Metabolic flux response to salt induced stress in the halotolerant yeast *Debaryomyces hansenii*

Keywords - *Debaryomyces hansenii* ; halotolerance; metabolic flux; growth parameters; enzyme sensitivity.

Subject Category - Physiology and Growth.

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This work is dedicated to the memory of Prof. Nicolau van Uden

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ABSTRACT

The toxic effect of NaCl and KCl, on growth of the marine yeast *Debaryomyces hansenii* on glucose or glycerol was studied. Above threshold value both salts inhibited specific growth rate, specific glucose and glycerol respiration rates and specific glucose fermentation rate, as well as biomass yields. The exponential inhibition constant, k, and minimum toxic concentration, c_{min}, were similar for all physiological parameters assayed. The effect of either salt in the specific activity of several glycolytic enzymes showed a similar inhibition pattern, although at much lower salt concentrations. In agreement with published results on glycerol phosphate dehydrogenase stimulation by salt, we present evidence in which a general glycolytic flux deviation could occur naturally during salt stress, due to the intrinsic sensitivity of the glycolytic enzymes responsible for the prosecution of glycolysis to intracellular ion concentrations.

INTRODUCTION

Debaryomyces hansenii, liker other osmotolerant yeasts exposed to osmotic stress, produces and accumulates glycerol as the major compatible solute (Gustafsson & Norkans, 1976; Adler & Gustafsson, 1980). Glycerol is assumed to be synthesised *via* the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by the cytoplasmic NAD-dependent glycerol 3-phosphate dehydrogenase, followed by dephosphorilation of glycerol 3-P to glycerol (Adler *et al.*, 1985). Nevertheless, it is not yet completely understood the way the yeast senses osmotic stress and deviates the metabolism to glycerol production.

Since *D. hansenii* is one of the most salt-resistant species of yeast, its study could contribute to a better understanding of the phenomenon of osmoregulation. The present work aims to elucidate how the main metabolic fluxes react to salt stress, shifting glucose metabolism towards glycerol production. For this purpose, we studied the influence of salt-induced stress on growth, glucose respiration and fermentation rates and glycerol respiration rates, as well as on intracellular sodium and potassium concentrations, and *in vitro* specific activity of some glycolytic enzymes, in particular, glyceraldehyde 3-P dehydrogenase (EC1.2.1.12), responsible for the prosecution of glycolysis at the metabolic crossroads that lead to glycerol production.

METHODS

Microorganism and media. *Debaryomyces hansenii* type strain IGC 2968 (CBS 767) was maintained on YEPD solid medium. Cells were grown in mineral liquid medium (van Uden, 1967) with 2% (w/v) glucose or glycerol, as the sole carbon and energy source, supplemented with NaCl or KCl at the desired concentrations.

Culture conditions. Growth was performed in a 1:1 ratio liquid:air, at 25° C and 170 rpm in an orbital shaker. Growth was monitored by OD_{640} (Baush & Lomb Spectronic 21) and by dry weight determinations. Samples of 10 ml were filtered through a GF/C 2.5 Whatman membranes, followed by extensive washing and drying overnight at 80° C. All determinations were done in duplicate or triplicate for calculation of maximum specific growth rates (μ_{max}) during exponential growth. Yield coefficients (Y) were based on dry weight determinations and substrate consumption in stationary phase. Glucose and glycerol concentrations in culture media were estimated enzymatically using, respectively, Boehringer Mannheim Biochemical Test Combinations nº 124036 and 148270. Enzymic performance in the presence of salt was tested by producing calibration curves for either reaction in the presence of 2 M NaCl or KCl. Specific substrate consumption rates for growth (μ_s) were calculated as the ratio μ_{max}/Y .

Measurement of respiratory and fermentative fluxes. Cells growing on either glucose or glycerol were harvested in mid-exponential phase by centrifugation at 7000 rpm, washed twice with and resuspended in ice-cold distilled water. Respiration was monitored with a Clark oxygen electrode linked to a YSI model 5300 oxygen monnitor connected to a flat-bed Kipp & Zonen recorder. The electrode was immersed in a water-jacketed chamber of 10 ml capacity kept at 25° C and provided with magnetic stirring. Th assay mixture was composed by 3 ml of fresh medium supplemented with 2% glucose or glycerol with different salt concentrations, and 0.5 ml of cell suspension corresponding to a final biomass concentration in the assay of 7.3 ±.2.0 mg dry weight ml^{-1} for the assays in glucose medium and 11.8 ± 3.0 mg dry weight ml^{-1} for the assays in glycerol medium. Oxygen consumption was monitored for 1-2 min and the gradient was used to calculate the initial respiration rate for the biomass present in the assay. The specific respiration rate was estimated dividing the value obtained for the biomass present in the assay.

Fermentation rates at 25° C, were determined by manometry (Umbreit *et al.*, 1964),

using a Warburg Constant Volume Respirometer (B. Braun model VL 166). Cells, were harvested as described above, except that they were resuspended in fresh medium containing 2% glucose to a final concentration of 3 ± 0.6 mg dry weight ml^{-1}. Assays were performed immediately after resuspension. Ethanol production by glucose-fermenting cells was monitored enzymically with alcohol dehydrogenase from Boehringer Mannheim.

Preparation of cell-free extracts. To prepare cell-free extracts, 80 ml of a mid-exponential phase culture (O.D. ≈ 0.8) were centrifuged at 7000 rpm for 5 min and the cells were kept frozen at -20° C. The extracts were obtained immediately before the enzyme assays by adding 0.5 ml buffer (10 mM triethanolamine, 1 mM dithiothreitol, 1 mM EDTA, pH 7.5; Blomberg & Adler, 1989) and ≈ 1 g 0.5 mm glass beads, to the frozen pellet followed by at least 15 cycles vortexing for 1 min with 1 min intervals in ice. followed by 1 min. intervals in ice. Glass beads were separated from extracts by decantating and centrifugation at 14 000 rpm for 15 min.

