BILIARY EXCRETION OF PROGESTERONE METABOLITIES AND BILE ACIDS IN DEVELOPING FOETAL LAMBS

by

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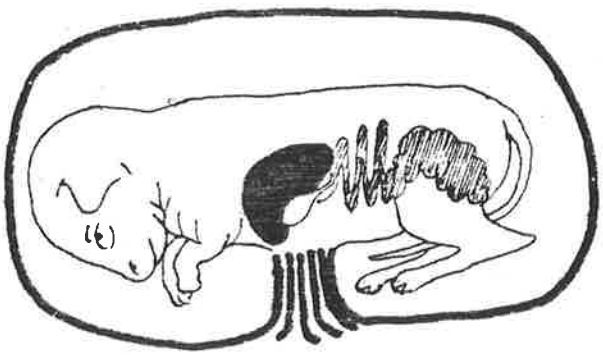

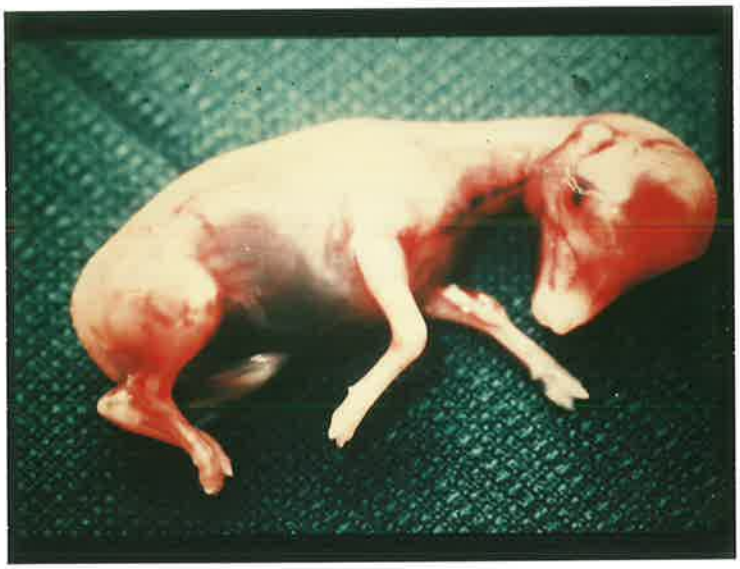

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I wish also to thank Dr. G. Phillipou and Dr. D. Johnson for indispensable help in steroid analyses involving GC and GC-MS.

DEDICATION

This thesis is dedicated firstly to my wife, Kathleen Butler, without whose support it would not have been possible and secondly, to the memory of my late father.

DECLARATION

I declare that the experiments reported in this thesis were carried out by myself except where due acknowledgment has been made. This thesis contains no material previously accepted for the award of any other degree, and to the best of my knowledge and belief, contains no material previously published or written, except where due reference is made in the text of this thesis.

R.N. BUTLER.

Abbreviations used

The abbreviations used in this thesis are defined in "Instructions to Authors" in Biochem J. (1975) 145 : 1-20. Non-standard abbreviations are defined in the text. The following steroids are quoted in the text:-

BILE ACIDS

Trivial Name	Systematic Name
Cholic Acid	5β-cholanic acid
Glycocholic Acid	5β-cholanic acid - 3α, 7α, 12α-triol-N-(carboxymethyl)-amide
Taurocholic Acid	5β-cholanic acid - 3α, 7α, 12α-triol-N-(2-sulphoethyl)-amide
Hyocholic Acid	5β-cholanic acid - 3α, 6α, 7α-triol
Chenodeoxycholic Acid	5β-cholanic acid - 3α, 7α-diol
Glycochenodeoxycholic Acid	5β-cholanic acid - 3α, 7α-diol-N-(carboxymethyl)-amide
Taurochenodeoxycholic Acid	5β-cholanic acid - 3α, 7α-diol-N-(2-sulphoethyl)-amide
Deoxycholic Acid	5β-cholanic acid - 3α, 12α-diol
Glycodeoxycholic Acid	5β-cholanic acid - 3α, 12α-diol-N-(carboxymethyl)-amide
Taurodeoxycholic Acid	5β-cholanic acid - 3α, 12α-diol-N-(sulphoethyl)-amide
Hyodeoxycholic Acid	5β-cholanic acid - 3α, 6α-diol
Ursodeoxycholic Acid	5β-cholanic acid - 3α, 7β-diol
Murocholic Acid	5β-cholanic acid - 3α, 6β-diol

Lithocholic Acid	5β-cholanic acid - 3α -ol
3β-5-cholenoic Acid	5 cholenic acid - 3β -ol
7-Ketolithocholic Acid	5β-cholanic acid - 3α -ol -7-one
7-Ketodeoxycholic Acid	5β-cholanic acid - 3α, 12α -diol-7-one

STEROIDS

DHEA	3β-hydroxy-androst-5-ene-17-one
progesterone	pregn-4-ene-3, 20-dione

Definition of Meconium

Meconium is defined in this thesis as the viscid, greenish black contents of the foetal intestine (particularly the distal small intestine) found therein at any time throughout gestation. It was so named by Aristotle who thought, because of its likeness to material obtained from the poppy (mekonion), that it kept the foetus asleep in the uterus. Hippocrates stated that the presence in the foetal intestine was proof of the fact that the "foetus sucks inside the womb".

Synonyms

Glucosiduronates ≡ Glucuronates ≡ Glucuronidates ≡ Glucuronide

SUMMARY

This thesis is concerned with aspects of the ontogeny of biliary excretion of steroid and sterol metabolites in the developing foetal lamb. The pattern of biliary excretion of progesterone metabolites and bile acids have been investigated from early foetal life (40 - 50 days gestation) until term, by analysis of samples of gallbladder bile, small intestinal contents and in some cases large intestinal contents. Analysis was carried out using high resolution chromatographic techniques for quantitation and identification coupled where possible with mass spectral analysis for structural confirmation.

It was found that :

(1) The only progesterone metabolites detected in foetal small intestinal (SI) meconium were pregnanediol glucosiduronates and these were found only at term. Despite the fact that the predominate progesterone metabolites in foetal blood are pregnanediol sulphates, no sulphates were found in S.I. meconium at any stage of gestation. Small amounts of free pregnanediols were found in several samples from early gestation. This may reflect the immaturity of this excretory pathway in early foetal life. Similar concentrations of pregnanediol glucosiduronates in both the small intestinal and large intestinal meconium suggested that these metabolites did not undergo an enterohepatic circulation.

(2) Whilst no conjugates of pregnanediols were detected in S.I. meconium until term, the fact that bile acids were found as early as 40 days gestation indicated that the biliary system was functional. The major bile acids present throughout gestation were cholic acid, chenode-

oxycholic acid and deoxycholic acid. The total bile acid concentration increased until term. The foetal liver was shown to be capable of synthesizing primary bile acids by at least 120 days gestation. Bile acids were found in amniotic fluid samples obtained at amniocentesis; these concentrations increased by up to 10 fold in amniotic fluid obtained from traumatised foetuses. Throughout gestation over 90% of the bile acids found in all compartments studied were conjugated with the amino acid taurine.

In common with other mammals having a long gestation period, the ovine foetus develops the ability to secrete bile acids quite early in gestation. Although the biliary system is functional, it appears that pregnanediols, either free or conjugated, are not secreted at high concentrations into the foetal intestine until just prior to term. This may be explained by the surge of circulating cortisol that occurs in the foetal lamb at this time.

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CHAPTER 1

INTRODUCTION
and
LITERATURE REVIEW

FIGURE 1

Diagramatic representation of Liver Lobule.
BD, bile duct; PV, prtal vein; HA, hepatic artery;
TPV, terminal portal venule; CV, central vein.

The circulating levels of progesterone and its metabolites in foetal sheep plasma depend on (1) the progesterone production rate and (2) the clearance rate of progesterone and its metabolites. This thesis is concerned with determining the contribution of the biliary secretory pathway to the metabolism of progesterone in the developing foetal lamb. The foetal liver is capable of reducing, oxidising and hydroxylating progesterone, however in terms of biliary excretion the most important metabolic function is the degree and type of conjugation present. In the first section of this thesis an analysis of the free, sulphated and the glucuronidates of pregnanediols in S.I. meconium is discussed with respect to the ontology of the foetal lamb. This is then related to the maturity of the biliary system as assessed by the relative concentration of the primary bile acids to the secondary bile acids throughout gestation.

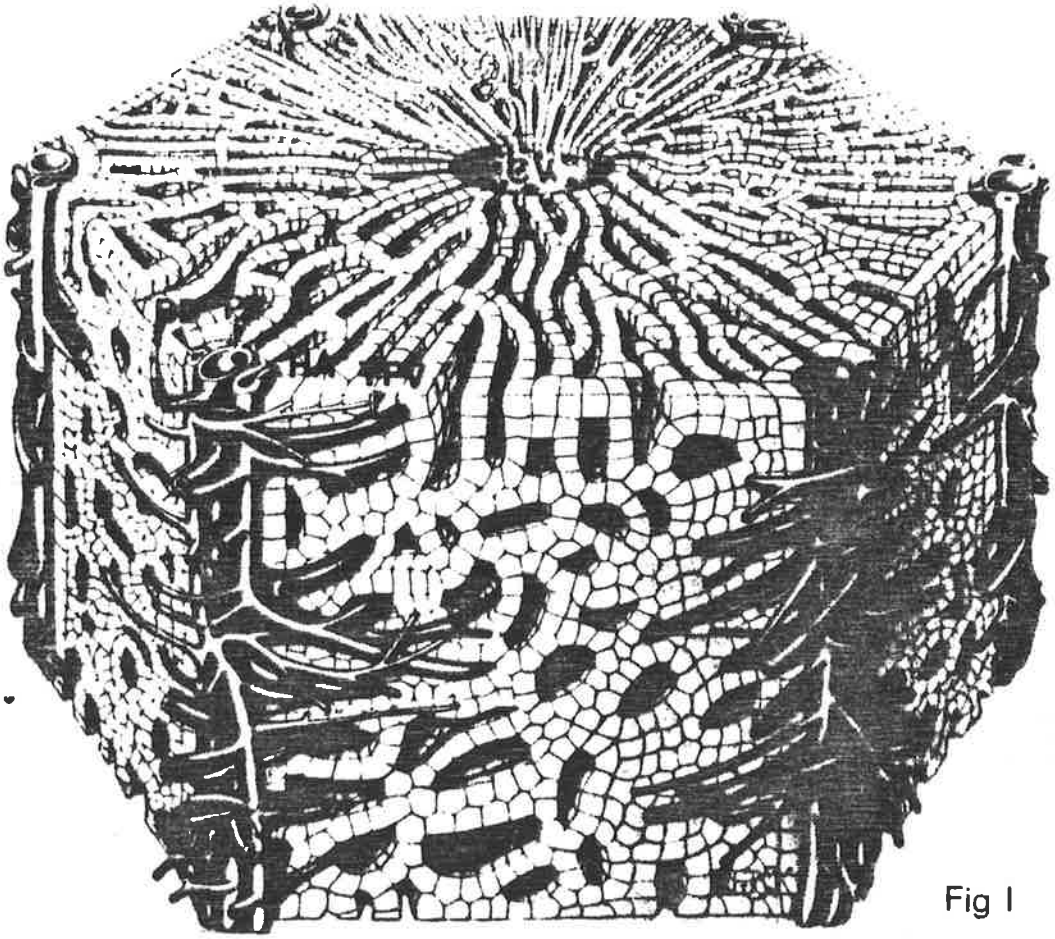


Fig I

Central in understanding the development of the gastro-intestinal tract in the foetus is a conception of the role played by the liver and its effluent connection with the small intestine, the biliary system. The liver produces many and varied metabolites quite early in gestation in large mammals. As this organ develops the number and variety of metabolites also increase. Some of these metabolites are excreted into the foetal intestine, perhaps to be reabsorbed, or on the other hand effectively sequestered out of the foetal circulation. Whilst certain molecules undergo mobilisation via the biliary route others are removed from the foetal peripheral system by the foetal kidneys or by exchange directly with the mother via the placenta.

Surprisingly little is known about the developmental state of gastrointestinal function, probably due to the fact that investigations in this area are severely impeded by technical and ethical constraints (this is particularly the case with respect to the human). Because of the limited accessibility and fragility of the foetal and neonatal organisms, the technical approach has been of necessity opportunistic and the results obtained using different methodological approaches may not be directly comparable. Species differences make extrapolation of results to human development difficult.

Concerning the biliary excretory pathway three primary models have been developed to describe the histological and/or functional units of the liver. The terms, classic lobule, portal lobule and liver acinus have been assigned to these units; these conceptual models are not conflicting but merely represent different interpretations of certain aspects of hepatic structure and function.

The lobules are composed of a continuous system of communicating parenchymal cell plates (see diagram Fig.1). The parenchymal cells throughout an entire lobule are interconnected and subdivided by sinusoids or lacunae. The sinusoids do not have the same structure throughout the whole liver. Since blood flows from the portal canals (hepatic artery, portal vein) into the liver lobule and through the sinusoids to the central vein, a lobular gradient concept has been postulated for the uptake of certain substances (e.g. bile acids).

Rappaport et al., (1954) proposed that the liver acinus is the microvascular unit of hepatic parenchyma (see Fig. 2, page 5). Within this unit, blood flows from a terminal portal venule and a terminal hepatic arteriole into sinusoids of the periportal area. The parenchymal cells are grouped into 3 concentric zones:-

> Those surrounding the terminal afferent vessels - the first to receive blood and nutrients (zone 1 in Fig. 2). The acinus periphery has a landmark, the terminal hepatic venules (zone 3). Between these two zones lies the intermediate zone 2 which has no boundaries of its own.

Whilst blood flows from zone 1 to zone 3 in this model, bile shows countercurrent movement, i.e. it flows towards zone 1. The primary units of the biliary system are the bile canaliculi. They are formed as very fine tubules between adjacent pairs of parenchymal cells, each cell contributing one half of the canaliculus. Numerous microvilli project into lumen (1μ in diameter). The Golgi apparatus of the cell is close to the canaliculus and ATPase activity in this area is high. This arrangement suggests the bile canaliculi are regions in which

active secretory processes occur. Under normal conditions, transfer of substances from blood to the bile canaliculi must occur by movement of water and solutes across the parenchymal cells. The bile canaliculi anastomose to form small bile ducts (ductules) which are 15 - 20μ in diameter and these ultimately form the hepatic bile duct which in certain species, such as the sheep, enters the gallbladder.

It is apparent from the preceding account of the microstructure of the liver that the processes involved in the transport of steroid metabolites into bile are markedly different from the mechanism of transport of steroids into urine. Both in the adult, foetus and neonate, the liver is a major site of steroid metabolism and conjugation, and the metabolites may be transferred back into the blood to be excreted by the kidney, or may cross the membrane of the canaliculus to be excreted in bile. Bile eventually passes into the small intestine either as hepatic bile (rat) or in those animals which have a gallbladder, (sheep, man etc.) as a mixture of gallbladder and hepatic bile. It is worthy of note here that much of the experimental work (adult) has been carried out using the rat, an animal that lacks a gallbladder. The significance of possession of a gallbladder is not clear, but it would seem to complicate whatever species differences that exist in delivery of bile to the intestine. This would be both in terms of frequency of entry of bile to the small intestine and the concentration of biliary contents (the gallbladder has a concentrating function).

Because of the minute size of the bile canaliculi, the composition of primary bile is not known, and so an exact

FIGURE 2

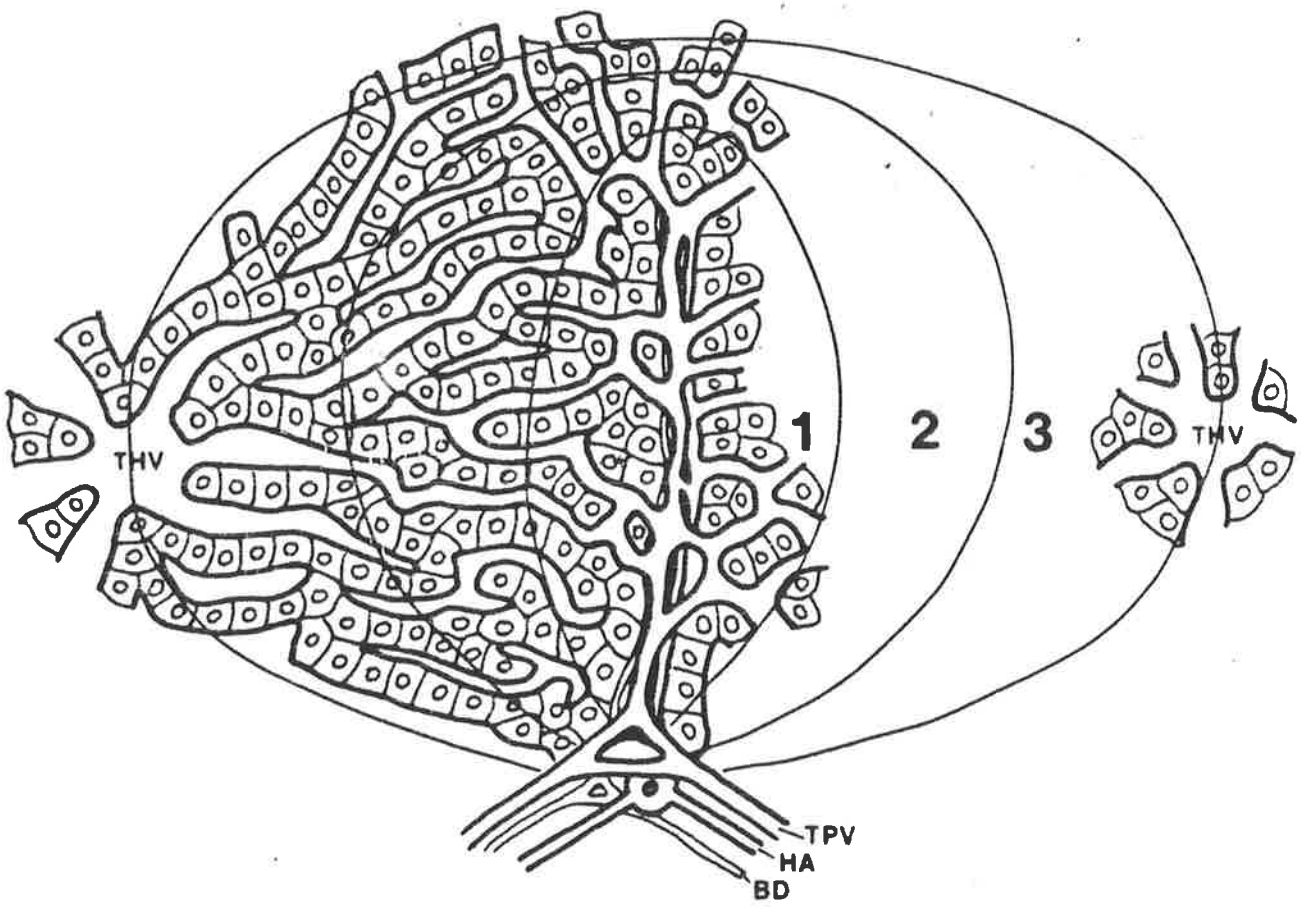
Diagram of liver acinus (after Gumucio and Miller, 1981)

THV - Terminal hepatic vein; TPV - Terminal portal vein,

HA - Hepatic artery; BD - Bile duct

explanation of the processes involved in bile secretion is not yet possible. Therefore the factors that influence the excretion of steroid hormones in bile in both adults and the foetus are obscure. From what has been described above it will be apparent that interpretation of the results obtained by analysis of steroids in bile is more complex than analysis of urine and other foetal fluids.

Fig 2



HEPATIC ACINUS

Furthermore analysis of meconium in foetal animals and faeces in neonates and adults is technically more difficult than analysis of urine. An advantage exists with respect to analysis of meconium c/w faeces however. Because the foetal gut is sterile the metabolites that are found in meconium should reflect those excreted in bile (relatively) unless some degree of reabsorption and/or metabolism by mucosal enzymes occurs.

Despite the limitations described, experiments have revealed some consistent patterns in excretion of steroid metabolites in adult animals. Although data for the foetus both with respect to steroid hormones and bile acids, are lacking, an understanding of the role played by the biliary system is beginning to emerge. Because of the paucity of foetal data much of the comparison presented here is of necessity with the adult animal or between species.

Ovariectomy of the pregnant ewe before about 50 days of gestation results in abortion (Casida and Warwick, 1945), however after this time abortion does not take place and pregnancy is maintained by placental production of progesterone (Linzell and Heap, 1968). In sheep the essential role that the placenta plays in the production of progesterone for the maintenance of pregnancy is well established, however those mechanisms which are responsible for increasing the production of progesterone by the placenta during gestation are not clearly defined.

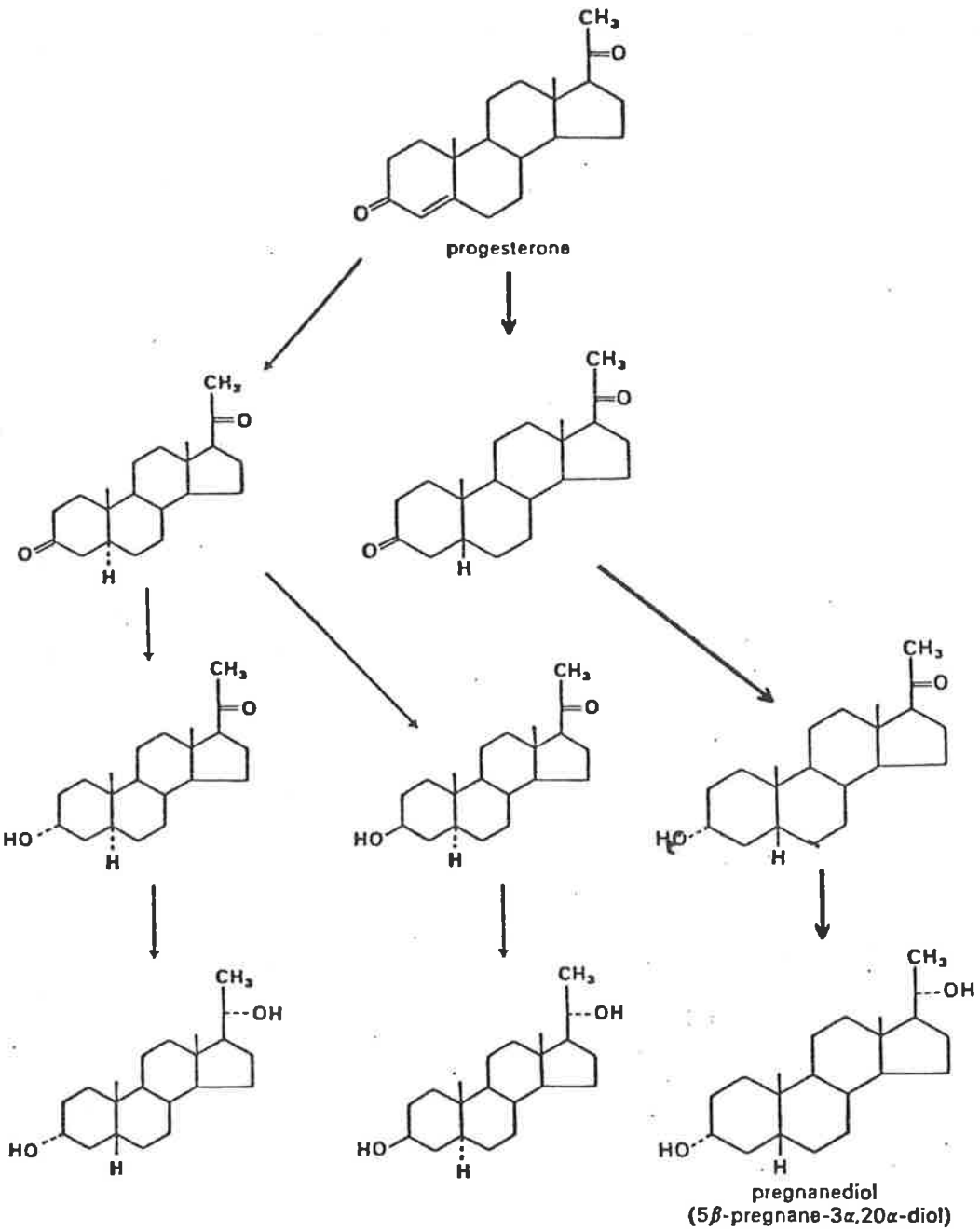
Foetal Metabolism of Progesterone

Whilst increased progesterone production as the foetus develops is essential for the maintenance of pregnancy, this results in the foetus being exposed to large amounts of

progesterone, which must be metabolised. In humans, placental production of progesterone is almost 300 mg/day, approximately 75 mg of which enters the foetal circulation. The rate of progesterone production in sheep appears to be much lower; it is estimated to range from 26 mg/day mid-gestation, to about 50 mg/day near term for a ewe bearing a single foetus. Over 80% of this production is accounted for by the conceptus (Bedford et al., 1972). The plasma levels of progesterone have been reported at < 2 ng/ml (Seamark, 1971; Strott et al., 1974b). Recent evidence suggests that in late pregnancy, under the influence of foetal cortisol, the placenta is also the source of the prepartum increase in unconjugated oestrogen (Challis, 1971; Currie, Wong, Cox and Thorburn, 1973; Flint, Anderson, Steele and Turnbull, 1975). Indeed it is now considered that the increase in foetal cortisol influences the onset of labour through its effects on placental steroidogenesis (Flint et al, 1975; Steele et al, 1976). These experiments suggest that under the influence of the increase in foetal cortisol at term, the sheep placenta becomes a "complete" endocrine gland capable of metabolizing pregnenolone to oestrogen. How does the sheep foetus deal with the progesterone (an unknown amount passes to the foetus) that enters its circulation? Progesterone is metabolised variously to gestagens 20α-hydroxy-preg-4-ene-3one (20αHP) and 20β Hydroxy-preg-4-ene-3one and 17α hydroxy-pregn-4-ene-3-20-dione. Nancarrow and Seamark (1968) demonstrated that the ovine foetal red blood cell dominates the initial metabolism of progesterone to 20α HP, this is catalysed by 20α hydroxysteroid dehydrogenase. Subsequent metabolism occurs mainly in the foetal

FIGURE 3

Metabolites of progesterone - The pregnanediols can exist as monosulphates, disulphates, glucuronides or mixed conjugates


progesterone
pregnanediol
(5β-pregnane-3α,20α-diol)

liver although testis, adrenal gland and the kidney are all capable of metabolizing progesterone. The foetal sheep liver also exhibits enzymes 5β-reductase, 3β hydroxysteroid dehydrogenase and 20β hydroxysteroid dehydrogenase (Anderson et al., 1970). In the foetal sheep liver, the 20β HSD enzyme predominates. The presence of 5β reductase indicates the likelihood of a high proportion of 5β metabolites in the sheep foetus. This differs from the human where a preponderance of 5α metabolites of progesterone is the case. Figure three shows the possible metabolites of progesterone after processing by the foetal liver.

