Ester Formation by Yeasts

2. FORMATION OF ETHYL ACETATE BY WASHED SUSPENSIONS OF *HANSENULA ANOMALA*

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Ester formation by growing cultures of certain yeasts has been reported by Davies, Falkiner, Wilkinson & Peel (1951) in the preceding paper. The amounts of ethyl acetate, ethanol and acetic acid present in a typical culture are given in Table 1.

Table 1. *Analysis of a culture of* Hansenula anomala

(Culture of vol. 100 ml. grown aerobically for 72 hr. at 25° on a medium containing 4% (w/v) glucose, salts and Difco yeast extract.)

Substance	Concentration ($M \times 10^{-3}$)
Acetic acid	5·8
Ethanol	35
Ethyl acetate	46

It is possible to calculate the concentration of ester which would be in equilibrium with the observed concentrations of ethanol and acetic acid, if the ester were formed by the condensation:

$$CH_3COOH + C_2H_5OH = CH_3COOC_2H_5 + H_2O.$$

Using the equilibrium data quoted by Parks & Huffman (1932), a value of 0·000015 M is obtained.

The concentration of ester actually found was 0·046 M. It may be concluded that the ester is not formed by the condensation indicated above but by some different process. The present communication reports investigation of the process involved in the formation of ester by washed suspensions of cells.

METHODS

Organism, growth media and preparation of washed suspensions. The organism used throughout was *Hansenula anomala* Sydow (no. 119) obtained from the Carlsberg Laboratory. The yeast was grown on the medium described by Davies *et al.* (1951). The sterilized medium was inoculated from a culture grown for 24 hr. at 25° on a similar medium but with 5% glucose. Except where otherwise specified, all cultures were grown for 48 hr. at 25°. Two growth methods were employed: when small quantities of yeast were required, cultures were grown in Roux bottles and the pellicle which formed was removed by filtration through several layers of muslin. Alternatively the yeast was grown in a stirred, aerated vessel as described by Mitchell (1949). Usually 2 l. of medium were contained in a 5 l. flask. This method produces a pellicle-free culture and was used when larger quantities of yeast were required. No significant differences in ester formation were observed between cultures grown by the two methods.

The yeast was separated from the pellicle-free culture by centrifugation, washed once with water and resuspended in water. The suspensions settled quite rapidly, consequently thorough mixing was necessary before removing samples. Dry weight determinations were made turbidimetrically, after the yeast had been dispersed by the addition of 10% (v/v) of 0·05 M-phthalate buffer pH 3 containing 0·1% Teepol X (Imperial Chemical Industries).

Ester determinations. The method used was developed from that described by Keenan (1945) for the determination of ethyl acetate in methanolic solution, which is based on the conversion of the ester to the corresponding hydroxamic acid by the action of alkaline hydroxylamine:

$$CH_3COOC_2H_5 + NH_2OH = CH_3CONHOH + C_2H_5OH.$$

The amount of hydroxamic acid formed is measured by the colour of its ferric complex in a manner similar to that used by Lipmann & Tuttle (1945) in the determination of acetyl phosphate. Consistent results could not be obtained when the method as described by Keenan was applied to aqueous solutions of ethyl acetate. A detailed investigation of the conditions affecting the colour intensity of the ferric complex and also of those affecting hydroxamic acid formation was therefore carried out.

Unknown to the author a similar investigation had been carried out by Hestrin (1949) in connexion with the determination of acetylcholine. As the author's results are in almost complete agreement with those of Hestrin no account will be published.

The procedure adopted was as follows: 2·0 ml. of 2·5 N-NaOH were added to 2·0 ml. of 2·0 M-NH_2OH.HCl, followed by 4·0 ml. of the sample containing up to 10 μmol. of ester. After standing for 5–10 min., 1·0 ml. of 5·6 N-HCl was added, followed by 1·0 ml. of 15% (w/v) $FeCl_3.6H_2O$ in 0·2 N-HCl, mixing after each addition. The colour intensity was measured using the Hilger 'Spekker' absorptiometer with Ilford no. 604 filters. A linear relationship between extinction coefficient and the amount of ester present was obtained up to 10 μmol. of ester. A separate calibration curve was constructed for each new batch of reagents, using a freshly prepared aqueous solution of ethyl acetate as standard. Determinations on aqueous samples of ester containing 6–10 μmol. were reproducible to within 3%.

