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Determination of ionic species formed during growth of *Escherichia coli* by capillary isotachophoresis

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ABSTRACT

The ionic species that are formed during the microbial growth of *Escherichia coli* were determined by capillary isotachophoresis as a function of the time of cultivation. This formation was indicated by the change in a sum parameter, the impedance of the nutrient broth, measured by a special electrode system. Based on the determination of the individual ions formed under the given conditions (identified as acetate, lactate, α-ketoglutarate, fumarate, ammonium and probably a simple amine), the change in conductivity was calculated and compared with that obtained by the impedance measurement of the bulk medium. From the results it can be concluded that the change in the sum parameter as a function of time is originated by the ions determined.

INTRODUCTION

Examinations of bacterial contamination are routine procedures in the fields of food hygiene, cosmetics, pharmaceuticals and medicine. However, also in fields in which microorganisms are used for production processes, in biotechnology, methods of bacterial examination are of high relevance. As conventionally used standard methods are very time consuming, the development of automatic methods is of general interest. The so-called impedance method is one of these. With this method, microbial metabolic processes that produce electrically measurable changes in the nutrient broth are used to detect bacteria. There, nutrients are converted by the metabolism into smaller, charged components, which contribute additionally to the transport of current. As a consequence, the electrical impedance of the nutrient medium will decrease.

At the Institute of Fundamentals and Theory of Electrotechnics a measuring system was developed, based on the so-called Impedance-splitting method (IS method [1]), which allows the separation of the impedance of the nutrient medium, Z_M, and that of the electrode system, Z_E. The latter is caused by ionic layers in the vicinity of the electrodes. Thus, two separate parameters are available for the determination and characterization of microbial growth. Which of them is used for routine examination, Z_M, Z_E or both, depends on the application and is discussed elsewhere [2–4].

A comparison of the registered time courses of the changes in Z_M and Z_E during microbial growth showed that both similar and contradictory time courses are observed. Large changes in Z_E with negligible changes in Z_M also occurred. To understand and interpret these different

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effects, it is necessary to know which nutrients are converted by the metabolism into which ionic substances.

Several metabolic pathways for the conversion of the different nutrients such as glucose, proteins, peptides, amino acids and lipids are possible. In addition, the bacteria are able to change the pathways during the growth depending on, *e.g.*, the oxygen content or the composition of the nutrient broth. It is therefore virtually impossible to predict the amount and also sometimes the species of the secreted molecules. As a consequence, chemical analysis is necessary to solve first the question of which molecules contribute most to the changes in the electrical conductivity, κ, and second to provide a basis for investigations on the more difficult questions of what happens near the surface of the measuring electrodes and what causes the changes in Z_E.

The aim of this work was to solve the first question for the bacterium *Escherichia coli*, which was chosen because it is a very common and well known species. After identification and determination of at least the main components, that are formed in the nutrient broth by the metabolism, attempts were made to calculate the contributions to κ of the different ions, their sum being compared with the changes in Z_M recorded by the IS method.

It can be expected that the ionogenic components formed during such metabolism are relatively simple organic acids and bases. These have been described in general for the different metabolic pathways in the biochemical literature [5–7] and are summarized in Table I, but the individual species formed under the conditions applied in this investigation cannot be predicted in detail. Therefore, the analysis of the cultivation media must focus on the determination of such ionic species.

This attempt was carried out by capillary isotachophoresis (ITP) with conductivity detection, which has been favourably applied to the investigation of other aspects of bacterial metabolism [8–12]. This method combines separation by electromigration (based on effective mobilities, u_{eff}) with the principle of electrical conductivity detection (which is based on the same analyte property). In contrast to capillary zone electrophoresis, this combination is favourable owing to the concentration adjustment in ITP, leading to relatively high concentrations of the sample components after separation, which are detected without the presence of a background electrolyte. Because this method is applied in a closed system without electroosmotic flow (EOF), the overall migration properties of the analytes, which are used for identification, are insensitive to wall adsorption effects compared with electrophoresis carried out in the presence of EOF (*e.g.*, micellar electrokinetic chromatography or zone electrophoresis with EOF). This is a decisive advantage especially when the samples contain complex matrices at high concentration and are injected directly, without further pretreatment.

