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ACETOLACTATE SYNTHETASE FROM BARLEY SEEDLINGS

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Abstract—Acetolactate synthetase has been isolated from barley seedlings. The enzyme requires TPP and either Mn^{2+} or Mg^{2+} for maximal activity. It has a broad pH optimum from pH 6·5 to 8·5. The major product of the reaction, with pyruvate as substrate, is acetolactate. The enzyme is inhibited by leucine plus valine acting in a co-operative manner and by the same amino acids, if added singly, at higher concentrations. The pair isoleucine plus leucine also co-operatively inhibit the enzyme, although isoleucine is relatively ineffective on its own. The inhibitions are greater at pH 6·5 than at pH 8·5. The results are consistent with the hypothesis that inhibition of the growth of barley seedlings by valine and leucine is due to their effect on acetolactate synthetase.

INTRODUCTION

RECENT experiments[1] have shown that valine, leucine and, to a much lesser extent, isoleucine are inhibitory to the growth of germinating barley embryos. The inhibition of leucine can be relieved by the addition of valine and isoleucine and that of valine by the addition of isoleucine. Similar inhibitory effects of valine on the growth of *Escherichia coli* K12[2] have been explained in terms of end-product inhibition of the acetohydroxyacid-synthesizing enzyme by valine.[3] This enzyme is responsible for the synthesis of both acetohydroxybutyrate, the precursor of isoleucine, and acetolactate, the precursor of valine and leucine.[4] The results with barley could be due to the operation of a similar mechanism but, in general, little is known of end-product inhibition in higher plants. Previous reports on acetolactate synthesis are contradictory with regard to its regulatory properties. Thus, although Davies[5] has isolated the enzyme from peas and shown it to be inhibited by valine and isoleucine, Satyanarayana and Radakrishnan[6] found no inhibition with a similar enzyme from *Phaseolus radiatus*. Neither of the results correlate with the observations on growth inhibition in barley. In an attempt to explain these results and to learn more of the regulatory systems in higher plants, acetolactate synthetase has been isolated from barley and a preliminary study of its regulatory properties made.

RESULTS

Initial studies showed that the activity and regulatory nature of acetolactate synthetase isolated in 0·05 M phosphate, 0·005 M EDTA (pH 7·5), varied considerably. Addition of $MgCl_2$, leucine and valine to the extraction buffer increases both the amount of activity and the degree of end-product inhibition of the enzyme. The preparation obtained using this extraction medium, followed by ammonium sulphate precipitation, only shows a small

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[2] H. E. UMBARGER and B. BROWN, *J. Bacteriol.* **72**, 241 (1955).
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[4] R. I. LEAVITT and H. E. UMBARGER, *J. Biol. Chem.* **236**, 2486 (1961).
[5] M. E. DAVIES, *Plant Physiol.* **39**, 53 (1964).
[6] T. SATYANARAYANA and A. N. RADAKRISHNAN, *Biochim. Biophys. Acta* **56**, 197 (1962).

stimulation by Mn^{2+} and TPP. However, after further resolution by 4 hr dialysis against 0·05 M EDTA, and passage over G-50 Sephadex, a definite requirement for TPP and either Mg^{2+} or Mn^{2+} can be demonstrated (Table 1). The enzyme, in common with that from other sources, has a wide pH optimum from around pH 6·5 to 8·5; the temperature optimum is about 40°.

TABLE 1. CO-FACTOR REQUIREMENTS FOR ACETOLACTATE SYNTHETASE

Reaction components	Rate (μmoles/hr/mg protein)
Complete	1·74
—Mn^{2+}	1·43
—TPP	1·12
—Mn^{2+} + Mg^{2+}	1·81
Complete + Mg^{2+}	1·74

The effects of the amino acids leucine, valine and isoleucine on the enzyme were investigated. Table 2 shows that leucine and valine are most effective in inhibiting activity at pH 6·5, although inhibition by valine is affected less by pH than leucine. The results also show that there is a synergistic effect when leucine and valine, or leucine and isoleucine are added together. This is not observed for the third pairing isoleucine and valine. Although these results and those of the *in vivo* studies[1] suggest that leucine and valine are acting in a co-operative fashion to inhibit acetolactate synthetase, they do not prove it. This is because if

