References and Notes

(1) This research was supported by a grant from the National Research Council of Canada and by Research Contract No. 1SQ5-0111 from Supply and Service Canada.
(2) The most common salt is *N*-methylphenazonium methylsulfate which is often referred to in the literature as PMS. We have chosen not to use this abbreviation because the phenomena we examine in this paper are independent of the anion. The abbreviation NMP^+ is specific to the cation.
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(4) (a) G. Hauska, *FEBS Letters*, **28**, 217 (1972); (b) G. Hauska, A. Trebst, and W. Draber, *Biochim. Biophys. Acta.*, **305**, 632 (1973); (c) G. Hauska and R. C. Prince, *FEBS Letters*, **41**, 35 (1974).
(5) A brief review is given by A. Trebst in *Proc. Int. Congr. Photosynth., 3rd*, I, 439–448 (Sept. 2–6, 1974). Elsevier Scientific Publishing Company, Amsterdam, The Netherlands, 1974.
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(9) This ESR spectrum is clearly due to the $NMPH^{+}\cdot$ cation radical and not the neutral $NMP\cdot$ radical. The gross hyperfine pattern can be analyzed in terms of two nearly equivalent nitrogens and four nearly equivalent protons (the three methyl protons and one N-H proton). By analogy with protonated and methylated nitrogens in other nitrogen heterocyclic radicals we expect that $a^N \sim a^H$. If the equivalences were exact then a nine-line pattern with intensities 1:6:17:30:36:30:17:6:1 would be expected which is close to the intensity ratios of the observed nine-line pattern. Further confirmation is obtained from the ESR spectrum in D_2O where the hyperfine pattern collapses to an eight-line spectrum as expected for one exchangeable hydrogen.
(10) A. Carrington and A. D. McLachlan, "Introduction to Magnetic Resonance", Harper and Row, New York, N.Y., 1967, p 228.
(11) Disproportionation of $NMPH^{+}\cdot$ limits its concentration[12] according to $2NMPH^{+}\cdot \rightleftharpoons NMP^{+} + NMPH + H^{+}$. Under our conditions (pH ≃7, $[NMP^+] \simeq 10^{-3}$ M), $[NMPH^{+}\cdot]_{max} \simeq 0.2\,[NMP^+]_0$.
(12) W. S. Zaugg, *J. Biol. Chem.*, **239**, 3964 (1964).
(13) The interpretation of these experiments rests on the assumption that we can consider these paramagnetic probes as point dipoles. Clearly they do have a finite size; however, even the $NMPH^{+}\cdot$ is small compared to the ~ 40-Å diameter of the micelle.
(14) C. A. Evans and J. R. Bolton, unpublished results.
(15) Of course, if NMPH were to be the proton translocating species, a two-electron oxidation would be necessary for a proton to be liberated. This would be only half as efficient for proton transfer as the scheme proposed in this paper.
(16) Contribution No. 168 from the Photochemistry Unit.

