Structure of the Product of the Enzymatic Reaction between Maleimides and Substituted 4-Carbon Amino Acids

SPECTROSCOPIC PROPERTIES OF HYDRAZONE DERIVATIVES

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SUMMARY

In elimination reactions, catalyzed by pyridoxal phosphate enzymes, from β- or γ-substituted 4-carbon amino acids, a transient intermediate is trapped by N-ethylmaleimide, leading to the accumulation of a new product in place of α-ketobutyrate. From examination of degradation products, this compound has tentatively been assigned the structure α-keto-3-[3′-(N′-ethyl-2′,5′-dioxopyrrolidyl)]butyric acid (KEDB). KEDB has now been isolated in pure form as its dinitrophenylhydrazone (DNPH) derivative, as has the corresponding compound, α-keto-3-[3′-(2′,5′-dioxopyrrolidyl)]butyric acid (KDB), formed when maleimide was substituted for N-ethylmaleimide. The molar specific radioactivity (compared with that of a labeled precursor) and ultraviolet absorbance of KEDB-DNPH were compatible with the molecular weight, 407, of the proposed structure, and the infrared spectrum indicated the presence of an intact succinimide ring. The presence or absence of optical activity could not be determined because of rapid racemization, as shown by interconversion of diastereoisomers, during the isolation procedure. The elementary composition of all the ions in a mass spectrum of KEDB-DNPH was determined. The peak of highest mass corresponded to the proposed structure less CO₂. However, the spectrum of α-ketobutyrate-DNPH also showed only a weak molecular ion, and an intense peak reflecting decarboxylation. The spectrum of KDB-DNPH served to distinguish fragments originating from DNPH, which were of the same mass as those from KEDB-DNPH, from those containing the succinimide group, which were 28 mass units lower. The elemental composition of fragments thus shown to be derived from the succinimide moiety (C₉H₅NO₄) of KEDB-DNPH, and containing 9, 8, or 6 carbon atoms, was consistent with plausible fragmentations of a structure in which N-ethylsuccinimide is connected with the carbonyl carbon of the α-keto acid moiety by —(CH₃)CH—, whereas the low abundance of γ-carbon fragments was evidence against a linkage through a —CH₂CH₂— bridge.

When maleimide, or an N-substituted derivative, was added to reaction mixtures containing a β- or γ-substituted 4-carbon amino acid and a pyridoxal phosphate enzyme which otherwise catalyzed elimination, a new kind of reaction occurred (1-4). The amino acid substituent (designated A since it could be any of several electronegative groups), as well as ammonia, continued to be liberated, but in place of α-ketobutyrate another compound was formed, which contained the elements of the maleimide (Reactions 1 and 2).

\[
\begin{align*}
\text{CH₃} & \quad A-\text{CH}-\text{CH(NH₂)COOH} + \text{H}_2\text{O} + \text{EM} \rightarrow \quad \text{AH} + \text{NH}_3 + \text{KEDB} \\
\text{CH}_3 \text{CH}_2\text{CH(NH₂)COOH} + \text{H}_2\text{O} + \text{EM} & \rightarrow \quad \text{AH} + \text{NH}_3 + \text{KEDB} 
\end{align*}
\]

If this product, labeled by either radioactive amino acid or maleimide, and isolated in trace amounts by chromatography, was treated with peroxide followed by strong acid, the radioactivity was recovered in the two diastereoisomers of α-methyl-β-carboxyglutaric acid (2, 4). From this result it was tentatively assigned the structure α-keto-3-[3′-(N′-ethyl-2′,5′-dioxopyrrolidyl)]butyric acid.

Since this assignment of structure was based on products isolated after a strong degradative procedure, it was desirable to confirm it by obtaining KEDB in pure form. Isolation of this labile compound was made possible by the recognition that it was an α-keto acid which formed a relatively stable DNPH derivative (4). Compound I (Fig. 1) was isolated from reaction mixtures containing O-succinylhomoserine, Neurospora cystathionase γ-cleavage enzyme, and EM. To help identify components in the mass spectrum which contained the succinimide moiety.

This paper presents the results of analyses of these derivatives.

