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BIOSYNTHESIS OF VALINE AND ISOLEUCINE IN PLANTS

I. FORMATION OF α-ACETOLACTATE IN *PHASEOLUS RADIATUS*

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SUMMARY

1. The presence of an enzyme system in plants catalyzing the formation of α-acetolactate from pyruvate has been demonstrated; the system in green gram *(Phaseolus radiatus)* has been partially purified and its characteristics have been studied.

2. Free acetaldehyde is formed as a product of the reaction and so the reaction is mainly diverted towards the formation of acetoin.

3. The system requires thiamine pyrophosphate and a divalent metal ion (Mn^{2+} or Mg^{2+}) for maximum activity. The optimum pH is around 6.0 and the optimum temperature is 60°.

4. The system is very labile in absence of pyruvate, Mn^{2+} and DPT.

5. The K_m values for pyruvate, Mn^{2+}, Mg^{2+} and DPT are $3 \cdot 10^{-2}$ M, $5 \cdot 10^{-5}$ M, $2 \cdot 10^{-5}$ M, and $3 \cdot 10^{-6}$ M respectively. The activation energy is 3540 cal/mole.

6. The enzyme is strongly inhibited by *p*-chloromercuribenzoate and the inhibition can be reversed partially by 2-mercaptoethanol, BAL or cysteine. Heavy metals, such as Hg^{2+} and Ag^{+}, are inhibitory but L-valine does not inhibit the reaction.

INTRODUCTION

In a previous report[1] evidence was provided for the existence of a biochemical pathway for the synthesis of valine and isoleucine in *Phaseolus radiatus*. The sequence of reactions leading to the formation of valine and isoleucine was shown to be identical with that in *Neurospora crassa*[2], *Escherichia coli*[3,4], yeast[5] and Salmonella[6].

The enzyme systems involved in the formation of α-acetolactate in *N. crassa* and *E. coli* have been partially purified and their properties studied[7]. In this report a partial purification and properties of the α-acetolactate-forming system of *Phaseolus radiatus* are described.

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Abbreviations: PCMB, *p*-chloromercuribenzoate; BME, 2-mercaptoethanol.

MATERIALS AND METHODS

Chemicals

Acetoin (Eastman Kodak) was purified and crystallized according to BERL AND BUEDING[8]. The pure compound was used as a standard and was determined essentially as described by WESTERFELD[9]. α-Acetolactate was prepared by the procedure of KRAMPITZ[10]. α-Napththol (Eastman Kodak) was purified by distillation under nitrogen and the product was recrystallized from aqueous ethyl alcohol. Analytical grade creatine (BDH) and sodium pyruvate (E. Merck) were employed.

Enzyme assay

Unless otherwise mentioned, the standard mixture for assay consisted of enzyme, 500 μmoles sodium pyruvate, 5 μmoles Mn^{2+}, 20 μg DPT, 100 μmoles sodium phosphate buffer (pH 6.0) in a total volume of 1.0 ml. After incubation for 2 h at 37°, the reaction was stopped by the addition, in order, of 0.1 ml of 1 M zinc sulphate and 0.1 ml of 1 M NaOH and made up to a volume of 2.0 ml and centrifuged. A portion (0.2 ml) of the supernatant fluid was used for the assay of acetoin directly. In another portion (0.2 ml) α-acetolactate was converted to acetoin by acidification with 5% trichloracetic acid (0.1 ml), heated at 60° for 10 min, cooled and neutralized with sodium bicarbonate (0.4 ml, 0.75 M), when the total acetoin was determined. The amount of α-acetolactate was obtained by difference. The specific activity of the enzyme is usually expressed as μmoles of acetoin formed in 1 h/mg of protein.

Protein was determined by the method of LOWRY *et al.*[11] using crystalline bovine serum albumin as standard.

Calcium phosphate gel was prepared according to the method of KEILIN AND HARTREE[12] and aged for at least 4 months before use.

