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On the Effects of Tricarboxylic Acid Cycle Intermediates on Sodium Transport by the Toad Bladder

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Summary. The effects of acetate, alpha-ketoglutarate, succinate and fumarate on sodium transport across the isolated bladder of the Colombian toad have been reinvestigated in bladders bathed in PO_4 Ringer's solution at pH 6.4. At this pH these substrates, when added to the serosal bathing medium in concentrations of 4 to 20 mM, stimulate sodium transport as measured by the short-circuit current; the stimulatory effect of succinate is synergistic with that of aldosterone. These findings support the conclusion that active sodium transport in the toad bladder is not linked to any specific step of the tricarboxylic acid cycle, and that the steps in the cycle prior to the formation of succinate do not play a unique role in the regulation of sodium transport by aldosterone.

Early studies of the relationship between metabolism and sodium transport in the toad bladder delineated and emphasized the role of the oxidative metabolism of pyruvate and certain other precursors of acetyl CoA in the transport process [2, 8, 11, 13]. On the assumption that the tricarboxylic acid (TCA) cycle was the likely major source of energy for sodium transport, Maffly and Edelman [8] studied the effects of a variety of TCA cycle precursors and intermediates on sodium transport as measured by the short-circuit current (S.C.C.) technique. They observed that addition of pyruvate, glucose, lactate and beta-hydroxybutyrate stimulated the S.C.C. but that addition of acetate or TCA cycle intermediates had no effect. These results were extended by Sharp and Leaf [11] who observed in aldosterone-treated bladders that pyruvate and substrates yielding pyruvate (glucose, lactate, and oxaloacetate), as well as beta-hydroxybutyrate and acetoacetate [13], stimulated sodium transport, whereas acetate and the TCA cycle intermediates (citrate, alpha-ketoglutarate, succinate, and fumarate) did not stimulate but rather produced a small depression of sodium transport. Similar substrate-specificity in aldosterone-treated bladders was subsequently reported by Fimognari, Porter and Edelman [7].

These observations led to the view that the sodium transport mechanism in the toad bladder might be linked to some specific part of the oxidative metabolic pathway [2, 7, 8, 11]. On the basis of inhibitor studies and of the observed substrate-specificity of the stimulatory effect of aldosterone – in particular, the failure to demonstrate substrate-aldosterone synergism with succinate or its precursors – Edelman and co-workers [3, 4, 6, 7] postulated that aldosterone might regulate sodium transport by 1) enhancing the activity of the TCA cycle at some point between the formation of citrate and the formation of succinate, thus increasing the rate of NADH production, or 2) increasing the rate of NADH oxidation. This hypothesis was challenged however by the demonstration that propionate, a fatty acid precursor of succinate, can provide the energy for aldosterone-stimulation of sodium transport [5].

It has been suggested that the failure to demonstrate stimulation of sodium transport on addition of certain substrates, e.g. succinate, may have merely reflected a failure of the substrate to penetrate in sufficient concentration into the epithelial cells under the experimental conditions used [6, 8, 12]. Previous studies of the effects of TCA cycle precursors and intermediates on the S.C.C. in the toad bladder have mostly been carried out at a pH of 7.2 to 8.2 [6, 7, 8, 11]. Since penetration of the epithelial cells by weak acids, and in particular by dicarboxylic acids, may be assumed to increase with decreasing pH [1], we have reinvestigated the effects of acetate, alpha-ketoglutarate, succinate and fumarate on the S.C.C., employing toad bladders bathed in Ringer's solution of pH 6.4. In addition, the effect of succinate in aldosterone-treated bladders has been reexamined.

Materials and Methods

Sodium transport across isolated hemibladders of the Colombian toad *Bufo marinus* was measured by means of the S.C.C. technique of Ussing and Zerahn [17]; this method has been shown to provide a valid measure of net sodium transport following the addition of substrate [12]. Hemibladders were mounted as diaphragms between glass chambers and bathed in a PO_4 Ringer's solution of pH 6.4 (Na 111, K 4.0, Ca 1.8, Cl 113, HPO_4 2.0 and H_2PO_4 2.0 mEquiv/liter; 220 mOsm/kg H_2O) [15], or in a HCO_3 Ringer's solution of pH 7.9 (Na 113.5, K 1.88, Ca 1.78, Cl 114.8, HCO_3 2.38; 215 mOsm/kg H_2O) [8]. Studies were made in both "freshly-isolated" and "overnight" bladders. "Freshly-isolated" bladders were those in which measurements of the S.C.C. were completed within 4 to 6 hr of sacrifice and mounting without addition of aldosterone [16]; we infer that the rate of sodium transport in freshly-isolated bladders from Colombian toads is under the influence of endogenous aldosterone since such bladders fail to respond to added aldosterone, whereas after overnight incubation stimulation by aldosterone can be regularly demonstrated [9]. "Overnight" bladders were those mounted the evening before the day of study and incubated overnight, with aldosterone added where specified;

