HYDROLYSIS OF BILE ACID CONJUGATES AND DEHYDROXYLATION OF CHOLIC ACID BY CLOSTRIDIUM BIFERMENTANS

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ABSTRACT

The transformation of bile acids by *Clostridium bifermentans* was studied with a view to developing a process whereby the bile acid conjugates of New Zealand mutton and beef gall may be converted to deoxycholic acid.

Statistically designed experiments were employed to maximise 7α-dehydroxylation of cholic acid to deoxycholic acid and to minimise the 7α-dehydrogenation of cholic acid to 7-ketodeoxycholic acid. Both transformations showed optima near pH 7. High deoxycholate yields were associated with conditions less favourable to strong growth and with relatively high electrode potentials. 7-ketodeoxycholic acid production was not as sensitive to environmental factors as was 7α-dehydroxylation and could not be eliminated merely by manipulating fermentation variables.

Studies on the 7α-dehydrogenation of cholic acid with washed resting-stage cells of *Cl. bifermentans* indicated several means of manipulating 7-ketodeoxycholate yields which were then tested using batch fermentation. In the presence of Zn^{++}ions, 7-ketodeoxycholate yields were reduced but dehydroxylation was completely inhibited. In the presence of EDTA, 7α-dehydrogenation was almost quantitative but deoxycholate yields were again nil. Both transformations were enhanced during aerobic incubation. The highest deoxycholate yield observed during the work (50 molar %) was obtained by sweeping the fermenter headspace with air.

Growing cells of *Cl. bifermentans* effected the near-quantitative hydrolysis of glycodeoxycholate, taurodeoxycholate and taurocholate within 48 h whilst glycocholate was 90% deconjugated. At substrate concentrations greater than 0.1% w/v however, taurine conjugates were less well hydrolysed.

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BILE ACID NOMENCLATURE

Throughout this work, bile acids will be referred to by the trivial names employed in "The Bile Acids" (Matschiner, 1971). These are listed below, together with the appropriate I.U.P.A.C. systematic chemical names (I.U.P.A.C. - I.U.B., 1969).

cholic acid - 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid
deoxycholic acid - 3α,12α-dihydroxy-5β-cholan-24-oic acid
chenodeoxycholic acid - 3α,7α-dihydroxy-5β-cholan-24-oic acid
ursodeoxycholic acid - 3α,7β-dihydroxy-5β-cholan-24-oic acid
lithocholic acid - 3α-hydroxy-5β-cholan-24-oic acid
7-ketodeoxycholic acid
- 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid
7-ketolithocholic acid
- 3α-hydroxy-7-oxo-5β-cholan-24-oic acid
glycocholic acid - 3α,7α,12α-trihydroxy-5β-cholan-24-oylglycine
glycodeoxycholic acid - 3α,12α-dihydroxy-5β-cholan-24-oylglycine
taurocholic acid - 3α,7α,12α-trihydroxy-5β-cholan-24-oyltaurine
taurodeoxycholic acid - 3α,12α-dihydroxy-5β-cholan-24-oyltaurine

Acids will be referred to by the suffixes "-ate" and "-oic acid" interchangeably, for example 7-ketodeoxycholic acid and 7-ketodeoxycholate.

ABBREVIATIONS

Abbreviations of bile acid names (from Eneroth and Sjövall, 1971):

C	cholic acid
D	deoxycholic acid
7KD	7-ketodeoxycholic acid
GC	glycocholic acid
GD	glycodeoxycholic acid
TC	taurocholic acid
TD	taurodeoxycholic acid

Abbreviations of units:

g	gramme
h	hour
l	litre
m	metre
M	moles per litre
min	minutes
Pa	Pascal (Newton per square metre)
rev	revolution
tonne	tonne (1,000 Kg)
s	second
V	volt

Other abbreviations:

A	coded value of pH in Experiment 1 (A = pH - 7.0)
ATCC	American Type Culture Collection
B	coded value of gas in Experiment 1 (B = 1 for H_2, B = 0 for N_2, B = -1 for N_2-CO_2, 9:1)
DMSO	dimethylsulphoxide
Ec	electrode potential relative to the saturated calomel electrode

Ec_{min}	minimum Ec observed during a fermentation
Ec_i	Ec prevailing at inoculation
$(\Delta Ec/\Delta t)_{max}$	maximum rate of decline of Ec during a fermentation
EDTA	ethylenediaminetetraacetic acid
Eh	electrode potential relative to the standard hydrogen electrode
F	ratio of mean sums of squares (i.e. F ratio)
H.P.L.C.	high performance liquid chromatography
Km	the Michaelis constant
L.M.C.	base 10 logarithm of the maximum cell count observed during a fermentation
ln	natural logarithm
log	base 10 logarithm
$\log K_1$	first formation constant of chelate complex
MS_{lof}	mean sum of squares due to lack of fit
MS_{pe}	mean sum of squares due to pure error
N	number of independent variables in an experiment design
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NCIB	National Collection of Industrial Bacteria
pCMB	parachloromercuribenzoate
R_f	T.L.C. mobility relative to solvent front mobility
T.L.C.	thin-layer chromotography
Tris	tris(hydroxymethyl)methylamine
$\hat{Y}$	estimated value of response variable being modelled

CHAPTER 1 PRELUDE

Currently in New Zealand, 140 tonnes of mutton and beef gall (75% solids), byproducts of the meat slaughtering industry, are processed annually. The component conjugated bile acids undergo severe alkaline hydrolysis and the resulting free bile acids are refined to realise $NZ 2 million in export earnings. Recently, alkaline hydrolysis, particularly of taurine conjugates, has been shown, on the 1 mg scale, to result in substantial loss of steroid material, thus limiting yields (Roseleur and van Gent, 1976). Experiments on the gram scale have shown 95% recovery of free cholic acid after the alkaline hydrolysis of glycocholic acid and 80-85% for taurocholate (Garland,1977). Losses of this magnitude are regarded as economically significant.

It was considered that if the cholanoylglycine and cholanoyltaurine hydrolase enzymes possessed by some micro-organisms could be effectively harnessed, a mild hydrolysis process might be developed offering potentially higher yields. Already conjugate hydrolases, prepared from clostridia, are commercially available as acetone powders for small-scale analytical deconjugation.

The ratio of cholic acid conjugate to deoxycholic acid conjugate in New Zealand mutton gall is *ca* 4:1. At the commencement of this project in 1977, deoxycholic acid had a market price of $30/kg and cholic acid $25/kg. The main aims of this work therefore were to develop a microbial process to hydrolyse the bile acid conjugates of mutton and beef gall, then to 7α-dehydroxylate the resulting cholic acid fraction to deoxycholic acid. It was also hoped that the investigation would add to current understanding of bile acid transformation.

Two major approaches were possible: firstly, the biochemistry could be studied in detail, each enzyme being isolated and characterised individually; secondly, the cell could be treated as a "black-box", environmental and chemical influences being investigated with intact cells. The second approach was adopted for several reasons:

(a) the availability of facilities and supervisory expertise,
(b) the reported instability of several of the isolated enzymes,
(c) the development gap between the biochemical approach and the envisaged industrial application, and

(d) the fact that a number of laboratories are already collecting the biochemical data and publishing in the cancer research literature.

By using an instrumented laboratory fermenter, it was hoped to provide a degree of control over the environment of growing pure cultures not available to other investigators in the field. This approach has an analogue in the intestine where, through the mediation of the gut flora, the same reactions proceed to completion with a minimum of side-product formation. While a simulation of gut conditions would not be attempted, it was hoped to identify some of the environmental factors which select for deconjugation and dehydroxylation while depressing competing dehydrogenations.

CHAPTER 2 INTRODUCTION AND LITERATURE REVIEW

This review seeks to organise a wealth of information gathered over half a century of bile acid research, into a form applicable to fermentation studies.

Since microbial bile acid transformations occur naturally in the intestinal tract of animals, much has been published in the medical and scientific journals, but very little in the technical or patent literature. Most workers have been interested in physiology (Midtvedt,1974), gall stone formation (Bokkenheuser *et al.*,1977), general steroid transformations (Carini *et al.*,1967), cancer research (Hill and Drasar, 1975; Macdonald and Rao, 1979; Owen *et al.*,1977; Hylemon and Stellwag, 1976), elucidation of aerobic bile acid metabolism (Hayakawa, 1973), and development of enzymatic analytical and diagnostic techniques (Macdonald *et al.*,1975a; Roseleur and van Gent, 1978; Yamamoto *et al.*, 1977).

In contrast, recent industrially-oriented reports of microbial bile acid transformation are rare: a patent issued to W.H. Saltzman (1975) and the 5-litre scale aerobic fermentation of cholic acid reported by Severino *et al.* (1969). Saltzman claimed that a particular strain of *Clostridium perfringens* can dehydroxylate a range of cholic acid derivatives to the corresponding chenodeoxycholate derivative in near quantitative yield. However, when the same strain and conditions as specified in the patent example were tested in this laboratory, no dehydroxylation was observed (Maddox, 1979).

In applying micro-organisms to the processing of mutton gall into deoxycholic acid, three groups of enzymes are important:

(a) the hydroxycholanoyl dehydroxylases, specifically the 7α-dehydroxylase,

(b) the competing hydroxycholanoyl dehydrogenases, particularly the 7α-dehydrogenase, and

(c) the conjugate hydrolases: cholanoylglycine and cholanoyltaurine hydrolase.

The literature on these three enzyme groups will be reviewed separately.

2.1 DEHYDROXYLATION

During their passage through the intestinal tract of animals, the primary bile acids are partially 7α-dehydroxylated to the secondary bile acids prior to reabsorption or passage in the faeces. (Drasar and Hill, 1974; Kellogg, 1973).

Even before any of the organisms responsible for dehydroxylation had been identified, Samuelsson (1960b) elucidated the mechanism of dehydroxylation *in vivo*. Having previously discounted the possibility of the 7-ketone acting as an intermediate (Bergström *et al.*, 1959), Samuelsson used a double labelling technique to demonstrate an initial diaxial *trans*-elimination of the 7α-hydroxyl group and the 6β-hydrogen atom yielding 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid, followed by *trans*-hydrogenation of the 6α and 7β positions to give deoxycholic acid. Not until 1977 did Ferrari *et al.* perform isotope trapping experiments with a cell-free extract of *Cl. bifermentans* which led them to conclude that the 6-ene intermediate is also involved in the *in vitro* 7α-dehydroxylation of cholic acid.

Over the period 1950 to 1970 a great deal of work was performed with germ-free and mono-contaminated laboratory animals. Mixed cultures of faecal isolates were also prepared and investigated. Much of this work has been well reviewed by Hayakawa (1973) and Midtvedt (1974).

In 1962, Portman *et al.* published the first report of a single faecal isolate capable of 7α-dehydroxylating cholic acid *in vitro*. Their organism, however, lost its activity after two transfers. Hayakawa and Kurokawa (1963) reported that 7α-dehydroxylated metabolites are formed from cholic acid by *Corynebacterium simplex*. Hattori and Hayakawa (1969) later isolated and identified a strain of *Bacteroides* from human faeces which 7α-dehydroxylated cholic acid. In 1966 Gustafsson *et al.* isolated eight strains of faecal anaerobes capable of forming lithocholic acid from chenodeoxycholic acid. These isolates were later identified as being of the tribe Lactobacilleae, and the activity of one strain was further characterised (Midtvedt, 1967; Midtvedt and Norman, 1968). Ferrari and Cocucci (1967) isolated from a highly selected mixed culture of human faecal organisms strains of *Cl. bifermentans*, *Escherichia coli* and *Pseudomonas aeruginosa* which all possessed dehydroxylase activity.

These organisms lost activity with successive subcultures, but retained activity in the mixed culture. The converse was observed by Hill and Drasar (1968) who found isolates of *Veillonella* which gained the ability to 7α-dehydroxylate after repeated subculturing in bile medium.

Carini *et al.* (1967) attempted to clarify the reasons for the loss of activity by fresh isolates, investigating the rôle of anaerobiosis, growth phase, development, vitamins, trace elements, energy sources, metabolic activators, and microbial filtrates of mixed cultures of intestinal micro-organisms. However, they were unsuccessful, concluding that the complex of physical, chemical and biological factors necessary for 7α-dehydroxylation is present *in vivo*, and can only be reproduced *in vitro* with mixed cultures. Carini also considered it "not unbelievable to think of a direct or indirect interference from viral agents." A decade later Tenneson *et al.* (1979) postulated a link between steroid degradative ability and either "stable" plasmids (activity was lost on mitomycin treatment) or non-compatible plasmids (activity was lost when selective pressure was removed, i.e. grown in steroid-free medium). These authors did not consider 7α-dehydroxylation, and worked with aerobic bacteria. The possibility exists, however, that the 7α-hydroxycholanoyl dehydroxylase of intestinal anaerobes is coded by plasmid material. Tenneson *et al.* (1979) considered deconjugating and dehydrogenating abilities to be coded on the main chromosome.

Since the first isolation and characterisation of a pure culture possessing a 7α-hydroxycholanoyl dehydroxylase, many isolates have been shown to catalyse the transformation. Hill and Drasar (1968) screened many isolates of faecal, intestinal and oral organisms for the activity. They found positive results in two strains of *Streptococcus faecalis* out of the 276 strains of facultative anaerobes tested, 8 out of the 237 strains of *Bacteroides*, 12 out of the 16 strains of *Clostridium*, and 8 out of the 47 strains of *Veillonella*. No activity was shown by 58 pseudomonads, 12 microaerophilic lactobacilli, 45 yeasts or 89 strains of *Bifidobacterium* spp. Aries *et al.* (1969) tested 60 clostridial faecal isolates from native Ugandans and 70 from inhabitants of the United Kingdom, finding 43% and 47% showed dehydroxylation activity respectively. These workers have also found activity in international type cultures (Aries and Hill, 1970b) and have concluded that although the dehydroxylase has not been detected in a high proportion of strains,

the capacity to produce the enzyme is widely distributed (Drasar and Hill, 1974; Hill *et al.*,1971). In contrast, Midtvedt (1974) considered that most authors have found the capability to be rare amongst intestinal organisms, citing Dickenson *et al.* (1971), who found no activity among 24 intestinal isolates belonging to 22 genera, and his own work (Midtved- and Norman, 1967) where none was found among 55 type stra ns of common intestinal organisms. Similarly Suzuki (1970) reported no activity in many strains isolated from the digestive tract of the mouse.

The approach of Midtvedt and Norman (1967) was adopted by Hayakawa and Hattori (1970), who demonstrated activity in both type cultures tested, *Cl. bifermentans* ATCC 9714 and *Cl. sordellii* NCIB 6929. Activity was demonstrated without the need for several subcultures in bile medium, shown by Hill and Drasar (1968) to elicit the activity in oral strains of *Veillonella*.

Very recently, a fungus has been shown to form deoxycholic acid from glycocholic acid. This novel discovery was made in this laboratory with *Curvularia coicis* (Johns, 1980).

Since much of the literature is medically-oriented and qualitative, yield data have not always been published. From those reports where data have been presented, a strong substrate concentration effect is apparent. The reports concerning growing cells are summarised in Table 2.1 overleaf. In addition, Aries and Hill (1970b) have studied the cell-free dehydroxylases of *Bacteroides*, *Cl. welchii*, *E. coli*, *Strep. faecalis* and two other clostridia and reported they were inhibited by a concentration of 6.7 mM bile acid. Drasar and Hill (1974) reiterated these and similar reports, saying "the enzyme is inhibited by substrate concentrations in excess of 6 mM (i.e. in excess of the normal level found in human faeces)."

Deoxycholate yields of up to 90% have been reported for other mixed cultures (Ferrari, 1967; Ferrari and Cocucci, 1967). Some very high dehydroxylation yields have also been observed for freshly isolated pure strains: 100% for *Cl. bifermentans*, for *E. coli* and for *Ps. aeruginosa* (Ferrari and Cocucci, 1967) and for Portman's short Gram negative rod (Portman *et al.*,1962). However, these strains all lost the activity within four or five transfers.

TABLE 2.1 7α-Dehydroxylation Yields Previously Published in the Literature.

Dehydroxylation Substrate	Concentration (mM)	Yield	Reference
cholic acid	0.01	92%	Midtvedt and Norman (1968) [a]
	0.10	27%	
	0.20	23.5%	
	0.40	18%	
	0.60	10.5%	
cholic acid	0.01	90%	Gustafsson *et al.* (1966) [a]
	0.10	19%	
	0.20	9%	
	0.40	5%	
	0.60	3%	
Na cholate	2.32 (0.1% w/v)	0%	Hayakawa and Hattori (1970)
	0.58 (0.025% w/v)	>0%	
cholic acid	0.37 (0.015% w/v)	100%	Portman *et al.* (1962) [b]
	2.45 (0.1% w/v)	50%	
cholic acid	.015 (6 µg/l)	39%	Bokkenheuser *et al.* (1969) [c]
	.061 (25 µg/l)	35%	
	.25 (100 µg/l)	33%	
	.98 (400 µg/l)	31%	

a Lactobacillus, 37°C, 7-day incubation

b Mixed caecal culture

c Isolate FA 1/146, 7-day incubation

Using their *Cl. bifermentans* SD 10 isolate, Ferrari and Aragozzini (1972) incubated 100 mg of cholic acid for 5 days, then isolated 26 mg of deoxycholic acid, 10 mg of 7-ketodeoxycholic acid and 23 mg of unreacted cholic acid. Hattori and Hayakawa (1969) recovered deoxycholic acid representing an 11.9% yield, and 85.5% unreacted cholic acid (7-ketodeoxycholate showed on thin-layer chromatography as a faint spot).

Most 7α-hydroxycholanoyl dehydroxylases studied have showed equal activity on cholic and chenodeoxycholic acids (Aries and Hill, 1970b; Drasar and Hill, 1974). Using growing cells of their strain II, Midtvedt and Norman (1968) showed that the two 7α-hydroxy acids were dehydroxylated equally when present together, but that more deoxycholate was formed than lithocholate when incubated separately. In complete contrast, Ferrari and Beretta (1977) reported the cell-free enzyme of *Cl. bifermentans* showed very little activity on chenodeoxycholic acid even if this bile acid had been present during cell production.

No *in vitro* study to date has demonstrated dehydroxylation of conjugated bile acids (Midtvedt, 1974; Drasar and Hill, 1974). However, glycocholate can be dehydroxylated without prior deconjugation *in vivo* in Man (Hepner *et al.* 1972, 1973).

The 7α-hydroxycholanoyl dehydroxylase is extremely labile, both in cell-free extracts and in whole cells, although some cell-free preparations have been made (Aries and Hill, 1970b; Macdonald *et al.* 1977; Ferrari and Beretta, 1977; Ferrari *et al.* 1977). Indeed Ferrari and coworkers report having electrophoretically separated the dehydroxylase activity of *Cl. bifermentans* SD 10 from two distinct dehydrogenases. However, this author makes no mention of dehydroxylase cofactor requirements (as yet unknown) despite having demonstrated activity in the highly refined enzyme.

Hydroxycholanoyl dehydroxylases which are active on the C-3 or C-12 hydroxyl groups have been rarely reported; certainly no such enzyme has been reported to have been isolated. Hayakawa and Samuelsson (1964) observed 12α-dehydroxylated metabolites amongst the degradation products of cholic acid after aerobic incubation with *Corynebacterium simplex*. However, these authors could not conclude whether removal of the 12-hydroxyl group occurred before or after ring A cleavage. Hill and Drasar (1967) reported observing the production of lithocholic acid from cholic

acid by intestinal isolates, but could not reproduce the reaction (Drasar and Hill, 1974). These workers further observed what may have been 5ξ-cholan-24-oic acid, indicating 3α-dehydroxylation had also occurred. Finally, Saltzman (1975) has claimed in a patent that cholic acid derivatives can be extensively 12α-dehydroxylated to the appropriate chenodeoxycholic acid derivatives through the agency of *Cl. perfringens*.

In summary, 7α-dehydroxycholanoyl dehydroxylase activity has been observed in a number of intestinal anaerobes, and a similar enzyme in some aerobic soil organisms. The activity is unstable both in cell-bound and cell-free states; pure cultures have been shown to lose or gain the ability with serial transfers. Yields of 7-deoxy product may be very high *in vivo*, in mixed cultures or with fresh isolates, and are lower at increased substrate concentrations. The few cell-free extracts prepared have shown pH optima near neutrality. Dehydroxylation of C-3 or C-12 hydroxyl groups of intact bile acids has been rarely reported.

2.2 DEHYDROGENATION

The long history of observed microbial oxidation-reduction of bile acids from the pre-1940 *in vitro* studies, through the large screening exercises performed by the British and Scandanavians in the 1960s, to the cell-free enzyme studies of the early 1970s has been well reviewed by Hayakawa (1973), Midtvedt (1974) and Drasar and Hill (1974). Several points are clear from these reviews:

(a) Organisms effecting the oxidation-reduction of bile acids are widespread in intestinal flora, including species of *Clostridium*, *Bacteroides*, *Eubacterium*, *Bacillus*, *Alkaligenes*, *Proteus*, *Lactobacillus*, *Ramnibacterium*, *Spherophillus*, *Streptococcus*, *Endosporus*, *Enterobacterium*, *Escherichia*, *Bifidobacterium* and *Pseudomonas*.

(b) Oxidation-reduction can be effected at the C-7, C-12 and C-3 positions, often by the same organism and often in the order given, resulting in di- and tri-keto compounds.

(c) Unlike dehydroxylation, dehydrogenation is reversible, with both epimeric hydroxyl derivatives sometimes being formed (although 7β-hydroxyl formation has not been observed *in vitro*).

(d) The extent of oxidation of conjugates or methyl esters of bile acids is usually small. (This conclusion must be modified in the light of later work, however.)

More recently, a number of reports have appeared characterising various isolated 3α, 7α and 12α-hydroxycholanoyl dehydrogenases, allowing a reasonable picture of the enzymes to be drawn up.

2.2.1 7α-Hydroxycholanoyl Dehydrogenases

Although Portman *et al.* (1962) obtained a cell-free extract of *E. coli* capable of producing 7-ketodeoxycholate from cholate in 1962, the first major study of cell-free 7α-dehydrogenases was performed by Aries and Hill (1970b). These authors found a NADP-dependent dehydrogenase in two strains of *Cl. welchii* with optima at pH 9.8-10.2 and with equal activity on cholic acid and chenodeoxycholic acid. However, their Km values were markedly different. A similar degree of activity, with identical substrate specificity, was extracted from *E. coli*. This enzyme was NAD-dependent with an optimum of pH 9.0-9.4. NAD-dependent enzymes were also extracted from two *Bacteroides* strains. These extracts showed 5-10% of the activity of the other anaerobes, with optima at pH 8.8-9.3 and 50% greater activity toward chenodeoxycholate than cholate. The *Bacteroides* enzyme was more heat stable and less prone to substrate inhibition than the clostridial enzyme. Both had reduction optima at pH 6.8-7.0. These workers also described the 3α and 12α-oriented activities in their extracts, and drew some general conclusions for hydroxycholanoyl dehydrogenases: the enzymes are inducible, activity increasing 70 to 95% when cells are grown in the presence of 0.01% cholate; little activity is shown on methyl esters, glycine or taurine conjugates, and the reaction always requires a nicotinamide cofactor and molecular oxygen.

In 1973 Macdonald *et al.* purified the NAD-dependent 7α-dehydrogenase of a named *E. coli* strain, finding it to be 7α-stereospecific, non-inducible and active on both conjugated and free bile acids.

The following year, Macdonald *et al.* (1974) screened cell-free extracts of 25 strains of *E. coli* for NAD-dependent 7α-hydroxycholanoyl dehydrogenase activity for use in the determination of 7α-hydroxy steroids (as the 3α-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* is used for total 3α-hydroxy bile acid determination). The activity was found in 96% of the strains tested.

In 1975, two groups independently published the results of screening studies of *Bacteroides fragilis* for 7α-hydroxycholanoyl dehydrogenases, each reporting a labile NADP-dependent activity together with the stronger NAD-dependent enzyme observed earlier by Aries and Hill (1970b). Macdonald *et al.* (1975b) demonstrated NAD-dependent dehydrogenase in 18 of the 20 strains tested. Of these 18, 16 also showed NADP-dependent activity which was more pronounced in the decline phase of growth. Both activities were non-inducible and 7α-stereospecific. Hylemon and Sherrod (1975) found both activities in most of the 9 strains of *B. fragilis* tested, and showed the NADP-dependent activity to be thermolabile and Mn^{++}-dependent, with an optimum at pH 8.5-9. The NAD-dependent enzyme had the same oxidation pH optimum but no metal ion requirement. Both activities increased into the stationary phase of growth, specific activity being low until glucose was depleted from the medium. This led the authors to speculate on an energy metabolism-linked rôle for dehydrogenation once other oxidisable medium compounds were expended. In contrast to Macdonald *et al.* (1975b), Hylemon and Sherrod (1975) found both activities were stronger if cholic acid was present during cell growth.

Macdonald *et al.* (1976) reported that the NADP-dependent 7α-dehydrogenase of *Cl. perfringens* is constitutive rather than inducible.

Midtvedt and Norman (1968) investigated dehydrogenase location in their lactobacillus-like isolate, and concluded that the enzyme was intracellular. Hylemon and Stellwag (1976) also investigated 7α-dehydrogenase location in *B. fragilis* and discovered a doubling of activity upon lysozyme treatment. Further, spheroplasts of *Eubacterium lentum* were 100-fold more active than the growing cells. Upon lysis, both activities were tightly membrane-bound.

In summary, the reports of 7α-dehydrogenase enzymes published over the last decade have indicated two principle enzyme types: an NAD-dependent and a less robust NADP-dependent activity. Some organisms possess both. The literature includes reports of constitutive and of inducible enzymes. Most have optima (in cell-free extracts) near pH 9, and are equally active on conjugated and free bile acids.

2.2.2 3α-Hydroxycholanoyl Dehydrogenases

Enzymic dehydrogenation of the 3α-hydroxyl group of bile acids has been well studied using the highly active 3α-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni*. This enzyme is available commercially, and is employed in several published assays for total bile acids (Bilton *et al.* 1977; Barnes *et al.* 1976; Mashige *et al.* 1976; Schwartz *et al.*, 1974; Sheltaway and Losowsky, 1975; Macdonald *et al.* 1975a). A similar enzyme, the 3α-hydroxysteroid dehydrogenase of *Flavobacterium dehydrogenans* has been applied to oxidation of the cholic acid 3α-hydroxyl group by Ferrari and Giannattasio (1970). *Mycobacterium mucosum* has also been shown to produce the activity (Severino *et al.*, 1969). Current research in bowel cancer should result in more publications since the introduction of a 3-keto moiety into the A-ring is a necessary prerequisite for Δ^1 and Δ^4 dehydrogenation yielding the unsaturated bile acid metabolites suspected of being carcinogens.

As for other dehydrogenases, most investigations of whole cell transformation have been screening exercises, while closer investigation of the activity has been with cell-free extracts. Hayakawa (1973) and Midtvedt (1974) have reviewed these screens showing that 3α-dehydrogenases are wide-spread amongst intestinal bacteria, including several species of *Clostridium*. Most 3-dehydrogenases are not stereo-specific, and the reduction of the 3-keto moiety to a 3β-hydroxyl is common but not universal.

In 1955 Hayaishi *et al.* isolated a crude NAD-dependent enzyme from *Escherichia freundii* which was active on only 3α-hydroxyl, 24-carboxyl bile acids. The next major study reported was that of Aries *et al.* (1970b), who prepared cell-free extracts of two strains of *Cl. welchii*, which were active on both the C-3 and C-7 hydroxyl groups of cholic acid and chenodeoxycholic acid. They showed an oxidation pH optimum on the C-3 hydroxyl of pH 10-10.5 and a reduction optimum of pH 6.4-7.8. A similar degree of 3α-dehydrogenase activity was also extracted from a *Bifidobacterium* species. This enzyme or enzyme mixture was active on both the C-3 (oxidation optimum pH 10.5-11.2, reduction optimum pH 6.4-7.8) and the C-12 hydroxyl group of cholate and deoxycholate. These enzymes were 3α-stereospecific and had a requirement for a 5β configuration and a 24-carboxyl group.

Macdonald *et al.* (1976) reported that 19 of 25 strains of *Cl. perfringens* possessed NADP-dependent 3α-hydroxycholanoyl dehydrogenase. The enzymes were constitutive with activity parallelling the growth curve, and showed a very sharp optimum at pH 11.3. They were active on both conjugated and free bile acids, on both 5α-H and 5β-H steroids, and were stereospecific for a 3α-hydroxy substituent.

In 1977 Macdonald *et al.* characterised a very stable NAD-dependent 3α-dehydrogenase from *Eubacterium lentum*. These authors found the activity to be very similar to those of *Ps. testosteroni* and *Cl. perfringens*, with an optimum at pH 11.3. It, too, was active on both conjugated and free bile acids, and on both 5α-H and 5β-H steroids, but was 3α-stereospecific. Enzyme production parallelled growth and was non-inducible; in fact activity decreased when cholate, deoxycholate and particularly chenodeoxycholate were present during growth. In contrast, Bilton *et al.* (1977) showed the *Pseudomonas* enzyme to be deoxycholate-inducible, since cells grown in the presence of that bile acid could oxidise several substrates rapidly without a lag, whereas uninduced cells showed lags of up to 10 h.

In summary, despite its importance in the catabolism of bile acids to possible intestinal carcinogens, the 3α-hydroxycholanoyl dehydrogenases of intestinal organisms have, until recently, been little studied. Several activities have now been partially characterised. These show differing similarities to the potent 3α-hydroxysteroid dehydrogenase of *Pseudomonas*. The enzymes of intestinal organisms may be either NAD- or NADP-dependent, and may or may not have a requirement for a C-24 carboxyl group. Most have optima near pH 11, and many are constitutive.

2.2.3 12α-Hydroxycholanoyl Dehydrogenases

Prior to 1974, the 12α-oriented enzyme was the least observed bacterial hydroxycholanoyl dehydrogenase (Drasar and Hill, 1974). In the only major study to that date, Aries *et al.* (1970b) had extracted a dehydrogenase from a *Bifidobacterium* species which was active on both the C-3 and C-12 positions. The pH optimum was pH 8.2-8.5 for the oxidation, and pH 5.8-6.4 for the reduction.

In 1976 Macdonald *et al.* observed an NAD-dependent 12α-hydroxycholanoyl dehydrogenase in 8 of 25 strains of *Cl. perfringens* tested. All activities were contaminated with a stronger 3α-dehydrogenase.

Both enzymes were constitutive and active against both conjugated and free bile acids. The C-12-oriented activity had a pronounced optimum at pH 10.5. The following year, Macdonald *et al.* (1977) reported a very similar NAD-dependent 12α-dehydrogenase from *Eubacterium lentum* with an optimum at pH 10.2. Again it was always associated with the C-3-oriented activity, and was active on both conjugated and free bile acids. The enzyme was not inducible; in fact, the presence of bile acid in the growth medium decreased enzyme yield, even when growth was not depressed.

In 1979 Macdonald *et al.* succeeded in detecting a 12α-hydroxycholanoyl dehydrogenase in an organism (*Clostridium* group P. strain ATCC 29733) which possessed no 3α- or 7α-oriented activity. The production of this NADP-dependent enzyme parallelled growth exactly and was constitutive. However, the presence of 1 mM dihydroxy bile acid in the growth medium inhibited enzyme production by 100% and growth by 90%. This enzyme showed an optimum at pH 7-8 and was active on both free bile acids and conjugates, with lower Km values for deoxycholate than cholate substrates. Harris and Hylemon (1978) produced a similarly uncontaminated 12α-oriented NADP-dependent enzyme preparation by purifying a *Cl. leptum* extract. This was again active on both free bile acids and glycine and taurine conjugates, with lower Km values for dihydroxy conjugates. The pH optimum was a little higher at pH 8.5-9.0. The activity was not induced by 0.1 mM cholic acid and there was no change in activity with growth phase.

Macdonald *et al.* (1978c) have also reported strong extractable NADP-dependent 12α-dehydrogenase activity in cell free extracts of mixed faecal cultures taken from three groups of American subjects, indicating that the NADP-dependent activity is probably more significant *in vivo* than is the NAD-dependent activity.

In summary, the 12α-hydroxycholanoyl dehydrogenase usually exists as a weaker activity in conjunction with 7α- and 3α-oriented enzymes. Optima (pH 7-9 for the NADP-dependent activity, pH 10-11 for the NAD-dependent activity) are still alkaline but generally lower than those of other oxidative activities. While distinct NAD- and NADP-linked enzymes exist, they are homogenous with respect to substrate specificity and in their ability to transform both free bile acids and conjugates. The enzymes are constitutive and are usually produced parallel to growth. The presence of bile acid in the growth medium can impair 12α-dehydrogenase production.

2.3 CHOLANOYLGLYCINE AND CHOLANOYLTAURINE HYDROLASES

The carbon-nitrogen bond of bile acid conjugates is unique in its great resistance to alkaline hydrolysis and to common proteolytic enzymes (Nair, 1973; Nair *et al.*, 1967). The conjugate hydrolases are a group of enzymes distinct from proteases and until recently were considered almost unique to intestinal organisms. In the intestine, bile acids are excreted as glycine or taurine conjugates and a proportion is re-absorbed by active transport in the terminal ileum (Lack and Weiner, 1973; Drasar and Hill, 1974). Others are hydrolysed and the free bile acid absorbed by passive diffusion in the colon. Any bile acids not absorbed are usually completely deconjugated (Midtvedt, 1974). In contrast, all faecal bile acids of germ-free animals are conjugated (Dickenson *et al.*, 1971; Kellogg, 1973).

Due to the obvious physiological importance of the reaction, many reports and reviews have appeared on the subject. Shimada *et al.* (1969) reviewed the major studies to that date and included the results of their own screening work involving 110 strains of 20 anaerobes and 9 aerobes. They reported 52 strains capable of deconjugation including both of the clostridia tested. Thus during 24 h growth at 37^0C, *Cl. perfringens* completely deconjugated glycocholate, taurocholate, glycodeoxycholate and taurodeoxycholate. Under identical conditions, *Cl. paraputrificium* catalysed the hydrolysis of both cholic acid conjugates. Shimida and colleagues also collated the results from 1006 bacterial strains; this included 39 clostridia of which 29 deconjugated glycocholate and taurocholate, and of the 16 tested against deoxycholate conjugates, 15 were found to be active.

These 16 strains were those of Hill and Drasar (1968), who further investigated the 15 active organisms, finding 87% to have a cell-bound enzyme. The hydrolases of *Bacteroides* and *Veillonella* were also reported to be chiefly intracellular, whilst only 34% of 29 active strains of *Bifidobacterium* showed cell-bound activity. These authors demonstrated optima of pH 6-6.5 and pH 6.5-7 for the enzymes of *Bifidobacterium* and *Streptococcus faecalis* respectively. The latter enzymes were found to be moderately O_2-stable in comparison with those of *Bacteroides*, which were very O_2-labile.

Few screening experiments have been reported since the review by Shimada *et al.* (1969). Aries *et al.* (1969) tested 130 human faecal isolates and reported that 95% of these hydrolysed taurocholate at substrate concentrations of both 0.05% w/v and 0.5% w/v. Dickenson *et al.* (1971) screened 124 strains from 22 genera, finding conjugate hydrolases in 83 strains representing 14 genera. Of those strains tested, 12 were clostridia; 8 of these catalysed glycodeoxycholate hydrolysis and 11 deconjugated taurocholate. These studies are included in the review of Lewis and Gorbach (1972). Gilliland and Speck (1977) screened anaerobic lactobilli for deconjugation, reporting that 19 of the 36 strains tested deconjugated glycocholate and 11 deconjugated taurocholate. These authors reported an optimum of pH 6 for deconjugation by growing cells and a requirement for anaerobic conditions. The activity was constitutive.

Recent work in this laboratory has demonstrated cholanoylglycine hydrolase activity in yeasts and fungi (Maddox and Chong, 1978). Subsequently, when *Cochliobolus intermedius* was further studied, it was discovered that it was able to grow with glycocholic acid as the sole nitrogen and carbon source. These workers have also tested a range of growing fungi against taurine conjugates, finding little activity (Chong *et al.*, 1980). In the light of most previous work, the occurrence of deconjugating activity in fungi is surprising, not only through their having no ecological link with bile acids (i.e. non-intestinal) but also because of their aerobic habit. Few other instances of aerobic deconjugation have been reported (Yesair and Himmelfarb, 1970). Most workers have used strict anaerobes and have stressed the anaerobic requirement of conjugate hydrolases(Aries and Hill, 1970a; Gilliland and Speck, 1977).

The first reported cell-free bile acid conjugate hydrolase activity was observed by Norman and Widström (1964) in the liquid phase of rat caecal and colon contents. The activity had an optimum of pH 6-7 with wide shoulders from pH 5-8. The authors concluded that the activity on taurocholate, taurodeoxycholate and glycocholate was sufficiently strong to account for all *in vivo* deconjugation.

Nair *et al.* (1965, 1967) published the first major characterisation of a cholanoylglycine and cholanoyltaurine hydrolase. The intracellular and constitutive enzyme from *Cl. perfringens* was purified 15-fold, and

showed an optimum of pH 5.6-5.8. It was active on both glycine and taurine conjugates of cholic, deoxycholic and chenodeoxycholic acids. with activity decreasing in the order dihydroxy > trihydroxy > monohydroxy. This work has resulted in the commercial availability (Sigma, Schwartz-Mann) of an acetone dried preparation of *Cl. perfringens* for analytical conjugate hydrolysis.

The effectiveness of hydrolysis using this acetone powder was compared with traditional alkaline hydrolysis by Roseleur and van Gent (1976) who obtained quantitative yields with the enzymic hydrolysis. Yamamoto *et al.* (1977) have successfully applied the same preparation to C-3, C-7 and C-12 keto bile acid conjugates, compounds which are normally unstable to alkaline hydrolysis. Yamamoto (1977) similarly hydrolysed a range of conjugates and their sulphates. More recently, Kobashi *et al.* (1978) have prepared an acetone dried powder of *Peptostreptococcus intermedius* with a strong cholanoyltaurine hydrolase activity but very little associated cholanoylglycine hydrolase. The powder was stable on storage and gave 100% yields within 1 h at 37^0C for a 0.2 mM substrate concentration.

Over the last decade, several studies have been made on a variety of isolated conjugate hydrolases. Aries and Hill (1970a) extracted activities from two strains of each of *Strep. faecalis*, *Cl. welchii*, *Bifidobacterium* spp. and *Bacteroides* spp. All except the bifidobacteria had intracellular or membrane-bound activities, while *Bifidobacterium* possessed an extra-cellular hydrolase active on glycodeoxycholate but not taurodeoxycholate. All enzymes showed optima of pH 5-6 on glycodeoxycholate except the *Strep. faecalis* extract, which had an optimum below pH 4.8. The bacteroides and clostridial enzymes were non-specific, even splitting some synthetic conjugates. The clostridial enzyme lost all activity over 4 months at -10^0C. In general, activity was greater on glycine than on taurine conjugates and greater on trihydroxy than dihydroxy conjugates. These workers also studied inhibition of the hydrolases by a range of compounds, observing (as did Nair *et al.* (1967)) inhibition by sulphydryl group inhibitors and by heavy metals.

Yesair and Himmelfarb (1970) studied six animal faecal isolates from different sources. From the aerobically-grown organisms, they extracted intracellular hydrolases which showed optima near pH 7 and

20% greater activity at 37°C than at 22°C. Hydrolysis of both glycine and taurine conjugates was complete within 24 h, but bile acids were ultimately degraded to non-steroidal products.

Hylemon and Stellwag (1976) investigated the cellular location of the conjugate hydrolases of two strains of *B. fragilis* and one *Eubacterium aerofaciens*. These authors demonstrated activity to be loosely membrane-bound, being released into the medium upon lysozyme treatment. There was greater activity in organisms harvested in stationary phase. Stellwag and Hylemon (1976) further characterised the enzyme of *B. fragilis* subsp. *fragilis*, and explained conflicting molecular weight determinations by postulating eight sub-units in the catalytically active enzyme. The optimum was pH 4.2 with the activity showing non-Michaels-Menton Kinetics on some substrates. Hydrolases were found only in those *Bacteroides* species which also possessed 7α-hydroxycholanoyl dehydrogenase activity.

Macdonald *et al.* (1976) tested cell-free extracts of *Cl. perfringens* finding 100% hydrolysis of taurocholate within 24 h for each of the 25 strains tested. In contrast, only one of 5 strains of *Cl. paraputrificum* showed such activity.

2.4 OCCURRENCE OF MULTIPLE TRANSFORMATION ABILITIES IN SINGLE ORGANISMS

In the gut, 7α-dehydroxylation is extensive, but 7α-dehydrogenation is slight. Bergström *et al.* (1959) showed that deoxycholic acid is formed from labelled cholic acid in the rabbit intestine with almost complete retention of the 7β-^{3}H label. At the same time the reduction of 7-ketodeoxycholate to cholate was found to proceed rapidly. This indicates the dehydrogenation equilibrium lies well to the reductive side *in vivo*. In contrast, Ferrari and Aragozzini (1972), who used washed and re-suspended resting cells of a pure culture of *Cl. bifermentans* SD 10, found almost complete loss of the 7β-^{3}H over 5 days' incubation with labelled cholic acid; of their recovered material, 17% was 7-ketone. Canonica *et al.* (1971) obtained similar results.

Hayakawa and Hattori (1970) discovered approximately equal quanti-

ties of 7-ketodeoxycholic acid and deoxycholic acid in cultures of *Cl. bifermentans* ATCC 9714, *Cl. sordellii* NCIB 6929 and *Bacteroides* 28S grown in Marcus and Talalay medium containing 0.025% sodium cholate. Midtvedt and Norman (1968) reported that keto derivatives were formed from cholic acid at all pH values studied, whilst dehydroxylation occurred only with an initial medium pH above 6.0. Bokkenheuser *et al.* (1969) found that 7α-dehydrogenation always accompanied 7α-dehydroxylation when their isolate FA 1/146 was grown with varying concentrations of cholic acid. Aries *et al.* (1969) found that all those bacteroides and clostridia tested which dehydroxylated also produced the 7-ketone; Stellwag and Hylemon (1978) in characterising the 7α-dehydroxylase in whole cells of *Cl. leptum* (formally *Bacteroides* 28S) reported no associated 7α-dehydrogenase or bile conjugate hydrolase activities. This contrasts with Hattori and Hayakawa (1969) who originally isolated *Bacteroides* 28S, and who found a small amount of 7-ketodeoxycholate contaminating the cholate dehydroxylase mixture. There are other reports of dehydroxylation without associated dehydrogenation. Ferrari and Pacini (1968) claimed that their mixed faecal culture converted cholate to deoxycholate quantitatively, and Ferrari and Cocucci (1967) isolated a strain of *Pseudomonas aeruginosa* and one of *Escherichia coli* which catalysed quantitative dehydroxylation. However the activity was lost after a single transfer in pure culture.

From this reading of the literature, there would appear to be no single bacterium which has been clearly shown to possess a stable 7α-hydroxycholanoyl dehydroxylase without at least one associated 7α-hydroxycholanoyl dehydrogenase. *In vitro* the oxidative activity is usually strongly expressed, except in some cases of mixed cultures or very fresh isolates. However, it would appear that 7α-dehydroxylation can occur with little concomitant oxidation at C-7 as indeed it does *in vivo*. Drasar and Hill (1974) state: "If the conditions are ideal for dehydroxylation, then very little 7-keto acid formation should take place."

The problem then is to manipulate the environment so that the appropriate combination of (unknown) factors sufficiently closely resemble the *in vivo* environment. No workers in the field have as yet attempted to use the resources of an instrumented fermenter; virtually all previous *in vitro* transformations have been performed in small bottles and flasks without pH control.

Faecal bile acids are 100% deconjugated, indicating that the bile conjugate hydrolases are widely distributed amongst intestinal organisms (Drasar and Hill, 1974). The numerous published screening studies have demonstrated this to be true, so that many organisms capable of further transforming bile acids can also hydrolyse the conjugates. Stellwag and Hyleman (1976) reported 7α-hydroxycholanoyl dehydrogenase activity in only those strains of *Bacteroides* possessing a bile conjugate hydrolase enzyme. In fact, *Bacteroides fragilis*, the most prevalent organism in human faeces, can be responsible for all the main transformations of bile acids (Hayakawa, 1973).

Aries *et al.* (1969) reported that many strains of *Bacteroides* spp., *Bifidobacterium* spp. and enterococci were able to degrade cholate and deoxycholate, and that strains of these genera could also hydrolyse bile acid conjugates. However, no enterobacteria were found able to deconjugate, even though many could metabolise free bile acids. Hill and Drasar (1968) discovered dehydroxylase activity in only those genera showing cholanoyl taurine hydrolase. With the exception of *Escherichia*, Dickenson *et al.* (1971) found the ability to transform cholic acid only in those genera capable of deconjugation.

Some selected isolates have received special attention in the literature. The organism isolated by Gustafsson *et al.* (1966) and tentatively identified by Midtvedt (1967) as being of the tribe Lactobacilleae, has been further studied (Midtvedt and Norman, 1968; Bokkenheuser *et al.* (1969). This lactobacillus-like isolate contained in addition to the 7α-dehydroxylase, enzymes capable of oxidising the hydroxyl groups at C-3, C-7 and C-12, but could not split taurocholate or glycocholate. Bokkenheuser and colleagues (1969) isolated an organism (strain FA 1/146) which was identical in these respects, but was not tested on conjugates. As mentioned above, Hattori and Hayakawa (1969) isolated *Bacteroides* 28S from human faeces. Stellwag and Hyleman (1976) reclassified *Bacteroides* 28S as *Cl. leptum* and studied it as washed cell suspensions. Perhaps the most numerous reports of a single isolate are those of the Italian workers and their isolate *Cl. bifermentans* SD 10 (Ferrari and Cocucci, 1967; Ferrari and Pacini, 1968; Carini *et al.*, 1967; Ferrari and Beretta, 1977; Ferrari *et al.*, 1977). Ferrari and Beretta (1977) observed cell-free extracts of *Cl. bifermentans* SD 10 to have, in addition to the 7α-dehydroxylase and 7α-dehydrogenase

abilities previously observed, the ability to rapidly deconjugate taurocholate and glycocholate. In addition, 3-keto- and 12-keto-containing cholate metabolites were detected. These authors showed two electrophoretically separable dehydrogenase activities in the extract.

2.5 CHOICE OF ORGANISM

The major project aim was to develop an industrial process to deconjugate and 7α-dehydroxylate the bile acids of mutton gall, using microbial cells. In selecting an organism or organisms to work with, two approaches were available:

(a) screening faecal isolates for organisms with the appropriate abilities, or

(b) using a strain named in the literature and previously demonstrated to have these abilities.

The former alternative has the disadvantages that:

(a) several workers have shown difficulty expressing dehydroxylase activity in screened organisms (Midtvedt and Norman, 1967; Dickenson *et al.*, 1971);

(b) isolation, purification and screening of faecal isolates is time-consuming, and

(c) since little was known of optimum conditions for dehydroxylation so early in the investigation, there was a danger that any data collected would poorly represent the true abilities of the strains.

For these reasons, the second of the two approaches was adopted.

The choice of organism then remained. *Clostridium bifermentans* was selected, since Ferrari and colleagues have published numerous reports on their own *Cl. bifermentans* isolate, and Hayakawa and Hattori (1970) have identified an international type strain possessing the 7α-hydroxycholanoyl dehydroxylase. Further, Ferrari and Beretta (1977) indicated that their strain possesses a cholanoyltaurine and cholanoylglycine hydrolase. The high incidence of deconjugating ability in clostridia (Shimada *et al.*, 1969; Lewis and Gorbach, 1972) suggested that *Cl. bifermentans* ATCC 9714 would probably also hydrolase conjugates.

2.6 CLOSTRIDIUM BIFERMENTANS

The taxonomy of *Cl. bifermentans* is somewhat confused. Although the American Type Culture Collection has classified strain ATCC 9714 as *Cl. bifermentans* for at least the last 10 years, Bergey's Manual (Smith and Hobbs, 1974) cites ATCC 9714 (NCIB 10717) as the reference strain for *Cl. sordellii*. Moore *et al.* (1966) included in their large study of clostridial fermentation patterns three strains of *Cl. bifermentans* and three of *Cl. sordellii*. Included in the latter group was ATCC 9714, which proved to be more similar to the two other *Cl. sordellii* than to the three *Cl. bifermentans* strains. Bergey's Manual differentiates between *Cl. bifermentans* and *Cl. sordellii* on the basis of urease activity which is produced by the latter only.

Prévot and Fredette (1966), while observing that both species have been isolated from gangrenous wounds, appear to consider *Cl. sordellii* alone to be pathogenic. Prévot deals at length with natural and experimental pathogenicity and with the highly active lecithinase toxin of *Cl. sordellii*, but makes no mention of pathogenicity or toxicity of *Cl. bifermentans*. This would imply that the strains used by Moore *et al.* were all *Cl. bifermentans*, since none showed toxicity to mice (although ATCC 9714 was not tested).

Hayakawa and Hattori (1970) failed in an attempt to trace the origins of strain ATCC 9714, which was first deposited as *Cl. sordellii*. Because of doubt concerning its true identity, these authors discounted the strain from their discussions on the possibility that separation of *Cl. sordellii* and *Cl. bifermentans* is justifiable on the basis of 7α-dehydroxylating ability. They also discounted the other dehydroxylating *Cl. bifermentans* strain, i.e. SD 10, stating: "It seems likely that Cocucci and Ferrari (1964) identified their strain as *Cl. bifermentans* according to Stewart's (1938) opinion." Stewart considered *Cl. sordellii* to be a pathogenic variant of *Cl. bifermentans*, and that they should be regarded as a single species *Cl. bifermentans*. Hayakawa (1973) reported an extension to his earlier work: 6 of 7 strains of non-pathogenic *Cl. sordellii* and none of the 16 strains of *Cl. bifermentans* tested showed *in vitro* 7α-dehydroxylation activity. The only *Cl. sordellii* to be negative was also the only one to be urease-negative.

Nomenclature *per se* is of little consequence to the current work. No attempt will be made to re-classify strains. The speciation adopted by ATCC and Ferrari, from whom the cultures were procured, will be retained.

From the viewpoint of an industrial process, a non-pathogenic organism is preferable, since wide safety margins are expensive. In the light of the confusion between sordellii/bifermentans, and the use of pathogenicity as a taxonomic criterion, there can be no preference established on these grounds.

Having decided which organism to use, the next step was to select a growth medium. Holland and Cox (1975) developed a fully synthetic medium which supported growth and sporulation of several *Cl. bifermentans* strains. Hayakawa and Hattori (1970), in their study with *Cl. bifermentans* ATCC 9714, used Marcus and Talalay Medium (Marcus and Talalay, 1956). Ferrari and colleagues also used this medium in some of their early studies (Ferrari and Cocucci, 1967), but later favoured Todd Hewitt Broth for *Cl. bifermentans* culture (Ferrari and Aragozzini, 1972; Ferrari *et al.*, 1977; Ferrari and Beretta, 1977). Midtvedt and Norman (1968) cite a number of studies in which Todd Hewitt Broth has been used for bile acid transformation work. Thus Todd Hewitt Broth was chosen for the current work, for several reasons:

(a) its extensive use by other workers,
(b) its availability as a standard commercial product, and
(c) its relative simplicity - an industrial fermentation could employ a very similar medium (protein digest, meat infusion, glucose syrup and salts).

2.7 INHIBITION OF BACTERIAL GROWTH BY BILE ACIDS

The inhibition of bacterial growth by bile acids has been long known and is the basis of several media selective for enteric organisms. In the last decade, several studies have been devoted to characterising the nature of the inhibition. A knowledge of these studies should be valuable in the development of a process for bile acid transformations, where organisms are cultured in the presence of bile acids.

Floch *et al.* (1972) reviewed the existing literature in this field, concluding that while conjugates were inhibitory to some organisms, free cholic and deoxycholic acids were more so. Anaerobes and Gram positive organisms were most affected. In the light of this conclusion, Floch and co-workers postulated an autoregulatory mechanism wherein gut bacteria inhibited their own growth by hydrolysing bile conjugates. They went on to determine minimum inhibitory levels of several bile acids, including in their study two strains of *Cl. perfringens*. These strains were inhibited by 5 mM glycocholic acid and 1 to 5 mM glycodeoxycholic acid. Both conjugates were hydrolysed and the released cholic acid was subsequently 7α-dehydroxylated. Floch and colleagues drew general conclusions from their study:

(a) dihydroxy bile acids are inhibitory at the 1-2 mM level, trihydroxy acids at 5 mM;
(b) increased bile acid concentration produced increased inhibition;
(c) greater initial cell density decreased inhibition for a given bile acid concentration; and
(d) the inhibitory effects of different bile acids are not necessarily additive.

Percy-Robb and Colley (1972) extended this work, and discovered bile acid toxicity to bacteria to be pH-dependent. These workers grew two strains of each of *Cl. welchii* and *Bacteroides* sp. in the presence of cholic acid in media of initial pH 7.2, 6.4 and 5.8. They found that at pH 5.8, cholic acid was toxic at 1.5 mM, but at higher pH values sensitivity was decreased. Consequently they proposed a homeostatic regulation system to prevent back-growth in the small intestine, where bile acid levels are *ca* 1 mM.

Binder *et al.* (1975) studied the inhibition of 11 strains of *Clostridium*, 15 of *Bacteroides* and 13 of *Lactobacillus*, and reported free bile acids to be more inhibitory than conjugates. Lithocholate had a greater effect than deoxycholate which was in turn more inhibitory than cholate. The inhibitory effect increased with decreasing pH. The addition of 5 mM of lecithin decreased the inhibitory effect of 10 mM cholic acid, especially for clostridia.

In the light of these findings, one fermentation variable worthy of study in this project will be the addition of bile acid transformation substrate before/after the completion of growth.

CHAPTER 3 METHODS

3.1 MELTING POINTS

All melting points were determined on either a Kofler hot-stage apparatus (Kofler, Reichert, Austria) and are corrected, or on a Leitz Dialux microscope with heating stage 350 (Ernst Leitz GMBH, Wetzlar, F.D.R.) and are uncorrected.

3.2 MATERIALS

3.2.1 Media

Cooked Meat Medium (Oxoid CM 81) was obtained from Oxoid Ltd., London, U.K., and Todd Hewitt Broth (BBL 11736) was obtained from Baltimore Biological Laboratories, Cockeysville, Maryland, U.S.A.

3.2.2 Chromatography Materials

Amberlite XAD-2 was obtained from Mallinckrodt, St.Louis, Missouri, U.S.A. Celite Hyflo Super-Cel, used for reverse phase partition chromatography, was supplied by Koch-Light Laboratories, Colnbrook, Buckinghamshire, U.K. Kieselgel DG (400 mesh), used for thin-layer chromatography (T.L.C.) was obtained from Riedel-de Haen AG, Seelze-hannover, F.D.R.

3.2.3 Bile Acids

Cholic acid, methyl 3α,7α-diacetoxy-12α-hydroxy-5β-cholan-24-oate and deoxycholic acid (pure and 98% pure) were obtained from New Zealand Pharmaceuticals Ltd., Palmerston North, N.Z. The cholic acid was recrystallised twice from water-saturated ethyl methyl ketone and once from methanol-water 98:2, before drying at 105^{0}C for 3 h.

The diacetate was recrystallised once from methanol and air dried at room temperature, whilst the lower grade of deoxycholic acid was recrystallised once from water-saturated ethyl methyl ketone and air dried at room temperature for use in subsequent syntheses. Pure deoxycholic acid was used as received, as a standard for high performance liquid chromatography.

3.2.3.1 7-Ketodeoxycholic acid

7-Ketodeoxycholic acid was prepared by the method of Fieser and Rajagopalan (1949). The reaction mixture could not be readily crystallised, and so was purified by repeated crystallisation of the diformate (Tserng and Klein, 1977). The diformate (10 g) was hydrolysed by refluxing for 4 h in a mixture of ethanol (50 ml) and 1.2 M NaOH (75 ml). The precipitate produced by the addition of 1 M H_2SO_4 at 50^{0}C was collected, recrystallised twice from water-saturated ethyl acetate, then dried for 3 h at 100^{0}C/0.2-0.4 kPa. The free acid (m.p. 176-178^{0}C; c.f. 170-171^{0}C, Hoehn and Linsk, 1945) had the equivalent weight of a mono-hydrate, (found: 423.6; calculated for $C_{24}O_5H_{38}.H_2O$:424).

3.2.3.2 3α,7α-Dihydroxy-5β-chol-11-en-24-oic acid

Methyl 3α,7α-diacetoxy-5β-chol-11-en-24-oate was prepared from methyl 3α,7α-diacetoxy-12α-hydroxy-5β-cholan-24-oate essentially by the method of Chen (1976) but omitting the hexane extraction of the crude 12α-mesylate. The 11-ene-diacetate (20.45 g) was subsequently hydrolysed by refluxing for 45 min in 1.8 M ethanolic NaOH (205 ml) and re-crystallised twice from water-saturated ethyl methyl ketone. This gave 3α,7α-dihydroxy-5β-chol-11-en-24-oic acid, m.p. 206-210^{0}C, for use as an internal standard in chromatographic analyses. A sample, when recrystallised from ethyl acetate had m.p. 209.5-212^{0}C (c.f. Nakada, 1963, 204-206^{0}C).

3.2.3.3 Conjugates

Sodium taurocholate, sodium taurodeoxycholate and glycodeoxycholic acid were prepared by the method of Norman (1955) while glycocholic acid was prepared by applying Norman's method for glycodeoxycholic acid to cholic acid.

Since the taurine conjugates were difficult to crystallise, their purity was checked by thin-layer chromatography (solvent system TLC1, refer section 3.6.4). Both were contaminated with unreacted free bile acid. To remove this, the taurocholate was dissolved in water, acidified to pH 3.5 with HCl, and the free bile acid extracted with n-butanol. Similarly, taurodeoxycholate was dissolved in water, acidified to pH 4.5, and extracted with ethyl acetate. In both cases,

the aqueous phase containing the conjugate was evaporated to dryness on the steam bath and the resulting gums were dissolved in boiling ethanol. Sodium hydroxide (2.0 M) was used to adjust the solutions to pH 8-9 (phenolphthalein) prior to filtration. Sodium taurocholate was crystallised twice from ethanol-ether 9:1, then dried for three days over P_2O_5 at 4-6 kPa. Sodium taurodeoxycholate was recrystallised twice from ethanol, then dried for 6 h at 80^{0}C and 4-6 kPa.

Both glycine conjugates were crystallised from aqueous ethanol. Glycocholic acid was then recrystallised three times from ethyl acetate-ethanol 9:1, while glycodeoxycholic acid was recrystallised once from ethanol and water, then twice from ethyl acetate and ethanol. Both products were dried for 5 h at 110^{0}C and 4-6 kPa.

All four conjugates were found to be homogenous by T.L.C. (solvent system TLC2, refer section 3.6.4). In addition, the glycine conjugates were subjected to H.P.L.C. (solvent system: methanol-0.17 M aq. acetic acid 80:20, μBondapak C_{18} column, 1.7 ml/min, refer section 3.6.5). Glycocholic acid was found to be pure, but glycodeoxycholic acid had a 0.9% deoxycholic acid contaminant.

Melting points were determined on the Kofler hot-stage apparatus, and are listed in Table 3.1.

TABLE 3.1 Melting Points of Synthetic Conjugates

Conjugate	Found ^{0}C	Literature ^{0}C	
Sodium taurocholate	171-174	130-145	Cortese and Bashau (1937)
		182-184	Tserng *et al.* (1977)
		225-235	Norman (1955)
Sodium taurodeoxycholate	171-174	172-173	Tserng *et al.* (1977)
		171-175	Norman (1955)
Glycocholic acid	166-169	165-168	Norman (1955)
Glycodeoxycholic acid	188-189	187-188	Norman (1955)

Since sodium taurocholate melts over wide temperature ranges (Cortase and Bashau, 1937; Norman, 1955), and since the melting point determined here was 10^0C below that of Tserng *et al.* (1977), the sodium taurocholate was compared by T.L.C. (system TLC2, ref. section 3.6.4) with authentic sodium taurocholate (Koch-Light Laboratories), and found to have the same R_f value.

3.2.4 Solvents

Solvents for crystallisation were generally B.D.H. A.R. grade (B.D.H. Chemicals New Zealand Ltd., Palmerston North, N.Z.). Chloroform for solvent extraction was purchased from Ajax Chemicals, Sydney, Australia, and was used as received. After use, chloroform was recovered by washing with water, drying over anhydrous $MgSO_4$ and redistilling. Recovered chloroform was stored containing 2% (v/v) ethanol. Methanol for glassware washing was purchased from I.C.I. (New Zealand) Ltd., Lower Hutt, N.Z., and redistilled before use. Hexamethylphosphoric triamide, used in the synthesis of 3α,7α-dihydroxy-5β-chol-11-en-24-oic acid, was "Aldrich-analysed" grade, obtained from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, U.S.A. 2-Ethylhexanol used in reverse phase partition chromatography was a Unilab laboratory reagent, Sydney, Australia, while tri-n-butylamine, used in conjugate syntheses, and dimethylsulphoxide (D.M.S.O.) were B.D.H. laboratory reagent grade.

All solvents for high performance liquid chromatography were redistilled in glass and stored in glass. Methanol was redistilled over 1% w/v NaOH, using a 1.5 m randomly-packed glass column, and water was distilled in stainless steel before redistillation. Acetic acid and column washing solvents were A.R. grade before redistillation.

All solvent ratios are reported on a volume to volume basis.

3.2.5 Gases and Other Chemicals

Hydrogen, oxygen-free nitrogen and mixtures of dry nitrogen and carbon dioxide were purchased from New Zealand Industrial Gases Ltd., Palmerston North, N.Z. The composition of nitrogen-carbon dioxide mixtures was checked periodically with an Orsat apparatus.

