ESTER FORMATION IN BREWERY FERMENTATIONS

By Hilary A. B. Peddie

(Brewing Research Foundation, Lyttel Hall, Nutfield, Redhill, Surrey RH1 4HY)

Esters are secondary products produced by brewing yeasts during the anaerobic metabolism of sugars and constitute one of the largest and most important groups of compounds affecting beer flavour. Many esters can be formed, the most important being ethyl acetate, isoamyl acetate, isobutyl acetate, 2-phenylethyl acetate and ethyl hexanoate. The odour threshold levels for ester detection are very low. Esters are especially important in high gravity brewing, where over production occurs causing unwanted solvent-like flavours. They are also important in low alcohol beer production due to the low levels produced, which can result in beers with little flavour. The factors influencing ester production are reviewed, together with the ways in which they can be used to control ester synthesis.

It is believed that acetate esters are synthesized by an enzyme called alcohol acetyl transferase (AAT) which uses as substrates an alcohol and acetyl co-enzyme A; the latter plays a central role in many intracellular reactions. However, esters can also be synthesized by esterase enzymes working in reverse. Several attempts have been made to locate the AAT enzyme and recent work suggests that it is located in either the yeast cell plasma membrane or in intracellular organelles called "vacuomes".

Key Words: *Ester, yeast, fermentation, review.*

INTRODUCTION

Many components are responsible for determining the flavour of beer, and esters are some of the most important[13,28,32,37,38]. Esters are positive flavour determinants, i.e. they provide beer with a desirable flavour[8,9,36]. Their sensory odour threshold levels are low[32], ranging from 0.2 ppm for isoamyl acetate to 15–20 ppm for ethyl acetate[8,31]. Esters are synthesized within the yeast cell[11,23,27] and, depending on their chain length, they diffuse into the surrounding medium[23,28,32].

The most significant[33,35] esters in beer are ethyl acetate (fruity, solvent-like), isoamyl acetate (isopentyl acetate) (pear drops), isobutyl acetate (banana), ethyl caproate (ethyl hexanoate) (apple) and 2-phenylethyl acetate (honey, fruity, flowery)[5,8,34,36].

THE FORMATION OF ESTERS

Esters can be formed via a chemical condensation reaction:

$$R'OH + RCOOH \rightarrow RCOOR' + H_2O$$
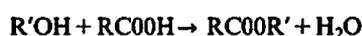
However, the concentration of esters found in beer is much too great to be produced in this way when the rate of reaction and the duration of fermentation is considered[8,34]. Nordström[21] showed that esters are probably formed via a biochemical pathway, the rate of which, in *Saccharomyces cerevisiae* (the yeast mainly used in these studies), could account for the concentration of esters found in the finished product[12]:

$$RCOOH + ATP + CoASH \rightarrow RCO^-SCoA + AMP + PPi$$
$$RCO^-SCoA + R'OH \rightarrow RCOOR' + CoASH$$
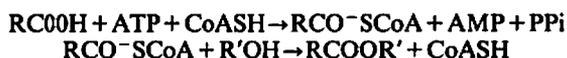
The compounds involved in ester production are ethanol or higher alcohols, fatty acids, co-enzyme A (CoASH) and an ester synthesizing enzyme which was first described by Nordström[17]. In later papers Nordström[18,21] proposed that alcohols become esterified by reacting with fatty acids which have undergone a previous activation by combining with CoASH. He concluded that although acetyl CoA can be formed by the oxidative decarboxylation of pyruvate[21] most of the other acyl CoA compounds come from acylation of CoASH by the action of acyl-CoA synthetase. It is in these activated forms that acyl-CoA compounds can act as the acyl donor[34]. As a result of the need for activation, ester synthesis is an energy requiring process.

It is important to stress the central role played in ester synthesis by acetyl CoA, since this is involved in many other reactions within the yeast cell (e.g. lipid biosynthesis, amino

acid biosynthesis, fatty acid biosynthesis and the TCA cycle)[1,17,18,19,21]. Figure 1 illustrates this. Any factors which govern production and consumption of this compound could be crucial for ester production[27,37].

