Inhibition of Diacetyl Synthesis by Valine and the Roles of α -Ketoisovaleric Acid in the Synthesis of Diacetyl by *Saccharomyces cerevisiae*

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SUMMARY

Valine did not inhibit the production of diacetyl from pyruvate by extracts of *Saccharomyces cerevisiae* that contained coenzyme A (CoA) and the co-factors necessary for forming acetyl-CoA. However, it did inhibit the production of diacetyl from glucose by a growing culture, though Ca-pantothenate, a precursor of CoA, was supplied. α -Ketoisovaleric acid enhanced the production of diacetyl from pyruvate and acetyl-CoA by extracts if mercaptoethylamine was present. Results indicate that α -ketoisovaleric acid has two roles in the synthesis of diacetyl by *S. cerevisiae*, a synthesis that generally requires an acidic environment, and that each is inhibited by excess valine. In addition to serving as an intermediate in the synthesis of acetyl-CoA, α -ketoisovaleric acid, which was not formed from valine by transamination at pH below 6.0, enhanced the activity of diacetyl synthetase.

INTRODUCTION

Diacetyl contributes off-flavour to beer (Shimwell & Kirkpatrick, 1939; West, Lautenbach & Becker, 1952) and some other alcoholic beverages (Goto & Iwano, 1968; Rankine, Fornachon & Bridson, 1969; Rodopulo & Pisarnitskii, 1969; Rodopulo, Pisarnitskii, Bezzubor & Egorov, 1969). Owades, Maresca & Rubin (1960) found that excess valine inhibits the production of diacetyl by *Saccharomyces cerevisiae*, and this has been confirmed by other investigators (Chuang & Collins, 1968; Inoue *et al.* 1968; Portno, 1966*a*). Since α -acetolactate is a precursor of valine in *S. cerevisiae* (McManus, 1954; Wang, Christensen & Cheldelin, 1955), Owades *et al.* (1960) suggested that α -acetolactate also is a precursor of acetoin, that acetoin is oxidized to diacetyl, and that valine might inhibit diacetyl synthesis by preventing the formation of α -acetolactate by a feed-back inhibition similar to that reported by Yates & Pardee (1956). However, subsequent investigations (Cowland & Maule, 1966; Portno, 1966*b*) showed that the addition of acetoin to constantly aerated fermenting wort did not increase the production of diacetyl.

Suomalainen & Ronkainen (1968) and Inoue *et al.* (1968) reported that synthetic α -acetolactate yielded diacetyl (and acetoin) non-enzymatically when heated at low pH. Chuang & Collins (1968), using a column chromatographic method recently developed by Speckman & Collins (1968) for separating acetoin and diacetyl, found that extracts of *Saccharomyces cerevisiae* did not form acetoin from α -acetolactate and did not form diacetyl from acetoin or α -acetolactate. Instead, the extracts, not having α -acetolactic acid decarboxylase, formed acetoin by condensation of hydroxyethylthiamine pyrophosphate with acetaldehyde, as originally reported by Juni (1952), and formed diacetyl by condensation of hydroxyethylthiamine pyrophosphate with acetyl-coenzyme A (CoA). Prolonged incubation of yeast

cell-free extracts, from which CoA had been removed, with Na-pyruvate and the co-factors necessary for the formation of α -acetolactate did not result in the formation of diacetyl, though the extracts formed acetoin (Chuang & Collins, 1968), undoubtedly from hydroxyethylthiamine pyrophosphate and acetaldehyde. Suomalainen & Ronkainen (1968) found that diacetyl was not formed at low concentrations of pyruvate unless acetyl-CoA was included in reaction mixtures.

The present study was undertaken to determine the way in which valine inhibits the formation of diacetyl by *Saccharomyces cerevisiae* and to relate this to the mechanisms used by the organism for forming diacetyl and acetoin.