short-circuiting was commenced in the morning, approximately 15 hr after sacrifice and mounting [9]. In all experiments paired hemibladders were employed, one serving as the experimental tissue and the other as the control. The test substrate was added only to the serosal bathing medium of the experimental hemibladders, while an equal volume of Ringer's solution was added to the mucosal medium. All substrates were added as sodium salts in solutions of pH and osmolality corresponding to those of the Ringer's solution used; concentrations of substrates specified in the text refer to final concentrations in the media bathing the bladders.

A quantitative index of the response to addition of a substrate was obtained by dividing the value of the S.C.C. recorded at time t after addition ($S.C.C._t$) by the value recorded just before addition ($S.C.C._o$) and factoring it by the equivalent values for the control hemibladder:

$$\frac{S.C.C._t}{S.C.C._o}\ \text{experimental} \Big/ \frac{S.C.C._t}{S.C.C._o}\ \text{control.}$$

Results in the text and the table are expressed as the percentage deviation of this value from unity $\pm$ the standard error for n number of pairs studied. Stimulation of the S.C.C. of the experimental hemibladders compared to the controls is indicated by + and inhibition by −. Values for p were calculated using Student's t test, with 0.05 or less considered to be significant. Where the S.C.C. is expressed in μamps, this is for an exposed area of 2.5 cm^2.

The following compounds were obtained commercially and used without further purification: glacial acetic acid (Baker and Anderson, Allied Chemical); alpha-ketoglutaric acid, disodium succinate, and disodium fumarate (Calbiochem).

Results

The Effects of Acetate, Alpha-Ketoglutarate, Succinate and Fumarate on the S.C.C. *at* pH 6.4

The effects of serosal addition of acetate, alpha-ketoglutarate, succinate and fumarate, in concentrations ranging from 4 to 20 mM, on the S.C.C. of hemibladders bathed in PO_4 Ringer's solution of pH 6.4 are shown in Table 1.

In "freshly-isolated" hemibladders, acetate stimulated the S.C.C. at concentrations of 6 mM and 20 mM, although in the experiments at 20 mM there was variability in the response. Alpha-ketoglutarate at both 8 mM and 20 mM produced a moderate stimulation of the S.C.C. Succinate stimulated mildly at 4 mM and markedly at 20 mM; the mean response to 20 mM succinate is depicted in Fig. 1. Fumarate at 4 mM had no effect on the S.C.C., while at 20 mM it caused a variable stimulation.

In "overnight" bladders, as in "freshly-isolated" tissue, 20 mM succinate markedly stimulated the S.C.C.

The Effect of Succinate in Aldosterone-Treated Bladders

Since serosal addition of succinate in PO_4 Ringer's solution of pH 6.4 resulted in a stimulation of the S.C.C., experiments were performed to

Table 1. Effects of acetate and TCA cycle intermediates on the S.C.C.

Compound	Concentration	Bladder preparation	n	Minutes after addition				
				15	30	60	90	120
Acetate	6 mM	Freshly-isol.	6	−2±6 n.s.	+27±7 <0.05	+46±18 =0.05	+49±19 <0.05	+43±17 =0.05
	20 mM	Freshly-isol.	8	+3±7 n.s.	+31±13 <0.05	+61±28 n.s.	+75±38 n.s.	+71±39 n.s.
Alpha-ketoglutarate	8 mM	Freshly-isol.	5	+7±1 <0.01	+13±3 <0.05	+24±5 <0.01	+31±6 <0.01	+36±6 <0.01
	20 mM	Freshly-isol.	8	+7±2 <0.02	+11±4 <0.05	+19±8 <0.05	+27±13 n.s.	+31±10 <0.02
Succinate	4 mM	Freshly-isol.	14	+2±1 n.s.	+6±3 n.s.	+15±4 <0.01	+18±6 <0.05	+18±8 <0.05
	20 mM	Freshly-isol.	7	+18± 7 <0.05	+51±16 <0.05	+120±21 <0.01	+159±24 <0.001	+205±37 <0.01
	20 mM	Overnight	6	−2±2 n.s.	+25±10 =0.05	+95±17 <0.01	+117±16 <0.001	+137±17 <0.001
Fumarate	4 mM	Freshly-isol.	6	−2±3 n.s.	0±4 n.s.	+4±8 n.s.	+6±7 n.s.	+5±7 n.s.
	20 mM	Freshly-isol.	9	+14±5 <0.05	+13±6 n.s.	+17±8 n.s.	+19±9 n.s.	+25±10 <0.05

Substrates were added to the serosal bathing medium of freshly-isolated hemibladders bathed in PO_4 Ringer's solution of pH 6.4; results are expressed as described in Materials and Methods.

