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Enzymatic Elimination from a Substituted Four-Carbon Amino Acid Coupled to Michael Addition of a β-Carbon to an Electrophilic Double Bond. Structure of the Reaction Product*

Martin Flavin and Clarence Slaughter

ABSTRACT: When *N*-ethylmaleimide was added to reaction mixtures containing β- or γ-substituted four-carbon amino acids and pyridoxal phosphate enzymes normally catalyzing elimination, ammonia and liberated substituent were formed as expected, but α-ketobutyrate did not appear. In its place a compound accumulated which contained the elements of *N*-ethylmaleimide and the four-carbon chain of the substrate. The portion of this reaction product corresponding to the first two carbons of the amino acid was shown to be present as an α-keto acid function. The rate of decomposition of the product obtained after oxidative decarboxylation was similar to the rate of hydrolysis of succinimide in dilute alkali. The two degradation products isolated after strong acid hydrolysis were shown to be identical with synthetic preparations of the diastereoisomers of α-methyl-β-carboxyglutaric acid. The original enzyme reaction product was also present as the corresponding two diastereoisomers, but it is not known whether these arose from spontaneous racemization. These results indicate that the structure of the enzyme reaction product was α-keto-3-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))-butyric acid.

This structure reflects a new kind of pyridoxal phosphate enzymatic reaction: the elimination of an electronegative substituent from a four-carbon amino acid, coupled to Michael addition of the third carbon to the electrophilic double bond of maleimide. Mechanisms were considered for the formation of the new reaction product.

In pyridoxal-P potentiated enzymatic reactions of the type termed "γ elimination" (reaction 1), it was found that, when *N*-ethylmaleimide (EM[1]) (or another maleimide derivative) was added to the reaction mixture (reaction 2), ammonia and the electronegative substituent "A" were liberated in expected amounts, but a large part of the α-ketobutyrate failed to appear (Flavin

$$ACH_2CH_2CH(NH_2)COOH + H_2O \longrightarrow AH + NH_3 + CH_3CH_2COCOOH \quad (1)$$

$$ACH_2CH_2CH(NH_2)COOH + H_2O + EM \longrightarrow AH + NH_3 + KEDB \quad (2)$$

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[1] Abbreviations used in this work: KEDB, α-keto-3-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl)) butyric acid, structure VII, formerly designated XMal; EDP, 2-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))-propionic acid, VIII, formerly YMal; HEDB, α-hydroxy-3-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))butyric acid, XI, formerly ZMal; KDB, α-keto-3-(3′-(2′,5′-dioxopyrrolidyl))butyric acid; KPDB, α-keto-3-(3′-(*N*′-phenyl-2′,5′-dioxopyrrolidyl))butyric acid; EM, *N*-ethylmaleimide. For KEDB and EDP, the subscripts 1 and 2 refer, respectively, to the electrophoretically slower and faster moving diastereoisomers.

and Slaughter, 1964a; Flavin, 1965a). In place of, and more or less equivalent to, the missing α-ketobutyrate, an unknown product, α-keto-3-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))butyric acid (KEDB), was isolated by chromatography, and shown by appropriate labeling to contain the carbons of the maleimide and the four-carbon chain of the substrate (Flavin and Slaughter, 1964a). This product did not contain pyridoxal-P. It did not result from prior spontaneous reaction of maleimide with substrate (though in the case of cystathionine, the substrate initially used, such a reaction did slowly occur). It was also not formed secondarily from the products of reaction 1; when substrate was replaced by similar *low* concentrations of ammonium α-ketobutyrate no KEDB could be detected (Flavin and Slaughter, 1964a; and see Discussion).

This report deals with the nature of reaction 2 as catalyzed by a cystathionine γ-cleavage enzyme purified from *Neurospora* (Flavin and Segal, 1964). This enzyme decomposes a number of substrates besides cystathionine (AH = cysteine in reactions 1 and 2) and, for convenience, the substrate employed was *O*-succinyl-DL-homoserine. Based on evidence obtained from degradation products, the previously unidentified product of reaction 2 has now been tentatively assigned the structure: α-keto-3-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))butyric acid (KEDB[1], structure VII).

Experimental Section

Synthesis of the 2-Diastereoisomers of α-Methyl-β-carboxyglutaric Acid. The procedure of Auwers *et al.* (1891) yielded a mixture of two diastereoisomers, of which the original authors were able to isolate only the less soluble, mp 183°. With some minor modifications, it was found possible to isolate also the more soluble, mp 147°, isomer in nearly pure form. Sodium (170 mg-atoms) was added in portions to a reflux flask containing a magnetic stirring bar and 100 ml of absolute ethanol. When the sodium had dissolved, a solution of 170 mmoles each of diethyl fumarate and diethyl methylmalonate, in 150 ml of ethanol, was added through a buret, with stirring. A condenser with a drying tube was attached, and the cloudy solution was refluxed with stirring for 2 hr. An orange color appeared in 20 min. The solution was chilled on ice and poured into 400 ml of ice-cold 0.25 M H_2SO_4, with addition of more acid to make the final pH 2. This solution was extracted three times with 400 ml of ether. The ether was extracted with saturated aqueous $NaHCO_3$, washed with water, dried over Na_2SO_4, and evaporated on a steam bath to a colorless oil which was fractionated by vacuum distillation. α-Methyl-α-carboxy-β-carboxyglutaric acid ethyl ester was recovered as the fraction boiling at 153° at 1.2 mm; 37 g (63%). The refractive index at 25° was 1.4408, and the tetraester reacted slowly and incompletely with hydroxylamine.