TABLE 2. INHIBITION OF ACETOLACTATE SYNTHETASE AT DIFFERENT pH'S BY VARIOUS COMBINATIONS OF AMINO ACIDS

Amino acids added	pH of reaction mixture	
	6·5	8·5
None	1·06	1·20
Leucine	0·73	1·20
Leucine + isoleucine	0·49	0·95
Leucine + valine	0·36	0·77
Leucine + valine + isoleucine	0·33	0·92
Isoleucine	1·02	1·22
Isoleucine + valine	0·83	1·06
Valine	0·84	1·06

Rates given as μmoles of acetolactate/mg protein/hr. All amino acids present at 5 mM final concentration. Those results underlined indicate synergistic inhibition by the combined amino acids.

the relationship between the concentration of inhibitor ([*I*]) and the degree of inhibition is not linear, but exponential, then doubling [*I*] will more than double the amount of inhibition. Thus, if leucine and valine act at the same site, then adding both at 5 mM ([*I*] = 10 mM) might merely produce the same result as doubling one of them. This has been investigated by studying inhibition curves and by comparing the pairs leucine plus valine and isoleucine plus leucine, with the amino acids added singly at the same total [*I*] in each case. From the resulting graphs the [*I*] giving 40 per cent inhibition with 0·05 M pyruvate as substrate has been

calculated and the values given in Table 3. Each experiment given in the table was done with a different preparation and these may vary slightly in the degree of desensitization occurring during isolation. The results clearly demonstrate that leucine and valine added together are effective at a much lower $[I]$ ($\frac{1}{10}$) than when either are added separately. The same holds true to a lesser extent for isoleucine plus valine but, as in the previous experiment, the pair isoleucine plus valine do not show any co-operative effects.

TABLE 3. CONCENTRATION (mM) OF INHIBITOR REQUIRED FOR 40% INHIBITION OF ACETOLACTATE SYNTHETASE

Amino acid combination	Experiment 1	2	3
Isoleucine	—	>10·0	>10·0
Valine	10·0	—	6·5
Leucine	3·5	2·1	—
Valine + leucine	0·35	—	—
Isoleucine + leucine	—	1·0	—
Isoleucine + valine	—	—	9·8

Concentration in mM, where two amino acids are present they are in equal amounts. The three experiments were carried out with different enzyme preparations.

Previous workers[5, 6] have shown that normal Michaelis–Menten kinetics can be demonstrated with respect to pyruvate concentration. Figure 1 also shows that at concentrations of pyruvate between 0·1 and 0·005 M a plot of 1/v against 1/[S] approximates to a straight line giving a K_m of 14·3 mM. The inhibition by leucine plus valine ($[I]=10^{-5}$ M) appears to be of the competitive type. However, the lowest concentration of pyruvate used in the experiment gave a rate in excess of 25 per cent of V_{max}. In view of the comments of Atkinson[7]

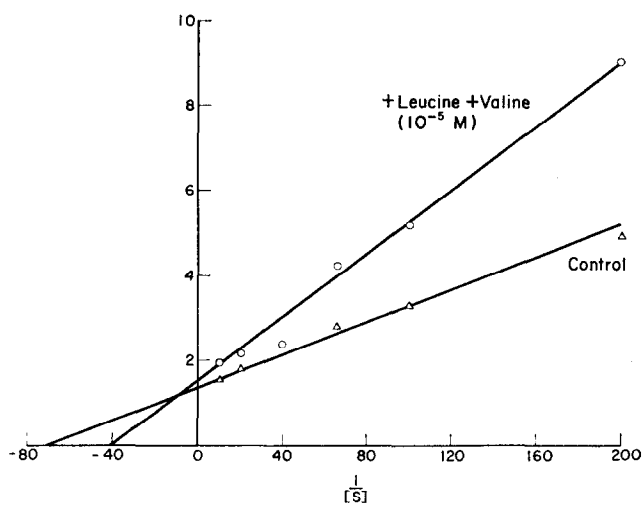

FIG. 1. RELATIONSHIP OF THE RATE OF ENZYME ACTIVITY TO HIGH PYRUVATE CONCENTRATION. Assay as in text except that the incubation was for 10 min only.

