Lipid metabolism of Saccharomyces cerevisiae and control of volatile ester synthesis in wort fermentations.

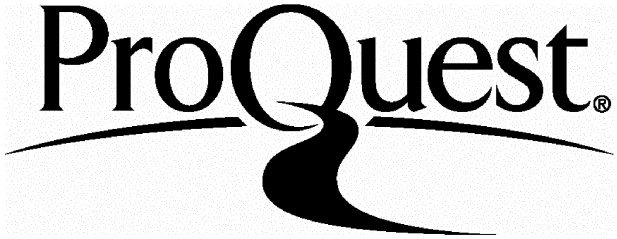
Thurston, Patrick Andrew

Award date:
1982

Awarding institution:
University of Bath

LIPID METABOLISM OF *SACCHAROMYCES CEREVISIAE* AND CONTROL OF VOLATILE ESTER SYNTHESIS IN WORT FERMENTATIONS

Submitted by
PATRICK ANDREW THURSTON
For the degree of Ph.D.
at the University of Bath
1982



SUMMARY

Summary

When added to wort, long chain fatty acids were incorporated into the lipids of Sacch. cerevisiae during fermentation. Linoleic or oleic, but not stearic acid significantly suppressed the formation, by yeast, of ethyl and iso-amyl acetates. Synthesis of these acetate esters was also suppressed when inocula enriched with linoleyl residues were used. Further effects of addition of linoleic acid were: inhibition of unsaturated but stimulation of saturated fatty acyl residues; a shift from synthesis of medium towards long chain acyl residues; enhancement of the conversion of squalene to lanosterol.

Syntheses of lipids (sterols; unsaturated and saturated fatty acyl residues) were most active during the initial two hours of fermentation when oxygen was present. Following this phase the specific rate of ethyl acetate synthesis increased to a constant level. Saturated fatty acyl residues and squalene continued to be formed but only until the mid-point of fermentation. At this point a further significant increase in the specific rate of ethyl acetate synthesis was observed. Increased rates of ester synthesis were not sustained but contributed significantly to the overall production of esters.

The ratio of acetyl-CoA to CoASH (the acetyl-charge)

increased and declined in parallel with specific rates of ester synthesis. It appears that specific rates of lipid syntheses determined the acetyl-charge of the cell. It is proposed that when lipid syntheses cease, a condition of 'acetyl-pressure' (i.e. a high acetyl-charge) develops and the cell responds by excreting acetate units (acetate esters). Studies using cerulenin, an inhibitor of fatty acid synthesis, support this view.

The suppression of ester synthesis by linoleic acid was found to be independent of intracellular availability of acetyl-CoA and seems likely to be a consequence of altered membrane lipid composition.

The results of these investigations suggest possible new procedures for control of ester synthesis in brewery fermentations.

CONTENTS

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

The work for this thesis was carried out at the Brewing Research Foundation, Nutfield, Surrey, between December 1978 and October 1981. I should like to thank, Professor Anthony Rose and Dr. Brian Kirsop for enabling me to register for a higher degree at the University of Bath, and Dr. Roy Tubb for his guidance and advice, especially during 'writing up'. Many friends and colleagues at the Foundation have also helped me over the last few years; notably Dr. David Quain. Mostly, I should like to thank my wife, Barbara, who not only typed this thesis but was always there when I needed her.

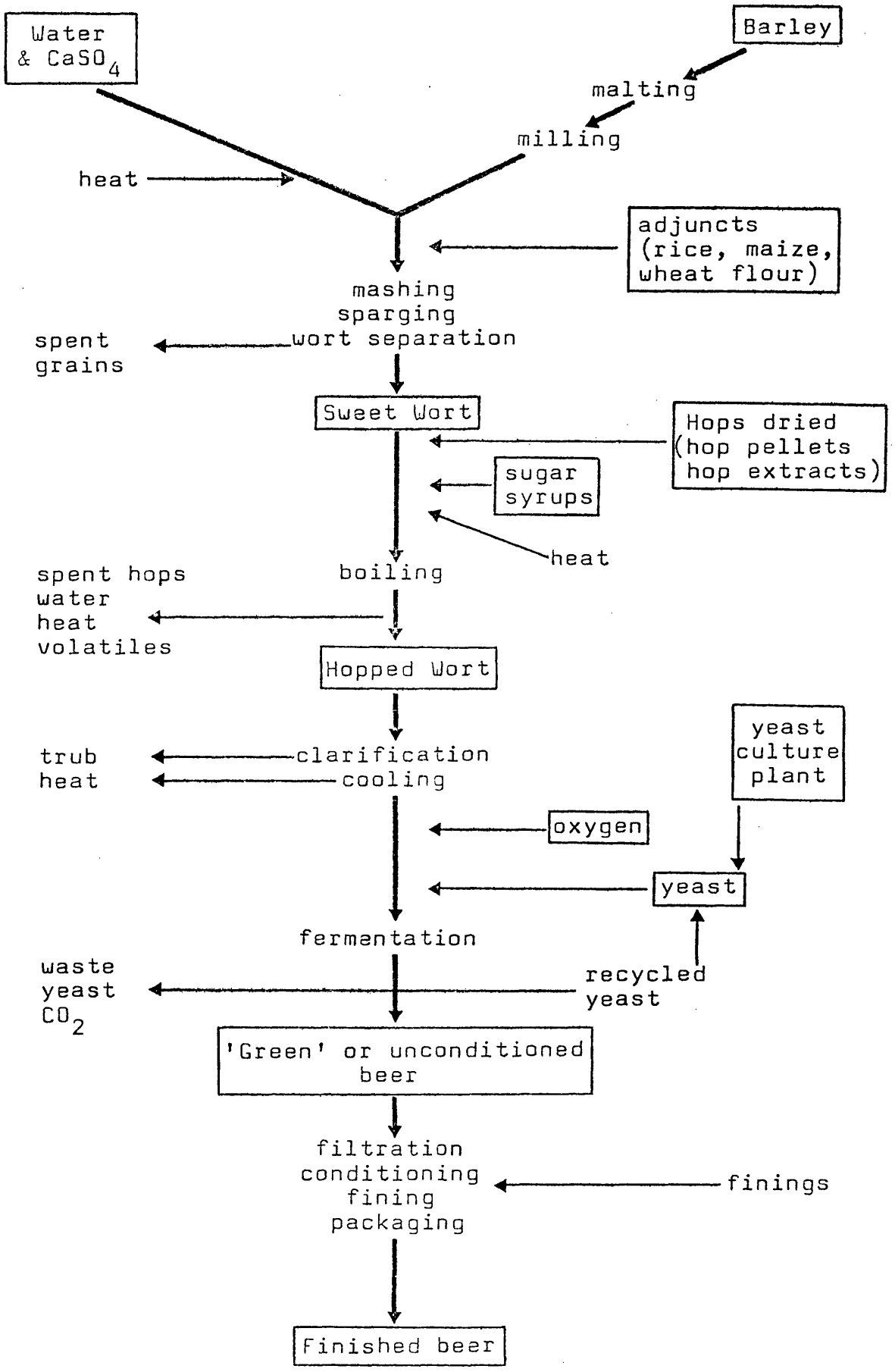
INTRODUCTION

1. Lipids and the Brewing Process

1.1 The Brewing Process

Beer has been brewed for thousands of years. In 1980 ca. 40 million barrels were brewed in the UK (Anon, 1981). The mechanics of brewing are remarkably simple, yet the biochemical processes vital to the production of beer are infinitely complex. There have always been several 'variations on a theme' as far as the production of beer is concerned, but there are certain fundamental operations that are always carried out. These are illustrated in Fig.1, (for reviews see MacLeod, 1977; Pollock, 1981). The basic raw material for brewing is barley (Hordeum vulgare - various varieties). Barley is ultimately a source of amino acids and fermentable sugars upon which yeast can grow and produce ethanol, but because barley is a seed, these components are in polymeric storage form. They are rendered soluble by the process of malting. In this process, barley is induced to germinate by steeping the grains in water at ca. 16^{o}C. In response to water and oxygen the embryo produces the hormone gibberellic acid which induces the synthesis of certain hydrolytic enzymes; endo-β-glucanase, α-amylase (β-amylase is present in the ungerminated grain) and peptidases. Endo-β-glucanase, and possibly other enzymes (Bamforth, 1981) break down the walls of the endosperm cells. These cells contain starch granules embedded in a protein matrix of hordein. Subsequently the amylases

Fig.1 The Brewing Process



and peptidases start to degrade starch and hordein respectively. The grains are normally steeped for about two days, during which their moisture content rises to approximately 45%; subsequently they are drained and 'cast' onto the malting floor where germination proceeds for up to a further five days. The complete solubilization of storage compounds is not required during malting since it continues at later stages in the brewing process. Malting involves a limited amount of seedling growth and is normally followed by kiln-drying. Kilning dries the grain (now malt) with hot air at 65^{o}-85^{o}C and reduces the moisture content to less than 3%. The seedling is killed as are any microorganisms on the grain surface, and the heat also promotes the formation of melanoidins — aromatic compounds which impart flavour and colour to beer. The malt must be kiln-dried to allow contamination-free storage. However, one consequence is that a certain proportion (between 4% and 30% depending on enzyme and kilning schedule) of the hydrolytic enzymes are inactivated. Following kilning the malt is milled and together with hot water is transfered to the mash-tun. The water generally has the bicarbonate ions removed from it by lime-treatment or ion-exchange resins and must possess or have artificially added, calcium ions which through a series of ionic interactions give rise to a pH of about 5·4 in the mash-tun. This is the best compromise between the pH optima of several hydrolytic enzymes, and thus yields maximal fermentable extract

from the malt. The object of mashing is to allow the malt enzymes to convert starch into dextrins and fermentable sugars, and proteins to amino acids and polypeptides. Mashing also solubilizes various other components, e.g. tannins, inorganic salts and vitamins. The amylolytic activity of most malts is more than sufficient to degrade the malt starch and thus can convert additional starch in the form of unmalted cereal adjuncts, e.g. maize, rice and wheat-flour which themselves contain no α-amylase. Adjuncts are often used because they are generally a cheaper source of carbohydrate than malt. There are two basic systems of mashing: (a) infusion mashing, where there is a uniform temperature (ca. 65°C) and a low ratio of water : grist. This gives rise to a thick mash with the grist floating on the surface. Soluble sugars, amino acids and vitamins diffuse out of the grist causing the specific gravity of the liquid to rise. After one to two hours the first-runnings of sweet-wort are drawn off through slots in the bottom of the mash-tun and the grains are washed or sparged with treated water at ca. 70°C, to remove residual soluble materials. (b) decoction mashing, where the ratio of water : grist is higher, the initial mash temperature lower (ca. 35°C) and the overall mash-time longer than for infusion-mashing. Portions of the mash are removed, heated to a higher temperature, then replaced, gradually increasing the temperature of the whole mash until it reaches as high as 75°C. When mashing is completed the mash is pumped

to a separate vessel, the lauter-tun, where sweet-wort is filtered off and the grains sparged. Mashing is central to the brewing process and changes in mashing conditions can have marked effects on beer properties (Hudson, 1973).

Sweet-wort is passed into the brew kettle and is boiled together with dried hops (Humulus lupulus) or hop-pellets, and any additional sugar syrups. Wort-boiling which lasts 1-2 hours serves to sterilize the wort, coagulate high molecular weight nitrogenous material and to extract and convert hop resins which impart bitterness and flavour to beer. Most important is the conversion of α-acids (e.g. humulone) to iso-α-acids which are responsible for the bitterness of beer. Important flavour compounds from hops are humulene and myrcene. Following wort-boiling, hops are separated from the wort in a hop-strainer and the remaining coagulum or trub removed in a whirlpool tank or by filtration (see MacLeod, 1977). The wort is then cooled by heat exchange, diluted to the appropriate specific gravity and aerated prior to addition to the fermentation tank. Wort is a complex growth medium for yeast (MacLeod, 1977). A typical profile of the carbohydrates would be: 1% glucose; 4% maltose; 1% maltotriose; 0·2% maltotetraose; 2% higher dextrins; plus traces of sucrose and fructose. The use of sugar syrups can of course alter this profile.

Two types of yeast (Sacch. cerevisiae) are used in brewing. Top-fermenting yeasts are generally used for the production of ales whereas those used in lager brewing generally tend to settle at the bottom of the fermentation vessel. The former taxonomic distinction between the two types of yeast was that lager yeasts (formerly classified as Sacch. uvarum) produce melibiase and are therefore able to fully ferment the trisaccharide raffinose. Ale-yeasts are only able to ferment one third of this sugar molecule. In the traditional batch brewery system yeast collected from a previous fermentation is added to wort at the rate of one pound per barrel (barrel = 36 gallons). Fermentation temperatures for ales and lagers are typically 15°C and 8°C respectively. Uptake of amino acids (Jones & Pierce, 1964) and sugars follows a sequential pattern and as the yeast converts sugars to ethanol and carbon dioxide, largely via the glycolytic pathway, the specific gravity of the fermenting wort falls; a process known as attenuation. In addition to the malt-derived nutrients, yeast requires one other, without which it will not ferment adequately. This is oxygen, and is required for the synthesis of essential lipids without which the cells cannot divide (Section 3). Yeast also synthesizes many other compounds during fermentation which although present in small amounts are important with respect to the flavour of beer (Section 2). At the end of primary fermentation partial separation of yeast from beer is brought about by

flocculation whereby cells aggregate together. After removal of the majority of the yeast, the beer is conditioned. This is essentially a storage-phase during which certain volatile fermentation products such as acetaldehyde and hydrogen sulphide (H_2S) are eliminated, the beer is carbonated and the yeast cells and particles are separated to yield a bright beer. Originally, and today for some traditional beers (so called 'real ales') this process is allowed to occur naturally at low temperatures. Now many beer are conditioned for short periods of time at elevated temperatures to volatilize H_2S and acetaldehyde. The beers are then cooled to precipitate any chill-haze material (polyphenols and polypeptides) and then artificially carbonated. Beers that have to be stored for some length of time have to be treated to render them free from bacterial contamination. This is accomplished by pasteurization (see Portno, 1968). Draught-beer is usually only flash-pasteurized (20 seconds at $70^{o}C$) whilst other beers are subjected to tunnel-pasteurization whereby the containers are sprayed with hot water for up to an hour. Although pasteurization stabilizes the beer it can damage its palate.

1.2 High-Gravity Brewing

In North America, for some time it has been an accepted practice to brew and ferment worts of relatively high specific gravity, then to dilute the beers to a specific gravity suitable for sale (Schaus, 1971; Anon,

1979). However, so called high-gravity brewing only takes place on a limited scale in the UK (Anon, 1979). Advantages of high-gravity brewing include: improved colloidal stability of beer leading to longer shelf-life (Whitear & Crabb, 1977; Whitworth, 1978); improved smoothness of taste and general flavour stability (Pfisterer & Stewart, 1975); higher adjunct levels can be used without loss of fermentability (Pfisterer & Stewart, 1975); reduced energy and labour costs (Hackstaff, 1977); and more efficient use of existing plant (Hackstaff, 1977; Whitworth, 1978; 1980). The last advantage listed above is where high-gravity brewing really comes into its own. For example, if demand (for beer) suddenly increased above the nominal capacity of the brewery, it could be met by brewing the same volume of beer of a higher gravity followed by dilution prior to packaging. Disadvantages include poorer extract yield and poorer hop-utilization. In addition, a certain amount of expenditure would be required for the change of process (Whitworth, 1980), though this is far less than would be incurred on new plant. One major capital cost of changing to high-gravity brewing is that involved in preparation of the dilution water. The water should be: sterile, bright, at beer pH, chilled (to ca. $2^{o}C$) and carbonated to the level of the recipient beer. It should also be, free from: oxygen (0·1p.p.m.), chlorine (0·02 p.p.m.) and significant amounts of heavy metals such as copper and iron. The pH can be adjusted with concentrated acid

(Whitear & Crabb, 1977) and techniques for sterilizing the water include UV irradiation, ultrafiltration and boiling. The methods for deaeration include: CO_2 washing of chilled water; boiling followed by carbonation during cooling; vacuum deaeration. For a discussion of methods of deaeration see Wilson (1977).

The solubility of oxygen in wort declines linearly as the specific gravity is increased (Baker & Morton, 1977) and therefore extra measures must be taken to ensure that adequate oxygen is added to the high-gravity fermentation. Often oxygen, rather than air, is used for this purpose, or the wort is aerated during the early stages of fermentation. Air can be added during active fermentation if shallow (ca. 2 metres deep) fermenters are used, where the dangers of gushing and consequent beer losses are much reduced (Whitworth, 1980). Alternatively oxygen-saturated water can be added (Rennie & Wilson, 1975).

If high-gravity brewing is to be used to achieve expanded output of a particular product, it is essential that the new process does not change the flavour of the final product. However, problems are often encountered with excessive formation of ethyl acetate and iso-amyl acetate resulting in beers with an unacceptably high 'fruity' aroma. These problems can be overcome to some extent if gravities above 1·060 are avoided (Whitworth,

1978), but above this gravity flavour-matching becomes increasingly difficult. The synthesis of esters in normal and high-gravity fermentations is discussed in Sections 2 and 3. Obviously if a new product is to be marketed the above problems do not apply, and high-gravity brewing is a more realistic proposition.

1.3 Fate of Lipids During the Brewing Process

Essentially all of the lipid material in wort is derived from the cereal grist (Forch & Runkel, 1974). Barley contains 2-3% lipid by weight (MacLeod & White, 1961; Banasik & Gilles, 1966; Holmberg & Sellmann-Persson, 1967; Hernandez et al., 1967; Blum, 1969; Morrison, 1978) although some varieties contain higher amounts (Price & Parsons, 1974; Parsons & Price, 1974; Bhatty & Rossnagel, 1980). The relative proportions of the lipid classes in barley are: triacylglycerols, 40-70% of the total lipids; phospholipids 9-20%; mono- and diacylglycerols 5-13%; glycolipids 10%; sterols plus steryl esters 10%; free fatty acids 3-7% (Holmberg & Sellmann-Persson, 1967; Blum, 1969; Price & Parsons, 1980; Bhatty & Rossnagel, 1980). The predominant fatty acid of the lipids is linoleic acid which represents 50% of the total fatty acids. Other acids present in the lipids are: palmitic (11-30%); oleic (8-28%); linolenic (4-6%) and stearic (1-4%) with traces of lauric, myristic, myristoleic, palmitoleic and arachidic (references as above). The glycolipids consist of mono- and digalactosyldiacylglycerols

(Zurcher, 1971) and sterylglycosides (Harwood, 1980). Predominant sterols of barley are the common plant sterols: β-sitosterol, stigmasterol and campesterol. During the germination of all seeds, a rapid mobilization of storage compounds takes place. During malting the total lipid content of the grain decreases between 25% and 40% (MacLeod & White, 1961; Hernandez et al., 1967) and the total fatty acid content by 50% (Krauss et al., 1972). The loss of lipid during malting has been attributed to a concomitant 3-fold increase in lipase activity (Hernandez et al., 1967) 68% of which is located in the embryo, the remainder in the aleurone layer (MacLeod & White, 1962). Phospholipase activity has also been detected in germinating barley (Acker & Geyer, 1968). No information is available on sterol metabolism during malting. Figures of 1-3% have been reported for the proportion of malt lipids that survive into sweet wort (Äyräpää et al., 1961; Krauss et al., 1972), though mashing conditions and lautering procedure are important in determining the precise levels. Klopper et al. (1975) showed that high concentrations of free fatty acids are present in the mash-tun. These levels are drastically reduced in the first-runnings, indicating that spent grains filter out the fatty acids. However, sparging washes out fatty acids and other lipids such as triacylglycerols and phospholipids from the grains (Jones et al., 1975). The collected sweet-wort contains 15-40mg free fatty acids per litre, linoleic acid contributing ca. 40% of this figure

(Klopper et al., 1975; Jones et al., 1975).

The lipid composition of spent grains has been analysed in some detail (Taylor et al., 1979). Greater than 30% of the total fatty acids were in free form. This is greatly in excess of the value for green-malt (Holmberg & Sellmann-Persson, 1967) and suggests either that lipolytic enzymes are active during mashing or that chemical hydrolysis of the complex lipids takes place. Phospholipase-B which is active in malting is not deactivated by kilning and its activity increases during mashing resulting in the release of fatty acids from phospholipids (Acker & Geyer, 1968). There are no reports of a similar hydrolysis of triacylglycerols during mashing.

A large proportion of the lipid material of sweet-wort is often eliminated by the boiling process in the form of coagulum or trub, which has been shown to be rich in lipids (Äyräpää et al., 1961; Klopper et al., 1975; Jones et al., 1975). Removal of trub in the whirlpool separator leaves the levels of free fatty acids in the wort at between 1·5 and 16·0mg l^{-1} depending on the original concentration in sweet-wort (Klopper et al., 1975; Jones et al., 1975). Hops contribute only a small amount of lipid to wort (Hildebrand et al., 1975).

After wort-cooling a 'cold-break' forms which can be separated by flotation. Removal of lipid (up to 50%)

by this means effectively reduces the high concentrations of fatty acids which result from certain sparging procedures (Klopper et al., 1975). A system which reclaims spent-grain liquor has been described (Coors & Jangaard, 1975). The liquor, which was lipid-rich was recycled back to the mash-tun but had no effect on fermentation or on beer quality. This demonstrates how effective the wort-boiling and cooling processes are at removing lipids from wort. The lipids that eventually find their way into wort are principally free fatty acids, mono-, di- and triacylglycerols and traces of phospholipid. There is little information on the fate of sterols in brewing. Forch (1977) quotes values of 0·1-0·34mg sterol (1 wort)$^{-1}$. Like the other lipids, sterols are removed with trub and in subsequent wort processing. Äyräpää et al. (1961) give the following values in mg l^{-1}

	sweet-wort	trub	beer
Steryl esters	0·1-0·2	0-0·2	0-0·1
Free sterols	0·2-0·4	0-0·1	0·01-0·02

Yeast is effective at removing certain lipids during fermentation and is able to incorporate fatty acids and sterols derived from spent grains (Taylor et al., 1979). Thus levels of lipids in beer are usually very low. Much of the research concerning the fate of lipids in brewing has been motivated by the possible detrimental effects they have on beer properties. For

example, low concentrations of mono- and diacylglycerols, mono- and digalactosyldiacylglycerols (0·2mg l^{-1}), and long chain unsaturated fatty acids, affect foam formation (Carrington et al., 1972; Krauss et al., 1972). These lipids will also repress gushing, and the use of an extract of spent grains as an anti-foaming agent in fermenters has been proposed (Roberts, 1976). There is general agreement in the literature (Krauss et al., 1972; Carrington et al., 1972; Klopper et al., 1975; Roberts, 1977; Roberts et al., 1978) that lipids normally encountered in beer have no detrimental effect on foam-stability. The main danger from lipids comes when they enter beer on or just prior to dispense, e.g. fats present on glassware, and oils in the compressed gases used to dispense beer (Roberts et al., 1978).

Volatile aldehydes formed during shelf-storage of packaged beer are a significant source of stale off-flavours (for a review see Hashimoto, 1981). Principle substances associated with a 'cardboard' off-flavour are trans-2-nonenal and 2-methyl furfural (Jamieson et al., 1969; Jamieson & Van Gheluwe, 1970; Drost et al., 1971). Drost et al. (1971) postulate that a tri-hydroxy C18 fatty acid is a precursor of stale flavours. Such a compound could be formed from an unsaturated fatty acid either by auto-oxidation, or by the action of lipoxygenases and isomerases (Galliard & Chan, 1980) during malting, but not during mashing since lipoxy-genase is readily destroyed by kilning (Lulai & Baker,

1975). However beer staling is rarely retarded by reducing the level of a suspected unsaturated fatty acid in beer (Hashimoto, 1981). In addition to the above effects, the presence of lipids in wort is known to affect the production of flavour compounds by yeast (Section 3).

2. Beer Flavour

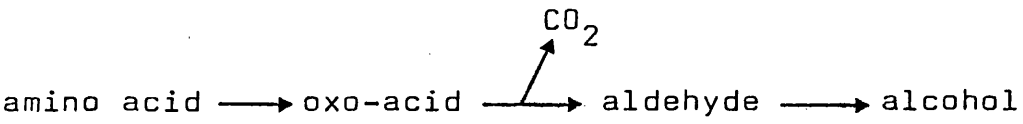
2.1 Measurement and Description of Beer Flavour

Assessment of beer flavour is subjective and in the brewing industry is generally carried out by panels of trained tasters (Clapperton, 1973, 1974, 1978; Clapperton & Piggot, 1979 a,b). The tasters use a recognized system of flavour terminology (Clapperton et al., 1976; Meilgaard et al., 1979) which comprises over one hundred descriptive terms. The system aims to provide terminology which allows all relevant personnel to communicate effectively about flavour, and to describe and define each separate flavour 'note' more precisely. Examples of terms used are, 'burnt' and 'toffee-like' for ales, and 'cabbagey', 'vegatable' and 'dimethylsulphide' (DMS) for lagers (Clapperton & Piggot, 1979a). This type of assessment can be supported by detailed chemical analysis. Many flavour notes can be attributed to specific compounds, for example: 'cardboard' (trans-2-nonenal); 'buttery' or 'butterscotch' (diacetyl); 'pear-drop' or 'banana' (iso-amyl acetate). Flavour compounds are often assigned threshold-values (Harrison, 1970), i.e. the concentration at which they can just be detected (tasted) in beer. However, precise values are of limited meaning since firstly, individuals differ in their ability to sense particular compounds and secondly, a taste-threshold depends on antagonistic and synergistic interactions with other flavour compounds.

For example, if several esters and alcohols are present in a beer at concentrations below their threshold values, collectively they can impart a fruity flavour to that beer (Engan, 1972). The formation of some important flavour compounds is discussed below, and that of others (i.e. diacetyl and DMS) can be found in Wainwright (1973) and Anness & Bamforth (1982).

2.2 Higher Alcohols

Amyl alcohols (2- and 3-methyl butanol), iso-butanol, n-propanol and 2-phenylethanol are present in beer at concentrations of ca. 60,15,15,25 mg l^{-1} respectively (Harrison, 1970). These alcohols, which impart a fragrant aroma, are formed from amino acids in the following manner (Äyräpää, 1971; Inoue, 1975):



Higher alcohol formation often parallels amino-acid utilization e.g. if leucine is added to a fermentation large amounts of 3-methyl butanol (iso-amyl alcohol) are formed (Engan, 1970). The formation of higher alcohols is likely to be important in maintaining the overall redox balance of the cell, just as glycerol formation is (Oura, 1977b).

2.3 Fatty Acids

Medium chain length fatty acids i.e. hexanoic (C_6),

octanoic (C_8), decanoic (C_{10}) and dodecanoic (C_{12}) have significant effects on beer flavour.

These acids are synthesized by yeast during fermentation (Taylor & Kirsop, 1977b) and impart a 'caprylic' or 'soapy-fatty' flavour (Clapperton & Brown, 1978). Such flavour notes can be detected in the majority of lager beers and ca. 20% of ales. The concentrations of C_8, C_{10} and C_{12} in beer are above the flavour threshold-values. The effects of these three fatty acids on beer flavour are additive (Clapperton, 1978). The concentration in beer of hexanoic acid, which has a sharper, more rancid odour than the other three acids (Clapperton & Brown, 1978), is generally lower than the flavour threshold (Clapperton, 1978).

2.4 Esters

With regard to flavour terminology, esters have been placed in the general group of 'aromatic' 'fragrant' 'fruity' 'floral' compounds and in a sub-group termed 'estery' (Clapperton et al., 1976; Meilgaard et al., 1979). Particular flavours associated with certain esters are: 'banana' or 'pear-drop' - iso-amyl acetate; 'apple-like' - ethyl hexanoate; 'light-fruity' 'solvent-like' - ethyl acetate. These three esters together with ethyl octanoate are the most significant with respect to beer flavour. The flavour-thresholds for ethyl acetate, iso-amyl acetate, ethyl hexanoate and ethyl octanoate are, 30,2,0·3 and 1 mg l^{-1} respectively (Harrison, 1970) and the actual levels found in beer

are 9-23, 0·9-3·7, 0·02-0·2 and 0·15-0·9mg l^{-1} respectively (Harrison, 1970; Äyräpää & Lindstrom, 1973; Norstedt et al., 1975; Engan & Aubert, 1977; White & Portno, 1979; Piendl & Geiger, 1980). Esters are formed by yeast during fermentation and not by direct esterification between acids and alcohols (Nordstrom, 1964a; 1965). For example, the non-enzymic reaction between acetic acid and ethanol is 1000-fold too slow to account for the amounts of ethyl acetate formed during fermentation. Furthermore, the amount of iso-amyl acetate formed is ca. 100-fold higher than the equilibrium value of the non-enzymic reaction (Nordstrom, 1964a; 1965).

Genera of yeast other than Saccharomyces form aromatic esters. Species of Pichia and Hansenula grown aerobically on glucose or ethanol produce ethyl acetate in large amounts. Hansenula anomala produces 25m moles ethyl acetate per 100 m moles glucose (Davies et al., 1951). Apparently the aerobic utilization of ethanol gives rise to the ethyl acetate (Peel, 1951; Tabachnick & Joslyn, 1953a,b). Further, once the supply of ethanol is exhausted, ethyl acetate is degraded (Peel, 1951). The optimum pH for ethyl acetate formation is 2·1-2·6 (Tabachnick & Joslyn, 1953a,b), oxygen must be present (Peel, 1951) and zinc ions have been found to be stimulatory (Miwa & Ueyama, 1961). Only traces of ethyl acetate are formed by H. anomala at pH 6·7 but addition of the esterase inhibitor diisopropylfluoro-

phosphate leads to high amounts of ethyl acetate. Therefore at pH 6·7, ethyl acetate is hydrolysed as it is formed. Ethyl acetate formation in these aerobic yeasts appears to be an energy-requiring process (Cantarelli, 1955) and it also requires pantothenate. On this basis Cantarelli (1955) proposed that acetate was activated to acetyl-CoA before becoming the acyl donor in the formation of ethyl acetate. In contrast to Hansenula and Pichia sp., Saccharomyces sp. form esters anaerobically. However, in agreement with Cantarelli (1955), Nordstrom (1962) found that pantothenate stimulated the synthesis of ethyl acetate by brewing strains of Sacch. cerevisiae implying that the acid moiety is a CoA-ester. Addition of short chain fatty acids e.g. butyrate, to the growth medium reduced the formation of ethyl acetate but lead to formation of ethyl butyrate. The inhibitory effect of butyrate on ethyl acetate could be overcome by provision of excess pantothenate, again implying the participation of coenzyme A in the reactions of ester synthesis. Whereas addition of short chain acids to the medium resulted in formation of their corresponding ethyl esters, acetic acid did not stimulate formation of ethyl acetate (Nordstom, 1961). From this observation Nordstrom concluded that acetyl-CoA is "not formed exclusively or to any great extent by activation of acetic acid but rather that it is formed in other processes" e.g. by the oxidative decarboxylation of pyruvate (Nordstrom, 1962; 1963; 1964b).

The role of acetyl-CoA in ethyl acetate synthesis was proved by Anderson and Howard (1976a,b) who reported that cell-free preparations of Sacch. cerevisiae form ethyl acetate from acetyl-CoA and ethanol but not from acetic acid and ethanol. Activity is stimulated by magnesium ions and is primarily located in the membraneous material sedimenting at 105,000G. In contrast with these reports Schermers et al. (1976) describe an esterase preparation from yeast which is capable of synthesizing ethyl acetate from ethanol and acetic acid. The same preparation could hydrolyse beer esters, the equilibrium between hydrolysis and synthesis being pH-dependent. Recently, the partial purification of alcohol acetyltransferase from brewer's yeast has been reported (Yoshioka & Hashimoto, 1981). The enzyme is membrane-located and catalyses the formation of iso-amyl acetate and ethyl acetate from the corresponding alcohols and acetyl-CoA. The enzyme is more active against iso-amyl alcohol than ethanol. However, in wort fermentations more (ca. 10-fold) ethyl acetate is formed than iso-amyl acetate because the ratio of ethanol to iso-amyl alcohol is so high (ca. 300:1). The formation of esters such as ethyl octanoate and ethyl hexanoate is presumably limited by the intracellular availability of the corresponding acyl-CoA compounds. Fatty actyl residues are released from the fatty acid synthetase complex as CoA esters (3·2·1) and can then be hydrolysed and excreted, or, esterified to alcohols. The figures for medium chain acids and their ethyl

esters quoted above, indicate that generally, more are excreted as free acids than as esters. The majority of evidence supports the view that acetyl-CoA is required for the formation of acetate esters. Thus, ester formation will be sensitive to the changes in physiology of yeast during batch fermentation, especially to changes in lipid metabolism since the syntheses of sterols and fatty acids require acetyl-CoA.

3. Lipid Metabolism of Sacch. cerevisiae

3.1 The Lipids of Sacch. cerevisiae

The lipids of yeasts (for extensive reviews see Hunter & Rose, 1971; Rattray et al., 1975; Kaneko et al., 1976) include the following: free fatty acids; glycerophospholipids; sphingophospholipids; sphingolipids (containing no phosphorus); acylglycerols; squalene; sterols and steryl esters. Strains of Saccharomyces sp. generally fall into a group of yeasts with a 'medium' lipid content (5-15% of the cellular dry weight) although both the total lipids present and the composition of these lipids can be greatly altered by the growth conditions (Hunter & Rose, 1971; Rattray et al., 1975). Quoted values (% of cellular dry weight) for the amount of lipid present in strains of Sacch. cerevisiae are: 7·2% (Kaneko et al., 1976); 12-14% depending on growth temperature (Hunter & Rose, 1972); 12-14% depending on supply of pantothenate in the growth medium (Hosono & Aida, 1974); and 8-15% (Hunter & Rose, 1971). In contrast some species of Candida and Rhodotorula can accumulate more than 30% of their dry weight as lipid; when grown under conditions of excess carbon and deficit of nitrogen, lipid accumulates to form up to 70% of the biomass (Ratledge, 1978).

3.2 Fatty acids and fatty acyl containing lipids

The majority of the fatty acyl residues in yeast are present in complex lipids e.g. acylglycerols, glycero-

phospholipids and steryl esters; only traces of free fatty acids are found in yeast. Rattray et al. (1975) reported that a total of 33 acids ranging from C_8 to C_{26} have been detected in Sacch. cerevisiae, although acids of 20 carbon atoms or more are in the minority (1-2%). Aerobically-grown yeasts contain large amounts of unsaturated fatty acids, e.g. palmitoleic and to a greater extent oleic acid.

Glycerophospholipids consist of glycerol-3-phosphate with each of the hydroxyl groups esterified to a fatty acid and the phosphate group esterified to a small polar molecule such as an amino alcohol. In yeasts glycerophospholipids can account for between 3% and 7% of the dry weight. Sacch. cerevisiae contains the following proportions of glycerophospholipids: phosphatidylcholine (PC) 40% (of total lipid phosphorus); phosphatidylethanolamine (PE) 22%; phosphatidylinositol (PI) 20% and phosphatidylserine (PS) 4% (Letters, 1968). Lysoglycerophospholipids i.e. the hydroxyl group at carbon atom 2 of glycerol is unesterified, have been detected in anaerobically grown Saccharomyces sp. (Letters, 1968; Brown & Johnson, 1971; Hunter & Rose, 1971).

Acylglycerols simply consist of a glycerol moiety to which 1,2 or 3 fatty acids are esterified. Mono-, di- and triacylglycerols have been detected in Sacch. cerevisiae (Barron & Hanahan, 1961; Kates & Baxter,

1962). Acylglycerols generally account for a similar proportion of the dry weight of yeast as phospholipids do. The triacylglycerol content of Sacch. cerevisiae increases in stationary-phase (Castelli et al., 1969) and during sporulation (Illingworth et al., 1973; Henry & Halvorson, 1973) and also when the cells are starved of phospate (Johnson et al., 1973) or inositol (Shafai & Lewin, 1968; Paltauf & Johnson, 1970). The large amounts of lipid that are accumulated by certain oleaginous yeast are largely in the form of triacylglycerols (Whitworth & Ratledge, 1974).

Other fatty acyl-containing lipids found in Sacch. cerevisiae are sphingolipids, cerebrosides, acyl glucoses, monogalactosyldiacylglycerol and sulpholipids (Brennan et al., 1970; Hunter & Rose, 1971; Steiner & Lester, 1972a; Smith & Lester, 1974; Tyorinoja et al., 1974; Becker & Lester, 1980).

3.2.1 Fatty acid biosynthesis in Sacch. cerevisiae

The biosynthesis of saturated fatty acids in yeast has been reviewed (Lynen, 1968; Schweizer et al., 1978; Lynen, 1980; for general reviews of fatty acid biosynthesis see Volpe & Vagelos, 1973; Bloch & Vance, 1977). Acetyl-coenzyme A (acetyl-CoA) is the ultimate source of all the carbon atoms of saturated fatty acids though of the eight acetyl units required for the formation of palmitic acid, only one is derived from acetyl-CoA

directly, the others coming from malonyl-CoA. Malonyl-CoA is formed in a reaction catalysed by the biotin-containing enzyme, acetyl-CoA carboxylase (Mishina et al., 1980). The overall reaction for the synthesis of palmitic acid, catalysed by fatty acid synthetase is:

$$\text{acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ \text{NADPH} + 14\ H^+ \longrightarrow$$
$$\text{palmityl-CoA} + 8\ \text{CoASH} + 14\ NADP^+ + 7\ CO_2 + 7\ H_2O$$

Acetyl-CoA carboxylase and fatty acid synthetase are located in the cytosol (Schweizer et al., 1978) and therefore acetyl-CoA must be made available at this intracellular location. Acetyl-CoA can be formed, in the cytosol of Sacch. cerevisiae, from acetate, CoASH and ATP, by the action of microsomal acetyl-CoA synthetase (Klein & Jahnke, 1968; 1971; 1979). Acetate may be formed by aldehyde dehydrogenase (Bradbury & Jakoby, 1971). Furthermore, intramitochondrial acetyl-CoA (formed by pyruvate dehydrogenase) can be transported to the cytosol by the action of carnitine acetyltransferase (Kohlhaw & Tan-Wilson, 1977). In this reaction acetylcarnitine is formed and transported across the mitochondrial membranes to the cytosol, where in reaction with CoASH, carnitine and acetyl-CoA are generated. Many yeasts generate cytosolic acetyl-CoA from intramitochondrial citrate; a reaction catalysed by ATP: citrase lyase (Boulton & Ratledge, 1981). However, Sacch. cerevisiae does not possess this enzyme (Kohlhaw & Tan-Wilson, 1977; Boulton & Ratledge, 1981).

Yeast fatty acid synthetase consists of multiple copies of two polypeptides (α and β) each with several enzymic activities. The two polypeptides or sub-units have been purified (Stoops & Wakil, 1978) and the overall molecular structure of the fatty acid synthetase complex is $\alpha 6/\beta 6$ (Schweizer et al., 1978), with a molecular weight of $2{\cdot}3 \times 10^6$ (Lynen, 1980). In Sacch. cerevisiae, long chain fatty acids are released from the fatty acid synthetase complex in the form of CoA thioesters (usually palmityl-CoA) and it is in this form that they undergo the reactions of elongation and unsaturation and will finally be used in the construction of complex lipids.

The elongation system of Sacch. cerevisiae can reversibly add one to three C-2 units to medium chain-length saturated or unsaturated fatty acids. It is malonyl-CoA independent i.e. acetyl-CoA serves as the donor of the C-2 units (Schweizer et al., 1978). The cellular location of the elongation system is not known for Sacch. cerevisiae.

The fatty acyl desaturase of Sacch. cerevisiae is a mixed-function oxygenase which introduces a double-bond between carbon atoms 9 and 10, into a variety of long-chain saturated acyl-CoA thioesters (Bloomfield & Bloch, 1960). The enzyme is located in the microsomal fraction and certain factors are required from the cytosol: NADPH, molecular oxygen and a protein factor

(Gurr & James, 1981).

Regulation of fatty acid synthesis is mediated in several ways. Formation of acetyl-CoA by acetyl-CoA synthetase (ACS) is inhibited by long chain acyl-CoA compounds (Satyanarayana & Klein, 1973) and by NADH, NADPH and AMP (Coleman & Bhattacharjee, 1976). Acetyl-CoA carboxylase in Sacch. cerevisiae is activated by citrate, isocitrate, α-glycerophosphate and Mg^{2+} ions (Rasmussen & Klein, 1968) and is inhibited by long chain acyl-CoA compounds (White & Klein, 1966). Addition of long chain fatty acids to the growth medium reduces the cellular content of acetyl-CoA carboxylase in Sacch. cerevisiae (Kamiryo & Numa, 1973) grown aerobically on sucrose. Like acetyl-CoA carboxylase, fatty acid synthetase is also inhibited by long chain acyl-CoA compounds (Lust & Lynen, 1968).

3.2.2 The formation of complex lipids in Sacch. cerevisiae

The biosynthesis of acylglycerols and glycerophospholipids in yeast has been reviewed (Hunter & Rose, 1971; Rattray et al., 1975). The first committed step is the synthesis of phosphatidic acid, whereby sn-glycerol-3-phosphate or dihydroxyacetone phosphate are acylated by acyl-CoA thioesters (Morikawa & Yamashita, 1978; Schlossman & Bell, 1978). The acyltransferases that catalyse the formation of phosphatidic acid are strongly inhibited by free fatty acids.

Triacylglycerols are synthesized from phophatidic acid by the action of phosphatidate phophorylase (yielding diacylglycerol) and diacylglycerol acyltransferase (Rattray et al., 1975). These reactions take place in the lipid particles of Sacch. cerevisiae (Christiansen, 1978, 1979).

All of the major glycerophospholipids in yeast (PS,PI, PG,PE,PC and cardiolipin) can be formed via cytidine diphophate diacylglycerol (CDP diacylglycerol), which itself is formed from phosphatidic acid (Hutchinson & Cronan, 1968). Reaction of serine, inositol or glycerol-3-phosphate with CDP diacylglycerol yields PS,PI and PG respectively; PE is the decarboxylation product of PS and can be sequentially methylated with S-adenosylmethionine to form PC. Furthermore, PC and PE can also be formed in reactions between diacyl-glycerol and CDP-choline or CDP-ethanolamine (Weiss & Kennedy, 1956; Steiner & Lester, 1972b; Waechter & Lester, 1973; Cobon et al., 1974). In some yeasts glycerophospholipids may undergo further modification such as exchange of headgroups or desaturation of fatty acyl chains (Rattray et al., 1975).

The complex lipids discussed above either have a structural or a storage function. The structural role consists of being an integral component of cellular membranes; the amphipathic lipids (glycerophospholipids and possibly sphingophospholipids) are ideally suited

to this role. Different types of lipid are not evenly distributed between the different cell membranes (Hunter & Rose, 1971). For example, cardiolipin is only synthesized and located in the mitochondrion or pro-mitochondrion (Paltauf & Schatz, 1969; Jakovic et al., 1971; Getz, 1972; Cobon et al., 1974). Non-structural lipids e.g. triacylglycerols (and also steryl-esters - see below) are probably located in low density vesicles (Hossack et al., 1973; Clausen et al., 1974; Cartledge et al., 1977; Hossack et al., 1977). The vesicles are rich in hydrolytic enzymes (Cartledge et al., 1977) and triacylglycerol synthesizing enzymes (Christiansen, 1978). Triacylglycerols probably serve as a source of fatty acyl moieties for glycerophospholipid synthesis in Sacch. cerevisiae (Taylor & Parks, 1979; Daum & Paltauf, 1980).

3.3 Biosynthesis of Sterols, Steryl esters and Squalene in Sacch. cerevisiae

The characteristic sterols of Sacch. cerevisiae are; ergosterol, 24(28)dehydroergosterol and zymosterol, though many other sterols have been detected in this organism (for reviews see Weete, 1973; Parks, 1978). Sterols can account for between 0·1 and 4% of the dry weight of yeast (Aries et al., 1977; Quain & Haslam, 1979a) and may be in free form or esterified to either fatty acids (Madyastha & Parks, 1969; Aries & Kirsop, 1978) or sugars (Adams & Parks, 1967).

All but one of the 28 carbon atoms of ergosterol are derived from acetyl-CoA. The pathway of sterol formation from mevalonate to lanosterol is not branched but there are a number of pathways in Sacch. cerevisiae by which lanosterol is transformed into ergosterol (Fryberg et al., 1972). Reactions leading to the synthesis of squalene from acetyl-CoA are shown in Fig.2.a. The formation of acetyl-CoA has beendiscussed earlier. Acetoacetyl-CoA thiolase and β-hydroxymethylglutaryl-CoA synthetase catalyse the first two reactions shown in Fig.2.a; both of these enzymes have been detected in the cytosol and mitochondria of Sacch. cerevisiae (Middleton & Apps, 1969; Kornblatt & Rudney, 1971; Shimizu et al., 1973; Trocha & Sprinson, 1976). The first committed step to the formation of sterols is the synthesis of mevalonate which is catalysed by β-hydroxymethylglutaryl-CoA reductase. This enzyme is located in promitochondria and mitochondria (Shimizu et al., 1973; Boll et al., 1975). Steps between mevalonate and squalene were elucidated by; Amdur et al. (1957), Rilling (1966), Rilling et al. (1971), Schechter & Bloch (1971), Nishino et al. (1978). The conversion of squalene to lanosterol requires molecular oxygen (Tchen & Bloch, 1957) and an epoxide is formed as an intermediate in this conversion (Barton et al., 1968). The two-step reaction is catalysed by squalene epoxidase and lanosterol cyclase. Little is known regarding the epoxidase of yeast though the cyclase is reported to be soluble, in contrast to that of the rat liver, and is

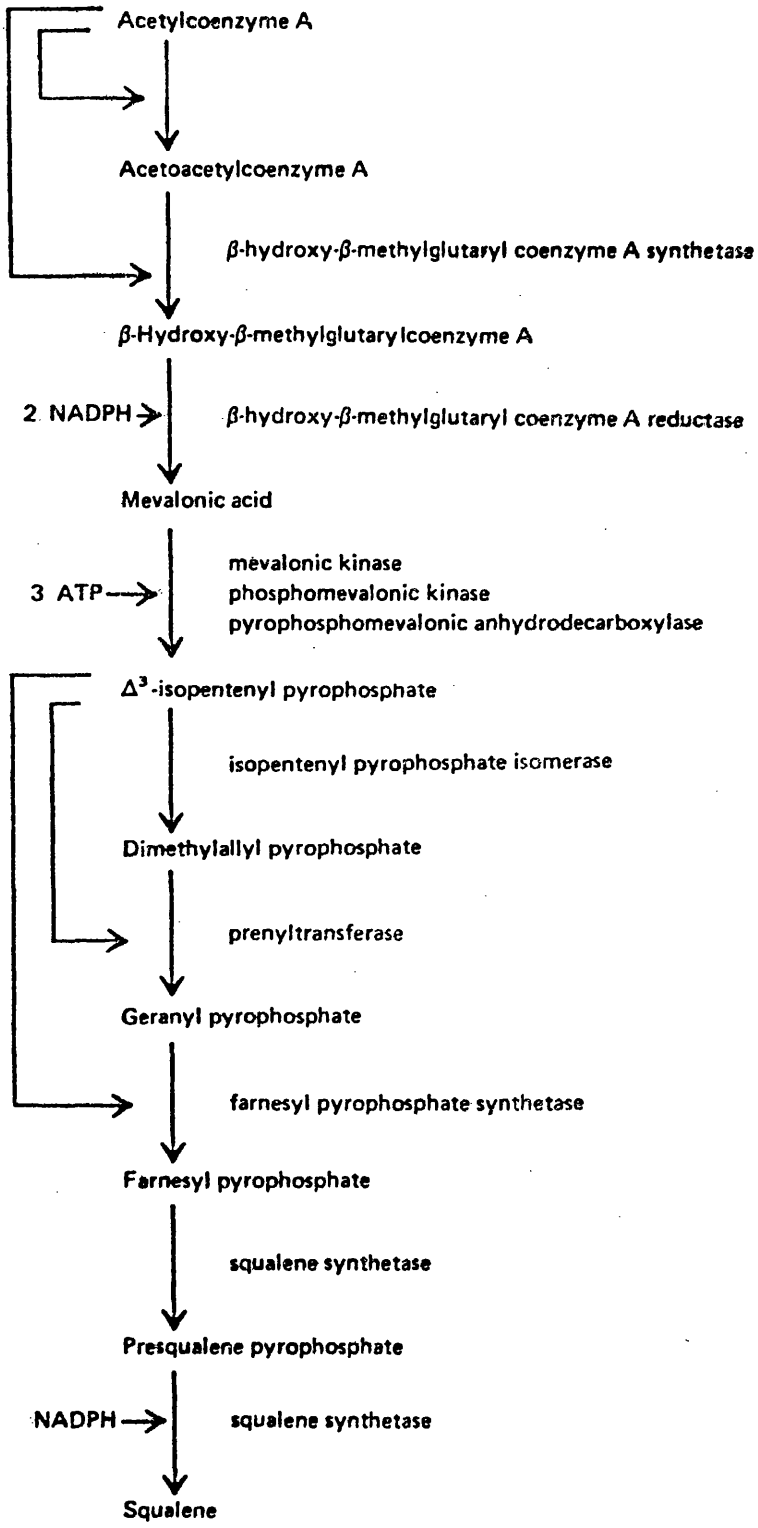


Figure 2.a

Conversion of acetyl-coenzyme A to squalene

stimulated by Triton X-100 (Yamamoto et al., 1969; Schechter et al., 1970).