Most of the work carried out on esters has concentrated on acetate esters as they are the most abundant[32]. Hence the enzyme involved, which has a molecular weight of 220,000, has been named alcohol acetyl transferase (AAT) by Yoshioka & Hashimoto[38]. However, there are many esters and each different activated fatty acid may have its own specific enzyme. In these circumstances, if several fatty acyl CoA compounds are present, competitive inhibition could occur and esterification of individual activated compounds would be inhibited by the others[14,18,27]. Competitive inhibition of this type is observed in practice. In addition, some fatty acids, which are not substrates for the enzyme (e.g. isovaleric acid, iso-butyric acid) can also act as inhibitors[17]. If several alcohols are present they can compete in a similar fashion[27]. Not surprisingly, alcohol acetyl transferase (AAT) is the most studied ester-synthesizing enzyme and so most of the ensuing discussion refers to this enzyme.

The enzymes involved in ester breakdown are termed esterases and catalyse the following reaction

$$RCOOR^1 + H_2O \rightarrow R^1OH + RCOOH$$

Some non-brewing yeasts synthesize esters using esterase

MALTOSE
↓
GLUCOSE
↓
PYRUVATE
↓
ESTERS ◄── ACETYL CoA ──► TCA CYCLE
↓
LIPIDS
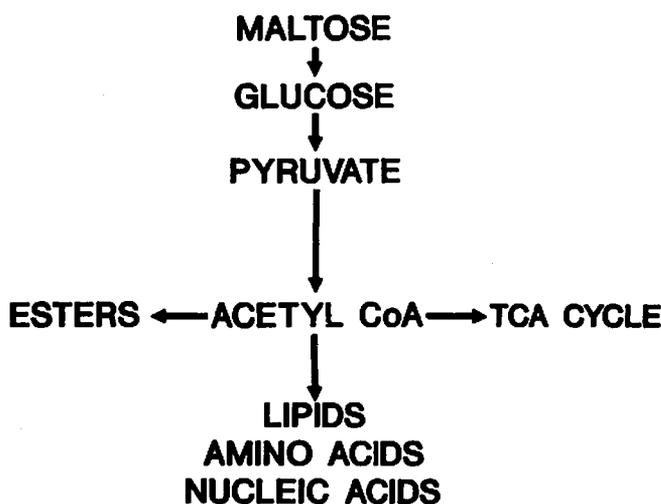**AMINO ACIDS**
NUCLEIC ACIDS

Fig. 1. The central role played by acetyl CoA in yeast metabolism.

enzymes working in reverse[32] and this occurs in the absence of CoASH[28]. One such yeast is *Hansenula anomala* which can synthesize up to 400 ppm ethyl acetate in this reaction[38]. *H. anomala* has been shown to produce less esters in the presence of acetyl co-enzyme A than in its absence[38]. The normal function of esterases is to hydrolyse esters and this has been suggested to be important in high gravity brewing where the breakdown of esters may help to limit the ester levels found in the beer in these circumstances. However, Schermers *et al.*[28] have suggested a role for esterases in ester synthesis by brewing yeasts since they found a positive correlation between the reverse activity of esterases found in different strains of *Sacch. cerevisiae* and the levels of ethyl acetate and isoamyl acetate produced.

One reason why acetate esters are so abundant could be that they are hydrolyzed by esterases at a much slower rate than other esters such as ethyl octanoate (caprylate)[32] (see Figure 2). However, since octanoyl CoA will ony be present at a very low concentration compared with acetyl CoA, substrate availability will limit the amount of ethyl octanoate which is produced.

