The Mechanism of the Formation of Tyrosol by *Saccharomyces cerevisiae*

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The mixture of higher alcohols which are known collectively as fusel oils, and which contribute to the flavour of many alcoholic drinks, have long been recognized as minor end-products of the metabolism of yeasts. The mechanism of the formation of these compounds is not, however, clear. The early work on this subject has been reviewed by Harden (1932) and what follows is a brief summary of the principal findings.

Felix Ehrlich, in a series of papers (Ehrlich, 1906, 1907*a*, 1911, 1912) showed conclusively that fusel oils were formed from amino acids without, apparently, the production of free ammonia. This work was complemented by that of Neubauer & Fromherz (1911), who studied in detail the conversion of phenylglycine into benzyl alcohol. They found that phenylglyoxylic acid and benzaldehyde were intermediates and, on the basis of these observations, they proposed the following reactions to account for the formation of fusel oil:

They also obtained evidence which suggested that the 2-hydroxy acid corresponding to the parent amino acid was not an intermediate, thus making it unlikely that the deamination was effected by hydrolysis.

Carboxylase, discovered by Neuberg & Hildersheimer (1911), was thought to account for reaction (2), and subsequent work has shown that many 2-oxo acids are decarboxylated by this enzyme. There are many reports (see Harden, 1932) showing that a large number of aldehydes are reduced to the corresponding alcohols by fermenting yeast cells (reaction 3).

Apart from the absence of ammonia, two other observations, which have been repeatedly confirmed, were made by the early workers. First, formation of fusel oil occurs only in the presence of glucose and, secondly, cell-free preparations of yeast (Ehrlich, 1907*b*; Pringsheim, 1906; Büchner & Meisenheimer, 1906), whilst able to ferment glucose, did not convert amino acids into the corresponding fusel oils even in the presence of glucose.

The major problems connected with formation of fusel oil thus appear to be: (a) the mechanism by which amino acids are deaminated anaerobically; (b) the fate of the amino groups; (c) the part played by glucose in the process.

Until now a major hindrance to the study of formation of fusel oil has been the lack of specific micro methods for the estimation of small amounts of higher alcohols in the presence of large amounts of ethanol. It occurred to us that 2-[*p*-hydroxyphenyl]ethanol (tyrosol), which Ehrlich (1911) showed to be produced from L-tyrosine, might be estimated by the phenol method of Folin & Ciocalteu (1927) and that, if so, it would be possible to study the conversion of L-tyrosine into tyrosol. Preliminary experiments showed that tyrosol could be estimated by the above method and, further, that it could be separated quantitatively from L-tyrosine. We were therefore in a position to study the mechanism of the formation of tyrosol and, in particular, the nature of the deamination reaction. Our results, a preliminary report of which has already been published (Senthesanmuganathan, 1956), suggest that the first step is a transamination between L-tyrosine and 2-oxoglutarate, giving *p*-hydroxyphenylacetaldehyde and L-glutamic acid. The former product is then decarboxylated by yeast carboxylase and the resulting aldehyde reduced to tyrosol (cf. Neubauer & Fromherz, 1911).

MATERIALS AND METHODS

Materials

Organism. The organism used throughout this work was *Saccharomyces cerevisiae* (baker's yeast strain 5 of the Departmental collection). Cultures were grown on the following medium (all quantities/100 ml.): glucose, 2 g.; KH_2PO_4 , 0.4 g.; $MgSO_4 \cdot 7H_2O$, 0.1 g.; $(NH_4)_2SO_4$, 0.2 g.; $CaCl_2$, 0.05 g.; H_3BO_3 , 10 mg.; ammonium molybdate, 4 mg.; $MnSO_4 \cdot 4H_2O$, 4 mg.; $ZnSO_4 \cdot 7H_2O$, 4 mg.; $CaSO_4 \cdot 5H_2O$, 4.5 mg.; $FeSO_4 \cdot 7H_2O$, 25 mg.; 400 µg. each of calcium (+)-pantothenate, nicotinic acid, pyridoxine hydrochloride, thiamine hydrochloride; 200 µg. each of folic acid, riboflavin, *p*-aminobenzoic acid; 2 µg. of biotin; 0.2 ml. of Tween 80. The Tween 80 was found essential for diffuse growth. The pH was adjusted to pH 6 and the medium sterilized by autoclaving at 15 lb./in.² for 10 min. Glucose was sterilized separately, either by autoclaving or

by filtration. Cultures were grown in conical flasks of a capacity two to three times the volume of the medium used. The inoculum was 5 ml. of a 24 hr. culture grown in the above medium. The culture was incubated at 25° for 36–42 hr. Stock cultures were maintained on slopes of the above medium, solidified with 2% (w/v) of agar but containing no Tween 80.

At the end of the growth period, the cells were harvested by centrifuging, washed twice with water and suspended in 0.067 M-phosphate buffer, pH 6, to a density of 70 mg. dry wt./ml. The phosphate buffers used in this work were mixtures of Na_2HPO_4 and KH_2PO_4 . Dry weight was determined turbidimetrically with the Hilger Spekker absorptiometer (1 cm. cell) with a neutral grey filter Ilford no. H508, and by reference to a standard graph relating optical density to dry weight.