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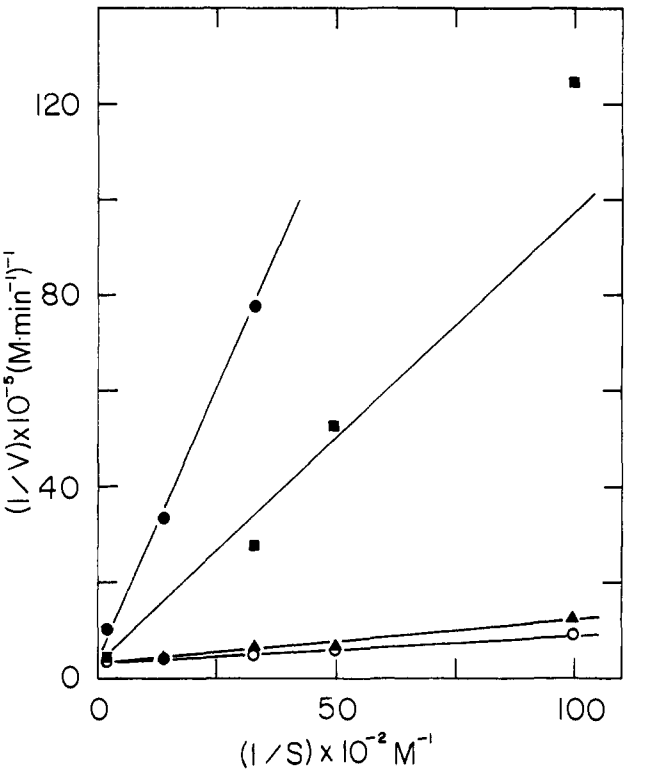
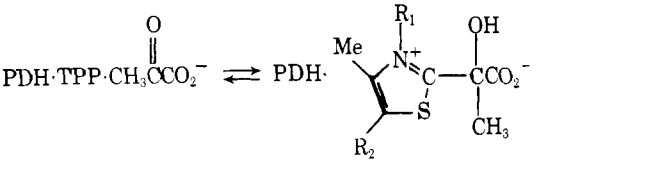
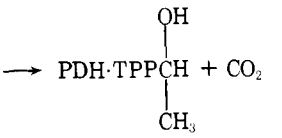


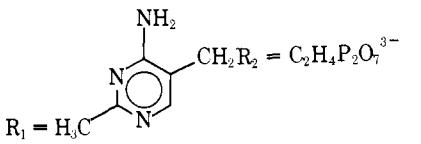
Figure 1. Lineweaver–Burke plot of the initial rate of conversion of pyruvate S to acetyl coenzyme A, catalyzed by *E. coli* PDH (3×10^{-3} units/mL) at 25 °C, pH 7.65 (0.05 M phosphate buffer) in the presence of MAP (concentrations (μm): ●, 1.5; ■, 0.6; ▲, 0.1; ○, 0.0), TPP (200 μM), coenzyme A (60 μM), NAD^+ (2 mM), and magnesium chloride (1 mM), according to the procedure of Reed and Mukherjee.[20] The appearance of NADH was followed at 340 nm with a Hitachi–Coleman recording spectrophotometer. The value of K_i, determined by a replot of the slopes of the lines in the figure (method in ref 21), is 5×10^{-8} M.



I



HETPP



Active Site Generated Analogues of Reactive Intermediates in Enzymic Reactions. Potent Inhibition of Pyruvate Dehydrogenase by a Phosphonate Analogue of Pyruvate[1]

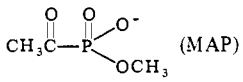
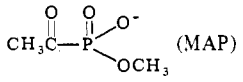
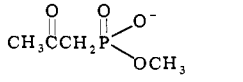
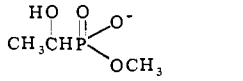
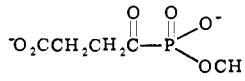
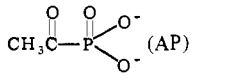
Sir:

Pyruvate dehydrogenases (PDH) are multienzyme complexes responsible for the conversion of pyruvate to acetyl coenzyme A.[2] The initial steps are catalyzed by a component enzyme (E_1) which promotes the decarboxylation of pyruvate. This requires the coenzyme, thiamine pyrophosphate (TPP).[3] A detailed mechanism has been proposed[4,5] that is based on an analogy to the mechanism proposed for condensation reactions which are catalyzed by thiazolines (eq 1).

