Metabolism of 1-aminoethylphosphinate generates acetylphosphinate, a potent inhibitor of pyruvate dehydrogenase

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The alanine analogue 1-aminoethylphosphinate [$\text{H}_3\text{C}-\text{CH}(\text{NH}_2)-\text{PO}_2\text{H}_2$] effectively inhibited anthocyanin synthesis in buckwheat hypocotyls and caused an increase in the concentrations of alanine and alanine-derived metabolites. Aminotransferase inhibitors partially alleviated the effects of the analogue. 1-Aminoethylphosphinate did not affect the growth of *Klebsiella pneumoniae* under anaerobic conditions, but under aerobic conditions it inhibited growth and caused the massive excretion of pyruvate. The analogue inhibited the pyruvate dehydrogenase complex *in vitro* in the presence of an aminotransferase activity. The transamination product of 1-aminoethylphosphinate, acetylphosphinate ($\text{H}_3\text{C}-\text{CO}-\text{PO}_2\text{H}_2$), was found to inhibit the pyruvate dehydrogenase complex in a time-dependent reaction that followed first-order and saturation kinetics and required the presence of thiamin pyrophosphate.

INTRODUCTION

In the majority of organophosphates found in Nature the phosphorus atom occurs at the +5 level of oxidation, i.e. as phosphoric acid, and is attached to the carbon-containing moiety of the molecule via either an O atom or, in a few cases, an N atom. Compounds having the C–P bond have, however, been isolated from biological materials [1]. Several synthetic and natural phosphonic analogues of amino acids, the aminoalkylphosphonic acids, have shown interesting biological activities [1,2]. Much less information is available on the corresponding aminoalkylphosphinic acids. Although the isolation of such compounds from *Streptomyces* species has been reported [3,4], Baylis *et al.* [5,6] initiated a programme for the systematic synthesis and biological evaluation of a series of 1-aminoalkylphosphonous acids as isosteres of the protein amino acids. (Although Baylis *et al.* [5,6] used the term ‘phosphonous acid’, the term ‘phosphinic acid’ is recommended by the IUPAC Nomenclature of Organic Chemistry. The IUPAC nomenclature is followed in the present paper.) Of these compounds, the alanine analogue (1-aminoethylphosphinate), in particular, was reported to exhibit antimicrobial as well as plant-growth-inhibiting activity [5,6]. Because of our continuing interest in the interference of organophosphonates with plant metabolism [7,8], we decided to investigate the mode of action of this compound in more detail. As a result of this investigation, we report here that its transamination product, acetylphosphinate, is a powerful inhibitor of the pyruvate dehydrogenase complex (PDC). Some of the results presented here were communicated in a poster at the 27th Harden Conference of the Biochemical Society on ‘The Biochemical Basis of Herbicide Action’ at Wye College, Kent, U.K., 14–19 September 1986.

EXPERIMENTAL

Materials

The stereoselective synthesis of 1-aminoethylphosphinate is described in ref. [6], and the synthesis of acetylphosphinate is described in ref. [9]. The phosphinates were placed at our disposal by CIBA-GEIGY AG, Basel, Switzerland. Biochemical reagents were obtained from Boehringer, Mannheim, Germany, and Sigma Chemie, Deisenhofen, Germany. Pyruvate decarboxylase from brewer's yeast, pyruvate dehydrogenase from bovine heart and lactate dehydrogenase [LDH-1 (H_4) isoenzyme] from bovine heart were supplied by Sigma Chemie, and alcohol dehydrogenase from yeast was obtained from Boehringer. Buckwheat (*Fagopyrum esculentum* Moench) seeds were obtained from Landwirtschaftliche Bezugs- und Absatzgenossenschaft, Bochum. Strain 1009/III of *Klebsiella pneumoniae* was supplied by Professor U. Winkler, Ruhr-University, Bochum, Germany.