METHODS

Organisms and cultural procedures. Two strains of *Saccharomyces cerevisiae* that produced both acetoin and diacetyl (Chuang & Collins, 1968) were used: *S. cerevisiae* 299 (University of California Food Science and Technology strain no. C299) and *S. cerevisiae* NCYC 1200. They were propagated routinely at 21 °C for 2.5 days in YED medium (yeast extract, 2 %; glucose, 2 %; KH_2PO_4 , 0.5 %; pH 5.95). Extracts were prepared from yeast grown at 21 °C for 2 days in YED medium containing 20 % (w/v) glucose (Chuang & Collins, 1968).

Extracts. The organisms were grown in 4 l Erlenmeyer flasks. Each flask contained 2 l broth and was shaken every 3 to 5 h during the incubation. The yeast was harvested at the middle of the logarithmic growth phase by centrifugation, washed twice with cold 0.2 M-phosphate-cysteine buffer (pH 5.8; 0.001 M with respect to L-cysteine), and resuspended in cold buffer. The yeast was disrupted by ballistic disintegration with a Mickle tissue disintegrator (H. Mickle, Gormshall, Surrey) operated at maximal amplitude for 1.5 h in a room maintained at 4 °C, and crude extract was separated from the disrupted yeast by centrifugation at 23000g at 2 °C for 30 to 40 min in a refrigerated centrifuge. Crude extract was used in some experiments. To purify it, 1 μmol of ethylenediaminetetraacetate (EDTA) was added per mg of protein in the extract to remove metal ions; then 15 ml of the extract (containing about 10 mg of protein/ml) were dialysed for 12 or 24 h against 100 volumes of 0.2 M-phosphate-cysteine buffer, pH 5.8. CoA was removed from dialysed extracts by treatment with an anion-exchange resin (acid-washed Dowex 1-X2). One volume of the extract was stirred slowly for 5 min with one-quarter volume of the resin. The pH was checked periodically and maintained between 7 and 7.3 by drop-wise addition of 0.1 M-tris (hydroxymethyl) aminomethane (tris) buffer. The resin was sedimented by centrifugation for 3 min at 5000g, and the supernatant fluid was removed with a Pasteur pipette.

Analytical procedures. The diacetyl and acetoin produced were separated by salting-out chromatography with a column (3.5 $\times$ 54 cm) packed with Dowex 1-X8 anion-exchange resin slurried in saturated Na_2SO_4 (Speckman & Collins, 1968). The products were determined by counting radioactivity or by the Westerfeld test (Westerfeld, 1945). Radioactivity was measured in a Nuclear-Chicago scintillation counter (Model 70034; Nuclear-Chicago Corporation, Des Plaines, Illinois, U.S.A.) with samples in 10 ml of Bray's solution (Long & Geiger, 1965), at an efficiency of approximately 75 %. A fraction collector (Redi-Rak, Stockholm, Sweden) was used to collect and distribute effluent during chromatographic separations. Acetoin was collected in 6 ml fractions; diacetyl was collected in 3 ml fractions.

Transamination was measured qualitatively by paper chromatography with a butanol + water + acetic acid mixture (5:4:1) as the elution solvent. The amount of glutamic acid produced from valine- α -ketoglutarate transamination was determined with glutamate decar-

boxylase (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.) by a manometric procedure (Gale, 1945).

CoA was measured by the phosphotransacetylase assay procedure described by Novelli (1955). Phosphotransacetylase was extracted from dried *Clostridium kluyverii* (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) by a procedure supplied by Sigma Chemical Co. (Form no. 482). One unit of phosphotransacetylase is that amount of enzyme required to catalyse the arsenolysis of 1 μ mole of acetyl phosphate in 15 min in the presence of 5 units of CoA (Novelli, 1955).

Acetyl-phosphatase activity of extracts was determined by incubating acetyl phosphate with extract at 28 °C for 15 min and determining the amount of acetyl phosphate that remained. Acetyl phosphate and acetyl CoA were determined with the hydroxamic acid assay method (Lipmann & Tuttle, 1945).

