The Reduction of 5-Oxodecanoic Acid by Normal Baker's Yeast

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1. A description is given of the course of the reduction of 5-oxodecanoic acid to 5-hydroxydecanoic acid by intact cells of baker's yeast and of the influence of pH on this reduction. As the pH of the medium is decreased the rate of uptake of the keto acid by yeast increases. However, the more the rate of uptake increases the more rapidly the yeast is poisoned by the keto acid or hydroxy acid or both. Consequently the optimum pH is at approx. 5. 2. In baker's yeast the conversion of 5-oxodecanoic acid takes place mainly in the mitochondria. 3. The conversion is strongly influenced by ATP, NADPH and Mg^{2+} or Mn^{2+} , and to a smaller extent by CoA-SH. The NADP can only partly be replaced by NADH. Thus the reduction seems to be NADPH-dependent. A mechanism for the conversion is proposed. 4. Like the reduction by intact cells, the reduction by an isolated mitochondrial fraction proceeds stereospecifically.

Optically active lactones of various δ -hydroxy fatty acids contribute considerably to the typical character of natural butter flavour (Boldingh & Taylor, 1962). These stereoisomers can be obtained in the form of their δ -hydroxy acids by reduction of the corresponding keto acids by certain micro-organisms, e.g. normal baker's yeast (*Saccharomyces cerevisiae*) (Tuynenburg Muys, Van der Ven & De Jonge, 1962a,b, 1963). Subsequently the hydroxy acids can easily be converted into lactones by heating *in vacuo* (Tuynenburg Muys *et al.* 1962a,b, 1963). To gain an insight into the character of the reduction in question and into the nature of the enzymes involved, the conversion of 5-oxodecanoic acid, with normal baker's yeast, has been further studied. The present paper gives a description of: the course of the reduction by means of intact cells; the localization of the conversion in the yeast cell; the reduction by means of an isolated mitochondrial fraction; the influence of various cofactors on the conversion mentioned. Part of the results of these experiments have already been published (Francke, 1963).

MATERIALS

Baker's yeast was obtained from the Royal Netherlands Fermentation Industries Ltd., Delft, The Netherlands. Unless stated otherwise the yeast was used immediately on receipt.

NAD⁺, NADH, NADP⁺ and NADPH were commercial products from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; CoA-SH (purity 75%), ATP (disodium salt tetrahydrate, purity 94-100%), GSH, DL-isocitric acid (trisodium salt, allo-free) and α -oxoglutaric

acid were from Sigma Chemical Co., St Louis, Mo., U.S.A.; fumaric acid was from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; AMP was obtained from Zellstofffabrik Waldhof, Wiesbaden, Germany; cocarboxylase was from F. Hoffmann-La Roche and Co. Ltd., Basel, Switzerland.

2,4-Dinitrophenylhydrazine, anhydrous Na₂CO₃, conc. HCl, KH₂PO₄ and succinic acid were analytical-grade preparations from E. Merck A.-G., Darmstadt, Germany; hydroxylamine hydrochloride, FeCl₃·6H₂O, K₂HPO₄ and citric acid were analytical-grade reagents obtained from British Drug Houses Ltd., Poole, Dorset. Commercial ether was made peroxide-free and distilled over KOH pellets.

Ballotini glass beads (grade 12) were supplied by H. Mickle, Mill Works, Gomshall, Surrey. The dialysis tubing was obtained from Kalle A.-G., Wiesbaden-Biebrich, Germany. Centrifugation was carried out in the cooled P15 K Eispirouette (Phywe A.-G., Göttingen, Germany).

2,4-Dinitrophenylhydrazine reagent. 2,4-Dinitrophenylhydrazine (250 mg.) was dissolved in 100 ml. of 2N-HCl at room temperature, after which the solution was kept at 4° for 16 hr. The solution was then filtered through a glass-filter crucible. The reagent was stored at 4°, at which temperature it could be kept for approx. 2 weeks.

5-Oxodecanoic acid. This acid was prepared by oxidizing 2-pentylcyclopentanone with chromic acid according to the method of Ruzicka, Seidel, Schinz & Pfeiffer (1948) for the oxidation of 1,1,7-trimethylcycloheptanone. The keto acid was purified by recrystallization from light petroleum (b.p. 40-60°) to m.p. 53.7-55.2° (corr.). The procedure was developed by G. Lardelli and A. P. de Jonge.

