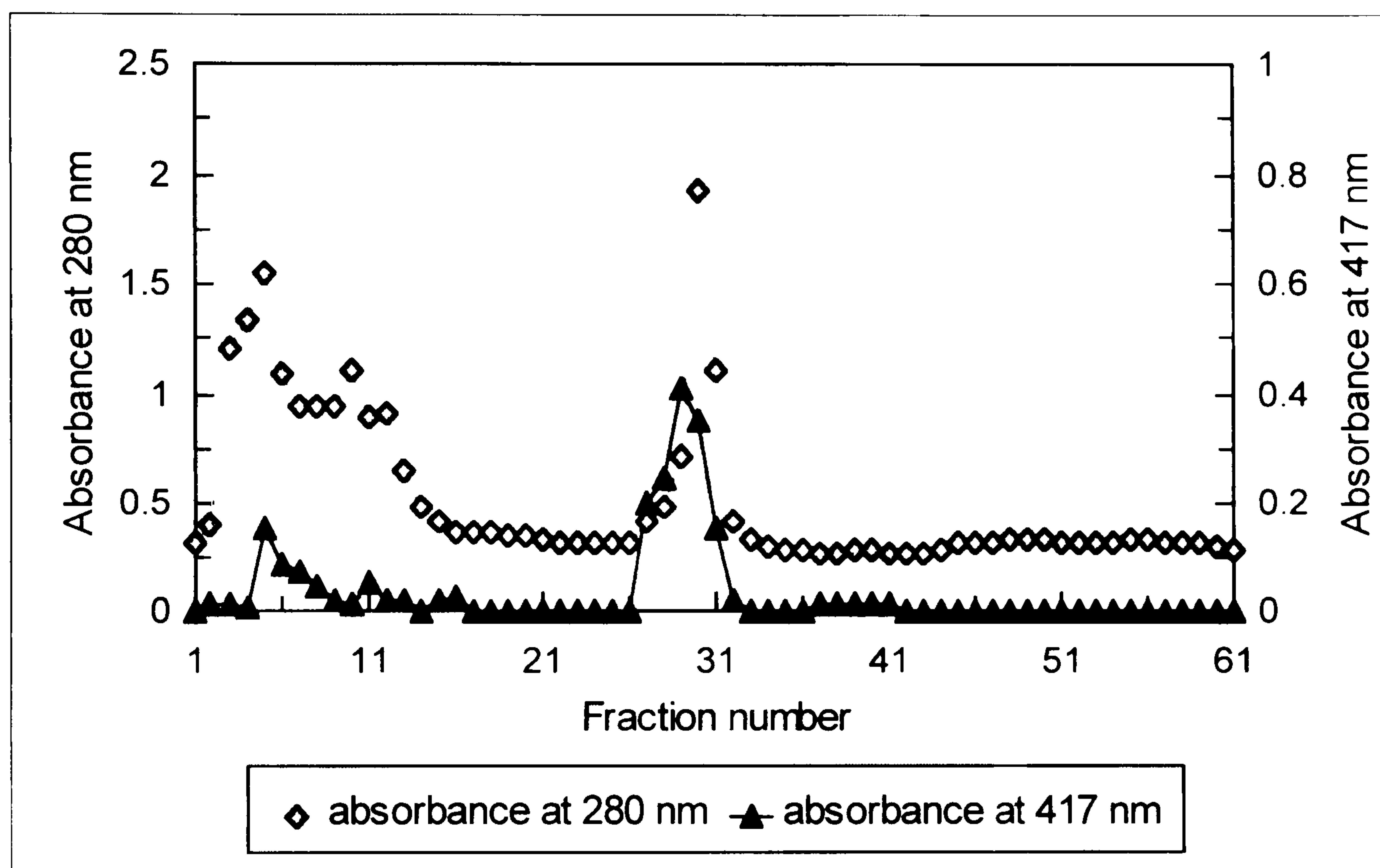

Figure 4.7.3a Elution profile of proteins eluting from the steroid 11α progesterone affinity column described. These were active post 200mM DEAE S15 protein fractions.

In Figure 4.7.3a the double peak represents unbound eluting protein. No hydroxylase activity was detected in these fractions. The second peak eluted at 150 mM NaCl and this fraction contained the hydroxylase activity. The progesterone hydroxylase had affinity for the substrate affinity column (Weibel *et al.*, 1972). Haemoprotein was detected by 417 nm absorbance. The 150 mM NaCl fraction contained all the 417 nm absorbance and

progesterone 16α hydroxylase activity. An encouraging sign here in terms of purification was the large haem peak which corresponded with the total protein peak containing the hydroxylase activity.

4.7.4 MIMETIC Ligand Affinity Chromatography of Post Steroid Affinity Chromatography Active Progesterone 16α Hydroxylase Fractions

This purification method followed the steroid affinity stage above, in the purification protocol.

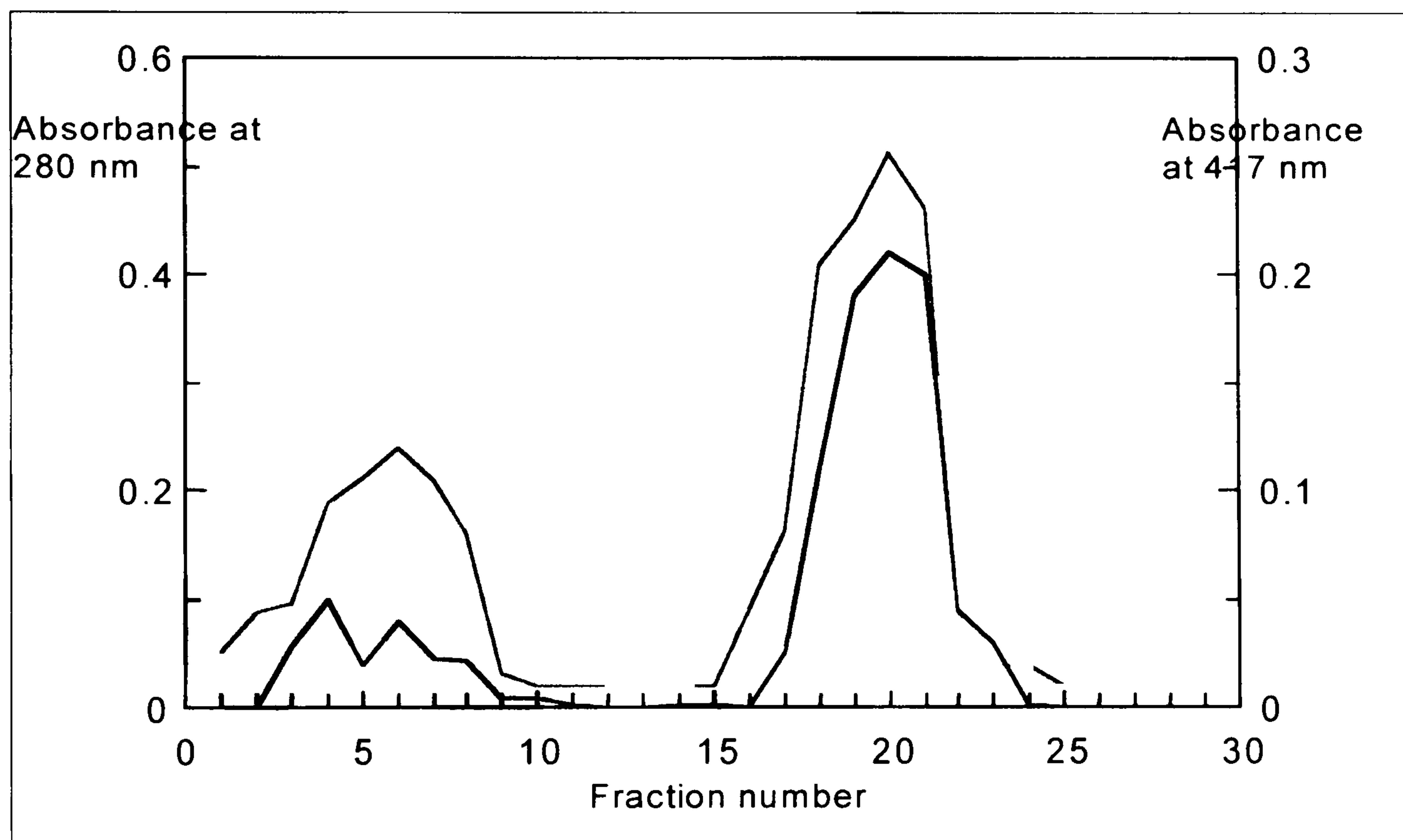

Figure 4.7.4a MIMETIC Ligand Blue I affinity column chromatography of active post steroid affinity chromatography fractions. The protein elution profile. The upper profile is total protein, A_{280} and the lower profile is haem A_{417}.

In Figure.4.7.4a, the first peak represents unbound protein eluted from the column. The second peak contained proteins eluted from the column at 120 mM NaCl and these fractions contained the progesterone 16α hydroxylase

activity. The protein eluted between fractions 18 - 22 contained pure P450 as judged by SDS polyacrylamide gel electrophoresis. The P450 concentration of the 120 mM NaCl fraction determined by the method of Omura & Sato, 1964, was 0.25 nmole. This represents a yield of 1.2 %. Total protein recovered, determined by the modified Bradford method, was 0.078 mg. Thus the specific content of the 120 mM NaCl MIMETIC Blue I column fraction was 3.21 nmol P450 / mg protein.

4.8 SDS PAGE of Proteins in Fractions from the above Chromatographic Stages of Purification

4.8.1 Polyacrylamide Gel Electrophoresis of Post MIMETIC Blue I 120 mM NaCl Fractions

Polyacrylamide gel electrophoresis was carried out as described in chapter 2 and gave an M_r of 63 x 10^3 for the P450. A 15 % gel was run with size marker protein ladders and the result is shown in Figure 4.8.1a. From left to right, lanes 1 and 2 show the two electron transfer proteins required by this P450 for reconstituted activity in the absence of $NaIO_4$. They are shown here for size comparison but their elucidation and purification are described in chapter 6.

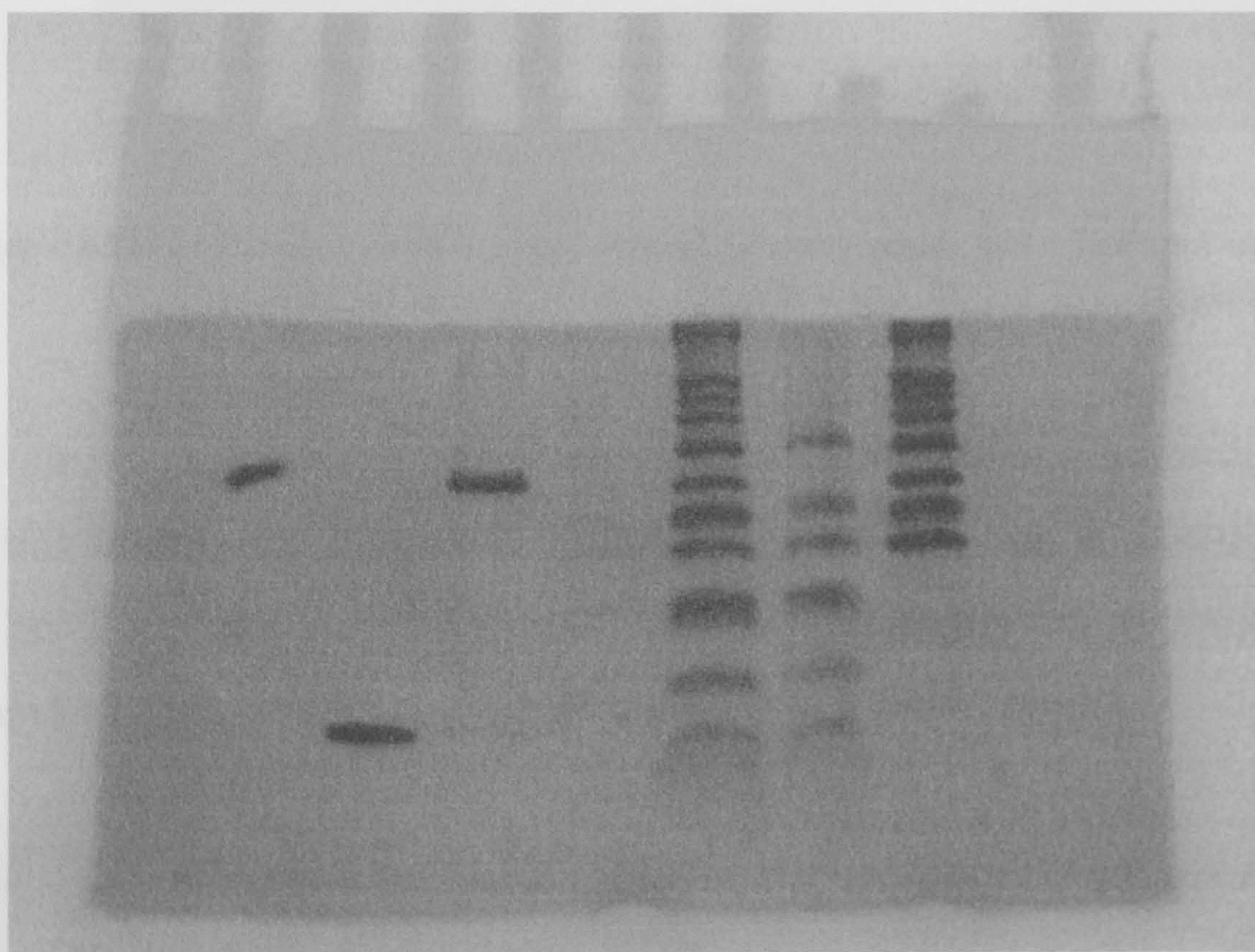

Figure 4.8.1a SDS-polyacrylamide gel of purified *S. roseochromogenes* 2β,16α hydroxylase cytochrome P450, roseodoxin and roseoredoxin reductase. From left to right: **Lane 1**, roseoredoxin reductase (3.1 μg M_r 65 x 10^3); **lane 2**, roseoredoxin (2.8 μg M_r 14 x 10^3); **lane 3**, progesterone 2β,16α-hydroxylase cytochrome P450 (4 μg M_r 63 x 10^3); **lane 4**, wide range protein size ladder M_r 205 x 10^3 - 14.2 x 10^3; **lane 5**, lower ranger protein size ladder M_r 66 x 10^3 -14.2 x 10^3; and **lane 6**, upper ranger protein size ladder M_r 45 x 10^3 -14.2 x 10^3. Lanes 4-6 contained 35 μg protein.

4.8.2 Haem Stain of the Progesterone 16α Hydroxylase P450 Post Polyacrylamide Gel Electrophoresis

A single sample of the purified progesterone 16α hydroxylase (approximately 5μg) was applied to a 10 % polyacrylamide gel prior to electrophoresis.

Figure 4.8.2a Photograph of a haem stained polyacrylamide gel. The spot indicates the staining of a single haem protein band in the gel, progesterone 16α hydroxylase P450 from *S. roseochromogenes*.

The electrophoretogram was visualised by haem staining according to the method described in chapter 2. A photograph of the result is shown in Figure 4.8.2a. The single band confirmed that the protein responsible for the $NaIO_4$ dependent transformation of progesterone, was a haem protein.

4.8.3 Two-Dimensional Gel Electrophoresis of Pure Progesterone 16α Hydroxylase Cytochrome P450

Two dimensional gel electrophoresis was employed in order to determine more precisely, the purity of the post MIMETIC Blue I, 120 mM NaCl

fraction progesterone 16α hydroxylase protein sample. Whereas SDS PAGE separates proteins on the basis of size, this procedure separates out any impurities on the basis of two parameters: isoelectric point and size, in the first and second dimensions respectively. The resulting 10 % slab gel was silver stained as described in chapter 2 and the result is shown in Figure 4.8.3a.

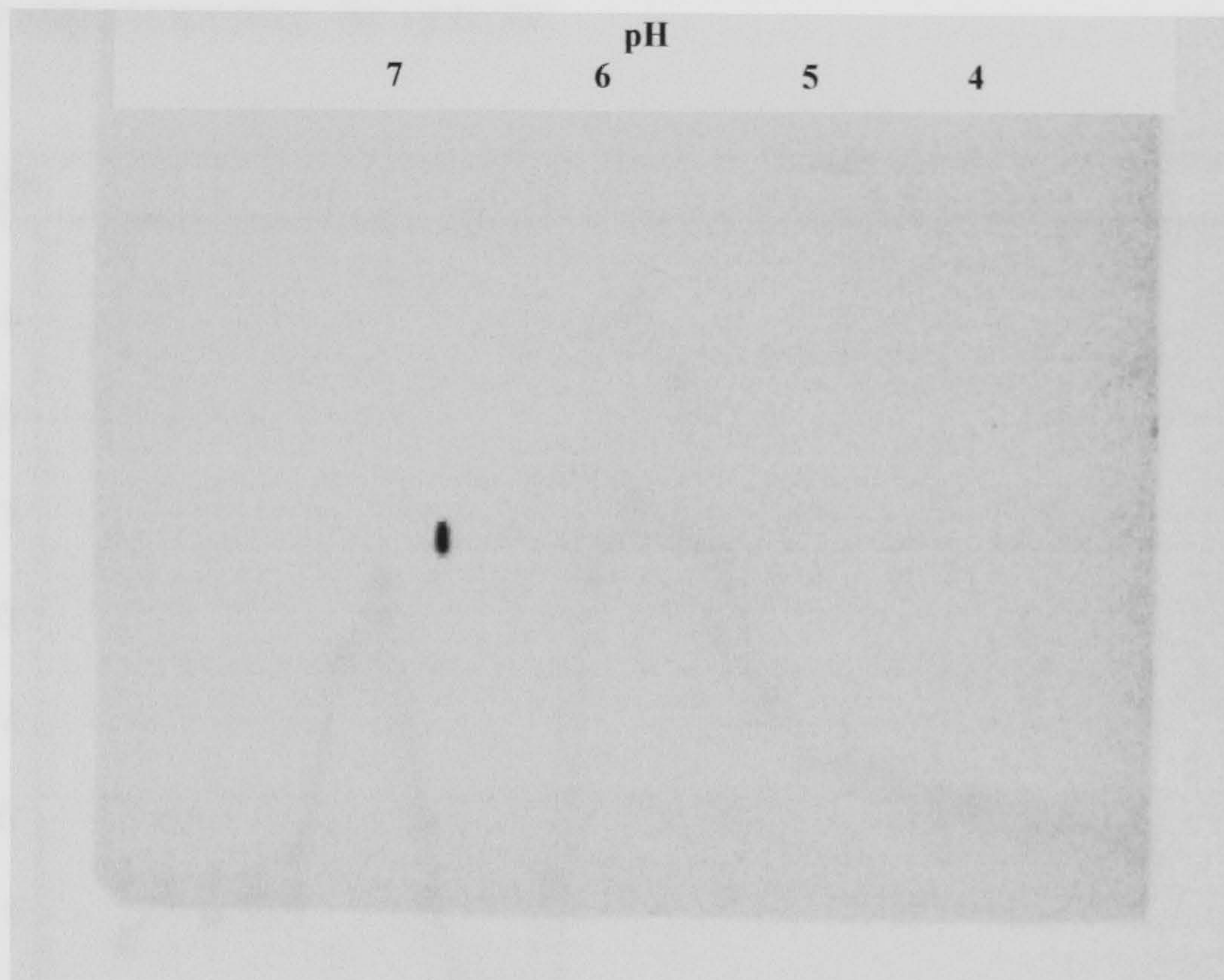

Figure 4.8.3a Photograph of a silver stained two dimensional polyacrylamide gel showing the progesterone hydroxylase cytochrome P450 of *S. roseochromogenes* has a pI of 6.6 and was highly pure.

The presence of a single silver stained band indicated a high degree of protein purity as silver staining is an extremely sensitive technique. Measurement of the position of the band in the second dimension gave a pI of 6.6 for this protein.

4.8.4 Dithionite Reduced Carbon Monoxide Difference Spectra of Partially Purified P450 from DEAE, Steroid Affinity and MIMETIC Chromatography

Co-ordinates were taken from each of the CO spectra obtained from the P450 purification stages described above and plotted as shown in Figure 4.8.4a., thereby superimposing the spectra.

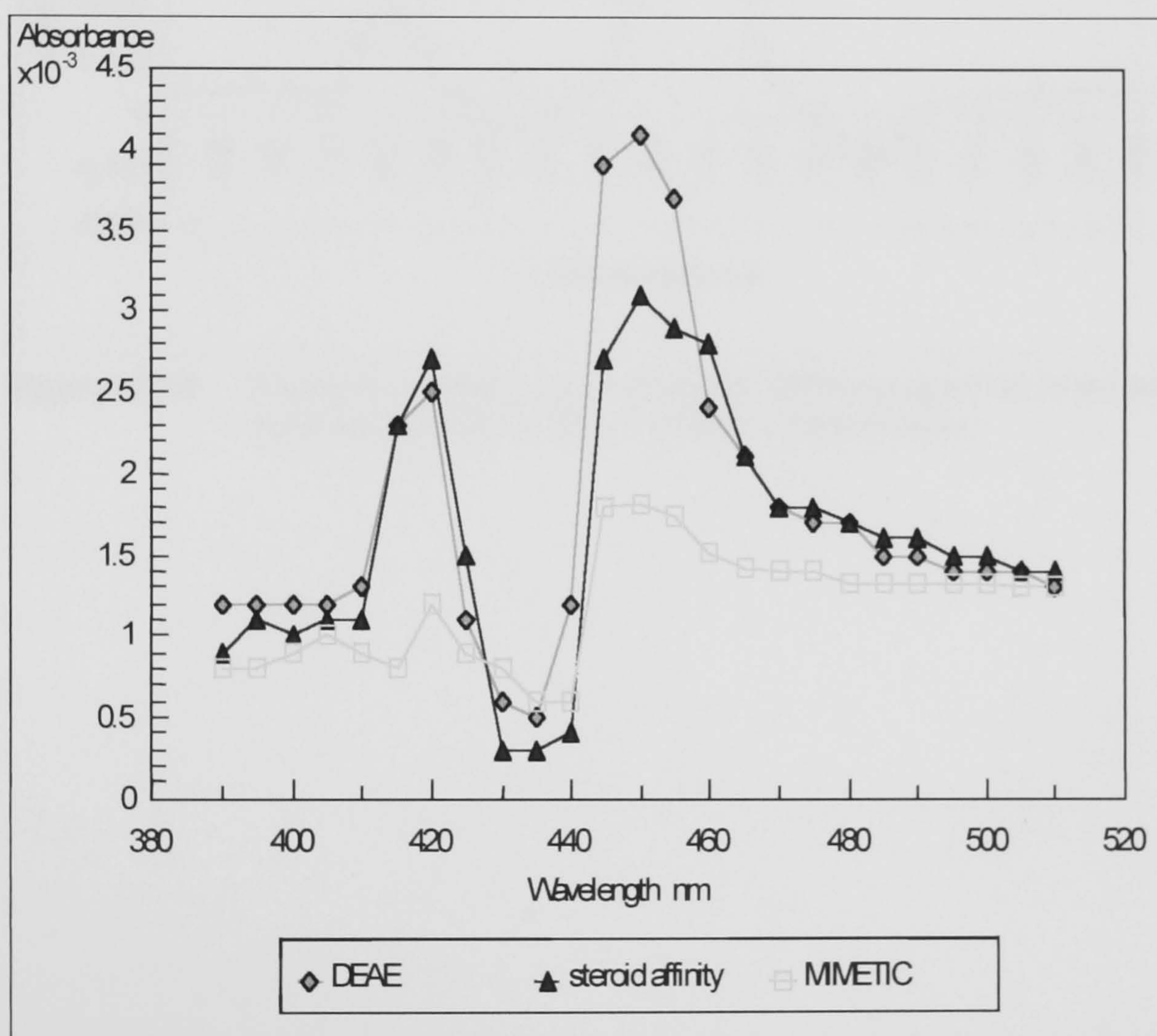

Figure 4.8.4a Reduced CO difference spectra of the three post purification stage active 2β,16α hydroxylase fractions. The profiles show a decline in P450 concentration with processing.

CO-difference spectra were examined at the three stages of purification. Spectra were not recordable for pre-DEAE preparations as these fractions were too turbid and consequently failed to give defined peaks against a general protein absorbance background. The spectra in Figure. 4.8.4a, were used to

estimate the P450 concentration at successive stages of the purification. These values are given in Table 4.8.5.

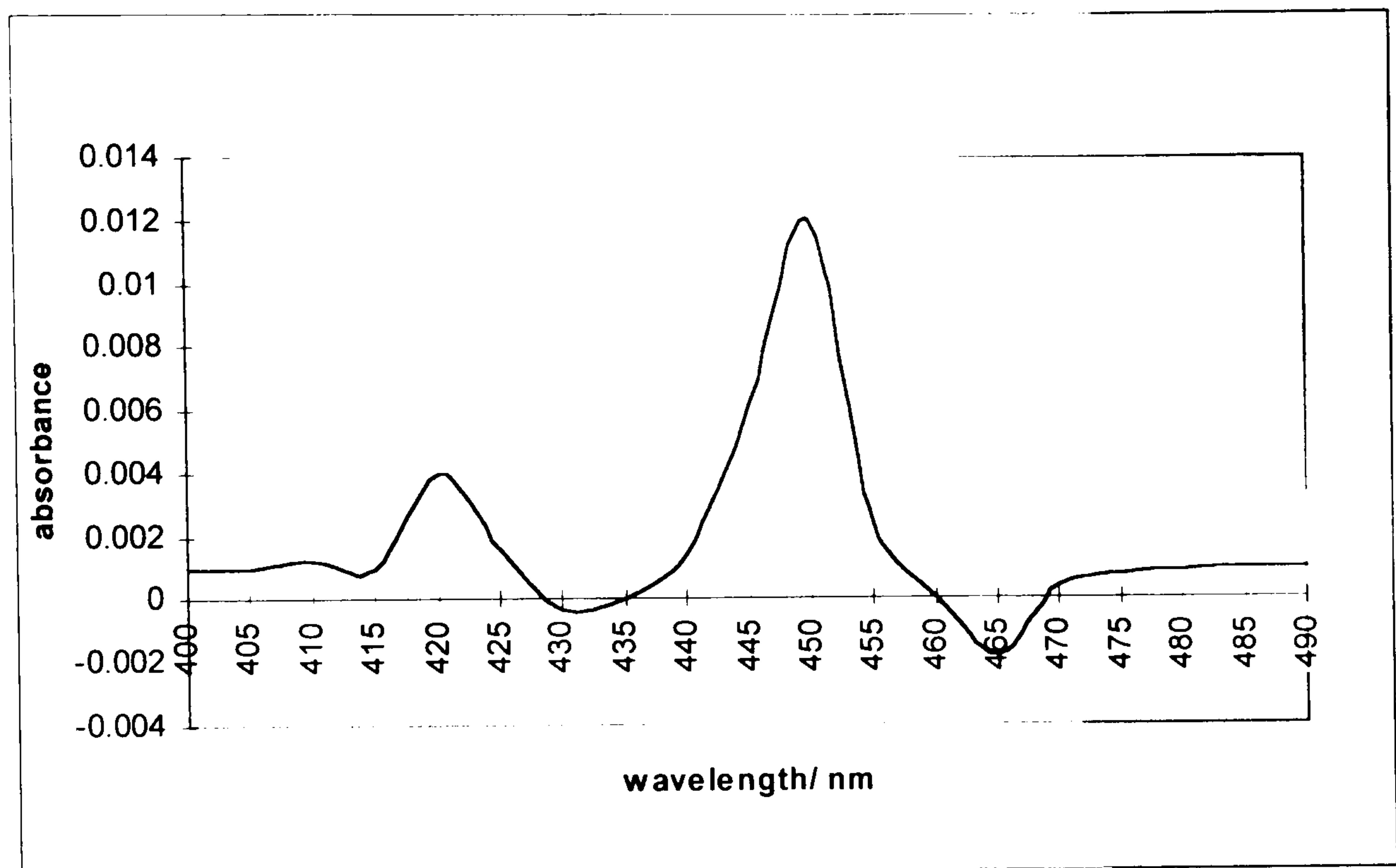

Figure 4.8.4b Dithionite reduced, carbon monoxide difference spectrum of the post 120 mM NaCl MIMETIC Blue 1 affinity column fraction.

4.8.5 Summary of *S. roseochromogenes*' 16α Progesterone Hydroxylase Cytochrome P450

The 16α hydroxylase P450 purification stages described above and their results are summarised in Table 4.8.5.

Purification Stage	Total Protein (mg)	P450 (nmol)	P450 (nmol/ mg protein)	Recovery (%)	Purification Factor
S15 extract	1,050	21.01	0.019	100	1
DEAE ion exchange (200 mM NaCl fraction)	42.59	20.02	0.47	95.29	24.7
Dialysis	42.53	18.1	0.43	86.14	22.6
Sepharose 6B 11α - progesterone affinity column (150 mM NaCl fraction)	2.8	3.3	1.18	15.71	62.1
Dialysis	2.79	2.94	1.05	13.99	55.3
MIMETIC Blue I affinity column (120 mM NaCl fraction)	0.08	0.27	3.46	1.29	182.1
Dialysis	0.08	0.25	3.21	1.2	168.9

Table 4.8.5 Purification of *S.roseochromogenes* progesterone 2β,16α hydroxylase cytochrome P450.

4.9.1 Sephadex G2000 Gel Filtration of Pure Protein to Determine the Size of the Progesterone 16α Hydroxylase

For confirmation of the 16α hydroxylase's molecular mass, gel filtration chromatography was performed as described in chapter 2.

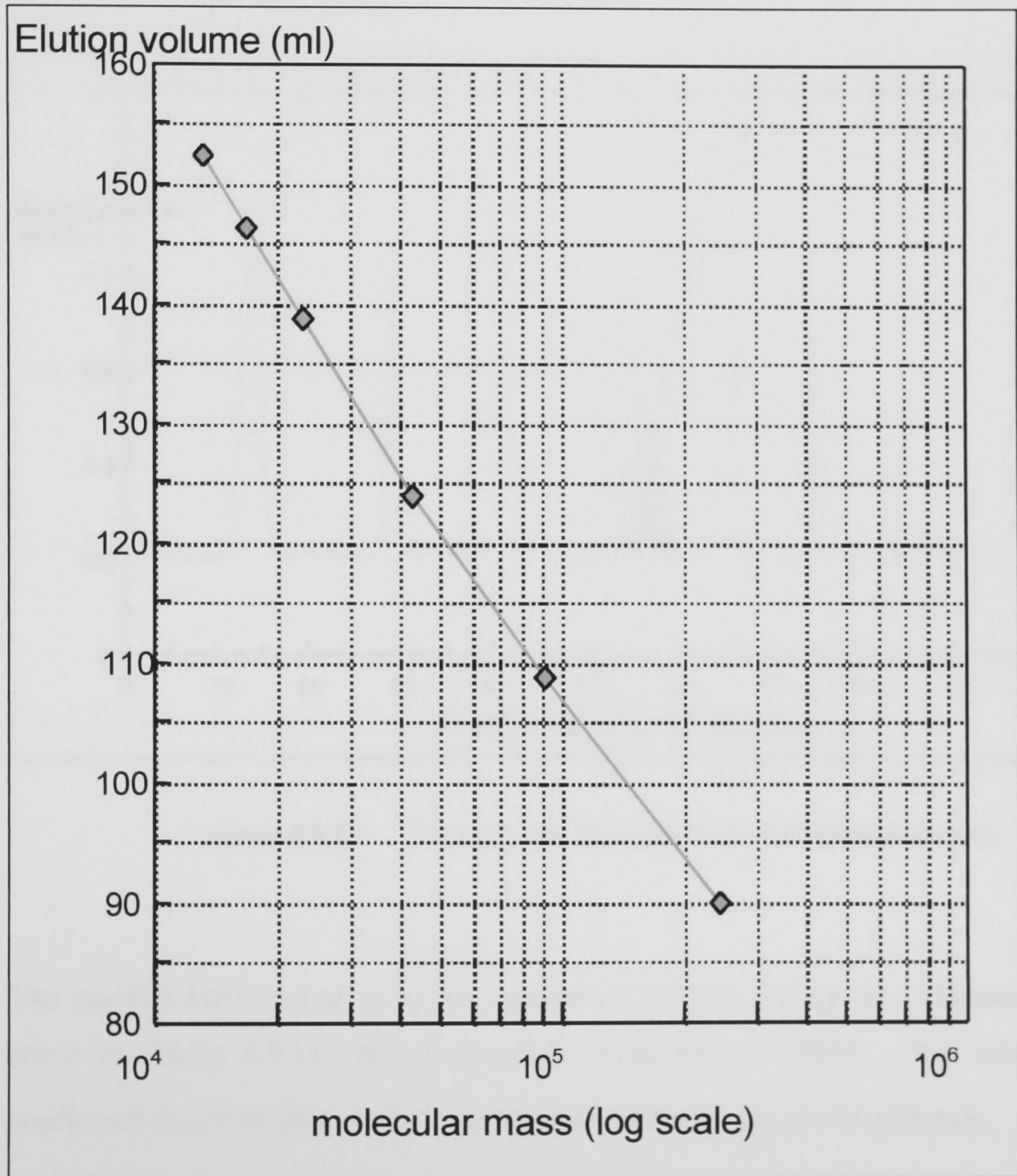

Figure 4.9.1a Calibration of the Sephadex column for molecular weight determination of progesterone 16α hydroxylase.

The standard curve in Figure 4.9.1a, shows the elution volumes from the Sephadex column of proteins in the range 13370 Da to 247500 Da. A sample

of purified steroid hydroxylase was applied to the calibrated column. The elution profile for the protein standards is shown in Figure. 4.9.1b. with the P450 sample peak superimposed. The P450 sample peak is the smaller peak marked 'S', at fractions 55-58 in Figure 4.9.1b.

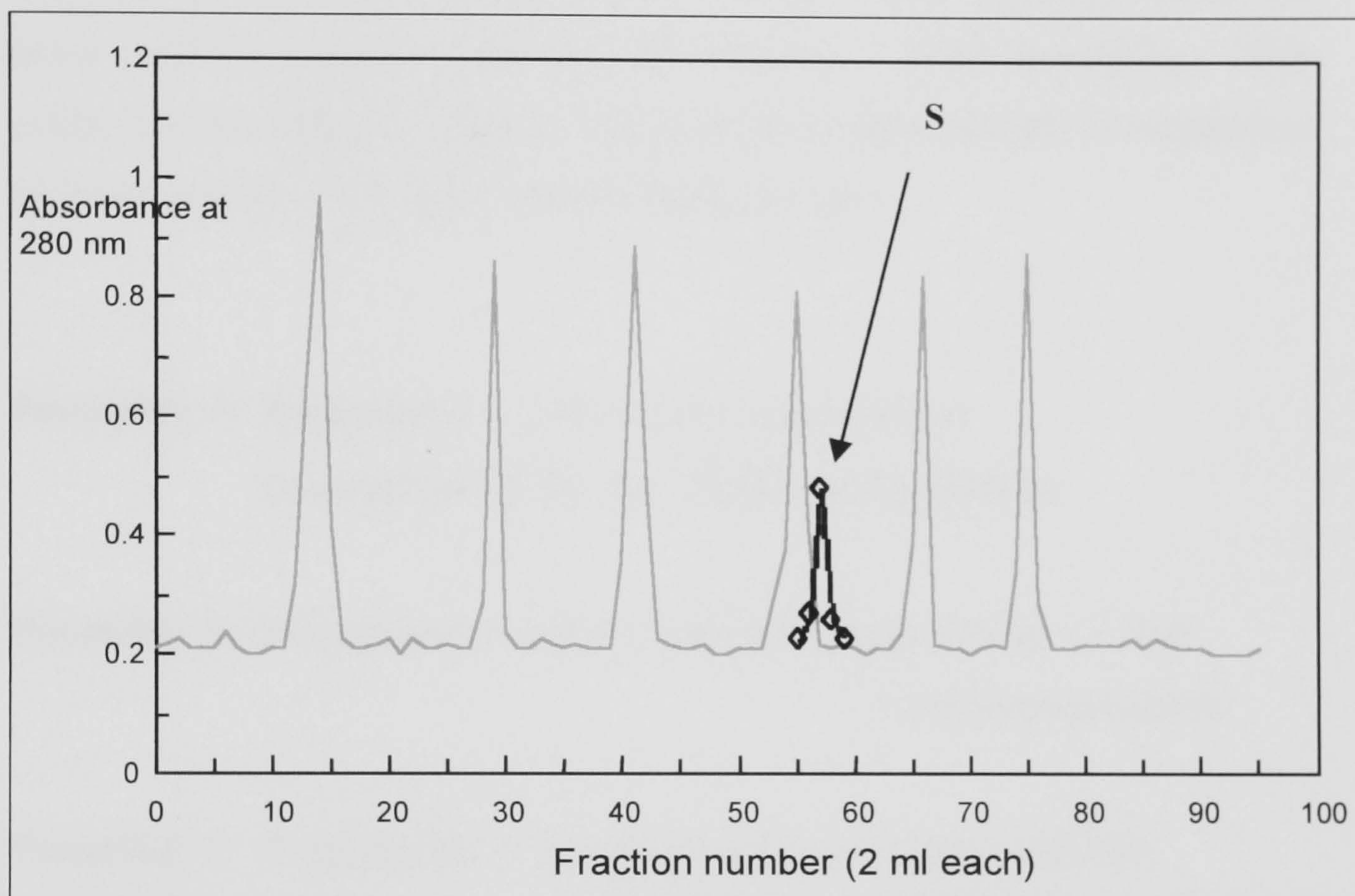

Figure 4.9.1b. Elution profile of proteins from the Sephadex column.

The purified P450 eluted in a total volume of 114 ml. Using the calibration curve in Figure 4.9.1a, this corresponds to an M_r of 63000. This value confirmed the estimation made by polyacrylamide slab gel electrophoresis.

4.9.2 Elucidation of the Pathway of 16α Monohydroxyprogesterone and 2β,16α Dihydroxyprogesterone Synthesis by *S. roseochromogenes* Progesterone 2β,16α Hydroxylase P450

There are three possible pathways by which the major and minor metabolites could be synthesised from progesterone. 2β monohydroxyprogesterone was never detected therefore removing the case for a fourth possibility. This evidence is described in chapter 5 along with the identification of metabolites by NMR analysis. The three possible pathways are :

Possibility 1: Progesterone → 16α-hydroxyprogesterone
Progesterone → 2β,16α-dihydroxyprogesterone

Possibility 2: Progesterone → 2β,16α-dihydroxyprogesterone → 16α-hydroxyprogesterone

Possibility 3: Progesterone → 16α-hydroxyprogesterone → 2β,16α-dihydroxyprogesterone

[4-^{14}C] Progesterone was incubated with purified progesterone 16α hydroxylase P450 to produce [4-^{14}C] 16α-hydroxyprogesterone, which was separated by TLC. 69 nM P450 was incubated for 5h with the radiolabelled 16α monohydroxylated product plus unlabelled progesterone each at 2mM. The 2β,16α-dihydroxyprogesterone contained the majority of the radiolabelled material. This result supports possibility 3 above, 16α monohydroxy dependent 2β hydroxylation of progesterone, as the pathway of biotransformation.

4.9.3 Reconstitution of the Progesterone 16α Hydroxylase P450 Hydroxylase System

Reconstitution of the 16α hydroxylase P450 was attempted with a view to determining the proteins required for restoration of catalytic competence. As exemplified in many other cases of P450 activity such as the system from *Pseudomonas putida* (Sligar *et al.*, 1974 and Gunsalus & Wagner, 1978), *Streptomyces carbophilus* (Matsuoka *et al.*, 1989), *Streptomyces griseus* (Trower *et al.*, 1989), *Streptomyces griseolus* (O'Keefe *et al.*, 1991) also the microsomal example of *Rhizopus nigricans* (Breskvar, 1983). Pure progesterone 16α hydroxylase P450, was combined with different post chromatographic column eluates to determine whether NADH dependent hydroxylation of progesterone could be supported naturally i.e: could catalytic competence of the P450 be restored by ancillary proteins replacing the unnatural peroxide shunt pathway. It was found that the first DEAE column peak eluate, by itself, restored catalytic competence to the hydroxylase. Since this fraction was not capable of hydroxylation alone I concluded that this fraction must contain one or more factors responsible for the transfer of electrons to the P450. This aspect of the work is developed further in chapter 6.

4.10 DISCUSSION

In chapter 3, 16α-monohydroxy- and 2β,16α-dihydroxyprogesterone were identified as the transformation metabolites produced by *S. roseochromogenes* cytochrome P450 during incubation with exogenous progesterone. Reported here in chapter 4 is the purification to homogeneity of the cytochrome P450 responsible for *in vitro* progesterone 2β,16α- hydroxylation.

Determination of P450 Concentration in Post MIMETIC Pure Protein Fractions

The purification table (Table 4.8) in section 4.8, shows a loss in the amount of P450 from one stage to the next as expected but more importantly there is a loss in P450 amount during dialysis. This is attributable to the denaturation of the protein from P450 to P420. This is why the spectra in 4.8.4a show an increasing P420 peak and a decreasing P450 peak at subsequent stages of the purification. Purified P450 appears to be at a higher concentration when determined by the dye-binding method, than when determined spectrophotometrically.

This thesis is the first report to identify unequivocally cytochrome P450 as a steroid hydroxylase enzyme in the *Streptomyces* genus.

P450 mechanisms of oxidation for substrates seems to be very similar. Evidence has accumulated that the crystal structure of the soluble P450 101 can be used as a guide for the elucidation of the active-site structure of membrane-bound cytochromes P450 (Koymans *et al.*,1993 [1]). Crystallisation of two other P450s is reported : $P450_{terp}$ & P450 102 ($P450_{BM3}$). P450 102 is catalytically self-sufficient, requiring only NADPH

and molecular O_2 to catalyse the hydroxylation of long-chained fatty acids. The enzyme contains haem, FAD and FMN in an equimolar ratio, with the haem located at the amino terminal domain (haem domain) and the FAD/FMN at the carboxyl domain (flavin domain). Thus, P450 102 resembles microsomal P450s which require an FAD/FMN-containing reductase for activity. Sequence comparisons with other P450s indicate that the haem domain of P450 102 more closely resembles membrane-bound eukaryotic P450s from family 4 than it does P450 101 ($P450_{cam}$). Therefore, P450 102 appears to be an excellent model for eukaryotic P450s with the distinct advantage that the enzyme is soluble and can be produced in large quantities in recombinant systems

Steroidal products were identified in chapter 3 by 1-D ^1H NMR. 2β mono hydroxyprogesterone was not found to be present after any incubation. Polyacrylamide gel electrophoresis of the pure protein showed that the P450 had an M_r of 63000. This was confirmed by Sephadex G2000 molecular sieve chromatography. Isoelectric focusing of the purified protein showed it to have a pI of 6.6. Bubbling CO through a solution of dithionite reduced P450 solution gave a characteristic absorption peak at 450 nm.
Cytochromes P450 (P450) are haem containing (Omura & Sato, 1962) monooxygenase proteins found in eukaryotes and in some prokaryotes (Lindenmayer & Smith, 1964, Smith *et al.*, 1992). In all cases they form part of a multicomponent enzyme system which catalyses the reductive cleavage of molecular oxygen (Sariaslani, 1991). This results in the oxygenation of substrate and the reduction of the other atom of oxygen to water. The multicomponent system in prokaryotes comprises a ferredoxin reductase and ferredoxin, which transfer electrons from NADH to the P450. This results in the reduction of the Fe^{3+} of the haem moiety to Fe^{2+}, thus allowing the binding of O_2 to give $Fe^{3+}.O^{2-}$ (White & Coon, 1980). A complete amino

acid sequence was not available for the P450 in question here. So what is the situation in *Streptomyces roseochromogenes* ?

It is interesting to note the reported purification of a P450 from a red alga. Where the first stage of purification was DEAE cellulose ion exchange chromatography as described here. Reconstitution was attempted as described here in section 4.9.3 and the approach used was very similar to that reported here in terms of the replacement of P450 with cytochrome c. Oxygenase activity was resolved in to three fractions, from extracts of the unicellular red alga *Cyanidium caldarium* by serial column chromatography through DEAE-cellulose, Reactive Blue 2-Sepharose and 2',5'-ADP-agarose. *In vitro* oxygenase activity required the presence of all three fractions, plus substrate, O_2, reduced pyridine nucleotide and another reductant. Fraction I has a visible absorption spectrum similar to that of ferredoxin and was bleached by dithionite reduction. Fraction I was replaceable by commercially available ferredoxin. Fraction III contained ferredoxin-linked cytochrome c reductase activity and could be partially replaced by spinach ferredoxin-NADP+ oxidoreductase. None of the three cell fractions could be replaced by bovine spleen microsomal haem oxygenase or NADPH-cytochrome P450 reductase (Cornejo & Beale, 1988). The rationale for the elucidation of the system in *S. roseochromogenes* commences in chapter 6.

Chapter 5

Spectrophotometric and Kinetic Analysis of *Streptomyces roseochromogenes* 2β,16α Hydroxylase Multicomponent Cytochrome P450 System

5.1 INTRODUCTION

Inhibitor and substrate induced spectral changes of characteristic reduced cytochrome P450 spectra were first observed by Remmer *et al.* in 1966. This work was carried out on hepatic microsomal P450s.

Spectral changes due to inhibitor and substrate binding are now recognised as characteristics of all P450s and can be used to determine the relative extent of interaction between ligand and protein (Jefcoate, 1978). This has recently been exemplified by the production of P450 type I substrate binding spectra from the fungus *Pleurotus pulmonarius*, in order to determine the substrate affinity of benzo[a]pyrene, where a K_s of 66 μM was obtained (Maspahy *et al.*, 1999).

Correlation has been made between the 'type', of spectrum and the spin state, or change in spin state of the Fe^{3+} ion of the P450s haem moiety. However, it is not always possible to predict the 'type' of spectrum a particular ligand will give rise to. Some ligands such as ketoconazole stabilise the haem moiety by N-co-ordination to the 6th ligand of the Fe^{3+} producing ferric iron. This would inhibit the electron accepting capability of P450 necessary to complete the catalytic cycle. This bonding replaces the loosely co-ordinated 6th ligand which is usually water (Malmström 1982).

Collection of kinetic parameters such as ligand binding and modelling of steroid biotransformation based upon data gathered through spectrophotometric analysis is vital in terms of the development of pharmaceutical steroids and other products with therapeutic activity, such as antifungal agents (Lamb *et al.*, 1999). The kinetics of various aspects of such reactions are of great importance, such as the dissolution kinetics of steroids (Chen & Wey 1990). Also the fact that enzymatic processes are usually conducted in the aqueous environment means that their kinetics may be dictated by solubility of substrate or ligand.

Binding Spectra of Partially Purified P450

It is a spectrophotometric property of cytochromes P450 that gives these enzymes their name. Reduced cytochrome P450, when exposed to carbon monoxide, gives a characteristic absorption maximum around 450 nm compared to non-treated reduced P450. This phenomenon was identified by Omura & Sato in 1963 while looking into the spectral properties of cytochrome b_5, from rabbit liver.

5.2 MATERIALS

Materials were as previously described in chapter 2.

5.3 METHODS

5.3.1 Production of Reduced Cytochrome P450 Carbon Monoxide Difference Spectra and Determination of Cytochrome P450 Concentration

The cytochrome P450 concentrations of purification fractions were measured from difference spectra in a split beam Pye Unicam PU8800 spectrophotometer. Equal volumes of a P450-containing fraction were pipetted into two matched glass cuvettes. A few grains of solid sodium dithionite were added to both fractions to reduce the P450. The cuvettes were gently inverted until the dithionite had dissolved, after which they were simultaneously scanned between 390 to 520 nm to obtain a zero baseline. Carbon monoxide was bubbled for 1.5 min into the test cuvette at a rate of 1 bubble/sec. The CO difference spectrum was then obtained by re-scanning between 390 to 520 nm.

To determine the concentration of cytochrome P450 in a particular fraction, the absorbance of that fraction was measured from the difference spectrum at 450 and 490 nm. The values obtained were applied to the formula:

$$\text{P450 concentration} = \frac{A450nm - A490nm}{\varepsilon_o}$$

Where ε_o is the millimolar absorbance coefficient of $P450_{450\text{-}490}$ and is 91 cm^{-1} mM^{-1} (Omura & Sato 1964[2]).

5.3.2. CO Difference Spectroscopy for *S. roseochromogenes* Progesterone 2β,16α Hydroxylase

As the degree or extent of P450 - CO binding interaction is used to determine the amount of P450 present in a sample, the CO difference spectra for the P450 of *S. roseochromogenes* were examined for different *in vitro* conditions.

To avoid repetition, the resulting spectra are shown in the result section along with the conditions under which the spectrum was obtained. This was carried out in order to attain optimum *in vitro* conditions for the purification of the progesterone 2β,16α hydroxylase enzyme.

5.3.3 Measurement of Substrate Binding with P450 by Optical Difference Spectroscopy

S. roseochromogenes P450 at varying concentrations was placed into matched silica cuvettes as described above and a baseline measured from 200 - 600 nm. Varying concentrations of progesterone from 0 - 5.0 pmol in 1.0 ml of 0.1 phosphate buffer were added to the test cuvette with an equal volume of buffer simultaneously added to the reference cuvette. The P450 concentration was 3.0

pmol. The spectra were then rescanned. The chart record paper was reversed to its original starting position prior to recording each assay. This was to permit superimposition of the spectra for the purposes of comparison. Which ever P450 sample was under investigation, spectra were recorded at increasing substrate concentration until a saturation effect was observed.

Spectral changes observed at ligand concentrations much higher than P450 concentration are of doubtful value because P450 may be affected by changes in structure by substrate such as the effect of camphor binding with $P450_{cam}$ (Tsai *et al.*, 1970) (and Jefcoate, 1978).

5.3.4 Measurement of Inhibitor Binding with P450 by Optical Difference Spectroscopy

This was performed in the same way as for substrate binding spectroscopy. In addition to this, assays which had been exposed to ketoconazole were then exposed to CO in the same way as described above to demonstrate the practically irreversible binding of ketoconazole to P450.

5.3.5 Determination of Ligand-P450 Dissociation Constant, K_d for *S. roseochromogenes* Progesterone 2β,16α Hydroxylase

To determine the ligand-P450 K_d , (i) absolute & (ii) difference spectra were performed and data plotted versus concentration of substrate. Substrates give Type I spectra. Pyridines, amines and azoles such as ketoconazole give Type II spectra generally. These compounds bind to the oxidised form of P450.

5.3.6 Substrate and Inhibitor Binding Spectroscopy

$$r/c = K_d n - K_d r \qquad \text{....equ. 5a.}$$

Where r = no. of bound ligands, c = no. of free ligands, K_d = dissociation constant, n = no. of binding sites.

