Microbial Aldonolactone Formation and Hydrolysis: Kinetic and Bioenergetic Aspects

H. J. Noorman,* J. L. L. Rakels, J. G. Kuenen, K. Ch. A. M. Luyben, and J. J. Heijnen

Department of Biochemical Engineering, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

From previous work, it was observed that during growth on mixtures of acetate and aldose sugars the growth yield of the bacterium *Acinetobacter calcoaceticus* was increased when aldonolactone hydrolysis occurred. In this paper, the underlying mechanism was investigated by studying the relationship between cellular yield and lactone hydrolysis in a quantitative way. The literature on the kinetics of acid formation from aldonolactones was reviewed. It appears that the reaction pattern is composed of lactone isomerization and hydrolysis steps which occur in series. In the acid pH range, lactone interconversion is the rate-limiting step for acid formation, whereas above pH 7 hydrolysis is slowest. Only for D-gluconolactone could the detailed mechanistic kinetics be evaluated. For a number of other aldonolactones, including D-xylono- and D-galactonolactone, an empirical kinetic equation was derived. The reaction rate constants of these lactones are much smaller than for gluconolactone. The kinetic model for the hydrolysis of xylonolactone was experimentally verified, by application of the electrical charge balance to measurement data from *A. calcoaceticus* cultures. Comparison of the published biomass yield data and the calculated rate of lactone hydrolysis revealed that they do not quantitatively agree. It can be concluded that the observed relation between biomass yield and lactone hydrolysis is only apparent. The results demonstrate the power of the electrical charge balance in the study of processes in which charged compounds are being converted.

Introduction

The use of microorganisms for the production of aldonic acids and aldonates from aldoses is widespread. The conversion rates, specificity, and product yields are high, making competition with chemical aldose oxidation commercially attractive. Intermediate compounds in the aldose conversion are the corresponding aldonolactones, which can be subdivided into aldono- γ - (or -1,4-) lactones, which are five-membered rings, and aldono- δ - (or -1,5-) lactones, which are six-membered rings (as an example the lactones of D-xylose are shown in Figure 1).

Most common is the use of β -D-glucose for the biochemical production of D-glucono- δ -lactone, D-gluconates, and D-gluconic acid (Röhr et al., 1983). These compounds have found a broad spectrum of applications in the food, pharmaceutical, chemical, and agricultural industries (Theander, 1980; Milsom and Meers, 1985).

Many microbial enzymes are able to catalyze the oxidation of sugars into lactones. Most important are the sugar oxidoreductases and oxidases, of which a large number were reviewed by Dixon and Webb (1979). From β -D-glucose, the δ -lactone is invariably formed. During the enzymatic oxidation of a number of other aldoses, such as D-arabinose, L-arabinose, D-galactose, L-xylose, and L-fucose, formation of γ -lactone was observed in all cases investigated.

Under physiologically moderate conditions, the lactones hydrolyze spontaneously, although the rate of hydrolysis is, in some cases, increased by the action of a specific lactonase (Jermyn, 1960; Dutta, 1975; Buchert and Viikari, 1988). It is generally observed that the γ -lactones are less readily hydrolyzed than the δ -lactones (Shafizadeh, 1958; Lemieux, 1964).

Figure 1. Configurations of D-xylono- γ - and D-xylono- δ -lactone.

The physiological function of the oxidation reaction has remained unclear for a number of years. This is because the sugar or the products can be partly metabolized. Consequently, the physiological effect of aldose oxidation cannot be separately studied. However, a recent study with *Acinetobacter calcoaceticus* shed more light on the subject (van Schie et al., 1987). This bacterium was grown on mixtures of acetate and aldose sugars, where the sugars were converted into the corresponding aldonolactones. It forms an ideal model system for the study of the oxidation reaction, for the microbe is not capable of aldose, aldonolactone, or aldonate uptake. Furthermore, the cells do not possess lactonase activity (B. J. van Schie, personal communication).

An important observation in this study was that the growth yield could be increased, when aldose sugars were supplied to the culture. More specific, an unexplained relation was found between the occurrence of D-xylono-, D-galactono-, 2-deoxy-D-glucono-, and D-gluconolactone hydrolysis and growth yield (Figure 2a,b). It was assumed that the bacterium forms a δ -lactone from the six-membered D-glucose, D-galactose, and 2-deoxy-D-glucose rings, and a γ -lactone from the five-membered D-xylose ring. At pH 7, the δ -lactones hydrolyze relatively fast, with concomitant cell yield increase (Figure 2a). On the

* To whom correspondence should be addressed.