NADPH-specific isocitrate dehydrogenase. This dehydrogenase was isolated from baker's yeast according to the method of Kornberg & Pricer (1951).

Cell-free homogenate. The cell-free homogenate was obtained by disintegrating the yeast in a mechanical shaker of the type used by Nossal (1953), and by centrifuging

off the intact cells and cell debris. The shaker, which was designed by J. IJlstra and H. van Kampen of the Laboratory for Biochemistry and Biophysics (Technological University, Delft, The Netherlands), was housed in a constant-temperature room at 0°. Into each cuvette were introduced 8 ml. of 0.9% KCl solution, 10 g. of Ballotini glass beads (grade 12) and 2 g. of yeast. After being closed, the tubes were cooled in ice-water for 15 min. Then the suspension was shaken twice for 30 sec. Between the two periods of shaking and after completion of the experiment the cuvettes were again cooled in ice-water for 10 min. The glass beads were removed by allowing them to settle followed by careful decantation of the crude homogenate. Intact cells and cell debris were removed by centrifuging the homogenate for 3 min. at 600g at 0°. Starting from 4 g. of yeast, 12.5 ml. of cell-free homogenate containing 40 mg. dry wt. of yeast material/ml. was obtained in this way. Dry weights were measured at 110°. From the microscopic picture of the crude homogenate (dilution 1:100) after staining with methylene blue, the percentage disintegration was estimated to be 60–70. The temperature of the suspension after destruction never exceeded 8–10°.

Mitochondrial fraction. The mitochondrial fraction was isolated in the following way. The crude homogenate obtained from one or more batches of 4 g. of yeast in the above-described way was centrifuged at 600g for 10 min. and the resultant supernatant was centrifuged at 10000g for 60 min. The sediment was washed twice with 0.02M-potassium phosphate buffer, pH 6.0 (5 ml. per portion of mitochondria obtained from 4 g. of yeast). The mitochondria were again isolated by centrifuging at 10000g for 60 min. All operations were carried out at 0°.

METHODS

Incubations. Cell suspensions were prepared as follows. In a 250 ml. stoppered flask 10 g. of glucose was dissolved in 100 ml. of 0.1M-citric acid–0.2M-potassium phosphate buffer at the required pH (McIlvaine, 1921) and 50 ml. of water. Then a solution of 360 mg. of 5-oxodecanoic acid in 30 ml. of water, brought to the required pH with 2N-KOH, was added and the flask rinsed with 10 ml. of water. After the resultant solution had been warmed to 30°, a suspension of 13 g. of pressed yeast in 10 ml. of water at 30° was added with stirring. Subsequently the flask was closed with a cork fitted with a glass tube for the removal of the CO₂ formed during fermentation, and placed in a thermostat at 30°. The moment the yeast was mixed with the solution to be investigated was regarded as zero time. The cell suspensions were incubated at 30° for 24 hr. The yeast was kept in suspension by stirring.

The incubation experiments with enzyme preparations were carried out in Thunberg tubes. The relevant enzyme solution was added at 0° after addition of the other components. The tubes were evacuated, flushed twice with nitrogen and evacuated again. After the tubes had been brought to 30° in a water bath at 35°, they were incubated at 30° for 3 hr. The reaction was stopped by adding 25% (v/v) H₂SO₄ to give pH 2. Protein was determined by the biuret method (Gornall, Bardawill & David, 1949).

Keto acid and hydroxy acid analyses. For the determination of the amounts of hydroxy acid and unconverted keto acid present in the medium at different times during the

experiments with the cell suspensions, 14 ml. samples of the suspension were pipetted off at given intervals and immediately centrifuged for 3 min. at 900g. As determination of the two acids in the same sample is difficult, the amounts of unconverted keto acid and formed hydroxy acid were determined in two separate 5 ml. samples. After acidification to pH 1 the samples were extracted with ether. The keto acid and hydroxy acid were determined respectively as the 2,4-dinitrophenylhydrazone in alkaline solution at 530 m μ and as the Fe(III)–hydroxamic acid complex at 520 m μ . The determination was carried out in 1 cm. cuvettes in the Unicam SP.500 spectrophotometer. The hydroxamic acid of the hydroxy acid can be easily obtained via the lactone, which gives a hydroxamic acid with alkaline hydroxylamine.