ITP can also be favourably applied in some instances rather than ion chromatography because, in contrast to the latter method, no stationary phase is present here to be contaminated and thus modified by the matrix. Solvent gradients for the elution of higher charged analytes are also unnecessary.

TABLE I

ANIONIC SUBSTANCES THAT MAY OCCUR IN THE NUTRIENT BROTH DURING THE GROWTH OF *E. COLI*, FORMED BY THE DIFFERENT METABOLIC PATHWAYS OR BY CELL LYSIS [5–7]

Monobasic	Dibasic	Tribasic
Formate	Oxalate	Citrate
Acetate	Fumarate	Phosphate
Propionate	Malate	
Butyrate	Tartrate	
Lactate	Malonate	
	α-Ketoglutarate	
	Adipate	
	Glutarate	

EXPERIMENTAL

Chemicals

The following chemicals were used for the preparation of the buffers: hydrochloric acid, butyric acid, acetic acid, morpholinoethane sul-

phonic acid (MES), β-alanine, tris(hydroxymethyl)aminomethane (Tris) and potassium hydroxide. All chemicals were of analytical-reagent grade (Merck, Darmstadt, Germany).

As nutrient broth for the bacteria, a mixture with the following common composition [13–16] was used (without sodium chloride): peptone from meat (tryptically digested, 7.8 g/l), peptone from casein (tryptically digested, 7.8 g/l), D-(+)-glucose monohydrate (1.0 g/l) and yeast extract (2.8 g/l).

In order to suppress electroosmosis, hydroxyethylcellulose (HEC) (Fluka, Buchs, Switzerland) was added to the leading electrolyte buffer solution. Water used for the preparation of the buffers was doubly distilled from a quartz apparatus.

Apparatus

For the measurement of the impedances, the BacTrac 4100 system (SY-LAB, Vienna, Austria), based on the impedance-splitting method, was used. It consists of an incubation block which allows temperature control from 4 to 65°C. The sample cells were made from glass (10 ml content) and were equipped with four stainless-steel electrodes for impedance measurement. A microprocessor-controlled electronic recorder registered data for the simultaneous determination of Z_M and Z_E on up to 40 sample cells. A personal computer was used to supervise up to six incubation blocks and showed on the screen the time courses of the relative decreases $-\Delta Z_M$ and $-\Delta Z_E$ (as a percentage) of the absolute values of Z_M and Z_E, respectively.

The concentration of bacteria (colony-forming units per ml; cfu/ml) was determined by the standard plate count method.

The pH of the nutrient broth was measured with a combined glass–calomel electrode (Portamess 654; Knick, Berlin, Germany) and absolute values of κ were additionally determined with a conductimeter (CG858; Schott, Hofheim, Germany).

Isotachophoretic measurements were carried out with a Trace 1 instrument (United Research, Vienna, Austria) equipped with a conductivity and a UV detector (at 254 nm) on-line. The separation capillary (20 cm × 200 μm I.D.) was made from a mixed polymer of polyethylene and polypropylene. Separation was carried out at a constant current of 50 μA. The injector was made of ceramic and had a constant volume of 0.20 μl. The control of the instrument and the data aquisition were done with a personal computer.

Procedure

The sample cells were filled with the described nutrient broth and inoculated with *E. coli* from an overnight culture, so that the initial bacterial concentration was about 10^6 cfu/ml. At the start and at 1-h intervals samples of the bacterial suspension were taken. One part of each sample was used for the determination of the number of bacteria (cfu/ml) and the other part was immeditately sterilized by using a membrane filter technique (Minisart Plus, 0.2 μm; Sartorius, Göttingen, Germany) to stop growth and metabolic activity. From this part the pH value, the absolute values of κ and the kind and concentration of the ions were determined, the latter by ITP (for the buffering electrolyte systems, see Table II).

