THE HYDROLYSIS OF BILE ACID CONJUGATES BY SELECTED FUNGI

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ABSTRACT

The presence of constitutive, intracellular and extracellular enzymes catalysing the hydrolysis of glycine bile acid conjugates in the fungus *Cercospora melonis* CBS 162.60 was demonstrated by the use of cell-free systems. Shake flask and fermenter studies were undertaken to determine environmental factors favouring high free bile acid yields. Two major factors were observed to reduce such yields. These were the binding of the bile acid to the mycelium and the degradation of free bile acids to non-steroidal products by the fungus.

Whole-cell cultures of *C. melonis* exhibited poor utilisation of taurine conjugates with no concomitant production of free bile acid. Incubation of synthetic bile conjugate analogues with *C. melonis* and the use of cell-free systems suggested that this was due to two major factors: firstly, the specificity of the extracellular enzyme for α-aminocarboxylic acid conjugates and secondly, the apparent inability of taurine conjugates to gain access to a constitutive, intracellular cholanoyl taurine hydrolase. It is proposed that the poor permeability of the fungal cell membrane is responsible. Hence, the low activity of whole-cell cultures of *C. melonis* on taurine conjugates suggests that an industrial process employing the fungal hydrolysis of gall is not feasible.

Comparative studies with *Curvularia fallax* IFO 8885 showed that it possessed superior specific hydrolase activity on glyco-deoxycholic acid compared to *C. melonis*, although this is not apparent from qualitative screening.

The abilities of *C. melonis*, *Curvularia coicis* IFO 7278 and *Aspergillus ochraceus* IFO 4071 (Wilhelm) to 7α-dehydroxylate cholic acid and its natural conjugates were investigated. Despite the presence of an apparently constitutive, intra-cellular 7α-hydroxycholanoyl dehydroxylase in these organisms, only low yields of dehydroxylated products were obtained with whole-cell cultures.

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BILE ACID NOMENCLATURE

The trivial names used for bile acids are given, followed by the abbreviations employed, in brackets, and their I.U.P.A.C. systematic chemical names (I.U.P.A.C.-I.U.B., 1969).

Cholic acid (CA) = 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid.

Chenodeoxycholic acid = 3α,7α-dihydroxy-5β-cholan-24-oic acid.

Dehydrocholic acid = 3α,7α,12α-trioxo-5β-cholan-24-oic acid.

Deoxycholic acid (DC) = 3α,12α-dihydroxy-5β-cholan-24-oic acid.

Lithocholic acid = 3α-hydroxy-5β-cholan-24-oic acid.

N-(α-alano)-deoxycholic acid (α-AD) = 3α,12α-dihydroxy-5β-cholan-24-oyl-α-alanine.

N-(β-alano)-deoxycholic acid (β-AD) = 3α,12α-dihydroxy-5β-cholan-24-oyl-β-alanine.

N-(α-aminomethanesulphonyl)-deoxycholic acid (Na-α-AMSD) = 3α,12α-dihydroxy-5β-cholan-24-oyl-α-aminomethanesulphonic acid.

Glycocholic acid (GC) = 3α,7α,12α-trihydroxy-5β-cholan-24-oylglycine.

Glycochenodeoxycholic acid = 3α,7α-dihydroxy-5β-cholan-24-oylglycine.

Glycodeoxycholic acid (GD) = 3α,12α-dihydroxy-5β-cholan-24-oylglycine.

Taurocholic acid (NaTC) = 3α,7α,12α-trihydroxy-5β-cholan-24-oyltaurine.

Taurochenodeoxycholic acid = 3α,7α-dihydroxy-5β-cholan-24-oyltaurine.

Taurodeoxycholic acid (NaTD) = 3α,12α-dihydroxy-5β-cholan-24-oyltaurine.

The term "free bile acid" denotes a bile acid with an unsubstituted C-24 carboxylic acid group. Sulphonic acid conjugates will be usually referred to as the sodium salt.

ABBREVIATIONS

Abbreviations of units:

amu	atomic mass units
^{o}C	degrees Celsius
d	day
g	gram
x g	gravitational acceleration (ms^{-2})
h	hour
l	litre
m	metre
M	mole per litre
m/e	mass: charge ratio
min	minute
mmol	millimole per litre
Pa	pascal (Newton per square metre)
psi	pound per square inch
rpm	revolutions per minute

Other abbreviations:

ACC	Akers Culture Collection of Imperial Chemical Industries Ltd.
ATCC	American Type Culture Collection
calcd.	calculated
CBS	Centraalbureau voor Schimmelcultures
D.O.	Dissolved oxygen
hplc	high performance liquid chromatography
HUT	Hiroshima University, Faculty of Engineering
8HQ	8-Hydroxyquinoline
I.D.	Internal diameter
IFO	Institute for Fermentation, Osaka
IMI	Commonwealth Mycological Institute
IR	Infra-red
Lit.	Literature
m.p.	melting point
R_f	Tlc mobility of a compound relative to the solvent front mobility
tlc	thin layer chromatography

CHAPTER 1

PRELUDE

1. PRELUDE

The free bile acids, cholic acid and deoxycholic acid, are used industrially overseas as feedstock for the chemical synthesis of corticosteroids, despite the development of the more efficient microbial route *via* progesterone. New Zealand currently exports $2 million worth of these acids. However, it would greatly benefit New Zealand if they were either further processed in this country to more valuable intermediates, or the present bile acid production process was made more efficient. In this regard, a microbial process would be more suited to New Zealand conditions than chemical conversion, mainly because of the cost of imported chemicals and the technology required for the latter.

The Biotechnology Department of Massey University has had a long involvement with the industrial processing of bile acids and investigations are presently being conducted into the feasibility of using anaerobic bacteria and/or fungi for further transformation. This thesis describes research performed using the latter microorganisms with particular emphasis on the hydrolysis of the bile acid conjugates found in cattle and sheep gall.

Although fungi have demonstrated multiple transformation abilities on a wide variety of steroid substrates, reports of their action on bile acids are scarce. However, from a commercial point of view, a fungal fermentation has several distinct advantages to one using anaerobic bacteria. These include the ease with which the mycelium can be separated from the fermentation broth, the relatively simple nutritional requirements of fungi and the relative "cleanness" of the fermentation liquor compared to that of anaerobic microorganisms.

Industrially, the hydrolysis of the bile acid conjugates in cattle and sheep gall is performed using sodium hydroxide at 120^{o}C for 3-6 hours (Garland, 1977). However, several

workers have reported considerable losses of bile acid during this alkaline hydrolysis procedure. Roseleur and van Gent (1976) found that after hydrolysis with 2M sodium hydroxide at 120°C for 3 hours, recoveries of cholic acid from sodium taurocholate were 40% and from glycocholic acid, 70%; recoveries of chenodeoxycholic acid from sodium taurochenodeoxycholate were 60% and from glycochenodeoxycholic acid, 40%. Nair and Garcia (1969) reported losses of 20% and 12% with glycocholic acid and sodium taurocholate respectively using an optimal hydrolysis scheme and they also found that dihydroxy cholanic acid conjugates were particularly vulnerable to losses. Garland (1977) gives recoveries of cholic acid as 95% and 80-85% from glycocholic acid and sodium taurocholate respectively. The free bile acids, however, are stable to alkaline hydrolysis (Roseleur and van Gent, 1976; Lepage *et al*, 1978). These data vary considerably but this may be due to the different scale of operation employed. Both Roseleur and van Gent and Nair and Garcia worked with milligram quantities with which losses due to experimental handling would be proportionately higher than those of Garland who used gram quantities. However, even the lower losses of product in an industrial alkaline hydrolysis process remain economically substantial. Consequently the development of an efficient microbial process may be advantageous, particularly if the resulting free bile acids could be further transformed to more valuable products in the same fermentation.

Initial work in this department has made an obvious distinction in the relative susceptibilities of glycine and taurine-conjugated bile acids to fungal hydrolysis. Therefore the present research programme was developed to investigate the hydrolysis of each type individually. In order for the research to be performed in depth the investigation was confined mainly to one organism, *Cercospora melonis*. Quantitative analysis of the hydrolysis of glycine conjugates was undertaken, and since glycodeoxycholic acid returned the poorer yield, work was concentrated on this conjugate with the aim of

maximising the productivity of the fermentation. During the work some properties of the hydrolase system and some unfavourable characteristics of the fermentation were investigated. Also, an analysis of the effects of selected environmental factors and substrate concentrations was performed. This research is presented in Chapter 4.

Simultaneously, the resistance of taurine-conjugated bile acids to fungal hydrolysis was studied using *C. melonis*. As these conjugates comprise *ca* 50% of the bile acid substrate in cattle and sheep gall, their recalcitrance would negate a fungal hydrolysis process. The failure to obtain their hydrolysis by the manipulation of selected fermentation variables led to a more fundamental attempt to identify the factors responsible for their recalcitrance. This work is contained in Chapter 5.

In Chapter 6, a comparative study of two fungi, *C. melonis* and *Curvularia fallax* is presented. It was conducted to obtain a comparison, albeit limited, of the variability of fungal hydrolase activity. Such knowledge could be useful in determining sensitive criteria for the screening of fungi for their hydrolysis ability.

The potential of fungi to modify the free bile acids into higher-value products was investigated to a lesser extent. Although both cholic acid and deoxycholic acid are used in the chemical synthesis of corticosteroids, deoxycholic acid is apparently the preferred starting material (Garland, 1977). Therefore, since the predominant product of the hydrolysis of cattle and sheep gall is cholic acid, fungal 7α-dehydroxylation was studied using both *Curvularia coicis* and *Aspergillus ochraceus*. This work is contained in Chapter 7.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. Bile Acids

2.1.1. Properties and Origin

Bile acids are a group of steroids based on the structure of 5β-cholanic acid (Figure 2.1). They are characterised by an acidic sidechain and, commonly, hydroxyl substituents at carbon 3, 6, 7 and 12 positions of the steroid ring nucleus. In vertebrates, the bile acids are synthesised from cholesterol and secreted into the bile fluid after conjugation by means of an amide bond with the amino acids glycine or taurine (Figure 2.2). They are amphiphilic molecules capable of a diverse range of physicochemical properties according to the degree of hydroxylation of the steroid nucleus and to the nature of the amino acid moiety in the conjugated form. The detergent nature of the conjugated bile acids is vital to their major role in vertebrates, namely, to aid in the absorption of triglycerides in the intestine. Table 2.1 presents some properties of the bile acid conjugates normally present in cattle and sheep gall and of their corresponding free bile acids. The physical chemistry of bile acids has been reviewed by Small (1971).

The major source of available bile acids in New Zealand is the gall of cattle and sheep slaughtered in the meat works. Gall is a mixture of compounds of which bile acid conjugates comprise 70-75% of the dry weight. The principal components are the sodium salts of taurocholic acid, taurodeoxycholic acid, glycocholic acid and glycodeoxycholic acid. The glycine:taurine ratio of cattle and sheep gall is usually one, while the cholic:deoxycholic acid ratio is 2.5 to 3.0 (Garland, 1977). Other constituents in the solids fraction include bilirubin pigments, phospholipids and mucin (Hoehn, 1964).

Table 2.1

Some properties of common bile acids (Small, 1971)

Bile acid[a]	pKa	pH of precipitation	C.M.C.[b] $mmol\,l^{-1}$	Aqueous solubility[c] $g\,l^{-1}$
CA	4.98	6.5	12	560
DC	5.30	6.92	5	334
GC	3.95	4.32	10	
GD	4.69	4.96	4	
TC	1.85		12	
TD	1.93		4	

a. Refer to Abbreviations.
b. Critical micellar concentration of the sodium salt.
c. As the sodium salt.

2.1.2. Bile Acids as Microbial Substrates

As substrates for microbial transformation, bile acids have problems in common with other steroid classes. The major one, from the viewpoint of the economic viability of a fermentation process, is the concentration at which they can be used in a fermenter. Preferably, this should be as high as possible. However, commercial steroid fermentations are limited to maximum concentrations of 1-5% w/v, while many operate at concentrations of only 0.05-0.10% w/v (Rhodes and Fletcher, 1966 ; Murray, 1976). The toxicity of bile acids to micro-organisms, their tendency to change physical state in solution due to pH and concentration effects, and the foaming property of their soluble salts below their critical micellar concentration are all characteristics which place limits on both their concentration in a fermentation and

FIGURE 2.1 5β-cholanic acid. The parent structure for bile acids.

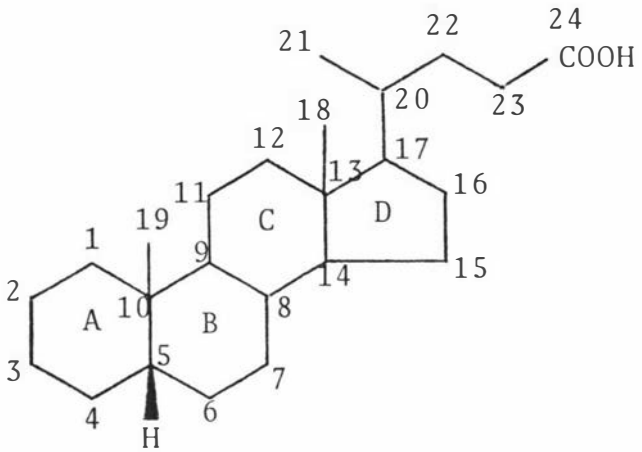
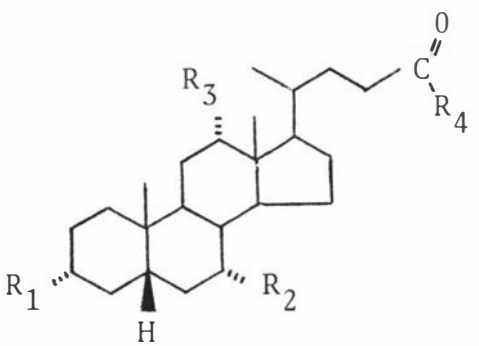


FIGURE 2.2 The structural formulae of some common bile acids.



R_1, R_3=OH; R_2=H; R_4=OH.	deoxycholic acid
R_1, R_3=OH; R_2=H; R_4=$NHCH_2COOH$.	glycodeoxycholic acid
R_1, R_3=OH; R_2=H; R_4=$NH(CH_2)_2SO_3H$.	taurodeoxycholic acid
R_1, R_2, R_3, R_4=OH.	cholic acid
R_1, R_2, R_3=OH; R_4=$NHCH_2COOH$.	glycocholic acid
R_1, R_2, R_3=OH; R_4=$NH(CH_2)_2SO_3H$.	taurocholic acid

the environmental conditions which can be employed during fermentation.

2.1.2.1. The toxicity of bile acids to microorganisms

The primary constraint on bile acid concentrations in a fermentation is their toxicity to the microorganism. No data have been reported for their toxic effect on filamentous fungi, although this aspect has been studied using bacteria. Yesair and Himmelfarb (1970) reported that the growth of aerobic bacteria was inhibited at levels of 1.25% w/v of conjugated bile acid, whereas concentrations as low as 0.2% w/v have been reported to suppress anaerobic bacteria (Shimada *et al*, 1969; Floch *et al*, 1971).

Binder *et al* (1975) reported that the toxicity of bile acids to bacteria was reduced by their conjugation with glycine or taurine, by greater hydroxylation of the steroid nucleus and by the substitution of keto for hydroxyl functions. This dependence of toxicity on the substituents of the steroid nucleus is important in the consideration of substrate concentrations since the products of a transformation may be more toxic than the substrate. In addition, Binder *et al* found that bile acid toxicity was pH dependent, increasing as pH decreased, suggesting that the toxicity of bile acids was inversely related to their degree of dissociation. Bile acid toxicity to microorganisms has been reported to be reduced in the presence of phospholipids, mucin and fatty acids, all of which are components of gall (Binder *et al*, 1975; Miyasazki, 1954.).

While the relevance of these data to filamentous fungi is not known, it is probable that the qualitative conclusions are applicable.

2.1.2.2. The physical state of bile acids in fermentation broth

Unlike other steroids, the aqueous solubility of bile acids is excellent if they are present as the ionised form. Their solubility is pH dependent and the free bile acids,

cholic and deoxycholic acids, precipitate from solution if the pH falls below 6.5 and 6.92 respectively (Table 2.1). However, these figures were derived from the behaviour of the pure bile acid in solution. Where both conjugated and free bile acids are present, as would be the case with the microbial hydrolysis of bile acid conjugates, the pH of precipitation is lowered significantly (Small, 1971). Even so, the pH of fungal fermentations often falls to values of pH 3-4 during growth, making the precipitation of any free bile acid present very likely. The effect of this on the transformation reaction is unknown although it has been found that the microbial transformation of steroids can be dependent to a significant degree on their physical state in the medium (Marsheck, 1971).

The physical state of bile acids in solution is also concentration dependent due to micelle formation by their soluble salts above a certain concentration, known as the critical micellar concentration (Table 2.1). For both dihydroxy and trihydroxy bile salts, this concentration lies within the range that might be used in a bile acid fermentation and, given high pH, their presence as micelles could be expected. Micelle formation by sterols is known to hinder their adsorption to hydrophobic, organic resins (Martin, 1977) and is thought to be a factor in preventing their facile degradation by microorganisms (Jones and Baskevitch, 1973). However, the effect of micelle formation on the transformation of bile acids has not been reported.

Another surface-active property of bile acids is their action as foaming agents when below their critical micellar concentration. For this reason, it may be necessary to operate above this concentration in highly aerated and agitated fungal fermentations if uncontrollable foaming and its concomitant problems of wall growth and the loss of fermenter contents are to be avoided.

In summary, the properties of bile acids impose conflicting constraints on their use as a substrate for microbial transformation. High bile acid concentrations are preferable since a higher economic return on investment is achieved

and concentrations above the critical micellar concentration minimise their property of foam production. Yet the toxicity of the bile acids to microorganisms limits their concentration in a fermenter and their formation of micelles above their critical micellar concentration may hinder their facile transformation by microorganisms. This problem is made more complex by the requirement for high-value bile acid products, which in many cases are more toxic to micro-organisms than the substrate. Since many steroid transformations appear to result from the operation of microbial detoxification mechanisms, this infers that, not only may the desired transformation not be performed, but that side-reaction products, such as bile acids with keto substituents or more hydroxyl functions, may be produced. However, the dependence of some of these bile acid properties on solution pH, suggests that pH control of the fermentation may help eliminate certain of these constraints.

2.2. The Microbial Hydrolysis of Bile Acid Conjugates

2.2.1. Bacterial Hydrolysis

Reports of the microbial hydrolysis of bile acid conjugates have almost exclusively related to the study of the anaerobic bacterial population of the intestinal tract. These bacteria have been found responsible for the conversion of conjugated bile acids to the corresponding free bile acids in the intestine of mammals to such an extent that only the latter are detected in the faeces. In contrast, bile acids recovered from the faeces of germ-free animals remain conjugated (Dickenson *et al*, 1971). Recent reviews by Hayakawa (1973) and Midtvedt (1974) summarise the results of extensive research undertaken to investigate the hydrolysis of bile acid conjugates by intestinal micro-organisms both *in vivo* and *in vitro*.

Whereas the hydrolysis of bile acid conjugates is a common transformation ability of intestinal bacteria it is quite rare for other bacteria (Hill and Drasar, 1968; Hill, 1976). In addition, there are few reported instances of the bacterial hydrolysis of bile acid conjugates under aerobic conditions

(Hayakawa, 1973).

In several cases, the bacterial hydrolase has been isolated and its properties determined (Nair *et al*, 1967; Hill and Drasar, 1968; Kobashi *et al*, 1978). For most, the enzyme was reported to be constitutive and intracellular, although Bifidobacteria elaborated extracellular hydrolases (Aries and Hill, 1970a). The enzymes are generally active on both taurine and glycine-conjugated bile acids but some are specific for either type (Midtvedt, 1974; Kobashi *et al*, 1978). Bacterial hydrolases are now commercially available, principally for the analytical determination of bile acid conjugates.

2.2.2. Fungal Hydrolysis

In contrast to the wealth of data published on the bacterial hydrolysis of bile acid conjugates, few data have been reported concerning fungal hydrolysis.

Grassmann and Basu (1931) reported the hydrolysis of glycocholic acid by an aqueous extract of *Aspergillus oryzae* after incubation at pH 8.0 for seventy hours. Sodium taurocholate was not hydrolysed under the same conditions as judged by titration with alkali. However, Furuta (1959) isolated cholic acid from the broth of *Aspergillus niger* grown on a modified Czapek medium containing 0.25% w/v sodium taurocholate. The yield of cholic acid was 7% after twenty days aerobic incubation at 28^{o}C. To isolate the bile acids, Furuta removed the mycelium by filtration, acidified the culture broth with dilute hydrochloric acid and extracted the resulting washed precipitate successively with ether, ethyl acetate and methanol. The products were obtained by crystallisation from these extracts. Furuta was unable to isolate sodium taurocholate from the spent medium and suggested that hydrolysis may have gone to completion. However, due to its very low pKa value, it is unlikely to have precipitated from the medium on acidification. If any was precipitated it would be primarily extracted by the methanol wash from which Furuta was unable to crystallise any bile acids. Consequently,

it is likely from the low cholic acid yield, the absence of other bile acid metabolites and his methodology, that he failed to detect the taurocholic acid remaining.

More recently, work in this laboratory (Maddox and Chong, 1978; Chong *et al*, 1980) has demonstrated the hydrolysis of both glycocholic and glycodeoxycholic acids to the corresponding free bile acids by various fungi. A substrate concentration of 0.05% w/v was used and the fungi were grown in shake-flask culture on a sucrose-peptone medium at 30°C (Table 2.2.). Under identical conditions of incubation, none of the fungi hydrolysed sodium taurocholate, using the appearance of cholic acid as the criterion for hydrolysis. Despite this, taurocholate losses of 5-25% were observed for most of the fungi. By using sodium taurocholate as the sole sulphur source for *Penicillium chrysogenum* a 30% yield of cholic acid was obtained after nine days incubation at 30°C. However, no hydrolysis was detected with the other fungi when tested under identical conditions.

The negative result of Chong *et al* (1980) with *A. niger* on sodium taurocholate contradicts that of Furuta (1959), but the reason for this discrepancy is not immediately clear. Similarly, the attempt by Chong *et al* to stimulate hydrolysis of the taurine-conjugated bile acid succeeded with only the one organism, suggesting that this substrate is resistant to the action of many fungi which are able to hydrolyse glycine-conjugated bile acids.

Reports of bile acid hydrolysis by yeasts are contradictory. Using a brewers' yeast, Takahashi (1939) isolated cholic acid at yields of 20% and 28% from taurocholic and glycocholic acids respectively after 40 days incubation at 37°C. However, Kobashi *et al* (1978) using washed cells of *Saccharomyces carlsbergensis* and a bakers' yeast, reported no activity on glycocholic acid. *S. carlsbergensis* did, however, hydrolyse taurocholic acid, monitored by the release of ninhydrin-positive material, while the bakers' yeast showed no such activity. Maddox and Chong (1978) tested three yeasts for their ability to hydrolyse glycocholic acid, glycodeoxycholic acid and sodium taurocholate

Table 2.2

The ability of fungi to hydrolyse bile acid conjugates in shake flask culture
(Maddox and Chong, 1978; Chong *et al*, 1980)

Organism	Bile acid substrate		
	GC	GD	NaTC utilisation (%)
Alternaria brassicola	+++	++	0
Aspergillus nidulans ATCC *11267*	++	++	13
Aspergillus niger IMI *75353*	+++	++	11
Botryodiplodia theobromae IFO *6469*	+		
Botrytis cinerea	+	+	21
Candida albicans	+	++	14
Cephalosporium spp.	+	+	14
Cercospora melonis CBS *162.60*	++++	++	11
Cladosporium resinae IMI *84419*	+	+	7
Cochliobolus geniculata IFO *6284*	++	++	12
Cochliobolus intermedius IMI *52980*	+++	++	13
Cochliobolus lunata IFO *5997*	++	++	11
Colletotrichum coccodes ATCC *12521*	++	++	9
Curvularia coicis IFO *7278*	++	++	7
Curvularia fallax IFO *8885*	+	++	12
Curvularia maculans IFO *6292*	+	++	0
Curvularia trifolii IFO *6692*	+	++	14
Fusarium moniliforme ACC *917*	+	++	11
Gibberella zeae IFO *5269*	++	++	26
Mucor hiemalis IMI *113136*	+	+	0
Penicillium chrysogenum	+	++	31
Penicillium roquefortii IMI *24313*	+	+	0
Saccharomyces cerevisiae	-	-	5
Torulopsis utilis IMI *33552*	+	+	5
Wojnowicia graminis CBS *160.73*	-	+	0

Experimental conditions were identical for the fermentation of all three substrates. The symbol notation is: ++++, hydrolysis complete after 11 days; +++, hydrolysis complete after 17 days; ++, hydrolysis complete after 26 days; +, partial hydrolysis after 26 days; -, no hydrolysis after 26 days. The glycocholic acid (GC) fermentation lasted 26 days, while those for glycodeoxycholic acid (GD) and sodium taurocholate (NaTC) lasted 23 days.

and found only weak activity on the glycine conjugates, if any, and none on sodium taurocholate (Table 2.2).

In summary, the ability of intestinal bacteria to hydrolyse both taurine and glycine bile acid conjugates *in vivo* and *in vitro* has been established and much is known about the enzymes responsible. However there are few reports of the hydrolysis of bile acid conjugates under aerobic conditions by either bacteria or fungi. In the case of the latter, the literature is sometimes contradictory but suggests that many filamentous fungi hydrolyse glycine-conjugated bile acids while taurine conjugates are recalcitrant.

2.3. Microbial Transformations of Free Bile Acids

Despite the volume of literature on the transformation of steroids by microorganisms, relatively little has been published concerning free bile acids (Charney and Herzog, 1967; Laskin and Lechevalier, 1974). As in the case of bile acid conjugate hydrolysis, investigations into the transformation abilities of intestinal bacteria have contributed much of the present knowledge, due mainly to their possible significance in intestinal cancer. Thus, enzymes capable of both reductive and oxidative modifications of the free bile acid molecule, both *in vivo* and *in vitro*, have been reported (Hayakawa, 1973; Hill, 1976). Of these, those catalysing the oxidoreduction of the α-hydroxyl functions at positions 3, 7 and 12 and the dehydroxylation at C-7 are the most common. The activity of these enzymes on the bile acid conjugates has been found to be minimal and their hydrolysis is regarded as a prerequisite for further transformation (Hill, 1976). No exceptions to this have been observed with aerobic bacteria, yeast or fungi, hydrolysis appearing to precede further modification of the bile acid (Furuta, 1959).

Reported instances of the microbial transformation of bile acids under aerobic conditions are less common. Both Yesair and Himmelfarb (1970) and Ferrari (1967) observed extensive degradation of a free bile acid mixture and cholic acid respectively in aerobic cultures of faecal bacteria,

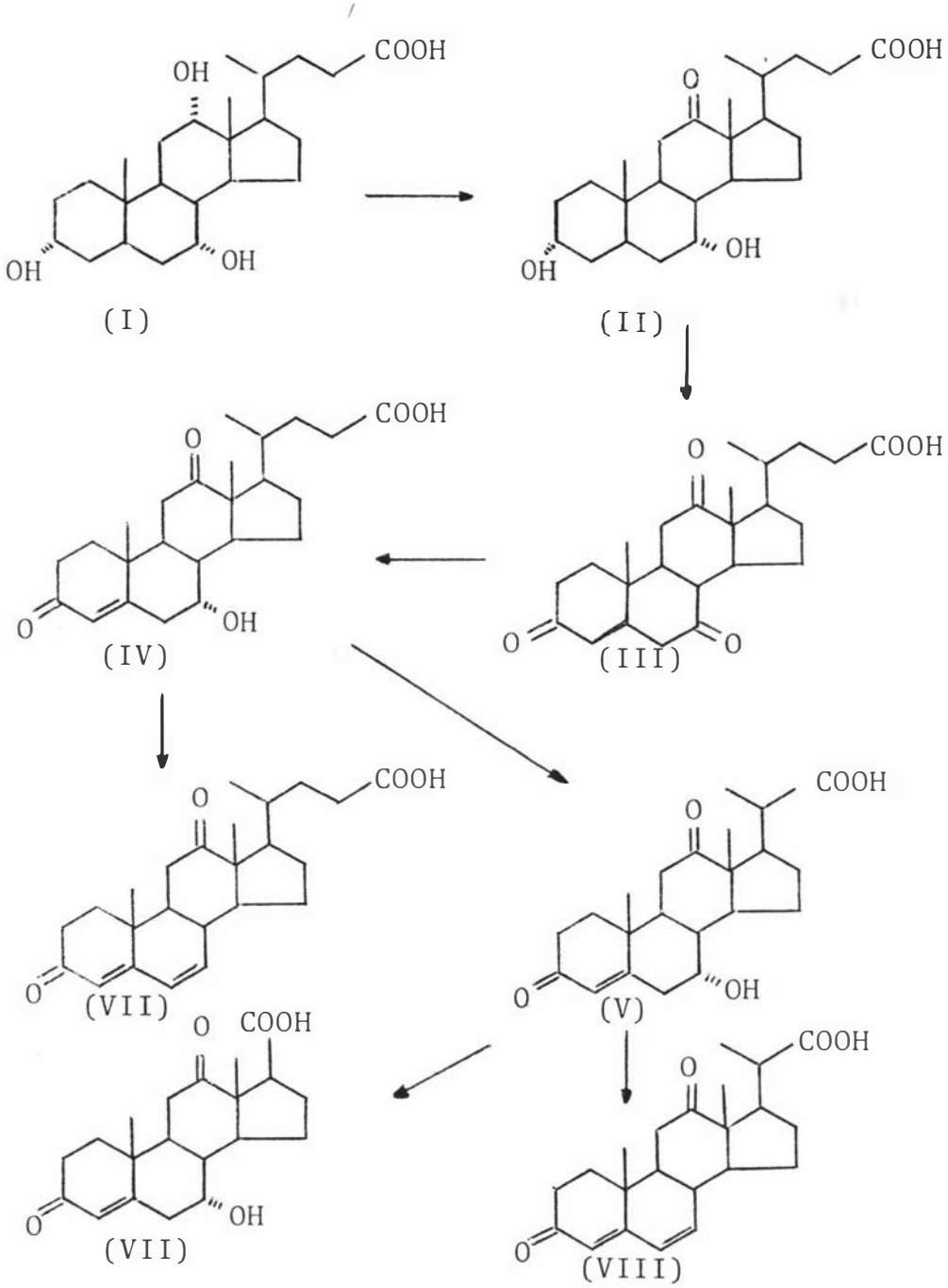
the former reporting the total utilisation of the bile acids within 72 hours. Isolation and identification of the degradation products was not undertaken in either case. Hayakawa (1973) has reviewed research on the degradation of cholic acid by aerobic bacteria, principally *Aerobacter aerogenes*, *Arthrobacter simplex*, *Streptomyces* species, *Corynebacterium equi* and *Mycobacterium mucosum*. He suggested that the microbial degradation of bile acids, *in vitro*, proceeded in a similar manner to that of other steroids. However, he also reported that many of these bacteria could not degrade dihydroxy bile acids.

Reports of fungal transformations of free bile acids are rare, despite their proven ability with other steroid classes. Since it is unlikely that studies have not been conducted, it would appear that little activity has been observed. Hayase *et al* (1958) reported that cholic, lithocholic and dehydrocholic acids are resistant to fungal action and work in this laboratory has observed similar recalcitrance with dihydroxy bile acids (Maddox and Chong, 1980).

2.3.1. Oxidative Transformations

A review by Furuta (1959) lists the products derived from the oxidation of cholic acid and some cholic acid derivatives by *Aspergillus*, *Penicillium* and *Gliocladium* species in shake flask culture. Multiple transformations of the substrate were usually observed and included the oxidation of the 3α- and 12α- hydroxyl substituents to keto functions and the degradation of the bile acid sidechain to C-22 and C-20 derivatives by a process analogous to β-oxidation. Furuta proposed a pathway for the progressive oxidation of cholic acid to a variety of unsaturated cholanic acid derivatives by Aspergilli (Equation 2.1) and concluded that side-chain degradation occurred only after the oxidation of the 3α- and 12α- hydroxyl functions to keto groups. In contrast, the oxidation of the 7α-hydroxyl substituent and the introduction of unsaturation at the 4(5) position in the steroid A ring, were not important for the oxidation of the side-chain. These oxidative transformations parallel those reported for bacteria (Hayakawa, 1973).

Equation 2.1.


COOH
OH
OH
OH
(I)
COOH
O
OH
OH
(II)
COOH
O
O
OH
(IV)
COOH
O
O
O
(III)
COOH
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(VII)
COOH
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OH
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O
OH
(VII)
COOH
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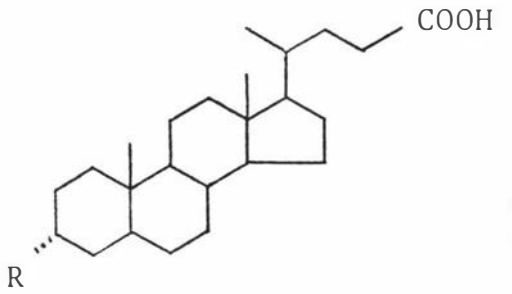
O
(VIII)

Only two instances of fungal hydroxylation of free bile acids have been reported. A patent by Dulaney and McAleer (1957) described the 11α-hydroxylation of lithocholic acid (IX) and 3-oxo-5β-cholanic acid (X) by *Aspergillus ochraceus*, but no yield figures were given. Hayakawa et al (1980) reported the 12β-hydroxylation of lithocholic acid by *Helicostylum piriforme* ATCC-8992 in 40% yield. The oxidation was performed over two days using three day old mycelium in deionised water with the substrate concentration at 0.05% w/v. It is surprising, given the proven ability of fungi to hydroxylate various steroids, that so few cases have been reported for the free bile acids. It suggests that some property of the bile acids, possibly their molecular structure, renders them resistant to such transformation.



R, α-OH (IX)
R, = O (X)

2.3.2. Other Transformations

In addition to the oxidative transformations described above, fungi have been reported to 7α-dehydroxylate and to introduce unsaturation into the ring nucleus of free bile acids. Products derived from the loss of the 7-oxygen group are listed in Table 2.3 together with the substrate, the fungus employed and other products isolated.

Clearly, 7-dehydroxylation occurs in several instances. However, only in the case of deoxycholic acid from cholic acid, reported by Furuta (1960), was 7α-dehydroxylation the sole transformation observed. Presumably the 7-keto group

Table 2.3

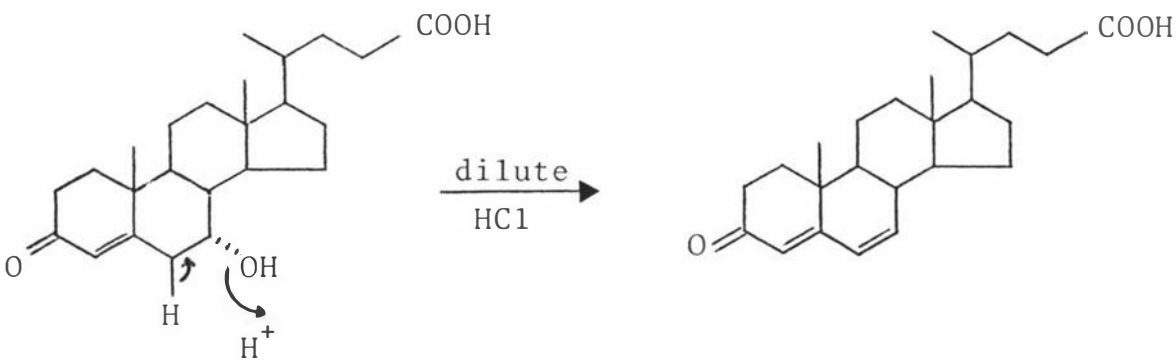
Reported fungal 7α-dehydroxylation, and concomitant products, of bile acids

Fungus	Substrate (Concentration)	Products (Those being 7α-dehydroxylated in italics)	Reference
Gliocladium species	Cholic acid (I)	*12α-hydroxy-3-oxo-chol-4,6-dienic acid*	Furuta (1959)
Aspergillus cinnamomeus HUT 2026	Cholic acid (0.6% w/v)	*12α-hydroxy-3-oxo-chol-4,6-dienic acid* *3,12-dioxo-chol-4,6-dienic acid (VII)* 3α,7α-dihydroxy-12-oxo-5β-cholanic acid (II)	Hasegawa (1959)
Aspergillus ochraceus (Wilhelm) IFO 4071	Cholic acid	*3α,12α-dihydroxy-5β-cholanic acid*	Furuta (1960)
Aspergillus niger	Cholic acid (0.4% w/v)	*3,12-dioxo-bisnorchol-4,6-dienic acid (VIII)* 7α-hydroxy-3,12-dioxo-eti-4-enic acid (VI)	Furuta (1959)
	3α,7α-dihydroxy-12-oxo-5β-cholanic acid (II)	*3,12-dioxo-bisnorchol-4,6-dienic acid (VIII)*	Furuta (1959)
	Dehydrocholic acid (II) (0.32% w/v)	*3,12-dioxo-chol-4-enic acid*	Furuta (1959)
Penicillium islandicum		*3,12-dioxo-bisnorchol-4,6-dienic acid* *3,12-dioxo-5α-bisnorcholanic acid* 3α,7α-dihydroxy-12-oxo-bisnorcholanic acid	Furuta (1959)

of dehydrocholic acid was reduced to a hydroxyl group prior to dehydroxylation.

Products listed in Table 2.3 with the conjugated 4, 6-diene structure may have lost their 7-oxygen function *via* a different mechanism to those with a saturated or 4-ene structure. Both Hasegawa (1959) and Furuta (1959) suggested that the 3-oxo-4-ene derivative (IV) (Equation 2.1) might be the actual fungal product, with the 7α-hydroxyl group being chemically eliminated to form the resulting 4, 6-diene compounds, 3, 12-dioxo-chol-4,6-dienic acid (VII) and a similar compound with side-chain degradation, 3, 12-dioxo-bisnorchol-4,6-dienic acid (VIII). In support of this, they reported that the 7α-hydroxy function of 7α-hydroxy-3-oxo-chol-4-enic acid (XI) was readily eliminated with dilute hydrochloric acid to form the corresponding 4,6-diene compound (XII) (Equation 2.2). However the isolation of deoxycholic acid from the fermentation of cholic acid with *A. ochraceus* reported by Furuta (1960) suggests that genuine fungal 7α-dehydroxylation does occur in some instances. Certainly, the bacterial 7α-dehydroxylation of cholic acid has been demonstrated *in vivo* by Samuelsson, (1960) to involve the enzymic introduction of a 6-ene function followed by hydrogenation to form deoxycholic acid and does not involve a spontaneous elimination of the 7α-hydroxyl function from a 3-oxo-4-ene derivative.

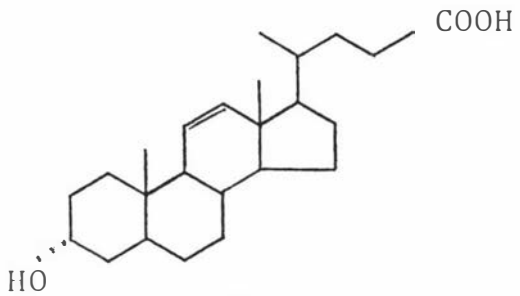
Equation 2.2.



(XI) (XII)

Fungi appear able to dehydrogenate the bile acid nucleus. Munro *et al* (1974) reported the isolation of 3α-hydroxy-

5β-chol-11-enoic acid (XIII) in low yield from the culture filtrate of a Curvularia species (IMI 52980). The substrate was not identified but was thought to have been a bile acid contaminant present in the peptone used in the medium, perhaps deoxycholic acid. If so, this would be a significant transformation since 12α-dehydroxylation of bile acids is a rare property of microorganisms, including those of the intestine (Hayakawa, 1973). Recently however, Saltzman (1976) has patented a method of producing chenodeoxycholic acid derivatives from those of cholic acid by use of the 12α-dehydroxylase activity of *Clostridium perfringens*.



(XIII)

The introduction of a 4-ene function by various fungi acting on cholic acid and some derivatives has been demonstrated by Furuta (1959) and Hasegawa (1959) (Table 2.3). However it was always conjugated with a 3-oxo group in the product. The production of a 3-oxo-cholanoyl-4(5)-dehydrogenase by some intestinal Clostridia under stringent anaerobic conditions has been observed. The enzyme is inducible and, once produced, can act under aerobic conditions (Hill, 1976). However, 4(5)-dehydrogenation is not a major degradative reaction in the intestine.

The presence of a 6-ene function in the bile acid products of fungal fermentations has been observed, but always in conjugation with a 3-oxo-4-ene system (Table 2.3). However, as mentioned above, chemical elimination to form the 6-ene function has been shown to occur spontaneously under mild acidic conditions (Hasegawa, 1959). Therefore, the existence of a fungal 6(7)-dehydrogenase has not been conclusively proven.

In conclusion, the literature available on the transformation of free bile acids by fungi demonstrates that they can perform, under aerobic conditions, many of those transformations reported for intestinal microorganisms. However, in comparison to other steroids, the bile acid molecule appears to be less susceptible to modification by fungal enzymes.

CHAPTER 3

METHODS

3. METHODS

3.1. Melting Points

The melting points were determined on a Kofler hot-stage microscope (Reichert Optische Werke AG., Vienna, Austria) or a Leitz Dialux Microscope with a heating stage 350 (Ernst Leitz, GmbH, Wetzlar, Germany), and are uncorrected.

3.2. Materials

3.2.1. Media

Potato dextrose agar and malt extract broth were obtained from Oxoid Ltd., London, England. The glucose-peptone medium was adapted from that described by Musgrave (1956) and included the trace element concentrations recommended by Hutner (1972) for fungal media (Table 3.1). Alterations in medium composition for certain experiments are detailed in the text. A sulphur-deficient medium employed in some experiments is detailed in Table 3.2.

3.2.2. Chromatography Materials

Kieselgel DG and Kieselgel DGF (both 400 mesh ASTM) were obtained from Riedel-De Haën AG., (Seelze-Hannover, Germany) for use in thin layer chromatography.

3.2.3. Bile Acids

3.2.3.1. Free bile acids

Cholic acid and deoxycholic acid were obtained from New Zealand Pharmaceuticals Ltd. (Palmerston North, New Zealand). They were recrystallised from water-saturated methyl ethyl ketone until homogenous by thin layer chromatography and dried at 105^{o}C for 4 hours. Chenodeoxycholic acid was purchased from Koch-Light Laboratories (Colnbrook, Buckinghamshire, England) and used as obtained.

Table 3.1

The glucose-peptone medium

Component	Concentration
D-glucose[a]	20 gl^{-1}
Bacteriological peptone[b]	5 gl^{-1}
$MgSO_4.7H_2O$[a]	0.25 gl^{-1}
K_2HPO_4	0.25 gl^{-1}
Iron as $(NH_4)_2Fe(SO_4)_2.6H_2O$[a]	42 mgl^{-1}
Zinc as $ZnSO_4.7H_2O$[a]	22 mgl^{-1}
Manganese as $MnSO_4.4H_2O$[a]	20 mgl^{-1}
Molybdenum as $(NH_4)_6Mo_7O_{24}.4H_2O$[c]	2.4 mgl^{-1}
Copper as $CuSO_4.5H_2O$[c]	1.0 mgl^{-1}

The medium was made to volume with distilled water and adjusted to pH 6.5 prior to sterilisation.

a. Analar grade, B.D.H. Chemicals Ltd., Palmerston North, New Zealand.
b. Oxoid Ltd., London, England.
c. May and Baker Ltd., Dagenham, England.

Table 3.2

The sulphur-deficient medium

Component	Concentration
D-glucose	20 gl^{-1}
glycine	3 gl^{-1}
K_2HPO_4	0.25 gl^{-1}
$MgCl_2.6H_2O$[a]	0.25 gl^{-1}
$FeCl_3.6H_2O$[a]	29.0 mgl^{-1}
$MnCl_2.4H_2O$[b]	18.0 mgl^{-1}
$ZnCl_2$.gran.[a]	10.4 mgl^{-1}
$(NH_4)_6Mo_7O_{24}.4H_2O$	5.4 mgl^{-1}
$CuCl_2.2H_2O$[a]	0.7 mgl^{-1}

The medium was made to volume with distilled water and adjusted to pH 6.5 prior to sterilisation.

a. B.D.H. Chemicals Ltd., Palmerston North, New Zealand.
b. May and Baker Ltd., Dagenham, England.

Methyl 3α,7α-diacetoxy-12α-hydroxy-5β-cholanate, 3α-hydroxy-12-oxo-5β-cholanic acid, and 3α,12α-dihydroxy-7-oxo-5β-cholanic acid were gifts from Dr R.P. Garland, Dr R. Chong and Dr R.H. Archer respectively. They were homogenous by thin-layer chromatography.

3α,7α-dihydroxy-12-oxo-5β-cholanic acid. Methyl 3α,7α-diacetoxy-12α-hydroxy-5β-cholanate (1g) was oxidised with Jones reagent in acetone (20ml) for 1h at room temperature. The reaction mix was diluted with distilled water (80ml), extracted with ethyl acetate (50ml) and washed with distilled water (2x30ml) prior to evaporation to dryness *in vacuo*. The resulting gum was subsequently hydrolysed by refluxing for 1h in 90% ethanol (20ml) containing potassium hydroxide (1g). The precipitate resulting from the addition of 1M hydrochloric acid was collected, recrystallised once from aqueous methanol and twice from ethyl acetate and dried for 3h at 105°C to give 140mg of white crystals m.p. 218-219°C (219°C, Wieland and Kapitel, (1932)), which were homogenous by tlc.

3.2.3.2. Glycine and taurine-conjugated bile acids

Glycocholic acid, sodium taurocholate and sodium taurodeoxycholate were synthesised by the method of Tserng *et al* (1977). Glycodeoxycholic acid was prepared using a combination of the methods of Norman (1955) and Tserng *et al* (1977). The mixed anhydride was produced as described by Norman and reacted with ethyl glycinate which was liberated from ethyl glycinate hydrochloride by excess tributylamine. The resulting mixture was treated as described by Tserng *et al* (1977) and glycodeoxycholic acid crystallised as fine needles from aqueous ethanol. All the conjugates were dried at 105°C for 3 hours and were homogenous by tlc and hplc analysis. The yields and melting points obtained are presented in Table 3.3.

3.2.3.3. Synthetic bile acid conjugates

Sodium N-(aminomethanesulphonyl)-deoxycholate

Aminomethanesulphonic acid (m.p. 188-189°C) was produced

Table 3.3

Data from the glycine and taurine bile acid conjugate syntheses

Substance	Crystallised from	Yield (%)	Melting Point (°C) Found. Literature.
sodium taurocholate	ethanol-ethyl acetate	86	180-2 182-4 (Tserng *et al*, 1977)
sodium tauro-deoxycholate	ethanol	47	171-3 172-3 (Tserng *et al*, 1977)
glycodeoxycholic acid	aqueous ethanol	98	192-3 191-2 (Tserng *et al*, 1977)
glycocholic acid	aqueous ethanol	75	131-3 132-4 (Norman, 1955). 140-2 (Tserng *et al*, 1977)

by the method of Lacoste and Martell (1955) (m.p. 190-4°C) and used in the synthesis of sodium N-(aminomethanesulphonyl)-deoxycholate by the procedure of Tserng *et al* (1977). The acid form of the conjugate was extremely hygroscopic and so it was crystallised from absolute ethanol-ethyl acetate as the sodium salt. The conjugate was homogenous by tlc and hplc analysis and its melting point is given in Table 3.4.

N-(α-alano)-deoxycholic acid

N-(α-alano)-deoxycholic acid was synthesised by the method

of Tserng *et al* (1977) for glycodeoxycholic acid. However, the reported hydrolysis conditions were insufficient for the complete hydrolysis of the N-(α-alano)-deoxycholate ethyl ester. Consequently, after this step, the reaction mixture was dissolved in ethyl acetate and washed with 0.5M sodium bicarbonate solution. The bicarbonate washes were back-extracted with ethyl acetate and then acidified with hydrochloric acid to yield a white gum. This was crystallised from 60% aqueous ethanol as white needles of *N-(α-alano)-deoxycholic acid*. m.p. 211-213°C, homogenous by tlc and hplc analysis. Recrystallisation from acetone-petroleum ether (60-80°C) did not alter the melting point.
Found: C, 67.7; H, 9.7; N, 2.9
Calcd: C, 67.3; H, 9.8; N, 2.9 for $C_{27}H_{45}NO_5.H_2O$.

N-(β-alano)-deoxycholic acid

The β-alanine ethyl ester hydrochloride was produced by the esterification of β-alanine using anhydrous hydrogen chloride gas in absolute ethanol. It was crystallised from ethanol-ether as white needles in 85% yield (m.p. 52-3°C; c.f. 58°C, Mitra (1938); 69-70°C, Pollock and Stevens (1965)). N-(β-alano)-deoxycholic acid was then synthesised by the method of Tserng *et al* (1977). As the melting point did not correspond to the value reported by Lee and Whitehouse (1963) who used Norman's (1955) method, it was also produced by that method for comparison. However, the N-(β-alano)-deoxycholic acid produced from both syntheses were identical with respect to melting point, tlc and hplc analysis and so the compound was chemically characterised (Table 3.4). N-(β-alano)-deoxycholic acid (Found: C, 69.4; H, 10.0; N, 3.0). $C_{27}H_{45}NO_5.\frac{1}{2}H_2O$ requires C, 68.6; H, 9.8; N, 3.0).

3.2.4. Solvents

For crystallisation, tlc mobile phases and the freeze-dry extraction procedure, the solvents were usually B.D.H. A.R. grade (B.D.H. Chemicals Ltd., Palmerston North, New Zealand).

Table 3.4

The characterisation of the synthetic conjugates

Substance[a]	Recrystallised from	tlc Rf	Melting Point (°C) Found	Melting Point (°C) Lit.[d]	Equivalent Weight Found	Equivalent Weight Calcd.
Sodium N-(amino-methanesulphonyl)-deoxycholate	ethanol-ethyl acetate	0.52[b]	183-5	175-180	-	-
N-(α-alano)-deoxycholic acid	aqueous ethanol	0.22[c]	211-13	-	486	481[e]
N-(β-alano)-deoxycholic acid	methanol	0.16[c]	208-9	192	474	472[f]

a. All dried at 100°C over P_2O_5 at 4-6 kPa.

b. Ethylene dichloride: acetic acid: water, 10:10:1. Rf's: NaTC, 0.26; NaTD, 0.42.

c. Iso-octane: ethyl acetate: acetic acid, 10:10:2. Rf's: GD, 0.16.

d. Lee and Whitehouse (1963).

e. $C_{27}H_{45}NO_5.H_2O$

f. $C_{27}H_{45}NO_5.\frac{1}{2}H_2O$

The chloroform used for solvent extraction was recovered, washed with water and dried over anhydrous $MgSO_4$ before being redistilled. It was stored for reuse in the presence of 2% v/v ethanol. Analytical grade solvents and distilled water were glass-redistilled for use in hplc and preparative tlc. Commercial grade methanol obtained from I.C.I. (NZ) Ltd., (Lower Hutt, New Zealand) was redistilled for use in the cleaning of glassware.

3.2.5 Other Chemicals

Chemicals used in fermentations and for the synthesis of bile acids were generally B.D.H. or May and Baker (Dagenham, England) analytical grade. Other chemicals and their source were:

B.D.H. Chemicals Ltd., (Palmerston North, New Zealand):
Lactose, 8-hydroxyquinoline, buffer tablets: pH 4.0, 7.0 and 9.2, phosphomolybdic acid. (Analar). taurine (Biochemical grade).