Phosphomolybdic acid ("Baker-analysed" grade) was obtained from

J.T. Baker Chemical Co., Phillipsberg, New Jersey, U.S.A. Sodium thioglycollate was purchased from Difco Laboratories, Detroit, Michigan, U.S.A., and dichlorodimethylsilane from the Pierce Chemical Company, Rockford, Illinois, U.S.A. B.D.H. Chemicals (New Zealand) Ltd. supplied tris(hydroxymethyl)methylamine, (Tris), 8-hydroxyquinoline and quinhydrone which were A.R. grade, and methanesulphonyl chloride, N-bromosuccinimide, ethyl chloroformate, sodium barbitone and disodium ethylenediaminetetraacetic acid (EDTA), which were all laboratory reagent grade. The enzyme inhibitors p-chloromercuribenzoate (pCMB) and dicumarol were purchased from Koch-Light Laboratories and the SIGMA Chemical Company (St. Louis, Missouri, U.S.A.) respectively.

Most other chemicals were A.R. or laboratory reagent grade, purchased from B.D.H. or May and Baker (New Zealand) Ltd., Lower Hutt, New Zealand.

3.3 ORGANISMS

Clostridium bifermentans ATCC 9714 was purchased from the American Type Culture Collection as a freeze dried specimen. *Cl. bifermentans* SD 10 was a gift from Professor A. Ferrari of the University of Milan. This strain was isolated from a highly selected mixed culture of human faecal organisms (Ferrari and Aragozzini, 1972), and was received as a freeze dried culture.

Both strains were reconstituted into and maintained in Cooked Meat Medium containing 0.05% w/v cholic acid. Subcultures were prepared approximately every twelve months.

3.4 STERILISATION OF MEDIA

Both Cooked Meat Medium for culture maintenance and Todd Hewitt Broth for small scale experiments and inoculum preparation were autoclaved in 25 ml McCartney bottles for 15 min at 121^{0}C.

3.5 CLEANING OF GLASSWARE

All glassware was routinely washed in hot tap water containing "Pyroneg" (Diversey-Wallace Ltd., Papatoetoe, Auckland, New Zealand), thoroughly rinsed in warm tap water and air dried at *ca* 60^{0}C. Glassware for use in analyses was further rinsed with methanol and air dried at room temperature. Items used for chemical syntheses were soaked in dilute NaOH prior to washing, and glassware used to store H.P.L.C. solvents were treated with hot chromic acid and rinsed several times with distilled water and the solvent to be stored.

3.6 ANALYTICAL METHODS

3.6.1 pH Measurement

Routine laboratory pH measurement was performed with a Metrohm Herisau pH Meter E520 (Metrohm Ltd., Herisau, Switzerland).

3.6.2 Dry Weight Determination

An aliquot of cell suspension was accurately pipetted into an aluminium moisture dish, previously stored in a desiccator and tared. Free water was removed on the steam bath. The residue was then dried for 16 h at 110^{0}C, cooled in a desiccator and the dish reweighed.

3.6.3 Cell Counts

An aliquot of live culture was counted directly using a haemocytometer with phase contrast microscopy. Spores were not distinguished from vegetative cells.

3.6.4 Thin-Layer Chromatography

Qualitative analytical T.L.C. was conducted on 10 mm x 20 mm glass plates with 0.25 mm layers of Kiesegel DG. Plates were activated by heating at 110^{0}C for 30 min before use. Development was by the tank saturation technique with one of the following solvent systems:

(a) system TLC1; 5:5:1, toluene-acetic acid-water, upper phase (Randerath, 1968).

(b) system TLC2; 10:10:1, ethylene dichloride-acetic acid-water (Gregg, 1966).

(c) system TLC3; 20:10:2, benzene-dioxan-acetic acid (Sjövall, 1964). The plates were visualised by spraying with 10% w/v ethanolic phosphomolybdic acid (Eneroth and Sjövall, 1971) and heating at 110°C for 5 to 10 min.

3.6.5 High Performance Liquid Chromatography

A Water Associates Model HPLC200 comprising a U6K septumless loop injector, model 6000A pump and R401 differential refractometer (Waters Associates Inc., Milford, Maryland, U.S.A.) was used in conjunction with a JJ Instruments CR600 twin-pen flat-bed recorder (J.J. Lloyd Instruments Ltd., Southampton, United Kingdom).

The instrument was operated at room temperature with either a Waters μBondapak C_{18} 3.9 mm dia. × 300 mm column, or a Bio-Rad Bio-Sil ODS10 4 mm dia. × 250 mm column, usually with a mobile phase flow rate of 2.0 ml/min.

3.6.5.1 Mobile phase

For samples containing free bile acids and glycodeoxycholic acid, the solvent systems used were mixtures of methanol and 0.17 M aqueous acetic acid, first used by New Zealand Pharmaceuticals Ltd., (Garland, 1977). For the μBondapak C_{18} column, a volume ratio of 77:23 was usually used, for the Bio-Rad column 79:21.

For conjugates, a solvent system based on that of Bloch and Watkins (1978) was used, i.e. a mixture of methanol, water and acetic acid, adjusted to a pH 4.7 with 10 M NaOH. For the μBondapak C_{18} column, a ratio of 70:30:3.27 was used; for the Bio-Rad column, 72:28:3.27.

3.6.5.2 Resolution

Retention volumes for the system methanol-0.17 M acetic acid, 79:21 are given in Table 3.2a. Deoxycholic acid is the last eluted bile acid; at 2.0 ml/min it is fully eluted within 12 minutes. With this system, the taurine conjugates cannot be separated from the solvent front, and glycocholic acid is difficult to separate from 7-ketodeoxycholic acid. The partial separation between glycodeoxycholic acid and cholic acid is insufficient to allow simultaneous quantitation.

Retention volumes for methanol-water-acetic acid 78:28:3.27 are given in Table 3.2b. Cholic acid and glycodeoxycholic acid are virtually co-eluted, but all other bile acids of interest show base-line separation. Deoxycholic acid is again the most slowly eluted, taking 22 minutes at 2.0 ml/min.

TABLE 3.2a Retention Volumes of Bile Acids

Bile Acid	Retention Time min	Retention Volume ml
7-ketodeoxycholic acid	2.7	5.3
cholic acid	5.1	10.2
glycodeoxycholic acid	5.8	11.5
3α,12α-dihydroxy-5β-chol-11-en-24-oic acid	6.8	13.7
deoxycholic acid	10.5	21.0

Conditions: Bio-Rad column, 2.0 ml/min, 79:21 methanol-0.17 M aq. acetic acid.

TABLE 3.2b Retention Volume of Bile Acids

Bile Acid	Retention Time min	Retention Volume ml
Na taurocholate	3.1	6.2
7-ketodeoxycholic acid	3.9	7.8
glycocholic acid	4.7	9.3
Na taurodeoxycholate	5.3	10.5
cholic acid	9.1	18.2
glycodeoxycholic acid	9.6	19.2
3α,12α-dihydroxy-5β-chol-11-en-24-oic acid	12.8	25.7
deoxycholic acid	19.9	39.8

Conditions: Bio-Rad column, 2.0 ml/min, 72:28:3.27 methanol-water-acetic acid

3.6.5.3 Operation

Injection volumes of 20 to 90 µl containing 0.1-1.1 µmoles of each bile acid provided suitable peak heights at 4× or 8× attenuation.

Each sample mixture included 3α,12α-dihydroxy-5β-chol-11-en-24-oic acid as internal standard, and a standard mixture of known composition was always injected in parallel.

3.6.6 Infra-Red Spectrophotometry

A glass disc containing 1% w/v bile acid in 75 mg of potassium bromide was prepared with a Beckman Evacuable KBr Minidie, Model DMO-1 (Beckman-Riic Ltd., Glenrothes, Fife, United Kingdom). This was scanned at slow speed and normal slit-width with a Perkin Elmer 12 720 spectrophotometer or with a Pye Unicam SP3 300 spectrophotometer.

3.7 CULTURE CONDITIONS

3.7.1 Small Scale Experiments

Culture maintenance, inoculum preparation and some deconjugation experiments were performed on a small scale. Medium (20 ml) was dispensed into 25 ml McCartney bottles and autoclaved (refer section 3.4). Inoculation was carried out using a sterile pasteur pipette with *ca* 0.5 ml of inoculum after media had cooled to *ca* 45^{0}C. Anaerobic conditions were adequately approximated by tightly screwing down the caps and incubating at 37^{0}C with no further special precautions.

3.7.2 Fermentation

3.7.2.1 Equipment

Figure 3.1 is a photograph, and Figure 3.2 a diagram of the assembled fermenter and ancillary equipment. Fermentations were conducted in a Microferm Laboratory Fermenter (New Brunswick Scientific Co. Ltd., New Brunswick, New Jersey, U.S.A.) using either the 7-litre vessel with 5 l working volume, or the 2-litre vessel with 1.25 l working volume. A modified head assembly with four electrode ports was constructed for the 2-litre vessel (Figures 3.3 and 3.4).

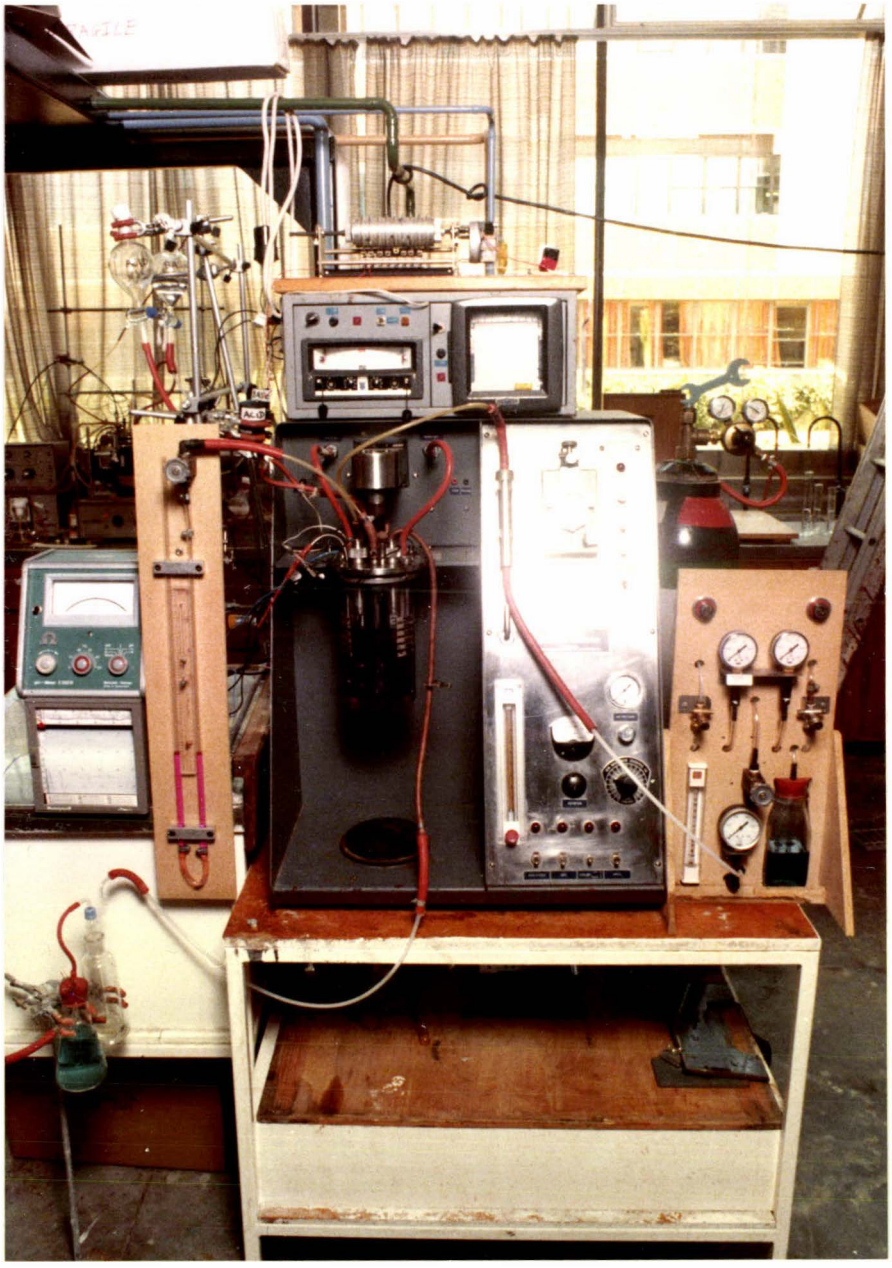

FIGURE 3.1 The Assembled Fermenter

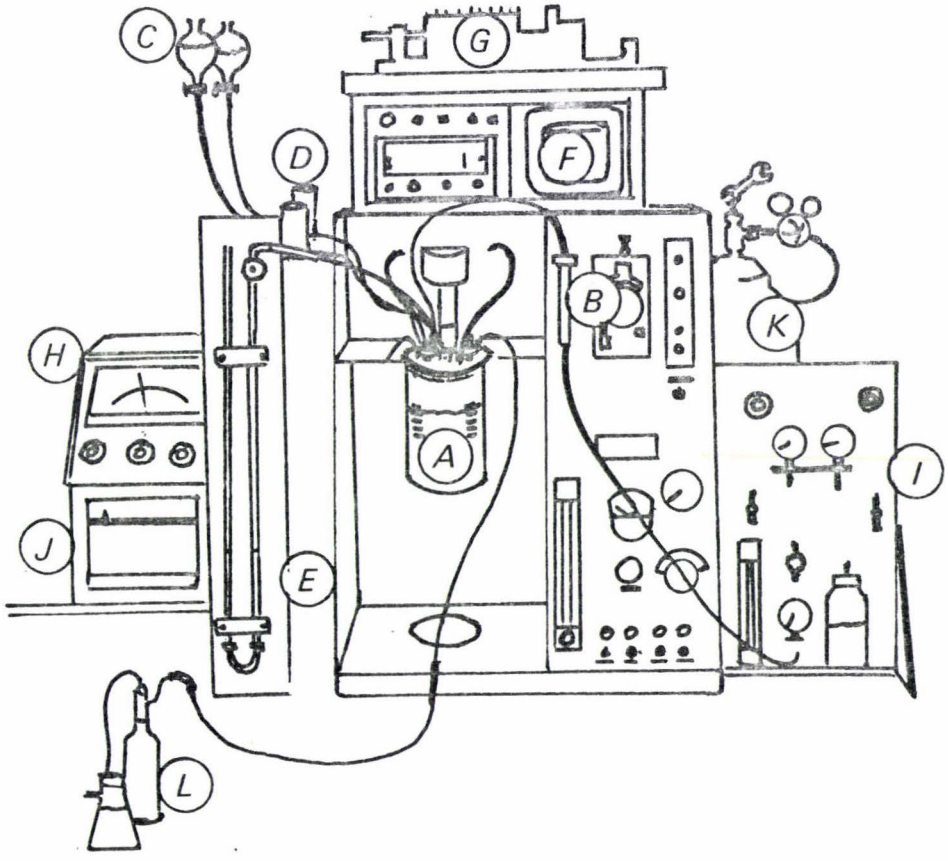

FIGURE 3.2 The Assembled Fermenter - Key to Figure 3.1

A 2-litre fermenter vessel mounted on Microferm services unit

B Inlet gas line with glass wool sterilising filter

C Acid/base reservoirs

D Acid/base solenoid valves

E Manometer to measure fermenter headspace pressure

F pH controller

G Time-switch allowing solenoids 1 s on-time in 30 s

H Electrode potential (Ec) meter

I Gas-mixer/flow regulator

J Chart recorder for pH/temperature/electrode potential signals

K Gas supply cylinder and regulator

L Effluent gas scrubber, set to maintain 0.5 kPa in headspace

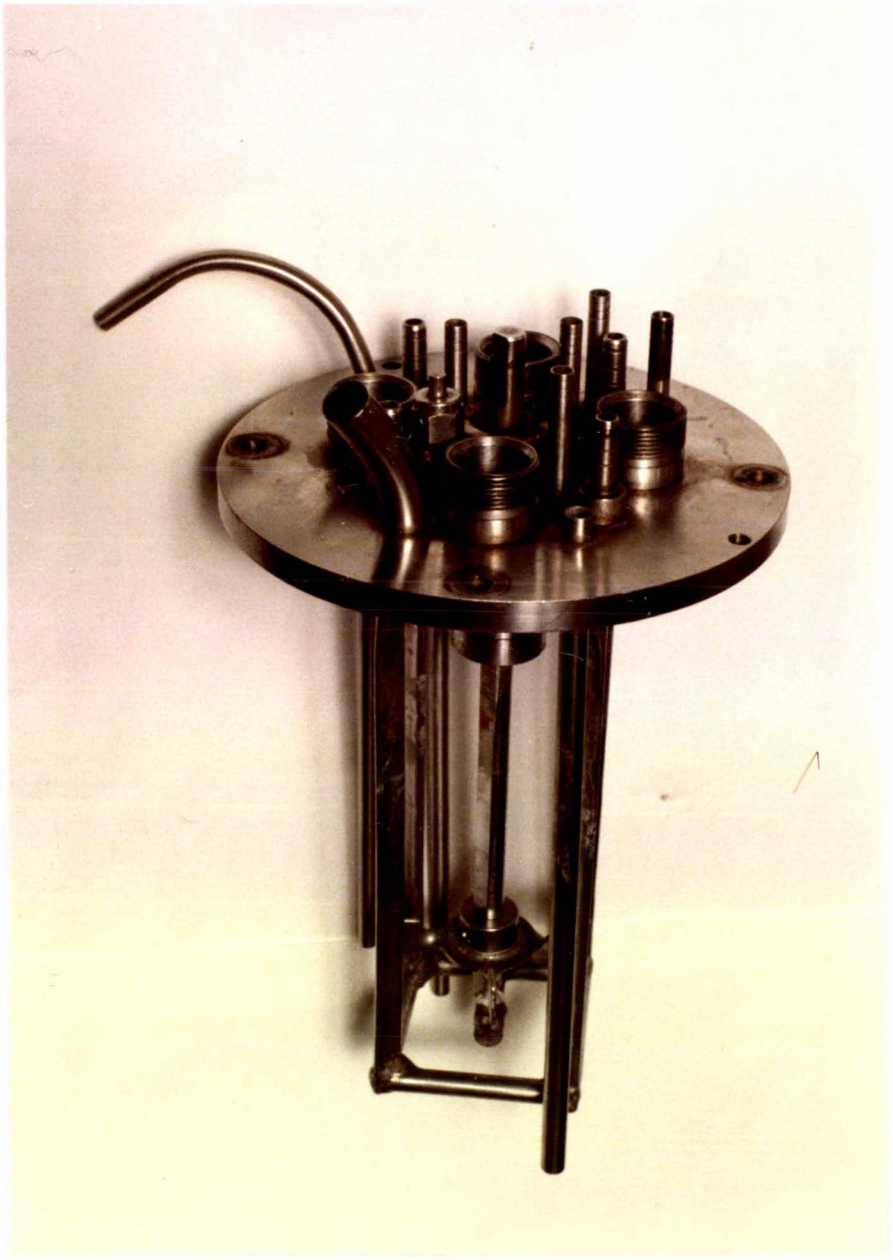

FIGURE 3.3 The 2-litre Fermenter Vessel Head

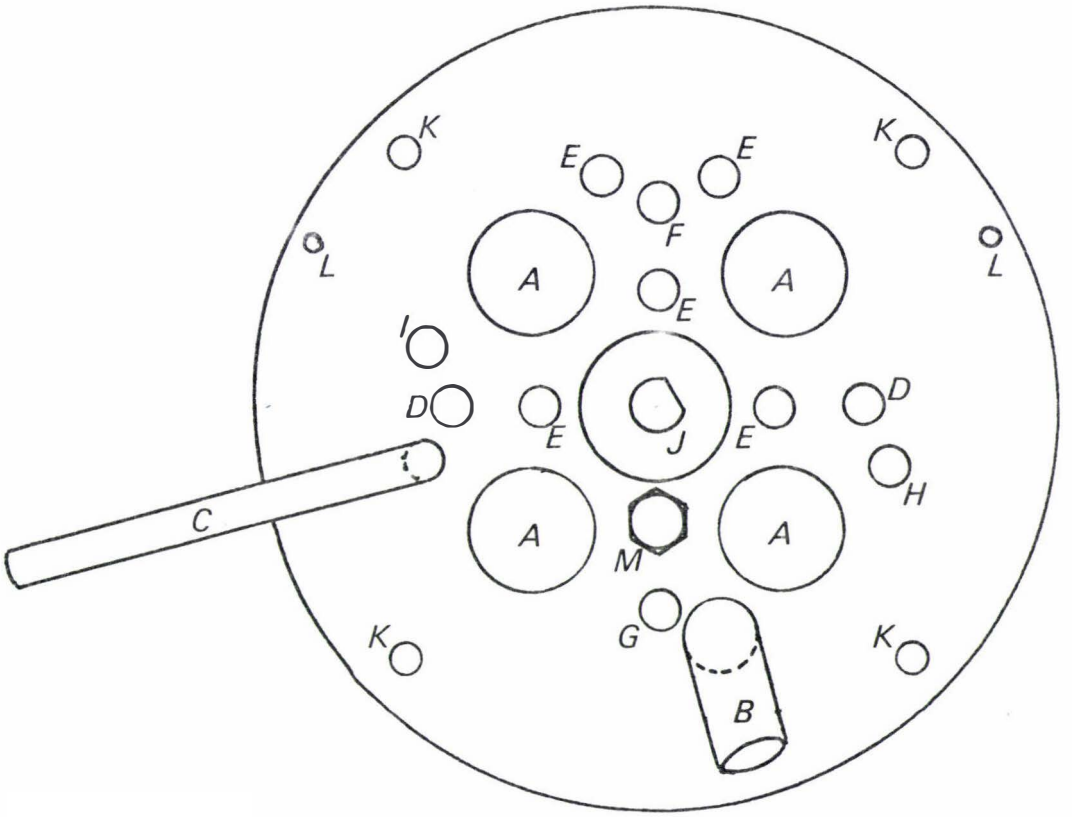

FIGURE 3.4 Plan of the 2-litre Fermenter Vessel Head

A	Electrode port
B	Filler tube
C	Sample tube
D	Water inlet/outlet for hollow-baffle heat-exchanger
E	Ports open to head space
F	Gas inlet to sparger
G	Gas inlet to head space
H	Thermometer well
I	Thermister well for temperature control
J	Bearing housing and impellor shaft
K	Holes for mounting bolts
L	Holes for locating pins

A two-channel gas mixer/regulator was built and is shown in Figures 3.5 and 3.6. The gas line was nylon pressure tube with rubber pressure tube connections. Headspace pressure was monitored by a water manometer connected via a valve to a port in the fermenter head. Effluent gas was expelled by sparging through 30 to 40 mm depth of 5 M NaOH which maintained a headspace pressure of *ca* 0.5 kPa.g.

For one-way pH control, the alkali solution was stored in a sterile dropping funnel and gravity-fed through an Alcon type ACDV P252 solenoid valve (Alexander Control Ltd., Sutton Coldfield, United Kingdom). For two-way pH control, both acid and base were gravity-fed through Flurocarbon Delta model DV2 122A2 solenoid valves (Delta Solenoid Valves, Anaheim, California, U.S.A.). Connecting lines were of surgical rubber hose, pre-soaked in acid or base to remove labile components. When using the 2-litre vessel, the solenoid valve signals were interrupted for 29 s in every 30 s with a cam-timer.

3.7.2.2 Instrumentation

One-way pH control was obtained with an E.I.L. pH transmitter 6320 (Electronic Instruments Ltd., Richmond, Surrey, United Kingdom), an E.I.L. 33 1070 030 toughened glass electrode and an E.I.L. 33 1320 210 laboratory sealed reference electrode. The same electrodes were used with an E.I.L. 91B pH controller for two-way control.

Difficulties were experienced with electrode drift in the highly proteinaceous media. The problem was controlled by frequently checking the pH of withdrawn samples, by soaking the glass electrode in 0.1 M HCl/pepsin solution overnight between runs, and by replacing the glass electrode approximately every twenty runs.

Electrode Potential (Ec) was monitored by a Metrohm Herisau E 350 B pH meter (Metrohn Ltd., Herisau, Switzerland) with the E.I.L. 33 1213 400 laboratory platinum electrode and an E.I.L. 33 1320 210 laboratory sealed (saturated calomel) reference electrode. Operation of the platinum electrode was periodically checked with saturated quinhydrone buffers (Radiometer, 1966).

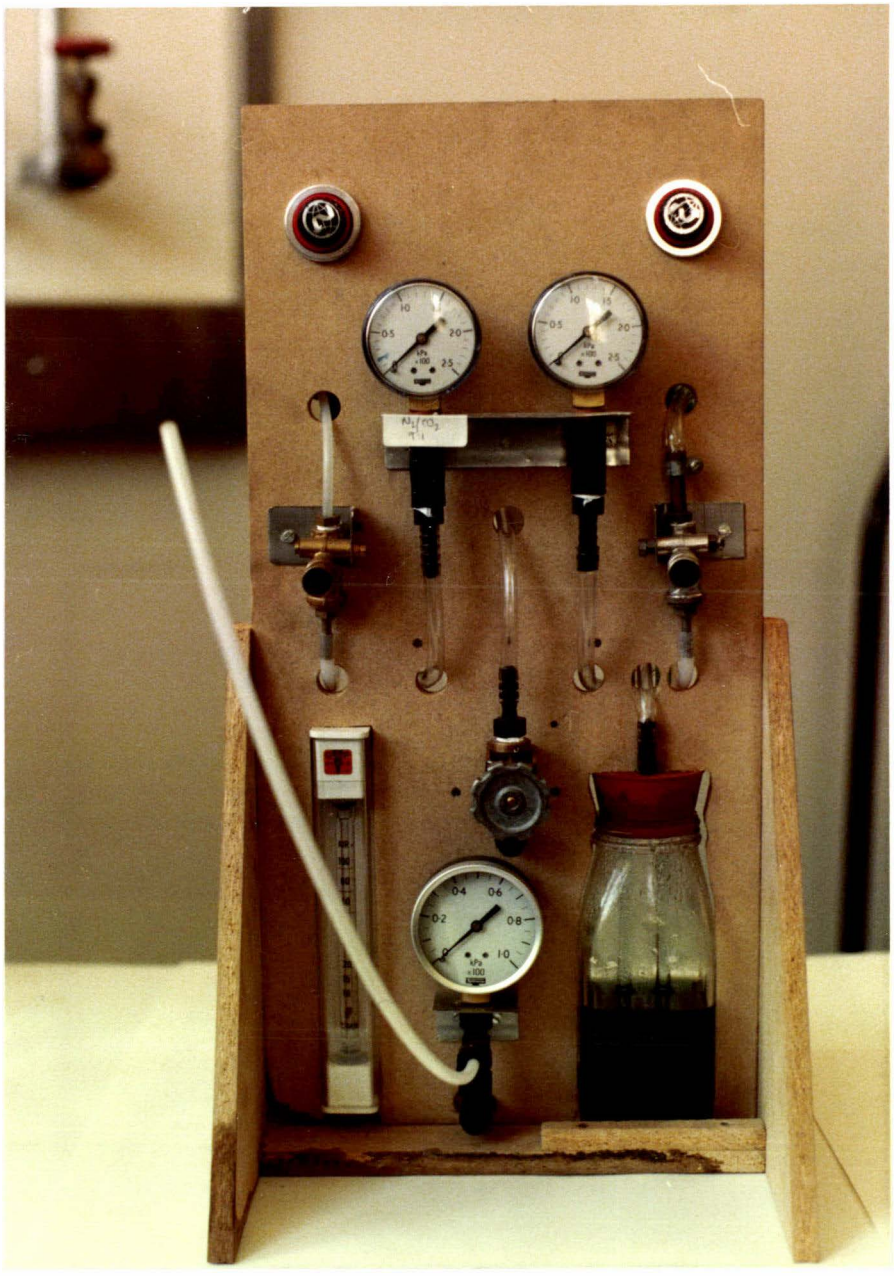

FIGURE 3.5 The Gas-Mixer/Flow-Regulator

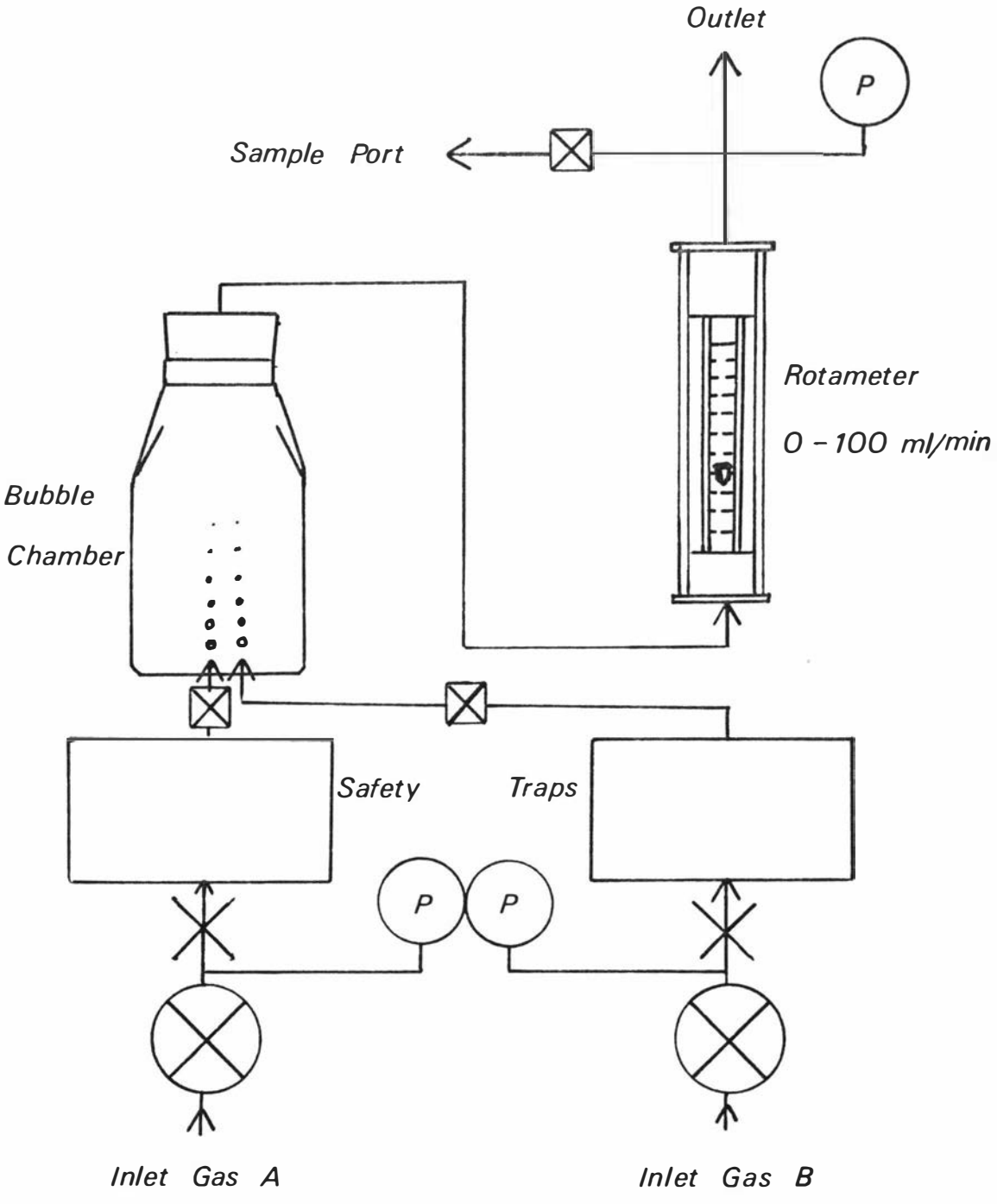

FIGURE 3.6 Schematic of Gas-Mixer/Flow-Regulator

Pressure Regulator

Needle Valve

Clamp on Rubber Pressure Tube

Pressure Gauge

Various combinations of pH, Ec and temperature (measured by copper/constantan thermocouple) were recorded throughout the work, using either a J.J. twin-pen flat-bed recorder model CR 600 (J.J. Lloyd Instruments Ltd., Southampton, United Kingdom) or a Honeywell Versaprint Type 121-1 (Honeywell Ltd., Bois d'Arcy, France).

3.7.2.3 Sterilisation

The fermenter vessel was assembled and filled with medium prior to autoclaving. Both the 7-litre and 2-litre vessels were sterilised (without the electrodes) by autoclaving for 15 min at 121^{0}C. All ports were plugged with non-absorbent cotton-wool and wrapped in aluminium foil. The sample line and gas inlet line were clamped closed.

The gas inlet filter was filled with fresh glass wool, wrapped in aluminium foil and sterilised in a hot air oven at 160^{0}C for 3 h.

Solutions of sulphuric acid, sodium hydroxide, sodium thioglycollate, EDTA and neutral solutions of bile acids were autoclaved for 15 min at 121^{0}C in conical flasks plugged with non-absorbent cotton-wool, wrapped in aluminium foil. Solutions of sodium hydrogen carbonate were sterilised by filtration through a 0.45 µm membrane (Oxoid, United Kingdom).

Electrodes and reservoirs, lines and solenoid values for acid and base addition were sterilised by soaking in 2% aq. formaldehyde for 30 min, then flushed with sterile distilled water prior to connection.

3.7.2.4 Inoculum preparation

A standard inoculum was prepared for each fermentation by sub-culturing into a tightly capped bottle of Todd Hewitt Broth containing 0.05% w/v cholic acid. After incubation for 48 or 72 h at 37^{0}C, this was used to inoculate a second bottle of Todd Hewitt Broth containing 0.05% w/v cholic acid. This bottle provided the appropriate volume of 24 h, 37^{0}C culture to inoculate the fermenter. In no case was the final inoculum further than 5 transfers away from the original freeze dried culture.

3.7.2.5 Operation

A single fermentation entailed:

(1) preparation of the inoculum,

(2) preparation of the appropriate volume of medium, together with additives, and the filling of the assembled fermenter vessel,

(3) sterilisation,

(4) insertion of the sterile, rinsed electrodes into the hot (*ca* 85^{0}C) vessel assembly and the fixing of this to the fermenter proper,

(5) connection of all lines and leads, and the starting up of systems (except cam-timer). Within 10 min of opening the autoclave, the medium would be under an anaerobic gas blanket and being cooled,

(6) allowing two hours for electrodes to equilibrate in the medium,

(7) withdrawal of a sample to check medium pH, so that the pH controller could be correctly set,

(8) inoculation and the starting of the cam-timer,

(9) operation for 1 to 7 days including periodic expulsion of samples under gas pressure,

(10) stopping the fermentation, sterilisation of the contents, disassembly and cleaning.

3.7.2.6 Sampling technique

At each sampling, the fermenter volume was recorded (the vessel was calibrated for volume at 35^{0}C, prior to the sample line being flushed and a sample withdrawn. The volumes of the medium flushed out and sampled were measured and recorded. A cell count was performed on the sample and the pH was determined.

Exactly 20.00 ml of sample was centrifuged (10 min at 3130 x g) with 600 mg of internal standard, added as a 0.2% w/v solution in 1M NaOH. The supernatant liquid was stored at 4^{0}C for up to 48 h before extraction and analysis.

3.8 WASHED CELL METHODOLOGY

3.8.1 Cell Production

Cells were produced by 16 h fermentation in the 2-litre vessel with Todd Hewitt Broth containing 0.01% w/v cholic acid. The medium was agitated at 200 rev/min, and its surface was swept with 20 ml/min N_2-CO_2, 9:1. Temperature was controlled at 37^{0}C, and pH at 6.9-7.1 with 0.5 M H_2SO_4 and 1 M NaOH. Ec was monitored and recorded.

3.8.2 Equipment

The cell-washing equipment is shown in Figures 3.7 and 3.9, and the incubation racks in Figure 3.8. The incubation racks could be either submerged in a water bath or placed in a controlled temperature room. Each had a manifold with allowed distribution of sterile gas to the tubes, and glass wool plugs in the exhaust lines which prevented back-contamination.

3.8.3 Harvesting and Washing

Cells were harvested using gas pressure to expel medium into two 500 ml screw-top centrifuge bottles, followed by centrifugation at 3000 x g and 25^{0}C for 10 min. The supernatant liquid was expelled under nitrogen pressure. Nitrogen pressure was then used to half-fill the centrifuge bottles with phosphate buffer (pH 7.2, 0.02M) which had recently been autoclaved for 5 min at 108^{0}C and cooled to 30^{0}C under nitrogen. Resuspension was by stirring with item (E) then with item (F) (Figure 3.8), under nitrogen. The centrifuge bottles were topped up with buffer and re-centrifuged at 3000 x g and 25^{0}C for 10 min. After decanting and resuspension as before, the samples were combined into one 500 ml centrifuge bottle and re-centrifuged at 3000 x g and 25^{0}C for 10 min. After the final decanting and resuspension, the slurry was made up to 50 ml in a sterile volumetric flask. This was stored for no longer than 1 h at 30^{0}C before use.

3.8.4 Incubation

The reaction mixture (20 ml, containing 0.05% cholic acid) was dispensed into 50 ml polypropylene centrifuge tubes which were then placed in the incubation racks. This assembly was autoclaved for 5 min at 108^{0}C and then cooled to incubation temperature either in a

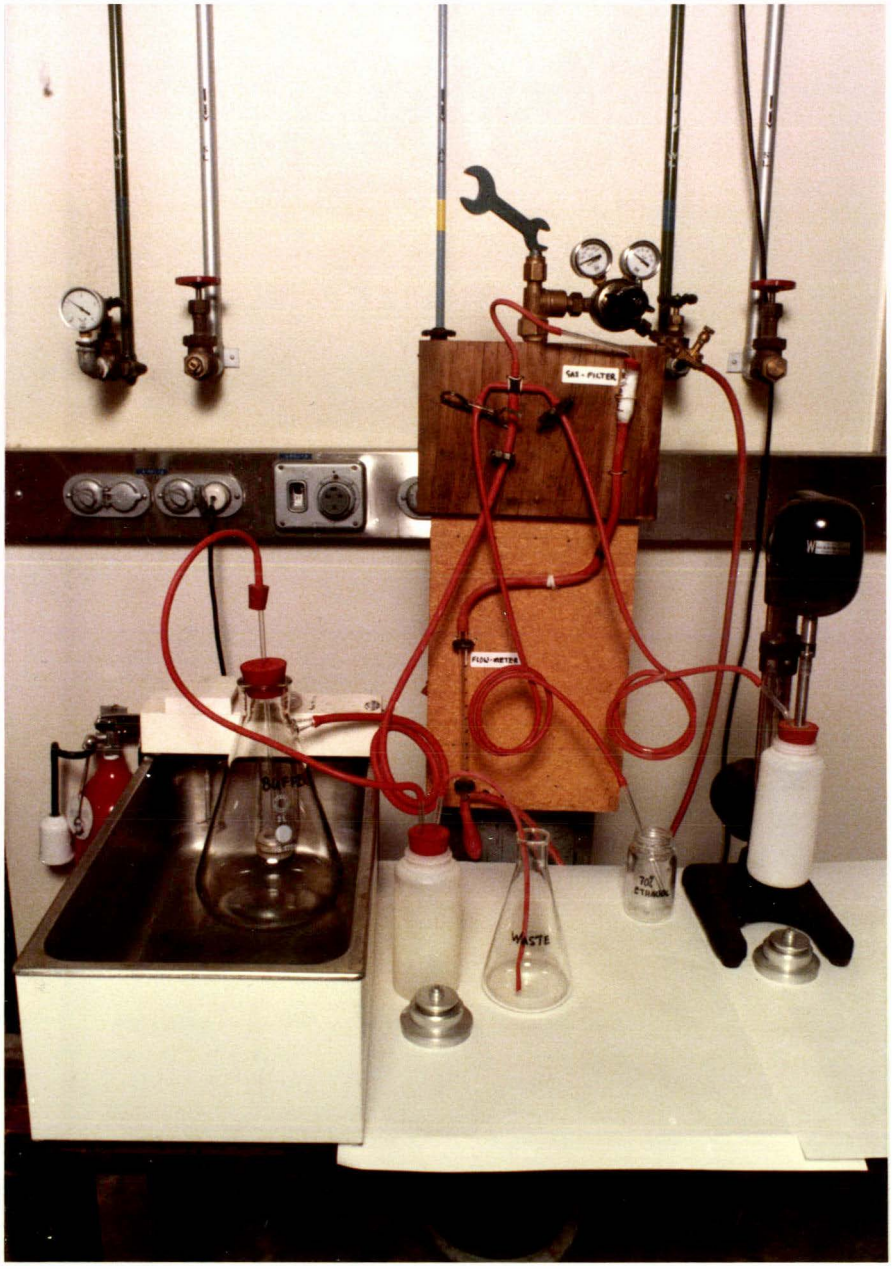

FIGURE 3.7 Cell Washing Equipment

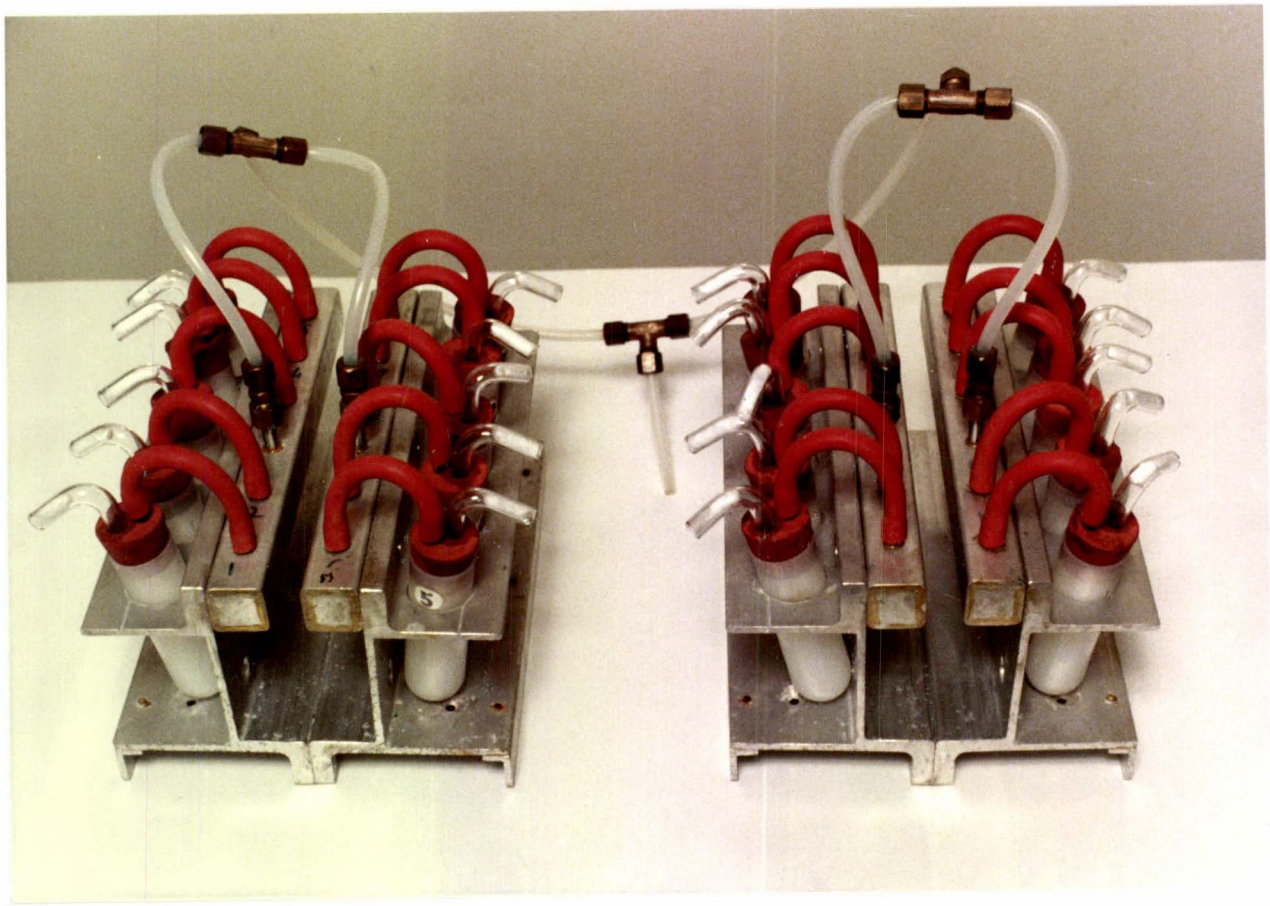

FIGURE 3.8 Washed Cell Incubation Rack

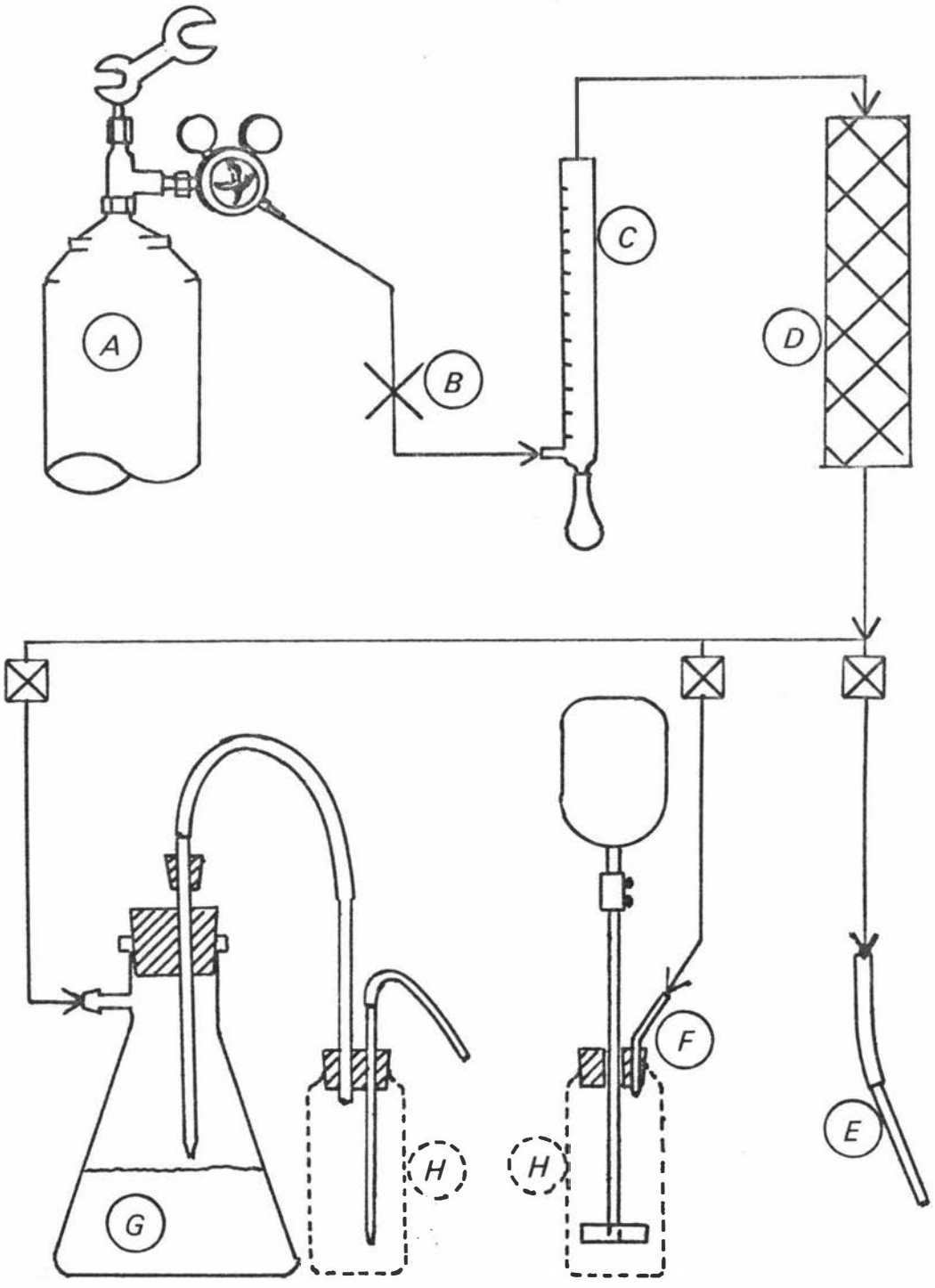

FIGURE 3.9 Cell Washing Equipment - Diagram

A Cylinder and regulator for oxygen-free nitrogen
B Needle valve
C Soap-bubble flow-meter
D Glass wool filter
E 200mm thick-walled glass tube, used as stirring rod
F Stirrer with nylon blade and provision for gas sweeping
G 2 l buchner funnel containing buffer at 30^{0}C
H 500 ml polypropylene centrifuge bottle

Symbols used are those defined in Figure 3.6

controlled temperature room at 30°C or in a water bath, with continuous flushing with sterile nitrogen gas.

After 1 h equilibration time, 2 ml of cell suspension was used to inoculate each tube, and triplicate 3 ml aliquots of the suspension were taken for dry weight determinations.

After incubation, 6.00 mg of H.P.L.C. internal standard were added to each tube as a 0.2% w/v solution in 0.05 M NaOH, and the suspensions were centrifuged at 6000 × g for 20 min. The supernatant liquid was decanted and stored at 4°C for no longer than 48 h before extraction for H.P.L.C. analysis.

3.9 EXTRACTION AND H.P.L.C. SAMPLE PREPARATION

3.9.1 Solvent Extraction

Cell-free culture supernatant liquid (*ca* 20 ml) was acidified to pH 2.0 with 5 M HCl, and extracted four times with chloroform (20 ml). The combined extracts were filtered through Whatman No. 54 filter paper, and evaporated to dryness *in vacuo*. The residue was dissolved in two 0.5 ml aliquots of methanol-0.17M aq acetic acid, 95:5 which were then combined and Swinney filtered (Millipore type FH, 0.5 μm filter). Filtered extracts were stored at -18°C in sealed tubes, then centrifuged briefly before the H.P.L.C. analysis.

3.9.2 Freeze-Dry Extraction

Cell-free culture supernatant liquid (*ca* 20 ml) was acidified to pH 4.5 with 2.5 M H_2SO_4, and freeze dried on a Virtis Bench-top Freeze Dryer Model 10.020 (The Virtis Co. Inc., New York, U.S.A.). The residue was extracted twice with 0.017 M methanolic acetic acid (15 ml), once at room temperature and then once at 60°C. The extracts were combined and filtered through Whatman No. 54 filter paper into a centrifuge tube containing acetone (20 ml). Centrifugation for 10 min at 3150 × g removed the insoluble material. The supernatant liquid was then evaporated to dryness *in vacuo*, and the residue dissolved and stored as for the solvent extract (refer section 3.9.1).

3.10 PRODUCT CHARACTERISATION

This section describes the fermentation of *Clostridium bifermentans* strains with various bile acid substrates, and the isolation and identification of transformation products.

All infra-red spectra are reproduced in Appendix 1. Melting points for subsection 3.10.1 were determined on the Kofler hot-stage apparatus, and for section 3.10.2 on the Leitz hot-stage apparatus.

3.10.1 Action of Strain ATCC 9714 on Cholic Acid

Cholic acid (0.05% w/v) in Todd Hewitt Broth (5 l) was incubated with *Cl. bifermentans* ATCC 9714 for 7 days at 37^{0}C in the 7-litre fermenter vessel. pH was controlled at not less than pH 7.0 with 1 M NaOH. The medium was agitated at 200 rev/min, and its surface was swept with N_2-CO_2,9:1.

Spent medium (4.5 l) was evaporated on a steam bath to 500 ml and then freeze dried. The residue was suspended in methanol (500 ml) and concentrated sulphuric acid was added until the solution was at pH 1. The resulting proteinaceous precipitate was removed by filtration, and the filtrate was refluxed for 1 h. Methanol and volatile methyl esters (of C_2 to C_6 acid fermentation products) were removed from the reaction mixture by evaporation *in vacuo*. The residue was suspended in water, extracted with ethyl acetate, and the organic layer evaporated to dryness *in vacuo*. After dissolution in methanol (25 ml), this residue was hydrolysed by adding 1 M NaOH (10 ml) and refluxing for 0.5 h. The solution was acidified and the bile acids extracted into chloroform. The chloroform extracts were evaporated to dryness and re-dissolved in methanol (25 ml). A 5 ml sample was subjected to reverse phase partition chromatography, as follows.

Celite Hyflo Super-Cel was washed by the method of Sjövall (1964) and then dried at 110^{0}C for 5 h. Silylation was achieved by contacting the Super-Cel (40 g) with dichlorodimethylsilane (10 ml) in anhydrous chloroform (40 ml). The hydrophobic Super-Cel was filtered and washed with chloroform followed by methanol. After drying at 110^{0}C for 2 days, it was stored in a dessicator. Chromatography of the 5 ml

sample was then performed according to Sjövall (1964), using a mobile phase of methanol-water (1:1) and a stationary phase of chloroform-2-ethylhexanol (1:1).

Fractions shown by T.L.C. to contain mainly deoxycholic acid were pooled and evaporated *in vacuo*. The residue was crystallised from water-saturated ethyl methyl ketone, then recrystallised twice from isopropanol-toluene 60:40. The toluene-deoxycholic acid complex was decomposed by boiling for 1 h in water. An authentic sample of deoxycholic acid was similarly crystallised from isopropanol-toluene and boiled in water. Both materials were dried over P_2O_5 for 4 h at 110°C *in vacuo*. Identity between the microbially produced (m.p. 168-171°C) and authentic deoxycholic acid (m.p. 171-173°C) was established by mixing melting point (m.p. 170-173°C) and superimposability of infra-red spectra.

Fractions containing mainly 7-ketodeoxycholic acid were pooled and evaporated *in vacuo*. The acid (50 mg) was crystallised twice from 0.001 M NaOH (75 ml) by adding 0.01 M HCl. A sample of authentic 7-ketodeoxycholic acid was similarly crystallised. Both materials were dried over P_2O_5 for 4 h at 110°C *in vacuo*, and identity was demonstrated by melting point (174-176°C for both) mixed melting point (172-176°C) and superimposability of infra-red spectra.

3.10.2 Action of Strain SD 10 on Conjugates.

Each of the four conjugates (final concentration 0.05%) was incubated in Todd Hewitt Broth (1.25 l) containing potassium fluoride (25 mM), 8-hydroxyquinoline (0.01 mM) and sodium thioglycollate (0.05% w/v) in the 2-litre fermentation vessel. The medium was agitated at 300 rev/min and sparged with N_2-CO_2, 9:1 at 20 ml/min. The temperature was controlled at 32°C; the pH was controlled between set-points 6.9 and 7.1 with 1.0 M $NaHCO_3$ and 0.5 M H_2SO_4, and the electrode potential was monitored. The conjugate substrates were added 6 h after inoculation, and the fermentation continued for a further 42 h, after which time deconjugation was shown by H.P.L.C. analysis to be complete.

For each conjugate, the spent medium (1.1-1.2 l) was autoclaved for 15 min at 121°C, cooled and adjusted to pH 10.5 with 1 M NaOH.

After filtration with Celite filter aid (0.5% w/v), the beer was passed down a column of the non-ionic macroreticular adsorbent XAD-2 as follows.

Amberlite XAD-2 (100 g) was washed according to Makino and Sjövall (1971), and poured into a glass column 23 mm dia. × 400 mm. The beers were metered through the column at 20 ml/h with a peristaltic pump (Type 10PP60, Quickfit Instrumentation, Stone, Staffordshire, U.K.). The column was then washed until neutral with distilled water (200-300 ml) prior to elution of bile acids with methanol (250 ml). Before re-use, the column was reconditioned by successive washings with acetone (400 ml) and water (600 ml).

The methanol eluate was evaporated *in vacuo* to near dryness and diluted to 40 ml with water. After acidification to pH 2 with 5 M HCl, the solution was extracted three times with chloroform (40 ml). The chloroform extracts were pooled and evaporated *in vacuo*.

Cholic acid was crystallised from the appropriate crude residues with methanol-water 9:1, then recrystallised twice from the same solvent. The product was dried over P_2O_5 for 4 h at 100^{0}C *in vacuo*. A similarly recrystallised authentic sample of cholic acid was used to identify both isolated materials by melting point and mixed melting point criteria (Table 3.3) and by superimposability of infra-red spectra.

The crude deoxycholic acid residues were crystallised from water-saturated ethyl methyl ketone and recrystallised from isopropanol-touene, 1:1. The toluene-deoxycholic acid complex was decomposed by boiling for 1 h in water. The product was dried at 100^{0}C over P_2O_5 for 3 h *in vacuo*. Melting point and mixed melting point data (Table 3.3), together with the superimposability of the infra-red spectrum on that of an authentic sample, confirmed the identity of the isolated material.

TABLE 3.3 Melting Points of Deconjugation Products

Product	Origin	Melting Point ^0C
cholic acid	authentic sample	199-200
cholic acid	from glycocholate	197.5-199.5
cholic acid	mixed authentic and from glycocholate	197-198.5
cholic acid	from taurocholate	198.5-199
cholic acid	mixed authentic and from taurocholate	198.5-199
deoxycholic acid	authentic sample	169.5-171
deoxycholic acid	from glycodeoxycholate	170-171.5
deoxycholic acid	mixed authentic and from glycodeoxycholate	171-172
deoxycholic acid	from taurodeoxycholate	169.5-171
deoxycholic acid	mixed authentic and from taurodeoxycholate	169-171

3.10.3 Action of Strain ATCC 9714 on Conjugates

Each conjugate (0.05% w/v) was fermented in Todd Hewitt Broth (20 ml) contained in 25 ml McCartney bottles for 48 h at 37^0C.

The entire contents of each bottle were extracted by the freeze dry method and the methanol in the final solution was removed by evaporation. The residues were dissolved in 0.1 M NaOH (10 ml), and the solution was allowed to stand over XAD-2 (1 g) for 24 h with occasional swirling. The resin was then washed with water until neutral and extracted with methanol (5 ml) for 24 h. The methanolic extract was decanted and evaporated to *ca* 0.5 ml, and aliquots were examined by T.L.C. (solvent systems TLC1, TLC2 and TLC3).

The products of glycodeoxycholic acid and taurodeoxycholic acid transformation showed R_f values identical to that of an authentic deoxycholic acid standard run in parallel. The products of glycocholic acid and taurocholic acid transformation showed spots of identical R_f to authentic cholic acid as well as faint spots corresponding to 7-ketodeoxycholic acid.

3.11 CALCULATIONS

3.11.1 H.P.L.C. Data Analysis

From the peak heights and known composition of the standard mixture injected in parallel with the sample mixture, a bile acid's response factor (R.F.) can be calculated:

$$R.F. = \frac{PHIS \times WU}{PHU \times WIS}$$

where PHIS = peak height of internal standard (mm)
PHU = peak height of unknown (mm)
WU = weight of unknown (mg)
WIS = weight of internal standard (mg)

For a sample containing X mg of internal standard, the unknown weight of the bile acid being analysed can be calculated:

$$\text{weight} = RF \times X \times \frac{PHU_{sample}}{PHIS_{sample}}$$

where PHU_{sample} = peak height of unknown in sample (mm)
$PHIS_{sample}$ = peak height of internal standard in the sample mixture (mm)

3.11.2 Calculation of Molar Compositions

For each sample, the fermenter volume at sample time was multiplied by the weight of each bile acid in the sample (as determined by H.P.L.C.) and divided by the sample volume analysed (20.00 ml), to give the weights of those bile acids in the fermenter. These were divided by molecular weights and the percentage molar compositions determined.

3.11.3 Mass Balance Calculation

Mass balance checks required an estimate of the extent of the bile acid pool (in moles) remaining in the fermenter. At the first sampling, the number of moles of substrate initially added was reduced by the fraction that the sample volume represented of the fermenter volume. The molar total was similarly reduced for subsequent samples. For each sample, the calculated molar content was compared with that determined analytically.

3.12 DISCUSSION OF METHODS

When bile acids were used to examine the H.P.L.C. column efficiencies, using the method of Johnson and Stevenson (1978), both columns were found to have greater than 600 theoretical plates. This means that peaks eluted within about 10 minutes of injection were narrow, making accurate area measurement by non-electronic means impossible without excessive chart use. For this reason, peak heights were used.

Figure 3.10 is the differential refractometer linearity characteristic for peak heights determined for the four free bile acids including the internal standard 3α,12α-dihydroxy-5β-chol-11-en-24-oic acid. Since the 7-ketodeoxycholic acid response deviates from a straight line at higher operating levels, the peak height ratio of the 7-ketone to the 11-ene varies from small injections to large. To overcome this problem, several standard mixtures were used, each with a different ratio of bile acid to 11-ene. The appropriate standard ratio was then available.

A complete error analysis was conducted using the estimates of uncertainly for calibrated glassware given by Vogel (1961). The 95% confidence limits for the ratios of cholic acid, deoxycholic acid and 7-ketodeoxycholic acid to 3α,7α-dihydroxy-5β-chol-11-en-24-oic acid were determined by statistically analysing 85 actual samples, each injected in duplicate.

These confidence limits and the linearity characteristic (Figure 3.10) were used in error calculation. It was found that cholic acid could be quantitated to ±5%, deoxycholic acid to ±8%, and 7-ketodeoxycholic acid to ±10%.

The error in mass balance checks increased as more samples were taken. The uncertainty was also greater in runs where the bile acid substrate was added after inoculation.

For a sample composition of 100% 7-ketodeoxycholic acid, with late substrate addition and six previous samples, the error is ±12.1%.