Although a Lineweaver-Burke plot could be used to determine the binding constant, equation 5a, the Scatchard equation takes account of the number of binding sites.

5.3.7 Effect of Ligand Structure on Binding to P450

P450 inhibitors are usually classified according to the modification that they produce on the maximum absorption of the Soret band. Most compounds oxidised by P450 produce a blue shift of the maximum of the Soret absorption, stabilising the high-spin form, whereas compounds having donor groups capable of binding to the metal move the Soret absorption to the red region, shifting the spin equilibrium to low spin.

X-ray structures have been reported for large numbers of adducts with non-natural substrates, for which different binding modes have been observed. The size, of the ligand molecule and its ability to interact with the protein residues determine the affinity. The nature, the size, and the steric crowding of the ligand determine also the displacement or the presence of the axial solvent ligand and its protonation state. The latter properties have been proposed to determine the spin state (low or high spin) of the iron (III) in the adducts.

5.4 RESULTS

5.4.1 CO Difference Spectroscopy and Determination of *S. roseochromogenes* 2β,16α Progesterone Hydroxylase P450 Concentrations

When CO was used for the determination of P450 presence, where it was passed through purification fractions of 1.0 ml at a rate of 1 bubble per sec. The profile below in Figure 5.4.1a was used to determine the P450 concentration in post MIMETIC Blue I affinity chromatography samples in the purification protocol for P450 described in chapter 4.

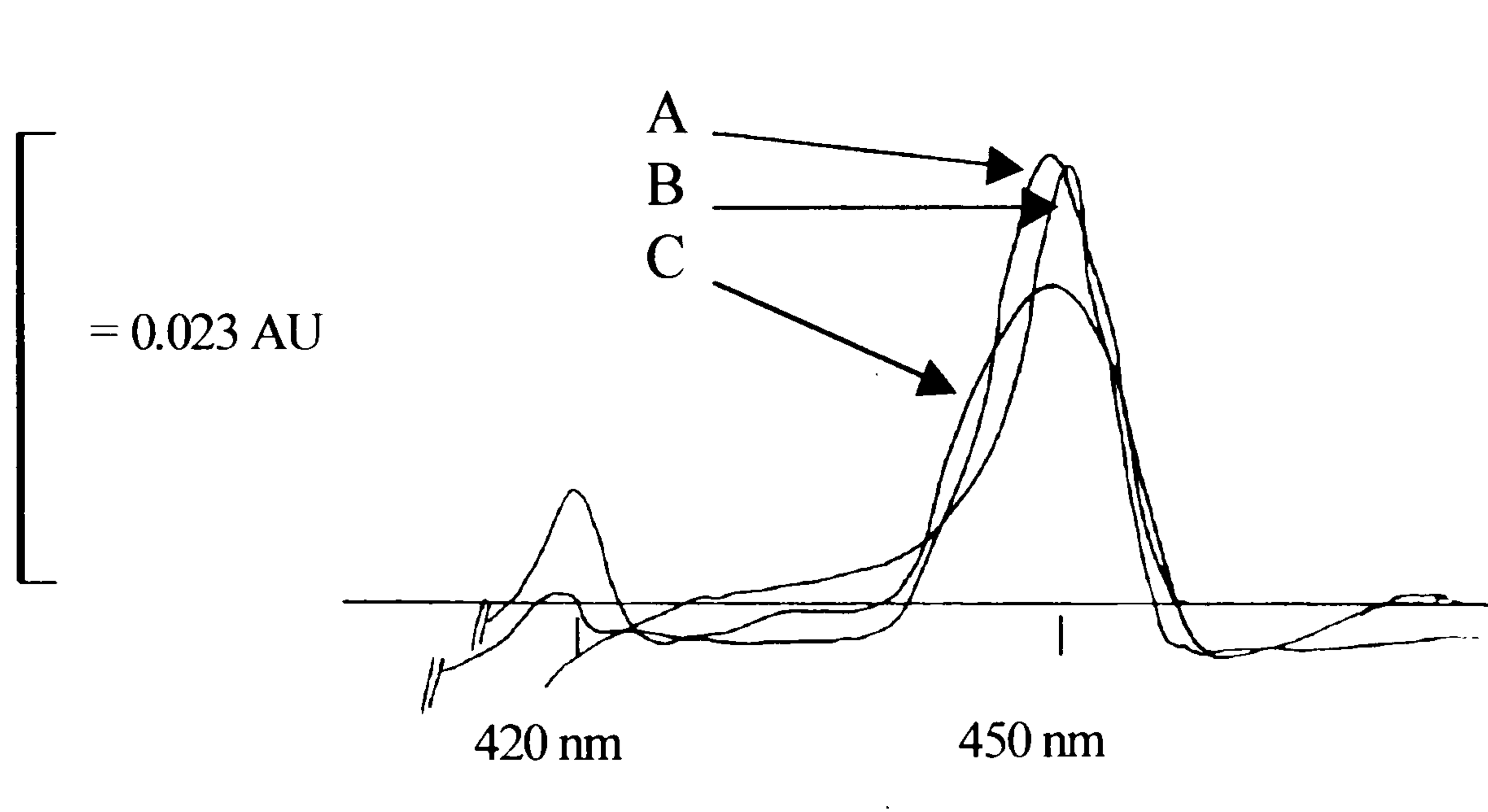

Figure 5.4.1a CO difference spectra prepared as described above. The P450 concentration was 0.25 μM and spectrum **A** represents the profile after 2 minutes of bubbling CO, at 1 bubble per second, through a 1.0 ml solution of post MIMETIC Blue I eluate in the P450 purification protocol. Profile **B** represents the same sample after 5 min of bubbling CO through the sample. Profile **C** represents an identical sample treated with CO but with the addition of ketoconazole to 0.1 μM. The 450 nm peak is shortened. Where AU = absorbance units.

5.4.2 Substrate Binding Difference Spectroscopy

With progesterone as substrate, Figure.5.4.2a was obtained with the component concentrations given in the legend. The P450 exhibited a typical set of type I substrate binding spectra with maxima at 384 nm, minima at 420 nm and an isosbestic point at 401 nm (Iba *et al.*, 1993).

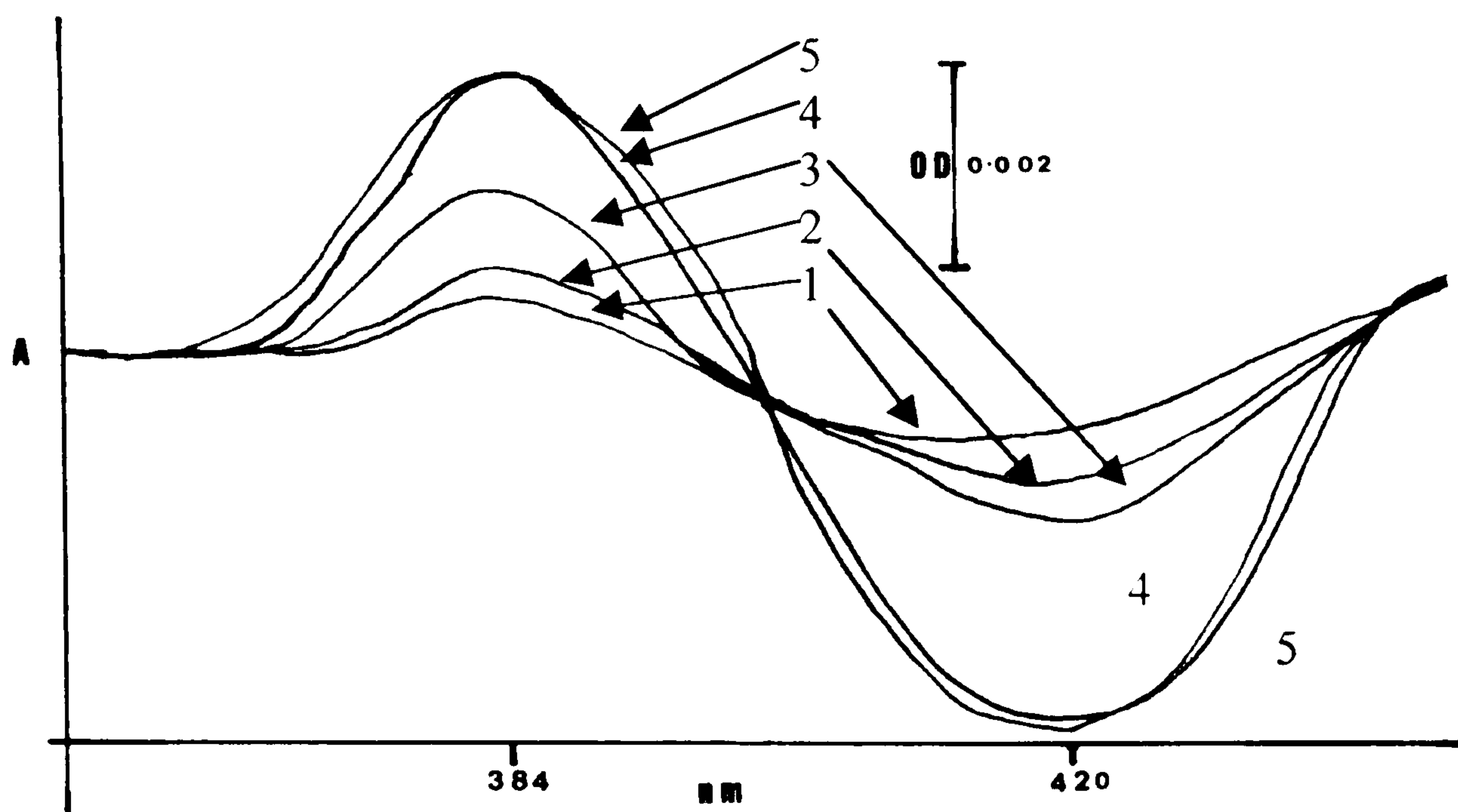

Figure 5.4.2a. *S. roseochromogenes* cytochrome P450 progesterone substrate binding spectra.

The amount of cytochrome P450 was 3.0 pmol in all incubations. Curve 1, 0.1 pmol progesterone; curve 2, 0.3 pmol progesterone; curve 3, 0.5 pmol progesterone; curve 4, 1.0 pmol progesterone and curve 5, 1.4 pmol progesterone. The assay volume was 1.0 ml in each case.

Data collected from substrate binding were used in the following sections for the determination of K_d values.

5.4.3 Determination of the Dissociation Constant (K_d) for the *S. roseochromogenes* Progesterone 2β,16α Hydroxylase P450 from Substrate Binding

According to Scatchard analysis as described in the introduction to this chapter, a K_d for progesterone of 1.95×10^{-8} M was obtained for this P450. The gradient of the chart in Figure 5.4.3a, gave the K_d value for progesterone binding.

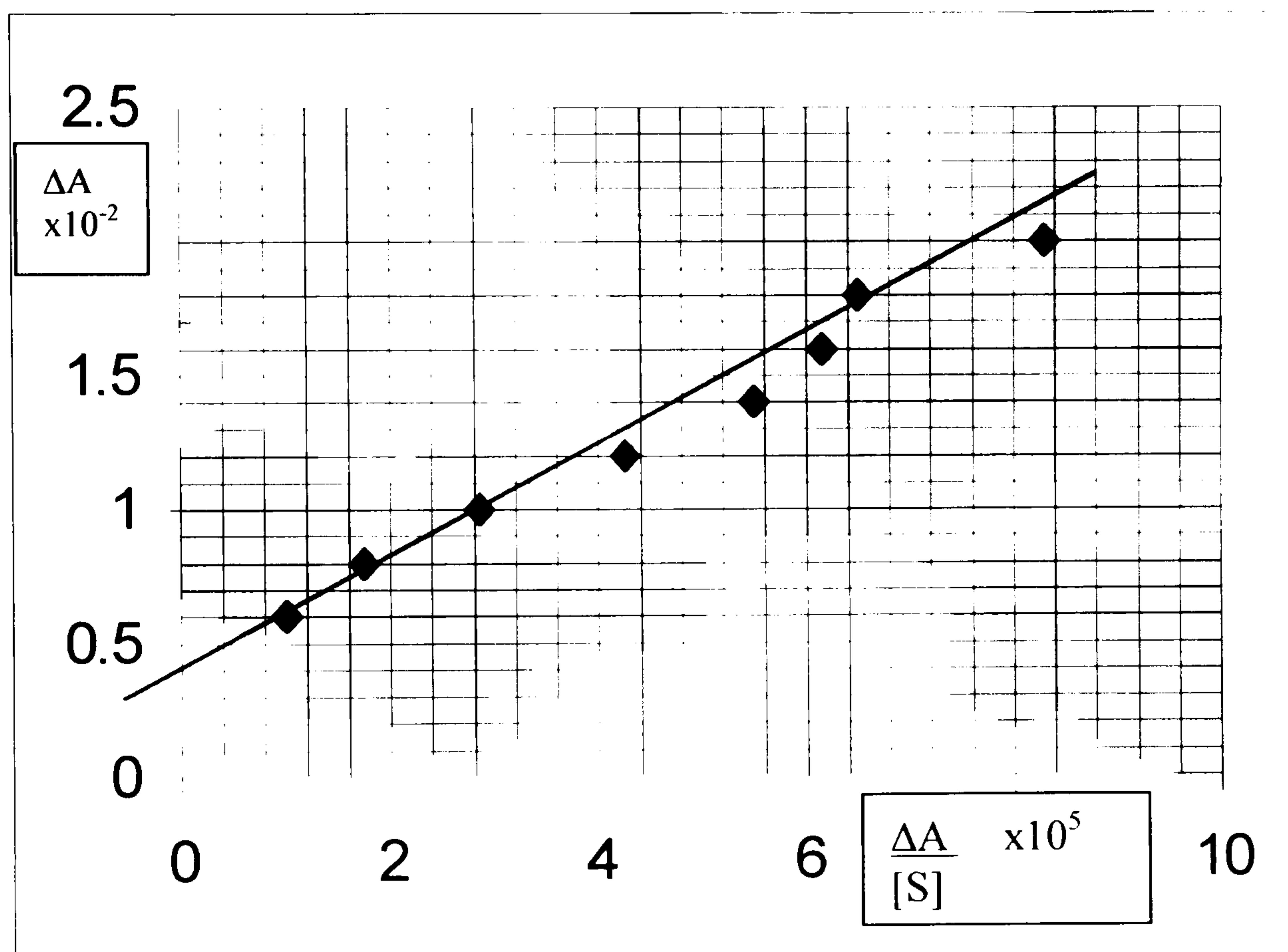

Figure 5.4.3a Chart showing ΔA vs ΔA/[S] for the binding of the substrate progesterone with *S. roseochromogenes* 2β,16α hydroxylase cytochrome P450. The gradient shown, gives a value for K_d of 1.95×10^{-8} M.

5.4.4 Determination of the Dissociation Constant (K_d) for the *S. roseochromogenes* Progesterone 2β,16α Hydroxylase P450 from Inhibitor Binding

As in the previous section, according to Scatchard analysis a K_d for ketoconazole of 1.36×10^{-7} M was obtained for this P450. The gradient of the chart in Figure 5.4.4a, gave the K_d value for ketoconazole binding.

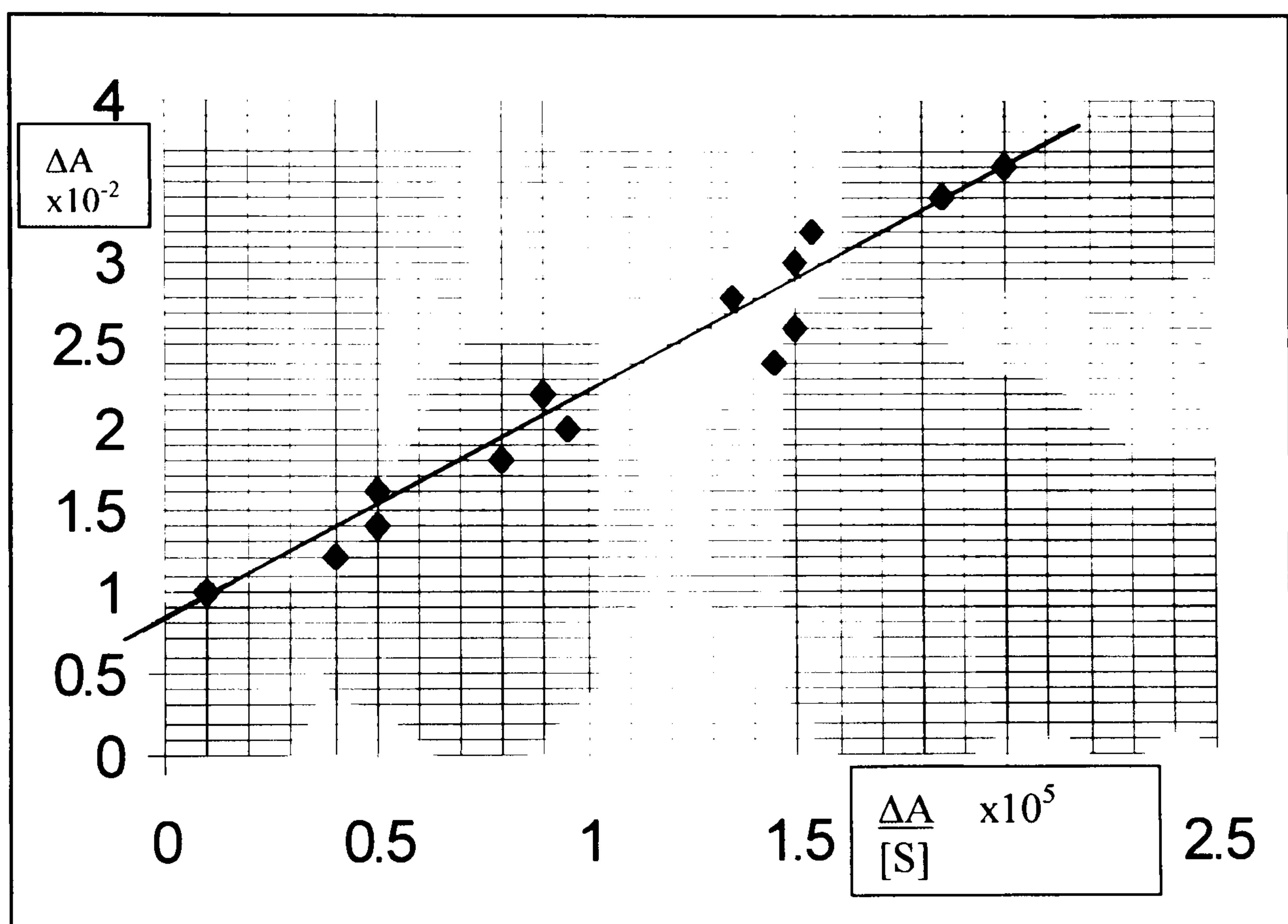

Figure 5.4.4a Chart showing ΔA vs ΔA/[S] for the binding of the inhibitor ketoconazole with *S. roseochromogenes* 2β,16α hydroxylase cytochrome P450. The gradient shown, gives a value for K_d of 1.36×10^{-7} M.

This K_d value must be greater than that for CO binding since bubbling CO through ketoconazole - saturated P450 did not remove the ketoconazole and no CO binding spectra were obtainable under such circumstances.

5.4.5 The *S. roseochromogenes* Progesterone 2β,16α Hydroxylase P450 Inhibitor Binding Spectra

Solutions of purified progesterone 2β,16α hydroxylase were set up as described above at 3.0 pmol in each 1.0 ml incubation, to examine the effect of ketoconazole concentration on the inhibitor binding spectra. The resulting spectra are shown in Figure 5.4.5a.

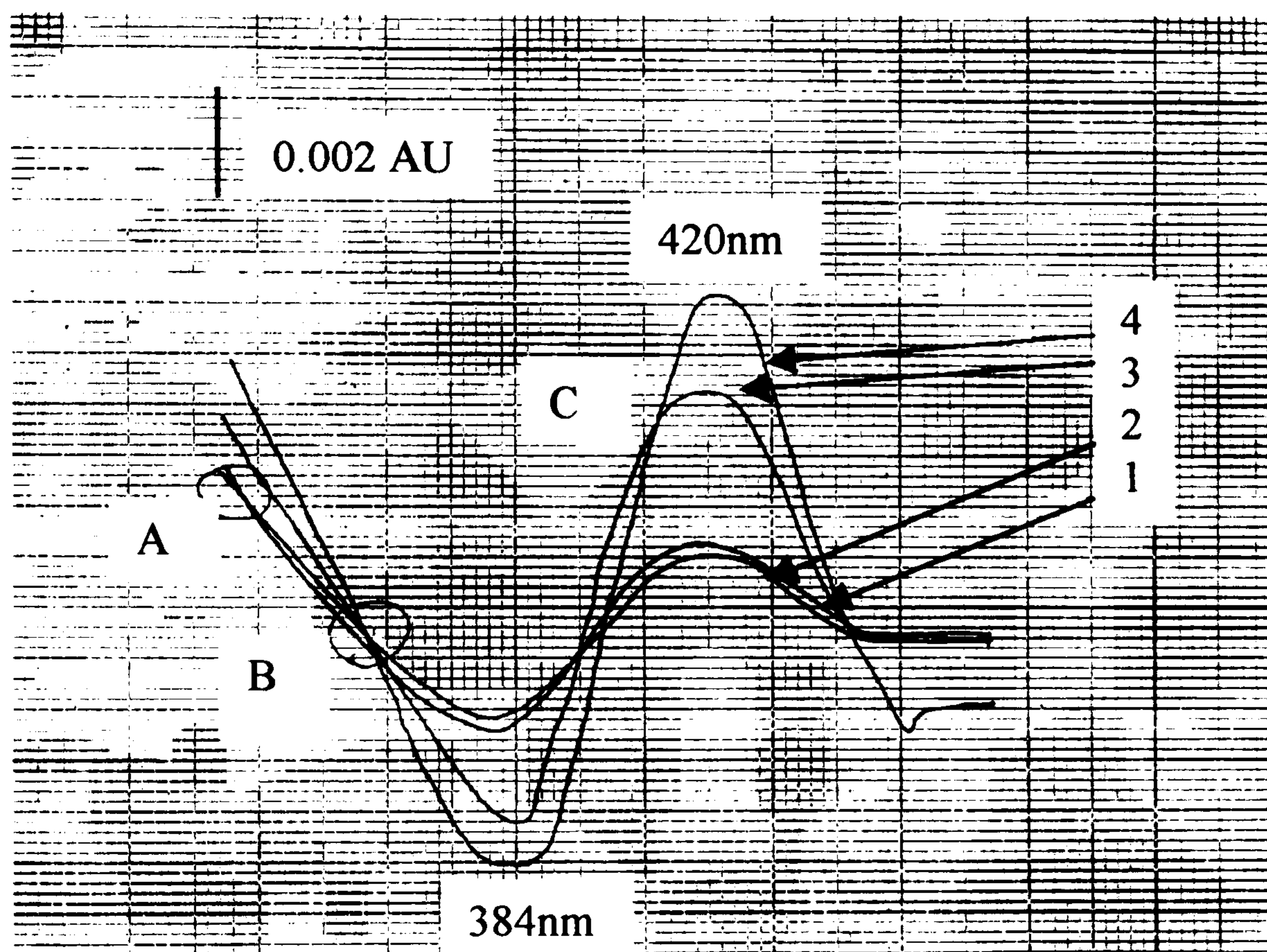

Figure 5.4.5a Ketoconazole binding spectra for the *S. roseochromogenes* cytochrome P450 where AU = absorbance units. The amount of progesterone 2β,16α hydroxylase was 3.0 pmol and ketoconazole amount was: spectrum 1, 0.1 pmol; spectrum 2, 0.1 pmol; spectrum 3, 0.3 pmol; spectrum 4, 0.5 pmol.

Points A,B and C in Figure 5.4.5a, where the spectra cross do not represent isosbestic points and the reason for the curves crossing at these points is unknown. These data gathered for ketoconazole binding did not allow for accurate Scatchard analysis.

5.4.6 Examination of the Kinetic Parameters of Models of Progesterone Biotransformation by *Streptomyces roseochromogenes*

Taking the Lineweaver-Burk equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{..........equ.5b.}$$

A Lineweaver - Burk plot and the values for V_{max}. and K_d , were obtained for each of the theoretical models for progesterone biotransformation, described below, where **L-B** = Lineweaver-Burk plot and **RP** = the roseoredoxin phenomenon, described in detail in chapter 7. When *S. roseochromogenes* cells were grown in the presence of progesterone and the roseoredoxin component purified from cells, it produced an altered product ratio when incubated in reconstitution assays. Namely the proportion of 2β,16α hydroxyprogesterone to 16α hydroxyprogesterone increased in these incubations compared to counterpart reconstitution assays where the roseoredoxin was taken from cells which had not previously been pre exposed to progesterone. Therefore each model described below was examined in two ways. One with roseoredoxin taken from progesterone pre incubated cells and one where roseoredoxin was taken from non progesterone pre incubated cells.
The theoretical models were examined using experimental data obtained.

Model 1:
Where the two transformation metabolites are treated as one, i.e. total transformation is considered, rather than the individual metabolites produced. The products in this case are therefore the 2β,16α hydroxyprogesterone plus the 16α hydroxyprogesterone.

L-B plot: The plot in figure 5.4.6a. shows the L-B plots as line 5 which is actually two lines. Table 5.1 shows this as the first and second rows of data in

the table. The kinetic parameters for this model are the same in the presence and absence of the roseoredoxin phenomenon. Theoretically this is because the overall production of metabolite is no different in either form of the model (+ or - RP) but the metabolite ratio is altered, a factor not considered in this model.

Model 2:

Where the two transformation metabolites are considered as separate reactions and as two alternative routes of transformation and hence analysed separately. The two transformation products are considered but the 16α hydroxyprogesterone is taken as the substrate for a second phase bioconversion to 2β,16α dihydroxyprogesterone i.e:

Step 1: progesterone ⇒ 16α hydroxyprogesterone

Step 2: 16α hydroxyprogesterone ⇒ 2β,16α dihydroxyprogesterone

L-B plot: Line 4 in Figure 5.4.6a is very close to line 5. The kinetic parameters of the production of 16α hydroxyprogesterone in the absence of RP, are similar to those for the overall biotransformation of progesterone given by model 1 (line 5). It is the second phase bioconversion to 2β,16α dihydroxyprogesterone in the absence of RP which give rise to line 1.

For the second phase, a greater proportion of the metabolites are dihydroxylated when the V_{max} is increased and the K_m is decreased (lines 1 and 2, Figure 5.4.6a.) as appears to be the case in the presence of RP.

Model ⇓	V_{max} (µmol min^{-1})	$1/V_{max}$ (µmol min)	$V_{max}/2$ (µmol min^{-1})	[S] at $V_{max}/2$ (mM)	1/[S] at $V_{max}/2$ (mM^{-1})	K_m (mM)	K_{cat}/K_m ($sec^{-1}M^{-1}$)
1 in the absence of RP	0.015	67	0.0075	0.6	1.67	0.6	1.53×10^5
1 in the presence of RP	0.015	67	0.0075	0.6	1.67	0.6	1.78×10^5
2 (step 1) in the absence of RP	0.0135	74	0.0068	0.5	2	0.5	1.50×10^5
2 (step 2) in the absence of RP	0.0015	667	0.0008	1.5	0.67	1.5	1.11×10^4
2 (step 1) in the presence of RP	0.0111	90	0.0056	0.7	1.43	0.7	8.93×10^4
2 (step 2) in the presence of RP	0.004	253	0.002	0.85	1.18	0.85	5.22×10^4

Table 5.1 Kinetic parameters of the various model components of the *in vitro* reconstituted biotransformation system of progesterone by *S. roseochromogenes* according to the models described. At $V_{max}/2$, K_m = [S] and -1/[S] = $-1/K_m$; the 'x' intercept in Figure 5.4.6a.
RP = 'roseoredoxin phenomenon' (see text for definition).

K_{cat}/K_m , Table 5.1, column 8, is a measure of the enzymatic efficiency and is different for each component part of the models described above.

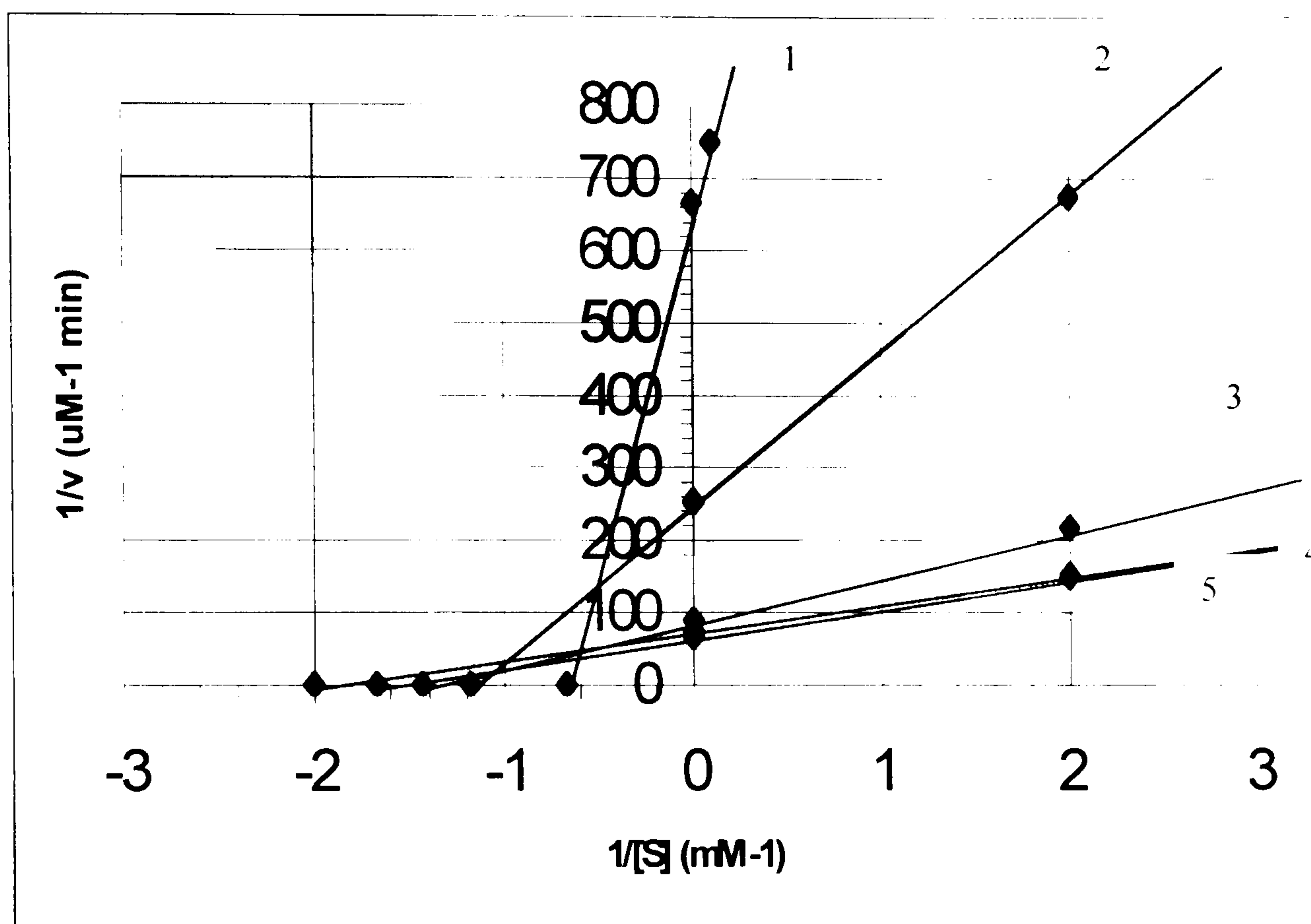

Figure 5.4.6a. Lineweaver-Burke (L-B) plot of the reactions described in the above models. Where line 5 represents two lines : that for model 1 in the presence and in the absence of the roseoredoxin phenomenon. The L-B plots are theoretically the same as there is no increase in yield, simply an altered product ratio (RP). Model 1 considers total transformation rather than the separate routes of transformation. Lines 1,2,3 and 4 represent the two stages of bioconversion described by model 2, both in the presence and absence of RP.

5.4.7 Absolute Spectrum for Purified *S. roseochromogenes* 2β,16α Hydroxylase, Roseoredoxin

The absolute absorbance spectrum of oxidised roseoredoxin showed maxima at 280 and 400 nm with a shoulder at 310 nm Fig.5.4.5a The absorbance profile was mainly preserved after reduction of the roseoredoxin with a 15 fold excess

of dithionite. Thus, the 280 and 400 nm maxima were retained but not the 310 nm shoulder. Reduction decreased the absolute absorbance above 300 nm.

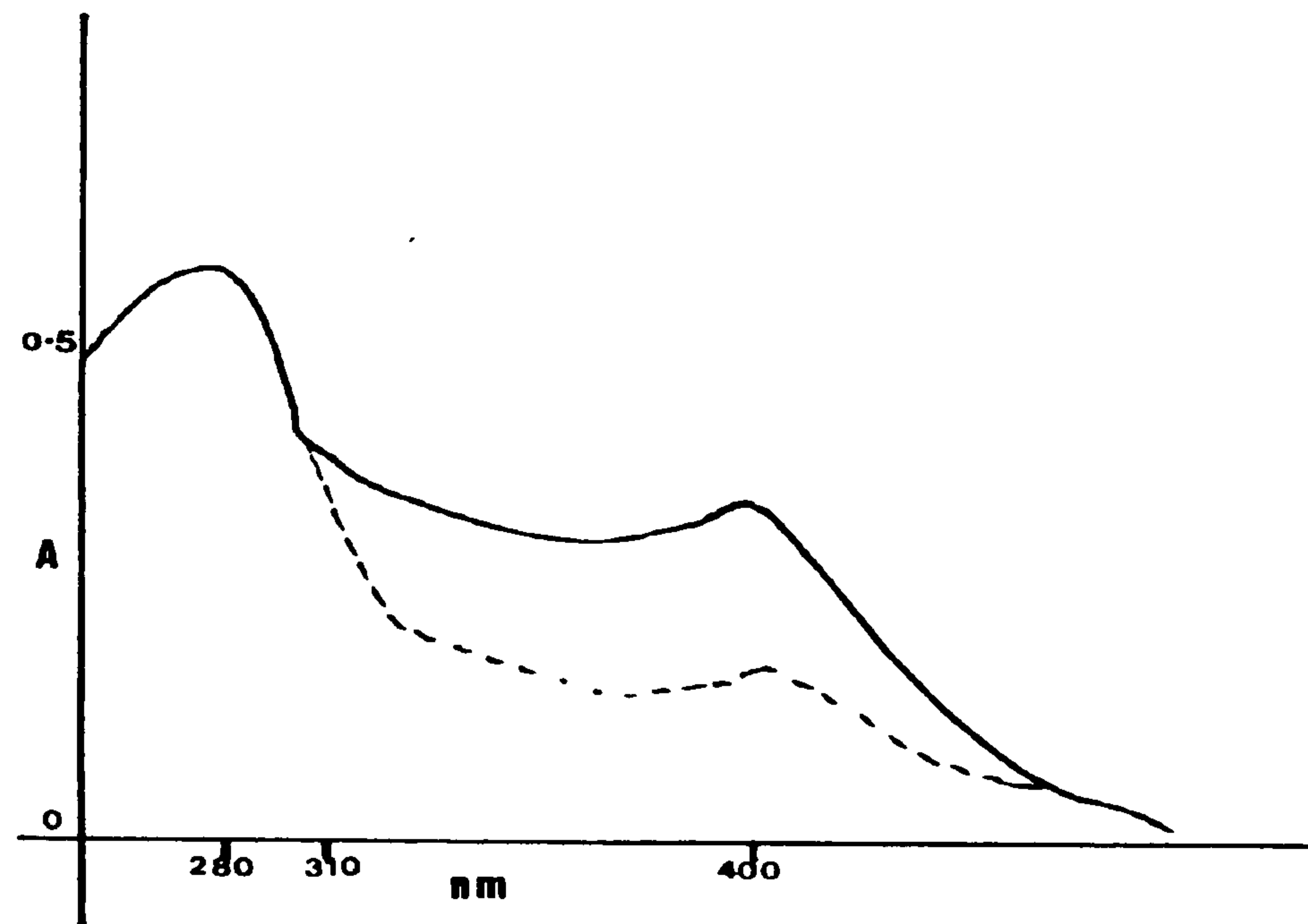

Figure 5.4.7a. Absorbance spectrum of *S. roseochromogenes* roseoredoxin. The solid line is the absorbance of 8.0 μM native oxidised roseoredoxin in 0.1 M K_2HPO_4 buffer pH 7, the dotted line is the absorbance of the reduced form treated with a 15 fold excess of sodium dithionite.

5.4.8 Absolute Spectrum for Purified *S. roseochromogenes* 2β,16α Hydroxylase, Roseoredoxin Reductase

The absolute absorbance spectrum of oxidised roseoredoxin reductase showed maxima at 272 , 374 and 446 nm Figure 5.4.6a. The absorbance profile was mainly preserved after reduction of the roseoredoxin with a 15 fold excess of dithionite.

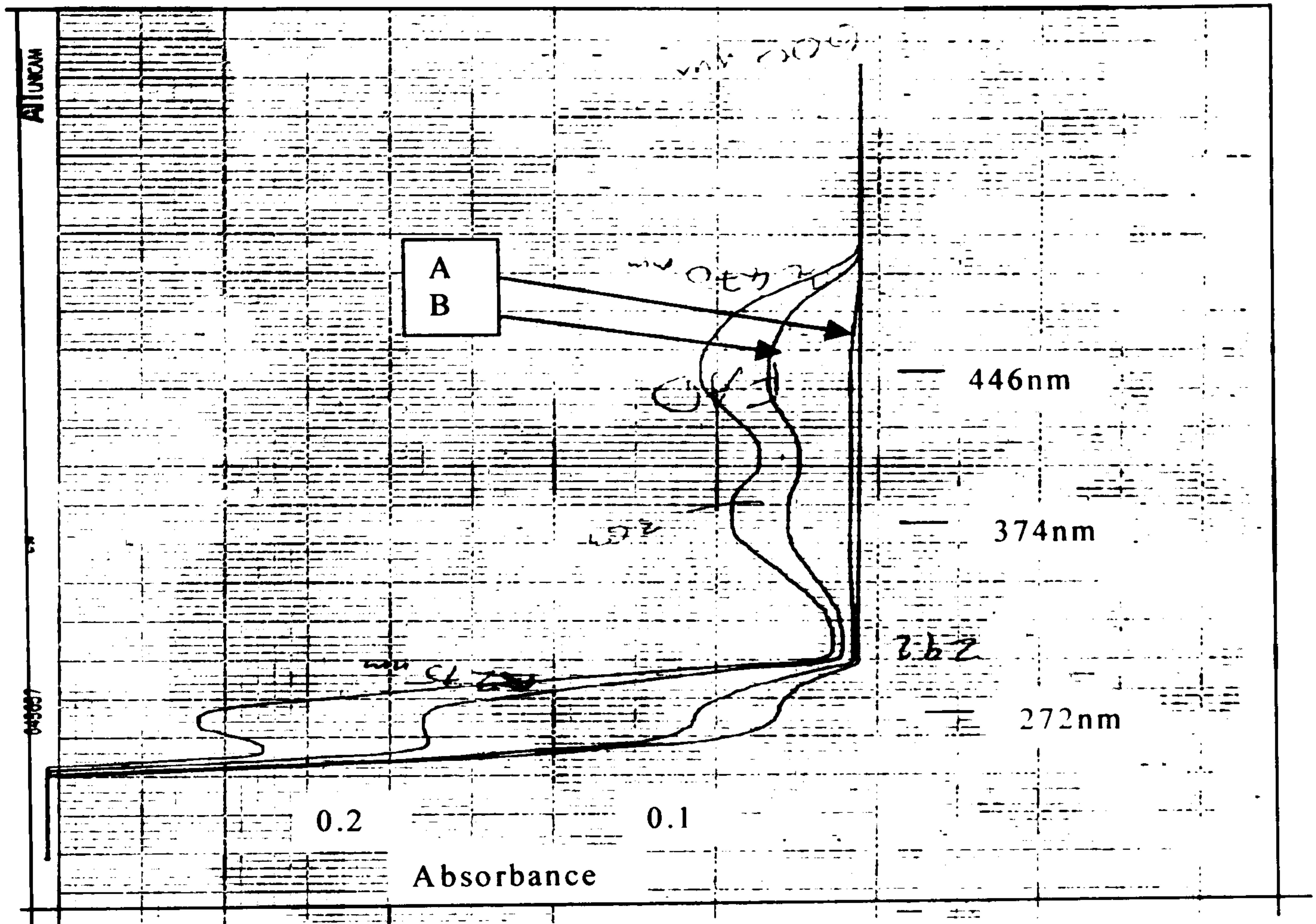

Figure 5.4.8a. Absorbance spectrum of *S. roseochromogenes* roseoredoxin reductase. The solid line is the absorbance of **A**, 5.0 μM; **B**, 7.0 μM native roseoredoxin reductase in 0.1 M K_2HPO_4 buffer pH 7 post reduction with 10 fold excess of sodium dithionite.

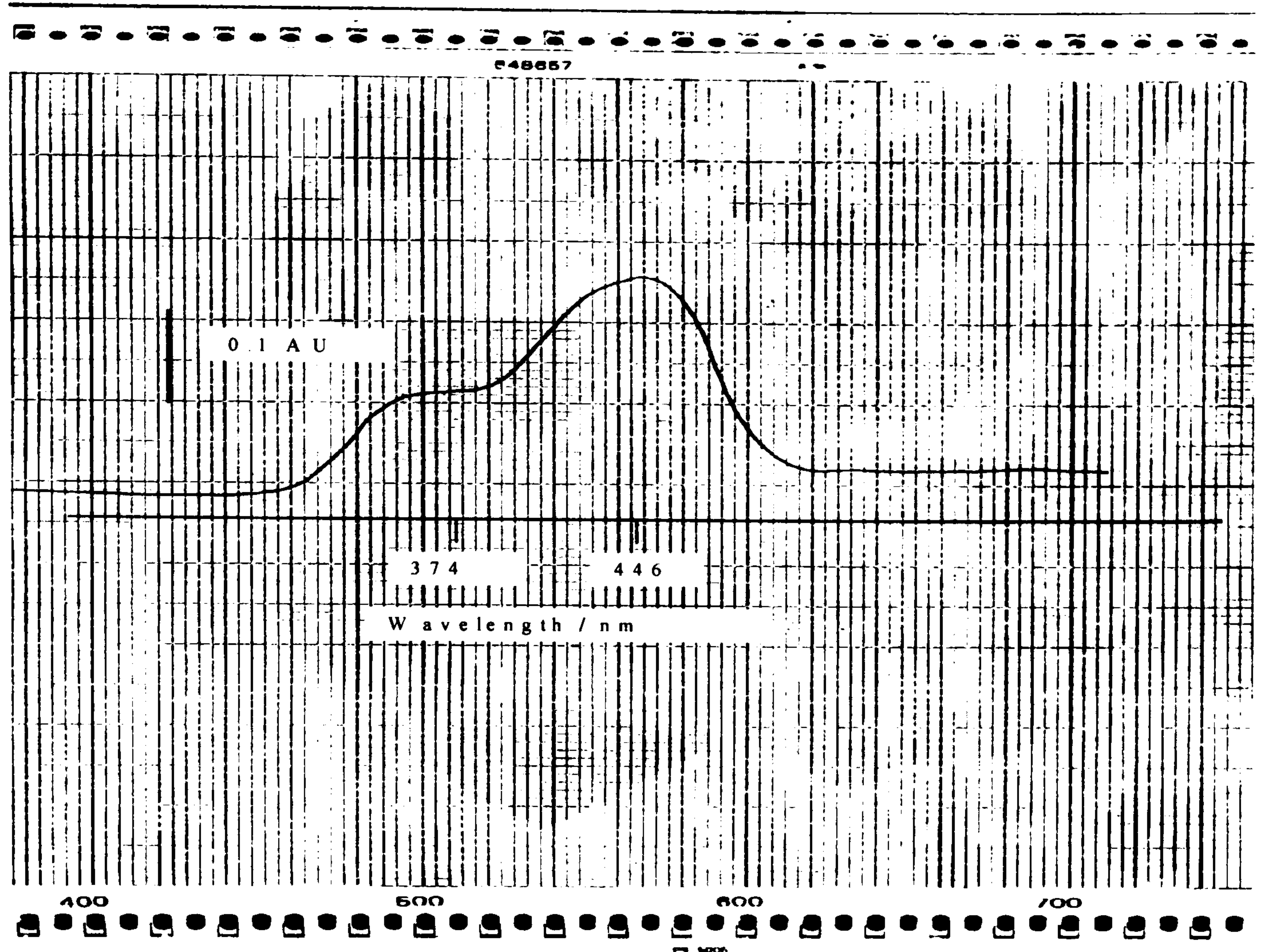

Figure 5.4.8b. Absolute absorbance spectrum of *S. roseochromogenes* roseoredoxin reductase.

The chart recording in Figure5.4.8b shows the 374 and the 446 nm peaks with the 374 peak as a shoulder of the 446 nm peak; these becoming separate artefacts upon reduction of the protein, as shown in Figure 5.4.8a.

The purification and characterisation is described in more detail in chapter 6. The kinetic analysis presented in this chapter incorporates this protein as the *in vitro* reductase component of the multicomponent P450 system. This is not necessarily representative of the *in vivo* scenario as the protein has reductase, i.e. electron transfer ability but may not exclusively be a 'roseoredoxin' reductase rather, this protein may transfer electrons to other recipient proteins.

5.5 DISCUSSION

5.5.1 The Importance of Kinetic Parameters

The type of analysis presented in this chapter is significant in terms of rationales for drug design and the development of modified enzymes for the metabolism of new drugs. The consideration of kinetic parameters is vital in terms of the relationship between an enzyme's efficiency and the half-life of a drug for example. V_{max}/K_m is particularly important in established strategies for the *in vitro* drug metabolism predictions from *in vitro* metabolite kinetic data. These relationships are often well established for the rat. However it has been recently claimed that these models are not useful for prediction of kinetics of drug metabolic behaviour in human tissue, because the dominant human P450 (CYP) 3A4 does not show classic hyperbolic metabolism rate v substrate concentration consistent with Michaelis - Menten kinetics, as predicted (Houston & Kenworthy, 2000). The human CYP 3A4 P450 (along with 3A5) has been implicated in the 16α hydroxylation of oestrone (an oestrogen) to the putative carcinogen 16α hydroxyoestrone (Zeqi *et al.*, 1998) . 16α hydroxylation of course being at the centre of the work presented here. The V_{max} for P450 (CYP) 3A4 is not maintained at high substrate concentrations and does not have a valid human model, despite the dissolution kinetic model for steroid substrates at high concentrations in the aqueous environment, postulated by Chen & Wey in 1990. The hydroxylation of oestrone by human P4503A4 has a K_m of 172 μM and by microsomes, a K_m of 154 μM for 16α hydroxylation. This contrasts with the *S.roseochromogenes* 16α hydroxylation of progesterone, where in the absence of the roseoredoxin phenomenon, K_m (reconstituted) = 0.0015 M and in the presence of the roseoredoxin phenomenon K_m = 0.0007 M (Table 5.1) a difference of an order of magnitude of 10^2 . The human P450 (CYP) 3A4 has a V_{max} of 0.238 mol min^{-1} for 16α

hydroxylation of oestrone by microsomes and 1.05 mol min^{-1} for the expressed P450 system (Zeqi *et al.*, 1998). This is in contrast to the 16α hydroxylation presented here of an order of magnitude of 10^{8} . It has been postulated that blocking the oestrone to 16α hydroxyoestrone pathway, could provide a therapeutic approach to diminishing the risk of oestrogen dependent breast cancer. Since, in the human system the rates are increased on average 6 fold in the presence of cytochrome b5 and V_{max} is not maintained as substrate concentration increases; is it possible that the reconstituted 16α hydroxylase system of *S. roseochromogenes,* presented in this work, could play a role in the examination of steroid 16α hydroxylation and its kinetics, to this end ?

5.5.2 The significance of the Substrate and Inhibitor Binding Spectra

The concentration of substrate within an assay and the extent of binding of P450 to a substrate are inversely correlated with protein concentration. Any relation between K_d (dissociation constant) and K_m (Michaelis constant) is invalid unless exactly the same protein concentration is used in each assay. Other errors affecting kinetic modelling frequently found in the experimental design are: (i) excessive consumption of substrates for the measurements of rates at low substrate concentration or (ii) there may be failure to estimate a significant concentration of P450-bound substrate. The latter can be calculated from the spectral response if Δε for the difference spectrum is known (typically 40-100 mM^{-1} cm^{-1} for P450s). Both problems are significant with high affinity substrates. Also distribution of a ligand between aqueous and non aqueous phases may require consideration in determining K_d and K_m values (Chen & Wey, 1990).

In titrations to calculate binding constants the extent of binding is generally quantified as ΔAbsorbance (peak to trough) and K_d is derived from

Lineweaver-Burk plots of $1/\Delta A$ vs $1/[S]$: K_d =1/intercept. Alternatively, Scatchard plot analysis of ΔA vs $\Delta A/[S]$ is plotted; K_d= slope. Scatchard analysis is better for calculation of dissociation constants because multiple binding sites and/or multiple P450s from dissociation curves can be detected and a wide range of substrates used.

Insolubility of substrate affects spectra in two ways: (i) turbidity - baseline curves up towards the UV, (ii) binding curves will saturate prematurely so that in extreme cases K_d reflects solubility in the aqueous-microsome medium rather than binding to P450. The solubility enzyme kinetics of 11β steroid hydroxylation by *Curvularia lunata* were examined in order to gain a better understanding of the mechanism of enzymatic transformation of steroids at high substrate level (Chen & Wey, 1990).

5.5.3 Measuring P450 Concentration using Spectra

n-Octylamine produces essentially identical difference spectra as CO with P450 so the spectra and calibration are applicable to octylamine-induced difference spectra (type IIa). The value ΔA (407-448nm) at saturation of octylamine also measures octylamine-bound P450 ($\Delta\varepsilon$ = 28cm^{-1} mM^{-1}). This value can be compared with total P450 calculated from a reduced-CO difference spectrum ($\Delta\varepsilon$ 450-490nm = 91cm^{-1} mM^{-1}). This value is used throughout this work.

ΔA 645-700nm can be used to measure P450 ($\Delta\varepsilon$ =4.5 cm^{-1} mM^{-1}) A 645nm absorbance peak is present only in the high-spin state; the response measures only the amount of high spin P450. It was relatively difficult to obtain this absorbance measurement and therefore this value was not used in this work.