Enzyme assays. Activities of glycolytic enzymes were determined *in vitro* in cell-free extracts, using the methods of Maitra & Lobo (1971). The various buffers were supplemented with 0-750 mM NaCl or KCl. All assays were done at 25° C and changes in NAD(H) or NADP(H) concentration were monitored by A_{340} using a Perkin-Elmer Lambda 2 UV/Vis spectrophotometer connected to a Epson II recording system. Protein concentrations were determined by the Lowry method modified by Peterson (1977). Coupling enzymes and substrates were obtained from Sigma. In all cases it was checked that coupling enzymes were not in limiting amounts in the presence of salt.

Determination of intracellular Na^+ and K^+ concentrations. These were determined in a Pu 7000 ICP Spectrometer (Unicam). Cells were collected in mid-exponential growth phase and centrifuged. The pellet was separated into two parts. One followed a

cycle of four washes in ice-cold ultrapure water and the other in water containing the same salt concentration as the growth medium, followed by a cycle of six washes in 4g $MgCl_2$ l^{-1} at 4°C. Cells were disrupted by incubation in 17 ml HNO_3 l^{-1} for 24 h. Samples were centrifuged and quickly filtered through 0.45 μm Millipore sterile filters prior to injection. Both $MgCl_2$ and HNO_3 solutions were prepared in ultrapure water and controlled as to ion content. Ultrapure water was obtained from a Permutit^R filtering device. Results were indexed, through dry weight determinations, to intracellular volume.

Intracellular volume measurement. This was determined as described by Lages and Lucas (1995) in the absence of salt and by incubating in salt solutions of the same molarity as that of the growth medium.

RESULTS

Growth parameters and biomass yields. Growth of *Debaryomyces hansenii* on glucose or glycerol, in the presence of different NaCl or KCl concentrations, was determined. In each assay, specific growth rate, μ_{max}, during exponential growth phase was determined. In addition, the duration of the lag phase, as well as final biomass (dry weight) obtained in stationary phase, and total substrate consumed were determined. Biomass yields, Y, and specific substrate consumption rates μ_s, were calculated. Results obtained are summarized in Tables 1 and 2 and illustrated in Fig. 1.

D. hansenii was able to grow up to 3.5-4 M of either NaCl or KCl in mineral medium. The toxic effect of both salts were similar. The monitored growth parameters decreased with increasing salt concentrations, except for duration of lag phase which increased with salt concentration. The pattern of salt inhibition was exponential after a minimum salt concentration, below which no pronounced effect was detectable. This biphasic

pattern fitted the general equation found in the literature to describe mathematically the toxic effect of alkanols on yeast growth parameters, metabolic fluxes and membrane processes (van Uden, 1985; 1989). A transposition of that equation gives: $\mu_{max_{[salt]}} = \mu_{max_0} e^{\pm k\,(c-c_{min})}$, where the c_{min} is the salt concentration below which its inhibitory effect was negligible, $\mu_{max_{[salt]}}$ is the maximum specific growth rate in an exponentially growing culture in the presence of a certain salt concentration, and μ_{max_0} is the maximum specific growth rate in the absence of salt. The evaluation of each salt toxic effect was thus performed comparing values of k and c_{min} (Table 3). Exponential inhibition was not detectable before 1.5 M and 2 M NaCl or KCl for, respectively, glucose- or glycerol-grown cells.

Respiration and fermentation fluxes. Cells of *D. hansenii* growing exponentially on glucose respired and fermented glucose simultaneously. Fermentation was monitored by manometry, by the production of CO_2, and confirmed by enzymic detection of ethanol. Specific respiration rates were measured both by oxygen electrode and manometric techniques. The latter technique was used to enable the establishment of a correlation between respiration and fermentation rates, expressed in the same units, μl O_2 consumed or CO_2 released min^{-1} (g dry weight)$^{-1}$. Specific glucose respiration and fermentation rates correlated in a proportion of 80 to 20% in mid-exponential glucose-grown cells. The inhibitory effect of increasing concentrations of NaCl or KCl on either specific glucose respiration or fermentation rates was detectable at lower salt concentrations than observed for the growth parameters presented above. The changes observed, exemplified in Fig. 2 and 3 respectively, fit the same mathematical reasoning explained above**.** The values of k and c_{min} for specific respiration and fermentation rates are presented in Table 3. Inhibition constant values did not diverge from the ones obtained for growth parameters, allowing the calculation of a mean value for all parameters studied in glucose- or glycerol-grown cells (Table 3). Nevertheless, k values calculated for Y were not included in this calculation since they represent an indirect

experimental result.

The toxic effect of either salt on respiratory flux was independent on the adaptation to the presence of either NaCl or KCl during growth on glucose. Cells grown on glucose in the presence of 0.5-3 M NaCl or KCl did not show a different inhibitory pattern on specific respiration rates from that described above, except when washes were performed in conditions isotonic to growth media, in which case a small generalized acquired resistance was observed (not shown). On the other hand, the effect of adaptation to salt during growth on glycerol resulted in an acquired resistance of respiratory flux, translatable into an approximately constant value for glycerol specific respiration rates in the presence of 0.5-3 M NaCl or KCl during assays for cell suspensions adapted during growth to either salt concentrations ≥1.5 M (not shown).