As far as is known the only compounds giving the colour reaction under these conditions are esters and acid anhydrides. When high concentrations of acetaldehyde

(e.g. 0·1 M) were present in the test sample, a slow increase in colour intensity was observed, extending over several minutes after addition of the HCl and $FeCl_3$ reagents. This was presumably due to a slow formation of hydroxamic acid from the acetaldehyde and was corrected for by taking successive colorimeter readings at known times after addition of the HCl and $FeCl_3$. The value at zero time was then deduced by extrapolation.

The absence of acyl phosphate in samples can be checked by reversing the order of addition of the NaOH and the HCl, as suggested by Hestrin (1949). No acyl phosphate has been detected in suspensions of *H. anomala* during ester formation.

Separation of the ester from reaction mixtures. Initially the separation was effected by carrying out a small-scale distillation in presence of excess $CaCO_3$. The sample, containing up to 20 μmol. of ester, was diluted to 15 ml., excess powdered $CaCO_3$ added and the first 10 ml. distillate collected in a receiver cooled in ice water. To minimize frothing, 2 drops of medicinal liquid paraffin were added to the mixture and distillation carried out in a wide boiling tube heated by a microburner. About 5 mg. of talc were also added to prevent bumping. A suitable sample of the distillate was taken for ester determination. The recovery of the ester in the distillate was not complete, but was constant at 93±7%. When this method of separation is used the determination is, as far as is known, specific for volatile esters.

It was subsequently found sufficient in most cases to remove the yeast from reaction mixtures by filtration and carry out determinations directly on the filtrate. The only substance in the filtrate known to interfere with hydroxamic acid determinations was phosphate (Lipmann & Tuttle, 1945). However, direct tests showed that, under the conditions used, the amount of phosphate present did not interfere with the determinations to any significant extent.

Total alcohol determinations. The method used was a modification of that due to Northrop, Ashe & Senior (1919), in which the alcohol is oxidized by $K_2Cr_2O_7$ and the residual $K_2Cr_2O_7$ determined iodometrically. A preliminary distillation in the presence of alkaline HgO was carried out to remove acids, aldehydes and ketones.

To the sample, containing up to 100 μmol. of alcohol, were added 1·0 ml. of 5 N-NaOH, 1·0 ml. of 10% (w/v) $HgSO_4$ in 2 N-H_2SO_4 and water to a total volume of 15 ml. The mixture was allowed to stand at room temperature in a stoppered flask for 15–20 min. to hydrolyse any ester present. It was then heated slowly to boiling point and 10 ml. distillate were collected. The distillate was added to a cooled mixture of 4·0 ml. of 1% (w/v) aqueous $K_2Cr_2O_7$ and 1·0 ml. of H_2SO_4 contained in a boiling tube. The tube was closed with a loose-fitting stopper or glass bulb, immersed in a boiling-water bath for exactly 20 min., then cooled in running water for about 5 min., 4·0 ml. of 10% (w/v) aqueous KI added and the liberated I_2 titrated against 0·03 N-$Na_2S_2O_3$. The method was calibrated empirically using a standard ethanol solution. A linear relationship between the $Na_2S_2O_3$ titre and the amount of ethanol present was obtained over the range 0–100 μmol. ethanol. Over this range there was found a decrement in titre of 0·00376 m-equiv. $Na_2S_2O_3$/μmol. of ethanol present. Duplicate determinations on 60–100 μmol. of ethanol agreed to within 3%.

Measurement of ester synthesis. Except where otherwise indicated, ester synthesis was measured as follows:

Total liquid volumes of 10·0 ml. containing buffer, washed yeast and substrate were incubated in 100 ml. Erlenmeyer flasks of uniform shape. The flasks were closed with rubber bungs to prevent loss of ester and were shaken in identical fashion throughout the incubation period. Variation of the rate of shaking produced a marked effect on the yield of ester. After incubation for 1 hr. the yeast was removed by filtration and determinations carried out as described above.

Under these conditions the amount of ester initially present could be neglected and the amount of ester found after incubation taken as the ester formed.

