and fish-liver oils for vitamin D, must be treated with reserve.

The experiments which have been described have shown that the treatment is ineffective in quantitatively removing vitamin A or, alternatively, in removing enough vitamin A to eliminate interference in the vitamin D determination. The procedure of Milas *et al.* (1941) completely destroys large amounts of vitamin D₃ and over 60 % of calciferol, even when large quantities of the latter are used. The safest solvent in which to carry out the condensation is ether, but unfortunately, although good recoveries of calciferol and vitamin D₃ have been obtained with this solvent, complete elimination of vitamin A is not possible within reasonable times of reaction. Condensation in benzene, as used by Pirlot & Rougir (1947), results in up to 20 % loss of calciferol and about 40 % loss of vitamin D₃.

The method of selective ultraviolet irradiation to remove vitamin A from vitamin D does not appear to be suitable for routine use. It is possible that, under carefully controlled conditions, small amounts of vitamin A (the bulk of which has been removed by other methods) can be successfully eliminated from vitamin D-containing material. Experiments have

shown that although vitamin A can be smoothly destroyed by filtered radiation in the 300–400 m μ . range, the irradiation, under similar conditions, of small amounts of vitamin D results in inevitable losses.

SUMMARY

1. The analytical purification of the vitamins D by the methods of maleic and citraconic anhydride condensation and by selective ultraviolet irradiation has been investigated.

2. The condensation method is not suitable for quantitative removal of vitamin A, and losses of vitamins D always occur in its use. Vitamin D destruction by the maleic anhydride-dioxan method is particularly severe.

3. Selective ultraviolet irradiation successfully destroys vitamin A, but the method cannot at present be used to separate vitamin A from vitamin D.

4. Methods for the analysis of vitamin D which include either of the above methods of purification are of doubtful value.

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Ester Formation by Yeasts

1. ETHYL ACETATE FORMATION BY *HANSENULA* SPECIES

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The ability to form traces of esters is a property of many yeasts and is partly responsible for the bouquet of wines; however, comparatively few species are able to form esters in considerable amounts. Prominent amongst these are the two species of *Hansenula*, *H. anomala* and *H. saturnus*. Very little in the way of quantitative work appears to have been done on ester formation by yeasts. Gomes (1945), in a study

of the microflora of the Douro valley, reported that a strain of *H. anomala* var. *sphaerica* (Naegeli) Dekker produced 9.59 g. total esters (as ethyl acetate) from 145 g. of total reducing sugars and at the same time ethanol formation was diminished. Gray (1949) isolated derivatives of acetaldehyde, ethanol and acetic acid from the products of the fermentation of glucose by *H. anomala* (Hansen)

Sydow, but his evidence that the ester formed by this organism is ethyl acetate is not conclusive. From the products of acid hydrolysis of the neutral fraction distilling at 55–70° he isolated crystalline derivatives of ethanol and acetic acid. The neutral distillate must undoubtedly have contained some free ethanol and therefore the isolation of ethanol derivatives does not prove conclusively that the ester was ethyl acetate. A study of the respiratory processes and dehydrogenases, particularly the ethanol dehydrogenase, of *H. anomala* was made by Usami (1942), but did not include any work on ester formation.

It was considered possible that *Hansenula* species might afford suitable material for studying the mechanism of ester formation in biological systems and the factors associated therewith. This paper deals with the qualitative and quantitative analysis of the products of the metabolism of glucose by *H. anomala* and *H. saturnus*.

EXPERIMENTAL

Organism

The strains of *H. anomala* and *H. saturnus* used in this work were obtained from the Carlsberg Laboratory, Copenhagen, through the kindness of Prof. O. Winge, and are nos. 119 and 56 respectively in the Carlsberg collection of yeasts.

Growth

The growth medium contained 4% (w/v) glucose (sterilized separately), 0.1% $\text{NH}_4\text{H}_2\text{PO}_4$, 0.1% $(\text{NH}_4)_2\text{HPO}_4$, 0.2% KH_2PO_4 , 0.2% Difco yeast extract and 10 ml./l. of a trace-element solution containing 2% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% NaCl , 0.05% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% $\text{MnSO}_4 \cdot 3\text{H}_2\text{O}$, 0.005% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1% (v/v) 0.1N- H_2SO_4 . For quantitative work the yeast was grown on 100 ml. of medium in a 1 l. Erlenmeyer flask fitted with a ground-glass joint and connected to a gas holder filled with oxygen, through (a) a sterilized cotton-wool filter, (b) a mercury seal, (c) a wash bottle containing 20% (w/v) aqueous KOH, (d) a second sterilized cotton-wool filter connecting with the gas holder. The culture vessel contained a 6 x 1 in. test tube holding 25 ml. 60% (w/v) aqueous KOH to absorb the CO_2 produced during the growth of the yeast. As the O_2 of the culture vessel was used up fresh O_2 was automatically drawn in from the gas holder and the composition of the gas phase in the culture vessel was therefore maintained constant during growth.