Inhibition of glycolytic enzyme activity. *In vitro* assays of glyceraldehyde 3-phosphate dehydrogenase activity in the presence of NaCl or KCl were performed. A maximum specific activity of 0.4 ± 0.1 U (mg total protein)$^{-1}$ was measured at a substrate concentration well above saturation. All the values obtained in the presence of salt were indexed to this one in terms of relative activity (Fig. 4). An equally exponential toxic effect of both salts on the enzyme maximum specific activity was observed, but for lower salt concentrations than before. Taking this result into account, all the glycolytic enzymes acting in glycolytic flux closer to glyceraldehyde 3-phosphate that did not require more than one coupling enzyme were assayed. The results are presented in Table 4.

Intracellular ion concentration. Considering that enzymes showed a much higher sensitivity to salt than did respiration and fermentation fluxes, intracellular concentrations of Na^+ and K^+ ions in cells grown in glucose with or without NaCl were determined (Fig. 5). To enable determination of concentrations, intracellular volumes for NaCl- or KCl-grown *D. hansenii* cells had to be determined. The value did not differ

significantly from cells grown in glucose without salt: 0.471 ± 0.110 μl (mg dry weight)$^{-1}$ (n=40). Intracellular potassium concentrations were generally higher than sodium concentrations, even in NaCl/-glucose-grown cells. No significant differences were obtained with different washing treatment of each sample (not shown). Cells cultivated at salt concentrations below 1-1.5 M NaCl or KCl, showed an increase in intracellular concentrations of Na^+ or K^+ followed by an exponential decrease above these salt concentrations. Nevertheless the equation used above to express salt toxicity does not fit results since no "plateau" of unchanged values in relation to control was observed.

DISCUSSION

From all the results presented, a general pattern of toxicity for either NaCl or KCl is apparent. Between each salt, no significant difference on general inhibitory effect could be found. This is well described by the kinetics of inhibition by both salts.

The generalized nature of the inhibition pattern on growth is in agreement with published results (Adler *et al*., 1985; Larsson & Gustafsson, 1987). The values for the inhibition constant k and c_{min} are very similar for all growth parameters studied. At 3 M NaCl or KCl we registered a biomass yield on glucose of about 53% of that in the absence of salt. In yet another strain, Prista & Madeira-Lopes (1995) registered a 60% reduction in Y in the presence of approximately 3M NaCl. In relation to the other parameters under study, the value found for the corresponding inhibition constant was smaller. This is understandable considering that Y is a relative unity. A striking difference was that growth yields on glycerol, obtained in the presence of salt concentrations above 2 M, decreased more steeply than that on glucose media. This is consistent with a double function of glycerol as carbon/energy source and as osmoprotectant.

Assuming that the effects observed on growth could be due to effects on main metabolic pathways, we proceeded studying glucose-specific respiration and fermentation rates and glycerol respiration rate. It should be pointed out that we confirmed the existence of simultaneous glucose respiration and fermentation. Authors have diverged on this matter and *D. hansenii* has been classified either as an exclusively (Gancedo & Serrano, 1989) or partially (Norkans, 1968; Barnett *et al*., 1990) respiratory yeast. The acquired resistance of glycerol respiration observed due to the presence of salt during growth was not observed in the case of glucose respiration. This reinforced the idea suggested above, that salt stress could be responsible for a metabolic diversion for glycerol production, on glucose media, or for glycerol retention, when this is the only carbon/energy source.

From the literature, the osmotolerance fo *D. hansenii* is not only a consequence of its capacity to maintain a low intracellular salt concentration, but also of the fact that the enzymes responsible for glycerol production are not only very tolerant to salt, but are even stimulated by its presence (Norkans, 1968; Nilsson & Adler, 1990). Results obtained with glyceraldehyde 3-phosphate dehydrogenase pointed to an exponential toxic effect of either salt on the maximum specific activity of the enzyme, although for lower salt concentrations than before. Glycerol 3-phosphate dehydrogenase is stimulated by salt concentrations up to 150 mM (Nilsson & Adler, 1990). At the same concentrations of NaCl or KCl, glyceraldehyde 3-phosphate dehydrogenase was strongly inhibited. From results obtained with other representative glycolytic enzymes, we observed a lower sensitivity for those leading to fructose 6-phosphate (F6P), and a higher sensitivity after fructose 1,6-*bis*phosphate (F1,6BP) in the glycolytic pathway. In *Saccharomyces cerevisiae*, albeit using a different technical approach, Singh and Norton (1991) described the accumulation of phosphorylated glycolytic intermediates after salt shock, when F6P and F1,6BP reached the highest intracellular levels.

The measured intracellular ion concentrations were much higher than those at which enzyme sensitivity was determined. Intracellular sodium and potassium concentrations

reached very high maximum values at external salt concentrations around 1M. Nevertheless, at 1.5 M NaCl, intracellular sodium and potassium concentrations were lower, and similar to those published by Burke and Jennings (1990). The approximately constant ratio between K^+ and Na^+, regardless of salt concentration during growth, is suggestive of the activity of an efficient transport system capable of maintaining K^+/Na^+ homeostasis. Taking into consideration these results, and the difference in relative intrinsic sensitivity of glycolytic enzymes in relation to the stimulation of glycerol 3-phosphate dehydrogenase observed by Nilsson and Adler (1990), it can still be reasoned that there is a salt-triggered shift in glycolytic flux in the presence of salt stress. Nevertheless, caution should be exercised in the interpretation due to the danger in extrapolating results from *in vitro* enzyme sensitivity assays to *in vivo* conditions, especially due to the presence of Cl^- ions, whose cytoplasmic concentration is unknown. In addition, although the internal cation concentrations appear to be much above the *in vitro* c_{min}, enzyme activity *in vivo* may be protected by cytoplasmic compartmentation, thereby decreasing the toxic effects of salt.