Wort composition: This can affect ester synthesis[8,9] and is especially important in high gravity brewing[24,37]. In an all-malt wort fermentation, where the carbohydrate : assimilable nitrogen (C : N) ratio is low, the yeast thrives until oxygen, the growth limiting factor, is used up[1]. However, although growth has ceased, nitrogenous compounds are abundant[1,24] and metabolites, including acetyl CoA, are still formed. Thus, within the cell there is an excess of acetyl CoA which stimulates ester synthesis[1].

When adjuncts are used, the C : N ratio is high and so when growth ceases there is no excess of nitrogen, indeed under these conditions this can be growth limiting[24]. Hence there is no excess of acetyl CoA and ester synthesis is less[5,24,37]. Yoshioka and Hashimoto[40] have suggested that, in adjunct worts, yeast may attempt to absorb more nitrogen, in the form of additional peptides not normally taken up during fermentation. The observed accumulation of unsaturated fatty acids in the cell membrane under these conditions[39], may encourage uptake of peptides. In addition, the accumulation of unsaturated fatty acids may inhibit the ester synthesizing enzyme directly[12,39].

The lipid content of the wort can affect the ester concentration of the final product[34]. It has been shown that incorporation of elevated levels of unsaturated fatty acids namely oleic, linoleic and linolenic acids, into the wort results in a decrease in ester synthesis[1,34,35,39]. This may be due to a change caused to the physical state of the membrane following incorporation of these fatty acids or may be due to a direct effect upon the synthetic enzyme[35,39]. Figure 3 shows the change in activity of the ester synthesizing enzyme in relation to time of fermentation and the effects of addition of various saturated and unsaturated fatty acids to the wort. There is no change in the activity of AAT in the presence of saturated fatty acids such
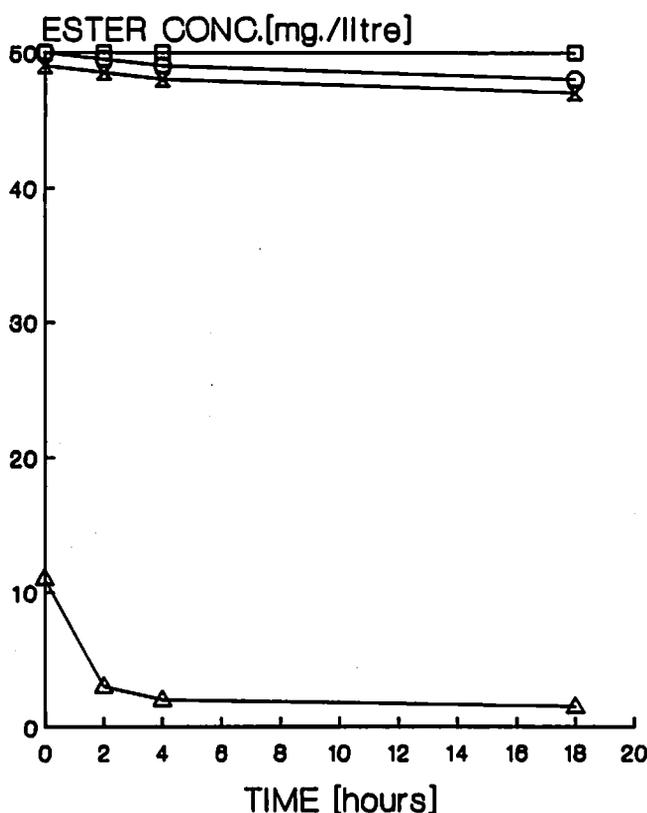

FIG. 2. The hydrolysis of different esters by esterase enzymes. Various esters were incubated with intact yeast and ethanol at pH 4 for the times indicated. □: ethyl acetate, O: isoamyl acetate, X: phenyl-ethyl acetate, △: ethyl caprylate. (redrawn from ref. 32).

It is clear from the above discussion that the prime function of esterases is still not known. In *Sacch. cerevisiae* it is uncertain whether their main role is in ester hydrolysis or ester synthesis.