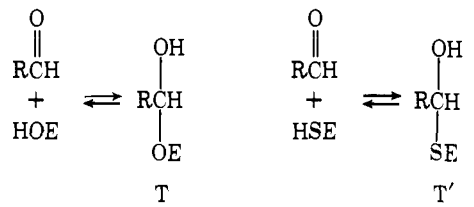
This mechanism requires the initial conversion of pyruvate to an enzyme-bound, covalent adduct of TPP, 2-lactyl TPP (I).[6] The intermediate is converted to an isolable product, hydroxyethyl TPP (HETPP). The involvement of I and the mechanism of its decarboxylation are of considerable interest but the properties of compounds related to I weigh against the isolation of I from an enzymic reaction.[7]

A promising method for experimental confirmation of the involvement of specific covalent intermediates has been developed by Westerik and Wolfenden[8,9] and by Thompson.[10] This is a modification of the use of transition state analogues.[11–13] The method is exemplified by the powerful inhibition of papain[8] and of elastase[10] by aldehydic analogues of peptide substrates. Peptides are normally cleaved in a catalytic step following addition of an enzymic hydroxyl or sulfhydryl group to the carbonyl moiety of the peptide via a supposed tetrahedral intermediate (T). However, it was proposed that the aldehyde forms readily and reversibly a hemiacetal or thiohemiacetal (T′) which may only revert to the starting species. Since the enzyme's catalytic function involves stabilization of normally reactive species, the powerful inhibition by aldehydes supports the existence of the proposed intermediate (T).

Table I. Effectiveness of Phosphonates as Inhibitors of PDH as Measured by Rate of Conversion of Pyruvate to Acetyl Coenzyme A in Presence (V_i) and Absence (V_o) of Listed Compounds

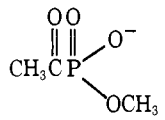
Compd	Concn, M	V_i/V_o[a]
 (MAP)	5×10^{-7}	0.82
 (MAP)	1×10^{-3}	~0.0
	1×10^{-3}	1.0
	1×10^{-3}	1.0
	1×10^{-3}	1.0
 (AP)	5×10^{-5}	0.83[b]

[a] Assay by the procedure of Reed and Mukherjee.[20] Pyruvate concentration is 0.1 mM for MAP and AP, 0.5 mM for others. [b] Effect is probably due to ~1% contamination by MAP from which this compound was made.



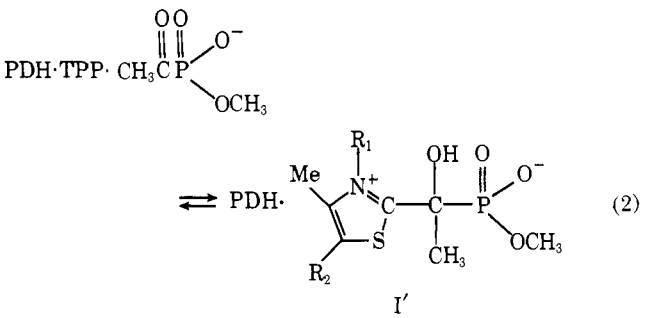
T T′

We have applied this method to demonstrate the existence of reactive intermediate I in the reaction catalyzed by the pyruvate decarboxylase (E_1) enzyme of *E. coli* PDH.[3] We prepared sodium methylacetylphosphonate (MAP) as a stable analogue of pyruvate. If this material binds to PDH and reacts



MAP

with TPP, the predictable properties of ketophosphonates[14,15] assure us that dephosphorylation will not take place under the reaction conditions (eq 2). We now find that MAP does compete with pyruvate for the active site of E_1 of PDH. The magnitude and specificity of inhibition suggest a common type of covalent adduct forms. The involvement of TPP in the overall PDH reaction suggests the nature of the addition product.



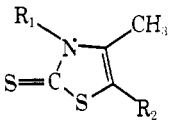
(2)

I′

MAP was prepared by the reaction of dimethyl acetylphosphonate[16] and sodium iodide in acetone.[17] the structure of MAP was confirmed by IR ($\nu_{C=O}$ 1670 cm^{-1}, $\nu_{P=O}$ 1234 cm^{-1}), ^1H NMR ($CH_3C(=O)$-, δ (D_2O, 1% DSS) 2.42 ($^3J_{PH}$ = 4.5 Hz), $POCH_3$, 3.57 ($^3J_{PH}$ = 10.4 Hz)), and analysis for $C_3H_6O_4PNa$ (CH, P). PDH from *E. coli* was purified by the method of Speckhard and Frey.[18] Assay of the pyruvate decarboxylase activity (E_1) of PDH was performed by Reed's procedures.[19,20]

