# A COMPARATIVE STUDY OF ACONITASE, FUMARASE, AND DPN-LINKED *ISO*CITRIC DEHYDROGENASE IN NORMAL AND RESPIRATION-DEFICIENT YEAST*

by

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It is a general belief that most, if not all, distinctive properties of any particular tissue can be explained by the uniqueness of its enzymic pattern. The acquisition of this pattern has frequently been postulated to be the result of a controlled segregation of cytoplasmic material.

The mutant "petite colonie" of *Saccharomyces cerevisiae* described by EPHRUSSI and co-workers (review in EPHRUSSI[6]) represents a working model for an inquiry into such a hypothesis. TAVLITZKI[34] and SLONIMSKI[28] have found that this mutant differs from normal yeast by a lack of respiratory ability, and SLONIMSKI AND EPHRUSSI[30] traced this deficiency to the absence of two respiratory enzymes, cytochrome oxidase and succinic dehydrogenase, linked to particulate cell material, separable by differential centrifugation. More recently, SLONIMSKI[29] has extended the investigation to several other enzyme systems and found that alpha-glycerophosphate dehydrogenase and DPN-cytochrome *c* reductase***, also linked to particulate material in the normal yeast, are missing in the mutant, while lactic, malic, and alcohol dehydrogenases, three enzymes apparently not linked to the sedimentable fraction of the cells, are present in both normal and mutant yeast. On the other hand, on purely genetic grounds, the mutation was postulated to consist in the loss or inactivation of a self-reproducing and particulate cytoplasmic component.

Variations of enzymic constitution similar to those caused by the mutation discussed can also be the result of environmental changes. WARBURG[35,36] observed that the respiration rate of baker's yeast is a function of the amount of air supplied during growth, and that resting yeast suspensions containing alcohol or acetate attain maximum respiration only after they have been shaken in air for several hours. EPHRUSSI AND SLONIMSKI[8] have found, in anaerobically grown baker's yeast, a loss of the ability to respire (but not to ferment) glucose, and have shown that this loss is accompanied by the absence of cytochromes *a*, *b*, and *c*, and by the appearance of cytochromes *a* 1 and *b* 1. When resting cells of such anaerobically grown yeast are aerated, the normal cytochrome components are restored, and with it the ability to respire.

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*** The following abbreviations will be used: DPN for diphosphopyridine nucleotide; TPN for triphosphopyridine nucleotide; and ATP for adenosine-triphosphate.

*References p. 686.*

In order to inquire further into the biochemical effects of the mutation it became of interest to investigate a. the activity and intracellular localization in the normal and mutant yeast of two enzymes known, in mammalian tissues, to catalyze reactions forming part of the tricarboxylic acid cycle, especially in view of the claims (*cf.* GREEN[11]; see, however, HOGEBOOM AND SCHNEIDER[14]) that these enzymes are also carried by particulate material, b. the activity of DPN-*iso*citric dehydrogenase, which catalyses the transformation of a cycle substrate without apparently being part of the cycle, and c. the changes these enzymes undergo as a function of oxygen supply. In addition to the results of this investigation the present paper includes the results of experiments with cell free extracts and dried cell preparations bearing on the controversial question of the existence of a tricarboxylic acid cycle in yeast.

## EXPERIMENTAL

### *Materials and methods*

The strains used were *Saccharomyces cerevisiae* 59 R (normal yeast, haploid, single spore isolate) and 59 RA (mutant yeast, isolated from acriflavine treated 59 R in January 1947[7]). Usually the yeasts were grown for 42 to 72 hours at 25° C or 28° C on touraillon agar in Petri dishes. A few times, organisms grown in rotating bottles in liquid medium A as described by EPHRUSSI AND SLONIMSKI[8], were used. When the organisms were to be grown anaerobically on solid media, the Petri dishes were placed in anaerobic jars or vacuum dessicators which were flushed out with oxygen free nitrogen ($< 0.005\%$ $O_2$) for one hour and then closed. An indicator tube containing glucose, sodium hydroxide, and reduced methylene blue was placed within the containers. Yeast which had been grown anaerobically was always examined by means of a Hartridge reversion spectroscope to see whether it exhibited the cytochrome spectrum typical of anaerobically grown yeast, *i.e.*, the bands *b* I at 557–559 m$\mu$ and *a* I near 581 m$\mu$[8]. The composition of the media was the following:

| *Liquid medium A* | | *Solid medium (touraillon agar)* | |
|---|---|---|---|
| $MgSO_4.7\ H_2O$ | 0.7 g | glucose | 30.0 g |
| $KH_2PO_4$ | 1.0 g | $(NH_4)_2HPO_4$ | 2.0 g |
| $CaCl_2$ | 0.4 g | $MgSO_4.7\ H_2O$ | 0.2 g |
| NaCl | 0.5 g | eau de touraillon | 3% |
| $(NH_4)_2SO_4$ | 1.2 g | agar | 20.0 g |
| $FeCl_3$ | 0.005 g | dist. water to 1 liter | |
| Glucose | 54.0 g | pH after sterilization | 5.8–5.9 |
| Yeast extract (Difco) | 10.0 g | | |
| dist. water to 1 liter | | | |
| pH after sterilization | 5.7–5.9 | | |

The two media were sterilized, respectively, for 60 minutes at 107° C and for 20 minutes at 120° C. For the manometric work standard Warburg techniques were employed. The temperature was 28° C, the gas phase, air. Aconitase and fumarase activities were determined by the method of RACKER[25] in which the variations of optical density at 240 m$\mu$ concomitant with the appearance or disappearance of unsaturated compounds are followed spectrophotometrically. The reaction follows a zero order course for many minutes after addition of the substrate and Q values were calculated on the basis of the initial rate. Enzyme activity was assayed on several levels of extract concentration and found to be proportional to the concentration. The reaction mixture consisted of 1.5 ml of 0.1 *M* phosphate buffer, pH 7.3; 0.45 ml of 0.2 *M* sodium citrate or 0.3 ml of 0.5 *M* sodium *l*-malate; 0.2 ml of a suitably diluted extract, and water to a final volume of 3.0 ml. The control cell contained no substrate. Silica cells of one cm lightpath were used.