Diversey-Wallace Ltd., (Papatoetoe, New Zealand):
Pyroneg(R) detergent.

Koch-Light Laboratories, (Colnbrook, England):
Celite HyfloSuper-Cel.

New Zealand Industrial Gases Ltd., (Palmerston North, New Zealand): Oxygen-free nitrogen gas.

Sigma Chemical Company, (St. Louis, Mo., U.S.A.):
α-Alanine ethyl ester hydrochloride, anthrone, β-alanine, glycine ethyl ester hydrochloride, N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ).

Swift Consolidated (NZ) Ltd., (Wellington, New Zealand):
Dow-Corning antifoam A.F. emulsion.

The Upjohn Company, (Kalamazoo, Michigan, U.S.A.):
Cycloheximide as Acti-dione(R) (85-100% w/w cycloheximide)

Most other chemicals were obtained as A.R. or laboratory grade from B.D.H. Chemicals Ltd., or May and Baker Ltd.

3.3. Organisms

The fungi used and their source culture collections were:

Aspergillus ochraceus (Wilhelm)	IFO 4071
Cercospora melonis	CBS 162.60
Cochliobolus intermedius	IMI 52980
Curvularia coicis	IFO 7278
Curvularia fallax	IFO 8885

All were maintained by subculture every 3 months on potato dextrose agar slopes, which were incubated at 30°C for 3-5 days and then stored at 4°C.

3.4. Sterilisation of Media

Potato dextrose agar and the glucose-peptone medium were sterilised by autoclaving at 121°C for 15 minutes. Malt extract broth was similarly sterilised at 115°C for 10 minutes. The bile acid conjugates were stable to this treatment as determined by tlc analysis.

In some cases, certain solutions were sterile-filtered through a 0.45 μm membrane filter (Oxoid Ltd., London, England) prior to their aseptic addition to cultures.

3.5. Cleaning of Glassware

All glassware was washed in hot Pyroneg (R) solution, rinsed in tap water and hot air dried. Prior to drying, fermentation equipment was rinsed with distilled water while glassware used for bile acid analysis was rinsed with methanol. Items used in the synthesis of bile acids or their isolation from fermentation medium were soaked in dilute sodium hydroxide after the detergent wash and tap water rinse. They

were then thoroughly rinsed with distilled water and hot-air dried. Glassware used for the storage of hplc solvents was treated in chromic acid after the detergent wash, then rinsed thoroughly with distilled water prior to drying.

3.6. Analytical Methods

3.6.1. pH Measurement

Solution pH was recorded from a Metrohm pH meter E520 (Metrohm AG., Herisau, Switzerland) which was regularly calibrated with standard pH buffer solutions.

3.6.2. Dry Weight Determination

A known volume of fungal culture was filtered through a pre-weighed filter paper on a buchner funnel. The fungal mass was washed thoroughly with distilled water and then dried at $105 \pm 2^{o}C$ to constant weight.

3.6.3. Total Carbohydrate Analysis

The total carbohydrate concentration in fermentation samples was determined by an adaptation of the anthrone method of Ghosh *et al* (1960). The sample was diluted to between 0 and 100 µg total carbohydrate per ml of solution. Two ml of an icecold, 0.2% w/v solution of anthrone in concentrated sulphuric acid were added to 1 ml of the diluted sample and thoroughly mixed. The samples were heated for 15 minutes in a boiling water bath and then cooled in an ice-bath. Two ml of 66% v/v sulphuric acid were added and the sample thoroughly mixed. The absorbance of the samples was read at 625 nm against a reagent blank using a Model 101 Hitachi spectrophotometer (Hitachi Koki Co. Ltd., Tokyo, Japan). Results were calculated from a standard curve using glucose in the range 0-100 $\mu g ml^{-1}$. All determinations were performed in duplicate.

3.6.4. Thin Layer Chromatography

Qualitative thin layer chromatographic analysis of samples for bile acid was performed under tank saturation conditions on glass plates (5 x 20 cm or 10 x 20 cm) coated with 0.25 mm silica gel which had been activated by heating at 105°C for 30 minutes prior to use. The solvent systems used to develop the plates were:

Part 1, for free bile acids and carboxylic acid conjugates:

a. isooctane:ethylacetate:acetic acid, 10:10:2 (Heftmann, 1967).
b. benzene:dioxan:acetic acid, 75:20:2 (Heftmann, 1967).
c. cyclohexane:ethylacetate:acetic acid, 7:23:3 (Heftmann, 1967).
d. isooctane:ethylacetate:acetic acid, 50:50:0.7 (Heftmann, 1967).
e. toluene:acetic acid:water-upper phase, 5:5:1 (Gänshirt *et al*, 1960).

Part 2, for conjugated bile acids:

f. ethylenedichloride:acetic acid:water, 10:10:1 (Gregg, 1966).
g. n-butanol:acetic acid:water, 10:1:1 (Gänshirt *et al*, 1960).

Chromatograms were visualised using 10% w/v phosphomolybdic acid in ethanol (Randerath, 1968) and heated at 105°C for 5 minutes. For the identification of the bile acids present, samples were run parallel to standard bile acids in at least three appropriate solvent systems. Where bile acid standards were not available, the published R_f values of Heftmann (1967) were used as a guide to the identity of unknown spots. If the presence of ketonic bile acids was suspected, a separate chromatogram was sprayed with methanol: sulphuric acid (1:1) and viewed under ultraviolet light (Heftmann, 1967).

For preparative thin layer chromatography approximately 70-100 mg of material was applied to a 20 x 20 cm, 1 mm thick, silica gel DG, tlc plate previously activated by heating at 105°C for 2 hours. The plates were developed under tank saturation conditions in a suitable solvent system. The resolved bands of material were then removed from the chromatogram and the bile acid eluted from the silica gel with methanol containing 1% v/v acetic acid (Shioda *et al*, 1969).

3.6.5. High Performance Liquid Chromatography

Quantitative analysis of samples for bile acid was performed using a Waters Associates model ALC/GPC 201 liquid chromatograph with a U6K septumless injector. A μBondapak C18 reverse-phase column (3.9 mm I.D. x 300 mm, Waters Associates Milford, Mass., U.S.A. or 4.0 mm I.D. x 250 mm, Bio-Sil ODS 10, Bio-Rad Laboratories, Richmond, California, U.S.A.) was used for all separations. Detection of the bile acids was with a Waters Associates R401 differential refractometer and the response was recorded on a CR 600 twin-pen, flat-bed chart recorder (J.J. Lloyd Instruments Ltd., Southampton, England). Analyses were conducted at ambient temperature with a solvent flow of 2.0 $mlmin^{-1}$, a refractometer attenuation of 4x or 8x and a chart speed of 10 cmh^{-1}.

For free bile acids and carboxylic acid conjugates, a mobile phase of methanol:1% v/v acetic acid (77:23) was used, while for sulphonic acid conjugates, the system of Bloch and Watkins (1978) was chosen (methanol:water, 65:35), although in some cases the ratio was increased to 73:27. The mobile phase was filtered and degassed prior to use through a 0.45 μm filter (Millipore Corporation, Bedford, Mass., U.S.A.). The retention volumes of the bile acids for the mobile phases used are presented in Table 3.5.

Samples containing a precise amount of an appropriate internal standard were injected in volumes of 30-80 μl

Table 3.5

The retention volumes of bile acids

Bile acid	Retention volume (ml) Mobile Phase 1	2
sodium taurocholate	-	6.0
sodium taurodeoxycholate	-	12.0
sodium N-(α-aminomethane-suphonyl)-deoxycholate	-	11.7
glycocholic acid	6.2	8.7
cholic acid	8.4	18.3
glycodeoxycholic acid	9.0	19.4
N-(α-alano)-deoxycholic acid	9.1	19.4
N-(β-alano)-deoxycholic acid	8.5	18.7
methyl 3α, 7α-diacetoxy-12α-hydroxy-5β-cholanate	11.5	28.3
deoxycholic acid	13.3	37.0

Mobile phase 1: 77:23 methanol:1% v/v acetic acid.
Conditions: μ-Bondapak C_{18} column, 2.0 $ml min^{-1}$, 30°C, 1800 psi.

Mobile phase 2: 73:27 methanol:water adaption of Bloch and Watkins (1978).
Conditions: Bio-Rad column, 2.0 $ml min^{-1}$, 25°C, 2200 psi.

and quantitated by peak height comparison to subsequent injections of a parallel standard of similar bile acid composition. All unknown samples were injected in duplicate, while parallel standards were in triplicate.

Where preparative hplc was employed to resolve and purify bile acid mixtures from fermentation broths the equipment, column and conditions described above were used. The bile acids were separated either as free acids, using a mobile phase of methanol:1% v/v acetic acid, 75:25, or as the methyl ester derivatives with the methanol:1% v/v acetic acid ratio at 72:28. Sample volumes of 0.2-1.0 ml were injected and the resolved fractions were pooled and checked for purity by tlc or analytical hplc analysis. Compounds separated in this way were crystallised from aqueous methanol.

3.6.6. Infra-red Spectrophotometry

A KBr disc containing 1% w/w bile acid, which had been dried at 100^{o}C over P_2O_5 under 0.2-0.4 kPa for 3 hours, in 75 mg potassium bromide was made with a model DMO-1 Beckman Evacuable KBr Minidie (Beckman-Riic Ltd., Glenrothes, Fife, U.K.). The disc was scanned at slow speed using a Perkin-Elmer 12-720 spectrophotometer.

3.6.7. Equivalent Weight Determination

The equivalent weight of a bile acid was determined by the method of Vogel (1959). An aqueous ethanolic solution containing a known weight of the bile acid was titrated with 0.05M sodium hydroxide, previously standardised against potassium hydrogen phthalate, using phenolphthalein indicator. All determinations were performed in duplicate.

3.6.8. Elemental Analysis

Elemental analysis of bile acid samples was performed by

the Chemistry Department of Otago University, New Zealand.

3.6.9. Mass Spectrometry

Mass spectrometric analysis of bile acid samples was performed by the Chemistry Department of Massey University, New Zealand. The samples were run by evaporation from a direct insertion probe on a MS 9 double-focussing Mass Spectrometer and the data was analysed by an A.E.I. DS 30 computer system.

3.7. Culture Conditions

3.7.1. Flask Culture

Experiments were predominantly conducted in 250 ml Erlenmeyer flasks containing 100 ml of medium. The bile acid substrate was initially present in the medium at a concentration of $0.5 g l^{-1}$, unless otherwise stated.

The inoculation procedure consisted of the aseptic addition of 1 ml per flask of a distilled water suspension of mycelia and spores taken from a potato dextrose agar slope of the fungus, which had been grown for a specified time at $30^{o}C$. The inoculated flasks were incubated at $30^{o}C$ for up to 25 days. Shake flasks were agitated either at 200 rpm on a New Brunswick Scientific Gyratory Shaker (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.A.), or at 250 rpm in a Gallenkamp Orbital Incubator (A. Gallenkamp and Co. Ltd., London, England). For sampling, whole flask contents were taken.

3.7.2. Fermenter Culture

For fermenter culture, a Multigen F2000 Benchtop culture apparatus (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.A.) was employed. A photograph of the

fermenter and its ancillary equipment is presented in Figure 3.1, while Figure 3.2 schematically illustrates its arrangement.

3.7.2.1. Equipment and instrumentation

The fermenter vessel was standard equipment for the culture apparatus and was constructed of pyrex glass with an ethylene-propylene head containing holes for the insertion of the various facilities required. The vessel head was held tightly in place by a stainless steel stopping plate bolted to a mounting ring. The vessel had a working volume of 1.5l and was volumetrically calibrated at 30^{o}C to facilitate the volumetric analysis of fermentation components.

Agitation was provided by an impellor assembly of 3, six-bladed turbines mounted vertically on the central impellor shaft. This was magnetically driven through the base of the fermenter vessel. The turbine heights on the shaft were adjustable but were normally fixed at heights of 33.5, 81.5 and 130 mm above the base of the vessel. The impellor speed control was calibrated in rpm by means of a stroboscope. A single baffle was used to ensure turbulent flow at the impellor speeds employed, as this is sufficient for small fermenters (Calam, 1969).

A solid-state temperature controller with a thermistor sensor maintained the fermenter temperature at 30$\pm$0.2^{o}C by means of a heating element operating against a constant cooling water flow. A thermometer was used as a visual check on the culture temperature.

A blower situated in the Multigen F2000 apparatus pumped air, *via* a needle control valve, to a Gap variable area flowmeter (Platon Flow Control Ltd., Basingstoke, Hampshire, England) which was calibrated from 100-1200 mlmin^{-1}. This metered air through a sterile, glasswool-packed filter before it was sparged through numerous, small holes at the

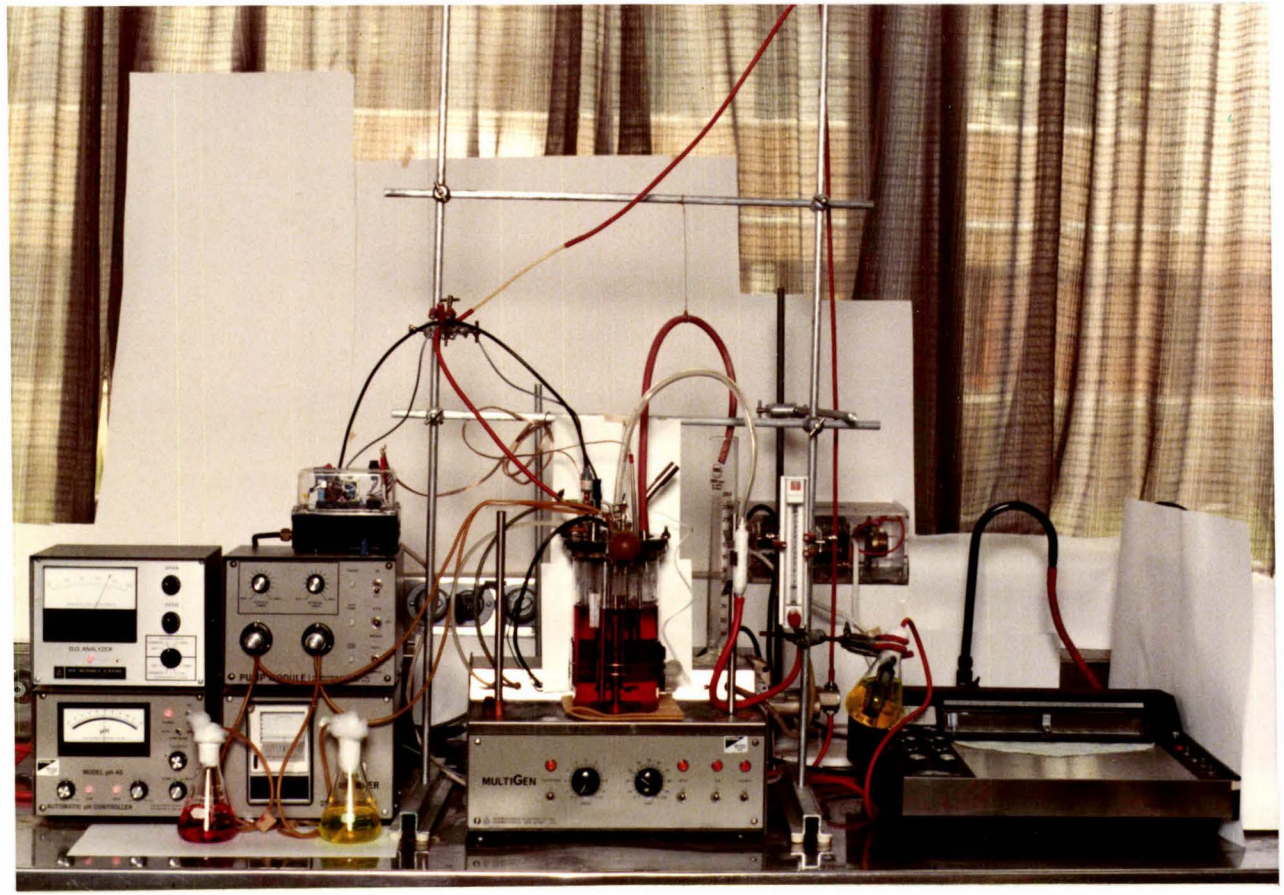


FIGURE 3.1 A photograph of the fermenter and its ancillary equipment.

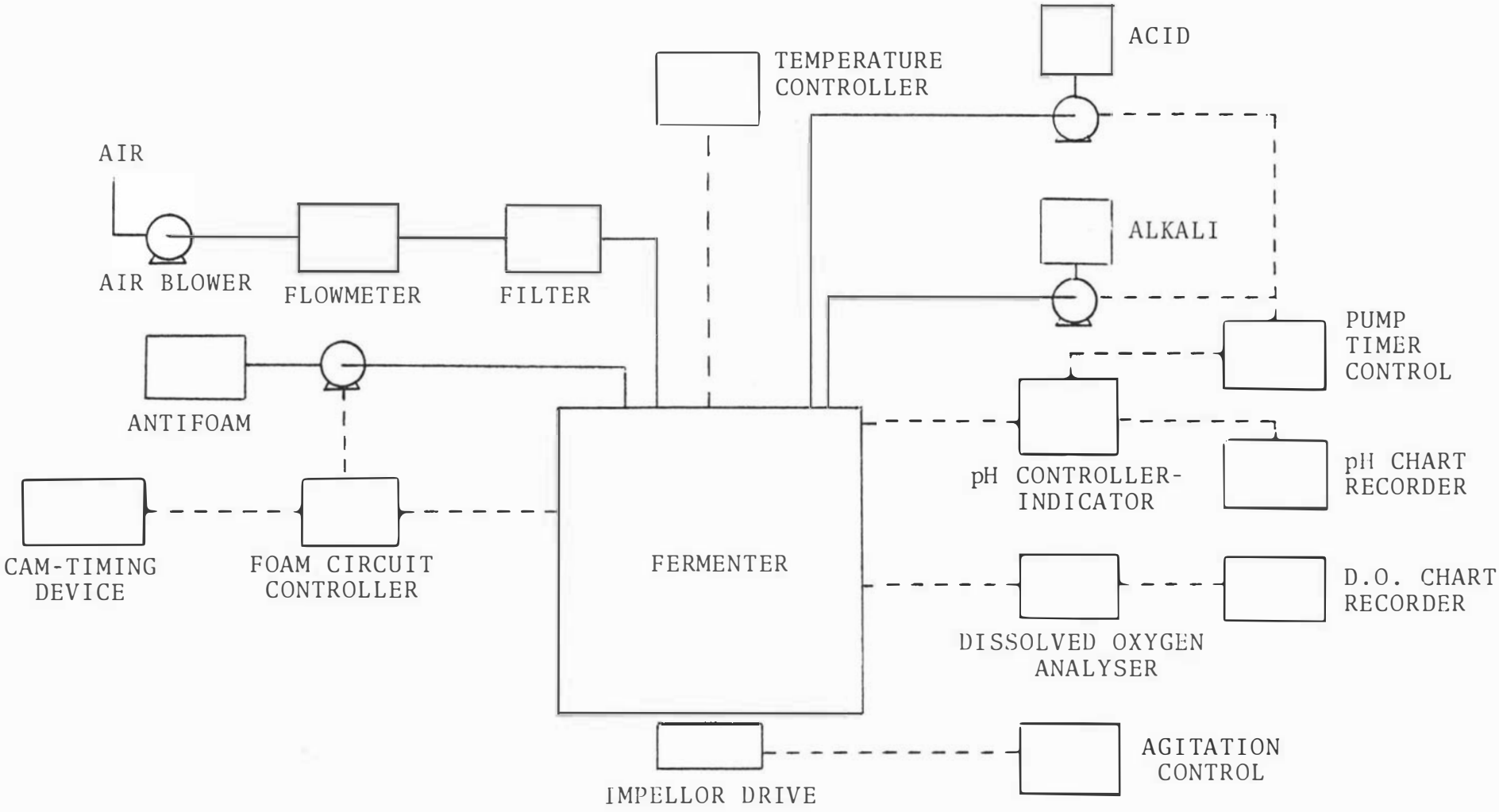


FIGURE 3.2 A schematic diagram of the fermenter and its ancillary equipment.

base of the hollow impellor shaft. The precision of the flow control was $\pm$ 25 $mlmin^{-1}$.

Exhaust gas was vented through a vertical tube with a sterile, non-absorbant cotton wool plug at the exit. The tube was passed into a 250 ml measuring cylinder to contain losses of fermenter contents, if they occurred.

The desired culture pH was maintained using an Automatic Mini pH Control System (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.A.) consisting of a pH 40 Controller module, a XpH 42 pump module and a pH 40 Recorder module with a XpH-75 Type 761-351B Ingold combination glass electrode (Ingold Electrodes Inc., Lexington, Mass., U.S.A.) to measure broth pH. Solutions employed to control pH were 0.5M sodium hydroxide and 0.5M hydrochloric acid. The pumps were controlled by a timing mechanism which followed a 20:10 second, pump on:off cycle as a safety measure. The fermentation pH was recorded on the recorder module strip chart.

The pH electrode was calibrated using pH 4.0, 7.0 and 9.2 buffer solutions prior to each fermentation. Occasionally the electrode required soaking for 2 hours in 0.1M hydrochloric acid, after a wash in detergent solution, to remove contaminating proteins. When not in use, the pH electrode was stored, after cleaning, in 3M potassium chloride solution.

All fermentation samples were monitored for pH to independently check the calibration of the fermenter pH control system, and any discrepancies were corrected.

Antifoam emulsion was included into the medium at a concentration of 0.1% w/v and an automatic foam control system was used to suppress the severe foaming generated by the high agitation and aeration rates employed during the fermentations. When foam contacted the teflon-sheathed, stainless steel sensor, the completed foam controller

circuit (Design Electronics, Palmerston North, New Zealand) activated a pump (M.P.L. Metering Pumps Ltd., London, England) to add a small dose of a sterile, 10% w/v aqueous antifoam solution to the fermenter. The destruction of the foam caused the circuit to be broken and the pump to stop. To avoid excessive addition of antifoam, the foam controller power input was activated 2 minutes out of every 15 minutes by a cam-timing device. Therefore two conditions had to be met to allow antifoam addition, first, the foam had to contact the sensor and second, the controller had to be actuated *via* the cam timer.

A galvanic, dissolved oxygen probe, Type M1016-0208 (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.A.) was used in conjunction with a model DO-40 Dissolved Oxygen Analyser (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.A.) and a 0-10 mV chart recorder (J.J. Lloyd Instruments Ltd., Southampton, England) to measure the dissolved oxygen tension in the fermenter. The system was calibrated prior to inoculation by flushing the fermenter vessel contents with oxygen-free nitrogen gas *via* the aeration system to obtain zero saturation conditions. Then the vessel was aerated vigorously for ten minutes to give 100% saturation, and the analyser and chart recorder spans set. When not in use, the dissolved oxygen probe was stored in distilled water.

3.7.2.2. Sterilisation methods

The fermenter vessel containing the medium, all items in the fermenter head except the pH and dissolved oxygen electrodes, the pH control solutions and all solution delivery systems were sterilised by autoclaving at 121^{o}C for 20 minutes. Any parts of piping open to the atmosphere were plugged with non-absorbent cotton wool and wrapped in aluminium foil prior to sterilisation. The 10% w/v antifoam solution was sterilised by autoclaving at 121^{o}C, for 15 minutes, 24 hours prior to fermenter assembly and resuspended overnight on a gyratory shaker.

The inlet air filter and any pipettes used to add solutions to the fermenter were sterilised by dry heat at 160°C for 2 hours. The pH and dissolved oxygen electrodes were sterilised by immersion in 2% v/v formaldehyde solution for 30 minutes followed by thorough rinsing with sterile, distilled water prior to their insertion into the fermenter vessel.

3.7.2.3. Inoculum preparation

The fungus required was subcultured on to a potato dextrose agar slope and incubated at 30°C for 72 hours. A 4 ml, sterile, distilled water suspension of mycelia and spores was then aseptically inoculated into 50 ml of sterile, glucose-peptone medium in a 150 ml Erlenmeyer flask and incubated at 30°C for 72 hours at 200 rpm on a gyratory shaker. The entire volume of culture, representing a 3.3% v/v inoculum, was then aseptically added to the fermenter vessel.

3.7.2.4. Operation

Each fermentation involved:

a. The preparation of the inoculum.
b. The preparation and sterilisation of the fermenter, medium and ancillary equipment.
c. The aseptic assembly of the fermenter and its ancillary equipment.
d. The calibration, sterilisation and insertion of the pH and dissolved oxygen electrodes.
e. The inoculation of the fermenter.
f. The operation of the fermenter in batch mode, usually for 6-8 days, according to the experimental conditions required. In some experiments, glucose was added to the culture during fermentation. Two modes of addition were used:
    1. A continuous mode in which a sterile-filtered solution of 400gl^{-1} glucose was added to the

fermenter by a reciprocating pump (M.P.L. Metering Pumps Ltd., London, England) set at 0.39 ml min^{-1} flow. The pump was actuated by a cam timing device on a cycle of 1.6 minutes in 15.

2. An incremental mode in which glucose solution prepared as above, was aseptically added to the fermenter manually as required.

g. The regular withdrawal of samples (*ca* 25 ml) from the fermenter for analysis. Prior to sampling, the sample system was flushed with approximately 5 ml of the culture to remove any resident "dead" volume. The fermenter, flush and sample volumes were recorded to enable volumetric analysis of fermentation components.

h. After the termination of the fermentation, the vessel and all equipment which was in contact with the fungal culture, was sterilised and cleaned.

3.7.2.5. Discussion

Pirt (1975) states that, due to problems of wall growth accretion which create a heterogeneous environment, a 2 l culture vessel is the minimum size practical when studying filamentous fungi in a fully-instrumented fermenter. The equipment used was chosen, recognising the potential problems, due to equipment constraints and the cost of the bile acid conjugate substrate. The long period of the fermentations often led to considerable wall growth accumulation. However, although this meant that the dry weight figures underestimated the actual biomass yields, the wall growth was not shown to affect bile acid metabolism significantly in terms of losses or yields of product. A duplicate fermentation of *Curvularia fallax* in the fermenter using 0.5gl^{-1} glyco-deoxycholic acid substrate yielded variation of dry weight and bile acid data of within $\pm$5%.

3.8. Cell-free Systems

3.8.1. Cell Production

The required fungus was subcultured on to a potato dextrose agar slope and incubated for 6 days at 30°C. This was then used to inoculate flasks containing glucose-peptone medium which were incubated for seven days at 30°C in a Gallenkamp Orbital Incubator at 250 rpm. No bile acid substrate was present in the medium. After this time, the flask contents were placed into 2 x 500 ml centrifuge tubes and centrifuged at 10,000 x g for 30 minutes, at 10°C, in a M.S.E. Hi-Spin 21 Centrifuge (M.S.E. Scientific Instruments, Sussex, England).

3.8.2. The Cell-free Filtrate

After centrifugation, the supernatant liquid was decanted from the tubes, adjusted to pH 6.5 with 1M sodium hydroxide and filtered successively through a 5.0 µm SM type filter (Millipore Corporation, Bedford, Mass., U.S.A.) and a sterile, 0.45 µm membrane filter. The sterile, cell-free filtrate was held at 4°C until used (no longer than 2 hours).

3.8.3. The Cell-free Extract

The mycelial mass remaining after centrifugation was re-suspended in 0.05M, pH 7.0 phosphate buffer and centrifuged at 7,500 x g for 15 minutes at 10°C. The supernatant liquid was discarded. The cell mass from each tube was suspended in 150 ml of phosphate buffer and homogenised in an ice-cooled Waring Blender (Waring Products Co., Winsted, Conn., U.S.A.) for 30 seconds at low speed, then 30 seconds at high speed. The homogenate was centrifuged at 7,500 x g for 15 minutes at 10°C to separate the cell debris from the cell-extract. The latter was decanted from the tubes and sterile filtered as for the cell-free filtrate.

3.8.4. Incubation Conditions

Twenty-five ml aliquots of either the cell-free filtrate or the cell-free extract solutions and 5 ml aliquots of a $6.0 g l^{-1}$ sterile, aqueous bile acid solution (pH 7.0) were aseptically transferred into sterile, 150 ml Erlenmeyer flasks to give a final bile acid concentration of $1.0 g l^{-1}$. A 5 ml sample was aseptically removed from each flask for pH and bile acid analysis and the remaining solution was incubated at $30^{o}C$ on a New Brunswick Scientific gyratory shaker. After 12 hours, each flask was analysed for pH and 1 ml of 1M sodium carbonate was added to inhibit further enzymic reaction. Two 10 ml samples were taken for bile acid analysis by tlc and hplc. Duplicate incubations were performed for each combination of bile acid and cell-free system tested.

3.9. Bile Acid Extraction and Sample Preparation

3.9.1. Solvent Extraction

The following bile acids were quantitatively estimated after chloroform extraction, unless otherwise stated: all free bile acids, glycodeoxycholic acid, N-(α-alano)-deoxycholic acid and N-(β-alano)-deoxycholic acid.

A known volume of culture filtrate (*ca* 20-25 ml) was acidified to pH 2 with 5M hydrochloric acid and extracted with chloroform (3 x 20-25 ml). The combined extracts were filtered and dried *in vacuo*. The residue was dissolved in 1-1.5 ml of 95% methanol containing a known amount (5-15 mg) of the internal standard, methyl 3α, 7α-diacetoxy-12-hydroxy-5β-cholanate and swinney-filtered (Millipore Corporation, Bedford, Mass., U.S.A.) prior to hplc analysis. For the qualitative detection of bile acids by tlc, the samples were not swinney-filtered and no internal standard was added.

3.9.2. Freeze-Dry Extraction

Glycocholic acid, and the taurine conjugates including sodium N-(aminomethanesulphonyl)-deoxycholate, were quantitatively extracted from fermentation samples using this method. Quantitative estimation of the bile acids listed in the previous section was also possible using this technique.

A known volume of the culture filtrate (*ca* 20-25 ml) was freeze-dried on a Virtis model 10-020 benchtop freeze-drier (Virtis Company Inc., Gardiner, N.Y., U.S.A.). The dried material was extracted with hot methanol containing 3% v/v acetic acid (2 x 15 ml) and the internal standard (5-15 mg), which was either glycocholic acid or sodium taurodeoxycholate. Non-bile acid material in the methanol extract was precipitated by the addition of acetone (20 ml) and removed by centrifugation. The supernatant liquid was decanted, filtered and evaporated to dryness *in vacuo*. The residue was dissolved in 1-1.5 ml of 95% methanol, swinney-filtered and analysed by hplc.

3.10. Product Characterisation

All infra-red spectra are reproduced in Appendix 1.

3.10.1. The Action of *C. melonis* CBS 162.60 on Glyco-deoxycholic acid

C. melonis CBS 162.60 was inoculated into glucose-peptone medium (2 x 100 ml) containing $10gl^{-1}$ glycodeoxycholic acid, and incubated at $30^{o}C$ and 250 rpm in a Gallenkamp Orbital Incubator.

After 20 days, the cultures were adjusted to pH 10 with 1M sodium hydroxide and slowly shaken for 30 minutes. The mycelium was removed by filtration and the filtrate (*ca* 200 ml) was acidified with hydrochloric acid to pH 2, and then

extracted with chloroform (4 x 200 ml). The combined chloroform extracts were evaporated to ¼ of the initial volume *in vacuo* and washed with distilled water (2 x 100 ml). The chloroform layer was then filtered and evaporated to dryness *in vacuo*. The residue was crystallised repeatedly from water-saturated methyl ethyl ketone to a constant melting point (169-171°C)*. The microbially-produced deoxycholic acid was dried over P_2O_5 at 100°C for 3 hours *in vacuo*. Authentic deoxycholic acid (m.p. 169-171°C) was crystallised from the same solvent and subjected to the same drying procedure as that used for the microbial product. They were identical by the criteria of mixed melting point (169-171°C) and the superimposibility of IR spectra.

3.10.2. The Action of *C. melonis* CBS 162.60 on Glycocholic acid

C. melonis CBS 162.60 was grown on glucose-peptone medium (2 x 100 ml) containing 5.0gl^{-1} glycocholic acid in shake flask culture at 30°C, on a New Brunswick Scientific gyratory shaker at 200 rpm.

After 15 days, the cultures were adjusted to pH 10 with 1M sodium hydroxide and slowly shaken for 30 minutes. The mycelium was removed by filtration and the filtrate (*ca* 200 ml) freeze dried on a Virtis bench-top freeze drier. The hygroscopic residue was dissolved in distilled water (30 ml), acidified to pH2 with hydrochloric acid and extracted with chloroform (4 x 50 ml). The pooled chloroform extracts were washed with distilled water (2 x

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* Normally, deoxycholic acid is crystallised from water-saturated methyl ethyl ketone as a fatty-acid complex which has melting points of 165-192°C (Sobotka & Goldberg, 1932). Although such a complexed form was obtained when deoxycholic acid was recovered from culture broths with initial substrate concentrations of 0.5gl^{-1}, the deoxycholic acid crystallised from this fermentation was not fatty-acid complexed. A possible explanation may be that the high deoxycholic acid concentrations present in the broth (*ca* 9.0gl^{-1}), due to the high yields of hydrolysis at the substrate concentration employed, were greatly in excess of the available fatty acid concentration.

50 ml) and then dried *in vacuo* to yield a white gum, which was homogenous on tlc with a R_f identical to that of authentic cholic acid. Crystals were obtained from a two-phase chloroform-water system as colourless needles and were dried over P_2O_5 at 100°C for 3 hours *in vacuo* (m.p. 197-9°C). Identity of the microbially-produced cholic acid with authentic cholic acid crystallised from the same solvent and similarly dried (m.p. 198-9°C) was established by mixed melting point (197-199°C) and the superimposibility of IR spectra.

3.10.3. The Action of *C. fallax* IFO 8885 on Glycodeoxycholic acid

C. fallax IFO 8885 was grown on glucose-peptone medium, containing $0.5gl^{-1}$ glycodeoxycholic acid, in shake flask culture at 30°C and on a New Brunswick Scientific gyratory shaker at 200 rpm.

After 20 days, the spent medium underwent normal sample preparation and *ca* 20 ml was subjected to the chloroform extraction method. The product of glycodeoxycholic acid hydrolysis exhibited an identical R_f value to that of authentic deoxycholic acid run in parallel in the tlc solvent systems a,b,c and e. Similarly an identical retention volume was observed by hplc analysis.

3.10.4. The Action of *C. coicis* IFO 7278 on Glycocholic acid

Three runs, each of 20 x 100 ml in 250 ml Erlenmeyer flasks, of *C. coicis* IFO 7278 grown on glucose-peptone medium containing $1.0gl^{-1}$ glycocholic acid, were incubated at 30°C for 11 days in a Gallenkamp Orbital Incubator at 250 rpm.

After incubation, the culture broth in each flask was adjusted to pH 10 with 1M sodium carbonate and slowly

shaken for 30 minutes. The mycelium was removed by filtration and the combined filtrates (*ca* 2 l per run) were freeze dried on a Virtis bench-top freeze drier. The hygroscopic residue was dissolved in hot, distilled water (100 ml), acidified with hydrochloric acid to pH 2 and extracted with chloroform (3 x 100 ml, 2 x 50 ml). The pooled, chloroform extracts were evaporated *in vacuo* to ¼ of their initial volume, washed with distilled water (2 x 100 ml) and taken to dryness *in vacuo* (0.35-0.45 g per run).

For the first two runs performed, preparative tlc using the solvent system isooctane:ethylacetate:acetic acid, 10:10:2, was employed to resolve the bile acid products, while for the third run, preparative hplc of the underivatised bile acids was used.

The preparative tlc fraction comprising mainly cholic acid was eluted from the silica gel and taken to dryness *in vacuo*. The material was crystallised as needles from aqueous ethanol until homogenous by tlc and dried over P_2O_5 at 100°C for 3 hours *in vacuo*. The microbially-produced cholic acid (m.p. 195-7°C) was identical to authentic cholic acid (m.p. 195-6°C) crystallised from aqueous ethanol and dried under the same conditions, by the criteria of mixed melting point (194-6°C) and the superimposibility of IR spectra.

The fraction eluted from preparative tlc as deoxycholic acid was taken to dryness *in vacuo*, then crystallised from water-saturated methyl ethyl ketone. The crystals were washed with chloroform prior to their recrystallisation from water-saturated methyl ethyl ketone to give 3 mg of crystals (m.p. 165-192°C) after drying at 105°C for 3 hours. They were homogenous by tlc with an identical R_f to that of authentic deoxycholic acid in all 5 solvent systems "a" to "e". Despite the tlc evidence of homogeneity, the melting point indicates that a substantial proportion of the product was present as the fatty acid-deoxycholic acid complex (m.p. 165-192°C, Sobotka and Goldberg, 1932). In this

case the criterion of tlc Rf is invalidated since the fatty acid-deoxycholic acid complex runs on the solvent front.

The dried, chloroform extract from Run 3 was, therefore, dissolved in methanol (10 ml), swinney filtered, and resolved by preparative hplc into a cholic acid fraction and a deoxycholic acid fraction. The latter was taken to dryness *in vacuo*. Tlc analysis of this fraction revealed substantial contamination from cholic acid and so it was subjected to a second preparative hplc separation. The resulting deoxycholic acid fraction was homogenous by both tlc and hplc analysis and had an identical hplc retention volume to an authentic deoxycholic acid standard run in parallel (32.0 ml). The microbial deoxycholic acid crystallised from a two-phase chloroform-water solvent to yield 3 mg of crystals. Its melting point was similar in behaviour to that of authentic deoxycholic acid also crystallised from the same solvent, both sintering at 140-5^{o}C, m.p. 169-171^{o}C. However, the microbial product was contaminated with a considerable amount of material which remained unmelted at 290^{o}C.

3.10.5. The Action of *C. melonis* CBS 162.60 on Glycodeoxycholic acid in the Fermenter at pH 7.5

C. melonis CBS 162.60 was inoculated into glucose-peptone medium (1.5l) containing 0.5gl^{-1} glycodeoxycholic acid and cultured in a fermenter at pH 7.5 and 30^{o}C. The aeration rate was reduced from 1 lmin^{-1} to 0.25 lmin^{-1} after 24h and glucose increments were added at appropriate intervals.

After 120h of fermentation, the culture was adjusted to pH 10 with 1M sodium hydroxide and slowly stirred for 30 minutes. It was then filtered to remove the mycelium and the filtrate was freeze-dried. The hygroscopic residue was redissolved in distilled water (200 ml), acidified to pH 2 with hydrochloric acid and extracted with chloroform (4x200 ml). The combined chloroform extracts were evaporated to ¼ of initial volume *in vacuo*, washed with distilled water (2x100 ml),

filtered, and evaporated to dryness *in vacuo* to yield 890 mg of gum.

The gum was dissolved in methanol and two drops of concentrated hydrochloric acid were added. After allowing to stand overnight, the mixture was neutralised with pyridine and then evaporated *in vacuo* to a brown oil. Diethyl ether was added and the resulting gum and liquid filtered through glass wool, the residue being washed thoroughly with diethyl ether. Tlc analysis of the filtrate demonstrated complete esterification of the bile acids present.

The ethereal filtrate was evaporated to dryness *in vacuo* and the residue was refluxed with Girard T reagent after the method of Fieser and Fieser (1967). The solution was then cooled, the ethanol removed *in vacuo* and the residue partitioned between diethyl ether and distilled water.

The aqueous phase containing the Girard T bile acid derivatives was treated with 2 ml of concentrated hydrochloric acid, allowed to stand overnight at room temperature and then extracted with ethyl acetate. The ethyl acetate layers were washed with distilled water, dried over anhydrous sodium sulphate and evaporated *in vacuo* to yield 80 mg of material. Tlc analysis demonstrated the presence of two side-products, metabolites A and B. These were then resolved by preparative hplc as the methyl ester derivatives as described in Section 3.6.5. to yield 7mg of white crystals (Fraction A) and 2mg of white needles (Fraction B), both from aqueous methanol. The fractions were partially characterised by the melting point of the crude crystals, tlc and hplc analysis and mass-spectrometry. The results are presented in Section 4.5.

3.11. Calculations

3.11.1. Hplc Data Analysis

Quantitation of bile acids by hplc was accomplished by peak height measurement using an internal standard in the sample and comparison with a parallel standard of a similar,

known bile acid composition.

From the parallel standard a response factor, F_B, was calculated for each component bile acid, B.

$$F_B = \left(\frac{IS}{B}\right)_P \Big/ \left(\frac{IS}{B}\right)_W$$

where:

$\left(\frac{IS}{B}\right)_P$, is the average of the ratio of internal standard:bile acid peak heights from triplicate injections.

$\left(\frac{IS}{B}\right)_W$, is the weight ratio of the internal standard:bile acid in the standard.

The response factors were used to calculate the weight of the bile acids in the sample.

$$B_W = IS_W * \left(\frac{B}{IS}\right)_{PX} * F_B$$

where: B_W, is the weight of the bile acid in the sample (mg).

IS_W, is the weight of the internal standard in the sample (mg).

$\left(\frac{B}{IS}\right)_{PX}$, is the ratio of the bile acid:internal standard peak heights from the sample chromatogram.

F_B, is the response factor for the bile acid.

For each sample, duplicate injections were performed, the B_W calculated for each and averaged for the final result. This procedure was repeated for each "unknown" bile acid present in the sample.

3.11.2. Mass Balances

For the mass balance of fermentation components, their concentration at any time, as determined by the quantitative analysis of a sample after compensation for changes in fermenter volume, was compared to their concentration in the fermenter at zero time. Mass balance data represent the total bile acid present after a given time of fermentation as estimated by hplc analysis, expressed as a percentage of the bile acid initially present.

3.11.3. Bile Acid Data Analysis

For each sample, the corrected weight of each bile acid in the sample was multiplied by the fermenter volume (1.5l) and divided by the sample volume. These were converted to a molar basis for the calculation of percentage data.

3.12. Discussion of Methods

The three synthetic bile acid conjugates produced were structural analogues of the natural compounds with respect to the amino acid moiety. The synthesis of sodium N-(aminomethanesulphonyl)-deoxycholate , with its α-amino, sulphonic acid structure, and N-(β-alano)-deoxycholic acid with its β-amino, carboxylic acid structure, has been previously reported by Lee and Whitehouse (1963) using the method of Norman (1955). The synthesis of N-(α-alano)-deoxycholic acid was however, novel.

The sodium N-(aminomethanesulphonyl)-deoxycholate produced by the route of Tserng *et al* (1977) was similar in melting point to that of the compound reported by Lee and Whitehouse (1963) (Table 3.4). The data from the equivalent weight determinations and elemental analysis of the N-(β-alano)-deoxycholic acid synthesised by the methods of both Norman (1955) and Tserng *et al* (1977) suggested that the conjugate was associated with solvent (Table 3.6). This may be responsible for its higher melting point (208-9^{o}C) compared to that of Lee and Whitehouse (1963) (192^{o}C). However a melting point of 188-190^{o}C was initially obtained for the conjugate crystallised from acetone and made by the procedure of Tserng *et al* (1977) but further crystallisation yielded the higher figure. The most probable form of N-(β-alano)-deoxycholic acid produced was the hemi-hydrate (βD.½$H_2$0), however, the elemental analysis and equivalent weight data were unable to differentiate between that and the other possible solvent associations contained in Table 3.6.

Table 3.6

Formulae suggested for N-(β-alano)-deoxycholic acid

Suggested Formula		Equivalent Weight	Elemental Analysis C(%)	H(%)	N(%)
Found		474	69.4	10.0	3.0
βD	Calcd.	463	69.7	9.8	3.0
$\beta D.\frac{1}{3}CH_3OH$	Calcd.	474	69.2	9.85	3.0
$\beta D.\frac{1}{3}\ H_2O$	Calcd.	469	69.1	9.8	3.0
$\beta D.\frac{1}{2}H_2O$	Calcd.	472	68.6	9.8	3.0

βD - N-(β-alano)-deoxycholic acid.

The data presented in Table 3.4 for N-(α-alano)-deoxycholic acid suggested that the hydrate was produced. The synthesis of this conjugate by the method of Tserng *et al* (1977) was facile except for the incomplete hydrolysis of the ethyl ester. This may have been due to the methyl function on the α-carbon of the alanine residue hindering the hydrolysis reaction. The bicarbonate wash successfully recovered the hydrolysed product free of esterified reactant.

A thorough error analysis of the procedure for the quantitation of bile acids by hplc was performed by Archer (1980) who found a variation within ± 10% in the worst case. Quantitation of cholic acid was accurate to ± 5% within 95% confidence limits. Except for cholic acid and glycodeoxycholic acid, all bile acids analysed by hplc were completely resolved from each other (Table 3.5). The hplc retention volumes of the alanine deoxycholic acid conjugates and of sodium N-(aminomethanesulphonyl)-deoxycholate were similar to glycodeoxycholic acid and sodium taurocholate respectively.

The variations in solubility among the bile acids studied precluded the use of one quantitative method for their analysis, therefore two methods were designed.

The chloroform extraction method was developed because of its rapidity, versatility and convenience for a large number of samples. The partition coefficients of glycocholic acid, glycodeoxycholic acid, cholic acid and deoxycholic acid between chloroform and water were determined to predict the expected recoveries of these bile acids. Close agreement was found, using these data, between the predicted and actual recoveries of the bile acids from glucose-peptone medium (Table 3.7).

However the partition coefficient of glycocholic acid was too high to realise acceptable recoveries, and the polar nature of the taurine conjugates rendered the use of solvent extraction unsuitable for their quantitation in fermentation samples. Consequently, the freeze-dry

Table 3.7

Recoveries of bile acids
by chloroform extraction

Bile Acid	Partition Coefficient[a] (D) mean	δ	Predicted Recovery[a] (%)	Recovery from medium[b] mean	δ
GC	42	-	6.8	-	-
GD	0.73	0.01	92.5	92.0	2.2
CA	0.77	0.15	91.8	90.3	2.4
DC	0.13	0.01	99.8	96.9	2.5

a. See Appendix 2.

b. From quadruplicate determinations using glucose-peptone medium containing $0.5 g l^{-1}$ bile acid.

δ The standard deviation.

extraction method was developed for these compounds. Table 3.8 presents recovery data for bile acids estimated by this technique.

Table 3.8

Recoveries of bile acid from medium by freeze-dry extraction

Bile Acid	Recovery (%) mean	δ
TC	100.0	1.3
GC	95.7	1.5
GD	95.2	-
CA	95.4	0.6
DC	101.5	-

δ, the standard deviation.

The freeze-dry extraction method gave acceptable recoveries for all bile acids studied and had advantages of simplicity, low cost and rapidity, once freeze-drying was completed. Generally, however, its samples for hplc analysis were more contaminated than those obtained from chloroform extraction and contained residual acetic acid.

CHAPTER 4

THE HYDROLYSIS OF GLYCINE BILE ACID CONJUGATES BY C. MELONIS

4. THE HYDROLYSIS OF GLYCINE BILE ACID CONJUGATES BY C. MELONIS

4.1. Introduction

Reports of the fungal hydrolysis of glycine conjugates, usually glycocholic acid, have demonstrated that a commercial process employing this ability of fungi may be feasible. However, only recently has any systematic research been performed (Maddox and Chong, 1978; Chong *et al*, 1980) and little is known concerning the quantitative aspects of this transformation. The aim of the work described in this chapter was to generate some quantitative data on this subject.

Two opposing approaches to the problem were considered. These were, firstly, the analysis of hydrolysis simply as a "capsule-system" without an attempt to study its component mechanisms, or secondly, a thorough biochemical analysis of the system. However, neither approach was entirely suitable, since the system was found to be too complex to justify the oversimplified basis of the former, while the equipment, expertise and time necessary for the latter, were not available. Consequently the programme attempted to reconcile both approaches to maximum advantage.

The work concentrated on the study of those factors significantly influencing the yield of the glycine conjugate hydrolysis. Obviously the choice of the microorganism to effect the transformation is of primary importance to the success of any fermentation process and involves a major part of a development programme. However, the extensive screening of microorganisms for their ability to hydrolyse glycine conjugates was considered to be outside the scope of this project. Hence, *Cercospora melonis* CBS 162.60 was selected for study due to its apparently superior performance compared to some other fungi. It yielded the most rapid hydrolysis of glycocholic acid in shake flask culture (Maddox and Chong, 1978) and gave the highest yield of deoxycholic acid in a fermenter trial of glycodeoxycholic

acid hydrolysis, when compared to *Curvularia fallax* IFO 8885 and *Cochliobolus intermedius* IMI 52980. The former was randomly selected for the comparison, while the latter gave a similar performance to *C. melonis* in shake flask culture on glycocholic acid (Maddox and Chong, 1978).

4.2. Shake Flask Studies

4.2.1. The Course of Hydrolysis

The hydrolysis of the glycine conjugates, each at a concentration of $0.5 g l^{-1}$ in glucose-peptone medium, was quantitatively monitored in shake flask culture to act as a basis for later work.

The mycelial dry weight, culture pH and total carbohydrate concentrations are presented in Figure 4.1. The presence of the glycine conjugates had no significant effect on the growth of the fungus compared to that of a control, and active growth ceased after 5 days of fermentation. The culture pH of the control gradually increased from pH 5.0 to pH 5.5 during the idiophase and, therefore, was considerably lower than that of cultures containing the glycine conjugates. The utilisation of carbohydrate by the fungus was virtually complete after 8 days of fermentation and was not affected by the presence of the conjugates.

The course of the hydrolysis of glycodeoxycholic acid and glycocholic acid are plotted in Figures 4.2 and 4.3 respectively. The mass balance data represent the bile acid accounted for by sample extraction, expressed as a percentage of the initial amount present. Cholic acid and deoxycholic acid accumulated as the sole products of the hydrolysis of glycocholic acid and glycodeoxycholic acid respectively. Both substrates were completely utilised after 12 days of fermentation. The maximum product yields were 50% and 22% for cholic acid and deoxycholic acid respectively, after 11 days of fermentation. Therefore, on the basis of this criterion, glycocholic acid hydrolysis was superior to that

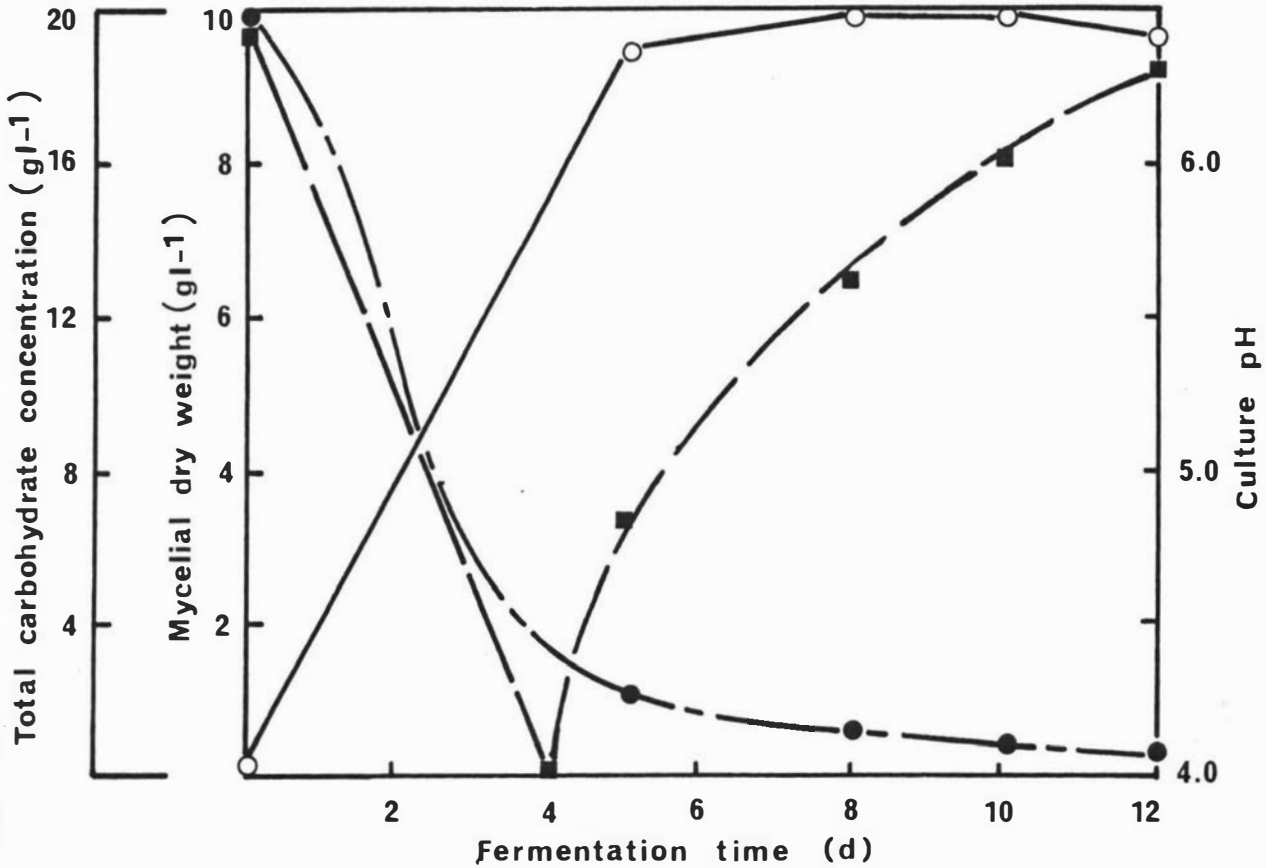


FIGURE 4.1 A typical shake flask culture of *C. melonis* in the presence of 0.5gl^{-1} glycine conjugates in glucose-peptone medium. Mycelial dry weight, ○——○; pH, ■— —■; total carbohydrate as glucose, ●— - —●. The initial pH was pH 6.5.