Enzyme purification

All the operations were carried out between 0–5°. Green gram *(P. radiatus)* seeds (200 g) were soaked for 12 h in running tap water and then germinated under diffuse light, after washing with glass-distilled water for 24 h at room temperature. The seeds were ground to a fine paste in a porcelain mortar with 200 ml of 0.1 M sodium phosphate buffer (pH 7.5), containing $5 \cdot 10^{-4}$ M cysteine, $5 \cdot 10^{-4}$ M Mn^{2+} and $5 \cdot 10^{-4}$ M sodium pyruvate. The presence of cysteine, Mn^{2+} and pyruvate all at a concentration of $5 \cdot 10^{-4}$ M, was found to stabilize the enzyme considerably during fractionation. These components were present in the buffers used in all such operations as dissolving the ammonium sulphate fractions, dialysis etc. (this is designated as fortified buffer). Homogenizing under these conditions in a blendor resulted in a considerable reduction of the specific activity of the enzyme while the total units were not affected. The paste was then centrifuged at 12 000 × *g* for 15 min, in a refrigerated centrifuge. The yellowish supernatant was drawn out by means of a syringe without disturbing the fatty layer floating on top. This fraction, which contained 4 · 10 g protein/100 ml, is designated as the crude extract (Step 1).

First ammonium sulphate fractionation

To 100 ml of crude extract were added 5 ml of 1 M $MnSO_4$ solution to remove

nucleic acids and 10 g of ammonium sulphate were added to give 20% saturation*. The mixture was stirred for 15 mins using a magnetic stirrer and was then centrifuged at 12 000 × *g* for 15 min. The precipitate was discarded and the supernatant fluid was brought to 45% saturation by the addition of 14.16 g of solid ammonium sulphate. The mixture was stirred for 30 min. After centrifugation at 12 000 × *g* for 15 min, the precipitate was suspended in 20 ml of 0.05 M 'fortified' phosphate buffer (pH 7.0). After stirring for 3–5 min, the suspension was centrifuged and a clear yellowish supernatant solution (43 ml, 645.1 mg of protein) was obtained (Step 2).

Dialysis

The fraction from Step 2 (42 ml) was dialyzed against 4 l of 0.2 M sodium-phosphate buffer (pH 7.5) for 1 h and then centrifuged at 12 000 × *g* for 15 min. The slight precipitate was discarded and the supernatant fluid (45.1 ml, 630.8 mg of protein) subjected to gel-fractionation (Step 3).

Gel-fractionation

The fraction from Step 3 (42 ml) was subjected to fractional absorption on calcium phosphate gel[14] (20 mg of solid/ml). The protein solution was added to a tube containing centrifuged gel (0.175 ml of gel/ml of enzyme solution), stirred for 10 min and centrifuged. The supernatant solution was transferred to a second tube containing the same quantity of centrifuged gel, stirred for 10 min and centrifuged. The supernatant fluid (45.1 ml, 315.40 mg of protein) from the second tube retained most of the activity (Step 4).

Second ammonium sulphate fractionation

The fraction (42 ml) from Step 4 was brought to 25% saturation by the addition of 5.63 g of $(NH_4)_2SO_4$, stirred for 20 min and centrifuged at 12 000 × *g* for 15 min. The precipitate was discarded and the supernatant fluid was brought to 35% saturation by the addition of 2.46 g of $(NH_4)_2SO_4$; after stirring for 30 min

TABLE I

PURIFICATION OF α-ACETOLACTATE-FORMING SYSTEM FROM *P. radiatus*

Step	*Total protein (mg)*	*Volume (ml)*	*Total activity (μmoles of acetoin/h)*	*Specific activity (μmoles acetoin/mg protein/h)*	*Recovery (%)*	*Fold purification*
1	4100	100.0	381.7	0.093	100	– –
2	645.1	43.0	314.2	0.487	82.31	5.23
3	630.8	45.1	290.7	0.461	76.15	4.95
4	315.4	45.1	213.5	0.677	55.94	7.27
5	54.3	12.3	185.1	3.410	48.50	36.62

* The percent saturations of ammonium sulphate are taken from a standard table[13] and are not corrected for temperature.

the suspension was centrifuged. The precipitate was suspended in 0.05 M sodium phosphate buffer ('fortified') (pH 7.0). This fraction (12.3 ml, 54.3 mg of protein) was used in most of the experiments as the purified preparation (Step 5) and the enrichment was about 36-fold over the crude extract with 48.5% recovery of the activity.

