

OF STEROIDS
STEROID METABOLISM BY INTESTINAL BACTERIA

A

Thesis submitted by

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ABSTRACT.

This thesis is concerned with some in vitro modifications of steroids by pure strains of intestinal bacteria isolated from human faeces. This work comprised of two projects. The first concerned the different mechanisms by which a double bond could be introduced into the steroid nucleus. The following enzymic reactions were demonstrated:-

- A. The introduction of a double bond in conjugation with a keto group.
- B. The introduction of a double bond by oxidative demethylation and aromatisation.
- C. The introduction of a double bond by a dehydration reaction.
- D. The introduction of a double bond in conjugation with other double bonds.

The major groups of bacteria, as listed in Table 1, were screened for the reactions listed above and it was found that reactions A, B and D were exclusively carried out by the lecithinase negative Clostridia. Strains of bacteria showing the reactions were further studied for their enzyme properties.

The second project concerned the metabolism of cholesterol by strains of intestinal bacteria in vitro and this was compared and contrasted with the in vivo metabolism of cholesterol in the large intestine of rats and guinea pigs. Cholesterol ^{14}C , was introduced directly into the caecum of the animals and the levels of radioactivity in the faeces and urine monitored. The in vitro studies showed the production of the cholesterol metabolites of coprostanol, coprostanone, 4-cholesten-3-one and small amounts of 1,4-cholestadien-3-one as well as some acid steroid as a result of a side chain cleavage reaction. These reactions were also demonstrated in vivo; in addition, the guinea pig showed production of radioactive oestrogens in the urine, probably synthesised in the colon from cholesterol.

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The work described in this thesis comprises part of a wider study on the epidemiology and aetiology of cancer of the colon.

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SECTION I

INTRODUCTION AND
AIMS OF THE PRESENT STUDY

INTRODUCTION

The epidemiology of colon cancer shows that this disease is highly correlated with fat in the diet (Wynder and Shigematsu, 1968; Drasar and Irving, 1973). It has been postulated that the high levels of fat in the diet promote the excretion of high levels of bile acid which are modified by the colonic flora to produce a carcinogen, possibly of the polycyclic aromatic type (Hill et al., 1971; Drasar and Hill, 1972). It has already been demonstrated that strains of intestinal bacteria can modify bile acids in vitro in several ways. They can carry out bile acid deconjugation reactions (Aries and Hill, 1970a), oxidoreductase reactions at the 3, 7 and 12 oxygen functions and 7 dehydroxylation reactions (Aries and Hill, 1970b). These types of reaction have been reviewed in full by Lewis and Gorbach, (1971).

It has been postulated that in order to form a compound of the polycyclic aromatic type only four types of desaturation reaction are needed and these can account for the theoretical full desaturation of the steroid nucleus (Hill, 1970). The four types of reaction all of which have already been demonstrated with soil organisms and mammalian systems are;

a) The introduction of a double bond in conjugation with an oxo group. This reaction has been shown with several soil organisms and has been discussed in two reviews (Talalay, 1957 and 1965).

b) Oxidative demethylation of a substrate with a 4-en-3-one configuration to produce a phenolic steroid. This type of reaction is common in placental and other endocrine preparations and has also been demonstrated using several soil organisms (Talalay, 1957 and 1965). In bacterial systems, this reaction often occurs in association with side chain cleavage at the C₁₇ position.

c) The production of metabolites with desaturation of rings A and B by the introduction of double bonds in conjugation with other double bonds. These compounds, termed equilins, are only produced by the horse placenta under normal conditions, although in vitro incubations of steroid substrates with human placental extracts have demonstrated the production of such compounds (Morand and Lyall, 1967).

d) The introduction of a double bond by a dehydration reaction. Hayakawa et al., (1958 and 1964), demonstrated the production of desaturated metabolites by such a mechanism using strains of soil organisms.

Although bile acid metabolism by intestinal bacteria has been little studied until recently, it has already been established in other fields that the flora plays an important role in drug metabolism, in enterohepatic circulation and in the detoxification mechanisms (see reviews by Smith, 1966 and Scheline, 1968).

Dietschy and Wilson (1970b), have reviewed the metabolism of cholesterol in the colon in the context of the overall catabolism of steroids in the body. It is already known that the intestinal bacteria have a profound effect on faecal neutral steroid metabolites in vivo (Kellog and Wostman, 1970), although attempts to demonstrate these reactions in vitro using single strains of intestinal bacteria have, not until now, been very successful (Snog Kjaer et al., 1956).

It has been the aim of this thesis to demonstrate the four types of desaturation reaction described above in vitro, using strains of human intestinal bacteria. In addition, the in vitro metabolism of cholesterol has been studied and contrasted with the in vivo metabolism in two experimental animals.

Introduction

1. Intestinal Microorganisms

Table 1 shows the major groups of microorganisms found in the faeces of healthy British subjects together with the average counts per gm wet weight faeces, (modified from Drasar and Williams, 1972). It can be seen that the aerobic organisms represent only about 1% of the total flora. The other 99% are made up of fastidious non-sporing anaerobic bacteria broadly grouped as Bifidobacteria (Gram-positive anaerobes) and Bacteroides (Gram-negative anaerobes). A relatively minor group of the anaerobes, numerically, are the spore forming Clostridia.

The bacteria isolated from faeces can be identified by their growth on selective media. The major genera, with their definitive characteristics (Drasar, 1967), are:-

Aerobic

Enterobacteria

All are catalase-positive, Gram-negative rods growing aerobically on MacConkey agar. In the large intestine these are made up mainly of Escherichia coli (96%), with the majority of the remainder being Aerobacter aerogenes. These are classified according to Edwards and Ewing (1962).

Streptococci

These are divided into three main groups all being catalase-negative, Gram-positive cocci, growing aerobically on blood agar.

a) Enterococci, usually in pairs or short chains and form intense red colonies on MacConkey agar, These grow on Dubos azide medium and form non-mucoid colonies on sucrose agar. This group includes three main species; Streptococcus faecium, Streptococcus faecalis and Streptococcus equinus (Diebel, 1964).

TABLE 1

Human Faecal Flora

<u>Major Bacterial Group</u>	<u>Species Often Isolated</u>	<u>Log Count per Gram wet wt faeces</u>
Enterobacteria	<u>Escherichia coli</u>	7.9
	<u>Klebsiella aerogenes</u>	
	<u>Proteus mirabilis</u>	
Streptococcus	<u>Streptococcus viridans</u>	6.5
	<u>Streptococcus faecalis</u>	
Lactobacillus	<u>Lactobacillus acidophilus</u>	6.6
	<u>Lactobacillus casei</u>	
Clostridium	<u>Clostridium perfringens</u>	5.7
	<u>Clostridium sporogenes</u>	
Veillonella	<u>Veillonella alcalescans</u>	5.0
	<u>Veillonella parvula</u>	
Bacteroides	<u>Bacteroides fragilis</u>	9.8
	<u>Bacteroides melaninogenicus</u>	
Bifidobacterium	<u>Bifidobacterium adolescentis</u>	9.3
Eubacterium	<u>Eubacterium bifforme</u>	6.5

Adapted from Drasar and Williams, (1972).

- b) Streptococcus salivarius, producing large ovoid colonies on sucrose agar.
- c) 'Viridans' Streptococci, do not grow on MacConkey agar or produce mucoid colonies on sucrose agar and are α -haemolytic on blood agar.

Aerobic bacilli

Catalase-positive, Gram-positive spore-forming rods, growing on nutrient agar aerobically at 37°C, the spores withstand heating at 70°C for 10 minutes.

Yeasts and filamentous fungi

Isolated on Sabourauds medium.

Anaerobic

Bacteroides

Gram-negative pleomorphic non-sporing rods, usually catalase-negative. All are oxygen-sensitive and form small colonies on rich blood agar.

Bifidobacteria

Gram-positive rods, catalase-negative and strictly anaerobic pleomorphic rods which show wide degrees of branching. Recent work has divided this group into three sections; Eubacterium sp., Propionibacterium sp. and Bifidobacterium sp. depending on patterns of sugar fermentation and acid production from glucose (Peach et al., 1972; Holdeman and Moore, 1972).

A differential medium for these two groups has been sought with only limited success. 0.03% China blue incorporated into reinforced Clostridial medium (Wiel Korstanje et al., 1970) showed Bacteroides as blue translucent colonies and Bifidobacteria as larger brown colonies. Sutter and Finegold (1971) attempted to use antibiotic sensitivities to select the more resistant Gram-negative Bacteroides.

Clostridia

Gram-positive, catalase-negative, spore-forming rods, which are neomycin-resistant. The spores are resistant to heating at 70°C for 10 minutes. These bacteria grow anaerobically on Willis and Hobbs egg yolk medium and are split into lecithinase-C-positive and lecithinase-C-negative strains on the basis of the opalescence or otherwise of colonies. At the species level strains are classified according to sugar fermentation patterns and acid products of glucose fermentation (Holdeman and Moore, 1972).

Veillonellae

Gram-positive rods, catalase -variable and growing on Rogosa agar containing 7.5ug/ml vancomycin.

Sarcinae ventriculae

Gram-positive, catalase-negative cocci, growing in a cubical arrangement in packets of eight cells. These have been isolated from the faeces of vegetarians only (Crowther, 1971a).

Until recently, classification and characterisation of the anaerobic bacteria had been little studied. Indeed, it was not until 1933 that Eggerth and Gagnon firmly established that the anaerobes were the dominant faecal and large intestinal organisms. Hungate (1950) set up a system for isolating and preserving anaerobic bacteria from the rumen and these methods were developed for faecal organisms by Drasar (1967 and 1968) and Drasar and Crowther (1970). Their technique involved the use of a glove box filled with 90% hydrogen and 10% carbon dioxide. This was used for the complete isolation of the fastidious oxygen-sensitive bacteria and all isolation steps eg. pouring of plates, serial dilution of faecal samples, plating and spreading, were carried out in this 'anaerobic cabinet'. Plates and broths were then directly transferred to an anaerobic vessel

and removed via an airlock from the isolator. The plates could then be incubated under strictly anaerobic conditions.

Environmental factors have been shown to significantly alter the numbers of bacteria per gm of faeces. Gorbach et al. (1967a) showed that the age of the subject affected the flora but showed no significant effect of diet. Crowther (1971b) however, found little change in the flora with age. Hill et al., (1971) showed that an increase in the numbers of strictly anaerobic bacteria was observed with people on a low fat or vegetarian type of diet.

2.

Intestinal Steroids

2.1. Bile Acids

At the present state of knowledge all evidence suggests that cholesterol is the obligatory precursor of primary bile acids (Borgstrom and Danielsson, 1968; Mosbach, 1972). The detailed steps and mechanisms of bile acid synthesis in the liver are now well characterised (Danielsson and Einarsson, 1968). In man the two primary bile acids ie. those synthesised in the liver from cholesterol, are 3 α , 7 α , 12 α -trihydroxy-5 β -cholan-24-oic acid (cholic acid) and 3 α , 7 α -dihydroxy-5 β -cholan-24-oic acid (chenodeoxycholic acid), (Danielsson, 1963). The bile acids are secreted into the bile duct as the taurine and glycine conjugates (Danielsson, 1963). In humans, the glycine conjugates are the most common, dominating over the taurine conjugates at a ratio of 3:1 (Hoffman, 1968). However in rats the taurine conjugates predominate at 20:1 (Haslewood, 1967).

Bile acids are extensively enterohepatically circulated (Mosbach, 1972). Borgstrom et al. (1963), have shown that 95% of bile acids are reabsorbed in the small intestine in their conjugated form. Portman et al. (1955), showed that this uptake was by an active transport mechanism and also that the free deconjugated bile acids competed for the uptake in this system. Of the other 5% of the bile acids, some are taken up passively by the large intestine (Dietschy and Wilson, 1970b) and the remainder are lost from the bile acid pool in the faeces. In man, the faecal loss of bile acids vary between 50mg and 2,000mg per day (Grundy et al., 1965; Eneroth et al., 1966a, b and 1968).

The size of the bile acid pool in humans has been estimated to be between 2 to 5g (Linsteadt, 1957) and this is recycled between six and ten times per day (Krone et al., 1968; Dietschy, 1972). In normal rats the pool

size of animals with a body weight of 200g has been calculated at between 14mg (Eppinger, 1920) and 20mg (Portman and Murphy, 1958). The loss of bile acids in the faeces is compensated for by the feed back controls to synthesis sites of primary bile acids in the liver (Mosbach, 1972). This worker estimated that man synthesises between 200mg to 500mg bile acid per day in roughly equal amounts of cholic and chenodeoxycholic acids. In rats, the rate of synthesis is between 4 to 5mg per day (Eriksson, 1960 and Gustafsson et al., 1960)

Diet has been shown to have a marked effect on bile acid pool size. Portman and Murphy (1958), showed that rats fed on a low fat and high starch synthetic diet had a bile acid pool size about one half that of a group of rats fed on Purina Chow. Excretion rates changed proportionally as did that of bile acid synthesis. Different levels of faecal steroids in populations have been shown to be correlated with high and low fat diets (Hill et al., 1971; Hill and Aries, 1970).

Although only two primary acids, cholic and chenodeoxycholic acids, are synthesised by the liver, biliary bile contains four major acid steroids (Table 2), being cholic, chenodeoxycholic and deoxycholic acids in the ratio of approximately 1.1: 1.0:0.6 (Sjovall, 1960), together with much smaller amounts of lithocholic acid (Hollstrom and Sjovall, 1961). Deoxycholic and lithocholic acids are produced from cholic and chenodeoxycholic acids respectively, as a result of a 7α dehydroxylation reaction carried out by the colonic flora (Lewis and Gorbach, 1972). These secondary bile acids are then taken up by the large intestine and subsequently transported to the liver in the hepatic portal vein.

Table 2 also shows the composition of the faecal bile acids. It can be seen that these acids are much more diverse than those excreted in the

TABLE 2

The Faecal and Biliary Acid Steroids

<u>Oxygen Function</u>			<u>Trivial Name</u>	<u>*Biliary Acids</u>	<u>Faecal Acids</u>
<u>C₃</u>	<u>C₇</u>	<u>C₁₂</u>			
α OH	α OH	α OH	Cholic acid	++	++
α OH	KETO	α OH		-	+
				-	
α OH	α OH	-	Chenodeoxycholic acid	++	++
α OH	-	α OH	Deoxycholic acid	+	++
β OH	-	α OH		-	+
α OH	-	KETO		-	+
β OH	-	KETO		-	+
α OH	-	-	Lithocholic acid	+	++
β OH	-	-		-	+
KETO	-	-		-	+
-	-	-	Cholanic acid	-	++

* Biliary acid steroids are excreted as the conjugates of Glycine and Taurine only.

+, indicates the presence of an acid steroid.

++, indicates the dominant acid steroids.

bile. All faecal bile acids have been deconjugated during their passage through the bowel. The major bile acids are 3 β -hydroxy-5 β -cholanic, 3 α -hydroxy-12-oxo-5 β -cholanic, 3 β ,12 α -dihydroxy-5 β -cholanic, 3 α ,12 α -dihydroxy-5 β -cholanic (deoxycholic) and 3 α -hydroxy-5 β -cholanic (lithocholic) acids.

Minor steroidal components of the faeces include 5 α 'allo' bile acids (Haslewood, 1967), unsubstituted cholanic acid (Hill, 1971) and a series of acids with mono, di and tri substituted acids with oxygen functions at the 3, 7 and 12 positions in both oxidised (keto) and reduced (hydroxyl) forms. Bile acids with unsaturated nuclei have not been widely reported. Norman and Sjovall (1958), isolated a compound from rat faeces, tentatively identified as a 4-ene-3-keto metabolite. Further studies on the metabolism of lithocholate by Norman and Palmer (1964), showed the presence of an unsaturated and unsubstituted bile acid metabolite.

To account for the modifications of the bile acids to form such a diverse array of faecal metabolites, the colonic flora has been implicated. Lewis and Gorbach (1972), reviewed deconjugation reactions, 3, 7 and 12 oxidoreductase reactions and 7 α dehydroxylase reactions, which have all been demonstrated in vitro using isolated strains of intestinal bacteria. As described in section 3.2., germ free animals have been used to demonstrate that bile acid metabolites arise in faeces as a direct result of intestinal bacterial metabolism.

2.2. Cholesterol

Intestinal cholesterol is derived from exogenous and endogenous sources. The exogenous form is taken in the diet, whilst endogenous cholesterol is synthesised principally by the liver and the intestinal wall (Dietschy, 1969). However, cholesterol synthesised in the liver and intestine are sub-

ject to different physiological and biochemical controls. Cholesterol synthesised in the liver and excreted in the bile is preferentially absorbed by the small intestine compared with that produced by the intestinal mucosa (Wilson and Reinke, 1968; Dietschy and Wilson, 1970b). To explain this phenomenon, it is thought that biliary cholesterol is excreted in micellar form with the bile acids and lecithin and is therefore already in an easily absorbable form. In contrast, mucosal cholesterol must be chelated by bile acids and converted to a micellar form before it can be absorbed. Schoenheimer (1931), suggested that cholesterol synthesised in the intestine was released through the lumen by the sloughing of mucosal cells. Such a process is quite credible as Leblond and Stevens (1948) showed that the turnover of mucosal cells in rats was only thirty hours.

The second major difference is that the liver is subject to stringent feed back control mechanisms in response to dietary cholesterol. In contrast, intestinal synthesis and secretion remains unchanged by such a stimulus (Dietschy and Wilson, 1970c). Although the difference could be of importance therapeutically, it is not as yet understood why this difference exists.

Dietary cholesterol reaches the intestine as both free sterol and as sterol esters (Treadwell and Vahouny, 1968). The esters are hydrolysed by the pancreatic esterase in the presence of bile acid. Dietary cholesterol, like that of intestinal origin is poorly absorbed.

Loss of cholesterol from the body pool occurs by two major routes. The first is the loss of neutral steroids in the faeces, accounting for 500mg to 1,500mg cholesterol per day. The major part is lost as the microbial degradation products, coprostanol along with smaller amounts of coprostanone and 4-cholesten-3-one (Grundy and Ahrens, 1969; Miettinen,

1965). Some evidence also exists for complete degradation of the steroid molecule under intestinal microbial influence (Grundy and Ahrens, 1968). The second route is in the synthesis of bile acids from cholesterol. Block et al., (1943) showed that isotopically labelled cholesterol could be converted to bile acids,,but it was not until 1952 that it was established that this was a major pathway for the excretion of cholesterol (Siperstein et al., 1952; Bergstrom, 1952).

3. Metabolism of intestinal steroids by intestinal bacteria

3.1. General aspects

As described in section 2.1. the difference in composition of bile acids in the bile and in the faeces has been attributed to the metabolic activity of the intestinal bacteria. A range of oxidoreductions, 7 α -dehydroxylations and deconjugation reactions has been demonstrated using intestinal bacteria (Lewis and Gorbach, 1972). In addition to the primary bile acids, cholic acid (3 α , 7 α , 12 α -trihydroxy-5 β -cholan-24-oic acid) and chenodeoxycholic acid (3 α , 7 α -dihydroxy-5 β -cholan-24-oic acid), conjugates of deoxycholic acid (3 α , 12 α -dihydroxy-5 β -cholan-24-oic acid) are found in significant concentrations in the bile (Sjovall, 1960; Gray et al., 1968) and small amounts of lithocholic acid (3 α -hydroxy-5 β -cholan-24-oic acid) in conjugated form (Hellstrom and Sjovall, 1961). These compounds are the product of a 7 α dehydroxylation in the large intestine by the colonic flora, and are returned to the liver by the portal blood system after absorption. Carey and Williams (1963), showed that if enterohepatic circulation is interrupted using a bile fistula, the products of 7 α -dehydroxylation disappear from the bile. Such modifications have been shown in vitro and are attributed to specific strains of intestinal bacte-

eria (Aries and Hill, 1970b).

Modification of steroids by the intestinal mucosa has been demonstrated. Norman and Palmer (1964), and Palmer (1967), showed the production lithocholate sulphate even under germ free conditions. Moser et al. (1966) isolated cholesteryl sulphate and attributed its production to the mucosa.

Unlike the bile acids, cholesterol metabolites do not undergo extensive enterohepatic circulation (Wilson and Reinke, 1968) and are not absorbed from the colon (Grundy and Ahrens, 1969). Thus, there is no accumulation of metabolites in the bile and all products formed from cholesterol during the passage through the gut are lost in the faeces.

3.2. Germ free animals

Germ free animals, i.e. animals delivered and reared under completely sterile conditions are a useful experimental tool in assessing the effects of intestinal bacteria on steroids and foreign compounds in the colon. By infection of these animals with single strains of intestinal bacteria, strains found to be metabolically active 'in vitro' can be assessed 'in vivo'. However, physiologically and anatomically germ free animals differ in many ways from their conventional counterparts (Luckey, 1963) and some care should be taken in interpreting results.

Kellog and Wostman (1969), showed substantial differences in the excretion of neutral and acid steroids in the faeces of germ free animals compared with their conventional controls. They found significantly reduced levels of neutral steroids, made up of cholesterol and lathosterol only, in germ free rats. The two common breakdown products of cholesterol, coprostanone and coprostanol, were not detected but were found in conventional controls. Similarly, the levels of faecal bile acids was signifi-

cantly lower and this fraction was made up of taurine and glycine conjugates of the primary bile acids only. This compared with the products of extensive degradation of faecal bile acids found in conventional animals (see section 2.1.). The latter contains no taurine or glycine conjugates.

This confirmed earlier work by Gustafsson et al. (1966) and other workers who observed a complete absence of faecal coprostanol and coprostanone in germ-free animals. Gustafsson et al. (1957), also showed that a quantitative recovery of taurine and glycine conjugates could be obtained from the faeces of germ free rats when fed with cholic-24- ^{14}C acid. On feeding the labelled steroid to conventional animals, the faecal bile acids contained a mixture of deconjugated and dehydroxylated steroids.

Contamination of germ-free animals with single strains of bacteria has been used to assess the enzymic ability of strains in vivo. Suzuki (1970), showed that germ-free mice contaminated with strains of strict anaerobes showed good deconjugation of the taurine and glycine bile salts. In contrast, contamination with strains of Escherichia coli, Vibrio and Shigella produced little or no deconjugation. Kellog et al. (1970), using a strain of Clostridium perfringens, showed an increase in cholesterol metabolism and complete deconjugation of faecal bile acids in germ-free rats and mice. Dickenson et al. (1971), tested strains of bacteria for their in vitro ability to degrade bile acids and then attempted to extrapolate to the in vivo situation by mono-contamination of germ-free animals. These workers, however, found that not all strains active in vitro were also metabolically active in vivo in this system. In the light of these results, some caution may be necessary when assessing such systems.

Antibiotic treatment of conventional animals has also been used to demonstrate a suppression of the metabolic activity of the intestinal bact-

eria. Hoffman et al. (1968), showed that neomycin-treated rabbits produced smaller amounts of allodeoxycholate ($3\alpha, 12\alpha$ -dihydroxy- 5α -cholan-24-oic acid, from allocholic acid ($3\alpha, 7\alpha, 12\alpha$ -trihydroxy- 5α -cholan-24-oic acid). As allo bile acids are not synthesised by the liver, their formation has been attributed to intestinal bacteria (Kallner, 1967b). Coleman and Baumann (1957a) showed that rats treated with penicillin, produced less of the neutral steroid coprostanol in the faeces and there was a significantly higher proportion of the parent compound cholesterol.

3.3. Bile acids

To account for the production of the common faecal bile acids from the primary amino acid conjugates excreted in the bile, four types of enzymic reaction have been postulated, all of these have been demonstrated in vitro. These are listed below.

- a) A deconjugation of the taurine and glycine conjugated acids to release amino acids and free bile acids. This reaction has been studied extensively in vivo (Norman and Sjoval, 1958) and in vitro by several workers using single strains of intestinal bacteria. These are reviewed by Lewis and Gorbach (1972). They partially isolated this enzyme and found that, depending on the particular group of bacteria it may be found in either the intracellular, extracellular or wall membrane of the bacterial cell.
- b) The oxidation of the steroid ring hydroxyl functions to a keto group. This reaction has been demonstrated in vivo by Norman and Sjoval (1958), and this has been shown in vitro to be carried out by most genera of intestinal bacteria as with a) above (Portman et al., 1962; Hill and Drasar, 1968 and Aries and Hill, 1970b).
- c) A dehydroxylation reaction which is able to remove the 7α hydroxy group

yielding deoxycholate from cholic acid and lithocholate from chenodeoxycholate. This reaction has been demonstrated in vivo by feeding experiments with labelled bile acids in rats (Norman and Sjovall, 1958 and 1960). Danielsson et al. (1963a), showed a similar effect in feeding experiments with human patients. In vitro, rat caecal contents were shown to dehydroxylate cholic acid and chenodeoxycholic acid to produce deoxycholic acid and lithocholic acid respectively. Aries and Hill (1970b), showed 7 α dehydroxylation by single strains of intestinal bacteria and were able to partially isolate and characterise the enzyme responsible.

d) A desaturation reaction producing a double bond in the steroid nucleus. This type of reaction is postulated to account for the production of 'allo' bile acids, steroids with a 5 α configuration. These have been isolated from the faeces of man (Haslewood, 1967) and from rabbits (Danielsson et al., 1967b). These bile acids are not synthesised in the liver. The introduction of a carbon 4-5 double bond linked to a 3-keto group is postulated, followed by a random resaturation of the intermediate to produce a mixture of 5 α and 5 β steroids. Kallner (1967b), obtained evidence using rat caecal contents that such a pathway exists in vitro.

Norman and Sjovall (1958), isolated an unsaturated and unsubstituted bile acid from faeces after feeding rats with labelled lithocholate. A more detailed appraisal of desaturation reactions occurs in section 4.

3.4. Cholesterol

As indicated in section 3.2., the importance of intestinal bacteria in the metabolism of cholesterol in the large intestine has been demonstrated using germ-free animals (Gustafsson et al., 1966; Kellog and Wostman, 1969). Germ-free animals had a faecal neutral steroid fraction

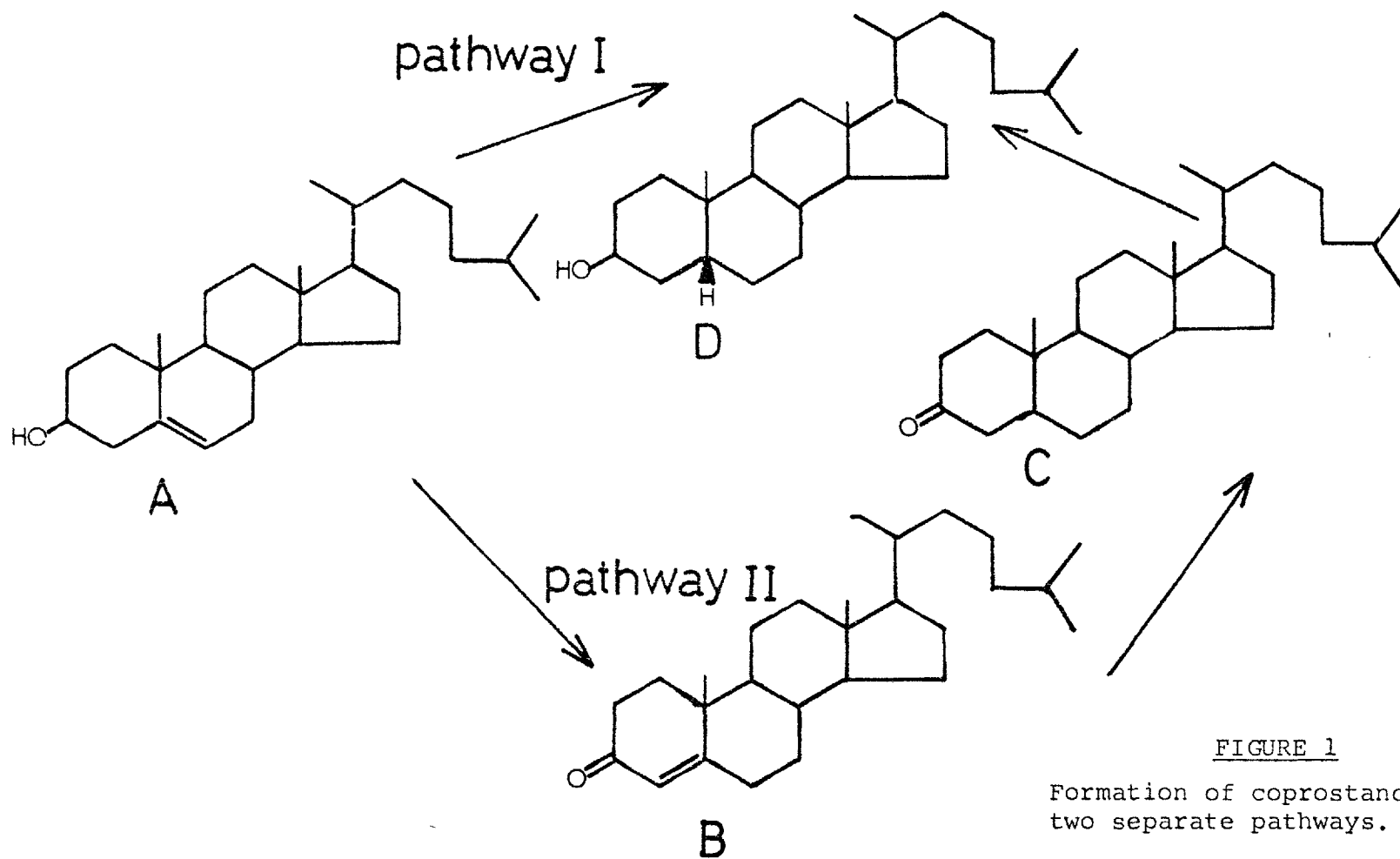


FIGURE 1

Formation of coprostanol by two separate pathways.

containing 89% cholesterol and 11% 7-dehydrocholesterol. Conversion of cholesterol to coprostanol has been demonstrated in vitro by Snog Kjaer et al. (1956). These workers were able to obtain an 80% conversion to coprostanol using human faecal specimens and an 89% conversion using a pure culture of an unidentified anaerobic organism. Coleman and Baumann (1957b), using rat caecal contents, studied a parallel reaction, the saturation of 5,7-cholestadien-3-ol to form 7-cholesten-3-ol; the latter is a major constituent of rat faeces. No other site of synthesis of coprostanol has been reported. Once formed, it is eliminated quantitatively in the faeces as it is an unabsorbable sterol (Iritanic and Wells, 1969).

