MECHANISM OF PHENYLACETYL CARBINOL SYNTHESIS BY YEAST

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The enzymatic mechanism by which microorganisms synthesize acetoin and other ketols has been the subject of much experimentation. Many bacteria form acetoin by the intermediate formation of α-acetolactate from two moles of pyruvate, followed by decarboxylation (Dolin and Gunsalus, 1951; Discherl and Höfmann, 1951; Happold and Spencer, 1952; Juni, 1952a). Yeasts, however, utilize free aldehyde. This fact and the demonstrated inability of brewers' yeast to decarboxylate α-acetolactate to acetoin (Juni, 1952b) indicate that the formation of ketols by yeast is through a mechanism distinct from that by bacteria.

Neuberg and Hirsch (1921) suggested that a special enzyme, "carboligase", brings about the formation of ketols in yeast. Neuberg and Hirsch (1921) and Discherl (1931) proposed the condensation of acetaldehyde or benzaldehyde with a "nascent" aldehyde to yield the corresponding ketol. Isotopic carbon studies by Gross and Werkman (1947) and by Juni (1952b) substantiate that a condensation of this general type occurs. Gross and Werkman (1947) found that the addition of C14 labeled acetaldehyde to a cell-free yeast juice in the presence of pyruvate resulted in the labeling of all carbon atoms of the acetoin formed. The heaviest labeling occurred in the carbinol end of the molecule. The addition of pyruvic acid-2-14C and unlabeled acetaldehyde to a cell-free enzyme preparation of brewers' yeast yielded acetoin with the heaviest labeling in the carboxyl portion of acetoin (Juni, 1952b). In both cases, the presence of labeled carbon in all carbon atoms could be accounted for by oxidation or reduction of some of the acetoin to a symmetrical molecule.

Singer and Pensky (1951) have reported that a purified α-carboxylase of wheat germ will synthesize acetoin from acetaldehyde, pyruvate, or both, when diphosphothiamin and Mg++ are present. Juni (1952b) was unable to separate carboxylase from the acetoin forming system of brewers' yeast.

The exact mechanism of condensation attributed to "carboligase" is not known. It seemed plausible to look upon ketol formation by yeast as an acetylation reaction. Since biological acetylations have been shown to be coenzyme A dependent, it appeared more than likely that the condensation occurred through the mediation of coenzyme A. Subsequent experimentation substantiated this hypothesis.

This paper reports the synthesis of phenylacetylcarbinol by an acetone powder of brewers' yeast by acetylation of benzaldehyde via a coenzyme A dependent system.

MATERIALS AND METHODS

Acetone powders were prepared from fresh pressed brewers' yeast (Standard Brands) by the method of Hochster and Quastel (1951). Nicotinamide was not added in order to permit the destruction of diphosphopyridine nucleotide.

One coenzyme A concentrate was kindly supplied by Dr. G. D. Novelli. Another less active concentrate was obtained from Krishell Laboratories, Portland, Oregon.

Pyruvic acid was purified by vacuum distillation and neutralized to bromthymol blue. Acetyl phosphate, as the lithium salt, was obtained from Krishell Laboratories.

Carbon dioxide evolution and O2 uptake were measured in the conventional Warburg apparatus at 28 C, with air as the gas phase. Retained CO2 was liberated by tipping in 0.5 ml N H2SO4. Green et al. (1942), reporting on the formation of acetoin and propion from pyruvic acid and α-ketobutyric acid, respectively, by pig heart

1 The authors have used the term acetylation to denote a -- C—C—CH3 linkage resulting from the condensation of aldehydes with acetyl-coenzyme A.
preparations, noted that the gas phase used had no effect on the reactions which were all completely anaerobic.

The following methods were employed for determination of substrates and products: acetyl phosphate by the method of Lipmann and Tuttle (1945); acetic acid, determined as volatile acid by steam distillation at pH 2 and titration with standard NaOH; lactic acid by the method of Barker and Summerson (1941); inorganic phosphate by the method of Fiske and Subbarow (1925); adenosine triphosphatase by the method of DuBois and Potter (1943); pyruvic acid, manometrically by ceric sulfate oxidation (Barron and Lyman, 1939). Lactic acid also is oxidized by ceric sulfate (Gordon and Quastel, 1939). The actual amount of unutilized pyruvic acid was found by correcting the result from ceric sulfate oxidation for the lactic acid formed.

