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Biosynthesis of Valine and Isoleucine

II. FORMATION OF α-ACETOLACTATE AND α-ACETO-α-HYDROXYBUTYRATE IN *NEUROSPORA CRASSA* AND *ESCHERICHIA COLI**

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The isotope studies of Strassman *et al.* (3, 4) first implicated α-acetolactate and α-aceto-α-hydroxybutyrate as biosynthetic precursors of valine and isoleucine, respectively. The observed accumulation of acetolactate by *Escherichia coli* (5, 6) and of the decarboxylation products of acetolactate and acetohydroxybutyrate by *Neurospora crassa* (7) when mutants of these organisms are grown with limiting amounts of valine and isoleucine is consistent with this role. More recently, the direct conversion of acetolactate to α-ketoisovaleric acid in crude extracts of yeast (8, 9) and to valine in cell-free extracts of *N. crassa* (1) has been demonstrated.

The formation of acetolactate from pyruvate in biological systems was first postulated by Krampitz (10) and has subsequently been demonstrated in crude cell-free extracts of *Aerobacter aerogenes* (11, 12), in *E. coli* (5, 6, 9, 13), and (contrary to earlier reports) in yeast (9). In contrast, the formation of α-aceto-α-hydroxybutyrate by cell-free enzymes has not been demonstrated. We report here formation of the latter product by cell-free preparations from *N. crassa*, together with a comparison of the properties of the partially purified, acetolactate-forming enzymes from *E. coli* and *N. crassa*. A preliminary account of some of this work has appeared elsewhere (2).

EXPERIMENTAL PROCEDURE

Methods—Acetoin was purified from the commercial product by the method of Berl and Bueding (14) for use as a standard, and determined essentially as described by Westerfeld (15). Pyruvate was determined by the method of Friedemann and Haugen (16). Quantitative recoveries were obtained in the presence of both acetoin and acetaldehyde. Carbon dioxide was measured manometrically in a Warburg apparatus. Acetaldehyde was determined by the method of Barker and Summerson (17).

α-Acetolactic acid was synthesized according to Krampitz (10). α-Aceto-α-hydroxybutyrate was prepared by this same general procedure, but ethyl iodide was used to prepare the intermediate ethyl α-acetobutyrate (b.p. 50–60° at 5 mm). From 190 g of the latter product, 40 g of pure ethyl α-acetoxy-α-acetobutyrate (yield, 15%; b.p. 87–88° at 5 mm) were obtained. It was redistilled once for analysis.

$C_{10}H_{16}O_5$ (216.2)
Calculated: C 55.54, H 7.46
Found: C 55.65, H 7.47

Sodium α-aceto-α-hydroxybutyrate was obtained by hydrolysis of the acetoxy ester with two equivalents of NaOH and lyophilization. The resulting mixture of this compound and sodium acetate was used without further purification. α-Acetolactate and α-aceto-α-hydroxybutyrate were assayed in the Warburg apparatus by determining the amount of CO_2 liberated on treatment with acid. α-Acetolactate was also determined by estimation of the acetoin formed after decarboxylation in acid.

Assay of Acetoin- and Acetolactate-forming Enzymes—Except where otherwise stated, the standard assay mixture consisted of enzyme, sodium pyruvate (0.5 mmole), Mn^{++} (0.5 μmole), thiamine pyrophosphate (20 μg), and potassium phosphate buffer (100 μmoles) of appropriate pH in a total volume of 1.0 ml. The mixtures were incubated for 1 hour at 37°. With preparations from *N. crassa*, the reaction was stopped by the addition of 0.1 ml of 2.5 N NaOH and acetoin was determined in an aliquot. Acetolactate loses CO_2 spontaneously in acid; for its determination a separate aliquot was acidified with sulfuric acid, heated at 60° for 15 minutes, cooled, and the total acetoin determined after neutralization of the acid. The amount of acetolactate was calculated by difference. With preparations from *E. coli* the reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid, the mixture was heated as before, and acetoin was determined in an aliquot. In agreement with other reports (6, 11) we find no detectable acetolactate decarboxylase in *E. coli* under our assay conditions; hence, the total acetoin found after acidification and heating corresponded to the amount of acetolactate formed. The enzyme activity is usually expressed as μmoles of acetoin or acetolactate formed per mg of protein per hour. Protein was determined by the method of Lowry *et al.* (11a).