Cell-free preparations. These were prepared with the Hughes press (Hughes, 1951). The paste of washed cells was packed into a cooled (–20°) press and crushed without abrasive. The resulting mass was extracted with 0.1 M-phosphate buffer, pH 6.2, 1 ml. of buffer being used for each 70 mg. dry wt. of cells. The extract was then centrifuged for 15 min. at 19 600 *g* in the high-speed head of an International refrigerated centrifuge, and the supernatant stored at –20° until required.

Extract of boiled yeast (EBY). This was prepared by boiling a mixture of equal parts of baker's yeast and water for 30 min. and clarifying by centrifuging. The preparation was stored at –20°.

L-Tyrosine. That obtained from L. Light and Co., Colnbrook, Bucks, was used throughout this work. The purity of the compound was checked both by paper-partition chromatography and by enzymic assay with tyrosine decarboxylase (Gale, 1945). For most experiments a mM-solution, adjusted to pH 6.2 with dil. NaOH, was used. Where larger amounts were required a fine suspension containing 16 μ moles/ml. was prepared by dissolving the L-tyrosine in aq. 1% (w/v) Na_2CO_3 and adjusting to pH 6.2 with dil. acetic acid.

p-Hydroxyphenylpyruvic acid. The azlactone was first prepared from hippuric acid and *p*-hydroxybenzaldehyde, as described by Erlenmeyer & Halsey (1899); this was then hydrolysed to *p*-hydroxyphenylpyruvic acid by the method of Mauthner (1909) as modified by Haworth (private communication). The hydrolysate was saturated with SO_2 , cooled to 2° and the precipitated benzoic acid filtered off. The filtrate, which contained the *p*-hydroxyphenylpyruvic acid, was acidified and boiled to expel the SO_2 . The keto acid precipitated on standing and after filtering off was recrystallized from water. A second sample was obtained from L. Light and Co.

p-Hydroxyphenylacetaldehyde. The free aldehyde could not be prepared by chemical means (cf. Langheld, 1909). It was found that cell-free extracts of *S. cerevisiae* prepared as described above would decarboxylate *p*-hydroxyphenylpyruvic acid, and this procedure was used to prepare small amounts of the aldehyde. To 5 ml. of the cell-free extract was added 10 ml. of a 0.01 M-solution of the sodium salt of the keto acid and the mixture incubated at 25° for 120 min. Proteins were precipitated by the addition of 5 ml. of 10% (w/v) trichloroacetic acid and the mixture was centrifuged. The supernatant was made alkaline (pH 10.5) with 10% Na_2CO_3 and extracted for 1 hr. with ether in a Kutscher-Stendel liquid-liquid extractor. The ether was

distilled off and the residue dissolved in 10 ml. of water. Determination of the phenol and the bisulphite binding material (for methods see below) indicated that 85–90% of the ether-extractable phenol (EEP) was in the form of the aldehyde.

2-[*p*-Hydroxyphenyl]ethanol (tyrosol). This was prepared by the biological method described by Ehrlich (1911), in which baker's yeast, sucrose and tyrosine are incubated together. Needle-shaped white crystals, m.p. 89–90°, were obtained.

Other 2-oxo acids. 2-Oxoglutaric acid was prepared by the method of Clutterbuck (1927). Crystalline sodium pyruvate was prepared as described by Robertson (1942) and oxaloacetic acid was the gift of Professor H. A. Krebs, F.R.S. Solutions of sodium oxaloacetate were prepared as described by Elsdon & Ormerod (1956).

Fructose 1:6-diphosphate. The barium salt was prepared by the method of Robison & Morgan (1930) and this was converted into the sodium salt by solution in dil. HCl and removal of Ba^{2+} ions by addition of a saturated solution of sodium sulphate. After centrifuging, the solution was adjusted to pH 6.2. Paper-partition chromatography (Eggleston & Hems, 1952) showed the presence of traces of hexose monophosphates.

Adenosine triphosphate. This was obtained as the barium salt from Schwarz Laboratories Inc., New York, N.Y., U.S.A.

Diphosphopyridine nucleotide. This was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Pyridoxal phosphate. This was given to us by Dr Morrison of Roche Products Ltd., Welwyn Garden City, Herts.

Methods

Experimental procedure. When gaseous exchanges had to be measured the reactions were carried out in Warburg manometers, otherwise Thunberg tubes or test tubes were used. Experiments with washed-cell suspensions were performed under an atmosphere of O_2 -free N_2 . The temperature was in all cases 25°. Reactions were started by the addition of either the cell suspension or the extract to the substrate-buffer mixture. At the end of the experiment 0.5 ml. of 10% (w/v) trichloroacetic acid was added to stop the reaction, to precipitate proteins and, in manometric experiments, to liberate the CO_2 bound by the buffer. In those experiments where the metabolism of tyramine was being studied 8N-HCl (0.5 ml.) was used in place of trichloroacetic acid and the mixture heated at 100° for 5 min. before centrifuging.

