Properties of Halophil Nicotinamide–Adenine Dinucleotide Phosphate-Specific Isocitrate Dehydrogenase

TRUE MICHAELIS CONSTANTS, REACTION MECHANISMS AND MOLECULAR WEIGHTS

By D. M. AITKEN* and A. D. BROWN

School of Microbiology, University of New South Wales, Kensington, N.S.W. 2033, Australia

(Received 16 May 1972)

True values of Michaelis constants of the NADP⁺-specific isocitrate dehydrogenase from *Halobacterium salinarium* were not very different from those of the apparent constants reported by Aitken *et al.* (1970). The true constants were affected by salt in a similar manner to that of the apparent constants obtained with NADP⁺ at fixed concentrations of 1.0–0.2 mM and *threo*-D₅-(+)-isocitrate at fixed concentrations of 2.0–0.125 mM. The response of apparent $V_{\max}$ to salt concentration was highly dependent on fixed substrate concentration in solutions of sodium chloride but much less so in solutions of potassium chloride. At several levels the results emphasize the difficulty of generalizing about the salt relations of a halophil enzyme without adequate attention to substrate concentration. The enzyme has at least two different reaction mechanisms depending on salt concentration. In its 'physiological' form (i.e. in 1.0 M-potassium chloride), and also in 1.0 M-sodium chloride, the reaction mechanism is ordered with NADP⁺ the first substrate added and NADPH the last product released. In 0.25 M-sodium chloride, however, the mechanism is different and is probably non-sequential. In 4.0 M-sodium chloride with low concentrations of either fixed substrate, there was evidence of a co-operative action of the variable substrate. The evidence suggests that salt participates in the reaction mechanism in two ways: one is the reversible addition to the enzyme in a manner analogous to that of a substrate; the other is dead-end complex-formation. The relative contributions of these two types of reaction determine whether salt activates or inhibits the enzyme. In addition, the inhibition caused by high concentrations of sodium chloride is more complex than the corresponding inhibition by potassium chloride. Gel-filtration experiments indicated that at very low salt concentrations the enzyme has an apparent molecular weight of about 70 800. In 'physiological' concentrations of potassium chloride the enzyme appears to be a dimer (mol.wt. 122 000–135 000) and, in 1.0–4.0 M-sodium chloride, it behaves as a trimer or tetramer (mol.wt. 224 000–251 000). A preliminary method of purifying the enzyme is described.

A preliminary investigation (Aitken *et al.*, 1970) showed several major differences between Na⁺ and K⁺ in their effects on the kinetics of crude preparations of a halophil isocitrate dehydrogenase. For example, apparent Michaelis constants responded differently to each ion as did apparent $V_{\max}$. Reaction velocities were higher in sodium chloride than in potassium chloride at optimum concentrations of each salt but were lower in high concentrations of sodium chloride than in potassium chloride. At high concentrations, potassium chloride acted as a linear non-competitive inhibitor of the enzyme. High concentrations of sodium chloride acted differently, however, and appeared to cause a change in the state of the enzyme. There was evidence that effects of salt

were modified in various ways by substrate concentration.

The present paper describes true Michaelis constants, the effects of fixed substrate concentration on the salt relations of apparent $V_{\max}$, the reaction mechanisms of the enzyme and effects of salt on these mechanisms, some preliminary determinations of molecular weight and a method of purifying the enzyme.

Materials and Methods

Enzyme preparation and assay

The enzyme was prepared from a colourless strain of *Halobacterium salinarium* as described previously (Aitken *et al.*, 1970). The enzyme was assayed and

* Present address: Department of Biology, University of Ottawa, Ottawa 2, Canada.

results of kinetic studies were evaluated statistically as previously. True Michaelis constants were obtained by extrapolating to infinite substrate concentration a plot of apparent values of these constants. The extrapolation was done statistically by a weighted least-squares method and use of the reciprocal of substrate concentration and a computer program as previously described (Aitken *et al.*, 1970). A weighting factor for each apparent constant was given by the computer program Hyper (Cleland, 1967). For these purposes fixed substrates were varied within the range 0.8–0.2 mM-NADP⁺ and 2.0–0.25 mM-DL-isocitrate (1.0–0.125 mM of the active isomer). Henceforth, unless specified otherwise, all isocitrate concentrations quoted are those of the active isomer [*threo*-D₅-(+)-isocitrate]. All reagents were as previously described except that twice-recrystallized sodium chloride was used in some comparative kinetic studies. No difference was found between the recrystallized salt and the untreated commercial reagent-grade product.