[7] D. E. ATKINSON, *Ann. Rev. Biochem.* **35**, 85 (1966).

and the fact that many other regulatory enzymes show sigmoid kinetics at low substrate concentrations, the relationship of rate to pyruvate concentrations down to 0·2 mM have been investigated. As expected, the dependence of rate upon substrate concentration at low levels of pyruvate shows a sigmoid relationship (Fig. 2).

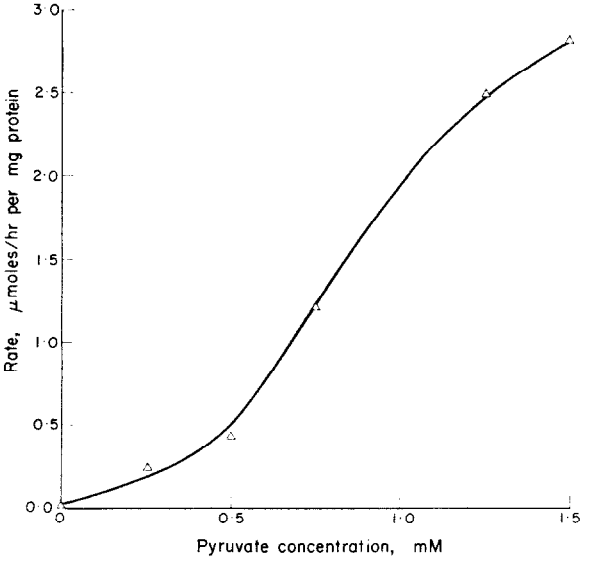

FIG. 2. RELATIONSHIP OF THE RATE OF ENZYME ACTIVITY TO LOW PYRUVATE CONCENTRATIONS. Assay as in text except that the incubation was for 10 min only.

Although all the results presented here have been done on shoot preparations, unpublished work (Garood and Miflin) has shown that the enzyme is also present in the roots and has very similar properties.

DISCUSSION

The end-product inhibition of acetolactate synthetase in barley is in agreement with the results in peas,[5] but differs from the enzyme reportedly present in *Phaseolus*.[6] The effect of leucine was not reported for the pea enzyme, but valine and, less effectively, isoleucine were inhibitory. Although Table 2 shows only a low level of inhibition for isoleucine, 30 per cent inhibition is obtained when the concentration is raised to 0·01 M.

Satayanarayana and Radakrishnan[6] were unable to obtain any evidence for feedback inhibition. Another difference between the pea and barley enzymes and the *Phaseolus* system is that the former produce only minor amounts of acetoin (in this study 3–15 per cent), whereas in the latter case the authors state that acetoin is the major reaction product. The difference may, in part, be due to the use of leucine and valine in the isolation procedure, thereby protecting the enzyme from partial destruction. Under conditions where it is imperfectly isolated it may be unable to bind the hydroxyethyl-TPP complex properly, which could then break down to yield free acetaldehyde and thus acetoin. Under these circumstances the enzyme may also lose its regulatory properties. The other consideration is that there is more than one enzyme system capable of producing acetolactate and the one reported in

Phaseolus[6] is not involved in amino acid biosynthesis. This situation is well described in *Escherichia coli*[8] where two enzymes, one with a pH optimum of 6·0 and one with a pH optimum of 8·0, are able to produce acetolactate. Halpern and Umbarger,[8] however, consider that only the pH 8·0 enzyme is involved in the synthesis of amino acids and that the pH 6·0 activity is due to part of the pyruvic oxidase complex described by Juni.[9] Only the pH 8·0 enzyme is subject to feedback control in *E. coli*. Davies[5] claims that his results also indicate that there are two enzymes forming acetolactate in his pea preparation. Although the barley system shows a similarly wide pH optimum, preliminary experiments have not indicated any of the great differences of K_m with pH that characterized the pea enzymes. The major difference in behaviour between pH 6·5 and pH 8·5 is in the degree of inhibition by the amino acids. This may merely reflect a different effect of pH on the inhibitor binding site compared to that on the substrate site rather than the presence of two enzymes. At present, therefore, there is no reason to suggest that there are two enzymes present in the barley preparations.

