

The 21-Dehydroxylation of Corticosteroids

by Eubacterium lentum

and

The Dehalogenation of 4-Chlorobenzoic Acid

by 4-Chlorobenzoate Dehalogenase

by

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## ABSTRACT

Two enzyme mechanisms were investigated: the 21-dehydroxylation of corticosteroids by Eubacterium lentum and the dehalogenation of 4-chlorobenzoic acid by Pseudomonas sp. CBS 3.

Chemical and enzymic methods of reduction of 21-oxo steroids were used to generate C-21-d<sub>1</sub> compounds of tetrahydrodeoxycorticosterone, with both predominant stereochemistries. It was found that during the dehydroxylation the pro-S hydrogen at the C-21 position was lost preferentially. This suggests that the enzyme removes the pro-S hydrogen during binding to the active site as the ene-diol.

To study the hydrolytic replacement of chlorine by hydroxyl, p-chlorobenzoic acid-d<sub>4</sub> was prepared and sent to Germany for an incubation with an enzyme preparation of 4-Chlorobenzoate Dehalogenase. Results suggests the possible loss of deuterium during the conversion of p-chlorobenzoate to p-hydroxybenzoate, from all four ring positions. Many methods of preparing the control compound p-hydroxybenzoic acid-d<sub>4</sub> were investigated.

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## INTRODUCTION

### The 21-Dehydroxylation of Corticosteroids

In the daily life of an organism, such as a human, many compounds are ingested and synthesized which need to be either deactivated after performing some function, or degraded to supply energy to the organism. To this end, the body has developed specialized organs to balance the need for synthesis and degradation as a function of the intake of exogenous materials. The gastrointestinal tract and the liver are two such organs that work in concert to maintain this balance.

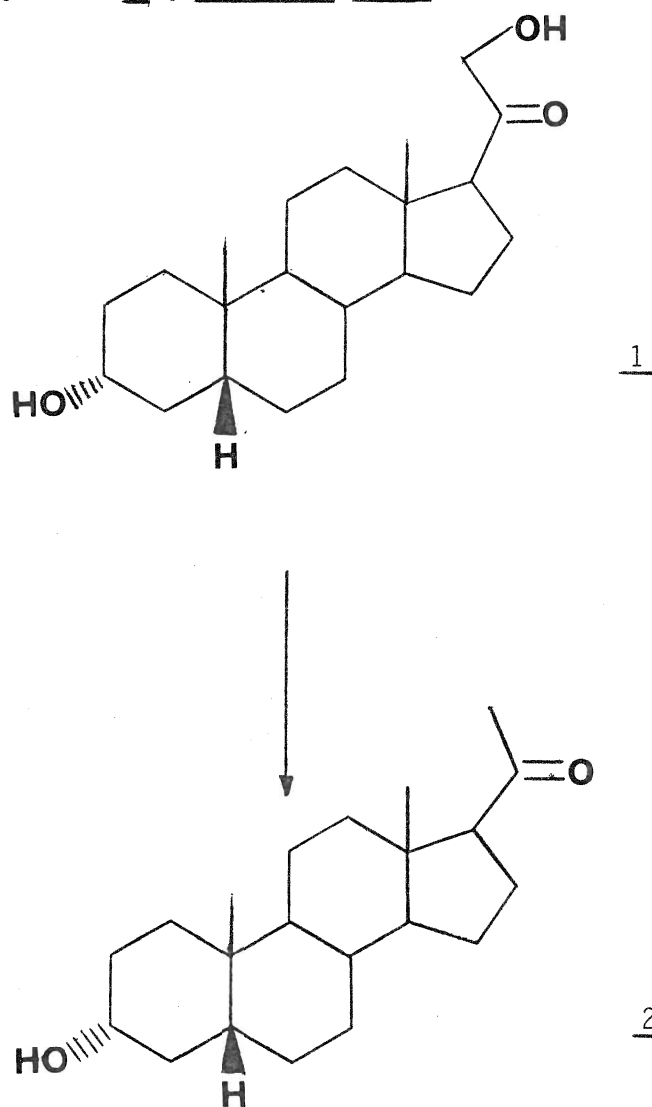
The gastrointestinal tract is mainly responsible for the absorption of ingested materials useful for building blocks, and energy sources such as sugars, proteins and fats. The liver on the other hand is responsible mainly for the balance of metabolism by the synthesis and degradation of endogenous and exogenous materials. The liver is often considered to be the purgative organ since it contains enzymes that detoxify or deactivate compounds to facilitate their removal from the body. For these reasons the liver and the gastrointestinal tract are linked in a bidirectional manner to allow for effective communication between the two and allow for circulation of most endogenous and ingested material through the liver and the

intestine.<sup>1</sup>

While the liver and its function are of great importance, considering the result of liver failure, the liver does not represent all of the possible degradative processes that a circulating compound may be exposed to.<sup>2</sup> Contained in the gastrointestinal tract between the jejunum and the caecum are approximately 400 different species of bacteria. There are so many cells that in the average adult they can total approximately one kilogram of cells. With such a large number of species, it is not surprising that a large number of reactions are catalysed by enzymes from these bacterial sources. A lot of work has gone into studying many of these processes since the substrates and products can be very important to the human host's metabolism. Hence, any study of human metabolism must take into consideration the metabolic capabilities of these commensal parasites.

An important area of research has been in the metabolism or transformation of steroid molecules by the gut flora, since many bile acids, steroid hormones and even cholesterol have been shown to be susceptible to transformation. There are many possible processes that can be carried out by these organisms on the steroid molecules. Some of the reactions include reductions, oxidations, epimerizations, side chain cleavages and dehydroxylations.<sup>2</sup> It is this last process that is important to this thesis since it will deal with the 21-dehydroxylation of tetrahydrodeoxycorticosterone 1 to pregnanolone 2 (Fig. 1).<sup>3</sup>

Figure 1: Conversion of tetrahydrodeoxycorticosterone 1 to Pregnanolone 2 by *Eubacterium lentum* 3





This process is of special importance to man since it represents a route for the deactivation of corticoid hormones (Fig. 2). Normally these hormones are synthesized in the adrenal cortex and released into the blood stream where they reach their target cells.<sup>4</sup> After performing their particular function they are again released into the blood stream where they arrive in the liver. Here the unsaturated A ring system is reduced to a 3-alpha hydroxyl and a 5-beta ring junction. This same process can be carried out by a number of fecal flora,<sup>5</sup> but it is felt that the major route for reduction of this ring system is by liver dehydrogenases. While in the liver transferases put hydrophilic groups on the alcohol functions at C-21 and C-3 to improve water solubility. Such groups are usually glucuronate or sulphonate and are removed by bacterial enzymes once the steroid has arrived in the gastrointestinal tract. In the gut the functional groups are removed by certain bacteria. It is in the gut that a bacterial species can perform the reductive removal of the hydroxyl group at the 21 position.<sup>6</sup> The identity of the particular species responsible for this reaction was not known for many years, while early investigations dealt with a culture capable of converting deoxycorticosterone 3 into 2 (Fig. 3).<sup>6</sup> The culture was named culture 116 but was not identified either as a pure culture or as a specific collection of species. It was merely grown by enrichment from continuous growth in the presence of 3.

Figure 2: Route for the deactivation and excretion of corticosteroids by the liver and the gastrointestinal tract flora<sup>4</sup>

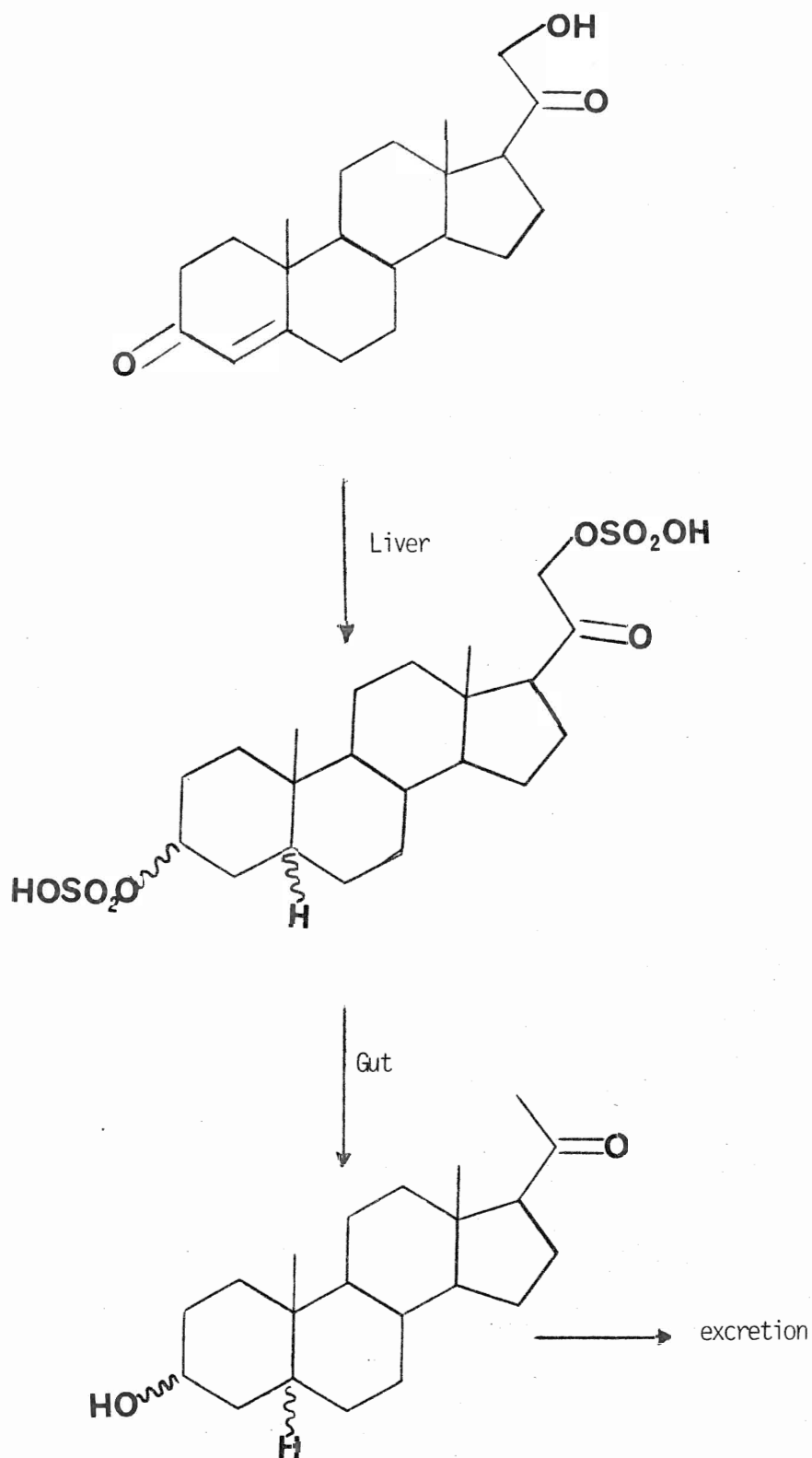
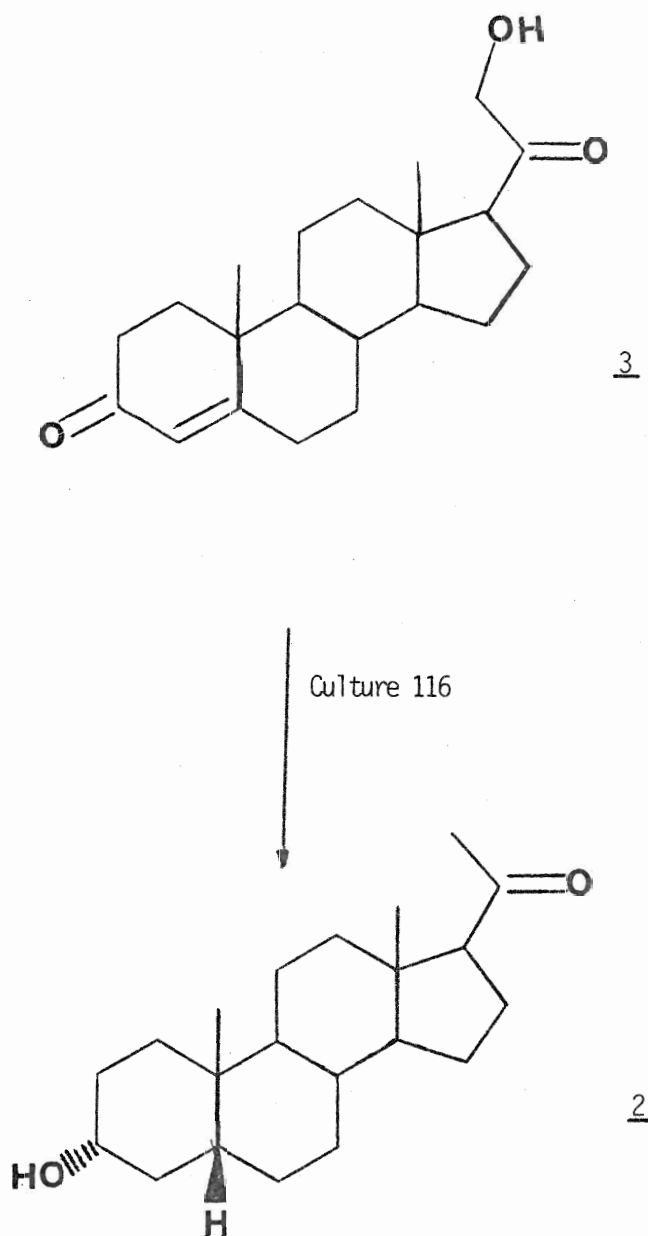


Figure 3:  
Conversion of deoxycorticosterone 3 to Pregnanolone 2 by the human fecal culture  
116



A species was soon identified that could dehydroxylate 1 and was identified as a common inhabitant of the human gut called Eubacterium lentum.<sup>7</sup> This species had much in common with the culture 116, however it required that the A-ring system of 3, be reduced to 1, before the dehydroxylation to 2 could occur. The E. lentum and the culture 116 both required a strong reducing environment and the presence of a reduced nicotinamide as well as the presence of a flavin cofactor.<sup>8</sup> These requirements are not unlike those of some other dehydroxylation reactions that have been studied, therefore an understanding of the mechanism of this process might be better served by an exploration of these other reactions.

The first reaction to be firmly understood was that of the 7- $\alpha$  dehydroxylation of cholic acid, 4, to deoxycholic acid, 5.<sup>9</sup> The enzyme that catalyses this process is synthesized by another bacterium found in the gut and is a Eubacterium sp. (Fig. 4). The mechanistic information was obtained through the use of radio labelled cholic acid molecules synthesized with known regio and stereospecificity of the label. The first substrate used was a tri-tritium compound with tritium present at both C-6 hydrogens and at the 8- $\alpha$  position (Fig. 5). Incubation with this substrate showed the loss of one tritium label. The question remained as to which of the carbons, 6 or 8, was involved for this loss of tritium. The deoxycholic acid formed from this reaction was then subjected to

Figure 4: Conversion of cholic acid 4 to deoxcholic acid 5 by the action of a  $7\alpha$ -dehydroxylase from a *Eubacterium* sp.<sup>9</sup>

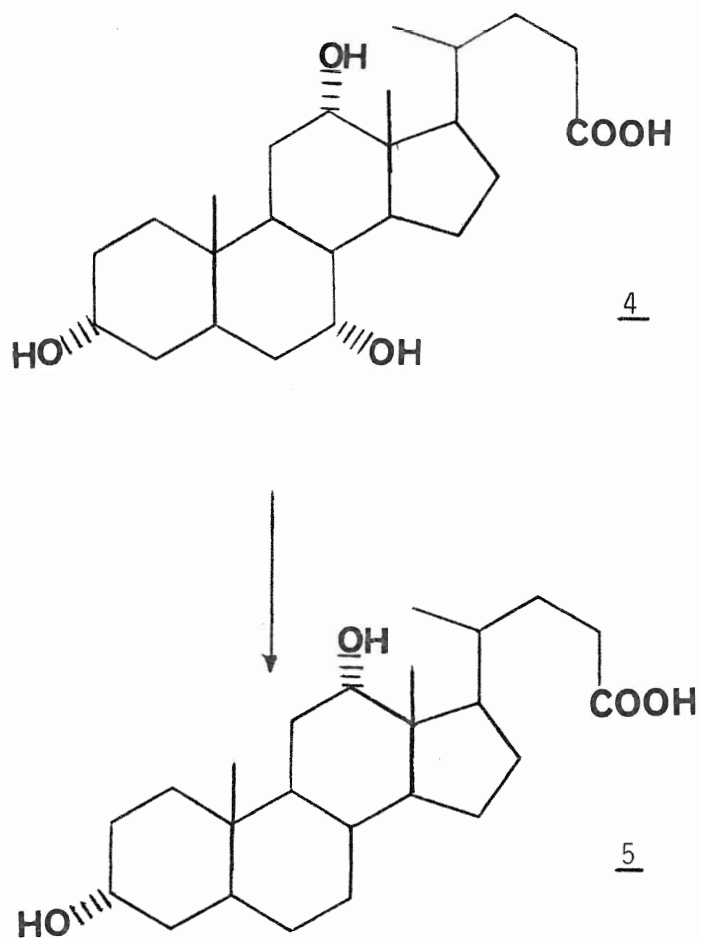
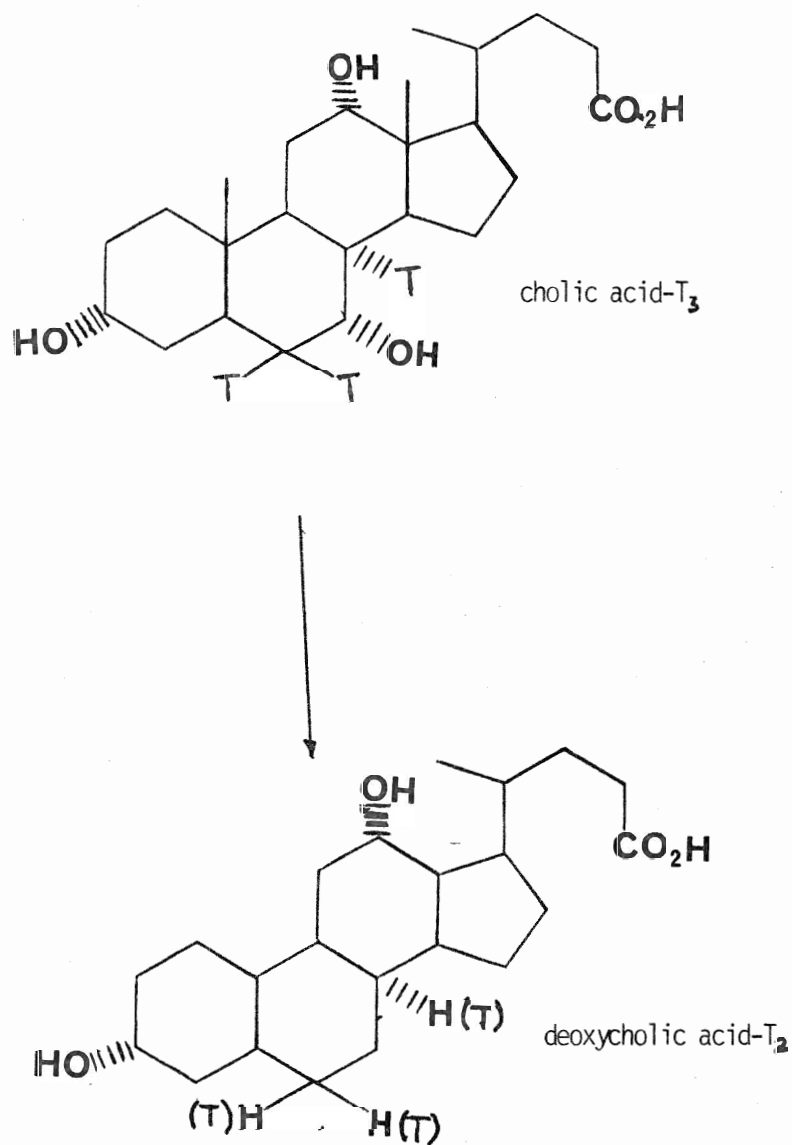


Figure 5: The loss of tritium from cholic acid- $T_3$  during  $\alpha$ -dehydroxylation generating deoxycholic acid- $T_2$  with unknown regiochemistry



another enzyme to generate delta-7-cholenic acid, 6 (Fig. 6). This process removed the 8-alpha hydrogen such that a loss of radioactivity relative to starting material would demonstrate that the 8-alpha hydrogen was not involved in the 7-alpha-dehydroxylation. The result of this reaction proved that the 8-alpha hydrogen was not involved in the original reaction, since the loss of one tritium was observed in the dehydrogenation reaction. Once it was established that the C-6 hydrogens were involved in the 7-alpha dehydroxylation, it remained only to ascertain which of the two was lost upon removal of the 7-alpha hydroxyl. This information was obtained from the use of singly tritiated cholic acid with the tritium in the 6-alpha position (Fig. 7). Use of this substrate in the dehydroxylation reaction showed the tritium to be present in the product, which suggested that the 6-beta hydrogen was the one removed in the process. A mechanism was proposed based on these results that involves the dehydration of ring B with the loss of the 7-alpha hydroxyl and the 6-beta hydrogen (Fig. 8). This loss of hydrogen was quite reasonable from a consideration of the stereoelectronics of elimination, since the hydrogen and the hydroxyl must be anti-periplanar for a smooth elimination. The 6-beta hydrogen and the 7-alpha hydroxyl are in such a relationship to each other, while the 6-alpha hydrogen is not. Once the molecule has been dehydrated to delta-6-cholic acid the double bond can be reduced to the unsaturated deoxycholic acid

Figure 6: Use of delta-7 dehydrogenase on deoxycholic acid- $T_2$  generating delta-7-cholenic acid- $T_1$  6, to distinguish loss of tritium from C-6 or C-8 during  $7\alpha$ -dehydroxylation

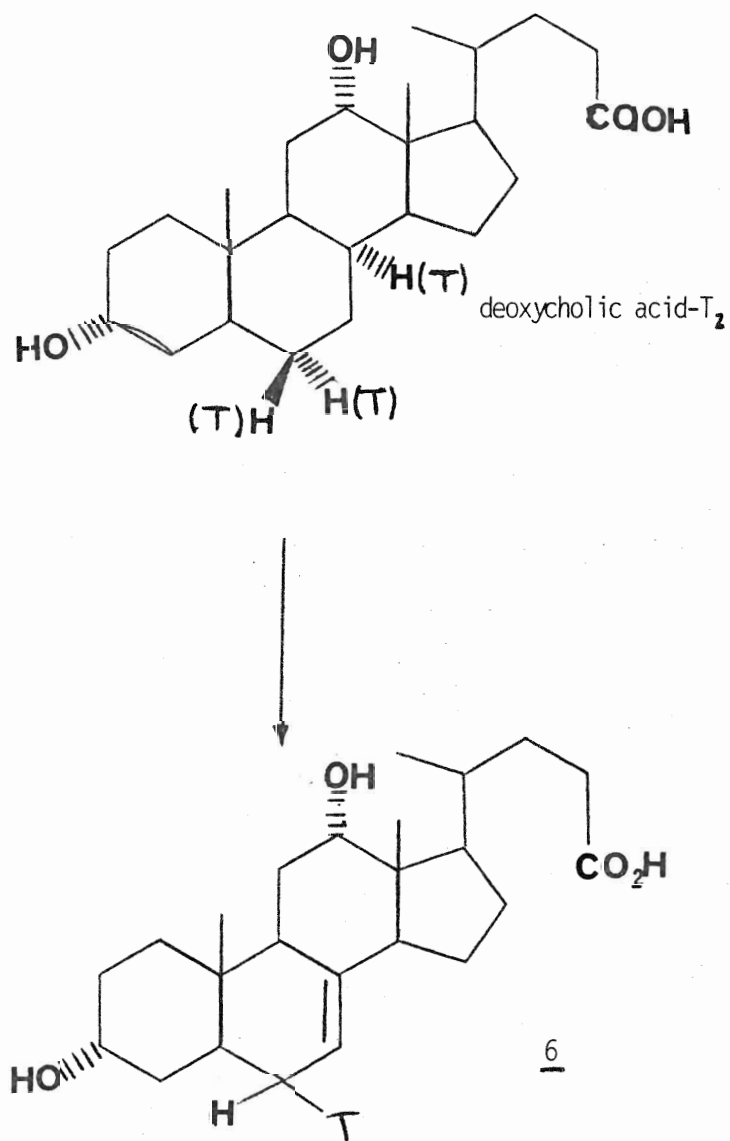




Figure 7: Use of cholic acid-6- $T_1$  to generate deoxycholic acid- $T_1$  to determine stereochemistry of the  $\alpha$ -dehydroxylation

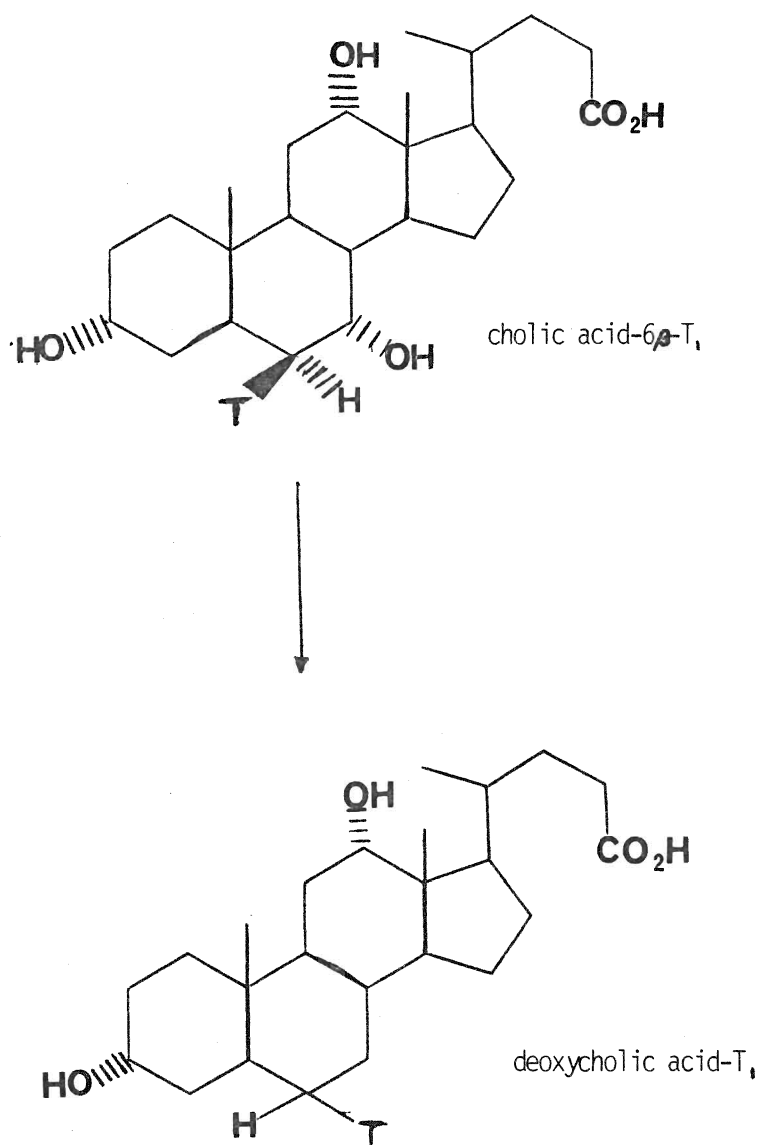
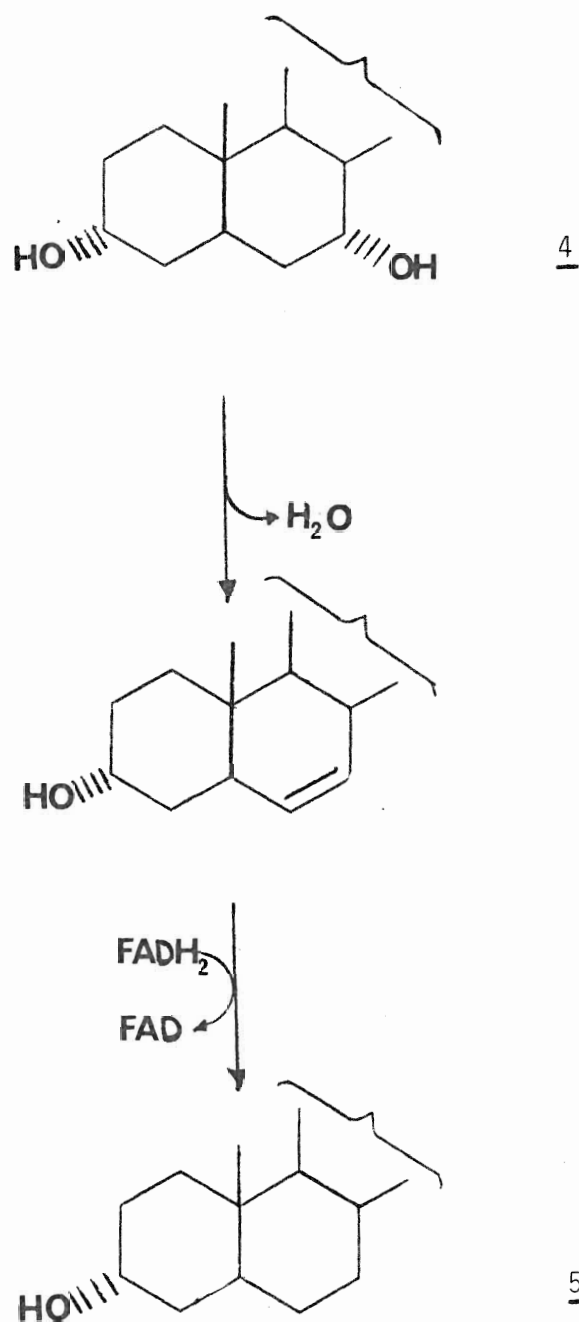


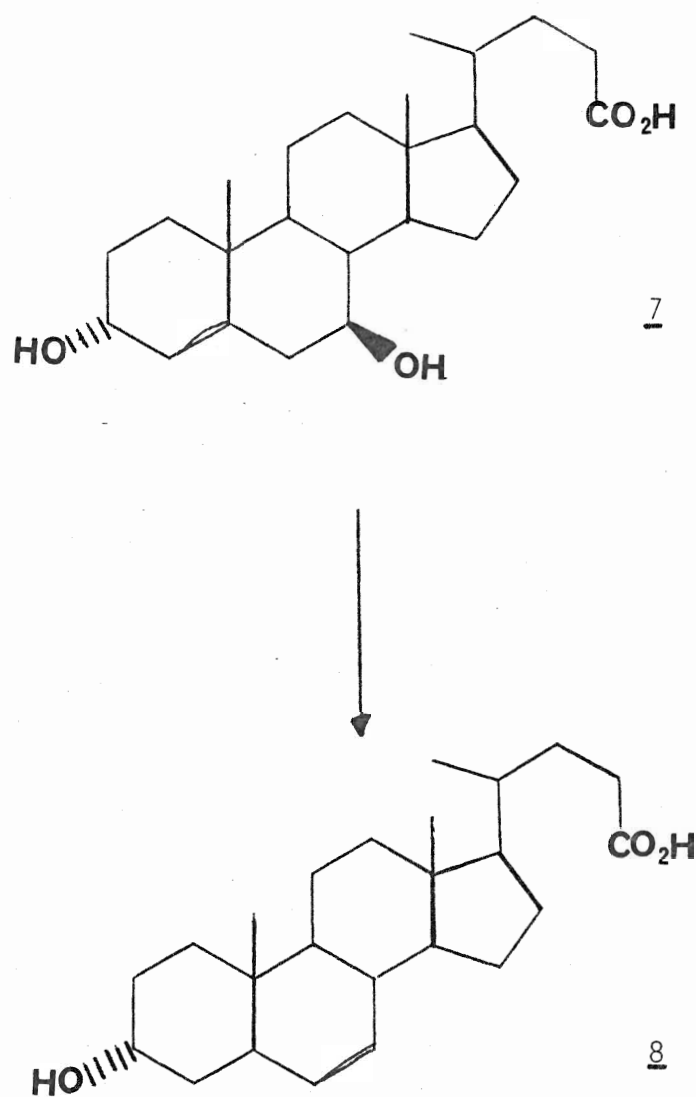
Figure 8: Proposed mechanism for the  $7\alpha$ -dehydroxylation of cholic acid 4 to deoxycholic acid 5



by a flavin cofactor.

A similar reaction, that has not yet been fully understood, is the 7-beta dehydroxylation that is found in the conversion of ursodeoxycholic acid 7 to lithocholic acid 8 (Fig. 9).<sup>10</sup> This reaction has also been shown to be carried out by a Eubacterium sp., but it is not yet known what the exact mechanism is. Initially it was thought that an enzyme inverted the configuration at C-7 to generate a 7-alpha hydroxyl and then the 7-alpha dehydroxylase carried out the removal of the hydroxyl group.<sup>9</sup> It was known that pure cultures of Clostridium absonum could do such an epimerization.<sup>11</sup> This reaction proceeded by a 7-keto intermediate which was detectable during the incubation. Use of a mixed cell culture with, 7, did produce the dehydroxylation product 8 without an observable amount of the 7-keto derivative. Also incubation of 7-keto lithocholic acid with a pure culture of Eubacterium sp. VPI 12708, which is capable of both 7-alpha and 7-beta dehydroxylation, did not lead to the formation of any chenodeoxycholic acid. The above data suggests that the 7-beta dehydroxylation proceeds by the direct removal of the hydroxyl without the inversion of configuration.<sup>10</sup> As well, experiments on the enzyme kinetics have suggested that the same enzyme carrying out the 7-alpha dehydroxylation also performs the 7-beta reaction. The fact that the 7-beta reaction is five times slower than the 7-alpha reaction may be due to the fact

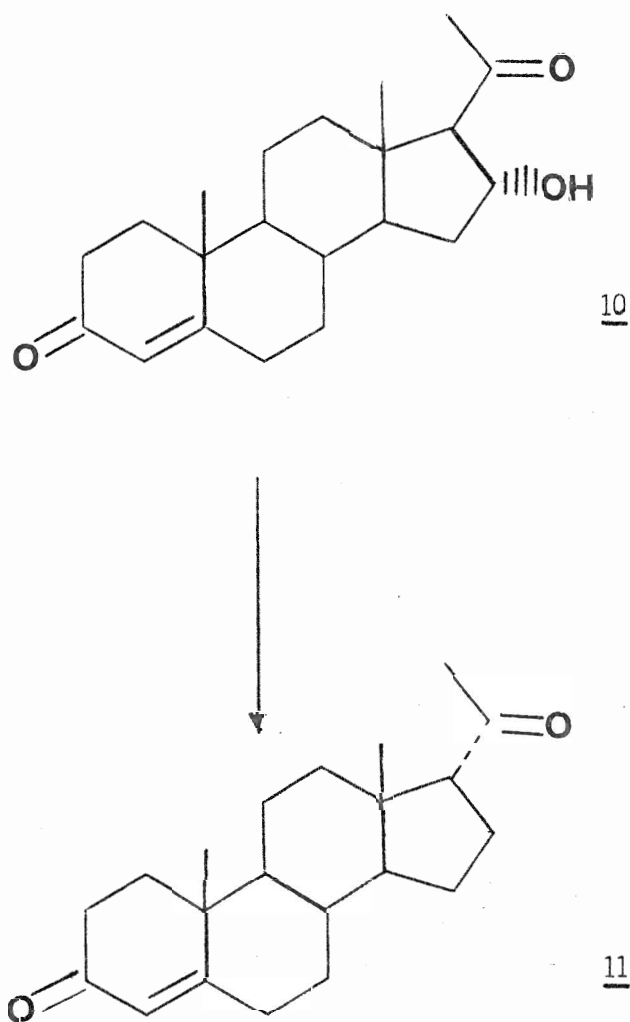
Figure 9: Conversion of ursodeoxycholic acid 7 to lithocholic acid 8 by 7 $\alpha$ -dehydroxylase from a Eubacterium sp..



that the initial dehydration does not have a good linear transfer of electrons. More work on this would need to be done, especially on the stereochemistry, before a firm understanding of this process can be achieved.