1 The abbreviations used are: KEDB, α-keto-3-[3′-(N′-ethyl-2′,5′-dioxopyrrolidyl)]butyric acid; KEDB₁, the diastereoisomer which is the weaker acid, and KEDB₂, the stronger; DNPH, 2,4-dinitrophenylhydrazone (or -hydrazone, when attached to the name of another compound); EM, N-ethylmaleimide; KDB, α-keto-3-[3′-(N′-phenyl-2′,5′-dioxopyrrolidyl)]butyric acid; KPDB, α-keto-3-[3′-(N′-phenyl-2′,5′-dioxopyrrolidyl)]butyric acid.
Concentrated with helium to 0.1 ml of pale yellow oil, which was and washed with a little methanol, and the filtrate and wash were concentrated, under reduced pressure and then with a stream of helium, to 1 ml. Solids were removed on a sintered glass filter.

Radioassay the extract, after filtration through a sintered glass funnel, contained 146 moles of KEDB. The methanol was concentrated with 200-ml portions of methanol for 20 min at 25°C. By radioassay the extract, after filtration through a sintered glass funnel, contained 146 moles of KEDB. The methanol was concentrated, under reduced pressure and then with a stream of helium, to 1 ml. Solids were removed on a sintered glass filter and washed with a little methanol, and the filtrate and wash were concentrated with helium to 0.1 ml of pale yellow oil, which was kept for 1 hour in a high vacuum desiccator. To the residue, 0.5 ml of cold water was added. The solids were filtered out and washed with cold water. The combined clear filtrates, 0.9 ml, containing 108 μmoles of KEDB, were placed in a centrifuge tube at 0°C, and 0.09 ml of 11 N HCl was added, followed by 15 ml of 0.015 N DNPH in 2 N aqueous HCl. A flocculent precipitate began to form after 10 min. After 150 min the precipitate was separated by centrifugation for 15 min at 14,000 rpm at 0°C. It was then washed by suspending it three times in 1.5 ml of cold water and centrifuging, and was dried overnight over P2O5 at high vacuum in a desiccator. The yield was 32.6 mg (80 μmoles for molecular weight 407) of an orange powder, m.p. 95-100°C (transition from solid to viscous liquid). The compound has been kept for 1 year at +10°C without decomposition.

By radioassay, confirmed by chromatography (see below), the supernatant contained an additional 29 μmoles of KEDB-DNPH. The latter was more soluble in water than α-ketobutyrate-DNPH. It was extracted into ethyl acetate, and most of the contaminating DNPH was removed by precipitation from aqueous methanol. Orange needles were observed in very concentrated methanol solution, but further attempts to recrystallize KEDB-DNPH yielded an oily material, in which chromatography revealed colored decomposition products. The remaining KEDB-DNPH was purified by thick layer silica gel chromatography. However, it could not be eluted without further decomposition.

Isolation of KEDB—KEDB was prepared in a reaction mixture incubated for 120 min at 30°C in a final volume of 90 ml, containing potassium phosphate, pH 7.6, 1800 μmoles; pyridoxal-P, 9 μmoles; O-succinyl-DL-homoserine, 900 μmoles; enzyme, 8 units; and EM-2,5-14C, 720 μmoles (5000 cpm per μmole). A low level of radioactivity made it possible to follow the reaction product throughout the isolation. 14C was measured by adding aliquots, together with 0.5 ml of 0.1 N NaOH, to planchets, which were dried on a steam bath. At 30 min intervals the pH of the reaction mixture was adjusted to 7.6, if necessary, by addition of KOH, and at the same time small aliquots were deproteinized and the amount of α-ketobutyrate liberated was determined; 20 μmoles was formed after 30 min, 32 after 60 min, and 40 after 120 min. The reaction was stopped by adding 9 ml of 1.5 M HClO4, and the pH of the supernatant (hereafter kept cold unless otherwise specified) was adjusted to 4.5 with KOH. After the settled precipitate of KClO4 was discarded, this solution could be stored frozen for several weeks without loss of KEDB.

