# THE MECHANISM OF YEAST RESPIRATION

## I. PREPARATION AND PROPERTIES OF ACTIVELY RESPIRING CELL-FREE YEAST EXTRACTS

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Despite the widespread use of yeast in biochemical investigation, the mechanism by which it converts carbohydrate through acetate to carbon dioxide remains unknown. The school of KREBS[1,2], using dry-ice frozen cells which were permeable to di- and tricarboxylic acids, has produced evidence against complete oxidation of acetate via the tricarboxylic acid cycle[3] (TAC)**. On the other hand, LYNEN AND NECIULLAH[4] showed several reactions of the TAC in frozen cells. WEINHOUSE AND MILLINGTON[5], studying the conversion of trideuteroacetate to succinate by fresh yeast cells, observed that the labelling of succinate was similar to that calculated assuming that the acetate had been metabolized via the TAC. MARTIUS AND LYNEN[6], LYNEN[7] and FOULKES[8] have all given evidence for the TAC in yeast, and all the individual steps have been described (Table I). HIRSCH[19] has also found high fumarase, aconitase, and *iso*citric dehydrogenase activities in yeast extracts.

TABLE I

OCCURRENCE OF TRICARBOXYLIC ACID CYCLE ENZYMES IN YEAST

| | |
|---|---|
| Acetate $\longrightarrow$ citrate | Intact cells in the presence of $Ba^{++}$ or $Mg^{++}$[5,9] |
| Acetate + OAA $\rightleftharpoons$ citrate | Extracts and crystalline enzyme[10] |
| Citrate $\rightleftharpoons$ *cis*-aconitate $\rightleftharpoons$ *iso*citrate | Extracts[8,11] |
| *iso*Citrate $\rightleftharpoons$ oxalosuccinate | Purified enzymes[12] |
| Citrate $\longrightarrow$ $\alpha$-ketoglutarate | Extracts[8] |
| $\alpha$-Ketoglutarate $\longrightarrow$ succinate | Intact cells (slowly) and extracts (faster)[13] |
| Succinate $\longrightarrow$ fumarate | Frozen cells[4] |
| | Intact cells at low pH[7] |
| | Isolated granules[14] |
| Fumarate $\rightleftharpoons$ malate | Extracts[15] |
| | Frozen and crushed cells[16] |
| Malate $\rightleftharpoons$ OAA | Frozen cells[14] |
| | Intact cells on free acids[17] |
| | Extracts[16] |
| OAA $\longrightarrow$ mixture of TAC acids | Extracts[18] |

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** The following abbreviations are also used: DPN and TPN, di- and triphosphopyridine nucleotide, respectively; DPNH, reduced DPN; TPP, thiamine pyrophosphate; A-5-P and ATP, adenosine 5'-mono- and triphosphates, respectively; CoA, coenzyme A; TRIS, tris(hydroxymethyl)aminomethane; and OAA, oxaloacetic acid.

*References p. 114/115.*

By using an efficient disintegrator, we have prepared cell-free extracts which oxidized endogenous and added substrates[18, 20, 21]; the optimum conditions for the preparation of such extracts are the subject of part of this report. Neither endogenous carbohydrate nor added acetate was oxidized completely to $CO_2$ and water. However, quantitative balance studies were conducted to see whether the TAC could account for the metabolism of various substrates. Since this work was completed, LINNANE AND STILL[22] have used the same high-speed disintegrator to prepare yeast particles claimed to resemble animal mitochondria.

## MATERIALS AND METHODS

*Yeast.* Effront baker's yeast, flown twice-weekly from Melbourne and stored at 2° in a humidor, was used throughout. The makers describe it as "a medium-fast baker's yeast, technically pure". We have found no microscopically visible contaminants, but plate cultures show occasional bacteria and wild yeasts. The actual strain used has no known number and was originally imported from Europe.

*Preparation of extracts.* The high-speed shaker is described elsewhere[20]. A mixture of 2 g yeast, 10 g Ballotini glass beads No. 12, and 10 ml disintegration medium was shaken for 10 sec at 0°. Usually, the material obtained from 6–7 disintegrations was pooled for one experimental run. 10 min centrifugation at 1000 *g* sufficed to remove glass, intact cells, and most cell debris. The resulting supernatant is the whole cell-free extract, *W*, about 6 ml of which was obtained from each disintegration.

Examination of extracts under phase-contrast illumination allowed distinction between intact and broken cells. Although our extracts contained small numbers of cell-wall fragments, contamination with intact cells was negligible.

Speed in preparation is essential to obtain actively respiring extracts: the first manometer reading was taken about 45 min after beginning disintegration. During all manipulations, the extracts were kept at 0–2° until incubated at 30°.

