(1985) *Nucleic Acids Res. 13*, 5027–5039.
Le Peuch, J. C., Ballester, R., & Rosen, O. M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6858–6862.
Martin-Perez, J., & Thomas, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 926–930.
Nick, H. P., Wettenhall, R. E. H., Hearn, M. T. W., & Morgan, F. J. (1985) *Anal. Biochem. 148*, 93–100.
Novak-Hofer, I., & Thomas, G. (1984) *J. Biol. Chem. 259*, 5995–6000.
Otaka, E., Higo, K., & Itoh, T. (1983) *Mol. Gen. Genet. 191*, 519–524.
Padel, U., & Soling, H.-D. (1985) *Eur. J. Biochem. 151*, 1–10.
Parker, P. J., Katan, M., Waterfield, M. D., & Leader, D. P. (1985) *Eur. J. Biochem. 148*, 579–586.
Perisic, O., & Traugh, J. A. (1983) *J. Biol. Chem. 258*, 9589–9592.
Sedmak, J. J., & Grossberg, S. E. (1977) *Anal. Biochem. 79*, 544–552.
Tabarini, D., Heinrich, J., & Rosen, O. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4369–4373.
Thomas, G., Martin-Perez, J., Siegmann, M., & Otto, A. M. (1982) *Cell (Cambridge, Mass.) 30*, 235–242.
Traugh, J. A. (1981) *Biochem. Actions Horm. 8*, 167–208.
Traugh, J. A., & Pendergast, A. M. (1986) *Prog. Nucleic Acid Res. Mol. Biol. 33*, 195–228.
Wettenhall, R. E. H., & Wool, I. G. (1972) *J. Biol. Chem. 247*, 7201–7206.
Wettenhall, R. E. H., & Howlett, G. J. (1979) *J. Biol. Chem. 254*, 9317–9323.
Wettenhall, R. E. H., & Cohen, P. (1982) *FEBS Lett. 140*, 263–269.
Wettenhall, R. E. H., & Morgan, F. J. (1984) *J. Biol. Chem. 259*, 2084–2091.
Wettenhall, R. E. H., & Quinn, M. (1984) *J. Chromatogr. 336*, 51–61.
Wettenhall, R. E. H., Cohen, P., Caudwell, B., & Holland, R. (1982) *FEBS Lett. 148*, 207–213.
Wettenhall, R. E. H., Chesterman, C. N., Walker, T., & Morgan, F. J. (1983) *FEBS Lett. 162*, 171–176.
Wittmann-Liebold, B., Geissler, A. W., Lin, A., & Wool, I. G. (1979) *J. Supramol. Struct. 12*, 425–433.
Wool, I. G. (1979) *Annu. Rev. Biochem. 48*, 719–754.
Zimmerman, C. L., Appella, E., & Pisano, J. J. (1977) *Anal. Biochem. 77*, 569–573.

Identification of 2-Enolbutyrate as the Product of the Reaction of Maize Leaf Phosphoenolpyruvate Carboxylase with (*Z*)- and (*E*)-2-Phosphoenolbutyrate: Evidence from NMR and Kinetic Measurements†

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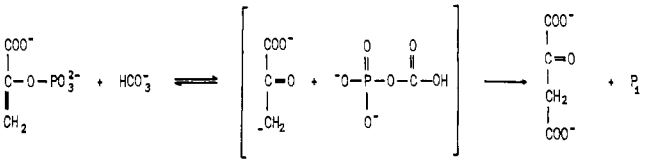
ABSTRACT: (*Z*)- and (*E*)-2-phosphoenolbutyrates were dephosphorylated at similar rates by phosphoenolpyruvate carboxylase purified from maize leaves, as determined from proton nuclear magnetic resonance measurements. The product of the reaction in D_2O was a mixture of 60–70% 2-oxo[3-H,D]butyrate, 25–30% 2-oxo[3-D_2]butyrate, and 5–10% 2-oxo[3-H_2]butyrate. The amounts of (*R*)- and (*S*)-2-oxo[3-H,D]butyrate in this mixture were determined by exchange at C-3 in D_2O catalyzed by pyruvate kinase as described by Hoving et al. [Hoving, H., Nowak, T., & Robillard, G. T. (1983) *Biochemistry 22*, 2832–2838]. Forty-five minutes after the addition of pyruvate kinase, the proportions of 2-oxo[3-H,D]butyrate and 2-oxo[3-D_2]-butyrate were 36–39% and 61–64%, respectively, indicating that the original mixture contained equal amounts of *R* and *S* enantiomers. In addition, a compound with properties similar to those of enolpyruvate was detected in solution during the action of phosphoenolpyruvate carboxylase on 2-phosphoenolbutyrate. This compound, most likely 2-enolbutyrate, presented maximum light absorption at 220–230 nm and was ketonized in a solution containing 80% D_2O and 20% H_2O (pH 7) with a rate constant of 1.33 min^{-1}. From these results, it is concluded that the actual product released from the active site of phosphoenolpyruvate carboxylase during the reaction with 2-phosphoenolbutyrate is the enolic form of 2-oxobutyrate and that protonation of this form takes place at random in solution.

Phosphoenolpyruvate (PEP)[1] carboxylase [orthophosphate: oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] catalyzes the irreversible β-carboxylation of PEP to yield oxaloacetate [for reviews, see O'Leary (1982) and Andreo et al. (1987)]. This enzyme is especially important in the so-called C_4 plants, where it participates in photosynthetic CO_2 fixation (Edwards et al., 1985), but is also present in all other types of plants, algae, and bacteria, with different functions and properties depending on the source (Utter & Kolen-

Scheme I: Two-Step Mechanism Postulated for PEP Carboxylase (O'Leary et al., 1981)



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[1] Abbreviations: PEP, phosphoenolpyruvate; PEB, 2-phosphoenolbutyrate; PEIV, 2-phosphoenolisovalerate; LDH, lactic dehydrogenase.

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brander, 1972; Latzko & Kelly, 1983).

