PRODUCTION OF L-MALIC ACID BY IMMOBILIZED BACTERIAL CELLS OF THE GENUS *Brevibacterium*

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A HPLC procedure was developed permitting an exact and rapid determination of L-malate, fumarate, succinate, and other dicarboxylic acids. The method was used to assay the products of fumarate metabolism in two *Brevibacterium* strains, obtained by screening, and showing a high fumarate conversion both in the cell suspension and in the immobilized system. Polyacrylamide and Ca-alginate were employed as polymeric matrices. The conditions were determined (pH, temperature, ion concentration, effect of inhibitors and detergents) under which L-malate is predominantly formed from fumarate and the conversion degree is the highest (8·60 mmol of L-malate h/g cells). The dependence of the conversion of various types of the polymeric matrix and the stability of the system in a continuous experimental arrangement were also examined. The results of these experiments will be utilized in L-malate technology.

L-Malic acid has received wide application especially in food industry, in the production of pharmaceuticals, and in metallurgy. Even though L-malic acid can be isolated from naturally occurring fruit juices or by the resolution of the racemic mixture prepared by chemical synthesis, considerable attention has been directed toward an economical preparation of L-malic acid by microbial cells. Ethanol[1], *n*-paraffins[2], acetate and propionate[3] have been examined as possible raw materials for the fermentation procedure. The first effort to prepare L-malic acid from fumarate on an industrial scale made use of *Lactobacillus brevis* cells[4]. The immobilization of fumarase from *Pseudomonas sp.*, the enzyme catalyzing the production of L-malic acid, was reported[5] in 1975.

The only procedure for L-malic acid production by microbial cells which has received so far technological application, is that of Yamamoto and coworkers[6] who used *Brevibacterium ammoniagenes* cells immobilized in polyacrylamide gel. The efficiency of the process has significantly increased recently[7–9], especially after the introduction of a new producing strain and of *kappa*-carrageenan as a new immobilization support. According to the authors the productivity of the immobilized cells has attained under optimal conditions the value of 42 kg of L-malic acid per h in a 1 000 l column.

As part of a general study on immobilized biological systems we decided to analyze in more detail the possibilities of preparation of L-malic acid by entrapped bacterial cells.

EXPERIMENTAL

Material

Cetyltrimethylammonium bromide (CTAB), sodium deoxycholate, Nonidet NP-40, and TWEEN 80 were from Serva (Heidelberg, F.R.G.), Triton X-100 from New England Nuclear (Dreieich, F.R.G.), heptanesulfonic acid from Fluka (Buchs, Switzerland), SAG (a mixture of polymethylsiloxane and emulsifiers) from Union Carbide, U.S.A. Na-alginate was purchased from Protan (Dramen, Norway). The remaining chemicals used (unless stated otherwise) were commercial preparations of analytical purity.

Methods

Microorganisms and conditions of their cultivation. The bacterial strains were obtained from a collection of CCEB, Institute of Entomology, Czechoslovak Academy of Sciences, Prague, and from the collection of this Institute. The medium used for the screening of strains showing a high fumarase activity contained 1% of Difco yeast extract, 0·5% of casamino acids, 0·8% of Difco nutrient broth (pH adjusted to pH 7). The medium used for the cultivation of strains showing a high fumarase activity contained 2% of corn steep liquor, 0·5% of diammonium citrate, 0·2% of KH_2PO_4, 0·05% of $MgSO_4.7\,H_2O$ and varying quantities (w/v) of fumarate and sodium malonate (pH adjusted to pH 7). Unless stated otherwise the microorganisms were grown for 24 h at 37°C with constant shaking.

