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Accumulation of Keto Acids during the Growth Cycle of *Escherichia coli*

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The accumulation of keto acids during the active growth of *Escherichia coli* has been followed by a colorimetric method. The keto acids were identified by thin layer chromatography. Also the influence of a low nitrogen content and the influence of the amino acids L-isoleucine, L-leucine, L-valine, and L-serine on the formation of keto acids in *E. coli* cultures were studied.

Pyruvic acid accumulates during the active growth cycle in a nitrogen-rich medium so that its content rises to a maximum in the acceleration phase, decreases and then rises to a second maximum at the end of the growth cycle at which stage small amounts of 2-oxoglutaric acid are secreted into the medium. Traces of other keto acids are also produced during the growth cycle.

More 2-oxoglutaric acid is formed in nitrogen-poor than in nitrogen-rich medium.

Added amino acids lead to the accumulation of the corresponding keto acids in the media already in the early phases of bacterial growth. L-Isoleucine, L-leucine, and L-valine promote the formation of the corresponding keto acids, but also the keto acids corresponding to the other two amino acids are formed in cultures containing one of these amino acids. These observations suggest that a common transaminase catalyzes the conversion of these amino acids.

Keto acids influence the formation of enzymes, for the repression of catabolic enzymes (catabolite repression [1] or metabolic repression [2]) varies with the concentration of pyruvic acid in the growth medium.[2,3] Catabolic enzymes are formed in the late phases of bacterial growth according to Gale [4] and hence the keto acid concentration would be expected to increase during these phases.

The influence of the nature of the nitrogen source on the accumulation of keto acids has been studied to only a limited extent, although it has been established that 2-oxoglutaric acid accumulates in nitrogen-deficient media.[5,6]

The aim of this study was to determine what keto acids are formed and to what extent during the growth of *E. coli* and to examine whether nitrogen sources such as ammonium salts and amino acids influence this formation. A study was also made of the influence of amino acids added to the medium on the accumulation of corresponding keto acids.

EXPERIMENTAL

Test organism and growth media. The wild strain of *Escherichia coli* employed as the test organism had been isolated as described previously.[7] It was transferred from an agar slant to an inoculation medium containing amino acids and vitamins in which it was allowed to grow without shaking in an incubator as described previously.[8] The cells in the inoculation medium were washed twice in 0.15 M sodium chloride and transferred to a simple medium containing 1.0 % D-glucose, 0.3 % monopotassium orthophosphate, 0.6 % dipotassium orthophosphate, 0.1 % ammonium sulphate, and 0.02 % magnesium sulphate heptahydrate. Owing to the method of inoculation the medium was not sterilized: the pH of the medium was 7. The growth took place under aerobic conditions in a shaker unit in an air thermostat in which six cultures were incubated simultaneously.

Determination of keto acids. The turbidities of samples taken at intervals from the cultures were measured with a Klett-Summerson colorimeter employing filter 62 (590—660 mμ). Each sample was then centrifuged in a Servall SS-1 bench centrifuge at 8—12000 *g* for 10 min at room temperature. The cell-free medium was added in 5-ml portions to test tubes to each of which was added 2.0 ml of a 0.2 % solution of 2,4-dinitrophenylhydrazine in 2 N HCl. After the contents had been mixed, the test tubes were left to stand at room temperature for 20 min. The hydrazones were extracted by shaking the mixture vigorously with 10 ml of ethyl acetate for 30 sec. A 0.5-ml volume of the organic phase was transferred to a second test tube and 5.0-ml of 2.5 % solution of KOH in 95 % ethanol was added to increase the color intensity. The color was measured 20 min later with the Klett-Summerson colorimeter employing filter 54 (520—580 mμ). Toluene was employed as the extracting solvent when the levels of monocarboxylic keto acids were determined.[9,10] The standard reference curve was one recorded with pyruvate because this was the dominating acid in the media to which no amino acids had been added.