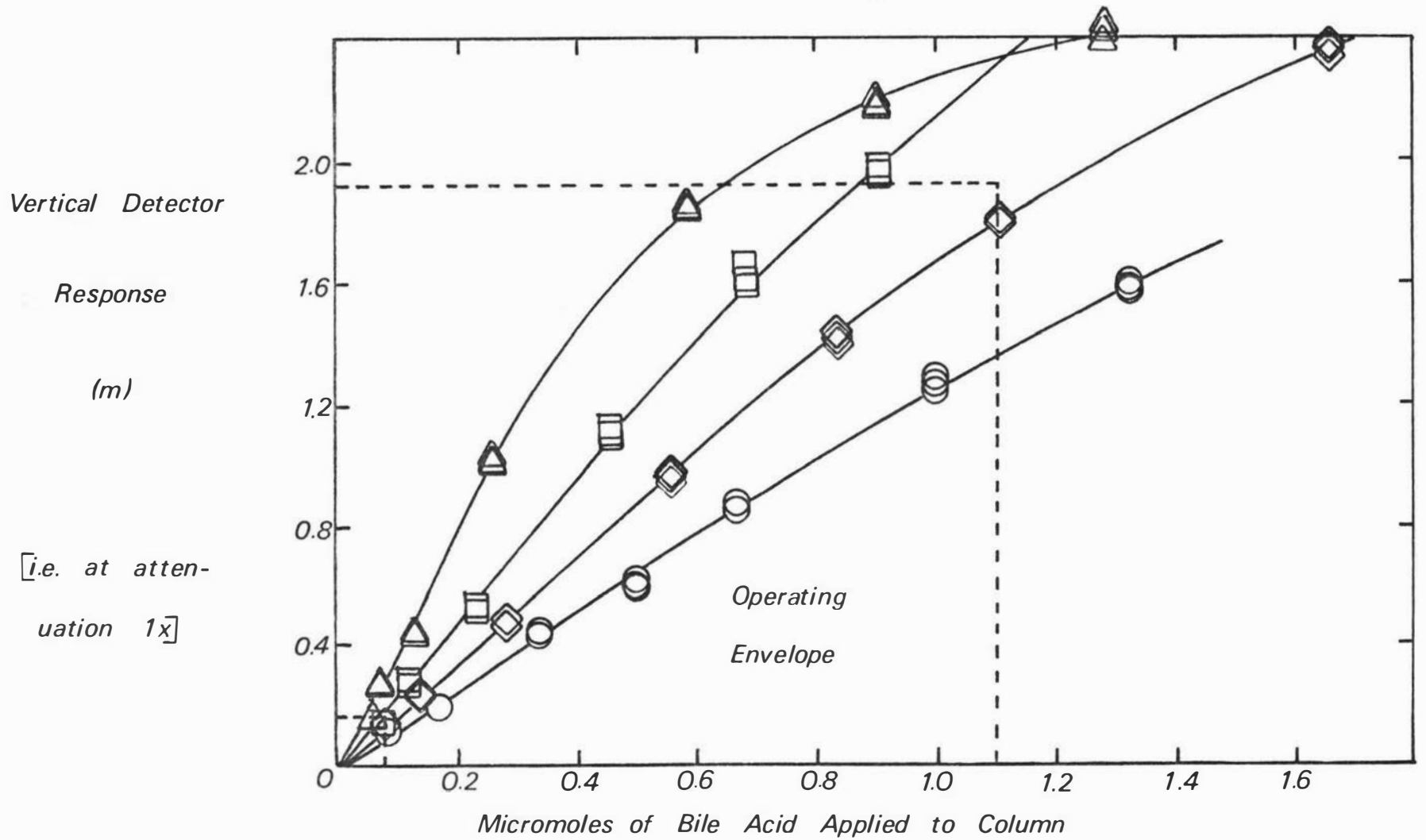

FIGURE 3.10 Differential Refractometer Linearity Characteristic

Deoxycholic acid (◯), 7-ketodeoxycholic acid (△), cholic acid (□), 3α,7α-dihydroxy-5β-chol-11-en-24-oic acid (◇)

For the first sample, containing only cholic acid, the substrate having been present in the original medium, an error of ±5.5% is estimated.

This error analysis took no account of uncertainties in extraction or transfer. Throughout the work, all data not mass balancing to within ±10%, except in special circumstances, were discarded. This occurred with fewer than 5% of the analyses made. indicating that any losses which occurred with either extraction method (solvent or freeze-dry) occurred identically to both the internal standard and to the bile acid being quantitated.

CHAPTER 4 EXPERIMENT 1 : EFFECT OF pH AND ATMOSPHERIC COMPOSITION ON CHOLIC ACID TRANSFORMATION

4.1 INTRODUCTION

A major part of any fermentation study is the identification of the important variables. It was planned to perform this exercise with a statistically designed screening experiment. However, some variables are of such importance that base values must be determined to provide a good starting point for screening. Two variables previously implicated in the literature as being important to dehydroxylation are pH and the gas with which the anaerobic atmosphere is maintained. The purpose of the experiment described in this chapter was to determine the approximate pH optimum and which of the several available gas compositions was superior.

4.2 EXPERIMENT DESIGN

Maddox and Richert (1977) have shown that in microbiology, in contrast with the chemical industry, complex interactions between variables are likely to be important. For this reason, and because of the efficiency with which information can be derived from relatively few trials, the statistical experimental design approach was adopted.

Since it was desired to determine the optimum pH for dehydroxylation (and not merely trends), and to determine which of the three gas compositions was superior, each variable (factor) needed to be tested at three levels. The basic 3^2 design was augmented with two replicate centre points to assist in significance and lack of fit testing.

The design used, therefore, was a fully randomised 3^2 factorial experiment with triplicate centre points. The three levels of pH were not less than pH 6.0, 7.0, and 8.0, which were coded in the design matrix as having values -1, 0, or 1 respectively. The three levels of gas were N_2-CO_2 9:1, oxygen-free nitrogen and hydrogen,

coded as -1, 0, and 1 respectively. The triplicate centre point was pH 7.0 with nitrogen. Table 4.1 lists the run order, pH, gases and design matrix.

TABLE 4.1 Design and Run Order, Experiment 1

Run Order	Gas (uncoded)	pH	Gas (coded)	pH
2	H_2	6.0	-1	-1
4	H_2	7.0	-1	0
8	H_2	8.0	-1	1
9	N_2	6.0	0	-1
7	N_2	7.0	0	0
6	N_2	7.0	0	0
3	N_2	7.0	0	0
5	N_2	8.0	0	1
11	N_2CO_2	6.0	1	-1
1	N_2CO_2	7.0	1	0
10	N_2CO_2	8.0	1	1

4.3 FERMENTATION CONDITIONS

Each of the eleven runs was conducted in the 7-litre fermenter vessel with *Clostridium bifermentans* ATCC 9714, using a 5 l working volume of Todd Hewitt Broth. Cholic acid substrate was added to the medium before sterilisation to a final concentration of 0.05% w/v. pH control was one-way using 1M NaOH and the medium surface was swept with the appropriate gas at 30 ml/min. After inoculation with a standard 24 h 20 ml inoculum in Todd Hewitt Broth containing cholic acid (0.05%), fermentation was continued for 7 days, during which time regular samples were taken for cell count determination and analysis of bile acid composition. The vessel contents were maintained at 37^0C throughout, and agitated at 200 rev/min.

4.4 RESULTS

Raw run data are presented diagrammatically in Figures 4.1 to 4.11. Each figure depicts the progress of transformation, the pH and cell count. In addition, the mass balance figure is given. Smooth curves were drawn to better estimate the 7-day bile acid compositions listed in Table 4.2.

TABLE 4.2 The Effect of pH and Gas on Cell Growth and Cholic Acid Transformation

Run Order	Gas	pH	7KD	C	D	Growth
			(molar percent yield)			(L.M.C.)
2	H_2	6	13.7	79.7	6.6	8.27
4	H_2	7	31.6	62.0	6.4	8.60
8	H_2	8	22.9	74.9	2.2	8.13
9	N_2	6	32.0	62.1	5.9	8.60
7	N_2	7	38.2	53.6	8.2	8.67
6	N_2	7	35.8	55.6	8.6	8.56
3	N_2	7	36.5	54.3	9.2	8.62
5	N_2	8	28.7	70.6	0.7	8.25
11	N_2CO_2	6	20.9	75.7	3.4	8.08
1	N_2CO_2	7	31.7	49.0	19.3	8.60
10	N_2CO_2	8	35.3	62.9	1.8	8.38

L.M.C. is the logarithm to base 10 of the maximum number of cells (including spores) observed during a run.

Bile acid values are 7-day yields.

The third significant figure in bile acid yields was preserved for use in computer regression programmes.

4.4.1 Statistical Analysis

The data in Table 4.2 were analysed by multiple linear regression using a commercial computing package, MINITAB (version II, July 1 1976, Pennsylvania State University). Statistically insignificant terms

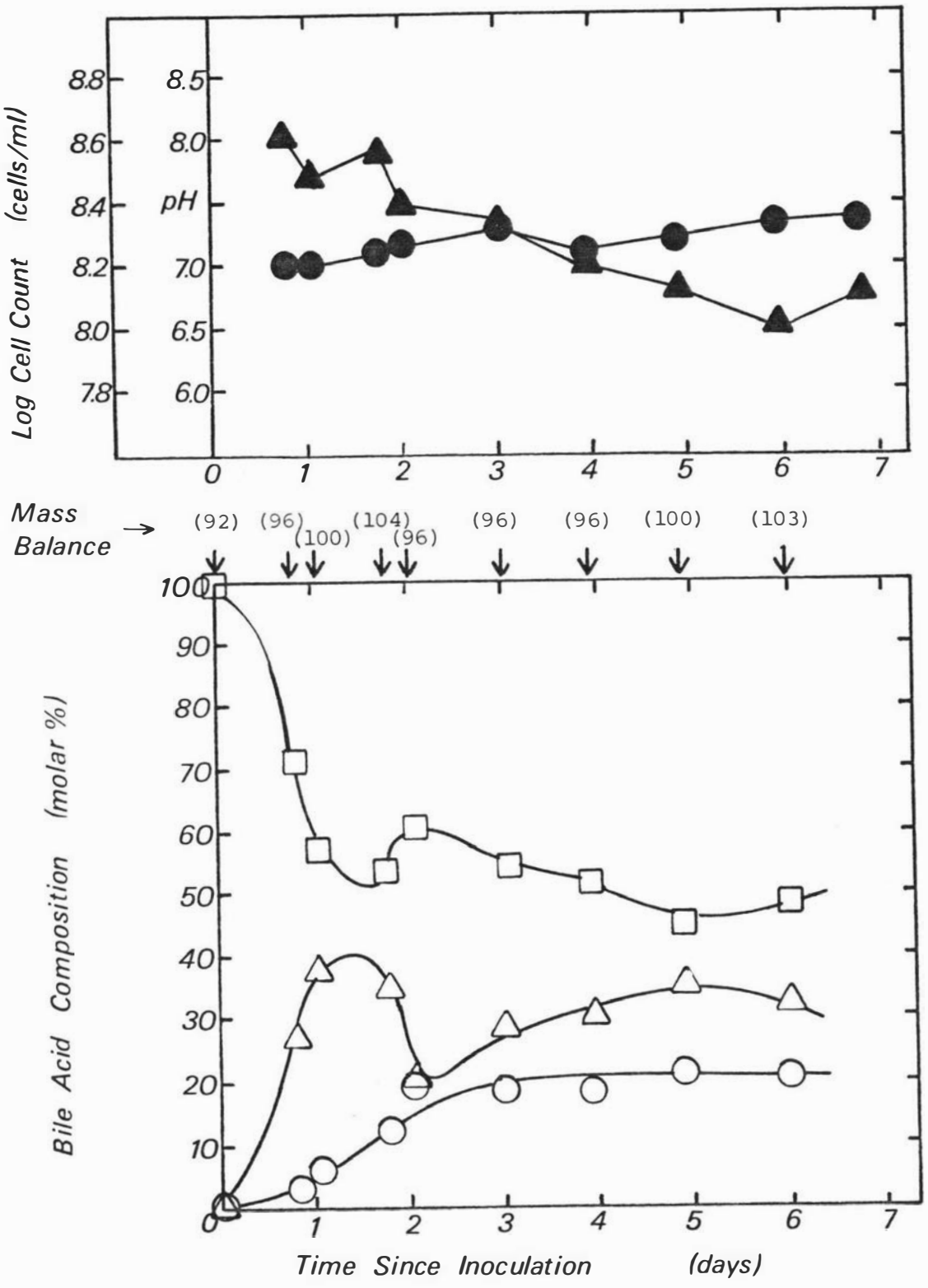

FIGURE 4.1 Course of Growth and Transformation for Experiment 1, Run 1

Deoxycholic acid (○), 7-ketodeoxycholic acid (△), cholic acid (□), pH (●), cell count (▲). The "Mass Balance" figure is defined in section 3.10.2. "Bile Acid Composition" is normalised to a 100% mass balance. Experimental conditions are given in the text.

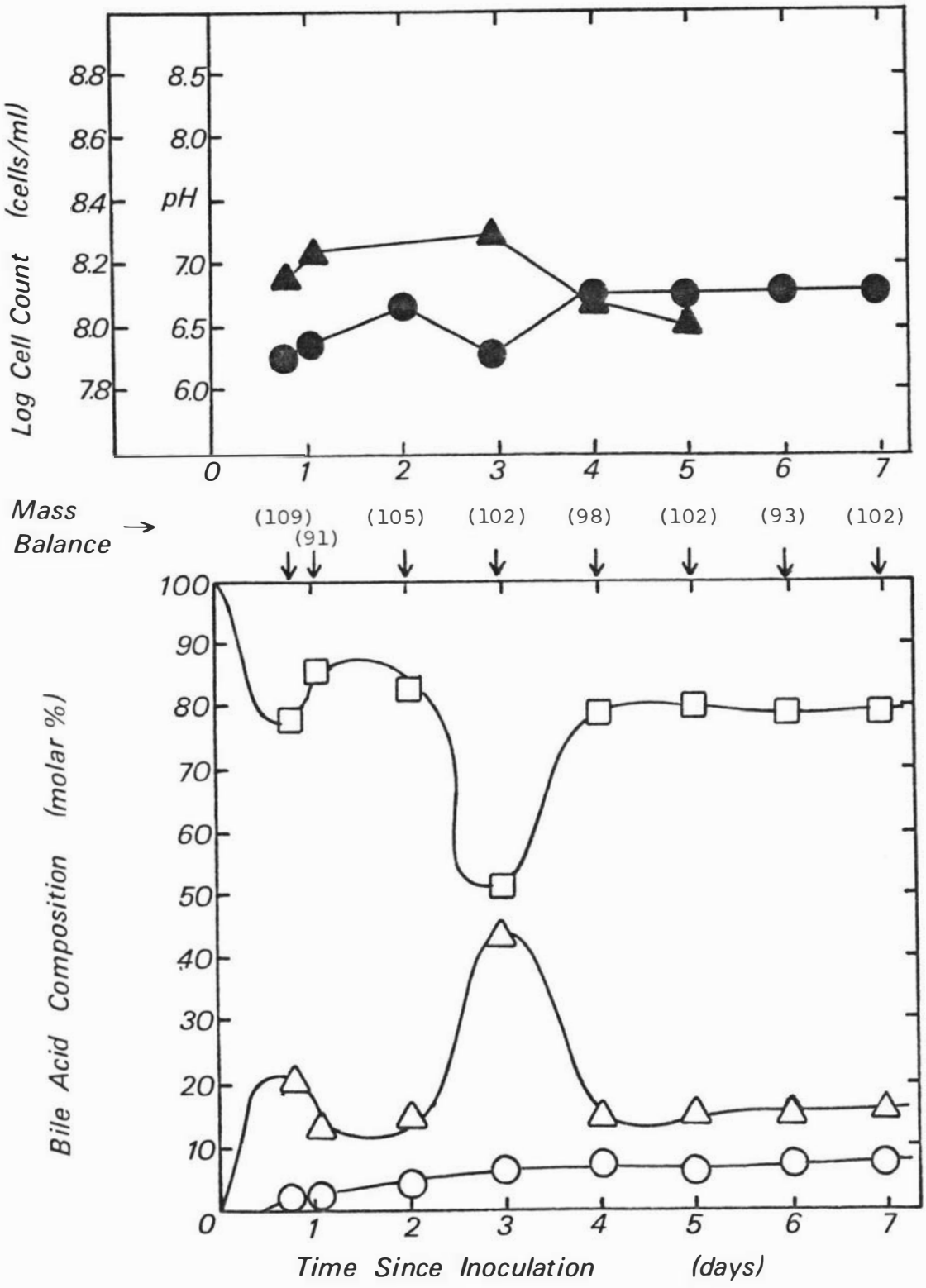

FIGURE 4.2 Course of Growth and Transformation for Experiment 1, Run 2

Legend as for Figure 4.1

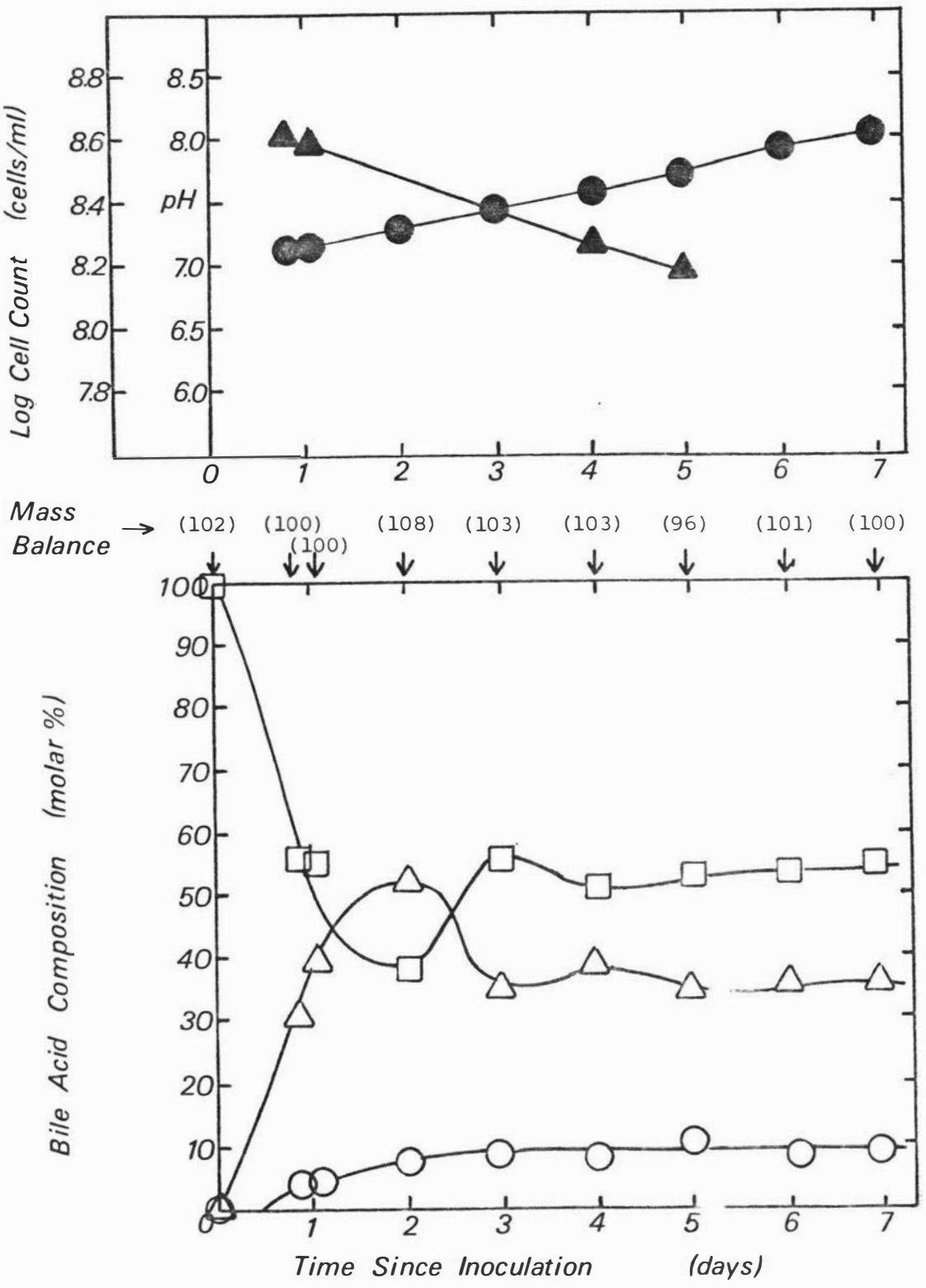

FIGURE 4.3 Course of Growth and Transformation for Experiment 1, Run 3

Legend as for Figure 4.1

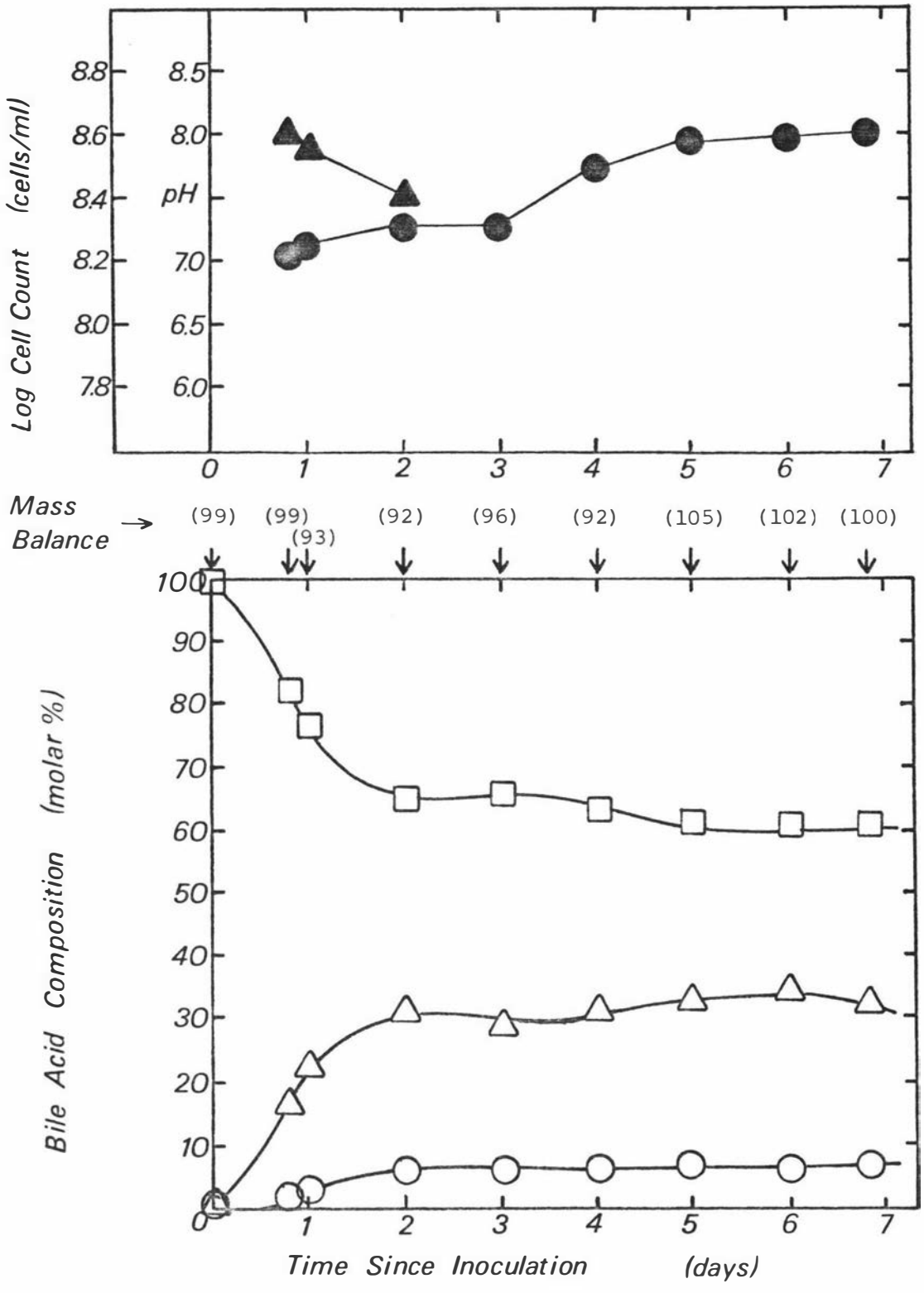

FIGURE 4.4 Course of Growth and Transformation for Experiment 1, Run 4

Legend as for Figure 4.1

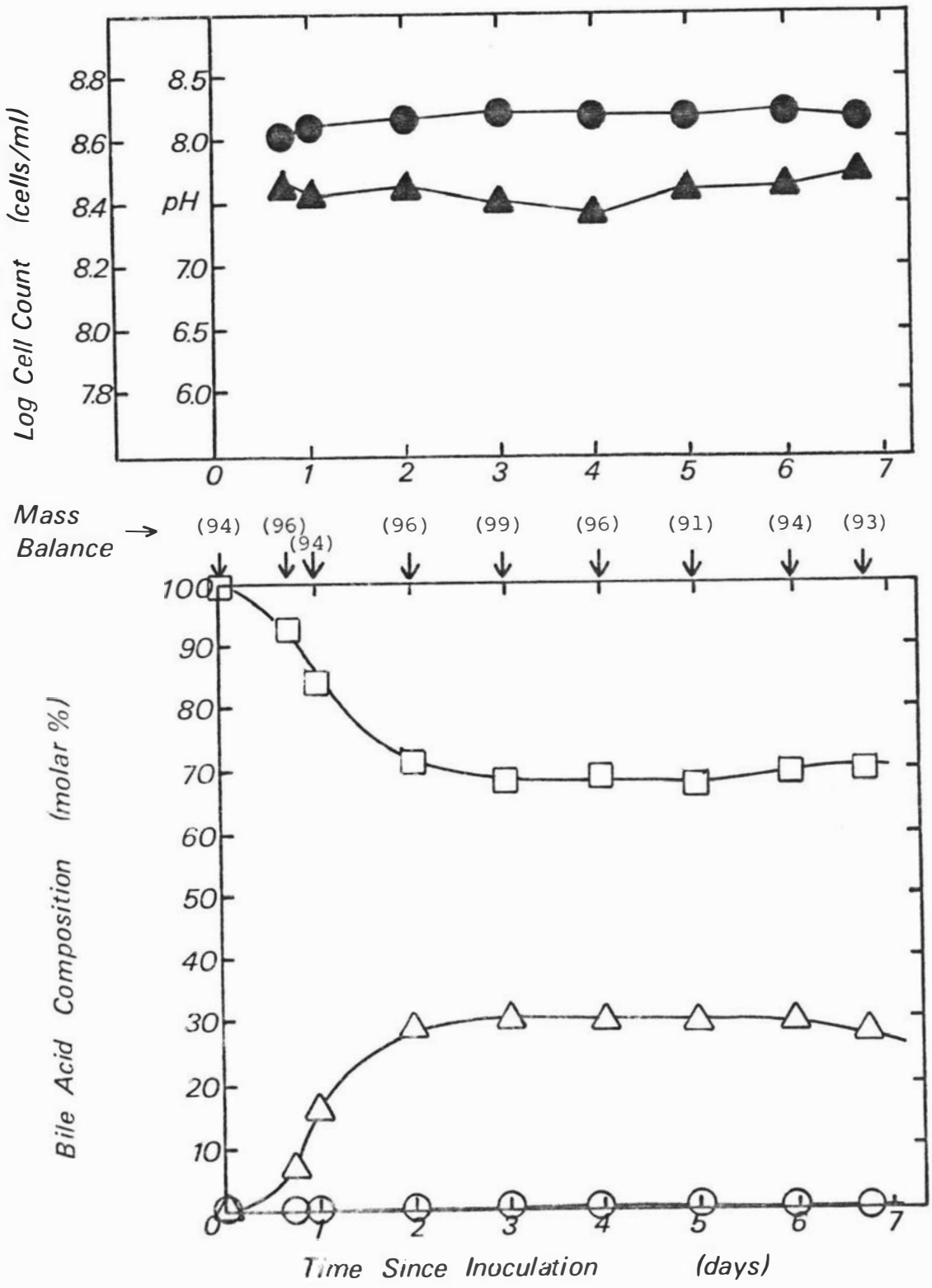

FIGURE 4.5 Course of Growth and Transformation for Experiment 1, Run 5

Legend as for Figure 4.1

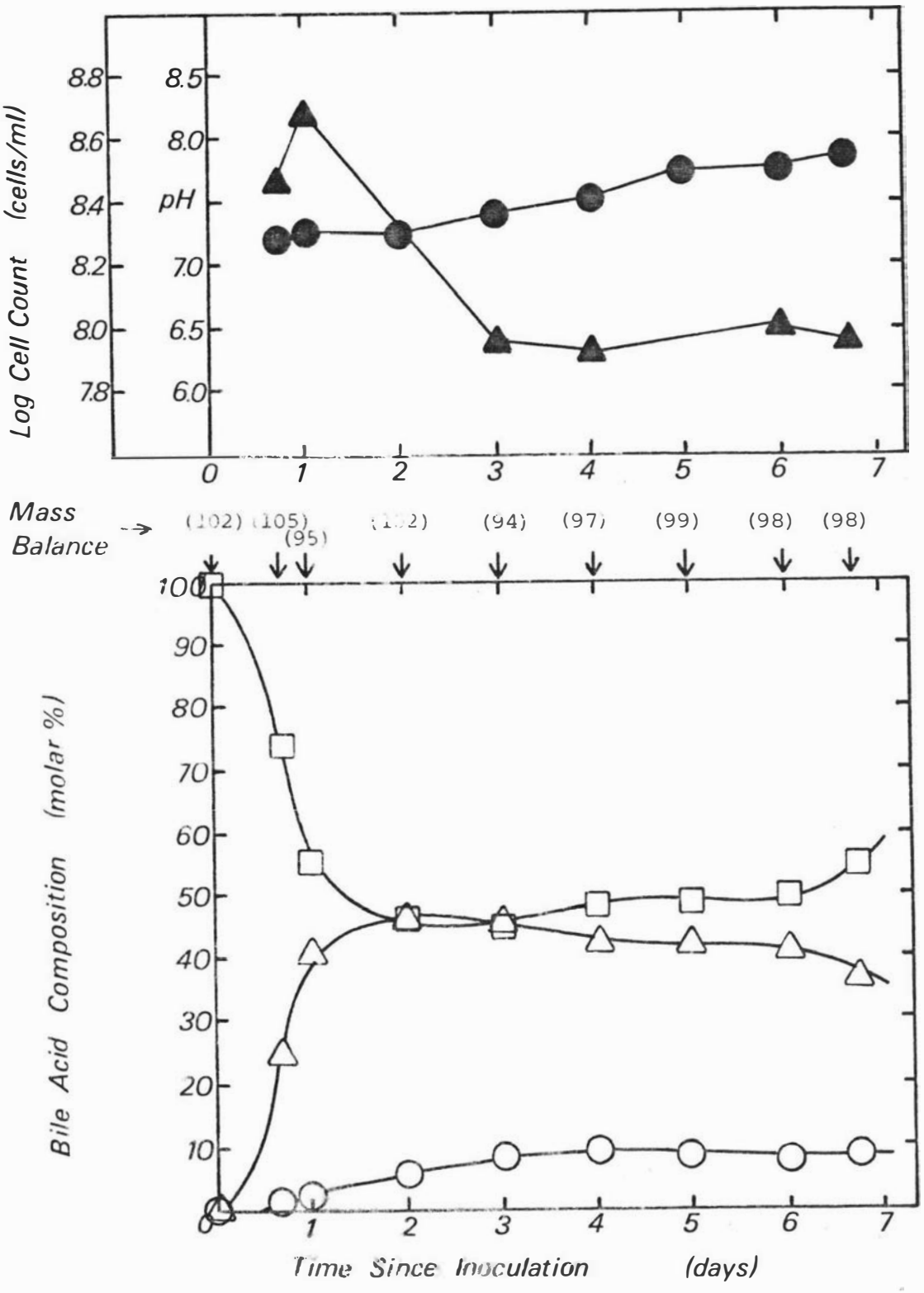

FIGURE 4.6 Course of Growth and Transformation for Experiment 1, Run 6

Legend as for Figure 4.1

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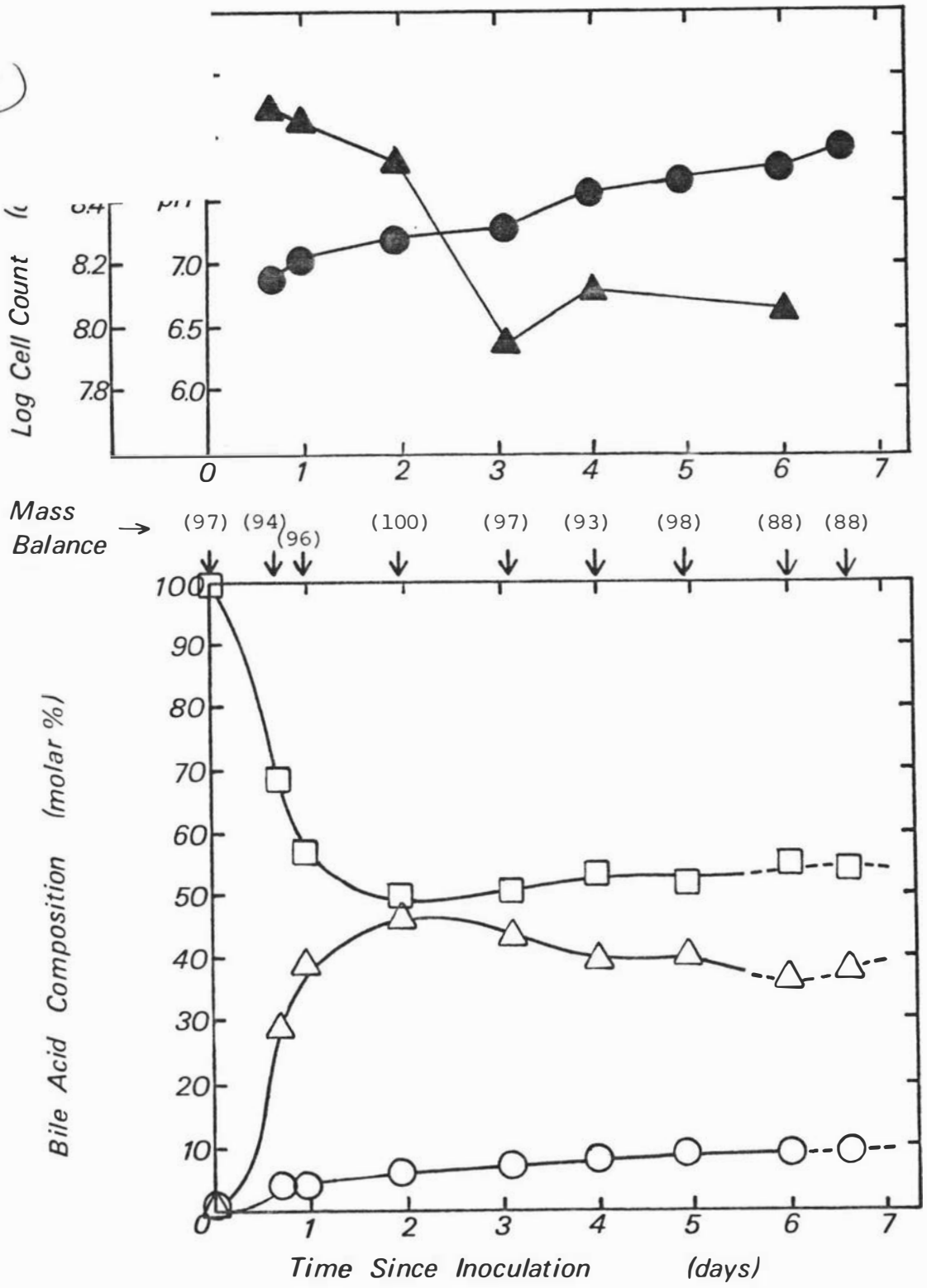


FIGURE 4.7 Course of Growth and Transformation for Experiment 1, Run 7

Legend as for Figure 4.1

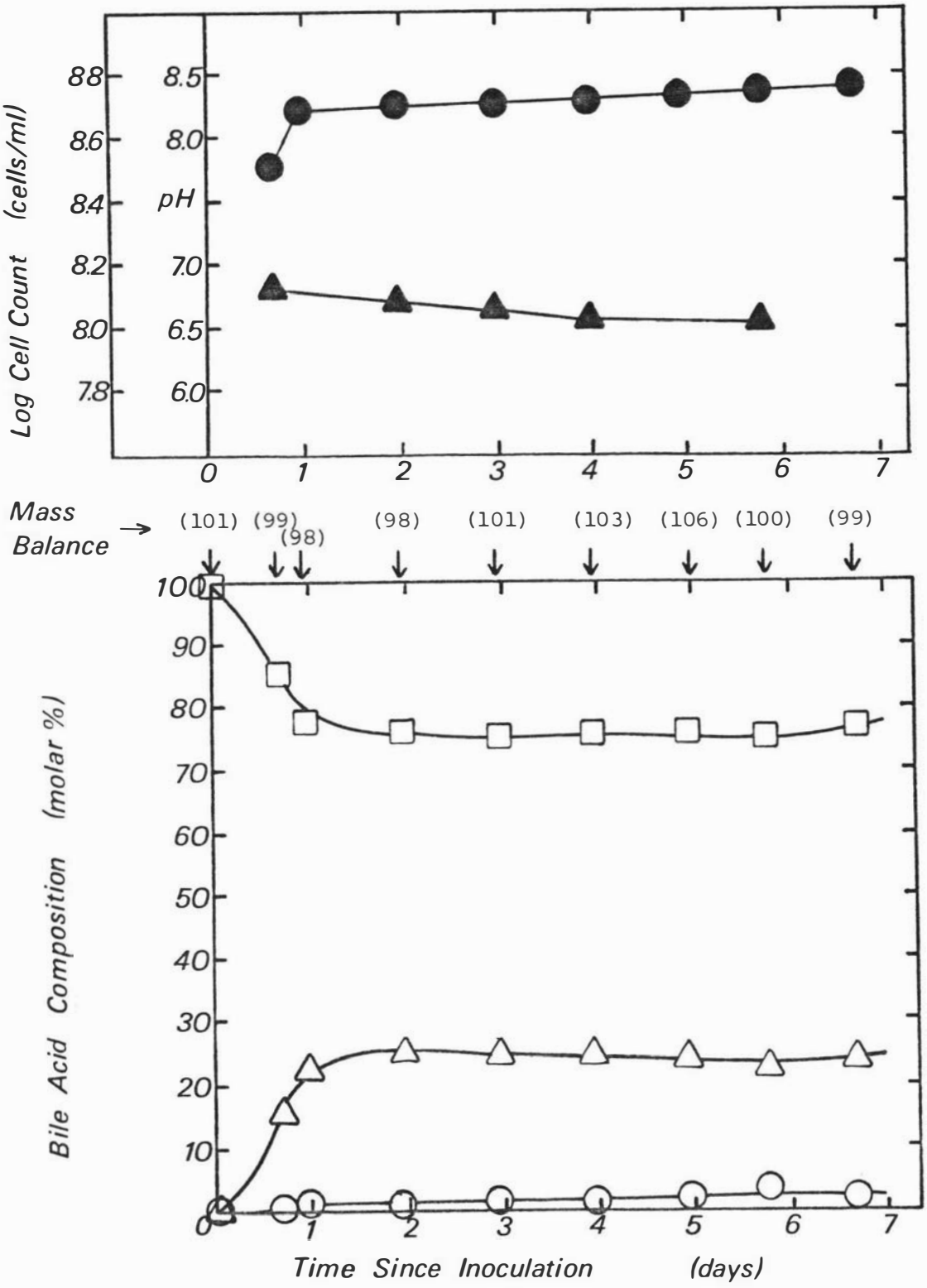


FIGURE 4.8 Course of Growth and Transformation for Experiment 1, Run 8

Legend as for Figure 4.1

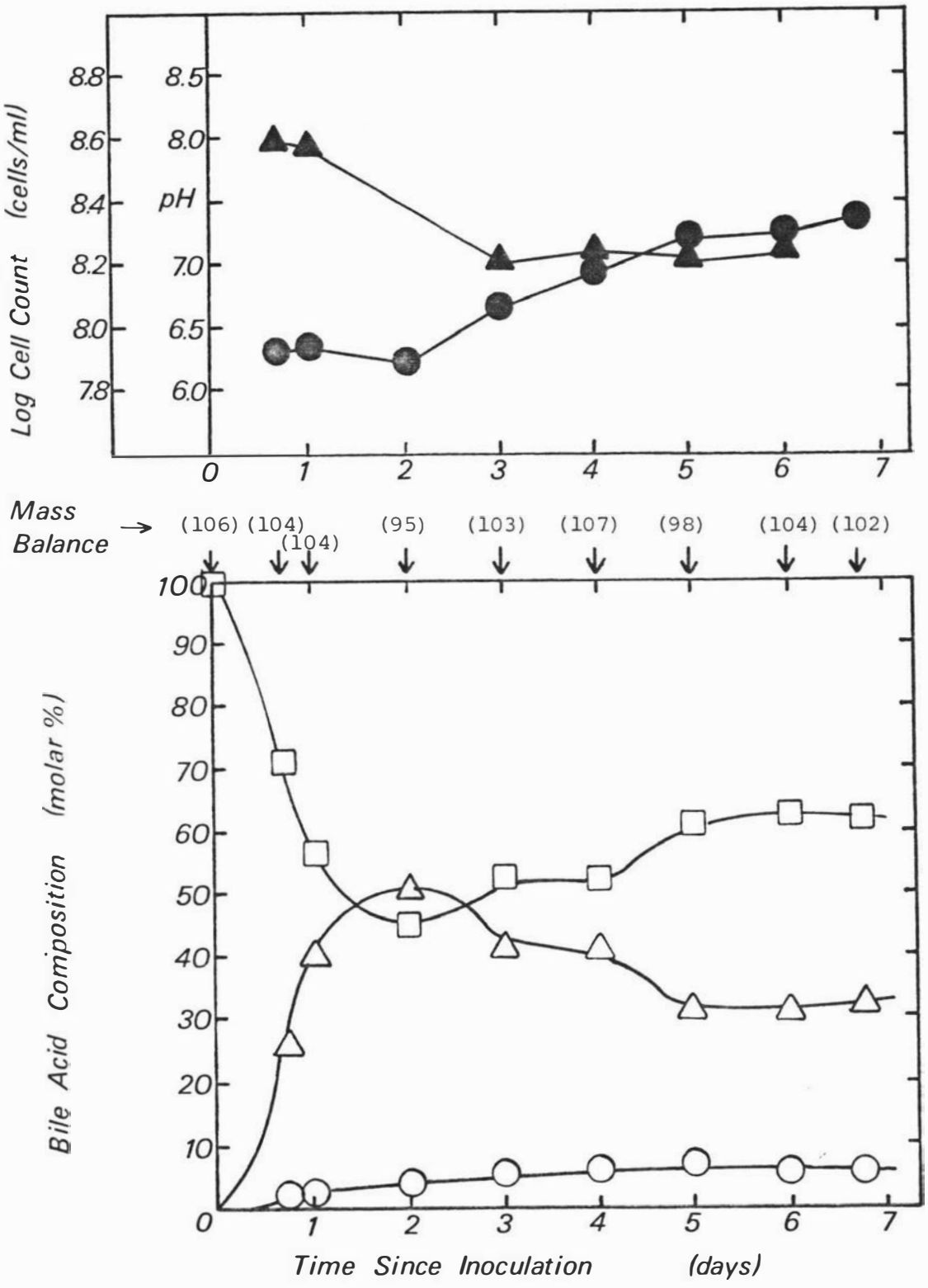


FIGURE 4.9 Course of Growth and Transformation for Experiment 1, Run 9

Legend as for Figure 4.1

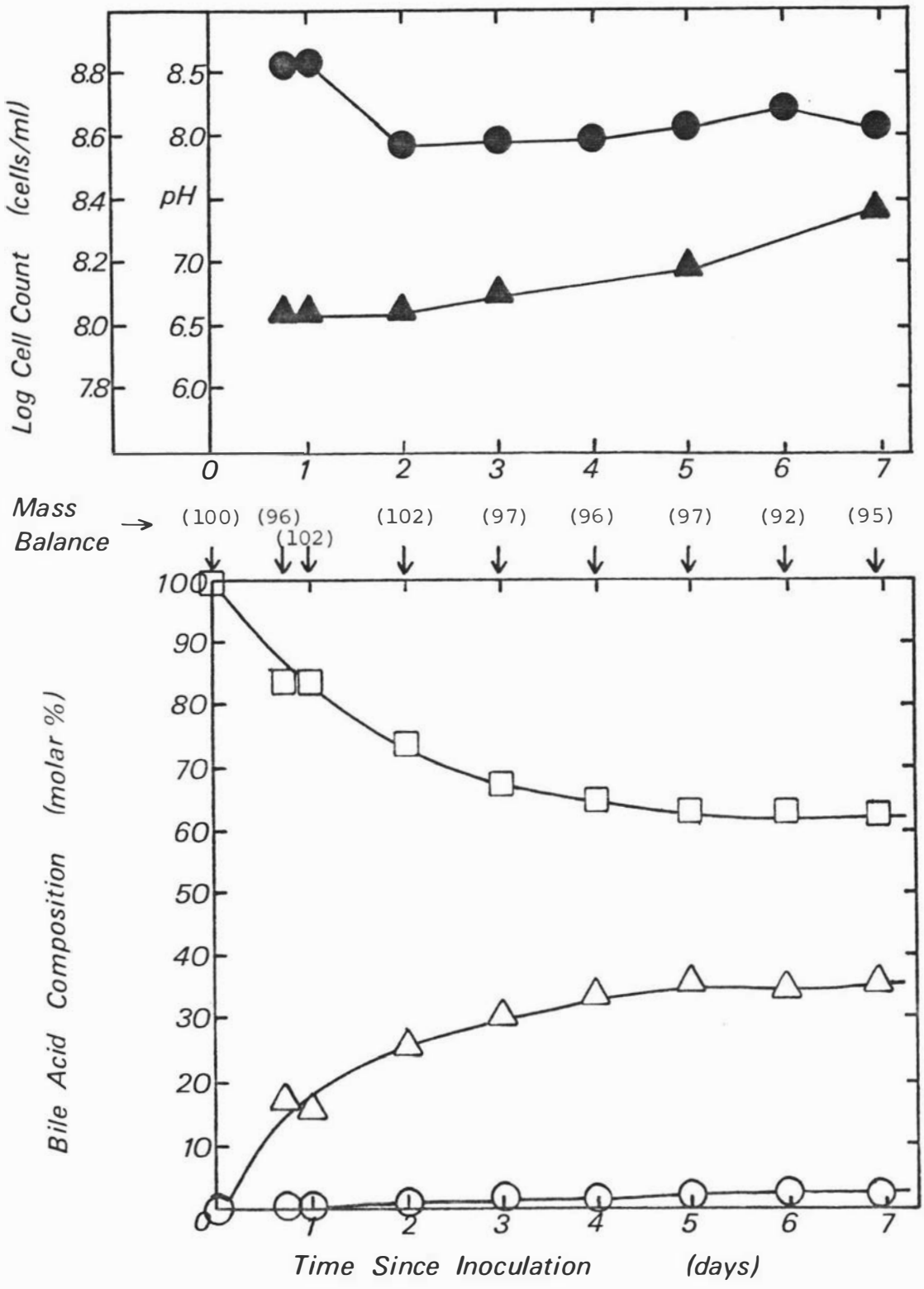


FIGURE 4.10 Course of Growth and Transformation for Experiment 1, Run 10

Legend as for Figure 4.1

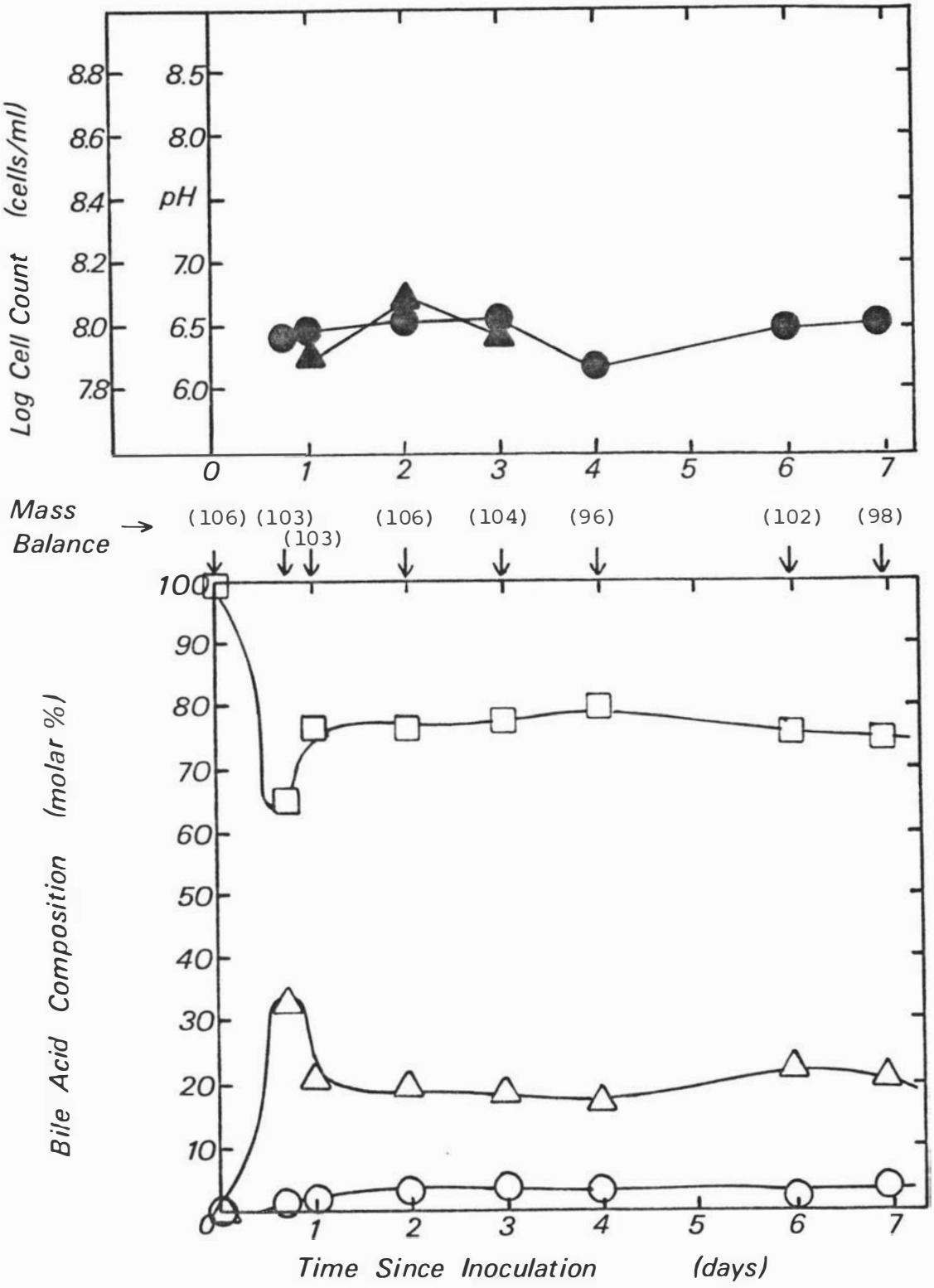


FIGURE 4.11 Course of Growth and Transformation for Experiment 1, Run 11

Legend as for Figure 4.1

(t-test) were rejected from the full model to establish a parsimonious model. The generalised full model has the form:

$$\hat{Y} = \beta_0 + \beta_A \cdot A + \beta_B \cdot B + \beta_{AB} \cdot A \cdot B + \beta_{AA} \cdot A^2 + \beta_{BB} \cdot B^2 + \beta_{AAB} \cdot A^2 \cdot B +$$

$$\beta_{ABB} \cdot A \cdot B^2 + \beta_{AABB} \cdot A^2 \cdot B^2$$

where $\hat{Y}$ is the value of the variable being regressed as predicted by the model. The β values are coefficients, A is the coded value of pH, B the coded value of gas constrained as integer and between -1 and 1 inclusive. β_0 is the grand average, or Y-intercept.

The full regressions are presented in Table 4.3. Asterisks (*) are used to indicate those coefficients determined to be significant by a t-test with the two degrees of freedom afforded by the replicate centre points. Parsimonious models, examples of residual plots and details of lack of fit tests appear in Appendix 2.

TABLE 4.3 Full Regression Models for Experiment 1

Term	7KD	C	D	ln(D)	L.M.C.
β_0	36.8***	54.5***	8.7***	2.16***	8.62***
β_A	-1.6	4.2*	-2.6**	-1.07***	-0.18*
β_B	-0.0	6.5**	-6.4***	-0.55***	-0.00
β_{AB}	-1.3	2.0	-0.7	-0.12	-0.11
β_{AA}	-6.5*	11.8***	-5.4***	-1.45***	-0.19
β_{BB}	-5.2*	1.0	4.2**	0.25*	-0.02
β_{AAB}	-4.8*	-2.5	7.4***	0.77***	-0.02
β_{ABB}	7.6**	-8.6**	1.1	0.63***	0.22*
β_{AABB}	-2.0	6.0*	-4.0**	0.16	-0.19

*** - statistically significant at the 1% level
** - statistically significant at the 2% level
* - statistically significant at the 5% level

Three residual plots were performed on each parsimonious model to check that the assumptions necessary for the regression were valid:

(a) Residual Plot 1. Standard residuals were plotted against run order to check the absence of time trends.

(b) Residual Plot 2. Standard residuals were plotted against the estimated value of the variable being modelled to check the equality of residuals.

(c) Residual Plot 3. Standard residuals were ordered and plotted against normal random numbers to check the normality of residuals.

Only in the case of deoxycholic acid yield was a residual plot abnormal. Here, residuals decreased with increasing estimated yield, as shown in Figure 4.12. For this reason, the natural logarithm of deoxycholate yield was regressed and a 6-term parsimonious model developed which showed adequate residual plots. An example of each of the two other types of residual plot is included in Appendix 2.

Lack of fit was tested for each parsimonious model by calculating the F ratio of the mean square due to lack of fit to the mean square due to pure error. In no case was the lack of fit significant at the 5% level.

The predictions of the three major parsimonious models are depicted in Figures 4.13 to 4.15. Since gas is a qualitative variable, the response surface is constrained to lines through the three integer points -1, 0, +1 on the gas axis.

Table 4.4 is the matrix of correlation coefficients between each pair of response variables.

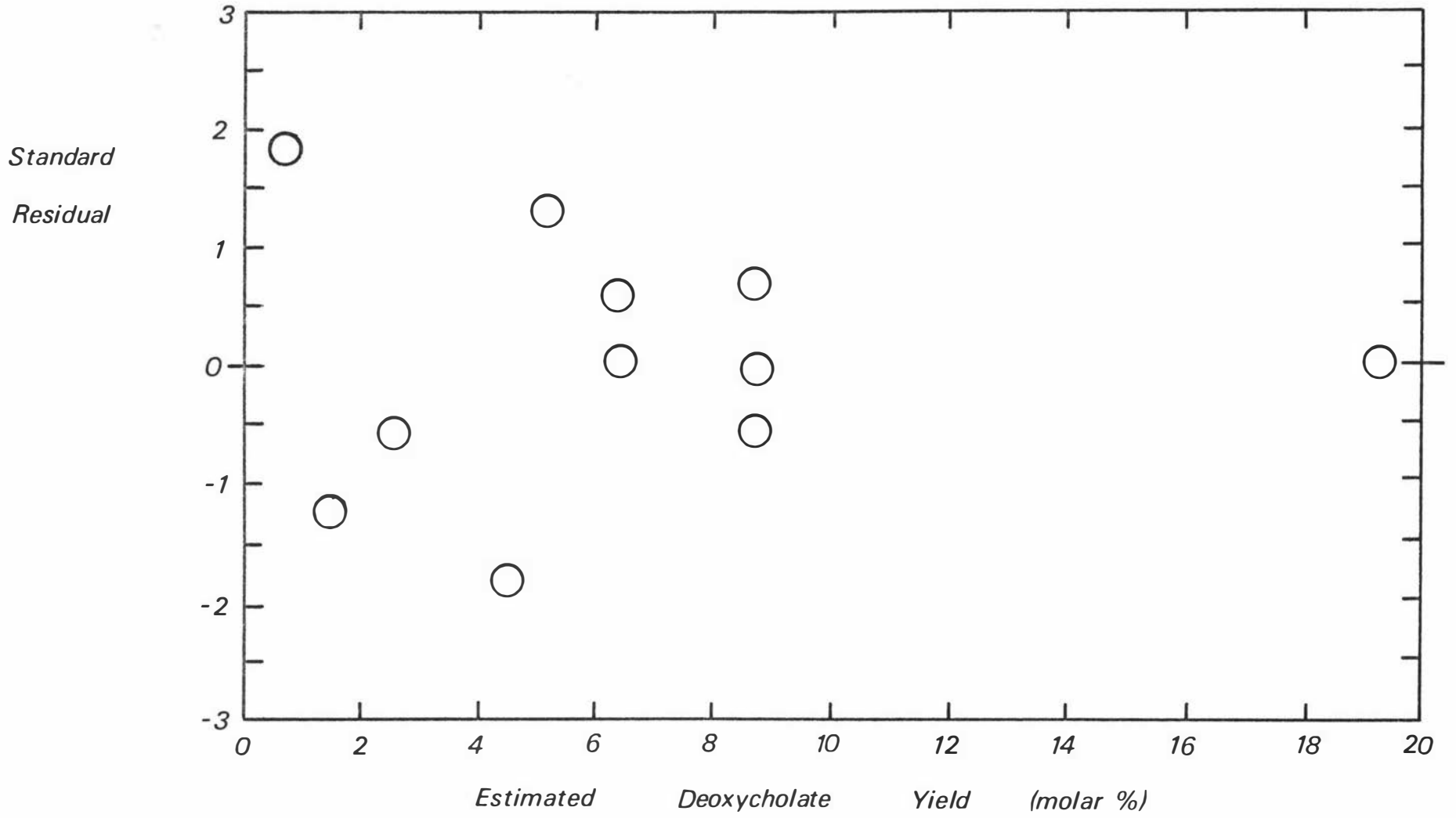


FIGURE 4.12 Residual Plot Number 2 for the Parsimonious Model for Deoxycholate Yield

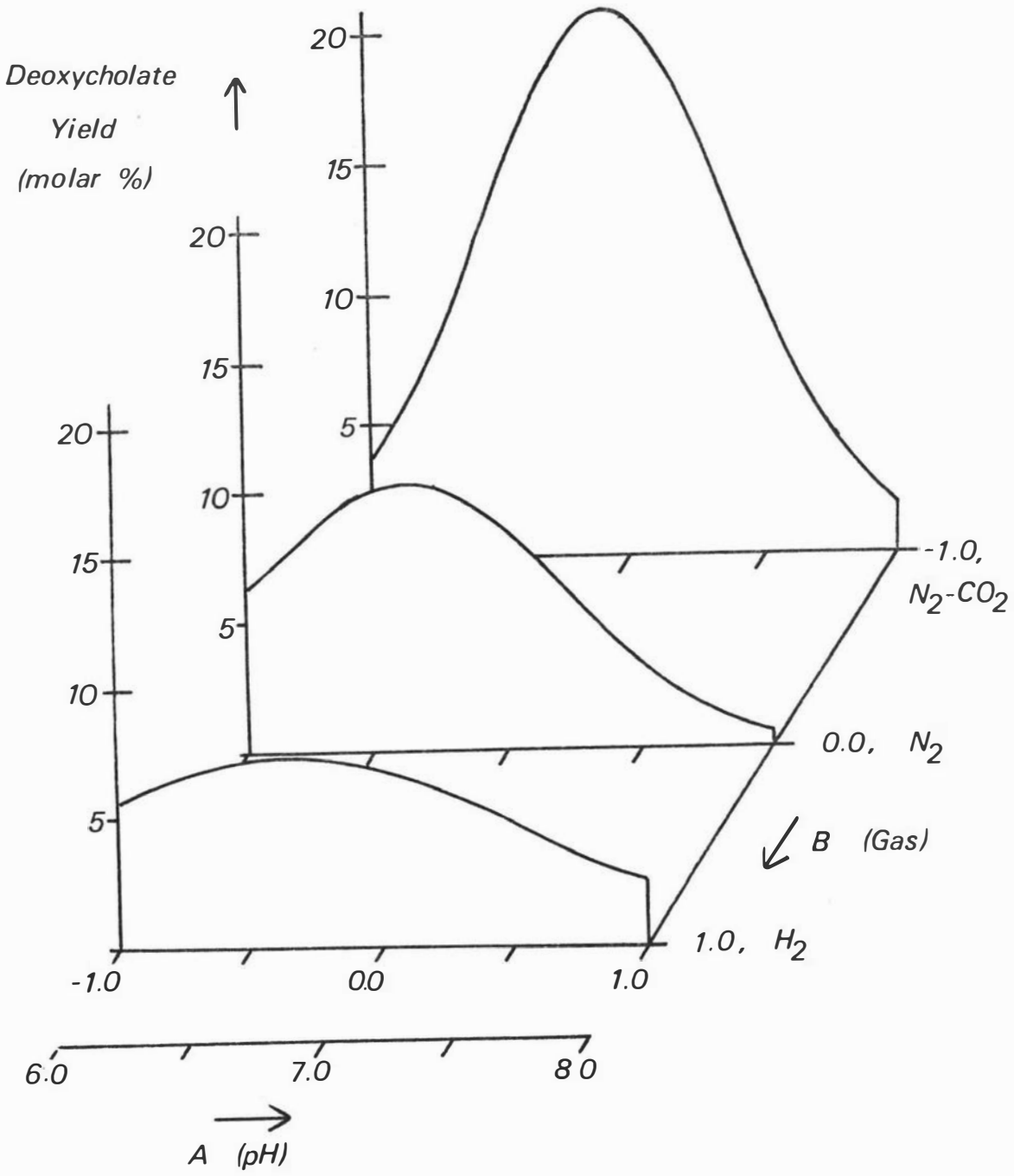


FIGURE 4.13 Predicted Deoxycholate Yield (via log-transform model)

The model from which this figure is drawn is presented in Appendix 2, Table A2.4.

