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Uptake of saccharin and related intense sweeteners by Streptococcus mutans NCTC 10449

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Summary: In a 1-octanol/phosphate buffer system, saccharin was much more lipophilic than would be inferred from its dissociation constant which, however, determined the partition behavior of acesulfame and cyclamate. The uptake of saccharin into Streptococcus mutans led to a 30 to 40-fold higher concentration of this intense sweetener within cells than in the incubation medium. Acesulfame and cyclamate were distributed between cells and medium essentially in a diffusion-controlled manner. The uptake of saccharin into S. mutans was found to depend strongly on simultaneous sugar fermentation, and in addition, on external pH, sweetener concentrations, and cell densities. Without glycolysis, caused, for example, by an exhaustion of added sucrose, too acidic external pH, or the addition of glycolysis inhibitors, the uptake of saccharin was diffusion-controlled as in the case of acesulfame and cyclamate. The uptake of saccharin was inhibited by a reversal of the direction of the lactate gradient from in → out to out → in. The activation energy of saccharin uptake into glycolyzing S. mutans was near 18 kJ/mol, while glycolysis itself required 82–98 kJ/mol as activation energy, depending somewhat on experimental conditions. Up to 100 attomol of saccharin per bacterial cell was observed. It was concluded that the cytomembrane of S. mutans was involved in mediating the inhibitory effects of saccharin by an antiport of saccharin into cells in exchange for lactate.

Zusammenfassung: In einem 1-Octanol/Phosphatpuffersystem war Saccharin viel stärker lipophil, als aus seiner Dissoziationskonstante abgeleitet werden kann; diese dagegen bestimmte die Verteilung von Acesulfam und Cyclamat. Die Aufnahme von Saccharin in S. mutans ergab eine 30- bis 40fache Anreicherung dieses Süßstoffs gegenüber dem Medium. Acesulfam und Cyclamat verteilten sich zwischen Zellen und Medium im wesentlichen nach einem diffusionskontrollierten Vorgang. Die Aufnahme von Saccharin in S. mutans erwies sich als von einer gleichzeitigen Zuckerfermentation abhängig, ferner auch vom Außen-pH, von der Süßstoff-Konzentration und der Zellzahl. Ohne Glykolyse – z. B. aufgrund des Verbrauchs der vorgelegten Saccharose, wegen eines zu sauren pH-Wertes im Medium oder wegen Anwesenheit von Glykolyse-Inhibitoren – war die Aufnahme von Saccharin ebenso nur diffusionskontrolliert wie die des Acesulfams und die des Cyclamats. Durch Zugabe von L-Lactat ins Medium, wodurch die Richtung des Lactat-Gradienten umgekehrt wurde, war die Saccharin-Aufnahme gehemmt. Die Aktivierungsenergie der Saccharin-Aufnahme betrug rund 18 kJ/Mol, während die Glykolyse selbst je nach Versuchsbedingungen 82–98 kJ/Mol erforderte. Bis zu 100 attomol Saccharin wurden pro Bakterienzelle gefunden. Daraus läßt sich schließen, daß die Cytomembran von S. mutans an der Vermittlung der Hemmeffekte von

Saccharin auf die Zuckervergärung – durch Antiport des Intensivsüßstoffs in die Bakterienzelle im Austausch gegen Lactat – beteiligt ist.

Key words: acesulfame, anion transport, cyclamate, saccharin, streptococcus mutans, sweetener

Schlüsselwörter: Acesulfam, Anionentransport, Cyclamat, Saccharin, Streptococcus mutans, Süßstoff

Introduction

Saccharin and the related sweeteners acesulfame and cyclamate have recently been described as inhibitors of bacterial glycolysis (10, 15, 16). Streptococcus mutans NCTC 10449 was the bacteria most frequently used in these studies due to its high potential for producing acid from sugars, an important factor in the etiology of dental caries. Inhibition of glycolysis by intense sweeteners in vitro should thus result in a cariostatic action. Caries were indeed suppressed in the animal model by intense sweeteners (11, 16, 18) under various experimental conditions.

Data on the inhibition of bacterial growth and metabolism by saccharin (1–7, 9, 12, 13) and on the inhibition of bacterial enzymes in vitro (1–3, 8) essentially support this laboratory's findings regarding the inhibition of glycolysis and the suppression of dental caries in the animal model (10, 11, 15, 16, 18), for which dose/effect relations were established.

When the carbon flux through the glycolytic chain was decreased by saccharin and related intense sweeteners, the investigation of anaerobic and aerobic fermentation products of S. mutans NCTC 10449 demonstrated a qualitative shift in the patterns of acids formed, concerning mainly the lactate : pyruvate ratio and the extent of formate production (20). A crossover analysis of glycolytic intermediary metabolites in S. mutans cells revealed that intense sweeteners interfere in vivo with some enzymes of the bacterial glycolytic chain (19).

Although all of these effects require the intracellular presence of saccharin and related intense sweeteners in S. mutans, intracellular concentrations of acesulfame, cyclamate, and saccharin had not been determined in any microorganism prior to the present study; the quantitative aspects of all studies previously reported were based, of course, on the concentrations of sweeteners in the incubation media. The solvent/water partition of the intense sweeteners and their uptake in Streptococcus mutans were therefore studied here.