Immobilization of microbial cells. The bacteria were immobilized immediately after isolation from the culture medium by centrifugation or ultrafiltration and washing with physiological saline by entrapment in polyacrylamide and Ca-alginate.

a) The bacteria were entrapped in the polyacrylamide matrix according to Yamamoto and coworkers[6] (w/v ratio of bacteria to the polymerization mixture 1 : 7). The resulting gel was disintegrated in a blender to particles of average size 1 mm which were subsequently washed with physiological saline.

b) The calcium alginate beads were prepared according to Kiersten and Bucke[10]; the suspension containing (in weight %) 2% of Na-alginate and 15% of bacterial cells (wet weight) was fed dropwise through a capillary 2 mm in diameter to a 2% aqueous solution of $CaCl_2$. The beads formed (diameter 4 ± 0·2 mm, average value obtained with 15 beads) were set aside for 5 h with constant stirring in the polymerization solution. The suspension was then dialyzed 5 h against physiological saline and the beads were either used or stored in 0·1 mol l^{-1} sterile fumarate solution at 4°C. When the cells were immobilized in alginate containing polyethyleneimine (PEI) the beads were placed in 0·30% (w/v) solution of polyethyleneimine and dialyzed against physiological saline afterwards.

If the bacterial cells were immobilized for experiments bound to proceed for more than 48 h the polymerization solutions were autoclaved for 10 min at 110°C before mixing with the suspension of the cells.

Unless stated otherwise, the immobilized cells were activated (increase in fumarase activity and suppression of succinate formation) by incubation in 1 mol l^{-1} sodium fumarate at pH 7·0 containing 0·02% of CTAB or 0·02% of SAG (ratio immobilized cells: sodium fumarate + detergent = 1/3) 20 h at 37°C with constant stirring. The immobilized cells activated by this procedure were dialyzed 5 h against physiological saline.

Standard fumarase activity assay. Unless stated otherwise the fumarase activity of intact and immobilized cells was determined in a reaction mixture containing the cells and 1 mol l^{-1} sodium fumarate (pH 7·0) at a ratio of 1 : 50 (wet cell weight/fumarate volume) at 37°C with constant stirring.

Determination of products of sodium fumarate conversion by microbial cells. Aliquots of 100 µl were removed from the reaction mixture at predetermined time intervals and subjected to ultrafiltration. Aliquot samples (10, 3 or 0·5 µl) were applied to the HPLC column. The dicarboxylic acids present in the mixture were analyzed by ion-pair or ion-exchange HPLC. The conditions of the separation and the detection procedure are given in Table I and in Fig. 1. L-Malate, fumarate, succinate and the other compounds which were present were identified by their retention times and quantitated by peak area measurement in a computing integrator (CI 100, Laboratorní přístroje, Prague). Uracil was used as an internal standard.

The determination of the number of immobilized cells in various gel types was effected by an indirect procedure by measuring protein content of the gel with subsequent extrapolation to cell weight[11].

Protein determination. The proteins were stained with Coomassie Brilliant Blue G-250 (ref.[12]).

RESULTS AND DISCUSSION

Screening of Microorganisms Showing High Fumarase Activity

A number of representatives of various bacterial genera were tested in order to find

TABLE I

Conditions of separation of selected dicarboxylic acids by HPLC

Characteristics	Method	
	A	*B*
Separation principle	Ion-pair chromatography	Ion-exchange chromatography
Column packing	Separon SIX C 18, manufactured by LP, Prague	Separon SIX-NH_2, manufactured by LP, Prague
Medium particle size	5 µm	5 µm
Column dimensions	3·3 × 150 mm, glass tube	3·3 × 150 mm, glass tube
Mobile phase	50 mmol l^{-1} $(NH_4)H_2PO_4$ + + 5 mmol l^{-1} n-$Bu_3N.H_3PO_4$ pH 2·05	0·15 mol l^{-1} $(NH_4)H_2PO_4$, pH 4·50
Flow rate of mobile phase	0·20, 0·45 ml/min	0·20, 0·45 ml/min
Column temperature	ambient	ambient
Detection	Model UVM4 Spectral UV Analyzer, Instrument Development Workshops, Czechoslovak Academy of Sciences, Model RIDK Refractometer, LP, Prague	Model UVM4 Spectral UV Analyzer, Instrument Development Workshops, Czechoslovak Academy of Sciences, Model RIDK Refractometer, LP, Prague