The pH of the solution was then brought to 2.30 with m H2SO4, and lyophilized overnight. The dry residue (1.7 g) was ground twice in a mortar with 20 ml of warm ethanol (or until no more radioactivity was extracted). The filtered extract was concentrated to 90 ml, 1350 moles of maleimide, 6.4 units of enzyme, and 900 μmoles of O-succinyl-DL-homoserine-4-H (2200 cpm per μmole; tritium in the L isomer only). By 20 min, the earliest assay, 14 μmoles of α-ketobutyrate had been formed, and there was no further increase (this enzyme fraction had previously been losing activity rapidly). The electrophoretic resolution of KEDB was poor, and it partially overlapped α-ketobutyrate (4). Just before DNPH was added, an aliquot was assayed for α-ketobutyrate; 0.49 μmole was still present, and there was no lactic dehydrogenase inhibitor in the solution. KEDB-DNPH, 3.9 mg (10.3 μmoles for molecular weight 407), was similar in appearance to KEDB-DNPH. The latter was more soluble in water than α-ketobutyrate-DNPH. It was extracted into ethyl acetate, and most of the contaminating DNPH was removed by precipitation from aqueous methanol. Orange needles were observed in very concentrated methanol solution, but further attempts to recrystallize KEDB-DNPH yielded an oily material, in which chromatography revealed colored decomposition products. The remaining KEDB-DNPH was purified by thick layer silica gel chromatography. However, it could not be eluted without further decomposition.

Isolation of KDB—KDB was isolated in poor yield by a procedure similar to that used for KEDB. The reaction mixture was incubated at 37°C for 120 min and contained, in a volume of 90, 1350 μmoles of maleimide, 6.4 units of enzyme, and 900 μmoles of O-succinyl-DL-homoserine-4-H (2200 cpm per μmole; tritium in the L isomer only). By 20 min, the earliest assay, 14 μmoles of α-ketobutyrate had been formed, and there was no further increase (this enzyme fraction had previously been losing activity rapidly). The electrophoretic resolution of KDB was poor, and it partially overlapped α-ketobutyrate (4). Just before DNPH was added, an aliquot was assayed for α-ketobutyrate; 0.49 μmole was still present, and there was no lactic dehydrogenase inhibitor in the solution. KDB-DNPH, 3.9 mg (10.3 μmoles for molecular weight 379), was similar in appearance to KEDB-DNPH, m.p. 117-121°C. Chromatography confirmed the presence of not more than 5% of α-ketobutyrate-DNPH.

Isolation of Separated Diastereoisomers of KEDB—The procedure was similar to that used for the mixed isomers, except that the paper electrophoresis was performed with the Gilson model D Electrophorator for 4 hours at 4300 volts at pH 2.4. The final aqueous solutions of the separated isomers, just before addition of DNPH, were examined for optical activity between 4000 and 6000 A with a Rudolph recording spectrophotopolarimeter. The concentrations, light paths, and magnitude of a paper electrophoretic blank were such that a specific rotation of ±20° might not have been reliably detected. The results were negative. However, repeated paper electrophoresis of these fractions (see below) showed that each had already undergone extensive isomerization to the other. The melting points of DNPH de-
Enzymatic products formed from O-succinylhomoserine, and maleimide or N-phenylmaleimide, as function of pH and maleimide concentration

The reaction mixtures were incubated for 2 hours at 30°C in a 1-ml volume containing potassium phosphate, 30 μmoles; pyridoxal-δ, 0.1 μ mole; O-succinyl-DL-homoserine-2,6-C, 6 μmoles; and enzyme, 0.1 unit. N-Phenylmaleimide was added as a 0.1 M solution in acetonitrile.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Amount (μmoles)</th>
<th>pH</th>
<th>Reaction pH</th>
<th>α-Keto-butyrate</th>
<th>KEDB</th>
<th>KPDB</th>
<th>Ratio of KEDB to KPDB</th>
</tr>
</thead>
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<tr>
<td>None</td>
<td>7.6</td>
<td>0.75</td>
<td>7.6</td>
<td>0.9</td>
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<td>Maleimide</td>
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<td>7.6</td>
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<td>0.29</td>
<td>0.3</td>
<td>0.3</td>
<td>0.9</td>
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<tr>
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<td>7.3</td>
<td>0.26</td>
<td>0.40</td>
<td>1.5</td>
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<tr>
<td>Maleimide</td>
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<td>7.6</td>
<td>0.18</td>
<td>0.33</td>
<td>1.8</td>
<td></td>
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<tr>
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<td>7.9</td>
<td>0.05</td>
<td>0.39</td>
<td>7.8</td>
<td></td>
<td></td>
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<tr>
<td>Maleimide</td>
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<td>0.52</td>
<td>10</td>
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<td>7.2</td>
<td>0.65</td>
<td>0.36</td>
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<tr>
<td>N-Phenylmaleimide</td>
<td>2</td>
<td>7.6</td>
<td>0.32</td>
<td>0.30</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chromatography of dinitrophenylhydrazone derivatives