Enzyme purification

A crude cell-free extract was prepared under the usual conditions from the organisms harvested from 7 litres of culture. The extract was dialysed overnight at 0°C against tris buffer (0.02 M, pH 7.75; 2.0 litres) containing sodium chloride (4.2 M) and sodium sulphate (0.1 M) (buffer 1). The non-diffusible fraction was then dialysed at 0°C for 6 h against 9 vol. of tris buffer (0.02 M, pH 7.75) containing sodium DL-isocitrate (4.0 mM), magnesium chloride (10 mM), sodium sulphate (0.1 M) and sodium chloride (0.42 M) (buffer 2). After dialysis an equal volume of saturated ammonium sulphate containing sodium DL-isocitrate (4.0 mM), magnesium chloride (10 mM) and sodium chloride (0.42 M) was added. The mixture was allowed to stand for 15 min at 0°C and then centrifuged at 27000g for 30 min at 0°C. The pellet was discarded. The supernatant fluid was dialysed overnight at 0°C against buffer 1 (2.0 litres). The non-diffusible fraction was concentrated by dialysis at 4°C against buffer 1 (2.0 litres) containing Carbowax 6000 (10%, w/v). The concentrated non-diffusible fraction was applied to a column (2 cm × 22 cm) of Sephadex G-100 and eluted at room temperature with buffer 1. Extinction (280 nm) of the effluent was continuously recorded; fractions (1.5 ml) were collected and assayed for isocitrate dehydrogenase activity. Active fractions were pooled and concentrated as above by dialysis against buffer 1 containing Carbowax 6000. The concentrated solution was then dialysed for 5 h against tris buffer (0.02 M, pH 7.75; 2.0 litres) containing potassium chloride (4.0 M) and sodium sulphate (0.1 M) and applied to a column (2 cm × 22 cm) of Sephadex G-100. The column was eluted at room

temperature with the same buffer. The active fractions were pooled and dialysed overnight against buffer 1 (2.0 litres) at 4°C. The non-diffusible fraction was dialysed for another 6 h at 4°C against tris buffer (0.02 M, pH 7.75) containing sodium DL-isocitrate (4.0 mM), magnesium chloride (10.0 mM), sodium chloride (1.0 M) and sodium sulphate (0.10 M) and transferred to a column (2.5 cm × 25 cm) of DEAE-Sephadex equilibrated with the same buffer. The column was eluted at room temperature with the same buffer containing a linear gradient of sodium chloride from 1.0 to 4.0 M. The active fractions were pooled and concentrated by dialysis at 4°C against Carbowax 6000 as described above. This represented the purified preparation.

Gel electrophoresis

Enzyme preparations were fractionated by electrophoresis in polyacrylamide gels with a discontinuous buffer system of the following overall composition: gel-solution 1 (acrylamide, 32 g; bis-acrylamide, 0.8 g; water to 100 ml)-solution 2 (tris, 18 g; tetramethylethylenediamine, 0.24 ml; HCl to pH 8.65; final volume, 100 ml)-solution 3 (riboflavin, 1 mg; ammonium persulphate, 40 mg; water to 100 ml)-solution 4 (1.0 M-sodium chloride) (1:1:1:2:0.04, by vol.).

The mixture was polymerized for 20–30 min in bright light. The upper (cathode) buffer contained (per litre): tris (5.16 g), glycine (3.48 g), HCl to pH 8.6, sodium DL-isocitrate (2.0 mmol). The lower (anode) buffer contained (per litre): tris (14.5 g), 1.0 M-HCl (60 ml, to give pH 8.07). The gels were subjected to a current of 4 mA/tube (diam. 5 mm) for 2 h at 0–4°C.

Before electrophoresis the enzyme preparation was dialysed for 3 h at 4°C against 200 vol. of tris buffer (0.02 M, pH 7.75) containing sodium DL-isocitrate (4.0 mM), magnesium chloride (10 mM) and sodium sulphate (0.1 M). After electrophoresis separate gels were stained specifically for isocitrate dehydrogenase and non-specifically for protein. The histochemical reagent used for the specific detection of the enzyme had the following composition: sodium DL-isocitrate (0.5 mM), NADP⁺ (0.5 mM), magnesium chloride (5.0 mM), sodium chloride (0.5 M), potassium cyanide (2.0 mM), phenazine methosulphate (0.8 mg/ml), Nitroblue Tetrazolium (4.0 mg/ml), tris buffer (pH 8.0, 0.1 M). The gels were incubated in this reagent for 1.0–1.5 h at room temperature in the dark and then stored in a solution containing methanol-acetic acid-water (5:1:5, by vol.). Gels were stained non-specifically for protein with a solution of Amido Black (1%, w/v) plus Nigrosine (0.1%, w/v) in acetic acid (7%, w/v); excess of stain was removed electrophoretically in acetic acid (7%, w/v).

Determination of apparent molecular weight

The apparent molecular weight of the isocitrate dehydrogenase was determined at various concentrations of sodium chloride and of potassium chloride by eluting from a column of Sephadex G-100 and comparing the elution volume with those of proteins of known molecular weight. The assumptions implicit in the method are discussed below.

The enzyme was dialysed at 4°C to equilibrium with the eluting buffer and applied to the column (2cm×22cm) already equilibrated with the same buffer. The enzyme and a mixture of protein 'markers' were separately eluted at room temperature from the column. Fractions containing the isocitrate dehydrogenase were identified by assaying the enzyme; proteins were detected non-specifically by their absorption at 280nm. Apparent molecular weight of the enzyme was interpolated from its position on a plot of log mol.wt. versus elution volume. All eluting buffers contained tris (0.02M, pH7.75) and sodium sulphate (0.1M) in addition to the following constituents: 1, sodium DL-isocitrate (4.0mM) and magnesium chloride (10mM); 2, sodium chloride (1.0M); 3, sodium chloride (4.0M); 4, potassium chloride (1.0M); 5, potassium chloride (4.0M).

Results

True Michaelis constants

True Michaelis constants at various salt concentrations are listed in Table 1. As found previously for the apparent constants, the K_m (NADP⁺) had a minimum value at low concentrations (0.25M or less) of sodium chloride and of potassium chloride. The values for K_m (isocitrate) had minima in about 0.75M-sodium chloride and in about 1.0M-potassium chloride. Values of the true constants were not

greatly different from those of the apparent constants previously determined with fixed concentrations of NADP⁺ and isocitrate of 1mM and 2mM respectively (Aitken *et al.*, 1970).