After reduction of progesterone and other gestagens by the foetal liver, the resulting pregnanediols are conjugated with either sulphuric acid or glucuronic acid. At what time this occurs in gestation and the subsequent partitioning of these conjugates to either the biliary tree or back to the peripheral circulation is not known in the foetal lamb.

The role of bile acids in the foetal biliary tract

The foetal lamb uses carbohydrates as a major source of energy (Alexander, Britton, Cohen and Nixon 1969) in contrast to its dam which absorbs very little carbohydrate from the gut, and which relies largely on short-chain fatty acids as an initial source of energy (Lindsay 1959). In some ways, the sheep foetus can be regarded as a "monogastric" animal, though the major determinant of nutrient availability must largely depend on the permeability of the syndesmochorial placenta to the various substrates. In the adult sheep, as in the human, efficient fat absorption depends on the co-ordinated functioning

of hepatobiliary, pancreatic and intestinal functions; the delivery of bile into the gut; pancreatic exocrine secretion; and optimal intestinal milieu and intestinal mucosal cell function. This thesis is concerned with the first of these functions with respect to both bile acid metabolism and progesterone metabolism throughout gestation.

There is a paucity of experimental data regarding the metabolism of progesterone metabolites in the biliary system and the intestine of the adult mammal. No studies have been carried out in the foetus of any species but man (Kinsella and Francis 1971, Huhtaniemi and Vihko 1973). Accordingly to provide a background, a brief account of current knowledge of progesterone biliary metabolism in the adult is presented.

Biliary excretion - Progesterone

Rodgers and McLellan in 1951 isolated pregnanediol from human bile after administration of 500 mg of progesterone. In 1958 Sandberg & Slaunwhite demonstrated that after intravenous injection of radioactive progesterone in patients with biliary fistula, approximately 30% of the radioactivity appeared in bile. Faecal excretion ranging from 4 - 50% of intravenously administered radioactive progesterone has been reported. Wiest et al., and Chang et al., identified the following radioactive metabolites in human bile after hydrolysis with β-glucuronidase:

5β -Pregnane-3α,20α -diol

5α -pregnane-3α,20α -diol

3α -hydroxy -5β -pregnane-20-one

In other studies most of the radioactivity could not be extracted from bile despite the fact that many hydrolytic procedures were used (Sandberg et al., 1958). The compounds identified were mainly liberated by β-glucuronidase hydrolysis. In 1969 Adlercreutz et al., using gas chromatography - mass spectrometry showed that the main metabolites were glucuronides, however some mono-sulphates and disulphates were detected. Whether progesterone itself is excreted in human bile has not been confirmed by modern analytical techniques. The same situation pertains to the ewe. A table of progesterone metabolites thus far detected in bile and their mode of conjugation has been presented by Taylor 1971 (see Table 1).

The main progesterone metabolites in non-pregnancy bile of humans are the glucuronide of 5β pregnane 3α-20α -diol and the disulphate of 5-pregnene-3β-20α-diol (Klopper & McNaughton, 1959) while in pregnancy bile 5β -pregnane- 3α, 20α -diol is the most abundant metabolite in all three conjugate fractions

Intestinal metabolism

It is generally accepted that orally administered progesterone has little biological effect. The exact mechanism that hinders biologically active progesterone reaching the main circulation has not been fully clarified. Rapid hepatic reductive metabolism of absorbed progesterone must play a role in decreasing bioavailability of orally administered progesterone. In the adult, metabolism by intestinal bacteria must also be involved, however as the foetal gut is sterile there would be no contribution from bacterial metabolism in utero. Once progesterone has crossed the intestinal wall into the portal circulation, its metabolism seems to be the same as if

TABLE 1

Progesterone Metabolites in bile (reproduced in part from Taylor, (1971)

Species and steroid	Type of preparation	Percent of dose in			Reference
		Bile	Urine	Feces	
Progesterone					
Rat	Normal	—	27	56	Shen *et al.* (1954)
	Chronic bile fistula	73	22	2	
	Ligated bile duct	—	80	10	
Rabbit	Normal	—	15–37	—	Cooke *et al.* (1963)
	Acute, cannulated bile duct	35–41	22–44	—	Taylor and Scratcherd (1963)
		31–44	13–31	—	Senciall and Thomas (1970)
Cat	Normal	—	0.6–3.0	53–59	Taylor and Scratcherd (1962)
	Acute, cannulated bile duct	26–27	0.5	—	Taylor and Scratcherd (1961)
Ewe	Normal	—	21	—	Stupnicki *et al.* (1969)
	Chronic bile fistula	70	26	—	
Baboon	Normal	—	34–41	14–17	Kulkarni *et al.* (1970)
Human	Normal	—	46–59	8–17	A. A. Sandberg and Slaunwhite (1958)
	T-tube drainage	20–35	38–70	3	
20α-Dihydroprogesterone					
Rabbit		30–51	15–23	—	Senciall and Thomas (1970)
20β-Dihydroprogesterone					
Rabbit		21–31	10–15	—	
Pregnenolone					
Rat	Normal, male	—	50	45	Eriksson and Gustafsson (1970a)
	female	—	45	50	
	Germfree, male	—	40	55	
	female	—	40	55	
17α-Hydroxyprogesterone					
Human	Normal	20	55	—	Slaunwhite and Sandberg (1961) Vermeulen *et al.* (1961)
	T-tube drainage	—	55	—	
17α-Acetoxyprogesterone					
Human	T-tube drainage	40	35	—	Slaunwhite and Sandberg (1961)
6α-Methyl-17α-acetoxyprogesterone					
Human	T-tube drainage	40	35	—	Peterson (1965)

administered parenterally. However, the enterohepatic metabolism of biliary progesterone metabolites does not seem to have been studied in man. This also is the case in the pregnant ewe and certainly the foetus. The major components of progesterone metabolites that are secreted via the biliary route in the adult are conjugated with either sulphuric acid or glucuronic acid, the latter apparently predominating.

In general, biliary excretion of organic compounds is influenced by several physico-chemical factors, namely:

(i) Molecular weight;

(ii) Polarity - an arrangement of polar groups that make the compound amphipathic is favoured, and,

(iii) Chemical structure.

The diverse chemical structures of "cholephilic" drugs encourage the speculation that the high molecular weights reflect a correlation with amphipathic behaviour rather than a specific requirement for molecular size or shape. A further compounding factor is that most of the organic solutes in bile are products of hepatic metabolism, so that the kinetics of solute transport from blood to bile are frequently complicated by changes in chemical configuration.

For appropriate biliary excretion to occur, a molecule must satisfy certain requirements regarding molecular weight and polarity.

Molecular Weight

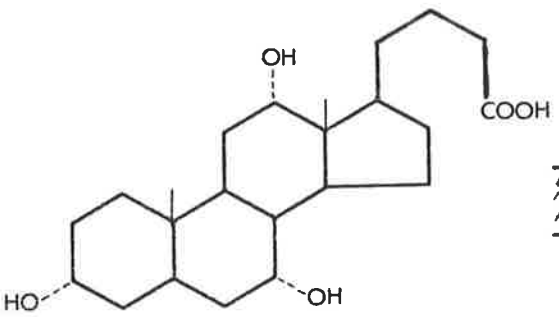
For man, the threshold molecular weight value for appreciable biliary excretion of organic anions is about 500 (Smith,1974), however species differences do exist.

FIGURE 4

The four major bile acids

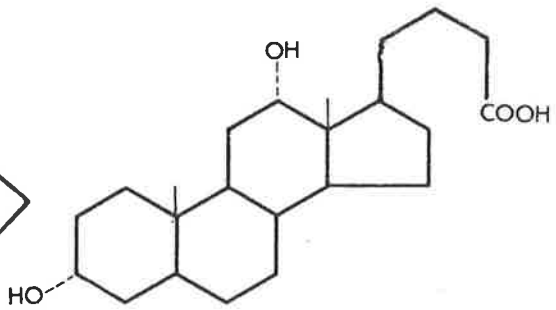
PRIMARY BILE ACID

SECONDARY BILE ACID

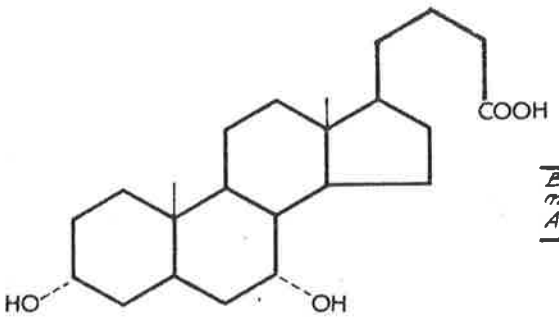


CHOLIC ACID

Bacterial metabolism in Adults

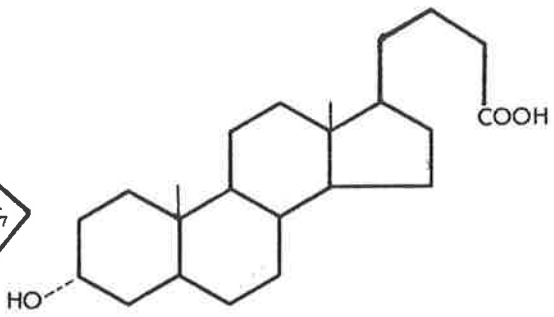


DEOXYCHOLIC ACID



CHENODEOXYCHOLIC ACID

Bacterial metabolism in Adults



LITHOCHOLIC ACID

The various naturally occurring and synthetic steroid hormones have molecular weights in the approximate range 270 - 330 and they are in general metabolised to conjugates having molecular weights between 400 - 500.

Polarity -

The compound should have in its molecular structure an appropriate polar group (usually a carboxylic, sulphonic or sulphate group) which allows the molecule at physiological pH to exist in the anionic form.

Chemical Structure

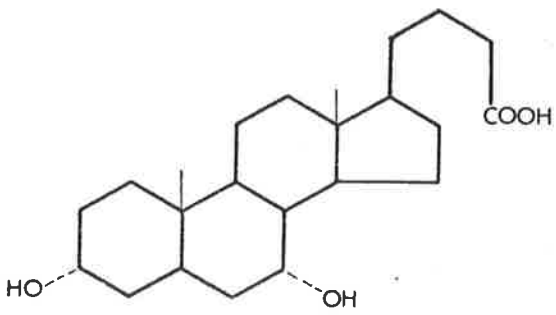
Minor structural changes which do not significantly effect molecular weight and polarity, may lead to marked changes in biliary excretion.

Hirom et al., (1972 b) suggested that the role of the structural factor may be to influence the relative relationship of the polar and non-polar parts of the molecule and also the shape of the molecule. Small alterations in chemical structure may greatly influence the extent of biliary excretion e.g. introduction of additional aromatic hydroxyl groups (Williams, Milburn, Smith & Williams 1965). Whether the apparent advantage conferred by an amphipathic structure relates to transport across the liver cell or to an association with micelles in the canalicular lumen is, however, not clear. Most drugs and many endogenous substances appearing in bile are glucuronides or other anionic products of intracellular conjugation - the relation between bile acid transport and their excretion appears to have received little attention.

The above considerations are based on studies carried out in adult animals, the situation in the foetus may be quite different, particularly in the earlier stages of gestation.

FIGURE 5

Ursodeoxycholic acid - present in trace amounts in isolated samples of foetal bile



URSODEOXYCHOLIC ACID

FIGURE 6

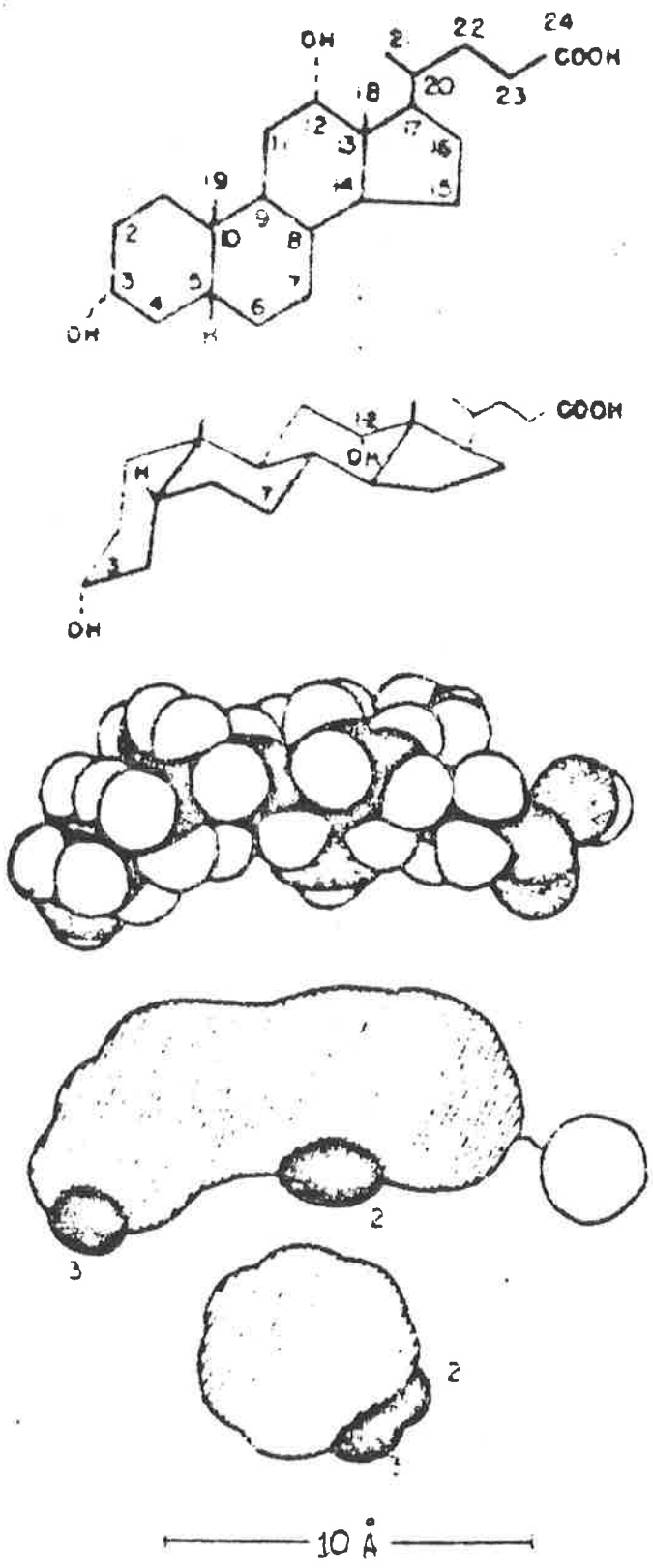
Different representations of deoxycholic acid

i Chemical formula

ii Perspective formula

iii Stuart-Breigleb space-filling model

iv Diagrammatic representation of the molecule in longitudinal and cross-section. Scale $10A^{0}$


OH
22
24
COOH
20
23
18
12
3
17
16
15
19
9
10
8
2
3
5
7
6
4
OH
H
COOH
12
OH
7
3
OH
2
3
2
10 Å

For example bile salt concentrations may well be too low to reach a critical micellar concentration (CMC) in early gestation.

Of the various metabolic pathways the most important from the point of view of biliary excretion are the conjugation processes, particularly glucuronic acid conjugation and glutathione conjugation and to a lesser extent glycine and sulphate conjugation. These however may be preceded by other metabolic reactions, chemically classified as oxidations, reductions or hydrolyses and these may be important in providing a reactive centre (e.g. hydroxyl, carboxyl, amino group) in the molecule at which a subsequent conjugation reaction can occur. Of the various metabolic conjugations by far the most important is glucuronide synthesis. This probably reflects the limited scope of other conjugation mechanisms in terms of the variety of chemical groups which can accept glucuronic acid, the availability of the conjugating agent and the numerous tissues that can effect glucuronide synthesis (Smith and Williams 1966).

The influence of bile salt concentration on bile flow in the adult.

At least two processes are responsible for total canalicular bile secretion.

(i) the active transport of bile acids into the canalicular lumen (Bile Salt - Dependent Secretion) BSDF. and

(ii) the production of the bile salt - independent canalicular fraction (BSIF) - unrelated to bile salt secretion.

Since the observation by Schiff that feeding bile caused an increase in bile flow, investigators recognized the importance of bile salts in stimulating the formation of hepatic bile.

Schanker and Hogeben in 1961 observed that intravenously administered, uncharged, inert, lipid-insoluble solutes, such as mannitol, passed rapidly into bile. Using such techniques Wheeler, Ross and Bradley demonstrated in dogs that the biliary clearance of mannitol was linearly related to bile acid output over a wide range of secretory rates. They observed that if the regression line of biliary clearance of mannitol was extrapolated upon bile acid secretory rate, there was a significantly positive intercept, which indicated that substantial canalicular secretion occurred in the absence of bile acid secretion.

However modern concepts are based on Sperber's (1963) observations that any osmotically active compound secreted into bile could move water and other diffusible solutes into bile (Sperber 1965). Representation of the relationship by a single linear regression analysis has been questioned because infusions of bile acids into the bile acid - depleted rat or rhesus monkey result in several regression lines, that progressively diminish in slope as the biliary bile acid concentration increases . It now appears clear that bile acids and other organic anions can modify the fraction of canalicular secretion formerly called bile acid independent, the mechanism remains to be defined (Boyer 1980). Indeed hydrocortisone injected intramuscularly in chronic fistula rats has been shown to increase bile flow without changing bile acid or electrolyte output (Butler, Lawson and Gehling 1980, unpublished observations). Whether these mechanisms are operative in the foetus is at present unknown. It has not yet been firmly established that in the early stages of development bile acid formation occurs along the same pathways it does in adult life.

Back et al.,(1973) confirmed earlier studies by Sharpe et al.,(1971) in human meconium and showed that in addition to the four common bile acids shown in figure 4,ursodeoxycholic acid and 3β-hydroxy-5-cholenoic acid may often be present. The finding of 3β-hydroxy-5cholenoic acid is of some significance as this bile acid has not been detected to date in the normal pregnant adult, or in cord blood, and it is therefore unlikely that its origin is the maternal liver or intestine. Amounts of both deoxycholic acid and 3βhydroxy-5cholenoic acid were significantly higher in the meconium samples from premature infants than in those from full term infants (Back et al.,1973). Deoxycholic acid has rarely been found in sterile samples of bile (Norman et al.,1972) or serum (Erb,1972) of full term babies and infants. Peric -Golia et al. in 1968 demonstrated that no deoxycholic acid was found in neonatal sheep bile during the first five days of life. Deoxycholic acid appeared only after the first week following birth and increased thereafter until the 10th to 12th month. Lithocholic acid has been detected. There is little to suggest that the foetal liver can form secondary bile acids directly, since [^{14}C] labelled cholesterol is not incorporated into foetal deoxycholic acid (Lester et al.,1973). There seems to be conflict regarding lithocholic acid (the other major adult secondary bile acid) however Murphy and Signer (1974) suggest that lithocholic acid may be involved in a synthetic pathway as an intermediate with 3β hydroxy-5 cholenoic acid which has been postulated as a minor synthetic pathway for bile acids in the adult human liver (Anderson et al.1972). The demonstration of the presence of lithocholic acid in the first week after birth

might resolve this question. The fact that lithocholic acid has been demonstrated to be hepatoxic in the adult suggests that it could be involved in a foetal synthetic pathway, but whether it is synthesized de novo in the foetus must await further studies. (Lester, 1980)

The role of bile acids in the foetal intestine.

Conjugated bile acids are excreted from the liver into the intestine via the common bile duct. The bile acids in adults are present in bile micelles i.e. water soluble polymolecular aggregates with detergent-like properties. (see Fig. 7).

In non-ruminant animals little digestion of dietary lipid occurs proximal to the small intestine and it is only after digesta mix with pancreatic biliary secretions, that digestion can be said really to begin. In the suckling lamb digestion appears to be similar to monogastric animals in that large amounts of monoglyceride and free fatty acids are present in contents of the small intestine. Milk lipids bypass the rumen by means of the oesophageal groove, and hence avoid the degradation and change that occurs in adult animals. Bile salts are a major factor in absorption of lipids in both the neonate and the adult of both ruminants and non-ruminants. Their role in absorption of non-dietary lipid (such as some steroid hormones) has been less comprehensively studied.

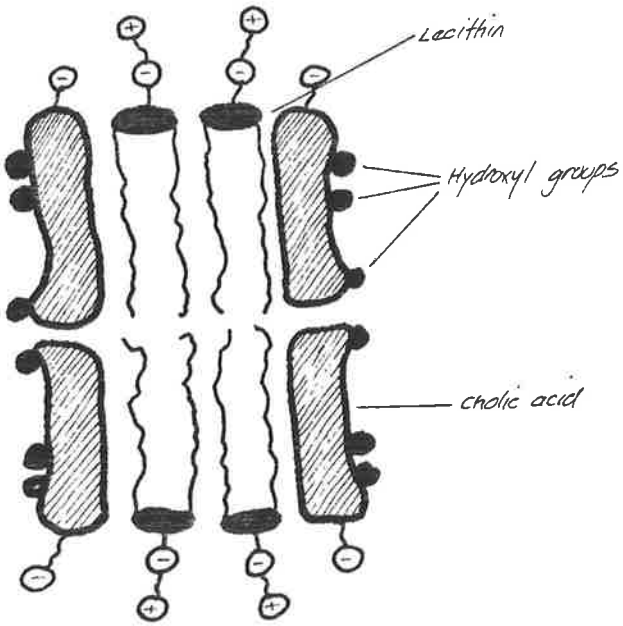
Processes of absorption of fat in the adult ruminant

The role of bile acids in lipid absorption of the foetal intestine is not known. Certainly the foetus begins swallowing amniotic fluid at around 80 days gestation and bile acids may play a part in facilating absorption of the lipids present in this liquor. It has been shown in the foetal dog that (Lester

FIGURE 7

Bile acid micelle

1



2

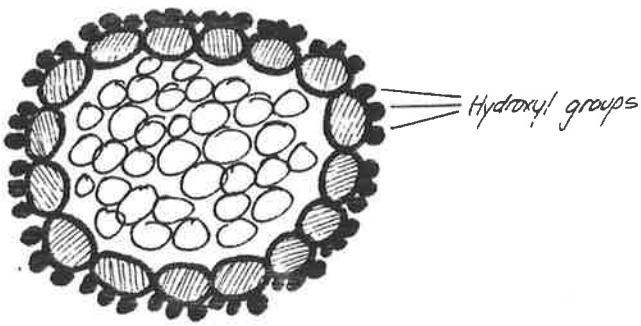
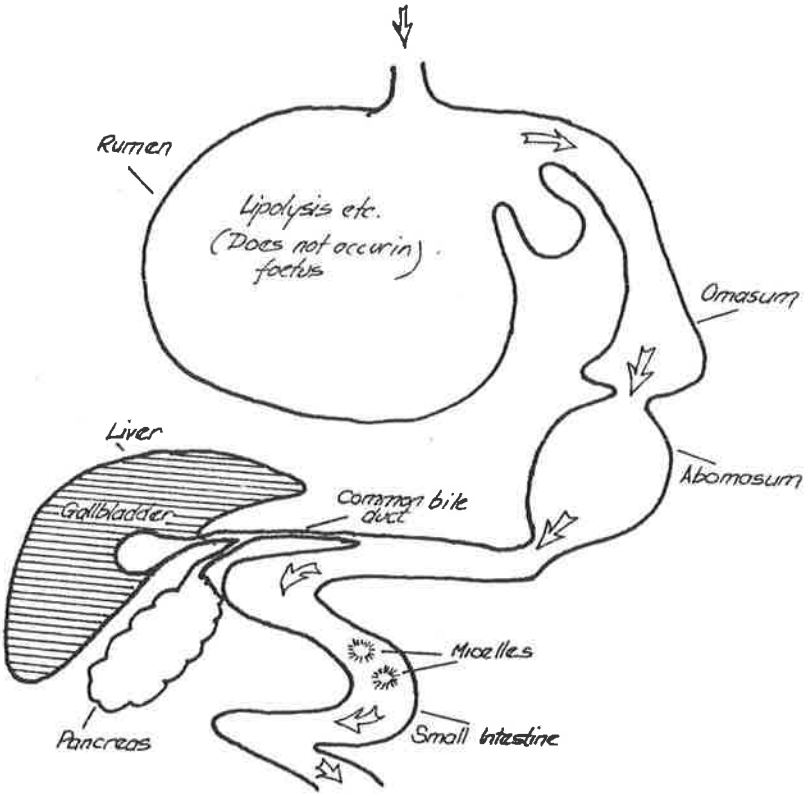


FIGURE 8

Diagrammatic representation of processes of fat absorption in the adult ruminant


Rumen
Lipolysis etc.
(Does not occur in)
foetus
Omasum
Liver
Abomasum
Gallbladder
Common bile duct
Micelles
Pancreas
Small intestine

et al., 1977) absorption of sodium taurocholate when measured in vitro in everted sacs and rings of jejunum and ileum, exhibits no significant difference. In the adult, active transport of bile acids occurs in the ileum but not in the jejunum. Sewell et al., in 1979 demonstrated that, in the near term, foetal sheep (as in the dog), bile salt uptake and excretion were well established. It would seem likely that reabsorption of bile acids by the foetal sheep intestine, as is the case in the dog, occurs by passive ionic diffusion only.