The summary of purification is given in Table I. Further attempts to purify the enzyme using DEAE-cellulose columns, or fractionation with ethanol or acetone, were unsuccessful and resulted in great reduction of specific activity.

Properties of the α-acetolactate-forming system

pH optimum: The purified enzyme exhibited optimum activity at pH 6.0 in phosphate buffer (Fig. 1). The activity at pH 6.0 was lowered in other buffers tried in the order, citrate < succinate < Tris–maleate < phosphate.

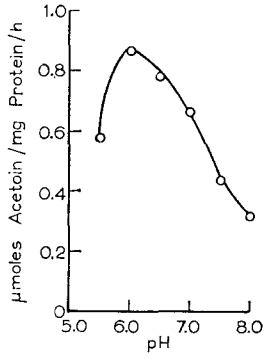

Fig. 1. pH optimum of the α-acetolactate-forming system of *P. radiatus*. Standard assay conditions with 0.35 mg of protein.

Effect of substrate concentration: (a) High concentrations of pyruvate were required for saturation of the enzyme. The K_m ($3 \cdot 10^{-2}$ M) was of the same order of magnitude (Fig. 2), as that reported previously for a similar enzyme from *E. coli*[7]. (b) Acetaldehyde, alone, in the range of $1–25 \cdot 10^{-4}$ M did not act as a substrate and in this respect the enzyme resembles the systems in *E. coli* and *N. crassa*. But, unlike these systems the preparations from green gram were able to form free acetaldehyde when pyruvate was used as substrate. Acetaldehyde strongly competes with pyruvate in the formation of α-acetolactate, so that the main product, when pyruvate is used as substrate, is acetoin. However, it has been shown previously[1] that α-acetolactate is definitely formed in this reaction. Many attempts to demonstrate the formation of acetoin by enzymic decarboxylation of α-acetolactate using purified preparations of green gram failed. So, acetoin formation in *P. radiatus* preparations is probably not linked to α-acetolactate formation.

Effect of DPT concentration: Purified preparations were devoid of activity unless supplemented by DPT. The effect of DPT concentration on the enzyme activity is given in Fig. 3 and the K_m value is $3 \cdot 10^{-6}$ M.

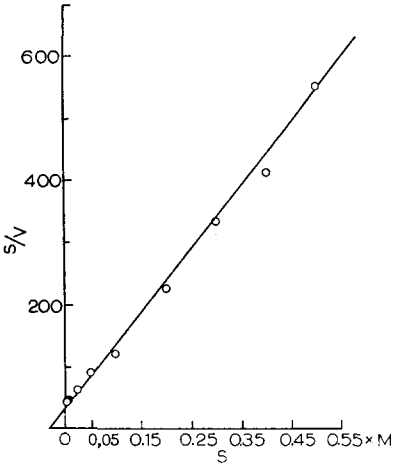

Fig. 2. The K_m value for pyruvate. Standard assay conditions with 0.32 mg of protein and the concentration of pyruvate varied.

Time-course: A linear relationship was obtained (Fig. 4) between the enzymic activity and the incubation period (up to 240 min).

Effect of enzyme concentration: UMBARGER AND BROWN[15] reported that a linear relationship between the concentration of the enzyme and the activity was not obtained in the systems catalyzing the formation of both α-acetolactate and α-

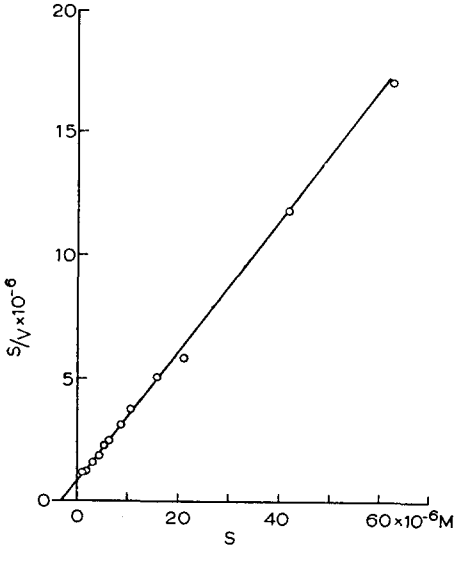

Fig. 3. The K_m value for thiamine pyrophosphate. Standard assay conditions with 0.38 mg of protein and the concentration of DPT varied.

