Acetolactate and Acetoin Synthesis in Ripening Peas[1]

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Introduction

The role of acetolactate as a precursor in valine biosynthesis was first indicated by the isotopic studies of Strassman et al. (18) and was subsequently supported by work on valine-less mutants of *Escherichia coli* (21, 22) and *Neurospora crassa* (24). The direct conversion of acetolactate to a number of valine precursors, and to valine itself, has since been demonstrated in cell-free extracts of a number of microorganisms (11, 15, 19, 25). A similar conversion of acetolactate to valine by crude extracts of spinach has been reported recently (27) and the enzymes responsible for this conversion demonstrated in cell-free extracts of *Phascolus radiatus* (16).

The formation of acetolactate from pyruvate has been observed in cell-free extracts of microorganisms (3, 5, 11, 14, 21) and pyruvate oxidase preparations from pigeon breast muscle (6). Such a synthesis has also been reported briefly by Satyanarayana and Radhakrishnan (16) for extracts of Phaseolus seedlings. The present communication reports on the properties of a partially purified preparation of acetolactate-forming enzymes from ripening pea seeds. The relationship between the enzymes responsible for acetoin and acetolactate synthesis is also considered.

Materials and Methods

Chemicals. Sodium pyruvate was prepared by neutralization of redistilled pyruvic acid (British Drug Houses) with $NaHCO_3$ at 0 to 5°. Stock solutions were stored at − 15° until used. Acetolactate was prepared by the method of Krampitz (7). Acetaldehyde was freshly distilled before use. Thiamine pyrophosphate (TPP) was obtained from the Sigma Chemical Co.

Plant Material. Ripening seeds of pea (*Pisum sativum* L. var. Onward) were obtained from locally grown plants and harvested immediately before use.

Assay of Acetoin- and Acetolactate-forming Enzymes. The standard assay mixture contained enzyme, sodium pyruvate (0.6 mmole), Mg^{++} (5 μmole), TPP (0.32 μmole) and either potassium phosphate buffer (0.3 mmole) or Tris-HCl buffer (0.3 mmole) of appropriate pH in a total volume of 3 ml. Incubation was for 1 hour at 30°, following which the reaction was stopped by the addition of 1 ml of *N* NaOH. For the determination of acetoin, 1 ml of reaction mixture was made up to 10 ml and an aliquot assayed by the method of Westerfeld (26). The determination of acetolactate was based on the fact that it is readily decarboxylated to acetoin on heating in dilute acid solution. A 1 ml sample of the reaction mixture was acidified with dilute H_2SO_4, heated at 70° for 10 minutes, cooled, neutralized and made up to 10 ml. An aliquot of this solution was taken for the determination of total acetoin, from which the amount of acetolactate was calculated by difference. The colors developed in the Westerfeld assay were measured in a Unicam SP.500 spectrophotometer at 503 mμ, using a zero-time sample as reference. Enzyme activity is generally expressed as μmoles of acetolactate or acetoin formed per mg protein per hour.

Other Assay Procedures. Acetolactate decarboxylase and CO_2 production in general were assayed by conventional manometric techniques. Acetaldehyde was estimated by the method of Barker and Summerson (1). Protein was determined by the method of Waddell (23).

Preparation of Acetolactate-forming Enzymes. Chilled, immature seeds (100 g) were blended in 300 ml of cold 0.1 M Tris-HCl, pH 7.5, and the resulting slurry strained through several layers of muslin. The filtrate was centrifuged for 15 minutes at 15,000 × *g* to remove cell debris and the supernatant fluid retained as the crude enzyme preparation. This was then subjected to $(NH_4)_2SO_4$ fractionation at 0 to 5°

The crude enzyme preparation (250 ml) was treated with 45 g of solid $(NH_4)_2SO_4$ and the inactive protein precipitate removed by centrifugation. To the supernatant fluid a further 15 g of $(NH_4)_2SO_4$ were added and the precipitate spun down as before. This precipitate contained the bulk of enzyme activity, and was resuspended, with the aid of a glass homogenizer, in 100 ml of 0.5 M potassium phosphate buffer, pH 7.5 The enzyme was reprecipitated from this solution by the addition of 24 g of $(NH_4)_2SO_4$, centrifuged and taken up in 50 ml of 0.02 M potassium phosphate, pH 7.5. This preparation was used as enzyme in the majority of experiments reported here.