Figure 2. Steady-state biomass yield on acetate as a function of the aldose/acetate net conversion rate ratio. Measurements were performed at $D = 0.15 \text{ h}^{-1}$, 30°C , and pH 7 for D-glucose, D-galactose, 2-deoxy-D-glucose, and D-xylose, and also at pH 8.2 for xylose [from van Schie et al. (1987)].

other hand, xylono- γ -lactone is not so easily hydrolyzed, and the energetics of the cell are not affected (Figure 2b). At pH 8.2, however, xylono- γ -lactone is assumed to hydrolyze more rapidly, and an increase of the growth yield is observed. These observations led to the hypothesis, that "acid production (i.e., lactone hydrolysis) is a prerequisite for in vivo energy generation from aldose sugars" (van Schie et al., 1987). As mentioned, an explaining mechanism could, however, not be given.

Aldose oxidation in *A. calcoaceticus* is mediated by the PQQ-dependent glucose dehydrogenase (EC 1.1.99.17; Dokter et al., 1987). However, to our knowledge it is not known whether the oxidation products are γ - or δ -lactones. By extrapolating from the observations with other enzymes (Dixon and Webb, 1979), it may be assumed that this enzyme also forms the γ -lactone, except in the case of β -D-glucose and probably 2-deoxy-D-glucose, where the δ -lactone is produced. This extrapolation, if correct, is contradictory with the assumption by van Schie et al. (1987), regarding the oxidation of D-galactose. The formation of a relatively unstable D-galactono- δ -lactone was proposed, to explain the increase in the cell growth yield at pH 7. More definite conclusions on this apparent contradiction await elucidation of the type of lactones formed by the PQQ-dependent glucose dehydrogenase.

The objective of this article is to quantitatively investigate the qualitative hypothesis of van Schie et al. (1987), that the bacterium only benefits from sugar oxidation when the lactone is hydrolyzed. To provide a general kinetic description of the overall pattern of aldonolactone interconversion and hydrolysis, published kinetic data on D-

gluconolactone and other aldonolactones will be reviewed. To test the validity of the model based on published data, some experiments involving D-xylonolactone hydrolysis under real culture conditions will be described.

Literature Survey on Aldonolactone Interconversion and Hydrolysis

In the following section the results of a literature survey on aldonolactone interconversion and hydrolysis are summarized. Detailed information can be found in the supplementary material for this paper.

D-Gluconolactones. Between 1958 and 1973, the D-gluconolactones-gluconate equilibria were studied by a number of authors, many of which drew different conclusions on the structure of the overall reaction pattern (Shafizadeh, 1985; Sawyer and Bagger, 1959; Jermyn, 1960; Takahashi and Mitsumoto, 1963; Shimahara and Takahashi, 1970; Mitchell and Duke, 1970; Skibsted and Kilde, 1971; Pocker and Green, 1973).

From the observations of Jermyn (1960) and Shimahara and Takahashi (1970), it can be concluded that the following equilibrium scheme is the most probable:

The γ -lactone and the δ -lactone can be isomerized, only the δ -lactone can be hydrolyzed, and only the acid can be lactonized. The equilibrium constants of interconversion, hydrolysis, and dissociation are denoted by K_L , K_H , and K_A , respectively.

The most probable values for the equilibrium constants, the first-order δ -lactone hydrolysis rate constant (k_h) and the first-order constant expressing the conversion of γ - into δ -lactone (k_L) are summarized in Table I. The equation for k_h includes catalysis by H^+ and OH^- ions. The addition of salts, especially phosphate (Pocker and Green, 1973), may further increase k_h .

The value of k_L could not be reduced to true H^+ and OH^- catalysis. The given equation was derived using different buffer solutions.

Following consideration of the limiting step for the formation of acid from glucono- γ -lactone, at neutral pH a regime switch takes place. In the acid pH range, the interconversion is rate limiting, whereas at high pH values the hydrolysis is the slowest reaction.

Other Aldonolactones. Published data on the equilibrium constants of lactones other than the gluconolactones are very scarce. According to Jermyn (1960), it can be assumed that the equilibria pattern of eq 1 is also valid for these aldonolactones. The available data do not cover the same pH range as that used with D-gluconolactone. Therefore, the calculation of the parameters of the complete mechanistical kinetic model cannot be done here. An exception may be made for the lactones from disaccharides such as lactose and maltose, as they can be considered as substituted D-gluconolactones, with the substituted group not greatly affecting the conformation of the molecule.