Materials and Methods

Chemicals

Acesulfame K was a gift from Dr. Rymon von Lipinski, Hoechst AG, Frankfurt, FRG; sodium cyclamate and sodium saccharin were commercial products of food quality.

Water-solvent partition of acesulfame, cyclamate and saccharin

The sweeteners were dissolved in 0.1 mol/l phosphate buffer at pH values from 7.0 to 0.5 (HCl added when necessary). Concentrations of acesulfame, cyclamate and saccharin were 50.0, 5.0, 0.5 and 0.05 mmol/l; 3 ml of a sweetener solution was added to 3 ml 1-octanol in a glass-stoppered test tube and shaken mechanically for 10 min. Aliquots of the aqueous phase were taken after phase separation and these were diluted when necessary for HPLC analyses of the sweeteners (see below). Upon finding less than 100 % of the respective sweetener in the aqueous phase, the organic phase was twice extracted with an equal volume of 1 % (w/v) sodium hydrogencarbonate for subsequent HPLC analysis.

HPLC analyses for acesulfame, cyclamate and saccharin

HPLC analyses were made according to Ziesenitz (17) with 0.004–0.008 NH_2SO_4 as eluent, depending on which sweetener was to be analyzed. A uv detector was used at 210 nm and calibrated with standard solutions of the sweeteners in the eluent, which had been read in the spectrophotometer Beckman DU-8 at 210 nm. HPLC uv detector and photometric readings were in agreement to within ± 5 %.

Growth conditions of S. mutans NCTC 10449

Were described in a previous paper (17).

Incubation for glycolysis and acid formation

The methods used here have been described in previous reports (16, 17); in most experiments pH stat techniques were applied. Under the conditions used for growth, detachment and suspension of S. mutans NCTC 10449, the relations existing between colony forming units (CFU), turbidity at 578 nm, and cell volume of saline-washed resting cells were as follows: 1×10^9 CFU ≙ OD_{578} 0.82 ≙ 2.6 µl. These data were used in calculating the intracellular concentrations of saccharin and related sweeteners in S. mutans.

Uptake of sweeteners into S. mutans

Incubations were performed as described above; cells were then separated by rapid centrifugation (2 min; 15 000 rpm) from the incubation medium and washed once with a salt solution (75 mmol/l each of NaCl and KCl, plus 2 mmol/l $MgSO_4$). Cells (for extraction of the sweeteners), incubation medium and washing fluid were heated for 10 min to 100 °C; this was followed by HPLC analyses (see above).

Thin-layer chromatography

for cyclohexylamine was done on silicagel 60, 0.2 mm thick, on alumina foil (Merck # 5553) using n-butanol/glacial acetic acid/H_2O = 4:1:5 as solvent; detection was made by means of the ninhydrin reagent Merck # 6758.

Results

Distribution of sweeteners between 1-octanol/phosphate buffer

In order to determine physicochemical characteristics of the intense sweeteners to be tested, acesulfame, cyclamate and saccharin were first examined as to their distribution between 1-octanol and phosphate buffer at pH values from pH 7.0 to pH 0.5. The results (shown in Fig. 1) furnish evidence for a distribution equilibrium very close to the dissociation

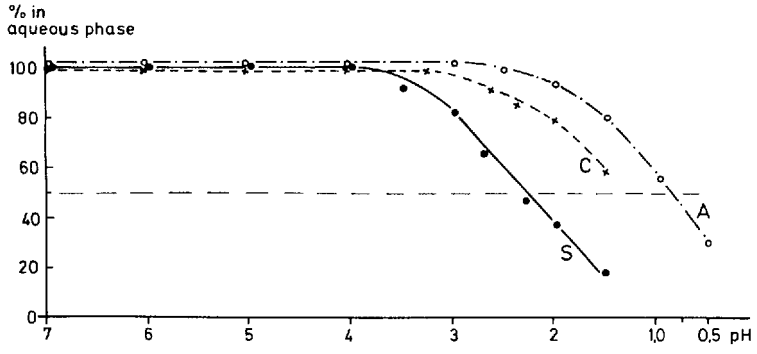

Fig. 1. Distribution of acesulfame (A), cyclamate (C), and saccharin (S) between 1-octanol and 0.1 mol/l phosphate buffer.

constant of the sweeteners in the case of acesulfame and cyclamate; pk_a: 0.76 for acesulfame, 1.70 for cyclamate.

The distribution equilibrium of saccharin between aqueous phase and organic phase was found at approximately pH 2.3, thus exhibiting a deviation from the pk_a of 1.5, and indicating a greater lipophilic tendency in the case of saccharin. From pH 4.0 to neutrality, all three intense sweeteners were exclusively present in the aqueous phase.