The only known cofactor required by the enzyme is Mg^{2+}, which can be replaced by other divalent cations (O'Leary et al., 1981). Bicarbonate is the active substrate for the carboxylation reaction (Cooper & Wood, 1971), which occurs from the *si* face of PEP (Rose et al., 1969). The mechanism of the reaction was thought to follow a concerted model for many years (Maruyama et al., 1966). Recently, this assumption was challenged, and a two-step mechanism was proposed by O'Leary et al. (1981) on the basis of isotope effect studies (Scheme I). Since then, additional evidence has been obtained favoring the stepwise mechanism (Hansen & Knowles, 1982; Fujita et al., 1984). The first step of the proposed reaction is the formation of carboxy phosphate and the enolate of pyruvate from the substrates; the second step is the carboxylation of the enolate to yield oxaloacetate and phosphate (O'Leary et al., 1981). No direct detection of any of the two intermediates has been reported, although methods are available to do this (Powers & Meister, 1976, 1978; Kuo et al., 1979; Wimmer et al., 1979), probably because the intermediates are consumed by the second step with no additional requirement, so that both steps cannot be separated.

PEP carboxylase also catalyzes a bicarbonate-dependent dephosphorylation of two PEP analogues, PEB and PEIV, presumably following a similar mechanism, but without carboxylation of the enolate from carboxy phosphate (Fujita et al., 1984; González & Andreo, 1986). Hydrolysis of carbamyl phosphate by the plant carboxylase, probably involving the formation of carboxy phosphate, has also been reported (González et al., 1987).

The present work was carried out to determine the stereospecificity of the PEP carboxylase catalyzed dephosphorylation of PEB, with the intention to get new information on the reaction mechanism. We have found that both diastereoisomers of PEB are consumed at similar rates by the carboxylase and that the reaction product is the enolic form of 2-oxobutyrate, indicating that the intermediate is neither carboxylated nor protonated by the enzyme action.

Materials and Methods

Enzyme Purification. PEP carboxylase was extracted and purified from *Zea mays* L. leaves as described by Iglesias et al. (1986). The purified enzyme showed a single protein band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and did not hydrolyze β-glycerophosphate, indicating that it was free from unspecific phosphatase activity. Specific activities with PEP and PEB as substrates were 12–15 and 0.6–0.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively, in the absence of thiol compounds [see Iglesias and Andreo (1984)].

NMR Measurements. Proton NMR determinations were carried out at 80.13 MHz on a Bruker WP80sy spectrometer in the FT mode with a presaturation sequence to eliminate the H_2O peak. Values in ppm were determined with a value of 4.6 for the resonance frequency of HDO set as reference. For these measurements, the carboxylase, stored as an ammonium sulfate precipitate, was dissolved in 200 μL of 50 mM potassium phosphate (pH 7.5) containing 20 mM $MgCl_2$ and desalted by filtration/centrifugation through Sephadex G-50 previously equilibrated with the same buffer (Penefsky, 1977). An aliquot of the filtrate, containing 250 μg of protein, was brought to 250 μL with buffer and lyophilized. The resulting powder was dissolved in 250 μL of D_2O and lyophilized again. The enzyme obtained by this procedure was dissolved in 500 μL of 100 mM PEB (potassium salt), 10 mM $NaHCO_3$, and 25 mM potassium phosphate (pD 7.5) in D_2O to start the reaction. Control experiments indicated that the enzyme retained full activity after this procedure.

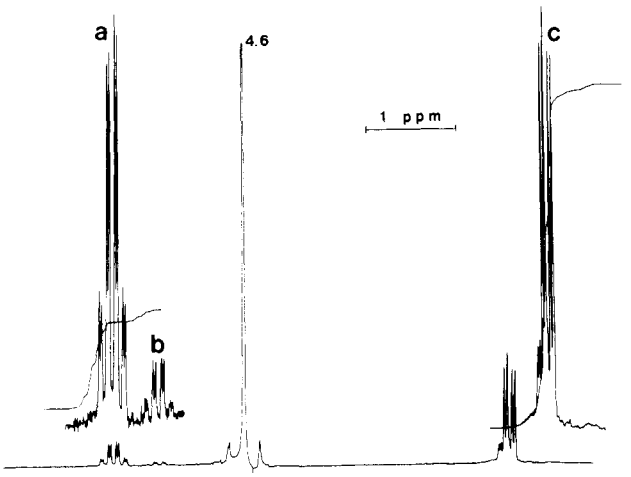


FIGURE 1: Proton NMR spectrum of the mixture of the potassium salts of (*E*)- and (*Z*)-PEB used in this study. The signals at around 6 ppm correspond to the vinyl proton of (*Z*)-PEB (a) and (*E*)-PEB (b). The signals at around 2 ppm are those of the methyl protons of the mixture (c). The peak at 4.6 ppm is HDO. The mixture was in 25 mM potassium phosphate (pD 7.5).

Spectrophotometric Measurements. Ultraviolet and visible light absorption measurements were performed in a Hitachi 150-20 spectrophotometer.

Protein Determination. Protein concentration was determined by the Coomassie Brilliant Blue dye binding method (Sedmak & Grossberg, 1977) with bovine serum albumin as standard.

Materials. 2-Oxobutyrate, rabbit muscle pyruvate kinase, and LDH were purchased from Sigma Chemical Co. (St. Louis, MO). Units of LDH are given in micromoles of NADH consumed per minute in a reaction mixture containing 2 mM 2-oxobutyrate and 0.16 mM NADH under the conditions employed in each assay. PEB (*EZ* mixture, monocyclohexylammonium salt) was a generous gift of Dr. A. E. Woods, Middle Tennessee State University. The mixture was converted to the potassium salt by passage through Dowex 50-W, hydrogen form, followed by neutralization with KOH (final pH 7.5). For NMR measurements a sample was lyophilized and dissolved in D_2O containing 25 mM potassium phosphate (pD 7.5). The values given as pD are meter readings; no isotope effect corrections were made. All other reagents were of analytical grade.

