UTILIZATION OF C\textsuperscript{14}-Labeled Pyruvate and Acetate by Yeast\(^*\)

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The metabolism of pyruvate and acetate has been widely investigated in a variety of organisms. Studies of pyruvate fermentation in yeast, as well as the aerobic metabolism of both compounds in animals and microorganisms, have established the importance of decarboxylation as an initial step in the anaerobic metabolism of pyruvate and of the central position of the Krebs tricarboxylic acid cycle as a major pathway of aerobic metabolism in many species.

On the other hand, with some important exceptions (1, 2), the mechanisms of conversion of pyruvate and acetate into the carbon skeletons of amino acids are less well understood. The work presented in this paper has been carried out in preparation for an investigation of these mechanisms, which will be considered in detail in forthcoming publications. Since pyruvate is known to be readily converted through decarboxylation to acetate or its equivalent, in the present study carbonyl-labeled pyruvate and carboxyl-labeled acetate were employed as substrates for bakers' yeast. A comparison of the over-all metabolism of the two substrates is made, with special reference to their relative utilization, the growth responses, production of ethanol, acetaldehyde, and CO\textsubscript{2}, and the distribution of radioactivity among these metabolites.

EXPERIMENTAL

Synthesis of C\textsuperscript{14}-Carboxyl-Labeled Acetic Acid and C\textsuperscript{14}-Carbonyl-Labeled Pyruvic Acid—Sodium acetate (137 mg.) with a specific activity of 3.70 \times 10^8 c.p.m. per mm was synthesized according to the method of Claycomb et al. (3). Yield, 74 per cent on the basis of barium carbonate. The acetate was later diluted as desired with unlabeled acetate for use as sub-

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strate in the fermentation experiments and also as starting material for the synthesis of pyruvic acid.

The carbonyl-labeled pyruvic acid was synthesized by the conversion of (diluted) carboxyl-labeled acetate to pyruvonitrile (4), and then to pyruvamide (5). The pyruvamide was immediately hydrolyzed in 2 N hydrochloric acid and the pyruvic acid isolated by ether extraction and distillation under reduced pressure (6). The fraction boiling at 60–63° at 10 mm. was collected. Purity of this fraction was confirmed by colorimetric assay (7). Yield, 4.35 gm. (42 per cent of theory, based on the sodium acetate used); activity, $1.85 \times 10^6$ c.p.m. per mm.

**Measurement of Radioactivity**—The methods used to measure the radioactivity of various types of samples were essentially the same as those described in a previous paper (8). Activities of sodium acetate, pyruvic acid, yeast, and carbon dioxide produced in the fermentation process were counted as barium carbonate and expressed as counts per minute after being corrected for background and self-absorption. Other samples, such as the yeast fermentation media, were deposited and counted directly in cupped metal planchets after evaporation. Appropriate aliquots (usually about 0.1 ml. each, with total activities of about 1000 c.p.m.) were used. Although the content of inorganic salts of the samples of media was relatively high and the resulting activities were thereby reduced owing to increased self-absorption, preliminary experiments indicated the method to be suitable for control and comparative purposes.

**Determination of Glucose**—This compound was determined iodometrically by the method of Shaffer and Somogyi (9).

**Determination of Pyruvic and Acetic Acids**—The colorimetric method of Friedemann and Haugen (7) was used for the assay of pyruvic acid in the fermentation medium. Acetate was determined by steam-distilling a 100 ml. aliquot of medium in the presence of sulfuric acid and magnesium sulfate. The distillate was titrated with standard alkali and the radioactivity of the neutral solution determined after direct deposition. The specific activity of the distilled acetic acid after fermentation indicated that it was not diluted by other volatile organic acids, nor by acetic acid from other sources.

**Determination of Ethanol and Acetaldehyde**—Ethanol produced during fermentation was analyzed by steam-distilling a 100 ml. aliquot of the medium in the presence of alkali and analyzing the distillate iodometrically (10). Radioactivity of the ethanol was determined after its oxidation to acetic acid, steam distillation of the latter compound, titration, and counting by direct deposition.