Tyrosine. The decarboxylase method of Gale (1945) was used for the estimation of this compound.

p-Hydroxyphenylpyruvic acid. This was estimated colorimetrically as the 2:4-dinitrophenylhydrazone, under conditions described by Goodwin & Williams (1952) for the estimation of pyruvic acid. The colour was measured with the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) and the blue filter provided. Whereas there was a linear relationship between the optical density and the amount of keto acid, the slope of the curve varied from determination to determination and, in consequence, a set of standards was always analysed with the unknowns.

Estimation of p-hydroxyphenylacetaldehyde, tyrosol and tyramine. These compounds give a blue colour with the phenol reagent of Folin & Ciocalteu (1927) under the conditions prescribed by these authors for the estimation of

phenol. The reagent, which was obtained from British Drug Houses Ltd., Poole, Dorset, was diluted 1:4 with water before use. The blue colour was measured with an EEL photoelectric colorimeter and the red filter provided with the instrument. With tyrosol there was a linear relation between the optical density and the amount of tyrosol present up to a level of $0.5 \mu\text{mole}$ of tyrosol in a total volume of 10 ml. Equimolecular amounts of tyrosol and tyramine gave the same optical density. Owing to our inability to prepare pure *p*-hydroxyphenylacetaldehyde, we were unable to compare this compound with tyrosol and we have assumed that it produces the same amount of colour/mole as tyrosol.

Since both tyrosine and *p*-hydroxyphenylpyruvic acid give a blue colour with the reagent, these compounds had to be removed before tyrosol, *p*-hydroxyphenylacetaldehyde or tyramine could be estimated. This was done by ether extraction. The protein-free solution was transferred to a Kutscher-Steucler extractor, made alkaline with an equal volume of 10% (w/v) Na_2CO_3 (final pH 10.5) and the mixture was saturated with NaCl. The alkaline solution was then extracted with freshly distilled ether for 30 min. The extraction apparatus and the conditions of operation were similar to those described by Krebs, Smyth & Evans (1940) for the extraction of succinic acid. Neither tyrosine nor *p*-hydroxyphenylpyruvic acid was extracted under these conditions. At the end of the extraction period the ether was distilled off, the residue dissolved in a measured volume of water and samples of the extract were analysed for phenols. It was found that tyrosol was completely extracted in 15 min., and a 30 min. period was chosen to provide a margin of safety. We were unable to measure precisely the extraction rate of *p*-hydroxyphenylacetaldehyde but semiquantitative experiments suggest that a 30 min. extraction period was adequate. Saturation with NaCl prevented foaming in the extractor and the consequent entrainment of tyrosine.

Since all non-acidic phenols are extracted we have used the term ether-extractable phenol (EEP) to designate the total amount of phenol, calculated as tyrosol, present in the extract. The term EEP is used when there is reason to believe that more than one phenol was present. Where both tyrosol and *p*-hydroxyphenylacetaldehyde were present and an estimate of each was required we proceeded as follows. The EEP in a sample of the extract was estimated as described above and a second sample was cooled to 2° in an ice bath and 1 ml. of 0.5% NaHSO_3 was added. The mixture was kept at 2° for 1 hr. to allow the formation of the bisulphite compound, the amount of which was then determined iodometrically in the usual way after treatment with solid NaHCO_3 . On the assumption that the two phenols give the same amount of colour with the phenol reagent, the amount of tyrosol in the mixture can be calculated. When tyramine was the substrate the solution, after deproteinizing with HCl, was extracted with ether without further treatment; the tyramine remained behind in the aqueous phase; this was then made alkaline with 10% Na_2CO_3 soln. and the tyramine extracted with ether.

Chromatography of phenolic compounds and amino acids. Tyrosol was readily separated from tyrosine by paper-partition chromatography with *n*-butanol-acetic acid-water (4:1:5, by vol.; upper phase) as solvent. After ascending development for 16 hr. the papers were dried at room temperature and sprayed first with 10% (w/v)

Na_2CO_3 and then with diazotized sulphanilic acid (Wickremasinghe & Fry, 1954). Tyrosol (R_F 0.93) and tyrosine (R_F 0.40) showed up as red spots on a yellow background. Amino acids on the paper were revealed by spraying with ninhydrin (Woiwod, 1949). L-Glutamic acid was estimated by the decarboxylase method of Gale (1945). 2-Oxoglutarate was estimated by the method of Krebs (1950).

Spectrophotometry. The reduction of *p*-hydroxyphenylacetaldehyde by extracts in the presence of reduced diphosphopyridine nucleotide was studied under conditions similar to those described by Racker (1950), the Unicam SP. 500 spectrophotometer being used to follow the change in optical density at $340 \text{ m}\mu$. The reduced diphosphopyridine nucleotide was prepared by treating diphosphopyridine nucleotide (DPN) with sodium dithionite and removing residual dithionite by aeration for 10 min.