Benzaldehyde, benzyl alcohol, and benzoic acid were determined by a modification of a method developed by Dr. Nelson Trenner of the Physical and Inorganic Research Department. Sodium bisulfite was added to the sample to bind the aldehyde and ketol. Benzoic acid then was extracted with petroleum benzine. Following this extraction benzyl alcohol was extracted with diethyl ether. The extracted sample was treated with excess sodium bicarbonate and the released aldehyde extracted with diethyl ether. Appropriate dilutions of these extracts were read in the Beckman spectrophotometer. The presence or absence of absorption peaks was noted at the following wavelengths: 241 mµ for benzaldehyde; 258 mµ for benzyl alcohol; 227 mµ for benzoic acid. Determinations were made from the density readings. This method permitted complete separation and quantitative determination of the above compounds.

Phenylacetylcarbinol was determined by titration with iodine. The endogenous reaction mixture served as a control. Since pyruvic acid also reacts with iodine, the value from the iodine titration had to be corrected for the amount of pyruvic acid remaining after the reaction. In order to substantiate the formation of ketol some samples were extracted with diethyl ether and assayed with potassium periodate. Each mole of phenylacetylcarbinol is degraded to benzaldehyde and acetic acid by one mole of periodate. Residual periodate was titrated as iodine liberated from potassium iodide.

Unless otherwise noted all reactions were carried out in pH 5.5 phosphate or phthalate buffers.

**EXPERIMENTAL RESULTS**

Intact yeast cells will form phenylacetylcarbinol from glucose or pyruvic acid and benzaldehyde. Quantitative conversion of benzaldehyde to phenylacetylcarbinol is never attained under normal fermentation conditions. A major portion

<table>
<thead>
<tr>
<th>COFACTOR ABSENT</th>
<th>Q (N)</th>
<th>PYRUVATE DECARBOXYLATED</th>
<th>LACTATE FORMED</th>
<th>BENZALDEHYDE REMAINING</th>
<th>BENZOIC ALCOHOL FORMED</th>
<th>CARINOL FORMED</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>929</td>
<td>15.7*</td>
<td>14.9*</td>
<td>0</td>
<td>0</td>
<td>18*</td>
</tr>
<tr>
<td>CoA</td>
<td>835</td>
<td>26.8</td>
<td>20.6</td>
<td>14.9*</td>
<td>0</td>
<td>3.1</td>
</tr>
<tr>
<td>DPN</td>
<td>553</td>
<td>17.2</td>
<td>13.6</td>
<td>13.6</td>
<td>4.4*</td>
<td>0</td>
</tr>
<tr>
<td>DPT</td>
<td>912</td>
<td>29.6</td>
<td>19.3</td>
<td>1.8</td>
<td>0</td>
<td>16.2</td>
</tr>
<tr>
<td>Mg++</td>
<td>1,000</td>
<td>32.0</td>
<td>20.2</td>
<td>5.4</td>
<td>0</td>
<td>12.6</td>
</tr>
</tbody>
</table>

* Numbers indicate micromoles.

of the aldehyde is reduced to benzyl alcohol. A definite competition for benzaldehyde by the ketol forming system and alcohol dehydrogenase exists since an increase in yield of phenylacetylcarbinol results in a decrease of the benzyl alcohol formed and vice versa. Normally a constant small portion of the benzaldehyde is oxidized to benzoic acid. Likewise a relatively constant amount is lost through aeration. An effort was made to gain a more concise understanding of these two competitive mechanisms and their interrelationships.