Another dehydroxylation that has been looked at mechanistically is the conversion of 16-alpha-hydroxyprogesterone 10 to 17-alpha-progesterone 11 by Eubacterium sp.144 (Fig. 10).<sup>12</sup> Considering the proposed mechanism for the 7-alpha dehydroxylation reaction, it is not difficult to visualize the events leading to the removal of the hydroxyl group and the inversion of the side chain. If the mechanism for this reaction follows the dehydration/reduction process then one would expect to see a delta-16-progesterone 12 intermediate.<sup>13</sup> This unsaturated system could then be reduced to give the 17-alpha side chain. Attempts at isolating an unsaturated intermediate were not very successful and even recovering added 12 from the medium was difficult.<sup>14</sup> The loss was not explained by the conversion to 11 since this only occurred with prolonged incubation. It was found that the intermediate was lost as a water soluble adduct with cysteine, which was a chemical conversion that occurred rapidly in the medium.<sup>14</sup> While the reactivity of this unsaturated carbonyl was not surprising, it demonstrated that it was present as an intermediate in the dehydroxylation reaction, but that it took a long time to convert the progesterone/cysteine adduct to the

Figure 10: Conversion of 16 $\alpha$ -hydroxyprogesterone 10 to 17 $\alpha$ -progesterone 11 by a *Eubacterium* sp.



unsaturated 11 (Fig. 11).

The pattern that has developed from a consideration of a few of the many dehydroxylations is that of a dehydration of the alcohol followed by a reduction of the unsaturated intermediate formed. The need for the two cofactors nicotinamide and flavine is not surprising considering the mechanisms for their types of reduction. The normal cell reducing equivalents are normally stored as the reduced nicotinamide which is used to carry out 2 electron reductions in the form of hydride (Fig. 12).<sup>15</sup> The flavin on the other hand can accept and deliver two electrons in more than one way. Evidence for the transfer of two electrons as the hydride has been observed as well as the transfer of two hydrogen atoms and the movement of electrons alone (Fig. 13).<sup>16</sup> It is for this reason that flavin cofactors can perform the role of intermediary in the transfer of electrons from a metabolic process to the hydrogenation of a double bond, which is normally not a hydride reduction. Since all of the dehydroxylation reactions looked at so far have required the reduction of a double bond the requirement of the flavin is apparent.

Looking back to the 21-dehydroxylation reaction, we see that it has many of the requirements and characteristics of the other dehydroxylation reactions mentioned. It is also carried out by an enzyme of bacterial origin in the gut and recently the species was identified as another species of *Eubacterium*-  
Eubacterium lentum .<sup>7</sup> The process also requires the presence

Figure 11: Proposed mechanism for the conversion of 10 to 11 via a delta-16 progesterone 12 intermediate

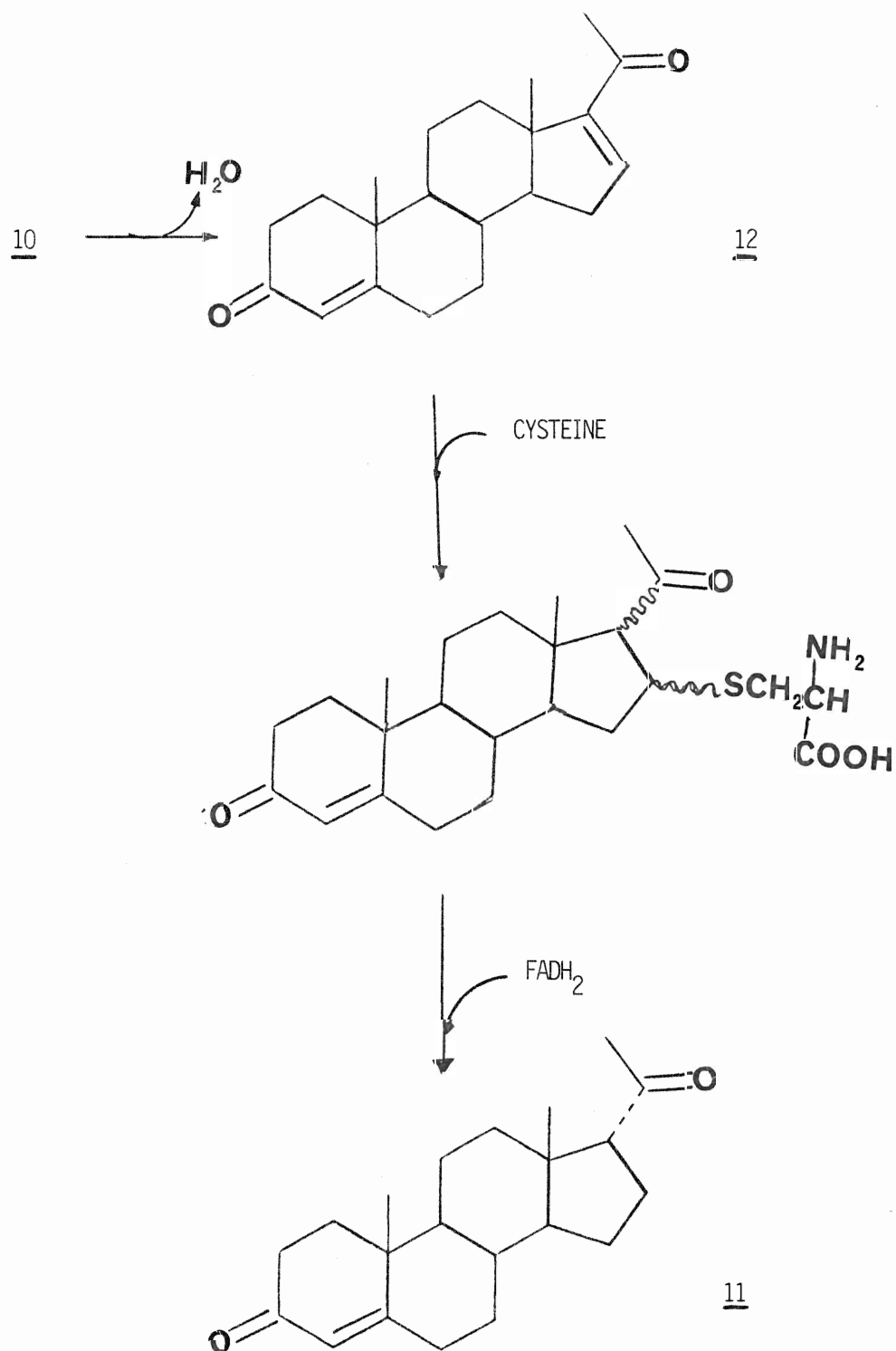
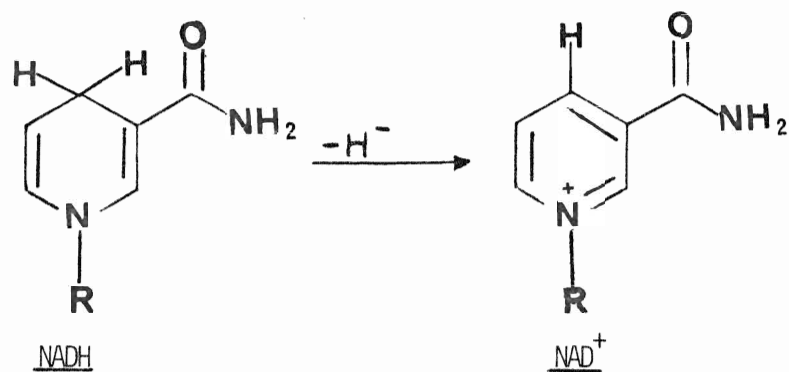
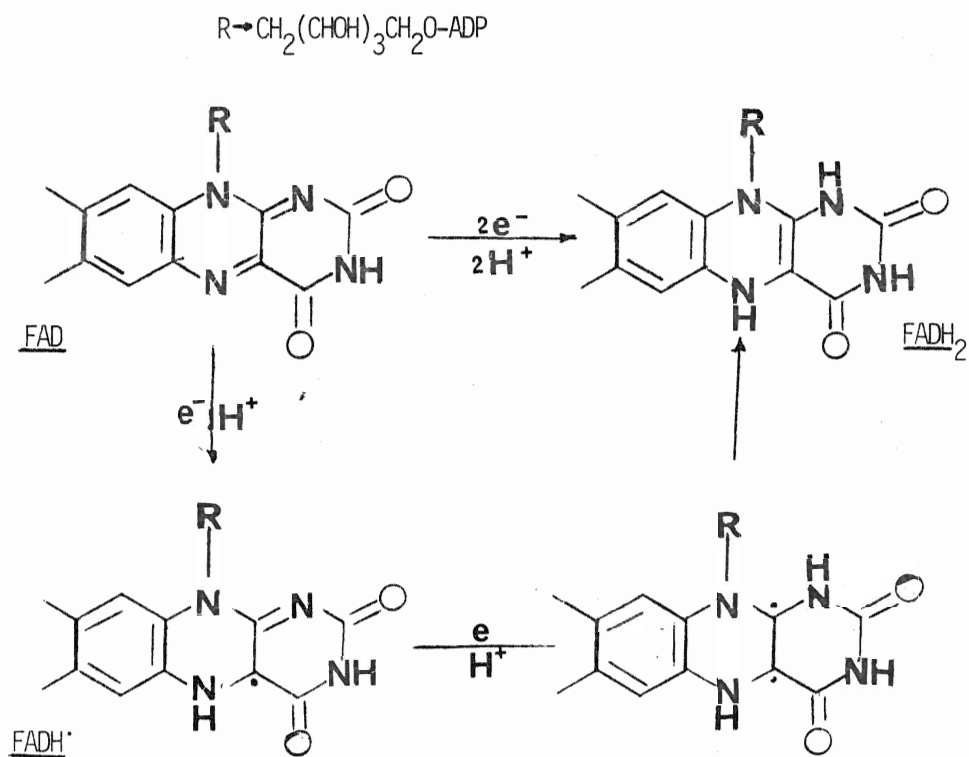




Figure 12: Redox reaction for NADH TO NAD<sup>+</sup>R  $\rightarrow$  ribose-ADPFigure 13: Redox reactions for the conversion of FAD to FADH<sub>2</sub>

of a reduced flavin mono-nucleotide, which shows that the reaction is another reductive dehydroxylation.<sup>8</sup> To investigate the involvement of the hydrogens on C-21, tritium labelled 3 was incubated, with both positions at C-21 labelled.<sup>18</sup> Experimental results showed a loss of label at this position, but due to inconsistencies in experiment a loss of one tritium was recorded sometimes, while more than one was lost other times. The investigators had to admit that no firm conclusion could be drawn from their results other than proof of the involvement of the C-21 hydrogens. Since these early experiments, more information has been obtained on the dehydroxylation reaction.

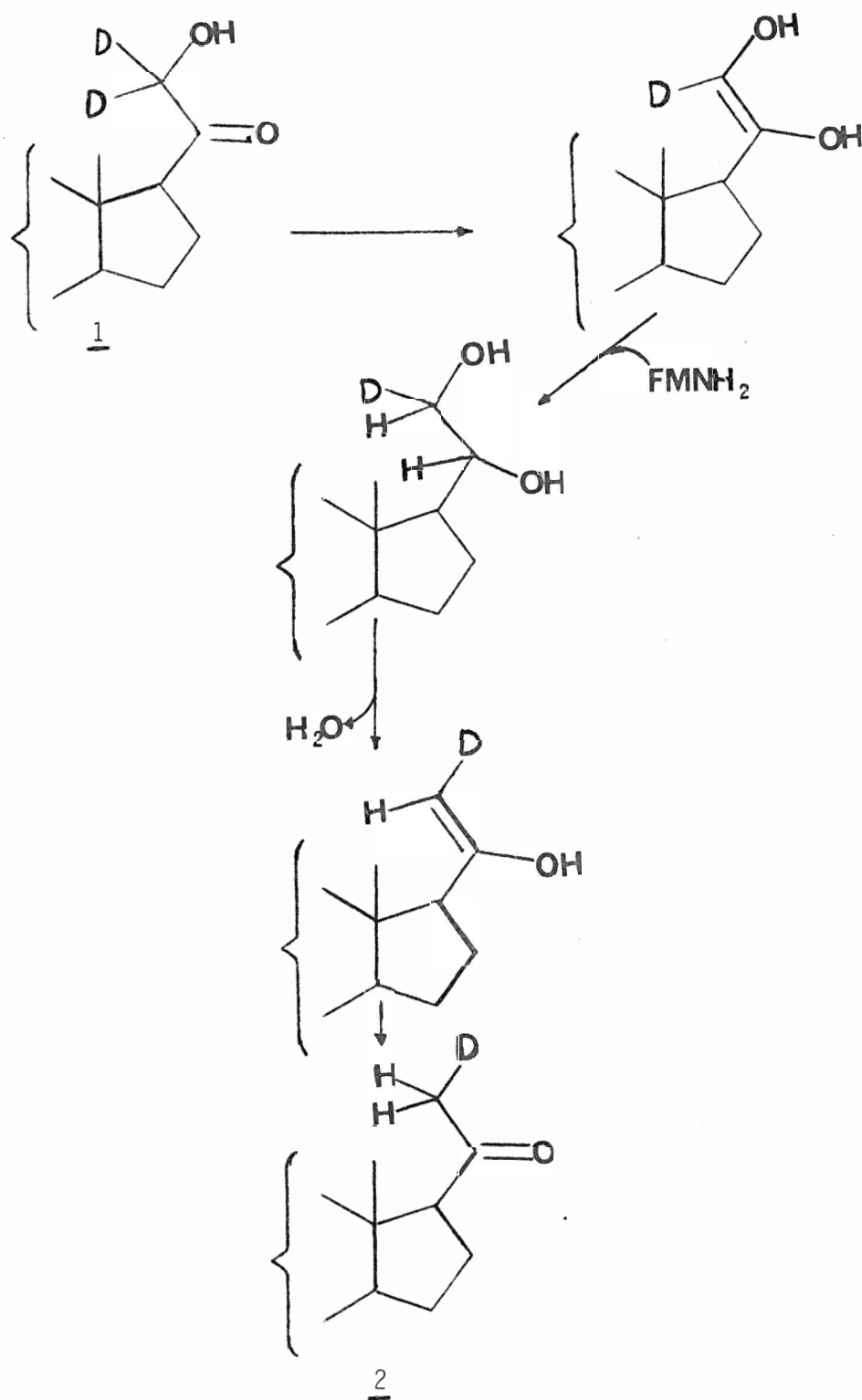
It is now known that there is a strict requirement for a specific A ring system.<sup>19</sup> Previously, when working with an unknown mixed culture, it was thought the A ring of 3 was unimportant.<sup>20</sup> It is now known that E. lentum will only take the saturated system of 1. It was shown for the mixed culture that there must also be a 20-ketone function, since incubations using a substrate with a 20-alcohol did not give rise to the dehydroxylated product.<sup>6</sup> There seems to be some confusion when the researchers compare their results with pure E. lentum. It may be necessary to assume, in this case, that E. lentum will also not dehydroxylate at C-21 in the presence of a 20-alcohol.

With the knowledge that the C-21 hydrogens were somehow

involved in the process, a study was undertaken using deuterium label. Holland and Reimland prepared 1 with deuterium at both of the C-21 positions and the 17-alpha position.<sup>3,21</sup> An incubation with E. lentum showed the loss of one hydrogen during the conversion to 2. A competitive incubation with the unlabelled compound showed an intermolecular isotope effect of  $k_H/k_D = 2.8$ , which demonstrated that the hydrogen was abstracted in a step that was at least partially rate determining. Based on this finding a mechanism was proposed which accounted for all of the observed requirements for the process (Fig. 14).<sup>21</sup> The substrate is thought to be bound to the enzyme as the enediol in the active site. It is known that the enzyme is inhibited by metal chelating agents which suggests the complexation of the enediol to some metal in the active site.<sup>22</sup> The C-21 hydrogen would then have been removed to form this enediol by some base in the active site. The double bond formed in this step could then be reduced by the flavin to generate a diol which could be dehydrated to form an enol. The enol would easily tautomerize to the 20-ketone and give the dehydroxylated product.

In this thesis the interest in this process centres on the stereochemistry of the initial abstraction reaction. If the abstraction is necessary for the binding of the substrate then perhaps it proceeds stereospecifically. The knowledge of the stereochemical course of the reaction might facilitate its prevention by some form of inhibition.

Figure 14: Proposed mechanism for the 21-dehydroxylation of 1 to 2 by Eubacterium lentum



In order to study this process a stereospecifically labelled 21-methylene group must be prepared with the use of deuterium labelling. Deuterium is a better label than tritium in this case because of the ease of analysis. It should be possible to generate a compound with nearly 100% label that can be easily monitored by simple techniques like deuterium nmr and mass spectrometry. The problem with the early study, using tritiated 3,<sup>18</sup> may have been due to their sensitivity in analyzing for their radioactive tracer. Locating the tracer would have been difficult using scintillation counting, hence they may have had some extra tritium incorporation at other sites of the molecule which induced errors.

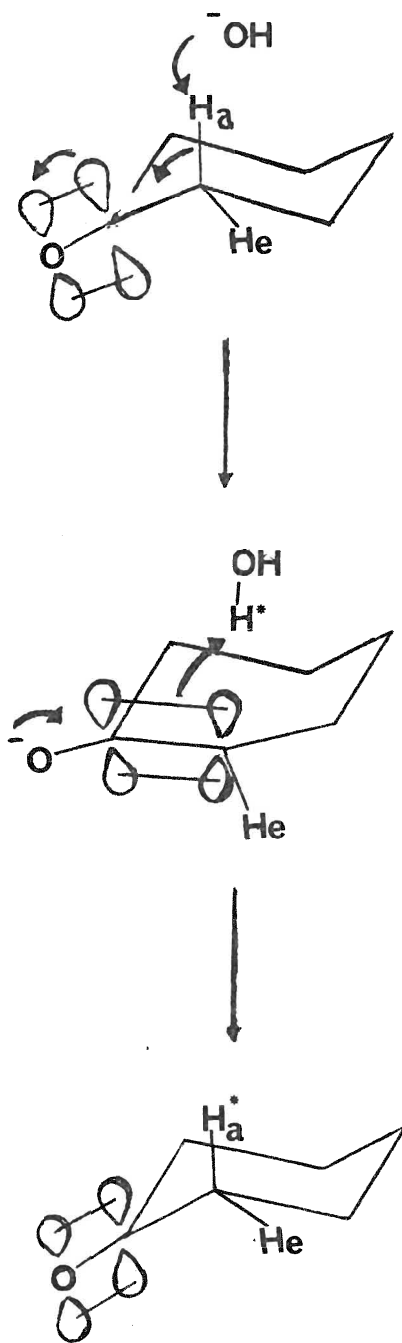
The task at hand is the replacement of one of the diastereotopic hydrogens at the C-21 position of 1, with deuterium, such that the absolute configuration is known in the final product. There are two types of approaches to this problem of replacement of a hydrogen by deuterium.

- 1) Reduction of a precursor with a specific reducing agent
- 2) Exchange of one of the positions in base or acid through an enol or enolate

The second method is based on the differences in steric and stereoelectronic properties of two alpha hydrogens. This difference is seen when a molecule containing alpha hydrogens has a predominant conformation. The conformation must be such that it gives both hydrogens a different orientation relative

to the carbonyl. A good example of such a difference can be seen between the axial and equatorial hydrogens of the chair conformation in a six-membered ring. A hydrogen removed from the axial position would allow for a linear transfer of electrons from the hydrogen to the carbonyl oxygen (Fig. 15). An equatorial relationship would not allow for this linear transfer of electrons since it is at an angle to the pi-system, hence the axial relationship is the one which leads to enolization. The molecule must be rigid enough to prevent the interconversion of the two hydrogens such that they become stereoelectronically equivalent. This criterion is usually met with six membered ring systems where one of the chair conformations is predominant and the rate of interconversion is slow. If the interconversion rate is slow, then the alpha-hydrogen with an axial relationship can be many times more likely to enolize than the equatorial hydrogen. For exchange purposes, the incoming hydrogen will also be oriented in the axial position because the pi-orbital will deliver the electrons in that direction. Performing the exchange of an alpha-hydrogen in the presence of a deuterium labelled solvent would then give exchange of the alpha position with deuterium. If however the molecule does not have the conformational rigidity of a six-membered ring or some other conformational restriction, then both positions of the methylene would be open for exchange and this method would be of little use in generating a

Figure 15: Preferred enolization of the axial hydrogen in a restricted chair conformation



stereospecifically labelled methylene. This is the case with the side chain of tetrahydrodeoxycorticosterone, which was prepared with both C-21 hydrogens labelled with deuterium by a base exchange.<sup>21</sup> This method would then not be useful for the generation of a mono deuterated species with stereoselectivity. This can be accomplished, however, using the other process of stereospecifically reducing a precursor.

This technique is the most common method for generating a chiral methylene and the approach can be looked at in two ways.

- a) Hydrogenolysis of a chiral precursor in an  $S_N2$  displacement
- b) Generation of a chiral alcohol

The preparation of the first type of molecule inevitably requires the second, meaning that a chiral secondary alcohol can be converted to some other functional group that can be removed by hydrogenolysis (e.g. halide, tosylate, etc.). Following the stereochemistry through the synthetic scheme, the configuration of the methylene could be known by comparison.<sup>23</sup> Also it is possible to measure an optical rotation of a chiral methylene due to the differences between the deuterium and the hydrogen in some cases.<sup>24</sup>

Alcohols can be prepared from non-chiral carbonyl precursor by reduction. There are many ways available to generate a chiral alcohol from this reduction and they can be grouped into three categories.

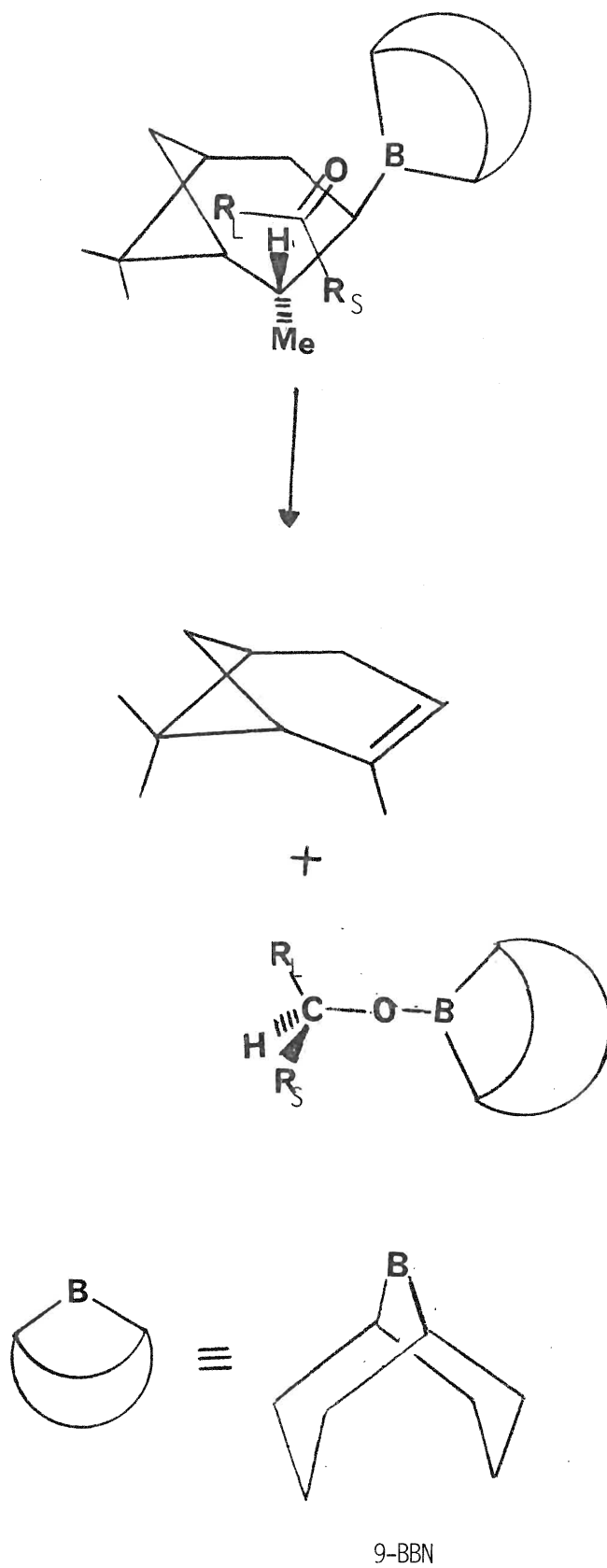


- 1) Induction of chirality from a chiral reducing agent
- 2) Relaying chirality from another part of the molecule
- 3) Use of reductive enzymes as catalysts

In the first case, chiral reagents are available that interact with the substrate in such a way as to generate a diastereomeric transition state. Since diastereomers have different properties they will also have different energies, hence one of the diastereomers might be favoured. In physical terms this means that because the carbonyl has two different groups attached to it there will be steric differences in the interaction of the molecule with a reducing agent of a defined geometry. In the simplest system the substrate will interact with the reducing agent in such a way as to place its small substituent near the large substituent of the reducing agent and its large substituent near the small substituent of the reagent. Common reagents contain boron or aluminum hydrides with one or more of the hydrogens replaced by chiral alcohol groups. Enantiomeric excesses of greater than 90% have been obtained using one of these reagents called Alpine Borane (Fig. 16.)<sup>26</sup> The reagent may not be suitable for all uses since it is very large and may be involved in steric hindrances that make its use with large molecules unfavourable.

In the second group a chiral reagent may or may not be employed since the chirality already present in the molecule is used to dictate where the hydride will be delivered. Since the products of reduction from both sides of the carbonyl will be

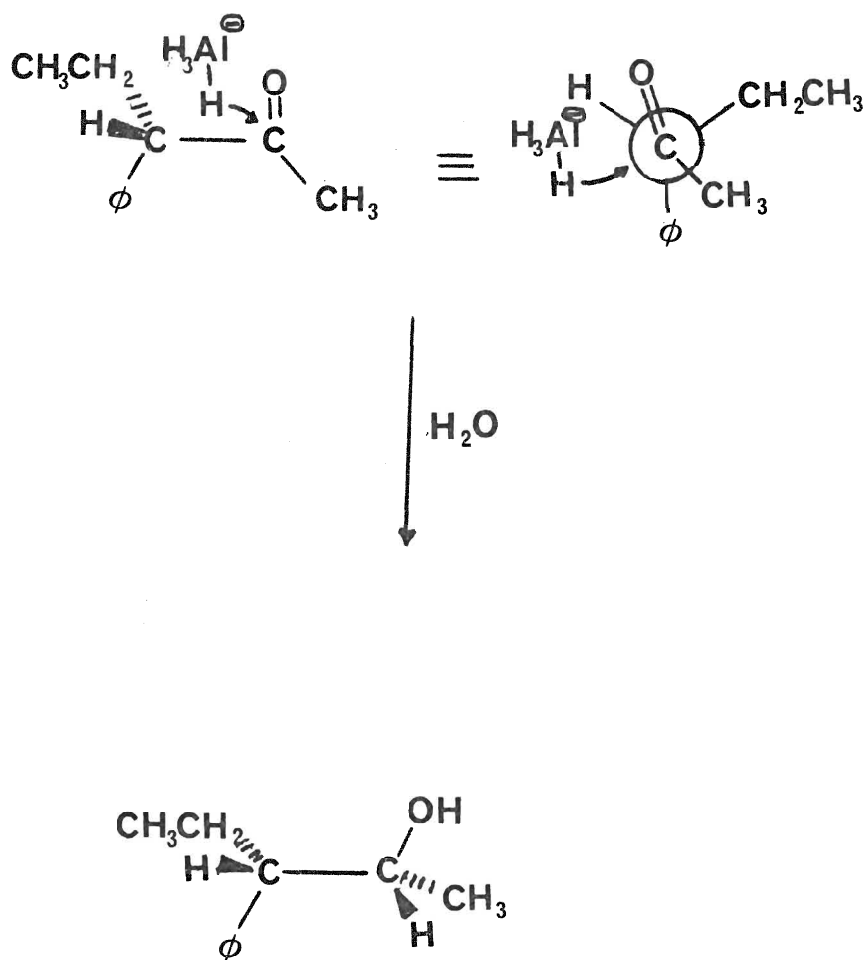
Figure 16: Use of a chiral reducing agent B-3-pinanyl-9-borobicyclo-[3.3.1]-nonane, Alpine Borane, to generate chiral alcohols



diastereomers, the ease of reduction of one or the other depends on the interaction of the molecule and the reducing agent just as in the first case. In this method however the fact that the starting material has chirality means that there will be a more favoured direction of attack of the reducing agent. This will lead to a product being enriched in one of the two possible diastereomers. The effect is best seen when the chiral centre is near the carbonyl so that the substituents of the asymmetric carbon can effectively influence the reduction. The principle at work is again a steric interaction between the starting material and the reducing agent (Fig. 17).<sup>27</sup> Rules have been established for acyclic ketones for predicting the predominant diastereomer, which are based on the hypothesis that the transition state geometry of the reduction resembles that of the starting ketone. The conformation of the ketone and the interactions between the reducing agent and the other groups of the molecule dictate which face of the carbonyl the reagent will approach.

The two procedures mentioned can now be seen to be basically the same in that they use the physical barriers of steric hindrance, either based on the substrate or the reagents original chirality, to favour the reduction from one side of the carbonyl over the other. Enzymes use this approach to generate specific stereo- and regiochemistries. Once a substrate is

Figure 17: Induction of chirality from one place in a molecule to developing one by steric hindrance



bound within the three dimensional active site, delivery of the hydride is usually to a specific carbonyl, with a single stereochemistry. Since the reducing agent is also bound specifically to the active site the enzyme functions, as a whole, like a large chiral molecule that uses the structure of the substrate to dictate which face the hydride will be delivered to. Horse liver alcohol dehydrogenase is an example of such an enzyme being used to investigate the substrate specificity for use in organic synthesis<sup>28</sup>. The enzyme can take into its active site many different molecules and reduce the carbonyls to alcohols in very high enantiomeric excesses. This enzyme is found to contain two binding pockets for the substrate that can accommodate either a large or small group of the substrate. The pockets have wide substrate specificity but not all carbonyls give 100% enantiomeric purity due to the substituent size differences or other functional groups present. At present Jones is investigating the use of site-directed mutagenesis to engineer enzymes with different binding pockets and hence different specificities.<sup>25</sup> Nature has done this for millions of years and developed enzymes that can perform perfect 100% stereospecific reductions, with their ideal substrates, and if these can be employed in a synthesis then there would be no doubt about the stereochemistry of the product or concern for its purity.

In this thesis, both enzymic and chemical methods will

be employed to stereoselectively reduce a 21-oxosteroid for use in the study of the 21-dehydroxylation reaction.

### Dehalogenation of 4-Chlorobenzoate

The extensive use of halogenated hydrocarbons as solvents, lubricants and pesticides, has led the world to a problem of pollution leaving few places in the world unaffected.<sup>29</sup> Normally, synthetic organic molecules have a short half-life in the environment due to chemical degradations from autoxidation and photochemical reactions, and more significantly, the actions of heterotrophic fungi and bacteria. However, the properties that make halogenated molecules so useful also makes them more resilient to environmental stress. This has led to the accumulation of these pollutants in the biosphere and in some cases the concentration and magnification in higher organisms. The concern for the presence of many of these compounds has been on the increase as more information about the toxicity to humans becomes available.<sup>30</sup> With the uncertainty of the efficiency of some chemical treatment plans, many microbiologists and bioorganic chemists have been investigating safer methods of degradation. One such method uses microorganisms to effect complete mineralization of the most hazardous compounds without the generation of toxic by-products.

In the environment most organic molecules are metabolized by the fungi, bacteria and algae present in a particular ecosystem. Since many organic molecules are similar

to naturally produced compounds, many enzymes synthesized by microorganisms have sufficient substrate flexibility to allow for unnatural compounds to be transformed.<sup>31</sup> However, the presence of an enzyme capable of transforming a new compound does not mean that it will be further degraded. In fact some compounds that are viewed as toxic, are in fact relatively harmless until they are activated biologically: e.g. polychlorobiphenyls (PCBs)<sup>32</sup> and polycyclic aromatic hydrocarbons (PAHs).<sup>33</sup> For a particular molecule to be completely degraded, there must be in place all the enzymes necessary for its metabolism through some natural pathway. This would require that either:

- 1) a given species of microorganism be complete with all of the genes necessary for the pathway
- 2) that one species contain some of the genes while other species contain the rest of the genes to facilitate a completely active metabolic pathway.

The process of evolution can play a simplifying role in this by making available genes and therefore enzymes that did not exist before, giving an organism the ability to degrade a new compound.<sup>34</sup> With these requirements in mind it is not surprising that the recent introduction of organo halides into the natural environment has not resulted in their complete removal. Despite this, bacteria and fungi are still being sought for the purpose of removing the halogenated compounds found in the environment.



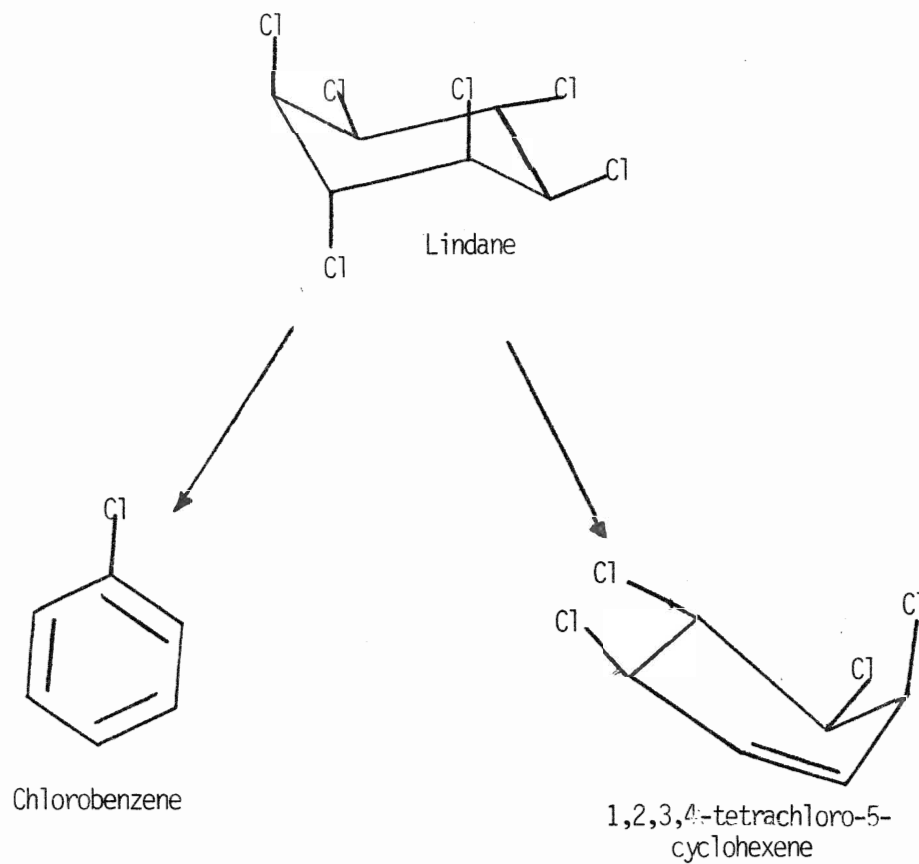
When a species of microorganism is found capable of effecting some transformation or perhaps a complete metabolism of a particular compound, the mechanisms by which the reactions occur is investigated. Many enzymes for transformation of halogenated compounds are known and their respective mechanisms of action worked out.<sup>29</sup> With some enzymes available and their genomic precursors isolated, molecular biologists have used recombinant DNA techniques to generate mutants, of some common species of bacteria, that can perform complete mineralization of some persistent pesticides ( 2,4-D, 2,4,5-T, DDT).<sup>34</sup> A review on the molecular genetic advances is beyond the scope of this thesis, but as it relates to the main topic of the dechlorination of p-chlorobenzoate it will be addressed. To understand this topic from both a biological and chemical view it is important to understand what types of reactions enzymes are known to perform on the rather stubborn halogenated compounds that we have introduced.