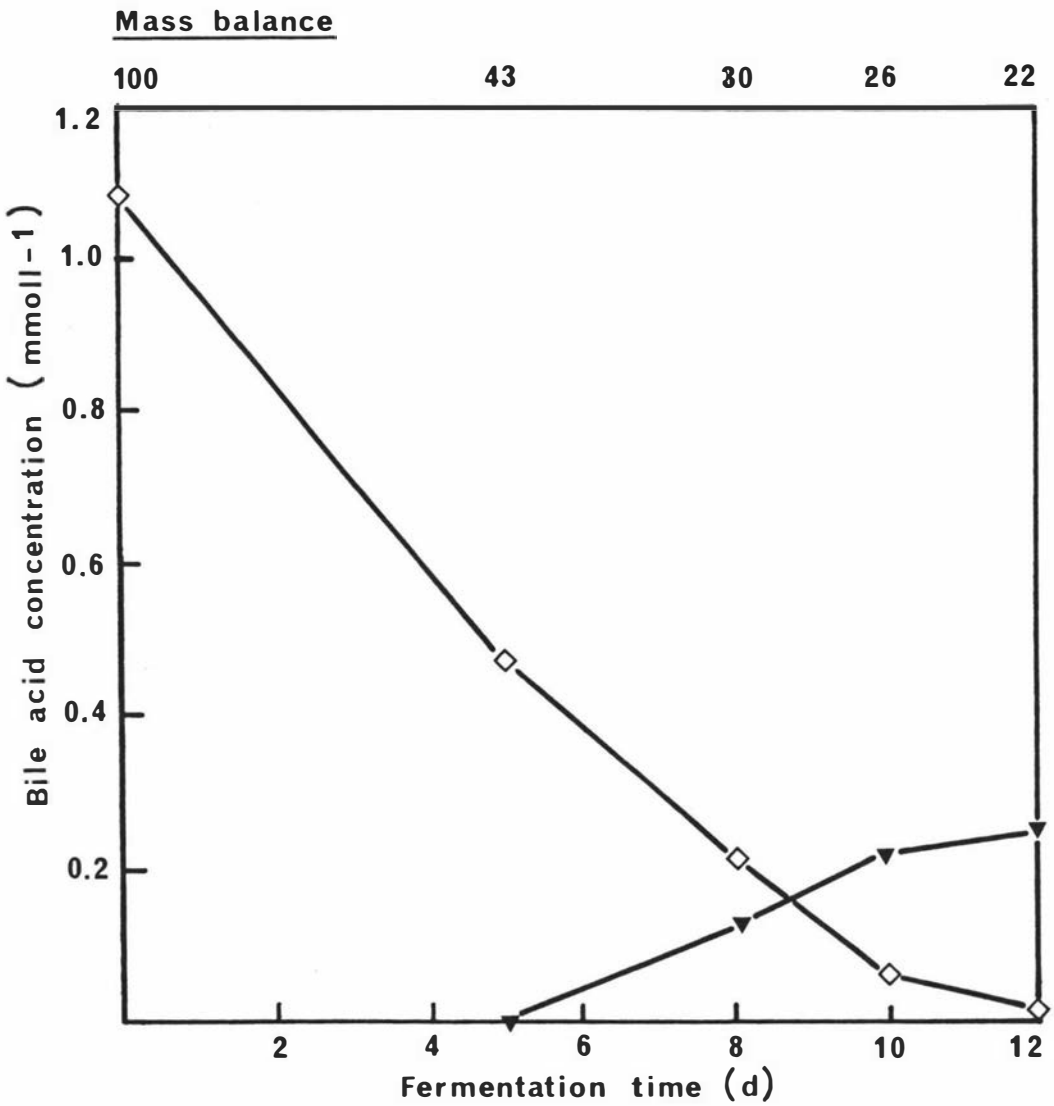


FIGURE 4.2 The hydrolysis of 0.5gl^{-1} glyco-deoxycholic acid by *C. melonis* in glucose-peptone medium. Glyco-deoxycholic acid, ◇——◇ ; deoxycholic acid, ▼——▼ .

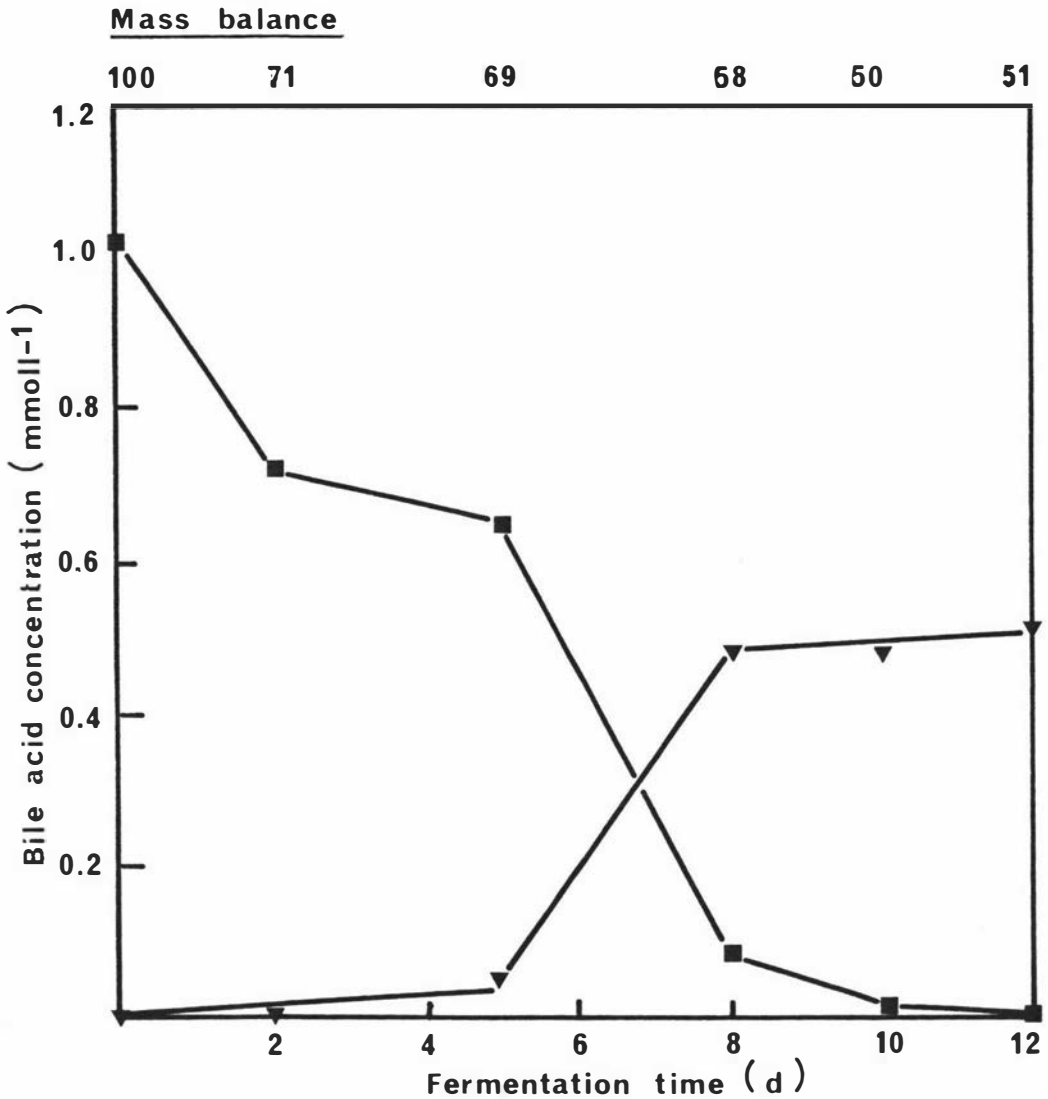


FIGURE 4.3 The hydrolysis of 0.5gl^{-1} glycocholic acid by *C. melonis* in glucose-peptone medium. Glycocholic acid, ■——■; cholic acid, ▼——▼.

of glycodeoxycholic acid.

The comparatively poorer yield of deoxycholic acid from glycodeoxycholic acid was due to the higher amounts of bile acid unaccounted for by the mass balance. Thus, after 10 days of fermentation, 74% of the bile acid initially present was unrecovered compared to 50% for glycocholic acid. Since the major portion of the losses occurred during active growth, the factors responsible appear to be growth-related. This disappearance of bile acid may constitute a major obstacle to the economic viability of a fermentation process using *C. melonis*.

4.2.2. The Time of Substrate Addition

In an attempt to eliminate the high losses of bile acid observed during growth when glycodeoxycholic acid was included in the medium prior to sterilisation, a sterile-filtered solution of the conjugate was added to cultures of *C. melonis* in which active growth had ceased (i.e. after 7 days incubation).

The mycelial dry weight and pH of the cultures, to which the glycodeoxycholic acid was added after 7 days, were not significantly different from those of cultures containing glycodeoxycholic acid during growth (Refer Figure 4.1). However, the absence of glycodeoxycholic acid from the medium during growth produced extremely viscous cultures which were difficult to manipulate. A similar observation was made in an unsuccessful fermenter trial, in which the conjugate was added after growth, the culture exhibiting a mucous growth which was gradually suppressed after the addition of the bile acid. It is likely that the glycodeoxycholic acid inhibits the elaboration of polysaccharide material by the fungus. Bile acids have been observed to inhibit the synthesis and secretion of mucopolysaccharides by fibroblasts (Trias *et al*, 1977) and to decrease the viscosity of mucus (Martin *et al*, 1978).

The bile acid data are depicted in Figure 4.4 with the corresponding mass balance figures. Hydrolysis was initiated within 24h of glycodeoxycholic acid addition, at a rate similar to that observed in control cultures, in which the conjugate was present prior to inoculation. Thus, complete utilisation of the conjugate was observed within 4 days of its addition to produce deoxycholic acid as the sole product at a maximum yield of 60%. This was significantly higher than the maximum yield of 22% achieved in the controls. Nevertheless, the loss of 40% of the bile acid, the majority within 48h of the addition of glycodeoxycholic acid to the grown cultures, was considerable and suggested that a non growth-related factor may also be responsible for losses of bile acid.

4.2.3. The Effect of Glycodeoxycholic acid Concentration on its Hydrolysis

An important consideration in the development of a steroid transforming fermentation is the effect of the steroid concentration on both the transforming microorganism and the transformation itself. From an economic viewpoint, the steroid concentration should be as high as possible and factors important in limiting bile acid concentrations in a fermentation have been discussed in Section 2.1.2. The following work was undertaken to observe the effect of the glycodeoxycholic acid concentration on its hydrolysis by *C. melonis*.

C. melonis was grown in glucose-peptone medium containing glycodeoxycholic acid at concentrations of $0.5gl^{-1}$, $1.0gl^{-1}$, $5.0gl^{-1}$ and $10gl^{-1}$. The mycelial dry weights achieved were unaffected by these concentrations of bile acid. However, there was a direct relationship between the concentration of bile acid and the corresponding culture pH, increasing concentrations causing an increase in the pH of the culture during the period of active hydrolysis. Bile acid precipitated during the growth of cultures containing $1.0gl^{-1}$ and higher concentrations but later resolubilised. This resulted in no apparent inhibition of growth.

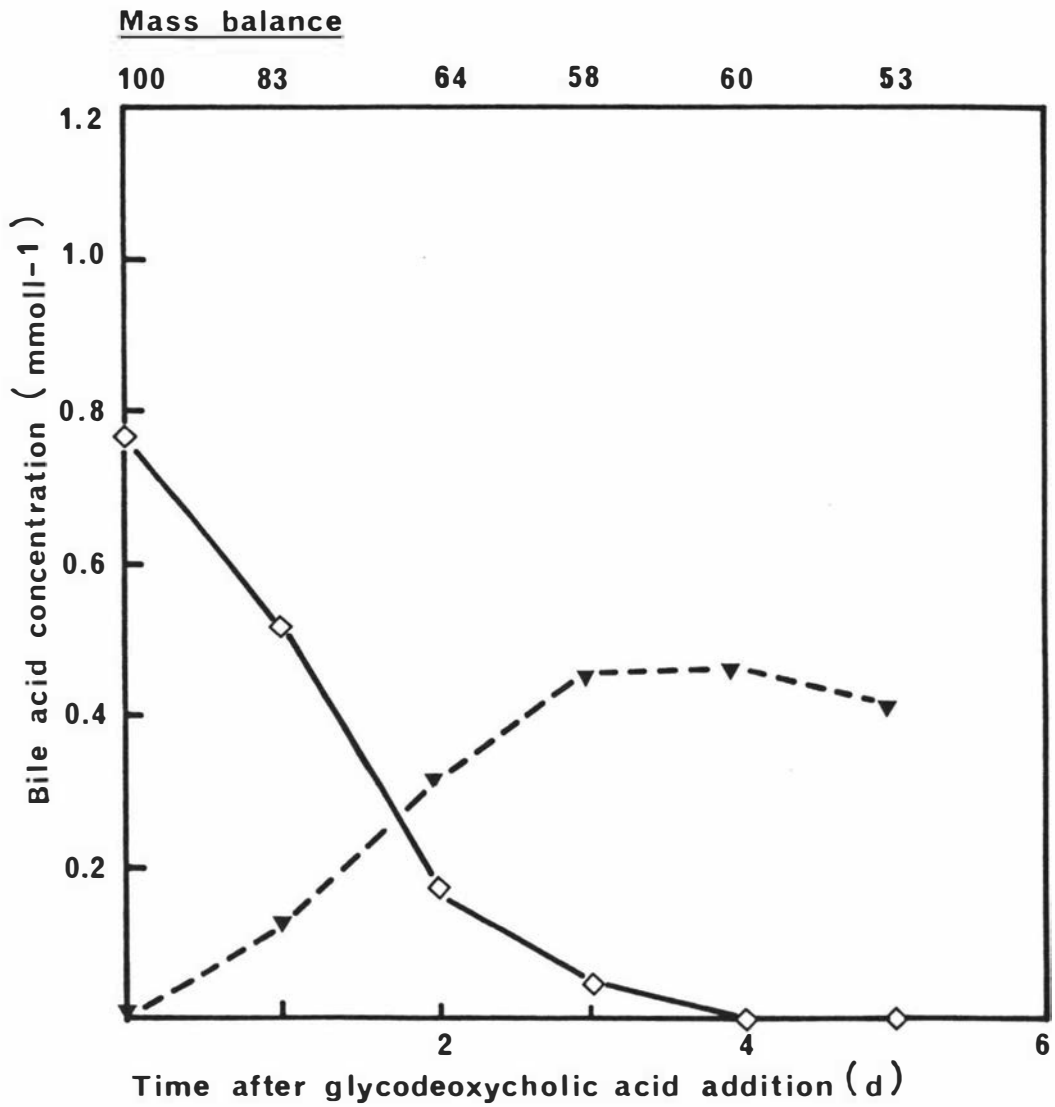


FIGURE 4.4 The hydrolysis of 0.5gl^{-1} glycodeoxycholic acid added to grown cultures of *C. melonis*. Glycodeoxycholic acid, ◇——◇ ; deoxycholic acid, ▼- - - -▼.

The utilisation of total carbohydrate was similarly unaffected by the bile acid concentrations employed compared to the control (Refer Figure 4.1).

The bile acid data are contained in Figures 4.2 and 4.5 to 4.7 for increasing concentrations of glycodeoxycholic acid. The course of hydrolysis was similar for each concentration of the conjugate, product accumulating after an initial lag of 4-5 days. Complete hydrolysis occurred within 15 days of fermentation at all the concentrations tested, except for the $10gl^{-1}$ concentration, where a residual, representing 4% of the initial level, remained. Deoxycholic acid was the sole product detected and the maximum yields obtained were 22%, 44%, 80% and 91% respectively for increasing concentrations of glycodeoxycholic acid. Therefore, increasing the concentration of the glycine conjugate dramatically increased the productivity of the hydrolysis.

This effect of increased substrate concentrations on the yield of hydrolysis resulted from the suppression of losses at the higher glycodeoxycholic acid concentrations. This is illustrated by the comparison of the mass balance data. The loss of bile acid at each concentration is presented in Table 4.1. It is apparent that the percentage loss declined

Table 4.1

The loss of bile acid at different glycodeoxycholic acid concentrations

Concentration of glycodeoxycholic acid		Bile acid losses after 15 days of fermentation	
Nominal (gl^{-1})	Actual ($mmoll^{-1}$)	(%)	($mmoll^{-1}$)
0.5	1.1	74	0.8
1.0	1.7	55	1.0
5.0	9.4	24	2.3
10	18.8	4	0.7

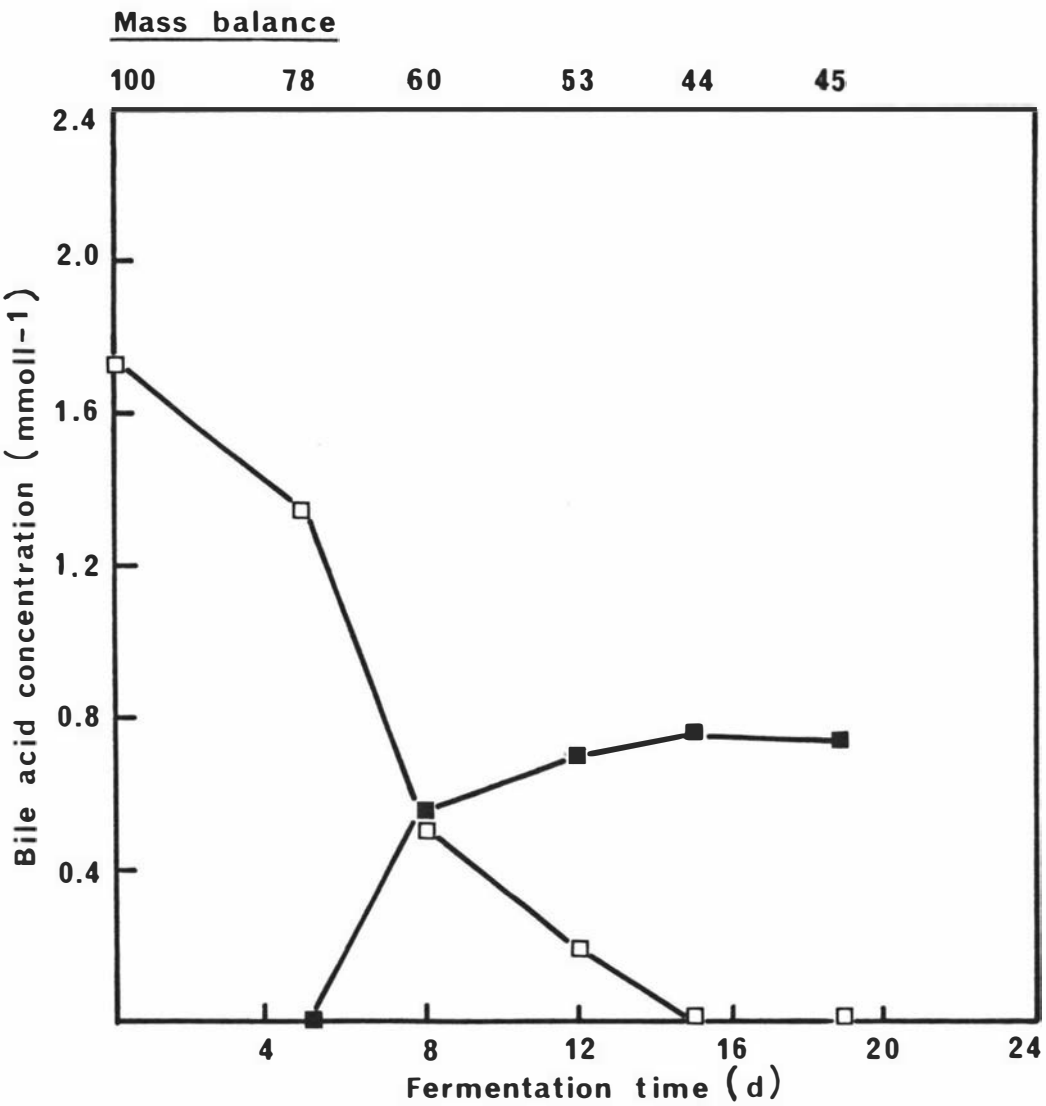


FIGURE 4.5 The hydrolysis of 1.0gl^{-1} glycodeoxycholic acid in shake flask by *C. melonis* grown on glucose-peptone medium. Glycodeoxycholic acid, □——□; deoxycholic acid, ■——■.

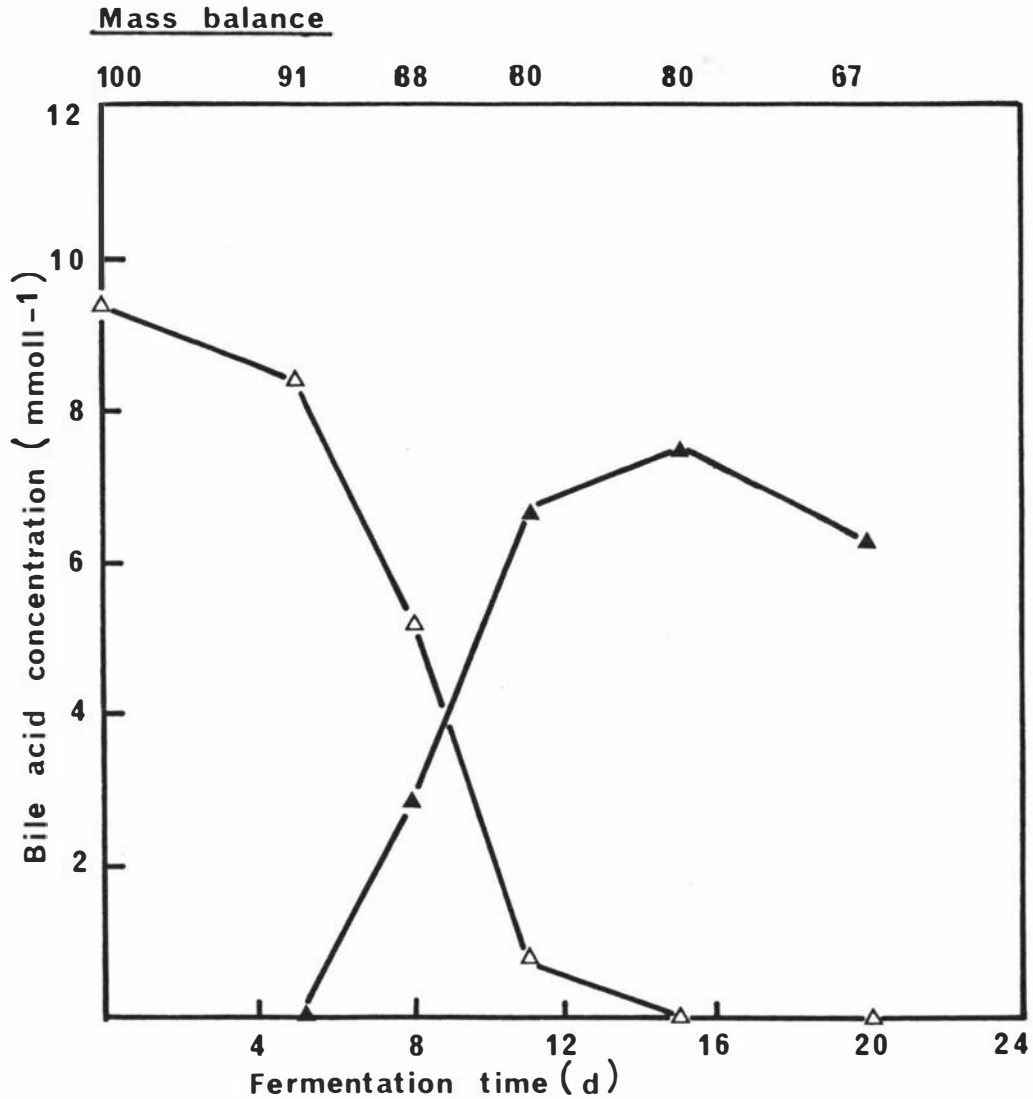


FIGURE 4.6 The hydrolysis of 5.0gl^{-1} glycodeoxycholic acid in shake flask by *C. melonis* grown on glucose-peptone medium. Glycodeoxycholic acid, △——△ ; deoxycholic acid, ▲——▲.

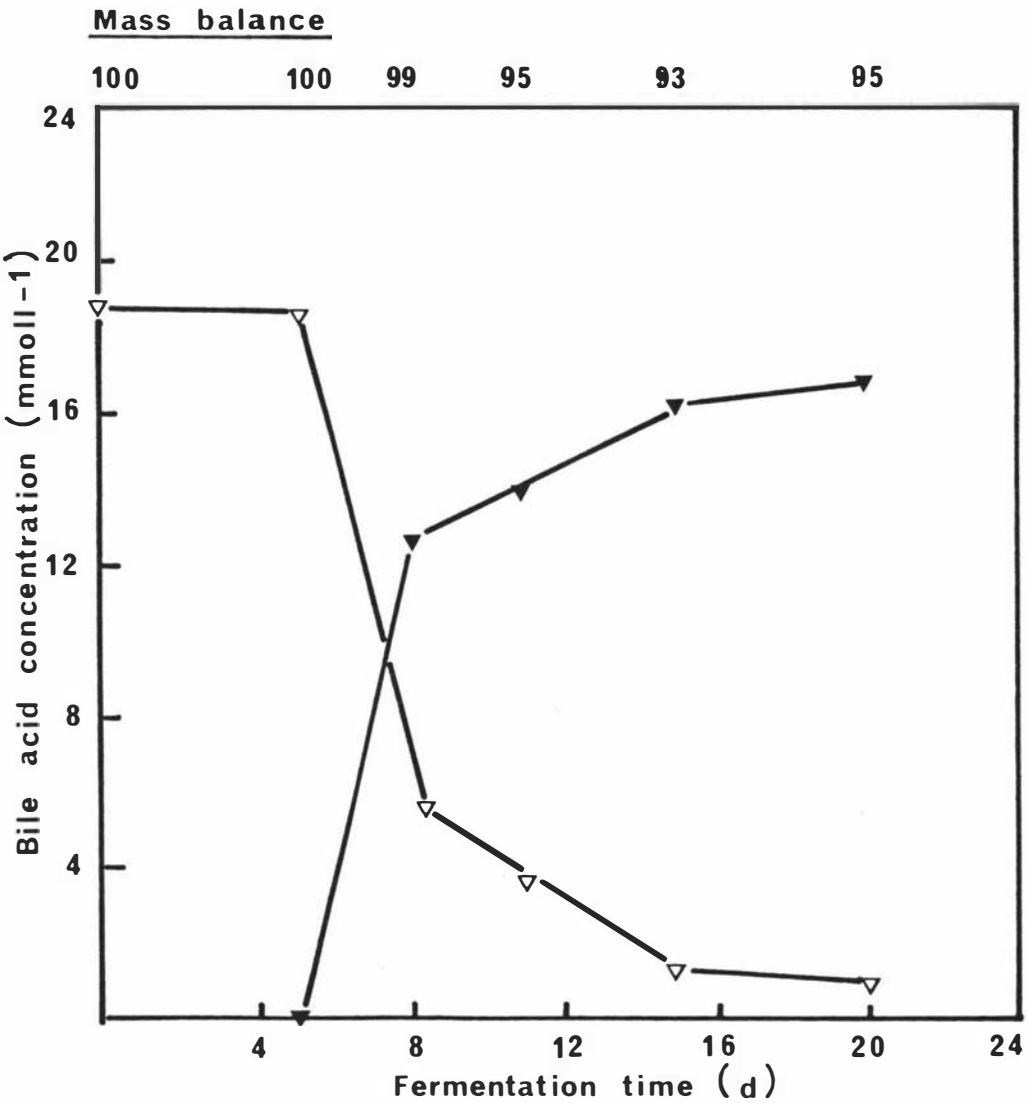


FIGURE 4.7 The hydrolysis of $10gl^{-1}$ glycodeoxycholic acid in shake flask by *C. melonis* grown on glucose-peptone medium. Glycodeoxycholic acid, ▽——▽; deoxycholic acid, ▼——▼.

at increasing concentrations, while the absolute loss reached a maximum at $5gl^{-1}$, then decreased.

As the same fungal dry weights were obtained, and as hydrolysis was completed in the same time interval for each glyco-deoxycholic acid concentration, it can be concluded that the specific rate of hydrolysis was concentration dependent. This is illustrated by the linear plot of Figure 4.8.

4.2.4. The Location of Activity

The location of enzyme activity determines which factors may regulate it. Intracellular enzymes are likely to be controlled not only by the internal regulatory mechanisms of the cell, but also by the chemical nature of the substrate and product insofar as this affects their ability to penetrate the cell membrane. Extracellular enzymes, however, will not be governed by permeability restraints, but they are usually more susceptible to regulation by the culture environment. To determine the location of the cholanoyl glycine hydrolase activity in *C. melonis*, cell-free systems were employed.

The data obtained are summarised in Table 4.2. No evidence of bile acid hydrolysis was detected in control incubations containing conjugates alone.

Table 4.2

The hydrolysis of glycine conjugates by cell-free systems of *C. melonis*.

		Extracellular activity		Intracellular activity	
	Substrate:	GC	GD	GC	GD
Products detected:	CA	+	-	+	-
	DC	-	+	+	+
	Others	-	-	+	+

The entries are the result of duplicate experiments.
Notation: +, product detected; -, product not detected.

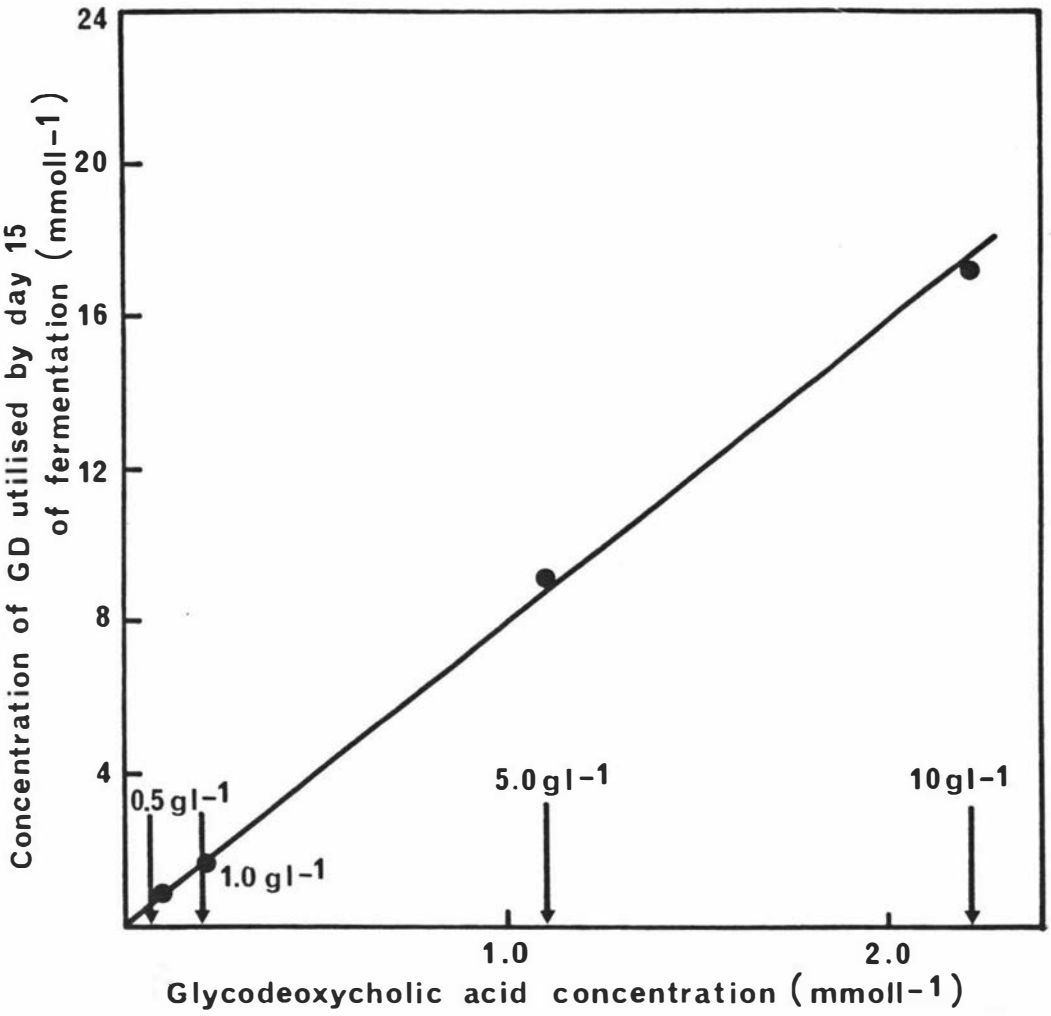


FIGURE 4.8 The "rate" of glycodeoxycholic acid utilisation vs. the initial substrate concentration in shake flask cultures of *C. melonis*.

Activity was present in the cell-free filtrate on both glycine conjugates, demonstrating the presence of extracellular hydrolase(s) in 7 day *C. melonis* cultures. With both conjugates, the yield of free bile acid was too low to be quantitated by hplc. In addition, intracellular hydrolase activity was observed on both substrates in the cell-free extracts, although again, the extent of hydrolysis was insufficient to allow accurate quantitation of the products. Such limited enzyme activity is not unusual in crude cell-free extracts due often to the instability of the enzyme and/or the presence of endogenous enzyme inhibitors (Kobashi *et al*, 1978; Chang and Sih, 1964).

During the production of the cell-free systems, the fungus was grown in the absence of bile acid. Therefore the data indicate that the hydrolase activity is constitutive.

The free bile acid resulting from the hydrolysis of its corresponding conjugate was not the sole product observed in the cell-free extract incubations. Deoxycholic acid was present in glycocholic acid incubations and, for both substrates, other less polar, unidentified bile acid derivatives were detected in low amounts. This indicates that *C. melonis* also possesses constitutive, intracellular, 7α-dehydroxylase and other steroid-modifying enzyme activity, although the level of such activity was low. The action of these enzymes on the products of hydrolysis may contribute to the losses of bile acid experienced during fermentation.

4.2.5. The Effect of Cycloheximide

The results from the cell-free system experiments indicate that the hydrolase activity in *C. melonis* is constitutive. To confirm this, 7-day cultures of the fungus were treated with cycloheximide (250 $\mu g ml^{-1}$), a well-known inhibitor of protein synthesis in eucaryotic cells (Vining, 1979; Jennings, 1976), 2.5h prior to the addition of glycodeoxycholic acid. This was performed in duplicate with appropriate control experiments.

Cycloheximide severely affected *C. melonis*. No growth was observed in duplicate flasks to which cycloheximide was added immediately after inoculation and the treatment of grown cultures with the antibiotic resulted in a rapid decline in their dry weight and an immediate increase in the culture pH (Figure 4.9).

The hydrolysis of glycodeoxycholic acid in the control experiments where the conjugate was added prior to inoculation or after 7 days of incubation proceeded identically to that of the experimental data presented in Figures 4.2 and 4.4 respectively. Also, the addition of cycloheximide to cultures actively hydrolysing glycodeoxycholic acid did not affect subsequent hydrolysis, and the resulting data were similar to those of cultures without cycloheximide treatment, in terms of the rate and yield of hydrolysis.

The bile acid data presented in Figure 4.10 demonstrate the effect of cycloheximide pretreatment of the cultures on the subsequent hydrolysis of glycodeoxycholic acid. Deoxycholic acid was the sole, major product observed. However, another metabolite was detected in trace amounts by tlc. It was not isolated for identification, but its R_f value (0.29) on tlc solvent system "a" (standard R_f values: glycodeoxycholic acid, 0.07; deoxycholic acid, 0.43.) was suggestive of the presence of an oxygen function at C-7, perhaps a keto substituent (Heftmann, 1967).

As the cycloheximide treatment did not prevent the hydrolysis of the glycodeoxycholic acid added subsequently, it can be concluded that the corresponding hydrolase activity is constitutive. This result confirms that obtained by the use of cell-free systems.

The treatment of the grown cultures with cycloheximide, prior to their incubation with glycodeoxycholic acid, promoted both the rate and the yield of the subsequent hydrolysis. Thus, whereas the specific rate of glycodeoxycholic acid disappearance in control cultures to which the conjugate was added after 7 days incubation was *ca* 1 μmol $h^{-1} g^{-1}$, that of cultures pre-

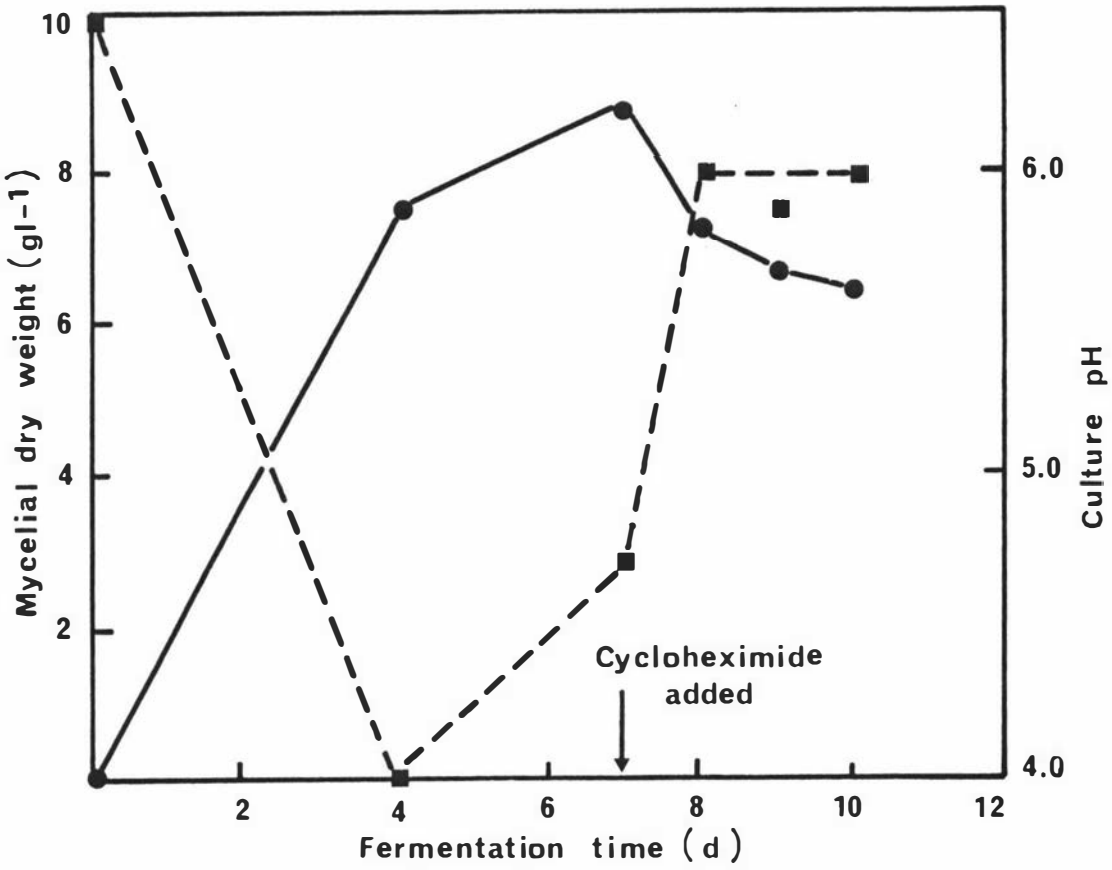


FIGURE 4.9 The effect of cycloheximide addition to grown cultures of *C. melonis* incubated in glucose-peptone medium. Mycelial dry weight, ●——●; culture pH, ■- - - -■.

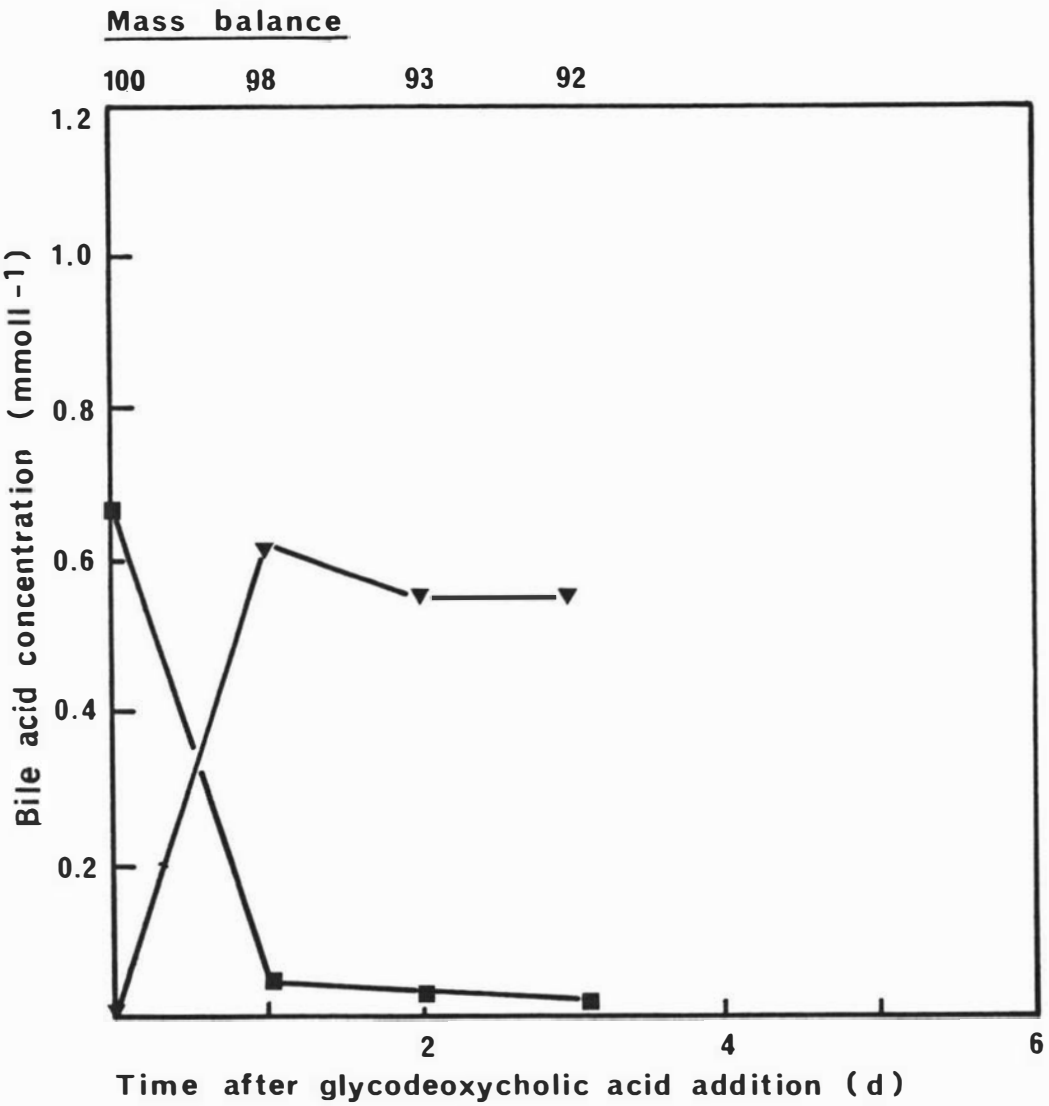


FIGURE 4.10 The hydrolysis of 0.5gl^{-1} glycodeoxycholic acid by cultures of *C. melonis* pretreated with cycloheximide. Glycodeoxycholic acid, ■———■; deoxycholic acid, ▼———▼.

treated with cycloheximide was *ca* 4 $\mu molh^{-1}g^{-1}$. A comparison of the rates of accumulation of deoxycholic acid in the two experiments demonstrates a corresponding difference (Refer Figures 4.4; 4.10). Since the rate increase was probably not due to the direct action of cycloheximide on the hydrolase(s) itself, this result implicates the strict regulation of the enzyme by the cell under "typical" culture conditions. Obviously, the potential exists for greatly superior rates of hydrolysis to those normally observed in shake flask culture.

Similarly, in contrast to the 60% yield obtained by the control (Figure 4.4), near quantitative yields of deoxycholic acid (92-98%) were obtained from the hydrolysis of glycodeoxycholic acid within 24h of its addition to cultures pretreated with cycloheximide. The mass balance data demonstrate that the antibiotic inhibited losses of bile acid. As the addition of cycloheximide to cultures previously incubated with glyco-deoxycholic acid was ineffective in preventing such loss, the above result suggests that the principal factor responsible is inducible.

4.2.6. Discussion

The results demonstrate that *C. melonis* possesses both intracellular and extracellular hydrolase activity for glycine conjugates. Furthermore, both activities are constitutive in that they do not require induction by the bile acid substrate. Thus, the hydrolase(s) was not suppressed by the pretreatment of the culture with cycloheximide.

When glycodeoxycholic acid was added to 7-day cultures of *C. melonis*, grown in the absence of bile acid, hydrolysis was observed within 24h (Refer Figure 4.4). This contrasted with the lag in hydrolysis of at least 4 days observed in cultures containing glycine conjugates from inoculation. Therefore some factor, or factors, appear to suppress hydrolysis during the tropophase. These may include, among others, a time requirement for the synthesis and elaboration of the extracellular hydrolase, if through the impermeability

of the cell-membrane to bile acids, the glycine conjugates are inaccessible to the intracellular hydrolase. In addition, catabolite regulation by glucose may contribute to determining the onset of hydrolysis, as the carbohydrate concentration was always near exhaustion prior to the accumulation of the hydrolysis product.

The specific rates of hydrolysis were not determined in shake flask cultures. However, they did not appear to vary significantly, except in the cases of increased concentrations of glycodeoxycholic acid or the pretreatment of grown cultures with cycloheximide. In the former instance, it appears that, if the hydrolase obeys Michaelis-Menton kinetics, then the rate of hydrolysis observed is not maximal, since it seems to be linearly related to substrate concentration at the levels employed (Figure 4.8). In addition, given the high probability of micelle formation by glycodeoxycholic acid at the $10gl^{-1}$ concentration, due to the culture pH of pH 7-8.5 during hydrolysis, the activity of the hydrolase(s) appears to be unaffected by such aggregation of the bile acid.

The stimulation of specific hydrolysis rates by cycloheximide further demonstrates their suppressed state under the normal culture environment. The effect of the antibiotic may be due to a number of factors. These may include the repression of an inducible, repressor molecule acting on the hydrolysis system or, alternatively, the effect of the antibiotic on the integrity of the cell-membrane (Jennings, 1976). The former would allow greater expression of the intracellular hydrolase, if it was previously hindered. The latter effect may allow the more rapid elaboration of the extracellular enzyme and/or the increased penetration of bile acid into the cell. Possibly, a combination of these factors may be operative. Many antibiotics are reported to increase the rates of certain steroid transformations, apparently by their effect on cell-membrane permeability (Martin, 1977).

Complete disappearance of the glycine conjugates was usually observed. Thus, even at glycodeoxycholic acid concentrations of $10gl^{-1}$, or with the addition of the conjugate in the

idiophase, over 95% of the substrate was utilised. This indicates that the cholanoyl glycine hydrolase is not particularly sensitive to product inhibition by the free bile acid. However, in most instances substantial losses of bile acid were observed, no steroidal side-products being detected. These losses were responsible for the poor free bile acid yields attained, especially in cultures containing low concentrations of glycodeoxycholic acid during growth.

The nature of the bile acid moiety influenced the extent of loss. Dihydroxy bile acids were considerably more susceptible than trihydroxy bile acids (Compare Figures 4.2 and 4.3). A similar dependence on bile acid structure was observed by Eastwood and Hamilton (1968) for the binding of bile acids by lignin. The binding of bile acids by biological macromolecules has also been observed for proteins (Bergstrom *et al*, 1975; Green *et al*, 1971) and for various fibres (Owen *et al*, 1975; Story *et al*, 1976) and binding may be an important factor in the loss of bile acid experienced in the present work.

When glycine conjugates were incubated with the fungus from inoculation, a high proportion of the loss occurred during growth. Yet the addition of glycodeoxycholic acid after growth also resulted in substantial losses of bile acid, although reduced in comparison. Thus, factors contributing to the loss of bile acid from the cultures are both growth- and non-growth related.

The markedly decreased loss of bile acid added to grown cultures pretreated with cycloheximide strongly suggests that one of the factors responsible is an inducible, bile acid-degrading enzyme system which rapidly metabolises the bile acid to non-steroidal product. Bile acid degradation has been observed in aerobic cultures of bacteria (Hayakawa, 1973) and fungi (Furuta, 1959) and follows a similar pathway to that of the microbial degradation of other steroids. The primary enzymes in these reactions are inducible and intracellular (Martin, 1977; Hill, 1976).

The formation of micelles by steroids has been reported to hinder their facile degradation by microorganisms (Jones and Baskevitch, 1973), while Hayakawa (1973) has reported that high bile acid concentrations (0.2% - 0.5%) inhibit their degradation in aerobic bacterial cultures. These may both, therefore, account for the results observed in the experiments employing different glycodeoxycholic acid concentrations. Hayakawa (1973) has reported that deoxycholic acid is more resistant to bacterial degradation than cholic acid. However, this does not appear to be the case in *C. melonis* cultures.

The constitutive activity of various bile acid-modifying enzymes in the cell-free extracts of *C. melonis* suggests that these enzymes may compete with the inducible degradative enzymes for bile acid. The absence of resultant side-products in whole-cell cultures suggests that either the bile acids are unable to penetrate the cell-membrane, or more likely, that such products are rapidly degraded to non-steroidal metabolites by the degrading enzymes.

4.3. The Loss of Bile Acid in *C. melonis* Cultures

In view of the large losses of bile acid observed in the shake flask cultures, it was considered desirable to attempt to account for this loss. Analysis of the fermentations suggested that the two factors that may be responsible, either individually or in combination, for such losses were the binding of bile acid to the mycelium and their utilisation by the fungus.