Eleven grams of ester and 20 ml of 6 N HCl were stirred under reflux for 7 hr with a short air condenser, which was periodically removed to ensure evaporation of ethanol. The volume was allowed to decrease to 15 ml toward the end. Complete solution of the oil phase and liberation of 1 equiv of CO_2 were both complete in 5 hr. After standing 1 to 3 days in an evaporating dish at 25° and scratching, the residual oil set to a mass of crystals, which was filtered and dried in a desiccator; mp 140–147°, 5.6 g (93% of theoretical yield for both isomers of α-methyl-β-carboxyglutaric acid).

Failure to obtain more than 100 mg of material melting above 147° by repeated fractional crystallization confirmed that the isomer melting at 183° was largely absent.[2] The above product was obtained in various crystalline forms (rectangular, rhomboid, hexagonal plates) from glacial acetic acid, but sharper melting material was obtained by one recrystallization from acetonitrile; 4.1 g, mp 145–147.5°. Paper electrophoresis (see Results) showed that this fraction contained a few per cent of the high melting isomer. The observed neutralization equivalent was 60.2; calcd 63.3. *Anal.* Calcd for $C_7H_{10}O_6$: C, 44.2; H, 5.3. Found: C, 44.19; H, 5.15.

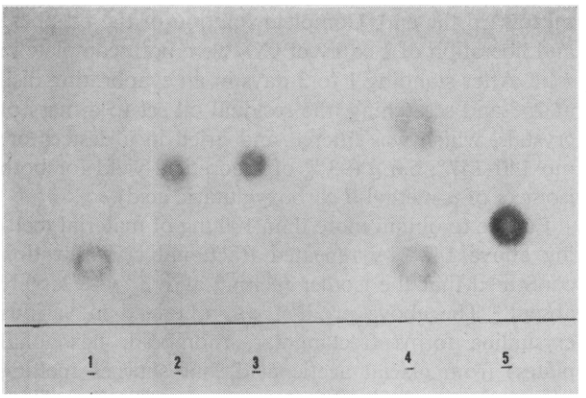
To obtain the isomer, mp 183°, ester was digested with 6 N HCl as above, but the volume was maintained constant, and after 6 hr the air condenser was replaced by a cold water condenser and reflux was continued for 10 days. On cooling, crystals separated at once, as described by Auwers *et al.* (1891); mp 181–183°, 2.31 g (38% of theoretical for both isomers). The melting point was not changed by recrystallization from water, and paper electrophoresis revealed no contamination by the low melting isomer.

As described in the Results section, samples, of enzymatic origin, of each isomer of [^{14}C]α-methyl-β-carboxyglutaric acid were recrystallized to constant specific radioactivities with the above synthetic isomers. Radioactivity was measured at constant sample thickness, 50 mg being applied to the planchets as a slurry in petroleum ether. After three recrystallizations, the labeled isomer of mp 147° was also used to prepare the *p*-bromophenacyl triester derivative. To 155 mg (0.82 mmole) of labeled acid in 1 ml of water adjusted to pH 6.4 with KOH was added 680 mg (2.45 mmoles) of *p*-bromophenacyl bromide dissolved in 15 ml of warm ethanol. After 3-hr reflux, KBr was filtered out, and the filtrate was concentrated and chilled to −10°. The precipitated product was washed on a filter with half-saturated $NaHCO_3$, followed by water; 300 mg (47%), mp 119–123°, 125–126.5° after three recrystallizations.

Other Preparations. DL-β-Carboxyadipic acid[3] was prepared from diethyl acetylsuccinate and acrylonitrile by the method of Tawney and Prill (1948) in 20% yield; mp 122.5–124.5° after one recrystallization from acetone–CCl_4. DL-[1-^{14}C]Homoserine was prepared from DL-[1-^{14}C]methionine, as previously described for

[2] In repeating this synthesis a decade after the original publication Michael (1901) was also unable to isolate any of the higher melting isomer.

[3] The authors are indebted to Dr. K. Freudenberg for a sample of D(+)-β-carboxyadipic acid (mp 102–103°). The chromatographic properties of the optically active compound were identical with those of the racemate.



