β-KETO ACID FORMATION AND DECOMPOSITION BY PREPARATIONS OF CLOSTRIDIUM KLUYVERI[1]

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When butyrate or vinylacetate is oxidized by enzyme preparations of *Clostridium kluyveri* in the absence of orthophosphate, acetoacetate is a major end product (Kennedy and Barker, 1951*b*). Indirect evidence was given also for the formation of a nonvolatile product during the oxidation of caproate under similar conditions, but the compound was not identified. If the oxidation of valerate and caproate occurs by a sequence of reactions similar to that involved in butyrate oxidation, the corresponding β-keto acids should accumulate in the absence of phosphate. In the present paper this expectation is shown to be correct.

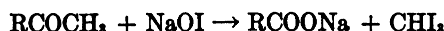
C. kluyveri preparations also catalyze a "phosphoroclastic" decomposition of acetoacetate yielding acetate and acetylphosphate (Stadtman and Barker, 1949*b*). We have found that the higher homologues, β-ketovalerate and β-ketocaproate, are decomposed in a similar manner. Some observations are reported on the relative rates of decomposition of the β-keto acids, the inhibitory action of fatty acids on the reaction, and the identity of the products.

METHODS

Dried cells of *C. kluyveri* were prepared by the method of Stadtman and Barker (1949*a*). Dilithium acetylphosphate was prepared by the method of Stadtman and Lipmann (1950), and β-keto acids by alkaline hydrolysis of the corresponding ethyl esters (Davies, 1943). Total acylphosphate was estimated colorimetrically according to Lipmann and Tuttle (1945), and the individual acylphosphates in mixtures were estimated by the method of Stadtman and Barker (1950). Edson's (1935) method was used to estimate β-keto acids, and the salicylaldehyde method was used to determine acetone derived from acetoacetate by application of a diffusion technique (Stadtman *et al.*, 1951). Ethyl methyl ketone, the product of decarboxylation of β-ketovalerate, does not give an appreciable color with salicylaldehyde.

Identification of β-keto acids. The method is based upon the following reactions.

$$RCOCH_2COOH \xrightarrow{acid} RCOCH_3 + CO_2$$



$$RCOCH_3 + NaOI \rightarrow RCOONa + CHI_3$$

The resulting fatty acid is identified by standard methods. In our procedure, a 10 ml sample containing at least 10 μM of the unknown β-keto acid is acidified to pH 2 with sulfuric acid and allowed to stand in a stoppered flask for 12 hours at 37 C. The sample is cooled then to 0 C, neutralized to pH 8 with 5 N KOH, and the ketone separated by distillation. To the distillate are added 5 ml of 1 N KOH and 5 ml of 0.2 N I_2-KI solution. After 10 minutes, 6 ml of 1 N H_2SO_4 are added, and the excess iodine reduced by addition of 0.15 M $NaHSO_3$. Then the solution is neutralized, concentrated, acidified to pH 3, and steam distilled. The fatty acid in the distillate is identified by Duclaux distillation or by paper chromatography (Kennedy and Barker, 1951*a*). It was shown that application of the method to acetoacetate, β-ketovalerate, and β-ketocaproate yields acetic, propionic, and butyric acids, respectively. The yield of propionic acid from β-ketovalerate was 63 per cent of theory in a typical experiment.

RESULTS AND DISCUSSION

Formation of β-keto acids by oxidation of valerate and caproate. β-Ketovalerate. n-Valerate was oxidized in the virtual absence of orthophosphate by cells of *C. kluyveri* in five Warburg vessels, each containing 30 μM of potassium valerate, 100 μM of Tris buffer 8.1, 0.2 ml of a 4 per cent solu-

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TABLE 1

Products of β-ketovalerate decomposition

Each Thunberg tube contained per ml 100 μM of potassium citrate buffer, 75 μM of potassium phosphate buffer, 33 mg of dried cells, and 30 μM of β-ketovalerate. Total volume, 3 ml. The mixtures were incubated *in vacuo* for 120 minutes at 26 C and then were analyzed. The volatile acid values include both free fatty acids and acylphosphates.