The types of compounds that have been introduced either as pesticides or industrial waste vary considerably from small molecules (chloroform, ethylene chloride) to polycyclic aromatics (PCBs, DDT) and from singly halogenated (2-chloropropionic acid) to polyhalogenated (lindane, pentachlorophenol).<sup>35</sup> These variations in chemical structure would therefore require variations in enzymic capabilities, since an enzyme capable of aromatic ring cleavage will not necessarily perform any reaction.

on a small chain hydrocarbon. Thus, wide reaction diversity in the metabolic pathways of halogenated organics has necessitated the development of many enzymes capable of performing either oxidation, reduction, hydrolysis, dehydrohalogenation, or dehydration,<sup>30</sup> all on compounds that may have never been produced naturally, and contain enough halogen to make them poorly reactive under normal environmental conditions. To look at 4-chlorobenzoate dehalogenase from a mechanistic point of view it would be necessary to look at other known reactions and pathways to draw a comparison. An identification of the requirements for other types of enzymes might lead to an intuitive understanding of this process.

Enzymes capable of reduction have been identified for many reactions but the one most descriptive is that for the removal of chlorine from hexachlorocyclohexane, commonly known as lindane. Anaerobic bacteria have been isolated that can convert lindane to 3,4,5,6-tetrachlorocyclohexene and to a greater extent chlorobenzene (Fig. 18).<sup>36</sup> Not enough research has been done on this particular system to determine the mechanism of this process. However, another system has been looked at that might give some insight. The liver microsome preparations from rat liver under anaerobic conditions also gives some of the same products with lindane.<sup>37</sup> The enzyme has been determined to be a cytochrome P-450 dependent mono-oxygenase, which normally

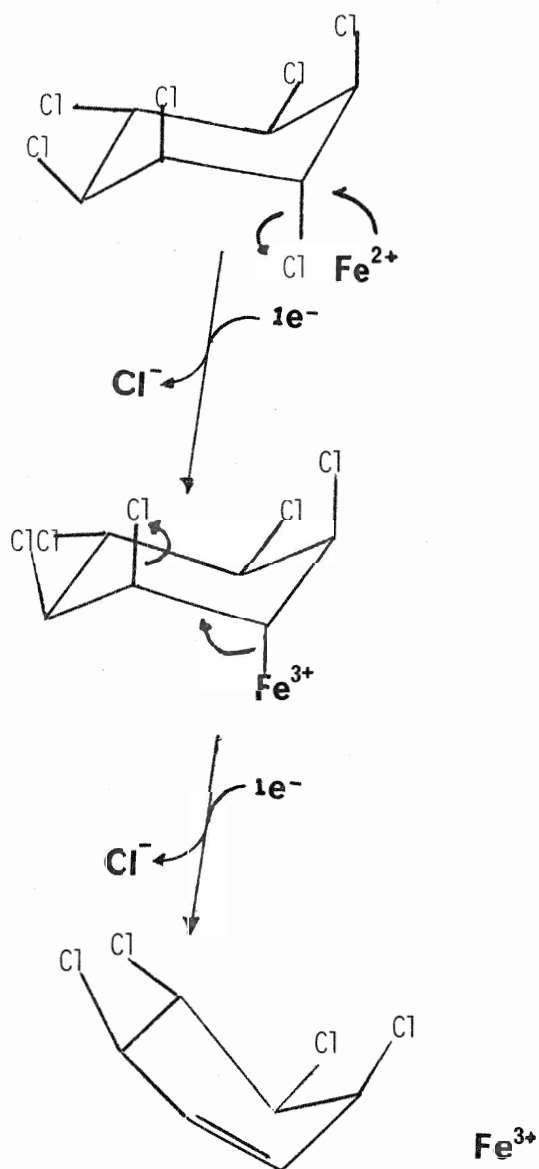
Figure 18: Anaerobic conversion of Lindane (1,2,4,5- ~~$\alpha$~~ -3,6- ~~$\beta$~~ -hexachlorocyclohexane) to chlorobenzene and 1,2,3,4-tetrachloro-5-cyclohexene



performs oxidations under aerobic conditions. Without the presence of oxygen the enzyme is capable of delivering electrons to an available oxidizing agent such as lindane. The mechanism determined for this process involves the removal of one chloride as an electron from the iron of the heme is delivered to the molecule (Fig. 19). The resulting homolytic cleavage of the carbon-chlorine bond generates a carbon based radical that combines with the iron. A second electron from the cytochrome electron transport chain eliminates the second chloride upon formation of the double bond. The formation of chlorobenzene was thought to be a result of a continuation of this process to give 5,6-dichlorocyclohexadiene, which was not identified due to its ease of conversion to the aromatic chlorobenzene. In the liver microsome study benzene itself was also identified as a product thought to be a result of a third reductive loss of chloride in the manner outlined above. Since the product composition of the bacterial reduction of lindane is similar, it may be fair to assume that the process of reduction is the same for both systems.

For most aliphatic compounds the mechanism of removal of the halide involves a simple nucleophilic substitution reaction where the halide is replaced by hydroxide.<sup>38,39</sup> These enzymes have been found to be important in the removal of halides from straight chain hydrocarbons ranging in size from  $C_1$  to  $C_{10}$ . A different set of enzymes is capable of displacing the halide

Figure 19: Proposed mechanism for the anaerobic dehalogenation of Lindane, by rat liver microsomes



from 2-halo acids. A look into the mechanism of action for the enzymes has shown there to be two different methods of carrying out the substitution reaction (Fig. 20).<sup>40</sup>

1) The displacement of the halide, after binding to the active site, by a sulphur group either as part of the protein (a cysteine residue) or by a reduced glutathione cofactor. The resulting thioether is then broken by a chemical reaction with water generating the alcohol.

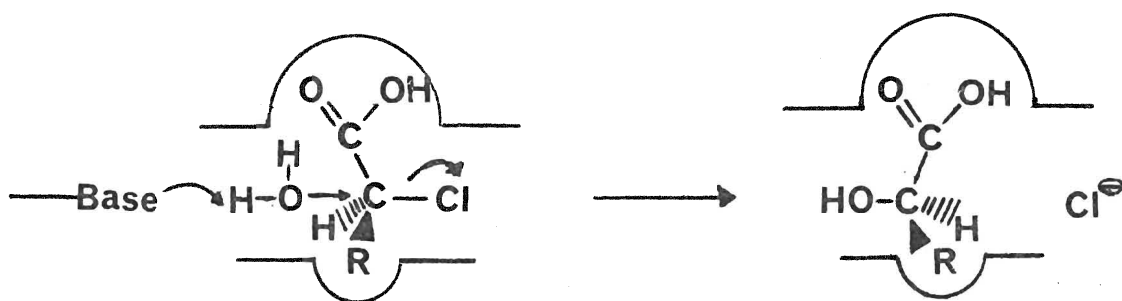
2) The direct displacement of the halide by water with the aid of a basic amino acid residue such as histidine.

Both mechanisms have been found to be employed for simple halogenated hydrocarbons as well as the 2-halo acids. However, no enzyme has been found capable of removing halide from both an alkane and an acid. This is probably due to differences in binding requirements between the 2-halo acid dehalogenases and the hydrocarbon dehalogenases.<sup>40</sup>

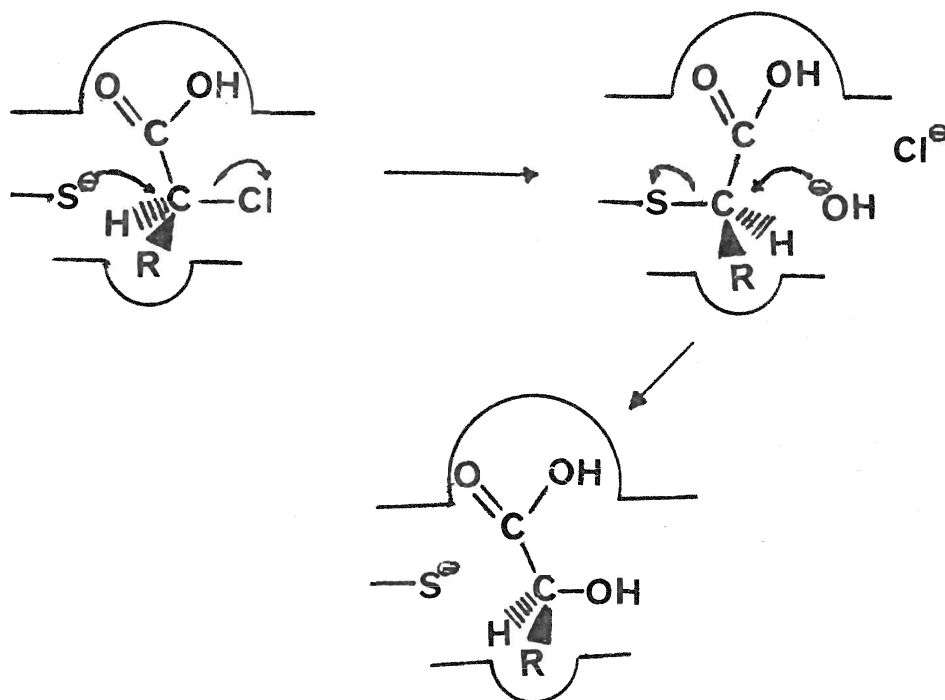
The two types of approaches looked at so far (reductive and hydrolytic) have been concerned mainly with the non-aromatic compounds and the way that organisms have developed to eliminate halide from the molecules. Once the halide has been eliminated the compounds are much more likely to be metabolized and hence removed completely. But in the case of aromatic compounds the

Figure 20: Proposed mechanisms for nucleophilic substitution of halogen by enzymes  
 A) direct displacement of halide by water B) displacement of halide by an enzymic sulphydryl group

A)



B)

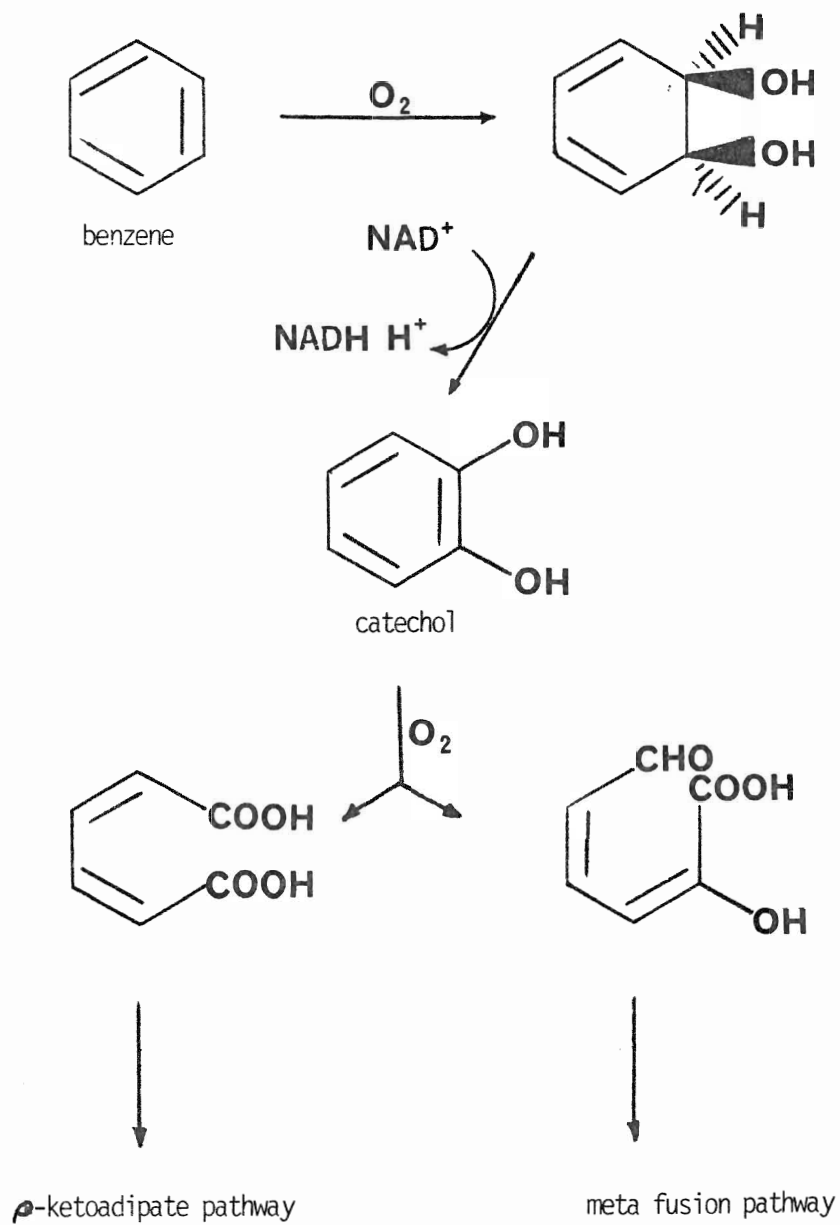


approach has usually been to break the aromatic ring and eliminate the halide at a later stage.<sup>31</sup> Once the molecule has been dehalogenated it is again possible to obtain further oxidation from an established metabolic pathway. For the most part then, organisms have developed enzymes for the oxidation of aromatic rings.

Many organisms have been isolated that contain enzymes capable of oxidizing many of the chlorine containing aromatic hydrocarbons. The genes for the pathways of degradation of di and trichlorophenoxy acetates, polychlorophenols, and chlorobenzoates have been isolated and used to transform other bacterial species.<sup>34</sup> The enzymes responsible for the aromatic ring cleavage have been found to be nearly the same enzymes responsible for the metabolism of naturally occurring aromatic rings. The general route for the cleavage is shown below for benzene (Fig. 21).<sup>41</sup> While some variation is possible, depending on the substitution of the ring, generalisations can be made. The majority of the processes investigated require a catechol system to allow for the dioxygenation and ring cleavage.<sup>34</sup> How the molecule generates the catechol depends on the type of substrate. A phenol would require the oxidation of the ortho position, other molecules like the PCBs and benzoates require an initial dioxygenation or two monooxygenations before a dioxygenase can cleave the ring. After ring cleavage cycloisomerase enzymes can form lactones that enable oxygen to



Figure 21: Oxidation of benzene to generate products of common metabolic pathways

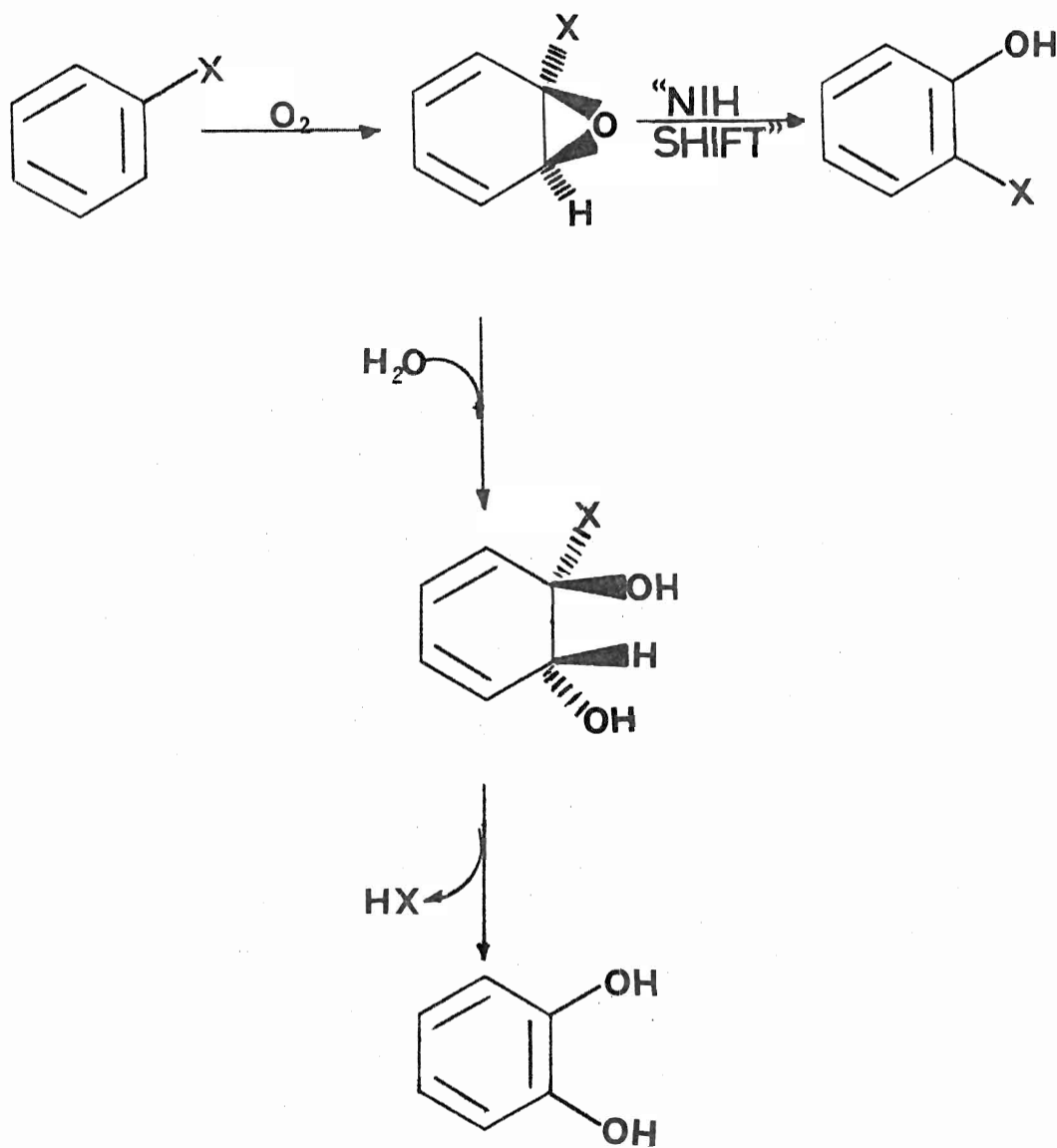


displace halide from either the 3 or 4 carbon. If the halide is present on the 2 or 5 carbon 2-halo acid dehalogenase can be employed to displace the halide, since the molecule is no longer aromatic. The most important stage in the process of aromatic ring degradation is then the oxidation of the ring. Enzymes are known that are capable of putting one or two oxygen atoms into an aromatic ring.

Monooxygenases are enzymes that can transfer one atom of oxygen from molecular oxygen into an aromatic ring. Enzymes like cytochrome P-450 dependent monooxygenases, have been identified and their mechanisms investigated (Fig. 22).<sup>42</sup> In the case of halogenated aromatics the oxygen is incorporated into the ring as an arene oxide.<sup>43</sup> If the arene oxide includes the carbon containing the halogen then the halogen is found to migrate to the other carbon of the arene oxide generating a phenol with the halogen in the ortho position, in a process called the "NIH shift". A diol can also be formed if the arene oxide is opened by a water molecule. A subsequent loss of HCl can regenerate the aromatic ring and the catechol required for ring cleavage. If the arene oxide opens with the halide shift then a second oxidation is required to generate the catechol.

Catechols can be formed by the action of the other type of oxygenases that incorporate both atoms of oxygen from molecular oxygen.<sup>41</sup> These enzymes generate a cis-diol directly

Figure 22: Routes for the oxidation of a substituted benzene by a monooxygenase



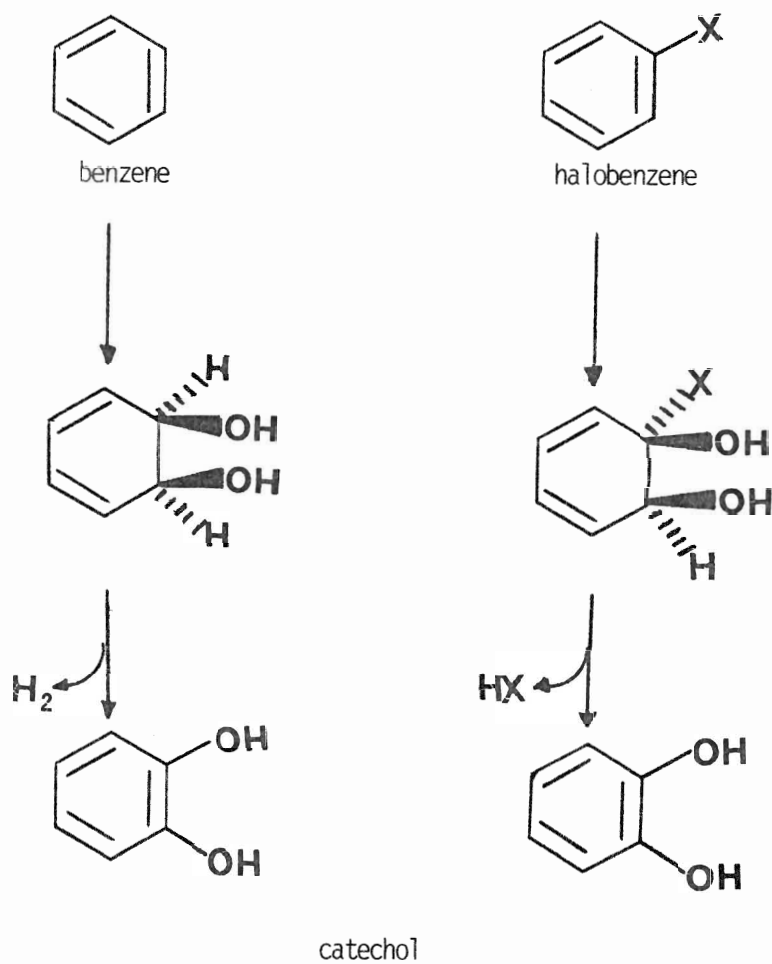
$X = Cl, Br$

and can lose either HCl or H<sub>2</sub> to generate the aromatic ring and hence the catechol (Fig. 23). These reactions are seen in many of the oxidations of the haloaromatic compounds mentioned including PCBs. Work done on the degradation of these rather stubborn compounds has shown that many of the isomers and congeners are susceptible to the action of the oxygenases.<sup>44</sup> However, there is still a problem of the higher chlorinated molecules that makes them resistant to the oxidative capabilities of these enzymes. Since the reaction of molecular oxygen or reactive intermediate is an electrophilic attack on the aromatic ring it appears that biphenyls with two or more chlorines per aromatic nucleus are poorly reacted or not affected at all. Work is in progress on the engineering super enzymes that will hopefully handle the most unreactive of the PCBs.

Another type of reaction seen for the aromatic system is that of replacement of the halogen with hydrogen directly. This is seen in DDT metabolism where the chlorines in the ring are removed in one step of a degradative pathway (Fig. 24).<sup>45</sup> The formation of diphenylketone from 2,2'-dichlorodiphenylketone can only be from reductive replacement perhaps in a way similar to the reductive removal of halogen from carbon tetrachloride.<sup>46</sup> In this case chloroform is generated from the action of cytochrome P-450 under anaerobic conditions.

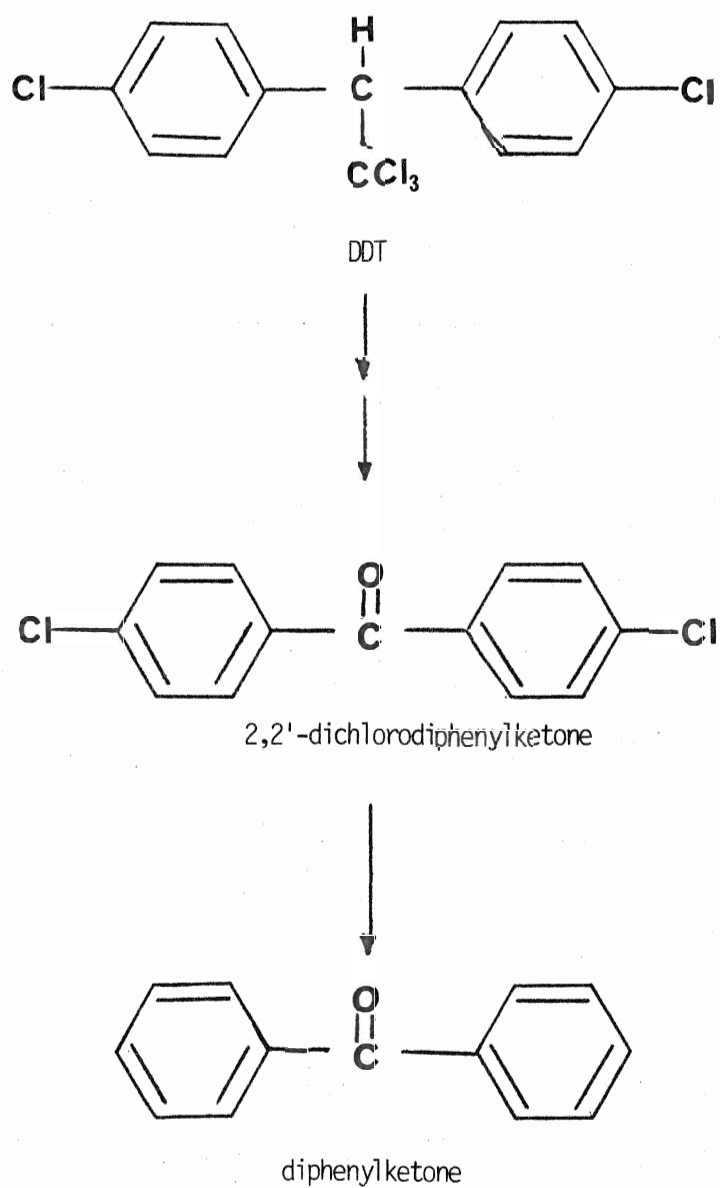
The most interesting observation of an aromatic halide

Figure 23: Oxidation of benzene and substituted benzene by dioxygenases to generate catechol in both cases



$X \equiv Cl, Br$

Figure 24: Replacement of halogen by hydrogen in a step of DDT metabolism converting metabolic 2,2'-dichlorodiphenylketone to diphenylketone

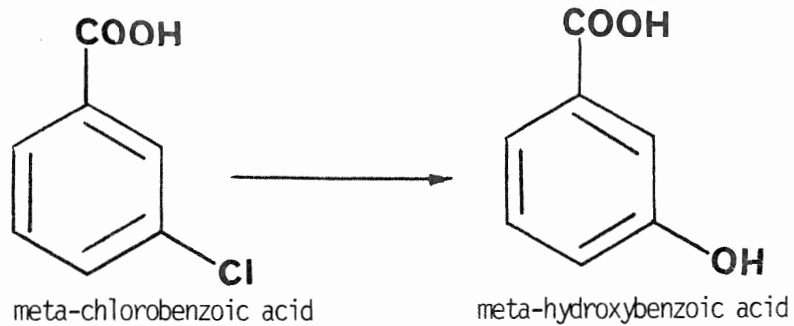


removal is that of the direct replacement of halide by hydroxyl. This process has been seen a number of times for the degradation of two halobenzoates (Fig. 25). It has long been known that the chlorine is removed from *m*-chlorobenzoate in the first step of its metabolic pathway.<sup>47</sup> However, not enough is known about the system to propose a mechanism. This is not the case for one of the enzymes responsible for the removal of halogen from *p*-halobenzoate.

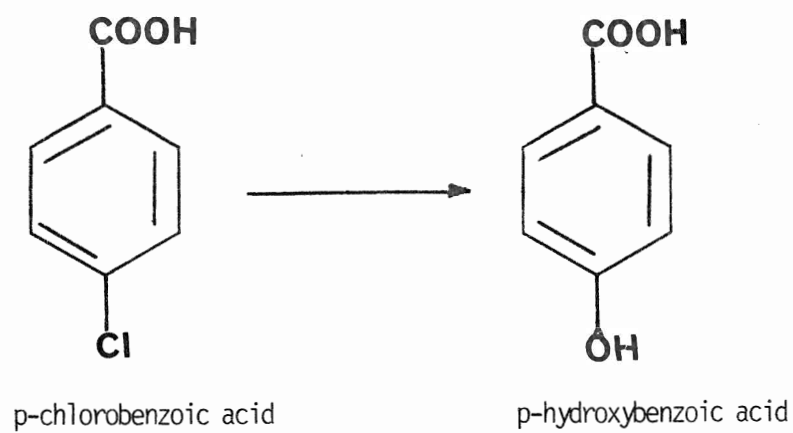
An enzyme was found in a species of bacteria known as Pseudomonas sp. CBS 3 that removed the halogen from both *p*-bromo and *p*-chlorobenzoate.<sup>48</sup> No details about the enzyme requirements were given, but it was found that the oxygen present in the product was derived from water not from molecular oxygen. This is a strange result considering most of the ring oxidations seen so far have been due to the action of an oxygenase. No reports have appeared, so far, relating to the removal of halogen from an aromatic nucleus by hydrolytic processes other than this report. The enzyme has been isolated and cloned into other species, but not enough information has been made available to date about the characteristics of the enzyme.<sup>49</sup> If it is true that the enzyme can hydrolytically remove the halogen from an aromatic ring then it should be possible to investigate this mechanism from the level of the substrate. To do this one must consider the types of hydrolytic reactions that can occur with an aromatic ring. Fortunately

Figure 25: Two examples of replacement of halogen by hydroxyl A) metachlorobenzoic acid to meta hydroxybenzoic acid B) p-chlorobenzoic acid to p-hydroxybenzoic acid

A)



B)





there are only two mechanisms that can be viewed as hydrolytic in the consideration of aromatic reactions.

The first involves the aromatic nucleophilic substitution reaction in which the halide is displaced in a two step reaction similar to the nucleophilic displacement reaction for acyl aliphatic compounds (Fig. 26). The reaction occurs when the ring also contains an electron withdrawing group in the ortho or para position relative to the leaving group.<sup>50</sup> The withdrawing group generates a slightly positive charge on the carbon with the leaving group which makes it electrophilic. The nucleophile attacks at this position generating a nonaromatic intermediate that can stabilize the negative charge by pushing the electrons onto the withdrawing group. Facile reactions are possible if the withdrawing capability is great as in the case of nitro substituents, while much stronger conditions are required for groups like carboxyl or acetyl.

The other reaction that could be viewed as hydrolytic is the process that involves a benzyne intermediate (Fig. 27). This intermediate is formed when a strong base removes the hydrogen from an aromatic ring ortho to the position of a leaving group such as chlorine. The resulting bond, commonly drawn as a triple bond, is very reactive and would quickly react with water to generate a phenol. The reaction usually requires strongly basic conditions to remove the hydrogen on the aromatic ring. For this reason the reaction is usually carried out in

Figure 26: Proposed mechanism for the nucleophilic aromatic substitution (NAS) of p-chloronitrobenzene to p-nitrophenol

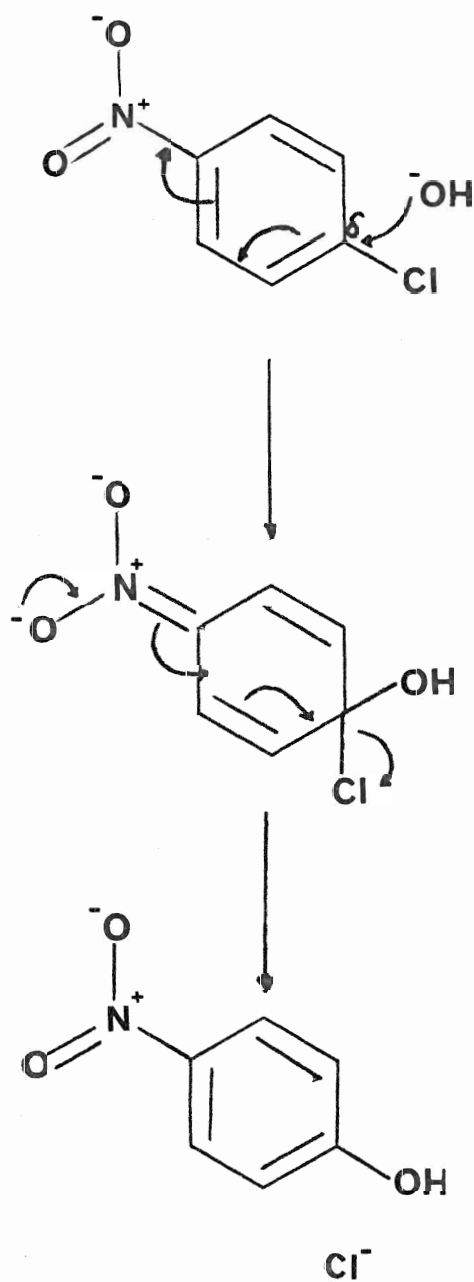
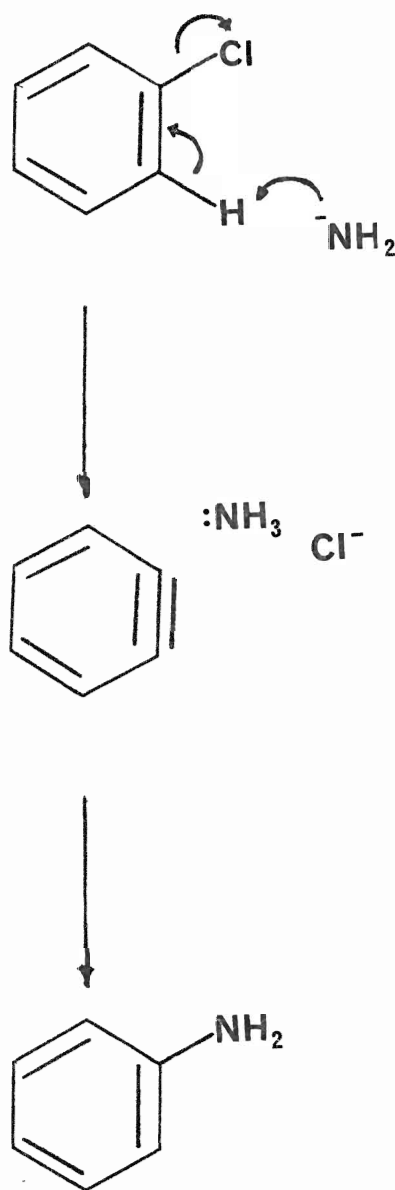


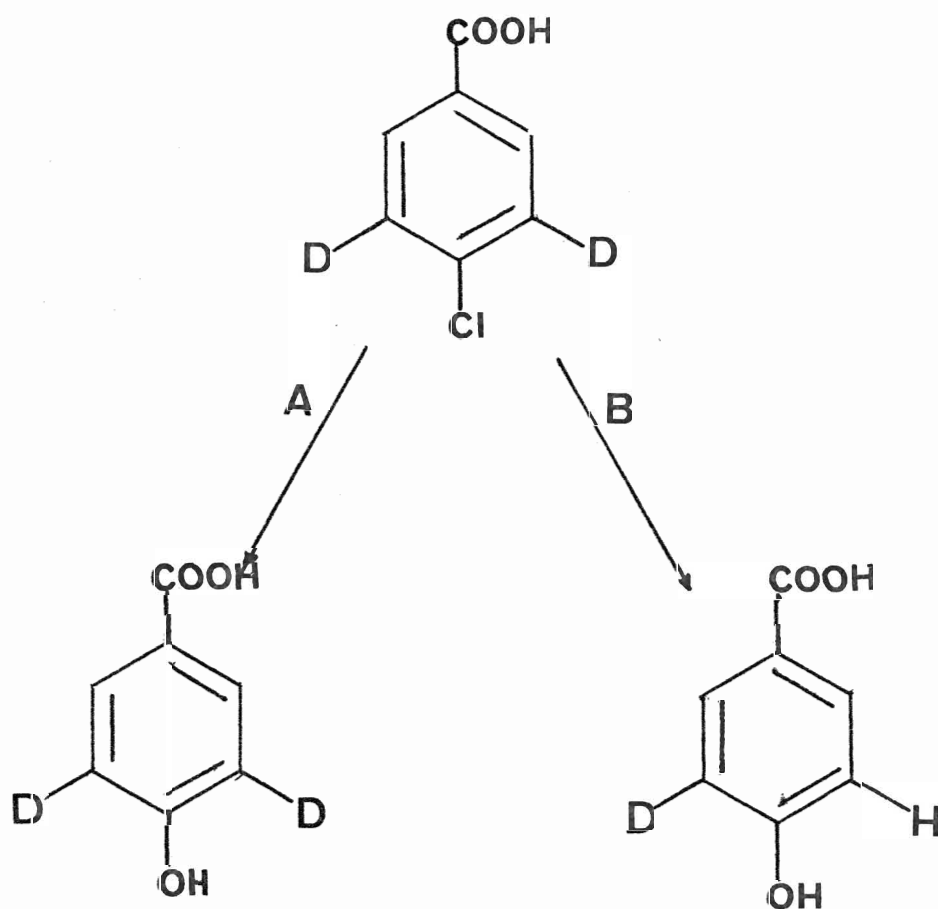
Figure 27: Generation of benzyne from chlorobenzene and its conversion to aniline by potassium amide in liquid ammonia



liquid ammonia with potassium amide as the base.<sup>51</sup>

To study the mechanism it is thought that incubation of a deuterated p-chlorobenzoate with deuterium label in the positions ortho to the chloro would differentiate between the two possible mechanisms (Fig. 28). A loss of one deuterium during the reaction would be suggestive of the benzyne mechanism, while no loss of deuterium would be suggestive of the nucleophilic aromatic substitution reaction. It is the aim of this thesis to prepare deuterated analogs of both p-chlorobenzoate and p-hydroxybenzoate. These substrates can then be sent to researchers in Germany for incubation with an enzyme preparation of 4-chlorobenzoate dehalogenase isolated from Pseudomonas sp. CBS 3.