As described under Results, evidence was obtained for the presence of 2 acetolactate-forming enzymes with different pH optima in the partially purified preparation. In a typical experiment, using the standard assay conditions, the crude enzyme preparation formed 0.1 and 0.13 μmole of acetolactate per mg protein per hour at pH 6 and pH 8.5 respectively. Under the same conditions the fractionated preparation formed 0.96 and 1.24 μmole of acetolactate per mg protein per hour at pH 6 and pH 8.5 respectively, representing approximately a 10-fold purification of both enzymes.

Preparation of Acetoin-forming Enzyme. Dry

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pea seeds were soaked in distilled water overnight at 0 to 5°. Fully imbibed seeds were rinsed in distilled water, blended in an equal volume of cold distilled water and the filtered slurry centrifuged for 15 minutes at 15,000 × *g*. The supernatant fraction (100 ml) was treated with 18 g of solid $(NH_4)_2SO_4$. The precipitated protein was then harvested by centrifugation and resuspended in 10 ml of 0.02 M potassium phosphate buffer, pH 6.5 This fraction contained most of the enzyme activity present in the preparation and was used in the experiments reported here without further purification. A fivefold purification of the acetoin-synthesising enzyme was generally achieved by this fractionation procedure.

Results

pH Optima and K_m Values of the Acetolactate-forming Enzymes. Although the pH-activity curve at a substrate concentration of 0.2 M showed an optimum between pH 7 and 7.5, a prominent shoulder was observed at pH 8.5, and lowering the pyruvate concentration to 0.004 M displaced the single optimum to between pH 8 and 8.5 (fig 1). In addition, a Linweaver-Burk double reciprocal plot of substrate concentration against activity at pH 7.5 showed a sharp deviation from linearity as the substrate concentration was increased (fig 2). No such discontinuities were evident at either pH 6 or pH 8.5, although the Linweaver-Burk plots were of markedly different slope (fig 3). These results are taken as indicating the existence of 2 distinct acetolactate-forming enzymes in the ripening pea preparation, one with a pH optimum between pH 8 and 8.5 with a K_m value of 3.2×10^{-3} M, the other with an optimum between pH 6 and 7 and a K_m value of 4.3×10^{-2} M. Attempts to separate the 2 enzymes by isoelectric precipitation, alcohol fractionation and adsorption on calcium phosphate gels were unsuccessful, leading to extensive denaturation of both components. Selective denaturation of the pH 8.5 enzyme by holding the preparation at pH 5.1 for 5 minutes at 0 to 5° prior to assay, a device used by Radhakrishnan and Snell (14) in their study of the corresponding enzymes from *E. coli,* also proved to be unsuccessful, resulting in the partial inactivation of both enzymes without resolution.

While the components of the acetolactate-synthesizing system in the fractionated ripening pea preparation could not be resolved, the data presented above indicates that the 2 enzymes can be studied independently in the same preparation since the pH 8.5 enzyme contributes little to the activity at pH 6, and the pH 6 enzyme is virtually inactive at pH 8.5. In the following experiments, the activity of the preparation at pH 6 and pH 8.5 is taken as being representative of the pH 6 and pH 8.5 enzymes, respectively.

Coenzyme and Metal Ion Requirements of the Acetolactate-forming Enzymes. In the absence of TPP the activity of both enzymes was greatly reduced, the pH 6 enzyme being slightly more sensitive to the omission than the pH 8.5 enzyme (table I).

