The Mechanism of the Formation of Higher Alcohols from Amino Acids by *Saccharomyces cerevisiae*

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Recent studies (SentheShanmuganathan, 1956; SentheShanmuganathan & Elsden, 1958) of the formation of tyrosol, one of the constituents of fusel oil, have established that in *Saccharomyces cerevisiae* the conversion of tyrosine into tyrosol is effected by the following reactions.

Tyrosine + 2-oxoglutarate →
p-Hydroxyphenylpyruvate + glutamic acid (1)

p-Hydroxyphenylpyruvate →
p-Hydroxyphenylacetaldehyde + CO_2 (2)

p-Hydroxyphenylacetaldehyde + reduced DPN →
p-Hydroxyphenylethanol (tyrosol)
+ oxidized DPN (3)

In reaction (1), the removal of the amino group is effected by transamination; 2-oxoglutarate was found to be essential for this step. This is followed by decarboxylation of the keto acid (reaction 2) to yield the aldehyde, which is reduced in the third stage to give the alcohol.

In the first investigation, tyrosine was chosen as a substrate because of the relative ease with which the intermediates and the product could be estimated (SentheShanmuganathan & Elsden, 1958). Many other amino acids give rise to fusel oils, and, having established the pathway of tyrosol formation, it was desirable to extend the work in order to decide whether other fusel oils were formed by a similar mechanism. This paper describes these investigations.

In experiments with growing cultures and whole cells, where glucose is an essential component, ethanol was always formed in addition to the alcohol corresponding to the amino acid added as substrate. The presence of ethanol made the estimation of other alcohols difficult. The cell-free extracts used would not reduce added aldehyde in the absence of a hydrogen donor and the amino acid is only converted as far as the corresponding aldehyde containing one less carbon atom than the parent compound. Moreover, there is no formation of ethanol by unrelated reactions or by endogenous metabolism. Consequently, the work reported in this paper has been done with cell-free extracts.

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Since the first reaction in the formation of fusel oil was found to be catalysed by transaminase it was thought desirable to study some of the properties of the transaminase with leucine as the substrate. Although transamination reactions have been studied extensively with preparations from animal tissues (Braunstein & Kritzman, 1937; Feldman & Gunsalus, 1950; Cammarata & Cohen, 1950; Rowsell, 1956*a*, *b*; Canellakis & Cohen, 1956), very little work has been done with yeast. Thus transamination reactions have been demonstrated only in three species of yeasts: *Torulopsis utilis* (Roine, 1947), *Saccharomyces fragilis* (Bigger-Gehring, 1955) and brewer's yeast (Holzer, Gerlach, Jacobi & Gnoth, 1958).

MATERIALS

Organism. The organism used throughout this work was *Saccharomyces cerevisiae* (strain 5, isolated in this Department). The method of growing the cells and the preparation of cell-free extracts have been described previously (SentheShanmuganathan & Elsden, 1958).

Amino acids. All the amino acids used (L. Light and Co. Ltd., Colnbrook, Bucks) were checked for purity by paper chromatography (Woiwod, 1949). Solutions of the amino acids were adjusted to pH 6·2 or 7·2 with 0·2 N-NaOH before use.

2-Oxo acids. The 2-oxo derivatives of caproic acid, *iso*caproic acid, valeric acid and *iso*valeric acid were prepared from their corresponding DL-amino acids by the D-amino acid oxidase method of Meister (1952). 2-Oxoglutarate was prepared by the method of Clutterbuck (1927). Oxaloacetate was a gift from Professor Sir Hans Krebs, F.R.S.; the sodium salt was prepared from it as described by Elsden & Ormerod (1956). Phenylpyruvate was obtained from L. Light and Co. Ltd. *p*-Hydroxyphenylpyruvate was prepared as described earlier (SentheShanmuganathan & Elsden, 1958). Indolylpyruvic acid was prepared from indole. Indole-3-aldehyde was synthesized as described by Boyd & Robson (1935). The azlactone was prepared from the aldehyde by condensing it with hippuric acid (Ellinger & Matsuoka, 1920) and the product hydrolysed with NaOH. The hydrolysate was poured into ten times its volume of ice-cold water and the solid precipitate filtered off. The supernatant was acidified and extracted with ether. After drying with anhydrous Na_2SO_4, the ether was evaporated under reduced pressure at room temperature, leaving a brownish white solid. The indolylpyruvic acid was separated from benzoic acid by extraction of the latter with light petroleum

(b.p. 60–80°). The crude product was purified and crystallized as described by Woods (1935).

Aldehydes. Butyric, *iso*butyric, valeric and *iso*valeric aldehydes (L. Light and Co. Ltd.) were distilled just before use. Aqueous solutions of the aldehydes were standardized by the $NaHSO_3$-binding method.

Alcohols. Pentan-1-ol, 3-methylbutan-1-ol, butanol and 2-methylpropan-1-ol (L. Light and Co. Ltd.) were distilled just before use.

Other compounds. Hexose diphosphate was prepared as described by SentheShanmuganathan & Elsden (1958). Diphosphopyridine nucleotide (DPN) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.) and crystalline yeast alcohol dehydrogenase from Boehringer und Söhne Mannheim, Germany. Reduced DPN was prepared from DPN by the alcohol dehydrogenase method of Pullmann, Colowick & Kaplan (1952). Pyridoxal phosphate was the gift of Dr A. L. Morrison of Roche Products Ltd., Welwyn Garden City, Herts.