DPN-linked *iso*citric dehydrogenase activity was determined according to KORNBERG AND PRICER[15]. The reaction cell contained 0.1 ml of 0.1 *M* $MgSO_4$; 0.1 ml of 0.005 *M* adenosine-5-phosphate (pH 7.0); 0.3 ml of 0.1 *M* KCN (pH 7.0); 0.2 ml of *iso*citrate varying between 1.28 and $1.66 \cdot 10^{-2}$ *M* in different experiments, and 0.3 ml of DPN, varying slightly in different experiments but containing *ca.* ½ micromole, 1.8 ml of 0.1 *M* phosphate buffer pH 7.3, and 0.2 ml of extract of a suitable dilution. The blank for measurement of endogenous DPN reduction contained no *iso*citrate, and both blank and test were read against a control containing no DPN and no *iso*citrate. In non-dialysed extracts, the endogenous DPN reduction was usually quite high, and the determinations were always repeated using dialysed extracts. Dialysis was against 0.02 *M* phosphate buffer of pH 7.3 in the icebox for 16–18 hours. KCN was added to prevent reoxydation of reduced DPN via the DPN-cytochrome

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*c* reductase-cytochrome oxidase system. The rate of reaction was found to be constant for at least 12 minutes and directly proportional to the concentration of the extract.

Cytochrome oxidase was determined manometrically[29], and citric acid by the method of PERLMAN *et al.*[23] The error in the determination of citrate left over never showed a deviation from the mean of more than ± 8%, and usually was well below 5%. Nitrogen was determined by a semi-micro Kjeldahl technique.

Extracts were prepared in the following manner. The harvested cells were centrifuged, washed twice in the cold with 0.1 *M* phosphate buffer, pH 7.3, resuspended in one volume of phosphate buffer, and broken by means of glass beads of a diameter between 0.05 and 0.15 mm on an ultra-rapid shaker (Towers) in the cold. The liquid was pipetted off, the residue washed several times with enough cold phosphate buffer to bring the total volume to 2 ½ × that of the original cell suspension, the washings added to the liquid, and the combined liquid portions centrifuged twice in the cold at *ca.* 3000 RPM (*ca.* 1700 g) to remove cell débris, unbroken cells, and glass beads. The supernatant is the extract.

Reagents: the ATP, obtained from Bios Laboratories as the dibarium salt, was converted to the potassium salt before use. The DPN used (General Biochemicals Inc.) was found to be *ca.* 35% pure, as determined by reduction with *iso*citrate in the presence of yeast DPN-*iso*citric dehydrogenase. *Iso*citric acid was purchased from Delta Chemical Works, *l*-malic acid from Eastman-Kodak, and crystalline adenosine-5-phosphoric acid from Sigma Chemicals and from General Biochemicals Inc.

## RESULTS

### *Influence of genotype and environment on aconitase, fumarase, and DPN-isocitric dehydrogenase in the normal and mutant yeasts*

The activities of the enzymes aconitase, fumarase, and DPN-linked *iso*citric dehydrogenase were studied in the normal and mutant yeast under varying environmental conditions. Aconitase and fumarase are an integral part of the tricarboxylic acid cycle system. Aconitase catalyzes the establishment of an equilibrium between citric, *cis*-aconitic, and *iso*citric acids (see, however, MARTIUS AND LYNEN[19]); fumarase catalyzes the reversible conversion of fumaric acid to malic acid. The DPN-linked *iso*citric dehydrogenase has recently been described by KORNBERG AND PRICER[15]; it catalyzes the breakdown of a substrate forming part of the cycle but its function in any cyclic system is at present unknown*.

Results from a number of experiments are recorded in Table I. It can be seen from the data that 1. aconitase, fumarase**, and DPN-*iso*citric dehydrogenase of normal and mutant yeast grown under anaerobic conditions are alike; 2. a change in growth conditions from anaerobiosis to aerobiosis leads to a marked increase in aconitase, fumarase, and DPN-*iso*citric dehydrogenase in the normal yeast, the extent of this increase being in the order mentioned, and 3. the mutation "petite colonie" leads to a partial conversion to the enzymatic pattern obtained in normal yeast grown under anaerobic conditions.

One is dealing here with differences in response to the presence or absence of oxygen. If one considers the variation of each enzyme separately, one finds that aconitase shows the greatest degree of variation with respect to both genotype and environment. Its activity in anaerobically grown cells represents only *ca.* 5% of that obtained in aerobically grown normal yeast. On the other hand, the mutant yeast grown aerobically

* The DPN-linked *iso*citric dehydrogenase described by these authors does not, contrary to the TPN-linked *iso*citric dehydrogenase system from pig heart and yeast, catalyze either the decarboxylation or the reduction of oxalsuccinate, and it is pointed out that a satisfactory scheme for the mechanism of the DPN-*iso*citric dehydrogenase reaction is not yet available.