Sensitivity of glucose or glycerol respiration and fermentation to inhibition by salt translates into an inhibition of growth as a whole, but it is not, at the same time, matched quatitatively by the inhibition of the enzymes responsible for glycolytic flux, which were more salt-sensitive.

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LEGENDS

Fig. 1. Variation in maximum specific growth rate (μ_{max}), of *D. hansenii* grown in mineral medium with 2% (w/v) glucose (○,●) or glycerol (△,▲), with NaCl (○,△) or KCl (●,▲) concentration in the growth media.

Fig. 2. (a) Example of a typical result obtained with the O_2 electrode measuring glucose respiration rates in the absence and in the presence of several NaCl concentrations in the assay buffer. Respiration rate in the absence of NaCl: 11.3 μl O_2_. min^{-1}. g^{-1} dry weight. (b) Variation of glucose (○,●) or glycerol (△,▲) specific respiration rates with assay NaCl (○,△) or KCl (●,▲) concentrations of a cell culture of *D. hansenii* recovered respectively from mineral medium with 2% (w/v) glucose (○,●) or glycerol (△,▲).

Fig. 3. (a) Example of a typical assay to measure specific fermentation rates in Warburg Apparatus, in the absence and in the presence of several NaCl concentrations (0 M, ○; 0.2 M, ●; 0.4 M, □; 1.0 M, ■; 1.3 M, △; 1.7 M, ▲). (b) Variation of glucose specific fermentation rate with assay NaCl (○) or KCl (●) concentration of a cell culture of *D. hansenii* I GC 2968 recovered from mineral medium with 2% (w/v) glucose.

Fig. 4. Relative activity of glyceraldehyde 3-P dehydrogenase with different NaCl (○) or KCl (●) concentrations (100% = 0.4 U mg^{-1} total protein).

Fig. 5. Intracellular concentrations of Na^+ (○,△) and K^+ (●,▲) in NaCl (○,●) and KCl (△,▲) glucose grown cells of *D. hansenii*. Results presented are mean values of independent experiments.

Table 1. Growth parameters of *D. hansenii* IGC 2968 in mineral medium with 2% (w/v) glucose, in the presence of several salt concentrations.

Salt concentration (M) [NaCl]	0	0.5	1	1.5	2	2.5	3	3.5	4
μ_{max} (h^{-1})	0.21±0.04 (7)	0.20±0.05 (3)	0.17±0.04 (5)	0.14±0.03 (5)	0.08±0.03 (6)	0.03±0.01 (4)	0.02 (2)	0.01 (1)	0 (2)
Lag phase (h)	16±7 (6)	16±5 (5)	17±5 (6)	24±6 (5)	28±5 (5)	56±12 (4)	100 (2)	n.d.	___
Y ($g.g^{-1}$)	0.17±0.01 (3)	0.17±0.03 (2)	0.14±0.01 (6)	0.13±0.01 (3)	0.1±0.02 (4)	0.11 (1)	0.09 (1)	0.08 (1)	___
μ_s ($g.h^{-1}.g^{-1}$)	1.24	1.18	1.21	1.08	0.80	0.27	0.22	0.13	___
[KCl]									
μ_{max} (h^{-1})	0.21±0.04 (7)	0.20±0.04 (3)	0.19±0.02 (3)	0.16±0.02 (3)	0.09±0.01 (3)	0.04±0.01 (3)	0.03±0.01 (3)	0.01 (1)	0.008 (1)
Lag phase (h)	16±7 (6)	8±2 (3)	12±3 (3)	14±3 (3)	15±4 (3)	18±5 (3)	31±5 (3)	100 (1)	550 (1)
Y ($g.g^{-1}$)	0.17±0.01 (3)	0.16 (2)	0.16 (2)	0.17 (2)	0.13 (2)	0.11 (2)	0.09 (2)	0.08 (1)	0.07 (1)
μ_s ($g.h^{-1}.g^{-1}$)	1.24	1.25	1.20	0.94	0.69	0.36	0.33	0.13	0.11

n.d. Not determinable due to the very low biomass yields attained.
Numbers of experiments are shown in parenthesis.
Standard deviation of medium values from less than three experiments are not presented.

Table 2. Growth parameters of *D. hansenii* IGC 2968 in mineral medium with 2% (w/v) glycerol. in the presence of several salt concentrations. Growth beyond 4M NaCl or KCl was not detected.

Salt concentration (M) [NaCl]	0	0.5	1	1.5	2	2.5	3	3.5	4
μ_{max} (h^{-1})	0.06±.005 (3)	0.04 (2)	0.08 (2)	0.07 (2)	0.06 (2)	0.03 (2)	0.02±0.01 (3)	0.014 (1)	0.006 (1)
Lag phase (h)	21 (2)	12 (2)	23 (2)	31 (2)	48 (2)	57 (2)	83 (2)	210 (1)	250 (1)
Y ($g.g^{-1}$)	0.33 (2)	0.28 (2)	0.29 (2)	0.30 (2)	0.24 (2)	0.34 (2)	0.074 (2)	0.07 (1)	0.04 (1)
μ_s ($g.h^{-1}.g^{-1}$)	0.18	0.14	0.28	0.23	0.25	0.09	0.27	0.20	0.15
[KCl]									
μ_{max} (h^{-1})	0.06±.005 (3)	0.054 (2)	0.07 (2)	0.06 (2)	0.05 (2)	0.03±0.006 (3)	0.02±0.006 (3)	0.007 (1)	0.005 (1)
Lag phase (h)	21 (2)	15 (2)	18 (2)	24 (2)	30 (2)	32 (2)	50 (2)	60 (1)	65 (1)
Y ($g.g^{-1}$)	0.33 (2)	0.23 (2)	0.29 (2)	0.25 (2)	0.21 (2)	0.11 (2)	0.08 (2)	0.032 (1)	0.03 (1)
μ_s ($g.h^{-1}.g^{-1}$)	0.18	0.23	0.24	0.24	0.24	0.27	0.25	0.22	0.17

Numbers of experiments are shown in parenthesis.
Standard deviation of medium values from less than three experiments are not presented.