The initial content of keto acid in the various suspensions was determined separately. To this end a cell suspension of pH 8.0 was prepared and the cell-free liquor immediately analysed for keto acid. As shown below, at a lower pH the amount of keto acid in solution decreases so rapidly during the first minutes of fermentation that a considerable consumption would occur during sampling.

Extraction of keto acid and hydroxy acid. (a) From the fermentation liquors. After acidification with 25% H₂SO₄ to pH 1, the samples to be investigated were extracted, those for the keto acid analysis 3 times and those for the hydroxy acid analysis 4 times, with equal volumes of ether. Subsequently the ether extracts for the keto acid analysis were washed 6 times and those for the hydroxy acid analysis 3 times with 0.1 vol. of water. The amounts of water used for washing the hydroxy acid extract were combined and extracted with an equal volume of ether. This ether extract was extracted with 0.5 ml. of water, after which the washed extract was added to the main ethereal hydroxy acid solution.

The ethereal keto acid solution was evaporated and the residue, in a 10 ml. measuring flask, taken up in 5 ml. of water to which 1 drop of 2N-KOH had been added. The solution was diluted to 10 ml. with 0.2N-H₂SO₄ and used for the keto acid determination. The further treatment of the ethereal hydroxy acid solution is described below.

The fermentation liquors contain a considerable amount of pyruvic acid, which interferes with the keto acid determination. Separate experiments showed, however, that the ethereal 5-oxodecanoic acid solution can be washed free of pyruvic acid in the way indicated above, whereas over 99% of the 5-oxodecanoic acid is retained in the ether. The quantitative removal of pyruvic acid (and the absence of other short-chain keto acids) was checked by paper chromatography of the 2,4-dinitrophenylhydrazone preparation with butan-1-ol–ethanol–water (5:1:4, by vol.) as solvent according to the method of Cavallini, Frontali & Toschi (1949).

(b) From the incubation mixtures. After being cooled to room temperature the mixtures were acidified with 25% H₂SO₄ to pH 1 and subsequently, both before and after the addition of 0.5 g. of KCl, were heated to 100° for 10 min. to precipitate the proteins present. After cooling the mixtures were centrifuged at 600g for 10 min. The sediment was extracted, for the keto acid analysis 3 times and for the hydroxy acid analysis 4 times, with 6 ml. portions of ether by stirring. In the final extraction the sediment was also thoroughly shaken with the ether. After each extraction the ether was carefully poured off and added to the cor-

responding supernatant, which was treated in the same way as were the fermentation liquors.

Determination of keto acid. A 0.5 ml. portion of the keto acid solution was pipetted into a small test tube. Then 0.5 ml. of 2,4-dinitrophenylhydrazine reagent was added and the solutions were carefully mixed. Neither the fermentation liquors from the experiments with intact cells nor the enzyme preparations gave a 2,4-dinitrophenylhydrazone value when incubated under the same conditions as in the experiments proper but without 5-oxodecanoic acid. Therefore for the blank experiment 0.5 ml. of 0.2N- H_2SO_4 was used as starting material, which received the same treatment as the actual sample. After closing, the tube was kept for 1 hr. at room temperature and then for 1 hr. at 0°. The contents of the tube were then transferred into a 25 ml. separating funnel, the tubes being rinsed with five 1 ml. portions of ether at 0°. The water-ether mixture was shaken for 2 min., after which the water layer was removed. The ethereal solution was first washed with 0.5 ml. of water and then extracted successively with one 3 ml., three 2 ml. and one 1 ml. portions of 5% (w/v) Na_2CO_3 solution. To 5 ml. of the resultant Na_2CO_3 extract 5 ml. of 0.3N- NaOH was added, immediately after which the extinction was measured at 530 $\text{m}\mu$ (molar extinction approx. 12000) against that of the blank. The total amount of keto acid in the initial sample was calculated from the extinction of this solution. The recovery was 98–102%.

Determination of hydroxy acid. The ethereal hydroxy acid solution of the extraction mentioned above was dried over 1 g. of anhydrous Na_2SO_4 . The Na_2SO_4 was filtered off and the filtrate collected in a 25 ml. pear-shaped flask. The Na_2SO_4 was washed on the filter with three 2 ml. portions of dry ether. The ethereal solution was evaporated *in vacuo* on a water bath at room temperature. Subsequently a water cooler was attached to the flask which was then heated *in vacuo* for 60 min. in boiling water. Model experiments showed that under these conditions the hydroxy acid is quantitatively converted into the lactone. After the apparatus had been cooled to room temperature, the cooler was rinsed with three 1 ml. portions of 96% (v/v) ethanol.