Preparation of Cell-free Extracts—*N. crassa* 16117[1] was grown on the medium of Vogel (18) supplemented with DL-valine (4 mmoles per liter) and DL-isoleucine (1.5 mmoles per liter). The cultures were shaken for 60 hours at 37°, then the mycelia were harvested by filtration through cheese cloth, washed with distilled water, and dried between folds of filter paper. The pads were ground under liquid nitrogen in a mortar and then homogenized in a TenBroeck apparatus with about twice the amount of distilled water or of 0.1 M phosphate buffer, pH 7.0. The result-

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[1] An *isoleucineless-valineless* mutant of *N. crassa*.

ing thick slurry was centrifuged for 30 minutes at 18,000 × *g* and the supernatant solution was separated carefully by means of a syringe from the uppermost fatty layer. These aqueous or buffer extracts were the source of the various enzyme preparations from *N. crassa* studied in this and a subsequent paper (19).

E. coli K-12 was grown either on the casein digest medium (20) or on the minimal salts-glucose medium of Davis and Mingioli (20) and harvested after shaking for 15 hours at 37°. The cells were washed once, then suspended in 0.1 M phosphate buffer pH 7.5 (4 g of wet packed cells per 20 ml of buffer), and treated for 20 minutes in a 10-kc Raytheon magnetostriction apparatus. The suspension was centrifuged for 30 minutes at 18,000 × *g*. The soluble extract was fractionated as described later.

Fractionation of Acetolactate-forming Enzyme from N. crassa—To 100 ml of the crude water extract (2000 mg of protein) of ground mycelium from a 60-hour culture of *N. crassa* 16117 were added 8.0 ml of 0.05 M $AlCl_3$. The pH fell to about 5.5. After 5 minutes, the heavy precipitate containing the enzyme was collected by centrifugation and suspended in 35 ml of 0.1 M Tris buffer pH 8.5, and solid ammonium sulfate was added to 50% saturation.[2] After 30 minutes, the mixture was centrifuged. The enzymatically active precipitate was suspended in 25 ml of 0.1 M phosphate buffer pH 7.5, again centrifuged to remove the insoluble residue, and the supernatant solution then was fractionated further with ammonium sulfate. The precipitate obtained between 20 and 30% saturation contained the acetolactate-forming enzyme together with some acetolactate decarboxylase. The fraction was reprecipitated two to three times with ammonium sulfate at 30% saturation, dissolving the precipitate each time in 20 ml of 0.1 M phosphate buffer pH 7.0. The initial crude extract formed 0.045 μmole of acetolactate and 0.6 μmole of acetoin per mg of protein per hour. The final preparation formed 0.77 μmole of acetolactate per mg of protein per hour and essentially no acetoin. The procedure thus effects little purification of the acetolactate-forming system,[3] but removes completely the acetolactate decarboxylase. When crude buffer extracts, rather than water extracts, of the mycelium were subjected to this same procedure, a 16- to 20-fold purification was achieved but the preparation was not free of the decarboxylase.

The decarboxylase-free enzyme of *N. crassa* showed a single broad pH optimum for acetolactate formation between 6.0 and 7.0 (Fig. 1). Crude extracts of the mycelium showed a similar pH optimum for formation of acetolactate plus acetoin, and this did not vary significantly with variation in the amount of valine added to the growth medium from which the mycelium was harvested (Fig. 1).

Fractionation of Acetolactate-forming Enzymes from E. coli—Like *A. aerogenes* (6, 12), but unlike *N. crassa*, *E. coli* shows two pH optima for the formation of acetolactate. Crude buffer extracts from cells grown on the minimal medium show high activity both at pH 8.0 and at pH 6.0. The activity at pH 8.0 was destroyed by adjusting the extract to pH 5.1 and keeping it at 0–5° for 3 to 5 minutes before the assay; the activity at pH 6.0 was unaffected by this treatment (Fig. 2). Attempts to purify the pH 8.0 enzyme by treatment with alumina or calcium phosphate gels, by absorption and elution from diethylaminoethylcellulose columns, or by ethanol fractionation resulted in almost complete destruction of activity. Reasonable recoveries of activity with a small degree of purification were obtained by collecting the fraction soluble in 10 but insoluble in 50% saturated ammonium sulfate. This same fraction also contained the pH