The main pathway for the conversion of lanosterol to ergosterol as quoted by Aries & Kirsop (1978), is shown in Fig.2.b. Like the epoxidation of squalene, the sequential removal of the three methyl groups (one at C-14 and two at C-4) from lanosterol requires molecular oxygen and NADPH, (Aoyama & Yoshida, 1978; Parks, 1978). This series of reactions results in formation of zymosterol, the overall reaction for which is:

$$\text{lanosterol} + 12\ \text{NADPH} + 12\ H^+ + 2\ \text{NAD}^+ + 9\ O_2 \longrightarrow$$
$$\text{zymosterol} + 12\ \text{NADP}^+ + 2\ CO_2 + 2\text{NADH} + 2\ H^+$$

Reaction 4, (Fig.2.b) is catalysed by the mitochondrial enzyme S-AM: Δ^{24}-sterol methyltransferase (Thompson et al., 1974) and accounts for the only carbon atom not derived from acetyl-CoA. This carbon atom comes from S-adenosyl methionine (Parks, 1958). Reactions 7, and 8, require molecular oxygen (Aries & Kirsop, 1978; Osumi et al., 1979) and result in the formation of 24(28)-dehydroergosterol. Reduction of the methylene group at C-24 yields ergosterol.

The principal site of regulation of the sterol biosynthetic pathway in Sacch. cerevisiae is HMG-CoA reductase (Kawaguchi, 1970; Berndt et al., 1973; Boll et al., 1975; Quain & Haslam, 1979a; Bard & Downing,

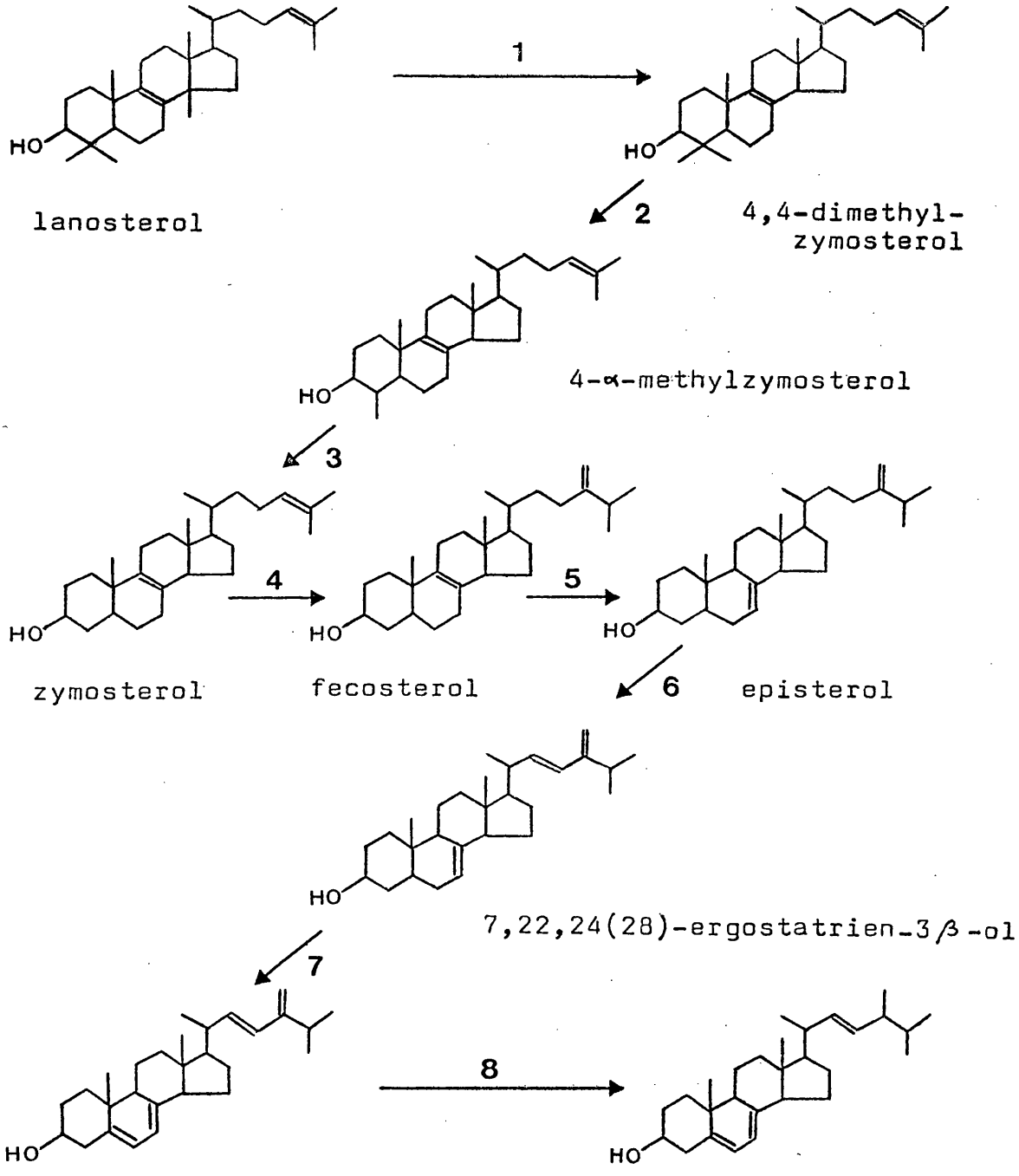


Figure 2.b

Biosynthesis of ergosterol from lanosterol in Sacch. cerevisiae

For details of reactions 1-8, see text.

1981). This enzyme is inhibited by coenzyme A (Tan-Wilson & Kohlhaw, 1978; Gilbert & Stewart, 1981). The specific activity of HMG-CoA reductase is low in anaerobic yeast but aeration leads to a six-fold increase in specific activity, with a concomitant increase in the total sterol content of the cells (Berndt et al., 1973; Boll et al., 1975). Further, catabolic derepression leads to increased sterol synthesis and higher specific activity of HMG-CoA reductase (Quain & Haslam, 1979a). It is interesting to note that the activities of acetoacetyl-CoA thiolase and HMG-CoA synthetase in anaerobically-grown yeast are reduced by the addition of ergosterol (Parks, 1978). These enzymes are not unique to the synthesis of sterols and therefore it might be expected that they would not be inhibited by ergosterol. Certain of the reactions of the sterol biosynthetic pathway are facilitated by cytosolic, non-enzymic proteins, termed sterol carrier proteins (Gaylor, 1974; Dempsey, 1974). These proteins are well characterized in mammalian systems and have also been reported in yeast (Dempsey & Meyer, 1977). It appears that sterol carrier proteins act within membranes and facilitate access of substrate to specific membrane-located enzyme sites (Saat & Bloch, 1976; Gavey & Scallen, 1978; Friedlander et al., 1980). This raises the possibility that a certain level of sterol carrier protein synthesis will be required for optimal rates of sterol synthesis; further, these steps may be influenced by the physical properties of the microsomal and

mitochondrial membranes.

The formation of sterols requires much energy, molecular oxygen and NADPH. For example, the formation of one mole of episterol from acetyl-CoA requires: 18 moles ATP; 26 NADPH and 10 O_2. Generation of NADPH is required by lipid syntheses at several intracellular locations: in the microsomes for squalene epoxidation, lanosterol demethylation and fatty acid desaturation; in the cytosol for fatty acid biosynthesis; in the promitochondria and mitochondria for synthesis of mevalonate. In the yeast cytosol NADPH can be formed via the hexose monophosphate shunt and via cytoplasmic NADP-linked isocitrate and acetaldehyde dehydrogenases (Polakis & Bartley, 1965). In yeast mitochondria, NADPH can be formed via transhydrogenation of NADH (Ernster & Lee, 1964), phosphorylation of NADH (Griffiths & Bernofsky, 1972) or by a mitochondrial glucose-6-phosphate dehydrogenase (Campbell & Bernofsky, 1979).

High oxygen tensions favour sterol synthesis and in such instances over 90% of the total sterol is in esterified form (Bailey & Parks, 1975). The esterified sterol is thought to be located in low density vesicles along with other lipid storage compounds as discussed above. Free sterols have a structural role in yeast membranes. Under anaerobic growth conditions the ratio

of free to esterified sterol increases (Hossack et al., 1977; Aries & Kirsop, 1978) and squalene accumulates to account for 1-2% of the cellular biomass (Jollow et al., 1968; Nurminen et al., 1975; Aries et al., 1977). The intracellular location of squalene is unknown in Sacch. cerevisiae. However it is interesting to note that squalene is located in the membrane fraction of Clostridium pasteurianium (Mercer et al., 1979) and more specifically, in the hydrophobic region of the membrane of certain halophilic bacteria (Lanyi et al., 1974). It remains an open question as to whether squalene can play a significant structural role in membranes of anaerobically grown Sacch. cerevisiae where the sterol concentration is low.

3.4 Role of Lipids in Yeast Membranes

The membranes of eukaryotic cells generally contain ca. 40% (w/w) lipid and 60% protein (Bretscher, 1973). Values quoted by Boulton (1965), Longley et al. (1968) and Suomalainen & Nurminen (1970) for yeast are not too dissimilar from this value though methods of isolation of membranes and analysis of lipids do generate some degree of variation between the reported values.

The hydrophobic nature of the lipid component makes all cells intrinsically impermeable to most polar or charged molecules. Therefore leakage of internal metabolites is prevented. However many charged molecules are required by the cell as nutrients. Entry of these

compounds is effected by the protein component of the cell-membrane. The most constant and well defined structural element of biological membranes is the lipid bilayer, which is composed of amphipathic lipids, e.g. glycerophospholipids. In a bilayer, the hydrophobic hydrocarbon chains of the fatty acyl residues associate with each other and the polar head groups interact with the aqueous phases of either the cytosol or the environment. The proteins of the membrane are either embedded in the fluid lipid bilayer or are loosely attached to the surface and thus there can be a high degree of asymmetry to membrane structure (Singer & Nicolson, 1972; Singer, 1974; Cherry, 1979; Op den Kamp, 1979). Lipids may also be distributed asymmetrically across the membrane (Bretscher, 1973; Bretscher & Raff, 1975; Bell et al., 1981; Van Deenan, 1981) and phospholipid exchange proteins may be required to achieve this asymmetry (Rothman & Kennedy, 1977). The fluidity of any given bilayer is dependent on temperature; generally an increase in temperature increases fluidity. The fluidity at any given temperature is dependent upon the nature of the fatty acyl moieties of the phospholipids; unsaturated lipids are more fluid than saturated lipids (Bretscher, 1973). In membranes of Sacch. cerevisiae sterols are important structural components. Sterols interact with the phospholipids and can influence the fluidity of the membrane (Van Deenan, 1972; Demel et al., 1972; Demel & DeKruyff, 1976; Lees et al., 1979). Generally sterols have a stabilizing effect on membranes;

they can liquefy membranes at low temperatures and rigidify them at higher temperatures (Demel & DeKruyff, 1976). It is thought that sterols react with a specific part of the hydrocarbon chains of phospholipids but do not affect the motion of the polar head-groups. The precise nature of the interaction is not known but is likely to involve hydrogen bonds between the sterol nucleus and water molecules bound to the phospholipids (Demel & DeKruyff, 1976).

Sterols and unsaturated fatty acids cannot be synthesized in the absence of oxygen. However, Sacch. cerevisiae will grow anaerobically if sterol and unsaturated fatty acids are provided in the growth medium (Andreasen & Stier, 1953;1954). It would appear that a variety of sterols can support the anaerobic growth of Sacch. cerevisiae (Proudlock et al., 1968; Hossack & Rose, 1976) though Nes et al. (1976; 1978) report a marked dependence on precise structural features of the sterol molecule as determined by growth-response and morphology. The C-3 hydroxyl group is obligatory for growth and the other groups are of the following relative importance: 24β-methyl- Δ^{22} grouping $>$ 24β-methyl group $>$ $\Delta^{5,7}$-diene system = Δ^{5}-bond $\doteq$ no double bond. However the nature of the unsaturated fatty acid supplied in the growth medium may determine whether or not a particular sterol supports anaerobic growth (Buttke et al., 1980). The sterol content of Sacch. cerevisiae can affect the activity of certain

promitochondrial enzymes (Cobon & Haslam, 1973) and depletion of sterols can lead to loss of coupled oxidative phophorylation due to increased proton entry into the mitochondria (Astin & Haslam, 1977).

A wide variety of unsaturated fatty acids can support anaerobic growth of Sacch. cerevisiae (Light et al., 1962) or that of unsaturated fatty acid auxotrophs of Sacch. cerevisiae (Wisnieski et al., 1970). Different acids support growth and respiration in the unsaturated fatty acid auxotroph KD115 (Resnick & Mortimer, 1966) with different efficiencies (Walenga & Lands, 1975). Sacch. cerevisiae also has a requirement for saturated fatty acids (Schweizer & Bolling, 1970; Henry, 1973). The level of unsaturated fatty acids in the mitochondrial lipids determines the capacity of the cells to carry out oxidative phosphorylation (Linnane & Crowfoot, 1975). The ability of KD115 to grow on non-fermentable substrates is lost below a value of 20% unsaturation (Haslam et al., 1971). Unsaturated fatty acids are not required for electron transport but are required if electron transport is to be coupled to oxidative phosphorylation. The nature, as well as the relative content, of unsaturated fatty acids influences oxidative phosphorylation and respiratury control (Walenga & Lands, 1975). Further, the activities of certain mitochondrial enzymes are also influenced by the level of unsaturated fatty acid (Janki et al., 1974; Watson et al., 1975). The effect of depletion of unsaturated

fatty acids on oxidative phosphorylation may well be a permeability effect similar to that caused by sterol depletion (Astin & Haslam, 1977) and similar to that on active transport across the plasma membrane (Haslam & Al Mahdawi, 1980).

3.5 Lipid Metabolism in Brewery Fermentations

3.5.1 The role of oxygen in wort fermentation

Yeast will not grow anaerobically in wort for more than several generations (David & Kirsop, 1972; 1973a,b). Since brewery fermentations are potentially anaerobic, a supply of oxygen is essential to ensure satisfactory fermentation (Kirsop, 1978). The requirement for oxygen reflects the need by yeast for sterols and unsaturated fatty acids (David & Kirsop, 1972; 1973a,b), the syntheses of which require molecular oxygen. Both of these lipids are components of the yeast plasma membrane and are essential for growth. The amount of sterols in yeast is of more importance than unsaturated fatty acids since worts may contain certain amounts of the latter compounds (David, 1974). Generally oxygen is supplied to a fermentation by aeration of wort prior to pitching (inoculation). The early phase of fermentation is the only stage at which significant contact between yeast and oxygen occurs; traces of oxygen may also be available during handling and storage of the yeast (see Quain & Tubb, 1982). Alternatively the majority of the required oxygen could be made available to yeast during the handling and storage procedures. Oxygen could be supplied to yeast slurries prior to pitching as, oxygen-saturated

water (Rennie & Wilson, 1975; Wilson, 1979) or by direct aeration of a yeast slurry with compressed air (Ahvenainen & Makinen, 1981). Aeration of yeast slurries prior to storage is reported to enhance viability (Hayduck *et al.*, 1910; Kirsop, 1974).

In high-gravity fermentations often pure oxygen rather than air is used to increase the concentration of dissolved oxygen in the wort since greater yeast growth is required to ferment the extra sugar (Searle & Kirsop, 1979).

Yeast strains differ in the magnitude of their oxygen requirement; some strains require as little as 2p.p.m. dissolved oxygen, others in excess of 30p.p.m. to fully attenuate 1·040 wort (Kirsop, 1974). Air-saturated wort contains *ca.* 8p.p.m. oxygen (Baker & Morton, 1977). There is probably a continuous range of oxygen requirement amongst yeast strains (Jakobsen & Thorne, 1980). Whilst adequate oxygen must be provided to satisfy the requirement of a particular yeast strain, too much oxygen may lead to more growth than is necessary to ferment the wort. To the brewer such extra yeast growth represents loss of fermentable sugar and also increases the amount of beer that is lost within the waste yeast. Therefore wort aeration or oxygenation requires careful control. In view of the above discussion it is perhaps surprising that Aries *et al.* (1977) and Kirsop (1978) report that only 5-15% of the oxygen, added to wort, is

used for the synthesis of sterols.

3.5.2 Lipid Composition of Yeast During Fermentation

Yeast harvested from the end of a wort fermentation can contain as little as 1μg sterol (mg dry weight)$^{-1}$. When inoculated into air-saturated wort the sterol content of the yeast increases rapidly to ca. 10μg (mg dry weight)$^{-1}$ (Aries & Kirsop, 1977). Oxygen is removed from the wort generally within the first few hours (Day et al., 1976; Aries & Kirsop, 1977) after which no de novo synthesis of sterols takes place. As cell-division proceeds, the concentration of sterol in the cells declines to a value of 1μg (mg dry weight)$^{-1}$ which is considered to be the growth-limiting value. Once this value is reached further cell-division does not occur and Haukeli & Lie (1975; 1976) report that when a value of 0·2-0·3μg sterol (mg dry weight)$^{-1}$ is reached, the absorption of wort nutrients and consequently the efficiency of growth are reduced. Obviously if the supply of oxygen to wort is severely limited due to technical problems in the brewery, then premature lipid-depletion may occur and the reduced uptake of amino acids and sugars could lead to inadequate attenuation.

The total sterol content of yeast grown in wort does not rise above 1% of the dry weight even in the presence of of excess oxygen (Aries & Kirsop, 1977; Aries et al., 1977). This is almost certainly because

sterol synthesis is subject to catabolite repression; sterols can account for up to 4% of the dry weight when Sacch. cerevisiae is grown on ethanol (1%) (Quain & Haslam, 1979a).

Recent evidence has suggested that glycogen catabolism provides the sole source of metabolic energy for lipid synthesis at the start of fermentation (Quain et al., 1981; Quain & Tubb, 1981). During the first few hours of fermentation there is no uptake of wort sugars but glycogen is dissimilated rapidly from ca. 40% to $<$ 10% of the dry weight of yeast. As discussed earlier, synthesis of sterols from acetyl-CoA requires much ATP, O_2 and NADPH. However, ATP is only required in the reactions leading to squalene formation. Squalene is present in pitching yeast in amounts generally high enough to provide carbon skeletons for sterol synthesis (Aries et al., 1977) and thus ATP is probably not directly required for sterol synthesis at the start of fermentation. However, the dissimilation of glycogen maybe essential in supplying NADPH which is required in the demethylation reactions leading to ergosterol biosynthesis. Glycogen is presumably catabolized via the hexose monophosphate shunt in order to generate NADPH. Synthesis of fatty acids and their subsequent desaturation, also requires NADPH and furthermore, glycogen dissimilation may supply ATP to fuel the synthesis of acetyl-CoA. Thus the amount of glycogen in the pitching yeast and the rate at which it can be dissimilated are

important in ensuring adequate synthesis of lipids and subsequent fermentation performance. Detailed information on the nature of sterols found in Sacch. cerevisiae in wort fermentations can be found in Aries et al. (1977) and Aries & Kirsop (1978).

Although David & Kirsop (1972) and David (1974) have shown that the concentration of sterols in yeast becomes growth-limiting before that of unsaturated fatty acids, the importance of unsaturated fatty acids in the functioning of the cell should not be overlooked; (Thompson & Ralph, 1967; Thompson, 1974; Aries et al., 1977). Like sterols, unsaturated fatty acids are only formed at the beginning of fermentation in the presence of oxygen and therefore are diluted out on a per cell basis as anaerobic cell-division proceeds. The percentage of unsaturated fatty acids rises from ca. 10% to ca. 50% soon after pitching; oleic and palmitoleic being the predominant acids, this value falls to ca. 10% by the end of fermentation (Aries et al., 1977). The composition and concentration of unsaturated fatty acids in yeast can also be affected by the lipid composition of the wort (3.5.3), carbohydrate composition of the wort and fermentation temperature (Pfisterer et al., 1977). Moreover, in response to anaerobic conditions synthesis of fatty acids in yeast shifts towards production of short chain (C_6-C_{12}) acids (Aries et al., 1977).

The cellular concentration of 0·1-0·2μg sterol (mg dry weight)$^{-1}$ is presumably growth-limiting because the functioning of the plasma membrane is much less efficient when depleted of sterols. Active transport of amino acids and sugars is accompanied by transport of protons (Eddy & Nowacki, 1971; Seaston et al., 1973; 1976; Deak, 1978) and requires that a gradient of protons across the plasma membrane is generated by a proton-translocating ATP-ase (Malpartida & Serrano, 1980). Depletion of unsaturated fatty acids, to less than 10% of the total fatty acids, impairs transport of amino acids and sugars possibly because under such conditions the proton gradient cannot be maintained (Haslam & Al Mahdawi, 1980); possibly depletion of sterols has similar effects. It is not known how a low level of sterol (or unsaturated fatty acid) in the cellular membranes of yeast, signals the cell to stop dividing, or whether a decline in active transport simply results in a general shutdown of metabolic activity thus promoting the cells to enter the so-called maintenance phase (Searle & Kirsop, 1979). A further factor to consider, especially in high-gravity fermentations is that a sterol-depleted membrane may lower the tolerance of the yeast to ethanol (Day et al., 1975).

3.5.3 Utilization of Wort Lipids by Yeast During Fermentation

Yeast removes lipids effectively from wort (Äyräpää et al.,

1961; Krauss *et al.*, 1972; Jones *et al.*, 1975; Klopper *et al.*, 1975; Chen, 1980). Taylor *et al.* (1979) have shown that addition to wort of lipids derived from spent-grains can obviate the need for oxygen during fermentation. The spent-grain lipids exert their effect through the synergistic action of free unsaturated fatty acids, sitosterol and phospholipids. Lipids derived from the cereal grist are potentially important in brewing. Taylor *et al.* (1979) state that only 1·5% of the lipid of malt would have to survive into the wort at pitching to preclude the need for oxygen. Although between 1% and 3% of malt lipid survives into sweet wort only *ca.* 0·1%-0·7% is present in the pitching wort (see 1.3). The levels of sterol (0·1-0·34 mg l^{-1}) are probably too low to exert a significant effect on yeast growth. Free fatty acids are present in wort in the range 1-16 mg l^{-1} of which $>55\%$ are unsaturated. The upper end of this range would be expected to stimulate yeast growth (Taylor *et al.*, 1979) but only if sufficient sterol and phospholipid are present. Moreover, the lipid content of wort may have important consequences with regard to beer flavour and foam formation.

3.5.4 Effects of Oxygen, Wort-lipids and other Effectors on the Production of Beer Flavour Compounds by Yeast

Changes in the brewing process often lead to problems with the flavour of beer. This is no more clearly illustrated than in high-gravity brewing where problems

are encountered with esters. If the original gravity is increased from 1·040 to 1·080, the final amounts of ethyl acetate and iso-amyl acetate are increased between 4 and 8-fold whereas the concentrations of higher alcohols are unaffected (Anderson & Kirsop, 1974). The reasons for the disproportionately high synthesis of acetate esters are unclear though in part they may be due to increased alcohol concentrations (Haukeli et al., 1973; Anderson & Kirsop, 1974). Certainly this problem is a major restraint on the wider application of high-gravity brewing. The following discussion relates to the problems with acetate esters in high-gravity brewing and also reviews the technological factors that affect production of flavour compounds, in general, during normal-gravity fermentations.

(a) Oxygen

The availability of oxygen during fermentation has a marked effect on the formation of flavoursome esters and medium chain length fatty acids. Lower than normal amounts (< 2 mg l^{-1}) of oxygen in wort at pitching result in high concentrations of ethyl acetate and iso-amyl acetate in beer (Cowland, 1967; Maule, 1967; Engan & Aubert, 1975). However, increasing the initial concentration of oxygen above that normally used has little effect on ester formation (Norstedt et al., 1975; White & Portno, 1979) presumably because yeast is limited in the amount of oxygen it can use (Aries et al., 1977). However if the oxygen is supplied, even at a low rate,

throughout fermentation, the synthesis of acetate ester, esters of C_8-C_{12} fatty acids and the free C_6-C_{12} acids are suppressed (Cowland & Maule, 1966; Norstedt et al., 1975; Anderson et al., 1975; White & Portno, 1979). Excessive formation of acetate esters in high-gravity fermentations can be overcome by addition of oxygen (Palmer & Rennie, 1974; Anderson & Kirsop, 1975a). Anderson & Kirsop also found that addition of oleic acid to wort, like oxygen would stimulate yeast growth and greatly suppress ester formation; the two processes being considered to be in competition. Oxygen does not have to be supplied throughout fermentation and a single oxygenation lasting two hours can eliminate disproportionate synthesis of esters if applied at a certain point in fermentation (Anderson & Kirsop, 1975b). For a 1·080 fermentation the most effective suppression is obtained if the wort is oxygenated when the specific gravity is in the range 1·063-1·044. The effect of oxygen is to virtually stop ester synthesis during the oxygenation period and to cause a diminished rate of ester synthesis subsequently. Oxygenation also has the beneficial effect of stimulating the rate of fermentation. However, there are two main drawbacks to this procedure:

(i) Growth is greatly stimulated and thus the amount of beer (high-gravity) lost in the waste yeast may be so high as to cancel out the economic advantages of high-gravity brewing.

(ii) Addition of oxygen to actively fermenting wort

(as compressed air or oxygen) is potentially hazardous since it can cause a sudden release of CO_2 gas which in turn can cause excessive foaming and spillage. However this problem can be overcome by adding the oxygen as oxygen-saturated water (Rennie & Wilson, 1975) or by incrementally filling the fermenter with batches of air or oxygen-saturated wort (White & Portno, 1979). Both procedures reduce the concentrations of esters in high-gravity fermentations.

Aerobically-grown yeast synthesizes less medium chain length fatty acids than anaerobically-grown (Jollow et al., 1968) and not surprisingly aeration of fermentation leads to less C_6-C_{12} fatty acids in yeast and beer (Taylor & Kirsop, 1977b). Evidence suggests that although aerobically-propagated yeast synthesizes lower amounts of C_6-C_{12} acids, it also excretes a lower proportion of these acids, therefore implying that the composition of the plasma membrane may influence the release of medium chain length acids into beer (Aries et al., 1977). Aeration reduces the levels of ethyl esters of C_6-C_{12} acids concomitantly with a reduction in the levels of the free acids, the notable exception being ethyl hexanoate which is unaffected (Norstedt et al., 1975).

(b) Wort lipids

Nordstrom (1964c) provided the first evidence that addition of C_{12}-C_{18} fatty acids stimulate the synthesis,

by yeast, of ethyl acetate. The data, (Nordstrom 1964c) although somewhat equivocal, suggested that exogenous fatty acids inhibited fatty acid synthesis thus increasing the availability of acetyl-CoA for ethyl acetate synthesis. These observations were extended by Äyräpää & Lindström (1973) who showed that trub from spent grains, when added to wort, reduced the ester content of beer. These workers found that long chain unsaturated fatty acids (which are present in trub; see 1.3) reduced the formation of acetate esters and ethyl esters of medium chain length fatty acids. The unsaturated fatty acids ($C_{16:1}$, $C_{18:1}$ and $C_{18:2}$) were also effective as their monoacylglycerols but not as di and triacylglycerols. Palmitic acid (C_{16}) was found to be without effect whereas stearic acid (C_{18}) stimulated the formation of esters, in agreement with Nordstrom (1964b). Äyräpää & Lindström (1973) drew the conclusion that if too much lipid material (containing free unsaturated fatty acids or monoacylglycerols) is allowed to pass into the pitching wort, beers with unacceptably low ester levels may be produced. However, such lipid material does have a use, in high-gravity fermentations as reported by Palmer & Rennie (1974). These workers found that the material in spent grain 'fines' reduced the synthesis of acetate esters by ca. 60% in fermentations of 1·070 wort, whereas barley lipid was without effect. This is because barley lipid contains small amounts of free fatty acids whereas ca. 30% of the fatty acids in spent grains are in the free form (Taylor et al.,

1979). These observations were extended and confirmed by Taylor et al. (1979) and White & Portno (1979) and though the significance of these findings to production scale brewing are unclear, lipids derived from spent grains offer a potentially powerful means of controlling the levels of esters in high-gravity fermentations.

In addition to suppressing the formation of acetate esters, free unsaturated fatty acids suppress formation of medium chain length fatty acids and their ethyl esters (Aries et al., 1977; Äyräpää & Lindström, 1977).

(c) Other effectors

Yeast strains vary greatly in their ability to form esters (Nykanen & Nykanen, 1977; Piendl & Geiger, 1980) and C_6-C_{12} fatty acids (Aries et al., 1977). Initial wort pH, hopping rate and pitching rate have little effect on the formation of esters though the levels of C_6-C_{12} acids increase if the initial wort pH is raised from 4·2 to 7·2 (Norstedt et al., 1975).

Generally an increase in the temperature of fermentation and maturation stimulates the formation of ethyl acetate and iso-amyl acetate (Piendl & Geiger, 1980) but different esters show different temperature dependencies (Norstedt et al., 1975; Engan & Aubert, 1977). For example, ethyl hexanoate and ethyl decanoate are unaffected, and ethyl dodecanoate reduced by raising the fermentation temperature from 10°C to 20°C whereas

formation of ethyl octanoate is optimal at 15°C (Norstedt et al., 1975).

Obviously original gravity plays a role in determining the levels of esters in beer (see 1.2). The means by which the gravity of wort is increased appears to greatly affect ester synthesis. Beers with acceptable levels of acetate esters can be produced by high-gravity fermentation, if the worts are prepared using adjuncts rather than an all-malt grist (Pfisterer & Stewart, 1975; Pfisterer et al., 1977; White & Portno, 1979). These adjunct worts have an increased carbon - nitrogen ratio. Less acetate esters and yeast mass are formed in fermentations of 1·070 wort containing 40% (w/v) glucose, than in 1·070 all-malt wort fermentations (Pfisterer & Stewart, 1975). This contrasts with the situation where oxygen reduces ester formation in high-gravity fermentations by stimulating yeast growth. Therefore there appears to be no direct relationship between yeast growth and formation of esters. Pfisterer & Stewart (1975) attempt to explain their results by the fact that Sacch. cerevisiae is a Crabtree-positive yeast (De Deken, 1966); glucose thus represses synthesis of TCA-cycle enzymes (some of which are active during fermentative growth; Oura, 1977a,b), more acetyl-CoA is then available for lipid synthesis (Pfisterer et al., 1977) and consequently less acetyl-CoA is available for acetate ester synthesis. However White & Portno (1979) have found that if such 'glucose

adjunct' worts are supplemented with a source of α-amino nitogen, glucose does not suppress ester synthesis. Thus the reduced level of assimilable nitrogen in adjunct worts appears to be the main effector in suppressing ester formation. Although the use of adjuncts appears to be an effective means of reducing excessive synthesis of acetate esters in high-gravity fermentations, this procedure does have drawbacks. If the nitrogenous material is diluted too severely, the fermentation may not attenuate completely (Harding & Kirsop, 1979). Furthermore, any contribution of the malt grist to flavour will of course be diminished.

Dilution of normal gravity wort (i.e. 1·040) with up to 25% glucose (w/v) (Engan, 1970), 30% maize extract or 15%-30% sucrose (Norstedt et al., 1975) has no effect on synthesis of ethyl and iso-amyl acetates. The addition of amyloglucosidase to wort leads to a medium with glucose as the sole carbon source. In such a medium disproportionately high levels of ethyl and iso-amyl acetate are formed (Hockney & Zealey, 1981). The hypothesis put forward to explain this observation is that glucose represses formation of TCA-cycle enzymes thus increasing the availability of acetyl-CoA for the synthesis of acetate esters.

4. Aims of the Present Study

It is clear from the preceeding discussion that fermentation and beer flavour are affected by the lipid metabolism of yeast and by the presence of lipids in wort. Many factors determine the quantity of lipid material in wort at pitching. Certainly, significant concentrations of free unsaturated fatty acids can be present and it is these compounds that reduce the production of flavour compounds (volatile esters and medium-chain fatty acids) by yeast. Thus, one objective of the present study is to establish the extent to which free unsaturated fatty acids inhibit ester synthesis, how they exert their effects and what effects they have on fermentation in general. Such information should enable evaluation of the feasibility of using sources of unsaturated fatty acids (e.g. trub or spent-grain lipids) to control synthesis of esters. Certainly, efficient control over ester synthesis during fermentation is desirable, especially in high-gravity brewing. Therefore, a further objective of the investigations, reported here, is to obtain knowledge regarding the regulation of acetate ester synthesis during fermentation. Here the aim is to provide information that can be applied to brewing so that existing procedures for controlling ester synthesis in high-gravity brewing can be optimized and novel control procedures can be developed. In order to achieve these objectives a study of the interrelationships between

lipid metabolism and ester synthesis during fermentation, and assessment of the intracellular availability of acetyl-CoA for ester synthesis were undertaken.

MATERIALS AND METHODS

5. Materials and Methods

5.1 Yeast strains

The following strains of *Saccharomyces cerevisiae* were all obtained from the National Collection of Yeast Cultures (present address - ARC Food Research Institute, Colney Lane, Norwich, NR4 7UA); NCYC 240, 1026, 1062, 1245. Cells were stored at 4°C in YM Broth (Difco) at a cell density of *ca.* 10^8 cells ml^{-1} and were sub-cultured every eight weeks.

5.2 Growth media

All-malt hopped wort of specific gravity 1·040 was used in all laboratory-scale wort fermentations. Wort was prepared by the pilot-brewery at the Brewing Research Foundation and was transfered into sterile stainless steel vessels (20 l) and stored at 4°C until required. Wort is a complex medium (see MacLeod, 1977) but typical figures (g l^{-1}) for the carbohydrate content of the wort used in these studies are: maltose, 54; dextrins, 24; maltotriose, 12·5; glucose, 7; fructose, 2. The pH of the wort was 5·2 and the total soluble nitrogen and free amino nitrogen contents were 830 and 160-200mg l^{-1} respectively (determined by the Analytical Section, Brewing Research Foundation).

In some studies, semi-defined growth media were used. These media contained Difco yeast extract (1%, w/v); Oxoid neutralized bacteriological peptone (0·5%, w/v);

and Saccharomyces salts (Wallace et al., 1968) which comprised per litre of medium; $(NH_4)_2SO_4$ (1·2g), NaCl (0·5g), $CaCl_2$ (0·1g), $MgCl_2$ (0·7g), KH_2PO_4 (1g) and $FeCl_3$ (3mg). Saccharomyces salts were prepared as a stock solution (5x concentration), adjusted to pH 5·4 and stored at 4°C until required. Carbon and energy sources used were: glucose (5% or 10% w/v), YEPG medium; glucose plus maltose (1% and 6·5% w/v respectively), YEPGM medium; ethanol plus glucose (1% v/v and 0·1% w/v respectively), YEPGE medium. When 10% glucose was used, the concentrations of all other components were doubled. Media and sugar solutions were autoclaved separately at 0·1 MPa for 20 minutes. For YEPGE medium, ethanol was added after the medium had been autoclaved and cooled.

5.3 Experimental conditions of yeast growth

To reproduce brewery growth conditions, yeast used in all experimental fermentations was propagated with limited access to air as follows. Initially, cells of a stock culture were inoculated into hopped wort (400ml) and then grown, with shaking, at 25°C for 72h. Transfer of gases took place asceptially through a non-absorbant cotton wool plug. Yeast was harvested from these cultures by centrifugation, and resuspended in sterile water in the ratio 1ml H_2O (g wet weight yeast)$^{-1}$. Air-saturated wort (1·5 l) containing 1ml l^{-1} silicone anti-foam (Dow Corning RD emulsion) was inoculated with 7·5 ml of the yeast/water suspension and fermented with

stirring at 20°C in a glass fermenter (2 litres nominal volume). Oxygen-free nitrogen was passed through the headspace of the fermenter throughout fermentation. After ca. 72h, yeast was harvested and mixed with sterile water as above and again inoculated into air-saturated wort, which was fermented as before. After 72h yeast was then used in experimental wort fermentations; the details of inoculation and fermentation were identical to the 'propagation fermentations'. Wort was air-saturated (in the fermenters) by stirring for 3h at 20°C (with no flow of N_2 through the headspace) prior to inoculation. Air-saturated 1·040 wort contained 8·25p.p.m. oxygen (= 0·258m moles O_2 l^{-1}); this is essentially the only oxygen that the yeast comes into contact with, during wort fermentations reported here. The dissolved oxygen is removed within 2h (Quain et al., 1981) though not all is removed as a consequence of yeast activity; a proportion is removed by the passage of nitrogen through the fermenter headspace.

The inoculum size (2·5g wet weight yeast l^{-1}) is equivalent to ca. 10 x 10^6 cells ml^{-1}. In most experiments NCYC 240 was used. Under the experimental conditions employed here this yeast fails to ferment maltotriose completely (Taylor et al., 1979). Consequently NCYC 240 will not attenuate below a specific gravity of 1·009.

For all experiments in semi-defined media, the inoculum was initially grown as above, except that Difco YM Broth (400ml) was used as the medium. For aerobic experiments, yeast from these cultures was taken as the inoculum. For anaerobic experiments the yeast was further propagated, anaerobically, in fermenters as described above for wort fermentation. Here, the medium used for propagation and experimentation were the same unless otherwise stated. Experimental details are given in appropriate legends but generally shake-flask experiments were performed at 25°C on a rotary shaker (180r.p.m.).

5.4 Measurement of flavour compounds in beer and fermenting wort

5.4.1 Esters and higher alcohols:-

The levels of esters and higher alcohols of beers (sections 6.1 and 6.2) were measured by the Analytical Section of the Brewing Research Foundation using a modification of the method of Maule (1967). A 500ml bottle containing sodium chloride (50g) was flushed with nitrogen, and then beer (100ml) plus internal standard solution (1ml of a solution containing 1ml methyl acetate; 1ml pentanol; 3ml ethanol; made up to 100ml with water) were added. The bottle was 'suba'-sealed and 50ml N_2 gas injected. After incubation in a water bath at 30°C for 30 min, 10ml headspace gas was removed and analysed by gas chromatography, on a Pye GCD equipped with a flame ionization detector,

using a 2·8m x 4mm i.d. glass column packed with 10% Carbowax 1540 on Chromosorb AW 60-80 mesh. The injection temperature was 150°C, detector temperature 200°C and the column temperature was held at 60°C for 7 min and then rapidly increased to 85°C. Carrier gas was oxygen-free nitrogen (20ml min^{-1}). Quantification of esters and alcohols was based on their peak areas relative to that of internal standard using calibration curves constructed from known amounts of the pure compounds. Ethyl and iso-amyl acetates were quantified using methyl acetate as the internal standard whereas for n-propanol, iso-butanol and iso-amyl alcohols, the peak areas relative to n-pentanol were used in calculations. Where many samples were taken during fermentation (sections 6.4 and 6.5) a small-scale headspace technique was used to measure the ethyl and iso-amyl acetate content of fermenting wort. The order of addition of reagents to a McCartney bottle was: sample (2ml or 1ml + 1ml water); internal standard solution (1ml containing 30µg methyl acetate); NaCl (3g). The bottle was then 'suba'-sealed and 20ml N_2 gas injected. After incubation at 30°C for 30 min, 10ml of headspace gas was removed and analysed by gas chromatography as described above, except that the injection temperature was 100°C, detector temperature 200°C and the column temperature was initially 60°C rising 2°C min^{-1} to 90°C; carrier gas was oxygen-free nitrogen (40ml min^{-1}). Quantification of esters in each sample was based on peak height relative to that

of the internal standard using calibration curves constructed of peak height ratios versus known amounts of each pure compound.

5.4.2 Fatty acids:-

The content of C_6-C_{10} fatty acids in beer was measured using the method of Taylor & Kirsop (1977a). The extraction mixture, in a stoppered flask, contained: beer (70ml); nonanoic acid solution (1·0ml ethanol containing 0·1µl nonanoic acid) as internal standard; ethanol (1·4ml); chloroform (1ml); hydrochloric acid (6M, 2·5ml) and sodium chloride (18·5g). The flask was shaken vigorously for 60 min at room temperature. After leaving the flask for sufficient time to allow the chloroform to settle out of solution, the bulk of the aqueous phase was decanted and the remaining contents (chloroform plus some aqueous phase) transfered to a glass centrifuge tube. The phases were separated by centrifugation, the aqueous layer discarded and chloroform released from the residual solid material by brief vigorous shaking. After further centrifugation the solid material formed a layer above the globule of chloroform. A portion (10µl) of the chloroform was analysed by gas chromatography, on a Pye GCD equipped with a flame ionization detector, using a 1·4m x 4mm i.d. glass column packed with 5% FFAP on Chromosorb GAW DMCS 80-100 mesh. Injection temperature was 250°C, detector temperature 280°C and the column temperature was held at 150°C for 11 min then increased by 8°C

min^{-1} to 235°C. The combined extraction and analysis procedure was calibrated by adding known amounts of pure fatty acids to wort or beer and measuring peak heights relative to that of nonanoic acid. Calibration curves were linear, and were used to quantify levels of C_6-C_{10} fatty acids in samples of beer.

5.5 Analyses of the lipid composition of yeast

Centrifuged yeast samples were either resuspended in a small volume of distilled water, frozen in liquid nitrogen and stored at 20°C or were washed x3 with distilled water under an atmosphere of nitrogen and freeze-dried, prior to lipid analyses.

5.5.1 Determination of fatty acyl residues in yeast:- Total C_6-C_{18} fatty acyl residues were extracted from yeast and measured according to the method of Taylor and Kirsop (1977a). Yeast (wet or freeze-dried ca. 100mg dry weight) together with 1mg tridecanoic acid, as internal standard, were saponified with potassium hydroxide (1M) in refluxing 90% (v/v) ethanol (10ml) for 60 min. The yeast debris was washed with a further 10ml ethanolic potassium hydroxide. The ethanolic extracts plus washings were combined, diluted to 70ml final volume with distilled water, acidified to pH 2 with 6M hydrochloric acid (3·5ml) and extracted into chloroform (2ml) by vigorously shaking the mixture on a flask shaker for 30 min. The chloroform phase was then removed and a portion subjected to gas chromatography

as described above (5.4.2) except that the column temperature was held at 190°C for 12 min and then increased by 16°C min^{-1} to 225°C. Fatty acids were quantified by comparison of peak heights relative to that of the internal standard, having previously calibrated the combined extraction and gas chromatography procedures with known amounts of fatty acids. Squalene was also measured in this way.

The amounts of free fatty acids in yeast were measured in a similar manner after extraction from yeast by refluxing with 90% (v/v) ethanol. Since solvents at room temperature are reported to activate lipolytic enzymes (Van den Bosch et al., 1967) boiling ethanol (90% v/v) was added directly to the yeast samples. The amount of HCl (6M) added prior to extraction into chloroform was reduced from 3·5ml to 0·1ml. This reflux and extraction procedure did not cause detectable hydrolysis of ethyl heptanoate and therefore presumably did not cause hydrolysis of complex lipids.

In certain figures and tables, values for a parameter termed % UFA are given. The % UFA of the total fatty acyl composition of yeast is defined as: mg unsaturated fatty acyl residues/mg total fatty acyl residues in yeast.