** The difference between fumarase activity in the normal and mutant yeast grown anaerobically is not statistically significant (t test).

*References p. 686.*

exhibits *ca.* 19% of the activity obtained in aerobically grown normal yeast. The corresponding figures for fumarase are *ca.* 17% and 36%, and for DPN-*iso*citric dehydrogenase *ca.* 38% and 44%. This indicates that the degree of variation is greatly different for different enzymes and this might be interpreted on the basis that the reactions mediated by these three enzymes are involved to a different degree in the overall aerobic metabolism of the cell. Further observations strengthening such a point of view will be given later on. It will be observed also that oxygen has a stimulating effect on aconitase of the mutant yeast, an organism in which the WARBURG-KEILIN system is absent; this is much less marked in the case of fumarase, and not at all the case with DPN-*iso*citric dehydrogenase. It is interesting to note in this connection that aconitase content in different animal tissues is generally parallel in extent to the amount of aerobic metabolism occurring in that tissue[19].

TABLE I

DETERMINATION OF FUMARASE, ACONITASE, AND DPN-LINKED *ISO*CITRIC DEHYDROGENASE ACTIVITIES OF NORMAL AND MUTANT YEAST

Data are expressed as specific activity; *i.e.*, $\frac{\text{Units}}{\text{mg extract N}}$, where one unit is defined as the amount of enzyme causing an increase in optical density of 0.001 per minute at 25° C; and as $Q_{\text{aconitate}}(N)$, $Q_{\text{fumarate}}(N)$, or $Q_{\text{isocitrate}}(N)$ values. $Q_{\text{aconitate (fumarate)}}(N)$ = microliters of aconitate (fumarate) formed per hour per mg of extract N. $Q_{\text{isocitrate}}(N)$ = microliters of *iso*citrate disappearing per hour per mg of extract N, as measured by DPN reduction.

Q values determined from:

$$\frac{\text{specific activity} \times 10^{-3} \times 60 \times 22.4 \times 10^{6} \times 3}{\text{molecular extinction coefficient}}$$

The molecular extinction coefficients are: sodium *cis*-aconitate at 240 mμ $3.54 \times 10^6$ ($cm^2 \times moles^{-1}$); sodium fumarate at 240 mμ $2.11 \times 10^6$ ($cm^2 \times moles^{-1}$); reduced DPN at 340 mμ $6.22 \times 10^6$ ($cm^2 \times moles^{-1}$).

Measurements were done at room temperature and corrected to values that would have been obtained at 25° C, assuming a $Q_{10} = 2$.

Yeast grown aerobically or anaerobically on touraillon agar in Petri dishes.

| *Yeast* | *Conditions of Growth* | *Specific activity (mean)* | | *Standard error of mean* | *Number of* independent experiments* | $Q_{\text{aconitate}}(N)$ *fumarate isocitrate* |
|---|---|---|---|---|---|---|
| normal | aerobiosis | aconitase | 528 | 21 | 6 | 602 |
| | | fumarase | 2624 | 296 | 5 | 5012 |
| | | *iso*citric dehydrogenase | 198 | 43 | 3 | 126 |
| normal | anaerobiosis | aconitase | 27 | 8 | 7 | 31 |
| | | fumarase | 393 | 46 | 7 | 751 |
| | | *iso*citric dehydrogenase | 68 | 8 | 2 | 43 |
| mutant | aerobiosis | aconitase | 98 | 16 | 6 | 112 |
| | | fumarase | 951 | 162 | 5 | 1816 |
| | | *iso*citric dehydrogenase | 87 | 16 | 3 | 57 |
| mutant | anaerobiosis | aconitase | 27 | 7 | 6 | 31 |
| | | fumarase | 483 | 41 | 5 | 923 |
| | | *iso*citric dehydrogenase | 83 | 13 | 2 | 55 |

* Each independent experiment comprises two to five determinations of which the average is taken.

*References p. 686.*

It is well known that inferences as to enzymic content from data based on activity measurements need caution. The difference observed between any two preparations can be due, *e.g.*, to the unequal distribution of inhibitors or stimulators, or of enzymes removing the reaction products being measured. In order to demonstrate that the differences observed between normal and mutant yeasts were not due to such factors, extracts from the two were mixed in different proportions and the resulting aconitase and fumarase activities measured. The activities appeared to be additive. It is clear, therefore, that the differences observed between normal and mutant yeasts are due to differences in enzymic content.

Aconitase is known to be quite unstable in aqueous solutions and very sensitive to dialysis[22]. This was confirmed in work with our extracts. Although activity remained constant for several days when the extracts were frozen in dry ice, a large part of it was lost on storage at 0° C. The decrease in activity was more rapid during dialysis at this temperature. Fumarase activity, on the contrary, remained unchanged on storage or dialysis.

Advantage was taken of this lability of aconitase in order to determine whether an enzyme transferring hydrogen directly from citrate to DPN exists in yeast. "Citric dehydrogenase" as well as aconitase activities were measured in extracts before and after dialysis. The determination of "citric dehydrogenase" was essentially similar to that of *iso*citric dehydrogenase except that sodium citrate (0.2 ml, 0.2 *M*) was used as substrate instead of *iso*citrate. Nondialysed extracts from aerobically grown normal yeast definitely exhibited "citric dehydrogenase" activity which was greatly diminished in the dialysed extracts. The fall in activity was roughly proportional to the loss in aconitase activity. "Citric dehydrogenase" activity in nondialysed extracts from anaerobically grown normal or mutant yeast was very small. It can be assumed, therefore, that a "citric dehydrogenase" as such does not exist in yeast (that it does not occur in various animal tissues has been well known for some time). The observed activity is obviously due to the conversion of citrate to *iso*citrate by aconitase and subsequent action of *iso*citric dehydrogenase.