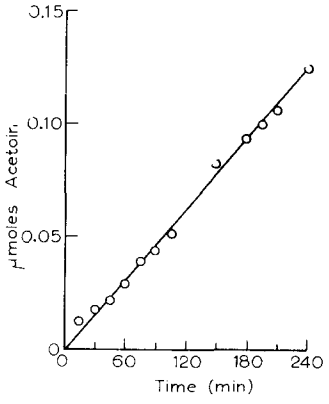

Fig. 4. α-Acetolactate formation by the purified enzyme from *P. radiatus* as a function of time. Standard assay conditions with 0.21 mg of protein.

aceto-α-hydroxybutyrate. In the green gram system, however, there was linearity over a wide range of protein concentration (Fig. 5).

Temperature optimum: The optimum temperature for enzyme activity was found to be 60° (Fig. 6). Heating the enzyme preparation alone (5 min at 60°) without the full complement of the standard assay mixture, resulted in complete loss of

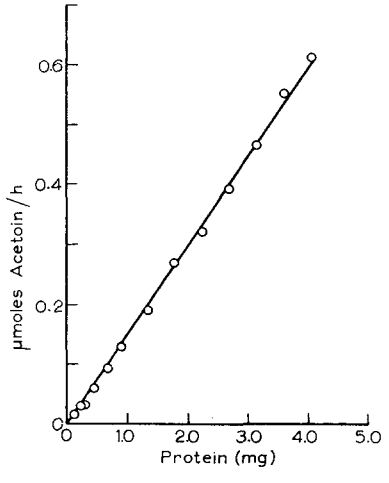

Fig. 5. α-Acetolactate formation by the purified enzyme from *P. radiatus* as a function of the concentration of the enzyme. Standard assay conditions with the amount of protein varied.

activity. So, in these studies the standard assay mixtures were incubated for varying periods at different temperatures. At any particular temperature employed there was a linear relationship between activity and time of incubation (Fig. 7). The optimum temperature was around 60° irrespective of the period of incubation

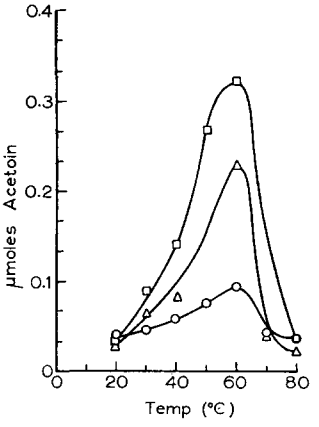

Fig. 6. Formation of α-acetolactate by the purified enzyme from *P. radiatus* as a function of temperature. Standard assay conditions at different temperatures and times of incubation; 10 min (○—○), 20 min (△—△) and 30 min (□—□) with 0.048 mg of protein. The enzyme used in this experiment and in those given in Figs. 7 and 8 was a highly purified (150-fold) preparation. This degree of purification was obtained in a single experiment when the final 25–35% saturated ammonium sulphate fraction was dialyzed against 0.02 M phosphate buffer (pH 7.5). The inactive protein precipitate, which was obtained during dialysis, was centrifuged off and the supernatant fraction of high specific activity was then used in these experiments. Repetition of this procedure did not give consistent results.

(Fig. 6). The Arrhenius plot (Fig. 8) gave an activation energy (μ) of 3540 cal/mole using the data obtained by incubating for 10 min. At incubation periods of 20 and 30 min, there was an apparent increase in the activation energy. In view of the observed linear relationship between enzymic activity and time of incubation at any given temperature, the increase in activation energy at longer incubation periods cannot readily be explained.

Stability: The presence of cysteine, pyruvate and Mn^{2+} (all at $5 \cdot 10^{-4}$ M)was

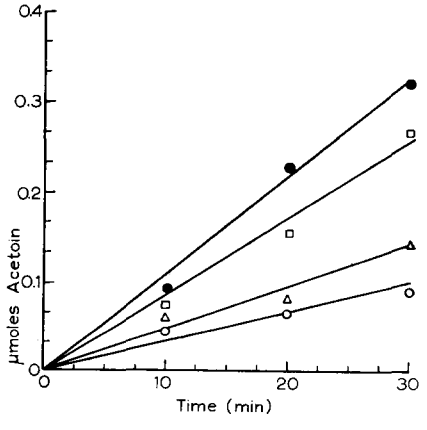

Fig. 7. α-Acetolactate formation as a function of time at different temperatures of incubation. Data from results given in Fig. 6; 30° (○—○), 40° (△—△), 50° (□—□) and 60° (●—●).