The reaction products were separated by thin layer chromatography on Silica Gel G, 0.5 mm thick, developed with chloroform-formic acid (99:1). The chromatograms were dried at 250°C, and samples were applied in methanol or ethyl acetate. Ultraviolet absorption spectra were determined in methanol, and infrared spectra in KBr pellets. α-Keto-butyrate was determined with lactic dehydrogenase (6); KEDB does not react under the standard conditions.

High resolution mass spectra of Compounds I, II, and III (Fig. 1) were determined in a CEC 21-100 double focusing mass spectrometer in the laboratory of Dr. K. Biemann. Because of their low volatility and relative thermal instability, the samples were introduced directly into the ion source of the instrument.

Materials—The enzyme used in all experiments was the Step 3 fraction of Neurospora cystathionine γ-cleavage enzyme (7). Availability of enzyme was the limiting factor in large scale preparations. Crystalline α-keto-butyric acid (Aldrich) assayed 100% with lactate dehydrogenase. DNPH-HCl (Eastman) was recrystallized from boiling 1-butanol, yielding large transparent needles, m.p. 201–203°C. The source or preparation has been described for maleimide, EM, and EM-2,5,6-C (1), and for O-succinyl-DL-homoserine, O-succinyl-DL-homoserine-2,4-C, and O-succinyl-DL-homoserine-2,6-C (8). N-Phenylmaleimide was a gift from Dr. P. O. Tawney, and N-ε-lysyl-β-chloro-α-aminoacyl-butyrate was a gift from Dr. M. Rabinovitz.

RESULTS AND DISCUSSION

The procedure for isolation of KEDB-DNPH was reproducible in yielding a pure product. The yield varied, because in some experiments the enzyme was inactivated before much of the substrate had reacted. Conditions of high pH and EM concentration, which favor formation of KEDB over α-keto-butyrate, also inactivate the enzyme (1). Because of the manipulations involved, the yield was worse in smaller scale preparations. Since all preparations appear to be mixtures of diastereoisomers, the observed 5 melting point ranges are not surprising. KEDB-DNPH could be heated above its melting point (to 120°C) without decomposition. It did not sublime below its melting point at 1 mm of pressure.

Indirect evidence had previously indicated that EM could be replaced in Reaction 2 by other maleimide derivatives (1). This was confirmed by electrophoretic separation of the reaction products from maleimide (KDB) and from N-phenylmaleimide.
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FIG. 2. Infrared spectra of the dinitrophenylhydrazone of KEDB and of related compounds

(KPDB). The effects of pH and maleimide concentration on the partition of substrate between the former and \( \alpha \)-ketobutyrate (Table I) were similar to those observed with EM (1). At relatively high pH, as much as 90% of the substrate that was decomposed could be converted to KDB (Table I). However, inhibition of the enzyme by the maleimide derivatives also increased at high pH, and was complete above pH 7.9. KDB-DNPH (Compound II, Fig. 1) was then isolated on a larger scale, principally so that its mass spectrum could be compared with that of KEDB-DNPH.

KEDB-DNPH and KDB-DNPH were readily separated and were homogeneous with respect to both radioactivity and color in three solvent systems (Table II). Their ultraviolet absorption spectra showed no significant differences from that of \( \alpha \)-ketobutyrate-DNPH (Table III). On the basis of their respective calculated molecular weights, the molar absorbances were the same as those of \( \alpha \)-ketobutyrate-DNPH, and the molar specific radioactivity of a weighed sample of KEDB-2',5'-\( \text{C} \)-DNPH was 105% of that of the EM-2',5'-\( \text{C} \) from which it was made. Another rough but convenient guide to purity was the absorbance ratio, \( A_{370}/A_{332} \); the ratio was 1 for DNPH and 2.4 for the pure hydrazones of KEDB, KDB, and \( \alpha \)-ketobutyrate. At 332 \( \mu \), the molar absorbance was about 11,000 for all four compounds.