microorganisms showing a high fumarase activity in the immobilized system, suitable for continuous production of L-malic acid. As obvious from Table II a relatively high fumarase activity under the conditions of the experiment was found in numerous species, in accordance with the observations of Japanese authors[13]. The most important criteria of selection of the appropriate bacterial species for additional experiments was the fumarase activity, possibly highest in the immobilized system, and the level (possibly lowest) of side product (succinate) formation. From this viewpoint two strains, *Brevibacterium imperiale* and *Brevibacterium sp. mal* appeared optimal. The results of our experiments also show that both in intact cells and in immobilized cells the detergent suppresses not only succinate formation but also significantly increases the degree of conversion of sodium fumarate into L-malic acid.

Conversion of Sodium Fumarate into L-Malic Acid as Function of Time

Fig. 2. shows the time profile of sodium fumarate conversion by immobilized cells of selected bacterial strains which had been activated by CTAB. It is evident that this

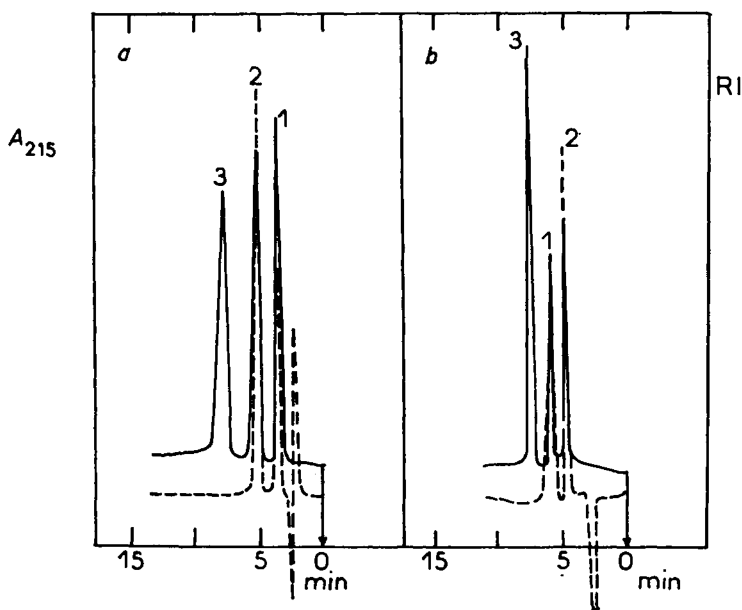

FIG. 1

Resolution of dicarboxylic acids by HPLC. The conditions are given in the legend to Table I. A 10 μl sample containing 125 μg of L-malate, 150 μg of succinate, and 0·45 μg of fumarate was applied to the column. (When refractometric detection was used the fumarate content was beyond the detection limit of the assay.) Sensitivity of detection: λ = 215 nm, refractometer scale X 32. 1 L-Malate, 2 succinate, 3 fumarate. *a* ion pair chromatography, *b* ion exchange chromatography

TABLE II

Conversion of sodium fumarate by selected bacterial species

Microorganism	L-Malate formation mmol/h/g cells[a]				Succinate formation μmol/h/g cells[a]			
	Intact cells		Immobilized cells		Intact cells		Immobilized cells	
	Without detergent	CTAB	Without detergent	CTAB	Without detergent	CTAB	Without detergent	CTAB
Brevibacterium amoniagenes	0·26	8·30	0·48	7·40	14	252	20	270
Brevibacterium saperdae	0·13	4·30	0·24	2·10	12	80	18	54
Brevibacterium fuscum	0·10	0·12	0·10	0·42	[b]	[b]	[b]	22
Brevibacterium protophormiae	0·14	0·62	0·15	0·90	[b]	82	43	108
Brevibacterium imperiale	0·10	6·60	1·15	8·20	[b]	70	[b]	82
Brevibacterium vitarumen	0·04	0·20	0·08	1·15	[b]	[b]	[b]	87
Alcaligenes metalcaligenes[c]	0·18	3·70	0·30	2·10	10	54	[b]	49
Corynebacterium sp.	0·12	1·06	0·43	3·05	12	180	58	240
Brevibacterium sp. mal.	0·20	6·80	1·20	8·60	[b]	61	[b]	90

[a] Wet cell weight; [b] beyond detection limit of the assay; [c] cultivation at 30°C.

enzymatic conversion follows the classical kinetics of product formation as a function of time. The slope of the linear part of the curve was determined at time $t = 10$ min.