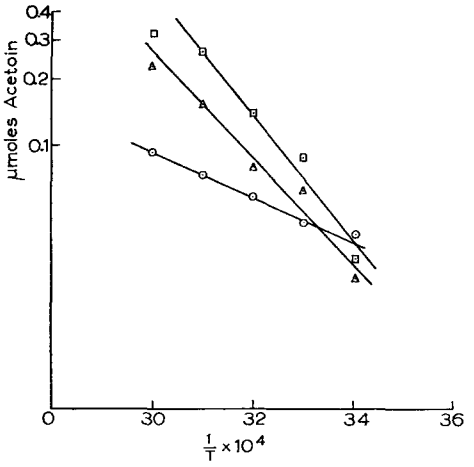

Fig. 8. The Arrhenius plot of α-acetolactate-formation. Data taken from Fig. 6. Incubation times: 10 min (○—○), 20 min (△—△) and 30 min (□—□).

found to be essential to obtain reasonable recovery of the enzyme activity at various stages of fractionation. In the absence of these components, the specific activities of the preparations were very low. However, a striking property of the enzyme system is its stability towards heat as stated above in presence of all the components of the standard assay mixture.

The purified preparations in the frozen state retained full activity for 7 days and had still 50% activity up to 30 days.

Metal requirement: Besides DPT, the enzyme requires a divalent metal ion (Mg^{2+} or Mn^{2+}) for full activity. The relative affinity constants for Mg^{2+} (Fig. 9)

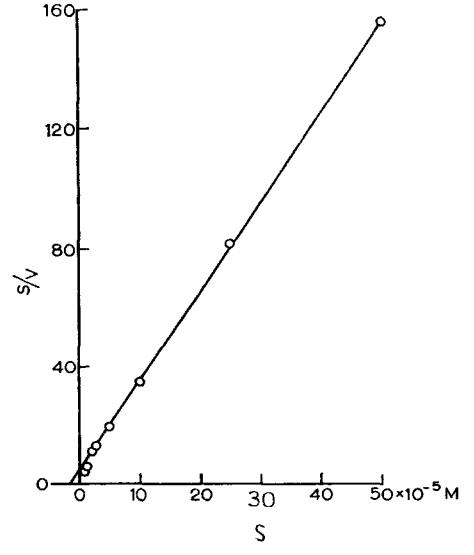

Fig. 9. The K_m value for Mg^{2+}. Standard assay conditions with the concentration of Mg^{2+} varied.

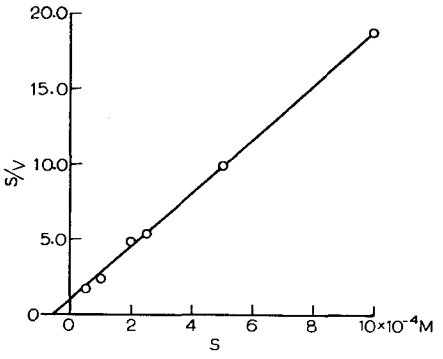

Fig. 10. The K_m value for Mn^{2+}. Data from Fig. 11.

and Mn^{2+} (Fig. 10) and the maximum activities of a series of divalent metal ions in Tris–maleate and phosphate buffers are summarized in Table II. Both Mg^{2+} and Mn^{2+} were quite effective in restoring the activity of the enzyme which was lost during dialysis against buffer containing a chelating agent (EDTA). At higher concentrations Mn^{2+} was found to be inhibitory (Fig. 11). The K_m values for Mn^{2+} and Mg^{2+} were $5 \cdot 10^{-5}$ M and $2 \cdot 10^{-5}$ M respectively.