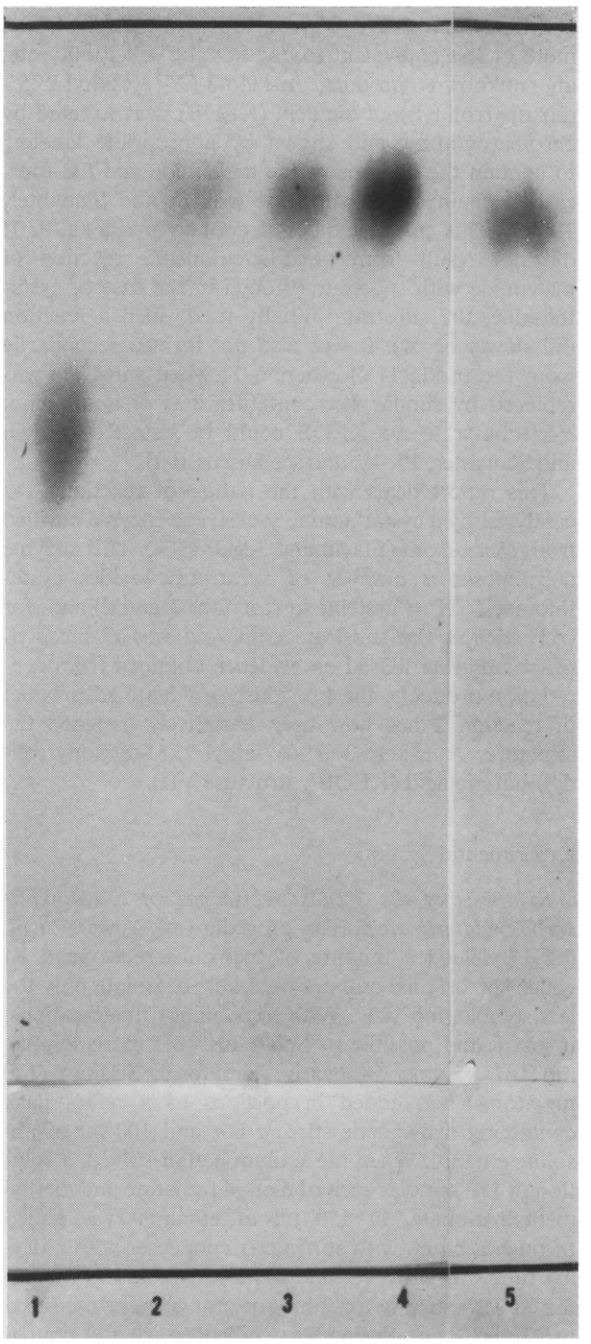


FIGURE 1: Photographs of radiograms of [2′,5′^{14}C]-KEDP after reaction. (A) (above) Photograph of radiograms obtained after low voltage paper electrophoresis of [2′,5-^{14}C]KEDB treated in various ways. Electrophoresis was in the eph. 1 system (Table II) for 2 hr; the origin was at the line near the bottom, and the anodic buffer compartment communicated with the top. Applied at: 3, [^{14}C]KEDB; 1, the same after ceric sulfate treatment (Experimental Section); 2, same as 3 followed by application to the same spot of the supernatant after neutralization of ceric sulfate; 4, a sample of [^{14}C]KEBD stored frozen for 6 months (4 and 5 are from a different electrophoresis paper, on which KEDB migrated slightly further); 5, [^{14}C]KEDB subjected to catalytic hydrogenation. (B) (right) Photograph of radiogram after paper chromatography in solvent 9 (Table II) of products formed from dinitrophenylhydrazine treatment of [2′,5′-^{14}C]KEDB, with and without prior treatment with hydrogen peroxide. The origin and solvent front are indicated by lines at bottom and top, respectively. The paper had been cut for exposure to X-ray film. Applied at: 4, [^{14}C]KEDB; 1, [^{14}C]KEDB treated with dinitrophenylhydrazine; 5, [^{14}C]EDP (KEDB treated with peroxide); 3, [^{14}C]EDP treated with dinitrophenylhydrazine; 2, [^{14}C]KEDB treated with 2 N HCl.

[2-^{14}C]homoserine (Flavin and Slaughter, 1964a). The electrolytic desalting step was omitted. The yield of crystals was 33%; an additional 27% was obtained amorphous but radiographically pure. Succinamic acid was prepared by the method of Jeffery and Vogel (1934); silver was removed with Dowex 50-H$^+$ followed by filtration of an acetone solution through Celite. The yield was 56% of stocky needles, mp 157.5–158°.

Procedures. Unless otherwise specified, paper chromatographic and electrophoretic procedures and solvents (No. 1–7), and methods for detecting compounds and radioactivity, were as previously described (Flavin and Slaughter 1964a). The electrophoretic systems, eph. 1 and 2 (see Table II), were pyridine–acetate buffers, pH as indicated, run in the relatively low voltage apparatus previously described (Flavin and Slaughter, 1964a). Runs were for 2–3 hr and results are expressed as distance migrated toward the anode in 100 min. The Gilson Model D (5000 v) electrophorator proved of great value for the separation of acidic diastereoisomers otherwise unresolvable by chromatography. The use of shallow buffer solutions allowed compounds to travel 120 cm from the origin before reaching the anodic buffer compartment. Results are expressed as actual

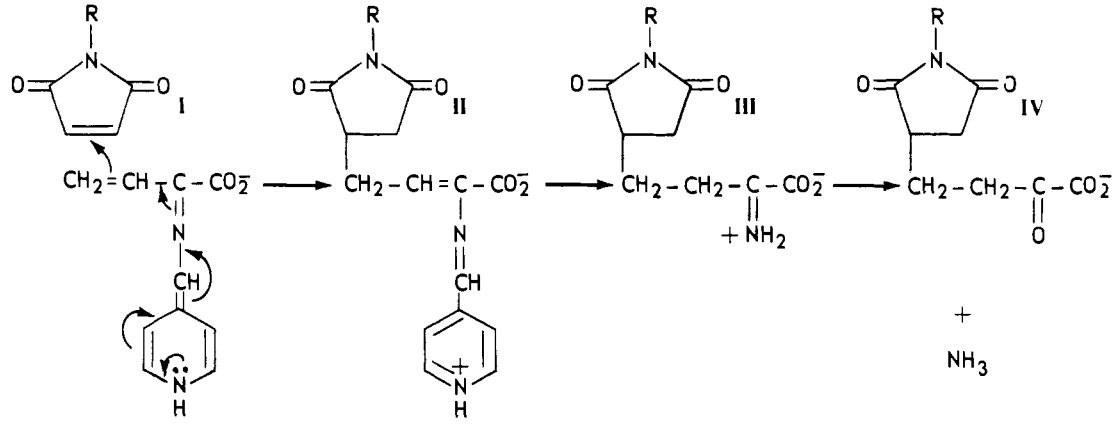


FIGURE 2: A possible structure for KEDB (IV) resulting from addition to EM of an intermediate with carbanion character on the γ-carbon (I). Pyridoxal-P ring substituents have been omitted for convenience.

migration distances in each experiment. Buffers used with this apparatus were: eph. G1, pyridine–acetate, pH 3.5; eph. G2, 4% formic acid plus a trace of pyridine, pH 2.0; eph. G3, 4% formic acid, pH 1.7.

KEDB was prepared with *Neurospora* cystathionine γ-cleavage enzyme according to reaction 2 and was isolated by several sequential chromatographic procedures, as previously described (Flavin and Slaughter, 1964a). To detect and estimate KEDB, either substrate or maleimide must be labeled. Homoserine was used in some experiments, but gives poor yields because of its low V_{max} and high K_M with this enzyme (Flavin and Segal, 1964). *O*-Succinylhomoserine is the most economical and effective substrate. α-Ketobutyrate, which is always formed together with KEDB, was determined with lactic dehydrogenase (Flavin and Slaughter, 1964b).