From the studies carried out to date it is obvious that comparisons of foetal bile acid metabolism between species must be made with caution. In many cases too little data has been produced for an adequate interpretation of its significance to be made. Several facts have however emerged from previous investigations. The most interesting of these is that throughout gestation in all species studied $> 90\%$ of bile acids in bile and meconium are conjugated with the amino acid taurine. Bile acid synthesis by the foetus begins in the latter half of gestation in all species studied. Precisely when this occurs has only been ascertained in the rat; (15 - 17 days gestation). Bile acid secretion in sub-human primates (Little et al., 1975) is not as mature as in the perinatal foetal dog or sheep. The quantity of bile acid in meconium is modified by unknown, changing rates of secretion and reabsorption. The relative proportions of bile acids in meconium dating from early gestation to the period just before birth are unknown (Lester 1980). This is the case in all species. The foregoing provides a background against which some aspect of biliary excretion of progesterone metabolites and bile acids are to be studied. These aspects are briefly elucidated in the following objectives.

Objectives

(1) To investigate the conjugates of progesterone metabolities in small intestinal meconium of the ovine foetus. To develop an integrated picture spanning early gestation until term and relating this to a parameter of intestinal and liver function, the bile acids. To analyse progesterone metabolites in amniotic fluid.

(2) To perform an analysis of non-sulphated bile acids both in bile and small intestinal meconium from early gestation until term; enabling some interpretation regarding maturity of the biliary system and the patterns of bile acids present.

(3) To carry out analysis of bile acids in samples of amniotic fluid in traumatised and non-traumatised foetal sheep.

CHAPTER 2

PROGESTERONE METABOLITES

CHAPTER 2

Progesterone Metabolites in Ovine Meconium and Amniotic Fluid

Introduction

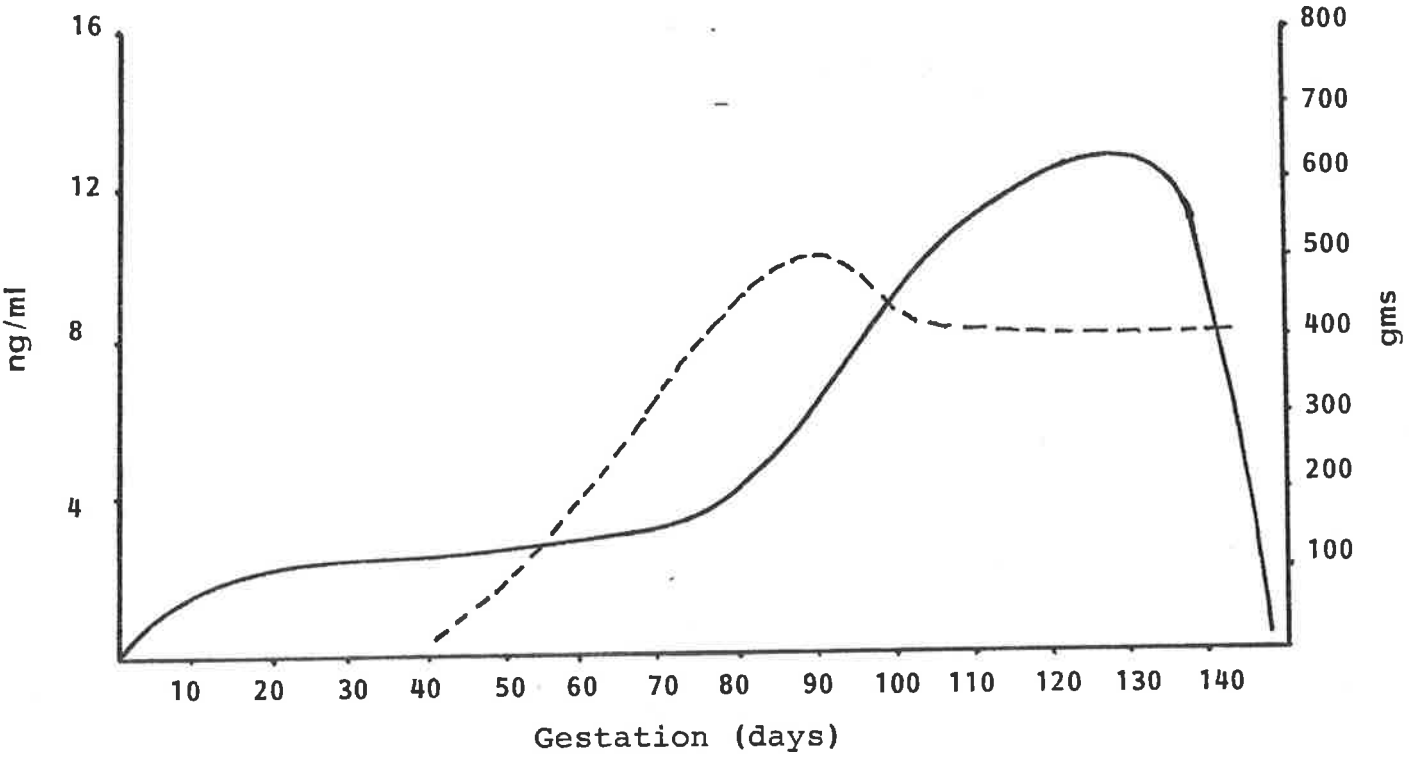
The physiological role of steroid hormone metabolites in the intestinal tract, both in the adult, neonate and the foetus is largely unknown (Adlercreutz and Martin, 1980). Both endogenous and exogenous natural and synthetic oestrogens and progestins are excreted to a considerable extent in bile and are at least partly reabsorbed by the intestine. One of the most important consequences of this enterohepatic circulation of steroids is the delaying effect it has upon their overall elimination from the body.

As pregnancy advances in the ewe the circulating progesterone levels increase, reaching a peak between 130 - 140 days gestation at which time they rapidly begin to drop (see Figure 9 Bedford et al., 1972b). As the maternal progesterone level rises the foetus has to deal with the increasing load of progesterone entering its circulation. One of the metabolic sequelae of this increased placental transfer of progesterone is a progressive increase in circulating levels of pregnanediol sulphates (Dolling and Seamark, 1979)

Seamark (1973) reported the presence of 5β-pregnane-3α-20α-diol ($\sim$ 1 ng/ml) and 5β-pregnane-3α-20β-diol in foetal sheep. Dolling and Seamark in 1979 showed that three progestagen metabolites occurred in foetal sheep blood, namely, 5β- pregnane-3β, 20β-diol, 5β-pregnane-3β-20α-diol and 5β-pregnane-3α-20α-diol, all present as sulphates in μg/ml

FIGURE 9

Placental weight and plasma progesterone levels in pregnant sheep as a function of gestational age, progesterone in ng/ml, placental weight in gm (redrawn from Bedford et al, 1972 b)

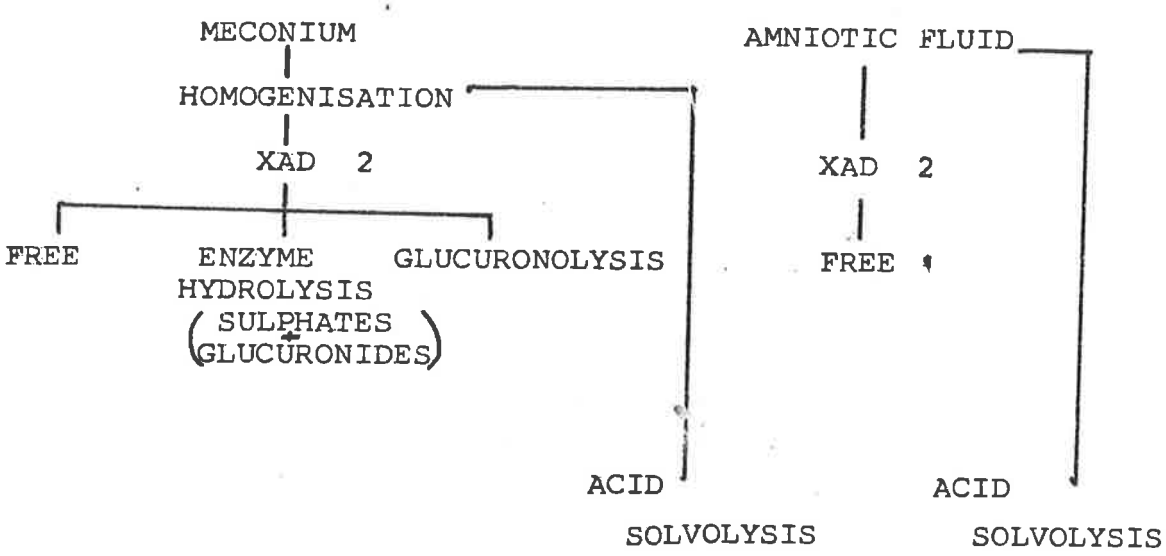

16
12
8
4
ng/ml
800
700
600
500
400
300
200
100
gms
10
20
30
40
50
60
70
80
90
100
110
120
130
140
Gestation (days)

concentrations. These occurred respectively in the ratio 1 : 2 : 1 and the fact that all the pregnanediols have a 5β configuration strongly suggested that the major site of progesterone metabolism in the ovine foetus was the liver. The major metabolite found, 5β-pregnane-3β- 20α-diol is consistent with the presence of the most active enzymes found in both the foetal blood and liver. This contrasts with the maternal organism where 5β-pregnane-3α-20α-diol is the major urinary metabolite (Stupnicki et al., 1968). No 5α-pregnanediols were found in foetal plasma. In the adult, pregnanediol formation takes place mainly in the liver. The same seems to be true in the foetus as incubation of progesterone with foetal liver homogenates yields sulpho-conjugates of both 5β-pregnane -3β-20β-diol and 20α-diol as major metabolites in the same proportions as are found in foetal blood (Nancarrow, 1969; Anderson, Pierrepoint, Griffiths and Turnbull, 1970). In view of the metabolism of pregnanediol sulphates by the liver and their high concentrations in foetal plasma it could be expected that these progesterone metabolites may be partially excreted via the biliary system. The purpose of this study was to investigate small intestinal meconium and amniotic fluid using high resolution chromatography. In meconium both free, sulphated and glucuronidated pregnanediols were analysed. However in amniotic fluid only free and sulphated pregnanediols were investigated. No studies have been carried out on foetal sheep meconium or gallbladder bile. In humans however, Francis and Kinsella in 1966 isolated and identified 5β- pregnane-3α, 20α-diol from pools of meconium excreted during the first 24 hours after birth; 85 - 95% of this fraction was sulphated and 5 - 15%

FIGURE 10

Workup procedures for pregnanediols

WORK UP PROCEDURES FOR PREGNANEDIOL ASSAY



glucuronidated. In 1973 Huhtaniemi, and Huhtaniemi and Vihko showed that in early to midterm human foetuses that glucuronides of neutral steroids predominated. Of the sulphates detected, only 12% were pregnanediols, whereas 40% of the glucuronides were pregnanediols.

Materials and Methods

Animals

Specimens were obtained from foetuses of Merino cross-bred ewes from the University of Adelaide Experimental Farm at Mintaro, South Australia, or from SAMCOR (abbatoirs).

Assay Procedures

The method for assay of the major pregnanediols in foetal sheep meconium and amniotic fluid was adapted from the procedure of Phillipou, Seamark and Cox (1978) for analysis of neutral urinary steroids (see Chapter 5 for further details).

Gas Chromatography

Trimethylsilyl derivatives of the steroids were formed as described in Chapter 5. Gas chromatography was carried out on a PYE 104A instrument fitted with a splitless injection system and an 18 m x 0.4 mm 1% SE 30 glass SCOT column (SGE, Melbourne, Australia). Conditions were as described in Chapter 5.

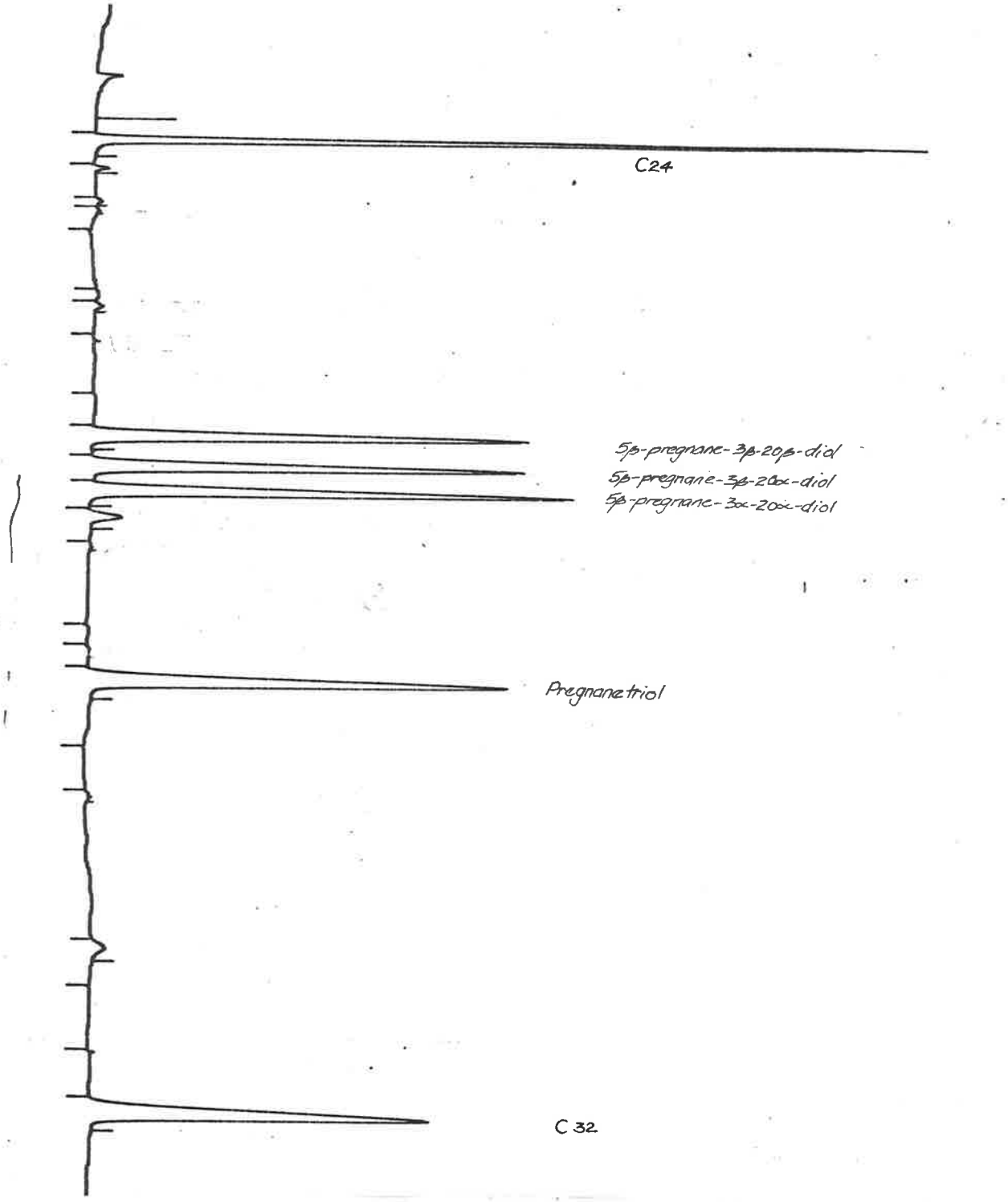
Identification of Steroid Conjugates

The following standards were run using the system with the corresponding retention indices (see Figure 11).

Pregnanediol	Retention Index
5β-pregnane-3β, 20β-diol *	2664
5β-pregnane-3α, 20β-diol	2681
5β-pregnane-3β, 20α-diol *	2696
5β-pregnane-3α, 20α-diol *	2710
5α-pregnane-3β, 20β-diol	2760
5α-pregnane-3β, 20α-diol	2784

FIGURE 11

Gas chromatography of pregnanediol standards.
Total time for analysis approximately 55 minutes


C24
5β-pregnane-3β-20β-diol
5β-pregnane-3β-20α-diol
5β-pregnane-3α-20α-diol
Pregnanetriol
C 32

The limit of sensitivity of the assay was 200 ng/ g of meconium wet weight. No 5α-pregnanediols were detected, the three 5β pregnanediols asterisked were subsequently investigated, identified and quantitated. A comparison of the GLC characteristics of meconium extracts with known steroids showed three peaks with retention indices corresponding 5β- pregnanediols:-

5β- pregnane-3β-20β-diol

5β- pregnane-3β-20α-diol

5β- pregnane-3α-20α-diol

Mass Spectra

Final confirmation of the identity of the pregnanediols in meconium was obtained by utilization of GLC-mass spectrometry. This was carried out in one sample from early gestation (free pregnanediols only) and from one sample at term (glucuronides). All three 5β- pregnanediols showed mass spectral characteristics (Base peak M/Z = 117) similar to authentic compounds. Figure 12 shows the mass spectrum of a peak with a retention index of 2710 (5β-pregnane 3α-20α-diol). The corresponding mass spectra of the authentic compound is shown in Figure 13.

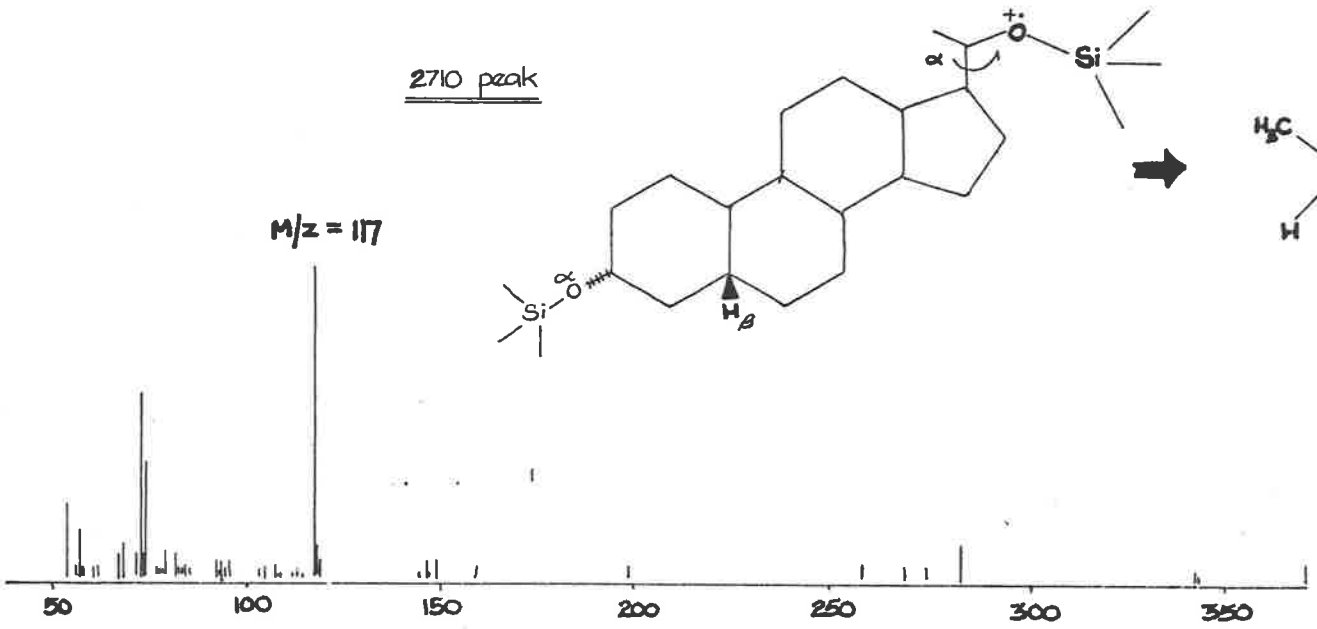
Results

A total of 20 foetuses were used in the study, 14 of these were obtained from the abbatoirs and samples from the remaining six were obtained at or after operation. Meconium from the distal small intestine was assayed in all animals. The foetuses ranged in age from 40 days gestation to term (147 days).

FIGURE 12

Mass spectrum of 5β-pregnanediol from meconium

2710 peak


m/z = 117
50
100
150
200
250
300
350

α
O
Si
α
O
Si
Hβ

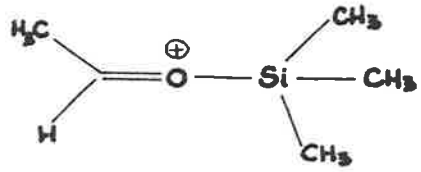

H3C
H
O
Si
CH3
CH3
CH3

FIGURE 13

Mass spectrum of authentic 5β-pregnane-3α, 20α-diol

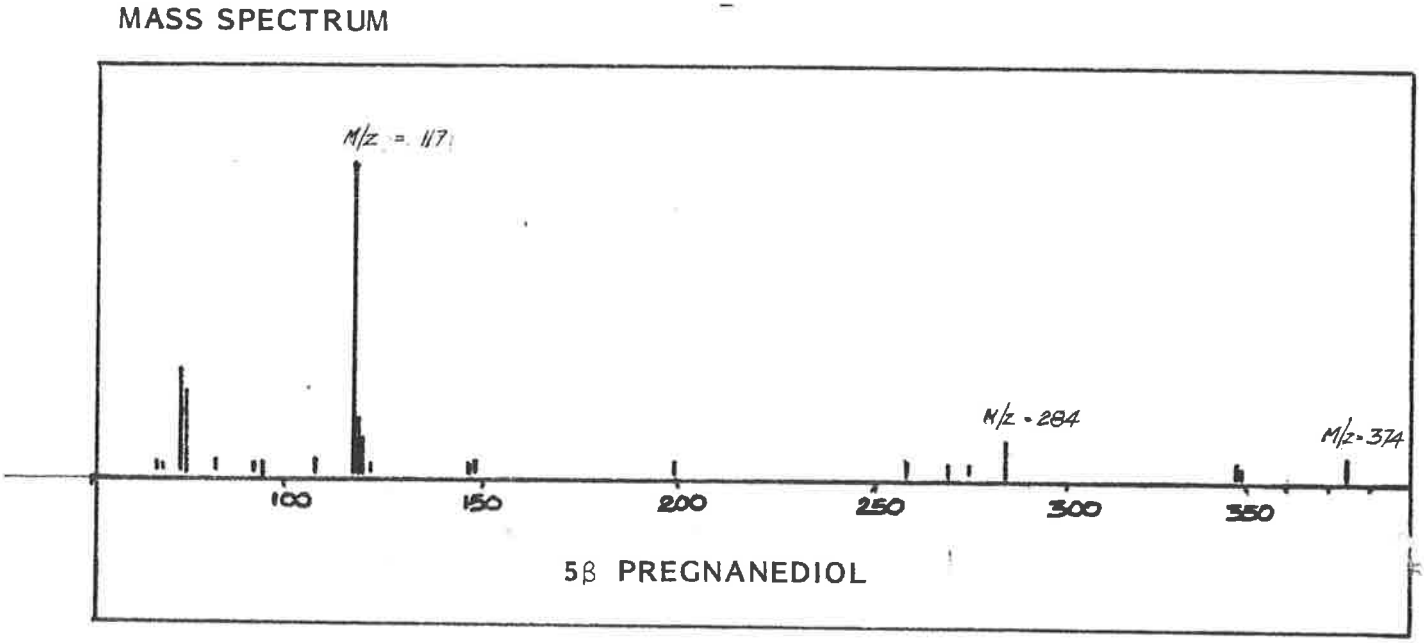


Fig 13. Mass spectrum of authentic 5β-pregnane-3α-20α-diol

Separation of pregnanediol isomers effectively using a packed column is not possible, so a support coated open tubular column with 1% SE30 was used. Using retention indices as described by Kovats (1965) identification and resolution of almost all the pregnanediol isomers is possible. The retention indices were calculated as methyl equivalents by interpolation between two alkane series compounds, n-tetracosane (2400 units) and n-dotrioacontane (3200 units). The values determined by this method were highly reproducible for a particular column, and were largely unaffected by minor changes in column temperature, gas flow etc.