The mechanism for the saturation of cholesterol to coprostanol has been studied extensively and the two favoured hypotheses are shown in Fig. 1. Pathway 1 involves a direct saturation reaction and Pathway 11, a two step process involving an isomerisation followed by a saturation reaction. Coprostanone, compound C, is found in small quantities in the faeces (Miettinen, 1965; Grundy and Ahrens, 1969). Using tritiated cholesterol ($3\text{ }^3\text{H}$), Rosenfeld et al. (1954), and Rosenfeld and Gallagher (1964), found evidence to support mechanism 1, the direct saturation. This was based on the indication that there was no loss of label from the 3 position in the formation of coprostanol. However, a lot of earlier work by Rosenheim and Webster (1941; 1943), supported mechanism 11 as they showed that coprostanone and 4-cholesten-3-one were readily converted to coprostanol in vivo. Later work by Bjorkhelm and Gustafsson (1971), using ($4\text{ }^3\text{H}$, $4\text{ }^{14}\text{C}$) cholesterol incubated with rat caecal contents, also supported mechanism 11 as the major pathway but indicated that some transformation may be occurring by direct saturation. The difference in mechanisms within the same incubation system probably indicates that different mechanisms are favoured by different

genera of intestinal bacteria.

Total degradation of the steroid nucleus has been implied from work by Grundy and Ahrens (1969), and Rosenfeld and Hellman (1971). In feeding experiments, radioactive cholesterol was lost from the faecal neutral steroid fraction and could not be identified in any steroidal separation.

4. Desaturation of the steroid nucleus

Although there are only a few reports of nuclear desaturation by intestinal bacteria, such reactions have been quite extensively reported in soil organisms and mammalian systems. Four types of reaction will be considered in this section:

- a) Introduction of a double bond in conjugation with a keto group.
- b) Oxidative demethylation of the carbon 19 group linked to the introduction of double bonds to aromatise ring A.
- c) Double bonds introduced in association with other double bonds.
- d) The introduction of a double bond by a dehydration reaction in conjugation with another double bond and keto group as a stabilising factor.

The advantage of studying soil organisms for such reactions is that many may use steroids as their sole source of carbon. Mammalian systems are useful when considering aromatisation reactions by endocrine organs in the production of oestrogens.

4.1. Introduction of a double bond linked to a keto group

Microorganisms which are able to introduce a double bond at the C₁-C₂ position and C₄-C₅ position (see Fig. 2), of a range of steroids with different side chain functions at the C₁₇ position, have been exten-

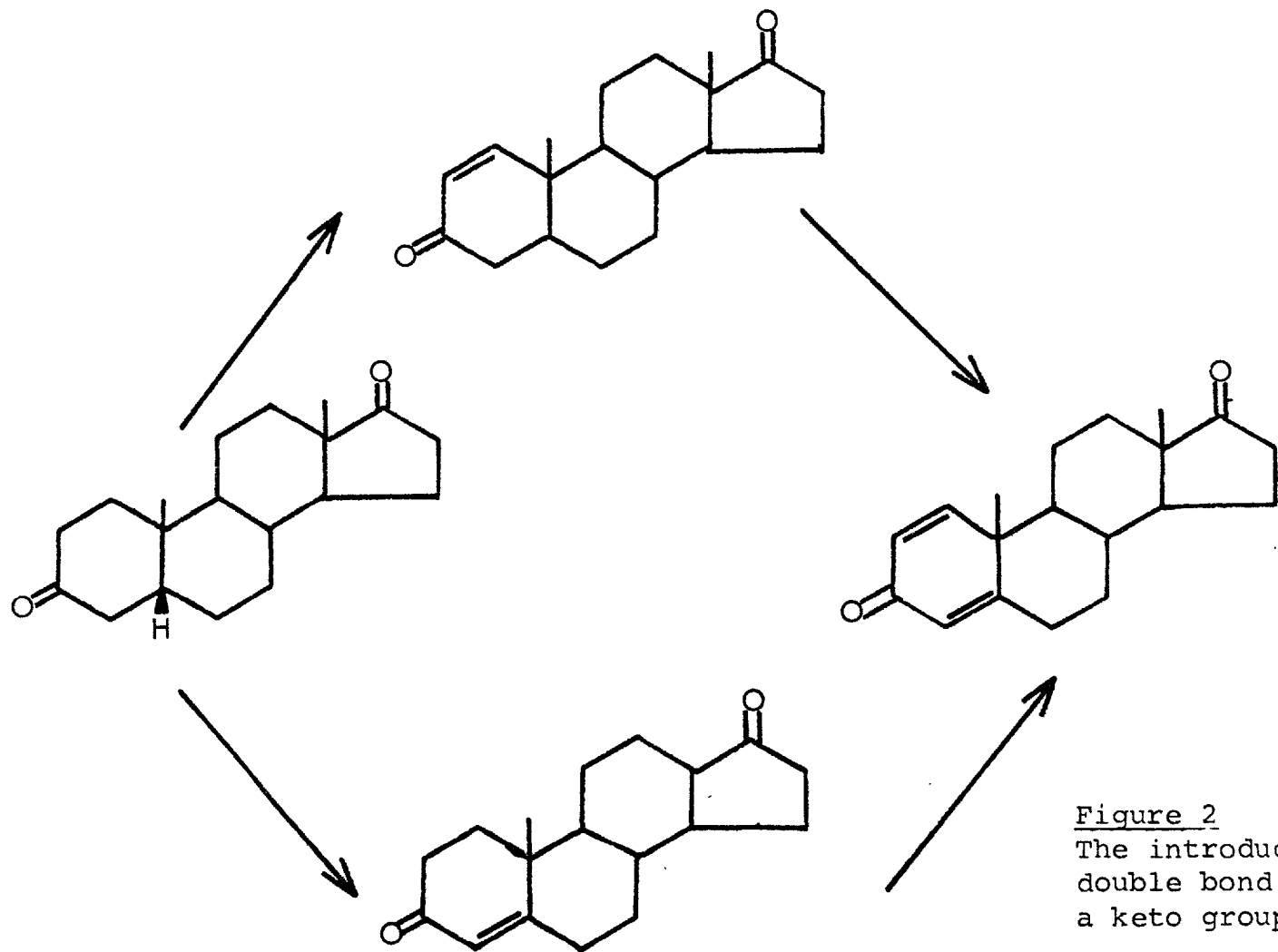


Figure 2
The introduction of a
double bond linked to
a keto group.

sively investigated. Fried et al. (1953), showed that Streptomyces lavendulae and Cylindrocarpum radicola possessed a Δ^1 dehydrogenase which also acted with side chain oxidation. A similar reaction was shown with Fusarium solani (Vischer and Wettstein, 1953). The latter workers also showed that this organism could introduce a C_4-C_5 double bond without side chain modification. Corynebacterium simplex (Nobile et al., 1955) and Didymella lycopersia (Kahnt et al., 1955) have also been shown to possess the Δ^1 dehydrogenase which acts without any associated side chain modification. A general review of these two reactions is given by Talalay (1957).

More recently, Alicyclobacillus faecalis (Wix et al., 1968) and Pseudomonas testosteroni (Davidson and Talalay, 1966) have been shown to possess the Δ^1 dehydrogenase. These enzymes were stereospecific and may act on 5α , 5β or 4-en-3-one substrates. Pseudomonas testosteroni was considered an ideal organism with which to study steroid degradation, as this organism will grow rapidly using C_{19} and C_{21} steroids as its sole carbon source (Levy and Talalay, 1959a). These workers have partly separated and purified a Δ^1 dehydrogenase as described above and the specific $\Delta^4(5\alpha)$ and $\Delta^4(5\beta)$ dehydrogenases (Levy and Talalay, 1959a and b).

The reactions described above all concern androstane or pregnane substrates, but there are a few reports of desaturation of bile acids. Hayakawa et al. (1957), showed that a strain of Streptomyces galatinosus could introduce a C_4-C_5 double bond into the 3-oxo bile acid. Halperin (1954), showed a similar reaction using a species of *Nocardia*. Hayakawa et al. (1958 and 1964) using Streptomyces rubescens and Corynebacteria simplex respectively, showed the formation of a Δ^4 intermediate from cholic acid.

The mechanism for these reactions has been studied in detail. Davidson and Talalay (1966), using a purified $\Delta^4-5\beta$ -dehydrogenase, found that this

enzyme was intracellular and strictly stereospecific for 5β steroids. They also found that a wide range of artificial electron acceptors could be substituted for NAD^+ or NADP^+ in the assay of the enzyme. The enzymic mechanism of the Δ^1 dehydrogenase isolated from Bacillus sphaericus has been studied using isotope exchange techniques (Hayano et al., 1961 and Ringold et al., 1963). They showed that the Δ^1 formation was a 1α - 2β trans-diaxial elimination which took place in two steps. The first was the formation of a C_2 - C_3 double bond by the enolisation of the 3-keto group, followed by the excision of an activated hydride ion forming the Δ^1 bond.

In mammalian systems, these reactions have been little studied. Placental preparations will readily saturate double bonds in steroids. The production of a Δ^4 bond by the mammalian systems occurs mainly by an isomerisation of the Δ^5 bond of cholesterol (Talalay, 1957 and 1965; Moras and Lyall, 1967). Microorganisms will also readily saturate double bonds in steroids under reducing conditions (Davidson and Talalay, 1966; Talalay, 1957 and 1965).

4.2. Oxidative demethylation and aromatisation

The production of phenolic steroids in the mammalian placenta and other hormonal tissue is of great importance in the production of 17β -oestradiol and oestrone, two essential female hormones. Zondek, in 1934, first realised that androgens may be the precursors of oestrogens. Meyer (1955a and b), was able to show, using placental preparations, that 4-androsten- 17β -ol-3-one and 4-androsten-3,17-dione could yield oestradiol and oestrone respectively. He also showed quantitative conversion of 19-hydroxy-4-androsten-3,17-dione to oestrone and a small amount of oestradiol in the placenta. Moreover, the 19-hydroxy substrate was also shown to be

an intermediate in bovine adrenal extracts in oestrogen production. This was a strong indication that in aromatisation reactions, there was a prior modification of the C_{19} methyl group (see Fig. 3).

Soil microorganisms have also been shown to utilise 19-hydroxy-4-androsten-3,17-dione, as a substrate in the production of phenolic steroids. Strains of *Nocardia* and *Pseudomonas* have both been shown to utilise this substrate, (Dodson and Muir, 1961; Sih, 1962). However, Brodie et al. (1968), have suggested that this mechanism is different to that occurring in mammalian systems. Using $1\beta(^3H)$ labelled 19-hydroxy-4-androsten-3,17-dione, they produced evidence that aromatisation occurs in mammalian systems concomitant with the excision of the oxidised methyl group. In the microbial systems studied a prior C_1-C_2 dehydrogenation was suggested. Morato et al. (1962), showed that in mammalian systems there was no loss of a $1\alpha(^3H)$ label which is associated with a C_1-C_2 , $1\alpha,2\beta$ transdiaxial elimination (see section 4.1.).

Townsley and Brodie (1968), also suggested that a nuclear dehydrogenase was not involved in mammalian oestrogen synthesis, as most steroid nuclear dehydrogenase enzymes have a requirement for an artificial electron acceptor such as phenazine methosulphate (Davidson and Talalay, 1966). Placental extracts did not have such a requirement although NADP and molecular oxygen were essential (Townsley and Brodie, 1966). This argument is upheld by the work of Mayer (1955b), who had difficulty in demonstrating the production of even small amounts of oestrone from 4-estren-3,17-diene and 1,4-androstadien-3,17-dione substrates.

Sih et al. (1967a), have shown the production of C_{22} acids from 19-hydroxy-4-cholesten-3-one using strains of *Nocardia restrictus*. He suggested that these compounds were intermediates in the total side chain cleavage

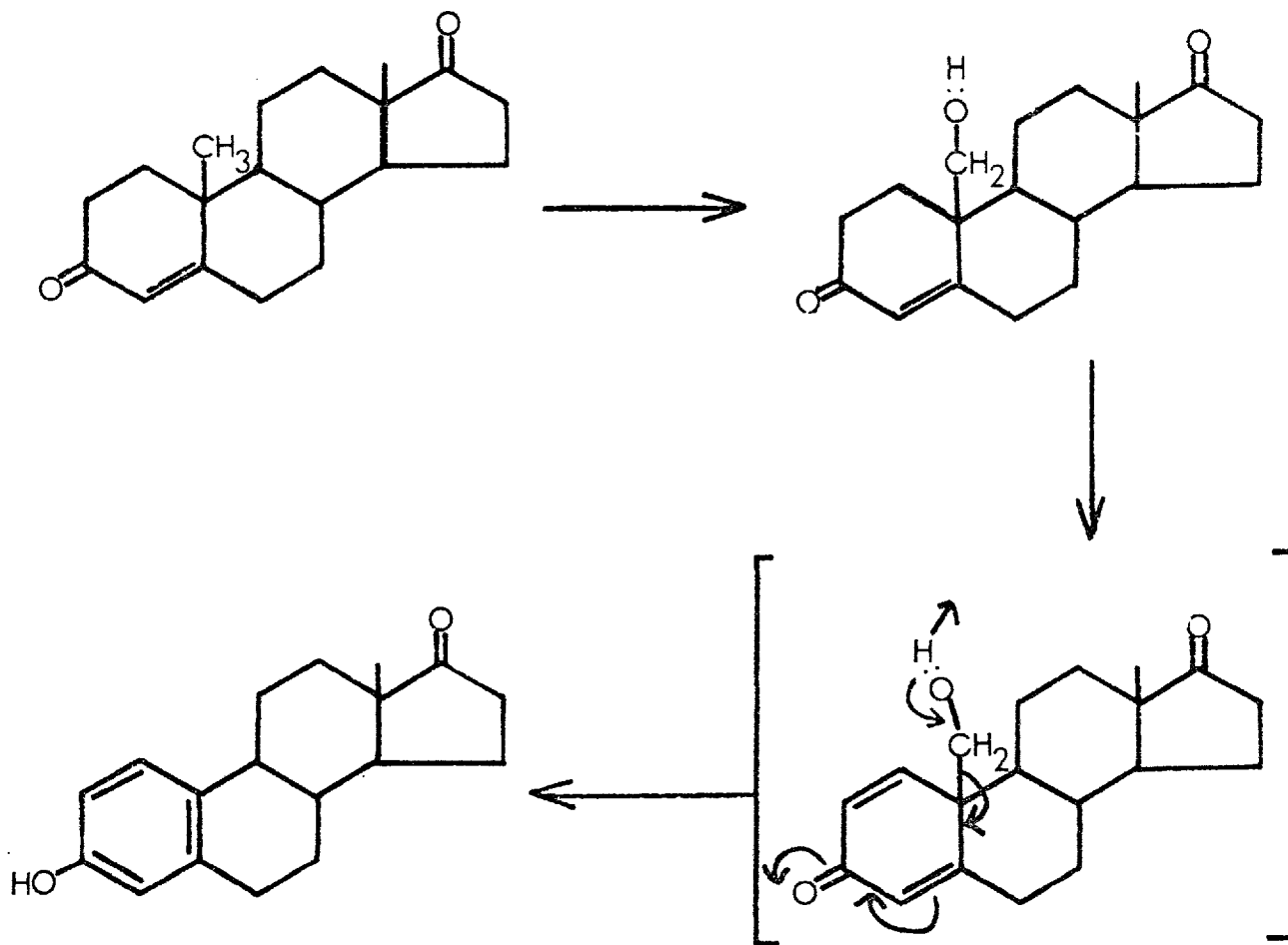


FIGURE 3 The introduction of a double bond linked to oxidative demethylation

of the cholesterol molecule, ultimately yielding oestrone and oestradiol. Sih et al. (1967b), have also shown that the same organisms in a parallel reaction can produce a cleavage in ring B of the steroid, yielding aromatic 'seco steroids'.

4.3. Formation of double bonds in conjugation with other double bonds

Phenolic steroids with varying degrees of desaturation in the B ring have been isolated from equine pregnancy urine (see Fig.4). Equilin(1,3,5, (10),7-estratetraen-3-ol-17-one) was first isolated by Girard et al. (1932a). The fully aromatised compound, equilenin (1,3,5(10),6,8-estrapenten-3-ol-17-one) was also isolated by the same workers from pregnant mares urine (Girard et al., 1932b). Korn et al. (1969), reported the production of a ring B aromatic product, 3B-hydroxy-5,7,9-estratrien-17-one from the $\Delta^{5,7}$ substrate using a phagocytic vacuole extract from an amoeba Acanthamoeba costellani. This compound has also been isolated in pregnant mares urine (Glen et al., 1958). Sachanick and Berlinen (1957), have reported equilin production under pathological conditions in man with a feminizing adrenal carcinoma.

Synthesis of equilin and equilenin in the placenta of the horse involves the $\Delta^{4,7}$ androstadiene as substrate (Starka and Bruer, 1966). Equilin formation could be demonstrated in human placental extracts from a similar substrate in vitro, but as yet there is no evidence for in vivo synthesis under normal conditions (Starka et al., 1966). Using 4,6-androstadien-3,17-dione, these workers also showed the production of dehydroestrone (1,3,5 (10),6-estratetraen-3,17-dione).

Vezina et al. (1969), have shown the production of similar metabolites using 19-hydroxy-4,7-androstadien-3,17-dione as substrate, by a range of

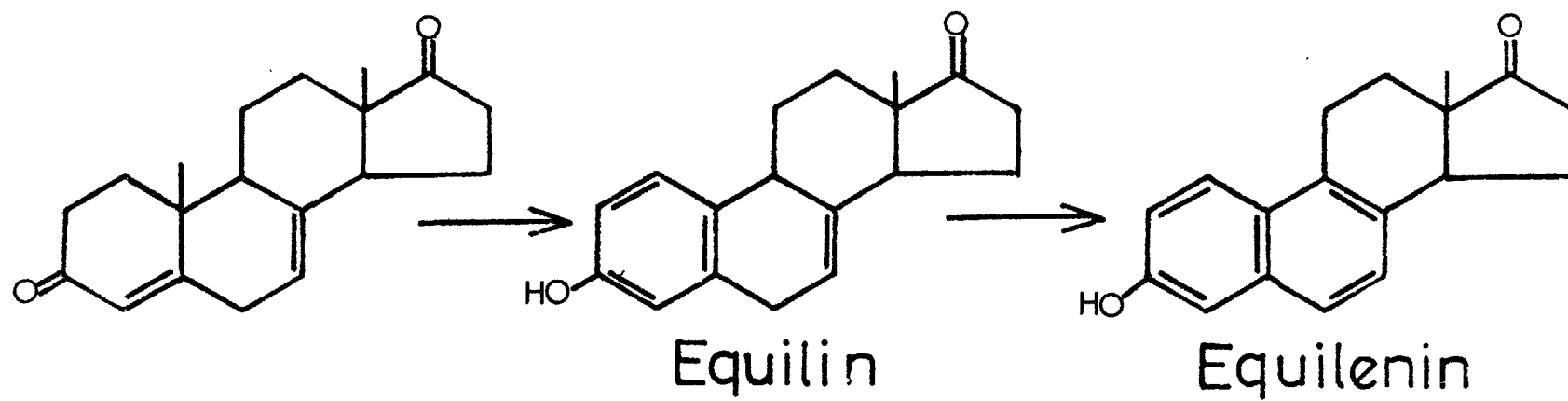


FIGURE 4

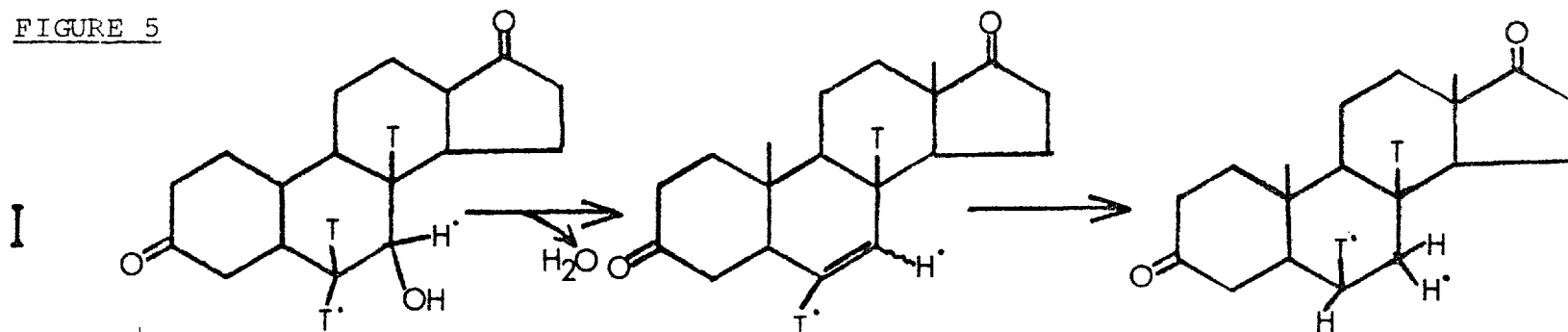
The introduction of a double bond linked to other double bonds

soil fungi and bacteria.

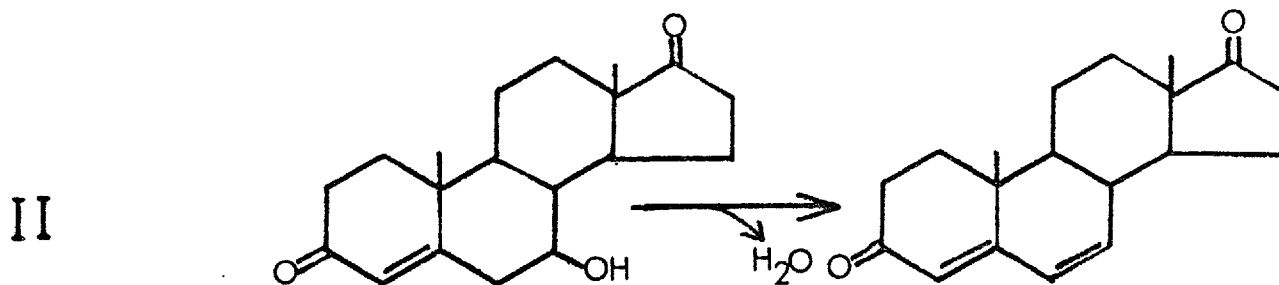
4.4. Introduction of a double bond by a dehydration reaction

Fig. 5 (scheme1) shows the proposed mechanism for the 7 α dehydroxylation of bile acids by intestinal bacteria (Samuelsson, 1960). Using 6 α , 6 β , 8 β tritiated cholic acid fed to rabbits and rats, the redistribution of label in the reisolated steroid obtained suggested a two step process. The first step was hypothesised as diaxial transelimination of water to produce a Δ^6 intermediate. This is followed by a reduction of the intermediate by the trans addition of the two equatorial hydrogens. This intermediate could be stabilised if the dehydration reaction occurred in conjugation with a 4-en-3-one substrate (Fig.5, scheme 2); such a reaction has been reported using Streptomyces rubescans and Corynebacteria simplex (Hayakawa et al., 1958 and 1964). Norman and Palmer (1964), investigating the metabolites from oral doses of radioactive lithocholate, isolated an unsaturated and unsubstituted steroid 'compound B'. It is possible that this reaction may arise from a dehydration reaction involving the 3 α hydroxyl group in a similar manner to that described above for the 7-dehydration.

FIGURE 5



The hypothesis for 7 α -dehydroxylation of a steroid (Samuelson 1960)



The introduction of a double bond by 7-dehydration reaction

5. Intestinal bacteria, bile acids and aetiology of cancer of the large bowel.

The work in this thesis forms part of a larger study on the epidemiology and aetiology of cancer of the large bowel.

5.1. Epidemiology

It is striking that cancer of the large bowel shows a marked geographical variation in incidence (Doll et al., 1966; Doll, 1967 and 1969). This variation is due to a low incidence in underdeveloped countries (eg. 0.6 per 100,000 population per year in Uganda), and a high incidence in the so-called westernised countries such as England (18.2 per 100,000), Scotland (31.2 per 100,000) and the U.S.A. (28.1 per 100,000). Japan and India represent intermediate incidence values at 4.9 and 5.7 cases per 100,000 population per year respectively. These differences do not seem explicable on a racial basis as epidemiological studies have shown that Japanese who become westernised due to prolonged domicile in California (Buell and Dunn, 1965) or following an increase in the standard of living in Japan (Wynder et al., 1969) have a higher incidence of colon cancer than 'normal' Japanese living in Japan. Geographical location can also be discounted as different ethnic groups in Hawaii and South Africa (Doll, 1969) and Singapore (Lee, 1973) show different levels of colon cancer incidence.

However, environmental factors have been closely linked with the incidence of this disease. In earlier studies, Wynder and Shigematsu (1967) showed a correlation with dietary fat intake, and Gregor et al. (1969), showed a correlation with protein intake. A positive correlation with refined carbohydrate and a negative correlation with fibre intake have also been associated with colon cancer incidence and a multitude of

'associated diseases' (Burkett, 1971). Drasar and Irving (1973) have shown a high correlation between colon cancer incidence and socio-economic class. The latter was evaluated using such indicators as income per capita, televisions, cars, etc.. A high correlation was obtained for several dietary factors; combined animal fats, total fat and protein, all being associated at a highly significant level.

Therefore, from the above evidence a dietary influence appears to be strong. The extensive searches for ingested carcinogens in, for example, cooking oils failed to account for this geographical variation; in fact most of the dietary carcinogens have been found in the food of peoples living in low incidence areas.

As the basis of the project at St. Marys Hospital, colon cancer was considered to be best correlated with dietary fat. With an increase in consumption of fat there is a stimulus to bile acid production, and it is then hypothesised that the small percentage of bile acid reaching the large intestine may be modified by the intestinal bacteria to produce a carcinogen-type compound (Hill et al., 1969 and 1971). Steroids, and in particular several bile acids, have been shown to be carcinogenic. Inhoffen (1953), reviewed the structural similarities between steroids and the potent carcinogens of the polycyclic aromatic type. Deoxycholic acid (Chiron, 1939; Cook et al., 1940; Badger et al., 1940; Salaman and Roe, 1956), bis nor-5-cholen-24-oic acid (Lacassagne et al., 1966) and apocholic acid (Lacassagne et al., 1941) have all been shown to be carcinogenic. Deoxycholate can in fact, be converted chemically to the potent carcinogen 20-methylcholanthrene via a non carcinogenic intermediate, dehydronorcholene (Cook and Haslewood, 1933 and Haddow, 1958). Druckery et al. (1941), have shown that gut bacteria can convert dehydronorcholene to an unidentified carcinogenic

product. Heiger (1969), has shown that injected cholesterol may also show carcinogenic properties.

5.2. Geographical variation and faecal bile acid and bacterial composition

To test the above hypothesis, the bacteriology and bile acid composition of faecal specimens from individuals from different countries were estimated (Hill et al., 1971). Faecal samples were collected from the different populations and stored under liquid nitrogen until they arrived at the laboratory. Geographical differences were found in the bacterial composition and especially the degree of steroid degradation.

Good correlations were obtained between the national incidence of colon cancer and the mean values for faecal deoxycholate, total bile acids and total neutral steroids. From this it appeared that dietary factors may be responsible for both the faecal steroid concentration and the degree of steroid degradation. Racial factors did not appear to cause any differential as black and white Americans from Atlanta, Georgia showed no significant differences in faecal composition. Vegans, a strict vegetarian sect in this country were taken as a control group for the English sample as their diet has a low fat content. It was found that both the total faecal bile acid steroid fraction and neutral steroid fraction was significantly lower than those of people on a mixed diet (Aries et al., 1971).

More direct evidence for the effect of fat intake on bile secretion was obtained by Hill (1971). Five healthy volunteers reduced their fat intake to 30g per day (normal level around 120g fat per day) for a period of two weeks (one individual for four weeks). An immediate drop in the level of faecal acid steroids and a slower drop in the level of faecal neutral steroids was observed. Urobilin, a marker compound derived from

biliary bilirubin, remained at a constant level throughout the experimental period. Danielsson (1963) has also shown that faecal bile acid composition may vary with dietary intake.

5.3. Modification of bile acids by intestinal bacteria

There is increasing evidence that individual strains of bacteria are implicated in vitro and in vivo in the modification of bile acids. Aries et al. (1969) and Aries and Hill (1970a), showed the deconjugation of conjugated bile acids to produce free acids by individual strains of intestinal bacteria. Aries and Hill (1970b), showed the modification of the 3 α , 7 α and 12 α hydroxyl groups to ketone functions and the complete removal of the 7 α hydroxyl group. Lewis and Gorbach (1972), extensively reviewed all the material available so far for the in vitro studies carried out on these reactions. Gustafsson et al. (1968), having isolated bacterial strains metabolically active in vitro, mono-contaminated germ-free rats to demonstrate the reactions in vivo. Dickenson et al. (1971), who carried out similar work have indicated that such an extrapolation from the in vitro to the in vivo situation is not always valid. Some strains active in vitro failed to show a similar reaction when contaminating a germ-free animal. Kellogg et al. (1971), also reported some similar discrepancies.

A geographical variation in the activity of the 7 α dehydroxylase enzyme has been described (Hill et al., 1970). High activity in a large number of strains was found in isolates from countries with a high incidence of cancer of the colon, in contrast to the low incidence areas such as Uganda and India.

Further enzymic degradation of the steroid nucleus by desaturation to form a carcinogen of the 20-methylcholanthrene type is theoretically possible

(Hill, 1970 and Hill et al., 1971). To account for this, four types of reaction have to be considered. These were detailed in the last section and are summarised below:

- a) Introduction of a double bond in conjugation with a keto group.
- b) Introduction of a double bond by a dehydration reaction.
- c) Oxidative demethylation and aromatisation.
- d) Introduction of a double bond in conjugation with other double bonds.

SECTION II

MATERIALS AND METHODS

1.

Bacteriological Methods

1.1. Growth media and cultural methods

Growth media unless otherwise stated were made up from dehydrated powders supplied by Oxoid Ltd.. Sterilisation of Todd Hewitt broth was for 10 minutes at ten pounds pressure but all other media were autoclaved at fifteen pounds pressure for 20 minutes. Solid media, which included Willis and Hobbs agar, blood agar, MacConkey agar and Dorset egg medium (Southern Group Hospitals Ltd.) were prepared as described by Cruikshank (1967).

'Rich Blood agar', used for the growth of the fastidious non-sporing anaerobes (species of *Bacteroides* and *Bifidobacteria*), contained 52.5g reinforced clostridial agar powder and 10g liver digest, made up to 1 litre with distilled water to which was added 100ml horse blood.

The liquid media used included Todd Hewitt broth and glucose broth as described by Cruikshank (1967). A peptone/tryptone enriched media was made as described by Hungate (1960). This contained yeast extract, resazurin, vital inorganic salts, cysteine hydrochloride and haemin in addition to peptone and tryptone.

Acetone-extracted calf brain medium (Coleman and Baumann, 1957b) was prepared using 5kg of fresh beef brain homogenised in a 'Waring' blender and extracted with ten litres of cold acetone for 24 hours. The acetone was decanted from the solid brain material and was extracted for 24 hours with a further ten litres of acetone. A total of eight extractions was required to remove all sterols from the solid residue. The powder was dried and made up as a 2% suspension with 0.5g K_2HPO_4 , 5 drops Tween 80 and 1ml of an inorganic salt solution per 100ml distilled water. The salts contained 10g magnesium sulphate, 0.5g sodium chloride, 0.5g ferrous

sulphate and 0.5g manganese sulphate in 250ml of distilled water. The suspension was dispersed by treatment for 20 seconds in an M.S.E. 100W ultrasonic disintegrator at an intensity of 6 μ M. This medium was used for the investigation of bacterial degradation of cholesterol.