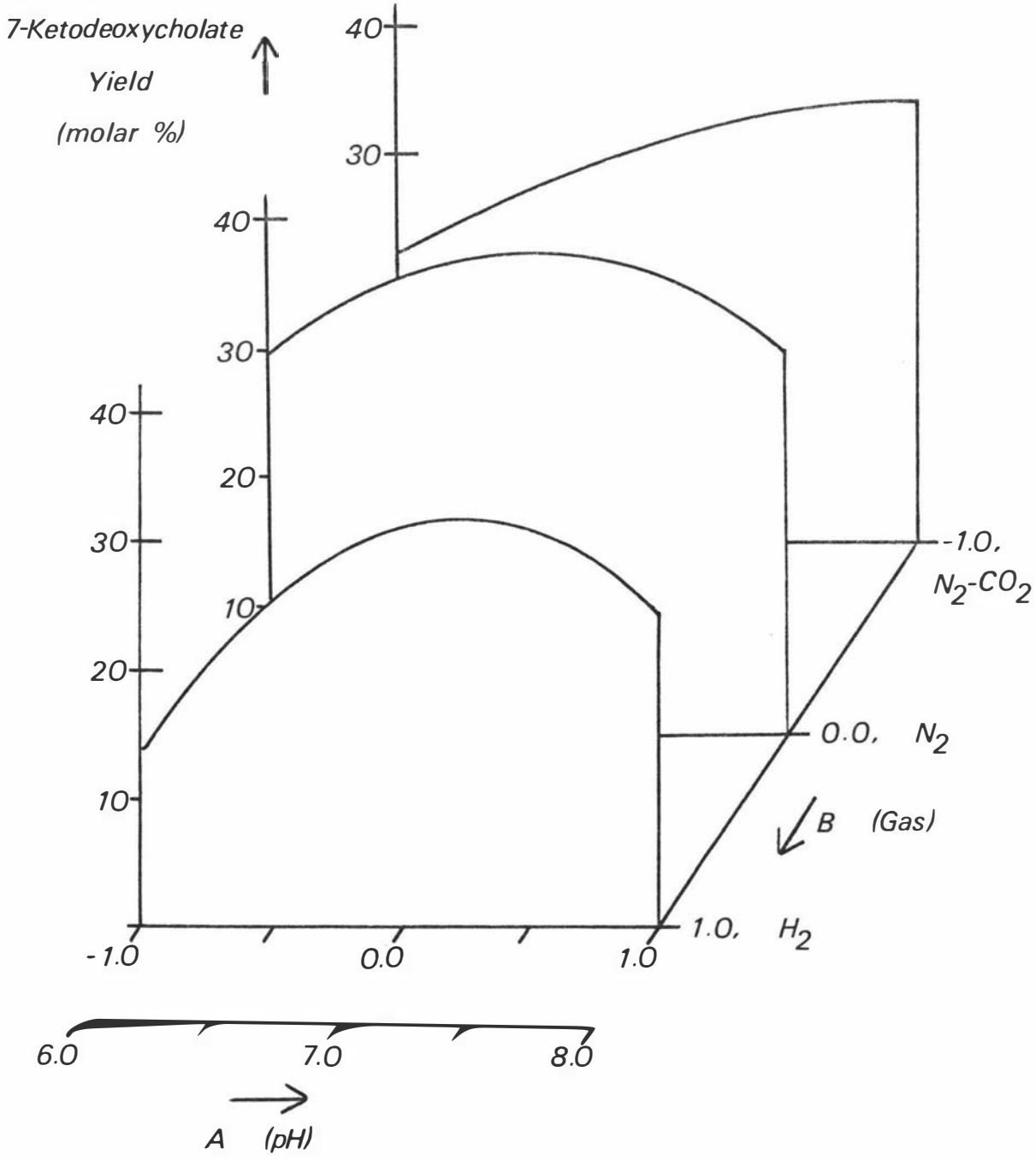


FIGURE 4.14 Predicted 7-Ketodeoxycholate Yield

The model from which this figure is drawn is presented in Appendix 2, Table A2.1

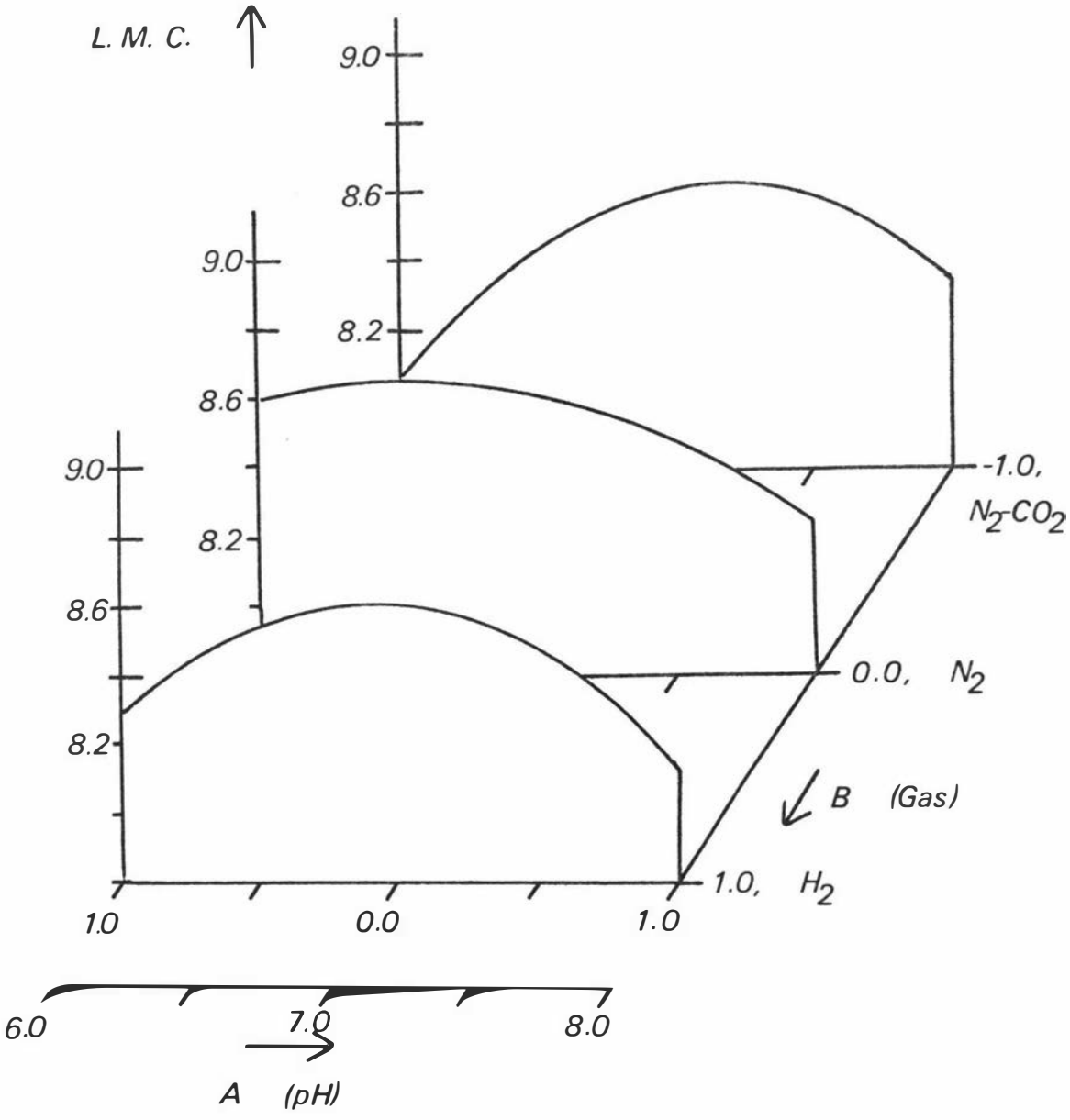


FIGURE 4.15 Predicted Cell Yield (as L.M.C., log of maximum observed number of cells per millilitre of culture)

The model from which this figure is drawn is presented in Appendix 2, Table A2.5.

TABLE 4.4 Matrix of Correlation Coefficients for the Pairs of Response Variables in Experiment 1

	7KD	C	D
C	-0.876***		
DC	0.266	-0.698*	
L.M.C.	0.772***	-0.884***	0.620*

*** - statistically significant at the 1% level
* - statistically significant at the 5% level

4.5 DISCUSSION OF EXPERIMENT 1

The experimental design approach adopted is empirical, and as such it is suited to the project aim. This means that any mechanistic discussion indulged in is speculative, but may assist in the identification of areas worthy of further research.

The regression models confirm what is intuitively obvious from the raw data; the preferred conditions for dehydroxylation lie very close to pH 7 with an atmosphere of N_2-CO_2, 9:1, whereas for dehydrogenation, an atmosphere of nitrogen, close to pH 7 is optimum.

If an experimental design with only two levels (i.e. a 2^2 factorial) had been selected, the dehydroxylation optimum might not have been detected, since the significant negative main effects for both variables would lead to the conclusion that greatest deoxycholate yields lay in the direction of low pH and hydrogen gas. The three-level design adopted allowed the elucidation of much curvature in both variables, reflected in the size of quadratic terms in all models, and evidenced by the ability of a logarithmic transformation to describe deoxycholate yield. The high peak for dehydroxylation at pH 7/N_2-CO_2 is manifest mainly in interactions involving quadratic terms. This peak is of great influence in the model, yet comprises only a single data point. The regression techniques used tend to weight such large outlying points, and in this case the apparent statistical signifi-

cance is enhanced by close agreement of the replicates. Moreover, this high value arose in the very first run, where different random influences may have been acting.

To check this value, and to substantiate the predicted maximum deoxycholate yield (which corresponded closely to run 1 conditions), run 1 was repeated. The results are depicted in Figure 4.16. The total degree of transformation was as predicted (50.6%, prediction 50.0%) and growth yield only a little high (L.M.C. 8.80, prediction 8.60). However, the yield of deoxycholate was lower than predicted *via* the logarithmic transformation (10.9%, prediction 20.3%) and 7-ketodeoxycholate yield correspondingly higher (38.5%, predicted 31.0%). The difference between the two pH $7/N_2$-CO_2 runs may reflect a change in the random influences affecting any experiment. This is particularly likely since the 11-run design was performed as a single randomised block over 3 months, and the repeat some weeks later on its own. Despite the difference, the repeat run still showed a higher deoxycholate yield than any of the other ten trials. Thus the basic conclusion still holds: dehydroxylation was greatest at pH 7 under the N_2-CO_2 mixture.

Most literature reports concerning dehydroxylation as a function of pH have dealt with rates of transformation. The current work has been concerned with yields, which are more relevant to the early stages of process development, and required fewer data points for adequate calculation. Only when deoxycholic acid yields become favourable will rates become of interest from a process viewpoint. This difference between yield optima and rate optima must be borne in mind when considering previously reported pH "optima".

The literature shows some disagreement concerning the dependence of dehydroxylation on pH. Drasar and Hill (1974) reviewed their own extensive faecal-isolate screening experiments, and similar experiments of their colleagues, all of which employed T.L.C. to detect 7α-dehydroxylation by harvested and resuspended cells of anaerobically grown organisms. They concluded that if the pH of the culture medium fell below pH 6.5, very little dehydroxylation could be detected. Midtvedt and Norman (1968) investigated growing cultures of their lactobacillus-

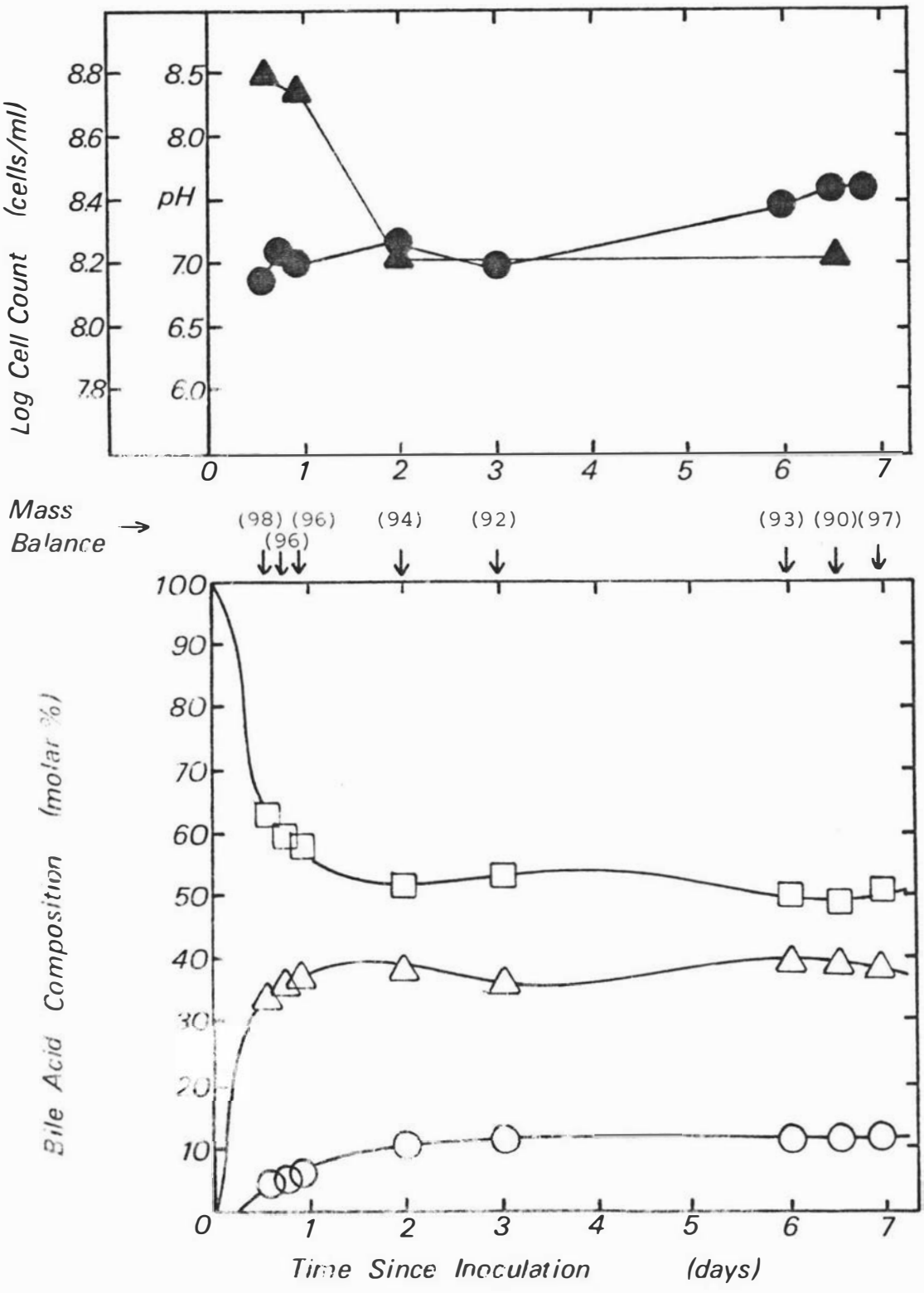


FIGURE 4.16 Course of Growth and Transformation for Experiment 1, Run 1 Repeat

Legend as for Figure 4.1

like isolate in chenodeoxycholate-containing medium, initially at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 or 8.0. These authors found growth at all initial pH values except at pH 5.0 and 5.5, but no dehydroxylation in those initially at pH 5.0, 5.5 or 6.0. Despite not having demonstrated dehydroxylation at any pH below 6.5, they concluded: "7α-dehydroxylation ..... was only present in media with a pH above 6.0".

Macdonald *et al.* (1978) reported a yield optimum of pH 8 for actively growing mixed faecal cultures, with activity evident between pH 5.5 and pH 9. Dehydroxylation was still pronounced at pH 9, and it was postulated by the author that a selective effect was imposed on the faecal populations by the initial hydrogen ion concentration.

Samuel *et al.* (1973) reported that cholic acid and chenodeoxycholic acid were 7α-dehydroxylated at maximal rate over a pH range of 5.0 to 8.0 in homogenised stool samples. It should be noted that since these faecal organisms were from clinical samples, they would have grown at physiological pH. Similar results have been obtained more recently by Stellwag and Hylemon (1978) with washed resting cells of *Cl. leptum* which had been grown in peptone-yeast-glucose medium (pH not stated). These authors found a broad rate optimum from pH 6.5 to 7.5, with activity expressed over a range from 5.0 to 9.0.

Of these five reported studies, that of Midtvedt and Norman (1968) is most similar in methodology to the current work, in that the transformation substrate was present during growth, and a pure culture was used. Thus both enzyme elaboration and enzyme activity occur under the same pH conditions. These authors do not, however, cite an optimum, but merely record the presence of the 7-deoxy product.

The experiment described in this chapter indicates a pH yield optimum near pH 7 for 7α-dehydroxylation of cholic acid by growing cells (although it must be noted that growth had ceased prior to dehydroxylation being expressed. Deoxycholate yields were very low when the pH was maintained above pH 8.0, but moderate when maintained

at not less than pH 6.0. These latter runs were effectively not pH controlled, since the lower limit of pH 6 was never reached. However, considerable time was spent below pH 6.5, until the pH rose later in the run, probably as a result of deamination. The deoxycholate yield was still rising during these periods below pH 6.5.

Published optima for cell-free preparations of 7α-dehydroxycholanoyl dehydroxylase are generally higher than pH 7, and generally refer to transformation rates. Ferrari and Beretta (1977) found 7α-dehydroxylase activity between pH 5.5 and pH 9, with an optimum between pH 7.0 and 8.5. The activity was present in *Cl. bifermentans* cells grown between pH 6.0 and 8.5. Aries and Hill (1970b) found that cell-free enzyme preparations of *Bacteroides fragilis*, three strains of *Cl. welchii*, two of other clostridia and one each of *E. coli* and *Strep. faecalis* showed pH rate optima between pH 7.0 and 8.0.

In the current work, at both pH 6 and pH 8, the general trend of greater dehydroxylation under hydrogen gas supports the recommendation of Drasar and Hill (1974) to flush the growth vessel vigorously with hydrogen and incubate in a hydrogen atmosphere. At pH 7, however, yields under nitrogen were slightly higher than under hydrogen, and under N_2-CO_2 higher still. Despite the empirical nature of the experiment, it is tempting to speculate that the influence of pH on this observed carbon dioxide effect might implicate the hydrogen carbonate ion, which exists above pH 6.4 (pKa of carbonic acid dissociation).

Actual hydrogen carbonate ion levels in the growth medium are difficult to calculate, since the system is so complex. Todd Hewitt Broth contains 0.25% w/v "Na_2CO_3", according to the BBL specifications, (BBL, 1968), but some may be lost on autoclaving. During a 20 ml-scale trial autoclaving of Todd Hewitt Broth, and of Todd Hewitt Broth containing 0.25% w/v added $NaHCO_3$, pH changes of pH 7.40 to 7.61 and pH 7.42 to 8.83 were observed for the respective samples. This indicates that at least some CO_2 is lost from solution during autoclaving.

During fermentation, the medium was swept with gas, and over time would equilibrate with the 10% CO_2 atmosphere. If equilibrium is reached, then hydrogen carbonate concentration can be calculated for

each pH. From the chart of Capri and Marais (1975), the concentrations of hydrogen carbonate ion in an anaerobic digestion medium are 1 mM and 11 mM at pH 6 and 7 respectively. By extrapolation, the hydrogen carbonate level at pH 8 will be *ca* 110 mM. Less than 1% of this will exist as carbonate ion at pH 8. Thus hydrogen carbonate could be associated with the boosting of dehydroxylation yields by CO_2 at pH 7. However, the reason for this effect not occurring at pH 8, where the hydrogen carbonate species is even more predominant, is unknown.

A fermentation run in the 2-litre fermentation vessel, sparged with N_2-CO_2, 1:1 produced deoxycholate and 7-ketodeoxycholate yields very similar to those observed in a control run with N_2-CO_2, 9:1. Thus the observed carbon dioxide effect appears not to be enhanced by atmospheric CO_2 levels greater than 10%. By referring again to the charts of Capri and Marais (1975), it can be seen that at pH 7, medium in equilibrium with the 50% CO_2 atmosphere will contain 53 mM hydrogen carbonate, compared with 11 mM for the 10% CO_2 atmosphere. Hence increasing the hydrogen carbonate levels above 10 mM, either by increasing the pH or by increasing the atmospheric CO_2 content, results in no further increase in deoxycholate yield.

An unusual mass balance phenomenon was observed with the 50% CO_2 level: from 6 to 21 h, 5 to 20% of the bile acid pool was unaccounted for in solution, but by 30 h levels had returned to normal. The bile acid was apparently reversibly associated in some way with the cells. The reason for this is unknown, and may merit further investigation.

7-Ketodeoxycholate yield is parsimoniously modelled by a regression equation exclusively involving terms based on quadratics. Although transformation was generally higher at pH 8 than pH 6, this main effect is not statistically significant. Dehydrogenation was not as sensitive to pH and gas environment as was dehydroxylation, and at least 13% of the cholic acid substrate always existed as the 7-ketone after 7 days incubation. The presence of this side product would pose an unattractively expensive separation problem in an industrial process. The 7-ketodeoxycholate also represents a loss of valuable steroid material; unreacted cholic acid can conceivably be recycled. The minimum 7-ketodeoxycholate yield was observed at pH 6 under hydrogen, but was accompanied by a low deoxycholate yield.

There was no way of obtaining high deoxycholate yields with little concurrent dehydrogenation merely by manipulating the two variables investigated. In fact there was always more 7-ketodeoxycholate than deoxycholate after 7 days incubation.

Dehydrogenation was maximal under nitrogen near pH 7. The effect of gas is not marked. Yields were lowest under hydrogen and highest under nitrogen. However, gas affected the shape of the pH response, particularly N_2-CO_2 where pH 8 resulted in greatest dehydrogenation. No study (which directly compares the effects of anaerobic gases on dehydrogenation) is reported in the literature.

Although Midtvedt and Norman (1968) reported 7-ketone formation by cells growing over the whole pH range tested (pH 5 to 8), 7α-hydroxycholanoyl dehydrogenases have been studied mainly as cell-free extracts. Aries and Hill (1970b) found an alkaline optimum, pH 8.8 or above, for demonstrably different enzymes from *Cl. perfringes*, *Bacteroides* and *E. coli*. Macdonald *et al.* (1973) characterised the NAD-dependent 7α-dehydrogenase of *E. coli*, finding a pH optimum of pH 9-10. Hylemon and Sherrod (1975) reported two distinct 7α-dehydrogenases in *Bacteroides fragilis* subsp. *thetaiotaomicron*; both had pH optima 8.5-9.0. In contrast, Macdonald *et al.* (1975b), when studying two *B. fragilis* enzymes, determined optima at pH 9.5-10.0 and pH 7.0-9.0.

In the current work, the dehydrogenation involving growing cells showed pH optima near neutrality, much lower than those reported for the free enzymes. Such differences between whole cell and isolated enzyme optima are common and are usually accounted for by postulating the existence of microenvironments around cell membranes or within cells where pH conditions in the immediate vicinity of an enzyme can be substantially different from those of the bulk solutions. Alternatively, other cell processes necessary for dehydrogenation such as NAD^+ or NADP+ regeneration, enzyme induction and synthesis or the function of permeability mechanisms might have lower pH optima. Apart from the finding of Ferrari and Beretta (1977) that cells grown between pH 6 and 8.5 produced extractable dehydrogenase activity, few data are available on the degree of enzyme production as a function of pH.

Growth yields throughout the 11 trials ranged over only 0.59 log units, a factor of 3.9. Again quadratic terms dominate the model, describing the pH optimum at pH 7. This is more marked under hydrogen and N_2-CO_2 than under nitrogen. From the table of correlation coefficients (Table 4.4) it is evident that growth and cholic acid use are correlated (99% level). Growth is also correlated with deoxycholate and 7-ketodeoxycholate yields (95% level each). This relationship is also apparent when the parsimonious model for the log of maximum observed number of cells is compared with those for transformations. Growth follows a similar trend to the transformations with high cell counts at pH 7 and having much curvature in the model. This trend is particularly apparent in the model for cholic acid remaining where the extent of transformation peaks at pH 7.

A pattern to the growth and transformation became apparent during this experiment. Exponential growth was usually complete by 6 to 9 hours with 7α-dehydrogenation becoming most rapid during the late phases of growth. After *ca* 12 h 7-ketodeoxycholate levels often declined with concomitant increase in cholic acid levels. Dehydroxylation usually commenced between 16 and 24 h. This order of events, and the visible reduction of the 7-keto acid which usually occurred before the onset of dehydroxylation agrees with the observations of Ferrari and Aragozzini (1972), Midtvedt and Norman (1968) and Bokkenheuser *et al.* (1969).

4.6 CONCLUSIONS

Growth, 7α-dehydroxylation and 7α-dehydrogenation all showed yield optima at pH 7, although the 7-ketone was still strongly produced at pH 8. Dehydrogenation and growth were generally strongest under nitrogen, while dehydroxylation was greatest under hydrogen except at pH 7, where nitrogen and especially N_2-CO_2, 9:1 gave greater yields. It is evident that CO_2 present in N_2 increases dehydroxylation and decreases dehydrogenation over a limited range.

In view of these results, it was decided that future screening work for fermentation variables should be conducted at pH 7 under N_2-CO_2, 9:1, since these conditions resulted in greatest deoxycholate yield. Dehydrogenation could not be prevented by manipulation of gas and pH within the ranges employed; in fact 7-ketodeoxycholate yield exceeded that of deoxycholate in all runs.

CHAPTER 5 EXPERIMENT 2: IDENTIFICATION OF FERMENTATION VARIABLES IMPORTANT TO DEHYDROXYLATION AND DEHYDROGENATION OF CHOLIC ACID.

5.1 INTRODUCTION

The aim of this project is to develop a process, based on microbial enzymes, for the hydrolysis and dehydroxylation of bile acids. In principle the simplest of such processes would be a single stage fermentation in which the required transformation was conducted in the same vessel as cell growth. In developing such a process, the major fermentation variables affecting cell growth, enzyme production and enzyme activity must first be identified.

The optimum convenient gas and pH combination for dehydroxylation having been indicated empirically, it was now possible to design an experiment specifically to determine which of the many other possible fermentation variables have a significant effect on the extent of dehydroxylation and dehydrogenation.

5.2 EXPERIMENT DESIGN

It was decided to use again the experimental design approach. The classical experimental approach would be to adjust each independent variable one at a time, holding the others at a base level, and compare the response of each with that of a control where all variables were at a base level. In a saturated statistically designed screening experiment for N (N being an odd number) independent variables (factors), each factor is at its high level for $(N + 1)/2$ runs and at its low level for the remaining $(N + 1)/2$ runs. This gives many more estimates of the individual effect of each variable. The sequence of high and low levels is defined by the design matrix. However, since more than one factor is adjusted to its high level for each run, the effects of any interactions in the system will appear confounded with the main effects of individual variables.

Plackett and Burman (1946) developed a series of orthogonal screening designs which, when "folded-over" (so that the N + 1 runs are performed twice, the second time with the high and low levels reversed), give estimates of main effects free of two-factor interactions. One Plackett-Burman fold-over design is presented in the form of a 2^{8-4} design of resolution IV by Box and Hunter (1961). The experiment requires 16 trials and allows the determination of fifteen contrasts: eight are main effects, each confounded with three-factor and higher interactions; seven are chains of four two-factor interactions, each confounded with four-factor and higher level interactions.

In the experiment described in Chapter 4, those trials which showed high 7-ketodeoxycholate or deoxycholate yields after 7 days had already exhibited high yields after 2 days. Thus it was decided that a 48 h fermentation time would adequately indicate the extent of transformation. Including preparation and analysis time, one such fermentation run could be performed each week. It was decided, therefore, that a 16 run experiment was the maximum size which could conveniently be handled, and the 2^{8-4} design was adopted.

This design will not individually screen both main effects and two-factor interactions; there is no economical way of doing this, since for 8 factors there are 28 two-factor interactions. Thus, despite the strong possibility that they might be important (Maddox and Richert, 1977; Chapter 4 of the current work), two-factor interactions must be regarded as secondary pieces of information.

A long list of possible fermentation variables was reduced to the eight accommodated by the design. These were:

1. Strain of organism. The only two strains of *Clostridium bifermentans* reported by previous workers to be capable of dehydroxylation were used: strain ATCC 9714 (Hayakawa and Hattori, 1970), used in Experiment 1, and strain SD 10 (Ferrari and Aragozzini, 1972).
2. Presence or absence of inhibitors. Drasar and Hill (1974) discussed cell-free dehydroxylases, citing complete inhibition by 30 mM Cu^{++}. They also reported partial inhibition of

dehydrogenase by 30 mM Cu^{++}. In the present experiment, 8-hydroxyquinoline was used as a chelating agent for cupric ion, to boost transformation. The formation constants (log K_1) of cupric and ferric 8-hydroxyquinolate are 12.2 and 12.3 respectively. These values exceed those for all other commonly encountered ions by several orders of magnitude (Dean, 1979). To allow adequate trace metal concentrations for other metabolic processes essential for growth, 8-hydroxyquinoline levels were limited to 10 μM.

In preliminary experiments, the presence of 25 mM fluoride ion allowed growth to proceed to almost the same final cell count as did its absence, but growth was noticeably slower; the organism was presumably under some stress. Thus fluoride was included to ascertain whether or not transformation yields were increased when growth conditions were less favourable. Fluoride (50 mM) has previously been shown to have no effect on rates of 7α-dehydroxylation or 7α-dehydrogenation (Drasar and Hill, 1974).

The two variables fluoride and 8-hydroxyquinoline, present for different purposes, were combined and group-screened as one variable.

3. Time of substrate addition. A batch fermentation is simpler when the transformation substrate is added before sterilisation and inoculation. However, bile acids have been shown to inhibit the growth of some organisms (Binder *et al.*, 1975). At the low level of this factor, cholic acid was added before inoculation and for the high level, 6 h after inoculation.
4. Gas delivery and agitation. The 2-litre fermenter vessel had provision to introduce gas either into the head-space or by a single nozzle directly below the impellor. Sparging with an impellor speed of 300 rev/min provided a thoroughly agitated medium in which the stripping of volatile waste products would be relatively rapid. The

alternative, surface sweeping at an impellor speed of 200 rev/min, provided just sufficient agitation to adequately mix in added chemicals, suspend organisms, and maintain a flow over electrode surfaces.

5. Temperature. Most transformation work with the mesophilic clostridia has been performed at 37^{0}C (Ferrari and Pacini, 1968; Hayakawa and Hattori, 1970; Ferrari and Aragozzini, 1972) with no other temperature being investigated. Temperature settings of 32^{0}C and 37^{0}C were chosen for this experiment to encompass a sufficient range to measure any temperature effect, without exceeding the recommended range of 30-37^{0}C cited in Bergey's Manual (Smith and Hobbs, 1974) for *Cl. bifermentans*.
6. Presence or absence of 0.05% w/v sodium thioglycollate. The presence of this reducing agent lowers the initial Redox Potential of microbiological media (Brewer, 1940). This variable was included to determine whether a lower starting Ec affected the relative extent of the reductive dehydroxylation and oxidative dehydrogenation reactions.
7. pH anion. To follow up the carbon dioxide effect observed in the experiment described in Chapter 4, the usual 1 M NaOH solution was replaced at the high level of this factor by 1 M $NaHCO_3$.
8. Inoculum size. A larger inoculum usually promotes more rapid growth and means fewer generations are required to achieve the same final cell count. Standard inocula of 20 ml or 50 ml were used to investigate any effect of this variable.

These eight factors and their high and low levels are summarised in Table 5.1, together with the run order (fully randomised) and design matrix.

TABLE 5.1 Design Matrix and Variables for Experiment 2

Run Order	A	B	C	D	E	F	G	H
4	-	-	-	-	-	-	-	-
3	+	-	-	+	+	-	+	-
14	-	+	-	+	-	+	+	-
7	+	+	-	-	+	+	-	-
12	-	-	+	-	+	+	+	-
16	+	-	+	+	-	+	-	-
15	-	+	+	+	+	-	-	-
2	+	+	+	-	-	-	+	-
9	-	-	-	+	+	+	-	+
10	+	-	-	-	-	+	+	+
5	-	+	-	-	+	-	+	+
8	+	+	-	+	-	-	-	+
11	-	-	+	+	-	-	+	+
13	+	-	+	-	+	-	-	+
1	-	+	+	-	-	+	-	+
6	+	+	+	+	+	+	+	+

Variable	Strain	Inhibitors	Substrate Addition time	Gas Delivery	Temperature	Thioglycollate	pH Anion	Inoculum Size
High (+) Level	SD 10	Present	6 h	Sparge (300 rev/min)	37^0C	0.05% w/v	HCO_3^-	50 ml
Low (-) Level	ATCC 9714	Absent	0 h	Sweep (200 rev/min)	32^0C	Absent	OH^-	20 ml

5.3 FERMENTATION CONDITIONS

Each of the sixteen runs was conducted in the 2-litre fermentation vessel with a 1.25 l working volume of Todd Hewitt Broth. The pH was controlled two-ways at setpoints 6.9 to 7.1, using 0.5 M H_2SO_4 and 1.0 M NaOH or 1.0 M $NaHCO_3$. The medium was sparged or swept with N_2-CO_2, 9:1 at 20 ml/min, and samples were taken regularly over the 48 h incubation period for cell count, pH determination and analysis of bile acid composition. Cholic acid substrate was added to a final concentration of 0.05% w/v. When substrate addition was prior to inoculation, the cholic acid was dissolved in the stochiometric quantity of 1 M NaOH and added to the medium before sterilisation. For late substrate addition the acid was similarly dissolved, diluted to *ca.* 20 ml and sterilised. 8-hydroxyquinoline was dissolved in 1 M HCl (*ca.* 0.5 ml) and together with potassium fluoride added to the medium before sterilisation. The electrode potential (Ec) was monitored throughout each run, and continuously recorded together with pH and temperature.

5.4 RESULTS

Raw run data are presented diagrammatically in Figures 5.1 to 5.16. Each figure depicts the progress of transformation, the pH, Ec and cell count. In addition, the mass balance figure is given. Smooth curves were drawn to better estimate the 48 h bile acid compositions listed in Table 5.2a. Table 5.2b lists important values of electrode potential.

The MINITAB multiple regression package was used to regress each of the response variables included in Tables 5.2a and 5.2b against the terms of the full model. The full regression models are shown in Table 5.3.

The terms of greatest statistical significance were isolated by 1/2-normal plotting (Daniel, 1959). An example plot is shown in Figure 5.17, (p.111). Those variables shown not to conform to a normal

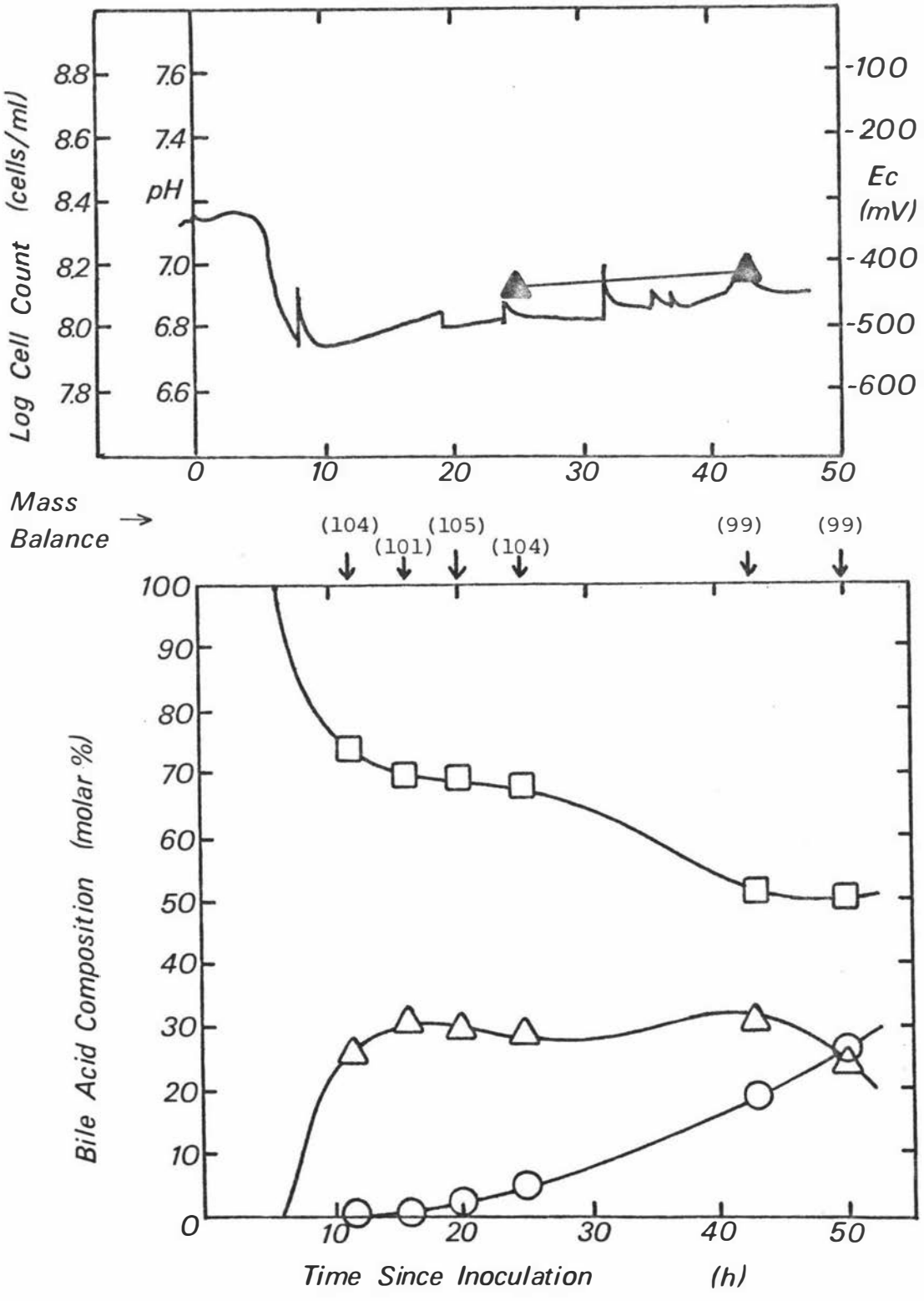


FIGURE 5.1 Course of Growth and Transformation for Experiment 2 Run 1

Deoxycholic acid (◯), 7-ketodeoxycholic acid (△), cholic acid (□), pH (●), cell count (▲). The electrode potential, Ec, is represented by a solid line. The "Mass Balance" figure is defined in section 3.10.2. "Bile Acid Composition" is normalised to a 100% mass balance. Experimental conditions are given in the text.

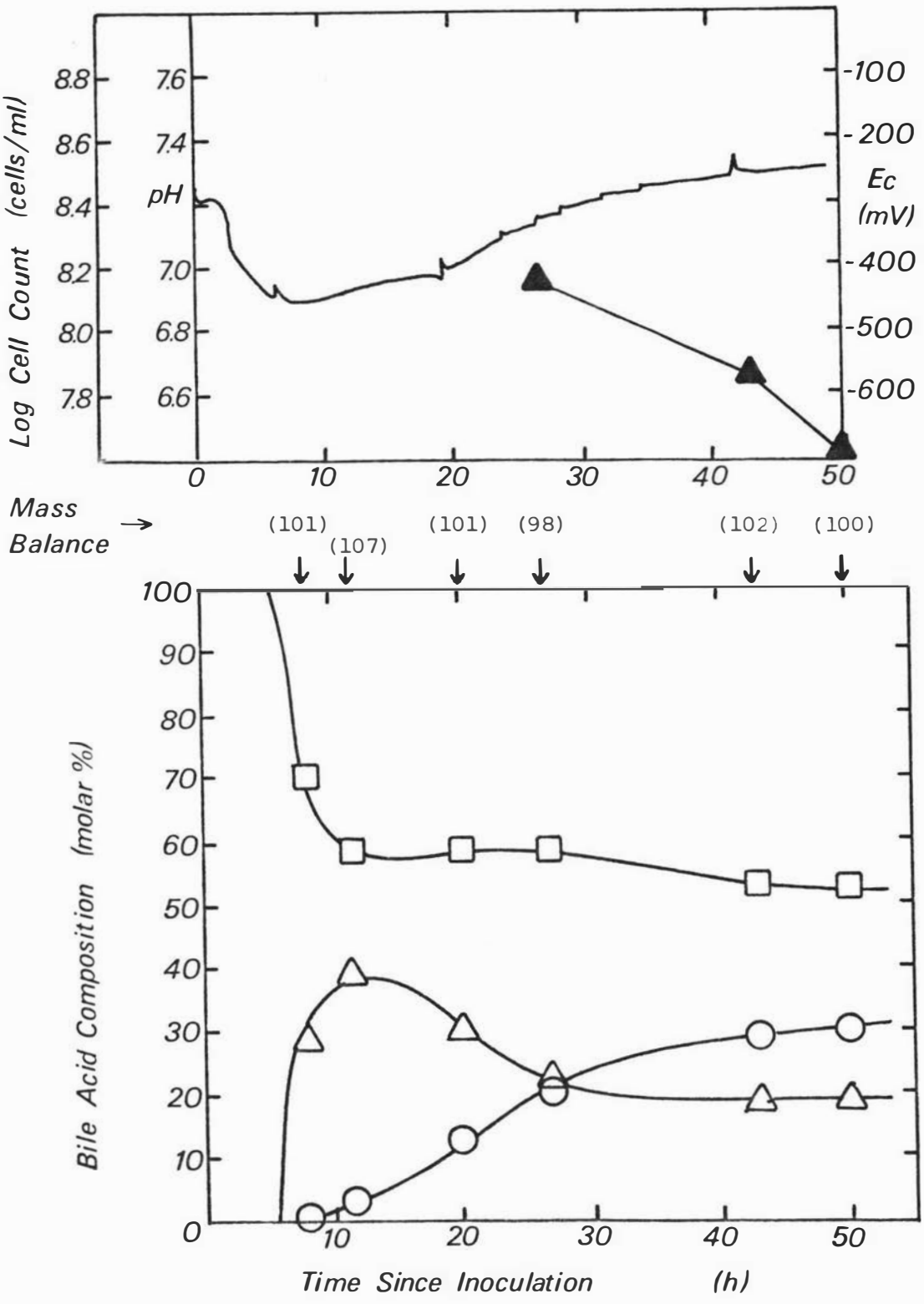


FIGURE 5.2 Course of Growth and Transformation for Experiment 2, Run 2

Legend as for Figure 5.1

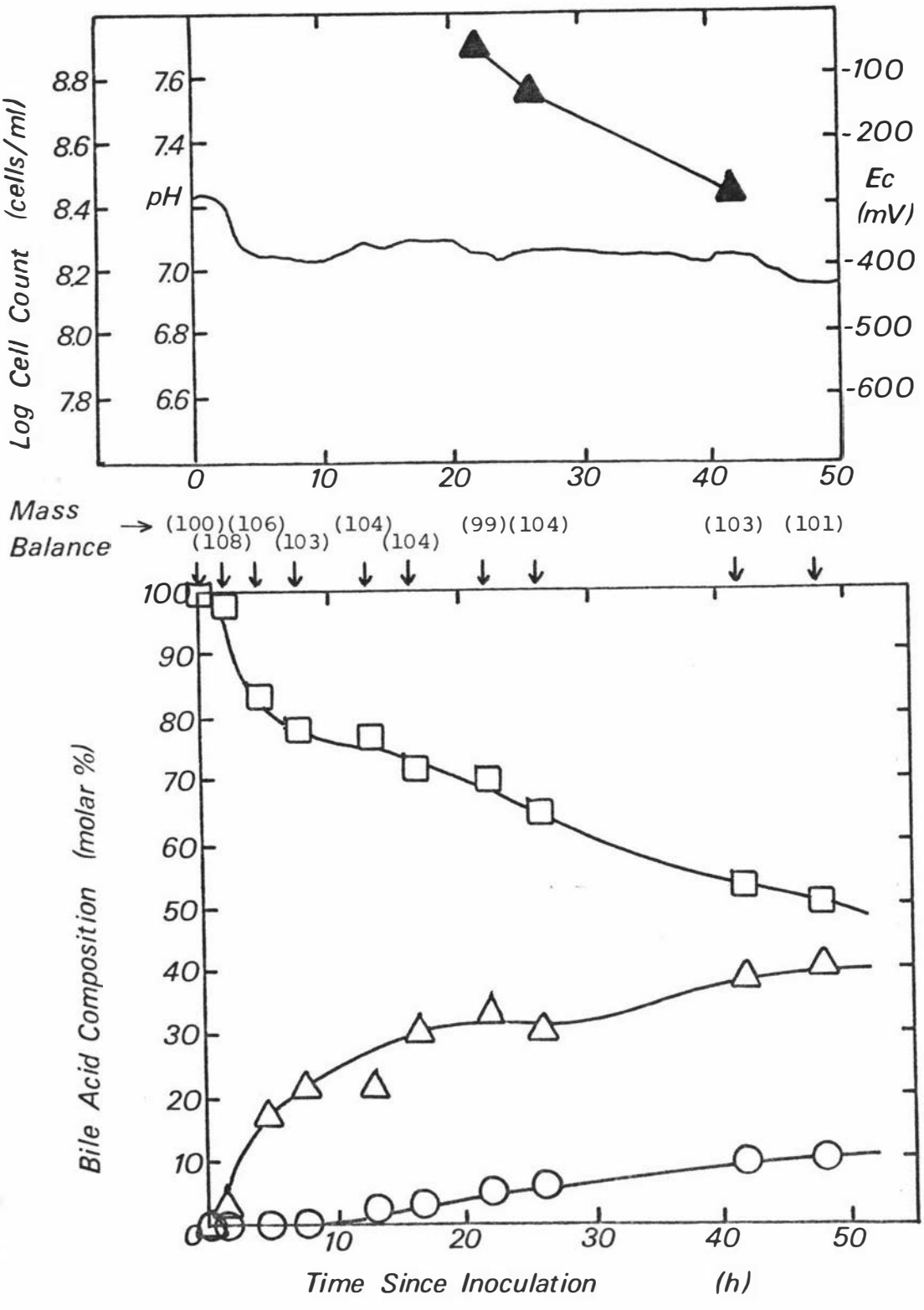


FIGURE 5.3 Course of Growth and Transformation for Experiment 2, Run 3

Legend as for Figure 5.1

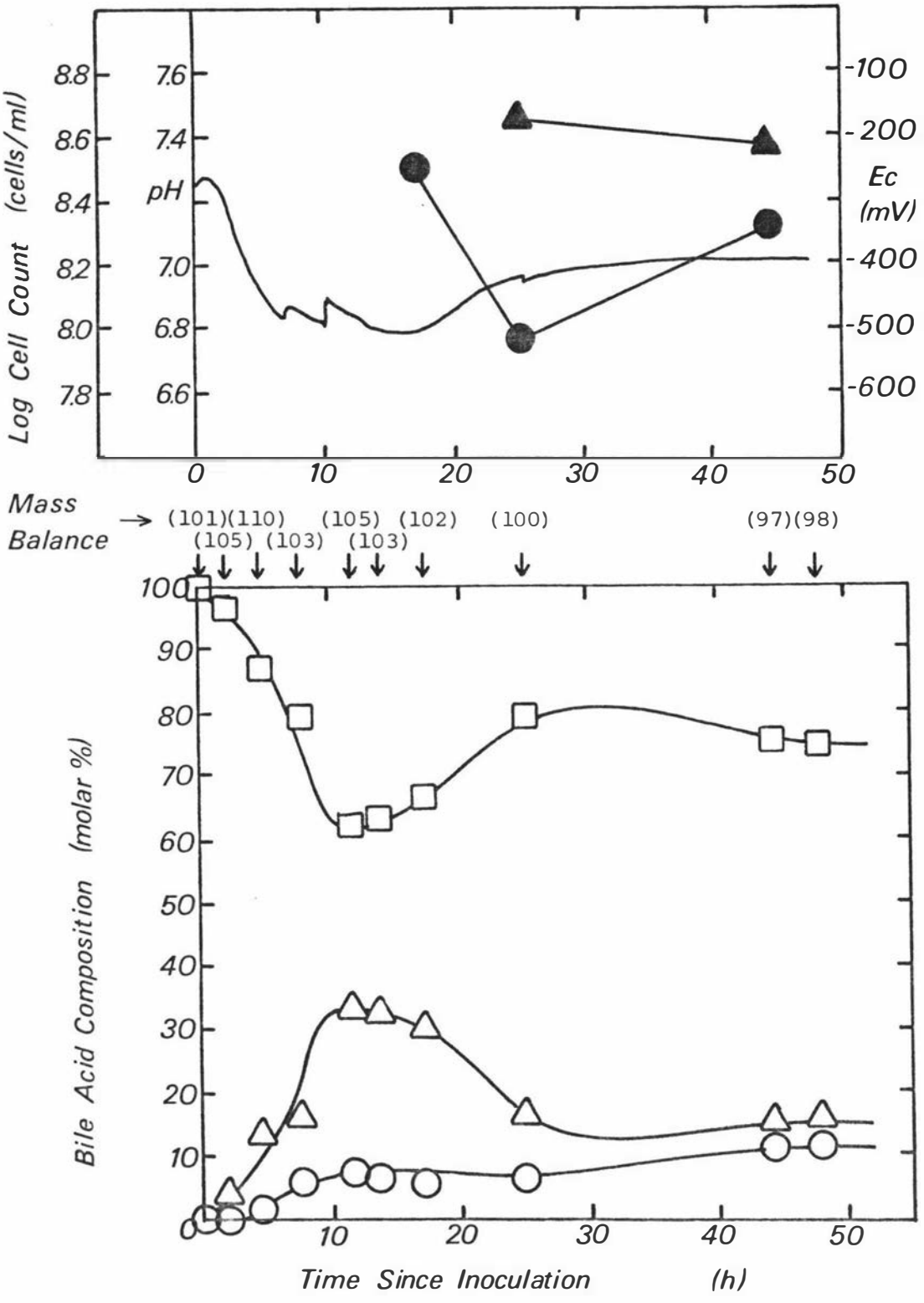


FIGURE 5.4 Course of Growth and Transformation for Experiment 2 Run 4

Legend as for Figure 5.1

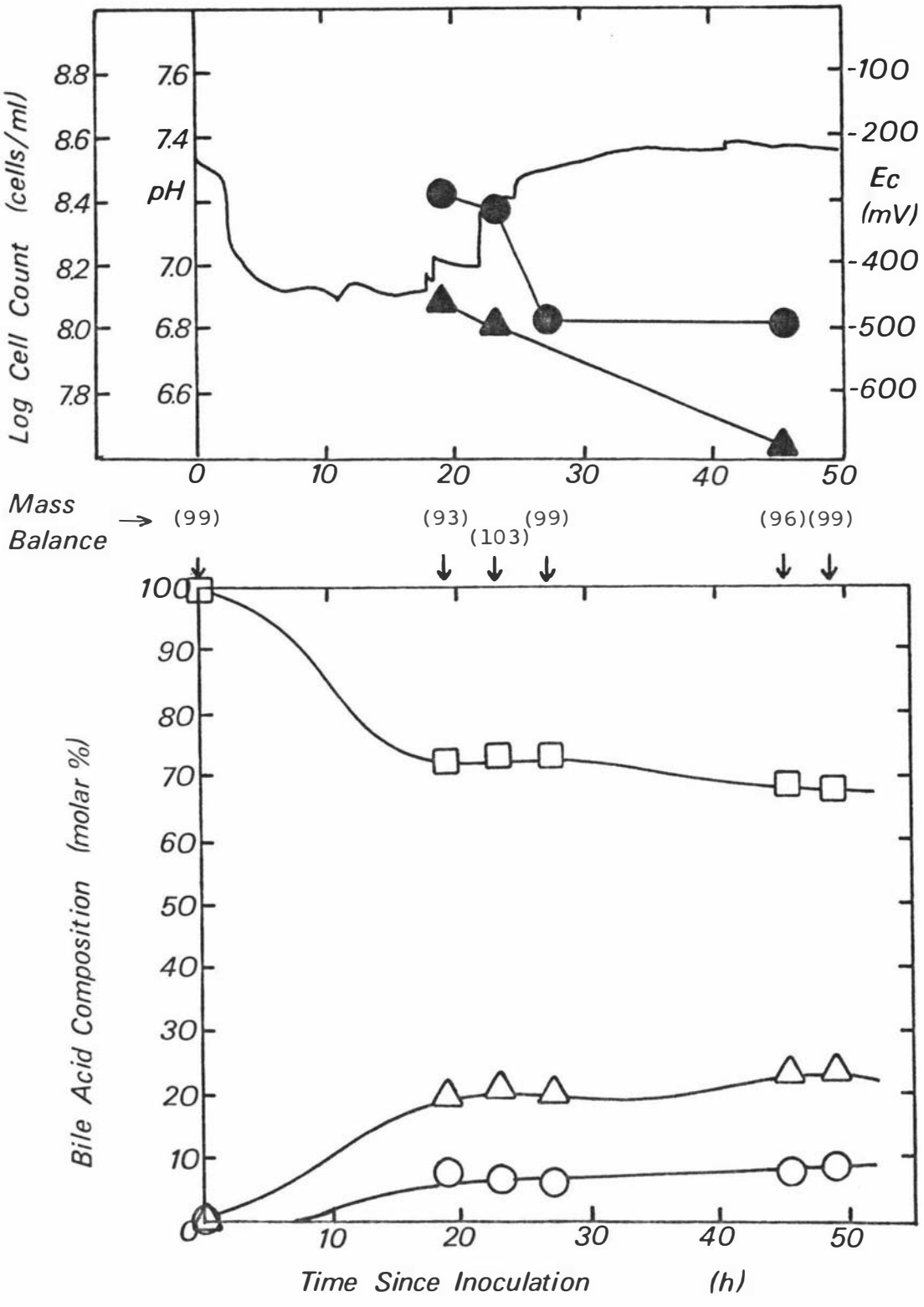


FIGURE 5.5 Course of Growth and Transformation for Experiment 2, Run 5

Legend as for Figure 5.1

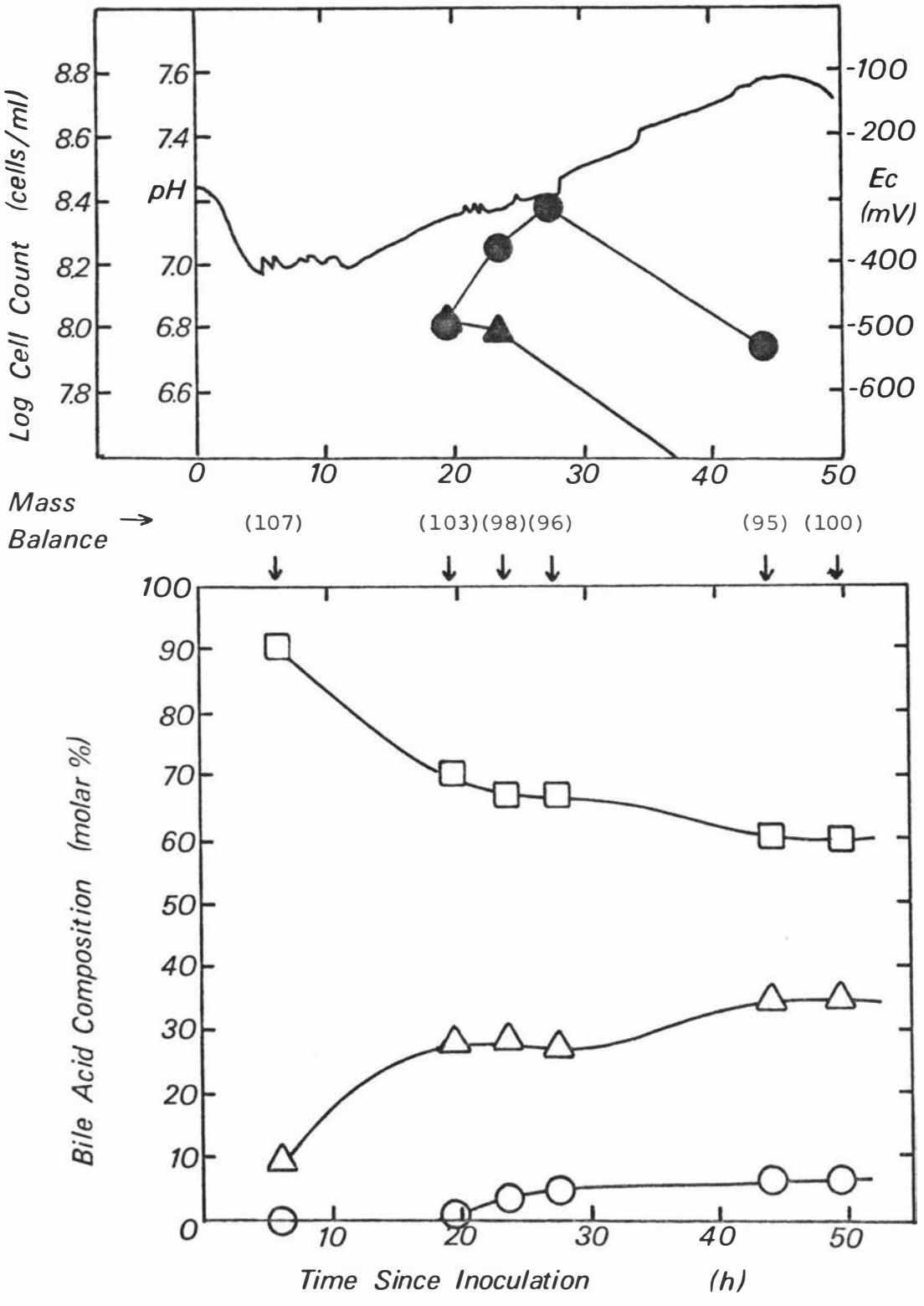


FIGURE 5.6 Course of Growth and Transformation for Experiment 2, Run 6

Legend as for Figure 5.1

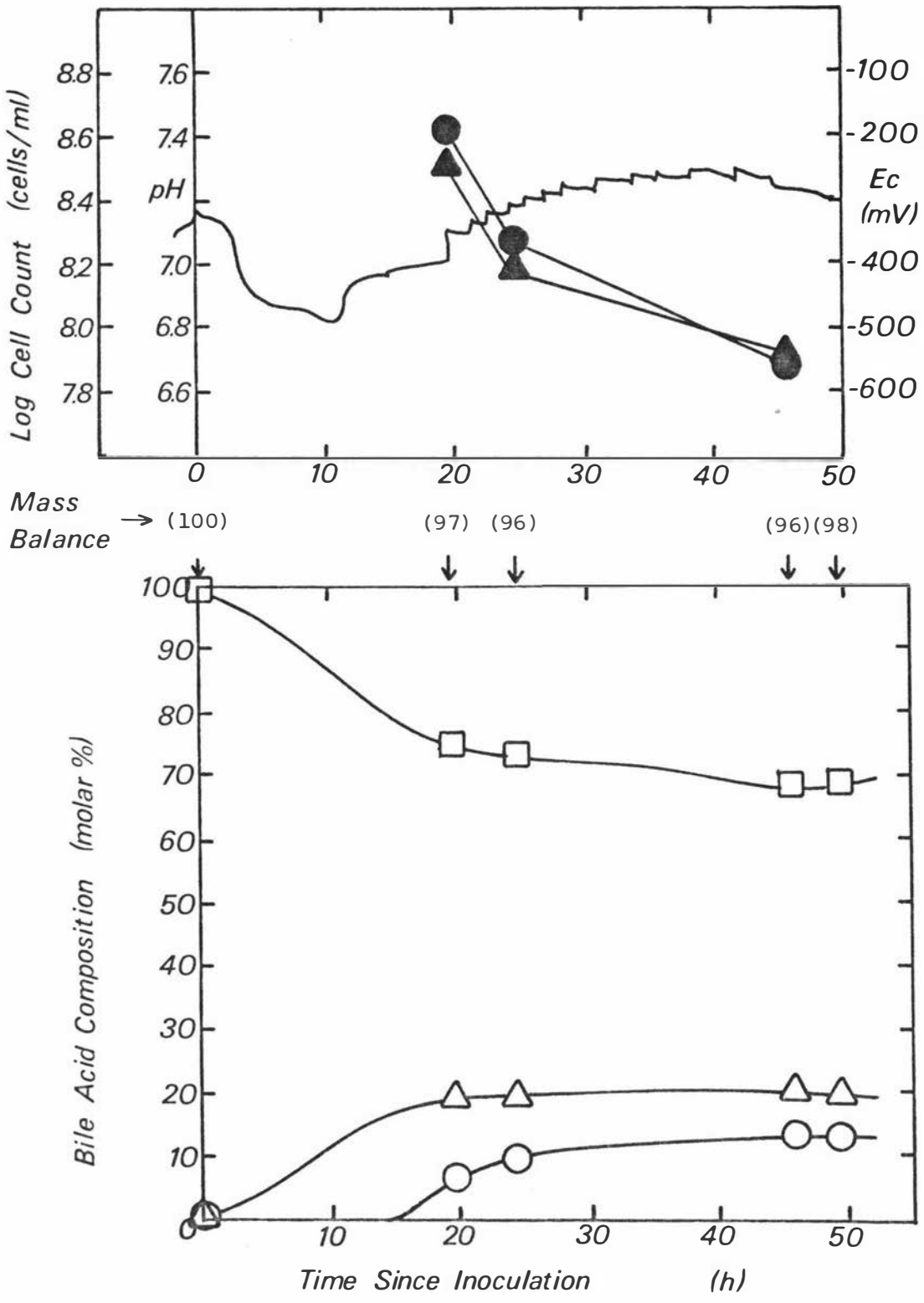


FIGURE 5.7 Course of Growth and Transformation for Experiment 2 Run 7

Legend as for Figure 5.1

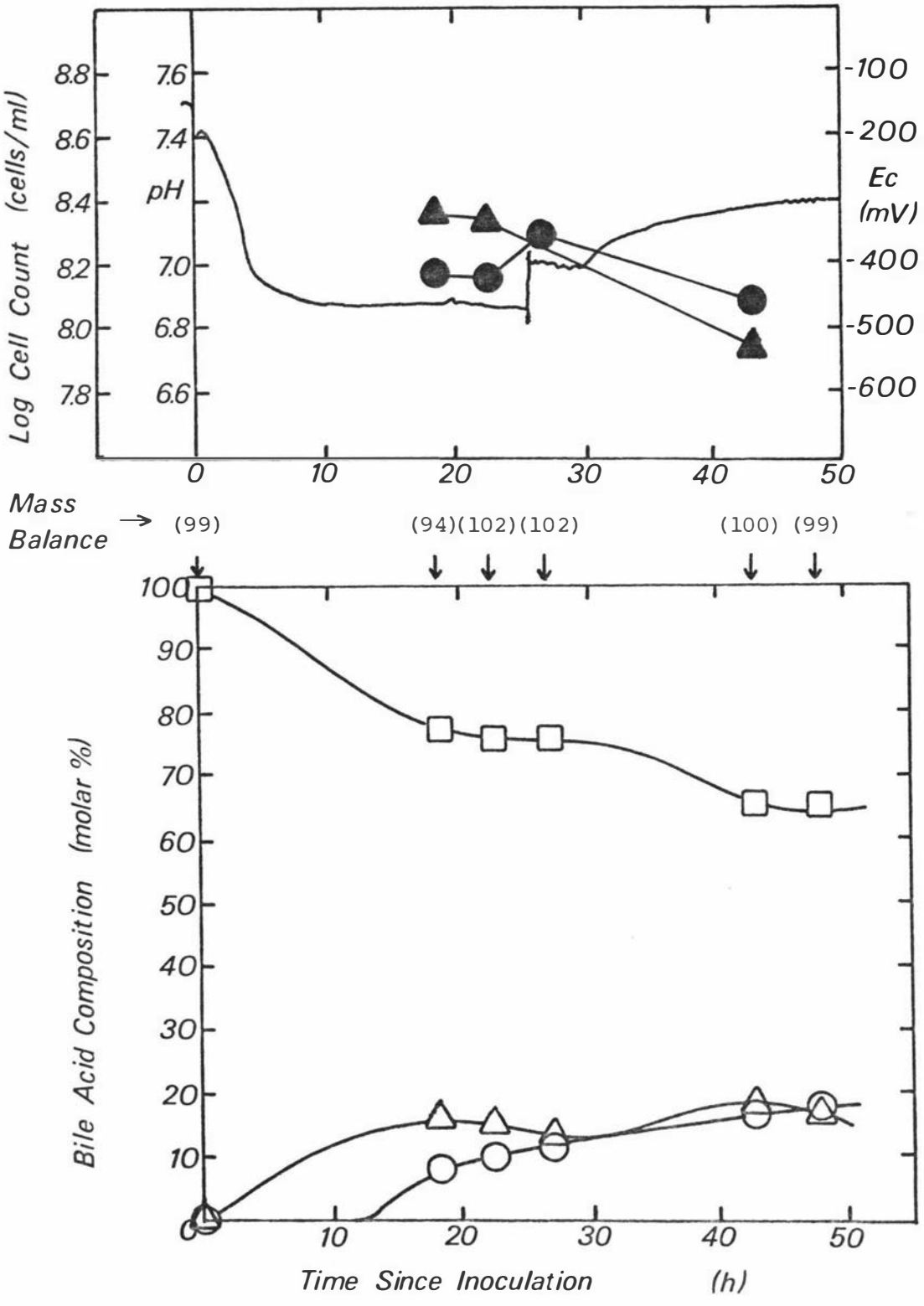


FIGURE 5.8 Course of Growth and Transformation for Experiment 2, Run 8

Legend as for Figure 5.1

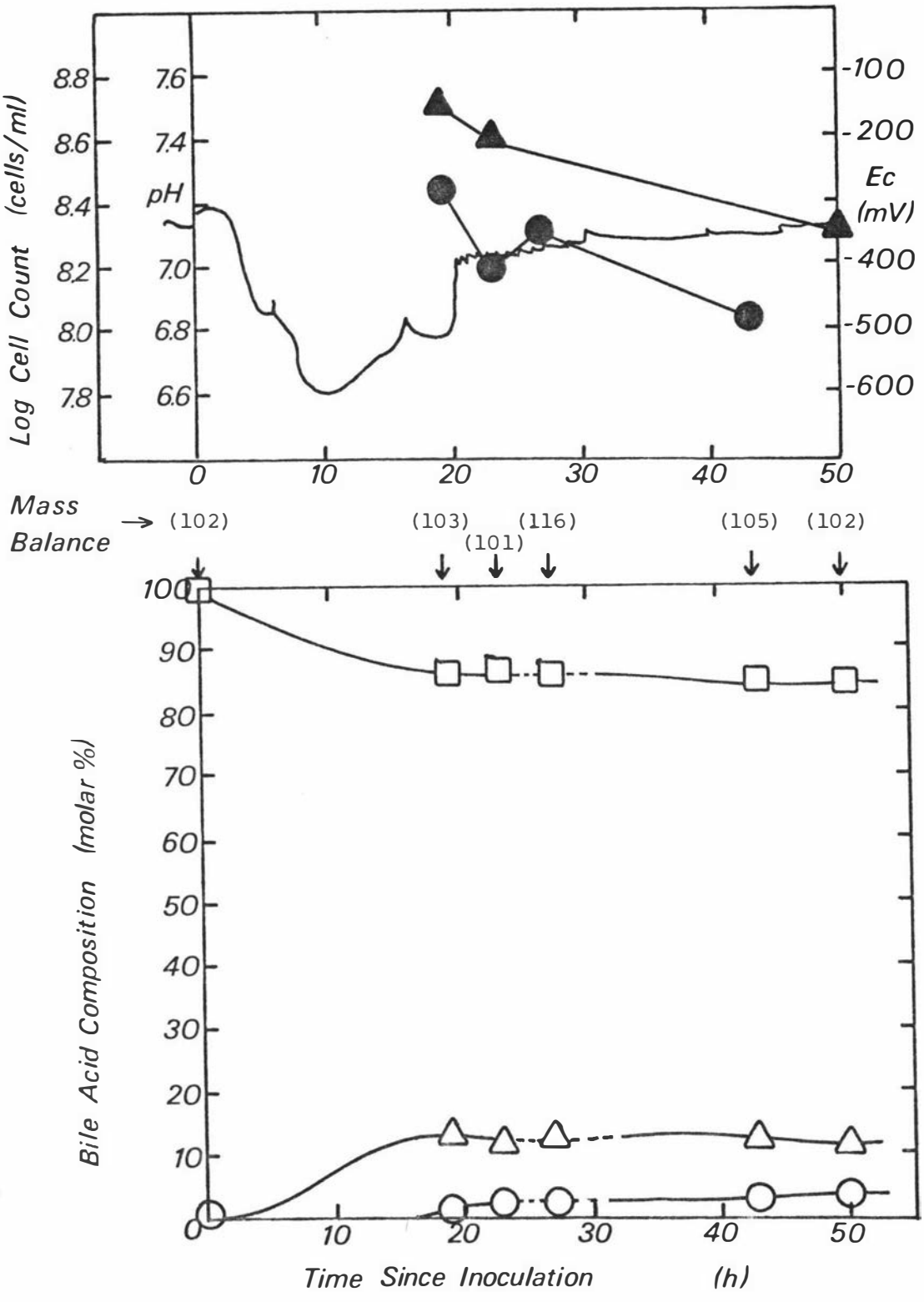


FIGURE 5.9 Course of Growth and Transformation for Experiment 2 Run 9

Legend as for Figure 5.1 Dashed line signifies unacceptable mass balance.