Acetate, where present in incubation mixtures, was added as a mixture of acetic acid and sodium acetate in such proportions as to give the required pH. In experiments at pH 2·8 where McIlvaine buffer was added, the standard citrate-phosphate solution at pH 2·5 was added, this giving a pH of 2·8 in the final volume.

RESULTS

The earlier experiments were carried out using ethanol in presence of 0·04 M-acetate as substrate. It was subsequently found that higher concentrations of acetate inhibit ester formation and several experiments were repeated using ethanol alone as substrate. The results were substantially the same in both series of experiments except that the pH optimum for ester formation is altered by the presence of acetate. Table 2 illustrates the ability of a washed suspension of *H. anomala* to form ester from ethanol in the presence of acetate. In the absence of ethanol no ester was formed. In the absence of acetate, ester formation was diminished, but this may have been due to the change in pH optimum found when ethanol alone replaced (ethanol + acetate) as substrate.

Table 2. *Formation of ester by a washed suspension of* Hansenula anomala

(The complete system comprised, in a total vol. of 5·0 ml.: McIlvaine buffer (pH 4·6), 1·5 ml.; acetate, 0·04 M; ethanol, 0·1 M; washed yeast, 80 mg. dry wt./ml. Incubated with shaking for 1 hr. at 25°.)

System	Ester formed (μmol.)
Complete system	230
Control (boiled yeast)	20
Ethanol absent	18
Acetate absent	128
Ethanol and acetate absent	14

Stability of activity. The ester-forming activity of washed suspensions was unstable. On keeping for 24 hr. at 1°, 50–75% of the initial activity was lost.

Effect of age of culture. The activity of washed suspensions in producing ester from (ethanol + acetate) increased with the period of growth of the cultures prior to harvesting, up to 48 hr. Cells harvested after 96 hr. were much less active (Table 3).

Dependence of ester formation on the presence of oxygen. Table 4 shows that ester formation from (ethanol + acetate) was dependent upon the presence of oxygen.

Table 3. *Effect of age of culture on ester formation by a washed suspension of* Hansenula anomala

(Yeast harvested after growth at 25° for the times indicated. Samples of washed cells of about 90 mg. dry weight together with McIlvaine buffer (pH 4·6), 3·0 ml., 0·04M-acetate and 0·15M-ethanol in a total vol. of 10·0 ml., incubated with shaking for 1 hr. at 25°.)

Age of culture (hr.)	Ester formed (μmol./100 mg. dry weight yeast)
12	0
24	6
48	98
96	25
336	7

Table 4. *Dependence of ester formation by a washed suspension of* Hansenula anomala *on the presence of oxygen*

(Krebs vessels contained, in a total vol. of 10·0 ml.: McIlvaine buffer (pH 4·6), 3·0 ml.; acetate, 0·04M; ethanol, 0·1M; washed yeast, 4·0 mg. dry wt./ml. Incubated with shaking for 1 hr. at 25°.)

Gas phase	Ester formed (μmol.)
N_2	5
Air	33
O_2	43

Effect of pH. The precise relationship between pH and ester formation is a little uncertain. With (ethanol + 0·04M-acetate) as substrate and in presence of McIlvaine buffer, ester formation proceeded at a maximum rate at pH 4·5–5·0 (Fig. 1). The situation was complicated, however, by the fact that acetate was inhibitory at concentrations above 0·05M.

With ethanol alone as substrate the optimum pH was found to vary with the nature of the buffer present. The results for glycine, phthalate, phosphoric acid-potassium dihydrogen phosphate, and McIlvaine citrate-phosphate buffers are shown in Fig. 1. Ester formation from ethanol alone was studied at pH 2·8 in presence of McIlvaine buffer.

Effect of concentration of yeast cells. Ester formation was found to be directly proportional to the concentration of yeast cells over the range 0–9 mg. dry wt./ml. both in presence of ethanol alone (at pH 2·8) and of (ethanol + 0·04M-acetate) (at pH 4·6).

Effect of ethanol concentration. Fig. 2 shows the effect of ethanol concentration on ester formation in presence and in absence of 0·04M-acetate at pH 4·6, and in the absence of acetate at pH 2·8. In presence of 0·04M-acetate at pH 2·8 ester formation was completely inhibited (Fig. 3). In each of the three cases studied, increasing the ethanol concentration above 0·2M produced little increase in ester formation.