Oxidative decarboxylation of KEDB to 2-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))propionic acid (EDP[1]) (structure VIII) could be accomplished by 20-min incubation at 25° at pH 5, after the addition of one-tenth volume of 30% H_2O_2, or by application of 3% H_2O_2 to the same spot as KEDB at the origin of a chromatogram. Alternatively KEDB was allowed to react for 30 min at 25° in a volume of 1 ml containing 0.3 ml of 1 M H_2SO_4 saturated with ceric sulfate. During this time a stream of helium was bubbled slowly through the solution and into a trap with 0.1 N NaOH; the CO_2 evolved was precipitated as $BaCO_3$ for radioassay. To isolate EDP, the reaction mixture was neutralized with KOH, the precipitate was discarded, and aliquots of the supernatant were chromatographed.

The dinitrophenylhydrazone of KEDB was prepared by incubating an aliquot for 30 min at 25° with 0.6 ml of 0.005 M dinitrophenylhydrazine in 2 N HCl. The solution was then extracted with CCl_4, and the latter was dried over $MgSO_4$ and then applied to a chromatogram. The same result was obtained by adding a dilute ethyl acetate solution of dinitrophenylhydrazine · HCl directly to the same spot as KEDB.

Acid treatment of EDP and other compounds was in sealed Pyrex vials; the contents were then evaporated to dryness in a desiccator. Alkali treatments were with KOH in stoppered plastic tubes. At the end of the reaction the pH was brought to 5 with perchloric acid, and precipitated $KClO_4$ was discarded before paper electrophoresis.

The rate of alkaline hydrolysis of succinimide was determined by following the disappearance of the shoulder absorption at 235 mμ. Aliquots were acidified for the determination because succinimide does not absorb at this wavelength at alkaline pH. The rate of alkaline hydrolysis of succinamate was followed by the decline in alkaline hydroxylamine reaction. The optimum color yield from succinamate was obtained when it was heated with alkaline hydroxylamine in a boiling water bath for 5 min.

Materials. The enzyme used in all experiments was the step 3 fraction of cystathionine γ-cleavage enzyme of *Neurospora* (Flavin and Segal, 1964). The source or preparation has been described of maleimide and derivatives (Flavin and Slaughter, 1964a) and ^{14}C and unlabeled *O*-succinylhomoserine (Flavin and Slaughter, 1965). Diethyl methylmalonate was obtained from Sapon Organic Chemicals, and diethyl acetylsuccinate from Aldrich.

Results

It was earlier observed that catalytic hydrogenation transformed KEDB into an electrophoretically separable, weaker acid (Flavin and Slaughter, 1964a). In anticipation of the results below the latter can now be tentatively called α-hydroxy-3-(3′-(*N*′-ethyl-2′,5′-dioxopyrrolidyl))butyric acid (HEDB) (structure XI). Exposure to bromine transformed KEDB to a still weaker acid, EDP (structure VIII). However, EDP also appeared spontaneously after long storage. These results are illustrated in Figure 1a, 4 and 5. It became probable that KEDB did not take up bromine, but that the latter was acting only as an oxidizing agent. This was confirmed by the finding that hydrogen peroxide, or

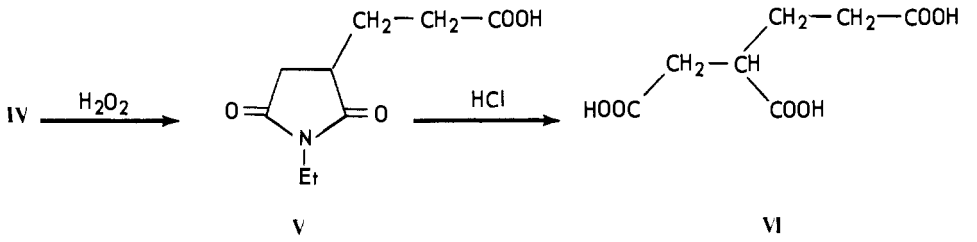


FIGURE 3: Formation of β-carboxyadipic acid (VI) by oxidation of compound IV, followed by acid hydrolysis.

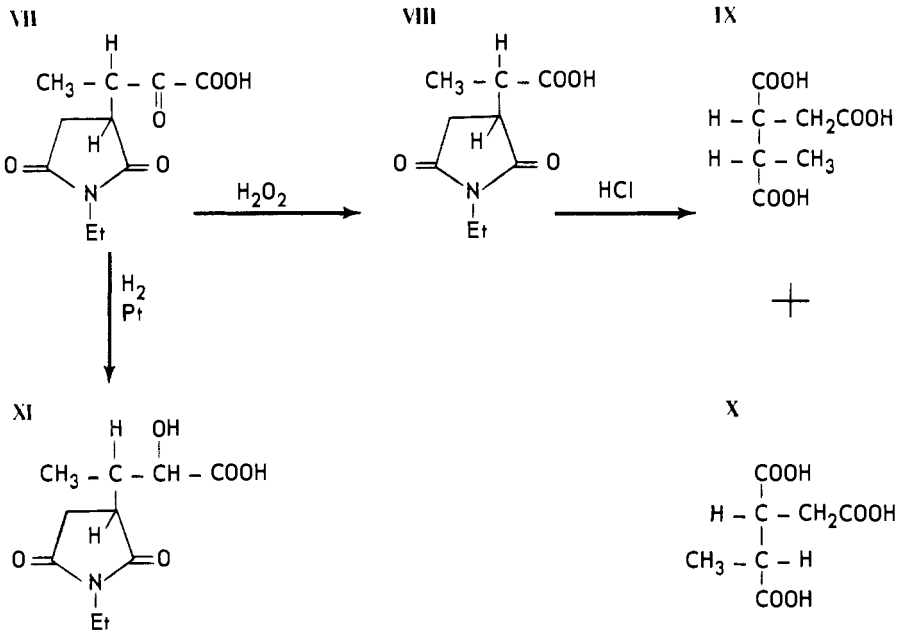


FIGURE 4: Products formed from KEDB (VII) after catalytic hydrogenation (XI, HEDB), and after oxidative decarboxylation (VIII, EDP), followed by acid hydrolysis (IX, *erythro*-, and X, *threo*-α-methyl-β-carboxyglutaric acid).

ceric sulfate, was also able to convert KEDB quantitatively to EDP (Figure 1a, 1–3).