Effects of substrate concentration on salt relations of the enzyme

As previously, the effects of salts were considered in two concentration ranges, namely a low range in which an increase in salt concentration enhanced enzyme activity (the 'activating' range) and a high range in which an increase in salt concentration decreased enzyme activity (the 'inhibitory' range). In the activating range of both salts, double-reciprocal plots with NADP⁺ as variable substrate intersected to the right of the ordinate (Fig. 1). This type of effect had already been encountered when isocitrate was used at a fixed initial concentration of 2mM (Aitken *et al.*, 1970). The present results showed the effect to persist at fixed concentrations of isocitrate down to 0.125mM. The concentration of NADP⁺ at which the intersection occurred ranged between 0.17 and 0.5mM in solutions of sodium chloride and between 0.25 and 0.5mM in solutions of potassium chloride.

The effects of varying isocitrate at several fixed concentrations of NADP⁺ were different. At high fixed concentrations of NADP⁺ the double reciprocal plots intersected 'conventionally' to the left of the ordinate but, with decreasing fixed concentrations of NADP⁺, the point of intersection moved progressively to the right and eventually crossed the ordinate (Figs. 2-5). The substrate concentrations at which the lines intersected to the right of the ordinate were: in sodium chloride, fixed concentration of NADP⁺, 0.2mM, isocitrate, 0.77mM; in potassium chloride, fixed concentrations of NADP⁺ 0.4 and 0.2mM,

Table 1. *True Michaelis constants in solutions of sodium chloride and of potassium chloride*

Because of non-linearity in double-reciprocal plots at high NaCl concentrations and low fixed substrate concentrations, true Michaelis constants were not determined above 2.5M-NaCl.

Concn. of NaCl (M)	$10^4 K_m$ (isocitrate) (M)	$10^4 K_m$ (NADP ⁺) (M)	Concn. of KCl (M)	$10^4 K_m$ (isocitrate) (M)	$10^4 K_m$ (NADP ⁺) (M)
0.25	1.41	0.20	0.25	1.03	0.37
0.50	1.20	0.40	0.50	0.81	0.64
0.75	0.92	0.76	0.75	0.67	0.78
1.0	1.03	1.24	1.0	0.34	1.00
1.5	1.26	1.69	1.5	0.39	1.19
2.0	2.05	1.81	2.0	0.55	1.72
2.5	2.26	2.23	2.5	0.71	1.64
			3.0	0.82	1.69
			4.0	0.85	1.98

Fig. 1. Double-reciprocal plots obtained by varying the concentration of NADP^+ and with a fixed concentration of 0.125 mM-isocitrate

(a), (b) Results obtained in sodium chloride solutions; (c), (d) the corresponding results for potassium chloride. The two left-hand figures (a, c) represent low ('activating') concentrations of the salt. These concentrations are, from top to bottom (right of intersection), 0.75, 0.50 and 0.25 M-sodium chloride and 1.0, 0.75, 0.50 and 0.25 M-potassium chloride. The two right-hand figures (b, d) represent high ('inhibitory') concentrations of salt and, from top to bottom (right of intersection), are 3.0, 2.5, 2.0, 1.5 and 1.0 M-sodium chloride and 4.0, 3.0, 2.5, 2.0 and 1.5 M-potassium chloride. The results are qualitatively similar to those obtained with isocitrate at fixed concentrations of 0.25, 0.50, 1.0 mM and, as reported by Aitken *et al.* (1970), 2.0 mM. Regressions for 4.0 and 5.0 M-sodium chloride were omitted from the present figure and those (not illustrated) for 0.50 and 0.25 mM-isocitrate because, under those conditions, the kinetics were not Michaelis-Menten. The other difference between this figure and those representing higher fixed concentrations of isocitrate is that, under all other conditions, the primary plots for high concentrations of sodium chloride intersected at obviously more than one point. Although the present figure is atypical in that respect, it has been shown because it represents the lowest experimental fixed concentration of isocitrate whereas the corresponding figure of Aitken *et al.* (1970) shows the highest. Arrows denote the direction of increasing concentration of sodium chloride or potassium chloride. The dimensions of $1/v$ in all the figures are $(\mu\text{mol/min per mg of protein})^{-1}$.

isocitrate 2.5 and 0.17 mM respectively. Furthermore, whereas increasing salt concentration increased the slope of reciprocal plots with NADP^+ as variable substrate, it decreased the slope with isocitrate as variable substrate.

In the inhibitory ranges of salt concentration, pronounced differences with potassium chloride and

with sodium chloride were evident. Under all experimental conditions in potassium chloride the primary double-reciprocal plots intersected at one point to the left of the ordinate (Figs. 1-5). The position of the point of intersection varied with the concentration of fixed substrate but, qualitatively, the results were as already described for NADP^+ and isocitrate used

Fig. 2. Double-reciprocal plots obtained by varying the concentration of isocitrate at a fixed concentration of 0.8 mM-NADP^+