The reaction flask and the ground joint of the cooler were washed with respectively five 1 ml. and one 1 ml. portions of ethanol. All the ethanol was collected in a 10 ml. measuring flask, which was made up to the mark. Then 0.5 ml. of the resultant solution was pipetted into a large test tube, which was immediately closed. For the blank experiment 0.5 ml. of 96% ethanol was used as starting material. This 0.5 ml. was used for the determination of hydroxy acid via the Fe(III) complex of the corresponding hydroxamic acid. The procedure was analogous to that used by Baggett, Engel & Fielding (1955) for the determination of the number of esterified hydroxyl groups in sterols. From the extinction at 520 $\text{m}\mu$ measured against that of the blank, the total amount of hydroxy acid in the initial sample was calculated. The recovery of the determination was 97–102%.

Identification of the hydroxy acid formed. The hydroxy acid formed in the experiments with intact cells had already been identified in the original screening tests (Tuyneburg Muys *et al.* 1962b, 1963). In all experiments with enzyme preparations, however, the hydroxy acid formed was invariably identified. This identification was carried out by paper-chromatographic analysis of the hydroxamic acid

of the hydroxy acid. To this end approx. 50 μg . of the lactone fraction to be investigated was applied to a strip of Whatman no. 1 paper next to 50 μg . of authentic 5-hydroxydecanoic acid lactone. Each spot was treated with 2 drops of alkaline hydroxylamine reagent (Baggett *et al.* 1955) and then chromatographed either with the benzene-propan-2-ol-water-acetic acid (5:2:5:1, by vol) system (R_f 0.60) or with the benzene-water-acetic acid (5:5:1, by vol.) system (R_f 0.33) by the method described by Keeney (1957). After development the strips were dried in air and subsequently sprayed with an aq. 1% (w/v) FeCl_3 solution.

RESULTS

Reduction by intact cells. Fig. 1 shows the course of the decrease in the amount of keto acid and of the increase in the amount of hydroxy acid in solution during incubation at pH 4.8. The conversion proceeds most rapidly during the first 2 hr. of incubation. Then the reaction rate decreases but the conversion goes on continuously. In the solution a material deficit of approx. 10% is observed after 30 min. incubation, which deficit then remains constant. At this stage of the conversion the disappearance of the keto acid results in the formation of a corresponding amount of hydroxy acid.

Fig. 1. Course of the reduction of 5-oxodecanoic acid by baker's yeast. ○, 5-Oxodecanoic acid in solution; Δ, 5-Hydroxydecanoic acid in solution.

Fig. 2. Influence of pH on the reduction of 5-oxodecanoic acid by baker's yeast. ●, pH 4.1; Δ, pH 4.8; ▲, pH 6.0; ○, pH 8.0.

After 24 hr. approx. 80% of the original amount of keto acid is present in the medium as hydroxy acid.

The influence of the pH on the conversion has been studied from the decrease in the amount of keto acid in solution starting from suspensions of pH 4.1, 4.8, 6.0 and 8.0 respectively (Fig. 2). Even on using buffered solutions in the experiments in which the initial pH of the incubation mixtures was respectively 6.0 and 8.0, the pH of the medium decreased considerably during the first 2 hr. of incubation owing to the formation of carbon dioxide during fermentation. For this reason the pH was continually adjusted in these experiments by adding 4.5N-potassium hydroxide. Corrections were made for the changes in volume due to the addition of alkali.

During the first hour of the conversion (Fig. 2) the keto acid disappears more rapidly from the medium the lower the pH. At pH 8 the decrease is very slight. Disappearance of the keto acid continued over a longer period at pH 4.8 than at the other pH values.

Localization of the conversion in the yeast cell. The various yeast fractions obtained on fractionation of 5 ml. of the cell-free homogenate by centrifuging according to Scheme 1 were incubated with the keto acid in the presence of NADP⁺ and DL-isocitric acid. These compounds were added in view of the results obtained in the experiments with an acetone-dried powder of baker's yeast (Francke, 1963).

Observed under the phase-contrast microscope the cell walls appear as empty vesicles in which, however, there are still some particles, of the size and shape of mitochondria, which are not removed even by repeated washings. So the cell-wall fraction is probably contaminated with mitochondria.