Results

Dephosphorylation of (E)- and (Z)-PEB by PEP Carboxylase. The proton NMR spectrum of the mixture of (*E*)- and (*Z*)-PEB that was used throughout this study is shown in Figure 1. The C-3 proton signals allow the calculation of the relative amount of each of the two isomers (Stubbe & Kenyon, 1971; Duffy et al., 1982; Hoving et al., 1983). On the basis of integrations of these signals, it was determined that the mixture was composed of 86% (*Z*)-PEB and 14% (*E*)-PEB.

When PEP carboxylase was added to the mixture in D_2O, together with $NaHCO_3$ and $MgCl_2$, the formation of products, 2-oxo[3-H,D]butyrate and 2-oxo[3-H_2]butyrate, was observed (Figure 2). This result confirms previous reports that indicate that PEB is not carboxylated, but dephosphorylated, by the action of PEP carboxylase (Fujita et al., 1984; González & Andreo, 1986). From spectra recorded every 15 min and the corresponding integrations, it was determined that the relative proportion of (*E*)- and (*Z*)-PEB remained almost constant during the dephosphorylation of PEB catalyzed by PEP

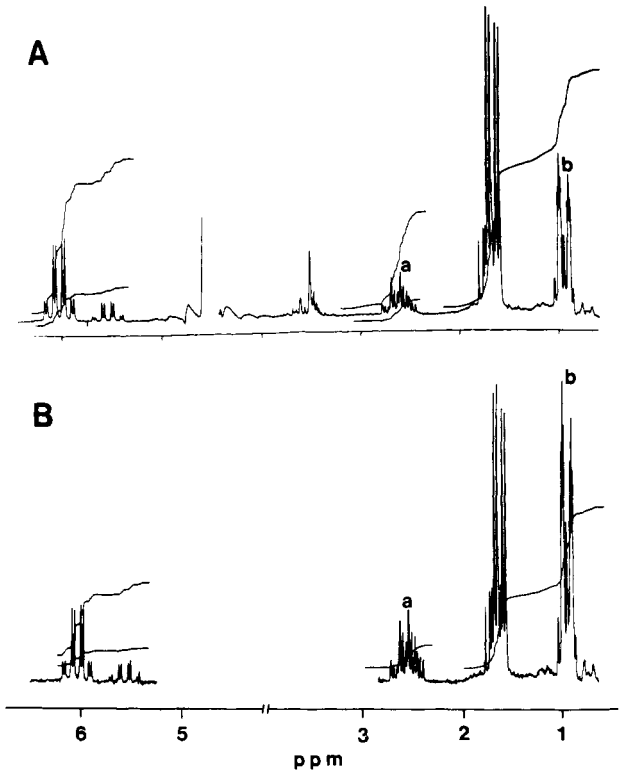


FIGURE 2: Proton NMR spectra of the mixture of (*E*)- and (*Z*)-PEB during PEP carboxylase action. Reaction conditions were 100 mM PEB, 10 mM $NaHCO_3$, 10 mM $MgCl_2$ and 250 μg of enzyme in 50 mM potassium phosphate (pD 7.5), incubated at 25 °C for (A) 2 and (B) 3 h. The signals indicated as a and b arise from the C-3 protons and methyl protons of 2-oxobutyrate, respectively.

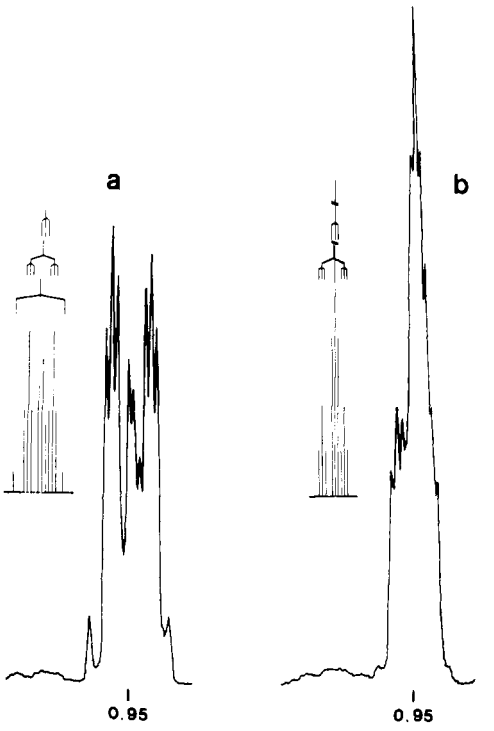


FIGURE 3: Methyl proton NMR signals during the pyruvate kinase catalyzed enolization of 2-oxobutyrate obtained after the action of PEP carboxylase on PEB. After dephosphorylation of PEB was completed (a), 2 mg of pyruvate kinase and 3.8 mg of KCl were added; spectrum b is 45 min after the addition of pyruvate kinase. Besides, a model of the signals that compose each spectrum is presented. These are a triplet (0.95 ppm, J_{H-H} = 7.33 Hz), a doublet (0.94 ppm, J_{H-H} = 7.33 Hz, J_{H-D} = 1.10 Hz), and a singlet (0.94 ppm, J_{H-D} = 1.10 Hz).

carboxylase (Figure 2). If the reaction mixture was allowed to stand for enough time (around 6 h), the signals corresponding to the isomers of PEB completely disappeared, and only the spectrum of 2-oxobutyrate was detectable, indicating that the reaction is rather irreversible under the conditions employed in this assay. The spectrum showed a singlet, doublet, and triplet at around 1 ppm for the methyl group (Figure 3a) and a multiplet near 2.6 ppm for the methylene group (not shown).