INITIAL pH	Δ KETO ACID	Δ VOLATILE ACID	Δ ACYLPHOSPHATE	μM ACETYL-P / μM PROPIONYL-P
	μM	μM	μM	
5.5	−3.4	7.1	2.2	1.00
6.1	−8.3	15.5	6.2	1.06
6.5	−11.1	21.5	9.4	0.88
7.0	−10.5	19.6	8.5	0.95

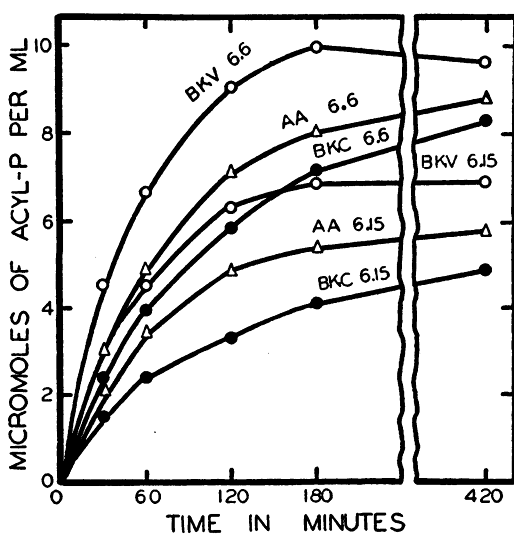


Figure 1. Decomposition of β-keto acids

Each of six Thunberg tubes contained per ml 100 μM of potassium citrate buffer, 80 μM of potassium phosphate buffer, and 30 mg of dried cells, lot YE 5A. In addition, each of two tubes received per ml 26 μM of acetoacetate (AA), β-ketovalerate (BKV), and β-ketocaproate (BKC), respectively. The final volume in each tube was 2.0 ml. The reaction mixture in one tube of each pair was adjusted to pH 6.15, that of the other to pH 6.6. The tubes were evacuated and incubated at 26 C. Samples were removed for acylphosphate estimation at the indicated intervals.

tion of crystalline catalase, and 50 mg of dried cells in a total volume of 2 ml. The reaction mixture was incubated in air at 37 C for 120 minutes by which time the rate of oxygen uptake had decreased markedly. The oxygen uptake per vessel was 13.1 μM, and the quantity of β-keto acid estimated by the aniline citrate method was 10.2 μM. The contents of the five vessels were combined, and the β-keto acid was identified by decarboxylation and oxidation of resulting ketone with alkaline iodine. The Duclaux values of the fatty acid isolated in 67 per cent yield agreed closely with those of propionic acid. The results demonstrate that β-ketovalerate is the main product of oxidation of valerate in the absence of phosphate.

β-Ketocaproate. *n*-Caproate was oxidized under the conditions already described. In 120 minutes 6.4 μM of oxygen were consumed and 4.8 μM of β-keto acid were formed per vessel. Duclaux distillation and paper chromatography of the fatty acid obtained in 63 per cent yield by the degradation procedure showed it to be *n*-butyric acid. Therefore the oxidation product of caproate must have been β-ketocaproate.

Cleavage of β-keto acids. Relative rates of decomposition. The decomposition of acetoacetate, β-ketovalerate, and β-ketocaproate by a suspension of dried cells was studied at pH 6.15 and 6.6. The reaction was followed by determining the production of acylphosphate. In the pH range used, approximately 0.8 mole of acylphosphate is formed per mole of β-keto acid decomposed (table 1).

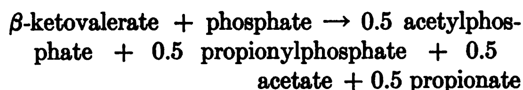
Figure 1 shows that at both pH levels β-ketovalerate is decomposed most rapidly, followed by acetoacetate and β-ketocaproate. The absolute rates with all three substrates are greater at pH 6.6 than at pH 6.15. This is somewhat at variance with the conclusions of Stadtman and Barker (1949*b*) that the rate is maximal at about pH 6.1. However, since these authors did not present any data for solutions between pH 6.1 and 7.0, there is no real discrepancy in the experimental results.

Products of β-keto acid composition. Since aceto-

acetate is converted to acetylphosphate and acetate, it can be expected that β-ketovalerate will give rise to an acylphosphate and a fatty acid. However, the identities of the products cannot be predicted. There are three possibilities; the products could be (a) propionylphosphate and acetate, (b) acetylphosphate and propionate, or (c) an equimolar mixture of all four compounds. In an attempt to distinguish between these possibilities, the decomposition of β-ketovalerate was carried out at a sufficiently low pH to retard greatly the secondary transfer of the phosphoryl group between acetate and propionate. Preliminary observations showed that at pH 6.5 and under the conditions of the following experiment approximately 3 hours were required to reach equilibrium in the phosphoryl transfer reaction, whereas at a lower pH the reaction was considerably slower.

Table 1 gives quantitative data on the products of decomposition of β-ketovalerate at four pH values between 5.5 and 7.0. For each mole of keto acid decomposed, roughly one mole each of acylphosphate and fatty acid was formed. The somewhat low yield of acylphosphate is probably the result of a secondary hydrolytic decomposition of acylphosphates (Stadtman and Barker, 1950).