FACTORS INFLUENCING ESTER SYNTHESIS

Many factors have a significant effect on ester production by *Sacch. cerevisiae* during fermentation:

Yeast strain: The strain of yeast chosen is important since each yeast strain produces its own characteristic ester profile and some yeast strains can produce much greater amounts of esters than others[8,18,22,23].
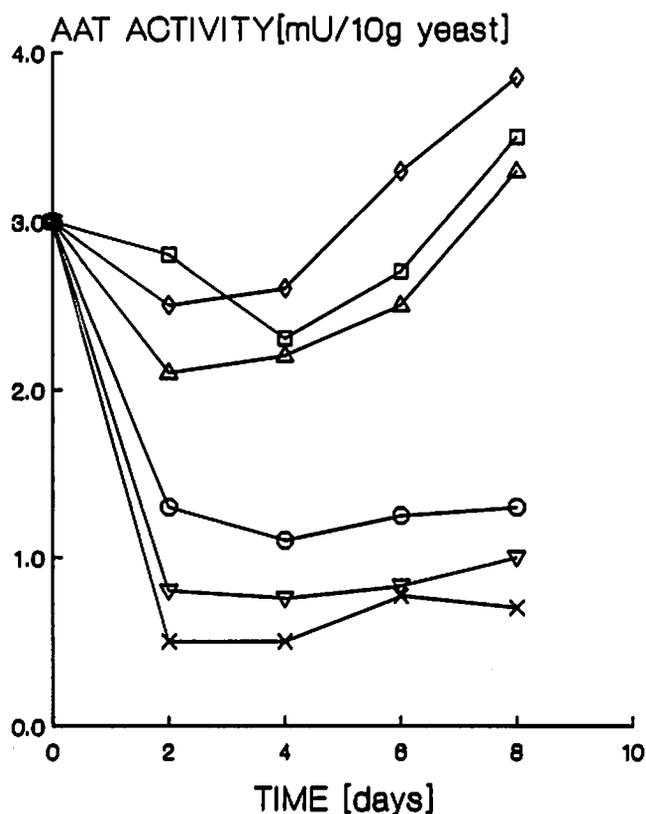

FIG. 3. The effect of the degree of saturation of membrane fatty acids on alcohol acetyl transferase (AAT) activity. Various fatty acids were incubated with intact yeast cells in a synthetic medium for the times indicated and the alcohol acetyl transferase activity measured. □: no addition, ◇: stearic acid, △: palmitic acid, O: oleic acid, ▽: linoleic acid, X: linolenic acid. (redrawn from ref. 39).

as stearic or palmitic acids when compared to the control (i.e. no addition). Upon addition of unsaturated fatty acids such as oleic, linoleic or linolenic acids there is a great reduction in the activity of AAT resulting in a reduction in the ester level[1,35,39]. With the exception of linoleic acid[35], most unsaturated fatty acids promote yeast growth during fermentation[1], and so there is a reduced availability of CoASH for ester synthesis[12]. If the membrane is enriched with linoleic acid it seems to prevent stimulation of the synthesis of esters[35], even though the intracellular level of CoA increases and that of acetyl CoA remains similar to the level in control cells[34].

Wort-aeration: This results in increased yeast growth[8] since oxygen is essential for key reactions in the biosynthesis of sterols and unsaturated fatty acids[3,6], which are needed for yeast growth. Increased yeast growth creates additional demand for acetyl CoA for that purpose and so the availability of acetyl CoA for ester synthesis is reduced; even a low rate of aeration during fermentations can strongly inhibit ester formation[1,2,11,14,17,33,37].