5.5.4 Determination of Inhibitor and Substrate K_d s for *S. roseochromogenes* Progesterone Hydroxylase P450

CO : P450 complexes show Soret peaks at 420-424nm suggesting interaction of haem iron and azole nitrogen. The binding of ketoconazole to lanosterol 14α demethylase $P450_{14DM}$ is very high, with an apparent K_d <0.01mM for example (Vanden Bossche *et al.*, 1993). Azoles also interact with the reduced form of $P450_{14DM}$. Absorption spectra suggest that the azole nitrogen still interacts with haem iron (Turi & Loper 1992).

Reduced P450:ketoconazole complexes are not readily converted to reduced P450:CO complexes suggesting the interaction of azole with P450 is strong in that the K_d is high for ketoconazole binding.

K_d values have often been used in the determination of rate limiting steps of P450 reactions. For example product release has been found to be a rate limiting step for several P450 reactions. The examination of azole antifungal agents in terms of their effects upon P450 activity continues to be vitally important because so many P450 related products are based upon these azole antifungals. The appearance of drug resistant strains of pathogens is a legitimate concern. For example the pathogen *Candida krusei* has a P450 which is inhibited by ketoconazole and itraconazole at lower concentrations than fluconazole but all three drugs interfered with the binding of CO to sterol 14α demethylase P450, in direct proportion to their inhibitory effect upon ergosterol biosynthesis. The 'slightly' lower K_d of the fluconazole was only partially responsible for its poor inhibitory effect (Venkateswarlu *et al.*, 1997). However, the sterol 14α demethylase P450 of a closely related organism, *Candida albicans*, was mutated in the haem binding domain by G464S, and the poor inhibitory effect of fluconazole was then purely attributable to a low K_d (Kelly *et al.*, 1999). Comparisons of such reactions with mammalian counterparts may well be useful in terms of biochemical drug modelling since

this enzyme catalyses the oxidative removal of C32 methyl of lanosterol in the biosynthesis of the fungal sterol. ergosterol. Ergosterol is the fungal counterpart to mammalian cholesterol as the mammalian analogue catalyses the identical transformation in cholesterol biosynthesis (Yoshida & Kumaoka. 1975).

The extent of binding or K_d can determine, indirectly, the rate of a reaction. In the oxidation of ethanol to acetic acid by P450 2E1 the K_{cat}/K_m value is at least one order of magnitude higher than in the oxidation to acetaldehyde (Bell Parikh & Guengerich, 1999). Determinations of K_d values are important in the collection of modelling data for P450 systems. Another example of the significance of K_d in terms of the mechanism of P450 catalysis is in the house fly (*Musca domestica*), P450 epoxidation of heptachlor, where the affinity of the P450 reductase for NADPH is 10 times higher than for $NADP^+$ (K_d of 0.31 and 3.3 μM, respectively). Such an affinity change during catalysis could account for a +30 mV shift of the redox potential of FAD (Murataliev *et al.*, 1999). In this mechanism, oxidation of the NADPH is required, hence its higher affinity. The high affinity of the reductase, an FMN semiquinone and the P450 for each other in the formation of a catalytic intermediate has evolved an extremely efficient reaction. The rate of oxidation of the one molecule of NADPH is rapid but the rate of oxidation of a second is too slow to account for the rate of catalysis, this is explained by the formation of two semiquinones (Murataliev & Feyereisen, 1999).

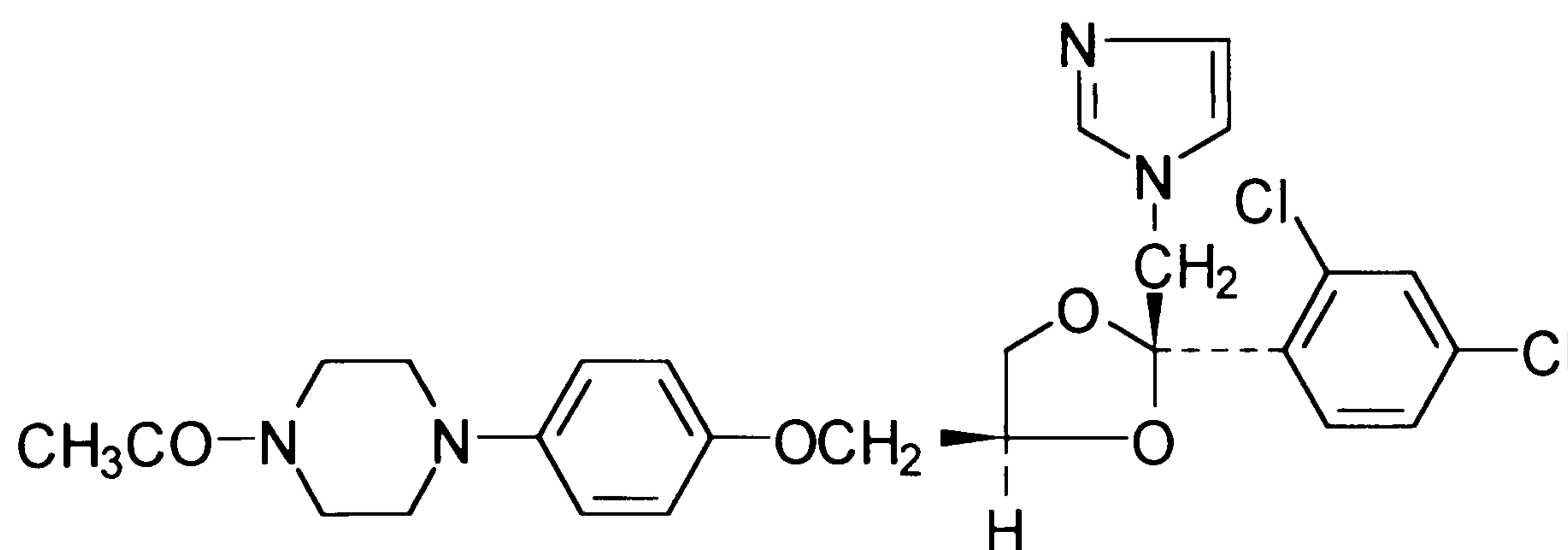

Figure 5A....... Ketoconazole.

5.5.5 Determination of the Dissociation Constant (Kd) for the *S. roseochromogenes* P450 from Inhibitor Binding

The usefulness of determining a ketoconazole K_d for this P450 is questionable in view of the binding spectra, points A, B and C, shown in Figure 5.4.5a above.

However, the binding of ketoconazole to this P450 appeared to be much stronger than to progesterone as might be expected for an inhibitor. This was in view of the fact that bubbling CO through the enzyme inhibitor complex failed to displace ketoconazole in favour of CO.

5.5.6 The Absolute Absorption Spectra of the Three Components of the 2β,16α Hydroxylase System.

One of the more characteristic spectral features of P450 is the large blue shift in the Soret visible absorption band from 419 - 390 nm when substrate is bound. Hence, from a P450 spectrum one can observe substrate free or substrate bound P450. The spectra here, of the P450 and of the reductase give little information except that the reductase spectrum showed the absorbance peaks associated with its FAD moiety. The P450 spectra do not represent a characteristic or particular type of spectra. The roseoredoxin spectrum however, does share characteristics with another known *Streptomyces* ferredoxin (Trower *et al.*, 1990), that of a 7Fe ferredoxin from *Streptomyces griseus*. The significance of this similarity is tenuous even though both proteins are from the same genus, because Fe/S redoxin spectra do not generally have common features and are always unique (Nicholls & Ferguson, 1992). Spectral characterisation would be better served by ESR or EPR.

5.5.7 The Two Models of Progesterone Biotransformation Presented in this Chapter

Considering models 1 and 2 and Table 5.1; if the roseoredoxin phenomenon is due to a fundamental difference in the electron transfer efficiency then one would expect that P450 might be reduced in its catalytic cycle at a different rate by the different forms of the transfer protein. This would affect the second hydroxylation (2β) in that the faster the reduction of P450 the greater the yield of the dihydroxy product. One would therefore expect V_{max} to increase in this instance but not K_m which is a measure of the binding affinity of the substrate. This should be unchanged in both scenarios because the P450 is unchanged. K_{cat}/K_m should change as efficiency changes. Data lines 4 and 6 in Table 5.1 show that the roseoredoxin phenomenon may be related to a change in reducing efficiency, K_{cat}/K_m are of the same order of magnitude and V_{max} is much larger, 0.004 μmol min^{-1} in the presence of the roseoredoxin phenomenon compared to 0.015 μmol min^{-1} in the absence of the phenomenon for the second hydroxylation. K_m however changes from 1.5 mM to 0.85 mM in the presence of the roseoredoxin phenomenon for the second hydroxylation (lines 4 and 6, Table 5.1).

Many drugs and xenobiotics combine with cytochrome P450 to produce a Type I difference spectrum characterised by maximum absorbancies at 390-395nm and minimum absorbancies at 416-420nm (Remmer *et al.*, 1966; Imai & Sato 1966; Schenkman *et al.*, 1967). Most compounds that produce the type I spectra are substrates for cytochrome P450-mediated monooxygenase reactions. Other compounds, which are frequently not substrates produce a Type II spectrum, which has a maximum absorbance at 425-435nm and a minimum absorbance between 390-400nm. The rather broad range of maximum absorbance may be due to a contribution of a small increment of a type I difference spectrum elicited by certain type II compounds e.g. aniline

(Schenkman *et al.,* 1972 & 1981). A binding spectrum with its maximum absorbance more narrowly defined at 418-420nm, referred to as "reverse Type I" difference spectrum (Schenkman *et al.,* 1972, Kumaki *et al.*, 1978), is qualitatively the near mirror image of the Type I spectrum.

The ferric form of P450 haem has five electrons in the d-orbital. Depending on the pairing of these electrons, P450 exists in either high-spin (five unpaired electrons, S=5/2) or low-spin (one unpaired electron, S=1/2) configuration. It is thought that high spin-P450 is penta-coordinated and low spin-P450 is hexa-coordinated. ESR studies have suggested that the Type I spectrum is generated when LS-P450 is converted to HS-P450. The Type II difference spectrum is a complex mixture of absorbance changes that involve the N atom in the haem of the P450 (Katagiri *et al.* 1968, Peterson, 1971; Waterman *et al* 1973, Schenkman *et al* 1981).

A variety of difference spectra are generated when perturbations of P450 are produced by Types I, II or reverse Type I compounds (Remmer *et al.*, 1966, Imai & Sato 1966, Schenkman *et al.,* 1967, 1972, Kumaki *et al* 1978) by carbon monoxide (Omura & Sato, 1964) or ethyl isocyanide (Imai & Sato, 1966 [2]), by changing temperature (Cinti *et al* 1979) or by using linoleic acid hydroperoxide (LAHP) to bleach P450 (Nerland *et al.*, 1981). With the exception of the difference spectrum produced when microsomes are bleached with LAHP, these spectra do not resemble the "absolute" spectra observed with purified, soluble P450, rather, the method is in contrast to others described as it applies exclusively to membrane bound P450s. Visualisation of the spectra obtained with LAHP is possible because, at certain concentrations LAHP bleaches P450 but spares cytochrome b_5, the only other haemoprotein present in carefully prepared microsomes (Nerland *et al.*, 1981). This procedure allows visualisation of a number of membrane-bound forms of P450; reduced, reduced and complexed with CO, oxidised, and oxidised and complexed with compounds that produce Types I, II and reverse Type I difference spectra

(Nerland *et al.*, 1981). These spectra closely resemble the "absolute" spectra observed with solubilised, purified P450.
The LHAP method can be used to determine the relative amounts of high spin- and low spin-P450 bound in rat hepatic microsomes with and without the addition of Type I compounds. Advantage is taken of the temperature dependency of the spin state of P450 (Cinti *et al.*, 1979) to determine the overall enthalpic and entropic changes for the spin equilibrium to generate computer-derived spectra of high spin (HS)- and low spin (LS)-form P450, and to construct a nomogram that allows direct estimation of the percentages of HS and LS forms of P450 at temperatures compatible with biochemical functions.

The extent to which theoretical models can be examined is limited here, in respect of the fact that the most up to date methodologies for kinetic analysis were not available. However, the work does allow for appreciation of the fact that many avenues are left open for development of this work. The models presented here could form the basis of mechanistic investigation of this P450 with more advanced techniques than spectrophotometric analysis. For example, Electron Paramagnetic Resonance (EPR) spectroscopy as used in the determination of single turnover kinetics of putidaredoxin (Brewer & Peterson, 1988) and more recently 'designed' probes were used to detect the presence of human P450 of CYP2D6 in poor metabolising individuals of MDMA (Ecstasy). Michaelis-Menten kinetics were obtained for demethylenation to show that the absence of a low K_m component for demethylenation by liver microsomes meant a lack of CYP2D6 (Kreth *et al.*, 2000).

The data presented in this chapter provide a basis for further work. Table 5.1 shows the kinetic parameters for the reactions considered in this work. In particular the measure of enzymatic efficiency by K_{cat}/K_m, shows how much more efficient the secondary hydroxylation of progesterone is in the presence of the roseoredoxin phenomenon compared to in its absence. This aspect of the

work is considered in detail in chapter 7. K_{cat}/K_m of 1.53×10^5 sec^{-1} M^{-1} for the overall bioconversion of progesterone to 2β,16α dihydroxyprogesterone (model 1), is many times less efficient than the theoretical maximum efficiency, which would give an order of magnitude of 10^8 for K_{cat}/K_m where this value is approaching the diffusion controlled association rate. The rate-limiting step was not determined here.

As outlined above the problems of obtaining accurate human P450 kinetic models for drug design are widely reported and arise from making *in vitro*/*in vivo* comparisons. Models are valid but only when unique adjustments are accounted for in any model by the use of appropriate validation techniques, such as dose response studies (Yu *et al.*, 1996). This year, in fact, the P450 mentioned above in the first section of this discussion (5.5.1), from CYP3A4, has been predicted to be involved in the drug interaction between triazolam (a psychotropic drug) and erythromycin (an antibiotic drug). Prediction by the pharmacokinetic model of a mechanism based inhibition of CYP3A4 was successfully borne out *in vivo* as a result of *in vitro* examination of each drug's pharmacokinetic parameters with recombinant CYP3A4 (Kanamitsu *et al.*, 2000).

Chapter 6

Reconstitution of the 2β,16α Progesterone Hydroxylase Cytochrome P450 System from *Streptomyces roseochromogenes*

6.1 INTRODUCTION

In chapter 4, it was shown that a factor or factors in the initial eluate of the first stage of P450 purification, was responsible for the restoration of NADH-dependent catalytic competence to the *S. roseochromogenes* progesterone 2β,16α hydroxylase P450. Only this fraction was capable of restoring the self supported hydroxylation of progesterone.

The genus Streptomyces is a rich source of cytochrome P450 monooxygenase enzymes that are involved in a wide variety of biosynthetic and xenobiotic transformation reactions. In *Streptomyces antibioticus*, this enzyme is responsible for C-8 epoxidation of the lactone ring of the antibiotic oleandomycin (Rodriguez, *et al.*, 1995). The DNA sequence of the *S. antibioticus* P450 is related to the eryF gene of *S. erythraea* (*Saccharopolyspora erythraea*) which codes for a soluble cytochrome P450 (CYP107) that stereospecifically 6-hydroxylates 6-deoxyerythronolide B to erythronolide B during erythromycin A biosynthesis (Shafiee & Hutchinson, 1987). In *S. carbophilus* P450sca hydroxylates compactin to pravastatin, a tissue-selective inhibitor of 3-hydroxy-3-methylglutamyl-coenzyme A reductase (Matsuoka, *et al.*, 1989). The sulphonyl urea herbicide chlorimuron ethyl is metabolised by two inducible *S. griseolus* P450s, P450SU1 (CYP105A1) and P450SU2 (CYP105B1) (O'Keefe, *et al.*, 1988). *S. griseus* contains a cytochrome P450 that is inducible by the isoflavenoid genistein present in soyaflour (Sariaslani & Kurtz, 1988). Extracts of this organism, prepared from soya flour-induced cells, and supplemented with spinach ferredoxin and ferredoxin-NADPH reductase are capable of aromatic benzylic, and alicyclic hydroxylation (Trower, *et al.*, 1988). The ChoP gene of *Streptomyces sp.* SA-COO encodes a cholesterol oxidase cytochrome P450 that has a high degree of homology with human and Pseudomonas P450s (Horii, *et al.*, 1989).

Steroid hydroxylation by Streptomyces has been reported. C2 and C4 hydroxylation of the phenolic steroid oestradiol is known to be P450 catalysed (Trower, *et al.*, 1988). Skeletal sites transformed in non-phenolic

steroids include ξ1, 2β, 6β, 7β, 9α 11α, 11β, 15α and 16α [for examples see Collingsworth *et al.* (1952), Fried (1956), Herzog *et al.* (1957), McAleer *et al.* (1958), Nazaki *et al.* (1965), Shirasaka & Tsuruta (1960), Smith *et al.* (1961), Vondrova & Capek (1963)]. Unlike in the oestradiol bioconversion the nature of these other steroid hydroxylases is unknown. However, by analogy with steroid hydroxylation in the bacterial species *Bacillus cereus* (Wilson, *et al.*, 1966) and *B. megaterium* (Berg, *et al.*, 1976), a reasonable assumption is that these hydroxylases are site-selective cytochrome P450 monooxygenases; see chapter 4.

In chapter 3 16α-monohydroxy and 2β,16α-dihydroxyprogesterone, were identified as the transformation metabolites produced by *S. roseochromogenes* during a 72 h incubation with exogenous progesterone. Also reported, in chapter 4, was the purification to homogeneity of the cytochrome P450 responsible for *in vitro* progesterone 2β,16α hydroxylation. The purification of the two endogenous electron transfer proteins, roseoredoxin and roseoredoxin reductase required to confer catalytic competence on progesterone 2β,16α hydroxylase P450, are discussed in this chapter.

6.2 MATERIALS

Materials were essentially as described in chapter 4.

6.3 METHODS

6.3.1 **Development of the Cytochrome c Assay for Electron Transfer Determination.**

In order to conserve stock P450 and to obtain 'same-day' results on the transfer of reducing power from the electron transfer factor/s present in the DEAE 52 column wash; this assay was developed, in which cytochrome c replaced the P450 component of the assay described in chapter 4. Electron

transfer was observed from the factor/s in the DEAE 52 eluate, to cytochrome c rather than observing this transfer to P450 by the transformation of progesterone. Instead of the $NaIO_4$ supported transformation of progesterone (as described in chapter 4), for the assay of P450 activity; this assay detects transfer of electrons from NADH, the natural electron donor, to cytochrome c.
In the assay to be described in this chapter, changes to the 'background' transfer of electrons by the addition of different cellular fractions was used as a measure of the presence or absence of *S. roseochromogenes* electron transfer proteins.

The advantages of developing a cytochrome c assay for the purification of the electron transfer factor/s from the DEAE 52 initial eluate, rather than using the purified P450 component of the hydroxylase system, are given in Table 6a.

Factors to be considered → **Assay ↓**	Analysis	Time	Availability	Expense
1. Using purified P450	Results are analysed by TLC post steroidal extraction from the assay mixture	Several hours for assay and extraction followed by running and drying of TLC plates	This P450 is not overexpressed and is taken from the purification protocol described in chapter 3, thus not very available	Expensive in time and the cost of purification materials
2. Using Cytochrome c	Results instantly observable as an increase in A_{550} with reduction of cyt. c.	Many assays may be repeated in one day	Commercially available in large quantities	Less processing to obtain results therefore less expense. Cyt.c is comparatively inexpensive

Table 6a. Table illustrating the advantages gained through development of this cytochrome c assay for the observation of electron transfer from roseoredoxin and roseoredoxin reductase.

6.3.2 Purification of Electron Transfer Proteins from *S. roseochromogenes.*

The roseoredoxin reductase (ferredoxin reductase) was purified from the flow-through wash of the DE-52 DEAE-cellulose column obtained in the first stage of P450 purification. This fraction was directly loaded onto a Whatman DE-32 DEAE-cellulose column and eluted with a linear 0-300 mM NaCl gradient made up in buffer B and 10% glycerol. Fractions (5 ml) were collected and assayed for cytochrome c reductase activity at 550 nm and for NADH oxidation at 340 nm. Also, the spectrum of each fraction was recorded from 260 to 460 nm and compared to that of a standard of authentic FAD.

Fractions which reduced cytochrome c, oxidised NADH, and contained FAD, were pooled and dialysed for 8 h against buffer B before loading on to a Cibacron Blue FG3A column and eluted with a linear 0-200 mM NaCl gradient in buffer B. Fractions (2.0 ml) were collected and assayed as described above. Active fractions that contained roseoredoxin reductase were pooled, dialysed against buffer B + 20% glycerol (buffer C), and stored at - 70 °C.

The roseoredoxin reductase DE-32 cellulose column fractions obtained above were used to purify roseoredoxin (ferredoxin), the second electron transfer protein in the *S. roseochromogenes* progesterone 2β,16α hydroxylase cytochrome P450 pathway. Roseoredoxin activity was assayed by measuring the rate of cytochrome c reduction in the presence of NADH and purified roseoredoxin reductase (Cibacron Blue fraction). The stimulation of the basal rate of electron transfer, from roseoredoxin reductase directly to cytochrome c, was used to measure roseoredoxin activity. Active fractions were pooled and dialysed as described above and then applied to an NAD-Sepharose affinity column. Proteins were eluted with a linear 0-100 mM KCl gradient in buffer B. KCl buffers were required due to loss of electron transfer activity in NaCl. Fractions were assayed for cytochrome c reductase activity as described above and the absorbance of

these fractions was measured at 280 and 414nm. Active fractions containing the highest A414/A280 ratios were pooled, dialysed against buffer C and stored at -70°C.

6.3.3 Measurement of *S. roseochromogenes* Roseoredoxin and Roseoredoxin Reductase Activity

Roseoredoxin activity was measured in a final volume of 1 ml containing 100 mM Na phosphate, 5.2 mM $MgCl_2$ buffer pH 7.2, 0.05 mM cytochrome c, 0.05 units roseoredoxin reductase and 0.2 mM NADH. (1 unit of roseoredoxin reductase is the amount of protein required to reduce 0.5 μmol of cytochrome c per min in the presence of excess roseoredoxin). The rate of cytochrome c reduction was measured at 550 nm against a control incubation without NADH. Cytochrome c reduction was measured to completion of the reaction at 25°C. The absolute amount reduced was calculated by measuring the absorbance difference between the test and control incubations at 550 nm. An absorbance coefficient of 29.9 mM^{-1} cm^{-1} was used to calculate reduced cytochrome c and 8.9 mM^{-1} cm^{-1} for oxidised cytochrome c.
Roseoredoxin reductase activity was measured as described above except that roseoredoxin was omitted from the incubations.

6.3.4 Cytochrome P450 Dependent Progesterone Hydroxylation: Natural NADH Dependent Verses $NaIO_4$ Supported Reactions

The progesterone 2β,16α-hydroxylase activity of purification fractions was determined using the $NaIO_4$ method, in the absence of natural electron transfer proteins (Goodman & Smith, 1961). Progesterone (4 mM) and $NaIO_4$ (1.5 mM) were added to 0.5 ml of fraction contained in metal capped Bijou bottles. Mixtures were made up to 1 ml with buffer B. Bottles were

fixed to a turn-table that was vertically rotated at 40 rpm for 2 h at 25°C. Steroid metabolites were extracted from the incubations by shaking the mixtures with chloroform (1 ml) for 1 min. The chloroform layer was removed and evaporated at 60°C. The residue was dissolved in methanol (10 μl) and spotted onto TLC plates. These were run and processed as described above.

In the natural reconstituted system where, 1.5 mM $NaIO_4$ was replaced by 2 mM NADH, along with 0.3 μM purified progesterone 2β,16α-hydroxylase cytochrome P450, 0.05 U roseoredoxin and 0.1 U roseoredoxin reductase, hydroxylation was absolutely specific for NADH. NADPH did not substitute at any concentration tested. The NADH optimum for hydroxylation in these assays was 2 mM.

Steroid metabolites synthesised in the above incubations were identified only by TLC because the tiny quantities of compound produced precluded structural determination by full chemical methods or by NMR. The TLC spots obtained co-chromatographed with spots of the authentic 16α monohydroxy- and 2β,16α dihydroxyprogesterone produced by intact *S. roseochromogenes*, even when the constituents of the solvent system were varied.

6.3.5 Detection and Stoichiometry of the Flavin Prosthetic Group

Fractions from the purification of roseoredoxin reductase, were examined for their FAD content. Only fractions which contained FAD were pooled prior to dialysis.

FAD was detected post dissociation from its protein, although in the first instance, samples were selected by their typical flavin spectra (200-600nm) where peaks at 272 nm, 374 nm and 446 nm were observed as shown in Figure 6.4.2a.

For the dissociation of FAD from protein, samples were boiled for 4 min in 0.1 M phosphate buffer, pH 7.2. The samples were cooled prior to

centrifugation in Centricon® concentrator tubes (Centricon-10) at 10000 rpm for 10 min. TLC was performed on the supernatant with 2 % w/v Na_2HPO_4 in water, as solvent (Fazekas & Sandor, 1971). Flavin presence was detected as a yellow fluorescence when observed under u.v. light at 364 nm. Protein concentrations were determined as described in chapter 2 in order to determine the FAD/protein stoichiometry for purified samples.

6.4 RESULTS

6.4.1 The Detection of Reducing Power in *S. roseochromogenes* Cell - Free Extracts, Utilising the Cytochrome c Assay

The spectra in Figure 6.4.1a. show the changes induced in the cytochrome c spectra by roseoredoxin and roseoredoxin reductase. Different cellular fractions were added to the cytochrome c assay described during the purification of the electron transfer proteins for the purpose of locating them resulting in the assay containing purified roseoredoxin and roseoredoxin reductase.

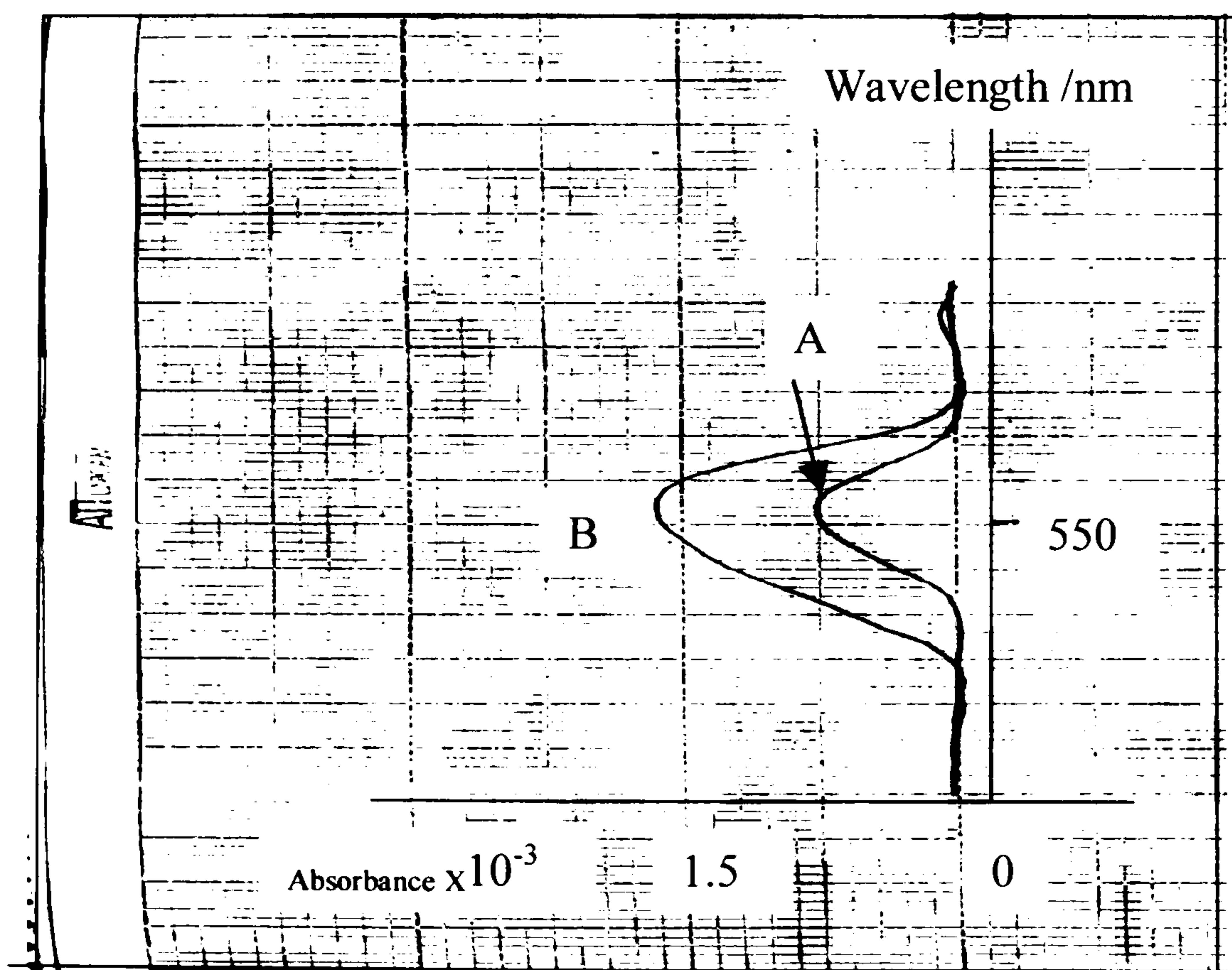

Figure 6.4.1a Cytochrome c reduction profile, absorbance v wavelength, showing increasing absorbance at 550 nm with increasing reduction by *S.roseochromogenes* roseoredoxin and roseoredoxin reductase. 0.5μmol cytochrome c was present with 1.1 units of roseoredoxin reductase and excess roseoredoxin. The reaction was initiated by the addition of NADH to 0.2mM. The final volume was 1.0 ml. Where A = spectrum 30s post NADH addition and B = spectrum 1 min post NADH addition.

Roseoredoxin reductase activity was measured as described above except that roseoredoxin was omitted from the incubations.

Curve B in Figure 6.4.1a represents 1.0 unit of cytochrome c reductase activity.

Roseoredoxin within the cytochrome c assays accounted for a 'leakage reduction rate of 0.1 units of cytochrome c reductase activity which had to be subtracted from final rates of reduction. This leakage is illustrated in Figure 6.4.1b.

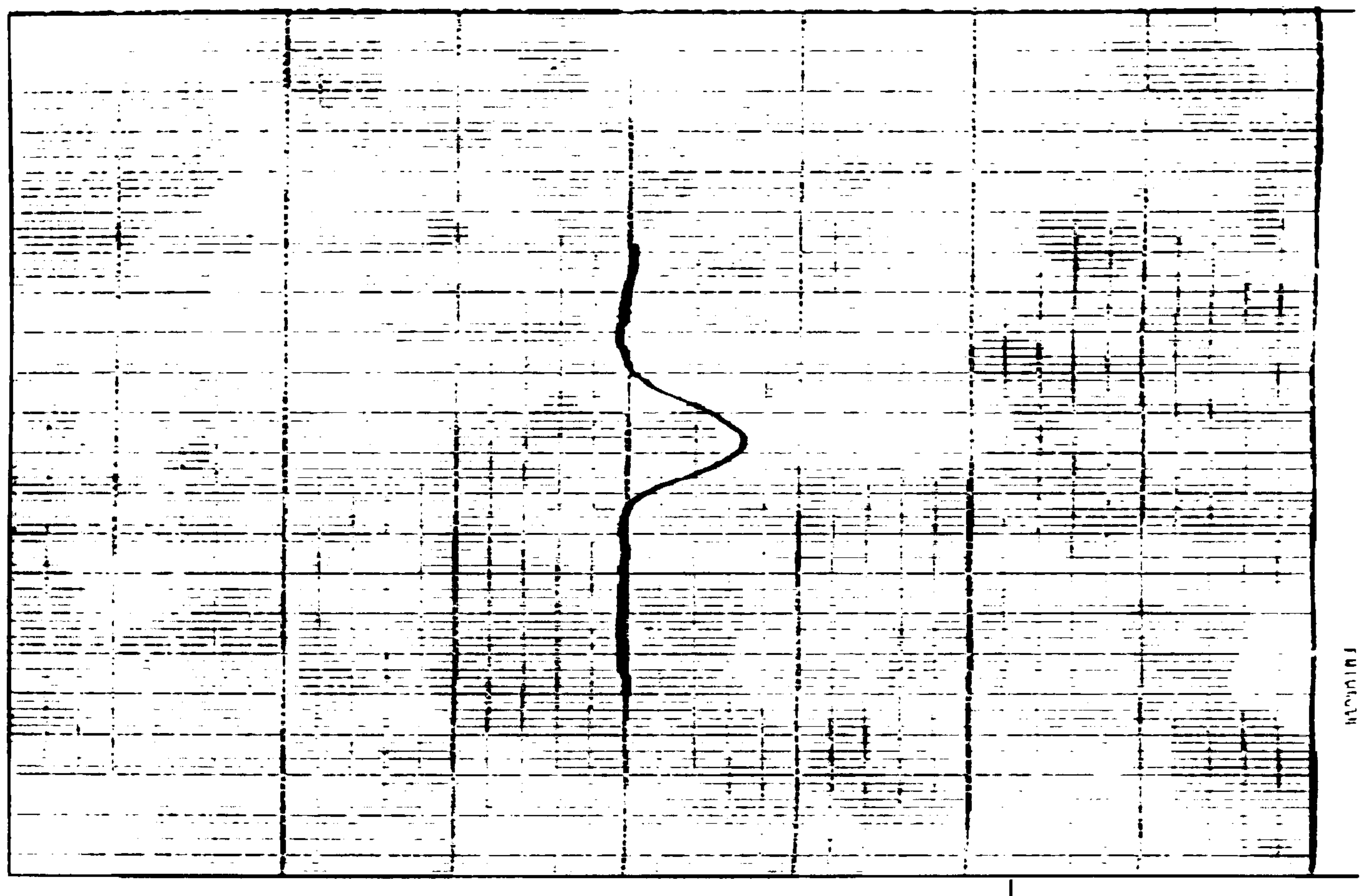

Figure 6.4.1b Cytochrome c reduction attributed to roseoredoxin in the absence of roseoredoxin reductase. This represents a leakage rate of 0.1 units of reductase activity. 0.5μmol cytochrome c was present with excess roseoredoxin. The reaction was initiated by the addition of NADH to 0.2mM. The final volume was 1.0 ml.

Figure 6.4.1b represents the maximum amount of electron transfer attributable to roseoredoxin. When cytochrome c was then replaced by *S. roseochromogenes*' cytochrome P450 and the components of the assay retained i.e. those illustrated in Figure 6.4.4a., progesterone hydroxylase activity was observed as shown in Figure 6.4.1c.

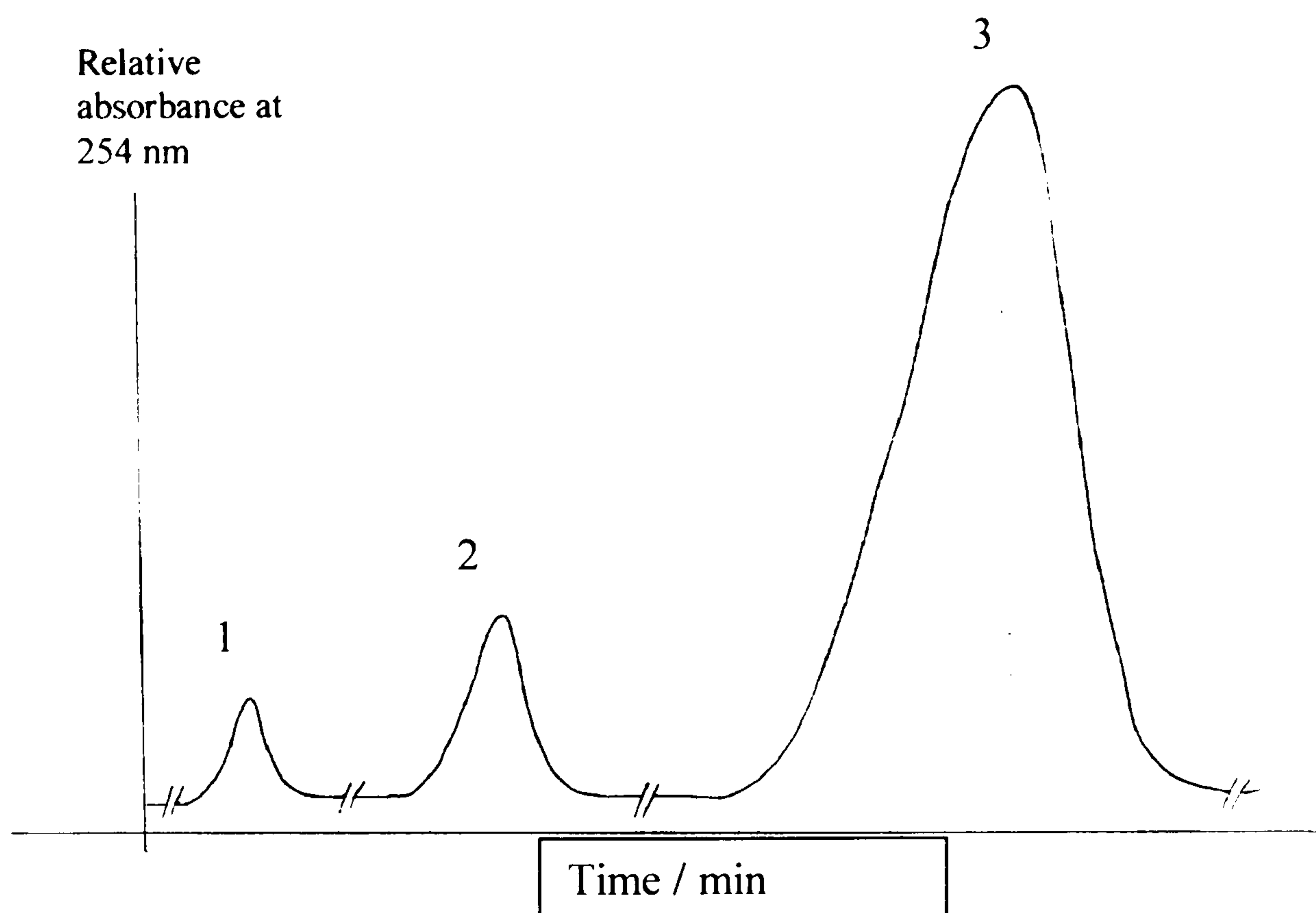

Figure 6.4.1c. HPLC trace of 6.0 h progesterone transformation incubation by reconstituted *S. roseochromogenes'* cytochrome P450 multicomponent hydroxylase system. Peak 1, 2β,16α-dihydroxyprogesterone (retention time 20 min); Peak 2, 16α-monohydroxyprogesterone (retention time 40 min)

The product ratios were compared to those obtained for the sodium periodate mediated progesterone P450 hydroxylations described in methods section 6.3.4. Although the initial rates of hydroxylation were found to be different, the turnover numbers were vastly different as shown in Table 6.4.1a.

Pathway	Initial rate of progesterone transformation (mmol progesterone /mmol P450/h)	Turnover (mol progesterone / mol P450)
Reconstituted	1.18	6.00
$NaIO_4$	1.62	0.45

Table 6.4.1a. Comparison of progesterone metabolism catalysed by the reconstituted natural cytochrome P450 dependent hydroxylation pathway and the $NaIO_4$ dependent pathway.

6.4.2 Identification of the Roseoredoxin Reductase Prosthetic Group

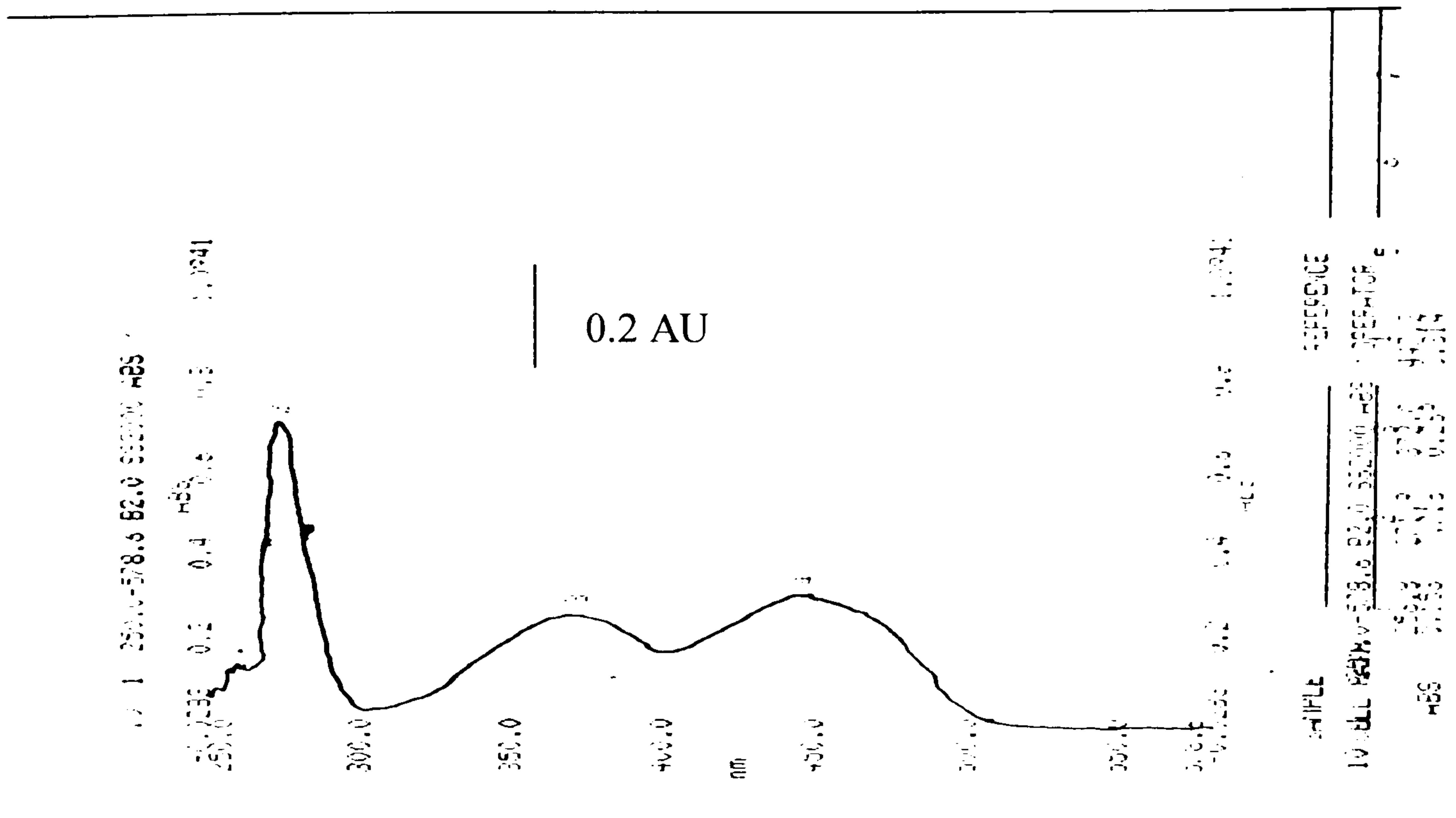

Figure 6.4.2a Absorbance spectrum (200-600 nm) of FAD dissociated from roseoredoxin reductase.

As discussed in section 6.3.5, such spectra as shown in Figure 6.4.2a were used to identify FAD during roseoredoxin reductase purification. U.V. visible analysis also revealed that the flavin and the flavoprotein gave the typical flavoprotein spectrum in the 350-500 nm range with peaks at 374 nm and 446 nm, with a trough at 400 nm.

6.4.3 TLC Analysis to Reveal the Nature of the Electron Transfer Protein's Prosthetic Group

TLC analysis revealed by a yellow fluorescent spot, that the flavin from roseoredoxin reductase, co-migrated with standard authentic FAD. A typical chromatogram is shown in Figure 6.4.3a.

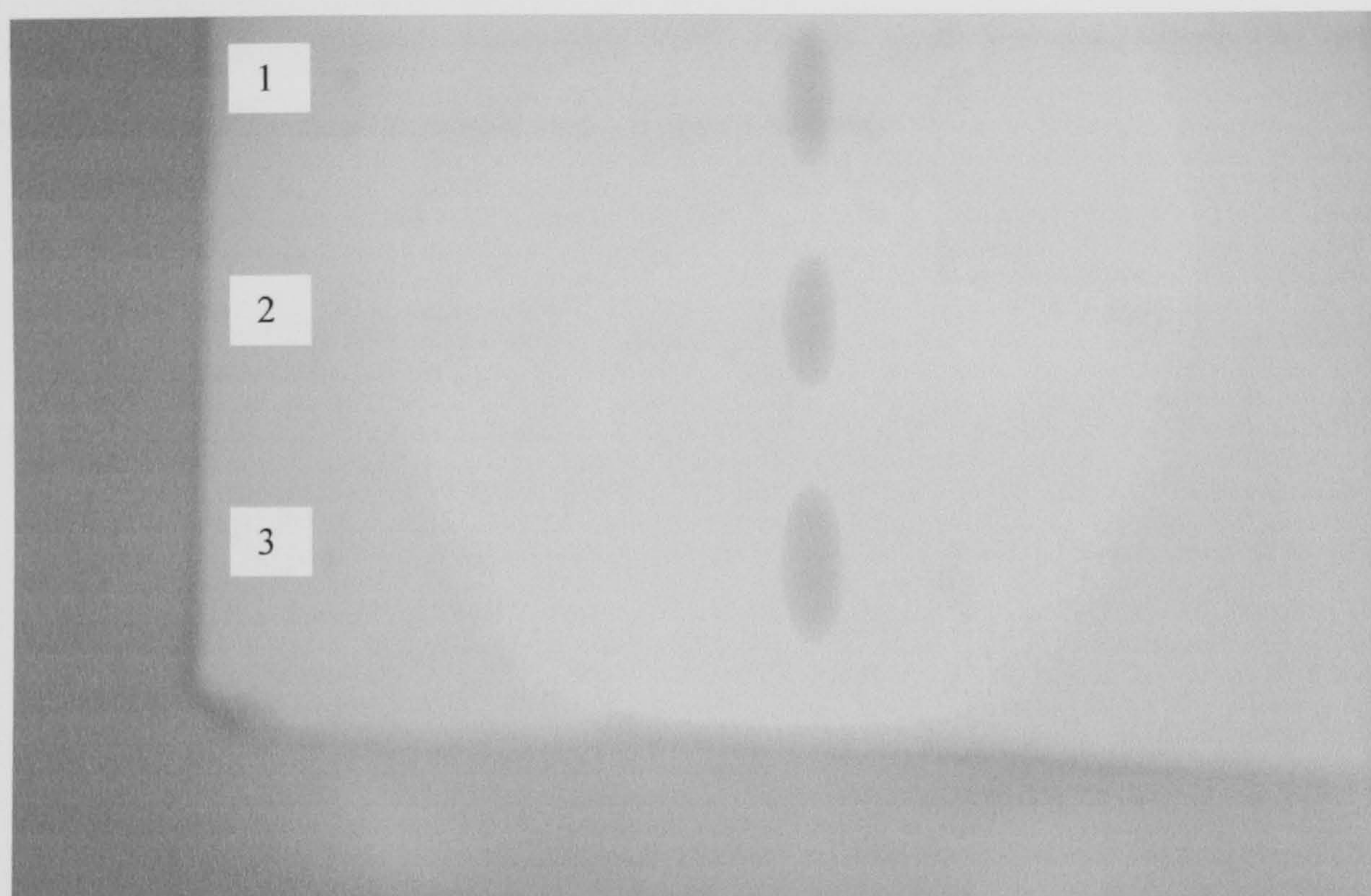

Figure 6.4.3a Photograph of a chromatogram visualised under uv light. Migration was from left to right. Lane 1- standard authentic FAD, Lanes 2 & 3 are samples of the roseoredoxin reductase FAD, illustrating co-migration.

6.4.4 Purification of the Progesterone 2β,16α-Hydroxylase Cytochrome P450 Electron Transfer Proteins Roseoredoxin Reductase and Roseoredoxin from *S. roseochromogenes*

The DE-52 DEAE-cellulose column flow-through and wash, obtained from the first stage of P450 purification, as described in section 4.9.3., was used as starting material for the purification of the electron transfer proteins roseoredoxin reductase and roseoredoxin. A two step procedure was used to purify to homogeneity both proteins. The data for these methods are shown in Tables 6.4.4a. (roseoredoxin reductase) and 6.4.4b. (roseoredoxin) respectively.

In the case of roseoredoxin reductase, a second DEAE column, containing DE32 cellulose, followed by dye affinity chromatography on Cibacron Blue FG3A resulted in a 258 fold purification of this particular protein. Roseoredoxin co-eluted with the roseoredoxin reductase in the DE32 300 mM NaCl fraction, but was separated from the latter protein by NAD-Sepharose affinity chromatography to give a final 163 fold purification. Both proteins gave single bands by SDS-PAGE. M_r values of 14 x 10^3 and 65 x 10^3 were determined from the SDS-PAGE gels for roseoredoxin and roseoredoxin reductase respectively, Figure 6.4.4a.

