THE SYNTHESIS, SOME DERIVATIVES, AND THE METABOLISM
OF \( \alpha, \gamma \)-DIKETO-\( n \)-OCTANOIC ACID

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It is generally believed that in the course of the biological oxidation of fatty acids either mono- or polyketonic acids are formed as intermediates preceding the breakdown to the ketone bodies and other fragments. However, such higher ketonic acids have never been isolated from mammalian tissues, and only a few compounds pertaining to this situation have been synthesized and described. It is apparent that in order to determine the detailed mechanism of fatty acid oxidation to the same degree which has been accomplished for carbohydrate metabolism the chemistry and physiology of such hypothetical intermediate ketonic acids must be approached experimentally.

Since an \( \alpha, \gamma \)-diketo acid (acetopyruvic acid) has shown marked metabolic activity in the in vitro experiments of Krebs and Johnson (1) and in the in vivo experiments of the author (2), it was decided to investigate the synthesis and metabolism of an 8-carbon homologue having the \( \alpha, \gamma \)-diketo configuration; i.e., \( \alpha, \gamma \)-diketo-\( n \)-octanoic acid. Although there is no definite experimental reason at present to suspect the biological occurrence of \( \alpha, \gamma \) oxidation of even chain fatty acids, Jowett and Quastel (3) have pointed out that this mechanism may well obtain in the case of the odd carbon acids, and that the possibility of its occurrence in the case of even carbon acids cannot be dismissed. The choice of the 8-carbon homologue was made, since it invited comparison in biological activity with its parent substance, octanoic acid, which represents the maximum effective chain length for in vitro work (due to solubility considerations (3)).

Since this compound has not been previously described, this report will consider first its synthesis, properties, and certain of its derivatives; studies on its intestinal absorption and metabolism will complete the report.

Synthesis, Properties, and Derivatives—Lower homologues have been prepared by the Claisen condensation of ethyl oxalate with the appropriate methyl ketone in the presence of sodium ethoxide, followed by saponification of the ester formed (4–7). Although the velocity of this condensation diminishes considerably with increasing size and complexity of the methyl ketone, it was found possible to effect the condensation of methyl \( n \)-butyl ketone and ethyl oxalate in 60 per cent yield by running the reaction at
higher temperatures and by recovering the product as the ethyl ester, rather than by the more usual isolation of the sodium derivative, which in this case was found to be quite soluble in the alcoholic medium.

That the condensation of methyl n-butyl ketone with ethyl oxalate actually took place with the methyl group, resulting in \( \alpha,\gamma \)-diketo-octanoic ester (I), and not with the \( \alpha \)-methylene group (which would result in \( \alpha \)-keto-\( \gamma \)-acetylhexanoic ester (II)), was demonstrated by a method successfully employed by Tracy and Elderfield (8) in the case of a lower homologue. The homogeneous ethyl ester formed in the Claisen condensation was allowed to react with phenylhydrazine, forming a substituted pyrazolecarboxylic ester. This was saponified and the alkyl side chain oxidized with alkaline permanganate. If the \( \alpha,\gamma \)-diketo configuration resulted in the original Claisen condensation, then the product of the permanganate oxidation should be 1-phenylpyrazole-3,5-dicarboxylic acid (III) (9); if the branched chain acid resulted, then the product of the oxidation should be 1-phenylpyrazole-3,4,5-tricarboxylic acid (IV) (10). Only the former compound could be isolated, and since the ester formed in the Claisen condensation was homogeneous this is proof of the \( \alpha,\gamma \)-diketo-octanoic acid structure (see Formulas I to IV).
Both the ester and the free acid react with hydrazine or substituted hydrazines to form pyrazole derivatives. Tracy and Elderfield (8) have shown that when the ethyl ester of a lower homologue reacts with phenylhydrazine, two isomeric pyrazoles are formed, depending on whether the α- or γ-carbonyl group is initially involved. Only one homogeneous product was grossly apparent with the 8-carbon homologue when semicarbazide or 2,4-dinitrophenylhydrazine were condensed with the free acid. In the case of condensations of the hydrazines with the ethyl ester homogeneous derivatives were isolated, but no special attempt was made to determine the presence of the other isomers nor to determine the configuration of the isolated derivatives.