Effect of acetate concentration. At pH 4·6, the addition of 0·008M-acetate produced 50 % increase in the production of ester, but the rate decreased with further increase in acetate concentration until complete inhibition was obtained in presence of about 0·1M-acetate. At pH 2·8, a small stimulation (7 %) was observed on addition of 0·01M-acetate. Further increase in acetate concentration produced inhibition which was complete at a lower concentration (0·04M) than at pH 4·6 (Fig. 3).

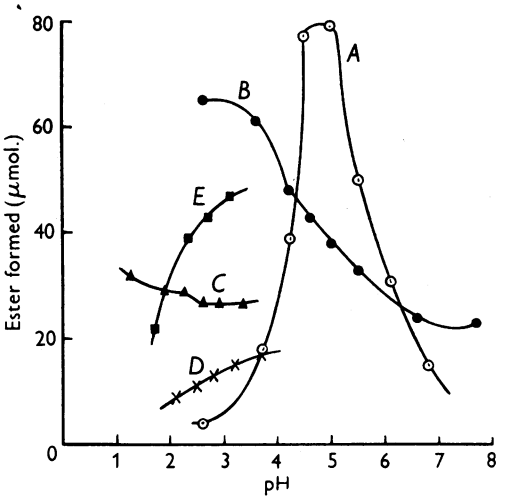

Fig. 1. Effect of pH on ester formation by washed suspensions of *Hansenula anomala*. Incubation mixtures contained in a total vol. of 10·0 ml.: ethanol, 0·2M; washed yeast, 40 mg. dry wt. (Exps. *A*, *C*, *D*, *E*) or 25 mg. dry wt. (Exp. *B*); buffers as follows: Exp. *A*, 0·04M-acetic acid-sodium acetate mixtures together with 3·0 ml. McIlvaine standard citrate-phosphate mixtures; Exp. *B*, 3·0 ml. McIlvaine standard citrate-phosphate mixtures; Exp. *C*, 3·0 ml. Sørensen standard glycine in NaCl-HCl mixtures (Clark, 1928); Exp. *D*, 0·25M-potassium phthalate buffer; Exp. *E*, 0·03M-H_3PO_4-KH_2PO_4 mixtures. Incubated with shaking for 1 hr. at 25°. pH of incubation mixtures determined using glass electrode. Since the activities of the yeast suspensions used varied somewhat in different experiments, only the shapes and not the heights of the different curves are comparable.

Effect of temperature. Ester formation occurred readily over the range 19–30° with an optimum at 25–30°. There was a sharp drop in activity at 37° (Table 5).

Ester formation from other substrates. When incubated for 1 hr. under conditions optimal for ester formation from ethanol alone, ester formation could be shown from equimolar concentrations of glucose, but not from methanol, *n*-propanol, *iso*-propanol, *sec.*-butanol, choline, glycol, or glycerol. Traces, of doubtful significance, were found in presence of *n*-butanol, *iso*butanol, *n*-amyl alcohol, and benzyl alcohol. When added to the normal

ethanol substrate at pH 2·8 or 4·6, 0·01 M-acetaldehyde stimulated, whereas 0·2 M-acetaldehyde inhibited ester production. No appreciable ester was formed from acetaldehyde alone, aerobically or anaerobically at either pH.

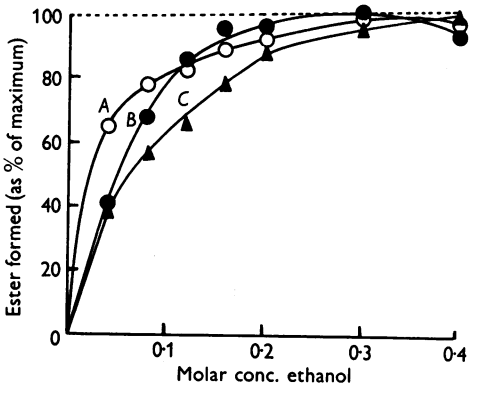

Fig. 2. Effect of ethanol concentration on ester formation by a washed suspension of *Hansenula anomala*. Incubation mixtures of total vol. 10·0 ml. contained: McIlvaine buffer (pH 2·5 or 4·6), 3·0 ml.; washed yeast, 60 mg. dry wt. (Exp. *A*, *C*) or 25 mg. dry wt. (Exp. *B*); acetate, 0·04 M (Exp. *C* only); ethanol as indicated. Incubated with shaking for 1 hr. at 25°. Exp. *A*: acetate absent, pH 2·8; Exp. *B*: acetate absent, pH 4·6; Exp. *C*: acetate present, pH 4·6.