Citrate disappearance in extracts from normal and mutant yeast grown aerobically and anaerobically was measured also. The results from some experiments are given in Table II. Citrate disappearance in extracts made from aerobically grown mutant yeast or from normal and mutant yeast grown anaerobically was always approximately ½ of that of the normal yeast grown aerobically. The fact that citrate disappears in the anaerobically grown normal and mutant yeasts in which aconitase activity is very low may be accounted for on the basis that two or more pathways for citrate disappearance exist in yeast. Experimental proof for this has recently also been given by FOULKES[9,10].

FOULKES[9] has reported stimulation of citrate disappearance through the addition of ATP and Mg ions to nondialyzed extracts of baker's yeast. No significant stimulation of citrate disappearance on the addition of ATP and Mg ions was observed in our experiments and, also in contrast to FOULKES[9], we observed no inhibition of citrate disappearance by 0.01 *M* cyanide. WEINHOUSE AND MILLINGTON[37], using *cis*-aconitate as substrate found $Q^{25^\circ C}_{citrate}$ values in baker's yeast of *ca.* 3–5, while FOULKES[9] reports $Q^{37^\circ C}_{citrate}$ values of 1000 (μl citrate metabolized/h/g fresh weight) which correspond, roughly, to a $Q^{37^\circ C}_{citrate}$ of 3 (μl citrate metabolized/h/mg dry weight). Since our values have been calculated on a mg nitrogen basis and can be converted to a dry weight basis by dividing by 10, it can be seen that they compare well with those found by the other authors.

*References p. 686.*

TABLE II

Citrate disappearance in extracts from aerobically and anaerobically grown normal and mutant yeast. Each flask (small Erlenmeyer) contained 5 mg of citric acid (23.8 micromoles) and, where added, 1 mg ATP and 100 $\mu$g of Mg ions. Flasks shaken at 28° C in Warburg bath for 40 minutes. Gas phase: air. Total volume: 4.0 ml; 2.0 ml of extract per flask were used, or, in case of the blank, 2.0 ml of boiled extract. Determinations done in duplicate or triplicate. Citrate determined by method of PERLMAN *et al.* (1944). $Q_{citrate}$ (N) defined as $\mu$l of citrate metabolized per hour per mg of extract Nitrogen at 28° C.

| *Exp. No.* | *Conditions under which cells were grown* | *Additions* | *Citrate metabolized* | | | |
|---|---|---|---|---|---|---|
| | | | *Normal yeast* | | *Mutant yeast* | |
| | | | *micromoles* | $Q_{citr.}$ *(N)* | *micromoles* | $Q_{citr.}$ *(N)* |
| 9 | Aerobically in liquid medium A; rotating bottle, compressed air added | Extract alone | 9.5 | 45.9 | | |
| | | Extract + ATP + Mg | 10.9 | 52.6 | | |
| | | Extract + ATP + Mg + 0.01 *M* KCN | 11.4 | 55.0 | | |
| | | Extract + ATP + Mg + 0.01 *M* Sodium arsenite | 8.6 | 41.7 | | |
| 10 | As in exp. 9 | Extract + ATP + Mg | 10.9 | 52.6 | | |
| | | Extract + ATP + Mg + 0.01 *M* Sodium arsenite | 9.8 | 47.3 | | |
| 11 | As in exp. 9 | Extract alone | | | 6.8 | 31.5 |
| | | Extract + ATP + Mg | | | 5.0 | 23.1 |
| | | Extract + ATP + Mg + 0.01 *M* KCN | | | 7.1 | 32.8 |
| | | Extract + ATP + Mg + 0.01 *M* Sodium arsenite | | | 4.6 | 21.3 |
| 17 | Aerobically on Petri dishes on Touraillon agar | Extract alone | 6.5 | 80.9 | 3.3 | 43.3 |
| 19 | As in exp. 17 | Extract alone | 5.4 | 61.7 | 2.6 | 30.5 |
| 23 | Anaerobically on Petri dishes, on Touraillon agar | Extract alone | 2.8 | 33.6 | 0.7 | 7.8 |
| 27 | As in exp. 23 | Extract alone | 3.2 | 29.9 | 3.5 | 34.4 |

*Increase in aconitase and fumarase in anaerobically grown yeast exposed to air*

EPHRUSSI AND SLONIMSKI[8] have shown that exposure of anaerobically grown normal yeast to air results in an "adaptive" synthesis of cytochromes *a*, *b*, and *c*, and a concomitant reestablishment of respiration. It has been shown above that both normal and mutant yeast also have higher aconitase and fumarase activities following growth under aerobic as compared with anaerobic conditions and that this is much more pronounced in normal than in mutant yeast. Several experiments were run to determine whether the increase in aconitase and fumarase activity under the influence of oxygen would take place also in non-proliferating cells or in cells growing at a minimal rate. Resting cell suspensions in phosphate buffer (pH 6.5, 0.05 *M*) were prepared from normal yeast grown anaerobically for 2½ days on touraillon agar in Petri dishes. The experiments were performed under three different conditions: in the presence of air, in the presence of air and glucose, in the presence of oxygen-free nitrogen gas and glucose. Glucose was added in a final concentration of 2%. The temperature was 28° C. Aeration tubes ("tubes à barbotage"; TAVLITZKI[34]) were used, and compressed air or nitrogen bubbled through the suspensions. Aliquot samples were removed from time to time, extracts prepared, and assayed for fumarase and aconitase activity. At the end of the experiments the suspensions were checked for contamination; none was encountered.