Glucose. Glucose was estimated by the colorimetric method of Nelson (1944) after removal of proteins with trichloroacetic acid as described above.

RESULTS

Formation of tyrosol by growing cultures of Saccharomyces cerevisiae

We first investigated the production of tyrosol by growing cultures of *S. cerevisiae*. Culture medium (192 ml.) containing 220 μmoles of L-tyrosine was placed in a 250 ml. conical flask and sterilized at 10 lb./in.^2 for 15 min. To this was added 8 ml. of a 50% (w/v) solution of glucose sterilized by filtration. The medium was inoculated with 6 drops of a 24 hr. culture and incubated at 25° . Samples (10 ml.) were withdrawn every 6 hr. and the turbidity, glucose and tyrosol contents estimated. The results are shown in Fig. 1. It will be seen that tyrosol formation was coincident with growth and that the amount of tyrosol formed was equivalent to the tyrosine added to the medium. It is important to emphasize at this point that tyrosol was the only product formed by whole cells;

Fig. 1. Growth of *S. cerevisiae*, glucose utilization and formation of tyrosol. Medium is described in the text. Temp. 25° . ●, Growth of *S. cerevisiae*; ▲, glucose used; ○, tyrosol formed.

neither *p*-hydroxyphenylpyruvic acid nor *p*-hydroxyphenylacetaldehyde was ever detected. Fig. 2 shows the effect of varying the glucose content of the medium on both the yield of cells

and the amount of tyrosol formed; both measurements were made after incubating for 48 hr., by which time growth had ceased.

Formation of tyrosol by suspension of washed cells

Effect of glucose. Under anaerobic conditions suspensions of washed cells rapidly fermented glucose and small amounts of tyrosol were formed. Addition of tyrosine plus glucose to the suspension increased the yield of tyrosol 3.5-fold, and in 60 min. some 50 % of the tyrosine added was converted into the alcohol (Table 1). If glucose was omitted, the yield of tyrosol from tyrosine was 13 % of that when glucose was present.

There is evidence (Taylor, 1947) that the transport of amino acids across the yeast-cell wall is an energy-requiring process. It seemed possible that the requirement for glucose in the conversion of tyrosine into tyrosol was, in part, connected with this process of active transport, the fermentation of glucose supplying the energy needed. Both sodium azide and 2:4-dinitrophenol (DNP) are known to inhibit active transport and, in consequence, the effect of these two compounds on the formation of tyrosol was studied. Table 2 shows that DNP at a concentration of 0.1 mM completely inhibited formation of tyrosol and 2 mM azide gave 86 % inhibition. Whereas these observations are in keeping with the above explanation, the possibility is not excluded that these compounds have a more direct effect on the process.

Effect of 2-oxo acids and L-glutamate. Transamination is a possible mechanism for the anaerobic deamination of amino acids and, because of this, we tried the effect of pyruvate, oxaloacetate and 2-oxoglutarate on the yield of tyrosol; L-glutamic acid, as an alternative source of 2-oxoglutarate, was also tested. The results of these experiments were difficult to interpret for, at best, the effects were small. It was obvious that, if further progress was to be made, the reaction had to be studied in cell-free systems where the amounts of the reactants and the conditions of the reaction could be more rigorously controlled.

Experiments with cell-free extracts

When tyrosine plus 2-oxoglutarate was added to cell-free extracts prepared with the Hughes press, EEP was produced over and above that formed from tyrosine alone. Neither pyruvate nor oxaloacetate replaced 2-oxoglutarate. Table 3 shows the effect of varying the concentration of 2-oxoglutarate on the amount of EEP formed from 1 μ mole of L-tyrosine. In subsequent experiments, a ratio of 2-oxoglutarate to L-tyrosine of 10:1 was used in the reaction mixture. Supplementing with pyridoxal phosphate further increased the amount

Fig. 2. Effect of glucose content of medium on formation of tyrosol and yield of cells. Medium is described in the text; tyrosine added (110.4 μ moles/100 ml.). Temp. 25°. Growth and formation of tyrosol were measured after incubation for 48 hr. ○, Cell yield; ●, tyrosol formed.

Table 1. *Formation of tyrosol by washed-cell suspensions of Saccharomyces cerevisiae*

The complete system contained: 2 ml. of cell suspension in 0.066 M-phosphate buffer, pH 6; 1100 μ moles of glucose, 5.5 μ moles of L-tyrosine, 2 ml. of 0.066 M-phosphate buffer, pH 6, and water to a total volume of 10 ml. Temp. 25°; gas phase N₂. Incubation time 60 min.