Chromatographic fractionation of the keto acids. The organic phase remaining after the 0.5-ml sample had been removed was transferred to an Erlenmeyer flask and evaporated to dryness on a hot plate. The residue was dissolved in the smallest possible volume of ethyl acetate for application onto a thin layer of Kieselgel G (according to Stahl) 0.17 mm thick. The activation of the layer and the chromatographic separation were carried out as described by Ronkainen.[11] The solvent was a mixture containing 0.104 mole of propionic acid in 100 ml of petroleum ether-ethyl formate. The spots were identified and photographed. The keto acids ketoleucines, ketovaline and phenylpyruvic acid used as reference compounds were prepared by transamination.[8] The formed keto acids were extracted with toluene and after the solvent had been evaporated, applied together with 2-oxoglutaric acid to a thin layer for chromatography.

RESULTS AND DISCUSSION

1. *Accumulation of keto acids during the growth cycle.* When keto acids in microbial cultures have been studied, large amounts of pyruvic acid have been found but mostly traces of 2-oxoglutaric acid, 2-oxoisovaleric acid (ketovaline) 2-oxo-β-methylvaleric acid (ketoisoleucine), and 2-oxoisocaproic acid (ketoleucine).[12,13] Keto acid content of old cultures of lactic and propionic acid bacteria and of baker's yeast has also been studied.[14] Except for pyruvic acid,[15] the keto acids present during different phases of cell growth have not been determined qualitatively or quantitatively. Pyruvic acid was found toward the end of the growth cycle in cultures of *Saccharomyces guillermondii* in amounts that varied with the iron content of the medium. A very important observation was that the endogenic pyruvic acid content was proportional to the content of the acid in the medium. It was hence expected that the distribution of keto acids would be the same in the cells and in the medium. Fig. 1 shows the levels of keto acids at different growth phases in growing cultures of *E. coli*. It will be seen that keto acids accumulate in the medium already in the early lag

and acceleration phases, but the total content of keto acids decreases, evidently owing to the vigorous growth, and finally increases again rapidly at the end of the growth cycle (curves 2 and 3). The second maximum was usually 4—5 times as great as the first maximum and occurred in the retardation phase. The levels at the first maximum varied in replicate experiments, evidently as a consequence of variations in the rate of cell growth. The second maximum may be due to an increased activity of the catabolic enzymes as proposed by Gale [4] or to retarded utilization of pyruvic acid. The levels of the keto acids extracted by toluene varied in parallel with the level of total keto acids extracted by ethyl acetate up to the retardation phase (curves 2 and 3). Thin layer chromatographic analyses revealed that the monocarboxylic keto acid frac-

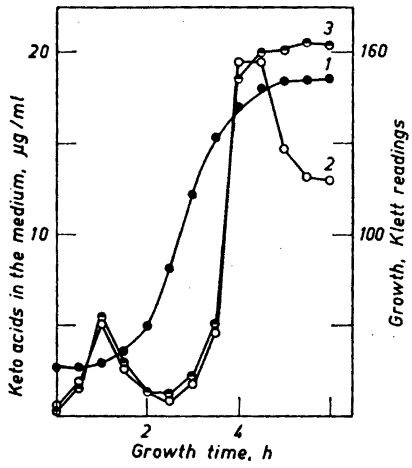

Fig. 1. Accumulation of keto acids during the active growth of *Escherichia coli*. Keto acid contents in duplicate 5-ml samples harvested at half-hour intervals from 500 ml of an aerobic *E. coli* culture were determined colorimetrically. The hydrazones of the monocarboxylic keto acids were extracted with toluene (curve 2) and those of all keto acids with ethyl acetate (curve 3). Curve 1 plots the growth as Klett scale readings (scale on right).