For the remaining lactones that have been considered, the following can be concluded: (1) at pH values below 7, the interconversion rate constant, k_L , is about 10–100 times lower than for D-gluconolactone; (2) at pH values above 7, the hydrolysis rate constant, k_h , is at least 2–10 times lower than for D-gluconolactone; (3) D-xylono-, L-gulono-, D-mannono-, and D-galactonolactone behave in a similar way; (4) at pH above 7, the hydrolysis is catalyzed by OH^- . From these conclusions, it is possible to obtain an empirical

Table I. Most Probable Expressions for the Hydrolysis and Interconversion of D-Gluconolactone

constant	units	T (°C)	pH	reference
$k_h = [9.0 \times 10^5 + (6.1 \times 10^6)a_h + (4.0 \times 10^2)a_h^{-1}] \exp[(-59 \times 10^3)/RT]$	s ⁻¹	20–45	2.0–8.6	Pocker and Green (1973)
$k_L = 5 \times 10^{-5} (3.2 \times 10^{-7})a_h^{-1}$	s ⁻¹	30	2.0–8.6	Pocker and Green (1973), Shimahara and Takahashi (1970), Takahashi and Mitsumoto (1963), Jermyn (1960), and Reese and Mandels (1960)
$K_A = 0.14$	mol·m ⁻³	25		Mitchell and Duke (1970)
$K_H = k_h/k_{-h} = 5.4 (\pm 0.2)$		25		Mitchell and Duke (1970)
$K_L = k_L/k_{-L} = 1.4 (\pm 0.1)$		25		Mitchell and Duke (1970)

kinetic equation that can be used to calculate the apparent rate of lactone hydrolysis over the pH range 4–10. After combination of the results, it follows for the apparent hydrolysis constant, k_h' , of D-xylono-γ- and D-galactono-γ-lactone, and possibly also for L-gulono-γ- and D-mannono-γ-lactone at 30 °C that

$$k_h'(\text{s}^{-1}) = 4 \times 10^{-6} + (1.0 \times 10^{-8})a_h^{-1} \quad (2)$$

The equilibrium constants of the acid dissociation are similar for all of the lactones studied. As an average value, it can be calculated that $K_A = 0.23$ (SD 0.05) mol·m⁻³. The values of the other equilibrium constants, K_H and K_L , are only known as a nonlinear combination.

Materials and Methods

Bacterial Strain. The aerobic, Gram-negative bacterium *A. calcoaceticus* LMD 79.41 was used. It was obtained from the Department of Microbiology and Enzymology at the Delft University of Technology. The cells were maintained on peptone–agar slants and subcultured monthly.

Culture Conditions and Growth Medium. Experiments were carried out in continuous culture in a 3 L double-walled bioreactor (Applikon) with a culture volume of about 2 L, at 30 °C, pH 7.0, and dilution rate 0.15 h⁻¹. The bioreactor was equipped with a special overflow device using electromagnetic valves, to prevent selective biomass removal (Noorman et al., 1991a). The cultures were fed with 60 C-mol·m⁻³ acetate as a carbon and energy source. Cultures were also grown in the presence of acetate and xylose, where the C-mol-based xylose/acetate feed ratio was 1.1 or 3.3. The pH was controlled (Applikon E561/3) by the addition of 1 N KOH or 1 N H₂SO₄. The temperature was kept constant by an external thermostat (Lauda MGW). The air flow rate was about 0.060 m³·h⁻¹ and controlled with a mass flow controller (Brooks 5850 TR, 0–15 L·min⁻¹). Air was sparged into the reactor and dispersed with two four-bladed turbine stirrers, where the stirrer speed was 1200 min⁻¹. The gas flowing out of the reactor was cooled in a condenser (2 °C) and dried (Perma Pure dryer PD-625-12P). The dissolved oxygen concentration never dropped below 25% of air saturation. The mineral salts medium contained (per liter of demineralized water) (NH₄)₂SO₄, 4.25 g; MgSO₄·7H₂O, 0.10 g; K₂HPO₄, 0.65 g; KH₂PO₄, 0.50 g; EDTA, 15 mg; ZnSO₄·7H₂O, 4.5 mg; MnCl₂·4H₂O, 1.0 mg; CoCl₂·6H₂O, 0.3 mg; CuSO₄·5H₂O, 0.3 mg; Na₂MoO₄·2H₂O, 0.4 mg; CaCl₂·2H₂O, 4.5 mg; FeSO₄·7H₂O, 3.0 mg; H₃BO₃, 0.1 mg. The feed medium contained 32 mg·L⁻¹ antifoam, a 30% silicone emulsion in water (BDH).