These results were obtained with [2′,5′-^{14}C]KEDB formed from labeled EM, and [2-^{14}C]KEDB formed from [2-^{14}C]homoserine behaved the same way (Table I). However, when [1-^{14}C]KEDB was treated with ceric sulfate, all the radioactivity was recovered as [^{14}C]CO_2. In experiment 3 of Table I, some of the labeled α-ketobutyrate which was formed from [1-^{14}C]homoserine in the same enzyme reaction mixture was also eluted from the electrophoresis paper (most of it had evaporated). Its radioactivity was also recovered as [^{14}C]CO_2 under the same conditions.

When KEDB was treated with DNP and then rechromatographed, all of the radioactivity migrated to a new position, which coincided with a yellow spot (Figure 1b). EDP was unchanged after the same treatment. These results were confirmed by chromatography in other solvents, which separate KEDB and EDP.

These experiments indicated that the first two carbons of the substrate were present as an α-keto acid function

TABLE I: Liberation of [^{14}C]CO_2 by Ceric Sulfate Treatment of KEDB Labeled in Different Positions.[a]

Expt	Enzyme Reaction Product	Source of Label	% ^{14}C Present Recovered as $BaCO_3$
1	[2′,5′-^{14}C]-KEDB	[2,5-^{14}C]EM	0.9
2	[2-^{14}C]-KEDB	[2-^{14}]Homoserine	2.0
3	[1-^{14}C]-KEDB	[1-^{14}C]Homoserine	100
	[1-^{14}C]α-Keto-butyrate	[1-^{14}C]Homoserine	91

[a] Isolation of enzyme reaction products and conditions for ceric sulfate oxidation are described in the text.

TABLE II: Paper Chromatographic R_F Values and Paper Electrophoretic Mobilities.

Compound	Chromatography Solvent[a] 2	8	9	Electrophoretic System[b] eph. 1[c] pH 3.5	eph. 2[c] pH 3.8	eph. G1[d] pH 3.5	eph. G2[e] pH 2.0	eph. G3[f] pH 1.7
α-Methyl-β-carboxyglutaric acid								
isomer mp 147° (from KEDB)	0.76	0.21		4.2	6.9	37		
(synthetic)	0.76	0.21		4.2	6.9	37	16	4.1
isomer mp 183° (from KEDB)	0.75	0.21		4.2	7.5	47		
(synthetic)	0.75	0.21		4.2	7.5	47		4.5
β-Carboxyadipic acid	0.69	0.20		2.6	3.8	28		
$KEDB_1$			0.89	10		70	72	43
$KEDB_2$			0.89	10		72	83	61
EDP_1		0.74	0.89	3.5		17	9	3
EDP_2		0.74	0.89	3.5		19	14	3
$WMal_{1a}$	0.77	0.53				24		
$WMal_{1b}$	0.77	0.53				26		
$WMal_{1c}$	0.77	0.53				30		
KDB				9.5				
KPDB				8				
KEDB dinitrophenylhydrazone			0.71					
Dinitrophenylhydrazine			0.94					
α-Ketobutyrate dinitrophenylhydrazone			0.71					
Pyruvate dinitrophenylhydrazone			0.61					
α-Ketobutyric acid				11.2				
N-Ethylmaleamic acid	0.86	0.74				27		3.2
Succinic acid	0.69	0.32		2.5	5.3	22		
Fumaric acid	0.83			9.4	12.5	84	33	9.7
Maleic acid	0.48	0.28		12.3	13.3			67
O-Succinylhomoserine				1.8		10		
N-Succinylhomoserine				4.7		34		3.5

[a] Chromatographic solvent mixtures: 2, isoamyl alcohol saturated with 4 M formic acid, descending; 8, ethanol–water–concentrated ammonia (7:1:2), ascending; 9, 1-butanol–ethanol–water (5:1:4), organic phase, descending. [b] The paper electrophoretic procedures are described in the text. Values are distance migrated toward the anode in centimeters per: [c] 100 min. [d] 300 minutes at 5000 v. [e] 300 min at 4000 v. [f] 360 min at 4000 v.

in KEDB, and suggested that a C–C bond might link the substrate and maleimide moieties of the latter. The properties of maleimide implied that such a bond might result from an attack on its electrophilic double bond by an enzyme-bound intermediate with carbanion character.[4] Intermediate I (Figure 2; for the proposed reaction sequence see Flavin and Slaughter, 1964a) seemed the best candidate, with potential carbanion character on the α and γ carbons. However, a bond to the α carbon would prohibit formation of the observed carbonyl function. A γ-carbon attack would yield II which could decompose to yield structure IV for KEDB. Oxidative decarboxylation of IV followed by imide hydrolysis should then yield β-carboxyadipic acid (VI, Figure 3). When KEDB was degraded in this way, no radioactive β-carboxyadipic acid was found in the products. A synthetic sample of the latter was easily separated[3] from what appeared, by the earlier chromatographic procedures, to be a single homogeneous product from KEDB (Table II).