Table I

Thiamine Pyrophosphate and Metal Ion Requirements of the Acetolactate-forming Enzymes

The basic reaction mixture contained sodium pyruvate (0.6 mmole), potassium phosphate (0.3 mmole) or Tris HCl (0.3 mmole) buffer, and ripening pea preparation (4.2 mg protein) in a total volume of 3 ml. Thiamine pyrophosphate (0.32 μmole), Mg^{++} (5 μmole) and Mn^{++} (0.5 μmole) were added as indicated.

Cofactors added	Acetolactate (μmole/mg protein hr)	
	pH 6	pH 8.5
None	0.06	0.18
Mg^{++}	0.1	0.26
TPP	0.19	0.39
TPP + Mg^{++}	0.91	1.04
TPP + Mn^{++}	1.17	0.94

Acetolactate formation by fractionated extracts at both pH values was considerably reduced in the absence of added metal ions (table I). Both Mg^{++} and Mn^{++} were effective in restoring activity, although the response to these ions differed at the 2 pH values. For the pH 6 enzyme Mn^{++} was the more effective activator. The reverse was true for the pH 8.5 enzyme which showed greatest activity in the presence of Mg^{++}.

Substrate Specificity and Products of Acetolactate-forming Enzymes. Under the standard assay conditions acetolactate was the major reaction product at both pH values, although some acetoin was also formed. Small amounts of acetaldehyde were also detected in reaction mixtures at pH 6. The ratio of acetoin to acetolactate was fairly constant under these conditions, especially at pH 8.5 where the amount of acetoin formed generally represented 5 to 10% of the total acetoin estimated after acidification. The ratio at pH 6 was rather more variable, but the amount of acetoin formed usually accounted for 10 to 20% of the total estimated.

The addition of acetaldehyde over a range of concentration from 0.01 to 0.05 M to reaction mixtures containing 0.02 M sodium pyruvate slightly increased acetoin formation relative to acetolactate at pH 6, but was without effect on the amount of acetolactate produced at either pH.

Acetaldehyde alone was not used as a substrate for acetoin synthesis by the ripening pea preparation at either pH.

Time Course of Acetolactate Formation and Effect of Enzyme Concentration. The rates of acetolactate production at both pH values were linear for at least the first 90 minutes of incubation. The rate of CO_2 evolution at pH 6 was linear for only the first 15 minutes after which it steadily declined, although total CO_2 production always exceeded that of acetolactate. This apparent lack of stoichiometry at pH 6 is to be expected in view of the observed formation of free acetaldehyde under these conditions.