Growth of organisms

Buckwheat seedlings were grown in the dark and hypocotyls were incubated as described in ref. [10]. *K. pneumoniae* was grown aerobically in 20 ml of a minimal medium [11] with 10 mM-glucose as carbon source at 30 °C on a rotary shaker at 100 rev./min. For growth under anaerobic conditions, 10 ml of minimal medium containing 50 mM-glucose and 5 mM-sodium thioglycolate were overlaid with 2 ml of liquid paraffin in test tubes. Growth was monitored by measuring A_{650} .

Extraction and assay of metabolites

Anthocyanins and soluble amino acids were extracted from buckwheat hypocotyls and assayed as described in

Abbreviations used: PDC, pyruvate dehydrogenase complex; TPP, thiamin pyrophosphate.

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Fig. 1. Inhibition of light-induced anthocyanin formation in excised buckwheat hypocotyls as a function of 1-aminoethylphosphinate enantiomer concentration

ref. [10]. Extraction and assay of pyruvate, lactate and ethanol followed the procedures given in refs. [12] and [13]. Supernatants of bacterial cultures were analysed by the same procedures.

Extraction and assay of enzymes

Pyruvate dehydrogenase. A mitochondrial fraction was prepared from hypocotyls of 6 day-old etiolated buckwheat seedlings according to the procedure given in ref. [14] and stored at -20°C . PDC was released from the mitochondria by treatment with 1% (w/v) Triton X-100 and assayed in the supernatant after centrifugation at 27000 g for 15 min. The incubation mixture for buckwheat PDC contained, in a volume of 1 ml, 100 μmol of potassium phosphate buffer, pH 7.6, 10 μmol of MgCl_2 , 2.5 μmol of NAD^+ , 0.8 μmol of TPP, 0.1 μmol of CoASH and 5 μmol of pyruvate. The reaction was started with the addition of pyruvate, and NADH formation was monitored spectrophotometrically at 340 nm at 30°C .

Bovine heart PDC was assayed as described in ref. [15], with, however, the omission of dithiothreitol.

Pyruvate decarboxylase and lactate dehydrogenase. The two enzymes were assayed according to the procedures given in refs. [16] and [13] respectively. Lactate oxidation was measured in medium containing 450 mM-glycine, 180 mM-hydrazine sulphate, 2.4 mM-EDTA and 1.5 mM- NAD^+ at pH 9.5, and pyruvate reduction in medium containing 300 mM-triethanolamine, 3 mM-EDTA and 0.2 mM-NADH at pH 7.6.

RESULTS

Interference of 1-aminoethylphosphinate with plant metabolism

Inhibition of light-induced anthocyanin synthesis in buckwheat hypocotyls had previously been instrumental in our laboratory in the elucidation of the mode of action of the phosphonate herbicide glyphosate as well as of the phenylalanine analogue (1-amino-2-phenylethyl)-

Table 1. Partial reversal of 1-aminoethylphosphinate-mediated inhibition of anthocyanin accumulation in buckwheat hypocotyls by aminotransferase inhibitors

Treatment	Anthocyanin (% of control)
0.3 mM-1-Aminoethylphosphinate	20
0.3 mM-1-Aminoethylphosphinate + 0.02 mM-amino-oxyacetic acid	60
0.3 mM-1-Aminoethylphosphinate + 0.3 mM-cycloserine	54
0.02 mM-Amino-oxyacetic acid	79
0.3 mM-L-Cycloserine	65

Fig. 2. Concentration of alanine in excised buckwheat hypocotyls incubated in 1 mM racemic 1-aminoethylphosphinate