4.3.1. The Binding of Bile Acids to Mycelium

The irreversible binding of steroids and sterols to micro-organisms, including fungi, has been reported by Buetow and Levedahl (1964). However, bile acids have apparently not been tested in this respect. In addition, although the binding of bile acids to various macromolecules has been studied, the applicability of these data to microbial systems is uncertain. Therefore experiments were performed to assess

the extent to which the mycelium of *C. melonis* bound bile acids.

Shake flasks containing glucose-peptone medium were inoculated with *C. melonis* and incubated at 30°C for 7 days, after which time the typical mycelial growth was present. The cultures were then autoclaved at 121°C for 15 minutes to inactivate enzyme activity and, after cooling, bile acid solution was added to each flask to a final concentration of $0.5 g l^{-1}$. Sodium taurocholate, glycocholic acid, glycodeoxycholic acid, cholic acid and deoxycholic acid were tested and controls, using uninoculated media, were included for comparison. All flasks were adjusted to pH 5 and reincubated for 1h to allow for equilibration of binding.

The weight of bile acid bound per g dry weight of mycelium was calculated from the difference in the bile acid recoveries between the inoculated test flask and the corresponding control. The data are presented in Table 4.3. It is assumed that the denaturation of the mycelial protein by autoclaving does not significantly affect its capacity to bind bile acid. Rudman and Kendall (1957) reported that the denaturation of albumin did not alter its ability to bind deoxycholic acid. Also, it is assumed that in a fermentation containing a mixture of bile acids, there exists a situation of competitive binding for the binding sites on the mycelium.

Table 4.3

Bile acid binding to mycelium of *C. melonis*

Bile acid tested	Bile acid bound per g dry weight of mycelium (mg)
GD	15
DC	14
GC	3
CA	8
NaTC	0.3

Clearly, bile acid is bound to the mycelium in a manner not reversed by the method of sample treatment, which comprised adjusting the culture to pH 10 with 1M sodium carbonate and slowly agitating it for 15 minutes prior to filtration to remove the mycelium. The results show, also, that the structure of the bile acid determines its susceptibility to binding. Thus, dihydroxy bile acids are bound to a greater degree than trihydroxy bile acids, whereas sodium taurocholate is bound to a negligible extent.

The maximum dry weight of $10gl^{-1}$ attained in shake flask cultures of *C. melonis* containing $0.5gl^{-1}$ of glycine conjugate (Refer Figure 4.1), was used to calculate the loss of bile acid due to mycelial binding predicted by these data. It is assumed that an identical binding situation existed in both the binding experiments and the actual cultures. Thus, the predicted bile acid loss in cultures hydrolysing $0.5gl^{-1}$ of glycodeoxycholic acid or glycocholic acid is 33% and 19% respectively, taking the greater binding of cholic acid compared to glycocholic acid into account. Since the actual maximum bile acid loss observed in such cultures was 74% and 50% for glycodeoxycholic and glycocholic acids respectively (Refer Figures 4.2, 4.3), the binding data demonstrate that, although the mycelial binding of bile acids is substantial in shake flask cultures of *C. melonis* hydrolysing glycine conjugates, the actual losses observed in such cultures exceed those accounted for solely by binding by at least a factor of 2 for both conjugates. Hence, some other factor must also contribute to the observed loss.

4.3.2. The Effect of 8-Hydroxyquinoline on Bile Acid Loss

The inability of the binding data to satisfactorily explain all the losses of bile acid observed in *C. melonis* cultures led to the use of 8-hydroxyquinoline to test the hypothesis that these losses may also derive from the degradation of the bile acid by the fungus.

Many microorganisms, including fungi, can utilize steroids as their sole source of carbon (Buetow and Levedahl, 1964)

and recently, *Cochliobolus intermedius* has been shown to utilise glycocholic acid as its sole nitrogen and carbon source, although this may not necessarily have involved the degradation of the bile acid moiety (Maddox and Chong, 1978). Initial attack on the steroid molecule has been shown, with bacteria, to result from the action of two independent enzyme systems, one acting on the steroid nucleus, the other on the sidechain. Whereas the former activity is common, there have been no reports of microorganisms able to selectively degrade the sidechain without concomitant oxidation of the nucleus, despite extensive screening programmes for such microorganisms (Martin, 1977). Descriptions of both pathways are included in reviews by Marsheck (1971) and Martin (1977).

The degradation of the ring nucleus of bile acids by bacteria has been reported to produce intermediates common to the breakdown of other steroids and, eventually, to intermediates of carbohydrate catabolism (Hayakawa, 1973). Hydrolysis appears to be a prerequisite for the degradation of conjugated bile acids, as they are inert to the modifying enzymes of both bacteria and fungi (Furuta, 1959; Hill, 1976; Tenneson *et al*, 1977). Bile acids are also vulnerable to sidechain degradation by both bacteria and fungi *via* a process analogous to β-oxidation (Furuta, 1959; Hayakawa, 1973; Tenneson *et al*, 1977).

In the present work, the degradation of the steroid nucleus was investigated in preference to that of the sidechain, although both may occur. This was for two reasons. First, the degradation of the steroid nucleus appears more ubiquitous than that of the sidechain in microbial cultures. Secondly, *C. melonis* has been reported to possess steroid 9α-hydroxylase activity (Kondo and Tori, 1964) and 1(2)-dehydrogenation has been demonstrated by various Fungi Imperfecti on steroids (Marsheck, 1971). Both of these enzymes are essential to the loss of steriod *via* metabolic degradation.

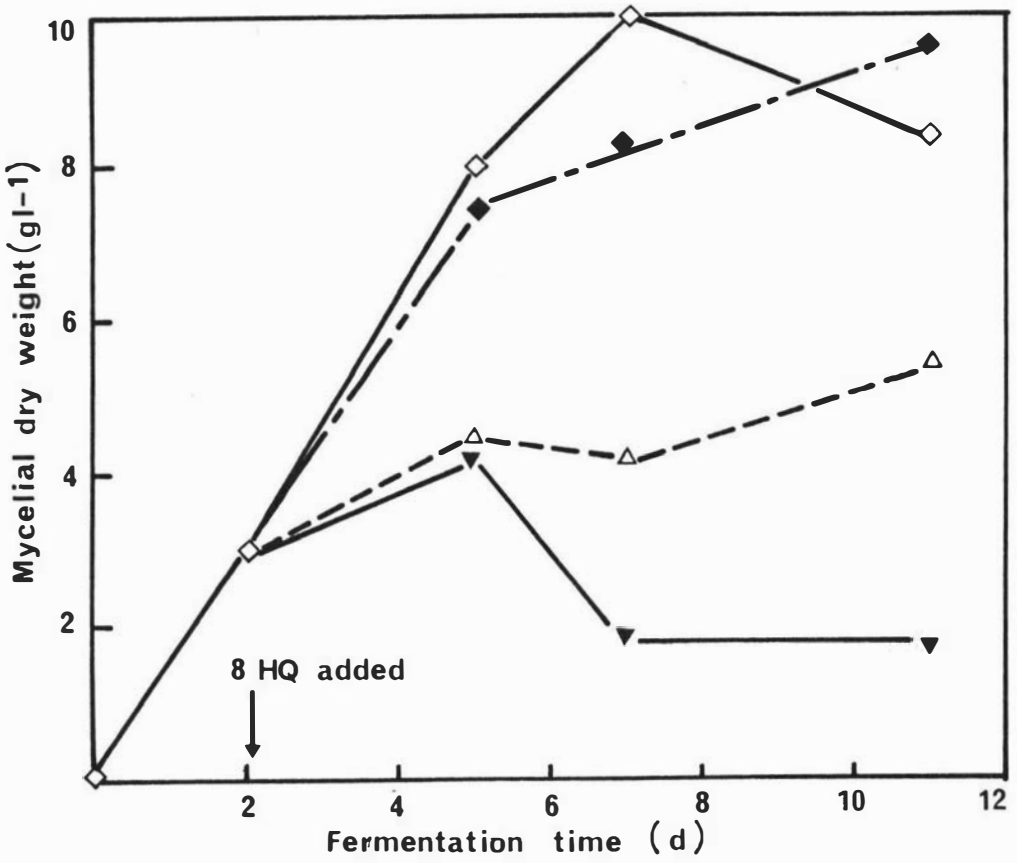
Lipophilic chelating agents, such as 8-hydroxyquinoline and α,α´-dipyridyl, have been successfully employed to inhibit the

9α-hydroxylation of steroids, at concentrations ranging from 0.25 to 0.69 $mmol\,l^{-1}$ (Whitmarsh, 1964; Wix *et al*, 1968). Therefore, the former inhibitor was used to attempt to reduce bile acid losses in *C. melonis* cultures.

Shake flask cultures of *C. melonis* were grown on glucose-peptone medium containing 0.5 $g\,l^{-1}$ glycodeoxycholic acid. After 52h incubation, a sterile-filtered, ethanolic solution of 8-hydroxyquinoline was aseptically added to the flasks to give final concentrations of 0.34 $mmol\,l^{-1}$, 0.68 $mmol\,l^{-1}$ and 1.36 $mmol\,l^{-1}$. Controls without added inhibitor were also run. Incubation was continued for the appropriate time and the flasks were then analysed for culture pH, dry weight and bile acid concentrations. The last were estimated by chloroform extraction with the modification that the combined chloroform extracts were back-extracted with 0.25M hydrochloric acid (25 ml) to remove the 8-hydroxyquinoline and then washed with distilled water (15 ml) prior to being evaporated to dryness *in vacuo*.

The presence of the 8-hydroxyquinoline severely inhibited the growth of *C. melonis* in direct relation to its concentration in the culture (Figure 4.11). This was probably a direct result of the chelation of metal ions essential to the growth of the fungus, by the inhibitor. Therefore, in contrast to the typical pH profile of the control and 0.34 $mmol\,l^{-1}$ flasks, those of the 0.68 $mmol\,l^{-1}$ and 1.36 $mmol\,l^{-1}$ concentrations of 8-hydroxyquinoline remained at pH 4 throughout further incubation.

The effect of the 8-hydroxyquinoline on the hydrolysis of glycodeoxycholic acid is illustrated in Figure 4.12 for the 0.34 $mmol\,l^{-1}$ concentration of inhibitor and in Figure 4.13 for inhibitor concentrations of 0.68 $mmol\,l^{-1}$ and 1.36 $mmol\,l^{-1}$. The hydrolysis of glycodeoxycholic acid in the control flasks paralleled that depicted in Figure 4.2. Hydrolysis of glycodeoxycholic acid, as judged by free bile acid production, was completely inhibited at 8-hydroxyquinoline concentrations of 0.68 $mmol\,l^{-1}$ and 1.36 $mmol\,l^{-1}$, and it was substantially delayed at an 8-hydroxyquinoline concentration of 0.34 $mmol\,l^{-1}$.



Notation:

The dry weight at various concentrations of 8-hydroxyquinoline (8HQ):

control, ◇——◇.

0.34mmoll^{-1}, ◆—–—◆.

0.68mmoll^{-1}, △– – –△.

1.36mmoll^{-1}, ▼———▼.

FIGURE 4.11 The effect of 8-hydroxyquinoline on the dry weight of *C. melonis* grown in glucose-peptone medium containing 0.5gl^{-1} glycodeoxycholic acid.

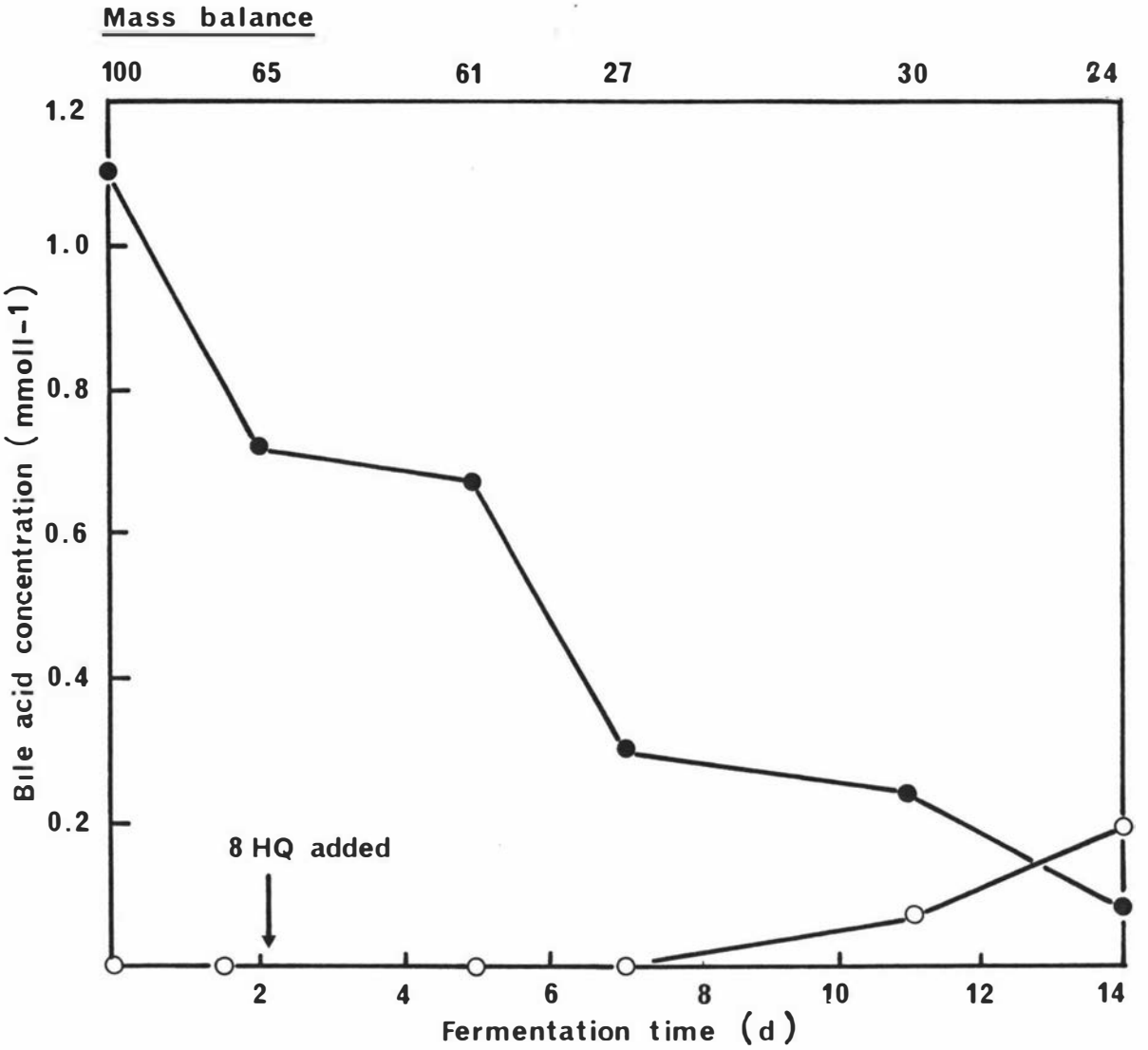


FIGURE 4.12 The effect of 0.34 mmoll^{-1} 8-hydroxyquinoline (8HQ) on the hydrolysis of 0.5gl^{-1} glycodeoxycholic acid by *C. melonis*. Glycodeoxycholic acid, ●——●; deoxycholic acid, ○——○.

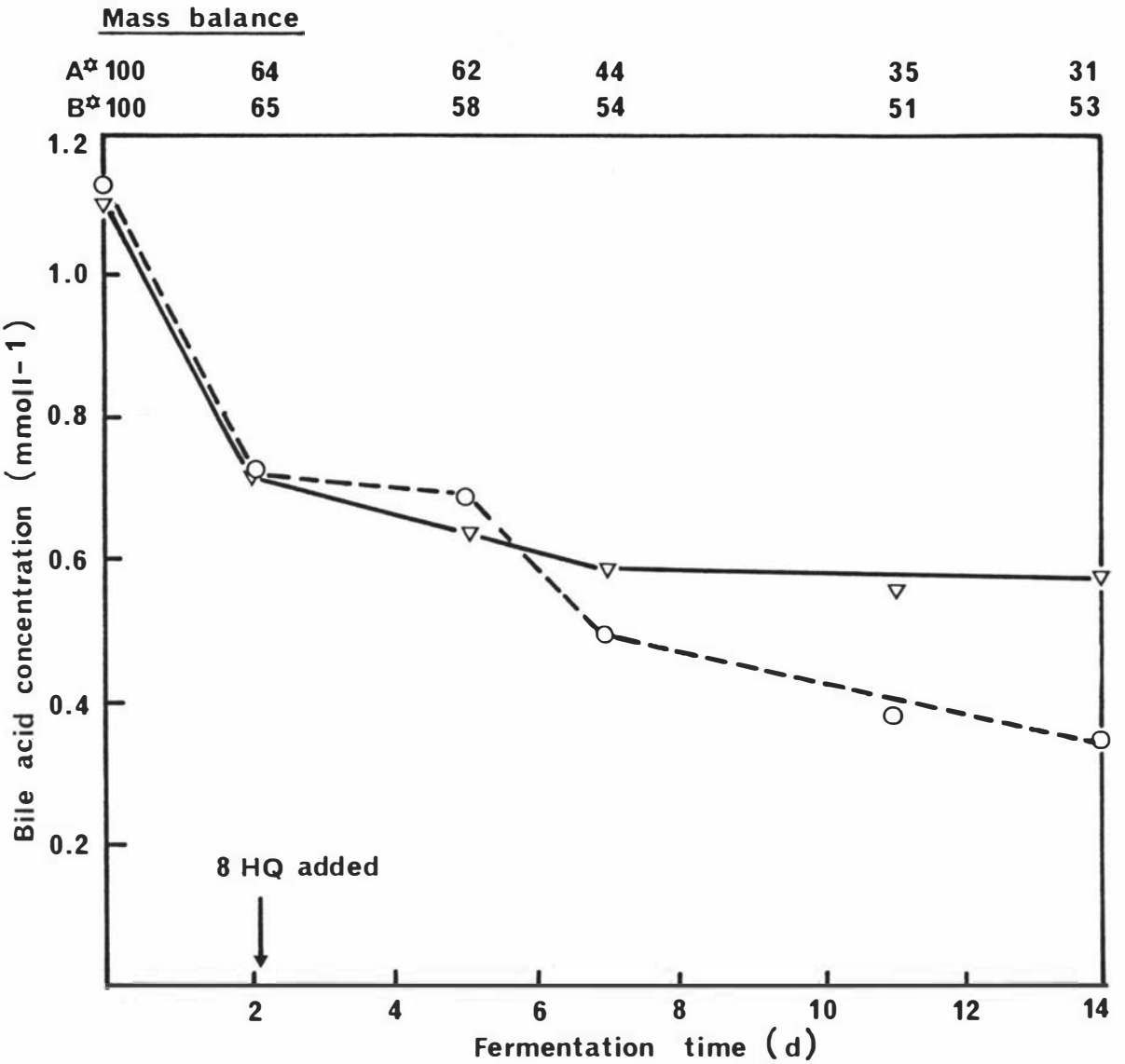


FIGURE 4.13 The effect of 0.68 mmoll^{-1} and 1.36 mmoll^{-1} 8-hydroxyquinoline (8HQ) on the hydrolysis of 0.5gl^{-1} glycodeoxycholic acid by *C. melonis*. Glycodeoxycholic acid with

*A: 0.68 mmoll^{-1} 8-hydroxyquinoline, ○---○.

*B: 1.36 mmoll^{-1} 8-hydroxyquinoline, ▽——▽.

Thus, although the maximum deoxycholic acid yields of the 0.34 $mmoll^{-1}$ inhibitor experiment and the control experiment were similar, the former had a lag of 8-10 days prior to deoxycholic accumulation compared to 5-6 days for the control.

The 8-hydroxyquinoline also affected the total bile acid losses in the cultures. Losses for increasing inhibitor concentrations were 78%, 76%, 69% and 48% respectively. Of this, *ca* 35% loss appeared to have occurred in the 52h prior to the addition of the 8-hydroxyquinoline. Clearly, the lower inhibitor concentration had no effect on the loss of bile acid, whereas the highest level achieved some measure of success in reducing losses, given that *ca* 35% loss had occurred prior to its addition.

Unfortunately, the effect of the 8-hydroxyquinoline on the mycelial dry weight and hence, on the amount of mycelium available for bile acid binding, renders difficult an unambiguous assignment of the reduced losses at the higher inhibitor concentrations to the inhibition of a degradative pathway. However, the reduction in the total observed loss at an inhibitor concentration of 1.36 $mmoll^{-1}$ (30%), compared to the control, exceeded that predicted by binding data (16%) due to the reduced dry weight. This provides some evidence that the postulated degradation of the bile acid was inhibited at this inhibitor concentration.

4.3.3. The Loss of Hydrolysis Product

Deoxycholic acid is particularly resistant to the degradative action of aerobic cultures of bacteria (Hayakawa, 1973) and there are no reported instances of deoxycholic acid being metabolised by fungi. Therefore an experiment was performed to assess the susceptibility of deoxycholic acid to loss in *C. melonis* shake flask cultures.

C. melonis was grown in glucose-peptone medium containing 0.5gl^{-1} deoxycholic acid. The mycelial dry weight obtained was not significantly different to that produced during

growth in the presence of glycodeoxycholic acid (Refer Figure 4.1). However, the culture pH rose to a final value of pH 8 after 14 days, which exceeds that of the corresponding glycodeoxycholic acid cultures by *ca* 2 pH units.

The bile acid data are depicted in Figure 4.14. Only deoxycholic acid was detected throughout the course of the fermentation. However, the data reveal that a loss of *ca* 50% of the bile acid occurred, largely during growth. The loss expected due to binding after 4 days of fermentation is 22%, compared to the 41% observed. Therefore deoxycholic acid is lost in considerable amounts from the fermentation and its utilisation by the fungus is implicated to contribute to this loss.

4.3.4. Discussion

The data confirm that the binding of bile acids to the mycelium was a predominant factor in the losses of bile acid observed in shake flask cultures. This is supported by the observation that the relationship between the bile acid structure and the extent to which bile acid was bound to the mycelium was very similar to that observed between the structure of the bile acid and its loss in shake flask cultures. The influence of the bile acid structure on the extent of binding implicates hydrophobic binding as the principal mechanism involved. Eastwood and Hamilton (1968) reported a similar relationship between bile acid structure and the binding of bile acids by lignin and they postulated hydrophobic binding as being responsible. This mechanism is thought to predominate in the binding of small molecules by biological macromolecules (Nair, 1976).

However, binding of the bile acids to mycelium does not account for the total losses observed. This discrepancy can be explained by hypothesising the simultaneous operation of a bile acid degrading pathway in the cultures. However, the attempt to prove this hypothesis by blocking the 9α-hydroxylase of the fungus with 8-hydroxyquinoline was largely unsuccessful.

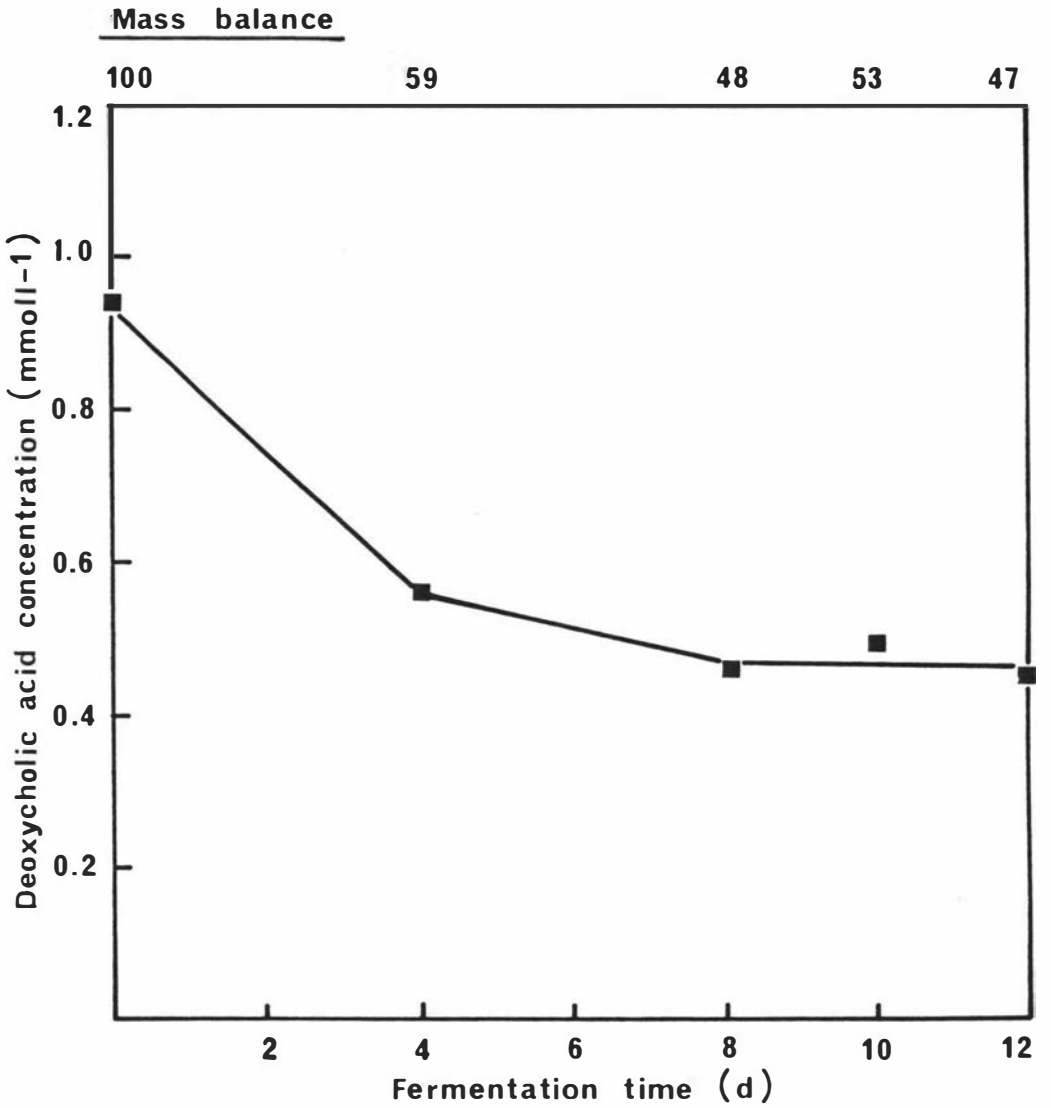


FIGURE 4.14 The disappearance of $0.5gl^{-1}$ deoxycholic acid from cultures of *C. melonis* grown on glucose-peptone medium.

Nevertheless, the marked reduction in bile acid loss in cultures containing a 1.36 mmoll^{-1} concentration of 8-hydroxyquinoline, suggests that degradative enzymes were inhibited. This result supports those of the cycloheximide experiments, which implicated the activity of inducible, free bile acid metabolising enzymes in *C. melonis* cultures. The inability of lower 8-hydroxyquinoline concentrations to suppress the steroid-degradative pathway has been observed by other workers (Martin, 1977).

Another factor reported to result in the loss of steroid from culture broths, is the modification of the steroid solubility by the microorganism to render them refractory to solvent extraction (Martin, 1977). However, the gathering of similar data from identical experiments, differing only in the bile acid extraction procedure, appears to discount the operation of this factor.

The inhibition of hydrolase activity by the 8-hydroxyquinoline suggests that the cholanoyl glycine hydrolase(s) may be a metalloenzyme.

4.4. Fermenter Studies

The principal aim of the work described in this section was to assess the effect of selected variables on the hydrolysis of glycodeoxycholic acid by *C. melonis*. It was hoped that the manipulation of these variables would result in high yields of deoxycholic acid and, in the absence of similar information in the literature, provide a quantitative description of glycine conjugate hydrolysis by a fungus.

The selection of significant variables was difficult as steroid transformations can be sensitive to many factors including trace element concentrations and the inoculum development procedure (Marsheck, 1971). The lack of basic knowledge concerning the hydrolysis of bile acid conjugates by fungi, therefore, necessitated the arbitrary selection of the type, number and the level of the variables for study.

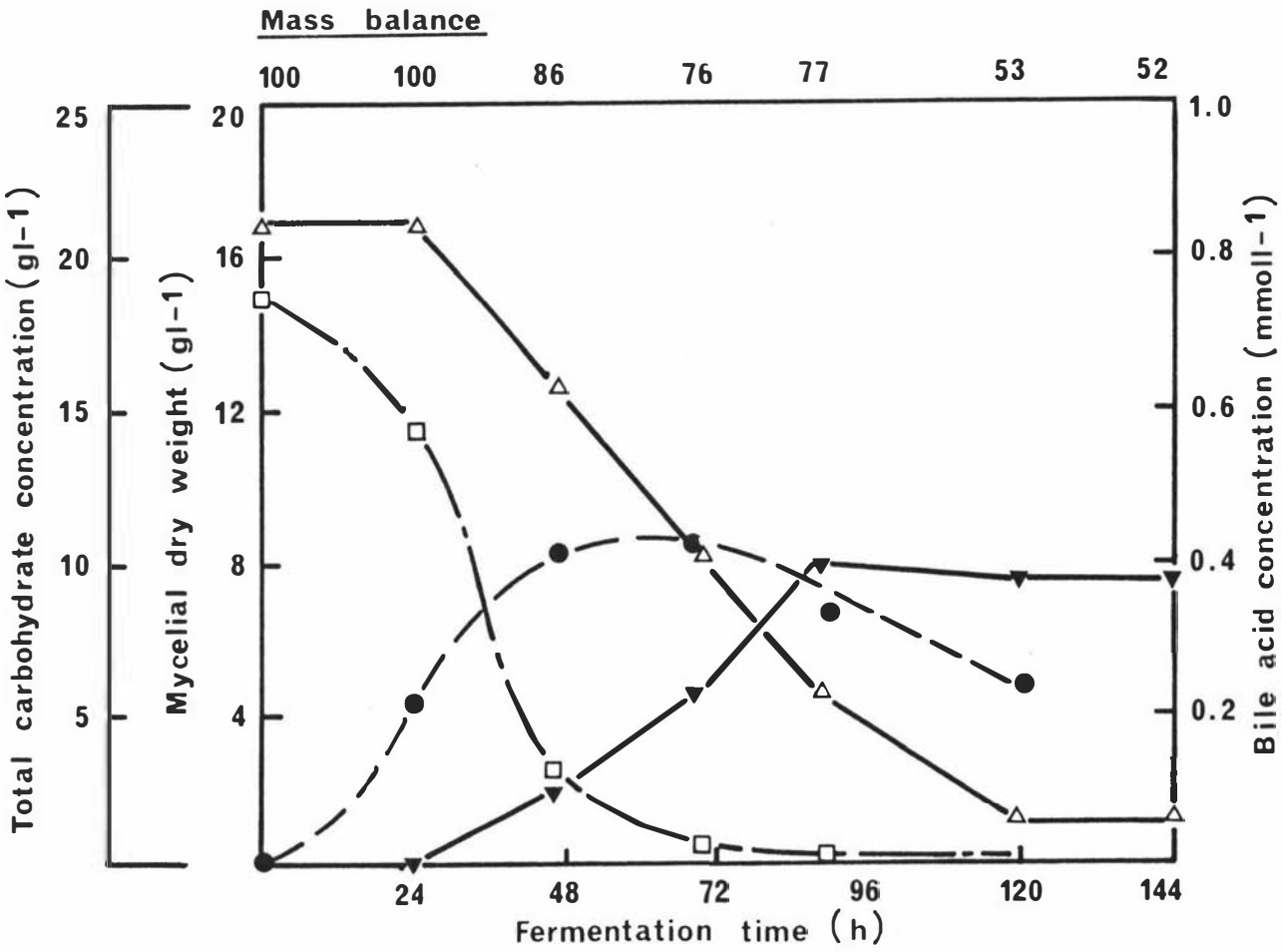
Hence, as the basis for future work, the following basic fermentation parameters were chosen. Glycodeoxycholic acid was employed, as it represented the "worst case" in view of the poor yields obtained in shake flask culture compared to glycocholic acid. It was included into the medium prior to sterilisation at $0.5gl^{-1}$ final concentration, the low concentration being used to reduce the requirements for substrate synthesis. Glucose-peptone medium was employed as it yielded good growth of the fungus in shake flask and was, peptone excepted, chemically well defined. A culture pH of pH 6.5 was chosen as the set value from shake flask data and, in addition, this pH is within the optimum range commonly observed for bacterial hydrolases (Hill, 1976). A temperature of $30^{o}C$, and agitation (750 rpm) and aeration rates were selected to ensure that these variables were not growth limiting.

The fermentations were operated in a batch mode and usually lasted 6-8 days. Foam control was achieved manually for early runs, while the automatic system was employed thereafter. Each fermentation was monitored for the culture pH, mycelial dry weight, total carbohydrate and bile acid concentrations by means of samples withdrawn daily.

4.4.1. The Course of Hydrolysis

Data describing the basic course of hydrolysis in the fermenter were obtained from a culture grown at pH 6.5 with an aeration rate of 1 $lmin^{-1}$ and are presented in Figure 4.15.

The maximum mycelial dry weight was attained with 60h of inoculation. The typical morphology in the fermenter comprised khaki mycelium during the initial 24-48h of fermentation after which time, the culture became thick and black. Microscopic examination of the mycelium showed growth as filamentous threads. The biomass flocculated on the addition of sodium carbonate during sample preparation and was readily filtered on a Buchner apparatus. These morphological characteristics were observed in all subsequent fermentations.



Notation:
dry weight, ●——●;
total carbohydrate as glucose, □——□;
glycodeoxycholic acid, △——△;
deoxycholic acid, ▼——▼.

FIGURE 4.15 The course of the hydrolysis of $0.5gl^{-1}$ glycodeoxycholic acid by *C. melonis* in the fermenter using glucose-peptone medium; pH 6.5; aeration rate of $1lmin^{-1}$.

Although the culture pH was controlled, the sequence and the rate of addition of pH control solutions were monitored throughout the fermentation. The rapid addition of alkali was required during the first 40h of fermentation after which time acid was added. The culture pH remained close to the controlled value of pH 6.5 after *ca* 96h. This sequence of addition of pH control solutions was observed for all the fermenter experiments conducted, except where lactose was substituted for glucose (Refer Section 4.4.3).

Total carbohydrate was exhausted within 60h of fermentation, which corresponded to the time of maximum dry weight production. Dissolved oxygen tension was not quantitated precisely, but it remained at high levels throughout the fermentation. Foaming was severe during the initial 48h of growth, but decreased in intensity thereafter.

Deoxycholic acid accumulated as the sole product within 48h of inoculation and towards the end of the tropophase. At this stage of the fermentation, the total carbohydrate concentration was low and the addition of alkali to the culture changed to that of acid. Hydrolysis continued until *ca* 120h after inoculation, when hydrolase activity ceased and a residual substrate level of 6% of the initial concentration remained. At this time, the yield of deoxycholic acid was maximal at 47% and a loss of 47% of the total bile acid initially present had occurred, this loss increasing reasonably linearly with the time of fermentation over the 24-120h period.

The specific rates of glycodeoxycholic acid disappearance and deoxycholic acid accumulation are depicted in Figure 4.16. These stayed constant during the principal period of hydrolysis at values of *ca* 1.2 $\mu molh^{-1}g^{-1}$ and 1.0 $\mu molh^{-1}g^{-1}$ respectively. However, after 96h of fermentation, the specific hydrolase activity declined rapidly, and presumably accounts for the glycodeoxycholic acid remaining unconverted after this time. The rate data did not fit a first order rate law.

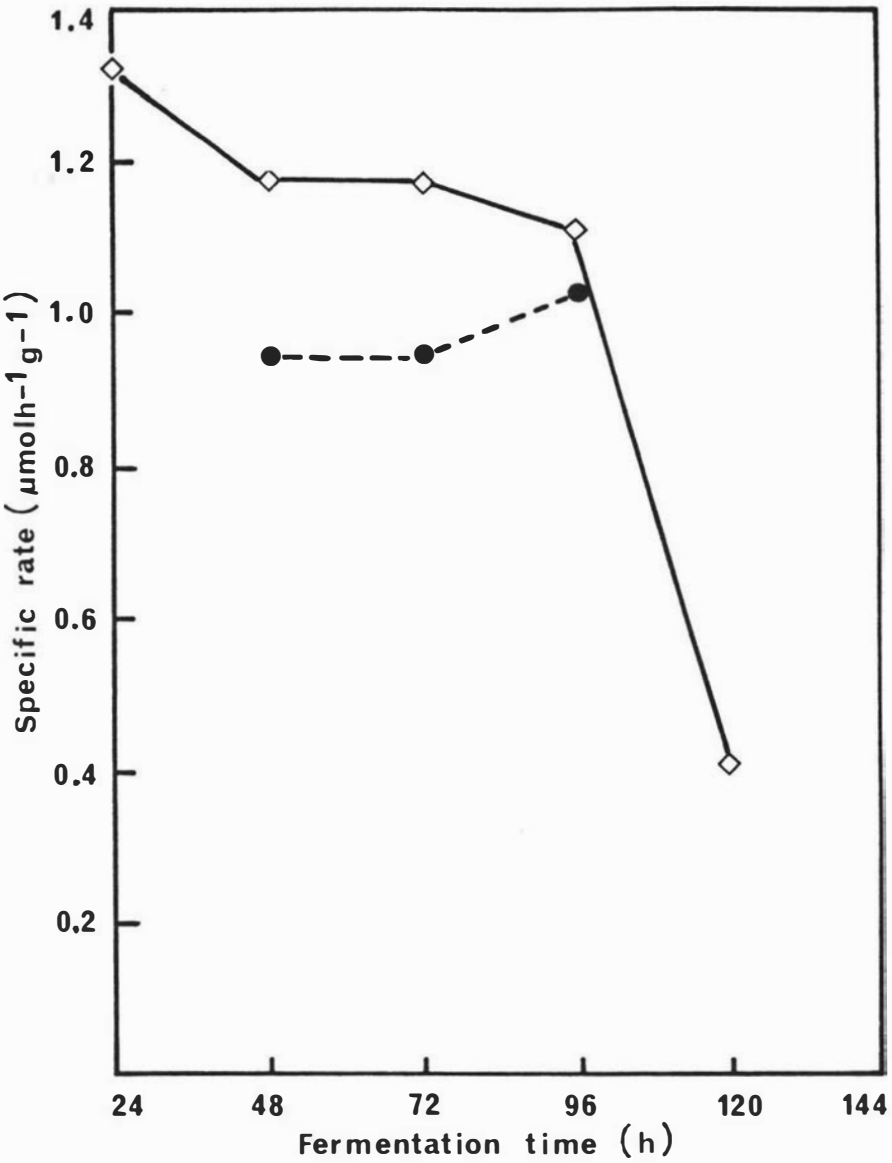


FIGURE 4.16 Specific rates of glycodeoxycholic acid disappearance and deoxycholic acid accumulation in the fermenter.
Conditions:
pH 6.5; aeration rate, 1lmin^{-1}.
Glycodeoxycholic acid disappearance, ◇———◇; deoxycholic acid accumulation, ●- - - - -●.

4.4.2. The Effect of Aeration

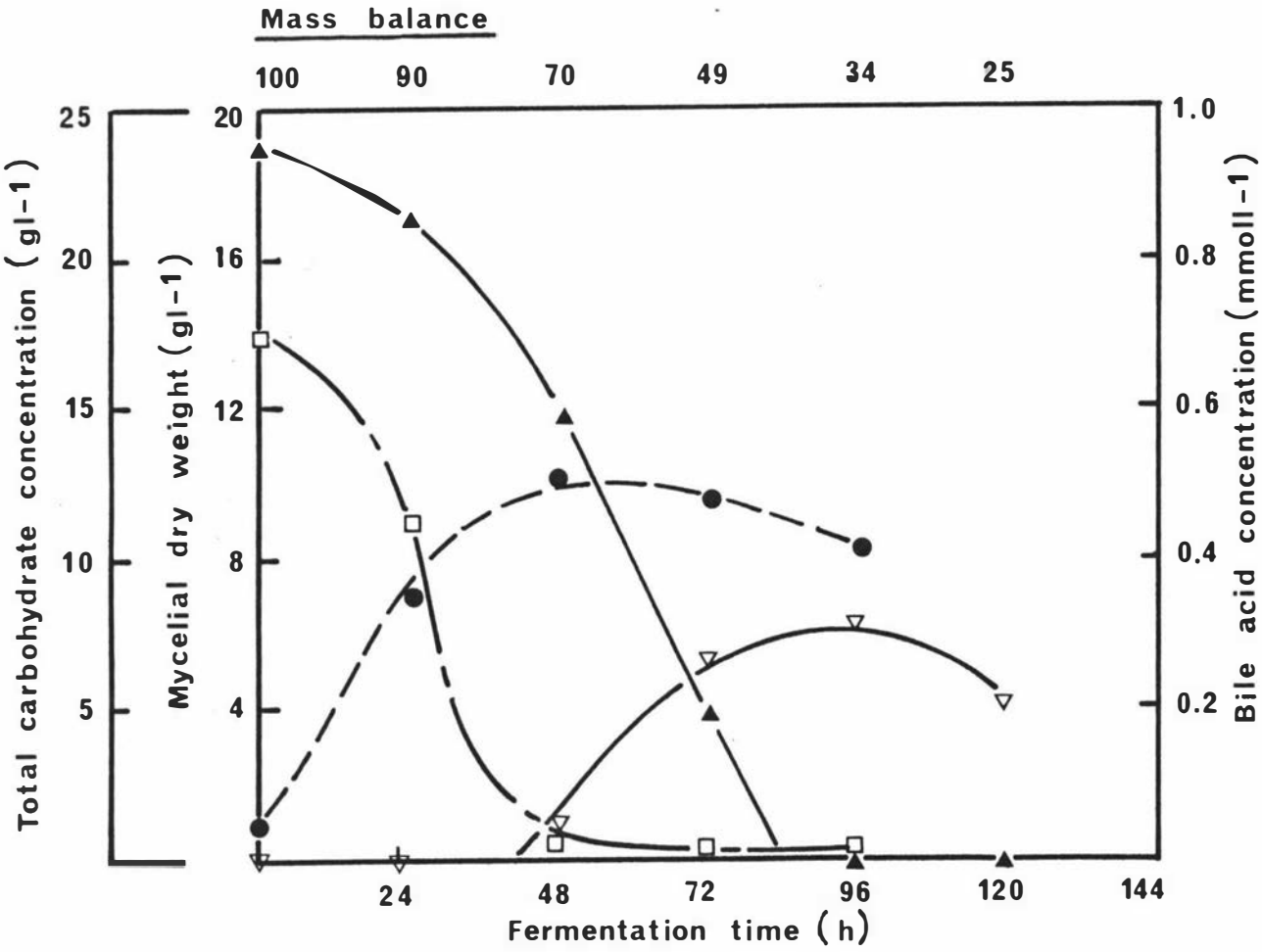
To ascertain the effect of the aeration rate on the hydrolysis of glycodeoxycholic acid, the rate employed was lowered to $0.25 l min^{-1}$ after 24h of fermentation. Otherwise, the fermentation conditions remained the same as for those of the base study. The data collected from this experiment are presented in Figure 4.17. The reduced aeration rate significantly decreased the dissolved oxygen tension during the fermentation, however, there was no corresponding difference in the dry weight or total carbohydrate concentrations compared to the base study.

Deoxycholic acid appeared as the sole product of hydrolysis within 48h of inoculation. A maximum yield of 34% was observed after 96h of fermentation, by which time the glycodeoxycholic acid had been completely utilised. Severe loss of bile acid occurred at a constant rate during the fermentation, 75% of the total bile acid being unaccounted for after 120h. The specific rates of glycodeoxycholic acid disappearance and of deoxycholic acid accumulation were relatively constant during the main period of glycodeoxycholic acid utilisation and values of 1.6 $\mu mol h^{-1} g^{-1}$ and 0.4 $\mu mol h^{-1} g^{-1}$ respectively were determined. The data deviated significantly from a first order rate equation.

4.4.3. The Effect of the Carbon Source

To observe the effect of a less readily utilisable carbohydrate as a carbon and energy source on the hydrolysis, lactose at a concentration of $40 g l^{-1}$ was substituted for glucose in the glucose-peptone medium. All other factors were held at the levels employed in the base study.

The data are depicted in Figure 4.18. The growth of *C. melonis* in the lactose-peptone medium was poor and most of the dry weight increase occurred during the first 24h of fermentation over which time lactose was utilised to a negligible extent. Subsequent utilisation of the lactose gave little increase in mycelial dry weight. The culture was characterised by the



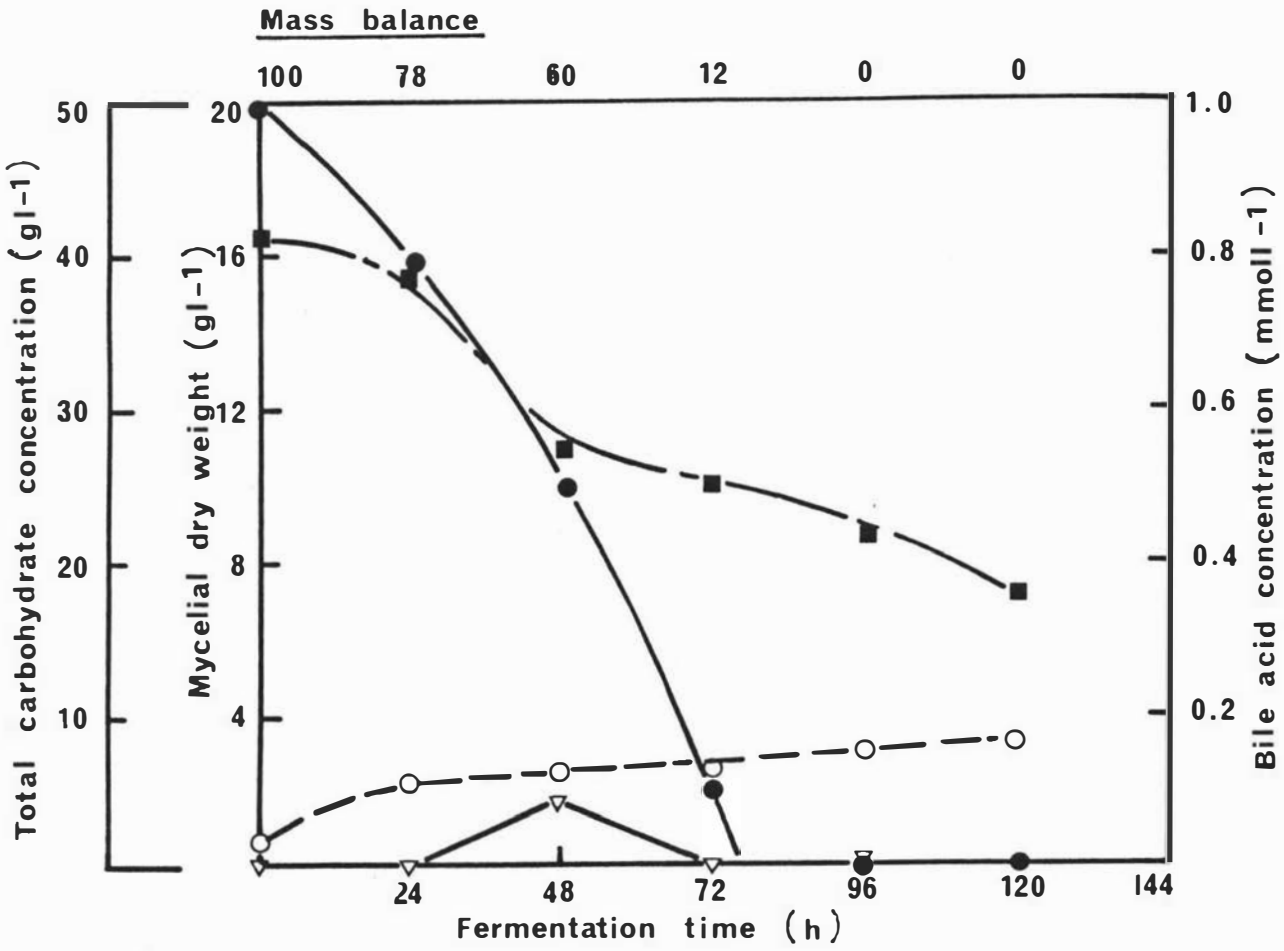
Conditions:

pH 6.5; Aeration rate, 1lmin^{-1} for 24h, then 0.25 1min^{-1}.

Notation:

dry weight, ●—— - ——●; total carbohydrate as glucose, □—— - ——□; glycodeoxycholic acid, ▲——▲; deoxycholic acid, ▽——▽.

FIGURE 4.17 The effect of reduced aeration on the hydrolysis of 0.5gl^{-1} glycodeoxycholic acid by *C. melonis* grown on glucose-peptone medium in a fermenter.



Conditions:
pH 6.5; aeration rate, 1lmin^{-1}.

Notation:
dry weight, ○-----○; total carbohydrate as lactose, ■——■; glycodeoxycholic acid, ●——●; deoxycholic acid, ▽——▽.

FIGURE 4.18 The effect of lactose on the hydrolysis of 0.5gl^{-1} glycodeoxycholic acid by *C. melonis* grown in lactose-peptone medium in a fermenter.

addition of pH control solutions in a reverse sequence to that of the base study. Therefore, it appears that the peptone in the medium was preferentially utilised as a carbon and energy source.

Hydrolysis of the glycodeoxycholic acid occurred, as observed by the transient appearance of deoxycholic acid after 48h of fermentation. However, both the conjugate and its hydrolysis product had completely disappeared by 96h of fermentation. The specific rate of glycodeoxycholic acid disappearance was high in comparison to the base study and remained relatively constant at a value of 5.6 $\mu mol h^{-1} g^{-1}$. The data for the utilisation of glycodeoxycholic acid did not fit a first order rate equation.

4.4.4. The Effect of Glucose Addition

The effect of sustained levels of glucose on the hydrolysis was studied in two experiments. The first employed the fermentation conditions of the base study, excepting that additional glucose was fed to the culture by an automatically-controlled pump at a predetermined rate. The second used the manual, incremental mode of glucose addition and, also, a reduced aeration rate of $0.25 l min^{-1}$ after 24h of fermentation. Otherwise, the variable levels were as for the base study.

The use of the combination of a high aeration rate ($1 l min^{-1}$) and a sustained glucose concentration resulted in a dry weight of $13 g l^{-1}$ within 48h of fermentation, which gradually increased to a maximum of $15 g l^{-1}$ after 144h. The total carbohydrate concentration was constant at $20 g l^{-1}$ until *ca* 72h of fermentation after which it rose to a peak of $50 g l^{-1}$ by 120h at which time the addition of glucose was terminated.

No quantitative analysis of bile acid concentration was performed due to the predominance of side-reactions in the fermenter. The hydrolysis of glycodeoxycholic acid was incomplete after 144h of fermentation and only low levels of deoxycholic acid were detected, principally over the 24-48h

period of fermentation after which time these disappeared. Two side-reaction products were observed after 72h of fermentation and they remained present throughout the course of the fermentation. They were identical, in terms of their chromatographic properties from tlc and hplc analysis, to the metabolites whose isolation and partial characterisation is described in Section 4.5.

The data derived from the experiment using a low aeration rate and a sustained glucose concentration are presented in Figure 4.19. The fungus attained a maximum dry weight of *ca* $15gl^{-1}$ within 48h of inoculation and maintained this mass until the final 24h of fermentation. Total carbohydrate was exhausted within 96h of fermentation and the dissolved oxygen tension paralleled that of the culture described in Section 4.4.2.