*Measurement of respiration.* Double-sidearm Warburg flasks of about 20 ml volume were used for the incubation. The substrates were placed in one sidearm, $H_2SO_4$ in the other, and the remaining components in the main compartment (the yeast extract being added last). Gas exchange was recorded in the usual manner. $CO_2$ evolution was measured at the end of the experiment by tipping in the $H_2SO_4$, and corrected for the bicarbonate content at zero time. Where $Q_{O_2}$ values are given they refer to $\mu$l $O_2$ taken up/mg dry wt/h at 30°, calculated from the mean of the first three 5-min readings. Dry weights were determined after drying the sample to constant weight at 130°.

*Substrates and cofactors.* These were the same as described previously[21].

*Estimations*

*Total hexose* was estimated by the anthrone method of TREVELYAN AND HARRISON[23]. More reproducible results were obtained when the reagent was made up 3 hours before use.

*Pentose* was determined by the orcinol method of MEIJBAUM, as described in UMBREIT *et al.*[24]. Undeproteinized samples or trichloracetic acid filtrates were used since tungstic acid interferes with the estimation.

*Acetate* was determined by the method of BARTLEY[25] with slight modifications. In addition to the precautions described by VON KORFF[26], the following notes may be useful in avoiding some of the difficulties. Different batches of AR. $Na_2SO_4$ gave widely different amounts of volatile acid on addition of $H_2SO_4$ alone. Samples that gave high blanks, and were therefore unsuitable for use, were heated for several days at 600° in a muffle furnace; this reduced the blank to a low figure. Attempts to use Celite, silica gel, or fused $Na_2SO_3$ in place of $Na_2SO_4$ were unsuccessful. The water in the receiving tube was neutralized to phenol red before the estimation. A good vacuum is essential.

*Pyruvate* was estimated, on samples deproteinized with 10% trichloracetic acid, by the method of FRIEDEMANN AND HAUGEN[27] or by spectrophotometric assay with lacticde hydrogenase and DPNH.

*Oxaloacetate* was estimated by the aniline citrate method of EDSON[28].

*Organic acids* were separated and estimated by silica gel chromatography. Several modifications were introduced into our earlier method[29]. The incubation mixture was acidified to make it 2.7 *N* with respect to $H_2SO_4$, adsorbed on Celite, and extracted with ether. We were unable to estimate citric acid by the earlier method, as this band merged into the $H_2SO_4$ coming off

*References p. 114/115.*

the column itself. The column also deteriorated visibly during development. By equilibrating the developing solvents against 0.05 $N$ $H_2SO_4$ all these difficulties were overcome; no $H_2SO_4$ was eluted and citric acid was estimated quantitatively. Equilibration with $H_2SO_4$ caused no increase in the blank values between the bands of known acids.

*Glutamic, aspartic and α-ketoglutaric acids* were estimated by the decarboxylase-transaminase method of KREBS[30].

*Diacetyl and acetoin* were determined qualitatively by the VOGES-PROSKAUER reaction[31]. Acetaldehyde was determined qualitatively by the appearance of a precipitate after microdiffusion into 2:4-dinitrophenylhydrazine; less than one μmole gives a visible precipitate.

## RESULTS

### *Effect of composition of disintegration medium on activity*

The $O_2$ uptakes of extracts prepared by 10 sec disintegration of yeast cells in media of differing compositions were measured with ethanol and succinate as substrates. Addition of fluoride and nicotinamide had no effect (Table II). As the composition of the medium seemed to have little influence on the oxidative ability of the extracts, most of the subsequent experiments were carried out in phosphate buffer pH 7.6 without further additions.

TABLE II

EFFECT OF DISINTEGRATION MEDIUM ON RESPIRATORY ACTIVITIES OF WHOLE EXTRACTS

Cells were disintegrated for 10 sec in 0.1 $M$ Na, K-phosphate buffer, pH 7.6, or other medium as shown. Each Warburg flask contained 3.0 ml $W$ and 400 μmole ethanol or sodium succinate in a final volume of 4.0 ml. Temp. 30°. The $O_2$ uptakes, expressed as μl/mg dry wt over the 90-min experimental period, have been corrected for the blank values.

| *Experiment* | *Composition of medium* | *$O_2$ uptake* | |
|---|---|---|---|
| | | *Ethanol* | *Succinate* |
| I | Buffer | 27 | 21 |
| | Buffer + 0.01 $M$ fluoride | 26 | 24 |
| | Buffer + 0.01 $M$ nicotinamide | 27 | 20 |
| | Buffer + fluoride + nicotinamide | 28 | 20 |
| II | Buffer | 26 | 22 |
| | 0.9% KCl | 22 | 18 |
| | Saturated lactose | 22 | 13 |
| | Water | 15 | 19 |

In a different series of experiments[32], 1% NaCl containing 0.01 $M$ versene and TRIS or glycylglycine buffer was also used successfully.