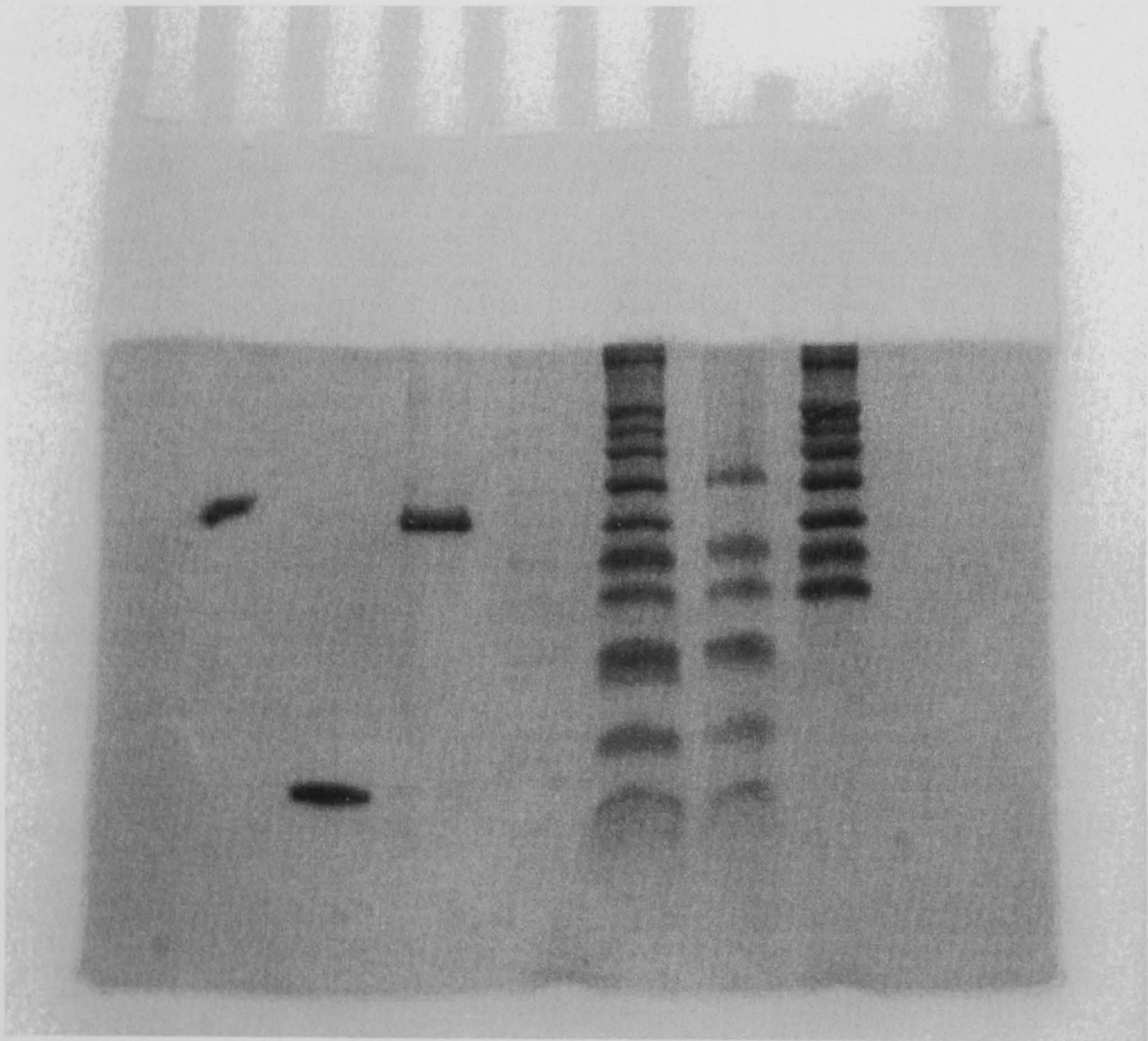

Figure 6.4.4a SDS-polyacrylamide gel of purified *S. roseochromogenes* 2β,16α hydroxylase cytochrome P450, roseoredoxin and roseoredoxin reductase.
Lane 1, roseoredoxin reductase (3.1 μg M_r 65 x 10^3); lane 2, roseoredoxin (2.8 μg M_r 14 x 10^3); lane 3, progesterone 2β,16α-hydroxylase cytochrome P450 (4 μg M_r 63 x 10^3); lane 4, wide range protein size ladder M_r 205 x 10^3 -14.2 x 10^3; lane 5, lower ranger protein size ladder M_r 66 x 10^3 -14.2 x 10^3; and lane 5, upper ranger protein size ladder M_r 45 x 10^3 -14.2 x 10^3. Lanes 4-6 contained 35 μg protein.

Table 6.4.4a. Purification of *S. roseochromogenes* roseoredoxin reductase

Purification Stage	**Total Protein (mg)**	**Total Roseoredoxin reductase (nmol)**	**Yield roseoredoxin reductase (nmol/mg protein)**	**Recovery Roseoredoxin Reductase (%)**	**Purification factor**
S15 Extract	1050	32	0.03	100	1
DEAE 52 Column Flow-through	800	30.5	0.04	95.31	1.3
DE 32 0-300 mM NaCl Fraction	6.84	19.96	2.92	62.38	97.3
Dialysis	6.84	18.44	2.70	57.63	90
Cibacron Blue FG3A 0-200 mM NaCl	1.88	14.55	7.74	45.47	258
Dialysis	1.88	13.03	6.93	40.72	231

Table 6.4.4b. Purification of *S. roseochromogenes* roseoredoxin

Purification Stage	Total Protein (mg)	Total Roseoredoxin (nmol)	Yield roseoredoxin (nmol/mg protein)	Recovery Roseoredoxin (%)	Purification factor
S15 extract	1050	51	0.05	100	1
DEAE 52 column Flow-through	800	39.10	0.05	76.67	1
DE 32 0-300 mM NaCl fraction	6.84	18.79	2.75	36.80	55
Dialysis	6.84	17.77	2.60	34.80	52
Sepharose NAD affinity 0-100 mM KCl fractions	1.95	9.81	5.03	19.20	101
A414/A280 fractions Pooled & dialysed	1.10	8.95	8.14	17.55	163

6.5 DISCUSSION

Streptomyces roseochromogenes, contains a cytochrome P450 (chapter 4) which, in conjunction with two indigenous electron transfer proteins, roseoredoxin and roseoredoxin reductase, hydroxylates exogenous progesterone firstly to 16α-hydroxyprogesterone and thereafter in a second phase bioconversion to 2β,16α-dihydroxyprogesterone. The progesterone 2β,16α-hydroxylase P450 and the two electron transfer proteins have been purified to homogeneity. In this purification process, a cytochrome c assay was developed, for two major reasons ; 1) in order to conserve valuable stock purified cytochrome P450 and 2), for ease of detection of electron transfer activity. A fully reconstituted assay would be very time consuming because it would require time for steroid hydroxylation, significant time for steroid extraction and finally, a relatively long HPLC analysis for each metabolite assay. By contrast the spectrophotometric cytochrome c assay is rapid and can be used to analyse multiple incubations.
Following this, the natural reconstituted incubation containing the three purified proteins (roseoredoxin reductase, roseoredoxin and cytochrome P450) and NADH, the natural electron donor, produced identical hydroxy-progesterone metabolites as produced by intact cells.

Comparison of Progesterone Metabolism Catalysed by a Reconstituted Natural Cytochrome P450 Dependent Hydroxylation Pathway and an $NaIO_4$ Dependent Peroxide Shunt Pathway

The roseoredoxin, roseoredoxin reductase and NADH requirement in the natural progesterone 2β,16α hydroxylase cytochrome P450 hydroxylation pathway was replaceable by $NaIO_4$. Peroxy and hydroperoxy compounds (XOOH) act in a shortened form of the catalytic cycle known as the "peroxide shunt" by replacing the requirement for NADH, the electron transfer proteins and molecular O_2, the terminal electron acceptor in the

natural pathway. When these two pathways were compared for progesterone transformation catalysed by highly purified *S. roseochromogenes* progesterone 2β,16α hydroxylase cytochrome P450 , it was found that the initial rate of hydroxylation was nearly 40% greater in the $NaIO_4$ peroxide shunt pathway (1.62 mmol progesterone converted/mmol P450/h) than in the natural reconstituted pathway (1.18 mmol progesterone converted/mmol P450/h). By contrast, the peroxide shunt pathway supported 13 fold fewer hydroxylation events per molecule of P450 (0.45 mol progesterone converted/mol P450) than the reconstituted pathway (6.0 mol progesterone converted/mol P450) (Table 6.4.1a.). These yield data show that in the reconstituted natural pathway, progesterone 2β,16α hydroxylase P450 supports multiple rounds of hydroxylation in contrast to a likely single round by a minority of P450s in the peroxide shunt pathway (Berrie *et al.*, 1999).

Examination of the Reconstitution of P450 Catalysis

Development of assays such as the natural reconstitution assay described in this chapter are important in terms of cloning strategies for cytochromes P450. Since P450s are multicomponent there is a need for them to be co-expressed with their electron transfer proteins in host vectors or for the P450 to become catalytically competent in combination with the hosts own reductase proteins. Alternatively, overexpressed P450 may be purified and detected by its addition to reconstitution assays as described in this chapter. An example of integration with host proteins upon reconstitution is in the case of cytochrome P450 NF a eukaryotic P450 expressed in eukaryotic host (a yeast). Cytochrome P450 NF, a member of the P450 IIIA subfamily, is the major contributor to the oxidation of the calcium-channel blocker, nifedipine in human liver microsomes. This drug is used in the treatment of hypertension. NF25 cDNA was expressed in *Saccharomyces cerevisiae*

using an expression vector. The yeast endogenous NADPH-cytochrome P450 reductase coupled efficiently with the heterologous P450 NF25 though its concentration was far lower than that of its analogue in human liver. Addition of rabbit liver NADPH-cytochrome P450 reductase increased the oxidation rates. Rabbit liver cytochrome b5 also caused an enhancement of catalytic activity of this particular P450 in a reconstituted system involving the protein purified from human liver. Furthermore, the level of the yeast endogenous cytochrome P450 (lanosterol 14α-demethylase) was found to be negligible compared to the heterologously expressed cytochrome P450 (30 times less). Therefore yeast microsomes containing P450 NF25 constitute a good functional model for studying the binding capacities and catalytic activities of this form of human hepatic cytochrome P450 (Renaud *et al.*, 1990).

Yeast P450s are also expressed in other yeast expression vectors such as the two P450s : P450 52A3 (P450Cm1) and 52A4 (P450Cm2), from the fungus *Candida maltosa*. Both the P450 proteins and the corresponding *C. maltosa* NADPH-cytochrome P450 reductase were separately produced by expressing their cDNAs in *Saccharomyces cerevisiae*. This reconstituted both active monooxygenase systems (Scheller *et al.*, 1996). This is an example of the expressed P450s being co-expressed along with their own reductase gene rather than utilising the host reductase. For this purpose, a multicopy plasmid was constructed that contained two independent expression units controlled by the galactose-inducible GAL10 promoter (Zimmer *et al.*, 1995).

The multicomponent co-translation of P450 systems is exemplified by the catalytically self sufficient 118 kDa, fatty acid oxygenase P450 from *Bacillus megaterium*. Even though the enzyme system is an exception to other P450s by being self sufficient its domains are nevertheless co-translated, each requiring the others for catalytic competence, just as in other P450s. A 4-base pair sequence (AAAG) is found to have a regulatory role in the expression of the BM-1 gene in *B. megaterium*. This sequence is found in all eukaryotic and prokaryotic systems which contain barbiturate

inducible proteins and is known as a Barbie box. This sequence would therefore necessarily need to be included or mutated to enable constitutive expression of this P450.

The above examples are of co-expression to restore catalytic competence to a P450, where reductase elements and regulatory factors are expressed with the P450 itself, thereby negating the need for a separate assay to establish the success of P450 cloning. Purification and characterisation of NADPH-cytochrome P450 reductase from the filamentous fungus *Rhizopus nigricans* was observed initially by its cytochrome c reductase activity, rather than utilising valuable purified P450 and observing 11α steroid hydroxylation (Makovec & Breskvar 1998). This meant the development of a cytochrome c assay such as the assay described in this chapter. Purified reductase contained approximately equimolar quantities of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) per mole of the enzyme as would be expected from such a eukaryotic P450 reductase. Not all cytochrome c (P450) reductase proteins transfer electrons to all P450s. Therefore there is a theoretical danger from obtaining false negative results of successful P450 expression. For example, the reductase in this example, from *R. nigricans*, was unable to replace the adrenodoxin reductase from the 11β steroid hydroxylase P450 from adrenal mitochondria (Makovec & Breskvar 1998).

As mentioned above, the 14α demethylation reaction in the biosynthesis of fungal sterols is important and the subject of many studies; *Ustilago maydis* (Lamb *et al.*,1998) and the grass, *Sorghum bicolor* (Bak *et al.*,1997) as is the benzopyrene hydroxylase system from *Pleurotus pulmonarius* (Maspahy *et al.*,1999). However, the P450 system from *Candida albicans*, was used in the generation of a soluble and catalytically active sterol 14αdemethylase reductase complex. The associated NADPH dependent reductase is also bound to the endoplasmic reticulum. A diglycine linker recognition site was used to remove the hydrophobic N terminal membrane anchor of 33 amino acids. Reconstitution of the soluble enzyme with yeast reductase and treatment with the suitable protease for the diglycine linker, resulted in a functional, soluble eukaryotic P450 14DM system. The purpose of this

reconstitution was to show that the membrane anchor serves to localise P450 14DM to the endoplasmic reticulum where the substrate is located but that the anchor is not essential in demethylase activity (Lamb *et al.*, 1999 [2]). Also a genetically engineered fused enzyme between rat cytochrome P4501A1 and yeast NADPH-P450 reductase was created by the formation of three expression plasmids, pAMC1 for rat P4501A1, pAMR2 for P4501A1 and yeast NADPH-P450 reductase. pAFCR1 was for a fused enzyme between P4501A1 and the reductase. After construction each was introduced into *Saccharomyces cerevisiae* AH22 cells for the purpose of comparing kinetic parameters of the zoxazolamine 6-hydroxylase reaction by the fused enzyme and reconstituted assay reactions. By dual-wavelength stopped-flow spectrophotometry, it was found that electrons were rapidly transferred from NADPH through FAD and FMN to the heme iron of the fused enzyme (Sakaki *et al.*, 1994).

A third type of 'artificial' P450 has been created where FAD or FMN were covalently bound to cytochrome P450 2B4. The most catalytically active was the conjugate of cytochrome P450 with FMN. This artificial single protein hydroxylase was able to initiate the demethylation of dimethylaniline and aminopyrine. It also initiated the *p*-hydroxylation of aniline. The conjugate of cytochrome P450 2B4 with FAD oxidised these substrates at a much slower rate (Uvarov *et al.*, 1994).

Most characterised P450 systems are eukaryotic. The most obvious physical difference between prokaryotic and eukaryotic systems is solubility. Bacterial P450s are soluble whereas the microsomal and mitochondrial P450s are membrane-associated proteins. In bacteria the three-component arrangement is most common but two component systems have been identified but are very rare. For example, in contrast to *Streptomyces roseochromogenes*, P450 from *Streptomyces carbophilus* catalyses the hydroxylation of mevastatin to pravastatin as a two rather than three component P450 monooxygenase. The NADH-cytochrome P450 reductase was purified from *S. carbophilus* as a single polypeptide with a molecular weight of 51 kDa. This alone reconstituted the *in vitro* hydroxylation with

cytochrome P450, NADH and O_2. This protein contained an FAD and FMN molecule. The FMN molecule was easily dissociated from the reductase (Serizawa & Matsuoka 1991). In contrast, $P450_{cam}$ putidaredoxin reductase contains only FAD and is specific for NADH and the $P450_{meg}$ megaredoxin reductase as discussed above in terms of reconstitution, contains only FMN and is specific for NADPH. The catalytically self-sufficient P450BM-3 is currently the only single-component P450-dependent monooxygenase known, other than those manufactured (for example, Lamb *et al.*, 1999 [2] and Sakaki *et al.*, 1994). It is a paradox that P450BM-3 is structurally more analogous to the P450 systems of liver microsomes than to bacterial P450 monooxygenases. Another difference between prokaryotic and eukaryotic P450s is function. Most known bacterial P450-dependent systems initiate the oxidation of recalcitrant carbon compounds so that the hosts can utilise them as sole carbon sources for growth (Fulco, 1991). Eukaryotic P450s are more associated with the removal of xenobiotics and biosynthesis.

$P450_{cam}$, described above is an exception to the prokaryotic P450s. In terms of other, prokaryotic P450 systems; ferredoxin electron transfer proteins have also been purified from *Streptomyces griseolus* which was found to contain two 7 kDa isoforms, designated Fd-1 and Fd-2. These proteins have 52% identity and both contain single [3Fe-4S] clusters (O'Keefe *et al.*, 1991). Both ferredoxins are active in reconstituted cell-free systems containing the SU1 P450 isoform, although Fd-2 is more effective. The genes for the ferredoxins and the sulfonylurea P450 monooxygenases are virtually contiguous. Thus P450SU1 and the downstream Fd-1 form a closed spaced pair, similarly P450SU2 and Fd-2. In *Streptomyces griseus* only a single ferredoxin encoded by SOY B has been putatively identified. The situation in *S. roseochromogenes* remains to be resolved.

Chapter 7

Preincubation of *Streptomyces roseochromogenes* with Progesterone, Induces a Post Translational Modification in the Electron Transfer Protein, Roseoredoxin

The Roseoredoxin Phenomenon

7.1 INTRODUCTION

There is now a wealth of compelling evidence identifying cytochrome P450 as responsible for steroid hydroxylation reactions in both bacteria and filamentous fungi e.g. in bacteria - 6β in *Bacillus thermoglucosidasius* (Sideso *et al.*, 1998) and 15β in *Bacillus megaterium* (Berg *et al.*, 1976): in filamentous fungi - 7α in *Phycomyces blakesleeanus* (Ahmed *et al.*, 1995); 11α in *Aspergillus fumigatus* (Smith *et al.*, 1994), *Aspergillus ochraceus* (Samanta & Ghosh, 1987), *Nectria haematococca* (Ahmed *et al.*, 1996) and *Rhizopus nigricans* (Breskvar *et al.*, 1987); 11β in *Cochliobolus lunatus* (Janig *et al.*, 1992); and 15α in *Penicillium raistrickii* (Irrgang *et al.*, 1997).

Bacterial cytochromes P450 are the terminal proteins in a soluble three component system (Type I system) that transfer electrons to acceptor molecular dioxygen. P450 catalyses the reductive cleavage of the dioxygen producing H_2O and an -OH group which is ultimately covalently bonded to enzyme-bound substrate by the P450 apoprotein. The stereochemistry of the hydroxylation is determined by the particular structural architecture of the catalytic active centre of the apoprotein. The electrons that drive the hydroxylation originate from NADH passing to P450 via intermediate redox proteins. In Type-I systems these proteins are, a low molecular weight ferredoxin-like redoxin that directly interacts with the P450 and a larger flavin-containing redoxin reductase that is a one electron transducer positioned between NADH and the redoxin. This soluble three component system has been identified in the $P450_{soy}$ system of *S. griseus* (Trower *et al.*, 1992) and in the sulfonylurea herbicide P450 monooxygenase system in *Streptomyces griseolus* (O'Keefe *et al.*, 1991) where the component proteins have been purified.

A cytochrome P450 was purified from *Streptomyces roseochromogenes* strain 10984 (chapter 4) which catalyses progesterone 16α hydroxylation and a

second phase 2β hydroxylation (Berrie *et al.*, 1999), the basis of this chapter. Indeed the subsequent publication is based upon the phenomenon (designated, the 'roseoredoxin phenomenon') described in this chapter.
Two other proteins, a redoxin and a redoxin reductase together with the electron donor NADH were absolutely required for these hydroxylations (Berrie *et al.*, 1999). Further to the reconstitution in chapter 6; it is shown here that the ratio of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone produced in a reconstituted cell-free system, containing all three highly purified protein components, depends on the growth history of the *S. roseochromogenes* and that the change in metabolite ratio is manifested by the roseoredoxin rather than the roseoredoxin reductase or progesterone 2β,16α-hydroxylase cytochrome P450.

7.2 MATERIALS

Streptomyces roseochromogenes strain 10984 was purchased from the National Collection of Industrial and Marine Bacteria (NCIB) Ltd., Aberdeen, Scotland. Media and general chemicals were purchased from the sources previously described in chapter 2. Cibacron Blue FG3A and NAD-Sepharose affinity gel were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

7.3 METHODS

7.3.1 Media, Culture Maintenance and Cultivation of *Streptomyces roseochromogenes*

These were as previously described in chapter 2.

7.3.2 Determination of Progesterone Metabolite Yields Produced by *Streptomyces roseochromogenes*

This was as previously described in chapter 4.

7.3.3 Purification of Progesterone 2β,16α -Hydroxylase Cytochrome P450 from *Streptomyces roseochromogenes*

This was as previously described in chapter 4.

7.3.4 *In vitro* Cytochrome P450 Dependent Progesterone Hydroxylation

The progesterone 2β,16α-hydroxylase activity was determined using 4 mM progesterone and 2 mM NADH, 0.3μM progesterone 2β,16α-hydroxylase cytochrome P450, 0.05 units of roseoredoxin and 0.1 unit of roseoredoxin reductase made up to 1 ml with 0.1 M Na phosphate, 5 mM EDTA, 0.25mM DTT (buffer A) and 10% glycerol in Bijou bottles. Bottles were fixed to a turn-table that was vertically rotated at 40 rpm for 2 h at 25°C. Steroid metabolites were extracted from the incubations by shaking the mixtures with 1 ml chloroform for 1 min. The chloroform layer was removed and evaporated to dryness at 60°C. The residue was dissolved in 10 μl methanol and separated on HPLC as described in chapter 2.

7.3.5 Purification of Roseoredoxin & Roseoredoxin Reductase Electron Transfer Proteins from *Streptomyces roseochromogenes*

A detailed description of the purification of these two proteins is given in chapter 6. Fractions which reduced cytochrome c. oxidised NADH and contained FAD, were pooled in this protocol for the purification of the two electron transfer proteins.

7.3.6 Effect of Cell Growth - History on Progesterone Metabolite Synthesis in *in vitro* Reconstituted Incubations

Cultures of *S. roseochromogenes* were incubated in the normal way prior to harvesting and the purification of the three progesterone hydroxylase cellular components, as previously described. Progesterone metabolite synthesis was compared between assays comprising the hydroxylase components from cells grown in the presence of 0.32 mM progesterone for 8 h prior to harvesting and assays comprising cellular components from cells grown in the absence of an 8 h pre-incubation with progesterone. Metabolite yields were determined essentially by HPLC analysis as described in chapter 2.

7.3.7 Separation of the Two Isoforms of *Streptomyces roseochromogenes* Roseoredoxin

A qualitative comparison was made between the protein components of the hydroxylase system in progesterone pre-incubated and control cells by two dimensional polyacrylamide gel electrophoresis.

Results are shown in Figures 7.4.6a, 7.4.6b and 7.4.6c.

The roseoredoxin obtained from the NAD-Sepharose affinity purification step was separated into two activity fractions on Whatman CM-52 cellulose by stepwise elution between 50-100mM KCl and 240-280mM KCl. The isoforms co-migrated in 10% SDS-PAGE (Figure 7.4.7a.).

7.3.8 Isoelectric Focussing of the Isoforms of *Streptomyces roseochromogenes*

An identical two-dimensional gel electrophoresis procedure for analysis of the roseoredoxin, was used, as described in the general analytical procedures section in chapter 2.

7.3.9 Measurement of *Streptomyces roseochromogenes* Roseoredoxin and Roseoredoxin Reductase Activity

Roseoredoxin activity and roseoredoxin activity were measured according to the procedure described in section 6.3.3.

7.3.10 SDS Polyacrylamide Gel Electrophoresis and Protein Concentration Determination

These were performed as previously described in the general analytical procedures section, in chapter 2.

7.3.11 To determine whether the difference between the two forms of roseoredoxin was attributable to a genetic change induced by the progesterone in the growth medium ?

It was deduced that there must be more than one form of the roseoredoxin, as shown below in section 7.4. Cells were cultured in the normal way in YMG medium, as described previously. To mature cell cultures cycloheximide was added to a concentration of 100 µg/ml. Incubation was continued for a further 8.0 h, after which progesterone was added to a concentration of 0.32 mM. The incubation was continued for a further 8.0 h prior to cell harvesting as described previously. To a second stationary phase culture, progesterone was added to a concentration of 0.32 mM but no cycloheximide was added to this second culture. The third culture contained 100 µg/ml cycloheximide and the fourth culture was incubated identically as above but with no additions.

Table 7.3a summarises these growth conditions.

Culture	Growth conditions
1	Cycloheximide followed by the addition of progesterone
2	Progesterone alone
3	Cycloheximide alone
4	No progesterone & no cycloheximide

Table 7.3a. Summary of the growth conditions described.

Cells were harvested as described previously. From these harvested cells, roseoredoxin, roseoredoxin reductase and cytochrome P450, were all purified to homogeneity.

7.3.12 Examination of Reconstitution Assays Comprising the Components Purified From Progesterone Pre-incubated and Control Cells

Reconstituted assays were performed as described in section 7.3.6. Assays were terminated after 40.0 min (the optimum incubation time determined in chapter 6). The steroidal metabolites were extracted and analysed by HPLC as described in chapter 2. Retention times and peak areas were compared.

Protein composition of reconstitution assays :

1. Roseoredoxin(a) + Roseoredoxin reductase(a) + P450(a)
2. Roseoredoxin(a) + Roseoredoxin reductase(a) + P450(p)
3. Roseoredoxin(a) + Roseoredoxin reductase(p) + P450(p)
4. Roseoredoxin(a) + Roseoredoxin reductase(p) + P450(a)
5. Roseoredoxin(p) + Roseoredoxin reductase(p) + P450(p)
6. Roseoredoxin(p) + Roseoredoxin reductase(p) + P450(a)
7. Roseoredoxin(p) + Roseoredoxin reductase(a) + P450(p)
8. Roseoredoxin(p) + Roseoredoxin reductase(a) + P450(a)

Where **(a)** represents component purified from cells grown in the absence of progesterone and **(p)** represents component purified from cells grown in the presence of progesterone. The same assays were established where components were purified from cells grown in the presence of progesterone but where cycloheximide had been added to cultures 8 h prior to the addition of progesterone. The components were also substituted for counterparts purified from cells grown in the presence of cycloheximide but in the absence of progesterone.

7.3.13 Time course of reconstitution assays comprising components purified from cells grown in the presence and absence of progesterone

Assays containing all three of the cellular factors (Figures. 7.4.1b. & 7.4.8.) were run incubated for 40 min. The assays were established as described in chapter 6 but each component substituted for its counterpart purified from cells pre-incubated with 0.32 mM progesterone. The assay compositions are listed in section 7.3.12, above.

7.4 RESULTS

7.4.1 Hydroxyprogesterone Metabolite Production by Intact *S. roseochromogenes* Cells

The HPLC trace of the hydroxyprogesterone metabolites produced in a 25 h whole cell transformation of progesterone by *S. roseochromogenes* is shown in Figure 7.4.1a. Two metabolites were identified. The minor peak 1 (retention time 14 min) was 2β,16α-dihydroxyprogesterone and the major peak 2 (retention time 27 min) was 16α-monohydroxyprogesterone. Peak 3 is untransformed substrate progesterone. The NMR identifying features of these compounds have been published, Smith *et al.* (1989) and Kirk *et al.* (1990).

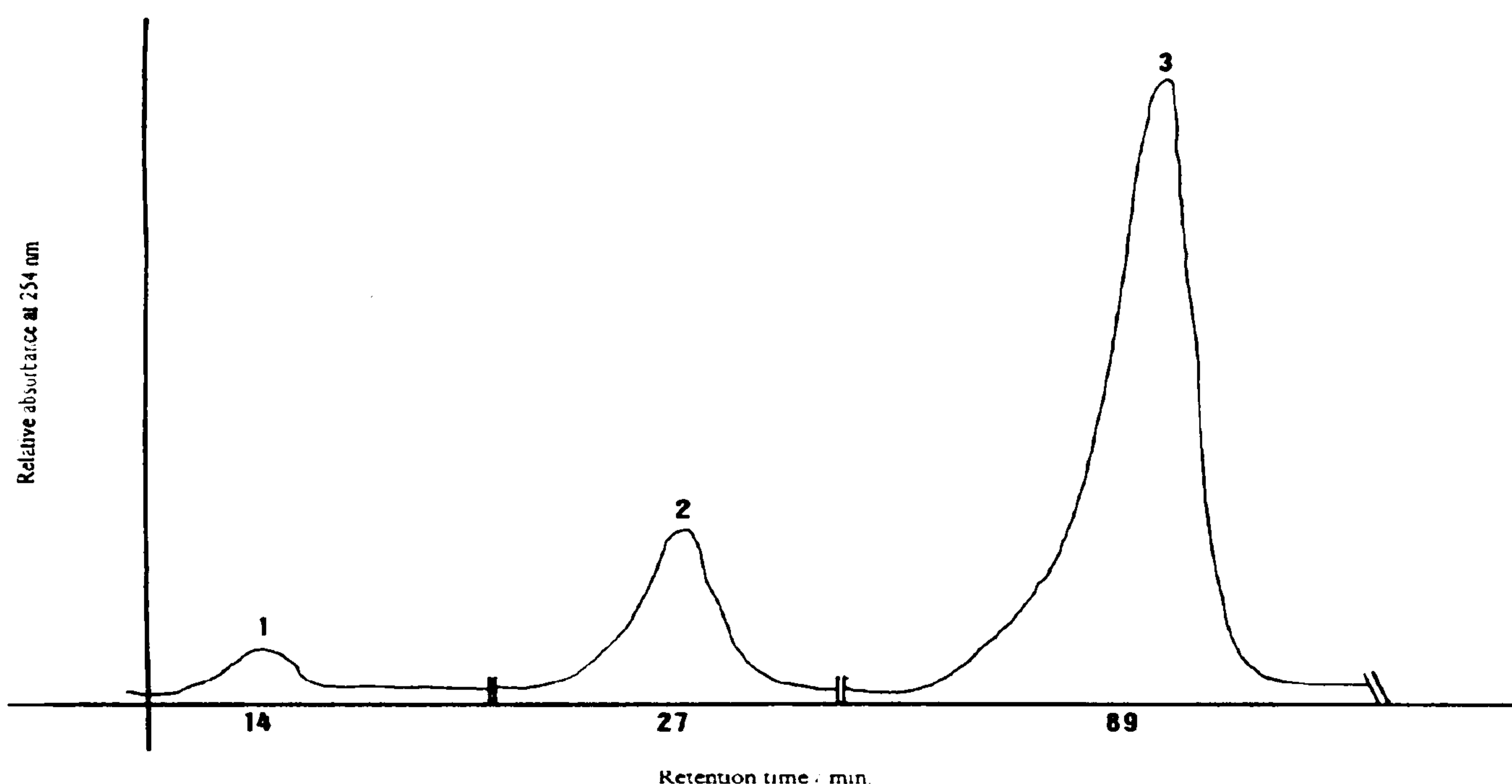

Figure 7.4.1a. HPLC of 25 h progesterone transformation incubation by *S. roseochromogenes*. From left to right, peak 1, 16α-dihydroxyprogesterone (retention time 14 min); Peak 2, 16α-monohydroxyprogesterone (retention time 27 min); Peak 3, progesterone (retention time 89 min)

Ratios of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone produced during this 25 h incubation are given in chapter 6. From 8 h onward in whole cell biotransformations, 2β,16α-dihydroxyprogesterone also accumulated. The ratio of 16α-monohydroxyprogesterone to 2β,16α-

dihydroxyprogesterone gradually declined from an initial value of 61 at 8 h to a final value of 3.6 from 21 h onward. This change in metabolite ratio reflected dihydroxylated metabolite enrichment consequent on second phase 2β-hydroxylation.

Assay after 40 min.	2β,16α-DHP % total transformation products	16α-HP % total transformation products	Ratio of 16α-HP to 2β,16α-DHP
1 Roseoredoxin(a) + Roseoredoxin reductase(a) + P450(a)	9.03	90.97	10.1
2 Roseoredoxin(a) + Roseoredoxin reductase(a) + P450(p)	8.81	91.19	10.4
3 Roseoredoxin(a) + Roseoredoxin reductase(p) + P450(p)	9.65	90.35	9.4
4 Roseoredoxin(a) + Roseoredoxin reductase(p) + P450(a)	8.95	91.05	10.2
5. Roseoredoxin(p) + Roseoredoxin reductase(p) + P450(p)	25.97	74.03	2.9
6 Roseoredoxin(p) + Roseoredoxin reductase(p) + P450(a)	26.03	73.97	2.8
7 Roseoredoxin(p) + Roseoredoxin reductase(a) + P450(p)	25.9	74.1	2.9
8 Roseoredoxin(p) + Roseoredoxin reductase(a) + P450(a)	26.36	73.64	2.8
	Minor	Major	

Table 7.4a. Result of HPLC analysis of the assays established in section 7.3.12

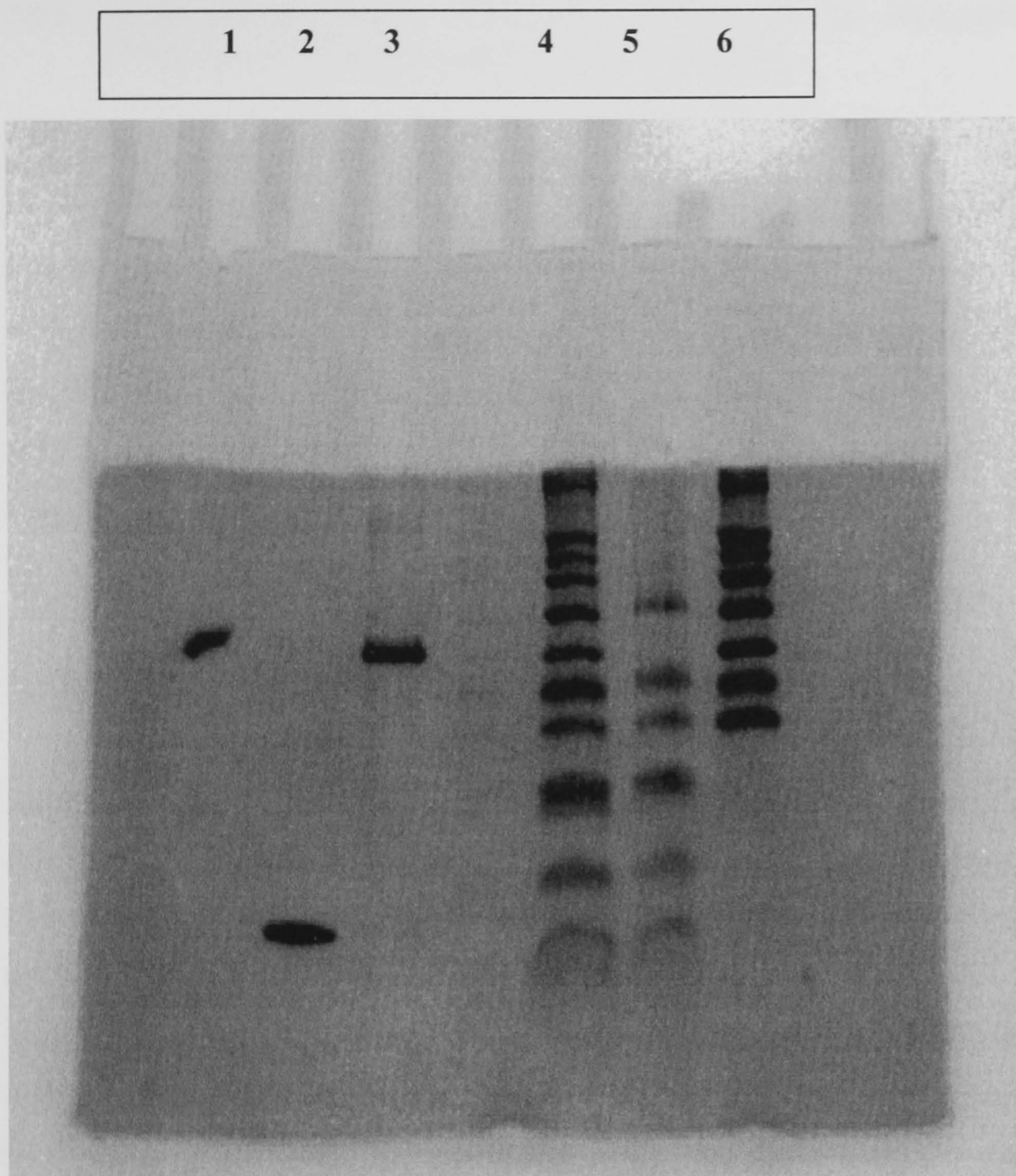

Figure 7.4.1b SDS-15% polyacrylamide gel of purified *S. roseochromogenes* 16α-hydroxylase cytochrome P-450, roseoredoxin and roseoredoxin reductase. From left to right, **lane 1**, roseoredoxin reductase (3.1 μg M_r 65 x 10^3); **lane 2**, roseoredoxin (2.8 μg M_r 14 x 10^3); **lane 3**, progesterone 16α-hydroxylase cytochrome P450 (4 μg M_r 63 x 10^3); **lane 4**, wide range protein size ladder M_r 205 x 10^3 - 14.2 x 10^3; **lane 5**, lower range protein size ladder M_r 66 x 10^3 - 14.2 x 10^3; and **lane 6**, upper range protein size ladder M_r 205 x 10^3 - 45 x 10^3. Lanes 4-6 contained 35 μg total protein.

7.4.2 Hydroxyprogesterone Metabolite Production in Reconstituted Progesterone Hydroxylation Cell-Free Systems

The two progesterone metabolites produced in whole cell transformations were also produced in reconstituted cell-free incubations containing highly purified progesterone hydroxylase cytochrome P450, roseoredoxin and roseoredoxin reductase. When all three proteins were from control i.e. non progesterone pre-incubated cells, a value of 10.1 was calculated for the relative synthesis of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone in a 40 min incubation (Table 7.4a, line 1).

7.4.3 Results of Steroidal Metabolite Analysis by HPLC

Each assay was initiated by the addition of NADH to a concentration of 0.2 mM in 0.1 M potassium phosphate buffer following addition of progesterone. Reaction times were initially varied from 0 - 40 min and reactions were quenched by the addition of 0.5 ml chloroform.

Table 7.4a. shows the results of reconstituted cell-free progesterone incubations containing purified roseoredoxin, roseoredoxin reductase and cytochrome P450. In Table 7.4a., the first column is the assay and the subsequent columns describe the HPLC result. The assays are represented thus :

(a) represents component purified from cells grown in the absence of progesterone and (p) represents component purified from cells grown in the presence of progesterone.

Table 7.4a. shows that assays 1,2,3 and 4 produced an approximate product ratio of 10 : 1 for 16α hydroxyprogesterone to 2β,16α dihydroxyprogesterone, respectively. Assays 5,6,7 & 8 produced an approximate product ratio of 3 : 1 for the same respective metabolites.

7.4.4. Effect of an 8 h Progesterone Pre-incubation on the Relative Synthesis of 16α-Monohydroxyprogesterone and 2β,16α-Dihydroxyprogesterone in Reconstituted Progesterone Hydroxylase Cell-Free Systems

The growth history of the cells was immaterial to the ultimate relative synthesis of the two hydroxylated progesterone metabolites only if the cell-free incubations contained either progesterone hydroxylase cytochrome P450 or roseoredoxin reductase (or both) from control or 8 h progesterone pre-incubated cells (Table 7.4a., lines 2,3&4). Thus relative synthesis ratios of 10.35, 10.17 and 9.36 respectively were obtained when the P450 and the roseoredoxin reductase originated from progesterone pre-incubated or control cells but only the roseoredoxin was from control cells. By contrast the relative synthesis of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone was 2.79 when the roseoredoxin originated from progesterone pre-incubated cells but the P450 and roseoredoxin reductase were from control or progesterone pre-incubated cells. No substantive difference in the product ratio produced (2.85) was noted if all the cell-free protein components were derived from progesterone pre-incubated *S. roseochromogenes* (Table 7.4a., line 5). Incubation time was not a factor in the roseoredoxin effect. Thus, although the absolute amount of both metabolites increased in incubations lasting from 2 to 40 min, the same metabolite ratios shown in Table 7.4a. were always obtained.

7.4.5 Effect of Cycloheximide Pre-Incubation on the Relative Synthesis of 16α-Monohydroxyprogesterone and 2β,16α-Dihydroxyprogesterone in Reconstituted *S. roseochromogenes* Progesterone Hydroxylation Cell-Free Systems Containing Control and Progesterone Pre-Incubated Roseoredoxin

The data in Table 7.4a. clearly show that the roseoredoxin component of the P450 electron transfer chain modulates the relative synthesis of 16α-monohydroxyprogesterone and 2β,16α-dihydroxyprogesterone. We examined whether progesterone pre-incubation induced *de novo* synthesis of a roseoredoxin isoform or whether a post-translational modification of the protein had occurred. Gene expression was inhibited in 8 h progesterone pre-incubated cells by blocking protein synthesis with 0.335 mM cycloheximide, a concentration in excess of that required to inhibit totally, cell growth. The progesterone hydroxylation profile of the reconstituted cell-free incubations containing roseoredoxin purified from these cycloheximide and progesterone pre-treated cells (16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone ratio 2.8) was identical to the progesterone only pre-incubated counterpart (ratio 2.8) contrasting the 10:1 ratio in the non pre-incubated cell-free assays (chapter 4). These results are consistent with post-translational modification of the roseoredoxin rather than there being multiple roseoredoxin genes.

7.4.6. Identification of Two Isoforms of *S. roseochromogenes* Roseoredoxin in Progesterone Pre-incubated Cells

The roseoredoxin from the NAD-Sepharose purification stage from control and progesterone pre-incubated cells was analysed by isoelectric focussing (IEF). Control, non progesterone pre-incubated cells, contained only the pI 7.45 roseoredoxin isoform (Figure 7.4.6a). By contrast the pI 5.6 isoform predominated in 8 h progesterone pre-incubated cells and only a minor pI 7.45

band was observed (Figure 7.4.6.b). Interestingly, both isoforms were present in approximately equal amounts in 4 h progesterone pre-incubated cells (Figure 7.4.6c). The two roseoredoxin isoforms were separable by CM-52 cellulose ion exchange chromatography (Table 7.4.7a.). Figure 7.4.7a in the next section, shows the two isoforms of roseoredoxin. 5.0 μg of roseoredoxin was loaded on each IEF gel in Figures 7.4.6a, 7.4.6b and 7.4.6c, prior to electrophoresis in the first dimension. These photographs show the isoforms observable following the second electrophoretic dimension.

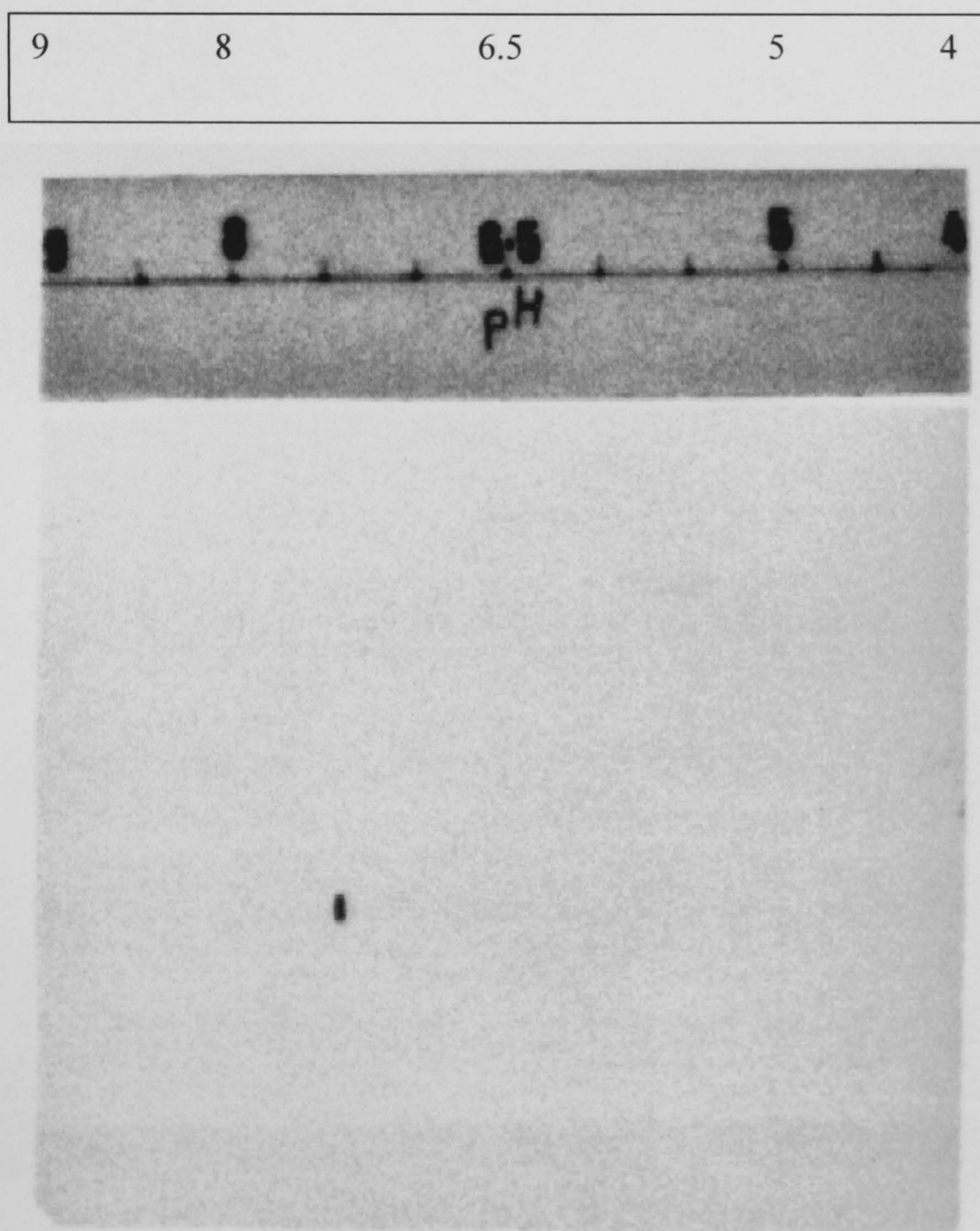

Figure 7.4.6a Roseoredoxin purified from control cells.

Figure 7.4.6a shows the form of pI 7.45 present alone. Here there was no pre-incubation of the *S. roseochromogenes* cells with progesterone prior to purification of the roseoredoxin.

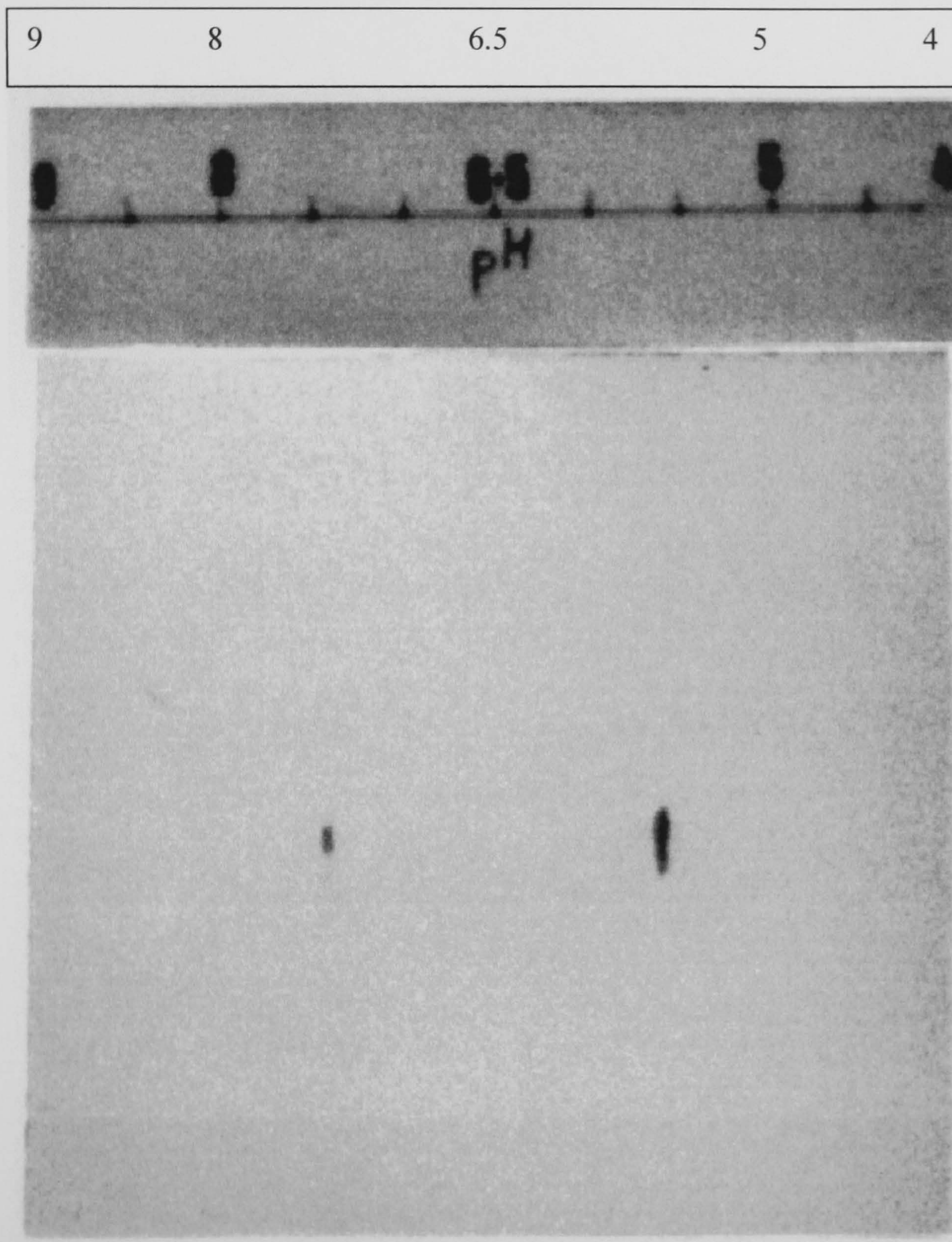

Figure 7.4.6b. Roseoredoxin purified from 8 h progesterone pre-incubated cells.

Figure 7.4.6b, shows a photograph of the second dimension. The spot corresponding to pI 5.6, of much greater intensity than that corresponding to pI 7.45. This shows that the roseoredoxin isoform of pI 5.6 is the predominant isoform produced under these conditions of progesterone pre-exposure.