A rough neutralization equivalent for KEDB was 400; that calculated for Structure I (Fig. 1) was 407.

The solid state infrared spectrum of KEDB-DNPH (Fig. 2)
amounts of KEDB are formed in Reaction 3 only with high concentration and pH (Table IV).

It should be emphasized that α-ketobutyrate is not the intermediate which reacts with EM in Reactions 1 and 2. Measurable amounts of KEDB are formed in Reaction 3 only with high concentrations of ammonia and α-ketobutyrate, and at high temperature and pH (Table IV).

Determination of the configuration present at the two asymmetrical centers of KEDB is important to an understanding of the mechanism of its formation (4). The presence of both diastereoisomers in the material first isolated (Table IV, Fraction 1) suggested that one or both centers were racemic, but isomerization has been found to occur so rapidly that it is possible that only one isomer is formed by the enzyme. In the latter case the apparent proportion of isomers was slightly different from that in the spectrum of succinimide. The band at 1692 to 1712 cm⁻¹, which was present in all four spectra, and contains contributions from both imide and carboxyl carbonyls, showed enhanced intensity in KEDB-DNPH compared with α-keto-butyrate-DNPH. These results confirm the presence of an intact succinimide ring in KEDB.

Further evidence concerning the structure of KEDB was obtained by mass spectrometry (for a general discussion of the interpretation of mass spectra, see References 9 and 10). The highest peak (Fig. 3, Peak E) in the mass spectrum of KEDB-DNPH was found to correspond to C₁₆H₁₇NO₆ (calculated 363.1179; found, 363.1166). Since KEDB-DNPH was known to be a free carboxylic acid, this ion must have been derived from a C₁₀H₂₈N₄O₄ compound by loss of CO₂, because the remainder of the mass spectrum requires the presence of both a dinitrophenyl hydrazine group (which accounts for 4 nitrogens and 4 oxygens) and also the N-ethylsuccinimide group (containing the 5th nitrogen and 2 more oxygens), as will be outlined below. The absence of added radiactivity recovered (in both isomers) in KEDB₁ and KEDB₂, by electrophoresis at pH 2; recoveries and isomerization

<table>
<thead>
<tr>
<th>Fraction eluted from electrophoretogram</th>
<th>Percentage of added radiactivity recovered</th>
<th>Percentage of recovered radiactivity present in KEDB₁</th>
<th>Percentage of recovered radiactivity present in KEDB₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. KEDB formed enzymatically*</td>
<td>17</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>2a. KEDB formed spontaneously*</td>
<td>26</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2b. KEDB₁ from 2a (after second electrophoresis)</td>
<td>100</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>2c. KEDB₁ from 2a (after incubation with enzyme and second electrophoresis)</td>
<td>80</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>2d. KEDB₂ from 2a (after second electrophoresis)</td>
<td>80</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>2e. KEDB₂ from 2a (after incubation with enzyme and second electrophoresis)</td>
<td>50</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

* The reaction mixture was maintained for 1 hour at 30°C at pH 7.5, and contained (per ml) DL-erythro-β-chloro-α-aminobutyrate, 10 μmoles; 2,5-¹C-EM, 5 μmoles; pyridoxal-P, 0.1 μmole; and 0.1 unit of Step 3 cystathionine γ-cleavage enzyme from Neurospora.

CH₃CH₂COCOOH + EM → KEDB (3)

This reaction requires ammonia as catalyst, but not enzyme (3). It should be emphasized that α-ketobutyrate is not the intermediate which reacts with EM in Reactions 1 and 2. Measurable amounts of KEDB are formed in Reaction 3 only with high concentrations of ammonia and α-ketobutyrate, and at high temperature and pH (Table IV).