In Figs. 2–5 (a) and (b) give results obtained with sodium chloride; (c) and (d) give the corresponding results with potassium chloride. The two left-hand panels (a and c) represent low ('activating') concentrations of salt and, from top to bottom (right of intersection), are 0.25, 0.50 and 0.75M-sodium chloride and 0.25, 0.50, 0.75 and 1.0M-potassium chloride. The right-hand panels (b and d) denote high ('inhibitory') concentrations of salt. The concentrations of sodium chloride from bottom to top (right of intersection) are 1.0, 1.5, 2.0, 2.5M and, where present, 3.0, 4.0 and 5.0M. The corresponding concentrations of potassium chloride, from bottom to top, are 1.5, 2.0, 2.5, 3.0 and 4.0M. The series of figures shows the point of intersection in the low range of salt concentration displaced from left to right across the ordinate as the fixed concentration of NADP^+ is lowered. Arrows indicate the direction of increasing concentration of sodium chloride or potassium chloride.

as fixed substrates at concentrations of 1 mM and 2 mM respectively. The consequence of these kinetic patterns are shown in secondary plots of slope and intercept (Fig. 6), from which it is apparent that, in the inhibitory range, potassium chloride acted as a linear non-competitive inhibitor under all experimental conditions.

It was reported previously that, in the inhibitory range of sodium chloride concentration, primary plots intersected at more than one point to the left of the ordinate when the fixed substrates were each

used at the concentration stated above. Secondary plots showed sodium chloride to be a linear non-competitive inhibitor at concentrations up to 2.5M; above that concentration a deviation from linearity was encountered (Aitken *et al.*, 1970). This type of result was also obtained under the present experimental conditions (Figs. 1–5) but, with decreasing concentration of each fixed substrate, the non-linear characteristics of the secondary plots became more pronounced and tended to occur at lower salt concentrations. Secondary plots of both slope and

Fig. 3. Double-reciprocal plots obtained by varying the concentration of isocitrate at a fixed concentration of 0.6mM-NADP⁺

For details see Fig. 2.

intercept were non-linear at fixed concentrations of NADP⁺ from 0.20 to 0.80mM (Fig. 7).

Secondary plots of slope were non-linear at fixed concentrations of isocitrate between 0.125 and 1.00mM, with the reservation that, with 1.00mM-isocitrate, the results might be interpreted as linear at salt concentrations below about 2M. The secondary plots of intercept at all fixed concentrations of isocitrate were linear at concentrations of NaCl below about 2.5M; above this concentration the departure from linearity was more pronounced at the lower fixed concentrations of isocitrate.

The fixed substrate concentration affected the salt relations of the apparent $V_{\max}$ in several ways (Fig. 8). The fixed concentration of NADP⁺ had a pronounced effect on the apparent $V_{\max}$ in sodium chloride but only a minor effect in potassium chloride. Lowering the fixed concentration of NADP⁺ from 0.8 to 0.2mM caused a reduction in the maximal value

of $V_{\max}$ in sodium chloride but had little effect on its value in potassium chloride. It also flattened the two curves until, with 0.2mM-NADP⁺, no maximum was encountered in either salt. It can be inferred that, under these conditions, apparent $V_{\max}$ had its greatest value at a salt concentration lower than 0.25M. The fixed NADP⁺ concentration had little effect on the salt concentration at which the two curves (sodium chloride and potassium chloride) intersected.

The fixed concentration of isocitrate also had a pronounced effect on apparent $V_{\max}$ in sodium chloride and a relatively minor one in potassium chloride. In sodium chloride, lowering the fixed concentration of isocitrate from 1.0 to 0.125mM caused a fall in the maximal value of $V_{\max}$ without flattening the curve in these regions. There was much less response in potassium chloride so that the combined effect of lowering the fixed concentration of isocitrate was to move the point of intersection of the two

Fig. 4. Double-reciprocal plots obtained by varying the concentration of isocitrate at a fixed concentration of 0.4 mM-NADP⁺

For details see Fig. 2.

curves (sodium chloride and potassium chloride) to the left, i.e. towards a lower salt concentration. It can be inferred therefore that at some fixed concentration of isocitrate less than 0.125 mM, apparent $V_{\max}$ in sodium chloride would be less than in potassium chloride at all salt concentrations.

The reaction mechanism and its salt-dependence

Initial velocity measurements with a series of fixed substrate concentrations were made over a range of concentrations of each salt. For this purpose NADP⁺ was used at fixed concentrations in the range 0.2–0.8 mM; isocitrate was used at fixed concentrations (of the active isomer) in the range 0.125–1.0 mM. Sodium chloride and potassium chloride were each used within the range 0.25–4.0 M. The results of these experiments are shown in Figs. 9–13.

With isocitrate as variable substrate, reciprocal

plots were parallel for all fixed concentrations of NADP⁺ in sodium chloride at concentrations of 0.25–0.75 M. In higher concentrations of the salt the plots intersected to the left of the ordinate (Figs. 9 and 10). With NADP⁺ used as variable substrate, the primary plots were parallel for 0.25 and 0.50 M-sodium chloride but intersected with higher concentrations of the salt (Figs. 10 and 11). In potassium chloride, when isocitrate was variable substrate, parallel plots were obtained only at a salt concentration of 0.25 M; at higher salt concentrations the characteristic intersecting pattern was obtained (Figs. 12 and 13). When NADP⁺ was variable substrate the primary plots were parallel with 0.25 and 0.50 M-potassium chloride and intersected for all higher concentrations of the salt (Fig. 13).