METHODS

Protein. Protein contents of preparations were determined by the turbidimetric method of Stadtman, Novelli & Lipmann (1951). It was assumed that a solution containing 0·1 mg. of protein/ml. gave an extinction of 0·1 at 340 mμ in the Unicam SP. 500 spectrophotometer.

Glutamic acid. The incubation mixtures were deproteinized and centrifuged. The supernatant was brought to pH 4·5 with 2N-acetic acid and the glutamate content estimated by the decarboxylase method of Gale (1945) with Warburg cups of approx. 10 ml. capacity.

2-Oxo acids. 2-Oxoglutarate was estimated by the transaminase method of Krebs (1950). Aliphatic 2-oxo acids were determined by yeast carboxylase (Meister, 1952) and *p*-hydroxyphenylpyruvate by the colorimetric method of Briggs (1922) as modified by Canellakis & Cohen (1956).

Qualitative test for glutamate and other amino acids. For this purpose the incubation mixtures were inactivated by heating in a boiling-water bath for 10 min. The denatured proteins were centrifuged off and equal volumes of the supernatant placed on paper chromatograms. The procedure of Woiwod (1949) was adopted for the separation of the amino acids. After the papers had been dried, the positions of the amino acids were located by spraying with 0·1% (w/v) ninhydrin in acetone (Dent, 1947).

Preparation of extracts for assay of transaminase activity. Washed whole cells were prepared (SentheShanmuganathan & Elsden, 1958) and crushed in the bacterial press (Hughes, 1951). The crushed mass was extracted with 12 ml. of 0·1M-phosphate buffer (0·072M-Na_2HPO_4–0·028M-KH_2PO_4), pH 7·2/g. dry wt. of cells (dry weight was determined turbidimetrically by using a Hilger Spekker absorptiometer with a neutral-grey Ilford filter no. H. 508) for 1 hr. at 37° and centrifuging at 19 600 *g* for 20 min. at −2°. The insoluble material (P) was resuspended in an equal volume of the same buffer for testing transaminase activity. The supernatants (pH 6·5–6·7) were brought to pH 7·2 with 0·2N-NaOH and dialysed against 0·02M-KCl at 1–2° for 16–18 hr. with continuous stirring to remove glutamate and other amino acids. The dialysed extract and the precipitate were tested for transaminase activity between 2-oxoglutarate and tyrosine or leucine; the precipitate was inactive. Such a dialysed extract contained 28 mg. of protein/ml.

Assay of transaminase activity. The formation of glutamate from 2-oxoglutarate and the appropriate amino acid was used as an index of transaminase activity.

Unless otherwise stated, incubations were in duplicate at 25° in test tubes which contained, in a total volume of 3 ml.: pyridoxal phosphate (0·04 μmole), enzyme preparation, 2-oxoglutarate, amino acid and 2 ml. of phosphate buffer, pH 7·2 [see Tables and Figures for concentrations]. The mixture was incubated for 10 min. at 25° and the reaction then started by addition of the amino acid (Canellakis & Cohen, 1956). Two controls, one without amino acid and the other without 2-oxoglutarate, were included in all experiments; no detectable glutamic acid was formed in either of the controls.

At the end of the incubation period 0·2 ml. of 50% trichloroacetic acid was added, the tubes were plunged into a boiling-water bath for 3 min. and the denatured proteins separated by centrifuging.

Manometry. When gas exchanges were measured, reaction mixtures were incubated in Warburg manometers with double side bulbs by conventional techniques. Reactions were initiated by the addition of the cell-free extracts from the side bulb. Unless stated otherwise, all manometric experiments were carried out at 25° in phosphate buffer, pH 6·2, in air. The reaction was stopped by the addition of 0·5 ml. of 10% trichloroacetic acid from the second side bulb. This served to liberate the bound CO_2, to inactivate the enzymes and to precipitate the proteins. The mixture was then centrifuged and the supernatant used for analysis.

RESULTS

When leucine, norleucine, valine or norvaline and 2-oxoglutarate were incubated with cell-free extracts reinforced with pyridoxal phosphate, CO_2

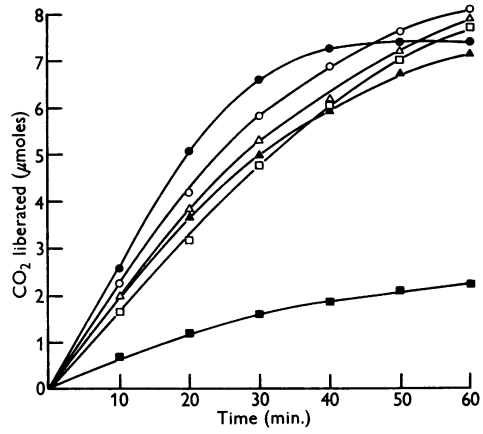

Fig. 1. Time course of CO_2 liberation in the system amino acid plus 2-oxoglutarate by cell-free extracts. Experiments were carried out in Warburg flasks with double side bulbs. The main compartment contained L-amino acid (10 μmoles), 2-oxoglutarate (100 μmoles), pyridoxal phosphate (0·01 μmole) and water to 1·7 ml. Reaction was initiated by the addition of extract (1 ml.) from one of the side bulbs. Gas phase, air; temp. 25°. ○, Valine; △, norvaline; □, norleucine; ●, *iso*leucine; ▲, leucine; ■, no amino acid added.

was liberated in excess of the amount formed in the absence of the amino acid (Fig. 1). When pyridoxal phosphate was omitted, the rate of CO_2 liberation decreased. At the pH used (6·2) there is some retention of CO_2, and the total CO_2 produced was obtained by the addition of acid at the end of the experiment. Since CO_2 retention was small, the rate of gas formation was used as a rough index of the progress of the reaction. 2-Oxoglutarate could not be replaced by either pyruvate or oxaloacetate.