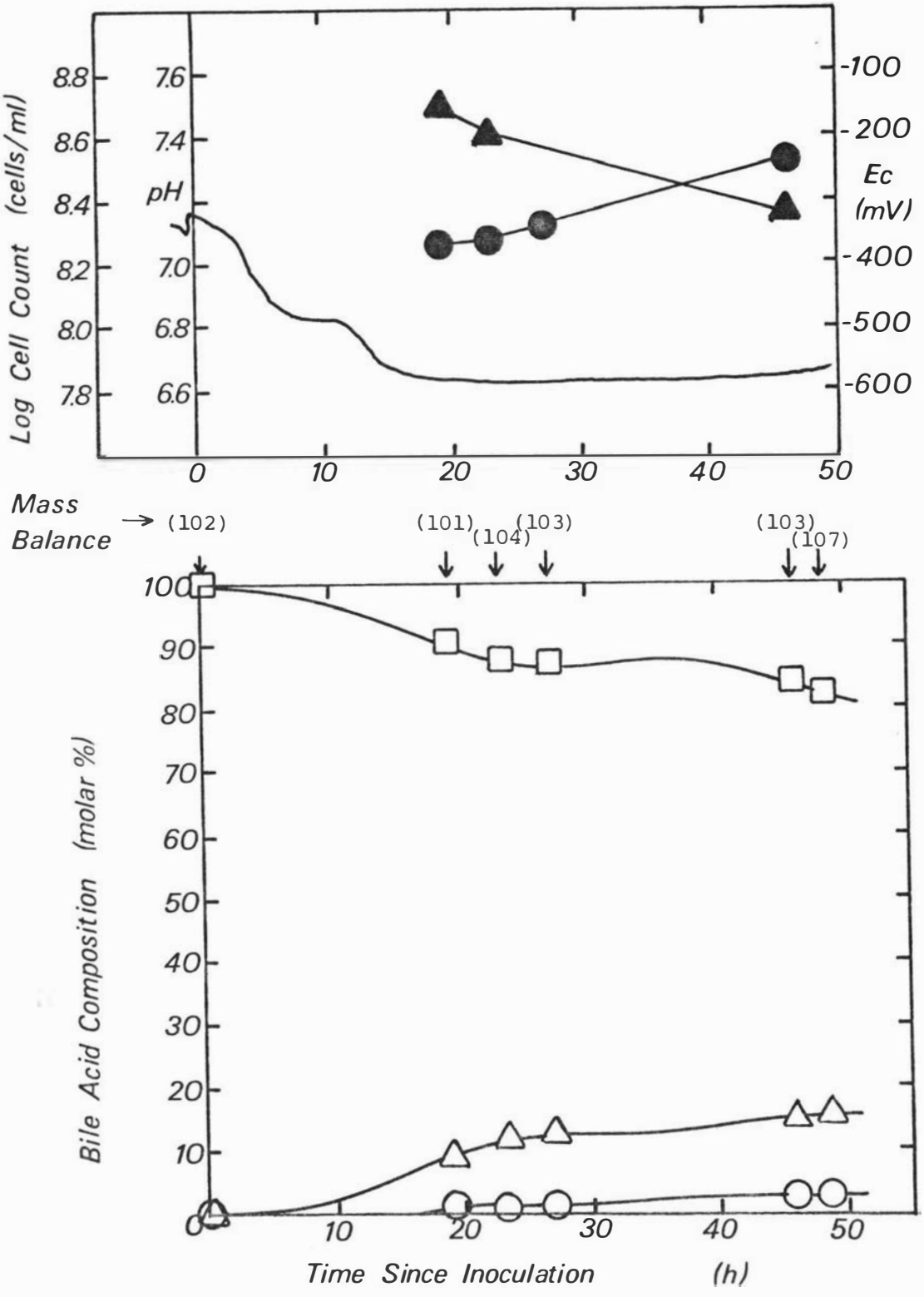


FIGURE 5.10 Course of Growth and Transformation for Experiment 2 Run 10

Legend as for Figure 5.1

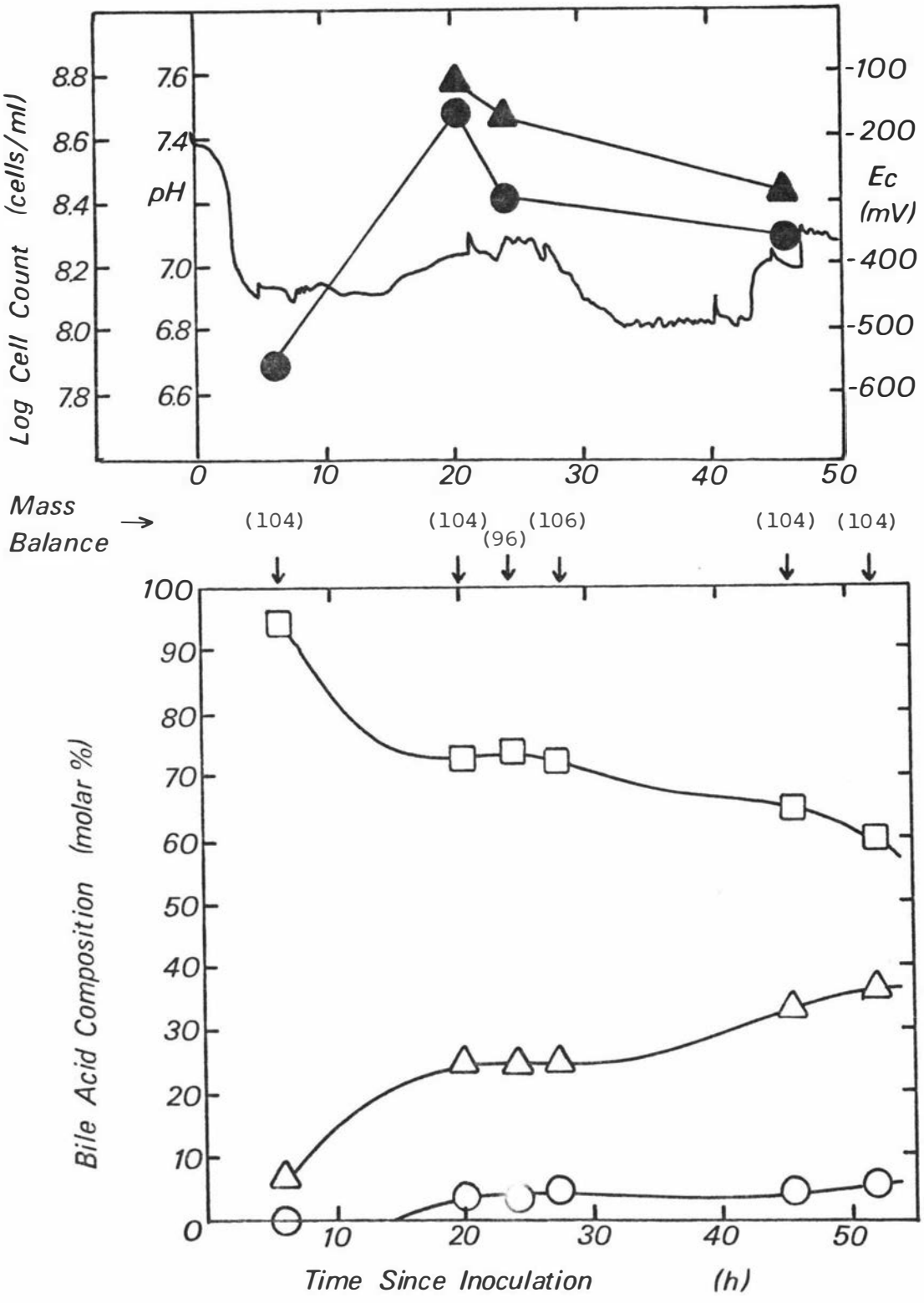


FIGURE 5.11 Course of Growth and Transformation for Experiment 2, Run 11

Legend as for Figure 5.1

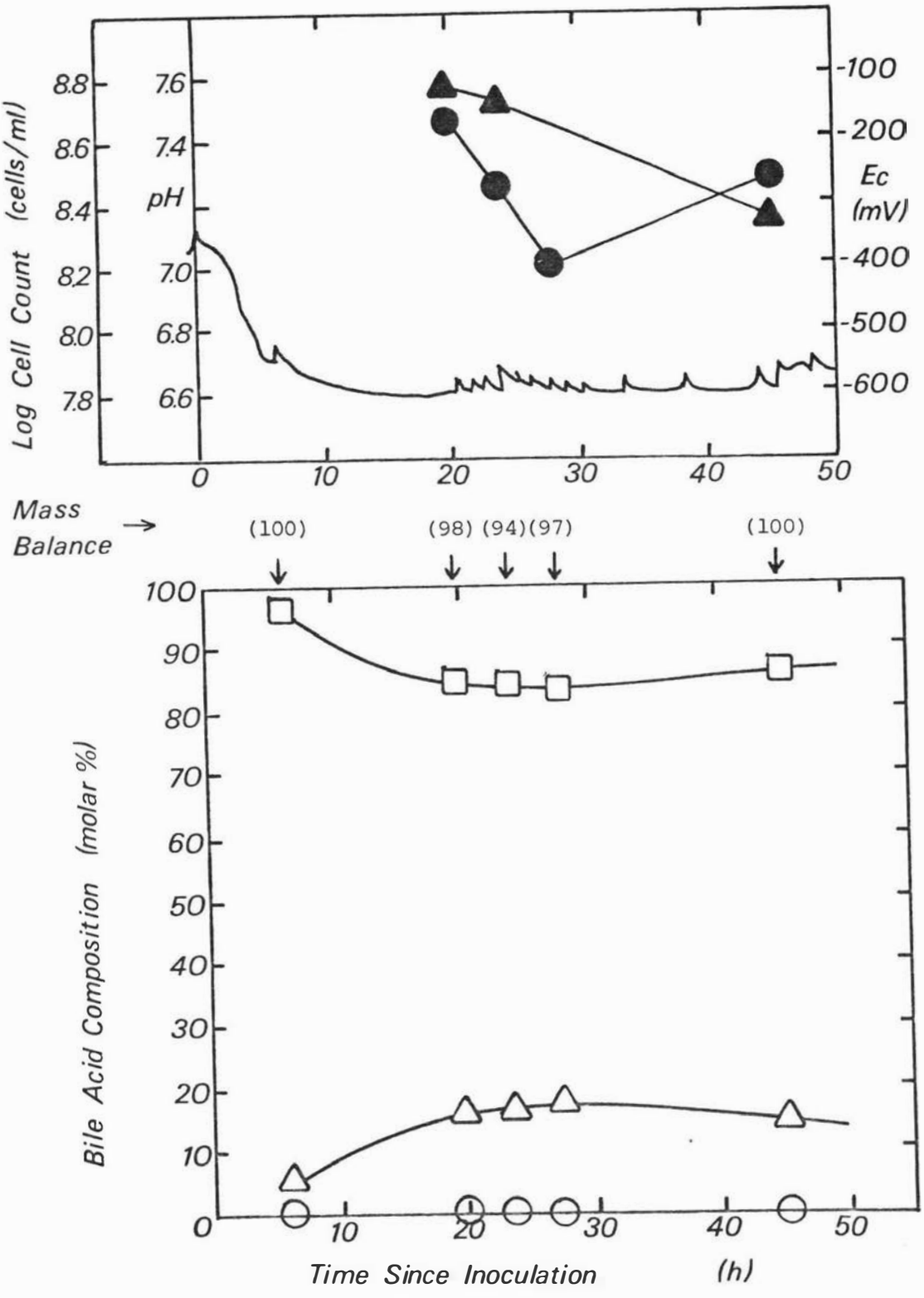


FIGURE 5.12 Course of Growth and Transformation for Experiment 2, Run 12

Legend as for Figure 5.1

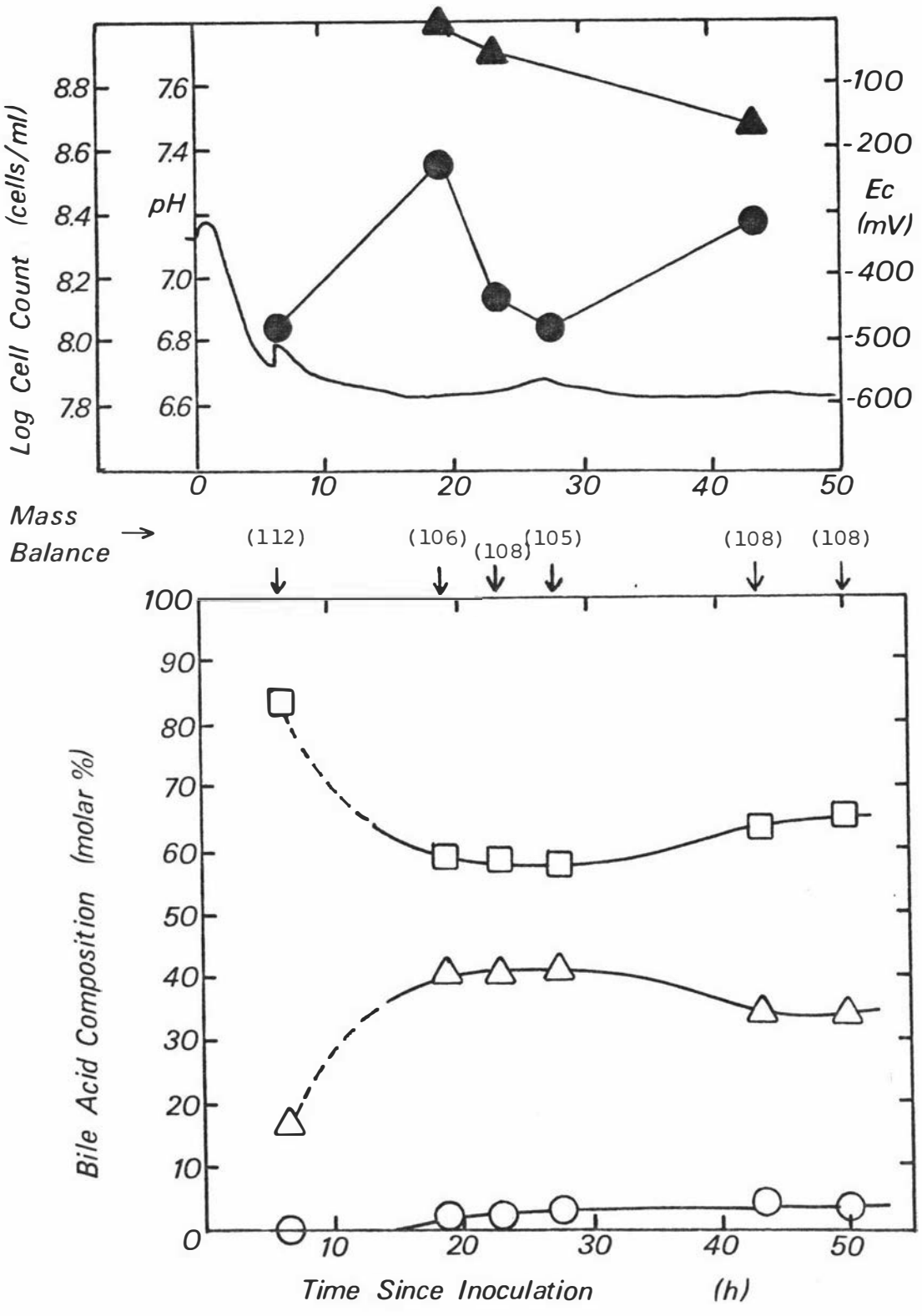


FIGURE 5.13 Course of Growth and Transformation for Experiment 2, Run 13

Legend as for Figure 5.1 Dashed line signifies unacceptable mass balance

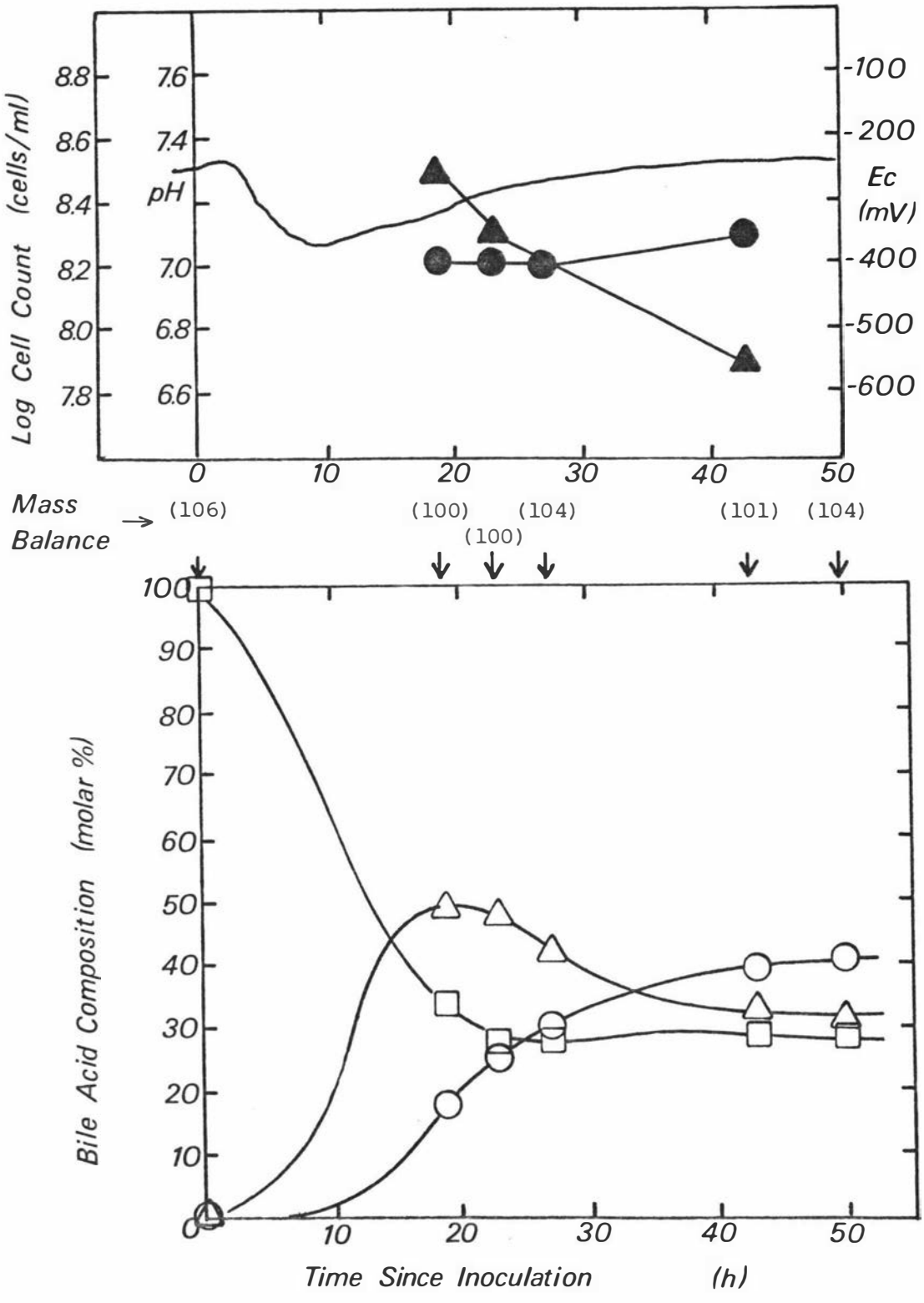


FIGURE 5.14 Course of Growth and Transformation for Experiment 2, Run 14

Legend as for Figure 5.1

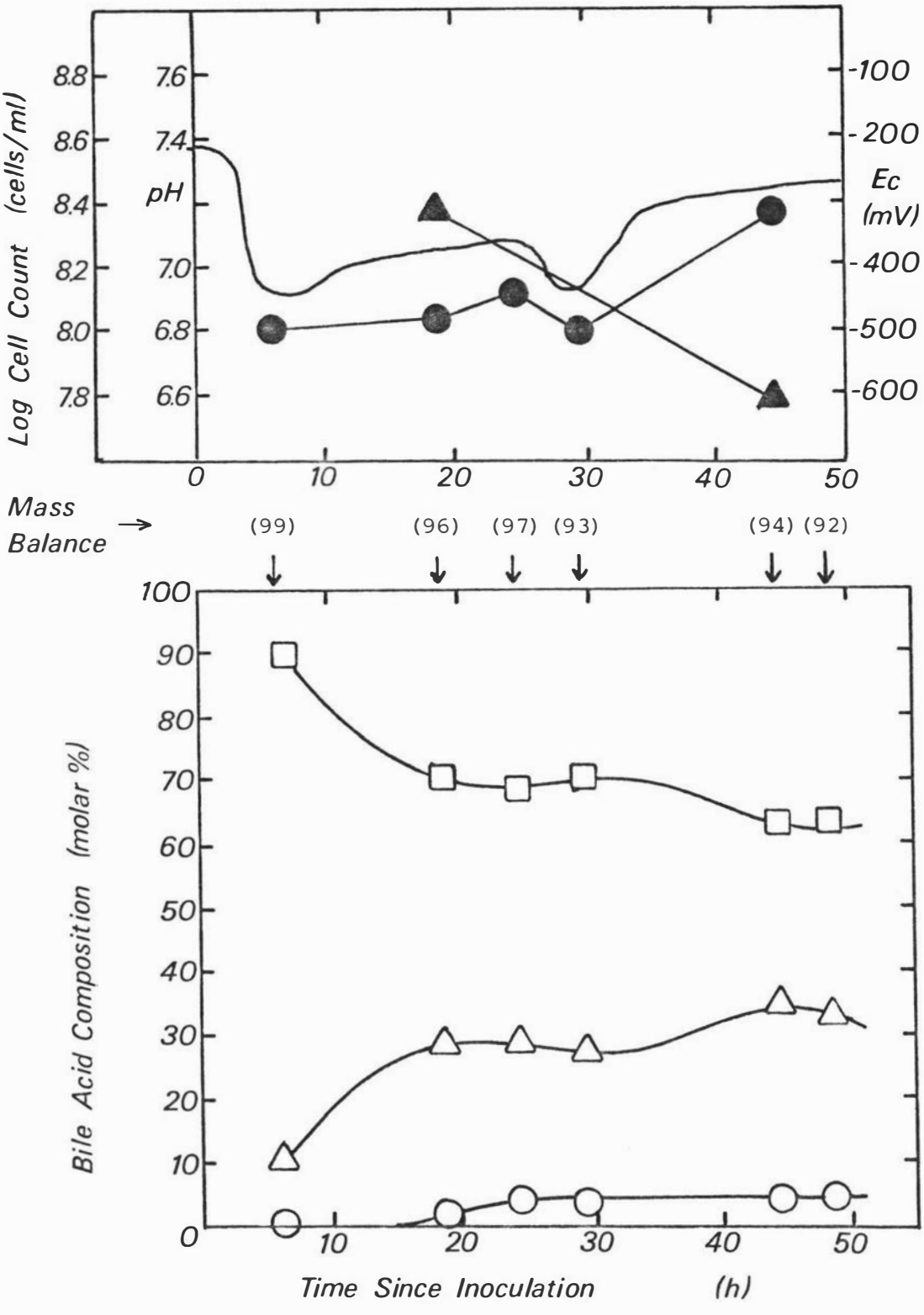


FIGURE 5.15 Course of Growth and Transformation for Experiment 2, Run 15

Legend as for Figure 5.1

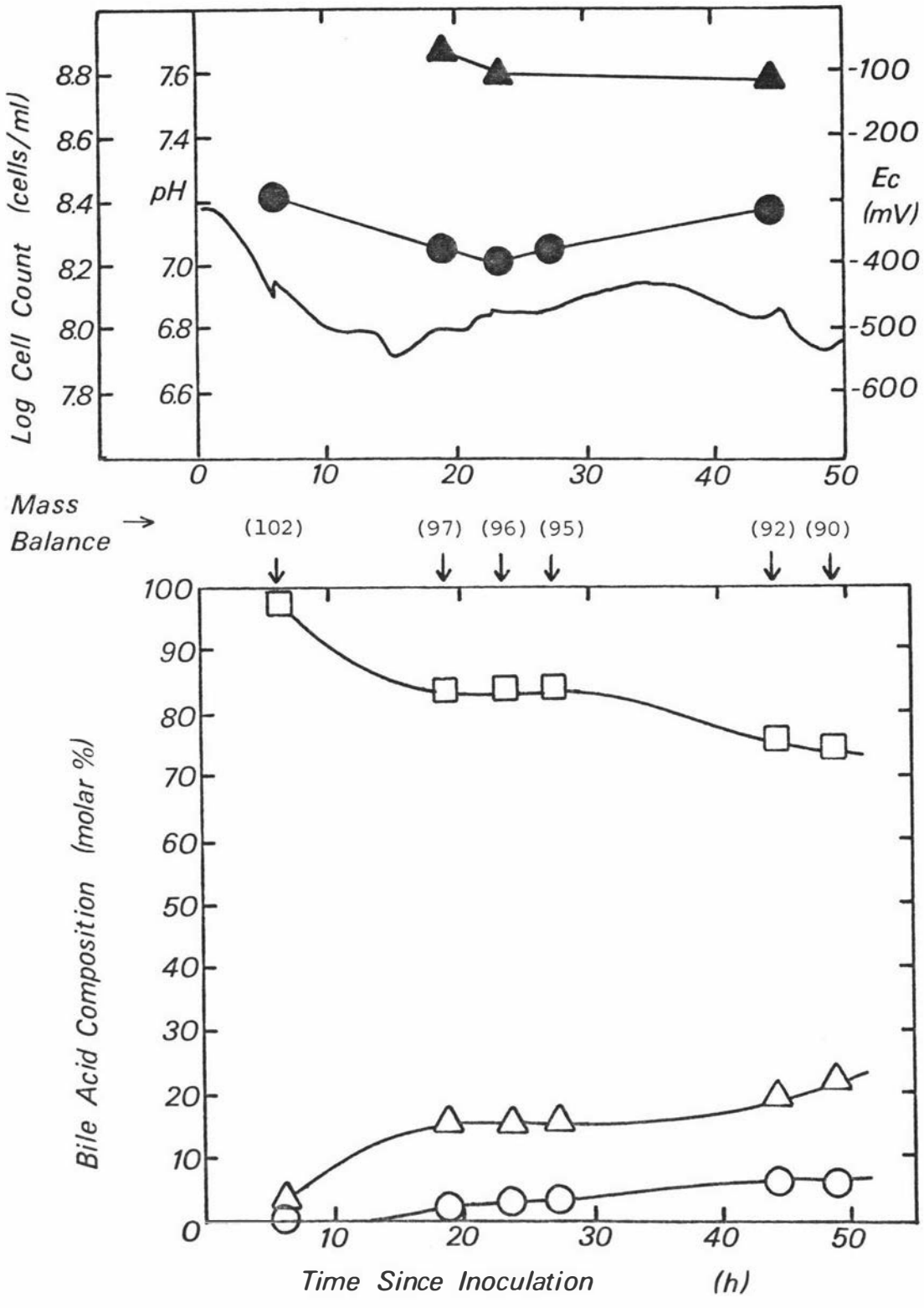


FIGURE 5.16 Course of Growth and Transformation for Experiment 2, Run 16

Legend as for Figure 5.1

distribution (they lay off the 1/2-normal plot line of best fit) were used to form a parsimonious model. Sufficient degrees of freedom were then available to test significance by the t-test. These models are presented in detail in Appendix 3, but the significance levels determined for the identified variables are indicated in Table 5.3.

TABLE 5.2a Raw Transformation and Growth Data for Experiment 2.

Run	Molar % Yield			L.M.C.
	7KD	C	D	
4	11.2	74.3	14.5	8.66
3	40.2	50.0	9.8	8.89
14	31.0	28.6	40.4	8.50
7	18.9	68.6	12.5	8.51
12	13.6	86.4	0.0	8.77
16	20.8	74.2	5.0	8.87
15	32.9	63.0	4.1	8.37
2	17.9	52.4	29.7	8.15
9	11.6	85.0	3.4	8.69
10	14.4	83.0	2.6	8.70
5	23.2	68.5	8.3	8.08
8	17.6	65.2	17.2	8.36
11	33.7	62.3	4.0	8.76
13	33.2	64.0	2.8	8.97
1	26.8	50.2	23.0	8.16
6	34.7	59.5	5.8	8.02

L.M.C. is the logarithm to base 10 of the maximum number of cells observed during a run.

Bile acid yields are those obtained after 48 h

The third significant figure in bile acid yields was preserved after normalization for use in computer regression programmes.

TABLE 5.2b Raw Electrode Potential Data for Experiment 2

Run	$(\Delta Ec/\Delta t)_{max}$ (mV/h)	Ec_{min} (mV)	Ec_i (mV)
4	-79	-510	-270
3	-57	-430	-280
14	-26	-370	-240
7	-51	-490	-320
12	-104	-600	-360
16	-39	-550	-310
15	-107	-450	-215
2	-114	-450	-295
9	-82	-600	-340
10	-79	-580	-330
5	-153	-450	-260
8	-198	-470	-195
11	-102	-500	-205
13	-72	-590	-335
1	-109	-530	-325
6	-45	-410	-280

$(\Delta Ec/\Delta t)_{max}$ is the maximum rate of electrode potential decline observed during a run.

Ec_{min} is the minimum electrode potential (mV) reached.

Ec_i is the electrode potential (mV) at inoculation.

TABLE 5.3 Coefficients from the Full Regression Models of Experiment 2

	Coefficient	Variable	%7KD Yield	ln(%7KD Yield)	%C Remaining	%D Yield	L.M.C.	$(\Delta Ec/\Delta t)_{max}$ (mV/h)	Ec_{min} (mV)	Ec_i (mV)
Ave.	β_0	Constant	23.9***	3.09***	64.7***	11.4***	8.52***	-88.6***	-499***	-285***
Main Effects	β_A	Organism	0.9	0.05	-0.1	-0.8	0.03	7	2	-8
	β_B	Inhibitors	1.5	0.11	-7.7***	6.2***	-0.26***	-12	46***	18**
	β_C	Time Subst.Addn.	2.8***	0.14*	-0.7	-2.1	-0.02	2	-11	-5
	β_D	Gas Delivery	4.0***	0.16*	-3.7*	-0.2	0.03	7	26**	27***
	β_E	Temperature	2.2**	0.08	3.4*	-5.6***	0.01	5	-4	-13
	β_F	Thioglycollate	-2.4**	-0.10	2.2	0.1	-0.00	22**	-18	-28***
	β_G	pH Anion	2.2**	0.09	-3.4*	1.1	-0.04	4	25**	3
	β_H	Inoculum Size	0.5	0.03	2.5	-3.1*	-0.06	-16*	-18	2
Two-Factor Interactions	$\beta_{AB+CG+DH+EF}$		-4.0***	-0.19**	4.5**	-0.6	-0.04	-8	-5	2
	$\beta_{AC+BG+EH+DF}$		-0.9	-0.04	-1.4	2.3	-0.04	12	8	-7
	$\beta_{AD+BH+CF+EG}$		-0.3	-0.02	1.4	-1.0	-0.05	-9	5	0
	$\beta_{AE+CH+BF+DG}$		4.9***	0.20***	-7.5***	2.7	0.03	21**	20*	3
	$\beta_{AF+GH+CD+BE}$		-0.1	0.00	4.5**	-4.3***	-0.03	7	6	12
	$\beta_{AG+BC+FH+DE}$		-0.1	-0.04	-0.0	0.2	-0.07	5	4	-7
	$\beta_{AH+BD+CE+FH}$		-0.3	-0.03	0.8	-0.5	0.02	-0	1	7

*** - significant at the 1% level as shown by the adopted parsimonious model
** - significant at the 2% level as shown by the adopted parsimonious model
* - significant at the 5% level as shown by the adopted parsimonious model

For each parsimonious model derived, the residual plots used in Chapter 4 were again employed to check equality, normality and the absence of time trends in the residuals. Only the model derived for 7-ketodeoxycholate yield showed an abnormal plot, residuals decreasing with increasing predicted yield (Figure 5.18). A new model was derived for the natural logarithmic transformation of 7-ketodeoxycholate yield which showed a better pattern of residuals.

The parsimonious models for log 7-ketodeoxycholate yield, deoxycholate yield and the three electrode potential variables all contained sufficiently few variables for them to be represented by 2^3 or 2^{4-1} designs which were replicated within the sixteen runs performed. This allowed testing for lack of fit. Only in the model for maximum rate of electrode potential decline ($(\Delta Ec/\Delta t)_{max}$) was there significant lack of fit at the 5% level. Growth (L.M.C.) was adequately described by a straight line (i.e. a one-term model).

Table 5.4 is the matrix of correlation coefficients for the various pairs of response variables.

TABLE 5.4 Matrix of Correlation Coefficients for the Pairs of Response Variables in Experiment 2.

	%7KD Yield	% C	% D	L.M.C.	$(\frac{\Delta Ec}{\Delta t})_{max}$	Ec_{min}	Ec_i
% C Remaining	-0.660**						
% DC Yield	0.041	-0.778**					
L.M.C.	-0.023	0.320	-0.406				
$(\Delta Ec/\Delta t)_{max}$	-0.264	0.193	-0.036	-0.371			
Ec_{min}	0.526*	-0.774**	0.590*	-0.546*	-0.055		
Ec_i	0.373	-0.420	0.247	-0.278	0.380	0.663**	
ln(7KDC Yld.)		-0.688**	0.089	-0.096	-0.218	0.545*	0.386

** - significant at the 1% level
* - significant at the 5% level

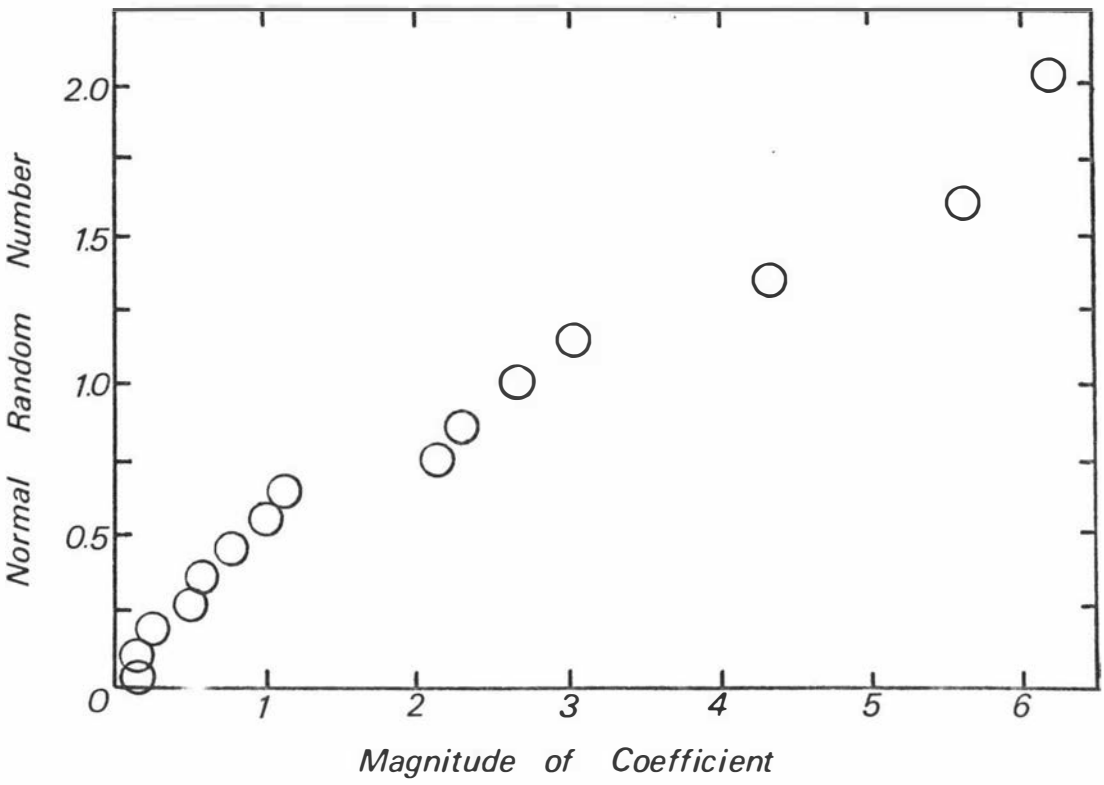


FIGURE 5.17 Half-normal Plot for Deoxycholate Yield from Experiment 2

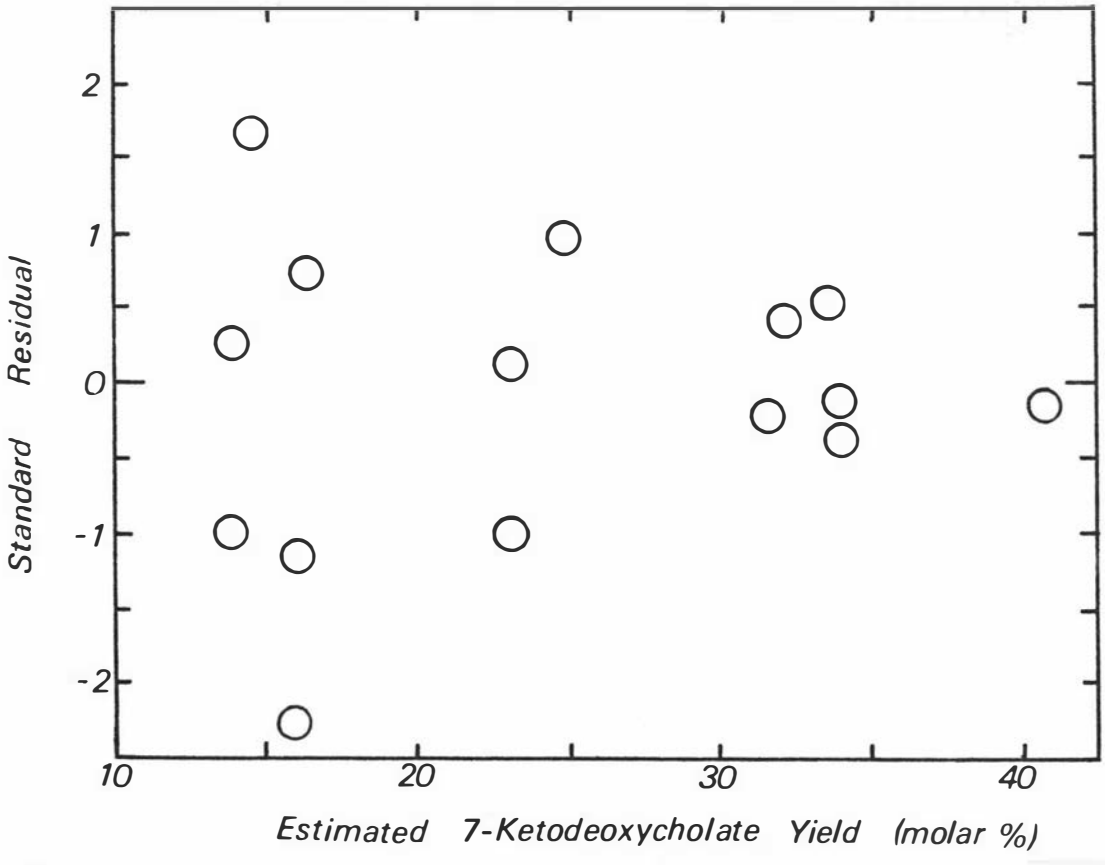


FIGURE 5.18 Residual Plot Number 2 for the Parsimonious Model for 7-Ketodeoxycholate Yield from Experiment 2

5.5 DISCUSSION OF EXPERIMENT 2

For most of the response variables investigated, at least one chain of two-factor interactions was identified as having an important effect. Since each such interaction contrast is the sum of four effects of variable sign, there may be other important two-factor interactions eclipsed by effects of opposite sign within the same chain. Furthermore, if two-factor interactions are so important, some three-factor interactions, which are confounded with main effects, may also be important. Although this is unlikely, the possibility must be borne in mind.

The inability of this design to discern the effects of individual interactions is a disadvantage; however, no design could do so in so few trials, least of all in a classical-type experiment.

The fact that each two-factor interaction contrast contains effects involving all eight independent variables means that no model involving such an interaction contrast can infallibly exclude any variable as being insignificant. From this point of view, the stated aim of the experiment may be impossible to achieve. However, it is possible to develop a scale of 'most likely to be important' to 'least likely to be important' variables.

The factors most likely to be important to dehydroxylation yields are temperature and the presence of inhibitors, followed by inoculum size. The largest of the interaction-chain effects is also highly significant. It is tempting to speculate that the inhibitor/temperature interaction is dominant here, since inoculum size partners a variable with an insignificant main effect.

A mechanistic explanation for these results is not available from the purely empirical experimental approach adopted, nor is it necessarily within the aim of the experiment. However, the observation that greater deoxycholate yields are achieved at the lower temperature with inhibitors being present, and with the smaller inoculum size, might indicate that dehydroxylation is favoured under conditions less suited to rapid growth. The non-significance of the correlation between

deoxycholate yield and L.M.C. suggests that the presence of inhibitors, the most important factor in determining deoxycholate yields, was also the only factor to significantly effect L.M.C.

The effect of the smaller inoculum size may be to increase the number of generations. As is the case with the presence of inhibitors and the lower temperature, smaller inoculum size would result in a longer growth period. Usually, exponential growth is complete within six to eight hours. The presence of inhibitors delayed completion to eight to ten hours. In all cases, dehydroxylation was seldom apparent before 18 h after inoculation. Probably it is during growth that the foundation of the dehydroxylating ability is being laid; the induction and/or production of cofactors, enzymes or co-enzymes is possibly limited to this period. Thus increased time spent in cell growth might conceivably increase dehydroxylating ability.

Optimum transformation under sub-optimal growth conditions has been reported for other systems. Macdonald *et al.* (1977) observed maximum production of extractable 3α- and 12α-hydroxycholanoyl dehydrogenase activities under sub-optimal growth conditions for an arginine-utilising *Eubacterium lentum*. Increased arginine levels resulted in greater cell yield and enzyme specific activity only up to a certain point. Further increases in arginine levels continued to improve growth yields, but resulted in a fall in dehydrogenase production.

In vivo, in the animal intestine, dehydroxylating organisms must compete in a crowded environment with many other organisms. Conditions for growth of one particular organism are presumably far from optimal, yet the 7α-hydroxy bile acids which are present at levels similar to those used in this experiment are largely dehydroxylated.

With regard to 7-ketodeoxycholate formation, no individual factor has as large an effect as temperature and inhibitors have on dehydroxylation. Here, gas delivery is the most likely factor to be important, yields being reduced by sweeping at 200 rev/min. Early substrate addition also assisted in decreasing 7-ketodeoxycholate yields, as did (to a lesser extent) low temperature, the presence of thioglycollate, and the use of OH^- ion as pH correctant. The relative unimportance of

inhibitors here is in sharp contrast to its effect on dehydroxylation. The fact that only one of the two transformations is boosted by the presence of inhibitors means that this variable probably does not act merely by increasing cell permeability to bile acids in general. As originally postulated, the inhibitors factor may indeed operate chiefly through 8-hydroxyquinoline chelating Cu^{++} ions which inhibit 7α-dehydroxylase to a greater extent than 7α-dehydrogenase (Drasar and Hill, 1974).

Fortunately, from the point of view of boosting deoxycholate while reducing 7-ketodeoxycholate yields, only two of the eight main effects have the same sign for both transformations. These are the presence of inhibitors, which boosts both transformations (although insignificantly for dehydrogenation), and pH anion; for which OH^- ion boosts both (although insignificantly for dehydroxylation). These two variables are also identifiable as showing two of the higher coefficients in the model for cholic acid utilisation. However, five of the seven two-factor interaction chains have the same sign for both transformation yield models reflected in three interaction chains being important to cholic acid utilisation. A model derived for the ratio of deoxycholate yield to 7-ketodeoxycholate yield showed low temperature and the presence of inhibitors to be most effective in producing a high ratio.

Perhaps the most prominent finding of the experiment is the insignificance of 'organism' to any model derived. Changing from the ATCC 9714 to the SD 10 strain produced no more than a 1% drop in deoxycholate yield or a 1% increase in 7-ketodeoxycholate yield, and increased cell yield by a factor of only 1.07. Since there is probably no significant difference between these strains insofar as the response variables investigated are concerned, any two-factor interaction involving factor 'A' is probably also negligible. The chains of two-factor interactions are therefore effectively reduced to length three.

During each trial of this experiment, the electrode potential was monitored. This value, Ec, is the potential difference (mV) between the polished platinum electrode and a saturated calomel electrode in electrical contact with it. It is an approximation to the true oxidation-reduction or redox potential.

Since the redox potential was not manipulated or controlled in this experiment, except through the variable thioglycollate, passive observations only are available. It is impossible therefore to conclude that a movement in electrode potential is a cause or an effect of a trend in transformation. It can be reported only that the two events occurred simultaneously.

Several response variables were defined in an attempt to determine the importance of redox potential in transformation. Of these, one - $(\Delta Ec/\Delta t)_{max}$ i.e. the maximum rate of decline of electrode potential - could not be adequately described. Models containing significant (5% level) terms showed significant (5% level) lack of fit, indicating the presence of important random influences not controlled in the experiment. The maximum rate of decline of electrode potential was governed mainly by the variables thioglycollate and inoculum size. Not unexpectedly, larger inocula increased the rate, and the presence of thioglycollate decreased the rate. This latter effect may be due to increased redox buffering or to decreased initial electrode potential, causing decreased scope for large rapid Ec decline (thioglycollate was highly significant in governing Ec_i, the electrode potential at inoculation). Of these two variables, only Ec_i is significantly correlated with any other response variable: runs with low initial electrode potentials also showed low electrode potential minima (Ec_{min}).

Strong correlations were observed between transformation and minimum electrode potential. The more negative the minimum Ec, the less transformation occurred. This result might be expected for dehydrogenation, an oxidative reaction requiring a supply of oxidised nicotinamide cofactor. The occurrence of greater dehydroxylation in trials where electrode potentials stayed relatively high was unexpected.

A greater degree of transformation has already been observed under conditions least conducive to rapid growth. There is also a significant correlation between maximum cell numbers observed (L.M.C.) and minimum Ec; greater growth parallels more negative Ec minima. The question then arose: is the association of low Ec with both strong growth and weak transformation a consequence of:

(a) strong growth depressing Ec, which in turn reduces activity of the enzyme systems involved, or

(b) better transformation occurring during weak growth, which incidentally results in less pronounced Ec minima?

This question is unanswerable within the context of the experiment; further work is required, involving redox control under different growth conditions.

The phenomenon of redox optima for dehydroxylation and dehydrogenation reactions has recently been observed by Bokkenheuser *et al.* (1977) with the 21-dehydroxylation of corticosteroids by *Eubacterium lentum*. At an Eh of -250 to -300 mV (equivalent to Ec = -494 to -544 mV), this organism catalysed the 21-dehydroxylation of tetrahydrodeoxycorticosterone to pregnalone, while at Eh -120 to -180 mV (Ec = -364 to -424) it oxidised the 3-hydroxy group of the same substrate to form dihydrodeoxycorticosterone. In the present experiment, the beginning of dehydroxylation often accompanied a rise in electrode potential to *ca* -400 mV. If the Ec rose past -250 mV, dehydroxylation had usually ceased.

Trial 14 of the current experiment produced yields of 41% for the 7-deoxy and 31% for the 7-keto products; the second highest deoxycholate yield was 30% in run 2. As in Experiment 1, this is a high single value. In fact, run 14 was performed twice, since on the first occasion pH control failed after 24 hours, by which time deoxycholate yield exceeded 30%, identical to the second attempt. The 24 h 7-ketodeoxycholate yield was a little higher however, at 55% compared with 44% on the second attempt. The L.M.C. values of the two replicates differed by only 0.07 log units, and the electrode potential curves were superimposable for the first 24 h.

The usual follow-up to a screening experiment is to incorporate those variables shown to be important into a more detailed design. This would involve three or more levels, and often extend variables past the limits used in the screen. In Experiment 2, the largest yield of deoxycholate observed was of the order of 40%, still accompanied by 30% of the 7-keto side-product. Although some advance has been made (in Experiment 1, deoxycholate yield never exceeded that of 7-ketodeoxycholate, and the highest deoxycholate yield was 19%), the target of *ca* 90% dehydroxylation with no dehydrogenation - dictated by probable process economics - was still distant. Thus, there was considered to

be little point in performing a detailed fine-tuning experiment at this stage of the project. The largest single main effect on dehydroxylation was that of inhibitors. When the inhibitors were present, yields were higher than the average 11.4% by 6.2%. Thús, a small increase in inhibitor concentration is unlikely to significantly enhance dehydroxylation.

A new approach was therefore required in the search for conditions which should stimulate dehydroxylation and for conditions which depress dehydrogenation. The next chapter describes a series of experiments which were conducted with the aim of discovering a selective inhibitor of the oxidative reaction.

5.6 CONCULSIONS

In their effect on transformation yields, growth, and electrode potentials, the two *Cl. bifermentans* strains ATCC 9714 and SD 10 were indistinguishable.

7α-Dehydroxylation was boosted, and 7α-dehydrogenation largely unaffected by the presence of fluoride and 8-hydroxyquinoline. These inhibitors also reduced growth, and were associated with less pronounced electrode potential minima.

Zero-time addition of bile acid substrate decreased the yield of the 7-keto and slightly increased that of the 7-deoxy product compared with 6-hour substrate addition. The presence of 0.05% w/v cholic acid during growth had no effect on cell yield.

Sweeping the medium surface with gas gave lower 7-ketodeoxycholate yields than sparging, while having little effect on dehydroxylation.

Higher deoxycholate yields were obtained at 32^{0}C than at 37^{0}C, accompanied by slightly lower 7-ketodeoxycholate yields. The ratio of the 7-deoxy to the 7-keto product was significantly higher at the

lower temperature, and the overall degree of transformation was increased.

The presence of thioglycollate reduced the rate of electrode potential decline, and depressed the initial potential, but only slightly depressed the minimum Ec. Thioglycollate also decreased 7α-dehydrogenation and thus total transformation to some extent.

Use of hydrogen carbonate ion rather than hydroxyl ion for pH control was associated with higher electrode potential minima and slightly greater 7-ketodeoxycholate yields.

Inoculum size had no effect on maximum growth yield. Smaller inocula boosted dehydroxylation yield, and was associated with a reduced rate of electrode potential decline.

A strong association was apparent between high electrode potential, weaker growth and high transformation yields, particularly of deoxycholate.

On several occasions, deoxycholate yield exceeded 7-ketodeoxycholate yield. This represents an advance over Experiment 1 results. Further, the highest 48-hour deoxycholate yield of 40% was double the highest 7-day yield of 19% achieved in Experiment 1. Despite using the best Experiment 1 condiditions throughout (pH 7.0/N_2-CO_2, 9:1) many Experiment 2 runs showed low deoxycholate yields. Hence these conditions do not necessarily guarantee deoxycholate yields of greater than 10%.

No conditions tested resulted in the elimination of 7α-dehydrogenation. A more drastic approach must be adopted to prevent totally any oxidative side-product formation.

CHAPTER 6 CHOLIC ACID TRANSFORMATION USING WASHED RESTING CELLS OF Cl. BIFERMENTANS ATCC 9714

6.1 INTRODUCTION

In Experiment 2, major fermentation variables were examined. Some were found to be more effective at increasing deoxycholate yield and decreasing 7-ketodeoxycholate yield than others. However, manipulating the variables within the confines of the experiment failed to produce yields approximating the 90-100% dehydroxylation or the 0% dehydrogenation desired.

Since dehydroxylation was always accompanied by dehydrogenation despite the range of environments tested, an expensive separation and purification operation would be involved in any industrial process based on these fermentation conditions. It was therefore considered necessary to seek an alternative method of preventing the oxidation without impairing dehydroxylation.

Since single-vessel batch fermentation, with its ease of operation and simplicity of plant had not so far been successful, the next step was to consider a separation of the process into two stages:

(a) growth of cells in a fermenter, followed by
(b) use of harvested resting-stage cells to effect the cholic acid transformation.

The use of harvested, washed and resuspended cells has the advantages that:

(a) transformation substrate comes into contact with buffer and with cells, rather than with complex media, thus simplifying subsequent purification, and
(b) compounds inhibitory to 7α-dehydrogenation but also damaging to cell growth can be included in the reaction mixture without any undesired effects.

In addition, in the context of the present research, several experimental trials could be performed with a single batch of cells. From a process point of view, there are also disadvantages:

(a) complexity - it is a two-stage operation, and

(b) plant - at least two controlled-environment vessels are required, as well as cell separation equipment.

Given the points made above, the series of experiments reported in this chapter was principally a search for an agent to inhibit dehydrogenation, since the production of the 7-keto side-product represents the greatest drawback in developing a microbial process for the production of deoxycholic acid.

6.2 FERMENTATION CONDITIONS AND CELL PREPARATION

Cell production, harvesting, washing and resuspension are described in section 3.8. A low level of cholic acid was included in cell production media to ensure enzyme induction. Growth conditions were selected to provide good cell yields and high dehydrogenase activity, and resulted in low levels of dehydroxylase. Cell harvesting, washing and incubation were performed under as anaerobic conditions as possible in the absence of a glove cabinet. Virtually no dehydroxylase activity was detected in washed cells, but whether this was entirely due to low initial levels of activity or to loss of activity during harvesting and washing is not clear. Spent cell production liquor was assayed for deoxycholate and yields of between 1% and 4% were found, indicating low initial dehydroxylase levels.