The hydrolysis of glycodeoxycholic acid proceeded in a similar manner to that of the base study. Thus, deoxycholic acid was the sole product observed, accumulating within 24h of inoculation. The maximum yield of product was 64% after 144h of fermentation, at which time a 6% residual of glycodeoxycholic acid remained. The loss of total bile acid was *ca* 35%, all of which was sustained in the first 72h after inoculation.

The specific rates of glycodeoxycholic acid disappearance and deoxycholic acid accumulation are plotted in Figure 4.20. These varied considerably during the course of the fermentation, attaining maximum values both in the initial and final 24h of fermentation. The rate of glycodeoxycholic acid utilisation deviated from first order kinetics over the final 24h of fermentation.

4.4.5. The Effect of pH

The influence of pH on the hydrolysis was studied at pH 5.5, pH 6.5 and pH 7.5 employing the conditions of the base study, amended to include an aeration rate of $0.25lmin^{-1}$ after 24h of fermentation and the incremental addition of glucose.

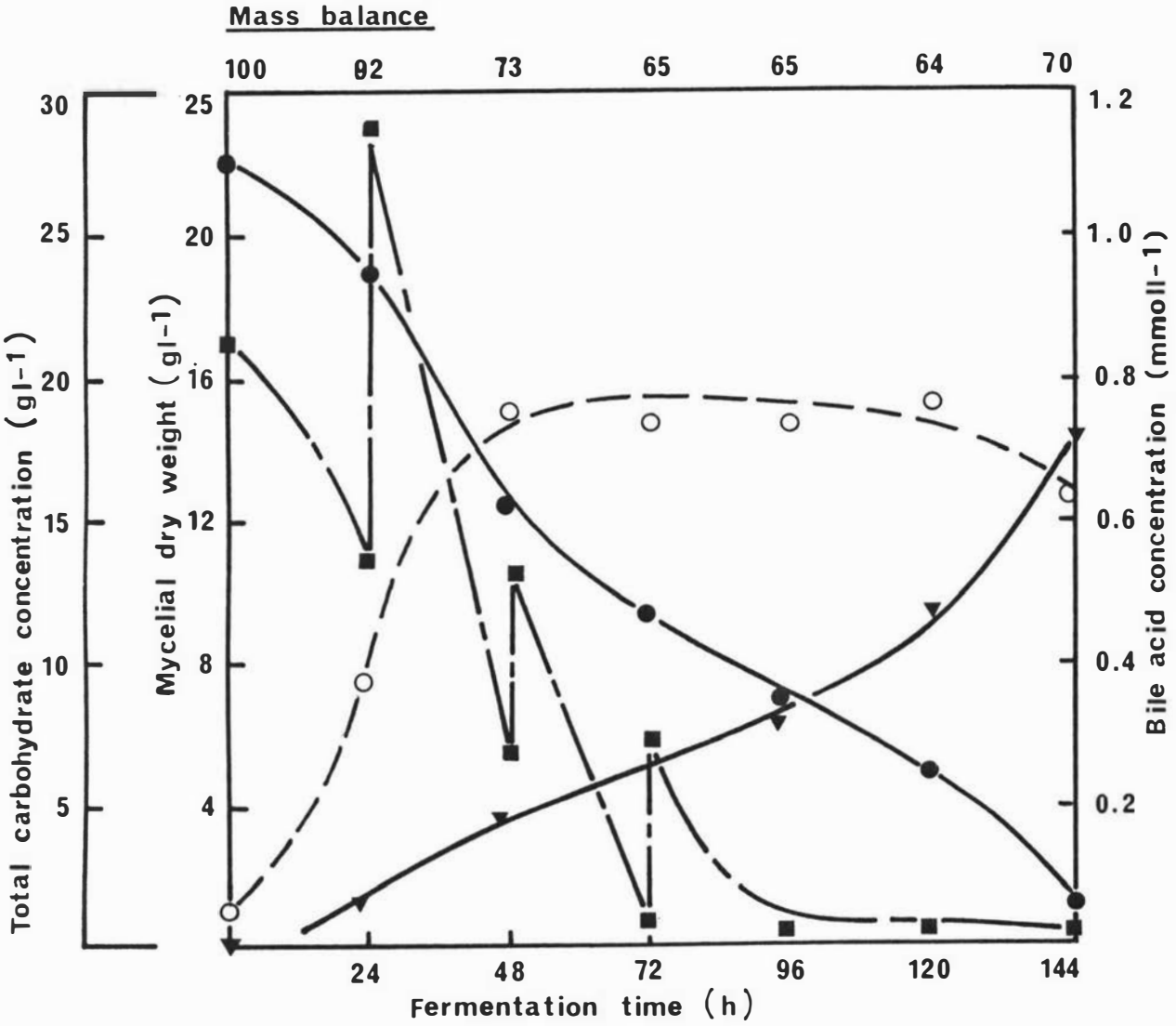


FIGURE 4.19 The effect of incremental glucose addition on the hydrolysis of 0.5gl^{-1} glycodeoxycholic acid by *C. melonis* grown on glucose-peptone medium in a fermenter.

Conditions:

pH 6.5; aeration rate, 1lmin^{-1} for 24h, then 0.25lmin^{-1}; 30g, 10g, and 10g of glucose added at 24h, 48h and 72h respectively.

Notation:

dry weight, O----O; total carbohydrate as glucose, ■---■; glycodeoxycholic acid, ●——●; deoxycholic acid, ▼——▼.

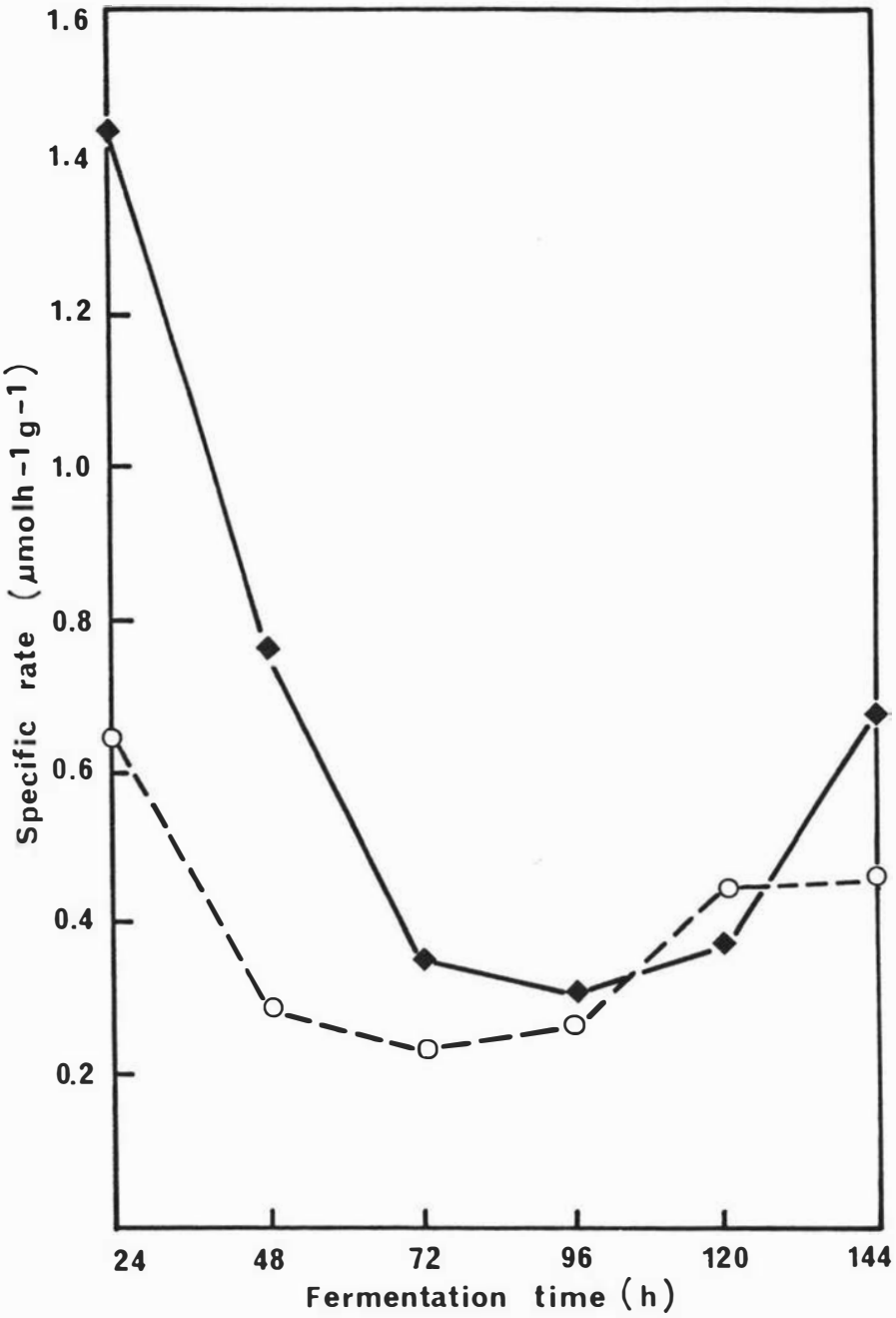


FIGURE 4.20 Specific rates of glycodeoxycholic acid disappearance and deoxycholic acid accumulation in the fermenter.
Conditions:
pH 6.5; aeration rate, 1lmin^{-1} for 24h, then 0.25lmin^{-1}, sustained glucose concentration.
Glycodeoxycholic acid disappearance, ◆———◆; deoxycholic acid accumulation, ○– – –○.

Results from the pH 6.5 fermentation have been presented in the previous section and this pH value gave the highest deoxycholic acid yield of those observed under varying pH conditions.

The data from the fermentations conducted at pH 5.5 and pH 7.5 are presented in Figures 4.21 and 4.22 respectively. In contrast to the corresponding pH 6.5 fermentation (Figure 4.19), the growth of the fungus was slower at both pH values. At pH 5.5, a similar dry weight maximum of $15gl^{-1}$ was obtained after 120h of fermentation, but the growth rate declined noticeably after 48h. The maximum dry weight of the culture at pH 7.5 was $12.5gl^{-1}$. Dissolved oxygen tension at both pH values paralleled that of the corresponding pH 6.5 culture. However, the total carbohydrate concentration in the pH 5.5 culture remained high in comparison, probably due to its slower growth. Thus, the glucose was not exhausted until late in the fermentation.

At pH 5.5, deoxycholic acid accumulated as the sole product of hydrolysis within 48h, giving a maximum yield of 30% after 192h of fermentation. At this time, 15% of the initial glycodeoxycholic acid remained. The loss of total bile acid proceeded at a constant rate throughout the fermentation to attain a maximum value of 55% by its termination.

The specific rates of glycodeoxycholic acid disappearance and deoxycholic acid accumulation were the lowest of those measured in cultures in which deoxycholic acid was the sole product (Figure 4.23). They were similar to those of the corresponding pH 6.5 culture except that the specific hydrolase activity degenerated with time. The rates of bile acid utilisation and accumulation did not conform to first order rate equations.

At a culture pH of pH 7.5, side-reactions were favoured and only trace amounts of deoxycholic acid were observed. In fact, hydrolysis appeared to be inhibited, as glycodeoxycholic acid remained as the major bile acid after 192h of fermentation. Quantitative bile acid analysis was not performed as the side-reaction products had similar retention volumes on hplc to

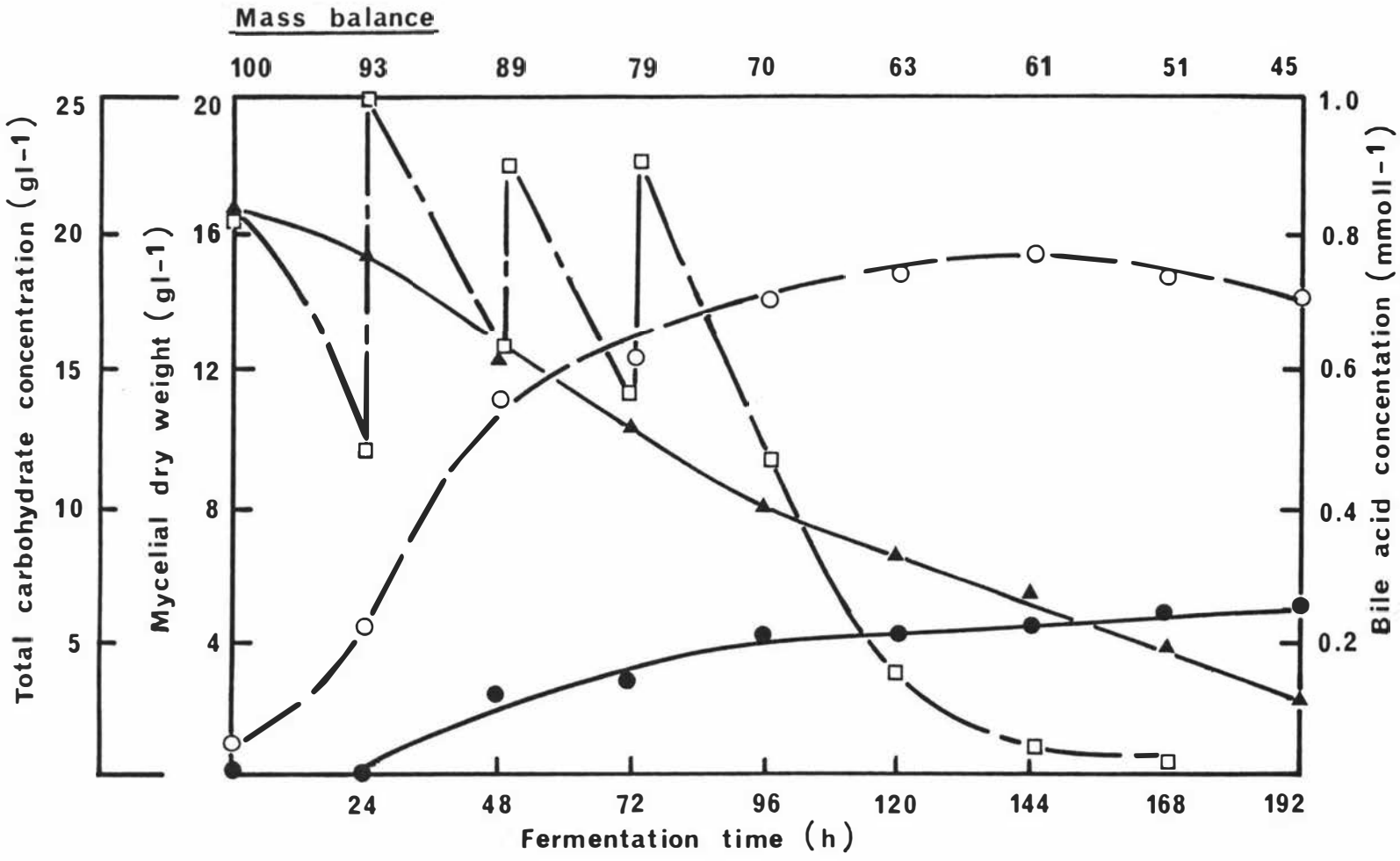


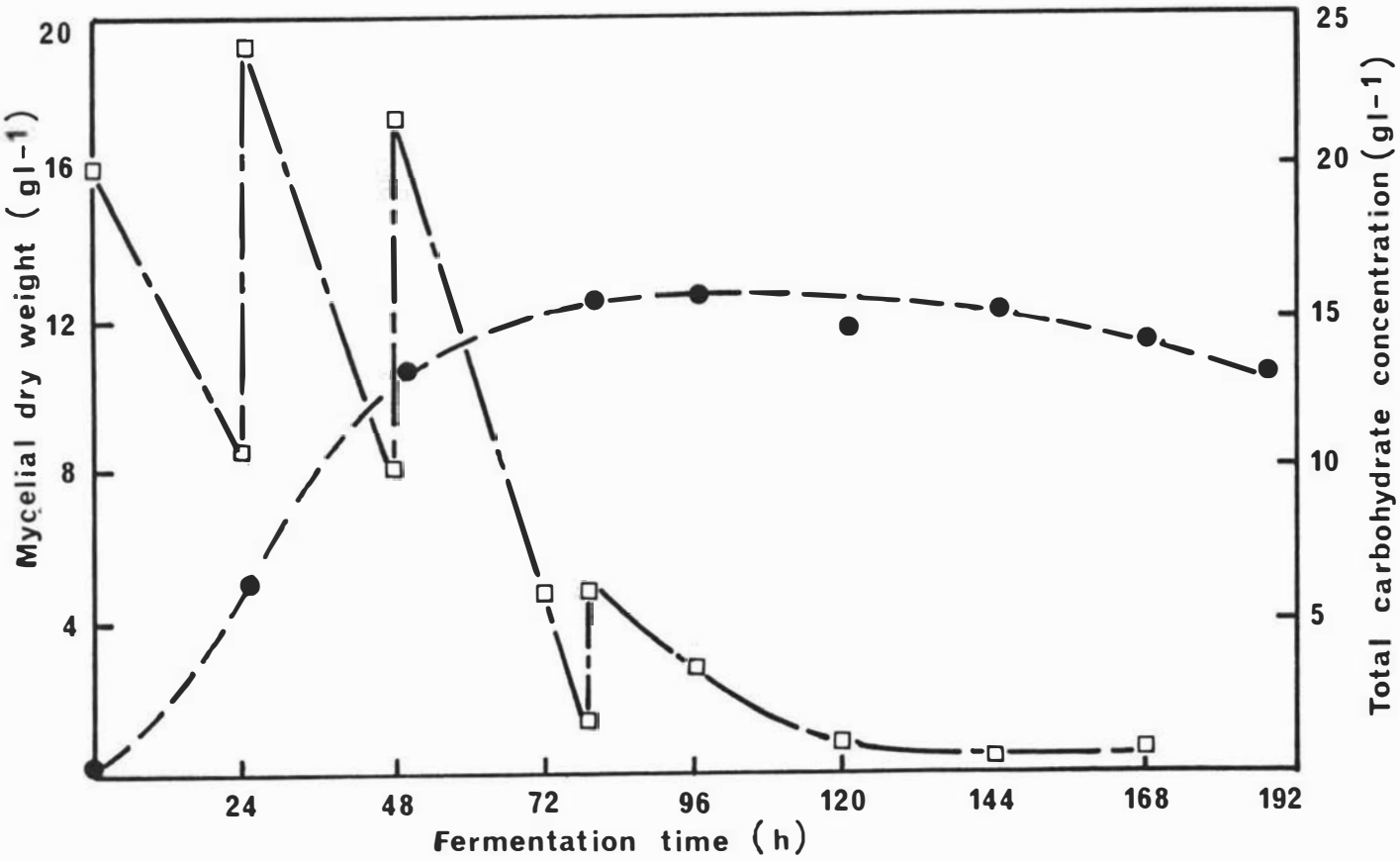
FIGURE 4.21 The hydrolysis of 0.5gl^{-1} glycodeoxycholic acid at pH 5.5 by *C. melonis* grown in glucose-peptone medium in a fermenter.

Conditions

pH 5.5; Aeration rate, 1lmin^{-1} for 24h, then 0.25l min^{-1}; 30g, 10g and 10g of glucos added at 24, 48 and 72h respect-ively.

Notation:

dry weight, O---O total carbohydrat as glucose, □———□; glyco-deoxycholic acid, ▲———▲; deoxy-cholic acid, ●———●.



Notation:
dry weight, ●----●;
total carbohydrate as glucose, □——□.

FIGURE 4.22 The growth and utilisation of glucose by *C. melonis* at pH 7.5 in a fermenter, in glucose-peptone medium containing 0.5gl^{-1} glycodeoxycholic acid; aeration rate, 1lmin^{-1} for 24h, then 0.25lmin^{-1}; 30g, 10g and 5g of glucose added at 24, 48 and 72h respectively.

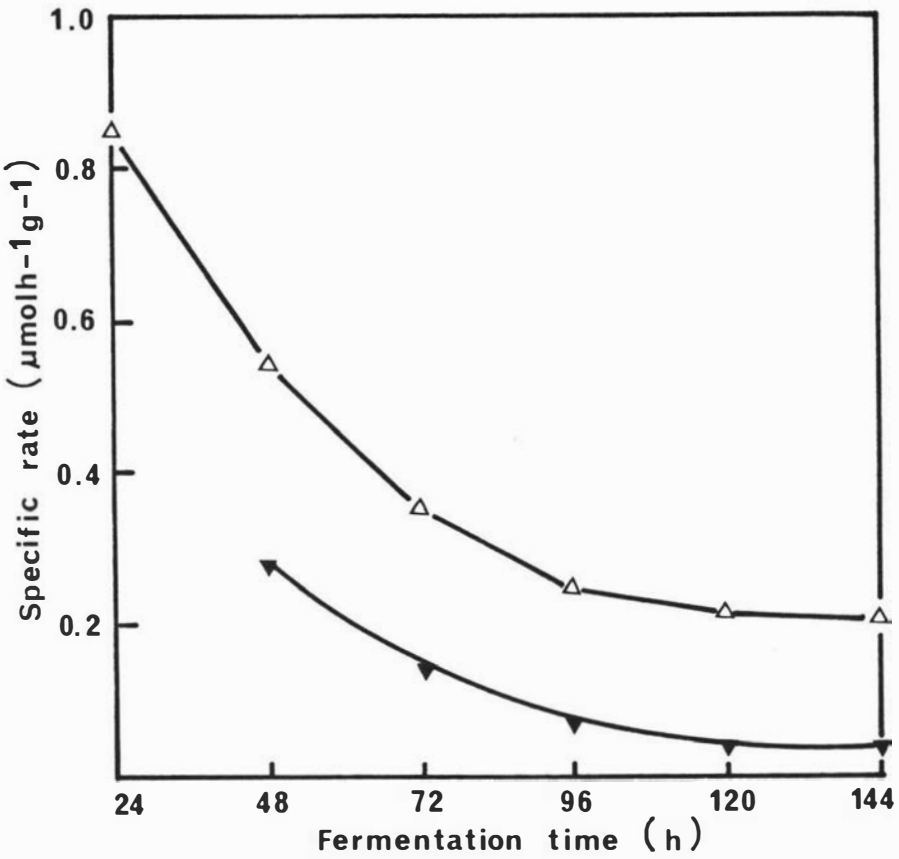


FIGURE 4.23 Specific rates of glycodeoxycholic acid disappearance and deoxycholic acid accumulation in the fermenter at pH 5.5.
Aeration rate, $1 l min^{-1}$ for 24h, then $0.25 l min^{-1}$; sustained glucose concentrations.
Glycodeoxycholic acid disappearance, △——△; deoxycholic acid accumulation, ▼——▼.

the usual internal standards employed. The isolation and partial characterisation of the two predominant bile acid side-products are described in Section 4.5.

4.4.6. Discussion

Good growth and biomass yields of *C. melonis* were obtained under most of the conditions employed in the fermenter. Incremental addition of glucose produced increased dry weight values, which were sustained throughout the fermentation. However, caution is required when interpreting dry weight data, particularly if the culture is nitrogen-limited, since an increase in dry weight may represent an increase in cell mass due to the conversion of excess carbon to storage compound, rather than cell proliferation (Borrow *et al*, 1961). The poor dry weight values obtained from lactose suggest that this carbohydrate is a poor carbon and energy source for the fungus. Similar results have been reported for the growth of some other *Cercospora* species on lactose (Dayal and Ram, 1967).

In general, dissolved oxygen tension did not appear to be growth-limiting. Thus, fermentations in which the aeration rate was reduced after 24h of fermentation produced similar dry weight values and growth rates to those in which the aeration rate was sustained at 1 $lmin^{-1}$.

The bile acid data demonstrate that the yield of deoxycholic acid was sensitive to each of the environmental factors tested (Figure 4.24). However, this may not necessarily reflect the sensitivity of the hydrolysis alone, but also that of the factors contributing to losses of bile acid. Hence, considerable ambiguity is introduced into any interpretation of the yield data, since the effect of a variable on the yield of deoxycholic acid may be the sum of its individual effects on both the hydrolysis of glycodeoxycholic acid and the factors responsible for bile acid loss. The same ambiguity is contained in the specific rates of product accumulation. However, the specific rate of glycodeoxycholic acid disappearance is more meaningful, provided it is assumed that

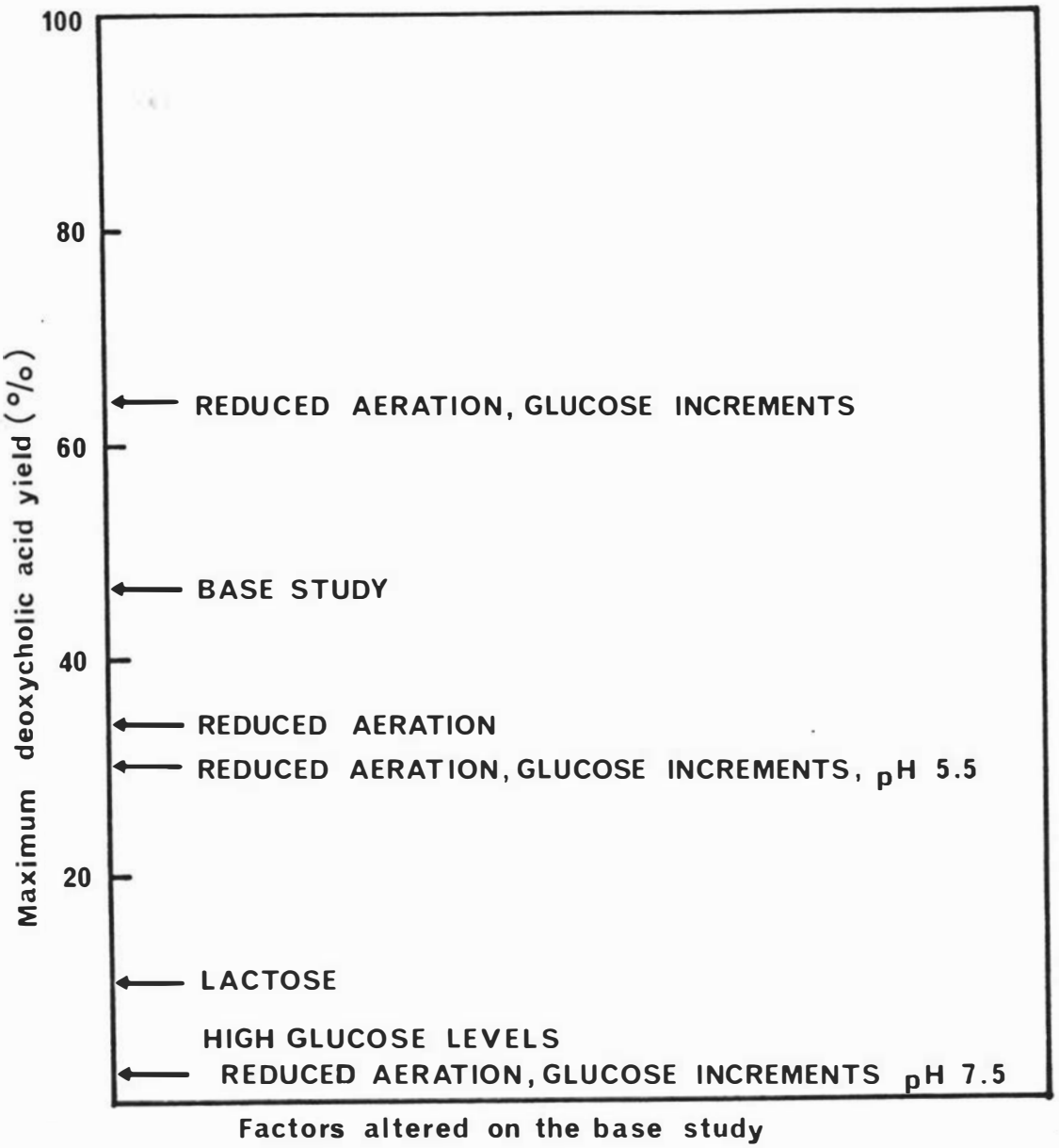


FIGURE 4.24 The effect of variables and their combinations on the maximum yield of deoxycholic acid from hydrolysis in the fermenter. The variable levels named indicate the change from the base study. Reduced aeration; $1 l min^{-1}$ for 24h, then $0.25 l min^{-1}$; lactose, at $40 g l^{-1}$; glucose increments are given in the text; high glucose levels are $> 20 g l^{-1}$. The base study variable levels are: aeration rate, $1 l min^{-1}$; pH 6.5; glucose-peptone medium.

hydrolysis is a prerequisite to further bile acid degradation.

A high aeration rate gave superior deoxycholic acid yields to the corresponding fermentation in which the aeration rate was reduced after 24h. The lower dissolved oxygen tension of the latter resulted in greater loss of bile acid compared to the former fermentation, whereas the hydrolysis of the conjugate was not significantly affected. This is demonstrated by the comparison of the specific rates of deoxycholic acid accumulation and glycodeoxycholic acid disappearance which were 1.0 and 1.2 $\mu molh^{-1}g^{-1}$ respectively for the base study and 0.4 and 1.6 $\mu molh^{-1}g^{-1}$ respectively for the lowered aeration experiment. Clearly, whereas the specific rate of hydrolysis (i.e. the specific rate of glycodeoxycholic acid disappearance) is similar for both, the specific rate of deoxycholic acid accumulation is markedly reduced for the latter. This suggests that the degradation of the free bile acid product is enhanced under conditions of reduced aeration in the fermenter.

The carbohydrate source, and its concentration in the case of glucose, significantly affected both hydrolysis and the loss of bile acid. The use of incremental glucose addition to maintain the level of carbohydrate at *ca* 5-20gl^{-1} for the first 84h after inoculation, gave the highest deoxycholic acid yield of all the fermenter experiments (Figure 4.24). In fact, the sustained glucose levels in that culture (Figure 4.19) offset the deleterious effect of reduced aeration rates on the yield of deoxycholic acid. This is a fortunate result, as the reduced aeration rates created a more manageable fermentation in terms of foam production and wall growth. The promotion of higher product yields under these conditions was due to the suppression of bile acid loss after 72h of fermentation, perhaps due to the catabolite repression of bile acid utilisation by glucose. The enzymes involved appear to be inducible in view of the inhibition of their activity by cycloheximide (Refer Section 4.2.5). Such repression would not be unexpected since the breakdown of bile acids by bacteria appears to produce intermediates of carbohydrate catabolism (Hayakawa, 1973).

However, the use of high glucose concentrations (> $20gl^{-1}$) and an aeration rate of 1 $lmin^{-1}$ stimulated the operation of side-reactions, to the detriment of deoxycholic acid production. The side-products appear to be oxidised derivatives of both glycodeoxycholic acid and deoxycholic acid (Refer Section 4.5) and may not necessarily be intermediates in the degradation of bile acid by *C. melonis*. Thus, the high glucose concentrations may repress the latter enzymes while the high aeration rates may promote the appearance of the oxidised side-products. Constitutive, steroid-modifying activity was observed in cell-free extracts of *C. melonis* (Refer Section 4.2.4.) and such products may be the result of their action. Hence, two pathways may operate on the hydrolysis product. An inducible, degradative pathway repressed by glucose and enhanced by low aeration rates, and a bile acid-modifying pathway constitutive to some degree and enhanced at higher aeration rates. The former appears the more active of the two.

Although the combination of high glucose levels and a high aeration rate resulted in oxidised side-products, these were not observed in the culture employing a high lactose concentration in combination with a high aeration rate. The hypothesised catabolite repression of the degradative enzymes by glucose may explain both this and the accelerated metabolism of bile acid in the lactose culture (Refer Figure 4.18), since lactose exerts no such repression. Hence, peptone was apparently utilised as the carbon source with a consequent release of the excess nitrogen as ammonia. Since no glucose was present to repress the degradative enzymes free bile acid was used as a carbon source, overriding perhaps, the oxidative, steroid-modifying pathway with the result that such products did not accumulate.

The utilisation of the bile acid by the fungus appeared to be the predominant factor in its loss in fermenter cultures. The binding of bile acids by the mycelium neither accounts for the observed sensitivity of the losses to environmental factors, nor correlates well with the actual losses when the data of Table 4.3 are used to predict the loss. This is

particularly well illustrated by the culture grown on lactose, in which the expected loss from binding 48h after inoculation is 8% compared to the 40% observed (Refer Figure 4.18). Thus, the relative contribution to bile acid loss by binding and degradation appear to differ significantly in fermenter and shake flask cultures, binding apparently predominating in the latter.

The specific rates of glycodeoxycholic acid disappearance were significantly lower in cultures with glucose increments added compared to the corresponding control cultures. This difference was not explained by the assumption that the additional dry weight concentrations of the former cultures were inert with respect to hydrolase activity and hence the apparent specific rate underestimated the true rate. This suggests that hydrolysis may be carbon catabolite regulated by glucose.

This hypothesis may explain the rapid increase in hydrolase activity after glucose exhaustion in the culture in which incremental glucose addition and a reduced aeration rate were employed (Figures 4.19, 4.20), and the poor extent of hydrolysis observed in the culture in which a high aeration rate and glucose concentration were used. In addition, such a proposal may also account for the significantly higher specific rate of glycodeoxycholic acid utilisation in cultures grown on lactose (i.e. 5.6 $\mu molh^{-1}g^{-1}$ compared to 1.2 $\mu molh^{-1}g^{-1}$ in base study), as this carbohydrate does not exert catabolite regulation (Demain *et al*, 1979). Catabolic regulation of extracellular enzyme synthesis and secretion is thought to be a primary mechanism in their regulation (Fogarty and Kelly, 1979) and, in view of the observation of extracellular hydrolase activity in *C. melonis* cultures, the sensitivity of hydrolysis to glucose concentrations is, perhaps, not surprising.

Under the conditions employed, a culture pH of pH 6.5 was optimum for hydrolysis both in terms of rate and yield (Figure 4.24). At pH 5.5, the rate of hydrolysis was significantly reduced compared to the corresponding pH 6.5

culture, although this may, in part, be due also to its higher glucose concentration. Given the pKa of glycodeoxycholic acid (pKa 4.69), the lower culture pH may have been expected to result in a greater proportion of undissociated molecules able to penetrate the cell membrane with a concomitant increase in the rate of hydrolysis. The absence of such a response suggests that the extracellular hydrolase activity may predominate in these cultures. The higher culture pH of pH 7.5 was particularly unfavourable for obtaining good yields of deoxycholic acid.

The pattern emerging from the data suggests that the cholanoyl glycine hydrolase may be a neutral, extracellular protease elaborated by the fungus, which is non-specific to the extent of allowing the hydrolysis of glycine-conjugated bile acids. This would explain the significantly higher specific hydrolysis activity in the lactose-peptone culture where it appears, from culture pH and lactose concentration data, that peptone was preferentially utilised as the carbon source. For this to occur, it can be expected that increased amounts of extracellular protease would be secreted to degrade the peptone compared to cultures in glucose-peptone medium in which there is a lesser requirement for peptone hydrolysis. Hence, the markedly higher specific rates of glycodeoxycholic acid hydrolysis in the presence of lactose, may reflect higher extracellular protease concentrations in the culture.

The inhibition of hydrolysis by 8-hydroxyquinoline (Refer Section 4.3.2.) also suggests, in conjunction with the above data, that the hydrolase may be a neutral protease. Unlike acid and alkaline microbial proteases, the neutral, extracellular proteases are zinc-containing metalloproteins and are inhibited by metal chelators (Fogarty and Kelly, 1979). Zinc is completely precipitated as a complex with 8-hydroxyquinoline at pH values of pH 4.4 or greater (Vogel, 1962) which are in the pH range observed in *C. melonis* cultures. Further support for the hypothesis that the cholanoyl glycine hydrolase is a neutral protease is provided by the hydrolase pH optimum of *ca* pH 6.5 in fermenter culture.

4.5. Bile acid Metabolites of Glycodeoxycholic acid

The main aim of the fermenter studies was to increase the yield of deoxycholic acid from hydrolysis. However, under some conditions, other products were formed. As these represent competing or subsequent reactions, it was considered desirable to attempt the isolation and identification of such products.

Bile acid products other than deoxycholic acid were observed under two sets of fermenter conditions. The first employed the base study conditions varied to include a culture pH of pH 7.5, glucose increments and an aeration rate reduced to 0.25 $1min^{-1}$ after 24h of fermentation. The second used the base study conditions with the exception that the glucose concentration was at a high level (> $20gl^{-1}$). In each, two metabolites were produced and the products of both fermentations were identical by the criteria of the tlc and hplc retention data.

The isolation and partial characterisation of metabolites A and B were performed as described in Section 3.10.5. The Girard T separation was poor; whereas 80 mg of material was present in the aqueous phase, 370 mg remained in the ether layer and tlc analysis demonstrated the presence of high concentrations of both metabolites together with glyco-deoxycholic acid and deoxycholic acid in the latter. This was unexpected since the chromatographic properties of both metabolites strongly suggested the presence of a keto function in the molecule.

The large losses of material during preparative hplc (*ca* 88%) hindered the complete characterisation of both metabolites. It appears that the compounds may be strongly adsorbed by the column packing material.

4.5.1. Fraction A

The melting point of the crystals (7 mg) from fraction A was

160-3°C. Tlc and hplc analysis demonstrated the presence of metabolite A only. Table 4.4 compares the tlc R_f and hplc retention volume data for metabolite A with those of standard bile acids. The metabolite had a slightly higher R_f than glycodeoxycholic acid in most tlc solvent systems and had a smaller retention volume on hplc. The former results suggested that metabolite A was conjugated with glycine. The latter was evidence of the presence of a keto function, since bile acids containing ketone functions are observed to have smaller retention volumes compared to those of the corresponding hydroxylated bile acids under the hplc conditions employed in this laboratory.

The mass spectrum of the methyl ester exhibited a low intensity molecular ion (m/e 461; $C_{27}H_{43}NO_5$) which had a similar fragmentation pattern to methyl glycodeoxycholate (Table 4.5). Thus, there was loss of molecules of water (M-18; M-18-172-18) and the sidechain (172 amu). The fragmentation pattern demonstrated that metabolite A was conjugated with glycine and had a single unsaturation equivalent in the steroid nucleus. The characteristic m/e 249 peak expected from the presence of a 12-keto function was not observed.

4.5.2. Fraction B

The crystals (*ca* 2mg) obtained as fraction B had a melting point of 108-110°C and were slightly contaminated with material with a melting point at 149-151°C. Tlc and hplc analysis indicated that metabolite B was the principal component with a small quantity of compound A present. The chromatographic analysis of this fraction indicated that metabolite B was a free bile acid (Table 4.4). It ran slightly above the 3α-hydroxy-12-oxo-5β-cholanic acid standard in all the tlc solvent systems used and on hplc was greatly retarded in comparison. The chromatographic data suggest that 12α-hydroxy-3-oxo-5β-cholanic acid is the most probable identity of metabolite B. The β-hydroxy epimers of deoxycholic acid are eliminated by the chromatographic data.

Table 4.4

Chromatographic properties[a] of metabolites A and B

Tlc solvent system	Bile acid	A	B	GD	DC	12-keto	3-keto	12β-OH	3βOH
a	free acid	0.20	0.61	0.15	0.56	0.58	0.60	0.57	0.57
b	free acid	0.03	0.55	0.02	0.27	0.53	0.55	0.47	0.42
c	free acid	0.22	0.73	0.13	0.60	0.72	-	-	-
d	free acid	0.0	0.22	0.0	0.09	0.17	0.20	0.15	-
a	methyl ester	0.22	0.63	0.17	0.58	0.60	-	-	-
Hplc retention volume (ml)	free acid	6.6	10.8	9.6	16.8	7.2	10.8		
	methyl ester	11.4	31.2	16.8	42.6	19.2			

[a] For tlc; Rf Values for 12β-OH, 3β-OH are taken from Heftmann (1967) and are adjusted for those obtained by standards.

12-keto = 3α-hydroxy-12-oxo-5β-cholanic acid;
3-keto = 12α-hydroxy-3-oxo-5β-cholanic acid;
12β-OH = 3α, 12β-dihydroxy-5β-cholanic acid;
3β-OH = 3β, 12α-dihydroxy-5β-cholanic acid.

The mass spectrum of the methyl ester of metabolite B exhibited a low intensity molecular ion (m/e 404; $C_{25}H_{40}O_4$) with one unsaturation equivalent in the bile acid nucleus (Table 4.5). The fragmentation pattern was characterised by the loss of molecules of water (M-18; M-18-115-18) and the sidechain (115 amu). The intensity of the m/e 249 peak (8%) was less than expected if a 12-keto group had been present (90%) and the sidechain had not been degraded.

4.5.3. Discussion

The mass spectrometry data suggest that metabolites A and B have an identical cholane nucleus containing one unsaturation equivalent, which chromatographic data suggest is a keto function. Metabolite A is conjugated with glycine and it is likely that metabolite B is the corresponding free bile acid. Enzymatic or alkaline hydrolysis of some metabolite A would conveniently establish whether this is the case, although keto bile acids suffer severe degradation during the latter procedure (Lepage *et al*, 1978). It is uncertain whether metabolite B was derived from the action of the hydrolase(s) on metabolite A or from deoxycholic acid *via* enzymic oxidation.

The identification of metabolite A as a glycine-conjugated derivative of deoxycholic acid is novel, particularly since substantial yields of 15-20% were obtained as estimated by hplc analysis. In most cases of microbial transformation reported, hydrolysis precedes further modification of the bile acid nucleus (Furuta, 1959; Hayakawa, 1973; Hill, 1976).

From the data obtained, metabolite B appears to be 12α-hydroxy-3-oxo-5β-cholanic acid. Mass spectrometry and tlc and hplc analysis results clearly eliminate the presence of a 12-keto function and the chromatographic data also rules out β-hydroxy epimers of deoxycholic acid. Thus, on tlc solvent systems "a" and "b", the free bile acid mono-β-hydroxy epimers have lower R_f values than 3α-hydroxy-12-oxo-5β-cholanic acid (Heftmann, 1967), whereas metabolite B has a higher R_f value

Table 4.5

Fragmentation patterns of metabolites A and B

Bile acid methyl ester standard	m/e	Intensity	Metabolite	m/e	Intensity
GD	463	6	A	461	20
	273	75		404	8
	255	100		402	7
				271	100
				253	46
DC	406	4			
	404	11			
	388	51	B	404	26
	370	67		386	25
	273	100		271	100
	255	97		253	23
12-keto	404	100			
	386	22			
	271	12			
	249	90			
	231	77			

12-keto = 3α-hydroxy-12-oxo-5β-cholanic acid.

than this compound (Table 4.4). The melting point of the majority of the material in fraction B (108-110°C) does not agree with published values for methyl 12α-hydroxy-3-oxo-5β-cholanate (m.p. 148-149°C, Tserng, 1978), but the latter are in agreement with the melting point (149-151°C) of the smaller quantity of material present in Fraction B. In view of the apparent absence of free bile acid contaminants in the mass spectrum of this fraction, the lower melting point may be due to solvation of the crystals.

The low extent of reaction of metabolite B with the Girard T reagent seems unusual if it is indeed the 3-keto derivative, as there should be no steric hindrance to retard the reaction. However, there is no published data regarding the formation of Girard T derivatives of 3-keto bile acids.

It is probable that metabolite A is N-(glyco)-12α-hydroxy-3-oxo-5β-cholanic acid. However, the synthesis of this compound has not been reported in the literature and therefore physical data are unavailable for confirmation.

3α-Hydroxy dehydrogenase activity has been observed on cholic acid and its derivatives in fungal cultures, where 3-keto-dihydroxy bile acids are the result of the initial attack on the cholic acid molecule (Furuta, 1959; Hasegawa, 1959).

4.6. Fermenter Studies with Glycocholic acid

The hydrolysis of glycocholic acid by *C. melonis* in shake flask culture gave superior product yields to those of glyco-deoxycholic acid hydrolysis. To determine if this difference in behaviour persisted in the fermenter a comparative experiment was performed.

4.6.1. The Course of Hydrolysis

The fermenter environment used for the glycodeoxycholic acid experiment in Figure 4.19 was employed for the comparison. The time courses of the fermentations were similar, except that the utilisation of total carbohydrate in the culture containing glycocholic acid was *ca* 50% that of the culture containing

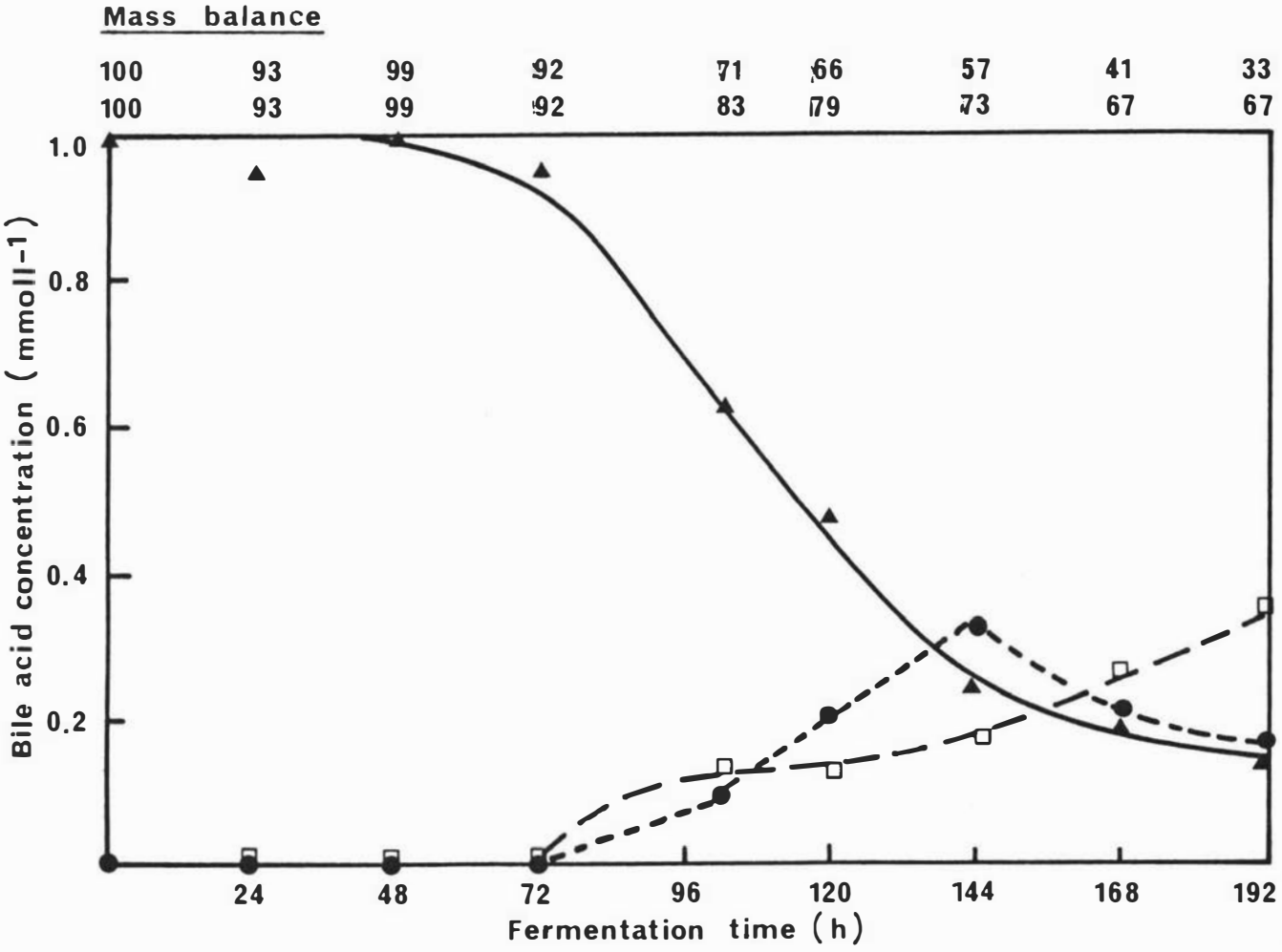
glycodeoxycholic acid between 24h and 120h of fermentation.

The quantitative bile acid data for the glycocholic acid experiment are displayed in Figure 4.25 for comparison with those of Figure 4.19. Two major bile acid products accumulated from glycocholic acid and trace amounts of two others were detected by tlc. Of the major products, one was cholic acid, while the other (compound X), was of an unknown structure (Table 4.6). As the hplc retention volume of compound X was similar to that of 3α,12α-dihydroxy-7-oxo-5β-cholanic acid, the latter was employed as a standard to obtain approximate quantitation of the unknown product. An attempt to isolate and characterise compound X *via* the formation of, and its separation as, a Girard T derivative using the procedure given in Section 3.10.5, was unsuccessful.

Both cholic acid and compound X accumulated simultaneously after 72h of fermentation, virtually no activity being apparent on glycocholic acid before this time. The cholic acid yield peaked at 30% after 144h and then declined, whereas the yield of compound X was static between 96h and 144h, after which time it increased to a maximum of 34%. A residual of 18% of glycocholic acid remained by the end of the fermentation. The mass balance demonstrates a maximum loss of bile acid of 33%, principally during the time of active hydrolysis. However, the glycocholic acid and the cholic acid concentration together was only 33% of the initial bile acid present, by the end of the fermentation.

The specific rates of glycocholic acid disappearance and product accumulation are presented in Figure 4.26. The specific hydrolysis activity was maximal 96h after inoculation, after which time it declined rapidly to be negligible after 144h of fermentation, despite the presence of unconverted glycocholic acid in the culture. The specific rate of cholic acid accumulation was constant during the 96-144h period of fermentation, whereas that of compound X increased towards the end of the fermentation.

The data from the final 48h of fermentation suggests that cholic



Conditions:
pH 6.5; aeration rate, 1.0 $l min^{-1}$ for 24h, then 0.25 $l min^{-1}$; 30g, 10g, 10g glucose added at 24, 48 and 72h respectively.

Notation:
Glycocholic acid, ▲——▲;
cholic acid, ●- - - -●;
compound X, □— — —□.

FIGURE 4.25 The hydrolysis of 0.5 $g l^{-1}$ glycocholic acid by *C. melonis* grown on glucose-peptone medium in a fermenter.

Table 4.6

Chromatographic properties of compound X

Bile acid	Tlc Rf. Solvent system "a"	"b"	Hplc retention volume (ml)
CA	0.17	0.04	13.3
7-keto	0.25	0.12	7.5
DC	0.51	0.23	25.0
X	0.29	0.15	9.2

Solvent front: 150mm

7-keto = 3α,12α-dihydroxy-7-oxo-5β-cholanic acid.