Growth Conditions

For the obligate anaerobic bacteria, broths were steamed before inoculation to remove dissolved oxygen from the medium. These bacteria were incubated at 37°C in a modified milk churn containing an atmosphere of 90% hydrogen, 10% carbon dioxide and fitted with a 'D' catalyst to remove remaining traces of oxygen. Lactobacilli were incubated in an atmosphere of 100% carbon dioxide (Drasar, 1967), as these bacteria have an absolute requirement for this gas.

Strains of bacteroides and bifidobacteria were stored in Robertsons cooked meat medium (Southern Group Hospitals Ltd.) with 10% v/v added glycerol at -60°C (Crowther, 1971b). Short term storage of these strains (three to four weeks) was possible in Robertsons cooked meat maintained at 4°C. It was important when thawing out the anaerobes from -60°C to subculture them quickly into further cooked meats. Failure to observe this procedure led to the loss of the strain. Clostridia were maintained in Robertsons cooked meat at 4°C.

Cultures, especially of the strict anaerobes, were regularly checked by Gram staining for contamination by other bacteria. Contaminated cultures were either plated out and the strain reisolated or in the case of bad contamination the strain was discarded.

1.2. Bacterial Strains

Clostridia: Lecithinase-negative Clostridia were isolated from human faecal specimens by the following technique. Serial dilutions were made from 10^{-2} - 10^{-5} in half strength Ringers medium and the tubes were heated for 10 minutes at 70°C to kill vegetative organisms and to activate the spores. By this treatment almost all genera were killed and only Clostridia and bacilli remained. 0.1ml of each dilution was spread on Willis and Hobbs plates and incubated at 37°C for 72 hours under anaerobic conditions. Fifteen colonies of lecithinase-negative bacteria were picked from a suitable plate and were streaked on individual Willis and Hobbs plates as a purification step. These were incubated anaerobically for a further 72 hours. From these plates a single colony was picked and inoculated both into 10ml of a 3% thioglycollate broth (Oxoid Ltd.) and onto a blood agar plate. Both were incubated aerobically for 24 hours, the reducing agents in the thioglycollate broth maintaining anaerobiosis without the aid of a hydrogen atmosphere, allows the growth of the anaerobes. The blood plate acted as an aerobic check and any growth after 24 hours indicated the presence of contaminating aerobic bacilli and the strain was discarded.

If no growth appeared in 24 hours the strain was identified according to the tests listed in Table 3. The method of classification of clostridial strains was that of Holdeman and Moore(1972). These strains were also tested for their ability to desaturate steroids (see Methods 6.1.)

Other Strains: N.C.T.C. 9343 a Bacteroides fragilis was provided by the National Collection for Type Cultures, Colindale.

All other strains were isolated by bacteriologists in this labora-

TABLE 3

Criteria for the Identification of Clostridia (after Moore and Holdeman
(1972).

<u>Test</u>	<u>Conditions</u>
<u>Sugar Fermentation to</u> <u>acid Products</u>	Aesculin, Fructose, Glucose, Positive test Lactose, Mannitol, Maltose, shown by bromo- Mannose, Xylose, Sucrose cresol indicator and Starch. Yellow when acid
<u>Glycoside Hydrolysis</u>	Aesculin — 1% Ferric chloride gives a black complex for hydrolysis. Starch — 1% iodine gives a black indicating no hydrolysis.
<u>Acid Products of Glucose</u> <u>Fermentation.</u>	Products of glucose fermentation separated by Gas Liquid Chromatography. Acidify the grown culture and extract with ether. Identification of acetic, butyric, propionic, caproic and other minor acids. After methylation of extract, lactic and succinic acids detected.
<u>Detecting Gelatine Hydrolysis</u>	Positive test shown by the digestion of a gelatine disc within three days from afresh inoculum of the strain into a Robertsons cooked meat medium, and incubation at 37°C.
<u>Indole Production from</u> <u>Tryptophan.</u>	Addition of 1 ml of Kovacs solution, one week after inoculation into a Robertsons cooked meat. A red complex in separated upper layer indicates an indole reaction.
<u>Gram Stain</u>	Appearance - a Gram positive pleomorphic rod

tory, used the methods described by Drasar (1967), and identified as described by Cato et al. (1970)

2. Detection and Identification of Steroids

2.1. Thin layer chromatography

Thin layer plates were prepared using silica gel G or silica gel 254 (Merck Ltd.). For qualitative experiments these were poured on 20cm x 20cm glass plates using an 'Unoplan' spreader (Shandons Scientific Co. Ltd., London.) set at 0.5mm thickness. For preparative experiments the thickness of the plates was 0.75mm. Plates were dried at 110°C for 2 hours after pouring and were reactivated for half an hour at 110°C just prior to use. Commercially prepared pre-poured plates were later used as they gave better resolution. These were Polygram Sil G and Polygram G U.V./254, thickness 0.25mm, supplied by Camblab Ltd., Cambridge.

Solvents used to develop thin layer plates were of two types,
a) those described by Eneroth (1963); they will be referred to using his nomenclature and have an S prefix.

b) those described by Aries (1970), namely

V ₁	Benzene/dioxane/water	100/10/1
V ₂	Trimethyl pentane/ethyl acetate/acetic acid	75/45/1
V ₃	Chloroform/methanol/hexane	99/1/1
BA	Chloroform/acetone/acetic acid/water	7/2/1/0.1
NS	Hexane/chloroform/acetone	10/9/1

V₁, V₂ and V₃ were used for the separation of androstanes and oestrogens. BA was used for general bile acid separation and NS for neutral steroid separations.

Table 4 shows the means for detection and visualisation of steroids on thin layer plates. Apart from the steroid specific sprays, a useful method of detection of certain unsaturated steroids was the absorbance of UV light under a Hanover 254 lamp.

Preparative and Quantitative T.L.C.

Preparative T.L.C. and quantitative estimations involved development of the plate, followed by an elution step. For preparative T.L.C. the sample was streaked along the length of the plate and marker spots placed at one end. After the compounds had been separated by the developing solvent, the steroids of the marker and the edge of the preparative strip were visualised using the appropriate spray (see Table 4). The strip containing the required compound was then removed from the plate and the steroid eluted from the gel by shaking with chloroform or ethanol. Preparative T.L.C. was used in the purification of chemically synthesised compounds or the separation and purification of bacterial metabolites; up to 500mg of a compound could readily be isolated by this procedure by pooling the eluent from twelve plates.

For quantitative T.L.C. a specific volume of sample was spotted on a thin layer plate from a micropipette, and the compound located using marker compounds either side of the unknown, and eluted as above.

Two Dimensional T.L.C.

On occasions it was necessary to use two dimensional chromatography when the usual method was insufficient to separate a metabolite from artifacts. In addition, two dimensional chromatography was used to identify purified metabolites; the metabolite was mixed with an authentic sample of a known compound and separation by chromatography proved lack of identity. This was used for the separation of phenolic steroids, solvent V_1 to

TABLE 4

Sprays for Visualising Steroids Separated by T.L.C..

<u>Spray.</u>	<u>Specificity</u>	<u>Colour</u>	<u>Reference</u>
<u>Potassium Ferricyanide</u>	Oestrogens,	Blue	Burton, (1953)
<u>Folins</u>	phenols with	Blue	Mitchell, (1953)
<u>Sulphanilic Acid</u>	free OH gp..	Red	Mitchell, (1953)
<u>p-Nitrofluorobenzene-</u> <u>diazoniumtetrafluoroborate</u> <u>(P.N.B.D.)</u>	Oestrogens Equilins	Brown Red	Vezina <u>et al.</u> , (1969)
<u>Anisaldehyde</u>	All Steroids	(see Aries 1970)	Kritevsky and Kirk, (1952)
<u>Phosphomolybdate</u>	Non specific	Black	Kritevsky and Kirk, (1952)
<u>U.V. Light</u>	Desaturated ketones	Black on on fluorescent U.V.254 plate	Bush, (1952)

develop the X axis and solvent V_3 for the Y axis.

Radioactive scanning of the T.L.C. plates

Thin layer chromatograms of radioactive products were scanned using a "Panex" radioactive scanner fitted with a propane/argon gas detector. This could be used in both quantitative or qualitative preparations.

2.2. Gas liquid chromatography

Bile acid methyl esters, neutral steroids, androstanes and oestrogens were separated on a Pye Unicam 104 Gas Chromatograph fitted with a flame ionisation detector and a 5ft x $\frac{1}{4}$ inch column of S.E.30 coated on 100-120 diatomite, at 230°C. Fatty acids produced by bacterial fermentation of glucose, used in the identification of Clostridia (see Table 3), were separated on a column of polyethylene glycol + 10% phosphoric acid packed on diatomite, at 130°C. Succinic and lactic acids had to be methylated with diazomethane, prior to separation, since the unesterified acids were degraded on the column.

2.3. Infra red spectroscopy (I.R.)

Infra red spectra were recorded on a Pye Unicam S.P. 1200 scanning spectrometer. Solutions of the purified compound were made at a concentration of about 10mg/ml in Aristar grade solvents (B.D.H.). Solvents used were carbon disulphide, chloroform, carbon tetrachloride and ethanol. The solutions were scanned in potassium bromide cells, pathlength 1mm and read against a blank containing solvent only. To obtain a base line and to establish how well the cells were matched a 'spectrum' of solvent against solvent was made prior to each run.

2.4. Ultra violet spectroscopy (U.V.)

U.V. spectra of absolute alcoholic solutions in quartz cells, path length 1cm were obtained using a Pye Unicam S.P. 8000 scanning spectrometer or a Pye Unicam S.P. 500 spectrophotometer

2.5. Spectrofluorimetry

Fluorimetric spectra were obtained on a Perkin Elmer 204 scanning spectrofluorimeter using quartz cells, 1cm x 1cm. Solutions were made in absolute ethanol at a concentration of approx. 4ug/ml.

2.6. Melting Points

Melting point estimations were carried out using a melting point block apparatus and were uncorrected.

2.7. Liquid scintillation counting

Aliquots of 0.1 - 1.0ml aqueous solution or the dried residues from an organic solution were added to 15ml of "Brays scintillation mixture" which contains 300g naphthylamine, 20g 2,5-diphenyloxazole (P.P.O.), 1g 1,4-di(2(5-phenyloxazolyl)) benzene (P.O.P.O.P.), 500ml anhydrous methyl alcohol, and 100ml ethenadiol made up to 5 litres with dioxane.

Blood samples were treated by placing 0.1ml of a sample into a vial with 1.5ml Solvene 100/Isopropanol mixture (1:1) to which was added 0.5ml 30% H_2O_2 . After shaking gently for one hour, 15ml HCl/Instagel mixture (Packard Ltd.) 0.5M HCl/Instagel 12:88 was added and the mixture shaken gently and then counted.

Tissue samples were prepared by adding 0.4ml of a 10% homogenate to 0.8ml water and 10ml Instagel and shaking gently; they were then ready to

be counted.

Scintillation vials were counted in a Packard Tricarb liquid scintillation counter (Packard Instruments Co.) and corrected for quenching by the internal channels ratio method (E. Bush, 1963).

2.8. Mass spectrometry

Samples of steroids were dissolved in analar methanol (B.D.H.) to a final concentration of 1 μ g/ml. The spectra were obtained using a direct probe injection onto a Varian CH₅ mass spectrometer, fitted with U.V. oscillographic recorder and photographic bar graph print out systems. Mass scans were from $m/e = 1$ to $m/e = 600$, at operating temperature between 90°C and 200°C, resolution from 500 up to 5,000 and ionisation energy of 70 eV. For some compounds (where stated) a clearer spectrum was obtained using a linked gas liquid chromatography system fitted with an S.E.30 column. Peaks were split by a standard vapour splitter, half the effluent from the column going to the mass spectrometer and half to the flame ionisation detector.

Trifluoroacetyl derivatives were prepared by their reaction with trifluoroacetic anhydride for 15 minutes and subsequent evaporation of excess reagent. Trimethylsilyl derivatives were prepared by the reaction with Bis-nor-trimethylsilyl acetamide; 0.1ml of the reagent was added per milligram of steroid. Derivatives were purified by thin layer chromatography.

The spectra obtained were used for confirmation of identity of a bacterial or chemically synthesised product by comparison with the commercially prepared sample. Alternatively they were also used for the analysis of unknown metabolites. Spectra were characterised by ascertain-

ing the mass of the parent ions and the major mass fragments. The "cracking" patterns of bacterial metabolites and authentic compounds were compared for confirmation of structure.

3. Synthesis of Bile Acids and Androstane Intermediates

Bile acids that had been chemically synthesised as described below, were purified by preparative thin layer chromatography in two solvents. The composition of the eluted compound was confirmed or identified by some or all of the previous techniques.

3.1. Bile acid methyl esters

The C₂₄ carboxyl group of the bile acids was methylated using diazomethane in ethereal solution. prepared by the method of De Boer and Bracker, (1954) and stored at 4°C for not more than three weeks.

The bile acids were treated with diazomethane until there was a persistent yellow colour in the solution, indicating excess unreacted methylating agent. The methyl esters were separated from the bile acids by T.L.C.

To recover the free bile acid, the methyl ester was saponified using 2N NaOH in 80% ethanol at room temperature overnight or at 80°C for one hour. The resulting solution was then acidified and the steroids extracted with one volume chloroform/methanol (2:1), followed by one volume of chloroform. The pooled extracts were evaporated to dryness to yield the free acid.

3.2. Oxo bile acids

3-oxo compounds were synthesised to be used as the substrate for enzymatic desaturation of steroids. Compounds of the androstane type as well as those with cholanyl and cholesteryl type side chains at the carbon 17 position were prepared by similar methods.

For parent compounds substituted only at the C₃ position (i.e..3 α -hydroxy-5 β -cholan-24-oic acid and 3 β -hydroxy-5 β -cholestanol) the chromic acid oxidation described by Bergstrom and Haslewood (1939) was used. 5g of bile acid were dissolved in 350ml glacial acetic acid and 25ml water and cooled to 4°C. 2.5g chromic acid in 25ml acetic acid and 25ml water were added slowly from a dropping funnel with stirring at 4°C. The reaction mixture was left for 17 hours (overnight) at that temperature, then 1.5 litres of water was added slowly with stirring to precipitate the bile acids. The precipitate was filtered under reduced pressure, dried and re-precipitated from methanol.

This method may be used on bile acids substituted with 7 α or 12 α hydroxyl groups, but the 3 position is oxidised only after prior production of the 7 and 12 keto groups. Consequently, a chromic acid oxidation of cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid) produced a mixture of products with varying degrees of oxidation.

Only fully oxidised products were used from this synthesis i.e. 3,12 dioxo, 3,7 dioxo and 3,7,12 trioxo derivatives from deoxycholic, cheno-deoxycholic and cholic acids respectively. All these compounds were separated as described by Eneroth; their relative metabolites were checked with previously synthesised compounds and the values given by Eneroth (1963). Infra red spectra of the compounds were compared with those of authentic compounds kindly supplied by Dr. G.A.D. Haslewood to Dr. V.

Aries of this department. The mass spectrum of the 3-keto-5 β -cholan-24-oic acid showed a parent ion of $m/e = 373$ equivalent to the molecular weight of the compound.

For the specific oxidation of the 3 α hydroxyl group of di- or tri-substituted bile acids, the Oppenheimer type oxidation described by Jones et al. (1949), was used. 1g of bile acid methyl ester was refluxed for 18 hours with 1.2g aluminium tertiary butoxide and 12.5ml methyl ethyl ketone in 30ml benzene. It should be emphasised that the reaction required extremely dry conditions to obtain a good yield. The presence of any water produces the hydroxide from the tertiary butoxide which inhibits the reaction. Therefore, the following precautions were taken to ensure dry conditions.

- a) Methyl ethyl ketone was dried by stirring overnight with anhydrous sodium carbonate, filtered and added directly to the reaction vessel.
- b) The methyl bile acid was dessicated overnight before dissolving in 120% of the required volume of benzene. By distillation of the extra 20% benzene a dry solution was obtained.
- c) All glassware was dried in an oven at 150°C for one hour and then allowed to cool in a dessicator.
- d) The reflux condenser was fitted with a calcium chloride drying tube.
- e) The methyl ethyl ketone tended to become discoloured with age. This was rectified by distilling the liquid over at 80°C and the clear distillate was then dried as in (a).

The refluxed solution was poured with stirring onto 20ml of 2N sulphuric acid. The benzene layer was then separated and washed twice with 2N sulphuric acid, twice with water, twice with 5% sodium carbonate and again with water. The solution was then dried overnight with sodium sulphate and evaporated to dryness.

Yields from both cholic acid and chenodeoxycholate varied from batch to batch, optimum yields for both being between 60% and 70%. Products were either separated on a 20g Alumina column as described by Berseus et al (1965) or by thin layer chromatography. These products were identified by comparison with authentic compounds by infra red spectroscopy, T.L.C. mobility and mass spectrometry. A useful guide was the red colour obtained on the T.L.C. with anisaldehyde reagent, usually characteristic of the 3-keto compounds (Aries, 1970).

3.3. Bile acids with β substituted hydroxyl groups

A solution of the keto acid was treated with an excess of sodium borohydride in ethanol at room temperature. The inorganic material was removed by filtration and the remaining ethanol solution was evaporated to leave a mixture of α and β substituted acids. This was used especially in the synthesis of isolithocholate (3 β -hydroxy-5 β -cholan-24-oic acid) from lithocholate (3 α -hydroxy-5 β -cholan-24-oic acid).

3.4. Unsaturated Steroids

Table 5 shows a summary of the unsaturated steroids, whose syntheses are described below, and some of the typical properties for those compounds used in their identification.

4-en- Δ^3 -one synthesis

Desaturation of the mono-substituted bile acid lithocholate, was by the bromination of the carbon 4 position (Reigal and Macintosh, 1944), followed by dehydrobromination with lithium carbonate and lithium bromide mixture (Joly and Warnant, 1958).

To introduce a C₄-C₅ double bond into a di- or tri-substituted bile

TABLE 5

The General Chemical and Physical Properties Used in the Identification
of Chemically Synthesised Desaturated Steroids.

<u>Compound</u>	<u>Absorbance</u>	<u>Colour With</u> <u>Anisaldehyde</u>	<u>UV. Spectrum</u>	<u>Extinction</u>	<u>Mpt °C</u>
	<u>Under</u> <u>U.V. 254 Lamp</u>		<u>λ_{max}, nm</u>	<u>Coefficient</u>	
1-androstene	+	Mauve	231	10,000	-
1-cholenic	++	Mauve	231	10,000	--
4-cholenic	++	Rust/Orange	240	15,000	-
1,4-androstadiene	++	Green	244	12,500	-
1,4-choladiene	++	Rust	244	12,000	-
1,4-cholestadiene	++	Brown/Orange	244	12,500	-
4,6-androstadiene	-	Red/Mauve	284	24,000	168-170
4,6-choladiene	-	Mauve	284	24,500	182-183
4,6-cholestadiene	-	Mauve	284	24,000	80-82

acid, a selenium dioxide oxidation was used (Berseus et al., 1965) since 7 α hydroxyl and 12 α hydroxyl groups are acid labile and would therefore not resist bromination (Kallner, 1967a).

Compounds were identified by their relative mobilities on thin layer chromatography and their absorbance under a U.V. lamp when run on fluorescent plates. Their U.V. spectrum gave $\lambda_{\text{max}} = 240\text{nm}$ $E = 15000$. Confirmation of the identification was by mass spectrometry.

1-en -3-one and 1,4-dien -3-one synthesis

This reaction was catalysed by freshly activated DDQ (2,3dichloro 5,6 dicyano 1,4benzoquinone) obtained by recrystallisation of the commercial product from petroleum ether (60-80°C fraction). The red crystals were filtered off under reduced pressure, dried and used the same day (otherwise the compound reverted to the brown inactivated form).

3-oxo-5 β -steroid was treated with 50% DDQ w/w in dry benzene under reflux for 18 hours. To ensure dry conditions a calcium chloride drying tube was fitted to the reflux condenser. The bile acid substrate was methylated prior to refluxing to protect the C₂₄ carboxyl group. The reaction mixture was then cooled and evaporated under reduced pressure. The residue was redissolved in diethyl ether and this solution was washed twice with 2N sulphuric acid, twice with 5% sodium carbonate and finally with water. The ether fraction was then evaporated again and the products separated by preparative T.L.C. using authentic compounds as markers. Bile acids were demethylated with 2N sodium hydroxide prior to separation.

The products produced in this reaction were 3-oxo-1-ene, 3-oxo-4-ene, 3-oxo-1,4-diene, 3-oxo-1,4,6-triene and 3-oxo-4,6-diene. An identical procedure was used for the synthesis of desaturated products from androstane, cholanoyl and cholesteryl substrates.

U.V. spectra of the products reflected the A ring structure and not that of the side chain. Hence, bile acids and their androstane analogues produced similar spectra. With the double bond at the '1' position $\lambda_{\max}$ was 231nm, $E = 10,000$. For the 4-ene and 1,4-diene $\lambda_{\max}$ was 240nm and 244nm ($E = 12,500$) respectively. Criteria for the identification of the 4,6-diene are given below. Relative mobilities of these compounds were compared with authentic compounds on thin layer chromatography. For the androstanes, V_1 and V_3 , and bile acids, S_{15} and V_1 developing solvents produced identical relative mobilities. The 1,4,6-triene was not characterised.

4,6-diene steroids

These were prepared by the method of Sondheimer et al. (1953 a and b). 1g of the 3β -hydroxy-5-ene steroid was heated under reflux for 24 hours with 10g activated manganese dioxide in 100ml benzene. The activated manganese dioxide was prepared by the method of Manceron, (1953). The activated compound was used directly after its synthesis for maximum efficiency.

The three unsaturated steroids prepared were (a) 3-oxo-4,6-choladien-24-oic acid from 3β -hydroxy-5-cholen-24-oic acid, (b) 3-oxo-4,6-cholestadiene from cholesterol (3β -hydroxy-5-cholestene) and (c) 4,6-androstadien-3,17-diene from 3β -hydroxy-5-androsten-17-one. Criteria for identification were T.L.C. mobilities, their U.V. - $\lambda_{\max} = 284\text{nm}$ ($E = 24,000$) together with their insensitivity to phenol specific sprays. The 4,6-androstadien-3,17-dione had a melting point of 172°C - 173°C . Djerassi et al. (1950) reported a melting point of 168°C - 170°C for this compound. The 4,6-cholestadien-3-one had a melting point of 80°C - 82°C and no depression was observed when a mixed melting point was carried out using the authentic compound (Swartz-

Mann Ltd.). Confirmation of the 3-oxo-4,6-choladien-24-oic acid was by mass spectrometry.

3.5. Methylated derivatives of 1,3,5(10)-estratrien-3,17 β -diol (oestradiol).

- a) 1-methyl-3-hydroxy-1,3,5(10)-estratrien-17 β -ol and 1-hydroxy-4-methyl-1,3,5,(10)-estratrien-17 β -ol.

These compounds were prepared by a diene-phenol rearrangement described by Drieding (1953). 0.5g of 3-oxo-1,4-androstadien-17 β -ol was dissolved in 6ml of 48% hydrogen bromide and was allowed to stand at room temperature (20°C) for five days. During this time a precipitate formed and on heating the mother liquor for 24 hours at 55°C a full black precipitate was obtained. On washing with water this turned grey-white. The two compounds were separated by heating the crude product at 100°C in 5N NaOH for two hours.

A precipitate of the alkali insoluble 1-hydroxy-4-methyl compound formed and was removed by filtration; acidification of the alkaline solution yielded the 1-methyl;3-hydroxy compound. Overall yield was up to 50% being distributed roughly equally between the two products.

The products were identified by their U.V. spectra $\lambda_{\max} = 280\text{nm}$ ($E = 2,000$) and their reaction with phenol specific sprays on a T.L.C. plate (blue spot with folins solution and ferric chloride spray reagent). I.R. spectra showed the distinctive large peak at $11.67\mu - 11.76\mu$ for the 1-methyl derivative and $12.28 - 12.45\mu$ for the 4-methyl derivative, both described by Drieding (1953). Relative mobilities in solvent V_1 were 0.5 for the 1-methyl and 1.5 for the 4-methyl derivatives (mobilities relative to testosterone). Mass spectra for both compounds showed a parent ion, $m/e = 286$, equivalent to their molecular weights. The main difference

in the spectra was that the 4-methyl group was more labile giving a much larger peak at $m/e = 271$ than the 1-methyl derivative.

Methylation of oestradiol with dimethyl sulphate

Using dimethyl sulphate the 3-methoxy, 17-methoxy and 3,17-dimethoxy derivatives of 1,3,5,(10)-estratrien-3,17-diol were obtained. 0.5g oestradiol was added to 0.5ml dimethyl sulphate (B.D.H.). For exhaustive methylation the reaction mixture was incubated at room temperature for two hours. For partial methylation to produce the 3-methoxy or 17-methoxy products, the reaction was stopped after 15 minutes by extraction with diethyl ether (Vogel, 1957).

Identification of these products is dealt with in detail in a later section (see Results 2.2.).

4.

Enzymological Methods

4.1. 3-oxo-5 β -steroid Δ^1 dehydrogenase and 3-oxo-5 β -steroid Δ^4 dehydrogenase

Screening

Species of Bacteroides, Bifidobacteria, Lactobacilli, Enterococci, Enterobacteria and Clostridia were screened for enzyme activity. Two enzyme assay systems were used, both having a) and anaerobic step and b) an aerobic step.

a) 50ml Todd Hewitt broth in a 150ml container was inoculated with 0.2ml bacterial suspension from a Robertsons cooked meat. The broth contained 0.01% 5 β -androstan-3,17-dione, added as 0.5ml of a 1% solution in ether as inducer. Broths were steamed before inoculation to remove the solvent and to aid dissolution of the substrate. Steaming also helps to reduce the E_h

by expelling dissolved oxygen thereby making the broth more conducive to the growth of strict anaerobes.

Broths were incubated anaerobically in converted milk churns (see Methods 1.1.) for 72 hours at 37°C. Enterobacteria and Enterococci were grown both aerobically and anaerobically.

b) After the above induction stage, assay was by two systems. The more commonly used was the "shaking" system. A further 0.01% substrate was added to the grown cultures and the pH was adjusted with sterile 2N NaOH to 7.5. The culture was then shaken for a further 72 hours on a "Brunswick" shaker (arbitrary setting 3.5). It was then extracted with one volume chloroform/methanol mixture (2:1) and a further volume of chloroform. The pooled extracts were evaporated to dryness and the residue dissolved in a small volume of solvent and spotted on a thin layer plate. Products were primarily identified at this stage by their mobility in developing solvent V_1 and compared with samples of authentic compounds.

The second assay system used a washed cell suspension of the grown culture. This assay system could detect the formation of metabolites in 6 hours, but incubation was for up to 24 hours to demonstrate maximum conversion rates. Bacterial cells from the anaerobic induction stage were harvested at 2,000g for 20 minutes, at 4°C, in an M.S.E. Mistral 4L centrifuge. The cells were resuspended in sterile physiological saline and again harvested by spinning for 40,000g.min. The bacterial pellet was resuspended in 0.05M sodium phosphate buffer pH 7, which contained 4mM steroid substrate and 1mM P.M.S. (phenazine methosulphate, Koch-Light Ltd.) as an artificial electron acceptor. The organisms were disrupted by ultrasonic treatment in this reaction mixture, then incubated for between 2 and 20 hours at 37°C. The resulting reaction mixture was extracted with one volume of chloroform

and the extract was analysed by T.L.C..

Due to the relative ease of handling, solubility and commercial availability, androstane substrates were used in preliminary screening. These were 5 β -androstan-3,17-dione, 1-androsten-3,17-dione and 4-androsten-3,17-dione. Strains shown to possess desaturating enzymes were then screened with the appropriate cholanyl or cholesteryl substrate with the same ring A configuration i.e. 3-oxo-5 β -cholan-24-oic acid, 3-oxo-1-cholen-24-oic acid, 3-oxo-4-cholen-24-oic acid and 3-oxo-5 β -cholestane.

Preparation and identification of metabolites

Strains shown to be strongly positive in the above assay systems were grown up in 4 litres of broth with 0.01% inducer at 37°C for 72 hours. Another 0.01% substrate was added and the culture was shaken anaerobically for a further 3 days. The shaker was custom built for this size of vessel. Metabolites were extracted as above with one volume chloroform/methanol (2:1) and one volume chloroform. The pooled extracts were rotary evaporated under reduced pressure at 75°C and the residue was analysed by preparative thin layer chromatography. The eluted compounds were identified and confirmed by I.R., U.V., fluorometric and mass spectra. If crystals could be produced, melting points and mixed melting point determinations were made.

Quantitative assay

Percentage yields under different conditions and pH optima were estimated for both assay systems. Desaturated androstanes and bile acids were eluted from a quantitative T.L.C. plate (see Methods 2.1.). Absorbance readings at 240nm were recorded, concentrations being obtained by relating optical density reading to the molecular extinction coefficients, calculated from authentic samples.

4.2. Aromatisation of Ring A

Screening

Species of Bacteroides, Bifidobacteria, Clostridia, Enterobacteria and Enterococci were screened using the methods described above for the 5 β -steroid-3-oxo- Δ^1 -dehydrogenase and 5 β -steroid-3-oxo- Δ^4 -dehydrogenase. This assay system however, required the use of 250ml of broth and the induced culture was shaken aerobically for 4 days. Concentrations of substrates were 0.01% for induction and a further 0.01% for the assay stage. Substrates used were the 4-en-3-one compounds in the androstane, cholanyl and cholesteryl series.

The extraction of the phenolic steroids was based on a method described by Kilshaw (1970), and first developed by Bulbrook and Greenwood (1957). Phenolic steroids were extracted from an acidified culture with one volume chloroform/methanol mixture (2:1) and one volume chloroform. The extracts were evaporated to dryness and redissolved in 1.6% caustic soda and filtered to remove insoluble material. The solution was then reacidified and extracted with chloroform again. This method enabled the extraction of phenolic steroids and their separation from all other steroid substrates and metabolites.

Initial identification of products was by thin layer chromatography in solvents V_1 and V_3 and by comparison of relative mobilities with authentic compounds. Visualisation of phenolic steroids was by the use of phenol specific sprays (see Table 4).

Enzyme preparation

A crude enzyme preparation was obtained by harvesting cells from a 250ml induced culture which had been grown up anaerobically for 72 hours and resuspending in 5ml sterile physiological saline. The cells were frozen

at -10°C overnight followed by sonication for 5 seconds. The incubation mixture contained 0.05ml enzyme preparation, 4mM P.M.S. (phenazine methosulphate), 4mM 4-androsten-3,17-dione and 1mM NAD^{+} or NADP^{+} . 0.05M sodium pyrophosphate buffer was used to adjust the pH to 8.5. Incubation of the total 0.5ml reaction mixture was at 37°C for 8 hours.