Catalytic Properties of Immobilized Cells

To determine the optimal conditions of L-malic acid production by immobilized bacterial cells some of the enzymatic characteristics of the detergent-activated cells were examined. The effect of pH on L-malic acid formation is shown in Fig. 3. The pH-optimum varies between 6·5 and 7·5 both for active cells and also for the immobilized cells. It is therefore obvious that the immobilization does not significantly affect the pH-optimum. No differences were observed, either, as regards the different polymeric matrices even though polyacrylamide represents a type of nonionic polymer and Ca-alginate is a polymer of ionic type. It appears therefore that the electrostatic theory of immobilized enzyme systems[14] postulating a shift of pH-optima of enzymes immobilized in an ionic polymerous matrix does not hold either partly or completely for immobilized cells[15]. The effect of temperature on L-malic acid formation is shown in Fig. 4. The optimum temperature varies between 55 and 60°C in all cases. The operational stability of immobilized cells kept at temperatures higher than 50°C, however, sharply decreases with time thus making the preparation of L-malic acid under these conditions uneconomical.

Because of the existence of the equilibrium attained in the fumarase catalyzed reaction K_{eq} ([L-malate]/[fumarate]) was determined at various temperatures with 1 mol l^{-1} sodium fumarate as substrate. The K_{eq}-value found at 37°C was 4·55 ± 0·1. Fig. 4 shows the logarithmic plot of K_{eq} *versus* $1/T$.

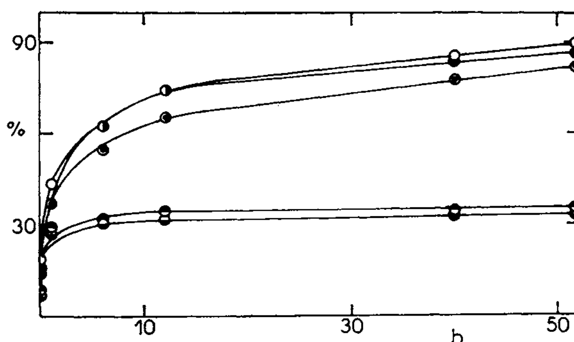

FIG. 2

Conversion of fumarate (in %) into L-malate as function of time. The slope of the curve was determined at $t = 10$ min. Calculated values of tg φ: ○ 3·58, ● 3·22, ⊙ 2·833, ◓ 1·76, ◒ 1·566. ○ ***Brevibacterium imperiale***, ● ***Brevibacterium amoniagenes***, ⊙ ***Alcaligenes metalcaligenes***, ◓ ***Brevibacterium vitarumen***, ◒ ***Brevibacterium saperdeae***.

Effect of Detergents on L-Malate and Succinate Formation

One drawback of the system studied by us is the formation of a side product, succinate, which – unlike fumarate – can be separated from L-malic acid only with difficulties. It is therefore necessary to establish conditions minimizing succinate formation. As follows from the studies of Japanese authors[6] the most advantageous way is the treatment of the immobilized system by a selective detergent. Such a treatment results not only in the suppression of succinate formation but also in a big increase of L-malate production leaving the stability of the system essentially unaffected. Table III shows the results of experiments in which we examined the effect of treatment of the cells immobilized in alginate with a number of different detergents. We assayed the induction of L-malate production, the suppression of succinate formation and the quantity of proteins released which should be an indirect measure of cells lysis caused by the detergent. The results of our study are in good agreement with those obtained by the Japanese authors[6]. CTAB, sodium deoxycholate, bile acids, and SAG are the best detergents from the results of our experiments. SAG