System	Tyrosol formed (μ moles)
Complete	3.8
Tyrosine omitted	1.1
Glucose omitted	0.5
Tyrosine and glucose omitted	0.3

Table 2. *Effect of sodium azide and 2:4-dinitrophenol on formation of tyrosol by suspensions of washed cells*

Experiments were carried out in Warburg manometers. The main compartment contained: L-tyrosine, 1 μ mole; glucose, 550 μ moles; 1 ml. of 0.066 M-phosphate buffer, pH 6; inhibitors (when added) and water to 4 ml. Side bulb contained 1 ml. of washed suspension (70 mg. dry wt. of cells) in 0.066 M-phosphate buffer, pH 6. Temp. 25°; gas phase N₂. Incubation time 60 min.

Additions	Tyrosol formed (μ mole)	Inhibition (%)
None	0.51	—
NaN ₃ (2 mM)	0.07	86
DNP (0.1 mM)	0	100

Table 3. *Effect of 2-oxoglutarate on the formation of ether-extractable phenol by cell-free extracts of Saccharomyces cerevisiae*

Experiments were carried out in Warburg manometers. Main compartment contained: L-tyrosine (1 μ mole); pyridoxal phosphate, 10^{-2} μ mole; 2-oxoglutarate as indicated and water to 2.1 ml. All additions were adjusted to pH 6.2. Side bulb contained 1 ml. of extract in 0.1M-phosphate buffer, pH 6.2. Temp. 25°; gas phase air. Incubation time 60 min.

2-Oxoglutarate added (μ moles)	EEP formed (as μ mole of tyrosol)
0	0.05
2	0.17
4	0.25
6	0.34
8	0.38
10	0.45
12.5	0.34
25	0.34

Fig. 3. *Effect of pyridoxal phosphate on formation of EEP by cell-free extracts. Experiments were carried out in Warburg manometers. Main compartments contained: 1.9 ml. of 0.1M-phosphate buffer, pH 6.2; 2-oxoglutarate (10 μ moles); tyrosine (1 μ mole); pyridoxal phosphate as given and water to 4 ml. Side bulbs contained 1 ml. of cell-free extract. Temp. 25°; time 60 min. Gas phase air.*

of EEP formed. Fig. 3 shows the effect of pyridoxal phosphate on formation of EEP in the presence of a fixed amount of 2-oxoglutarate. It will be noted that, even at 20 μ M, the system was not saturated. The optimum pH for formation of EEP was pH 6.2 (Fig. 4).

Products of the reaction. The stimulation of formation of EEP by 2-oxoglutarate and pyridoxal phosphate supports the view that transamination is involved in the conversion of tyrosine into EEP. If this is the case, then it is to be expected that glutamate will be formed in an amount equivalent to the EEP produced. The phenol produced is extracted by ether from an alkaline solution and hence it is not acidic; it could be either tyrosol itself or *p*-hydroxyphenylacetaldehyde. An experiment was carried out in which the amount of tyrosine disappearing and the amounts of the products (glutamate, EEP and CO_2) were measured. In addition, the amount of bisulphite binding material (assumed to be *p*-hydroxyphenylacetalde-

Fig. 4. *Effect of pH on formation of EEP by cell-free extracts. Experiments were carried out in test tubes. Reaction mixture contained: 1.9 ml. of 0.1M-phosphate buffer; tyrosine (1 μ mole); 2-oxoglutarate (10 μ moles); pyridoxal phosphate (10^{-2} μ mole) and water to 4 ml. Reaction was started by addition of 1 ml. of cell-free extract. Temp. 25°; time 60 min. The pH of the system was measured at the end of the experiment.*

Table 4. *Balance sheet of the reaction*

Experiments were carried out in Warburg manometers with double side-bulb cups. The main compartment contained: L-tyrosine, 16 μ moles; 2-oxoglutarate, 160 μ moles; pyridoxal phosphate 2×10^{-2} μ mole; all additions were adjusted to pH 6.2; water to 2.7 ml. First side bulb contained 1 ml. of cell-free extract in 0.1M-phosphate buffer, pH 6.2; second side bulb contained 0.5 ml. of 10% (w/v) trichloroacetic acid. Temp. 25°; gas phase air. Reaction was stopped after 90 min. by addition of trichloroacetic acid.

System	Glutamate (μ moles)	CO_2 (μ moles)	EEP (as μ moles of tyrosol)	Aldehyde (μ moles)	Tyrosine (μ moles)
(1) Complete	+12.2	+16.5	+9.0	+9.3	-8.0
(2) Tyrosine omitted	+3.8	+9.4	+1.2	+2.2	0
(1) - (2)	+8.4	+7.1	+7.8	+7.1	-8.0

hyde) in the EEP fraction was estimated. The results are given in Table 4. It will be seen that both glutamate and CO_2 were formed and that most of the EEP was present as bisulphite binding material. If the appropriate control values are deducted, there is a stoicheiometric relationship between the tyrosine used and the products formed. The CO_2 produced in the absence of tyrosine is probably derived from the 2-oxoglutarate, which is slowly decarboxylated by yeast carboxylase. The amount of 2-oxoglutarate which takes part in the transaminase reaction is small compared with the total added, and for this reason it could not be determined accurately.