Figure 28: Two possible routes for the hydrolytic replacement of chlorine by hydroxyl by *Pseudomonas* sp. CBS 3 A) Nucleophilic aromatic substitution B) With a benzyne intermediate. A comparison using p-chlorobenzoic acid-3,5-d<sub>2</sub>



## EXPERIMENTAL

### Apparatus, Materials and Methods

Melting points were obtained using an Electrothermal melting point apparatus and are uncorrected. Infrared spectra were obtained with an Analect FX-6260 FTIR spectrophotometer interfaced with an Analect MAP-66 data system.  $^1\text{H}$  NMR spectra were obtained on either a Bruker WP-80 CW NMR, using deuterated chloroform as solvent and tetramethylsilane as internal standard, or a Bruker AC-200 FT NMR and Bruker AM-500 MHz FT NMR using deuterated chloroform as solvent and internal standard. Carbon-13 NMR spectra were obtained at 50.3 MHz using a Bruker AC-200 FT NMR using deuterated chloroform as the solvent and the internal standard. The deuterium NMR spectra were obtained at 30.7 MHz using either chloroform or acetone as the solvent and internal standard. The electron impact mass spectra were obtained using an AEI MS 30 mass spectrometer interfaced to a Kratos DS55. The fast atom bombardment mass spectra were obtained with the same machine fitted with an Ion Tech. Ltd. saddle field gun operating at 7 KeV using a 3-nitrobenzyl alcohol or glycerol matrix. Capillary GC/mass spectra were obtained using an HP 5890A GC with an HP5970 mass selective detector interfaced to an HP 9000/300 computer. Centrifugation was done using either a Sorvall RC-5 with a GSA head or an IEC

B-20A with an IEC 870 head. Liver homogenates were obtained using a Waring blender. Incubations were done in either a Hotpack convection incubator 35°C or a Gyrotory Water Bath Shaker G76 at 37°C.

Column chromatography was performed on Silica gel, Merck ASTM-Kieselgel 60, mesh 230-400. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60-F254 (0.2 mm thick). Preparative TLC was performed on the same type of plate as the analytical TLC. The plates were examined under UV light or visualized with iodine absorption.

The live culture Escherichia coli was obtained from Dr. B.J. Barclay, Department of Biological Sciences, Brock University. The freeze-dried cultures of Eubacterium lentum ATCC 25559 and Clostridium paraputrificum ATCC 25780 were obtained from the American Type Culture Collection laboratories. A live culture of Eubacterium lentum was also obtained from ATCC.

## PREPARATION OF SUBSTRATES

### Preparation of labelled 21-hydroxypregnanes

#### Pregn-4-ene-3,20-dione-17 $\alpha$ -ol-21-al     13

A 25 mL solution of 0.005M cupric acetate in anhydrous methanol was added, all at once, to 25 mL solution of 350 mg of commercial 14 in anhydrous methanol. The solution was stirred with air bubbling through at rate of approximately one bubble per second. The reaction was allowed to proceed for 45 minutes as prescribed by Lewbart and Mattox.<sup>52</sup> After this time 50 mg of EDTA (disodium salt) in 3 mL of water was added and the solution was allowed to stir for an additional 15 minutes. At this time the the methanol was removed under reduced pressure and the residue was diluted with 50 mL of water. The aqueous solution was extracted with dichloromethane (3\*25mL). The organic extract was washed with 5% sodium bicarbonate (3\*25mL) and water (3\*25mL) and dried over anhydrous sodium sulphate. After evaporation of the solvent, 330 mg of a yellow foam remained which later solidified to a yellow solid, M.P. 100-105°C with decomposition. The <sup>1</sup>H nmr of 13 compared tp 14 showed the loss of the 21-methylene AB at delta 4.5 ppm and the presence of two signals for the 18-methyl group one at 0.758 ppm and the other at 0.707 ppm, neither of which overlapped with the

Note: the symbol \* represents multiplication



18-methyl signal for starting material at 0.725 ppm. There was also the presence of small peak in the aldehyde region of the spectrum at 9.4 although it did not integrate to 1 hydrogen. There was another peak at 4.9 ppm due to the hydrogen of a gem-diol at C-21. This peak also was less than one hydrogen. This nmr suggests a mixture of free aldehyde and the hydrate. FAB mass spectrum of the product showed the presence of both aldehyde and hydrate having peaks at (M+H) 345 and (M+H) 363 respectively. There was also the presence of starting material at (M+H) 347 which was approximately 4% of the mixture.

Pregn-4-ene-17,21-diol-3,20-dione     14

The acetone powder of commercial beef liver was prepared, following a procedure of Monder and White,<sup>53</sup> by homogenizing the liver in four volumes of cold acetone (-20°C) for one minute and filtering. The process was repeated and after filtering for the second time the solid remains of the liver was allowed to dry at room temperature, open to the air for 24 hours at which time it was ground up and stored at -20°C. This first step in the isolation is known to be sufficient for testing for the presence of 21-hydroxysteroid-NADH oxidoreductase.

To 75 mL of 0.1 M sodium phosphate buffer pH 7.4<sup>†</sup>, 1.0 g

†

See Appendix III

of acetone powder was added and the mixture was stirred for 15 minutes to extract out the enzyme. To this mixture 14.6 mg of 13 , in 1.0 mL 95% ethanol, was added slowly and 34.0 mg of NADH was added all at once. The heterogeneous reaction mixture was stirred slowly for one hour at room temperature. At this time the powder was removed by filtration and washed with ethyl acetate (2\*20mL). The aqueous solution was extracted with ethyl acetate (2\*30mL). The organic extracts were combined, dried over anhydrous sodium sulphate and evaporated leaving a 15.0 mg residue. TLC analysis of the residue showed a spot for starting material along with a number of unknown spots, but no product was observed.

The procedure was repeated using an acetone powder prepared from fresh beef liver. Again the TLC showed only starting material and other unknown without the presence of product.

To remove the complication of the multiple protein composition of the reaction mixture Monder and Whites<sup>53</sup> purification procedure was followed three more steps to achieve their 4 fold enzyme activity increase.

To 100 mL of 0.1 M sodium phosphate buffer pH 7.4, 14 g of the acetone powder from fresh beef liver was added. The mixture was stirred for 1 hour at room temperature. The flask

was cooled to 4°C and centrifuged for 4 minutes at 2000\*g. The precipitate was discarded and the supernatant was topped up to 100 mL by the addition of phosphate buffer. The solution was brought to 20% saturation of ammonium sulphate by the addition of 10.6 g of solid ammonium sulphate. The solution was put on ice for 15 minutes and then centrifuged for 5 minutes at 2000\*g. The precipitate was discarded and the supernatant (90 mL) was brought to 50% saturation by the addition of 15.8 g of ammonium sulphate. The precipitate was centrifuged at 2000\*g for 6 minutes and the supernatant was discarded. The precipitate was resuspended in 15 mL of 1 mM EDTA pH 7.0 and brought to 57°C for 12 minutes. The solution was quickly cooled to 4°C and then centrifuged at 3000\*g for 10 minutes. The supernatant containing the 21-hydroxysteroid NADH oxidoreductase was stored at 4°C until needed.

To 100 mL of phosphate buffer, 5 mL of this enzyme extract was added and 50 mg of NADH was added with stirring. To this solution a 1.5 mL solution of 25 mg of 13, in 95% ethanol, was added slowly to avoid precipitation. The solution was allowed to stir for 2 hrs, during which time the solution went from being turbid to nearly clear. The aqueous solution was then extracted with ethyl acetate (3\*25mL), dried over anhydrous sodium sulphate and evaporated leaving a 20 mg residue. The residue was shown to contain the desired product 14, by comparative TLC. The <sup>1</sup>H nmr showed the presence of the

21-methylene AB signals at delta 4.5 ppm and the C-18 methyl signal of 14 was also present. There was no peak for the C-18 methyl signal of 13, and no spot on TLC corresponding to starting material.

5 $\beta$ -Pregnan-3 $\alpha$ -ol-20-one-21-al 15

Following the procedure outlined for 14, 35 mg of 1 was dissolved in 2.5 mL methanol and 2.5 mL of 0.005M cupric acetate in methanol was added all at once. Bubbling air through the reaction for 1 hour followed by the workup procedure, afforded 30 mg of a slightly yellow solidified foam. TLC showed predominantly a single spot different from starting material, with some starting material also present. The  $^1\text{H}$  nmr showed a peak for the 21 methylene at delta 4.1 ppm, which integrated 1:9 with respect to the C-3-alpha hydrogen. There was a small aldehyde peak at 9.2 ppm integrating 1:10 with respect to the C-3 hydrogen. A broad peak between 4.6 and 4.9 ppm was present which integrated 1:2.6 with respect to the C-3 hydrogen. There were two C-18 methyl peaks, one at 0.594 ppm overlapped with the 18-methyl of the starting material, while there was another smaller peak at 0.724 ppm which did not correspond to the starting material. I.R. Showed two peaks for the carbonyl stretch at  $1722\text{ cm}^{-1}$  and  $1711\text{ cm}^{-1}$ . Starting material had

carbonyl stretch at  $1709\text{ cm}^{-1}$ .

5 $\beta$ -Pregnan-3 $\alpha$ ,21-diol-20-one     1

To 100 mL of phosphate buffer pH 7.4, 7 mL of enzyme extract was added followed by 40.0 mg of NADH. To this solution 20 mg of the compound mixture prepared above, in 1 mL of 95% ethanol was added giving a turbid solution. Three hours of stirring at room temperature did not cause an observable decrease in turbidity. The medium was extracted into ethyl acetate (3X25mL). After drying over sodium sulphate the ethyl acetate was evaporated to give a 27 mg residue. Analysis of the residue by TLC and  $^1\text{H}$  nmr did not show the presence of 1, although TLC showed the presence of the starting material 15

Repetition of this reaction, at  $37^\circ\text{C}$ , did not show any product upon extraction after 2 hours of stirring.

Pregn-4-ene-3,20-dione-21-al     16

To 36 mL of dry methanol, 500 mg of 3 was added along with 36 mL of the cupric acetate solution. To this solution air was bubbled through for one hour. The reaction was quenched with 70 mg of EDTA in 4.2 mL of water and the reaction worked up as described previously. After evaporation of the

dichloromethane 380 mg of a yellow oil was obtained which showed two spots on TLC, both different from starting material. Recrystallization was attempted from an acetic acid/water mixture and from a hexane/ethyl acetate mixture. Neither method gave crystalline product as the ratio of components seemed to change by TLC analysis. Column chromatography was attempted using 3:2 hexane:ethyl acetate eluent, which afforded 210 mg of a white solid, M.P. 180-200°C with decomposition, and 100 mg of a yellow oil. The yellow oil turned into a white solid within days of the reaction. The TLC of this product was the same as that for the solid isolated from the column.

The white powder had the following spectral properties: Mass spectrum showed a molecular ion  $m/e$  316. The  $^1\text{H}$  nmr showed no signal for the C-21 methylene at  $\delta$  4.1 ppm and no aldehyde signal at 9.2 ppm. The carbon-13 spectrum is shown in table 1 with the spectrum of starting material. The I.R. showed two carbonyl stretching peaks at  $1729\text{ cm}^{-1}$  and  $1657\text{ cm}^{-1}$  different from the starting material values of  $1709\text{ cm}^{-1}$  and  $1666\text{ cm}^{-1}$ . The compound was assigned structure 24.

The reaction was repeated and the progress was followed by TLC, until only one spot less polar than starting material was visible after 50 minutes. The reaction was then quenched and 390 mg of yellow oil was recovered. The product 16

Table I: Carbon-13 peaks for compounds, 3 , and, 24

peak no.	compound <u>3</u> (ppm)	compound <u>24</u> (ppm)
1	35.6	35.8
2	33.8	33.9
3	199.1	199.5
4	123.7	123.9
5	170.4	171.0
6	32.6	32.8
7	31.8	32.0
8	35.4	35.8
9	53.5	53.8
10	38.4	38.7
11	20.8	20.9
12	38.2	38.0
13	44.5	44.0
14	55.8	55.5
15	24.4	24.4
16	22.8	23.4
17	59.0	55.0
18	13.3	13.3
19	17.3	17.4
20	210.0	178.8
21	69.3	-

was quickly stored under Argon and placed in the  $-20^{\circ}\text{C}$  freezer until needed.

5 $\beta$ -Pregnan-3 $\alpha$ ,21-diol-20-one -21-d<sub>2</sub>,17 $\alpha$ -d<sub>1</sub> 1 -d<sub>3</sub>

To 1 mL of methanol-d<sub>1</sub>, 100 mg of unlabelled starting material 1 was added.<sup>55</sup> To this solution was added another solution containing 210 mg of tris(hydroxymethyl)methylamine (TRIS) in 10 mL of 50:50 D<sub>2</sub>O/MeOD. The flask was sealed and then placed in an oven set at  $60^{\circ}\text{C}$  for 24 hours. After this time the flask was cooled to room temperature and opened. The solution was acidified to pH 5.5 using 38% deuterium chloride in D<sub>2</sub>O. The methanol was removed under reduced pressure and the residue extracted with ethyl acetate (3X25mL). The extract was dried over anhydrous sodium sulphate and evaporated leaving 98 mg of white solid. The TLC showed the product to be pure and identical in R<sub>f</sub> value to starting material. <sup>1</sup>H nmr showed the presence of some 21-methylene hydrogens. Integration of this peak indicated the presence of 20% of the unlabelled compound. To remove more of the hydrogen from this position the reaction was repeated on this same sample giving 95 mg of product. Comparative TLC again showed the product to be pure. The <sup>1</sup>H nmr of the product showed 90% of the hydrogen had been replaced at the 21-methylene position.



Pregn-4-ene-3,20-dione-21-ol -21-d<sub>2</sub>,17 $\alpha$ -d<sub>1</sub> 3 -d<sub>3</sub>

To 3 mL of methanol-d<sub>1</sub>, 500 mg of 3 was added and the solution was added all at once to a 50 mL solution of 50:50 D<sub>2</sub>O:MeOD with 1.05 g of TRIS. The reaction flask was sealed and stored at 60°C for 28 hrs. After this time the reaction was worked up as previously mentioned, and gave 490 mg of product showing single spot on TLC, identical to that of starting material. The <sup>1</sup>H nmr showed a small peak at 4.1 ppm which when integrated showed that 70% of the hydrogen had been exchanged for deuterium at the C-21 position. This same sample was then run through the same procedure for 18 hours. After workup only 390 mg were isolated, however the product showed only one spot on TLC identical to that of starting material. The C-21 methylene was nearly completely absent from the <sup>1</sup>H nmr spectrum which when integrated showed the compound to be 80% deuterium at the methylene position. Deuterium nmr showed two major peaks, one at delta-4.1 ppm for the C-21 methylene group and another broad peak, centred at 2.4 ppm for the C-17 methine position and the C-2 methylene. The integral for these two peaks showed a 2:1.5 ratio respectively. A small peak was visible for the C-4 methine hydrogen at 5.7 ppm which integrated 0.5:10 with respect to the peak at 4.7 ppm. The FAB mass spectrum showed a complex set of peaks between m/e 330 and 336.

Pregn-4-ene-3,20-dione-21-al -21-d<sub>1</sub>, 17 $\alpha$ -d<sub>1</sub> 16 -d<sub>2</sub>

To 28 mL of dry methanol-d<sub>1</sub>, 380 mg of 3 -d<sub>3</sub> was added with 28 mL of a 0.005 M cupric acetate in methanol-d<sub>1</sub>. The reaction was followed as described for 75 minutes and worked-up. After evaporation 350 mg of a yellow oil was isolated and put under argon for storage at -20°C. The deuterium nmr showed a large peak at delta 4.7 ppm for the gem-diol hydrogen at C-21 and a peak at 4.1 ppm for unreacted starting material, which integrated 0.7:10 with respect to the peak at 4.7 ppm. The peak at 2.4 ppm for the C-17 methine now integrated 1.1:1 with respect to the C-21 deuterium. The peak at 5.7 ppm integrated 1:10 with respect to the C-21 deuterium.

21-(R)- Pregn-4-ene-3,20-dione-21-ol -21-d<sub>1</sub>, 17 $\alpha$ -d<sub>1</sub> 17

(a) To a flask containing 100 mL sodium phosphate buffer, 5 mL of enzyme extract (containing 21-hydroxysteroid NADH oxidoreductase) and 220 mg of NADH was added. To this solution a 2 mL solution of 100 mg. 16 -d<sub>2</sub>, in 95 % ethanol, was added slowly with stirring. The mixture was allowed to stir for one hour, during which time the turbidity decreased. After the hour

the medium was poured into a continuous extractor with ethyl acetate. After 48 hours the ethyl acetate was evaporated and the residue showed the presence of product by TLC. The residue was chromatographed with 3:2 hexane ethyl acetate giving 52 mg of product. The  $^1\text{H}$  nmr at 500 MHz showed only one peak in the region of the C-21 methylene at  $\delta$  4.130 ppm. The I.R. showed carbonyl peaks identical to those of authentic 3. FAB mass spectrum showed a complex set of peaks between  $m/e$  330 and 336.

(b) The title compound was also synthesized from 16 - $\text{d}_2$  and  $\text{NaBH}_4$  by a method of Monder and Orr.<sup>56</sup> Following this procedure, 200 mg of 16 - $\text{d}_2$  was added to 5 mL of pyridine followed by 6.1 mg of  $\text{NaBH}_4$ . The reaction immediately turned to a yellow/orange colour as the yellow colour from the starting material vanished. After 15 minutes of stirring at  $4^\circ\text{C}$  the reaction mixture was poured into 100 mL of water. The aqueous solution was extracted with ethyl acetate (4\*25mL). The ethyl acetate extract was washed with 2M tartaric acid (3\*50mL) and then with water until neutrality was obtained. The ethyl acetate was dried over anhydrous sodium sulphate and evaporated giving a yellow residue. TLC analysis showed the presence of the product and absence of the starting material. The product was obtained by chromatography which afforded 109 mg of material. High resolution  $^1\text{H}$  nmr at 500 MHz showed two singlets in the region

of the C-21 methylene signals. One peak was seen at delta 4.167 ppm and the other at 4.130 ppm in a 1:2 ratio. The I.R showed peaks for carbonyls identical to those of 3 . The FAB mass spectrum showed a complex set of peaks between m/e 331 and 336.

21-(S)- Pregn-4-ene-3,20-dione-21-ol -21-d<sub>1</sub> 18

(a) Following the same procedure described above, 250 mg of 16 was dissolved in 5 mL of dry pyridine and 7.9 mg of NaBD<sub>4</sub> was added. After 15 minutes of stirring the reaction mixture was poured onto water and extracted with ethyl acetate. After chromatography 160 mg of product was obtained, which was shown by high resolution <sup>1</sup>H nmr at 500 MHz to have two peaks in the region of the C-21 methylene signals. One peak was seen at delta 4.167 ppm and the other was at 4.130 ppm in a 3.2:1 ratio. I.R. showed the carbonyl peaks to be identical to those of 3 . The FAB mass spectrum was less complicated having predominantly only one deuterium per molecule. The pattern between m/e 330 and 334 was similar to that for 3 between m/e 329 and 333. See table II for list of peaks and relative intensities.

Table II: FAB Molecular ion peak patterns for 3 and 18

m/e	% Intensity of base peak	
	compound <u>3</u>	compound <u>18</u>
329	10	-
330	12	11
331	100	24
332	26	100
333	9	27
334	-	6

(b) Starting with unlabelled 16 it was necessary to generate a deuterated  $\text{NAD}^+$  molecule to be used in a hydride transfer from ethanol to the dehydro steroid. Following a procedure from Colowick and Kaplan,<sup>57</sup> 200 mg of  $\text{NAD}^+$  was dissolved in 3.5 mL of  $\text{D}_2\text{O}$  with 232 mg of KCN. To this solution, 95 mL of a 5 N KOH/ $\text{D}_2\text{O}$  solution was added and the mixture was allowed to stand for 2 hrs. During this time the solution had changed from a clear colourless solution to deep reddish/orange colour. At this time the solution was poured into a solution of 1.12 g of potassium dihydrogen phosphate in 34 mL of  $\text{D}_2\text{O}$ . Argon was passed through the solution for one hour to remove the HCN and give a final pH of 6.5.

The above solution was then used in the reduction of  $\text{NAD(D)}^+$  to  $\text{NAD(D)H}$  by a modified procedure of Rafter and Collowick.<sup>58</sup> To the solution of  $\text{NAD(D)}^+$ , 3 mg (840 units) of yeast alcohol dehydrogenase and 25 mL of ethanol were added. This solution was adjusted to pH 8.8 by the addition of a 5 N KOH solution (approximately 1.5 mL). This solution was then

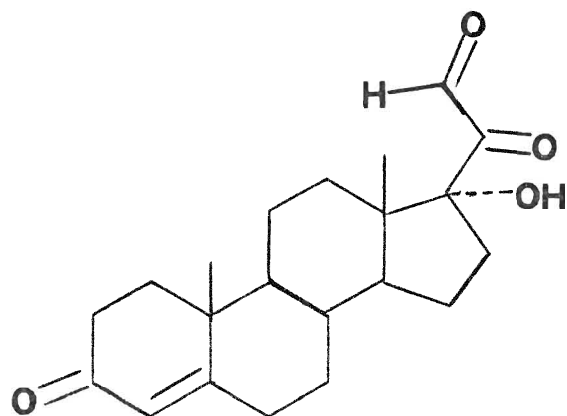
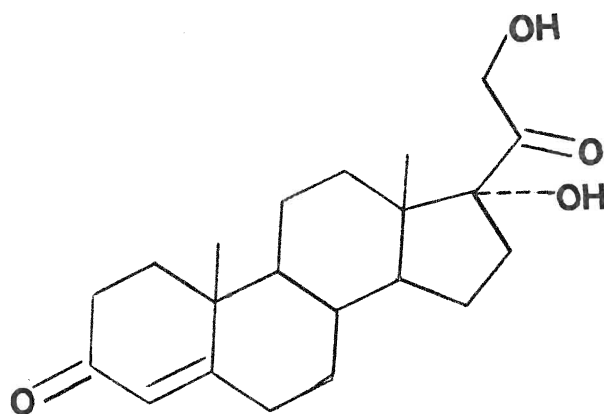
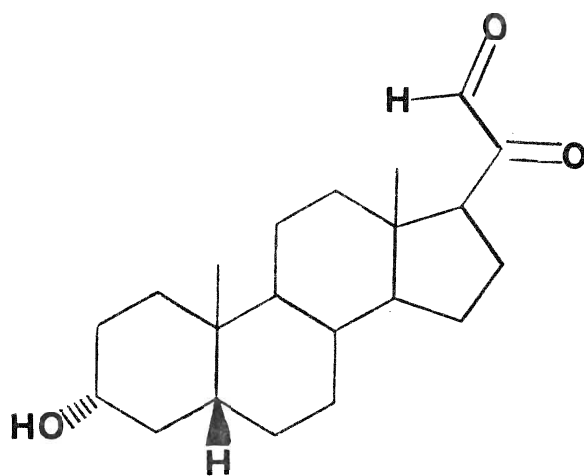
topped up to 100 mL by the addition of phosphate buffer pH 7.4. To this solution 3 mL of the beef liver enzyme extract, containing 21-hydroxysteroid NADH oxidoreductase, was added and following that 80 mg of, 16, in 2 mL of ethanol. This solution was stirred for 3 hours after which time the medium was placed in the continuous extractor with ethyl acetate for 48 hours. After this time the ethyl acetate was evaporated leaving a 70 mg residue which showed product by TLC. The product was isolated by column chromatography with 3:2 hexane:ethyl acetate giving 23 mg of product. The  $^1\text{H}$  nmr showed no peak at 4.1 for the C-21 methylene hydrogen.

The reaction was repeated following the same procedure except for the step involving the addition of the liver enzyme extract. Before this addition was made the pH was adjusted to 7.5 by the addition of phosphoric acid. Following the remainder of the steps 17 mg of product was isolated after chromatography which again showed no peak for the C-21 methylene position in the  $^1\text{H}$  nmr.

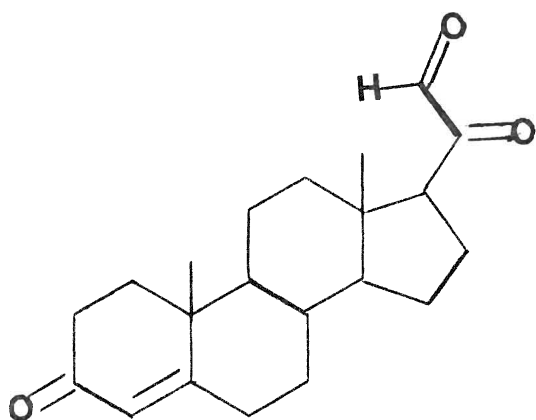
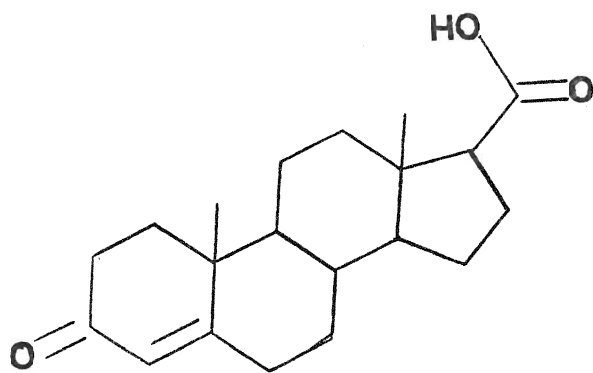
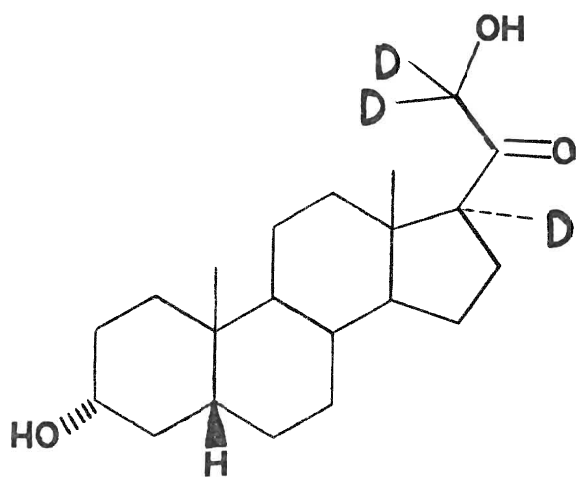
5 $\beta$ -Pregnan-3 $\alpha$ -ol-20-one -21-d<sub>3</sub>, 17 $\alpha$ -d<sub>1</sub> 19 63

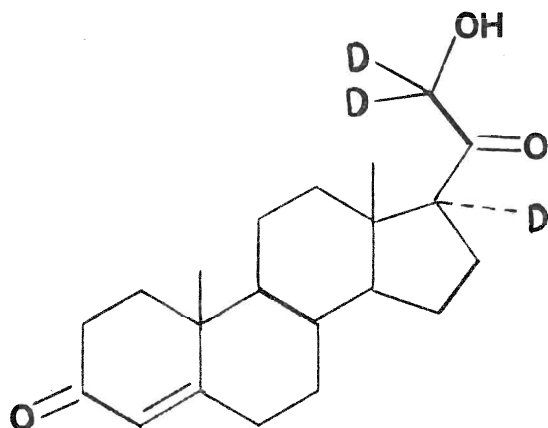
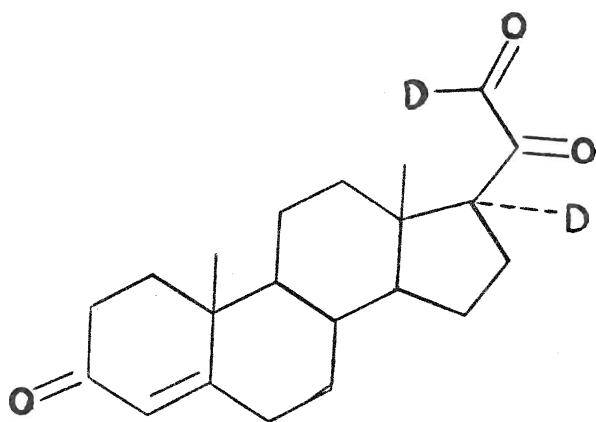
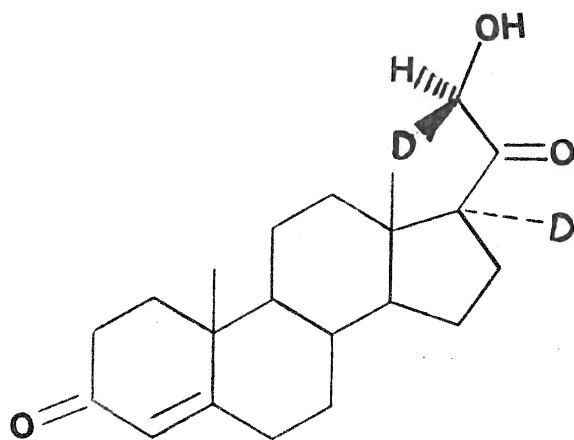
To 5 mL of dry benzene, 100 mg of unlabelled starting material, 19, was added. A solution of 100 mg of tetrabutylammonium bromide in 10 mL of 5% NaOD/D<sub>2</sub>O was added to

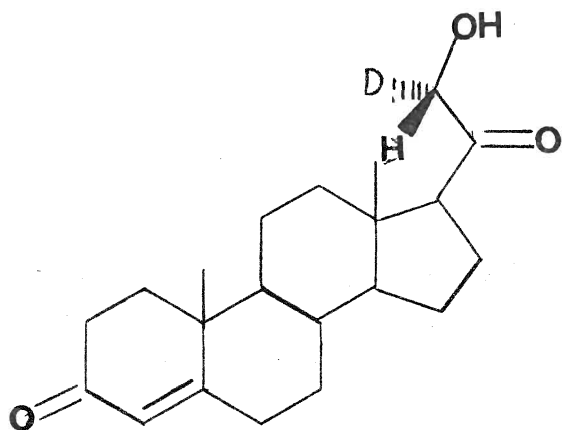
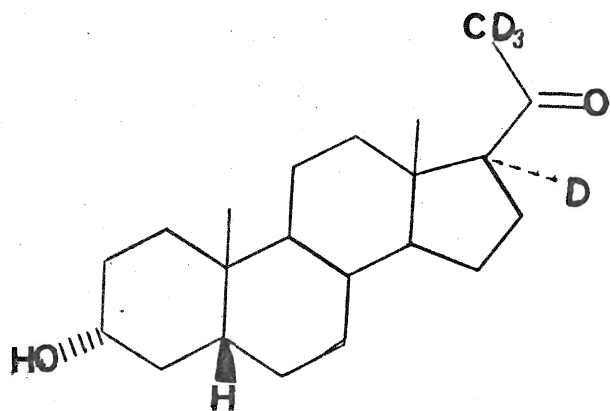
the benzene solution and the two phase reaction mixture was refluxed under argon for 48 hours. At this time the aqueous layer was poured off and the organic layer was washed with pH 6.68 buffer. The organic layer was dried over anhydrous sodium sulphate and evaporated leaving 75 mg of a white crystalline material, which had a single spot on TLC identical to that of starting material. The E.I. mass spectrum showed the peak cluster for  $M-H_2O$  302(13.8), 303(13.8), 304(91.4), 305(26.3), with no peaks apparent for the unlabelled compound having  $M-H_2O$  300(100), 301(25.5)

131415



16241-d<sub>3</sub>

3-d<sub>3</sub>16-d<sub>2</sub>17

1819

### Preparation of deuterium labelled Benzoic acids

#### p-Toluidine-3,5 -d<sub>2</sub>

To 20 mL of 2.5% sodium deuterioxide in D<sub>2</sub>O, 500 mg of p-toluidine was added with 100 mg of nickel on alumina.<sup>59</sup> The heterogeneous mixture was refluxed for 24 hours at which time 50 mL of ether was poured into the flask. The catalyst was removed by gravity filtration. The aqueous layer was separated and the ether layer dried over anhydrous sodium sulphate and evaporated. TLC analysis showed a single spot identical with that of starting material. The <sup>1</sup>H nmr showed a spectrum identical to that of starting material with no apparent deuterium exchange at the ortho positions to the amino group. The whole aromatic region integrated to four hydrogens relative to the methyl signal.