Identification of enzyme products

Isolation of metabolites for identification and assay was similar to that given for the enzymes in the previous section (Methods 4.1.). Phenolic steroids were extracted as in the screening experiments described above. Metabolites eluted from a preparative T.L.C. plate were characterised by their I.R. spectra, U.V. spectra, spectrofluorimetry and mass spectra. Where authentic samples were available their T.L.C. mobility was compared and their reaction with phenol specific sprays and anisaldehyde spray reagent analysed.

Quantitative assay was similar to that described for the Δ^4 dehydrogenase and Δ^1 dehydrogenase in the previous section (see Methods 4.1.). However, phenolic steroids were estimated spectrophotometrically at O.D. 280nm.

4.3. Aromatisation of ring A and B

Screening

Two assay systems were used to screen for equilin production, one being an agar plate method which could be used as a mass screening assay and the other a broth assay similar to that described in the last two sections.

The plate screening test was adapted from Vezina et al. (1969). Willis and Hobbs egg yolk medium was poured into 10cm petri dishes so that the

agar just covered the plate. When solid, a further layer of the medium containing dissolved substrate (4,6-androstadien-3,17-dione or 4,6-chol-estadien-3-one) was poured onto the base layer. Concentrations of substrate varied between 10 and 20mg per plate.

Point inoculations of the egg yolk plate were made using colonies freshly grown up on 'rich blood' agar plates. The Willis and Hobbs plates were inoculated in duplicate and incubated anaerobically for 72 hours. One set of plates was then sprayed directly with 1% p-nitrobenzenediazoniumtetrafluoroborate (Kodak Ltd.) in 50% acetic acid. Metabolites were visualised as a coloured halo around the colony. Steroids with a phenolic ring A but a fully saturated ring B gave a brown colour. Steroids with a phenolic ring A and a partial desaturation of ring B gave an orange colour and those with full aromatisation of ring A and B gave a deep red colouration. Colonies showing a positive reaction were excised from the duplicate plates, extracted with chloroform and the extract separated by thin layer chromatography and compared with authentic compounds. This eliminated any plate artifacts, the compounds being visualised on a thin layer plate using the same specific equilin spray.

Metabolites were characterised by their relative mobilities on thin layer plates and by their characteristic U.V. and mass spectra. These properties were compared with those of authentic samples.

The broth assay was similar to that described in Methods 4.1. and 4.2. 250ml Todd Hewitt broth was inoculated and grown up for 72 hours anaerobically in the presence of 0.01% inducer. The pH of the broth was then adjusted to 8 and the culture shaken for a further 72 hours aerobically. The chloroform extract was evaporated to dryness, separated by thin layer chromatography and the metabolites identified as described above.

4.4. 7-dehydration of 3-oxo-7 α -hydroxy-4-cholen-24-oic acid

Conditions for this reaction were identical to those given by Aries and Hill (1970) and Aries (1970), for the 7 α dehydroxylation of cholic acid. Strains of bacteria found to be positive for this reaction were screened for their ability to dehydrate 3-oxo-7 α -hydroxy-4-cholen-24-oic acid. The organisms were grown in Todd Hewitt or glucose broth, containing 0.03% substrate and the washed cell suspension obtained by spinning down the induced culture, was incubated at 37°C under anaerobic conditions for 48 or 72 hours with 0.1% substrate.

The steroids were extracted from the reaction mixture with one volume chloroform/methanol (2:1) and one volume chloroform. The pooled extracts were evaporated to dryness, redissolved and analysed by T.L.C.. Criteria for identification were T.L.C. mobilities, U.V. spectra, fluorimetric spectra, mass spectra and melting point determinations.

Inhibition studies were carried out by the introduction of inhibitory ions into the assay system.

5.

Cholesterol Metabolism

5.1. In vitro metabolism

Screening

Strains were screened for cholesterol degradation using the acetone extracted calf brain medium described by Coleman and Baumann (1957b). The cholesterol substrate was used at a concentration of 100mg per 100ml broth. The substrate was injected in a benzene solution and the broth was then steamed to evaporate off all the solvent and solubilise the cholesterol. Tween 80, added to the broth, aided the solubilization of the cholesterol substrate. The strain was incubated anaerobically for up to 6 days in a 20ml broth. The resulting culture was extracted with chloroform and spotted on a thin layer plate. Percentage yields of coprostanol (3 β -hydroxy-5 β -cholestanol) were estimated by gas liquid chromatography using an S.E.30 column (see Methods 2.2.). A more precise screening for minor metabolites was carried out using 1 μ Ci of ¹⁴C cholesterol (Radiochemical Centre, Amersham), added to the 20ml broth containing 0.1% cold cholesterol. Extracted steroids were separated by thin layer chromatography and radioactive peaks located by a Panax radioactive strip scanner.

The extraction procedure for the radioactive incubations was adapted from Grundy, Ahrens and Miettinen (1965). This provided both an acid and neutral steroid fraction. The freeze dried residue obtained from the grown broth suspension was incubated for one hour with 1N NaOH in 90% alcohol. This was then filtered and washed with 20ml 1N NaOH in 50% alcohol and 20 ml water. The filtrate was then extracted with 3 x 1 volume petroleum ether (boiling range 60-80°C) and evaporation of the extracts produced the neutral steroid fraction.

The remaining aqueous phase was acidified with 5ml conc. HCl and extracted with one volume chloroform/methanol (2:1) and two volumes chloroform. The residue produced from evaporating the pooled extracts provided the acid steroid fraction.

Both fractions were analysed by T.L.C. using solvent NS for neutral steroids and BA, S₁ and S₁₀ for acid steroids. The T.L.C. plates were scanned for radioactive peaks. All relative mobilities were compared with those of authentic compounds. Spots were visualised by spraying with 10% phosphomolybdate spray reagent in alcohol, (see Table 4).

Purification and identification of metabolites

500ml cultures were grown in acetone extracted calf brain medium containing 0.1% cholesterol substrate and 5 μ Ci ¹⁴C labelled substrate maintained at constant pH during growth in a Metrohm Herisau pH stat.

A large inoculum of faeces or a purified strain grown in Robertsons cooked meat was added to the broth, incubated at 37°C and maintained under anaerobic conditions by bubbling carbon dioxide and hydrogen gases through the medium. After 24 hours, the pH was set at the desired value and aliquots of 20ml were removed from the system at 12 hour intervals, extracted and analysed by T.L.C.. After 6 days the remaining culture was extracted into acid and neutral steroid fractions as described above and analysed by T.L.C..

5.2. In vivo metabolism

¹⁴C cholesterol, labelled at carbon 4 was introduced into the caecum of experimental animals by a simple surgical procedure. Dose levels varied from 2 μ Ci to 10 μ Ci substrate. The animals were 250g male Sprague-Dawley rats and 300g albino Guinea pigs.

Animals were anaesthetised during the operation with diethyl ether. A half inch incision was made longitudinally in the lower middle right abdomen and the caecum was exposed. The labelled cholesterol was dispersed in sterile physiological saline and then injected into the proximal end of the caecum. The animals were then sewn up, allowed to recover, and placed in "Metabowl" metabolism cages. Twenty-four hour samples of faeces and urine were collected for up to 5 days. At the end of this time the animals were killed, the liver, gall bladder and any bile were removed as were the contents of the caecum and large intestine. A sample of blood was taken from the heart.

The liver and blood were prepared for scintillation counting as described in Methods 2.7. Faeces and caecal contents were separated into neutral and acid steroid fractions as described in the previous section (Methods 5.1.), and the fractions obtained were evaporated to dryness and redissolved in 10ml chloroform. 0.5ml acid steroid and 0.1ml neutral steroid solution were added to scintillation vials. As chloroform has strong quenching properties, all solvent was evaporated prior to the addition of 15ml scintillation fluid. Urine samples were either directly counted by the addition of 0.5ml urine to 15ml scintillation fluid or extracted prior to counting. Urine samples were treated prior to extraction, with 2N NaOH for one hour to hydrolyse the steroid conjugates. Phenolic steroids were then extracted as described in Method 4.2. (adapted from Kilshaw, 1970).

All extracts were analysed by T.L.C. for quantitative as well as qualitative estimations. The T.L.C. plates were scanned to locate labelled metabolites and subsequently sprayed with phosphomolybdate spray reagent to visualise cold steroids. Compounds were purified by elution from preparative T.L.C. plates and characterised by I.R., U.V. and mass spectra.

Sulphate and Glucuronide conjugates

Conjugates were assayed by incubation with a preparation of sulphatase hydrolytic enzyme (from Helix pomatia; Sigma Chemicals) and β -glucuronidase (Sigma Chemicals). Incubations at pH 5.5 were for 0, 30, 60, 120 and 180 minutes. The reaction was stopped by spotting an aliquot on a T.L.C. plate and drying at 110°C. Thin layer plates were scanned for changes in the location of radioactive peaks.

6. Classification of lecithinase-negative Clostridia and their Relationship to Steroid Desaturation, Diet and Cancer of the Colon.

Lecithinase-negative Clostridia were isolated and identified according to methods 1.1. These bacteria were isolated from faecal specimens from individuals in Uganda, Hong Kong, Scotland and England. Faecal samples were collected in the country of origin, stored in liquid nitrogen and shipped to St. Marys Hospital. Here they were stored at -60°C.

People from Uganda and Hong Kong represent individuals on a low fat diet and low colorectal cancer incidence. People from Scotland and England are representative of a high fat diet and high colon cancer incidence (see Hill et al. 1971). A parallel study was carried out comparing the populations of Clostridia isolated from the faeces of colon cancer patients and from a control group of patients in the same hospital. This was part of a larger study carried out in collaboration with Dr. T.A. Meade at the Northwick Park Hospital. Each Clostridium was characterised by the method of Holdeman and Moore, (1972). Each strain was also screened for its ability to desaturate steroids as described in Methods 2.1.

SECTION III

RESULTS AND DISCUSSION

SECTION III A

NUCLEAR DESATURATION
OF STEROIDS

1. 3-oxo-5 β - Δ^1 -dehydrogenase and 3-oxo-5 β - Δ^4 -dehydrogenase

1.1. Introduction

Incubation of 5 β -androstane-3,17-dione with a range of bacterial strains at pH 7.4 produced two metabolites. Separated by thin layer chromatography, these compounds were tentatively identified as being unsaturated by their fluorescence under U.V. light. In developing solvent V_1 they had relative mobilities R_T (relative to testosterone) of 1.0 and 1.65. Both metabolites reacted with anisaldehyde spray giving a blue and green colour respectively, and these properties were identical to those of authentic samples of 4-androsten-3-one-17 β -ol and 4-androsten-3,17-dione respectively.

At pH 9.5, incubation with the same substrate produced a third desaturated metabolite and the other two described above were present at only minimal levels. Separation by T.L.C. gave a relative mobility of 1.4 for this compound in solvent V_1 and a green colour with anisaldehyde spray reagent. This behaviour was identical to that of authentic 1,4-androstadien-3,17-dione.

1.2. Identification of products

All metabolites were purified by preparative T.L.C. and eluted from the plate for further characterisation. Table 6 shows a summary of the U.V. spectra, molecular extinction coefficients, infra red spectra, mass spectra and relative mobilities for the metabolites isolated. These properties were identical to those of commercially available authentic samples. The infra red spectra of Δ^4 compounds showed a single narrow based peak at 5.95 μ m, indicative of carbon-carbon double bond bending vibrations. These were distinguished from the $\Delta^{1,4}$ compounds, which had a double peak at 5.95

TABLE 6

Criteria Used to Characterise Desaturated Steroid Metabolites.

<u>CRITERIA</u>	<u>Androstane Series</u>			<u>Cholanoyl Series</u>	
	Δ^1	Δ^4	$\Delta^{1,4}$	Δ^4	$\Delta^{1,4}$
<u>U.V. absorption spectrum</u>					
Maximum λ_{max} nm	231	240	244	238	244
Extinction Coefficient	10,000	15,000	12,500	15,000	12,500
<u>Infrared Spectrum</u>					
Major Peaks		5.95	6.00	5.95	6.00
		5.72	5.95	5.72	5.95
			5.72		5.72
<u>Mass Spectrum</u>					
Molecular Ion		286	284	371	369
Major Fragments m/e:		244	159	357	296
		202	150	320	260
		187	146	305	233
		149	133	288	223
		148	122	279	168
		124	105	277	149
		107	91	149	128
		106		122	105
		92		106	91
		91		97	
<u>T.L.C. Behaviour</u>					
R_T Solvent V_1	2.00	1.65	1.40	1.38	1.20
Solvent V_3	1.5	2.00	1.60		
Solvent S_1				1.36	1.21
Solvent S_{15}				1.44	1.32
<u>Colour with Anisaldehyde</u>					
	Mauve	Brown/ Green	Green	Orange / Rust	Rust

and 6.00 μ m. The parent ion from the mass spectrum gives an indication of the molecular weight of the metabolites and the cracking pattern shows a typical distribution of major peaks for steroids. Fluorimetric spectra of Δ^4 and $\Delta^{1,4}$ compounds were not distinguishable; both had an $E_{\max}$ of 235nm and $A_{\max}$ 265nm. However, this did distinguish these compounds from saturated steroids and phenolic products.

The metabolite, 4-cholesten-3-one was characterised by its U.V. spectrum $\lambda_{\max} = 240\text{nm}$, $E = 15,000$ and its mass spectrum which gave a parent ion $m/e = 384$ and a large peak at $m/e = 255$, equivalent to the loss of a side chain fragment, C_8H_{16} . 1,4-cholestadien-3-one was characterised by its U.V. spectrum $\lambda_{\max} = 244\text{nm}$, and a mass spectrum with a parent ion $m/e = 382$. Both metabolites showed similar properties in T.L.C. behaviour in solvents V_1 and NS when compared with authentic compounds.

1.3 Substrate specificity

Three clostridial strains which showed the Δ^1 and Δ^4 dehydrogenase activity were tested for their ability to desaturate other substrates. These were strains c.c. 493 (Clostridium butyricum) and c.c. 633 and c.c. 510 (Clostridium paraputrificum). Table 7 shows the percentage conversions to the desaturated products after 72 hours aerobic shaking for strain c.c. 493. These values were estimated from the O.D._{240nm} of the chloroform extracts of the culture. It was concluded that both enzymes in all three of these strains had an absolute requirement for substrates with the 5β configuration since 5α -androstan-3,17-dione and 3-oxo- 5α -cholan-24-oic acid were not desaturated. 5β compounds with variations in the carbon 17 side chain structure, as shown in Table 7 were all equally good substrates for both the Δ^1 and Δ^4 dehydrogenase enzymes assayed at pH 9.5 and pH

TABLE 7

Substrate Specificity for the Introduction of a Double Bond at the C4-5
and C1-2 Position by Clostridium butyricum, C.C. 493.

<u>SUBSTRATE</u>	<u>*% Yield Δ^4</u> <u>Metabolite</u>	<u>*% Yield $\Delta^{1,4}$</u> <u>Metabolite</u>
3-oxo-5 β -cholan-24-oic acid	10.2	6.5
3-oxo-7 α -5 β -cholan-24-oic acid	10.5	-
5 β -androstan-3,17-dione	12.6	8.7
5 β -androstan-3-one-17 β -ol	12.6	8.6
5 κ -androstan-3,17-dione	0.1	0.0
5 κ -androstan-3-one-17 β -ol	0.0	0.0
5 κ -cholestan-3-one	0.0	-
5 β -cholestan-3-one	8.8	-
1-androsten-3,17-dione	-	12.6
1-androsten-3-one-17 β -ol	-	12.4
4-androsten-3,17-dione	-	0.0
4-androsten-3-one-17 β -ol	-	0.0

*The production of the Δ^4 metabolite was assayed at pH 7.5.

*The production of the $\Delta^{1,4}$ metabolite was assayed at pH 9.5.

7.5 respectively. Both enzymes were inducible and could not be detected in cultures grown in the absence of substrate.

The activity of the Δ^1 dehydrogenase could not be demonstrated using 4-androsten-3,17-dione as substrate over a range of pH values. In spite of the oxidising conditions of the assay, there was the reduced metabolite, 5 β -androstan-3,17-dione produced. During screening procedures for $\Delta^{1,4}$ production, a Δ^1 intermediate was never isolated, although such compounds could be used as substrates for the Δ^4 dehydrogenase. This seemed to indicate that the Δ^1 dehydrogenase had a lower specific activity than that of the Δ^4 dehydrogenase. This is investigated further in section 1.4.

1.4. Activity and properties of the enzymes in washed cell suspensions

Both strains c.c. 493 and c.c. 633 were used to determine the conditions required for the desaturation of steroids in washed cell suspension. 5 β -androstan-3,17-dione was used as substrate throughout these investigations. As described in Results 1.1, it was found that both enzymes were inducible and required prior induction with substrate in the growing phase. The Δ^4 dehydrogenase required the presence of an artificial electron acceptor, 1mM phenazine methosulphate. Any decrease in this concentration markedly reduced enzymic conversion, but increasing the concentration to 5mM and 10mM did not further enhance steroid desaturation. The optimal pH in this system for the Δ^4 dehydrogenase was 7.4. A similar optimum was obtained when using the alternate aerobic shaking assay (see Methods 4.1.). NAD⁺, NADP⁺, FMN and FAD could not be used as alternative electron acceptors but did not inhibit the efficiency of phenazine methosulphate.

For the production of $\Delta^{1,4}$ steroids, inferring the activity of both the Δ^1 and Δ^4 dehydrogenases, in washed cell suspension, prior sonic

disruption for 5 seconds of the bacterial cells was required. This was an optimal value, as both no sonication or more than 20 seconds disruption brought about complete inactivation of the enzyme system. The Δ^1 dehydrogenase also required the presence of an artificial electron acceptor, 4mM phenazine methosulphate, to obtain maximum activity. The pH optimum for the washed cell suspension was 8.5; at lower pH values the 4-androsten-3,17-dione metabolite was the major product, presumable due to the Δ^4 dehydrogenase activity described above. Figure 6 shows the relative production of the $\Delta^{1,4}$ and Δ^4 metabolites at different pH values. At no time was a Δ^1 intermediate isolated in this assay system.

Saturation of the C_4-C_5 double bond is carried out under reducing conditions by several genera of intestinal bacteria and was described by Aries (1970). However, using 1,4-androsten-3,17-dione as substrate and incubating under anaerobic, and therefore reducing conditions, resulted in the production of 1-androsten-3,17-dione as the major metabolite, together with small amounts of the Δ^4 and 5 β products. The Δ^1 product could only be demonstrated on thin layer chromatography after disruption of the cells by sonic disintegration or chloroform extraction. Thus the cell membrane seems to act as a permeability barrier to this metabolite, although it is permeable to the Δ^4 and $\Delta^{1,4}$ analogues. If the sonicated suspension was subsequently incubated with 3mM phenazine methosulphate, it could be demonstrated, by taking aliquots at time intervals, that the Δ^1 product was converted with almost 100% efficiency to the $\Delta^{1,4}$ product in 4 hours.

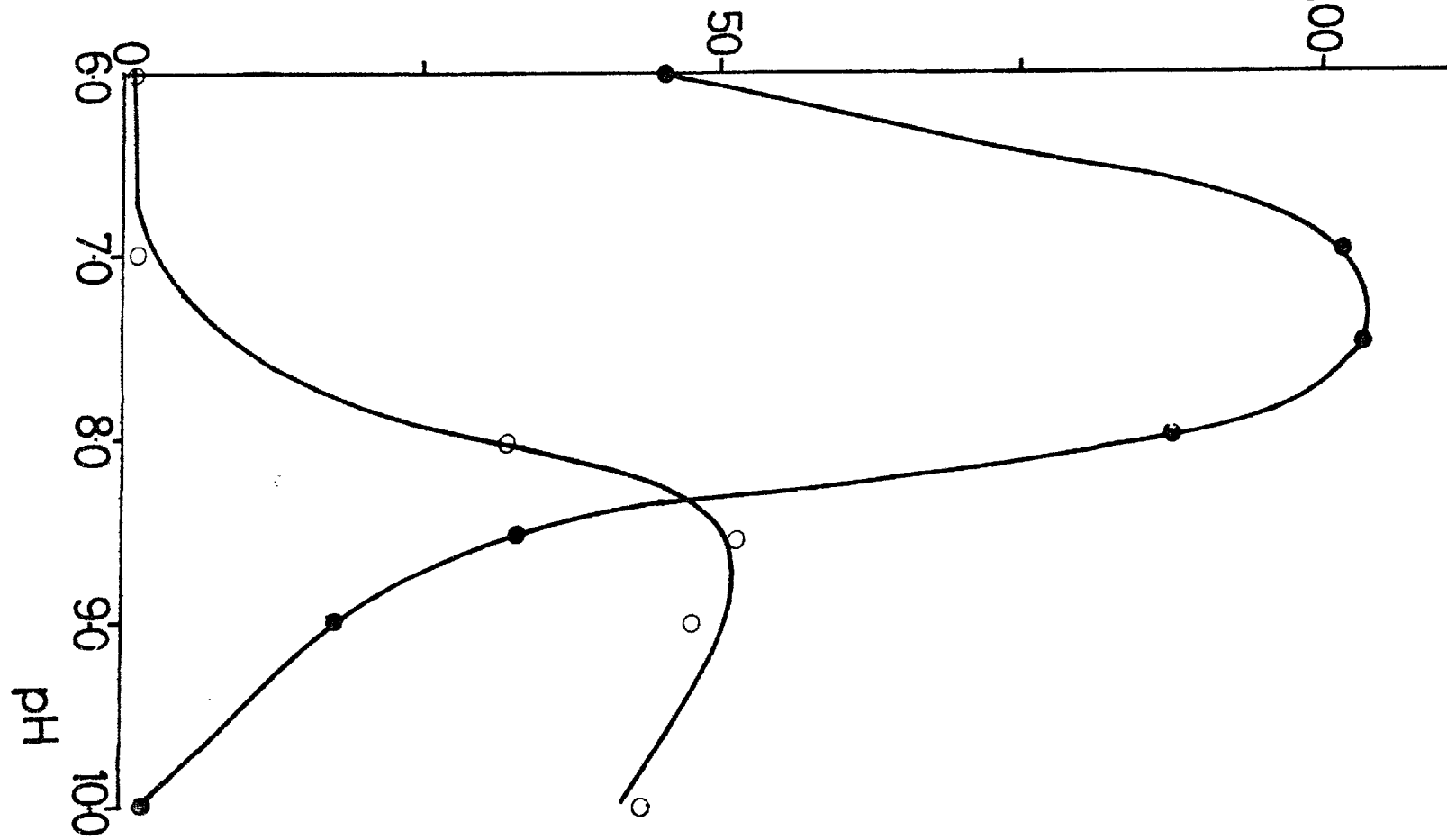
The Δ^1 and Δ^4 dehydrogenase enzymes could be induced by a range of saturated steroids. 5 β -cholestan-3-one, 3-oxo-5 β -cholan-24-oic acid, 3-oxo-7 α -hydroxy-5 β -cholan-24-oic acid, 5 β -androstan-3,17-dione and 5 β -androstan-3-one-17 β -ol all showed inductive properties. All these compounds

Fig. 6. Effect of pH on the production of 4-androsten-3,17-dione and 1,4-androstadien-3,17-dione.

Δ^4 production (●—●)

$\Delta^{1,4}$ production (○—○)

Activity $\text{nM}/10^8 \text{ cells/hr.}$



appeared to induce the same enzyme, as subsequent incubation with a variety of substrates showed similar specificities, (see Results 1.3.).

An attempt to separate the Δ^1 dehydrogenase from cellular debris was unsuccessful. Intracellular material, obtained by sonic disintegration for 30 seconds and separating the clear supernatant from the particulate fraction by spinning for 18,000g.min. at 4°C, showed no Δ^1 dehydrogenase activity, but a little Δ^4 dehydrogenase activity.

1.5. Screening

Preliminary screening of bacterial strains for the Δ^4 dehydrogenase by Aries (1970), showed that this enzyme was confined to about 30% of the lecithinase-negative Clostridia. Extended screening for this enzyme confirmed these results with one small exception. Of 100 strains of lecithinase-positive Clostridia screened for this reaction, three were positive; these were identified as Clostridium welchii.

In screening for Δ^1 activity it was found that only strains positive for the Δ^4 dehydrogenase described above were positive for this enzyme. It was found that all strains possessing the Δ^4 dehydrogenase also possessed the Δ^1 dehydrogenase.

1.6. Discussion

The presence of these two enzymes has previously been described for soil organisms. 3-oxo-5 β -steroid- Δ^4 -dehydrogenase from Pseudomonas testosteroni was described by Davidson and Talalay (1966). 3-oxo-5 β -steroid- Δ^1 -dehydrogenase was also demonstrated in this organism. Alcaligenes faecalis (Wix et al., 1968) and Bacillus sphaericus (Hayano et al., 1960), also showed similar enzyme activity, In contrast to the enzymes of clostri-

dial origin, a 5α - Δ^1 dehydrogenase was also isolated from these organisms. Also, Pseudomonas testosteroni was shown to produce the $\Delta^{1,4}$ product from a Δ^4 substrate (Levy and Talalay, 1959a and b; Davidson and Talalay, 1966). However, these authors indicated that a 5β configuration was preferred for both Δ^1 and Δ^4 dehydrogenase and hypothesised that the organism presented with 4-androsten-3,17-dione as substrate, followed a metabolic sequence of saturation followed by C_1 - C_2 desaturation and then C_4 - C_5 desaturation. Because of the considerably lower activity of the clostridial enzymes this could not be demonstrated with the intestinal bacterial strains used.

The requirement of steroids with 5β configuration for the enzymes isolated from bacteria of intestinal origin, is not surprising when it is considered that the major sources of steroid 'substrate' in the gut are the bile acids and coprostanol, all of which possess such a configuration. The conversion of cholic acid and other bile acids to their 3-keto derivatives has been demonstrated in vitro by Aries and Hill (1970b). The presence of such compounds in faecal steroid fraction (Danielsson et al., 1963), suggests that a similar reaction occurs in vivo. Such compounds are of a suitable configuration for nuclear desaturation reactions as has been demonstrated in vitro in this section. Proof that such desaturation reactions occur in vivo is however, more tenuous. The presence of allo bile acids in faeces (Haslewood, 1967), has led to the hypothesis that a Δ^4 dehydrogenase is implicated in their synthesis. Kallner (1967a), has demonstrated that rat caecal contents can convert deoxycholic acid to allodeoxycholic acid via an unsaturated intermediate. The saturation of a double bond at the C_4 - C_5 position has shown to be random, 5β and 5α products being produced (Aries, 1970).

The results presented suggest that the Δ^4 dehydrogenase and Δ^1

dehydrogenase may be two different enzyme systems located in different parts of the bacterial cell. The Δ^4 dehydrogenase appears in the supernatant of the disrupted cells and its location in the intact cell is probably intracellular. The Δ^1 dehydrogenase was absent from this supernatant and was inactivated very effectively after minor disruptions of the cell. It appears from this observation, that the enzyme is located in the cell membrane, disruption causing a loss of conformation and therefore activity. This would account for failure to purify the Δ^1 dehydrogenase. The requirement for minor cellular disruption to demonstrate enzymic activity in a washed cell suspension, suggests that the membrane is impermeable to the enzyme substrate; the need to sonically disrupt to release 1-androsten-3,17-dione produced by reduction of the 1,4-androstadiene substrate, indicates that the membrane is also impermeable to the enzyme product.

The complete absence of detectable levels of a Δ^1 intermediate in the reduction of steroid substrate, also gives rise to several problems. On one hand, it can be argued that the Δ^1 dehydrogenase is of much lower activity than the Δ^4 dehydrogenase and so is the limiting step in this process. This may be inferred by the lower percentage conversion to $\Delta^{1,4}$ product (8%), than the Δ^4 product (12%), under optimal conditions in the same incubation time. This is also supported by the accumulation of Δ^1 compounds in the resaturation of the $\Delta^{1,4}$ substrate and the apparent irreversibility of the Δ^1 dehydrogenase when incubated with the Δ^1 substrate in resaturation experiments.

The second explanation, is that the cell contains two species of Δ^4 dehydrogenase enzymes. The pH optima for Δ^4 and $\Delta^{1,4}$ production are 7.5 and 8.5 respectively. Cell free incubation for the Δ^4 dehydrogenase,

which was located intracellularly and carried out by Aries, (1970), showed from the pH activity profile that the enzyme was almost completely inactive at pH 8.5. If the production of the $\Delta^{1,4}$ metabolite is, as results suggest, the product of a Δ^1 and Δ^4 dehydrogenase acting stereospecifically on the 5β steroid, a second species of Δ^4 dehydrogenase is strongly suggested, being active at pH 9.5. The inhibition of $\Delta^{1,4}$ production on disruption of the cell, suggests that both the Δ^1 and Δ^4 dehydrogenases responsible for $\Delta^{1,4}$ production, are located in the cell membrane in close proximity. This would explain both the failure to isolate a Δ^1 intermediate in a desaturation sequence, as this compound may be transferred directly to the Δ^4 dehydrogenase. This may also explain the failure to completely resaturate a $\Delta^{1,4}$ substrate; the Δ^1 compound formed is not released from the enzyme complex, until sonic disruption of the cell has occurred.

The properties of such a cell membrane Δ^4 dehydrogenase would compare well with that isolated from *Pseudomonas testosteroni* (Davidson and Talalay, 1966), which was also a cell membrane enzyme and had a pH optimum of 8.5. The Δ^1 dehydrogenase, also isolated by the same authors, had a similar pH optimum and was also located in the cell membrane.

2. Oxidative Demethylation and Aromatisation

2.1. Screening

Table 8 shows the results from screening five different groups of intestinal bacteria for phenolic products, using 4-androsten-3,17-dione as substrate. Two phenolic metabolites were detected by the reaction with phenol-specific potassium ferricyanide spray, after separation on T.L.C. in developing solvent V_1 . Compound Y was produced by about 60% of the strains of Enterobacteria and 20% of the strains of lecithinase-negative Clostridia, both at a yield of about 1%. This compound had a relative mobility (cf. testosterone) of 1.92 in solvent V_1 and its reaction with anisaldehyde gave a brown/purple colouration. This was identical in behaviour to the properties of authentic oestrone (1,3,5(10)-estratrien-3-ol-17-one). In parallel screening experiments using 4-androsten-3-one-17 β -ol as substrate, compound Y was not detected, but about a 1% yield of another phenolic product, compound X, was detected. This had a T.L.C. mobility of 1.12 in developing solvent V_1 and this was identical to oestradiol (1,3,5(10)-estratrien-3,17-diol).

Metabolite B, produced by about 30% of lecithinase-negative Clostridia at about an 8% yield, showed a much faster T.L.C. mobility than compound X or compound Y. In solvents V_1 , V_2 and V_3 , T.L.C. mobilities were 2.10, 2.65 and 1.59 respectively.