Course of ester formation from ethanol alone. Fig. 4 shows the progress curve for ester formation from ethanol alone at an initial concentration of 0·2 M: the production is approximately linear for the first 2 hr.

Table 5. *Effect of temperature on ester formation by a washed suspension of* Hansenula anomala

(Duplicate mixtures were incubated with shaking for 1 hr. at the various temperatures. Incubation mixtures of total vol. 10·0 ml. contained: McIlvaine buffer, 3·0 ml.; ethanol, 0·2 M; acetate, 0·04 M (Exp. 1 only); washed yeast, 4·0 mg. dry weight/ml. (Exp. 1) or 4·5 mg. dry weight/ml. (Exp. 2).)

Temperature (°)	Ester formed (μmol./100 mg. dry wt. yeast) Exp. 1 (pH 4·6; substrate: ethanol + acetate)	Exp. 2 (pH 2·8; substrate: ethanol)
19	58	47
25	110	60
30	78	69
37	10	16

When smaller initial concentrations of ethanol were used in the presence of an adequate oxygen supply, ester was found to accumulate until the free alcohol had almost disappeared. Beyond this point the ester disappeared very rapidly (Fig. 5). This behaviour was later used to obtain a sample of the ester relatively free from ethanol.

Identification of the ester formed. The identification of the ester is based on indirect evidence and depends upon the separation and identification of the acid and alcohol moieties.

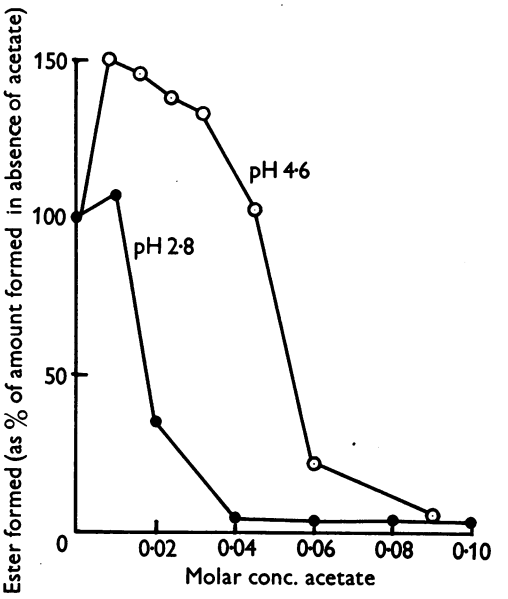

Fig. 3. Effect of acetate concentration on ester formation by a washed suspension of *Hansenula anomala*. Incubation mixtures of total vol. 10·0 ml. contained: McIlvaine buffer (pH 2·5 or 4·6), 3·0 ml.; washed yeast, 45 mg. dry wt. (Exp. at pH 2·8) or 80 mg. dry wt. (Exp. at pH 4·6); ethanol, 0·2 M; acetate as indicated. Incubated with shaking for 1 hr. at 25°.

The ester could not be readily separated from the unchanged free ethanol in the reaction mixture. Consequently it was desirable to stop the reaction at a point where the free ethanol concentration was as low as possible relative to the ester concentration. Under the conditions used the amount of ester present decreases rapidly when the free alcohol in the mixture has almost disappeared. In these circumstances, the relatively slow alcohol determinations were of little use for determining the optimum incubation time. The procedure adopted, therefore, was to follow the course of the reaction by ester determinations until the amount of ester present began to fall. At this point the reaction was stopped and the ester separated.

The reaction mixture contained in 200 ml. water: 1·32 g. dry weight of washed yeast; 60 ml. of McIlvaine buffer (pH 2·5); approximately 7 mmol. of ethanol. This mixture was contained in a 5 l. flask closed with a rubber bung and rotated in the apparatus described by Mitchell (1949). Samples were removed periodically and the ester present immediately determined. Total alcohol was also determined on some of the samples.