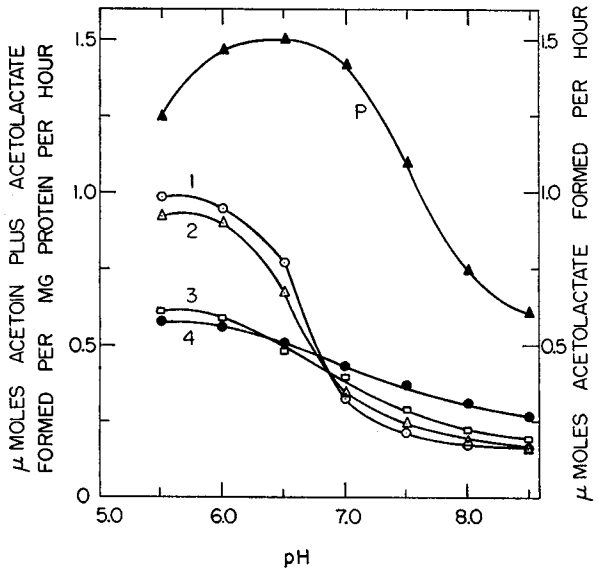

FIG. 1. Acetolactate- and acetoin-forming activity of crude (*Curves 1* to *4*) and purified (*P*) extracts of *N. crassa* as a function of pH. *Curve P*, acetolactate formation (right ordinate) by 2.4 mg of the decarboxylase-free enzyme from *N. crassa* 16117 in 1.5 ml of assay mixture. *Curves 1* to *4*, specific activities (left ordinate) for acetoin plus acetolactate formation, under standard conditions, by mycelial extracts from cultures grown as follows: *Curves 1*, wild type (Em 5256) on minimal salts-glucose medium; *2*, as in *Curve 1* with added DL-valine (470 mg per liter) and DL-isoleucine (200 mg per liter); *3*, isoleucineless-valineless mutant 16117 on minimal medium plus DL-valine (15 mg per liter) and DL-isoleucine (5 mg per liter); *4*, as in *Curve 3* but with increased levels (30 and 10 mg per liter, respectively) of DL-valine and DL-isoleucine.

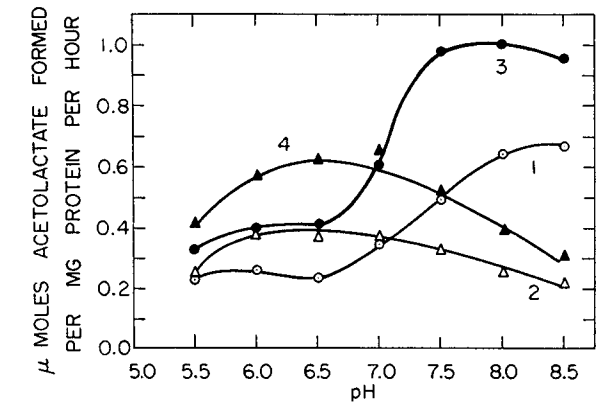

FIG. 2. The effect of pH on acetolactate formation by extracts of *E. coli*. *Curves 1*, crude extract; *2*, crude extract held at pH 5.1 and 0–5° for 5 minutes; *3*, fraction of crude extract insoluble between 10 and 50% saturation with ammonium sulfate; *4*, same as *Curve 3* but held at pH 5.1 for 5 minutes. The increase in specific activity of preparations at pH 6.0 after treatment at pH 5.1 is due to precipitation of inert protein; no increase in total units at pH 6.0 occurs.

[2] The per cent saturations of $(NH_4)_2SO_4$ are calculated from a standard table (21) and are not corrected for temperature. The fractionations were carried out at 0–5°.

[3] It is assumed that all of the acetoin formed arises by decarboxylation of acetolactate, since there is evidence for an alternate route for acetoin formation in this organism.

TABLE I

Variation of acetolactate formation by crude extracts of E. coli with pH and cultural conditions of original cells

Strain of *E. coli*	Medium	Acetolactate formed μmoles per mg of protein per hour	
		pH 6.0	pH 8.0
K-12	Casein digest	0.445	0.21
	Minimal	0.43	1.35
K-12, mutant 413*	Casein digest	0.39	0.32
	Minimal + valine and isoleucine	0.17	0.66

* An isoleucineless-valineless mutant supplied by Dr. E. A. Adelberg and lacking the ability to convert α,β-dihydroxyisovalerate to α-ketoisovalerate. The minimal medium for this mutant was supplemented with 30 mg each of valine and isoleucine per liter.

TABLE II

Fractionation of pH 6.0 enzyme from E. coli

Fraction	Volume	Protein	Acetolactate formed
	ml	*mg/ml*	*μmoles/mg protein/hr*
1. Crude extract*	20	25.5	0.33
2. Ammonium sulfate fractions			
a. 0–20%	10	4.4	0.52
b. 30–40%	5	4.9	0.63
c. 40–50%	10	7.3	1.01

* From cells grown in a casein-digest medium.