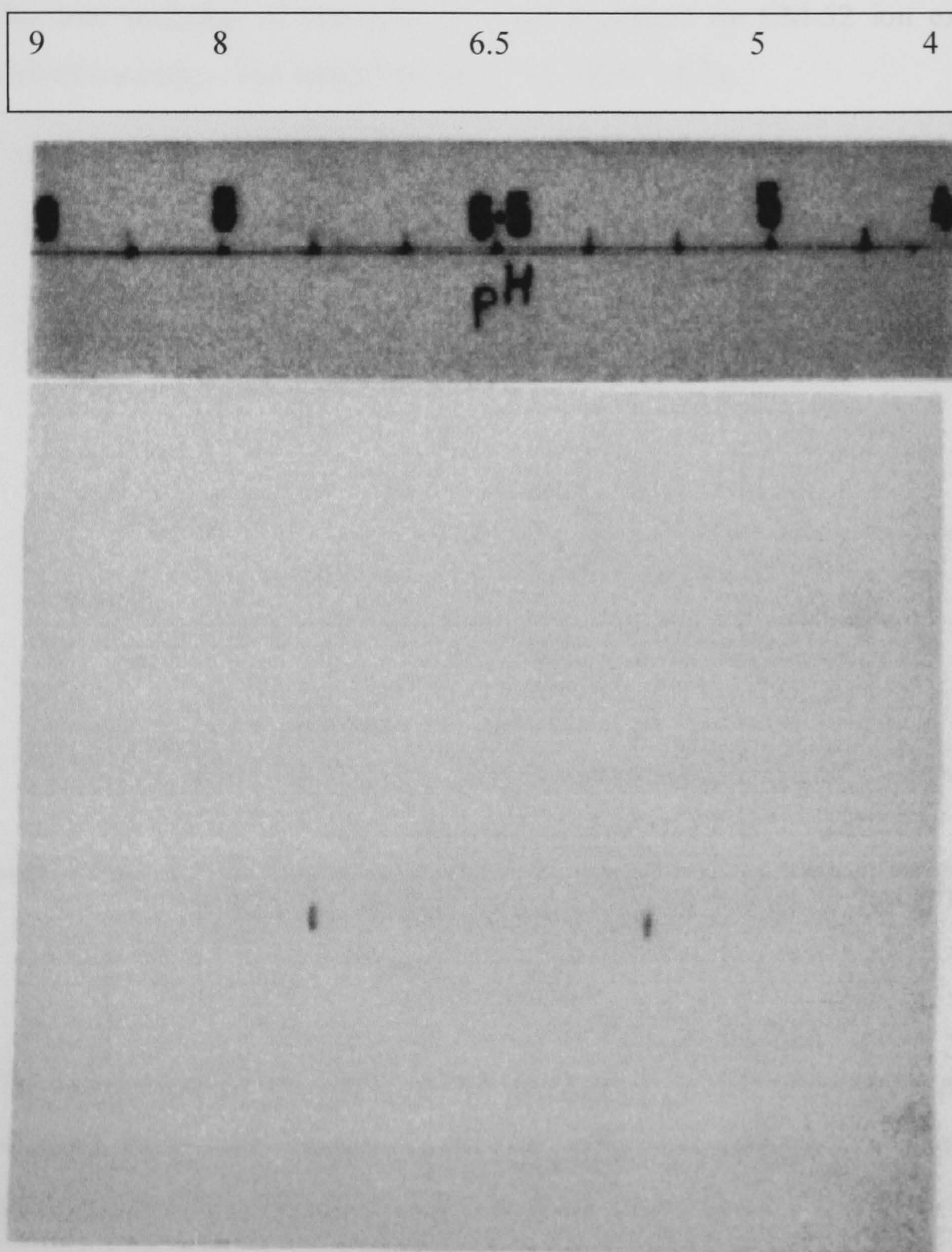

Figure 7.4.6c. Roseoredoxin purified from 4 h progesterone pre-incubated

The photograph in Figure 7.4.6c shows the two spots in the second dimension corresponding to pI 7.45 and pI5.6, to be of relatively equal intensity and therefore appear to be present in equal amounts.

7.4.7. **Isoforms of Roseoredoxin**

The two isoforms of roseoredoxin were separated by CM-52 ion exchange chromatography. The results are shown in Table 7.4.7a.

Purification Stage	Total Protein (mg)	Total Roseoredoxin (nmol)	Yield roseoredoxin (nmol/mg total protein)	Recovery roseoredoxin (%)
CM cellulose 50-100 mM KCl fraction	2.0	79.2	39.6	55.4
CM cellulose 240 - 280 mM KCl fraction		63.7	31.85	44.6

Table 7.4.7a. Separation two isoforms of *S. roseochromogenes* roseoredoxin by CM-52 cellulose ion exchange chromatography as described in section 7.3.6 and 7.3.7.

The photograph in Figure 7.4.7a. shows the two isoforms of roseoredoxin from separate fractional ranges from the CM 52 column. They were indistinguishable at this stage as they were of the same molecular mass.

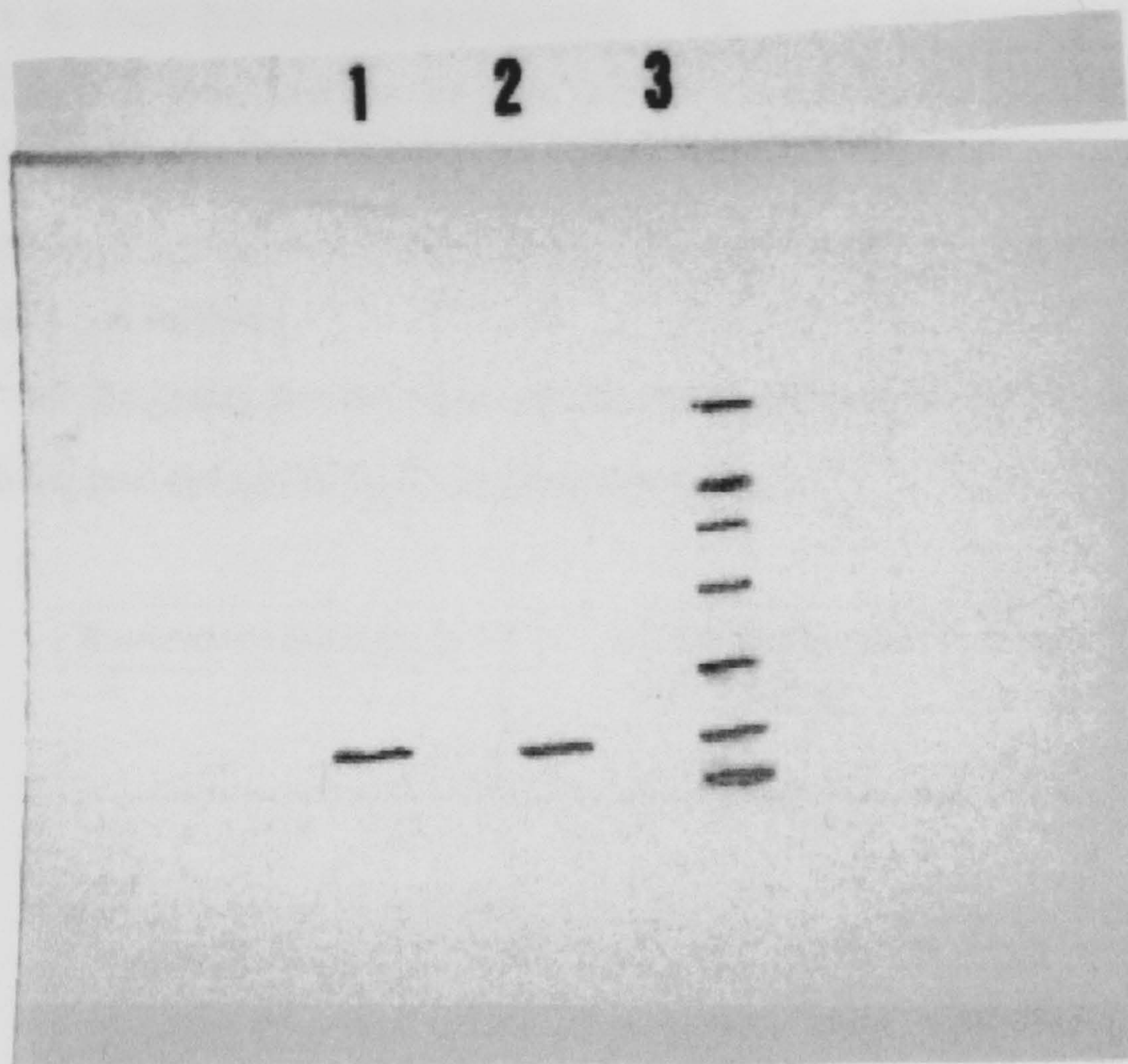

Figure 7.4.7a. 10 % polyacrylamide gel of *S. roseochromogenes'* roseoredoxin isoforms.

Lane 1, 50-100 mM KCl roseoredoxin isoform fraction from CM 52 (2.8 μg M_r 14 x 10^3)
Lane 2, 240-280 mM KCl roseoredoxin isoform fraction from CM 52 (2.8 μg M_r 14 x10^3)
Lane 3, protein ladder (35 μg total protein, M_r 66 x 10^3 - 14.2 x 10^3 (M_r x 10^3 from the top of the gel to bottom: 66, BSA; 45, chicken egg ovalbumin; 36, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase; 29, bovine erythrocyte carbonic anhydrase; 24, bovine pancreas trypsinogen; 20, soybean trypsin inhibitor and 14.2, bovine milk α-lactalbumin.

7.4.8. Isoelectric Focussing of the Isoforms of *Streptomyces roseochromogenes'* Roseoredoxin

The same procedure for two-dimensional gel electrophoresis was used here, as described in the general analytical procedures section in chapter 2. The two cellular components were separated from each other by two dimensional polyacrylamide gel electrophoresis. In the first dimension they were separated on the basis of their pI. The second dimension did not separate the two components but showed that they were of the same relative molecular mass,

14000Da as they had co-electromigrated. The three photographs, Figures 7.4.6a, 7.4.6b & 7.4.6c, show the two isoforms separable by 2D-IEF. These results indicate that the longer the pre-incubation period of cells with progesterone, the greater the conversion of the constitutive roseoredoxin to the modified pI 5.6 isoform.

Figure 7.4.8 illustrates the two-dimensional electrophoresis of the whole 2β and 16α hydroxylase system from *S. roseochromogenes*.

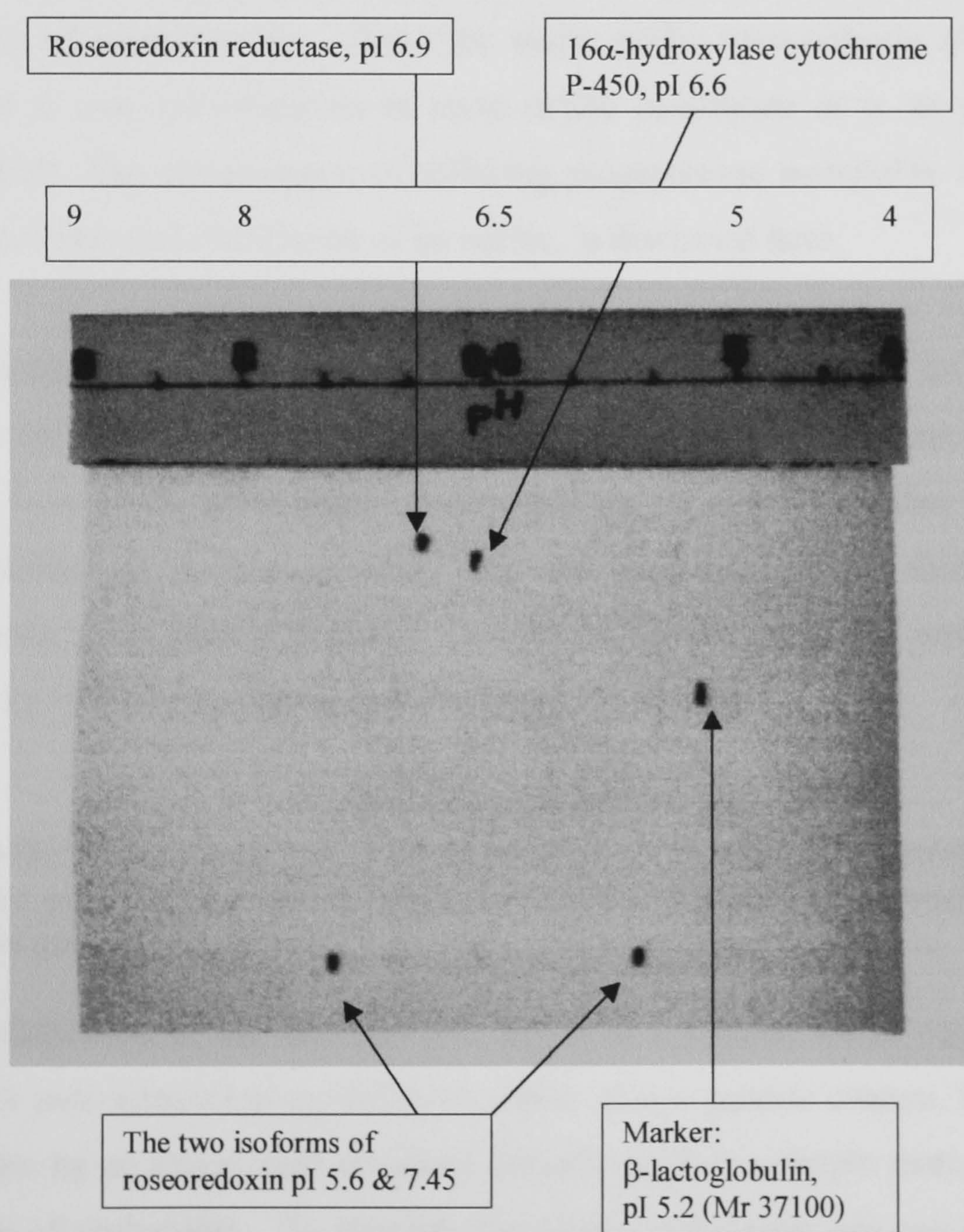

Figure 7.4.8 2-D, IEF gel of *S. roseochromogenes* roseoredoxin, roseoredoxin reductase and 2β,16α-hydroxylase cytochrome P-450 :
roseoredoxin reductase pI 6.9, 16α-hydroxylase cytochrome P-450 pI 6.6, β-lactoglobulin pI 5.2 (M_r 37,100), and the two isoforms of roseoredoxin pI 5.6 and 7.45. The gel was silver stained.

7.5 DISCUSSION

Isoelectric focussing of the two proteins, roseoredoxin and roseoredoxin reductase in the work presented here, revealed that the roseoredoxin is separable into two fractions. One having a pI of 7.45 and the other 5.6, initially raising doubts over its purity. However, the presence of two spots, only on a two-dimensional electrophoretogram lead to the conclusion of the presence of two forms of roseoredoxin. Does the more acidic roseoredoxin allow for growth of *S. roseochromogenes* in more acidic conditions or is its presence mechanistic? The phenomenon of differing progesterone metabolite ratios by the two isoforms and elucidation of its nature, is discussed here.

Roseoredoxin was the common cellular factor which according to its history, provided the phenomenon of an altered progesterone metabolite ratio. More than one form of the same protein meant that the progesterone either initiated the production of another protein i.e.: an inducible protein thereby the phenomenon being genetic in nature. Or a change to the expressed protein was initiated i.e.: a post translation modification.

7.5.1 Is the difference between the two forms of roseoredoxin attributable to a post translation modification, induced by the progesterone in the growth medium?

The demonstration of the fact that two forms of a cellular factor co-exist by virtue of a post-translation modification rather than a genetic change, has been shown here by an experiment designed initially around a simple mathematical evaluation of probability. To simplify the whole experiment one can envisage three positions, say, A,B & C. Any one position can be filled with either of two possibilities, say, position A could be A1 or A2 etc. There are therefore $2^3 = 8$ possible incubations of progesterone preincubated and non pre-incubated P450,

roseoredoxin and roseoredoxin reductase. This is how the roseoredoxin was found to be responsible for the observed metabolite ratio change from whole cell ratios obtained from cells grown in the absence of progesterone.
No other cellular factor effected this change. Hence the term 'the roseoredoxin phenomenon'.

7.5.2 Why should there be an increase in the number of hydroxylation events occurring per unit time for the hydroxylase system pre exposed to progesterone?

Clearly, the number of hydroxylation events increases in reconstitution assays (Berrie *et al.*, 1999) due to the post translation modification described above. However, the reason why this should occur is unclear. The fact that cytochromes P450 are generally regarded as xenobiotic metabolisers (Sariaslani, 1991), (Taylor *et al.*, 1999), (Trower *et al.*, 1988) may point to a possible role for this phenomenon. In this example progesterone is not a substrate *S. roseochromogenes* would normally encounter in its natural environment and increasing the hydroxylase ability of this P450 system, may, from an evolutionary point of view be one mechanism employed to deal with such xenobiotics.

7.5.3 Why should there be a difference between roseoredoxin from cells grown in the presence of progesterone and roseoredoxin from cells grown in the absence of progesterone?

In contrast with that discussed above, electron transfer protein isoforms can exist as constitutive and distinct genetically different cellular factors, probably serving a multifunctional role. In the case of *S. griseolus* (Omer *et.al.,* 1990) two 7 kDa ferredoxin isoforms co-exist, designated Fd-1 and Fd-2. These proteins have 52% mutual identity and both contain single [3Fe-4S] clusters (Nagy *et.al.*, 1995). Both ferredoxins function in reconstituted cell-free

systems containing the SU1 isoform of P450 although Fd-2 is the more active protein. The genes for the ferredoxins and the sulfonylurea P450 monooxygenases are virtually contiguous. Thus P450SU1 and the downstream Fd-1 form a closed-spaced pair, similarly P450SU2 and Fd-2. This is in contrast to the findings presented here because in *S. roseochromogenes*, only one of the roseoredoxin isoforms is constitutive. The fact that a second isoform exists is dependent upon the environmental history of the cells and is a post translation modification of the same protein, not a genetic change.

Ferredoxin-like iron sulphur containing proteins required for cytochrome P450 monooxygenase activity have been identified in a wide variety of bacteria e.g. *Bacillus megaterium* ATCC 13368 (Rauschenbach *et.al.*, 1993), *Bradyrhizobium japonicum* (Tully *et.al.*, 1998), *Pseudomonas incognita* (Peterson *et.al.*, 1992), *Pseudomonas putida* (Koga *et.al.*, 1989), *Rhodococcus* SP strain NI86/21 (Nagy *et.al.*, 1995), *Saccharopolyspora erythraea*, CA340 (Shafiee & Hutchinson, 1988), *Streptomyces griseolus* (O'Keefe *et.al.*, 1991), *Streptomyces griseus* (Trower *et.al.*, 1993), *Xanthobacter* sp., (Trickett *et.al.*, 1991). Where sequence analysis has been performed, the ferredoxin-like protein genes seem to be constituents of operons containing respectively the P450 gene and the redoxin reductase gene (the significance of this was discussed in chapter 6). However, in this case for *S. roseochromogenes*, we do not know this.

Less in known about the situation in *S. griseus* where only a single ferredoxin encoded by SOY B has been putatively identified (Trower *et.al.*, 1993).

Results here suggest that a novel system for ferredoxin-like protein expression, namely post-translational modification, probably operates in *Streptomyces*. In control cells grown in a standard medium a single, mildly basic and soluble form of roseoredoxin (pI 7.45) is present. This protein, like its published bacterial counterparts forms the middle component of a natural cytochrome P450 electron transfer chain. *In vitro*, this *S. roseochromogenes* system

predominantly catalyses 16α monohydroxylation of progesterone and as a rather minor secondary reaction 2β hydroxylation of the primary product. By contrast, pre-incubating the organism with progesterone results in the appearance of a second but more acidic, soluble form of roseoredoxin (pI 5.6). This modified form increases the 2β hydroxylation capability of the cytochrome P450, evinced by a greater hydroxylation turnover number (Berrie *et al.*, 1999) of the P450 resulting in a decline in the 16α-monohydroxy to 2β,16α-dihydroxprogesterone ratio. That a supra optimal growth inhibitory dose of cycloheximide added to the cells for 8 h prior to the progesterone pre-incubation had no effect on the appearance of the pI 5.6 isoform is strongly consistent with a progesterone dependent post translational modification of the roseoredoxin rather than for transcriptional activation of a second gene. There is no literature precedent for the regulation of ferredoxin activity by post-translational modification.

Bacterial responses to changes in their environment are largely controlled by signal transduction systems that contain two central enzymatic components, a protein kinase that uses adenosine triphosphate to phosphorylate itself at a histidine residue and a response regulator that accepts phosphoryl groups from the kinase. This conserved phosphotransfer chemistry operates in diverse systems to provide different regulatory outputs (Stock *et al.*, 1989). What is the nature of the post-translational modification in roseoredoxin from *S. roseochromogenes* and how does it modulate the activity of the P450? The covalent attachment of an acidic group such as phosphate is an obvious candidate to account for the 1.85 pH unit decrease of the roseoredoxin pI value. This enhanced 2β hydroxylation capacity of the P450 directed by the modified roseoredoxin isoform must be a consequence of a change in the efficiency of electron transfer from NADH to the P450.

Chapter 8

Structural Analysis of the *S. roseochromogenes* Progesterone 2β,16α Hydroxylase P450 and Ancillary Electron Transfer Proteins

8.1 INTRODUCTION

8.1.1 Crystallisation by Vapour Diffusion

This is the most commonly used technique for crystallisation and was first used in the crystallisation of tRNA (Hampel *et al.*, 1968).

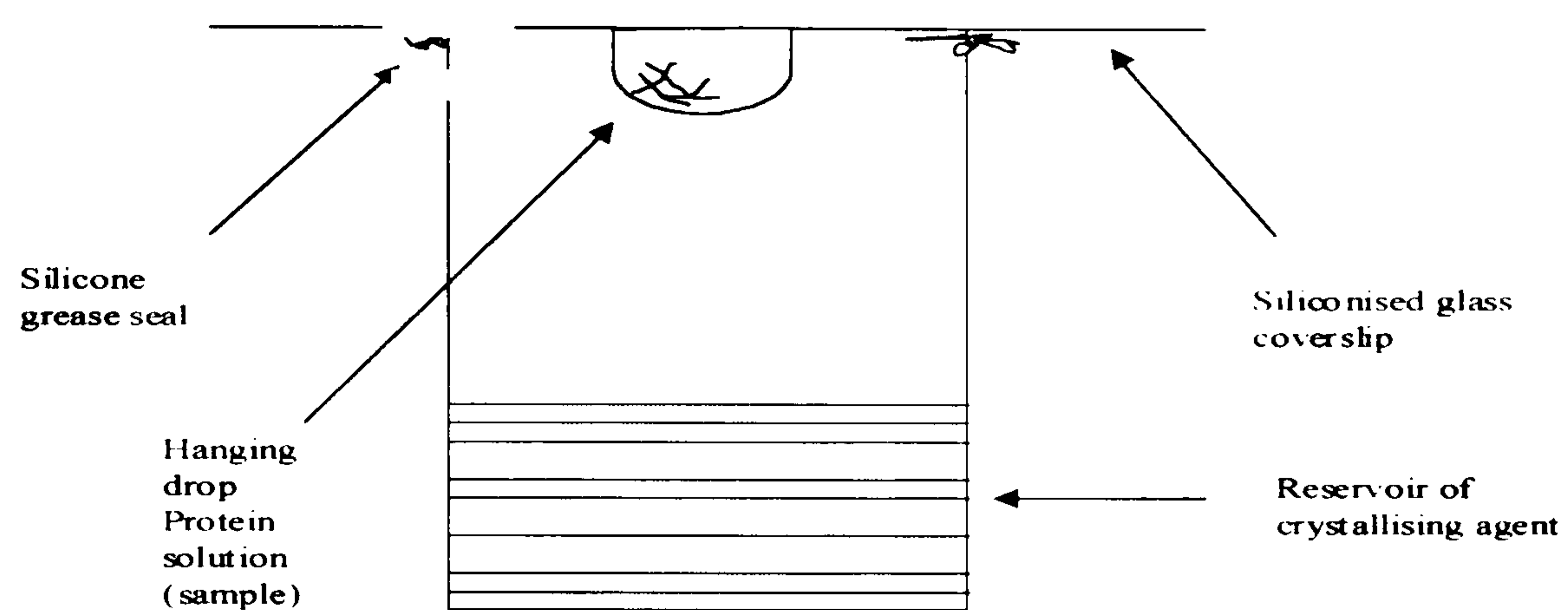

Figure 8.1a Representation of the hanging drop set up for vapour diffusion. Each well in a Linbro box has a capacity of 2 ml and an internal diameter of 16 mm.

The principle of vapour diffusion crystallisation is indicated in Figure 8.1a. A droplet containing the macromolecule to be crystallised, buffer, crystallising agent and additives, is equilibrated against a reservoir containing a solution of crystallising agent at a higher concentration than the droplet. If equilibration occurs by diffusion of water from the hanging drop then all components of the drop become more concentrated with species possessing a vapour pressure higher than water, exchange occurs from the reservoir to the drop. In this case the drop volume will increase and the constituents become less concentrated.

The aim in any type of crystallisation trial is to achieve a supersaturated solution, which will nucleate and give rise to crystals which are as large as possible (Stura & Wilson, 1990).

8.1.2 Protein Crystals

A protein crystal tends to be a rather open three-dimensional lattice with 30 - 90 % of the crystal volume occupied by water molecules. Relatively little protein surface area is involved in protein - protein contact. Rather, each protein molecule is surrounded by a very ordered arrangement of water molecules by virtue of their hydrogen bonding networks. The types of interactions involved in crystal formation are like those which stabilise protein structure i.e. hydrogen bonding, ion pairs, hydrophobic interactions and even metal co-ordination. With membrane proteins, the crystal lattice also has protein - detergent and detergent - detergent micelle like interactions. As a result all protein crystals are soft and sensitive to environmental factors such as humidity.

8.1.3 The use of Crystal Screen™.

On examination of Table 8a. it can be seen that a range of 50 relatively broad conditions were used in this method known as sparse matrix sampling (Jancarik & Kim , 1991). If crystals are obtained in any one of the trials, the conditions within that trial can be varied to attempt to increase the size or number of crystals. Two variables can be chosen, such as pH and PEG concentration, then new trials are set up with these two components which are varied around the deduced and projected conditions of the initial incomplete factorials.

Table 8a. Crystal Screen Reagent Formulation

Trial	Salt	Buffer	Precipitant
1	0.02 M calcium chloride dihydrate	0.1 M sodium acetate trihydrate pH 4.6	30 % v/v 2-methyl-2,4-pentanediol
2	None	None	0.4 M potassium sodium tartrate tetrahydrate
3	None	None	0.4 M mono-ammonium dihydrogen phosphate
4	None	0.1 M tris-hydrochloride pH 8.5	2.0 M ammonium sulphate
5	0.2 M tri-sodium citrate dihydrate	0.1 M HEPES - Na pH 7.5	30 % v/v 2-methyl-2,4-pentanediol
6	0.2 M magnesium chloride hexahydrate	0.1 M tris-hydrochloride pH 8.5	30 % w/v polyethylene glycol 4000
7	None	0.1 M sodium cacodylate pH 6.5	1.4 M sodium acetate trihydrate
8	0.2 M tri sodium citrate dihydrate	0.1 M sodium cacodylate pH 6.5	30 % v/v iso-propanol
9	0.2 M ammonium acetate	0.1 M tri-sodium citrate dihydrate pH 5.6	30 % w/v polyethylene glycol 4000
10	0.2 M ammonium acetate	0.1 M sodium acetate trihydrate pH 4.6	30 % w/v polyethylene glycol 4000
11	None	0.1 M tri sodium citrate dihydrate pH 5.6	1.0 M mono-ammonium dihydrogen phosphate
12	0.2 M magnesium chloride hexahydrate	0.1 M HEPES - Na pH 7.5	30 % v/v iso-propanol

13	0.2 M tri sodium citrate dihydrate	0.1 M tris-hydrochloride pH 8.5	30 % w/v polyethylene glycol 400
14	0.2 M calcium chloride dihydrate	0.1 M HEPES - Na pH 7.5	28 % w/v polyethylene glycol 400
15	0.2 M ammonium sulphate	0.1 M sodium cacodylate pH 6.5	30 % w/v polyethylene glycol 8000
16	None	0.1 M HEPES - Na pH 7.5	1.5 M lithium sulphate monohydrate
17	0.2 M lithium sulphate monohydrate	0.1 M tris-hydrochloride pH 8.5	30 % polyethylene glycol 4000
18	0.2 M magnesium acetate tetrahydrate	0.1 M sodium cacodylate pH 6.5	20 % w/v polyethylene glycol 8000
19	0.2 M ammonium acetate	0.1 M tris-hydrochloride pH 8.5	30 % v/v iso-propanol
20	0.2 M ammonium sulphate	0.1 M sodium acetate trihydrate pH 4.6	25 % w/v polyethylene glycol 4000
21	0.2 M magnesium acetate tetrahydrate	0.1 M sodium cacodylate pH 6.5	30 % v/v 2-methyl-2,4-pentanediol
22	0.2 M sodium acetate trihydrate	0.1 M tris-hydrochloride pH 8.5	30 % w/v polyethylene glycol 4000
23	0.2 M megnesium chloride hexahydrate	0.1 M HEPES - Na pH 7.5	30 % v/v polyethylene glycol 400
24	0.2 M calcium chloride dihydrate	0.1 M sodium acetate trihydrate pH 4.6	20 % v/v iso-propanol
25	None	0.1 M imidazole pH6.5	1.0 M sodium acetate trihydrate
26	0.2 M ammonium acetate	0.1 M tri-sodium citrate dihydrate pH 5.6	30 % v/v 2-methyl-2,4-pentanediol

27	0.2 M tri-sodium citrate dihydrate	0.1 M HEPES - Na pH 7.5	20 % v/v iso-propanol
28	0.2 M sodium acetate trihydrate	0.1 M sodium cacodylate pH 6.5	30% w/v polyethylene glycol 8000
29	None	0.1 M HEPES - Na pH 7.5	0.8 M potassium sodium tartrate tetrahydrate
30	0.2 M ammonium sulphate	None	30 % w/v polyethylene glycol 8000
31	0.2 M ammonium sulphate	None	30 % w/v polyethylene glycol 4000
32	None	None	2.0 M ammonium sulphate
33	None	None	4.0 M sodium formate
34	None	0.1 M sodium acetate trihydrate pH 4.6	2.0 M sodium formate
35	None	0.1 M HEPES - Na pH 7.5	0.8 M mono-sodium dihydrogen phosphate
36	None	0.1 M tris-hydrochloride pH 8.5	8 % w/v polyethylene glycol 8000
37	None	0.1 M sodium acetate trihydrate pH 4.6	8 % w/v polyethylene glycol 4000
38	None	0.1 M HEPES - Na pH 7.5	1.4 M tri-sodium citrate dihydrate
39	None	0.1 M HEPES - Na pH 7.5	2 % v/v polyethylene glycol 400, 2.0 M ammonium sulphate

40	None	0.1 M tri-sodium citrate dihydrate pH 5.6	20 % v/v iso-propanol. 20%w/v polyethylene glycol 4000
41	None	0.1 M HEPES - Na pH 7.5	10% v/v iso-propanol, 20%w/v polyethylene glycol 4000
42	0.05 M mono-potassium dihydrogen phosphate	None	20 % w/v polyethylene glycol 8000
43	None	None	30 % w/v polyethylene glycol 1500
44	None	None	0.2 M magnesium formate
45	0.2 M zinc acetate dihydrate	0.1 M sodium cacodylate pH 6.5	18 % w/v polyethylene glycol 8000
46	0.2 M calcium acetate hydrate	0.1 M sodium cacodylate pH 6.5	18 % w/v polyethylene glycol 8000
47	None	0.1 M sodium acetate trihydrate pH 4.6	2.0 M ammonium sulphate
48	None	0.1 M tris-hydrochloride pH 8.5	2.0 M mono-ammonium dihydrogen phosphate
49	1.0 M lithium sulphate monohydrate	None	2 % w/v polyethylene glycol 8000
50	0.5 M lithium sulphate monohydrate	None	15 % w/v polyethylene glycol 8000

It has been noted in general but not exclusively, that relatively long equilibration times give better quality crystals in that they are more robust[§]. Any crystals observed may potentially be just salt crystals. This can be avoided by using low concentration phosphate buffer for example. A destructive method to test for salt crystals is to use a needle to press a crystal. A salt crystal will be resistant and maintain its structure but a protein crystal will easily crumble. A non destructive method would be to use a protein stain.

Prior to being able to observe an X-ray diffraction pattern, the crystals should be examined for birefringence. Polarising lenses are placed in the microscope above and below the plane of the crystal sample and turned relative to each other while observing the crystals through the upper polarising lens. If the crystals have two refractive indices they will change colour as the polarising lens is rotated. The crystals are said to be birefringent.

8.1.4 The Phase Diagram

The solubility of a protein depends upon various factors as mentioned. The saturated macromolecule solution is in equilibrium with the crystallised

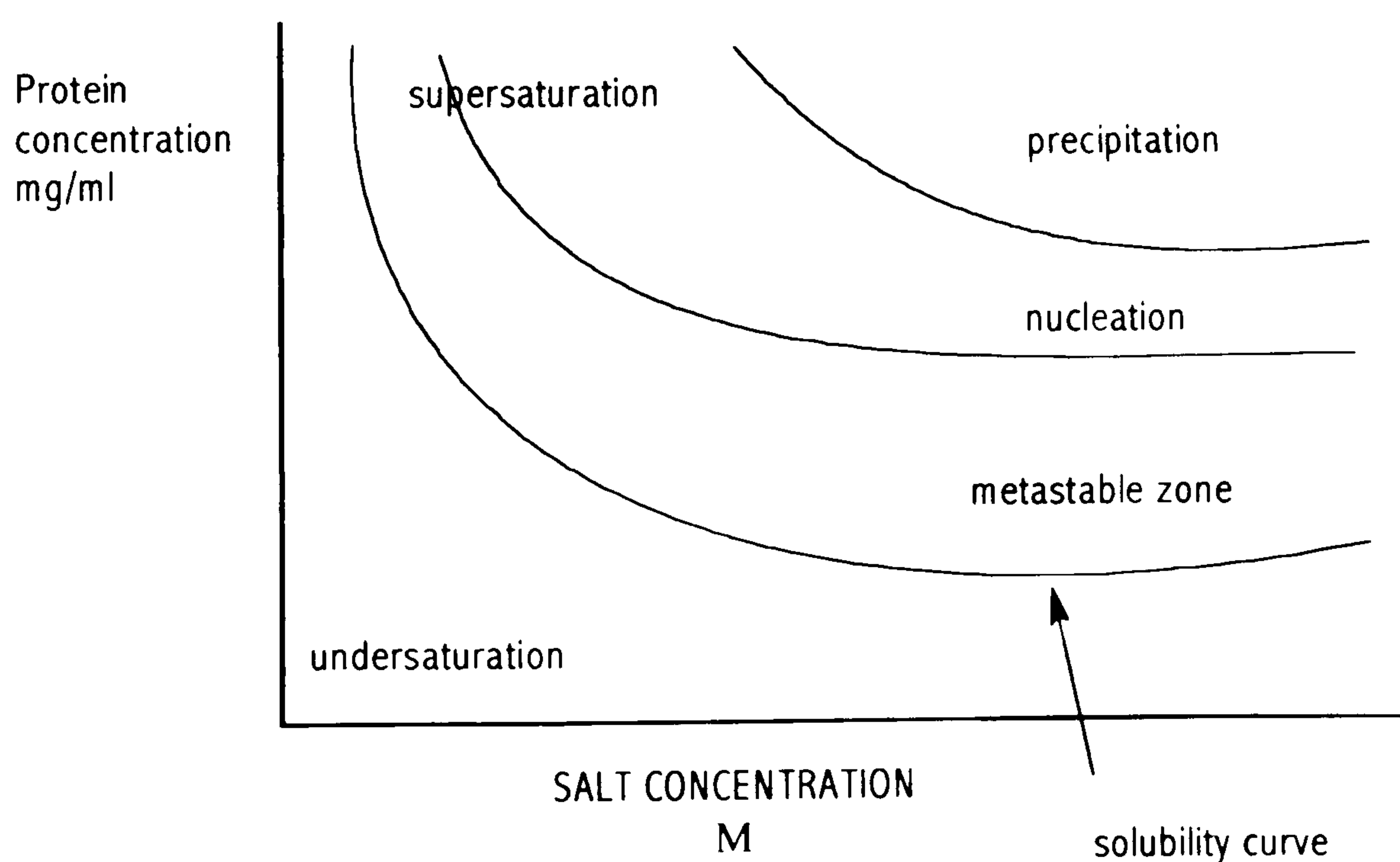

Figure 8.1.4a Solubility diagram for a crystallising protein.

[§] Personal communication from Professor R.W. Pickersgill of QMW.

macromolecule, adding crystal macromolecule would not dissolve the protein whereas adding reservoir solution would dissolve the crystals. In Figure 8.1.4a, below the solubility curve, the solution is undersaturated. No crystallisation will occur. Above the solubility curve effectively excess macromolecule will appear as a solid phase. Zones of nucleation within the hanging drop are required to form as the centres of crystal growth (Riès-Kautt & Ducruix, 1999).

The purpose of obtaining crystals is to gain knowledge of the protein structure through analysis of the X-ray diffraction pattern obtained when an X-ray beam is focussed on a crystal. This in conjunction with the protein's amino acid sequence can be used to gain an understanding of the quaternary structure of the proteins and possibly how they interact with each other as well as binding of substrates and ligands. These lines of research have been pursued extensively with the first P450 crystal structure to be mapped, that of $P450_{cam}$ from *Pseudomonas putida.* This has been developed by the high resolution crystal structure of $P450_{cam}$ (Poulos *et al.*, 1987). In crystal structural studies, binding of the substrate, camphor to the type I binding site without its binding to the type II binding site of P450cam was elucidated (Poulos *et al.*, 1985). Binding of 5-exo-hydroxycamphor to both $P450_{cam}$ binding sites forming binary complexes have also been observed through this methodology (Narasimhulu *et al.*, 1998).

8.1.5 X-Ray Analysis

Crystal quality is hugely important. Quality in this case is judged by the ability to diffract. Crystals may degenerate in the X-ray beam; they may be unstable or not diffract or have a large unit cell dimension. In these cases the crystals may not be suitable for full structural determination. X-rays are used because of the wavelength of this type of radiation, *ca.* 1.5 Å. By comparing diffraction distances from varying angles of incident X-ray beams, a pattern of electron density can be derived. Regions of high

electron density within a protein structure, correspond to the areas where the atoms are located. These areas will diffract the X-rays through a greater angle than X-rays incident upon an area of low electron density. This scattering is not random and can be explained mathematically, by Bragg's law.

8.2 MATERIALS

Molykote Silicone grease was obtained from Dow Corning, Munich, Germany. Linbro boxes[ℵ], were obtained from Flow Laboratories, Virginia, USA. Pre-siliconised glass coverslips, and Crystal Screen Kit were from Hampton Research, California, USA.

8.3 METHODS

8.3.1 Setting up Vapour Diffusion Trials

Silicon grease was applied to the full circumference of each of 50 Linbro box well rims. To each of the wells, 1.0 ml of the corresponding crystal screen solution was added, in turn from solution 1 to solution 50. The formulation of each of the 50 solutions is shown in Table 8a.
To each of 50 pre-siliconised glass coverslips, 20 μl of the protein solution was added (concentration = 5mg/ml) in the centre. To each of these drops, 20 μl of the corresponding crystal screen solution was added, thereby doubling the volume of the drop. This process was carried out six drops at a time rather than all 50 at once to reduce evaporation of the protein drops.

Once the 40 μl drop was prepared, the coverslip was placed over its corresponding Linbro box well, containing the correct Crystal Screen solution. Thus the drop in the centre of the coverslip was hanging over the

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[ℵ] Commercially available Linbro boxes are normally used for tissue culture but with a slight modification they are ideal for crystal trials. See methods.

reservoir solution in an airtight environment created by the silicon grease seal around the rim.

Once all 50 wells were sealed with their corresponding hanging drops, the lids to the Linbro boxes were prepared. To each inner corner of each of the lids, a small rolled-up piece of parafilm was firmly lodged. The lids were then placed on the Linbro boxes thereby creating space for the glass coverslips by virtue of the elevation by the parafilm.

The crystal trials were then placed in a thermostatically controlled environment at 17° C and left with minimum agitation while checking the drops every few days.

Once broad parameters have been set for the crystallisation of a protein, it is then desirable to narrow down the matrix components in order to find the optimum conditions for crystallisation. This may be performed on successively finer grids. This systematic screening of conditions was used to show that most proteins crystallise over a range of 1.5 to 2 pH units and 20 % saturation of ammonium sulphate solutions (Weber, 1990).

8.4 RESULTS

8.4.1 On careful inspection through a microscope after 1 month, the first set of trials had not given rise to any sign of crystallisation. The trials were repeated using protein samples twice as concentrated as the original trials i.e. 5.0 mg/ml instead of 2.5 mg/ml.

8.4.2 The fresh trials were left at 17° C for one month and crystals appeared in trials 14 and 15 of the roseoredoxin samples. Photographs of these are shown in Figure 8.4.2a and Figure 8.4.2b. In trial 6 of the

cytochrome P450 trials, successful crystallisation was achieved. This result is shown in Figure 8.4.2c.

In both samples that crystallised, the crystals were isomorphous. Neither crystal sample was birefringent.

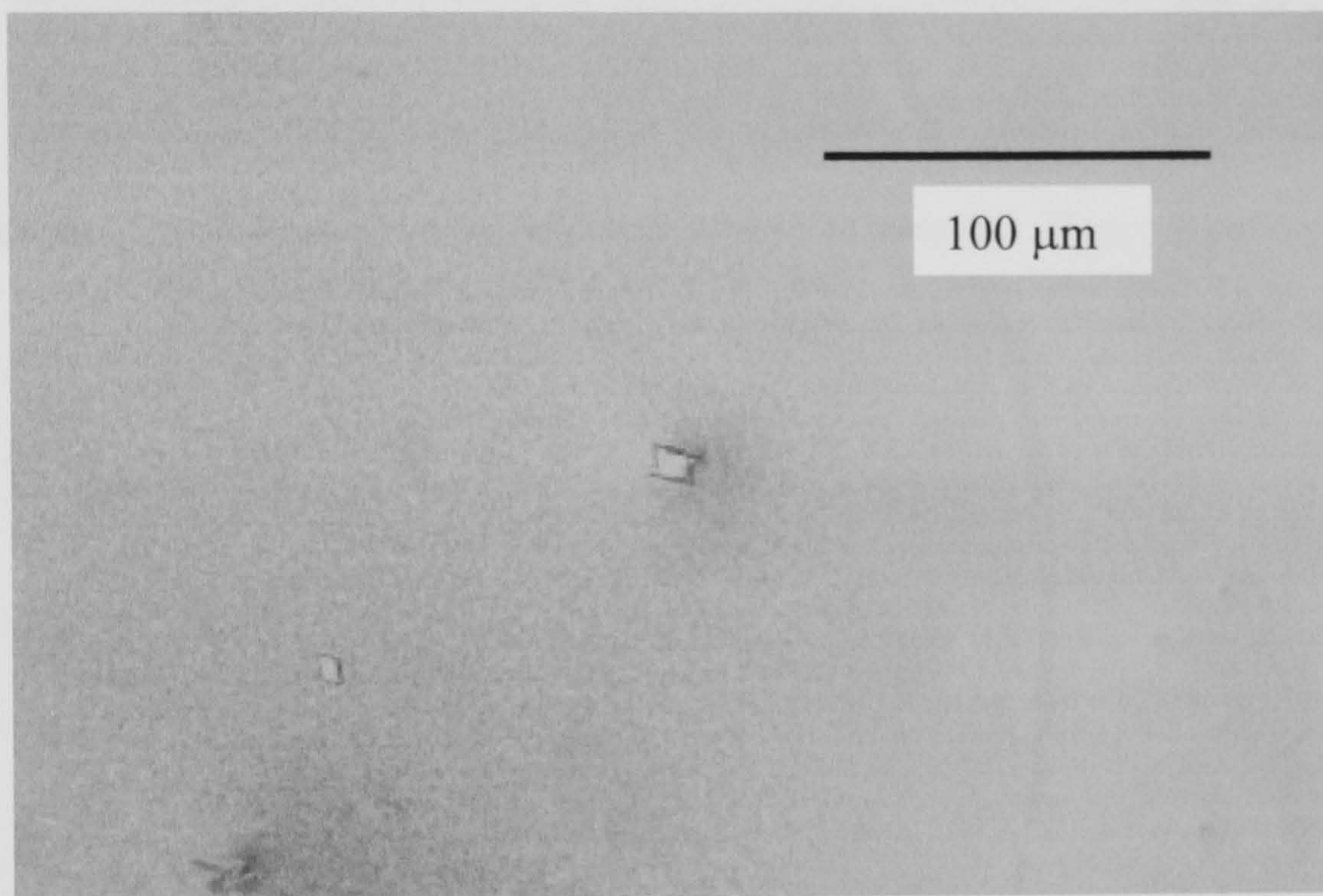

Figure 8.4.2a A photograph of crystals from trial 14 of table 8a. These crystals are of the roseoredoxin protein purified from *S. roseochromogenes*.

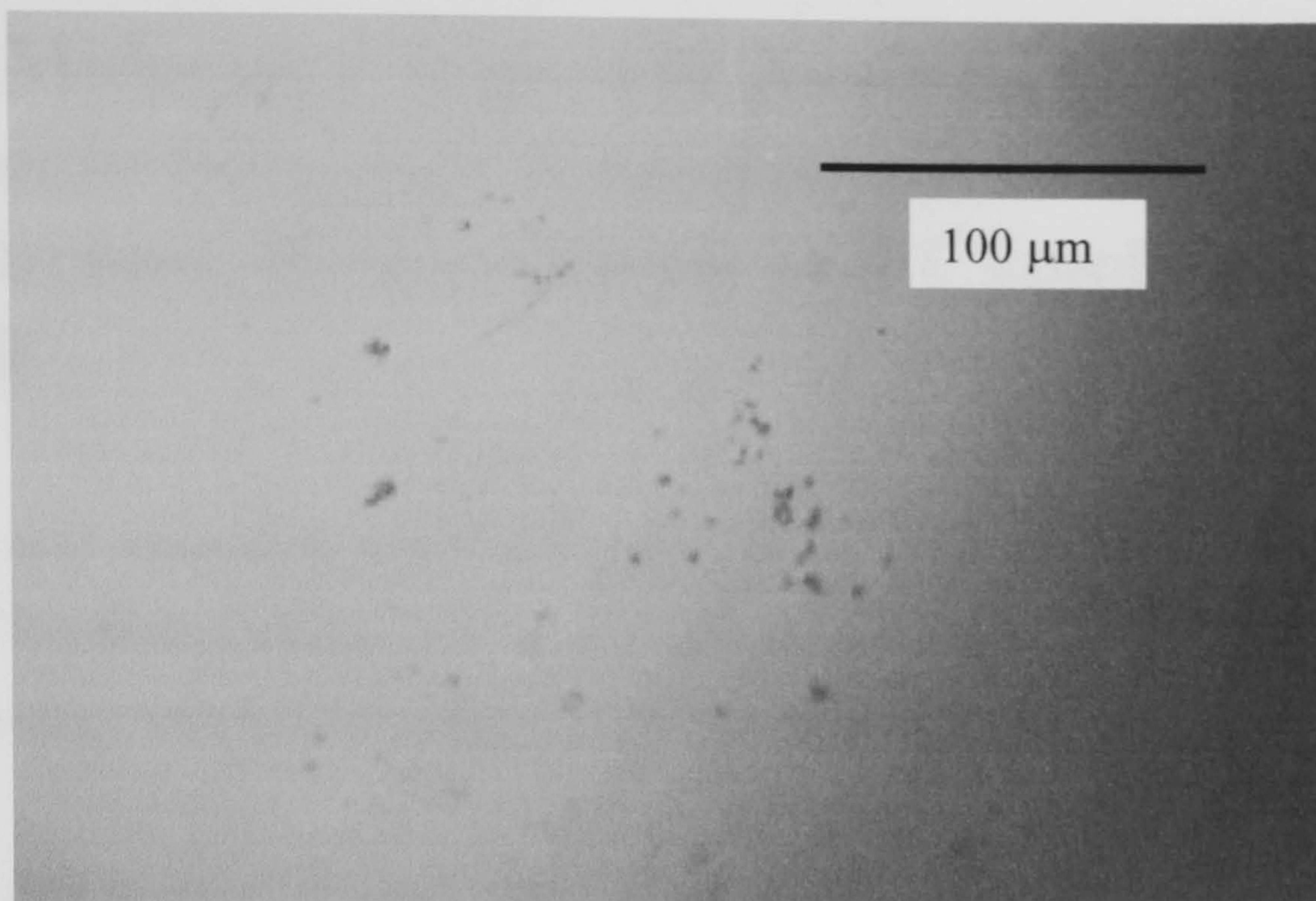

Figure 8.4.2b A photograph of crystals from trial 15 of table 8a. These crystals are of the roseoredoxin protein purified from *S. roseochromogenes*. The crystals produced here were not as clear as in trial 14, shown in Figure 8.4.2a.

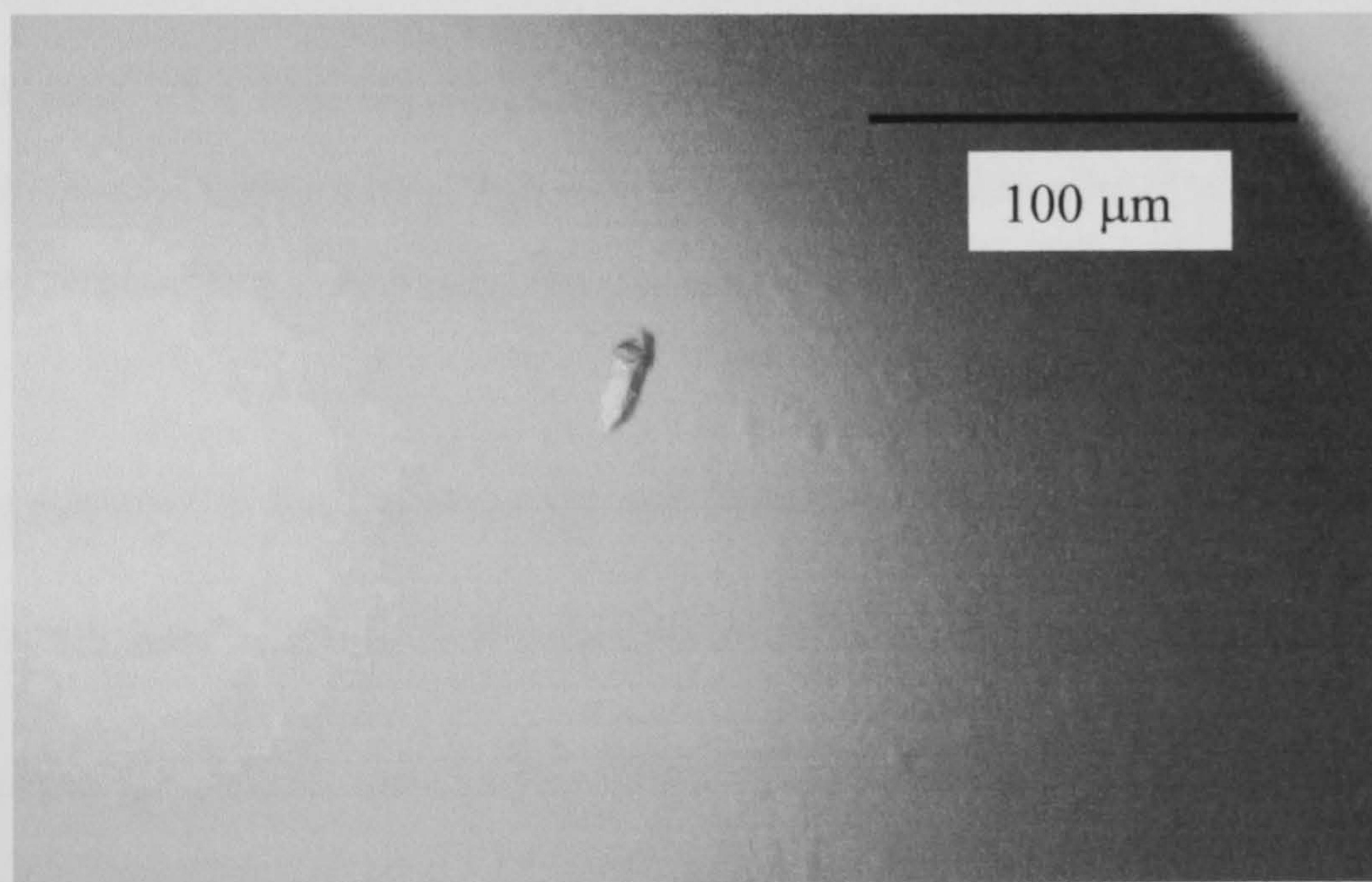

Figure 8.4.2c A photograph of a crystal from trial 6 of table 8a. This crystal is cytochrome P450 purified from *S. roseochromogenes*.