Uptake mechanisms for intense sweeteners into bacterial cells

Activation energy of saccharin uptake

An initial ascertainment of the properties of the uptake of saccharin in sucrose-fermenting, glucose-adapted S. mutans NCTC 10449 was per-

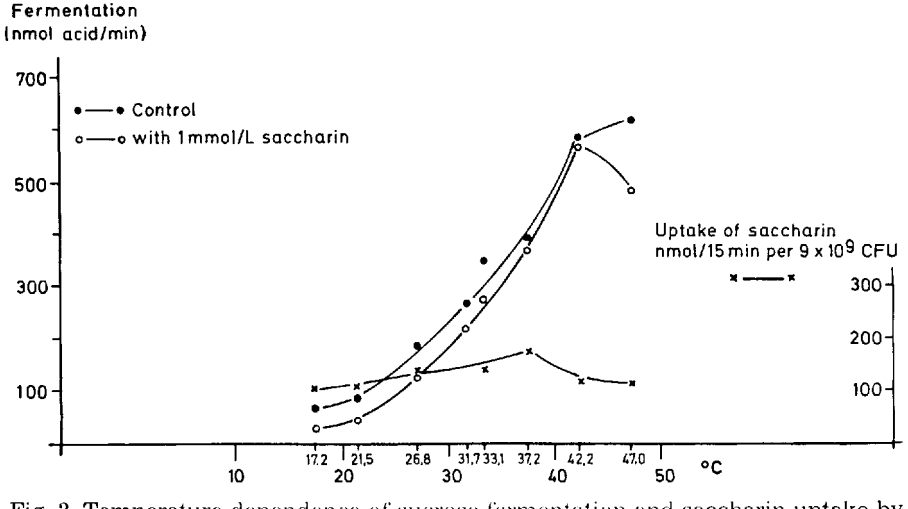

Fig. 2. Temperature dependence of sucrose fermentation and saccharin uptake by S. mutans NCTC 10449 cells at pH 4.5. Experimental conditions were those of Table 1.

Table 1. Activation energy of sucrose fermentation and saccharin uptake in S. mutans NCTC 10449.

Conditions		Activation energy			
Sucrose (mmol/l)	Saccharin (mmol/l)	pH 7.0		pH 4.5	
		(kcal/mol)	(kJ/mol)	(kcal/mol)	(kJ/mol)
Fermentation					
20	–	19.5	81.5	16.9	70.6
20	5	20.6	86.0	–	–
20	1	–	–	23.4	97.8
Saccharin uptake					
20	5	4.3	18.1		
20	1	–	–	4.3	18.1

pH stat experiments; 37 °C; anaerobic. Initial fermentation rates without saccharin were determined at each temperature, then saccharin was added and the fermentation rates as well as saccharin uptake were determined.
S. mutans NCTC 10449; glucose-adapted; 1.1×10^{10} CFU/assay at pH 7.0 and 9×10^{9} CFU/assay at pH 4.5.

formed by determining the temperature dependence of sucrose fermentation and saccharin uptake (Fig. 2). This permitted a calculation of the activation energy involved in these processes. The uptake of saccharin into bacterial cells was found to be non-temperature-dependent. These data (Fig. 2) served as a basis for the activation energy data at pH 7.0 and 4.5 as listed in Table 1.

Relatively high values were found for the activation energy of sucrose fermentation, understandable in light of the complexity of the fermentation process. Most notably, at pH 4.5 the activation energy of sucrose fermentation rose by 40 % in the presence of saccharin. At 4.3 kcal/mol, the

Table 2. Influence of sucrose fermentation on accumulation of intense sweeteners in S. mutans NCTC 10449.

Sweetener (mmol/l)	Concentration of intense sweeteners			
	Resting cells		Fermenting cells[1])	
	(mmol/l) intracellular	Factor of accumulation internal/external	(mmol/l) intracellular	Factor of accumulation internal/external
Acesulfame (7)	6.9	1.0	9.6	1.4
Cyclamate (3.5)	1.2	0.34	1.1	0.31
Saccharin (0.5)	0.08	0.16	3.0	6.0

Anaerobic incubation in pH stat for 15 min at pH 4.5 with and without sucrose; 37 °C; 1.2×10^{10} CFU/assay.
[1]) 20 mmol/l sucrose.

activation energy of saccharin uptake amounted to only 21 % of the activation energy of sucrose fermentation at pH 7.0; at pH 4.5, this value was 18 %. The activation energy of saccharin uptake was independent of pH. For this reason, enzymatically-catalyzed, energy-dependent mechanisms do not appear to play a significant role in saccharin uptake.

Metabolic requirements

Sucrose fermentation

S. mutans NCTC 10449 showed a distinct differentiation in concentrating the intense sweeteners acesulfame, cyclamate and saccharin (Table 2). When fermenting sucrose at pH 4.5, S. mutans accumulated 1.4 times more acesulfame than without fermentation. Sucrose fermentation did not affect the uptake of cyclamate. By contrast, sucrose-fermenting S. mutans was found to accumulate 38-times as much saccharin as non-fermenting streptococci.

Results essentially similar to those obtained for pH 4.5 were found by examining the accumulation of acesulfame, cyclamate, and saccharin at pH 7.0 (Fig. 3); in the course of these analyses, two concentrations of the intense sweeteners, varied by a factor of 5, were tested, each in the presence and in the absence of fermentation. It was only in the case of acesulfame that a tendency was observed towards inverse reaction at pH 7.0 in comparison to pH 4.5, in that somewhat less acesulfame was found in the S. mutans NCTC 10449 fermenting sucrose than in the non-fermenting bacteria (Fig. 3).

Cyclamate was not detectable in once-washed Streptococcus mutans (Fig. 3, top). If the washing fluid was included, then cyclamate concentrations were found which were analytically definite, but were independent of whether or not S. mutans was fermenting sugar (Fig. 3, bottom).