It was now necessary to consider the possibility of a β-carbanion attack on maleimide, yielding structure VII for KEDB (Figure 4). The two diastereoisomers of α-methyl-β-carboxyglutaric acid (IX and X) were synthesized. Since it is now known which is the *erythro* (IX) and which the *threo* (X) isomer (Alder and Soll, 1949), they will be designated by their respective melting points, 147 and 183°. In several chromatographic systems which did not resolve these isomers they migrated together with the products obtained by decarboxylation followed by strong acid hydrolysis of KEDB (Table II).

[4] Maleimides undergo radical-induced polymerization (Tawney *et al.*, 1961), so a free radical attack on the double bond is another possibility. However, there has not been evidence for unpaired electron intermediates in pyridoxal-P mediated reactions.

TABLE III: Acid and Alkali Hydrolysis Products[a] from Peroxide Treated KEDB Labeled in Various Positions

Expt	Source of Label in KEDB	Hydrolysis conditions			% Recovered ^{14}C Present in					
		Time (min)	Temp (°C)	Solvent	EDP	$WMal_{Ia}$	$WMal_{Ib}$	$WMal_{Ic}$	α-Methyl-β-carboxyglutaric Acid Isomer	
									mp 147°	mp 183°
1	[2,5-^{14}C]EM	4	25	pH 12.5	83					
		5	25	0.05 N KOH	69					
		30	45	0.05 N KOH	45	9.8	9.9	35	0.63	0.53
		120	60	0.5 N KOH	12				3	2
2	[2,5-^{14}C]EM	4000	120	6 N HCl					51	49
3	*O*-Succinyl[2-^{14}C]homoserine	4000	120	6 N HCl					53	47

[a] Hydrolysis products were separated by eph. G1 (Table II), and their amounts were determined by radioassay after elution.

In the eph. G1 system, which separated the synthetic isomers, the KEDB product was also resolved to two components of mobilities identical with those of the synthetic isomers. These were present in equal amounts in acid hydrolysates from both [2-^{14}C]KEDB and [2′,5′-^{14}C]KEDB (Table III).

Various mild alkaline treatments of EDP (VIII) also yielded small amounts of the two isomers of α-methyl-β-carboxyglutaric acid, and at least three still unidentified components, $WMal_{Ia-c}$, presumably isomeric amides (Table III). In 1 M NaOH at 45° the pseudo-first-order rate constant for the hydrolysis of succinimide was found to be 0.064 min^{-1}, and that of succinamate 0.015 min^{-1}. The rate of disappearance of EDP in alkali (Table III) was closer to that of succinimide, suggesting the presence of an intact imide ring.

The two radioactive products isolated from KEDB in experiment 2 of Table III were recrystallized three times with the respective carrier synthetic isomers without change in specific radioactivity, as was the *p*-bromophenacyl triester prepared from the isomer of mp 147° (Table IV). In the synthesis of α-methyl-β-carboxyglutaric acid it was observed that the isomers were interconverted when refluxed in 6 N HCl. This isomerization was found to be complete in 17 hr at 120°. The same equilibrium mixture, containing about 60% of the isomer of mp 183°, was reached in 17 or 70 hr, starting with either isomer (Table V). Strong evidence for the identity of the synthetic isomers with the KEDB hydrolysis products was provided by the fact that, when either of the latter was treated in the same way, all the radioactivity was recovered as the same equilibrium mixture of both isomers (Table V).

Discussion

The results indicate that the product of the enzymatic reaction between γ-substituted amino acids and maleimides is compound VII, KEDB. The portions of this reaction product originating from the first and second carbons of the amino acid were shown to be present as a carboxyl and carbonyl group, respectively. The alkali lability of the weaker acid (EDP, VIII) obtained after oxidative decarboxylation was compatible with retention of an intact *N*-ethylsuccinimide ring. Hydrolytic conditions expected (Smyth *et al.*, 1964) to liberate ethylamine from the latter decomposed EDP, and all of the radioactive label present, whether initially incorporated from the amino acid or from EM, was recovered as a mixture of the two isomers of α-methyl-β-carboxyglutaric acid. In a subsequent paper[5] further confirmation of structure VII will be reported, derived from spectroscopic studies of the original compound isolated as its dinitrophenylhydrazone.

Partial alkaline hydrolysis of EDP yielded at least three other components besides small amounts of α-methyl-β-carboxyglutaric acid. This is consistent with

[5] M. Flavin and S. Tsunakawa, manuscript in preparation.

the possible formation of four isomeric *N*-ethylmonoamides from VIII. KEDB has two asymmetric centers, so two diastereoisomers will be present if it is racemic at one or both of these. As isolated, both KEDB and EDP were homogeneous by chromatography, but could be resolved to two components by high voltage electrophoresis which resolved diastereoisomeric mixtures of other acids (Table II). However this result does not prove that both diastereoisomers of KEDB are formed directly by the enzyme because interconversion of the isomers has been found to be very facile.[5] The degradations reported here employed mixtures of these components, and of course yielded both isomers of α-methyl-β-carboxyglutaric acid (apart from the fact that in strong acid the latter are also interconverted).

TABLE IV: Recrystallization of Labeled Enzyme Reaction Products with Synthetic Isomers of α-Methyl-β-carboxyglutaric Acid.

Expt	Crystal Fraction	Crystallization Solvents	Specific Radioactivity (cpm × 50 mg)
1. Isomer mp 147°, enzyme product + carrier	Coprecipitate		740
	Recrystallization 1	Acetonitrile	610[a]
	Recrystallization 2	Acetonitrile	860
	Recrystallization 3	Acetonitrile	800
2. Isomer mp 183°, enzyme product + carrier	Coprecipitate		720
	Recrystallization 1	Water	660[a]
	Recrystallization 2	Methanol–chloroform	800
	Recrystallization 3	Methanol–chloroform	860
3. *p*-Bromophenacyl triester prepared from third crystals of expt 1	Amorphous ppt		230
	Recrystallization 1	Acetone–ethanol	230
	Recrystallization 2	Benzene–carbon tetrachloride	250
	Recrystallization 3	Benzene–carbon tetrachloride	230

[a] These lower values were due to the fact that the crystals were not ground to a powder before plating.