On incubating with Janus green as described by Linnane & Still (1955), the mitochondrial fraction showed the specific staining of mitochondria. This fraction oxidizes succinic acid and isocitric acid, and, to a smaller extent, also fumaric acid (Table 1).

Plate 1 shows a representative part of the picture

Scheme 1. Fractionation of the whole cell-free homogenate.

under the electron microscope. The particles were fixed with osmic acid at pH 7.0, dehydrated and embedded in polyester resin (Vestopal W; W.M. Jaeger, Vésenaz, Switzerland) according to Kellenberger's 'standard' technique (Ryter, Kellenberger, Birch-Andersen & Maaløe, 1958). Thin sections were cut with a glass knife (LKB Ultratome 4800; LKB-Produkter A.B., Stockholm, Sweden) and examined with the Siemens Elmiskop I (Siemens und Halske A.-G., Berlin, Germany). The electron micrograph was taken at an initial magnification of 15000 $\times$ and enlarged photographically. The micrograph shows the internal membranes characteristic of the mitochondrial structure (Palade, 1953). However, the particles are considerably swollen and in many of them the cristae have vanished. Mitochondria debris is clearly seen on the micrograph. Nuclei, which have a much greater size and a different structure, and storage granules, with a greater electron density and lack of internal structure (Agar & Douglas, 1956), are completely absent. The double-membrane debris visible on the micrograph is undoubtedly derived from damaged mitochondria, since the internal cellular membranes are much thicker (Agar & Douglas, 1956). There are also vesicle-like bodies connected to double membranes, which are, however, also seen in the mitochondrial debris. Membranous filaments resembling the endoplasmic reticulum occurring in the cells of higher organisms are not distinctly present on the micrograph. Therefore the vesicle-like bodies and the proteinous material is thought to be derived from damaged mitochondria, formed either during disintegration and isolation or by the fixation procedure.

The cell-free homogenate, the $S_{800}^{6.5}$ fraction and the 'soluble fraction' were investigated as such. After isolation, the cell walls and mitochondria

Table 1. *Oxidation of Krebs-cycle substrates by the mitochondrial fraction*

Standard Warburg technique was used; the temperature was 30°. Each manometer cup contained 2.25 ml. of 0.9% KCl in 0.03M-potassium phosphate buffer, pH 7.0, containing AMP (5 μ moles), NAD⁺ (0.1 μ mole), NADP⁺ (0.1 μ mole), thiamine pyrophosphate (0.5 μ mole), MgCl₂ (1 μ mole), MnCl₂ (0.5 μ mole), cysteine (10 μ moles), substrate (30 μ moles) (DL-isocitric acid, however, 60 μ moles) and 0.75 ml. of mitochondrial suspension in the same KCl-phosphate solution; the protein content was 4.4 mg. Oxygen consumption was measured for 20 min.

Substrate	O ₂ uptake (μ l./mg. of protein/hr.)
None	9
Succinate	78
Fumarate	14
Isocitrate	30

EXPLANATION OF PLATE 1

Electron micrograph of a thin section of the mitochondrial fraction fixed with osmic acid at pH 7.0.
Magnification 45 000 $\times$.

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Table 2. *Reducing power of the various yeast fractions*

The composition of the incubation mixtures was as follows: 5 ml. of yeast fraction and 1 ml. of 0.06M-potassium phosphate buffer, pH 6.0, containing 5-oxodecanoic acid (30 μ moles); NADP⁺ (1 μ mole) and DL-isocitric acid (40 μ moles). Before incubation the pH of the mixtures was adjusted to 6.0. The mixtures were incubated *in vacuo* for 3 hr. at 30°.

Expt. no.	Fraction investigated	Protein content (mg./ml.)	Hydroxy acid formed (μ moles)
1	Cell-free homogenate	18.3	12.6
2	S ₈₀₀ ⁵ fraction	16.6	13.8
3	'Soluble fraction'*	13.1	3.0
4	Cell-wall fraction*	1.4	2.1
5	Cell wall fraction + 'soluble fraction'*	14.6	3.9
6	Mitochondrial fraction	3.8	12.6

* In these experiments NADPH-specific isocitrate dehydrogenase was added (40 μ g. per mixture).

were washed twice with 5 ml. of potassium phosphate buffer, pH 6.0, and then suspended in 5 ml. of 0.9% potassium chloride solution in 0.02M-phosphate buffer, pH 6.0. The cell-wall fraction was also investigated in combination with the 'soluble fraction'. In these experiments the cell walls were suspended in the corresponding 'soluble fraction'. NADPH-specific isocitrate dehydrogenase, required for the regeneration of the NADPH, was added separately to ensure its presence in the cell wall plus 'soluble fraction'. The results are given in Table 2.