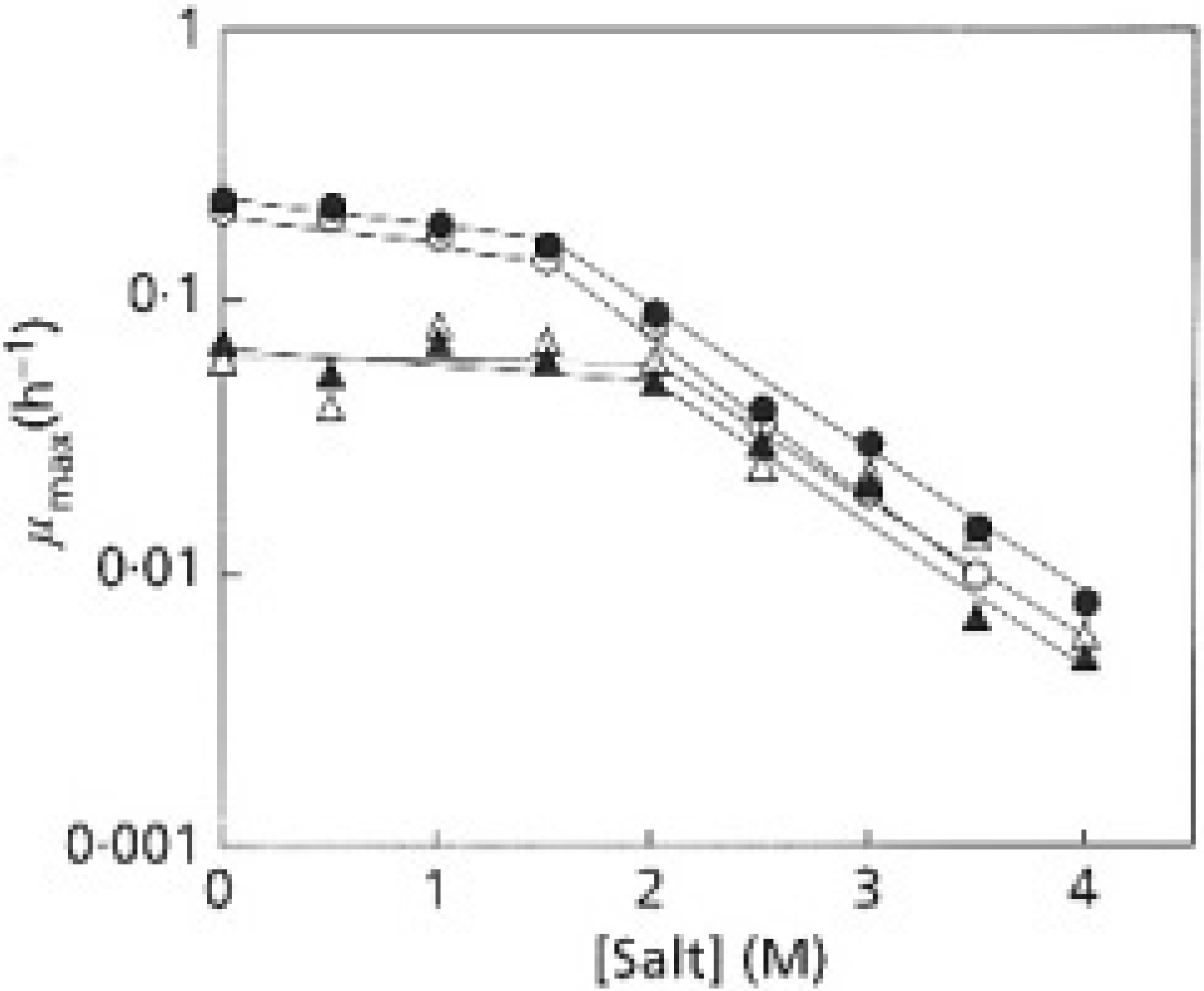
Table 3. Inhibiton constants (k) and salt minimum inhibitory concentrations (c_{min}) of all parameters under study. Calculations were performed using the medium values presented in Tables 1 and 2.