*References p. 686.*

TABLE III

Variation of fumarase and aconitase activities in a resting suspension of anaerobically grown normal yeast in the presence of oxygen.

Data are expressed as specific activity (see Table I for definition).

| Time (min) | Aconitase | | | Fumarase | | |
|---|---|---|---|---|---|---|
| | Air plus glucose | Air alone | $N_2$ plus glucose | Air plus glucose | Air alone | $N_2$ plus glucose |
| 0 | 4 | 4 | 4 | 232 | 232 | 232 |
| 130 | 26 | 20 | | 353 | 275 | |
| 220 | 42 | 45 | | 366 | 339 | |
| 345 | 70 | 60 | | 448 | 386 | |
| 540 | | 65 | 17 | | 364 | 274 |

It can be seen from the results recorded in Table III, that aeration results in an increased formation of aconitase and fumarase in resting cells. It will be noted that the increase in aconitase activity is much more marked than that of fumarase; this was observed in all the experiments run. There were some indications that the aconitase finally formed is proportional to that present originally.

That the cells had adapted, as far as cytochrome content was concerned, could be seen from the fact that the strong cytochrome *b*1 band (555 mμ at −170° C) present in the anaerobically grown cells disappeared, and the cytochrome *c* and *b* bands (546.5 mμ and 559 mμ, respectively, at −170° C) appeared during the process of aeration. The organisms which were exposed to a current of oxygen free nitrogen retained the original *b*1 band. It should be noted, however, that exposure to air of anaerobically grown cells in the aeration tubes used here does not lead to the same degree of respiratory activity as that displayed by cells grown in air; the $Q_{O_2}$ obtained in this manner represents roughly 50–60% of that obtained during aerobic growth.

These experiments were repeated in the presence of added ammonium ions in order to see whether conditions which allow a small amount of growth (ammonium sulfate was added in a concentration corresponding to a little less than the nitrogen content of the cells) would lead to a more pronounced increase in aconitase and fumarase activity. Actually, the results obtained were not greatly different from those recorded in the absence of added nitrogen. It can be concluded that prolonged growth in the presence of air is necessary to permit appearance of maximal aconitase and fumarase activity.

It will have been noticed that when growth conditions are changed from that of anaerobiosis to that of aerobiosis, fumarase increases approximately 7 times in the normal yeast, while aconitase increases 20 times. Although the total activity of these enzymes in the adaptation experiments remains below that obtained during aerobic growth, aconitase increases to a much greater extent than fumarase activity. This might be interpreted by assuming that fumarase participates in the strictly anaerobic as well as in the aerobic metabolism of yeast, while aconitase is involved predominantly in aerobic metabolism.

*Localization of enzyme activities by differential centrifugation*

Since the work of GREEN[11,12] it has been widely assumed that "cyclophorase" of mammalian tissues, of which fumarase and aconitase are integral parts, is associated with a sedimentable fraction of homogenates. HOGEBOOM AND SCHNEIDER[14] have shown, however, that TPN-linked *iso*citric dehydrogenase, another member of the tricarboxylic acid cycle system, is associated almost entirely with the soluble fraction of mouse liver

*References p. 686.*

TABLE IV

Differential centrifugation of extracts from normal and mutant yeast and determination of fumarase, aconitase, DPN-linked *iso*citric dehydrogenase, and cytochrome oxidase activities in extracts and fractions. Organisms grown aerobically for 72 hours on touraillon agar on Petri dishes. Preparation of extracts and fractionation done with 0.1 *M* phosphate buffer, pH 7.3. Centrifugal force employed: *ca.* 31,000 g for one hour. Particles washed once at same centrifugal force and for same time. Activity expressed in units per ml of extract, or in units per ml of fraction. The latter (as well as the mg-N-per-ml figures) are calculated on basis that fractions are contained in same volume as original extract. Specific activity = units/mg N. For definition of unit of fumarase, aconitase, and DPN-*iso*citric dehydrogenase see Table I. Activity in case of cytochrome oxidase = $\mu$l $O_2$ taken up per 1 ml of extract per hour; specific activity = activity/mg N.

| *Organism and fraction* | *Fumarase* | | | *Aconitase* | | | *DPN-isocitric dehydrogenase* | | | *Cytochrome oxidase* | | | *mg N per ml* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Total activity* | *% of total* | *Specific activity* | *Total activity* | *% of total* | *Specific activity* | *Total activity* | *% of total* | *Specific activity* | *Total activity* | *% of total* | *Specific activity* | |
| Normal yeast | | | | | | | | | | | | | |
| Whole extract | 4787 | 100 | 1930 | 1328 | 100 | 535 | 385 | 100 | 155 | 707 | 100 | 285 | 2.48 |
| Supernatant | 4470 | 93 | 2829 | 732 | 55 | 463 | 356 | 92 | 225 | 1 | <1 | <1 | 1.58 |
| Particles | 490 | 10 | 557 | 74 | 6 | 86 | 28 | 7 | 32 | 759 | 107 | 863 | 0.88 |
| Mutant yeast | | | | | | | | | | | | | |
| Whole extract | 1428 | 100 | 562 | 180 | 100 | 71 | 206 | 100 | 81 | 0 | 0 | 0 | 2.54 |
| Supernatant | 1270 | 89 | 655 | 80 | 44 | 41 | 166 | 81 | 86 | | | | 1.94 |
| Particles | 140 | 10 | 226 | 34 | 19 | 55 | 6 | 3 | 10 | | | | 0.62 |

homogenates, and consequently consider the view that the cyclophorase complex is entirely associated with mitochondria as unjustified.