TABLE III

Effect of metal ions on acetolactate-forming enzymes of N. crassa and E. coli

Metal ion added	Acetolactate formed	Metal ion added	Acetolactate formed
A. Enzyme from *N. crassa**			
5 × 10⁻⁴ M	*μmoles*	*5 × 10⁻⁴ M*	*μmoles*
None	0.25	Co^{++}	0.92
Mn^{++}	1.90	Zn^{++}	0.34
Ca^{++}	1.35	Ni^{++}	0.31
Mg^{++}	1.25	Fe^{++}	0.17
Fe^{+++}	1.05	Cd^{++}	0.16
B. pH 6.0 enzyme from *E. coli*†			
Undialyzed		Dialyzed	
None	0.91	None	0.52
Mn^{++}	1.21	Mn^{++}	0.71
Mg^{++}	1.16	Mg^{++}	0.71

* A preparation (2.4 mg of protein per ml of assay mixture) free of acetolactate decarboxylase (see text) was used under standard assay conditions but with Mn^{++} omitted.

† The preparation used was Fraction 2c, Table II. Either 1.46 mg of undialyzed protein, or 1.07 mg of dialyzed protein was used per assay. Dialysis was against 0.01 M sodium ethylenediaminetetraacetate for 24 hours.

6.0 enzyme (Fig. 2). Narrower range fractionation resulted in substantial loss of the enzyme.

Preparations of the pH 6.0 enzyme free of the pH 8.0 enzyme were readily obtained either by inactivation of the latter enzyme by treatment at pH 5.1, or by fractionation of extracts from cells grown in the casein digest medium. As originally noted by Umbarger and Brown (6), cells grown under the latter conditions contain reduced amounts of the pH 8.0 enzyme (Table I). Although the pH 6.0 enzyme was stable to fractionation with ammonium sulfate, only a modest increase in activity was obtained (Table II).

Properties of Acetolactate-forming Enzymes from N. crassa and E. coli

1. Metal Ion Requirements—Several divalent metal ions restored activity of the purified *N. crassa* enzyme to varying degrees (Table III). At low concentration Mn^{++} was by far the most active, but it inhibited at higher concentrations. At higher concentrations activity with added Mg^{++} equaled that obtained with Mn^{++} (Fig. 3).

The pH 6.0 enzyme from *E. coli* was activated only slightly by Mg^{++} or Mn^{++} (Table III). The pH 8.0 enzyme, in contrast, was well resolved with respect to metals (Fig. 4). Like the enzyme from *Neurospora*, this enzyme is activated by both Mn^{++} and Mg^{++}. Unlike the *Neurospora* enzyme, it is only slightly more sensitive to Mn^{++} than to Mg^{++}, and high concentrations of Mn^{++} are not inhibitory.

2. Thiamine Pyrophosphate Requirements—None of the three acetolactate-forming enzymes showed activity in the absence of thiamine pyrophosphate. For the enzyme from *N. crassa*, half-maximal activity was obtained at a cofactor concentration of 3×10^{-6} M.

3. Substrate Specificity and K_m Values—a. Pyruvate Only—Acetolactate formation by crude extracts of *N. crassa* could be detected only at relatively high (>0.02 M) concentrations of

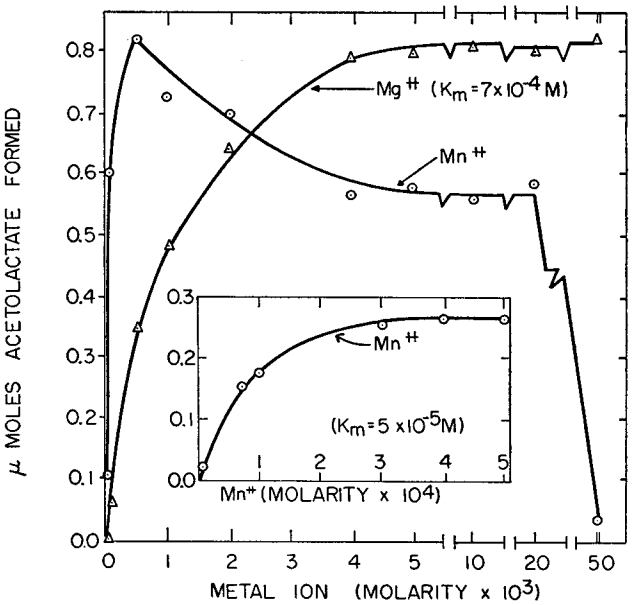

FIG. 3. Effect of Mn^{++} and Mg^{++} on the formation of α-acetolactate by the enzyme from *N. crassa*. The insert shows the effects in a separate experiment of lower concentrations of Mn^{++}. Standard assay conditions (2.24 mg of protein per ml of assay mixture) but with concentration of metal ions varied.