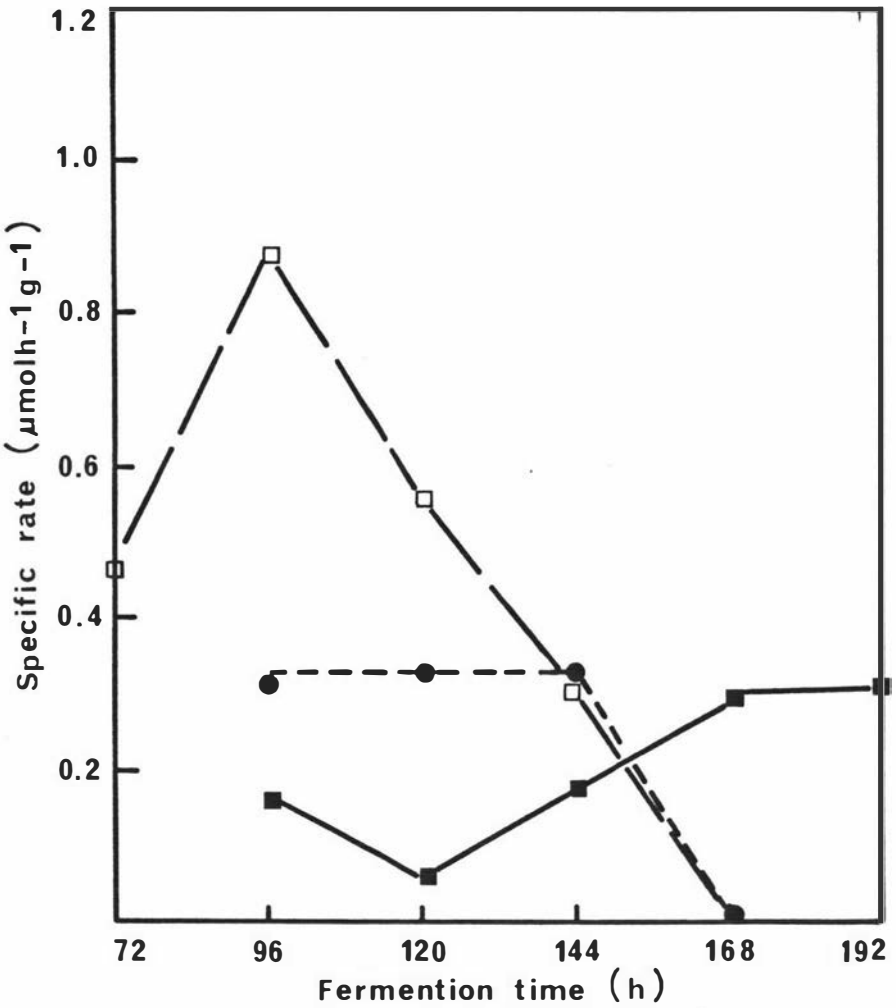


FIGURE 4.26 Specific rates of glycocholic acid disappearance and product accumulation in a fermenter. Conditions: pH 6.5; aeration rate, 1 $lmin^{-1}$ for 24h, then 0.25$lmin^{-1}$; glucose increments at 24, 48 and 72h. Glycocholic acid disappearance, □——□; cholic acid accumulation, ●– – –●; compound X accumulation, ■——■.

acid was directly modified to compound X. Thus, the concentration of compound X increased by 0.17 $mmol\,l^{-1}$ during this time, corresponding to an equivalent decrease in cholic acid concentration. In addition, the specific rate of compound X accumulation exceeded that of glycocholic acid disappearance during the final 48h of fermentation (Figure 4.26). The rate of glycocholic acid disappearance and the combined product accumulation both fitted first order rate equations, although the individual rates of product accumulation deviated significantly from first order kinetics.

4.6.2. Discussion

The accumulation of significant yields of compound X, in addition to cholic acid, from glycocholic acid in the fermenter contrasts with the hydrolysis of this conjugate in shake flask cultures (Refer Figure 4.3), and with the fission of glycodeoxycholic acid in the corresponding fermenter culture, in which cholic acid and deoxycholic acid respectively were the sole products of fungal action. Thus, whereas superior yields of cholic acid were obtained from glycocholic acid hydrolysis in shake flask cultures compared to those of deoxycholic acid from glycodeoxycholic acid, the reverse is true for the fermenter conditions employed.

The factor(s) responsible for the operation of the side-reactions in the fermenter culture containing glycocholic acid is unknown. However, cholic acid has been reported as being more vulnerable than deoxycholic acid to modification in aerobic cultures of bacteria (Hayakawa, 1973) and transformation of cholic acid to various oxidised derivatives has been observed in fungal cultures by Furuta (1959) and Hasegawa (1959). Thus compound X may represent an initial stage of modification of the cholic acid.

The assignment of a tentative structure for compound X is difficult, despite the evidence from the fermentation data that it is an immediate derivative of cholic acid. The tlc data suggest that compound X is not conjugated with glycine, but it does not appear to be a monoketodihydroxy derivative

of cholic acid, in contrast to the metabolite derived by the action of *C. melonis* on deoxycholic acid (Refer Section 4.5). Thus, 3α,12α-dihydroxy-7-oxo-5β-cholanic acid and 3α,7α-dihydroxy-12-oxo-5β-cholanic acid standards had Rf values consistently lower than compound X in the tlc solvent systems employed ("a" to "e"), while in solvent system "b" compound X had an Rf lower than deoxycholic acid, whereas 7α,12α-dihydroxy-3-oxo-5β-cholanic acid has an Rf higher than deoxycholic acid (Heftmann, 1967).

Similarly, a β-orientation of one hydroxyl function is inconsistent with the tlc data and the Rf value of compound X on the tlc solvent systems employed is too low to suggest that dehydroxylation has occurred (Heftmann, 1967). In addition, compound X probably retains an undegraded sidechain, since such modification has not been observed by mass-spectrometric analysis of bile acid metabolites recovered from *C. melonis* cultures. Hence, the identity of compound X awaits its isolation as crystals and further characterisation.

The hydrolysis of glycocholic acid in the fermenter was also characterised by a considerable lag prior to its metabolism and by the decline of hydrolase activity after 144h of fermentation. The reason for the first observation is unclear, since in the corresponding glycodeoxycholic acid culture, losses occurred within 24h of inoculation and fermentation conditions were similar for both cultures. The second observation confirms that active hydrolysis rarely continues beyond *ca* 144h in fermenter cultures. Similar declines in activity after this time were noted in glycodeoxycholic acid fermentations (Refer Figures 4.16, 4.23).

Otherwise, however, in terms of total product yield and specific rates of hydrolysis, the action of *C. melonis* on glycocholic acid in the fermenter was similar to that on glycodeoxycholic acid.

4.7. Summary

The data demonstrate the presence of intracellular and

extracellular, constitutive cholanoyl glycine hydrolase in cultures of *C. melonis* under a variety of fermentation conditions. Both glycocholic acid and glycodeoxycholic acid are hydrolysed by this activity, presumably by the same enzyme. The hydrolysis is a genuine activity of the fungus and is not the result of the pH conditions of the fermentation, as proved by the aseptic incubation of the glycine conjugates at pH values of 2.5, 7.0 and 8.5 in glucose-peptone medium for 25 days, without evidence of their hydrolysis.

Whether the intracellular and extracellular enzymes are the same is unclear. Nor are their relative contributions to the hydrolysis known. However, at the normal culture pH observed in the experiments, the glycine conjugates will exist mainly as anions, to which the cell membrane is largely impermeable. As permeability did not appear to be a significant factor governing the hydrolysis of glycine conjugates, it seems probable that the extracellular enzyme may be largely responsible for the hydrolysis observed. This may explain the sensitivity of the hydrolysis to environmental parameters, especially culture pH and glucose concentration.

It is tempting to speculate that the hydrolysis of glycine conjugates may be catalysed by an extracellular, neutral protease elaborated by the fungus to degrade the peptone in the medium. Such proteases are widespread in fungi and hence, a protease acting on the amino acid at the carboxyl terminal of a peptide or protein may indiscriminately cleave the glycine moiety from the bile acid conjugate. The observation that high free bile acid concentrations did not product-inhibit hydrolysis and that there was equivalent activity on both cholic acid and deoxycholic acid conjugates would not be unexpected for such a protease.

Although complete hydrolysis occurred under most conditions, the free bile acid yield was variable. In shake flask cultures, the binding of bile acids by the mycelium caused large losses of the dihydroxy bile acid at low substrate concentrations. Trihydroxy bile acids were less susceptible

to binding and losses were accordingly less in shake flask. However, in fermenter cultures, this factor did not contribute significantly to the reduced bile acid yields. The data strongly suggest the presence of an inducible, free bile acid degrading pathway in cells of *C. melonis*, its activity being the prominent factor in bile acid loss in fermenter cultures. These enzymes appear to be catabolite-repressed by glucose, inhibited by high glycodeoxycholic acid concentrations and stimulated at low dissolved oxygen tension.

Some constitutive bile acid modifying enzymes also exist in *C. melonis* cells but their activity in whole cell cultures was observed only in the fermenter at pH 7.5 or at high glucose and aeration levels.

CHAPTER 5

THE HYDROLYSIS OF TAURINE BILE ACID CONJUGATES BY C. MELONIS

5. THE HYDROLYSIS OF TAURINE BILE ACID CONJUGATES BY C. MELONIS

5.1. Introduction

The reported resistance of taurine bile acid conjugates to fungal hydrolysis (Chong *et al*, 1980) presents a serious obstacle to the development of an industrial hydrolysis fermentation. Hence, work was undertaken to investigate the problem. *C. melonis* was employed since it had been extensively studied with respect to its action on glycine conjugates. Sodium taurocholate was used as the test compound in the majority of the experiments owing to its predominance in gall.

Initial attempts to demonstrate the hydrolysis of sodium taurocholate by *C. melonis* involved the manipulation of selected environmental factors. However, the failure to achieve the hydrolysis of the taurocholate led to an attempt to identify the factors responsible.

5.2. The Use of Taurocholate as a Sole Sulphur Source

Sulphur is an essential growth element for microorganisms and most fungi utilise it preferentially as the sulphate ion (Lilly, 1965). Consequently, the inclusion of sodium taurocholate into a synthetic medium as the sole source of sulphur would require the fungus to utilise the sulphonic acid group of the taurine moiety for sulphur, and possibly also result in the hydrolysis of the conjugate. This rationale was used successfully with *Penicillium chrysogenum* by Chong *et al* (1980), although the other fungi tested remained unable to perform the reaction.

To observe the response of *C. melonis* under these conditions, experiments were conducted in shake flask using the basal medium defined in Table 3.2. Sodium taurocholate (final concentration, $1.5 g l^{-1}$) and/or taurine (final concentration, $25.0 mg l^{-1}$) were added as required. Controls were run using

the same basal medium, except that the trace elements were substituted as their sulphate salts, as in the glucose-peptone medium (Table 3.1).

The growth data are depicted in Figure 5.1. No growth of *C. melonis* was observed in a control medium containing no source of sulphur. Therefore, sulphur was required for the growth of the fungus. In addition, growth in control flasks was unaffected by the presence of either taurocholate, or the combination of taurocholate and taurine, in the medium. *C. melonis* grew in flasks containing taurocholate as the sole sulphur source and also in those with the taurine supplement, although both had an increased lag phase compared to the control flasks. Therefore, it can be concluded that taurocholate was utilised as a source of sulphur by *C. melonis*.

This is confirmed by the results of the taurocholate analyses (Figures 5.2 and 5.3). Utilisation of the taurocholate occurred in all the experiments, totalling 18% of the initial amount present when it was employed as the sole sulphur source. Despite this, however, no cholic acid, or other bile acids, were detected by tlc or hplc analysis. Thus, the experiment did not achieve its aim.

5.3. The Effect of Lactose on the Action of *C. melonis* on Taurocholate

When glycodeoxycholic acid was incubated with *C. melonis* grown in a fermenter on a lactose-peptone medium, the complete disappearance of bile acid was observed within 96h of fermentation (Refer Figure 4.18). To ascertain whether a similar result occurred with taurocholate the conjugate was added to lactose-peptone medium, at a concentration of $0.5 g l^{-1}$, and an experiment conducted using the conditions described in Section 4.4.3.

The mycelial dry weight and total carbohydrate concentrations are plotted in Figure 5.4. Between 20h and 40h of fermentation, base was added to control the culture pH, in contrast to the identical fermentation with glycodeoxycholic

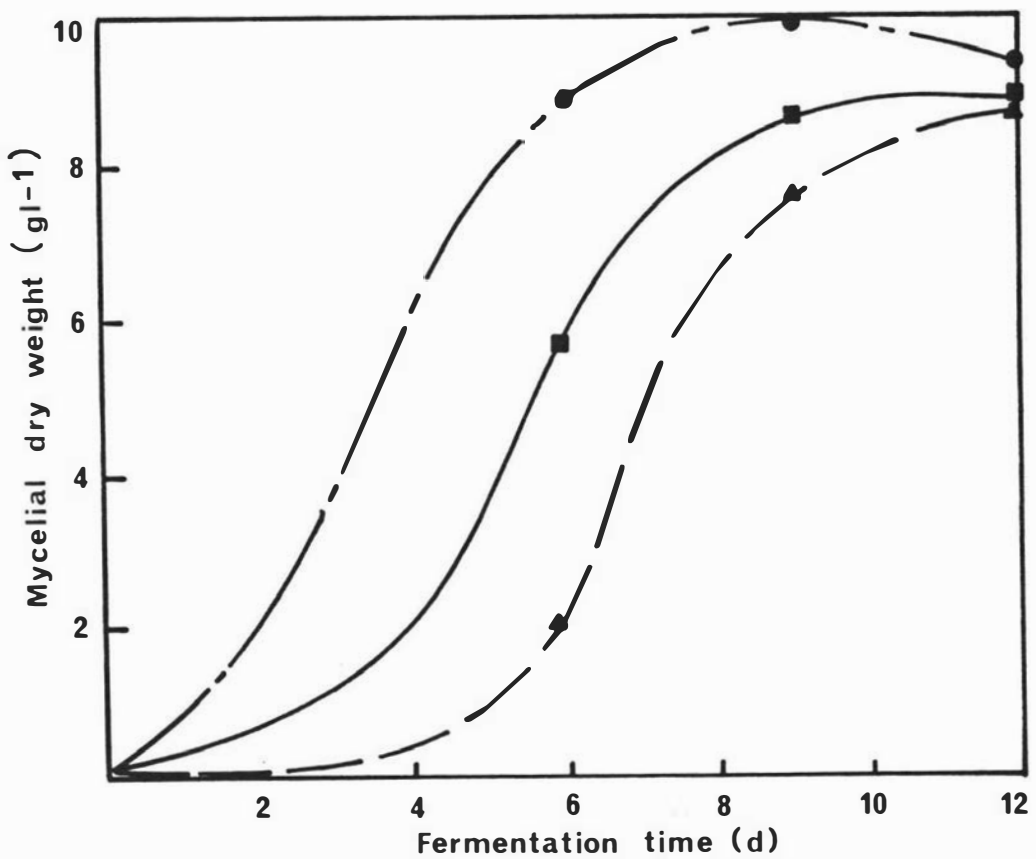


FIGURE 5.1 The growth of *C. melonis* in shake flask using taurocholate with, and without, taurine as a sole sulphur source. Mycelial dry weights on: taurocholate ($1.5gl^{-1}$), ■——■; taurocholate ($1.5gl^{-1}$) and taurine ($25.0mgl^{-1}$), ▲– –▲; Controls (both), ●—·—●.

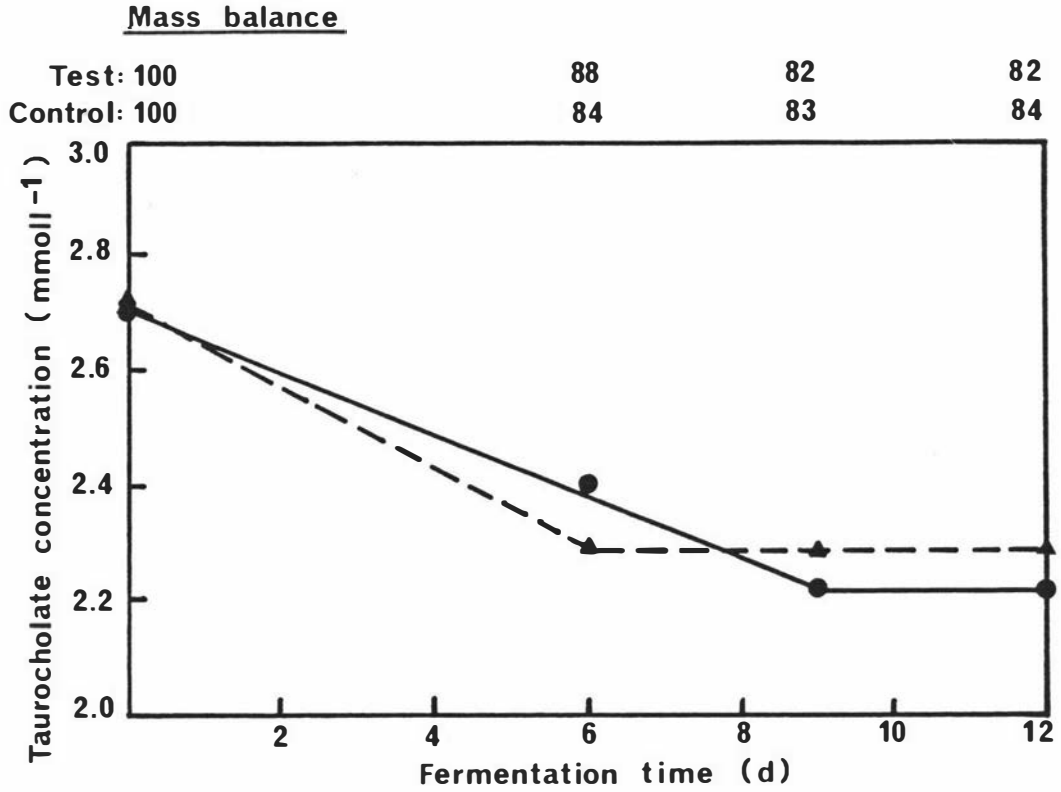


FIGURE 5.2 Taurocholate concentrations in *C. melonis* cultures: with taurocholate as sole sulphur source, ●——●; control, ▲---▲.

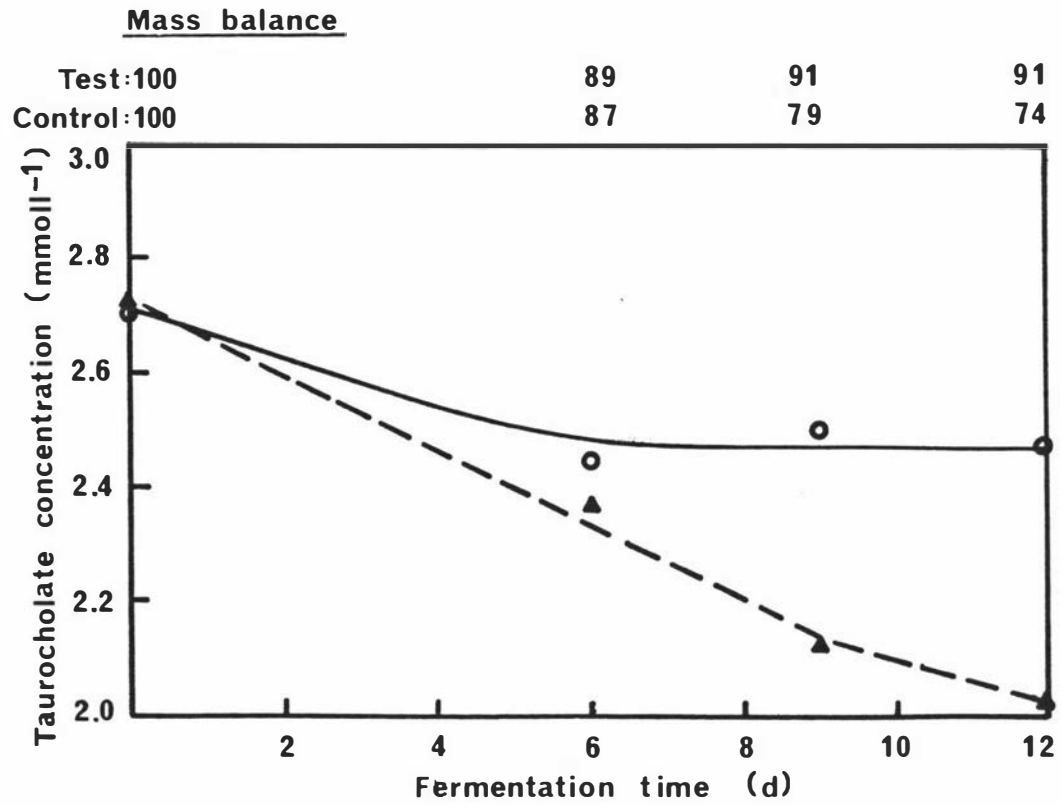


FIGURE 5.3 Taurocholate concentrations in *C. melonis* cultures: with taurocholate and taurine as sulphur source, ○——○; control, ▲---▲.

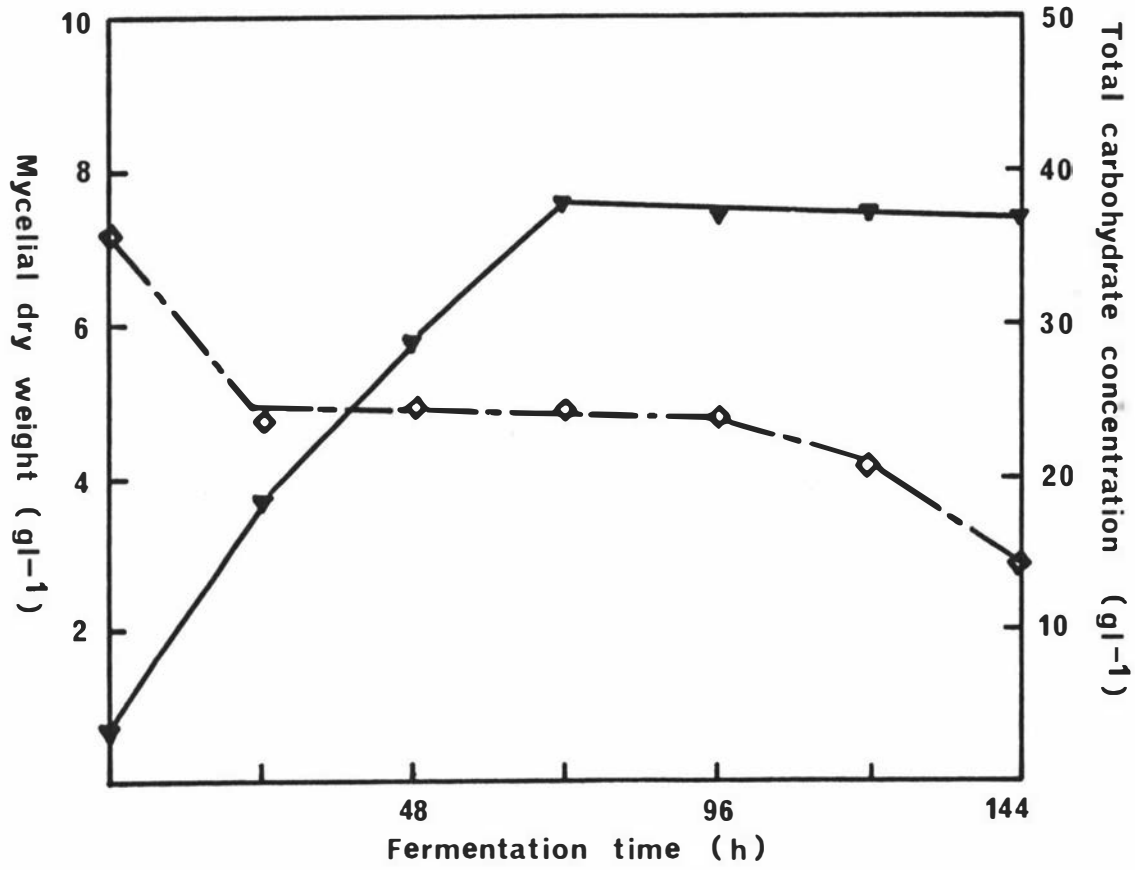


FIGURE 5.4 The mycelial dry weight of *C. melonis* and lactose concentrations in the presence of 0.5gl^{-1} taurocholate in a fermenter. Dry weight, ▼——▼ ; total carbohydrate as lactose, ◇—--◇.

acid where acid was added. *C. melonis* grew to a maximum dry weight of $8gl^{-1}$ 72h after inoculation. Lactose was primarily utilised during the initial 24h of fermentation. The reason for the marked difference in growth and lactose utilisation in this experiment compared to that observed for the culture containing glycodeoxycholic acid (Refer Figure 4.18) is unclear. It may be related to the presence of the different bile acid conjugates.

The taurocholate concentration in the culture broth was unaltered at 1.0 $mmoll^{-1}$ throughout the fermentation and no cholic acid, or other bile acids, were detected. Therefore, despite a fermentation environment which promotes the rapid utilisation of glycodeoxycholic acid, the incubation of taurocholate with *C. melonis* under the same conditions resulted in no hydrolysis or utilisation of the conjugate.

5.4. Co-fermentation of Taurocholate and Glycodeoxycholic acid

Since the cholanoyl glycine hydrolase is now known to be constitutive and extracellular, it was suspected that the lack of taurine conjugate hydrolysis may be due to the substrate-specificity of the extracellular enzyme activity. To confirm this, the two types of bile acid conjugate were incubated together in shake flask culture, each at a concentration of $0.5gl^{-1}$. Appropriate controls, including those containing solely glycodeoxycholic acid at concentrations of $0.5gl^{-1}$ and $1.0gl^{-1}$, were run for comparison.

The broth pH, mycelial dry weight and total carbohydrate concentrations of cultures containing both conjugates were not significantly different to those of the control containing $0.5gl^{-1}$ glycodeoxycholic acid (Refer Figure 4.1).

The bile acid data from the co-fermentation experiment are depicted in Figure 5.5. Those of the controls containing $0.5gl^{-1}$ and $1.0gl^{-1}$ glycodeoxycholic acid have been presented in Figures 4.2 and 4.5 respectively. Deoxycholic acid

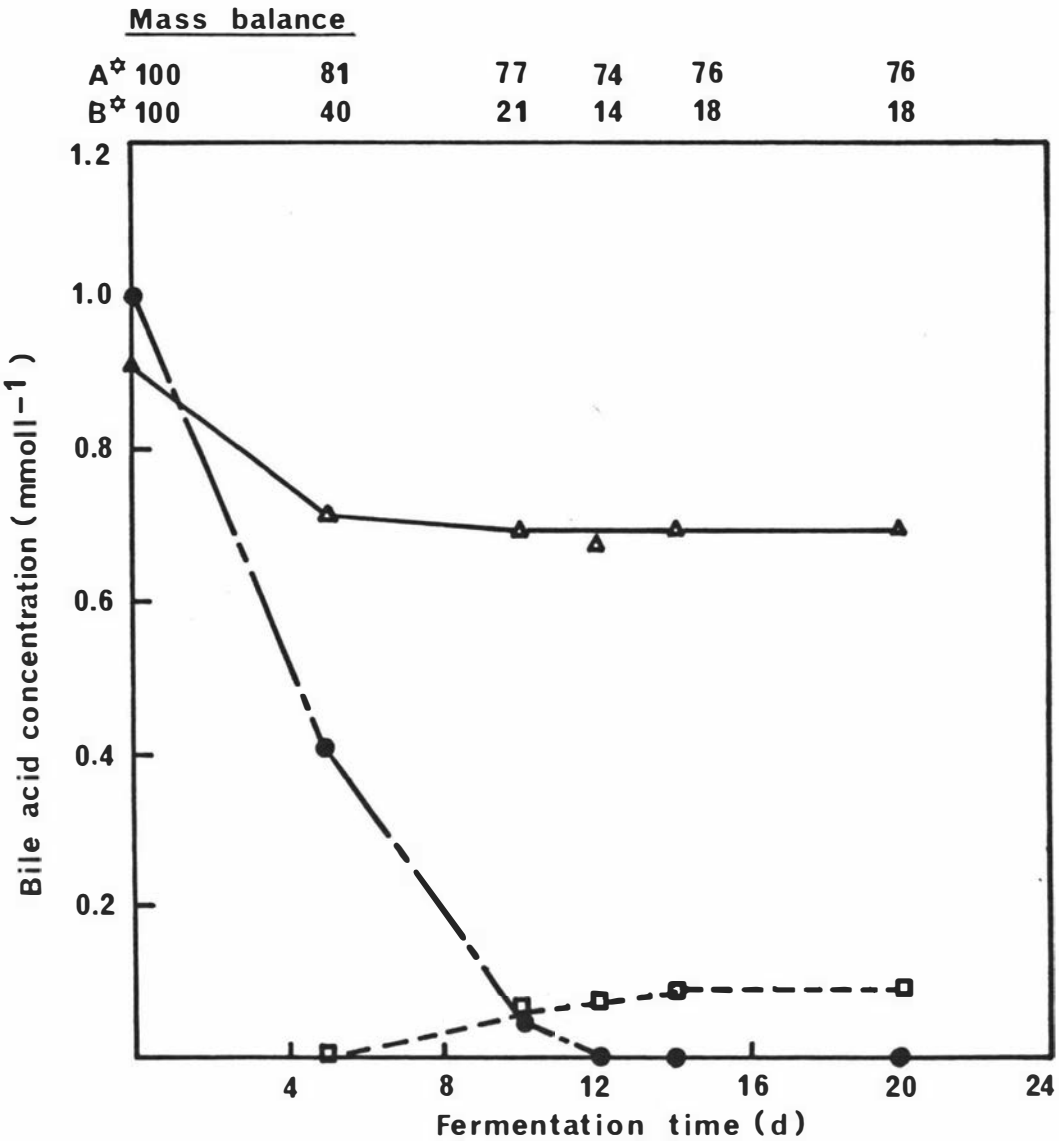


FIGURE 5.5 Bile acid concentration in shake flask cultures of *C. melonis* containing 0.5gl^{-1} each of taurocholate and glycodeoxycholic acid in glucose-peptone medium. Taurocholate, △——△; glycodeoxycholic acid, ●—--—●; deoxycholic acid, □---□.

* A - taurocholate only.

* B - glycodeoxycholic and deoxycholic acids only.

was the sole product detected by tlc and hplc analysis. Therefore, the taurocholate was not hydrolysed by *C. melonis* as judged by the absence of cholic acid, despite active hydrolysis of glycodeoxycholic acid by the culture. However, a loss of 24% of the taurine conjugate from the cultures was observed, mainly during mycelial growth.

The presence of sodium taurocholate in the cultures appeared to have no effect on the hydrolysis of glycodeoxycholic acid or on the extent of the loss of non-taurine conjugated bile acids when compared to the $0.5gl^{-1}$ glycodeoxycholic acid control (Compare Figures 4.2 and 5.5). Thus, deoxycholic acid yields were 18% and 22% respectively and the loss of bile acid comprised 82% and 78% respectively. The hydrolysis of the glycodeoxycholic acid also followed a similar time course in the co-fermentation and control cultures. Hence it appears that the taurocholate is not a substrate for the extracellular hydrolase of *C. melonis* and does not inhibit its action on glycodeoxycholic acid.

5.5. The Utilisation of Various Amino acids as Sole Nitrogen Sources by *C. melonis*

The hydrolysis of glycocholic acid, but not taurocholate, by *C. melonis* strongly suggested that the structure of the amino acid moiety of the bile conjugate was an important factor in the reaction. It is possible that the ability of *C. melonis* to hydrolyse bile acid conjugates is related to its ability to utilise the amino acid moiety as a nitrogen source and that the fungus can not utilise taurine. The few fungi tested in this respect have not been able to utilise taurine as their sole carbon or nitrogen source (Braun and Fromageot, 1962). Consequently, it was decided to study the structural requirements for the use of the amino acid moiety as a nitrogen source by the fungus and the structural specificity of the hydrolase(s) with respect to the amino acid moiety of the conjugate. The first was undertaken by testing the ability of *C. melonis* to utilise glycine, taurine, and some amino acid analogues of these two, as sole nitrogen sources.

Thus, glycine, taurine, α-alanine, β-alanine and α-aminomethanesulphonic acid were tested as sole sources of nitrogen for the growth of *C. melonis* in shake flask cultures, using bacteriological peptone for comparison. Glucose-peptone medium was used with the amino acids substituted for the peptone at a concentration calculated to provide 0.56g of nitrogen per litre of medium. A control containing no nitrogen was run.

As mycelial dry weight was used as the criterion of the utilisation of the amino acids, the inoculation procedure was altered to ensure a uniform inoculum. Thus, two, eight-day potato dextrose agar plate cultures of *C. melonis*, grown at 30°C, were washed with sterile, distilled water and the resulting spore suspensions were combined and used to inoculate each flask after thorough mixing.

The dry weight data are presented in Figure 5.6. The control without added nitrogen reached a dry weight of $0.2gl^{-1}$ after 12 days. Therefore, it is evident that *C. melonis* can utilise all the amino acids as its sole nitrogen source with the exception of taurine, which gave a dry weight of only $0.7gl^{-1}$.

Testing for the microbial utilisation of a compound as a sole nitrogen source, using the criterion of growth, has often led to invalid conclusions due to the pH inhibition of growth resulting from the use of the compound (Pateman and Kinghorn, 1976). However, only in the case of α-aminomethane-sulphonic acid may growth have become pH inhibited due to the utilisation of the amino acid. All other cultures were of pH values conducive to fungal growth.

The fungal dry weight obtained from the carboxylic amino acids were similar to that of the peptone control, although there was an inexplicable lag of 5 days prior to subsequent rapid growth with α-alanine. However, the utilisation of α-aminomethanesulphonic acid resulted in a significantly lower production of dry weight. The results from the utilisation of the carboxylic amino acids by *C. melonis* agree

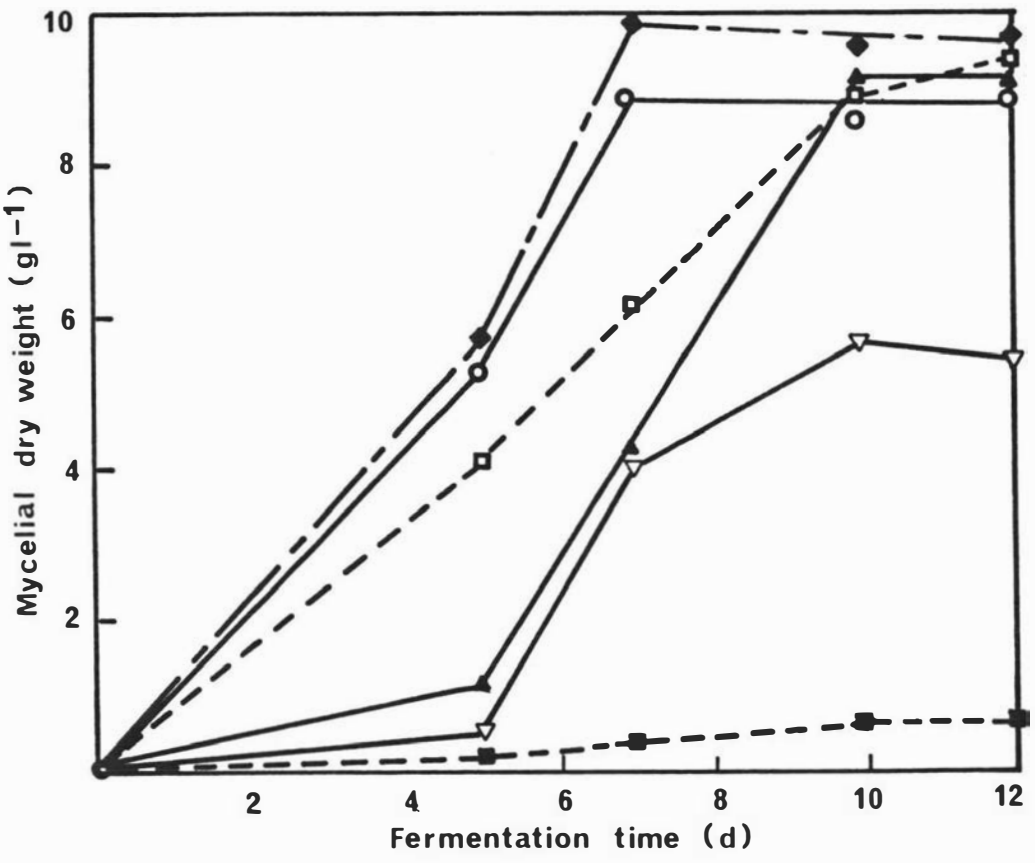


FIGURE 5.6 The growth of *C. melonis* on various amino acids provided as sole nitrogen sources. Peptone, ○——○; glycine, □- - -□; α-alanine, ▲——▲; β-alanine, ◆—- -◆; α-aminomethane-sulphonic acid, ▽——▽; taurine, ■- - -■.

with those obtained by workers testing them with other *Cercospora* species (Dayal and Ram, 1968; Berger, 1963; Stavely and Nimmo, 1968). Thus, whereas *C. melonis* can utilise the amino acid product from the hydrolysis of glycine conjugates, taurine cannot be so used.

5.6. The Influence of the Amino acid Moiety on Bile Conjugate Hydrolysis

C. melonis has been demonstrated to utilise various amino acids as sole nitrogen source, excepting taurine. Therefore, its ability to hydrolyse the bile acid conjugates of these amino acids was studied to determine the influence of the structure of the amino acid moiety on conjugate hydrolysis by whole-cell cultures of the fungus.

Hence, the effects of two components of the taurine structure, namely the sulphonic acid group and the β-position of the amine group, were investigated separately by the use of structural analogues of sodium taurodeoxycholate and glycodeoxycholic acid. In addition, the ability of *C. melonis* to hydrolyse the α-alanine conjugate was studied to further determine the specificity of the hydrolase.

Experiments were performed in shake flask using glucose-peptone medium containing $0.5 g l^{-1}$ of the appropriate bile acid conjugate. The bile conjugates tested were sodium taurodeoxycholate, sodium N-(α-aminomethanesulphonyl)-deoxycholate, glycodeoxycholic acid, N-(α-alano)-deoxycholic acid and N-(β-alano)-deoxycholic acid.

The growth of *C. melonis* in cultures containing each conjugate was generally similar. In the presence of carboxylic acid conjugates, the dry weight profiles were similar to that of glycodeoxycholic acid (Refer Figure 4.1). However, cultures containing sulphonic acid conjugates reached their maximum dry weight *ca* 3 days later. The culture pH values exhibited similar trends to that of cultures containing glycodeoxycholic acid (Refer Figure 4.1).

The bile acid data for cultures containing N-(α-alano)-deoxycholic acid, N-(β-alano)-deoxycholic acid, sodium N-(α-aminomethanesulphonyl)-deoxycholate and sodium tauro-deoxycholate are depicted in Figures 5.7 to 5.10 respectively. The hydrolysis of glycodeoxycholic acid paralleled the data portrayed in Figure 4.2. Hydrolysis, as determined by the appearance of deoxycholic acid, occurred for N-(α-alano)-deoxycholic acid and glycodeoxycholic acid only. The authenticity of the deoxycholic acid produced by the hydrolysis of the former was confirmed by tlc and hplc analysis. Sodium N-(α-aminomethanesulphonyl)-deoxycholate, sodium tauro-deoxycholate and N-(β-alano)-deoxycholic acid were not hydrolysed, although some losses occurred. Thus, both the sulphonic acid group and the β-amine structure appear to contribute to the resistance of taurine conjugates to hydrolysis by whole-cell cultures of *C. melonis*.

The hydrolysis of N-(α-alano)-deoxycholic acid was significantly different to that of glycodeoxycholic acid. A 50% yield of deoxycholic acid was observed within 5 days of inoculation and no conjugate remained after 8 days. This contrasts with the hydrolysis of glycodeoxycholic acid, where the maximum yield of deoxycholic acid was only 22%, none accumulating before 4-5 days of fermentation, and conjugate remained until *ca* 11 days (Refer Figure 4.2). Consequently, N-(α-alano)-deoxycholic acid appears to be a superior substrate for hydrolysis.

Losses of bile acid from the fermentation were observed for all conjugates. The loss profile from cultures containing N-(α-alano)-deoxycholic acid was particularly unusual, 74% of the loss occurring over the 5-8 day period of fermentation. Clearly, this loss is not growth related. However, the extent of total bile acid loss was similar to that of cultures containing glycodeoxycholic acid, being 64% and 78% respectively. The results demonstrate that the bile acid conjugates hydrolysed by *C. melonis* suffered higher losses than recalcitrant conjugates.

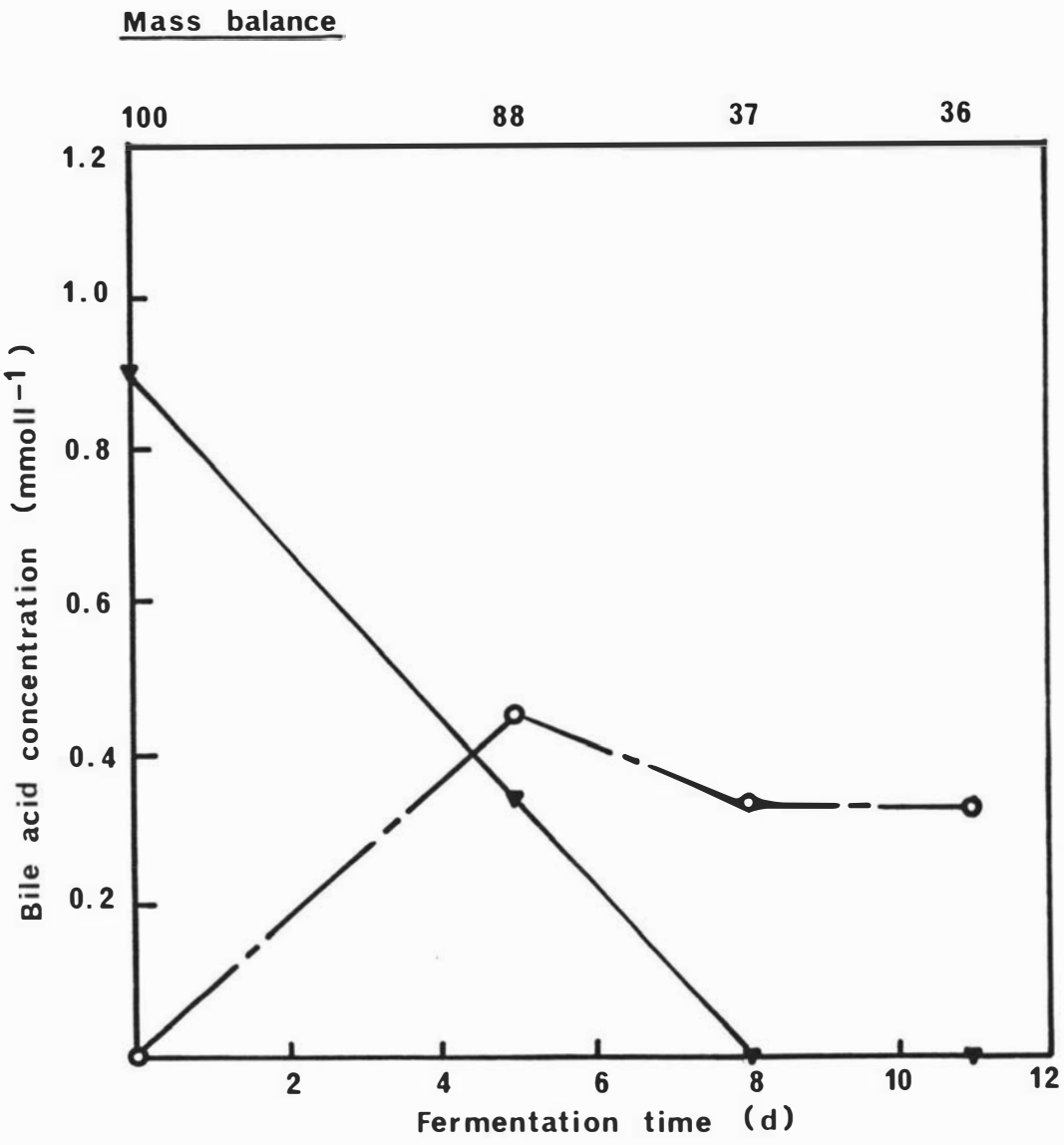


FIGURE 5.7 The hydrolysis of 0.5gl^{-1} N-(α-alano)-deoxycholic acid by *C. melonis* grown in shake flask cultures on glucose-peptone medium. N-(α-alano)-deoxycholic acid, ▼——▼; deoxycholic acid, O- - -O.

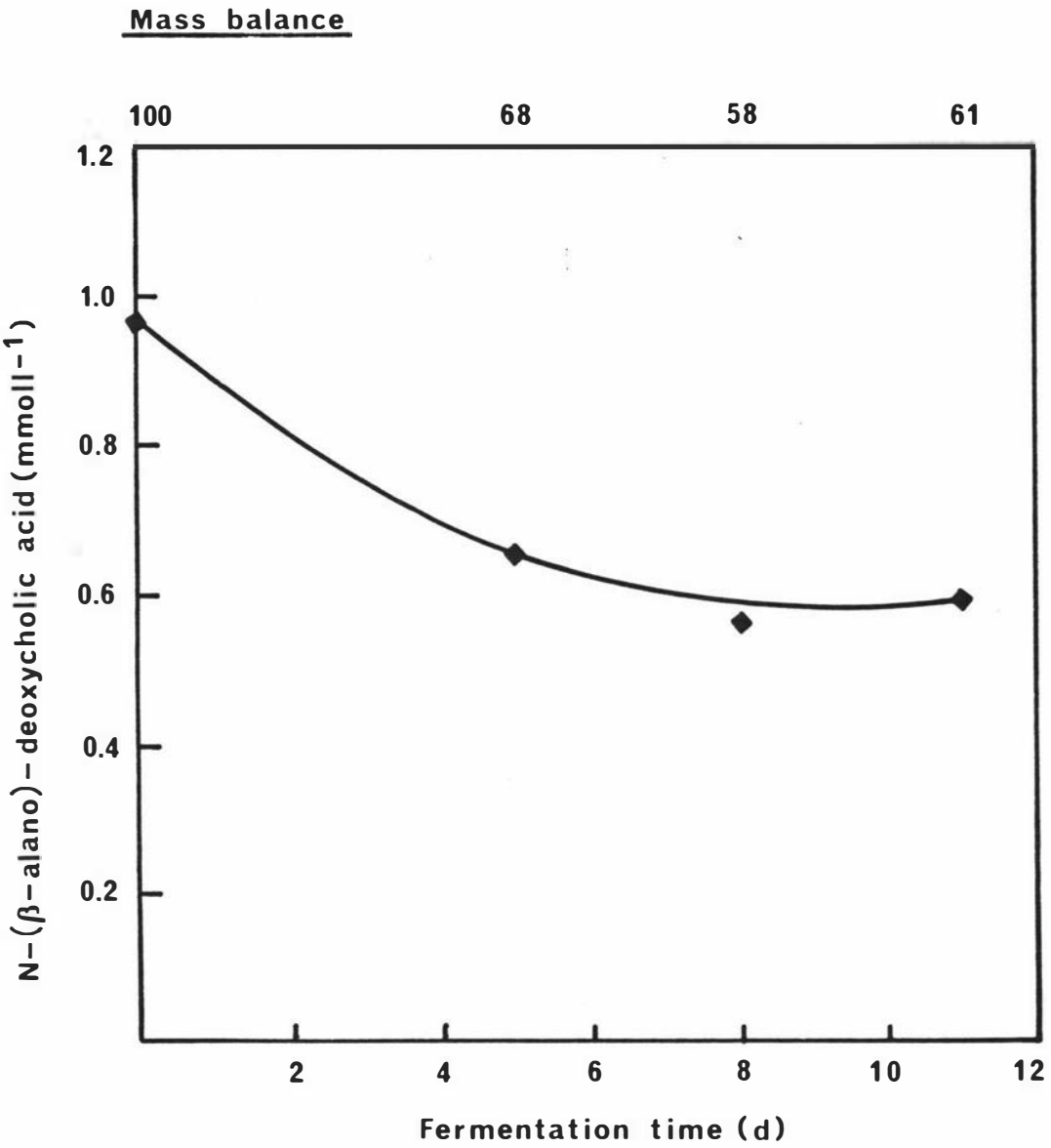


FIGURE 5.8 The concentration of 0.5gl^{-1} N-(β-alano)-deoxycholic acid in shake flask cultures of *C. melonis* grown on glucose-peptone medium.

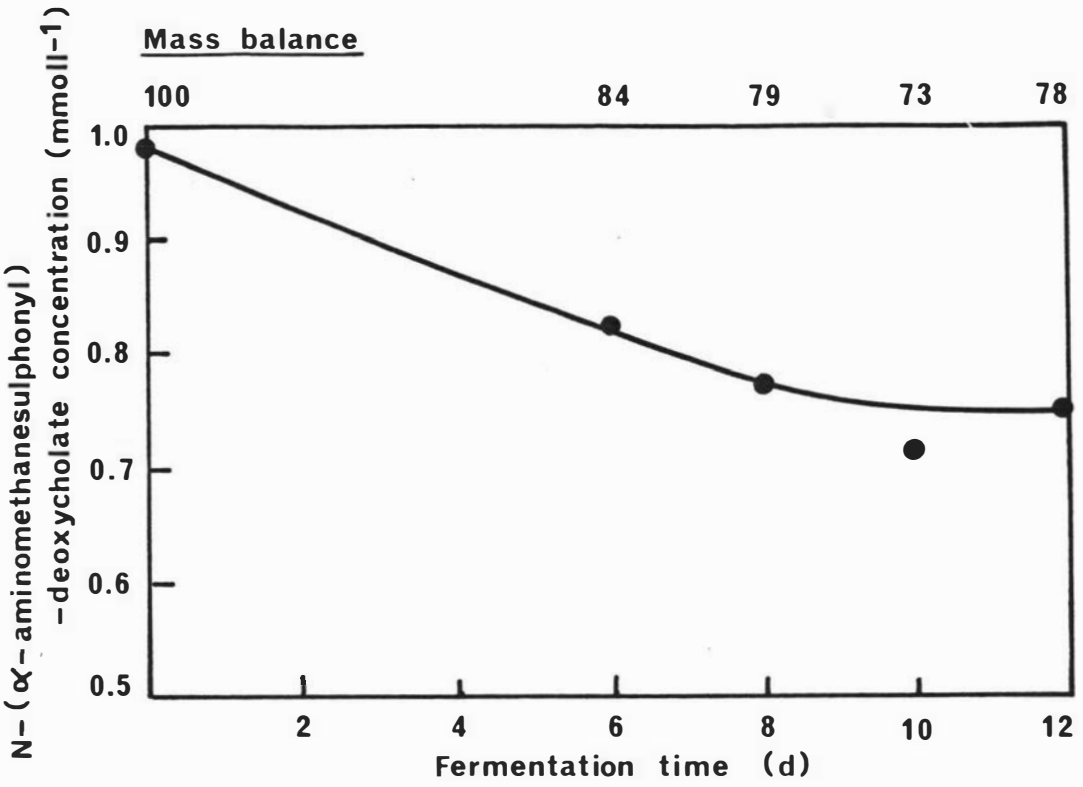


FIGURE 5.9 The concentration of N-(α-aminomethane sulphonyl)-deoxycholate in *C. melonis* shake flask cultures grown on glucose-peptone medium.

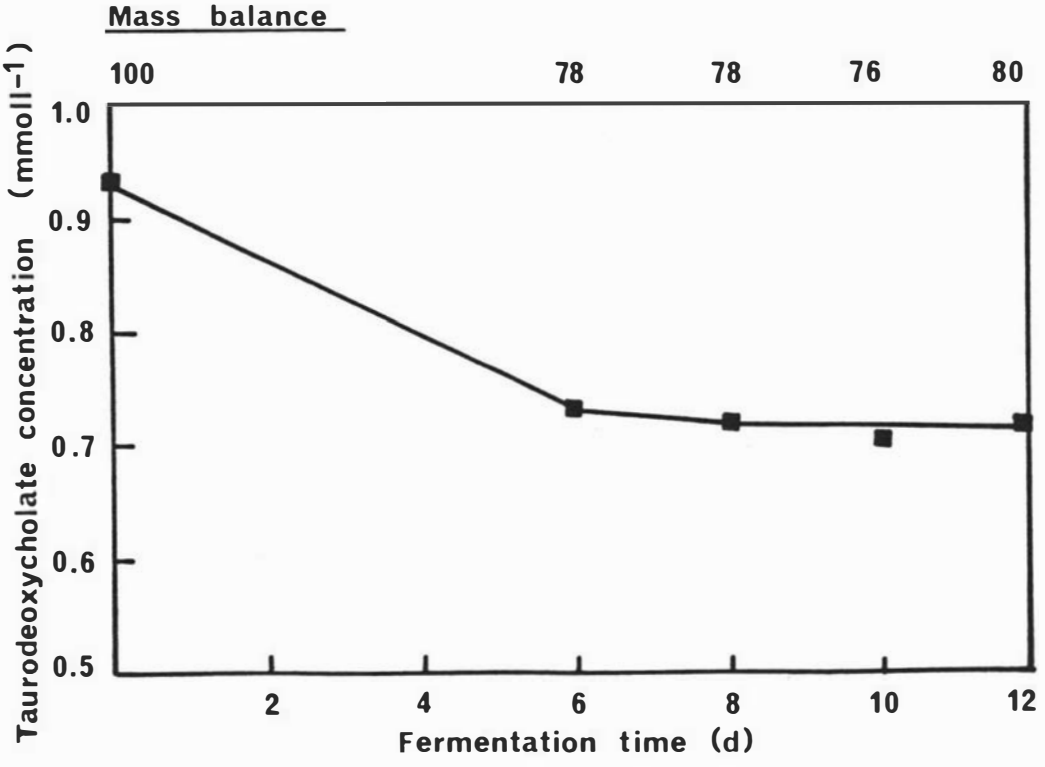


FIGURE 5.10 The concentration of sodium taurodeoxycholate in *C. melonis* shake flask cultures grown on glucose-peptone medium.