After both one and two hours of incubation, acetyl and propionylphosphates were found in equimolar quantities within the accuracy of the method. The data indicate that the free fatty acids also were produced in approximately equivalent amounts. Therefore the reaction may be written as follows:

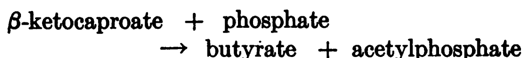

β-ketovalerate + phosphate → 0.5 acetylphosphate + 0.5 propionylphosphate + 0.5 acetate + 0.5 propionate

The formation of equimolar amounts of acetyl and propionylphosphates and the corresponding fatty acids, under conditions excluding the rapid interconversion of acylphosphates and fatty acids, may be interpreted in more than one way. In view of the well established role of coenzyme A in the activation and cleavage of β-keto acids (Stern *et al.*, 1953; Lynen *et al.*, 1952; Green *et al.*, 1953; Stadtman, 1953*b*), it is possible that β-ketovalerate is converted to β-ketovaleryl-coenzyme A which then is cleaved by reaction with free coenzyme A under the influence of the enzyme β-ketothiolase (Lynen, 1953) to yield equimolar amounts of propionyl-coenzyme A and acetyl-coenzyme A. Approximately half of the coenzyme A derivatives are converted then by the enzyme phosphotransacetylase (Stadtman, 1952) to the corresponding acylphosphates. The remainder of the propionyl and acetyl-coenzyme A must be used according to this hypothesis to supply the energy needed to convert β-ketovalerate to its coenzyme A derivative. The free fatty acids would be formed in this process.

The above hypothesis accounts for the observed equimolar yields of acetyl and propionyl phosphates and the complete inhibition of β-keto acid decomposition by arsenate (Stadtman and Barker, 1950). In the presence of phosphotransacetylase, arsenate causes a rapid breakdown of acetyl and propionylphosphates and coenzyme A derivatives and thereby should prevent the conversion of β-keto acid to its coenzyme A derivative.

An alternative but less attractive hypothesis is that β-ketovalerate, rather than its coenzyme A derivative, undergoes a thioclastic cleavage with coenzyme A, forming propionyl-coenzyme A and acetate. These products then could react under the influence of the enzyme coenzyme A-transphorase (Stadtman, 1953a), which is active in the pH range used in the experiments, to give an equilibrium mixture of acetate, propionate, and their coenzyme A derivatives. The latter then would be converted slowly to the acylphosphates. The main objection to this interpretation is that it does not readily account for the inhibitory action of arsenate on β-keto acid decomposition since no preliminary activation is required.

An experiment similar to that with β-ketovalerate was done with β-ketocaproate at pH 6.4. In 180 minutes, 9.7 μM of keto acid were decomposed, and 5.1 μM of acetyl phosphate and 20.8 μM of volatile acid were formed. Only a trace of butyryl hydroxamic was detected after the acylphosphate in the reaction mixture was allowed to react with hydroxylamine. The free fatty acid was not identified specifically, but presumably it consisted mainly of butyric acid plus some acetic acid formed by hydrolysis of acetyl phosphate. The over-all reaction may be written


β-ketocaproate + phosphate → butyrate + acetylphosphate

These results are not inconsistent with those obtained with β-ketovalerate since it is known that

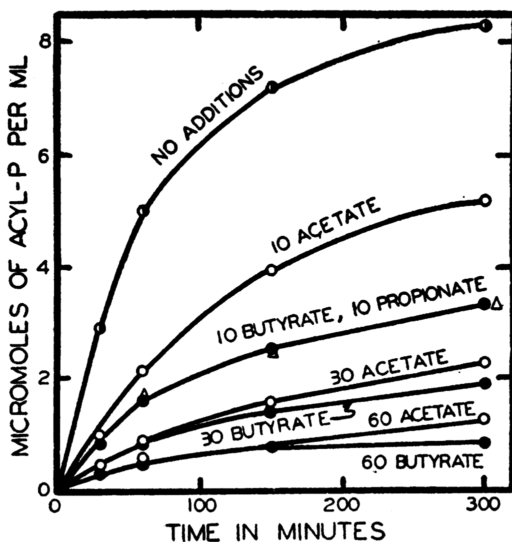


Figure 2. Inhibition of acetoacetate decomposition by fatty acids.

Each Thunberg tube contained per ml, 100 μM potassium citrate, 80 μM potassium phosphate, 29 μM acetoacetate, 30 mg dried cells, lot YE 5A, and the indicated quantities of fatty salts. The total volume was 2.0 ml and the initial pH 6.6. Temperature, 26 C. In a separate experiment involving 4 levels of propionate, the quantity of acetoacetate decomposed was shown to be approximately equivalent to the acylphosphate formed.