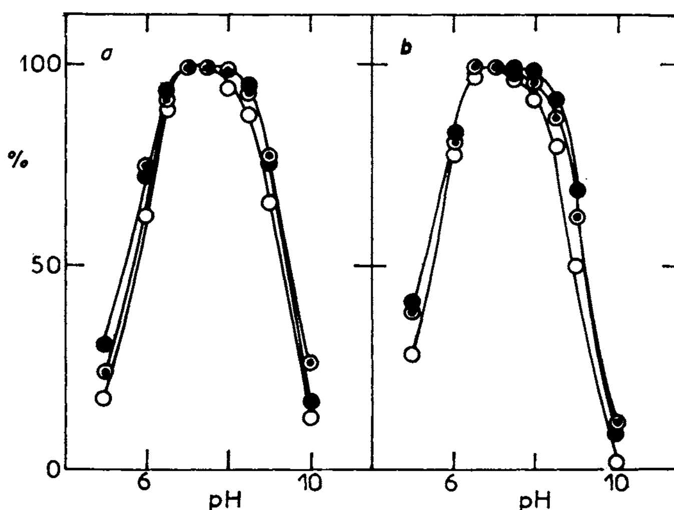

FIG. 3

Conversion of fumarate into L-malate, expressed as relative activity, at various pH-values. Microorganisms used: *a* ***Brevibacterium amoniagenes***, ***b Brevibacterium sp. mal.*** Composition of reaction mixture: intact cells + 0·02 CTAB or activated immobilized cells resuspended in 0·5 mol . . l^{-1} sodium fumarate in 0·1 mol l^{-1} buffer solution. Buffers used: acetate (pH 5·0), MES – [2-(N-morpholino)ethane sulfonic acid] (pH 6·0–7·0), HEPES – [(N-hydroxyethylpiperazine--N'-2-ethanesulfonic acid)] (pH 7·0–8·5), CHES – [cyclohexylaminoethanesulfonic acid] (pH 9·0–10·0); ● intact cells , ⊙ cells immobilized in Ca-alginate, ○ cells immobilized in polyacrylamide

seems as the most promising one from the economical point of view even though its use (obviously because of its high hydrophobicity) requires a vigorous shaking of the system during its activation. The marked difference in the quantity of proteins liberated by the detergents between intact cells and cells immobilized in Ca-alginate could be ascribed either to a higher stability of the immobilized system and/or to the ability of alginate gel to retain the proteins liberated because of its pore size.

Stimulating Effect of Malonate and Fumarate on Bacteria Growth and Their Fumarase Activity

The studies on the effect of a number of carbon sources in the culture medium on the fumarase activity of *Brevibacterium flavum* carried out by Takata and coworkers[13] showed a remarkable stimulating effect of relatively high malonate concentrations,

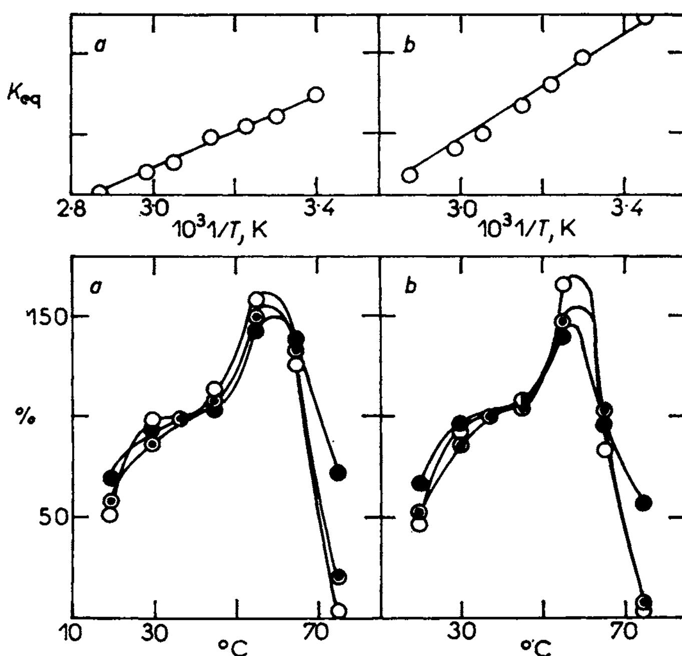

FIG. 4

Conversion of fumarate into L-malate, expressed as relative activity, as function of temperature. The equilibrium constant K_{eq} (L-malate/fumarate) was determined for the individual temperatures using 1 mol l^{-1} fumarate as substrate, the concentration of L-malate and fumarate were determined at the moment the reaction reached equilibrium. Microorganisms used: *a Brevibacterium amoniagenes*, *b Brevibacterium sp. mal.* See Fig. 3 for symbols.