When the amount of ester present began to fall, excess powdered $CaCO_3$ and about 5 mg. of talc were added, and all but 30–40 ml. distilled, the receiver being cooled in ice water. Samples of the distillate were removed for alcoho land ester

determinations and then 10 ml. of 5N-NaOH added to the remainder which was left overnight to complete the hydrolysis of the ester. 10 ml. of 10% (w/v) $HgSO_4$ in 2N-H_2SO_4

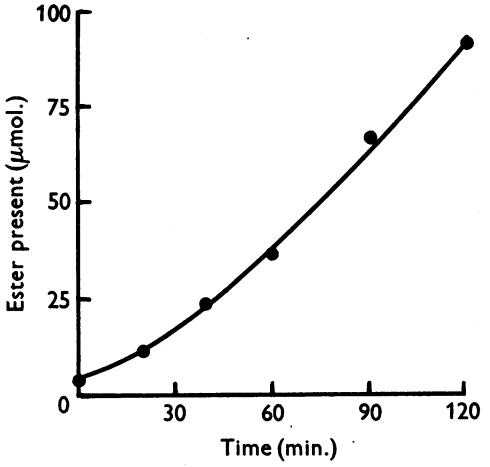

Fig. 4. Progress curve of ester formation by a washed suspension of *Hansenula anomala* in presence of excess ethanol. Duplicate incubation mixtures of total vol. 10·0 ml. contained: McIlvaine buffer (pH 2·5), 3·0 ml.; ethanol, 0·2M; washed yeast, 60 mg. dry weight. Incubated with shaking at 25°, one of the duplicate mixtures being used for ester determination at each of the times indicated.

and 20–30 ml. water were then added and the whole distilled, 100 ml. distillate being collected. This distillate (*A*), now free from aldehydes, ketones and formic acid, contained the alcohol moiety of the ester together with any residual ethanol.

To the residue, containing the Na salt of the volatile acid moiety of the ester, were added in turn, 100 ml. of water, about 5 mg. of talc, 50 g. of $MgSO_4.7H_2O$, 10 ml. of 20N-H_2SO_4, and a further 80 ml. of water with careful mixing. The whole was heated slowly to boiling and distilled to the point of crystallization. The distillate was diluted to 250 ml. and the acid present determined by titration of a 25 ml. sample. The remainder was neutralized, concentrated and examined chromatographically as described by Elsden (1946).

To the distillate *A* were added 2 g. of powdered $K_2Cr_2O_7$ and 10 ml. of 20N-H_2SO_4, and the mixture was heated for 30 min. under reflux in a boiling-water bath. After cooling, the condenser was washed down with 50 ml. of water, talc was added, followed by 50 g. of $MgSO_4.7H_2O$ and the whole distilled to the point of crystallization. The distillate was diluted to 250 ml. and the acid present determined by titration of a 25 ml. sample. The remainder was neutralized, concentrated and examined chromatographically. Table 6 shows the recovery of acids from the original distillate.

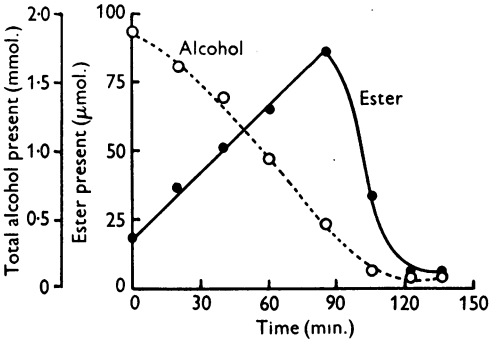

Fig. 5. Formation and disappearance of ester when a washed suspension of *Hansenula anomala* acts upon a small amount of ethanol under highly aerobic conditions. Incubation mixture of total vol. 60 ml. contained: McIlvaine buffer (pH 2·5), 18 ml.; ethanol, about 0·03M; washed yeast, 312 mg. dry weight. The mixture was incubated in a closed 5 l. flask, and rotated in the apparatus described by Mitchell (1949). Samples were removed periodically for determinations of ester and total alcohol.