A Beckman spectrophotometer, model DB, was used for all spectrophotometric determinations, and pH was measured with a Radiometer (Copenhagen, Denmark) pH meter, model 22. A Klett-Summerson colorimeter, model 800-3, was used with filter no. 54 for Westerfeld analysis. Protein was determined by the biuret method (Gornall, Bardswill & Davis, 1949).

Calcium *d*-pantothenate, α -ketoisovaleric acid, DL-thioctic acid (lipoic acid), adenosine-triphosphate (ATP), sodium pyruvate, CoA, TPP, and NAD were obtained from Calbiochem, Los Angeles, California, U.S.A. Acetyl-CoA was synthesized from acetic anhydride and CoA with the method of Stadtman (1957). Mercaptoethylamine was obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Acetoin and diacetyl were obtained from Eastman Organic Chemicals Co., Rochester, New York, U.S.A. Acetoin, obtained as the crystalline dimer, was washed with ether until free from diacetyl, as shown by both column and gas chromatography. [3-¹⁴C]Sodium pyruvate (specific activity varied with time of purchase) and [1-¹⁴C]glucose (specific activity, 7.08 mCi/mmol) were obtained from New England Nuclear Corporation, Boston, Massachusetts, U.S.A. Other chemicals were analytical grade.

RESULTS

Effect of α -ketoisovaleric acid on diacetyl biosynthesis

The suppression of α -acetolactic acid synthetase by valine (Magee & deRobichon-Szulmajster, 1968) should suppress the formation of α -ketoisovaleric acid, a remote precursor of pantothenic acid, a constituent of coenzyme A (Cohen, 1968). Consequently, the involvement of α -ketoisovaleric acid in diacetyl biosynthesis was studied by attempting to produce CoA from α -ketoisovaleric acid and other necessary substrates and attempting to use the CoA in the formation of acetyl-CoA and diacetyl from radioactive pyruvic acid. In step 1 of the procedure, α -ketoisovaleric acid, 100 μ mol; formaldehyde, 100 μ mol; MgSO₄, 4.5 μ mol; 0.1 M-phosphate buffer, pH 6.0; and crude extract (4.8 to 13.1 mg protein) were mixed and permitted to react for 10 min at 27 °C. β -Alanine (100 μ mol) and ATP (50 μ mol) then were added, the mixture was permitted to react for 10 min at 27 °C, mercaptoethylamine (100 μ mol) was added, an additional reaction time of 4 h at 27 °C was allowed, the reaction mixture (3.0 ml; pH 5.9 to 6.0) received 1.0 ml of cold 5% trichloroacetic acid to stop the reaction, precipitated protein was removed by centrifugation, and the mixture was adjusted to pH 4.65 to 4.75 by addition of 4 or 5 drops of 2.5 N-NaOH. In step 2, this mixture, the presumed source of CoA, was added to a mixture of sodium pyruvate, 100 μ mol; plus 2 μ Ci [3-¹⁴C]sodium pyruvate, 2.13 mCi/mmol; NAD, 2 μ mol; lipoic acid, 0.2 μ mol; TPP, 0.13 μ mol; MgSO₄, 4.5 μ mol; 0.1 M-phosphate buffer, pH 4.5; and

Fig. 1. Incorporation of radioactive pyruvate into diacetyl by extract of *Saccharomyces cerevisiae* 299 with (▲) and without (●) inclusion of α -ketoisovaleric acid in the reaction mixture. The protein content of the extract added in step 1 (coenzyme A not removed) was 12.8 mg; 25.6 mg were added in step 2. (For detailed conditions see text.)

dialysed (24 h) cell-free extract from which CoA had been removed (5.6 to 26.2 mg protein). The final volume was 7.5 ml; pH was 4.9 to 5.0. This final mixture was permitted to react for 2 h at 27 °C, 1.0 ml of cold 5 % trichloroacetic acid was added to stop reactions, and precipitated protein was removed by centrifugation. The diacetyl and acetoin produced were separated by column chromatography and the incorporations of radioactive pyruvate were determined. Results of one experiment with and without addition of α -ketoisovaleric acid are shown in Fig. 1.