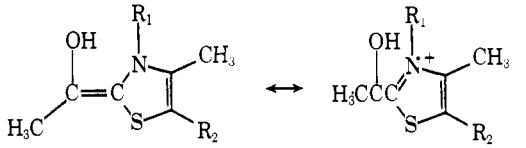
In Figure 1, the results of studies of inhibition of PDH by MAP are presented. It is clear that MAP is a powerful competitive inhibitor against pyruvate (K_i[21] $\simeq 5 \times 10^{-8}$ M). Furthermore, the inhibition is structurally specific for the 2-keto acid anion (Table I). Unlike pyruvate, MAP is not a substrate of PDH. No reaction to give products from MAP is observed, although the dephosphorylation product would be the same as that from decarboxylation. The inhibition is reversible since the enzymes activity remains when it is incubated with MAP. The remarkable stability of the MAP·TPP·PDH complex (as indicated by K_i) is characteristic of covalent activated intermediate analogues.[8-10] For comparison, K_m for pyruvate is 10^{-4} M and K_s for TPP is 10^{-5} M.[22] The structure of I′ is further supported by our isolation of the nonenzymic reaction product of thiamine and MAP. Spectroscopic data (NMR, IR) are consistent with a structure analogous to I′ (the pyrophosphoryl group is absent since thiamine and not TPP was used).

Our results suggest that I is a reactive intermediate in the reaction catalyzed by PDH. If the transition state for decarboxylation is not high in energy compared to I, then I′ qualifies as a transition state analogue. Gutowski and Lienhard found that thiaminethiazolone pyrophosphate (TTPP) combines with PDH to form a stable adduct.[22] The adduct does not bind TPP



TTPP

and is fully dissociable only when PDH is denatured. These authors suggest that the transition state for decarboxylation of I should resemble the conjugate base (J) of HETPP.[5,22] This



J_1 J J_2

transition state resembles TTPP if J_1, in which charge separation is eliminated at the cost of loss of aromaticity, is of major importance. We know that J is a high energy species since HETPP is a very weak acid.[22,23]

We suggest an alternative mechanism in which the transition state for decarboxylation resembles I, and J is avoided by concerted protonation, as carbon dioxide leaves I, by a suitably positioned Brønsted acid (on the side opposite CO_2^-). This would explain the failure of enzymes to promote proton exchange in HETPP,[24] the stereoselectivity of production of HETPP[24] (which is then due to stereospecifity), and the high rate constant for enzymic decarboxylation compared to a

model system lacking an internal general acid.[7] Condensation reactions of HETpp would proceed with inversion and internal general base catalysis to avoid J, in analogy to reactions of acetyl coenzyme A.[25]

We are further examining the enzymatic and nonenzymatic reactions we have discussed to elucidate in detail the processes involved in catalysis by TPP of reactions involving pyruvate.

Acknowledgment. We thank Professor P. A. Frey, of The Ohio State University, for helping us obtain PDH and for interesting discussions.

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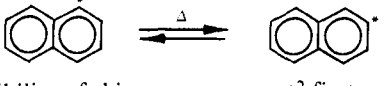
Automerization of Naphthalene

Sir:

We wish to report a ^{13}C-labeling experiment which reveals the scrambling of α and β carbon atoms in naphthalene at high temperatures[1] (eq 1).