For both enzymes, acetolactate formation was pro-

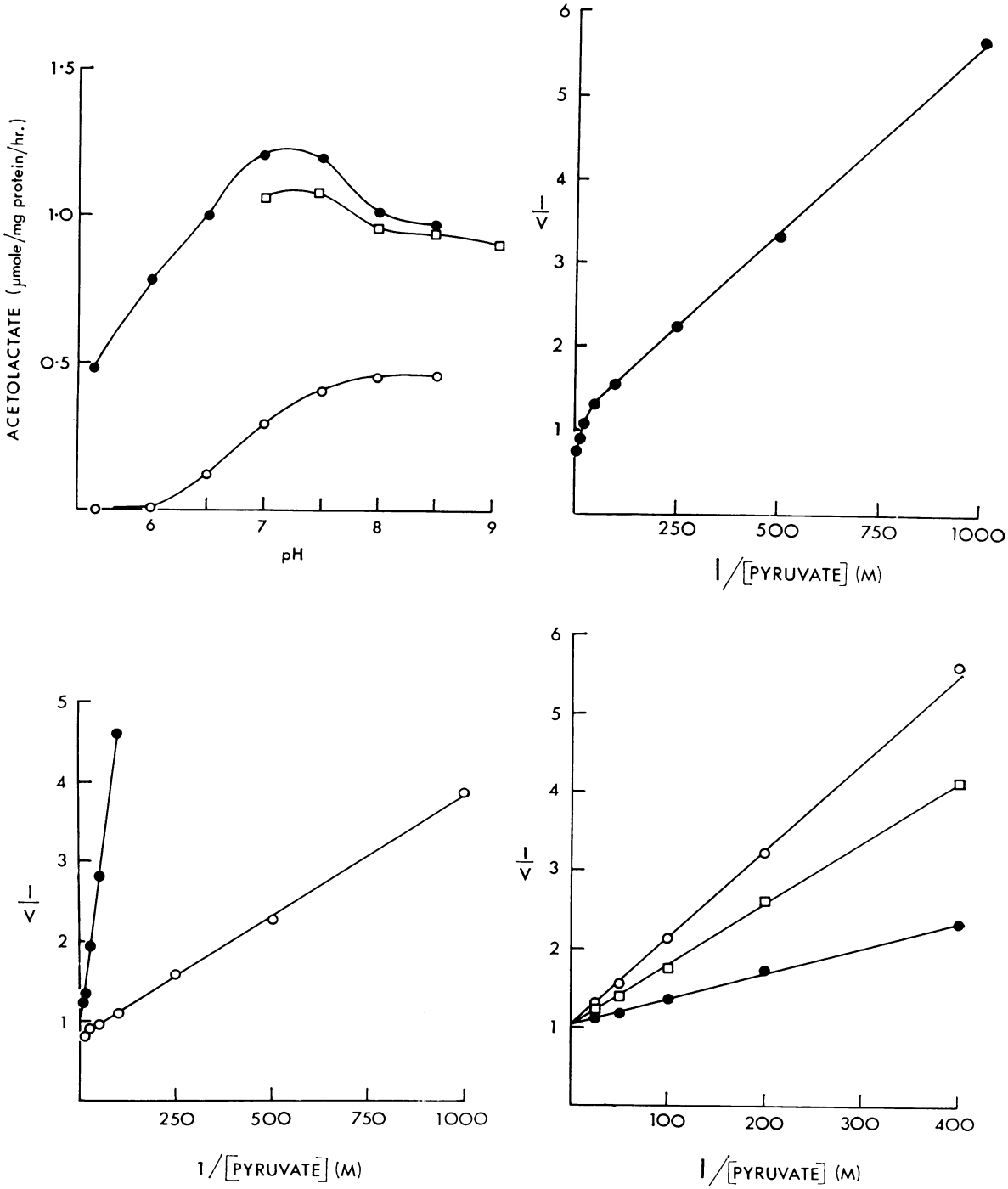

FIG. 1 (*upper left*). Effect of pH on acetolactate formation at 2 substrate concentrations. Standard assay conditions with pH and pyruvate concentration varied as shown. ● 0.2 M pyruvate (0.1 M potassium phosphate); □ 0.2 M pyruvate (0.1 M Tris-HCl); ○ 0.002 M pyruvate (0.1 M potassium phosphate).

FIG. 2 (*upper right*). Effect of pyruvate concentration on acetolactate synthesis by the ripening pea preparation. Standard assay conditions at pH 7.5 (0.1 M potassium phosphate) with pyruvate concentration varied.

FIG 3 (*lower left*). Effect of pyruvate concentration on acetolactate synthesis by the ripening pea enzyme at 2 pH values. Standard assay conditions with pyruvate concentration varied. ● pH 6 (0.1 M potassium phosphate); ○ pH 8.5 (0.1 M Tris-HCl).

FIG. 4 (*lower right*). Effect of L-valine on the activity of the pH 8.5 acetolactate-forming enzyme. Standard assay conditions with pyruvate concentration varied. ● no valine; □ 1×10^{-3} M L-valine: ○ 5×10^{-3} M L-valine.

portional to enzyme concentration over the range tested (0.6 – 2.4 mg protein per ml of assay mixture).