TABLE III

Effect of various detergent types on sodium fumarate conversion by *Brevibacterium imperiale* cells

Detergent	Concentration %	Intact cells			Immobilized cells		
		L-Malate formation mmol/h/g	Succinate formation μmol/h/g	Proteins[a] liberated μg ml^{-1}	L-Malate formation mmol/h/g	Succinate formation μmol/h/g	Proteins[a] liberated μg ml^{-1}
None	—	0·10	[b]	86	1·15	[b]	6
CTAB	0·02	9·06	70	215	8·20	82	50
SDS	0·02	8·40	108	70	7·30	150	14
Sodium cholate	0·2	9·65	42	40	5·40	71	8
Sodium deoxycholate	0·2	10·20	45	88	8·06	62	8
TRITON X-100	0·02	6·40	420	126	6·90	345	34
TWEEN 80	0·02	1·25	140	96	5·80	180	28
Bile acids	0·2	6·70	51	113	8·35	60	16
Lauryl sarcosinate	0·02	7·75	180	62	7·16	195	10
Heptanesulfonic acid	0·02	0·69	47	135	3·12	75	19
Nonidet	0·02	0·72	54	120	1·29	108	26
SAG	0·002	7·90	57	56	7·80	25	10
SAG	0·02	8·20	23	23	8·25	24	2
SAG	0·2	6·40	20	91	5·17	41	11

[a] The proteins in the reaction mixture were determined after completion of the reaction; [b] beyond the detection limit of the assay method.

a strong inhibitor of succinic dehydrogenase (and also a weak inhibitor of fumarase). We examined this effect in more detail. As shown in Fig. 5 the optimal concentration of malonate in the culture medium which yields microorganisms of highest fumarase activity is 2·5%. We have not determined as yet whether this fact is due to an increased induction of fumarase formation in the growing bacterial cells or to an increased permeability of the cell wall for the substrate. On the other hand, the presence of fumarate in the culture medium is without any effect on the fumarase activity of the growing microorganisms.