phosphonic acid [7,8]. The synthesis of the flavonoid carbon skeleton proceeds via the condensation of a phenylpropanoid unit, namely the CoA ester of a hydroxycinnamic acid, with three acetate units derived from malonyl-CoA [17] (see the Discussion section), and therefore requires an adequate supply of high-energy substrates from two metabolic pathways. 1-Aminoethylphosphinate was found to be a potent inhibitor of light-induced anthocyanin synthesis in excised buckwheat hypocotyls, the (R)-enantiomer [having the configuration of (S)-alanine] being about 35-fold more potent than the (S)-enantiomer (Fig. 1). In contrast with our previous experience with other inhibitors of anthocyanin synthesis [7,18], it was not possible to alleviate the inhibition mediated by 1-aminoethylphosphinate by the addition of direct precursors of anthocyanin synthesis [shikimic acid, phenylalanine, (hydroxy)cinnamic acid(s), naringenin, dihydroquercetin] or any other metabolites (protein amino acids, glucose, citric acid-cycle intermediates, pyruvate, acetate). However, the aminotransferase inhibitors amino-oxyacetic acid and L-cycloserine, even though they are inhibitors of anthocyanin synthesis in their own right [18], significantly decreased the inhibitory activity of 1-aminoethylphosphinate (Table 1). This result suggested the involvement of aminotransferase(s)

Table 2. Effect of a 24 h incubation in 1 mM-1-aminoethylphosphinate on concentrations of metabolites in buckwheat hypocotyls

	Concentration ($\mu\text{mol/g}$ fresh wt.)		Increase (fold)
	Control	1-Aminoethylphosphinate	
Pyruvate	0.22	2.53	11.5
Lactate	0.21	2.12	10.1
Ethanol	1.43	2.15	1.5
Alanine	0.08	1.65	20.6
Valine	0.05	0.19	3.7
Leucine	0.04	0.11	2.8
Isoleucine	0.07	0.08	1.2

in the action of 1-aminoethylphosphinate. In agreement with evidence previously cited for pea seedlings [6], buckwheat hypocotyls treated with the inhibitor accumulated massive amounts of alanine (Fig. 2). In addition to alanine, the hypocotyls accumulated a number of other metabolites that have in common their metabolic origin from pyruvate, as well as pyruvate itself (Table 2). Similar results were obtained with mung-bean seedlings and leaves from tomato, wheat and pea plants, as well as with cultured cells from various plant species (results not shown).

Effect of 1-aminoethylphosphinate on *K. pneumoniae*

Because of the reported antibacterial activity of 1-aminoethylphosphinate [5,6], its action was also investigated in the facultatively anaerobic bacterium *K. pneumoniae*. Fig. 3 shows that, under aerobic growth conditions, 1-aminoethylphosphinate greatly delayed the

onset of exponential growth and decreased the growth rate, as well as the growth yield considerably. Concentrations lower than $1 \mu\text{M}$ did not affect growth, but the generation time was doubled in the presence of $20 \mu\text{M}$ -1-aminoethylphosphinate. Although during growth in the absence of the inhibitor a small and transient increase in the concentration of pyruvate in the growth medium was observed, pyruvate was excreted at a constant rate during the lag phase of growth in the presence of 1-aminoethylphosphinate, and its concentration drastically increased to approx. 10 mM during the exponential phase of growth. As the initial concentration of glucose in the growth medium was 10 mM , these data indicate that about 50% of the carbon utilized by the bacteria was excreted as pyruvate. No re-utilization of pyruvate by the bacteria during the stationary phase of growth was observed. The excretion of pyruvate during growth of *K. pneumoniae* in the presence of 1-aminoethylphosphinate bears a striking resemblance to the behaviour of a *Bacillus subtilis* mutant that lacks PDC activity [19,20]. Parallel to the increase of pyruvate in the growth medium, the concentration of alanine was increased about 80-fold from $1.4 \mu\text{M}$ (control) to $110 \mu\text{M}$ (1-aminoethylphosphinate; stationary phase), while valine concentrations were increased from $0.2 \mu\text{M}$ to $7.9 \mu\text{M}$. No accumulation of lactate, ethanol, leucine and isoleucine was observed. Neither lactate, pyruvate nor citric acid-cycle intermediates, when added to the growth medium in the presence of 1-aminoethylphosphinate, were able to alleviate the inhibition of growth by the compound. Of the protein amino acids, only alanine (both enantiomers) and glycine fully antagonized the effect of 1-aminoethylphosphinate. We assume that these amino acids, the uptake of which is known to be mediated by the same transport system [21], interfered with the uptake of the inhibitor. When the bacteria were grown under anaerobic conditions, 1-aminoethylphosphinate, even at 1 mM

Fig. 3. Effect of 1-aminoethylphosphinate on aerobic growth and pyruvate excretion of *K. pneumoniae*

Bacteria were grown in the absence (○ and △) and in the presence (● and ▲) of 0.3 mM racemic 1-aminoethylphosphinate in a minimum salt medium with glucose as carbon source. Growth (○ and ●) and pyruvate concentrations in the medium (△ and ▲) were monitored.