The Effect of L-*Valine and* L-*Isoleucine on Acetolactate Synthesis.* The activity of the pH 8.5 enzyme was inhibited by L-valine and this inhibition was competitive (fig 4). The K_i for inhibition by valine was estimated at 7×10^{-4} M. L-Isoleucine also acted as an inhibitor of the pH 8.5 enzyme but with a lower affinity for the active site, the K_i for isoleucine inhibition being 3.6×10^{-3} M. The pH 6 enzyme was, in contrast, little affected by the presence of L-valine or L-isoleucine at concentrations as high as 5×10^{-3} M.

Changes in Enzyme Content during Ripening of the Pea Seed. The relative amounts of the 2 acetolactate-forming enzymes changed considerably during the ripening period, the pH 8.5 enzyme showing relatively greater activity in the very young seed (table II, stage 1), and the pH 6 enzyme predominating at maturity (stage 3). The activity, per unit fresh weight of both enzymes increased greatly during early development, reaching a maximum prior to the onset of full maturity (stage 2). Maturation and dehydration were accompanied by a sharp and progressive decrease in the content of both acetolactate-forming enzymes which was paralleled by an increase in the amount of acetoin formed under the standard assay conditions at pH 6, culminating in the condition in the fully dehydrated seed (stage 5) where acetolactate formation was negligible although considerable amounts of acetoin were formed.

Table II

Changes in Enzyme Content during Ripening

Ten grams of each sample were ground in a chilled mortar with 30 ml of distilled water. The resulting slurry was strained through muslin, centrifuged for 15 minutes at 17,000 × *g* and the clear supernatant fluid used as enzyme without further purification. One ml of each preparation was used in 3 ml of the standard assay mixture.

Stage*	Avg fr wt g/seed	Acetolactate (μmole/g fr wt hr)		Acetoin (μmole/g fr wt hr)	
		pH 6	pH 8.5	pH 6	pH 8.5
1	0.28	1.66	4.2	0.6	0.45
2	0.78	8.1	10.5	1.95	0.6
3	1.05	5.3	4.6	2.2	0.5
4	0.98	2.2	1.2	2.8	0.33
5	1.1	0.15	0.0	3.6	0.15

* Seeds were harvested at various stages of maturation and graded as follows: Stage 1, immature small; Stage 2, immature intermediate; Stage 3, maximum fresh weight attained, seed still attached to funicle and pod fresh; Stage 4, seed detached from funicle and pod withered; Stage 5, seeds allowed to dry in air and then soaked in distilled water overnight at 0 to 5°.

Acetolactate decarboxylase could not be demonstrated in the seed preparations at any stage of development, and thus the possibility of acetoin production by decarboxylation of acetolactate can be disregarded. It seems likely therefore that the formation of acetoin from pyruvate during the final stages of development was due to the formation of a third enzyme with α-carboxylase activity.

The Effect of pH on Acetoin Synthesis. From figure 5 it will be seen that acetoin synthesis from pyruvate by dormant seed preparations was optimal at pH 6.5. A similar pH-activity curve was obtained when an equimolar mixture (0.05 M) of pyruvate and acetaldehyde was supplied as substrate.