Products of the reaction. The production of CO_2 which occurred when amino acids were incubated with the extract, 2-oxoglutarate and pyridoxal phosphate suggested that transamination followed by decarboxylation of the resulting 2-oxo acid to yield the aldehyde was occurring. This was tested by analysing the reaction mixture for glutamate and aldehyde at the end of the experiment. An approximate estimate of the amount of aldehyde was obtained by steam-distillation into thiosemicarbazide, and the thiosemicarbazenes formed were determined by their absorption at 260 mμ. Although recoveries with pure aldehydes or with aldehydes produced from 2-oxo acids by the action of purified yeast carboxylase were quantitative, recoveries of aldehydes added to reaction mixtures were only in the order of 80 % (Table 1). The CO_2 production in the absence of added amino acid was probably due to the slow decarboxylation of 2-oxoglutarate by the cell-free extract. Table 2 shows that the CO_2 produced, corrected for that of the control, was equivalent to the glutamate formed. The recoveries of the aldehydes were, as expected, low.

In order to confirm that aldehydes were in fact produced in the reaction, the aldehyde formed when leucine was incubated with 2-oxoglutarate and pyridoxal phosphate was isolated as the 2:4-dinitrophenylhydrazone. The procedure was as follows. To 100 ml. of the extract in 0·1 M-phosphate buffer, pH 6·2, and containing 0·5 μmole of pyridoxal phosphate, was added 50 ml. of the substrate mixture containing 2 m-moles of DL-leucine and 10 m-moles of 2-oxoglutarate (pH of the substrate mixtures was preadjusted to 6·2 with dilute NaOH soln.). A sample (3 ml.) of the incubation mixture was transferred to a Warburg manometer. The incubation was carried out at 25° for 5 hr., by which time gas output in the manometer had ceased.

At the end of the incubation period the pH of the mixture was adjusted to 1·0 with 5 N-HCl and the precipitated proteins were separated by centrifuging at 0° for 10 min. at 19 600 *g*. The supernatant was adjusted to pH 9·0 with dilute sodium hydroxide soln. to prevent distillation of succinic semialdehyde, and steam-distilled. The distillate (50 ml.) was collected in a saturated solution of 2:4-dinitrophenylhydrazine (50 ml.) in 2 N-HCl, the receiver being kept in an ice bath. The mixture was left at 0° overnight. The derivative was purified as described earlier for *p*-hydroxyphenylacetaldehyde (SentheShanmuganathan & Elsden, 1958). The melting point of the compound was 122–124° and the mixed melting point with an authentic sample of *iso*valeraldehyde 2:4-dinitrophenylhydrazone (m.p. 122–123°) was 121–123° (Found: C, 49·5; H, 5·56; N, 21·12. $C_{11}H_{14}O_4N_4$ requires C, 49·6; H, 5·26; N, 21·05 %).

The control experiment which contained no DL-leucine gave no precipitate with 2:4-dinitrophenylhydrazine under these conditions.

Tests with 2-oxo acids. If transamination is the first step in the conversion of the amino acids into their corresponding alcohols, the intermediates will be the corresponding 2-oxo acids and aldehydes containing one less carbon atom than the parent amino acid. 2-Oxocaproic acid, 2-oxo*iso*caproic acid, 2-oxovaleric acid and 2-oxo*iso*valeric acid, which would be expected to arise from norleucine, leucine, norvaline and valine respectively, were tested and all were found to give CO_2 and aldehyde with extracts at pH 6·2 and at 25° (Table 3).

Purified carboxylase prepared from *S. cerevisiae* (strain 5) by the method of Green, Herbert & Subrahmanyan (1941) also decarboxylated these

Table 1. *Effect of interfering substances present in the experimental system on the recovery of butyraldehyde*

The experimental system contained, in a total volume of 10 ml.: 3·59 μmoles of butyraldehyde, 2ml. of extract or 160 μmoles of 2-oxoglutarate or both at pH 6·2. Incubated at 25°; gas phase, air. Samples were withdrawn at intervals of 30 min., deproteinized with trichloroacetic acid and stored at 0° until required. After adjustment to pH 9·0 with 0·1 N-NaOH the aldehyde was steam-distilled in a Markham apparatus. The distillate (50 ml.) was collected in a tube containing 10 μmoles of thiosemicarbazide in 2 ml. of 0·5 M-acetate buffer, pH 4·0. After standing for 30 min. at 37° the thiosemicarbazones were determined by their absorption at 260 mμ.

	Extract		2-Oxoglutarate		Extract + 2-oxoglutarate	
Time (min.)	Aldehyde found (μmoles)	Recovery (%)	Aldehyde found (μmoles)	Recovery (%)	Aldehyde found (μmoles)	Recovery (%)
0	2·3	64	3·59	100	3·18	88
30	2·3	64	3·00	83	3·12	87
60	1·9	52	2·64	73	2·37	66

four 2-oxo acids almost quantitatively (Table 4) to yield the aldehyde and CO_2. The aldehydes produced in these experiments were also estimated by the thiosemicarbazone method. The carboxylase of the strain of *S. cerevisiae* used in these experiments had a specific activity of 6·1 units/mg. of protein [1 unit: that amount of enzyme required to catalyse the liberation of 100 μl. of CO_2 in 3 min. under the conditions specified by Green *et al.* (1941)]. *p*-Hydroxyphenylpyruvic acid was not decarboxylated by the purified enzyme when 0·694 mg. of protein (4·2 units of activity) was present. However, when 4 mg. of protein (24·4 units of activity) was used, *p*-hydroxyphenylpyruvic acid was

Table 2. *Products of the reaction from amino acids plus 2-oxoglutarate by undialysed cell-free extract*

All experiments were carried out in Warburg vessels with double side bulbs. The main compartment contained L-amino acid (10 μmoles), 2-oxoglutarate (100 μmoles), pyridoxal phosphate (0·01 μmole). One of the side bulbs contained extract (1 ml.) in 0·1 M-phosphate buffer, pH 6·2; the second side bulb contained 0·5 ml. of 10% trichloroacetic acid. Total vol., 3 ml. Incubation period, 60 min. Gas phase, air; temp. 25°.