In order to determine the distribution of aconitase, fumarase, and DPN-*iso*citric dehydrogenase within the yeast cell we fractionated the extracts by differential centrifugation. The desiderata for such procedures and criteria for the evaluation of results have been given by SCHNEIDER[26] and HOGEBOOM AND SCHNEIDER[14]. Two principal sources of error in such studies, namely transfer of material from particles to supernatant, and adsorption of soluble material unto particles have recently been reemphasized by STILL AND KAPLAN[32], and by BEINERT[2].

The extracts were centrifuged at about 31,000 g with the aid of the multispeed attachement of the refrigerated International Centrifuge. A very light pellicle of fatty material is always found on the surface of the liquid after centrifugation; the particulate material at the bottom of the tube is very compact and can be removed and dispersed only with difficulty. The two fractions obtained consist of 1. residue, here called particulate fraction, and corresponding, probably, to the large granules (mitochondria)* and the microsomes[4,5], and 2. supernatant. Enzyme assays were always carried out on the original extract as well as on the fractions. The results from a typicale xperiment are given in Table IV. For comparison's sake, data on cytochrome oxidase activity from the same experiment are also included in order to show the validity of the fractionation procedure. As pointed out by CHANTRENNE[3] and SLONIMSKI AND EPHRUSSI[30], cytochrome oxidase activity is associated with particulate material within the yeast cell.

It can be seen that fumarase, aconitase, and DPN-*iso*citric dehydrogenase activity

* For discussions concerning the occurrence of mitochondria in yeast, see[3,20,21,30].

*References p. 686.*

in the normal and mutant yeast are found mainly in the supernatant fraction. It will be noted, in the case of aconitase, that recovery data, and therefore the data on specific activity, are rather low. This is due to the instability of aconitase; a considerable amount of activity was lost during the fractionation procedures; recombination of the supernatant and particulate fractions did not restore activity to the level found in the whole extract. This experiment was repeated several times, with cultures grown on liquid as well as solid media; essentially similar results were obtained throughout. SCHNEIDER[26] has pointed out that the medium in which the cells are disrupted has a profound effect on the cytological and biochemical properties of the particulate components isolated, and that the phenomena of adsorption and release of enzymes which may occur during the process of fractionation can be tested to some extent by using different suspending media. Isotonic lactose as suspending medium was, therefore, also employed; baker's yeast does not metabolize lactose. In this experiment, the particulate fraction was not washed, and fumarase and aconitase activities in the particulate fraction were in the neighbourhood of 20%. Aconitase seemed more stable when lactose was used and total recoveries were higher. During this experiment, citrate disappearance in the extract and fractions was measured also and found to take place mainly in the supernatant fraction.

It appears from the data given in Table IV that at least two enzymes associated with the tricarboxylic acid cycle in animal tissues, aconitase and fumarase, are associated to a large extent with the supernatant fraction in yeast. Whether the activity of these enzymes found in the particulate fraction is actually associated with this fraction in the cell, or whether some enzyme has been adsorbed unto the particles during the fractionation procedure is difficult to determine.

*Oxidation of some tricarboxylic acid cycle intermediates by extracts and dried cell preparations*

It was attempted, without success, to obtain preparations which would catalyze all the reactions of the KREBS tricarboxylic acid cycle. Several observations seem, however, to be of interest in this connection and will be mentioned briefly.

Rapidly dried cells were prepared in addition to extracts. In extracts from the normal yeast, the endogenous respiration was found to be quite high, corresponding, approximately, to an initial $Q_{O_2}(N)$ 100, which fell, however, somewhat after the first 20 minutes. Addition of citrate (3 micromoles), malate (6 micromoles), pyruvate (6 micromoles), and glucose (2.5 micromoles) resulted in no significant increase in respiration over the endogenous rate at pH 7.3, even in the presence of added ATP and Mg ions. Succinate (6 micromoles), on the other hand, gave during the first 20 minutes after addition, an increase of *ca.* 35% over the endogenous respiration, a figure which dropped to *ca.* 15%* at the end of 60 minutes. It should be noted that in homogenates made from animal tissues, oxidation of succinate has been found to occur at a rate which is independent of the rates of other reactions of the tricarboxylic acid cycle[24]. The system oxidizing succinate is relatively simple, since no pyridine nucleotides or flavo-proteins are apparently involved[27,33]. Extracts made from the mutant yeast show a very low, cyanide nonsensitive respiration, both endogenous and in the presence of the above-mentioned substrates[29].

When pyruvate is added in the presence of one of the acids of the KREBS cycle (malate) no catalytic effect of the latter on the oxidation of the former is observed.

* Statistically significant, as compared with the endogenous respiration, by t test on the 1% level.