**Formation of phenylacetylcarbinol from pyruvic acid and benzaldehyde**. Acetone powders of yeast were prepared, as described, to eliminate diaphosphopyridine nucleotide since this coenzyme mediates the reduction of benzaldehyde. These acetone powders in the absence of added cofactors produce no ketol, but decarboxylate pyruvic acid to acetoldehyde. Upon the addition of a number of cofactors, diphosphothiamin, Mg++, dipho-
phopyridine nucleotide, and coenzyme A, benzaldehyde in the presence of pyruvic acid is converted to phenylacetylcarbinol. No detectable amounts of benzyl alcohol or benzoic acid are formed under optimal conditions. Benzaldehyde when present in amounts in excess of pyruvic acid results in the formation of benzyl alcohol. Preferential reduction of pyruvic acid occurs when this compound is in excess. If any of the aforementioned cofactors is absent, there is a decrease in the amount of phenylacetylcarbinol formed. This is shown in table 1.

| Levels | Amounts | of phenylacetatecarbinol | were inhibitory to decarboxylation. Diphos-
|-------|---------|--------------------------|-----------------------------------------|
| phenopyridine nucleotide was equally effective at 0.0003 M and 0.001 M concentrations. Coenzyme A was required in catalytic amounts, the optimal level being 20 units per ml. Synthesis of phenylacetylcarbinol is dependent on the amount of coenzyme A present as shown in figure 1.

Preliminary experiments indicated that one acetone powder, 3-10, required adenosine triphosphate for the synthesis of phenylacetylcarbinol. Another preparation, 5-19, required neither adenosine triphosphate nor adenyl acid. Re-analysis of the former preparation revealed that adenylic acid could substitute for adenosine triphosphate. It is probable that adenosine triphosphate served only as a source of adenyl acid since this acetone powder possessed some adenosine triphosphatase activity. The level of adenyl acid or adenosine triphosphate for optimal activity was 0.0005 M. Greater or lesser amounts prevented or inhibited the over-all reaction. The exact role of adenyl acid is unknown. Experiments showed that for every mole of adenyl acid present approximately two moles of inorganic phosphate disappeared. Spectrophotometric analysis of the acetone powders showed the presence of four times as much adenine in acetone powder 5-19 than in acetone powder 3-10.

The molar ratios of pyruvic acid decarboxylated, and lactic acid and phenylacetylcarbinol formed, are essentially 1:1:1 as shown in table 1. All of the pyruvic acid and benzaldehyde utilized could be accounted for in recovery experiments by the products listed. Insignificant amounts of acetaldehyde were formed in the complete system. The products formed and their molar ratios conform to the equation for the dismutation of pyruvic acid to lactic acid and the active acetyl intermediate, acetyl-coenzyme A (Korkes et al., 1951). However, diphosphopyridine nucleotide probably does not serve as the direct H+ carrier to bring about the reduction of pyruvic acid in this system. Since the formation of phenylacetylcarbinol is coenzyme A dependent, acetylation of benzaldehyde by the acetyl-coenzyme A intermediate is a possibility.

**Formation of phenylacetylcarbinol from acetyl phosphate and benzaldehyde.** If phenylacetylcarbinol is synthesized by the acetylation of benzaldehyde, acetyl phosphate or acetate might serve as a source of the acetyl group. Acetone powders were incubated with acetyl phosphate and co-
enzyme A in one case and with sodium acetate, coenzyme A, and adenosine triphosphate in another in pH 5.5 phthalate buffer. Table 2 shows that phenylacetylcarbinol is formed from acetyl phosphate but not from acetate. No acetyl phosphate formation could be detected when acetate and adenosine triphosphate were incubated with

**TABLE 2**

Formation of phenylacetylcarbinol from acetyl phosphate

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>SUBSTRATE IN µM</th>
<th>µM CARBINOL FORMED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial Remaining</td>
<td></td>
</tr>
<tr>
<td>Acetyl phosphate</td>
<td>50</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.8</td>
</tr>
<tr>
<td>Acetate</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

**TABLE 3**

Inhibition of phenylacetylcarbinol formation by arsenate

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>ACETYL PHOSPHATE IN µM</th>
<th>µM CARBINOL FORMED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial Remaining</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>0.01 M arsenate</td>
<td>50</td>
<td>&lt;1</td>
</tr>
<tr>
<td>0.02 M arsenate</td>
<td>50</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

The effect of pH on the formation of phenylacetylcarbinol from acetyl phosphate is shown in figure 2. Phthalate buffer, m/15, was used for experiments at pH 4.5 and 5.5 and phosphate buffer, m/50, for pH 6.0 to 7.5. Previous experiments have indicated that m/50 phosphate buffer does not prevent phenylacetylcarbinol formation. The optimal pH for phenylacetylcarbinol synthesis was found to be between 4.5 and 5.5. Above pH 5.5 the yield of carbinol rapidly decreases.