In analyses reported in sections 6.1 and 6.2, freeze-dried cells were used. Whilst the values reported in

these sections are in agreement with other workers (Aries et al., 1977; Äyräpää & Lindström, 1977) they are substantially lower than those reported when wet cells have been extracted (e.g. see 6.5). To determine whether the differences were purely quantitative or whether 'qualitative differences' had also arisen, (e.g. differences in % UFA values) ten identical samples of an aqueous suspension of yeast were analysed for total fatty acyl residues and compared with ten samples of the same suspension subsequent to freeze-drying. Further, rehydrated freeze-dried cells were analysed. Cells were rehydrated by adding 1ml H_2O to 100mg dry weight yeast, under an atmosphere of nitrogen for 1h. Results are presented in Table 1. The values of all of the fatty acyl residues from the freeze-dried cells were significantly lower (at a probability of 0·05) than those from wet cells. However, the values of all the fatty acyl residues from rehydrated freeze-dried cells were not significantly different ($p = 0·05$) from wet cells as can clearly be seen from Table 1. The % UFA values for wet, freeze-dried and rehydrated freeze-dried cells respectively were 31·4; 30·2; 32·8. The corresponding values for the ratio of fatty acyl residues $\geqslant C_{16} : < C_{16}$ were 5·1, 4·8, and 5·3. Therefore although extraction of fatty acyl residues from freeze-dried cells may be relatively inefficient, the spectrum of acyl residues is in good agreement with that obtained after extraction of wet cells. Further, the efficiency of extraction of freeze-dried cells can be

Table 1. Comparison of extraction of total fatty acyl residues from wet, freeze-dried or rehydrated freeze-dried cells

	Fatty acid μg (mg dry weight yeast)$^{-1}$											
	C_6	C_8	C_{10}	C_{12}	C_{14}	$C_{14:1}$	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	Total
1. $\bar{x}$ (10)	0·067	0·67	2·05	0·67	0·21	Tr	7·56	2·29	3·94	4·74	0·173	22·38
$\pm$	0·01	0·018	0·05	0·021	0·01	Tr	0·26	0·065	0·226	0·267	0·015	2·23
2. $\bar{x}$ (10)	0·05	0·60	1·68	0·45	0·153	Tr	5·34	1·60	3·45	3·49	0·11	16·85
$\pm$	0·005	0·024	0·062	0·08	0·006	Tr	0·154	0·065	0·12	0·109	0·016	0·573
3. $\bar{x}$ (10)	0·051	0·635	1·98	0·63	0·198	Tr	7·38	2·37	4·02	4·92	0·14	22·2
$\pm$	0·003	0·03	0·057	0·034	0·008	Tr	0·194	0·097	0·13	0·176	0·008	0·439

1. Wet cells 2. Freeze-dried cells 3. Rehydrated freeze-dried cells (see text). $\bar{x}$ (10) refers to mean of ten determinations and $\pm$ values represent 95% confidence limits. Tr = trace amounts detected.

increased by rehydration, prior to saponification, as described above.

5.5.2 Determination of the sterol content of yeast:-
Total sterols were released from yeast by direct saponification and measured by gas chromatography as described by Aries & Kirsop (1977). Freeze-dried or centrifuged wet yeast (ca. 100mg dry weight) was saponified with potassium hydroxide (40% w/v) by refluxing at 100°C under an atmosphere of nitrogen for 2h. The reflux mixture contained 50µg cholesterol as internal standard. After cooling, water (10ml) and ethanol (3ml) were added and the sterols extracted with 3 x 15ml petroleum ether (b.p. 40°-60°C). The extracts were dried over anhydrous sodium sulphate, evaporated to dryness in a rotary evaporator and then redissolved in chloroform (ca. 200µl). Extracts were analysed by gas chromatography on a Pye GCD equipped with flame ionization detector using a 1·4m x 4mm i.d. glass column packed with 3% OV1 on Diatomite CQ. The injection temperature was 280°C, detector temperature 310°C and the column temperature 230°C. Carrier gas (oxygen-free nitrogen) flow rate was 50ml min^{-1}. Sterols were identified on the basis of retention time relative to internal standard (Aries, unpublished observations).

5.5.3 Determination of the fatty acyl composition of the lipid classes of yeast:-
Freeze-dried cells (ca. 200mg dry weight) were refluxed

in ethanol (10ml) for 30 min under an atmosphere of nitrogen. As for free fatty acid determinations, boiling ethanol was added directly to the yeast samples to inactivate hydrolytic enzymes. Following cooling and centrifugation, the supernatant was stored at -20°C. The cell debris was extracted with chloroform/methanol (15ml; 2:1 v/v) by vigorously shaking the mixture in a stoppered tube on a flask shaker for 60 min. Following filtration through a glass filter (Whatman GF/C 2·1cm diameter) the filtrate was pooled with the ethanol extract and the cell debris further extracted with chloroform/methanol twice more. Pooled extracts were washed with 0·88% KCl (w/v) to remove non-lipid compounds, dried over anhydrous sodium sulphate and rotary evaporated under vacuum to dryness. Lipids were redissolved in chloroform (ca. 500µl), applied to a silica gel GF plate, 2mm thickness x 20cm x 20cm (Shandon Anachem Uniplate). Plates were developed in hexane: diethyl ether: acetic acid (70:30:1, v/v/v) until the solvent-front reached the top of the plate. Reference compounds were chromatographed on one edge of each plate and bands of lipid could be detected under UV light and/or by spraying the reference compounds and the edge of the sample bands with a solution of 1% (w/v) iodine in methanol. Six lipid classes were detected (in ascending order); phospholipids; monoacylglycerols; sterols; diacylglycerols plus free fatty acids (FFA/di); triacylglycerols; steryl esters plus squalene. The bands of fatty acyl containing lipids

were removed from the plate and 1mg tridecanoic acid added as internal standard. Lipids were eluted with chloroform and in the case of phospholipid, methanol. All fractions were rotary evaporated to dryness and redissolved in either ethanol (FFA/di), ethanolic potassium hydroxide (acylglycerols and phospholipids) or 1ml chloroform plus ethanolic potassium hydroxide (steryl esters). The phospholipid, acylglycerol and steryl ester fractions were saponified and extracted as described above (5.5.1) for total fatty acyl residues in yeast. The FFA/di fraction was treated as described for determination of free fatty acids. After gas chromatography the FFA/di fraction was saponified and the difference between free and total values was taken as the fatty acyl residues in diacylglycerols.

5.6 Extraction and measurement of acetyl-CoA and CoASH in Sacch. cerevisiae

Accurate measurement of intracellular metabolite concentrations requires that yeast be harvested and its metabolism quenched as rapidly as possible (for reviews on extraction of metabolites from yeast see Gancedo & Gancedo, 1973; Saez & Lagunas, 1976). Typical procedures involve rapid membrane filtration of culture sample followed by quenching in perchloric acid (PCA) (Van Wijk & Konijn, 1971; Quain & Haslam, 1979b); alternatively removal of a volume of culture directly into concentrated PCA may be more convenient (Weibel et al., 1974) although problems with quenching of

fluorescence by growth medium may be encountered here. Release of metabolites is usually effected by a freeze-thaw procedure (Van Wijk & Konijn,1971; Weibel et al., 1974; Quain & Haslam, 1979b). Such methods have proved successful for the measurement of numerous metabolites in yeast but as yet no published data are available concerning acetyl-CoA. However, acetyl-CoA and CoASH have been extracted and their levels measured in other tissues (Herrera & Frienkel, 1967; Lumbers et al., 1969; Hansford, 1974; Brass & Hoppel, 1980, 1981).

Initially the extraction of acetyl-CoA and CoASH from yeast was attempted using PCA by the procedure of Quain & Haslam (1979b). However no acetyl-CoA or CoASH could be detected in the neutralized PCA extracts when assayed either spectrophotometrically or fluorimetrically using the method of Allred & Guy (1969) (see below). Notably, fluorimetric analysis was impeded by; high background fluorescence, and unstable rates of fluorescence increase.

However, early reports showed that coenzyme A can be extracted from yeast with hot water (Kaplan & Lipmann, 1948; Klein & Lipmann, 1953; Stadtman & Kornberg, 1953; Hosono & Aida, 1974). Therefore attempts were made to extract acetyl-CoA and CoASH from yeast using hot water; such attempts were successful and the following procedure was routinely used.

A volume of culture containing 10-20mg dry weight yeast was harvested by membrane filtration (Millipore Type HA, 0·45μm, 5cm diam.) and the filter immersed in liquid nitrogen for ca. 2s. The total length of time from removal of sample from culture, to immersion of filter plus cells in liquid nitrogen was ca. 10s. The frozen filter plus cells was then transfered to a pre-cooled beaker and stored on ice. Cells were washed from the filter with 1ml hot (95°C) water, the beaker being held in a boiling water bath while this procedure was carried out. The suspension of cells was then transfered to a glass tube and stored on ice. The filter was further washed with two or three 0·5 volumes of hot water which were bulked together with the initial washings, and stored on ice. Subsequently the tube, containing the cell suspension, was capped with a glass sphere and heated in a boiling water bath for 10min. After cooling, the cell suspension was centrifuged, the supernatant (hot water extract) decanted and its volume determined (usually ca. 2ml). Dithiothreitol (100mM) was added to the hot water extract to give a final concentration of 1mM, to prevent oxidation of CoASH (Hansford, 1974). Hot water extracts were stored on ice and assayed for acetyl-CoA and CoASH within 2h. Acetyl-CoA but not CoASH is destroyed by prolonged heating (Dawson et al., 1978). The optimum length of time for heating the cell suspension was found to be 10 min (Fig.3.a). Presumably, heating the cell suspension for a shorter period of time does not completely

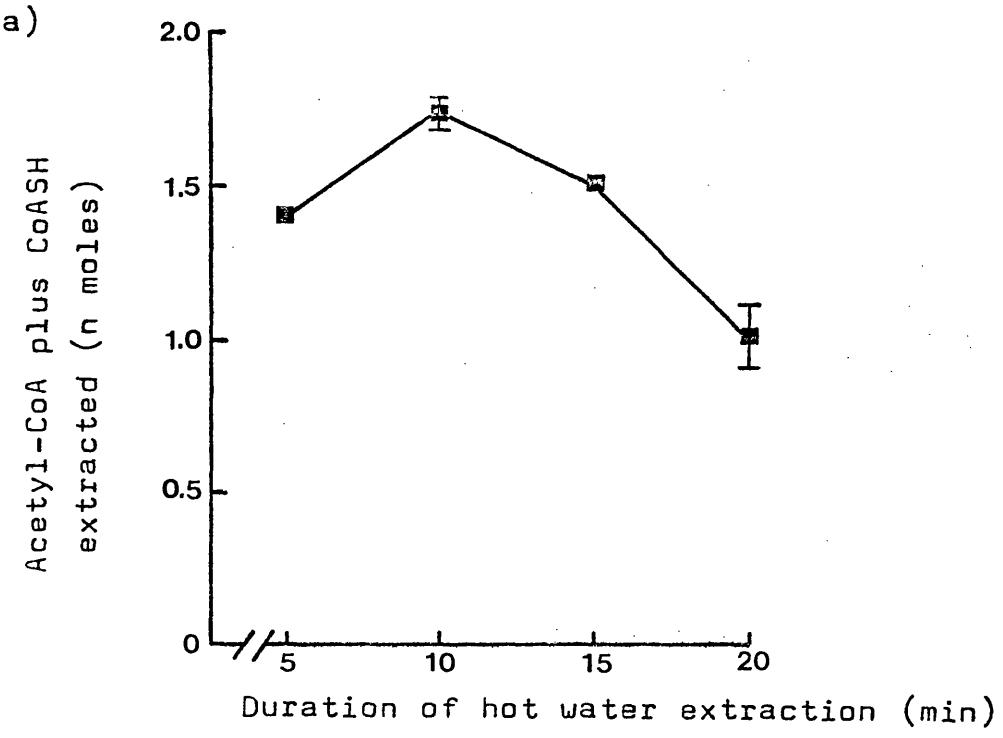
Figure 3.

Extraction and measurement of acetyl-CoA and CoASH in Sacch. cerevisiae

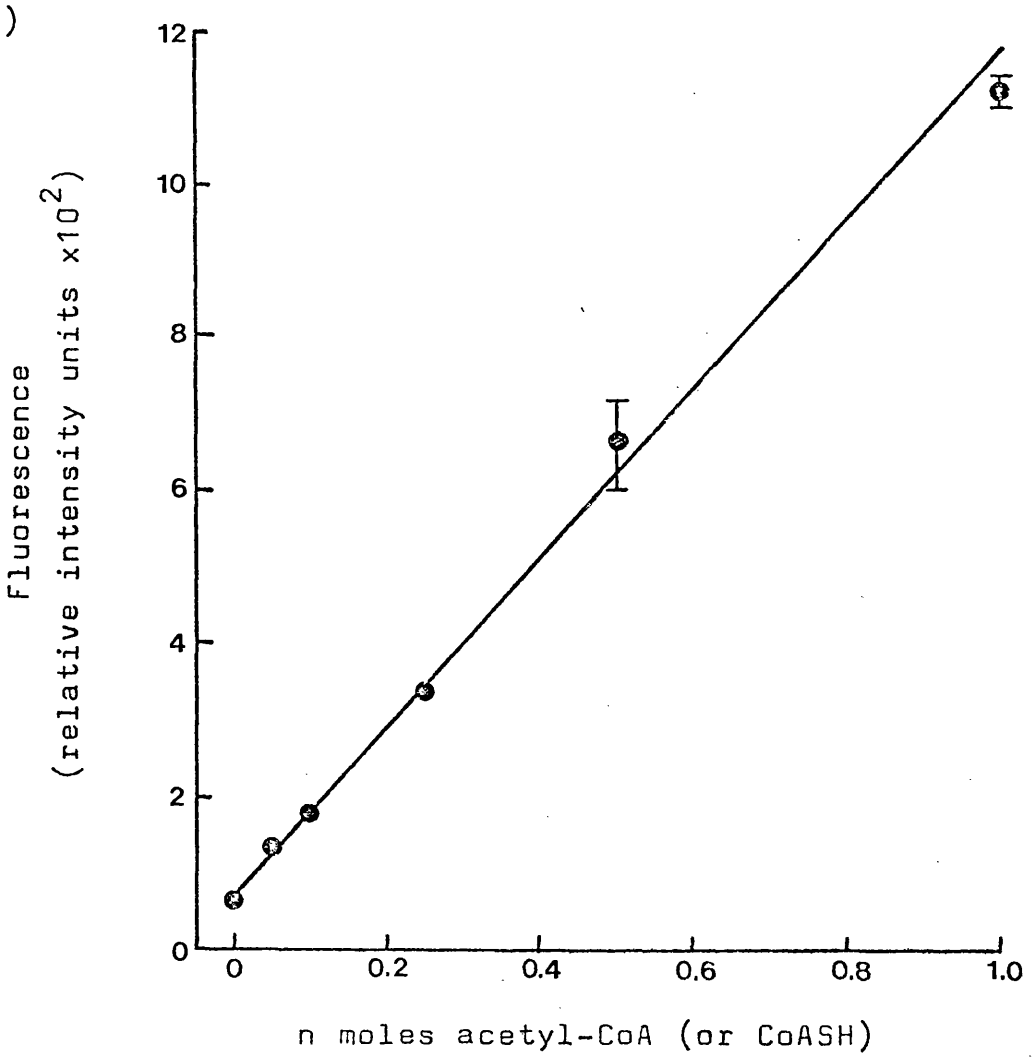
(a) Optimum length of time for extraction, with hot water, of acetyl-CoA and CoASH from yeast. Yeast samples subjected to hot water extraction for varying lengths of time as indicated in figure. Points represent mean of two determinations on duplicate hot water extracts. Bars show range of values obtained.

(b) Calibration curve for assay of acetyl-CoA and CoASH. All points are mean of duplicate determinations of a known amount of acetyl-CoA (or CoASH). Bars show range of values obtained.

(a)


Acetyl-CoA plus CoASH
extracted (n moles)
2.0
1.5
1.0
0.5
0
5
10
15
20
Duration of hot water extraction (min)

(b)


Fluorescence
(relative intensity units x10^2)
12
10
8
6
4
2
0
0
0.2
0.4
0.6
0.8
1.0
n moles acetyl-CoA (or CoASH)

extract CoASH and acetyl-CoA from the cells; whilst heating for longer periods results in degradation of acetyl-CoA. The method of Allred & Guy (1969) was used to determine the amounts of acetyl-CoA and CoASH in the hot water extracts. This assay recycles CoASH through the following reaction sequence:

acetyl phosphate + CoASH $\longrightarrow$ acetyl-CoA + phosphate

acetyl-CoA + oxaloacetate $\longrightarrow$ citrate + CoASH

malate + NAD^+ $\longrightarrow$ oxaloacetate + NADH

Both CoASH and acetyl-CoA participate in these reactions and the rate of NADH formation is thus proportional to the concentration of CoASH plus acetyl-CoA since all other substrates are supplied in excess. Normally 100-500µl hot water extract was assayed. A reaction premix was constructed in the following proportions: 0·5ml imidazole buffer (Sigma fluorimetric grade, 400mM, pH 7·2); 0·1ml potassium chloride (1M); 0·1ml malate (0·2M); 0·1ml acetyl phosphate (0·08M); 0·1ml NAD (0·02M); malate dehydrogenase (1·6 units); citrate synthase (3·5 units). Usually 'premix' sufficient for 10 assays (9ml) was made up and stored on ice. The reaction mix contained: 0·9ml 'premix'; 0·1ml dithioerythritol (0·02M) and sufficient hot water extract plus water to give a final volume of 1·7ml. After incubation of reaction mix at 30°C for 2 min, 0·1ml of phosphotransacetylase (30 units) was

added to start the reaction. Each sample was assayed with and without an internal standard (usually 0·25 n moles acetyl-CoA per assay) because the hot water extracts were slightly inhibitory towards the cycling reactions, as are neutralized PCA tissue extracts (Herrera & Frienkel, 1967; Hansford, 1974; Van Broekhoven et al., 1981). Acetyl-CoA was measured separately by first reacting all of the CoASH with N-ethylmaleimide (0·1ml, 0·02M, freshly prepared) for 5 min at 30°C. Prior to assay the excess N-ethylmaleimide was reacted with 0·2ml dithioerythritol (0·02M) for a further 5 min at 30°C. The concentration of CoASH was calculated from the difference in the rates of NADH formation obtained with and without pretreatment of samples with N-ethylmaleimide. Rates of NADH formation were followed fluorimetrically.

Fluorescence was monitored using an Aminco fluorocolorimeter (J4-7439) equipped with a UV-lamp (G.E. no. F4T4/BL), a Corning 7-60 primary filter and a combination of a Wratten 2A, plus a Turner 48 (460nm) narrow pass filter as the secondary filter. Rates of increase in fluorescence were followed and recorded over a period of 10-15 min per assay. Rates were linear. Acetyl-CoA and CoASH were quantified using a calibration curve constructed of rate of increase in fluorescence versus amount of coenzyme A (either CoASH or acetyl-CoA can be used). The calibration curve (Fig.3.b) was linear over the range 0 - 1·0 n moles coenzyme A and as

little as 50 p moles could be detected. In the absence of either CoASH or acetyl-CoA a low rate of NADH formation was detected, in agreement with Allred & Guy (1969). These workers attribute this observation to the presence of small amounts of either CoASH or acetyl-CoA in the phosphotransacetylase preparation.

To assess the reproducibility of the method (extraction plus measurement), four samples (3ml) were removed from a standard laboratory-scale fermentation that had reached stationary-phase. Values for the mean of four determinations $\pm$ the standard error of the mean were $95{\cdot}5 \pm 5{\cdot}3$ and $141{\cdot}8 \pm 7{\cdot}3$ p moles (ml culture)$^{-1}$ for acetyl-CoA and CoASH respectively.

The acetyl-CoA and CoASH content of yeast was found to be respectively in the range 15-60 and 20-100 f moles (μg dry weight)$^{-1}$ though data are generally presented on the basis of cell number (6·6). Data are also expressed as the acetyl-charge which is defined by Hampsey & Kohlhaw (1981) as [acetyl-CoA] / [acetyl-CoA + CoASH]. No data are available in the literature for comparison of the values for the acetyl-charge presented in 6·6. Published values for the coenzyme A content of yeast are in Lipmann units (Kaplan & Lipmann, 1948; Klein & Lipmann, 1953; Stadtman & Kornberg, 1953; Hosono & Aida, 1974). Values quoted are in the range 30-300 units (mg dry weight yeast)$^{-1}$. Since 1mg pure coenzyme A is equivalent to 413 units (Dawson et al., 1978) these

values represent 95-950 f moles coenzyme A (μg yeast)$^{-1}$; the higher values were obtained from yeast grown aerobically in the presence of 200μg calcium pantothenate l^{-1} (Klein & Lipmann, 1953).

The above method was developed in collaboration with Dr. D E Quain (Fermentation Department, Brewing Research Foundation).

5.7 Measurement of acetyl-CoA hydrolysing activity in cell-free extracts of yeast

Yeast was harvested from media by centrifugation and the yeast pellet washed into a Braun homogenization bottle with the minimum of phosphate buffer (ca. 2ml, 100mM, pH7). Pre-cooled glass beads (0·45-0·55mm diam) equivalent to the cell wet weight were added and the cells disrupted at 0 to 4^{o}C using a Braun homogenizer for 2 x 30s periods. Glass beads and unbroken cells were removed by centrifugation; the supernatant stored on ice and assayed for acetyl-CoA hydrolysing activity immediately, according to the method of Prass et al. (1980) except that 0·15mM acetyl-CoA was used. The reaction mixture contained in 1ml: Tris HCl buffer pH8 (30mM); 5,5′-dithiobis-(2-nitrobenzoic acid) (0·2mM); cell-free homogenate (5-20μl, containing 300-500μg protein). The reaction was initiated by addition of acetyl-CoA (0·1mM). Rate of reaction was followed by measuring rate of increase in absorbance at 412nm against a control cuvette which contained all of the

reactants except acetyl-CoA. Rates of CoASH formation were calculated using the ε value for 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at 412nm as $13{\cdot}6 \times 10^3$ $mole^{-1}$ cm^{-1} (Prass et al., 1980). All assays were in triplicate. Protein in cell-free extracts was determined using the Biuret procedure.

5.8 Other analytical procedures

5.8.1 Yeast dry weight:-

Yeast dry weight was generally used as a measure of the concentration of yeast in cultures. Samples (usually 5ml) were centrifuged in a bench centrifuge (full speed) for ca. 2 min and the resulting pellets washed x3 with deionized water (5ml). The washed pellets were transfered quantitatively to pre-weighed aluminium caps which were re-weighed after drying in an oven at 105°C for 48h.

5.8.2 DNA:-

The DNA content of yeast samples was measured according to the method of Stewart (1975). An equal volume (2ml) of ice-cold 0·5M perchloric acid (PCA) was added to a cooled suspension (2ml) of washed yeast (10-40mg dry weight) and incubated on ice for 15min. Following centrifugation (at 4°C) the pellet was resuspended in ice-cold water (2ml) and 0·5M PCA (2ml) and the above procedure repeated. Pelleted cells were then resuspended in 0·5M PCA (1·5ml) and heated for 15 min at 70°C. The heated suspension was then centrifuged, the

supernatant collected and the pellet extracted at 70°C twice more. Supernatants were pooled and made up to 5ml with 0·5M PCA and assayed for deoxyribose using diphenylamine.

Diphenylamine reagent consisted of 1·5g diphenylamine dissolved in 100ml concentrated glacial acetic acid plus 1·5ml sulphuric acid (18M). Just prior to use, 500µl of acetaldehyde solution (16mg ml^{-1} in H_2O) was added per 100ml reagent.

Diphenylamine reagent (2ml) plus sample (1ml PCA extract) were mixed and incubated at 25°C for ca. 20h. The absorbance of the samples was read at 600nm against a reagent blank. A standard curve was constructed using salmon testes DNA, in the range 10-100µg DNA. The calibration curve was linear. To prepare salmon testes DNA for use in construction of standard curve, an equal volume of DNA stock solution (200µg DNA ml^{-1} in 5mM NaOH) was added to 1M PCA and incubated at 70°C for 15 min. All samples were analysed in duplicate.

5.8.3 Protein:-

The protein content of whole cells was measured using the Biuret procedure (Herbert et al., 1971). 2M NaOH (1ml) was added to a washed cell suspension (1ml, 10mg dry weight) and heated in a boiling water bath for 5 min. Samples were then cooled and each was mixed with 0·1M $CuSO_4$ (1ml). The colour was allowed to

develop for 5 min and then the precipitate was removed by centrifugation. Absorbance of samples was measured against a reagent blank at 555nm. A standard curve in the range 0-8mg protein was constructed using bovine serum albumin.

The protein content of cell-free extracts of yeast (used for assay of acetyl-CoA hydrolysing activity) was also measured using the biuret procedure as above except that 1ml diluted cell extract (usually a 50-fold dilution) was incubated with the 2M NaOH. All assays were in triplicate.

5.8.4 Glycogen:-

The glycogen content of yeast was measured using the specific, enzymic method of Quain (1981). This method involves extraction of glycogen with sodium carbonate, followed by perchloric acid, and then hydrolysis of the glycogen with amyloglucosidase to glucose which was then estimated enzymically (Bergmeyer et al., 1974). All analyses on glycogen extracts were in duplicate.

5.8.5 Glucose:-

Cell-free medium was assayed for glucose using the glucose oxidase method (Perid Kit, Boehringer) or using hexokinase and glucose-6-phosphate dehydrogenase (Bergmeyer et al., 1974). When using the glucose oxidase method a calibration curve in the range of 10 to 100µg glucose was constructed for each series of analyses.

5.8.6 Specific gravity :-

Progress of wort fermentations was followed by monitoring the specific gravity of the fermenting wort after removal of cells by centrifugation. Either the equilibrium drop method of Ault (1954) or a Digital Density Meter (Paar DMA 45) was used.

5.8.7 Oxygen:-

The concentration of dissolved oxygen in wort was determined using a Yellow Springs Oxygen Meter (Model 54A) and electrode.

5.8.8 Yeast cell counts:-

Cell numbers in cultures were determined using a Thoma haemocytometer. However, microscopic counting of cells was found to be inconvenient for routine analyses of large numbers of samples such as were obtained in experiments reported in 6.6.1. The DNA content of yeast (NCYC 240) was found to be in the range 55-70 fg cell^{-1} and in agreement with Rose (1976) it was concluded that determination of total DNA of the culture provides a useful and accurate measurement of cell number. Therefore cell numbers were determined in this manner as indicated in relevant sections.

5.9 Calculations

5.9.1 Consumption of oxygen for syntheses of sterols and unsaturated fatty acids :-

The information presented in Table 15 concerning

consuption of oxygen for the syntheses of sterols and unsaturated fatty acids was derived from data in Table 3.b and Fig.4.c by applying the following criteria:

(i) Different amounts of oxygen are required for the synthesis of different sterols. Only 1 mole O_2 is required for the synthesis of 1 mole lanosterol from squalene butthe amounts of oxygen required for the synthesis of other sterols are: 4-α-methylzymosterol, 7 O_2; zymosterol, 10 O_2; fecosterol, 10 O_2; episterol, 10 O_2; 24(28)-dehydroergosterol, 12 O_2; ergosterol, 12 O_2. The synthesis of 1 mole of palmitoleic ($C_{16:1}$) or oleic ($C_{18:1}$) acid requires 1 mole O_2.

(ii) Small amounts of sterols and unsaturated fatty acids were added to the cultures in the yeast inocula. These amounts were subtracted from those presented in Table 3.b and Fig.4.c so that all subsequent calculations were based on the amounts of lipids synthesized during fermentation.

(iii) All values for lipids in $\mu g\ ml^{-1}$ were converted to $\mu moles\ ml^{-1}$. The amount of oxygen (μmoles) required for the synthesis of these amounts of lipids was then calculated by applying the premise stated in (i).

5.9.2 Utilization of acetyl-CoA for lipid syntheses during fermentation :-

Calculations were performed on data from certain experiments in 6.2 to estimate the amount of acetyl-CoA utilized by syntheses of fatty acids, sterols and ethyl acetate, and to see whether addition of linoleic acid

to wort affected acetyl-CoA utilization by these syntheses.

Values given in Tables 6, 11 and 13 for ethyl acetate, fatty acids and sterols respectively were converted from $\mu g\ ml^{-1}$ to $\mu moles\ ml^{-1}$ and then the following criteria were applied:

(i) that 1μmole of acetyl-CoA is required for the synthesis of 1μmole of ethyl acetate

(ii) that 3μmoles of acetyl-CoA are required for the synthesis of 1μmole of hexanoic acid and so on up to stearic and oleic acids the syntheses of which require 9μmoles of acetyl-CoA

(iii) that 18μmoles of acetyl-CoA are required for the syntheses of 1μmole of squalene (C_{30}) and therefore 1μmole of any sterol, even though some sterols contain less than thirty carbon atoms. 18μmoles and not 15μmoles are required because of the decarboxylation step catalysed by pyrophosphomevalonate decarboxylase (Fig.2.a). The net reaction therefore is three acetate units forming one isopentyl unit and six of the latter forming the squalene skeleton.

The values for fatty acids and sterols in Tables 11 and 13 include the amounts of those lipids which were added to the fermentation in the yeast inoculum. Consequently, for calculation of acetyl-CoA consumption these small amounts were subtracted from the values shown. No account was taken of acetyl-CoA consumed by other

syntheses e.g. citrate or acetate production.

5.10 Materials:-

All inorganic salts and reagents, organic solvents and carbohydrates were of Analar grade or of the highest purity available and were obtained from B.D.H. Laboratory Chemicals Division, Poole, England. Fatty acids and lipids, acetyl phosphate, acetyl-coenzyme A (trilithium salt), deoxyribonucleic acid (salmon testes), diphenylamine, 5,5′-dithiobis-(2-nitrobenzoic acid), dithioerythritol, dithiothreitol, N-ethylmaleimide, imidazole (fluorimetric grade), L-malic acid, oxaloacetic acid, phosphotransacetylase (EC 2.3.1.8) were purchased from Sigma Chemical Company, London, England. Adenosine triphosphate, nicotinamide adenine dinucleotide (free acid of oxidized form and disodium salt of reduced form), nicotinamide adenine dinucleotide phosphate (disodium salt, oxidized form), citrate synthase (EC 4.3.1.7) and malate dehydrogenase (EC 1.1.1.3.7) came from The Boehringer Corporation (London) Ltd, Lewes, East Sussex, England. Compressed gases and gas-liquid chromatography stationary-phases were obtained from British Oxygen Company, Brentford, Middx, England, and Perkin-Elmer, Beaconsfield, Bucks, England, respectively.

RESULTS

6.1 General Effects of Supplementing Wort Fermentations with Fatty Acids

To examine the effects of exogenous fatty acids on fermentation and production of beer flavour compounds, the wort was supplemented with stearic, oleic or linoleic acid at a concentration of 50μg ml^{-1} prior to inoculation. All fermentations attenuated to a specific gravity of 1·009 although biomass production was ca. 20% higher in fermentations supplemented with linoleic acid.

6.1.1 Effects on production of beer flavour compounds

The concentrations of acetate esters, fusel alcohols, and medium chain length fatty acids in beer were measured (Table 2). The production of the two acetate esters was reduced by addition of oleic or linoleic acid, the latter being more effective. Linoleic acid reduced the concentration of ethyl acetate by 70% and that of iso-amyl acetate by 80%. Addition of stearic acid had the reverse effect; it increased production of ethyl and iso-amyl acetates by ca. 20%. The content of C_6-C_{10} fatty acids of beer was reduced by 50% by oleic acid and to a lesser extent by linoleic acid; stearic acid had no such effect. In general, fusel alcohol production was stimulated by each of the three acids.

6.1.2 Uptake and incorporation of fatty acids into the lipids of Sacch. cerevisiae

The presence of long chain fatty acids, particularly

Table 2. Effect of addition of long chain fatty acids, to wort, on the production of flavour compounds by Sacch. cerevisiae during wort fermentation

Addition	Fatty Acids C_6-C_{10}			Esters		Fusel Alcohols		
	C_6	C_8	C_{10}	Ethyl-acetate	Iso-amyl acetate	n-Propanol	Iso-butanol	Amyl alcohols
None	3·4±0·3	4·6±0·3	0·5 ±0·08	20·6±0·8	1·0±0·1	27·0±1·0	18·5±0·5	46·8±4·4
18:0	3·5	4·6±0·1	0·5	24·3±2·9	1·2±0·2	40·0±0·4	26·0±1·0	69·7±3·2
18:1	1·3±0·2	2·7±0·08	0·4 ±0·06	11·4±0·9	0·6±0·1	50·0±5·5	55·1±5·1	119·3±9·2
18:2	2·8±0·4	3·2±0·07	0·44±0·10	6·7±0·6	0·2	37·1±1·6	31·4±2·0	81·0±4·8

Wort supplemented with either stearic (18:0), oleic (18:1) or linoleic (18:2) acid at a concentration of 50µg ml^{-1}. Beers analysed ca. 60h after inoculation. All values are in µg $(ml\ beer)^{-1}$ and represent the mean of duplicate determinations from two separate fermentations. Errors (±) represent the range of determinations.

unsaturated fatty acids in wort, significantly affected the production of certain important groups of flavour compounds by yeast. The fatty acid composition of yeast from such fermentations was determined, in order to investigate how exogenous acids may exert such effects.

The data of Table 3.a indicate that the exogenous acids were taken up by yeast. This is particularly evident in the case of supplementation with linoleic acid, since Sacch. cerevisiae does not synthesize this acid. However, Sacch. cerevisiae synthesizes both stearic and oleic acid and therefore where these acids have been added to wort their uptake by yeast is not as readily apparent as it is in the case of linoleic acid. However the amount of oleic acid in yeast, from fermentations supplemented with this acid, is much higher than in the control yeast. The same is true for stearic acid, in yeast from fermentations supplemented with stearic acid. Therefore when oleic and stearic acid are added to wort they are almost certainly taken up by yeast. However, the difference between values from supplemented and control fermentations can be taken only as an indication of the amount of exogenous acid incorporated.

The free fatty acid content of all yeast samples except those from stearic acid-supplemented fermentations was very low (Table 3.c). Therefore essentially all of the fatty acids in yeast, including exogenously derived

Table 3.a Total fatty acyl composition of yeast from fermentations supplemented with C_{18} fatty acids

Addition	Fatty acid - μg (mg dry weight)$^{-1}$										
	C_6	C_8	C_{10}	C_{12}	C_{14}	$C_{14:1}$	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$
None	0·03	0·45	2·5 ±0·1	1·1 ±0·1	0·3 ±0·05	Tr	5·5 ±0·5	0·86 ±0·7	4·2 ±0·5	2·5 ±0·2	-
18:0	0·04 ±0·01	0·4	2·5 ±0·1	1·1	0·3	0·01	4·6 ±0·4	0·75 ±0·4	8·8 ±0·7	2·4 ±0·3	-
18:1	0·02	0·17 ±0·01	1·1 ±0·1	0·5	0·6 ±0·05	Tr	4·2 ±0·25	0·5 ±0·1	3·4 ±0·8	9·1 ±0·5	-
18:2	0·02	0·2 ±0·03	1·3	0·55 ±0·05	0·4 ±0·1	Tr	4·4 ±0·2	0·4	2·8 ±0·2	0·8 ±0·1	3·0 ±0·1

Wort supplemented with C_{18} fatty acids as detailed in legend to Table 2. Yeast samples removed during stationary-phase. Analyses performed on freeze-dried yeast. All values are mean of single determinations from two separate fermentations. Errors (±) represent the range of values obtained.

Table 3.b Total fatty acyl composition of yeast from fermentations supplemented with C_{18} fatty acids - summary of data presented in Table 3.a

Addition	Sum of $C_{6-18:2}$ fatty acids $\mu g\ (mg\ yeast)^{-1}$	Sum $C_{6-18:2}$ $\mu g\ (ml\ culture)^{-1}$	% UFA	Sum $C_{6-18:1}$ synthesised by yeast $\mu g\ (ml\ culture)^{-1}$
None	17·5	59·8	19	59·8
18:0	20·9	62·3	15	*
18:1	19·6	65·8	49	*
18:2	13·9	61·5	30	48·2

Experimental and analytical details as in Table 3.a. For calculation of % UFA see section 5.5.1. Data are also expressed in $\mu g\ (ml\ culture)^{-1}$. These values are calculated by multiplying the values for total amount of fatty acyl residues in yeast by the yeast dry weight $(mg\ ml^{-1})$ of the culture. Therefore expression of the fatty acyl content of yeast on a $\mu g\ ml^{-1}$ basis takes into account differences in growth between cultures. Values do not include acids (i.e. C_6-C_{10}) released into beer.

* These values cannot be accurately determined (see text).

Table 3.c Free fatty acid composition of yeast from fermentations supplemented with C_{18} fatty acids

Addition	C_6-$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	Sum μg (mg yeast)$^{-1}$	Sum μg (ml culture)$^{-1}$
None	0·73 ±0·06	0·55 ±0·06	0·25 ±0·13	-	1·53 ±0·14	5·25
18:0	0·52 ±0·05	5·2 ±1·9	Tr	-	5·74 ±1·95	17·1
18:1	0·51 ±0·02	0·26	0·63 ±0·1	-	1·4 ±0·12	4·7
18:2	0·3 ±0·02	0·12 ±0·02	0·04 ±0·01	0·09 ±0·01	0·55 ±0·06	2·43

Details of experimentation as in Table 3.a

See section 6.5 for measurement of free fatty acids in yeast.

All values are mean of single determination from two separate fermentations; ± values represent the range of values obtained.

oleic or linoleic acid, are in saponifiable form (e.g. as phospholipids or acylglycerols). However, a high percentage of the stearic acid in yeast from fermentations supplemented with this acid appeared to be in the free form. It was not removed from the yeast by washing (see methods section) but neither was it esterified in the form of complex lipids.

Expression of data on a μg (ml culture)$^{-1}$ basis shows that synthesis of fatty acids (C_6-$C_{18:1}$) by yeast was reduced by 20% in fermentations supplemented with linoleic acid (Table 3.b). Such an inhibitory effect on total fatty acid synthesis by the addition of stearic and oleic acid cannot be observed directly since yeast synthesizes both of these acids. However, it is clear that oleic acid like linoleic acid, reduced the formation of medium chain length fatty acids (C_6-C_{10}) by $>$50% (Tables 1 and 3.a).

Thin layer chromatography (TLC) of yeast lipids was used to establish that the exogenous fatty acids were incorporated into the functional lipids of yeast i.e. the phospholipids. Six classes of lipid could be separated using TLC: sterols; steryl esters plus squalene; monoacylglycerols; free fatty acids plus diacylglycerols; phospholipids; triacylglycerols. The fatty acyl composition of each class (excluding sterols) was determined. The overall amount of fatty acyl residues in the phospholipids as a percentage of the

total fatty acyl residues varies slightly for the different yeasts but is ca. 40% (Table 4). The most striking feature of this analysis is that the % UFA of the phospholipid fraction of all the yeasts was very similar to that of the overall % UFA. Further, the percentage of the total linoleic acyl residues in the yeast, that was present in the phospholipids (42·9%) was very similar to the percentage of the total fatty acyl residues in the phospholipids. These data suggest that yeast does not or possibly cannot radically alter its membrane lipid fatty acyl composition from that of its lipids as a whole. Therefore by supplementing wort with fatty acids, the fatty acid composition of yeast phospholipids can be manipulated. This approach is used in 6.2 where varying amounts of linoleic acid were added to wort in order to study the effect of yeast membrane lipid unsaturation on production of beer flavour compounds. The TLC data also indicate that determination of the total fatty acyl composition of yeast by direct saponification provides a good indication of the fatty acyl composition of the phopholipids. Thus in later sections where the % UFA of the total fatty acids was increased by addition of linoleic acid to wort it is assumed that the % UFA of the phospholipids was increased similarly. It should be noted that the overall % UFA of the lipids from the TLC plates (Table 4) was higher than that derived by direct saponification (Table 3.b) e.g. the control yeast lipids contained overall 19% UFA when analysed

Table 4. Comparison of certain aspects of the fatty acyl composition of yeast phospholipids with the overall fatty acyl composition

Addition	Fatty acyl residues in phospholipid fraction (% of total fatty acyl residues)	Overall % UFA residues of yeast lipids	% UFA residues of phospholipid fraction	18:2 present in phospholipid fraction (% of total 18:2 in yeast)
None	38·6	24·9	24·0	-
18:0	42·4 ± 2·7	26·0 ± 3·9	24·7 ± 3·9	-
18:1	54·8 ± 22·6	48·5 ± 6·3	49·3 ± 8·6	-
18:2	42·5 ± 1·1	37·3 ± 0·4	33·8 ± 1·6	42·9 ± 0·4

Values are mean of at least two determinations.

Errors (±) represent the range of values obtained.

For abbreviations (18:0, 18:1, 18:2) see Table 2.

by direct saponification (Table 3.b) but 24·9% UFA when analysed by TLC. However, this does not affect the reasoning of the above arguments.

6.1.3 Effects on the synthesis of sterols

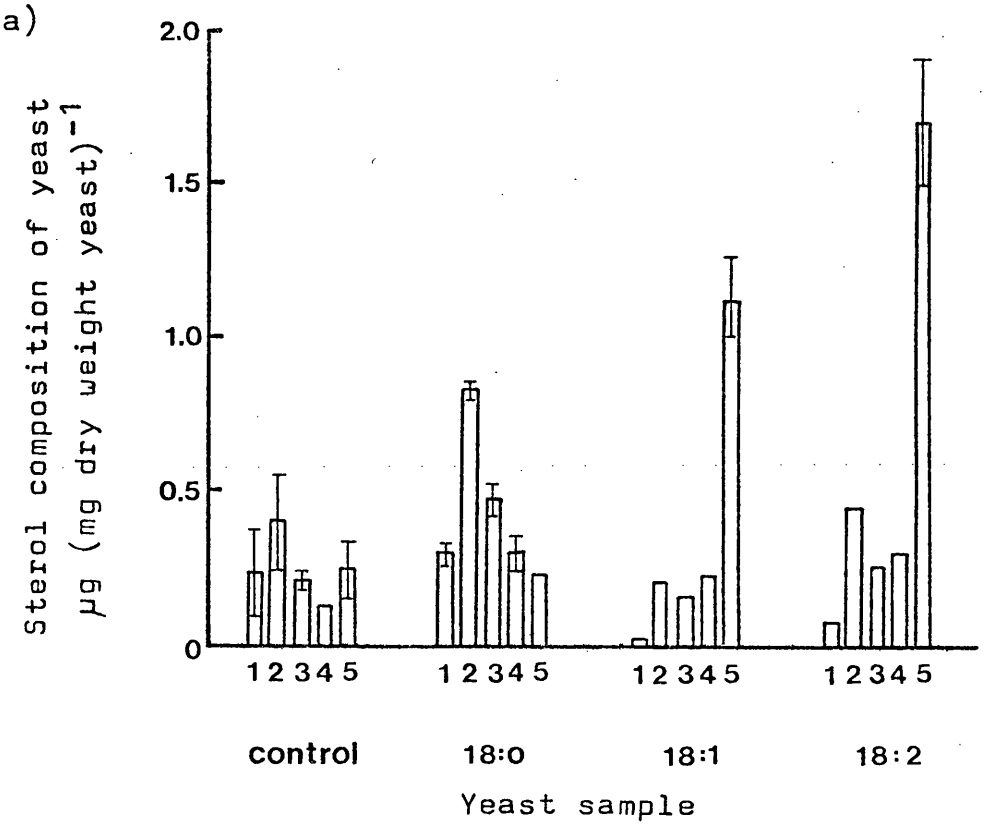
The total sterol content of yeast from unsupplemented (i.e. control) fermentations was ca. 1μg (mg dry weight)$^{-1}$ (Fig 4.b) in agreement with other workers (Aries et al., 1977; Aries & Kirsop, 1978). Of this, ergosterol was the predominant sterol followed by lanosterol. Zymosterol, fecosterol and 4-α-methylzymosterol were also detected. Expression of this data on a μg (ml culture)$^{-1}$ basis showed that ca. 4μg sterol ml^{-1} were synthesized (Fig 4.d). Yeast synthesized more sterol when stearic acid was added to wort. The sterol content of the yeast was doubled (Fig 4.b) and the pattern of sterols was similar to the control yeast (Fig 4.a). Less growth (ca. 10%) occurred in the cultures supplemented with stearic acid and ca. 6μg sterol ml^{-1} were formed (Fig 4.d). Sterol synthesis was stimulated by the addition of unsaturated fatty acids, linoleic acid being more effective than oleic acid (Fig 4.a,b,c,d) in this respect. In particular the synthesis of lanosterol was stimulated, and in these fermentations on a μg ml^{-1} basis (Fig 4.c) lanosterol was increased between 6 and 8-fold by linoleic acid. Overall the amount of sterol formed by the linoleic acid-supplemented cultures was ca. 3-fold greater than control cultures (Fig 4.d). Both oleic and linoleic acids substantially reduced the

Figure 4.

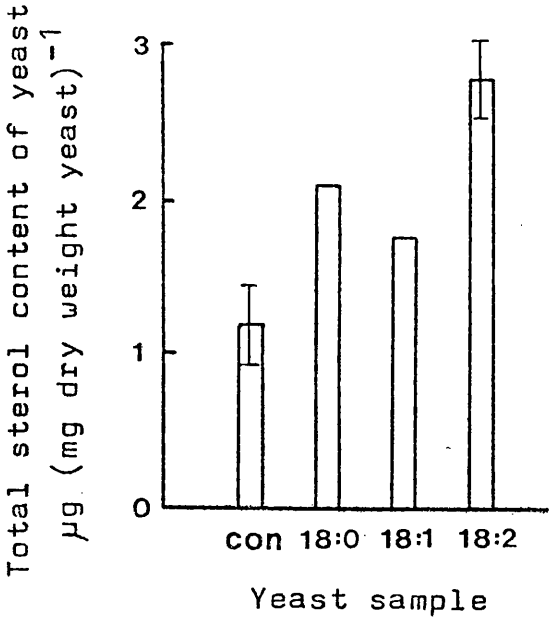
Effect of exogenous fatty acids on the sterol content of yeast from wort fermentations. Experimental details as in Table 3. Analyses performed on freeze-dried yeast. All values are mean of single determinations on samples from two separate cultures. Bars indicate range of values obtained.

In Fig.4.a the histograms numbers 1,2,3,4,5 refer to; zymosterol, ergosterol, fecosterol plus 4-α-methylzymosterol, episterol and lanosterol respectively.