Chromatographic examination showed that the acid moiety of the ester and the acid derived by oxidation of the free and ester-bound alcohol each gave rise to a single band on the chromatogram which was only slightly mobile in chloroform containing 1% (v/v) *n*-butanol, but could be washed down the column with chloroform containing 5% (v/v) *n*-butanol. When authentic acetic acid was added to each of the two derived acids and the mixtures tested on the chromatogram the behaviour was identical with that observed with the two derived acids alone.

The chromatographic evidence, considered in conjunction with the data on the recovery of acids from the hydrolysis of the ester and oxidation of the alcohols (Table 6), indicates that only one ester is formed and that this ester is ethyl acetate.

Table 6. *Recovery of volatile acids derived from the residual alcohol and the ester formed from ethanol by a washed suspension of* Hansenula anomala

(The incubated mixture was distilled and determinations of ester and total alcohol were carried out on the distillate. The volatile acids subsequently derived from the acid moiety of the ester, and from the residual alcohol together with the alcohol moiety of the ester were determined (see Text).)

Substance	Method of determination	Amount found (μmol.)
Ester after incubation	Conversion to hydroxamic acid	533
Ester-bound acid recovered	Titration with NaOH	530
Total alcohol after incubation	Oxidation by $K_2Cr_2O_7$	640
Acid recovered after oxidation of free and ester-bound alcohol	Titration with NaOH	625

DISCUSSION

The results obtained show that, as with growing cultures of the yeast, the amounts of ester formed by washed suspensions are too large to be accounted for by the reversal of a simple esterase reaction, indicating a more exergonic mechanism of ester formation. This is confirmed by the fact that, under anaerobic conditions, no ester is formed from ethanol plus acetate or from acetaldehyde, showing that oxidation is necessary for the synthesis of ester.

In the case of ester formation from (ethanol + acetate), there is no evidence that the acid moiety of the ester is derived from the added acetate. The marked stimulation of ester production followed by inhibition, occurring when the acetate concentration is increased at pH 4·6, may be due to an alteration of the external pH required to give optimal internal conditions for ester formation from ethanol alone. This explanation would also account for the effect of increasing the acetate concentration at pH 2·8 and the behaviour in presence of different buffers. The problem of the origin of the acetate moiety of the ester could be readily investigated by suitable isotope experiments.

It is possible that oxidation of ethanol may lead to the formation of a labile acetyl derivative (cf. Stadtman & Barker, 1949; Black, 1950) which then reacts with further ethanol to produce the ester. This hypothesis can only be tested by investigation of ester synthesis in a cell-free system. Attempts to prepare such a system are being made.

SUMMARY

1. Under aerobic conditions, washed suspensions of *Hansenula anomala* form ester from ethanol, alone or in presence of acetate.

2. No ester is formed in the absence of oxygen.

3. The ester formed is ethyl acetate.

4. The ester formed disappears after exhaustion of the ethanol substrate.

5. The effects of pH, age of culture, ethanol concentration and acetate concentration on ester formation have been investigated.

6. A number of alcohols have been tested but ester is formed at an appreciable rate only from ethanol or glucose. Acetaldehyde does not give rise to any ester, but in low concentrations acetaldehyde stimulates ester formation in presence of ethanol.

I wish to express thanks to my colleagues, in particular to Dr R. Davies and Dr E. F. Gale, for advice, criticism and encouragement during the course of this work. My thanks are also due to the Medical Research Council for a training grant, and to Trinity College, Cambridge, for an exhibition.

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Comparative Studies of 'Bile Salts'

2. PYTHOCHOLIC ACID

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It was shown in Part 1 (Haslewood & Wootton, 1950*b*) that the bile of three species of Boidae contained a new acid, $C_{24}H_{40}O_5$, then called by us pythocholic acid, which was easily converted into pythocholic lactone, $C_{24}H_{38}O_4$. In the African python at least, this acid, conjugated with taurine, formed the main constituent of the bile salts.

The work now described had two aims; to explore further how far pythocholic acid is characteristic of the bile of the Boidae, and to elucidate as far as possible the chemistry of the new acid.

A preliminary communication on this work has appeared (Haslewood & Wootton, 1950*a*).

RESULTS

Biological. Approximate figures for the content of pythocholic acid in the total (hydrolysed) 'bile acids' from the bile of seven species of Boidae are