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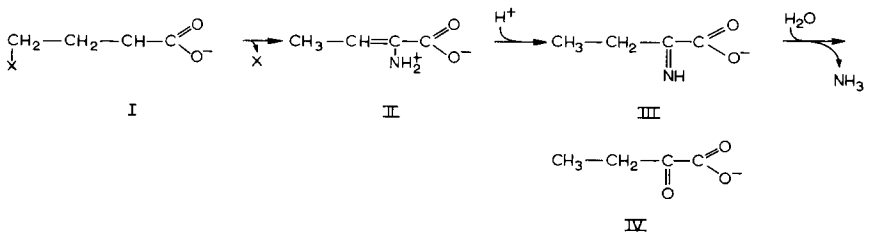
The formation of an enzyme-bound β-carbanion in the enzymic conversion of L-homoserine to α-ketobutyrate*

Pyridoxal phosphate participates in a number of reactions in which a γ-substituted amino acid is converted to the corresponding α-keto acid with elimination of the γ substituent. The generally accepted mechanism of action[1] of pyridoxal phosphate predicts that an enamine is initially formed and is then converted to the keto acid. The following represents the postulated reaction sequence (intermediates preceding the enamine (II) have been omitted):



A consequence of the proposed enamine intermediate is that, if the reaction is carried out in ^2H_2O, the product should contain deuterium in the β position. This has recently been verified for a pyridoxal phosphate dependent γ elimination reaction[2]. Evidence for the formation of an intermediate enamine has been obtained for a related reaction[3]. The enamine can spontaneously convert to the keto acid and could therefore be the final reaction product of the enzymatic reaction. In that case, the β-proton will be incorporated directly from the solvent. Alternatively, the ketonization of the enamine could be enzyme-catalyzed and then the β-proton is added to an enzyme bound intermediate. It is not known at what stage of the reaction the β-proton is introduced.

We have carried out the following experiment which establishes that in the conversion of homoserine to α-ketobutyrate the β-proton is added to an enzyme-bound intermediate. Homoserine was converted to α-ketobutyrate by the action of rat liver homoserine dehydratase in deuterated solvent. According to the proposed reaction sequence, deuterium will be introduced into the β position of α-ketobutyrate. If the introduction of the β-proton (deuteron in ^2H_2O) is enzyme catalyzed, deuterium will be introduced stereospecifically and the β carbon will be asymmetric. If the β proton is introduced non-enzymatically, which would happen if the enamine spontaneously converts to the keto acid, the β-carbon will be racemic. To avoid loss of deuterium, α-ketobutyrate was not isolated, but it was decarboxylated to propionic acid, which was then isolated. The decarboxylation does not affect the asymmetry of the β-carbon of α-ketobutyrate. Asymmetrically labeled α-deutero-α-ketobutyrate** will be con-

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** The conversion of homoserine to α-ketobutyrate could lead to β,γ-dideutero-2-ketobutyric acid. However, a recent[2] report indicates that in the conversion of *O*-succinylhomoserine to ketobutyrate in ^2H_2O, only 20% of the γ-hydrogen was derived from the solvent. One atom of deuterium was introduced into the β position of ketobutyrate as expected. It is therefore possible that the α-ketobutyrate, produced in our experiment, contains between zero and one

TABLE I

SPECIFIC OPTICAL ROTATION OF SODIUM α-DEUTEROPROPIONATE DERIVED FROM ENZYMATICALLY FORMED α-KETOBUTYRATE

The optical rotation was determined with a Cary model 60 spectropolarimeter in a cell with 1.0-cm path length. The sodium propionate concentration was 16 mg/ml.

λ (mμ)	*Sodium 2-deutero-propionate**	*2(R)-Deutero-propionate***
300	+ 2.9	
280	+ 3.9	− 3.9
270	+ 5.0	− 5.0
260	+ 6.4	− 6.1
250	+ 8.5	− 8.3
240	+12.5	−12.3

* Derived from enzymatically formed α-ketobutyrate.
** Prepared synthetically[5]. 0.92 atom deuterium/molecule.

verted to asymmetrically labeled α-deuteropropionate. α-Deuteropropionate was isolated, purified and its optical rotatory dispersion spectrum was determined. The optical rotation at various wavelengths is given in Table I. The magnitude of the rotation is equal at all wavelengths to that of synthetically prepared sodium 2(*R*) deuteropropionate[5]. The negative rotation indicates that the enzymatically formed compound has the (*S*) configuration. Sodium propionate from a control experiment in H_2O was not optically active.

These results show that the β-proton is added to an enzyme-bound intermediate. Therefore, the free enamine cannot be a reaction product. Both, chemical considerations and experimental evidence are consistent with the existence of an intermediate enamine at some stage of the reaction. Therefore, we suggest that the enamine is formed in the generally accepted way[1], but is not released from the enzyme. The enzyme then catalyzes its ketonization, and the ketamine or keto acid is the final product of the enzymic reaction. An enzyme catalyzed ketonization is not without precedent. In the conversion of 2-keto-3-deoxyarabonic acid to α-ketoglutarate semialdehyde, an enzyme-bound enol is formed[6]. Its conversion to the corresponding ketone is enzyme-catalyzed. The results obtained with homoserine dehydratase corroborate the conclusions reached by FLAVIN AND SLAUGHTER[7] that an enzyme bound β-carbanion occurs in a γ-elimination reaction.

Experimental. The enzyme was prepared according to GREENBERG[4]. The purification was carried through the first ethanol fractionation, which yielded an enzyme preparation with a specific activity of 89 units/mg. Before use, the enzyme was precipitated with $(NH_4)_2SO_4$ and then taken up in ^2H_2O. The following reaction mixture was used: 1800 units enzyme, $7.5 \cdot 10^{-3}$ M pyridoxal phosphate, $7.5 \cdot 10^{-3}$ M mercaptoethanol, 0.15 M DL-homoserine, $7 \cdot 10^{-3}$ M EDTA, 0.05 M potassium phosphate buffer, p^{2}H 7.5. Total volume, 50 ml. The solvent was ^2H_2O. The reaction was

atom of deuterium in the γ position. The absence or presence of deuterium in the γ position does not affect our argument and was not investigated. We will refer to the ketobutyrate obtained in ^2H_2O as β-deutero-2-ketobutyrate and to the propionic acid derived from it as α-deuteropropionic acid, although molecules may contain an additional deuterium atom.

allowed to proceed 60 min at 37°. Analysis of an aliquot indicated that 1700 μmoles of α-ketobutyrate had been formed. The reaction mixture was chilled in an ice bath and 0.6 ml H_2O_2 were then added to decarboxylate α-ketobutyrate. After 1 h 4 mg catalase were added to destroy the excess H_2O_2. After 30 min the pH of the solution was brought to approx. 10, and the solution was taken to dryness on a rotary evaporator. Propionic acid was then isolated by silicic chromatography[8]. The column fractions were titrated with NaOH. Based upon titration, 955 μmoles of sodium propionate were recovered. Sodium propionate was recrystallized from ethanol. Its identity was established by its elution volume from the silicic acid column, by its infrared spectrum, and NMR spectrum. The spectrum of the β-hydrogens was no longer a triplet, but was converted to a doublet, indicating the presence of deuterium in the α position. The optical rotatory dispersion spectrum (Table I) is also that expected from α-deuteropropionate.

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