### *Substrates oxidized*

Ethanol, succinate, lactate, α-ketoglutarate, *iso*citrate, *cis*-aconitate, citrate, pyruvate, and glutamate, when added to cell-free extracts, all caused appreciable increases in $O_2$ uptake over the blank value[18]. Malate, fumarate, alanine, formate and glycollate were oxidized slowly. Acetate, glycine, aspartate and formaldehyde were not oxidized. For the purpose of comparing the effects of various factors on the respiration of the extracts, succinate and ethanol were the most commonly used substrates, the former representing an oxidation independent of pyridine coenzymes, and the latter one requiring DPN.

*References p. 114/115.*

*Cofactors required for various oxidations*

Succinate, lactate, and ethanol were oxidized at rates that were not increased by the addition of cofactors other than $MnCl_2$; the latter seemed to be required for all reactions tested. Although yeast ethanol dehydrogenase is a DPN-linked enzyme, sufficient DPN for maximal activity was present in the extract itself. The oxidation of citrate, α-ketoglutarate, and glutamate required added DPN/TPN and TPP. Although A-5-P has been implicated in citrate oxidation in yeast[12], it had no effect in our system.

*Effect of disintegration period*

It seemed likely from our previous studies that the disintegration period would affect the respiratory activities of yeast extracts. In fact, all oxidations tested were decreased by disintegrating cell suspensions for 90 seconds; this was most marked in the case of lactate and least in the case of ethanol (Table III). The comparative inactivity of 90-sec extracts was not due to excessive heating during disintegration, since the capsule and contents were cooled after 45 sec and then shaken for a further 45 sec. The temperature did not rise above 20° and was at that level for very short periods only. It is more likely that the co-ordination between the dehydrogenases and the electron transport system, which is necessary for aerobic oxidations and which is found in 10-sec extracts, is impaired in extracts prepared by longer disintegration.

TABLE III

EFFECT OF DISINTEGRATION PERIOD ON RESPIRATORY ACTIVITIES OF WHOLE EXTRACTS

Cells were disintegrated in 0.1 *M* phosphate buffer, pH 7.6. Each Warburg flask contained 3.0 ml *W* and 400 μmole substrate in a final volume of 4.0 ml. Temp. 30°. The figures given are means of three separate experiments, and represent μl $O_2$ consumed/mg dry wt over the 90-min experiment period corrected for the blank values.

| *Disintegration period (sec)* | *$O_2$ uptake* | | |
|---|---|---|---|
| | *Ethanol* | *Succinate* | *Lactate* |
| 10 | 27 | 24 | 19 |
| 30 | 20 | 24 | 10 |
| 90* | 18 | 14 | 4.5 |

* Two 45-sec periods, capsule and contents being cooled after the first.

*Blank respiration*

Yeast extracts prepared in phosphate buffer pH 7.4–7.6 have a very low blank respiration ($Q_{O_2}$ = 1–2), which can be markedly stimulated by the addition of ATP or A-5-P. Of DPN, TPN, and TPP, only the addition of the latter caused any significant stimulation. A final concentration of 0.01 *M* fluoride inhibited $O_2$ uptake in the stimulated system by 50% and $CO_2$ output by much more; but with iodoacetate, $O_2$ uptake was inhibited more than $CO_2$ output (Table IV). Two μmoles of ATP or 4 μmoles of A-5-P usually caused large increases in $O_2$ uptake and $CO_2$ output ($R.Q.$ = 1). The effects of ATP and A-5-P were not additive, but both showed peculiar concentration effects. Whereas the above amounts usually sufficed for full stimulation, half these amounts caused none. The respiration of a few preparations was not stimulated by 2 μmoles of ATP but fully stimulated by 4 μmoles (Table IV, experiment III).

A similarly high respiration rate could be caused by decreasing the pH of the disintegration medium to 6.5–7.1 (Table V). The $R.Q.$ of this process was generally 1–2.

*References p. 114/115.*

TABLE IV

EFFECT OF COFACTORS AND INHIBITORS ON THE BLANK RESPIRATION OF WHOLE EXTRACTS

Each Warburg flask contained 3.0 ml *W* (45–50 mg dry wt) 10 μmole $MnCl_2$ and other additions as indicated, in a final volume of 4.0 ml. Temp. 30°. Incubation period 60 min. In experiment IV, the complete system contained 3.0 ml *W*, 4 μmole ATP, 10 μmole $MnCl_2$, 50 μg TPN, 50 μg DPN, and 100 μg TPP.

| *Experiment* | *Disintegration medium* | *Other additions* | *Total $O_2$ uptake (μl)* | *Total $CO_2$ output (μl)* |
|---|---|---|---|---|
| I | 0.1 *M* phosphate, pH 7.6 | — | 96 | 86 |
| | | 4 μmole ATP | 615 | 588 |
| | | 4 μmole ATP, 40 μmole NaF | 316 | 72 |
| | | 4 μmole ATP, 4 μmole iodoacetate | 99 | 149 |
| | | 4 μmole A-5-P | 610 | 530 |
| II | 0.1 *M* phosphate, pH 7.6 | — | 84 | 92 |
| | | 2 μmole ATP | 573 | 550 |
| | | 4 μmole A-5-P | 571 | 447 |
| | | 4 μmole A-5-P, 40 μmole NaF | 217 | 72 |
| | | 4 μmole A-5-P, 4 μmole iodoacetate | 58 | 77 |
| III | 0.1 *M* phosphate, pH 7.4 | — | 67 | — |
| | | 2 μmole ATP | 83 | — |
| | | 4 μmole ATP | 412 | — |
| | | 4 μmole A-5-P | 77 | — |
| | | 8 μmole A-5-P | 361 | — |