Loss of the recalcitrant conjugates was growth-related, and the nature of the amino acid moiety was important in determining their vulnerability. Thus, loss of the carboxylic acid conjugate, N-(β-alano)-deoxycholic acid, totalled *ca* 40% compared to *ca* 22% for the two sulphonic acid conjugates. The binding study described in Section 4.3.1. predicts that glycodeoxycholic acid will be bound by mycelium to the extent of *ca* 30% in cultures of $10gl^{-1}$ dry weight and containing $0.5gl^{-1}$ conjugate. Therefore, the losses observed in cultures containing N-(β-alano)-deoxycholic acid were probably due to this factor. However, the binding data showed that taurocholate was negligibly bound. A possible explanation for the loss of the sulphonic acid conjugates may be that limited hydrolysis occurs but that the free bile acid product is lost either by binding to the mycelium or by being rapidly degraded.

5.7. Enzyme Location Studies

Cell-free systems of *C. melonis* were employed to investigate the hypothesis that the resistance of the taurine conjugates may, in part, be due to their lack of transport into the cell, where an intracellular, cholanoyl taurine hydrolase may be located.

The following bile acid conjugates were tested using both a cell-free extract and a cell-free culture filtrate: N-(α-alano)-deoxycholic acid, N-(β-alano)-deoxycholic acid, sodium N-(α-aminomethanesulphonyl)-deoxycholate, sodium taurodeoxycholate and sodium taurocholate. Duplicate experiments were run.

The results are summarised in Table 5.1 and, in addition, those of the glycine conjugates from Section 4.2.4. are included for comparison. No hydrolysis was detected in controls run without fungal cell material.

The identification of deoxycholic acid in the cell-free

Table 5.1

The hydrolysis of bile acid conjugates by cell-free systems of *C. melonis*

Bile acid	Intracellular activity Product			Extracellular activity Product	
	CA	DC	Others	CA	DC
GD	-	+	+	-	+
GC	+	+	+	+	-
α-AD	-	+	+	-	+
β-AD	-	-	-	-	-
Na α-AMSD	-	+	+	-	-
NaTD	-	+	+	-	-
NaTC	+	+	+	-	-

Notation: + , the product was detected;
- , the product was not detected.

extract incubations of sodium N-(α-aminomethanesulphonyl)-deoxycholate and sodium taurodeoxycholate and, likewise, of cholic acid from sodium taurocholate, represent evidence that *C. melonis* possesses constitutive cholanoyl taurine hydrolase activity. In contrast to the cholanoyl glycine hydrolase activity, the cholanoyl taurine hydrolase is intracellular only. However, the product yields from such activity were insufficient to permit quantitation by hplc analysis or identification by isolation and chemical characterisation.

With regard to the carboxylic acid conjugates, N-(β-alano)-deoxycholic acid was not hydrolysed either intracellularly

or extracellularly. This appears to be an unusual result, since β-alanine is the carboxylic acid analogue of taurine. The reason for such a discrepancy is not clear.

Whether the one intracellular enzyme catalyses the hydrolysis of both the sulphonic acid and the α-aminocarboxylic acid conjugates cannot be determined from the available data. However, the specificity of the extracellular enzyme activity for the glycine and α-alanine conjugates indicates that there exist at least two, discrete hydrolases in *C. melonis*. Thus, the data strongly suggest that two factors are responsible for the resistance of taurine conjugates to hydrolysis in whole-cell cultures of *C. melonis*. First, the impermeability of the cell membrane to these conjugates and, secondly, the inability of the extracellular hydrolase to act on them.

As with glycodeoxycholic acid, N-(α-alano)-deoxycholic acid was hydrolysed in both the cell-free extract and the cell-free filtrate incubations. However, superior deoxycholic acid yields, 14% in the cell-free extract and 5% in the cell-free filtrate, were obtained in contrast to those from the glycine conjugate, which were too low to be quantitated. This confirms that the methyl sidechain of the α-alanine moiety appears to enhance hydrolysis compared to the glycine conjugate. Furthermore, it demonstrates that an α-amino-carboxylic acid structure for the amino acid moiety is important for conjugate hydrolysis by the extracellular hydrolase of *C. melonis*.

The production of deoxycholic acid from taurocholate implies the presence of constitutive 7α-hydroxycholanoyl-dehydroxylase activity in the crude cell-free extract. Previous experiments indicated a similar activity on glycocholic acid by crude cell-free extracts of *C. melonis*.

5.8. The Effect of Cycloheximide

The incubation of sulphonic acid conjugates with *C. melonis*, in shake flasks, resulted in losses of bile acid of *ca* 10-20%

during mycelial growth without the concomitant observation of free bile acid as the product of hydrolysis (Figures 5.9 and 5.10). Chong *et al* (1980) observed similar losses of bile acid, without the appearance of cholic acid, during the incubation of sodium taurocholate with other fungi. Binding by the mycelium can be disregarded as a factor in such loss since binding experiments have demonstrated that sodium taurocholate is bound by fungal mycelium to a negligible extent, a result for which the sulphonic acid function appears primarily responsible.

Therefore, as *C. melonis* possesses a constitutive, intracellular cholanoyl taurine hydrolase, a possible explanation may be that taurine conjugates penetrate the cell to a limited extent and hydrolysis then occurs. However, such hydrolysis is not detected because the free bile acid product is rapidly metabolised by degradative enzymes, whose presence was implicated in the previous chapter.

Assuming that these degradative enzymes are inducible, cycloheximide was employed to test this hypothesis by repressing the enzymes and thus allowing free bile acid to accumulate. It was also envisaged that the antibiotic might increase the permeability of the cell to the taurocholate and so enhance its hydrolysis by the intracellular cholanoyl taurine hydrolase.

C. melonis was grown in shake flask in glucose-peptone medium. After 7 days incubation, a sterile-filtered, aqueous solution of cycloheximide was aseptically added to a final concentration of 250 $\mu g ml^{-1}$. A sterile-filtered, aqueous solution of sodium taurocholate was then aseptically added 2.5h later to a final concentration of $0.5 g l^{-1}$. Controls were run omitting the addition of cycloheximide.

The addition of cycloheximide to the cultures had an identical effect on mycelial dry weight and culture pH to that observed for cycloheximide-treated cultures in Section 4.2.5 (Refer Figure 4.9). The bile acid data are presented in Figure 5.11.

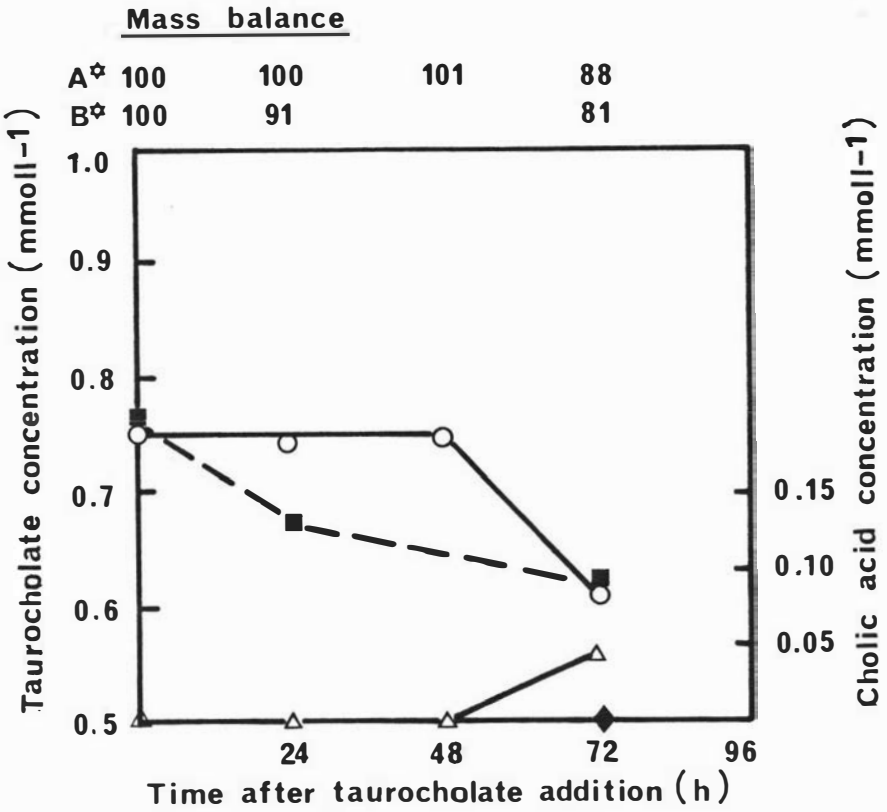


FIGURE 5.11 The effect of cycloheximide on the hydrolysis of 0.5gl^{-1} taurocholate by *C. melonis* grown in glucose-peptone medium. Cycloheximide-treated cultures (A*): taurocholate, O———O; cholic acid, Δ———Δ; control without cycloheximide (B*); taurocholate, ■———■; cholic acid, ◆.

In the presence of cycloheximide, a 7% yield of cholic acid was observed 3 days after the addition of sodium taurocholate. The cholic acid was characterised by its identical behaviour to authentic cholic acid on tlc and hplc. No other bile acid products were detected. No cholic acid or other bile acid products were observed in the absence of cycloheximide, although a loss of 19% of the sodium taurocholate had occurred after 3 days compared to a 12% loss by the same time in the cycloheximide-treated cultures.

Thus, the data provide the first instance of the hydrolysis of taurocholate by whole cells of *C. melonis* and confirm the discovery of constitutive cholanoyl taurine hydrolase activity in *C. melonis*. Furthermore, the data strongly suggest that the reason for the apparent lack of hydrolysis of taurine conjugates, as judged by the accumulation of free bile acid product, is the rapid degradation of such product by inducible enzymes.

5.9. Discussion

The results from the use of the cell-free systems and cultures treated with cycloheximide demonstrate that *C. melonis* possesses a constitutive cholanoyl taurine hydrolase capable of catalysing the hydrolysis of both of the primary taurine conjugates of cattle and sheep gall. However, the observed resistance of the taurine conjugates in whole-cell cultures, as judged by the absence of free bile acid product, appears to be due to the intracellular location of this enzyme, acting in combination with two other factors. These are the permeability of the cell wall/membrane and the degradation of free bile acid by the fungus.

Cell-free studies have demonstrated the elaboration of an extracellular enzyme by the fungus, catalysing the hydrolysis of glycine, but not taurine, conjugates. Given their marginal penetration into the fungal cell, this may explain the recalcitrance of the latter conjugates, despite environmental conditions conducive to the hydrolysis of the former, as in

the case of the co-fermentation experiment and the use of a lactose-peptone medium. Both the β-amino and the sulphonic acid components of the taurine structure eliminated taurine conjugates as substrate for the extracellular hydrolase, demonstrated by the inactivity of whole-cell cultures and the cell-free culture filtrate on N-(β-alano)-deoxycholic acid and N-(α-aminomethanesulphonyl)-deoxycholate.

In the previous chapter it was suggested that the extracellular hydrolase may be a neutral protease secreted by the cell to degrade the peptone in the medium. The results from the incubation of various synthetic bile acid conjugates with *C. melonis* further support this hypothesis in view of the specificity of the extracellular activity for an α-aminocarboxylic acid structure of the amino acid moiety. Consequently, the inactivity of the extracellular hydrolase on taurine conjugates requires their penetration into the cell for hydrolysis.

However, it appears that the cell membrane is largely impermeable to taurine conjugates, thus restricting their transport into the cell and, hence, their availability to the intracellular cholanoyl taurine hydrolase. This is not unexpected, as the taurine conjugates exist in the dissociated form at virtually all values of culture pH due to their low pKa value (Refer Table 2.1). Dissociated organic acids diffuse into cells at low rates (Rothstein, 1965). The growth-related loss of 10-20% of the taurine conjugates may reflect their greater ability to enter the cell at the low pH conditions predominating during the growth of the culture.

The results from the use of cycloheximide strongly suggest that the "lost" taurine conjugate is hydrolysed in the cell, but that the free bile acid product is degraded by intracellular, inducible enzymes at a rate equal to its production. Consequently, no product accumulates to be detected. The repression of such enzymes by the pretreatment of the culture with cycloheximide results in the appearance of cholic acid from taurocholate. Nevertheless, a loss of bile acid of 12% still occurred in the cycloheximide-treated cultures.

This may be due to the binding of cholic acid to the mycelium after taurocholate hydrolysis. The binding data predicts a 12% loss of cholic acid under the experimental conditions employed (Refer Table 4.3).

This explanation accounts for the low losses of sulphonic conjugates in whole-cell cultures of *C. melonis* compared to glycine conjugates. Thus, the combination of the negligible binding of sulphonic acid conjugates to the mycelium and the poor penetration of the cell membrane by such conjugates and, hence, restricted hydrolysis and degradation, restrains the losses to 10-20%.

The hypotheses proposed above also explain the data from the experiment employing taurocholate as the sole sulphur source. Thus, the fungus utilised the sulphonic acid group of the taurine moiety as a source of sulphur, presumably *via* hydrolysis, since the deamination of taurine is reported to precede its desulphurisation in fungi (Garreau, 1941; Braun and Fromageot, 1962). However, the restriction on the entry of the taurocholate into the cell imposed by the cell membrane and subsequent metabolism of the cholic acid may explain the lack of apparent hydrolysis.

It is tempting to postulate the existence of two intracellular enzymes in *C. melonis* cells, one specific for α-aminocarboxylic acid conjugates, the other specific for sulphonic acid conjugates. The former may or may not be identical to the extracellular hydrolase. The latter may be an amidase exhibiting no dependence on an α- or β-amino structure of the amino acid moiety of conjugates, demonstrated by the hydrolysis of N-(α-aminomethanesulphonyl)-deoxycholate and taurodeoxycholate by cell-free extracts of *C. melonis*. The postulation of two such discrete, intracellular enzymes may explain the lack of both extracellular and intracellular hydrolysis of N-(β-alano)-deoxycholic acid by *C. melonis*, despite its ability to readily utilise β-alanine as a sole nitrogen source.

Although the 40% loss of N-(β-alano)-deoxycholic acid was double that of the sulphonic acid conjugates, it was significantly less than the 65-75% loss experienced with α-aminocarboxylic acid conjugates. This loss was probably due to binding by the mycelium, to which carboxylic acid-deoxycholic acid conjugates appear to be susceptible. Losses of this order are predicted from the binding data obtained for glycodeoxycholic acid (Refer Table 4.3). In view of the recalcitrance of the β-alanine conjugate to hydrolysis, the lower losses observed from its incubation with the fungus, compared to those of the hydrolysed carboxylic acid conjugates, suggests that hydrolysis is a prerequisite for further degradation of the bile acid.

CHAPTER 6

THE HYDROLYSIS OF BILE ACID CONJUGATES BY CURVULARIA FALLAX

6. THE HYDROLYSIS OF BILE ACID CONJUGATES BY CURVULARIA FALLAX

6.1. Introduction

A characteristic feature of steroid transformations is the specificity of their occurrence among microorganisms. Significant variations in transformation capabilities exist even within different strains of the same species. The microbial hydrolysis of bile acid conjugates is no exception. Among the intestinal bacteria, the ability to hydrolyse conjugates is widespread; however, there are several instances of variations in substrate specificity (Hill, 1976; Kobashi *et al*, 1978). Similarly, fungi vary considerably in their ability to hydrolyse bile conjugates. While most whole-cell cultures can split only glycine conjugates (Chong *et al*, 1980), they differ significantly in the time required to achieve complete hydrolysis of glycocholic acid (Maddox and Chong, 1978).

From the viewpoint of developing an industrial hydrolysis fermentation such variability must be recognised in order to select the most productive organism. Hence, screening tests must be able to distinguish between desirable and deleterious features of the organisms with respect to the envisaged process. For this reason, work was undertaken to study the hydrolysis of bile acid conjugates by *Curvularia fallax* IFO 8885 to provide a comparison with *C. melonis*. *C. fallax* exhibited poor activity on glycocholic acid compared to *C. melonis* in the qualitative screen performed by Maddox and Chong (1978). Hence, from a comparison of quantitative data from both fungi, it was hoped to delineate some criteria for a screening programme for the adequate assessment of the hydrolysis capabilities of fungi.

6.2. Shake Flask Studies

6.2.1. The Hydrolysis of Glycodeoxycholic acid at Various Concentrations

C. fallax was grown in glucose-peptone medium containing glycodeoxycholic acid at final concentrations of $0.5gl^{-1}$, $1.0gl^{-1}$, $5.0gl^{-1}$ and $10gl^{-1}$.

The mycelial dry weight and bile acid concentration data are presented in Figures 6.1 to 6.4 for increasing bile acid levels respectively. The pH of the cultures was similar during fermentation, although it was affected by the growth lags experienced at the higher bile acid concentrations. In general, however, the culture pH fell to values of pH 3-4 during growth and then rose rapidly to *ca* pH 8 after 12 days of fermentation.

The growth of *C. fallax* was severely retarded by concentrations of glycodeoxycholic acid of $1.0gl^{-1}$ or more, although the maximum dry weights attained were similar for all concentrations. Bile acid precipitated from cultures containing $0.5gl^{-1}$, $1.0gl^{-1}$ and $5.0gl^{-1}$ of glycodeoxycholic acid during growth but later redissolved.

Deoxycholic acid was the sole product observed in all of the cultures, the maximum yields being 76%, 80%, 44% and 60% for increasing substrate levels respectively. Hydrolysis was delayed by the inhibited growth of *C. fallax* at high concentrations of glycodeoxycholic acid. Thus no deoxycholic acid accumulated until after 16 days of fermentation at the $5.0gl^{-1}$ concentration. Furthermore, complete utilisation of glycodeoxycholic acid occurred only at low substrate concentrations, whereas substrate representing *ca* 35% of the initial concentration remained after 23 days of fermentation in cultures containing $5.0gl^{-1}$ and $10gl^{-1}$ of bile acid. In the case of the former level, this appears to be due to the severe inhibition of growth observed in the cultures.

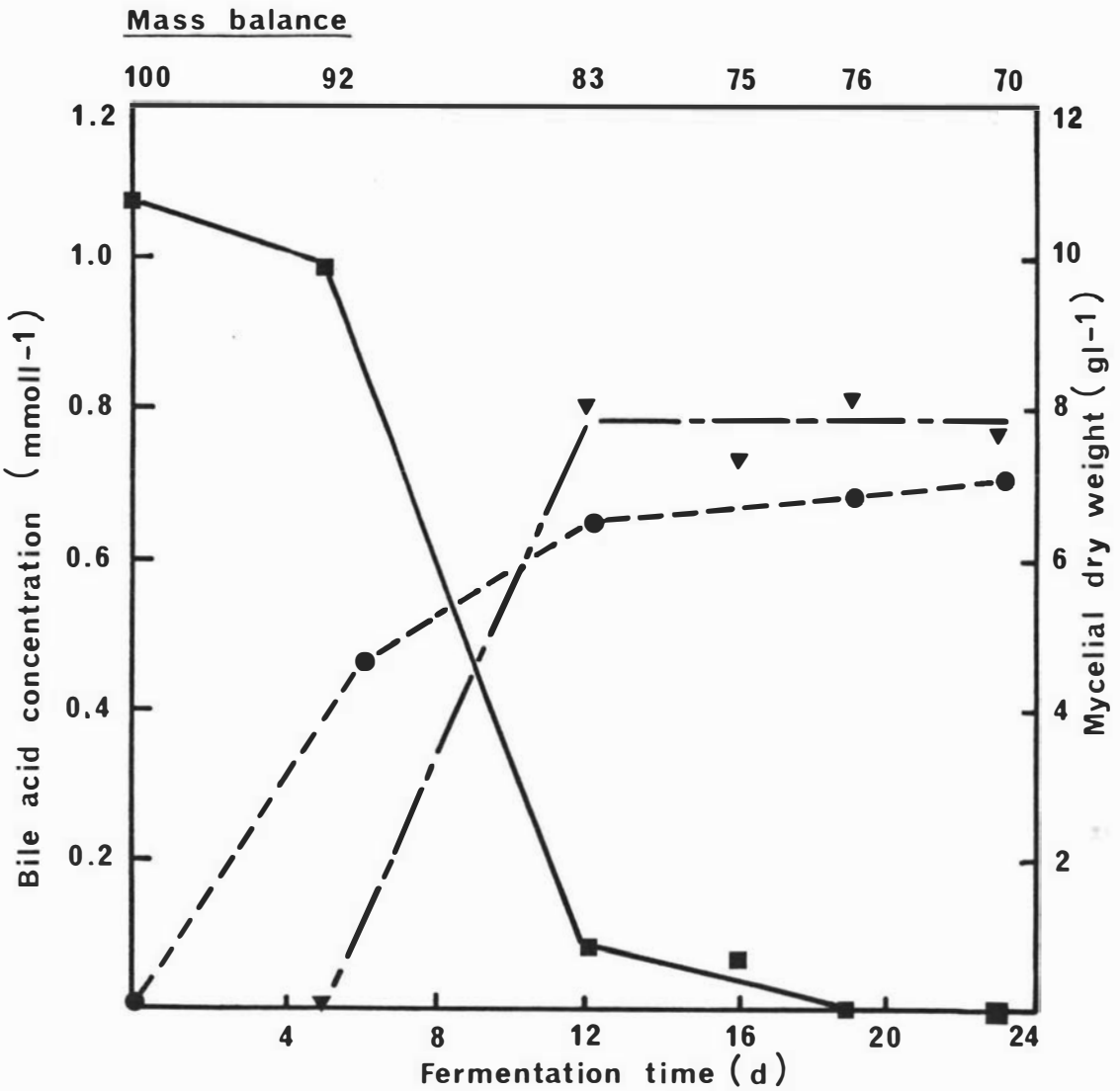


FIGURE 6.1 The hydrolysis of 0.5gl^{-1} glycodeoxycholic acid by *C. fallax* in shake flasks containing glucose-peptone medium. Glycodeoxycholic acid, ■——■; deoxycholic acid, ▼— - —▼; dry weight, ●- - - -●.

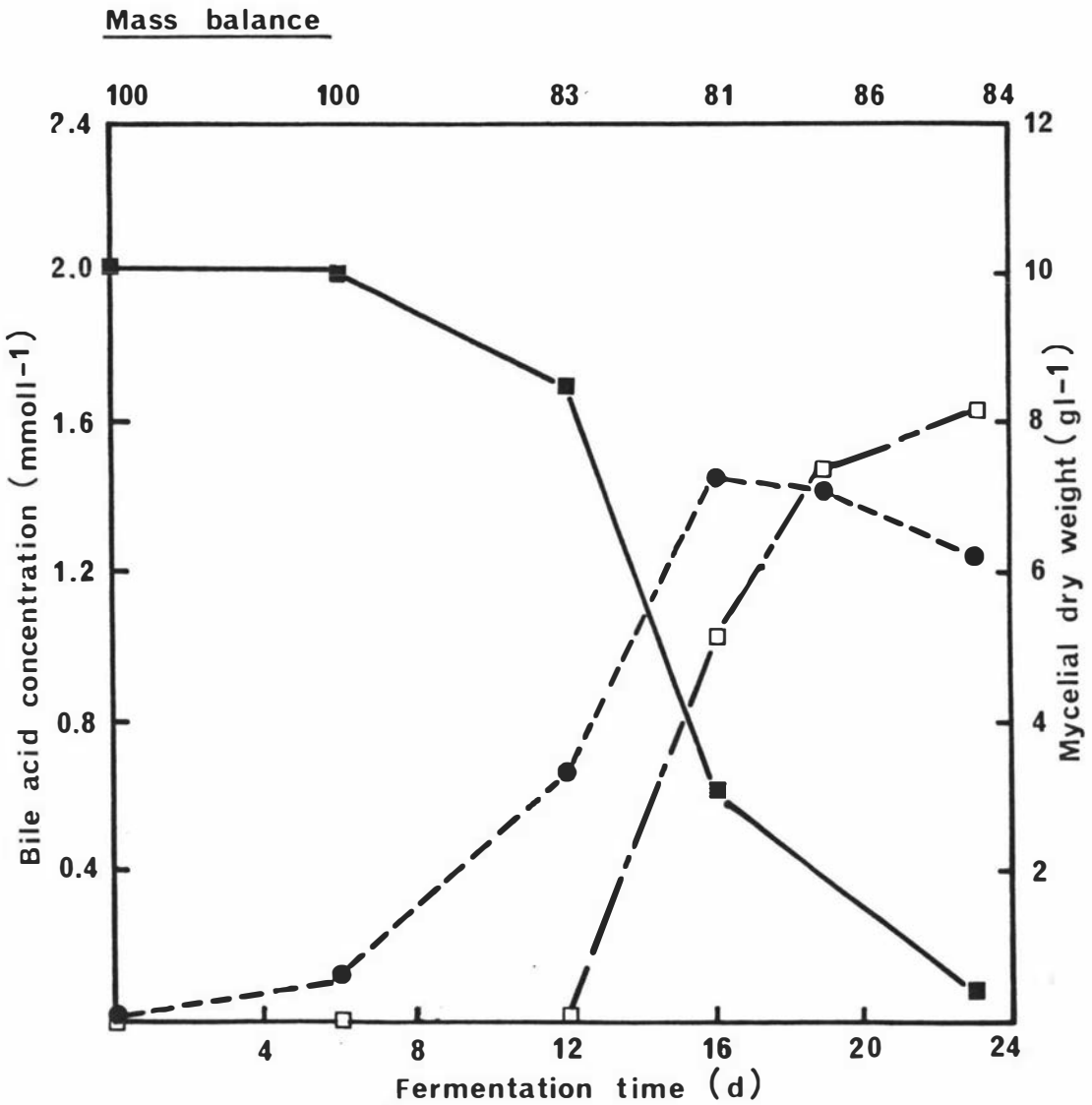


FIGURE 6.2 The hydrolysis of 1.0gl^{-1} glycodeoxycholic acid by *C. fallax* in shake flasks containing glucose-peptone medium. Glycodeoxycholic acid, ■——■; deoxycholic acid, □— - —□; dry weight, ●- - - -●.

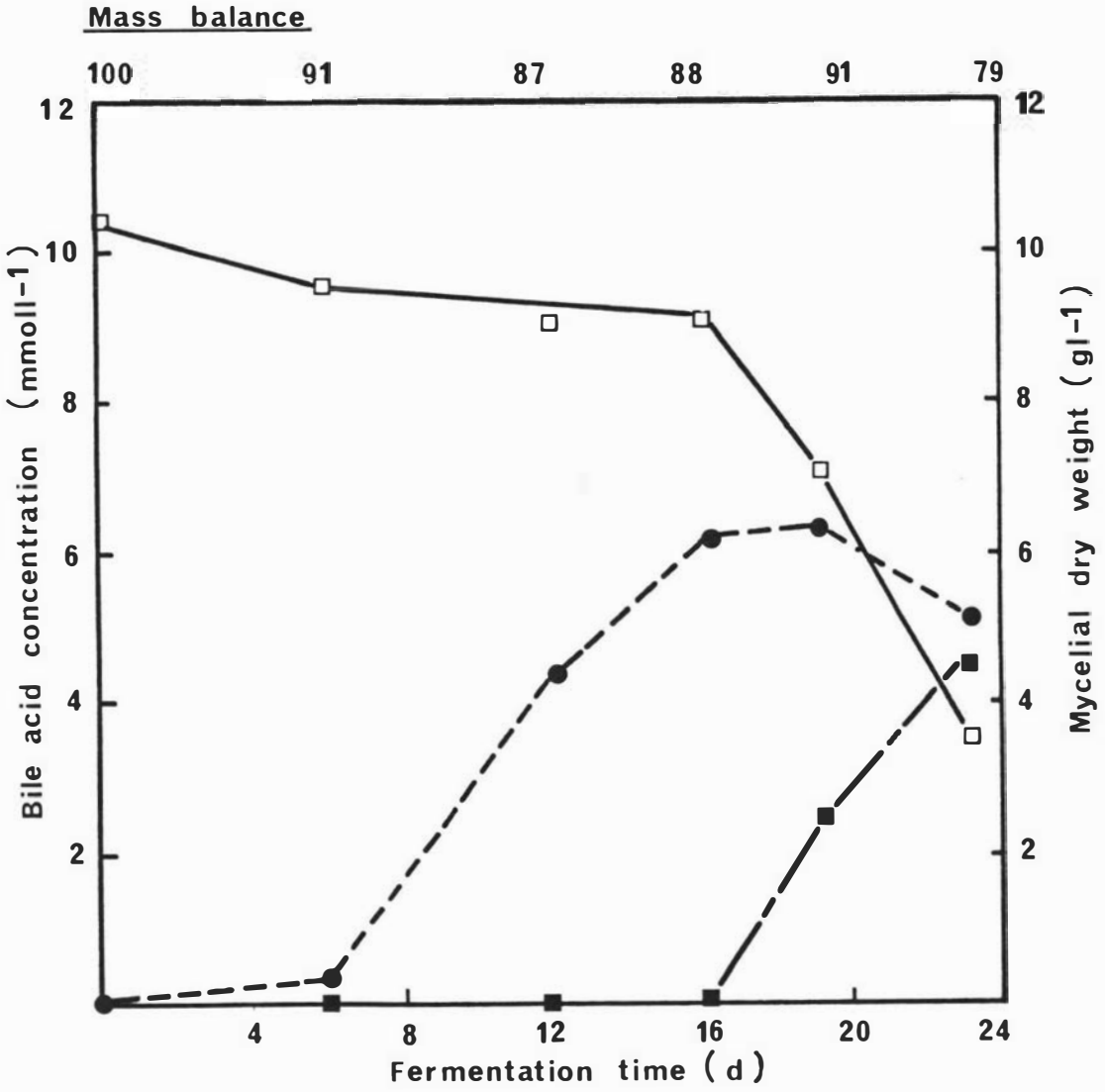


FIGURE 6.3 The hydrolysis of 5.0gl^{-1} glycodeoxycholic acid by *C. fallax* in shake flasks containing glucose-peptone medium. Glycodeoxycholic acid, □——□; deoxycholic acid, ■— - —■; dry weight, ●- - - -●.

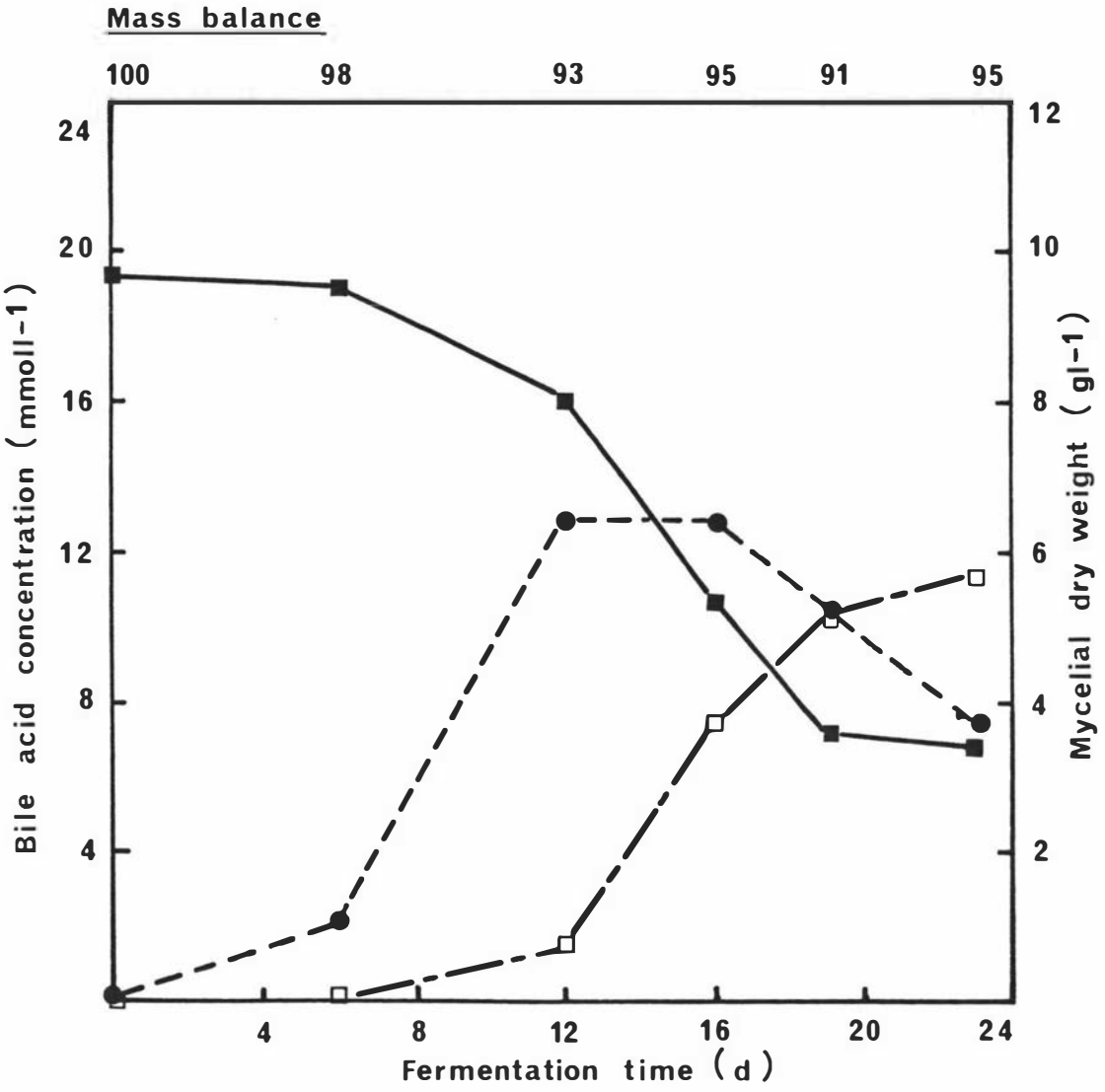


FIGURE 6.4 The hydrolysis of $10gl^{-1}$ glycodeoxycholic acid by *C. fallax* in shake flasks containing glucose-peptone medium. Glycodeoxycholic acid, ■———■; deoxycholic acid, □– – –□; dry weight, ●– – – –●.

Nevertheless, the mass balance data demonstrate that bile acid losses were inversely related to the glycodeoxycholic acid concentration. In all cultures the greater proportion of loss appeared to be growth-related. The actual loss of bile acid was 0.32 $mmol\,l^{-1}$, 0.36 $mmol\,l^{-1}$, 2.1 $mmol\,l^{-1}$ and 1.4 $mmol\,l^{-1}$ for increasing substrate concentrations respectively. Thus, the loss peaked at the 5.0 $g\,l^{-1}$ substrate concentration.

The absolute deoxycholic acid yields were higher with increasing concentrations of glycodeoxycholic acid, being 0.8 $mmol\,l^{-1}$, 1.6 $mmol\,l^{-1}$, 4.6 $mmol\,l^{-1}$ and 11.4 $mmol\,l^{-1}$ respectively. However, the percentage yields were poorer at high substrate concentrations, due to the incomplete hydrolysis of the conjugate.

The specific rate of hydrolysis appeared to increase with increasing glycodeoxycholic acid concentration. A linear relationship is obtained from the plot of glycodeoxycholic acid utilisation after 23 days of fermentation vs. the substrate concentration employed (Figure 6.5). However, this does not correct for the severe lag in hydrolysis observed at the 5.0 $g\,l^{-1}$ glycodeoxycholic acid concentration. If it is assumed that complete utilisation of the conjugate can occur within 23 days of incubation (*i.e.* the lag is ignored), a non-linear plot is obtained (dashed line, Figure 6.5) which suggests that the hydrolase(s) was becoming saturated at substrate concentrations approaching 5.0 $g\,l^{-1}$.

Possible factors contributing to the reduction in hydrolase activity at higher substrate concentrations are inhibition of the enzyme(s) by the substrate or product and/or a decay in its synthetic activity towards the late stages of fermentation. Simple product inhibition was ruled out, in the case of the 1.0 $g\,l^{-1}$ and 10 $g\,l^{-1}$ glycodeoxycholic acid concentrations, by the non-linearity of the data on a plot from Chen *et al* (1962). Insufficient data were available to analyse for substrate inhibition.

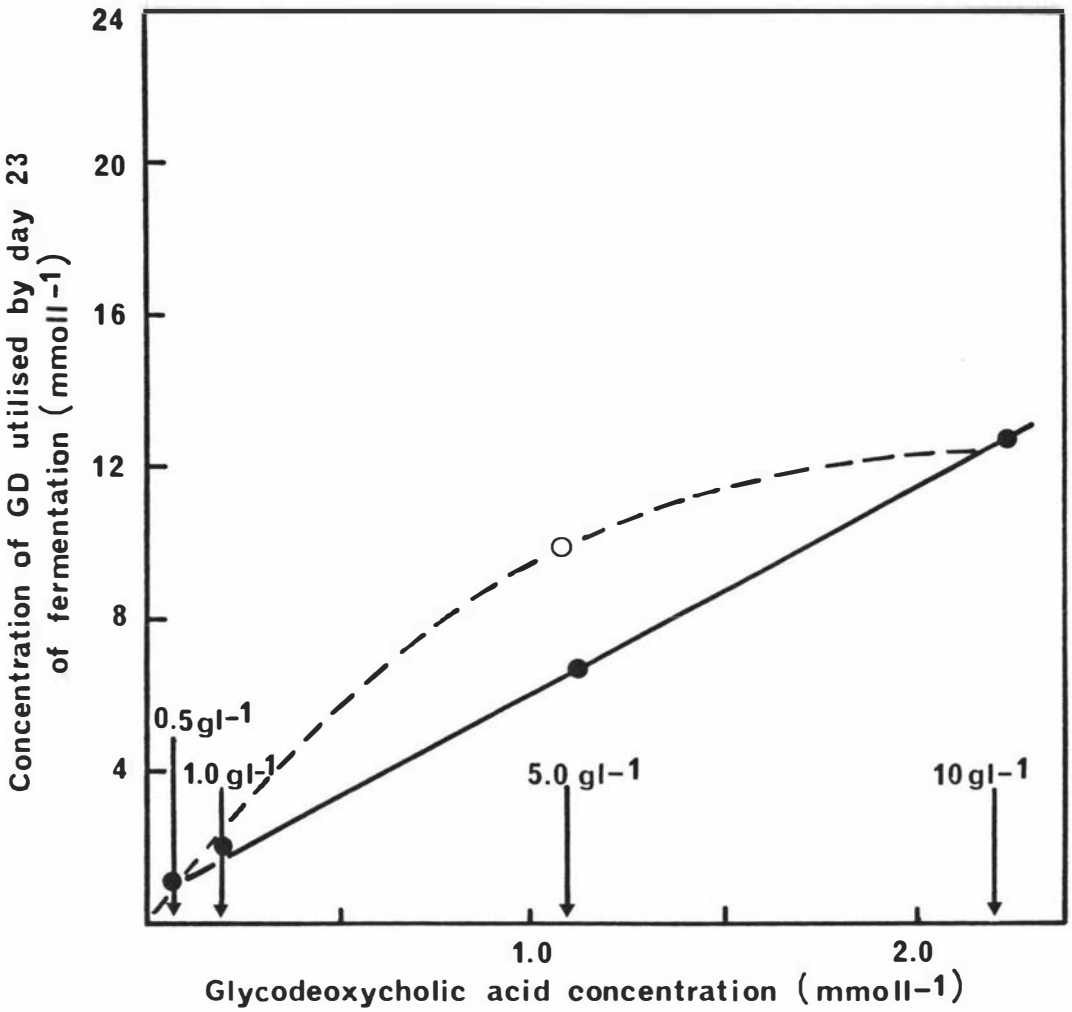


FIGURE 6.5 The extent of glycodeoxycholic acid utilisation vs. the initial substrate concentration in shake flask cultures of *C. fallax*. Actual data, ●——●; Assuming complete utilisation of glycodeoxycholic acid at $5.0gl^{-1}$ after 23 days, ○- - - -○.

6.2.2. The Co-fermentation of Taurocholate and Glycodeoxycholic acid

Like *C. melonis*, *C. fallax* can not hydrolyse taurocholate in shake flask culture, although some utilisation of the conjugate is observed (Chong *et al*, 1980). Therefore, to assess both the resistance of taurocholate to hydrolysis in *C. fallax* cultures actively hydrolysing glycodeoxycholic acid, and the effect of the taurine conjugate on the glycine conjugate hydrolysis, the two conjugates were cofermented each at a concentration of $0.5gl^{-1}$. Appropriate control experiments, with and without bile acid, were performed.

The cultures produced pH profiles and mycelial dry weights that were not significantly different to those previously described for shake flask cultures containing $0.5gl^{-1}$ of glycodeoxycholic acid (Refer Section 6.2.1; Figure 6.1). Therefore, the presence of the taurocholate in the cofermented cultures had no effect on the growth of *C. fallax*, despite a combined bile acid concentration of $1.0gl^{-1}$.

The bile acid data from the cofermented cultures are plotted on Figure 6.6. The hydrolysis of the $0.5gl^{-1}$ glycodeoxycholic acid in the control experiment paralleled that portrayed in Figure 6.1. The $0.5gl^{-1}$ taurocholate control cultures produced no cholic acid or other bile acids over the course of the fermentation, although a loss of 10% of the taurocholate was observed after 16 days of incubation. Likewise, no cholic acid was detected in the cofermentation cultures, despite the accumulation of deoxycholic acid from the hydrolysis of glycodeoxycholic acid. Thus, cofermentation of the taurocholate with glycodeoxycholic acid did not alter its recalcitrance.

The taurine conjugate had no significant effect on the hydrolysis of glycodeoxycholic acid compared with the corresponding control experiment. Thus, a deoxycholic acid yield of 68% was achieved after 16 days of fermentation. However, the loss of 20% taurocholate was double that of the taurocholate control. In conclusion, it appears that

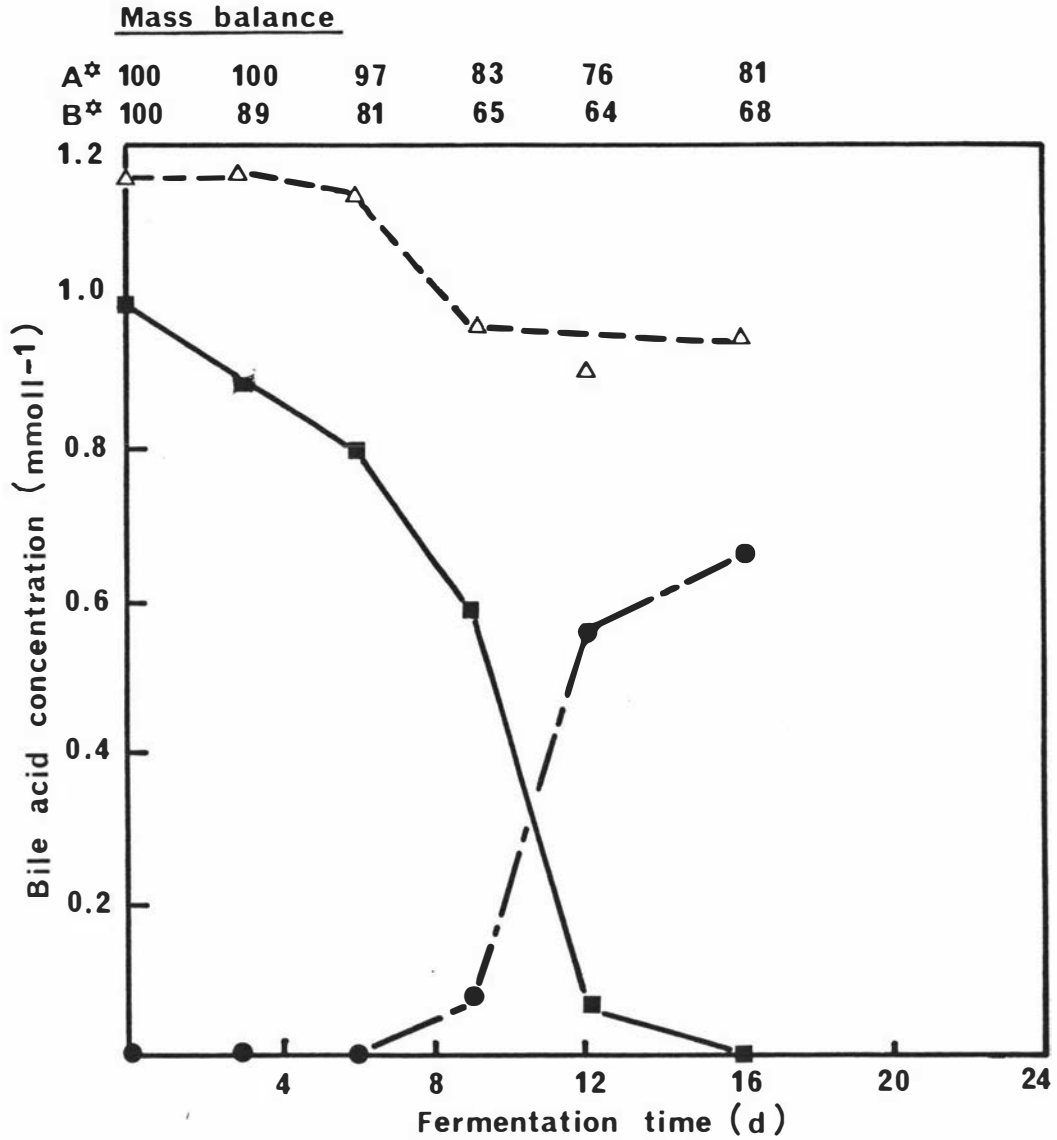


FIGURE 6.6 The effect of $0.5 g l^{-1}$ taurocholate on the hydrolysis of $0.5 g l^{-1}$ glycodeoxycholic acid by shake flask cultures of *C. fallax* grown in glucose-peptone medium. Glycodeoxycholic acid, ■——■; deoxycholic acid, ●—·—●; taurocholate, △– – –△.

* A, taurocholate only.
B, glycodeoxycholic and deoxycholic acids only.

C. fallax elaborates a substrate-specific cholanoyl glycine hydrolase.

6.3. Fermenter Studies

The hydrolysis of $0.5gl^{-1}$ glycodeoxycholic acid was studied in fermenter culture employing the conditions described for the base study of *C. melonis* in the fermenter (Refer Section 4.4.1.). The mycelial dry weight, total carbohydrate and bile acid concentrations during the fermentation are depicted in Figure 6.7.

The growth of *C. fallax* was poor, attaining a maximum dry weight of barely $2gl^{-1}$ after 96h of fermentation. A duplicate fermentation gave identical results. The factor(s) responsible is not clear, since the medium used gave good dry weight yields of *C. fallax* in shake flask cultures. The pH profile of the fermentation lagged that of the corresponding *C. melonis* culture by *ca* 48h, although the sequence of addition of the pH control solutions was similar.

Deoxycholic acid was the sole bile acid product detected, at a maximum yield of 32% within 144h of inoculation. At this time, a residual concentration of glycodeoxycholic acid, representing *ca* 19% of the initial substrate concentration, remained. The loss of bile acid throughout the fermentation amounted to 58% after 168h, the major proportion of the loss occurring during the 24-96h period of fermentation.

The specific rates of glycodeoxycholic acid disappearance and deoxycholic acid accumulation are depicted in Figure 6.8. The former declined significantly with the age of the culture, its value at 120h being only 21% of that after 24h of fermentation. The disappearance of glycodeoxycholic acid from the culture fitted a first order rate equation. However the plot of deoxycholic acid accumulation deviated markedly from first order rate kinetics.

In conclusion, the hydrolysis by *C. fallax* in the fermenter

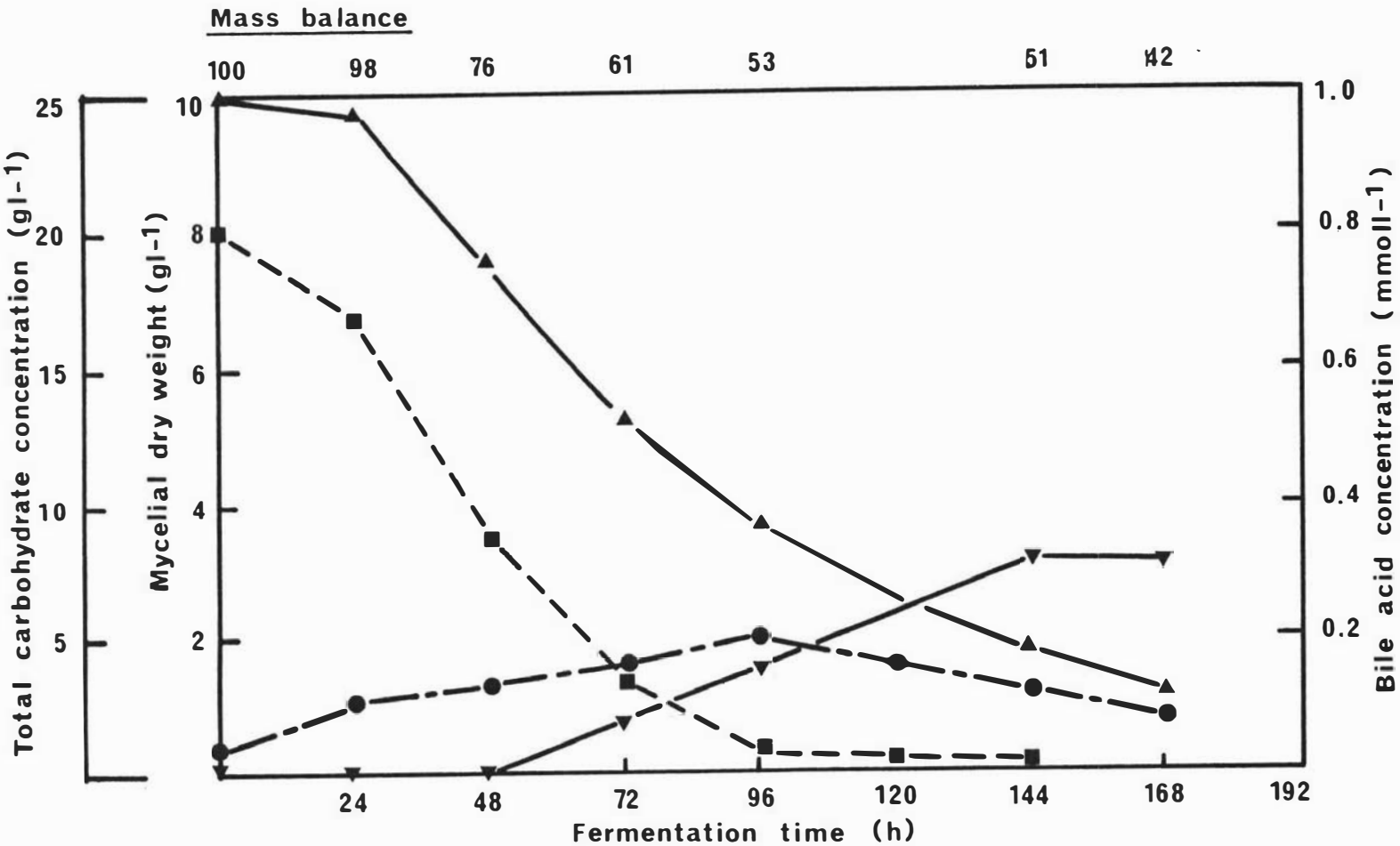


FIGURE 6.7 The hydrolysis of 0.5gl^{-1} glycodeoxycholic acid in a fermenter by *C. fallax* grown in glucose-peptone medium. Aeration rate, 1.0lmin^{-1}; pH 6.5; agitation, 750 rpm. Mycelial dry weight, ●——●; total carbohydrate as glucose, ■- - - -■; glycodeoxycholic acid, ▲——▲; deoxycholic acid, ▼——▼.