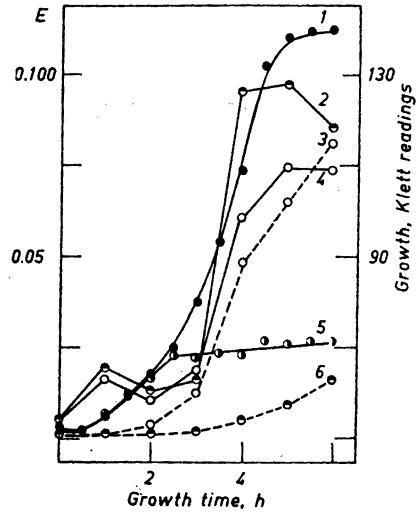

Fig. 2. Accumulation of pyruvic and 2-oxoglutaric acids during the growth of *Escherichia coli* in nitrogen-poor and nitrogen-rich media. 1, growth curve in nitrogen-rich medium; 2, concentration of pyruvic acid in nitrogen-rich medium; 3, concentration of 2-oxoglutaric acid in nitrogen-poor medium; 4, concentration of pyruvic acid in nitrogen-poor medium; 5, growth curve in nitrogen-poor medium and 6, concentration of 2-oxoglutaric acid in nitrogen-rich medium. Spots containing the keto acids on the thin layer chromatogram were extracted with ethyl acetate and color intensities of the pyruvic and 2-oxoglutaric acid spot extracts were measured with Beckman DU spectrophotometer using 1-ml cuvettes. The samples were treated identically and thus the OD readings obtained (scale on left) are comparable to each other.

tion consisted for the most part of pyruvic acid (Fig. 4), whereas 2-oxoglutaric acid was found only in the later phases of the growth cycle. The pronounced accumulation of pyruvic acids is in accordance with previous results obtained with *Streptococcus violeceus* and *Saccharomyces guillermondii*.[15]

2. *Effect of nitrogen deficiency on the accumulation of keto acids.* When *E. coli* was grown in a medium of low ammonium sulphate content (50 μg/ml), it was found that much more 2-oxoglutaric acid was formed than when the bacterium grew in a medium rich in nitrogen (1000 μg/ml) (curves 3 and 6 in Fig. 2). The formation of pyruvic acid (curves 2 and 4 in Fig. 2) followed the curves in Fig. 1, and hence pyruvic acid was the dominating keto acid in the medium.

Thin layer chromatograms of the keto acids precipitated by hydrazine from media in which the bacterial growth had progressed to the later phases of the growth cycle (Fig. 4) revealed that other keto acids, primarily ketoleu-

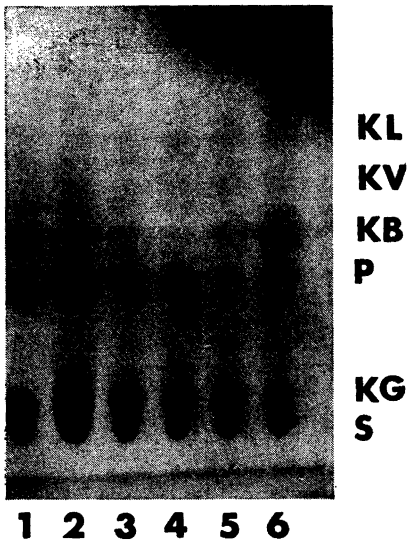

Fig. 3. Accumulation of keto acids during the growth of *Escherichia coli* in a medium to which 5 mM L-leucine had been added before inoculation. 5 ml samples were taken at hourly intervals and the hydrazones of the keto acids were extracted with ethyl acetate and separated by thin layer chromatography. KL, ketoleucine(s); KV, ketovaline; KB, ketobutyric acid; P, pyruvic acid; KG, ketoglutaric acid, and S, starting line. The growth curve was closely similar in this experiment than the curve 1 in Fig. 2.