After fractionation, the activity of the cell-free preparation is found in the mitochondrial fraction. The specific activity of the 'soluble fraction' is low as compared with that of these particles. The cell-wall fraction has a specific activity about half that of the mitochondrial fraction. Combining the cell walls with the 'soluble fraction' does not increase the activity with respect to that of the 'soluble fraction' as such.

Reduction by the mitochondrial fraction. The mitochondrial fraction obtained from 1.6 g. of yeast was suspended in 4 ml. of 0.06M-potassium phosphate buffer, pH 6.0, and incubated *in vacuo* for 3 hr. at 30° with 30 μ moles of 5-oxodecanoic acid dissolved in 2 ml. of 0.06M-potassium phosphate buffer, pH 6.0 (protein content 3.0 mg./ml.). After incubation the residual amount of keto acid was 76%, and 23% of hydroxy acid had been formed. Storing the mitochondrial fraction in 0.002M-phosphate buffer, pH 6.0, for 30 hr. at 0° did not influence its activity. The R_f found by paper chromatography for the hydroxamic acid of the hydroxy acid to be identified corresponded to

that of the hydroxamic acid obtained from authentic 5-hydroxydecanoic acid lactone.

For the determination of the specific rotation of the lactone obtained via reduction with the mitochondrial fraction the preparation obtained from 64 g. of yeast was suspended in 200 ml. of 0.06M-potassium phosphate buffer, pH 6.0. The resultant suspension was divided into 5 ml. portions, and each portion was incubated with 1 ml. of phosphate buffer, pH 6.0, 30 μ moles of 5-oxodecanoic acid, 3 μ moles of NADP⁺ and 50 μ moles of DL-isocitric acid (final protein content 3.0 mg./ml.). After incubation the mixtures were combined and the hydroxy acid was extracted in the same way as from the samples for the hydroxy acid determination. After evaporation of the ether the crude hydroxy acid fraction, which also contained the unconverted keto acid and a considerable amount of fatty acids from the yeast, was dissolved in 25 ml. of 0.5N-sodium hydroxide. The unsaponifiable residue was then removed by extracting the soap solution four times with 10 ml. of light petroleum (b.p. 40–60°). After acidification of the soap solution the hydroxy acid was again extracted and the ethereal solution evaporated *in vacuo*. The residue was heated *in vacuo* for 1 hr. at 100° to convert the hydroxy acid into its lactone. Subsequently the crude lactone was fractionated via short-path distillation at 0.005 mm. Hg. The fraction formed between 90° and 110° was collected. The free acids present in this fraction were removed by treatment with triethanolamine (Tuynenburg Muys *et al.* 1963). A yield of 82 mg. of substance was obtained in this way; the content of 5-hydroxydecanoic acid lactone was 72%. This fraction was once more subjected to short-path distillation. Finally, a yield of 53 mg. of substance having a lactone content of 83% was obtained. This fraction was dissolved in 1.5 ml. of methanol. In 1.2 ml. of the resultant solution the rotation was determined in a Bellingham and Stanley polarimeter. A value of +3.25° was found (temperature 22°; D line); consequently $[\alpha]_D^{22} = +55.5^\circ$. This value corresponds with that of pure lactone prepared by using intact cells (Tuynenburg Muys *et al.* 1963). By gas-liquid chromatography it was found that the 17% of impurities of our preparation obtained from the experiments with isolated mitochondria consist of non-rotating material such as normal fatty acids and 5-oxodecanoic acid.

Influence of various cofactors. The influence of various cofactors on the conversion was ascertained from reduction experiments with a dialysed mitochondrial fraction. The fraction obtained from 64 g. of yeast was suspended in 40 ml. of 0.002M-potassium phosphate buffer, pH 6.0, and dialysed against a flow of 0.002M-phosphate buffer, pH 6.0 (50–60 l. in all), for 30 hr. at 0°. Then the suspension

Table 3. *Influence of various cofactors on the conversion of 5-oxodecanoic acid by baker's yeast*

The composition of the incubation mixtures was as follows: 4 ml. of mitochondrial suspension and 2 ml. of 0.06 M-potassium phosphate buffer, pH 6.0, containing 5-oxodecanoic acid (30 μ moles) and the cofactors and co-substrates indicated below. The amounts of cofactors and co-substrate added were: NAD⁺, 2 μ moles; NADP⁺, 2 μ moles; NADH, 6 μ moles; NADPH, 6 μ moles; ATP, 30 μ moles; CoA-SH, 1.5 μ moles; GSH, 70 μ moles; DL-isocitric acid (trisodium salt), 50 μ moles; MgCl₂, 4 μ moles; MnCl₂, 4 μ moles. The protein content was 3.0 mg./ml. Before incubation the pH of the mixtures was adjusted to 6.0. The mixtures were incubated *in vacuo* for 3 hr. at 30°.