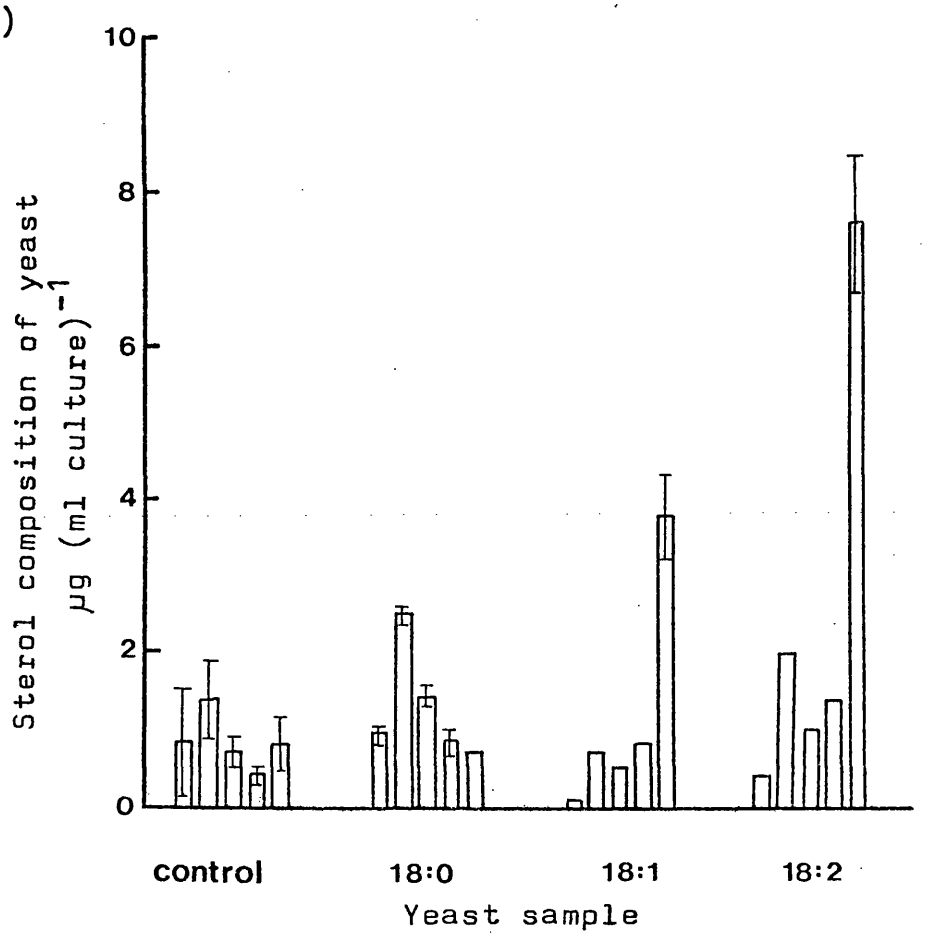
(a)


Sterol composition of yeast
μg (mg dry weight yeast)$^{-1}$
2.0
1.5
1.0
0.5
0
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
control
18:0
18:1
18:2
Yeast sample

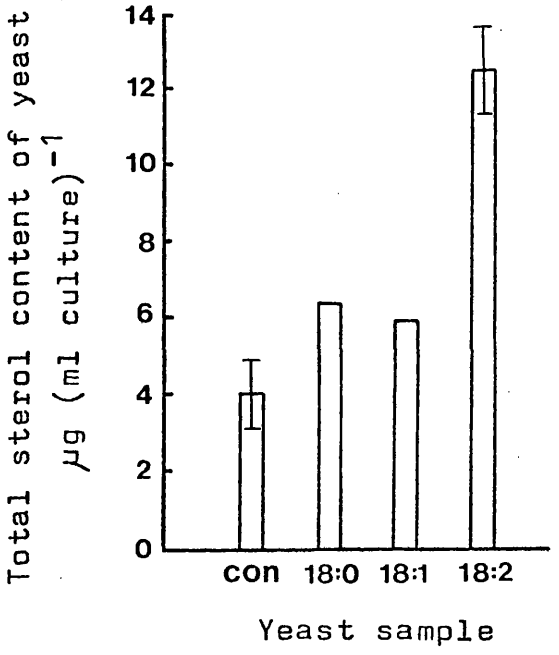
(b)


Total sterol content of yeast
μg (mg dry weight yeast)$^{-1}$
3
2
1
0
con
18:0
18:1
18:2
Yeast sample

(c)



(d)



All experimental and analytical details as in Fig.4.a and b

formation of squalene. Overall, because squalene is in excess of sterol in anaerobic yeast, the amount of squalene plus sterol was reduced (Table 5). This indicates that less acetyl-CoA was consumed by the sterol biosynthetic pathway. Addition of stearic acid to wort, had no such effect (Table 5).

Table 5. Effect of addition of C_{18} fatty acids to wort on the formation of squalene by Sacch. cerevisiae

Addition	μg squalene (mg dry weight yeast)$^{-1}$	μg squalene (ml culture)$^{-1}$	μg squalene + sterol (ml culture)$^{-1}$
None	7·50±1·20	25·72±4·12	29·8 ±5·32
18:0	7·62±0·57	22·70±1·70	29·0 ±1·70
18:1	2·42±0·09	8·13±0·30	14·0 ±0·33
18:2	1·97±0·44	8·83±1·97	21·37±3·0

Experimental details as in Table 3. Analyses performed on freeze-dried yeast. All values are the mean of single determinations from two separate fermentations. Errors (±) represent the range of values obtained. Data are also expressed as μg (ml culture)$^{-1}$ and are calculated by multiplying values for μg (mg dry weight yeast)$^{-1}$ by the dry weight (yeast) of the culture (mg ml^{-1}).

6.2 Alteration of the % UFA of Yeast by Supplementing Wort with Varying Concentrations of Linoleic acid - Effects on Beer Flavour and Lipid Synthesis

The effects of unsaturated fatty acids on beer flavour and synthesis of lipids by yeast were further investigated by altering the unsaturated fatty acid content of NCYC 240. This was achieved by supplementing the wort with various concentrations of linoleic acid. This acid was used rather than oleic acid since it is not synthesised by Sacch. cerevisiae and therefore its accumulation into the lipids of yeast can easily be followed. Further, it is the major unsaturated fatty acid in barley and therefore can be naturally present in wort in varying amounts; although the wort used in all of these studies contained only traces of linoleic acid.

6.2.1 Effects on synthesis of beer flavour compounds

The formation of acetate esters and of medium chain lenth fatty acids was inhibited significantly when low concentrations, ca. 5-10µg ml^{-1} of linoleic acid were added to wort (Table 6). Above a concentration of 50µg ml^{-1}, little further reduction was observed. Therefore linoleic acid is more effective than oleic acid, at reducing the synthesis of beer flavour compounds; oleic acid gives maximum effect at 140µg ml^{-1} (Äyräpää & Lindström, 1977).

Production of iso-amyl alcohols, and to a lesser

Table 6. Effect of addition of various amounts of linoleic acid to wort, on the production of beer flavour compounds

Addition Linoleic acid $\mu g(ml\ wort)^{-1}$	Fatty acids C_{6-10}			Esters		Fusel alcohols		
	C_6	C_8	C_{10}	Ethyl acetate	Iso-amyl acetate	n-Propanol	Iso-butanol	Amyl alcohols
None	1·95	5·63	0·72	39·1	2·4	20·4	19·3	54
5	1·77	4·74	0·62	36·6	3·2	21·2	17·3	56
10	2·60	3·07	0·60	31·1	2·1	25·9	19·2	64
15	1·83	3·53	0·54	25·7	1·9	22·7	15·4	63
25	1·32	2·98	0·41	17·6	1·0	29·2	15·8	68
50	1·14	2·20	0·50	9·9	0·3	29·2	16·7	70
100	0·78	2·24	0·53	7·5	0·3	30·6	18·3	74
150	0·95	2·06	0·40	6·9	0·1	35·1	24·2	86

Analyses performed on beers after ca. 60h of fermentation. All values are in $\mu g\ (ml\ beer)^{-1}$ and are mean of single analyses from duplicate fermentations. Range of values not quoted but generally in the range ± 5% (see Table 2)

extent n-propanol, was stimulated, perhaps due to the stimulation of growth (Anderson & Kirsop, 1974) by linoleic acid. The final biomass from these fermentations was increased by ca. 20% at the higher concentrations of linoleic acid used.

Linoleic acid also repressed the formation of ethyl acetate when added mid-way through a fermentation, though the effect was not as marked as when added to wort prior to inoculation (Fig.5). Addition of 50μg linoleic acid ml^{-1} at 20h resulted in a reduction (ca. 55%) in the rate of ethyl acetate formation within 2h, and this then remained low throughout fermentation (Fig.5). Linoleic acid was incorporated into saponifiable lipids very rapidly (Table 7) a process which requires coenzyme A to activate the fatty acid (Weiss & Kennedy, 1956; Numa, 1981). Previously it has been suggested (Anderson & Kirsop, 1974) that consumption of coenzyme A for activation of exogenously derived fatty acids restricts acetyl-CoA synthesis and therefore reduces the formation of acetate esters. However, since linoleic acid was rapidly incorporated into yeast lipids the requirement for coenzyme A must be transient and therefore cannot account for the extensive repression of ethyl acetate synthesis by this acid. This was also apparent in studies where pitching yeasts enriched with linoleic acid were used (see below). Here the process of uptake and incorporation has been eliminated since the linoleic acid is present in the

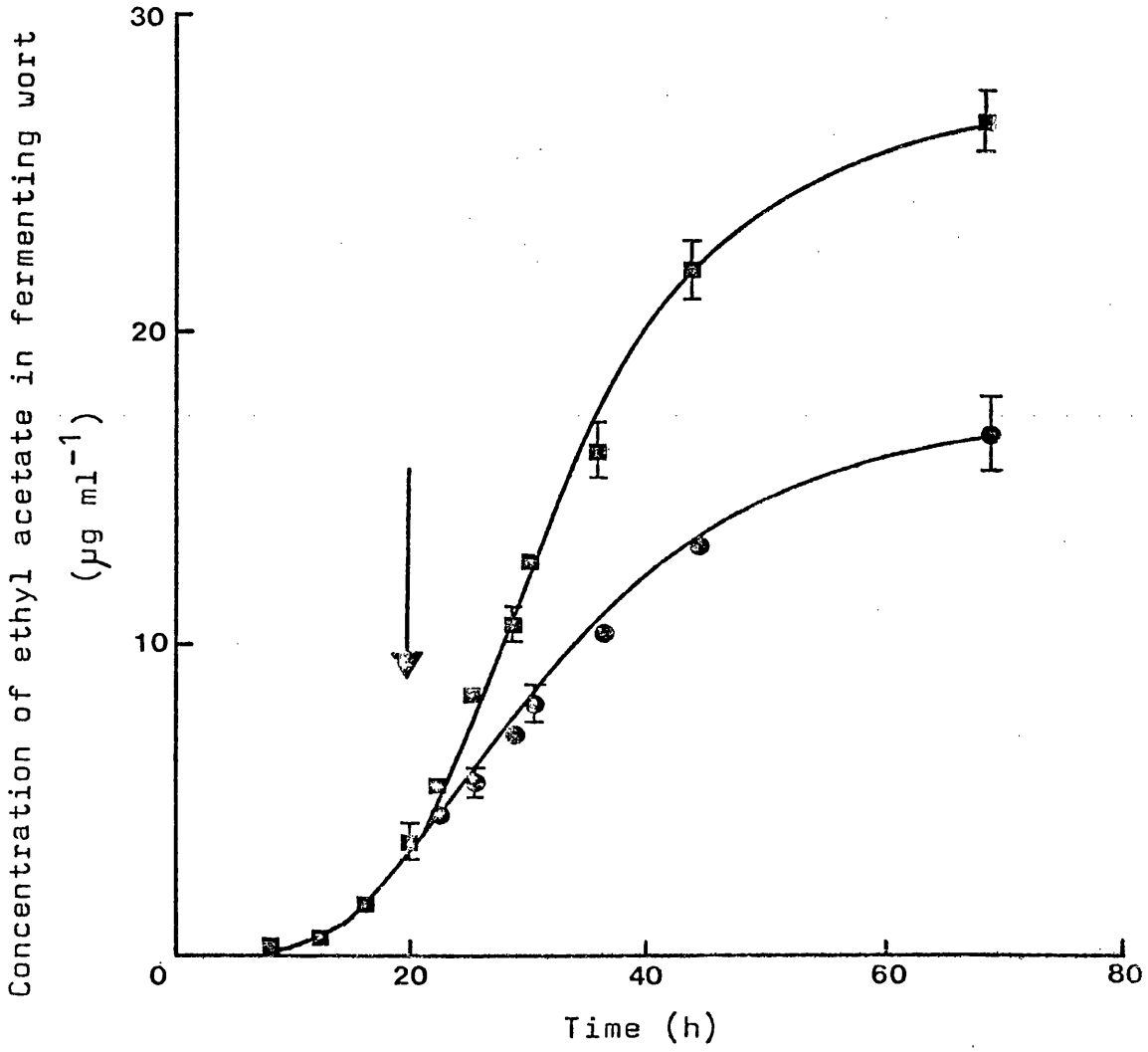


Figure 5.

Effect of linoleic acid on the rate of ethyl acetate formation during fermentation. Fermentations of all-malt wort supplemented with either 4ml ethanol l^{-1} containing 50mg linoleic acid (●-●) or 4ml ethanol l^{-1} (■-■). Point of additions is arrowed. All points are the mean of duplicate determinations from duplicate fermentations; bars show range of values obtained.

Table 7. Rate of incorporation of exogenous linoleic acid into lipids of Sacch. cerevisiae

		Time after addition of linoleic acid (h)			
		0·5	2	5	52
Linoleic acid	Free	0·12	0·08	0·10	0·06
μg (mg dry weight yeast)$^{-1}$	Total	5·40	9·40	7·60	5·60

Experimental details as in Fig.6.

The low levels of free linoleic acid in the yeast show that $>98\%$ of this acid is in esterified form.

All values are average of at least two determinations.

yeast already esterified into complex lipids. Four yeast strains (NCYC 240, 1062, 1026, 1245) were enriched with linoleic acid by adding 1mg linoleic acid $(ml\ wort)^{-1}$ to the propagation medium. The extent to which the fatty acyl residues of the four yeast strains were enriched is shown in Table 8. The content of linoleic acid in yeast varied with yeast strain from ca. 55-100μg $(mg\ dry\ weight)^{-1}$. Therefore since the inoculum size is 0·3mg dry weight ml^{-1}, linoleic acid is added (as yeast lipids) at between 17 and 30μg ml^{-1}. The concentrations of acetate esters in beers produced by these enriched yeasts, were much lower than in those produced by unenriched yeasts (Table 9). This reduction varied with yeast strain from ca. 60% - 75% in the case of ethyl acetate, whilst iso-amyl acetate was reduced by between 69% and 84%. This suppression of acetate ester synthesis is comparable with that obtained when linoleic acid is added directly to wort (Table 6).

Strain NCYC 240 was also enriched with stearic or oleic acid. The extent to which the yeast lipids were enriched by these acids is shown in Table 10.a. Enrichment with stearic acid caused NCYC 240 to release more medium chain length fatty acids into beer and synthesize more ethyl acetate although the synthesis of iso-amyl acetate was unaffected (Table 10.b). Enrichment with oleic acid had little effect on the synthesis of ethyl acetate or medium chain length fatty acids;

Table 8. Total fatty acyl composition, of four strains of Sacch. cerevisiae enriched with linoleic acid, and of the un-enriched yeasts

Yeast Strain	Fatty Acid - μg (mg dry weight)$^{-1}$												
	C_6	C_8	C_{10}	C_{12}	C_{14}	$C_{14:1}$	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	sum $C_{6-18:1}$	Total
NCYC 240 control	Tr	1·0	3·2	1·7	0·4	Tr	8·2	1·5	4·0	1·4	nd	21·4	21·4
+ 18:2	Tr	0·3	1·1	1·0	2·2	Tr	17·0·	1·0	6·5	0·8	88·3	29·9	118·2
NCYC 1062 control	Tr	0·2	3·5	1·6	0·1	Tr	7·9	1·8	4·7	1·5	nd	21·3	21·3
+ 18:2	Tr	0·3	2·6	1·8	2·3	Tr	16·3	1·0	6·2	1·4	56·4	31·9	88·3
NCYC 1026 control	Tr	0·3	2·1	1·7	0·5	Tr	5·7	1·3	3·4	1·3	Tr	16·3	16·3
+ 18:2	Tr	0·2	1·5	1·4	3·1	Tr	19·4	1·2	8·0	1·3	103·1	36·1	139·2
NCYC 1245 control	Tr	0·3	2·8	2·0	0·5	Tr	6·1	1·1	3·3	1·0	Tr	17·1	17·1
+ 18:2	Tr	0·2	2·0	1·7	2·7	Tr	15·8	1·0	6·9	1·1	79·0	31·4	110·4

For details of enrichment (indicated above as +18:2), see text. Values are the mean of at least two determinations. Ranges of values not shown but were ca. ±5% (for reproducibility of analyses of fatty acyl composition, see 5·5). Tr - trace detected. nd - not detected.

Table 9. Production of flavour compounds by four strains of Sacch. cerevisiae enriched with linoleic acid

Yeast Strain	Fatty acids C_{6-10}			Esters		Fusel alcohols		
	C_6	C_8	C_{10}	Ethyl acetate	Iso-amyl acetate	n-Propanol	Iso-butanol	Amyl alcohols
NCYC 240 control	1·8	2·5	0·3	26	5·1	19	26	92
18:2-enriched	1·1	1·9	0·2	10	1·0	30	48	110
NCYC 1062 control	1·7	3·4	0·9	17	1·6	23	41	71
18:2-enriched	1·7	3·5	1·1	7	0·5	30	56	101
NCYC 1026 control	2·1	3·4	0·6	27	3·5	22	50	88
18:2-enriched	0·8	1·8	0·3	7	0·9	22	55	106
NCYC 1245 control	1·7	3·6	0·7	27	3·2	35	56	100
18:2-enriched	0·9	2·1	0·5	7	0·5	26	60	111

For details of enrichment see text. All figures in $\mu g\ ml^{-1}$ and are mean of duplicate determinations from duplicate fermentations. Range of these determinations was not $> 5\%$.

Table 10.a Fatty acyl composition of Sacch. cerevisiae enriched with stearic or oleic acid

Acid used for enrichment	μg Fatty acid (mg dry weight yeast)$^{-1}$										
	C_6	C_8	C_{10}	C_{12}	C_{14}	$C_{14:1}$	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	SUM
None	Tr	1·0	3·2	1·7	0·4	Tr	8·2	1·5	4·0	1·4	21·4
18:0	Tr	0·9	3·5	1·6	0·4	Tr	7·2	1·4	138·0	nd	153·0
18:1	Tr	0·3	1·1	1·6	2·5	Tr	12·3	0·8	5·6	119·0	143.2

For details of enrichment see text.

All values are mean of at least two determinations.

For reproducibility of analyses see section 5.5.

Table 10.b Flavour compounds of beers produced by Sacch. cerevisiae enriched with either oleic or stearic acid

Acid used for enrichment	Fatty acids C_{6-10}			Esters		Fusel Alcohols		
	C_6	C_8	C_{10}	Ethylacetate	Iso-amyl acetate	n-Propanol	Iso-butanol	Amyl-alcohol
None	1·8	2·5	0·29	26	5·1	19	20	92
18:0	2·5	4·2	0·56	40	4·9	32·	25	74
18:1	2·2	2·9	0·47	23	3·0	23	32	87

All figures in $\mu g\ ml^{-1}$ and are mean of single determinations on duplicate beer samples.

Range of values not quoted but generally in the range $\pm$ 5% (see Table 2).

synthesis of iso-amyl acetate was inhibited by ca. 40% (Table 10.b).

6.2.2 Effects on synthesis of fatty acids

Results of these experiments confirm some of the earlier observations (6.1); for example, the synthesis of C_6-C_{10} fatty acids was reduced by addition of linoleic acid (5-150µg ml^{-1}) to wort (Table 11). Quantitatively there was no substantial effect on total synthesis of fatty acids, low levels of linoleic acid resulting in a slight reduction (Table 11). The uptake of linoleic acid was incomplete in all cases and as discussed in the methods section this was probably due to inefficient fatty acid extraction. However, the yeasts' content of linoleic acid was proportional to the amount added to wort (Fig.6) and in all cases, greater than 95% of it was in esterified form.

The data in Table 8 (i.e. fatty acid composition of yeasts enriched with linoleic acid) show that growth of yeast in the presence of a high concentration of linoleic acid (1mg ml^{-1}) had quite marked effects on fatty acid synthesis. The amounts of unsaturated fatty acids, on a µg (mg dry weight)$^{-1}$ basis, synthesized by NCYC 240 and 1062 were reduced by 38% and 27% respectively. No reduction was observed for the other two strains. Overall, due to extensive uptake of linoleic acid, the % UFA for these yeasts was ca. 75% (NCYC 1245, 1026 and 240) or 65% (NCYC 1062) which is

Table 11. Synthesis of fatty acyl residues by Sacch. cerevisiae in fermentations supplemented with various amounts of linoleic acid

Addition of linoleic acid $\mu g\ ml^{-1}$	μg fatty acid $(ml\ culture)^{-1}$											Sum	$C_{6-18:1}$ in Yeast	$C_{6-18:1}$ Yeast + C_{6-10} * Beer
	C_6	C_8	C_{10}	C_{12}	C_{14}	$C_{14:1}$	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$			
None	0·1	1·5	10·5	3·0	0·7	Tr	15·6	2·4	11·3	3·1	nd	48·2	48·2	56·5
5	0·1	2·1	10·9	4·3	0·7	nd	13·7	2·5	8·2	2·6	1·5	46·6	45·1	52·2
10	0·1	1·7	8·9	3·3	0·6	nd	12·8	2·3	7·6	2·3	3·3	42·9	39·7	46·0
15	0·1	1·6	8·1	2·6	0·9	nd	12·3	2·2	7·9	2·8	4·6	43·1	38·4	44·4
25	0·09	1·2	7·0	2·7	1·0	nd	14·0	1·7	10·0	3·2	8·1	49·2	41·0	50·4
50	0·09	0·9	6·0	2·6	1·9	nd	19·5	1·7	12·4	3·8	13·1	62·0	48·9	52·8
100	0·07	0·9	4·8	2·5	2·3	nd	22·3	2·1	11·9	4·9	22·1	73·9	51·8	55·3
150	0·1	0·8	4·5	2·2	2·9	nd	22·2	3·2	13·6	4·9	44·0	98·4	54·4	57·8

Analyses performed on freeze-dried yeast (fermentations sampled after ca. 60h). Values represent mean of two determinations. For typical range of values for this type of analysis see section 5.5. Tr - trace detected nd - not detected * - value taken from table 6.

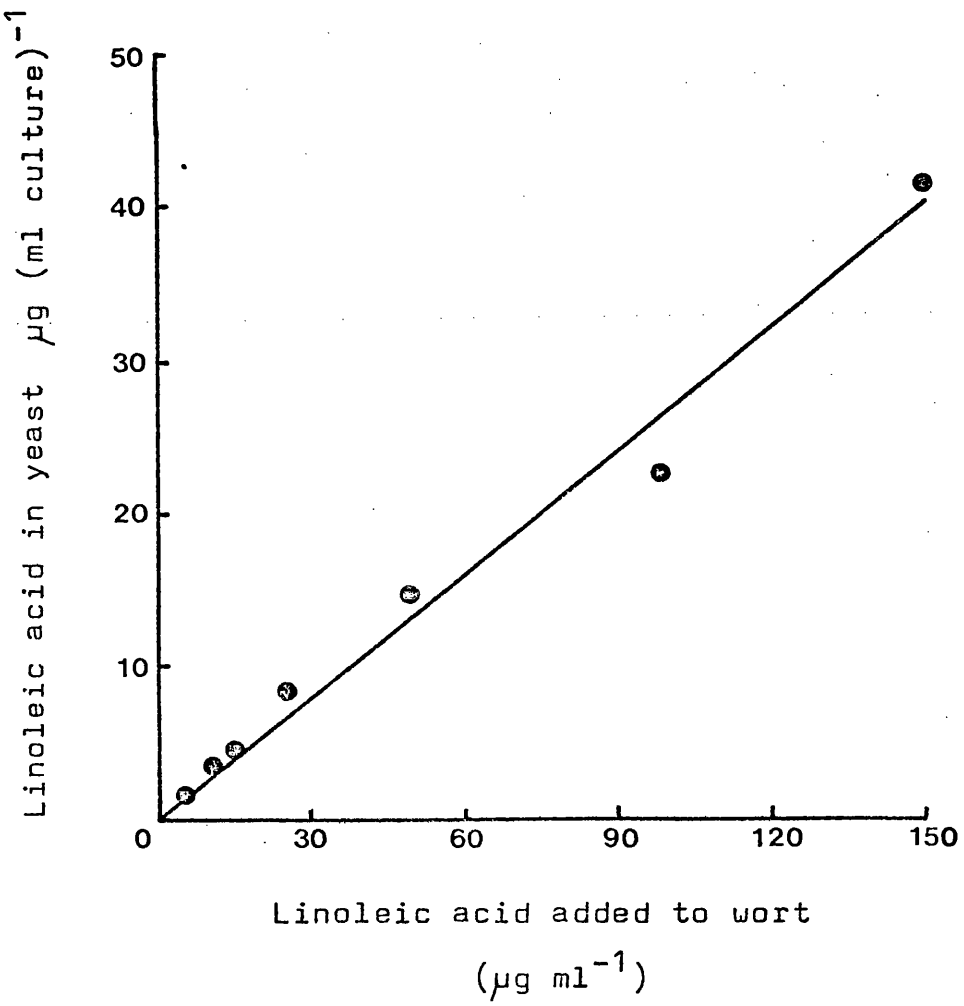


Figure 6.

Relationship between concentration of linoleic acid added to wort and the amount of linoleic acid in yeast. Data are derived from Table 11. Line drawn by linear regression; $r^2 = 0{\cdot}9742$

significantly lower than reported for aerobically-grown yeast (Jollow, et al., 1968; Hunter & Rose, 1972; Johnson & Brown, 1972). This was due to the fact that, rather than suppressing fatty acid synthesis, the high concentration of exogenous linoleic acid actually stimulated synthesis of fatty acids; particularly of palmitic acid. Hence this differs from the situation where Sacch. cerevisiae is grown aerobically, where addition of fatty acids to the growth medium represses fatty acid synthesis (Kamiryo & Numa, 1973; Meyer & Schweizer, 1976). As shown in Table 8, on a μg (mg dry weight)$^{-1}$ basis, palmitic acid was increased by 95%, 145%, 51% and 64% for NCYC 1245, 1026, 240 and 1062 respectively. These effects possibly reflect a need to maintain a certain level of saturated fatty acyl residues in the yeast lipids since a very high level of unsaturated residues may be deleterious to membrane function under these (anaerobic) conditions where the cellular concentrations of sterol are low (Fig.4.a,b). Notably certain fatty acid auxotrophs, and cells starved for fatty acid by addition of cerulenin will not grow when supplemented solely with unsaturated fatty acids (Henry & Keith, 1971; Otoguro et al., 1981). These data suggest that Sacch. cerevisiae can exert some control over its fatty acid composition in response to environmental conditions.

Yeast excretes or releases medium chain length fatty acids into beer (Taylor & Kirsop, 1977b; Aries et al.,

1977). Data taken from Tables 2,3,6 and 11 have been used to calculate the % of the total C_6-C_{10} acids formed by yeast, that are excreted into beer (Table 12). Yeasts with different fatty acid compositions, and therefore different membrane lipid compositions (see 6.1.2), excrete or release similar percentages of the total medium chain length fatty acids produced. The values for % excretion shown in Table 12 are in excellent agreement with those of Aries *et al.* (1977) for NCYC 240 though results of these workers suggest that membrane lipid composition may be important. They report that in fermentations conducted with aerobically propagated yeast, the percentage of C_6-C_{10} fatty acids excreted was *ca.* 50% lower than in fermentations where the yeast had been anaerobically propagated. The authors postulate that this effect is a reflection of the permeability of the plasma membrane, arising from alterations in the sterol and fatty acid composition of the yeasts propagated by different methods, However results presented here (Table 12) suggest that fatty acid composition does not play such a role. Thus, linoleic and oleic acid affect the amounts of C_6-C_{10} acids in beer by reducing synthesis of these acids rather than by affecting their release from the yeast cell. The effect of unsaturated fatty acids on suppression of medium chain length fatty acids is similar to that of oxygen (Suomalainen & Keranen, 1968; Jollow *et al.*, 1968; Sumper *et al.*, 1969). In fact Sumper (1974) considers the effect of oxygen to be

Table 12. Effect of addition of C_{18} fatty acids to wort on the excretion of medium chain length fatty acids

(a)

Addition	C_6-C_{10} fatty acids in beer	C_6-C_{10} fatty acids in yeast	% in beer
None	9·5	10·1	48
18:0	8·6	8·9	49
18:1	4·4	4·3	50
18:2	6·4	6·9	48

(b)

Addition of linoleic acid ($\mu g\ ml^{-1}$)	C_6-C_{10} fatty acids in beer	C_6-C_{10} fatty acids in yeast	% in beer
0	8·3	12·1	40
5	7·13	13·1	35
10	6·27	10·7	37
15	5·76	9·8	37
25	4·71	8·3	36
50	3·84	7·0	35
100	3·71	5·8	39
150	3·41	5·4	39

Data in Table 12.a taken from Tables 2 and 3.a. All C_{18} fatty acids added to wort at a concentration of 50$\mu g\ ml^{-1}$. Abbreviations as in Table 2.

Data in Table 12.b taken from Tables 6 and 7.

Values in both tables are μg (ml culture)$^{-1}$.

For reproducibility of analyses see original data.

mediated by the increased amounts of unsaturated fatty acids formed in the presence of oxygen. Long chain acyl-CoA esters bind to and inhibit acetyl-CoA carboxylase *in vitro* (Sumper, 1974). This leads to reduced availability of malonyl-CoA and thus fatty acid synthetase (FAS) activity is inhibited. Saturated fatty acyl-CoA esters are more effective inhibitors than esters of unsaturated fatty acids. As a consequence of such inhibition of FAS, medium chain length fatty acids are released prematurely. Under anaerobic conditions when synthesis of unsaturated fatty acids does not take place, this situation would be particularly marked and thus medium chain length acids are abundant. In the experiments reported here, anaerobic conditions were employed, but where exogenous unsaturated fatty acids were taken up by the yeast a lower degree of inhibition of acetyl-CoA carboxylase presumably took place and less medium chain length acids were produced.

6.2.3 Effects on synthesis of sterols

Sterol metabolism was substantially affected by the addition of linoleic acid to wort (Table 13). In these experiments the amount of squalene in yeast was reduced by up to 70%. The lanosterol content of yeast was increased 3-fold and total sterol synthesis *ca*. 2-fold. Overall, the amount of sterol plus squalene was reduced (by 40%) by linoleic acid; a concentration of 25µg linoleic acid ml^{-1} gave maximal effect.

Table 13. Effects of addition of various amounts of linoleic acid to wort on the synthesis of sterols and squalene by Sacch. cerevisiae

Addition of Linoleic acid $\mu g\ ml^{-1}$	Sterol μg (ml culture)$^{-1}$							
	Squalene	Zymosterol	Ergosterol	Fecosterol+ 4-α-methyl-zymosterol	Episterol	Lanosterol	Total Sterol	Sterol + Squalene
None	32·2	0·44	1·98	1·01	1·41	1·06	5·9	38·1
5	22·6	0·67	2·02	1·33	1·42	1·56	7·0	29·6
10	19·0	0·83	2·40	1·74	1·78	2·15	8·9	27·9
15	14·1	0·33	1·97	1·10	1·70	2·90	8·0	22·1
25	11·3	0·41	1·67	0·98	1·78	2·36	7·2	18·5
50	10·8	0·73	2·32	1·90	1·97	2·98	9·9	20·7
100	12·2	0·90	2·60	1·98	2·04	2·98	10·5	22·7
150	13·3	0·51	1·66	0·97	1·66	2·80	7·6	20·9

Experimental and analytical details as in Table 11. All values are mean of at least two determinations.

6.2.4 Consumption of acetyl-CoA by lipid syntheses during fermentation

The synthesis of ethyl acetate like that of fatty acids and of sterols consumes acetyl-CoA. The amounts of acetyl-CoA consumed by these biosyntheses were calculated from the data above and are presented in Table 14. Overall consumption of acetyl-CoA was reduced by linoleic acid. Possibly this reflects a reduction in acetyl-CoA synthesis although increased utilization of acetyl-CoA by alternative pathways (not accounted for here) cannot be ruled out. It is evident that at the lower levels of linoleic acid addition a constant proportion (ca. 13%) of the 'total' acetyl-CoA was utilized for ethyl acetate synthesis. When the concentration of linoleic acid was increased above 25μg ml^{-1} the total amount of acetyl-CoA consumed remained approximately constant, but the proportion consumed by ethyl acetate synthesis decreased from 13% to 3%. Therefore low levels of linoleic acid may inhibit synthesis of ethyl acetate by reducing acetyl-CoA synthesis, but higher levels of linoleic acid may directly affect the ester-synthesizing enzyme.

In following sections (6.5 and 6.7) both the intracellular levels of acetyl-CoA and the dynamic relationship between lipid syntheses and acetate ester synthesis are monitored throughout fermentations supplemented with linoleic acid. These studies throw further light on possible mechanisms by which linoleic acid suppresses ester synthesis.

Table 14. Effect of linoleic acid on the consumption of acetyl-CoA for the biosynthesis of fatty acids, sterols and ethyl acetate in Sacch. cerevisiae

Addition of Linoleic acid $\mu g\ ml^{-1}$	μ moles acetyl-CoA (ml culture)$^{-1}$				
	Fatty Acids	Sterols*	Ethyl acetate	Sum	% consumed by ethyl acetate biosynthesis
None	1·34	1·57	0·44	3·35	13·1
5	1·24	1·19	0·42	2·85	14·7
10	1·08	1·12	0·35	2·55	13·7
15	1·04	0·86	0·29	2·19	13·2
25	1·12	0·71	0·20	2·03	9·8
50	1·30	0·80	0·11	2·21	5·0
100	1·47	0·89	0·085	2·44	3·5
150	1·55	0·81	0·078	2·44	3·2

All data derived from Tables 6,11 and 13. For calculations see section 6.9.

* including squalene

6.3 Further Investigations on the Effect of Linoleic Acid on Sterol Synthesis

6.3.1 Consumption of dissolved oxygen for lipid syntheses

In the investigations described above, one of the most marked effects of addition of C_{18} fatty acids to wort, was on sterol synthesis. The amount of oxygen consumed for the syntheses of sterols and unsaturated fatty acids during the fermentations described above have been calculated (see Methods section) and are presented in Table 15. Yeast utilized less oxygen for the synthesis of unsaturated fatty acids when stearic, oleic or linoleic acid was added to wort, least unsaturated fatty acid being formed in the presence of linoleic acid (Table 15). Yeast used more oxygen for sterol synthesis when stearic or linoleic acid was present but less when oleic acid was added to wort. Overall use of oxygen for lipid syntheses was 20% and 30% higher in the linoleic and stearic acid supplemented fermentations respectively, and 45% lower in the presence of oleic acid. The formation of additional sterol in the presence of exogenous linoleic and stearic acid cannot, therefore, be explained solely by an 'oxygen-sparing' effect caused by the suppression of unsaturated fatty acid synthesis. Thus stearic acid and linoleic acid, added to wort, enhance the ability of the yeast to utilize dissolved oxygen. Since air-saturated 1·040 wort contains ca. 8µg O_2 ml^{-1}, yeast only uses ca. 50% of the

Table 15. Effect of addition of C18 fatty acids to wort on the consumption of oxygen for the synthesis of sterols and unsaturated fatty acids by Sacch. cerevisiae

Addition to wort	Sterol synthesis		Unsaturated fatty acid (UFA) synthesis		Total oxygen consumed for lipid synthesis $\mu g\ ml^{-1}$
	Total sterol synthesized $\mu g\ ml^{-1}$	Oxygen consumed $\mu g\ ml^{-1}$	Total UFA synthesized $\mu g\ ml^{-1}$	Oxygen consumed $\mu g\ ml^{-1}$	
None	3·6	2·90	10·4	1·20	4·1
18:0	5·9	4·53	8·4	0·97	5·5
18:1	4·9	1·66	5·5	0·65	2·3
18:2	11·6	4·49	4·6	0·54	5·0

Data derived from Table 3 and Fig 4. Values in $\mu g\ ml^{-1}$ refer to $\mu g\ (ml\ culture)^{-1}$. Oxygen consumption was not measured but was calculated as detailed in section 5. Abbreviations of fatty acids (18:0, 18:1, 18:2) as in Table 3.

available oxygen. The figures concerning sterol synthesis in Table 15 require some explanation here. For example, when oleic acid was added to wort, the yeast synthesized more sterol; 4·9μg ml^{-1} compared with 3·6μg ml^{-1} in control cultures. However, much less oxygen was consumed for sterol synthesis in the former situation. This is because different sterols vary greatly in the amount of oxygen required for their synthesis. Thus, although the yeast in oleic acid-supplemented fermentations formed 4.0μg sterol (ml culture)$^{-1}$ a large proportion of this sterol was lanosterol the synthesis of which only requires a tenth of the oxygen that ergosterol synthesis does. Similarly the yeast in linoleic acid-supplemented fermentations formed twice as much sterol as yeast in fermentations supplemented with stearic acid, yet similar amounts of oxygen were consumed. Again this is because the sterol composition of the two yeast populations differed markedly (for original data see Fig.4).

6.3.2 Sterol synthesis by yeast during anaerobic/aerobic transition - effect of enriching yeast with linoleic acid

The effect of linoleic acid on sterol synthesis in yeast was further investigated by aerating yeast, which had previously been propagated anaerobically with or without linoleic acid. In these experiments yeast was grown in YEPGM medium. Once stationary-phase had been reached cultures were aerated (by submerged glass-sinter)

at a rate of 0·15 culture volumes min^{-1}. Formation of sterols was followed over the next 4h (Fig.7). Prior to aeration there were 5·9 and 8·5μg sterol ml^{-1} in the control and linoleic acid-supplemented cultures respectively. Table 16 shows the fatty acyl composition of yeast from these cultures at this point. The % UFA of yeast from the linoleic acid-supplemented cultures was ca. 3-fold higher than control yeast. More sterol was formed in the linoleic acid-supplemented cultures compared with the control cultures, over the first hour of aeration. However, this difference was not maintained and after 4h aeration both types of culture had synthesized similar amounts of sterol (Fig.7).

The importance of glycogen dissimilation for sterol synthesis at the start of brewery fermentations has been discussed in Section 3.5.2; the relationship between glycogen dissimilated and sterol formed appears to be stoichiometric. During the period of aeration in the experiments described above, there was extensive dissimilation of glycogen, and depletion of squalene (Fig.8). Both types of culture dissimilated similar amounts of glycogen. However the stoichiometric relationship between glycogen dissimilation and sterol synthesis over the first hour differs between the two types of culture since between two and three times more sterol was formed by the linoleic acid-enriched yeast. Yeast in the control cultures initially contained more

Table 16. Total fatty acyl composition of Sacch.cerevisiae grown in YEPGM medium with and without linoleic acid

Additions to growth medium	Fatty acyl composition of yeast - μg (mg dry weight)$^{-1}$													
	C_6	C_8	C_{10}	C_{12}	C_{14}	$C_{14:1}$	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	Sum μg mg^{-1}	Sum μg ml^{-1}	%UFA
None	Tr	1·1	3·7 ±0·2	1·9	0·7	0·1	9·7 ±0·4	1·5 ±0·1	3·7 ±0·1	2·0 ±0·2	-	24·4 ±1·0	99·3 ±4·1	14·8 ±0·6
18:2	Tr	0·4	1·5	1·2	1·5	0·2 ±0·1	11·0	1·0	4·4 ±0·2	2·2 ±0·1	13·2 ±0·9	36·6 ±1·6	183·4 ±8·0	45·4 ±0·4

Linoleic acid (18:2) added prior to inoculation at a concentration of 50 μg ml^{-1}. Cultures sampled for lipid analyses at 48h, just prior to initiation of aeration. All values are mean of at least two determinations; ± figures represent the range of values obtained. Values in μg ml^{-1} refer to μg (ml culture)$^{-1}$ and were obtained by multiplying μg mg^{-1} figures by the cell dry weight of the culture (mg ml^{-1}).

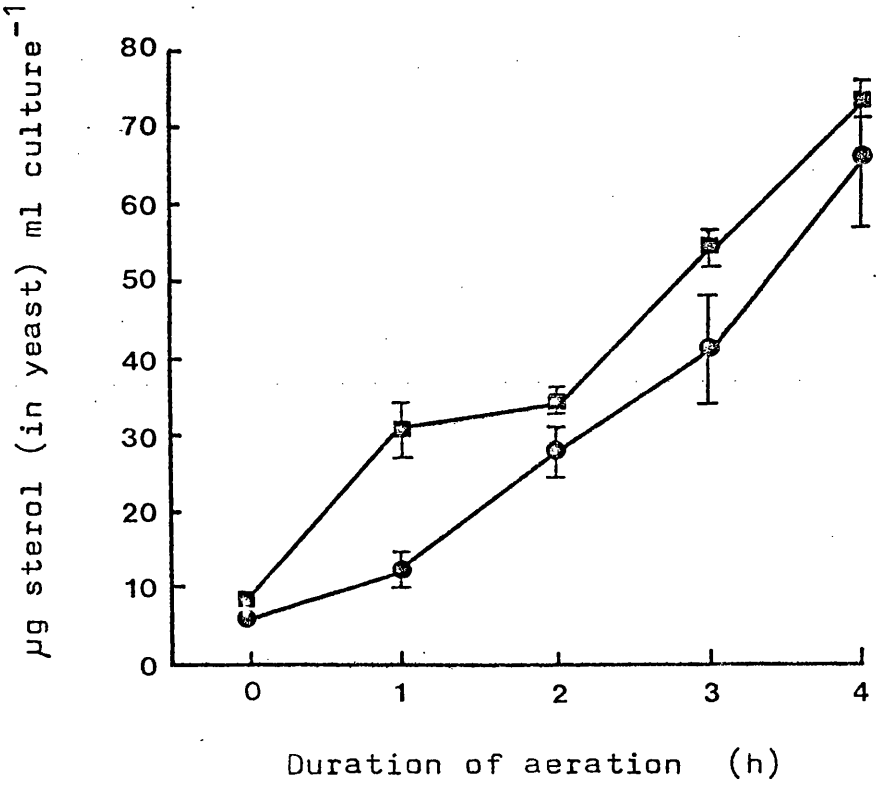
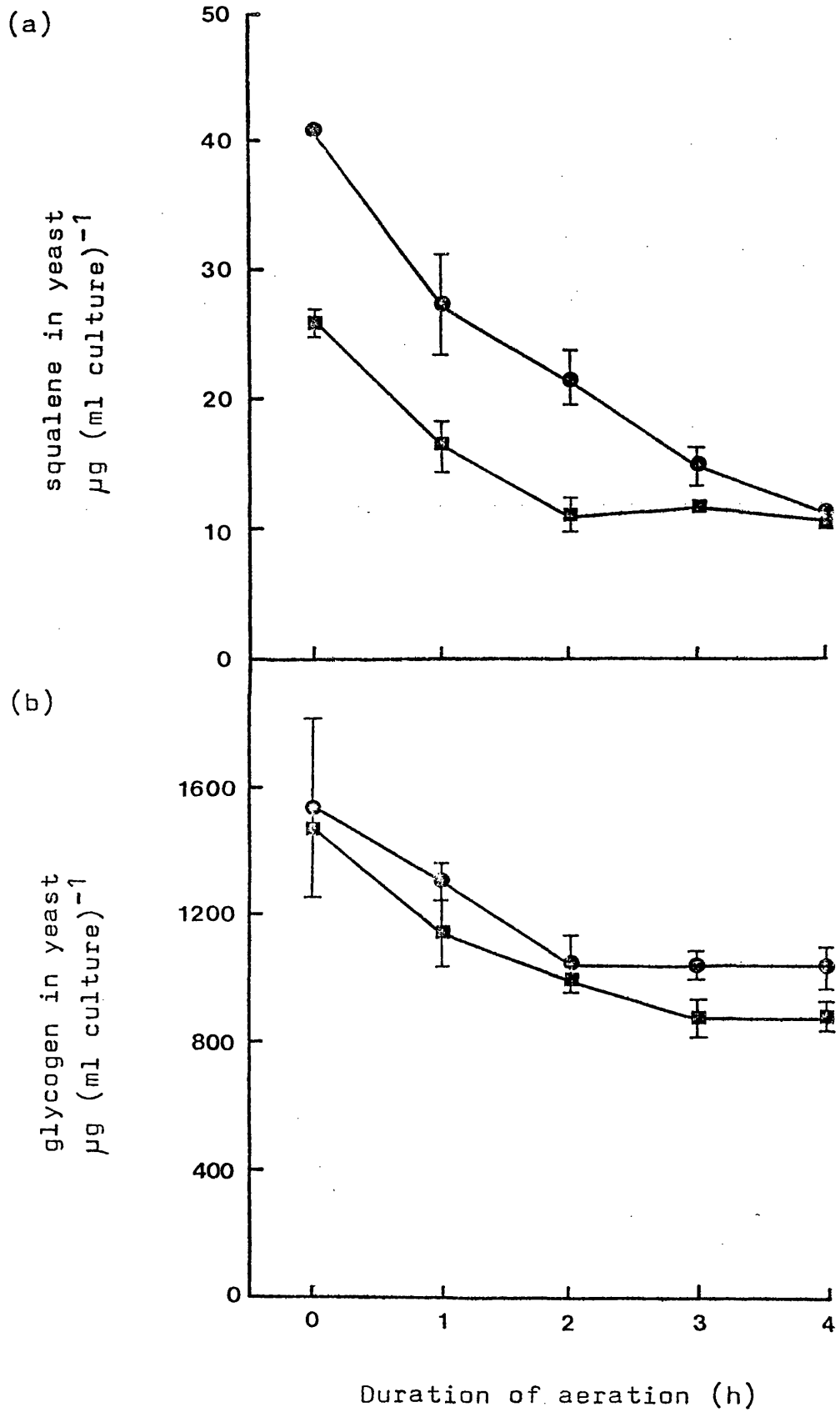


Figure 7.

Formation of sterols by Sacch. cerevisiae during an anaerobic/aerobic transition. Stationary-phase YEPGM cultures (with and without 50µg linoleic acid ml^{-1} added prior to inoculation) were aerated and sterol synthesis was followed over the next 4h. Control cultures (●–●); linoleic acid-supplemented cultures (■–■). All points are mean of single determinations from duplicate cultures. Bars represent range of values obtained.

Figure 8.

Dissimilation of squalene and glycogen during an anaerobic/aerobic transition. Symbols and experimental details as in Fig.7. Values for glycogen are the mean of two determinations on samples from two separate cultures. Those for squalene are the mean of single determinations on samples from two separate cultures. Bars show range of values obtained.


(a)
50
40
30
20
10
0
squalene in yeast
µg (ml culture)⁻¹
(b)
1600
1200
800
400
0
glycogen in yeast
µg (ml culture)⁻¹
0
1
2
3
4
Duration of aeration (h)

squalene as expected (section 6.2.3) and more was utilized (for sterol synthesis) over the 4h aeration phase. However, there was more sterol formed overall than could be accounted for in terms of decline in squalene. Therefore synthesis of squalene must have taken place during this period. Synthesis of DNA and protein also took place during the aeration period; the rates of each were similar in the two types of culture (Fig.9).

Aeration induced the synthesis of both saturated and unsaturated fatty acids; less unsaturated fatty acid being formed by the linoleic acid-enriched yeast as expected (Fig.10). The % UFA increased from 15% to 50% in the control cultures over the first 2h of aeration. In the linoleic acid-supplemented cultures the overall % UFA increased from 45% to 54% over the same period. The final % UFA values were similar in both types of culture (Fig.10a).