2.2. Identification of products

Compound X and Y

Compound X and Y were tentatively identified as oestradiol and oestrone respectively, by their relative mobilities in solvents V_1 , V_2 and V_3 (see

TABLE 8

Screening of Bacterial Groups for the Production of two Phenolic Metabolites
from the Substrate, 4-androsten-3,17-dione.

<u>Bacterial Group</u>	<u>No. Strains Tested</u>	<u>Production of</u>	
		<u>Metabolite Y</u>	<u>Metabolite B</u>
Bacteroides	40	0	0
Bifidobacteria	40	0	0
Enterococci	15	0	0
Enterobacteria	26	16	(2)?
Clostridia: Lecithinase +ive	95	0	3
Lecithinase -ive	15	4	6

Table 9). They produced a brown/purple colouration with anisaldehyde spray reagent and a blue colour with the phenol specific sprays, Folins and potassium ferricyanide spray reagents. Their U.V. spectra ($\lambda_{\max} = 280\text{nm}$ and E about 3,000) was comparable to the authentic compounds and ring A phenolic steroids in general. Spectrofluorimetric assay showed both the bacterial metabolites and authentic compounds to have an excitation maximum, $E_{\max} = 285\text{nm}$ and emission maximum, $A_{\max} = 330\text{nm}$. Mass spectra analysis showed a parent ion of $m/e = 272$ for compound X and $m/e = 270$ for compound Y. The base peak for both compounds was $m/e = 147$. Figure 7 shows the mass spectrum of T.M.S. oestrone for both the bacterial metabolite and that of authentic origin.

Metabolite B

Preliminary analytical characterisation of this compound indicated that it was a phenolic steroid with a mass of 286 and more lipophilic than oestrone or oestradiol.

The mobility on a thin layer plate indicated its lipophilic character. In developing solvents V_1 , V_2 and V_3 , the relative mobility (cf. testosterone) was faster than that of oestradiol and oestrone (see Table 9). A phenolic steroid was indicated by its blue colouration on reaction with the phenol specific sprays, Folins and potassium ferricyanide. Its U.V. spectrum ($\lambda_{\max} = 282\text{nm}$, $E = 2,800$) and its fluorimetric spectrum ($E_{\max} = 283\text{nm}$, $A_{\max} = 330\text{nm}$) were typical of a phenolic steroid. The mass spectrum shown in Figure 8 shows a molecular ion of $m/e = 286$. This indicates that not demethylation, but a transmethylation has occurred, as this compound has a molecular weight of 14 more than oestradiol. The T.F.A. derivative of this compound gave a parent ion of $m/e = 456$, equivalent to the addition of two trifluoroacetyl groups, but with the loss of a methyl

TABLE 9

Comparison of T.L.C. Behaviour of Three Bacterial Metabolites and
Oestrone and Oestradiol.

<u>T.L.C. Solvent</u> <u>Relative Mobility R_F</u>	<u>Bacterial Metabolite</u>			<u>Oestrone</u>	<u>Oestradiol</u>
	<u>B</u>	<u>X</u>	<u>Y</u>		
V_1	2.10	1.12	1.92	1.92	1.12
V_2	2.65	1.66	2.50	2.51	1.66
V_3	1.59	0.69	1.38	1.38	0.69

Colour With:-

Anisaldehyde	Purple	Purple	Purple	Purple	Purple
Potassium ferricyanide	Blue	Blue	Blue	Blue	Blue

Relative mobilities are measured against Testosterone,
(4-androsten-3-one-17 β -ol)

Fig. 7. Mass spectra of T.M.S. derivatives of phenolic steroids, using a G.L.C./Mass spectrometer system.

- A) Mass spectrum of T.M.S. derivative of authentic oestrone (1,3,5(10)-estratrien-3-ol-17-one)
- B) Mass spectrum of T.M.S. derivative of bacterial metabolite, compound Y.

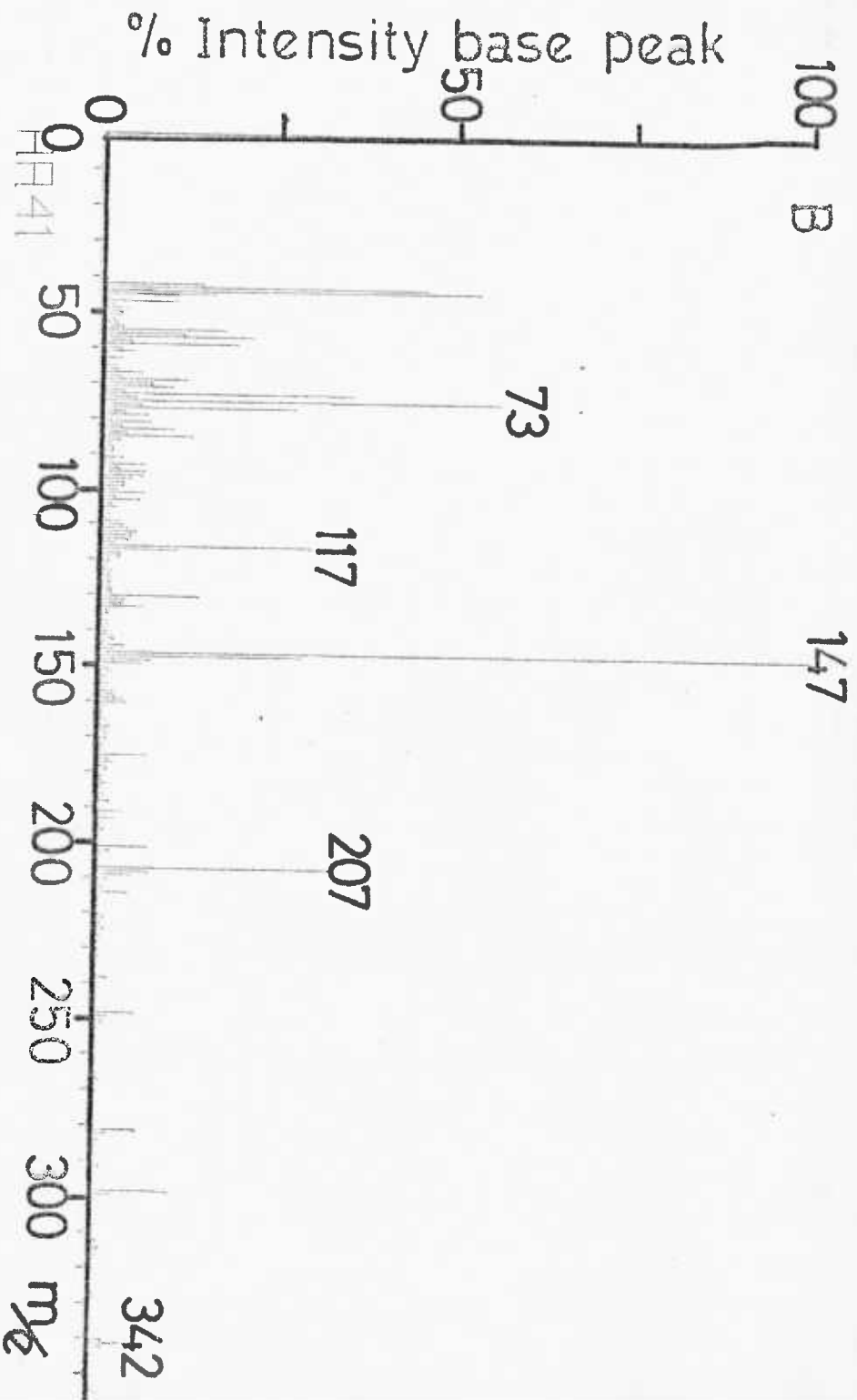
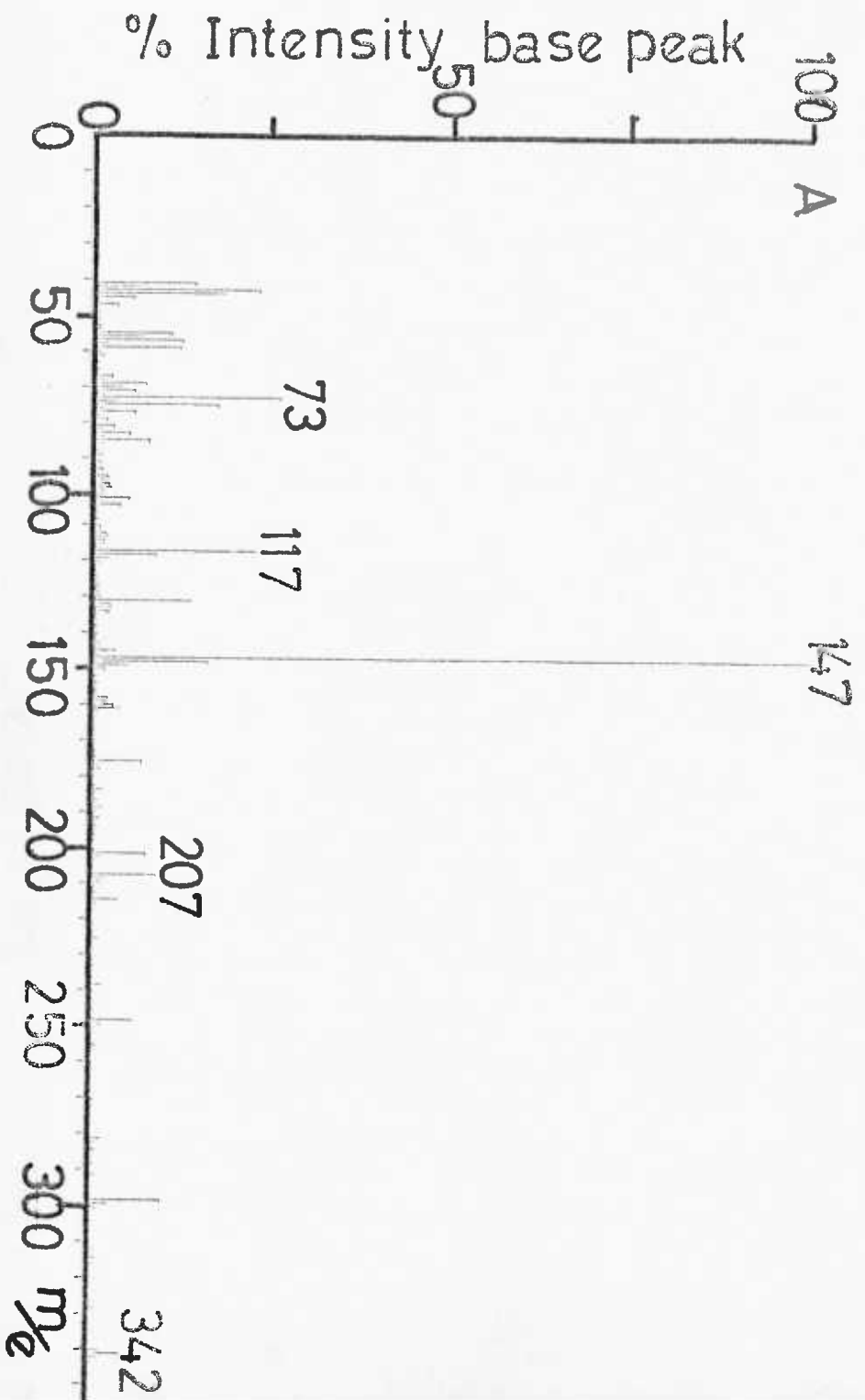


Fig. 8. Mass spectra of phenolic steroids using direct probe injection at 150°C.

- A) Mass spectrum of 17 β -methoxy-1,3,5(10)-estratrien-3-ol synthesised by the methylation of oestradiol.
- B) Mass spectrum of bacterial matabolite B.

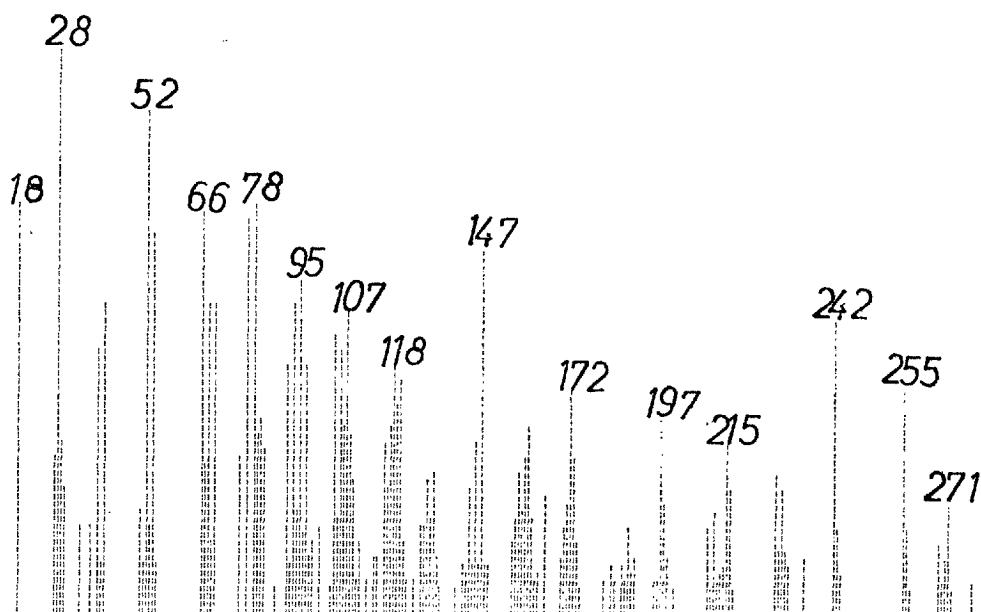
A

286

% Intensity parent ion

100

50



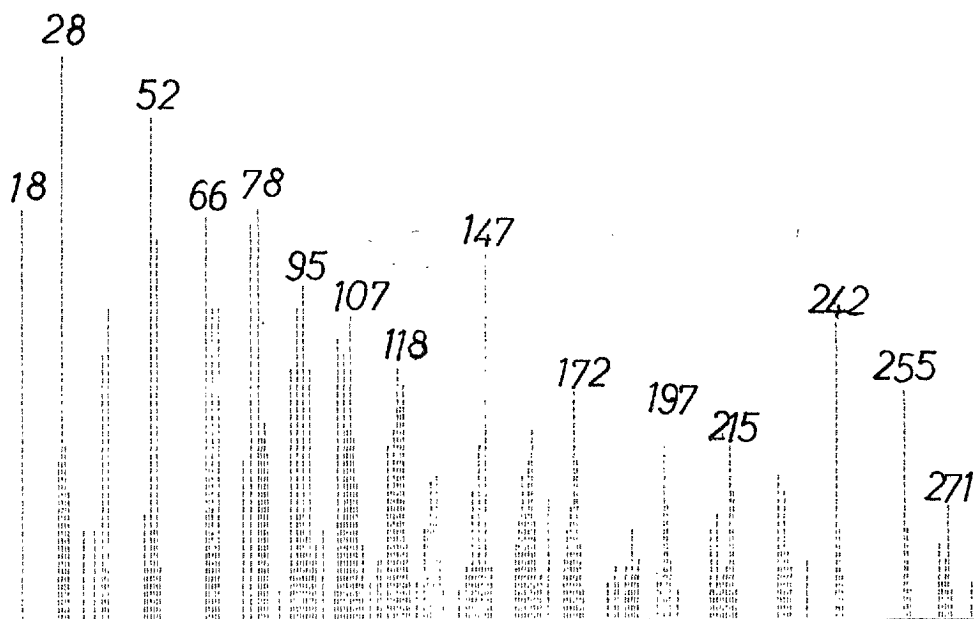
B

286

% Intensity parent ion

100

50



0

50

100

150

200

250 m/e

group. This could be explained by the acid hydrolysis of a methoxy group in the preparation of the trifluoroacetyl derivatives.

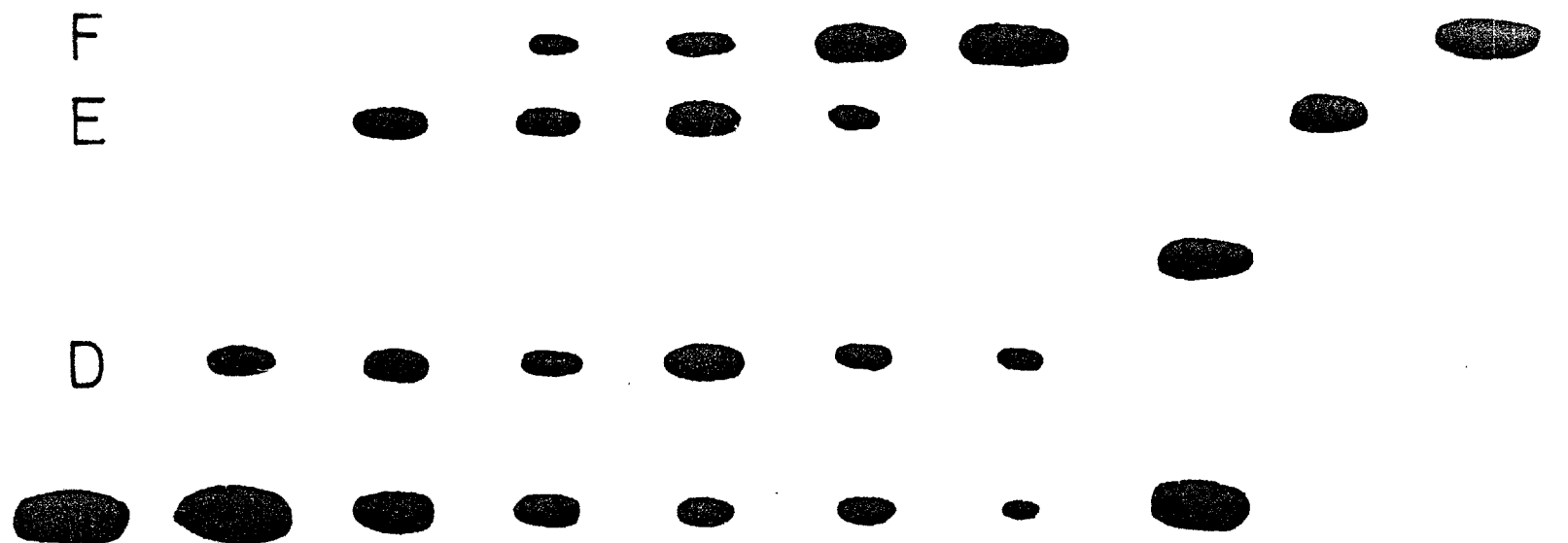
4-androsten-3,17-dione can be aromatised chemically, via the 1,4-dien-3-one and a rearrangement to 1-hydroxy-4-methyl-1,3,5(10)-estratrien-17B-ol and 3-methyl-1-hydroxy-1,3,5(10)-estratrien-17B-ol; neither of these compounds corresponded in T.L.C. mobility to metabolite B. Methylation of oestradiol with dimethyl sulphate produced three products, and their production was followed with time, as shown in the thin layer chromatographic analysis of Fig. 9 . These compounds were termed D, E and F. The exhaustive methylation product was compound F and so was identified as the 3,17-dimethoxy derivative. Mass spectral analysis showed a parent ion of $m/e = 300$ which supported this conclusion. This was confirmed chemically as the incubation of both compound D and E with dimethyl sulphate produced a single product, compound F. Moreover, incubation of metabolite B with dimethyl sulphate produced a single product, also identified as compound F.

Compound C was the first methylation product to be detected on incubation of oestradiol with the methylating agent as seen in Fig 9 . This had only a weak reaction with phenol specific sprays, but with anisaldehyde produced a strong colouration . Its relative mobility in solvent V_1 (cf. testosterone) was 1.15 which was identical to the methylation product, produced by the weaker methylating agent, diazomethane, on incubation with oestradiol.

Compound E had a T.L.C. mobility (cf. testosterone) in V_1 of 2.10, identical to that of metabolite B. Subsequent mass spectral analysis of this compound showed it to have an identical spectrum to that of the bacterial metabolite (see Fig. 8). This compound could not be produced on

Fig. 9. Thin layer chromatogram, separated in developing solvent V_1 , showing the methylation products produced with time after the incubation of 1,3,5(10)-estratrien-3,17-diol with dimethyl sulphate.

- (a) Authentic 1,3,5(10)-estratrien-3-ol-17-one and 1,3,5(10)-estratrien-3,17-diol.
- (b) Metabolite B (17 β -methoxy-1,3,5(10)-estratrien-3-ol).
- (c) The methylation product after exhaustive incubation of metabolite B with diazomethane.



0 1 5 15 30 60 180 (a) (b) (c)

Reaction products (methylation time in min.)

incubation of diazomethane with oestradiol. Diazomethane only methylates acidic groups and would therefore modify the active phenolic group, but not the hydroxyl at the 17 position; further, the product of diazomethane methylation, compound D, had only a very weak reaction with the phenol-specific spray. It was therefore identified as 3-methoxy-1,3,5(10)-estratrien-17 β -ol. The other monomethyl product, compound E, which was identical to the bacterial metabolite B, had a free phenolic hydroxyl group (since it reacted with ferric chloride spray), and was therefore tentatively identified as 17 β -methoxy-1,3,5(10)-estratrien-3-ol. The melting point of B was 234-235°C compared with a mixed melting point of 233-235°C for B and E. These values are very close to the reported melting point of 17 β -methoxyoestradiol (Neudart and Ropke, 1965; Coombs and Roderick, 1965). Neudart and Ropke (1965), also reported that this compound had a U.V. $\lambda_{\text{max}} = 281\text{nm}$, E = 2,100 (compound B had U.V. $\lambda_{\text{max}} = 282\text{nm}$, E = 2,300).

2.3 Further investigations of enzymic production of 17 β -methoxy-1,3,5(10)-estratrien-3-ol

It was not possible to obtain a completely cell free system for the production of phenolic steroids, but a crude enzyme preparation was obtained as described in Methods 4.2. Cells harvested from the induced culture had to be frozen overnight at -10°C followed by sonic disruption for 5 seconds. Both these steps were required to split the cell open in such a way, that the enzymes could be demonstrated in an active form. The intracellular fluid, the supernatant obtained after spinning down the cellular debris, showed no activity. The enzyme preparation required the presence of both an artificial and a natural electron acceptor. Natural acceptors which were used were 1mM NAD⁺ or 1mM NADP⁺. The artificial electron

acceptor was 4mM phenazine methosulphate; other acceptors such as cytochrome C or menadione were ineffective. Inhibition of this enzyme system could be demonstrated using 0.25M Cu^{2+} , 0.1M Ca^{2+} , 0.1M F^- , 0.05M N_3^- , 0.05M iodoacetate, 0.006M periodate and 0.3M formaldehyde. It was not inhibited by as much as 40mM E.D.T.A.. This enzyme system was inducible and was not active unless the bacteria were grown up in the presence of a substrate inducer.

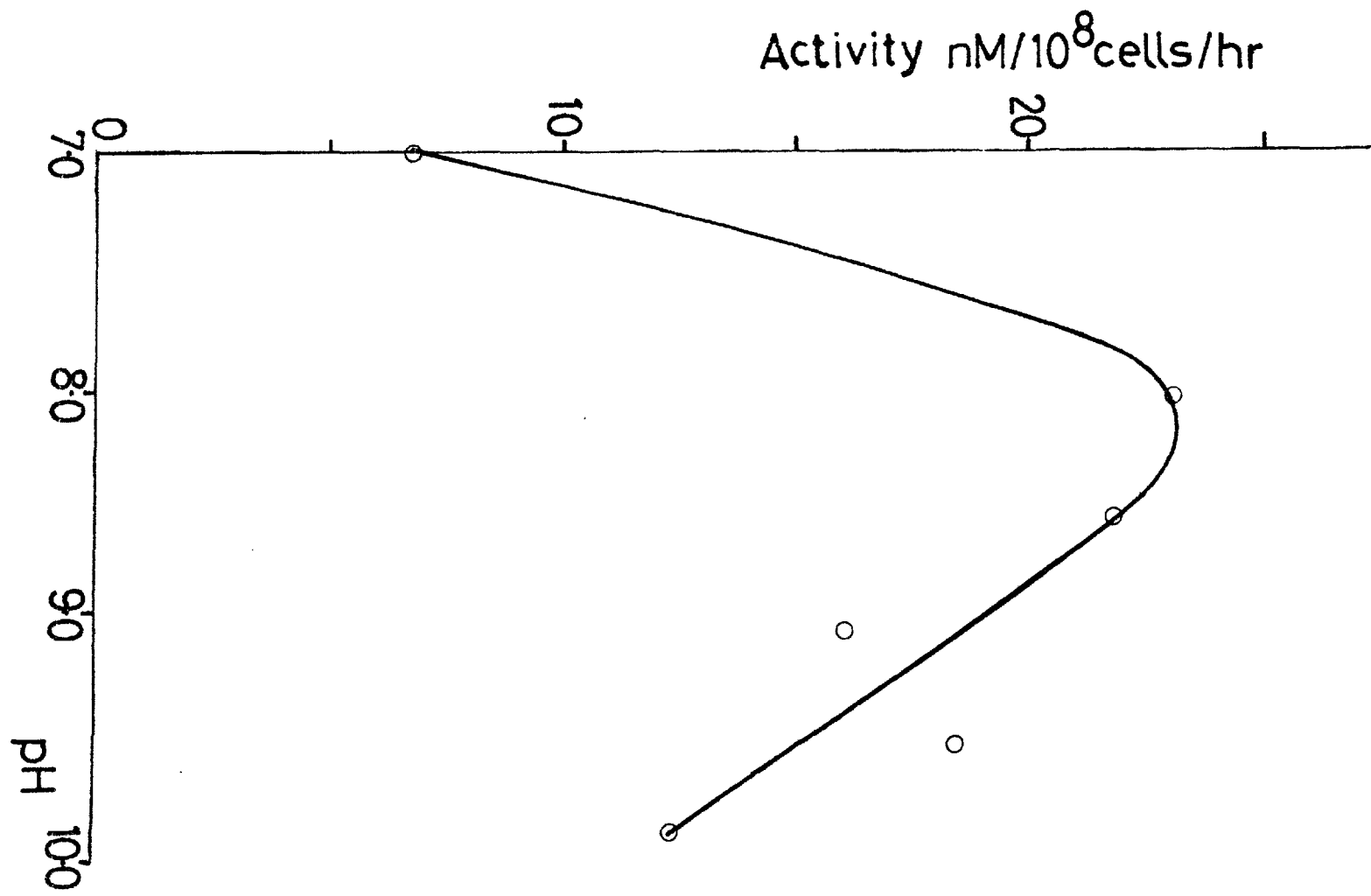
A pH optimum of 8.5 was obtained using this enzyme preparation. In the aerobic shaking assay system the pH optimum was 8.25 with a conversion of about 7% after 72 hours shaking (see Fig. 10). Using either assay system, incubation of oestradiol or oestrone as substrate, did not produce any methylated products, although some 17-oxidoreductase activity was observed.

2.4. Substrate specificity

Strains c.c. 493 (Clostridium butyricum) and c.c. 633 (Clostridium paraputrificum) were tested for their ability to degrade different substrates with a 4-en-3-one configuration and variation in functions at the carbon 17 position.

2.41. 4-cholesten-3-one: Incubation of this substrate with induced cultures of c.c. 633 produced five phenolic metabolites, not present in control cultures. Their thin layer chromatographic behaviour in solvent V_1 gave mobilities (relative to testosterone, R_T) of 0.52, 1.25, 1.41, 1.54 and 1.70. The R_T of oestradiol is 1.25. The eluted compounds all gave a U.V. spectrum $\lambda_{\text{max}} = 280\text{nm}$, E about 2,400. Fluorimetric scans gave similar spectra for all five metabolites, $E_{\text{max}} = 285\text{nm}$, $A_{\text{max}} = 330\text{nm}$. All metabolites were produced at a percentage yield of 5% or less. The compound isolated with $R_T = 1.25$ had a mass spectrum with parent ion $m/e = 272$ and a

Fig. 10. Effect of pH on the production of metabolite B by bacterial cells in washed cell suspension.



base peak $m/e = 147$. This spectrum was identical to that of authentic oestradiol. The compound $R_T = 1.70$ had a mass spectrum with parent ion $m/e = 368$. This mass fits the structure for the compound 1,3,5(10)-cholestatrien-3-ol and was tentatively identified as such. Other large peaks in the spectrum were at $m/e = 297$ ($-C_5H_{11}$), $m/e = 255$ ($-C_8H_{17}$) and $m/e = 147$ (ring A and ring B fragment). Identification of the other three unknown metabolites was inconclusive. These showed parent ions of $m/e = 325$, 340 and 345. The spectra, however, were by no means pure; possibly the samples purified by T.L.C., still contained contaminants, or else the compound may lack stability in mass spectral analysis. If these parent ions are representative of the compounds, partial side chain degradation associated with aromatisation is suggested.

C.C.493, the other strain tested, produced only three metabolites of phenolic nature. Their relative mobilities were 0.52, 1.25 and 2.10 on a thin layer plate, using solvent V_1 . They all had phenolic properties, producing a blue spot with potassium ferricyanide spray reagent and U.V. spectrum λ_{max} about 280 nm and $E = 2,300$. The metabolite with $R_T = 2.10$ was identical in behaviour to metabolite B and compound E described in the previous section (Results 2.3.). This compound was identified as 17 β -methoxy-1,3,5(10)-estratrien-3-ol and its mass spectrum confirmed this (see Fig. 8). The metabolite with an $R_T = 1.25$ was identified from its mass spectrum as being oestradiol, being of identical cracking pattern to the authentic oestrogen. The third product, $R_T = 0.52$ was produced at less than 0.5% conversion and was not further characterised

2.42. 11 β , 17 α , 21-trihydroxy-4-pregnen-3,20-dione (cortisol): In addition to the 1mM substrate added to the induced culture, 1 μ Ci of carbon 4 ^{14}C cortisol was added. When the chloroform extract was separated by T.L.C. in

solvent V_1 and V_3 , three peaks were traced by the radioactive scanner. In the solvent V_1 , the parent peak had a low mobility running close to the origin and the two other metabolites showed mobilities relative to the parent compound of 1.86 and 2.70. These radioactive metabolites were phenolic, giving a blue spot with potassium ferricyanide and Folin's spray reagent. Percentage yield of these two compounds, was estimated by the area under the radioactive peaks and was 4.5 and 3.5% respectively. On elution from the T.L.C. plate they both gave a U.V. spectrum $\lambda_{\max} = 280\text{nm}$ and a fluorimetric spectrum $E_{\max} = 285\text{nm}$ and $A_{\max} = 335\text{nm}$. They were not however, further characterised, but were tentatively identified as aromatisation metabolites of unknown structure.