Fig. 4. Effect of 1-aminoethylphosphinate on anaerobic growth of *K. pneumoniae*

Bacteria were grown in the absence (○) and in the presence (●) of 1 mM racemic 1-aminoethylphosphinate.

concentration, did not inhibit growth at all (Fig. 4), indicating that the compound interfered with metabolic reaction(s) specifically required for aerobic growth. A direct effect of the compound on protein synthesis is therefore unlikely. The ineffectiveness of 1-aminoethylphosphinate under anaerobic conditions, as well as the observation that bacteria grown aerobically in its presence behave similarly to bacteria lacking PDC, strongly suggested that the activity of this enzyme is affected by 1-aminoethylphosphinate or one of its metabolites. The decreased inhibition by 1-aminoethylphosphinate of anthocyanin synthesis in buckwheat hypocotyls in the presence of aminotransferase inhibitors (Table 1) pointed to its transamination product, acetylphosphinate, as a likely candidate for the actual PDC inhibitor. In consequence, the effect of 1-aminoethylphosphinate, in the absence and in the presence of an appropriate aminotransferase, as well as of acetylphosphinate, on pyruvate dehydrogenase *in vitro* was tested.

Effect of 1-aminoethylphosphinate and acetylphosphinate on PDC activity

PDC is a multienzyme complex consisting of multiple copies of the three enzymes pyruvate dehydrogenase (EC 1.2.4.1), dihydrolipoamide acetyltransferase (EC 2.3.1.12) and dihydrolipoamide dehydrogenase (EC 1.8.1.4). It catalyses the following overall reaction [22]:

Fig. 5. Effect of amino-oxyacetic acid on the inactivation of buckwheat PDC by 1-aminoethylphosphinate

Buckwheat hypocotyl extract (58 pkat of PDC activity, 1.26 mg of protein) was incubated with 0.3 mM racemic 1-aminoethylphosphinate in the standard reaction mixture without pyruvate (see the Materials and methods section) in the absence (○) and in the presence (●) of 0.3 mM-amino-oxyacetic acid. At the times indicated, pyruvate was added and the remaining PDC activity was determined.

The initial reaction is the TPP-dependent decarboxylation of pyruvate, giving rise to hydroxyethyl-TPP and CO_2 . Although a wealth of information is available on the structure and reactions catalysed by PDC from bacteria and animals [22,23], knowledge of higher plant PDC, which occurs in two (mitochondrial and plastid) forms, is much less detailed [24–26].

In initial experiments, the effect of 1-aminoethylphosphinate on PDC activity in a crude mitochondrial fraction prepared from etiolated buckwheat hypocotyls was assessed. The compound inhibited the overall reaction catalysed by PDC in a time-dependent manner, and the inhibition was suppressed by the simultaneous presence of amino-oxyacetic acid (Fig. 5). At 0.3 mM concentration, the (*R*)-enantiomer produced 50 % inhibition within 3.5 min, whereas the (*S*)-enantiomer required 8.4 min. The inhibition required the presence of TPP during a preincubation and was effectively blocked by pyruvate (results not shown). Although an apparent K_i of 0.4 mM was determined for 1-aminoethylphosphinate by analysis of inhibition kinetics [27], this value cannot be considered meaningful, because the actual concentration of the presumed inhibitory agent, acetylphosphinate, could not be computed. An 1-aminoethylphosphinate-dependent amination of 2-oxo[^{14}C]glutarate by the crude extract was demonstrable under the incubation conditions of the PDC assay (results not shown), and thus provided evidence for our conclusion that acetylphosphinate was formed during the incubation.