#### p-Chlorotoluene

Following a procedure from Koviak and Bruce,<sup>60</sup> 27 g of ferric chloride was added to 25 g of toluene with 2.0 g of AlCl<sub>3</sub> as catalyst. The mixture was stirred at 50°C for 3.5 hours during which time the evolved HCl was trapped by a solution

containing 10.8 g of NaOH in water. The reaction was terminated when the trap solution approached neutrality. The reaction mixture was poured over 200 g of ice and the resulting aqueous solution was steam distilled. The distillate was extracted with hexane (4\*50mL) and dried over magnesium sulphate. The GC/MS showed the 13.1 gram extract to be mixture of toluene and p-chlorotoluene.

p-Chlorotoluene -d<sub>7</sub>

Following the same procedure outlined for the unlabelled compound, 25 g of toluene-d<sub>8</sub> was mixed with 25 g of FeCl<sub>3</sub> and 1.8 g AlCl<sub>3</sub> and the mixture was allowed to react for 3.5 hours. at 50°C. Following steam distillation and extraction, 14.0 g of extract was obtained which gave 2.6 g of p-chlorotoluene from a constant boiling fraction of 155°C. The GC/mass spectrum showed the fraction to be mostly a chlorotoluene-d<sub>7</sub> with a residual amount of toluene-d<sub>8</sub>. The GC could not distinguish between ortho and para chlorotoluene, which have only a 2 degree boiling difference. No measure of the amount of the ortho isomer was made at this stage.

p-Chlorobenzoic acid -d<sub>4</sub>

Following a procedure in Vogel<sup>61</sup> for the conversion of p-chlorotoluene to p-chlorobenzoic acid, 2.6 g of p-chlorotoluene-d<sub>7</sub> was mixed with 65 mL of D<sub>2</sub>O and 7.0 g of potassium permanganate. The mixture was refluxed for 24 hours after which time the manganese dioxide was filtered off. The aqueous solution was acidified with 5% HCl which precipitated the product. The product was isolated by filtration and recrystallized three times from aqueous ethanol to remove any ortho isomer, which resulted in the isolation of 230 mg of product with a melting point, in a sealed capillary, of 235-241°C. A sample obtained from others and prepared by a different method gave an identical melting point.

Deuterium exchange under weak acid and high temperatures

General Procedure<sup>62</sup>

To a medium walled glass tube 200 mg of substrate and 10 mL of a 1% DCl/D<sub>2</sub>O solution were added. The solution was degassed by a repetition of freezing at liquid nitrogen

temperature and the pumping off of gas that was evolved upon warming. After four rounds of freezing and thawing no further gas evolution was seen. The tube was sealed under vacuum and sealed in a high temperature and pressure autoclave with water outside the glass tube to equalize pressure. Reactions took place at 280°C between 48 and 72 hours after which time the tube was removed, cooled and opened. The contents of the tube were poured out and the organic material(s) extracted with ether. The products were analysed.

p-Hydroxybenzoic acid -d<sub>4</sub>

Exchange of this compound was attempted a number of times for periods of time between 48 and 72 hours. The product was dissolved in the solution and attempts at precipitating it were unsuccessful. Initially deuterium nmr showed the presence of aromatic deuterium signals as the aromatic proton signals disappeared from the <sup>1</sup>H nmr. The signal in the deuterium nmr at delta 7.2 ppm was thought to be hydrogens ortho to the phenolic carbon, while the smaller peak at 8.2 ppm was thought to be the hydrogens ortho to the carbon with the carboxyl, since longer reaction time made this peak increase in intensity, in the deuterium spectrum. It was observed that gas was evolved upon opening two of the tubes after 48 and 60 hours and two tubes

left for 72 hours exploded sometime during the reaction. TLC analysis of the samples showed them to contain more than one component one of which corresponded to phenol and another corresponded to p-hydroxybenzoic acid. The other components were not identified.

Anisaldehyde -d<sub>4</sub>

In the tube, 200 mg of the title compound was reacted for 72 hours at 280°C. After this time the tube was opened and gas evolved from the clear colourless solution. A reddish black precipitate was also present, approximately 100 mg, that was not soluble in ether, chloroform or acetone and had a melting point >300°C.

p-Hydroxybenzaldehyde -d<sub>4</sub>

To see if the same result would be obtained from the demethyl derivative of anisaldehyde, 150 mg of the title compound was reacted for 72 hours. The same reddish black precipitate was present in the clear colourless solution. No attempt was made to identify the substance.



### Hydrolysis of p-Hydroxybenzonitrile

Following a procedure in Vogel,<sup>63</sup> 1.0 g of p-hydroxybenzonitrile was dissolved in 10 mL of 10% sodium hydroxide. The solution was refluxed for 24 hours, after which the solution was cooled and acidified with concentrated HCl. The precipitate was filtered by suction giving 630 mg of a beige powder. The melting point of the material was determined to be 205-215°C (lit. 215°C).<sup>64</sup> TLC analysis of the product showed only a spot corresponding to p-hydroxybenzoic acid, no spot for the starting material was observed. The <sup>1</sup>H nmr showed only an aromatic AB system identical to that of p-hydroxybenzoic acid.

### p-Hydroxybenzonitrile

a) Following a procedure of Holland and Sultana,<sup>65</sup> the title compound was prepared from 194 mg of p-bromophenol and 100 mg of cuprous cyanide in 2.2 mL of distilled N,N-dimethylformamide. The mixture was stirred and refluxed for 3.5 hours under an atmosphere of argon. After this time the solution was cooled to room temperature and a solution of 1.6 g ferric chloride in 3.3 mL of water with 1 mL of concentrated HCl was added. The mixture was then heated on a water bath at 55°C for a further 3 hours. After this time the reaction mixture was

extracted, while still warm, with ether (3\*25mL), dried over anhydrous sodium sulphate and evaporated. The 130 mg residue was shown by TLC to be a mixture of the product p-hydroxybenzonitrile and the starting material. The composition was determined, by integration of the aromatic AB doublets in the  $^1\text{H}$  nmr, to be 70 % p-hydroxybenzonitrile.

b) Following the same procedure for p-bromophenol, 143 mg of p-chlorophenol was mixed with 100 mg of cuprous cyanide in 2.2 mL of DMF. After refluxing under argon for 5 hours the reaction was treated with 1.6 g of  $\text{FeCl}_3$  in 3.3 mL of water and 1.1 mL of concentrated HCl. After heating for another 3 hours and extraction with ether, 123 mg of a slightly yellow oil was obtained. TLC and  $^1\text{H}$  nmr showed that only the starting material p-chlorophenol was present, no spot or peaks were visible for p-hydroxybenzonitrile.

p-Bromophenol -d<sub>4</sub>

Following the general procedure for the high temperature, weak acid deuterium exchange reaction, 250 mg of p-bromophenol was reacted with 13 mL of 1%  $\text{DCl}/\text{D}_2\text{O}$  for 48 hours at  $280^\circ\text{C}$ . The resulting brown oil was extracted with ether (4\*25mL) and after drying and evaporation 197 mg of the brown

oil was obtained. TLC analysis showed the oil to be a complex mixture of products, one of which corresponded to p-hydroxyphenol. Capillary gas chromatography of the starting material showed the sample to be pure p-bromophenol while the chromatogram of the product mixture showed at least 5 major components. With the mass spectrometer linked to the G.C. the peaks were identified as phenol, o-bromophenol, p-bromophenol, monochlorophenol, dibromophenol as well as more complex phenolic materials. Since all the compounds were deuterated identification was difficult. Quantification of the mixture was not attempted by G.C. and the deuterium nmr showed a complex multiplet between  $\delta$ -6.8 ppm and 7.8 ppm. It was difficult to identify the peaks for p-bromophenol.

p-Hydroxybenzonitrile -d<sub>4</sub>

Following the general procedure, 200 mg of p-hydroxybenzonitrile was reacted with 13 mL of 1% DCl/D<sub>2</sub>O for 72 hours at 280°C. At this time it was found the tube had exploded.

p-Hydroxytoluene -d<sub>4</sub>

To 12 mL of 1% DCl/D<sub>2</sub>O, 200 mg of the unlabelled p-hydroxytoluene was added and the mixture was heated for 72 hours at 280°C. The brown oil that was isolated after extraction was found to be a complex mixture of products by TLC analysis one of which corresponded to p-hydroxytoluene. The other spots were not identified.

p-Methoxytoluene

To 40 mL of 95% ethanol, 5.1 g of KOH and 10.8 g of p-hydroxytoluene were added. The mixture was refluxed for 1 hour and then 15 g of methyl iodide was added down the condenser and this mixture was heated under reflux for a further 2 hours. After this time the solution was poured onto 100 mL of water and the aqueous solution was extracted with ether (3\*25mL). The ether extract was washed with 20% sodium hydroxide (4\*20mL) and water (2\*30mL). After drying and evaporation, 6.5 g of a slightly yellow liquid was obtained. TLC analysis showed only one spot less polar than starting material. The <sup>1</sup>H nmr showed two methyl signals singlets, one at delta 3.8 ppm and the other

at 2.4 ppm which integrated 1:1.

p-Methoxybenzoic acid

Following the procedure for the oxidation of p-chlorotoluene, 1.0 g of p-methoxytoluene was added to 26 mL of water and 3.1 g of  $\text{KMnO}_4$ . The mixture was refluxed for 12 hours and worked up as described previously. After evaporation of the ether, 480 mg of product was obtained which showed a single spot on TLC identical to that for authentic p-methoxybenzoic acid. The  $^1\text{H}$  nmr showed the loss of the singlet at  $\delta$  2.4 ppm, but the retention of the singlet at 3.8 ppm, which integrated 3:4 with respect to the aromatic AB at 7.2 ppm. The e.i. mass spectrum showed an  $M^+$  152.

p-Methoxytoluene -d<sub>4</sub>

Following the deuterium exchange procedure, 200 mg of p-methoxytoluene was added to 10 mL of the 1%  $\text{DCl}/\text{D}_2\text{O}$  solution and heated for 72 hours at  $280^\circ\text{C}$ . Isolation of 149 mg of product showed the presence of p-methoxytoluene by TLC but also p-hydroxytoluene as well as some more polar material.

Comparison of the  $^1\text{H}$  nmr and deuterium nmr showed the exchange was complete at the aromatic hydrogens but also a peak was present in the deuterium nmr at delta 2.4 ppm. Integration showed that 9% of the hydrogen had exchanged at this position. Using this value an integration of the proton methyl signals showed that 9% of the methoxy methyl had been removed. Repetition of this reaction showed variation in the amount of demethylation for the same reaction time. An attempt was made to remove the phenol from the mixture. The product from one run (195 mg) was dissolved in 50 ml ether and washed with 20 % NaOH (3\*25mL). After evaporation of the ether only 11 mg of p-methoxytoluene was obtained.

#### Salicylic acid

Following a procedure of Hurtley,<sup>66</sup> 2.0g of o-chlorobenzoic acid was dissolved in 50 mL of water as its sodium salt. To this solution, 510 mg of cupric acetate and 5.0g of sodium acetate were added and the solution was refluxed for 16 hours. At this time the reaction was cooled and 5% HCl was added until the solution was pH 1. The white precipitate was filtered off and dried. The E.I. mass spectrum showed the presence of peaks for salicylic acid based on comparison with authentic salicylic acid and o-chlorobenzoic acid. See table

III for a brief list of peaks.

Table III: Important peaks in the E.I. mass spectra for o-chlorobenzoic acid, salicylic acid and the reaction mixture

m/e	% intensity of base peak		
	o-chlorobenzoic acid	salicylic acid	rxn mix
156	67		60
139	100	5	100
138		53	5
120		100	10
111	40		42
92	5	70	13

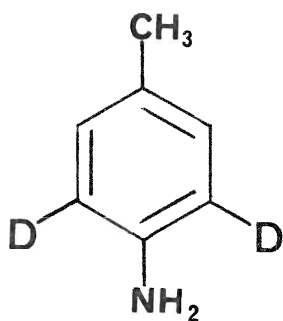
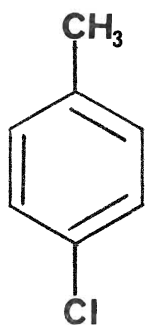
p-Hydroxybenzoic acid

(a) Following the same procedure mentioned above, 2.0 g of the sodium salt of p-chlorobenzoic acid, 5.0 g of sodium acetate and 510 mg of cupric acetate were dissolved in 50 mL of water. The mixture was refluxed for 72 hours at which time a blue green precipitate was observed. The reaction was stopped and worked-up as mentioned above. The white precipitate was obtained as the blue/green precipitate dissolved in the acidic solution. The E.I. mass spectrum was identical to that of starting material. No peaks for p-hydroxybenzoic acid were observed.

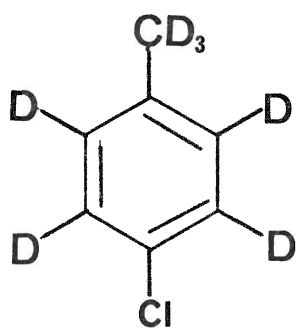
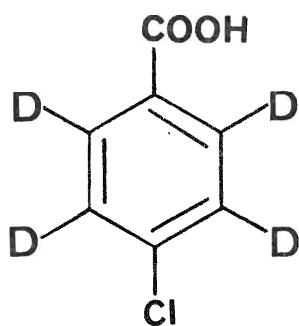
(b) The reaction was repeated but the blue green precipitate was isolated by filtration. After drying, 290 mg of the

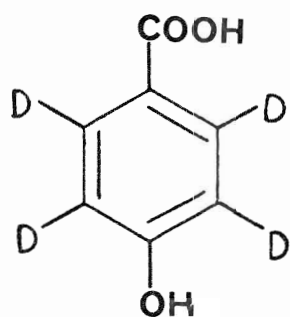
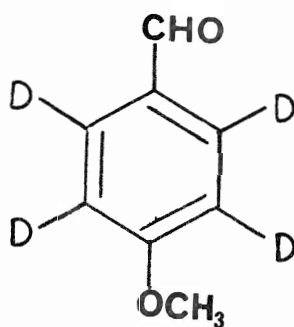
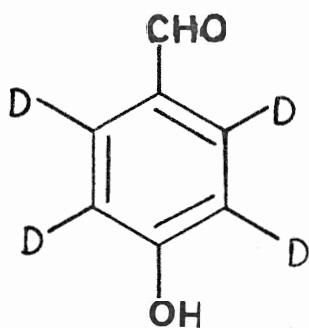
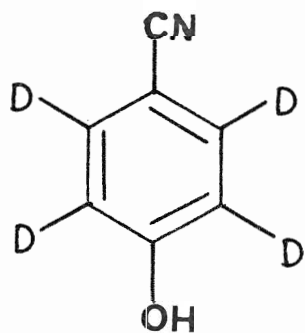
precipitate were obtained. Attempts at obtaining mass spectra failed as the compound would not ionize in E.I. to give a reliable ion current. FAB was attempted with glycerol, glycerol/acetic acid, and nitrobenzyl alcohol matrices. None gave sufficient ion currents for analysis. The I.R. spectrum showed three large peaks at  $1542\text{ cm}^{-1}$ ,  $1587\text{ cm}^{-1}$ , and  $1597\text{ cm}^{-1}$ . The aromatic C-H stretch appeared at  $3603\text{ cm}^{-1}$ .

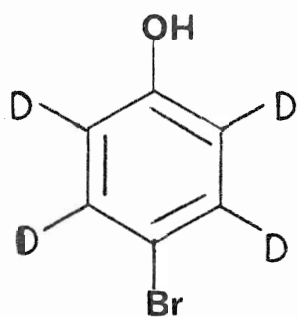
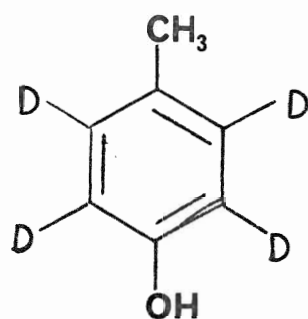
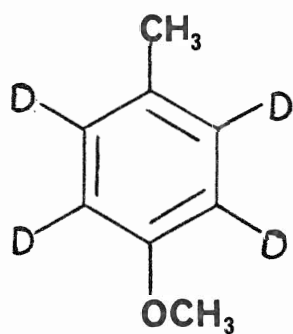
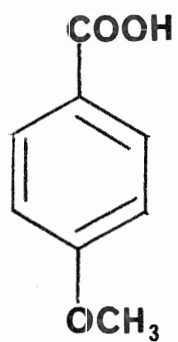


p-Toluidine-3,5-d<sub>2</sub>

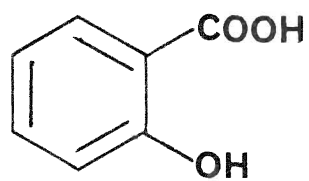
p-Chlorotoluene

p-Chlorotoluene-d<sub>7</sub>p-Chlorobenzoic acid-d<sub>4</sub>

p-hydroxybenzoic acid-d<sub>4</sub>p-anisaldehyde-d<sub>4</sub>p-hydroxybenzaldehyde-d<sub>4</sub>p-hydroxybenzonitrile-d<sub>4</sub>

p-bromophenol-d<sub>4</sub>p-hydroxytoluene-d<sub>4</sub>p-methoxytoluene-d<sub>4</sub>

p-methoxybenzoic acid



salicylic acid

## Microbial Incubations

### Preparation and Maintenance of Cultures

For the dehydroxylation of Pregn-4-en-3,20-dione-21-ol, three bacterial species were needed; Clostridium parapurificum ATCC 25780, Eubacterium lentum 25559 and Escherichia coli . Each species was maintained separately. E . coli was plated out on Nutrient agar (Difco 0001) and stored at 4°C. The culture was grown as an overnight culture at 37°C on a rotary shaker in liquid nutrient broth (5g beef extract, 3 g peptone per Litre of distilled water). E . lentum and C . paraputrificum were both maintained on supplemented Brain Heart Infusion Broth (sBHIB) under an Argon atmosphere, prepared as follows.

The sBHIB was prepared in a 500 mL side arm flask containing 9.25 g of dehydrated BHIB (Difco) and 130 mg of cysteine hydrochloride in 250 mL of distilled water. The flask was lightly stoppered and autoclaved at 15 psi, 121°C for 20 minutes. After autoclaving, the side arm and the top of the flask were fitted with rubber septa (the temperature was not allowed to drop below 95°C while putting on the septa). With the septa in place a continuous stream of argon was passed through the medium through the top septum via a 3 foot long stainless steel tube (18 gauge). When the flask and its

contents cooled to room temperature the tube was removed. The freeze-dried culture obtained from ATCC was rehydrated with purified, sterile water (1 mL) and the suspension was added through the septum with a sterile syringe into the medium. The culture was then placed in an incubator at 35°C. To maintain viability the culture was subcultured every 14 days.

Both C . paraputrificum and E . lentum were initially grown in this way before they were combined to form a mixed culture. After the failure of the mixed culture each species was grown separately. Growth of E . lentum proved difficult so that three more freeze-dried cultures and a live culture were obtained from ATCC. A list of the changes associated with the SBHIB medium preparations or growth conditions, for each of the new culture is shown below.

- 1) The medium was boiled and cooled under argon before autoclaving in an attempt to improve the anaerobic nature of the medium. This did not lead to viability.
- 2) Boiling before autoclaving was again performed but fritted glass tubes were used for passing argon instead of the stainless steel tubes. No viability was observed.
- 3) Two ten mL tubes were fitted with side arms and to them 5 mL of anaerobic medium, prepared as in 2, was added. The rehydrated culture was added 0.5 mL to each tube. No growth was observed after one week.
- 4) A culture revived at ATCC was received as a growing culture

in 6 mL of chopped meat medium (ATCC medium 593). A two mL inoculum was added to 250 ml of sBHIB medium, prepared as in 2. After one day the flask was cloudy with bacteria cells, similar in morphology to those of E . lentum .

#### General procedure for incubation

The growth medium was prepared as described above, but to this medium a 2 mL inoculum of the stock culture (at least 7 day old) of either C . paraputrificum or E . lentum (or both) was added via syringe. Also a 2 mL inoculum of an overnight culture of E . coli was added to the medium. The steroid substrate was dissolved in 1 mL of 95% ethanol and added slowly, with stirring to avoid precipitation, via a syringe. After inoculation of the substrate (60-200 micrograms per mL of medium) the septa were wrapped with parafilm and the flasks placed in the incubator at 35°C. The incubations with C . paraputrificum were found to give off a foul smelling gas, so to avoid the septum being popped off, a sterile syringe was used to relieve the pressure every two days. After the incubation time had elapsed the flask was opened and 200 ml of methanol was added. The flask was allowed to stand undisturbed for 4 hours while the bacterial cell components settled out. After this the

medium was filtered by gravity to remove these components. The filtered precipitate was discarded and the filtrate was placed on the rotary evaporator to remove the methanol. After this the aqueous solution was extracted with dichloromethane (3\*50mL). The extract was filtered through cellulose and dried over anhydrous sodium sulphate. The crude residues were analysed and purified by chromatography as required.

#### Incubations with Substrates

Pregn-4-en-3,20-dione-21-ol      3

To a mixed culture of E . coli and C . paraputrificum, 25 mg of 3 was added and the incubation carried out for 144 hours. This afforded 40.1 mg of a medium extract which was shown to contain 5 $\beta$ -Pregnan-~~3 $\alpha$~~ ,21-diol-20-one by TLC with no spot corresponding to starting material. Preparative TLC in 1:1 hexane:ethyl acetate gave 10.2 mg of product which had an <sup>1</sup>H nmr identical to that of authentic 1 .

Pregn-4-en-3,20-dione-21-ol -21-d<sub>2</sub>-17 -d<sub>1</sub>; 3 -d<sub>3</sub>

To a mixed culture of C . paraputrificum and E . coli, 25 mg of the title compound was added and incubated for 144



hours. After extraction of the medium a 37 mg residue was obtained that was shown to contain the reduced product 1 by TLC. Preparative TLC gave 7.5 mg of product which had a signal in the deuterium nmr for the C-21 methylene. The  $^1\text{H}$  nmr showed a peak in the region of the C-21 methylene at delta 4.1 ppm. Integration relative to the C-3 hydrogen showed only 26% hydrogen was present at the C-21 position.

Pregn-4-en-3,20-dione-21-ol 3

To a mixed culture of C . parapatrificum , E . lentum and E . coli , 30 mg of the starting material was added. The incubation proceeded for 168 hours at which time the medium was extracted giving 53. mg of extract. Analysis of the extract by TLC showed the presence of the intermediate 1 . There was no spot for starting material 3 or the anticipated product 2 .

5 $\beta$ -Pregnan-3 $\alpha$ ,21-diol-20-one 1

To a mixed culture of E . lentum and E . coli , 25 mg of the substrate was added and incubated for 168 hours. Much of the substrate precipitated out during the addition. Precipitate was also present after the incubation time had elapsed. After extraction the 36.8 mg residue was found to contain starting material by comparative TLC. No trace of the anticipated

product 2 was observed. Other attempts were made that involved decreasing the substrate concentration and the extension of the incubation time. None of these changes was seen to cause the desired transformation.

Pregn-4-en-3,20-dione-21-ol -21(R)-d<sub>1</sub> ; 17

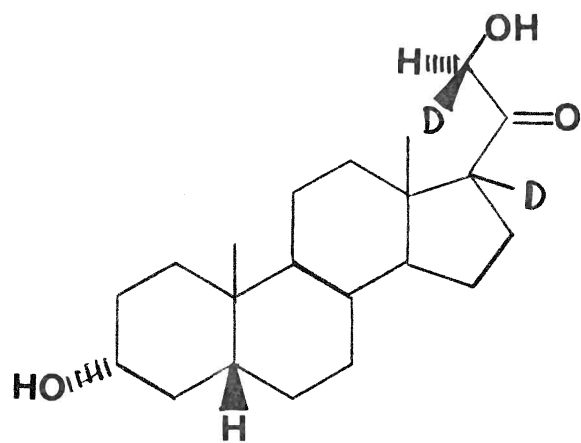
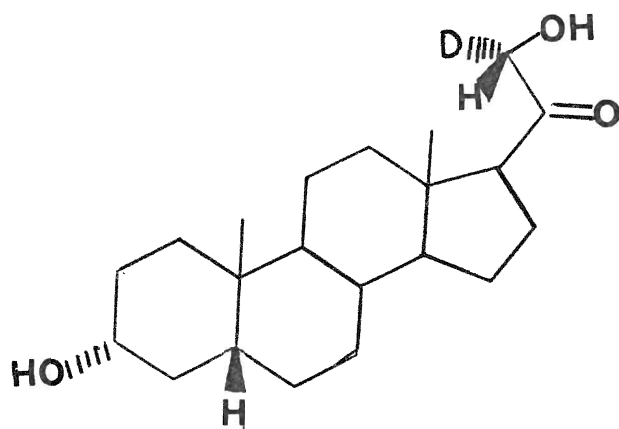
A mixed culture of C . paraputrificum and E . coli was prepared in two separate flasks from a medium prepared by bubbling nitrogen through the medium. Incubations with both 17a and 17b gave no reduced product. Repetition using medium prepared with argon also gave only starting material. A new culture was obtained and was used on the 19.1 mg of 17b only, since very little of 17a (12.0 mg) was left after the reisolations. The incubation of 17b with the new culture generated 4.0 mg of 20 , which was isolated by preparative TLC.

Pregn-4-en-3,20-dione-21-ol -21(S)-d<sub>1</sub> ; 18

With the same problems mentioned above the substrate 18 (26.0 mg) was incubated with the active culture of C . paraputrificum which gave, after column chromatography, 6.0 mg of 21 .

5 $\beta$ -Pregnan-3 $\alpha$ ,21-diol-20-ones 1 , 20 and 21

The stock culture of E . lentum that had been induced from growth in the presence of 1 was used to inoculate the medium containing 10 mg of 1 (200 mL). After 160 hours the medium was worked up as described previously. The 42 mg medium extract was found to contain no starting material 1 by comparative TLC. Since the medium showed the presence of 2 , by TLC, the medium was used to inoculate two other flasks containing 150 mL of medium, for use with the labelled substrates 20 and 21 . After 192 hours the flasks were worked-up and analysed separately by comparative TLC. In both cases there was no sign of starting material, but only a spot corresponding to the product 2 . Both products 22 and 23 were isolated by preparative TLC, which gave approximately 200 micrograms of 22 and 300 micrograms of 23 . The EI mass spectra and the high resolution <sup>1</sup>H nmr at 500 MHz were obtained for both compounds, which also showed the presence of a non-polar, high molecular weight impurity. The values obtained from the spectra will be shown in the Results and Discussion section.

20

21

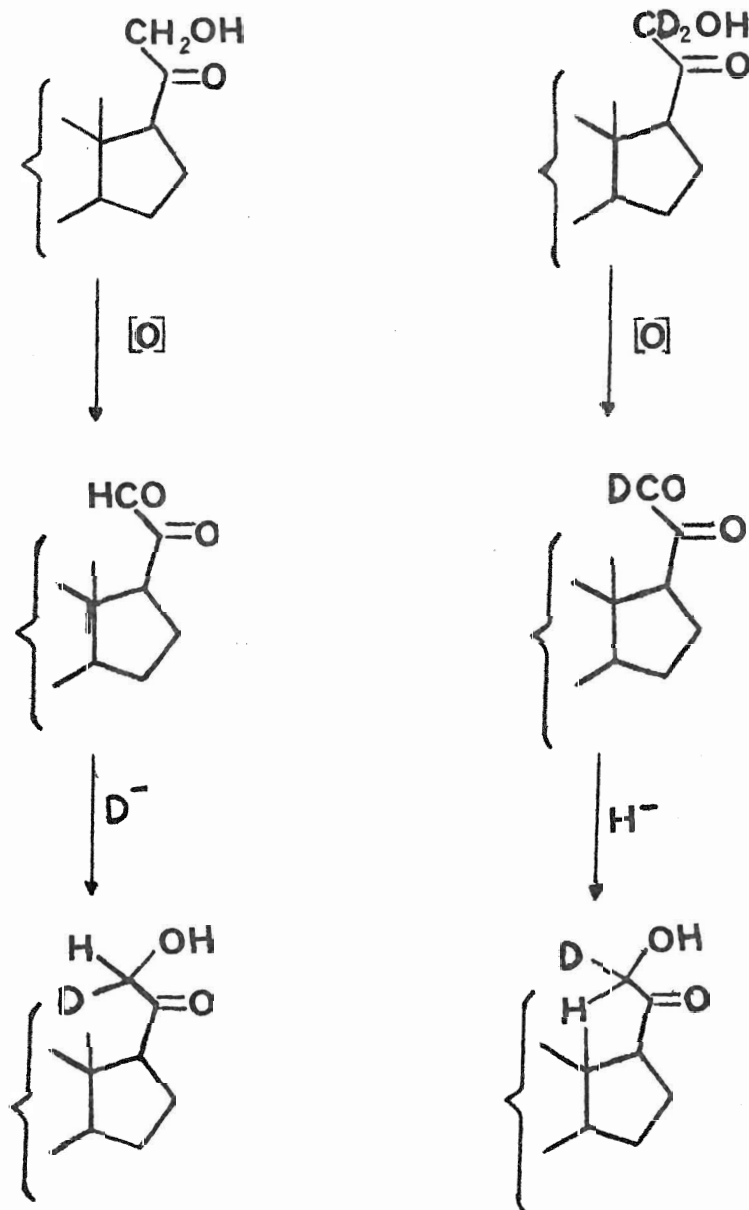
## RESULTS AND DISCUSSION

### The 21-Dehydroxylylation of Corticosteroids

To generate the singly labelled hydroxy methyl groups at C-21 of 1 , a scheme was devised that could give both isomers (Fig. 29). The reduction of a C-21 aldehyde by either enzymic or chemical means would generate a C-21-d<sub>1</sub> if the reduction was performed using deuteride on an unlabelled aldehyde or hydride on a labelled aldehyde. It was known that an enzyme existed that could do such a reduction on C-21 aldehydes, giving an alcohol in a highly stereoselective manner. Since the stereoselectivity of the enzyme is known, it was possible to generate a chiral methylene of known stereochemistry. The enzyme is found in the liver and kidneys of most mammals, with the highest concentration in the livers of sheep and cows. Since beef liver was readily available and the enzyme is known to be reasonably stabile, liver from a commercial source was obtained.

To check for 21-hydroxysteroid dehydrogenase activity the substrate with the highest known activity was prepared. The C-21 aldehyde of corticosterone 13 was obtained by the copper acetate catalysed air oxidation of 14 , a reaction which is specific for alpha-hydroxy carbonyls. The reduction could be

Figure 29: Schemes for the generation of both configurations of C-21-d<sub>1</sub> of 3



easily followed by nmr due to the appearance of the characteristic AB pattern of the C-21 methylene of 14 which was in a region of the spectrum where few interferences were present. The presence of the AB doublets between delta 4.2 and 4.7 ppm as well as the comparative TLC evidence could be taken as evidence for the activity of the dehydrogenase. Sufficient activity of the enzyme was obtained from commercial beef liver only after the enzyme was purified following a procedure of Monder and White.<sup>53</sup> The reason that this purification procedure was required in order to increase enzyme activity is thought to be due to either the large pieces of acetone powder absorbing the substrate preventing it from getting to the enzyme in the time frame of the experiment, or the enzyme not being extracted out of the powder in the time frame of the experiment. Extraction of the enzyme and precipitation and denaturation of other protein components made for a cleaner and more reliable enzyme preparation.

Having prepared an active enzyme preparation, it was necessary to test its effectiveness towards the required substrate. Following the same oxidation procedure 1 was converted to 15 and the reduction attempted. Without the apparent reduction of 15 back to 1, it was assumed that the enzyme would not work on this substrate. A possible explanation for this result is the need for a delta-4, 3-ketone of the A-ring in the substrate. This requirement is seen in another

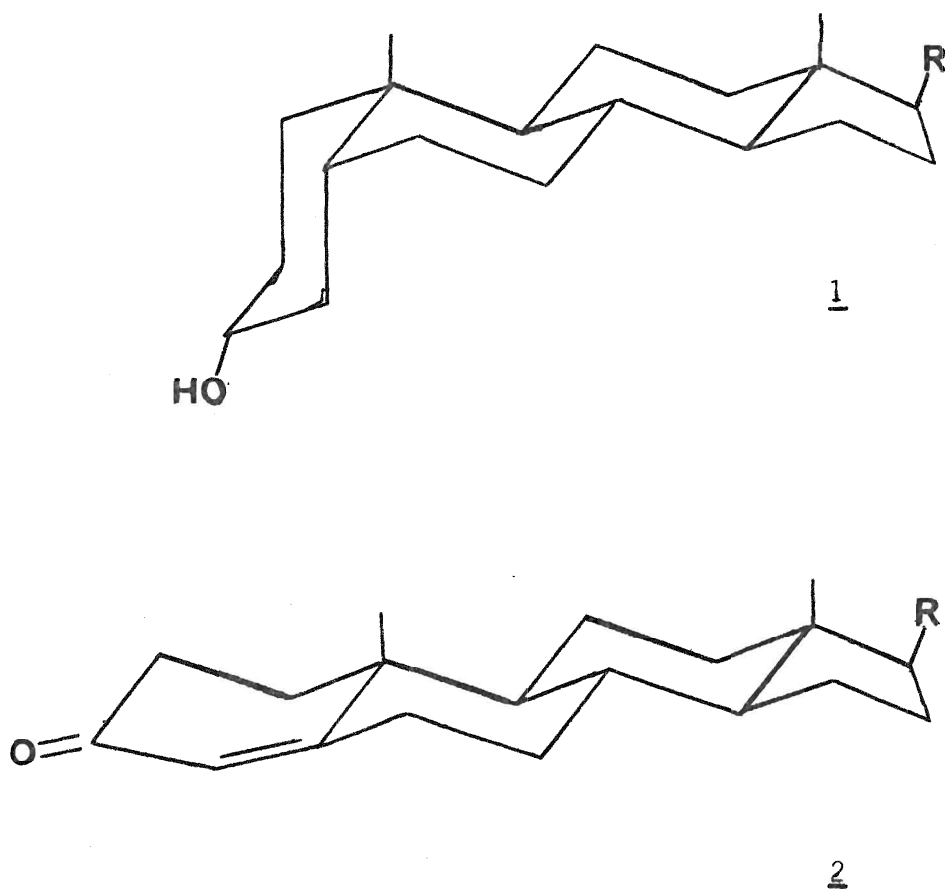
steroid side chain dehydrogenase, that of 20-keto dehydrogenase.<sup>5</sup> Since no examples of reduction of C-21 with substrates having anything but the unsaturated A-ring system have been found in the literature and with the apparent inability of the enzyme to perform it in this case, it is reasonable to assume that 15 is not a substrate for the enzyme. Looking at the shapes of the two types of molecules, with respect to their A-rings it is not surprising that there may be some differences in binding (Fig. 30).