2.43 11 α -hydroxy-4-androsten-3,17-dione: In contrast to the above substrate, using $1\mu\text{Ci}$ 11 α -hydroxy-4-androsten-3,17-dione in a 1mM solution of cold compound, produced no phenolic products when incubated with induced cultures of c.c. 493 and c.c. 633. When the products were analysed by T.L.C. in solvents V_1 , V_2 and V_3 and the plates scanned, one metabolite showed up as a radioactive peak at about a 15% yield. This was found to be the product of the saturation of the C_4-C_5 double bond. It did not react with Folin's reagent or ferricyanide spray, but produced a blue/purple spot with anisaldehyde spray reagent. Unlike the parent compound this metabolite did not show absorption properties under a U.V. light, further indicating the loss of a double bond. Relative mobilities of the metabolite in solvents V_1 , V_2 and V_3 were identical to authentic 11 α -hydroxy-5 β -androstan-3,17-dione.

2.44. 3-oxo-4-cholen-24-oic acid: using 3-oxo-4-cholen-24-oic acid as substrate, three products were obtained, each with U.V. spectra $\lambda_{\max} = 280\text{nm}$ and $E = 2,300$, typical of phenolic steroids. All three showed a blue colour

reaction on a T.L.C. plate, when sprayed with Folins reagent and potassium ferricyanide reagent. In solvent V₁ they had relative mobilities, R_T , of 2.10, 1.92 and 1.25. The compounds with $R_T = 2.10$ and 1.25 had properties indistinguishable from those of 17 β -methoxyoestradiol and oestradiol respectively, and were characterised as above. The third product $R_T = 1.92$ was obtained at a low yield (about 0.1%). The mass spectrum showed a parent ion of $m/e = 356$. From this spectrum and its other phenolic properties the metabolite was tentatively identified as 3-hydroxy-1,3,5(10)-cholatrien-24-oic acid. Characteristic peaks were found at $m/e = 343$ ($-\text{CH}_3$), $m/e = 236$ ($-\text{CH}_3 + \text{OH}$) and a large peak common to oestradiol was also identified at $m/e = 147$ (ring A and ring B fragment).

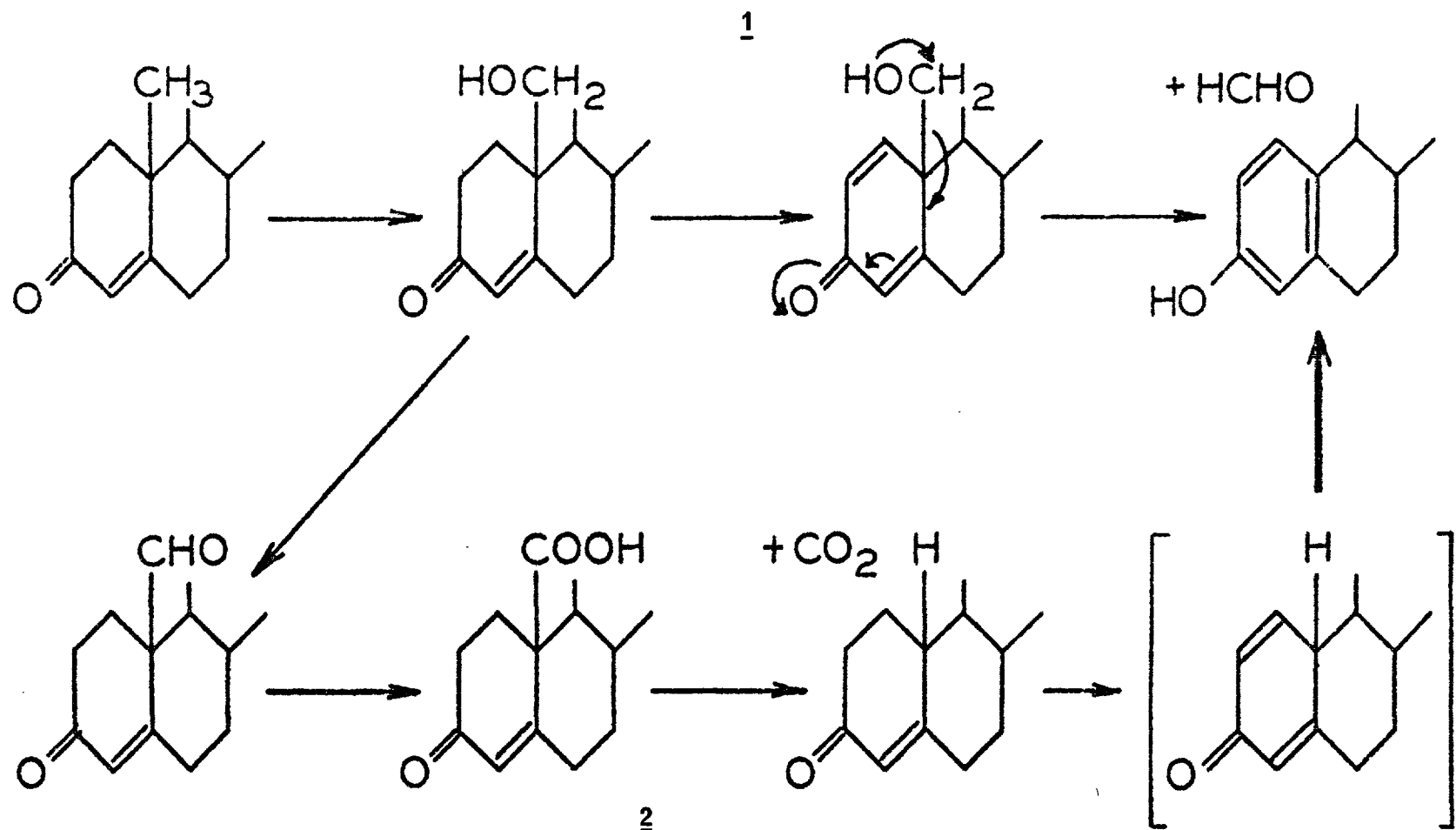
2.45. 1,4-androstadien-3,17-dione: No phenolic products could be obtained using this compound as substrate.

2.5. Discussion

In considering the mechanism by which Clostridia aromatise steroids, it is necessary to look to the mechanisms of related reactions in other bacterial systems and mammalian placental preparations. Talalay (1965;1957)

has proposed two mechanisms to explain aromatisation reactions carried out by these systems. Both mechanisms (see Fig. 11) postulate the oxidative modification and elimination of the C₁₉ methyl group and the production of a C₁-C₂ double bond, prior to aromatisation. Talalay suggested that the 19-hydroxy intermediate was essential in oestrogen production. Meyer (1955a) showed that 19-hydroxy-4-androsten-3,17-dione was essential in the oestrogen production by mammalian placental extracts. However, Hayano et al. (1960), have shown that Bacillus sphaericus does not utilise 19-hydroxy steroids in aromatisation reactions.

Fig. 11. Two alternative mechanisms for the oxidative demethylation and aromatisation of steroids (from Talalay, 1957).



In support of pathway 1 (see Fig. 11), Sneedon and Turner (1955), have demonstrated the elimination of formaldehyde when placental extracts were incubated with certain ouabain derivatives. Meyer (1955b), whilst not producing positive evidence for pathway 1 strongly disputed the possibility of the alternate hypothesis. Using human placental preparations, he was unable to show significant conversion of 4-estren-3,17-dione or 1,4-androstadien-3,17-dione to oestrone. The only evidence for pathway 2 was inferred from related work by Olson and Block (1956). They showed that rat liver homogenates, when incubated with lathosterol (4,4¹,14-trimethyl-8,24-cholestadien-3-ol) labelled with ¹⁴C at the methyl groups, released labelled carbon dioxide; none of the label was trapped in formaldehyde or amino acids such as serine. They made the rather dangerous generalisation that all demethylation reactions may follow the same pathway. Brodie et al. (1968), presented evidence that suggested that the bacterial mechanisms of aromatisation by *Nocardia* and *Pseudomonas* operated on pathway 1, whilst mammalian systems had a mechanism similar to pathway 2. This argument was based on observations that suggested that aromatisation reactions with a concomitant expulsion of the C₁₉ methyl group involved the loss of the 1B hydrogen. On the other hand, a prior C₁-C₂ dehydrogenation has been shown to be associated with the elimination of the 1α hydrogen group (Morato et al., 1962 and Ringold et al., 1963). Sih et al. (1968b), showed a series of aromatisation reactions using the soil organism *Nocardia restrictus*. Using a substrate of 19-hydroxy-4-cholesten-3-one, he identified a series of phenolic products, which were produced in close association with side chain cleavage.

The aromatisation reactions demonstrated with both *Clostridia* and *Enterobacteria*, when taken in the context of the above hypotheses appear

to follow pathway 1, as suggested by Brodie et al. (1968), for other bacterial systems. The failure to produce oestrogens from a $\Delta^{1,4}$ substrate supports this and in addition, the requirement of NAD^+ and phenazine methosulphate may be significant. This suggests that at least two enzymes may be involved, a demethylase together with a Δ^1 dehydrogenase rather than the single 'aromatase', which is associated with mammalian systems, which require NADP^+ and oxygen (Townsend and Brodie, 1966); artificial electron acceptors could not be substituted for NADP^+ in this system. This is in contrast to the nuclear dehydrogenations (eg. Davidson and Talalay, 1966), which require artificial electron acceptors such as menadione or phenazine methosulphate. Therefore, the requirements of both natural and artificial acceptors in the aromatisation of steroids by the intestinal bacteria, indicated that the demethylation step may be accompanied by a separate desaturation at the $\text{C}_1\text{-C}_2$ position. Inhibition of the crude enzyme preparations by such a wide range of cations and anions also suggests that more than one enzyme is involved in this system. However, the removal of either electron acceptor did not appear to cause the build up of an intermediate metabolite, but caused complete inhibition.

The production of 17β -methoxy oestradiol by the clostridial strains adds a further complication to the mechanism. The methylation step appears to be closely linked to the aromatisation reaction and could not be demonstrated except as part of this enzymic system. Oestrone and oestradiol were not methylated by the bacteria, but on the other hand, side chain cleavage products from 4-cholesten-3-one and 3-oxo-4-cholen-24-oic acid substrates did show partial methylation at the C_{17} position. It is debatable whether the methylase acts by selectively removing the C_{10} methyl group released during aromatisation, transferring it to the C_{17}

position, or by drawing randomly from the metabolic pool of carbon donors. This could be resolved by selective labelling at the C₁₉ position and measuring the specific activity of substrate and metabolite.

The production of phenolic metabolites, following side chain cleavage has been previously reported by Sih et al. (1968b), using the soil organism Nocardia restrictus. He showed the production of phenolic C₂₂ acids from 19-hydroxy-4-cholesten-3-one and these were identified as intermediates in the production of oestrone. I have demonstrated complete side chain degradation associated with aromatisation of similar substrates using intestinal organisms. It is likely, that the compounds which were not characterised, were of a similar intermediate nature. As described above one of these compounds had a mass spectrum with a parent ion of $m/e = 326$. This is identical to that of the compound 3-hydroxy-19-nor-bis-1,3,5,(10)17(20)-cholaten-22-oic acid, identified by Sih, which also had a parent ion of $m/e = 326$.

The common factor in all the aromatisation reactions observed as being carried out by strains of intestinal bacteria, was that the substrate had to be of the 4-en-3-one configuration, but that the nature of the carbon 17 side chain was unimportant; aromatisation reactions were demonstrated using androstane, cholanyl, cholesteryl and pregnane type steroids. The only 4-en-3-one substrate that was not aromatised was the 11 α -hydroxy-4-androsten-3,17-dione. Here, it is possible that the 11 α hydroxyl group, sterically at right angles to the plane of the steroid nucleus, could cause steric hinderance and inhibition in the enzyme. This is in contrast with cortisol, which has an 11 β hydroxyl group and was metabolised to two phenolic metabolites.

3.

Introduction of Further Double Bonds

Preliminary results have shown that strains of lecithinase-negative Clostridia, capable of aromatising 4-androsten-3,17-dione, are also able to aromatise 4,6-androstadien-3,17-dione. Screening techniques, carried out by incorporating the substrate into egg yolk agar plates (see Methods 4.3.), showed that 5 out of 22 strains of lecithinase-negative Clostridia, produced a phenolic product. After spraying the plates with equilin spray reagent, a red halo appeared around positive colonies. This colouration is due to a complex between the equilin and the spray reagent, P.N.B.D.. Further analysis of the agar around the colonies from a duplicate, by thin layer chromatography, showed that two of these strains were false positive, the compounds reacting with the P.N.B.D. not having a T.L.C. mobility similar to that of any of the authentic markers. Strain c.c.510 and c.c. 498 (Clostridium paraputrificum) and c.c.600B (Clostridium oedamatiens) produced compounds with relative mobilities on T.L.C. in solvents V_1 , V_2 and V_3 identical to those of authentic markers. Strains c.c.510 and c.c. 600B, produced metabolites equivalent to authentic 1,3,5(10),7-estratetraen-3-ol-17-one (equilin) and strain c.c. 510 produced a metabolite equivalent to 1,3,5(10),6,8-estrapenten-3-ol-17-one (equilenin). Both metabolites were visualised by their absorbance on a fluorescent U.V.₂₅₄ T.L.C. plate under a U.V. lamp. Each compound produced a blue spot with phenol specific sprays and a red colouration with the equilin specific spray, P.N.B.D.. Most distinctive were the U.V. spectra, which are unique to these compounds. The estrapentene gave seven peaks between 220nm and 350nm and the estratetrene, only four peaks. The criteria used for identification are summarised in Table 10. The mass spectra for these compounds are given in Figs. 12 and 13.

TABLE 10

Comparison of the Chemical and Physical Properties of *Equilin and
**Equilenin.

<u>Compound</u>	<u>Colour with</u> <u>P.N.B.D.spray</u>	<u>R_n in Solvent</u>			<u>U.V. Spectrum, λ_{max} nm.</u>
		<u>V₁</u>	<u>V₂</u>	<u>V₃</u>	
<u>Authentic*</u> Equilin	Orange	2.50	4.50	2.13	230, 262, 271, 305.
<u>Authentic**</u> Equilenin	Red	2.40	4.02	1.89	230, (258), 269, 280, 291 327, 340.
<u>Bacterial Metabolites;</u>					
Strain C.C. 510	Red	2.40	4.00	1.91	230, (258), 271, 280, 291, 327, 340.
Strain C.C. 600B	Orange	2.50	4.50	2.11	230, 262, 271, 305.
Strain C.C. 498	Orange	2.48	4.50	2.10	230, 262, 271, 305.
<u>Substrate; 4,6-diene.</u>	None	2.25	3.85	1.22	220, 284.

*Equilin, proper name; 1,3,5(10),7-estratetraen-3-ol-17-one.

**Equilenin, proper name; 1,3,5(10),6,8-estrapenten-3-ol-17-one.

Fig. 12. Mass spectra of T.M.S. derivatives of phenolic steroids, using a G.L.C./Mass spectrometer system.

- A) Mass spectrum of T.M.S. derivative of authentic equilin (1,3,5(10),7-estratetraen-3-ol-17-one)
- B) Mass spectrum of T.M.S. derivative of bacterial metabolite, identified as equilin.

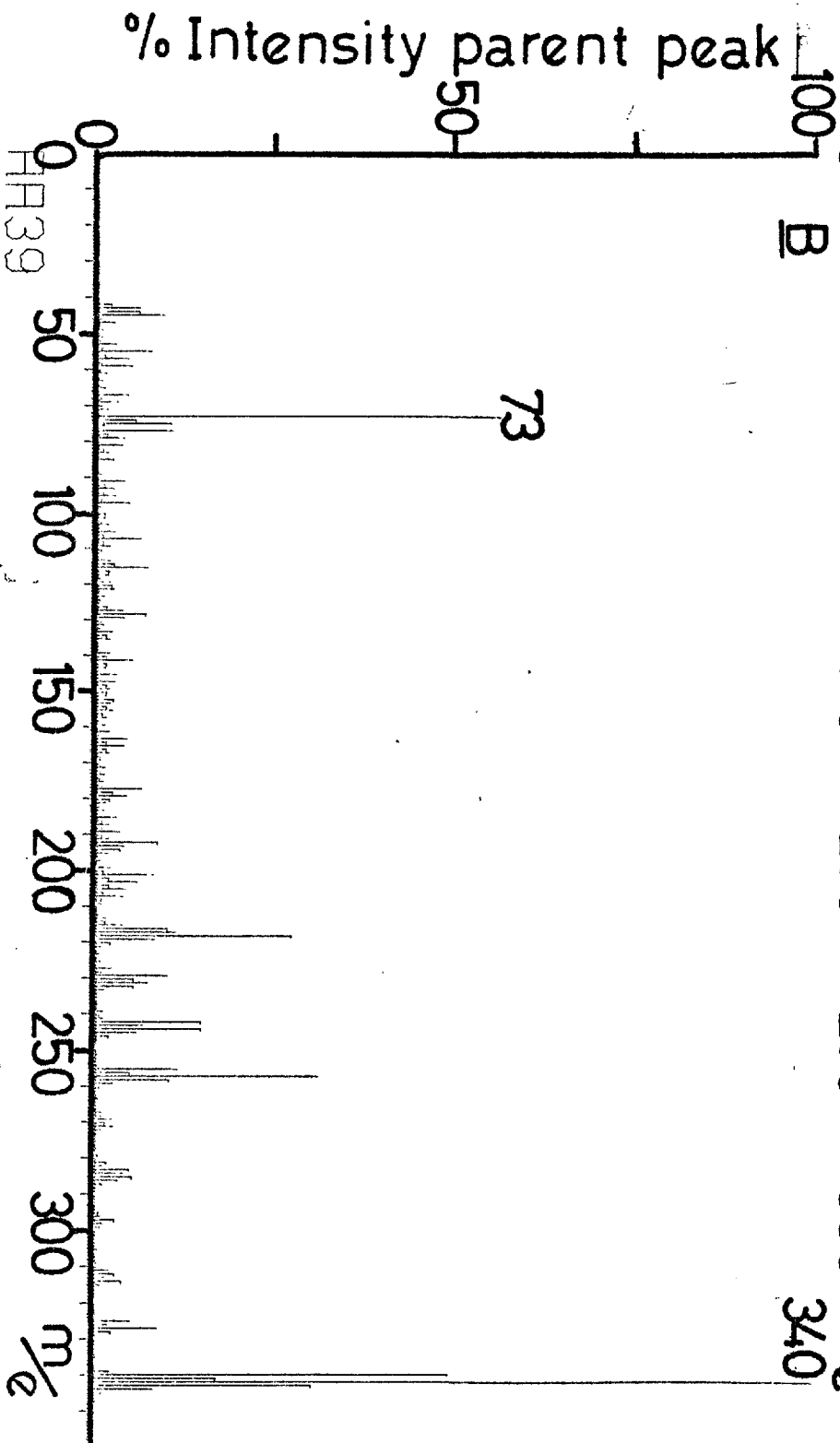
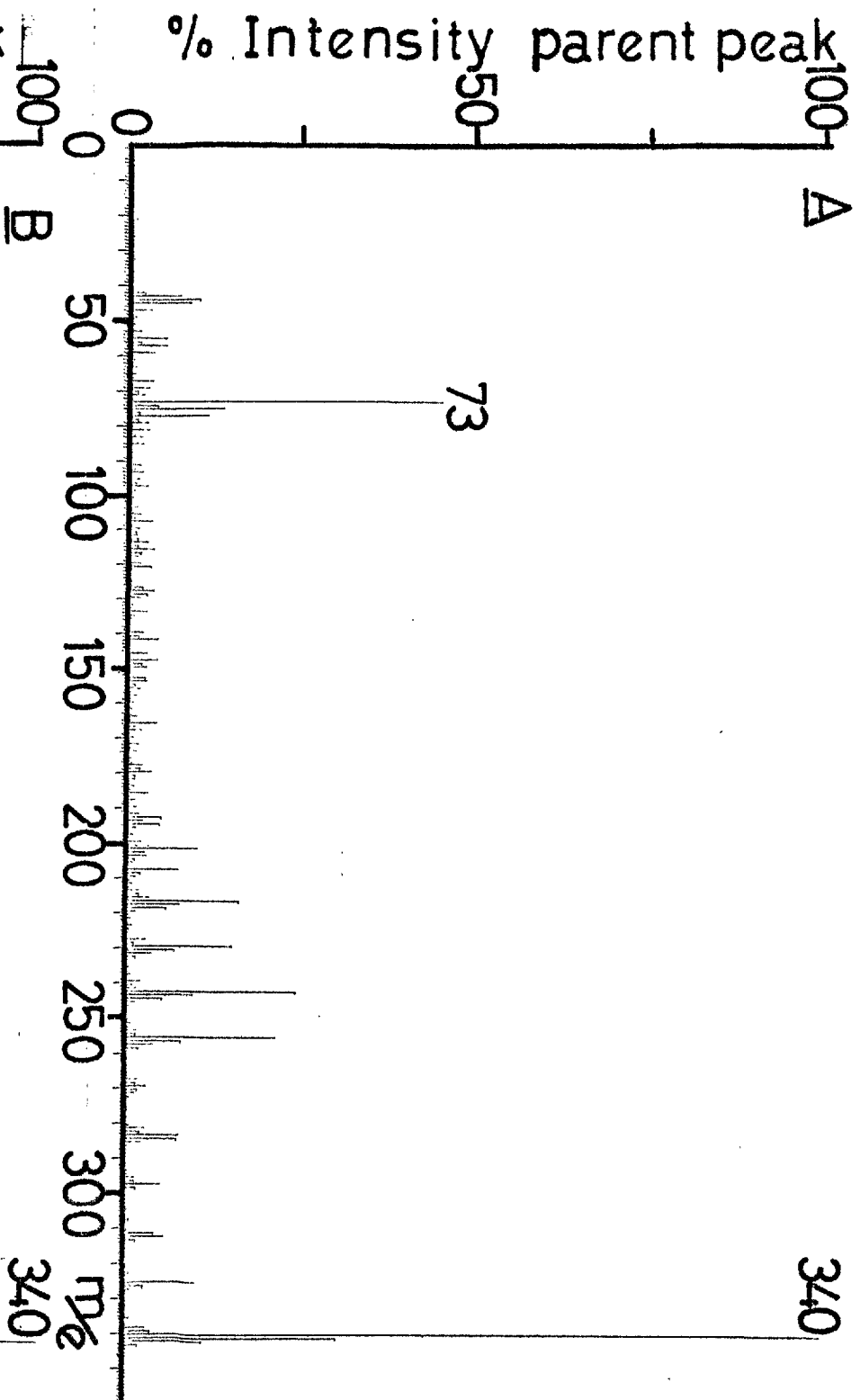
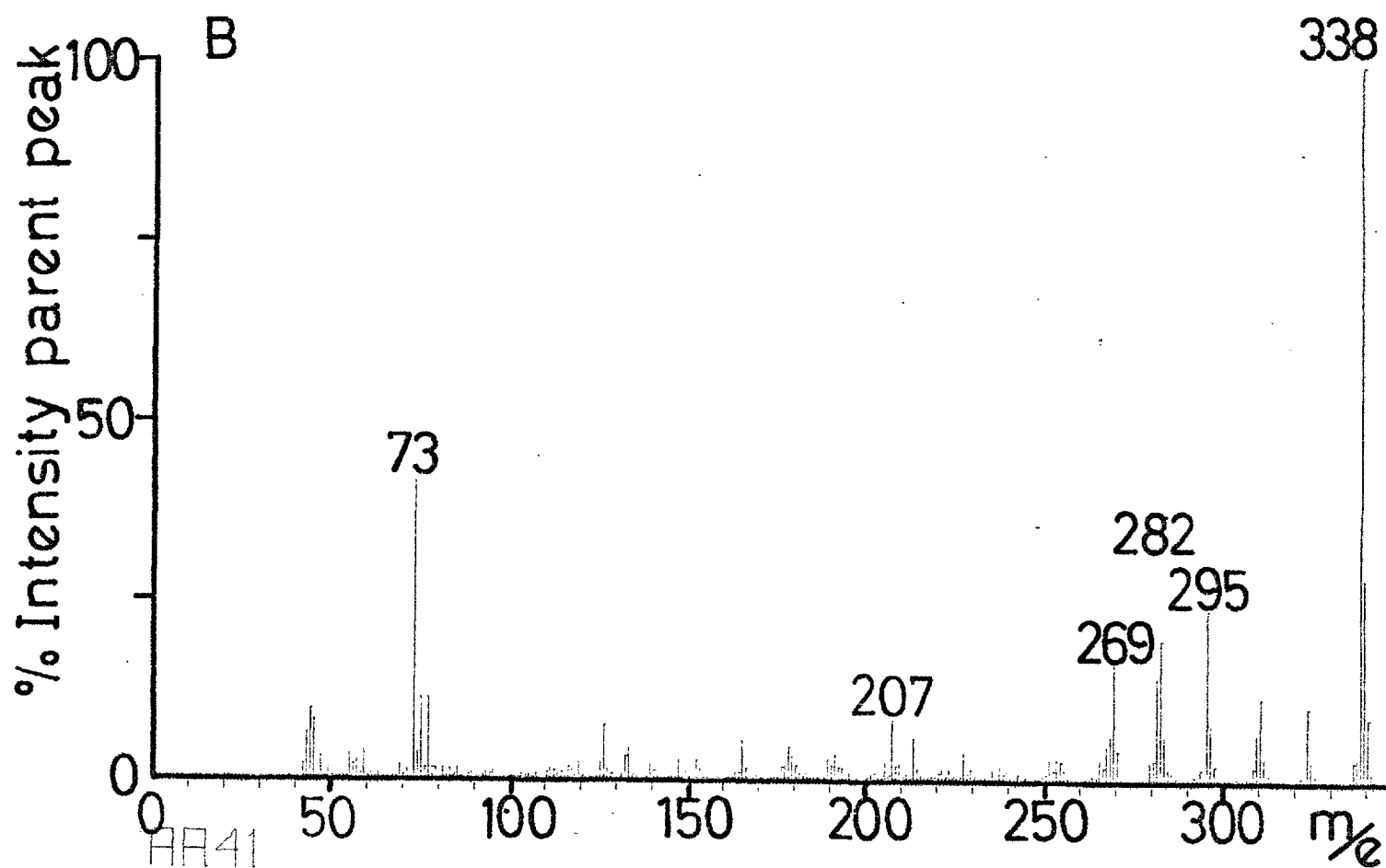
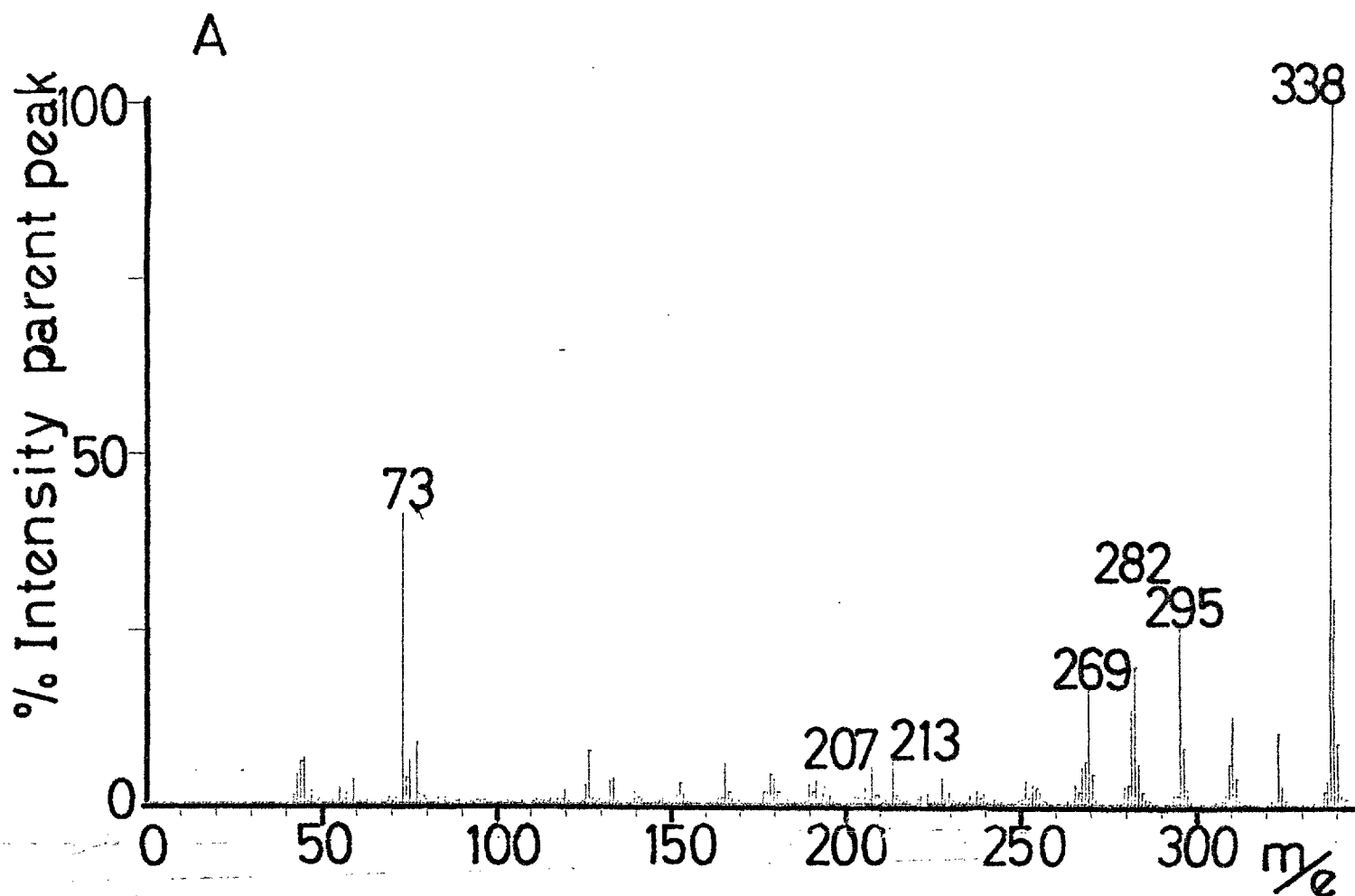


Fig. 13. Mass spectra of T.M.S. derivatives of phenolic steroids, using a G.L.C./Mass spectrometer system.

- A) Mass spectrum of T.M.S. derivative of authentic equilenin (1,3,5(10),6,8-estrapenten-3-ol-17-one).
- B) Mass spectrum of T.M.S. derivative of bacterial metabolite, identified as equilenin.



The T.M.S. derivative of the estratetrene gave a prominent parent ion at $m/e = 340$ and the estrapentene at $m/e = 338$. The same strains also produced a phenolic steroid when incubated with 4,6-cholestadien-3-one. The U.V. spectra of these compounds obtained on elution from T.L.C. plates was very similar to those obtained from androstane substrates. The compounds also showed a positive reaction with phenol and equilin specific sprays, and were tentatively identified as the cholesterol analogues of equilin and equilanin. These compounds were not further characterised.