Details of the sterol composition of the yeast throughout the aeration-phase are not shown. The 'anaerobic' yeasts had different sterol compositions notably the linoleic acid enriched yeast had two to three times more lanosterol as would be expected from the data of sections 6.1 and 6.2. However this difference was not maintained after 4h aeration; in fact the control yeast contained more lanosterol than the linoleic acid enriched yeast. After aeration, zymosterol became the predominant sterol whereas it was a minor component in

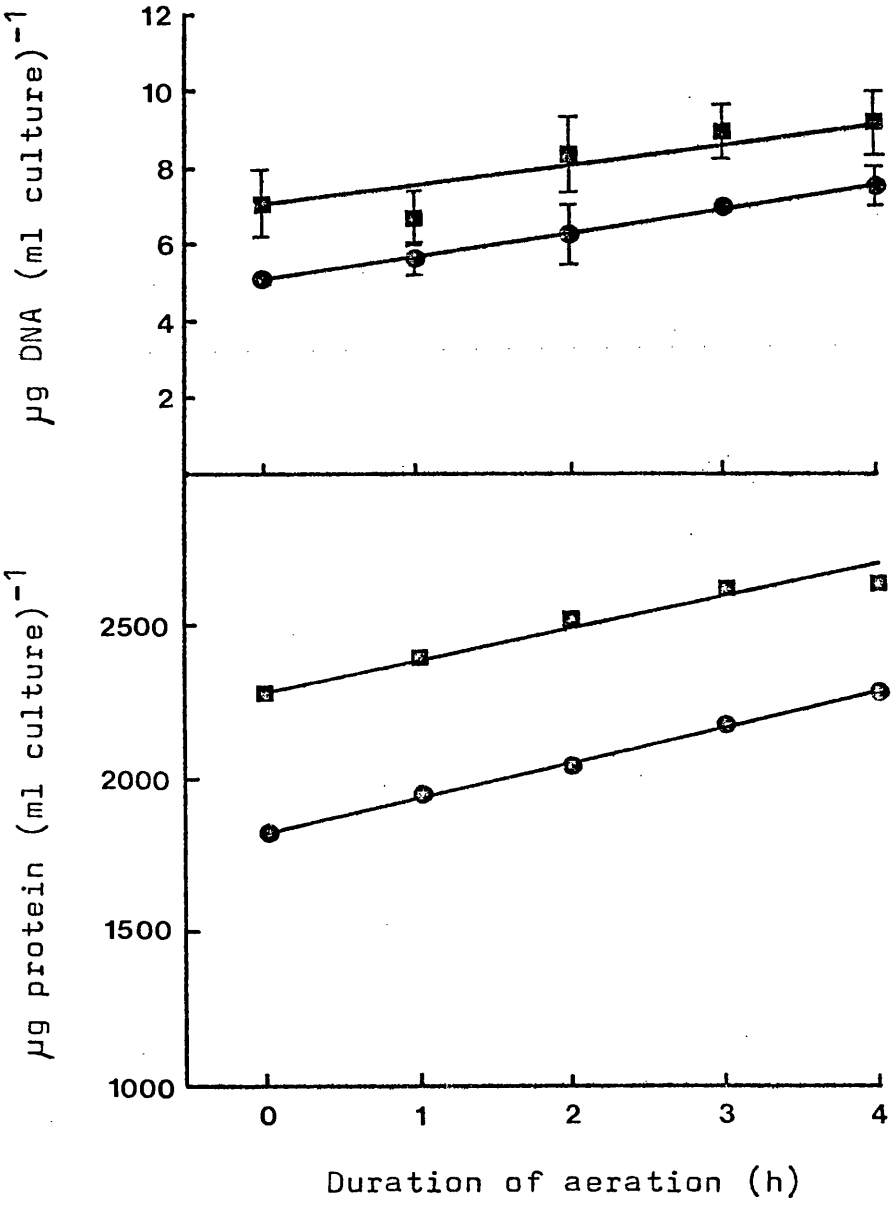


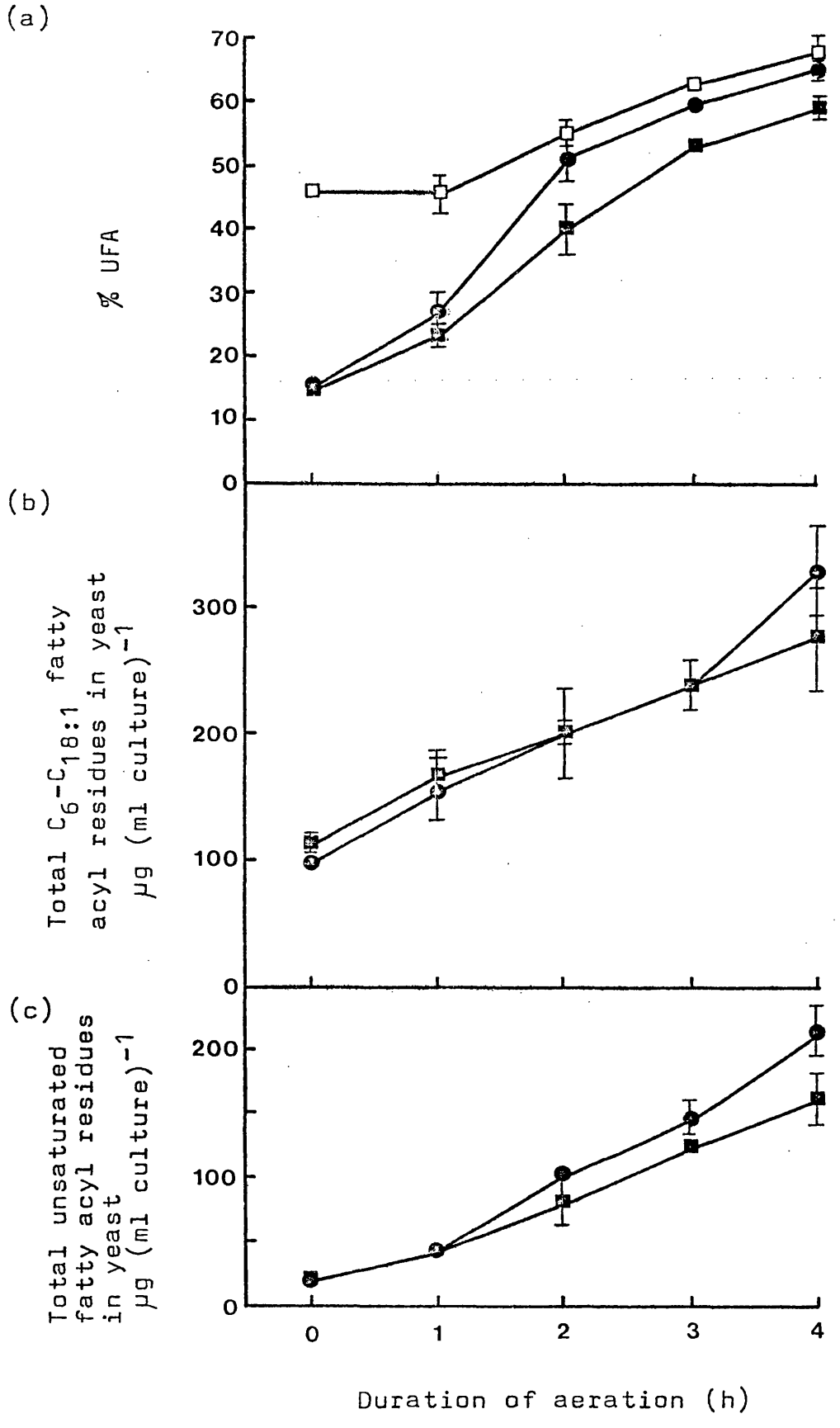
Figure 9.

Synthesis of DNA and protein during the phase of aeration. Experimental details and symbols as in Fig.7. Values for DNA represent mean of four determinations and those for protein are the mean of six determinations. Bars show range of values obtained.

Figure 10.

Synthesis of fatty acyl residues by Sacch. cerevisiae during an anaerobic/aerobic transition. Symbols and experimental details as in Fig.7. Values are mean of single determinations from two separate cultures. Bars show range of values obtained.

In Fig.10.a data are also expressed in terms of C_6-$C_{18:2}$ acyl residues for the 18:2-supplemented cultures (□—□); this represents endogenously produced acyl residues (i.e. C_6-$C_{18:1}$) plus exogenously derived residues (i.e. $C_{18:2}$).


(a)
% UFA
70
60
50
40
30
20
10
0
(b)
Total C_6-$C_{18:1}$ fatty acyl residues in yeast µg (ml culture)$^{-1}$
300
200
100
0
(c)
Total unsaturated fatty acyl residues in yeast µg (ml culture)$^{-1}$
200
100
0
0
1
2
3
4
Duration of aeration (h)

the anaerobic yeasts. Presumably a large proportion of this zymosterol was in esterified form (Aries et al., 1977; Aries & Kirsop, 1978; Quain & Haslam, 1979a).

6.4 Relationship Between the Syntheses of Lipids and Acetate Esters during Fermentation

In the experiments reported in this section, specific rates of ester synthesis were measured to see if changes in these can be associated with specific events during fermentations. Specific rates of ester synthesis are likely to be correlated with changes in the formation of lipids since both utilize acetyl-CoA. Therefore, lipid syntheses were also monitored throughout fermentation.

6.4.1 Fermentation and growth parameters

The final specific gravity of these fermentations was 1·009, and in terms of fall of specific gravity the mid-point (ca. 1·025) occurred at 20h. Fermentations were essentially complete by 45h (Fig.11.a). The rate and extent of fermentation were virtually unaffected by the addition of linoleic acid to wort, although the final yeast mass was increased by ca. 10% due largely to increased protein synthesis (Fig. 11.b). Between 10 and 20h a maximum specific growth rate of $0{\cdot}125h^{-1}$ was obtained (equivalent to a doubling-time of 5·54h) after which the specific growth rate declined sharply (Fig.11.b). Cell-division stopped at ca. 24h; the final cell number was ca. 90×10^6 cells ml^{-1}. However cell mass continued to increase due to accumulation of protein (Fig.11.b) and glycogen (Quain et al., 1981).

Figure 11.

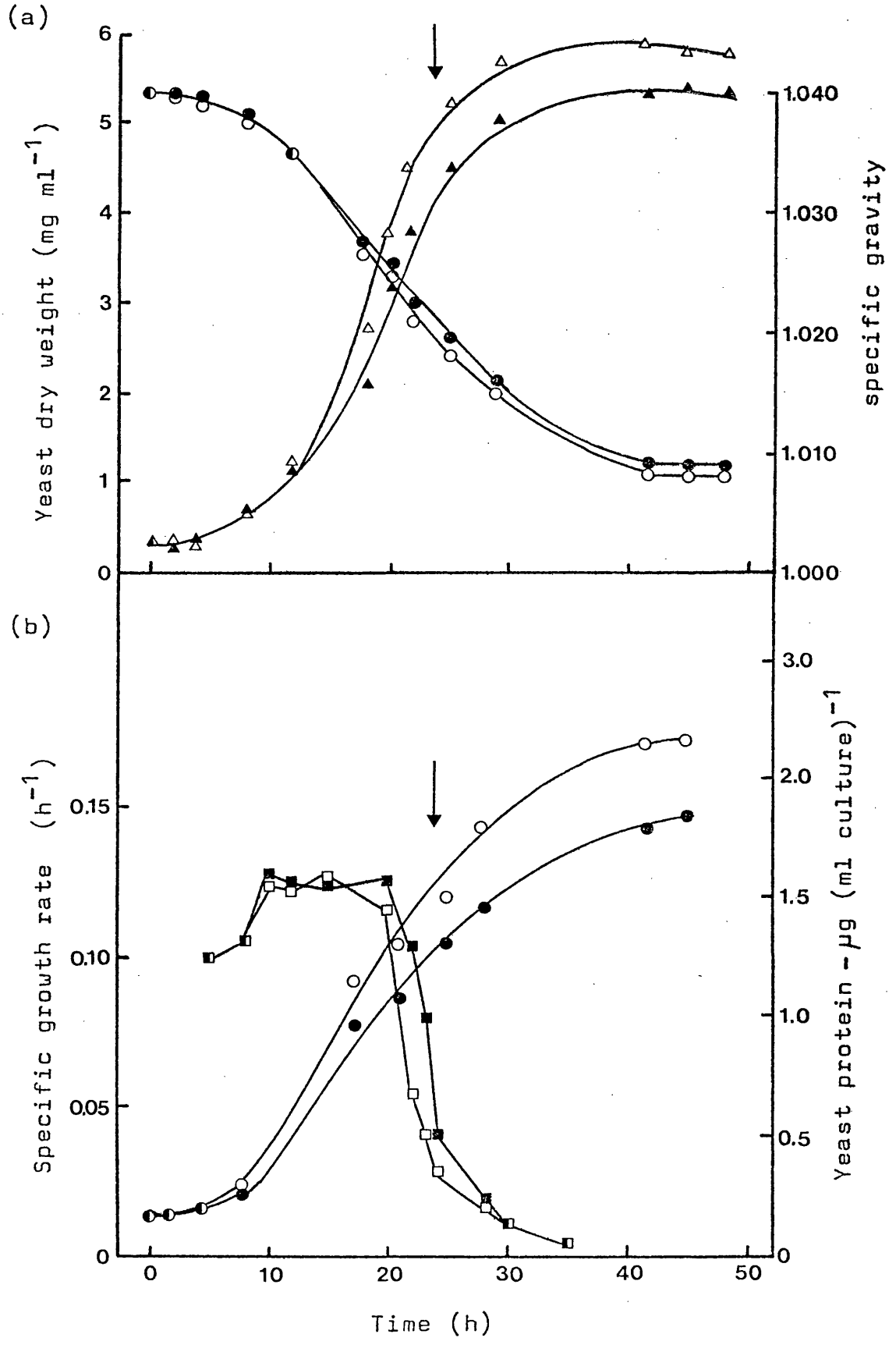
Fermentation and growth data for 1·040 all-malt wort fermentation by NCYC 240.

(a) Specific gravity (●,○) and cell dry weight (▲,△).

(b) Specific growth rate (■,□) and yeast protein (●,○).

Open symbols represent fermentations supplemented with linoleic acid (50μg ml^{-1}). Cessation of cell division (↓) coincided with cessation of DNA synthesis (not shown). Measurement of the DNA content of the culture is thus a convenient indicator of cell division and also cell number (Quain *et al.*, to be published).

Values for specific gravity are the mean of single measurements on two separate cultures whilst those for cell dry weight and protein are the mean of triplicate determinations on two separate cultures.


(a)
Yeast dry weight (mg ml^{-1})
specific gravity
6
5
4
3
2
1
0
1.040
1.030
1.020
1.010
1.000
(b)
Specific growth rate (h^{-1})
Yeast protein - µg (ml culture)$^{-1}$
0.15
0.10
0.05
0
3.0
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
Time (h)

6.4.2 Formation of acetate esters during fermentation

The final concentration of ethyl acetate and iso-amyl acetate in these fermentations was 23 ± 0·5μg ml^{-1} and 1·5μg ml^{-1} respectively. Both esters were synthesized predominantly in the latter half of fermentations (Fig.12). Supplementing wort with linoleic acid reduced the formation of ethyl and iso-amyl acetates by ca. 80%. Figure 13 shows the specific rates of formation of these esters. During the first half of fermentation the specific rate of ethyl acetate formation was ca. 0·2μg h^{-1} (mg dry weight)$^{-1}$ (Fig.13.a). Reliable values could not be calculated between 0h and 8h, as only a trace amount of ethyl acetate was present during this period. The specific rate of ethyl acetate formation increased more then two-fold between 20h and 26h and then declined rapidly. If this increase had not occurred, the concentration of ethyl acetate in the beer would have been ca. 16μg ml^{-1} (70% of the actual concentration). Therefore this burst of ester formation contributed significantly to the overall production of ethyl acetate in beer. In contrast, in fermentations supplemented with linoleic acid the specific rate of ethyl acetate formation did not increase at 20h, and at all times the specific rate was lower than in unsupplemented fermentations (Fig.13.a). This figure also shows that the rate of ethyl acetate formation per cell increases at 20h. This could not be concluded directly from rates expressed on a dry weight basis because the dry weight of a cell changes throughout fermentation

Figure 12.

Formation of ethyl acetate and iso-amyl acetate during fermentation of 1·040 all-malt wort. Closed symbols represent control fermentations; open symbols represent fermentations supplemented with linoleic acid (50μg ml^{-1}). All points represent the mean of duplicate determinations from each of two fermentations. Bars represent range of values obtained.

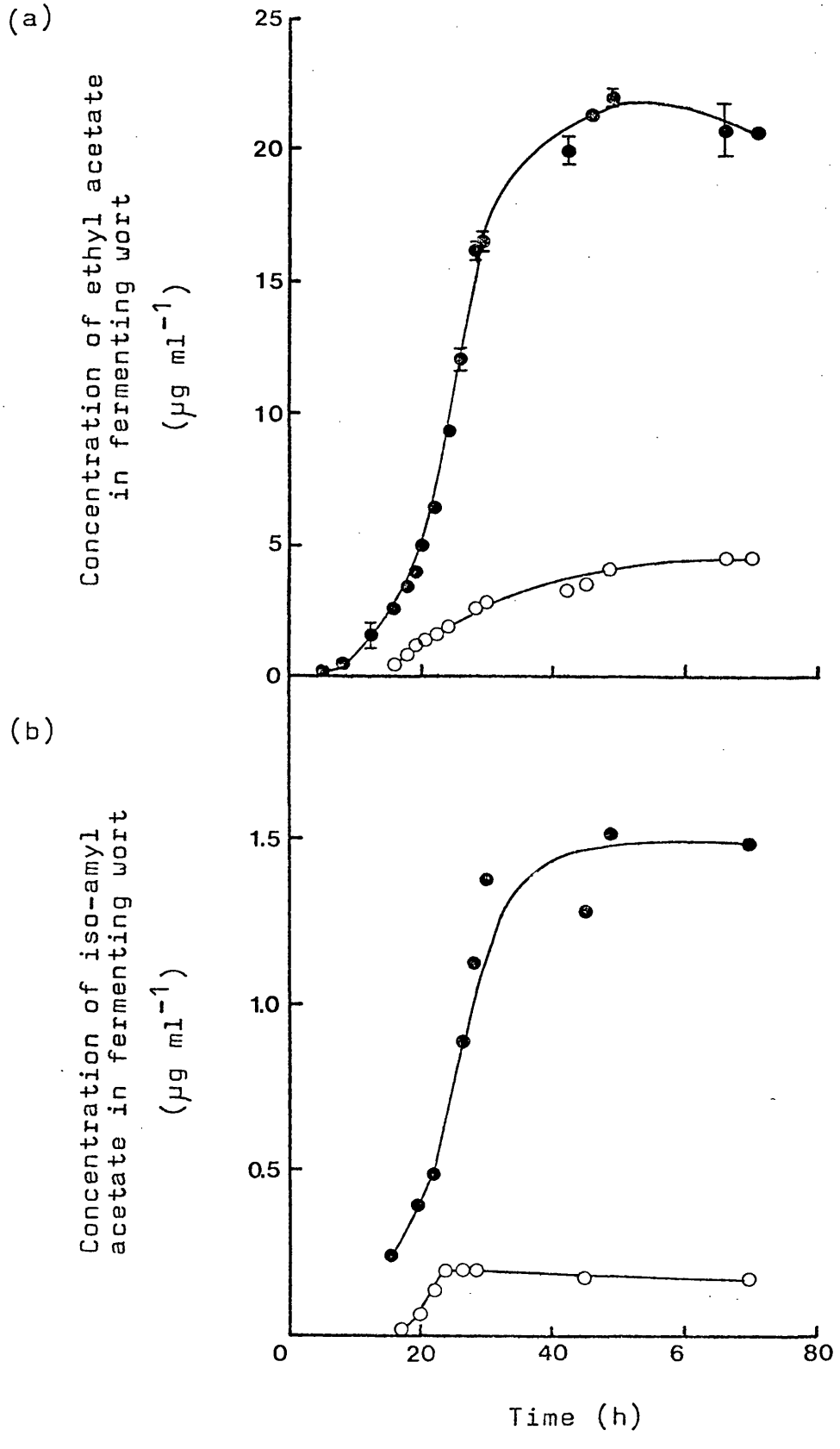
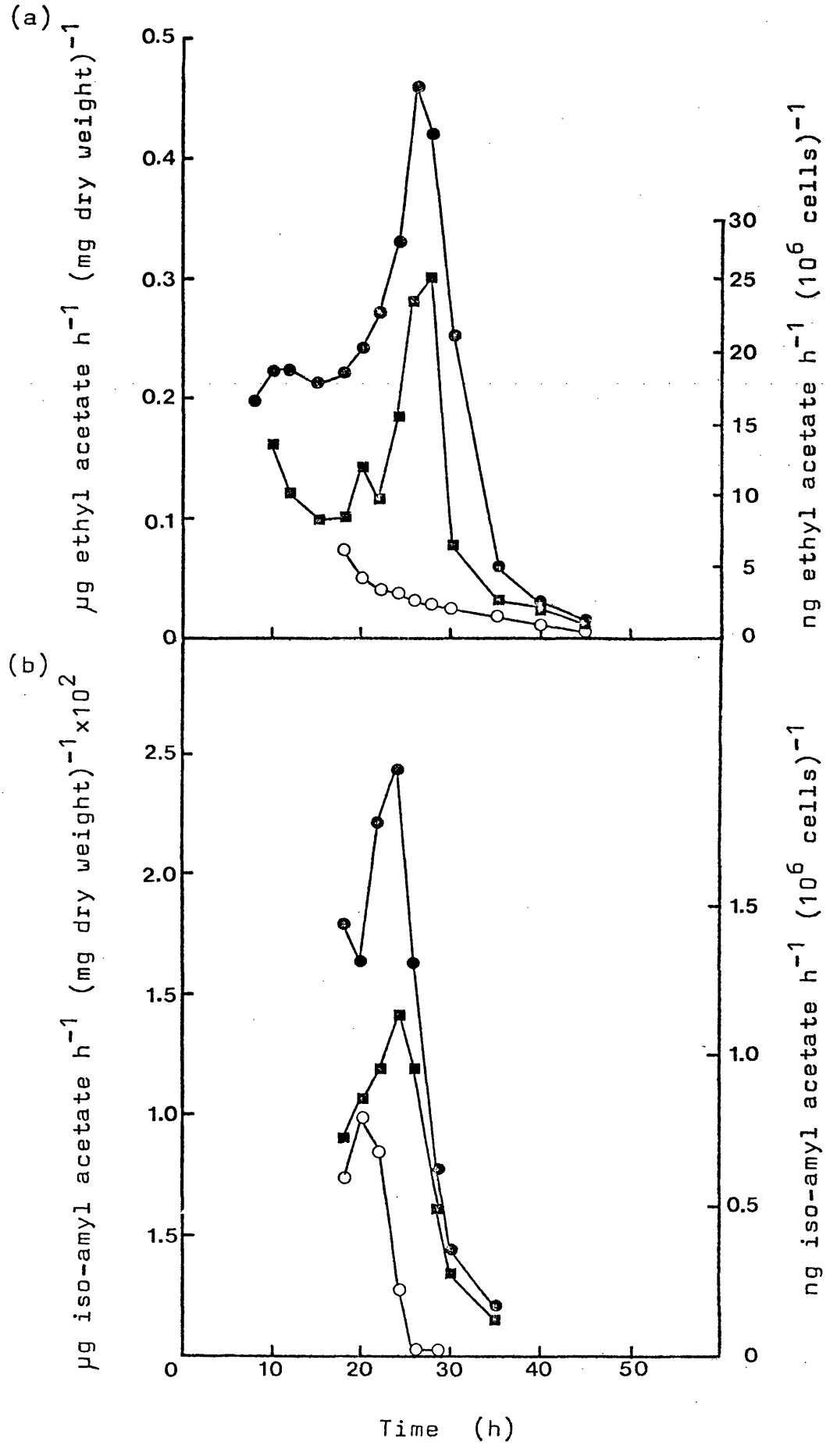

(a)
Concentration of ethyl acetate in fermenting wort (μg ml⁻¹)
25
20
15
10
5
0
(b)
Concentration of iso-amyl acetate in fermenting wort (μg ml⁻¹)
1.5
1.0
0.5
0
20
40
6
80
Time (h)

Figure 13.

Specific rates of formation of ethyl acetate and iso-amyl acetate during fermentation. Specific rates are on basis of yeast dry weight (●, ○) or cell number (▬). Closed symbols represent control fermentations; open symbols represent fermentations supplemented with linoleic acid. Rates of ester formation were calculated by drawing tangents to the plots present in Fig.12. These rates were then divided by cell dry weight or cell number to give an expression of specific rate.


(a)
μg ethyl acetate h⁻¹ (mg dry weight)⁻¹
ng ethyl acetate h⁻¹ (10⁶ cells)⁻¹
0.5
0.4
0.3
0.2
0.1
0
30
25
20
15
10
5
0
(b)
μg iso-amyl acetate h⁻¹ (mg dry weight)⁻¹ x10²
ng iso-amyl acetate h⁻¹ (10⁶ cells)⁻¹
2.5
2.0
1.5
1.0
1.5
1.5
1.0
0.5
0
0
10
20
30
40
50
Time (h)

(Quain et al., to be published).

The specific rate of formation of iso-amyl acetate (on the basis of dry weight or cell number) sharply increased between 20 and 24h and then rapidly declined (Fig.13.b). The extent of the duration of this increase was smaller than that for ethyl acetate. Without such a burst of production the final concentration of iso-amyl acetate would have been 1·35μg ml^{-1}. Addition of linoleic acid reduced the specific rates of formation of iso-amyl acetate throughout fermentation (Fig.13.b).

6.4.3 Synthesis of fatty acids during fermentation

Desaturation, elongation and new synthesis of fatty acids occurred rapidly upon inoculation of anaerobically propagated yeast into air-saturated wort (Fig.14.a,b, c,d). The fatty acyl content of the yeast (Fig.14.b) reached a maximum value after ca. 4h. The ratio of long chain ($\geqslant C_{16}$) to shorter chain ($< C_{16}$) fatty acids increased from a value of ca. 3·5 to ca. 14 during the early phase of fermentation when oxygen was present, but then declined as reported by Aries et al. (1977) (Fig.14.d). The synthesis of unsaturated fatty acids requires molecular oxygen (Bloomfield & Bloch, 1960); during the period that this was available (i.e. the first few hours), the % UFA increased from 20% at inoculation to ca. 56% (Fig.14.c). No unsaturated fatty acids were formed after 4h, because of the anaerobic fermentation system. Thereafter the pool of

Figure 14.

Synthesis of fatty acids by yeast and changes in the fatty acyl composition of yeast during fermentation.

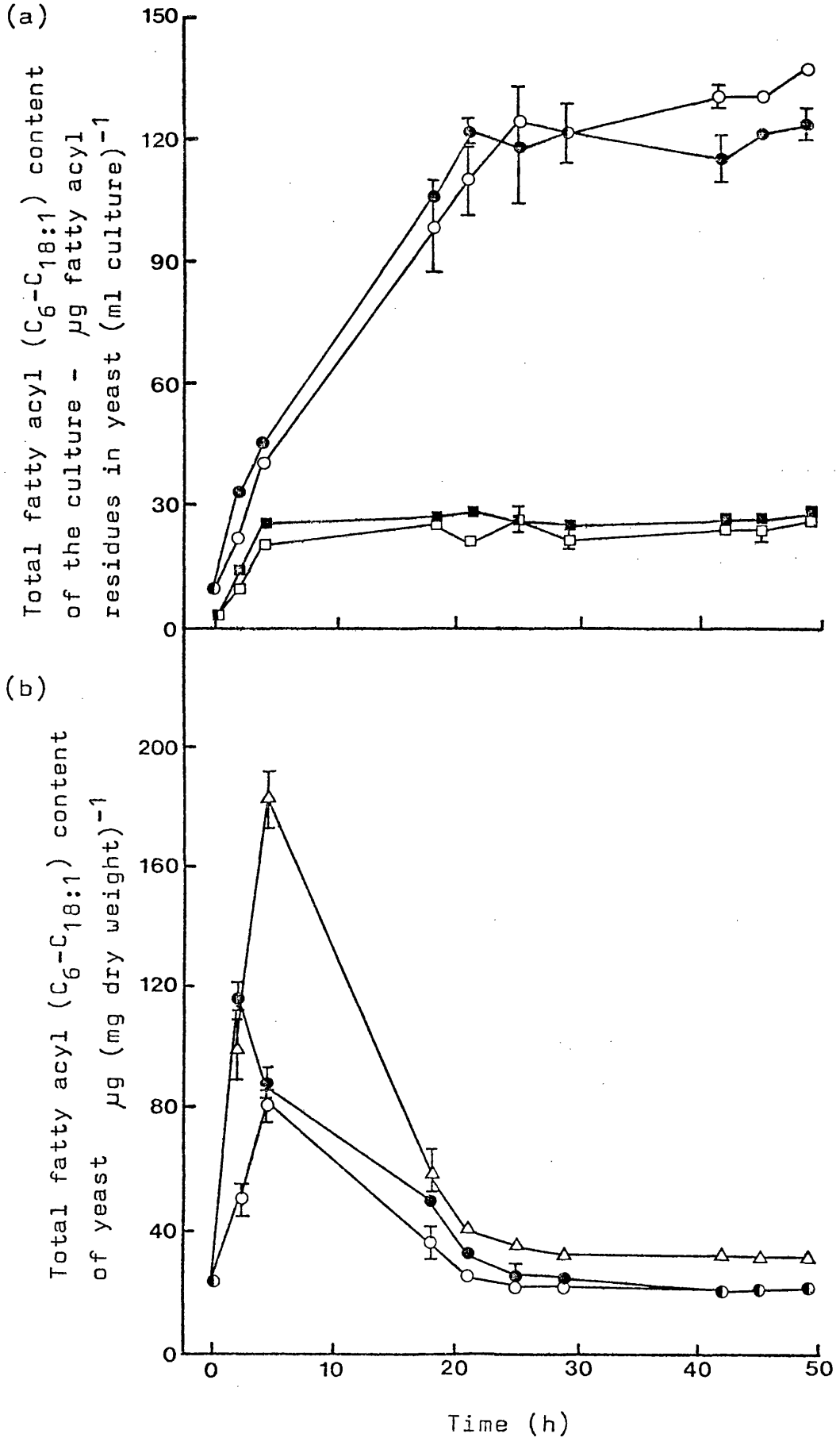
(a) Amount of C_6-$C_{18:1}$ fatty acids (as acyl residues in yeast) in the culture (●,○) and the total amount of unsaturated fatty acids (as acyl residues in yeast) in the culture (■,□).

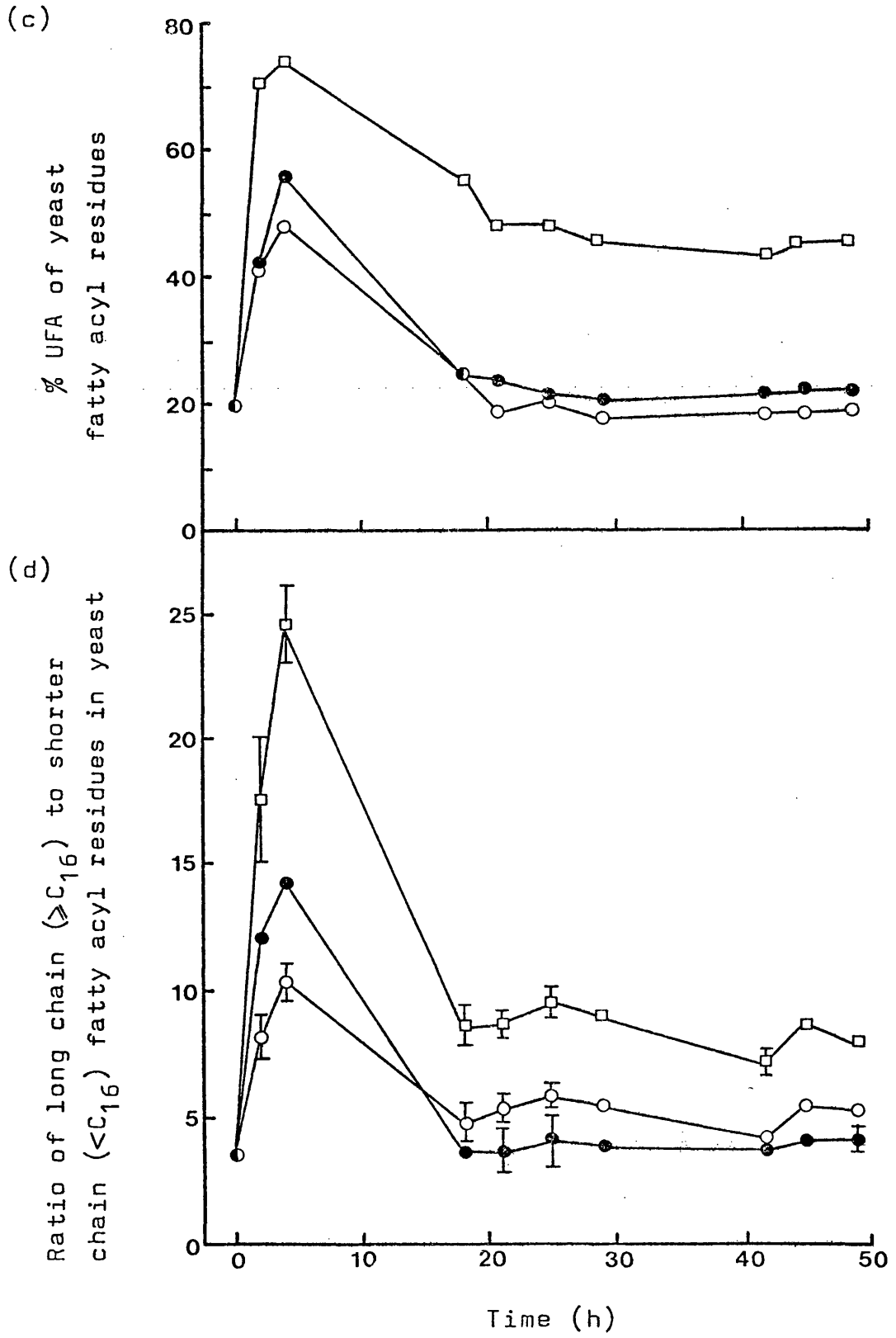
(b) Total amount of C_6-$C_{18:1}$ fatty acyl residues (●,○) and C_6-$C_{18:2}$ fatty acyl residues in yeast(△—△).

(c) Change in the % UFA of yeast fatty acyl residues during fermentation (●,○).

(d) Change in the ratio of long chain ($\geqslant C_{16}$) to shorter chain ($< C_{16}$) fatty acyl residues in yeast during fermentation (●,○). This ratio was calculated on a weight basis rather than a molar one.

Closed symbols represent control fermentations; open symbols represent fermentations supplemented with linoleic acid (50μg ml^{-1}). In (c) and (d) open squares represent data expressed in terms of C_6-$C_{18:2}$ fatty acyl residues in yeast from cultures supplemented with linoleic acid. All points represent the mean of single analyses from two fermentations. Bars show range of values obtained.


(a)
Total fatty acyl (C_6-$C_{18:1}$) content of the culture - μg fatty acyl residues in yeast (ml culture)$^{-1}$
150
120
90
60
30
0
(b)
Total fatty acyl (C_6-$C_{18:1}$) content of yeast μg (mg dry weight)$^{-1}$
200
160
120
80
40
0
0
10
20
30
40
50
Time (h)


(c)
% UFA of yeast fatty acyl residues
80
60
40
20
0
(d)
Ratio of long chain (≥C16) to shorter chain (<C16) fatty acyl residues in yeast
25
20
15
10
5
0
0
10
20
30
40
50
Time (h)

unsaturated fatty acyl residues was diluted out by growth and the % UFA declined to its former level (Fig.14.c).

The synthesis of saturated fatty acyl residues continued beyond the first few hours of fermentation resulting in up to a three-fold increase in the total fatty acid content of the culture between 4h and 20h (Fig.14.a). During this period yeast biomass increased six-fold (Fig.11.a) and consequently the amount of C_6-$C_{18:1}$ fatty acids in the yeast was halved (Fig.14.b). Consideration of these data presented (Fig.11.a and 14.a) suggests that the specific rate of fatty acid synthesis was very high during the first 4h of fermentation but then rapidly declined over the next few hours and continued to gradually decline. Synthesis of saturated fatty acids did not continue throughout fermentation but ceased at ca. 20h (Fig.14.a). This corresponded to the approximate mid-point of fermentation in terms of specific gravity (Fig.11.a).

In fermentations supplemented with linoleic acid (50µg ml^{-1}) the acid was rapidly taken up by yeast and incorporated into saponifiable lipids. Here ca. 70% of the amount added to wort was taken up within 4h and 100% within 18h (probably much earlier) of fermentation. Consequently the fatty acyl composition was substantially altered as described in section 6.2.1. The % UFA was much higher than in the control yeasts throughout

fermentation (Fig.14.c) as was the ratio of long chain to shorter chain acids (Fig.14.d). When the % UFA and the above ratio are expressed in terms of C_6-$C_{18:1}$ fatty acyl residues it can be seen that linoleic acid slightly reduced the synthesis of oleic and palmitoleic acids and as described above, that of medium chain length fatty acids (Fig.14.c and d). Unlike the control cultures the synthesis of saturated fatty acyl residues did not cease at 20h but continued albeit at a very low rate (Fig.14.a). Thus linoleic acid marginally stimulated overall fatty acid synthesis which is in contrast to the results reported in section 6.2.2. The values reported for total fatty acids in Fig.14 are higher than in sections 6.1 and 6.2 because the method of extraction differs (see 5.5). However the overall conclusion drawn in 6.2.2 that linoleic acid (5 to 150µg ml^{-1}) neither inhibits nor stimulates fatty acid synthesis by yeast under anaerobic growth conditions is still tenable.

6.4.4 Synthesis of squalene during fermentation

The squalene content of yeast dropped dramatically during the first hour after inoculation, remained at a low level for the next two hours (Fig.15.c) and was then re-accumulated. The initial drop presumably represents the conversion of squalene to sterols at a faster rate than squalene itself could be formed. After 3h squalene synthesis continued in the absence of sterol synthesis though consideration of data in Fig.11 and

Figure 15.

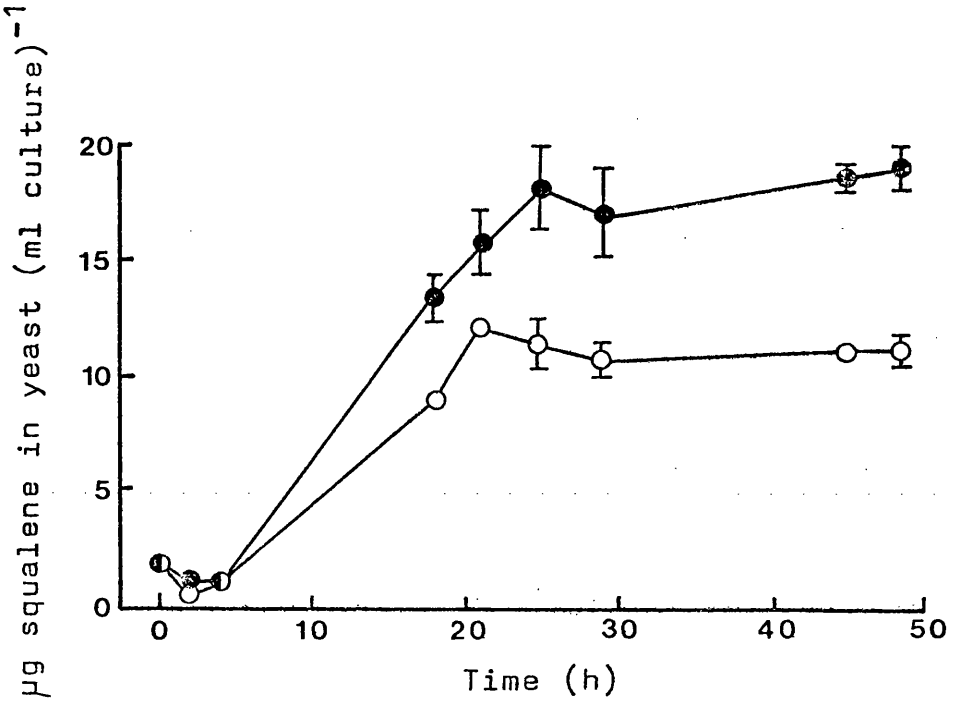
Synthesis of squalene by yeast during fermentation.

(a) Amount of squalene in the culture; (●—●) control fermentations, (o—o) fermentations supplemented with linoleic acid (50μg ml^{-1}).

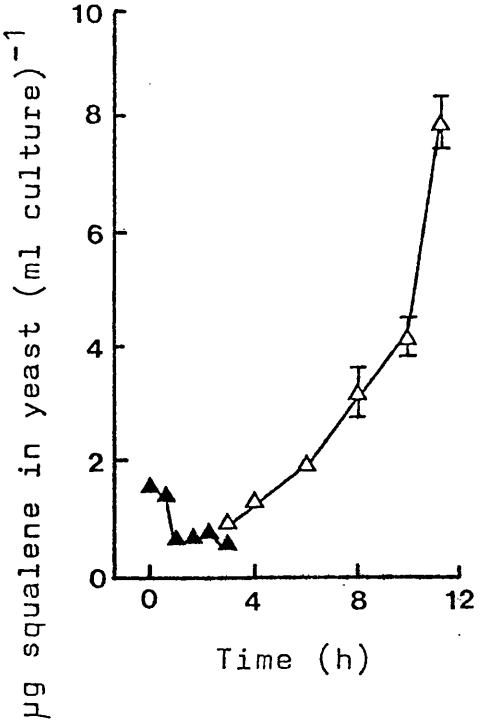
(b) and (c) Data are from separate experiments to those in (a), and represent control fermentations. Open symbols represent the 'change-over' of one set of cultures to another set. Large volume samples were required during this early phase of fermentation because of the low cell dry weight of yeast present. Therefore sampling had to occur from two new cultures after ca. 3h. Analyses were performed on all four cultures at 3h to measure the reproducibility between the two pairs of cultures.

All values are mean of at least single determinations from two separate cultures and bars show range of values obtained.

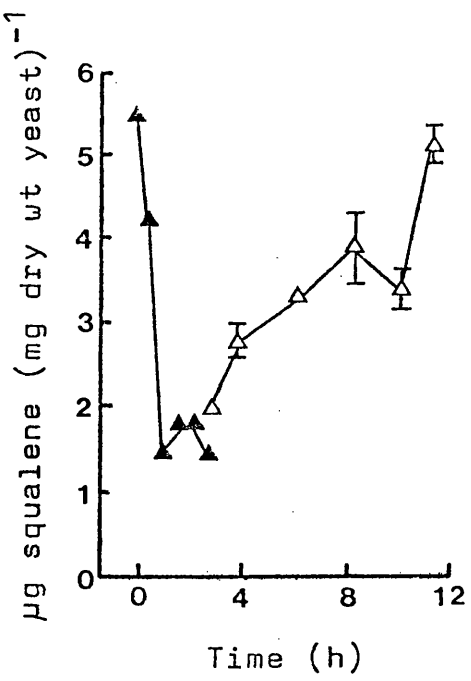
(a)


μg squalene in yeast (ml culture)$^{-1}$
20
15
10
5
0
0
10
20
30
40
50
Time (h)

(b)


μg squalene in yeast (ml culture)$^{-1}$
10
8
6
4
2
0
0
4
8
12
Time (h)

(c)


μg squalene (mg dry wt yeast)$^{-1}$
6
5
4
3
2
1
0
0
4
8
12
Time (h)

15.a suggest that the specific rate of squalene synthesis declined after 3h. However, in these fermentations squalene synthesis stopped after ca. 20h (Fig. 15.a) thus coinciding with the cessation of fatty acid synthesis (Fig.14.a) and the induction of acetate ester synthesis (Fig.13.a and b). The same pattern of synthesis was observed in cultures supplemented with linoleic acid, although the total amounts synthesized were ca. 50% lower, in agreement with previous results (sections 6.1 and 6.2).

6.5 Pertubation of Lipid Syntheses by Cerulenin - Effects on the Synthesis of Acetate esters by Sacch. cerevisiae

Cerulenin (CRL) is an antibiotic which prevents growth by inhibiting the biosyntheses of fatty acids and sterols (for review see Omura, 1976). More specifically CRL inhibits the β-keto-acyl carrier protein synthetase in Escherichia coli (D'Agnolo et al., 1973) but does not affect either fatty acid elongation or desaturation in Sacch. cerevisiae (Awaya et al., 1975). The site at which CRL inhibits sterol synthesis in cell-free extracts of Sacch. cerevisiae is β-hydroxymethylglutaryl-CoA synthetase (Ohno et al., 1974) though Greenspan et al. (1977) report that CRL does not prevent sterol synthesis in vivo. Cerulenin-inhibition can be 'reversed' by the provision of fatty acids in the growth medium (Awaya et al., 1975). In the experiments reported here the effects of CRL on the syntheses of esters and lipids were studied to establish: (i) if cessation of fatty acid synthesis is in fact responsible for the stimulation of acetate ester synthesis, as suggested in 6.4 and (ii) whether CRL-induced inhibition of fatty acid synthesis can be used as an experimental system for studying the regulation of ester synthesis.

6.5.1 Preliminary studies

Yeast NCYC 240 was grown aerobically on YM Broth for 45h. Then shake flasks containing YEPG 5% (75 ml per

250 ml flask) were inoculated with 10μg dry weight yeast ml^{-1} (ca. $0{\cdot}25 \times 10^6$ cells ml^{-1}). Certain of the flasks contained Tween 80 (3mg ml^{-1}) and where indicated CRL was added 18h after inoculation. After 40h cultures were sampled for biomass, yeast fatty acyl composition and ethyl acetate (Table 17).

Addition of CRL (2μg ml^{-1}) inhibited growth of Sacch. cerevisiae NCYC 240. Supplementation with Tween 80 did not fully reverse CRL-inhibition of growth; these cultures (CRL-reversed cultures) formed ca. 30% less biomass than the control cultures (Table 17). The fatty acyl content of yeast from the CRL-reversed cultures was approximately double that of CRL-inhibited cultures. However, the % UFA of the CRL-reversed yeast was 99%, indicating that the cells were starved for saturated fatty acid; and this accounts for the incomplete reversal of CRL-inhibition by Tween 80. This argument is supported by Table 18 which shows that full reversal of CRL-inhibition can be achieved by addition of Tween 80 plus palmitic acid. Further, a report has appeared recently (Otoguro et al., 1981) which confirms and expands these observations and suggests that membrane functions (e.g. energy transduction and nutrient transport) in Sacch. cerevisiae require certain amounts of saturated fatty acid.