Chirality of 2-Oxo[3-H,D]butyrate Formed by the Action of PEP Carboxylase on (E)- and (Z)-PEB. When the dephosphorylation of (*E*)- and (*Z*)-PEB was completed, a mixture of 2-oxo[3-H,D]-, 2-oxo[3-D_2]-, and 2-oxo[3-H_2]butyrate was obtained, as indicated by their methyl proton NMR signals shown in Figure 3a. These signals are a triplet, a doublet, and a singlet arising from the species with two, one, and no ^1H at C-3, respectively. The formation of 2-oxo[3-H,D]butyrate and 2-oxo[3-H_2]butyrate would be the consequence of addition of a deuteron or a proton, respectively, to the enolate intermediate presumably formed during catalysis. Formation of the species with two ^1H's at C-3 was consistently observed in several experiments.[2] The double-deuteriated compound must have arisen by exchange with solvent of one C-3 proton from 2-oxo[3-H,D]butyrate.

The relative amounts of the three forms of 2-oxobutyrate were determined in two ways. First, the region of the spectrum corresponding to the methyl protons was amplified, and then, the different peaks were cut and weighed. The values obtained for triplet, doublet, and singlet were compared with the ratio of the areas (obtained from an integrated spectrum) of the signal of methyl protons to that of C-3 protons. This relation was 3:0.8 and indicates the average amount of protons at C-3 relative to the three protons of the methyl group.[3] By these procedures, the following percentages were calculated: 60–70% doublet, 25–30% singlet, and 5–10% triplet.

To determine the stereospecificity, if any, of protonation (or deuteriation) at C-3, it was necessary to measure the amount of *R* and *S* enantiomers of 2-oxo[3-H,D]butyrate in the final mixture. This could be done by the procedure described by Hoving et al. (1983), which is based on the stereospecificity of the pyruvate kinase catalyzed enolization of 2-oxobutyrate. These authors determined that the kinase catalyzes the exchange of the *pro-R* proton of the C-3 atom of the keto acid. According to this procedure, treatment of the *S* enantiomer of 2-oxo[3-H,D]butyrate in D_2O with pyruvate kinase would result in the formation of 2-oxo[3-D_2]butyrate. Treatment

[2] We have used 99% D_2O to perform the reaction. However, we observed that the amount of HDO (peak at 4.6 ppm) was increased by the addition of enzyme, probably because not all the H_2O was exchanged with D_2O by the procedure described under Materials and Methods. This, together with the expected isotope effect for the reaction (Kuo et al., 1979), explains why 5–10% of 2-oxo[3-H_2]butyrate was present in the final mixture. In fact, we have observed an enrichment in the double-protonated form when the H_2O level was increased, although we did not make accurate measurements of this effect. Furthermore, with the value of k_{H_2O}/k_{D_2O} obtained by coupling the reaction to that of LDH (4.1; see the text), 2% ^1H in the initial conditions would be enough to account for the amount of 2-oxo[3-H_2]butyrate that was obtained.

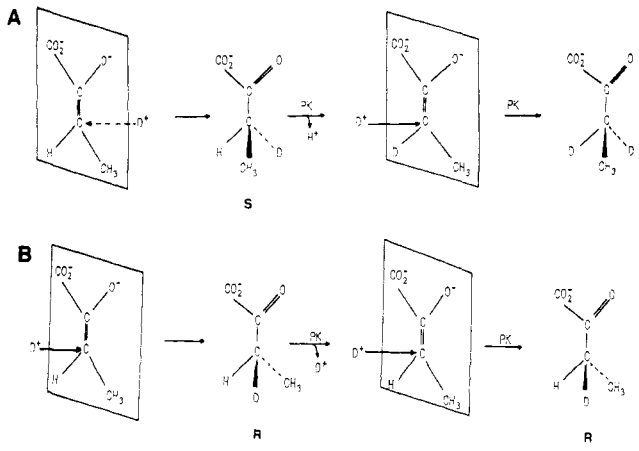
[3] As an example, the area of the most downfield peak of the triplet is 2–2.5% of the total. Since this peak represents around 25% of the total area of the triplet, it can be calculated that 8–10% of the signal corresponds to 2-oxo[3-H_2]butyrate. With this value fixed and a 3:0.80 ratio of methyl to C-3 protons, the relative amounts of 2-oxo[3-H,D]butyrate and 2-oxo[3-D_2]butyrate can be calculated. These values are 60–64% and 28–30%, respectively. This procedure was repeated with all the peaks of the spectrum, and the given amounts are the extreme values that were obtained.

Table I: Action of Pyruvate Kinase on the Mixtures of 2-Oxobutyrate That Would Have Arisen from Deuteriation of the Enolate (86% *Z*–14% *E*) from Different Faces

	initial (%)[a]				final (%)[c]		
		H,D					
deuteriation from	D_2[b]	*R*	*S*	H_2	D_2	H,D	H_2
(i) 2*re* face	25–30	8–10	52–60	5–10	77–90	13–20	nd[d]
(ii) 2*si* face	25–30	52–60	8–10	5–10	33–40	57–70	nd
(iii) random	25–30	30–35	30–35	5–10	55–65	35–45	nd
experimental[e]	25–30	60–70		5–10	61–64	36–39	nd

[a] Before the addition of pyruvate kinase; values were calculated from the methyl proton signal of the NMR spectrum. Proportions of the *R* and *S* enantiomers were calculated assuming deuteriation of the 86% *Z*–14% *E* mixture in each case. [b] D_2, H,D, and H_2 stand for the species of 2-oxobutyrate with two, one, and no deuterium atom at C-3. [c] After complete action of pyruvate kinase. Initial D_2 plus (*S*)-H,D yield final D_2; H_2 plus (*R*)-H,D produce H,D (see the text for details). [d] Not detectable. [e] Conditions as in Figure 3.

Scheme II: Deuteriation of (*Z*)-2-Enolbutyrate from the 2*re*,3*si* Face (A) or 2*si*,3*re* Face (B) Followed by Exchange at C-3 Catalyzed by Pyruvate Kinase



of the *R* enantiomer of 2-oxo[3-H,D]butyrate by pyruvate kinase enolization would not produce any change.

Assuming that deuteriation of the enolate intermediate could take place either specifically from one face or randomly from both faces, (i) deuteriation of (*Z*)-PEB (or the *Z* isomer of the enolate) from the 2*re*,3*si* face would result in formation of 2-oxo[3(*S*)-H,D]butyrate, (ii) deuteriation from the 2*si*,3*re* face would yield the *R* enantiomer (Scheme II) [deuteriation of (*E*)-PEB would produce the opposite results], and (iii) random activation from both faces would yield achiral (*RS*)-2-oxo[3-H,D]butyrate.