8.5 Molecular Sequence Analysis of *S. roseochromogenes* Roseoredoxin Reductase, Roseoredoxin and Cytochrome P450

Amino acid sequencing was performed by Dr. A. Carne of The Institute of Cancer Research, Fulham, London, on an automated sequenator. The amino acid residues were automatically analysed by HPLC.

8.5.1 *Streptomyces roseochromogenes* Roseoredoxin Reductase

No sequence was obtained for this component of the hydroxylase system. Upon re-examination of the protein post sequence analysis by SDS-PAGE, there was a lack of a distinct band indicating degradation of the sample. In these circumstances accurate sequence analysis was not possible due to the presence of artefactual small peptides contaminating the HPLC analysis.

8.5.2 *Streptomyces roseochromogenes* Roseoredoxin

The longest sequence obtained for this protein was:

X-N-L-E-F-P-E-R-I-K-M-L-K-D-Y-E-X-V-A-E-G-L-.......

8.5.3 *S. roseochromogenes* Progesterone 2β,16α Hydroxylase Cytochrome P450

A relatively short sequence of eleven amino acid residues was obtained for this protein:

V-G-V-S-I-N-K-G-V-H-F-......

8.5.4 Using Amino Acid Sequence Databases Accessible on the Internet to Determine the Uniqueness of Amino Acid Sequences Obtained

The European Bioinformatics Institute (EBI) maintains and distributes the European Molecular Biology Laboratory (EMBL) Sequence database, Europe's primary sequence data resource. The EBI also maintains and distributes the SWISS-PROT Protein Sequence database (Rodriguez Tomé *et al.*, 1996). The EBI network services include database searching and sequence similarity searching facilities accessible via the EBI website. Searches performed for each sequence obtained were BLAST and SWISS-PROT.

The 'PDBFinder' (Protein Databank) database was also used to search for similar sequences to those obtained from sequencing here (swift.embl-heidelberg.de).

The amino acid sequences for the roseoredoxin and the progesterone 2β,16α hydroxylase P450 were entered into database searches as described in the methods section of this chapter. The cytochrome P450 sequence received zero matches and is therefore unique.

8.6 DISCUSSION

Crystal Structure

The current crystals are too small for X-ray analysis but it is anticipated that further refinement of the crystallisation conditions will give crystals large enough for diffraction trials. In addition to the single protein crystals, it is anticipated that combinations of the proteins may be co - crystallised in order to gain some insight into the way the structures interact in the hydroxylase system. Full amino acid sequences would greatly facilitate this

work in order that residues may be identified from X-ray diffraction patterns.

Cytochrome P450 haemoproteins comprise a large gene superfamily that catalyse monooxygenase reactions in the presence of a redox partner. Mammalian members are membrane bound and therefore have resisted structure-function analysis by means of X-ray crystallographic methods. However, soluble P450s from bacteria have been used to build a model of the $P450_{arom}$, which catalyses the conversion C19 steroids to oestrogens. This is a complex and poorly understood aromatisation (Graham-Lorence *et al.*, 1995). The 3-D model was based upon the structures of $P450_{cam}$, $P450_{terp}$ and $P450_{BM-3}$. From this model a redox partner binding site was postulated.

Detailed structural information is available for $P450_{cam}$ and its X-ray structure determined to a resolution of 1.63 Å (Poulos *et al.*, 1987). Two highly conserved regions are found between P450 101 ($P450_{cam}$) and eukaryotic P450s. In modelling, conserved mammalian P450 sequences are aligned with conserved sequences from P450 101 (Koymans *et al.*, 1993 [2]). This method is ambiguous and imprecise as a result of the assumption of tertiary structures of eukaryote P450s being similar and the presence of a membrane binding segment at their amino termini. The most highly conserved region between prokaryote and eukaryote P450s, is the haem binding domain. The second most conserved being the helix I region; the oxygen binding site which constitutes part of the substrate binding site (Koymans *et al.*, 1993[2]).

The Sequences Presented in this Chapter

The sequences presented in this chapter are too short for any type of structural analysis to be performed. This is because a peptide, when part of a large protein will take on a different shape due to non local factors within the protein influencing the factors which may exist within the peptide thereby invalidating any structural analysis of the peptide. Existing

approaches to protein secondary structure prediction from the amino acid sequence usually rely on local
residue interactions and the secondary structural state of the central residue. An attempt has been made to produce an algorithm for secondary structure prediction based on recognition of potentially hydrogen-bonded residues in a single amino acid sequence. The unique feature of this approach involves database-derived statistics on residue type occurrences in different classes of β-bridges to delineate interacting β-strands. The α-helical structures are also recognised on the basis of amino acid occurrences in hydrogen-bonded pairs. The algorithm has a prediction accuracy of 68% in three structural stages and relies only on a single protein sequence as input (Frishman & Argos,1996).

The Significance of Physical Modelling of Cytochromes P450 Based upon their Amino Acid Sequences

P450s all appear to take on a similar structural fold even though they frequently have less than 20 % sequence identity and catalyse different classes of reaction upon binding a diverse range of substrates. Therefore the regions within P450 sequences which do not have sequence identity (80 %) must share some other common factor or factors.

The Haem Binding Site
⊗

It is now universally accepted that a cysteinyl residue at alignment position 564 (Nelson & Strobel, 1989) corresponding to 'C' -357 in P450 101 (the $P450_{cam}$), provides the fifth axial haem ligand in all P450s. The cysteinyl residue is part of a polypeptide chain that contains some other highly conserved and invariant residues ('F'-557, 'G'-560, and 'G'-566). In

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⊗ Amino acid letter designations are given in the following footnotes →

eukaryotic P450s the P450 101 residues 'H'-355 and 'L'-358 (Aligned positions 562 & 565) are changed into the functionally conservative residues 'R' and 'I'/'V' respectively.[⊗]

'H'-355 of P450 101 is in a position to either hydrogen bond or interact ionically with one of the haem proprionic acids (Poulos *et al.*, 1987). Highly conserved 'R'-562 may have a similar function in all eukaryotic P450s. The 3-d structure surrounding the fifth haem ligand 'C' may be very similar in all eukaryotic P450s. The conservation in tertiary structure almost certainly relates to the critical role played by the Fe-S bond in the catalytic mechanism of all cytochromes P450.

Site-directed mutagenesis has been used to investigate the functional role of amino acid residues around the haem binding domain. These experiments performed on rat liver P450 1A2 confirmed that the Cysteine-thiolate bonding to the haem iron atom ('C'-456 = Aligned position 564 of P450 101) (Shimizu *et al.*, 1989) and that 'R'-454 (Aligned position 562) interacts with the haem proprionate side chain similarly to 'H'-355 in P450 101. Hydrophobic residues such as 'F'-449, 'L'-451, 'G'-452, 'I'-457 and 'G'-458 of P450 1A2 (Aligned positions 557, 559, 560, 565 and 566) are located next to the axial thiolate ligand to hold the haem in the active site (Shimizu *et al.*, 1988).

The Molecular Oxygen Binding Site

This highly conserved domain occurs at the central region of helix I that forms the O_2 binding pocket in P450 101 (Nelson & Strobel, 1987, 1988 & 1989). Highly conserved amino acid residues are found at alignment positions 396 ('A'/'G') and 400 ('T'). Also, amino acid residues at positions 393, 394, 395 and 405 are all hydrophobic in character, whereas a negatively charged amino acid ('E' or 'D'), a hydroxy amino acid ('T' or 'S')

[⊗] A, Alanine C,Cysteine D, Aspartic acid E, Glutamic acid
F, Phenylalanine G, Glysine H, Histidine I, Isoleucine K, Lysine
L, Leucine *....continued...*→

and an aromatic residue ('F', 'Y' or 'W') are conserved at positions 399, 403 and 407 respectively.⊗

In P450 101 a localised distortion and widening of the distal helix I between 'G'-248 and 'T'-252 (Aligned positions 396 & 400) provide a pocket for molecular O_2. Thus, high sequence homology around 'T'-252 residue in P450 101 and the corresponding 'T' at alignment position 400 in eukaryotic P450s indicates a similar O_2 binding domain in this latter group of proteins.

It has been suggested that some of the residues around the O_2 binding site might form part of the substrate binding site, influencing the binding and orientation of the substrates. Residues 'N'-310 to 'F'-325 of P450 1A2 (Alignment positions 391-406) have been mutated (Shimizu *et al.*, 1989). CO-difference spectra were strongly influenced by these mutations agreeing with the proposal (Shimizu *et al.*, 1989) that the region 'N'-391 to 'F'-406 forms part of the substrate binding site in this particular P450. The catalytic activities of these mutants toward benzphetamine, 7-ethoxycoumarin and testosterone are dependent on the nature of the mutation (Furuya *et al.*, 1989[2] and Hiroya *et al.*, 1991).

Threonine 301 of $P450_{cam}$

Substitution of the nearly invariant 'T' at alignment position 400 by 'A' in P450 1A2 yielded a mutant enzyme without activity toward benzphetamine but enhanced activity toward 7-ethoxycoumarin and testosterone. Substitution of invariant 'G' at alignment position 397 for 'E' enhanced activity for both benzphetamine and 7-ethoxycoumarin (Furuya *et al.*, 1989[1]). These data are unexpected because both 'T'-319 and 'G'-316 of P450 1A2 stabilise the oxygen binding pocket (Poulos, 1986). Substitution of these residues for functionally different amino acid 'E' or 'A' would be expected to result in catalytically inactive or weakly active mutants.

⊗ M, Methionine N, Asparagine P, Proline Q, Glutamine R, ArginineS, Serine T, Threonine V, Valine W, Tryptophan Y, Tyrosine.

This is significant because if one looks at the model of $P450_{arom}$ discussed above, which was built based on a "core structure" identified from the structures of the soluble, bacterial P450s ($P450_{cam}$, $P450_{terp}$, and $P450_{BM-3}$) rather than by molecular replacement. From this model it was postulated by Graham-Lorence *et al* (1995) that a membrane-associated hydrophobic region of aliphatic and aromatic residues is involved in substrate recognition and a redox-partner binding region that may be unique compared to other P450s, as well as residues involved in active site orientation of substrates and inhibitor of $P450_{arom}$, (vorozole). From this model it was proposed that a scheme for the reaction mechanism involves a "threonine switch" determining whether oxygen insertion into the substrate molecule involves an oxygen radical or a peroxide intermediate (Graham-Lorence *et al.*, 1995). Changing the invariant 'T'-301 (Aligned position 400) of P450 2C2 into 'H', 'V' 'I', 'L', 'Q', 'C' or 'N' diminished or abolished the hydroxylase activity of P450 2C2 toward substrates laurate and caproate (Imai & Nakamura, 1988 & 1989)(Imai, *et al.*, 1989 & 1990). Substituting 'T'-301 for 'S' diminished laurate ω-1 hydroxylase activity in P450 2C2, whereas the corresponding caproate activity was higher than in the wild-type enzyme (Imai, *et al.*, 1989 & 1990). A double mutant 'T'-301 to 'S' and 'T'-302 to 'V' resulted in laurate ω-hydroxylase activity, which was not detectable in the wild-type P450 (Imai, *et al.*, 1989 & 1990). Thus, 'T'-301 & -302 of P450 2C2 (Aligned positions 400 & 401) may play an important role in determining substrate specificities and recognising fatty acid substrate chain length, resulting in hydroxylation either at the ω or ω-1 position or both.

The 16α steroid hydroxylation reaction (the reaction investigated in this work) has also been investigated by mutation. Mutants of P450 2C14 (**testosterone 16-α hydroxylase**) have been constructed in which 'T'-301 (Aligned position 400) is replaced by 'S', 'V' or 'H'. The 'V' & 'H' mutants have diminished or no hydroxylase activity towards progesterone or testosterone, whereas the 'S' mutant has 50% less activity towards testosterone and slightly increased activity toward progesterone (Imai &

Nakamura 1989). These studies taken with the data on P450 2C2 point to a hydroxyl group ('T' or 'S') as being necessary for efficient catalysis.
Changing 'T'-252 (Aligned position 400) of P450 101 for 'A', 'V', 'C', 'P' or 'G' uncoupled catalytic activity and resulted in H_2O_2 production, suggesting that the nearly invariant 'T' at alignment position 400 might have different functions in different P450s (Furuya *et al.*, 1989[2]).
As discussed above, for these reasons of ambiguity and a membrane binding domain in eukaryotic P450s, it seems that this type of modelling has only limited uses. It appears that comparison of primary sequences is the only accurate stage in this modelling approach. The problem of the prokaryote and eukaryote comparative model is important if modelling techniques are to be developed for the purpose of drug design. For example, the aromatase, $P450_{arom}$ catalyses the conversion of androgens to oestrogens in a variety of cells such as ovarian granulosa, testes, placenta, adipose tissue and various centres of the brain. The extragonadal synthesis of oestrogens has a major role in the pathophysiology of various breast carcinomas and endometrial adenocarcinoma (VandenBossche *et al.*, 1994). This catalysis is in conjunction with an NADH $P450_{arom}$ reductase. Misalignment in the eukaryote modelling approach may prevent 1/. Active site determination and 2/. Reductase binding site elucidation because $P450_{arom}$ has an endoplasmic reticulum binding domain. The development of $P450_{arom}$ inhibitors would reduce the need to prescribe steroidal P450 inhibitors in the treatment of some carcinomas.

The Aromatic Region

The amino acid primary sequences of membrane-bound P450s have a highly conserved region which is not shared by P450 101 (Ouzounis & Melvin, 1991). This domain is called the "aromatic region" because it is characterised by its peculiar amino acid sequence from alignment position 513-525 (Nelson & Strobel, 1989). The sequence is expressed as A_1-X-X-

P-X-X-A_2-X-P-X-B-A_3, where A_1=an aromatic amino acid. P=Proline. B=either'R' or 'H', X= a weakly conserved amino acid.

Three P450 1A2 mutants have been constructed to elucidate the function of this aromatic region and the individual amino acids it comprises (Furuya *et al.*, 1989[2]). Residues 'F'-425, 'P'-427 and 'F'--430 (Aligned positions 520, 522 and 525 respectively) were all changed into 'L' and their effect on catalytic activity of P450 1A2 toward benzphetamine and ethoxycoumarin was examined. Benzphetamine activity accounted for only 7% of wild-type, ethoxycoumarin activity, this was increased to 2.5 fold with the 'L' replacement mutation (Furuya *et al.*, 1989[2]).

Mutants in which amino acid 'W'-419, 'P'-422 and 'R'-429 (Aligned positions 513, 516 & 524 respectively) were changed to 'L' resulted in failure of haem to bind to the apoprotein., suggesting that the aromatic region indirectly contributes to the haem binding at least in P450 1A2 (Furuya *et al.*, 1989[1]). ESR spectra of mutants F425L and P427L support this suggestion as they displayed different g values compared to wild-type P450 1A2 (Sotokawa *et al.*, 1990). This aromatic region seems to be a characteristic of membrane-bound P450s because this sequence is not readily apparent in P450 101. As the sequence is not apparent in the soluble $P450_{cam}$, this region might therefore help to orientate the haem toward the membrane surface with the substrate binding site facing the membrane surface but in the absence of a suitable eukaryotic model these points cannot easily be resolved. Problems with alignment based modelling are not limited to the differences between prokaryotic and eukaryotic sequences. There are differences between microsomal and mitochondrial P450s. At the beginning of the L helix, all mitochondrial P450s contain two positively charged residues, usually 'R' (Peterson & Graham, 1998)

The functions of the amino acid residues, which fall into other conserved regions, are largely unknown. these include the 'P'-'G'-'P' region at the N-terminus, almost invariant 'G' at alignment positions 110 & 118, and a highly conserved W-X-X-X-B region at positions 177-181 where W=tryptophan, X=a weakly conserved residue, and B=Arginine or Lysine

(Gonzales, *et al.*, 1989). Using nanosecond fluorometry , it has been shown that 'W'-121 of P450 2B2 (Aligned position 177) is not situated in the vicinity of the substrate binding site. The highly conserved region from alignment positions 177-181 is probably not part of the active site in membrane-bound P450s. It has been suggested that this sequence might be involved in the interaction with redox proteins (Aoyama *et al.*, 1989).
Despite these 'best-fit' scenarios in various domains of eukaryotic P450s, there are examples of minor changes having a large impact on selectivity and therefore limitations in modelling substrate interaction and transition state in eukaryotes, on P450 101 ($P450_{cam}$) (Linberg & Negishi , 1989) (Aoyama *et al.*, 1989) (Johnson *et al.*, 1992). However, such modelling may be useful in discerning more general structural features and should provide insight into aspects of electron transfer protein binding and oxygen activation (Guengerich, 1992).
A method for attributing a measure of reliability to a residue pair in an optimal alignment of two protein sequences has been proposed (Mevissen & Vingron, 1996). Validation techniques are based on a database of structurally correct sequence alignments (Pascarella & Argos, 1992) and shows that correctly aligned parts of sequence alignments systematically receive high scores by this measure. The higher the sequence similarity between two sequences, the larger the fraction found of the correct parts of the alignment. These observations were used to design a program that draws a reliability curve along an optimal alignment indicating the chances for each residue pair to be aligned correctly (Mevissen & Vingron, 1996). Again, this method has its limitations as the reliability curve only gives a probability of alignment. Eukaryotic P450 structural determinations based upon amino acid sequences are still being modelled on the 3-D crystal structure of P450BMP, the haem domain of P450BM-3. For example, the construction of a three-dimensional model of human CYP2E1 based on CYP102. Interactive docking of a number of human CYP2E1 substrates was found to be consistent with their known metabolism. For example the production of *N*-acetyl-*p*-benzoquinone imine, a hepatotoxin from

acetaminophen (paracetamol) at high concentrations due to its high K_m. (Tonge *et al.*, 1998). Site directed mutagenesis has also been employed as detailed above to examine substrate regioselectivity and active site composition along with the functions of hydrogen bonding within the active site of CYP2E1 (Lewis *et al.*, 2000).

P450 3D Modelling - The way Forward

Discussed above are the problems associated with amino acid sequence alignment. The fundamental issue is not the alignment but the absence of a eukaryotic model other than one derived from P450BM-3 and $P450_{cam}$. This situation however is currently under review. The development of a structurally determined microsomal P450 has been reported (Graham & Peterson, 1999).

A variant of microsomal P4502C3 (progesterone 21 hydroxylase) has been constructed which lacks the problematic membrane binding segment of its N-terminus residues 3-20. This variant was designated P4502C3d and expressed at high levels in *E.coli*. Also contained in this variant was the substitution of alanine for 'D' at position 2. Also serine was substituted for 'H'24 and 'G'25 to introduce a restriction site. These modifications are likely to facilitate attempts to crystallise the catalytic domains of microsomal P450s (von Wachenfeldt *et al.*, 1997). Deletion of the N-terminal membrane-spanning domain from microsomal P450s 2C3 and then also 2C5 generated the enzymes, 2C5dH and 2C3dH. The two proteins are tetramers and dimers, respectively. Each of four substitutions, N202H, I207L, S209G, and S210T, diminished the aggregation of P450 2C5dH and produced a monomeric enzyme. The N202H and I207L mutations also diminished the stimulation of catalytic activity by phospholipid and reduced the binding of P450 2C5dH to phospholipid vesicles. The modified enzymes exhibit rates of progesterone 21-hydroxylation that are similar to that of P450 2C5dH. These P450s are suitable for crystallisation and structural determination by x-ray diffraction studies (Cosme & Johnson, 2000).

For the amino acid sequencing of the proteins presented in this work, there is some sequence alignment with a protein, β xylanase for the roseoredoxin protein suggesting a hypothetical link but no such structural, even primary, comparisons, as discussed above can be made here. The P450 matched no other known P450 sequences. This *S. roseochromogenes* P450 protein is unique.

Chapter 9

General Discussion

There is now a wealth of compelling evidence identifying cytochrome P450 as responsible for steroid hydroxylation reactions in both bacteria and filamentous fungi e.g. in bacteria - 6β in *Bacillus thermoglucosidasius* (Sideso *et al.*, 1998) and 15β in *Bacillus megaterium* (Berg *et al.*, 1976), in filamentous fungi - 7α in *Phycomyces blakesleeanus* (Ahmed *et al.*, 1995); 11α in *Aspergillus fumigatus* (Smith *et al.*, 1994), *Aspergillus ochraceus* (Samanta & Ghosh, 1987), *Nectria haematococca* (Ahmed *et al.*, 1996), and *Rhizopus nigricans* (Breskvar *et al.*, 1987); 11β in *Cochliobolus lunatus* (Janig *et al.*, 1992); and 15α in *Penicillium raistrickii* (Irrgang *et al.*, 1997). Despite the long history and extensive literature on microbial steroid hydroxylation no such role has so far been indisputably assigned to P450 for this reaction in *Streptomyces* species. Therefore, this work is the first report to identify unequivocally cytochrome P450 as a steroid hydroxylase enzyme in the *Streptomyces* genus.

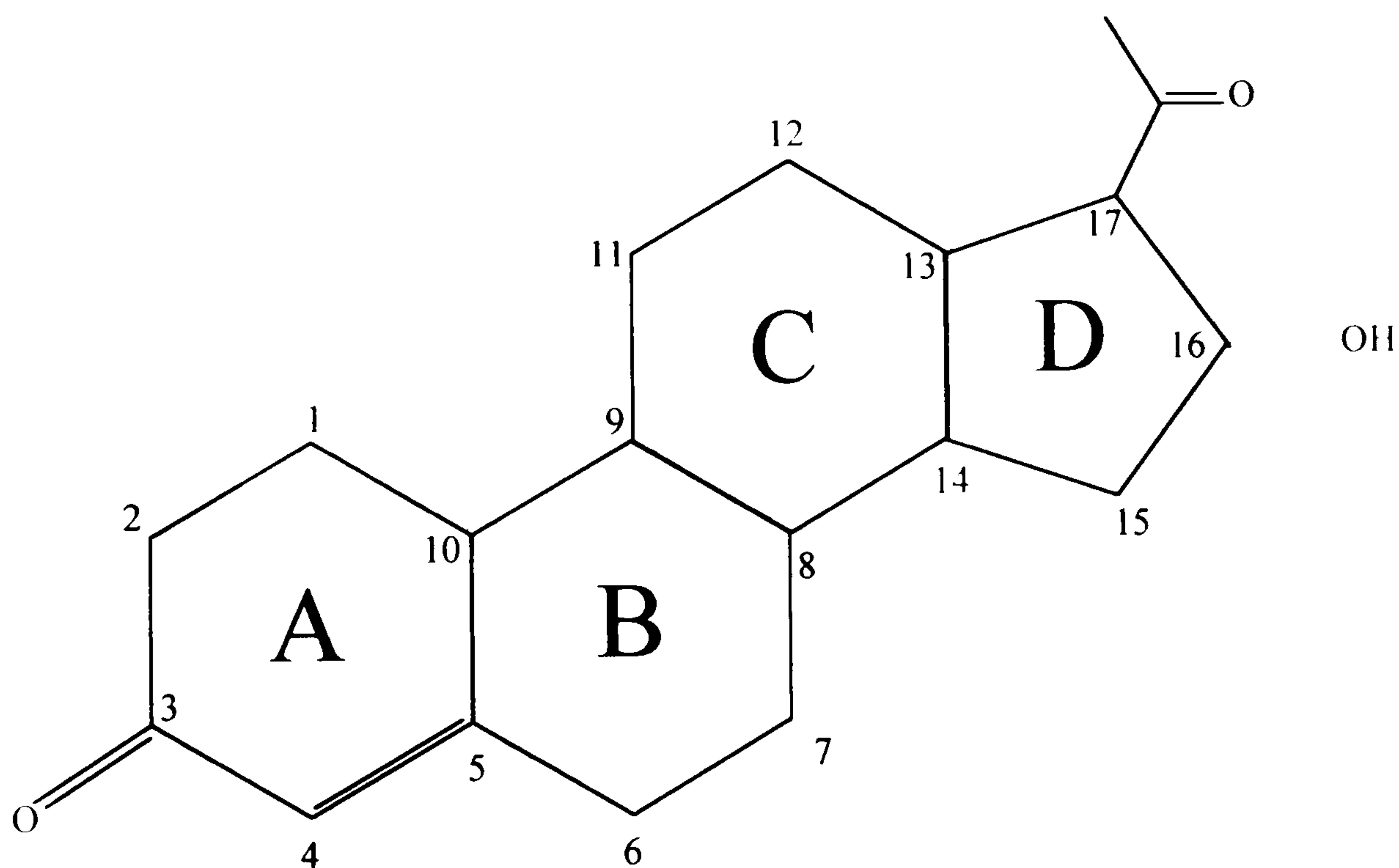

Figure 9.1 16α hydroxyprogesterone, the first product of the progesterone hydroxylase. The 16th carbon position of the perhydrocyclopentanophenanthrene ring system is hydroxylated as determined by NMR.

Steroid 16α hydroxylation was first identified 40 years ago during the search for micro-organisms capable of efficient and mild stereospecific

access of the 16 site of the steroid nucleus (Thoma *et al.*, 1957) (Goodman & Smith, 1961). Microbial access of this site was eventually employed for the synthesis of a new generation of highly potent, synthetic, anti-inflammatory pharmaceuticals exemplified by triamcinalone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione) developed to replace natural corticosteroids found to cause mineralocorticoid contraindications.

Here, the results of incubations with radiolabelled 16α hydroxyprogesterone showed that the monohydroxyprogesterone (Figure.9.1) is substrate for the production of the dihydroxyprogesterone (Figure.9.2). 2β hydroxyprogesterone was never identified as a product of this enzyme system.

When two solutions of purified protein were reduced with sodium dithionite and CO bubbled through one and the absorbances of each compared, a characteristic P450 spectrum was obtained with the absorption peak at 450 nm. When ketoconazole, a known P450 inhibitor, was added to incubations, no hydroxylase activity was observed. The results obtained show that this steroid hydroxylase is a cytochrome P450.

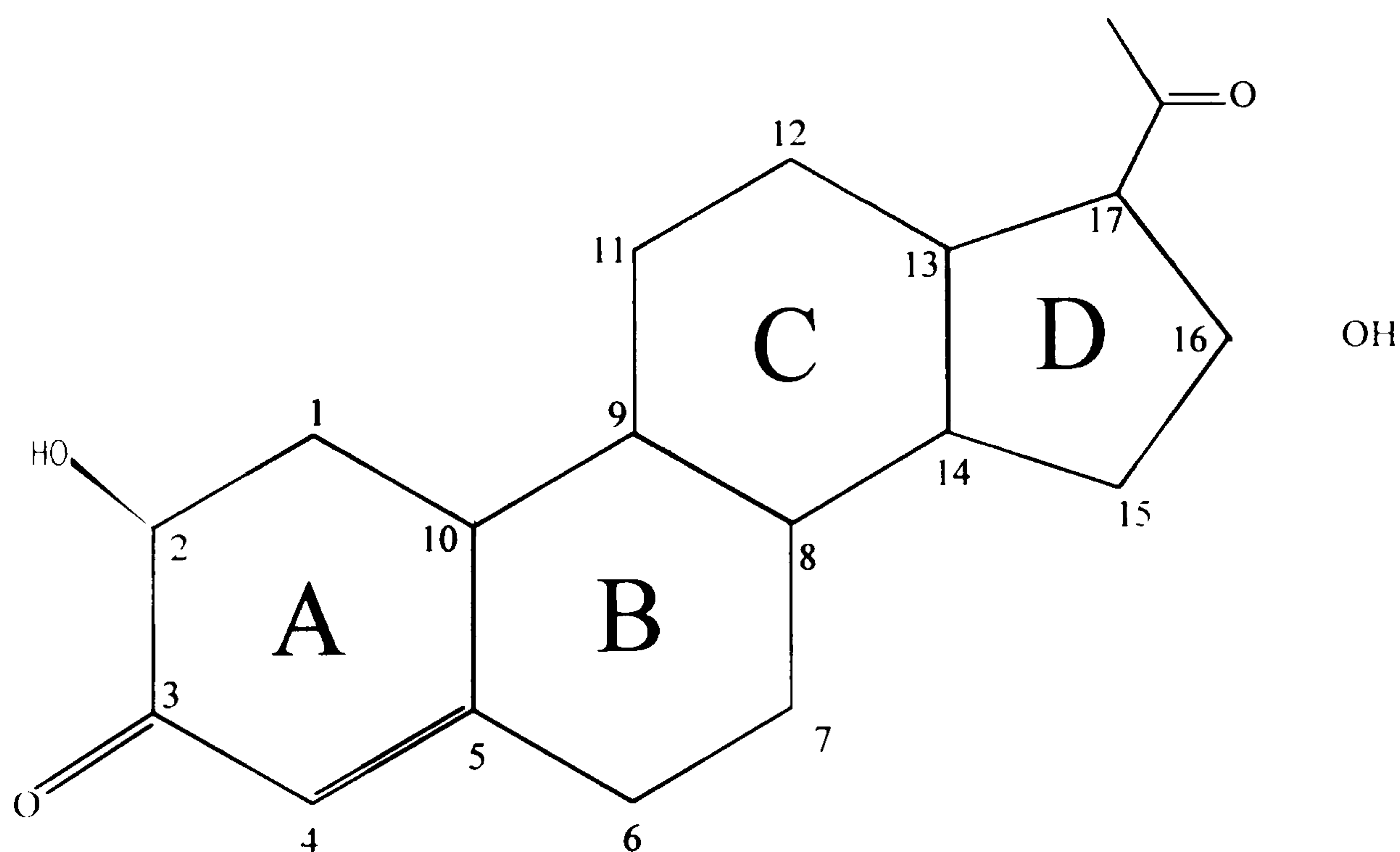

Figure 9.2 2β,16α dihydroxyprogesterone, the second product of the progesterone hydroxylase. The molecule is hydroxylated at opposite ends.

When the progesterone molecule is reversed and capsized, as shown in Figure 9.3 (180° horizontally and 180° vertically), one can see that the two

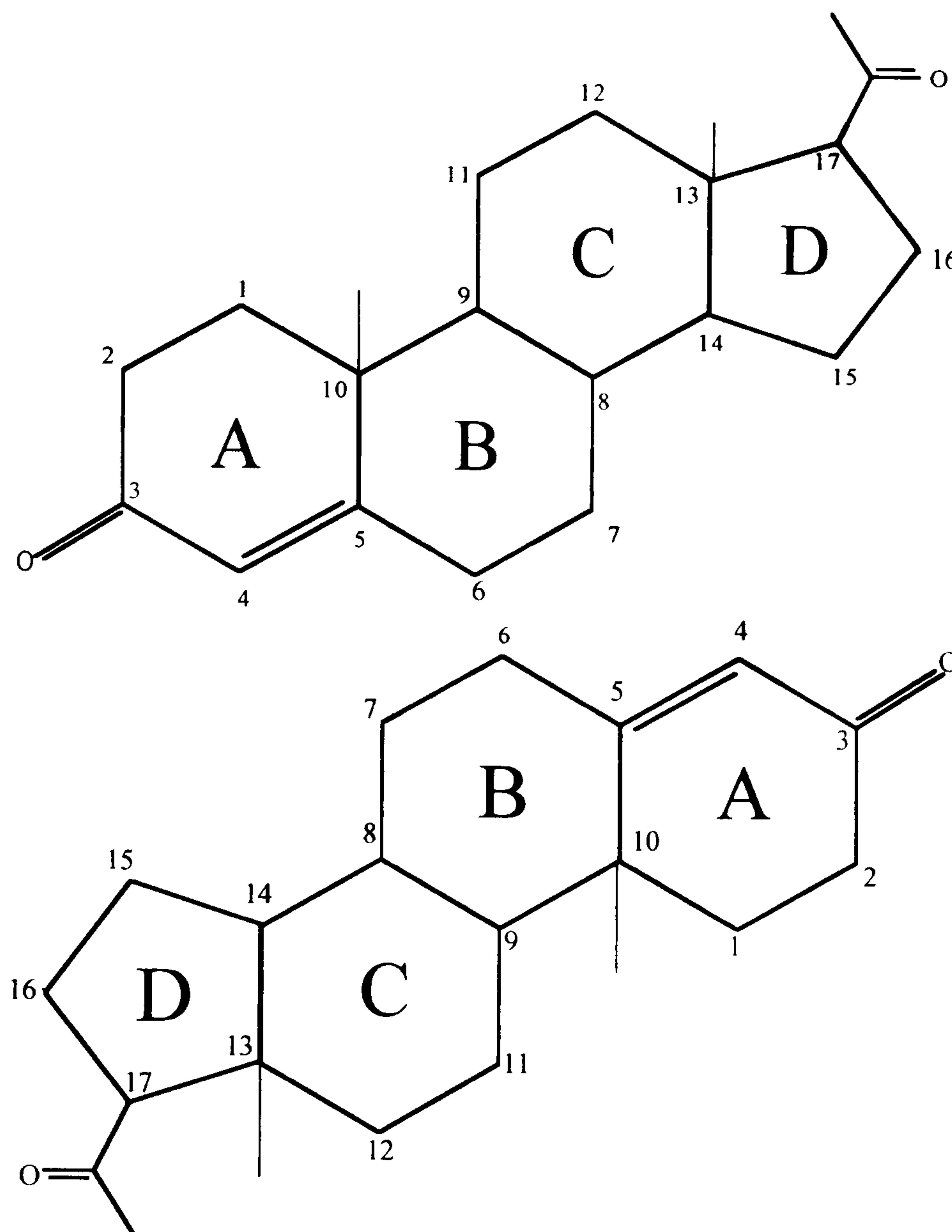

Figure 9.3 View of progesterone turned through 180° horizontally and 180° vertically (reverse capsized). One can see how the two replaceable protons become virtually spatially coincident.

replaceable protons, 2β and 16α, become virtually spacially coincident. This P450 from *S. roseochromogenes* is capable of hydroxylating progesterone in those two orientations. The amount of dihydroxyprogesterone present is greater than that expected were mono- hydroxylations entirely random. From this it appears that the P450 has a greater affinity for the monohydroxylated progesterone than for the progesterone. This broad

substrate specificity and catalytic activity, is characteristic of cytochromes P450. This may confer upon micro-organisms which express them, a selective advantage in terms of the production of secondary metabolites from recalcitrant carbon compounds and in the case of *Streptomyces* species, the production of antibiotic compounds.

S. roseochromogenes 10984 possesses strong progesterone 2β and 16α hydroxylation activities. The time course of progesterone transformation showed 16α hydroxylation to be the primary event and 2β hydroxylation to be a second phase reaction using 16α monohydroxyprogesterone as substrate. Thus, 16α monohydroxyprogesterone was first detected in the culture medium after 6 h of incubation and 2β,16α-dihydroxyprogesterone appeared 2 h thereafter at 8 h. That this is the true route of progesterone bioconversion was shown when *S. roseochromogenes* transformed exogenous 16α-hydroxyprogesterone to 2β,16α-dihydroxyprogesterone. Moreover, free 2β-monohydroxyprogesterone was never detected in this system even in transformation incubations containing 20 mg of progesterone and a metabolite detection threshold of under 0.1 mg of steroid (Berrie *et al.*, 1999).

With the purified P450 protein, reconstitution of the system was attempted. Purified P450 protein in combination with DEAE column peak 1 eluate (chapter 4), was capable of NADH dependent hydroxylation of progesterone, yielding the same products as in Figures 9.1 and 9.2, although less efficiently than in assays containing all three purified proteins found in the natural system.

A turnover number of *ca* 0.02/min was calculated for *S. roseochromogenes* P450 catalysed progesterone hydroxylation in the reconstituted pathway and a comparable value of *ca* 0.03/min for the $NaIO_4$ dependent pathway. Both numbers are now the lowest reported for a purified P450 being an approximate order of magnitude lower than the previous bottom of the table turnover numbers of 0.6/min reported for the steroid 15β-hydroxylase cytochrome P450 ($P450_{meg}$) of *Bacillus megaterium* (Berg A. *et al.*, 1979)

and of 1.8/min and 2.1/min reported for procene-II and 7-ethoxy coumarin hydroxylation respectively catalysed by a crude fraction of *S. griseus* P450 (Trower *et al.*, 1988). The reason for this catalytic inefficiency is unclear but it may be connected with the fact that progesterone is almost certainly not the physiological substrate for the *S. roseochromogenes* P450. Due to this paradox, a meaningful comparison of the true efficiency of this enzyme with its bacterial counterparts cannot be made until a natural substrate is identified. However, these data show that in the natural pathway highly pure *S. roseochromogenes* P450 catalyses multiple cycles of hydroxylation even with the unnatural steroid substrate.

The major monohydroxylated metabolite, 16α-hydroxyprogesterone was produced in 3.6 fold excess to the minor metabolite 2β,16α-dihydroxyprogesterone. In a reconstituted system containing highly purified progesterone 2β,16α-hydroxylase cytochrome P450 and electron transfer proteins, ferredoxin-like redoxin (roseoredoxin) and redoxin reductase (roseoredoxin reductase), both metabolites were produced but in a 10:1 ratio. When *S. roseochromogenes* was pre-incubated for 8 h with 0.32mM progesterone and the purified components of the hydroxylase system incubated as before, the ratio of 16α-hydroxyprogesterone to 2β,16α-dihydroxyprogesterone produced, decreased to 2.8 : 1, virtually identical to the ratio in whole cell transformations. Reconstitution assays containing all possible combinations of hydroxylase proteins purified from progesterone pre-incubated and control cells showed that the roseoredoxin was solely responsible for the observed changes in *in vitro* metabolite ratios. The fact that the lower 16α-hydroxyprogesterone to 2β,16α-dihydroxyprogesterone ratio was also obtained when *S. roseochromogenes* was exposed to 0.335 mM cycloheximide for 8h prior to the progesterone pre-incubation, pointed to post translation modification of the roseoredoxin. Separation of two isoforms of roseoredoxin by isoelectric focusing supported this proposition.

In contrast to steroid hydroxylation, cytochrome P450 has been identified in several *Streptomyces* pathways of secondary metabolism. Thus, this enzyme

participates in oleandomycin biosynthesis in *Streptomyces-antibioticus* (Rodriguez *et al.*, 1995), the hydroxylation of compactin to pravastatin by *Streptomyces carbophilus* (Matsuoka *et al.*, 1989), and in a wide variety of xenobiotic transformations in *Streptomyces griseus* (Sariaslani *et al.*, 1986; Trower *et al.*, 1988).

Cytochrome P450 genes have been cloned from *Streptomyces* species, *S. griseolus* and *S. griseus*. In *S. griseolus*, genes for the herbicide-inducible cytochromes P450, P450SU1 and P450SU2, have been sequenced and the amino acid sequence of segments of the encoded proteins deduced. These proteins were found to possess high sequence identity to the *Pseudomonas putida* camphor hydroxylase P450 (P450 101, $P450_{cam}$) particularly in the haem binding domain (Omer *et al.*, 1990). A DNA segment carrying the structural gene encoding $P450_{soy}$ (soyC), has been cloned from *S. griseus*. It is noteworthy that this cytochrome P450 also has high sequence conservation with $P450_{cam}$ in the haem binding region (Trower *et al.*, 1992). Ferredoxin electron transfer proteins have also been purified from the above two *Streptomyces* species. *S. griseolus* was found to contain two 7 kDa isoforms, designated Fd-1 and Fd-2. These proteins have 52% identity and both contain single [3Fe-4S] clusters (O'Keefe *et al.*, 1991). In *S. griseolus* only a single ferredoxin encoded by SOY B has been putatively identified. The situation in *S. roseochromogenes* remains to be resolved.

Ferredoxin-like iron sulphur containing proteins required for cytochrome P450 monooxygenase activity have been identified in a wide variety of bacteria e.g. *Bacillus megaterium* ATCC 13368 (Rauschenbach *et al.*, 1993), *Bradyrhizobium japonicum* (Tully *et al.*, 1998), *Pseudomonas incognita* (Peterson *et al.*, 1992), *Pseudomonas putida* (Koga *et al.*, 1989), *Rhodococcus* Sp strain NI86/21 (Nagy *et al.*, 1995), *Saccharopolyspora erythraea*, CA340 (Shafiee & Hutchinson, 1988), *Streptomyces griseolus* (O'Keefe *et al.*, 1991), *Streptomyces griseus* (Trower *et al.*, 1993), *Xanthobacter* sp., (Trickett *et al.*, 1991). Where sequence analysis has been performed, these ferredoxin-like genes seem to be constituents of operons containing also the P450 and the redoxin reductase gene.

In the case of *S. griseolus'* Fd-1 and Fd-2 (O'Keefe *et al.*, 1991). Both ferredoxins function in reconstituted cell-free systems containing the SU1 isoform of P450 although Fd-2 is the more active protein. The genes for the ferredoxins and the sulfonylurea P450 monooxygenases are virtually contiguous. Thus P450SU1 and the downstream Fd-1 form a closed-spaced pair; similarly P450SU2 and Fd-2 pair.

Results here for *S. roseochromogenes* suggest that a novel system for ferredoxin-like protein expression, namely post-translational modification, may well operate in *Streptomyces*. In control cells grown in a standard medium a single, mildly basic and soluble form of roseoredoxin (pI 7.45) was present. This protein, like its published bacterial counterparts forms the middle component of a natural cytochrome P450 electron transfer chain. *In vitro* this *S. roseochromogenes* system predominantly catalyses 16α monohydroxylation of progesterone and as a rather minor secondary reaction, 2β hydroxylation of the primary product. By contrast, pre-incubating the organism with progesterone results in the appearance of different, second, more acidic, soluble form of roseoredoxin (pI 5.6). This modified form increases the 2β hydroxylation capability of the cytochrome P450. This is achieved by increasing the hydroxylation turnover number of the P450 thereby increasing the number of 2β hydroxylation events. The result is seen as a decline in the 16α-monohydroxy to 2β,16α-dihydroxprogesterone ratio. That a growth inhibitory dose of cycloheximide added to the cells for 8 h prior to the progesterone pre-incubation had no effect on the appearance of the pI 5.6 isoform is strongly consistent with a progesterone dependent post translational modification of the roseoredoxin rather than for transcriptional activation of a second gene.

Transcriptional regulation of the P450 per se is a more usual regulatory P450 process. Regulation of P450 catalysis ultimately by post translational modification of a P450 reductase, is a unique mechanism in P450 regulation. There is no literature precedent for the regulation of ferredoxin activity by post-translational modification.

What is the nature of the post-translational modification of roseoredoxin and how does it modulate the activity of the P450? The covalent attachment of an acidic group such as phosphate is an obvious candidate to account for the 1.85 pH unit decrease in the pI value of the roseoredoxin. This enhanced 2β hydroxylation capacity of the P450 stimulated by the modified roseoredoxin isoform could be accounted for by increased electron shuttling from the roseoredoxin reductase to the P450. This improved shuttling could result from enhanced interaction of the roseoredoxin with its adjacent electron transfer protein partners or an inherently superior electron transfer velocity of the modified roseoredoxin. The results of NMR experiments involving cytochrome $P450_{cam}$ (CYP101) and its Fe_2S_2 ferredoxin electron transfer partner, putidaredoxin, indicate that conformational gating of the electron transfer complex between these two proteins may be important in their redox reactions (Pochapsky *et al.*, 1996). For example, the enzymatic removal or modification of the C-terminal tryptophan of putidaredoxin is known to cause a much reduced rate of enzymatic activity in the reconstituted camphor hydroxylase system (Sligar *et al.*, 1974) and (Davies & Sligar, 1992).

Comparison of the properties of microsomal NADPH-P450 reductase and the flavoprotein domain of P450BM-3 (BMR) has revealed a significant difference in the mechanism of reduction of the haemoprotein P450 by these flavoproteins (Sevrioukova & Peterson, 1995). Microsomal NADPH-P450 reductase transfers electrons to the haemoprotein by shuttling between hydroquinone and semiquinone forms of the FMN, delivering one electron per cycle. Since the microsomal NADPH-P450 reductase has evolved as part of a multi-enzyme system, this type of mechanism may permit regulation of the steps of the P450 reaction via variation in the affinity of the reductase for different P450s. This may provide a clue as to the possible role of the post translational modification of the P450 reductase presented in this work. Is the post translational modification involved in an altered reductase / P450 affinity? Thereby altering electron transfer efficiency and ultimately the rate of progesterone hydroxylation. The reductase domain

has evolved along with a haem domain. This enzyme was found to utilise the fastest and simplest way to reduce the haem iron, with the FMN moiety of BMR shuttling between the semiquinone and oxidised states. There are differences in the intermediates involved in the reduction of P450s by these two enzymes but the domain structures and mode of interaction between the reductase and P450s has been maintained over evolutionary time (Sevrioukova & Peterson, 1995).

Most P450 systems which have been characterised in terms of their full multicomponent nature have been NADPH dependent and mammalian such as rat hepatic P450s. Elucidation of the mechanism of catalysis incorporating the three proteins purified here, has been limited by procedures available. However, a huge potential for further work has been released. Indeed, crystallisation trials and sequence analysis have already been initiated for this enzyme system - see chapter 8. Mechanistic investigation in terms of molecular changes in the protein interactions would require access to procedures such as magnetic circular dichroism (Andersson *et al.*, 1997) or pulse radiolysis (Kobayashi K. *et al.*, 1990).

These investigations for *S. roseochromogenes'* progesterone hydroxylase, remain as potential for further investigation. Amino acid sequences for the two proteins most discussed in this work; P450 and roseoredoxin, were obtained. These were short sequences but long enough to design a probe for a DNA library for the purpose of gene identification. Once the P450 gene has been identified, overexpression through cloning will enable further investigation of this complex enzyme system to progress. The cloning vector of choice may need to express the roseoredoxin and the reductase in order to support hydroxylation by the P450. This issue was discussed in chapter 6.

It appears that the roseoredoxin here is primarily the P450 reductase in view of the evidence that its isoform had such a significant effect upon catalysis of the progesterone 2β hydroxylase efficiency. The three proteins presented here are therefore probably the three proteins which constitute the 2β,16α

hydroxylase system of *S. roseochromogenes in vivo*. The roseoredoxin phenomenon would probably not have been identified if the roseoredoxin had been a non specific reductase.

Considering the initial aims of this work as set out in chapter 1, the points have been addressed progressively chapter by chapter as the nature of this 2β and 16α progesterone hydroxylase P450 system was revealed. For example, P450 in *Streptomyces* in chapter 3, cell-free biotransformation in chapter 4, P450 enzymatic modelling in chapter 6 and approaches to multicomponent reconstitution and P450 regulation in chapters 6 and 7. The potential for cloning strategy development and three-dimensional structural analysis was alluded to in chapter 8. It has been the intention by discussion at the end of each chapter to contextualise the findings presented, thereby prompting routes for further investigation.

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Microbial transformations of steroids-XI. Progesterone transformation by *Streptomyces roseochromogenes*–purification and characterisation of the 16α-hydroxylase system

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Abstract

Streptomyces roseochromogenes, NCIB 10984, contains a cytochrome P450 which, in conjunction with two indigenous electron transfer proteins, roseoredoxin and roseoredoxin reductase, hydroxylates exogenous progesterone firstly to 16α-hydroxyprogesterone and thereafter in a second phase bioconversion to 2β,16α-dihydroxyprogesterone. The progesterone 16α-hydroxylase P450 and the two electron transfer proteins have been purified to homogeneity. A reconstituted incubation containing these three purified proteins and NADH, the natural electron donor, produced identical hydroxy-progesterone metabolites as in intact cells.

Peroxy and hydroperoxy compounds act in a shortened form of the cycle known as the 'peroxide shunt' by replacing the natural pathway requirement for the electron donor NADH, the electron transfer proteins and molecular O_2, the terminal electron acceptor. In an $NaIO_4$ supported incubation, the initial rate of progesterone hydroxylation was marginally higher (1.62 mmol progesterone/mmol P-450/h) than in the reconstituted natural incubation (1.18 mmol progesterone/mmol P-450/h) but the product yield was significantly lower, 0.45 mol hydroxyprogesterone produced/mol P-450 compared to 6.0 mol hydroxyprogesterone produced/mol P-450. These yield data show that in the reconstituted natural pathway, progesterone 16α-hydroxylase P450 supports multiple rounds of hydroxylation in contrast to a likely single oxygenation by a minority of P450s in the peroxide shunt pathway. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Streptomyces roseochromogenes*; Progesterone 16α-hydroxylation; Cytochrome P450; Cytochrome P450 electron transfer proteins

1. Introduction

The genus *Streptomyces* is a rich source of cytochrome P450 monoxygenase enzymes that are involved in a wide variety of biosynthetic and xenobiotic transformation reactions. In *Streptomyces antibioticus*, this enzyme is responsible for C-8 epoxidation of the lactone ring of the antibiotic oleandomycin [1]. The DNA sequence of the *S. antibioticus* P450 is related to the eryF gene of *S. erythraea* (*Saccharopolyspora erythraea*) which codes for a soluble cytochrome P450 (CYP107) that stereospecifically 6-hydroxylates 6-deoxyerythronolide B to erythronolide B during erythromycin A biosynthesis [2]. In *S. carbophilus* $P450_{sca}$ hydroxylates compactin to pravastatin, a tissue-selective inhibitor of 3-hydroxy-3-methylglutamyl-coenzyme A reductase [3]. The sulphonyl urea herbicide chlorimuron ethyl is metabolised by two inducible *S. griseolus* P450s, $P450_{SU1}$ (CYP105A1) and $P450_{SU2}$ (CYP105B1) [4]. *S. griseus* contains a cytochrome P450 that is inducible by the isoflavenoid genistein present in soyaflour [5]. Extracts of this organism, prepared from soya flour-induced cells and supplemented with spinach ferridoxin and ferridoxin-NADPH reductase are capable of aromatic benzylic and alicyclic hydroxylation [6]. The *ChoP* gene of *Streptomyces* sp.