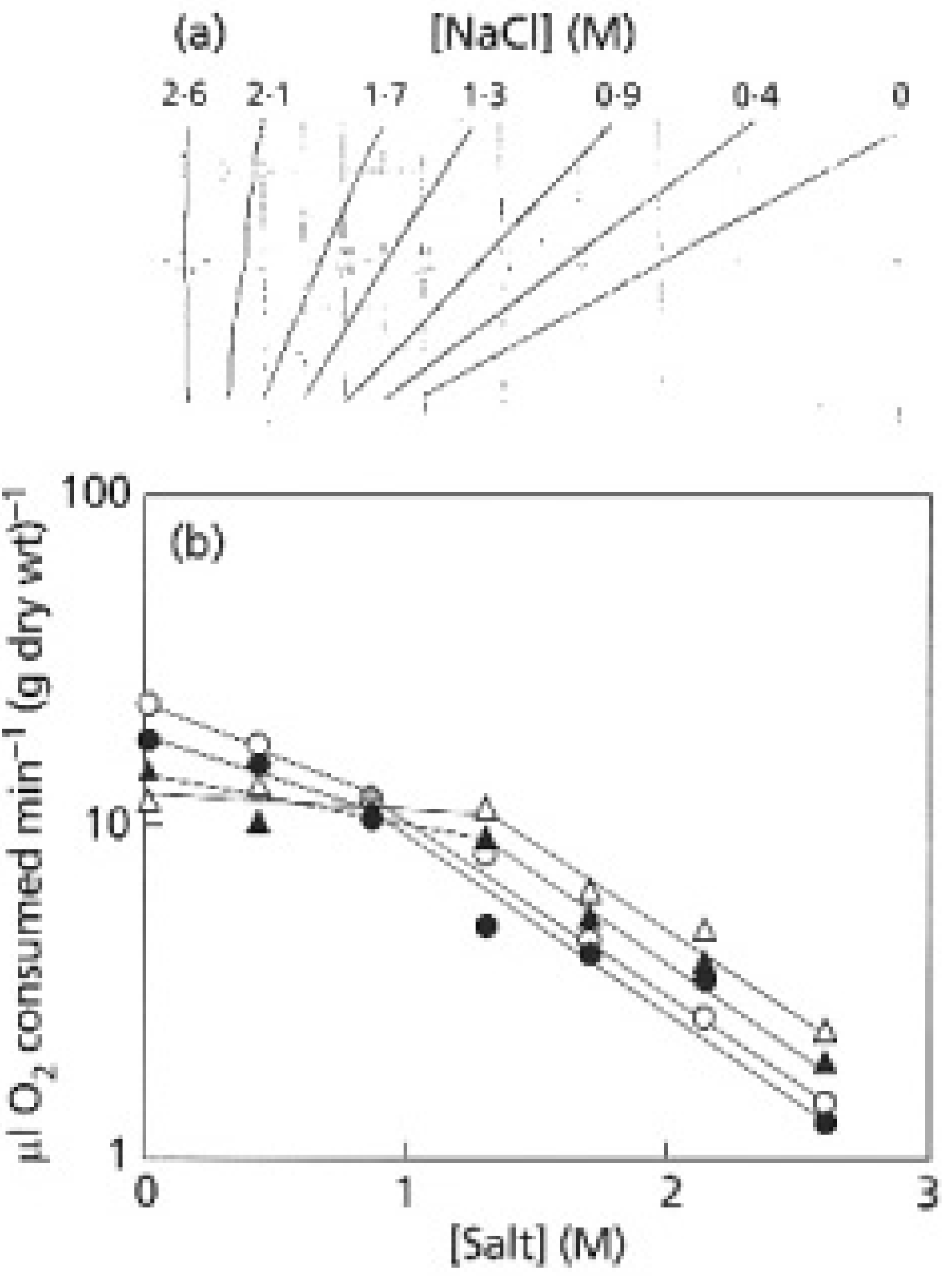
	k (M^{-1})		c_{min} (M)	
	NaCl	*KCl*	*NaCl*	*KCl*
GLUCOSE MEDIA				
μ_{max}	- 1.3	- 1.3	1.5	1.5
μ_s	- 1.5	- 0.6	1.5	1.5
Total biomass attained	- 0.6	- 1.2	1.5	1.5
Total glucose consumed	- 0.6	- 0.8	1.5	1.5
Y	- 0.2 *	- 0.3 *	0	1.5
Respiration rate	- 1.3	- 1.1	0.9	0.9
Fermentation rate	- 1.1	- 0.9	0.2	0.2
Media ± s.d.	*1.1 ± 0.4*	*1.0 ± 0.2*		
GLYCEROL MEDIA				
μ_{max}	- 1.1	- 1.2	2.0	2.0
Total biomass attained	- 1.3	- 1.7	2.0	2.0
Total glycerol consumed	- 0.5	- 0.5	0	0
Y	- 1.0 *	- 1.0 *	2.0	2.0
Respiration rate	- 1.0	- 1.2	1.3	1.3
Media ± s.d.	*1.0 ± 0.3*	*1.2 ± 0.4*		