Parallel experiments using Todd Hewitt broth as growth medium and an aerobic shaking assay, produced low yields of 1,3,5(10),7-estratetraen-3-ol-17-one from 4,6-androstadien-3,17-dione substrate. This reaction was only demonstrated with strains c.c. 498 and c.c. 600B; strain c.c. 510, although positive using the plate assay system, was negative in broth culture. A further strain of Clostridium paraputrificum, c.c. 633, produced a phenolic metabolite with a T.L.C. relative mobility, R_T , of 0.71 in developing solvent V_1 . It reacted with phenol specific and equilin specific sprays and its U.V. spectrum showed maxima at 230, 269, 272, 280 and 288nm. This compound was tentatively identified as 1,3,5(10),6-estratetraen-3-ol-17-one, based on a previous report by Starka et al., (1966), who described a similar compound with a U.V. spectrum identical to that of the above metabolite.

Discussion

The production of estratetrenes from the 4,6-dien-3-one substrate is merely an extension of the previous section, being the aromatisation of a 4-en-3-one type substrate, with a double bond already present in ring B. Production of the estrapentene is a manifestation of a further reaction,

since the organisms have introduced a double bond NOT in conjugation with a keto group or as a result of a demethylation, but in conjugation with an aromatic ring A.

Of the strains producing an estratetrene, in one the ring B double bond remains at the C_6-C_7 position whilst in the other two there is a migration to the C_7-C_8 position. The question is, why should this migration occur? On simple theoretical principles the C_6-C_7 should be favoured, since it is in conjugation with ring A, whilst the C_7-C_8 double bond involves a migration to a non-conjugated position. Apparently, however, enzymic aromatisation in this bacterial system favours this rearrangement in ring B.

Equilins are only found naturally in the urine of pregnant mares, (Klyne, 1955). Sachanick and Berlinen (1957), however, have demonstrated equilin production from a case of feminizing adrenal carcinoma in a male patient. Synthesis of equilin in the horse placenta (Starka and Breuer, 1966), and by microbial soil organisms (Vezina et al., 1969), occurs from the $\Delta^{5,7}$ substrate and not the $\Delta^{4,6}$ substrate as demonstrated with the clostridial systems. 1,3,5(10),6-estratetraen-3-ol-17-one has been produced in vitro only; using a 4,6-androstadien-3-one-17 β -ol substrate human placental preparations have shown this reaction (Starka et al., 1966).

4. Production of a Double Bond by a Dehydration Reaction

Samuelsson (1960), showed that the 7α -dehydroxylation of cholic acid involved the formation of an unstable Δ^6 intermediate, which was rapidly reduced to deoxycholic acid. No-one has reported the isolation of the Δ^6 intermediate, but such a product might be stabilised in conjugation with a 4-en-3-one group; a number of strains known to dehydroxylate cholic acid were therefore incubated with such a substrate.

Using 3-oxo- 7α -hydroxy-4-cholen-24-oic acid as substrate, several strains of intestinal bacteria showed the production of 3-oxo-4,6-choladien-24-oic acid. Strains which were shown to be positive were: Bacteroides fragilis (N.C.T.C.9343), Clostridium welchii (c.c.273 and c.c.63) and E.coli (E.B.C.109). The 4,6-dienewas only produced when cells were grown above pH 6.5 and the reaction was inhibited by the presence of 30mM Cu^{2+} , 3mM periodate and 30mM iodoacetate ions. The enzyme was induced with cholic acid and chenodeoxycholate ($3\alpha,7\alpha$ -dihydroxy).

The 3-oxo-4,6-choladien-24-oic acid metabolite was identified by its U.V. spectrum ($\lambda_{\text{max}} = 284\text{nm}$ and $E = 26,000$) and its fluorimetric spectrum ($E_{\text{max}} = 275\text{nm}$ and $A_{\text{max}} = 317\text{nm}$). This was identical to the chemically synthesised compound. A melting point estimation gave a value of $181-183^\circ\text{C}$ and a mixed melting point caused no depression of this value. The compound gave a red/brown colouration with anisaldehyde spray reagent, but did not give a blue colouration with phenol specific potassium ferricyanide spray reagent.

Percentage yield of the metabolite was low and varied between 3% and 8% at pH 7.0. A further metabolite was detected, which was a dehydroxylation product having a T.L.C. mobility equivalent to 3-oxo-4-cholen-24-oic

acid. This was produced at about a 3% yield.

Discussion

These results, although very preliminary, have demonstrated that a double bond may be introduced at the C₆-C₇ position as a result of a dehydration reaction. The strain shown to be positive for this reaction and the conditions for the conversion are identical to those for the 7-dehydroxylation reaction demonstrated by Aries (1970) and Aries and Hill (1970b). It is therefore reasonable to assume that the same enzyme is involved and that Samuelsson's hypothesis, that a desaturated intermediate is formed in the 7-dehydroxylation is demonstrated by the formation of the stable 4,6-dien-3-one metabolite. A similar reaction has been demonstrated by Hayakawa et al. (1959 and 1964), using strains of *Corynebacteria*. This hypothesis is further supported by the small amounts of dehydroxylated product isolated.

The pattern of inhibition, identical to that of the 7-dehydroxylase, suggests that the active site requires the involvement of sulphhydryl groups; periodate, iodoacetate and cuprous ions all modify these groups in proteins.

SECTION III B

CHOLESTEROL METABOLISM,
IN VIVO AND IN VITRO.

5. Cholesterol Metabolism in Vivo in Rats and Guinea Pigs

Since cholesterol is only poorly absorbed, if at all, from the large intestine, the metabolism of this neutral steroid was followed by the direct introduction of radioactive cholesterol into the caecum of rats and guinea pigs. 24 hour samples of urine and faeces were obtained from the animals and the distribution of radioactivity was estimated both quantitatively and qualitatively. The distribution of radioactive counts in the various steroidal fractions isolated is given in Table 11. The neutral, acid and urine fractions are represented by the mean and standard deviations of the sum of the total counts obtained over 72 hours from each animal. It can be seen that the majority of the activity remains in the neutral steroid fraction, although in the rat acid steroid extracts a significant level of counts was obtained. Table 12 shows the average distribution of activity in the isolated fractions for 24 hour samples in the two experimental models. It can be seen that within the first 48 hours, 90% of the counts had been eliminated in the faeces and that after 72 hours only a very low level of residual activity was present in the caecal and colonic contents. If the animals were kept in the metabolic cages for an additional 24 hours, the activity of faecal and urinal steroids in both guinea pigs and rats fell to a negligible level.

5.1 Qualitative and Quantitative Estimation of the Neutral Steroid Fraction

Rats: Extracts of neutral steroid fractions separated by T.L.C. in NS developing solvent were scanned for radioactive peaks. Three large peaks and two minor peaks were resolved using this solvent and their relative mobilities to cholesterol, R_G , were 0.00, 1.00, 1.28, 1.81 and 2.09. The

TABLE 11

The Distribution of Radioactive Counts Recovered from Rats and Guinea Pigs Over a Three Day Period, after the Introduction of 5 μ Ci Cholesterol Directly into the Caecum.

	<u>RATS</u>	<u>GUINEA PIGS</u>
No. Animals Tested.	7	5
Faeces:-		
Neutral Steroids %	87.7 $\pm$ 4.64	88.25 $\pm$ 4.38
Acid Steroids %	6.27 $\pm$ 4.15	0.75 $\pm$ 0.26
Urine, Total Counts %	0.32 $\pm$ 1.80	1.65 $\pm$ 0.43
Unrecovered %	5.86 $\pm$ 2.04	9.35 $\pm$ 0.43

TABLE 12

Distribution of Radioactive Counts Recovered from Rats and Guinea Pigs in Each 24 Hour Sample After the Introduction of 5 μ Ci ^{14} C Cholesterol into the Caecum by Direct Injection.

A. RATS

<u>Time,Hours</u>	<u>Neutral Steroids %</u>	<u>Acid Steroids %</u>	<u>Urine %</u>
24	56.0	80.0	55.0
48	37.3	17.6	17.7
72	6.3	1.8	27.3
Caecum & Colon	0.4	0.6	-
<u>Total</u>	100.0	100.0	100.0

B. GUINEA PIGS

<u>Time,Hours</u>	<u>Neutral Steroids %</u>	<u>Acid Steroids %</u>	<u>Urine %</u>
24	60.5	30.0	19.2
48	27.7	40.7	26.8
72	11.5	29.0	54.0
Caecum & Colon	0.3	0.3	-
<u>Total</u>	100.0	100.0	

The percentages stated are mean values obtained from all animals tested.

latter two compounds represented the minor peaks and co-chromatographed with authentic samples of 5 β -cholestan-3-one and 4-cholesten-3-one respectively. The latter was also able to absorb U.V. light when separated on a fluorescent T.L.C. plate and illuminated with a Hanover U.V.₂₅₄ lamp; this is indicative of an unsaturated ketone. The U.V. spectrum for this compound gave a $\lambda_{\text{max}} = 244\text{nm}$. The peak with $R_G = 1.28$ co-chromatographed with coprostanol (5 β -cholestan-3 β -ol). The purified compound, eluted from a preparative T.L.C. gave a melting point of 99-101°C and a mixed melting point did not depress this value. The I.R. spectrum of the authentic and isolated compounds were identical, giving a triplet of sharp peaks at 3.25, 3.30 and 3.35 μm , followed by two broader peaks at 3.40 and 3.60 μm . The compound with $R_G = 1.00$, was the parent peak, cholesterol. The compound which did not leave the origin, having an $R_G = 0.00$, was not identified. This compound appeared in none of the 24 hour, 50% of the 48 hour and all of the 72 hour samples, suggesting that some of the cholesterol substrate had been progressively degraded over a three day incubation, possibly to non-steroidal products. This peak accounted for between 5 and 24% of the total activity of the neutral steroid fraction and varied greatly with individual animals. This did not effect the relative concentrations of the four other metabolites which reached an equilibrium point at the levels given in Table 13. This shows the percentage distribution of counts between cholesterol, coprostanol, coprostanone and 4-cholesten-3-one; the conversion to coprostanol from cholesterol varied between 50 and 65%.

Guinea Pigs: The neutral steroid fraction isolated from the guinea pig faeces showed significant variations from that of the rat (see Table 13). The conversion to coprostanol was higher at approximately 69% and did not show much variation among individual animals. An equilibrium in the neutral

TABLE 13

Relative Concentrations of the Faecal Neutral Steroids of the
Rat and Guinea Pig.

<u>Neutral Steroid</u>	<u>Rat</u>	<u>Guinea Pig</u>
*5-cholesten-3 β -ol (cholesterol.)	35.0	26.7
*5 β -cholestan-3 β -ol (coprostanol.)	57.3	69.0
*5 β -cholestan-3-one (coprostanone.)	2.0	0.0
*4-cholesten-3-one	5.7	4.3

* Values are expressed for each compound as the percentage of the total neutral steroid fraction and are mean values.

steroid metabolites was gained within 24 hours and remained constant at 48 and 72 hours. The presence of low concentrations of 4-cholesten-3-one was detected, but there appeared to be no 5 β -cholestan-3-one in the faecal extracts. The major difference with the guinea pig compared to the rat, was that when the neutral steroid fraction was separated by T.L.C. in NS developing solvent, there was no radioactive peak detectable at the origin ($R_G = 0.00$) in any of the faecal extracts.

5.2. Qualitative and quantitative estimation of the acid steroid fraction

Guinea pigs had a very low activity profile in the acid steroid fraction as seen from Table 11. Pooled acid steroid extracts showed a small radioactive peak at the origin of a thin layer plate run in solvent S_1 . The acid steroid fractions isolated from rat faeces, when separated by T.L.C. in solvent S_1 , showed a single peak when scanned for radioactivity which remained at the origin. When run in a more hydrophilic solvent, chloroform/methanol/ammonia (70:30:1) the compound still remained at the origin, but in solvent 20% KCl/acetic acid (100:1) a single peak was detected at the solvent front.

5.3. Incubation of rat faecal acid steroids with sulphatase

Fig. 14 shows the radioactive profiles from T.L.C. analyses of aliquots of acid steroid fractions after incubation with sulphatase for various time intervals. Two peaks were formed which migrated from the origin when analysed by T.L.C. in developing solvent S_{15} ; these were termed peak S and T, the latter being at roughly twice the concentrations of the former. An enzyme-negative control did not produce peaks S and T. Exhaustive incubation with sulphatase enzyme left a residual peak of about 15%, which

Fig. 14. Radioactive profiles of rat faecal acid steroid fraction separated by T.L.C. in solvent S_1 , at time intervals after incubation with sulphatase enzymes.

Peak A The parent peak with $R_L = 0.00$

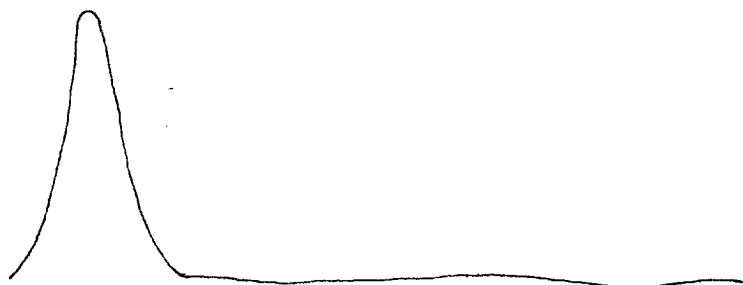
Peak S Identified as 3α -hydroxy- 5β -cholan-24-oic acid
with $R_L = 1.00$

Peak T Identified as 3β -hydroxy- 5β -cholan-24-oic acid
with $R_L = 1.15$

(R_L - Relative mobility c.f. lithocholic acid)

TIME MINS.

0



30



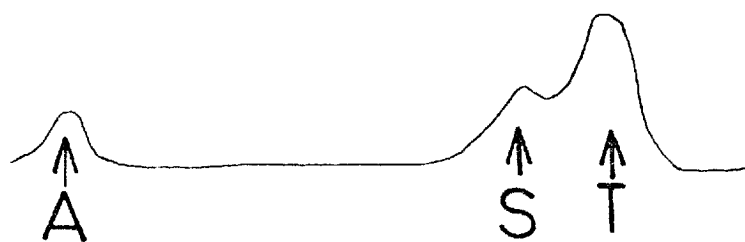
60



90



180



did not migrate from the origin of the T.L.C. plate.

In developing solvent S_{15} , peak S had a mobility relative to a lithocholate marker of 1.00 and peak T had a relative mobility of 1.15. The latter co-chromatographed with an authentic sample of 3β -hydroxy- 5β -cholan-24-oic acid (isolithocholate). Peak S had a T.L.C. behaviour of lithocholate and as can be seen from Fig. 14, represented about 50% of peak T. Both had mass spectra with a parent ion of $m/e = 374$, but showed extensive degradation from electron bombardment. After methylating the carboxyl group with diazomethane, both compounds gave a parent ion of $m/e = 388$.

It was concluded that there was overall conversion of 1.8% and 3.5% to the sulphates of lithocholate and isolithocholate respectively, from cholesterol in the lower intestine.

5.4. Qualitative estimation of urine extracts

Due to the low radioactive counts obtained in urines, pooled extracts were required to examine molecular species. From Table 11, it can be seen that there was a higher percentage of counts in the urine of guinea pigs ($\bar{x} = 1.65\%$), than in that of rats ($\bar{x} = 0.32\%$). The radioactive material isolated from rats was not identified and was probably non steroidal; it could not be extracted with chloroform from the acidified urine, even after extensive acid hydrolysis or incubation with sulphatase or glucuronidase enzymes. This material was probably produced by extensive nuclear degradation of the steroid.

Prior incubation with sulphatase and β -glucuronidase followed by acidification and subsequent extraction with chloroform, removed between 30 and 45% of the radioactivity from the pooled guinea pig urines. The chloroform extracts were analysed by T.L.C. in developing solvents V_1 and

V₂. Radioactive scanning showed two small peaks with R_T in V₁ of 1.25 and 1.92 and in V₂ of 1.90 and 2.50. These values were identical to those of authentic oestradiol and oestrone respectively. These compounds gave a blue colour reaction with phenol specific sprays and their mass spectra obtained after eluting the compounds from a preparative T.L.C. plate confirmed this structure. Oestrone gave a parent ion of m/e = 270 and oestradiol m/e = 272. Both showed major peaks at m/e = 255, 242 and 147; the mass spectrum for oestradiol is given in Fig. 7. Both metabolites represented less than 1% of the total dose of cholesterol.

5.5. Enterohepatic circulation

Two guinea pigs, which had 10 μ Ci radioactive cholesterol introduced into the caecum, were sacrificed after 24 hours. The radioactive counts of the 24 hour sample were at normal levels (see Table 12) and accounted for 55% of the dose. A further 35% of the activity was recovered from the caecum and large intestinal contents but negligible counts were obtained from the blood or bile (obtained from the gall bladder). The liver contained a small amount of activity, approximately 0.3 and 0.5% of the total dose in the two animals. A total of 1.8% of the dose was obtained from extraintestinal sources and represented that part of the dose which had been detected as being absorbed from the gut, either before or after intestinal bacterial metabolism.

5.6. Discussion

The preceding experiments indicate differences between the metabolism of cholesterol in two experimental animal models. The rat acted as a negative control system for the urinary excretion of phenolic steroids

derived from the intestinal cholesterol. Sandberg et al. (1967), have shown that oestrogens are excreted almost exclusively in rat bile as their glucuronide conjugates. The lack of phenolic steroids detected in the rat urine in my study, supports these workers results. In contrast, guinea pig urines contain phenolic steroids, which could be shown to be metabolites of intestinal cholesterol and it is possible that the oestrogens were formed in the large intestine and produced from cholesterol by the intestinal bacteria. Previous work in this thesis (see Results 2) has demonstrated that such reactions occur in vitro; the following points also support the hypothesis that such a reaction may also occur in vivo:-

a) Gustafsson et al. (1966), have shown that cholesterol, coprostanol and related steroids, with a similar side chain are not absorbed from the large intestine. These workers suggest that for any significant uptake from the colon a degree of side chain degradation is necessary. Such a reaction could be the ~~bacterially~~ catalysed aromatisation associated with side chain cleavage; this has been demonstrated in vitro using strains of intestinal bacteria (see Results 2.4).

b) Point (a) is supported by the very high recovery rates of radioactivity in the faeces of the experimental animal. Most of the activity could still be identified in the neutral steroid fraction and the 'interrupted' experiments also suggested that after 24 hours very little enterohepatic circulation was occurring. A minimal amount of activity was shown in the liver, but it is to be expected as this is the site for glucuronide and sulphate conjugation of oestrogens, prior to excretion in the urine. Thus, it is unlikely that cholesterol is being transported from the gut and converted to oestrogens by mammalian enzymes in other organs; if this were the case, a high blood count would be expected. In these experiments,

activity could not be detected in the blood, even at minimal levels.

c) Oestrogens, especially oestrone, are isolated from the urine of males of most species including man (Roberts et al., 1968).

Analysis of steroids in the neutral fraction showed the presence of the four most common compounds, cholesterol, coprostanol, coprostanone and 4-cholesten-3-one. The three latter metabolites have previously been described in rat faeces (Kellog and Wostman, 1969) and have been shown by these workers to be produced from cholesterol by the intestinal bacteria. Hill and Aries (1971), have shown the presence of these compounds in human faecal extracts and that, depending upon diet, conversion to coprostanol may vary from 52-72%. Coprostanone represented 1-15% of the neutral steroid fraction of human faecal extracts. These observations do at least illustrate some similarities in cholesterol metabolism between man and the experimental animals investigated.

Analysis of the acid steroid fraction revealed the presence of an extremely sulphatase sensitive compound, extracted from rat faeces. Sulphated conjugates of steroids are usually present as semi-esters, generally water soluble and acid sensitive. Palmer (1967) has shown, however, that for complete hydrolysis the sulphate conjugates of lithocholic acid require three days at room temperature in 2N HCl. He also showed that 50% of a dose of ^{14}C lithocholic acid given orally to human patients was recovered in the bile as the sulphate conjugates of taurolithocholate, glycolithocholate and to a lesser extent lithocholate. The sulphation was assumed to have taken place in the liver; however, Baulieu (1963), demonstrated the sulphation of bile acids by the large intestinal mucosa in human patients. Norman and Palmer (1964), have shown the presence of lithocholate sulphate in human faeces after oral administration of labelled lithocholic acid. It

is likely that the faecal isolithocholate and lithocholate sulphates observed in the rat in my experiments, were the result of mucosal sulphation of the mono-substituted acids, which had been produced by bacterial action. This is likely because these acids are poorly absorbed in the colon and if the sulphation were the result of hepatic metabolism, then the majority of the lithocholate produced would be unsulphated. Lithocholate and isolithocholate are probably produced via a β -keto intermediate, formed during the side chain degradation of cholesterol. Saturation and sulphation of this intermediate would produce the mixture of isomeric metabolites observed. Due to the highly ionic nature of the sulphate conjugates, absorption from the lumen of the colon is minimal. In contrast to the rat, the guinea pig produced no faecal bile acid sulphates, although the guinea pig liver is known to produce sulphate conjugates; therefore, the lack of faecal sulphates presumably reflects the lack of an intestinal mucosal sulphate ester synthetase.

6.

Cholesterol Metabolism In Vitro

Preliminary screening in vitro for cholesterol metabolism was carried out using inocula of fresh faecal specimens in the brain medium described in Methods 1.1. All the ten samples tested showed an almost 100% conversion to a metabolite, which on separation on T.L.C. in developing solvent NS, had a mobility relative to the substrate, cholesterol, of 1.28. This co-chromatographed with an authentic sample of 5β -cholestan- 3β -ol (coprostanol).

Further screening, using single strains of intestinal bacteria, showed the production of a further three metabolites in a small proportion of strains. These had R_G values of 1.89, 2.09 and 2.20 and these are identical to authentic samples of 5β -cholestan-3-one, 4-cholesten-3-one and 1,4-cholestadien-3-one respectively.

6.1. Identification of products

The compound with $R_G = 1.28$, was tentatively identified by its similar T.L.C. behaviour to authentic samples of coprostanol. On recrystallisation of the metabolite from methanol, a melting point estimation of $99-101^\circ\text{C}$ was obtained. A mixed melting point estimation gave no depression of this value. Its G.L.C. retention time on an S.E. 30 column at 230°C was 82 minutes; this was identical to that of an authentic marker. G.L.C. of a mixture of the metabolite and authentic coprostanol gave a single peak. The I.R. spectrum obtained from both the metabolite and the commercially prepared sample were identical and were as described in the previous section (see Results 5.1). The metabolite with R_G values of 2.09 and 2.20 were tentatively identified as 4-cholesten-3-one and 1,4-cholestadien-3-one

respectively, by their U.V. and mass spectra as detailed in Results 1.2. Coprostanone was tentatively identified by its T.L.C. mobility only. All metabolites were confirmed as being derived from cholesterol by a later incubation using radioactive substrate in the same experimental system and active peaks were obtained at $R_C = 1.89, 2.09$ and 2.20 , the same as described above.

6.2. Conditions for the degradation of cholesterol

For the production of coprostanol extremely anaerobic and reducing conditions were required. Cysteine hydrochloride (0.01%) was added as a reducing agent and effected an increase in the percentage conversion to coprostanol on incubation with faecal suspensions over 5 days from 80% to 98%. The optimal pH for this reaction was between 7.0 and 7.5; the addition of a small amount of Tween 80 was essential, probably as an aid to increasing the solubility of the cholesterol substrate. The acetone-extracted calf brain medium was most effective in aiding bacterial metabolism when freshly prepared, but its efficiency was lost after about 4 weeks at room temperature. Further extraction of the medium with chloroform caused complete inhibition of the bacterial degradation of cholesterol. A marked substrate inhibition on the production of coprostanol was obtained if the cholesterol concentration was more than 0.3%. At higher concentrations, conversion fell by 50% and at 0.6% a complete inhibition was observed.

Further studies carried out using a 500ml reaction vessel linked to a pH-stat provided a more sensitive method for assessing cholesterol metabolism. Faecal suspensions and single strains were introduced into the vessel and the cholesterol degradation followed using a radioactive

substrate in 0.1% cold substrate. The pH was maintained at 7.3 and the anaerobic conditions were maintained by bubbling carbon dioxide and hydrogen gases through the reaction mixture. Subsequent extraction of the reaction mixture after 5 days into acid and neutral steroid fractions and separation on T.L.C., showed an 80% conversion to coprostanol by faecal suspensions and 40% by a *Bacteroides* strain, U.S. 17.5. This compared with 98% and 62% respectively with the bottle method described above.

Radioactive scans of the neutral steroid fraction, separated by T.L.C. in developing solvent NS, confirmed the production of the metabolites, coprostanol, coprostanone, 4-cholesten-3-one and 1,4-cholestadien-3-one which showed radioactive peaks at relative mobilities, R_f , of 1.89, 2.09 and 2.20, identical to the authentic samples. Additionally, of the ten faecal samples tested, two showed a small amount of activity in the acid steroid fraction. Separated in solvent S_{15} , two small peaks were obtained at a yield of less than 0.1% and these were identical to authentic markers of lithocholic (3α -hydroxy- 5β -cholan-24-oic acid) and isolithocholic (3β -hydroxy- 5β -cholan-24-oic acid). These compounds were not further characterised due to lack of material. Using the single strain U.S. 17.5, only coprostanol could be demonstrated in this assay system.

6.3. Screening

The results from screening individual strains of intestinal bacteria for cholesterol degradation are given in Table 14. The assay was carried out under optimal conditions as described above at a pH of 7.3. The best conversion was obtained using a strain of *Bacteroides fragilis*, U.S.17.5, which showed a 62% conversion to coprostanol in vitro. It was necessary to compare each culture extract to a control as cholesterol showed up to

TABLE 14

Screening of Bacterial Groups for the Conversion of Cholesterol to Coprostanol

<u>Bacterial Group</u>	<u>No. Strains Tested</u>	<u>No. Positive</u>	<u>Range of % Yield</u>
Bacteroides	18	12	20-60
Bifidobacteria	12	9	20-60
Enterococci	20	0	0
Enterobacteria	20	0	0
Clostridia: Lecithinase +ive	15	8	10-15
Lecithinase -ive	15	9	10-15

an 8% spontaneous, chemical chemical conversion to coprostanol during incubation.

The only metabolite produced by the non-sporing anaerobic bacteria was coprostanol. However, 25% of the Clostridia screened, showed the production of coprostanone, 4-cholesten-3-one and 1,4-cholestadien-3-one as well as approximately a 15% yield of coprostanol. The minor metabolites, when present, were at yields of less than 0.5%.

6.4. Discussion

The conversion of cholesterol to coprostanol has been previously reported using faecal suspensions in vitro (Snog Kjaer et al., 1956). He showed up to a 98% conversion to coprostanol using human faecal preparations although isolated strains could only show a 10% yield of this metabolite. He did report that one strain of unidentified anaerobic bacterium showed an 80% conversion, but he was unable to reproduce this finding. Coleman and Baumann (1957b), demonstrated an analagous reaction using 7-dehydro-cholesterol which gave the metabolite, 5 β -cholesten-7-en-3 β -ol on incubation with rat caecal contents. Here again, as above, these workers did not demonstrate this reaction with single strains of intestinal bacteria.

The above results (section 6.1), demonstrate significant levels of conversion of cholesterol to coprostanol, especially in non-sporing, strict anaerobes. These results were obtained using the same culture medium as was used by the workers described above; no other culture system that I tested showed any good cholesterol degradation. The strict anaerobiosis and the high E_h under which the bacteria were grown, probably aided their growth and metabolic activities. It is significant that when the same strains were tested in the pH stat, the percentage conversion dropped to

66% of the level measured in bottled media. This was probably due to the difficulty in obtaining anaerobic conditions in the pH stat reaction vessel; it can be seen from Table 14 that the bacteria that are most active metabolically, were the strictly anaerobic groups, the organisms most sensitive to the presence of small amounts of toxic oxygen. Species of *Bacteroides* and *Bifidobacteria* showed the greatest ability to degrade cholesterol and to a lesser extent, species of *Clostridia*, which showed a much lower conversion rate to coprostanol. These observations are in agreement with those made by Snog Kjaer et al. (1956), who hypothesised that, since penicillin feeding to experimental animals suppressed coprostanol formation, the most active group must be the non-sporing anaerobic bacteria, which were at that time still penicillin-sensitive.

The other metabolites identified, 4-cholesten-3-one, 1,4-cholestadien-3-one and coprostanone were all isolated from clostridial extracts. There is evidence for the production of these compounds being formed in vivo in the large bowel by intestinal bacteria, from cholesterol substrate. Gustafsson et al. (1966) and Kellog and Wostmann (1969), have shown that germ-free animals do not have these faecal metabolites compared with those present in conventional animals, and cholesterol is eliminated undegraded from the germ-free animal.

The production of lithocholate and isolithocholate from cholesterol by intestinal bacteria in vitro has not been previously reported. From the previous section (see Results 5.3.), a parallel reaction was demonstrated in the large intestine of the rat. Both in vitro and in vivo, the percentage yield was very small. However, in contrast to the in vivo system, no sulphated bile acids were isolated (not surprisingly, as this conjugation reaction has been associated with the intestinal mucosa). The mechanism by

which the C_{24} bile acids are formed from cholesterol is still rather obscure, especially the formation of the 3 α -hydroxy-5 β -cholan-24-oic acid (lithocholate), which involves the inversion of the hydroxyl group at the three position in its formation from cholesterol. Sih et al. (1968a and b), have shown that soil organisms are capable of degrading cholesterol isomers to form small amounts of C_{24} bile acid, among many other metabolites. Therefore, the intestinal bacteria may be following a similar mechanism in the side chain cleavage of the cholesterol molecule.

The mechanism of inversion of the hydroxyl group may be as described for the in vivo production of 3 α -hydroxy-5 β -cholan-24-oic acid in the previous section of results (5.6). The formation of a 3-keto intermediate followed by the random resaturation at this position would produce a mixture of 3 α and 3 β metabolites. Bjorkhelm and Gustafsson (1971), presented evidence that coprostanol formation occurred via 3-keto and 4-en-3-one intermediates, which fits in well with the above hypothesis. It is noteworthy that these two metabolites were identified in the neutral steroid extracts, both from cultures containing faecal suspensions or single strains of Clostridia; this lends support to the above hypothesis for coprostanol formation (see also Fig.1, pathway 11). In contrast, these intermediates were not isolated from cultures containing the non-sporing anaerobes which may suggest a direct saturation reaction (see Fig. 1, pathway 1).

The success in demonstrating coprostanol formation by intestinal bacteria appears to depend upon the nature of the medium, an acetone calf brain powder. Rosenheim and Webster (1941) and Coleman and Baumann (1957b), both attributed its activity to the cerebroside content. My results tend to support this hypothesis, as further extraction of the acetone powder with chloroform brought about complete inactivation of the medium.

In summary, to demonstrate a good in vitro formation of coprostanol from cholesterol, the acetone calf brain medium must be incubated under highly anaerobic conditions and with a high E_h . Solubilisation of the substrate was important, but the presence of too much Tween 80 inhibits bacterial growth. In addition to coprostanol, faecal suspensions can be demonstrated to produce 4-cholesten-3-one, 1,4-cholestadien-3-one, 5 β -cholestan-3-one and C_{24} bile acids as products of side chain cleavage. Single strains of Clostridia have also been used to demonstrate the production of the former three metabolites.