To get around this problem it was necessary to impose one more step on the preparation of the C-21-d<sub>1</sub> compounds by starting with the unsaturated compound 3. This can be converted to 1 by the reductive capabilities of another obligate anaerobe found in the gastrointestinal tract, Clostridium paraputrificum.<sup>5</sup>

The aldehyde 16 was prepared following the same procedure for 13, but in this case the product seemed unstable and the attempts at purification were unsuccessful. The apparent instability was observed as a rapid loss of the alpha-dicarbonyl yellow colour being replaced by a white material. The white material obtained was identified as the C-20 acid compound 24 based on its <sup>13</sup>C nmr spectrum and its mass spectrum. In the <sup>13</sup>C nmr two changes were apparent from a comparison of the <sup>13</sup>C nmr of 3 (Table 1). The peak for the C-20 ketone at 210 ppm and the C-21 hydroxymethylene at 69.3 ppm were both absent from the



Figure 30: The stereochemical view of the steroid nucleus for both 1 and 2

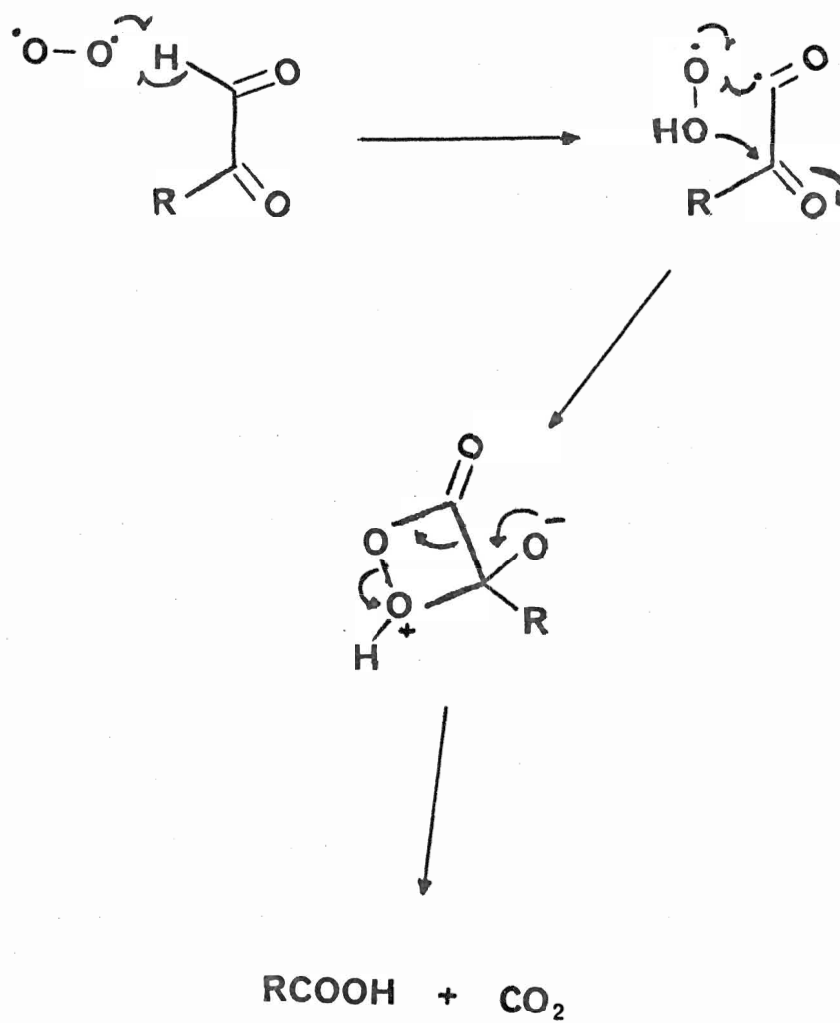


spectrum of 24 . In this spectrum a peak was seen at 178.8 ppm, which is the expected chemical shift of an acid group. Also the mass spectrum showed a molecular ion of 316 which would be the molecular weight of 24 . A probable mechanism for the formation of 24 from the intermediate 16 is shown in Figure 31, which is in line with an old observation that alpha-keto aldehydes can decompose in the presence of molecular oxygen to give CO<sub>2</sub> and a carboxylic acid.<sup>67</sup>

With the realization that the product 16 was being lost as the acid 24 , it was decided that the product would be isolated from the reaction mixture as soon as the conversion from 3 was complete. The product was quickly extracted and stored under argon at -20°C. Use of this compound with the enzyme extract containing 21-hydroxysteroid dehydrogenase showed the anticipated conversion back to 3 . The reaction was conveniently monitored by the transition from a turbid solution, present when the substrate was dissolved in the medium, to a nearly clear solution as the aldehyde was converted to the alcohol. This result made it possible to proceed with the preparation of the labelled compounds, however, two more substrates were required before this could be performed.

The first was the aldehyde 16 with deuterium present at the C-21 position and the second was a nicotinamide cofactor with a deuterium at the C-4 position on the B-face of the

Figure 31: Possible mechanism for the oxidation of an alpha-keto aldehyde generating an acid and  $\text{CO}_2$



nicotinamide ring to insure that deuterium was delivered to the unlabelled aldehyde 16 . With both of these substrates it could have been possible to generate both isomers of C-21-d<sub>1</sub> by using 21-hydroxysteroid dehydrogenase.<sup>56</sup>

The first substrate requirement was met by the preparation of 3 -d<sub>3</sub> and its oxidation to the aldehyde 16 -d<sub>2</sub>. The alcohol 3 -d<sub>3</sub> was prepared from the deuterium exchange of unlabelled 3 in the presence of deuterium oxide and methanol-d<sub>1</sub>. The use of tris(hydroxymethyl)methylamine (TRIS) generated a sufficiently basic environment (pH 8.5) to allow for the reversible enolate exchange of the acidic hydrogens. To effect the exchange of the C-21 hydrogens it was only necessary to use a weakly basic environment. The acidity of these hydrogens is based on the fact that they are not only alpha to a carbonyl, giving resonance stabilization of the enolate anion, but also the hydroxyl group at C-21 may give inductive stabilization to the anion. The C-17 hydrogen is also known to exchange under these conditions but not at the same rate since methine hydrogens are less acidic than methylene hydrogens and because the C-17 does not have the added inductive effect of a hydroxyl.

Unlike the substrate 1 , which contains only C-17 and C-21 as alpha positions to a carbonyl, the substrate 3 also contains enolizable hydrogens at the C-2 methylene, C-4 methine and the C-6 methylene, due to the C-3 keto group. While the

exchange reaction for 1 only shows exchange at C-21 and C-17, after the first reaction some residual hydrogen is seen in the  $^1\text{H}$  nmr for the C-21 methylene. To obtain 90-95% incorporation at C-21 it is necessary to perform the reaction again on the same sample. For 1 this is not a problem because only two positions in the molecule are open for exchange. If 3 is used, which has more than these two positions, exchange in the steroid nucleus becomes apparent. In the deuterium nmr a peak at 5.7 ppm, for the C-4 hydrogen, becomes visible while the peak at 2.4 ppm, which is for the C-17 hydrogen in 1 becomes broader and larger relative to the C-21 signal as the C-2 and C-6 hydrogens exchange. Since all of these peaks increase with continued reaction under the exchange conditions, a compromise had to be reached to minimise the amount of deuterium in the molecule outside of the C-21 position. This is the reason the reaction was not carried fully through the second 24 hours. The result obtained was only 80% incorporation at the C-21 position but minimal incorporation at C-4. Unfortunately the deuterium signals in the nmr at 30.7 MHz are not resolved well enough to distinguish between a peak at 2.2 ppm and a peak at 2.4 ppm; therefore it can not be known, from the deuterium nmr, how much of the nucleus contains deuterium. The FAB mass spectrum of the compound shows a complex molecular ion cluster of about 6 peaks. The cluster was unlike the cluster for unlabelled 3 (Table II), in that the cluster was like a 1:2:4:4:2:1 sextet.

Attempts at deconvoluting the cluster by hand were unsuccessful, but it was possible to make some assumptions and use the BMASABD program on the Kratos computer. The values obtained for the compounds 3 -d<sub>3</sub>, 17 & 18 are shown in Table IV. The assumptions made for the analysis are that the molecular ion cluster for 3, which contains M-1 and M-2 peaks of certain intensity, will be consistent despite the presence of deuterium in the molecule. This means, that the losses of hydrogen seen in the FAB mass spectrum of unlabelled material will be the same for labelled material and will have the same relative intensity for each labelled species.

Table IV: The summary of deuterium composition for 3 -d<sub>3</sub>, 17a, 17b, 18

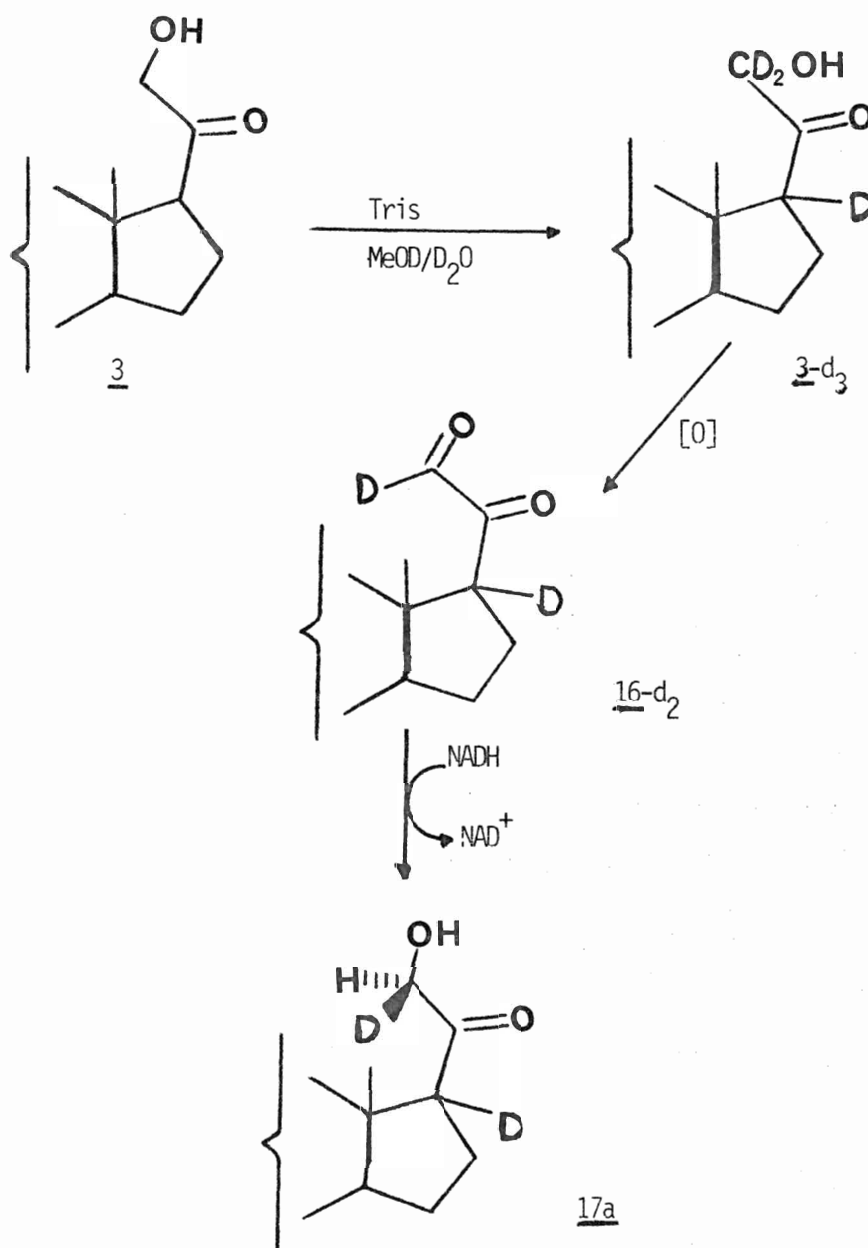
deuterium content	compound			
	<u>3</u> -d <sub>3</sub>	<u>17a</u>	<u>17b</u> (%)	<u>18</u>
d <sub>0</sub>	3	5	0	11
d <sub>1</sub>	15	22	18	89
d <sub>2</sub>	36	42	45	0
d <sub>3</sub>	30	34	38	0
d <sub>4</sub>	15	0	0	0

As can be seen from Table IV, compounds 3 -d<sub>3</sub>, 17a and 17b show a large proportion of deuterium in other parts of the molecule outside of C-21. This would cause interferences in the final analysis of the incubation of 22, since the loss of deuterium at C-21 might be masked by a potential

loss of deuterium at other positions. Use of  $^1\text{H}$  nmr in isolating the C-21 methyl signal proved more effective in the analysis of the product of the final incubation, since it does not involve the deuterium of the other positions.

Having obtained the deuterium labelled alcohol 3, it was possible to convert the alcohol to the deuterium labelled aldehyde and then to reduce the aldehyde to the singly C-21- $\text{d}_1$  species by reduction using the enzyme 21-hydroxysteroid dehydrogenase or sodium borohydride (Fig. 32).

The first conversion of 3 - $\text{d}_3$  to 16 - $\text{d}_2$  was followed by  $^2\text{H}$  nmr, analysis of which showed the loss of 95% of the C-21 hydroxy methylene signal at 4.1 ppm and the appearance of the C-21 gem-diol at 4.7 ppm. The other signals at 5.7 ppm and 2.4 ppm were also present. The reduction of 16 - $\text{d}_2$  by the enzyme was seen by the loss of the  $^1\text{H}$  signal at 4.7 ppm and the rise of the C-21 methylene signal generating 17a. The nmr showed that the C-21 methylene was in fact oxidized and reduced back to the C-21 hydroxyl. It also showed that some deuterium was lost in the process of reduction at the A-ring sites, since the 5.7 ppm signal was no longer present. It is possible that the bonding at the substrate involves the  $\Delta^4$ , 3-keto function allowing for a reversible enolate formation. The mass spectral analysis showed the molecule to contain no  $\text{d}_4$  species, which was the minimum for the labelled alcohol 3 - $\text{d}_3$ , which suggests that a replacement of one of the C-21 hydrogens had occurred.

Figure 32: Scheme for the preparation of 17a starting from 3



The  $^1\text{H}$  nmr for 17a showed a single peak in the region of the spectrum corresponding to the AB pattern of unlabelled 3. By comparison of the two spectra (Fig. 33, Fig. 34), the singlet was found at the chemical shift of the right half of the AB pattern.

This chemical shift was determined from equations which established the unlabelled pattern as an AB pattern<sup>68</sup> and determined the chemical shifts of the uncoupled hydrogens.<sup>69</sup> See Figure 35 for definitions of the parameters.

$$\delta_A - \delta_B = \sqrt{(v_4 - v_1)(v_3 - v_2)} \quad (2)$$

$$I_1 / I_2 = (2C+J)/(2C-J) \quad (1)$$

By measuring the observed chemical shifts of the peaks in the spectrum the relative intensities for outside and inside peaks can be calculated. Agreement between the calculated peak ratios and the observed ratios is sufficient to establish a pattern as an AB pattern. Since the chemical shift difference between AB is very small it is expected that the AB pattern should generate a complex spectrum at 200 MHz, with the outside peaks smaller than the inside peaks. Calculation using equation 1 shows that the ratio inside/outside should be approximately 20:1. The actual value was not easily determined since the

Figure 33: The AB pattern for 3 at 4.1 ppm with coupling to the C-21 hydroxyl

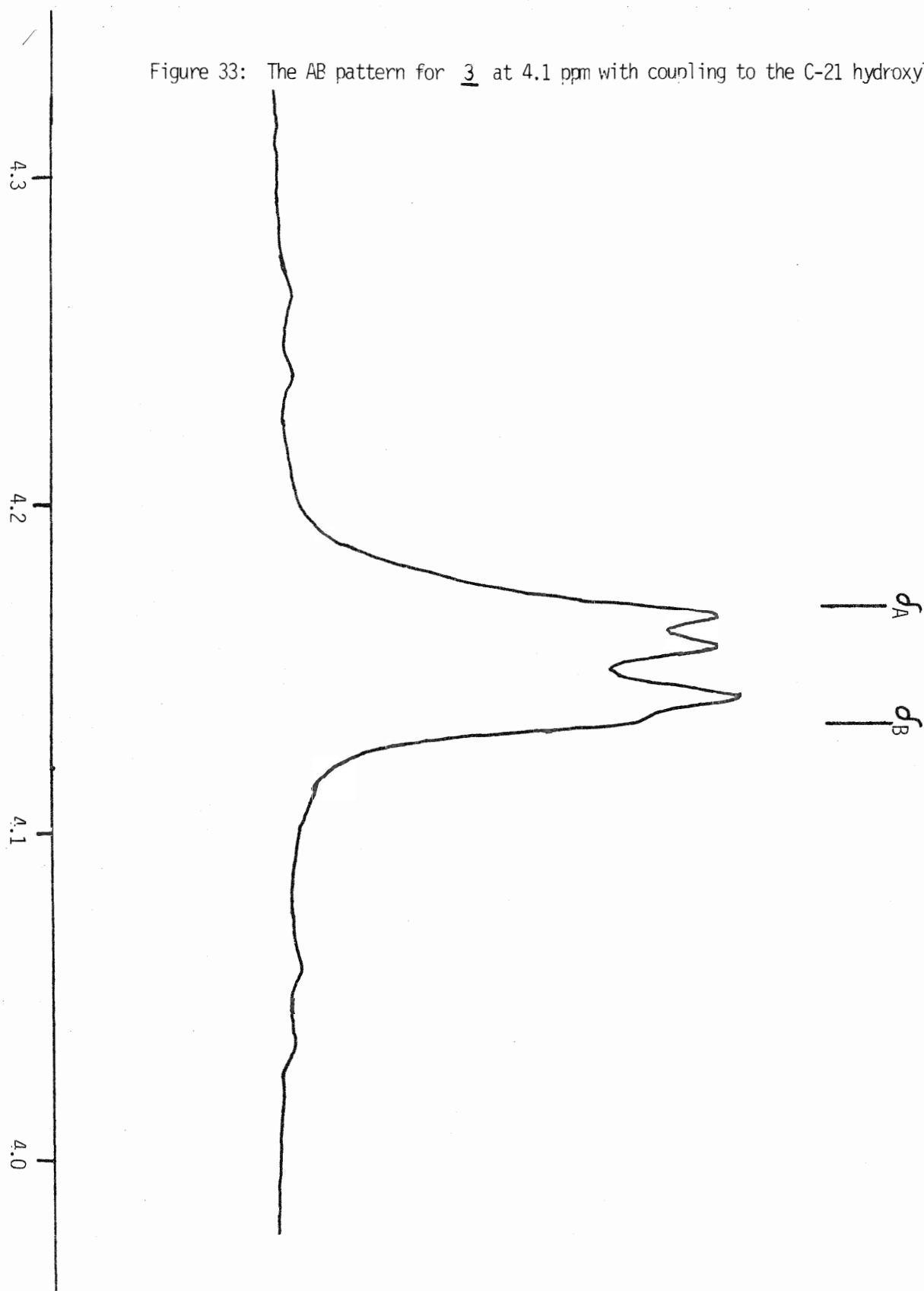


Figure 34: The high resolution nmr at 500 MHz for the AB region of 17a

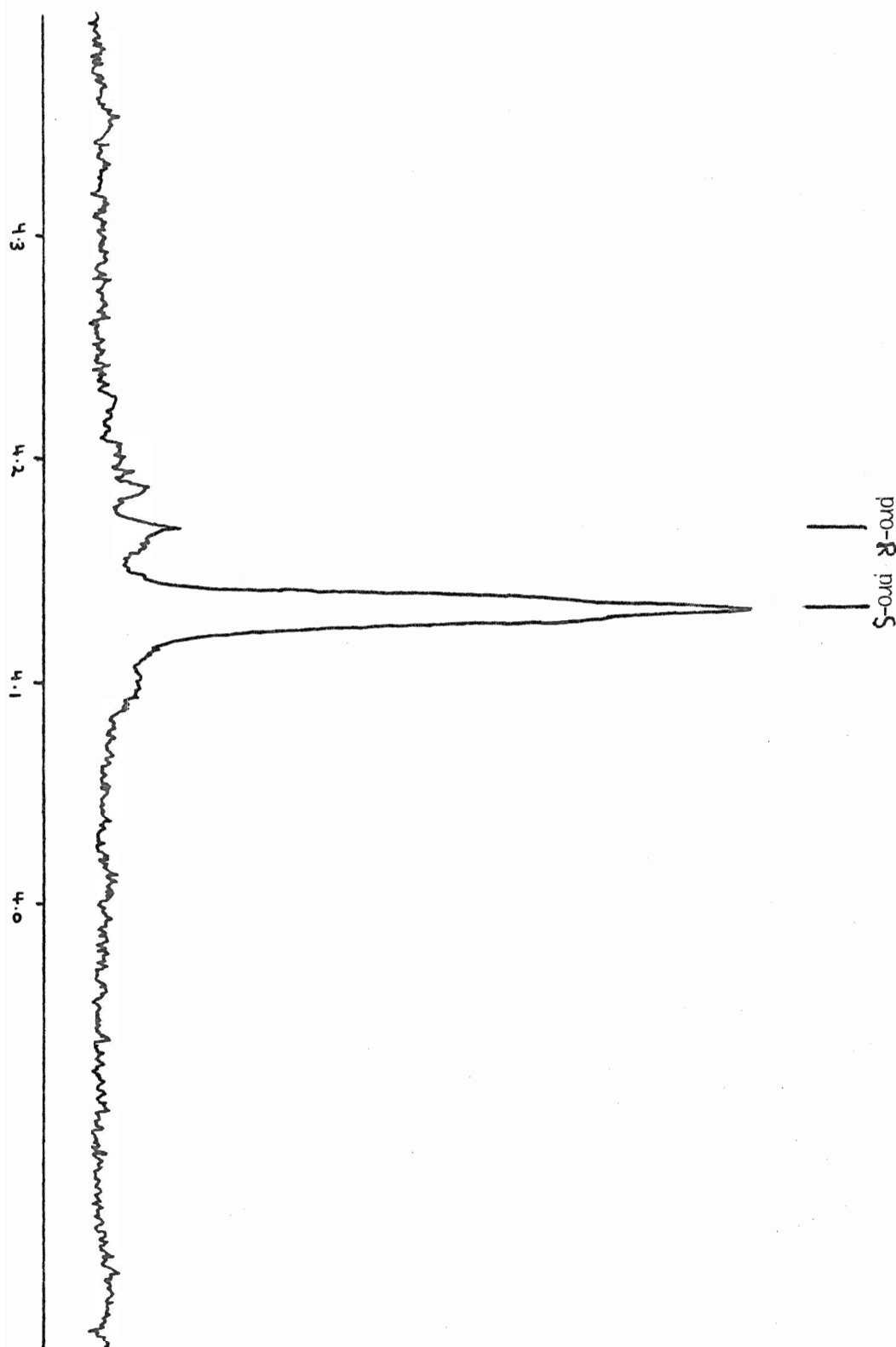
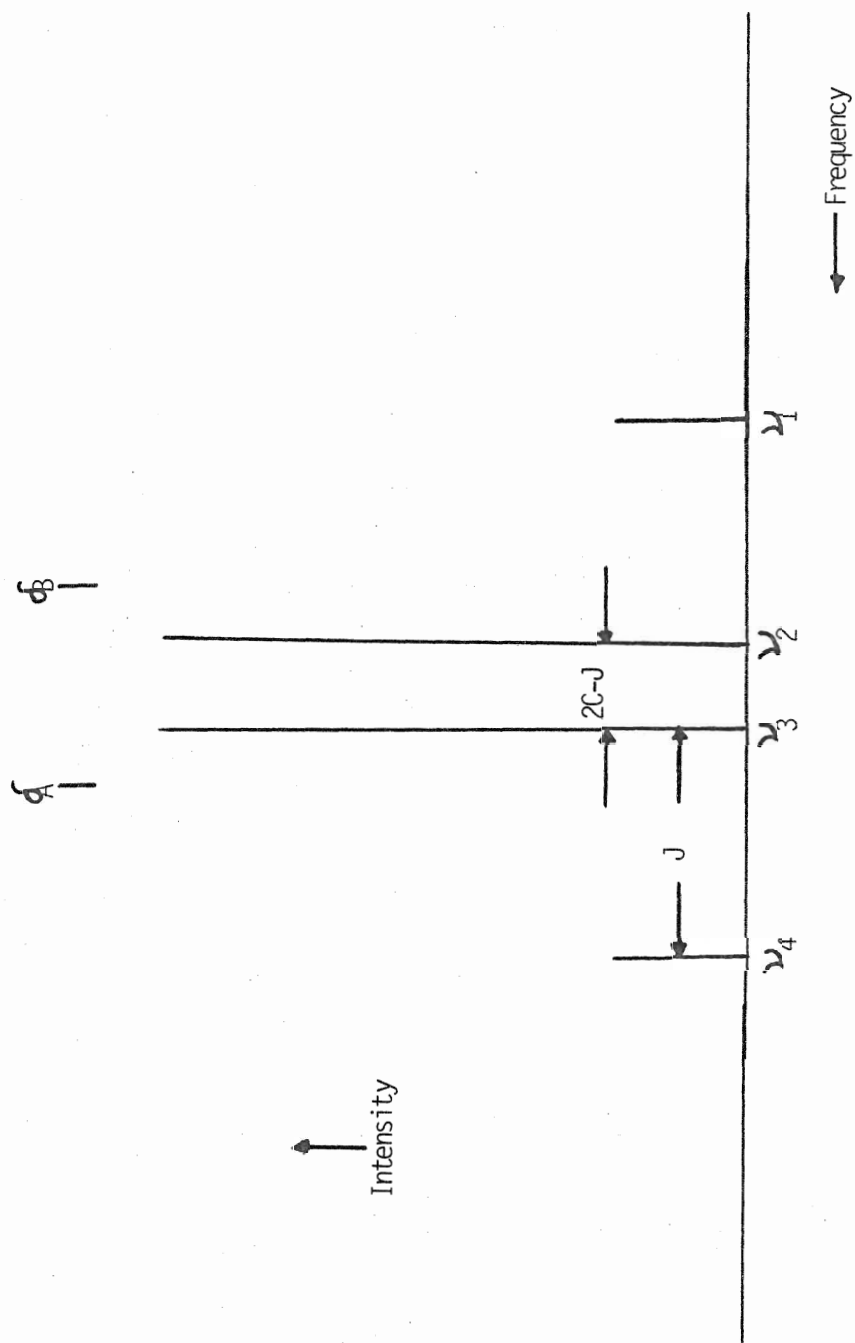


Figure 35: A schematic representation for a theoretical complex AB pattern with the parameters for equations 1 and 2



inside peaks were so close and the outside peaks were so small. However from a qualitative standpoint, the pattern does seem to resemble the theoretical AB pattern, if the coupling to the hydroxyl is removed. From this spectrum it is possible to establish the chemical shift of the A proton and the B proton for the methylene. Using equation 2 it is possible to establish the chemical shift based on the chemical shifts of the two outside peaks and inside peaks.

The chemical shifts determined are:

$$H_A = 4.167 \text{ ppm}$$

$$H_B = 4.124 \text{ ppm}$$

The difference of 8.5 Hz is observable in the 200 MHz spectrum of 3 but not refined well enough to establish the ratio of  $H_A/H_B$  in the cases of the singly deuterated substrates 17a , 17b , and 18

The enzymically prepared compound 17a showed only one peak at 4.130 ppm (Fig. 34), which is close enough to the  $H_B$  shift. The substrate 17b , prepared chemically, showed 2 peaks (Fig. 36), one at 4.130 and 4.167 ppm in a 2:1 ratio. The singly deuterated compound 18 also showed two peaks (Fig. 37), one at 4.130 and the other at 4.167 in a 1:3.2 ratio.

Since the enzyme used in compound 17a is known to reduce at the pro-S position the centre generated by the

Figure 36: The high resolution nmr at 500 MHz for 17b in the region of the C-21 methylene

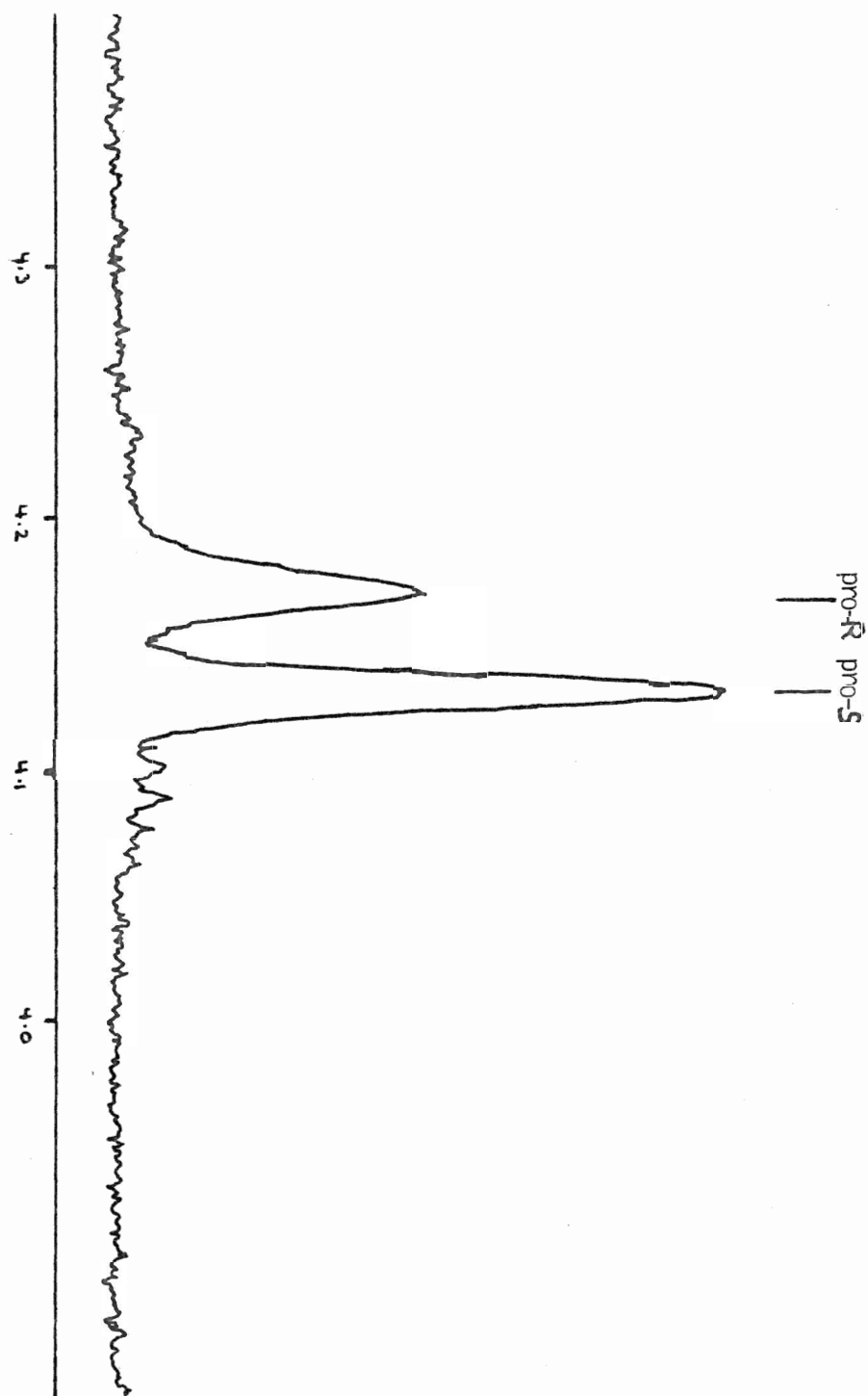
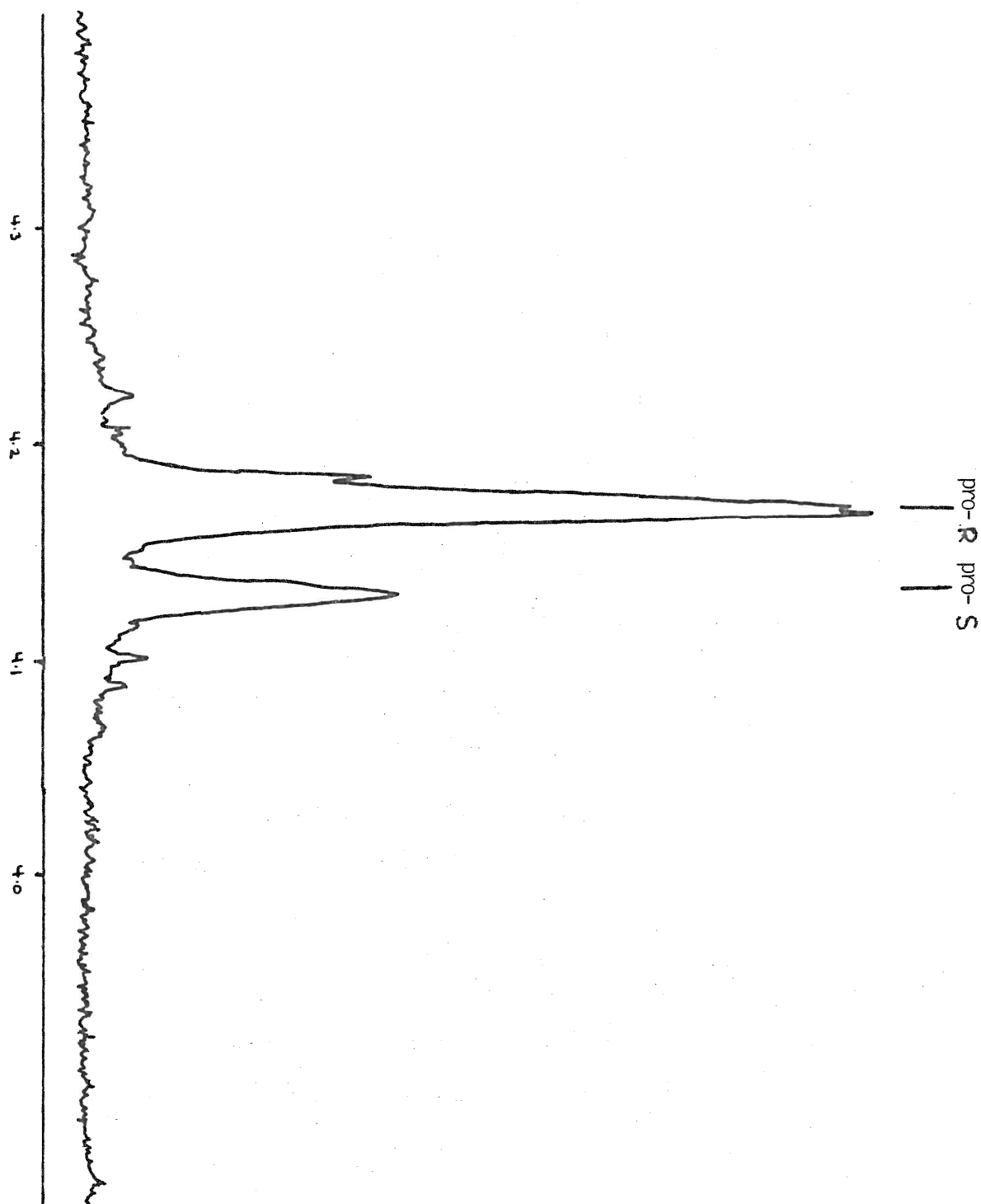


Figure 37: The high resolution nmr at 500 MHz for 18 in the region of the C-21 methylene



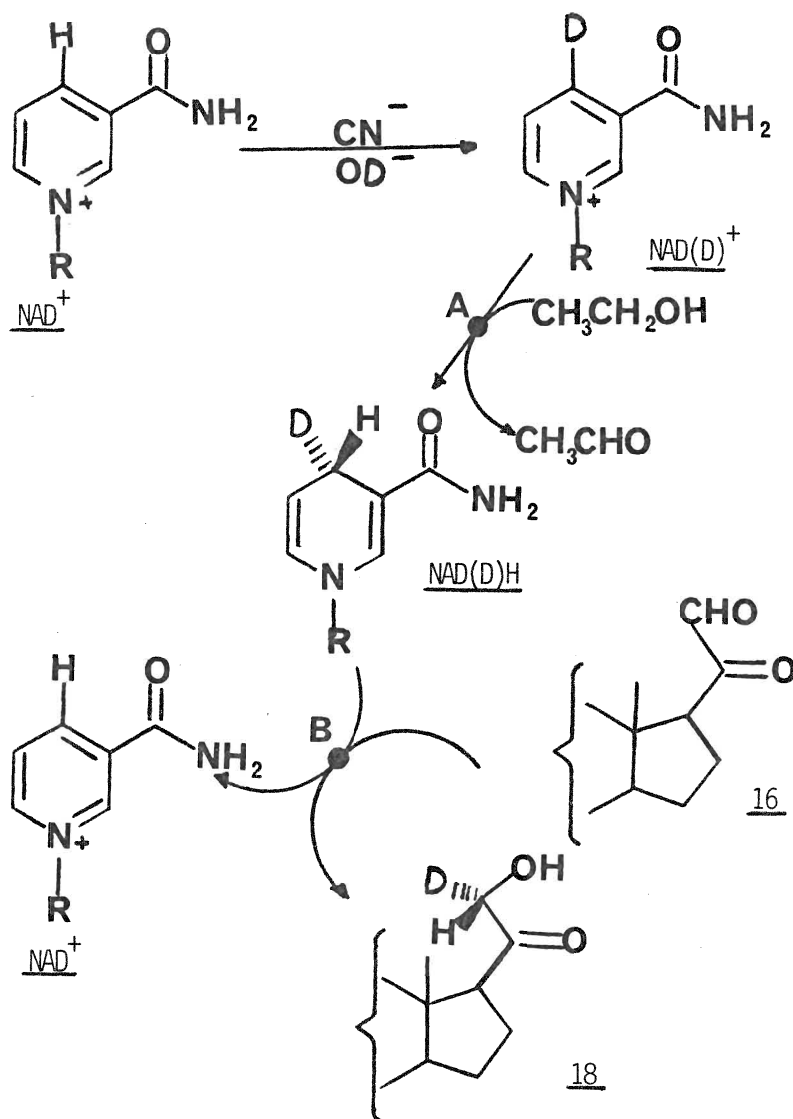
reduction is defined as an R configuration. This gives a comparison to the other compounds since it can be used as a reference. The  $H_B$  is then defined as the pro-R hydrogen and  $H_A$  as the pro-S hydrogen. From this it can be shown that compound 17a is a nearly pure isomer of C-21-d<sub>1</sub>. The compound 17b is a mixture of C-21 R and S isomers in a ratio of 2:1 and compound 18 is also a mixture of C-21 R and S in a ratio of 1:3.2.