Amino acid	Initial rate of CO_2 liberation (μmoles/10 min.)	Total CO_2 liberated (μmoles)	Glutamic acid formed (μmoles)	Aldehyde recovered (μmoles)
None	0·75	2·4	2·1	0·9
Leucine	2·00	12·6	12·0	6·3
Isoleucine	2·55	10·6	9·9	5·5
Norleucine	1·75	13·4	12·9	7·5
Valine	2·25	11·3	10·5	5·0
Norvaline	2·00	9·7	9·5	4·3
Alanine	0·75	2·4	2·1	—

Table 3. *Decarboxylation of 2-oxo acids by undialysed cell-free extracts of* Saccharomyces cerevisiae *and products of the decarboxylation*

All incubations were carried out in Warburg vessels with double side bulbs until CO_2 evolution ceased. The main vessel contained the 2-oxo acid at pH 6·2, one of the side bulbs contained 1 ml. of extract in 0·1 M-phosphate buffer, pH 6·2, the other contained 0·5 ml. of 10% trichloroacetic acid. Total volume, 3 ml. Temp. 25°; gas phase, air; incubation period, 30 min.

2-Oxo acid	Substrate added (μmoles)	Initial rate of CO_2 liberated (μmoles/3 min.)	Total CO_2 liberated (μmoles)	Recovery of aldehyde (μmoles)
None	—	0·30	0·73	0·90
Pyruvic acid	6·5	3·8	6·72	4·9
2-Oxovaleric acid	11·65	5·8	11·70	6·1
2-Oxo*iso*valeric acid	12·0	3·8	7·94	5·0
2-Oxocaproic acid	12·5	5·8	12·24	6·3
2-Oxo*iso*caproic acid	13·65	3·8	11·60	7·8
p-Hydroxyphenylpyruvic acid*	10·0	0·31	5·23	—

* The incubations were carried out for 60 min.

Table 4. *Decarboxylation of 2-oxo acids by purified carboxylase and estimation of aldehydes by the thiosemicarbazone method*

Incubations were carried out in Warburg vessels with double side bulbs. The purified carboxylase [6·1 units of activity/mg., cf. Green, Herbert & Subrahmanyan (1941)] equivalent to 0·694 mg. of protein, was used for the test from Expts. 1–7. The 2-oxo acid was kept in the main vessel in citrate buffer, pH 6·0, and the carboxylase was in the side bulb in the same buffer, pH 6·0. Gas phase, air; temp. 25°; incubation period, 30 min.

Expt. no.	2-Oxo acid	Substrate added (μmoles)	Initial rate of CO_2 liberated (μmoles/3 min.)	Total CO_2 liberated (μmoles)	Aldehyde recovered (μmoles)
1	None	—	0	0	0·9
2	Pyruvic acid	16·0	5·6	16·2	—
3	2-Oxovaleric acid	12·5	4·1	12·4	11·9
4	2-Oxo*iso*valeric acid	12·5	2·5	11·8	11·0
5	2-Oxocaproic acid	15·0	2·8	13·4	13·0
6	2-Oxo*iso*caproic acid	12·5	2·0	11·4	11·4
7	*p*-Hydroxyphenylpyruvic acid	10·0	0	0	—
8	*p*-Hydroxyphenylpyruvate*	10·0	1·2	4·8	—

* In Expt. no. 8, the amount of carboxylase used was equivalent to 4 mg. of protein; incubation period, 60 min.

decarboxylated (Table 4) slowly. The purified carboxylase obtained by Green *et al.* (1941) and the cell-free preparation of the yeast obtained by Meister (1952) also did not catalyse the decarboxylation of this keto acid.

Reduction of the aldehydes by reduced diphosphopyridine nucleotide. The oxidation of reduced DPN by dialysed extract in the presence of butyric, *iso*butyric, valeric and *iso*valeric aldehydes at pH 8·8 was investigated spectrophotometrically. The reduced pyridine nucleotide was oxidized in all cases but the rate of oxidation decreased with increasing length of the carbon chain of the aldehyde (Fig. 2). Similar observations were made with *p*-hydroxyphenylacetaldehyde (SentheShanmuganathan & Elsden, 1958). The reverse reaction, reduction of DPN in the presence of the corresponding alcohols, was also demonstrated.

The oxidation of reduced DPN by the aldehydes in the presence of crystalline yeast alcohol dehydrogenase was also investigated (Fig. 3). It was found that the rate of oxidation of reduced DPN (as measured by the change in extinction at 340 mμ/min.) by butyric aldehyde was about seven, four and two and a half times that of *iso*butyraldehyde, *iso*valeraldehyde and valeraldehyde respectively. *p*-Hydroxyphenylacetaldehyde was also found to be capable of oxidizing reduced DPN in the presence of the crystalline enzyme.