Analytical Methods. Measurement of Biomass. Cooled 200-mL samples of culture were centrifuged for 20 min at 10 000 rpm (Sorvall superspeed RC2-B). The pellets were washed three times with ice-water, frozen, and lyophilized until constant weight.

Measurements of Acetate, Xylonolactone, Xylolate, and Xylose. Cooled 5-mL samples of culture were centrifuged, and the acetate content of the supernatant was determined with HPLC (Waters) using an HPX-87H column (eluent 10 meq of H₂SO₄, 60 °C). Detection was spectrophotometric at 214 nm. During the same run, the total concentration of xylonolactone plus xylolate was determined. We were not able to develop a method where these two compounds can be separately determined. Instead, an indirect method using the electrical charge balance was applied, which is described below. The xylose concentration in the supernatant was determined with HPLC (Waters), using an HPX-87P column (eluent water, 85 °C). Detection was refractometric.

Measurement of Dissolved Oxygen, and Oxygen and Carbon Dioxide in the Gas Phase. The oxygen concentration in the culture was measured using a galvanic electrode (Biolafitte MG2). The mole fraction of oxygen in the off-gas was analyzed paramagnetically (Servomex 1100A, Model C). The gas-phase carbon dioxide mole fraction was determined with a Beckman Model 864 infrared analyzer.

Measurement of pH and Acid and Alkali Addition Rate. pH was monitored with a standard glass electrode (Ingold 465-35-90-K9). The acid and alkali containers were both positioned on a digital balance (Mettler PE 6000 and 4000).

On-Line Monitoring. pH, dissolved oxygen concentration, weight of acid and alkali containers, and gas-phase oxygen and carbon dioxide concentration were continuously monitored and stored by the bioreactor data-acquisition program Biowatch (Hellings 1989), which was run on a PC (Olivetti M240).

Elemental Analysis. The carbon, nitrogen, and hydrogen contents of the dry cells were determined on a Perkin Elmer CHN analyzer.

Conservation Constraints. When (bio)chemical conversion processes are studied, it is important to use the laws of conservation of elements and electrical charge. They constrain the number of degrees of freedom of the system, leading to a number of linear relations (balances) among net conversion rates, which can be used for many purposes (Noorman et al., 1991b). These equations are very useful to check the consistency of the measured rates and to detect possible gross measurement errors. For the studied *Acinetobacter* system, four different relations are used: (1) the carbon balance; (2) a linear combination of the elemental balances (also known as degree of reduction balance); (3) the sugar balance (the aldose carbon skeleton is conserved); (4) the electrical charge balance (Heijnen, 1981). The electrical charge balance will be used to calculate the rates of xylonolactone and xylolate formation and to test whether the proposed kinetic expression for D-xylonolactone hydrolysis (eq 2) is correct or not. The other balances serve to check the consistency of the data.

For the derivation of the four relations, the elemental composition of the relevant compounds, their electrical

charge, and the metabolic route of D-xylose oxidation have to be known. Because the metabolic pathway of D-xylic acid formation simply comprises a single enzymatic step, the only unknown factor is the elemental composition of the biomass. Fewson (1985) gives the following for another *Acinetobacter* strain (standard deviation between parentheses):

Me represents the metals present as trace elements, such as Ca^{2+} , Zn^{2+} , Fe^{3+} , Cu^{2+} , and Mn^{2+} , with a mean molar mass of about $55 \text{ g}\cdot\text{mol}^{-1}$. The molar mass of the cell is $25.5 \text{ g}\cdot\text{C}\cdot\text{mol}^{-1}$.

To investigate whether this composition also represents *A. calcoaceticus* LMD 79.41, the hydrogen/carbon ratio and nitrogen/carbon ratio were determined from bacteria grown in steady state in continuous culture over the specific growth rate range $0.1\text{--}0.6 \text{ h}^{-1}$.

For growth in the presence of acetate, the following was found (standard deviation between parentheses):

and if xylose was also present, the following was found:

The conclusion can be drawn that the H/C and N/C ratios are not dependent on the presence of xylose and the specific growth rate, and this is in agreement with the results given by Fewson (1985). It is, therefore, assumed that the complete elemental composition given by Fewson is also valid for this *Acinetobacter* strain, under the given experimental conditions.