The free diketo acid was found to be liquid at room temperature and only very slightly soluble in water. It formed readily soluble sodium and potassium salts. The barium, magnesium, calcium, copper, lead, silver, and mercury salts were insoluble as expected. The diketo acid was labile to alkali, decomposing into methyl butyl ketone and oxalic acid in the presence of excess NaOH. At pH 8.0 at 0° a 0.05 M solution showed noticeable decomposition in 2 days, as evidenced by the odor of methyl butyl ketone which developed on standing.

The general method for the synthesis of α,γ-diketo esters was employed in an attempt to synthesize a 20-carbon homologue. A sample of hexadecanone-2 (prepared by the dry distillation (11) of a mixture of the barium salts of acetic and margaric acids, the latter being of questionable purity) was condensed with ethyl oxalate in the presence of sodium ethoxide by using a 24 hour period under a reflux. Elementary analysis of the ester formed agreed with the calculated values within the error of the determinations, but a constant melting point could not be obtained. This was probably due to the contamination of the original margaric acid by lower and higher homologues which would be carried through into the final condensation product as homologous α,γ-diketo esters. The difficulties of detecting and removing such impurities are of course well known. The synthesis of higher homologues is apparently perfectly feasible by this method and rests only on the availability of the appropriate methyl ketones.

Metabolism—The experiments described on the biological utilization of this compound are perhaps not very promising when considered from the point of view of in vitro work on surviving tissue slices. The compound (in solution as the sodium salt) was not absorbed readily from the alimentary canal, nor did it affect in any way the basal $Q_{o_2}$ of surviving rat tissue slices. It must be noted that the compound forms almost insoluble salts with calcium and magnesium and for this reason the usual Krebs-Ringer-phosphate buffer was substituted by an isotonic phosphate-saline buffer. That the compound was not freely diffusible into the slices as a probable consequence of this solubility behavior was shown when it was found that broken cell
preparations, such as fine minces and homogenates, could oxidize the compound, albeit slowly, whereas the whole slice showed no extra oxygen uptake in its presence.

The compound was found to be decarboxylated by yeast carboxylase at an extremely low rate, and indeed acted as an inhibitor in pyruvate decarboxylation in the same system. Anaerobic experiments with mammalian tissues showed no dismutation effects as described by Krebs and Johnson for pyruvic acid (12).

The failure of the compound to be metabolized to any great extent contrasts sharply with the metabolic activity of acetopyruvic acid, its lower homologue (1, 2), and may indicate that it is not a normal metabolite. However, caution must be maintained in this regard because the experiments have called attention to perhaps the greatest problem in experimentation in vitro on fatty acid oxidation; namely, the technical problem of solubility, diffusion, and physical state of the lipid investigated. Although it is known that liver slices oxidize endogenous 16- and 18-carbon fatty acids (13), those same compounds invariably reduce the basal respiration of slice and cell-free preparations (14) when added to these as their slightly soluble alkali salts. From these considerations, it would perhaps be presumptive to rule out the possibility of $\alpha,\gamma$-diketo intermediates on the basis of the exploratory work here reported. It is quite clear, however, that the diketo acid is relatively inert compared to its parent substance, octanoic acid.

EXPERIMENTAL

*Synthesis and Derivatives of $\alpha,\gamma$-Diketo-octanoic Acid*

**Ethyl $\alpha,\gamma$-Diketo-n-octanoate**—As starting material for the Claisen condensation, n-butyl methyl ketone was prepared via the acetoacetic ester synthesis (15); the ethyl oxalate was an Eastman product.

To 2.42 gm. of sodium dissolved in 25 ml. of absolute ethanol, maintained at boiling temperature under a reflux, a mixture of 10.0 gm. of n-butyl methyl ketone and 14.6 gm. of ethyl oxalate (both dried over Na$_2$O$_2$) was added dropwise with stirring over a period of 30 minutes. After the addition was complete, the turbid mixture was refluxed for 2 hours. The whole reaction mixture was then poured over 100 gm. of crushed ice, immediately followed by 10 ml. of concentrated H$_2$SO$_4$. The mixture was quickly and thoroughly stirred, the ester collecting as an oily layer. The whole mixture was then extracted with benzene, the extract dried by one shaking with anhydrous Na$_2$SO$_4$, and the benzene removed in vacuo. The crude ester was then distilled in vacuo, collecting the fraction from 120–145° at 13 mm. The ester after two more distillations was found to boil at 138–139° at 13 mm. The yield of the pure product was 55 to 60 per cent of the theoretical. The
pure ester has a yellow color, resembling the lower homologous ethyl esters in this respect. No other major reaction product could be isolated.