When pyruvate kinase and KCl were added to the mixture containing the products of the PEP carboxylase reaction in the above-mentioned proportions (Figure 3a; Table I), a rapid change of the methyl proton signal of 2-oxobutyrate was observed. In fact, after 15 min the singlet signal increased, while the doublet decreased and the triplet became undetectable. The ratio of the areas of the methyl proton signal to that of C-3 protons moved to 3:0.39. After this period, only slow changes were detected, and 30 min later, the methyl proton signal still showed singlet and doublet structures (Figure 3b) and the ratio was 3:0.36.[4] These results indicate that the final mixture was composed of 36–39% 2-oxo[3-H,D]butyrate and 61–64% 2-oxo[3-D_2]butyrate (Table I). Subtracting the 5–10% of 2-oxo[3-H,D]butyrate which must have arisen from exchange of the initial 2-oxo[3-H_2]butyrate and the 25–30% of 2-oxo[3-D_2]butyrate which was already present in the mixture before the addition of pyruvate kinase, the results indicate that approximately equal amounts (30–35%) of the single- and double-deuteriated forms came from the original 2-oxo[3-H,D]butyrate (Table I). Hence, similar proportions of *R* and *S* enantiomers had been formed by the action of PEP carboxylase, indicating that the addition of deuterium by this enzyme was nonstereoselective. Table I also shows the final proportions of isotopically substituted 2-oxobutyrate that would have been obtained if addition of deuterium had been stereoselective from either the 2*re* or the 2*si* face. The experimental values do not agree with these proportions.

[4] An upfield shift in the methyl proton signal of 2-oxobutyrate is expected for deuteriation at C-3 (Hoving et al., 1983). This shift is evident from the spectra of Figures 2 and 3 (for example, note that the singlet is not centered between the doublet peaks in Figure 3b). The shift is 0.55 and 1.10 Hz for the single- and double-deuteriated forms, respectively, which is very low compared with J_{H-H} (7.33 Hz).

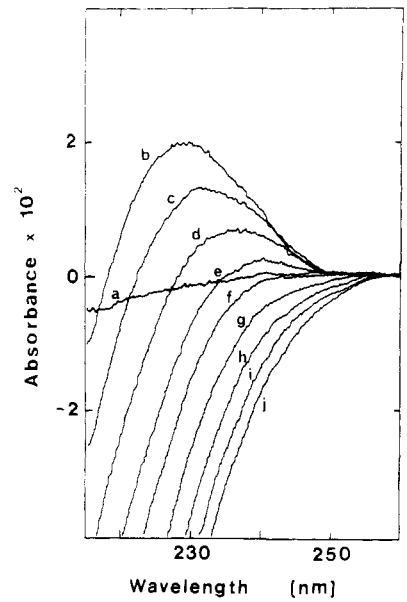


FIGURE 4: Absorption spectral changes during the dephosphorylation of PEB by PEP carboxylase. Sample and reference cuvettes contained 20 mM potassium phosphate (pH 7), 10 mM $NaHCO_3$, 100 μM PEB, and 50 μg of enzyme in a volume of 1 mL. Reaction was started by the addition of 2.5 μL of 1 M $MgCl_2$, and then spectra were recorded every 30 s from curve b (30 s) to curve j (270 s). (a) Base line. Scan speed was 200 nm·min^{-1}. Temperature was 25 °C.

The most likely explanation for these results is that the enolate is released from the active site of the enzyme and that the addition of deuterium takes place in solution. The following experiments were carried out in order to detect if the enolic form of 2-oxobutyrate was present in solution during PEB dephosphorylation by PEP carboxylase.

Formation of Enolate during PEP Carboxylase Catalysis. When $MgCl_2$ was added to a mixture containing PEB, PEP carboxylase, and $NaHCO_3$, an increase in absorbance between 220 and 240 nm was observed after the first 30 s, followed by a decrease below 230 nm at longer times (Figure 4). Clearly, a compound that absorbs around 230 nm was formed

as a consequence of the dephosphorylation of PEB, but its concentration did not increase continuously during the reaction. The decrease below 230 nm was assigned to the disappearance of PEB (Woods et al., 1970). The observed changes in absorbance can be fit to

$$\text{PEB} \xrightarrow{v} \text{2-enolbutyrate} \xrightarrow{k_2} \text{2-oxobutyrate} \quad (1)$$

assuming that 2-enolbutyrate is the compound with an absorption peak between 220 and 230 nm. The related compound enolpyruvate has an absorption peak at 225 nm (Kuo et al., 1979). When PEB was replaced by PEIV, a compound that is also dephosphorylated by PEP carboxylase, similar spectral changes were observed (not shown).

Figure 5A shows the time-dependent absorbance changes at different wavelengths after the dephosphorylation of PEB was started by the addition of $MgCl_2$. The formation of 2-enolbutyrate is also clear from this figure. According to eq 1, the concentration of 2-enolbutyrate will increase until the rate of its ketonization equals the rate of its formation. The absorbance changes at a fixed wavelength will be described by

$$A_{obsd} = vt(\epsilon_{KB} - \epsilon_{PEB}) + (\epsilon_{enol} - \epsilon_{KB})(1 - e^{-k_2 t})v/k_2 \quad (2)$$

where ϵ_{KB}, ϵ_{PEB}, and ϵ_{enol} are the extinction coefficients of 2-oxobutyrate, PEB, and 2-enolbutyrate, respectively. When the steady state is reached, the change in absorbance will be

$$A_{obsd} = v(\epsilon_{KB} - \epsilon_{PEB})t + (\epsilon_{enol} - \epsilon_{KB})v/k_2 \quad (3)$$

and will be given only by changes in the concentrations of PEB and 2-oxobutyrate. By extrapolation to $t = 0$ of this line, a value that is directly proportional to $\epsilon_{enol} - \epsilon_{KB}$ can be obtained. Figure 5B shows these values for wavelengths between 215 and 245 nm and indicates that the intermediate has an absorption peak at around 230 nm, in agreement with the reported spectrum of enolpyruvate (Kuo et al., 1979). Furthermore, assuming a value of 10 000–14 000 $M^{-1}\cdot cm^{-1}$ (Kuo et al., 1979) for $\epsilon_{enol} - \epsilon_{KB}$ at 230 nm, the steady-state concentration of 2-enolbutyrate (v/k_2) falls between 5 and 7 μM, which is consistent with the amount calculated by coupling this reaction to the reduction of 2-oxobutyrate by LDH (see below). Then, the detected amount of 2-enolbutyrate accounts for all of the product formed, indicating that the enolate is the actual form released from the active site of the enzyme.