Diacetyl was produced, and the amounts produced were smaller (and about the same) if either α -ketoisovaleric acid (Fig. 1) or formaldehyde, β -alanine, ATP, and mercaptoethylamine were left out of the reaction mixture. Similar results were obtained with an extract of *Saccharomyces cerevisiae* NCYC1200. Diacetyl production was stimulated whether the β -alanine, ATP, and mercaptoethylamine were added at the time of adding the α -ketoisovaleric acid and formaldehyde or later, as indicated above. Less diacetyl was produced when the amounts of extract were reduced, and none was produced in the absence of extract, indicating that the diacetyl was produced enzymatically.

Synthesis of diacetyl in the presence of valine

An experiment similar to that reported in Fig. 1 was run with and without addition of 50 μ mol of valine with the α -ketoisovaleric acid and formaldehyde. Immediately before separating the radioactive diacetyl from radioactive acetoin, 400 μ g of cold diacetyl were added to the reaction mixture. Subsequently, separated diacetyl and acetoin were determined both by tracing radioactivity and colorimetrically by the Westerfeld method. Diacetyl synthesis was not prevented by the addition of valine.

Valine inhibition in the presence of Ca-pantothenate

Since valine did not inhibit the formation of diacetyl in the above experiment, it appeared that valine might influence the formation of diacetyl in growing cultures merely by inhibiting

α -acetolactic acid synthetase and the subsequent formation of CoA. If this were the only suppressive action of valine affecting the formation of diacetyl, the suppression should not occur in growing cultures furnished excess Ca-pantothenate for the production of CoA. We previously had found that the addition of 0.02 % Ca-pantothenate enhanced the production of diacetyl by growing cultures of *Saccharomyces cerevisiae* (Chuang & Collins, 1968).

The influence of 0.1 % valine on the production of diacetyl from radioactive glucose by *Saccharomyces cerevisiae* NCYC 1200 in YED medium (containing 20 % glucose) with and without the addition of 0.02 % Ca-pantothenate was studied. Addition of Ca-pantothenate did not restore diacetyl production to the level normally found with this culture. No diacetyl was detected in the culture that lacked pantothenate, and the culture that received pantothenate in addition to valine produced only a small amount. It was apparent from the results that the influence of valine on the production of diacetyl *in vivo* could not be attributed entirely to inhibition of CoA synthesis.

Determination of CoA

Results had shown that α -ketoisovaleric acid and at least one of the other substrates necessary for the synthesis of CoA were important in the synthesis of diacetyl by *Saccharomyces cerevisiae* (Fig. 1), but they had not shown specifically that CoA was produced, because some CoA was present in the extract used in step 1 of the experiment.

Phosphotransacetylase from dried *Clostridium kluyverii* was used for determining CoA according to the procedure of Novelli (1955). With crude extract of *Saccharomyces cerevisiae* used in the assay in place of CoA, acetyl phosphate was decomposed. However, boiling the extract for 3 min or more considerably reduced this activity, which indicated the presence of acetyl-phosphatase in addition to some CoA. We then assayed dialysed extract from which CoA had been removed for acetyl-phosphatase. In a 1.0-ml reaction mixture containing 6.0 μ mol of acetyl phosphate, 1.0 M-tris buffer (0.1 ml, pH 8.0), 0.1 M-cysteine-HCl (0.1 ml), extract (0.1 ml), and water (0.6 ml), 2.4 μ mol of acetyl phosphate were decomposed in 15 min at 28 °C. None was decomposed when extract that had been boiled for 3 min was used.

Simultaneously with assaying the crude extract for CoA, we assayed a reaction mixture in which CoA was presumed to have been produced. Results showed that CoA had not been produced.