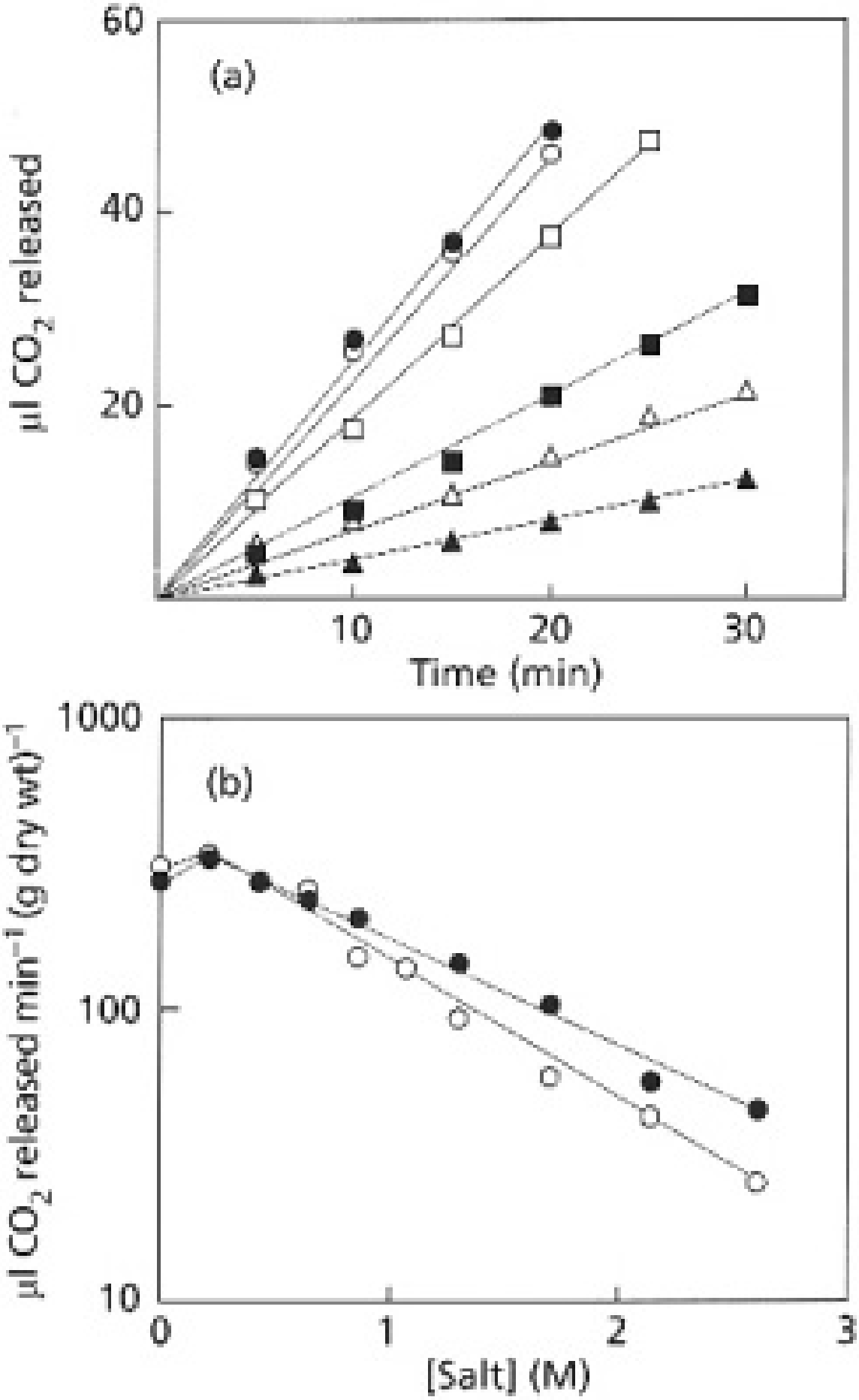
* Values not included in media calculations.

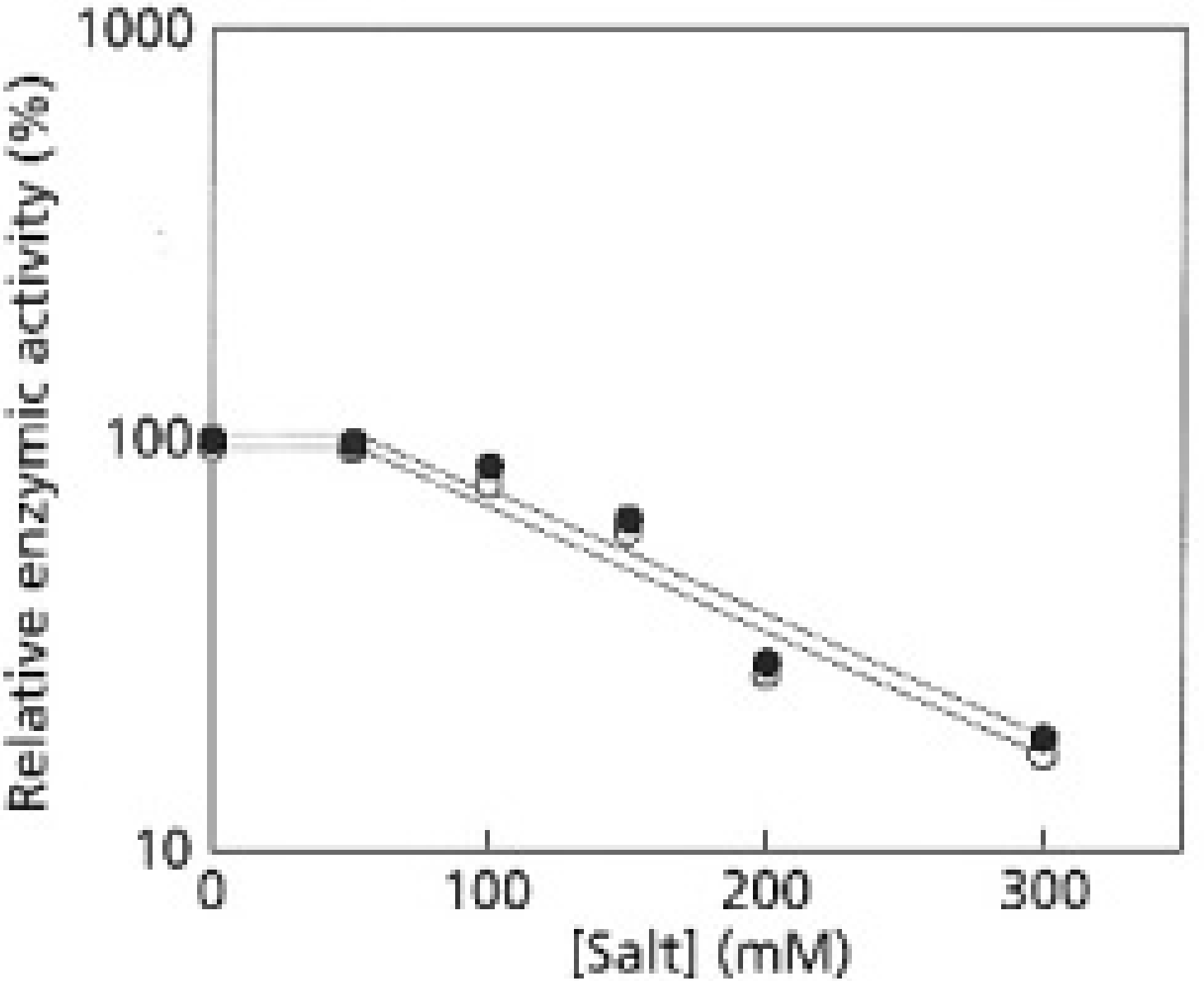
Table 4. Inhibiton constants (k) of glycolytic enzymes assayed *in vitro* in the presence of NaCl or KCl in the assay buffer.