TABLE V

EFFECT OF pH ON THE BLANK RESPIRATION OF WHOLE EXTRACTS

Cells were disintegrated in 0.1 *M* phosphate buffer at the pH shown. Each Warburg flask contained 3.0 ml *W* and 10 μmole $MnCl_2$. 2 μmole ATP or 4 μmole A-5-P was added as indicated, the final volume being 4.0 ml. Temp. 30°. Incubation period 60 min.

| *Experiment* | *pH of disintegration medium* | *pH of extract* | *pH of mixture after incubation* | *Dry wt of 3.0 ml extract* | *Other additions* | *$O_2$ uptake μl)* |
|---|---|---|---|---|---|---|
| I | 7.0 | 6.8 | 6.8 | 61 | — | 556 |
| | | | | | ATP | 658 |
| | | | | | A-5-P | 768 |
| II | 6.8 | 6.8 | 6.7 | 50 | — | 455 |
| | | | | | ATP | 477 |
| | | | | | A-5-P | 493 |
| III | 6.5 | 6.3 | 6.2 | 57 | — | 458 |
| | | | | | ATP | 525 |

*Nature of the blank respiration*

ATP-stimulated respiration was accompanied by a large decrease in anthrone-reacting material, measured as hexose. This decrease was apparently not due to utilization of free hexoses or reducing disaccharides, since the amount of reducing sugar present showed little change. In fact, the most likely substance to be disappearing is glycogen, which occurs abundantly in yeast cells. For each mole of hexose removed, about 1.5

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moles of $O_2$ were taken up and about 1.5 moles of $CO_2$ produced. Pyruvic and acetic acids accumulated, but the amounts of each were insufficient to account for the disappearance of carbohydrate (Table VI). Little pyruvate or acetate was formed in the unstimulated blank at pH 7.4, and pentose formation could account for the decrease in hexose; but pentose formation was too small to account for hexose disappearance during stimulated respiration. No evidence was obtained for the formation of sedoheptulose, acetoin, diacetyl, or acetaldehyde. During respiration at pH 6.8, one mole of $O_2$ was taken up and 1–2 moles of $CO_2$ produced for each mole of hexose removed. Using $^{14}C$-glucose it has been found that some phosphate esters accumulate. This aspect is being further investigated.

TABLE VI

PRODUCTS FORMED BY WHOLE EXTRACTS DURING BLANK RESPIRATION

Cells were disintegrated in 0.1 *M* phosphate buffer, pH 7.6 or 6.8. Each Warburg flask contained 3.0 ml *W*, 10 μmole $MnSO_4$, 100 μg TPP, 50 μg TPN, and 50 μg DPN, and 4 μmole ATP where indicated. The final volume was 4.0 ml. Temp. 30°. Duplicates were stopped at zero and 60 min by the addition of 0.1 ml 10 *N* $H_2SO_4$. Values shown represent changes in μmole/flask over the 60-min period.

| *Experiment* | *pH of buffer* | *Addition* | $O_2$ | $CO_2$ | *Products of reaction* | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *Hexose* | *Pentose* | *Pyruvate* | *Acetate* |
| I | 7.6 | — | — 6.5 | 6.0 | — 3.5 | 4.1 | 0.2 | 0.0 |
| | 7.6 | ATP | —28.7 | 34.0 | —19.8 | 1.4 | 6.0 | 17.0 |
| II | 6.8 | — | —20.0 | 30.4 | —20.1 | 1.6 | 3.3 | 14.9 |
| | 6.8 | ATP | —19.4 | 32.8 | —18.8 | 3.8 | 2.8 | 15.3 |

The amounts of $O_2$ consumed and $CO_2$ produced were greater than those calculated from the amounts of pyruvate and acetate formed. Also, these amounts of pyruvate and acetate only accounted for about half the hexose utilized. These observations might be explained if a portion of the hexose were removed by a non-oxidative process while the remainder were oxidized via acetate and the TAC; however, this is unlikely, since malate oxidation was slow and other TAC intermediates were not oxidized significantly beyond malate. The amount of these intermediates and of glutamic and aspartic acids did not alter significantly during the incubation period. By comparison, although $^{14}C$-acetate was utilized up to 50 %, very little was converted to $CO_2$ and most of the label was accounted for in TAC acids and glutamate[33]. Unless the operation of the cycle during this blank respiration differs from that during utilization of added acetate, the carbohydrate deficit cannot be accounted for by the TAC, and the mechanism of carbohydrate conversion to $CO_2$ remains to be elucidated.