Table 3. Stereospecific conversion of 1-aminoethylphosphinate into a PDC inhibitor by alanine aminotransferase

Bovine heart PDC (113 pkat) was incubated with 1 mM-1-aminoethylphosphinate in the standard reaction mixture without pyruvate (see the Materials and methods section) containing, in addition, 1 mM-2-oxoglutarate as amino group acceptor and 40 nkat of alanine aminotransferase as indicated. After 25 min, pyruvate was added and the remaining PDC activity was determined.

Incubation mixture	Activity remaining (% of control)
Alanine aminotransferase absent	
(<i>R</i>)-1-Aminoethylphosphinate	100
(<i>S</i>)-1-Aminoethylphosphinate	109
Alanine aminotransferase present	
(<i>R</i>)-1-Aminoethylphosphinate	11
(<i>S</i>)-1-Aminoethylphosphinate	95

Fig. 6. Effect of pyruvate and TPP on the inactivation of bovine heart PDC by acetylphosphinate

Bovine heart PDC (113 pkat) was incubated in 50 mM-potassium phosphate buffer, pH 8.0, containing 1 mM-MgCl₂, 0.13 mM-CoASH, 0.1 μM-acetylphosphinate and further additions given below for the indicated periods and then assayed for the enzymic activity after addition of the missing substrate or cofactors. ○, 2.5 mM-NAD⁺ and 0.2 mM-TPP present during preincubation; reaction started with 2 mM-pyruvate. △, 2.5 mM-NAD⁺ present during preincubation; reaction started with 0.2 mM-TPP and 2 mM-pyruvate. □, 0.2 mM-TPP and 2 mM-pyruvate present during preincubation; reaction started with 2.5 mM-NAD⁺.

Because of the low activity and lability of PDC in the mitochondrial fraction from buckwheat hypocotyls, subsequent analyses were carried out with the commercially available PDC from bovine heart. As was expected, 1-aminoethylphosphinate did not affect the

Fig. 7. Inactivation of bovine heart PDC as a function of acetylphosphinate concentration after 5 min incubation

Conditions were as described in Fig. 6 legend (reaction started with pyruvate).

activity of purified PDC unless alanine aminotransferase was present in the incubation mixture (Table 3). The PDC inhibitor was generated by the aminotransferase only from the (*R*)-enantiomer. Chemically synthesized acetylphosphinate, indeed, inhibited PDC at low concentrations in a time-dependent manner, and the loss of activity followed first-order and saturation kinetics (Figs. 6 and 7). The inhibition was apparently irreversible, as judged by dilution experiments [28] (results not shown), and required the presence of TPP and Mg²⁺, whereas it was prevented by pyruvate (Fig. 6). When dialysed overnight against 50 mM-potassium phosphate buffer, pH 7.4, containing 0.2 mM-EDTA, 1 mM-MgCl₂ and 0.32 mM-dithiothreitol, the uninhibited enzyme lost 20 % of its activity, whereas the inhibited enzyme, which had lost 98 % of its activity during the preincubation with TPP and acetylphosphinate, regained 56 % of its activity (corrected for the loss of activity of the control). Because of the apparent slow dissociation of the enzyme-inhibitor complex it is permissible to apply the equation for enzyme-catalysed irreversible inactivation to the analysis of the time-dependent loss of PDC activity in the presence of acetylphosphinate and TPP:

where E = free enzyme, I = free inhibitor, E·I = enzyme inhibitor complex and E-I = inactivated enzyme. Time-dependent inactivation of the enzyme as function of the inhibitor concentration follows the equation [27]:

$$t_{\frac{1}{2}} = \frac{\ln 2}{K_{\text{inact.}}} \left(1 + \frac{K_i}{[I]} \right)$$

Fig. 8. Determination of kinetic constants for the inactivation of PDC by acetylphosphinate

Conditions were as described in Fig. 6 legend (reaction started with pyruvate).