Table 3. Influence of fermentation inhibitors on saccharin uptake in S. mutans NCTC 10449 at pH 4.5.

Sucrose (mmol/l)	(20 mmol/l) substances added (mmol/l)	% inhibition of fermentation	% uptake of saccharin in 15 min	µmol/l saccharin intracellular
Saccharin (mmol/l)				
0.5	79.0 DMSO	7	6.7	973
0.5	0.05 Nigericin in DMSO	92	0.022	7.0
0.5	0.05 Gramicidin in DMSO	94	0.033	9.3
0.5	0.5 Fluoride[1])	>99	<0.4	<0.26

pH stat experiment at pH 4.5; 37 °C; anaerobic preincubation for 10 min; afterwards saccharin and nigericin or gramicidin added, respectively, anaerobic incubation with inhibitors for 15 min.

[1]) Fluoride was used without DMSO; cells were preincubated with fluoride for 5 min, then sucrose and saccharin were added. [DMSO = Dimethylsulfoxide]

Cyclamate was not degraded by S. mutans into cyclohexylamine and sulfate (thin-layer chromatograms not shown).

In the case of saccharin, the results obtained for pH 7.0 were very similar to those found at pH 4.5, in that sucrose fermentation raised saccharin uptake at 0.3 mmol/l by a factor of 130, and at 1.5 mmol/l by a factor of 250, respectively.

Further proof of the increase of saccharin uptake in the presence of sucrose fermentation was furnished by the use of fermentation inhibitors

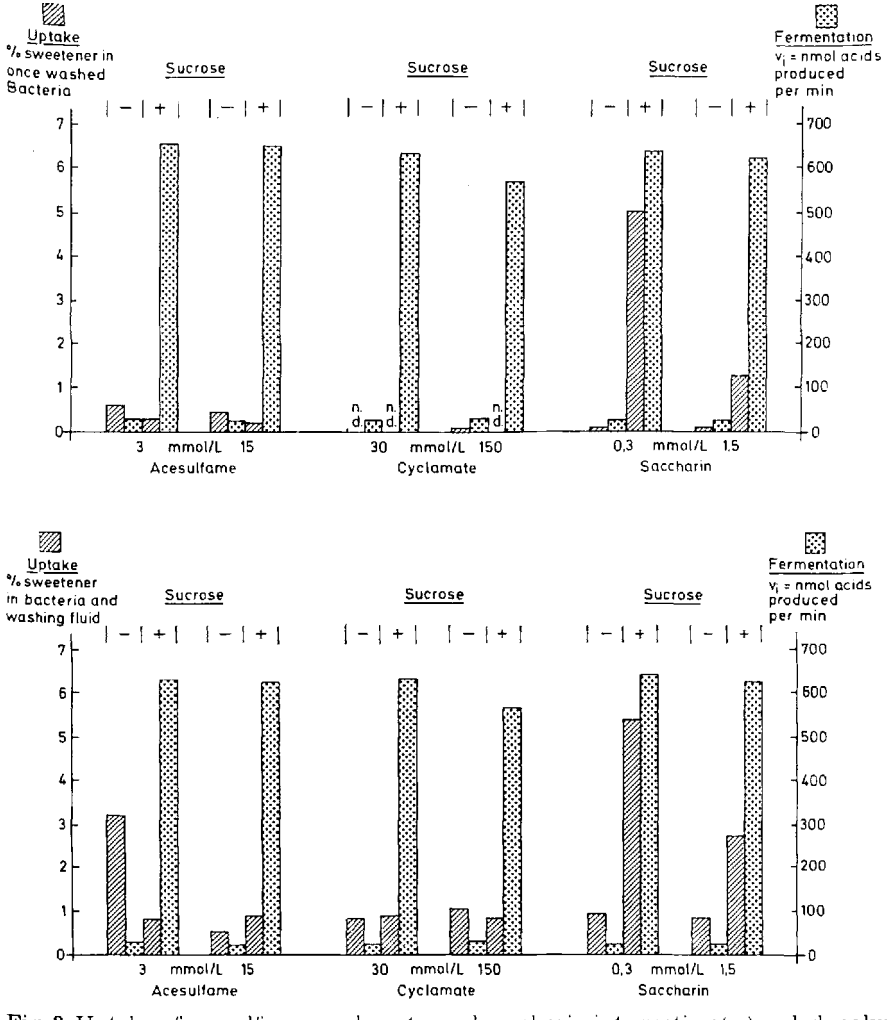

Fig. 3. Uptake of acesulfame, cyclamate, and saccharin into resting (−) and glycolyzing (+) cells of S. mutans NCTC 10449 at pH 7.0. When added, 20 mmol/l sucrose; anaerobic at 37 °C; 18 min; pH stat at pH 7.0. Upper panel: percent uptake of sweeteners into once saline-washed cells; lower panel: percent sweetener in once saline-washed cells plus the washing fluid (viz. Materials and Methods).

Table 4. Influence of length of a fermentation-free interval on saccharin uptake in S. mutans NCTC 10449.