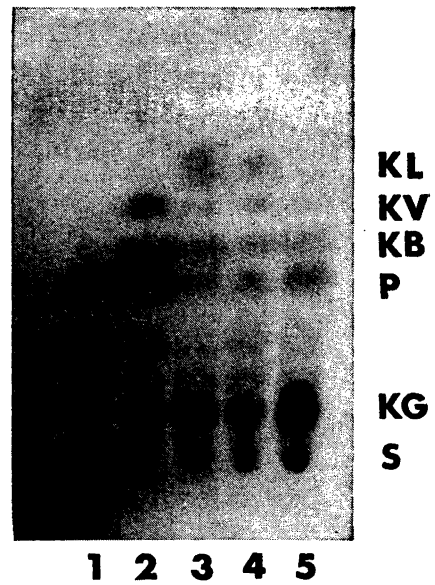

Fig. 4. Accumulation of keto acids in cultures of *Escherichia coli* to which L-isoleucine, L-leucine, or L-valine had been added before inoculation. 100 ml samples were harvested from the cultures at the end of the exponential phase and the keto acids determined as described in the text to Fig. 3. 1, keto acids in a medium containing 1 mg of ammonium sulphate per ml (nitrogen-rich medium), but no amino acids added; 2, 5 mmoles of L-valine added per litre; 3, 5 mmoles of L-leucine present per litre; 4, 5 millimoles of L-isoleucine present per litre and 5, keto acids in a medium containing 50 μg of ammonium sulphate per ml (nitrogen-poor medium).

cines and ketovaline, are precipitated together with 2-oxoglutaric acid from nitrogen-deficient media. These observations confirm the significance of transamination and glutamic acid in amino acid synthesis. Less pyruvic acid was produced in the nitrogen-deficient medium (curve 4) than in the nitrogen-rich medium (curve 2); this may have been due to different rates of bacterial growth or to the promotion of glycolysis by the ammonium ion.[15,16]

3. *Effect of amino acids on the keto acid formation.* In the study of the effect of amino acids on the keto acid formation, 5 mmoles/l of L-isoleucine, L-leucine, L-valine, and L-serine were separately added to cultures of *E. coli* at different stages of bacterial growth. A typical thin layer chromatogram of the keto acids formed in a growing culture of *E. coli* is shown in Fig. 3. The amino acid present in the culture which contained 0.1 % ammonium sulphate was L-leucine. Keto acids accumulated in the culture already in the acceleration phase and their concentration was a maximum at the end of the growth cycle. The keto acids were ketoleucine (and probably also ketoisoleucine whose spot was difficult to distinguish from the ketoleucine spot), ketovaline and, probably, 2-oxobutyric acid. The amount of pyruvic acid also rose to a clear maximum in the lag and acceleration phases (Fig. 1). The curves in Fig. 3 show either that the amino acid is decomposed at least to the corresponding keto acid during the active growth phase, which observation deviates from the previous view that the metabolism of a cell during the active growth is a one-way system that leads directly to the final products,[17] or that the added amino acid represses the synthesis of leucine(s) and valine so that the corresponding keto acids accumulate in the culture. Results similar to those which were obtained with L-leucine were obtained with other amino acids, though the formation of ketoleucines in the presence of L-valine was rather poor. Especially L-serine strongly promoted the formation of pyruvic acid.

L-Isoleucine, L-leucine, and L-valine separately added to *E. coli* cultures led to the formation not only of the corresponding keto acid but also of the acids corresponding to the other two amino acids. The accumulation of ketoleucines and ketovaline in the cultures containing these amino acids is shown in Fig. 4. These keto acids were isolated for the chromatography from cells removed from cultures at the end of the growth cycle. Fig. 4 shows that L-isoleucine and L-leucine when separately added to cultures to give a concentration of 5 mM both led to the formation of ketovaline, 2-oxoisovaleric acid. The levels of ketoleucines were lower in the culture containing L-valine, but still observable. All three amino acids led also to the formation of 2-oxobutyric acid. It has been previously shown that the biosynthesis of isoleucine, leucine and valine is affected by common enzymes including aminotransferase.[8,18,19]

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