Abbreviations: PAGE; polyacrylamide gel electrophoresis; P450; cytochrome P450.

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SA-COO encodes a cholesterol oxidase cytochrome P450 that has a high degree of homology with human and *Pseudomonas* P450s [7].

A plethora of *Streptomyces* species have been widely reported as excellent steroid hydroxylators. C2 and C4 hydroxylation of the phenolic steroid oestradiol is known to be P450 catalysed [6]. Skeletal sites transformed in non-phenolic steroids include ξ1, 2β, 6β, 7β, 9α 11α, 11β, 15α and 16α [for examples [8–15]] but unlike in the oestradiol bioconversion the nature of these other steroid hydroxylases is unknown. However by analogy with steroid hydroxylation in the bacterial species *Bacillus cereus* [16] and *B. megaterium* [17] a reasonable assumption is that these hydroxylases are site-selective cytochrome P450 monooxygenases.

In this paper we identify 16α-monohydroxy and 2β,16α-dihydroxyprogesterone as the transformation metabolites produced by *S. roseochromogenes* during a 72 h incubation with exogenous progesterone. We also report the purification to homogeneity of the cytochrome P450 responsible for in vitro progesterone 16α hydroxylation and the two endogenous electron transfer proteins, roseoredoxin and roseoredoxin reductase.

2. Methods

2.1. Materials

Streptomyces roseochromogenes strain 10984 was purchased from the National Collection of Industrial and Marine Bacteria (NCIB) Ltd., Aberdeen, Scotland.

Media and general chemicals were purchased from the sources previously described [18]. Epoxy activated Sepharose 6B was purchased from Pharmacia Biotech Ltd., St. Albans, Herts., UK; MIMETIC Blue 1 A6XL affinity resin was obtained from Affinity Chromatography Ltd., Freeport, Ballasala, Isle of Man, UK and Cibacron Blue FG3A and NAD-Sepharose affinity gel from Sigma Chemical Co., Poole, Dorset, UK.

2.2. Media, culture maintenance and cultivation of S. roseochromogenes strain 10,984

S. roseochromogenes was grown at 25°C on yeast extract-malt extract-glucose (YMG) agar slopes and plates. YMG agar contained yeast extract (4 g), malt extract (10 g), glucose (4 g) and agar (15 g)/l of deionised H_2O and pH 7.2. The organism was stored at 4°C and sub-cultured every three months. The agar was omitted from liquid YMG.

2.3. Steroid transformation by S. roseochromogenes strain 10,984

Steroid transformations were performed essentially as previously described for filamentous fungi [18]. For small-scale analytical experiments, cells were grown at 25°C for 48 h with continuous shaking (100 rpm in an orbital incubator) in 50 ml YMG in 500 ml conical flasks containing coiled wires to aid aeration and dispersal of biomass. After an initial 24 h of growth, progesterone (0.25 ml of a 20 mg/ml stock solution in ethanol) was added to each culture flask. After a further 24 h, cells were harvested by centrifugation in an 8 × 50 ml angle rotor spun for 30 min at 8000 rpm and 4°C in a Sorvall 5B centrifuge. The supernatant was decanted and extracted with two equal volumes of chloroform. The organic layers were collected, combined and evaporated to dryness.

To obtain sufficient pure metabolites for structure determination, progesterone transformation incubations were increased fourfold.

2.4. Determination of progesterone metabolite yields produced by S. roseochromogenes strain 10,984

Yields of progesterone metabolites produced by *S. roseochromogenes* were determined by TLC and HPLC. In the TLC method exactly 5 mg of progesterone was added to each incubation and dried transformation products were dissolved in exactly 100 μl HPLC-grade methanol. Equal A_{242} absorbance units of sample contained in *ca* 5 μl were spotted onto fluorescent high-performance Kieselgel 60 F_{254} TLC plates, which were developed in an ethyl acetate/ether/toluene (4:3:3 by volume) solvent system. Steroids were viewed under UV light and plates were photographed. Individual spots were scraped from the TLC plate and eluted in HPLC-grade methanol. Dried steroids were re-dissolved in exactly 1 ml of methanol and the UV absorbance at 242 nm was measured. The absolute amount of steroid present was determined by reference to a calibration curve.

In the HPLC method, progesterone metabolites were separated in 60% aqueous methanol on an analytical reverse-phase Whatman Partisil PXS 5/25 ODS column. Column effluent was passed through an Pye Unicam PU 4020 UV detector set at 254 nm. Metabolite concentrations were calculated from the areas of the individual peaks eluting from the column measured on a Hewlett Packard Integrator.

2.5. Purification and structure determination of progesterone metabolites produced by S. roseochromogenes strain 10,984

Metabolites for structure determination were puri-

fied as described above from scaled-up incubations. Analyses were by ^{1}H NMR spectroscopy on a Bruker WH400 MHz spectrometer as previously described [18]. 16α-Hydroxyprogesterone was identified by the 'fingerprint' method [18], which involved superimposing spectra of authentic monohydroxy steroid standards on the spectrum of putative 16α hydroxyprogesterone. This assignment was confirmed by measuring chemical shifts of key substituent protons of 16α-hydroxyprogesterone and comparing these values with those in authentic monohydroxyprogesterones [19]. A full range of dihydroxyprogesterone spectra was not available to permit identification of 2β,16α-dihydroxyprogesterone by fingerprinting. The structure of this metabolite was assigned by the chemical shift calculation method described above for 16α-hydroxyprogesterone and by a full 2-D ^{1}H COSY spectrum analysis (homonuclear correlation spectroscopy) [18].

2.6. *Purification of progesterone 16α-hydroxylase cytochrome P450 from S. roseochromogenes strain 10,984*

S. roseochromogenes was grown in 200 ml batches as described above. Cell pellets were resuspended in buffer A (0.1 M Na phosphate, 10 mM EDTA, pH 7.2) containing 10% glycerol. Washed cells were harvested and the pellets obtained were pressed between several sheets of Whatman 3MM chromatography paper to remove excess moisture and the resulting cake was stored at −70°C.

Cells were disrupted by adding an equal mass of acid-washed sand to partially thawed biomass and the mixture was suspended in 1.5 ml ice-cold buffer A + 10% glycerol/g cell biomass. The mixture was blended at full speed in an ice-cold MSE propeller style homogeniser in six bursts of 30 sec duration. The slurry was centrifuged at 15,000 rpm for 30 min at 4°C to remove particulate matter. The supernatant (S15) was decanted and stored at −70°C.

All purification procedures were performed at 0–4°C. S15 fraction (10 ml) was loaded onto a Whatman DE52 DEAE-cellulose anion exchange column (100 ml) previously washed and equilibrated in buffer B (0.1 M Na phosphate, 5 mM EDTA, 0.25 mM DTT and 10% glycerol). The column was washed with 2 bed volumes of buffer B before proteins were eluted stepwise in buffer B containing 100, 200 and 300 mM NaCl. Each fraction was assayed for progesterone 16α-hydroxylase activity as described below.

The 200 mM DE-52 fraction, containing progesterone 16α-hydroxylase activity, was dialysed overnight against two changes of buffer B. This fraction was then loaded onto an 11α-hydroxyprogesterone coupled Sepharose 6B affinity column (11 ml) which was washed and equilibrated in buffer B. Unbound protein was washed from the column in two bed volumes of buffer B. Bound proteins were eluted in three separate fractions of buffer B containing 100, 150 and 200 mM NaCl. The 150 mM NaCl fraction contained the hydroxylase activity. This fraction was dialysed as described above.

The dialysed progesterone affinity fraction was loaded onto a MIMETIC Blue 1 A6XL column (15 ml). This column was washed with buffer B followed by buffer B containing 100 mM NaCl to remove all non-specifically bound protein. Progesterone 16α-hydroxylase activity was eluted with 120 mM NaCl in buffer B.

2.7. *Cytochrome P450-dependent progesterone hydroxylation*

The progesterone 16α-hydroxylase activity of purification fractions was determined using the $NaIO_4$ method as previously described [23–25]. Progesterone (4 mM) and $NaIO_4$ (1.5 mM) were added to 0.5 ml of fraction contained in metal capped Bijou bottles. Mixtures were made up to 1 ml with buffer B. Bottles were fixed to a turn-table that was vertically rotated at 40 rpm for 2 h at 25°C. Steroid metabolites were extracted from the incubations by shaking the mixtures with chloroform (1 ml) for 1 min. The chloroform layer was removed and evaporated at 60°C. The residue was dissolved in methanol (10 μl) and spotted onto TLC plates. These were run and processed as described above.

In the natural reconstituted system in which the $NaIO_4$ was replaced by NADH, purified progesterone 16α-hydroxylase cytochrome P450, roseoredoxin and roseoredoxin reductase (e.g. Table 6), hydroxylation was absolutely specific for NADH. NADPH did not substitute at any concentration tested. The NADH optimum for hydroxylation in these assays was 2 mM.

Steroid metabolites synthesised in the above incubations were identified only by TLC because the tiny quantities of compound produced precluded full chemical or NMR structural analysis. The TLC spots obtained co-chromatographed with spots of the authentic 16α-monohydroxy- and 2β,16α-dihydroxyprogesterone produced by intact *S. roseochromogenes*.

2.8. *Production of reduced cytochrome P450 carbon monoxide difference spectra and determination of cytochrome P450 concentration*

The cytochrome P450 concentrations of purification fractions were measured from difference spectra in a split beam Pye Unicam PU8800 spectrophotometer. Equal volumes of a P450-containing fraction were pipetted into two matched glass cuvettes. A few grains

of solid sodium dithionite were added to both fractions to reduce the P450. The cuvettes were gently inverted until the dithionite had dissolved, after which they were simultaneously scanned between 390 to 520 nm to obtain a zero baseline. Carbon monoxide was bubbled for 1.5 min into the test cuvette at a rate of 1 bubble/s. The CO difference spectrum was then obtained by re-scanning between 390 to 520 nm.

To determine the concentration of cytochrome P450 in a particular fraction, the absorbance of that fraction was measured from the difference spectrum at 450 and 490 nm. The values obtained were applied to the formula:

$$\text{P450 concentration} = \frac{\text{A450}_{\text{nm}} - \text{A490}_{\text{nm}}}{\varepsilon_{\text{o}}}$$

where ε_o is the millimolar absorbance coefficient of $P450_{450-490}$ and is 91 cm^{-1}/mM^{-1}.

2.9. Purification of electron transfer proteins from S. roseochromogenes strain 10,984

The roseoredoxin reductase (ferredoxin reductase) was purified from the flow-through wash of the DE-52 DEAE-cellulose column obtained in the first stage of P450 purification. This fraction was directly loaded onto a Whatman DE-32 DEAE-cellulose column and eluted with a linear 0–300 mM NaCl gradient made up in buffer B and 10% glycerol. Fractions (5 ml) were collected and assayed for cytochrome c reductase activity at 550 nm and for NADH oxidation at 340 nm. Also, the spectrum of each fraction was recorded from 260 to 460 nm and compared to that of a standard of authentic FAD.

Fractions which reduced cytochrome c, oxidised NADH and contained FAD, were pooled and dialysed for 8 h against buffer B before loading on to a Cibacron Blue FG3A column and eluted with a linear 0–200 mM NaCl gradient in buffer B. Fractions (2.0 ml) were collected and assayed as described above. Active fractions that contained roseoredoxin reductase were pooled, dialysed against buffer B + 20% glycerol (buffer C) and stored at −70°C.

The roseoredoxin reductase DE-32 cellulose column fractions obtained above were used to purify roseoredoxin (ferredoxin), the second electron transfer protein in the *S. roseochromogenes* progesterone 16α-hydroxylase cytochrome P450 pathway. Roseoredoxin activity was assayed by measuring the rate of cytochrome c reduction in the presence of NADH and purified roseoredoxin reductase (Cibacron Blue fraction). The stimulation of the basal rate of electron transfer, from roseoredoxin reductase directly to cytochrome c, was used to measure roseoredoxin activity. Active fractions were pooled and dialysed as described above and then applied to an NAD-Sepharose affinity column. Proteins were eluted with a linear 0–100 mM KCl gradient in buffer B. KCl buffers were required due to loss of electron transfer activity in NaCl. Fractions were assayed for cytochrome c reductase activity as described above and the absorbance of these fractions was measured at 280 and 414 nm. Active fractions containing the highest A414/A280 ratios were pooled, dialysed against buffer C and stored at −70°C.

2.10. Measurement of S. roseochromogenes roseoredoxin and roseoredoxin reductase activity

Roseoredoxin activity was measured in a final volume of 1 ml containing 100 mM Na phosphate, 5.2 mM $MgCl_2$ buffer pH 7.2, 0.05 mM cytochrome c, 0.05 units roseoredoxin reductase and 0.2 mM NADH. (1 unit of roseoredoxin reductase is the amount of protein required to reduce 0.5 μmol of cytochrome c per min in the presence of excess roseoredoxin). The rate of cytochrome c reduction was measured at 550 nm against a control incubation without NADH. Cytochrome c reduction was measured to completion of the reaction at 25°C. The absolute amount reduced was calculated by measuring the absorbance difference between the test and control incubations at 550 nm. An absorbance coefficient of 29.9 mM/cm was used to calculate reduced cytochrome c and 8.9 mM/cm for oxidised cytochrome c.

Roseoredoxin reductase activity was measured as described above except that roseoredoxin was omitted from the incubations.

2.11. SDS-polyacrylamide gel electrophoresis

SDS-PAGE (15% by wt) was performed according to the method of Laemmli [20] except that the running buffer contained glycerol (10% by volume). Gels were run at 125 V for 3 h, stained with Coomassie Brilliant Blue and if appropriate, over-stained with silver [21].

2.12. Protein determinations

Protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hemel Hempstead, Herts., UK).

2.13. Coupling of 11α-hydroxyprogesterone to Sepharose 6B

Freeze dried epoxy-activated Sepharose 6B (1 g) was swollen by suspending in distilled water (100 ml) and gently stirring for 5 min. The gel was harvested by filtration onto a scintered glass funnel under vacuum. The gel was then washed for 1 h with distilled water (1200 ml) before coupling to 11α-hydroxyprogesterone.

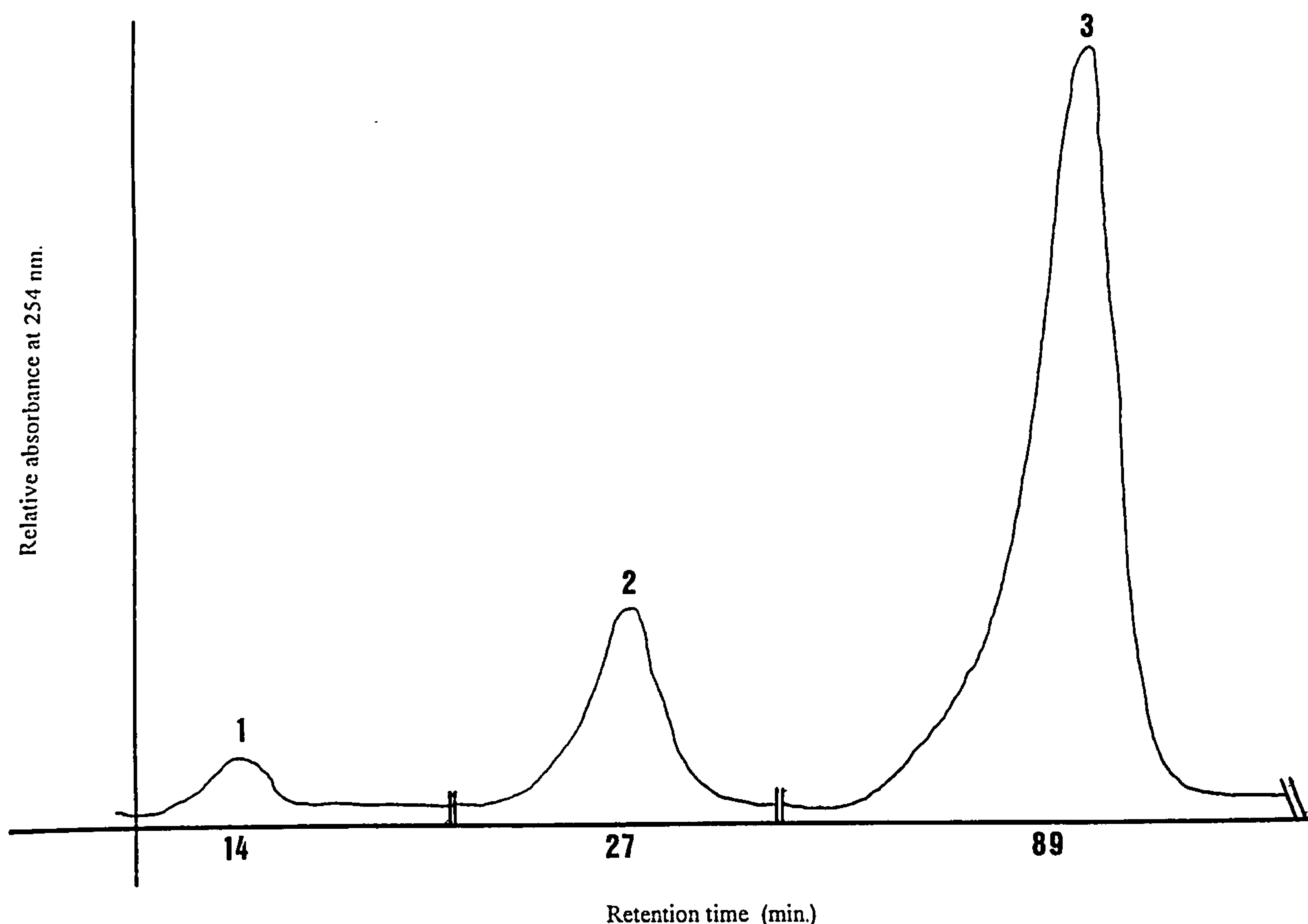

Fig. 1. HPLC of 25 h progesterone transformation incubation by *S. roseochromogenes*. From left to right, peak 1,2β,16α-dihydroxyprogesterone (retention time 14 min); Peak 2, 16α-mono-hydroxyprogesterone (retention time 27 min); Peak 3, progesterone (retention time 89 min).

The coupling was performed by adding Sepharose 6B to 1.8 mg of 11α-hydroxyprogesterone dissolved in coupling buffer containing 100 ml dimethylformamide + 100 ml 0.1 M Na phosphate buffer pH10. The suspension was incubated for 16 h at 32°C. After washing the coupled gel with copious phosphate buffer, unreacted epoxy groups were blocked by incubation in 1.0 M ethanolamine at 40°C for 10 h. The gel was again washed with phosphate buffer and the slurry poured into a glass column. The column was washed in turn with three bed volumes of 0.1 M acetate, 0.1 M NaCl buffer pH 4.0, 0.1 M borate, 0.5 M NaCl buffer pH 8.0 and finally with buffer B.

Table 1
Progesterone and its 2β and 16α-hydroxylated derivatives and HPLC peak 1 progesterone metabolite[a]

Compound	4-H	17α-H	18-H	19-H	21-H	C*H*OH
Progesterone	5.73	2.54	0.67	1.20	2.13	
2β-Hydroxy-progesterone	5.82 (0.09)	2.54 (0.00)	0.67 (0.00)	1.18 (−0.02)	2.13 (0.00)	4.20 (2α-H) (1.85)
16α-Hydroxy-progesterone	5.75 (0.02)	2.54 (0.00)	0.68 (0.01)	1.19 (−0.01)	2.18 (0.05)	4.86 (16β-H) (2.67)
2β,16α-Dihydroxy-progesterone[c]	5.84 (0.11)	2.54[b] (0.00)	0.68 (0.01)	1.17 (−0.03)	2.18 (0.05)	4.20 (2α-H) (1.85) 4.86 (16β-H) (2.67)
HPLC peak 1	5.83 (0.10)	2.54[b] (0.00)	0.67 (0.00)	1.18 (−0.02)	2.18 (0.05)	4.19 (2α-H) (1.84) 4.88 (16β-H) (2.69)

[a] δ, Relative to Me_4Si. Increments relative to progesterone in parentheses.
[b] δ calculated from the summed individual group increments, relative to progesterone.
[c] doublet.

Table 2
Production of 16α-monohydroxy- and 2β,16α-dihydroxyprogesterone by *Streptomyces roseochromogenes*

Time (h)	Total metabolite (% initial progesterone)	2β,16α-DHP (mM)	2β,16α-DHP (% initial progesterone)	2β,16α-DHP (% total metabolites)	16α-HP (mM)	16α-HP (% initial progesterone)	16α-HP (% total metabolites)
1–5	0	0	0	0	0	0	0
6	0.28	0	0	0	0.011	0.275	100
7	0.75	0	0	0	0.03	0.75	100
8	1.53	0.01	0.25	16.3	0.061	1.28	83.66
9	1.95	0.01	0.25	12.8	0.078	1.7	87.18
10	2.90	0.02	0.5	17.2	0.116	2.4	82.76
11	3.75	0.04	1.0	26.7	0.15	2.75	73.33
12	6.6	0.054	1.35	20.5	0.264	5.25	79.55
13	11.05	0.062	1.55	14.0	0.442	9.5	85.97
14	11.8	0.072	1.80	15.3	0.472	10	84.75
15	14.75	0.1	2.50	17.0	0.59	12.25	83.05
16	15.9	0.11	2.75	17.3	0.636	13.15	82.7
17	17	0.13	3.25	19.1	0.68	13.75	80.88
18	19.25	0.17	4.25	22.1	0.77	15	77.92
19	19.85	0.174	4.35	21.9	0.794	15.5	78.09
20	21.50	0.22	5.50	25.6	0.86	16	74.42
21	23.45	0.258	6.45	27.5	0.938	17	72.49
22	24.08	0.263	6.58	27.3	0.963	17.5	72.67
23	24.95	0.278	6.95	27.9	0.998	18	72.14
24	25.28	0.281	7.03	27.8	1.011	18.25	72.19

3. Results

3.1. *Identification of progesterone metabolites produced by S. rosechromogenes strain 10,984*

Two metabolites were purified from a 25 h progesterone transformation incubation and are shown in the HPLC trace in Fig. 1. These metabolites were identified by ^{1}H NMR spectroscopy as 2β,16α-dihydroxyprogesterone (Fig. 1 peak 1, retention time 14 min) and 16α-monohydroxyprogesterone (Fig. 1 peak 2, retention time 27 min). The NMR identifying features of 16α-hydroxyprogesterone have been described previously [22].

3.2. *NMR data for 2β,16α-dihydroxyprogesterone*

The ^{1}H NMR spectrum of 2β,16α-dihydroxyprogesterone has not been previously published. Therefore, we identified this compound by matching the measured values for the chemical shifts of key identifying signals of peak 1 metabolite to theoretical values calculated for 2β,16α-dihydroxyprogesterone (Table 1). The calculations involved summing the published values of the key identifying protons in 2β- and 16α-monohydroxyprogesterone [19]. 2D COSY analysis confirmed the structure. The features of structural significance of 2β,16α-dihydroxyprogesterone are summarised below.

Peak 1 retained the fundamental signals of the progesterone skeleton, i.e. 4-H (s[1], δ 5.83), 18-H_3 (s, δ 0.67), 19-H_3 (s, δ 1.18), 21-H_3 (s, δ 2.18) and displayed the features of dihydroxylation with two clearly separated mid-field multiplets between δ 4.9 and δ 4.2 attributable to methine protons of secondary hydroxylated carbon atoms. The shape and spectral position of these peaks was consistent with 2β,16α-dihydroxylation. This transformation was confirmed by the following spectral data. The characteristic triplet of 17α-H in progesterone was coincident with a predicted doublet (δ 2.54), consequent on splitting by the 16β-H. 16α-Hydroxylation was confirmed by the strong correlation of the 17-H in the COSY with the distinctive mid-field multiplet (2.69 ppm) at δ 4.88 extant at lower field than any other CHOH proton of a hydroxyprogesterone as a result of the proximity of the 16β-H to the 20-oxo group. Correlation of COSY cross peaks of the mid-field multiplet CHOH signal at δ 4.19 with lα-H at δ 2.4, which was significantly shifted to low field (0.71 ppm) relative to progesterone and to 1β-H (δ 1.56) and unusually shifted to high field (−0.48 ppm) relative to progesterone, confirmed 2β-hydroxylation.

[1] s, singlet; m, multiplet.

3.3. *Time course of progesterone transformation by S. roseochromogenes strain 10,984*

16α-Hydroxyprogesterone, the major metabolite of progesterone transformation, first appeared in the culture medium after 6 h of incubation (Table 2). This metabolite was actively produced throughout the entire 24 h incubation but the rate of production significantly slowed after 22 h. A second phase metabolite, 2β,16α-dihydroxyprogesterone, was first detected in the culture medium 2 h after the first appearance of 16α-hydroxyprogesterone. This compound steadily accumulated during the next 13 h of transformation but at less than half the rate of synthesis of 16α-hydroxyprogesterone. Production also virtually ceased at 21 h. 2β-Hydroxyprogesterone, the counterpart monohydroxy metabolite to 16α-hydroxyprogesterone, was not observed at any time during the 24 h transformation period. At the end of transformation, 25% of substrate progesterone had been converted into hydroxylated products of which *ca* 72% was 16α-hydroxyprogesterone and *ca* 28% was 2β,16α-dihydroxyprogesterone.

3.4. *Optimisation of progesterone hydroxylation by S15 homogenates of S. roseochromogenes strain 10,984*

The conditions summarised below were established for optimal periodate hydroxylation of progesterone by *S. roseochromogenes* S15 supernatants (data not shown). The most active extracts were obtained from mature cultures grown for about 35 h and from cells disrupted by blending for 3 min with an equal mass of sand in bursts of 30 s, interspersed with 30 s resting on ice. Protease inhibitors in the blending buffer had no significant effect on hydroxylation, whereas the addition of 10% glycerol stimulated hydroxylation nearly 3-fold. EDTA concentrations to 10 mM were slightly stimulatory (30%) but were strongly inhibitory at higher concentrations. At 30 mM EDTA, hydroxylation was totally inhibited. A similar effect was observed with DTT. The optimum concentration that gave 50% stimulation was 0.5 mM and 3 mM DTT inhibited hydroxylation by 50%. The pH activity profile was extremely sharp, the optimum being pH 7.2. Hydroxylation was severely inhibited either side of this value (30% respectively at pH 7.0 and 7.6). In contrast the $NaIO_4$ oxidant optimum was broad and flat from 1.5 mM to 5 mM. A true optimum for progesterone concentration could not be determined due its poor solubility in incubation buffer.

Thus, varying the concentration from 0.5 to 7.5 mM (beyond the solubility limit) had virtually no effect on metabolite production.

Using these optimal conditions, hydroxylation was linear for up to 18 minutes at 25°C and was pro-

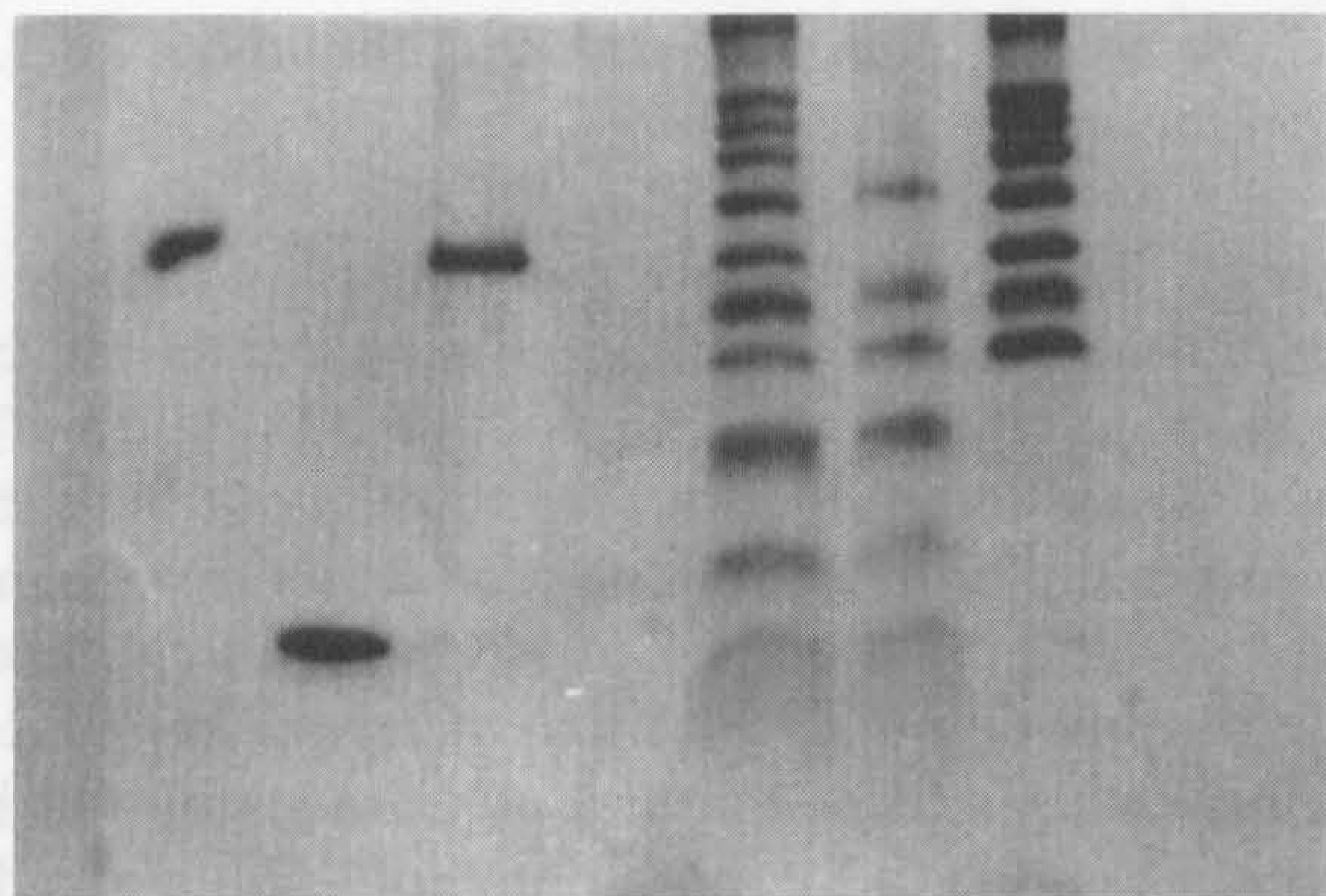

Fig. 2. SDS-polyacrylamide gel of purified *S. roseochromogenes* 16α-hydroxylase cytochrome P-450, roseoredoxin and roseoredoxin reductase. From left to right, lane 1, roseoredoxin reductase (3.1 μg M_r 65 × 10^3); lane 2, roseoredoxin (2.8 μg M_r 14 × 10^3); lane 3, progesterone 16α-hydroxylase cytochrome P450 (4 μg M_r 63 × 10^3); lane 4, wide range protein size ladder M_r 205 × 10^3–14.2 × 10^3; lane 5, lower range protein size ladder M_r 66 × 10^3–14.2 × 10^3; and lane 6, upper range protein size ladder M_r 205 × 10^3–45 × 10^3. Lanes 4–6 contained 35 μg total protein.

portional to S15 concentrations between 1 to 6 mg protein/ml. Hydroxylation was 50% inhibited by 1 μg/ml ketoconazole and 100% inhibited by 4 μg/ml. This ketokonazole inhibition is supportive of cytochrome P450 as the progesterone 16α-hydroxylase enzyme.

3.5. Purification of progesterone 16α-hydroxylase cytochrome P450 from S. roseochromogenes strain 10984

A three step procedure was used to purify progesterone 16α-hydroxylase cytochrome P450 to homogeneity judged by SDS-PAGE and silver staining (Fig. 2). $NaIO_4$ was used in the hydroxylation assays *in lieu* of NADH and the electron transfer proteins required in the natural P450 pathway. The details of the P450 purification are summarised in Table 3. *S. roseochromogenes* S15 extract was applied to a DE52 DEAE-cellulose column, which was eluted by a step 50–350 mM NaCl gradient. The hydroxylase activity was in the 200 mM NaCl fraction. This fraction contained less than 5% of the total protein applied to the column.

After dialysis, the DE52 fraction was applied to a progesterone affinity column and eluted with a NaCl step-gradient. The fractions obtained were assayed for haemoproteins by absorbance at 417 nm as well as for general protein (absorbance at 280 nm). Over 95% of the general protein applied to this particular column failed to bind and eluted in the flow-through and wash fractions. These fractions contained virtually no haemoprotein. The haemoprotein was found to bind moderately tightly to the progesterone affinity ligand and, together with the remaining 6.5% of protein, eluted in

Table 3
Purification of *S. roseochromogenes* progesterone 16α hydroxylase cytochrome P450

Purification stage	Total protein (mg)	P-450 (nmol)	P-450 (nmol/mg protein)	Recovery (%)	Purification factor
S15 extract	1050	21.01	0.019	100	1
DEAE ion exchange (200 mM NaCl fraction)	42.59	20.02	0.47	95.29	24.7
Dialysis	42.53	18.1	0.43	86.14	22.6
Sepharose 6B 11α-progesterone affinity column (150 mM NaCl fraction)	2.8	3.3	1.18	15.71	62.1
Dialysis	2.79	2.94	1.05	13.99	55.3
MIMETIC Blue 1 affinity column (120 mM NaCl fraction)	0.08	0.27	3.46	1.29	182.1
Dialysis	0.08	0.25	3.21	1.2	168.9

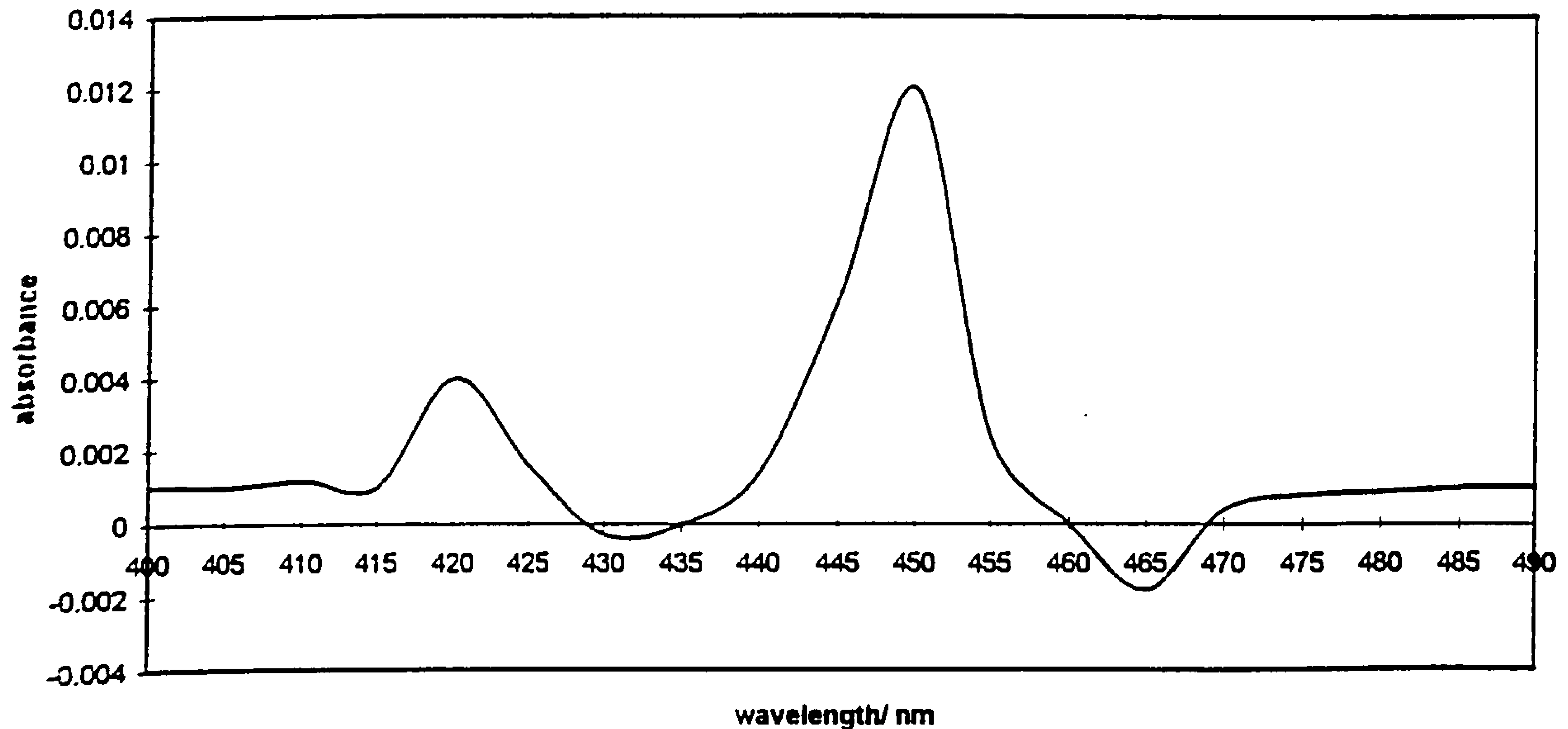

Fig. 3. Dithionite reduced, carbon monoxide difference spectrum of the 120 mM NaCl MIMETIC Blue 1 affinity column fraction. Each cuvette contained 0.79 mg P450.

150 mM NaCl. This fraction contained all the hydroxylase activity.

In the third purification step, the 150 mM NaCl progesterone affinity fraction was applied to a MIMETIC Blue 1 affinity column. This column also proved to be extremely efficient as virtually 100% of the 417 nm absorbing material initially applied, i.e. the haemoprotein, was bound but only 3% of the total protein. All bound protein and progesterone 16α-hydroxylase activity eluted in the 120 mM NaCl fraction. A single protein band was seen for this fraction on SDS-PAGE (Fig. 2). Single protein bands were also observed on haem-stained and Coomassie blue-stained 5% native acrylamide gels and on 3% isoelectric focusing gels (results not shown). An M_r value of 63×10^3 was determined for the P450 from the SDS-PAGE and a pI value of 6.6 from the isoelectric focusing gels.

Based on CO difference spectroscopy, a 182 fold purification and a yield of 1.3% native P450 was calculated for this simple purification procedure. A small minority of the cytochrome P450 was in inactive P420 form (Fig. 3).

3.6. Purification of the progesterone 16α-hydroxylase cytochrome P450 electron transfer proteins roseoredoxin reductase and roseoredoxin from S. roseochromogenes strain 10,984

The DE-52 DEAE-cellulose column flow-through and wash, obtained from the first stage of P450 purification, was used as starting material for the purification of the electron transfer proteins roseoredoxin reductase and roseoredoxin. A two step procedure was used to purify to homogeneity both proteins. The data for these methods are shown in Table 4 (roseoredoxin reductase) and Table 5 (roseoredoxin) respectively.

In the case of roseoredoxin reductase, a second DEAE column, containing DE32 cellulose, followed by dye affinity chromatography on Cibacron Blue FG3A resulted in a 258 fold purification of this particular protein. Roseoredoxin co-eluted with the roseoredoxin reductase in the DE32 300 mM NaCl fraction, but was separated from the latter protein by NAD-Sepharose affinity chromatography to give a final 163 fold purification. Both proteins gave single bands in SDS-PAGE (Fig. 2). M_r values of 14×10^3 and 65×10^3 were determined from the SDS-PAGE gels for roseoredoxin and roseoredoxin reductase respectively (Fig. 2).

3.7. Comparison of progesterone metabolism catalysed by a reconstituted natural cytochrome P450 dependent hydroxylation pathway and an $NaIO_4$ dependent peroxide shunt pathway

The roseoredoxin, roseoredoxin reductase and NADH requirement in the natural progesterone 16α-hydroxylase cytochrome P450 hydroxylation pathway is replaceable by an organic or inorganic peroxy or hydroperoxy compound (XOOH) such as $NaIO_4$. When these two pathways were compared for progesterone transformation catalysed by highly purified *S. roseochromogenes* progesterone 16α hydroxylase cytochrome P450, it was found that the initial rate of hydroxylation was nearly 40% greater in the $NaIO_4$ peroxide shunt pathway (1.62 mmol progesterone converted/mmol P-450/h) than in the natural reconstituted pathway (1.18 mmol progesterone converted/mmol P-

Table 4
Purification of *S. roseochromogenes* roseoredoxin reductase

Purification stage	Total protein (mg)	Total roseoredoxin reductase (nmol)	Yield roseoredoxin reductase (nmol/mg protein)	Recovery roseoredoxin reductase	Purification factor
S15 extract	1050	32	0.03	100	1
DEAE 52 column flow-through	800	30.5	0.04	95.31	1.3
DE 32 0-300 mM NaCl fraction	6.84	19.96	2.92	62.38	97.3
Dialysis	6.84	18.44	2.70	57.63	90
Cibacron Blue FG3A 0-200 mM NaCl fraction	1.88	14.55	7.74	45.47	258
Dialysis	1.88	13.03	6.93	40.72	231

Table 5
Purification of *S. roseochromogenes* roseoredoxin

Purification stage	Total protein (mg)	Total roseoredoxin (nmol)	Yield roseoredoxin (nmol/mg total protein)	Recovery roseoredoxin (%)	Purification factor
S15 extract	1050	51	0.05	100	1
DEAE 52 column flow-through	800	39.10	0.05	76.67	1
DE 32 0-300 mM NaCl fraction	6.84	18.79	2.75	36.80	55
Dialysis	6.84	17.77	2.6	34.80	52
SepharoseNAD affinity 0-100 mM KCl fraction	1.95	9.81	5.03	19.20	101
A414/A280 fraction pooled and Dialysis	1.10	8.95	8.14	17.55	163

Table 6
Comparison of progesterone metabolism catalysed by the reconstituted natural cytochrome P450 dependent hydroxylation pathway and the $NaIO_4$ dependent pathway

Pathway	Initial rate of progesterone transformation (mmol progesterone/mmol P-450/h)	Turnover (mol progesterone/mol P-450)
Reconstituted	1.18	6.00
$NaIO_4$	1.62	0.45

450/h). By contrast, the peroxide shunt pathway supported 13 fold fewer hydroxylation events per molecule of P450 (0.45 mol progesterone converted/mol P-450) than the reconstituted pathway (6.0 mol progesterone converted/mol P-450) (Table 6).

4. Discussion

S. roseochromogenes was first identified as catalysing steroid 2β- and 16α-hydroxylation 40 yr ago during the search for microorganisms capable of efficient and mild stereospecific access of the 16 site of the steroid nucleus [e.g. [23–24]]. Microbial access of this site was eventually employed for the synthesis of a new generation of highly potent, synthetic, anti-inflammatory pharmaceuticals exemplified by triamcinalone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione) developed to replace natural corticosteroids found to suffer mineralocorticoid contraindications.

S. roseochromogenes 10,984 possesses strong progesterone 2β- and 16α-hydroxylation activities. The time course of progesterone transformation (Table 2) shows 16α-hydroxylation to be the primary event and 2β-hydroxylation to be a second phase reaction using 16α-monohydroxyprogesterone as substrate. Thus, 16α-monohydroxyprogesterone was first detected in the culture medium after 6 h of incubation and 2β,16α-dihydroxyprogesterone appeared 2 h thereafter at 8 h. That this is the true route of progesterone bioconversion was shown when *S. roseochromogenes* transformed exogenous 16α-hydroxyprogesterone to 2β,16α-dihydroxyprogesterone. Moreover, free 2β-monohydroxyprogesterone was never detected in our system even in transformation incubations containing 20 mg of progesterone and a metabolite detection threshold of under 0.1 mg of steroid.

There is now a wealth of compelling evidence identifying cytochrome P450 as responsible for steroid hydroxylation reactions in both bacteria and filamentous fungi e.g. in bacteria — 6β in *Bacillus thermoglucosidasius* [25] and 15β in *Bacillus megaterium* [17]: in filamentous fungi — 7α in *Phycomyces blakesleeanus* [26]; 11α in *Aspergillus fumigatus* [27], *Aspergillus ochraceus* [28], *Nectria haematococca* [29] and *Rhizopus nigricans* [30]; 11β in *Cochliobolus lunatus* [31]; and 15α in *Penicillium raistrickii* [32]. Despite the long history and extensive literature on microbial steroid hydroxylation no such role has so far been indisputably assigned to P450 for this reaction in *Streptomyces* species. Therefore, this paper is the first report to identify unequivocally cytochrome P450 as a steroid hydroxylase enzyme in the *Streptomyces* genus.

A turnover number of *ca* 0.02/min was calculated for *S. roseochromogenes* P450 catalysed progesterone hydroxylation in the reconstituted pathway and a comparable value of *ca* 0.03/min for the $NaIO_4$ dependent pathway. Both numbers are now the lowest reported for a purified P450 being an approximate order of magnitude lower than the previous bottom of the table turnover numbers of 0.6/min reported for the steroid 15β-hydroxylase cytochrome P-450 ($P\text{-}450_{meg}$) of *Bacillus megaterium* [33] and of 1.8/min and 2.1/min reported for precocene II and 7-ethoxy coumarin hydroxylation respectively catalysed by a crude fraction of *S. griseus* P450 [6]. The reason for this catalytic inefficiency is unclear, but it may be connected with the fact that progesterone is almost certainly not the physiological substrate for the *S. roseochromogenes* P450. Thus, a meaningful comparison of the true efficiency of this enzyme with its bacterial counterparts cannot be made until a natural substrate is identified. However these data show that in the natural pathway highly pure *S. roseochromogenes* P450 catalyses multiple cycles of hydroxylation even with the unnatural steroid substrate.

In contrast to steroid hydroxylation, cytochrome P450 has been identified in several *Streptomyces* pathways of secondary metabolism. Thus, this enzyme participates in oleandomycin biosynthesis in *Streptomyces antibioticus* [1], the hydroxylation of compactin to prevastatin by *Streptomyces carbophilus* [3] and in a wide variety of xenobiotic transformations in *Streptomyces griseus* [4–6].

Cytochrome P450 genes have been cloned from only two *Streptomyces* species, *S. griseolus* and *S. griseus*. In *S. griseolus*, genes for the herbicide-inducible cytochromes P450, P450SU1 and P450SU2, have been sequenced and the amino acid sequence of segments of the encoded proteins deduced. These proteins were found to possess high sequence identity to the *Pseudomonas putida* camphor hydroxylase P450 (P450 101, $P450_{cam}$) particularly in the haem binding domain

[34]. A DNA segment carrying the structural gene encoding P-450_{soy} (soyC), has been cloned from *S. griseus*. It is noteworthy that this cytochrome P450 also has high sequence conservation with $P450_{cam}$ in the haem binding region [35]. It would be interesting to compare the primary sequence of the *S. roseochromogenes* progesterone 16α-hydroxylase cytochrome P450 with those of these other *Streptomyces* P450s.

Ferredoxin electron transfer proteins have also been purified from the above two *Streptomyces* species. *S. griseolus* was found to contain two 7 kDa isoforms, designated Fd-1 and Fd-2. These proteins have 52% identity and both contain single [3Fe-4S] clusters [36]. Both ferredoxins are active in reconstituted cell-free systems containing the SU1 P-450 isoform, although Fd-2 is more effective. The genes for the ferredoxins and the sulfonylurea P450 monooxygenases are virtually contiguous. Thus P-450SU1 and the downstream Fd-1 form a closed spaced pair, similarly P-450SU2 and Fd-2. In *S. griseolus* only a single ferredoxin encoded by SOY B has been putatively identified. The situation in *S. roseochromogenes* remains to be resolved.

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NMR spectroscopy was performed under the ULIRS WH-400 NMR service at Queen Mary and Westfield College by Mr Peter Haycock and at Kings College by Mrs Jane Hawkes.

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Microbial transformations of steroids-XII. Progesterone hydroxylation profiles are modulated by post-translational modification of an electron transfer protein in *Streptomyces roseochromogenes*

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Abstract

When *Streptomyces roseochromogenes* strain 10984 was incubated with exogenous progesterone for 25 h the major monohydroxylated metabolite, 16α-hydroxyprogesterone was produced in 3.6 fold excess to the minor metabolite 2β,16α-dihydroxyprogesterone. In a reconstituted system containing highly purified progesterone 16α-hydroxylase cytochrome P-450, and electron transfer proteins ferredoxin-like redoxin (roseoredoxin) and redoxin reductase (roseoredoxin reductase), both metabolites were produced but in a 10:1 ratio. When *S. roseochromogenes* was pre-incubated for 8 h with 0.32 mM progesterone and the purified components of the hydroxylase system incubated as before, the ratio of 16α-hydroxyprogesterone to 2β,16α-dihydroxyprogesterone produced decreased to 2.8:1, virtually identical to the ratio in whole cell transformations. Reconstitution assays containing all combinations of hydroxylase proteins purified from progesterone pre-incubated and control cells showed that the roseoredoxin was solely responsible for the observed changes in in vitro metabolite ratios. The fact that the lower 16α-hydroxyprogesterone to 2β,16α-dihydroxyprogesterone ratio was also obtained when *S. roseochromogenes* was exposed to 0.335 mM cycloheximide for 8 h prior to the progesterone pre-incubation, pointed to post-translation modification of the roseoredoxin. Separation of two isoforms of roseoredoxin by isoelectric focusing supported this proposition. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Microbial transformation; Progesterone hydroxylation; Electron transfer protien

1. Introduction

There is now a wealth of compelling evidence identifying cytochrome P450 as responsible for steroid hydroxylation reactions in both bacteria and filamentous fungi e.g. in bacteria-6β in *Bacillus thermoglucosidasius* [1] and 15β in *Bacillus megaterium* [2]: in filamentous fungi-7α in *Phycomyces blakesleeanus* [3]; 11α in *Aspergillus fumigatus* [4], *Aspergillus ochraceus* [5] *Nectria haematococca* [6], and *Rhizopus nigricans* [7]; 11β in *Cochliobolus lunatus* [8]; and 15α in *Penicillium raistrickii* [9].

A plethora of *Streptomyces* species has been widely reported as excellent steroid hydroxylators but the nature of the enzyme responsible for these conversions was until very recently unproven. The skeletal sites of non-phenolic steroids transformed include ξ1, 2β, 6β, 7β, 9α 11α, 11β, 15α and 16α [for examples see [10–17]]. By analogy with steroid hydroxylation in the bacterial species *Bacillus cereus* [18] and *B. megaterium* [2] the tacit assumption was that these hydroxylases are site-selective cytochrome P450 monooxygenases. Indirect evidence supported this assumption in the case of C2 and C4 hydroxylation of the phenolic steroid oestradiol by extracts of *Streptomyces griseus* [19]. Recently a cytochrome P450 has been purified from *S. roseochromogenes* strain 10984 which catalyses progesterone 16α hydroxylation and a second phase 2β hydroxylation [20].