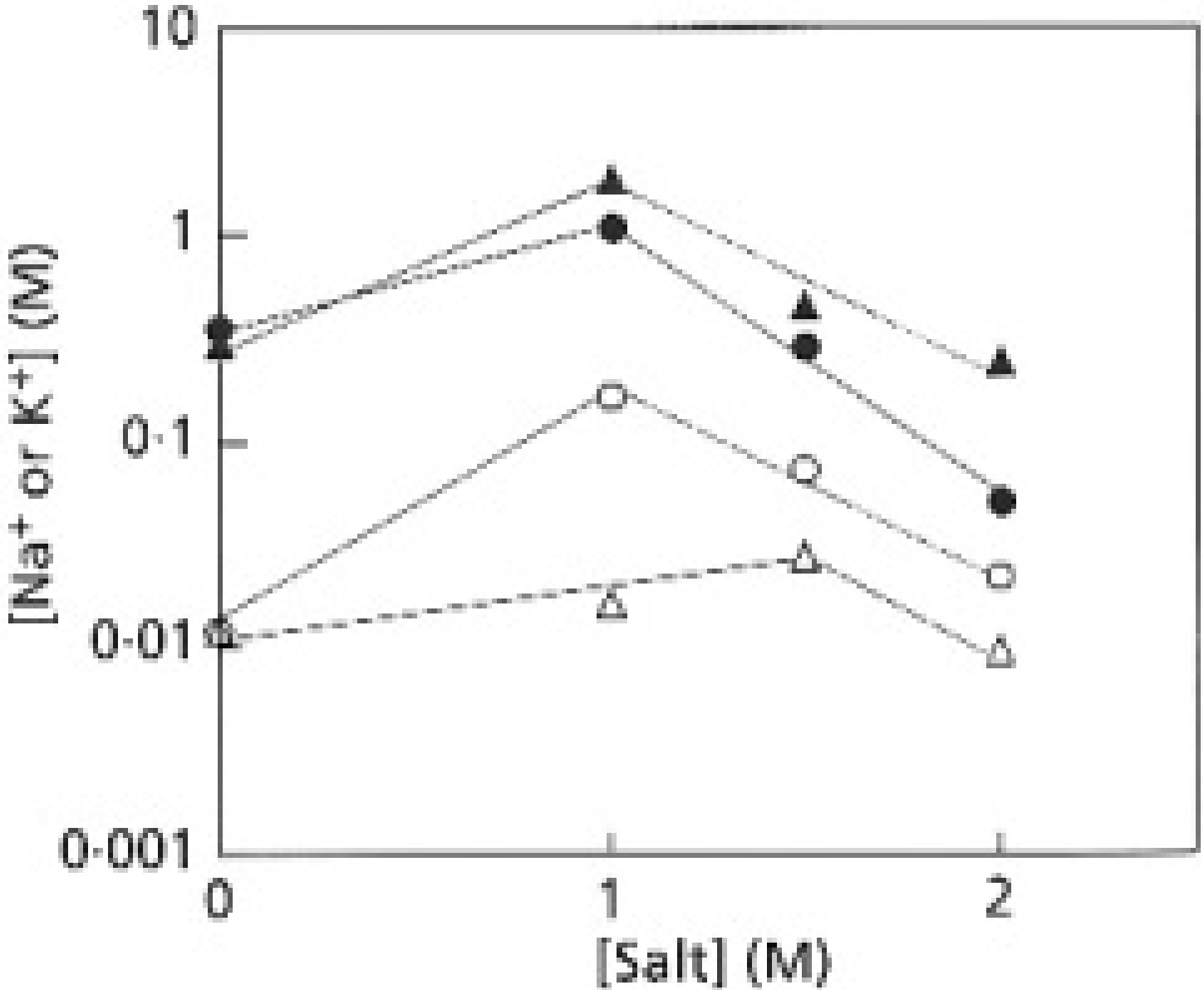
Enzyme assayed	k (M^{-1})		% Activity left at 250mM	
	NaCl	*KCl*	*NaCl*	*KCl*
Hexokinase	- 1.6	- 2.3	61.2	60.5
Glucose 6-P isomerase	- 3.8	- 3.3	58.0	61.3
Aldolase	- 6.9	- 4.1	15.0	45.5
Triose-P isomerase	- 4.8	- 4.0	30.6	32.8
Glyceraldehyde 3-P dehydrogenase	- 6.8	- 6.8	23.2	24.8
P-glycerate kinase	- 5.5	- 4.5	58.4	61.2


1
0·1
0·01
0·001
μ_{max} (h^{-1})
0
1
2
3
4
[Salt] (M)


(a)
[NaCl] (M)
2·6
2·1
1·7
1·3
0·9
0·4
0
(b)
100
10
1
0
1
2
3
[Salt] (M)
μl O2 consumed min−1 (g dry wt)−1


(a)
60
40
20
µl CO_2 released
10
20
30
Time (min)
(b)
1000
100
10
µl CO_2 released min^{-1} (g dry wt)$^{-1}$
0
1
2
3
[Salt] (M)


1000
100
10
Relative enzymic activity (%)
0
100
200
300
[Salt] (mM)


10
1
0·1
0·01
0·001
[Na+ or K+] (M)
0
1
2
[Salt] (M)