The identification of the structure of KEDB has revealed the existence of a new type of pyridoxal-P potentiated enzymatic reaction, which can be regarded as elimination from four-carbon amino acids coupled to Michael addition of a β-carbanion to an electrophilic double bond.[4] Although this paper concerns the structure of KEDB, it is appropriate to summarize here the current status of results which bear on the nature of this reaction and the identity of the intermediary carbanion which reacts with maleimide. The early observation (Flavin and Slaughter, 1964a) that maleimide did not react with any intermediate in β elimination from three-carbon amino acids (reaction 3),

$$ACH_2CH(NH_2)COOH + H_2O \longrightarrow AH + NH_3 + CH_3C(=O)COOH \quad (3)$$

The recent observation (Flavin, 1965b) that KEDB was formed during β elimination from four-carbon amino acids (reaction 4) has ruled out the first hypothesis, and focussed attention on the terminal intermediates related to aminocrotonate which would be common to both β and γ elimination.

$$ACHCH(CH_3)(NH_2)COOH + EM + H_2O \longrightarrow AH + NH_3 + KEDB \quad (4)$$

Also it was recently found (Flavin, 1965b) that, when high concentrations (but not the low concentrations formed enzymatically from amino acids) of α-ketobutyrate and ammonia were incubated with EM in neutral aqueous solution, small amounts of KEDB were formed. Reaction 5 required ammonia, but not pyridoxal-P or enzyme.

$$CH_3CH_2COCOOH + EM \longrightarrow KEDB \quad (5)$$

Ammonia cannot be replaced, under these conditions,

TABLE V: Acid-Catalyzed Interconversion of Synthetic and Enzymatically Formed Isomers of α-Methyl-β-carboxyglutaric Acid.

Initial Synthetic Isomer (mp, °C)	Treatment	Proportion[a] of Added Compound Recovered in Isomer mp 147°	mp 183°
147	None	10+	1+
147	30 min at 45°, 0.05 N KOH	10+	1+
147	1000 min at 120°, 6 N HCl	3+	5+
147	4000 min at 120°, 6 N HCl	3+	5+
183	None	0	10+
183	1000 min at 120°, 6 N HCl	4+	5+
183	4000 min at 120°, 6 N HCl	4+	5+
Initial ^{14}C Isomer from KEDB			
147	1000 min at 120°, 6 N HCl	42%	58%
147	4000 min at 120°, 6 N HCl	44%	56%
183	1000 min at 120°, 6 N HCl	39%	61%
183	4000 min at 120°, 6 N HCl	41%	59%

[a] The proportions of products formed from synthetic isomers were estimated visually from sprayed electropherograms (Flavin and Slaughter, 1964a). The products from the isomers isolated from KEDB are expressed as per cent of the added radioactivity which was recovered in eluates from the electropherogram after locating radioactive spots with X-ray film.

by primary or secondary amines, but this might be due to rapid addition of the latter to EM.[6] Pyruvate reacts with EM as extensively as α-ketobutyrate does, yielding an analog of KEDB from which tricarballylic acid can be isolated after oxidation and acid hydrolysis. The catalytic role of ammonia in reaction 5 has not yet been determined.

In any case, in comparing reaction 5 with the enzymatic formation of KEDB from amino acids (reactions 2 and 4), two observations must be considered. The first is the fact that reaction with EM requires alkyl substitution on the β carbon in the latter case (reaction 4), but does not require it in the nonenzymatic reaction. The second is that the relative yield of KEDB varies greatly with different enzymes catalyzing reaction 2.[7] Enzyme control implies either that (a) free aminocrotonate is not the intermediate that reacts with EM or (b) it is, but different enzymes catalyze reaction 1 by different pathways, in some of which free aminocrotonate is not an intermediate at all. The following alternative schemes could account for the observed results in either of these two ways.

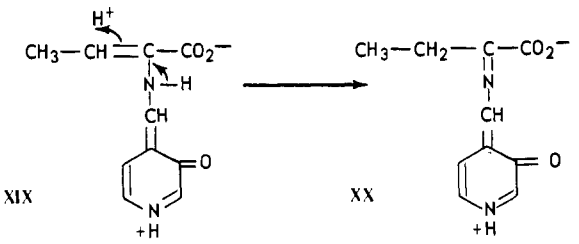
One possibility is illustrated in Figure 5. In conventional schemes free aminocrotonate is liberated from XII by hydrolysis. Electron withdrawal into the pyridine ring in XII potentiates the formation of threonine (XII → XIV). However, because the ring has a phenolic substituent, it also has the potentiality to donate electrons. Structures XIIa → XV → XVI show a resultant path[8] in which free vinylamines are not intermediates in the formation of keto acids. Compound XII could be decomposed by hydrolysis by some enzymes, but by XIIa → XV by others, or the same enzyme might decompose XIIa in different ways depending on whether the substituent was hydrogen or methyl. On the other hand, XII could itself react with maleimide by XII → XVII → XVIII (Flavin, 1965a).

A simpler alternative is suggested by considering that the steps by which amino acid substrates have been proposed to bind to transaminase might be the same, in reverse, as those by which vinylamino acid products are liberated in reactions 1, 3, and 4. The latter would then occur as follows: the bond to coenzyme in XII would be broken through transaldimination by an unprotonated enzyme lysine amino group to yield the protonated lysine–coenzyme Schiff base and unprotonated, enzyme-bound vinylamino acid (Jencks and Cordes, 1963); the latter would not escape from the enzyme until after it had undergone enzyme-directed

[6] N. E. Sharpless and M. Flavin, manuscript in preparation.