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Bacterial cytochromes P450 are the terminal proteins in a soluble three component system (Type I system) that transfer electrons to acceptor molecular dioxygen. P450 catalyses the reductive cleavage of the dioxygen producing H_2O and an –OH group which is ultimately covalently bonded to enzyme-bound substrate by the P450 apoprotein. The stereochemistry of the hydroxylation is determined by the particular structural architecture of the catalytic active centre of the apoprotein. The electrons that drive the hydroxylation originate from NADH passing to P450 via intermediate redox proteins. In Type-I systems these proteins are a low molecular weight ferredoxin-like redoxin that directly interacts with the P450, and a larger flavin-containing redoxin reductase that is a one electron transducer positioned between NADH and the redoxin. This soluble three component system has been identified in the $P450_{soy}$ system of *S. griseus* [21] and in the sulfonylurea herbicide P450 monooxygenase system in *S. griseolus* [22] where the component proteins have been purified.

During a 25 h incubation of exogenous progesterone with *S. roseochromogenes* strain 10984, 16α-monohydroxyprogesterone is produced in a first phase of transformation followed shortly thereafter in a second phase by 2β,16α-dihydroxyprogesterone [20]. A cytochrome P450 was purified from this organism and was shown in vitro to synthesise the whole cell metabolites 16α-hydroxyprogesterone and 2β,16α-dihydroxyprogesterone. Two other proteins, a redoxin and a redoxin reductase together with the electron donor NADH were absolutely required for these hydroxylations [20].

In this paper we show that the ratio of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone produced in a reconstituted cell-free system containing all three highly purified protein components depends on the growth history of the *S. roseochromogenes* and that the change in metabolite ratio is manifested by the roseoredoxin rather than the roseoredoxin reductase or progesterone 16α-hydroxylase cytochrome P450.

2. Methods

2.1. Materials

S. roseochromogenes strain 10984 was purchased from the National Collection of Industrial and Marine Bacteria (NCIB) Ltd., Aberdeen, UK.

Media and general chemicals were purchased from the sources previously described [23]. Cibacron Blue FG3A and NAD-Sepharose affinity gel were from Sigma Chemical Co., Poole, Dorset, UK.

2.2. Media, culture maintenance and cultivation of S. roseochromogenes strain 10984

S. roseochromogenes was grown at 25°C on yeast extract–malt extract–glucose (YMG) agar slopes and plates. YMG contained yeast extract (4 g), malt extract (10 g), glucose (4 g) and agar (15 g/l) of deionised H_2O and pH 7.2. The organism was stored at 4°C and subcultured every 3 months. The agar was omitted from liquid YMG. Growth of *S. roseochromogenes* was completely inhibited by 100 μg/ml cyclohexnimide.

In pre-incubation experiments, 0.32 mM progesterone was added to the growth medium for 8 h prior harvesting the cells.

2.3. Determination of progesterone metabolite yields produced by S. roseochromogenes strain 10984

Yields of progesterone metabolites produced in whole cell and cell-free incubations were determined by HPLC as described previously [20]. Incubations were extracted twice for 5 min by vigorously mixing the aqueous fraction with equal volumes of chloroform. The organic layers were collected, combined, evaporated to dryness and dissolved in HPLC grade methanol. The metabolite mixtures were dissolved and separated in 60% aqueous methanol on an analytical reverse-phase Whatman Partisil PXS 5/25 ODS column. Column effluent was passed through a Pye Unicam PU 4020 UV detector set at 254 nm. Metabolite concentrations were calculated from the areas of the individual peaks eluting from the column measured on a Hewlett Packard Integrator. The individual metabolites were identified by their retention time on HPLC compared to authentic standards of 16α-hydroxyprogesterone and 2β,16α-dihydroxyprogesterone.

2.4. Purification of progesterone 16α-hydroxylase cytochrome P450 from S. roseochromogenes strain 10984

S. roseochromogenes progesterone 16α-hydroxylase cytochrome P450 was purified as previously described [20]. With progesterone as substrate (Fig. 1), the P450 exhibited a typical set of type I substrate binding spectra with maxima at 384 nm, minima at 420 nm and an isosbestic point at 401 nm [[24] and references therein]. Scatchard analysis of these binding curves gave a K_d value for progesterone of 1.95×10^{-8}M.

2.5. Cytochrome P450-dependent progesterone hydroxylation

The progesterone 16α-hydroxylase activity was determined using 4 mM progesterone and 2 mM NADH, 0.3 μM progesterone 16α-hydroxylase cytochrome P450,

0.05 units of roseoredoxin and 0.1 units of roseoredoxin reductase made up to 1 ml with 0.1 M Na phosphate, 5 mM EDTA, 0.25 mM DTT (buffer A) and 10% glycerol in Bijou bottles. Bottles were fixed to a turn-table that was vertically rotated at 40 rpm for 2 h at 25°C. Steroid metabolites were extracted from the incubations by shaking the mixtures with 1 ml chloroform for 1 min. The chloroform layer was removed and evaporated to dryness at 60°C. The residue was dissolved in 10 μl methanol and separated on HPLC as described above.

2.6. Purification of roseoredoxin and roseoredoxin reductase electron transfer proteins from S. roseochromogenes strain 10984

The roseoredoxin reductase was purified before roseoredoxin from the flow-through wash of the DE-52 DEAE-cellulose column obtained in the first stage of P450 purification [20]. This fraction was directly loaded onto a Whatman DE-32 DEAE-cellulose column and eluted with a linear 0–300 mM NaCl gradient made up in buffer A and 10% glycerol. Fractions (5 ml) were collected and assayed for cytochrome *c* reductase activity at 550 nm and for NADH oxidation at 340 nm. Also, the spectrum of each fraction was recorded from 260 to 460 nm and compared to that of a standard of authentic FAD.

Fractions which reduced cytochrome c, oxidised NADH, and contained FAD, were pooled and dialysed for 8 h against buffer A before loading on to a Cibacron Blue FG3A column and eluted with a linear 0-200 mM NaCl gradient in buffer A. Fractions (2.0 ml) were collected and assayed as described above. Active fractions that contained roseoredoxin reductase were pooled, dialysed against buffer A + 20% glycerol (buffer C), and stored at −70°C. The purification data are shown in Table 1(a) and the 15% polyacrylamide gel electrophoresis analysis in Fig. 2.

The roseoredoxin reductase DE-32 cellulose column fractions obtained above were used to purify roseoredoxin, the second electron transfer protein in the *S. roseochromogenes* progesterone 16α-hydroxylase cytochrome P450 pathway. Roseoredoxin activity was assayed by measuring the rate of cytochrome *c* reduction in the presence of NADH and purified roseoredoxin reductase (Cibacron Blue fraction). The stimulation of the basal rate of electron transfer, from roseoredoxin reductase directly to cytochrome c, was the measure of roseoredoxin activity. Active fractions were pooled and dialysed as described above and then applied to an NAD-Sepharose affinity column. Proteins were eluted with a linear 0–100 mM KCl gradient in buffer A. KCl buffers were required due to loss of electron transfer activity in NaCl. Fractions were assayed for cytochrome *c* reductase activity as described above and the absorbance of these fractions was measured at 280 and 414 nm. Active fractions containing the highest A414/A280 ratios were pooled, dialysed against buffer C and stored at −70°C. The final purified roseorëdoxin solution was pale yellow.

The purification data are shown in Table 1b and the gel analyses in Fig. 2, Fig. 3 and Fig. 4. The absolute absorbance spectrum of oxidised roseoredoxin showed maxima at 280 and 400 nm with a shoulder at 310 nm Fig. 5. The absorbance profile was mainly preserved after reduction of the roseoredoxin with a 15 fold excess of dithionite. Thus, the 280 and 400 nm maxima

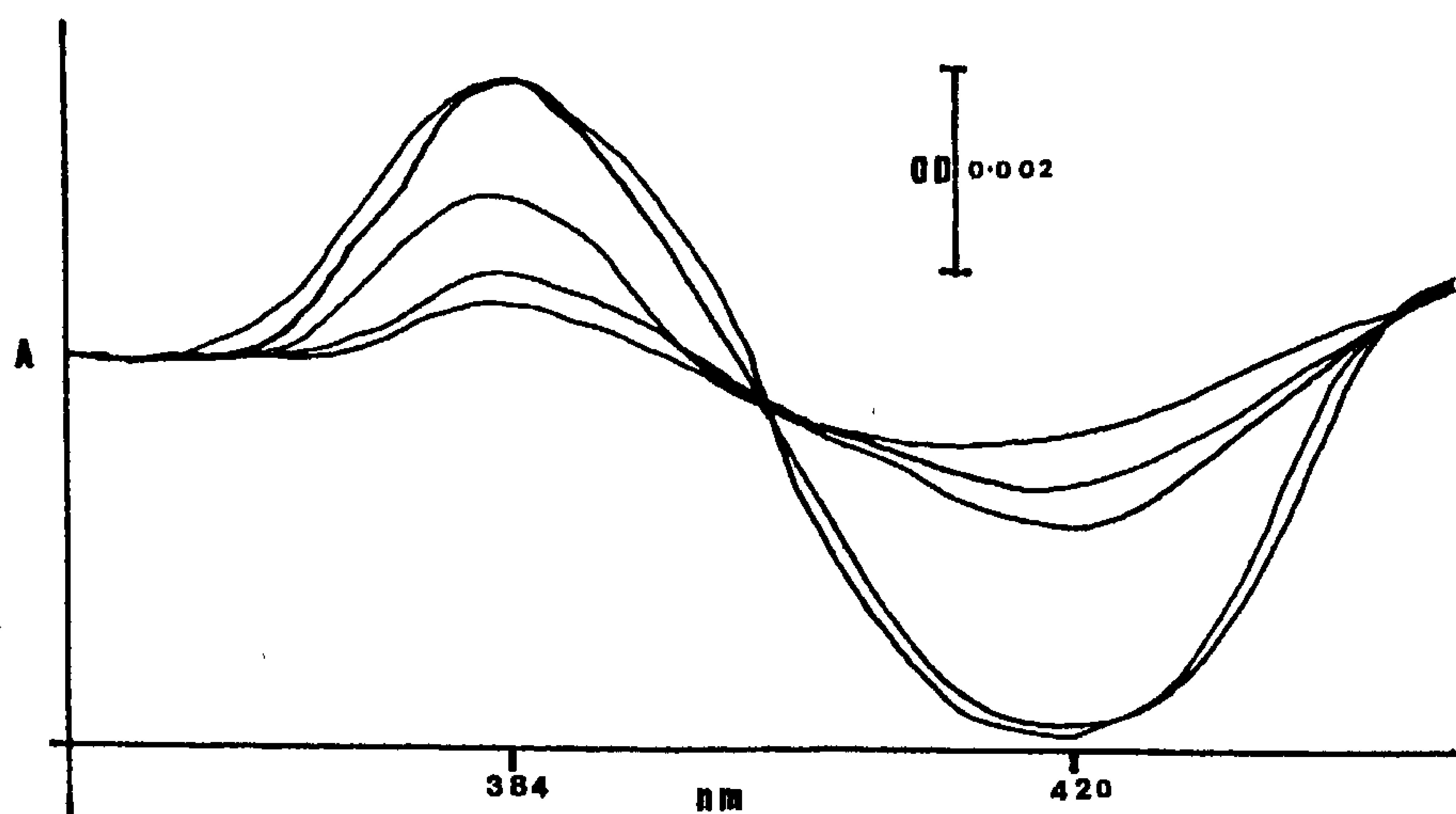

Fig. 1. *S. roseochromogenes* cytochrome P450 progesterone substrate binding spectra. The amount of cytochrome P450 was 3 pmol in all incubations. Curve 1, 0.1 pmol progesterone; curve 2, 0.3 pmol progesterone; curve 3, 0.5 pmol progesterone; curve 4, 1.0 pmol progesterone and curve 5, 1.4 pmol progesterone.

Table 1

Purification stage	Total protein (mg)	Total roseoredoxin reductase (nmol)	Yield roseoredoxin reductase (nmol/mg protein)	Recovery roseoredoxin reductase (%)	Purification factor
(a) Purification of *S.roseochromogenes* roseoredoxin reductase					
S15 extract	1050	32	0.03	100	1
DEAE 52 column flow-through	800	30.5	0.04	95.31	1.3
DE 32 0-300mM NaCl fraction	6.84	19.96	2.92	62.38	97.3
Cibacron Blue FG3A 0-200 mM NaCl fraction, dialysed	1.88	13.03	6.93	40.72	231
		Total roseoredoxin (nmol)	Yield roseoredoxin (nmol/mg protein)	Recovery roseoredoxin (%)	
(b) Purification of *S.roseochromogenes* roseoredoxin					
S15 extract	1050	51	0.05	100	1
DEAE 52 column Flow-through	800	39.10	0.05	76.67	1
DE 32 0–300 mM NaCl fraction	6.84	18.79	2.75	36.80	55
Sepharose-NAD affinity 0–100 mM KCl fraction	1.95	9.81	5.03	19.20	101
Pooled Abs 414/A280 fraction, dialysed	1.10	8.95	8.14	17.55	163

were retained but not the 310 nm shoulder. Reduction decreased the absolute absorbance above 300 nm. These spectra are typical of a 7 Fe-containing ferredoxin [25].

2.7. Separation of two isoforms of S. roseochromogenes roseoredoxin

The roseoredoxin obtained from the NAD-Sepharose affinity purification step was separated into two activity fractions on Whatman CM-52 cellulose by elution at 50–100 mM KCl and 240–280 mM KCl. The isoforms co-migrated in 10% SDS-PAGE (Fig. 3).

2.8. Measurement of S. roseochromogenes roseoredoxin and roseoredoxin reductase activity

Roseoredoxin activity was measured in a final volume of 1 ml containing 100 mM Na phosphate, 5.2 mM $MgCl_2$ buffer pH 7.2, 0.05 mM cytochrome c, 0.05 units roseoredoxin reductase and 0.2 mM NADH. (1 unit of roseoredoxin reductase is the amount of protein required to reduce 0.5 μmol of cytochrome c per min in the presence of excess roseoredoxin). The rate of cytochrome *c* reduction was measured at 550 nm against a control incubation without NADH. Cytochrome *c* reduction was measured to completion of the reaction

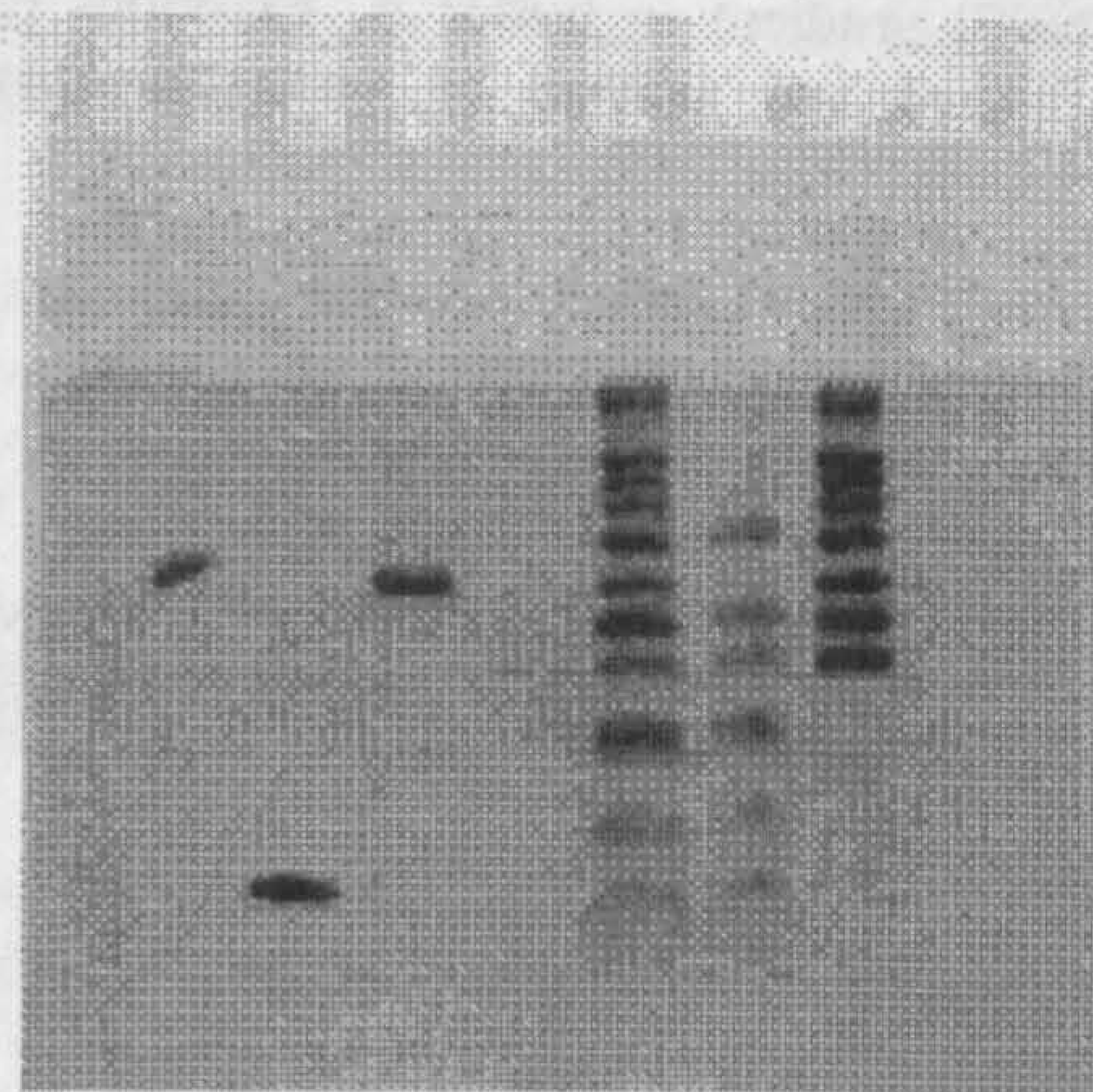

Fig. 2. SDS-15% polyacrylamide gel of purified *S. roseochromogenes* 16α-hydroxylase cytochrome P-450, roseoredoxin and roseoredoxin reductase. From left to right, lane 1, roseoredoxin reductase (3.1 μg M_r 65×10^3); lane 2, roseoredoxin (2.8 μg M_r 14×10^3); lane 3, progesterone 16α-hydroxylase cytochrome P450 (4 μg M_r 63×10^3); lane 4, wide range protein size ladder M_r 205×10^3–14.2×10^3; lane 5, lower range protein size ladder M_r 66×10^3–14.2×10^3; and lane 6, upper range protein size ladder M_r 205×10^3–45×10^3. Lanes 4–6 contained 35 μg total protein.

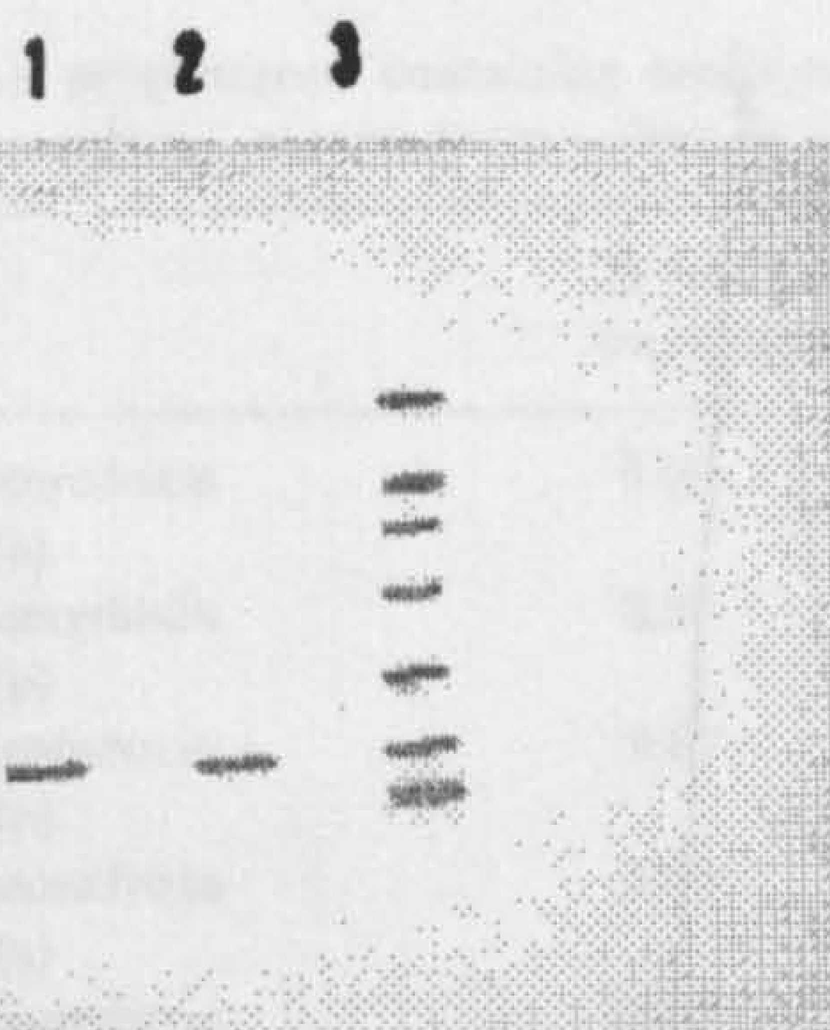

Fig. 3. 10% SDS-polyacrylamide gel of purified *S. roseochromogenes* roseoredoxin isoforms. Lane 1, 50–100 mM KCl roseoredoxin isoform fraction from CM 52 (2.8 μg M_r 14 × 10^3); Lane 2, 240–280 mM KCl roseoredoxin isoform fraction from CM 52 (2.8 μg M_r 14 × 10^3); lane 3, protein ladder (35 μg total protein, M_r 66 × 10^3–14.2 × 10^3 (M_r × 10^3 from the top of the gel to bottom: 66, BSA; 45, chicken egg ovalbumin; 36, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase; 29, bovine erythrocyte carbonic anhydrase; 24, bovine pancreas trypsinogen; 20, soybean trypsin inhibitor and 14.2, bovine milk α-lactalbumin.

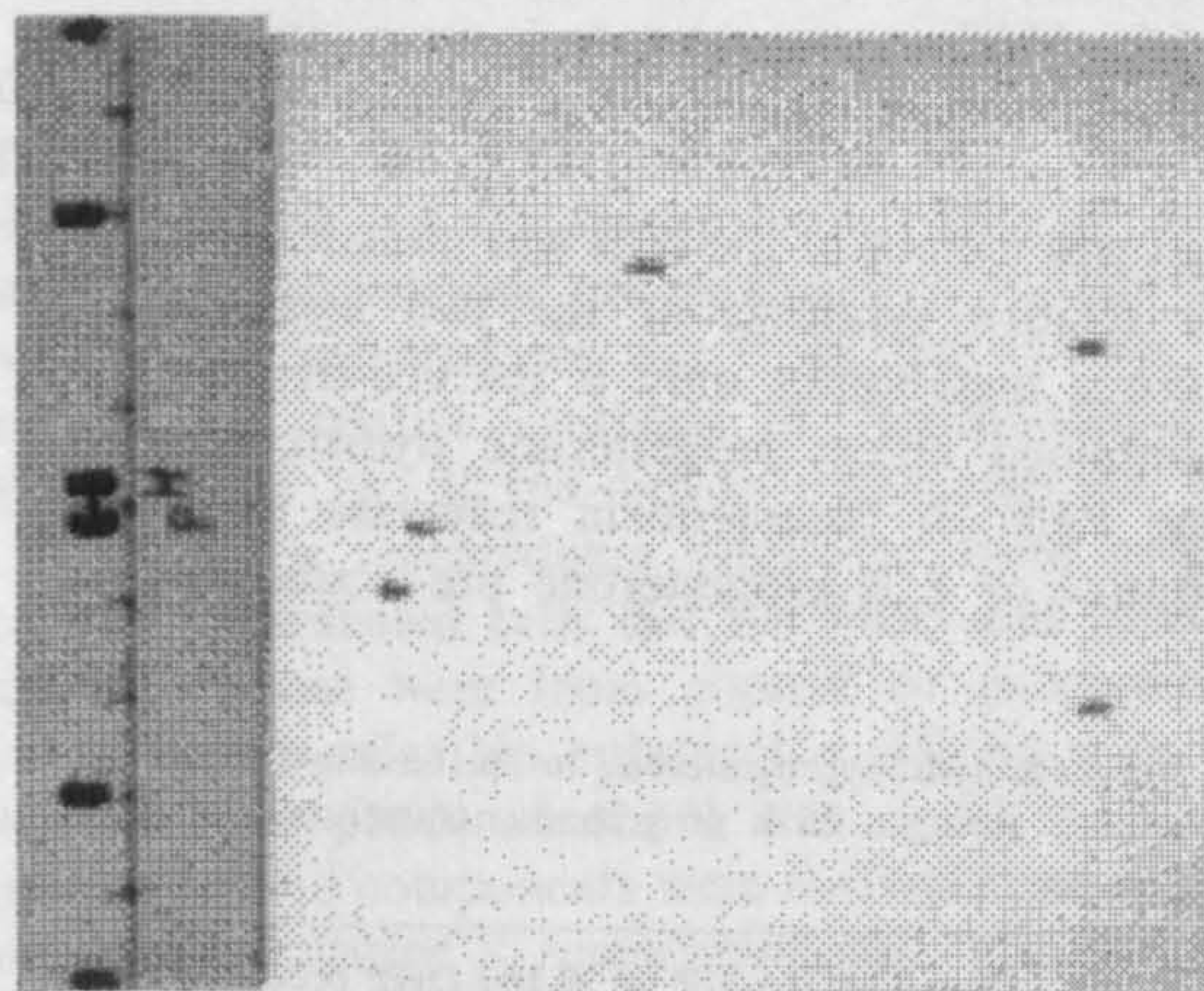

Fig. 4. 2-D, IEF gel of *S. roseochromogenes* roseoredoxin, roseoredoxin reductase and 16α-hydroxylase cytochrome P-450. Clockwise left to right, roseoredoxin reductase pI 6.9, 16α-hydroxylase cytochrome P-450 pI 6.6, β-lactoglobulin pI 5.2 (M_r 37,100), and the two isoforms of roseoredoxin pI 5.6 and 7.45. The gel was silver stained.

at 25°C. The absolute amount reduced was calculated by measuring the absorbance difference between the test and control incubations at 550 nm. An absorbance coefficient of 29.9 mM^{-1} cm^{-1} was used to calculate reduced cytochrome *c* and 8.9 mM^{-1} cm^{-1} for oxidised cytochrome c.

Roseoredoxin reductase activity was measured as described above except that roseoredoxin was omitted from the incubations.

2.9. Polyacrylamide gel electrophoresis

SDS-PAGE (10 and 15% w/v) was performed according to the method of Laemmli [26] except that the running buffer contained glycerol (10% v/v). Gels were run at 125 V for 3 h, stained with Coomassie Brilliant Blue, and if necessary, over-stained with silver [27].

Isoelectric focusing was carried out on the NAD-Sepharose affinity gel fractions described above in a Biorad Mini Protean electrophoresis apparatus with a Bio-Rad rod gel rack attachment, essentially according to the method of Harrington et al. [28]. Separation in the first dimension was by electrofocusing using 5% acrylamide rod gels containing equal concentrations of ampholines pH 4–9 and pH 5–8 (0.5 ml/6 ml gel solution). The second dimension was 10% acrylamide, SDS slab gels.

2.10. Protein determinations

Protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hemel Hempstead, Herts., UK).

3. Results

3.1. Hydroxyprogesterone metabolite production by intact S. rosechromogenes cells

The HPLC trace of the hydroxyprogesterone metabolites produced in a 25 h whole cell transformation of progesterone by *S. roseochromogenes* is shown in Fig. 6. Two metabolites were identified. The minor

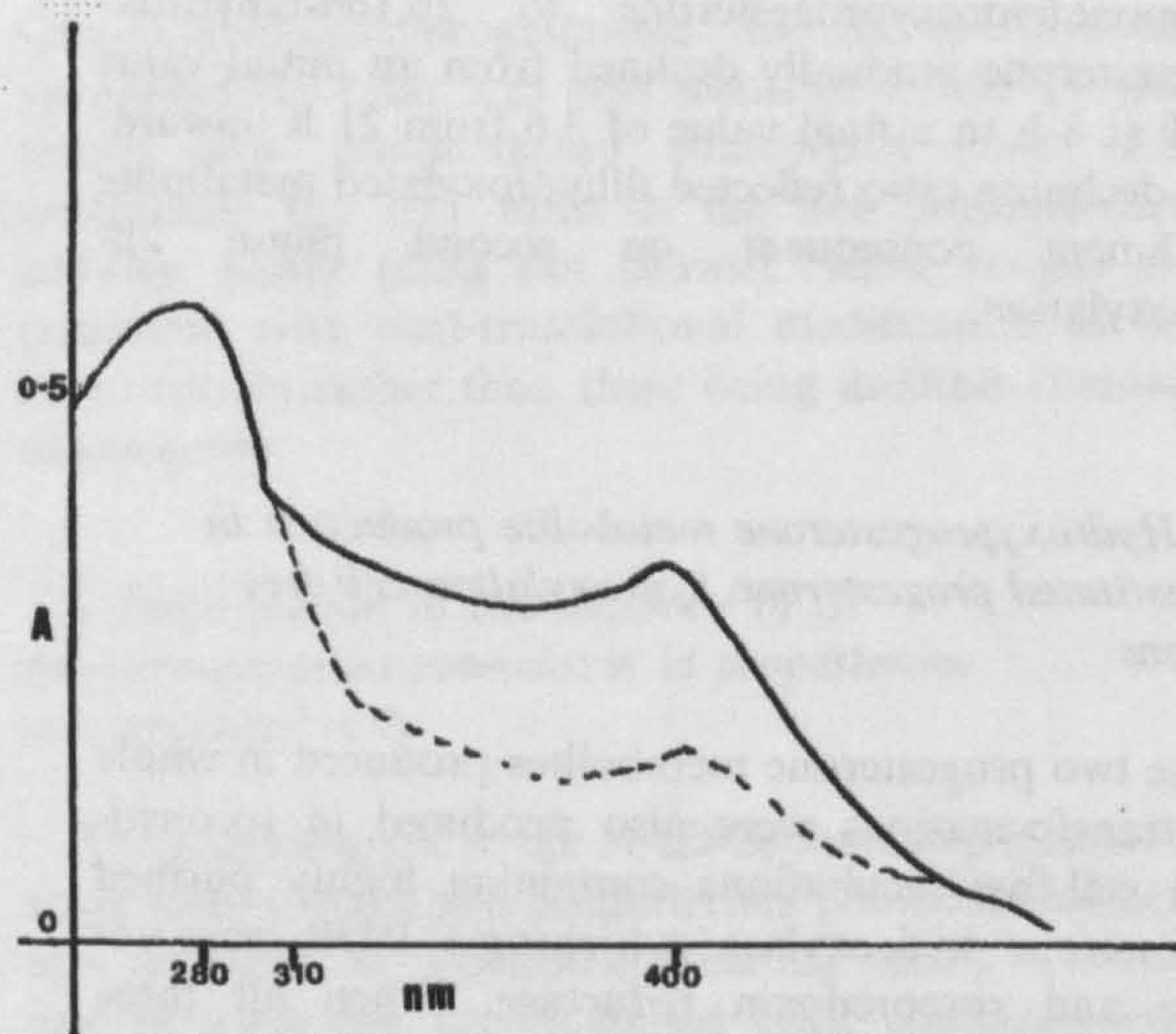

Fig. 5. Absorbance spectrum of *S. roseochromogenes* roseoredoxin. The solid line is the absorbance of 8 μM native oxidised roseoredoxin in 0.1 M K_2HPO_4 buffer pH 7, the dotted line is the absorbance of the reduced form treated with a 15 fold excess of sodium dithionite.

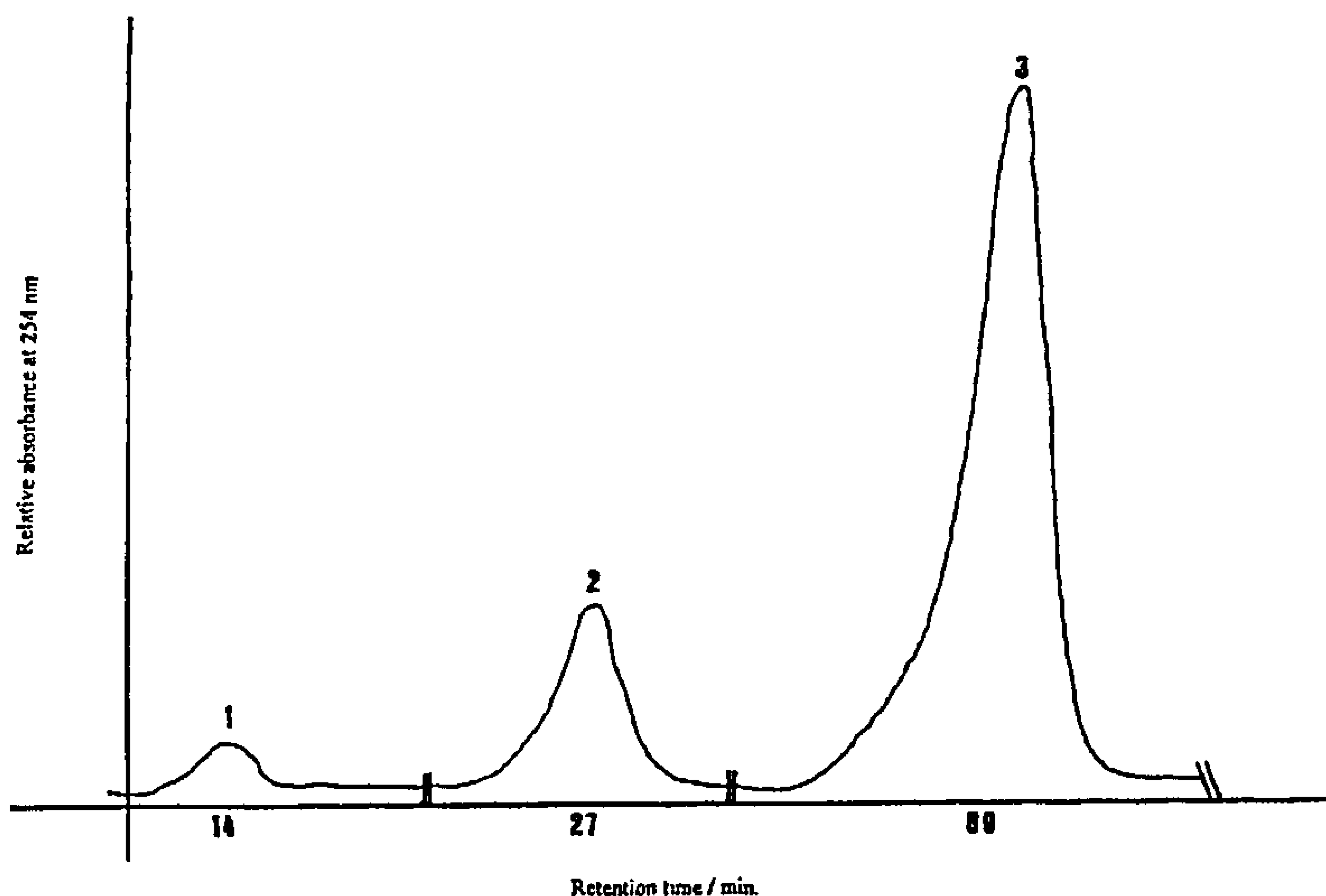

Fig. 6. HPLC of 25 h progesterone transformation incubation by *S. roseochromogenes*. From left to right, peak 1, 16α-dihydroxyprogesterone (retention time 14 min); Peak 2, 16α-monohydroxyprogesterone (retention time 27 min); Peak 3, progesterone (retention time 89 min).

peak 1 (retention time 14 min) is 2β,16α-dihydroxyprogesterone and the major peak 2 (retention time 27 min) is 16α-monohydroxyprogesterone. Peak 3 is untransformed substrate progesterone. The NMR identifying features of these compounds have been published [29,30].

Ratios of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone produced during this 25 h incubation are shown in Table 2. From 6 to 7 h only 16α-monohydroxyprogesterone was detected in the culture medium. From 8 h onward 2β,16α-dihydroxyprogesterone also accumulated. The ratio of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone gradually declined from an initial value of 61 at 8 h to a final value of 3.6 from 21 h onward. This declining ratio reflected dihydroxylated metabolite enrichment consequent on second phase 2β-hydroxylation.

3.2. *Hydroxyprogesterone metabolite production in reconstituted progesterone hydroxylation cell-free systems*

The two progesterone metabolites produced in whole cell transformations were also produced in reconstituted cell-free incubations containing highly purified progesterone hydroxylase cytochrome P450, roseoredoxin and roseoredoxin reductase. When all three proteins were from control i.e. non progesterone pre-incubated cells, a value of 10.07 was calculated for the relative synthesis of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone in a 40 min incubation (Table 3, line 1).

3.3. *Effect of an 8 h progesterone pre-incubation on the relative synthesis of 16α-monohydroxyprogesterone and 2β,16α-dihydroxyprogesterone in reconstituted progesterone hydroxylation cell-free systems*

The growth history of the cells was immaterial to the ultimate relative synthesis of the two hydroxylated progesterone metabolites only if the cell-free incubations contained either progesterone hydroxylase cytochrome P450 or roseoredoxin reductase (or both) from control or 8 h progesterone pre-incubated cells

Table 2
Ratio of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone produced during a 25 h progesterone transformation by *S. roseochromogenes*

Time (h)	Ratio of 16α-HP to 2β,16α-DHP produced
1.5–5	No products detectedced
6–7	16α-HP only
8	61
9	78
10	58
11	3.75
12	4.9
13	7.1
14	6.5
15	5.9
16	5.8
17	5.2
18	4.5
19	4.7
20	3.9
21	3.6
22	3.7
23	3.6
25	3.6

Table 3
The effect of growth in progesterone containing medium on hydroxyprogesterone metabolite synthesis in reconstituted cell free incubations containing purified roseoredoxin, roseoredoxin reductase and cytochrome P-450[a]

Assay after 40 min	2β,16α-DHP % total transformation products	16α-HP % total transformation products	Ratio of 16α-HP to 2β,16α-DHP
Roseoredoxin(a) + Roseoredoxin reductase(a) + P-450(a)	9.03	90.97	10.1
Roseoredoxin(a) + Roseoredoxin reductase(a) + P-450(p)	8.81	91.19	10.4
Roseoredoxin(a) + Roseoredoxin reductase(p) + P-450(p)	9.65	90.35	9.4
Roseoredoxin(a) + Roseoredoxin reductase(p) + P-450(a)	8.95	91.05	10.2
Roseoredoxin(p) + Roseoredoxin reductase(p) + P-450(p)	25.97	74.03	2.9
Roseoredoxin(p) + Roseoredoxin reductase(p) + P-450(a)	26.03	73.97	2.8
Roseoredoxin(p) + Roseoredoxin reductase(a) + P-450(p)	25.9	74.1	2.9
Roseoredoxin(p) + Roseoredoxin reductase(a) + P-450(a)	26.36	73.64	2.8
Whole cells	Minor	Major	

[a] (a) is the protein purified from cells grown in the absence of progesterone and (p) is the protein purified from cells grown in the presence of progesterone. (HP, hydroxyprogesterone, DHP, dihydroxyprogesterone). The three proteins were substituted with counterparts purified from cells grown in the presence of cycloheximide but in the absence of progesterone. Identical results to those in the table were obtained.

(Table 3, lines 2, 3 and 4). Thus relative synthesis ratios of 10.35, 10.17 and 9.36, respectively were obtained when the P450 and the roseoredoxin reductase originated from progesterone pre-incubated or control cells but only the roseoredoxin was from control cells. By contrast the relative synthesis of 16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone was 2.79 when the roseoredoxin originated from progesterone pre-incubated cells but the P450 and roseoredoxin reductase were from control or progesterone pre-incubated cells. No substantive difference in the product ratio produced (2.85) was noted if all the cell-free protein components were derived from progesterone pre-incubated *S. roseochromogenes* (Table 3, line 5). Incubation time was not a factor in the roseoredoxin effect. Thus, although the absolute amount of both metabolites increased in incubations lasting from 2 to 40 min, the same metabolite ratios shown in Table 3 were always obtained (data not shown).

3.4. *Effect of cycloheximide pre-incubation on the relative synthesis of 16α-monohydroxyprogesterone and 2β,16α-dihydroxyprogesterone in reconstituted S. roseochromogenes progesterone hydroxylation cell-free systems containing control and progesterone pre-incubated roseoredoxin*

The data in Table 3 clearly show that the roseoredoxin component of the P450 electron transfer chain modulates the relative synthesis of 16α-monohydroxyprogesterone and 2β,16α-dihydroxyprogesterone. We examined whether progesterone pre-incubation induced *de novo* synthesis of a roseoredoxin isoform or whether a post-translational modification of the protein occurs. Gene expression was inhibited in 8 h progesterone pre-incubated cells by blocking protein synthesis with 0.335 mM cycloheximide, a concentration in excess of that required totally to inhibit cell growth. The progesterone hydroxylation profile of the reconstituted cell-free incubations containing roseoredoxin purified from these cycloheximide and progesterone pre-treated cells (16α-monohydroxyprogesterone to 2β,16α-dihydroxyprogesterone ratio 2.8) was identical to the progesterone only pre-incubated counterpart (ratio 2.8) contrasting the 10:1 ratio in the non pre-incubated cell-free assays (data not shown). These results are consistent with post-translational modification of the roseoredoxin rather than there being multiple roseoredoxin genes.

3.5. *Identification of two isoforms of S. roseochromogenes roseodoxin in progesterone pre-incubated cells*

The roseoredoxin at the NAD-Sepharose purification stage from control and progesterone pre-incubated cells was analysed by isoelectric focusing (IEF). Control, non progesterone pre-incubated cells, contained only the pI 7.45 roseoredoxin isoform (Fig. 7a). By contrast the pI 5.6 isoform predominated in 8 h progesterone pre-incubated cells and only a minor pI 7.45 band was observed (Fig. 7b). Interestingly, both isoforms were

present in approximately equal amounts in 4 h progesterone pre-incubated cells (Fig. 7c). The two roseoredoxin isoforms were separable by CM-52 cellulose ion exchange chromatography (Table 4).

4. Discussion

Ferredoxin-like iron sulphur containing proteins required for cytochrome P450 monooxygenase activity

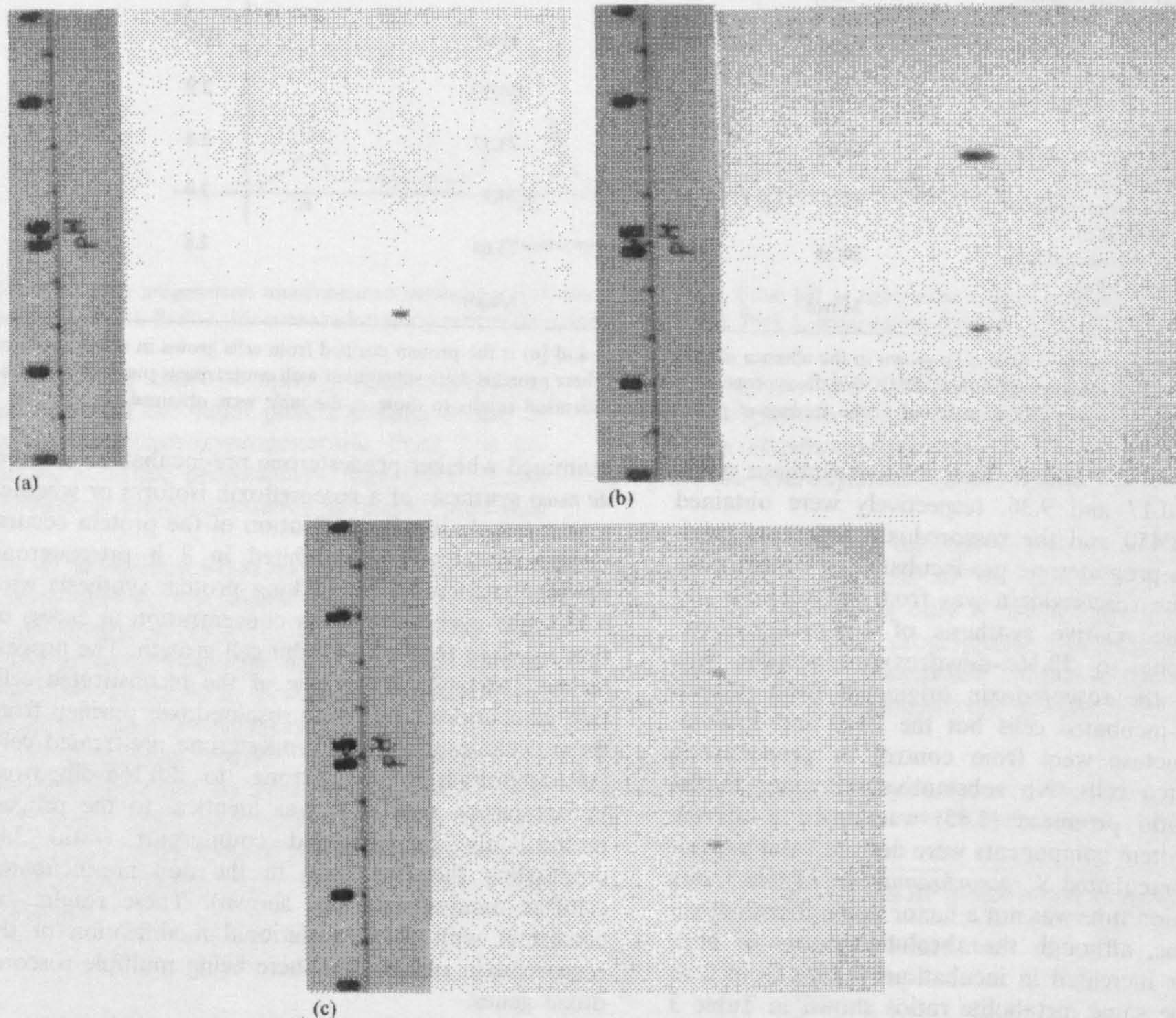

Fig. 7. IEF electrophoresis of *S. roseochromogenes* roseoredoxin purified from control and progestrone pre-incubated cells. 5 μg of roseoredoxin protein was loaded on each IEF gel. 7a, roseoredoxin purified from control cells; 7b, roseoredoxin purified from 8 h progesterone pre-incubated cells; 7c, roseoredoxin purified from 4 h progesterone pre-incubated cells.

Table 4
Separation of two isoforms of *S. roseochromogenes* roseoredoxin by CM-52 cellulose ion exchange chromatography

Purification Stage	Total protein (mg)	Total roseoredoxin (nmol)	Yield roseoredoxin (nmol/mg total protein)	Recovery roseoredoxin (%)
(A) CM cellulose 50–100 mM KCl fraction	2.0 mg protein applied to column and purified into A and B fractions	79.2	39.6	55.4
(B) CM cellulose 240–280 mM KCl fraction		63.7	31.85	44.6

have been identified in a wide variety of bacteria e.g. *Bacillus megaterium* ATCC 13368 [31], *Bradyrhizobium japonicum* [32], *Pseudomonas incognita* [33], *Pseudomonas putida* [34], *Rhodococcus* SP strain NI86/21 [35], *Saccharopolyspora erythraea*, CA340 [36], *S. griseolus* [22], *S. griseus* [37], *Xanthobacter* sp. [38]. Where sequence analysis has been performed, these ferredoxin-like genes seem to be constituents of operons containing also the P450 and the redoxin reductase gene.

In the case of *S. griseolus* two 7 kDa isoforms, designated Fd-1 and Fd-2 have also been purified. These proteins have 52% mutual identity and both contain single [3Fe–4S] clusters [22]. Both ferredoxins function in reconstituted cell-free systems containing the SU1 isoform of P-450 although Fd-2 is the more active protein. The genes for the ferredoxins and the sulfonylurea P450 monooxygenases are virtually contiguous. Thus P-450SU1 and the downstream Fd-1 form a closed-spaced pair; similarly P-450SU2 and Fd-2 pair.

Less in known about the situation in *S. griseus* where only a single ferredoxin encoded by SOY B has been putatively identified [37].

Our results suggest that a novel system for ferredoxin-like protein expression, namely post-translational modification, may well operate in *Streptomyces*. In control cells grown in a standard medium a single, mildly basic, and soluble form of roseorodoxin (pI 7.45) is present. This protein, like its published bacterial counterparts forms the middle component of a natural cytochrome P450 electron transfer chain. In vitro this *S. roseochromogenes* system predominantly catalyses 16α monohydroxylation of progesterone and as a rather minor secondary reaction, 2β hydroxylation of the primary product. By contrast, pre-incubating the organism with progesterone results in the appearance of different, second, more acidic, soluble form of roseredoxin (pI 5.6). This modified form increases the 2β hydroxylation capability of the cytochrome P450. This is achieved by increasing the hydroxylation turnover number of the P450 thereby increasing the number of 2β hydroxylation events. The result is seen as a decline in the 16α-monohydroxy to 2β,16α-dihydroxprogesterone ratio. That a growth inhibitory dose of cycloheximide added to the cells for 8 h prior to the progesterone pre-incubation had no effect on the appearance of the pI 5.6 isoform is strongly consistent with a progesterone dependent post-translational modification of the roseodoxin rather than for transcriptional activation of a second gene. There is no literature precedent for the regulation of ferredoxin activity by post-translational modification.

What is the nature of the post-translational modification and how does it modulate the activity of the P450? The covalent attachment of an acidic group such as phosphate is an obvious candidate to account for the 1.85 pH unit decrease in the pI value of the roseodoxin. This enhanced 2β hydroxylation capacity of the P450 stimulated by the modified roseoredoxin isoform could be accounted for by increased electron shuttling from the roseoredoxin reductase to the P450. This improved shuttling could result from enhanced interaction of the roseoredoxin with its adjacent electron transfer protein partners or an inherently superior electron transfer velocity of the modified roseoredoxin. The results of NMR experiments involving cytochrome $P450_{cam}$ (CYP101) and its Fe_2S_2 ferredoxin electron transfer partner, putidaredoxin, indicate that conformational gating of the electron transfer complex between these two proteins may be important in their redox reactions [39]. For example, the enzymatic removal or modification of the C-terminal tryptophan of putidaredoxin is known to cause a much reduced rate of enzymatic activity in the reconstituted camphor hydroxylase system [40,41].

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