Although previous work with regulatory enzymes has suggested that a sigmoid relationship between rate and substrate concentration is common in such systems, there is no evidence, in the present work, to prove that the sigmoidal relationship reflects the regulatory nature of the enzyme. Davies and Corbett (quoted in Davies[10]) have shown that the decarboxylation of glyoxylate by pyruvic decarboxylase shows sigmoid kinetics indicative of a second-order reaction in which glyoxylate reacts a second time with the enzyme substrate complex. Acetolactate synthetase is a closely related enzyme with which pyruvate reacts to form hydroxyethyl-TPP which, in turn, reacts with a second molecule of pyruvate to form acetolactate. Thus the results in Fig. 2 may reflect the complexity of the enzyme catalysed reaction rather than the regulatory properties of the enzyme.

Although the results in this paper relate only to the synthesis of acetolactate, there is reason to believe that the same enzyme carries out the synthesis of acetohydroxybutyrate. The inhibition of growth by valine and leucine, the relief of the former by isoleucine and the latter by valine and isoleucine, coupled with the co-operative effect of isoleucine and leucine on acetolactate synthetase, provide strong circumstantial evidence that the enzyme is responsible for the synthesis of both of the hydroxy acids. Such a situation has been shown to be true for *E. coli*[4] and *Neurospore crassa*.[11, 12]

The regulation of acetolactate synthetase in barley differs from that in *E. coli*,[13] *Salmonella typhimurom*,[14] *Neurospora*,[12]pea,[5] and *Phaseolus radiatus*[6] in that the enzyme is subject to co-operative feedback by the pairs isoleucine plus leucine and valine plus leucine. A similar type of co-operative feedback has been described for aspartyl kinase in bacteria,[15] although in this case the modifiers are not effective on their own. The co-operative system is advantageous to the plant in that it enables it to overcome some of the difficulties inherent in regulating branched synthetic pathways by only one end-product. Such regulation has the potential disadvantage that overproduction of the regulatory end-product could lead to the discontinuation of the synthesis, and a consequent shortage, of the other end-products. The

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[15] P. Datta and H. Gest, *Nature* **203**, 1259 (1964).

co-operative system described here partly avoids this in that maximum feedback regulation requires the presence of two out of the three amino acids.

There is good correlation between the previous observations on *in vivo* inhibition of growth[1] and the *in vitro* inhibition of acetolactate synthetase. Thus leucine, valine and isoleucine are inhibitory to both processes in decreasing order of effectiveness and leucine and valine act in a co-operative manner in both systems. The results presented in this paper are, therefore, consistent with the hypothesis that the inhibitory effects of leucine, valine and isoleucine on the growth of excised barley embryos are due to their inhibition of acetolactate synthetase.

EXPERIMENTAL

Barley seeds were treated and germinated over a culture medium as described previously.[16] After growth for 5–7 days shoots were removed and ground in cold 0·05 M phosphate, 0·005 M EDTA, 0·005 $MgCl_2$, 0·001 M valine and 0·001 M leucine, pH 7·5 in a pestle and mortar. The resultant brei was filtered through nylon cloth and centrifuged at 20,000 × *g* for 5 min. To 1 vol. of supernatant was added $\frac{1}{3}$ vol. of saturated $(NH_4)_2\ SO_4$ (pH 7·5) and the preparation stood at 2° for 5 min and then centrifuged. The precipitate was discarded and a further $\frac{1}{3}$ vol. $(NH_4)_2\ SO_4$ added to give 40% saturation. The resulting precipitate was collected as above and taken up in 0·005 M phosphate and 0·001 $MgCl_2$ (pH 7·0) and passed over a G25 sephadex column. The resulting preparation was used as the source of the enzyme in the majority of the following experiments.

Acetolactate synthetase was assayed by incubating the enzyme in 0·05 M pyruvate, 20 mM phosphate buffer, pH 7·0, 0·5 mM $MnSO_4$, 0·32 mM TPP. The reaction was stopped by the addition of 0·2 ml 0·3 N $ZnSO_4$ and 0·2 ml 1 N NaOH. The resultant precipitate was removed by centrifugation and two 0·5 ml aliquots of the supernatant taken. One of these was acidified and heated at 60° for 15 min to decarboxylate the acetolactate to acetoin. The other was used to estimate the formation of acetoin during the reaction. The amount of acetolactate formed being determined by the difference between the two tubes. Acetoin was determined colorimetrically by the method of Westerfield.[17]

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