Pressure: Reports on the effects of pressure on ester formation are somewhat inconsistent[15,16,22,23]. Nordstedt *et al.*[16] and Miedaner *et al.*[15] have suggested that, when pressure is increased, acetate ester synthesis is reduced. However when pressure is increased, yeast growth is also reduced and so there should be a high availability of acetyl CoA for ester synthesis and yet ester synthesis is decreased. This is in contrast to the effects of limiting oxygen, when yeast growth associated processes are reduced and acetyl CoA becomes available for ester synthesis which increases. Posada *et al.*[25,26] have observed that increased pressure causes a decrease in ethyl acetate levels but an increase in those of isoamyl acetate and 2-phenylethyl acetate. In general, however, it seems that application of pressure results in inhibition of ester synthesis and decreased yeast growth[11,12].

Pitching rate: Maule[14] observed that when pitching rate is increased four-fold, ester synthesis is reduced. However, for modest increases of pitchng rate (i.e. two-fold) only the concentration of ethyl acetate is affected[8].

Suspended solids: The presence of trub in the wort at pitching can influence ester production. Trub is of variable composition but often contains zinc and lipids, both of which are stimulatory to yeast growth. Schisler *et al.*[29] have suggested that trub stimulates yeast growth because of the presence of these nutrients. As a result of accelerated yeast growth, ester synthesis is reduced since availability of acetyl CoA for this purpose is reduced.

Siebert *et al.*[30] have observed that other solids such as activated carbon and diatomaceous earth can also cause a reduction in ester synthesis, most probably by a direct physical effect. The suspended solids may stimulate carbon dioxide removal from the fermentation vessel as they create a larger surface area from which carbon dioxide can be evolved. This phenomenon is important to yeast since carbon dioxide can act as an inhibitor of yeast growth[16]. By reducing carbon dioxide levels the solids stimulate yeast growth and hence less acetyl CoA is available for ester production, and consequently ester levels are reduced[30].

Temperature: When temperature is elevated, the concentration of esters produced during the fermentation is also increased[8,21,33]. For example, a 15°C temperature increase from 10°C to 25°C can result in a 75% increase in ester concentration[8]. This increase in temperature may make the membrane more fluid and so affect the activity of the AAT enzyme, if it is membrane bound. An increase in membrane fluidity may allow more ester to diffuse into the medium. AAT is also known to be unstable at higher temperatures[4]. However, the higher temperature may simply increase enzyme activity. Engan and Aubert[9] have shown that the effect of temperature differs with both ester type and temperature range.

TIMING OF ESTER SYNTHESIS

Thurston[34] has suggested that during fermentation there are two inductions of ester synthesis. At the beginning of fermentation, ester synthesis is very slow due to the high metabolic demand for acetyl CoA for yeast growth[33,39]. At this time oxygen and acetyl CoA are rapidly consumed in production of unsaturated fatty acids and sterols. Immediately following this an equilibrium is established between acetyl CoA consumption for fatty acid and sterol synthesis and for ester production. This represents the first induction of ester synthesis and occurs after about 8 hours of fermentation. When fatty acid and sterol synthesis finally stop there is a peak in cellular acetyl CoA levels and also in the acetyl charge (the ratio between [acetyl-CoA] and [acetyl CoA + CoASH]) and at this point the second induction of ester synthesis occurs. This happens about the midpoint of fermentation (between 20 and 30 h) and is relatively short-lived[33,34,37]. However, it does contribute significantly to the overall ester level. At the point of maximum specific rate of ethyl acetate synthesis about 80% of the CoASH is in the acetyl form[34].

THE REASONS FOR ESTER SYNTHESIS BY YEAST CELLS

Esters could simply be overspill products from sugar metabolism during fermentation and may be of no advantage to the yeast cell, simply leaving the cell by diffusion. However, there may be a reason for ester production. Fatty acids with a chain length between C8–C14[27] are toxic to yeast and exhibit strong anti-microbial activity; and if they happen to be unsaturated this intensifies the effect. Esters may be formed to remove, from the yeast cell, these toxic fatty acids[17,19]. Esters of shorter chain fatty acids (e.g. C2–C6) would be produced by the same detoxification processes. Another reason for ester formation could be to reduce the acetyl charge, as it is essential for the yeast cell to maintain a balance between acetyl CoA and CoASH[34].