*References p. 686.*

Dried cell preparations were made from "starved" yeast. The latter was prepared by bubbling air through washed yeast suspensions in $M/15$ phosphate buffer at pH 4.5 for 19 hours. Addition of succinate, lactate, glucose, and pyruvate results in an increase in respiration, while citrate and malate are not respired at pH 7.3. When phosphate buffers of pH 4.5 are employed, malate again is not oxidized, while addition of lactate and pyruvate results in relatively strong increases in oxygen uptake (see Table V)

TABLE V

Respiration of dried cell preparations from normal yeast; 30 $\mu$moles of substrate and 20 mg dry weight of cells per WARBURG vessel; temperature 28° C.

| *Substrate* | *Final pH* | *$\mu$l $O_2$ taken up per mg dry weigt during* | |
|---|---|---|---|
| | | *1st hour* | *2d hour* |
| Endogenous | 5.5 | 8.2 | 5.1 |
| Citrate | 6.2 | 10.6 | 5.4 |
| Succinate | 5.8 | 10.3 | 6.6 |
| Malate | 5.7 | 8.9 | 5.4 |
| Lactate | 5.7 | 12.9 | 13.6 |
| Pyruvate | 6.1 | 14.7 | 16.1 |

When the same experiment is performed at pH 1.8, there is no activity. Whether the increase in activity due to the addition of citrate at pH 4.5 is significant could not be decided because it was found that the endogenous respiration of the dried preparations is a function of pH, with a maximum near pH 6.0, and because the final pH was somewhat higher in the vessels containing citrate than those in which the endogenous respiration was measured. Experiments, in which the final pH in all vessels was alike, showed a very slight increase in oxygen uptake due to the addition of citrate, an increase the significance of which is somewhat doubtful*.

BARRON *et al.*[1] have shown that the endogenous respiration of washed, resting baker's yeast is independent of pH within the 1–10 range. The drying process employed here obviously abolishes the ability of yeast to maintain a relatively constant intracellular pH regardless of environmental conditions; it is likely that this is accompanied by a change of permeability of the cell wall. BARRON *et al.*[1] working with resting cells, had found that at pH 1.08 only pyruvate is oxidized, while at pH 2.3, 2.9, and 3.9 pyruvic, acetic, citric, and succinic acids are oxidized, and that oxidation of citric acid (for which a net $Q_{O_2}$ of 1.5 was considered significant) ceases at pH 5.0. The two other acids of the tricarboxylic acid cycle (malic and alpha-ketoglutaric acids) were not oxidized at pH 2 and 6, and this was ascribed to the nonpenetration of these two substrates.

## DISCUSSION

In mammalian tissues, the enzymes which mediate the reactions of the tricarboxylic acid cycle constitute a catalytic system whose major importance in tissue respiration is now established beyond doubt. In yeast, however, the existence of such a cyclic mechanism is still a matter of controversy[16, 19]. A major obstacle lying in the path of elucidation of this problem has been the impermeability of the cell wall to several of the intermediates involved[17, 18]. It is well known from work on more or less crude enzyme preparations that many, if not all, of the individual reactions which are part of the cycle can be effected, but no system mediating the complete oxidation of added cycle

* $Q_{O_2} = 0.9$; results of t test show that P is between 0.01 and 0.05.

*References p. 686.*

intermediates to $CO_2$ and water, similar to that from mammalian tissues, has been prepared from yeast.

It has been shown above that malate does not increase oxygen uptakes in either extracts or dried cells, and stimulation by citrate is very small, if significant at all. This can be due either to the fact that these substrates are not a part of those lying on the main path of hydrogen transport in the yeast cell, or to the fact that our preparations are deficient in one or more of the catalysts taking part in such a system. We think that the first interpretation may be the correct one, for the following reasons: A complete respiratory system exists both in extracts and dried cells as is shown by the high endogenous respiration of these preparations; it is comparable in activity to that occurring in intact cells. The breakdown of endogenous reserves by yeast is a purely respiratory process[31] and it is usually assumed that the decomposition of stored reserve material in yeast proceeds by the same mechanism as the respiration of added substrates. Whether one stipulates that the endogenous respiration in extracts and dried preparations from normal yeast is the same as that occurring in intact yeast or not, the fact remains that some sort of terminal respiration system remains intact. An increase in respiration occurs following the addition of several substrates involved in the tricarboxylic acid cycle, but not of others. In dried preparations, addition of succinate, pyruvate, lactate, and glucose results in increased oxygen uptakes, while malate gives no such increase and citrate only a very low one. It seems doubtful that the latter can be ascribed to non-penetration. This is further supported by the observation that addition of equal amounts of succinate to extracts and dried cells results in identical final oxygen uptakes.

It is clear from our data concerning the mutant yeast that KREBS cycle reactions may take place in yeast devoid of succinic dehydrogenase and the WARBURG-KEILIN system. The aim in establishing metabolic schemes such as the tricarboxylic acid cycle was precisely to explain the mechanism of oxygen consumption resulting from the metabolism of the intermediary compounds formed by glycolysis. In the mutant yeast grown aerobically as well as in normal and mutant yeast grown anaerobically, two of the enzymes forming an integral part of the cycle exist, though their activity is reduced. However, respiration in such cells is very low, and an important carrier system is absent. The conclusion from this would appear to be that the tricarboxylic acid cycle if it does exist at all in yeast (which we consider by no means established*) might conceivably be integrated into a system of reactions which is not a part of respiration but of fermentation or reductive synthetic reactions.