Figs. 14–16 show product-inhibition patterns in sodium chloride (0.25 and 1.0 M) and in potassium chloride (1.0 M). In the lower concentration of

Fig. 5. Double-reciprocal plots obtained by varying the concentration of isocitrate at a fixed concentration of 0.2 mM-NADP^+

For details see Fig. 2. The order of regressions in (c) is the same as in Figs. 2–4, when viewed on the left of the intersection.

sodium chloride, α -oxoglutarate was competitive with each substrate; NADPH was competitive with isocitrate but not NADP^+ . In 1.0 M solutions of each salt, however, the inhibition pattern was different. NADP^+ and NADPH were competitive; all other combinations gave non-competitive inhibition by the product.

There was also a change in the type of kinetics of the enzyme at a high concentration (4.0 M) of sodium chloride and low fixed substrate concentration, when plots of initial velocity against substrate concentration were sigmoidal. The corresponding Hill plots had the following values for slope: with NADP^+ as fixed substrate at 0.2 mM and 0.4 mM , slopes 1.7 and

1.3 respectively; with isocitrate as fixed substrate at 0.125 mM and 0.25 mM , slopes 1.8 and 1.4 respectively.

Apparent molecular weight

The results of the elution estimates of molecular weight are shown in Fig. 17. From this figure the following values for the apparent molecular weight were obtained; in 1.0 M -potassium chloride, 122 000; in 4.0 M -potassium chloride, 135 000; in 1.0 M -sodium chloride, 224 000; in 4.0 M -sodium chloride, 251 000; in the buffer system without added potassium or sodium chloride (buffer 1), 70 800. Addition of sodium DL-isocitrate (4.0 mM) to buffers containing sodium

Fig. 6. Secondary plots of slopes and intercepts from Figs. 1–5 (and related figures which have not been shown) against concentration of potassium chloride

(a) and (b) Results obtained with NADP^+ at fixed concentrations of (top to bottom) 0.20, 0.40, 0.60 and 0.80 mM. (c) and (d) Results obtained with isocitrate at fixed concentrations of (top to bottom) 0.125, 0.25, 0.50 and 1.0 mM. Arrows indicate the direction of increasing concentrations of NADP^+ or of isocitrate.

chloride did not affect the elution volume of the enzyme.

Purification of the enzyme

Gel electrophoresis of crude cell-free preparations of the enzyme always gave a single band migrating about 1 cm, detectable with the histochemical reagent and many bands with Amido Black. After purification the preparation gave a single band of identical mobility with each reagent. Table 2 shows the effect of each purification step on the specific activity of the enzyme; it will be noted that there was only a 31-fold increase in specific activity in the whole process.

Discussion

The kinetic studies can be interpreted in terms of enzyme reaction mechanisms but the system is very

complex and there are limitations on the conclusions that can be drawn. One minor limitation is the omission from the product-inhibition studies of CO_2 , one of the three products of the reaction. A complicating factor is the evidence (see below) that salt apparently behaves, at least at the purely kinetic level, as a reactant adding reversibly to the enzyme system. Thus in any analysis in which salt concentration is a variable the enzyme behaves for all practical purposes as one with three substrates and four products.

Nevertheless, evidence has been obtained about the nature of the reaction mechanism that operates under 'physiological' conditions and also evidence of a change in mechanism at very low salt concentrations. The reciprocal plots for different fixed concentrations of each substrate gave intersecting patterns at high salt concentrations and parallel patterns at low salt concentration (Figs. 9–13). If the

Fig. 7. Secondary plots of slopes and intercepts from Figs. 1–5 (and related figures which have not been shown) against concentration of sodium chloride

(a) and (b) Results obtained with NADP^+ at fixed concentrations of (top to bottom) 0.20, 0.40, 0.60 and 0.80 mM. (c) and (d) Results obtained with isocitrate at fixed concentrations of (top to bottom) 0.125, 0.25, 0.50 and 1.0 mM. At high concentrations of sodium chloride and low fixed substrate concentration, the kinetics were not Michaelis–Menten. Arrows indicate the direction of increasing concentrations of NADP^+ or of isocitrate.

patterns at low salt concentration are in fact parallel, as we believe, and not imperceptibly converging, then the enzyme has a different mechanism at high ('physiological') salt concentrations from that at very low salt concentrations. The product-inhibition experiments (Figs. 14–16) confirm a difference in mechanisms in, on the one hand, 1.0 M salt and, on the other, 0.25 M-sodium chloride. The intersecting initial-velocity patterns indicate a sequential reaction mechanism, which is usual for the pyridine nucleotide-linked dehydrogenases. A parallel pattern usually signifies a non-sequential mechanism of the so-called Ping-Pong type (Cleland, 1970).

The product-inhibition experiments at the higher salt concentration were indicative of an ordered

reaction in which NADP^+ was the first substrate added and NADPH was the last product released. Although the initial-velocity studies in 0.25 M salt indicated a non-sequential mechanism, product inhibition, within its limitations, suggested a rapid-equilibrium random mechanism in which one dead-end complex is formed (Cleland, 1963). Non-sequential mechanisms do not always give classical Ping-Pong inhibition patterns, however, and this is conspicuously so if the enzyme has more than one catalytic site (Cleland, 1970). It is sufficient for our present purposes to state that at very low salt concentrations the mechanism is different from the physiological one and is probably non-sequential. A change in the mechanism of an enzyme is unusual but

Fig. 8. Effects of salt concentration on apparent $V_{\max}$ at several concentrations of each fixed substrate

(a)–(d) Results obtained with NADP^+ at fixed concentrations of (left to right) 0.80, 0.60, 0.40 and 0.20 mM. (e)–(h) Results obtained with isocitrate at fixed concentrations of (left to right), 1.0, 0.50, 0.25 and 0.125 mM. o, Sodium chloride; ●, potassium chloride.

not unknown. Sanwal *et al.* (1965) reported that AMP changed the mechanism of the NAD-specific isocitrate dehydrogenase of *Neurospora* from random to ordered.