Further information on the nature of the reaction product was obtained by measuring the dephosphorylation of PEB coupled to the reduction of 2-oxobutyrate by LDH (Stubbe & Kenyon, 1971). If the product is the enolic form of 2-oxobutyrate, according to eq 4, and assuming that all steps

$$\text{PEB} \xrightarrow{V_m} \text{enolbutyrate} \xrightarrow{k_2} \text{2-oxobutyrate} \underset{\text{NADH}}{\overset{k_3}{\rightleftharpoons}} \underset{\text{NAD}^+}{\text{2-hydroxybutyrate}} \quad (4)$$

are irreversible and that the ketonization of 2-enolbutyrate is rate-determining, the rate of product formation will be

$$\frac{dp}{dt} = V_m(1 - e^{-k_2 t}) = V_m(1 - e^{-t/\tau}) \quad (5)$$

This expression predicts that when following NADH consumption in the presence of LDH coupled to the dephosphorylation of PEB by PEP carboxylase, the curve of product formation will show a lag, the length of which will depend on the rate of ketonization (k_2). The steady-state portion of the progress curve can be described by $p = V_m t - V_m\tau$. This line allows the calculation of $\tau = k_2^{-1}$ and of the steady-state concentration of enolbutyrate ($V_m\tau$) by extrapolation to the time and absorbance axis, respectively. A similar approach (which is based on the fact that the enolic forms of the α-keto acids are not used as substrates by LDH) has been used by Kuo et al. (1979) to detect the production of enolpyruvate from PEP promoted by phosphatase.

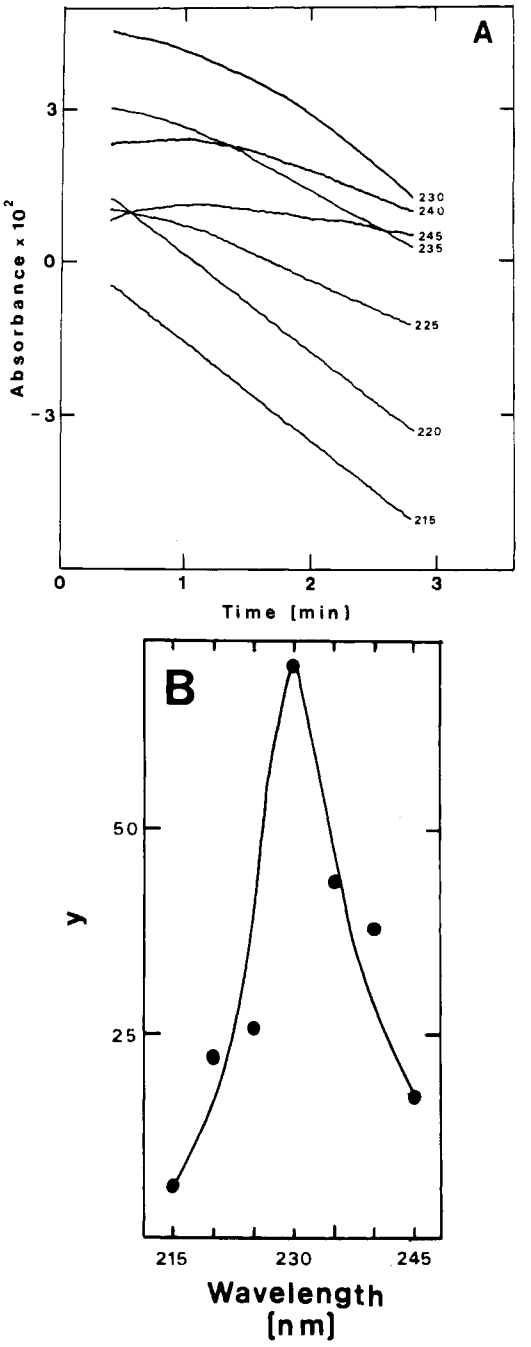


FIGURE 5: (a) Changes in absorbance observed during the action of PEP carboxylase on PEB. Reaction conditions were the same as in Figure 4. The absorbance reading was set to zero before the addition of $MgCl_2$. Recording was started when $MgCl_2$ was added. The part of the traces that is not shown (from 0 to 0.4 min) is the time required to mix the components in the cuvette. (B) Values of $y = (\epsilon_{enol} - \epsilon_{KB})v/k_2$ at different wavelengths were calculated as described in the text.