Fermentation (without saccharin)		Uptake of saccharin at external concentration of 20 µmol/l			
Length of fermentation (min)	Fermentation-free interval before saccharin added (min)	pH 7.0		pH 4.5	
		% uptake	µmol/l (intracellular)	% uptake	µmol/l (intracellular)
15[a])	0	3.58	36.6	12.70	130.3
13[b])	2	0.68	6.9	5.58	57.1
10[c])	5	1.48	15.1	0.88	9.0
7[d])	8	0.43	4.4	0.48	4.9
0[e])	15	0.43	4.4	<0.03	<0.26

pH stat experiment; anaerobic; 37 °C; 1.5×10^{10} CFU/assay. Length of fermentation period was determined by quantity of sucrose added.
[a]) 5 mmol/l sucrose
[b]) 1 mmol/l sucrose
[c]) 0.3 mmol/l sucrose
[d]) 0.1 mmol/l sucrose
[e]) sucrose-free.

(Table 3). When sucrose fermentation was strongly inhibited by means of nigericin or gramicidin, and also by fluoride, the accumulation of saccharin in S. mutans was stopped.

Fermenting S. mutans very quickly accumulated saccharin (Fig. 4); after pre-incubation for 8 min with sucrose, the half-time of the intracellular saccharin uptake was approximately 0.3 min.

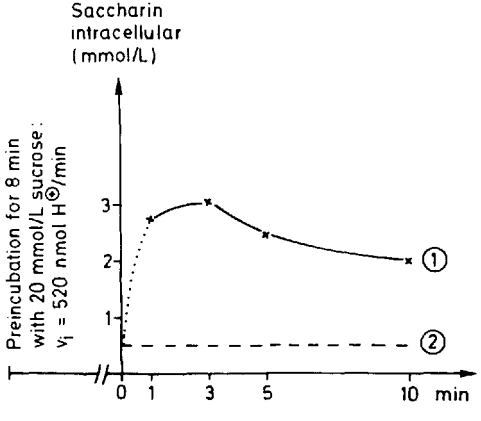

Fig. 4. Time elapsed for saccharin uptake in S. mutans NCTC 10449 at pH 4.5 during sucrose fermentation (20 mmol/l). Addition of saccharin (0.5 mmol/l) after 8 min of fermentation; 1.2×10^{10} CFU/assay. 1) intracellular concentration of saccharin (mmol/l), 2) concentration of saccharin in incubation medium (mmol/l).

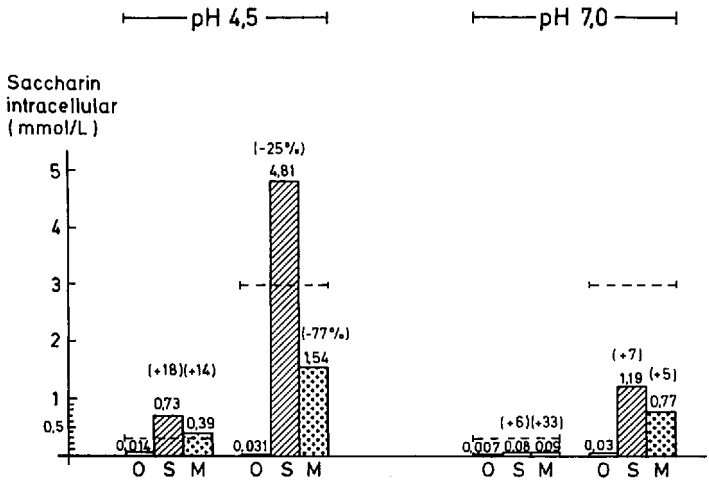

Fig. 5. Effects of sucrose and of maltose fermentation on saccharin uptake in S. mutans. pH stat, anaerobic; 37 °C; 20 mmol/l disaccharide; 2×10^{10} CFU/assay; S. mutans NCTC 10449. O = without sugar; S = with 20 mmol/l sucrose; M = with 20 mmol/l maltose.
(+ or −) = % of rate of fermentation with saccharin.
- - - - - = saccharin concentration in incubation medium.

When a fermentation-free period was established before adding saccharin, it was seen that the length of the fermentation-free interval determined the extent of saccharin uptake (Table 4). In this experiment, an interval of approximately 2–3 min was needed for reduction of the intracellular saccharin concentration to half the original level after fermentation was completed.

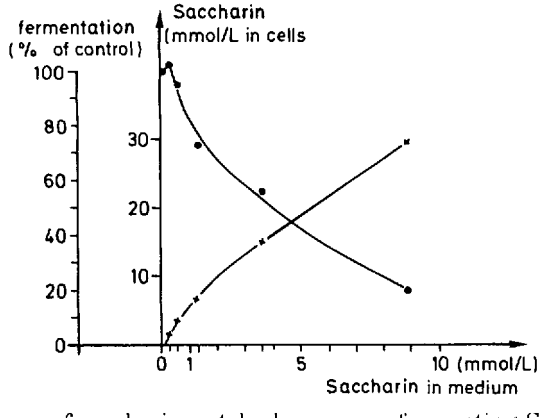

Fig. 6. Dependence of saccharin uptake by sucrose-fermenting S. mutans cells on the external concentration of saccharin as well as independence of saccharin uptake from fermentation inhibition via saccharin (viz. Table 5 on experimental conditions). ●——● = fermentation, ×——× = concentration of saccharin in S. mutans.