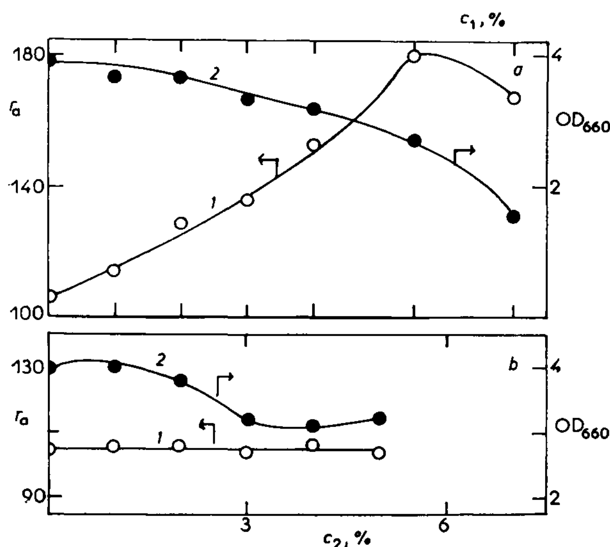

FIG. 5

Stimulating effect of malonate (*a*) and fumarate (*b*) on growth of bacteria and their fumarase activity. Microorganism: *Brevibacterium sp. mal*, the corresponding quantity of sodium malonate or fumarate was added to the medium and the pH was adjusted to 7·0. The equilibrium constants (K_{eq}) were determined as described in the legend to Fig. 4. Symbols: r_a fumarase activity expressed in μmol/h/g dry cell weight, c_1 malonate concentration in culture medium, c_2 fumarate concentration in culture medium; *1* fumarase activity, *2* cell growth expressed in OD_{660}. Calculated K_{eq}-values: *a* $c_1 =$ $= 0\%$ — 4·50, 0·5% — 4·56, 1·0% — 4·90, 1·5% — 5·60, 2·0% — 6·80, 2·5% — 7·33; *b* $c_2 = 0\%$ — 4·50, 1% — 4·52, 2% — 4·52, 3% — 4·57, 4% — 4·59, 5% — 4·62.

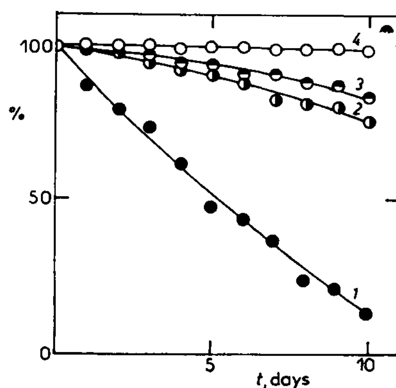

FIG. 6

Transformation of sodium fumarate in column of immobilized *Brevibacterium sp. mal* cells. The microorganisms were grown 48 h at 37°C. Column: 0·8 × 0·6 cm; flow rate of substrate: 10 ml/h; substrate: 1 mol . . l^{-1} sodium fumarate (pH 7·0); temperature of the flowing substrate and of the column were kept at 37°C. *1* intact cells, *2* cells immobilized in polyacrylamide, *3* cells immobilized in Ca-alginate, *4* cells immobilized in Ca-alginate + 0·30% of PEI.

Effect of Ions on L-Malic Acid Formation

The effect of some ions in the reaction mixture containing either intact or immobilized cells on L-malic acid formation is summarized in Table IV. A fact deserving interest is the increase of the conversion degree in the presence of K^+- and Cu^{2+}-ions in the reaction mixture. It remains to be shown whether this stimulating effect is operational at the level of the enzyme itself or whether the presence of these ions affects substrate transport or the permeability of the bacterial cell wall. An investigation of the effect of these ions on purified fumarase will be necessary to elucidate these problems.

Comparison of Stability of Fumarase Activity of Intact and Immobilized Cells

In an effort to compare the efficiency of intact cells and of cells immobilized in the various types of polymeric matrices under the conditions of the enzyme reaction the stability of fumarase activity in these systems was examined as a function of time. Both the intact and the immobilized cells were activated in the same manner by CTAB. The results of these experiments are shown in Fig. 6. It is obvious that the stability of the immobilized cells is much higher than the stability of the intact cells. Of the different polymerous matrices used for the immobilization the highest stability was observed with Ca-alginate containing 0·3% of PEI. The lower stability of the Ca--alginate gel is caused by its partial dissolving (detected refractometrically), most likely due to the constant action of relatively high fumarate concentration. The latter obviously acts as an agent chelating Ca^{2+}-ions. The dissolution is the highest during

TABLE IV

Effect of some ions present in reaction mixture on fumarate conversion by *Brevibacterium sp. mal* cells

Addition	Concentration mol	L-Malate formation mmol/h/g cells	
		Intact cells	Immobilized cells
Li-fumarate	1	11·80	10·20
K-fumarate	1	13·40	13·70
Na-fumarate	1	11·80	10·20
Na-fumarate/NaCN	$1/1 . 10^{-4}$	7·50	4·10
Na-fumarate/$CuSO_4$.5 H_2O	$1/1 . 10^{-4}$	15·29	14·37
Na-fumarate/$HgCl_2$	$1/1 . 10^{-4}$	[a]	[a]
Na-fumarate/malonate	1/0·5	[a]	[a]

[a] Beyond the detection limit of the assay.

the first 48 h of passage of the substrate through the system. The lower stability of the acrylamide gel is caused, besides others, also by the substantially lower size of the particles (approximately 1 mm) and thus by a higher leakage of biological material.

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