pyruvate. At lower concentrations only acetoin was formed. High pyruvate concentrations were at first assumed to suppress activity of acetolactate decarboxylase. However, preparations free of the decarboxylase showed this same high requirement for pyruvate, the K_m being 4.6×10^{-2} M. Both the pH 6.0 and the pH 8.0 enzyme from *E. coli* show similarly high K_m values of 4.15×10^{-2} M and 2.24×10^{-2} M, respectively. The relation of substrate concentration to activity of the pH 8.0 enzyme from *E. coli* is shown in Fig. 5; similar data, not shown, were obtained for the other two enzymes. A similarly high requirement for pyruvate has been reported for the acetolactate-forming enzymes of *A. aerogenes* (12).

b. Pyruvate Plus Acetaldehyde—In the presence of equimolar (0.01 M) amounts of pyruvate and acetaldehyde, preparations from *N. crassa* free of acetolactate decarboxylase formed only acetoin. When the concentration of pyruvate was increased and that of acetaldehyde was held at 0.01 M, both acetoin and acetolactate were formed. The formation of acetolactate is competitive with that of acetoin, as shown in Fig. 6. In the presence of 5×10^{-3} M pyruvate, the K_m value for acetaldehyde for acetoin formation was determined to be 5.8×10^{-3} M; the value of K_I as measured by inhibition of acetolactate formation was 2.0×10^{-3} M.

c. Acetaldehyde Only—Acetaldehyde alone can serve as a substrate in the synthesis of acetoin by extracts of wheat germ (22), of pig heart (23, 24), and of filariae (14). In extracts of *N. crassa* and of *E. coli*, no free acetaldehyde could be detected even when pyruvate was added, and no detectable amounts of acetoin were formed when acetaldehyde alone was supplied as substrate.

d. Pyruvate Plus α-Ketobutyrate—By analogy with the formation of acetolactate from pyruvate, it was postulated (4) that α-aceto-α-hydroxybutyrate was formed by condensation of acetaldehyde (formed from pyruvate) with α-ketobutyrate. Because acetolactate is formed with pyruvate alone as substrate, identification of the very similar compound, α-aceto-α-hydroxybutyrate, is rendered difficult. However, the acid decarboxylation products of these β-keto acids, acetoin and acetoethylcarbinol, form chromatographically distinct pteridines when allowed to react with 2,4,5-triamino-6-hydroxypyrimidine (7). The pteridines formed in this way from a reaction mixture containing the decarboxylase-free, acetolactate-forming enzyme from *N. crassa*, pyruvate, and α-ketobutyrate were dissolved in dilute ammonia and chromatographed alongside authentic standards. *n*-Propanol-1% ammonia (2:1) was the solvent (7) and the pteridines were detected by their fluorescence under ultraviolet light. Two separate zones were present in the incubation mixtures with R_F values of 0.65 and 0.50, corresponding exactly to the pteridines formed from authentic samples of acetylethylcarbinol and acetoin, respectively. Ultraviolet spectra in 0.1 N NaOH of the eluted pteridine zones were identical with those of the authentic standards. Quantitative determinations were not made, since it was not certain that formation of the two pteridines was either quantitative, or occurred in equal yield. Rough visual compari-

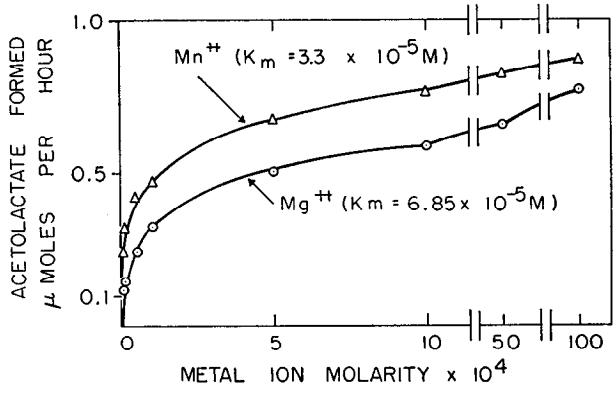

FIG. 4. Activation by metal ions of acetolactate formation by the pH 8.0 enzyme of *E. coli*. Standard assay conditions were used; the enzyme preparation (1.09 mg of protein per ml of assay mixture) was that fraction soluble at 30 but insoluble at 50% saturation with ammonium sulfate.