Ag^{+} and Hg^{2+} ions strongly inhibited the reaction. A dialyzed preparation with low specific activity exhibited only 6.8% and 6% of the original activity in the presence of Ag^{+} and Hg^{2+} ions, respectively, at a concentration of $5 \cdot 10^{-4}$ M. The in-

TABLE II

EFFECT OF METALLIC IONS

*Metal**	*μmoles of acetoin/mg of protein/h*	
	*Tris***	*Phosphate****
None	0.18	0.22
Mn^{2+}	0.67	0.58
Mg^{2+}	0.67	0.67
Co^{2+}	0.25	0.62
Zn^{2+}	0.23	0.36
Cd^{2+}	0.22	0.25
Ni^{2+}	0.19	0.31
Cu^{2+}	0.18	0.08
Fe^{2+}	0.18	0.21
Fe^{3+}	0.17	0.21
Ba^{2+}	0.16	0.18
Sn^{2+}	0.15	0.15
Ca^{2+}	0.14	0.17

* All metals were used at a final concentration of $5 \cdot 10^{-4}$ M.

** 2 ml of enzyme (11 mg of protein) mixed with EDTA (35 μmoles) was dialysed against 500 ml of 0.02 M Tris buffer (pH 8.0) containing EDTA ($1 \cdot 10^{-1}$ M) for 2 h. It was further dialysed against 500 ml of 0.02 M Tris buffer (pH 8.0) for 4 h with three changes.

*** 4 ml of enzyme (32 mg of protein) mixed with EDTA (102 μmoles) was dialysed against 500 ml of 0.02 M phosphate buffer (pH 7.5) containing EDTA ($1 \cdot 10^{-1}$ M). It was further dialysed against 2 l of 0.02 M phosphate buffer (pH 7.5) for 15 h with buffer every 2 h up to a total of 14 l. The buffer always contained pyruvate, cysteine and BAL (all at $5 \cdot 10^{-4}$ M).

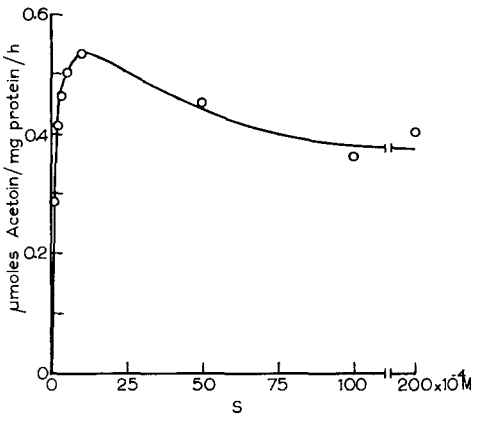

Fig. 11. Activation of α-acetolactate formation by Mn^{2+}. Standard assay conditions with the concentration of Mn^{2+} varied.

hibition was apparent even in the presence of Mn^{2+} ($5 \cdot 10^{-4}$ M), the activity being 58% (for Ag^{+}) and 62.5% (for Hg^{2+}) of the original activity.

Inhibition studies: The enzyme was strongly inhibited by PCMB and to a small extent by iodoacetate (Table III). The inhibition by PCMB was partially reversed by BME, BAL and cysteine (Table IV). Arsenite, sulphite and cyanide were not inhibitory.

Effect of L-*valine:* α-Acetolactate formation was inhibited by L-valine in *E. coli*[15,17] and in *A. aerogenes*[16]. There was no such inhibition in the green gram system at

TABLE III

EFFECT OF PCMB AND IODOACETATE

Standard assay conditions except that the enzyme was preincubated with the inhibitor for 5 min prior to the addition of substrate and cofactors. Control tubes containing the enzyme alone were incubated for the same length of time.

Inhibitor	*Inhibitor concentration (M)*	*μmoles of acetoin/mg protein/h*	*Percent activity*
PCMB	—	1.05	100
(reaction at	$5 \cdot 10^{-4}$	0.18	17.5
pH 6.0)	$5 \cdot 10^{-5}$	0.18	17.5
	$1 \cdot 10^{-5}$	0.76	72.5
	$1\text{–}5 \cdot 10^{-6}$	0.99	93.7
Iodoacetate	—	1.13	100
(reaction at	$5 \cdot 10^{-3}$	1.11	97.6
pH 6.0)	$5 \cdot 10^{-4}$	1.11	97.6
	$5 \cdot 10^{-5}$	1.13	100
Iodoacetate	—	0.86	100
(reaction at	$5 \cdot 10^{-3}$	0.62	71.7
pH 7.0)	$5 \cdot 10^{-4}$	0.83	95.6
	$5 \cdot 10^{-5}$	0.86	100