*Oxidation of 2- and 3-carbon compounds*

Yeast extracts at pH 7.4 showed a large increase in $O_2$ uptake when incubated with ethanol, but no increase in $CO_2$ output. All the ethanol oxidized appeared as acetic acid, one mole of $O_2$ being consumed for each mole of acetate formed (Table VII). Addition of OAA and ATP did not promote more complete oxidation of the ethanol, but much OAA was reduced to malate + fumarate, apparently by coupling between ethanol and aldehyde dehydrogenases and malic dehydrogenase.

*References p. 114/115.*

Pyruvate, acetate, and acetyl phosphate were not oxidized by extracts supplemented with CoA, TPN, DPN, TPP, and $Mn^{++}$, nor did addition of ATP and OAA promote oxidation of these compounds.

TABLE VII

OXIDATION OF ETHANOL BY WHOLE EXTRACTS

Cells were disintegrated in 0.1 *M* phosphate buffer, pH 7.4. Each Warburg flask contained 3.0 ml *W*, 10 μmole $MnSO_4$, 10 μg CoA, and 4 μmole ATP where indicated. The added substrates were 200 μmole ethanol and 40 μmole OAA. The final volume was 4.0 ml. Temp. 30°. Values shown represent changes in μmole/flask over the 60 min period.

| *Substrate* | *Addition* | *Products of reaction* | | | | |
|---|---|---|---|---|---|---|
| | | *$O_2$* | *$CO_2$* | *Acetate* | *Pyruvate* | *Fumarate + malate* |
| — | — | — 6 | 6 | 0 | 0.0 | 0 |
| Ethanol | — | —31 | 7 | 28 | 0.0 | 0 |
| OAA | — | — 5 | 30 | 4 | 2.3 | 8 |
| Ethanol + OAA | — | —24 | 15 | 30 | 0.4 | 15 |
| — | ATP | —29 | 34 | 10 | 19 | 2 |
| Ethanol + OAA | ATP | —25 | 60 | 5 | 22 | 12 |

*Oxidation of TAC acids*

A series of balance experiments was performed in order to determine the products of oxidation of the main TAC acids. Disintegration was carried out in 0.1 *M* phosphate buffer, pH 7.4. In general, 3.0 ml 10-sec *W* was incubated for 60 min with 20–40 μmoles of substrate together with cofactors, and the reaction stopped by tipping in 0.1 ml 10 *N* $H_2SO_4$. Cups were set up in duplicate and one of each pair was stopped at zero time. The reaction in vessels containing no added substrate was stopped similarly at zero time and 60 min.

During citrate oxidation, no increase in pyruvate, acetate, glutamate, or aspartate occurred, and the disappearance of anthrone-reacting material was equal to that in the blank. The disappearance of citrate could be completely accounted for by its oxidation to the succeeding TAC acids (Table VIII). The observed gas exchange,

TABLE VIII

OXIDATION OF CITRATE BY YEAST EXTRACTS

Cells were disintegrated in 0.1 *M* Na, K-phosphate buffer, pH 7.4. Each Warburg flask contained 3.0 ml *W*, 10 μmole $MnSO_4$, 100 μg TPP, 50 μg DPN, 50 μg TPN, in a final volume of 4.0 ml. Temp. 30°.

| *Incubation period (min)* | *Substrate* | *Products of reaction (μmole)* | | | | | | *$O_2$ uptake (μatom)* |
|---|---|---|---|---|---|---|---|---|
| | | *Citrate* | *α-Ketoglutarate* | *Succinate* | *Fumarate* | *Malate* | *$CO_2$* | |
| 0 | — | 0.3 | 1.5 | 0.9 | 0.7 | 1.6 | — | — |
| 60 | — | 0.4 | 0.3 | 1.1 | 1.6 | 3.4 | 13.0 | —32.6 |
| | Change | + 0.1 | —1.2 | +0.2 | +1.1 | +1.8 | — | — |
| 0 | Citrate | 20.5 | 1.5 | 0.8 | 0.7 | 1.8 | — | — |
| 60 | Citrate | 6.8 | 6.2 | 5.8 | 2.7 | 5.7 | 39.5 | —54.2 |
| | Change | —13.7 | +4.7 | +5.0 | +2.0 | +3.9 | — | — |
| Change due to substrate | | —13.8 | +5.9 | +4.8 | +0.9 | +2.1 | +26.5 | —21.6 |