Now we are able to set up the balances. The carbon balance reads

$$-r_{\text{As}} = r_{\text{Ax}} + r_{\text{Ac}}^{\text{G}} + r_{\text{Ab}} \quad (3)$$

A linear combination of the elemental balances leads to the degree of reduction balance:

$$-r_{\text{As}} - 0.1r_{\text{Aa}} = 1.065r_{\text{Ax}} - r_{\text{Ao}} \quad (4)$$

The sugar balance reads

$$-r_{\text{Aa}} = r_{\text{Alp}} \quad (5)$$

For the electrical charge balance, it can be derived for glucose that

$$-r_{\text{An}} + 0.5r_{\text{As}} + r_{\text{Ab}} + r_{\text{Aoh}} - r_{\text{Ah}} - 0.167r_{\text{Aa}}f + 2r_{\text{Ap1}} + \\ r_{\text{Ap2}} + 2r_{\text{Asu}} - r_{\text{Ak}} - 2r_{\text{Amg}} - r_{\text{Ana}} + r_{\text{Acl}} - 2r_{\text{Ame}} = 0 \quad (6)$$

In the case of xylose, the factor 0.167 should be changed in 0.2. f is the fraction of the lactone that is hydrolyzed and subsequently dissociated into the aldinate and H^+ . This fraction can be calculated by solving the state equations of the system and using the kinetic expression for the rate of lactone hydrolysis. From the assumptions that (1) the acid dissociation equilibrium is established very quickly, (2) lactonization does not occur (which is true for pH values above 5), and (3) the aldose that enters the reactor is immediately converted to the lactone by the microorganism (indeed, during all experiments, no residual aldose could be detected), in the case of glucose, f is equal to

$$f = k_{\text{h}}/[D + k_{\text{h}} + k_{\text{-L}} - k_{\text{-L}}k_{\text{L}}/(D + k_{\text{L}})] \quad (7)$$

For xylose, the detailed kinetics are not known, and f must be calculated using the apparent hydrolysis rate constant,

k_{h}' :

$$f = k_{\text{h}}'/(D + k_{\text{h}}') \quad (8)$$

Equation 6 can be simplified by elimination of the rate of H^+ conversion, and by using the following elemental balances:

$$\text{N balance:} \quad -r_{\text{An}} = 0.22r_{\text{Ax}} \quad (9)$$

$$\text{P balance:} \quad -r_{\text{Ap1}} - r_{\text{Ap2}} = 0.017r_{\text{Ax}} \quad (10)$$

$$\text{S balance:} \quad -r_{\text{Asu}} = 0.014r_{\text{Ax}} \quad (11)$$

$$\text{K balance:} \quad -r_{\text{Ak}} = 0.0058r_{\text{Ax}} \quad (12)$$

$$\text{Na balance:} \quad -r_{\text{Ana}} = 0.0014r_{\text{Ax}} \quad (13)$$

$$\text{Mg balance:} \quad -r_{\text{Amg}} = 0.0026r_{\text{Ax}} \quad (14)$$

$$\text{Cl balance:} \quad -r_{\text{Acl}} = 0.0005r_{\text{Ax}} \quad (15)$$

$$\text{Me balance:} \quad -r_{\text{Ame}} = 0.0002r_{\text{Ax}} \quad (16)$$

It follows that

$$-0.5r_{\text{As}} - r_{\text{Ap1}} = r_{\text{Ab}} + r_{\text{Aoh}} + 0.19r_{\text{Ax}} - 0.167r_{\text{Aa}}f \quad (17)$$

It should be noted that r_{Aoh} represents the rate of OH^- conversion. The rate of acid/alkali addition to keep the pH constant is equal to this rate. If r_{Aoh} is positive, acid is added to the system, and if r_{Aoh} is negative, alkali is added. The same procedure applied for xylose gives

$$-0.5r_{\text{As}} - r_{\text{Ap1}} = r_{\text{Ab}} + r_{\text{Aoh}} + 0.19r_{\text{Ax}} - 0.2r_{\text{Aa}}f \quad (18)$$

The net consumption rates of acetate, $-r_{\text{As}}$, xylose, $-r_{\text{Ax}}$, and oxygen, $-r_{\text{Ao}}$, the formation rates of xylonate plus xylonolactone, r_{Alp} , biomass, r_{Ax} , the rate of carbon dioxide transfer into the gas phase, r_{Ac}^{G} , and the rate of acid/alkali formation, r_{Aoh} , were determined experimentally. The rate of bicarbonate formation, r_{Ab} , was calculated from the kinetics and thermodynamics of the reactions involving CO_2 in water (Noorman et al., 1992). For this, it was estimated that the volumetric carbon dioxide transfer coefficient, $(k_{\text{La}})_c$, is about 180 h^{-1} . The rate of HPO_4^{2-} conversion, r_{Ap1} , can be calculated using the phosphate dissociation equilibrium:

with equilibrium constant $K_{\text{P}} = 6.5 \times 10^{-5} \text{ mol}\cdot\text{m}^{-3} (30^\circ\text{C})$. It follows that

$$r_{\text{Ap1}} = D[C_{\text{p,in}}K_{\text{P}}/(K_{\text{P}} + a_{\text{h,in}}) - C_{\text{p}}K_{\text{P}}/(K_{\text{P}} + a_{\text{h}})] \quad (20)$$