[7] Under the same reaction conditions, it was shown that the fraction of either homoserine or cystathionine decomposed by cystathionine γ-cleavage enzyme from liver that could be recovered as KEDB was only 10% of the amount that was formed by the comparable *Neurospora* enzyme (Flavin and Slaughter, 1964a). A cystathionine γ-synthetase purified from *Salmonella* (Kaplan and Flavin, 1965a), which decomposes *o*-succinylhomoserine by reaction 1 in the absence of cysteine, yields only 0.1% as much KEDB by reaction 2 as the *Neurospora* enzyme does from the same substrate (M. Kaplan and M. Flavin, manuscript in preparation). This amount is so small that it could be formed secondarily from α-ketobutyrate by reaction 5.

[8] Spectroscopic evidence has suggested that the Schiff base of aminocrotonate might exist in solution as the quinoid form XIX (Martell, 1963). Enzymatic catalysis of α-ketobutyrate formation could then occur by the simple prototropic shift, XIX → XX.



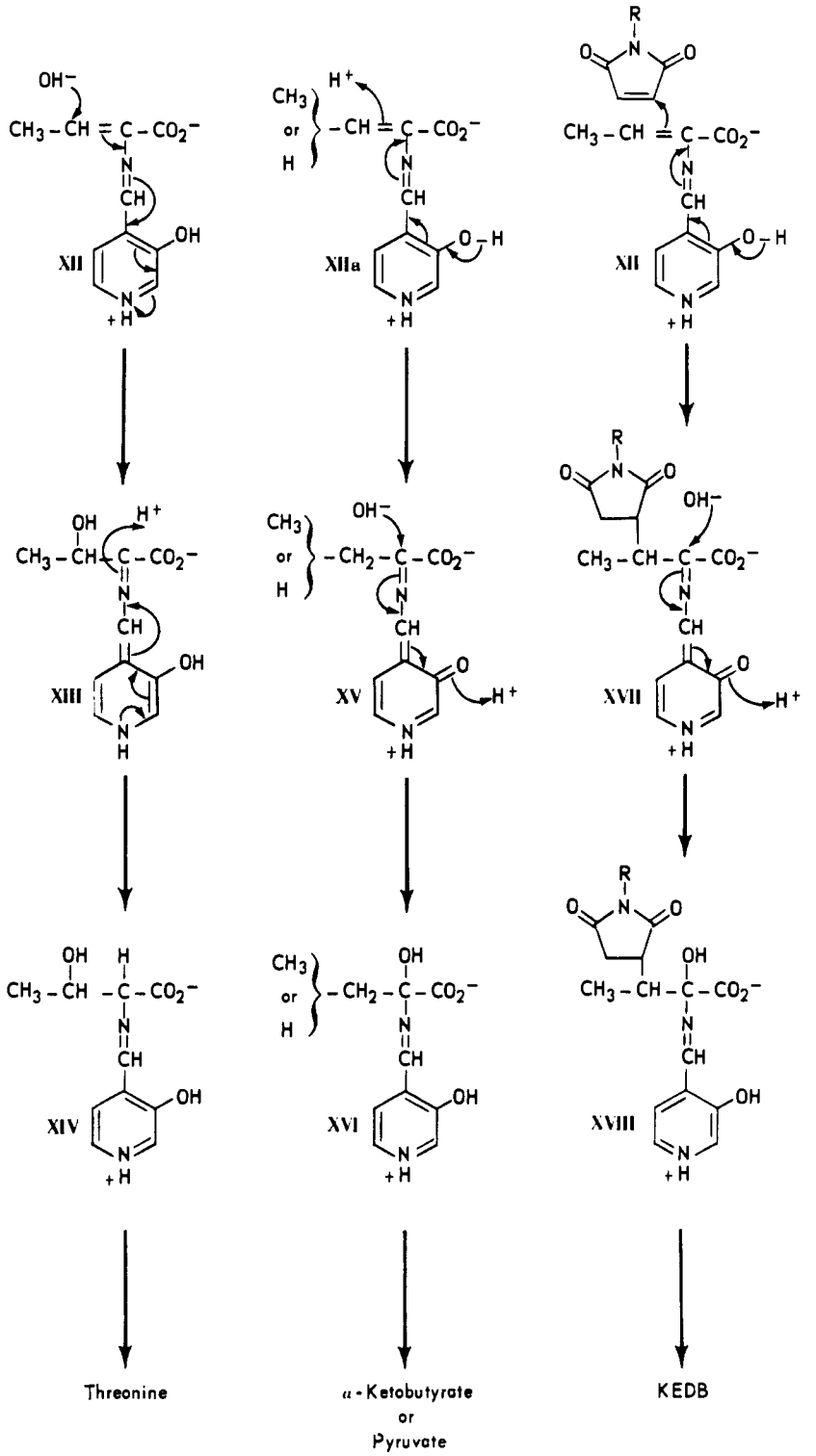


FIGURE 5: Alternative mechanisms for the formation of threonine (XII → XIV), α-ketobutyrate and pyruvate (XIIa → XV → XVI), and KEDB (XII → XVII → XVIII). Some pyridoxal-P ring substituents have been omitted for convenience.

protonation (Hammes and Fasella, 1963; Bruice, 1964; Jenkins, 1964). The enzyme-bound unprotonated vinylamino acid would be a reactive carbanion, comparable to the common anion of a keto–enol pair (Szmuszkovicz, 1963), and suited to undergo alkylation by maleimide.

Here also, however, free aminocrotonate might be the intermediate which reacts with maleimide. If in some

cases (*i.e.*, three-carbon amino acids with all enzymes and four-carbon with cystathionine γ-synthetase[7]) a proton was transferred from enzyme directly to carbon, rather than nitrogen, of the bound vinylamino acid, the first free intermediate would be an imino acid rather than aminocrotonate or aminoacrylate. The imino acid would have no potentiality to react with maleimide.

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