Isolation of the 2:4-dinitrophenylhydrazone of p-hydroxyphenylacetaldehyde. To 100 ml. of a cell-free extract of *S. cerevisiae* in 0.1M-phosphate buffer (pH 6.2) and containing 0.2 μ mole of pyridoxal phosphate was added 100 ml. of the substrate mixture, containing 1 m-mole of L-tyrosine and 10 m-moles of 2-oxoglutarate (the substrate mixture was adjusted to pH 6.2 with dil. NaOH). A sample (3 ml.) of the substrate mixture was transferred to a Warburg manometer and the evolution of the CO_2 followed manometrically at 25°; the remainder of the mixture was incubated at the same temperature. Evolution of gas ceased after 8 hr. and, after acidifying to pH 1 with N-HCl, the mixture was heated for 30 min. on a boiling-water bath to precipitate the proteins, cooled and centrifuged. The supernatant was adjusted to pH 10.5 with saturated aq. Na_2CO_3 and extracted for 3 hr. with freshly distilled ether in a liquid-liquid extractor. The ether extract so obtained was dried over anhydrous Na_2SO_4 and the ether distilled off, leaving a reddish syrup. This was stirred up with 15 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in N-HCl and left overnight at 0°. The reddish precipitate was filtered off, washed with N-HCl, water, saturated Na_2CO_3 , water, N-HCl and water, in that order, and dried *in vacuo* over CaCl_2 . The crude product (m.p. 158°) was recrystallized from aq. ethanol and, after four recrystallizations, brown needle-shaped crystals with a constant m.p. (179–180°) were obtained (Found: C, 53.35; H, 3.53; N, 17.5. Calc. for $\text{C}_{14}\text{H}_{12}\text{O}_5\text{N}_4$: C, 53.2; H, 3.79; N, 17.70).

Effect of sodium azide and 2:4-dinitrophenol. The experiments recorded in Table 2 showed that formation of tyrosol by whole cells was inhibited by both NaN_3 and DNP. In contrast with whole cells, neither NaN_3 nor DNP inhibited the reaction catalysed by extracts. It would thus appear that the enzymes concerned with the conversion of tyrosine into EEP are not affected by these inhibitors. It should be noted that, in the experiments with whole cells, no EEP was formed in the presence of either NaN_3 or DNP, and hence these

compounds do not act by inhibiting the reduction of *p*-hydroxyphenylacetaldehyde to tyrosol for, if they did, the aldehyde would be expected to accumulate.

Tyramine as a possible intermediate. The experiments so far described suggest that two reactions, a transamination and a decarboxylation, are involved in the conversion of tyrosine into *p*-hydroxyphenylacetaldehyde, but they do not indicate the order in which these reactions occur. If the first step is decarboxylation, then tyramine would be the first intermediate and it would then be necessary to postulate a transamination between the amine and 2-oxoglutarate in order to account for the formation of *p*-hydroxyphenylacetaldehyde and glutamate. On the other hand, if a transamination is the first reaction then *p*-hydroxyphenylpyruvate will be the first intermediate, and this will be decarboxylated, giving *p*-hydroxyphenylacetaldehyde.

When tyramine was added either to growing cultures of *S. cerevisiae* or to washed suspensions in the presence of glucose, insignificant amounts of tyrosol were formed and the base was quantitatively recovered from the medium at the end of the experiment. Likewise, when tyramine was added to cell-free extracts of *S. cerevisiae* reinforced with 2-oxoglutarate and pyridoxal phosphate, negligible amounts of EEP were produced (Table 5). These experiments eliminate tyramine as an intermediate.

p-Hydroxyphenylpyruvic acid as an intermediate. Cell-free extracts of our strain of *S. cerevisiae* slowly decarboxylate *p*-hydroxyphenylpyruvate (Fig. 5). The EEP formed was mainly accounted for as *p*-hydroxyphenylacetaldehyde. It is of interest that Green, Herbert & Subrahmanyam (1941) reported that their purified preparation of yeast carboxylase did not attack *p*-hydroxyphenylpyruvate. Washed cells, in the presence of glucose, converted *p*-hydroxyphenylpyruvate into tyrosol (see Table 6).

Reduction of p-hydroxyphenylacetaldehyde. This compound is reduced to tyrosol by washed-cell suspensions of *S. cerevisiae* in the presence of glucose (Table 6). It was next found that cell-free extracts oxidize reduced diphosphopyridine nucleotide (DPNH) on addition of *p*-hydroxyphenylacetaldehyde, the reaction being followed spectrophotometrically at 340 μ . Finally, the reduction of the aldehyde was coupled to the glycolytic system. Cell-free extracts rapidly reduce DPN on addition of hexose diphosphate. If, when the DPN was fully reduced, *p*-hydroxyphenylacetaldehyde was added, the DPNH was oxidized. The results of such an experiment are shown in Fig. 6. A quantitative reduction of the aldehyde was never observed, but whether this was due to

destruction of the aldehyde, which is known to be unstable, is not clear. The reverse reaction, the oxidation of tyrosol by extracts in the presence of DPN, was also demonstrated.