In some experiments the yeast was grown anaerobically, in which cases the culture vessel, after inoculation with the yeast, was flushed out with a rapid stream of N_2 for 10 min. and then closed with a glass stopper.

The temperature of incubation was 25° and at the end of the incubation period the yeast was filtered off through a sintered-glass filter (Pyrex, porosity 3), dried at 100° and weighed.

Analytical methods

Carbon dioxide produced during growth. This was determined by titrating the KOH in the boiling tube against

standard acid using phenolphthalein and methyl orange successively as indicators.

Glucose. The culture filtrate was boiled with 1% of Actibon charcoal (Hopkins & Williams) for 30 sec., allowed to stand for 10 min. and filtered. Glucose in the filtrate was estimated by the method of Shaffer & Somogyi (1933).

Volatile fatty acids were estimated either by the method of Friedemann (1938) or by distillation in the apparatus of Markham (1942) followed by titration in a CO_2 -free atmosphere using phenol red as indicator.

Acetaldehyde was estimated by direct distillation into NaHSO_3 solution in the lactic acid apparatus of Friedemann, Cotonio & Shaffer (1927).

Glycerol was estimated using the procedure described by Platt & Glock (1943) for the estimation of inositol. A longer period of incubation was found to be necessary to ensure complete removal of the glucose (2.5–3 hr.). The results are only very approximate because of the rather uncertain correction which must be made for glycerol produced during the removal of the residual glucose.

Acetic acid, ethanol and ethyl acetate in mixtures. The estimation of these substances in mixtures containing all three was based on the methods described by Friedemann (1938) for the estimation of volatile alcohols and fatty acids. Direct application of these methods is not possible because they would involve hydrolysis of the ester. *Free acetic acid:* to 2 g. CaCO_3 were added 20 ml. of the culture filtrate and 100 ml. water. The mixture was gently warmed under reflux for 5 min. to complete the formation of calcium acetate and 100 ml. was then distilled into a receiver cooled in ice (distillate A). The residue was thoroughly cooled, 180 ml. water, 50 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 ml. 10% (w/v) HgSO_4 in 2N- H_2SO_4 were added followed by 2 ml. 20N- H_2SO_4 and the mixture distilled. Distillate (200 ml.) was collected and titrated against standard alkali in a CO_2 -free atmosphere using phenol red as indicator. *Ethyl acetate:* distillate A contained ethyl acetate and free ethanol. To A was added 10 ml. 20N- H_2SO_4 and the mixture refluxed for 30 min. over an open flame. It was then thoroughly cooled, the condenser washed down with 90 ml. water, 50 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 ml. 10% (w/v) HgSO_4 in 2N- H_2SO_4 added and the mixture distilled. Distillate (200 ml.) was collected and the acid titrated as above. This is the acid component of the ester and is therefore equivalent in amount to the ester present. *Ethanol* was estimated as the difference between the total alcohol as determined by the method of Friedemann and the alcohol present as ester. Table 1 is representative of the results obtained in this way with known mixtures of acetic acid, ethanol and ethyl acetate. Acetaldehyde at a concentration of 1 mg./ml. did not interfere with these estimations.

Table 1. *Analysis of a known mixture of acetic acid, ethanol and ethyl acetate*

(For methods used see text.)

	In mixture (mg./ml.)	Found (mg./ml.)	Recovery (%)
Acetic acid	1.27	1.16	91
Ethanol	3.95	3.67	93
Ethyl acetate	0.90	0.80	89

Carbon built into cell substance. Determinations of total carbon in *H. anomala* grown for varying lengths of time were made using the wet ashing method of Van Slyke & Folch (1940). Values of 47.2 and 47.3% were obtained for cells

harvested after 72 and 360 hr. The carbon built into cell substance was therefore obtained by multiplying the dry weight of yeast by 0.47.