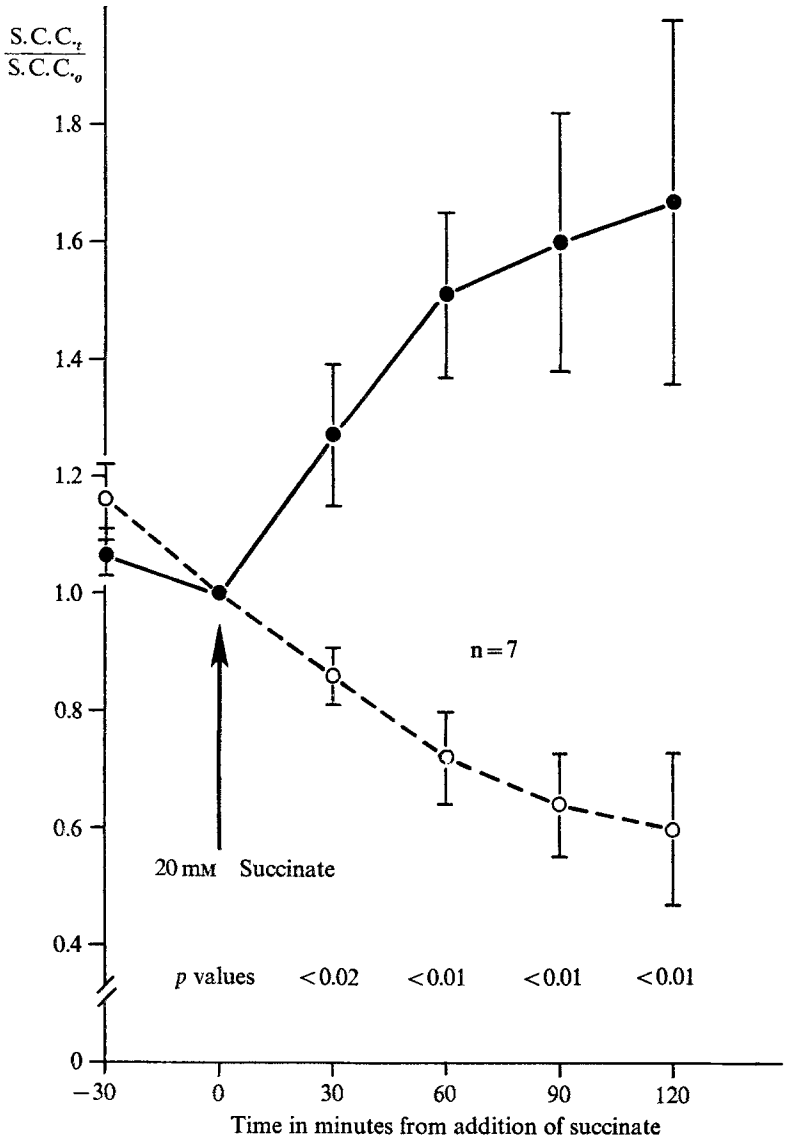

Fig. 1. Effect of 20 mM succinate on the S.C.C. (PO_4 Ringer's solution, pH 6.4.) The S.C.C. was measured in paired freshly-isolated hemibladders; at time zero, 20 mM succinate (•—•) was added to the serosal bathing medium of one of each pair of hemibladders. The effects are expressed as the mean values of the ratio $\frac{\text{S.C.C.}_t}{\text{S.C.C.}_o}$, plotted for the experimental and control (o---o) hemibladders; p values were calculated for the mean differences between paired hemibladders. Succinate markedly stimulated the S.C.C.

determine if succinate-aldosterone synergism could be demonstrated using this bathing solution. The studies were carried out on "overnight" hemibladders, using a modification of the method developed by Porter and