DISCUSSION

We stated in the introduction that the three main problems associated with formation of fusel oil by *S. cerevisiae* are: the mechanism by which amino acids are deaminated anaerobically, the fate of the amino group and the part played by glucose. The experiments we have described are concerned solely with the conversion of L-tyrosine into tyrosol, and we believe that we can provide a complete description of the mechanism in biochemical terms.

Table 5. *Tests of tyramine as an intermediate in formation of tyrosol*

Expt. 1. Reactions were carried out in Warburg manometers. Total volume in each cup was made up to 5 ml. Side bulbs contained in all cases 1 ml. of washed-cell suspension (70 mg. dry wt. of cells in 0.1M-phosphate buffer, pH 6.2). Main compartment always contained 0.5 ml. of 0.1M-phosphate buffer, pH 6.2, plus, where necessary: tyrosine (10 μ moles), tyramine (10 μ moles), glucose (550 μ moles) and water to 4 ml. Gas phase N_2 ; temp. 25°. Time 60 min.

Expt. 2. Reactions were carried out in Warburg manometers. Total volume in each cup was 3.7 ml. Side bulbs contained 1 ml. of cell-free extract in 0.1M-phosphate buffer, pH 6.2. Main compartment always contained 0.5 ml. of 0.1M-phosphate buffer, pH 6.2, and 10^{-2} μ mole of pyridoxal phosphate plus, where necessary: tyrosine (10 μ moles); tyramine (10 μ moles); 2-oxoglutarate (100 μ moles) and water to 2.7 ml. Gas phase air; temp. 25°. Time 60 min.

	EEP (as μ moles of tyrosol)	Tyramine remaining (μ moles)
Expt. 1		
(a) Cells	0.14	—
Cells + glucose	0.28	—
Cells + tyrosine	0.21	—
Cells + tyrosine + glucose	1.40	—
(b) Cells	0.14	0.26
Cells + glucose	0.21	0.52
Cells + tyramine	0.21	9.80
Cells + tyramine + glucose	0.42	9.80
Expt. 2		
(a) Extract	0.26	—
Extract + 2-oxoglutarate	0.52	—
Extract + tyrosine	0.39	—
Extract + tyrosine + 2-oxoglutarate	4.16	—
(b) Extract	0.52	0.26
Extract + 2-oxoglutarate	0.78	0.52
Extract + tyramine	0.52	9.90
Extract + tyramine + 2-oxoglutarate	0.91	9.90

Fig. 5. Decarboxylation of pyruvate (O) and *p*-hydroxyphenylpyruvate (●). Experiments were carried out in Warburg manometers with double side-bulb cups. Main compartment contained 8 μ moles of substrate and water to 2.5 ml. In the first side bulb was 1 ml. of cell-free extract in 0.1M-phosphate buffer, pH 6.2; the second side bulb contained 0.5 ml. of 10% (w/v) trichloroacetic acid. When gas formation ceased the acid was added from the side bulb to liberate bound CO_2 .

Table 6. *Conversion of intermediates into tyrosol by washed suspension of Saccharomyces cerevisiae*

Reactions were carried out in Thunberg tubes. Side bulbs contained 1 ml. of washed-cell suspension in 0.066M-phosphate buffer, pH 6 (70 mg. dry wt. of cells/ml.). Main compartment contained 2 ml. of 0.066M-phosphate buffer, pH 6, and, where indicated, tyrosine (8 μ moles), *p*-hydroxyphenylpyruvic acid (10 μ moles; sodium salt), *p*-hydroxyphenylacetaldehyde (4.5 μ moles); water to 4 ml. Gas phase N_2 ; temp. 25°. Time 60 min.

	EEP (as μ moles of tyrosol)	Aldehyde in EEP (μ moles)
Cells + glucose	0.28	0.27
Cells + glucose + tyrosine	1.54	0.40
Cells + glucose + hydroxyphenylpyruvate	1.96	0.65
Cells + glucose + hydroxyphenylacetaldehyde	4.34	1.06

The experiments show that the first step is a transamination between L-tyrosine and 2-oxoglutarate, yielding *p*-hydroxyphenylpyruvate and glutamate. The former product is then decarboxylated by yeast carboxylase with the formation of *p*-hydroxyphenylacetaldehyde and carbon dioxide. The aldehyde so formed is then reduced to tyrosol with DPNH as the hydrogen donor. Experiments, which will be published later, show that the

alcohol dehydrogenase catalyses this reaction. The overall reaction can now be written:

L-Tyrosine + 2-oxoglutarate + DPNH

Transaminase carboxylase
alcohol dehydrogenase

→ tyrosol +
L-glutamate + CO₂ + DPN

These reactions account both for the deamination of the amino acid and for the absence of ammonia from amongst the end products. That a transaminase could also act as deaminase was first proposed by Braunstein & Bychkov (1939), Braunstein & Azarkh (1945), to explain the deamination of L-amino acids by animal tissues. In our case, transamination is followed by decarboxylation of the keto acid produced, whereas in Braunstein's example the second reaction is the oxidation of the glutamate by the glutamic dehydrogenase.