The total phosphate concentration in the culture, C_{p} , is calculated from the phosphorus balance:

$$0.017r_{\text{Ax}} = D(C_{\text{p,in}} - C_{\text{p}}) \quad (21)$$

Nonideality of the Solution. The ionic strength of the fermentation medium was $0.16 \text{ mol}\cdot\text{L}^{-1}$, giving an ion activity coefficient, γ , of 0.75 (Noorman et al., 1992). This coefficient plays an important role in the calculation of r_{Ab} and r_{Ap1} . The formation of bicarbonate is dependent on the apparent equilibrium constant, K_{C}' , of the following reaction:

When the activity of the H^+ ions is being measured with the ion-selective standard glass electrode, the value of this constant follows from

$$K_{\text{C}}' = K_{\text{C}}\gamma^{-1} \quad (23)$$

where K_{C} is the true equilibrium constant (i.e., at ionic strength zero). The same reasoning can be applied to the water equilibrium constant, K_{W} . The apparent phosphate equilibrium constant, K_{P}' , is dependent on the ionic

Table II. Measured and Calculated Net Conversion Rates for the Experiments Described in This Paper^a

D (h ⁻¹)	pH	f	$-r_{As}$	r_{Ax}	$-r_{Ao}$	r_{Ac}^G	$-r_{Aa}$	r_{Alp}	r_{Aoh}	r_{Apl}	r_{Ab}
0.155	7.00	— ^b	9.2	2.7	7.1	7.4	— ^b	— ^b	4.0	-0.1	0.3
0.165	6.97	— ^b	9.8	2.5	7.5	7.4	— ^b	— ^b	3.4	-0.1	0.3
0.166	6.89	— ^b	9.8	2.3	6.2	7.1	— ^b	— ^b	3.6	-0.1	0.2
0.167	7.24	— ^b	9.7	2.4	6.3	6.7	— ^b	— ^b	3.3	-0.3	0.4
0.167	7.17	— ^b	9.7	2.4	6.0	6.8	— ^b	— ^b	3.5	-0.2	0.4
0.167	7.05	— ^b	10.0	2.7	6.0	6.7	— ^b	— ^b	3.6	-0.2	0.3
0.156	7.00	0.71	9.1	2.3	7.3	7.2	9.9	10.1	2.4	-0.1	0.3
0.167	6.97	0.68	9.8	3.0	9.0	6.4	34	34	0.0	-0.1	0.2
0.174	7.00	0.68	10.2	3.3	9.3	6.4	35	35	-0.7	-0.2	0.3

^a f was calculated from eqs 2 and 8. In the case that r_{Aoh} is positive, acid is added to keep the pH constant, and if r_{Aoh} is negative, alkali is added. All r values have the units C-mol-m⁻³·h⁻¹ or mol-m⁻³·h⁻¹. ^b No xylose was present.

strength according to (Stumm and Morgan, 1970)

$$K_P' = K_P \gamma^{-3} \quad (24)$$

where the factor 3 is the difference between the squares of the charges of the two phosphate ions H₂PO₄⁻ and HPO₄²⁻.

Results and Discussion

Testing the Kinetic Equation for Xylonolactone Hydrolysis. Nine steady states were obtained in continuous culture. From the raw measurements, the net conversion rates were calculated and summarized in Table II. Their consistency was checked using the four balances and the kinetic expression for xylonolactone hydrolysis. The results can be found in Table III. The balances are expressed by dividing the right-hand side of eqs 3–5 and 18 by the left-hand side. For the electrical charge balance, this only involves the experiments where acid is added to the reaction mixture to keep the pH constant. If alkali was added to the medium, the following ratio was used (using D-xylose):

$$(r_{Ab} + 0.19r_{Ax} - 0.2fr_{Aa})/(-0.5r_{As} - r_{Apl} - r_{Aoh}) = 1 \quad (25)$$

Representation of the balances in the given forms will lead to the most accurate check of the data consistency, because the values of both the numerator and denominator are maximal. Theoretically, all ratios should equal 1.