TABLE II

BUFFERING ELECTROLYTE SYSTEMS USED FOR THE ISOTACHOPHORETIC MEASUREMENTS

System	Leading ion	Concentration (mol/l)	Counter ion	pH	HEC added (%, w/w)	Terminating ion	Concentration (mol/l)	Counter ion	pH	Additive
Anionic, pH 7.5	Cl^-	0.01	Tris	7.5	0.05	MES	0.01	Tris	6.0	None
Anionic, pH 3.5	Cl^-	0.01	β-Alanine	3.5	0.05	Butyrate	0.01	β-Alanine	4.0	None
Cationic	K^+	0.01	Acetate	5.4	0.05	Tris	0.01	Acetate	5.0	None

RESULTS AND DISCUSSION

Identification of the ions formed by the metabolism of E. coli

In Figs. 1 and 2, typical isotachopherograms obtained from the nutrient broth at the beginning and end of the incubation time after 8 h are shown for the anionic and cationic modes. It can be seen that at the start of the growth a number of steps can be observed for the anions, but only one step between the leading and terminating electrolytes occurs in the cationic mode. The occurrence of such steps is obvious, because the nutrient medium (see *Chemicals*) contains a variety of different ionic substances that may appear in the mobility region under consideration.

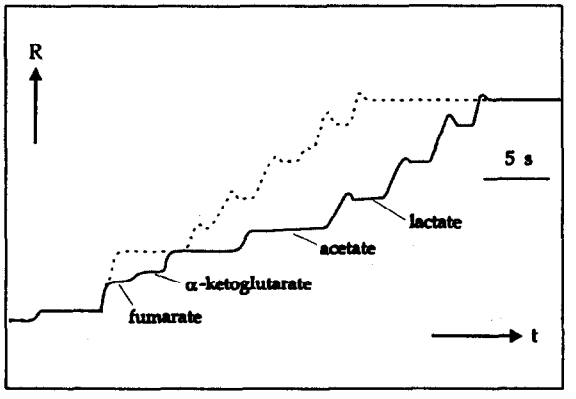

Fig. 1. Isotachopherograms of anions of the nutrient medium of *E. coli* at the beginning of the incubation period (dotted line) and after 8 h of growth (solid line). The electropherograms were measured at pH 7.5 of the leading electrolyte with a conductivity detector. The humps occurring at the front of the upper four zones are caused by substances migrating in the "enforced" mode. For conditions, see Experimental. R = Electrical resistance; t = time.

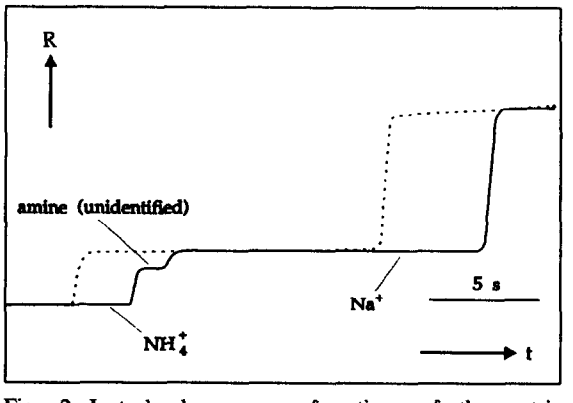

Fig. 2. Isotachopherograms of cations of the nutrient medium of *E. coli* at the beginning of the incubation period (dotted line) and after 8 h of growth (solid line). The electropherograms were measured at pH 5.4 of the leading electrolyte with a conductivity detector. R = Electrical resistance; t = time.

Identification of the components was attempted by comparison of the step heights of the zones in the isotachopherogram of the samples with those obtained from (pure) reference compounds according to Table I. The measured step heights were not used for a final identification, however, but they allowed a preselection of the analytes. Based on this selection, a closer identification was carried out by adding the corresponding reference ions to the samples.