Figure 6 shows a progress curve on NADH consumption during PEB dephosphorylation promoted by PEP carboxylase and coupled to the LDH reaction in 80% D_2O at pH 7. The activity increased exponentially with time (see first-derivative trace), and the transient time (τ) of the process was 0.75 min. The reaction was carried out in a D_2O-enriched medium be-

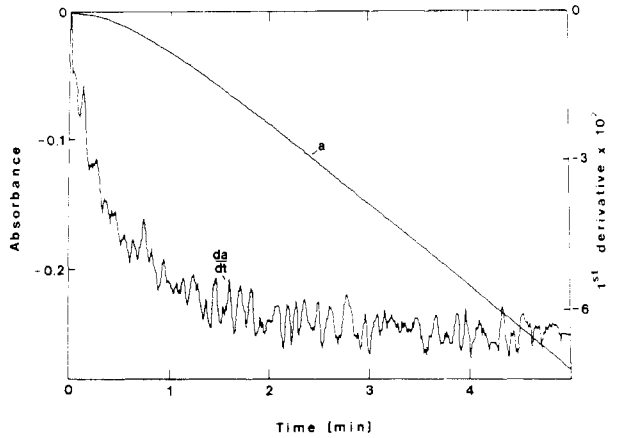


FIGURE 6: Coupling of the PEP carboxylase catalyzed enolization of PEB to the LDH reaction. Reaction components were 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH (pH 7), 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.16 mM NADH, 45 units of LDH, 1.25 mM PEB, and PEP carboxylase (50 μg) in a mixture of 80% D_2O and 20% H_2O at 25 °C. Recording was started when PEB was added to begin the reaction. (a) Absorbance trace; (da/dt) first-derivative trace of line a.

cause the ketonization is much slower under these conditions. In fact, the transient time fell to 0.30 min when D_2O was replaced by H_2O, indicating that proton transfer is rate-limiting. From these transient times, rates of ketonization of 3.3 min^{-1} and 0.8 min^{-1} can be calculated for the reaction in 100% H_2O or D_2O, respectively, corresponding to a ratio k_{H_2O}/k_{D_2O} = 4.1. Such an effect is expected for the protonation (deuteriation) of the enolic form in solution (Kuo et al., 1979). In each case, the transient times observed were at least 1 order of magnitude higher than those predicted for the system

$$\text{PEB} \rightarrow \text{2-oxobutyrate} \rightarrow \text{2-hydroxybutyrate} \quad (6)$$

Thus, we assume that the observed transient times are good measurements of the rate of ketonization of the intermediate, most likely 2-enolbutyrate.

Discussion

PEP carboxylase from maize leaves catalyzes the dephosphorylation of the two diastereoisomers of PEB at similar rates as indicated by the fact that their relative proportions did not change during their conversion to 2-oxobutyrate. According to this, it can be concluded that the maximum velocities are the same for both isomers. This is consistent with the fact that the activities displayed by the carboxylase from different sources toward either PEIV (a compound with two methyl groups at C-3) or a mixture of (*E*)- and (*Z*)-PEB are of the same order of magnitude (Fujita et al., 1984; González & Andreo, 1986). However, differences in K_m for (*E*)- and (*Z*)-PEB may exist.

The product of the dephosphorylation in D_2O seems to be an equimolar mixture of (*R*)- and (*S*)-2-oxo[3-H,D]butyrate. Our results using the pyruvate kinase catalyzed enolization of 2-oxobutyrate indicate this fact, or at least that deuteriation of the enol intermediate is rather nonstereoselective. One limitation of the method used for determining the stereospecificity of the reaction is that 2-oxobutyrate undergoes keto–enol tautomerization, and so unspecific exchange with solvent at C-3. This fact does not allow an exact calculation of the extent of specificity of the reaction. The presence of 25–30% of 2-oxo[3-D_2]butyrate after complete dephosphorylation indicates that some product molecules had undergone unspecific exchange at C-3 before the addition of pyruvate kinase. The exchange reduces the expected difference in the amounts of *R* and *S* enantiomers for the case of stereospecific deuteriation of the enolate (Table I). Even assuming that 20% of the total were molecules of 2-oxo[3-H,D]butyrate that had undergone exchange, the expected values after the addition of pyruvate kinase for stereospecific deuteriation would be significantly different from the experimental results (i.e., 50–60% of the single-deuteriated form for deuteriation from the *si* face and 20–25% for deuteriation from the *re* face). The amount of 2-oxo[3-H,D]butyrate generated by unspecific exchange should be well below 20%.[5] Thus, we conclude that the stereospecificity of deuteriation of the enolic form is either zero or very low.

Two possibilities should be mentioned regarding the way in which the intermediate becomes protonated. The most likely is the one that assumes that the intermediate is released from the active site of PEP carboxylase; the other possible explanation is that protonation from both faces occurs when the intermediate is still bound to the enzyme. Kuo and co-workers (Kuo et al., 1979) have established that the actual product of the action of either alkaline or acid phosphatase on PEP is enolpyruvate. Furthermore, they have characterized several properties of this compound, such as its absorption spectrum and lifetime in solution both in H_2O and D_2O. Assuming that the related compound 2-enolbutyrate, which would be formed during PEB dephosphorylation promoted by PEP carboxylase, would have similar properties, we have carried out a set of experiments to detect if the enol was present in solution. As mentioned under Results, a compound with similar properties to those of enolpyruvate could be detected by us. Thus, we assume that 2-enolbutyrate is the actual product of the reaction. The fact that 2-enolbutyrate is released from the enzyme explains the lack of stereoselectivity observed in the NMR experiments.

To our knowledge, this is the first report showing direct detection of one of the intermediates proposed by O'Leary and co-workers (O'Leary et al., 1981) for the PEP carboxylase reaction. Although the finding has not been made with the normal reaction, there is enough evidence that supports the idea that the carboxylation of PEP and the dephosphorylation of PEB proceed by similar mechanisms (Fujita et al., 1984; González & Andreo, 1986). Moreover, our results suggest that the multiple ^{18}O incorporation from $HC^{18}O_3^-$ into P_i observed by Fujita et al. (1984) for the reaction of *Escherichia coli* PEP carboxylase with PEB can be attributed to the longer lifetime of the intermediate at the active site.

It is likely that carboxylation is prevented by incorrect binding of the enolate owing to its methyl group. Studies with other PEP analogues and with separated (*E*)- and (*Z*)-PEB may be useful to gain more information about this question. Such studies are currently in progress in our laboratory.