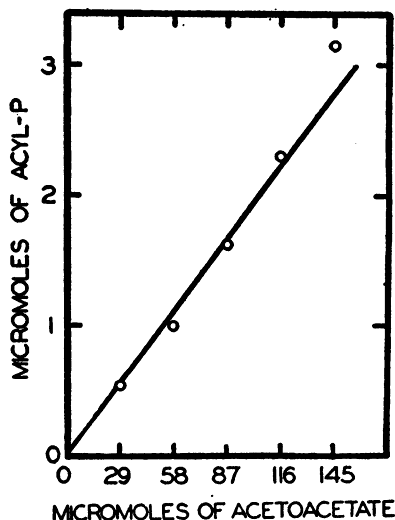


Figure 3. Competitive inhibition of acetoacetate decomposition by propionate.

The conditions are the same as described under figure 2 except that 30 μM of potassium propionate and the indicated amounts of acetoacetate were present per ml of reaction mixture. The incubation time was two hours. The ordinate refers to the quantity of acetylphosphate formed per ml. A control mixture containing 29 μM of acetoacetate and no propionate formed 6.4 μM of acetylphosphate per ml.

butyryl phosphate is not formed from butyryl-coenzyme A and phosphate by the phosphotransacetylase present in extracts of *C. kluyveri* (Stadtman, 1952). Under these circumstances the butyryl-coenzyme A, which may be a primary product of β-ketocaproate cleavage, must be decomposed completely by transferring its coenzyme A group to acetate, either directly or through the mediation of β-ketocaproyl-coenzyme A.

Inhibition of β-keto acid decomposition by fatty acids. Acetate strongly inhibits the decomposition of acetoacetate (Stadtman and Barker, 1949*b*). We have observed a similar but greater inhibition, at equal inhibition concentrations, by propionate and butyrate (figure 2). The latter compounds are about equally effective. Isobutyrate, *n*-valerate, and *n*-caproate are also strongly inhibitory whereas alanine, β-alanine, succinate, and methyl ethyl ketone are inert. From these results it appears that only fatty acids inhibit acetoacetate decomposition.

In order to determine whether the inhibition of acetoacetate breakdown by propionate is competitive, an experiment was performed with a constant level of propionate, 30 μM per ml, and varying levels of acetoacetate, 29 to 145 μM per ml. The reaction was followed by the appearance of acylphosphate. The data given in figure 3 show that inhibition by propionate is competitive, increasing levels of acetoacetate causing a reversal of the inhibition. However, even a fivefold increase in acetoacetate concentration causes only a 50 per cent reversal.

The effect of acetate, propionate, and butyrate on the rate of decomposition of β-ketovalerate also was tested. All three acids were inhibitory, but the effect was somewhat less than with acetoacetate.

To obtain information concerning the site of the inhibitory action of fatty acids on β-keto acid decomposition, the effect of acetate and propionate on butyrate oxidation was studied. Butyrate oxidation to acetate by *C. kluyveri* extracts is known to involve the formation of acetoacetyl-coenzyme A as an intermediate (Stadtman, 1953*b*). Since acetoacetyl-coenzyme A is also a probable intermediate in acetoacetate

decomposition, it is possible that the paths of butyrate oxidation and acetoacetate decomposition converge at this point. It was reasoned that if acetate or propionate causes inhibition of butyrate oxidation to the same extent as acetoacetate breakdown, a site of action between acetoacetyl-coenzyme A and the final products would be indicated. Actually it was found that propionate stimulates rather than inhibits butyrate oxidation. Therefore the site of acetate and propionate inhibition of β-keto acid breakdown cannot be on the path of butyrate oxidation.

In view of the above results and the competitive nature of the inhibition, it appears probable that fatty acid salts retard β-keto acid breakdown by interfering with the conversion of free β-keto acids to their coenzyme A derivatives.

SUMMARY

The oxidation of *n*-valerate and *n*-caproate by dried cells of *Clostridium kluyveri* in the absence of orthophosphate is shown to result in the formation of β-ketovalerate and β-ketocaproate. These keto acids, like acetoacetate, are decomposed to fatty acids and acylphosphates. β-Ketovalerate yields equimolar quantities of acetyl and propionylphosphates and the corresponding fatty acids under conditions that preclude a rapid exchange of the phosphate group between acylphosphates and free fatty acids. β-Ketocaproate yields only acetylphosphate and a fatty acid, presumably butyric acid. Propionate and butyrate, like acetate, strongly inhibit the decomposition of β-keto acids, but acetate and propionate do not inhibit butyrate oxidation. The results are discussed in relation to possible mechanisms of β-keto acid formation and breakdown.

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