It can be concluded from Table III that, according to the 95% confidence limits, most of the averaged balances are closed. Two exceptions are the degree of reduction balance resulting from growth in the presence of xylose and the electrical charge balance when growth took place without xylose. The recovery of the former balance, being 0.95, is acceptable. The latter, however, only gives a recovery of 0.87. Because the rate of acid addition is the most important contribution to this balance, the value of this measurement is probably underestimated.

The results do not allow a rejection of the kinetic model for the hydrolysis of xylonolactone, as derived from published data.

An experimental check of the kinetic model for D-gluconolactone hydrolysis has not been made, because it is known that, after prolonged cultivation, *A. calcoaceticus* starts to consume gluconate (van Schie et al., 1989).

From Table II it can be calculated that the yield values obtained in this study differ from the published values. In contrast to van Schie et al. (1987), we found that at pH 7.0 the oxidation of xylose also leads to an increased biomass yield. In addition, part of the previous experiments have recently been repeated but could not be reproduced. The reasons for this are not clear and are the subject of present investigations. However, it is beyond the scope of this article to discuss these observations in more detail.

Table III. Individual Balances, Averaged Balances, and 95% Confidence Regions for the Averaged Balances, Using the Results Given in Table IV^a

	balance			
	carbon	degree of reduction	sugar	electrical charge
Data for Acetate				
no. 1	1.13	1.08		1.02
no. 2	1.04	1.04		0.82
no. 3	0.97	0.88		0.85
no. 4	0.99	0.91		0.81
no. 5	0.99	0.88		0.86
no. 6	0.96	0.88		0.85
av	1.01	0.95		0.87
95% conf	0.05	0.07		0.06
Data for Acetate Plus Xylose				
no. 1	1.07	0.97	1.02	0.96
no. 2	0.98	0.93	1.00	1.07
no. 3	0.98	0.94	1.00	0.94 ^b
av	1.01	0.95	1.01	0.99
95% conf	0.05	0.02	0.01	0.02

^a The values were calculated from eqs 3–5 and 18, by dividing the right-hand side by the left-hand side. In the case that no measurement errors have been made, all balances should equal 1. ^b Calculated from eq 25.

Role of Lactone Hydrolysis in Energy Transduction by *A. calcoaceticus*. It was argued (van Schie et al., 1987) that the growth of *A. calcoaceticus* only increased if the produced lactone was hydrolyzed into the corresponding acid. Two observations supported this correlation. The first is that the rate of acid formation of a number of aldonolactones at different pH values in batch cultures qualitatively agreed with the yield increase in continuous culture. The second is that from continuous culture data on the rate of acid/alkali addition for pH control (Table IV) the value of the measured rate of aldonic acid formation was calculated. This was done by comparing the rate of acid or alkali addition in cultures that grew in either the absence or presence of sugar. The difference between the two was attributed to the lactone hydrolysis reaction. These results give a very good correlation with the cell yield increase data.

To understand the possible mechanism behind the apparent correlation between lactone hydrolysis and biomass yield increase, it was investigated whether the relationship is quantitative. A simple mechanism would suggest a direct linear correlation. Figure 3 shows the comparison between the biomass yield increase data and the fraction of hydrolyzed lactone. For gluconolactone hydrolysis, catalysis by 7.4 mol-m⁻³ phosphate in the medium is taken into account using the catalytic rate constants given by Pocker and Green (1973). The standard deviation of the measurements was calculated from the results obtained with xylose at pH 7.0: $Y''_{ex} = 0.235 \pm 0.012$. Thus, the relative measurement error is 5%. From the figure, it appears that there is no quantitative agreement between the biomass yield and the rate of lac-

Table IV. Calculated (van Schie et al., 1987), Maximal, and Predicted Rates of Aldonic Acid Formation at $D = 0.15 \text{ h}^{-1}$ ^a

aldose	ald/ac ratio	pH	calcd rate (mol·m ⁻³ ·h ⁻¹)	maximal rate (mol·m ⁻³ ·h ⁻¹)	pred rate (mol·m ⁻³ ·h ⁻¹)
glucose	1.5	5.0	0.17	2.25	1.25
glucose	1.5	7.0	2.02	2.25	1.87
xylose	0.92	7.0	0.06	1.65	1.18
xylose	1.25	8.2	2.24	2.25	2.19

^a The calculated rate results from the acid/alkali addition data. The predicted rate of gluconic acid formation was calculated from Table I and eq 7, and for the rate of xylonic acid formation, eqs 2 and 8 were used. The maximal acid formation rate was calculated with $f = 1$.