(1)

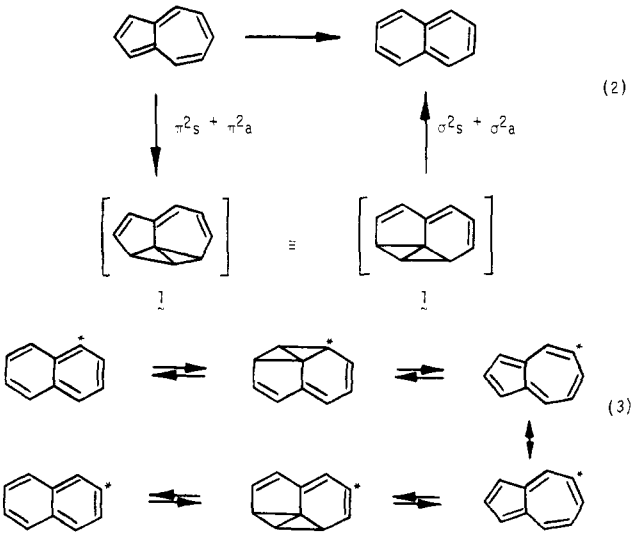
The possibility of this rearrangement[2] first occurred to us during our studies on the thermal isomerization of azulene to naphthalene, a venerable mechanistic puzzle in the chemistry of nonbenzenoid aromatic hydrocarbons.[4] One plausible mechanism for the latter transformation involves initial conversion of azulene to tetracyclic triene **1** followed by ring opening of the bicyclobutane intermediate to naphthalene (eq 2). The symmetry allowed nature of both steps[5] lends special appeal to this pathway.[6]

Reversibility of this process would provide a pathway for scrambling the α and β carbon atoms of naphthalene (eq 3). Note as a further consequence of the transformations proposed in eq 2 and 3 that the two angular carbon atoms (γ) are predicted to retain their original identity and never migrate to the α or β positions of naphthalene.

Table I. Distribution of the ^{13}C Label[a] (±4%) in the Pyrolysate of Naphthalene-*1*-^{13}C As a Function of Contact Time

Contact time, sec[b]	α, %	β, %	γ, %
0.0[c]	100%	0	0
1.0	78	18	4
2.0	71	26	3
5.0	58	40	2
8.0	55	43	2
11.0	52	46	2

[a] These values[11] have been corrected for the natural abundance of ^{13}C. [b] The "contact time" was calculated from the known rate at which naphthalene passed through the apparatus[10] and the volume of the hot zone. [c] This entry refers to the material before pyrolysis.[9]



(2)

(3)

From the reported activation parameters for the azulene → naphthalene isomerization[7] and the known difference in free energy between these two species,[8] one can estimate for the reverse reaction a $\Delta G^{\ddagger}$ of 86 kcal/mol at 1035 °C, a temperature at which isomerization should proceed to an observable extent in a flow system. Azulene should not accumulate to any significant extent during the equilibration, of course, since the thermodynamic stability of naphthalene greatly exceeds that of the nonbenzenoid isomer.[8]

To test the above predictions, we slowly sublimed naphthalene-α-^{13}C[9] through a quartz pyrolysis apparatus[10] heated to 1035 ± 5 °C. Collection of the pyrolysate in a liquid nitrogen trap and analysis[11] of the recovered naphthalene for distribution of the ^{13}C label gave the results summarized in Table I.[13]

These data clearly reveal scrambling of the α and β carbon atoms in naphthalene at high temperatures.[14] Furthermore, within experimental error (±4%), the angular carbon atoms (γ) retain their original identity and remain unlabeled even after several half-lives of the α–β scrambling. Both of these experimental observations stand in complete harmony with the mechanistic scheme outlined in eq 3.

Isomerization of naphthalene-*1*-^{13}C to naphthalene-*2*-^{13}C under these conditions follows the rate law for a unimolecular reversible reaction with $K_{eq} = 1$ (Figure 1).[15] From the plot in Figure 1 (slope = $2k$), one can thus calculate for the automerization of naphthalene at 1035 °C an experimental $\Delta G^{\ddagger}$ of 86 kcal/mol,[16] a value in striking agreement with that estimated for the thermal isomerization of naphthalene to azulene.

It is tempting to conclude that naphthalene does indeed isomerize reversibly to azulene by the mechanism proposed in