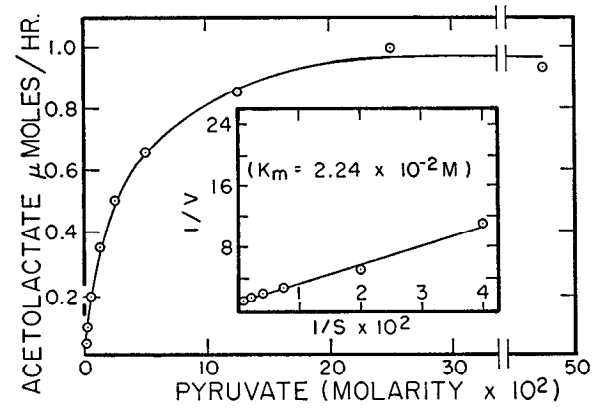

FIG. 5. Relation of acetolactate formation by the pH 8.0 enzyme of *E. coli* to pyruvate concentration. The insert shows a Lineweaver-Burk plot of the same data. Standard assay conditions with pyruvate varied and 1.09 mg of protein per ml. The enzyme preparation was that used in Fig. 4.

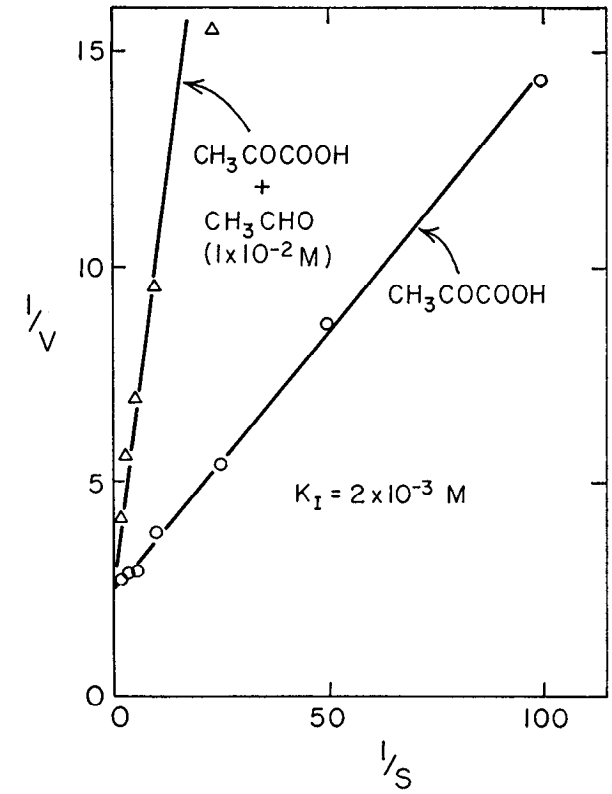

FIG. 6. Competitive inhibition by acetaldehyde of acetolactate formation by the purified enzyme from *N. crassa*. Standard assay conditions, with 1.9 mg of protein per ml and varying amounts of pyruvate.

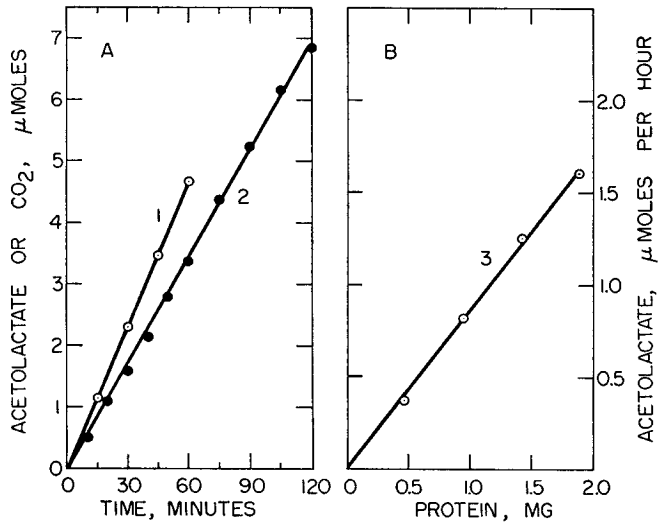

FIG. 7. Acetolactate formation by the purified enzyme from *N. crassa* as a function of time (*A*) and enzyme concentration (*B*). *Curve 1*, acetolactate formation under standard assay conditions with purified enzyme (4.8 mg of protein in 2.0 ml volume). *Curve 2*, CO_2 formation under standard assay conditions with purified enzyme (2.4 mg of protein in 2.0 ml volume). The two curves are from separate experiments. *Curve 3*, acetolactate formation under standard conditions.