A further experiment served to test whether the highly stimulatory effect of sucrose fermentation on saccharin concentration was a sucrose-specific phenomenon, or whether this effect could also be observed together with the fermentation of other sugars, such as maltose. Figure 5 proves that, both at 0.3 and 3.0 mmol/l saccharin in the incubation medium, maltose fermentation served to increase the uptake of saccharin at pH 4.5 by a factor of 28 to 50. At pH 7.0, there was a discernible effect only at 3 mmol/L of saccharin in the medium; maltose fermentation led to a 25-fold increase in saccharin uptake by S. mutans NCTC 10449. Under the experimental conditions chosen for this experiment, sucrose and maltose fermentation were greater in the presence of 0.3 mmol/l of saccharin than in the saccharin-free control, indicated in parentheses (Fig. 5).

Saccharin uptake was tested primarily using intense sweetener concentrations which had no inhibitory effect on the fermentation of sugars, in consistence with earlier data (15, 16). The question of what dependence might exist between saccharin uptake and the concentration of saccharin in the medium was therefore closely allied with the question of whether inhibiting fermentation to a sub-maximal degree could also have an influence on saccharin uptake stimulated by fermentation.

Figure 6 shows that saccharin concentrations in bacterial cells rose in proportion with, but not linear to, the saccharin concentration in the incubation medium in the range between 0.7 and 8.9 mmol/l. The numerical values of these measurements are found in Table 5. Furthermore, inhibition of fermentation by 80 % was not seen to impair the concentration-dependent rise in saccharin uptake during sucrose fermentation (Fig. 6).

The pH-dependence of saccharin accumulation in S. mutans was examined first in the absence of fermentation at a level of 20 µmol/l of saccharin in the incubation medium. A clear pH-dependence was not discernible (detailed data not shown); from pH 5.5 to pH 4.0; however, the concentration of saccharin in the washing fluid rose to more than double the original level, indicating that saccharin was more easily washed out of non-fermenting cells at an acidic pH than at neutrality.

Table 5. Dependence of saccharin uptake upon its concentration at pH 4.5 in S. mutans (viz. also Fig. 6).

Saccharin in medium (mmol/l)	Saccharin intracellular		Factor of accumulation internal/external	Inhibition of fermentation (%)
	(nmol/10^{10} CFU)	(mmol/l)		
0	0	0	–	reference
0.27	45	1.7	6.6	+ 3
0.53	85	3.3	6.1	– 5
1.30	178	6.9	5.3	–28
3.60	397	15.3	4.3	–44
8.88	757	29.1	3.3	–80

S. mutans NCTC 10449; 1×10^{10} CFU/assay; 20 mmol/l sucrose; pH 4.5; 37 °C; anaerobic; pH stat experiment. After fermenting with sucrose for 8 min, saccharin was added at the final concentration shown and incubation then continued for 15 min.

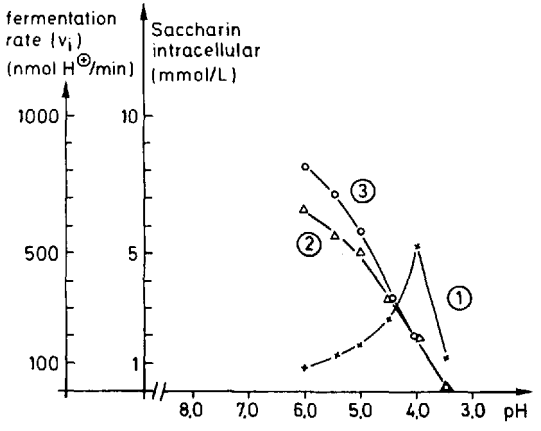

Fig. 7. pH-dependence of saccharin uptake during sugar fermentation (20 mmol/l sucrose) and 0.5 mmol/l saccharin in incubation medium. Cells were pre-incubated anaerobically for 8 min, saccharin was then added and cells were incubated for another 18 min. S. mutans NCTC 10449; 1×10^{10} CFU/assay; 37 °C; anaerobic; pH stat experiments.

1) intracellular saccharin concentration (mmol/l);
2) fermentation rate (v_i) in pre-phase (nmol $H^{\oplus}$/min);
3) fermentation rate (v_i) during saccharin uptake (nmol $H^{\oplus}$/min).

The pH-dependence of saccharin uptake during sugar fermentation was tested between pH 6.0 and pH 3.48 at an external saccharin concentration of 0.5 mmol/l (Fig. 7). Between pH 6.0 and 4.0, the uptake of saccharin increased fivefold, and fell sharply at more acidic pH values, at which point no more fermentation occurred.

It became especially apparent in this experiment (Fig. 7) that a rise in the rate of fermentation occurred at low concentrations of saccharin (0.5 mmol/l) when a pH value near neutrality was approached; a similar effect was already indicated in Fig. 5 at 0.3 mmol/l of saccharin.

During sucrose fermentation, saccharin uptake by S. mutans occurred in proportion with, but not linear to, the concentration of cells (Table 6);

Table 6. Accumulation of saccharin at various concentrations of streptococcal cells.

CFU/assay ($\times 10^8$)	Saccharin intracellular			
	nmol/CFU in assay		(mmol/l)	
	pH 7.0	pH 4.5	pH 7.0	pH 4.5
9	3.4	16	1.45	6.85
110	50	187	1.75	6.54
350	139	804	1.53	8.85

S. mutans NCTC 10449; 20 mmol/l sucrose; 37 °C; anaerobic; pH-stat experiments. Saccharin (5 mmol/l) was added after preincubating for 5 min; incubation with saccharin lasted 10 min.