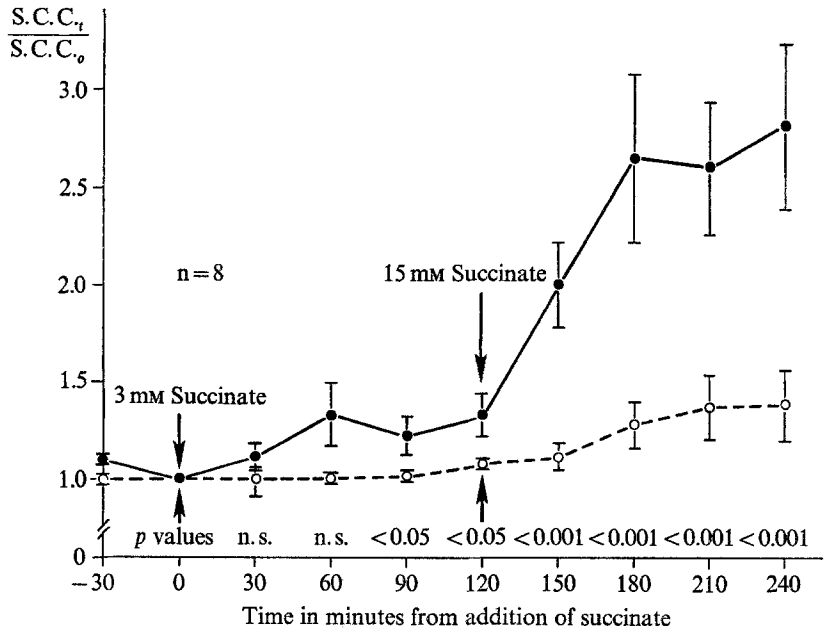

Fig. 2. Effect of aldosterone on the response of the S.C.C. to succinate. Eight hemibladder pairs were incubated overnight in pH 6.4 PO_4 Ringer's solution containing 5 mM glucose. One of each pair was incubated with 7×10^{-8} M aldosterone (•—•), the other was not (o---o). The next morning (time zero) 3 mM succinate was added to the serosal bathing media of both halves of each pair of hemibladders; at time 120 min, the succinate concentration was raised to 15 mM. Results are expressed as in Fig. 1. Values for p refer to the differences between the eight paired hemibladders relative to time zero. Succinate stimulated the aldosterone-treated tissues to a much greater degree than their non-aldosterone-treated controls

Edelman [9]. Both members of each pair of hemibladders were incubated overnight in PO_4 Ringer's solution of pH 6.4 containing 5 mM glucose, with the addition of 7×10^{-8} M aldosterone to the solution bathing one member of each pair. The next morning the bathing media were changed to fresh Ringer's solution without glucose, but with aldosterone remaining in the solution bathing the experimental hemibladders. Two and one-half hours later the mean S.C.C. of the aldosterone-treated tissues was 17 ± 4 μamps and that of the controls 11 ± 2 μamps ($n = 8$). Addition of 3 mM succinate at this time to the serosal medium of both members of each pair of hemibladders resulted in mild stimulation of the S.C.C. of the aldosterone-treated hemibladders compared to that of the nonaldosterone controls, as shown in Fig. 2; when the succinate concentration was raised to 15 mM the aldosterone-treated hemibladders were stimulated markedly but the non-aldosterone controls only mildly.

Dependence of the Stimulatory Effect of Succinate on the Composition of the Bathing Medium

To demonstrate that the response to succinate was dependent on the composition of the bathing medium, the effect of succinate was compared in paired aldosterone-treated hemibladders bathed in two different Ringer's solutions. Paired hemibladders were mounted and incubated overnight, one of each pair in PO_4 Ringer's solution of pH 6.4 and the other in the HCO_3 Ringer's solution of pH 7.9 used in our previous negative study [8]; both solutions contained 5 mM glucose and 7×10^{-8} M aldosterone. The next morning fresh Ringer's solutions of the same composition were substituted, containing aldosterone but no glucose; two hours later the S.C.C. was lower in the hemibladders incubated in the Ringer's solution of pH 6.4 (12 ± 3 μamps *vs.* 46 ± 13 μamps, $n = 6$, $p < 0.001$). Addition of 10 mM succinate at this time to the serosal medium resulted in substantial stimulation of the S.C.C. in all six hemibladders bathed in the pH 6.4 Ringer's solution and no rise in the S.C.C. in any of the paired hemibladders bathed in the Ringer's solution of pH 7.9. Fig. 3*a* shows an example of the response in one pair of hemibladders with the S.C.C. plotted directly in μamps; the mean response in six pairs of hemibladders is depicted in Fig. 3*b*.