Calculation:

$$\frac{(RT^{*}\ \text{internal std.} - RT\ C24) - (RT\ \text{Internal Std} - RT\ \text{unidentified Peak})}{(RT\ C32 - RT\ C24)} \times 800 + 2400$$

= RETENTION INDEX

*RT = Retention Time

Validation of assay

Four work up procedures were used to ascertain whether the metabolites were either free pregnanediols, sulphated pregnanediols or glucosiduronated pregnanediols. No consideration was given to mixed conjugates. The flow chart shown in Figure 10 details the rationale in the pre-chromatography workup.

The recovery from these hydrolytic and extraction procedures was greater than 80% for the three pregnanediol isomers analysed.

Identification of pregnanediols found in small intestinal meconium.

A comparison of GLC characteristics of small intestinal meconium with known steroids indicated three peaks (in some cases) corresponding to the pregnanediols 5β-pregnane-3β-20β-diol, 5β-pregnane-3β-20α-diol and 5β-pregnane-3α. 20α-diol. (see table 2). These peaks were identified by comparison of retention indices (Kovats, 1965; Phillipou et al., 1978) with those of reference steroids. Final confirmation of identity of the pregnanediols detected was carried out (using samples from term foetuses and one mid-gestation foetus) by GLC-mass spectrometry. Meconium samples were extracted as before, derivatised and run on the GC-MS (Hewlett-Packard 5992 B). Three peaks were present in each case having the same retention indices as the pregnanediols previously found. The mass spectra showed a base peak at M/Z of 117 and a fragmentation pattern corresponding well with authentic 5β-pregnanediol giving peaks at M/Z 284 and M/Z 374.

At no time during gestation were pregnanediol sulphates detected in specimens of small intestinal meconium at levels >200 ng/g. Free pregnanediols on the other hand were detected in three specimens. No consistent pattern emerged. When the presence of pregnanediol glucurosiduronates was investigated none were detected in any samples prior to the perinatal period. In the three term samples analysed however, concentrations of the three major pregnanediols detected averaged 54.15 μg/g in toto. The 5β-pregnane-3β-20α diol and 5β-pregnane-3α-20α-diol

TABLE 2

Concentrations of pregnanediol glucosiduronates in foetal sheep meconium (distal part of small intestine) at term.

PREGNANEDIOL GLUCURONIDES

μg/g WET WEIGHT MECONIUM (distal small intestine)

Sample	5β-pregnane-3β-20α-diol	5β-pregnane-3α-20α-diol	5β-pregnane 3β-20β-diol
EWE 100	4.21	35.25	ND
EWE 102	16.90	33.90	ND
EWE 251	48.10	10.50	13.60

ND - none detected (limit of sensitivity 200ng/g meconium)

isomers were present in almost equal amounts. In only one specimen was the 3β-20β isomer detected. No free pregnanediols were detected in 2 of the term specimens; however when detected in both early gestation and one term sample, all isomers were present. The concentration was higher in earlier specimens when detected. The one term specimen in which free pregnanediols were detected in the distal small intestinal meconium had none in the large intestinal meconium. Glucuronides were also investigated in this sample of large intestinal meconium and showed comparable concentrations with small intestinal meconium levels. Sulphates were not investigated in this sample.

Amniotic Fluid

Neither free pregnanediols nor sulphated pregnanediols were detectable in any samples of amniotic fluid analysed.

Discussion

The data presented provides evidence for the presence of three progestagen metabolites in perinatal foetal meconium. These metabolites are conjugated with glucuronic acid. Small amounts of free pregnanediols were also shown to be present, whether their presence is artifactual or represents a true result of biliary secretion is not possible to explain from these data. However the concentration of free pregnanediols found in the two samples from earlier gestation were higher. The conjugates of 5β- pregnanediol have been shown to occur in foetal sheep blood in μg/ml concentrations. In the adult, 5β- pregnanediols are formed mainly as end products of progesterone catabolism, and in vivo studies with radioactive

tracers have shown these pathways to be operative in the conceptus (Nancarrow, 1969). Furthermore the enzymes involved, including two specific oxidoreductases catalysing the reversible reduction of the 3-OXO and 20-OXO group, a 4-ene-5β reductase catalysing the irreversible reduction of the A ring of progesterone, and the specific alkyl sulpho-transferases involved in conjugate formation, are widely distributed in both foetal and placental tissue (Cox, 1975).

Pregnanediol formation in-the adult takes place mainly in the liver. The same is probably true in the foetus as incubation of progesterone with foetal liver homogenates leads to the formation of sulpho-conjugates of both 5β pregnane 3β-20β diol and 20α-diol as major metabolites in the same proportions as found in foetal blood (Nancarrow,1969; Anderson, Pierrepoint, Griffiths and Turnbull, 1970). Nancarrow also showed the presence of pregnanediol glucuronides. Interestingly 5β-pregnane-3α-20αdiol was present in the highest concentration in term foetal meconium. The results here, although preliminary, suggest that 5β orientation may confer a preferential advantage for biliary excretion of a molecule. This is in agreement with studies in the foetal human (Francis and Kinsella 1966; Huhtaniemi, 1973). Dolling and Seamark showed that circulating 5β-pregnane-3α-20α-diol in both control and nephrectomised sheep foetuses was present in much lower concentrations than the other two isomers. This metabolite was found in foetal sheep meconium in the highest concentration at term; it may be that this isomer of pregnanediol is diverted to excretion by the biliary system, at least in the perinatal period.

Huhtaniemi (1973) detected no monosulphates of pregnanediols and

only small amounts of disulphates compared with relatively high levels of glucuronidates in foetal human bile. It appears that no studies of neutral steroid composition of bile in the last trimester has been done in the human foetus. Francis and Kinsella analysed meconium excreted in the first 24 hours after birth and detected relatively higher levels of sulphated pregnanediols (predominantly 5β) than pregnanediol glucosiduronates. The progesterone estimated to enter the foetal sheep circulation is considerably less than that estimated for the human foetus. Both pregnanediol sulphates and glucuronides may be present in foetal sheep meconium at levels lower than those detectable by the assay used and an analysis of foetal sheep bile extracts from larger volumes would answer this. The finding of high levels of pregnanediol glucosiduronates in foetal sheep meconium suggests that the foetal sheep may differ in its conjugation and excretory pathways compared with the human foetus. The finding of Huhtaniemi (1973) that only disulpated pregnanediols occurred in bile of the human foetus suggests that the pregnanediol sulphates found in the sheep plasma may be monosulphated, thereby favouring partitioning into the plasma compartment rather than into the biliary space. It is puzzling that Huhtaniemi (1973) reported a much higher concentration of glucosiduronates in human foetal bile up to mid gestation yet Francis and Kinsella (1966) found relatively lower levels of glucosiduronates to sulphates in meconium from human foetuses in the first 24 hours of life. In a later report (Kinsella and Francis 1971) analysis of meconium revealed only trace

amounts of 5β-pregnane-3α ,20α-diol compared with the much higher levels (5β -pregnane-3α-20α-diol) reported in 1966. The heterogeneity of the foetal sheep samples analysed, both intrinsically, and in terms of their position in the G.I. tract (distal small intestine) may give rise to some errors, particularly where only small samples were taken (as in this thesis).

Presence of free pregnanediols in samples from early gestation could be due to the immaturity of the liver and biliary system at this time. There is no simple explanation for the finding of some free pregnanediols at term - this occurred in only one term sample and it was noted that there were no free pregnanediols in the large intestinal meconium. This suggests that the free pregnanediols when present are efficiently absorbed by the foetal small intestine. Pregnanediol glucuronides were found in similar concentrations in both the large and small intestinal meconium of one term foetus suggesting that the glucosiduronates are either not reabsorbed or only absorbed inefficiently.

The concentration of cortisol in the plasma of chronically catheterized foetal lambs begins to increase significantly 10 - 15 days before parturition and reaches the highest levels during the last 2 - 3 days of intrauterine life (Bassett and Thorburn, 1969; Nathanielsz, Comline and Silver and Paisley, 1972). Induction of liver enzymes and general maturation of the foetal liver would explain the presence of pregnanediol glucosiduronates. Their presence in foetal meconium compared with pregnanediol sulphates may possibly be explained on the basis of an increased molecular weight and perhaps different polarity and chemical structure, thereby favouring biliary excretion.

It is not surprising that no free pregnanediols or pregnanediol sulphates were detected in amniotic fluid of foetuses up until 135 days gestation, particularly if their origin is from foetal meconium (it is likely that in the 14 specimens obtained from the abbatoirs that some meconium was released into the amniotic fluid; see Chapter 4). Because no amniotic fluid was obtained from term specimens (n = 3) it was not possible to carry out an analysis, however it may be that between 135 days and term, pregnanediol glucosiduronates may be present in amniotic fluid, particularly in cases of foetal distress.

CHAPTER 3

BILE ACIDS

IN

BILE AND MECONIUM

CHAPTER 3

Bile acids in Adult bile and foetal Gall bladder bile throughout gestation.

INTRODUCTION

Meconium is a viscid colourless, greenish black, sterile material containing little fat or protein, and consisting of predominantly carbohydrate-containing substances - this characteristic colour is due to bile pigments such as bilirubin (Feldman 1920; Harries, 1978). Because of its sterility, in contrast to faeces in the adult, its components represent enteric metabolites derived from the maternal-foetal placental unit. The mean pH of meconium is 6.1. The precise origin of the components of meconium has not been defined. Nevertheless the two most important sources must be the amniotic fluid and the secretions of the foetal alimentary tract. The tissue debris in meconium is largely derived from desquamated cells from mouth, skin and the alimentary tract.

The developmental metabolism of bile acids can be partly studied by the analysis of bile acid patterns in meconium. With respect to steroids, studies of the metabolite pattern in meconium have revealed differences between the composition of steroid excretion in the newborn and adult. Only a few reports exist dealing with the analysis of the composition of bile acids in meconium (Sharp, Peller and Carey, 1971; Lavy, Burstein, Gut, 1977; Watkins and Brown 1977, Back and Ross, 1973). In the foetal sheep 40 - 60% of bile acids exist as sulphate conjugates (Watkins and Brown 1977). In general a higher degree of sulphation of secondary

bile acids was the case. Back and Walter (1980) in human meconium (collected at term) showed that 8% of cholic acid and 21% of chenodeoxycholic and hyocholic acids are found also in the sulphate fraction.

Patterns of bile acids in meconium and gallbladder bile.

As previously mentioned metabolism of bile acids can be partly studied by the analysis of bile acid patterns in bile and meconium. Employing gas liquid chromatography and mass spectrometry an analysis of the non-sulphated bile acids present in foetal lambs has been made. The rationale for measuring bile acids in this thesis was as a criteria of maturity of the biliary system. Because all studies reported to date (Smallwood, Jablonski and McWatts 1973; Peric-Golia et al. 1978) indicate that deoxycholic acid is totally derived from the maternal circulation, the ratio of this dihydroxy bile acid with the primary bile acids was used as an index of foetal maturity of the biliary excretory pathway. This was done in both bile and meconium because an unknown amount of bile acids may be absorbed at different times throughout gestation. The pattern of bile acids present in meconium was investigated, the major bile acids being conclusively identified using GC-MS. Where no authentic standard was available a tentative indication of whether the peak on the gas chromatogram was a bile acid was made by inspecting the fragmentation pattern in its mass spectrum. Where the amounts of bile acids were too small for a mass spectral analysis to be performed its retention characteristics on the gas chromatogram were taken

as a tentative identification (Almē, Bremmelgaard, Sjovall, 1977).

Materials and Methods

All materials and methods used in this chapter are described fully in Chapter 5 of this thesis. All the samples used were obtained from abbatoir specimens or at operation.

Results and Discussion

Eighteen samples of meconium and twelve samples of gallbladder bile were obtained (see Table 3). Five samples of gallbladder bile from adult sheep were also obtained (at operation ——— pregnant sheep) and analysed. The results are shown in Table 4. As can be seen from Table 3 bile acids were found in the foetal sheep gallbladder as early as forty days gestation. This differs from the estimation of around 110 days gestation of Alexander et al.,1969. The concentrations of the three major bile acids found in foetal gallbladder are shown in Table 5. The pattern of bile acids found in meconium was qualitatively similar to that of gallbladder bile. Because the samples of meconium were from different parts of the gastrointestinal tract (some distal small intestine and a few large intestine) and because reabsorption of bile acids would differ from these sites no quantitation was carried out. The relative molar percentage of bile acids in adult bile was 39 ± 6.7% (1 standard error of the mean) for cholic acid, 6.6 ± 1.66% for chenodeoxycholic acid and 54.4 ± 6.17% for deoxycholic acid. The major bile acids in adult sheep bile are cholic and deoxycholic acid with chenodeoxycholic acid being a relatively minor component. This is quite different from the relative proportions of foetal bile acids where

TABLE 3

Samples assayed for non-sulphated bile acids

TABLE 3

SAMPLES ASSAYED FOR

NON-SULPHATED BILE ACIDS

Conceptus Age (Range) days	Wt. Kg.	Sex	Estimated Actual Conceptus Age	Small Intestinal Meconium	G.B. Bile
50					
1	.025g	M	40	●	●
50 - 100					
2	.200	M	60	●	●
3	.300	M	78	●	●
4	.500	M	90	●	
100-140					
5	.720	M	100	●	
6	1.75	M	122	●	●
7	1.3	M	116	●	●
8	1.15	M	108	●	●
9	1.84	M	125	●	●
10	1.14	F	108	●	
11	1.21	F	110	●	
12	1.55	M	117	●	
13	1.25	M	112	●	
14	2.45	M	135	●	●
15	2.37	M	130	●	●
TERM					
16 100	3.35	M		●	●
17 102	3.91	M		●	●
18 251	3.16	M		●	●
TOTAL				18	12

TABLE 4

Bile Acids in Adult Gallbladder Bile

TABLE 4

Adult Gallbladder Bile Concentrations (μmol/ml) and Relative Molar Percents

SAMPLE	CHOLIC ACID	CHENODEOXYCHOLIC ACID	DEOXYCHOLIC ACID
1	12.7 (49)	0.6 (2)	12.9 (49)
2	15.6 (55)	1.3 (5)	4.4 (40)
3	1.3 (40)	0.4 (12)	1.6 (48)
4	3.2 (35)	0.6 (6)	5.6 (59)
5	2.0 (16)	1.1 (8)	9.9 (76)
$\bar{x} \pm SE$	6.96 ± 2.98	0.80 ± 0.±7	6.88 ± 2.01

In brackets relative molar % - calculated by summing the three major bile acid concentrations and taking a %.

$\bar{x} \pm SE$	39 ± 6.7%	6.6 ± 1.66%	54.4 ± 6.17%

FIGURE 14

Relative molar percent of cholic acid ●—● and deoxycholic acid △----△ in gallbladder bile throughout gestation

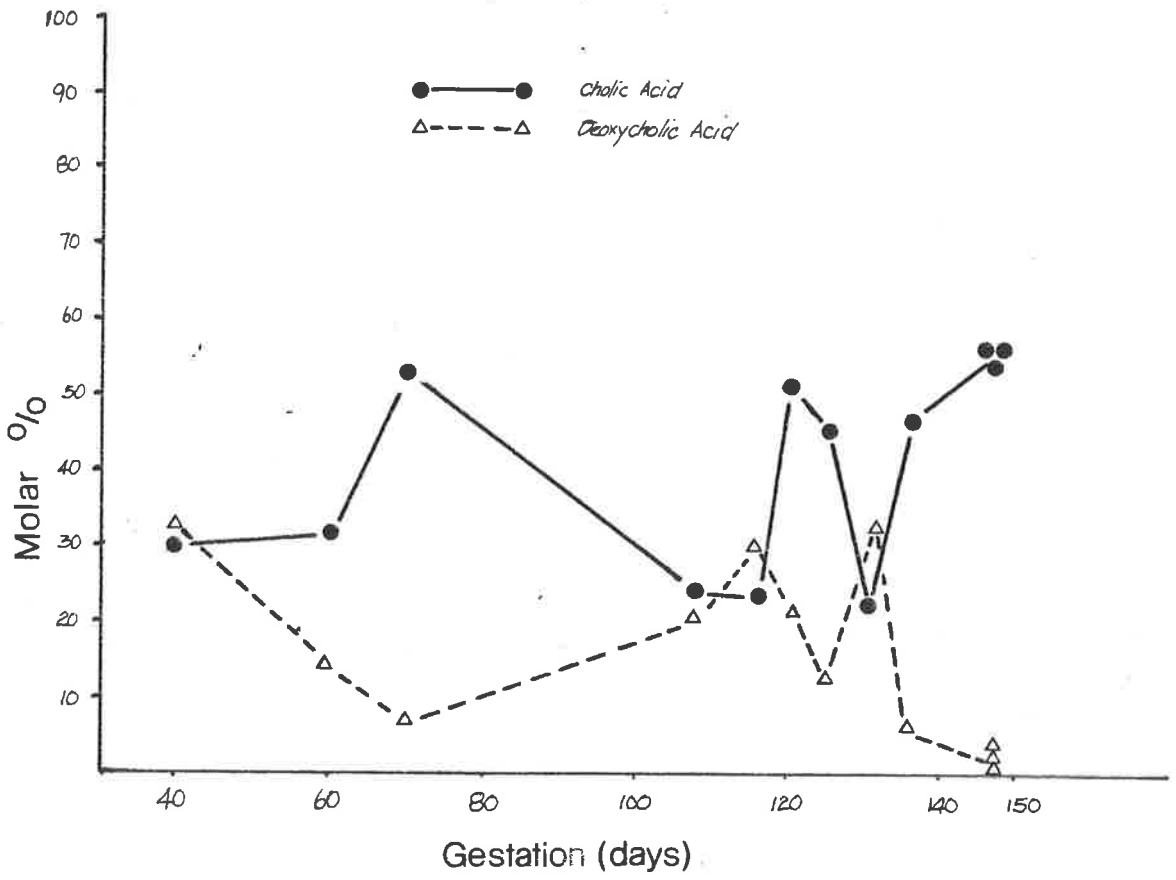
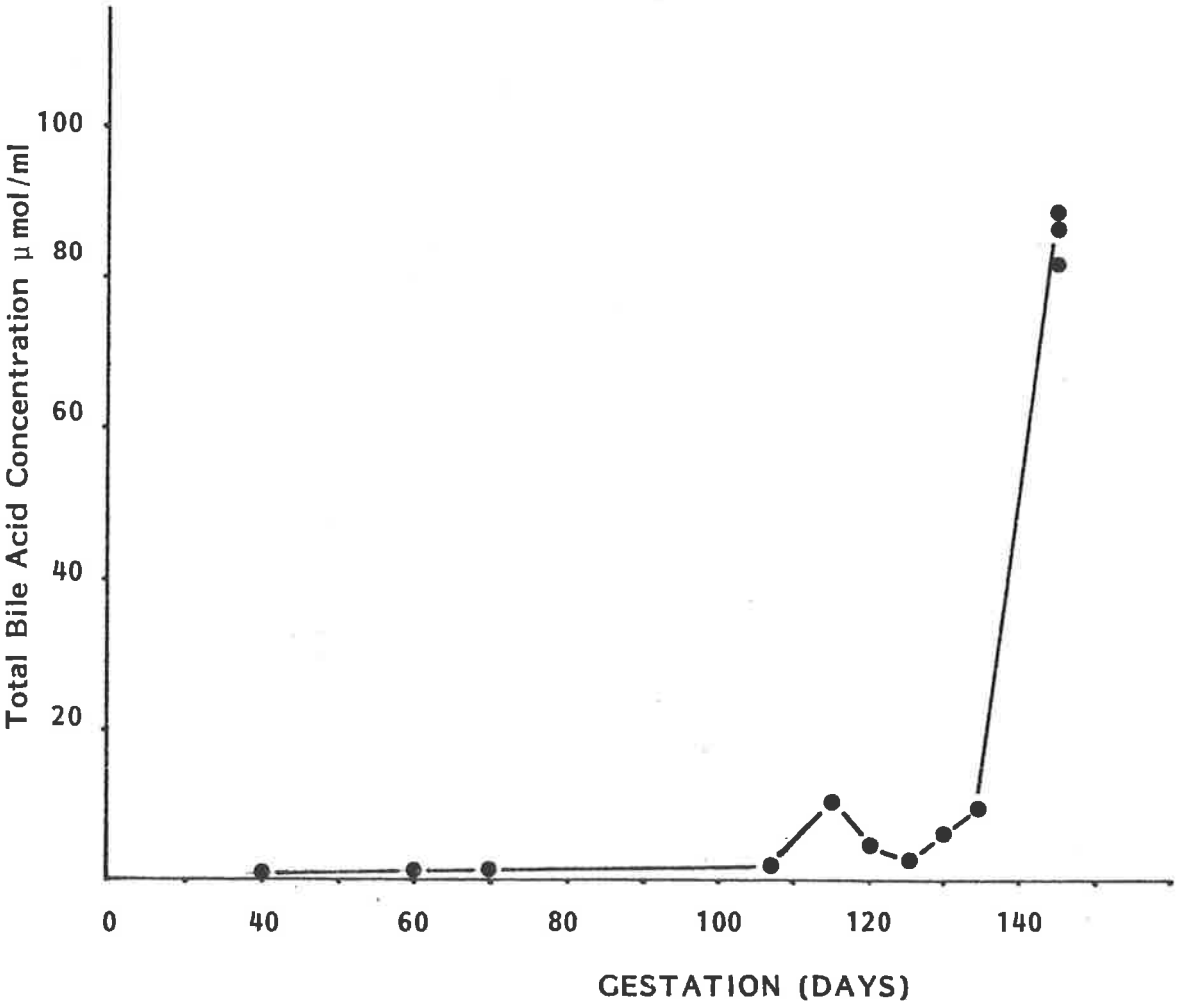

100
90
80
70
60
50
40
30
20
10
Molar %
Cholic Acid
Deoxycholic Acid
40
60
80
100
120
140
150
Gestation (days)

FIGURE 15

Change in total bile acid concentration in gallbladder bile of foetal lambs throughout gestation (40 days gestation to term).


Total Bile Acid Concentration µmol/ml
100
80
60
40
20
0
40
60
80
100
120
140
GESTATION (DAYS)

chenodeoxycholic acid accounts for 43.3 ± 2.16% of the major bile acids in bile, cholic acid for 41.25 ± 3.9% and deoxycholic acid only 15.3 ± 3.54%. When the relative molar percent of deoxycholic acid is followed throughout gestation it can be seen from Figure 14 that while it remains relatively stable up until around 120 - 130 days gestation, thereafter it drops to around 2 ± 0.58%. Comparing the relative molar percent of cholic acid at this time a rise is seen at term from 36.56 ± 4.1% in the first 135 days to 55.33 ± 0.67%. This change is most likely due to a large increase in the synthesis of the primary bile acids just prior to parturition rather than any significant drop in deoxycholic acid transfer from the ewe to the foetus. In fact the concentration of non-sulphated deoxycholic acid increases from 0.74 ± 0.31 µmol/1ml (n = 9) in the first 135 days of gestation up to 1.62 ± 0.49 µmol/ml (n = 3) at term. Figure 15 shows the large increase in total bile acid concentration that occurs between 10 - 12 days prior to parturition and at time of birth.

Profiles of Bile acids in Bile and Meconium

Figure 16 shows a representative chromatogram from a sample of foetal bile at around 120 days gestation. Peaks corresponding to lithocholic acid, deoxycholic acid, chenodeoxycholic acid and cholic acid were all present (peaks 1, 4, 6 and 9 respectively). In addition two other unknown peaks were present (3 and 5). Peak 5 was shown to have a mass spectrum diagnostic of a dihydroxy bile acid. Trace amounts of bile acids tentatively identified

TABLE 5 GALLBLADDER BILE THROUGHOUT GESTATION

Conc. µmol/ml

DAYS GESTATION	CHOLIC	CHENODEOXYCHOLIC	DEOXYCHOLIC
40	0.009 (30)	0.011 (37)	0.008 (33)
60	0.26 (31)	0.45 (54)	0.12 (14)
70	0.50 (53)	0.38 (40)	0.06 (7)
108	0.28 (24)	0.66 (56)	0.24 (20)
116	2.17 (25)	3.82 (45)	2.55 (30)
120	1.6 (51)	0.85 (27)	0.67 (22)
125	1.17 (45)	1.13 (43)	0.3 (12)
130	1.4 (23)	2.62 (43)	2.09 (34)
135	3.8 (47)	4.8 (47)	0.61 (6)
TERM	50 (56)	38.7 (43)	0.7 (1)
TERM	50 (56)	36.2 (41)	2.34 (3)
TERM	45.1 (54)	36.8 (44)	1.82 (2)
$\bar{x}$ ± SE	41.25 ± 3.90	43.3 ± 2.16	15.3 ± 3.54

FIGURE 16

Gas Chromatogram of gallbladder bile (meconium gave a similar profile) from a 120 day old foetus.