Fig. 9. Kinetic analysis of pyruvate decarboxylase inhibition by acetylphosphinate (Lineweaver-Burk plot)

where $t_{1/2}$ = time required for 50% inactivation and K_i = inhibitor constant = dissociation constant of E·I. A secondary plot of $t_{1/2}$ for PDC inactivation versus $1/[\text{acetylphosphinate}]$ (Fig. 8) gave an apparent rate constant for inactivation ($K_{\text{inact.}}$) of $0.55 \pm 0.14 \text{ min}^{-1}$ and a K_i value of $0.33 \pm 0.08 \mu\text{M}$. Thus acetylphosphinate is obviously a very strong inhibitor of mammalian PDC.

Effect of acetylphosphinate on pyruvate decarboxylase

The pyruvate dehydrogenase activity of PDC and pyruvate decarboxylase both catalyse the TPP-dependent decarboxylation of pyruvate and the intermediate formation of hydroxyethyl-TPP [22,29]. It was therefore of interest to study the effect of acetylphosphinate on pyruvate decarboxylase. The inhibition of the yeast enzyme was fully reversible and linearly competitive towards pyruvate (Fig. 9). An apparent K_i value of 2.7 mM was determined from a Dixon plot. This K_i value has the same order of magnitude as the apparent K_m value of the enzyme for pyruvate (4 mM), and acetyl-

phosphinate must therefore be considered a relatively weak inhibitor of pyruvate decarboxylase.

Generation of acetylphosphinate from 1-hydroxyethylphosphinate

The claim [30] that 1-hydroxyethyl- and 1-hydroxy-aminoethyl-phosphinic acids have fungicidal properties led us to speculate that oxidation of these compounds to acetylphosphinate *in vivo* and subsequent inhibition of PDC might be involved in their mode of action. Lactate dehydrogenase would be expected to be one of the enzymes capable of catalysing the oxidation. As shown in Table 4, bovine heart lactate dehydrogenase indeed catalysed the dehydrogenation of 1-hydroxyethylphosphinate as well as the hydrogenation of acetylphosphinate, even though its affinity for these substrates was substantially lower than for lactate and pyruvate respectively.

DISCUSSION

A screening programme in our laboratory for inhibitors of the biosynthesis of shikimate-derived aromatic compounds in plants utilizes the light-induced synthesis of anthocyanin pigments in buckwheat hypocotyls as a rapid and simple test system [7]. Of the two aromatic carbon rings of the anthocyanin structure, one is derived from shikimate via phenylalanine and cinnamate, and the other is formed via the acetate-malonate pathway (Scheme 1). 1-Aminoethylphosphinate, included in the routine screening programme, attracted our attention because it inhibited anthocyanin synthesis very effectively (Fig. 1). As the inhibition could not be reversed by phenylpropanoid intermediates, it seemed unlikely that the inhibitor interfered directly with the shikimate pathway. Accumulation of pyruvate and pyruvate-derived metabolites in tissues treated with 1-aminoethylphosphinate (Table 2 and Fig. 2), as well as the massive excretion of pyruvate by bacteria grown in the presence of the compound (Fig. 3), suggested that 1-aminoethylphosphinate interfered with the oxidative decarboxylation of pyruvate by PDC. Further evidence for the involvement of PDC inhibition in the action of 1-aminoethylphosphinate came from the observation that the compound did not inhibit the anaerobic growth of *K. pneumoniae* (Fig. 4). PDC, from both a plant and an animal source, was subsequently found to be subject to inhibition by 1-aminoethylphosphinate provided that an aminotransferase activity was present in the incubation mixture (Fig. 5 and Table 3). The involvement of a transamination reaction in the inhibition by 1-aminoethylphosphinate of anthocyanin synthesis in buckwheat hypocotyls had previously been indicated by the antagonizing effect of aminotransferase inhibitors (Table 1). Acetylphosphinate, but not 1-aminoethylphosphinate, was in fact found to be a very potent inhibitor of purified bovine heart PDC, with a K_i value of $0.33 \mu\text{M}$ (Fig. 8). Likewise, highly purified PDC from *Escherichia coli* is strongly inhibited by acetylphosphinate (B. Laber & N. Amrhein, unpublished work). The loss of activity of PDC in the presence of acetylphosphinate follows first-order and saturation kinetics (Figs. 6 and 7) and is irreversible, on the basis of the results of dilution experiments. However, more than 50% of the lost activity can be regained by extensive dialysis, indicating an essential reversibility of the inactivation. The requirement for both TPP and

Scheme 1. Scheme of the metabolic pathways investigated in the elucidation of the mode of action of 1-aminoethylphosphinate
Metabolites surrounded by a broken line accumulate in the presence of the inhibitor.