The intersection of reciprocal plots to the right of the ordinate, as shown in Figs. 1–5, was encountered in the activation range of salt concentration. The net stimulation caused by salt under these conditions can be explained in kinetic terms if salt is regarded as a reactant that adds reversibly as a ‘substrate’ in the reaction sequence. Under these conditions, however, salt increased slope with NADP^+ as variable substrate but decreased it when isocitrate was variable. This difference implies in part (cf. Cleland, 1970) that the substrate-like addition of salt occurs after NADP^+ and before isocitrate. Such an interpretation supports the basic conclusion from product inhibition that NADP^+ is the first substrate added.

The inhibitory action of salt revealed when NADP^+ was variable, however, requires another point of addition of salt in the reaction sequence. The evidence suggests that salt forms a dead-end complex with the free enzyme before the addition of the first substrate,

Scheme 1. Representation of the ‘physiological’ reaction mechanism

NADP^+ . By using Cleland’s (1963) type of formulation the ‘physiological’ reaction mechanism can therefore be depicted in the way shown in Scheme 1. This mechanism is virtually equivalent to substrate inhibition once the substrate-like role of salt is recognized. Indeed, intersecting plots of the type shown in Figs. 1–5 are characteristic of substrate inhibition under some circumstances (Cleland, 1970). The effects depicted in Figs. 1–5 occurred over a range of salt concentration wide enough to include

Fig. 9. Reciprocal plots obtained at various fixed concentrations of NADP^+ and of sodium chloride

The major purpose of Figs. 9–13 is to show the evidence for parallelism in some of the plots. An additional set of results (for various fixed concentrations of isocitrate at potassium chloride concentrations of 1.0–4.0M) gave entirely intersecting plots and have not been illustrated. The fixed substrate concentrations are depicted in all five figures by the following symbols. NADP^+ : $\circ$, 0.2mM; $\blacktriangle$, 0.4mM; $\triangle$, 0.6mM; $\bullet$, 0.8mM. Isocitrate: $\circ$, 0.125mM; $\blacktriangle$, 0.25mM; $\triangle$, 0.5mM; $\bullet$, 1.0mM. In this figure the concentrations of sodium chloride were (M): (a) 0.25; (b) 0.5; (c) 0.75; (d) 1.0; (e) 1.5; (f) 2.0. Arrows indicate the direction of increasing concentrations of NADP^+ .

both the 'physiological' mechanism (Scheme 1) and the 'unphysiological' one, which functions at very low salt concentrations and which we have suggested is non-sequential.

In the high, inhibitory, range of salt concentration, potassium chloride was a linear non-competitive inhibitor under all experimental conditions. This is a logical consequence of Scheme 1 with dominance of the dead-end complex at high salt concentrations. Scheme 1 can therefore be assumed to function in all but very low concentrations of potassium chloride.

There was a difference, however, in the inhibition caused by high concentrations of sodium chloride, which gave non-linear replots of slope and intercept

under certain circumstances (Fig. 7 and Aitken *et al.*, 1970). The non-linearity of sodium chloride inhibition is affected by substrate concentration and was discussed by Aitken *et al.* (1970) in terms of a change in the state of the enzyme. It can also be interpreted in kinetic terms with the inference that sodium chloride (or Na^+) adds more than once in an inhibitory manner in the reaction sequence (see Cleland, 1970). Heyde & Morrison (1970) reported slope-parabolic, intercept-linear inhibition by sodium chloride of the reverse ATP-creatine phosphotransferase reaction and explained this by a proposed reaction of one Cl^- with the free enzyme and one with each other enzyme form. With the halophil

Fig. 10. Reciprocal plots obtained at various fixed concentrations of substrate and of sodium chloride

The sodium chloride concentrations were (M): (a) 2.5; (b) 3.0; (c) 4.0; (d) 5.0; (e) 0.25; (f) 0.5. The changing fixed substrate was NADP^+ in (a)–(d) and in (e)–(f) it was isocitrate. Plots obtained with lower concentrations of NADP^+ have been omitted because they were non-linear (see the text). See Fig. 9 for further details. Arrows indicate the direction of increasing concentrations of substrate.

isocitrate dehydrogenase, however, the conditions for multiple addition of sodium chloride are affected by substrate concentration as is the change in state of the enzyme discussed by Aitken *et al.* (1970).

The proposed change in state is possibly a change in molecular weight. The assumption underlying the molecular-weight estimations is that salt did not affect the apparent molecular weight of the markers used in the gel-filtration experiments. The experimental justification for this assumption is that a plot of $\log$ (theoretical mol.wt.) of the markers against their elution volumes was a straight line under all conditions; there were no aberrant points nor was there any change in the order of elution of the markers.

Thus if salt did change the molecular weight of the markers it changed them all uniformly and in the opposite direction from the changes deduced for the isocitrate dehydrogenase. This is improbable.