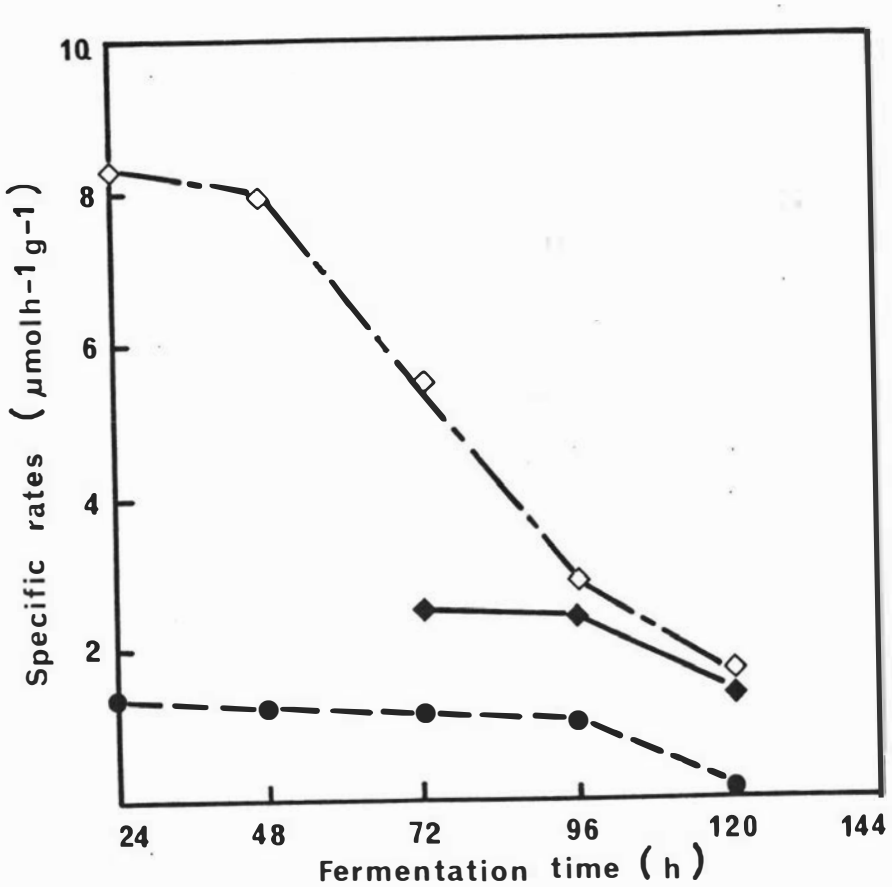


FIGURE 6.8 The specific rates of glycodeoxycholic acid hydrolysis by *C. fallax* in a fermenter. Glycodeoxycholic acid specific rate of disappearance, ◇——◇; specific rate of deoxycholic acid accumulation, ◆——◆. The specific rate of glycodeoxycholic acid disappearance in the corresponding *C. melonis* culture, ●----●, is included for comparison.

gave low deoxycholic acid yields. However, despite the poor mycelial growth, the rate of hydrolysis was comparable to that of *C. melonis* due to the high specific rate of hydrolysis observed.

6.4. Discussion

The data demonstrate that *C. fallax* is superior to *C. melonis* in its ability to hydrolyse glycodeoxycholic acid efficiently. Thus, in shake flask culture, at a conjugate concentration of $0.5 gl^{-1}$, maximum deoxycholic acid yields of 76% and 22% were attained after 12 days of fermentation for *C. fallax* and *C. melonis* respectively, with no substrate remaining in either culture. In the fermenter, the reverse is true, the maximum yields of deoxycholic acid being 32% and 47% respectively. The difference in yield in the fermenter between the two fungi was due to the higher residual glycodeoxycholic acid remaining in *C. fallax* cultures (*ca* 19%) compared to *ca* 6% for *C. melonis*. (Compare Figures 4.15 and 6.7).

However, notwithstanding this, the specific rates of glycodeoxycholic acid disappearance and deoxycholic acid accumulation from the fermenter cultures show that those of *C. fallax* were a factor of at least 3 greater than those of *C. melonis* (Compare Figures 4.16 and 6.8). Thus, the specific rates of glycodeoxycholic acid disappearance were *ca* 4 $\mu molh^{-1}g^{-1}$ and *ca* 1.2 $\mu molh^{-1}g^{-1}$ for *C. fallax* and *C. melonis* respectively. This clearly demonstrates that *C. fallax* is capable of superior specific rates of glycodeoxycholic acid hydrolysis compared to *C. melonis*. The decline in specific hydrolysis activity of the former with culture age compared to the relatively constant activity of the latter, suggests that the hydrolase system of *C. fallax* is, as a whole perhaps, less stable than that of *C. melonis*.

The superiority of *C. fallax* compared to *C. melonis* with regard to the yield of hydrolysis product, was not observed in the qualitative screens reported by Maddox and Chong

(1978) and Chong *et al* (1980). Therefore, it seems important that, in order to select the best organism, quantitative screening for bile acid hydrolysis is required.

C. fallax, however, was more sensitive to adverse culture conditions, particularly bile acid concentrations, than *C. melonis*. Whereas the growth of the latter was unaffected by glycodeoxycholic acid concentrations of up to $10gl^{-1}$, the growth of *C. fallax* was inhibited at concentrations of $1.0gl^{-1}$. Furthermore, *C. fallax* grew poorly in fermenter culture, despite good dry weight yields on the same medium in shake flask culture. Hence, in the fermenter, its superior specific hydrolysis capability was offset by its low biomass yields.

Both fungi gave similar hydrolysis profiles with respect to the culture age in shake flask and the fermenter. Neither exhibited side-reactions or the hydrolysis of taurocholate. Thus, in several respects, the hydrolysis of glycodeoxycholic acid by the two organisms was similar and this suggests that their hydrolase enzymes may be comparable in their cellular compartmentalisation and in their specificity.

The markedly superior deoxycholic acid yields obtained from *C. fallax* in shake flask cultures hydrolysing $0.5gl^{-1}$ glycodeoxycholic acid, compared to those from the corresponding *C. melonis* cultures, were due to the reduced loss of bile acid in the former. Thus, losses of bile acid were 23% and 78% respectively. Binding assays performed by the procedure described in Section 4.3.1. revealed that the weights of glycodeoxycholic acid and deoxycholic acid bound per g dry weight of *C. fallax* are 14 mg and 27 mg respectively. These data predict a maximum loss by binding of bile acid by the mycelium of *ca* 38% in shake flask cultures of *C. fallax* containing $0.5gl^{-1}$ glycodeoxycholic acid, assuming hydrolysis occurs. This figure accounts for the losses observed in such cultures (Figure 6.8), in contrast to the case for *C. melonis*, and therefore suggests that *C. fallax* does not further metabolise the free bile acid product.

However, the binding of bile acid by the mycelium predicts losses of only 11% for the fermenter culture, in which the observed bile acid loss was 58% (Figure 6.7). This strongly suggests that an anologous situation to that of *C. melonis* fermenter cultures exists for the loss of bile acid, namely, that metabolic degradation of the free bile acid is primarily responsible. The reason for the apparent absence of this factor in shake flask cultures of *C. fallax* is unclear.

In conclusion, the bile acid transforming abilities of *C. melonis* and *C. fallax* differ significantly in aspects which would have a marked influence on their employment in an industrial process. *C. fallax* possesses higher specific hydrolase activity on glycodeoxycholic acid than *C. melonis* and, given higher dry weights, would probably achieve faster reaction. Under shake flask conditions, *C. fallax* gave higher product yields than *C. melonis* and appeared not to further degrade the free bile acid. These are desirable features for a process organism. However, *C. fallax* suffered greater inhibition from high glycodeoxycholic acid concentrations.

Obviously, no simple screening experiment can hope to determine all these data. However, for these fungi, a shake flask run containing $5.0 g l^{-1}$ glycodeoxycholic acid in which dry weight and quantitative bile acid data were obtained would provide information useful for their comparison by the above criteria. Since scale-up is easier from fermenter data, promising organisms should be compared in fermenter culture.

CHAPTER 7

FUNGAL 7α-DEHYDROXYLATION

7. FUNGAL 7α-DEHYDROXYLATION

7.1. Introduction

The presence of 7α-hydroxycholanoyl dehydroxylase activity in fungi offers the potential for the conversion of the major hydrolysis product of New Zealand cattle and sheep gall, cholic acid, to the apparently more valuable deoxycholic acid, possibly in the same fermentation in which hydrolysis occurs. Preliminary screening of fungi in this laboratory for 7α-hydroxycholanoyl dehydroxylase activity showed that it was not commonly expressed in whole cell fermentations. However, deoxycholic acid was detected, by tlc, from the incubation of glycocholic acid with *Curvularia coicis* IFO 7278.

Other reports of fungal 7α-dehydroxylation are confined to the few instances recorded by Furuta (1959). Although there is probably a discrete 7α-hydroxycholanoyl dehydroxylase in fungi reported to possess such activity, 7α-dehydroxylation occurred as the sole transformation only in the conversion of cholic acid to deoxycholic acid by *Aspergillus ochraceus* (Wilhelm) IFO 4071, reported by Furuta (1960).

Therefore, studies were now conducted to investigate this potentially valuable transformation, employing *C. coicis*. In addition, *A. ochraceus* (Wilhelm) IFO 4071 was studied for comparison.

7.2. Dehydroxylation using Whole Cells of *C. coicis*

7.2.1. The Course of Dehydroxylation

Duplicate experiments were performed in shake flasks with *C. coicis* grown in glucose-peptone medium containing $1.0 g l^{-1}$ glycocholic acid. An uninoculated control was run for comparison.

The dry weight, total carbohydrate concentrations and the

culture pH data are presented in Figure 7.1. As the duplicate experiments yielded parallel data, they are represented as a single curve for each parameter. The dry weight peaked at $8gl^{-1}$ after 7 days incubation, by which time the total carbohydrate in the medium was exhausted.

The bile acid data are depicted in Figure 7.2. The control cultures contained only the substrate after 11 days incubation. *C. coicis* initiated hydrolysis of glycocholic acid towards the end of growth (*ca* day 5) and complete conversion was attained after 10 days of fermentation to give a 92% yield of cholic acid. Deoxycholic acid was observed in *ca* 3% yield after 11 days incubation. Its concentration did not increase further, despite prolonged incubation. The appearance of deoxycholic acid after day 10 suggests that the 7α-hydroxycholanoyl dehydroxylase acted on cholic acid in preference to the cholic acid conjugate. The loss of total bile acid during the course of the fermentation was negligible.

7.2.2. The Effect of Substrate

To determine whether 7α-dehydroxylation was performed on cholic acid, this bile acid was incubated with *C. coicis* in shake flasks of glucose-peptone medium at a concentration of $0.5gl^{-1}$. A control containing glycocholic acid was also run.

The dry weight of both the control and the cholic acid-containing cultures peaked at $6gl^{-1}$, after 4 days incubation. This was significantly earlier than cultures containing $1.0gl^{-1}$ glycocholic acid (Figure 7.1). Despite this, the control cultures yielded bile acid data similar to those given in Figure 7.2.

However, cultures containing cholic acid exhibited no 7α-hydroxycholanoyl dehydroxylase activity and no bile acids, other than cholic acid, were detected. A loss of *ca* 8% cholic acid had occurred after 21 days incubation, probably due to binding to the mycelium. Hence, in shake flask

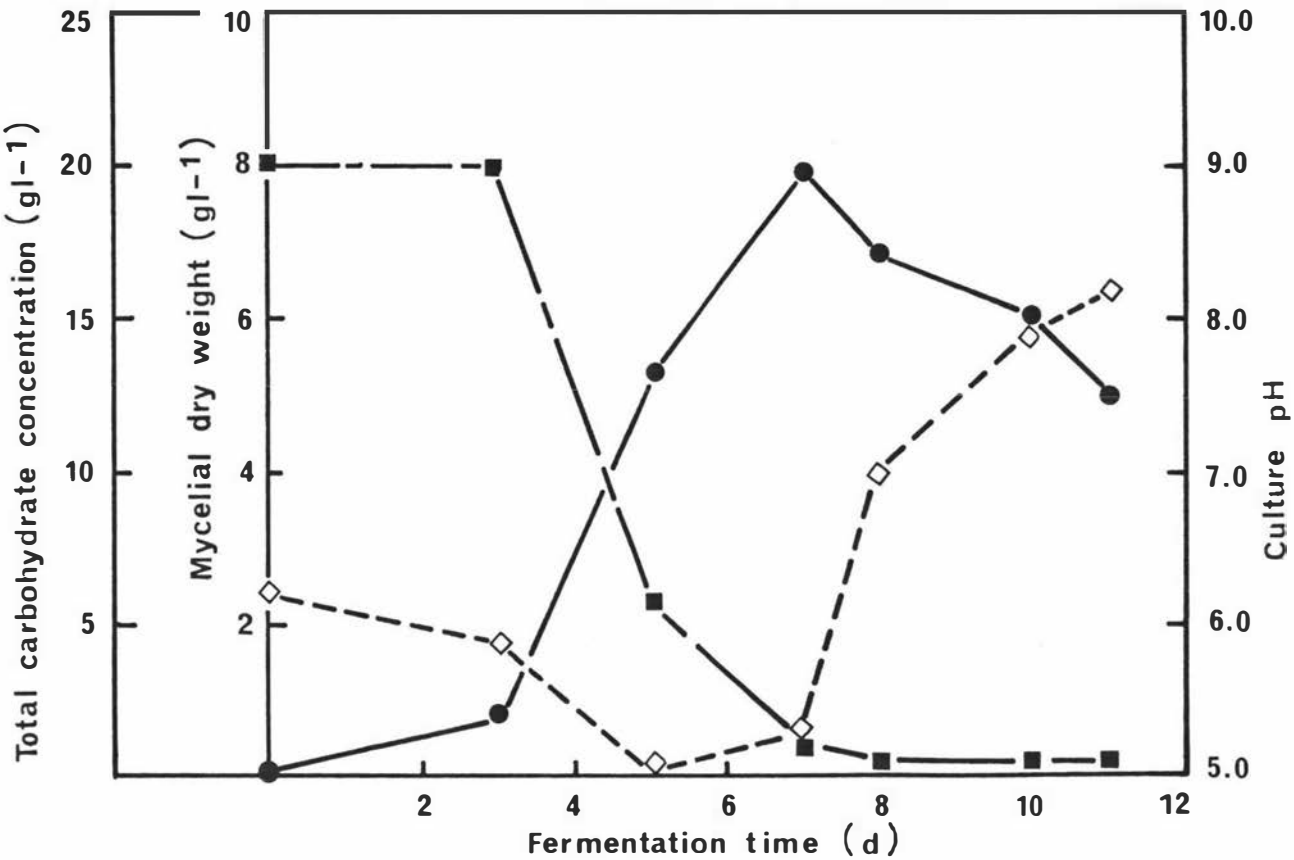


FIGURE 7.1 The growth of *C. coicis* in shake flask cultures of glucose-peptone medium containing 1.0gl^{-1} of glycocholic acid. Mycelial dry weight, ●——●; culture pH, ◇– – –◇; total carbohydrate as glucose, ■– – –■.

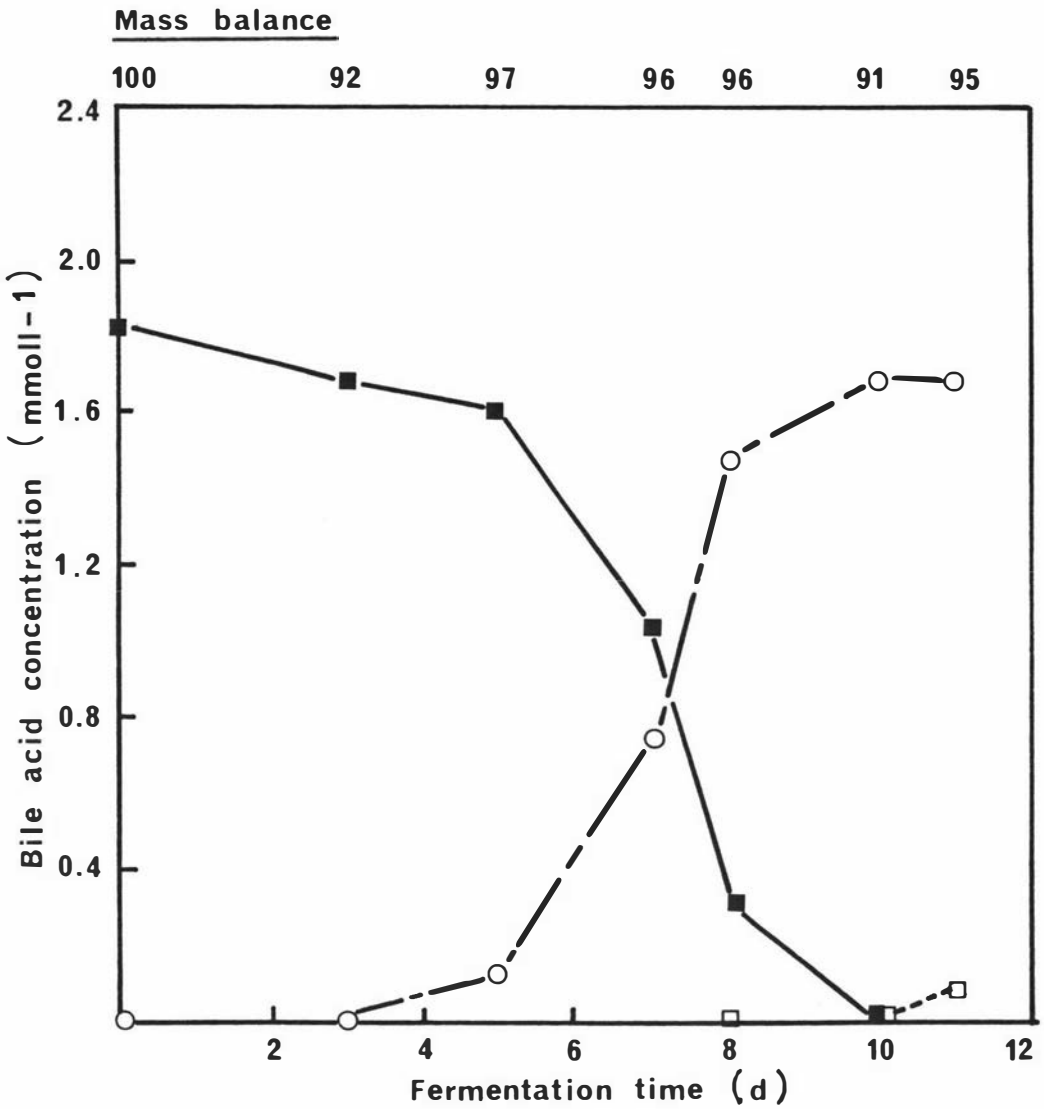


FIGURE 7.2 The hydrolysis and 7α-dehydroxylation of 1.0gl^{-1} glycocholic acid in shake flask cultures of *C. coicis* grown in glucose-peptone medium. Glycocholic acid, ■———■; cholic acid, ○— - —○; deoxycholic acid, □— — — —□.

cultures of *C. coicis*, glycocholic acid was the preferred substrate for 7α-dehydroxylation.

7.2.3. The Effect of the Method of Incubation

To study the effect of the method of incubation of *C. coicis* cultures on 7α-dehydroxylation, flasks containing glucose-peptone medium with $0.5gl^{-1}$ of glycocholic acid or cholic acid were inoculated and incubated in still culture.

The mycelial dry weight of the still cultures increased at a slower rate than that of the corresponding shake flasks, peaking at $7gl^{-1}$ after 10 days incubation. Culture pH data showed a similar lag. The growth of the fungus was not affected by the type of bile acid present.

In still cultures containing cholic acid, no other bile acids were detected, despite incubation of the cultures for 30 days. A 12% loss was observed by this time. Thus, the incubation of cholic acid with *C. coicis* in still culture did not promote 7α-dehydroxylation.

In still cultures containing glycocholic acid, hydrolysis began after 10 days incubation, but no deoxycholic acid was detected, despite an incubation time of 21 days. However, very low concentrations of a compound, identical to authentic glycodeoxycholic acid on tlc using solvent systems "a", "c" and "e" to "g", were observed after 10 days of fermentation. The yields of this compound remained low despite further incubation. Thus whereas cholic acid was inert in *C. coicis* cultures, glycocholic acid was transformed to low quantities of glycodeoxycholic acid and deoxycholic acid in still and shake flask culture respectively. It is probable that the glycodeoxycholic acid produced in still cultures of *C. coicis* would be hydrolysed eventually.

7.3. Dehydroxylation using Whole Cells of *A. ochraceus*

7.3.1. The Effect of the Substrate

The 7α-hydroxycholanoyl dehydroxylase activity of *A. ochraceus* grown in glucose-peptone medium was studied in shake flask culture, employing glycocholic acid or cholic acid as the substrate at a concentration of $1.0 g l^{-1}$. The fungus grew initially as a thick, cream-coloured mycelium, which degenerated after *ca* 9 days of incubation into a thin, brown suspension.

Despite the incubation of cultures containing cholic acid for 28 days, no evidence of 7α-dehydroxylation was observed. However, those containing glycocholic acid exhibited both the hydrolysis of the conjugate and the production of deoxycholic acid within 6 days of inoculation although the yield of the latter was too low for quantitation.

7.3.2. The Effect of the Method of Incubation

The incubation of flasks, identical to those of the previous section, as still cultures, resulted in the fungus growing as a white, surface felt on which tan spores appeared 10 days after inoculation. Similar results to those described for the shake flask cultures were obtained with respect to the transformation of cholic and glycocholic acids. Thus, no 7α-dehydroxylation of the former was observed, despite an incubation period of 50 days. Glycocholic acid was weakly hydrolysed after 10 days of fermentation, and low levels of deoxycholic acid were detected by this time. They remained too low for quantitation.

7.4. Dehydroxylation using Cell-free Systems

The incubation of cell-free extracts of *C. melonis* with cholic acid conjugates during the study of its hydrolase activity, revealed a low level of constitutive 7α-hydroxycholanoyl

dehydroxylase activity, although such activity was not observed in whole-cell cultures of the organism. The paradoxical resistance of cholic acid to 7α-dehydroxylation by *C. coicis* and *A. ochraceus*, despite activity on glycocholic acid, led to the use of cell-free systems of these fungi to assess whether these demonstrated activity on cholic acid itself.

Cultures were grown in shake flask using glucose-peptone medium without bile acid present. Cell-free culture filtrates and cell-free extracts were then prepared and incubated with cholic acid, glycocholic acid and sodium taurocholate. Appropriate controls were run.

The results of the cell-free system incubations using *C. coicis* and *A. ochraceus* are compared to those of *C. melonis* in Table 7.1.

The cell-free extracts of all 3 fungi exhibited weak 7α-hydroxycholanoyl dehydroxylase activity on each of the bile acids tested, except for *C. coicis* in the case of taurocholate. Thus, for all, the activity was constitutive and intracellular, the latter demonstrated by the absence of 7α-dehydroxylation products in the cell-free culture filtrate. The substrate for the enzyme in all cases may, in fact, be cholic acid, despite the apparent lack of activity on this bile acid in whole-cell cultures. Thus, in all instances where deoxycholic acid was observed in cell-free extracts containing a cholic acid conjugate, the extract also possessed hydrolase activity for that conjugate. Where hydrolase activity was absent, as in the case of the incubation of taurocholate with *C. coicis* cell-free extracts, 7α-hydroxycholanoyl dehydroxylase activity was also absent. However, glycodeoxycholic acid was detected in the cell-free extract of *C. coicis* incubated with glycocholic acid, a result unique to this organism.

Whereas both *A. ochraceus* and *C. melonis* possess an intracellular hydrolase(s) active on both glycocholic acid and

Table 7.1

Products from the incubation of bile acids with the cell-free systems

Fungus	Cell-free system	Bile acid substrate CA	GC	NaTC
C. melonis	Filtrate		CA	-
	Extract	DC	CA	CA
		OT	DC	DC
			OT	OT
C. coicis	Filtrate	-	CA	-
	Extract	DC	CA	-
			GD	
			DC	
A. ochraceus	Filtrate	-	CA	-
	Extract	DC	CA	CA
		OT	DC	DC
			OT	OT

Notation: -, no products detected;
OT, other products.

taurocholate, that of *C. coicis* was specific for glycocholic acid. Furthermore, the bile acid degradative products observed in the cell-free extracts of *C. melonis* and *A. ochraceus* were absent from those of *C. coicis*. Hence, *C. coicis* appears to possess significantly different bile acid transforming abilities to those of the other two organisms.

7.5. Discussion

Intracellular 7α-hydroxycholanoyl dehydroxylase is present in each of the three fungi tested and is principally constitutive. However, even when whole-cell cultures are incubated with bile acid from inoculation, the yields of the 7α-dehydroxylated product remain very low. The usual product, deoxycholic acid, was clearly distinguished from chenodeoxycholic acid by the use of the tlc solvent system "a" with the authentic standards. Thus, 7α-dehydroxylation was the transformation observed. The preference of the fungal enzyme for 7α-dehydroxylation compared to 12α-dehydroxylation is not unexpected in view of the accessibility of the relatively exposed 7α-hydroxyl function compared to the 12α-hydroxyl group, which is protected by the steroid sidechain, especially by the C-21 methyl function (Atkinson and Blickenstaff, 1974).

Despite the occurrence of 7α-dehydroxylation in the cell-free extracts of the three fungi, it is clearly not expressed to any large extent in whole-cell cultures. There may be several factors contributing to this limited expression. Severe suppression of the enzyme by cellular regulatory mechanisms may be responsible, particularly since the intracellular release of high yields of the more toxic 7α-dehydroxylated product may damage the cell. However, a more likely factor may be the limited permeability of the cell membrane to bile acids. Thus, the situation may be analogous to that of taurocholate hydrolysis with *C. melonis*, where, even though an intracellular enzyme exists, the substrate is not readily available to the enzyme.

To explain the dehydroxylase activity of whole-cell cultures of *C. coicis* and *A. ochraceus* on glycocholic acid, but not cholic acid, it may be hypothesised that with these cultures, whereas glycocholic acid achieves some slight penetration into the cells, none occurs with cholic acid. This may also account for the low losses of bile acid in cultures of *C. coicis*, since the degrading enzymes are probably also intracellular. With *C. melonis*, it has previously been shown

that the high losses of bile acid in whole-cell cultures are, in part, due to degradative enzymes. This clearly assumes that bile acids can enter the cell to a major extent. In this case, the 7α-dehydroxylated product may be rapidly degraded by the fungus and so not accumulate.

Although the time-course data with whole-cell cultures of *C. coicis* and *A. ochraceus* may suggest that hydrolysis precedes the 7α-dehydroxylation of cholic acid conjugates, the detection of glycodeoxycholic acid in still cultures and cell-free extracts of *C. coicis* containing glycocholic acid provides evidence that this may not always be the case. In addition, it gives support to the suggestion that it is glycocholic acid, rather than cholic acid, which penetrates into the cell in the instance of *C. coicis*. This dehydroxylation of glycocholic acid appears to be the first instance of conjugate dehydroxylation. Known bacterial 7α-hydroxycholanoyl dehydroxylases exhibit no activity on glycocholic acid (Aries and Hill, 1970b; Hayakawa, 1973).

The 7α-hydroxycholanoyl dehydroxylase of the fungi catalysed the reaction under aerobic conditions. Similar results were found by Furuta (1959; 1960) with other fungi. In contrast, the enzyme isolated from strictly anaerobic intestinal bacteria requires stringent anaerobic conditions for its expression (Aries and Hill, 1970b; Hill, 1976), although recently Archer (1980) has observed that aerobic conditions actually favour the 7α-dehydroxylation of cholic acid by whole cells of *Clostridium bifermentans*.

The results also provide data from two further organisms concerning the hydrolysis capabilities of fungi. Whereas cell-free systems of *A. ochraceus* possess activity similar to that of *C. melonis*, the intracellular activity of *C. coicis* is significantly different, being specific for glycocholic acid. Whether this result demonstrates the absence of the cholanoyl taurine hydrolase in *C. coicis* or whether this activity was simply not expressed is unclear. However, the result further supports the view that fungi, in general, may possess two discrete, intracellular hydrolases, one specific

for α-aminocarboxylic acid conjugates, the other specific for sulphonic acid conjugates.

In conclusion, the data suggest that although the ability to 7α-dehydroxylate cholic acid may be common to a number of fungi, the poor expression of this activity in fungal whole-cell cultures renders a process utilising this fungal transformation unlikely without considerable further work.

CHAPTER 8

FINAL DISCUSSION

8. FINAL DISCUSSION

Data have been obtained describing the hydrolysis of several bile acid conjugates by *C. melonis*, *C. fallax* and *C. coicis* under various environmental conditions.

The products of the hydrolysis of glycocholic acid by *C. melonis* and *C. coicis* and similarly of glycodeoxycholic acid by *C. melonis* have been characterised by the criteria of melting point, mixed melting point and the IR spectra comparison of the free bile acid crystals. The chromatographic identity of these products, using tlc and hplc analysis, with authentic standards, further confirms the transformations observed. The product of glycodeoxycholic acid hydrolysis by *C. fallax*, and of sodium taurocholate and sodium taurodeoxycholate hydrolysis by *C. melonis*, were confirmed by both tlc and hplc identity with the corresponding standards.

Data describing the fungal 7α-dehydroxylation of cholic acid and its conjugates were also obtained. The product of the 7α-dehydroxylation of glycocholic acid, after its hydrolysis, by *C. coicis* was not isolated in sufficient quantity to allow its characterisation by the usual criteria employed due to its formation of complexes with fatty acids and its low yield. However its chromatographic behaviour on tlc and hplc was identical to authentic deoxycholic acid and its melting point was characteristic of a deoxycholic acid-fatty acid complex. The products of 7α-dehydroxylation in cultures of *C. melonis* and *A. ochraceus* were identified by their coincidence with deoxycholic acid in several tlc solvent systems.

Data have been obtained describing the ability of fungi to hydrolyse glycine conjugates. *C. melonis*, *C. coicis* and *A. ochraceus* possess both intracellular and extracellular cholanoyl glycine hydrolase activity. In addition, the enzyme(s) is constitutive. The extracellular activity does not appear to be due to leakage of the intracellular enzyme from the cells. If cell lysis were responsible extracellular activity should also have been observed on taurine conjugates, which was not the case. Furthermore, the centrifugation

conditions employed in the preparation of the cell-free systems were sufficient to remove cell wall/membrane material from the supernatant liquid (Aries and Hill, 1970a); therefore only "free" activity was present in the cell-free filtrate.

A major reason for investigating fungal hydrolysis of bile acid conjugates was the considerable loss of bile acid observed during alkaline hydrolysis. However, the yield of free bile acid from the hydrolysis of glycine conjugates by *C. melonis* was not quantitative. Three factors are responsible for the observed losses. These are:

- The binding of the bile acid conjugate and the free bile acid to the mycelium.
- The rapid degradation of the free bile acid product by an inducible enzyme system to give non-steroidal products.
- Under certain conditions, the conversion of the conjugate and/or the free bile acid to keto derivatives.

The relative contribution of these factors to the losses of bile acid observed varies depending on the organism, the environmental conditions and the type of bile acid conjugate employed.

Previous work has shown taurine conjugates to be resistant to hydrolysis by whole-cell cultures of fungi, except for two instances involving *Aspergillus niger* (Furuta, 1959) and *Penicillium chrysogenum* (Chong *et al*, 1980). The current research has demonstrated the presence of intracellular, but not extracellular, cholanoyl taurine hydrolase activity in *C. melonis* and *A. ochraceus*. The enzyme is constitutive.

In whole-cell shake flask cultures of *C. melonis* and *C. fallax* there is some utilisation of the taurine conjugates, but no free bile acid is observed. It is probable that, since taurocholate is bound to the mycelium to a negligible extent, this loss is due to two factors which act on the free bile

acid produced by the hydrolysis of the low amounts of taurine conjugate that can penetrate into the cell. These factors are:

- The binding of the free bile acid to the mycelium.
- The rapid degradation of the free bile acid by an inducible, intracellular enzyme system to give non-steroidal products.

The poor hydrolysis of taurine conjugates by whole-cell cultures of *C. melonis* is due to the lack of extracellular activity on these conjugates. The taurine conjugates are apparently unable to penetrate into the cell to any great extent and, hence, be hydrolysed by the intracellular enzyme. In elucidating the specificity of the extracellular hydrolase by the use of analogues of the natural conjugates, it became apparent that this enzyme may be a neutral protease. This suggestion is supported by the specificity of the enzyme for α-aminocarboxylic acid conjugates and its possible requirement for a metal ion.

Further work is needed to test this hypothesis, particularly in view of the statement by Nair *et al* (1967) that the peptide bond of the bile acid conjugates is resistant to the action of most of the known proteolytic enzymes. Such work might include the incubation of fungal proteases, particularly neutral enzymes, with bile acid conjugates to observe whether hydrolysis occurs. The isolation and purification of the extracellular hydrolase from *C. melonis* and its incubation with protein would also be useful.

Although the cholanoyl glycine hydrolase appears to be constitutive, if the enzyme is a protease it is possible that it was induced by the peptone used in the medium to grow the fungus. Inducible and constitutive extracellular proteases have been reported in cultures of *Neurospora crassa* (Cohen *et al*, 1975) and *Aspergillus nidulans* (Cohen, 1973) respectively. To obtain more information it would be useful to grow *C. melonis* in a medium containing a non-peptide/protein nitrogen source to assess the effect of this on glycine conjugate hydrolysis.

Work has been performed to study fungal 7α-dehydroxylation. Low levels of intracellular, and apparently constitutive, activity were found in the organisms employed. However, in whole-cell cultures, little or no activity was observed, possibly due to permeability constraints on the access of the bile acid to the intracellular enzyme.

Much of the published literature regarding the microbial transformation of bile acids concerns the action of intestinal bacteria on these compounds. The current work has demonstrated that significant differences in transformation ability exist between fungi and intestinal bacteria. Regarding the hydrolysis of bile acid conjugates, bacterial hydrolases generally exhibit activity on both glycine and taurine conjugates (Hill and Drasar, 1967; Nair *et al*, 1967; Hayakawa, 1973), although some exceptions have been reported (Kobashi *et al*, 1978). Also, bacterial hydrolases are predominantly intracellular (Hill, 1976) and exhibit no requirement for metal ions (Nair *et al*, 1967; Aries and Hill, 1970a). Further whole-cell cultures of anaerobic bacteria are inhibited at glycodeoxycholic acid concentrations of *ca* 0.2% w/v (Shimada *et al*, 1969) although the isolated hydrolase is not inhibited by substrate excess (Hill, 1976). Consequently, it appears that intestinal bacteria are significantly more permeable to bile acids than fungi, and that the hydrolase enzymes are probably different.

A similar difference is apparent in the 7α-dehydroxylation abilities of intestinal bacteria and fungi. The bacterial enzyme is almost entirely inducible; it is highly active in the intestine and is produced, and is active, only under strictly anaerobic conditions (Hill, 1976). In contrast, the fungal 7α-hydroxycholanoyl dehydroxylase is produced and is active in an aerobic environment.

Under anaerobic conditions, the intestinal bacteria appear to achieve quantitative conversions of bile acid (Archer, 1980). However, aerobically-grown, bacterial cultures appear to rapidly degrade bile acid to non-steroidal products (Yesair and Himmelfarb, 1970; Hayakawa, 1973). In this respect,

therefore, they behave in a similar manner to fungi.

In conclusion, the major aim of this work was to assess the feasibility of a fungal hydrolysis process, including the possibility of converting cholic acid to deoxycholic acid. Unfortunately, the poor hydrolysis of taurine conjugates by whole-cell cultures cannot be easily overcome by the manipulation of the fermentation conditions and, hence, negates a fungal hydrolysis process. Furthermore, the limited expression of 7α-hydroxycholanoyl dehydroxylase activity by whole-cell cultures similarly rules out a process based on the conversion of cholic acid to deoxycholic acid. Nevertheless, the inherent variation in transformation abilities observed in different fungi during this work suggests that further screening and, perhaps, mutation programmes, may produce fungi exhibiting a superior performance to those employed.

CHAPTER 9

SUMMARY

9. SUMMARY

Work has been performed to assess the feasibility of a fungal process for the hydrolysis of bile acid conjugates present in cattle and sheep gall from New Zealand meatworks. The data obtained demonstrate that such a process is not feasible with the fungi studied, due to the limited ability of whole-cell cultures to hydrolyse taurine conjugates.

C. melonis has been shown to possess enzymes catalysing the hydrolysis of both glycine and taurine conjugates. The activity on glycine conjugates is both intracellular and extracellular and is constitutive. The extracellular activity appears to be due to a metallo enzyme with a pH optimum of pH 6.5 and a substrate requirement for an α-aminocarboxylic acid structure. The enzyme may be a neutral protease secreted to degrade the peptone in the medium, and which also hydrolyses the glycine conjugates. The fungus completely utilised both glycocholic acid and glycodeoxycholic acid, at a concentration of $0.5 g l^{-1}$, to give product yields of 50% and 22% respectively after 11 days in shake flask culture. In fermenter culture, a maximum yield of 65% deoxycholic acid was obtained from $0.5 g l^{-1}$ glycodeoxycholic acid after 6 days of fermentation.

Losses of the bile acids are attributed to two major factors. First, the binding of the bile acids to the fungal mycelium; secondly, the further metabolism of the free bile acid. In shake flask culture, yields of deoxycholic acid from glycodeoxycholic acid were increased by adding the conjugate after growth was complete (60%), pretreating grown cultures with cycloheximide (92-98%) or increasing the conjugate concentration (91% at $10 g l^{-1}$). In fermenter cultures, the highest yields were obtained using high aeration rates ($1 l min^{-1}$) or incremental glucose additions to maintain the glucose concentration at $10\text{-}20 g l^{-1}$.

The cholanoyl taurine hydrolase of *C. melonis* is intracellular and constitutive. Whether it is the same enzyme as the intracellular cholanoyl glycine hydrolase is unclear. However, it

may be a discrete enzyme with a substrate requirement for a sulphonic acid structure. The primary factors responsible for the lack of observed hydrolysis of taurine conjugates in whole-cell cultures are the specificity of the extracellular hydrolase, the apparent impermeability of the cell-membrane to these conjugates and also the degradation and binding of the free bile acid produced.

C. melonis and *C. fallax* exhibit similar hydrolysis abilities, but the latter has been shown to possess markedly superior specific activity. However, this was counter-balanced by the poorer growth of *C. fallax*. The results demonstrate the requirement for quantitative screening of organisms to determine their true potential regarding hydrolysis.

Cell-free extracts of *C. melonis*, *C. coicis* and *A. ochraceus* exhibited low levels of constitutive 7α-dehydroxylase activity on cholic acid and its conjugates. However, the expression of such activity in whole-cell cultures was observed only with *C. coicis* using glycocholic acid as substrate. Thus, with *C. coicis*, deoxycholic acid was detected from glycocholic acid at a maximum yield of 3%. Hence a fungal process to convert cholic acid to the apparently more valuable deoxycholic acid is not presently feasible using the fungi studied.

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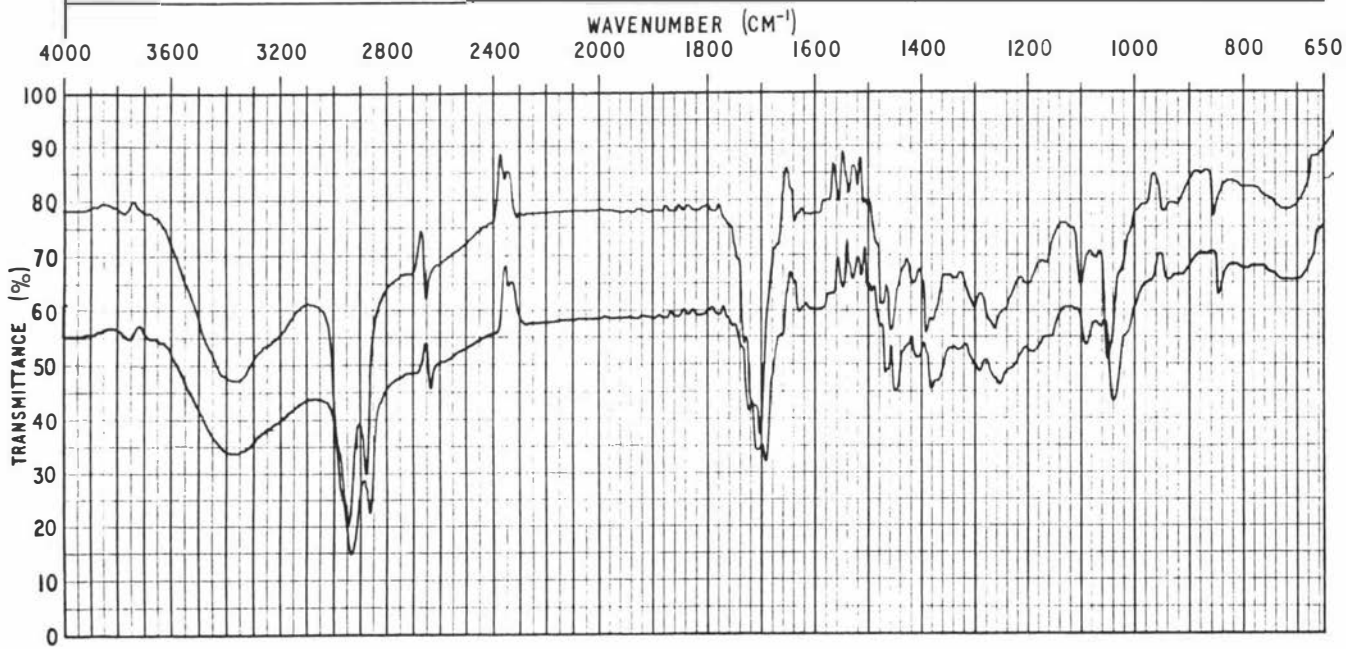
NO. 5-100-0309

REMARKS	ORIGIN	PERKIN-ELMER
Characterisation of the product of hydrolysis of glycodeoxycholic acid by *C. melonis* CBS 162.60. P.E. 720 Normal slit width Slow scan.	PURITY Recrystallised from water-saturated methyl ethyl ketone and dried. PHASE Solid, as KBr Disc CONCENTRATION 1% w/w in 75mg KBr THICKNESS DATE OPERATOR	SPECTRUM NO. SAMPLE 1 Upper line: Deoxycholic acid, authentic SAMPLE 2 Lower line, Deoxycholic acid from glyco-deoxycholic acid.



NO. 5-100-0309

REMARKS
Characterisation of the product of hydrolysis of glycocholic acid by *C. melonis* CBS 162.60.

P.E. 720

Normal slit width

Slow scan

ORIGIN

PURITY Recrystallised from 2-phase chloroform-water and dried.

PHASE Solid, as KBr Disc

CONCENTRATION 1% w/w in 75mg KBr

THICKNESS

DATE

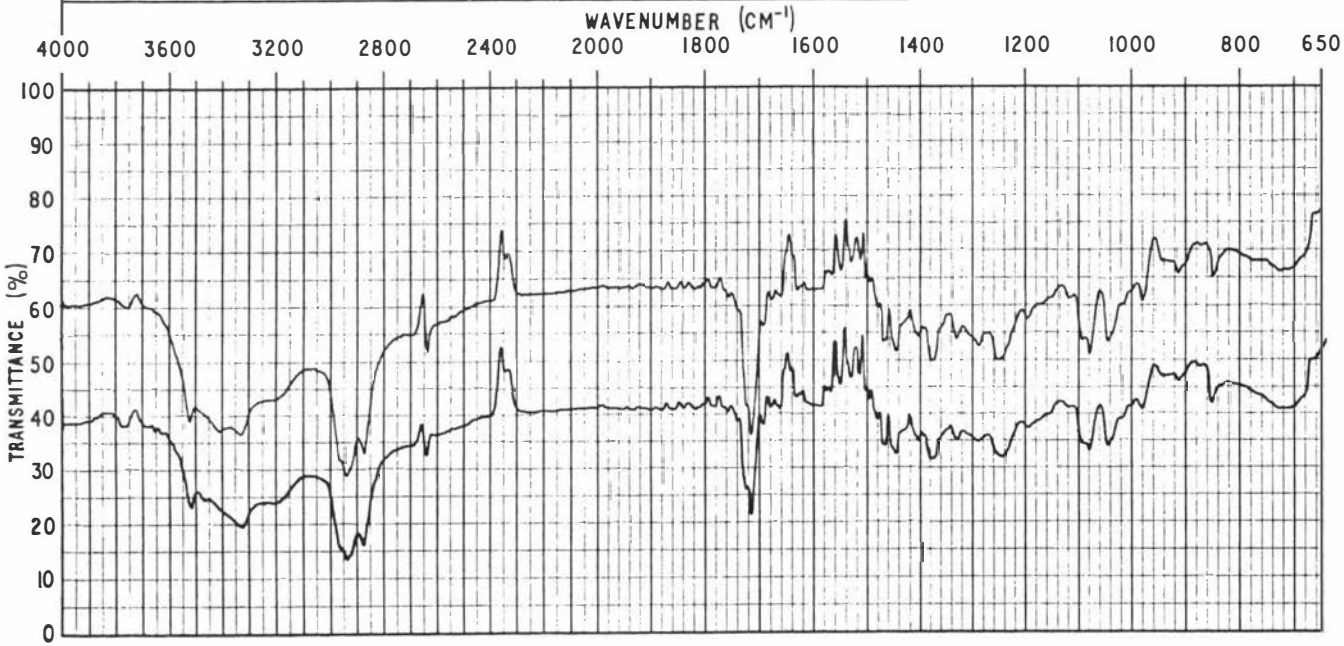
OPERATOR

PERKIN-ELMER

SPECTRUM NO.

SAMPLE 1 Upper line:
Cholic acid, authentic

SAMPLE 2 Lower line:
Cholic acid from glycocholic acid.



NO. 5-100-0309

REMARKS

Characterisation of the product of hydrolysis of glycocholic acid by *C. coicis* IFO 7278.

P.E. 720

Normal slit width

Slow scan

ORIGIN

PURITY Recrystallised from aqueous ethanol and dried.

PHASE Solid, as KBr Disc

CONCENTRATION 1% w/w in 75mg KBr

THICKNESS

DATE

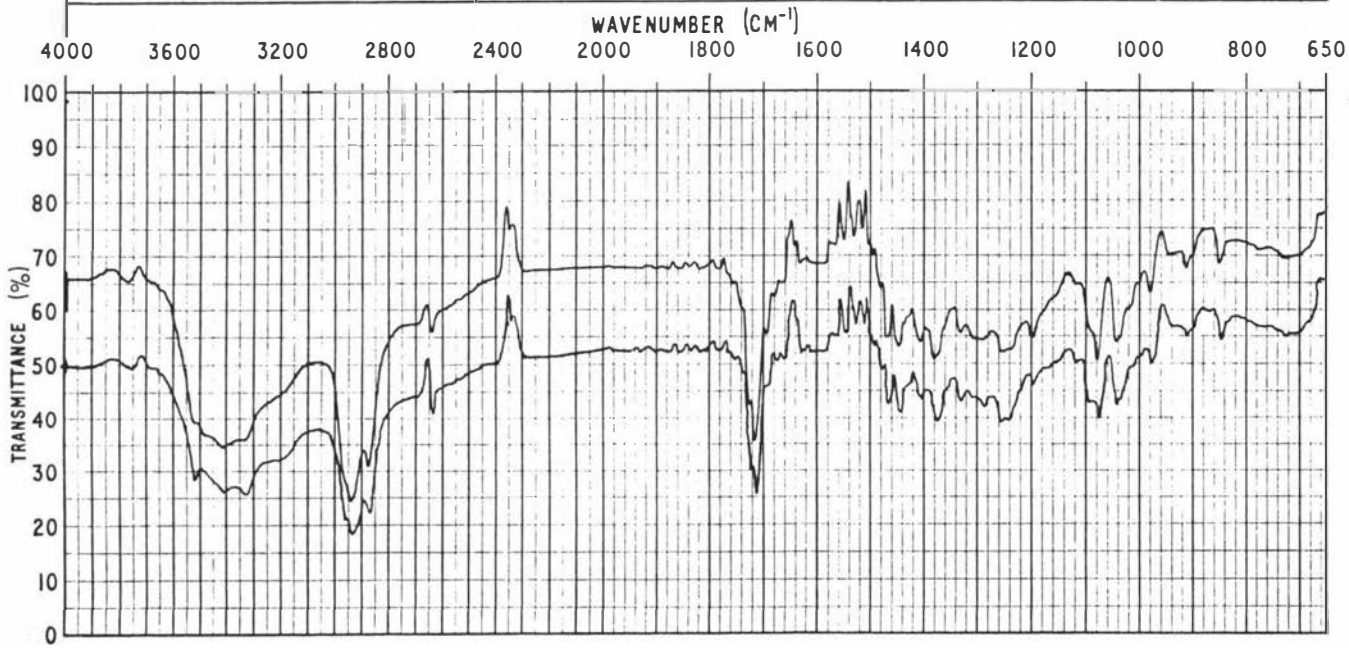
OPERATOR

PERKIN-ELMER

SPECTRUM NO.

SAMPLE 1 Upper line: Cholic acid from glycocholic acid.

SAMPLE 2 Lower line: Cholic acid, authentic



APPENDIX 2.

The Determination of Bile Acid Partition Coefficients

(Refer Table 3.7).

The bile acid (25.0 mg) was dissolved in dilute sodium hydroxide (50 ml) and acidified to pH 2 with 5M hydrochloric acid. The solution was shaken for 0.5 minutes with chloroform (50 ml) in a separating funnel and allowed to equilibrate for 1 hour. The chloroform layer was then evaporated to dryness *in vacuo* and the residue analysed for bile acid by hplc. The concentration of the bile acid in the aqueous phase was calculated as the difference between the initial weight of bile acid dissolved in the distilled water and the weight of bile acid detected in the chloroform phase by hplc. The partition coefficient, D, was calculated as the mean of quadruplicate determinations, excepting glycocholic acid.

$$D = \frac{A}{B}$$

where:

A, is the weight of bile acid in the aqueous phase (mg)
B, is the weight of bile acid in the chloroform phase (mg)

Recoveries of the bile acids from an aqueous solution could be predicted using the equation,

$$\frac{X_c}{X_o} = 1 - \left(\frac{DV}{DV+v}\right)^n \qquad \text{(Vogel, 1962)}$$

where:

X_c, is the weight of bile acid in the chloroform phase (mg).
X_o, is the initial weight of bile acid in the aqueous phase (mg).
D , is the partition coefficient.
n , is the volume of the chloroform phase (ml).
v , is the volume of the chloroform phase (ml).
V , is the volume of the aqueous phase (ml).