The effect of the mutation "petite colonie" consists in an extensive modification of the enzymatic pattern of the cell. As far as has been studied, it involves the loss of cytochrome oxidase, succinic dehydrogenase, alpha-glycerophosphate dehydrogenase, and DPN-cytochrome *c* reductase, an increased amount of cytochrome *c*, the acquisition of a DPN-independent malic-cytochrome *c* reductase and a new cytochrome component, $a_1$[29,30], and a diminution of aconitase, fumarase, and DPN-*iso*citric dehydrogenase**.

* Obviously, the presence in yeast of individual enzymes similar to those mediating intermediary steps in the KREBS cycle in mammalian tissues by no means establishes the existence of the cycle as a whole.

** All these changes are interpreted as being the direct result of the mutation "petite colonie". It should be noticed that this conclusion is drawn from the comparison of the normal yeast strain on one hand and, on the other, the mutant strain isolated from it several years previously. It is conceivable that a more recently isolated mutant strain would have given somewhat different results.

*References p. 686.*

Under anaerobic conditions of growth normal and mutant yeast seem to be alike, and the differences manifest themselves only under aerobic growth conditions. Thus if for the moment we consider the normal yeast as one kind of tissue, mutant yeast could well be regarded as a new kind of tissue. The bearing this has on attempts to establish models for the processes of differentiation is obvious.

The mutation "petite colonie" seems to induce a partial conversion towards the enzymatic constitution which is exhibited by normal yeast cultured under anaerobic conditions; but while in the normal yeast the ability to respire can be restored easily by changing the cultural conditions, the loss of this ability in the mutant yeast seems, from the evidence obtained so far, to be irreversible and thus corresponds to a change in the potencies of the cell.

From all the evidence obtained so far it appears that enzymes which are linked to a particulate fraction in the normal yeast are lost in the mutant, while non-linked enzymes are not lost, although their activity may be greatly reduced. It would thus appear that in the case of the mutant yeast one deals with particularly favorable material for the study of the intracellular distribution of enzymes and of the problem of the relation between cell chemistry and cell structure.

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SUMMARY

A comparative study of three enzymes, aconitase, fumarase, and DPN-linked *iso*citric dehydrogenase was made in normal baker's yeast and in the mutant "petite colonie" grown under varying conditions of oxygen supply. When both normal and mutant yeasts are grown anaerobically, aconitase, fumarase, and DPN-*iso*citric dehydrogenase activities in the two strains are alike. When grown in the presence of air, all three enzymes show increased activity, but under such conditions distinct differences between the two strains become apparent: the increases in the normal strain are much more pronounced than in the mutant strain. Aeration of non-proliferating suspensions of anaerobically grown normal yeast which is known to re-establish ability to respire also leads to an increased formation of aconitase and, to a lesser extent, fumarase, but in order to obtain their full complement of these enzymes, extensive growth of the cells in the presence of air seems necessary. By differential centrifugation all three enzymes were found to be associated mainly with the supernatant fraction in both normal and mutant yeast. The function of these enzymes in the metabolism of yeast is discussed.

RÉSUMÉ

Les activités des enzymes aconitase, fumarase et déshydrogénase *iso*citrique liée au coenzyme I ont été étudiées dans la levure de boulangerie normale et le mutant "petite colonie" ayant proliféré en aérobiose et en anaérobiose. Les activités des 3 enzymes sont identiques dans les deux levures lorsque celles-ci ont proliféré en anaérobiose. Lorsqu'elles ont proliféré en aérobiose, les deux levures présentent des activités enzymatiques accrues, mais cette augmentation est plus prononcée chez la levure normale que chez la levure mutante. L'aération de suspensions non proliférantes de levure normale ayant proliféré en anaérobiose, qui restaure l'abilité respiratoire de la levure, conduit également à l'augmentation de l'activité de l'aconitase et, à un moindre degré, de la fumarase. Cependant, la récupération de l'activité totale de ces enzymes requiert une multiplication aérobie prolongée. La mesure des activités des 3 enzymes dans les diverses fractions des broyats de levures séparées par centrifugation différentielle montre qu'ils se trouvent surtout dans le liquide surnageant. Le rôle des enzymes étudiés dans le métabolisme de la levure est discuté.

*References p. 686.*

ZUSAMMENFASSUNG

Die Aktivität der Enzyme Aconitase, Fumarase und an DPN gebundene *iso*citrische Dehydrogenase in normaler Bäckereihefe und in dem Mutanten "petite colonie" wurde bei aerobem und anaerobem Wachstum untersucht. Die Aktivitäten der 3 Enzyme sind nach anaerobem Wachstum in beiden Hefen gleich. Nach aerobem Wachstum zeigen beide Hefen erhöhte Enzym-Aktivitäten, aber diese Erhöhung ist bei der normalen Hefe ausgesprochener als bei dem Mutanten. Bei Suspensionen nicht proliferierender normaler Hefe, welche anaerob gewachsen war, stellt Lüftung die Fähigkeit zur Atmung wieder her und erhöht auch die Aconitase-Aktivität und in geringerem Masse die Fumarase-Aktivität. Zur vollständigen Wiederherstellung der Aktivität dieser Enzyme ist aber ein längeres Wachstum in Gegenwart von Sauerstoff nötig. Die Aktivität der drei Enzyme wurde in den verschiedenen Fraktionen von zentrifugiertem Hefebrei gemessen; hierbei zeigte sich, dass sich die Enzyme bei beiden Hefen hauptsächlich in der überstehenden Flüssigkeit vorfinden. Die Rolle, welche die untersuchten Enzyme im Metabolismus der Hefe spielen, wird erörtert.

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