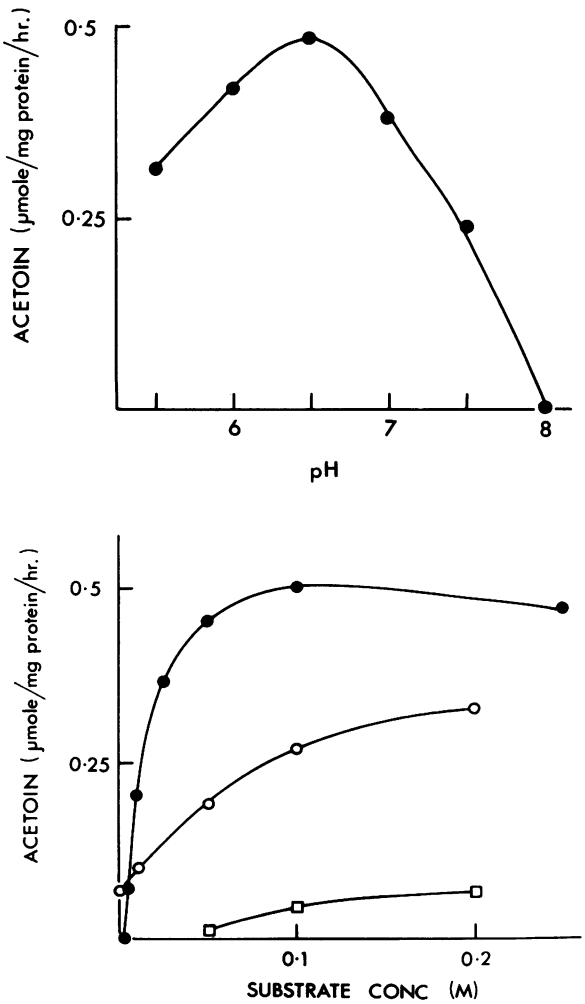

FIG. 5 (*upper*). Effect of pH on acetoin synthesis by the preparation from dormant pea seed. Standard assay conditions with pyruvate as substrate (0.2 M) and pH varied as shown.

FIG. 6 (*lower*). Effect of pyruvate and acetaldehyde on acetoin synthesis by the preparation from dormant pea seed. ● pyruvate only; ○ acetaldehyde in presence of 5×10^{-3} M pyruvate; □ acetaldehyde only.

Cofactor Requirements for Acetoin Synthesis. The acetoin-forming enzyme was poorly resolved with respect to TPP and metal ions, the omission of both cofactors from the standard assay medium resulting in only a 20% decrease in activity. Reaction mixtures deficient in either TPP or Mg^{++} still retained 90 to 95% of the activity observed in the complete assay medium.

Substrate Specificity and Products of Acetoin-forming Enzyme. With pyruvate alone as substrate, significant amounts of acetoin were formed only at relatively high concentrations (>0.01 M), while concentrations in excess of 0.1 M were slightly inhibitory and no acetoin was formed below 0.001 M (fig 6). Under the standard assay conditions (0.2 M sodium pyruvate), the rate of CO_2 and acetaldehyde production greatly exceeded that of acetoin, although the rates of formation of all 3 products were not linear with time. Following an initial rapid burst, the rate of CO_2 production declined slowly over the first 60 minutes of incubation while the rate of acetoin synthesis, initially quite low, gradually increased over the same time period. It would appear that an accumulation of free acetaldehyde is required before active synthesis of acetoin from pyruvate can take place. Acidification of the reaction mixtures following incubation generally resulted in an increase in the amount of acetoin estimated, but the amount of acetolactate thus indicated never exceeded 10% of the total acetoin. Gassing the standard assay mixture with nitrogen during incubation and the addition of aldehyde-complexing agents such as semicarbazide and dimedone to the system failed to increase acetolactate production.

The addition of acetaldehyde stimulated acetoin synthesis at all pyruvate concentrations tested, although the response was most marked at relatively low pyruvate concentrations (fig 6).

A small but significant amount of acetoin was produced when acetaldehyde was supplied as the sole substrate at high concentrations (fig 6).

Discussion

An interesting feature of the above results is the demonstration of at least 3 different enzymes capable of decarboxylating pyruvate in plant tissue extracts. Some indication of the reasons for the diversity of products observed in the above systems may be obtained from the results of recent work on the nature of the α-carboxylase reaction (2, 3, 8, 9). The decarboxylation of pyruvate by cell-free extracts of yeast involves the formation of first α-lactyl-2-thiamine pyrophosphate (activated pyruvate) from which α-hydroxyethyl-2-thiamine pyrophosphate (activated acetaldehyde) is derived by decarboxylation (4). This latter hydroxyethyl derivative is also produced by wheat germ preparations (2). Some of the reactions thought to be involved are shown in figure 7, which is a modification of the scheme suggested by Holzer and Beaucamp (4).