The role of glucose remains to be explained. It is clear that the essential reactions can take place in cell-free extracts if 2-oxoglutarate is supplied. In whole cells, on the other hand, the reactants are L-tyrosine and glucose. There are two obvious functions of glucose: first, it provides a source of DPNH by glycolysis; secondly, it is the ultimate source of the 2-oxoglutarate. That this must be so is clear from the fact that yeast can synthesize all its amino acids from glucose and ammonia, and since 2-oxoglutarate and glutamate are interconvertible it is clear that the amino acceptor (2-

oxoglutarate) can be synthesized anaerobically from glucose.

The formation of tyrosol by whole cells is inhibited by both sodium azide and 2:4-dinitrophenol, and this suggests that the passage of tyrosine into the cell is a process requiring energy. If this is so, it would be an additional reason for the requirement of glucose for production of fusel oil by whole cells. It is of interest that Taylor (1947) found that the uptake of glutamate was a process requiring energy.

So far, we have restricted this discussion to the conversion of tyrosine into tyrosol, but it seems probable that the mechanism we have proposed will account for formation of fusel oil in general. Roine (1947) and Bigger-Gehring (1955) have studied the transaminase systems of yeasts. The latter author worked with *Saccharomyces fragilis* and found that a number of amino acids participate in a transamination with 2-oxoglutarate as the amino acceptor. Thus at least in this species the deaminating system is present. It is also well known that yeast carboxylase decarboxylates a wide range of 2-oxo acids (see Green *et al.* 1941). Table 4 shows that when cell-free extracts of *S. cerevisiae* were incubated with tyrosine and 2-oxoglutarate at pH 6.2, more carbon dioxide was produced than in the presence of 2-oxoglutarate alone. Although the reaction was slow, it was possible to follow its time course manometrically. We have now found that cell-free extracts produce carbon dioxide from a number of amino acids in the presence of 2-oxoglutarate, thus providing a simple method for studying the first steps in the reaction. Bigger-Gehring (1955) incubated her reaction mixtures in Warburg manometers, but no carbon dioxide was formed. This is not surprising, since carboxylase is inactive at above pH 7, and Bigger-Gehring worked at the optimum pH for transamination (pH 7.8).

SUMMARY

1. Washed-cell suspensions of *Saccharomyces cerevisiae* form tyrosol from L-tyrosine anaerobically in the presence of glucose. The presence of glucose is essential.

2. Formation of tyrosol by whole cells is inhibited by 2:4-dinitrophenol and sodium azide.

3. Cell-free extracts of *S. cerevisiae* incubated with 2-oxoglutarate and tyrosine form *p*-hydroxyphenylacetaldehyde, glutamic acid and carbon dioxide. The reaction is stimulated by pyridoxal phosphate.

4. Cell-free extracts of *S. cerevisiae* decarboxylate *p*-hydroxyphenylpyruvate.

5. Cell-free extracts oxidize reduced diphosphopyridine nucleotide on addition of *p*-hydroxyphenylacetaldehyde.

Fig. 6. Oxidation of DPNH by *p*-hydroxyphenylacetaldehyde. Reaction mixture (3.3 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 *p*-hydroxyphenylacetaldehyde was added. Optical density at 340 mμ was measured. Arrows indicate time of addition of *p*-hydroxyphenylacetaldehyde in the amounts indicated.

6. It is suggested that the formation of fusel oil involves the successive action of a transaminase, carboxylase and the alcohol dehydrogenase.

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Thermodynamic Quantities for the Dissociation Equilibria of Biologically Important Compounds

7. THE SECOND ACID DISSOCIATION OF GLYCEROL 1-PHOSPHATE*

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Glycerol 1-phosphate is widely distributed in animals, being a constituent of a large number of phospholipids, and it has been suggested that it is a precursor in the biosynthesis of lecithins (Kornberg & Pricer, 1952). It also occurs as an intermediate in the conversion of glycerol into glyceraldehyde phosphate. These enzyme-catalysed reactions are probably highly sensitive to the charge on the glycerophosphate ion, so accurate information about its dissociation constants would be necessary in any kinetic investigations. Further, it is of interest to compare its dissociation constant and the related thermodynamic quantities with

those for the isomeric glycerol 2-phosphate, which have already been determined (Ashby, Crook & Datta, 1954*b*).

EXPERIMENTAL

The apparatus and technique for the measurements in cells without liquid junction were, in general, the same as have been described previously (Ashby, Crook & Datta, 1954*a*; Ashby, Clarke, Crook & Datta, 1955). Two stock solutions were made containing disodium glycerol 1-phosphate and HCl. In these solutions the proportions of disodium glycerol 1-phosphate to sodium hydrogen glycerol 1-phosphate were 0.7077:1 (stock 1) and 1.4883:1 (stock 2). Twelve diluted solutions were made from each stock solution, the ionic strengths being from 0.02 to 0.09.

* Part 6: Clarke & Datta (1956).