Cerulenin-reversed cultures produced between 35% and 80% more ethyl acetate but not iso-amyl acetate, per

Table 17. Effect of cerulenin on growth, and syntheses of fatty acids and ethyl acetate by Sacch. cerevisiae

Additions	Biomass (mg ml^{-1})	Fatty acyl content of yeast ($\mu g\ mg^{-1}$)	%UFA	Ethyl acetate		Iso-amyl acetate	
				$\mu g\ ml^{-1}$	$\mu g\ mg^{-1}$	$\mu g\ ml^{-1}$	$\mu g\ mg^{-1} \times 10^{2}$
None	9·0±0·2	104±4	90	3·9 ±0·2	0·43	0·36±0·04	4·0
Fatty acids	8·8	103±2	90	3·7 ±0·4	0·42	0·24	2·7
CRL + Fatty acids	6·4±0·3	39±2	99	4·9 ±0·2	0·77	0·26	4·0
CRL	1·8±0·1	20±2·5	94	0·75±0·15	0·42	0·17±0·1	9·4

Fatty acid supplementation = 3mg Tween 80 $(ml\ culture)^{-1}$. Cerulenin ($2\mu g\ ml^{-1}$) added 18h after inoculation. Values for biomass are mean of triplicate determinations; those for ethyl and iso-amyl acetates are mean of two determinations and those for the fatty acyl content of yeast are the mean of single determinations from two separate cultures. Errors (±) show range of values obtained. Values for μg (ester) mg^{-1} are obtained by dividing the $\mu g\ ml^{-1}$ values by the biomass (mg ml^{-1}) values and therefore represent μg ester formed $(mg\ dry\ wt\ yeast)^{-1}$ produced.

Table 18. Effect of cerulenin on growth and syntheses of fatty acids and ethyl acetate by Sacch. cerevisiae

Additions	Biomass (mg ml^{-1})	Fatty acyl content of yeast ($\mu g\ mg^{-1}$)	%UFA	Ethyl acetate	
				$\mu g\ ml^{-1}$	$\mu g\ mg^{-1}$
None	8·7±0·13	128±9	91	4·7 ±0·3	0·54
Fatty acids	9·2±0·04	200±0	75	4·1 ±0·3	0·44
CRL + Fatty acids	8·5±0·10	145±4	81	6·2 ±0·2	0·73
CRL	1·4±0·15	39±6	95	0·85±0·1	0·60

Experimental and analytical details as in Table 17, except:

(i) Fatty acid supplementation consisted of 3mg ml^{-1} Tween 80 plus 1mg ml^{-1} palmitic acid.

(ii) Concentration of iso-amyl acetate in the culture medium was not determined.

mg dry weight yeast, than did control cultures (Tables 17 and 18). Cerulenin-inhibited cultures produced ca. 3-fold more iso-amyl acetate than control cultures (Table 17). The acetyl-CoA used for the increased synthesis of ethyl acetate in CRL-reversed cultures is probably insignificant compared with the amount not used for fatty acid synthesis. This implies that inhibition of fatty acid synthesis does not cause a sustained 'overflow' of acetyl-CoA formation. Any 'overflow' is probably manifested as a transient increase in acetate ester formation (and other acetyl-CoA utilizing reactions), as suggested by the observations of 6.4.2.

6.5.2 Effect of inhibition of fatty acid synthesis on specific rate of formation of ethyl acetate

The growth medium used in this study was YEPG 10% supplemented with heptadecanoic acid (0·1mg ml^{-1}) dispersed in Triton X-100 (0·25% v/v). Heptadecanoic acid was used rather than palmitic acid because Sacch. cerevisiae does not synthesize significant amounts of fatty acids with an odd-number of carbon atoms. Therefore fatty acid synthesis could be followed in the presence of uptake of an exogenous source of fatty acid. Air was passed through the headspace of the culture vessels (containing 1·5 l medium) at a low rate sufficient to displace the headspace every 30min. This procedure was carried out to prevent fatty acid synthesis stopping due to lack of oxygen as it does in wort

fermentation (6.4). Propagation and size of inoculum were as described above (6.5.1). Cerulenin was added 18h after inoculation.

(a) Effect of cerulenin on growth and syntheses of fatty acids and squalene

At the point of CRL addition, the cultures were in logarithmic growth phase (Fig.16). Synthesis of fatty acids ceased immediately upon addition of CRL (Fig.17). After 3-4h, despite the presence of heptadecanoic acid in the growth medium, the growth rate declined.

Analysis of the fatty acyl composition of yeast revealed that heptadecanoic acid was taken up, but clearly the amount added was insufficient to maintain reversal of CRL-inhibition for long. Alternatively there may have been other inhibitory effects of CRL. Thus the cultures to which CRL was added can be considered to have three phases of metabolism: normal logarithmic growth ; growth where CRL had inhibited fatty acid synthesis but exogenous fatty acid was available; CRL-induced stationary phase.

Sterol synthesis was not monitored, though squalene synthesis was. On a μg (mg dry weight)$^{-1}$ basis the level of squalene in the yeast increased from *ca.* $1\mu g$ mg^{-1} to *ca.* $3\mu g$ mg^{-1} in the control cultures (on a μg ml^{-1} basis this is 0·6 to $30\mu g$ ml^{-1}). Squalene synthesis in the CRL cultures increased from 0·64μg ml^{-1}

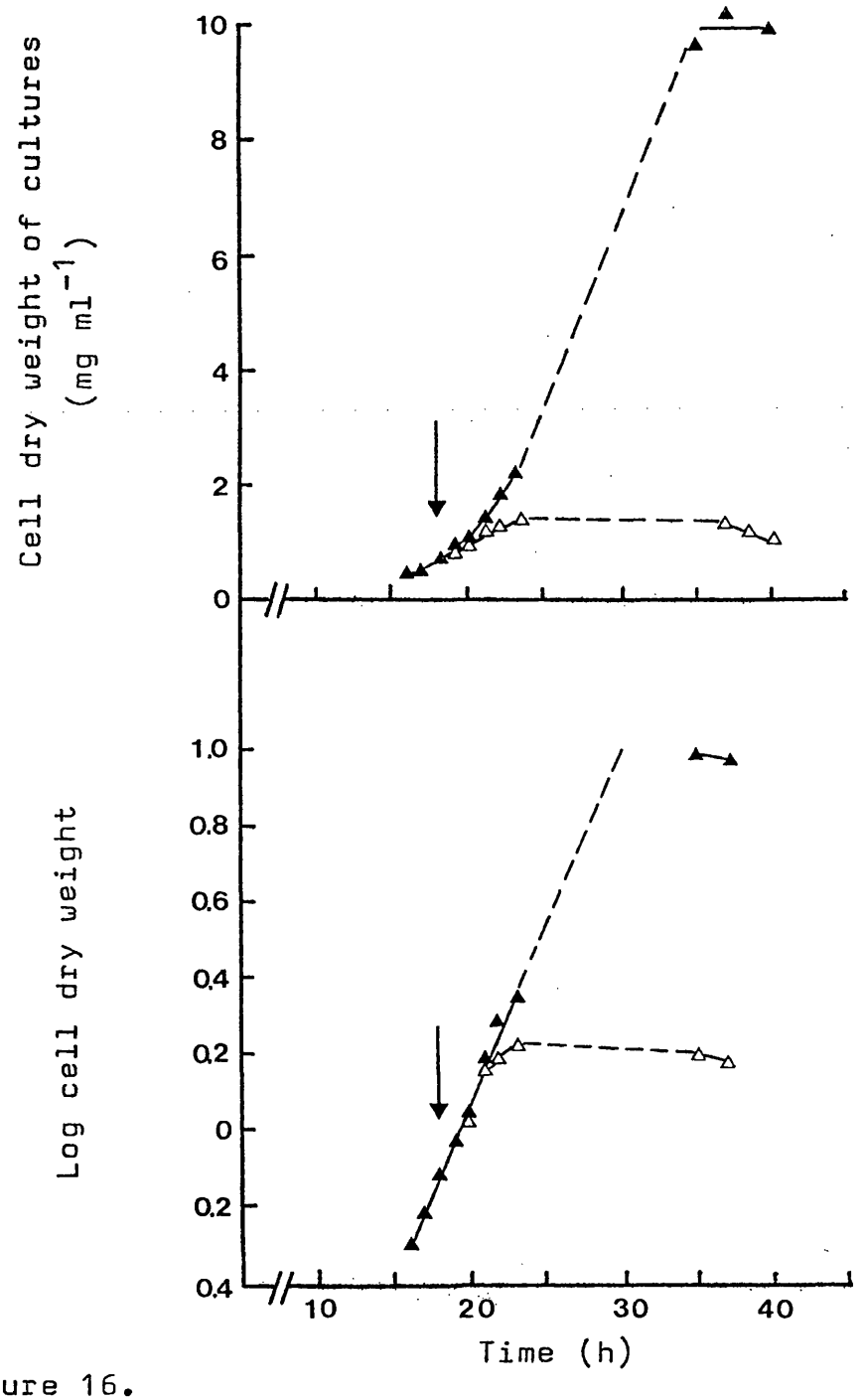


Figure 16.

Effect of cerulenin on growth. For experimental details see text. Closed symbols represent control cultures; open symbols represent cultures to which cerulenin ($2\mu g\ ml^{-1}$) was added (↓). All values are the mean of triplicate determinations from each of two cultures.

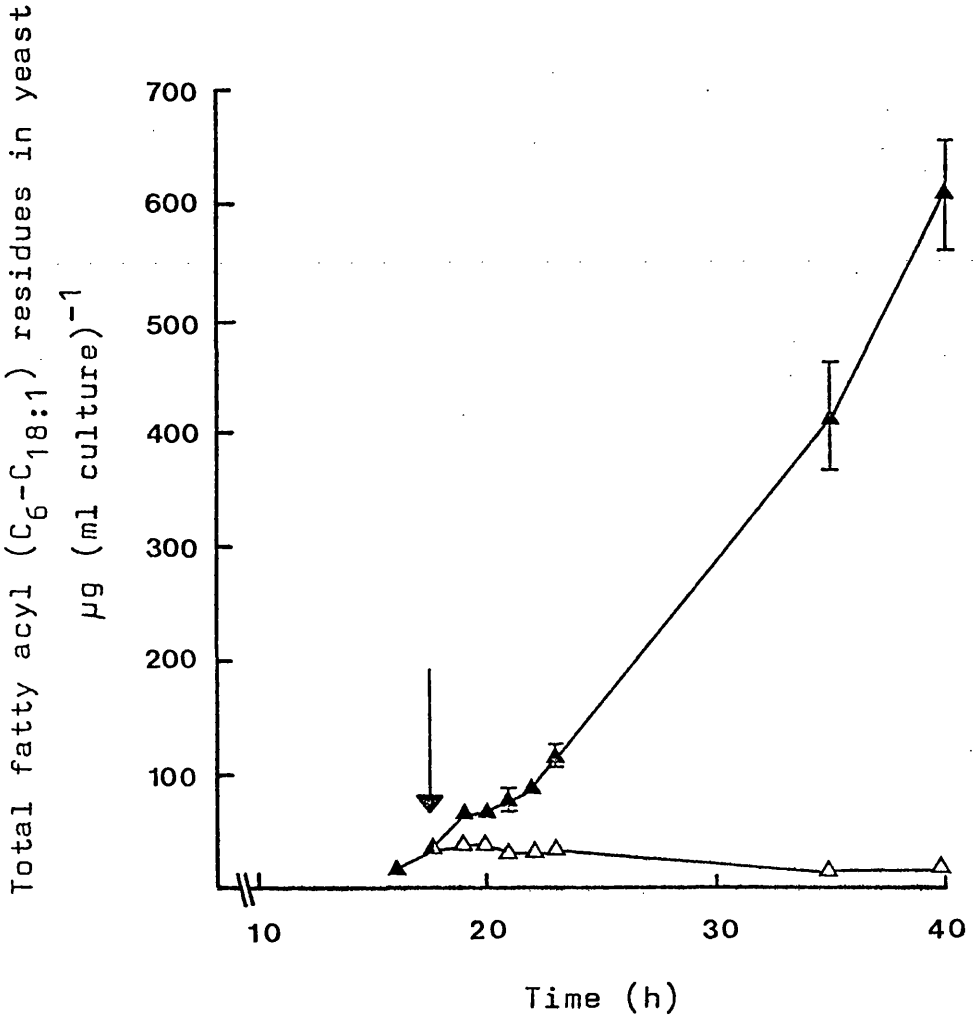


Figure 17.

Effect of cerulenin on the synthesis of fatty acids by yeast. Experimental details and symbols as in Fig.16. All points are mean of single analyses from two separate cultures. Bars represent range of values obtained.

at the point of CRL addition to 1·8μg ml^{-1} after 40h. Therefore, in agreement with Greenspan et al. (1977) CRL does not inhibit squalene (and sterol) synthesis to the same extent as fatty acid synthesis. Since oxygen is present, utilization of squalene for sterol synthesis will occur. Therefore the measurements of squalene do not indicate the degree of squalene synthesis per. se. Squalene accumulated substantially in the yeast in the control cultures possibly because at the high cell density (ca. 10mg ml^{-1}) oxygen became limiting for sterol synthesis.

In control cultures glucose was exhausted after ca. 35h, whereas CRL cultures used only 25% of the available glucose (Fig.18). However glucose was taken up after addition of CRL, indicating that this particular membrane function was partially intact. The CRL-inhibited cells extensively dissimilated their endogenous reserves of carbohydrate (i.e. glycogen), suggesting that uptake of glucose was not able to fully support the maintenance phase metabolism of this yeast (Table 19). The glycogen present in control cells accounted for ca. 10% of the total biomass which is much lower than the level found in anaerobically grown yeast (Chester, 1963; Quain et al., 1981).

(b) Effect of cerulenin on the synthesis of ethyl acetate

The availability of oxygen throughout the experiment maintained active lipid synthesis and the % UFA of the

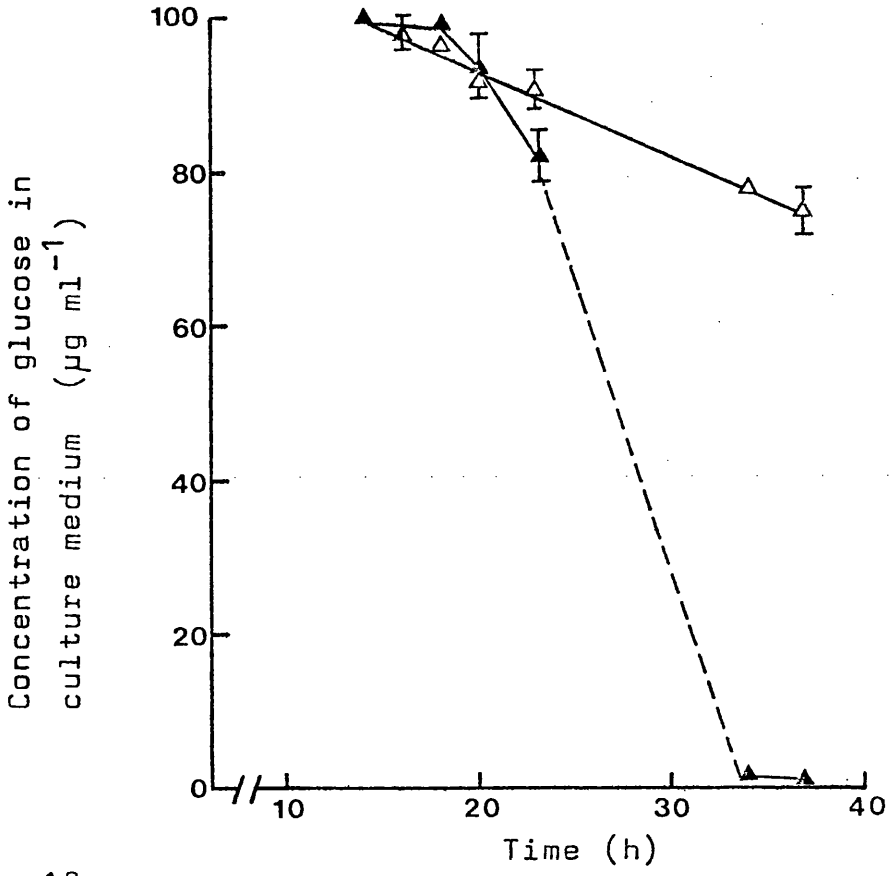


Figure 18.

Effect of cerulenin on uptake of glucose by yeast. Symbols and experimental details as in Fig.16. Values are mean of duplicate determinations on two separate cultures. Bars show range of values obtained.

yeast sample	Glycogen content of yeast µg (mg dry wt)$^{-1}$	
	Alkali soluble glycogen	Acid soluble glycogen
Control	4·8 ± 0·9	89·3 ± 9·0
+ CRL	10·2 ± 0·3	8·0 ± 0·2

Table 19. Effect of cerulenin on dissimilation of glycogen reserves in yeast. Samples taken after 40h. Values are mean of duplicate determinations from each of two cultures. Errors (±) show range of values obtained.

yeast lipids was high (ca. 90%). Consequently the amounts of ethyl acetate formed were much lower than in wort fermentations (Fig.19; see 6.1-6.4). The addition of CRL had no detectable effect on the rate of ethyl acetate formation over the next 5h (Fig.19) and only a slight effect on the specific rate of ethyl acetate synthesis (Fig.20). During the logarithmic phase of growth (12-20h) the specific rate of ethyl acetate synthesis declined linearly from 0·17 to 0·05 $\mu g\ h^{-1}$ (mg dry weight)$^{-1}$ and remained at this low level in the control cultures. Despite the inhibition of growth by CRL, these cells continued to synthesize ethyl acetate (Fig.19). Consequently the specific rate of ethyl acetate formation substantially increased (Fig.20). Cells in the CRL-inhibited state produced on a per mg dry weight basis, 6-fold more ethyl acetate than did control yeast cells. However, during the analysis of the culture medium for ethyl acetate, the presence of another volatile compound was noted. On the basis of retention time (relative to the internal standard, methyl acetate) and peak enhancement this compound was identified as acetaldehyde. Cerulenin-inhibited cultures produced relatively large amounts of this compound the synthesis of which was stimulated immediately upon the addition of CRL (Fig.21). Thus under these conditions stimulation of ethyl acetate synthesis is a more secondary response to inhibition of fatty acid synthesis.

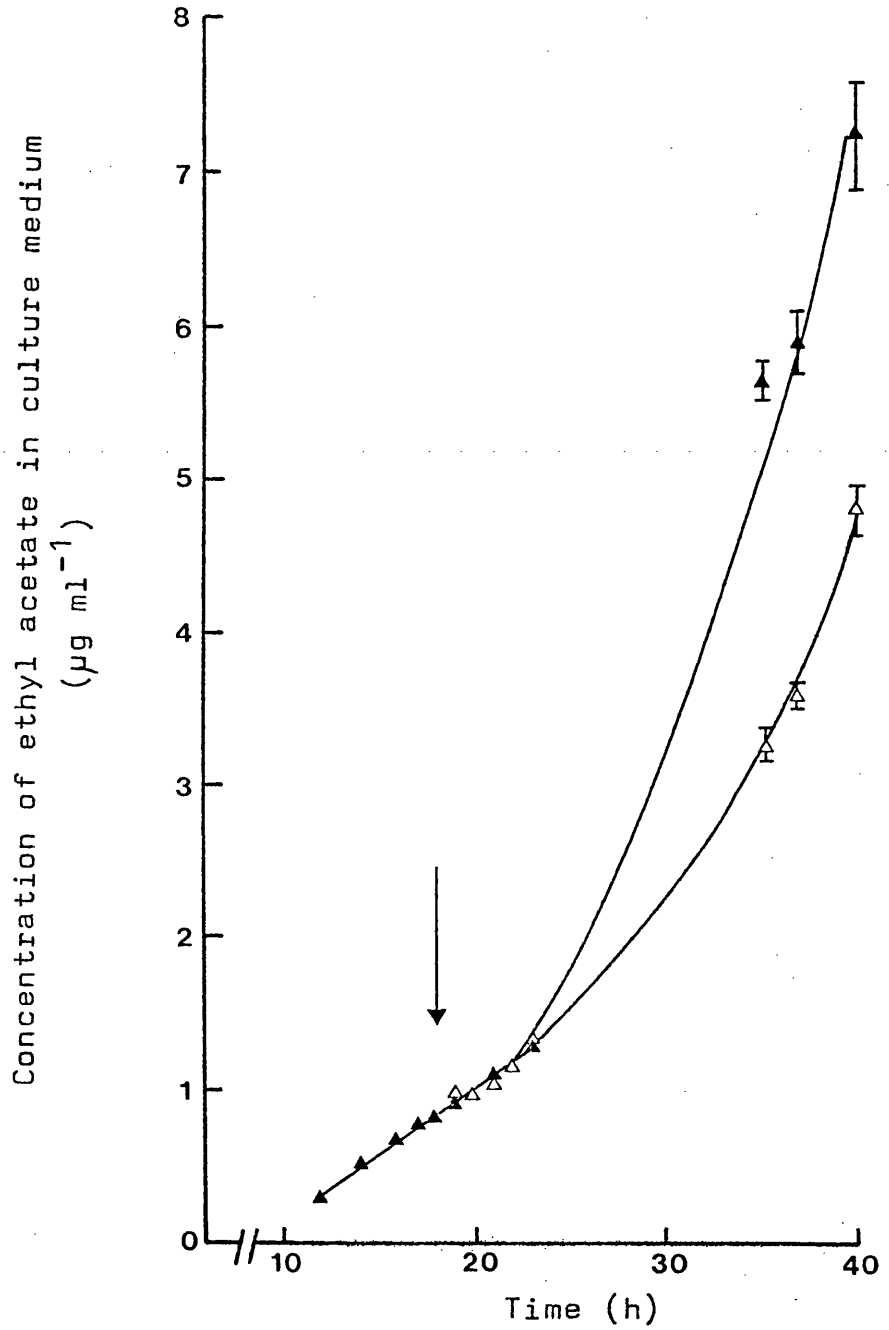


Figure 19.

Effect of cerulenin on the synthesis of ethyl acetate by yeast. Experimental details and symbols as in Fig.16. All points represent mean of duplicate determinations from two cultures. Bars show range of values obtained.

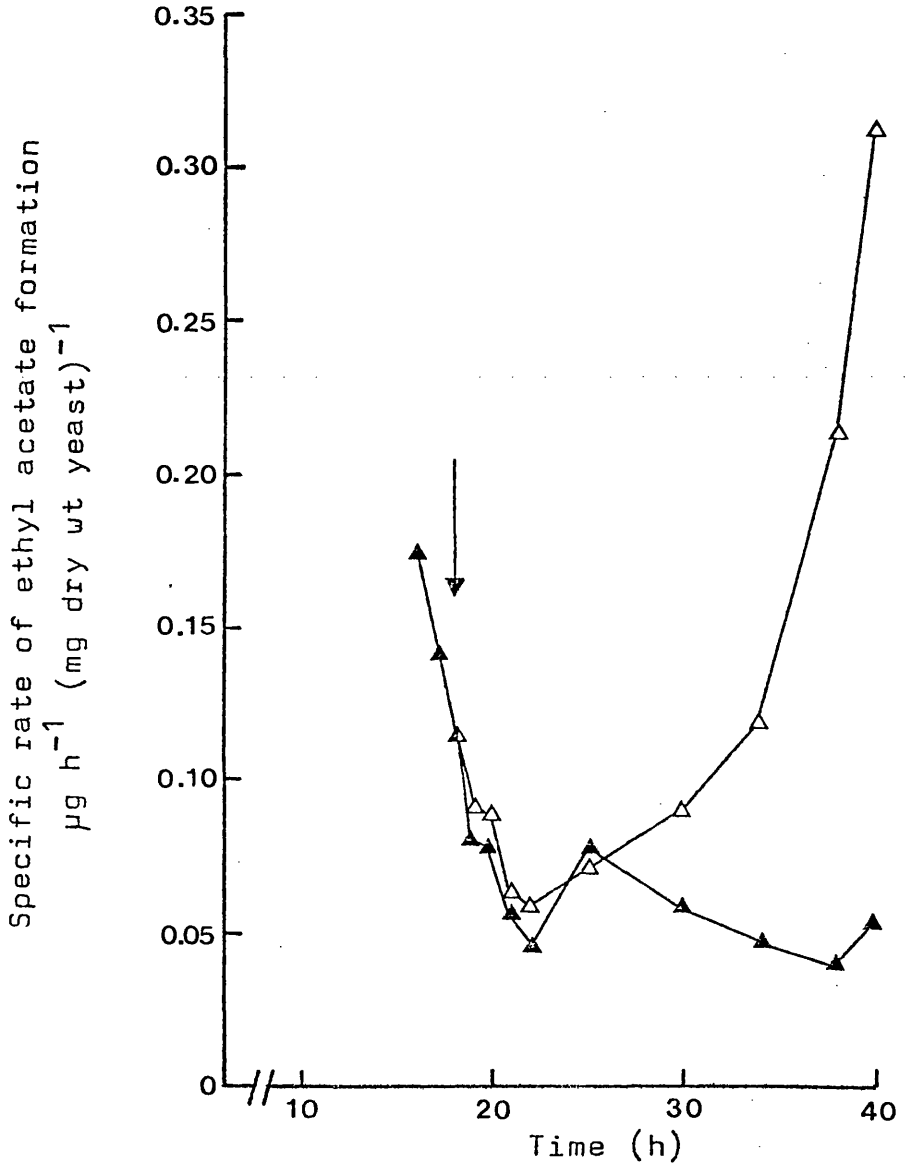


Figure 20.

Effect of cerulenin on the specific rate of formation of ethyl acetate. Experimental details and symbols as in Fig.16. Data derived from Fig.19.

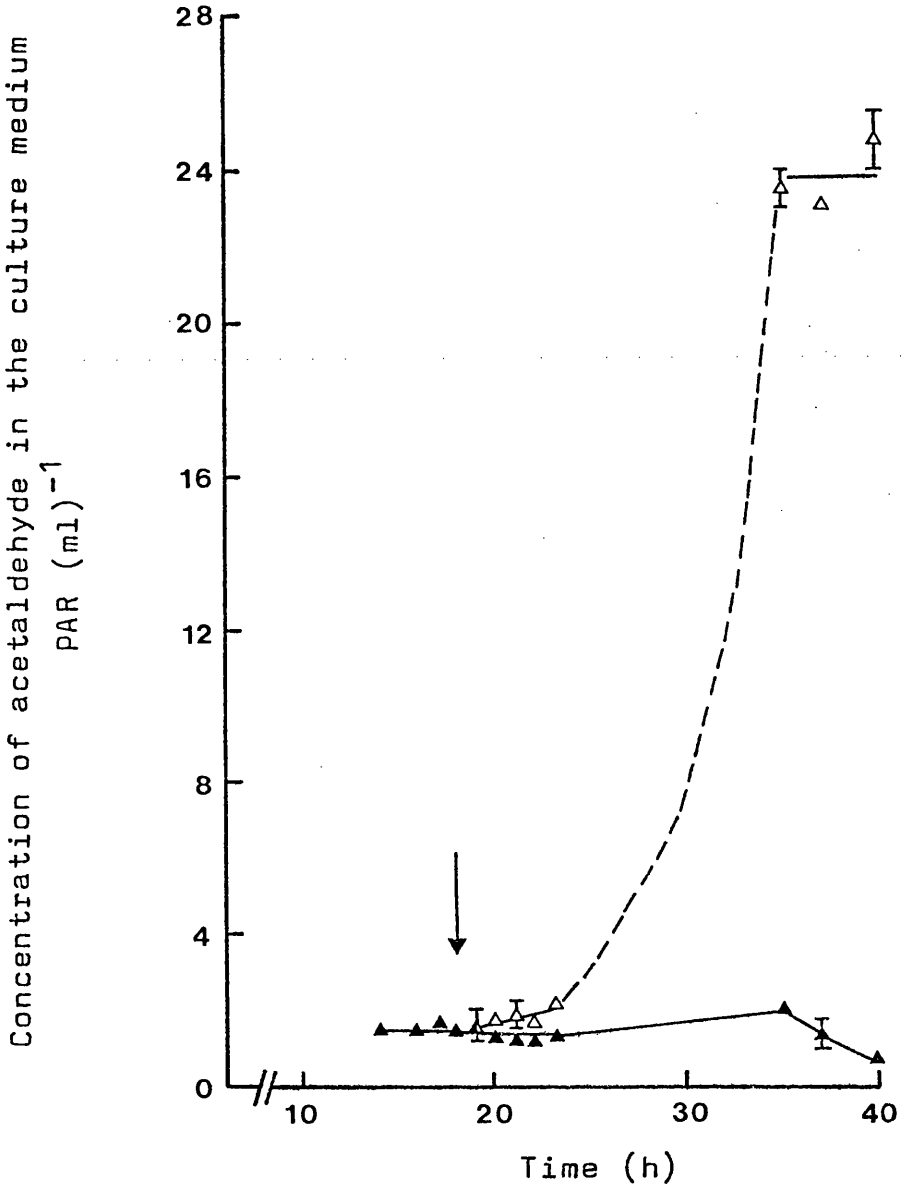


Figure 21.

Effect of cerulenin on the synthesis of acetaldehyde. Experimental details and symbols as in Fig.16. Peak area ratio (PAR) calculated using a Pye Unicam calculating integrator. All points plotted are the mean of duplicate determinations from two cultures. Bars show range of values obtained.

6.6 The Acetyl-charge and the Concentrations of acetyl-CoA and CoASH in Sacch. cerevisiae

6.6.1 The acetyl-charge and the concentrations of acetyl-CoA and CoASH during fermentation of wort

Once a satisfactory method for the measurement of acetyl-CoA and CoASH in yeast had been developed, the levels of these two metabolites in NCYC 240 were monitored during fermentation of 1·040 wort. Particular attention was paid to the latter half of fermentation during which the specific rate of ester synthesis changes most markedly. The following parameters were also monitored: specific gravity; yeast biomass; and the amount of DNA and protein in the yeast. In all respects the course of fermentations were in excellent agreement with those reported in other sections. Fermentations to which linoleic acid (50μg ml^{-1}) had been added were also studied.

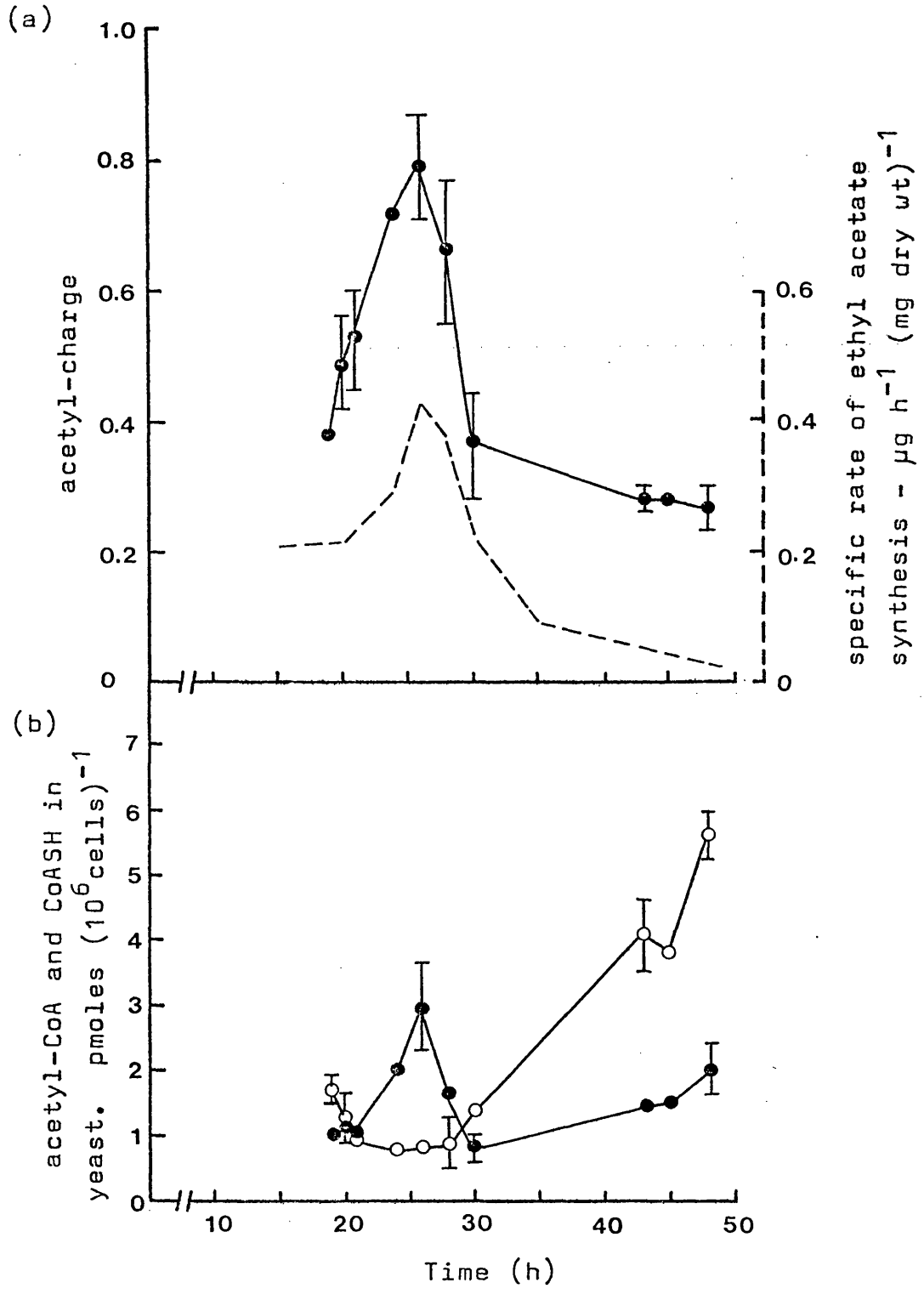
The relationship between acetyl-CoA and CoASH is expressed here as the acetyl-charge (as defined by Hampsey & Kohlhaw, 1981) i.e. [acetyl-CoA] / [acetyl-CoA + CoASH] . The acetyl-charge (which is unit-less) rose from 0·38 at 19h to 0·8 at 26h and then declined to less than 0·3 in stationary-phase. The 'peak' in acetyl-charge, clearly corresponded to the peak in specific rate of formation of ethyl acetate (Fig.22.a) The acetyl-charge, whilst describing the relationship between acetyl-CoA and CoASH does not indicate the

Figure 22.

The acetyl-charge and the intracellular pool size of acetyl-CoA and CoASH in Sacch. cerevisiae during fermentation of 1·040 all-malt wort.

(a) For definition of acetyl-charge see text. This figure shows the relationship between acetyl-charge (●—●) and the specific rate of formation of ethyl acetate (----) during 1·040 all malt wort fermentation. Data for specific rates of ethyl acetate formation are taken from Fig.13.a. Values for acetyl-charge are mean of single determinations (of CoASH and acetyl-CoA) from each of two fermentations. Bars represent range of values obtained.

(b) Intracellular amounts of acetyl-CoA (●—●) and CoASH (o—o). Cell number was determined by measurement of the DNA content of the cultures as described in Materials and Methods. All DNA values used in the calculations were the mean of duplicate determinations from two separate cultures. These values were converted to give values for the cell number of each culture from which the data in Fig.22.b could be calculated. All points are mean of two determinations and bars show range of values obtained.


(a)
1.0
0.8
0.6
0.4
0.2
0
acetyl-charge
0.6
0.4
0.2
0
specific rate of ethyl acetate synthesis - μg h⁻¹ (mg dry wt)⁻¹
(b)
acetyl-CoA and CoASH in yeast. pmoles (10⁶cells)⁻¹
7
6
5
4
3
2
1
0
10
20
30
40
50
Time (h)

absolute levels of these two metabolites. However, these data are presented in Fig.22.b and Fig.24.a. Between 20h and 26h the intracellular level of CoASH remained relatively constant whereas that of acetyl-CoA increased three-fold. Thus the change in acetyl-charge was due to an increase in the pool-size of acetyl-CoA rather than a change in the degree of acetylation of a finite amount of coenzyme A. These data are also expressed on a concentration (μM) basis (Fig.24) so that comparison can be made with data for other metabolite levels and with *in vitro* studies. As can be seen the concentration of acetyl-CoA varied from 7 to 20μM between 20 and 26h. The levels of both metabolites rose quite markedly during the stationary-phase while the acetyl-charge remained at a low and constant value (Fig.22).

In fermentations supplemented with linoleic acid the increase in the acetyl-charge between 20 and 26h (Fig.23) was not so pronounced; the acetyl-charge was 0·45 at 20h and increased to a maximum of 0·68 at 26h. The intracellular levels of acetyl-CoA during this phase of fermentation (Fig.23 and 24) were only slightly lower than in control fermentations (Fig.22 and 24). However the intracellular level of CoASH was higher in yeast which had taken up linoleic acid, this accounting for the lower acetyl-charge. During the stationary-phase the intracellular pool of CoASH increased markedly, though in contrast to control fermentations, the pool

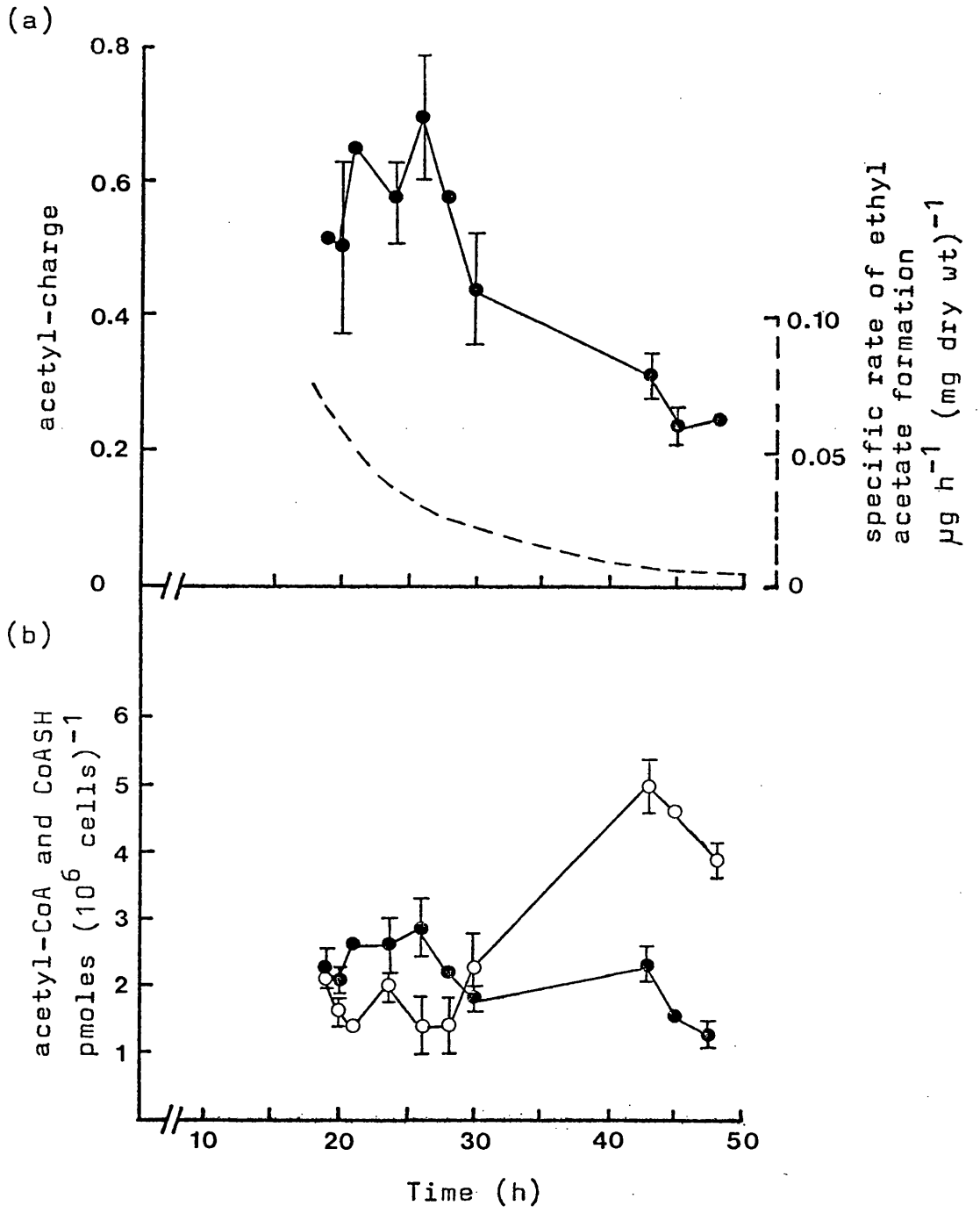


Figure 23.

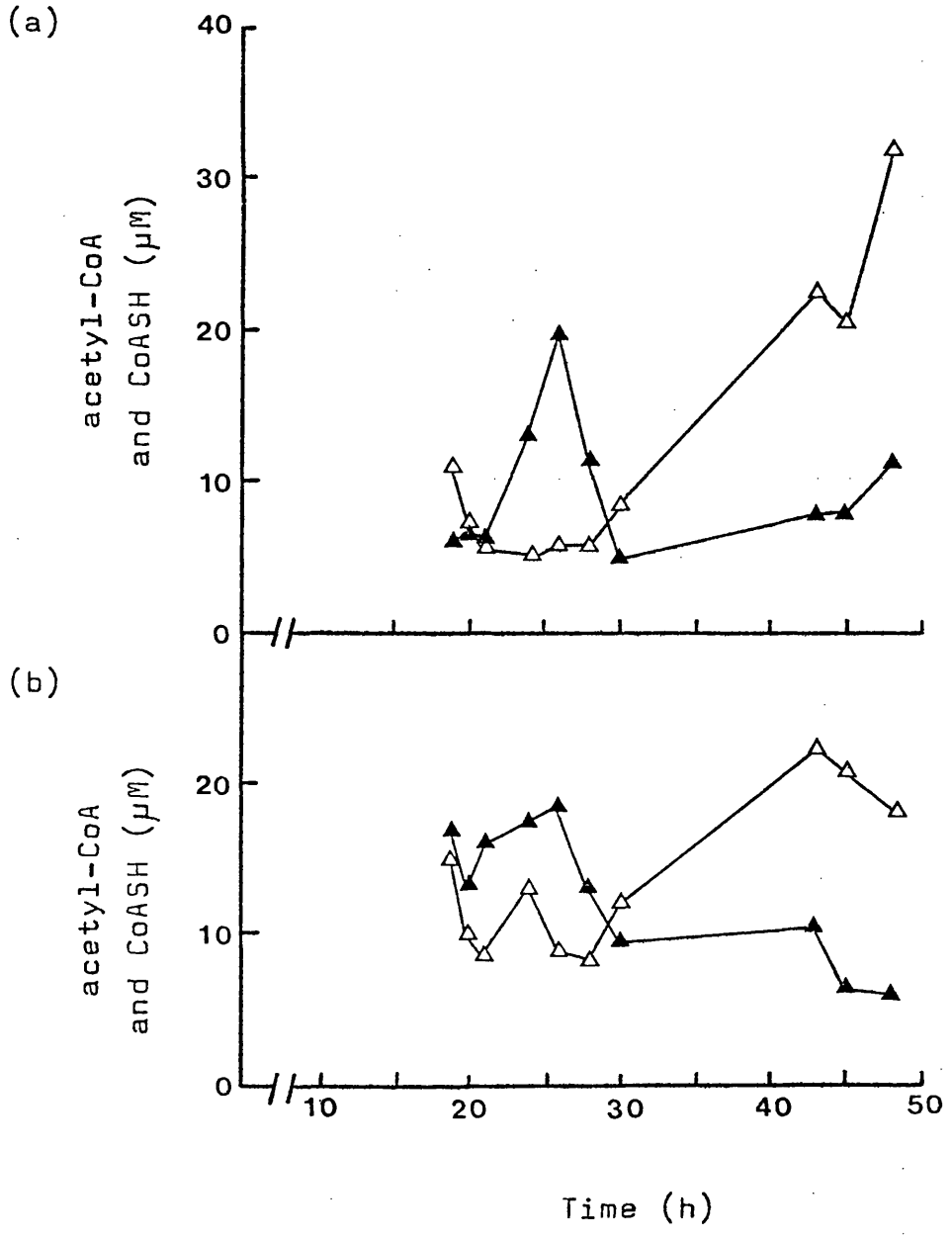
The acetyl-charge and the intracellular pool-size of acetyl-CoA and CoASH, during fermentation of wort supplemented with linoleic acid (50μg ml^{-1}). All symbols, experimental and analytical details as in Fig.22.

Figure 24.

Intracellular concentrations of acetyl-CoA and CoASH during fermentation of wort.

(a) control fermentations; (b) fermentations supplemented with linoleic acid (50μg ml^{-1}). Open symbols represent acetyl-CoA, closed symbols, CoASH.

Data are derived from Fig. 22 and 23. The 'cell-water' volume was taken as 3μl (mg dry wt)$^{-1}$ (Gancedo & Gancedo, 1973). For further comments on this method of estimating the intracellular concentration of metabolites see 6.6.3.


(a)
40
30
20
10
0
acetyl-CoA
and CoASH (μM)
(b)
20
10
0
acetyl-CoA
and CoASH (μM)
10
20
30
40
50
Time (h)

of acetyl-CoA declined slightly (Fig. 23 and 24). The acetyl-charge was correspondingly low (ca. 0·25). There was no clear-cut relationship between the specific rate of ethyl acetate synthesis and the acetyl-charge in fermentations supplemented with linoleic acid. Some of the data concerning intracellular levels of acetyl-CoA and specific rates of ethyl acetate synthesis are summarized in Table 20. As can be seen, during the period following cessation of lipid synthesis (i.e. 20h to 26h) the specific rate of ethyl acetate synthesis appears to be independent of both the acetyl-charge and intracellular concentration of acetyl-CoA in fermentations supplemented with linoleic acid. In such fermentations the acetyl-charge does not reach 0·8 but the intracellular acetyl-CoA concentration is comparable with that of the control fermentations during the 20-26h period (see also Fig.24). However, the specific rates of ethyl acetate synthesis are very much lower in the linoleic acid supplemented fermentations. Thus the effect of linoleic acid would appear to be exerted at a site other than one which affects the intracellular concentration of acetyl-CoA.