Influence of α -ketoisovaleric acid and mercaptoethylamine on the activity of diacetyl synthetase

It was apparent that the enhancement of diacetyl synthesis from pyruvate *in vitro* when α -ketoisovaleric acid and other substrates were added to reaction mixtures (Fig. 1) could not be attributed to the synthesis of CoA. We had found α -ketoisovaleric acid to enhance diacetyl production by extracts only in the presence of formaldehyde, β -alanine, ATP, and mercaptoethylamine, but we had not determined which of the latter four compounds was required or which enzyme was stimulated. Consequently, we tested separately and together the influences of α -ketoisovaleric acid and mercaptoethylamine, which has a sulphhydryl group, on diacetyl synthetase, the enzyme that forms diacetyl from hydroxyethylthiamine pyrophosphate and acetyl-CoA (Chuang & Collins, 1968). Dialysed extract from which CoA had been removed was used as the source of diacetyl synthetase, because the enzyme is unstable and attempts to purify it were unsuccessful.

The results in Fig. 2 show that the addition of α -ketoisovaleric acid and mercapto-

Fig. 2. Influence of α -ketoisovaleric acid in the presence of mercaptoethylamine on activity of the diacetyl synthetase in a cell-free extract (dialysed; coenzyme A removed) of *Saccharomyces cerevisiae* NCYC 1200. Reaction mixture contained: Na-pyruvate, 100 μ mol; [$3\text{-}^{14}\text{C}$]Na-pyruvate, 4 μ Ci (2.13 mCi/mmol); acetyl-CoA, 25 μ mol; TPP, 0.13 μ mol; MgSO_4 , 4.5 μ mol; extract, 27 mg protein; and 0.1 M, pH 4.5, phosphate buffer, with (●) and without (○) the addition of α -ketoisovaleric acid (100 μ mol) and mercaptoethylamine (100 μ mol). Total volume, 7.5 ml. Temperature, 27 °C. Reaction time, 2 h.

ethylamine did considerably increase the synthesis of diacetyl from radioactive pyruvate and acetyl-CoA. Simultaneously, less acetoin was produced. When α -ketoisovaleric acid and mercaptoethylamine were tested separately, diacetyl synthesis was not stimulated.

Diacetyl not produced from acetoin in the presence of α -ketoisovaleric acid and mercaptoethylamine

In several experiments the amounts of acetoin produced by extracts of *Saccharomyces cerevisiae* had been somewhat lower upon the inclusion of α -ketoisovaleric acid and mercaptoethylamine in reaction mixtures, simultaneously with the production of larger amounts of diacetyl. Consequently, an experiment was run to determine whether α -ketoisovaleric acid and mercaptoethylamine had enhanced diacetyl production by making possible the oxidation of acetoin to diacetyl.

We had found the production of diacetyl by *Saccharomyces cerevisiae* 299 to be highly dependent on stage of growth and that crude extracts prepared from yeast grown 2.5 days or longer in YED medium containing 20 % (or 2 %) glucose did not produce detectable diacetyl. Consequently, we tested such an extract to determine whether the inclusion of α -ketoisovaleric acid and mercaptoethylamine in the reaction mixture would result in the production of diacetyl from the acetoin produced from pyruvate. Considerable acetoin was produced, but diacetyl was not detected (Fig. 3), and it was apparent that the inclusion of α -ketoisovaleric acid and mercaptoethylamine in reaction mixtures had not made possible the oxidation of acetoin. We attribute the reductions in production of acetoin that in several experiments had occurred simultaneously with increases in the production of diacetyl to competition for the hydroxyethylthiamine pyrophosphate that the extracts formed from pyruvate.