It can be seen from Fig. 2 that prior to the incubation only one long zone for cations was observed, which was identified as sodium. This is surprising because, in contrast to the common recipes [13–16], no sodium chloride was added to the nutrient broth. The result of the isotachophoretic measurement was, however, confirmed by atomic emission spectrometry.

After the 8-h incubation period, a second zone occurred in the mobility range between sodium and potassium, the leading ion. As this ion has no UV absorbance at 254 nm (the wavelength of the detector), it must be a simple organic cation, probably an amine, but no further information about the nature of this substance was found in the literature. The formation of another cation can also be observed indirectly from the isotachopherogram, because the time of appearance of the first ion after the leading ion was shifted with high reproducibility to higher values for the samples taken after 8 h, compared with the initial solution. This must be the result of the migration of a (non-UV-absorbing) cation formed, which has about the same ionic mobility as potassium. This is most plausibly ammonium, which can be indirectly determined in this way. This assumption was supported by the expected increase in the length of the leading zone when NH_4^+ standard was added to the samples before injection.

It can be seen from Fig. 1 that after 8 h of cultivation the lengths of four zones of anions

increase remarkable. An approach to the identification of the ionic components of this solution will thus focus on these zones. According to the anions that can be expected (given in Table I), identification was carried out at two different pH values of the leading electrolyte, namely 3.5 (not shown) and 7.5. It was found that the same number of anions in about the same amount was observed in the isotachopherograms at both pH values. This result allows the conclusion that the substances detected are not amino acids, because these compounds would not migrate as anions at pH 3.5 owing to their zwitterionic character.

In both anionic electrolyte systems the relative step heights (with respect to acetate) were determined for the anions listed in Table I and were compared with the step heights of those zones which increased with incubation time. For final identification reference components were added to the samples, as already mentioned.

The results of this procedure allowed the identification of the compounds formed as fumarate, α-ketoglutarate, acetate and lactate. The most pronounced increase in zone length was observed for acetate and lactate.

Quantification

The determination of those components which showed an increase in concentration in the nutrient broth during the growth of *E. coli* was carried out in the usual way using calibration graphs obtained from the solutions of the different pure reference compounds. These graphs relate the zone length in the isotachopherogram to the concentration of the analyte. Based on these graphs, the increase in concentration was determined over an 8-h period by taking samples at 1-h intervals as described under *Procedure*. The results of the measurements of the concentrations are given in Table III. The change within the first 3 h was too small for the anions to allow quantification. The maximum increase in the concentrations for both anions and cations is *ca.* 10^{-3} mol/l. It can be seen from Figs. 1 and 2 and Table III that the sum of the increase in concentration, Δc, relative to the initial concentration of ionic components at the beginning of growth ($t = 0$) is about 25%. The precision of the measurements (expressed by the relative standard deviation) was very high for subsequent injections, namely in the range of a few tenths of percent. The long-term precision was more than one order of magnitude lower (5–7%).

It can also be seen from Table III that the sum of the increase in cations is higher than that for anions. This is obvious because the different species stem from different metabolic pathways. Electroneutrality is established, however, as the

TABLE III

INCREASE IN CONCENTRATION, Δc, OF DIFFERENT IONIC COMPONENTS FORMED DURING AN 8-h CULTIVATION OF *E. coli* DETERMINED BY ITP