LOCATION OF ESTER SYNTHESIZING ENZYMES

Yoshioka and Hashimoto[38] believe that the AAT enzyme is bound to the plasma membrane. They have attempted to isolate the plasma membrane and then assay ester synthesizing enzyme activity; the cell lysates prepared were also assayed for enzyme activity. Table 1 quotes the results obtained. The fraction claimed to be the plasma membrane had the greatest specific enzyme activity[38]. However, it is not certain that this fraction was actually plasma membrane since no assay was carried out for a marker enzyme representative of plasma membranes, such as vanadate sensitive ATPase[38].

TABLE I.   Alcohol Acetyl Transferase activity in a cell membrane preparation and in cell lysates (data from ref. 38)

Fraction	Specific enzyme activity (mU)
Precipitate after 3rd lysis (Cell membrane fraction)	54.0
Supernatant after 1st lysis	21.1
Supernatant after 2nd lysis	3.1
Supernatant after 3rd lysis	1.2

In contrast to this, Howard and Anderson's[10] results suggested that the AAT is located in subcellular fractions which sediment at 105,000 × g when a 3000 × g supernatant is centrifuged. Their data is in agreement with recent work by Malcorps and Dufour[13] who believe that the plasma membrane has little or no AAT activity and that the activity resides in cellular "vacuomes". The latter work entailed fractionating an ale yeast homogenate by differential centrifugation. Certain marker enzymes were chosen which were representative of the different cellular organalles as follows:

Plasma membrane—PM ATPase (oligomycin insensitive)
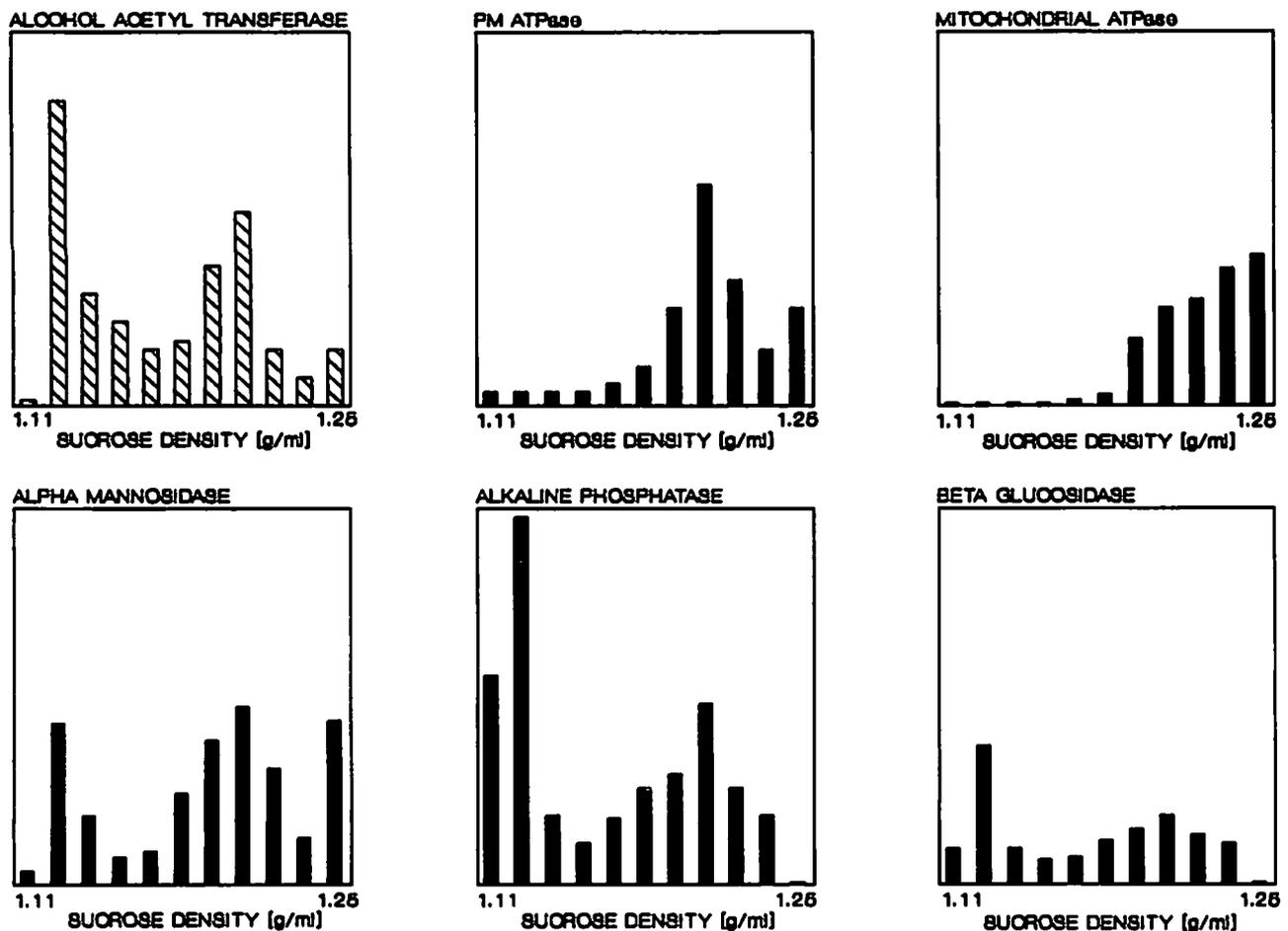Mitochondrial membrane—Mitochondrial ATPase (oligomycin sensitive)