6.3 RESULTS AND DISCUSSION

Several individual trials were conducted, each involving a separate batch of cells. This subsection reports the results of selected trials.

The cell yield and electrode potential curve during cell production for each trial are presented in Appendix 4. Cell yields of the cell slurries averaged 17.0 mg/ml dry weight, ranging from 13.2 to 20.8 mg/ml. This corresponds to 0.66 to 1.04 mg/ml dry weight of fermentation medium.

6.3.1 Time Scale and Reproducibility of the Reaction

An initial experiment was performed to investigate the time-scale of transformation and also to check the point-to-point reproducibility within a single run. Cholic acid was added to a concentration of 0.05% w/v in pH 7.0, 0.02 M phosphate buffer. Incubation was at 35^0C under oxygen-free nitrogen. The results of this run are depicted in Figure 6.1.

7-Ketodeoxycholate yields of 8 to 10% were achieved under these conditions, with most of the transformation being complete within two hours. No deoxycholate was detected. In the zero-time sample cells were in contact with cholic acid substrate for 5 to 10 min before the pH was raised to *ca* pH 12 and the cells removed by centrifugation. Appreciable transformation had occurred even within this short period. In all subsequent experiments, zero-time samples were taken within seconds of inoculation with the result that this sample showed no transformation.

As is apparent from the clustering of individual determinations in Figure 6.1, reproducibility from tube to tube was acceptable.

6.3.2 Effect of Aerobic Incubation

A trial was conducted to investigate the effect of atmospheric oxygen on the extent of dehydrogenation. Reaction mixtures were either left open to the atmosphere or continuously flushed with oxygen-free nitrogen. Incubation was at 30^0C with 0.05% w/v cholic acid in 0.02 M pH 7.2 phosphate buffer. The results are depicted in Figure 6.2. The mass balance is also shown, and indicates that there was appreciable loss of detectable steroid material under aerobic conditions. There are several possible explanations for this:

(a) aerobic degradation by *Cl. bifermentans* to non-steroid products,
(b) formation of di- or tri-keto products which would be eluted at the HPLC solvent front and so not be detected, or
(c) contamination of all 48 h aerobic incubation mixtures by steroid-degrading organisms (the tubes were open to the atmosphere in a non-sterile environment).

Again, 7-ketodeoxycholate formation had virtually ceased within two

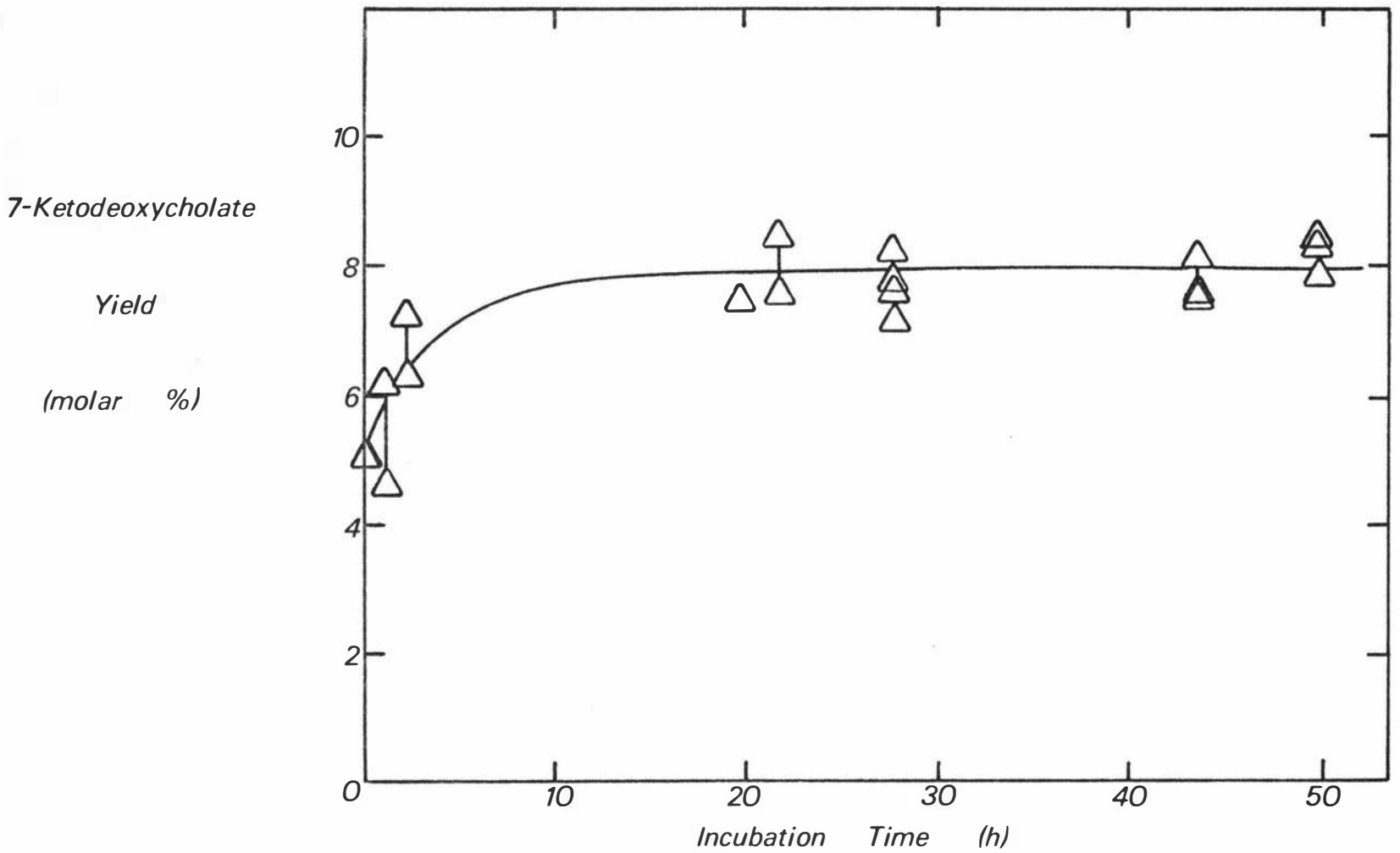


FIGURE 6.1 Time Scale of Dehydrogenation by Whole Resting Cells

Experimental conditions are described in the text (section 6.3.1)

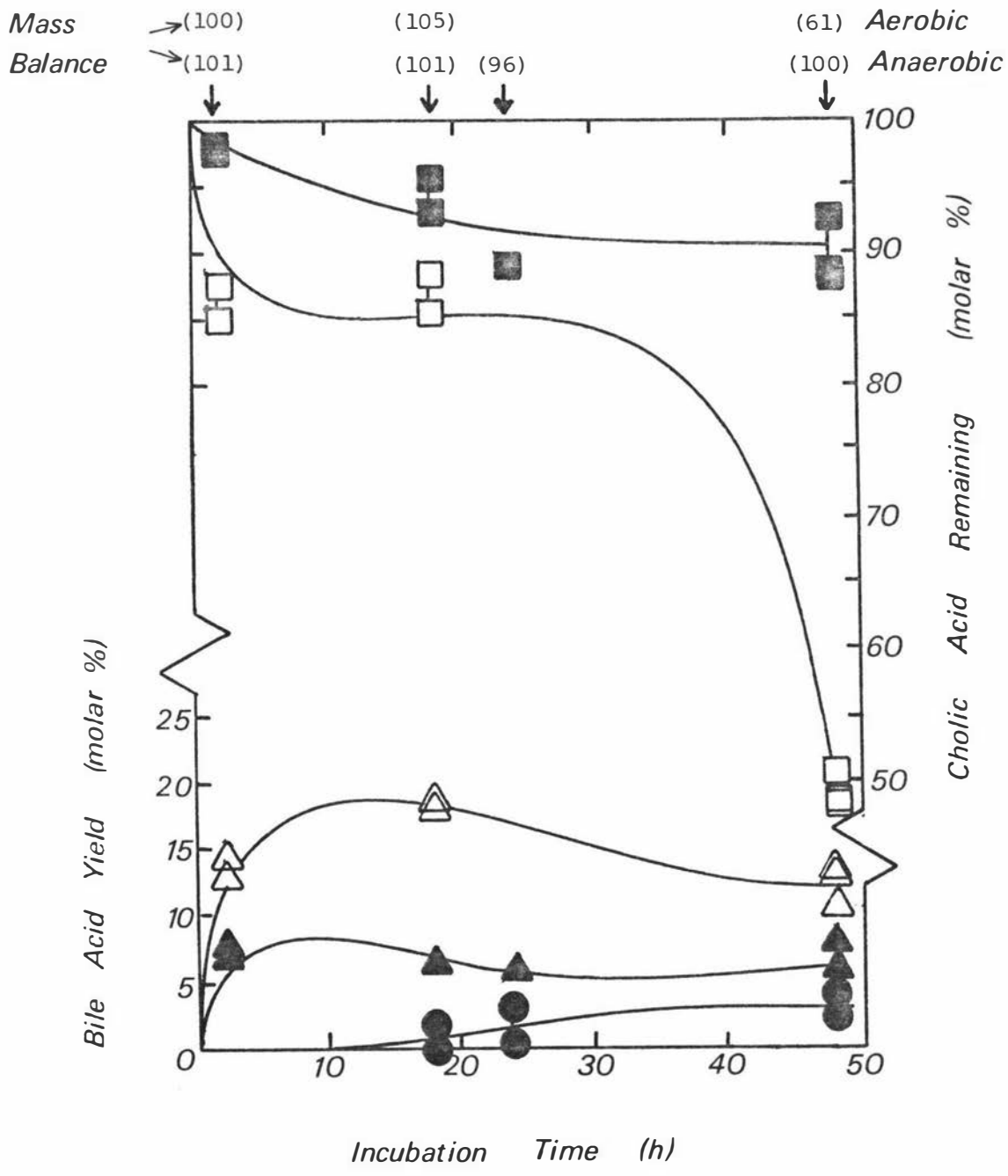


FIGURE 6.2 Effect of Aerobic Incubation on Dehydrogenation by Whole Resting Cells

Deoxycholic acid (○,●), 7-ketodeoxycholic acid (△,▲), cholic acid (□,■). Hollow symbols for aerobic incubation, solid symbols for anaerobic incubation. Yields are not normalised. Experimental conditions are described in the text (section 6.3.2).

hours, with the yield under anaerobic conditions similar to that in Figure 6.1 Under aerobic conditions, however, yields were approximately double. This agrees with the observations of Aries and Hill (1970b) who found molecular oxygen to be necessary for cell-free dehydrogenation by extracts of several anaerobes, and of Sherrod and Hylemon (1977). These latter workers incubated whole cell suspensions of the fastidious anaerobe *Bacteroides thetaiotaomicron* with a number of electron acceptors. When they incubated aerobically, conversion of cholate to 7-ketodeoxycholate proceeded at approximately 10% conversion per hour over the four hours investigated. A control incubated under nitrogen stopped dehydrogenating after 1 h at 8% conversion. Another electron acceptor, fumarate (under anaerobic conditions) promoted conversion to a total of 30% over four hours, with conversion rate increasing with time.

Some deoxycholate production was observed in this trial, but notably not under aerobic conditions. It is apparent from the results of this run that to minimise dehydrogenation, transformation conditions should be oxygen-free.

6.3.3. Effect of Atmosphere, pH, Incubation Time and Temperature

This section reports the combined results of two trials which investigated the effects of atmosphere, incubation time and temperature on the pH profile of whole cell cholate dehydrogenation. In the first trial, incubation was at 37^0C, with five tubes being flushed with oxygen-free nitrogen, and five with N_2-CO_2, 9:1. Each group of five contained 0.05% w/v cholic acid in 0.02 M buffer at pH 8.50, 7.75, 7.00, 6.25, or 5.50. The two high pH values employed Tris-HCl buffer, the lower values phosphate buffer. The results are presented in Figure 6.3. The second trial was conducted at 30^0C with ten tubes being flushed with N_2-CO_2, 9:1 directly from the cylinder; the other ten were flushed with N_2-CO_2, 9:1 which was continuously de-oxygenated by passage over heated copper turnings. This was to check if trace oxygen levels in the gas mixture (prepared with industrial dry nitrogen, not oxygen-free nitrogen) affected dehydrogenation. Half of each group of ten tubes was incubated for 1 h, the other half for 20 h. Each group of five contained 0.05% w/v cholic acid in 0.1 M buffer at pH 8.50, 7.75, 7.00, 6.25 or 5.50. The same buffers were used as in

the first trial, except that maleate buffer was used at pH 5.5. The results of this trial are plotted in Figure 6.4. The final pH of the 1 h mixtures was within 0.1 units of the initial pH except for those initially at pH 8.50, which had dropped to 8.26. The final pH of the 20 h mixtures was within 0.2 units of the initial pH except for those initially at pH 7.75, which had dropped to *ca* pH 7.2, and those initially at pH 8.50 which had dropped to pH 7.5.

From Figure 6.3 it is apparent that under oxygen-free nitrogen, the pH yield optimum is between pH 6.0 and 6.5. The optimum appears to be nearer neutrality under N_2-CO_2, 9:1. However, acidification by CO_2 of those mixtures with higher initial pH values occurred during incubation. The differences between the two gases are not marked, either in terms of maximum yield or of optimum pH. The results at 30^0C, in Figure 6.4, are very similar, with peak yields of *ca* 15% occurring between pH 6.3 and 7.0. Yields were slightly lower after 20 h than after 1 h, indicating some reduction of the ketone had occurred after 1 h. Taking into account pH drift, the 20 h mixtures would result in curves very similar to the 1 h curves.

As the earlier discussion in Chapter 4 indicated, cell-free dehydrogenases show highly alkaline pH optima (*ca* pH 10). The relatively low pH yield optima determined here probably reflects the differences between cell-bound and cell-free enzyme activities, and differences between maximum rates and final yield achieved. It is conceivable that the reactions involved in 7-ketodeoxycholate formation continue for a longer period between pH 6.3 and pH 7.0 than at other pH values. The dehydrogenation pH yield optimum reported here is slightly more acid than that determined for growing cells in Experiment 1, and may indicate that greater enzyme production occurs under neutral or mildly alkaline conditions.

The trials reported in this section have revealed little difference between the temperatures 30^0C and 37^0C, or between the gasses oxygen-free nitrogen, N_2-CO_2, 9:1 and oxygen free N_2-CO_2, 9:1. Little increase, indeed some decrease, in 7-ketodeoxycholate yield occurred after the first hour of incubation.

6.3.4 Effects of Common Enzyme Inhibitors and Other Miscellaneous Chemicals

This section reports the combined results of several trials. In each trial various chemicals (including organic solvents, electron acceptors, common enzyme inhibitors, metal ions and chelating agents) were investigated for their effects on the 7α-dehydrogenation relative to duplicate control samples.

Many of the compounds tested have been implicated in the literature as having effects on similar steroid transformations. Fumarate was included as a potential electron acceptor as mentioned in section 6.3.2. Ohlson *et al.* (1978) have reported that the presence of organic solvents can increase the rate of hydroxysteroid dehydrogenation by increasing the steroid permeability of cell walls and/or membranes. Barnes *et al.* (1976) and Bilton *et al.* (1977), using *Pseudomonas*, have shown that nuclear steroid dehydrogenase can be directly coupled to nitrate reduction. (Bergey's Manual (Smith and Hobbs, 1974) states that for *Cl. bifermentans* and *Cl. sordellii*, nitrate reduction is variable.) Glucose was included to test the possibility that steroid dehydrogenation is linked to energy metabolism, in which case the presence of an alternative energy supply may depress transformation. On the other hand, glucose may have a stimulatory effect if an initial energy source is necessary to assist dehydrogenation. Other compounds tested included p-chloromercuribenzoate (pCMB), dicumarol, EDTA and several divalent metal ions. Each of these was employed in the search for an inhibitor of 7α-dehydrogenation, possibly by poisoning nicotinamide cofactor metabolism.

The electron transport inhibitor dicumarol and the sulphydryl enzyme inhibitor pCMB were added to incubation mixtures as DMSO solutions after autoclaving. For these two compounds, results are expressed as % of the DMSO-control yield.

All compounds mentioned so far were tested in pH 7.2 0.02 M phosphate buffer. However, some metal phosphates are insoluble, and also some polybasic carboxylic acid buffer compounds are chelating agents. For these reasons, metal salts were dissolved in 0.04 M pH 7.0 barbitone-acetate buffer to a final concentration of 0.05 M.

These solutions were adjusted to pH 7 if necessary with 1 M NaOH. This resulted in some precipitation of hydroxides of Cu^{++} and Sn^{++}, reducing the effective concentration of these ions in the supernatant liquid. Excessive precipitation of hydroxide at pH 7 prevented the testing of some other ions such as Fe^{+++}, Pb^{++} and Hg^{++}.

In all trials reported in this section, incubation was at 30^0C with 0.05% cholic acid for 48 h under oxygen-free nitrogen. The results are reported in Table 6.1, which includes details of reaction conditions. All tests were performed in duplicate unless otherwise indicated. (Table 6.1 overleaf)

The presence of 1% acetone resulted in a near doubling, and of 5% acetone a near tripling of the 7-ketodeoxycholate yield observed in the control. The presence of DMSO had a similar, though less marked, effect. Nitrate also showed a marked stimulatory effect, approximately doubling the control yield. Glucose and fumarate, alone and in combination, had little net effect, glucose being slightly stimulatory, and fumarate - if anything - slightly inhibitory.

Sodium thioglycollate, the only reducing agent tested, lowered control activity by 65%, presumably by depriving the cells of many of the potential electron acceptors, which might otherwise have been available.

These results indicate that *Cl. bifermentans* can probably link nitrate reduction and possibly the reduction of other electron acceptors such as O_2 to steroid dehydrogenation. This process can be depressed somewhat by addition of suitable reducing agents. The results involving solvents confirm the work of Ohlson *et al.* (1978), and may implicate a permeability barrier as being a limiting factor in steroid dehydrogenation by whole cells.

The uncoupling reagent, dicumarol, and the sulphydryl group inhibitor, pCMB, had no great effect on whole cell dehydrogenation, yields being of the order of those observed for the control. There is, however, no indication as to the effective intracellular inhibitor concentration.

TABLE 6.1 Effects of Miscellaneous Compounds on Cholate Transformation by Whole Cells

Compound	Concentration	% of Control 7-Ketodeoxycholate yield	% Mass Balance
acetone[a]	1% v/v	188	97
acetone[a]	5% v/v	276	98
DMSO	1% v/v	133	94
DMSO	5% v/v	175	102
KNO_3[c]	0.1% w/v	198	100
glucose	0.1% w/v	127	101
fumarate	4 mM	81	100
glucose + fumarate	0.1% + 4 mM	121	98
Na thioglycollate[a]	0.05% w/v	34	96
pCMB	10^{-4}M	119	100
pCMB	10^{-5}M	117	99
pCMB	10^{-6}M	111	102
pCMB	10^{-7}M	92	101
dicumarol	10^{-4}M	108	100
dicumarol	10^{-5}M	112	99
dicumarol	10^{-6}M	119	103
dicumarol	10^{-7}M	89	97
EDTA[b]	50 mM	264	101
$SnCl_2 \cdot 2H_2O$[b]	50 mM	178	92
$MgSO_4 \cdot 7H_2O$[b]	50 mM	160	102
$BaCl_2 \cdot 2H_2O$[b]	50 mM	105	101
$MnCl_2 \cdot 4H_2O$[b]	50 mM	73	118
$CuCl_2 \cdot 2H_2O$[b]	50 mM	66	89
$CoCl_2 \cdot 6H_2O$[b]	50 mM	65	114
$ZnSO_4 \cdot 7H_2O$[b]	50 mM	45	99
$CdCl_2 \cdot \frac{1}{2}H_2O$[b]	50 mM	26	103

(a) - single determination only. All other results are averages of duplicates.

(b) - normalised to mass balance of 100% to aid comparison. These results were obtained using 0.04 M pH 7 barbiturate-acetate buffer. All others were with 0.02 M pH 7.2 phosphate buffer.

(c) - internal standard degraded on acidification prior to extraction. Yield extracted by assuming 100% mass balance.

Aries and Hill (1970b) investigated the effect of several enzyme inhibitors on cell-free extracts of bacteroides and clostridial 7α-hydroxycholanoyl dehydrogenase (including periodate and iodoacetate but excluding pCMB and dicumarol). In reviewing this work, Drasar and Hill (1974) concluded the incomplete action of the inhibitors indicated that the enzyme does not possess readily denatured or complexed groups at the active site. The present results do not contradict this conclusion.

There was weak dehydroxylation apparent during the experiment with dicumarol and pCMB. The latter compound completely inhibited deoxycholate formation at the 10^{-4}M level, and by 20% at 10^{-5} M. Dicumarol inhibited dehydroxylation by 30% at 10^{-4}. At all other inhibitor concentrations, deoxycholate yields were at the order of the (very low) control yields.

These results indicate that neither dicumarol nor pCMB can be used to inhibit whole cell dehydrogenation; nor would these compounds act without inhibiting dehydroxylation.

The hydroxides of copper and tin precipitated heavily when centrifuged with alkaline internal standard solution. This resulted in an overall loss of bile acid, probably by adsorption to the precipitating hydroxide, and is reflected in the low mass balance figures. Alternatively tin and copper cholanoates may have been partially insoluble. The samples containing Mn^{++} and Co^{++} showed high mass balance figures which may have been caused by impaired extraction of the internal standard. The yields of metal ion- and EDTA-containing samples have been normalised to a 100% mass balance to aid comparison between those with high and those with low mass balance.

Aries and Hill (1970b) investigated the effects of metal ions on the cell-free 7α-hydroxycholanoyl dehydrogenase of *Bacteroides* and *Clostridium*, and reported partial inhibition by 30mM Cu^{++} and Mg^{++}. The partial inhibition by copper observed in the current work is in agreement with these workers, but the stimulation by Mg^{++} is in direct contrast. This may reflect a difference between whole cells and cell-free extracts.

The stimulation of dehydrogenation by EDTA in this experiment is an important finding, and may offer a method for greatly stimulating 7-ketodeoxycholate formation, should this ever be desired. This effect has two possible explanations:

(a) One or more metal ions may inhibit whole cell dehydrogenation at the low levels remaining associated with the cells after harvesting and washing. If these were chelated, yields might be expected to be higher. The inhibition observed by several metal ions in this experiment supports this explanation.

(b) EDTA may disrupt the cholate permeability barrier as has been shown in the *Escherichia* cell wall, presumably by chelating divalent metal ions responsible for maintaining murein structural integrity (Burman *et al.*, 1972; Nordstom *et al.*, 1970; Hylemon and Stellwag, 1976).

Aries and Hill (1970b) also reported an effect of EDTA on cell-free 7α-hydroxycholanoyl dehydrogenase. These workers observed that EDTA enhanced dehydrogenation in extracts containing no added NAD^+, but had no effect in those extracts to which exogenous NAD^+ had been added. They concluded that EDTA was probably somehow responsible for releasing nicotinamide cofactor from the small membrane particles present in the extract.

Several of the metal ions tested showed inhibitory effects, that of Cd^{++} being the greatest. However, cadmium is one of the very toxic cumulative heavy metal poisons, and hence undesirable in an industrial effluent. Zinc, the second most inhibitory metal ion, would be preferable.

6.4 CONCLUSIONS

Although 7α-hydroxycholanoyl dehydrogenase activity was lower in washed cell suspension than in the fermentation cultures of Experiments 1 and 2, the activity proved robust. A range of possible inhibitors was tested, but only the presence of Zn^{++}, Cd^{++} or the reducing agent sodium thioglycollate markedly depressed dehydrogenation. Conversely, the potential electron acceptors nitrate and molecular oxygen boosted

oxidation as did the presence of some organic solvents, Sn^{++}, Mg^{++} and, most markedly, EDTA.

The pH yield optimum of 7α-dehydrogenation by whole resting cells was between pH 6 and pH 7, and yields were largely unaffected by temperature or the presence of CO_2.

These results offer no method of entirely preventing formation of the 7-ketodeoxycholate side-product, although yields might be further decreased if thioglycollate, Zn^{++} or Cd^{++} levels were increased. Zinc ion and thioglycollate were subsequently tested in fermentations (see Chapter 7).

Several experiments reported in this chapter have indicated methods by which 7α-dehydrogenation could be boosted. It seems possible that high 7-ketodeoxycholate yields could be obtained in the absence of deoxycholate production, if this were desired.

The cell production conditions employed were not optimal for 7α-dehydroxylation activity. Despite its presence at low levels in the 16-hour fermentations, dehydroxylase activity was observed in only two of the thirteen cell suspensions used. Hence activity appears to be very sensitive to the cell washing procedure. It was partly for this reason that no trials were performed with washed cells produced under conditions better suited to dehydroxylation. It is therefore very difficult to extrapolate the results of these batch washed-cells experiments to the industrial process situation; no conclusions can be drawn in this respect. Further research in the area of 7α-dehydroxylation of cholic acid by washed cells with a view to an industrial process would probably have merit only if very anaerobic conditions could be provided, perhaps through the use of a glove cabinet.

CHAPTER 7 EFFECT OF AEROBIC INCUBATION AND MISCELLANEOUS COMPOUNDS ON CHOLIC ACID TRANSFORMATION DURING BATCH FERMENTATION

7.1 INTRODUCTION

This chapter reports a series of experiments designed to apply the results of the washed cell work to batch fermentation. The practice used in the washed cell experiments of growing cells in the presence of only small quantities of transformation substrate was retained. However, instead of the cells being harvested and resuspended, cholic acid and the compound under test were added directly to the fermentation medium after growth was complete. This represents a compromise in complexity between washed cell transformation and growing cell transformation. Some advantages of the former method are retained in the latter: conditions beneficial to transformation, but harmful to growth, are not employed until near stationary phase, and only one controlled environment vessel is required. However, some of the advantages of washed cell transformation are lost: steroid substrate comes into contact with the divers microbial growth metabolites in the spent medium, and only one experiment is possible per batch. Further, the method adopted was considered worthwhile because dehydroxylation commenced only after growth was complete (see Chapters 4 and 5).

7.2 FERMENTATION CONDITIONS

Fermentations were based almost exactly on the conditions described for Experiment 2. Where it was desired that the 7α-dehydroxylase be dominant, conditions were based on Experiment 2, run 14. Where it was desired that the 7α-dehydrogenase be dominant, conditions were based on Experiment 2, run 13. Any modifications to these conditions are reported with the appropriate fermentation in the following Results and Discussion section (7.3).

7.3 RESULTS AND DISCUSSION.

7.3.1 Effect of Very Late Substrate Addition

From the fermentation profiles of Experiment 1 (Figures 4.1 to 4.11) and Experiment 2 (Figures 5.1 to 5.16), it was apparent that little dehydroxylation occurred before 18 h after inoculation, while dehydrogenation had usually peaked much earlier. Towards the end of the exponential phase (6 to 8 h), 7-ketodeoxycholate levels were rising rapidly, but by 18 h some of the 7 keto acid had disappeared, sometimes with a concomitant increase in cholic acid levels. Since mass balances were acceptable, the possibility of diketone formation was ruled out. Thus it is probable that by 18 h the rate of ketone reduction exceeds the rate of hydroxyl oxidation.

It was considered that if low levels of cholate are sufficient to induce the synthesis of 7α-dehydroxylase within 18 h, then further cholic acid added after that time might be dehydroxylated without delay. This would mean that dehydroxylation would be active without much of the cholic acid substrate being oxidised. Fermentation conditions were based on those of Experiment 2, run 14 (i.e. 2-litre vessel, 1.25 l working volume, 2-way pH control at pH 7 with H_2SO_4 and $NaHCO_3$ 32^0C, sparged with 20 ml/min N_2-CO_2, 9:1 at an impellor speed of 300 rev/min, 10 μM 8-hydroxyquinoline, 25 mM potassium fluoride, 0.05% w/v sodium thioglycollate, 6 h substrate addition, strain ATCC 9714, 20 ml inoculum size) with the following modifications: cholic acid was added to the medium (1.25 l) to a final concentration of 0.05% w/v; 0.015 g was present before inoculation, 0.010 g was contained in the inoculum itself, and 0.600 g was added after 18 h incubation. Fermentation was then continued for a further 52 h with regular sampling for cell count, pH determination and bile acid analysis. Electrode potential and temperature were monitored as usual.

The results of this fermentation are depicted in Figure 7.1. Yields were generally low, with 16% 7-ketodeoxycholate and 10% deoxycholate present after 70 h incubation (52 h incubation with substrate). As usual, 7α-dehydrogenation began almost immediately after substrate addition, but despite the presence of 0.002% w/v (0.05 mM) cholic acid since inoculation, dehydroxylation still showed a 24 h lag after

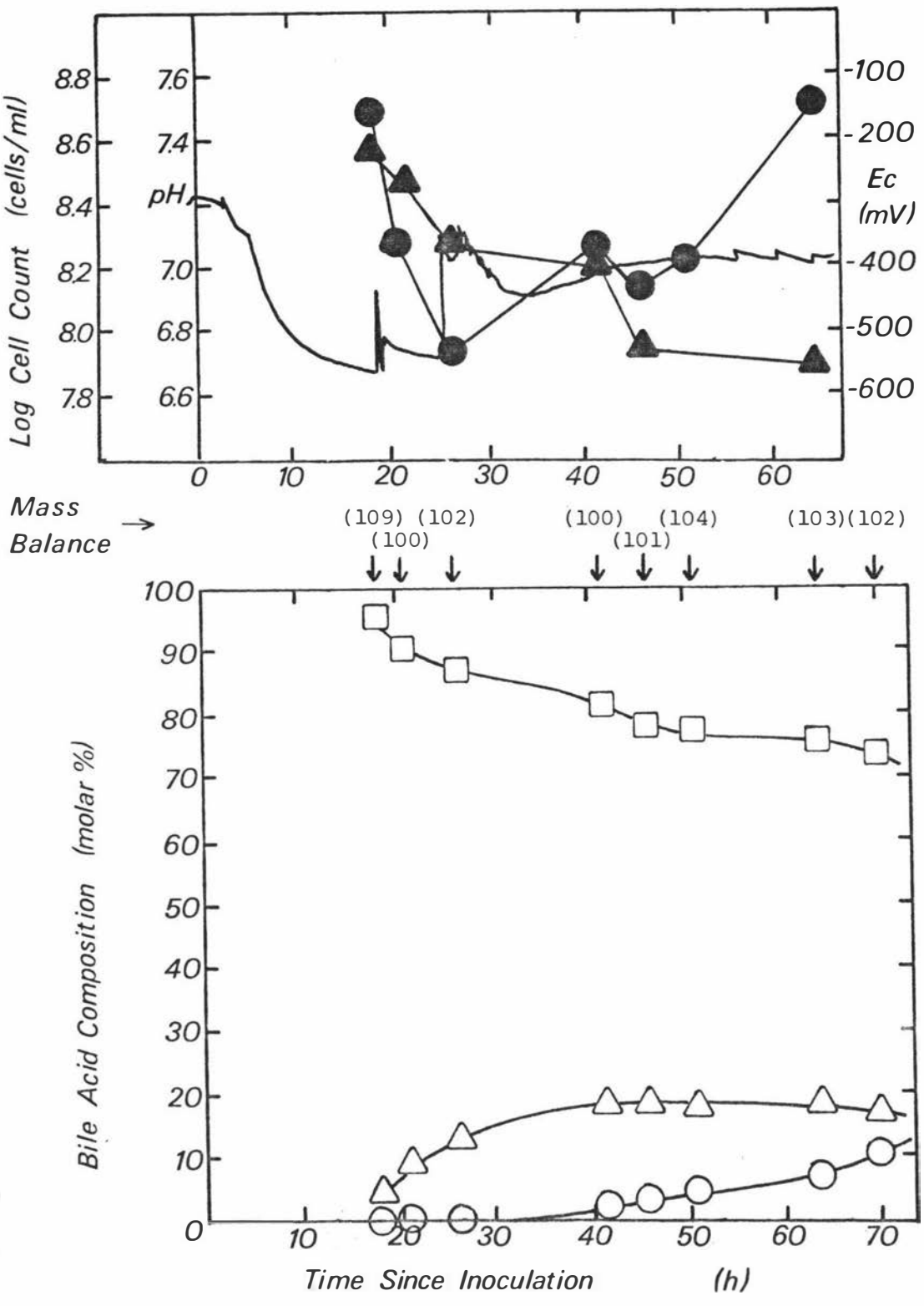


FIGURE 7.1 Course of Growth and Transformation for Fermentation with 18-hour Substrate Addition

Deoxycholic acid (○), 7-ketodeoxycholic acid (△), cholic acid (□), pH (●), cell count (▲). The electrode potential, Ec, is represented by a solid line. The "Mass Balance" figure is defined in section 3.10.2. "Bile Acid Composition" is normalised to a 100% mass balance. Experimental conditions are described in the text (section 7.3.1).

substrate addition.

In Experiment 2, run 14, it was shown that these fermentation conditions are conducive to dehydroxylation. If cholic acid had been added before inoculation, deoxycholate production would have been expected by 18 h. Hence it is apparent that in this fermentation 0.002% cholic acid failed to induce the enzyme.

Several workers have reported 7α-hydroxycholanoyl dehydroxylase to be inducible. Aries and Hill (1970b) demonstrated cell-free 7α-dehydroxylase activity in bacteria grown in the presence of bile acid substrate, but no activity when grown in its absence. Stellwag and Hylemon (1978) reported that the specific activity of 7α-dehydroxylase in bacteria cultured in the presence of 0.1 mM sodium cholate was 6- to 8-fold higher when compared to the specific activity in bacterial cultures grown in the absence of sodium cholate. In the current work, a long lag was observed before cholic acid was dehydroxylated, regardless of when the addition was made. Furthermore, 50 μM cholic acid failed to induce the dehydroxylase while 1225 μM succeeded. This is in contrast to Midtvedt and Norman (1968), who observed strong 7α-dehydroxylation by a lactobacillus grown in the presence of 10 μM chenodeoxycholic acid.

The very long lag, even after substrate addition, may indicate that conditions are far from optimal for induction. In Experiments 1 and 2, fermentation conditions were manipulated, but no marked reduction in the lag was observed. Conceivably, a better inducer than cholic acid could be found if sufficient compounds were screened. Alternatively, the lag may not be a function of dehydroxylase induction, but may be due to the development of other cell machinery for whole cell dehydroxylation.

It must be concluded that the technique of very late substrate addition was not successful in reducing the yield of 7-ketodeoxycholate relative to that of deoxycholate. Dehydrogenation was not prevented, dehydroxylation showed a long lag, and yields were low.

7.3.2 Effect of Zn^{++} Ion

In section 6.3.4, Zn^{++} ion was reported to reduce dehydrogenation yields of washed resting cells to 45% of the control yield. In the present experiment, Zn^{++} ion was tested on freshly grown cells in the fermenter.

Fermentation conditions were again based on Experiment 2, run 14, to favour dehydroxylation, but fluoride was omitted from the medium since ZnF_2 is only sparingly soluble. Cholic acid (0.015 g) was added prior to inoculation, and the balance (0.600 g) was added together with $ZnSO_4 \cdot 7H_2O$ (1.80 g, to give a final concentration of 5 mM) 6 h after inoculation. The fermentation was conducted for 48.5 h in total, with regular sampling for cell count, pH determination and bile acid analysis.

The progress of fermentation and growth are described in Figure 7.2. The 7-ketodeoxycholate yield was indeed low, being 11% compared with 31% for Experiment 2, run 14. This represents a 65% inhibition, which is similar to the 55% obtained for washed resting cells. However, dehydroxylation - usually strong under these conditions - was completely inhibited. This occurred despite the relatively high electrode potentials observed which, in the light of the Experiment 2 results, might be expected to boost dehydroxylation. Some lowering of deoxycholate yield could be due to the absence of fluoride, but based on the regression data from Experiment 2, this would not explain all the decrease.

Hence, although Zn^{++} ion had the inhibitory effect on dehydrogenation predicted from the washed cell experiments, the concomitant total inhibition of dehydroxylation prohibits its use in selecting for the latter reaction.

7.3.3 Effect of High Thioglycollate Levels

Section 6.3.4 described a marked inhibition of cholic acid dehydrogenation by washed resting cells in the presence of sodium thioglycollate. To further investigate this effect, sodium thioglycollate was added to a final concentration of 0.15% w/v in the 2-litre fermenter. (In Experiment 2, a concentration of 0.05% was used).

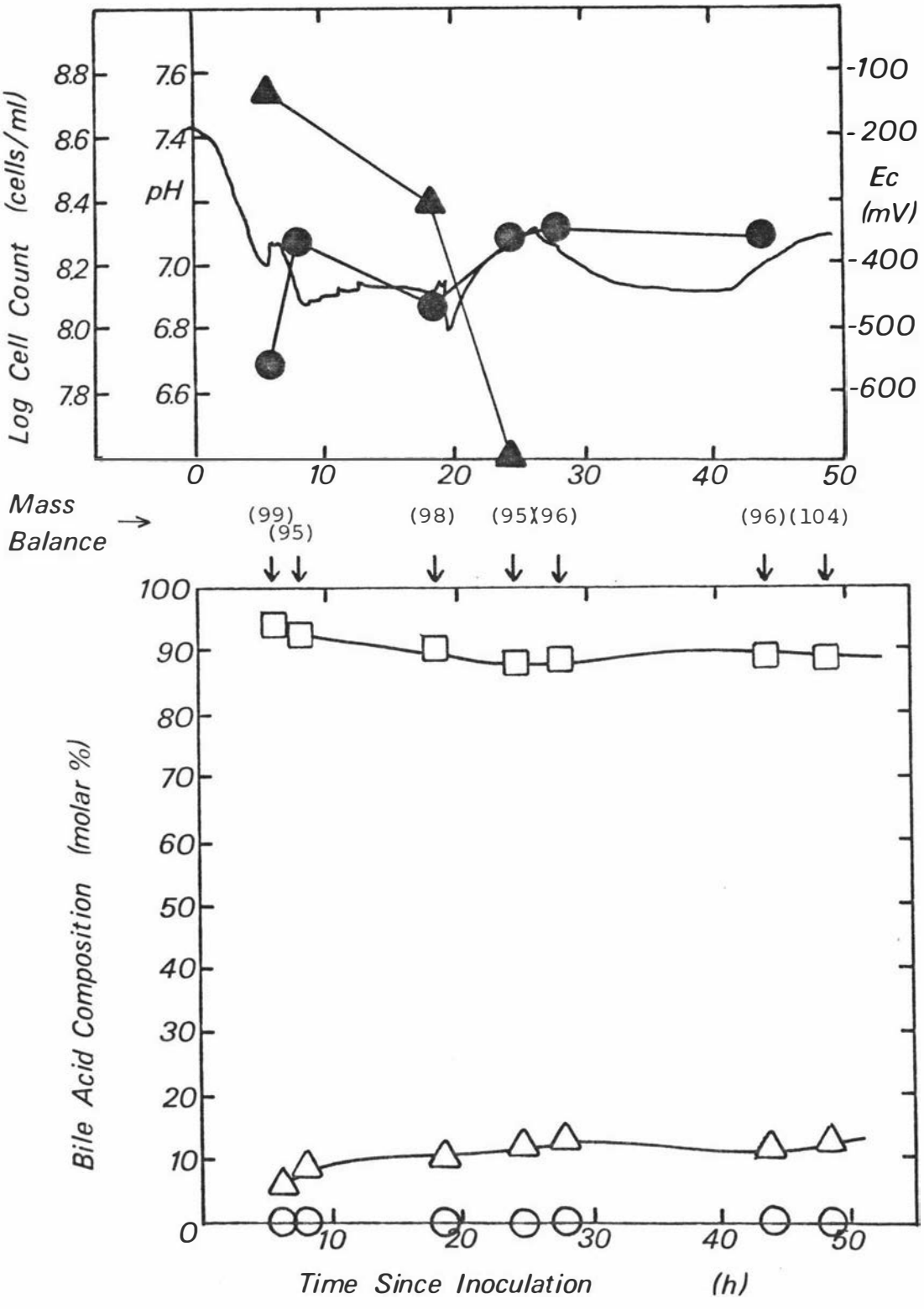


FIGURE 7.2 Course of Growth and Transformation for Fermentation with 6-hour Addition of Zn^{++} Ion to 5mM

Legend as for Figure 7.1. Fermentation conditions are given in the text (section 7.3.2).

Conditions were based on Experiment 2, run 14, with the following modifications: sodium thioglycollate (0.625 g) and cholic acid (0.015 g) were present in the 1.25 l of medium before inoculation. After 6 h incubation, cholic acid (0.600 g) and sodium thioglycollate (1.25 g) were added. Incubation was continued for a further 43 h, with sampling and analysis as usual.

The fermentation data are plotted in Figure 7.3. Dehydrogenation was indeed depressed over Experiment 2, run 14, by some 40%, while the deoxycholate yield after 48 h was 18%. It was noticed that the presence of the thioglycollate did not depress the electrode potential, rather it apparently introduced extra redox buffering capacity (poising), preventing the Ec minimum falling below -500 mV and maintaining the potential below -350 mV even after 48 h incubation. This appears to have had the effect of delaying dehydroxylation, which was apparent at low levels until *ca* 24 h, after which it increased. By 48 h, there was still very strong activity which may have resulted in high deoxycholate yield, had incubation been continued.

Thus high levels of thioglycollate in the transformation medium appear to buffer the electrode potential. 7α-Dehydrogenation was depressed to some extent, but insufficiently to ease the separation problem posed by a fermentation medium containing three bile acid species.

7.3.4 Effect of EDTA on Cholic Acid Transformation

This section reports two fermentations which were designed to follow up the results obtained with washed resting cells and reported in section 6.3.4. In that experiment, 0.05 M EDTA was found to boost 7-ketodeoxycholate yield by 264%. In both fermentations reported here, transformation substrate (cholic acid) and modifier (EDTA) were added after growth, lest the presence of EDTA impair growth.

In the first of the fermentations, conditions were based on Experiment 2, run 13, which had a 48-hour 7-ketodeoxycholate yield of 33%, accompanied by only 3% deoxycholate. Fermentation conditions in Experiment 2 were: 2-litre vessel, 1.25 l working volume, 2-way pH

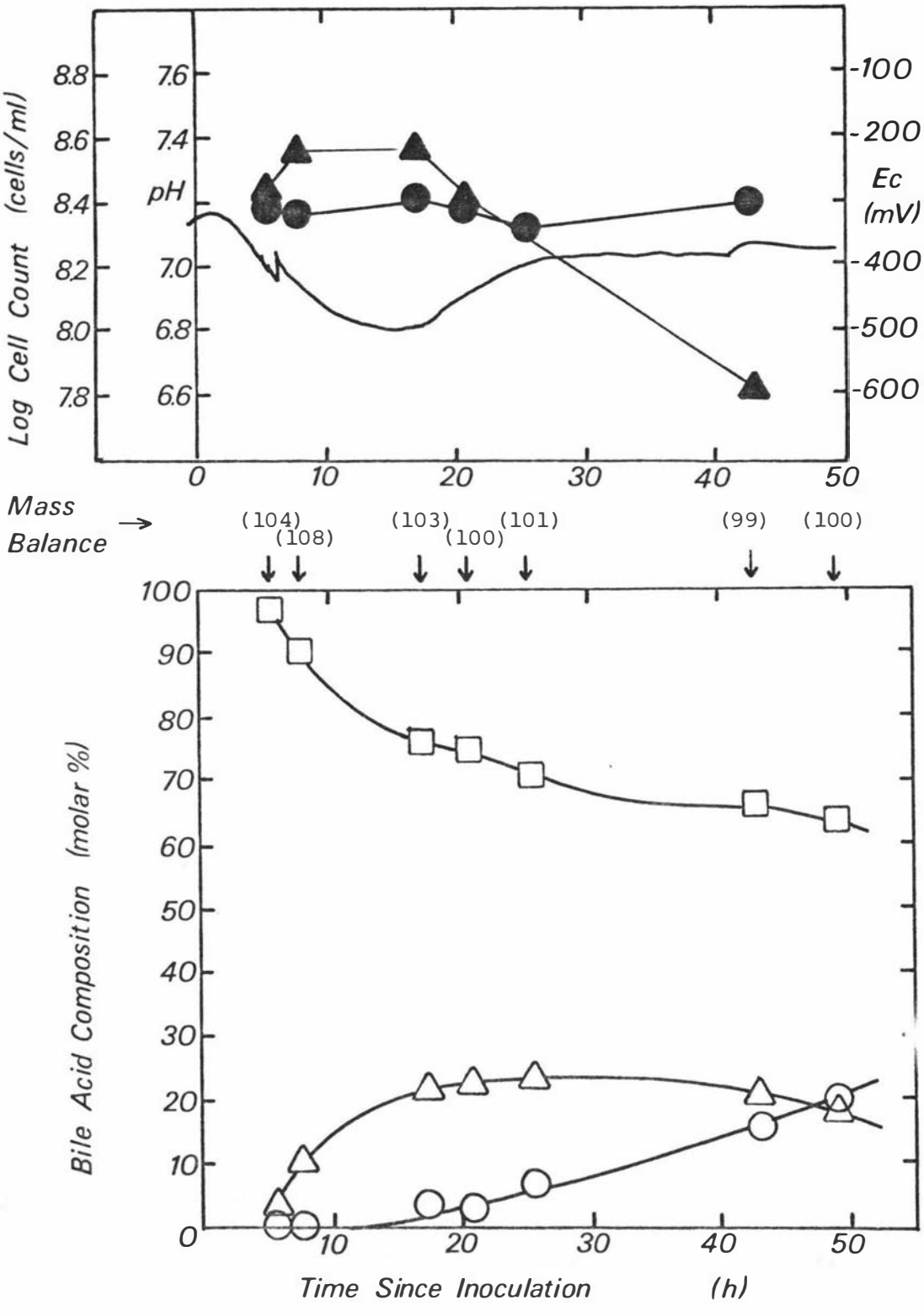


FIGURE 7.3 Course of Growth and Transformation for Fermentation with 6-hour Addition of Sodium Thioglycollate to 0.15% w/v

Legend as for Figure 7.1. Fermentation conditions are given in the text (section 7.3.3).

control at pH 7 with NaOH and H_2SO_4, 37^{0}C, swept with 20 ml/min N_2-CO_2, 9:1 at an impellor speed of 200 rev/min, strain SD 10, 50 ml inoculum size, 6-hour substrate addition and no thioglycollate or inhibitor present. The conditions now used were identical except that strain ATCC 9714 was used in a 20 ml inoculum. Cholic acid (0.015 g) was added before inoculation. After 6 h incubation, disodium EDTA (9.306 g) and cholic acid (0.600 g) were added to give final concentrations of 20 mM and 0.05% w/v respectively. Fermentation was continued for a total of 48 h with regular sampling. The second fermentation was based on the Experiment 2 conditions which gave greatest deoxycholate yield, i.e. run 14, with the following modifications: cholic acid (0.015 g) was added before inoculation, and after 6 h incubation, disodium EDTA (9.306 g) and cholic acid (0.600 g) were added.

The results of these two fermentations are depicted in Figures 7.4 and 7.5. In the first, 7α-dehydrogenation was rapid and nearly complete. There was net oxidative activity over the whole 42 h available for transformation. In this time, 97% of the cholic acid substrate was oxidised with no accompanying dehydroxylation or overall loss of steroid. Although the control run (Experiment 2, run 13) itself showed a low 48-hour deoxycholate yield, dehydroxylation here appears to be completely inhibited. This finding was corroborated by the second of the two trials in which there was no dehydroxylation under conditions which usually promoted high yields of the 7-deoxy product. Thus, EDTA would appear to have some direct effect upon the dehydroxylase activity, possibly by chelating an essential metal ion. The 100% inhibition of whole cell dehydroxylation is in contrast to the report of Aries and Hill (1970b) that the cell-free 7α-dehydroxylase of several human intestinal strains was not inhibited by 30 mM EDTA.

Although the 7-ketodeoxycholate yield of 61% in the second fermentation was not as high as the 97% yield observed in the first, it was double that observed in the control (31% in Experiment 2, run 14). Thus, in both fermentations, EDTA enhanced 7α-dehydrogenation. The possible reasons for this observed effect have been discussed in section 6.3.4.

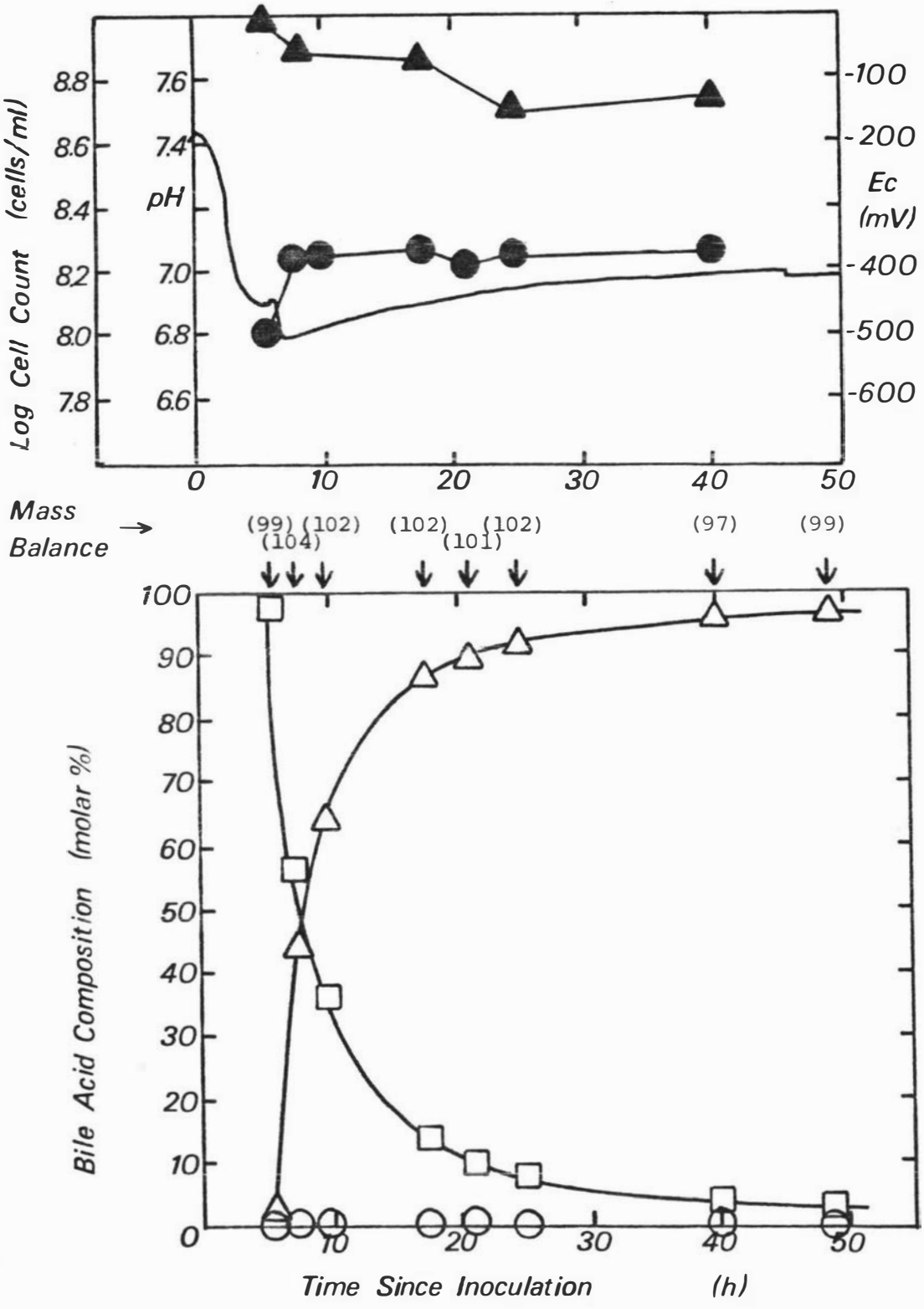


FIGURE 7.4 Course of Growth and Transformation for Fermentation with 6-hour Addition of EDTA. (a).

Legend as for Figure 7.1. Fermentation conditions are given in the text (section 7.3.4), and were suited to 7α-dehydrogenation. Strain ATCC 9714 was used.

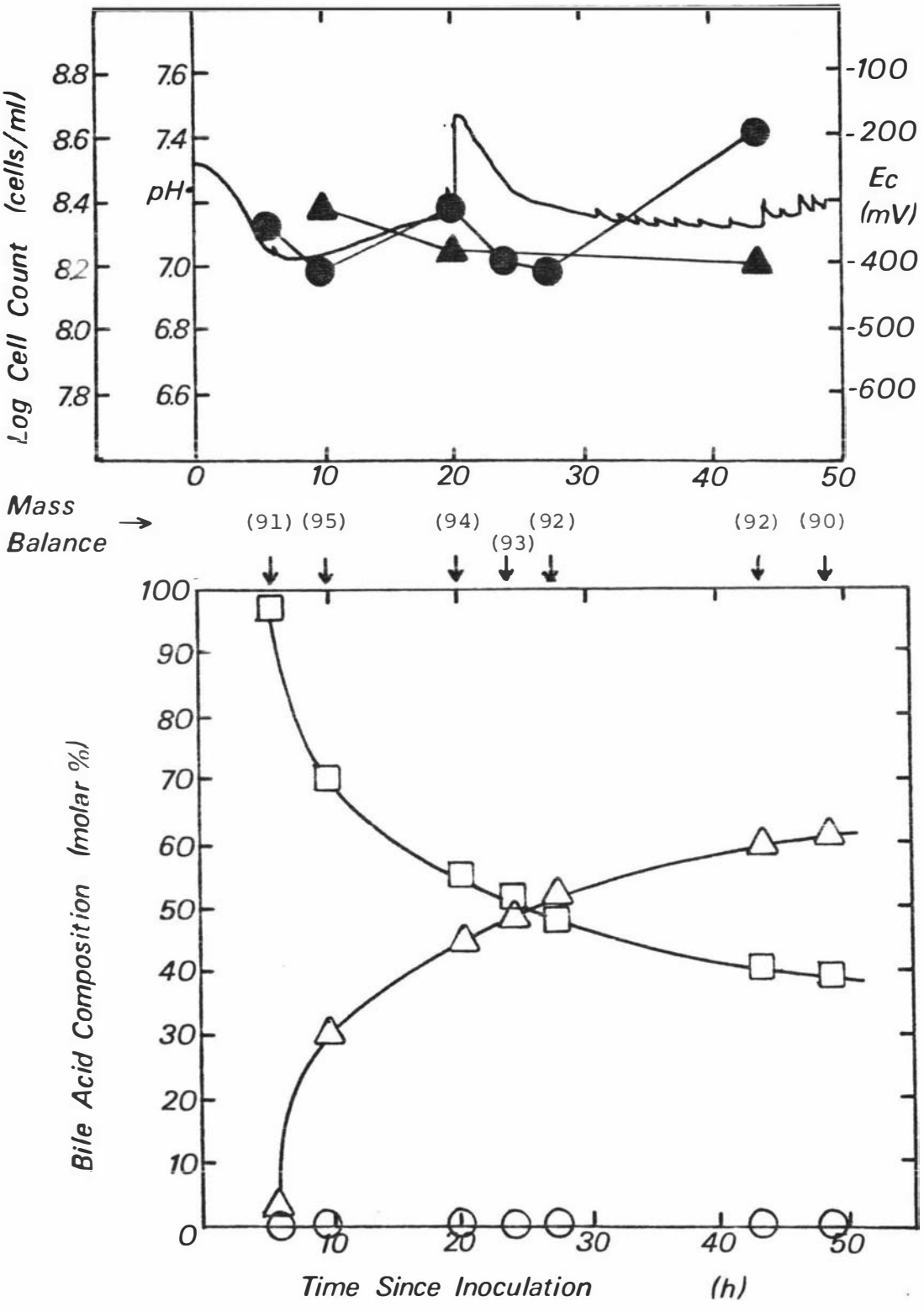


FIGURE 7.5 Course of Growth and Transformation for Fermentation with 6-hour Addition of EDTA. (b).

Legend as for Figure 7.1. Fermentation conditions are given in the text (section 7.3.4), and were suited to both C-7 transformations. Strain ATCC 9714 was used.

The ability to 7α-dehydrogenate selectively in such high yields adds flexibility to the present project, and provides one possible solution to the product separation problem. With simple mixtures of cholic acid and its 7-ketone, the oxidised product could be relatively easily separated for further transformation.

7.3.5 Effect of Air on Cholic Acid Transformation

Reported in section 6.3.2 is the observation that aerobic incubation doubled the extent of 7α-dehydrogenation of cholic acid by washed resting-stage cells. This current section reports three fermentations in which the medium surface was swept with air after cell growth was largely complete.

In the first of these three fermentations, conditions suitable for both C-7 transformations were employed, so that the effect of aerobic conditions on the yields of both 7-ketodeoxycholate and deoxycholate could be simultaneously observed. Fermentation conditions were as for Experiment 2, run 14, except that 8-hydroxyquinoline was (inadvertently) omitted, and substrate addition was late. Cholic acid (0.015 g) was present in the medium (1.25 l) before inoculation, and for the first 7.5 h of incubation the medium was sparged with N_2-CO_2, 9:1 (20 ml/min) at an impellor speed of 300 rev/min. At the 7.5 h mark, cholic acid (0.600 g) was added, and sparging was stopped. The medium surface was now swept with air at 20 ml/min, and the impellor speed was reduced to 200 rev/min. Fermentation was continued in this fashion for a further 42.5 h.

The second of these fermentations was performed under almost identical conditions. On this occasion, however, 8-hydroxyquinoline was included, strain SD 10 was employed, and sampling was more frequent.

Conditions for the third fermentation were based on Experiment 2, run 13 (which had a 48-hour 7-ketodeoxycholate yield of 32% accompanied by only 3% deoxycholate), except that a small inoculum size was used. As for the previous two fermentations, cholic acid (0.600 g) was added after 7.5 h incubation, and sparging with N_2-CO_2, 9:1 (20 ml/min) at an impellor speed of 300 rev/min was replaced by sweeping with air (20 ml/min) at 200 rev/min. Fermentation was continued for

a further 41.5 h with regular sampling for cell count, pH determination and bile acid analysis.

The results of these fermentations are shown in Figures 7.6, 7.7 and 7.8 in the order already described.

In some respects, the first fermentation was unsatisfactory in that some data points were lost, pH control failed near the end of the run, and mass balances were poor. However, both transformation products were present in high yields. As expected from the washed-cell experiments, 7α-dehydrogenation was enhanced, yields being *ca* 30% greater than for the control (Experiment 2, run 14). Unexpectedly, however, dehydroxylation was also pronounced, with the highest yield yet observed in the project, i.e. 50%.

The progress of the second fermentation is depicted in Figure 7.7. Here, the 48-hour deoxycholate yield was *ca* 40%; this is less than in the first fermentation, but still approximately equal to the previous highest yield observed. The two fermentations together indicate that aerobic incubation does allow strong dehydroxylation, and may even enhance it. In this second fermentation however, 7-ketodeoxycholate yield was only of the order of the control yield.

The third fermentation in the series (Figure 7.8) was conducted under conditions suited to high 7-keto and low 7-deoxy product yields. 7-Ketodeoxycholate production was indeed strong, with a 48-hour level of 64%. The effect of air on 7α-dehydrogenation was more pronounced under these conditions than under those more suited to dehydroxylation. In addition, the deoxycholate yield was much higher here (at 10%) than that observed in the control run (3% for Experiment 2, run 13).

To date, most workers using anaerobic bacteria for 7α-hydroxylation have stressed the need for strictly anaerobic conditions, both during growth and transformation (Drasar and Hill, 1974). Aries and Hill (1970b) reported that the 7α-hydroxycholanoyl dehydroxylase enzymes from all eight strains studied were active only in an anaerobic environment.