Attempts at preparing the pure S isomer from the enzymic reduction of the undeuterated aldehyde 16 were difficult using the in situ NADH coupling procedure (Fig. 38). The problem that arises in this procedure is that during the reduction of  $NAD^+$  to NADH the enzyme ethanol dehydrogenase requires an environment of pH 8.8-9.0. This environment is a good environment for the enolate mediated exchange of C-21 methylene hydrogens as in the case of the TRIS buffer. Once the deuterium from the  $NAD(D)^+$  had been transferred to the C-21 position of the aldehyde 16 to generate the C-21-S-d<sub>1</sub> compound, the basic environment could have been sufficient to exchange the hydrogen at the C-21 position.

To check this possibility, the medium was made pH 7.4, after sufficient time to generate the reduced nicotinamide. Under these conditions it should not have been possible to exchange even the very acidic C-21 methylene hydrogens. Again,



Figure 38: The scheme for the in situ coupling of deuterium from  $\text{NAD}^+$  to 18

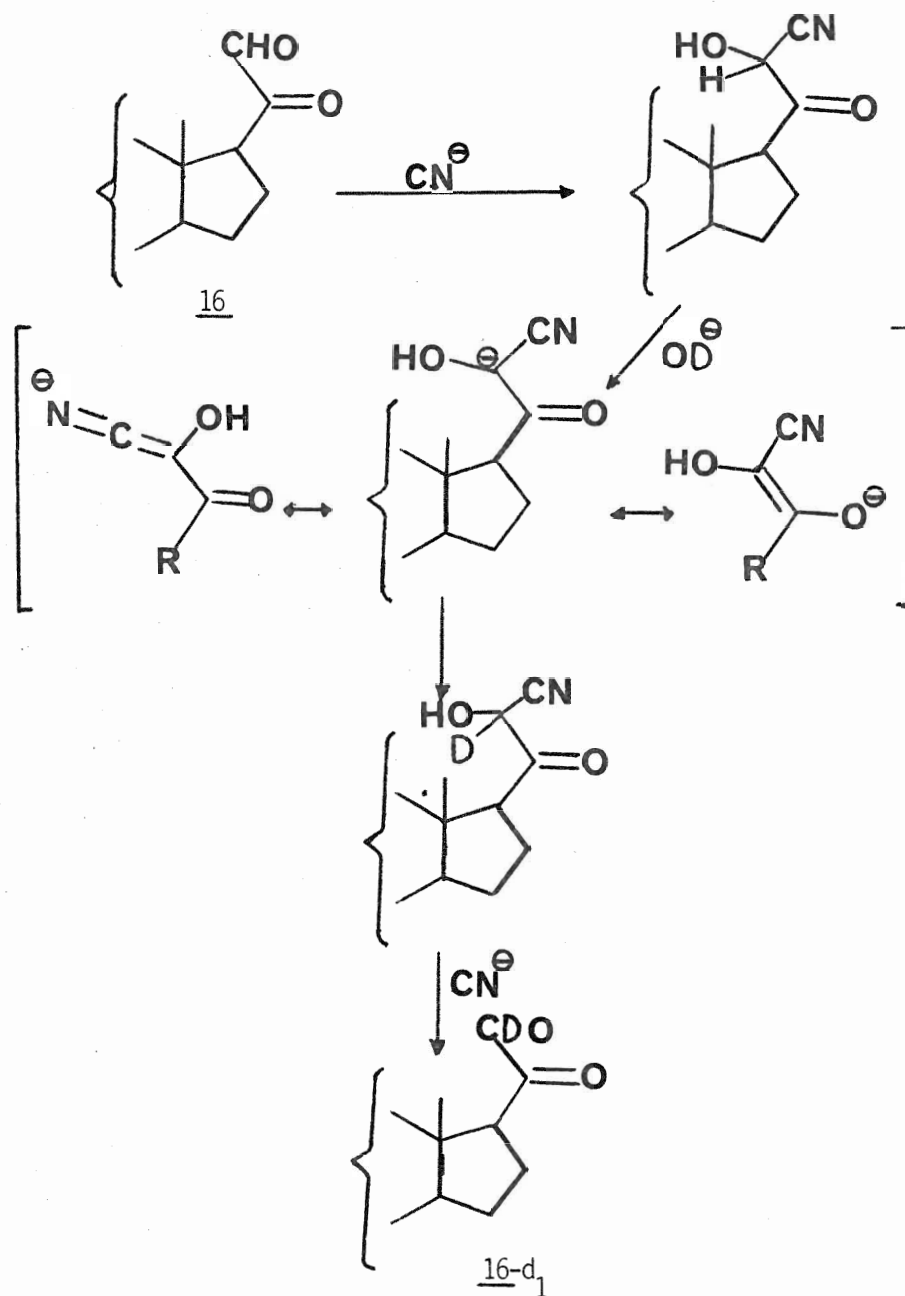


A) Yeast alcohol dehydrogenase    B) 21-hydroxysteroid dehydrogenase

however, intensity decrease of any proton signal at the chemical shift for the C-21 position in the  $^1\text{H}$  nmr was indicative of some sort of exchange reaction. If the reaction was that of exchange after the reduction we would not have expected the exchange to occur at pH 7.4. Also, by looking at the remaining C-21 methylene signal ( $^1\text{H}$  nmr) of a product of reversible exchange,  $\underline{3}$ - $\text{d}_3$ , it was apparent that the exchange was not equal for both C-21 hydrogens. While the 200 MHz nmr was used, which can not resolve the two AB signals to the baseline, it did compare well with the C-21 methylene signal for 18 (Appendix I). So it appeared that of the 20 % remaining hydrogen of C-21 most was in the pro-R position. This suggests that there is enough of a stereochemical difference between the pro-R and S hydrogens to allow for an observable difference in enolization rate. If this is true then the pro-R position is the slower of the two. Since the product of enzyme reduction would generate a configuration with the hydrogen in this less reactive position, it is unlikely that it would exchange so rapidly in the time frame of the reduction reaction. Therefore some other process must be occurring.

Since it is possible that cyanide may still be present in a catalytic amount, even after the treatment with the phosphate and argon, that it might react with the very reactive C-21 carbonyl of 16 to generate a cyanohydrin (Fig. 39). This would convert the aldehyde hydrogen into an alpha-hydrogen in

Figure 39: Possible explanation for the formation of C-21-d<sub>1</sub> of 16 by the cyanide catalysed exchange of the aldehyde hydrogen



the same way that cyanide catalyses the benzoin condensation. In this case the anion generated by deprotonation would be very stable due to the C-20 ketone as well as the C-21 nitrile group. It is possible that at pH 7.5 the reaction might still proceed, given enough reaction time. The resulting equilibrium reaction could put a deuterium in the C-21 position and after an enzymic reduction would give C-21-d<sub>2</sub>. Also the low yield of the reaction might be due to side reactions not unlike the benzoin condensation in which the enolate acts as a nucleophile. Without more time to investigate these possibilities this reaction was no longer looked at. Since other substrates were already prepared that showed opposite configurations at C-21, 17 and 18 it was decided to move on to the incubations.

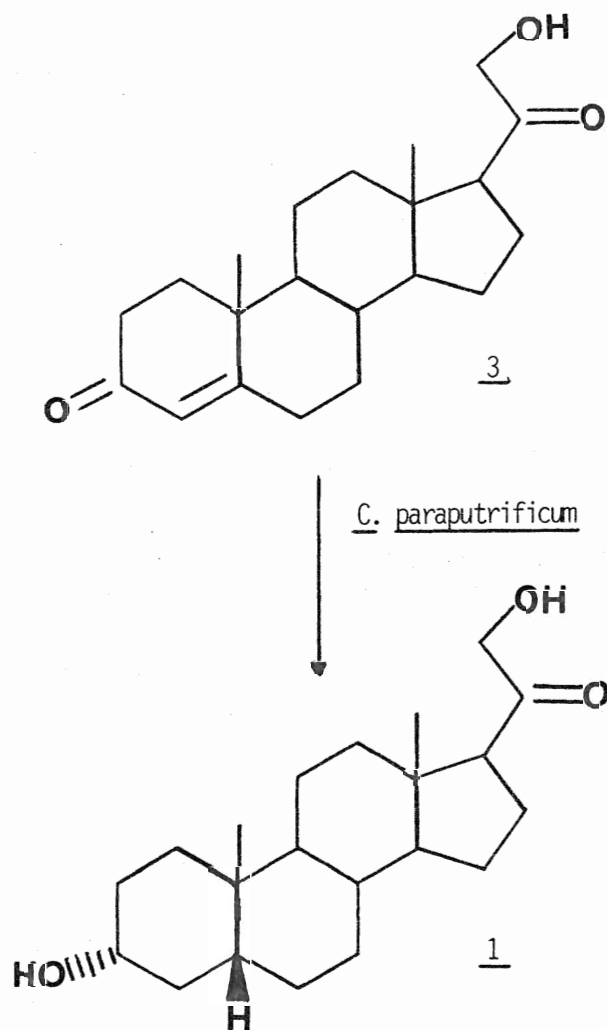
### Incubations with C-21 Labelled Corticosteroids

Before the incubations with E . lentum could be performed it was necessary to convert the C-21-d<sub>1</sub> derivatives of 3 into derivatives of 1 (Fig. 40). A species of bacteria is known to perform this reduction, Clostridium paraputrificum , so it was obtained. It was found to grow very rapidly in the medium that was used for the E . lentum , which minimized the problems associated with working with an unknown microorganism.

Control incubations performed initially showed the ease of conversion from 3 to 1 . But there was no certainty that the C-21 position would not be affected by the reduction of the unsaturated A-ring system. An incubation performed on the compound 3 -d<sub>3</sub> showed the conversion to derivative of 1 , but <sup>1</sup>H nmr showed that about 6% of the deuterium at C-21 may have been lost during the conversion. Within the error of the integration this may not be a significant loss. This problem will be addressed later.

In the beginning the whole transformation was to be done as a mixed culture containing C . paraputrificum , E . lentum and E . coli . The first two were grown together, while the E . coli was only to be added during the incubation to insure that the medium remained anaerobic over the two week period. Since the C . paraputrificum and the E . lentum are obligate anaerobes they do not remain viable in the presence of oxygen

Figure 40: The conversion of 3 to 1 by the bacterium Clostridium paraputrificum



and would therefore not perform their roles. The E . coli being an facultative anaerobe can live under anaerobic as well as aerobic conditions and could therefore serve to keep the oxygen concentration low in the incubation flask. The first attempts at a mixed incubation showed only conversion of 3 to 1 without the desired dehydroxylation of 1 to 2 . Attempts at conversion of 1 to 2 without the presence of C . paraputrificum also showed no change in 1 . Also an analysis of the incubation medium by Gram staining showed it to contain only E . coli , which suggested that the E . lentum was nonviable. More cultures were obtained and it was decided that the conversion of 3 to 2 would be performed as two separate incubations to avoid the problem of one species outgrowing another.

The new cultures of E . lentum were approached differently than the C . paraputrificum in the way that they were rehydrated and grown. By changing the way that the medium was prepared and the cell concentrations it was hoped that viability would result. With the failure of each alteration it was decided to obtain an actively growing culture from the culture collection. This culture obtained was found to grow very fast in the sBIHB medium that had been used all along, which suggests that rehydration and initial growth of this species is more complicated than initially expected. With the actively growing culture of E . lentum it only remained to

show its 21-dehydroxylase activity by the conversion of 1 to 2 .

Initial attempts showed only the recovery of starting material 1 , which suggested that either the medium was not anaerobic enough, since E . coli was not used because of contamination concerns, or that the enzyme was not a constitutive one, meaning it required induction. The culture was then maintained on a medium that contained 400 micrograms per mL of 1 to insure induction and was subcultured every 7 days. After two subcultures the culture was found to be capable of converting the substrate 1 into the dehydroxylated product 2 . It was then necessary to convert the deuterated derivatives of 3 into the derivatives of 1 by means of the other bacterial species C . paraputrificum .

Having maintained the culture for a long period of time it was found to be inactive in terms of the reduction of 3 to 1 . A new culture was obtained that initially converted the commercial sample but attempts at converting the deuterated compounds 17 and 18 resulted only in the reisolation of starting material. Differences in the growth medium during the incubation were suggestive of some contamination, which indicated that some other species was present other than the C . paraputrificum . A new culture was obtained that was capable of transforming the substrates 17 a and 18 , which generated 20 and 21 respectively. Due to the poor recovery in



reisolation of substrates in the failed incubations (see Experimental) there remained too little of the substrate 17a to use further, therefore only the two substrates prepared chemically were used.

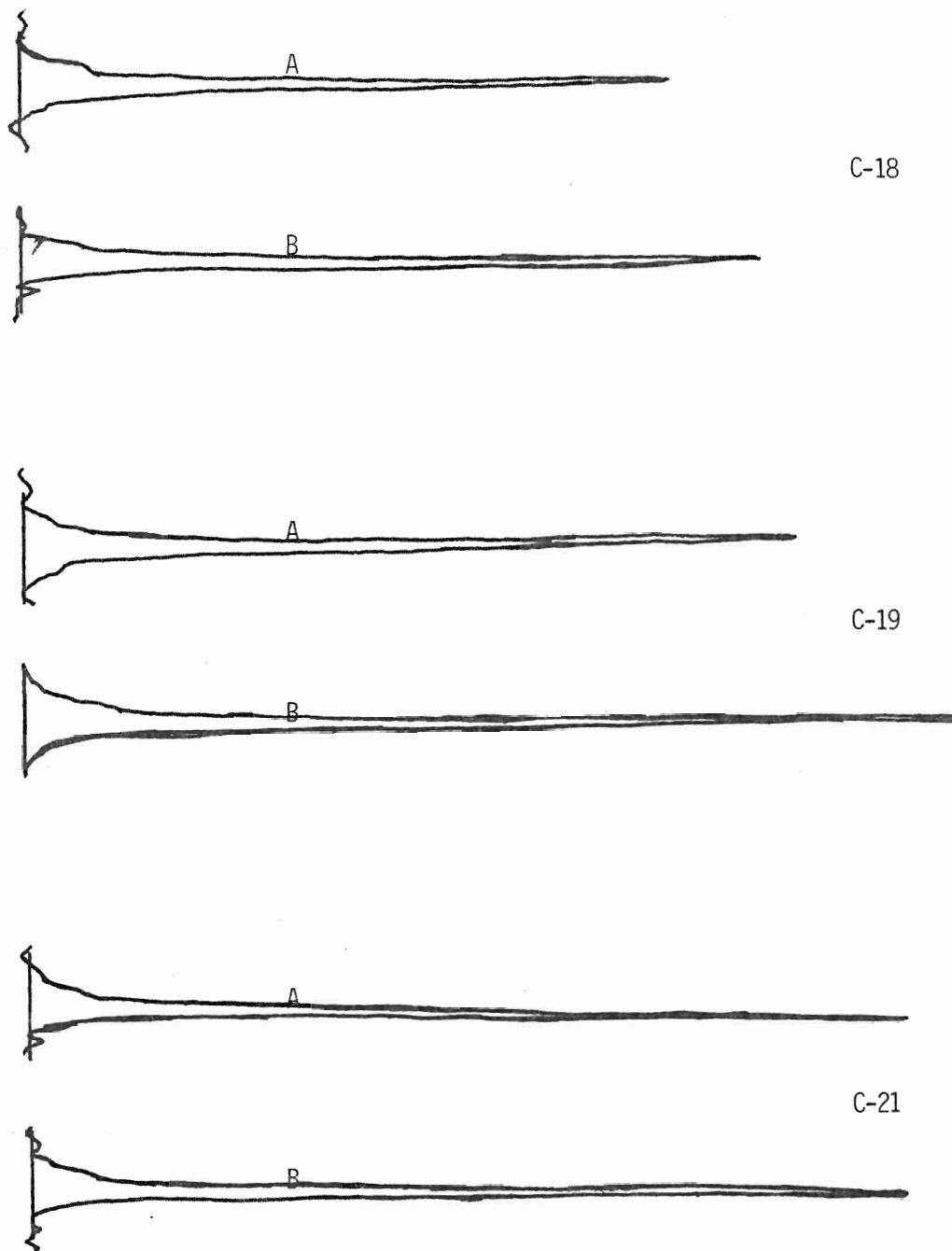
Having obtained the deuterated derivatives of 1 the incubations with E. lentum were then performed which gave the desired products compounds 22 and 23, from 20 and 21 respectively. These were obtained in an impure state because of the tailing of the large non-polar impurity on the preparative TLC. Fortunately the impurity was not large enough to impede the analysis of the compounds by EI mass spec. and high resolution  $^1\text{H}$  nmr at 500 MHz.

The nmr could easily distinguish the three methyl signals of the steroid molecule for each of the compounds 22 and 23 (Fig. 41).

C-21	2.07 ppm
C-19	0.90 ppm
C-18	0.56 ppm

The singlets were clear of all other peaks so that their peak heights and integrals could be measured (Table V). Normally it is possible to obtain an integral from the computer of the nmr machine, however if the baseline is sloped by the presence of large peaks nearby then the electronic integrals become inaccurate. Because the samples were initially to be run on deuterium nmr they were prepared in non-deuterated solvent.

Figure 41: The isolated methyl for A) 23 B) 22



Because of this, large ethanol peaks at 1.1 ppm were present, which made the electronic integrals for the C-19 and C-18 peaks unreliable. Fortunately the ethanol signals left the peak heights undisturbed and since they are symmetrical the results are expected to be reliable as a measure of peak intensity.

Table V: The values for the peak heights for the three methyl signals of 22 and 23

Signal	Peak Height
Compound <u>22</u>	(mm)
C-21	123
C-19	131
C-18	105
Compound <u>23</u>	
C-21	123
C-19	109
C-18	92

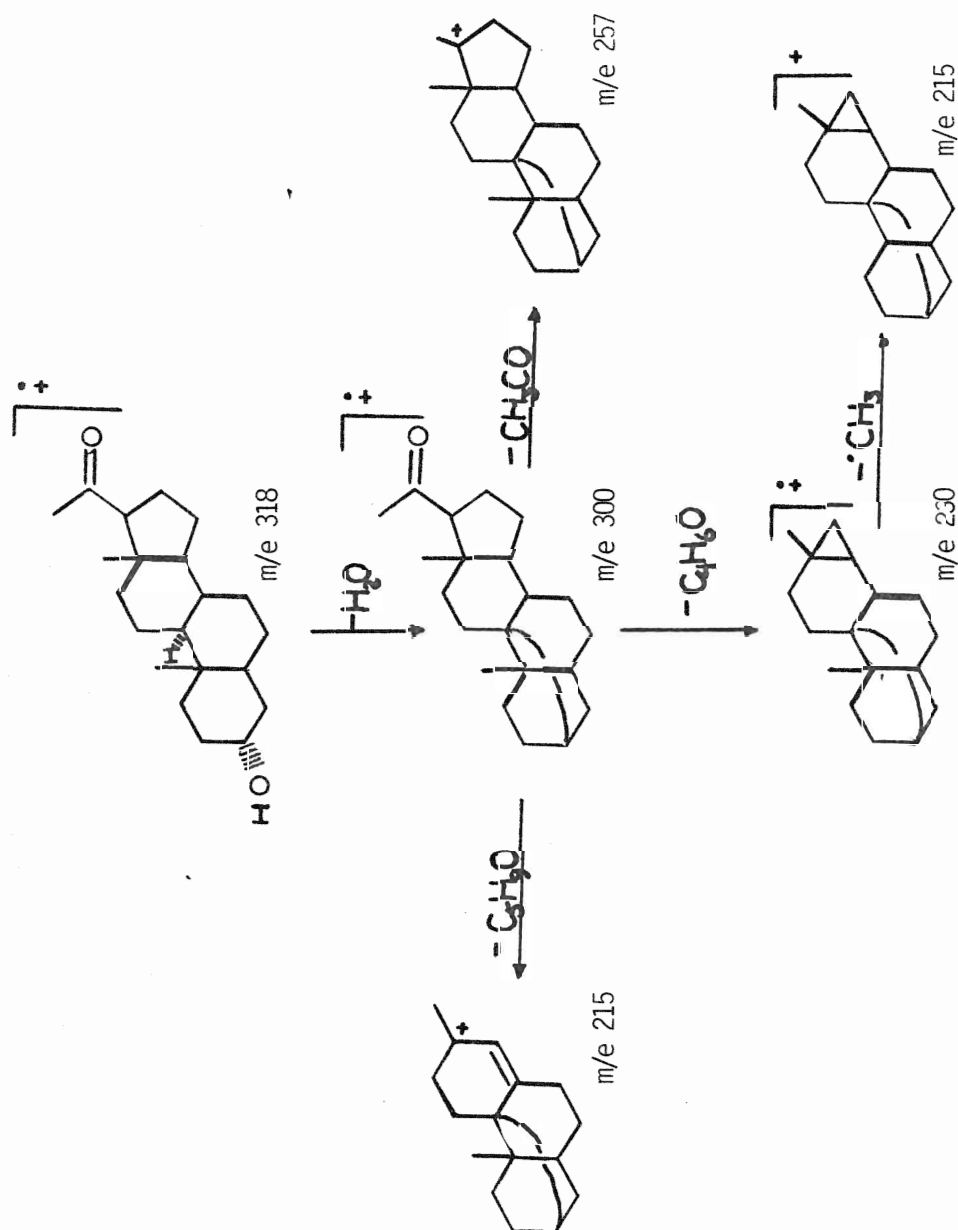
Since 21 contained deuterium only in the C-21 position, the product of the incubation 23 should have only one deuterium or none. This means that the EI mass spectrum should contain either a  $d_0$  or  $d_1$  or some mixture of the two. Any deuterium that is present must be in the C-21 position and nowhere else. Looking at the mass spectrum we see the molecular ion at 318 as well as the M+1 peak at 319. Comparison of the two peaks and accounting for the carbon-13 contribution to M+1 there is approximately 7% of a  $d_1$  species present. This information can be used to interpret the nmr signals to account

for the peak height and deuterium content of the C-21 position of 22 , which has a more complex mass spectrum.

In an attempt to interpret the mass spectrum of 22 , different fragments were analysed for their deuterium content in hopes of determining how much was present at C-21. See Figure 42 for a condensed fragmentation analysis for the compound 2 . Due to the impurities in the sample, that were most prominent at the low mass end of the spectrum, the actual deuterium content could only be estimated by comparison of the impurity pattern of 22 to that of 23 , which contained no deuterium in the steroid nucleus, to the pattern of the fragments for the commercial sample (Appendix II). The validity of performing this comparison is not very great for a number of reasons.

The first one is due to the fact the deuterium present in the molecule may change the relative intensity of peaks because of isotope effects. Then considering that both 22 and 23 did not contain the same relative amount of impurities it is difficult to assign an increase in intensity to either impurity or deuterium. Also the spectrum of 2 was obtained on a different day possibly under slightly different conditions (temperature and pressure), which might have given different relative intensities for the peaks in the clusters of interest. Since the molecular ion was free of interferences it was possible to determine the deuterium content of the whole molecule fairly accurately. The fragment analysis is, much less

Figure 42: Selected fragmentation pattern used for the analysis of compounds 22 and 23



reliable, but it suggests that deuterium is present at the C-21 position.

Table VI: Estimates for the deuterium content of 22 obtained by the mass spec. fragmentation comparison to 23 and 2

Deuterium content	M	M-H <sub>2</sub> O-CH <sub>3</sub> CO (%)	M-H <sub>2</sub> O-C <sub>5</sub> H <sub>9</sub> O
d <sub>0</sub>	46	68	82
d <sub>1</sub>	34	15	14
d <sub>2</sub>	16	17	4
d <sub>3</sub>	4	-	-

By comparing the value for d<sub>0</sub> between the molecular ion and the fragment in which the C-21 group is lost, it can be seen that the amount of d<sub>0</sub> increases. Since it is expected that every molecule containing deuterium in the steroid nucleus contained deuterium in the side chain, an increase in the d<sub>0</sub> population should be accounted for by the decrease in d<sub>3</sub> and the changes in the other deuterium populations. Since this relationship is not seen in these numbers it becomes apparent that the original assumptions were not valid. Therefore the mass spectrum can not be used to determine the amount of deuterium at the C-21 methyl group. It depends, therefore, on the interpretation of the methyl signals of the nmr.

Since the mass spectrum of 23 showed that the molecule had 7% d<sub>1</sub> it must be assumed that the methyl signal corresponding to this position must be smaller than it should be

by 7/3 of a percent. The actual intensity of the peak without any deuterium can be calculated by the empirical equation 3

$$\frac{X}{100} * Y / 3 = Y - Z \quad (3)$$

Where      X is percent of  $d_1$   
              Y is the true intensity  
              Z is the observed intensity

Use of this equation for the peak height gives the true intensity of the C-21 methyl of 23 to be 126 mm. Next the peak heights of the other two methyl signals, C-19 and C-18 were compared to those of 22 and the ratios of the peaks were obtained ( 23 / 22 ).

C-19      0.83

C-18      0.88

With an average ratio between spectra of 0.855 this number was used to calculate the expected intensity of the C-21 methyl signal for 22 with no  $d_1$ . This gave a value of 147 mm which is obviously larger than the observed. Using equation 3 the percent  $d_1$  can be calculated. The number obtained from this calculation shows approximately 50% of the molecules contain deuterium at the C-21 position. The summary of the findings with the corresponding substrate compositions are shown in Table

VII. Taking these values back to the substrate stereochemistry, predictions can be made on the expected values based on two theoretical approaches.

Based on the study of Riemland<sup>19</sup> it is expected that one of the hydrogens is lost from the C-21 position during the dehydroxylation reaction. By comparing the rates of hydrogen loss versus deuterium loss in a competitive incubation it was found that hydrogen would be lost 2.8 times as fast as deuterium. If the mechanism does indeed involve a loss of hydrogen as the molecule is bound in the ene-diol form, then the loss can be either of two possible types;

- 1) Non-stereospecific
- 2) Stereospecific

If the reaction follows the first type in which there is no stereochemical difference, then the product after dehydroxylation should contain deuterium irrespective of the stereochemistry. Using the value for the isotope effect the product could contain nearly 2.8 times as much  $d_1$  at C-21 as  $d_0$ . Since this is independent of the orientation of the deuterium, the product from 20 should have a similar deuterium composition to that of 21. A rough estimate, based on the value of the isotope effect would give approximately 75%  $d_1$  and 25%  $d_0$  at the C-21 position. This estimate assumes that there is no  $d_0$  in the starting material, but if there is the numbers



Table VII: Summary of deuterium composition at the C-21 position for compounds 20 , 21 , 22 and 23

Compound	$d_o$	$d_1$	
		R	S
<u>20</u>	0%	66%	33%
<u>21</u>	12%	21%	67%
	$d_o$		$d_1$
<u>22</u>	50%		50%
<u>23</u>	93%		7%

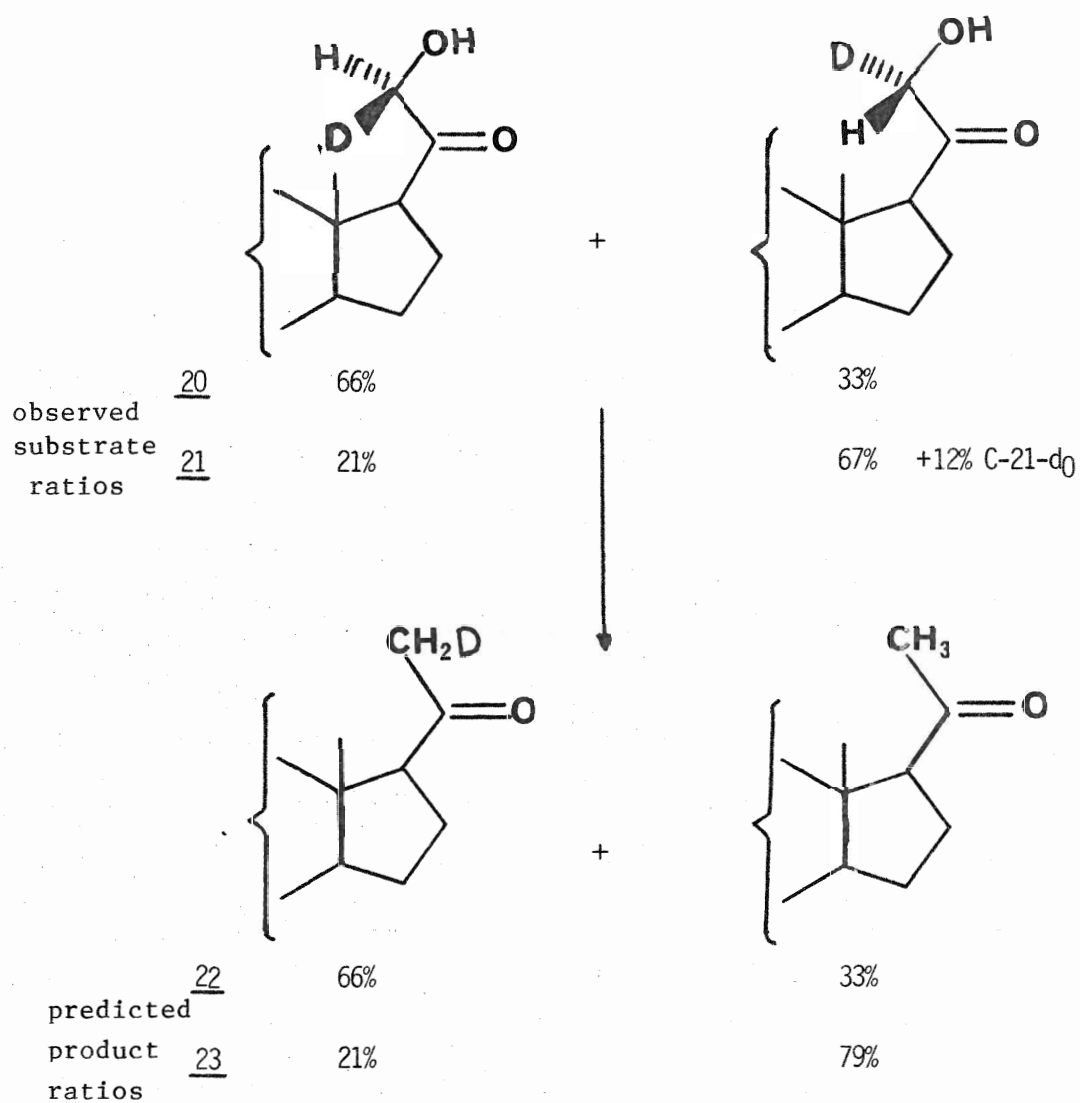
would be adjusted depending on the amount.

If the mechanism follows the second type in which the hydrogen abstraction is stereospecific the products deuterium composition at C-21 will be related to the stereochemical orientation of the deuterium in the starting material (Fig. 43).

In the case of 21 the compound was 88%  $d_1$  at C-21 with the predominant isomer having an S configuration. Since most of the deuterium was lost during the dehydroxylation it would imply that this hydrogen would be the one to be lost. If this were the case then we would expect to find 21% of 23 to be  $d_1$ . Also the compound 20, which is assumed to be 100% C-21- $d_1$  should generate a product 22 with 66%  $d_1$  at the C-21 position. Looking at the values obtained from the actual incubations we see that 23 actually contains 14% more  $d_0$  than expected and 22 about 16% more. This can almost be accounted for by looking at some control experiments.