Figure 3. Comparison of biomass yield data, Y''_{sx} (van Schie et al., 1987), and fraction of total hydrolyzed D-xylonolactone- and D-gluconolactone, f (this work), as a function of pH. Blank denotes the cell yield for cells growing in the absence of aldose (van Schie et al., 1987). The glucose/acetate net conversion rate ratio is 1.5; the xylose/acetate net conversion rate ratio is 1.25 at pH 7.0 and 2.0 at pH 8.2. Conditions: $D = 0.15 \text{ h}^{-1}$; 30°C . Symbols: $\square$, glucose; $\circ$, xylose.

tone hydrolysis. For glucose, the hydrolysis rate is already quite significant at low pH, and therefore the yield value of $0.24 \text{ C-mol-C-mol}^{-1}$ at about pH 5 cannot be approached using the model. On the other hand, the biomass yield increase between pH 5 and 6 is stronger than the model predicts. For xylose, the data point at pH 7 is below the theoretical curve, indicating that the lactone hydrolyzes more quickly than expected according to the hypothesis.

The agreement of the rate of acid/alkali addition and the biomass yield increase (Table IV) is probably coincidental, due to the presence of an error in the calculations. This is concluded from the amount of acid production which can be expected according to the kinetic expressions (also shown in Table IV). For glucose at pH 5.0 and xylose at pH 7.0, there is a large difference between the calculated and the predicted aldonic acid formation rates. The other results agree with the model prediction. By application of the electrical charge balance, it is found that the difference is associated with the rate of alkali addition, equal to $-r_{Aoh}$, suggesting that this variable has been miscalculated.

Conclusions

The hypothesis of van Schie et al. (1987) that "the formation of acid from the lactones is essential in the beneficial effect of sugars on the growth yield" has been reconsidered. Using the kinetic equations for the formation of acid from aldonoalactones, it can be concluded that

the relationship between lactone hydrolysis and yield increase is not sufficiently quantitative and the mechanism behind the observed relationship needs further study. The direct cause of the yield increase is more likely to be found in the aldose oxidation reaction and its coupling to the cellular energetics.

It can be concluded from the presented experiments that, for the study of biochemical processes in which electrically charged compounds are formed or consumed, the electrical charge balance is a very useful tool. It was successfully applied to test data consistency and to detect measurement errors, and it is of vital importance to quantify the unknown rate of acid formation from the lactone. We conclude that its infrequent appearance in the literature, possibly because of its the apparent complexity at first glance, is certainly not justified.

Notation

a_i	activity, mol·m ⁻³
$a_{i,in}$	activity in feed, mol·m ⁻³
C_p	total phosphate concentration, mol·m ⁻³
$C_{p,in}$	total phosphate concentration feed, mol·m ⁻³
D	dilution rate, h ⁻¹
f	fraction of lactone that is hydrolyzed
I	ionic strength, mol·L ⁻¹
k_h	true hydrolysis rate constant, s ⁻¹
k_h'	apparent hydrolysis rate constant, s ⁻¹
k_{-h}	lactonization rate constant, s ⁻¹
$(k_{LA})_c$	volumetric CO ₂ transfer coefficient, h ⁻¹
k_L	interconversion rate constant, s ⁻¹
k_{-L}	interconversion rate constant, s ⁻¹
K	overall constant of lactone interconversion
K_A	acid dissociation equilibrium constant, mol·m ⁻³
K_C	CO ₂ hydration equilibrium constant, mol·m ⁻³
K_H	hydrolysis equilibrium constant
K_L	lactone interconversion equilibrium constant
K_P	phosphate equilibrium constant
r_{Ai}	net conversion rate, mol of C-mol·mol ⁻³ ·h ⁻¹

Greek Symbol

γ	activity coefficient
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Subscripts

a	aldose
b	bicarbonate
c	carbon dioxide
cl	chloride
h	proton
k	potassium
lp	aldonoalactone plus aldinate
me	metal
mg	magnesium
n	ammonium
na	sodium
o	oxygen
oh	alkali
p	aldinate
p1	hydrogen monophosphate
p2	dihydrogen monophosphate
s	acetate
su	sulfate
x	biomass

Superscript

G	gas phase
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Supplementary Material Available: A literature overview on aldonolactone interconversion and hydrolysis, of which a summary is given in the text, comprised of 10 pages of text, 5 tables, and 5 figures (18 pages). Ordering information is given on any current masthead page.

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