α-Keto-3-[3'-(N'-ethyl-2',5'-dioxopyrrolidyl)]butyric Acid

* N. E. Sharpless and M. Flavin, unpublished results.
of a molecular ion was not too surprising since the high resolution mass spectrum of α-ketobutyrate-DNPH itself (Compound III, Fig. 1) showed only a weak molecular ion and an intense peak corresponding to the molecular ion less CO₂.

The determination of the elemental composition of all the ions in this spectrum (Fig. 3) revealed that many of them had a high nitrogen and oxygen content and corresponded to those found in dinitrophenylhydrazine itself as well as in the spectrum of α-ketobutyrate-DNPH, i.e. the ions C₆H₅N₂O₂ (F), C₆H₇N₃O₃ (G), and C₆H₈N₃O₄ (H). The presence of these ions confirms that Compound 1 is an unmodified dinitrophenylhydrazone; thus the C₄H₄N₄O₄ portion of the molecule must be accounted for by a DNPH residue. It then remained to assign the residual atoms, namely, C₆H₁₁N₂O₄ of which 2 carbons, 2 oxygens, and 1 hydrogen had to be the carboxyl group plus the carbon atom of the former carbonyl.

Conclusive evidence for the presence of an unmodified N-ethylsuccinimidy l group could be deduced from the elemental composition of the peaks at m/e 127 (Fig. 3, A, C₄H₄NO₂), 128 (A + hydrogen), 138 (B, C₆H₇NO₂), 140 (B + 2 hydrogens), and 153 (C, C₆H₈NO₂), of which those at m/e 127 and m/e 153 were much more abundant than the others. These fragments must have been derived by loss of the part of the molecule attached to the succinimide moiety with transfer of a hydrogen toward it,
resulting in the C$_4$H$_9$NO$_2$ ion (A in Fig. 4, upper) as well as by cleavage of the $\alpha,\beta$ bond with rearrangement of a hydrogen from a $\gamma$ carbon to the carbonyl group. This process, which is often observed in the mass spectrum of carboxylic acids, would then lead to the C$_8$H$_{10}$NO$_2$ ion (C in Fig. 4, lower).

Consideration of these results seemed to require that the N-ethylsuccinimidyl part of the molecule be connected with the carbonyl carbon of the $\alpha$-keto acid moiety by a group that totaled C$_2$H$_4$, and that could be $\text{CH}_2-\text{CH}_2-$ or $\text{CH}(\text{CH}_3)-$. The low intensity of the succinimidyl fragment with a total of 7 carbon atoms (Fig. 3) would tend to eliminate the former possibility, since cleavage of a carbon-carbon bond between two methylene groups should be a rather facile process and give rise to a much more abundant ion of the composition C$_7$H$_{10}$NO$_2$. The structure with a free terminal methyl group (Compound I, Fig. 1) could yield a C$_7$H$_{10}$NO$_2$ ion only after prior loss of the methyl group, or in another equally unfavorable rearrangement process, and is thus in better agreement with the spectrum. This evidence against the presence of a $\text{CH}_2-\text{CH}_2-$ group in this part of the molecule is consistent with the failure to detect any $\beta$-carboxyadipic acid in the hydrolysate of KEDB (4).

The mass spectrum of KDB-DNPH resembled that of KEDB-DNPH with the exception that all peaks due to fragments containing the succinimidyl group were found 28 mass units (C$_2$H$_4$) lower, while those originating from the dinitrophenylhydrazone moiety remained the same.

Corollary evidence for the presence of a dinitrophenylhydrazone of an $\alpha$-keto acid could be deduced from the quite intense peak at m/e 180 in the spectrum (Fig. 3, Peak D) of KEDB-DNPH, and m/e 152 in the spectrum of KDB-DNPH. The elemental compositions were found to be C$_9$H$_{15}$N$_2$O and C$_9$H$_{15}$N$_2$O$_2$, respectively, and their formation can be explained as shown in Fig. 5.

In conclusion, these results indicate that KEDB-DNPH has Structure I shown in Fig. 1.

Acknowledgments—The authors are indebted to Dr. K. Bie mann for discussion of the mass spectra, and to H. K. Miller for the infrared and optical rotatory dispersion measurements.

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