There is therefore evidence of a correlation between apparent molecular weight of the enzyme and the reaction mechanisms discussed above. Very low salt concentrations that are associated with the supposedly non-sequential mechanism are also associated with an enzyme molecular weight of about 70 800. In potassium chloride (1 M and 4 M), when Scheme 1 operates, apparent molecular weights of about 122 000 and 135 000 were obtained. It is likely therefore that the 'physiological' state of the enzyme is a dimer in

Fig. 11. Reciprocal plots obtained at various fixed concentrations of isocitrate and of sodium chloride

The sodium chloride concentrations were (M): (a) 0.75; (b) 1.0; (c) 1.5; (d) 2.0; (e) 2.5; (f) 3.0. See Fig. 9 for further details. Arrows indicate the direction of increasing concentrations of isocitrate.

Fig. 12. Reciprocal plots obtained at various fixed concentrations of NADP^+ and of potassium chloride

The potassium chloride concentrations were (M): (a) 0.25; (b) 0.5; (c) 0.75; (d) 1.0; (e) 1.5; (f) 2.0. See Fig. 9 for further details. Arrows indicate the direction of increasing concentration of NADP^+ .

Fig. 13. Reciprocal plots obtained at various fixed concentrations of substrate and of potassium chloride

The potassium chloride concentrations were (M): (a) 2.5; (b) 3.0; (c) 4.0; (d) 0.25; (e) 0.5; (f) 0.75. In (a)–(c) the changing fixed substrate was NADP^+ , and in (d)–(f) it was isocitrate. See Fig. 9 for further details. Arrows indicate the direction of increasing concentrations of fixed substrate.

Fig. 14. Product-inhibition experiments in 0.25M-sodium chloride

In Figs. 14–16 are shown the results of product-inhibition experiments in which NADP^+ and isocitrate were separately varied at several fixed concentrations of NADPH or α -oxoglutarate. The fixed concentrations of NADP^+ and isocitrate were 0.1 mM and 0.25 mM respectively. (a) and (c) NADPH was added at the following concentrations (mM): $\blacktriangle$, 0; $\circ$, 0.0625; $\triangle$, 0.125; $\bullet$, 0.25. (b) and (d) α -Oxoglutarate was added at the following concentrations (mM): $\blacktriangle$, 0; $\square$, 0.5; $\triangle$, 1.0; $\bullet$, 2.0. Arrows indicate the direction of increasing concentrations of product.

Fig. 15. Product-inhibition experiments in 1.0M-sodium chloride

The fixed concentrations of NADP^+ and isocitrate were 0.6mM and 0.5mM respectively. (a) and (c) See Fig. 14 for concentrations of added NADPH. (b) and (d) α -Oxoglutarate was added at the following concentrations (mM): $\blacktriangle$, 0; $\circ$, 0.25; $\square$, 0.5; $\triangle$, 1.0; $\bullet$, 2.0. Arrows indicate the direction of increasing concentrations of product.

which the monomer has a molecular weight in the region of 70000. The apparent molecular weights of 224000 and 251000 in sodium chloride (1M and 4M) suggest that the interaction of this salt with the enzyme as discussed above is associated with a trimer or a tetramer.

The complex inhibition by sodium chloride as well as the non-Michaelis kinetics observed at high sodium chloride and low substrate concentrations therefore are related to the enzyme's being in the highest state of polymerization encountered experimentally. The ability of high substrate concentrations to modify the type of inhibition caused by sodium chloride suggests that high substrate concentrations might also be able to depolymerize the 'sodium enzyme' to the dimer. For economic reasons, this hypothesis was not tested, although as stated in the Results section, 4.0mM-isocitrate did not affect elution volume in sodium chloride. Molecular weights reported for NADP-specific isocitrate dehydrogenases from other sources are: pig heart, 64000 (Moyle & Dixon, 1956); *Azotobacter vinelandii*, 80000

(Chung & Franzen, 1969), 78000 (Barrera & Jurtschuk, 1970); *Escherichia coli*, 80000 (Reeves *et al.*, 1972). Barrera & Jurtschuk (1970) believe that the *A. vinelandii* enzyme is a polymer in which the monomeric units have a molecular weight of 15000–20000.

The change in enzyme mechanism at very low salt concentrations is of enzymological interest but dubious physiological significance. The bacterium cannot remain intact, let alone survive, at the low salt concentration required for the non-sequential reaction mechanism and the monomeric form of the enzyme.

The intracellular concentration of potassium in halobacteria is normally within the range 4–5 molal but, according to Christian & Waltho (1962), sodium does not exceed about 1.4 molal in stationary-phase *Halobacterium salinarum*, nor, according to C. E. Armstrong (unpublished work) does it exceed about 0.9 molal in our colourless strain of *H. salinarum* in its exponential growth phase. Ginzburg *et al.* (1970), however, have reported that intracellular sodium can reach 3 molal in the early exponential phase of a

Fig. 16. Product-inhibition experiments in 1.0M-potassium chloride

The fixed concentrations of NADP^+ and isocitrate were as in Fig. 15. (a) and (c) See Fig. 14 for concentrations of added NADPH. (b) and (d) α -Oxoglutarate was added at the following concentrations (mM): $\blacktriangle$, 0; $\circ$, 0.25; $\triangle$, 1.0; $\bullet$, 2.0. Arrows indicate the direction of increasing concentrations of product.

species of *Halobacterium*. There is thus a possibility that circumstances might sometimes be encountered that give rise to the sigmoidal kinetics of isocitrate dehydrogenase in very high sodium chloride and low substrate concentrations. One other halophil enzyme, threonine deaminase, has been reported to give sigmoidal kinetics in high concentrations of sodium chloride (Lieberman & Lanyi, 1972). In that case the co-operative substrate effects were overcome by ADP; there was evidence for several forms of the enzyme. Effects of potassium chloride were not investigated. Speculation about the physiological significance of sigmoidal kinetics, however, is not warranted at present.