Acetaldehyde was assayed only by measurement of the radioactive dimedon solution in which it was trapped. The moles of acetaldehyde
present were calculated, assuming that this compound had the same specific activity as the pyruvic acid added. This seemed justified, since the ethanol produced had the same specific activity.

Apparatus—The apparatus consisted of a 3 liter 3-necked round bottom flask, with attachments as follows: A mercury-sealed paddle stirrer was attached in the center neck, together with an outlet tube for gas and leads for a pair of electrodes to be used in determining the pH of the medium. A small dropping funnel was inserted through each side neck for adding acid or base as needed, together with a ½ inch stainless steel baffle to promote turbulent motion of the medium. Entering also through one side neck was an aeration tube with a sintered tip extending to the bottom of the flask beneath the stirrer, whereas the other side neck was equipped with a tube for the removal of samples during fermentation.

The rate of aeration was determined by passing the inlet gas through a manometer type flow meter. The gas was then led through a soda lime tower and finally filtered through a sterilized cotton tube before entering the culture flask. Acetaldehyde and carbon dioxide leaving the flask were dispersed through a sintered glass tube into 500 ml. of saturated dimedon solution and then scrubbed through two 225 ml. portions of 3 N NaOH.

Growth of Yeast—The organism, Saccharomyces cerevisiae, was obtained by isolation from a cake of Fleischmann's bakers' yeast. Stock cultures were carried on malt-agar slants.1

Organisms to be used for metabolic studies were carried through three stages of growth, as follows: (1) A loop of cells (about 5 mg.) from the stock slant was inoculated into 50 ml. of sterile medium containing malt extract 200 gm., KH₂PO₄ 2 gm., and urea 1 gm. per liter. (2) After incubation for 16 hours at 30°, this 50 ml. culture was used to inoculate the remaining 950 ml. of medium. The pH during the second incubation period was maintained at 4.0 to 4.5 through occasional addition of NH₄OH. After completion of growth, the culture was centrifuged and the cells washed twice with cold distilled water. (3) 10 gm. of wet cells were inoculated into 1 liter of a medium2 containing glucose as the source of carbon. The culture was stirred at 400 r.p.m., gassed with oxygen, air, or nitrogen at 100 ml. per minute, and maintained at pH 4.0 to 4.5 through addition of 3 N NaOH. After 4 hours incubation at 30°, at which time all of the glucose was utilized (see Fig. 1), the cells were centrifuged

1 The composition of the agar was Blue Ribbon malt extract 10 gm., KH₂PO₄ 0.2 gm., agar 1.5 gm., tap water to 100 ml.; pH 4.8.
2 The composition of this medium was glucose 18 gm., (NH₄)₂SO₄ 2.5 gm., NaCl 2 gm., KHPO₄ 2 gm., MgSO₄·7H₂O 250 mg., CaCl₂·2H₂O 250 mg., H₂BO₃·1 mg., ZnSO₄·1 mg., MnSO₄·4H₂O 1 mg., FeCl₃·1 mg., TiCl₃·0.5 mg., CuSO₄·5H₂O 0.1 mg., KI 0.1 mg., Bacto-yeast extract 100 mg., distilled water to 1000 ml. (see also Snell et al. (11)).
and washed twice with cold distilled water. Such cells were uniformly viable and were essentially free of glucose (and presumably also polysaccharides). They were able at this stage to utilize pyruvate readily (Fig. 1), whereas organisms that had not been depleted of glucose would not metabolize pyruvate.

**Incubation with Pyruvate or Acetate**—Immediately following the 4 hour incubation period with glucose, the yeast cells were centrifuged and washed as before. The entire crop (4.0 gm., dry weight equivalent) was inoculated into 1 liter of fresh medium, in which the glucose was replaced by 20 mM of labeled pyruvate or acetate. The system was incubated as above for 4 hours in oxygen or 5 hours in nitrogen to allow complete utilization of pyruvate. Cells were then harvested by centrifugation, washed twice with distilled water, dried *in vacuo* over sodium hydroxide, and retained for subsequent degradation and analysis.