Expt. no.	Cofactors, metal ions and substrates added	Activity (% of that of complete system)
1	Blank	7
2	NADP ⁺ , DL-isocitric acid, ATP, CoA-SH, Mg ²⁺ , Mn ²⁺ and GSH	100
3	Complete system—NADP ⁺	29
4	Complete system—DL-isocitric acid	64
5	Complete system—ATP	32
6	Complete system—CoA-SH	68
7	Complete system—Mg ²⁺	75
8	Complete system—Mn ²⁺	64
9	Complete system—Mg ²⁺ —Mn ²⁺	29
10	Complete system—GSH	89
11	NADPH, ATP, CoA-SH, Mg ²⁺ , Mn ²⁺ and GSH	100
12	NADPH, ATP, CoA-SH, Mn ²⁺ and GSH	89
13	NADPH, ATP, CoA-SH, Mg ²⁺ and GSH	86
14	NADPH, ATP, CoA-SH and GSH	25
15	NADH, ATP, CoA-SH, Mg ²⁺ , Mn ²⁺ and GSH	29
16	Complete system, but mitochondrial suspension boiled for 10 min.	7

was diluted to 160 ml. with 0.06 M-potassium phosphate buffer, pH 6.0, and thoroughly homogenized in an all-glass Potter-Elvehjem homogenizer. The resultant suspension was incubated with the keto acid in 4 ml. portions in the presence of various cofactors and metal ions, either with or without the addition of DL-isocitric acid (Table 3).

After dialysis the capacity to reduce keto acid had disappeared but was restored by the addition of NADP⁺, DL-isocitric acid, ATP, CoA-SH, Mg²⁺, Mn²⁺ and GSH. The combination NADP⁺ and DL-isocitric acid can be replaced by NADPH but not by NADH. The enzymic character of the conversion is clear from Expt. 16: on heating the mitochondrial suspension the activity disappeared.

In all cases the isolated hydroxy acid was identical with 5-hydroxydecanoic acid, and after

lactonization the relevant fractions contained no other substances giving a hydroxamic acid with alkaline hydroxylamine, as appeared from the paper-chromatographic analysis.

DISCUSSION

The material deficit that, on reduction with intact cells, was found in the solution after 30 min. incubation is due to the fact that part of the keto acid and the hydroxy acid is retained in the yeast. If after 3 and 24 hr. incubation both the solution and the yeast are investigated for keto acid and hydroxy acid, no material deficit can be detected.

As the pH of the medium increases the conversion of keto acid proceeds less rapidly and the amount converted decreases (Fig. 2). It is known that pyruvic acid (Barron, Ardao & Hearon, 1950) and lactic acid (Rothstein, 1956) can enter the yeast cell only in the non-dissociated form. Presumably a similar phenomenon occurs with 5-oxodecanoic acid. The pK of this acid is 5.10 at 25° (determined potentiometrically), so that at pH 4.1, 4.8, 6.0 and 8.0 respectively 91, 66, 11 and 0.13% of the acid is in the undissociated form. The initial rate of uptake of the keto acid at pH 4.1, 4.8 and 6.0, studied under the same conditions as in the incubation experiments but without glucose and at 25°, was found to be 0.68, 0.44 and 0.23 μ mole/min./g. wet wt. of yeast respectively. At pH 8.0 no uptake could be detected. The initial uptake at pH 6.0 is relatively high, but the other values are in agreement with the relative concentrations of undissociated acid at the various pH values. At pH 8.0 the uptake in the incubation experiments can be explained from the fact that during fermentation the pH of the outer region of the yeast cell, which is normally 5.8, is markedly decreased (Conway & Downey, 1950).