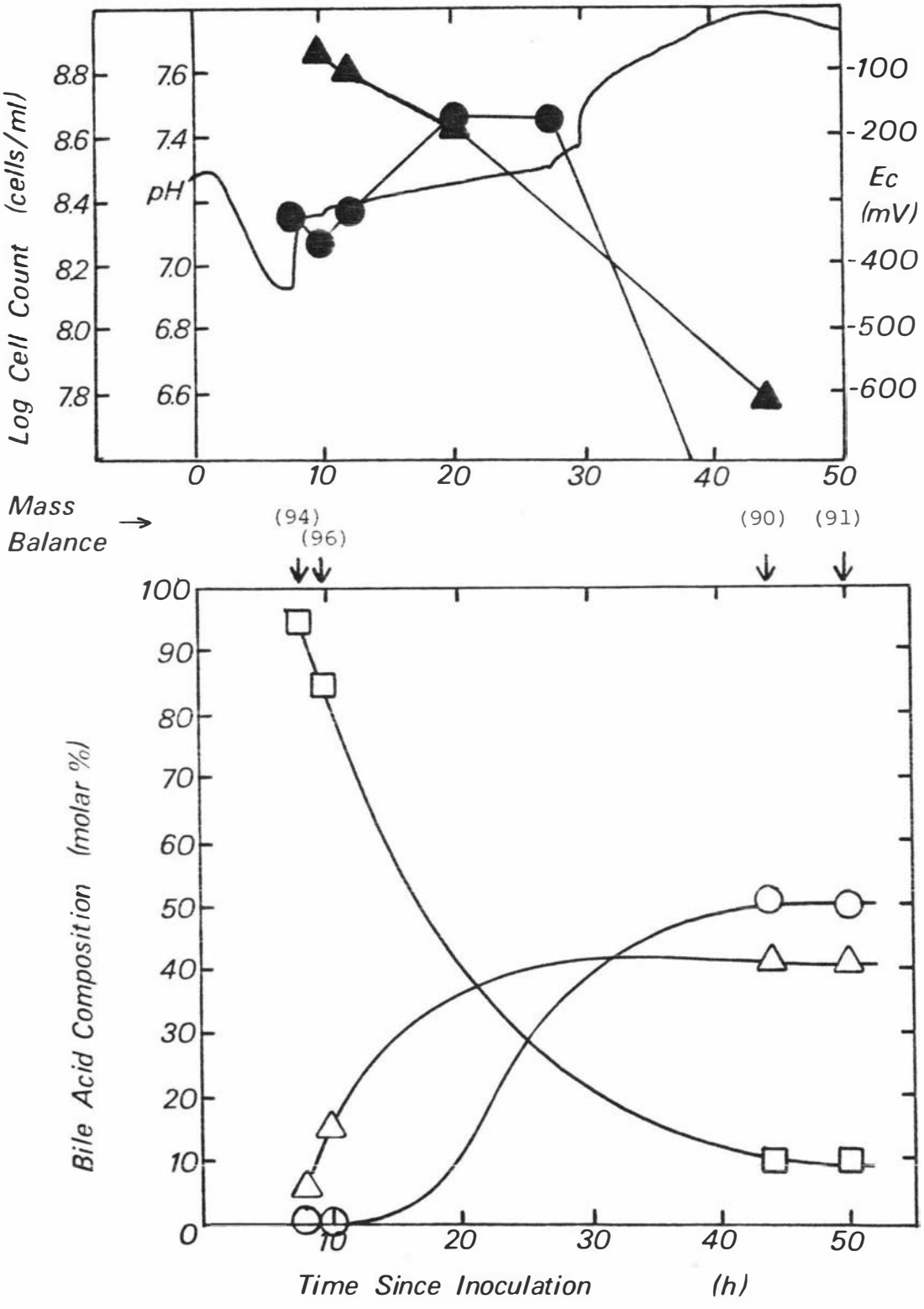


FIGURE 7.6 Course of Growth and Transformation for Fermentation with Air-Sweeping. (a).

Legend as for Figure 7.1. Fermentation conditions are given in the text (section 7.3.5), and were suited to both C-7 transformations. Strain ATCC 9714 was used.

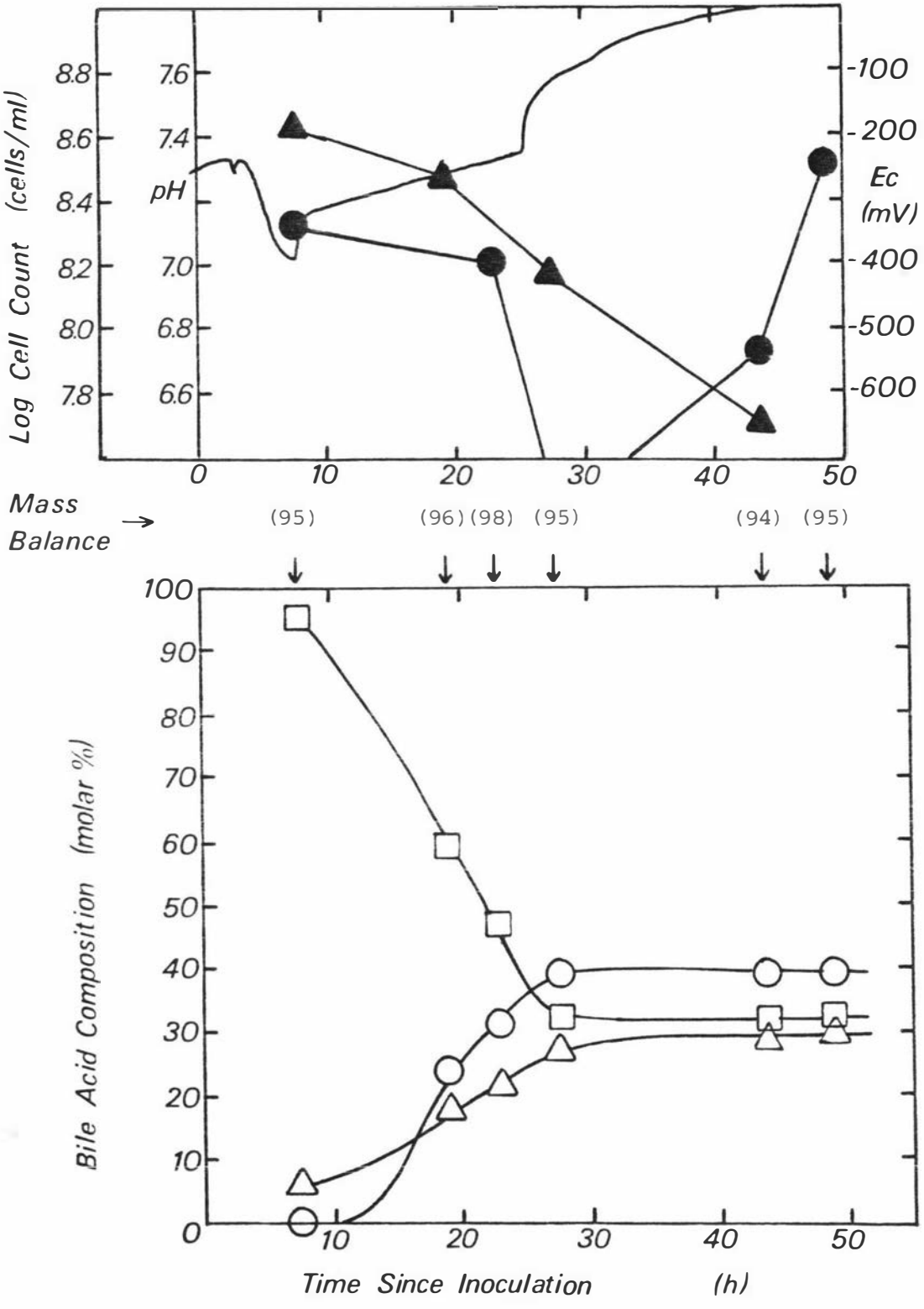


FIGURE 7.7 Course of Growth and Transformation for Fermentation with Air-Sweeping. (b).

Legend as for Figure 7.1. Fermentation conditions are given in the text (section 7.3.5), and were suited to both C-7 transformations. Strain SD 10 was used.

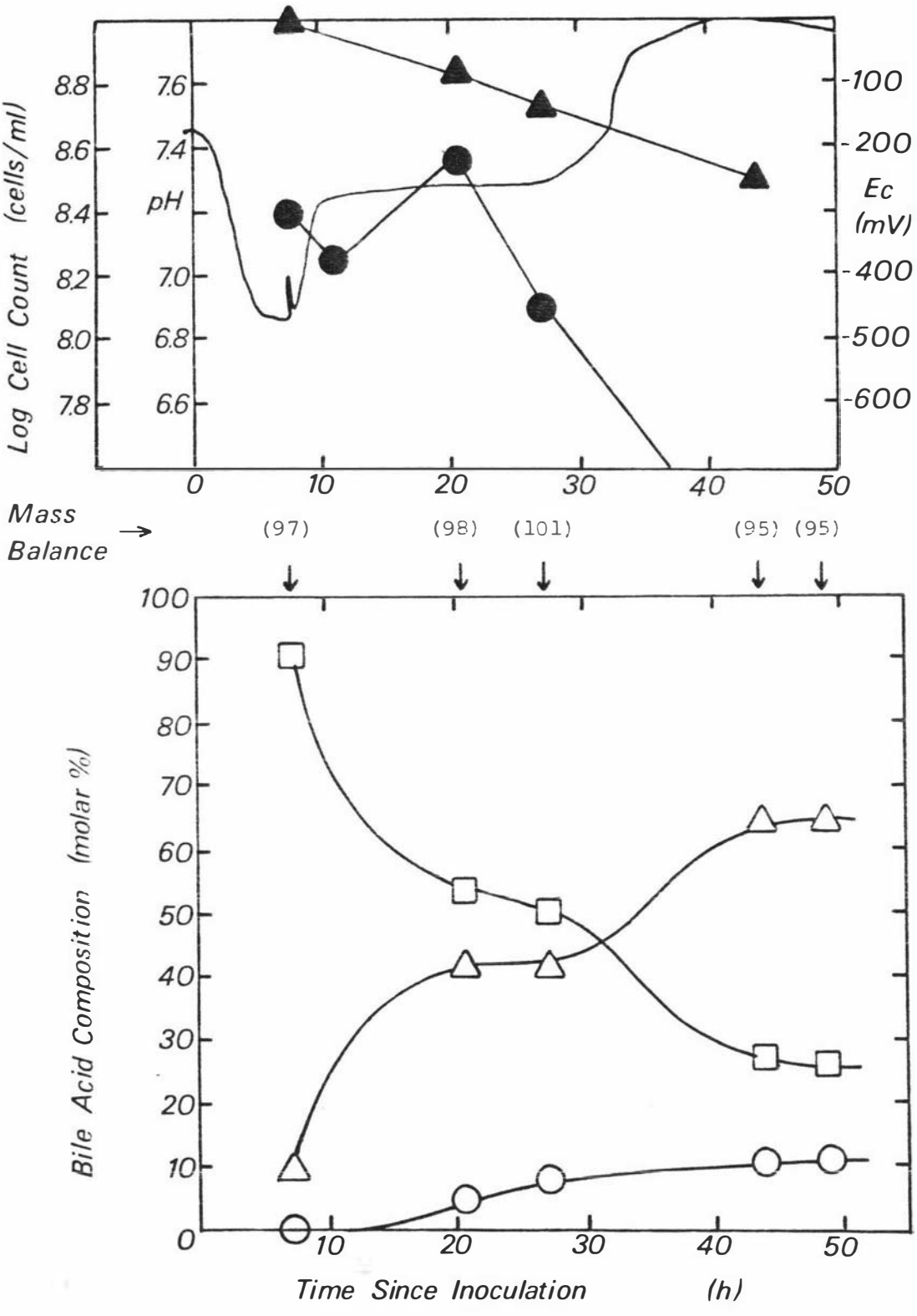


FIGURE 7.8 Course of Growth and Transformation for Fermentation with Air-Sweeping. (c).

Legend as for Figure 7.1. Fermentation conditions are given in the text (section 7.3.5), and were suited to 7α-dehydrogenation. Strain SD 10 was used.

There have been instances of aerobic 7α-dehydroxylation reported for aerobically growing organisms. Hayakawa and Samuelsson (1964) found 7-deoxy bile acids amongst the degradation products when *Corynebacterium simplex* was grown aerobically with sodium cholate as the sole carbon source. Very similar results were reported for a *Myobacterium* by Severino *et al.*, (1968). However, it is probable that these products arise by a different mechanism to be discussed later. More recently, aerobically growing fungi have been demonstrated to produce deoxycholate from glycocholate (Johns, 1980). Further, Ferrari and Beretta (1977) found the cell-free 7α-hydroxycholanoyl dehydroxylase of *Cl. bifermentans* SD 10 to be active when incubated aerobically.

The current observation appears to be the first where an intact obligate anaerobe dehydroxylates cholic acid under aerobic conditions, let alone with higher yields than for anaerobic incubation.

The occurrence of higher yields is not entirely surprising in the light of the redox optimum apparent in Experiment 2. The electrode potential of the fermentation described in Figure 7.6 increased rapidly as expected when air was introduced, but only to -330 mV. Over the next 18 h, the Ec rose only slowly to -260 mV. This apparent redox buffer zone is not evident during growth; the rapidly declining Ec of the growing cultures shows no point of inflexion near -280 mV. Thus the "buffering" appears not to be a property of the medium *per se*. It is probably therefore a property of the cells, which may be able to metabolise molecular oxygen at a sufficient rate to neutralise the diffusion from the swept surface. A similar observation was made by Samuel *et al.* (1973), who reported no significant drop in the rate of deoxycholate production by faecal homogenates when incubated in air, but found that those shaken in air showed markedly depressed activity.

All three fermentations reported in this section showed very similar electrode potential profiles. The few differences appeared to be related to the cell yield. In the first, second and third fermentations the 7.5-hour cell yields were 7.6×10^8, 4.1×10^8 and 1.0×10^9 cells/ml respectively. It is notable that the "buffered period" (during which there is a slow rise in electrode potential

through *ca* -280 mV) which in each run started immediately after air addition, is longer in those runs with greater cell density. Further, the rate of electrode potential rise was greater where fewer cells were present after 7.5 h. This supports the hypothesis that the electrode potential is poised near -280 mV by cellular action.

The observed differences in cell density noted here are probably due to the presence or absence of fluoride ion and 8-hydroxyquinoline. The third fermentation contained neither, and yielded most cells. The first fermentation contained fluoride alone, and produced a moderate cell density in comparison with the second trial, which contained both inhibitors. Although there were several differences in conditions between the first two fermentations, the absence of 8-hydroxyquinoline in the first (which showed higher transformation yields) might indicate that its rôle is not as direct as surmised in Chapter 5.

Unfortunately, the pH control failed during all three air-swept runs. Since this failure coincided to some extent with the observed decrease in cell numbers 12 to 20 h after exposure to air, with the rapid increase in Ec 16 to 22 h after exposure, and with the ceasing of 7α-dehydroxylation, it is difficult to determine why transformation ceased. If the cause could be determined and prevented, dehydroxylation could possibly be continued longer, and deoxycholate yields increased still further.

Since aerobic incubation has proved effective in increasing transformation yields, further work in the area is warranted. Future trial fermentations could be designed to prolong the period spent near -280 mV, possibly by sweeping with air for no more than 12 h, and possibly by adding sodium thioglycollate near the 24 h mark.

If yields could be further increased, cholic acid utilisation would approach 100%. This would allow a second possible solution to the product separation problem involved in an industrial process. With only two bile acid species present, separation should be less expensive. The total bile acid content of the spent medium could be subjected to Wolff-Kishner reduction (Fieser and Rajagopalan, 1949), resulting in a single product: deoxycholic acid.

7.4 CONCLUSIONS

Some results from the experiments with washed resting cells have been successfully applied to the larger scale, and some not. The addition of substrate to the fermenter as late as 18 h after inoculation failed to limit dehydrogenation selectively. The incorporation of Zn^{++} ion into the medium after 6 h incubation did limit dehydrogenation, but entirely inhibited dehydroxylation. Incorporation of 0.15% w/v sodium thioglycollate after 6 h also depressed dehydrogenation and delayed dehydroxylation. This agent appeared to add redox buffering capacity to the medium. EDTA, similarly added after incubation for 6 h, greatly enhanced 7α-dehydrogenation and also totally inhibited dehydroxylation. Surprisingly, introduction of air into the fermenter after 7.5 h incubation enhanced both transformations. The observation of enhanced dehydroxylation by an aerobic atmosphere is unique for an intact anaerobe.

Two methods of achieving almost quantitative transformation have been elucidated:

(a) Use of EDTA will allow the transformation of cholic acid to 7-ketodeoxycholic acid in 97% yield, and

(b) Use of air will allow the extensive transformation of cholic acid to a near equal mixture of deoxycholic and 7-ketodeoxycholic acids.

These two discoveries are merely first observation. For industrial application, much more work would be required, particularly in optimising the duration of air-sweeping and the reduction of EDTA concentrations to less expensive levels.

No method is yet available for eliminating 7α-dehydrogenation whilst simultaneously allowing 7α-dehydroxylation to proceed.

CHAPTER 8 HYDROLYSIS OF BILE ACID CONJUGATES BY CLOSTRIDIUM BIFERMENTANS.

8.1 INTRODUCTION

The results reported in the previous four chapters have concerned C-7 transformations. The current chapter reports experiments performed on the hydrolysis of glyco- and tauro-cholate and glyco- and tauro-deoxycholate, by growing or freshly grown cells of *Cl. bifermentans*.

It was obvious from preliminary studies that near quantitative hydrolysis of all four conjugates could be achieved within 48 hours incubation. This meant that in developing a process to produce deoxycholic acid from mutton gall, the initial deconjugation step was unlikely to pose a major problem. For this reason, most attention was focussed on 7α-dehydroxylation and 7α-dehydrogenation so that optimal conditions for the C-7 transformation could then be applied to the deconjugation.

8.2 FERMENTATION CONDITIONS

Initially two sets of small-scale experiments were performed with strain ATCC 9714. Both employed Todd Hewitt Broth with conjugate added to the appropriate final concentration before inoculation. The basic methodology for this is described in section 3.7.1. Incubation was at 37^0C for 48 h, after which time the final pH was measured and the alkaline internal standard solution added. Spent cultures with high bile acid content were diluted so that a 20 ml sample contained no greater than 20 mg of bile acid (excluding internal standard). Centrifugation and extraction by the freeze dry method were conducted as described in sections 3.7.2.6 and 3.9.2. Samples containing glycodeoxycholic acid were extracted by the chloroform extraction method, section 3.9.1.

Also performed were four fermentations in the 2-litre vessel, each with a working volume of 1.25 l. These employed strain SD 10 since strain ATCC 9714 suddenly failed to grow in the small fermentation

vessel, and remained weak for some months. This condition applied only to the fermenter and not to the small scale culture, and it applied only to the ATCC 9714 strain. Bacteriophage attack was suspected.

These four runs, one with each conjugate at a final concentration of 0.05% w/v, were performed under the following conditions: 32^0C, two-way pH control at pH 7.0 with 0.5 M H_2SO_4 and 1.0 M $NaHCO_3$ 10 μM 8-hydroxyquinoline, 25 mM KF, 0.05% w/v sodium thioglycollate, sparged with N_2-CO_2, 9:1 (20 ml/min) and agitated at 300 rev/min. No bile acid was present before inoculation with a 24 h (20 ml) culture in Todd Hewitt Broth containing 0.05% w/v cholic acid. After 6 h incubation sterile bile acid conjugate (0.625 g) was added. Fermentation was continued for a further 42 h with regular sampling for pH, cell count determination, and bile acid analysis.

Finally, two 1.25 l fermentations were performed using a mixture of 0.025% w/v sodium taurocholate and 0.025% glycocholic acid (i.e. total final substrate concentration of 0.05%). The first fermentation was performed under the conditions of Experiment 2, run 13 (which yielded 33% 7-ketodeoxycholate and 3% deoxycholate from cholic acid), with the following modifications: no bile acid was present before inoculation with a 20 ml inoculum containing cholic acid (0.05% w/v); after 6 h incubation, sodium taurocholate (0.3125 g), glycocholic acid (0.3125 g) and disodium EDTA (9.306 g) were added as sterile neutral solutions. This gave a final EDTA concentration of 20 mM. The second of these two fermentations was performed under the conditions of Experiment 2, run 14 (which yielded 31% 7-ketodeoxycholate and 40% deoxycholate from cholic acid), with the following modifications: no bile acid was added before inoculation; after 7.5 h incubation, sodium taurocholate (0.3125 g) and glycocholic acid (0.3125 g) were added as sterile neutral solutions. Simultaneously, sparging with N_2-CO_2, 9:1 was replaced by sweeping with air (20 ml/min) and agitation speed was reduced to 200 rev/min. After 19 h incubation, this change was reversed, anaerobic sparging being restored.

All fermentation samples containing glycocholate, taurocholate or taurodeoxycholate were extracted by the freeze dry method. Those containing glycodeoxycholate as the sole conjugate were extracted by chloroform extraction.

8.3 RESULTS

Raw data from the small-scale experiments are presented in Appendix 5.

Table 8.1 contains the processed results from the first experiment which was performed to obtain a preliminary indication of the extent of hydrolysis. This involved incubating each of the four conjugates (0.05% w/v in 20 ml) at 37^{0}C for 48 h. Each conjugate was incubated in duplicate, and for each an uninoculated control was also analysed. Since mass balances on the small-scale were poor, results were normalised to a 100% mass balance for ease of comparison.

TABLE 8.1 Yield of Deconjugation after 48 h

Conjugate		Normalised [a] Molar % Deconjugation	% Mass Balance
Glycocholic acid	control	0	88
	test [b]	93	90
Glycodeoxycholic acid	control	0	90
	test [b]	97	95
Na taurocholate	control	0	98
	test [b]	97	90
Na taurodeoxycholate	control	0	95
	test [b]	98	96

Reaction conditions are as described in the test.

a % deconjugation is the sum total of % yield of all deconjugated products.

b mean of two duplicates.

The second experiment on small-scale was performed to investigate the effect of substrate concentration on the extent of hydrolysis. The processed results are depicted in Figures 8.1 and 8.2. In the former, the normalised molar % deconjugation (i.e. total of all deconjugated products) is plotted against substrate concentration.

In Figure 8.2, the total number of millimoles of bile acid conjugate hydrolysed (calculated from normalised molar yields) in a particular 20 ml reaction mixture is shown to vary as an effect of substrate concentration. The abscissa of Figure 8.2 is calibrated in mM, not % w/v, since the conjugates have unequal molecular weights (0.10% w/v corresponds to 2.13 mM, 2.20 mM, 1.86 mM and 1.92 mM for glycocholic and glycodeoxycholic acids, sodium taurocholate and sodium taurodeoxycholate respectively).

The progress of fermentation and transformation of the six fermentations in the 2-litre fermentation vessel are shown in Figures 8.3 to 8.8.

8.4 DISCUSSION

From the results of the small-scale experiments (Table 8.1) it is clear that at a substrate concentration of 0.05% w/v, hydrolysis of three of the conjugates was nearly quantitative within 48 h. Glycocholic acid was least well hydrolysed, some 7% remaining after 48 h. The batch fermentations showed similar trends, in that glycocholic acid was again least well hydrolysed (87%), while the other three conjugates were almost quantitatively hydrolysed (Figures 8.3 to 8.6). However, when substrate concentrations were increased to levels greater than 0.10%, deconjugation was impaired (Figures 8.1 and 8.2) The extent of this was affected by both the amino acid moiety and the presence of a 7α-hydroxyl substitute. Hydrolysis of both taurine conjugates was more sensitive to substrate concentration than was that of the glycine conjugates. The effect of the 7α-hydroxyl group was less well defined; whereas glycocholate hydrolysis was more sensitive to substrate concentration than that of glycodeoxycholate, taurocholate hydrolysis was less sensitive than that of taurodeoxycholate.

At a concentration of 0.05% w/v, the taurine conjugates were hydrolysed most rapidly, the reaction being 90% complete within 14 h of substrate addition. Glycocholate was most slowly hydrolysed, showing a 3-5 h lag before deconjugation commenced. The taurine conjugate hydrolysis also showed a lag, but of shorter duration,

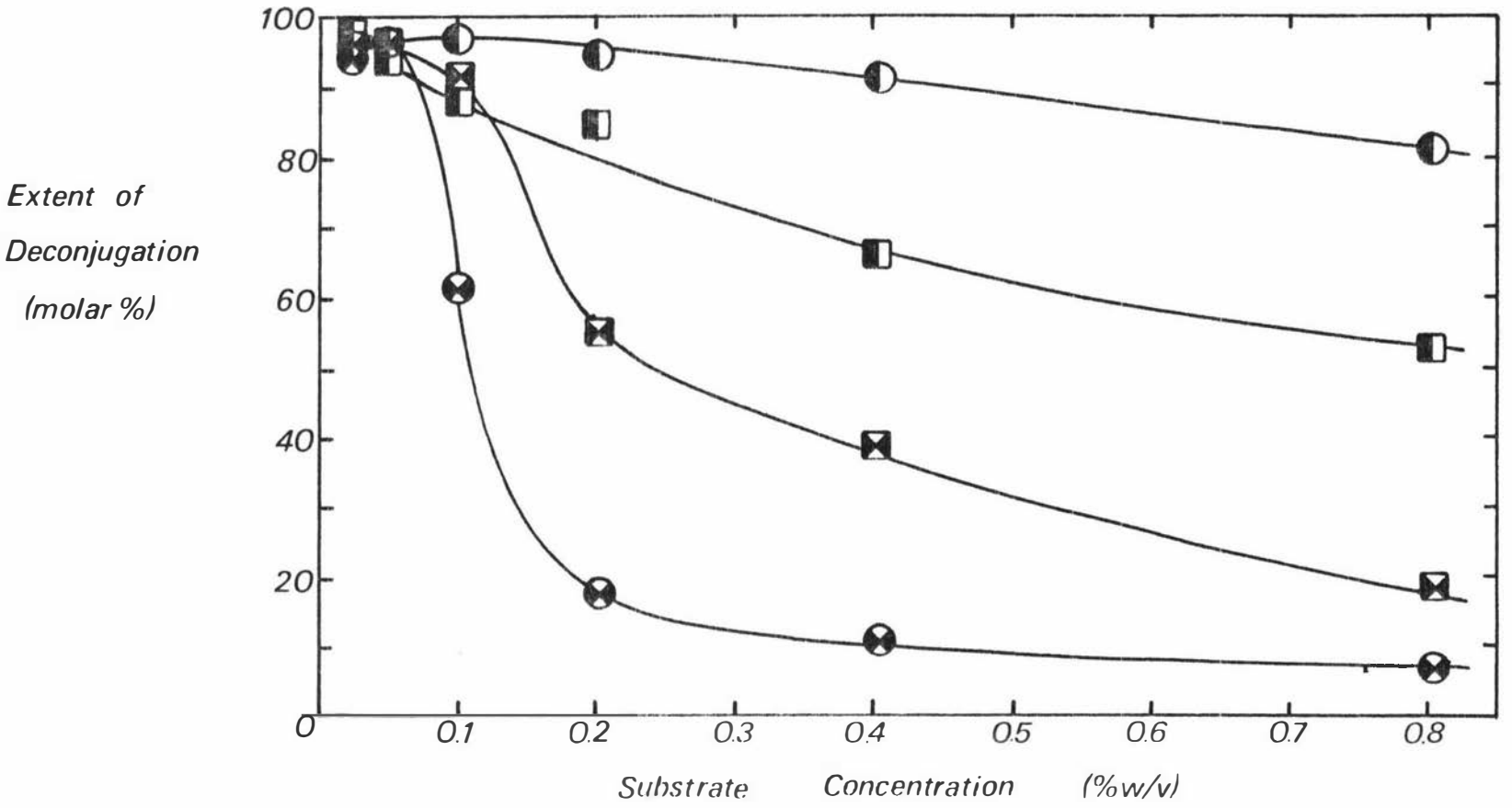


FIGURE 8.1 Effect of Substrate Concentration on Deconjugation Yields

Glycocholate (◧), glycodeoxycholate (◐), taurocholate (⊠), taurodeoxycholate (⊗). Experimental conditions are described in the text.

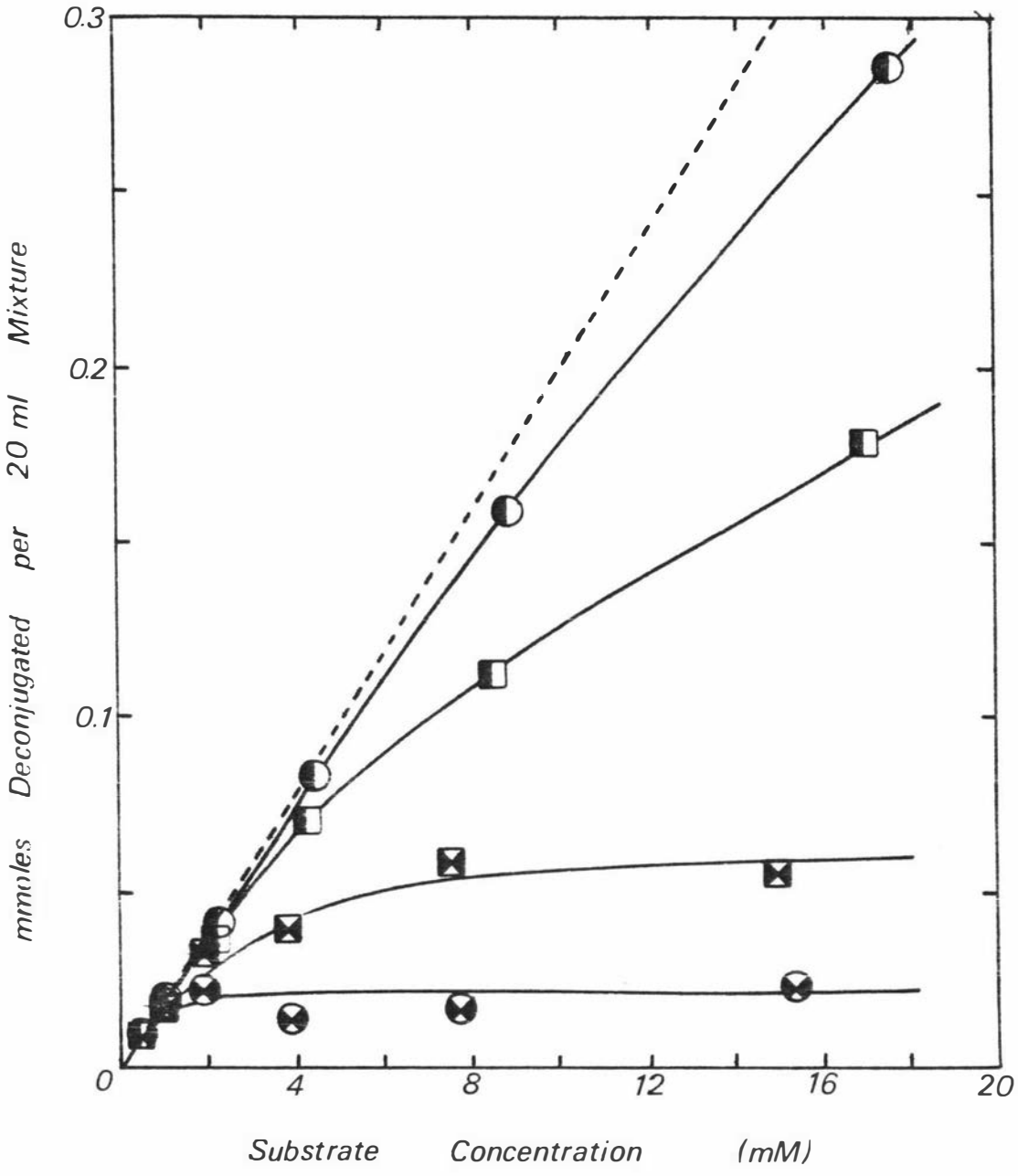


FIGURE 8.2 Effect of Substrate Concentration on Total Extent of Deconjugation

Legend as for Figure 8.11. The dashed line is the assymptote for 100% deconjugation.

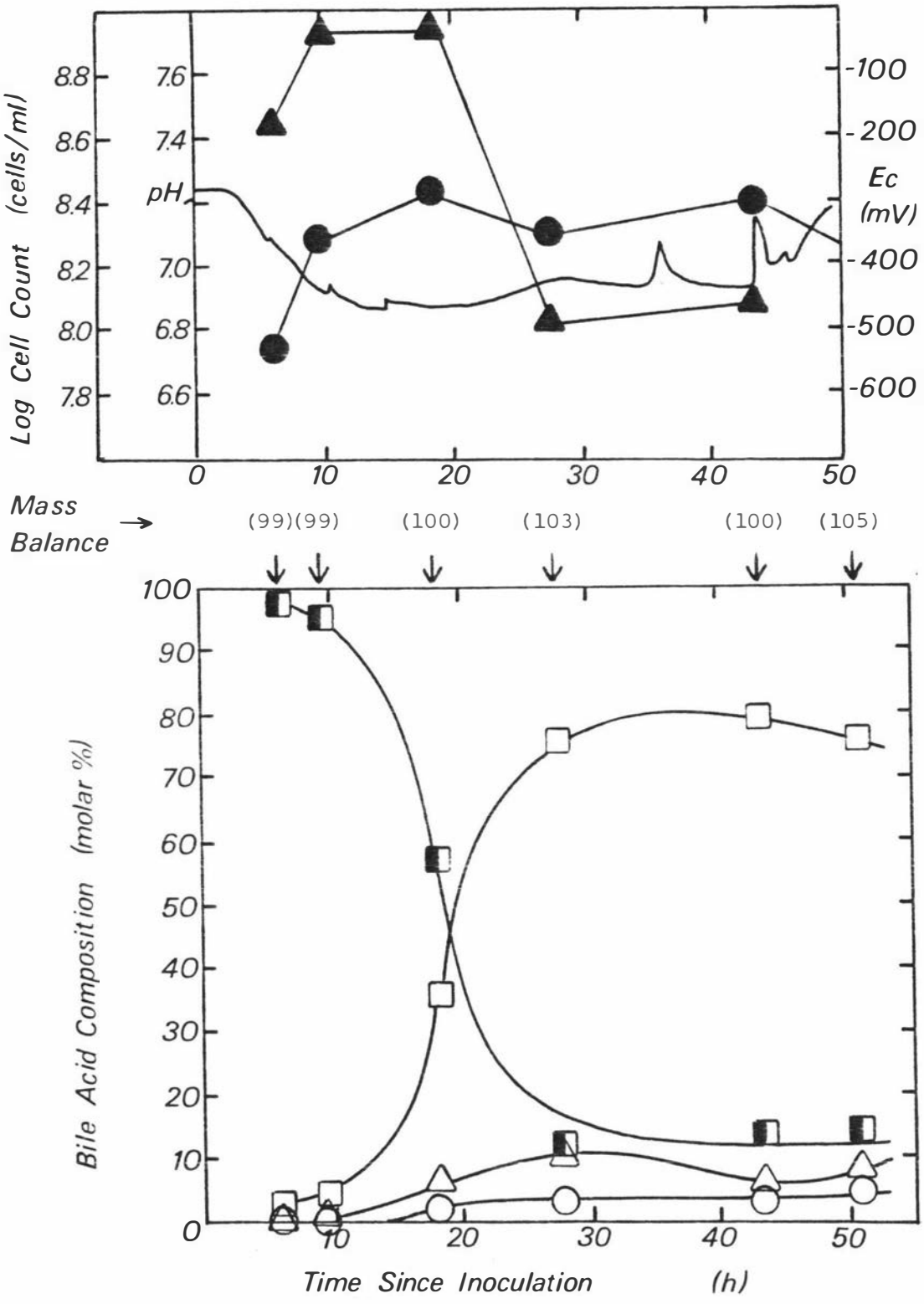


FIGURE 8.3 Course of Growth of Strain SD 10 and Transformation of Glycocholic Acid in Batch Fermentation

Deoxycholic acid (○), 7-ketodeoxycholic acid (△), cholic acid (□), glycocholic acid (◧), pH (●), cell count (▲). The electrode potential, Ec, is represented by a solid line. The "Mass Balance" figure is defined in section 3.10.2. "Bile Acid Composition" is normalised to a 100% mass balance. Experimental conditions are given in the text.

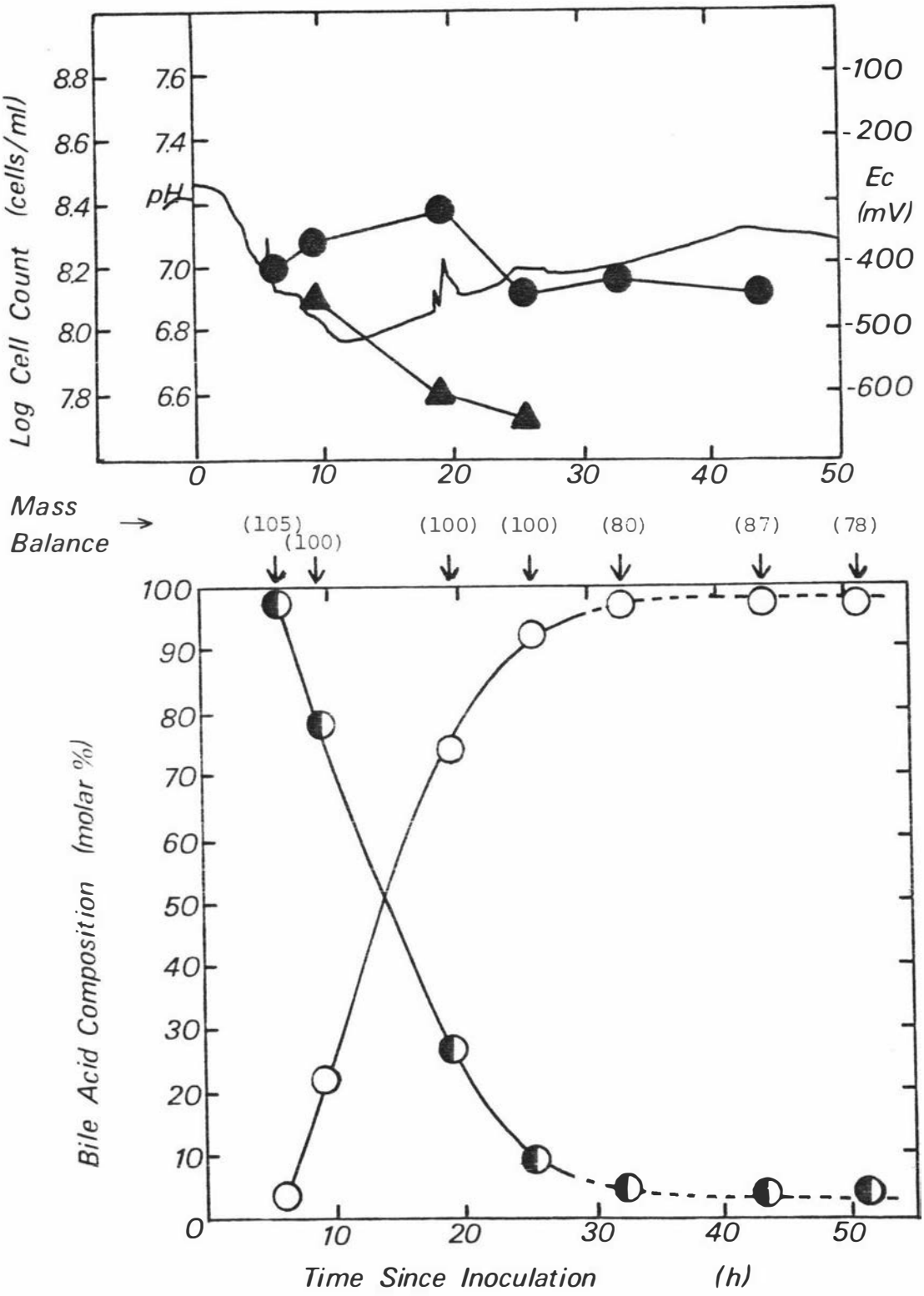


FIGURE 8.4 Course of Growth of Strain SD 10 and Transformation of Glycodeoxycholic Acid in Batch Fermentation

Glycodeoxycholic acid (◐). Remaining legend is as for Figure 8.3. A dashed line signifies unacceptable mass balance.

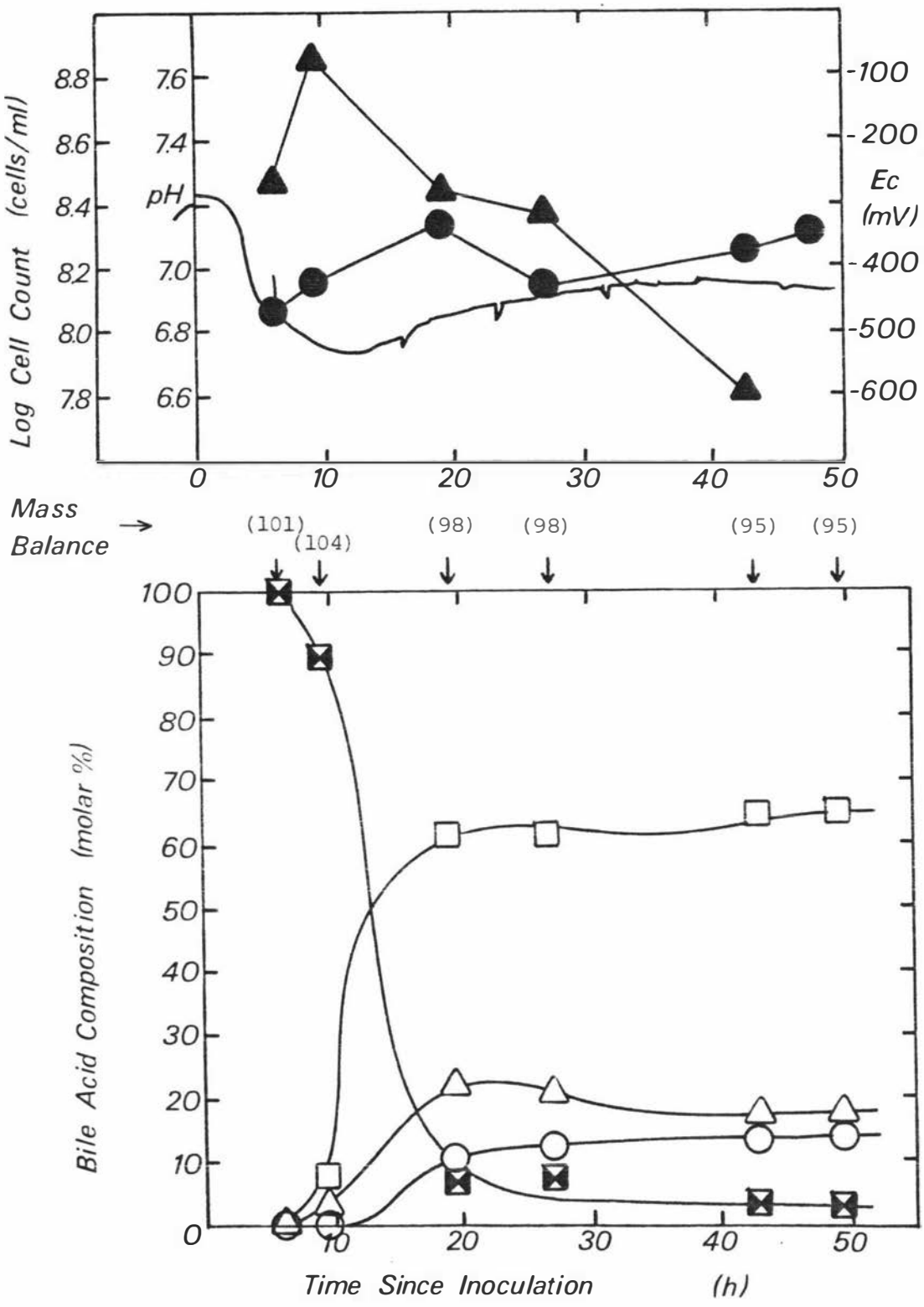


FIGURE 8.5 Course of Growth of Strain SD 10 and Transformation of Sodium Taurocholate in Batch Fermentation

Taurocholate (⊠). Remaining legend is as for Figure 8.3.

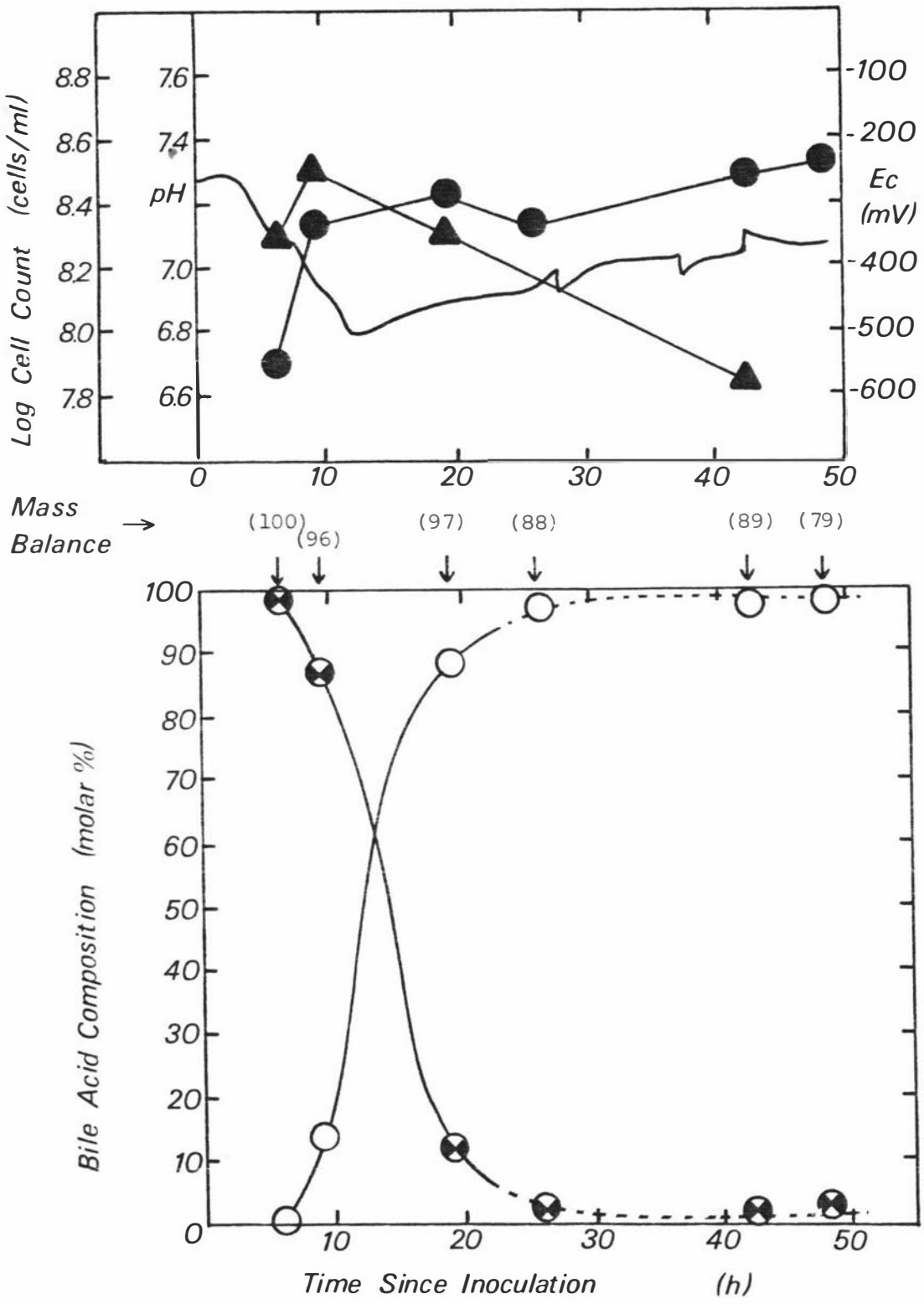


FIGURE 8.6 Course of Growth of Strain SD 10 and Transformation of Sodium Taurodeoxycholate in Batch Fermentation

Taurodeoxycholate (⊗). Remaining legend as for Figure 8.3.
A dashed line signifies unacceptable mass balance.

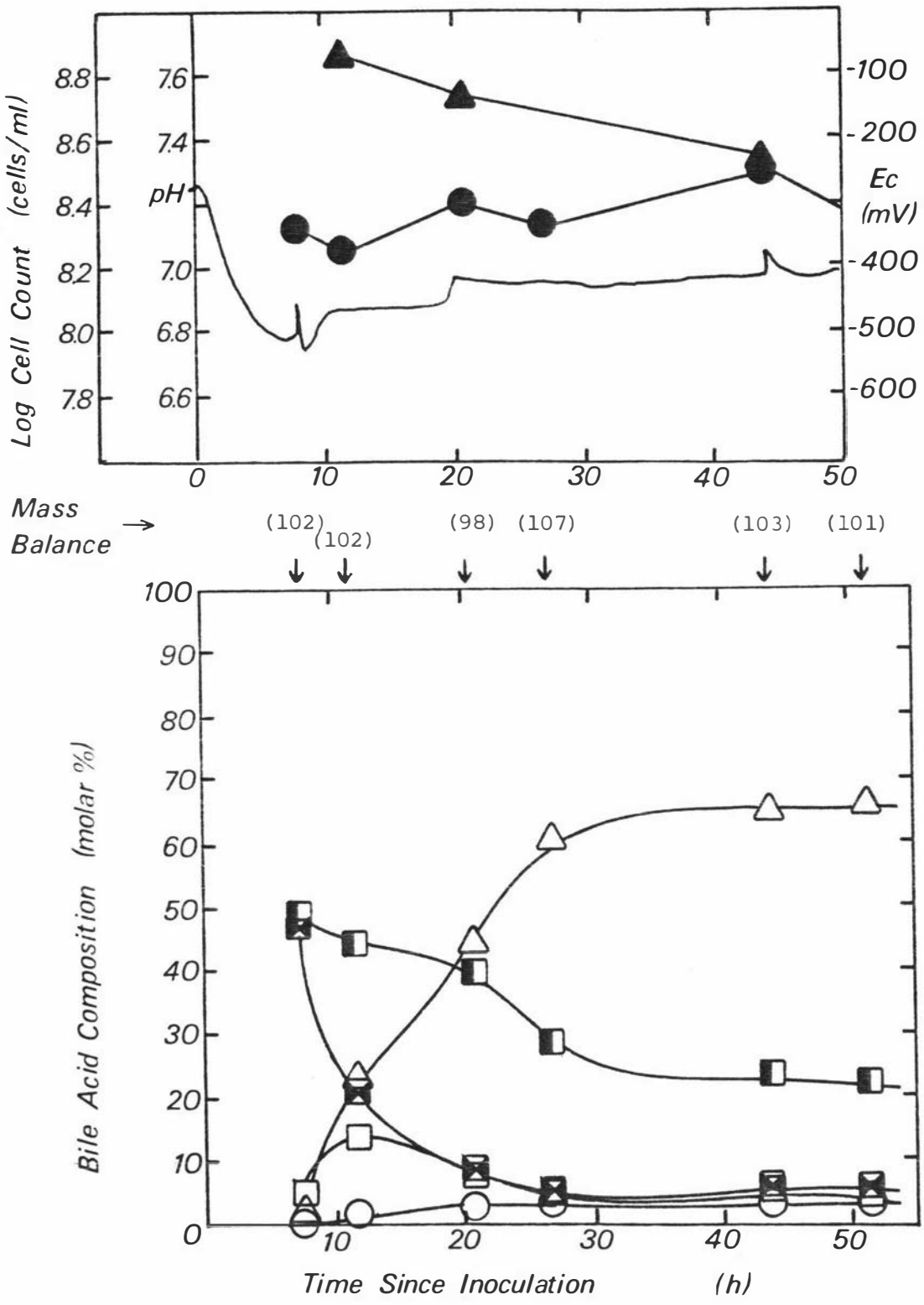


FIGURE 8.7 Course of Growth of Strain SD 10 and Transformation of Glycocholic Acid and Sodium Taurocholate in Batch Fermentation with 6-hour Substrate Addition

Taurocholate (⊠). Remaining legend as for Figure 8.3. Fermentation conditions were suited to 7α-dehydrogenation.

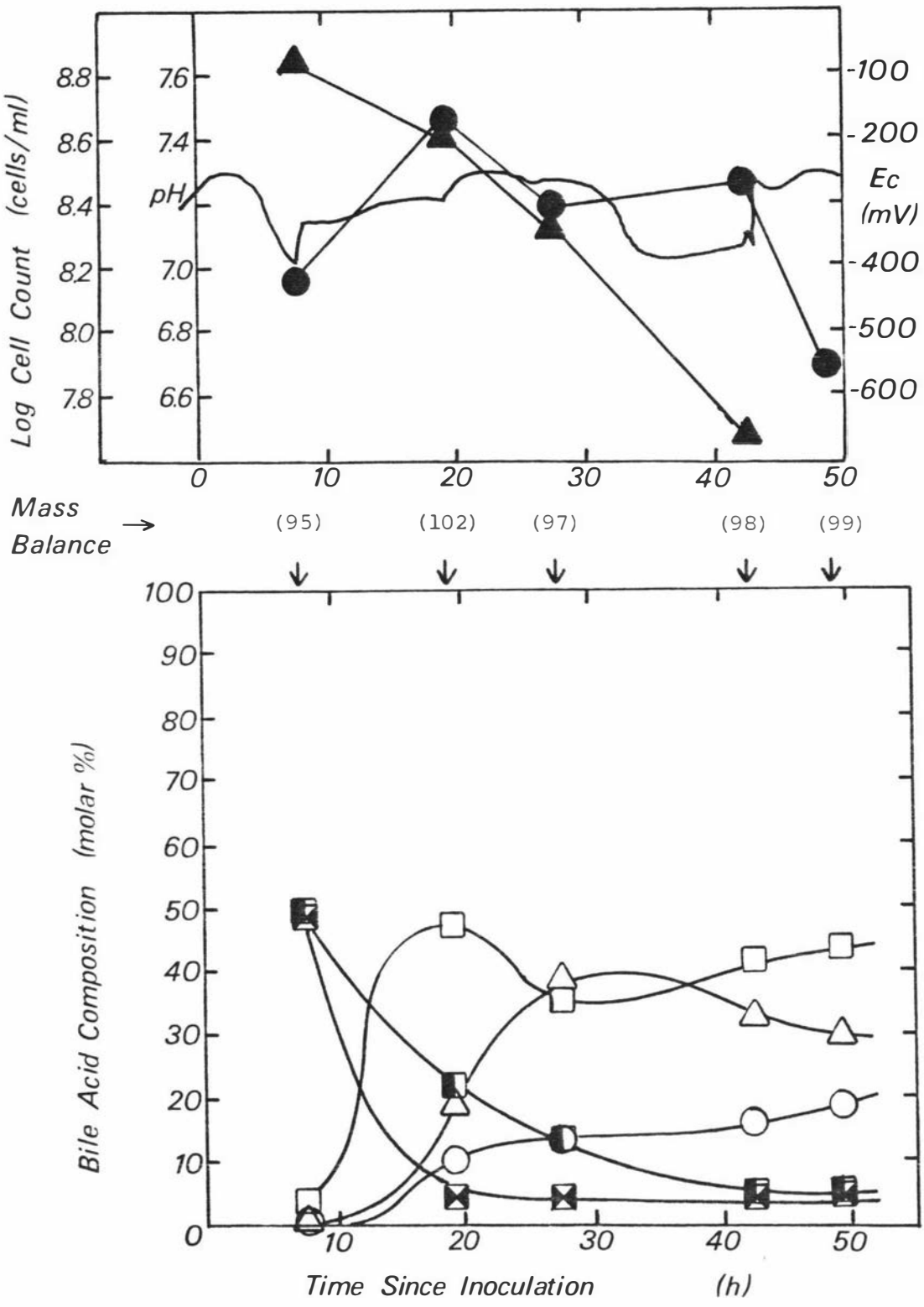


FIGURE 8.8 Course of Growth of Strain SD 10 and Transformation of Glycocholic Acid and Sodium Taurocholate in Batch Fermentation with Air-Sweeping from 7.5 to 19 Hours

Taurocholate (⧓). Remaining legend as for Figure 8.3. Fermentation conditions were suited to both C-7 transformations.

whilst glycodeoxycholate hydrolysis commenced immediately. If the conjugate hydrolases are constitutive, as reported in the literature (Nair *et al.* 1967, Aries and Hill, 1970a), then the presence of a lag might indicate transport of bile acid to and from the enzyme site to be rate limiting. Alternatively, the lag may reflect the increase in hydrolase activity into stationary phase reported for *Bacteroides fragilis* subsp. *fragilis* by Stellwag and Hylemon (1976).

One hypothesis which might explain the higher rate of taurine conjugate hydrolysis and its greater sensitivity to substrate concentration is the presence of two distinct enzymes, cholanoylglycine hydrolase and cholanoyltaurine hydrolase. This might also explain the different effect of a 7α-hydroxyl substituent on substrate concentration sensitivity of taurine and glycine conjugate hydrolysis. However, the existence of two separate enzymes is not supported by the several literature reports of isolated enzymes which have been partially purified by column chromotography, since taurine and glycine conjugate hydrolase activities were simultaneously eluted (Nair *et al.* 1967; Aries and Hill, 1970a; Stellwag and Hylemon, 1976).

A second hypothesis is that the cell wall/membrane is more permeable to taurine conjugates. This might allow higher intracellular substrate concentration, which at 0.05% w/v bulk solution substrate concentration would allow greater conversion rates, but which at higher levels would allow the build-up of intracellular bile acid concentrations to inhibitory levels. Inconsistent with this idea is the absence of a lag for glycodeoxycholate hydrolysis. Furthermore, it is unlikely that the more polar, more highly ionised taurine conjugates should diffuse more easily through the cell wall/membrane structure.

It may be that some unknown effect applies specifically to glycocholic acid hydrolysis. It is alone in its relative stability with respect of hydrolysis by these strains of *Cl. bifermentans*. If glycocholate hydrolysis were less sensitive to substrate concentration than glycodeoxycholate hydrolysis, then a pattern would be apparent which would agree with most reports in the literature. Most workers (Shimada *et al.* 1969; Stellwag and Hylemon, 1976; Aries and Hill, 1970a) have found the hydrolytic activity of cell-free extracts or growing

cells to be greater on cholic than on deoxycholic acid and conjugates. This trend applies over the range of taurine-conjugate concentrations tested in the current work (Figures 8.1 and 8.2), but not to the glycine conjugates. If a specific unknown effect does indeed apply to glycocholic acid which is resulting in its increased stability to hydrolysis, this might also account for its dev ation from the general trend described in the literature.

The small-scale experiments reported in this chapter show that the hydrolysis of taurine conjugates by growing cells of *Cl. bifermentans* is limited at high substrate concentrations. This was not due to inhibition of growth as monitored by turbidity and pH changes. It is also apparent from Figure 8.2 that the total number of mmoles of substrate hydrolysed was not reduced at the higher concentrations. Rather, a plateau was reached. In the case of sodium taurodeoxycholate, 0.02 mmoles of conjugate was hydrolysed wherever at least this quantity was present in the 20 ml culture volume. This could be indicative of product inhibition exerted by free taurine. In their work, Aries and Hill (1970a) included a study of product inhibition. The cell-free enzymes of two clostridial strains were inhibited by 8.5 mM free bile acid; the inhibition was effected equally by cholic and deoxycholic acids, and was exerted on the hydrolysis of all conjugates. However, glycine and taurine (present at 20 mM) enhanced rather than inhibited hydrolysis of all conjugates. In addition, Nair *et al.* (1967) reported competitive product inhibition exerted by cholic acid on the partially purified enzyme of *Cl. perfringens*. If taurine is responsible for some product inhibition in the current work, it would be in contrast to the findings of Aries and Hill. Furthermore, the data presented in Figures 8.3 to 8.6 plot poorly on the coordinates of Chen *et al.* (1962), indicating that simple product inhibition is not occurring.

Most reported studies of the effect of substrate concentration on deconjugation have been concerned with <u>rates</u> of hydrolysis rather than <u>yields</u>. Stellwag and Hylemon (1976), working with partially purified *Bacteroides* enzyme, reported that only three of the conjugates tested were hydrolysed at maximum velocity at substrate concentrations up to 4 mM. The velocity of taurodeoxycholate hydrolysis dropped to

zero by 4 mM. It is this conjugate which showed greatest sensitivity to substrate concentration in the current work.

From the results of the two fermentations involving conjugate mixtures (Figures 8.7 and 8.8), it is apparent that the presence of air did not impair deconjugation, but EDTA restricted hydrolysis of glycocholate. Cholic acid released in the presence of EDTA was rapidly 7α-dehydrogenated. However, cholic acid released in the presence of air was not transformed as rapidly; after 27.5 h, 7-ketone reduction exceeded 7α-hydroxyl oxidation, and the rate of 7α-dehydroxylation decreased after 19 h incubation.

The observation that hydrolysis of glycocholic acid, but not taurocholate was impaired in the presence of EDTA (55% deconjugation) may support the earlier suggestion that this conjugate is uniquely difficult to hydrolyse. A further experiment to test the effect of EDTA on glycodeoxycholate hydrolysis would clarify whether this effect pertains to the glycine moiety or is specific to glycocholate itself. The rapid and extensive conversion of released cholic acid to 7-keto-deoxycholate was as expected from sections 6.3.4 and 7.3.4, but despite this 94% conversion, only 65% of the added bile acid conjugate finished as 7-ketodeoxycholate. Five percent remained as taurocholate and 22% as glycocholate. Addition of EDTA after (say) 12 h contact with bile conjugate substrate may increase total 7-ketodeoxycholate yield.

The observation that the presence of air had no detrimental effect on cholic acid conjugate hydrolysis is in contrast with the report of Aries and Hill (1970a), who showed that in culture, the clostridial strains tested produced the conjugate hydrolase only under strictly anaerobic conditions, although the atmospheric requirements for growth were less rigorous.

In section 7.3.5, a fermentation was reported which was performed under the conditions of Experiment 2, run 14, with strain SD 10 and air-sweeping. This run achieved a 48-hour deoxycholate yield of 40%. The yield now achieved under similar conditions (Figure 8.8) was 19%. However, there is no proof that this difference is due to the use of

conjugated rather than free cholic acid substrate, the return from air-sweeping to anaerobic conditions at the 19-hour mark, or to the high (out-of-control) pH reading after 19 h incubation. Although the 7-ketodeoxycholate yields were identical between the two fermentations, in the one being currently described a net reduction of the 7-keto acid occurred some time after the air-flow was stopped, while in the run described in Chapter 7, oxidation occurred throughout.

In the two fermentations with mixed cholate conjugate substrates, neither conjugate was ever hydrolysed to yields greater than 91%. Since each was present at 0.025% w/v, *ca* 0.1 mmole of each remained unreacted after 48 h. This is approximately the same quantity remaining in those runs where a single conjugate was added to 0.05% and was 95-97% deconjugated. This might indicate that some conjugate is always unavailable for hydrolysis, yet is extractable for analysis.

Cl. bifermentans was chosen for this project partly because it is able to catalyse both the hydrolysis of conjugates and the dehydroxylation at C-7. If the deconjugation alone were desired, other organisms might be preferable. Organisms capable of selectively catalysing the hydrolysis of conjugates without further metabolising the free bile acid have been reported in the literature (Dickenson *et al.* 1971). Furthermore, organisms have been reported with the capability of selectively hydrolysing either only taurine conjugates (Kobashi *et al.* 1978) or, more commonly, only glycine conjugates (Aries and Hill, 1970a; Shimada *et al.* 1969).