Table 4. Kinetic parameters of the reaction of lactate dehydrogenase with its substrates and phosphorus analogues

Direction of reaction	Substrate	K_m (mM)	Relative V
$H_3CCH(OH)R^* \rightarrow H_3CC(O)R$	$R = -CO_2H$	2	1
	$R = -PO_2H_2$	75	0.012
$H_3CC(O)R \rightarrow H_3CCH(OH)R$	$R = -CO_2H$	0.17	1
	$R = -PO_2H_2$	67	0.46

* Racemic compounds were used.

Mg^{2+} for the inactivation of PDC by acetylphosphinate, as well as the protective effect of pyruvate, show the involvement of the enzyme's active site in the inactivation process. It is known that Mg^{2+} is required for the binding of TPP to PDC [22].

Even though the fate of acetylphosphinate during the reaction with PDC has not been demonstrated, for lack of the radiolabelled compound, the experimental criteria available so far support the hypothesis that acetylphosphinate is an enzyme-activated inhibitor of PDC. In the reaction catalysed by PDC, pyruvate is initially converted into an enzyme-bound covalent adduct of TPP, lactyl-TPP, which, after decarboxylation, can be isolated as hydroxyethyl-TPP [22]. Dephosphorylation of acetylphosphinate during its interaction with PDC and TPP can be excluded, because the dephosphorylation product would be the same as the product of pyruvate decarboxylation. We therefore assume that the activated intermediate has a structure analogous to lactyl-TPP. It was previously reported that acetylphosphonate inhibits the *E. coli* PDC with a K_i of approx. $4 \mu M$ (cited in [31]), and that its monoanionic methyl ester is an 80-fold

stronger inhibitor ($K_i = 0.05 \mu M$ [32]). The structure of acetylphosphinate corresponds to that of methyl acetylphosphonate in that it is also monoanionic ($-OCH_3$ replaced by $-H$). The enzymic formation of a phosphonate analogue of lactyl-TPP that forms a tight complex with PDC was indeed suggested as the mode of action of methyl acetylphosphonate [32]. Acetylphosphinate and methyl acetylphosphonate exhibit further similarities in being a poor (acetylphosphinate) or not an inhibitor of yeast pyruvate decarboxylase (Fig. 9; [33]). Even though acetylphosphinate can be generated *in vitro* from 1-hydroxyethylphosphinate (Table 4), this compound produced little, if any, inhibition of light-induced anthocyanin synthesis in buckwheat hypocotyls, even at 1 mM concentration. Likewise, acetylphosphinate was a very ineffective inhibitor *in vivo* when compared with 1-aminoethylphosphinate (B. Laber & N. Amrhein, unpublished work). The reason for the lack of activity of acetylphosphinate and 1-hydroxyethylphosphinate *in vivo* in the buckwheat system is unknown, but may be related to low rates of uptake and transport of the two compounds, as well as conditions unfavourable for the oxidation of 1-hydroxyethylphosphinate to acetylphosphinate in the cells. Application of 1-aminoethylphosphinate is therefore at present the only suitable way to generate acetylphosphinate capable of interacting with PDC.

Note added in proof (received 25 September 1987)

At the time this work was in the press we were informed by Dr. A. C. Baillie (Schering Agrochemicals Ltd., Saffron Walden, Essex, U.K.) that, while engaged in the design of pyruvate dehydrogenase inhibitors as herbicides, he and his colleagues prepared acetylphosphinate and found it to be a potent inhibitor of pyruvate dehydrogenase from pea mitochondria [34].

We are grateful to Dr. Baillie for making these results available to us.

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