*References p. 114/115.*

TABLE

OXIDATION OF α-KETOGLUTARATE

Conditions as

| *Incubation period (min)* | *Substrate* | | *Products of* | | | | |
|---|---|---|---|---|---|---|---|
| | | | *Citrate* | *α-Ketoglutarate* | *Succinate* | *Fumarate* | *Malate* |
| 0 | — | | 0.1 | 0.1 | 0.9 | 0.2 | 0.8 |
| 60 | — | | 0.1 | 0.1 | 0.5 | 0.1 | 0.6 |
| | | Change | 0.0 | 0.0 | — 0.4 | — 0.1 | — 0.2 |
| 0 | α-Ketoglutarate | | 0.1 | 39.1 | 0.8 | 0.1 | 0.3 |
| 60 | α-Ketoglutarate | | 0.1 | 28.0 | 2.8 | 1.4 | 4.4 |
| | | Change | 0.0 | —11.1 | + 2.0 | + 1.3 | + 4.1 |
| Change due to substrate | | | 0.0 | —11.1 | + 2.4 | + 1.4 | + 4.3 |
| 0 | Succinate | | 0.1 | 0.2 | 37.8 | 0.1 | 0.4 |
| 60 | Succinate | | 0.1 | 0.6 | 22.8 | 3.6 | 9.4 |
| | | Change | 0.0 | + 0.4 | —15.0 | + 3.5 | + 9.0 |
| Change due to substrate | | | 0.0 | + 0.4 | —14.6 | + 3.6 | + 9.2 |

TABLE

OXIDATION OF FUMARATE AND

Conditions as

| *Incubation period (min)* | *Substrate* | | *Products of* | | | | |
|---|---|---|---|---|---|---|---|
| | | | *Citrate* | *α-Ketoglutarate* | *Succinate* | *Fumarate* | *Malate* |
| 0 | — | | 0.1 | 0.1 | 1.3 | 0.8 | 1.1 |
| 60 | — | | 0.1 | 0.1 | 0.3 | 0.9 | 1.1 |
| | | Change | 0.0 | 0.0 | — 1.0 | + 0.1 | 0.0 |
| 0 | Fumarate | | 0.1 | 0.1 | 1.2 | 39.6 | 1.1 |
| 60 | Fumarate | | 0.1 | 0.7 | 3.4 | 9.1 | 28.6 |
| | | Change | 0.0 | 0.6 | + 2.2 | —30.5 | + 27.5 |
| Change due to substrate | | | 0.0 | + 0.6 | + 3.2 | —30.6 | + 27.5 |
| 0 | Malate | | 0.1 | 0.0 | 1.3 | 0.8 | 38.8 |
| 60 | Malate | | 0.1 | 0.6 | 2.7 | 8.6 | 28.0 |
| | | Change | 0.0 | + 0.6 | + 1.4 | + 7.8 | —10.8 |
| Change due to substrate | | | 0.0 | + 0.6 | + 2.4 | + 7.9 | —10.8 |

IX

AND SUCCINATE BY YEAST EXTRACTS

in Table VIII

| reaction (μmole) | | | | | | $O_2$ uptake (μatom) |
|---|---|---|---|---|---|---|
| Pyruvate | Acetate | Glutamate | Aspartate | Carbohydrate (as hexose) | $CO_2$ | |
| 2.9 | 1.6 | 8.8 | 4.5 | 63 | — | — |
| 5.3 | 1.6 | 15.4 | 2.0 | 60 | 3.5 | — 6.5 |
| + 2.4 | 0.0 | + 6.6 | — 2.5 | — 3 | — | — |
| 2.9 | 1.6 | 8.8 | 4.5 | 63 | — | — |
| 2.8 | 3.8 | 18.2 | 3.1 | 60 | 10.3 | —19.6 |
| — 0.1 | 2.2 | + 9.4 | — 1.4 | — 3 | — | — |
| — 2.5 | + 2.2 | + 2.8 | + 1.1 | 0 | + 6.8 | —13.1 |
| 2.8 | 1.6 | 8.8 | 4.3 | 63 | — | — |
| 4.9 | 1.9 | 15.2 | 2.6 | 60 | 4.0 | —22.6 |
| + 2.1 | + 0.3 | + 6.4 | — 1.7 | — 3 | — | — |
| — 0.3 | + 0.3 | — 0.2 | + 0.8 | 0 | + 0.5 | —16.1 |

X

MALATE BY YEAST EXTRACTS

in Table VIII

| reaction (μmole) | | | | | | $O_2$ uptake (μatom) |
|---|---|---|---|---|---|---|
| Pyruvate | Acetate | Glutamate | Aspartate | Carbohydrate (as hexose) | $CO_2$ | |
| 0.1 | 1.5 | 6.0 | 3.7 | 49 | — | — |
| 0.1 | 0.9 | 6.3 | 2.3 | 44 | 3.8 | — 7.6 |
| 0.0 | — 0.6 | + 0.3 | — 1.4 | — 5 | — | — |
| 0.1 | 1.5 | 6.0 | 3.7 | 49 | — | — |
| 0.4 | 1.3 | 1.3 | 2.8 | 44 | 9.7 | —13.6 |
| + 0.3 | — 0.2 | — 4.7 | — 0.9 | — 5 | — | — |
| + 0.3 | — 0.4 | — 5.0 | + 0.5 | 0 | + 5.9 | — 6.0 |
| 0.1 | 1.4 | 6.0 | 3.7 | 49 | — | — |
| 0.4 | 0.9 | 1.8 | 2.3 | 44 | 8.2 | —12.4 |
| + 0.3 | — 0.5 | — 4.2 | — 1.4 | — 5 | — | — |
| + 0.3 | + 0.1 | — 4.5 | 0.0 | 0 | 4.4 | — 4.8 |