8.5 CONCLUSIONS

All four of the major bile acid conjugates of mutton gall can be hydrolysed by *Cl. bifermentans* strains SD 10 and ATCC 9714. The limiting substrate concentration for single conjugates is 0.1% w/v; above this level, the taurine conjugates are hydrolysed in only low yields. A further limitation is the rate and extent of glycholate hydrolysis: 87-93% yield over 30-48 h. Hydrolysis of the other three conjugates is nearly quantitative within this period (at least at 0.05% concentration).

This reaction rate is probably adequate for an industrial process. However, the limitation on substrate concentration has important economic ramifications: large volume fermenters would be required, and large volumes of beer would need to be processed and then disposed of.

Thus, high hydrolysis yields (except for glycocholate) have been obtained. Further experimentation to "fine-tune" transformation conditions, and a strain development programme to decrease the sensitivity to substrate concentration, might increase the applicability of microbial conjugate hydrolysis to industry.

CHAPTER 9 GENERAL DISCUSSION

A series of experiments has been performed to obtain information useful in developing a process, based on microbial cells, for the hydrolysis of bile acid conjugates in New Zealand mutton and beef gall, and to 7α-dehydroxylate the released cholic acid to deoxycholic acid. Screening experiments were not used to search for an organism possessing the requisite enzymes, since little was known of optimal conditions for expressing the 7α-hydroxycholanoyl dehydroxylase. Instead, an organism was selected from those reported in the literature. *Clostridium bifermentans* ATCC 9714 was the only international type strain previously shown to have, or to be very likely to have, the requisite activities. This strain was procured, together with a second strain of the same organism, *Cl. bifermentans* SD 10. The latter was isolated by Italian workers, who successfully demonstrated its 7α-dehydroxylase and conjugate hydrolase activities. Both strains of *Cl. bifermentans* also possessed the (undesirable) ability to catalyse the 7α-dehydrogenation of cholic acid to 7-ketodeoxycholate. The occurrence of an organism possessing the 7α-hydroxycholanoyl dehydroxylase with no associated 7α-hydroxycholanoyl dehydrogenase appears to be exceedingly rare.

The products of 7α-dehydroxylation and 7α-dehydrogenation of cholic acid by *Cl. bifermentans* SD 10, and of 7α-dehydroxylation of cholic acid by *Cl. bifermentans* ATCC 9714, have already been characterised by Ferrari and Aragozzini (1972) and by Hayakawa and Hattori (1970) respectively. The products from strain SD 10 were identified by comparative infra-red spectrophotometry and mass spectometry, while Hayakawa and Hattori used mixed melting point and infra-red spectra comparison of the crystalline methyl deoxycholate with authentic ester.

Hayakawa's work is repeated, confirmed and extended here (see section 3.10.1), using different techniques: both products were isolated and characterised as the free acids.

There are only two reports in the literature of *Cl. bifermentans* possessing bile acid conjugate hydrolase enzymes. Ferrari and Ara-

gozzini (1972) used comparison of T.L.C. mobilities in one solvent system to demonstrate that strain SD 10 produced cholic acid from sodium taurocholate, and Ferrari and Beretta (1977), in a short communication, reported deconjugation of taurocholate and glycocholate by a cell-free extract of the same strain.

The current work (see section 3.10.2) provides conclusive evidence for the deconjugation of the four major cholic and deoxycholic acid conjugates. Furthermore, *Cl. bifermentans* ATCC 9714, hitherto not tested for deconjugation, has been shown to be active on all four conjugates.

Further evidence for the identity of all products from both strains is the coincidence of H.P.L.C. peaks with peaks of standard compounds.

The three reactions having been demonstrated to occur, preliminary experiments were conducted. Deconjugation was extensive and rapid. However, yields of deoxycholic acid from free cholic acid were initially very low; furthermore, significant production of the 7-ketodeoxycholate side-product occurred. This latter compound represented a loss of steroid substrate, and, as the third steroid species present, would pose an expensive separation problem to an industrial process based on cholate dehydroxylation.

For these reasons, attention was first devoted to increasing deoxycholate yields while eliminating 7-ketodeoxycholate. This aim was reasonable, since *in vivo*, in the animal intestine, extensive bile acid dehydroxylation occurs with relatively little concomitant production of oxidised metabolites (Drasar and Hill, 1974). No previous workers have employed an instrumented fermenter to provide close control of several fermentation variables, enabling the effects of individual variables to be elucidated either separately or simultaneously.

Statistically designed experiments were chosen to determine in the first instance the optimum combination of pH and atmospheric composition, and then to screen eight other fermentation variables,

in an attempt to identify the most important ones. Each experimental trial was a single batch fermentation, and each item of data was a 48-hour or 7-day yield of deoxycholate or 7-ketodeoxycholate. Yields were used rather than rates, since:

(a) for an industrial process, a high yield is of paramount importance - a short period at high rate may result in little product;

(b) rates require several data points for accurate calculation, particularly where a lag precedes transformation; and

(c) if rates vary greatly within a single fermentation, the time of measurement is critical.

The statistically designed experiments indicated that the optima for 7α-dehydroxylation and 7α-dehydrogenation of cholic acid were near pH 7. The presence of carbon dioxide in the nitrogen atmosphere was associated with increased yields of deoxycholate. Dehydroxylation was enhanced at low temperatures (32^0C compared with 37^0C) and in the presence of 8-hydroxyquinoline and fluoride ion. There was an association between high transformation yields (particularly of deoxycholate), weaker growth and less pronounced electrode potential minima.

Despite these findings, no single factor or combination of factors, varied within the confines of the experiment, resulted in greater than 40% deoxycholate yield or (not simultaneously) less than 11% 7-ketodeoxycholate yield. While 7α-dehydrogenation is occurring, it is unlikely that a commercial process could be based on the dehydroxylation.

To characterise whole-cell dehydrogenation further, with a view to finding a specific inhibitor, a series of experiments was performed with washed, resting-stage cells. No agents were found which, in moderate concentrations, completely inhibited oxidation. Some agents caused a decrease in 7-ketodeoxycholate yield, others caused an increase. Dehydroxylation activity, already low before cell harvesting, appeared to be impaired upon cell washing.

Application of the findings from washed cells to batch fermentation provided two methods which could, after further experimentation, result

in no more than two bile acid species being present in the final reaction mixture:

(a) EDTA enhanced dehydrogenation and completely inhibited dehydroxylation. Only 7-ketodeoxycholic acid together with *ca* 3% cholic acid remained after 48 h.

(b) Sweeping the fermenter head-space with air after growth had ceased enhanced both transformations. Very little cholic acid remained to contaminate the deoxycholate and 7-ketodeoxycholate products.

In a final group of experiments, deconjugation was investigated. All four major mutton and beef gall conjugates, when present at 0.05% w/v, were hydrolysed at greater than 95% yield with the exception of glycocholate, which was 87-93% deconjugated over 48 h. Taurine conjugate hydrolysis was impaired at substrate concentrations in excess of 0.1% w/v. Glycocholate deconjugation was impaired in the presence of EDTA whilst taurocholate deconjugation was unaffected. Each of these conjugates could be hydrolysed under aerobic conditions.

Throughout this project, 7-ketodeoxycholate has been treated as an unwanted side-product. It does, however, have some value, in that the 7-ketone is an intermediate in the chemical synthesis of deoxycholic acid from cholic acid. Deoxycholic acid can be produced from 7-ketodeoxycholic acid by Wolff-Kishner reduction (Fieser and Rajagopalan, 1949). Indeed, Fieser's work was performed with industrial 7α-dehydroxylation of cholic acid in mind. If cholic acid were converted totally to 7-ketodeoxycholate or to a mixture of the 7-keto acid and deoxycholic acid, then by Wolff-Kisnner reduction of the crude bile acid extract, deoxycholic acid would be produced as the sole product.

It has been reported (Anon, 1979) that ursodeoxycholic acid has therapeutic properties regarding human gallstones. Provided that the necessarily extensive clinical trials are successful, the market for ursodeoxycholate should increase significantly. Any process involving the conversion of cholic acid to ursodeoxycholic acid would benefit from the added-value economics. It is conceivable that 7-ketodeoxycholate could be converted to ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid) chemically by:

(a) protection of the 3α-hydroxyl and carboxylic acid groups,

(b) elimination of the 12α-hydroxyl group by a modification of the method of Nakada (1963),

(c) hydrogenation of the 11-ene,

(d) selective reduction of the 7-ketone to the 7β-hydroxyl by a modification of the Samuelsson (1960a) method, and finally

(e) deprotection of the 3α-hydroxyl and carboxylic acid groups.

The present method of ursodeoxycholate synthesis is via chenodeoxycholate, which is in turn prepared from methyl 3α,7α-diacetoxy-12α-hydroxy-cholanoate. Chenodeoxycholate is selectively oxidised at C-7 and reduced to give the 7β-hydroxyl. The older method of 12α-hydroxyl elimination with phosphorous oxychloride and pyridine (Nakada, 1963) suffers from low yields, probably because of facile *trans*-elimination of acetic acid from the 7α-acetate group (Chen, 1976). Chen proposed a newer method of elimination via the 12-mesylate. This has the advantage of higher yields, but employs mesyl chloride and hexamethylphosphoric triamide, both expensive reagents.

By inverting the sequence and eliminating after C-7 oxidation, the possibility is presented of avoiding the *trans*-elimination of acetic acid while having a function which will be subsequently needed for generation of the 7β-hydroxyl group.

It should be noted that a simple chemical method for the synthesis of 7-ketodeoxycholic acid from cholic acid is available from the selective oxidation with N-bromosuccinimide (Feiser and Rajagopalan, 1949). Several methods for purifying the crude reaction product have also been published (Hoehn and Linsk, 1945; Jervois *et al.*, 1951; Gauthier, 1953), some of which are in the patent literature.

Further, some organisms possess particularly active 7α-hydroxycholanoyl dehydrogenase enzymes (Macdonald *et al.*, 1973, 1974, 1975b). Such organisms could convert cholic acid to 7-ketodeoxycholic acid, possibly without the need for high concentrations of EDTA. However, it is not known whether these particular organisms possess deconjugating ability.

In both the chemical and biological cases, if 7-ketodeoxycholic acid were to be produced from mutton and beef gall, some deoxycholic acid would also be present, since glycodeoxycholic acid and taurodeoxycholic acid are part of the gall mixture.

This discussion on 7-ketodeoxycholate use is speculative. Within the context of the original project aim, the 7-keto acid was an unwelcome side-product. Despite the environmental manipulations and screen for inhibitors, no method of entirely preventing its formation has been discovered. There are, however, three possible methods which could be investigated:

(a) Ferrari and Pacini (1968) developed a highly selected mixed culture capable of entirely 7α-dehydroxylating cholic acid with only trace dehydrogenation. Use of this or of a similar mixed culture may have industrial application. However, obtaining reproducibility on a small scale may be difficult.

(b) Hattori and Hayakawa (1969) isolated *Bacteroides* 28S which possesses only a weak 7α-dehydrogenase but an active 7α-dehydroxylase. A second organism would probably be required for preliminary deconjugation.

(c) A mutation programme might produce a strain of *Cl. bifermentans* without the ability to synthesise an active 7α-hydroxycholanoyl dehydrogenase. Such a programme was considered to be beyond the scope of this project.

All of these areas would be worthy of further research in the continued development of a selective dehydroxylation process.

Although the project aim was not achieved in that selective 7α-dehydroxylation of cholic acid was not successful with *Cl. bifermentans* alone, the deconjugation step was successful, at least at low substrate concentration. *Cl. bifermentans* could conceivably be used in a microbial deconjugation process if it could be ensured that no further transformation occurred. This might be possible, since the clostridial conjugate hydrolase appears to be more stable than the 7α-dehydrogenase or the 7α-dehydroxylase. While only low yields of 7-ketodeoxycholate (and very low yields of deoxycholate) were achieved with washed cells as described in Chapter 6 of this study, acetone dried

cells of *Cl. welchii* are capable of quantitatively hydrolysing conjugates (Roseleur and van Gent, 1976). A possible area for future research is the preparation of acetone powders of *Cl. bifermentans* with the aim of preserving the deconjugation activity, but destroying the C-7 hydroxyl-transforming activities.

The finding that taurine conjugates (at less than 0.1% w/v) can be almost quantitatively hydrolysed is particularly important in the light of the report by Roseleur and van Gent (1976) that these conjugates are substantially degraded during alkaline hydrolysis. After 6 h reaction time at 120^{0}C, less than 50% recovery was observed from the alkaline hydrolysis of sodium taurocholate, while a 70-75% recovery was observed for glycocholic acid. Recent work has shown that fungal hydrolysis offers a second alternative method of deconjugation. However, while fungal hydrolysis of glycine conjugates is nearly complete, taurine conjugates are poorly hydrolysed (Maddox and Chong, 1978; Chong *et al.*, 1980).

If a simple deconjugation process were desired, *Cl. bifermentans* would probably not be the organism of choice. A strain would be required which possessed no bile acid-transforming enzyme other than the conjugate hydrolases, and which showed less sensitivity to substrate concentration. To process three 1-tonne, 48-hour batches of 60% solids gall per week at only 0.1% w/v total bile acid concentration (as is the limitation for *Cl. bifermentans*) would required a 300 m^3 fermenter volume.

In this study, cells have been treated as "black boxes" and an approach adopted whereby the environment of whole cells has been manipulated. It was considered that other laboratories are better equipped to investigate the biochemistry of bile acid transformations, but that a knowledge of their results should be useful to this project. Indeed, some insight has been gained into dehydrogenation and deconjugation in this manner. Unfortunately, little work has been performed with cell-free 7α-hydroxycholanoyl dehydroxylases, since these enzymes are very labile in both the cell-free and cell-bound states (Drasar and Hill, 1974). In the current work, 7α-dehydroxylase activity has been found to be very sensitive to a number of factors, some of which are still unidentified.

From the few studies of 7α-dehydroxylase enzymology which have been published, it is apparent (Ferrari *et al.*, 1977) that the cell-free *in vitro* mechanism is identical to that elucidated *in vivo* by Samuelsson (1960b); firstly the 6β-hydrogen and 7α-hydroxyl group are eliminated and secondly the 6-ene intermediate is *trans*-hydrogenated at the 6α and 7β positions.

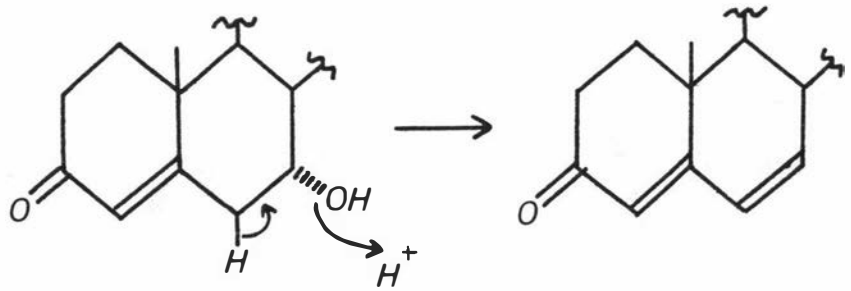
The second reaction step, being a hydrogenation, might be expected to benefit from low redox potential conditions where electron donors predominate. It is this step which gives the dehydroxylation its net reductive character, and probably partially accounts for the literature emphasis on strict anaerobiosis. However, the second reaction appears to be extremely rapid (Ferrari *et al.*, 1977). If the hydrogenation is not rate limiting, the apparent need for strict anaerobiosis is not so obvious and the occurrence of strong dehydroxylation at moderate electrode potentials is not so surprising. The reason for enhanced 7α-dehydroxylation under aerobic conditions (section 7.3.5) is not yet clear.

Further research involving redox control independent of dissolved oxygen control might assist in clarifying the rôles of these two parameters.

The only case where a 6-ene intermdiate has been isolated was during the aerobic transformation of cholic acid by *Arthrobacter simplex* (Hayakawa and Samuelsson, 1964). Hayakawa *et al.* (1969) proposed an aerobic degradation pathway in which the 4,6-dien-3-one intermediate arose:

Cholic acid → 7α-hydroxy-3-keto C_{24} acids → 7α-hydroxy-4-en-3-keto C_{24} acids → 4,6-dien-3-keto C_{24} acids → 4-en-3-keto C_{24} acids → further degradation

It is conceivable that the 7α-hydroxyl group is eliminated chemically in this case. In mildly acidic conditions:



The fact that a 6-ene intermediate has been isolated only on this one occasion may be indicative of the relative stability of the 4,6-dien-3-one structure to dehydrogenation. Together, the possibility of chemical elimination and the relative stability of the olefinic intermediate suggest a real difference between the aerobic 7α-dehydroxylation mechanism of *Arthrobacter* spp. and *Mycobacterium* spp. and the anaerobic 7α-dehydroxylation of intestinal bacteria.

If, as is the aim of this project, an industrial process were to be developed using *Cl. bifermentans* to 7α-dehydroxylate cholic acid, then the sensitivity of dehydroxylation to substrate concentration would pose a problem. Midvedt and Norman (1968) and Gustafsson *et al.* (1966) have demonstrated marked reduction in yields of the 7-deoxy product in response to increased substrate concentration. Similar results were obtained in preliminary experiments in the current work. If a substrate concentration of 0.05% were employed, $1m^3$ of reaction volume would be required for each 0.5 Kg of bile acid. (For the New Zealand annual gall throughput, in 48-hour batches, 600 m^3 of fermenter volume would be required). The expense of plant and of product recovery would be prohibitive.

Costs would also be higher in a process where steroid substrate came into contact with the many contaminating compounds contained in spent microbiological media. More desirable, from this viewpoint, would be a process employing washed, dried or immobilised cells in buffer solutions.

Hence, this project has not achieved its aim of developing an industrial process, but it has provided some novel and sometimes puzzling data relating to the bile acid transformations involved, and has suggested areas for future work.

CHAPTER 10 GENERAL CONCLUSIONS

Studies have been performed on the transformation of bile acids by *Clostridium bifermentans* for the purpose of developing a process whereby bile acid conjugates of New Zealand mutton gall may be converted to deoxycholic acid.

Statistically designed experiments proved successful in identifying which fermentation variables most affected yields of deoxycholic and 7-ketodeoxycholic acid from cholic acid. Both products were formed in greatest yield near pH 7. Deoxycholate yield was highest in the presence of carbon dioxide, fluoride ion and 8-hydroxyquinoline as well as being higher at 32^0C than at 37^0C. Strong 7α-dehydroxylation was associated with relatively weak growth and relatively high electrode potentials. 7-Ketodeoxycholate production was not as sensitive to environmental factors as was deoxycholate production, and could not be eliminated by merely manipulating fermentation variables.

Studies on the 7α-dehydrogenation of cholic acid with washed resting-stage cells of *Cl. bifermentans* indicated that 7-ketodeoxycholate yields could be reduced by incubating in the presence of Cd^{++} or Zn^{++} ions or sodium thioglycollate, while higher yields were obtained in the presence of air or EDTA. In batch fermentation Zn^{++} ion reduced 7-ketodeoxycholate yields as expected, but completely inhibited dehydroxylation. EDTA increased 7α-dehydrogenation sufficiently to allow a 97% yield of 7-ketodeoxycholate with 3% of the added cholic acid remaining after fermentation for 48 h. Aerobic incubation (after cell growth was complete) increased 7-ketodeoxycholate yields as expected, but also supported strong dehydroxylation. A 50% yield of deoxycholate (the highest observed during the work) was obtained by sweeping the fermenter headspace with air. This is an unexpected finding for a reaction hitherto considered to be strictly anaerobic.

Studies of bile acid conjugate hydrolysis during incubation with growing cells of *Cl. bifermentans* revealed that near quantitative deconjugation can be obtained for glycodeoxycholate, taurocholate and

taurodeoxycholate, and *ca* 90% deconjugation for glycocholate, within 48 h. At substrate concentrations greater than 0.1% w/v however, the taurine conjugates were less well hydrolysed. Hydrolysis of glycocholate and taurocholate was unimpaired by aerobic incubation, while EDTA partially inhibited deconjugation of glycocholate but not of taurocholate.

The low yields of 7α-dehydroxylation (less than 50% deoxycholic from cholic acid), the great sensitivity of this transformation to environmental factors and the inability to eliminate the side-product 7-ketodeoxycholic acid require that further work be done before an industrial process can be based on this research. Several avenues of enquiry have been proposed.

The rapidity and near-completeness of hydrolysis of the four bile acid conjugates provide the basis of an industrial process, so long as further transformation can be controlled, product extraction methods developed, and the limited substrate concentrations tolerated.

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REMARKS

Characterisation of the product of 7α-dehydroxylation of cholic acid by *Cl. bifermentans* ATCC 9714

P.E. 720

Normal slit width

Slow scan

ORIGIN

PURITY Recrystallised via toluene complex, and dried

PHASE Solid, as KBr Disc

CONCENTRATION 1% w/w in 75 mg KBr

THICKNESS

DATE

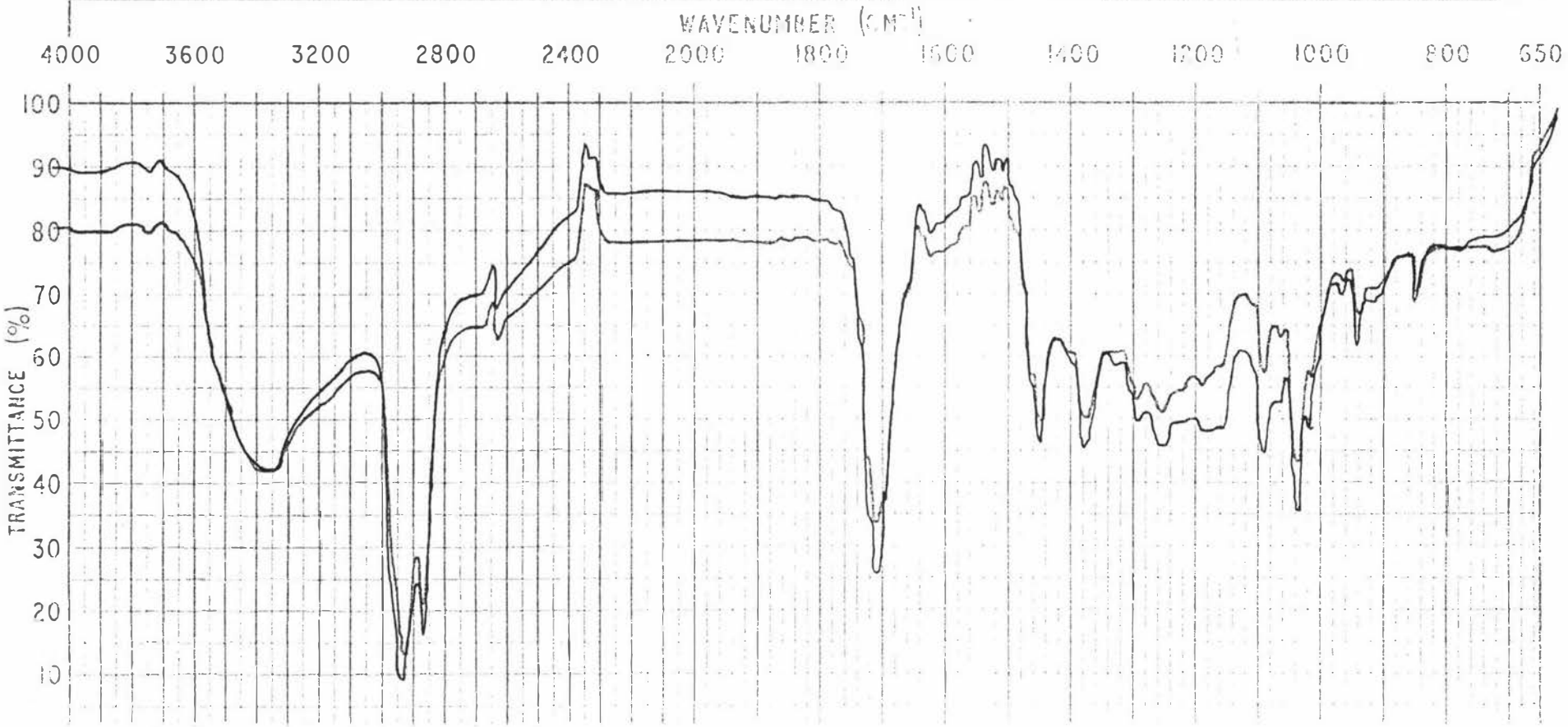
OPERATOR R.H.A.

PERKIN-ELMER

SPECTRUM NO.

SAMPLE 1 Light, upper line: Deoxycholic acid, microbially produced

SAMPLE 2 Dark, lower line: Deoxycholic acid, authentic



REMARKS

Characterisation of the product of 7α-dehydrogenation of cholic acid by *Cl. bifermentans* ATCC 9714

P.E. 720

Normal slit width

Slow scan

ORIGIN

PURITY Recrystallised from water, and dried

PHASE Solid, as KBr Disc

CONCENTRATION 1% w/w in 75 mg KBr

THICKNESS

DATE

OPERATOR R.H.A.

PERKIN-ELMER

SPECTRUM NO.

SAMPLE Dark, upper line: 7-ketodeoxycholic acid, authentic

SAMPLE Light, lower line: 7-ketodeoxycholic acid, microbially produced

REMARKS

Characterisation of the product of hydrolysis of glycodeoxycholic acid by *Cl. bifermentans* SD 10

P.E. 720

Normal slit width

Slow scan

ORIGIN

PURITY Recrystallised via toluene Complex, and dried

PHASE Solid, as KBr Disc

CONCENTRATION 1% w/w in 75 mg KBr

THICKNESS

DATE

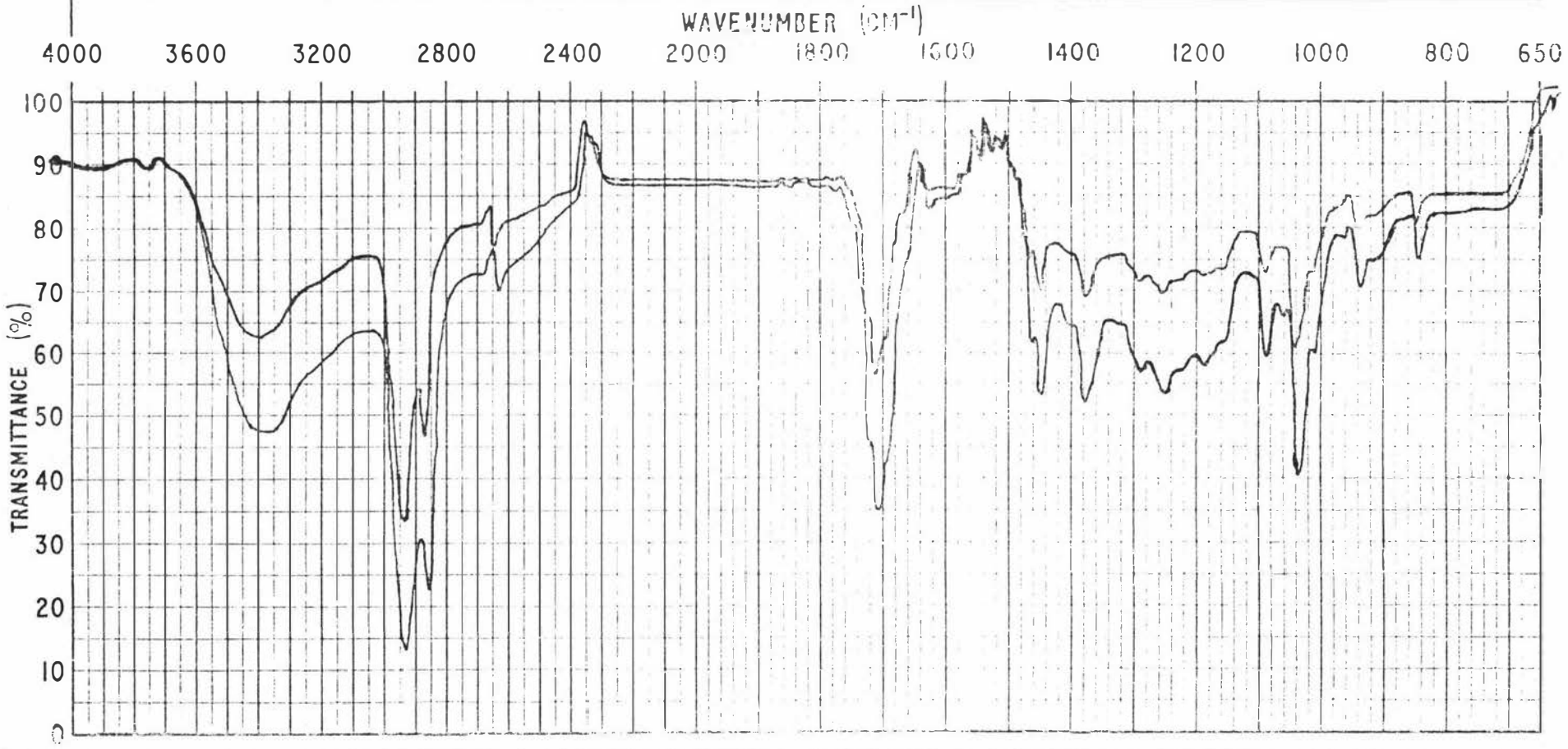
OPERATOR R.H.A.

PERKIN-ELMER

SPECTRUM NO.

SAMPLE 1 Upper line: Deoxycholic acid, authentic

SAMPLE 2 Lower line: Deoxycholic acid from glyco-deoxycholic acid



REMARKS

Characterisation of the product of hydrolysis of sodium tauro-deoxycholate by *Cl. bifermentans* SD 10

P.E. 720

Normal slit width

Slow scan

ORIGIN

PURITY Recrystallised via toluene complex, and dried

PHASE Solid, as KBr Disc

CONCENTRATION 1% w/w in 75 mg KBr

THICKNESS

DATE

OPERATOR R.H.A.

PERKIN-ELMER

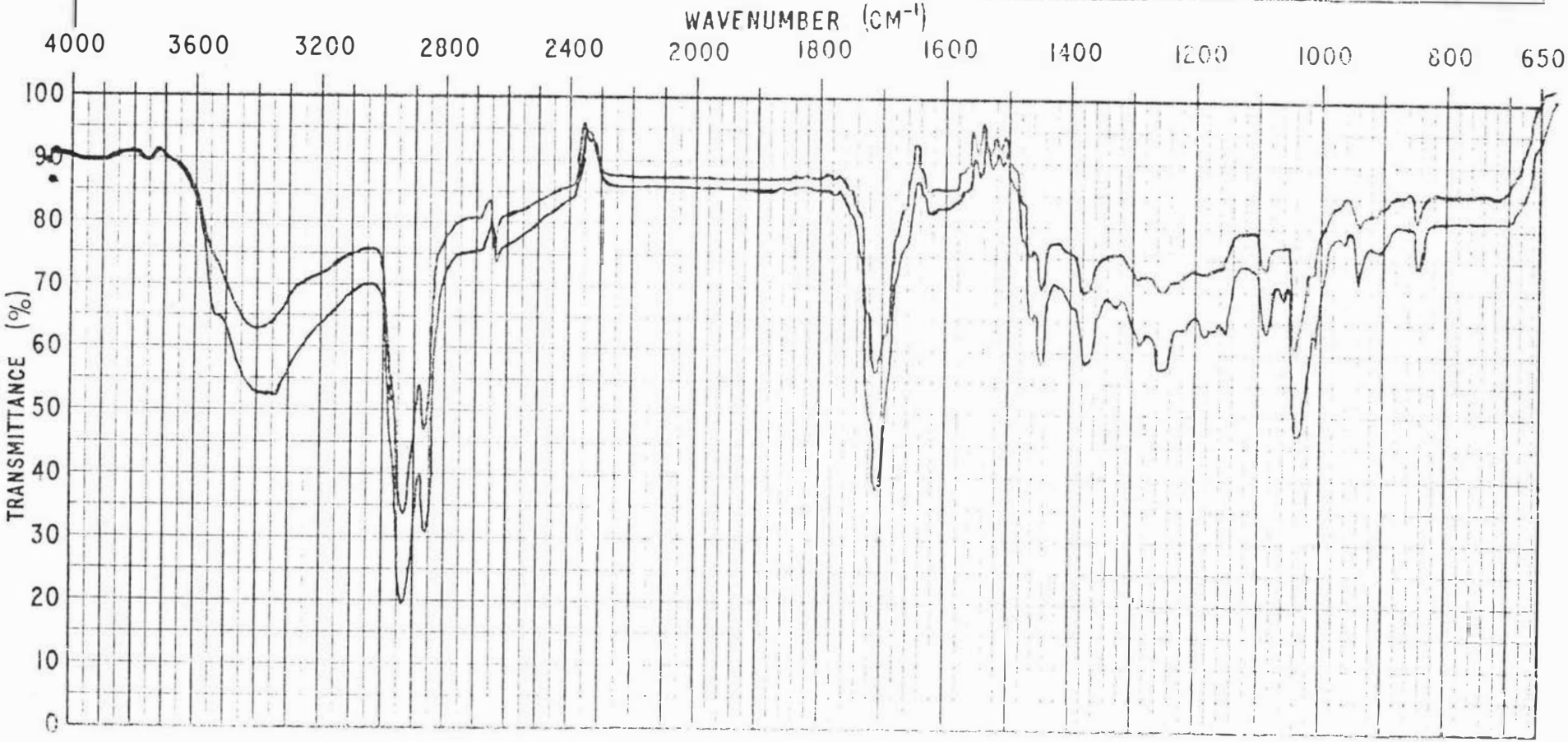
SPECTRUM NO.

SAMPLE : Upper line:

Deoxycholic acid, authentic

SAMPLE 2 Lower line:

Deoxycholic acid from sodium taurodeoxycholate



Characterisation of the product of hydrolysis of glycocholic acid by *Cl. bifermentans* SD 10

Pye Unicam SP3 300

Spectraset 7

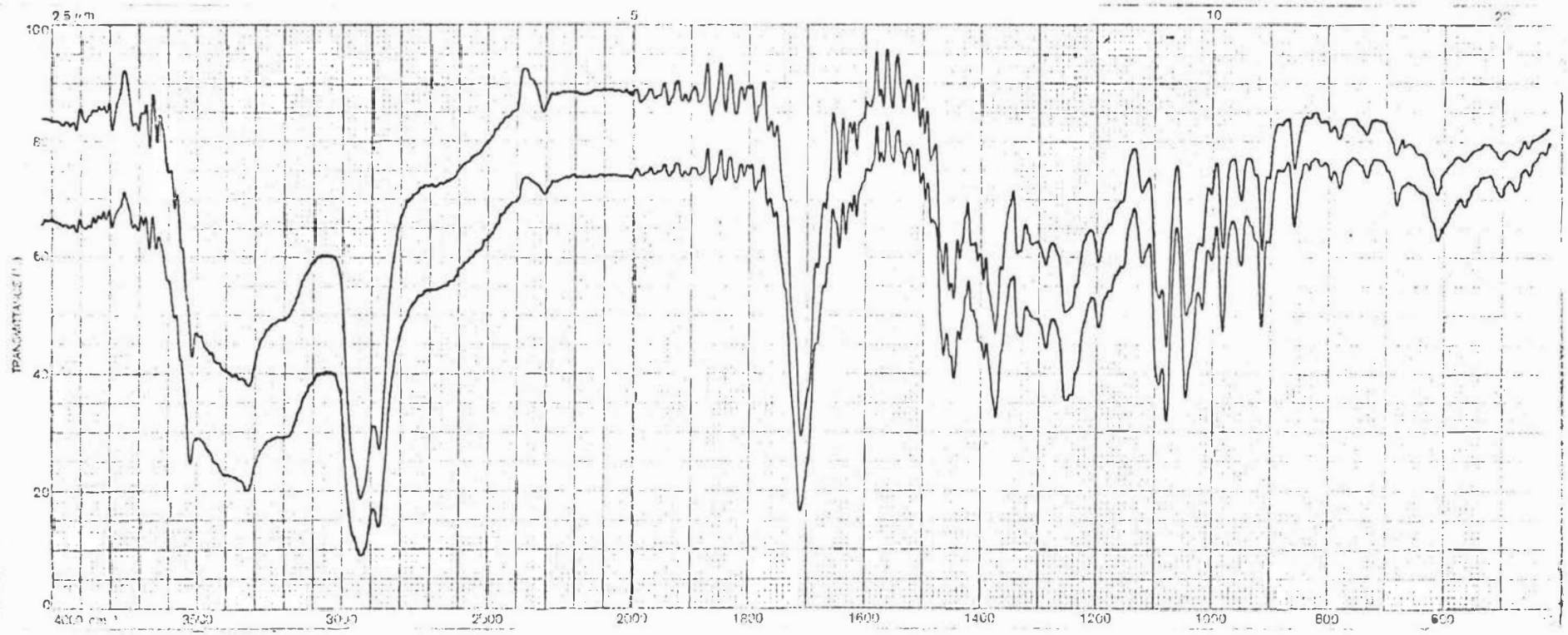
PURITY Recrystallised from methanol, and dried

PHASE Solid, as KBr disc

CONCENTRATION 1% w/w in 75 mg KBr

SAMPLE 1 Upper line: Cholic acid, microbially produced

SAMPLE 2 Lower line: Cholic acid, authentic



Characterisation of the product of hydrolysis of sodium taurocholate by *Cl. bifermentans* SD 10

Pye Unicam SP3 300

Spectraset 7

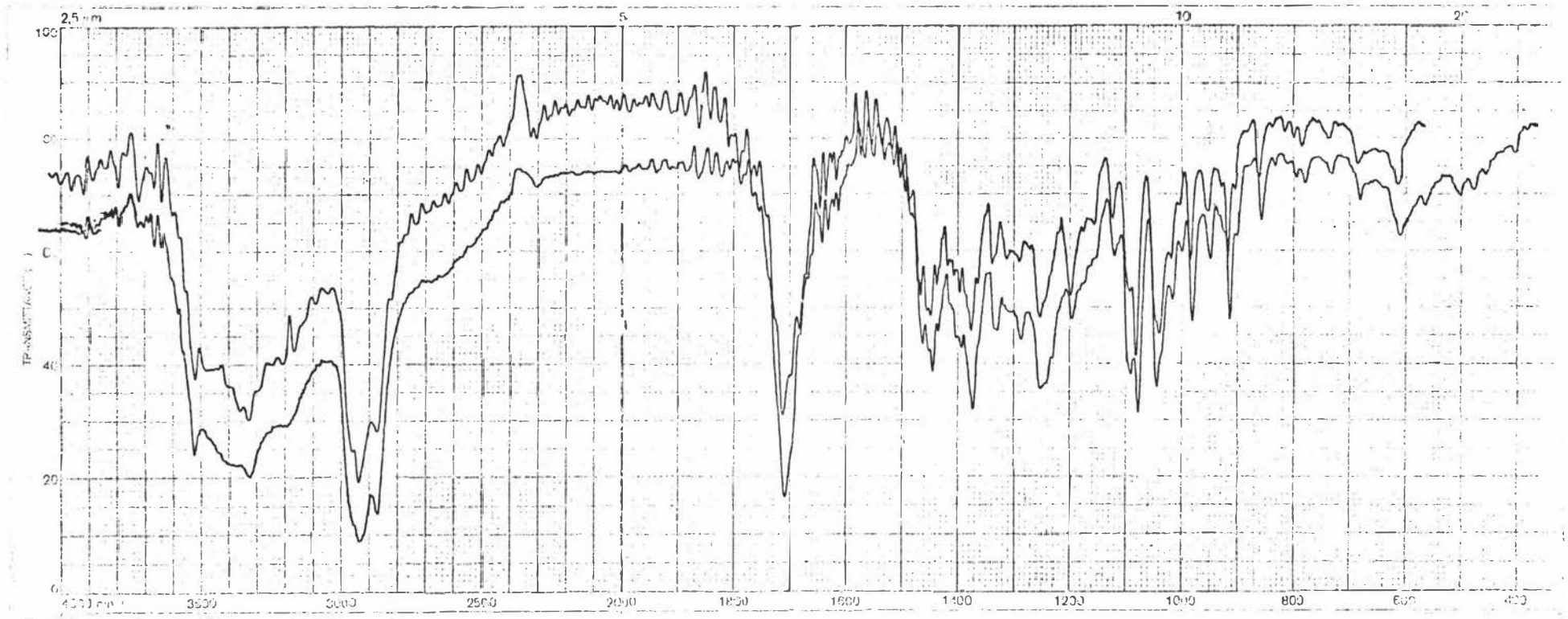
PURITY Recrystallised from methanol, and dried

PHASE Solid, as KBr disc

CONCENTRATION 1% w/w in 75 mg KBr

SAMPLE 1 Upper line: Cholic acid, microbially produced

SAMPLE 2 Lower line: Cholic acid, authentic



APPENDIX 2 Parsimonious Models and Regression Statistics for Experiment 1

TABLE A2.1 Parsimonious Model and Regression Statistics for 7-Ketodeoxycholate Yield

Parsimonious model:

$$\hat{Y} = 37.2 - 7.5A^2 - 6.2B^2 - 4.9A^2B + 5.9AB^2$$

Note: A, B and $\hat{Y}$ are defined in "Abbreviations".

Coefficient of Determination:

$r^2 = 97.0\%$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	4	562.91	140.73
Residual	6	17.70	2.95
Total	10	580.61	

Test for Lack of Fit:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	2	3.05	1.53
Lack of Fit	4	14.65	3.66
Residual	6	17.70	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{3.66}{1.53} = 2.40$$

As $F_{4,2,0.05} = 19.2$, the lack of fit is not significant at the 5% level.

TABLE A2.2 Parsimonious Model and Regression Statistics for Cholic Acid Remaining

Parsimonious model:

$$\hat{Y} = 54.9 + 4.2A + 4.8B + 11.4A^2 - 8.6AB^2 + 7.0A^2B^2$$

Coefficient of Determination:

$$r^2 = 97.4\%$$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	5	1023.61	204.72
Residual	5	27.59	5.52
Total	10	1051.20	

Test for Lack of Fit:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	2	2.06	1.03
Lack of Fit	3	25.53	8.51
Residual	5	27.59	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{8.51}{1.03} = 8.26$$

As $F_{3,2,0.05} = 19.2$, the lack of fit is not significant at the 5% level.

TABLE A2.3 Parsimonious Model (discarded) and Regression Statistics for Deoxycholic Acid Yield

Parsimonious model:

$$\hat{Y} = 8.7 - 1.9A - 6.4B - 5.4A^2 + 4.2B^2 + 7.4A^2B - 4.0A^2B^2$$

Coefficient of Determination:

$$r^2 = 98.4\%$$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	6	258.50	43.08
Residual	4	4.08	1.02
Total	10	267.58	

Test for Lack of Fit:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	2	0.51	0.26
Lack of Fit	2	3.57	1.79
Residual	4	4.08	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{1.79}{0.26} = 7.00$$

As $F_{2,2,0.05} = 19.0$, the lack of fit is not significant at the 5% level.

TABLE A2.4 Parsimonious Model and Regression Statistics for the Natural Logarithm of Deoxycholic Acid Yield

Parsimonious model:

$$\hat{Y} = 2.12 - 1.07A - 0.55B - 1.36A^2 + 0.34B^2 + 0.77A^2B + 0.63AB^2$$

Coefficient of Determination:

$$r^2 = 99.1\%$$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	6	8.5322	1.4229
Residual	4	0.0769	0.192
Total	10	8.6091	

Test for Lack of Fit:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	2	0.0067	0.0033
Lack of Fit	2	0.0702	0.0351
Residual	4	0.0769	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{0.0351}{0.0033} = 10.52$$

As $F_{2,2,0.05} = 19.0$, the lack of fit is not significant at the 5% level.

TABLE A2.5 Parsimonious Model and Regression Statistics for the Base Ten Logarithm of the Maximum Number of Cells Observed. (L.M.C.)

Parsimonious model:

$$\hat{Y} = 8.61 - 0.18A - 0.11AB - 0.18A^2 + 0.22AB^2 - 0.21A^2B^2$$

Coefficient of Determination:

$$r^2 = 91.4\%$$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	5	0.46292	0.09258
Residual	5	0.00730	0.00146
Total	10	0.47022	

Test for Lack of Fit:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	2	0.0061	0.0030
Lack of Fit	3	0.0012	0.0004
Residual	5	0.0073	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{0.0004}{0.0030} = 0.1355$$

As $F_{3,2,0.05} = 19.2$, the lack of fit is not significant at the 5% level.

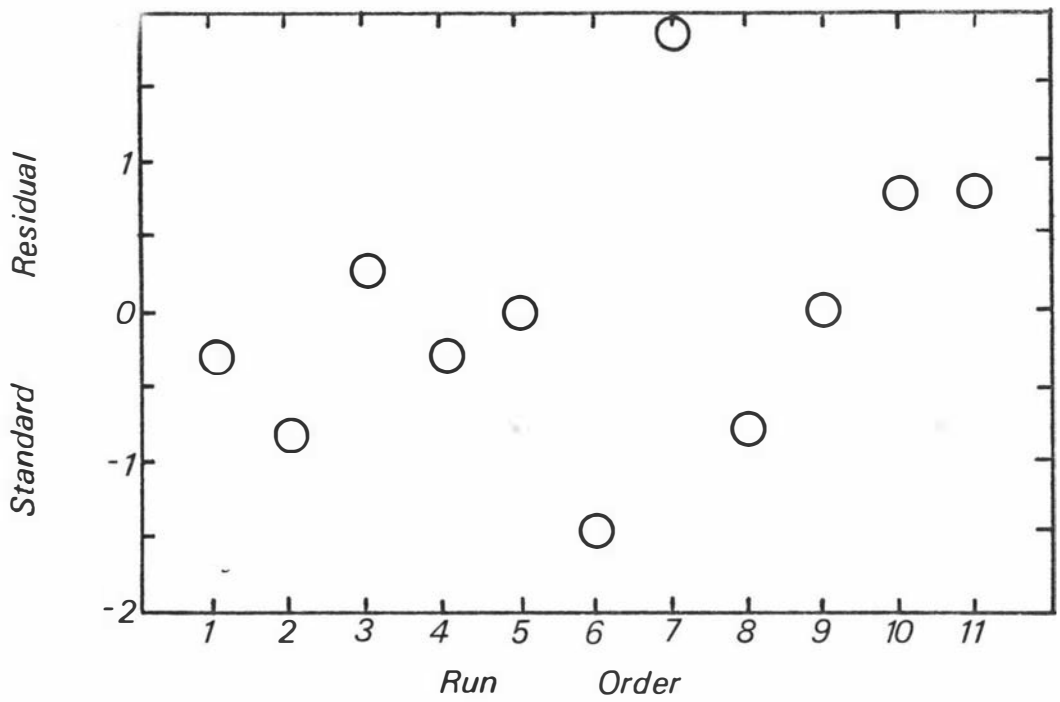


FIGURE A2.1 Residual Plot Number 1 for the Parsimonious Model for 7-Ketodeoxycholate Yield

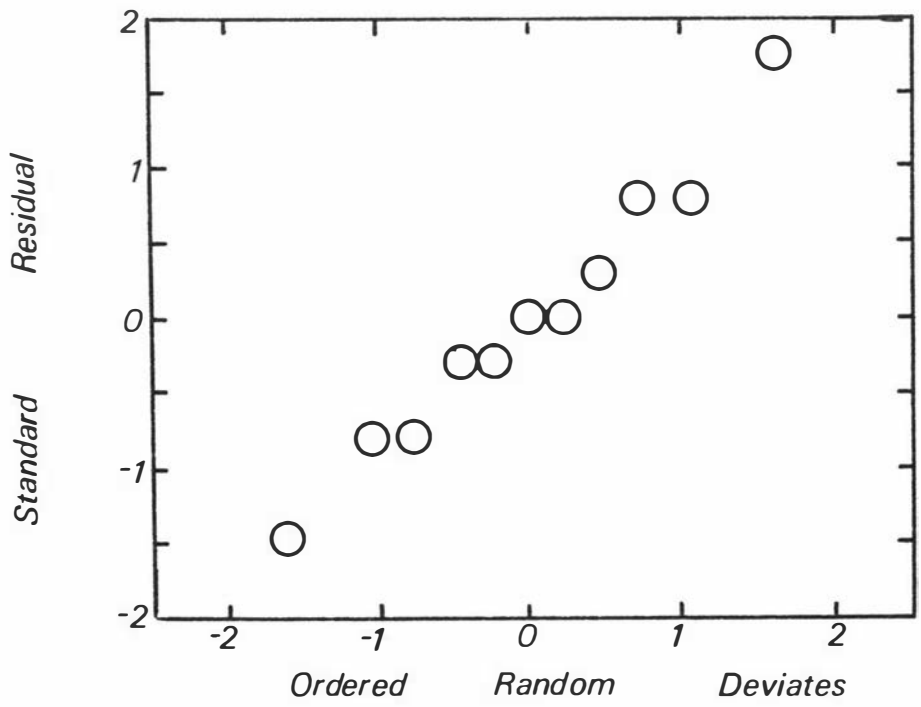


FIGURE A2.2 Residual Plot Number 3 for the Parsimonious Model for L.M.C. (i.e. log of maximum observed cell count)

APPENDIX 3 Parsimonious Models and Regression Statistics for Experiment 2

TABLE A3.1 Parsimonious Model and Regression Statistics for 7-Ketodeoxycholate Yield

Parsimonious model:

$$\hat{Y} = 23.9 + 2.8C + 4.0D + 2.2E - 2.4F + 2.2G - 4.0(AB+CG+DH+EF) + 4.9(AE+CH+BF+DG)$$

Coefficient of Determination:

$r^2 = 94.7\%$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	7	1254.09	179.16
Residual	8	70.27	8.78
Total	15	1324.36	

Note: It should be remembered that the main effects A,B,C *etc.* and first order interaction contrasts (AB+CG+DH+EF), (AC+BG+EH+DF) *etc.* are confounded with second order and higher interactions. This applies to all models presented in Appendix 3.

TABLE A3.2 Parsimonious Model and Regression Statistics for the Natural Logarithm of 7-Ketodeoxycholate Yield

Parsimonious model:

$$\hat{Y} = 3.09 + 0.14C + 0.16D - 0.19(AB+CG+DH+EF) + 0.20(AG+BC+FH+DE)$$

Coefficient of Determination:

$r^2 = 73.7\%$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	4	1.9450	0.4862
Residual	11	0.6935	0.0630
Total	15	2.6385	

Test for Lack of Fit:

If the experiment is regarded as a fully replicated 2^{4-1} (I=CDGH) with significant main effects for C and D and significant interactions for CG confounded with DH and for CH confounded with DG, then the sum of squares due to pure error can be calculated from the replicates:

$SS_{pe} = 0.5373$

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	8	0.5373	0.0672
Lack of Fit	3	0.1560	0.0520
Residual	11	0.6935	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{0.0520}{0.0672} = 0.774$$

As $F_{3,8,0.05} = 4.07$, the lack of fit is not significant at the 5% level.

TABLE A3.3 Parsimonious Model and Regression Statistics for the Cholic Acid Remaining at 48 Hours

Parsimonious model:

$$\hat{Y} = 64.7 - 7.7B - 3.7D + 3.4E - 3.4G + 4.5(AB+CG+DH+EF) - 7.5(AE+CH+BF+DG) + 4.5(AF+GH+CD+BE)$$

Coefficient of Determination:

$r^2 = 92.3\%$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	7	3088.5	441.2
Residual	8	258.7	32.3
Total	15	3347.2	

TABLE A3.4 Parsimonious Model and Regression Statistics for Deoxycholate Yield

Parsimonious model:

$$\hat{Y} = 11.4 + 6.2B - 5.6E - 3.1H - 4.3(AF+GH+CD+BE)$$

Coefficient of Determination:

$r^2 = 82.7\%$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	4	1565.5	391.4
Residual	11	327.2	29.7
Total	15	1892.8	

Test for Lack of Fit:

If the experiment is regarded as a fully replicated 2^3 (I=BEH), with significant main effects for B, E and H and significant interaction BE then the sum of squares due to pure error can be calculated from the replicates: $SS_{pe} = 206.8$

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	8	206.8	25.85
Lack of Fit	3	120.4	40.13
Residual	11	327.2	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{40.13}{25.85} = 1.55$$

As $F_{3,8,0.05} = 4.07$, the lack of fit is not significant at the 5% level.

TABLE A3.5 Parsimonious Model and Regression Statistics for the Base 10 Logarithm of Maximum Observed Cell Counts (L.M.C.)

Parsimonious model:

$\hat{Y} = 8.53 - 0.26B$

Coefficient of Determination:

$r^2 = 76.1\%$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	1	1.0816	1.0816
Residual	14	0.3388	.0242
Total	15	1.4204	

TABLE A3.6 Parsimonious Model and Regression Statistics for the Maximum Rate of Electrode Potential Decline $\left(\frac{\Delta E}{\Delta t}\right)_{max}$

Parsimonious model:

$$\hat{Y} = -88.6 + 21.7F - 16.4H + 20.9(AE+CH+BF+DG)$$

Coefficient of Determination:

$$r^2 = 64.8\%$$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	3	18863	6288
Residual	12	10265	855
Total	15	29128	

Test for Lack of Fit:

If the experiment is regarded as a fully replicated 2^{4-1} (I=BCFH), then SS_{pe} = 3103.25 with 8 degrees of freedom.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	4	7161.8	1790.4
Lack of Fit	8	3103.2	387.9
Residual	12	10265	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{1790.4}{387.9} = 4.64$$

As $F_{4,8,0.05} = 3.84$, the lack of fit is significant at the 5% level.

TABLE A3.7 Parsimonious Model and Regression Statistics for Minimum Electrode Potential

Parsimonious model:

$$\hat{Y} = -499 + 46B + 26D + 25G + 20(AE+CH+BF+DG)$$

Coefficient of Determination:

$$r^2 = 80.7\%$$

Analysis of Variance Summary:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	4	61650	15412
Residual	11	14725	1339
Total	15	76375	

Test for Lack of Fit:

If the experiment is regarded as a fully replicated 2^3 (I=BDG), then SS_{pe} = 8900 with 8 degrees of freedom.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	8	8900	1112
Lack of Fit	3	5825	1941
Residual	11	14725	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{1941}{1112} = 1.75$$

As $F_{3,8,0.05} = 2.07$, the lack of fit is not significant at the 5% level.

TABLE A3.8 Parsimonious Model and Regression Statistics for Initial Electrode Potential (Ec_i)

Parsimonious model:

$$\hat{Y} = -285 + 18B + 27D - 28F$$

Coefficient of Determination:

$$r^2 = 76.8\%$$

Analysis of Variance:

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Regression	3	29667	9889
Residual	12	8956	746
Total	15	38623	

Test for Lack of Fit:

If the experiment is regarded as a 2^3 (I = BDF), then $SS_{pe} = 7450$ with 8 degrees of freedom.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Pure Error	8	7450	931.25
Lack of Fit	4	1506	376.5
Residual	12	8956	

$$\rightarrow \frac{MS_{lof}}{MS_{pe}} = \frac{376.5}{931.2} = 0.40$$

As $F_{4,8,0.05} = 3.84$, the lack of fit is not significant at the 5% level.

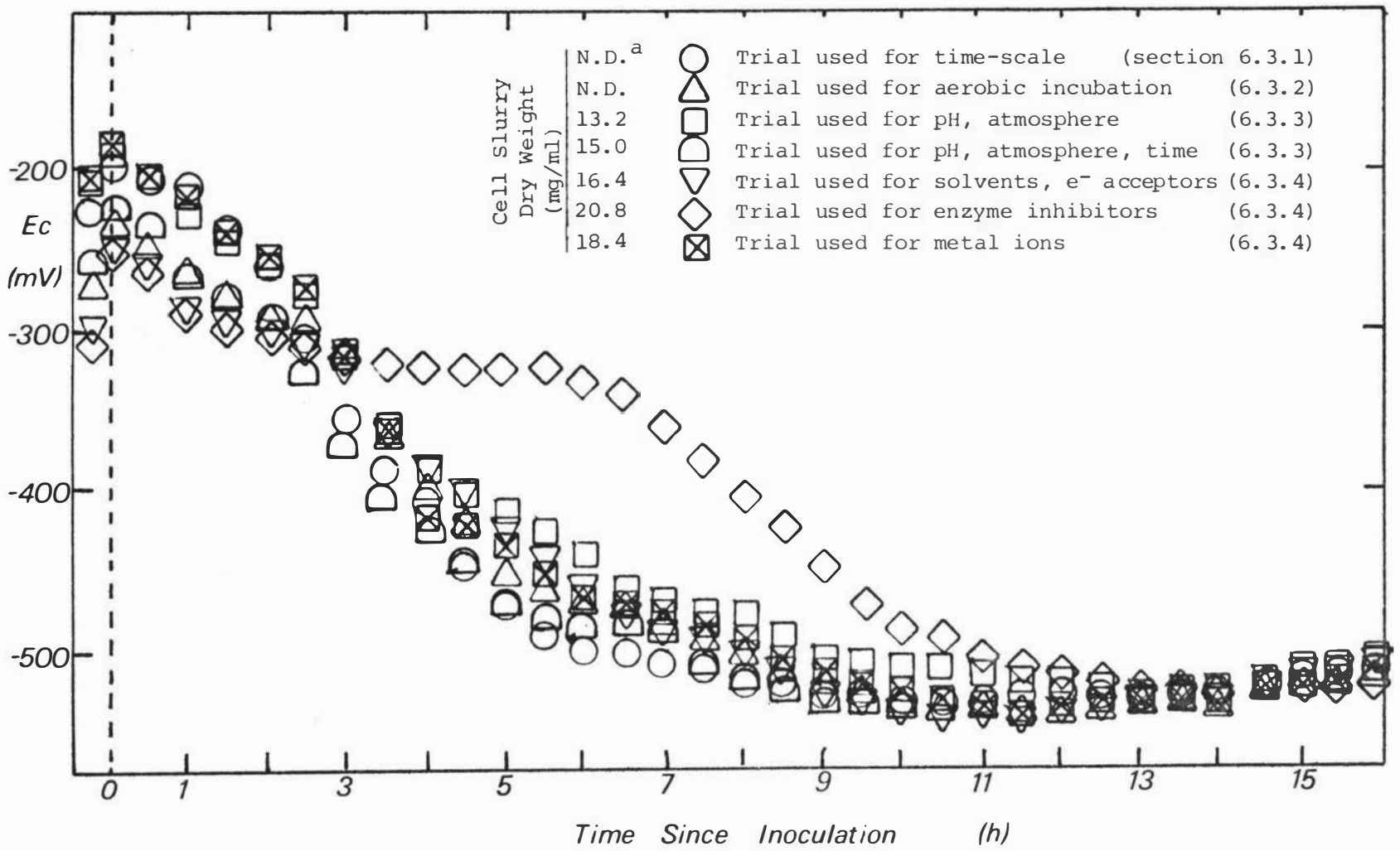


FIGURE A4.1 Electrode Potential Curves and Dry Weights of Cell Suspensions for Washed Cell Production Runs

(a) N.D. - not determined. Fermentation conditions are described in the text (section 6.2)

APPENDIX 5 Raw Data for Deconjugation Experiments Conducted on the Small Scale

TABLE A5.1 Extent of Transformation of Conjugate over 48 h

Conjugate		Product			Total
b	c	7KD	C	D	
GC	88	0	0	0	88 [a]
GC	4	12	72	1	89
GC	8	11	70	1	90
GD	90			0	90 [a]
GD	3			90	93
GD	4			94	98
TC	98	0	0	0	98 [a]
TC	2	14	71	3	90
TC	3	12	70	4	89
TD	95			0	95 [a]
TD	2			94	96
TD	2			95	97

All values represent the level of bile acid determined after 48 h, and are expressed as molar % of the bile acid originally added.

The "Total" column is effectively the mass balance figure. Each row represents one 20 ml-scale trial.

a - control (not inoculated). Other values are duplicates.

b - abbreviations are those listed at the beginning of this thesis.

c - this column lists the levels of conjugate remaining unhydrolysed at 48 h.

TABLE A5.2 Effect of Substrate Concentration on Transformation of Conjugates After 48 h

Level % w/v	Conjugate b	c	Product 7KD	C	D	Total	pH [d]	Growth [e]
0.80	GC	44	4	44	0	92	6.01	(5)
0.40	GC	32	4	60	0	96	5.84	(4)
0.20	GC	15	4	76	0	96	6.00	(4)
0.10	GC	11	7	71	1	90	5.96	(3)
0.05	GC	6	11	75	0	92	6.06	(4)
0.025	GC	2	11	84	5	102	6.07	(5)
0.80	GD	17			74	91	6.64	(5) [f]
0.40	GD	8			85	93	6.63	(5) [f]
0.20	GD	4			81	85	6.63	(3) [f]
0.10	GD	2			71	73	6.68	(2)
0.05	GD	3			85	88	6.48	(1)
0.025	GD	3			89	92	6.18	(1)
0.80	TC	73	2	15	0	90	6.14	(6)
0.40	TC	60	4	28	0	91	6.11	(5)
0.20	TC	42	5	46	0	92	6.08	(4)
0.10	TC	7	6	70	1	84	6.09	(4)
0.05	TC	2	9	73	2	87	6.11	(4)
0.025	TC	3	15	72	2	92	6.17	(5)
0.80	TD	95			8	103	6.12	(4)
0.40	TD	91			11	102	6.12	(4)
0.20	TD	86			18	104	6.04	(5)
0.10	TD	38			59	97	6.08	(5)
0.05	TD	2			91	93	6.19	(4)
0.025	TD	5			87	93	6.29	(4)

Legend is as for Table A5.1

d - Final pH (Initial pH *ca* 7.7)

e - Growth is measured on an arbitrari 1 to 6 scale of visible turbidity.

f - Reaction mixture was partially gelatinised, inflating the apparent turbidity.