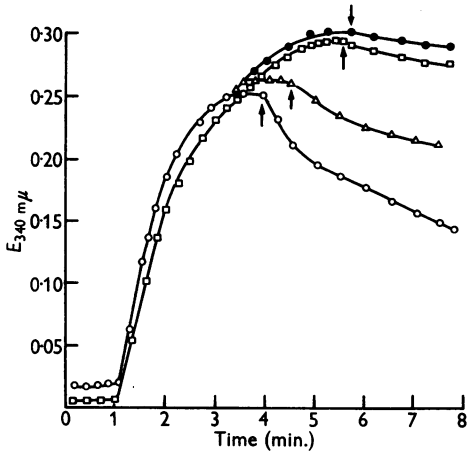

Fig. 2. Oxidation of reduced DPN by aliphatic aldehydes by crude cell-free extracts of *S. cerevisiae*. The reaction mixture (3 ml.) contained pyrophosphate buffer, pH 8·8 (30 μmoles), cobalt sulphate (1 μmole), hexose diphosphate (10 μmoles), DPN (0·3 mg.) and 0·5 ml. of extract. When DPN was reduced, aldehyde was added as indicated by the arrows. The reference cell contained no DPN. ○, Butyraldehyde (1·35 μmole); △, *iso*butyraldehyde (1·37 μmole); □, valeraldehyde (1·14 μmole); ●, *iso*valeraldehyde (1·33 μmole). The amounts of aldehyde added were estimated by the $NaHSO_3$-binding method.

Properties of leucine-2-oxoglutarate transaminase of Saccharomyces cerevisiae

Effect of pH. The pH optimum was found to be between 7·2 and 7·4 (Fig. 4). There was a sharp drop in activity below pH 7·0 but above this value the effect was relatively small over the range studied. For subsequent experiments, pH 7·2–7·4 was chosen, with a buffer concentration of 0·067 M-phosphate. Bigger-Gehring (1955) found that the pH optimum for the leucine-2-oxoglutarate transaminase of *Saccharomyces fragilis* was 8·0. It is difficult to advance an explanation for such a big variation in the pH optimum, for the two organisms belong to the same genus. The extracts were prepared by the same method (Hughes, 1951), the only difference is that Bigger-Gehring incubated her preparations anaerobically at 37° for 2 hr. whereas in these experiments the incubations were at 25° for 30 min.

Effect of 2-*oxoglutarate concentration.* The amount of glutamate formed in 30 min. increased with increasing concentration of 2-oxoglutarate up to 2 μmoles/μmole of leucine (Fig. 5). Above this

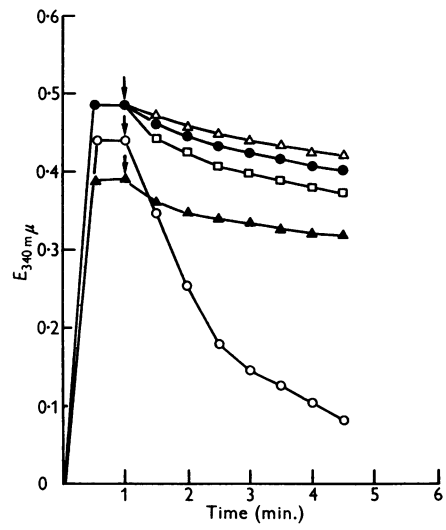

Fig. 3. Oxidation of reduced DPN by aldehydes in the presence of crystalline alcohol dehydrogenase. The reaction mixture (3 ml.) contained pyrophosphate buffer, pH 8·8 (30 μmoles), reduced DPN (0·1 ml. giving an extinction of 0·5 at 340 mμ) and crystalline alcohol dehydrogenase (75 μg.). Aldehydes (0·1 ml.) were added as indicated by the arrows. The reference cell contained no reduced DPN. ○, Butyraldehyde (1·35 μmole); △, *iso*butyraldehyde (1·37 μmole); □, valeraldehyde (1·14 μmole); ●, *iso*valeraldehyde (1·33 μmole); ▲, *p*-hydroxyphenylaldehyde [0·665 μmole estimated as tyrosol (cf. SentheShanmuganathan & Elsden, 1958)]. The amounts of aldehyde added were estimated by the $NaHSO_3$-binding method.

concentration there were signs of inhibition; therefore in all subsequent experiments the amount of 2-oxoglutarate was fixed at 2 μmoles/μmole of amino acid.

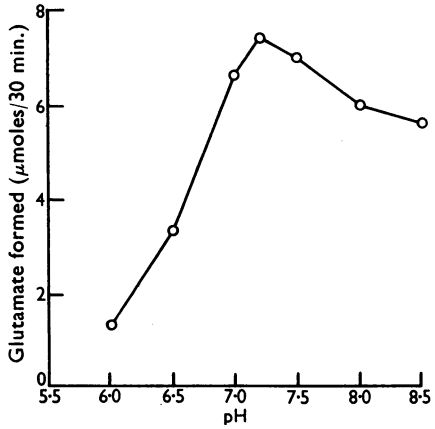

Fig. 4. Effect of pH on the formation of glutamate from leucine plus 2-oxoglutarate by dialysed cell-free extracts of *S. cerevisiae*. Experiments were carried out in test tubes. The complete system (3 ml.) contained pyridoxal phosphate (0·04 μmole), enzyme preparation, 2-oxoglutarate (20 μmoles) and amino acid (10 μmoles) in 0·067 M-phosphate buffer of the desired pH. The reaction was started by the addition of the substrate, amino acid, after first incubating the constituents for 10 min. Two controls, one without the amino acid and the other without 2-oxoglutarate, were included; no glutamic acid was detected in either. The pH remained constant throughout. Temp. 25°, incubation period, 30 min.; gas phase, air.