Fig. 3. Production of acetoin from pyruvate by crude extract prepared from *Saccharomyces cerevisiae* 299 grown 2.5 days in YED medium (see text) containing 20 % glucose. Reaction mixtures contained: Na-pyruvate, 100 μ mol; [$3\text{-}^{14}\text{C}$]Na-pyruvate, 2 μ Ci (2.13 mCi/mmol); NAD, 2 μ mol; lipoic acid, 0.2 μ mol; TPP, 0.13 μ mol; MgSO_4 , 4.5 μ mol; 0.1 M-phosphate buffer, pH 4.5; α -ketoisovaleric acid, 100 μ mol; mercaptoethylamine, 100 μ mol; and extract, 28.2 mg protein. Total volume, 7.5 ml. Temperature, 27 $^\circ\text{C}$. Reaction time, 4 h.

Transamination reactions of *Saccharomyces cerevisiae* 299

We studied the transamination of amino acids, which is known to occur in yeasts (Bigger-Gehring, 1955), because it was important to determine whether *Saccharomyces cerevisiae* could form α -ketoisovaleric acid from valine. The forward reaction leading to the formation of valine was tested first. A reaction mixture (3 ml) containing 10 μ mol of the amino acid to be tested (20 μ mol if the DL form was used), 10 μ mol of α -ketoisovaleric acid, 0.1 M phosphate buffer (pH 7.5, containing 20 μ g pyridoxal phosphate/ml), and dialysed (24 h) extract (about 4 mg protein) was incubated at 37 $^\circ\text{C}$ for 2 h in an atmosphere of nitrogen and then heated to boiling to stop the reaction. The amino acids in samples of the reaction mixtures were determined by paper chromatography. Most of the 20 amino acids tested transferred their amino groups to α -ketoisovaleric acid and formed valine.

Reversibility of the transamination reaction, i.e. the formation of α -ketoisovaleric acid from valine, was more important in our study. This was tested with concentrations of reactants and assay conditions similar to those given above. Reaction mixtures contained valine and one keto acid (α -pyruvic acid, α -ketoglutaric acid, or oxaloacetic acid), buffer which contained pyridoxal phosphate, and extract. Paper chromatography indicated that glutamic acid (the product of valine and α -ketoglutaric acid) was the only amino acid formed. Alanine (the product expected from valine and α -pyruvic acid) and aspartic acid (expected from valine and oxaloacetic acid) were not found.

The effect of pH on the α -ketoglutaric acid-valine transamination reaction was studied quantitatively (with glutamate decarboxylase) and qualitatively (by paper chromatography). Each method showed that the α -ketoglutaric acid-valine transamination reaction did not occur at pH values below 6.0.

DISCUSSION

Much attention has been given to determining the pathway by which diacetyl is formed and how diacetyl synthesis in *Saccharomyces cerevisiae* is inhibited by addition of 0.02 % or more valine (Owades *et al.* 1960). Valine is formed in *S. cerevisiae* from α -ketoisovaleric acid, and addition of valine has been found to suppress the synthesis of α -acetolactate by α -acetolactic acid synthetase (Magee & deRobichon-Szulmajster, 1968). The inhibition of α -acetolactate synthesis by valine in growing cultures of *S. cerevisiae* could result in suppression of α -ketoisovaleric acid formation, reduced synthesis of pantothenic acid, and less formation of CoA. The net result of this could be a severe limitation on the available acetyl-CoA and decreased diacetyl production, since the synthesis of diacetyl in growing cultures depends on the availability of acetyl-CoA not required for cell synthesis (Chuang & Collins, 1968). Our findings, showing that valine did not inhibit the formation of diacetyl from pyruvate by extracts that contained CoA and the co-factors necessary for forming acetyl-CoA, and that addition of a precursor of CoA, pantothenate, to cultures of *S. cerevisiae* enhanced their production of diacetyl (Chuang & Collins, 1968), support this explanation and indicate, also, that α -acetolactate is not directly involved in the synthesis of diacetyl. The latter conclusion is supported by results showing that the addition of α -ketoisovaleric acid (and mercaptoethylamine) enhanced the production of diacetyl from radioactive pyruvate and acetyl-CoA. If diacetyl had been formed directly from α -acetolactate in this experiment, non-radioactive α -acetolactate produced from α -ketoisovaleric acid would have diluted the radioactive α -acetolactate produced from pyruvate, and the results would have indicated a decrease in diacetyl production.