Analysis—C₁₁H₁₆O₄. Calculated, C 59.96, H 8.09; found, C 59.78, H 8.29

Proof of Structure—The ethyl ester was first condensed with phenylhydrazine to yield a pyrazole derivative. A mixture of 2.0 gm. of the ethyl ester formed in the Claisen condensation, 1.3 gm. of phenylhydrazine, and 3 ml. of glacial acetic acid was refluxed for 10 hours, poured on ice, and extracted with ether. The ether extract was dried over Na₂SO₄ and the ether removed in vacuo. No attempt was made to separate the possible isomeric pyrazolecarboxylic esters, since their simultaneous presence has no bearing on the final result (8).

The residue was then saponified by refluxing with 1 equivalent of 1 N NaOH for 2 hours. The mixture was filtered hot, acidified, and extracted with ether. The extract was dried and evaporated to yield a yellow oil, which was completely soluble in dilute NaOH and insoluble in dilute HCl.

The crude acid was then subjected to permanganate oxidation. The acid was dissolved in a minimum volume of 1 N NaOH and brought to boiling temperature under a reflux. Through a dropping funnel 4.7 per cent aqueous potassium permanganate was added dropwise, with continuous refluxing, until no more decolorization took place. Approximately 4 to 5 moles of KMnO₄ were required. After 2 hours at 100°, the slight excess of KMnO₄ was reduced by the addition of H₂O₂ and the hot mixture filtered. The clear, slightly alkaline filtrate was concentrated to a small volume and acidified with concentrated HCl. White needles separated on cooling. The substance was treated with norit and recrystallized to give a pure product having a melting point of 265°,¹ which was not altered on further recrystallization. The yield was 1.3 gm., or 57 per cent calculated from the original ethyl ester.

The two possible pyrazole derivatives which might result from this series of reactions (see Formulas I to IV) are 1-phenylpyrazole-3,5-dicarboxylic acid, m.p. 266° (9), and 1-phenylpyrazole-3,4,5-tricarboxylic acid, m.p. 138° (10). The obvious identity of the product obtained with the first possibility was proved by synthesis of the former from ethyl acetopyruvate (9). The melting point of the authentic sample was 266°; the mixed melting point with the product obtained was 265°. Since the ester formed in the Claisen condensation was homogeneous, only one product, α,γ-diketo-n-octanoic ester, was formed.

α,γ-Diketo-n-octanoic Acid—The free acid was obtained by saponification of the ester. To 50 ml. of 1.0 N NaOH were added 5 gm. of the ethyl ester and the mixture shaken vigorously for 3 minutes, forming a slightly turbid

¹ All melting points have been corrected for stem exposure.
yellow solution. It was immediately acidified with 10 N H$_2$SO$_4$, yielding a turbid white emulsion which on standing and cooling in ice water separated into two layers. The lower layer is the crude free acid, which has a melting point just below room temperature. The lower layer was separated and extracted several times with small portions of warm water to remove traces of oxalic acid and the ketone. The free acid was then carefully brought into solution as the sodium salt by suspending it in a small quantity of water with high speed stirring and adding dropwise 4 N NaOH (carbonate-free) until solution was effected. The pH should not rise above 8 in order to avoid hydrolysis to oxalic acid and butyl methyl ketone. The solution was then shaken with a small amount of norit and filtered. An excess of saturated barium acetate solution was then added. The barium salt separated as slightly yellow needles from dilute solutions or in an amorphous form from strong solutions. After standing overnight at 0° it was filtered off, washed with a small volume of ice water, and allowed to dry at room temperature. It was then washed with a very small amount of cold ether and dried over P$_2$O$_5$ in vacuo. The yields averaged 40 to 50 per cent and could be improved by reworking the mother liquor of the barium salt and the ether washings.

**Analysis**—C$_{16}$H$_{32}$O$_6$Ba. Calculated, Ba 28.6; found, Ba 28.4

The free acid could not be readily purified by vacuum distillation, since extensive decomposition usually resulted. Likewise, recrystallization at low temperatures was found inconvenient although possible. The barium salt was soluble in ether and absolute alcohol.

To obtain the free acid in solution as the neutral sodium salt (as for the biological experiments described later) a weighed quantity of the barium salt was suspended in water and the equivalent amount of sodium sulfate solution was added. The mixture was then homogenized either in a Waring blendor or the device of Potter and Elvehjem (16) at intervals over the course of a day. The barium sulfate was centrifuged off and the supernatant shaken with acid-washed norit, filtered, neutralized, and made up to volume, yielding a clear yellow solution.