Preparation of 3:5-dinitrobenzoates from alcohols. The method is a modification of that described by Reichstein (1926).

Reagents. (1) Pure anhydrous pyridine prepared by distillation over alkaline permanganate followed by distillation over anhydrous BaO. (2) Crystallizable benzene stored over fused CaCl₂. (3) Ether stored over Na. (4) 3:5-Dinitrobenzoyl chloride freshly prepared from 3:5-dinitrobenzoic acid and PCl₅. Carefully heat a mixture of equal weights of the acid and PCl₅ until all PCl₅ has been driven off. Dissolve the residue in the dry benzene and filter through a glass filter (porosity 1); prepare just enough for immediate use.

Method. To 1 mol. of the alcohol dissolved in a little dry benzene add 1.5 mol. of 3:5-dinitrobenzoyl chloride as a 7.5% (w/v) solution in dry benzene. Mix well and add 3 mol. of anhydrous pyridine slowly and with vigorous shaking. The mixture is kept overnight at room temperature and is then diluted with 4 vol. of dry, alcohol-free ether and extracted once with 2N-HCl, the extract being discarded. It is then extracted repeatedly (at least four times) with equal volumes of 2N-NaOH until the white precipitate is dissolved, the extracts again being discarded. It is extracted once more with 2N-HCl and washed four times with water, ether being added from time to time to keep up the volume. Finally the solution is evaporated to dryness *in vacuo* and the product recrystallized from 60% (v/v) aqueous ethanol, washed with a large volume of water and dried over P₂O₅. Starting with 160 mg. of ethanol a final yield of 75% of the theoretical was obtained. The method gave no reaction with ethyl acetate that had been stored over fused CaCl₂.

Identification of products of metabolism of glucose

Cultures of *H. anomala* grown aerobically for 72 hr. were filtered through Whatman no. 2 paper and the clear filtrate was used for the work described below.

Acetaldehyde. The culture filtrate (3 l.) was distilled through a 50 cm. fractionating column fitted with a reflux head and the first 5 ml. of distillate were collected in 25 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl. This fraction had distilled below 30°. The precipitate which formed was washed successively with N-HCl, saturated aqueous Na₂CO₃, water, N-HCl and finally water. It was recrystallized from 80% (v/v) aqueous ethanol (orange plates) and dried over P₂O₅ *in vacuo* for several days. M.p. 157–160° (uncorr.). The mixed m.p. with a sample of acetaldehyde 2:4-dinitrophenylhydrazine (m.p. 155–158°) prepared from freshly distilled acetaldehyde was 155–157°. (Found: C, 43.1; H, 3.7; N, 24.7. Calc. for C₈H₈N₄O₄: C, 42.8; H, 3.6; N, 25.0%.)

Acetic acid. The culture filtrate (12 l.) was adjusted to pH 8.0 with 15N-NaOH and evaporated *in vacuo* to 400 ml. Water (500 ml.) was added followed by 10N-HCl until the solution was red to thymol blue (pH 1–2). The solution was distilled to dryness *in vacuo*, the distillate being collected in NaOH solution. The distillate was brought to pH 8.0 with H₂SO₄ and evaporated to dryness on a boiling-water bath. The dry residue was moistened with water, excess anhydrous KHSO₄ added and the mass extracted with ether four times. The ether extracts were pooled and titrated with N-NaOH using phenol red as indicator. The neutral solution was

diluted to 100 ml. and examined for volatile fatty acids on silica gel columns as described by Elsdon (1946) and by Moyle, Baldwin & Scarisbrick (1948). Only one band was obtained, in the position corresponding to acetic acid. The addition of acetic acid to the unknown acid again resulted in only one band in the acetic acid position, whereas the addition of propionic acid gave rise to a second, faster moving band. No evidence was obtained, therefore, for the presence of any volatile fatty acid other than acetic acid in the culture filtrate.

A portion of the neutralized volatile acid solution was acidified, distilled and the distillate shaken with excess AgCO₃. The mixture was filtered, evaporated to small bulk and left at 0° to crystallize. The product was recrystallized twice from water and dried over P₂O₅ *in vacuo*. It crystallized as shining white plates. (Found: C, 14.9; H, 1.9; Ag, 64.2. Calc. for C₂H₃O₂Ag: C, 14.4; H, 1.8; Ag, 64.7%.)