An important consideration in regards to the above sequence of events in cells of *Saccharomyces cerevisiae* is the possible formation of α -ketoisovaleric acid from valine by transamination. Our results with extract of the yeast indicate that α -ketoisovaleric acid was not formed from valine and α -pyruvic acid or oxalacetic acid and that it was not formed from valine and α -ketoglutaric acid at pH values below 6.0. These results possibly explain why the inhibitory effect of valine on diacetyl production in growing cultures of *S. cerevisiae* at low pH (Chuang & Collins, 1968) is not circumvented by the transamination of valine to physiologically significant levels of α -ketoisovaleric acid. Conway & Downey (1950) determined by three methods the intracellular pH of yeast and found it to be 5.8. The intracellular pH increased slightly during fermentation, but the pH of the outer metabolic region (the thick wall) became markedly acidic.

Results show that α -ketoisovaleric acid, in addition to its role as a remote precursor of CoA, has another important function in the formation of diacetyl by *Saccharomyces cerevisiae* that is influenced by the suppressive action of valine on α -acetolactic acid synthetase. α -Ketoisovaleric acid in the presence of a strong reducing agent, mercaptoethylamine, enhanced the formation of diacetyl by extracts by stimulating the activity of diacetyl synthetase. That α -ketoisovaleric acid is involved in the control of diacetyl synthesis in growing cultures of *Saccharomyces cerevisiae* is indicated by our finding that the addition of pantothenic acid to growing cultures relieved only slightly the inhibition of diacetyl production that was caused by the addition of valine. We did not determine whether the stimulation of diacetyl synthetase by α -ketoisovaleric acid and mercaptoethylamine was the result of direct action or indirect, i.e. a result of larger amounts of hydroxyethylthiamine pyrophosphate being made available for use in diacetyl synthesis by an inhibition of acetoin production from hydroxyethylthiamine pyrophosphate and acetaldehyde. However, conclusion that there was direct action on diacetyl synthetase is supported by results showing that acetoin was produced

Fig. 4. Diacetyl synthesis in *Saccharomyces cerevisiae* as influenced by α -ketoisovaleric acid, valine, or pantothenic acid. The following abbreviations are used: pyruvate (PVA), thiamine pyrophosphate (TPP), tricarboxylic acid cycle (TCA cycle), and coenzyme A (CoA).

in all experiments and by results showing that suppression of α -ketoisovaleric acid formation by the addition of valine does not stimulate the production of acetoin *in vivo* simultaneously with inhibiting the production of diacetyl (Chuang & Collins, 1968).

The enhancement of diacetyl synthesis required both α -ketoisovaleric acid and mercaptoethylamine, and we assume that mercaptoethylamine was important merely as a means of providing strong reducing conditions for the activity of diacetyl synthetase. The possibility that a single enzyme, possibly with different subunits and activators, is used by the yeast to combine different substrates with hydroxyethylthiamine pyrophosphate in the synthesis of diacetyl, acetoin, and α -acetolactate has not been excluded.

The relationships between activation of diacetyl formation by α -ketoisovaleric acid, stimulation of diacetyl formation by pantothenate, inhibition of diacetyl formation by excess valine, and inability of excess valine to inhibit acetoin formation that we propose are shown in Fig. 4. When respiration in yeast stops as a result of over-growth or is repressed by anaerobic conditions or the presence of glucose (unpublished data), the yeast, with activation by α -ketoisovaleric acid (a key intermediate that connects carbohydrate metabolism with fatty acid synthesis and the synthesis of valine), apparently forms diacetyl as an initial step in removing part of the pyruvate and acetyl-CoA that are not needed for synthesizing fatty acids or for the tricarboxylic acid cycle.

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