In yeast and wheat germ preparations the hydroxyethyl group readily dissociates, or is displaced by pyruvate from the coenzyme-apoenzyme complex to yield free acetaldehyde (reaction 3) which can then be accepted by a further activated acetaldehyde residue to form acetoin (reaction 5). Such a series of reactions would constitute the carboligase system of Neuberg (12). In wheat-germ preparations acetaldehyde alone serves as a substrate for acetoin synthesis, and this again has been shown to involve the preliminary formation of activated acetaldehyde (reaction 4). It should be noted, however, that the formation of free acetaldehyde is not a prerequisite for acetoin formation from pyruvate in extracts of *N. crassa* (14). In a number of important properties, including pH optimum and its ability to form acetoin from pyruvate and acetaldehyde, alone and in combination, the acetoin-forming enzyme of the dormant pea seed resembles the wheat-germ enzyme studied by Singer and Pensky (17) and Carlson and Brown (2). An important point of difference is the relative insensitivity of the pea seed enzyme to added TPP and metal ions. A TPP independent type of decarboxylation is thus indicated, but in view of the accumulated evidence on the nature of the carboxylase reaction, it seems more likely that in the pea seed preparation the coenzyme is firmly bound to the apoenzyme and is not released by the purification procedures used. A similar insensitivity to the same cofactors has been reported for the pH 6 acetolactate-synthesizing enzyme of *Aerobacter aerogenes* (3).

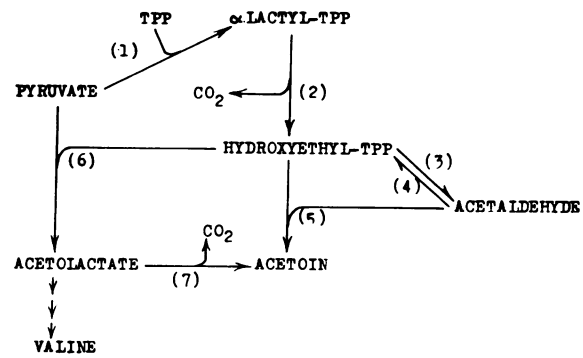

FIG. 7. Scheme showing possible relationships between acetoin and acetolactate synthesis [modified from Holzer and Beaucamp, (4)].

Where the hydroxyethyl group does not dissociate, or is not displaced from the coenzyme-apoenzyme complex, acetolactate formation can take place by transfer of the hydroxyethyl group to pyruvate (reaction 6). However, observations on the formation of acetoin in the ripening pea preparations raises the question as to whether the acetolactate-forming systems are capable of carboligase activity, one criterion of which would be stimulation of acyloin formation in the presence of free aldehydes. On this basis it would appear that the pH 8.5 enzyme of ripening peas is incapable of such direct acetoin synthesis, and that the acetoin which is formed at this pH arises by decarboxylation of acetolactate (reaction 7), presumably a nonenzymic process since an active acetolactate decarboxylase could not be demonstrated in the preparations. Acetoin production at pH 6 is, on the other hand, slightly stimulated by acetaldehyde and carboligase activity is thus indicated. In this respect the pH 6 enzyme resembles the corresponding enzyme from *N. crassa* (14) but unlike that enzyme free acetaldehyde did not act as a competitive inhibitor

of acetolactate formation. There is room for doubt therefore as to whether the bulk of the acetoin synthesized by the pea preparation at pH 6, especially in the presence of added acetaldehyde, was in fact due to the activity of the acetolactate-forming enzyme. It is possible that the relatively crude preparations contained traces of the acetoin-forming enzyme characteristic of the dormant seed, and that this was responsible for the carboligase activity observed.