Ethyl acetate and ethanol. It is extremely difficult to separate ethyl acetate and ethanol from each other in aqueous solution on a small scale because of the formation of azeotropic mixtures. Therefore identification of ethyl acetate has been done by indirect methods. It has been shown that the acid component of the ester-alcohol mixture is exclusively acetic acid and that the alcohol component is exclusively ethanol. It therefore follows that the ester must be ethyl acetate.

Culture filtrate (3 l.) was adjusted to pH 8.0 with aqueous NaOH and 300 ml. were distilled through a fractionating column as described previously, the first 5 ml. being discarded. The neutral distillate was refractionated twice, in the final distillation the fraction distilling below 81° being collected (distillate B). A portion of B was dried over anhydrous BaO and redistilled. This material (0.2 ml.) was esterified with 3:5-dinitrobenzoyl chloride as described above. The product had m.p. 93–93.7° (uncorr.). The mixed m.p. with an authentic sample of ethyl 3:5-dinitrobenzoate (m.p. 93–94°) was 93–93.5°.

Analysis of B showed it to consist of 30% free ethanol, 60% ester and 10% water. A portion of B was hydrolysed by heating at 100° for 30 min. in 0.25N-NaOH and half of the mixture was distilled off (distillate C). The residue was neutralized and distilled from HgSO₄ and H₂SO₄ as described by Friedemann for the estimation of volatile acids. The distillate was neutralized and examined for fatty acids on the silica gel chromatogram. Only one band was observed, corresponding to acetic acid.

C, which contains the alcohols liberated from ester combination as well as those originally uncombined, was oxidized with K₂Cr₂O₇ as described by Friedemann. The volatile acids produced were recovered by distillation and examined on the silica gel chromatogram. Again only one band was observed, corresponding to acetic acid.

These results indicate that the ester produced when *H. anomala* grows on glucose is very largely ethyl acetate. The presence of other esters in traces cannot be excluded, although none was detected.

A small amount of unidentified non-volatile, ether-soluble acid was formed in an amount equivalent to about 2 ml. of 0.1N-NaOH/mol. glucose metabolized. It gave no reaction with succinic dehydrogenase and no tests for pyruvic or lactic acids, but was not further investigated. Neither pyruvic acid nor lactic acid could be detected in the culture filtrates by a variety of colour tests nor, in the case of pyruvic acid, by the use of carboxylase.

Table 2. *Products of the aerobic and anaerobic growth of Hansenula species on a yeast extract-salts-4 % (w/v) glucose medium at 25°*

	mmol. product/100 mmol. glucose utilized				
	<i>H. anomala</i>		<i>H. saturnus</i>		
	Aerobic 3 days	Anaerobic 4 days	Aerobic		Anaerobic 6 days
Residual glucose	22.0	2.8	14.3	3.3	29.0
CO ₂	237.0	176.0	291.0	338.0	220.0
Ethyl acetate	25.1	1.1	3.6	4.2	1.4
Ethanol	19.0	87.0	13.1	2.3	124.0
Acetic acid	3.2	2.9	1.2	0.4	1.0
Acetaldehyde	0.05	0.23	0.1	0.08	0.36
Glycerol	2.7	5.6	7.0	8.5	10.6
Cell carbon (mg. atoms)	95.0	32.0	137.0	209.0	41.0
Percentage glucose carbon accounted for	81	67	84	92	92

Quantitative analysis of the cultures

The products of the aerobic and anaerobic growth of *H. anomala* and *H. saturnus* on a glucose-yeast extract-salts medium are shown in Table 2.

The dependence of ethyl acetate formation on aerobic growth conditions is clearly shown in the case of *H. anomala*. *H. saturnus* appears to possess a much more active oxidation system than *H. anomala*, as is shown by the smaller amounts of ester and ethanol and the larger amounts of CO₂ and cell carbon that are formed aerobically. Anaerobically the main product with both yeasts is ethanol and there is a much smaller proportion of glucose carbon built into cell substance.

SUMMARY

1. Ethyl acetate, ethanol, acetic acid and acetaldehyde have been identified as products

of the metabolism of glucose by *Hansenula anomala*.

2. *H. anomala* produces about 25 mmol. ethyl acetate/100 mmol. glucose utilized under aerobic growth conditions.

3. *H. saturnus* produces more carbon dioxide and cell substance and less ethyl acetate and ethanol than does *H. anomala* under similar growth conditions.

4. The formation of ethyl acetate is an aerobic process.

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