**RESULTS AND DISCUSSION**

**Utilization of Glucose-Pyruvate Mixtures**—Attempts to obtain utilization of isotopic pyruvate in the presence of non-isotopic glucose by growing....
cultures failed, since activity of the medium and the chemically determined pyruvate content remained virtually undiminished until the glucose was fully utilized (diauxie (12)). Shortly thereafter, the yeast cells became radioactive coincident with pyruvate disappearance. Sperber and Runnström (13) observed similar inhibition of pyruvate uptake by glucose in other yeasts, although Brechot and Haag (14) obtained utilization of pyruvate after the 4th day of a 13 day fermentation of glucose.

Although added pyruvate did not equilibrate with metabolizing glucose intermediates, pyruvic acid is formed from glucose during fermentation (14–16). The curves in Fig. 1 indicate the extent of its formation under oxygen, air, or nitrogen atmosphere. Practically no free pyruvate accumulated under anaerobic conditions, but with increasing oxygen tensions up to 4 mM accumulated from 100 mM of glucose. The aerobic accumulation may have been caused in part by reduced cocarboxylase activity at high oxygen tensions (17).

Inspection of the glucose utilization curve reveals that the reaction was of zero order, i.e., it was independent of substrate concentration until the latter reached a very low value. This behavior is consistent with the belief that glucose enters the cell by active enzymatic transfer. The rate of anaerobic utilization (not shown in Fig. 1) was practically identical with the aerobic rate, indicating that this transfer is unaffected by oxygen.

Utilization of Pyruvate and Acetate—The curves in Fig. 2 relate the rates of utilization of pyruvate and acetate, with the glucose-grown yeast described in the experimental section together with 20 mM of either substrate in 1 liter of medium. Pyruvate was completely removed in less than 4 hours in the presence of oxygen, although about 5 hours were needed for full utilization anaerobically. (This may be due to reduced permeability in the absence of oxygen (18).) Acetate, on the other hand, was only 39 per cent removed at the end of 4 hours (5.4 hours calculated for 50 per cent utilization). Also, whereas pyruvate, like glucose, disappeared according to a zero order reaction (with a slope practically identical to that of the glucose curve), the acetate utilization rate decreased uniformly with time. The data fit best a reaction of second order, and hence the rate depends not only upon the concentration of acetate but also of another reactant. This would suggest that acetate may have entered the cell through a non-enzymatic process such as diffusion, and that a condensing partner (e.g., oxalacetate) may have been limiting. The latter possibility recalls the observation (19) that fumarate enhances the rate of utilization of acetate by yeast.

Metabolism of Pyruvate and Acetate—Further comparisons between pyruvate and acetate in yeast are made in Table I, where determinations of several metabolic products are presented. As expected (20), no ethanol
appeared in the acetate medium. Also as expected, ethanol production from pyruvate was greater anaerobically than in oxygen; up to 4 mM were produced from 20 mM of substrate. The specific activity of the ethanol from each experiment was virtually the same as that of the pyruvate used \((1.8 \times 10^6 \text{ c.p.m. per mM})\); so it may be concluded that all of the ethanol arose directly from pyruvic acid. Acetaldehyde was found only in the aerobic pyruvate medium, but this is not surprising, inasmuch as this compound would serve as a major hydrogen acceptor under anaerobic conditions.

A major difference in acetate and pyruvate consumption by the organism is indicated by comparison of the yeast crops. Over 400 mg of net growth (dry weight) took place in both of the pyruvate experiments, corresponding to incorporation of about 17 mM of carbon, or the equivalent of 5 to 6 mM of pyruvate. On the other hand, the organism failed to grow significantly on acetate (20 mg recorded gain). In spite of this, 39 per cent of the acetate disappeared from the medium, 9 per cent was incorporated into the yeast, and 24 per cent appeared as respiratory CO\(_2\). The specific activity of the CO\(_2\) formed was only one-half of the carbon in the acetate.
used (one-fourth that of the acetate), and it thus appears that considerable exchange occurred between the products of acetate metabolism and intermediary cellular metabolites. Similar turnover evidently occurred in both pyruvate experiments, since the specific activity of the carbon was diluted