TABLE

METABOLISM OF OXALOACETATE

Conditions as in Table VIII

| *Incubation period (min)* | *Substrate* | *Products o* | | | | |
|---|---|---|---|---|---|---|
| | | *Citrate* | *α-Ketoglutarate* | *Succinate* | *Fumarate* | *Malate* |
| 0 | — | 0.1 | 0.2 | 1.1 | 0.4 | 1.2 |
| 60 | — | 0.1 | 0.1 | 0.6 | 0.4 | 1.1 |
| | Change | 0.0 | — 0.1 | — 0.5 | 0.0 | — 0.1 |
| 0 | OAA | 0.1 | 0.2 | 1.2 | 0.4 | 1.1 |
| 60 | OAA | 0.1 | 6.7 | 4.0 | 3.4 | 8.9 |
| | Change | 0.0 | + 6.5 | + 2.8 | + 3.0 | + 7.8 |
| Change due to substrate | | 0.0 | + 6.6 | + 3.3 | + 3.0 | + 7.9 |

however, departed somewhat from that expected. This was most likely due to the participation of unknown reactions in the blank respiration, which was quite rapid. In this experiment, the yeast extract was prepared in a medium containing both $Na^+$ and $K^+$ ions. By using a medium which contained no $K^+$ ions the blank respiration was reduced markedly, and disintegrations for subsequent experiments were therefore carried out in sodium phosphate buffer, pH 7.4.

With α-ketoglutarate as the substrate, two different types of results were obtained with apparently similar extracts. In one type, very little $O_2$ uptake or $CO_2$ evolution could be measured, and nearly all the α-ketoglutarate which disappeared could be accounted for as glutamate. In the other, oxidation occurred, and the products of the reaction are shown in Table IX. Although the changes were small, it can be seen that the products formed from α-ketoglutarate were glutamate, succinate, fumarate, and malate. Two reasons can be suggested for the formation of glutamate in this experiment, but not in the previous one. Firstly, the concentration of free α-ketoglutarate differed widely. Secondly, added α-ketoglutarate should be more readily available to the soluble transaminases, whereas that formed by a particulate enzyme complex would more likely be oxidized further.

Succinate was oxidized to fumarate and malate, but a small discrepancy in the balance was always found (Table IX). Subsequently it was shown that some further oxidation of malate occurred. The products of this oxidation were not apparent until fumarate and malate were used as substrates (Table X).

In both of these cases, some oxidation of malate to OAA occurred, but no increase in OAA could be detected. It seemed possible that transamination might account for a loss of OAA, but aspartate was apparently not the product. These extracts were known to exhibit high oxaloacetic carboxylase activity, and the most likely sequence of reactions therefore seemed to be: decarboxylation of OAA to pyruvate, transamination between pyruvate and endogenous glutamate with the formation of alanine and α-ketoglutarate, and finally, oxidation of some of the latter to succinate. This hypothesis was further tested by measuring the products formed from added OAA (Table XI).

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XI

BY YEAST EXTRACTS

Substrate: *ca.* 40 μmole Na oxaloacetate.

| *reaction (μmole)* | | | | | | *$O_2$ uptake (μatom)* |
|---|---|---|---|---|---|---|
| *OAA* | *Pyruvate* | *Acetate* | *Glutamate* | *Aspartate* | *$CO_2$* | |
| 0.0 | 0.8 | 0.8 | 9.3 | 3.5 | — | — |
| 0.0 | 0.1 | 0.8 | 9.6 | 2.9 | 2.2 | — 4.4 |
| 0.0 | — 0.7 | 0.0 | + 0.3 | — 0.6 | — | — |
| 32.6 | 0.8 | 0.8 | 9.3 | 3.5 | — | — |
| 5.4 | 2.0 | 0.8 | 0.0 | 2.7 | 20.2 | — 6.3 |
| —27.2 | + 1.2 | 0.0 | — 9.3 | — 0.8 | — | — |
| —27.2 | + 1.9 | 0.0 | — 9.6 | — 0.2 | + 18.0 | — 1.9 |

Although it is difficult to assess this experiment quantitatively, since no reliable method for estimating alanine was available, the results are in keeping with the suggested reaction scheme. As further evidence, it was found that, when $CH_3 \cdot CO \cdot {}^{14}COOH$ was added as substrate under the same conditions, significant incorporation into alanine occurred.

