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# The effect of antimycin A on the operation of Krebs cycle in baker's yeast

The Krebs (citric acid) cycle is the main pathway of acetate oxidation in baker's yeast[1-3]. The working of the cycle under anaerobic conditions has been little studied and no observations have been described with baker's yeast[3], although some reactions of the cycle would occur anaerobically in yeast as indicated by the synthesis of glutamic acid and other amino acids[4]. In the present study, metabolic conditions similar to those prevailing under anaerobiosis have been produced with antimycin A, an inhibitor of electron transport[5], and the operation of the cycle has been followed with [2-$^{14}$C]acetate which labels all the intermediates of the cycle. The observations reported below confirm the partial operation of the Krebs cycle in baker's yeast under anaerobic conditions[7], but other reactions, not requiring oxygen, must take place in order to regenerate oxaloacetic acid. The materials and experimental methods employed have been described elsewhere[2,6].

Antimycin A (0.25 $\mu$g/ml) completely inhibits acetate oxidation by baker's yeast (4.9 mg per ml suspension). However, in the presence of excess of antibiotic, [2-$^{14}$C]-acetate is incorporated by the cells and glucose stimulates the incorporation (Table I). Similar results are obtained with baker's yeast under $H_2$ (Table I) and with brewer's yeast. The anaerobic incorporation of [2-$^{14}$C]acetate does not exceed 12% of the aerobic (Table I) and very little $^{14}$C appears in $CO_2$ (less than 4% of $^{14}$C in cells), which is in contrast with the high radioactivity of the aerobic $CO_2$ (71–52% of $^{14}$C in cells).

TABLE I

ANAEROBIC INCORPORATION OF [2-$^{14}$C]ACETATE BY BAKER'S YEAST

*Expt. A*: 20 mg of yeast, 4.2 mM [2-$^{14}$C]acetate (specific activity, $8.6 \cdot 10^4$ counts/min/$\mu$mole), pH 4.4, and additions as shown. Total volume, 2 ml. Incubation for 35 min in Warburg manometers at 30°; equilibration for 10 min before addition of substrates. $Q_{O_2}$ ($\mu$l $O_2$/mg dry wt./h) values: yeast with acetate, 30; acetate and antimycin A, 1.6; glucose, 25; glucose and antimycin A, 0.8. *Expt. B*: 59 mg of yeast; 6.3 mM [2-$^{14}$C]acetate (specific activity, $3.4 \cdot 10^4$ counts/min/$\mu$mole), pH 4.0; 0.15 ml 5 N NaOH in the central well of the Warburg vessel. Incubation for 45 min. Other conditions as in Expt. A. After incubation, 1.5-ml samples of yeast suspension were mixed with 9 ml methanol; 300 $\mu$l methanolic suspension were evaporated on aluminum cups and counted. In Expt. B, the carbonate fixed in 5 N NaOH was precipitated as $BaCO_3$ and counted for $^{14}$C.

| *Expt.* | *Gas phase* | *Additions* | *Total $^{14}$C fixed in cells (counts/min)* | *Total $^{14}$C in $BaCO_3$ (counts/min)* |
|---|---|---|---|---|
| A | Air | Antimycin A (10 $\mu$g) | 8 600 | — |
| A | Air | Antimycin A (10 $\mu$g) + 5 mM glucose | 42 600 | — |
| B | Air | Antimycin A (50 $\mu$g) | 2 700 | — |
| B | Air | Antimycin A (50 $\mu$g) + 5 mM glucose | 4 930 | 193 |
| B | $H_2$ | None | 2 300 | 29 |
| B | $H_2$ | 5 mM glucose | 6 500 | 42 |
| B | Air | None | 76 500 | 39 600 |
| B | Air | 5 mM glucose | 57 200 | 40 600 |

Fig. 1 shows the kinetics of [2-$^{14}$C]acetate incorporation with $N_2$ or antimycin A. With antimycin A and glucose (Expt. B), $^{14}$C fixation and $CO_2$ evolution follow a

similar course and at the end of the incubation, the distribution (%) of $^{14}C$ in the cell fraction soluble in methanol–water was: tricarboxylic acids, 3.3; α-oxoglutaric acid, 0.6; succinic acid, 16.0; fumaric acid, 0.8; malic acid, 4.8; glycolic acid, 0.3; glutamic acid, 57.8; glutamine, 0.6; proline, 4.7; arginine–ornithine, 6.3; aspartic acid, 0.6; alanine, 1.2; hexose phosphates, 0.8 and phosphoglyceric acid, 0.3. The soluble fraction represented 63% of the total $^{14}C$ incorporated by the cells. The percentage of $^{14}C$ in glutamic, aspartic, dicarboxylic, and tricarboxylic acids did not vary significantly throughout the incubation period, except the increase of the amount in succinic acid from 12 to 16% in the 5–100-min interval. Similar distribution patterns were obtained in Expts. A, A′ and B′. The relatively high labeling of succinic acid among the Krebs-cycle intermediates is in contrast with the $^{14}C$ distribution after aerobic oxidation of [2-$^{14}C$]acetate where the tricarboxylic acids have 19–14% and succinic acid 3.3–4.1% of $^{14}C$ in the methanol–water extract[7].