Tuynenburg Muys *et al.* (1962b) showed that relatively high concentrations of 5-oxo acid or 5-hydroxy acid or both are poisonous to the yeast. Consequently a decrease in the pH in the reduction of 5-oxodecanoic acid by intact cells has two effects: a favourable effect with regard to the uptake of keto acid by the yeast cell and an unfavourable effect with regard to the poisoning of the yeast as a result of the increased uptake of the acid. Consequently in the reductions mentioned the optimum pH is approx. pH 5.

The existence of mitochondrial organelles in baker's yeast has been demonstrated in various ways (Mudd *et al.* 1951; Agar & Douglas, 1956; Linnane & Still, 1955). According to Agar & Douglas (1956) they are rod-shaped and have a maximum length of about 1.5 μ and an average diameter of 0.2–0.3 μ , and the internal membranes run parallel to the long axis of the mitochondrion. In the electron micrograph the profiles are pre-

dominantly circular or elliptical, as is to be expected if the particles are randomly distributed in the preparation. The fraction in question shows the specific staining and oxidizing properties of mitochondria. From the electron microscopy, in my opinion the fraction designated as mitochondrial fraction consists of mainly mitochondrial material. The percentage of intact mitochondria, however, is probably low.

The activity of the cell-wall fraction and the 'soluble fraction' is probably due to contamination with respectively mitochondria and enzymes originating from these particles. This conclusion is based on the appearance of the cell walls under the phase-contrast microscope (see the Results section). The protein content of the cell walls of baker's yeast is very low (6–7% of the dry weight; Roelofsen, 1953; Falcone & Nickerson, 1956), so that a slight contamination with mitochondria will give a high specific activity. The release of enzymes from the mitochondria involved in the conversion of 5-oxodecanoic acid during disintegration was clearly demonstrated by shaking washed mitochondria in the same way as intact cells. After centrifugation, the supernatant of this preparation had a considerably higher activity (about one-fourth of that of the original particles) than in the normal washing procedure. The combined 'soluble' and cell-wall fraction is incapable of fully converting the keto acid, since there is no increase in activity with respect to that of the 'soluble fraction' as such. In my opinion therefore the conversion of 5-oxodecanoic acid in yeast takes place mainly in the mitochondria. The increase in activity after removal of the cell-wall fraction from the whole

cell-free preparation, which gives the $S_{600}^{6.5}$ fraction, cannot be explained for the moment.

ATP and Mg^{2+} (or Mn^{2+} ; these ions are interchangeable) strongly influence the conversion; CoA-SH also has a stimulating effect, although to a smaller extent. For these reasons it seems likely that the keto acid is not converted as such, but as the CoA thio ester. Since the combination $NADP^+$ and DL-isocitric acid can be completely replaced by NADPH the activating effect of isocitric acid is probably connected with the regeneration of NADPH required for the reduction. In the experiments with an acetone-dried powder of baker's yeast fumaric acid had the same effect as DL-isocitric acid (Francke, 1963). Nossal, Hansen & Ladd (1957) showed, however, that after incubation of fumarate with a baker's yeast preparation that corresponds to the $S_{600}^{6.5}$ fraction from my experiments not only malate but also other Krebs-cycle substrates were formed. So it is also possible that in the experiments with the acetone-dried powder D-isocitric acid is formed from fumaric acid. The source of the reducing power in Expt. 4 (Table 3) in which isocitric acid was omitted is not known. The NADPH cannot be replaced by NADH, so that the reduction seems to be NADPH-dependent. The low activity that occurs on the addition of NADH may also be due to the fact that during incubation NADPH is formed from the added NADH, for in all these experiments ATP was used.

In all experiments with enzyme preparations the hydroxy acid could be isolated from the mixtures in the theoretically expected amount without additional hydrolysis. If the keto acid is converted as the CoA thio ester the final step must be a splitting

off of the CoA·SH from its ester with the hydroxy acid. This can take place either via a deacylation or via a transthioylation.

In view of the above facts I suggest for the conversion the reaction sequence shown in Scheme 2. It seems likely that the enzymes playing a part in these reactions will either strongly resemble those involved in the normal fatty acid metabolism or even be identical with them. Consequently the first and the last reactions are presumably catalysed by enzymes of respectively the thiokinase (or CoA·SH-transferase) type and the deacylase (or CoA·SH-transferase) type. For the time being the actual reducing enzyme has, on the analogy of the β -oxo acid reductase, been designated reductase. However, to ascertain the true character of these enzymes, they must first be isolated.

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