Formation of glutamate with time. The progress of transamination of 2-oxoglutarate with leucine was found to be linear with time over the period 0–40 min. (Fig. 6).

Transamination with other amino acids. Under the optimum conditions obtained for the leucine-2-oxoglutarate system, the transfer of amino groups from a number of amino acids to 2-oxoglutarate was tested with both dialysed and undialysed extract of *S. cerevisiae* after an incubation period of 60 min. Of the 21 amino acids tested, only 15 gave rise to glutamate in the presence of undialysed extract and 10 with the dialysed preparation (Table 5). The activity varied considerably from amino acid to amino acid. With undialysed extract, maximal activity was obtained with aspartic acid, whereas leucine and phenylalanine showed a slightly slower rate; the activities of isoleucine, norleucine, valine, norvaline, methionine and tyrosine were approximately half that of aspartate. Tryptophan and histidine showed the least activity and the other amino acids tested (Table 5) did not transaminate at all.

Effect of pyridoxal phosphate on the formation of glutamate. The results (Table 6) show that pyridoxal phosphate stimulates the synthesis of glutamate and that the degree of activation varies with different amino acids. This was most marked when valine was the substrate. The variable response to the cofactor could mean that more than one transaminase is involved.

Reversibility of the transaminase reactions. A mixture containing the 2-oxo acid (20 μmoles)

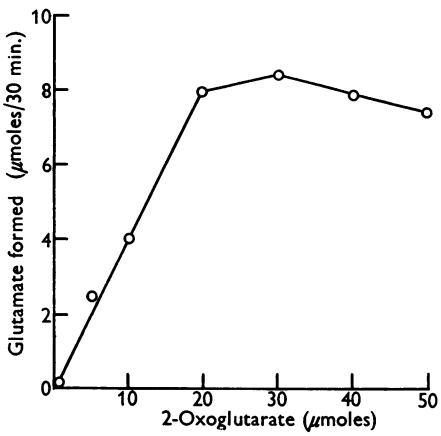

Fig. 5. Effect of 2-oxoglutarate on the formation of glutamate from leucine plus 2-oxoglutarate by dialysed cell-free extracts. Experimental details were as described in Fig. 4. Incubations were made at 25° in 0·067 M-phosphate buffer, pH 7·2. The amounts of 2-oxoglutarate added were varied and the glutamate formed in 30 min. was estimated. No glutamate was formed when the amino acid or 2-oxoglutarate was omitted.

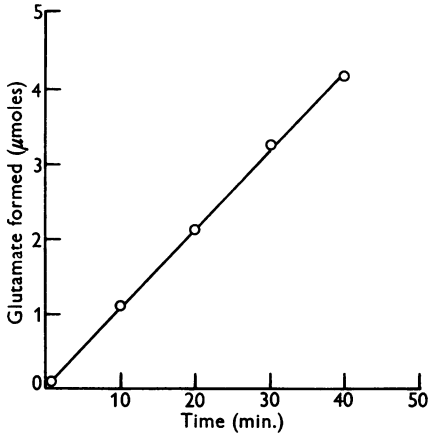

Fig. 6. Progress curve of glutamate formation from leucine plus 2-oxoglutarate by dialysed cell-free extracts. For experimental details see Fig. 4. Incubations were made at 25° in 0·067 M-phosphate buffer, pH 7·2. Glutamate was estimated at 10 min. intervals. No glutamate was produced when the amino acid or 2-oxoglutarate was omitted.

corresponding to the amino acid was incubated with glutamic acid (10 μmoles), pyridoxal phosphate (0·04 μmole) and dialysed extract at 25° for 30 min. in 0·1 M-phosphate buffer, pH 7·2. The control experiment contained no glutamate. At the end of the experimental period the proteins were denatured by heating in a boiling-water bath for 10 min. and separated by centrifuging. Equal volumes of the supernatant were placed on paper chromatograms. The transamination reactions, involving aspartic acid, phenylalanine, tyrosine, tryptophan, leucine, norleucine, valine and norvaline, were reversible. Alanine was not synthesized from pyruvate and glutamate.

Table 5. *Formation of glutamate from 2-oxoglutarate and amino acids by dialysed and undialysed cell-free extracts of* Saccharomyces cerevisiae

Experiments were carried out in test tubes and the system contained, in a total volume of 2·5 ml.: 15 μmoles of the L- or 30 μmoles of the DL-amino acid, 30 μmoles of 2-oxoglutarate, pyridoxal phosphate (0·04 μmole), 1 ml. of 0·1 M-phosphate buffer, pH 7·2, and 1 ml. of extract in 0·1 M-phosphate buffer, pH 7·2. All solutions were adjusted to pH 7·2.