The distribution of $^{14}C$ in glutamic and succinic acid after incorporation of [2-$^{14}C$]-acetate has been established by degradation of samples isolated from the experimental material described in Fig. 1. In glutamic acid, radioactivity in C-1, C-2, C-3 and C-5 was counted after decarboxylation, or after degradation of the C-2–C-3 fragment with the Schmidt reaction[8]. Labeling of C-4 was calculated from the difference between total $^{14}C$ activity and the sum of activities counted in the other carbon atoms. The following values (%) were obtained. Expt. A (20-min incubation): C-1, 2 ± 0.1*;

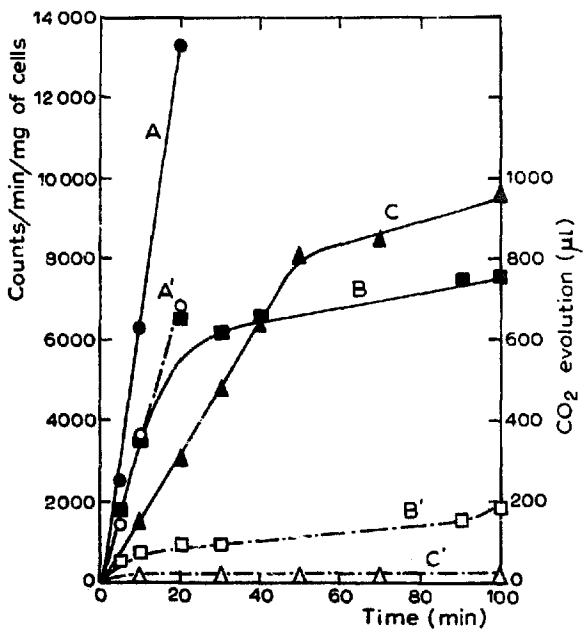


Fig. 1. Anaerobic incorporation of [2-$^{14}C$]acetate by baker's yeast. Yeast, 12.8 mg/ml; 6.1 mM [2-$^{14}C$]acetate (specific activity: $8.3 \cdot 10^4$ counts/min/μmole); pH 4.4. Total volume, 10 ml. Temp., 20°. Expt. A (●), $^{14}C$ incorporation with 5 mM glucose under $N_2$; Expt. A′ (○), control without glucose. Expt. B (■), $^{14}C$ incorporation with 5 mM glucose and antimycin A (10 μg/ml); Expt. B′ (□), control without glucose. Expt. C (▲), $CO_2$ production of 2 ml yeast suspension, with acetate, glucose and antimycin A, measured in Warburg manometers, under conditions identical with those in Expt. B; Expt. C′ (△), control without glucose. Expts. A and A′ were performed in the closed reaction vessels described in ref. 2. After incubation, samples (1 ml) of reaction mixture were added to 9 ml of methanol and aliquots of the methanolic yeast suspension counted for $^{14}C$.

* S.E. of the mean. Measurements were carried out in duplicate or triplicate.

C-2, 18 ± 1.1; C-3, 34 ± 1.0; C-4, 44 ± 1.1; and C-5, 2 ± 0.7. Expt. B (20-min incubation): C-1, 0 ± 0.2; C-2, 31 ± 10; C-3, 31 ± 4.0; C-4, 38 ± 7; and C-5, 0. Expt. B (100 min incubation): C-1, 0 ± 0.2; C-2, 24 ± 1.5; C-3, 44 ± 5; C-4, 31 ± 5; and C-5, 0 ± 0.1. In the succinic acid obtained from Expt. B, the percentage of $^{14}C$ in the methylene carbons was (in parentheses time (min) of incubation) 96 ± 3.9 (20), 98 ± 1.9 (30), 100 ± 9.4 (60), and 77 ± 9.5 (90), whereas in Expt. A the corresponding values were 82 ± 3.5 (10) and 87 ± 4.5 (20). The incorporation of $^{14}C$ in the methylene carbons of succinic acid is higher than in the aerobic oxidation of [2-$^{14}C$]acetate where it reaches the 66.6% value required by the Krebs-cycle model[6].

