the mechanism by which molybdenum becomes toxic in this mold, as well as to study in detail the control of molybdenum uptake by methionine.

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[1] L. F. GRAY AND L. J. DANIEL, *J. Nutrition*, 53 (1954) 43.
[2] R. VAN REEN AND M. A. WILLIAMS, *Arch. Biochem. Biophys.*, 63 (1956) 1.
[3] K. SIVARAMA SASTRY, A. RAMAIAH AND P. S. SARMA, *Biochim. Biophys. Acta*, 30 (1958) 438.
[4] P. R. ADIGA, K. SIVARAMA SASTRY, V. VENKATASUBRAMANYAM AND P. S. SARMA, *Biochem. J.*, in the press.
[5] A. T. DICK AND J. B. BINGLEY, *Australian J. Exptl. Biol. Med. Sci.*, 29 (1951) 459.

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Biosynthesis of valine and isoleucine in plants

The bulk of evidence available in recent years points to the following sequence of reactions leading to the biosynthesis of valine: pyruvate → α-acetolactate → α,β-dihydroxyisovalerate → α-ketoisovalerate → valine. In the case of isoleucine biosynthesis an analogous sequence in which α-aceto-α-hydroxybutyrate is an intermediate has also been shown. WAGNER *et al.*[1] demonstrated the conversion of α-acetolactate and α-aceto-α-hydroxybutyrate to α,β-dihydroxyisovalerate and α,β-dihydroxy-β-methylvalerate, respectively, using cell-free extracts of *Neurospora crassa*. On the basis of the existence in these extracts of a reductase which catalysed the conversion of synthetic α-keto-β-hydroxyisovalerate and α-keto-β-hydroxy-β-methylvalerate to the corresponding dihydroxy acid precursors of the two amino acids, these authors considered that these ketohydroxy compounds were intermediates in the conversion of α-hydroxy-β-ketoacids to the dihydroxy acids. However, RADHAKRISHNAN *et al.*[2] clearly demonstrated that in the case of *Escherichia coli* and *Neurospora crassa* this conversion is catalysed by a "reductoisomerase" which is distinct from the reductase. The participation or otherwise of the α-keto-β-hydroxy compounds in this reaction is still unknown. The above sequence of reactions has been amply confirmed by other workers in *E. coli*[3], in yeast[4] and in *Salmonella*[5].

In this report evidence is provided for the existence of enzyme systems involved in the biosynthesis of valine and isoleucine in plants. Seeds of green gram (*Phaseolus radiatus*) germinated for 24 h were ground in a mortar, the slurry centrifuged at 12000 × *g* for 15 min and the supernatant (crude extract) was used as the enzyme source. The enzyme system catalysing the conversion of pyruvate to α-acetolactate has been partially purified and the crude extract used for demonstrating the presence of other systems.

The α-acetolactate-forming system is extremely labile and the presence of a a metal (Mn^{2+}, $5 \cdot 10^{-4}$ *M*) and a thiol compound (cysteine or β-mercaptoethanol, $5 \cdot 10^{-4}$ *M*) is required for stabilisation throughout the procedure of fractionation.

By a fractional $(NH_4)_2SO_4$ precipitation and calcium phosphate gel treatment about 20-fold enrichment was achieved. Free acetaldehyde is a product of the reaction, in contrast to the reactions catalysed by the enzyme systems of *E. coli* and *N. crassa* and thus the reaction is mainly diverted towards the formation of acetylmethylcarbinol (acetoin). Acetaldehyde alone ($1 \cdot 10^{-4}$ *M* to $2.5 \cdot 10^{-3}$ *M*) is not a substrate in this reaction (*cf.* SINGER AND PENSKY[6]). The formation of α-acetolactate was indicated by assaying for acetoin by the procedure of WESTERFELD[7] by decarboxylation of α-acetolactate to acetoin by acid treatment. The difference between the amounts of acetoin before and after acid treatment though quite consistent was always small (approx. 10% of total acetoin). However, the formation of α-acetolactate can be unequivocally demonstrated by partial elimination of acetoin by volatilisation. In Table I are given the results of an experiment showing the formation of α-acetolactate.

The purified enzyme required the presence of thiaminepyrophosphate. Mn^{2+} and Mg^{2+} increased enzyme activity (K_m for Mn^{2+}, $5 \cdot 10^{-5}$ *M*) while Ag^+ and Hg^{2+} are inhibitory. As in other systems previously studied[8], the K_m for pyruvate ($2 \cdot 10^{-2}$ *M*) was very high. The purified preparation was devoid of α-acetolactate decarboxylase activity and so the free acetoin is derived mainly from the condensation of free acetaldehyde with the "enzyme–aldehyde" complex by competing with pyruvate. Removal of acetaldehyde by aeration of reaction mixtures simply reduced the total quantity of acetoin formed without correspondingly increasing the amount of α-acetolactate.

The next step in the sequence, the conversion of α-acetolactate and α-aceto-α-hydroxybutyrate to the corresponding dihydroxy acids was shown by incubating the dialysed crude extract, substrate (100 μmoles), glucose-6-phosphate (50 μmoles), TPN (0.5 μmoles), sodium phosphate buffer (pH 7.5, 125 μmoles) in a total volume of 2.5 ml for 7 h at 37°. The crude extract contained glucose 6-phosphate dehydrogenase activity. By using the procedures given by WAGNER *et al.*[1] the formation of α,β-dihydroxyisovalerate and α,β-dihydroxy-β-methylvalerate from the corresponding β-ketoacids was clearly demonstrated.