Like *E. coli* (14, 21) and *A. aerogenes* (3) the ripening pea preparations contain 2 acetolactate-synthesizing enzymes. In the present work, since the 2 enzymes were not resolved in the purification technique, the evidence for their presence is based upon the large differences in their pH optima, K_m values and sensitivity to L-valine, and supported by the observation that the content of the 2 enzymes varied independently during the ripening process. While acetolactate synthesis has previously been demonstrated by Satyanarayana and Radhakrishnan (16) in partially purified preparations from Phaseolus, it is clear that their system differs considerably from that described above for ripening pea seeds. The enzyme described by the Indian workers had a requirement for high pyruvate concentrations, functioned optimally at pH 7 and, most significantly, produced large quantities of acetaldehyde and acetoin, acetolactate being a relatively minor product of the reaction, usually accounting for about 10% of the total acetoin. Clearly, the enzyme from seedlings of Phaseolus is very similar to the acetoin-forming enzyme of the dormant pea seed, and neither enzyme can be regarded as being primarily concerned with acetolactate biosynthesis.

The pH 8.5 acetolactate-forming enzyme from pea seeds is competitively inhibited by L-valine, the end product of the reaction sequence which it initiates. The phenomenon of end product inhibition has been reported for a number of biosynthetic pathways in microorganisms and animals (20). Apart from the present demonstration of a similar process in ripening pea seed preparations, the only other reference to its occurrence in plants is the report of Neumann and Jones (13) on the inhibition of aspartic transcarbamylase by cytidylic and uridylic acids in extracts of lettuce seedlings. End product inhibition in the case of valine biosynthesis is especially interesting since evidence indicates that the high-pH acetolactate-forming enzyme is also concerned in the biosynthesis of isoleucine, where α-ketobutyrate replaces pyruvate as the activated acetaldehyde acceptor for the formation of α-aceto-α-hydroxybutyrate (10). Thus the position of end product inhibition as a feedback control mechanism in response to valine overproduction is complicated by the fact that such a mechanism would also reduce the rate of isoleucine biosynthesis. Similarly, overproduction of isoleucine would have an adverse effect on the rate of valine biosynthesis. The presence of a second, low-pH enzyme would not overcome this difficulty since this enzyme, at least in *N. crassa* (14) is capable of both acetolactate and α-aceto-α-hydroxybutyrate synthesis. Furthermore, the corresponding enzymes in pea seeds and *A. aerogenes* (3) are not subject to end-product inhibition.

The relationship between the 3 carboxylase enzymes during development of the pea seed is of some interest. The similarity of the acetoin-forming enzyme and the pH 6 acetolactate-forming enzyme with respect to effective pH range and requirement for high pyruvate concentrations suggests that some of the changes taking place during maturation of the seed could be explained in terms of enzyme modification rather than de novo synthesis. It is possible that the acetoin-forming enzyme is derived from the pH 6 acetolactate-forming enzyme, and that one of the changes occurring during this transformation is the firmer binding of coenzyme to apoenzyme. The observed insensitivity to metal ions which accompanies this phenomenon may indicate involvement of metal ions in the linking mechanism.

Summary

Extracts of ripening pea seeds, fractionated with ammonium sulfate, catalyzed the formation of acetolactate from pyruvate. Small amounts of acetoin were also produced. Evidence was obtained for the existence of 2 distinct acetolactate-forming enzymes in the preparation. A high-pH enzyme showing maximum activity in the pH range 8 to 8.5 had a K_m value for pyruvate of 3.2×10^{-3} M. The other, low-pH enzyme, was estimated to function optimally between pH 6 and 7, and had a K_m value for pyruvate of 4.3×10^{-2} M. Both enzymes required thiamine pyrophosphate and either Mg^{++} or Mn^{++} for maximal activity. The pH 8.5 enzyme was competitively inhibited by L-valine and L-isoleucine.

The content of the acetolactate-forming enzymes changed considerably during the ripening period, reaching a maximum prior to the onset of full maturity. Both enzymes were virtually absent from the dormant seed, but this material did contain a third enzyme with carboxylase activity which was characterized by the ability to form acetoin and acetaldehyde when supplied with pyruvate at high concentrations. Acetoin synthesis from pyruvate by this enzyme was stimulated by the addition of acetaldehyde and some acetoin was also formed when acetaldehyde was the sole substrate. The acetoin-forming enzyme was only slightly stimulated by thiamine pyrophosphate and metal ions.

Acknowledgments

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