Time (h)	Δc (mol/l)					
	Cations		Anions			
	Ammonium	Unidentified amine	Fumarate	α-Ketoglutarate	Acetate	Lactate
0	0	0	0	0	0	0
1	$3.3 \cdot 10^{-4}$	–[a]	–	–	–	–
2	$5.2 \cdot 10^{-4}$	–	–	–	–	–
3	$8.8 \cdot 10^{-4}$	–	–	–	–	–
4	$2.4 \cdot 10^{-3}$	–	$1.7 \cdot 10^{-4}$	$2.4 \cdot 10^{-4}$	$1.0 \cdot 10^{-3}$	–
5	$2.9 \cdot 10^{-3}$	–	$7.0 \cdot 10^{-4}$	$9.6 \cdot 10^{-4}$	$2.8 \cdot 10^{-3}$	–
6	$3.2 \cdot 10^{-3}$	–	$1.1 \cdot 10^{-3}$	$1.3 \cdot 10^{-3}$	$5.3 \cdot 10^{-3}$	$3.6 \cdot 10^{-4}$
7	$3.2 \cdot 10^{-3}$	$8.4 \cdot 10^{-4}$	$1.3 \cdot 10^{-3}$	$2.1 \cdot 10^{-3}$	$6.5 \cdot 10^{-3}$	$1.1 \cdot 10^{-3}$
8	$3.7 \cdot 10^{-3}$	$1.7 \cdot 10^{-3}$	$1.3 \cdot 10^{-3}$	$2.2 \cdot 10^{-3}$	$7.8 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$

[a] Dashes indicate concentrations too low to be determined.

free amines and acids released undergo protolysis reactions with the various buffering components in the broth (proteins, peptides, etc.).

Comparison of the measured and calculated conductivity changes

From the results of the ITP measurements given above, the corresponding changes in the conductivity, κ, can be calculated, based on the increase in concentration, c_i, as given in Table III, and the effective mobility, u_{ieff}, of the components of interest, i, at the pH of the nutrient broth, by $\kappa = \Sigma\ Fc_i u_{ieff}$, F being the Faraday constant.

The time dependence of the pH of the nutrient medium is shown in Fig. 3: the pH remains constant at the first 3 h, but then decreases steeply from the initial value of 6.8 by more than one unit to 5.6 after 8 h, indicating the formation of acidic constituents.

A similar shape is observed for the dependence of the number of organisms as a function of time, as shown in Fig. 3: after an initial time

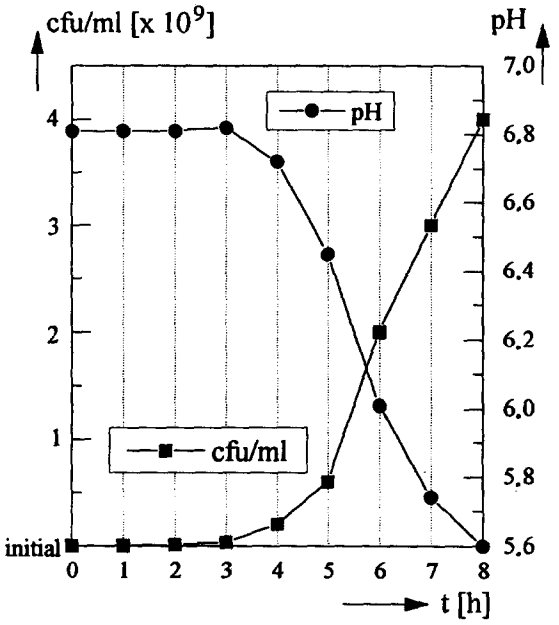

Fig. 3. Change in pH of the nutrient medium (●) and increase in the number (cfu/ml) of *E. coli* bacteria (initial concentration *ca.* 10^6 cfu/ml) (■) as a function of the time (t) of cultivation.

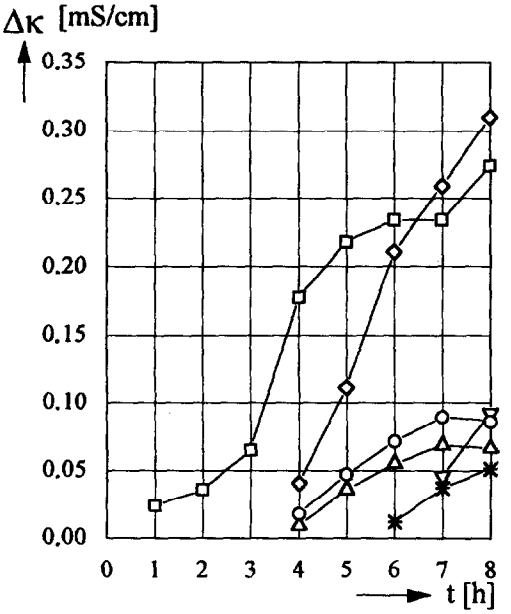

Fig. 4. Calculated increase in conductivity ($\Delta\kappa$) due to the formation of the individual ionic compounds during the cultivation of *E. coli*, based on the results of the isotachophoretic measurements. □ = Ammonium; ▽ = unidentified cation (plausibly a simple amine); ◇ = acetate; △ = fumarate; ○ = α-ketoglutarate; * = lactate. t = Time.