Amino acid	Glutamate (μmoles/10 mg. of enzyme protein in 60 min.) Undialysed preparation	Dialysed extract
None	2·8	0
DL-Aspartic acid	15·3	10·6
DL-Leucine	11·4	8·6
DL-Isoleucine	9·7	6·6
DL-Phenylalanine	10·9	6·8
DL-Valine	10·0	6·8
DL-Norleucine	10·0	6·1
DL-Norvaline	10·5	4·3
DL-Tyrosine	8·8	4·2
DL-Methionine	9·0	3·4
DL-Tryptophan	4·7	0·8
DL-Histidine	4·7	0
DL-Serine	4·2	0
L-Proline	3·9	0
L-Hydroxyproline	3·8	0
DL-Threonine	3·7	0
DL-Alanine	2·8	0
L-Arginine	2·8	0
α-Aminobutyric acid	2·8	0
L-Cystine	2·8	0
L-Ornithine	2·8	0
DL-Lysine	2·8	0

Table 6. *Effect of pyridoxal phosphate on the formation of glutamate from 2-oxoglutarate and amino acids by dialysed cell-free extracts*

The system (2·5 ml.), containing the 2-oxoglutarate (20 μmoles), pyridoxal phosphate (0·04 μmole), 1 ml. of 0·1 M-phosphate buffer, pH 7·2, and 0·5 ml. of extract, was incubated for 10 min. before the substrate (10 μmoles) was added. Temp. 25°; period of incubation, 60 min.

Amino acid	Glutamate (μmoles/10 mg. of protein enzyme in 60 min.) Without coenzyme	With coenzyme
None	0	0
DL-Aspartic acid	5·3	10·6
DL-Leucine	3·4	8·4
DL-Phenylalanine	1·7	6·2
DL-Tyrosine	1·0	3·3
DL-Valine	0·6	5·3

DISCUSSION

Transamination in yeast was first demonstrated by Roine (1947) in *Torulopsis utilis*. He found that a direct transfer of amino groups of alanine, aspartic acid, valine, leucine and isoleucine to 2-oxoglutarate was catalysed by extracts of the yeast. Similar observations were made by Bigger-Gehring (1955) with *S. fragilis*. In the present study it has been found that dialysed extracts of *S. cerevisiae* catalysed the transfer of amino groups from 10 amino acids to 2-oxoglutarate. 2-Oxoglutarate could not be replaced with either pyruvate or oxaloacetate; these findings are in agreement with those of Roine (1947) and Bigger-Gehring (1955). In contrast to the observations of these authors, alanine was not transaminated with 2-oxoglutarate by extracts of the yeast employed under the conditions of the standard test used (i.e. 0·1 M-phosphate buffer, pH 7·2). Bigger-Gehring made all her tests at pH 8·0 and this may explain this difference of activity.

It is seen from Table 2 that the rate of liberation of carbon dioxide from the mixture of amino acid and 2-oxoglutarate by extracts of *S. cerevisiae* is greater than that obtained when the amino acid was omitted. Moreover, the rate of carbon dioxide liberation from the 2-oxo acids (Table 3) corresponding to the amino acids is very much greater than the liberation of carbon dioxide from the amino acid plus 2-oxoglutarate by a similar extract. These results indicate that the carboxylase in yeast is probably responsible for the decarboxylation reaction (2). This was confirmed by the observation that the carboxylase purified from this strain of yeast catalysed the almost quantitative decarboxylation of all the α-oxo acids tested (Table 4).

Of the 2-oxo acids tested *p*-hydroxyphenylpyruvate was least attacked; it is possible that the reaction does not go to completion because the product of the decarboxylation, *p*-hydroxyphenylacetaldehyde, inhibits the enzyme system. This possibility was not investigated any further. Meister (1952) did not observe decarboxylation of *p*-hydroxyphenylpyruvic acid by extracts of yeast, which decarboxylated other 2-oxo acids.

Though *p*-hydroxyphenylpyruvate was not decarboxylated by the same amount of the purified carboxylase as used for the other 2-oxo acids, it was

found that decarboxylation did not take place at a very slow rate if larger amounts of the purified enzyme were used (Table 4). The purified enzyme had 6·1 units of enzyme activity/mg. of protein when assayed under the standard conditions employed by Green *et al.* (1941). In contrast with the present findings, the carboxylase preparation (6·07 units/mg. of protein) obtained by Green *et al.* (1941) from dried brewer's-ale yeast did not catalyse the decarboxylation of this 2-oxo acid. Therefore the present results may indicate the presence of a specific decarboxylase in *S. cerevisiae* (strain 5) for *p*-hydroxyphenylpyruvate and that this is different from the classical pyvuric acid yeast carboxylase.

The third enzyme, which is responsible for reaction (3), is probably yeast alcohol dehydrogenase. The evidence for this hypothesis is supported by the present studies and by the results of Barron & Levine (1952) and Elisuzaki & Barron (1957). Butyric, *iso*butyric, valeric and *iso*valeric aldehydes were tested for their ability to oxidize reduced DPN in the presence of cell-free extracts (Fig. 2). Such studies showed that only butyraldehyde is reduced at an appreciable rate by reduced DPN, whereas the others were reduced very slowly. The reverse reaction, the oxidation of alcohols by DPN in the presence of extracts, was also studied and similar observations were made.

Experiments with crystalline alcohol dehydrogenase confirmed the observations with extracts. Tyrosol was not oxidized by DPN by crystalline alcohol dehydrogenase in the present studies.

The presence of a new alcohol dehydrogenase, alcohol dehydrogenase II, in yeast has been claimed by Elisuzaki & Barron (1957). They have evidence that the new alcohol dehydrogenase did not reduce DPN in the presence of 2-methylpropan-1-ol, but it had higher activity with propanol and butanol than the classical alcohol dehydrogenase. On the other hand, the classical alcohol dehydrogenase showed a slightly higher activity with propan-2-ol, butan-2-ol and ethylene glycol. The oxidation of different alcohols by the intact yeast cells was also studied by Barron & Levin (1952), and they found that the yeast cell could bring about the oxidation of ethanol, propanol, butanol and 2-methylpropan-1-ol. Present studies also showed that the extracts and crystalline alcohol dehydrogenase could oxidize pentyl and *iso*pentyl alcohols at about the same rate as 2-methylpropan-1-ol. Tyrosol was not oxidized by the crystalline alcohol dehydrogenase in the presence of DPN, and it is not possible to say at present whether the oxidation of tyrosol and the pentyl alcohols by DPN could be effected by alcohol dehydrogenase II. It is therefore suggested from the observations of Barron & Levine (1952), Elisuzaki & Barron (1957) and the writer that the third step in the formation of fusel oil from amino acids is effected by either the classical alcohol dehydrogenase or alcohol dehydrogenase II, or by both. Further studies are required to confirm this hypothesis.