SECTION III C

LECTITHINASE-C- NEGATIVE CLOSTRIDIA.

7. Lecithinase-Negative Clostridia and Steroid Desaturation

Hill et al. (1971), hypothesised that the aetiology of cancer of the colon may be associated with the extensive desaturation of bile acids in situ in the colon to produce a carcinogen type molecule. In the following study, populations of Clostridia are examined along with their ability to perform one of these desaturating reactions.

7.1. Geographical Survey

Results of classification of strains of lecithinase-negative Clostridia and the distribution of 3-oxo-5 β -steroid- Δ^4 -dehydrogenase from strains isolated from individuals in Scotland, England, Uganda and Hong Kong are given in Tables 15, 16, 17 and 18. Each table lists the eight most common isolates for each country and their ability to form a Δ^4 metabolite from the saturated substrate (for more detail see Results 1). The column listed as 'others' includes the rare isolates, only identified once or twice from the total sample tested. These included for the Scottish strains, Cl. plagarum, Cl. sporogenes, and Cl. sphenoides. The Ugandan strains were represented by Cl. oroticum, Cl. rectum and Cl. aurant-obutyricum. From the English individuals there were many more 'rare' strains isolated, probably due in part to the large sample number; these included Cl. tyrobutyricum, Cl. cochlearium, Cl. putrificum, Cl. plagarum, Cl. sporophaeroides, Cl. glycolicum, Cl. fallax, Cl. sphenoides, Cl. septicum, Cl. ramosum and Cl. inulinum.

Apart from the general taxonomic variations shown by the four countries and the presence of the minor species listed above, there are two other significant differences shown in the tables between the countries

TABLE 15

The Distribution of Strains of Lecithinase Negative Clostridia Isolated from Scottish Faecal Samples, and their Ability to Introduce a C₄-C₅ Double Bond into a Steroid Substrate.

	<u>No. Strains(Y)</u>	<u>% of Total isolated(T)</u>	<u>No. Strains Desaturating Steroid (X)</u>	<u>* $\frac{X}{Y} \times 100$</u>
<u>Total No. Strains(T).</u>	67	100.0	31	44.25
<u>Cl. paraputrificum</u>	27	40.0	25	93
<u>Cl. chauvoei</u>	13	19.5	1	7
<u>Cl. indolis</u>	8	12.0	5	38
<u>Cl. cadaveris</u>	6	9.0	0	0
<u>Cl. felsinium</u>	5	7.5	0	0
<u>Cl. pasteurianum</u>	1	1.5	0	0
<u>Cl. sartagoformum</u>	1	1.5	0	0
<u>Cl. stricklandii</u>	0	0.0	-	-
<u>Others</u>	3	4.5	0	0
<u>Unclassified</u>	3	4.5	0	0

* $\frac{X}{Y} \times 100$: This represents the percentage of each strain isolated that possesses the ability to desaturate a steroid substrate.

TABLE 16

The Distribution of Strains of Lecithinase Negative Clostridia Isolated from English Faecal Samples, and their Ability to Introduce a C₄-C₅ Double Bond into a Steroid Substrate.

	<u>No. Strains(Y)</u>	<u>% of Total isolated(T)</u>	<u>No. Strains Desaturating Steroid (X)</u>	<u>* X/Y X 100</u>
<u>Total No. Strains(T).</u>	110	100.0	39	33.8
<u>Cl. paraputrificum</u>	37	30.0	30	91.0
<u>Cl. chauvoei</u>	7	0.5	0	0.0
<u>Cl. indolis</u>	18	16.5	5	28.0
<u>Cl. cadaveris</u>	4	3.8	1	25.0
<u>Cl. felsinium</u>	8	7.5	0	0.0
<u>Cl. pasteurianum</u>	0	-	0	0.0
<u>Cl. sartagoformum</u>	10	9.0	2	20.0
<u>Cl. stricklandii</u>	1	0.9	0	0.0
<u>Others</u>	12	11.0	0	0.0
<u>Unclassified</u>	19	12.2	1	5.2

* X/Y X 100: This represents the percentage of each strain isolated that possesses the ability to desaturate a steroid substrate.

TABLE 17

The Distribution of Strains of Lecithinase Negative Clostridia Isolated from Ugandan Faecal Samples, and their ability to Introduce a C₄-C₅ Double Bond into a Steroid Substrate.

	No. Strains(Y)	% of Total isolated(T)	No. Strains Desaturating Steroid (X)	*X/Y X 100
<u>Total No. Strains(T)</u>	54	100.0	4	7.5
<u>Cl. parapatrificum</u>	4	7.5	4	100.0
<u>Cl. chauvoei</u>	6	11.0	0	0.0
<u>Cl. indolis</u>	3	5.5	0	0.0
<u>Cl. cadaveris</u>	4	7.5	0	0.0
<u>Cl. felsinium</u>	5	9.0	0	0.0
<u>Cl. pasteuranium</u>	4	7.5	0	0.0
<u>Cl. sartagoformum</u>	2	3.3	0	0.0
<u>Cl. stricklandii</u>	2	3.3	0	0.0
<u>Others</u>	5	9.0	0	0.0
<u>Unclassified</u>	19	35.5	0	0.0

*X/Y X 100: This represents the percentage of each strain isolated that possesses the ability to desaturate a steroid substrate

TABLE 18

The Distribution of Strains of Lecithinase Negative Clostridia Isolated
from Hong Kong Faecal Samples, and their ability to Introduce a C₄-C₅ Double
Bond into a Steroid Substrate

	<u>No. Strains(Y)</u>	<u>% of Total isolated(T)</u>	<u>No. Strains Desaturating Steroid (X)</u>	<u>*X/Y X 100</u>
<u>Total No. Strains(T)</u>	16	100.0	2	12.25
<u>Cl. paraputrificum</u>	1	6.3	1	100.0
<u>Cl. chauvoei</u>	1	6.3	0	0.0
<u>Cl. indolis</u>	0	0.0	0	0.0
<u>Cl. cadaveris</u>	0	0.0	0	0.0
<u>Cl. felsinium</u>	3	18.8	0	0.0
<u>Cl. pasteurianum</u>	2	12.5	0	0.0
<u>Cl. sartagoforum</u>	0	0.0	0	0.0
<u>Cl. stricklandii</u>	0	0.0	0	0.0
<u>Others</u>	0	0.0	0	0.0
<u>Unclassified</u>	9	56.2	1	11.0

* X/Y X 100: This represents the percentage of each strain isolated that possesses the ability to desaturate a steroid substrate.

with high colon cancer incidence (ie. Scotland and England) and those with low incidence (Uganda and Hong Kong). The first difference is that the dominant lecithinase-negative species of *Clostridium* in the high incidence countries is the butyric acid producing *Clostridium paraputrificum*, which accounts for 40% of the Scottish isolates and for 25% of the English isolates, but in the low incidence countries it represents only a low proportion of the total strains, being 7.5% and 6.5% for Uganda and Hong Kong respectively. Not too much significance should be attached to the Hong Kong figures, as the total sample represents only 16 strains. A similar but less pronounced trend was shown with the incidence of *Clostridium indolis*. As the percentage of total strains this represents 12% in Scottish, 16.5% in English but only 5.5% in Ugandan and 0.0% in Hong Kong strains.

The second major taxonomic difference was observed in the unclassifiable category. A strain was deemed unclassifiable if it deviated by more than two sugar reactions from any species in the identification format given in the Anaerobe Laboratory Manual (Holdeman and Moore, 1972). Ugandan strains showed 56.25% in this category and Hong Kong 35.5%. In contrast, the westernised countries, Scotland and England, had only 4.5 and 17.25% respectively, of unclassifiable strains.

Although in this study it was felt that absolute *Clostridial* counts were not required, it is noteworthy that of the 30 Ugandan samples that were tested for *Clostridia*, 11 did not show the presence of any lecithinase-negative *Clostridia*, although some lecithinase-positive colonies were grown. This compares with the isolation of lecithinase-negative *Clostridia* from 100% of the Scottish and English samples.

7.2. Steroid desaturation

The right two rows of Tables 15, 16, 17 and 18 shows the numbers of strains of lecithinase-negative Clostridia possessing the 5β -steroid- Δ^4 - dehydrogenase and the proportion of positive strains for each species. Figure 15 shows that there is a linear relationship between the percentage of strains possessing the enzyme in the total national sample and the national incidence of cancer of the colon (taken from Doll, 1969). Whether this relationship is real or only coincidental, it shows that strains isolated from high incidence countries possess a greater ability to desaturate steroids than those isolated from low incidence countries.

It can also be seen from Tables 15, 16, 17 and 18 that Cl. paraputrificum is the dominant strain, possessing the Δ^4 dehydrogenase. Over 90% of strains of this species from all four countries possessed the enzyme. Cl. indolis, a closely related species, showed some enzyme activity with 38% of the English strains and 28.5% of the Scottish strains possessing the dehydrogenase. However, none of the Cl. indolis of Ugandan origin showed this enzyme activity. Other isolated individual strains of Cl. shauvoei, Cl. spenoides, Cl. cadaveris, and Cl. sartagoformum were shown to possess Δ^4 dehydrogenase activity, but these were not typical of their species; all showed low percentage yield of metabolite (less than 8% conversion rate), compared with an average 15% conversion by Cl. paraputrificum strains. Of the 8 active Cl. indolis strains, 5 showed a strong activity and 3 only a weak activity.

7.3. Cancer patient survey

Having demonstrated differences between populations of high and low incidence of cancer of the colon, the opportunity arose to examine some

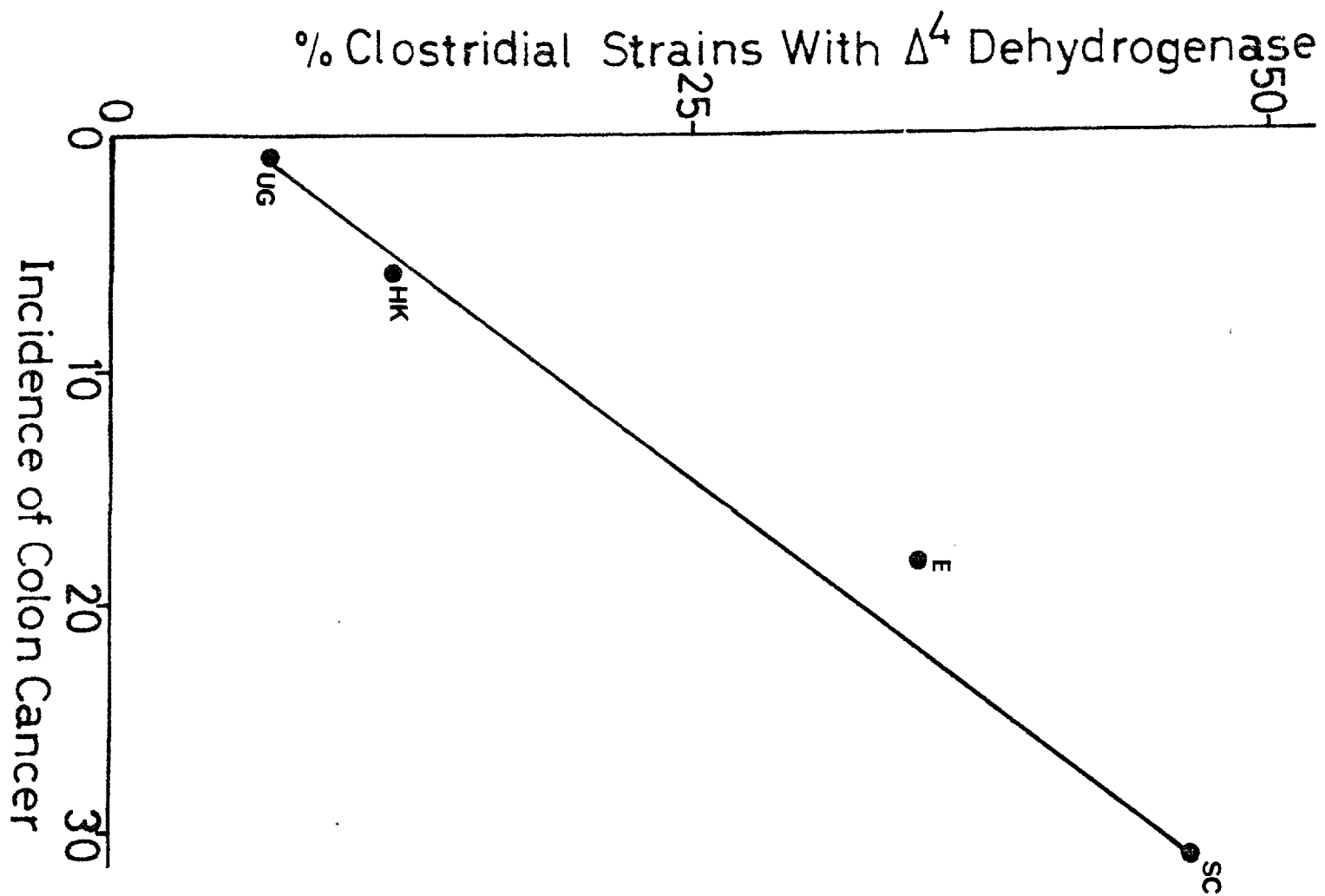
Fig. 15. Relationship of national incidence of colon cancer (cases per year per 100,000 population), to the ability of lecithinase-negative Clostridia to introduce a C_4-C_5 double bond.

HK - Hong Kong

UG.- Uganda

E - England

SC - Scotland



cancer patients in comparison with control groups. The results of this survey are summarised in Table 19. The 48 strains of Clostridia were isolated from 8 cancer patient faecal samples, and 72 strains of Clostridia from 23 control patients. The latter were split into three groups. Group 1 were patients admitted to hospital with miscellaneous conditions which included fractures, varicose veins, gall stones etc.. Group 2 were patients with non-cancerous complaints of the lower intestine and Group 4 had diseases of the upper intestine, two individuals having stomach carcinoma. The Table shows the distribution of strains of Cl. paraputrificum and Cl. indolis both of which show significant variations between cancer and control groups. In the colon cancer group, 47% of the lecithinase-negative strains isolated were Cl. paraputrificum, compared with 26%, 9% and 28% for the control groups, 1,2 and 4 respectively. All other strains were distributed in the cancer and control groups in a similar manner to that shown in Table 16 for England. These species are listed in Table 19 under the category 'Others'.

62.5% of the total strains isolated from the cancer patient group showed ⁴ dehydrogenase activity, 100% of the Cl. paraputrificum, 40% of the Cl. indolis and 25% of the 'Other' strains carrying out this reaction. 80% of the latter group possessing this activity were deemed unclassifiable. In contrast, the control group showed steroid desaturation in only 32% of the total isolates, about one half of that of the cancer group. In the control group, desaturating ability was shown by 88% of Cl. paraputrificum, 40% of Cl. indolis and only 10% of 'other' strains.

Table 20 shows the four categories into which the cancer and control groups have been placed. The four groups were designated using two factors at arbitrary levels. These were, (a) if the individual possessed more or

TABLE 19

Comparison of Strains of Lecithinase Negative Clostridia Isolated from
Colon Cancer Patient Faecal Samples and Three Control Groups.

	<u>Cancer Patients</u>	<u>Control Patients</u>		
		<u>Group 1</u>	<u>Group 2</u>	<u>Group 4</u>
<u>Cl. paraputrificum</u>	23(23)	19(16)	1(1)	5(5)
<u>Cl. indolis</u>	5(2)	13(5)	1(0)	1(1)
<u>Others</u>	20(5)	40(4)	10(0)	12(1)
<u>Total No. Strains</u>	48(30)	72(25)	12(1)	18(7)
<u>% Total Strains with Δ^+ Dehydrogenase Enzyme.</u>	62.5	33.8	8.3	39.0
<u>Mean Acid Steroid concn., mg/gm dry wt.</u>	6.18	4.72	4.45	3.22

() Numbers in parentheses indicate the strains possessing Δ^4 dehydrogenase activity.

For classification of Control Groups, see text.

TABLE 20

Categorisation of Cancer Patients and Controls According to Faecal Acid Steroid Concentration and Numbers of Lecithinase Negative Clostridia with 3-oxo-5 β -Steroid- Δ^4 - dehydrogenase activity

			<u>Control Patients</u>		
		<u>Cancer Patients</u>	<u>Gp. 1</u>	<u>Gp. 2</u>	<u>Gp. 4</u>
<u>Group A:</u>	More than 5.9 mg acid Steroid /gm. dry wt. faeces	12	1	0	0
+	More than 50% Clostridia with desaturating ability				
<u>Group B:</u>	More than 5.9 mg acid Steroid /gm. dry wt. faeces	0	2	2	1
+	Less than 50% Clostridia with desaturating ability				
<u>Group C:</u>	Less than 5.9 mg acid Steroid /gm. dry wt. faeces	2	11	1	1
+	More than 50% Clostridia with desaturating ability				
<u>Group D:</u>	Less than 5.9 mg acid Steroid /gm. dry wt. faeces	0	11	2	3
+	Less than 50% Clostridia with desaturating ability				
TOTAL		14	25	5	5

For classification of Control Groups, see Text.

less than 50% Clostridial isolates possessing the Δ^4 dehydrogenase and (b) the total bile acid concentration at a level more or less than 5.9mg/gram dry weight faeces. This represents the relative substrate concentration that may be present in the colon and the relative number of bacteria which may desaturate this substrate. These bile acid concentrations were kindly supplied by Dr. M.J. Hill and were estimated by the method of Grundy and Ahrens (1969). Both values of total bile acids and the percentage Clostridia desaturating the steroid are arbitrary and the levels were chosen so that the cancer patients are seen almost exclusively in category 1 (see Table 20). Only an extended cancer patient study will reveal whether or not such a classification is an artifact.

7.4. Discussion

When assessing the results obtained from this study, there are certain limitations which should be borne in mind. Firstly, although it was intended that ten strains should be isolated from each stool specimen, in practice, due to losses in the isolation procedure, contamination etc. this was not possible. Repeated re-isolation of a specimen led to a reduction of bacterial viability in that specimen by freezing and thawing. Therefore, to obtain statistically meaningful numbers of strains from each individual is a problem which could be overcome either by more refined isolation techniques or by picking many more strains per patient in the initial isolation. Secondly, there is a problem of selectivity in the isolation of bacterial strains. It was found that many colonies grown on primary isolation, failed to grow on sub-culturing. The tendency would then be for the results to be biased towards the more robust species. To minimise this species selectivity, all strains were, on initial isolation,

immediately inoculated into a Robertsons cooked meat (an extremely good culture medium for fastidious anaerobic bacteria), which acted as a reservoir of viable organisms in the event of loss of viability during subsequent identification stages. An additional problem arises using Robertsons cooked meat, because contamination of this rich medium can easily occur with the resultant loss of strain purity.

Within these limitations however, the results show certain geographical trends and differences between individuals with colon cancer and the control groups. Hill et al. (1971) have already shown a geographical variation, associated with the levels of 7 α -dehydroxylase enzyme produced by intestinal bacteria. A high percentage of faecal strains of bacteria of western origin showed this reaction compared with those from countries such as Uganda and India. This, if it can be extrapolated, showed a positive correlation between faecal enzyme levels and national incidence of colon cancer. A similar correlation has been demonstrated in this geographical survey between the proportion of faecal strains showing Δ^4 dehydrogenase activity and colon carcinoma. This variation is not due, as with the 7 α -dehydroxylase, to the proportion of certain strains performing the reaction, but to the variation in numbers of the specific species Cl. paraputrificum, the major lecithinase-negative Clostridium, contributing to steroid desaturation. It can be seen that the proportion of all other identified species remains reasonably constant for the different countries, and apart from the few unusual strains, none showed Δ^4 dehydrogenase activity.

The number of unclassifiable strains from Uganda and Hong Kong was much higher than that from England or Scotland. This is not surprising, as the classification of these bacteria was carried out on faecal isolates

of people in the U.S.A. and, therefore, a westernised diet. It is a reasonable assumption that some of the Clostridia put in the group considered unclassifiable have evolved and mutated from their relatives of western origin under the influence of a different diet. Although the strains were considered unclassifiable, the identification techniques place them in homogenous groups, rather than as a random distribution of strains.

The study involving cancer patients showed an extrapolation of the geographical survey. 62% of the strains isolated from cancer patients possessed the ability to desaturate steroids, compared with 32% of the strains from controls. This high proportion of strains in the cancer patients was due to both elevated numbers of Cl. paraputrificum and to a number of unclassifiable bacteria, which showed the Δ^4 dehydrogenase activity. Whether this phenomenon is due to cause or effect is debatable, but a prospective study now being started involving the collection of 10,000 faecal specimens is hoped to provide the answer.

It is important that further screening of these isolated Clostridia is carried out for further desaturating reactions, apart from the Δ^4 dehydrogenase. Such screening should include reactions for oxidative demethylation and aromatisation, equilin formation and C₆-C₇ dehydration as described in the previous sections. Once this has been demonstrated, then a theoretical potential to produce a polycyclic aromatic carcinogen from bile acids will have been demonstrated (see Hill et al. 1971).

SECTION IV

FINAL DISCUSSION

8.

Final Discussion and Conclusions

Results in this thesis have shown that intestinal bacteria possess the ability to desaturate steroids in vitro by four different mechanisms. Three of these reactions i.e. oxidative demethylation, desaturation linked to a keto group and desaturation linked to other double bonds, were almost exclusively carried by the lecithinase-C-negative Clostridia, a minor group of the gut flora. Drasar and Williams (1972), have shown that Clostridia are present in faeces at levels of 10^4 - 10^6 cells per gram, compared with total counts of 10^{10} - 10^{11} per gram faeces. In other words, the Clostridia represent about 0.001% of the total flora. Moreover, the reactions studied were carried out principally by one species of Clostridium; Clostridium paraputrificum.

These four types of reaction have not been previously demonstrated using intestinal microorganisms. In each of the preceeding sections, the results have been discussed in the context of parallel systems in soil organisms and mammalian tissue extracts. Similarities and differences in these systems have been pointed out. The main advantage in studying soil organisms is that the steroid substrate for the reaction, which is under scrutiny can be used as the sole source of carbon. However, in studying the same steroid transformations using intestinal bacteria, the medium required to support growth was often complex and the steroid transformation only a side reaction.

The four desaturating reactions show some common properties in that all of the reactions are stereospecific, especially for the structure at the C₅ position. This specificity does not appear to extend to the side chain at the C₁₇ position, since similar reactions could be demonstrated

using substrates in the androstane series (no side chain), pregnane series (two carbon side chain), bile acid series (five carbon side chain) and cholesteryl series (eight carbon side chain). The Δ^1 and Δ^4 dehydrogenase enzymes had a requirement for the 5 β configuration of the steroid nucleus. The aromatisation and 7-dehydration reactions both had a requirement for the 4-en-3-one configuration and the equilin formation required a 4,6-dien-3-one.

Attempts to isolate the Δ^1 dehydrogenase, Δ^4 dehydrogenase and aromatising enzymes were not successful. Washed cell suspensions which were partially disrupted could carry out all three reactions. Each enzyme had a requirement for artificial electron acceptors and in the case of the aromatisation reaction, NAD^+ was also required. Any further disruption of the cells brought about complete inactivity of the Δ^1 dehydrogenase and aromatisation enzymes, in both the intracellular fraction and cell wall fraction. To explain this phenomenon, it may be hypothesised that these enzymes, or enzyme complexes are present as structural components in the protoplast membrane and any disruption of the cell caused a loss in active conformation.

There is no direct evidence that these reactions occur in vivo. Faecal steroid analyses have not shown the presence of any desaturated steroids, although to account for the presence of the 5 α 'allo' bile acids, unsaturated intermediates have been implicated (Kallner et al., 1967a). Moreover, these enzymes have been demonstrated in vitro using oxidising conditions comparable to the systems shown for soil organisms and mammalian tissue preparations, although the group of bacteria responsible for these metabolic activities, the Clostridia, are strictly anaerobic organisms. Therefore, it may be hypothesised that if these reactions occur in vivo they act as a

detoxification mechanism to mop up small amounts of lethal oxygen present in the gut. Alternatively, or even supplementary to this hypothesis, these oxidation reactions in vitro or in vivo, may be a subsidiary energy producing process. The aromatisation reaction was shown to involve the utilisation of NAD^+ or NADP^+ when washed cell suspensions of bacteria were used as an enzyme source. The reduced co-enzyme produced in this reaction is vital to energy producing processes and could be utilised in a cytochrome system for anabolic processes. It is not known if molecular oxygen is a terminal acceptor in Clostridial cytochrome systems, but it is known that the bacteria cannot tolerate aerobic conditions.

Only minute quantities of desaturated steroid metabolite would be expected to be produced in vivo, if the yields from the in vitro assays described in this thesis are similar to those of in vivo desaturating reactions. Therefore, when considering the production of carcinogens of the poly cyclic aromatic type from bile acids by intestinal bacteria, (Hill et al., 1971) and the aetiology of colon cancer, one would expect the production of such compounds to be minimal since they would be the products of a chain of reactions, each of low yield.

Workers have previously attempted to ascertain with varying success whether exposure of poly cyclic aromatic carcinogens to the colon could cause in situ tumours. Wibecke et al. (1969), tested 20-methyl cholanthrene and related compounds by acute and chronic rectal smearing, but did not find production of local tumours. However, Berenblum et al. (1956), showed the production of local sarcomas by chronic exposure of 20-methyl cholanthrene to the rectum. Horava and von Haan (1963), showed the production of colon carcinomas in mice, a proportion of which were invasive. Of course, extrapolation of these results to man is not acceptable

primarily due to different species susceptibility. The exposure of the carcinogen to man is also different, the production of such a compound being over many years causing prolonged exposure over a lifetime. Such a prolonged chronic dosing appears to be probable, as this disease is associated with the older age groups of the population (Doll, 1969).

The distribution of strains of lecithinase-negative Clostridia and their ability to desaturate steroids has been shown to vary significantly in faeces from individuals living in different countries (see Results 7.1). Countries with a low incidence of cancer of the colon show poor steroid nuclear desaturating ability compared with high incidence countries, which show a much higher enzyme desaturating activity. Within Britain, isolates from faeces of cancer patients have above average nuclear desaturating ability. In understanding the aetiology of colon cancer, it is important to ascertain what stimulates the growth of the metabolically active desaturating Clostridia and especially Clostridium paraputrificum and Clostridium indolis. Crowther (1971c), attempted to statistically link 16 factors associated with gut flora and their inter-relationships. However, he did not demonstrate any significant correlations between lecithinase-negative Clostridia and faecal bile acids or faecal neutral steroids. It is possible however, that the stimulating factor may be a partially degraded bile acid.

The in vitro studies of cholesterol metabolism (see Results 6), is an extension of the work by Snog Kjaer et al. (1956). These workers demonstrated in vitro saturation of the Δ^5 double bond, but failed to demonstrate this reaction with individual identified strains of intestinal bacteria. Using the medium described by Coleman and Baumann (1957b), high yields of coprostanol from cholesterol were obtained, using single strains of intestinal bacteria. Coprostanone and 4-cholesten-3-one were also

identified as products of in vitro cholesterol metabolism and have been implicated in the conversion of cholesterol to coprostanol (Bjorkhelm and Gustafsson, 1971). Using the larger culture system in the pH-stat, small amounts of side chain cleavage were demonstrated producing mono-substituted bile acids.

The in vivo cholesterol metabolism experiments (see Results 5) in guinea pigs and rats confirmed that the cholesterol metabolites described above were produced in the large intestine. Side chain cleavage was demonstrated in the colon of the rat producing monohydroxy-cholanic acids, detected as their sulphate esters. The small amounts of oestrogen excreted in the urine after the introduction of radioactive cholesterol directly into the colon of guinea pigs, may well be of relevance in the aetiology of cancer of the breast. Mammary tumours may be induced by oestrogens (Cutts, 1965) and these compounds have been shown to maintain or stimulate the growth of certain tumours induced by poly cyclic aromatic carcinogens (Jellinck, 1965).

It has also been shown that like colon cancer, the epidemiology of cancer of the breast has a correlation with socio-economic class within countries and that the western world has a much higher incidence of this cancer (Doll et al., 1970). If fat, and in particular cholesterol in the diet, is implicated in the causation of this cancer, this hypothesis can be explained by the in vitro and in vivo production of oestrogens from cholesterol by intestinal bacteria. To further support this hypothesis Wynder (1968), has shown that surgical procedures including the removal of ovaries and adrenals in cases of breast cancer does not in all cases suppress oestrogen excretion in the urine post-operatively. It is possible that these oestrogen levels are maintained by the production of phenolic

steroids in the colon from cholesterol from intestinal bacteria. To support this hypothesis Wynder (1968), also stated that Japanese living on a low fat diet, had a higher survival rate after such surgical procedures.

The formation of acid steroids from cholesterol in the rat colon and in in vitro experiments by side chain cleavage may explain the loss of radioactive label from the neutral steroid fraction in in vivo experiments, observed by Grundy and Ahrens (1969). They noted that when cholesterol which was radioactively labelled, was introduced by tube into the top of the colon of two patients, a proportion of label was lost from the neutral steroid fraction during transit through the colon. However, any extrapolation of this kind from an experimental model to man must be carefully regarded.

CONCLUSIONS

Conclusions

The conclusions to be drawn from the work in this thesis are as follows. By the demonstration of four types of nuclear desaturation reaction by strains of intestinal bacteria, the theoretical potential of the colonic flora to produce a carcinogen of the polycyclic aromatic type has been realised (Hill, 1970). Some of the metabolites produced from bile acids in vitro may in fact be carcinogenic when tested in animals for tumour formation. The study on the lecithinase-negative Clostridia also demonstrated that the faecal flora of people living in countries with a low incidence of colon cancer have a smaller proportion of strains able to desaturate steroids than that of high incidence countries. In the comparison of cancer patients and controls, the cancer patients have been shown to have by far the greater potential to desaturate steroids. Whether this is a cause or effect has yet to be demonstrated.

Studies of cholesterol metabolism both in vivo and in vitro have confirmed and extended the observations of previous workers. Identified strains of intestinal bacteria were demonstrated to convert cholesterol to a range of degradation products. As well as the production of coprostanol, coprostanone and 4-cholesten-3-one, cholesterol side chain cleavage by the gut bacteria was also demonstrated leading to the formation of monohydroxy bile acids in the intestine. Preliminary work also showed the sulphation of steroids in the rat colon and the production of oestrogen conjugates in the urine of guinea pigs as a result of cholesterol metabolism in the colon.

These studies have shown that in addition to providing a wide range of faecal bile acid metabolites, the gut flora are also capable of produc-

ing a range of phenolic steroids by a combination of side chain cleavage and nuclear desaturation of faecal steroids, which include a range of steroid hormones. The significance of this metabolic potential has still to be determined.

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