1. Lithocholic acid
2. 3β,5-cholenoic acid (tentative identification)
3. Unknown
4. Deoxycholic acid
5. Unknown (confirmed that this is a dihydroxy bile acid by mass spectrometry)
6. Chenodeoxycholic acid
7. Ursodeoxycholic acid
8. Hyocholic acid
9. Cholic acid
10. 7-keto-lithocholic acid (internal standard)

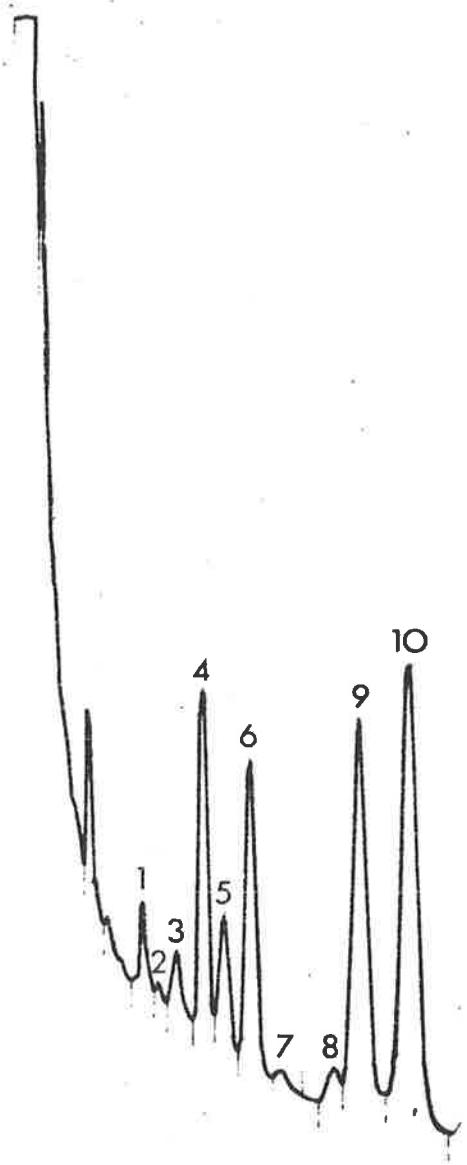

1
2
3
4
5
6
7
8
9
10

FIGURE 16a

Gas Chromatogram of bile acid standards

1. Lithocholic acid
2. Deoxycholic acid
3. Chenodeoxycholic acid
4. Cholic acid
5. I.S. 7-keto-lithocholic acid

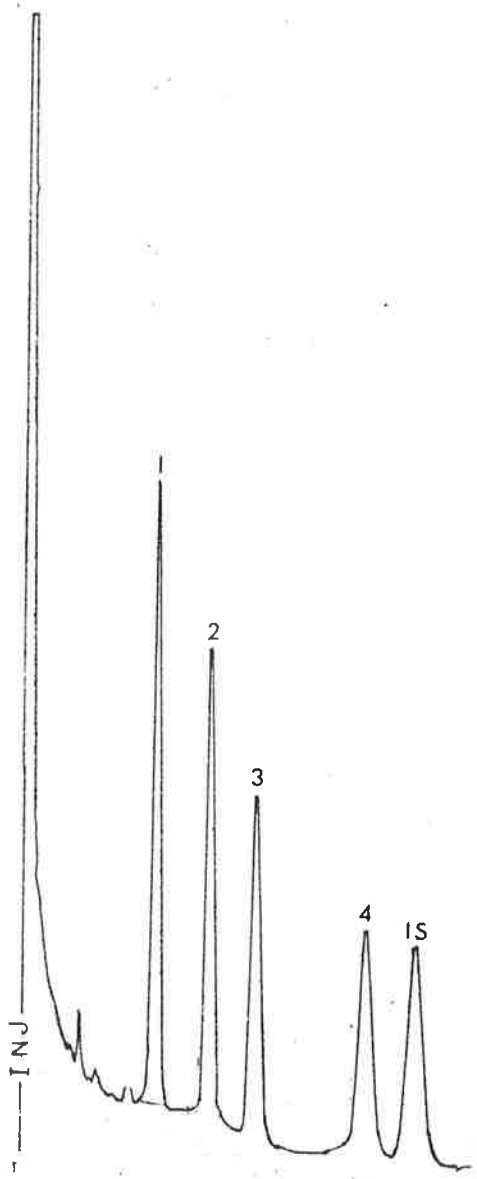
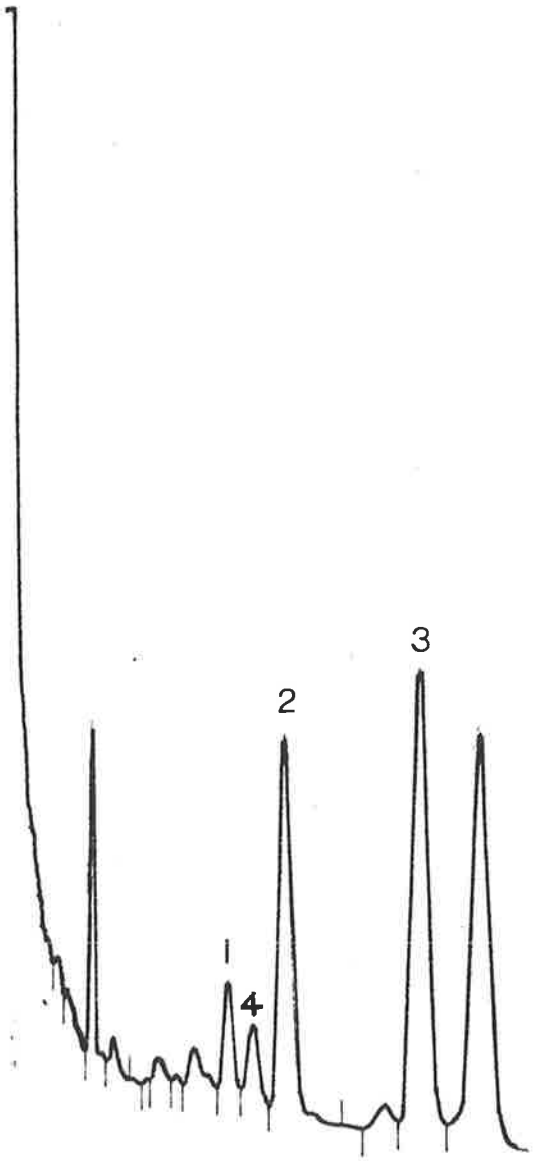

INJ
1
2
3
4
IS

FIGURE 17

Gas Chromatogram used for Mass Spectra

1. Deoxycholic acid
2. Chenodeoxycholic acid
3. Cholic acid
4. Unknown


3
2
1
4

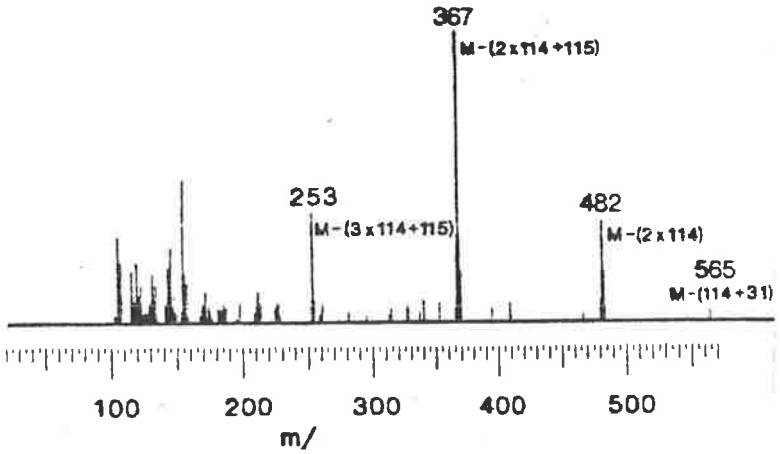
by their relative retention times were also present and these were 3β-OH-5-cholenoic acid (peak 2), ursodeoxycholic acid (peak 7) and hyocholic acid (peak 8). Hyocholic acid was present in most chromatograms of bile and meconium and also amniotic fluid.

Mass Spectra

Figure 17 shows the gas chromatographic elution profile of bile acid methyl ester trifluoroacetates from a sample of meconium. The major peaks corresponded to deoxycholic acid, chenodeoxycholic acid and cholic acid. The identity of these peaks was confirmed by comparing their retention times with authentic standards and by mass spectrometry. The peak with the retention time of cholic acid on mass spectrometry gave a fragmentation pattern shown in Fig. 18. There was a peak at M/Z 367 with other peaks occurring at M/Z482 and 253. The spectrum corresponded well with that of authentic cholic acid. The peak with the retention time of chenodeoxycholic acid gave a peak at M/Z 370 and other peaks at M/Z484 and 255. This mass spectrum corresponded well with authentic chenodeoxycholic acid. The peak with the retention time of deoxycholic acid (Figure 19) gave peaks at M/Z367, 482 and 253 corresponding well with authentic deoxycholic acid. The unknown peak (peak 2) gave a spectrum diagnostic of a dihydroxy bile acid, (see figure 20). No authentic bile acid was available to absolutely identify this bile acid. Other peaks were of insufficient concentration to generate an adequate mass spectrum.

FIGURE 18

Mass spectrum of Cholic Acid


367
M-(2x114+115)
253
M-(3x114+115)
482
M-(2x114)
565
M-(114+31)
100
200
300
400
500
m/

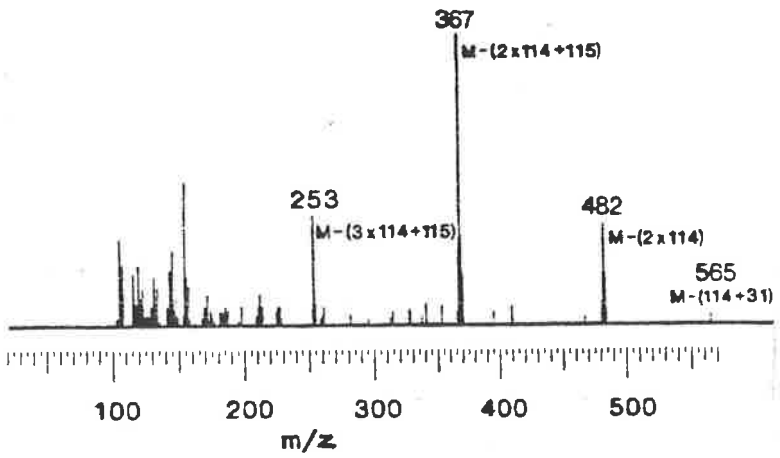

367
M-(2x114+115)
253
M-(3x114+115)
482
M-(2x114)
565
M-(114+31)
100
200
300
400
500
m/z

FIGURE 19

Mass spectrum of Deoxycholic Acid

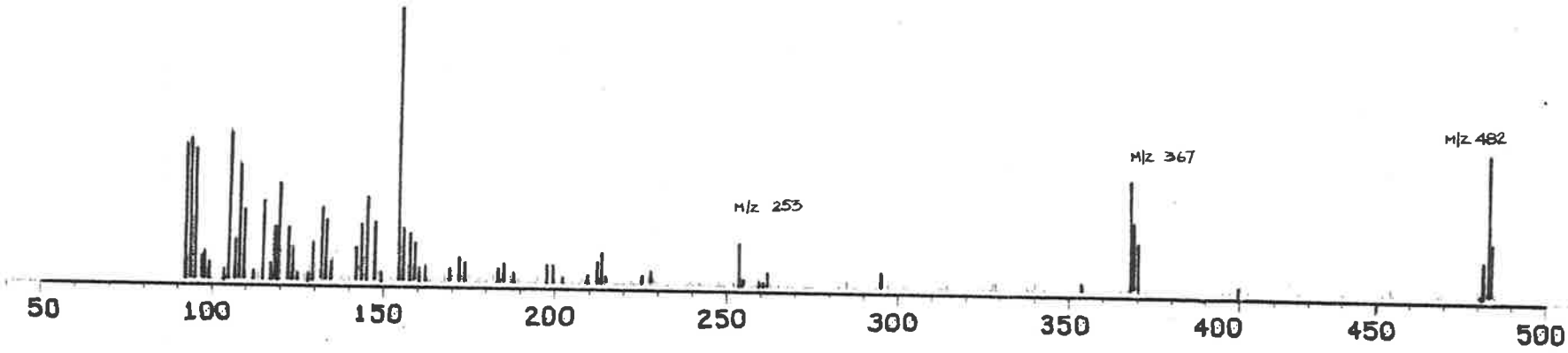
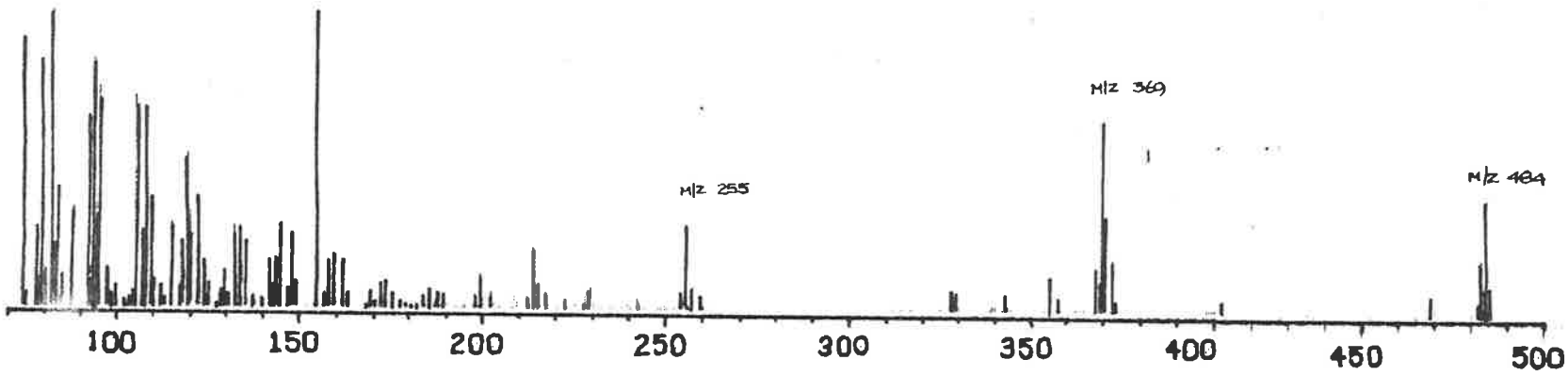

m/z 253
m/z 367
m/z 482
50
100
150
200
250
300
350
400
450
500

FIGURE 20

Mass spectrum of Unknown



255
(M-2TFA + carboxyl side chain)

369
(M-TFA + carboxyl side chain)

484
(M-TFA)

Thin Layer Chromatography - Results

Thin layer chromatography was carried out on selected samples of bile and meconium throughout gestation. Qualitatively (see Figure 21) it is obvious that most of the bile acids were conjugated with taurine throughout gestation. In order to quantitatively assess the relative percentage of bile acids conjugated with taurine throughout the gestation period a more sensitive thin layer chromatographic method was employed (HPTLC) allowing direct measurement using densitometry. (see Figure 22). At all times during gestation > 90% of the major bile acids were conjugated with taurine.

Discussion

The significance of a predominance of taurine conjugation in mammals during the foetal period is not known (Hayes 1976). All animals studied to date exhibit this pattern. The observations made here are in agreement with previous work by Peric-Golia et al.,1968 and Hardy et al., 1980. It has been shown in foetal organ culture that addition of taurine to the medium promotes preferential conjugation of bile acids with taurine (DeBelle et al.,1976). It may be that this conjugation pattern favouring taurine rather than glycine reflects the availability of taurine in utero.

FIGURE 21

Diagram of Qualitative Thin Layer Chromatography (TLC) of foetal bile on silica gel plates with a concentrating zone

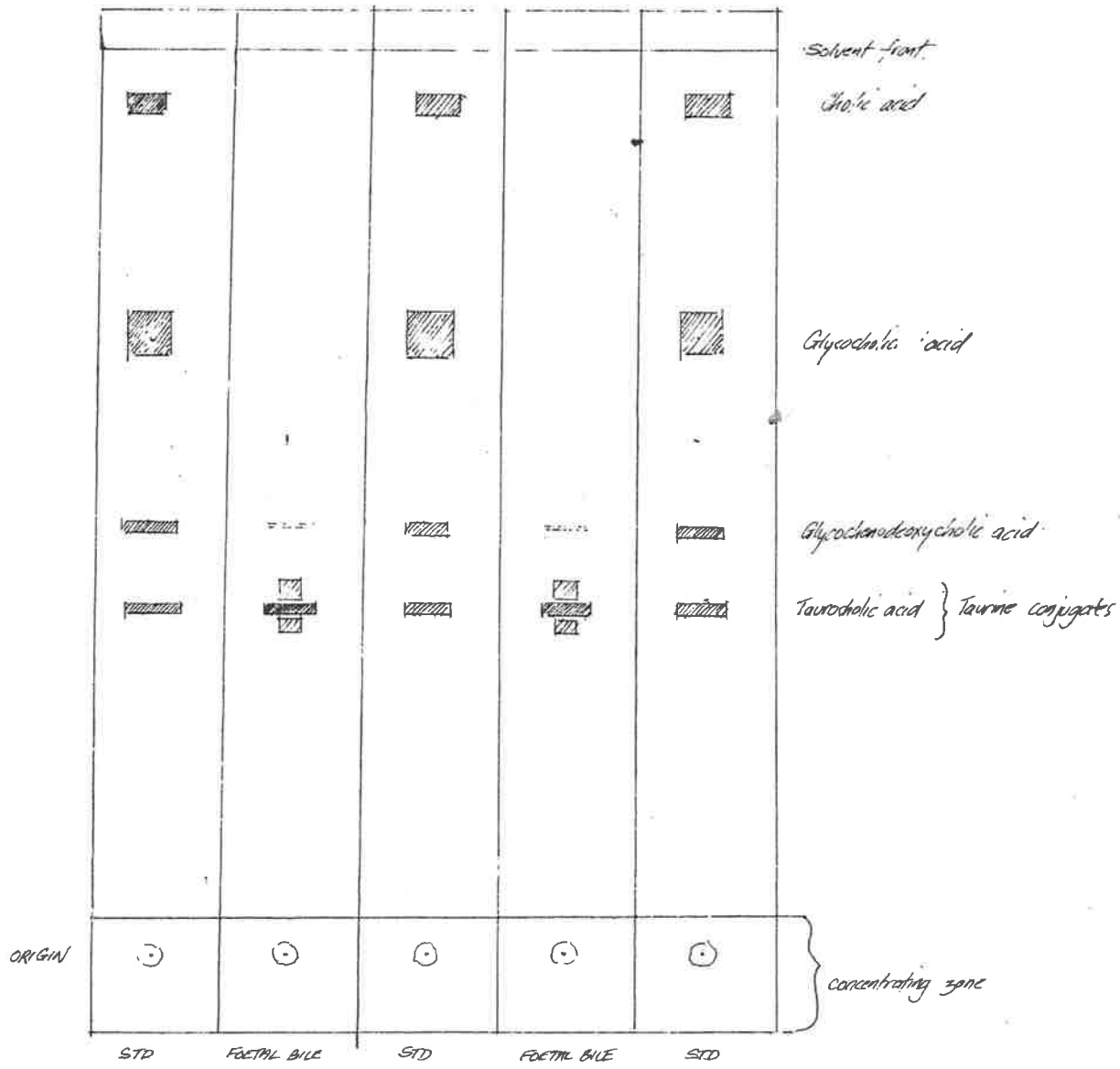
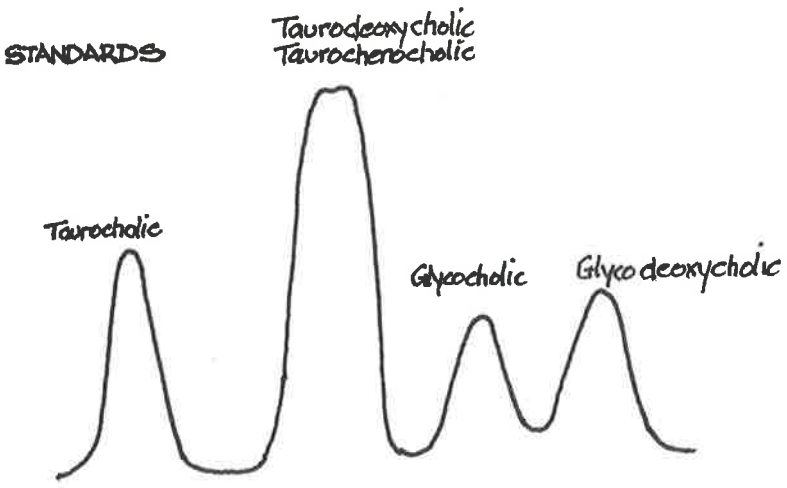
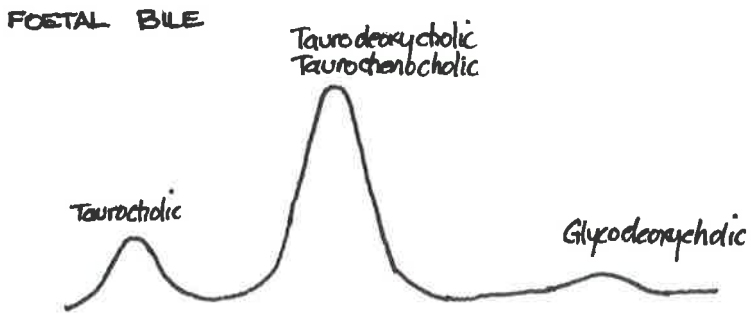

Solvent front
Cholic acid
Glycocholic acid
Glycochenodeoxycholic acid
Taurocholic acid
Taurine conjugates
ORIGIN
concentrating zone
STD
FOETAL BILE
STD
FOETAL BILE
STD

FIGURE 22

Photograph of High Performance Thin Layer Chromatography of foetal bile (for conjugated bile acids) and example of densitometry

FOETAL BILE STD





CHAPTER 4

BILE ACIDS IN AMNIOTIC FLUID

CHAPTER 4.