**DISCUSSION**

The coenzyme requirements, the products formed, and their molar ratios permit the formulation of the following equations as a mechanism of synthesis of phenylacetylcarbinol from pyruvic acid and benzaldehyde:

\[
\text{Acetyl phosphate} + \text{benzaldehyde} \rightarrow \text{phenylacetylcarbinol}
\]

\[
\text{Acetyl phosphate} + \text{arsenate} \rightarrow \text{phenylacetylcarbinol}
\]

\[
\text{Acetyl phosphate} + \text{arsenate} + \text{coenzyme A} \rightarrow \text{phenylacetylcarbinol}
\]
Phenylacetylcarbinol synthesis from acetyl phosphate may be preceded by the formation of the acetyl-coenzyme A complex, according to the reaction found by Stadtman (1952).

(3) \[ \text{acetyl phosphate} + \text{CoA} \rightarrow \text{acetyl CoA} + \text{phosphate} \]

The reaction then proceeds as in equation (2). The existence of phosphotransacetylase in yeast has not been proven (Stadtman et al., 1951). Whether such an enzyme is active in yeast at the low pH of the reaction or whether some other mechanism of acetyl phosphate utilization exists is unknown.

In the mechanism proposed for phenylacetylcarbinol formation, acetylation of benzaldehyde should yield a product of the oxidation level of a diketone (CH₃COCOC₆H₄); yet the product obtained is a hydroxyketone. Evidence is lacking to indicate whether the reaction sequence is diketone formation followed by reduction or whether reduction precedes acetylation. It might be postulated that diketone formation followed by reduction of a carbonyl group should result in a mixture of carbinols (CH₃COCHOHCO₆H₄, CH₃CHOHCO₆H₄). Since isotopic studies conclusively show that free aldehyde always forms the carbinol portion of the molecule and since we were unable to detect any compound with the benzoyl grouping, the possibility exists that reduction may have preceded condensation. The reduction of a diketone to give only one hydroxyketone, on the other hand, can well be attributed to enzyme specificity. In this case the sequence, diketone formation followed by reduction, is plausible. Further experimentation is necessary to pinpoint the locus of the reduction process.

Although this mechanism does not rule out other mechanisms of carbinol formation, the participation of coenzyme A is of special interest.

Acetylation of aldehydes adds to the numerous other biological reactions in which coenzyme A is intimately involved.

The dismutation of pyruvic acid to lactic acid and the acetyl-coenzyme A complex in yeast differs from that described for Escherichia coli by Korkes et al. (1951) in that diphosphopyridine nucleotide is not linked directly in the transfer of H⁺ to pyruvic acid by lactic dehydrogenase.

Benzaldehyde in excess of pyruvic acid is reduced to benzy alcohol. This is understandable since diphosphopyridine nucleotide is involved in both the dismutation reaction and reduction of aldehydes. It is on this account that a competition for benzaldehyde by the systems forming phenylacetylcarbinol and benzy alcohol exists.

**ACKNOWLEDGMENTS**

The authors are indebted to Dr. J. H. Quastel for his helpful recommendations. The technical assistance of Miss Dorothy Carkowski in performing many of these experiments is gratefully acknowledged.

**SUMMARY**

A mechanism by which yeasts synthesize phenylacetylcarbinol from pyruvic acid and benzaldehyde was found to be a dismutation of pyruvic acid to lactic acid and an acetyl-coenzyme A complex, which then condenses with benzaldehyde. The coenzyme requirements are carboxylase, Mg⁺⁺, diphosphopyridine nucleotide, and coenzyme A. The carbinol also can be synthesized from acetyl phosphate and benzaldehyde in the presence of coenzyme A. The optimal pH of this acetylation reaction lies between 4.5 and 5.5.

A competition for benzaldehyde by the carbinol synthesizing system and alcohol dehydrogenase exists. The point of interrelationship between
these two systems is their requirement for di-
phosphopyridine nucleotide.

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