6.6.2 Acetyl-CoA hydrolysing activity of cell-free extracts of yeast during fermentation of wort

Saccharomyces cerevisiae possesses an acetyl-CoA hydrolase (EC 3.1.2.1) (Kohlhaw & Tan-Wilson, 1977). Since the intracellular levels of acetyl-CoA and CoASH fluctuate during fermentation (6.6.1) and appear to

Table 20. Relationship between intracellular concentrations of acetyl-CoA and specific rates of ester synthesis

Time after inoculation (h)	acetyl-CoA μM		Acetyl-charge		Specific rate of ethyl acetate synthesis μg h^{-1} (mg dry weight)$^{-1}$	
	Control	+18:2	Control	+18:2	Control	+18:2
20	7·2	12·8	0·48	0·50	0·24	0·05
26	19·8	18·3	0·79	0·69	0·46	0·03

+ 18:2 refers to cultures supplemented with linoleic acid (50 μg ml^{-1}); data taken from Fig.13,22,23,24.

determine the specific rate of acetate ester synthesis, the acetyl-CoA hydrolysing activity of yeast during fermentation was measured. Samples of yeast were removed from fermentations at the times indicated in Fig.25, washed, homogenized, and acetyl-CoA hydrolysing activity measured in crude homogenates by the method of Prass et al. (1980). This method monitors acetyl-CoA dependent release of thiol groups; these react with DTNB to give a yellow colour, the absorbance of which is measured at 412nm. The specific activity of acetyl-CoA hydrolysis is expressed on a dry weight rather than a protein basis to enable strict comparison with the specific rate of ethyl acetate synthesis (Fig. 25.a). The specific activity (and total activity) of acetyl-CoA hydrolysis increased from 20h onwards as did the specific rate of ester synthesis (Fig.25). However the activity of acetyl-CoA hydrolysis remained at this elevated level for the remainder of fermentation whereas the specific rate of ester synthesis declined. There was very little difference in total and specific activity of acetyl-CoA hydrolysis between control cultures and those supplemented with linoleic acid.

6.6.3 Effect of varying growth conditions on the acetyl-charge of Sacch. cerevisiae

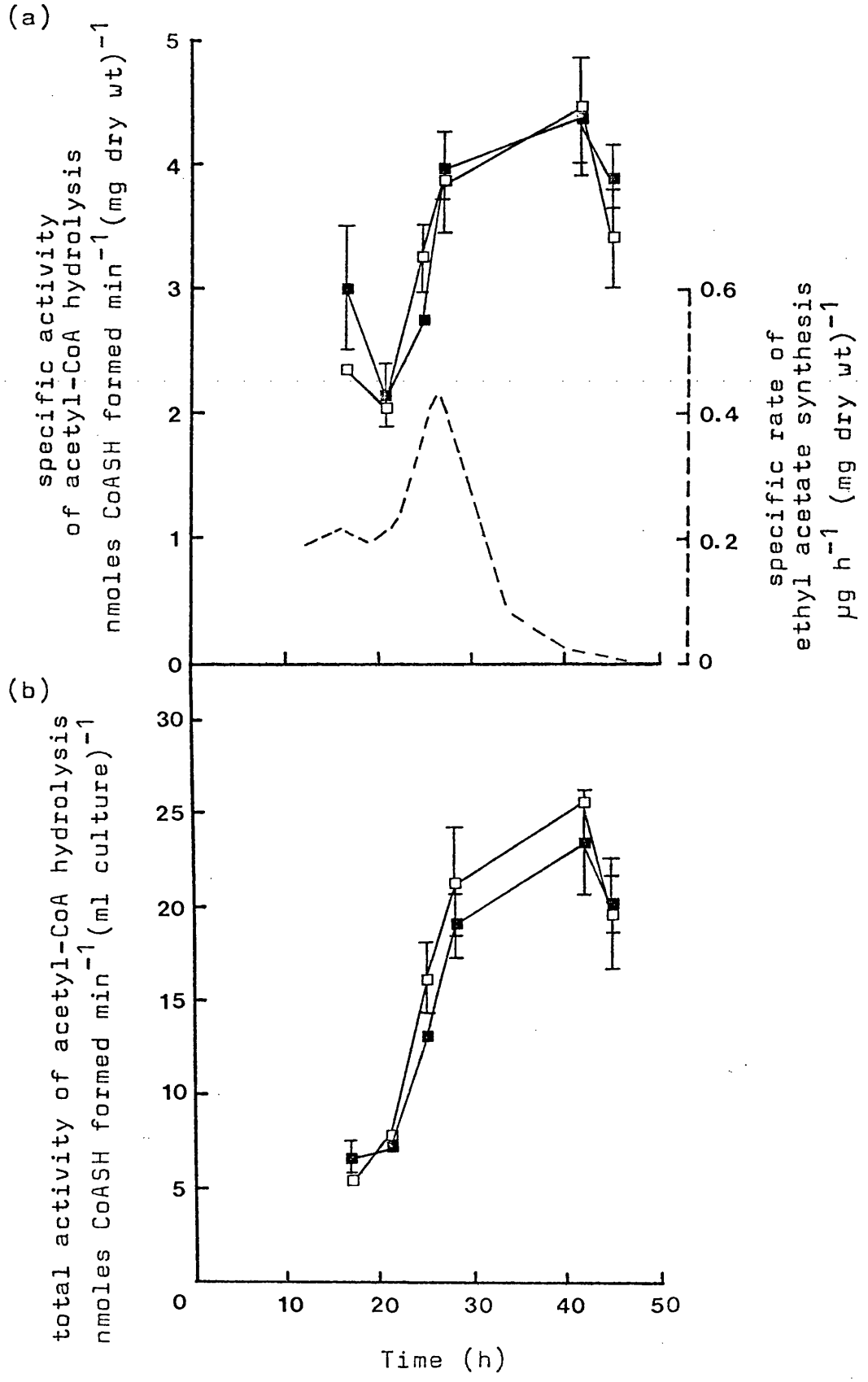
From previous studies (section 6.1) it is clear that the acetyl-charge can vary within wide limits. It was decided to extend these observations with a view to ascribing some metabolic significance to the acetyl-

Figure 25.

Specific activity and total activity of acetyl-CoA hydrolysis in cell-free extracts of Sacch. cerevisiae during fermentation of 1·040 all-malt wort.

(a) Specific activity of acetyl-CoA hydrolysis in yeast from control fermentations (■—■) and those supplemented with linoleic acid (50μg ml^{-1}) (□—□). Data for specific rate of ethyl acetate synthesis in control fermentations (----)are taken from Fig.13.a.
(b) Total activity of acetyl-CoA hydrolysis.
Symbols as in (a).

All points in (a) and (b) represent mean of triplicate determinations on samples taken from two separate fermentations. Bars show range of values obtained.


(a)
specific activity
of acetyl-CoA hydrolysis
nmoles CoASH formed min^{-1}(mg dry wt)$^{-1}$
specific rate of
ethyl acetate synthesis
μg h^{-1} (mg dry wt)$^{-1}$
(b)
total activity of acetyl-CoA hydrolysis
nmoles CoASH formed min^{-1}(ml culture)$^{-1}$
Time (h)

charge. Saccharomyces cerevisiae NCYC 240 was grown aerobically and anaerobically on YEPG (5%) medium and aerobically on YEPGE medium; and the intracellular concentrations of acetyl-CoA and CoASH were determined. Samples were taken from cultures in late-logarithmic phase. Results are presented in Table 21. The acetyl-charge of the ethanol-grown cells was substantially lower than in yeast of the two other physiological states. The intracellular concentrations of acetyl-CoA and CoASH are in relatively good agreement with the data presented in 6.6.1.

Representation of this type of data (Table 21) requires careful consideration. For example, comparison of the intracellular level of CoASH in ethanol-grown cells, with that in the anaerobic glucose-grown cells shows it to be 4 to 5-fold higher in the former cells. However when compared on a μM basis the cells have only a two-fold higher CoASH content. This arises because (a) the intracellular concentration was calculated on the premise that 1mg dry weight cells has a 'cell water' volume of 3μl (Gancedo & Gancedo, 1973) and (b) the biomass of 10^6 ethanol grown cells was lower than that of 10^6 anaerobic cells. Unequivocal concentrations can only be determined if the intracellular 'water' volume is measured for each yeast sample; this was not done. In fact the 'cell water' volume does change during anaerobic batch culture (Beaven et al., 1982). The observations reported in Table 21 are only of a

Table 21. Intracellular concentrations of acetyl-CoA and CoASH of Sacch. cerevisiae grown under different conditions

Growth conditions	Acetyl-charge	CoA pmoles (10^6 cells)$^{-1}$			Concentration of CoA - μM		
		Acetyl-CoA	CoASH	Sum	Acetyl-CoA	CoASH	Sum
1 Aerobic glucose-grown	0·61	1·44±0·24	0·93±0·09	2·37±0·33	12·1	7·9	20·0
2 Anaerobic glucose-grown	0·53	0·99±0·3	0·89±0·08	1·88±0·38	18·4	16·3	34·7
3 Aerobic ethanol-grown	0·20	0·93±0·03	4·02±0·04	4·95±0·07	7·5	32·5	40·0

The experimental conditions of growth were as follows:

1 YEPG (5%) medium inoculated with $0{\cdot}25 \times 10^6$ cells ml^{-1} (propagated in YM Broth). Flasks shaken at 180 r.p.m. at $28^{\circ}C$; samples removed after 24h.

2 Air-saturated medium as in 1, inoculated with 10×10^6 cells ml^{-1} (anaerobically propagated). Cultures stirred under an atmoshpere of oxygen-free nitrogen at $20^{\circ}C$. Samples removed after 24h.

3 YEPGE medium inoculated with 10^6 cells ml^{-1} (propagated in YM Broth). Cultural conditions as in 1, samples removed after 40h.

Values in 1 and 2 are mean of four measurements; those in 3 are mean of two.
($\pm$) shows the range of values obtained.

preliminary nature; a more detailed report is in preparation (Quain & Thurston, 1982). It is pertinent to note here that resuspension of aerobic glucose-grown cells in a medium in which ethanol is the sole carbon source, results in the acetyl-charge falling from ca. 0·6 to ca. 0·3 within 4h. The significance of the differences in the acetyl-charge between the different physiological states is discussed in section 7.2.

DISCUSSION

7.1 Regulation of Acetate Ester Synthesis in Sacch. cerevisiae

The specific rates of synthesis of ethyl and iso-amyl acetates change quite markedly during fermentation of 1·040 wort. In this section a marked increase in specific rate will be referred to as an induction of ester synthesis (this term is not intended to imply new synthesis of ester-synthesizing enzyme). In the wort fermentation system used here, there are two inductions of ester synthesis. There is also a notable decline in the specific rate of ester synthesis following the second induction. Possible reasons for this pattern of ester synthesis and the implications with regard to control of the formation of esters are discussed below.

7.1.1 Induction of acetate ester synthesis

The finding that the specific rate of ethyl acetate synthesis, after only 8h into fermentation, was up to ten times higher than in the stationary-phase of fermentation suggests that ethyl acetate formation must be induced at the start of fermentation (prior to 8h). A second induction occurs at the mid-point of fermentation.

The yeast inoculum is in a physiological state termed by Searle & Kirsop (1979) as maintenance-phase. In this state (at the end of fermentation) it derives energy from the utilization of small amounts of

exogenous sugars (e.g. maltotriose) and also from the slow dissimilation of glycogen (Quain et al., 1981). When inoculated into air-saturated wort, intense metabolic activity ensues. This is characterized by extensive lipid synthesis and rapid dissimilation of glycogen, the latter process being considered to fuel the former (Quain et al., 1981; Quain & Tubb, 1981). The dissimilation of glycogen should lead to the formation of ethanol, elevation of the energy-charge (Ball & Atkinson, 1975) and synthesis of acetyl-CoA. Following the initial rapid burst of lipid synthesis the specific rate of fatty acid synthesis rapidly declines. Thus acetyl-CoA would then be more readily available for ethyl acetate synthesis and may account for (in part at least) the first induction of ethyl acetate synthesis.

Whereas ethanol is formed as soon as appreciable glycolytic flux is obtained, the formation of iso-amyl alcohols (2- and 3-methylbutanol) requires a supply of leucine and iso-leucine (Äyräpää, 1971; Inoue, 1975). These amino acids have been placed in 'group B' by Jones & Pierce (1964) i.e. uptake does not commence immediately upon inoculation but would be complete before the end of fermentation. Therefore, temporally, the formation of iso-amyl alcohol would not be expected to parallel the formation of ethanol. Differences in the specific rate of formation between iso-amyl acetate and ethyl acetate may possibly be explained on this basis. For example, iso-amyl acetate synthesis may not be induced

as early on in fermentation as ethyl acetate. It is not possible from the studies described here to ascertain if this is so, since iso-amyl acetate was present in trace amounts during early phases of fermentation.

The second induction of acetate ester synthesis occurs at the mid-point of fermentation. This phase of induction, although short-lived, contributes significantly to the overall production of ethyl acetate in beer. As described above there is an inverse relationship between lipid syntheses (squalene and saturated fatty acids) and acetate ester synthesis; when lipid syntheses cease, ester synthesis is induced. The finding that acetate esters are synthesized at a constant specific rate between 8h and 20h indicates that this route of acetyl-CoA consumption is in equilibuium with the demands of lipid synthesis (although the specific rates of fatty acid and squalene syntheses are gradually declining at this stage). However when lipid syntheses stop, the overall metabolic demand for acetyl-CoA seems likely to decrease and shift the acetyl-CoA : CoASH ratio in favour of acetyl-CoA, thereby stimulating the rate of formation of esters. This was confirmed by the measurements made of intracellular levels of acetyl-CoA and CoASH during fermentation. Cessation of lipid synthesis does in fact cause the acetyl-charge and the intracellular concentration of acetyl-CoA to increase transiently and is almost certainly responsible for this induction of ester synthesis. At the point of

maximum specific rate of ethyl acetate synthesis, 80% of the coenzyme A is in the form of acetyl-CoA (long chain acyl-CoA esters and other CoA compounds were not measured). Elevation of the acetyl-charge to this level did not greatly induce synthesis of iso-amyl acetate. Possibly this was because a relatively high level of iso-amyl alcohol production and a high acetyl-charge did not concomitantly exist.

Since cessation of lipid synthesis apparently promotes the induction of ester synthesis, it is pertinent to consider why lipid synthesis does stop at the mid-point of fermentation. During fermentation, cell-division stops shortly after lipid synthesis does, though cell mass continues to increase due to accumulation of protein and glycogen (Quain et al., 1981). Cell division does not occur once the sterol content of the cells has become diluted by growth to ca. 0·1μg mg^{-1} (David & Kirsop, 1973; David, 1974). Cessation of lipid synthesis is almost certainly related to this growth-limiting cellular concentration of sterol. The biochemical signals for shutdown of lipid synthesis are not known, but it is likely that the syntheses of lipids and DNA, and the mechanics of cell division are co-ordinately controlled. Certainly in the sterol requiring prokaryote, Mycoplasma capricolum, availability of cholesterol appears to be coupled with phospholipid biosynthesis which may be coordinately controlled with protein biosynthesis (Dahl et al., 1981).

Notably, the intracellular pools of both acetyl-CoA and CoASH increase significantly during stationary-phase; the acetyl-charge remains at a low level (ca. 0·3). This increase may reflect a general release of coenzyme A from other sources e.g. fatty acyl-CoA esters, once lipid synthesis stops. It could be considered advantageous for the cell to develop a high level of CoASH during stationary-phase since when challenged with oxygen, upon reinoculation, there will be a high availability of substrate for the synthesis of acetyl-CoA which in turn is required for the synthesis of lipids. During stationary-phase the concentration of acetyl-CoA, in the cell, is relatively high though the specific rate of ester synthesis is exceptionally low. Thus, there is no absolute correlation between the intracellular concentration of acetyl-CoA and acetate ester synthesis throughout fermentation, although this relationship does hold between ca. 18h and 30h. Possibly the acetyl-charge is a controlling factor here rather than the intracellular level of acetyl-CoA per se.

7.1.2 Decline in the specific rate of ester synthesis.

The increases in both the acetyl-charge and the specific rate of acetate ester synthesis are transient. If they had remained at an elevated level, the final concentration of acetate esters in beer would have been very much higher. The rapidity with which the acetyl-charge declines from ca. 0·8 to 0·4, possibly reflects

the cell's need to maintain a balance between CoASH and acetyl-CoA such that they are present in approximately equimolar amounts. The acetyl-charge and the specific rate of ester synthesis may decline for one or more of the following reasons:

(i) Acetyl-CoA, as its concentration increases inhibits its own synthesis. To assess the significance of such feedback inhibition requires consideration of the possible routes of formation of acetyl-CoA. This has already been briefly considered. In yeast growing anaerobically on brewer's wort, the most likely route of acetyl-CoA formation is microsomal acetyl-CoA synthetase (ACS) (Klein & Jahnke, 1968). Another potential source of acetyl-CoA is via the action of pyruvate dehydrogenase (PDH) which is present in Sacch. cerevisiae (Polakis & Bartley, 1965; Wais et al., 1973; Kresze & Ronft, 1981a,b). Pyruvate dehydrogenase is generally considered to be a mitochondrial enzyme (Denton & Halestrap, 1979). Mitochondrial development is repressed under anaerobic growth conditions (Wallace & Linnane, 1964) but purification of PDH from brewer's yeast (Wais et al., 1973) provides evidence that potentially PDH (located in the promitochondria) is active during wort fermentation. If this is so, then acetyl-CoA may be transported out of the mitochondria via the action of carnitine acetyltransferase (Kohlhaw & Tan-Wilson, 1977). The relative importance of ACS and PDH as a supply of acetyl-CoA for ester synthesis is not known; neither is the cellular

location of ester synthesis. Certainly the PDH of Sacch. cerevisiae is subject to feedback inhibition by acetyl-CoA (Kresze & Ronft, 1981a) and if PDH does supply acetyl-CoA for ester synthesis such feedback inhibition may be one reason why the intracellular concentration of acetyl-CoA does not remain at a high level. Acetyl-CoA synthetase activity may decline during the phase when the specific rate of ester synthesis falls as a result of a drop in the energy-charge of the cell, since ACS activity is ATP-dependent.

(ii) As acetate esters are formed and the acetyl-charge declines, the increasing concentration of CoASH may inactivate the ester-synthesizing enzyme as it does other acetyl-CoA utilizing enzymes in Sacch. cerevisiae (Tracy & Kohlhaw, 1975; Tan-Wilson & Kohlhaw, 1978; Gilbert & Stewart, 1981).

(iii) Other control mechanisms may come into play with regard to the decrease in the intracellular pool of acetyl-CoA. Here hydrolysis of acetyl-CoA could be important. Acetyl-CoA hydrolysing activity increases significantly at 20h and remains at a high level for the remainder of fermentation. The method employed for measuring acetyl-CoA hydrolysis does not exclusively measure acetyl-CoA hydrolase since crude homogenates were assayed. If sufficient amounts of oxaloacetate and carnitine were present in the homogenates, citrate synthase (EC 4.1.3.7) and carnitine acetyltransferase (EC 2.3.1.7) activities may well be measured since both activities release CoASH from acetyl-CoA. Work in this

laboratory (Thurston & Quain, unpublished) has shown that anaerobically-grown Sacch. cerevisiae does contain carnitine and possess carnitine acetyltransferase and citrate synthase activities. The concentrations of substrates used in the assay of these two enzymes were 3mM and 2mM for carnitine and oxaloacetate respectively. However, only small volumes of crude homogenates are required for the assay of acetyl-CoA hydrolysis and therefore, for carnitine acetyltransferase and citrate synthase to account for a significant proportion of the activity reported in 6.6.2, unrealistically high concentrations of carnitine and oxaloacetate would have to be present in yeast. Thus a significant proportion of the activity reported in 6.6.2, is probably acetyl-CoA hydrolase (EC 3.1.2.1). Kohlhaw & Tan-Wilson (1977) state that the affinity of yeast acetyl-CoA hydrolase for acetyl-CoA is much lower than other acetyl-CoA utilizing enzymes in Sacch. cerevisiae. Therefore it may function as a coarse control mechanism and correct any gross imbalances in the acetyl-charge. Such an imbalance occurs at the mid-point of fermentation. Indeed, the existence of acetyl-CoA hydrolase in mammalian tissues is well documented (Knowles et al., 1974; Costa & Snoswell, 1975a,b; Robinson et al., 1976; Grigat et al., 1979; Prass et al., 1980). Knowles et al. (1974) view the acetyl-CoA hydrolase as a means of relieving what they term 'acetyl-pressure'. For example in rat-liver cells, if the TCA-cycle is restricted by lack of ADP or pyruvate, acetyl-CoA

would tend to accumulate. Hydrolysis of acetyl-CoA and release of acetate into the bloodstream would enable an oxidizable substrate to be redistributed to other tissues. The fact that the mammalian acetyl-CoA hydrolase is ATP-stimulated (Prass et al., 1980) further supports this view.

The activity of other acetyl-CoA consuming enzymes, e.g. citrate synthase, may also increase as described above for acetyl-CoA hydrolase during fermentation. This was not studied. Citrate synthase is a mitochondrial enzyme (Hampsey & Kohlhaw, 1981) and an increase in activity would only reduce ester synthesis, by restricting availability of acetyl-CoA, if mitochondrially generated acetyl-CoA is a significant contributor to ester synthesis. It is evident from the preceeding discussion that investigation of the relative importance of ACS and PDH in forming acetyl-CoA in anaerobically growing yeast is an area worthy of study.

7.1.3 A metabolic role for ester synthesis in Sacch. cerevisiae

The proposal that acetyl-CoA hydrolysing activities play a role in controlling imbalances in the acetyl-charge, suggests that acetate ester synthesis may also fulfil such a role, since it too hydrolyses acetyl-CoA and generates free CoASH. It is difficult to assess the relative importance of the hydrolysing activity and ester synthesis since:

(i) the acetyl-CoA hydrolysing activities (6.6.2) were measured *in vitro*; and (ii) the reduction in the size of the acetyl-CoA 'pool' cannot be stoichiometrically correlated with the formation of acetate esters. The acetyl-CoA 'pool' values (6.6.1) do not represent a finite amount of acetyl-CoA to be utilized. There is probably a continual state of formation and utilization of acetyl-CoA; therefore the reduction in the size of the acetyl-CoA 'pool' represents a decline in the ratio of the acetyl-CoA synthesizing : utilizing activities.

The second induction of ester synthesis follows exponential-phase and preceeds stationary-phase. This period has been termed 'idiophase' by Tempest and co-workers (for example see Leegwater *et al.*, 1982) and is the phase during which secondary metabolites (e.g. antibiotics) are often produced and excreted. Here ethyl acetate behaves as such an 'idiolite' and may be produced in response to an imbalance in the acetyl-charge. However, when fatty acid synthesis, and consequently exponential growth, were inhibited by cerulenin, no immediate excretion of ethyl acetate occurred. This was possibly because growth conditions were aerobic and resulted in a high degree of fatty acyl unsaturation in the yeast lipids, a situation which is not conducive to ester synthesis (see below). However, the cerulenin-inhibited cells did release relatively large amounts of acetaldehyde. Possibly, inhibition of fatty acid synthesis by cerulenin leads to overproduction of

acetyl-CoA which may have been controlled by hydrolysis to acetate and CoASH via the action of acetyl-CoA hydrolase. Since the cells were in a fermentative mode of metabolism, acetate may well have been used to oxidize NADH with the concomitant formation of acetaldehyde. These findings suggest that when fatty acid synthesis stops (or is inhibited), a condition of 'acetyl-pressure' develops. This can be alleviated by the excretion of acetyl units in the form of secondary metabolites such as ethyl acetate or acetaldehyde.

An alternative view is that ester synthesis may have no role and esters may be formed coincidentally by the enzymes which synthesize complex lipids. These enzymes may form more esters when, (i) the acetyl-charge increases and (ii) when there are no competing substrates i.e. fatty acyl-CoA esters.

7.1.4 Suppression of ester synthesis by unsaturated fatty acids

Addition of unsaturated fatty acids to wort, or enrichment of the yeast inoculum with unsaturated fatty acyl residues, greatly suppresses the formation of acetate esters. In linoleic acid-supplemented fermentations no induction of acetate ester synthesis takes place, and the specific rates of synthesis of esters are very low throughout fermentation (section 6.4). Collectively the data presented here suggest that the effect of unsaturated fatty acids is at the level of the membrane.

Certainly the linoleic acid is incorporated into the phospholipids of Sacch. cerevisiae and presumably alters the physical properties of the membrane.

The relationship between lipid synthesis and ester synthesis is not so well defined in fermentations to which linoleic acid has been added. At the mid-point of fermentation, squalene synthesis ceases but fatty acid synthesis continues, albeit at a low rate. This low rate of fatty acid synthesis may be sufficient to compete with ester synthesis for acetyl-CoA and thus suppress ester synthesis. This factor is possibly unimportant to the main argument however, for the following reasons. During the phase of fermentation when rates of lipid synthesis were similar in control and linoleic-acid supplemented cultures (i.e. at ca. 17h) the specific rates of ethyl acetate synthesis in the former cultures were very much higher than those in the latter cultures. Further, the specific rate of ester synthesis appears to be independent of both the acetyl-charge and the intracellular concentration of acetyl-CoA in yeast from fermentations supplemented with linoleic acid. Thus the effect of linoleic acid would appear to be exerted at a site other than one which affects the intracellular concentration of acetyl-CoA. Certainly uptake of linoleic acid increases the % UFA of the yeast phospholipids and would be expected to change the physical properties of the membranes (i.e. to increase the fluidity). Many enzymes in Sacch.

cerevisiae are regulated by membrane lipid composition (Cobon & Haslam, 1973; Janki et al., 1974; Watson et al., 1975; Haslam & Al Mahdawi, 1980). The ethyl acetate synthesizing activity of a brewing strain of Sacch. cerevisiae is reported to be primarily located in the membrane-rich sub-cellular fraction sedimenting at 105,000g (Anderson & Howard, 1976a) and more recently the purified enzyme has been shown to be membrane-located (Yoshioka & Hashimoto, 1981). Thus, modulation of the enzyme's activity by membrane lipid composition seems likely; a high % UFA in the membrane lipids prevents stimulation of acetate ester synthesis even though availability of acetyl-CoA is high.

Alternatively there may have been a direct effect on the ester-synthesizing enzyme by free linoleic acid. Yoshioka & Hashimoto (1981) found that the alcohol acetyltransferase was inhibited $>$ 95% by free linoleic acid (2mM) and oleyl-CoA. Generally $>$ 98% of the linoleic acid in yeast from fermentations described in section 6, was in the esterified form. Whether or not the small amounts of free acid were responsible for the extensive suppression of acetate ester synthesis observed in these studies cannot be determined.

Addition of stearic acid to fermentation resulted in a slight stimulation of acetate ester synthesis. This corresponded to only a slight increase in the saturated fatty acid content of the yeast lipids. Therefore,

activity of the ester synthesizing enzyme appears to be correlated well with the degree of unsaturation of the yeast lipids. Establishment of an *in vitro* ester synthesizing system may throw more light on this mode of regulation. A further factor worthy of consideration is that enrichment of yeast phospholipids with linoleyl residues may affect the transport of leucine and isoleucine. These amino acids are precursors of the iso-amyl alcohol moiety of iso-amyl acetate. Therefore, differences in the formation of this ester between control fermentations and those supplemented with linoleic acid may be partially caused by such a transport phenomenon.

7.1.5 Control of acetate ester levels in beer

The information discussed above explains why certain technological factors affect the formation of acetate esters (see Section 3) in normal gravity fermentations. Moreover, the information can be related to procedures used to control disproportionate synthesis of acetate esters in high-gravity brewing (Sections 1 and 3). Possible new methods of controlling ester synthesis in brewing can be tentatively proposed, although knowledge regarding the precise reasons for disproportionate synthesis are lacking.

Up to 30% of the ethyl acetate formed in 1·040 fermentation is the result of the second induction of ester synthesis. Thus a certain degree of control (i.e.

reduction) of acetate ester synthesis can be achieved if this induction-phase is minimized or prevented. Oxygenation is more effective in reducing acetate ester synthesis at certain points, in high-gravity fermentations, than at others. This is probably because induction of ester synthesis does occur in high-gravity brewing and so oxygenation just prior to induction is more effective than at any other time during fermentation. Oxygen presumably enables lipid synthesis to proceed for the duration of fermentation and thus there is no phase during which the acetyl-charge reaches a high level (e.g. 0·8). There are drawbacks to oxygenation of high-gravity fermentations as discussed in Section 3 i.e. growth is greatly stimulated and thus yeast wetting losses are increased; furthermore practical aspects of oxygenation may cause difficulties. However, knowledge of specific rates of formation of ester synthesis during high-gravity fermentations may allow further optimization of oxygenation procedures to control disproportionate ester synthesis.

Potentially larger reductions (than cited above) in the levels of acetate esters in normal gravity fermentations can be made by suppressing the 'non-induced' rate of synthesis of esters. This involves affecting the proportion of acetyl-CoA that is used for acetate ester synthesis throughout fermentation. Such procedures may or may not prevent induction. Addition of linoleic acid reduces the non-induced rate, and abolishes the induction

of acetate ester synthesis; consequently the formation of acetate esters is reduced by $>$ 80%. Certainly addition of linoleic acid to wort would appear to be a potential means of controlling ester synthesis in high-gravity fermentations. Use of this method of control firstly requires that an economic source of unsaturated fatty acids be available. Possibly spent-grains are such a source (Taylor *et al.*, 1979). Only *ca.* 0·1% to 0·7% of the total malt-lipid survives into the pitching wort but the concentration of free fatty acids can be in the range 1-16mg l^{-1} of which $>$ 55% are unsaturated. Therefore it is clear that the cereal grist contains quantities of unsaturated fatty acids far in excess of that required to control ester synthesis. It is also noteworthy that the cereal grains contain more than enough sterols to obviate the need for oxygen in fermentation (Taylor *et al.*, 1979). However, obtaining the lipids in a form which can be conveniently added to fermentation is a problem which has yet to be overcome. Further, the effects of fermenting worts with relatively high lipid concentrations, on beer properties would have to be extensively investigated before this method of control could be employed. Advantages of such a procedure are (i) that only several mg l^{-1} of linoleic acid significantly suppress acetate ester formation and (ii) the amount of additional yeast growth obtained as a result of addition of linoleic acid is much less than would be obtained if oxygen was used to suppress ester formation to the same extent.

The mechanism by which linoleic acid exerts its effect on ester synthesis suggests that manipulation of fermentation temperature may offer a new method for controlling ester synthesis (in both high and normal-gravity fermentations). The data presented by Norstedt et al. (1975) show that syntheses of different esters have different temperature optima. Possibly, elevation of fermentation temperature beyond these optima would reduce ester synthesis because of changes in the physical properties of the yeast membranes. Higher temperatures would be expected to increase membrane fluidity (as would incorporation of linoleic acid into membranes). An additional advantage may be a reduction in fermentation time.

Carbon dioxide (CO_2) is known to suppress formation of acetate esters in normal-gravity fermentations (Section 3). On the basis of data presented in section 6.7, where the availability of acetyl-CoA was shown to directly determine the specific rate of synthesis of acetate esters, a hypothesis can be proposed to explain the effect of CO_2. Higher than normal concentrations of CO_2 (or HCO_3^-) such as might occur in deep cylindroconical vessels (Norstedt et al., 1975) may stimulate acetyl-CoA carboxylase and pyruvate carboxylase (Haarasilta, 1978; Haarasilta et al., 1979). The former enzyme consumes acetyl-CoA directly and forms malonyl-CoA; the latter enzyme forms oxaloacetate which together with acetyl-CoA are substrates in the reaction catalysed

by citrate synthase. Citrate synthase activity has been detected in anaerobically grown Sacch. cerevisiae. Thus less acetyl-CoA may be available for acetate ester synthesis either throughout fermentation; or the effect may only be marked during the induction-phase. The reduction of beer ester levels by increased CO_2-pressure is generally far less than by addition of unsaturated fatty acids to wort (Norstedt et al., 1975). Whether or not CO_2-pressure offers an effective or realistic means of controlling ester formation in high-gravity brewing is open to question and investigation.

Related to the above hypothesis are the observations concerning the effect on ester synthesis of addition of amyloglucosidase (AMG) to wort (Hockney & Zealey, 1981). Addition of AMG results in a wort where the fermentable carbon source is almost totally glucose. Fermentation of such wort leads to disproportionately higher levels of acetate esters. This result suggests that ester synthesis is controlled by catabolite-repression. The high concentrations of glucose are suggested by these workers to repress the formation and therefore activity of TCA-cycle enzymes during fermentation, thus increasing the availability of acetyl-CoA for ester synthesis.

As discussed briefly in Section 3, beers with acceptable levels of acetate esters can be produced by high-gravity brewing if worts are prepared using adjuncts rather than an all-malt grist. The reasons for this

are unclear (White & Portno, 1979) though it is likely that adjunct worts , which contain less nitrogenous material than all-malt worts, consequently contain lower levels of pantothenate. Therefore, less coenzyme A (CoA) may be formed by yeast (Hosono & Aida, 1974) and ester synthesis, presumably along with all other acetyl-CoA and CoA requiring reactions, is suppressed. This method of high-gravity brewing does have drawbacks (Section 3).

Other methods by which ester synthesis could be controlled would probably involve strain selection or genetic manipulation. For example, Sacch. cerevisiae has been found to possess an active acetyl-CoA hydrolase (EC 3.1.2.1) during fermentation (6.1.2). Possession of a high specific activity of this enzyme with a low Km for acetyl-CoA could prevent any imbalances in the acetyl-charge and therefore may prevent or minimize induction of ester synthesis. There may be other activities which use acetyl-CoA that could be activated or 'switched on' if the intracellular level of acetyl-CoA increased. The degree to which acetate ester synthesis could be inhibited by such mechanisms may, however, only be confined to that produced by induction (i.e. 30% of the total). Thus these mechanisms could not be as effective as those which directly affect the ester synthesizing enzyme (e.g. linoleic acid). Alternatively, strains of brewing yeast could be sought (or produced) that have a high 'esterase' activity. So instead of controlling

synthesis of esters, control could be exerted at the level of hydrolysis of esters. Sacch. cerevisiae possesses at least two electrophoretically distinguishable esterases (Campbell et al., 1972; Wohrmann & Lange, 1980; see also Wheeler & Rose, 1973; Parkkinen et al., 1978). Substrates most commonly used to detect esterase activiry are p-nitro-phenyl acetate and naphthyl acetate. The only reports of hydrolysis of typical 'beer esters' by yeast are Schermers et al. (1976) and Suomalainen (1981). Schermers et al. (1976) describe an esterase preparation from Sacch. cerevisiae which is capable of synthesizing ethyl acetate from ethanol and acetic acid at pH 4·0, but which extensively hydrolyses this ester at higher pH values (>6). From the data presented it is difficult to assess whether such an esterase could be of value in high-gravity brewing. To be of use, the pH optima for the hydrolytic activity would have to be lowered towards typical beer pH (i.e. pH 4). Concerning the synthesis of ethyl acetate by 'reverse esterase' action it is pertinent to note that Schermers et al. (1976) show a correlation between 'esterase' activity and the amount of acetate esters that several brewing yeasts produce during fermentation; the higher the 'esterase' activity, the greater the amounts of esters formed. The correlation between the intracellular levels of acetyl-CoA and ester synthesis suggests that this is a more significant route of acetate ester formation than 'reverse esterase' action.

7.2 Physiological Significance of the Acetyl-charge

The work described in this thesis has established that the acetyl-charge of Sacch. cerevisiae varies between 0·2 and 0·8 depending on growth-phase and growth conditions. During anaerobic growth on wort, the acetyl-charge changes quite markedly for the reasons discussed above. Cells growing aerobically on ethanol (1%) have a lower acetyl-charge (ca. 0·2) than when grown aerobically on glucose. To attempt to explain this observation requires consideration of, how Sacch. cerevisiae uses ethanol, and what physiological state, growth on ethanol induces.

Saccharomyces cerevisiae is subject to the phenomenon of catabolite repression (for review see Linnane & Haslam, 1970). Growth on relatively high concentrations of glucose results in cells with poorly developed mitochondria and low activities of many hydrolytic and oxidative enzymes. However, cells grown (aerobically) on non-fermentable carbon sources (e.g. ethanol) should possess mitochondria which are fully developed morphologically (Wallace & Linnane, 1964) and which have an operative TCA-cycle and respiratory chain (Chapman & Bartley, 1968; Perlman & Mahler, 1974). Further, availability of oxygen permits synthesis of sterols which will also be 'derepressed' under these growth conditions (Quain & Haslam, 1979a). So, one possible reason for the low acetyl-charge is that the overall

metabolic demand for acetyl-CoA is high. Secondly, and more importantly, when ethanol is the sole carbon source input of carbon into the central metabolic pathways must be in the form of acetyl-CoA, presumably via the reaction sequence:

ethanol ⟶ acetaldehyde (Eltayeb & Berry, 1977) ⟶ acetate (Black, 1956; Bradbury & Jakoby, 1971) ⟶ acetyl-CoA (Klein & Jahnke, 1971; 1979). There are at least two isoenzymes of acetyl-CoA synthetase (ACS) in Sacch. cerevisiae (Klein & Jahnke, 1979); one mitochondrially-located, the other, microsomal. The relative activities of the two isoenzymes are not known for cells grown on ethanol, but the mitochondrial enzyme is probably predominant. A further factor to consider is the role of pyruvate dehydrogenase (PDH). Growth on ethanol requires that gluconeogenesis takes place in order to form structural polysaccharides. Thus, carbon must be withdrawn from the TCA-cycle, and the glyoxylate cycle must function to replenish the 'loss' of carbon (Dunze et al., 1969; Szabo & Avers, 1969; Perlman & Mahler, 1970; Parish, 1975). Oxaloacetate is decarboxylated to yield phophoenolpyruvate (PEP), a reaction catalysed by cytoplasmically-located PEP-carboxykinase (Haarasilta & Taskinen, 1977; Maitra & Lobo,1978) and gluconeogenesis ensues (Maitra & Lobo, 1978). Little pyruvate should be formed from PEP since activity of pyruvate kinase is low under such growth conditions (Barwell & Hess, 1971). If the activity of pyruvate kinase was high, a futile cycle would occur:

oxaloacetate ⟶ PEP ⟶ pyruvate ⟶ oxaloacetate, the last reaction being catalysed by pyruvate carboxylase, which is active in ethanol-grown cells (Haarasilta & Oura, 1975). Consequently although PDH may be present in the yeast mitochondria, it will have a low activity due to lack of substrate and little acetyl-CoA would thus be formed by this route when ethanol is the sole carbon source. Some organisms (e.g. E.coli) possess a CoASH-linked aldehyde dehydrogenase which forms acetyl-CoA from acetaldehyde and CoASH (Shore & Fromm, 1981). Whether or not this route of acetyl-CoA synthesis is open to Sacch.cerevisiae growing on ethanol is not known. Thus, it is evident that some details of acetyl-CoA formation and the subcellular locations at which it is formed are unclear. However, a low acetyl-charge in ethanol-grown cells would appear to be due to the nature of utilization of ethanol and the resultant high metabolic demand for acetyl-CoA.

When cells are grown aerobically in the presence of high concentrations of glucose (e.g.10%), sterol synthesis (Quain & Haslam, 1979a) and mitochondrial development and functioning are 'repressed' (Chapman & Bartley, 1968) though some respiration does occur (Lagunas, 1976). Metabolic demand for acetyl-CoA should be less under these conditions than when ethanol is the sole carbon source, and this possibly accounts for the higher acetyl-charge. It is pertinent to note

that the mechanism of formation of acetyl-CoA may differ between the glucose-grown and ethanol-grown cells. In the glucose-grown cells although mitochondrial development is restricted, PDH may be more active since pyruvate will be formed from glucose (though much of the pyruvate will be utilized by pyruvate carboxylase - Polakis & Bartley, 1965). Acetyl-CoA would also be formed by ACS in the glucose-grown cells.

The physiology of cells grown anaerobically on glucose bears certain similarities to that of aerobic glucose-grown cells except that sterol and unsaturated fatty acid synthesis would be absent, as would respiration (Chapman & Bartley, 1968). Again, metabolic demand for acetyl-CoA should be lower than in cells grown on ethanol and may account for the higher acetyl-charge. The acetyl-charge does of course vary during anaerobic growth as described earlier and is less than 0·3 in stationary-phase cells.

From the foregoing discussion, the acetyl-charge, to some extent appears to be an indicator of the physiological state of the organism. Kohlhaw and his colleagues (Tracy & Kohlhaw, 1975; Kohlhaw & Tan-Wilson 1977; Tan-Wilson & Kohlhaw, 1978; Hampsey & Kohlhaw, 1981) suggest that the acetyl-charge may also be an important regulator of metabolism. These workers present a model which describes how CoASH-inactivation of certain acetyl-CoA consuming reactions, in conjunction

with a low energy-charge, governs the distribution of acetyl-CoA between anabolic and catabolic pathways. The salient points of the argument are: (i) The mitochondrial acetyl-CoA consuming enzymes - isopropyl-malate synthase (EC 4.1.3.12) and homocitrate synthase (EC 4.1.3.21), which catalyse the first committed steps in leucine and lysine biosynthesis, respectively - are reversibly inactivated by CoASH. This inactivation is reversed by ATP. (ii) HMG-CoA reductase which again is a mitochondrial enzyme and controls the use of acetyl-CoA for sterol synthesis, is also inhibited by CoASH (or CoA disulphide). (iii) Citrate synthase is not inhibited by CoASH, but is, by ATP (see also Parvin, 1969). Therefore if the acetyl-charge of the mitochondria is high, all of the above enzymes will be active and the energy charge will be relatively high (the activity of citrate synthase will of course, be regulated by the concentration of ATP - Parvin, 1969). However, if operation of the TCA-cycle becomes limited by lack of acetyl-CoA i.e. the acetyl-charge is low, CoASH will inactivate the enzymes catalysing anabolic reactions, but will not inactivate citrate synthase. Therefore if the supply of acetyl-CoA becomes limited it can be diverted towards catabolism and hence towards ATP production. The *in vitro* effector concentrations cited by Hampsey and Kohlhaw (1981) are 100μM for CoASH and mM for ATP. In the experiments reported in section 6.6.3 the intracellular concentration of CoASH in the ethanol-grown cells was estimated to be

ca. 30μM though of course the mitochondrial concentration may have been different. From the above model one might predict that, in ethanol-grown cells, the anabolic acetyl-CoA consuming reactions would be in a state of CoASH-inactivation and that the majority of acetyl-CoA was used to form citrate. However, this may not be so, because: (i) The energy-charge may be high. A low energy-charge and acetyl-charge are not necessarily concomitant and thus any potential CoASH-inactivation may be minimized (see above). (ii) The mitochondrial acetyl-charge may differ from that of the whole cell.

Several yeast enzymes other than the ones mentioned above are known to be regulated by either CoASH or acetyl-CoA. The anaplerotic enzyme pyruvate carboxylase (EC 6.4.1.1) which is particularly active in anaerobic Sacch. cerevisiae (Haarasilta, 1978; Haarasilta et al., 1979) is activated by acetyl-CoA (Young et al., 1969). The PDH of Sacch. cerevisiae like the mammalian PDH (Denton & Halestrap, 1979) is competitively inhibited by acetyl-CoA with respect to CoASH (Kresze & Ronft, 1981a) the K_i being 18μM. Further, the availability of CoASH may limit fatty acid synthesis, since in Sacch. cerevisiae, CoASH is the obligatory acceptor for palmityl residues in the termination reaction (Weithman, 1974; cited by Sedgwick & Smith, 1981). An excellent example of the in vivo regulation of an enzyme activity by the acetyl-charge, is the ester synthesizing enzyme of Sacch. cerevisiae.

It is relevant to note that the acetyl-charge is variable within other biological systems. For example values of between 0·2 and 0·5 in rat liver can be calculated from the data of Brass & Hoppel (1980; 1981) the lower acetyl-charge being characteristic of tissues obtained from fed rather than fasted rats. However, Hansford (1974) reports that the acetyl-charge of the blowfly flight-muscle is the same before and after flight (calculated value os 0·4). Further, citrate lyase in Rhodopseudomonas gelatinosa is regulated by acetylation / deacetylation (Gottschalk, 1982).

With regard to future work, it would be of interest to investigate the effects of different acetyl-charge values, in vitro on key regulatory enzymes (e.g. phosphofructokinase, fructose-1,6-biphosphatase).

7.3 Effects of Exogenous Fatty Acids on Lipid Synthesis in Sacch. cerevisiae

Acetyl-CoA is the precursor to both the synthesis of lipids and of acetate esters. Therefore, certain effects that addition of linoleic acid to wort has on the syntheses of lipids and esters, have been discussed above with respect to consumption of acetyl-CoA. Certain other effects of linoleic acid, such as: inhibition of short chain fatty acid synthesis; stimulation of saturated fatty acid and inhibition of unsaturated fatty acid synthesis have been discussed in relevant sections. The effects of exogenous long chain fatty acids on sterol synthesis in yeast warrant further discussion.

7.3.1 Effects of exogenous fatty acids on sterol synthesis

Regarding the effects of linoleic and stearic acid on sterol synthesis, two observations merit discussion here:

(i) Linoleic acid (5-150μg ml^{-1}) significantly reduced the flux of carbon (acetyl-CoA) through the sterol biosynthetic pathway i.e. the total amount of sterol plus squalene is reduced by up to 50%.

(ii) Despite the above effect, more sterol is formed i.e. the conversion of squalene to lanosterol, in particular, is enhanced by linoleic acid and overall, more oxygen is consumed for lipid syntheses by yeast when

linoleic acid has been added to wort. Stearic acid also enhances oxygen consumption by yeast.

HMG-CoA reductase (EC 1.1.1.34) is the primary site of regulation of the sterol biosynthetic pathway in Sacch. cerevisiae (Kawaguchi, 1970; Berndt et al., 1973; Boll et al., 1975; Quain & Haslam, 1979a; Bard & Downing, 1981). As such, the suppression by linoleic acid, of this pathway may be the result of allosteric modulation of HMG-CoA reductase by linoleyl-CoA or even the free acid. Certainly in mammalian systems the incorporation of labelled acetate into cholesterol is inhibited by fatty acyl-CoA esters (Haas et al., 1978). Further, in vitro studies show that HMG-CoA reductase is inhibited by fatty acyl-CoA esters, the unsaturated fatty acyl esters being more effective than the saturated compounds (Haas et al., 1978; Lehrer et al., 1981). The rationale for such a regulatory mechanism may be that high intracellular levels of unsaturated fatty acid are indicative of high levels of oxygen and therefore of sterols; consequently acetyl-CoA is diverted away from sterol synthesis to other pathways. Clearly in wort fermentations supplemented with linoleic acid, a high level of unsaturated fatty acid is not concomitant with a high availability of oxygen. This is, in a sense, an 'artificial' situation, but the yeast still responds by suppressing the sterol biosynthetic pathway. An additional possibility is that squalene synthesis itself is inhibited by linoleic acid. Squalene synthetase is

microsomally located in Sacch. cerevisiae (Nishino et al., 1978) and as such may be affected by the lipid composition of the membrane. Alternatively direct allosteric modulation of the enzymes' activity by linoleic acid cannot be discounted.