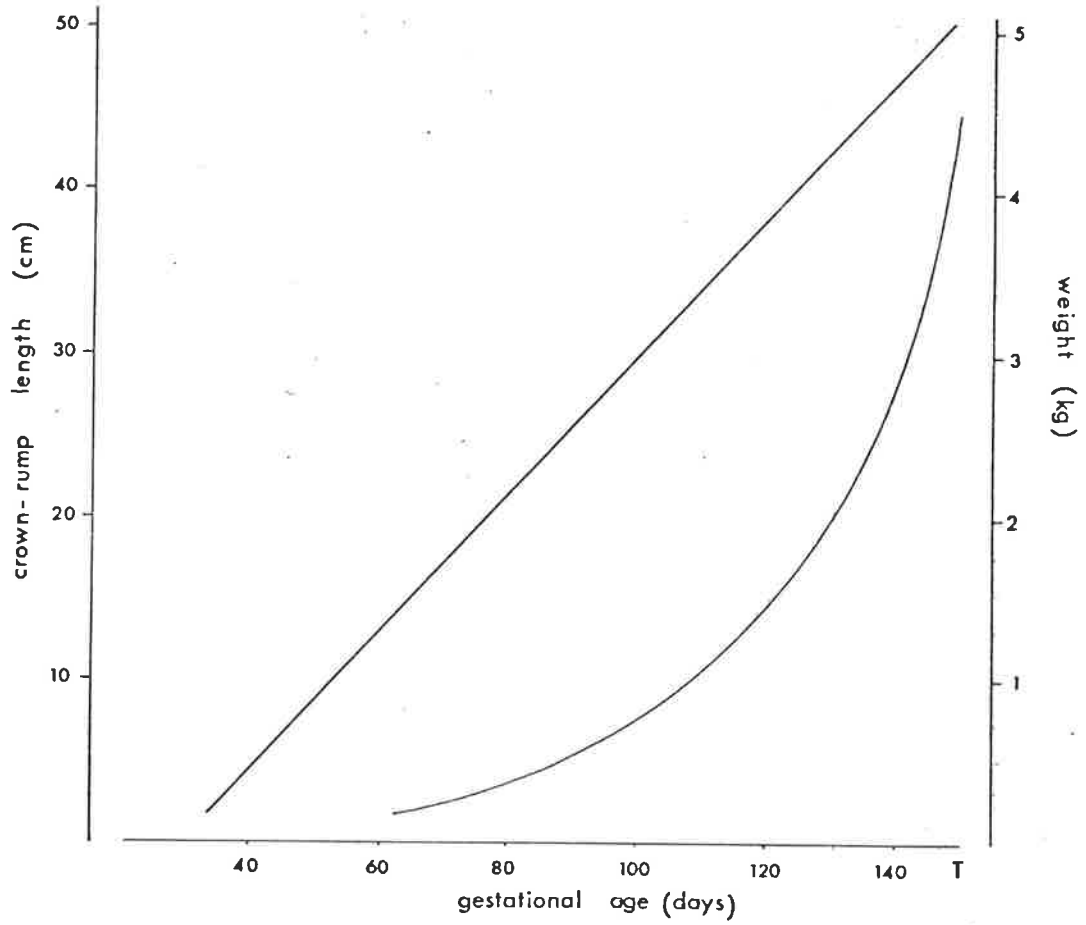
Bile Acids in Amniotic Fluid

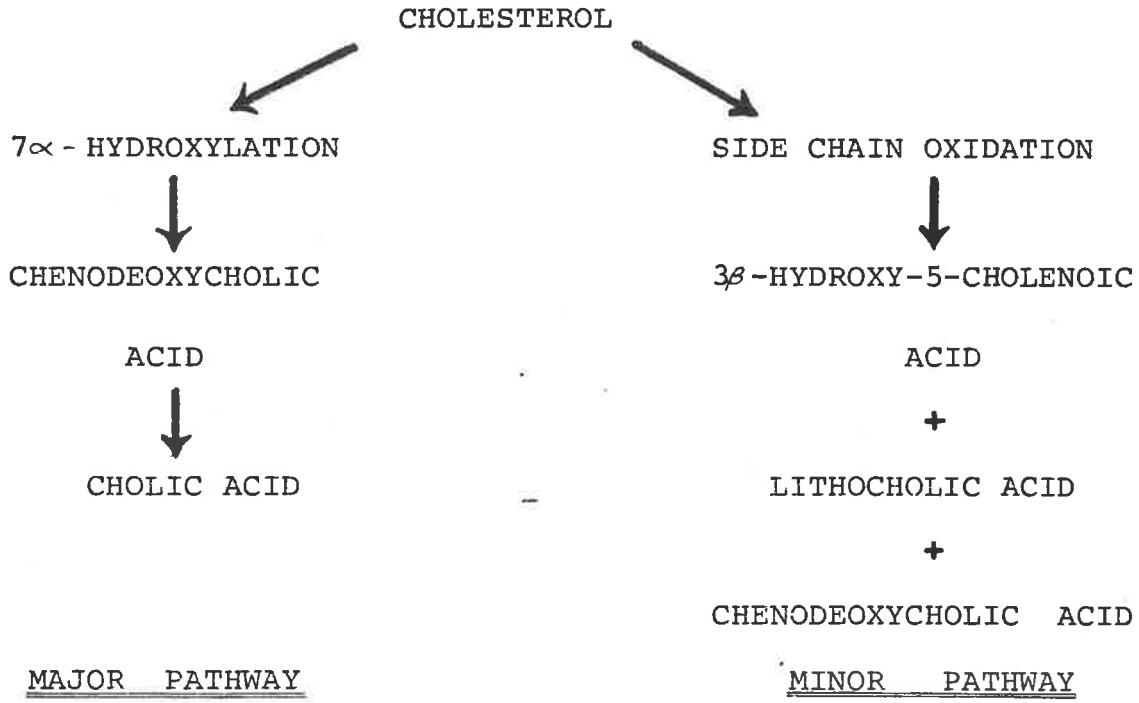
Synthesis of bile acids is a specific function of the liver which develops during foetal life (Daniellson and Rutter, 1968); DeBelle, Brown, Blacklow, Donaldson and Lester 1973; Little, Gavaler, Van Thiel, DeBelle and Lester 1976) and undergoes maturation (in varying degrees depending on the species) during the perinatal period (Little, Smallwood, Lester, Piasecki and Jackson 1975; Watkins, Szczepanik, Gould, Klein and Lester 1975). The first report that bile acids occurred in trace amounts in human amniotic fluid appeared in 1971. More recently it has been demonstrated that they can be measured quantitatively in human fluid (Dēlēze, Sidiropoulos and Paumgartner 1977). The total bile acid concentration during the third trimester of pregnancy ranged between 1.4 to 2.4 μmol/l. This work investigated the presence of a bile acid suggested to be unique to the foetal compartment, 3β-hydroxy-5-cholenoic acid. This bile acid was initially found in considerable quantities in human meconium by Back et al. (1973) and is formed by a pathway that begins with oxidation of the cholesterol side chain rather than 7α-hydroxylation of the cholesterol nucleus.

FIGURE 23

The Relationship of Fetal Crown-rump Length and Weight to Gestational Age in the Ovine Fetus

Fetal crown-rump length (cm) and gestational age (days) show a linear relationship. Fetal weight (kg) and gestational age (days) shows a curvi-linear relationship.


50
40
30
20
10
crown-rump length (cm)
5
4
3
2
1
weight (kg)
40
60
80
100
120
140
T
gestational age (days)



No studies detailing normal bile acid levels in sheep amniotic fluid have been reported. Furthermore bile acid levels in amniotic fluid, if raised, should indicate a degree of foetal distress or intestinal obstruction. Whether this is a sensitive index of foetal distress or an early indicator of stress is discussed.

Materials and Methods

Six normal amniotic fluid samples were obtained either by amniocentesis or at the beginning of an operation (see Chapter 5). Six samples of amniotic fluid were also obtained from abattoir specimens. These were obtained within 10 minutes of slaughter of the ewe. All amniotic fluid was collected from pregnant ewes of $>$ 100 days gestation. Conceptus age was estimated from the graph shown, using foetal weight.

To ascertain whether manipulations at operation and after would sufficiently traumatize the foetus, a pregnant ewe (115 days gestation) was chronically intubated with a Vinyl 4 PVC cannula in the amniotic sac. The foetal femoral vein was exposed as detailed in Chapter 5 and cannulated with a PVC Vinyl 2 catheter. These were both attached to the skin after exteriorization of the venous cannula and maintained in an aseptic condition by placing a three-way stopcock at the end of each cannula and placing this in a pre-sterilized plastic bag. When samples were collected the stopcock port was swabbed with alcohol and after collection a new sterile bag placed over it. Amniotic fluid samples were cultured on blood agar and nutrient agar plates and in thioglycollate medium. These were incubated at 37°C in 95% CO_2 and aerobically for 24 and 48 hours. The thioglycollate was subcultured at 24 hours and incubated in an anaerobic jar. The samples were checked for turbidity by visually comparing with the basal sample, increase in turbidity taken as indicating meconium staining or infection.

Bile acids were measured as previously by gas liquid chromatography on 3% SP-2401 (GaschromQ. 100/120 mesh) : column temperature 250°C, isothermal, 280°C injection temperature, detector temperature 300°C. Carrier gas flow 30 ml/min. Initially nitrogen was used but helium gave much better resolution, particularly between hyocholic acid and cholic acid. Extraction was initially carried out by two methods as detailed in Chapter 5. The simpler method was used throughout as recovery experiments showed

comparable results between the two methods. Even though this method did not clean up the sample quite as well (with respect to the solvent front) it did not interfere with quantitation. Only non-sulphated bile acids were investigated in this study.

RESULTS

The results in table 6 show a range of bile acids in amniotic fluid of 1.18 μmol/l - 2.10 μmol/l. The primary bile acids chenodeoxycholic acid and cholic acid were present in approximately equal amounts on a molar percent basis. Deoxycholic acid was also present in all samples analysed. Peaks corresponding to hyocholic acid were also seen in all normal samples (RRT = 0.81, 7 ketolithocholic acid used as internal standard). In two samples an unidentified peak with a RRT of 0.70 was evident. This peak occurred only in these samples of amniotic fluid and was not found in meconium or bile. A trace of another unidentified peak occurred (RRT = 0.53). This peak was also found in meconium and bile and was shown to be a dihydroxy bile acid.

FIGURE 24

Time course of bile acid concentrations in the amniotic fluid after cannulation of foetal femoral vein and the amniotic sac

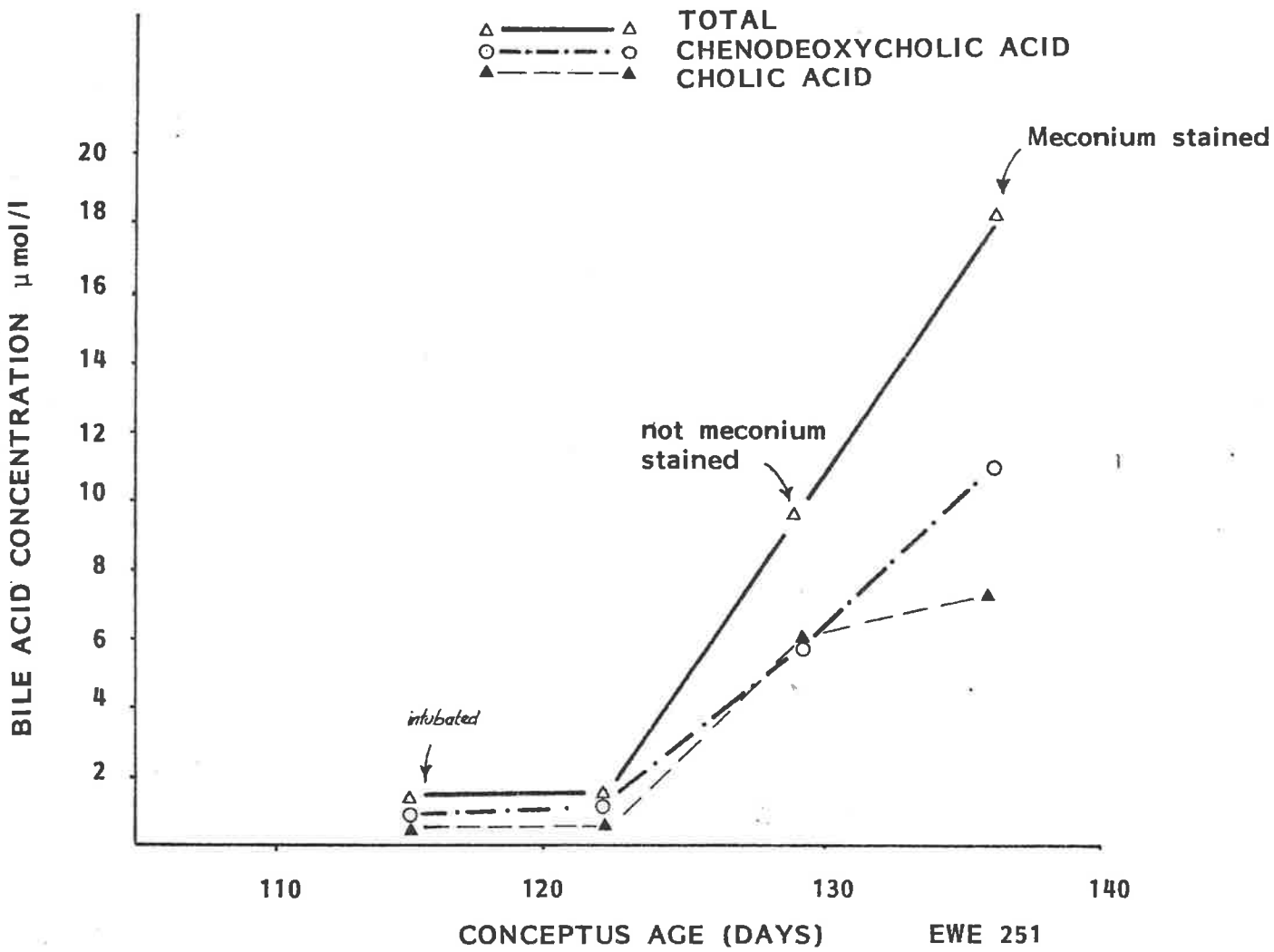

TOTAL
CHENODEOXYCHOLIC ACID
CHOLIC ACID
Meconium stained
not meconium stained
intubated
BILE ACID CONCENTRATION µmol/l
20
18
16
14
12
10
8
6
4
2
110
120
130
140
CONCEPTUS AGE (DAYS)
EWE 251

TABLE 6 Bile acid concentrations and relative molar percentages of the three major bile acids in amniotic fluid of normal pregnant ewes.

		Relative Molar Percent		
Sample	Total Bile Acids (μmol/l)	Deoxycholic Acid	Chenodeoxycholic Acid	Cholic Acid
1	1.4	31	43	26
2	1.59	31	30	39
3	2.1	12	43	39
4	1.99	13	37	51
5	1.21	26	41	33
6	1.18	4	40	56
TOTAL	9.5	117	234	244
MEAN ±SE	1.58 ± 0.16	20± 4.64	39± 2.02	41± 4.55

TABLE 7 Bile acid concentrations and relative molar percentages of the three major bile acids in amniotic fluid from abbatoir specimens.

		Relative Molar Percent		
Sample	Total Bile Acids (μmol/l)	Deoxycholic Acid	Chenodeoxycholic Acid	Cholic Acid
1	13.2	9	44	47
2	28.8	16	22	62
3	20	11	33	56
4	15.6	8	40	52
5	4.2	21	71	8
6	6.9	9	33	58
TOTAL	88.7	74	243	283
MEAN ± SE	14.8 ± 3.66	12.3 ± 2.09	40.5 ± 6.82	47.2 ± 8.11

TABLE 8. Bile acid concentrations in amniotic fluid after cannulation of foetal femoral vein and amniotic sac.

EWE 251.

GESTATION (Days)	115	122	129	136
Chenodeoxycholic Acid (μ mol/l)	0.8	1.0	4.81	11.03
Cholic Acid (μ mol/l)	0.62	0.51	4.96	7.35
TOTAL	1.42	1.51	9.77	18.38

As can be seen from table 7 the total bile acid concentration from amniotic fluid obtained from abattoir specimens was higher by a factor of 9.4. This was so, even though samples 5 and 6 were from foetuses with a conceptus age around 100 days. All other samples were 120 days or more. The relative molar percent of the three major bile acids did not change in the case of chenodeoxycholic acid, rose slightly for cholic acid and dropped by almost 50% in the case of deoxycholic acid.

Intubation of Pregnant Ewe

As can be seen from table 8 and Fig. 24 by the end of week two, the primary bile acid concentration had risen by a factor of 6.5. At this stage the amniotic fluid still appeared clear. However at week 3 the amniotic fluid was turbid to the naked eye, suggesting meconium staining. At no time were any organisms detected in the amniotic fluid samples.

Discussion

In the overall context of investigating the various compartments of the foetal lamb gastrointestinal tract, it was deemed important that a base line for bile acid levels should be established for ovine amniotic fluid. This is the first report of measurement of bile acids in ovine amniotic fluid. The results reported here suggest that trauma of various degrees will cause release of meconium into the amniotic fluid. Indeed in some cases at operation, when cannulae were being inserted into foetal blood vessels meconium was seen to issue from the foetal anus. As the foetus matures the bile acid pool increases and the concentration of bile acids in the large intestine also increases. It would seem then, that as the foetus approaches term, so too would the sensitivity of bile acid measurement in amniotic fluid as an index of foetal dysfunction (this would also depend on the relative increase in the volume of amniotic fluid). Initially it was intended to relate the presence of progesterone metabolities in amniotic fluid to the bile acid concentration and so provide an indication of the source of these metabolites. As no progesterone (pregnanediols) metabolities were found in amniotic fluid this was not useful in this regard. Nonetheless, for future measurement of metabolites in amniotic fluid (particularly where foetal distress is suspected) it could be a useful parameter to measure.

More generally, the preliminary experiment reported here suggests that what was considered to be a relatively minor perturbation of the foetal organism (at least after a reasonable

post-operative recovery period) may well induce more trauma than previously realised. It may be that the particular animal used developed foetal intestinal obstruction, (distal to the opening of the common bile duct) as has been reported by Délèze, Sidiropoulos and Paumgartner (1977) in two human pregnancies. This foetus was delivered vaginally but died soon after birth.

CHAPTER 5

MATERIALS AND METHODS

CHAPTER 5

MATERIALS AND METHODS

MATERIALS

Chemicals

All reagents and solvents were of analytical grade. Solvents were redistilled before use and stored in dark bottles. They were purchased from either Ajax Chemical Limited Australia (UNNAR, ANALAR); Merck (Germany); or May and Baker (England).

Solvents used were :

Diethyl ether	Acetonitrile
Ethyl acetate	Ethanol
Chloroform	Dichloromethane
Acetone	Pyridine
Heptane	
HCl	
H_2SO_4	
KOH	

Enzymes

1. Helix Pomatia (10.5 IU/ml -glucuronidase and 8.06 IU/ml aryl sulphatase) purchased from Calbiochem, Sandiego, U.S.A.
2. Glucuronidase (ex E. coli) 50,000 Fishman units A-grade - activity 1.39 x 10^6 Fishman units/g - purchased from Calbiochem, Australia.

Resin

1) Amberlite XAD2 - obtained from BDH, Poole, England.
2) Amberlite XAD7 - obtained from Chemical Dynamics Corp. South Plainfield N.J. U.S.A.

Thin Layer Chromatography

1) Concentrating plates 20 cm x 20 cm (obtained from Merck)

2) High performance thin layer chromatographic plates (obtained from Merck)

Steroids

Steroids were purchased from Sigma, Calbiochem/Behring or Steraloids. All steroids used were > 99% pure. Steroids used for GLC-MS were all obtained from Steraloids, in the case of bile acids all were unconjugated. Conjugated bile acids were obtained from either Sigma or Calbiochem/Behring.

Progesterone Metabolites

5β-pregnane-3β-20β-diol
5β-pregnane-3α,20β-diol
5β-pregnane-3β-20α-diol
5β-pregnane-3α-20α-diol
5α-pregnane-3α-20α-diol
5α-pregnane-3β-20β-diol
5α-pregnane-3β, 20α-diol

5β-pregnane-3α, 17α, 20α-triol

Bile Acids

Cholic Acid
Chenodeoxycholic Acid
Deoxycholic Acid
Lithocholic Acid
Hyocholic Acid
Hyodeoxycholic Acid
Ursodeoxycholic Acid
5β-cholanic Acid
murocholic Acid
3β-5- cholenoic Acid
Taurocholic Acid
Taurochenodoxycholic Acid
Taurodoexycholic Acid
Taurolithocholic Acid

Reference Steroids

Dehydroepiandrosterone sulphate

Cholesterol sulphate	Glycocholic Acid
Cholesterol glucuronide	Glycochenodeoxycholic Acid
	Glycodeoxycholic Acid
	Glycolithocholic Acid
Radioactive reference steroids	7-ketolithocholic Acid

(6,7- ^{3}H) (estriol-16-β-O-glucuronide)

(6,7- ^{3}H) estrone sulphate

4- ^{14}C-dehydroepiandrosterone sulphate

(obtained from Radiochemical Centre, Amersham, England)

Glassware

Glassware was rinsed in hot water and any residue removed with a bottle brush immediately after use. After hot water acetone was used, then alcohol and finally a distilled water wash. The glassware was then either dried in a hot air oven or left to air dry at room temperature overnight. It was important in all assays to keep glassware scrupulously clean as any left over residues interfered in the chromatographic system. Particular care needed to be taken when assaying amniotic fluid with the same glassware as was used previously for bile or meconium (the levels in meconium being much higher) to avoid cross contamination.

Agents for derivatisation

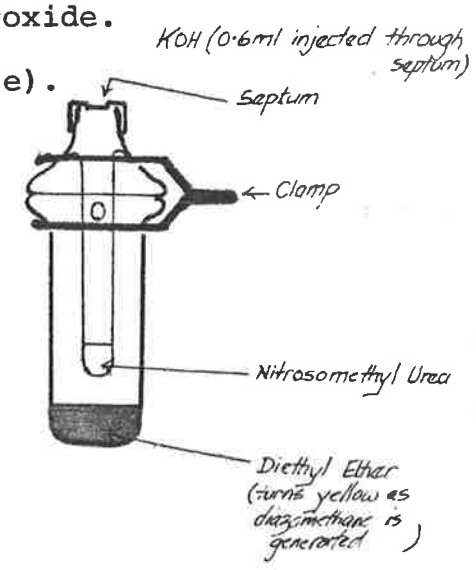
1) BSTFA with 1% TMCS.

   BSTFA - bis (trimethylsilyl) trifluoracetamide

   TMC - trimethylchlorosilane

2) Diazomethane

   Produced using a diazomethane generator (Pierce, U.S.A.) To an amount of nitrosomethylurea (in generator as shown in diagram was added 0.6 ml potassium hydroxide. Carried out at 0°C (i.e. on ice).



3) TFAA -

   - Trifluoracetic anhydride purchased from Sigma.

PROGESTERONE METABOLITES

Assay for Pregnanediols in bile and meconium and amniotic fluid.

The method for assay of pregnanediols in foetal sheep meconium was according to Phillipou et al. (1978) for the analysis of neutral urinary steroids, with modifications. The volume of gall-bladder bile was insufficient for assay (in many cases, particularly in early gestation, gallbladder volume was $\ll 0.5$ ml, in the order of 100 μl). In the case of meconium 1 gm/1 ml, of deionized water sample was homogenised using a ground glass homogenizer. 1 ml of this sample was used for analysis. At the beginning of each analysis 5 - 10 μg of an internal standard 5β-pregnane-3α, 17α, 20α-triol was added. This was used to assess recovery. Solvolysis, hydrolysis and ethyl acetate extraction were carried out as described in the section on extraction of samples. The efficiency of enzymatic hydrolysis and the solvolysis procedure were assessed using 4-^{14}C-DHEA-SO_4 and 4-^{14}C-oestrone-SO_4 giving a recovery of $>90\%$. The assumption that the pregnanediol sulphates were solvolysed in similar quantitative manner was made. The use of Lipidex 5,000 was found not to be necessary when processing bile or meconium as was reported by Phillipou et al.

Isolation

After weighing 1 gm of meconium (wet) and homogenizing this in an equal volume of deionized water the sample was then diluted to 10 ml with deionized water and this was applied to ∿ 1g of XAD2 (wet). This non-ionic resin was prepared by sequentially washing it with litre volumes of ethanol, methanol, acetone and finally deionized water until the acetone could no longer be smelt. Originally an XAD2 column (12 cm x 1 cm) was used, however a batch method of using XAD2 in plastic tubes (capped) was shown to be as reliable and more convenient. After application of the sample to the resin the tubes were rotated at room temperature for 60 minutes to ensure adequate mixing of the resin with the sample. A similar resin XAD7 was also used with similar efficacy. Both these non-ionic resins have been used in extraction of bile acids from serum and urine. After 60 minutes rotation the supernatant was removed with a hooked pipette attached to a suction apparatus (taking care not to suck up resin particles) and the resin washed with 15 ml deionized water. The adsorbed steroids and steroid conjugates were then eluted with methanol (45 ml) and the solvent removed on a rotary evaporator at 40°C. For reuse the resin was regenerated by washing as described previously.

Hydrolysis and Extraction

The dried residue containing the steroids was treated as follows :-

(1) For analysis of free pregnanediols the residue was derivatized as in the next section.

(2) The residue was suspended in acetate buffer (5 ml, 0.2 mol, pH 4.4), the Helix Pomatia extract (0.4 ml) added and the mixture incubated overnight (approx. 16 hr) at 49°C. The mixture was then extracted with ethyl acetate (2 x 15 ml), and the organic extract washed with sodium bicarbonate solution (15 ml 10% aqueous) and dried over anhydrous sodium sulphate. Prior to evaporation of the solvent, n-tetracosane and n-dotriacontane were added as internal standards.

(3) Glucuronidase - The dried residue was suspended in acetate buffer as above and 2,500 Fishman units of glucuronidase (ex E. coli Calbiochem, Australia) added and the mixture incubated overnight (approx. 16 hours) at 49°C (Phillipou, Seamark and Street, 1979).

In addition to enzymatic hydrolysis for both sulphates and glucosiduronates, acid solvolysis was carried out in all samples (specifically for sulphates).

Acid Solvolysis

0.5 ml of bile, 7 ml H_2O, 5.0 g of ammonium sulphate were placed in a ground glass stoppered test tube together with 0.75 ml of 50% H_2SO_4, shaken vigorously and left for 20 minutes at room temperature. This was then extracted with (1 x 25 ml ethylacetate and then 2 x 10 ml of ethyl acetate (if emulsion formed was centrifuged for 10 minutes at 2,000 r.p.m.). This extract was transferred to a stoppered conical flask and incubated at 37°C in a shaking water bath for at least 24 hours (Klopper, 1962). After incubation the extract was washed with 3 x 10 ml NaOH (pH 10), 2 x 10 ml distilled deionized water,

dried over sodium sulphate (anhydrous) and evaporated to dryness using a rotary evaporator. 10μg of n-tetracasane and n-dotriacontane were added to all samples and standards prior to evaporation.

Derivatisation - TMS

After extraction with ethyl acetate and drying of this fraction under a stream of N_2, acetonitrile (100 μl) plus BSTFA with 1% TMCS (100 μl) was added and vortexed. The reaction mixture was incubated at 60°C for 2 hours in a heating block. This was dried under a stream of dry N_2 and reconstituted with 50 μl of petroleum spirits, pyridine, hexamethyldisilizane (HMDS), 98 : 1 : 1. This solvent mixture was chosen because it stabilizes the TMS derivatives.

GLC on Capillary Column (SE30)

A Pye 104A instrument was used. This was fitted with a splitless injection system (SGE, Melbourne, Australia) and a 20 m x 0.5 mm, SE30 glass SCOT column (SGE). Sample injection volume was 1 μl. The injector temperature was 250°C, column temperature 215 - 250°C programmed at 1°C per minute; average linear gas flow (N_2) was 16 - 20 cms/sec. In practice the column showed about 18,000 effective plates. Sample injection volume was always 1 μl.