of about 3 h (where the number increases, however, by 2.5 orders of magnitude, a fact that is not visible from the bilinear plot in Fig. 3), a steep increase can be seen simultaneously with the decrease in pH.

TABLE IV

CHANGES IN CONDUCTIVITY, $\Delta\kappa$, OF THE NUTRIENT BROTH DURING THE BACTERIAL GROWTH AS CALCULATED FROM THE ITP RESULTS AND MEASURED WITH A CONDUCTIMETER

Time (h)	$\Delta\kappa$ (mS/cm)	
	Calculated	Measured
0	0	0
1	0.02	0.05
2	0.04	0.06
3	0.07	0.12
4	0.25	0.26
5	0.41	0.38
6	0.58	0.54
7	0.69	0.74
8	0.79	0.79

The result of the calculation of κ, which is based on the increase in the isotachophoretic zone lengths with time, considering the change in the pH of the nutrient broth, is shown for the different components in Fig. 4. It can be seen that in agreement with the pH and the cfu/ml *versus* time courses, $\Delta\kappa$ remains nearly zero during the first 3 h and then increases steeply, the most dominant increases stemming from acetate on the anionic side and from NH_4^+ at the cationic side, which finally contribute about 0.3 mS/cm each, compared with the initial conditions.

The values obtained for the calculated changes in κ by summing the individual contributions of the ions and the changes in κ obtained by conductimetric measurements are given in Table IV. The values exhibit a high linear correlation ($r = 0.996$).

For a comparison with the relative decrease $-\Delta Z_M$ (as percentage), measured by the impedance-splitting method, the relative decrease in the specific resistance was calculated for the two conductivities shown in Table IV. The results are shown in Fig. 5 and agree over the entire time period to within about 10%, which can be considered as an excellent correlation. From these findings it can be concluded that those species formed during the bacterial growth which are determined by ITP are in fact identical with those which cause the change $-\Delta Z_M$ during the incubation period. The change $-\Delta Z_M$ as a bulk property can indeed be interpreted by the changes in the concentration of the individual ions determined.

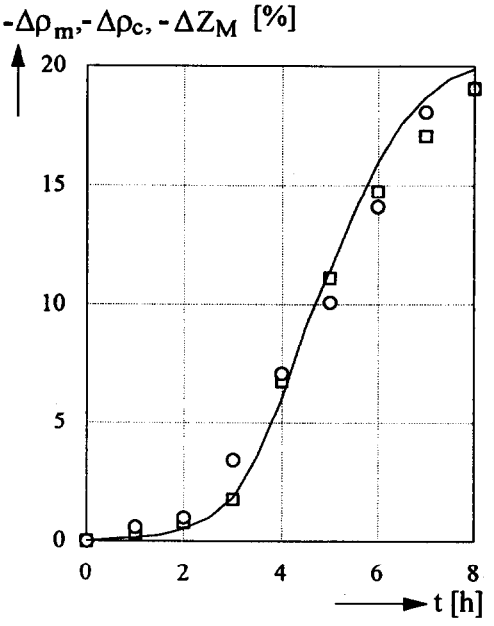

Fig. 5. Relative decreases in the measured and calculated specific resistances ($\bigcirc = -\Delta\rho_m$ and $\square = -\Delta\rho_c$, respectively) and the decrease $-\Delta Z_M$ of the impedance of the medium (solid line) as a function of time (t) of cultivation.

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