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[5] In control experiments, in which 2-oxobutyrate was incubated in D_2O under the same conditions used for the dephosphorylation of PEB, we found that 25% of the ^1H's at C-3 were exchanged by deuterium after 7 h. This is in good agreement with the amount of 2-oxo[3-D_2]butyrate found after dephosphorylation (25–30%). In addition, a deuterium–deuterium exchange at C-3 that would provoke racemization of the mixture is expected. This exchange should be much lower than 25% due to the existence of an isotope effect. In fact, Hoving et al. (1983) detected 20% exchange of the ^1H's in 2-oxo[3-H,D]butyrate by solvent deuterons but did not detect racemization (deuterium–deuterium exchange) of this compound under the same conditions, as evident from the spectra in Figure 4 of their work.

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References

Andreo, C. S., González, D. H., & Iglesias, A. A. (1987) *FEBS Lett. 213*, 1–8.
Cooper, T. G., & Wood, H. G. (1971) *J. Biol. Chem. 246*, 5488–5490.
Duffy, T. H., Saz, H. J., & Nowak, T. (1982) *Biochemistry 21*, 132–139.
Edwards, G. E., Ku, M. S. B., & Monson, R. K. (1985) in *Photosynthetic Mechanisms and the Environment* (Barber, J., & Baker, N. K., Eds.) Vol. 7, pp 287–327, Elsevier, Amsterdam.
Fujita, N., Izui, K., Nishino, T., & Katsuki, H. (1984) *Biochemistry 23*, 1774–1779.
González, D. H., & Andreo, C. S. (1986) *Z. Naturforsch., C: Biosci. 41C*, 1004–1010.
González, D. H., Iglesias, A. A., & Andreo, C. S. (1987) *Biochem. J. 241*, 543–548.
Hansen, D. E., & Knowles, J. R. (1982) *J. Biol. Chem. 257*, 14795–14798.
Hoving, H., Nowak, T., & Robillard, G. T. (1983) *Biochemistry 22*, 2832–2838.
Iglesias, A. A., & Andreo, C. S. (1984) *Plant Physiol. 75*, 983–987.
Iglesias, A. A., González, D. H., & Andreo, C. S. (1986) *Planta 168*, 239–244.
Kuo, D. J., O'Connell, E. L., & Rose, I. A. (1979) *J. Am. Chem. Soc. 101*, 5025–5030.
Latzko, E., & Kelly, G. J. (1983) *Physiol. Veg. 21*, 805–815.
Maruyama, H., Easterday, R. L., Chang, H.-C., & Lane, M. D. (1966) *J. Biol. Chem. 241*, 2405–2412.
O'Leary, M. H. (1982) *Annu. Rev. Plant Physiol. 33*, 297–315.
O'Leary, M. H., Rife, J. E., & Slater, J. D. (1981) *Biochemistry 20*, 7308–7314.
Penefsky, H. (1977) *J. Biol. Chem. 252*, 2891–2899.
Powers, S. G., & Meister, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3020–3024.
Powers, S. G., & Meister, A. (1978) *J. Biol. Chem. 253*, 1258–1265.
Rose, I. A., O'Connell, E. L., Noce, P., Utter, M. F., Wood, H. G., Willard, J. M., Cooper, T. G., & Benziman, M. (1969) *J. Biol. Chem. 244*, 6130–6133.
Sedmak, J., & Grossberg, S. (1977) *Anal. Biochem. 79*, 544–552.
Stubbe, J. A., & Kenyon, G. L. (1971) *Biochemistry 10*, 2669–2677.
Utter, M. F., & Kolenbrander, H. M. (1972) *Enzymes (3rd Ed.) 6*, 117–168.
Wimmer, M. J., Rose, I. A., Powers, S. G., & Meister, A. (1979) *J. Biol. Chem. 254*, 1854–1859.
Woods, A. E., O'Bryan, J. M., Mui, P. T. K., & Crowder, R. D. (1970) *Biochemistry 9*, 2334–2338.

Carbanicotinamide Adenine Dinucleotide: Synthesis and Enzymological Properties of a Carbocyclic Analogue of Oxidized Nicotinamide Adenine Dinucleotide†

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ABSTRACT: The dinucleotide carbanicotinamide adenine dinucleotide (carba-NAD), in which a 2,3-dihydroxycyclopentane ring replaces the β-D-ribonucleotide ring of the nicotinamide ribonucleoside moiety of NAD, has been synthesized and characterized enzymologically. The synthesis begins with the known 1-aminoribose analogue (±)-4β-amino-2α,3α-dihydroxy-1β-cyclopentanemethanol. The pyridinium ring is first introduced and the resultant nucleoside analogue specifically 5′-phosphorylated. Coupling the racemic carbanicotinamide 5′-mononucleotide with adenosine 5′-monophosphate produces two diastereomeric carba-NAD analogues which are chromatographically separable. Only one diastereomer is a substrate for alcohol dehydrogenase and on this basis is assigned a configuration analogous to D-ribose. The reduced dinucleotide carba-NADH was characterized by fluorescence spectroscopy and found to adopt a "stacked" conformation similar to that of NADH. The analogue is reduced by both yeast and horse liver alcohol dehydrogenase with K_m and V_{max} values for the analogue close to those observed for NAD. Carba-NAD is resistant to cleavage by NAD glycohydrolase, and the analogue has been demonstrated to noncovalently inhibit the soluble NAD glycohydrolase from *Bungarus fasciatus* venom at low concentrations (≤100 μM).

Nicotinamide adenine dinucleotide (NAD) is essential to all cellular metabolism as a cosubstrate in biological hydride-transfer reactions. More recently it has become clear that NAD has a second role in metabolic regulation. NAD is known to serve as a substrate for a series of related enzymatic reactions in which the adenosine diphosphate ribose (ADP-ribose) moiety is transferred to a nucleophilic acceptor or to water with concomitant release of nicotinamide (Ueda & Hayaishi, 1985; Hayaishi & Ueda, 1982). The reaction is driven by the exothermic hydrolysis of the pyridinium–N-glycosidic bond of NAD (Hayaishi & Ueda, 1977). The prototypical enzymes in this family are the NAD glycohydrolases, which catalyze the hydrolysis of NAD to yield nicotinamide, ADP-ribose, and a proton. The NAD glyco-

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