**Alkaline Degradation of Free Acid**—The free acid was found to undergo alkaline hydrolysis to n-butyl methyl ketone and oxalic acid. The free acid was refluxed with 3 equivalents of 2 N NaOH for 2 hours. Treatment of a portion of the neutralized mixture with a 0.1 per cent solution of 2,4-dinitrophenylhydrazine in 2 N HCl yielded the 2,4-dinitrophenylhydrazone of n-butyl methyl ketone, m.p. 106°; authentic sample, m.p. 106°; mixed m.p. 106°. The oxalic acid formed was identified as its calcium salt and subsequent permanganate titration. At pH 7.4 the sodium salt is stable in solution at 0° for several days. The hydrolysis can easily be detected by the odor of the methyl butyl ketone formed.
Copper Ethyl α,γ-Diketo-η-octanoate—2 gm. of the purified ester were dissolved in 20 ml. of ethanol. To this solution was added 1 equivalent of cupric acetate dissolved in the minimum required volume of boiling water. A dark green precipitate formed which was filtered off and dried in air. The copper derivative was found to be soluble in organic solvents and was quite volatile, both indications of a chelate ring structure. It was recrystallized from an ethanol-water mixture, yielding dark green needles. The compound melted at 135–137° after some discoloration. Yield, 80 per cent.

Analysis—C_{10}H_{20}O_{2}Cu. Calculated, Cu 13.77; found, Cu 14.00

1-(2,4-Dinitrophenyl)-5(3)-butylpyrazole-3(5)-carboxylic Ethyl Ester—1 gm. of ethyl α,γ-diketo-octanoate in alcoholic solution was treated with 1 gm. of 2,4-dinitrophenylhydrazine in 30 ml. of ethanol plus 5 ml. of concentrated HCl. A yellow precipitate formed immediately. After the mixture was heated for 30 minutes and cooled, the precipitate was filtered off and washed with ethanol and then water. It was recrystallized from ethanol, to give yellow needles melting at 186–187°. Yield, 74 per cent.

Analysis—C_{14}H_{18}O_{4}N_{4}. Calculated, C 53.05, H 5.01; found, C 52.98, H 5.22

1-(2,4-Dinitrophenyl)-5(3)-butylpyrazole-3(5)-carboxylic Acid—The sodium salt of the keto acid in aqueous solution was treated with 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N HCl, yielding a flocculent precipitate composed of pale yellow needles. The compound was recrystallized from toluene. It began to decompose at 185° with volatilization and formed a clear melt at 204°. Yield, 89 per cent.

Analysis—C_{6}H_{14}O_{4}N_{4}. Calculated, C 50.28, H 4.22; found, C 50.22, H 4.12

1-Carboxamide-5(3)-butylpyrazole-3(5)-carboxylic Acid—The sodium salt of the keto acid in aqueous solution formed a voluminous white precipitate in almost quantitative yield when treated with aqueous semicarbazide hydrochloride. This precipitate formed almost immediately in the cold or on slight warming, and was so insoluble it could be used as a roughly accurate gravimetric estimate of the concentration of the acid. The compound was filtered off and dried in vacuo over H_{2}SO_{4}. It was found to be labile to heat and water, as was the lower homologue previously described (17). The compound was obtained analytically pure without further treatment. Decomposition began at 80–82° and a clear melt was formed at 160–165°.

Analysis—C_{6}H_{18}O_{4}N_{4}. Calculated, C 51.18, H 6.20; found, C 51.10, H 6.33

5-Butylpyrazole-3-carboxylic Acid—This was formed by hydrolysis of 1-carboxamide-5-butylpyrazole-3-carboxylic acid by a method previously reported (17). The compound was recrystallized from water to yield
colorless prisms, which melted at 166-167° after drying over H₂SO₄ in vacuo.

Analysis—C₃H₁₀O₄N₂. Calculated, C 57.12, H 7.19; found, C 57.01, H 7.28

The compound was also obtained from α,γ-diketo-octanoic acid by reaction, in aqueous medium, with hydrazine sulfate. Its identity was confirmed by elementary analysis and mixed melting point tests.