The data summarized above demonstrate the anaerobic operation of the Krebs cycle up to the succinate–fumarate step. This reaction depends on the activity of the electron-transport system[3] which is inhibited by antimycin A. In this manner the antibiotic prevents the completion of the cycle, which is essential for the appearance of the methyl carbon atom of acetate in C-1 of glutamic acid and in respiratory $CO_2$. With antimycin A, the oxaloacetate required for the synthesis of citrate can be formed (i) by carboxylation of phosphopyruvate[2,9] which is consistent with the effect of glucose on [2-$^{14}C$]acetate incorporation, and (ii) from malate. Synthesis of [3-$^{14}C$]malate from [2-$^{14}C$]acetate and glyoxylate (malate synthetase reaction[10]) would account for the initial incorporation of $^{14}C$ into fumaric, malic and aspartic acids, alanine, and the labeling of glutamic acid C-2. Furthermore, equilibration of [3-$^{14}C$]malate with fumarate yields [2,3-$^{14}C_2$]malate. Glyoxylate is supplied by the isocitrate lyase reaction[11], and with isocitrate derived from [2-$^{14}C$]acetate and [3-$^{14}C$]-oxaloacetate (malate formed) the reaction yields [2-$^{14}C$]glyoxylate and [2,3-$^{14}C_2$]-succinate. Condensation of [2-$^{14}C$]glyoxylate and [2-$^{14}C$]acetate would constitute an extra source of [2,3-$^{14}C_2$]malate, and according to the Krebs-cycle model, the latter $^{14}C$ labels glutamic acid C-3 and the carboxyl groups of succinic acid. The postulated association of the Krebs and glyoxylate cycles (*cf.* also ref. 3) explains, at least qualitatively, the results presented above, and is also in agreement with the formation of radioactive glyoxylate and glycolate from [2-$^{14}C$]acetate, observed with baker's yeast by BOLCATO *et al.*[14].

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## Phosphatase reactions during tissue extractions

In a recent paper, ULLRICH AND CALVIN[1] showed that phosphatase in a spinach-chloroplast preparation was not immediately inactivated by cold methanol, and could catalyse the formation of methyl phosphate when inorganic phosphate was present. In this laboratory, methyl and ethyl phosphates were detected in extracts from plant tissues (*e.g.* potato-tuber slices) killed in boiling 80% methanol or 80% ethanol, and it appeared likely that these esters had also resulted from phosphatase-catalysed reactions. A partly purified potato phosphatase was prepared in order to test this. 50 g potato-tuber tissue was homogenized in 150 ml 0.03 M acetate buffer (pH 4.9) plus 0.005 M cysteine, and centrifuged (3000 × *g*, 15 min, 0–5°). The protein precipitating from the supernatant between 33% and 66% saturation with $(NH_4)_2SO_4$ at pH 6.5 was dissolved in distilled water, and from this solution was collected the protein fraction which precipitated between 40 and 60% saturation with $(NH_4)_2SO_4$ at pH 6.5. This precipitate was dissolved in distilled water and dialysed against several changes of distilled water and then, for 60 h, against 0.01 M acetate buffer (pH 4.9) plus 0.001 M $MgSO_4$. The dialysate was centrifuged (3000 × *g*, 15 min) and the supernatant (20 ml) frozen till required. It hydrolysed 83 μmoles *p*-nitrophenylphosphate per h per ml enzyme, at pH 4.9 and 22°. This represented 15% of the phosphatase activity of the original homogenate. In the presence of chloroform as a bacteriocide, this phosphatase lost less than 25% of its activity during 5 days at room temperature. The phosphatase was equally stable in 20% methanol.

The phosphatase synthesized a range of alkyl phosphates, including methyl-, ethyl-, *n*-propyl- and *n*-butyl- (but not *tert.*-butyl-) phosphates from the corresponding alcohols plus inorganic phosphate. Similar findings have been reported for other phosphatases[2,3]. Similarly, the phosphatase synthesized a mixture of glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate and mannose 6-phosphate (ratio 72:17:6:5) when in the presence of glucose and inorganic phosphate, and synthesized α-glycerophosphate and β-glycerophosphate (ratio 8:1) from glycerol and inorganic phosphate. All of the above reactions were also catalysed by a commercial alkaline phosphatase (EC 3.1.3.1) (*cf.* MORTON[4]).

When the partly purified potato phosphatase solution was dripped into boiling 80% methanol, inactivation was complete in less than 10 sec, and no methyl phosphate could be detected. However, when disks of potato-tuber tissue, 1 mm thick by 1 cm diameter, were plunged into the boiling 80% methanol and boiled for 3 min, a moderate amount of methyl phosphate was formed. Killing the tissue in 80% ethanol likewise produced ethyl phosphate. All this can be taken as evidence that