Both the α-ketoglutaric oxidase and the malic dehydrogenase systems are DPN-dependent and appear to be DPN-linked in these extracts.

## DISCUSSION

It has not yet been shown whether the TAC is quantitatively important for oxidation of acetate of whether another significant mechanism exists. KREBS[1,2] favours the second alternative, and three major findings support his claim that the TAC cannot be the main pathway for acetate oxidation: (1) depending on the degree of temperature shock, acetate and succinate oxidations are influenced differently, (2) malonate completely inhibits succinate oxidation but does not affect acetate oxidation, (3) carbon from $^{14}C$-labelled acetate does not become incorporated into added, unlabelled TAC intermediates during oxidation of acetate to $CO_2$.

Two technical difficulties have hitherto prevented the solution of this problem. The fresh cell at neutral reaction is incapable of oxidizing added di- or tricarboxylic acids, so that their oxidation rates cannot be studied. Also, yeast cells are resistant to disintegration and many procedures employed to break the cell walls may destroy the respiratory mechanism at the same time.

There is now no doubt that all the steps of the KREBS tricarboxylic acid cycle are present in yeast. These steps function not only individually but as a sequential process, even in cell-free extracts. In these extracts, cyclic activity appears to be impaired at several points, one of which is the malate oxidation step. The reason for this is not known. Although the balances obtained with single TAC intermediates cannot be considered precisely quantitative (probably for technical reasons), each acid is largely

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recovered as succeeding members of the cycle or amino acids directly derivable from them. This means that no member is participating significantly in other reactions such as may be found in certain bacteria. Preliminary isotope studies with labelled acetate[33] have confirmed these observations and have failed to indicate any other pathway of acetate utilization.

Although all our results point to the KREBS cycle as the terminal respiratory mechanism, acetate is not oxidized appreciably by cell-free extracts in spite of being incorporated into the TAC acids. If the condensation reaction to form citrate is indeed rate-limiting, it is unlikely that the carbohydrate which disappears during the blank respiration is oxidized through the cycle. Moreover, the $O_2$ uptake and $CO_2$ evolution are insufficient to account for complete combustion. Carbohydrate utilization results in the accumulation of pyruvate and acetate, but not of the TAC acids.

The mechanism of carbohydrate breakdown in these extracts therefore remains unknown. It may be the EMBDEN-MEYERHOF pathway or the hexosemonophosphate "shunt" (perhaps acting as a cyclic mechanism). The latter would explain the formation of small amounts of pentose. No sedoheptulose has been detected, but such an unfractionated system may not form measurable amounts of this substance.

Further work on the respiratory mechanisms of yeast is in progress.

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SUMMARY

1. Respiring cell-free baker's yeast extracts have been prepared by 10-sec high-speed mechanical disintegration.

2. Increasing the disintegration period reduces the oxidative ability of the extracts.

3. Whole extracts have a low blank respiration at or above pH 7.4. At pH 6.5–7.1 a high blank respiration is found. Addition of small amounts of ATP or A-5-P to the blank at pH 7.4 also induces this high respiration.

4. The blank respiration is due to catabolism of polysaccharide. The *R.Q.* of the reaction is about 1 or sometimes higher. Considerable amounts of pyruvate and acetate are formed, but these are insufficient to account for either carbohydrate disappearance, $O_2$ uptake, or $CO_2$ formation. Some pentose formation occurs.

5. Various acids of the KREBS tricarboxylic acid cycle are oxidized by the extracts, succinate most and malate least rapidly.

6. Quantitative balances of the metabolism of each of the KREBS cycle acids show that they can largely be accounted for by the reactions of the cycle. Only small amounts of citrate are formed from oxaloacetate, acetate and CoA. Considerable amounts of glutamate are formed from $\alpha$-ketoglutarate but not from citrate.

7. The results are discussed in the light of the KREBS cycle being a major pathway of yeast respiration. Other pathways exist and may account for the high blank respiration observed under certain conditions.

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# FATTY ACID UPTAKE AND ESTERIFICATION IN ADIPOSE TISSUE*

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The rate of mobilization of fat from adipose tissue and its deposition is not determined solely by diffusion of these substances from the tissue to the blood and vice versa. Thus, increased mobilization during hunger[1] or after treatment with pituitary extracts[2] is not brought about by decreased fat concentration in the blood, but rather takes place in spite of increased blood fat levels.

Evidence for the participation of tissue metabolism in the transport of fat into the cells was obtained by studying the uptake of fat by adipose tissue *in vitro*. It was found[3,4] that isolated adipose tissue removes fat from serum and other lipid media, provided that fat-depleted tissue is used. This uptake of fat disappears when heat-killed tissue is used or when metabolic poisons, such as fluoride or cyanide are added. Also, uptake was observed only with neutral fat and fatty acids but not with phospho-

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*References p. 120.*