FIG. 4. Sucrose density gradient enzyme profiles of a 12,000 × g pellet obtained from an ale yeast homogenate. The different enzyme activities were used as indicators of different cellular fractions (see text). (redrawn from ref. 13).

Vacuolar membrane—alpha mannosidase
Vacuolar compartment—alkaline phosphatase
Secretory vesicles—beta glucosidase

The fraction which pelleted at 12,000 × g during differential centrifugation was layered onto a sucrose gradient and subjected to further centrifugation. The results obtained are shown in Figure 4. Malcorps and Dufour[13] could not associate the AAT with any one particular marker enzyme when comparing the enzyme profiles. Association of AAT with the mitochondrial membrane was ruled out by isopycnic centrifugation as its profile was very different from the AAT profile. A plasma membrane location was ruled out on the grounds of magnesium effects on the plasma membrane during differential centrifugation which caused a shift in the oligomycin insensitive ATPase enzyme profile; the AAT profile, under similar conditions was unaffected. As a result, Malcorps and Dufour[13] suggest that AAT is associated with subcellular structures of varying size collectively known as "vacuomes" which are widely distributed within the yeast cell.

CONCLUSIONS

In summary, there exists controversy as to the location of the ester synthesizing enzyme in yeast cells and there is some disagreement about the more important factors affecting ester synthesis. From the 1960's until the early 1980's it was felt that factors affecting availability of acetyl-CoA were the most important, since any reduction in the availability of this compound results in a decrease in ester synthesis[1,8,11,12,17,18,19,20,21]. However, work performed by Yoshioka and Hashimoto between 1982-84[38,39,40,41] suggests that factors which change the physical state of the plasma membrane may also be of im-

portance since when the lipid composition of the membrane is changed, the activity of the ester synthesizing enzyme is altered. The most recent work by Malcorps and Dufour in 1987[13] suggests that the enzyme activity is not plasma membrane bound at all and that the ester synthesizing enzyme is located within cellular vacuomes. However, it is likely that no single factor controls ester synthesis; both direct physical and biochemical effects on AAT and substrate availability could be of equal importance. Clearly more experimentation is required to clarify these points if the mechanisms involved in ester synthesis are to be fully elucidated.

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