The evidence contained in this paper, as well as in the earlier publication of Aitken *et al.* (1970), is quite consistent that K^+ is a far more favourable ion for the function of isocitrate dehydrogenase than is Na^+ . This is evident, not only from simple kinetic parameters such as apparent $V_{\max}$ (Fig. 8) but also from the more complex nature of the inhibition

caused by sodium chloride and the associated additional polymerization of the enzyme. Apparent $V_{\max}$ was not only much less affected by concentration of potassium chloride than of sodium chloride but, in potassium chloride, it was also relatively unaffected by fixed substrate concentration.

Indeed there are some remarkable similarities between the action of potassium chloride on the halophil enzyme and the action of any of several polyols on the NADP-specific isocitrate dehydrogenase of yeast. Some polyols accumulate to a high intracellular concentration in the sugar-tolerant (or 'osmophilic') yeasts and their presence is responsible for the ability of these yeasts to grow at low water activities (Brown & Simpson, 1972). The similarities between K^+ and the polyols has led Brown & Simpson (1972) to designate as 'compatible solutes' substances that accumulate intracellularly, and which, at a high concentration, have only a slight inhibitory action on an enzyme.

The present work has also illustrated consistently

Table 2. Steps in the purification of isocitrate dehydrogenase

The yield and degree of purification of the enzyme are shown for each of the steps described in the text. A unit of activity is the reduction of 1 nmol of NADP⁺/min. Specific activity is expressed as units/mg of protein.

	Vol. (ml)	Activity (units/ml)	Total activity (units)	Protein (mg/ml)	Specific activity	Yield (%)	Purification
1 Cell-free extract	33	2640	87000	59.0	44.8	100	1
2 50% satd. (NH ₄) ₂ SO ₄ supernatant	130	515	67000	2.5	206	77	4.6
3 Sephadex G-100 eluent in 4.2M-NaCl	16.5	2450	40500	6.0	407	46.5	9.1
4 Sephadex G-100 eluent in 4M-KCl	12.0	1010	12100	0.9	1120	13.9	25.0
5 DEAE-Sephadex eluent	7.0	486	3400	0.35	1400	3.9	31.0

Fig. 17. Estimation of the apparent molecular weight of the enzyme by elution from Sephadex G-100

The markers, in order of increasing molecular weight, were cytochrome *c*, myoglobin, trypsin, deoxyribonuclease, haemoglobin and D-amino acid oxidase. The eluting buffers (see the text) were: (a) ●, buffer 1; ▲, buffer 2; ■, buffer 3. (b) ●, Buffer 1 (as in a); ▲, buffer 4; ■, buffer 5. The positions for isocitrate dehydrogenase are marked with arrows.

the significance of substrate concentration as a factor able to modify, sometimes extensively, the effect of a salt on an enzyme. This is important, not only in evaluating reports of the salt relations of any enzyme but also, of course, in understanding details of the mechanism of salt-enzyme interactions. The ability of a substrate at millimolar concentration to

modify effects caused by a salt at a concentration of several molar has extensive implications about regulatory mechanisms in halophilic bacteria. Whether or not methods of enzyme kinetics are adequate to answer the unresolved questions about enzyme mechanisms in the presence of such high salt concentrations might be open to some doubt. It is clear, however, that a useful extension of the work will require the application of physicochemical methods, which in turn will require a purified enzyme. The exploratory method of purification described, although giving only a 31-fold increase in specific activity, shows that the enzyme can be obtained with little difficulty in a state of reasonable purity.

References

- Aitken, D. M., Wicken, A. J. & Brown, A. D. (1970) *Biochem. J.* **116**, 125-134
- Barrera, C. R. & Jurtshuk, P. (1970) *Biochim. Biophys. Acta* **220**, 416-429
- Brown, A. D. & Simpson, J. F. (1972) *J. Gen. Microbiol.* in the press
- Christian, J. H. B. & Waltho, J. A. (1962) *Biochim. Biophys. Acta* **65**, 506-508
- Chung, A. E. & Franzen, J. S. (1969) *Biochemistry* **8**, 3175-3184
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104-137
- Cleland, W. W. (1967) *Advan. Enzymol. Relat. Areas Mol. Biol.* **29**, 1-32
- Cleland, W. W. (1970) *Enzymes*, 3rd. edn., **2**, 1-65
- Ginzburg, M., Sachs, L. & Ginzburg, B. Z. (1970) *J. Gen. Physiol.* **55**, 187-207
- Heyde, E. & Morrison, J. F. (1970) *Biochim. Biophys. Acta* **212**, 288-299
- Lieberman, M. L. & Lanyi, J. K. (1972) *Biochemistry* **11**, 211-216
- Moyle, J. & Dixon, M. (1956) *Biochem. J.* **63**, 548-552
- Reeves, H. C., Daumy, G. O., Lin, C. C. & Houston, M. (1972) *Biochim. Biophys. Acta* **258**, 27-39
- Sanwal, B. D., Stachow, C. S. & Cook, R. A. (1965) *Biochemistry* **4**, 410-421