TABLE IV

Stoichiometry of acetolactate and acetoin formation by purified enzyme from N. crassa

Trial*	Initial pyruvate	μMoles loss or gain					
		Found				Theory	
		Acetoin	Acetolactate	Pyruvate	CO_2	CO_2	Pyruvate
1	0.5 M	4.4	4.8		14.6	13.6	
2	0.04 M	7.0	2.8	−17.7			−19.6

* *Trial 1*, standard assay mixture with 6.1 mg of protein per ml. CO_2 evolution was followed. After 4 hours at 37°, acetoin and acetolactate were determined.

Trial 2, standard assay mixture except for reduced pyruvate concentration and 6.1 mg of protein per ml. Pyruvate, acetoin, and acetolactate were determined after 4 hours of incubation at 37°.

son indicated that the pteridine formed from acetoin was present at about 10 times the concentration of that from acetylethylcarbinol. No acetylethylcarbinol was formed in the presence of acetaldehyde and α-ketobutyrate as substrates, or in the absence of α-ketobutyrate with pyruvate as substrate.

4. Time-Course and Effect of Enzyme Concentration—The rates of acetolactate and carbon dioxide formation from pyruvate by the enzyme from *N. crassa* were followed in separate experiments. These rates were linear for at least 1 hour (Fig. 7). Product formation was directly proportional to enzyme concentration.

5. Stoichiometry—The over-all reactions involved in the formation of acetolactate and acetoin are as follows:

$$2\ \text{Pyruvate} \rightarrow \alpha\text{-Acetolactate} + CO_2 \quad (1)$$

$$\alpha\text{-Acetolactate} \rightarrow \text{Acetoin} + CO_2 \quad (2)$$

The data of Table IV demonstrate that the required stoichiometry exists. Although the preparations of *N. crassa* contained very little acetolactate decarboxylase, considerable acetoin was formed during the long incubation periods used for these experiments. When 0.5 M pyruvate was used, it was difficult to determine the disappearance of pyruvate accurately. For these reasons, the experiments were performed at high levels of pyruvate for measurement of CO_2 evolution, and at low levels for measurement of pyruvate disappearance.

6. Inhibition of Enzyme Activity—The formation of acetolactate from pyruvate by preparations from *N. crassa* is inhibited completely by 10^{-4} M $HgCl_2$ or $AgNO_3$ and to a much smaller extent (17.5%) by 10^{-4} M $CuSO_4$. No inhibition was observed at these concentrations of *p*-chloromercuribenzoate or iodoacetate.

DISCUSSION

The formation of acetoin from pyruvate has been observed in several microorganisms (11), in plants (22), and in higher animals (23, 24). In bacteria it is formed mainly by the decarboxylation of α-acetolactate, and the present results indicate this is also the route of its formation in extracts of *N. crassa*. Singer and Pensky (22) prepared a highly purified enzyme from wheat germ and showed that it catalyzed both the decarboxylation of pyruvate to acetaldehyde and condensation of acetaldehyde to acetoin. Acetolactate is thus not an obligatory intermediate in acetoin formation by this tissue. There is also no evidence for an intermediate role of acetolactate in acetoin formation by pig heart (13, 24) or filariae (14) since these tissues, like wheat germ, convert acetaldehyde directly to acetoin. The enzyme system from *N. crassa* studied here does not form acetoin directly from acetaldehyde, but forms it by decarboxylation of α-acetolactate. No acetaldehyde is formed from pyruvate by the extracts. Even in the presence of added acetaldehyde, pyruvate is still required for acetoin formation, but is diverted directly to the latter product without appearance of acetolactate as an intermediate. Free acetaldehyde is thus not a precursor of acetolactate (or of acetohydroxybutyrate) in *N. crassa*.