Identification of pregnanediols

Identification of steroids was achieved by comparison of retention indices, determined for reference steroids. The retention index values were calculated as previously reported (Kovats E. (1965) for urinary steroids (Phillipou 1978) by interpolation between n-tetracosane (2,400 index units), 5β pregnane-3α ,17α , 20α -triol (3 x TMS, 2800 index units)

and n-dotriacontane (3200 index units). The retention indices for the 5β -pregnanediols examined are listed in table 9.

Quantitation of pregnanediols

Peak areas were measured using a Hewlett-Packard 3380A integrator, 5β- pregnane-3α, 17α, 20 α-triol was used as the internal standard. Four standards were chromatographed prior to running samples (1μg, 2μg, 5μg, 7μg,) containing 5 μg of internal standard. The ratio of peak areas was plotted vs. micrograms of pregnanediol and this was linear up to 10μg of pregnanediol. The three pregnanediols gave equal responses. The same concentration of internal standard was added to samples as was added to the standards - thus the sample value could be directly read from the standard curve in μg /ml. (volume of sample used usually 1 ml). Coefficient of variation for the three pregnanediols when added to pooled duodenal contents from one foetus and assayed 10 times was equal to 8, 9 and 12%. Limit of sensitivity of the assay was 200 ng/g wet weight meconium.

Reproducibility of pregnanediol estimation (spiked) from meconium (n = 10).

1. 5β-pregnane-3β-20β-diol μg/g

5.341	5.121	5.201	5.179	4.981
5.636	5.240	5.19	4.910	4.066

$\bar{x}$ 5.09 SD = 0.41 CV = 8.1%.

2. 5β-pregnane-3β-20α-diol μg/g

5.265	5.381	4.09	4.73
5.601	5.120	4.93	5.32
5.06	5.78		

$\bar{x}$ = 5.13 SD = 0.481 CV = 9%.

3. 5β -pregnane 3α -20α -diol

μg/g

5.70	4.12	4.21	5.61
5.55	5.32	4.84	4.36
4.52	4.61		

$\bar{x}$ = 4.88 SD = 0.61

CV = 12%

TABLE 9

Retention Indices for Pregnanediols

RETENTION INDEX				RESPONSE (PD/PT)		
ββ	βα	αα				
2653	2680	2710	2857	1.136	2.071	
2654	2680	2708	2855	1.030	2.200	
2657	2683	2713	2860	0.281	0.669	
2659	2687	2705	2860	0.928	2.894	0.086
2658	2685	2703	2859	0.685	1.636	0.055
2656	2683	2705	2860	0.628	3.906	0.130
2658	2685	2709	2861	0.169	0.421	
2657	2683	27 0	2681	0.144	0.021	
2660	2686	2714	2861	0.317	0.754	
2658	2684	2702	2860	0.683	1.356	0.116
2657	2683	2711	2860	0.107	0.493	
2658	2685	2706	2860	1.712	3.746	0.078
2655	2682	2704	2858	0.196	0.446	0.101
2664	2691	2711	2869	0.850	1.966	0.164
2670	2697	2716	2870	0.665	2.710	0.140

REPEATED SAMPLES.

PD ≡ Pregnane diol

PT ≡ Pregnane triol

RETENTION INDEX				RESPONSE (PD/PT)		
AB	Bα	dα				
2654	2680	2699	2858	0.664	0.664	0.697
2657	2683	2703	2860	0.662	0.651	0.709
2659	2685	2705	2861	0.569	0.578	0.602
2663	2689	2708	2866	0.695	0.711	0.740
2664	2691	2711	2867	0.653	0.654	0.737
2661	2687	2707	2867	0.674	0.711	0.684
2663	2690	2710	2866	0.632	0.637	0.643
2663	2689	2709	2865	0.602	0.595	0.657
2665	2691	2711	2867	0.652	0.641	0.685
2663	2689	2709	2865	0.650	0.640	0.677
2662	2689	2709	2866	0.689	0.676	0.716
2664	2690	2710	2867	0.634	0.622	0.667
2665	2691	2711	2867	0.693	0.678	0.706
2670	2697	2717	2873	0.646	0.648	0.678
2663	2689	2709	2868	0.667	0.667	0.715

STANDARDS

PD ≡ Pregnanediol

PT ≡ Pregnanetriol

BILE ACIDS

Assay for bile acids in bile, meconium and amniotic fluid

Between 20 - 50 μl of bile was used for assaying bile acids. A similar volume was used from meconium after homo-genisation as in the previous section. Either 2 ml or 4 ml of amniotic fluid was used for assay.

Bile and Meconium

Methods for assaying bile acids in bile by gas liquid chromatography have been well documented (Eneroth, Sjövall 1970). Recovery experiments showed 90% recovery of ^{14}C glychocholic acid in both bile and meconium.. Recovery of taurine conjugates in amniotic fluid was slightly lower with a mean of 75.6 ± 2.4 %.

Bile acids were hydrolysed after adding an internal standard (either 7 ketolithocholic acid - 50 nmoles, or 7 ketodeoxycholic acid) to the sample. Hydrolysis was carried out in 2N NaOH in teflon tubes in a pressure cooker at 115 psi and at a temperature of 120°C for 3 hours. After cooling and acidification (with 0.4 ml HCl) the samples were extracted with 2 x 15 ml diethyl ether, dried over anhydrous sodium sulphate and rotary evaporated or evaporated under a stream of N_2.

Derivatization

This was carried out in a 5 ml reactivial (Pierce). Methylation was performed using freshly generated diazomethane at room temperature for 15 minutes. 100 μl of TFAA was then added to the dried, methylated residue and reacted for 30 minutes at 50°C. This was then dried under nitrogen and made up to volume (50μl) with chloroform and 1μl injected on the GLC.

Gas Liquid Chromatography

1 μl was injected into the gas chromatograph (PYE 104 flame ionisation gas-liquid chromatograph). Glass columns (3 m x 2 mm ID) were silanized and packed with 3% SP-2401 on Supelcort 100 - 120 mesh (Supelco inc.) with helium as carrier gas (30 ml/min). The injection temperature was 280°C, the flame ionization detector temperature was 300°C; and the column temperature was 250°C. Under these conditions the retention time for 7-keto-lithocholic acid was around 10 minutes. Peak height ratios of bile acid to internal standard were determined, and samples were quantitated by comparison with standards analyzed simultaneously. Bile acid profiles were calculated for bile with the relative concentrations of deoxycholic acid, cholic acid and chenodeoxycholic acid expressed as relative molar percent.

Meconium

The concentration of bile acids in meconium increased in samples obtained more distal to the foetal stomach. This variability being due to different degrees and differential absorption of bile acids in the small intestine. Because of this and because only small samples were taken of small intestinal meconium (i.e. the whole contents were not weighed and processed for analysis) a meaningful quantitative result was not obtained. Consequently the meconium samples were used qualitatively, comparing the pattern with gallbladder bile (where both samples were obtained from the same foetus). Analysis of the mass spectra

of some of the peaks that appeared in the gas chromatograms was carried out using a 3% SP-2401 column of 2 metres in length and internal diameter of 2 mm. The mass spectrometer used was a Hewlett Packard

The method used for analysis of bile acids in all samples detected only non-sulphated molecules

Amniotic fluid

Duplicate aliquots of 2 ml amniotic fluid along with 10 nmoles of 7-keto lithocholic acid as internal standard were extracted with 1 g of the nonionic resin, Amberlite XAD-7 according to the batch procedure of Barnes and Chitranukroh. The methanol in the eluate from the resin was removed in vacuo using a rotary evaporator, and the bile acids were suspended in 2 ml of 2 N NaOH and transferred to teflon tubes for chemical hydrolysis at 120°C for 3 hours in and autoclave. Upon cooling, the solution was acidified to pH1 with concentrated HCl and the free bile acids were vigorously extracted with two aliquots of 5 ml of diethyl ether. The combined extracts were dried down under a stream of N_2 and dissolved in 0.5 ml of methanol : water (7:3 vol/vol) and extracted twice with 1 ml of n-heptane. The aqueous methanol was then transferred to small vials and evaporated to dryness under N_2 at 50°C before derivatization (methylation and trifluoroacetylation, Ross et al 1977). It was found that omission of XAD-7 extraction and heptane extraction produced in most cases just as reliable recoveries. This simplified

extraction procedure, essentially the same as for bile acids in bile was subsequently used.

Validation of Bile Acid Assay in Amniotic Fluid

For free bile acids recovery relative to internal standard was complete. For the conjugated bile acid, mean recovery was only 80%. The precision of the assay was investigated by replicate analysis (N = 5) of pooled amniotic fluid and the mean coefficient of variation for the three major bile acids was 5.6%.

Thin Layer Chromatography (TLC)

Normal thin layer chromatography was carried out on selected samples to obtain a qualitative assessment of the mode of conjugating of the bile acids throughout gestation. To obtain sharper bands (i.e. better resolution) 20 cm x 20 cm silica gel G plates with concentrating zones were used (Merck).

The solvent system employed was iso-amylacetate : propionic acid : n-propanol : water (4 : 3 : 2 : 1 V/V) as described by Hofmann in 1962.

High performance Thin Layer Chromatography (HPTLC)

To determine the relative proportion of taurine to glycine conjugates high performance thin layer chromatography was employed using the method of Shepherd, Bunting, Khan, Hill, Soldin and Gall 1978. The solvent system used was a modification of the system used by Shepherd et al for conjugated bile acids :

n-Butanol : glacial acetic acid : water

(70 : 10 : 20) Time approximately 120 min for a run.

Colour development was achieved after air drying by spraying the plates evenly with a 1 : 1 mixture of 10% phosphomolybdate and 10% ceric ammonium sulphate, air drying, and oven heating the plates at 100°C for 5 minutes.

Quantitation was carried out using a filter of wavelength 620 nm using a 0.4 x 5 mm slit and the density of each bile acid recorded on a chart recorder in relation to a known standard. The concentration was calculated by simple ratio.

SURGERY

1) Animals

Merino crossbred ewes of known mating date were obtained from the University of Adelaide's Experimental Farm at Mintaro, South Australia. The pregnant ewes were transported to The Animal House at The Queen Elizabeth Hospital one week before surgery. The ewes were housed under conditions of 12 hour light and 12 hour dark at constant temperature (25°C) and maintained on 800 gm of lucerne chaff daily with water ad libitum.

2) Preparation of the ewe

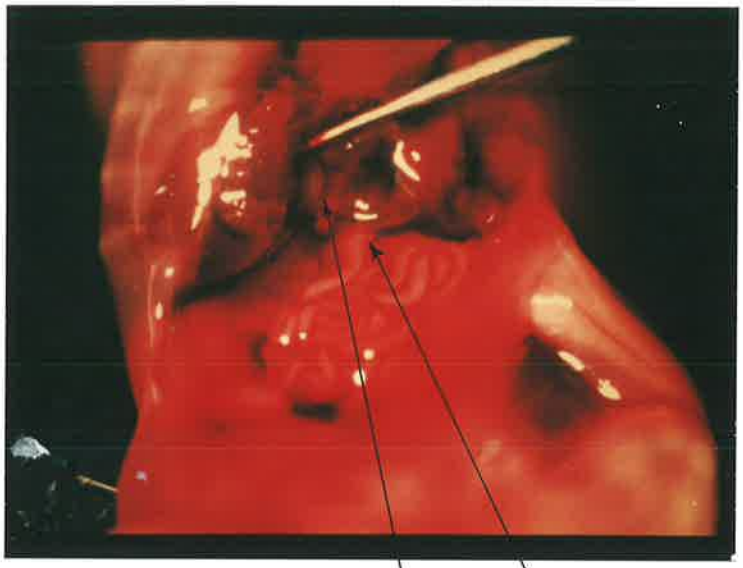
Fasted ewes 90 - 120 days pregnant were sedated with Rompun (0.4 - 0.8 ml, 0.008 ml/kg) injected intramuscularly. The wool covering an area over the 3rd and 4th sacral vertebra was closely clipped and then swabbed with chlorhexidine/cetrimide solution and lumbosacral anaesthesia was then induced with 10 ml lignocaine (2% xylocaine) using a technique similar to that described by Hopcroft (1967).

As soon as the ewes lost sensory response in the hind legs all the wool covering the abdomen and area around the groin was clipped with both coarse and very fine clippers. The clipped area was then thoroughly cleaned with an antiseptic detergent solution (Sapoderm), dried with a clean towel and then soaked with alcoholic chlorhexidine/cetrimide solution. The ewe was then placed on its back on a curved slatted wooden stretcher. Once secured on the operating table the abdomen was again cleaned with chlorhexidine/cetrimide solution.

FIGURE 25

Photograph of foetal common bile duct

50 days



Gallbladder

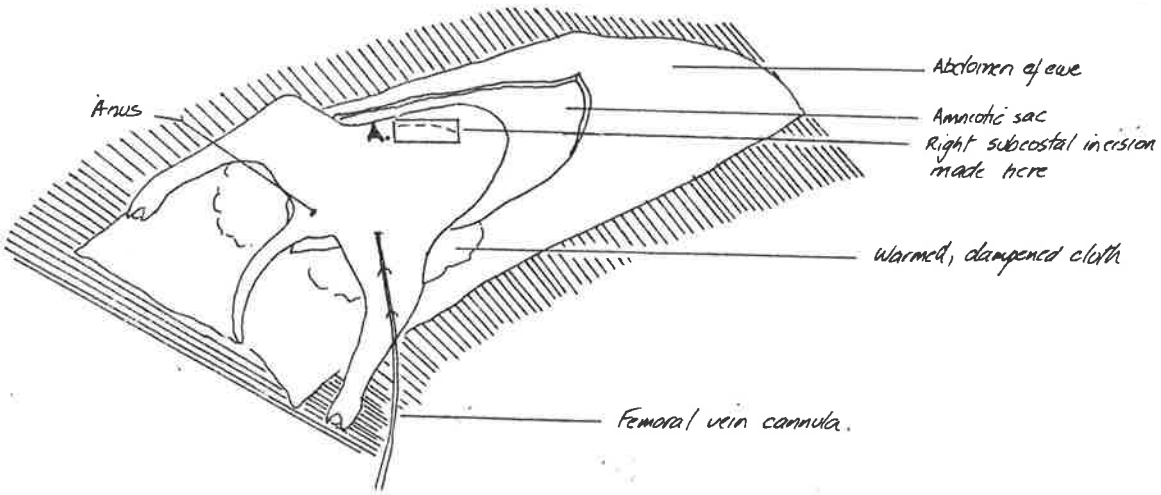
Severed Common Bile Duct. ~0.5cm

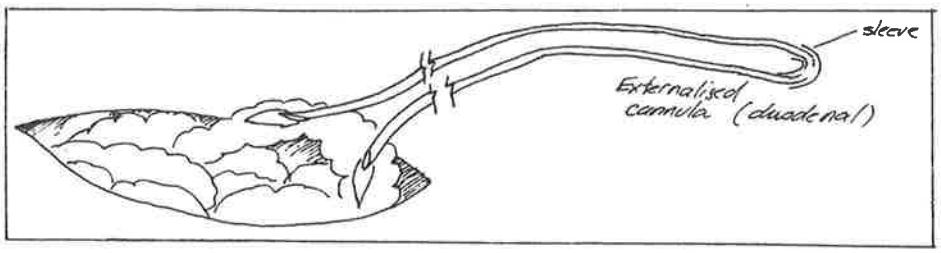
3) Cannulation

A number of experiments were attempted where either the foetal duodenum was cannulated (just distal to the ampulla of vater) or the foetal gall bladder cannulated. Most surgery was attempted at 100 - 120 days gestation. In only 2 cases was a successful cannulation maintained for >5 days and one of these died on day 7, the other went to term. Both of these were foetal duodenum cannulations (with foetal femoral vein cannulation). The foetal liver is tremendously fragile and friable at 100 - 120 days gestation and tends to haemorrhage very easily. In almost all attempts to cannulate the foetal common bile duct the foetus was lost due to inordinate haemorrhage of the liver. This was caused in the main by the inaccessibility of this structure in the foetal lamb. It is very short and lies right under the liver, necessitating reflection of this organ for access. Its length i.e. from gallbladder to duodenum is of the order (at 100 - 120 days gestation) of 2 - 3 cms and much shorter earlier (see Fig. 25). To cannulate any of the above structures the foetus was partially removed as per figure 26 and swathed with dampened cloths. After cannulation of the femoral vein a right subcostal incision was made, if possible just below the lower margin of the right lobe of the the liver. The foetus was tilted back to partially reflect the liver and expose the duodenum and gall bladder. In practice this was very difficult. In experiments to cannulate the duodenum some intestine was allowed to emerge from the

FIGURE 26

Diagrammatic representation of foetal vein and duodenal cannulations


Anus
A.
Abdomen of ewe
Amniotic sac
Right subcostal incision made here
Warmed, dampened cloth
Femoral vein cannula.


sleeve
Externalised cannula (duodenal)

DETAIL A.

incision and the duodenum was then cannulated as per figure 26.

Strict aseptic techniques were maintained during all procedures. Sterile gloves and gowns were worn with hats and masks, all instruments were steam sterilized immediately prior to commencing the operation. All cannulae were sterilized (in plastic bags) with ethylene oxide and prepared prior to commencing surgery. Preparation involved placing disposable three way stop cocks on square tipped needles inserted into catheters. The tip of the catheter was cut at 45° for easier insertion into vessels or intestine. The catheters were filled with sterile normal saline. Sterile drapes were placed over the ewe leaving only a small area around the site of incision. The ewes head was also draped. Laparotomy was then performed with an infraumbilicular longitudinal incision (11 - 12 cms) approx. 3 cms lateral to the midline. The foetus was palpated and the hind legs grasped and placed in apposition with the least vascular area of the uterus (intercotyledonary area). A small incision was made in the uterus, clamped, and enlarged until the foetal hind quarters could be removed as in figure 26. Operating time was usually 60 - 80 minutes.

CHAPTER 6

GENERAL DISCUSSION

CONCLUSIONS

CHAPTER 6

General Discussion

To establish a picture of the course of events, both endocrine (progesterone metabolites) and digestive, (bile acids) that occur in the biliary compartment in the foetal lamb it is convenient to divide the gestation period into two phases :

1. The period prior to 120 days gestation and
2. that from 120 days until birth.

The changes that take place during the first 120 days gestation tend to be gradual (Cox, 1975). The conceptus begins to synthesize and in many cases secrete various hormones into the maternal system. Some hormones such as cortisol, thyroid hormones and higher molecular weight compounds have little or no transplacental passage. In the maternal circulation progesterone rises only slightly from levels of about 2 ng/ml during the luteal phase to about 3 ng/ml by day 70; during this time progesterone concentration is related to the number of corpora lutea present (Bassett et al., 1969; Bassett and Thorburn, 1973). Progesterone concentrations rise to a plateau of around 12 ng/ml at 120 days gestation. In the foetus progesterone concentrations are low, about 0.5 ng/ml at 90 days and 1.5 ng/ml by day 120; they are difficult to assess owing to the very rapid metabolism of this steroid by foetal red blood cells (Nancarrow and Seamark, 1968; Findlay and Seamark, 1969). This is not the case in the human foetus. Seamark in 1973 showed the principle metabolite (after red blood cell

transformation to 20α and 20β -dihydroprogesterone) in foetal and placental tissues was 5β-pregnane-3α-20α-diol, reaching concentrations of 0.5 µg/ml by day 120.

The data presented in this thesis suggest that biliary excretion of pregnanediol metabolites does not play a major role in the overall progesterone metabolism of the foetal lamb. It is only in the perinatal period just prior to parturition that any appreciable concentrations (> 200 ng/ g of pregnanediols [glucosiduronates]) are found in the foetal small intestinal contents. During this period many endocrine changes occur, some of great rapidity and mostly they are derived from the foetus. Liggins et al. in 1966 showed the clear involvement of the foetal hypophysialadrenal axis in the initiation of parturition in the sheep. Dolling in 1979 showed that the level of circulating pregnanediol sulphates was around 1 µg/ml at 110 days of gestation and increased slowly during the next 25 days to a level of ∿2 µg/ml at 135 days gestation, finally increasing to ∿ 4 µg/ml at 142 days gestation. Thereafter the levels dropped toward 1 µg/ml 3 - 4 days prior to term.

An increase of pregnanediols in plasma indicates either a decrease in progesterone clearance or an increase in production. Progesterone production by the pregnant ewe has been well investigated. Thompson and Wagner in 1974 showed the importance of the ovary and placenta in progesterone production and to a lesser extent the adrenal in late gestation. Total progesterone production in the ewe reaches

a maximum at about 135 days gestation (Bedford et al., 1972a) after which it declines slowly until about 5 days prior to term. Even though total production decreases, placental production rises, increasing by 10% between 120 - 140 days of gestation reaching a maximum at 5 days prior to term (Mattner and Thorburn 1971). This progesterone production more correctly reflects the sum of both placental and foetal contributions. It is not known what proportion of placental progesterone production enters the foetus but the studies of Stupnicki and Stupnicki in 1970 showing that clearance is $\sim$5 times slower than the mothers suggest that in the foetus, only a small portion is placental derived. Linzell and Heap in 1968 showed that the umbilical arterial progesterone levels were higher than umbilical vein levels, suggesting a net flow from the foetus to the placenta. These foetuses were probably stressed (Dolling 1979) suggesting the adrenal gland to be the source of progesterone. Certainly the foetal adrenal shows an abrupt increase in weight (Comline and Silver, 1961; Liggins, 1969a) after day 135 at a time when the foetal pregnanediol levels increase sharply (Dolling and Seamark 1979). At about day 140 - 142 foetal cortisol levels begin to rise with a concomitant decrease in circulating pregnanediol levels and also a drop in progesterone levels in plasma (Figures 27 and 28).

Although no pregnanediols were measured in samples from foetuses > 135 days gestation the finding of pregnanediols in small intestinal meconium of three term foetuses suggests that from day 135 → parturition the role of the biliary pathway

FIGURE 27

Levels of pregnanediol, (5β-pregnane-3α,20α-diol) progesterone and cortisol in foetal plasma in serial samples collected near term (arrow represents time of birth). The samples were collected from a cannula implanted in the femoral artery of the foetus and were analysed by GC-MS using multiple ion monitoring (unpublished data of Seamark).