Table 7. Influence of acetate and lactate in incubation medium on saccharin uptake in S. mutans NCTC 10449 at pH 4.5.

Fermentation			Uptake of saccharin	Saccharin intracellular	Decrease in saccharin uptake	Inhibition of fermentation
Sucrose (mmol/l)	Saccharin (mmol/l)	Acids (mmol/l)	(nmol/l)	(mmol/l)	(%)	(%)
20	1.0	–	108	4.2	reference	– 9
20	1.0	2.0 acetate	94	3.6	−14	−13
20	1.0	10.0 L-lactate	65	2.5	−40	−32

S. mutans NCTC 10449; 1×10^{10} CFU/assay; 37 °C; 15 min; anaerobic; pH stat experiment at pH 4.5.

taking the uptake data as a basis for calculating the intracellular concentration of saccharin as mmol/l, the average concentration was approximately 1.6 at pH 7.0, and 7.4 mmol/l of saccharin at pH 4.5, while cell density varied almost 40-fold. Thus, saccharin accumulation was shown to be essentially independent of cell concentration in this experiment.

Saccharin uptake in glycolyzing streptococci was also examined adding lactate or acetate to the incubation medium at a pH of 4.5 (Table 7). Acetate (2.0 mmol/l) reduced sucrose fermentation and saccharin uptake by 13 % and 14 %, respectively. L-lactate (10 mmol/l) inhibited fermentation by 32 % and inhibited saccharin uptake by 40 %.

Discussion

The concentrations of intense sweeteners analyzed in S. mutans permitted a first differentiation to be made between the three sweeteners acesulfame, cyclamate, and saccharin. Saccharin was accumulated by S. mutans intracellularly to a concentration far greater than that of the medium whenever sugar was being fermented simultaneously. This fermentation-dependent saccharin accumulation in S. mutans could well be independent of the chemical nature of easily fermentable carbohydrates, as maltose fermentation facilitated the accumulation of saccharin very similar to that observed during sucrose fermentation (Fig. 5). Due to the relatively low level of activation energy observed (Table 1), the uptake process for saccharin into streptococci rules out the involvement of enzymatically catalyzed reactions here.

The uptake of saccharin into bacterial cells was definitely influenced by the simultaneous occurence of sugar fermentation (Fig. 3, Tables 2 and 4); on the other hand, experimentally added lactate or acetate led to an inhibition of fermentation and to a lower saccharin accumulation (Table 7). These findings make an antiport-mechanism of the model saccharin → in, while lactate → out appear probable for saccharin accumulation in S. mutans. Such an antiport mechanism for the uptake of saccharin into streptococci was not even affected by an inhibition of sucrose fermentation up to 80 %, since a stoichiometric analysis of mol of lactate formed per

mol of saccharin taken up by streptococci showed inhibition of fermentation of 80 % to be still compatible with an antiport mechanism.

The partition equilibrium in an organic/aqueous phase system (Fig. 1) was governed by the dissociation constants (10) of acesulfame and cyclamate, but not of saccharin, which displayed a more lipophilic character than would be inferred from the existence of the undissociated saccharin molecules; pk_a values of acesulfame (0.76), cyclamate (1.7), and saccharin (1.5) yield undissociated sweetener molecules at pH 7.0 of 6×10^{-4} % acesulfame, 5.4×10^{-3} % cyclamate, and 3.5×10^{-3} % saccharin, and make it highly unlikely that the uptake of intense sweeteners at neutral pH concerns the undissociated species. At pH 4.0, which is frequently reached in glycolyzing S. mutans, less than $^1/_{10}$ of a percent of acesulfame, and less than 1 % of cyclamate and saccharin exist in their undissociated forms. The severalfold increased uptake of saccharin at pH 4.5, compared to pH 7.0 (Table 4), may reflect a contribution to its uptake from its dissociation behavior, but would have to be > 100-fold stronger at pH 4.5 than at neutral pH if the uptake were dependent on the dissociation properties of saccharin. Thus, saccharin and related intense sweeteners exhibited a distribution behavior between resting S. mutans cells and the incubation medium which was essentially diffusion-controlled (Fig. 3) in the absence of sugar fermentation.

However, saccharin behaved quite differently when its distribution was determined between glycolyzing S. mutans cells and incubation medium (see above). It did not matter whether the external concentration of saccharin was chosen at inhibitory (e.g., Figs. 3, 5; Tables 5, 6) or at non-inhibitory concentrations (e.g., Figs. 4–6; Tables 2, 4, 5); as long as the inhibition was incomplete (Fig. 6), the dependence of the uptake process on glycolysis was clearly seen. At nearly 100 % inhibition (Table 3), however, the uptake of saccharin was negligible and similar to that of resting cells. The dependence on glycolysis (Table 4) was time-dependent in that an interval of as little as a few minutes between the end of acid production and the uptake experiment sufficed to eliminate the effect of glycolysis.