The presence of the dihydroxy acid dehydratase was established by using as substrates, α,β-dihydroxyisovalerate and α,β-dihydroxy-β-methylvalerate (kindly supplied by Dr. E. A. ADELBERG). The ketoacids were estimated colorimetrically[9]

TABLE I

FORMATION OF α-ACETOLACTATE IN *Phaseolus radiatus*

The reaction mixture consisted of purified enzyme (2 mg protein), sodium pyruvate (500 μmoles), DPT (20 μg), Mn^{2+} (5.0 μmoles), sodium phosphate buffer (pH 7.0, 100 μmoles) in a total volume of 1.0 ml. After incubation for 2 h at 37°, the reaction was stopped by the addition of 0.1 ml of 1 *N* $ZnSO_4$ and 0.1 ml of 1 *N* NaOH, made up to 2.0 ml and centrifuged. In Expt. 1, 0.1 ml of the supernatant was used as such for assay; in Expt. 2 and Expt. 3, 0.5 ml and 0.25 ml were subjected to high vacuum (< 1 mm) for 1 h for partial removal of acetoin and made up to 2.0 ml and 1.0 ml, respectively. 0.2-ml aliquots were used for assay.

Expt.	*Acetoin**	*Acetoin + α-acetolactate*	*α-Acetolactate (by difference)*
1	2.39	3.55	1.16
2	1.39	2.55	1.16
3	0.39	1.57	1.18

* Values expressed as μmoles formed in the reaction mixture.

TABLE II

DIHYDROXY ACID DEHYDRATASE IN *Phaseolus radiatus*

The reaction mixture consisted of crude extract, 0–80% $(NH_4)_2SO_4$ precipitate fraction, dialysed (13 mg protein), α,β-dihydroxyisovalerate (unresolved, 10 μmoles) or α,β-dihydroxy-β-methylvalerate (natural form, 10 μmoles), Mg^{2+} or Fe^{2+} (5.10^{-3} *M*), sodium phosphate buffer (pH 7.5, 100 μmoles) in a total volume of 1.0 ml. Incubation time was 3 h at 37°. The reaction was stopped by adding 0.5 ml of trichloroacetic acid, centrifuged and supernatant used for the assay of ketoacids.

Reaction	*Ketoacids formed**		
	—	Mg^{2+}	Fe^{2+}
α,β-Dihydroxyisovalerate → α-ketoisovalerate	0.34	1.27	0.66
α,β-Dihydroxy-β-methylvalerate → α-keto-β-methylvalerate	0.37	1.46	0.45

* Values expressed as μmoles formed in the reaction mixture.

(Table II) and further characterised by conversion to dinitrophenylhydrazones and catalytic reduction with Adam's catalyst to the respective amino acids[10] which were identified by paper chromatography[11]. The presence of the dehydratase activity in plants has been recently reported[12,13].

The last step, conversion of ketoacids to amino acids by transamination, was demonstrated in a reaction mixture containing the dialysed crude extract, α-keto-isovalerate or α-keto β-methylvalerate (20 μmoles), L-glutamic acid (20 μmoles), pyridoxal phosphate (10 μg), sodium phosphate buffer (pH 8.0, 100 μmoles) in a total volume of 1.0 ml and paper-chromatographic identification of the corresponding amino acids formed.

Further work on the purification of all the enzyme systems is currently in progress.

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1 R. P. WAGNER, A. N. RADHAKRISHNAN AND E. E. SNELL, *Proc. Natl. Acad. Sci. U.S.*, 44 (1958) 1047.
2 A. N. RADHAKRISHNAN, R. P. WAGNER AND E. E. SNELL, *J. Biol. Chem.*, 235 (1960) 2322.
3 H. E. UMBARGER, B. BROWN AND E. J. EYRING, *J. Biol. Chem.*, 235 (1960) 1425.
4 M. STRASSMAN, J. B. SHATTON AND S. WEINHOUSE, *J. Biol. Chem.*, 235 (1960) 700.
5 F. B. ARMSTRONG AND R. P. WAGNER, *J. Biol. Chem.*, 236 (1961) 2027.
6 T. P. SINGER AND J. PENSKY, *Biochim. Biophys. Acta*, 9 (1952) 316.
7 W. W. WESTERFELD, *J. Biol. Chem.*, 161 (1945) 495.
8 A. N. RADHAKRISHNAN AND E. E. SNELL, *J. Biol. Chem.*, 235 (1960) 2316.
9 T. E. FRIEDEMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
10 A. MEISTER AND P. A. ABENDSCHEIN, *Anal. Chem.*, 28 (1956) 171.
11 K. V. GIRI, A. N. RADHAKRISHNAN AND C. S. VAIDYANATHAN, *Anal. Chem.*, 24 (1952) 1677.
12 R. L. WIXOM, S. K. WADE AND G. B. HOWELL, *Federation Proc.*, 20 (1961) 11.
13 M. KANAMORI, J. SHERWOOD AND J. W. BLANKENSHIP, *Federation Proc.*, 20 (1961) 11.

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