The incubation of 3 - $d_3$  with C. paraputrificum was shown to give the product of reduction at the A-ring with no apparent loss of deuterium at the C-21 position. Since there is an assumption that the value of 6% loss of deuterium, during the transformation, is within experimental error, then one can assume this. However, this incubation may not be a valid control since the incubations of 17b and 18 were actually performed three times. If an incubation of one week does not

Figure 43: The expected result of an incubation with E. lentum with a stereospecific hydrogen abstraction of both C-21 isomers

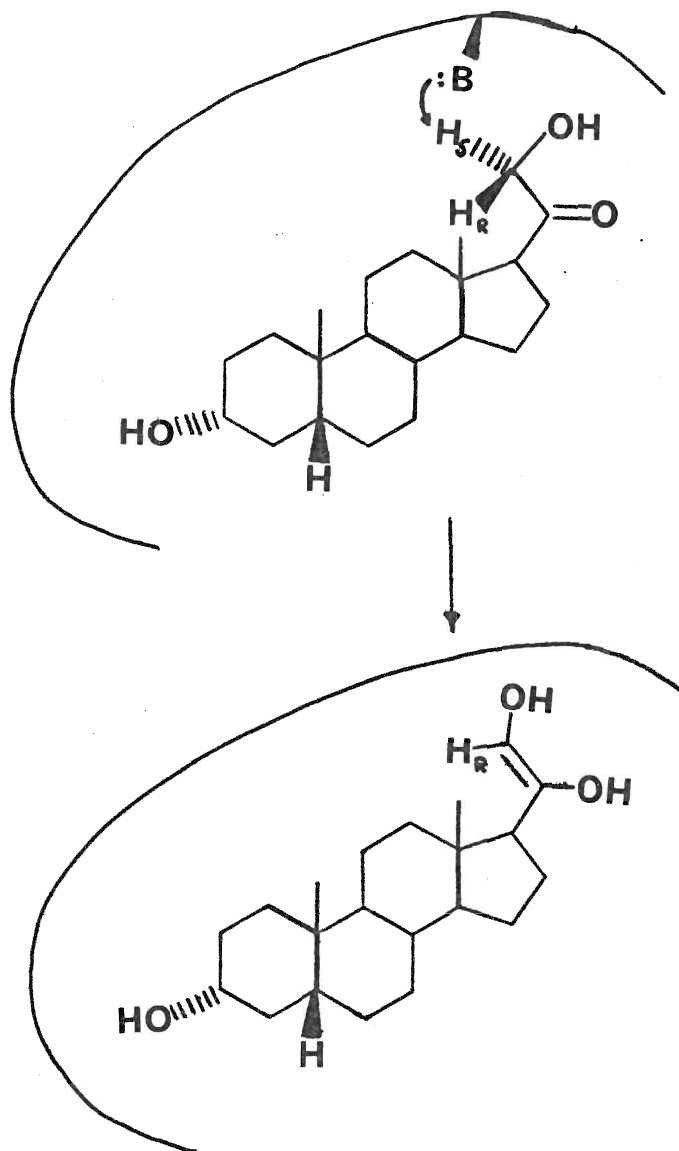


give a loss of deuterium at C-21 of any significance then perhaps after three weeks there might be a significant loss. The result is that a value of between 5 to 10% may be expected for the loss of deuterium at the C-21 position during the reduction at the A-ring. Also the incubation performed by Riemland<sup>19</sup> using the deuterated analog of 1 showed a 9% greater amount of  $d_0$  than expected. This was thought to be due to some unaccounted for 'side reaction'.

The net result of having these two possible sources of deuterium loss makes the numbers obtained for the deuterium content of 22 and 23 reasonable. It can be assumed that within errors the numbers fit into a scheme involving a stereospecific loss of one deuterium at C-21 at the pro-S position. This loss is not unexpected considering this position lies on the least hindered side of the molecule and would be more accessible to a basic group on the enzyme in the active site as the substrate was being bound (Fig. 44). This reasoning is based on the fact that the chemical and enzymic reductions and the chemical exchange of the C-21 position seem to favour the pro-S side of the molecule. This demonstrates that it may be the least hindered of the two hydrogens.

In summary, it appears that the enzyme responsible for the 21-dehydroxylation of corticosteroids removes one hydrogen stereospecifically in the binding of the substrate to the active site. Based on these experiments it appears that the

Figure 44: The proposed stereochemical hydrogen abstraction during binding of 1 to 21-dehydroxylase



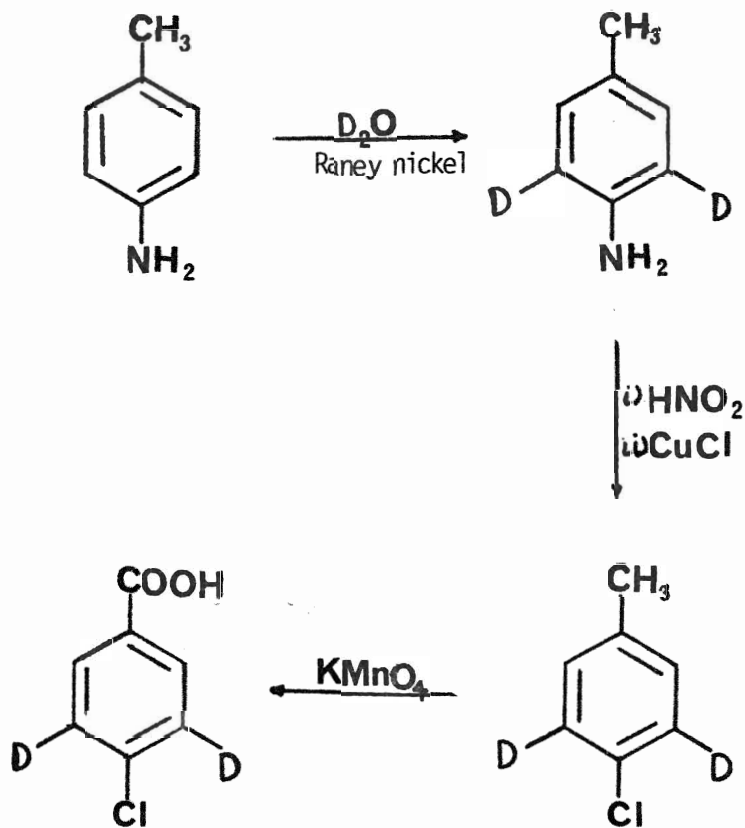
hydrogen that is abstracted is in the pro-S position of the C-21 methylene.

### The Dehalogenation of p-Chlorobenzoic acid

For the investigation of the dehalogenation of p-chlorobenzoic acid it was decided to prepare the deuterated analogs of the starting material, p-chlorobenzoic acid, and the product, p-hydroxybenzoic acid, with deuterium in the meta positions relative to the carboxyl group.

The first attempt was designed to use an aniline derivative, p-toluidine, which could be converted to p-chlorotoluene by the Sandmeyer reaction and the carboxyl formed by the simple oxidation of the methyl group (Fig. 45). To obtain deuterium in the ring a reaction was found that involved the use of a Raney nickel catalyst and deuterium oxide.<sup>59</sup> The reaction is reported to give exchange at the ortho positions relative to the amino group which would have been the ideal positions, since after the Sandmeyer reaction the deuterium would be ortho to the chloro substituent. The reaction seemed simple enough and since an active Raney nickel catalyst was available it was attempted. With the failure of this reaction the communication was looked at closer and it was found that the specific catalyst was identified as 20% nickel on kieselghur. This was different from the catalyst used, which was simply a Raney nickel catalyst. Rather than investigating ways of making 20% nickel on kieselghur and/or reinvestigating the deuterium exchange reaction, another approach was sought.

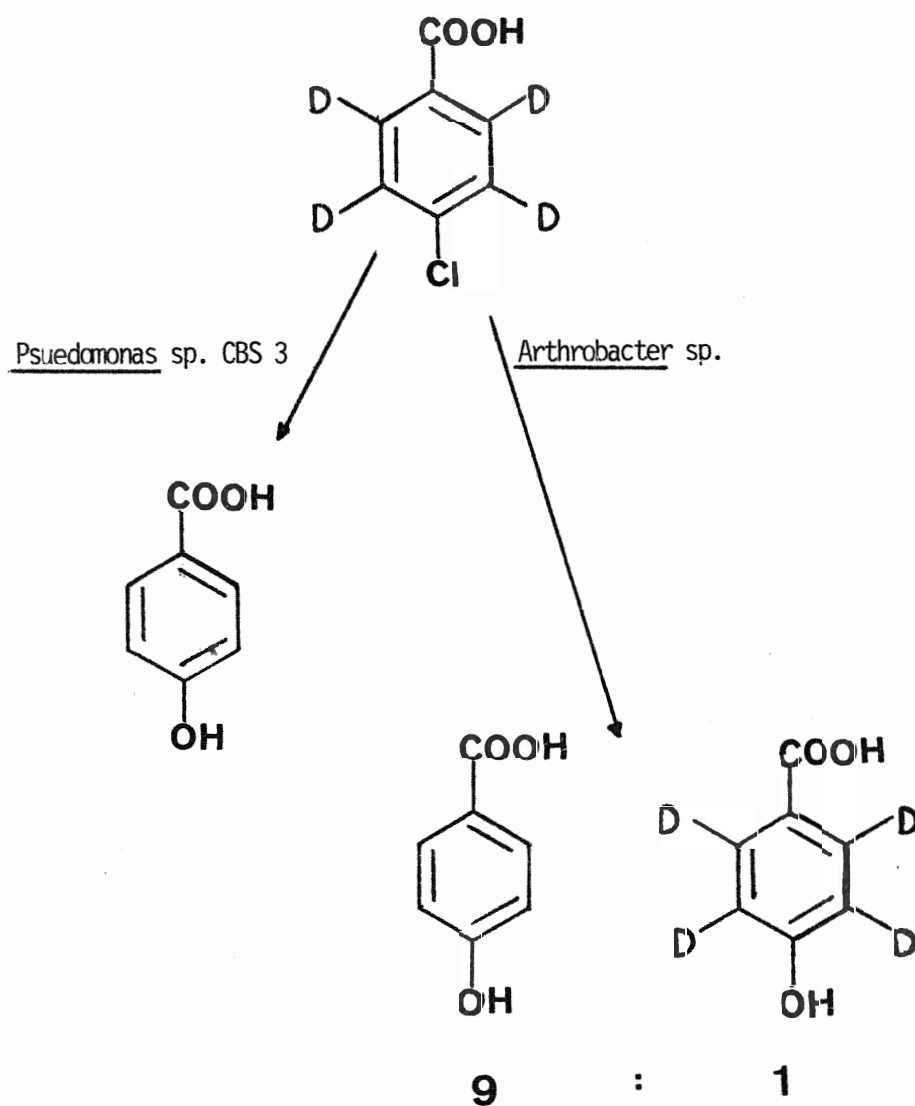
Figure 45: Scheme for the preparation of deuterium labelled p-chlorobenzoic acid from p-toluidine





The next approach used readily available deuterated toluene in which the deuterium was present in all five of the ring positions as well as the three positions of the methyl group. By performing a chlorination reaction and oxidizing the methyl it was possible to obtain a deuterated analog of p-chlorobenzoic acid. The difference between the initial objective and the final product was only the presence of the extra deuterium in the positions ortho to the carboxyl group. Since the purpose of the deuterium in the ring is to see if one is lost during the dehalogenation the extra deuterium in the ring should not have been a problem. Any theoretical mechanisms identified these positions as potentially unreactive. After the product p-chlorobenzoic acid had been prepared it was found to have an identical melting point to that of a sample prepared by another worker. Since there was concern that our sample might contain some ortho isomer, even after many recrystallizations, a comparison was made to the sample prepared by Prof. N.H. Werstiuk from Mc.Master University. Prof. Werstiuk's sample was prepared from a commercial sample of p-chlorotoluene that should have contained little to no contamination of the ortho isomer. Our sample was sent to researchers in Germany for an incubation with an enzyme preparation from Pseudomonas sp. CBS 3, that is found to perform the dehalogenation reaction.<sup>48</sup> The results obtained from this incubation as well as an incubation with another bacterial

Figure 46: Incubations of p-chlorobenzoic acid with enzyme preparations from two bacterial species



species known to perform the same transformation, an Arthrobacter sp., are shown in Figure 46. At this time the preparation of the control compound p-hydroxybenzoic acid-d<sub>4</sub> was being attempted.

With the success of Werstiuk at using the HTDA process for the preparation of p-chlorobenzoic acid-d<sub>4</sub>, it was felt that this process might be useful in the preparation of the p-hydroxybenzoic acid. Since Werstiuk had noted that a direct attempt to exchange p-chlorobenzoic acid under the conditions of the reaction resulted in the explosion of the reaction tube he found it necessary to perform an indirect exchange, using p-chlorotoluene.<sup>70</sup> Attempts at the direct exchange of p-hydroxybenzoic acid also met with some side reaction that resulted in the explosion of the reaction tube. It was apparent that an indirect approach was needed that could utilize a compound less likely to degrade under the conditions of the reaction. The compound would have to give a fairly clean reaction since the actual amount that could be used at any given time was small due to the size of our autoclave. Obtaining a sufficient amount of deuterated intermediate it would have to be possible to convert this compound to p-hydroxybenzoic acid by reactions that would not exchange the ring hydrogens. Since the problem with the p-chlorobenzoic acid exchange reaction was thought to be a decarboxylation under the high temperature

conditions it was possible that p-hydroxybenzoic acid was also decarboxylating. Since the acid solution is degassed before reaction and sealed under vacuum, the appearance of gas, as the reaction tube was opened in one case, can not be from any other source.

To avoid this, p-hydroxybenzaldehyde was used since the aldehyde could easily be subsequently converted to the acid by air oxidation. The resulting reaction generated a highly stable dark red material the formation of which could have been the result of an intermolecular condensation reaction similar to that for the thermoplastics called 'novolaks' which are prepared from phenol and formaldehyde under acidic conditions.<sup>71</sup> Since the same reaction seemed to occur in the case of the p-methoxy derivative it is possible that demethylation can occur under the conditions of the reaction.

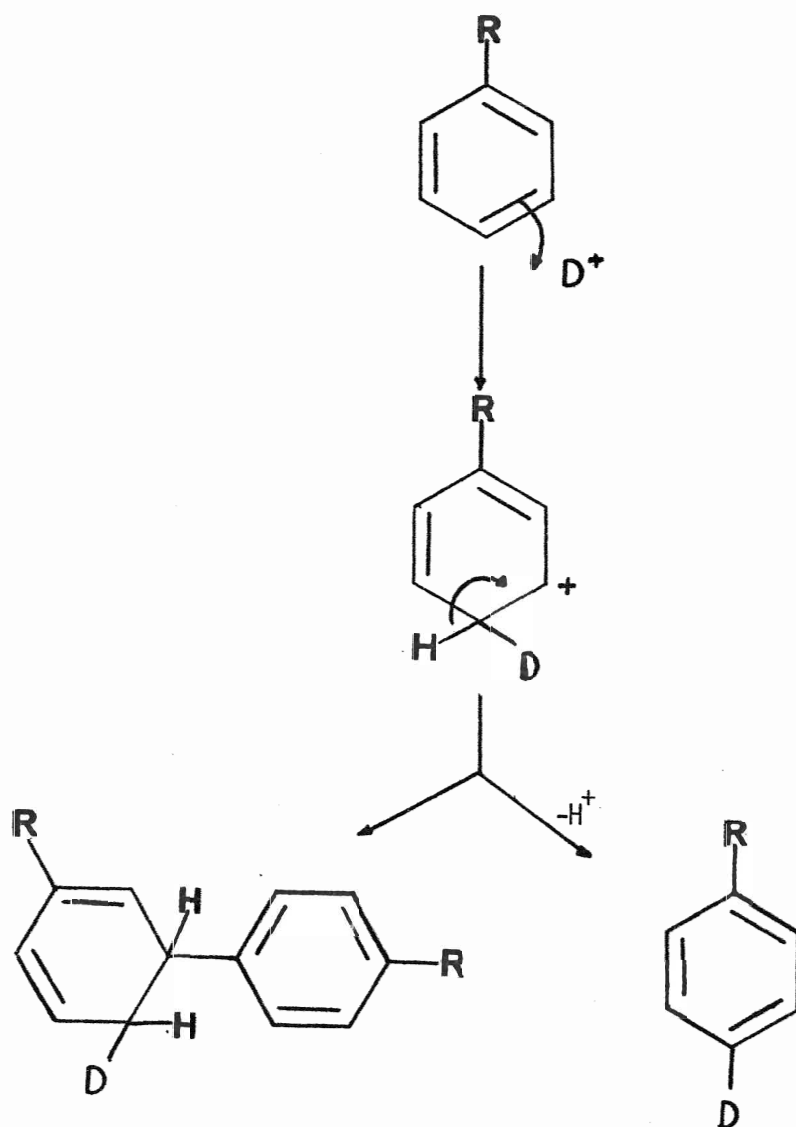
Another approach involved the use of a nitrile that was to be converted to the acid by hydrolysis. The hydrolysis was performed under basic conditions with concentrated base. It was uncertain whether the compound would hydrolyse under the acidic conditions of the HTDA process. However, the resulting explosion of the reaction tube in the attempt to carry out the exchange was evidence for this occurrence.

A reaction was found that involved the conversion of an aryl bromide to a nitrile, which could be used to get around this problem. The reaction would convert p-bromophenol to

p-hydroxybenzonitrile but when the bromophenol was subjected to the exchange reaction many side products were apparent, too many in fact to make this approach useful. To get around this p-chlorophenol could have been used since Werstiuk has used aryl chlorides as substrates. However, the conversion to the nitrile was found to be ineffective.

The next approach was very similar to the one used for the p-chlorobenzoic acid preparation, in which the chlorotoluene was converted to the acid. The only problem with this, in the case of the phenol, was that the phenol functionality had to be protected during the oxidation reaction due to its reactivity. The oxidation could be performed in the presence of a methoxy group so it only remained to either put in the methyl group before or after the deuterium exchange reaction. Using p-cresol (p-hydroxytoluene) in the HTDA process, many side reactions must have been possible because the resulting extract was very messy. A possible explanation might be that the presence of the two activating groups on the ring make it reactive enough for the exchange, but also reactive to other electrophiles in the reaction medium (Fig. 47). During the exchange reaction the ring becomes protonated in accordance with the mechanism of electrophilic aromatic substitution. Normally the loss of hydrogen generates the deuterium labelled aromatic ring. If, however, the protonated intermediate was very stable it may be long lived enough to act as an electrophile on another molecule

Figure 47: Possible mechanism for the exchange of hydrogen during the HTDA process with possible side reaction

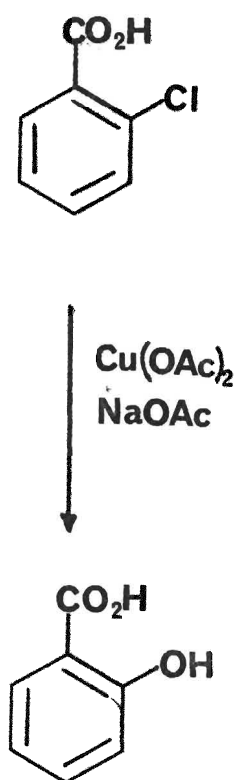


p-cresol. A continuation of this process could generate quite large complicated molecules. This process may not be likely at normal reaction temperatures, but at  $280^{\circ}\text{C}$  it might indeed be possible. To decrease the chances of this possibility the methoxy derivative was used, but as noted before the methyl can be somewhat labile. As was seen from the reaction on this substrate, the methoxy methyl must be labile, since a similar product mixture was seen. This approach was abandoned at this point.

Other schemes were tried in an attempt to prepare this compound but none were successful. With time being a factor this line of research was terminated without the preparation being achieved. This is of little consequence considering the fact that the researchers in Germany no longer communicated with us.

To investigate the plausibility of the nucleophilic aromatic substitution (NAS) mechanism the literature was searched for such an example on p-chlorobenzoic acid. One reaction was found that saw the conversion of o-bromobenzoic acid to salicylic acid under mild conditions in a very short period of time.<sup>66</sup> While the reaction was not as easy for the o-chlorobenzoic acid, the chlorine could still be eliminated by this reaction (Fig. 48). The reaction, which required the presence of copper acetate as a catalyst was thought to proceed by the NAS mechanism with the copper complexing the halide making it a better leaving group.<sup>66</sup> A problem arose when

Figure 49: Conversion of o-chlorobenzoic acid to salicylic acid under weakly basic conditions





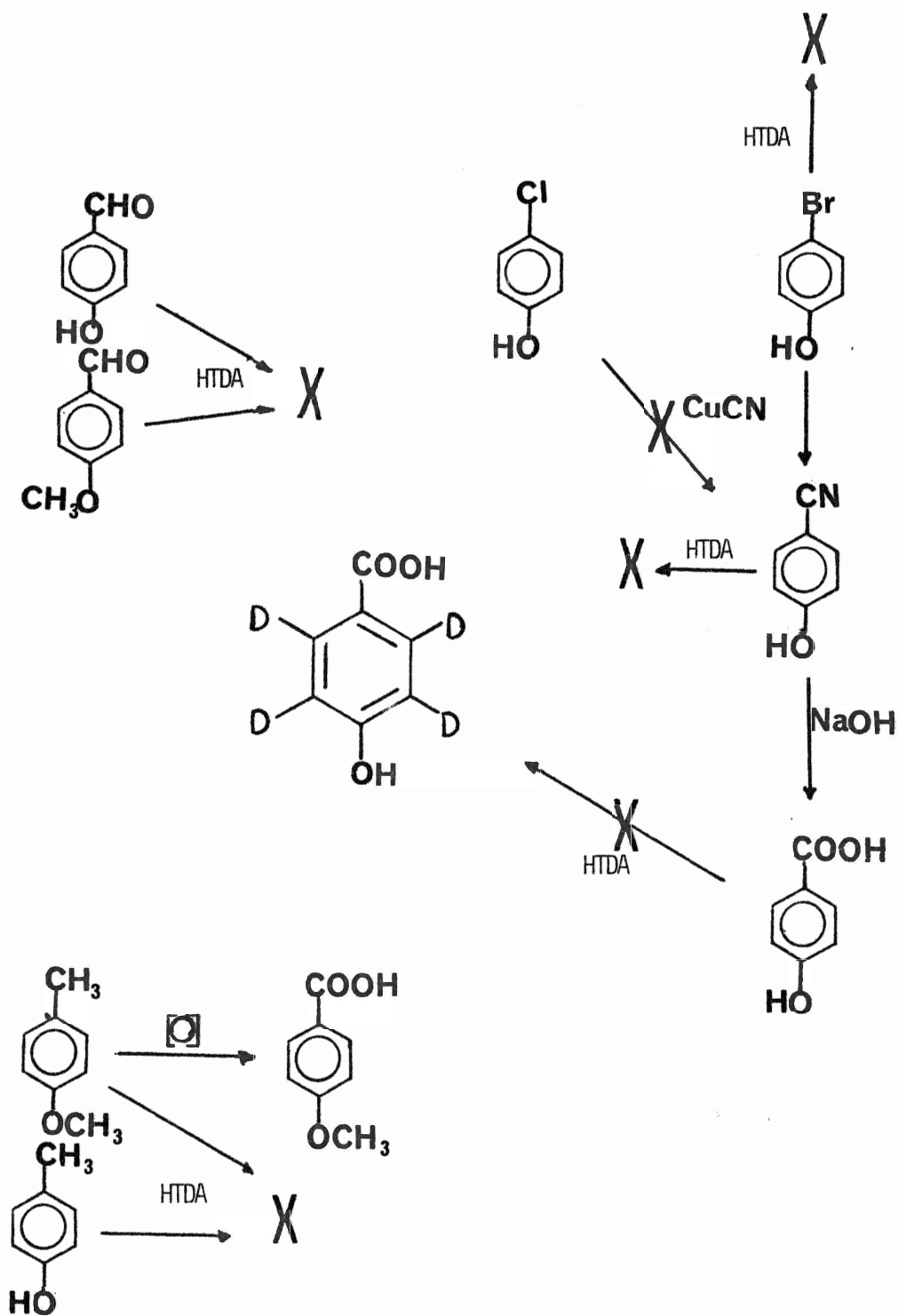
p-chlorobenzoic acid was used, that may account for the lack of reaction. When the cupric acetate was added to the solution of the p-chlorobenzoate and sodium acetate, a blue-green precipitate formed. This precipitate was difficult to identify by mass spectrometry, but the IR showed what appeared to be a complexed acid group. There were three peaks in the region of this vibration, which was around  $1580\text{ cm}^{-1}$  all of which were large and sharp. Since there were no other peaks in the normal acid carbonyl region of  $1680\text{ cm}^{-1}$  and there were peaks for an organic component it is quite possible that the p-chlorobenzoate precipitated out as some complex of copper. Since these benzoate copper compounds can be very complex, ranging in complexity from dimers to polymers,<sup>72</sup> it is difficult to say exactly what the compound is. However, since the catalyst is known to be essential, being lost as a precipitate would certainly prevent the reaction. However, the reaction of o-chlorobenzoic acid shows that strong withdrawing groups like nitro groups are not always necessary for a reaction to proceed by the NAS mechanism. In fact, if the leaving group is good enough (bromo) the reaction is quite facile.

This fact tends to support the idea that the reaction might be following such a mechanism in the enzymic case because enzymes usually only do chemistry that is already possible without enzymes. An enzyme usually does it faster, with regio and stereoselectivity and at pH's that would make it impossible

under straight chemical conditions. This does not, however, explain the results of the incubations with the deuterated p-chlorobenzoic acid. Since it is so difficult to exchange the hydrogens of an aromatic ring even under drastic conditions it is difficult to rationalize a mechanism for their complete removal, in the case of the enzymic dehalogenation. Since the business end of the molecule contains a phenolic group after the transformation it is possible that some exchange reaction is possible while the molecule is still in the active site. The pH of the medium is reported to be 7.0, therefore after the transformation exchange is unlikely even considering these hydrogens are at the reactive ortho positions relative to the hydroxyl group. Exchange in the HTDA process still requires 4 days at 280°C to effect complete exchange at the same position in phenol alone.<sup>62</sup> Even if this were possible it would, at best, account for the removal of two of the aromatic hydrogen, but would not account for the apparent loss of the hydrogens ortho to the carboxyl. Exchange at this position in benzoic acid usually requires 7 to 8 days at 280°C.<sup>62</sup> Unfortunately not enough information has been obtained about the enzyme preparation and incubations to get a clear picture. It is entirely possible that the preparation of the enzyme involves the isolation of native p-hydroxybenzoic acid. This would certainly explain the result obtained from the *Arthrobacter* sp. enzyme in which 90% d<sub>0</sub> and 10% d<sub>4</sub> was observed. Without the

appearance of some intermediate forms of deuterium label an exchange reaction of either substrate or product is unlikely if not physically impossible. Until more reactions are performed including our suggestion of an incubation with 50% D<sub>2</sub>O or some other known amount of deuterated solvent, the mechanism and the truth about the process will remain a mystery.

Figure 49: Schemes for the preparation of p-hydroxybenzoic acid- $d_4$  using the HTDA process



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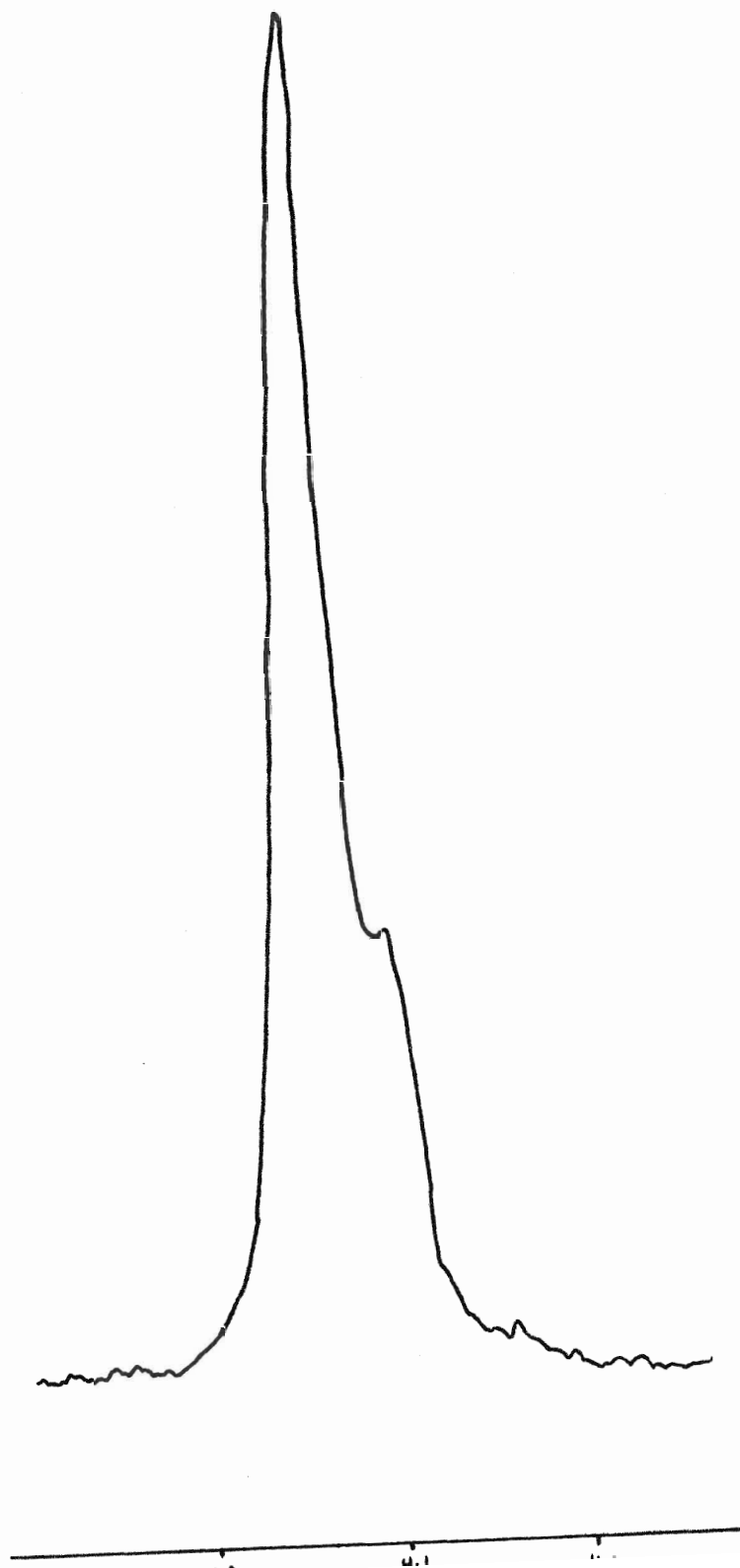
## Appendix I

The high resolution nmr at 200 MHz for the C-21 methylene signal for the following compounds.

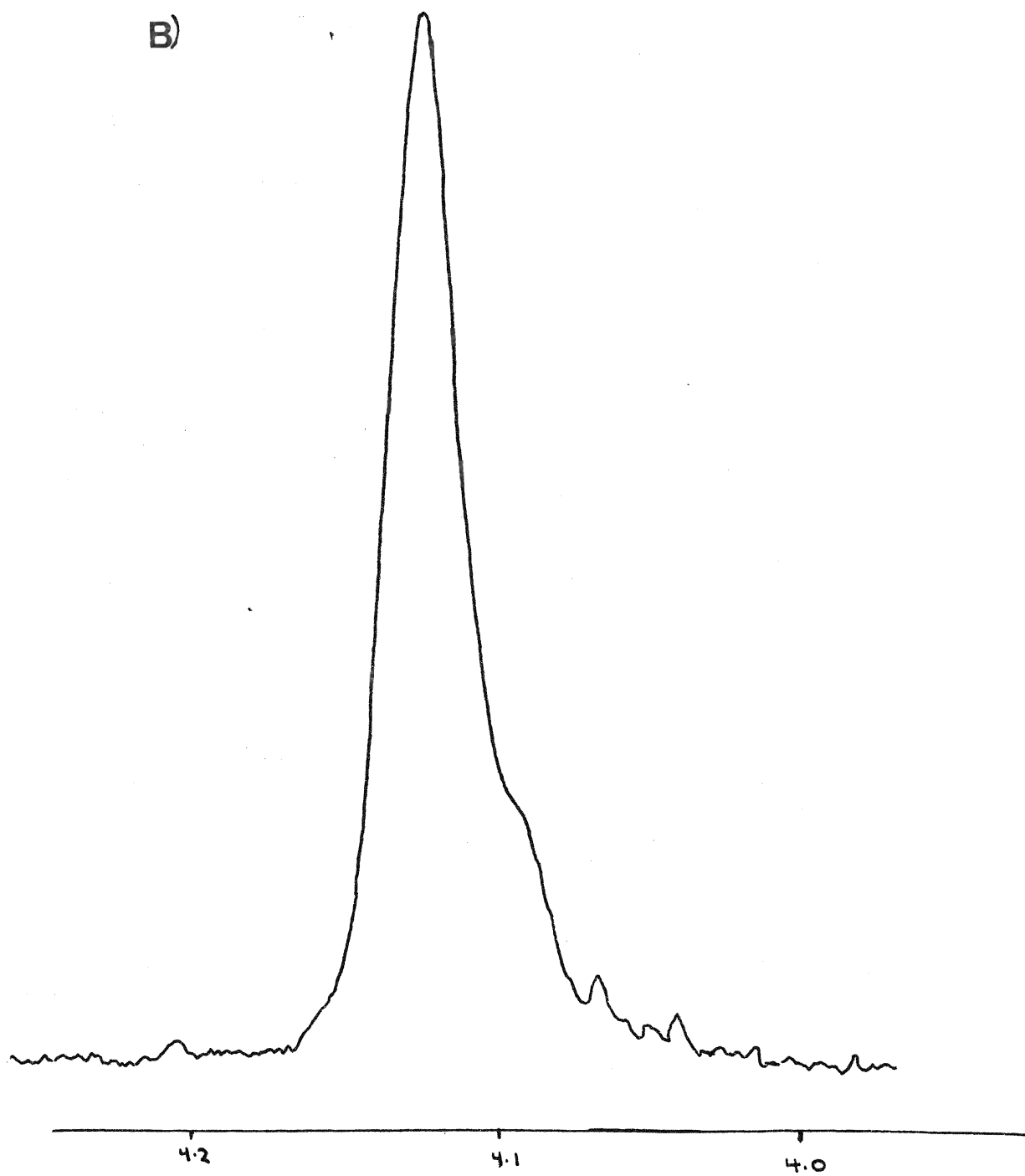
A) 18

B) 3-d<sub>3</sub>

A)



B)



## Appendix II

The normalized peak intensities of two fragments of 2 , 22 and 23 from their E.I. mass spectra for the estimation of deuterium content of 22 .

The value for the the largest peak of  $m/e$  215, corresponding to  $d_0$  of the the  $M-C_5H_9O$  fragment for 22 and 23 , was normalized to that of 2 . The difference between 2 and 23 was viewed as the contribution of impurity. Assuming the same relative amount of impurity in 22 , the excess was viewed as the contribution by deuterium. The estimates obtained for the content of deuterium in the nucleus of 22 are shown in Table VI

$m/e$	% intensity		
	<u>2</u>	<u>23</u>	<u>22</u>
215	100	100	100
216	27	28	45
217	17	24	29
257	22	29	28
258	11	14	20
259	6	8	15

The numbers were subtracted across the columns to determine the amount of deuterium contribution in the fragmentation clusters for 22

m/e	correction for the presence of deuterium	
215	100	-d <sub>0</sub>
216	17	-d <sub>1</sub>
217	5	-d <sub>2</sub>
257	28	-d <sub>0</sub>
258	6	-d <sub>1</sub>
259	7	-d <sub>2</sub>

The corrected numbers for each fragment are considered to be abundancies which can be converted to the percent seen in Table VI

### Appendix III

The method of preparing the sodium phosphate buffer used in the enzymic reduction of the 21-oxo steroids and 21-hydroxysteroid dehydrogenase



Since the pH of the buffer was on the basic side of neutral then it was assumed that the two species of sodium phosphate were to be the mono basic and the dibasic phosphates.

The two equations used to set up the amount of each phosphate species needed were:

$$\text{pH} = \text{pK}_a + \log\left(\frac{[\text{Na}_2\text{HPO}_4]}{[\text{NaH}_2\text{PO}_4]}\right)$$

$$[\text{Na}_2\text{HPO}_4] + [\text{NaH}_2\text{PO}_4] = 0.1 \text{ M}$$

The pH is 7.4 and the  $\text{pK}_a$  of this dissociation step is 6.8 hence the two equations can be solved for the two unknowns. Any deviation from the desired pH can be compensated by the addition of NaOH or phosphoric acid