These diverse results are readily correlated by extension of a scheme suggested by Singer and Pensky (22). According to this view, all degradative reactions of pyruvate *in vivo* have in common the formation of an enzyme-acetaldehyde complex the fate of which varies, depending upon the enzyme and its environment, as shown in Fig. 8. The enzyme from *N. crassa*, studied here, apparently forms a nondissociable enzyme-acetaldehyde complex

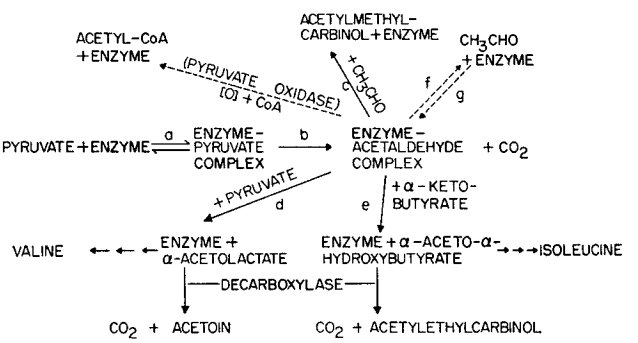

FIG. 8. Interrelationships between formation of acetaldehyde, acetoin, α-acetolactate and related products. Reactions observed in *N. crassa* in this and a preceding study (1) are indicated by solid arrows.

(Reaction $a + b$) which can pass the bound group either to added acetaldehyde to yield acetoin (c), or to pyruvate to yield α-acetolactate (d), or to α-ketobutyrate to yield α-aceto-α-hydroxybutyrate (e). The aldehyde-enzyme complex must be nondissociable because no free acetaldehyde is formed from pyruvate (f). Similarly, the complex cannot be formed from enzyme plus acetaldehyde (g), for in the latter event acetoin would be formed directly from acetaldehyde ($g + c$), as in wheat germ and pig heart.

Because activity of the pH 8.0 enzyme is decreased when organisms containing it are grown in the presence of valine, Umbarger and associates (6, 12) ascribe the principal role in biosynthesis of valine to the acetolactate formed by this enzyme. These authors also conclude (12) that when *Aerobacter aerogenes* grows at low pH values, and appears to lack the pH 8.0 enzyme, the pH 6.0 enzyme also can supply acetolactate for valine synthesis. *N. crassa* contains only the latter enzyme, which must therefore function in valine synthesis by this organism. Since its activity is not repressed by presence of valine, the type of feed-back control mechanism suggested for valine biosynthesis in *E. coli* (6) and *A. aerogenes* (12) must not be present in *N. crassa*.

SUMMARY

Procedures are described for obtaining the acetolactate-synthesizing enzymes of *Escherichia coli* and *Neurospora crassa* in cell-free form, and for separating this enzyme in *N. crassa* from an active acetolactate decarboxylase. *E. coli* contains two acetolactate-forming enzymes with pH optima at 6.0 and 8.0; only the former is stable at pH 5.1. *N. crassa* contains only one acetolactate-forming enzyme; this has a broad optimum between pH 6.0 and 7.0. The pH 8.0 enzyme extractable from *E. coli* is decreased when the organism is cultured in the presence of valine; the pH 6.0 enzyme from *E. coli* and that from *N. crassa* are unaffected by this procedure. Thiamine pyrophosphate is essential for the activity of all three enzymes; the pH 8.0 enzyme from *E. coli* and the enzyme from *N. crassa* also required added metal ions for activity. Mn^{++} was the preferred activator, but Mg^{++} also is highly active. Ca^{++}, Fe^{+++}, and Co^{++} also activated the fungal enzyme. All three enzymes require high pyruvate concentrations for activity. Although the fungal enzyme forms no acetoin from acetaldehyde alone, addition of the latter together with pyruvate results in acetoin formation without the intermediate formation of acetolactate. Concentrations (moles per liter) of activators and substrates permitting half-maximal rates of acetolactate formation by the fungal enzyme are: thiamine pyrophosphate, 3×10^{-6}; Mn^{++}, 5×10^{-5}; pyruvate, 4.6×10^{-2}; acetaldehyde (K_I), 2.0×10^{-3}. When α-ketobutyrate *and* pyruvate are added, the enzyme preparation from *N. crassa* forms a mixture of α-acetolactate and α-aceto-α-hydroxybutyrate. These results are interpreted in terms of a thiamine pyrophosphate-dependent decarboxylation of pyruvate to yield an acetaldehyde-enzyme complex that can pass the aldehyde residue to acetaldehyde to yield acetoin, to pyruvate to yield acetolactate, or to α-ketobutyrate to yield α-aceto-α-hydroxybutyrate, but cannot dissociate to free acetaldehyde and enzyme. Accordingly, free acetaldehyde is not a precursor of valine or of isoleucine in this organism.

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