Metabolism

In these experiments the sodium α,γ-diketo-octanoate employed was that obtained from a triply precipitated barium salt and in most cases some made from the singly precipitated barium salt was used also for comparison. There was no discernible difference in the behavior of these preparations in the biological experiments, minimizing the possibility of interference by trace impurities.

Absorption from Intestinal Tract—Four rats, weighing 150 to 200 gm., were fasted for 24 hours and each then given 5.0 ml. of 0.05 M sodium diketooctanoate by stomach tube. The urine and feces were collected separately for the next 12 hours and the animals were then sacrificed. The entire alimentary canal of each rat was then removed, emptied, and washed out with a stream of warm water. The feces, intestinal contents, and washings from all four rats were then combined, homogenized in a Waring blender, acidified, and extracted with ether. A similar group of four rats was treated in identical manner, 0.05 M sodium bicarbonate being substituted for the keto acid solution, and the feces and intestinal contents worked up in the same way.

The ether extracts were evaporated to dryness in vacuo and taken up in a few ml. of warm ethanol. Saturated sodium bicarbonate solution was added with stirring until the solution was neutral. It was then filtered and treated with an excess of solid semicarbazide hydrochloride and sodium acetate.

The characteristic insoluble derivative of α,γ-diketo-octanoic acid appeared in the extract from the animals fed the compound. After standing at 0° for several hours it was filtered off, washed with water, and dried in vacuo. Its melting point and neutralization equivalent (in alcohol) showed it to be quite pure. Its weight corresponded to a recovery of 67 per cent of the keto acid administered.

It was converted to 5-butylpyrazole-3-carboxylic acid by boiling with acid (17) and recrystallized. Mixed melting point tests proved the identity of the compound.

The control extract yielded 3 to 4 mg. of amorphous material after treatment with semicarbazide. It was soluble in excess water and was not iden-
tical with the derivative of α,γ-diketooctanoic acid or that of butyl methyl ketone, as proved by mixed melting point tests.

Treatment of the pooled urines of the two groups with semicarbazide yielded no insoluble material.

The only toxic manifestation noted after administration of the diketo acid was a slight diarrhea.

Reaction with Yeast Carboxylase—The method of Krebs and Johnson (1) was used in a determination of the degree of decarboxylation of α,γ-diketooctanoic acid, pyruvic acid, and an equimolecular mixture of the two by a Lebedev extract of brewers’ yeast. The compounds were tested in a final concentration of 0.01 M. In 4 hours only 3 per cent of the theoretical yield of CO₂ could be obtained from α,γ-diketooctanoic acid. Pyruvic acid was completely decarboxylated in 30 minutes by the same preparation. When both compounds were present together, the decarboxylation of pyruvic acid was inhibited 98 per cent.

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<th>TABLE I</th>
<th>Oxidation of α,γ-Diketooctanoate by Minced and Homogenized Liver</th>
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<td>Preparation</td>
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Oxidation by Tissue Slices—In these experiments rat tissue slices, prepared in the usual manner (8 to 20 mg. of dry weight) were equilibrated with 2.0 ml. of phosphate-saline buffer of pH 7.4 at 38° in Warburg vessels. The contents of the side bulb (either saline or 0.05 M sodium diketooctanoate) were then tipped in and measurements of oxygen uptake taken over a 2 hour period. The results, expressed as Qₒₒ values (c.mm. of O₂ taken up per mg. of dry tissue per hour), showed no significant difference between control tissues and those in the presence of the diketo acid. Either the acid is not oxidized by the tissues or it is unable to diffuse into the cells. The tissues examined were rat brain, liver, heart, kidney, diaphragm, and testis. Buffers containing Mg++ or Ca++ precipitate the diketo acid readily, even from very dilute solution.

Anaerobic Experiments with Rat Liver Slices—The anaerobic dismutation of α-keto acids, especially pyruvic acid, to yield CO₂ has been studied
The synthesis of $\alpha,\gamma$-diketo-octanoic acid was achieved by using an extension of available methods for lower homologues. The structure of the product was demonstrated by appropriate experiments.

2. Several derivatives of pyrazole nature were obtained from the free acid and ethyl ester by reaction with substituted hydrazines.

3. The degree of intestinal absorption of sodium $\alpha,\gamma$-diketo-octanoate was found to be quite small.

4. Experiments with surviving rat tissue slices showed no evident biological utilization of the compound, possibly due to its low diffusibility into the slices. In support of this interpretation, it was found that broken cell preparations of rat liver were able to oxidize the compound at a low rate.
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