TABLE IV

REVERSAL OF PCMB INHIBITION

Expt.	*Additions**	*Molar concentration of —SH compound*	*μmoles of acetoin/mg of protein/h*	*Activity (%)*
1	—	—	1.63	100
	PCMB	—	0.44	27
	PCMB +	$5 \cdot 10^{-3}$	1.22	75
	2-Mercapto-	$5 \cdot 10^{-4}$	1.06	65.5
	ethanol	$5 \cdot 10^{-5}$	0.63	38.7
2	—	—	1.85	100
	PCMB	—	0.41	22.2
	PCMB +	$5 \cdot 10^{-3}$	1.16	62.8
	2,3-dimer-	$5 \cdot 10^{-4}$	0.88	47.5
	captopropanol	$5 \cdot 10^{-5}$	0.45	24.4
3	—	—	1.63	100
	PCMB	—	0.41	25.2
	PCMB +	$5 \cdot 10^{-3}$	0.97	59.5
	Cysteine	$5 \cdot 10^{-4}$	0.93	57.2
		$5 \cdot 10^{-5}$	0.41	25.2
4	—	—	1.38	100
	PCMB	—	0.31	22.4
	PCMB +	$5 \cdot 10^{-3}$	0.47	34.4
	GSH	$5 \cdot 10^{-4}$	0.41	29.7
		$5 \cdot 10^{-5}$	0.41	29.7

* Standard assay conditions, except that the enzyme was preincubated with a PCMB concentration of $5 \cdot 10^{-5}$ M for 5 min, after which the —SH compound was added. The incubation was then continued for another 10 min before the addition of the substrate and the cofactors. Control tubes containing the enzyme alone were incubated for the same length of time.

concentrations of DL-valine of $5 \cdot 10^{-5}$ M to $5 \cdot 10^{-3}$ M. In this respect, it resembles the system from *E. coli* k-12/V3, a valine-resistant strain.

Germination of the seeds in the presence of DL-valine ($1–3 \cdot 10^{-1}$ M) did not reduce the activity of the enzyme.

Germination of the seeds in presence of pyruvate ($1–3 \cdot 10^{-1}$ M) did not enhance the enzymic activity.

DISCUSSION

The results which are presented in this paper clearly demonstrate the formation of α-acetolactate from pyruvate in *P. radiatus*. As in the enzyme system reported by SINGER AND PENSKY[18,19] from wheat-germ, the main product of the reaction is acetoin, the decarboxylation product of α-acetolactate. However, the formation of α-acetolactate in *P. radiatus* was established previously[1]. Free acetaldehyde is formed in the green gram system and, in the absence of pyruvate, acetaldehyde alone does not lead to the fromation of acetoin. Acetaldehyde competitively inhibits the formation of α-acetolactate and, in its presence, the reaction is mainly diverted towards the formation of acetoin. Since, we were unable to detect the presence of α-acetolactic decarboxylase in purified preparations from *P. radiatus*, α-aceto-

lactate does not appear to be an obligatory intermediate in the formation of acetoin and in this respect this system resembles that of wheat germ[18]. On the basis of these two studies it would appear that the enzyme–acetaldehyde complex in plant systems dissociates to free acetaldehyde to a greater extent compared with bacterial and fungal enzymic systems. However, it is possible that adequate quantities of α-acetolactate needed for the biosynthesis of valine are made available by stabilization of the complex by mechanisms not yet known.

The purified enzyme shows a requirement for a divalent metallic ion (Mg^{2+} or Mn^{2+}) and is inhibited by Ag^{+}, Hg^{2+}, and by PCMB. The inhibition due to PCMB can be partially reversed by sulfydryl reagents like BME, BAL or cysteine.

The presence of L-valine, either during germination or in the standard incubation mixture, had no inhibitory effect. Feed-back control mechanisms suggested for valine synthesis in *E. coli*[15] and *A. aerogenes*[16] may not be operative in higher plants.

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