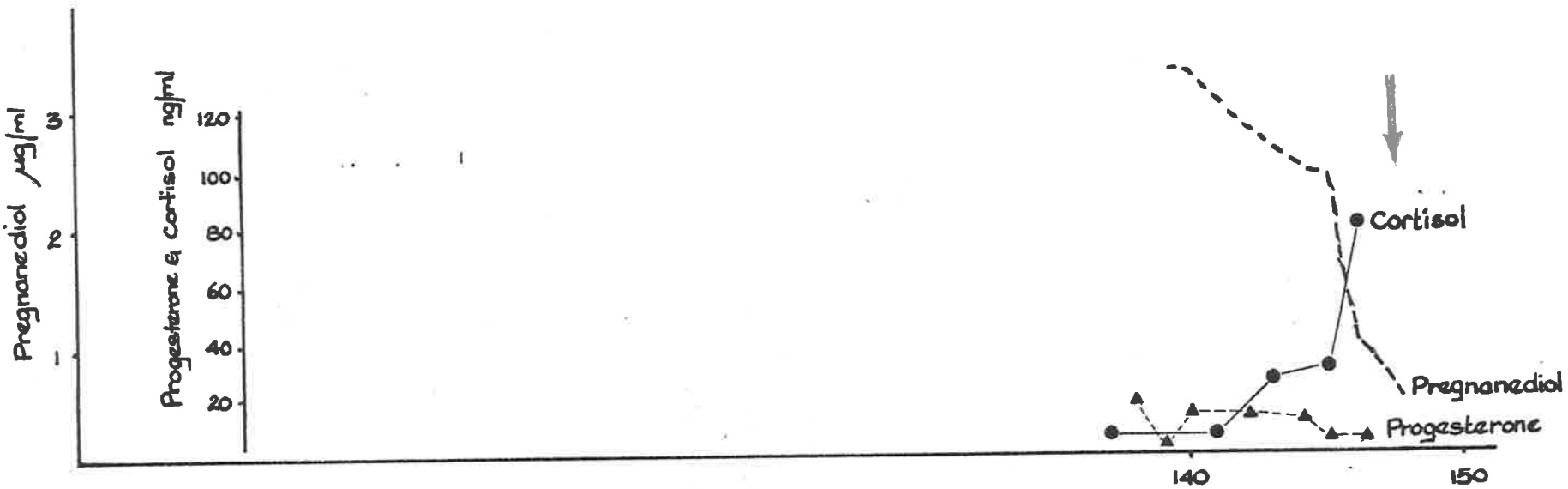
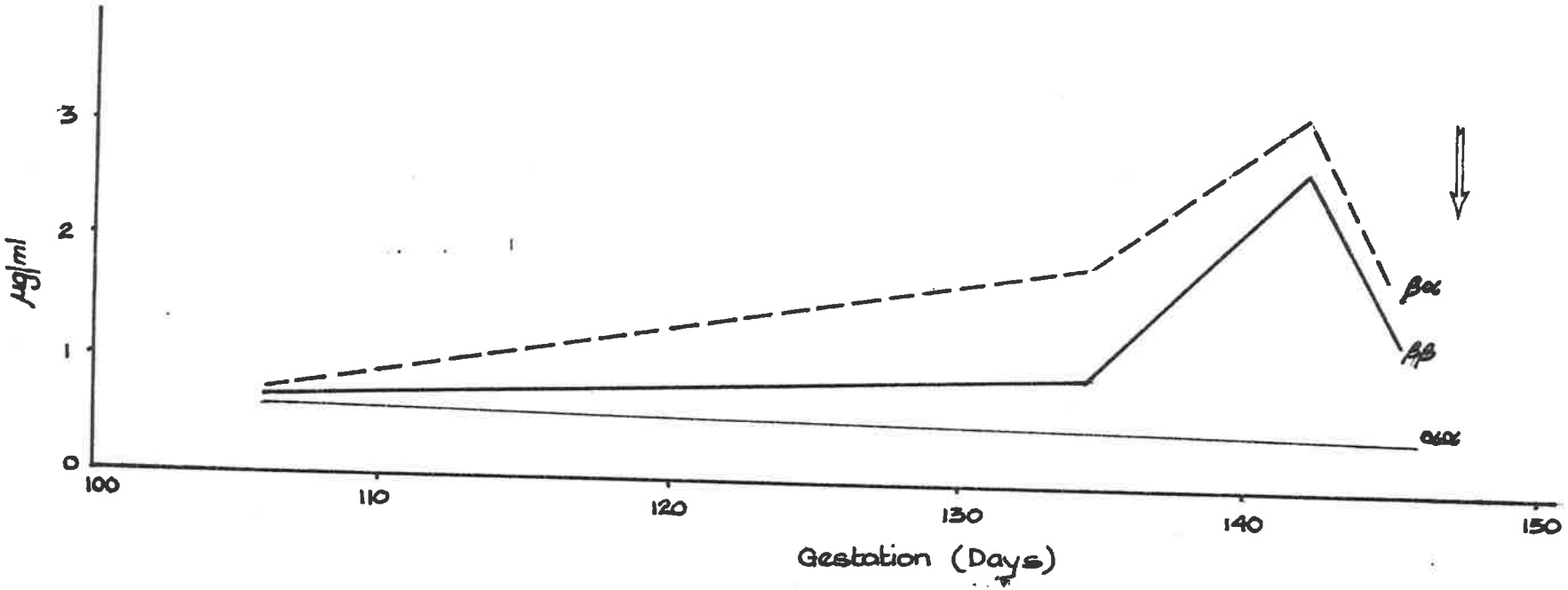

Pregnanediol µg/ml
3
2
1
Progesterone & cortisol ng/ml
120
100
80
60
40
20
Cortisol
Pregnanediol
Progesterone
140
150

FIGURE 28

Changes in foetal plasma pregnanediol levels in sham operated foetuses (from Dolling, 1977)

Chapter 6.



increases. The extent and significance of this in relation to the marked decline in foetal plasma pregnanediol sulphates is not known. However general maturation of foetal sheep liver, with an increase in UDP glucuronyl transferase activity, may enable a shift in the relative proportion of conjugation of the pregnanediols from sulphates to glucuronides. This could result in preferential biliary secretion of pregnanediol glucuronides due to increases in molecular weight and changes in polarity. The fact that pregnanediol sulphates were not found at levels > 200 ng / g in foetal meconium does not preclude the possibility that lower levels of this conjugate may be secreted. Reabsorption of pregnanediol sulphates may occur and analysis of bile samples (provided sufficient volume is obtained) should resolve this question. The work of Dolling and Seamark in 1979 showed that pregnanediol sulphates in nephrectomised and infected animals increased up to 10 times the levels found in sham operated foetuses. If the biliary pathway were able to handle these molecules it might be expected the increase in pregnanediol sulphates in plasma would not be so great. The type of sulphation was not determined in these studies i.e. whether mono or disulphates predominated.

No pregnanediol sulphates have been found in amniotic fluid of pregnant sheep (unpublished observations, Greenway, Seamark & Phillipou) and this was confirmed throughout gestation in this study. This is not surprising because these metabolites were not detected in foetal meconium and the foetuses used in this study were obtained from the abbatoirs and consequently would have been traumatised to

various degrees. This trauma would result in foetal intestinal contents entering the amniotic fluid (see Chapter 4).

The rise in pregnanediol sulphates in foetal plasma begins around day 135 in foetal lambs (see Figure 28). This is assumed to be related to increased foetal adrenal production of progesterone as this gland matures. An increase in bile acid levels in gallbladder bile is also evident between day 135 and term and this most likely reflects maturation of the liver and biliary system. A preliminary experiment reported in this thesis indicates that the foetal sheep liver is synthesising primary bile acids at around 120 days gestation. Although the model used in this experiment was not as rigorous as that used by Hardy et al., (1980) the estimate of a synthetic rate of 0.12 μmol/kg/hr is not necessarily inconsistent with the term estimate of 0.35 μmol/kg/hr reported by Hardy et al. The total maternal to foetal bile acid transfer has been estimated to be 0.004 μmol/kg/hr in the foetal sheep (Sewell, Smallwood, Hardy and Hoffman, 1977).

Early Foetal Development

It was noted that bile acids were present in the foetal sheep gallbladder as early as 40 days gestation. The earliest reported finding of bile acids in the biliary system of the human foetal gallbladder is 14 - 16 weeks gestation (Bongiovani, 1965). Presence of bile acids in intestinal contents of the foetal sheep soon after 40 days gestation indicates that secretion of bile acids occurs quite early in gestation; whether synthesis of primary bile

acids occurs at this time is not known. At this time and indeed throughout gestation the bulk of intestinal contents increased towards the distal intestine. In the rat foetus, bile acids were found as early as 5 days (Gestation period = 22 days) using radioimmunoassay (Little et al.,1979). This predates formation of the foetal liver in this species, however the situation in the sheep foetus at a comparable stage of gestation is quite different (as with other large mammals). Because the greater part of the bile acid pool at 19 days gestation is found in the liver of the foetal rat and soon after birth this is reversed, Little et al., suggested that secretion begins after synthesis and remains a persistently, if progressively less, immature function through to parturition. Smallwood has shown that in both the foetal dog and sheep the bile acid excretory pathway is remarkably mature at birth, more so than in the primate. The results presented in this thesis suggest that this higher degree of maturity is reflected by the early presence of bile acids in the sheep foetal gallbladder and meconium indicating secretory function and the relatively early acquisition of bile acid synthetic capacity by the foetal sheep liver (Smallwood, 1973). Whether secretion begins after the commencement of synthetic activity awaits further studies of the compartmentalization of the bile acid pool in early gestation.

Perinatal Development

The data in this thesis showing the large increase in the relative amounts of primary bile acids to a secondary bile acid at term, are in agreement with the studies of Hardy et al., 1980. This rise in foetal sheep coincides with the rapidly increasing circulating cortisol levels in foetal lambs at this time. Cortisol has been shown to be involved in general maturation of liver function in vitro (DeBelle et al., 1976). The large increase in bile acid concentration in the gallbladder could also in part be due to the increasing ability of the gallbladder to concentrate its contents.

The adult pattern of the major bile acids presented a markedly different pattern from that of foetal bile acids. In adult sheep, deoxycholic acid constituted 54.4 $\pm$ 6.17%, chenodeoxycholic acid 6.6 $\pm$ 1.66% and cholic acid 39 $\pm$ 6.7% compared with the foetal pattern where deoxycholic acid represented only 15.3 $\pm$ 3.4% chenodeoxycholic acid 43.3 $\pm$ 2.16% and cholic acid 41.25 $\pm$ 3.9%. These results essentially agree with those of Hardy et al., (1980) in that foetal bile showed a much greater proportion of chenodeoxycholic acid than adult bile. It is interesting to note that the relative molar percent of deoxycholic acid in the 3 term samples was of a similar magnitude to that shown by Hardy et al., (1980) in neonates.

The pattern of non-sulphated bile acids was the same in bile and meconium. A number of minor

peaks, some tentatively identified as particular bile acids were noted in certain samples. The three major bile acids were absolutely identified using GLC-MS. In addition a fourth peak was shown to be a dihydroxy bile acid - however no authentic standard was available to absolutely identify it. Four other peaks occurred, one unknown, one corresponding to hyocholic acid (this was seen in most samples), ursodeoxycholic acid and a trace in 3 instances of 3β(OH)-5-cholenoic acid. It will require a specific search for sulphated bile acids to confirm the presence of 3β(OH)-5-cholenoic acid. Watkins et al. in 1977 also reported several minor peaks in foetal meconium which they did not conclusively identify as this bile acid.

Over 90% of the bile acids (non-sulphated) were conjugated with taurine throughout gestation. This appears to occur in all species studied. The significance of this preferential conjugation is not known. The best known function attributed to taurine to date has been its conjugation with bile acids (Hayes, 1976). The foetal supply of taurine is ample and it has been shown that availability of taurine results in preferential conjugation with this amino acid. The other conjugating amino acid glycine may be required for protein synthesis as taurine is a non-protein amino acid. Recent work in rats (Yousef, Tuchweber, Vonk, Massē, Audet and Roy 1981) has shown that although lithocholic acid, glycolithocholic acid and the sulphates of these are cholestatic agents, the tauro conjugate is not. Lester (1980) suggests that the perinatal liver is normally subject to cholestasis and that cholestasis per se may be a governing factor in the control of pathways of bile acid synthesis. If

the work of Yousef et al. is confirmed in other species, the predominance of taurine conjugates of foetal bile acids may be a protective mechanism, effectively minimising the apparent foetal cholestasis.

Future work

Progesterone metabolism

It is obvious from the foregoing that the physiological role of steroid hormone metabolism in the foetal intestinal tract is largely unknown. The contribution of biliary secretion of progesterone metabolites in foetal steroid hormone metabolism just prior to term warrants further investigation. The great species differences that have been found for both the extent of biliary and faecal excretion of progesterone and oestrogen metabolites and the nature of the metabolites found should be emphasized. This fact together with the paucity of information on adult sheep biliary secretion of progesterone metabolites and its significance, makes studies in the foetus of this species more difficult, both in terms of delineating the most profitable area of investigation and in interpreting results. Accordingly an examination of both adult and neonatal biliary excretion of progesterone metabolites would clarify the possible contribution of this excretory pathway in the total development of the sheep.

It is believed that the maximal velocity of biliary elimination of organic anions is restricted by the capacity of the bile secretory process (Erlinger and Dhumeaux, 1974) in the adult mammal. It is not known whether this is the case

during foetal development (although results reported in this thesis would suggest that the secretory process has limited capacity until close to term). Studies of excretion of both progesterone and oestrogen metabolites via the biliary pathway would be of value in this regard, particularly in the latter one third of gestation up until term and through the early neonatal period. Much of the contribution of the bile acids in this period and in early life have been studied (Smallwood et al; Lester et al; Hardy et al), however no correlations have been made between the steroid hormones and the bile acids.

Furthermore it has been reported that oestrogen and its metabolites exert a cholestatic effect on the adult liver. In certain women (particularly Chileans and Scandinavians) so called benign cholestasis of pregnancy occurs (Back, Sjovall, Sjovall 1974; Fisher, Magmusson and Miyai 1971). The effect on the foetus of liver dysfunction in the mother is not known (Reid, Ivey, Rencouret and Storey 1976), however it is important that more studies in this area are initiated. One study in rats (Hassan and Subbiah, 1980) has shown a paradoxical and significant decrease in the bile acid content in the foetus of rats with a ligated bile duct.

Very little is known regarding the extent and specificities of transfer of both primary and secondary bile acids across the placenta although Hassan and Subbiah in 1980 and Lester et al (1972) suggest that transfer of the primary bile acids across the placenta is small. In 1980 Sewell et al. showed that the ewe at term contributes little

if any taurocholate to the foetal pool.

In 1960, Holsti showed that when significant amounts of lithocholic acid (hepatotoxic) is present in maternal serum, because of extensive transfer of this bile acid across the placenta, serious metabolic consequences might occur in the developing foetus. Indeed a study (Silverberg, Solomon, Ehrlich 1971) concerned with the effect of feeding lithocholic acid to pregnant hamsters indicated liver damage in neonates.

It has been held that sulphation is a method of detoxification for both steroid hormones and bile acids. Recent work is forcing a re-analysis of this "dictum". Yousef et al (1981) have shown that not only is lithocholic acid both free and conjugated with glycine, cholestatic, but also the sulphates of this monohydroxy bile acid exhibit a cholestatic effect. Perhaps of more significance, is the finding by this group that the taurine conjugate and its sulphate exerted no cholestatic effect. This coupled with the fact that taurine conjugates predominate in the foetus promote speculation that this sulphonic acid may confer some degree of protection with respect to cholestasis. With the advent of new techniques such as HPTLC, but more particularly HPLC, analysis of both bile and serum profiles of bile acids for taurine/glycine ratios in disease states and in experimental animals after perturbing the system, would seem a fertile area for future research.

CONCLUSIONS

(1) Excretion of pregnanediols in foetal bile does not constitute a major contribution to overall foetal progesterone metabolism. Only in the perinatal period are conjugates of pregnanediol excreted into the foetal intestine in appreciable amounts. No pregnanediol sulphates are present in foetal amniotic fluid at any time during gestation.

(2) The most significant rise in bile acid synthesis by the foetal liver occurs in the last 10-12 days of gestation although synthetic activity is evident by at least 120 days.

(3) The bile acid pattern in the non-sulphated fraction of bile acids is dominated by high proportions of the primary bile acids and deoxycholic acid. However small amounts of other bile acids are present.

(4) Amniotic fluid levels of bile acids increase in traumatised foetuses.

APPENDIX

APPENDIX

Bile acid washout curve - Preliminary assessment of foetal liver synthetic capacity.

Introduction

Until recently little was known about bile salt metabolism during early mammalian development, except for information derived from limited observations on the bile salts in gall-bladder bile and intestine of the near term foetus and early neonate (Encrantz & Sjövall, 1959; Peric-Golia & Socic, 1968). Smallwood et al. in 1972 studied aspects of the endogenous foetal bile salt in the dog and obtained data on the foetal hepatic bile salt excretory capacity. In 1973 Smallwood, Jablonski and McWatts using [^{14}C] cholesterol injected into the umbilical vein of foetal sheep in the latter half of gestation observed the incorporation of label into foetal bile acids. They concluded that the foetal sheep in the second half of gestation synthesises taurocholic acid and taurodeoxycholic acid. Estimates of foetal hepatic synthesis rate have been made in the near term foetal dog (0.4 to 0.8 μmol kg $^{-1}$ h $^{-1}$; Lester, 1980) and foetal sheep (0.35 μmol kg $^{-1}$h $^{-1}$; Hardy et al., 1980). Sewell et al. have shown using sodium [^{14}C] taurocholate that the near term foetal sheep exhibits comparable bile salt transport characteristics. Bile acid washout curves of doexycholic acid should provide an estimate of the rate of transfer of this secondary bile acid from the maternal to the foetal compartment.

Materials and Methods

A pregnant sheep (Ewe 59) was treated as detailed in Chapter 5 and the foetal duodenum was cannulated distal to the point of entry of the common bile duct. The cannula was

exteriorized and retraced to the duodenum,distal to the first point of cannulation. The animal was then allowed to recover from the operation (7 days). At the time of the experiment the estimated age of the conceptus was 122 days. On the day of the experiment (the ewe being fasted overnight) a chronic fistula was created and bile collected at 2 hourly intervals for 8 hours and then for a further 10 hours. Total collection time was 18 hours. At the end of the experiment the bile was redirected to the duodenum. No account was taken of pancreatic secretions in this model. Bile acids were measured by GLC as detailed in Chapter 5.

Results

Washout curves for the sum of cholic acid and chenodeoxycholic acid and for deoxycholic acid were generated using the MODFIT computer program depicted in Figs. 28 and 29. From these an estimate of synthetic rate of the primary acids can be made (however no allowance was made for maternal to foetal transfer of these bile acids that may have contributed to this estimate). Similarly from the curve for deoxycholic acid shown in Fig. 29 an estimate of the basal rate of transfer of this bile acid to the foetus was made.

Primary bile acids = 0.12 μmol per $kg^{-1}hr^{-1}$

Deoxycholic acid (Secondary Bile acid) = .0043 μmol $kg^{-1}hr^{-1}$.

Discussion

These results are in good agreement with the radioactive tracer studies of Smallwood et al. confirming that the foetal sheep is capable of synthesizing the primary bile acids at 122 days gestation. The contribution of maternal primary bile acids to the bile salt output is probably low. The fact that the

FIGURE 29

Washout curve - Cholic Acid + Chenodeoxycholic Acid

Modfit - computer fitted exponential decay curve

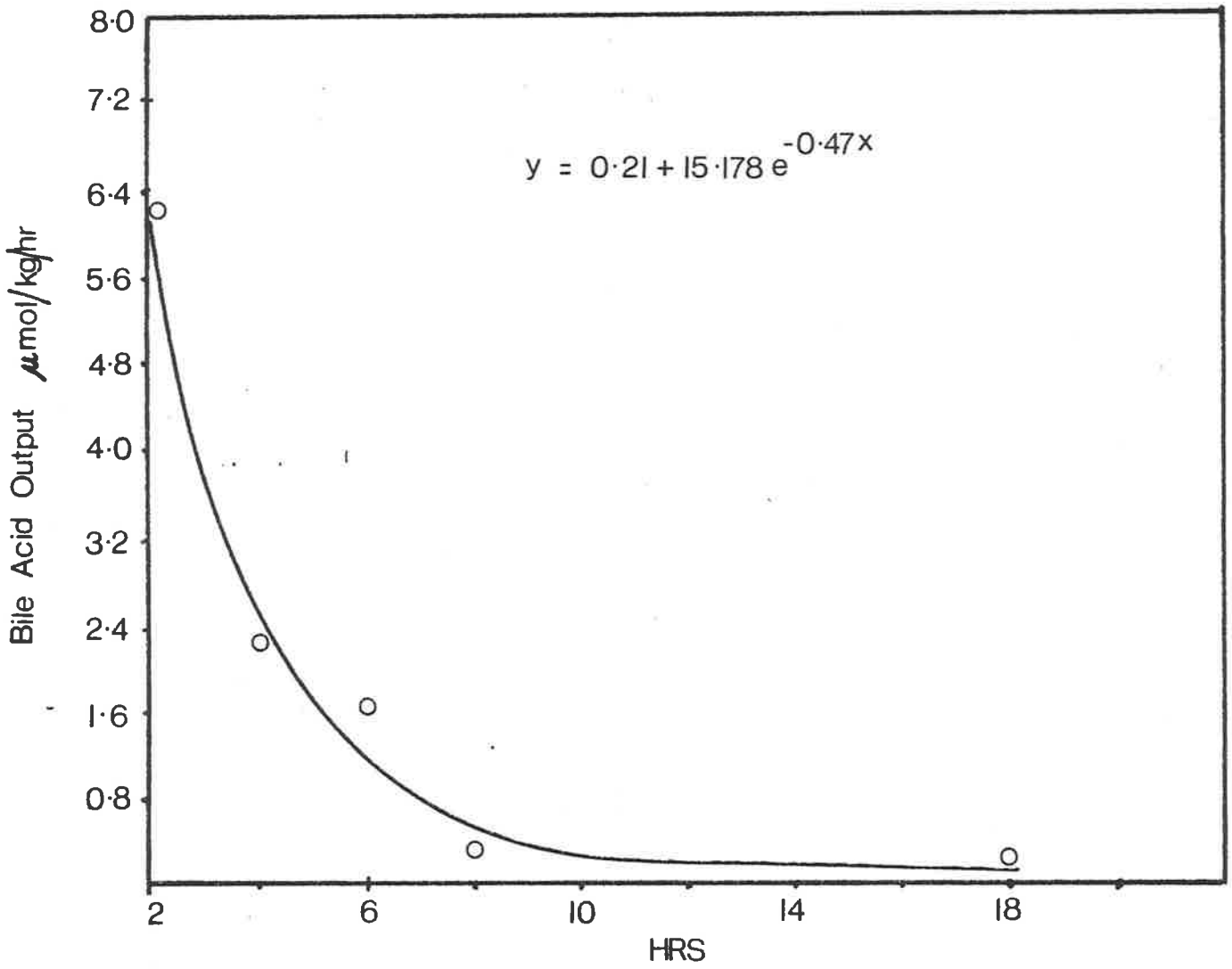
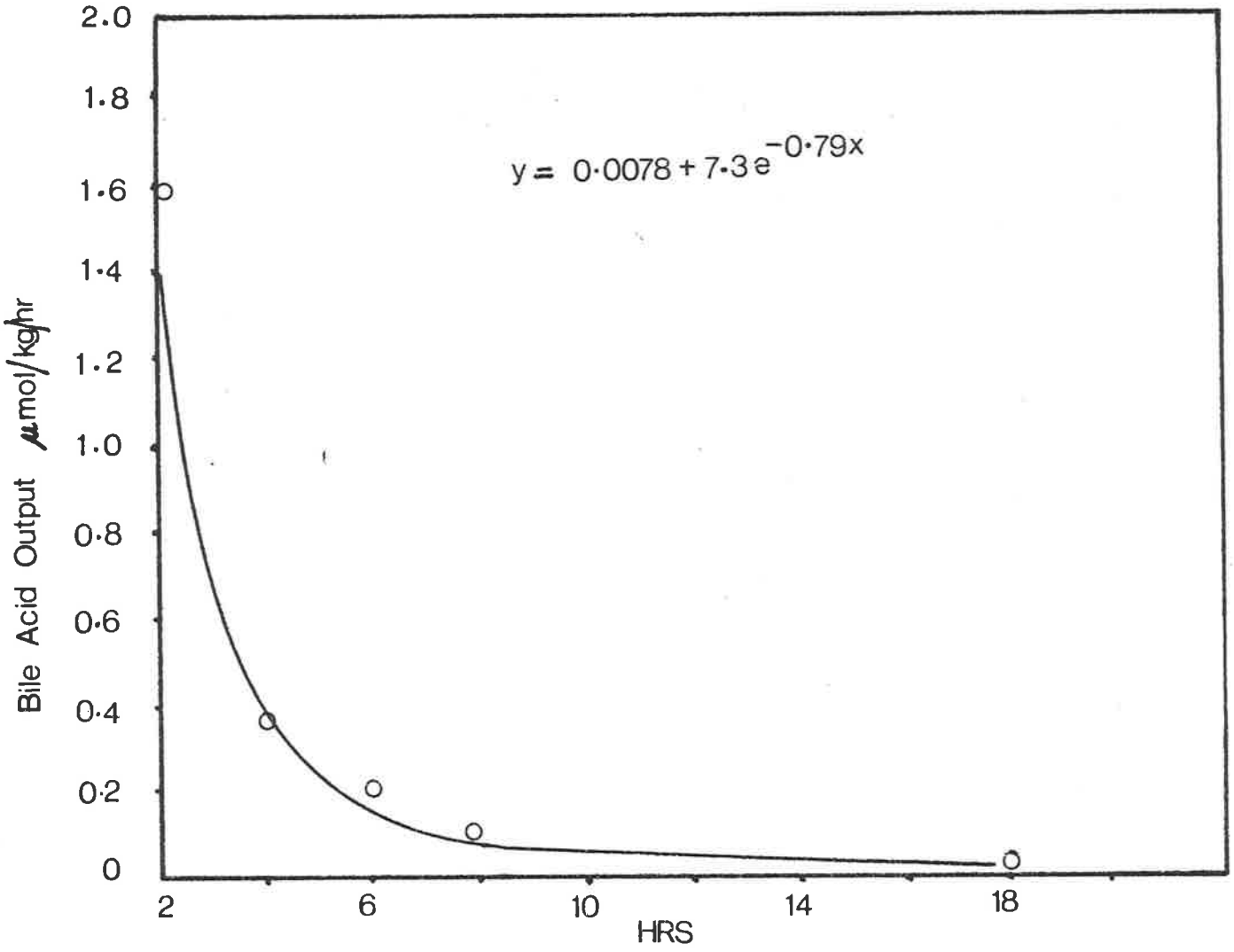

8·0
7·2
6·4
5·6
4·8
4·0
3·2
2·4
1·6
0·8
Bile Acid Output μmol/kg/hr
y = 0·21 + 15·178 e^-0·47x
2
6
10
14
18
HRS

FIGURE 30

Washout curve - deoxycholic acid

Modfit - computer fitted exponential decay curve


2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Bile Acid Output μmol/kg/hr
y = 0·0078 + 7·3e^−0·79x
2
6
10
14
18
HRS

foetal plasma bile salt concentrations in foetal sheep (unpublished observations) are higher than adult levels as has been shown in the dog (Smallwood et al.,1972), and that ^{14}C-cholate infused into the maternal circulation showed minimal accumulation in the foetal bile supports this contention. The estimated bile salt synthetic rate in this preliminary report is somewhat lower than that reported for the near term foetal dog (0.37 mol $kg^{-1}hr^{-1}$ and for the near term foetal sheep 0.35 mol $kg^{-1}hr^{-1}$). The estimated transfer of deoxycholic acid from this experiment was 0.0043 mol kg^{-1} hr^{-1}. Sewell et al. estimated total maternal to foetal bile acid transfer to be 0.004 mol kg^{-1} hr^{-1}.

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