From the previous (SentheShanmuganathan & Elsden, 1958) and present studies it has been concluded that the mechanism of fusel oil formation is of a general nature. The first step in the reaction sequence is a transamination and is most probably catalysed by different transaminases. This is followed by a decarboxylation yielding the aldehyde and carbon dioxide. The reduction of the aldehyde is finally effected by alcohol dehydrogenase via DPN as hydrogen carrier, e.g.

$$\text{Leucine} + \text{2-oxoglutarate} \xrightarrow{\text{transaminase}} \text{2-Oxo}\textit{iso}\text{caproic acid} + \text{glutamic acid} \quad (4)$$

$$\text{2-Oxo}\textit{iso}\text{caproic acid} \xrightarrow{\text{carboxylase}} \textit{iso}\text{Valeraldehyde} + CO_2 \quad (5)$$

$$\textit{iso}\text{Valeraldehyde} + \text{reduced DPN} \xrightarrow{\text{alcohol dehydrogenase}} \textit{iso}\text{Pentyl alcohol} + \text{oxidized DPN} \quad (6)$$

It is relevant to comment on the pH for optimum activity of the various individual enzymes involved in the conversion of tyrosine into tyrosol. The transaminase was found to have an optimum pH of 7·8 (SentheShanmuganathan, 1958), the carboxylase pH 6·0 and alcohol dehydrogenase acts only at a pH above 7·8. In fact all the experiments for alcohol dehydrogenase activity were carried out at pH 8·8. In contrast with these pH optima, the combined reaction transaminase and carboxylase was found to possess an optimum pH of 6·2. It is, however, difficult to advance an explanation of why in experiments with growing cultures and washed cells the conversion of tyrosine into tyrosol could be effected at pH 6·0, a pH at which alcohol dehydrogenase is inactive. This can only be explained on the basis that within the cell the entire system is so orientated that the pH would not be a limiting factor.

One more question remains to be answered, namely, why does yeast form fusel oil? Thorne (1941, 1950) studied the nutrient value of single amino acids and found that though they functioned in the same way of supplying ammonia to yeast they varied considerably among themselves in their nutrient values. Such an assimilation of amino acids probably occurs by the enzymes aspartase (Quastel & Woolf, 1926), glutamic acid dehydrogenase (Euler, Adler & Ericksen, 1937) or glutamic dehydrogenase and transaminase in combination, thus:

$$\text{Glutamic acid} \rightleftharpoons \text{2-Oxoglutarate} + NH_3$$

$$\text{Amino acid}_1 + \text{2-oxoglutarate} \rightleftharpoons \text{2-Oxo acid}_1 + \text{glutamic acid}$$

The availability of nitrogen from an amino acid will, however, depend on the extent to which each amino acid can transaminate with 2-oxoglutarate. From the present studies and those of Bigger-Gehring (1955) there are indications that the ease with which an amino acid can undergo transamination parallels the ease with which the amino acid can be utilized as source of nitrogen, as in the experiments of Thorne (1941).

It seems probable that the real significance of fusel oil formation is that it is a mechanism whereby yeast can utilize amino acids as a source of nitrogen. When simple sources of nitrogen such as ammonium salts and asparagine are available in the medium, fusel oil formation is very much reduced (Ehrlich, 1907), but when amino acids serve as the sole source of nitrogen the production of fusel oil occurs at a very high rate.

SUMMARY

1. Dialysed crude cell-free extracts of *Saccharomyces cerevisiae* catalyse the transfer of amino groups from aspartic acid, leucine, norleucine, isoleucine, valine, norvaline, methionine, phenylalanine, tyrosine and tryptophan to 2-oxoglutarate.

2. The transamination of aspartic acid, phenylalanine, leucine, valine and tyrosine with 2-oxoglutarate is stimulated by the addition of pyridoxal phosphate.

3. When leucine, norleucine, valine or norvaline is incubated with 2-oxoglutarate in the presence of extracts of *S. cerevisiae*, glutamate, carbon dioxide and aldehyde are produced. Addition of pyridoxal phosphate to this system stimulates the rate of formation of carbon dioxide.

4. 2-Oxo*iso*valeric acid, 2-oxovaleric acid, 2-oxo*iso*caproic acid and 2-oxocaproic acid are rapidly decarboxylated by both cell-free extracts and purified yeast carboxylase to give carbon dioxide and their corresponding aldehydes.

5. *p*-Hydroxyphenylpyruvate is decarboxylated very slowly with large amounts of purified yeast carboxylase.

6. Extracts oxidize reduced diphosphopyridine nucleotide on the addition of *p*-hydroxyphenylacetaldehyde, butyraldehyde, *iso*butyraldehyde, valeraldehyde or *iso*valeraldehyde. Crystalline alcohol dehydrogenase also behaved in a similar manner. The rate of reduction decreased with increasing length of carbon chain.

7. The mechanism proposed for the conversion of tyrosine into tyrosol appears to be a general one for the formation of all the constituents of fusel oils.

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