It is to be concluded from the activation energies in the uptake of saccharin (about 4 kcal/mol) and of glycolysis in the absence or presence of saccharin, both at neutral and acidic pH of 17–22 kcal/mol (Table 1) that no cellular energy such as ATP or phosphoenolpyruvate is spent for the uptake of saccharin; rather, facilitated diffusion should be held responsible for concentrating saccharin 30 to 40-fold within S. mutans cells. As the driving force, an antiport of saccharin$^{\ominus}$ *vs* lactate$^{\ominus}$ is derived from the data of Table 7: saccharin can be enriched within cells as long as lactate and perhaps other acidic fermentation products are discharged into the incubation medium. Intracellular concentrations of saccharin ranged from about 6 attomol[*])/cell to 75 amol/cell at pH 4.5 (Fig. 3), depending on the external concentration of saccharin; about 3–4 amol/cell of saccharin was found at pH 7.0.

In the studies reported above, saccharin had to be differentiated from acesulfame and cyclamate in regard to its partition between organic/aqueous phases and its distribution between cells/medium. When incubat-

[*]) attomol = 10^{-18} mol

ing S. mutans with acesulfame and cyclamate, it is to be expected that the concentrations of intense sweetener given in incubation medium are about the same as those in the cytoplasma of bacterial cells. In contrast, substantially higher intracellular concentrations are to be expected with saccharin than those given for incubation media or a cariostatic treatment (14). By the antiport-driven entry of the saccharin anion into bacterial cells, the intracellular accumulation results in a disappearance, or perhaps even a reversal of, the difference in the molar inhibitory potencies between acesulfame and saccharin (15); acesulfame was found to be about six times less potent than saccharin in inhibiting glycolysis, calculated on the basis of external (medium) concentrations. When the data of this paper were taken together, inhibition of acid formation might well occur at a smaller intracellular concentration of acesulfame than that of saccharin.

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References

1. Best GM, Brown AT (1987) Interaction of saccharin with hexitol metabolism by Streptococcus mutans. Caries Res 21:204–214
2. Brown AT, Best GM (1986) A proposed mechanism for the effects of saccharin on glucose metabolism by Streptococcus mutans. Caries Res 20:406–418
3. Brown AT, Best GM (1988) Apparent synergism between the interaction of saccharin, acesulfame K and fluoride with hexitol metabolism by Streptococcus mutans. Caries Res 22:2–6
4. Grenby TH, Saldanha MC (1986) Studies of the inhibitory action of intense sweeteners on oral microorganisms relating to dental health. Caries Res 20:7–16
5. Linke HAB, Chang CA (1976) Physiological effects of sucrose substitutes and artificial sweeteners on growth pattern and acid production of glucose-grown Streptococcus mutans strains in vitro. Z Naturforsch 31c:245–251
6. Linke HAB (1977) Growth inhibition of glucose-grown cariogenic and other streptococci by saccharin in vitro. Z Naturforsch 32c:839–843
7. Linke HAB (1983) Adherence of Streptococcus mutans to smooth surfaces in the presence of artificial sweeteners. Microbios 36:41–45
8. Linke HAB, Kohn JS (1984) Inhibitory effect of saccharin on glycolytic enzymes in cell-free extracts of Streptococcus mutans. Caries Res 18:12–16
9. Linke HAB, Doyle GA (1985) Effect of saccharin on growth and acid production of glucose-grown pathogenic and oral bacteria. Microbios 42:163–173
10. Pfeffer M, Ziesenitz SC, Siebert G (1985) Acesulfame K, cyclamate and saccharin inhibit the anaerobic fermentation of glucose by intestinal bacteria. Z Ernährungswiss 24:231–235
11. Siebert G, Ziesenitz SC, Lotter J (1987) Marked inhibition of caries in the sucrose-challenged rat by a mixture of nonnutritive sweeteners. Caries Res 21:141–148
12. Slee AM, Tanzer JM (1981) The effect of sodium saccharin on the growth of cariogenic and other selected plaque-forming streptococci. In: Ayer W (ed) Fifth Annual Conference on Foods, Nutrition and Dental Health, pp 25–36. American Dental Association, Chicago

13. Tanzer JM, Slee AM (1983) Saccharin inhibits tooth decay in laboratory model. J Amer Dent Assoc 106:331–333
14. Tanzer JM, Grant L, McMahon T (1988) Bicarbonate-based dental powder, fluoride, and saccharin inhibition of dental caries associated with Streptococcus mutans infection of rats. J Dent Res 67:969–972
15. Ziesenitz SC, Siebert G (1986) Nonnutritive sweeteners as inhibitors of acid formation by oral microorganisms. Caries Res 20:498–502
16. Ziesenitz SC (1987a) Süßstoffwirkungen auf Mundbakterien und Karies. Dtsch zahnärztl Z 42:S113–S123
17. Ziesenitz SC (1987b) Zuckervergärung durch Mundbakterien. Dtsch zahnärztl Z 42:S30–S38
18. Ziesenitz SC (submitted) Anticariogenic effect of a combination of acesulfame, cyclamate and saccharin depends on sucrose concentrations fed to rats
19. Ziesenitz SC, Friedrich S (submitted) Identification of the glycolytic enzymes from Streptococcus mutans NCTC 10449 inhibited by saccharin
20. Ziesenitz SC, Siebert G (submitted) Inhibition of fermentation in Streptococcus mutans NCTC 10449 by the intense sweeteners acesulfame, cyclamate and saccharin

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