### Table 1

**Analysis of Fermentations of Pyruvate and Acetate by Yeast**

<table>
<thead>
<tr>
<th>Fermentation constituents</th>
<th>Acetate Oxygen</th>
<th>Pyruvate Oxygen</th>
<th>Pyruvate Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radioactivity</td>
<td>Radioactivity</td>
<td>Radioactivity</td>
</tr>
<tr>
<td></td>
<td>Amount</td>
<td>Total c.p.m.</td>
<td>Specific activity c.p.m.</td>
</tr>
<tr>
<td></td>
<td>gm.</td>
<td>x 10^5</td>
<td>x 10^6</td>
</tr>
<tr>
<td>Substrate</td>
<td>1.64</td>
<td>37.0</td>
<td>1.85 x 100</td>
</tr>
<tr>
<td>Medium</td>
<td>22.7</td>
<td>61</td>
<td>1.1</td>
</tr>
<tr>
<td>Acids and non-volatile*</td>
<td>0</td>
<td>0</td>
<td>0.026</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.082†</td>
<td>5.18†</td>
<td>0.8</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>8.8</td>
<td>0.46</td>
<td>1.70</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>24.1</td>
<td>1.70</td>
<td>18.4</td>
</tr>
<tr>
<td>Yeast</td>
<td>0.2</td>
<td>0.44</td>
<td>13.2</td>
</tr>
<tr>
<td>Radioactivity recovery</td>
<td>94</td>
<td>0.2</td>
<td>0.2†</td>
</tr>
</tbody>
</table>

* Chiefly acetic acid.
† Calculated from the radioactivity, assuming that the specific activities of acetaldehyde and pyruvic acid were equal.
‡ Calculated by taking the value for carbon content of yeast as approximately 50 per cent.

from $6.2 \times 10^4$ to $4.8 \times 10^5$ and $3.1 \times 10^5$ c.p.m. per mM in the aerobically and anaerobically respired CO₂.

The close similarity of several features of aerobic and anaerobic pyruvate metabolism is somewhat unexpected. The anaerobic values have been checked in separate experiments with non-isotopic pyruvate, by means of nitrogen passed through four alkaline pyrogallol absorbing towers; the yield of CO₂, the rate of utilization of pyruvate, and the formation of pyruvate from added glucose were virtually identical with the amounts listed here. A part of the energy needed under anaerobic conditions for the synthesis of new yeast may have come from intermediates stored
within the cells at the end of the culturing period in glucose. Whatever the major source of biosynthetic energy may have been, it evidently was not limiting, since the anaerobic growth equaled the aerobic. Finally, the results indicate that, although anaerobic conditions were favorable to ethanol production, there was an even greater diversion of pyruvate into yeast growth (5 to 6 mM) than into ethanol (4 mM). Details of this conversion into aspartic acid and other amino acids will be presented elsewhere.

SUMMARY

The utilization by bakers' yeast of CH₃C¹⁴OOCOOH and CH₃C¹⁴OOH has been studied.

Although pyruvate could not be metabolized in the presence of glucose, it was readily assimilated when the yeast was first grown on glucose and then transferred to a pyruvate medium. Under these conditions, pyruvate was utilized much more rapidly than acetate, and according to a different kinetic pattern; disappearance of pyruvate proceeded linearly with time according to a zero order reaction, whereas the rate of acetate utilization diminished with time, approximating a reaction of the second order.

No acetaldehyde was formed from acetate or from anaerobic pyruvate fermentation. Ethanol was formed anaerobically from pyruvate, the specific activities indicating that it arose directly, without dilution from other sources.

About one-third of the carbon atoms from pyruvate was assimilated into the yeast cells, whether in oxygen or nitrogen. Acetate did not produce growth; nevertheless about one-tenth of the total radioactivity was incorporated into the yeast, thus indicating a considerable degree of exchange between the products of acetate metabolism and intermediary cellular metabolites.

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