Downing et al. (1980) consider that conversion of squalene to lanosterol may also be a point of regulation of sterol biosynthesis in Sacch. cerevisiae. Addition of linoleic acid to wort stimulated this conversion. This oxygen requiring reaction is catalysed by two enzymes: squalene epoxidase and lanosterol cyclase. In the experimental system used here, the only oxygen available to the yeast is that which is dissolved in the wort. Measurement of loss of oxygen from uninoculated wort shows that oxygen is removed mechanically by the passage of nitrogen through the headspace of the fermenter and possibly by chemical oxidation of wort components (Aries & Kirsop, 1977; Quain et al., 1981). Therefore yeast lipid syntheses are in competition with other processes for a finite amount of oxygen. As stated above, addition of linoleic acid represses the synthesis of unsaturated fatty acids. However, the stimulation of sterol synthesis by linoleic acid cannot be explained on the basis of an 'oxygen-sparing' effect, caused by the suppression of unsaturated fatty acid synthesis, since more oxygen per se was used for lipid syntheses. In standard 1·040 fermentations, lipid syntheses account for ca. 50% of the available oxygen.

This figure is far larger than reported by Aries & Kirsop (1977) but calculation of oxygen consumption from data presented by these workers does in fact lead to a figure nearer to 50% than the 5-15% generally quoted (Aries & Kirsop, 1977; Kirsop, 1977). Addition of linoleic or stearic acid to wort raises the figure to ca. 60%. Both of these acids must have exerted their effects very quickly i.e. during the first 2h after inoculation when oxygen is available. Linoleic acid is taken up and incoporated into yeast lipids very rapidly during this early phase. These acids may lower the Km for oxygen so that a more rapid and efficient utilization of the available oxygen occurs. An alternative explanation may be that the experimental conditions were not totally anaerobic since commercial oxygen-free nitrogen can contain traces of oxygen. Thus there may have been a very slow conversion of squalene to lanosterol during the fermentations. The conditions are anaerobic enough to prevent any synthesis of unsaturated fatty acids after 4h. Even if traces of oxygen are available, the presence of linoleic acid in yeast enhances utilization of oxygen for sterol synthesis; thus linoleic acid does have a real effect on the conversion of squalene to lanosterol. It is difficult to envisage how or why linoleic acid should do this. Possibly, high levels of linoleic acid in the yeast cell are indicative of fluid membranes and this signals the cell to produce more sterol to rigidify the membranes.

The effect of enrichment of yeast lipids with linoleic acid on sterol synthesis was investigated by subjecting cells, propagated anaerobically with and without linoleic acid, to aeration. Initially upon aeration there was a greater rate of total sterol synthesis in linoleic acid-enriched cells. The difference, in rate of sterol synthesis, between control and linoleic acid-enriched cells corresponded to the phase when these cultures had greatly different % UFA values; once the % UFA values were similar, comparable rates of sterol synthesis were observed. This evidence does suggest that the % UFA of the yeast lipids affects the conversion of squalene to lanosterol and other sterols. Synthesis of sterols requires a supply of energy and NADPH. Work in this laboratory has shown that this can be supplied by endogenous reserves of glycogen; indeed, at the start of a brewery fermentation, glycogen catabolism may provide the sole source of energy and NADPH for the synthesis of sterols and unsaturated fatty acids (Quain et al., 1981; Quain & Tubb, 1981). Further the relationship between glycogen dissimilated and sterol formed is stoichiometric (Quain & Tubb, 1981). However, enrichment of yeast lipids with linoleic acid did not cause a more rapid dissimilation of glycogen. Therefore the stoichiometric relationship between glycogen dissimilation and sterol synthesis would appear to be affected by the amounts of unsaturated fatty acids in the yeast. Thus, if worts with a relatively high unsaturated fatty acid content are routinely fermented,

the unsaturated fatty acid content of the pitching yeast may be unusually high and in such an instance the glycogen content of the yeast may not be as important as stated above.

The effect of unsaturated fatty acid enrichment of yeast on aerobic induction of sterol synthesis has also been reported by Boll et al. (1980). These workers used a strain of Baker's yeast, which when propagated anaerobically, would not form sterol, upon aeration, unless a carbon source was supplied in the medium. This particular observation may be attributable to the fact that Baker's yeast catabolizes its glycogen much more slowly than brewer's yeast under aerobic conditions (Chester, 1964). If glucose is supplied in the aeration medium extensive synthesis of sterols takes place and there is a concomitant increase in specific activity of HMG-CoA reductase. If anaerobic growth is supplemented with linolenic, linoleic or oleic acids, both the aerobic induction of HMG-CoA reductase and sterol synthesis occurs at a several-fold greater velocity. Boll et al. (1980) attribute these findings to the fact that provision of unsaturated fatty acid in anaerobic growth leads to a completion of the protein synthesizing apparatus. Unsaturated fatty acid-supplemented cells showed enhanced capacity to incorporate leucine into acid-precipitable protein. Thus HMG-CoA reductase is induced to a greater extent than in unsaturated fatty acid-depleted cells as is protein synthesis in general.

No such observations were made in 6.3.2. Possibly this is because: (i) a brewing strain of yeast was used in 6.3.2 and (ii) oxygen may have been limiting at a cell density of *ca.* 5mg ml^{-1}. Certainly the rate of sterol synthesis by anaerobically-propagated yeast is affected by the % UFA of the yeast lipids.

Fermentations supplemented with unsaturated fatty acid had slightly faster attenuation rates and greater biomass production but little or no increase in extent of fermentation. This latter observation implies that addition of, for example, linoleic acid, although directly supplying unsaturated fatty acid and indirectly supplying more sterol (lanosterol), does not satisfy the lipid-requirement of NCYC 240 to the extent that it permits the yeast to use more of the residual maltotriose. Provision of air (or oxygen) or source of sterol plus unsaturated fatty acid does allow complete utilization of maltotriose under these fermentation conditions (Taylor *et al.*, 1979). Lanosterol is thus a relatively inadequate sterol with respect to the overall functioning of the cell as suggested by other workers (Nes *et al.*, 1978; Buttke & Bloch, 1980).

BIBLIOGRAPHY

Acker, L. & Geyer, J. (1968) Brauwissenschaft 21, 222.

Adams, B.G. & Parks, L.W. (1967) Journal of Cellular Physiology 70, 161.

Ahvenainen, J. & Makinen, V. (1981) Proceedings of the European Brewery Convention Congress, Copenhagen p285.

Allred, J.B. & Guy, D.G. (1969) Analytical Biochemistry 29, 293.

Amdur, B.H., Rilling, H.C. & Bloch, K. (1957) Journal - American Chemical Society 79, 2646.

Anderson, R.G. & Howard, D. (1976a) Journal of the Institute of Brewing 82, 70.

Anderson, R.G. & Howard, D. (1976b) Journal of Applied Chemistry and Biotechnology 26, 107.

Anderson, R.G. & Kirsop, B.H. (1974) Journal of the Institute of Brewing 80, 48.

Anderson, R.G. & Kirsop, B.H. (1975a) Journal of the Institute of Brewing 81, 111.

Anderson, R.G. & Kirsop, B.H. (1975b) Journal of the Institute of Brewing 81, 296.

Anderson, R.G., Kirsop, B.H., Rennie, H. & Wilson, R.J.H. (1975) Proceedings of the European Brewery Convention Congress, Nice p243.

Andreasen, A.A. & Stier, T.J.B. (1953) Journal of Cellular and Comparative Physiology 41, 23.

Andreasen, A.A. & Stier, T.J.B. (1954) Journal of Cellular and Comparative Physiology 43, 271.

Anness, B.J. & Bamforth, C.W. (1982) Journal of the Institute of Brewing (in press)

Anon. (1979) Brewing and Distilling International 9, 22.

Anon. (1981) The Brewer 67, (Jan) p2.

Aoyama, Y. & Yoshida, Y. (1978) Biochemical and Biophysical Research Communications 85, 28.

Aries, V. & Kirsop, B.H. (1977) Journal of the Institute of Brewing 83, 220.

Aries, V. & Kirsop, B.H. (1978) Journal of the Institute of Brewing 84, 118.

Aries, V., Kirsop, B.H. & Taylor, G.T. (1977) Proceedings of the European Brewery Convention Congress, Amsterdam, p255.

Astin, A.M. & Haslam, J.M. (1977) Biochemical Journal 166, 287.

Ault, R.G. (1954) Brewers Guild Journal 40, 391.

Awaya, J., Ohno, T., Ohno, H. & Omura, S. (1975) Biochimica et Biophysica Acta 409, 267.

Äyräpää, T. (1971) Särtryckur Kemisk Tidskrift 85, 1.

Äyräpää, T., Holmberg, J. & Sellmann-Persson, G. (1961) Proceedings of the European Brewery Convention Congress, Vienna, p286.

Äyräpää, T. & Lindström, I. (1973) Proceedings of the European Brewery Convention Congress, Salzburg, p271.

Äyräpää, T. & Lindström, I. (1977) Proceedings of the European Brewery Convention Congress, Amsterdam p507.

Bailey, R.B. & Parks, L.W. (1975) Biochimica et Biophysica Acta 176, 858.

Baker, C.D. & Morton, S. (1977) Journal of the Institute of Brewing 83, 348.

Ball, W.J. & Atkinson, D.E. (1975) Journal of Bacteriology 121, 975.

Bamforth, C.W. (1981) Proceedings of the European Brewery Convention Congress, Copenhagen, p335.

Banasik, O.J. & Gilles, K.A. (1966) Cereal Science Today 11, 98.

Bard, M. & Downing, J.F. (1981) Journal of General Microbiology 125, 415.

Barron, E.J. & Hanahan, D.J. (1961) Journal of Biological Chemistry 231, 493.

Barton, D.H.R., Gosden, A.F., Mellows, G. & Widdowson, D.A. (1968) Chemical Communications (Royal Chemical Society), 1067.

Barwell, C.J. & Hess, B. (1971) FEBS Letters 19, 1.

Beavan, M.J., Charpentier, C. & Rose, A.H. (1982) Journal of General Microbiology, 128 (in press)

Becker, G.W. & Lester, R.L. (1980) Journal of Bacteriology 142, 747.

Bell, R.M., Ballas, L.M. & Coleman, R.A. (1981) Journal of Lipid Research 22, 391.

Bergmeyer, H.U., Berndt, E. & Schmidt, F. (1974) in "Methods of Enzymatic Analysis" (Bergmeyer, H.U. Ed). 3, 1196. Verlag Chemie, Wanheim/Academic Press, London and New York.

Berndt, J., Boll, M., Löwell, M. & Gaumert, R. (1973) Biochemical and Biophysical Research Communications 51, 843.

Bhatty, R.S. & Rossnagel, B.G. (1980) Cereal Chemistry 57, 382.

Black, S. (1956) Archives of Biochemistry 34, 86.

Bloch, K. & Vance, D. (1977) Annual Review of Biochemistry 46, 385.

Bloomfield, D.K. & Block, K. (1960) Journal of Biological Chemistry 235, 337.

Blum, P.H. (1969) Brewers Digest Oct, 58-63.

Boll, M., Löwell, M. & Berndt, J. (1980) Biochimica et Biophysica Acta 620, 429.

Boll, M., Löwell, M., Still, J. & Berndt, J. (1975) European Journal of Biochemistry 54, 435.

Boulton, A.A. (1965) Experimental Cell Research 37, 434.

Boulton, C.A. & Ratledge, C. (1981) Journal of General Microbiology 127, 169.

Bradbury, S.L. & Jakoby, W.B. (1971) Journal of Biological Chemistry 246, 6929.

Brass, E.P. & Hoppel, C.L. (1980) Biochemical Journal 190, 495.

Brass, E.P. & Hoppel, C.L. (1981) Analytical Biochemistry 110, 77.

Brennan, P.J., Flynn, M.P. & Griffin, P.F.S. (1970) FEBS Letters 8, 343.

Bretscher, M.S. (1973) Science 181, 622.

Bretscher, M.S. & Raff, M.C. (1975) Nature 258, 43.

Brown, C.M. & Johnson, B. (1971) Antonie van Leeuwenhoek 37, 477.

Buttke, T.M. & Bloch, K. (1980) Biochemical and Biophysical Research Communications 92, 229.

Buttke, T.M., Jones, S.D. & Bloch, K. (1980) Journal of Bacteriology 144, 124.

Campbell, I.C., Gilmour, R.H. & Rous, P.R. (1972) Journal of the Institute of Brewing 78, 491.

Campbell, W.H. & Bernofsky, C. (1979) Molecular and Cellular Biochemistry 25, 33.

Cantarelli, C. (1955) Annales De Microbiologie 6, 219.

Carrington, R., Collet, R.C., Runkin, I.R. & Halek, G. (1972) Journal of the Institute of Brewing 78, 243.

Cartledge, T.G., Rose, A.H., Belk, D.M. & Goodhall, A.G. (1977) Journal of Bacteriology 132, 426.

Castelli, A., Barbaresi, G. & Bertoli, E.I. (1969) Italian Journal of Biochemistry 18, 91.

Chapman, C. & Bartley, W. (1968) Biochemical Journal 107, 455.

Chen, C.H. (1980) Proceedings of the American Society of Brewing Chemists 38, 148

Cherry, R.J. (1979) Biochimica et Biophysica Acta 559, 289.

Chester, V.E. (1963) Biochemical Journal 86, 152.

Chester, V.E. (1964) Biochemical Journal 92, 318.

Christiansen, K. (1978) Biochimica et Biophysica Acta 530, 78.

Christiansen, K. (1979) Biochimica et Biophysica Acta 574, 448.

Clapperton, J.F. (1973) Journal of the Institute of Brewing 79, 495.

Clapperton, J.F. (1974) Journal of the Institute of Brewing 80, 164.

Clapperton, J.F. (1978) Journal of the Institute of Brewing 84, 107.

Clapperton, J.F. & Brown, D.G.W. (1978) Journal of the Institute of Brewing 84, 90.

Clapperton, J.F., Dalgliesh, C.E. & Meilgaard, M.C. (1976) Journal of the Institute of Brewing 82, 7.

Clapperton, J.F. & Piggot, J.R. (1979a) Journal of the Institute of Brewing 85, 271.

Clapperton, J.F. & Piggot, J.R. (1979b) Journal of the Institute of Brewing 85, 275.

Clausen, M.K., Christiansen, K., Jensen, P.K. & Behnke, O. (1974) FEBS Letters 43, 176.

Cobon, G.S., Crowfoot, P.D. & Linnane, A.W. (1974) Biochemical Journal 144, 265.

Cobon, G.S. & Haslam, J.M. (1973) Biochemical and Biophysical Research Communications 52, 320.

Coleman, J.S. & Bhattacharjee, J.K. (1976) Canadian Journal of Microbiology 22, 762.

Coors, J.H. & Jangaard, N.O. (1975) Proceedings of the European Brewery Convention Congress, Nice, p311.

Costa, N.D. & Snoswell, A.M. (1975a) Biochemical Journal 152, 161.

Costa, N.D. & Snoswell, A.M. (1975b) Biochemical Journal 152, 167.

Cowland, T.W. (1967) Journal of the Institute of Brewing 73, 542.

Cowland, T.W. & Maule, D.R. (1966) Journal of the Institute of Brewing 72, 480.

D'Agnolo, G., Rosenfeld, I.S., Awaya, J., Omura, S. & Vagelos, P.R. (1973) Biochimica et Biophysica Acta 326, 155.

Dahl, J.S., Dahl, C.E. & Bloch, K. (1981) Journal of Biological Chemistry 256,87.

Daum, G. & Paltauf, F. (1980) Monatshefte für Chemie 111, 355.

David, M.H. (1974) Journal of the Institute of Brewing 80, 80.

David, M.H. & Kirsop, B.H. (1972) Proceedings of the American Society of Brewing Chemists p14.

David, M.H. & Kirsop, B.H. (1973a) Journal of General Microbiology 77, 529.

David, M.H. & Kirsop, B.H. (1973b)Journal of the Institute of Brewing 79, 20.

Davies, R., Falkiner, E.A., Wilkinson, J.F. & Peel, J.L. (1951) Biochemical Journal 49, 58.

Dawson, R.M.C., Elliot, D.C., Elliot, W.H. & Jones, K.M. (Eds) (1978) Data for Biochemical Research, 2nd edn Clarendon Press, Oxford, pp193-195.

Day, A., Anderson, E. & Martin, P.A. (1975) Proceedings of the European Brewery Convention Congress, Nice, p377.

Day, A., Webb, T.J.B. & Martin, P.A. (1976) 5th International Fermentation Symposium, Berlin, p468 (Dellweg, H. Ed) Printed by: West Kreuz-Druckerei und Verlag, Berlin/Bonn.

Deak, T. (1978) Archives of Microbiology 116, 205.

De Deken, R.H. (1966) Journal of General Microbiology 44, 149.

Demel, R.A., Bruckdorfer, K.R. & van Deenen, L.L.M. (1972) Biochimica et Biophysica Acta 255, 321.

Demel, R.A. & DeKruyff, B. (1976) Biochimica et Biophysica Acta 457, 109.

Dempsey, M.E. (1974) Annual Review of Biochemistry 43, 967.

Dempsey, M.E. & Meyer, C.M. (1977) Federation Proceedings American Societies for Experimental Biology 36, 779.

Denton, R.M. & Halestrap, A.P. (1979) Essays in Biochemistry 15, 37.

Downing, J.F., Burrows, L.S. & Bard, M. (1980) Biochemical and Biophysical Research Communications 94,974.

Drost, B.W., van Eerde, P., Hoekstra, S.F. & Strating, J. (1971) Proceedings of the European Brewery Convention Congress, Estoril, p451.

Dunze, W., Neumann, D., Gancedo, J.M., Atzpodien, W. & Holzer, H. (1969) European Journal of Biochemistry 10, 83.

Eddy, A.A. & Nowacki, J.A. (1971) Biochemical Journal 122, 701.

Eltayeb, Y. & Berry, D.R. (1977) FEMS Microbiology Letters 2, 57.

Engan, S. (1970) Journal of the Institute of Brewing 76, 254.

Engan, S. (1972) Journal of the Institute of Brewing 78, 33.

Engan, S. & Aubert, O. (1977) Proceedings of the European Brewery Convention Congress, Amsterdam, p591.

Ernster, L. & Lee, C. (1964) Annual Review of Biochemistry 33, 729.

Forch, M. (1977) Monatsschrift für Brauerei 30, 124.

Forch, M. & Runkel, U.D. (1974) In "European Brewery Convention Wort Symposium, Zeist, Monograph 1" p258.

Friedlander, E.J., Caras, I.W., Lin, L.F.H. & Bloch, K. (1980) Journal of Biological Chemistry 225, 8042.

Fryberg, M., Oehlschlager, A.C. & Unrau, A.M. (1972) Biochemical and Biophysical Research Commumications 48, 593.

Galliard, T. & Chan, H.W.S. (1980) In "Biochemistry of Plants Vol 4, Lipids: Structure and Function" (Stumpf, P.K. Ed) Chap 5, pp131-161 Academic Press.

Gancedo, J.M. & Gancedo, C. (1973) Biochimie 55, 205.

Gavey, K.J. & Scallen, T.J. (1978) Journal of Biological Chemistry 253, 5746.

Gaylor, J.L. (1974) In "Biochemistry of Lipids", Vol 4, (Goodwin, T.W. Ed) pp1-37, University Park Press, Baltimore.

Getz, G.S. (1972) In "Membrane Molecular Biology" (Fox. C.F. & Keith, A.D. Eds) pp386-438, Sinauer Associates Inc, New York.

Gilbert, N.F. & Stewart, M.D. (1981) Journal of Biological Chemistry 256, 1782.

Gottschalk, G. (1982) Biochemical Society Bulletin 4,(2).

Greenspan, M.D., Mackow, R.C. & Omura, S. (1977) Lipids 12, 729.

Griffiths, M.M. & Bernofsky, C. (1972) Journal of Biological Chemistry 247, 1473.

Grigat, K.P., Koppe, K., Seufert, C.D. & Soling, H.D. (1979) Biochemical Journal 177, 71.

Gurr, M.I. & James, A.T. (1981) In "Lipid Biochemistry" 3rd Ed, p42, Chapman & Hall, New York.

Haarasilta, S. (1978) Analytical Biochemistry 87, 306.

Haarasilta, S. & Oura, E. (1975) European Journal of Biochemistry 52, 1.

Haarasilta, S., Oura, E. & Suomalainen, H. (1979) Archives of Microbiology 122, 121.

Haarasilta, S. & Taskinen, L. (1977) Archives of Microbiology 113, 159.

Haas, F.H., Carter, W.J. & Wynn, J.O. (1978) Biochimica et Biophysica Acta 531, 158.

Hackstaff, B.W. (1977) Master Brewers Association of America, Technical Quarterly 15, 1.

Hampsey, M.D. & Kohlhaw, G.B. (1981) Journal of Biological Chemistry 256, 3791.

Hansford, R.G. (1974) Biochemical Journal 142, 509.

Harding, S.A. & Kirsop, B.H. (1979) Journal of the Institute of Brewing 85, 171.

Harrison, G.A.F. (1970) Journal of the Institute of Brewing 76, 486.

Harwood, J.L. (1980) In "Biochemistry of Plants Vol 4, Lipids: Structure and Function" (Stumpf, P.K. Ed) pp1-161, Academic Press, London and New York.

Hashimoto, N. (1981) In "Food Science & Technology Vol 2, Brewing Science," (Pollock, J.R.A. Ed)

p348, Academic press.

Haslam, J.M. & Al Mahdawi, S.A.H. (1980) Biochemical Society Transactions 8, 34.

Haslam, J.M., Proudlock, J.W. & Linnane, A.W. (1971) Bioenergetics 2, 351.

Haukeli, A.D., Jacobsen, T. & Lie, S. (1973) Master Brewers Association of America, Technical Quarterly 10, 47.

Haukeli, A.D. & Lie, S. (1975) Journal of the Institute of Brewing 81, 58.

Haukeli, A.D. & Lie, S. (1976) Journal of the Institute of Brewing 82, 161.

Hayduck, F., Deknicke, J. & Wustenfeld, H. (1910) Journal of the Institute of Brewing 16, 314.

Henry, S.A. (1973) Journal of Bacteriology 116, 1293.

Henry, S.A. & Halvorson, H.O. (1973) Journal of Bacteriology 114, 1158.

Henry, S.A. & Keith, A.D. (1971) Chemistry and Physics of Lipids 7, 245.

Herbert, D., Phipps, P.J. & Strange, R.E. (1971) In "Methods in Microbiology 5B" (Norris, J.R. & Ribbons, D.W. Ed) p244, Academic Press, London and New York.

Hernandez, H.H., Banasick, O.J. & Gilles, K.A. (1967) Proceedings of the American Society of Brewing Chemists, p24.

Herrera, E. & Frienkel, N. (1967) Journal of Lipid Research 8, 515.

Hildebrand, R.P., Kavanagh, J.E. & Clarke, B.J. (1975) Brewers Digest 50, 58.

Hockney, R.C. & Zealey, G.R. (1981) Biotechnology Conference, Eastbourne Paper 59.

Holmberg, J. & Sellmann-Persson, G. (1967) Proceedings of the European Brewery Convention Congress, Madrid, p213.

Hosono, K. & Aida, K. (1974) Journal of General and Applied Microbiology - Tokyo 20, 47.

Hossack, J.A., Belk, D.M. & Rose, A.H. (1977) Archives of Microbiology 114, 137.

Hossack, J.A. & Rose, A.H. (1976) Journal of Bacteriology 127, 67.

Hossack, J.A., Wheeler, G.E. & Rose, A.H. (1973) In "Yeast Mould and Plant Protoplasts" (Villnueva, J.R., Garcia-Acha, I., Gascon, S. & Uruburu, F. Ed) pp211-217, Academic Press, London and New York.

Hudson, J.R. (1973) Proceedings of the European Brewery Convention Congress, Salzburg, p422.

Hunter, K. & Rose, A.H. (1971) In "The Yeasts, Vol 2, Physiology and Biochemistry of Yeasts" (Rose, A.H. & Harrison, J.S. Ed) Academic Press, London and New York.

Hunter, K. & Rose, A.H. (1972) Biochimica et Biophysica Acta 260, 639.

Hutchison, H.T. & Cronan, J.E. (1968) Biochimica et Biophysica Acta 164, 606.

Illingworth, R.F., Rose, A.H. & Beckett, A.J. (1973) Journal of Bacteriology 113, 373.

Inoue, T. (1975) Reports of the Research Laboratories, Kirin Brewery Co. Ltd. 18, 13.

Jakobsen, M. & Thorne, R.S.W. (1980) Journal of the Institute of Brewing 86, 284.

Jakovic, S., Gatz, G.S., Rabinowitz, M., Jakob, H & Swift, H. (1971) Journal of Cellular Biology 48, 490.

Jamieson, A.M., Chen, E.C. & Van Gheluwe, J.E.A. (1969) Proceedings of the American Society of Brewing Chemists, p123.

Jamieson, A.H. & Van Gheluwe, J.E.A. (1970) Proceedings of the American Society of Brewing Chemists, p192.

Janki, R.M., Aithal, H.N., McMurray, W.C. & Tustanoff, E.R. (1974) Biochemical and Biophysical Research Communications 56, 1078

Johnson, B. & Brown, C.M. (1972) Antonie Van Leeuwenhoek 38, 137.

Johnson, B., Brown, C.M. & Minnikin, D.E. (1973) Journal of General Microbiology 75, x.

Jollow, D., Kellerman, G.H. & Linnane, A.W. (1968) Journal of Cellular Biology 37, 221.

Jones, M. & Pierce, J.S. (1964) Journal of the Institute of Brewing 70, 307.

Jones, M.D., Cope, R. & Rainbow, C. (1975) Proceedings of the European Brewery Convention Congress, Nice, p669.

Kamiryo, T. & Numa, S. (1973) FEBS Letters 38, 29.

Kaneko, H., Hosohara, M., Tanaka, M. & Itoh, T. (1976) Lipids 11, 837.

Kaplan, N.O. & Lipmann, F. (1948) Journal of Biological Chemistry 174, 37.

Kates, M. & Baxter, R.M. (1962) Canadian Journal of Biochemistry and Physiology 40, 213.

Kawaguchi, A. (1970) Journal of Biochemistry (Tokyo) 67, 219

Kirsop, B.H. (1974) Journal of the Institute of Brewing 80, 252.

Kirsop, B.H. (1978) Brewers Digest (July), 28.

Klein, H.P. & Jahnke, L. (1968) Journal of Bacteriology 96, 1632.

Klein, H.P. & Jahnke, L. (1971) Journal of Bacteriology 106, 596.

Klein, H.P. & Jahnke, L. (1979) Journal of Bacteriology 137, 179.

Klein, H.P. & Lipmann, F. (1953) Journal of Biological Chemistry 203, 95.

Klopper, W.K., Tuning, B. & Vermiere, H.A. (1975) Proceedings of the European Brewery Convention Congress, Nice, p659.

Knowles, S.E., Jarret, I.G., Filsell, O.H. & Ballared, F.J. (1974) Biochemical Journal 142, 401.

Kohlhaw, G.B. & Tan-Wilson, A (1977) Journal of Bacteriology 129, 1159.

Kornblatt, J.A. & Rudney, H. (1971) Journal of Biological Chemistry 246, 4424.

Krauss, G., Zurcher, C. & Holstein, M. (1972) Monatsschrift Für Brauerei 25, 113.

Kresze, G.B. & Ronft, H. (1981a) European Journal of Biochemistry 119, 573.

Kresze, G.B. & Ronft, H. (1981b) European Journal of Biochemistry 119, 581.

Lagunas, R. (1976) Biochimica et Biophysica Acta 440, 661.

Lanyi, J.K., Plachy, W.Z. & Kates, M. (1974) Biochemistry 13, 4914.

Lees, N.D., Bard, M., Kemple, M.D., Haak, R.A. & Kleinhans, F.W. (1979) Biochimica et Biophysica Acta 533, 469.

Leegwater, M.P.M., Neijssel, O.M. & Tempest, D.W. (1982) Journal of Chemical Technology and Biotechnology 32, 92.

Lehrer, G., Panini, S.R., Rogers, D.H. & Rudney, H. (1981) Journal of Biological Chemistry 256, 5612.

Letters, R. (1968) In "Aspects of Yeast Metabolism" (Mills, A.K. Ed) pp309-319, Blackwells Scientific Publications Ltd, Oxford and Edinburgh.

Light, R.J., Lennarz, W.T. & Bloch, K. (1962) Journal of Biological Chemistry 237, 1793.

Linnane, A.W. & Crowfoot, P.D. (1975) In "Membrane Biogenesis" (Tzagoloff, A. Ed) pp99-104, Plenum Press, London and New York.

Linnane, A.W. & Haslam, J.M. (1970) In "Current Topics in Cellular Regulation Vol 2" (Horecker, B.L. & Stadtman, E.R. Ed) p101, Academic Press, London and New York.

Longley, R.P., Rose, A.H. & Knights, B.A. (1968) Biochemical Journal 108, 401.

Lulai, E.C. & Baker, C.W. (1975) Proceedings of the

American Society of Brewing Chemists 33, 154.

Lumbers, J., Threfell, C.J. & Stoner, H.B. (1969) Analytical Biochemistry, 31, 21.

Lust, G. & Lynen, F. (1968) European Journal of Biochemistry 7, 68.

Lynen, F. (1968) In "Aspects of Yeast Metabolism" (Mills, A.K. Ed) p271, Blackwells Scientific Publications Ltd, Oxford and Edinburgh.

Lynen, F. (1980) European Journal of Biochemistry 112, 431.

MacLeod, A.M. (1977) In "Economic Microbiology, Vol 1" (Rose, A.H. Ed) p43, Academic Press (London) Ltd.

MacLeod, A.M. & White, H.B. (1961) Journal of the Institute of Brewing 67, 182.

MacLeod, A.M. & White, H.B. (1962) Journal of the Institute of Brewing 68, 487.

Madyastha, P.B. & Parks, L.W. (1969) Biochimica et Biophysica Acta 176, 858.

Maitra, P.K. & Lobo, Z. (1978) Archives of Biochemistry and Biophysics 185, 535.

Malpartida, F. & Serrano, R. (1980) FEBS Letters 111, 69.

Maule, D.R. (1967) Journal of the Institute of Brewing 73,351.

Meilgaard, H.C., Dalgliesh, C.E. & Clapperton, J.F. (1979) Journal of the Institute of Brewing 85,38.

Mercer, E.I., Modi, N., Clarke, D.J. & Morris, J.G. (1979) Journal of General Microbiology 111, 437.

Meyer, K.H. & Schweizer, E. (1976) European Journal of Biochemistry 65, 317.

Middleton, B. & Apps, D.K. (1969) Biochimica et Biophysica Acta 177, 276.

Mishina, M., Roggenkamp, R. & Schweizer, E. (1980) European Journal of Biochemistry 111, 79.

Miwa, D. & Ueyama, H. (1961) Hakko Kugaku Zasshi 39, 410.

Morikawa, M. & Yamashita, S. (1978) European Journal of Biochemistry 84, 61.

Morrison, W.R. (1978) In "Advances in Cereal Science and Technology Vol 2" (Pomeranz, Y. Ed) pp221-348, American Association of Cereal Chemists Inc. Minnesota.

Nes, W.R., Adler, J.H., Sekula, B.C. & Krevitz, K. (1976) Biochemical and Biophysical Research Communications 71, 1296.

Nes, W.R., Sekula, B.C., Nes, W.D. & Adler, J.H. (1978) Journal of Biological Chemistry 253, 6218.

Nishino, T., Takatsuji, H., Hata, S. & Katsuki, H. (1978) Biochemical and Biophysical Research Communications 85, 867.

Nordstrom, K. (1961) Journal of the Institute of Brewing 67, 173.

Nordstrom, K. (1962) Journal of the Institute of Brewing 68, 398.

Nordstrom, K. (1963) Journal of the Institute of Brewing 69, 142.

Nordstrom, K. (1964a) Svensk Kemisk Tidskrift 76, 510.

Nordstrom, K. (1964b) Journal of the Institute of Brewing 70, 209.

Nordstrom, K. (1964c) Journal of the Institute of

Brewing 70, 233.

Nordstrom, K. (1965) Brewers Digest (Nov) p60.

Norstedt, C., Bengtsson, A., Bennet, P., Lindström, I. & Äyräpää, T. (1975) Proceedings of the European Brewery Convention Congress, Nice, p581.

Numa, S. (1981) Biochemical Society Transactions 9, 9.

Nurminen, T., Konttinen, K. & Suomalainen, H. (1975) Chemistry and Physics of Lipids 14, 15.

Nykanen, L. & Nykanen, I. (1977) Journal of the Institute of Brewing 83, 30.

Ohno, T., Awaya, J., Kesado, T. & Omura, S. (1974) Biochemical and Biophysical Research Communications 57, 1119.

Omura, S. (1976) Bacteriological Reviews 40, 681.

Op den Kamp, J.A.F. (1979) Annual Review of Biochemistry 48, 805.

Osumi, T., Nishino, T. & Katsuki, H. (1979) Journal of Biochemistry (Tokyo) 85, 819.

Otoguro, K., Awaya, J., Tanaka, H. & Omura, S. (1981) Journal of Biochemistry (Tokyo) 89, 523.

Oura, E. (1977a) Process Biochemistry 12, 19.

Oura, E. (1977b) In "EUCHEM Conference on Metabolic Reactions in the Yeast Cell in Anaerobic and Aerobic Conditions, Helsinki," pp23-24.

Palmer, A.K. & Rennie, H. (1974) Journal of the Institute of Brewing 80, 447.

Paltauf, F. & Johnson, J.M. (1970) Biochimica et Biophysica Acta 218, 424.

Paltauf, F. & Schatz, G. (1969) Biochemistry 8, 335.

Parish, R.W. (1975) Archives of Microbiology 105,187.

Parkkinen, E., Oura, E. & Suomalainen, H. (1978) Journal of the Institute of Brewing 84, 5.

Parks, L.W. (1958) Journal of the American Chemical Society 80, 2023.

Parks, L.W. (1978) CRC Critical Reviews in Microbiology 6, 301.

Parsons, R.G. & Price, P.D. (1974) Lipids 9, 804.

Parvin, R. (1969) In "Methods in Enzymology, Vol 13" (Lowenstein, J.M. Ed) p16, Academic Press, London and New York.

Peel, J.L. (1951) Biochemical Journal 49, 62.

Perlman, P.S. & Mahler, H.R. (1974) Archives of Biochemistry and Biophysics 162, 248.

Pfisterer, E., Hancock, I. & Garrison, I. (1977) Journal - American Society of Brewing Chemists 35, 49.

Pfisterer, E. & Stewart, G.G. (1975) Proceedings of the European Brewery Convention Congress, Nice, p255

Piendl, A. & Geiger, E. (1980) Brewers Digest (May) p26.

Polakis, E.S. & Bartley, W (1965) Biochemical Journal 97, 284.

Pollock, J.R.A. (Ed) (1981) "Food Science & Technology, Vol 2, Brewing Science" Academic Press (London) Ltd

Portno, A.D. (1968) Journal of the Institute of Brewing 74, 291.

Prass, R.L., Isohashi, F. & Utter, M.F. (1980) Journal of Biological Chemistry 255, 5215.

Price, P.D. & Parsons, P.G. (1974) Lipids 9, 560.

Price, P.D. & Parsons, P.G. (1980) Journal of Agricultual and Food Chemistry 28, 875.

Proudlock, J.W., Wheeldon, L.W., Jollow, D.J. & Linnane, A.W. (1968) Biochimica et Biophysica Acta 152, 434.

Quain, D.E. (1981) Journal of the Institute of Brewing 87, 289.

Quain, D.E. & Haslam, J.M. (1979a) Journal of General Microbiology 111, 343.

Quain, D.E. & Haslam, J.M. (1979b) Journal of General Microbiology 113, 195.

Quain, D.E., Thurston, P.A. & Tubb, R.S. (1981) Journal of the Institute of Brewing 87, 108.

Quain, D.E. & Tubb, R.S. (1982) Master Brewers Association of America, Technical Quarterly 20, in press

Rasmussen, R.K. & Klein, H.P. (1968) Biochemical and Biophysical Research Communications 28, 415.

Ratledge, C. (1978) In "Primary Products of Metabolism. Economic Microbiology Vol 2" (Rose, A.H. Ed) pp263-302, Academic Press London.

Rattray, J.B.M., Schibeci, A. & Kidby, D.K. (1975) Bacteriological Reviews 39, 197.

Rennie, H. & Wilson, R.J.H. (1975) Journal of the Institute of Brewing 81, 105.

Resnick, M.A. & Mortimer, R.K. (1966) Journal of Bacteriology 92, 597.

Rilling, H.C. (1966) Journal of Biological Chemistry 241, 3233.

Rilling, H.C., Poulter, C.D., Epstein, M.W. & Larsen, B.

(1971) Journal of the American Chemical Society 93, 1783.

Roberts, R.T. (1976) Journal of the Institute of Brewing 82, 96.

Roberts, R.T. (1977) Brewers Digest (June) p50.

Roberts, R.T., Keeney, P.J. & Wainwright, T.W. (1978) Journal of the Institute of Brewing 84,9.

Robinson, J.B.(Jr), Mohan, D.E. & Koeppe, R.E. (1976) Biochemical and Biophysical Research Communications 71, 959.

Rose, A.H. (1976) In "Chemical Microbiology An Introduction to Microbial Physiology, 3rd Edn" pp375-376, Butterworth & Co. Ltd., London.

Rothman, J.E. & Kennedy, E.P. (1977) Proceedings - National Academy of Sciences USA 74, 1821.

Saat, Y.A. & Bloch, K. (1976) Journal of Biological Chemistry 251, 5155.

Saez, M.J. & Lagunas, R. (1976) Molecular and Cellular Biochemistry 13, 73.

Satyanarayana, T. & Klein, H.P. (1973) Journal of Bacteriology 115, 600.

Schaus, O.O. (1971) Master Brewers Association of America, Technical Quarterly 8, 7.

Schechter, I. & Bloch, K. (1971) Journal of Biological Chemistry 246, 7690.

Schechter, I., Sweat, F.W. & Bloch, K. (1970) Biochimica et Biophysica Acta 222, 463.

Schermers, F.H., Duffus, J.H. & MacLeod, A.M. (1976) Journal of the Institute of Brewing 82, 170.

Schlossman, D.M. & Bell, R.M. (1978) Journal of Bacteriology 133, 1368.

Schweizer, E. & Bolling, H. (1970) Proceedings - National Academy of Sciences USA 67, 660.

Schweizer, E., Werkmeister, K. & Jain, M.K. (1978) Molecular and Cellular Biochemistry 21, 95.

Searle, B.A. & Kirsop, B.H. (1979) Journal of the Institute of Brewing 85, 342.

Seaston, A., Carr, G. & Eddy, A.A. (1976) Biochemical Journal 154, 669.

Seaston, A., Inkson, C. & Eddy, A.A. (1973) Biochemical Journal 134, 1031.

Sedgwick, B. & Smith, S. (1981) Archives of Biochemistry and Biophysics 208, 365.

Shafai, T. & Lewin, L.M. (1968) Biochimica et Biophysica Acta 152, 787.

Shimizu, I., Nagai, J., Hatanaka, H. & Katsuki, H. (1973) Biochimica et Biophysica Acta 296, 310.

Shore, C.C. & Fromm, A.J. (1981) Biochemistry 20, 7494.

Singer, S.J. (1974) Annual Review of Biochemistry 43, 805.

Singer, S.J. & Nicolson, G.L. (1972) Science 175, 720.

Smith, S.W. & Lester, R.L. (1974) Journal of Biological Chemistry 249, 3395.

Stadtman, F.R. & Kornberg, A. (1953) Journal of Biological Chemistry 203, 95.

Steiner, M.R. & Lester, R.L. (1972a) Biochimica et Biophysica Acta 260, 222.

Steiner, S. & Lester, R.L. (1972b) Journal of Bacteriology 109, 81.

Stewart, P.R. (1975) Methods in Cell Biology 12, 111.

Stoops, J.K. & Wakil, S.J. (1978) Biochemical and Biophysical Research Communications 84, 225.

Sumper, M. (1974) European Journal of Biochemistry 49, 469.

Sumper, M., Oesterhelt, D., Riepertinger, C. & Lynen, F. (1969) European Journal of Biochemistry 10, 377.

Suomalainen, H. (1981) Journal of the Institute of Brewing 87, 296.

Suomalainen, H. & Keranen, A.J.A. (1968) Chemistry and Physics of Lipids 2, 296.

Suomalainen, H. & Nurminen, T. (1970) Chemistry and Physics of Lipids 4, 247.

Szabo, A.S. & Avers, C.J. (1969) Annals - New York Academy of Sciences 168, 302.

Tabachnick, J. & Joslyn, M.A. (1953a) Plant Physiology 28, 681.

Tabachnick, J. & Joslyn, M.A. (1953b) Journal of Bacteriology 65, 1.

Tan-Wilson, A. & Kohlhaw, G.B. (1978) Biochemical and Biophysical Research Communications 85, 70.

Taylor, F.R. & Parks, L.W. (1979) Biochimica et Biophysica Acta 575, 204.

Taylor, G.T. & Kirsop, B.H. (1977a) Journal of the Institute of Brewing 83, 97.

Taylor, G.T. & Kirsop, B.H. (1977b) Journal of the Institute of Brewing 83, 241.

Taylor, G.T., Thurston, P.A. & Kirsop, B.H. (1979) Journal of the Institute of Brewing 85, 219.

Tchen, T.T. & Bloch, K. (1957) Journal of Biological Chemistry 226, 921.

Thompson, C.C. (1974) In " European Brewery Convention Wort Symposium, Zeist, Monograph 1" p285.

Thompson, C.C. & Ralph, D.J. (1967) Proceedings of the European Brewery Convention Congress, Madrid, p177.

Thompson, E.D., Bailey, R.B. & Parks, L.W. (1974) Biochimica et Biophysica Acta 334, 116.

Tracy, J.W. & Kohlhaw, G.B. (1975) Proceedings - National Academy of Sciences USA 72, 1802.

Trocha, P.J. & Sprinson, D.B. (1976) Archives of Biochemistry and Biophysics 174, 45.

Tyorinoja, K., Nurminen, T. & Suomalainen, H. (1974) Biochemical Journal 141, 133.

Van Broekhoven, A., Peeters, M.C., Debeer, L.J. & Mannoerts, G.P. (1981) Biochemical and Biophysical Research Communications 100, 305.

Van Deenen, L.L.M. (1972) Chemistry and Physics of Lipids 8, 366.

Van Deenen, L.L.M. (1981) FEBS Letters 123, 3.

Van den Bosch, H., Van der Elzen, H.M. & Van Deenan, L.L.M. (1967) Lipids 2, 279.

Van Wijk, R. & Konijn, T.M. (1971) FEBS Letters 13, 184.

Volpe, J.J. & Vagelos, P.R. (1973) Annual Review of Biochemistry 42, 21.

Waechter, C.J. & Lester, R.L. (1973) Archives of Biochemistry and Biophysics 158, 401.

Wainwright, T.W. (1973) Journal of the Institute of Brewing 79, 451.

Wais, U., Gillmann, U. & Ullrich, J. (1973) Hoppe-Seylers Zeitschrift für Physiologische Chemie 354, 1378.

Walenga, R.W. & Lands, W.E.M. (1975) Journal of Biological Chemistry 250, 9121.

Wallace, P.G., Huang, M. & Linnane, A.W. (1968) Journal of Cell Biology 37, 207.

Wallace, P.G. & Linnane, A.W. (1964) Nature 201, 1191

Watson, K., Bertoli, E. & Griffiths, D.E. (1975) Biochemical Journal 146, 401.

Weete, J.D. (1973) Phytochemistry 12, 1843.

Weibel, K.E., Mor, J.R. & Feichter, A. (1974) Analytical Biochemistry 58, 208.

Weiss, S.B. & Kennedy, E.P. (1956) Journal - American Chemical Society 78, 3550.

Weithman, K.J. (1974) Dissertation, University of Munich (cited by Sedgwick & Smith, 1981)

Wheeler, G.A. & Rose, A.H. (1973) Journal of General Microbiology 74, 189.

White, D. & Klein, H.P. (1966) Journal of Bacteriology 91, 1218.

White, F.H. & Portno, A.D. (1979) Proceedings of the European Brewery Convention Congress, Nice, p447.

Whitear, A.L. & Crabb, D. (1977) The Brewer (Feb) p60.

Whitworth, C. (1978) In "European Brewery Convention Fermentation and Storage Symposium, Zoeterwoude, Monograph V," p155.

Whitworth, C. (1980) The Brewer (Nov) p339.

Whitworth, D.A. & Ratledge, C. (1974) Process Biochemistry 9, 14.

Wilson, R.J.H. (1977) Proceedings of the European Brewery Convention Congress, Amsterdam, p343.

Wilson, R.J.H. (1979) In "European Brewery Convention Fermentation and Storage Symposium, Zoeterwoude, Monograph V," p17.

Wisnieski, B.J., Keith, A.D. & Resnick, M.R. (1970) Journal of Bacteriology 101, 160.

Wohrmann, K. & Lange, P. (1980) Journal of the Institute of Brewing 86, 174.

Yamamoto, S., Lin, K. & Bloch, K. (1969) Proceedings - National Academy of Sciences USA 63, 110.

Yoshioka, Y. & Hashimoto, N. (1981) Agricultural and Biological Chemistry 45, 2183.

Young, M.R., Tolbert, B. & Otter, M.F. (1969) In "Methods in Enzymology Vol 13," (Lowenstein, J.M. Ed) p250 Academic Press, London and New York.

Zurcher, C.H. (1971) Monatsschrift für Brauerei 24, 276.

APPENDIX

List of Publications

Parts of the work in this thesis have been published:

Thurston, P.A., Taylor, R. & Ahvenainen, J. (1981) Effects of linoleic acid supplements on the synthesis by yeast of lipids and acetate esters. Journal of the Institute of Brewing 87, 92-95.

Thurston, P.A., Quain, D.E. & Tubb, R.S. (1981) The control of volatile ester biosynthesis in Saccharomyces cerevisiae. Proceedings of the European Brewery Convention Congress, Copenhagen, pp197-206.

Thurston, P.A., Quain, D.E. & Tubb, R.S. (1982) Lipid metabolism and the regulation of volatile ester synthesis in Saccharomyces cerevisiae. Journal of the Institute of Brewing 88, 90-94.

Other publications:

Taylor, G.T., Thurston, P.A. & Kirsop, B.H. (1979) Influence of lipids derived from malt spent grains on yeast metabolism and fermentation. Journal of the Institute of Brewing 85, 219-227.

Quain, D.E., Thurston, P.A. & Tubb, R.S. (1981) The structural and storage carbohydrates of Saccharomyces cerevisiae: changes during fermentation of wort and a role for glycogen catabolism in lipid biosynthesis. Journal of the Institute

of Brewing 87, 108-111

Thurston, P.A. & Tubb, R.S. (1981) Screening yeast strains for their ability to produce phenolic off-flavours: a simple method for determining phenols in wort and beer. Journal of the Institute of Brewing 87, 177-179.