Discussion

In contrast to earlier findings [7, 8, 11], these studies demonstrate that acetate and several TCA cycle intermediates, including succinate, can stimulate sodium transport by the toad bladder. Stimulation of the S.C.C. was observed on addition of acetate, alpha-ketoglutarate, succinate and fumarate to the serosal medium of isolated hemibladders bathed in PO_4 Ringer's solution at pH 6.4, using concentrations in the range of 4 to 20 mM. Furthermore, the stimulatory effect of succinate in bladders incubated in this Ringer's solution was found to be synergistic with that of exogenous aldosterone.

That the response of the S.C.C. to succinate varies with the composition of the bathing medium was clearly demonstrated in the experiments in which paired aldosterone-treated hemibladders were bathed in two different Ringer's solutions: succinate stimulated only the S.C.C. of the tissue bathed in the PO_4 Ringer's solution of pH 6.4. The two Ringer's solutions differed in their concentrations of potassium, bicarbonate and phosphate as well as in pH; however, in their negative studies with succinate at pH 7.6 to 7.8, both Sharp and Leaf [11] and Fimognari *et al.* [7] employed solutions with

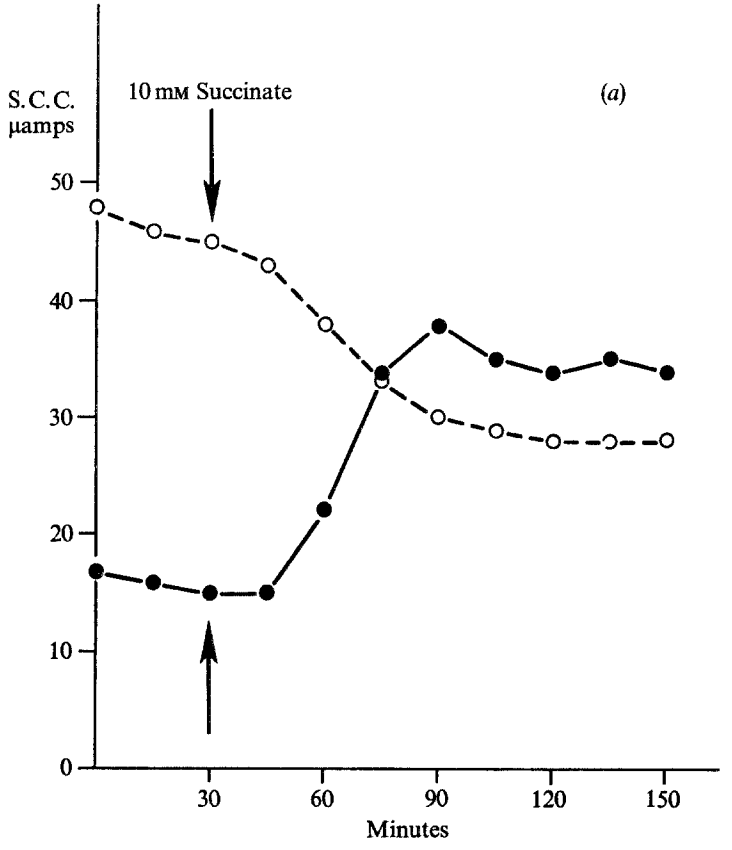

Fig. 3*a*

Fig. 3. Effect of composition of the bathing medium on the response of the S.C.C. to succinate. Six hemibladder pairs were incubated overnight, one of each pair in PO_4 Ringer's solution of pH 6.4 (•—•), the other in HCO_3 Ringer's solution of pH 7.9 (o---o); both solutions contained 5 mM glucose and 7×10^{-8} M aldosterone. The next morning 10 mM succinate was added to the serosal medium of all tissues. (*a*) An example of the response in one pair of hemibladders with the S.C.C. plotted in μamps; (*b*) The mean response in six pairs of hemibladders; results are plotted as in Fig. 1. Succinate stimulated only those hemibladders bathed in PO_4 Ringer's solution at pH 6.4

a potassium concentration (3.5 mEquiv/liter) similar to that of our pH 6.4 Ringer's solution (4.0 mEquiv/liter) and Sharp and Leaf [11] obtained comparable substrate responses whether phosphate or bicarbonate was present in the bathing medium. We therefore conclude that the difference in the response of the S.C.C. to succinate observed in the paired hemibladders is attributable to the difference in pH of the solutions employed. We infer that pH differences similarly account for the differing responses to the other

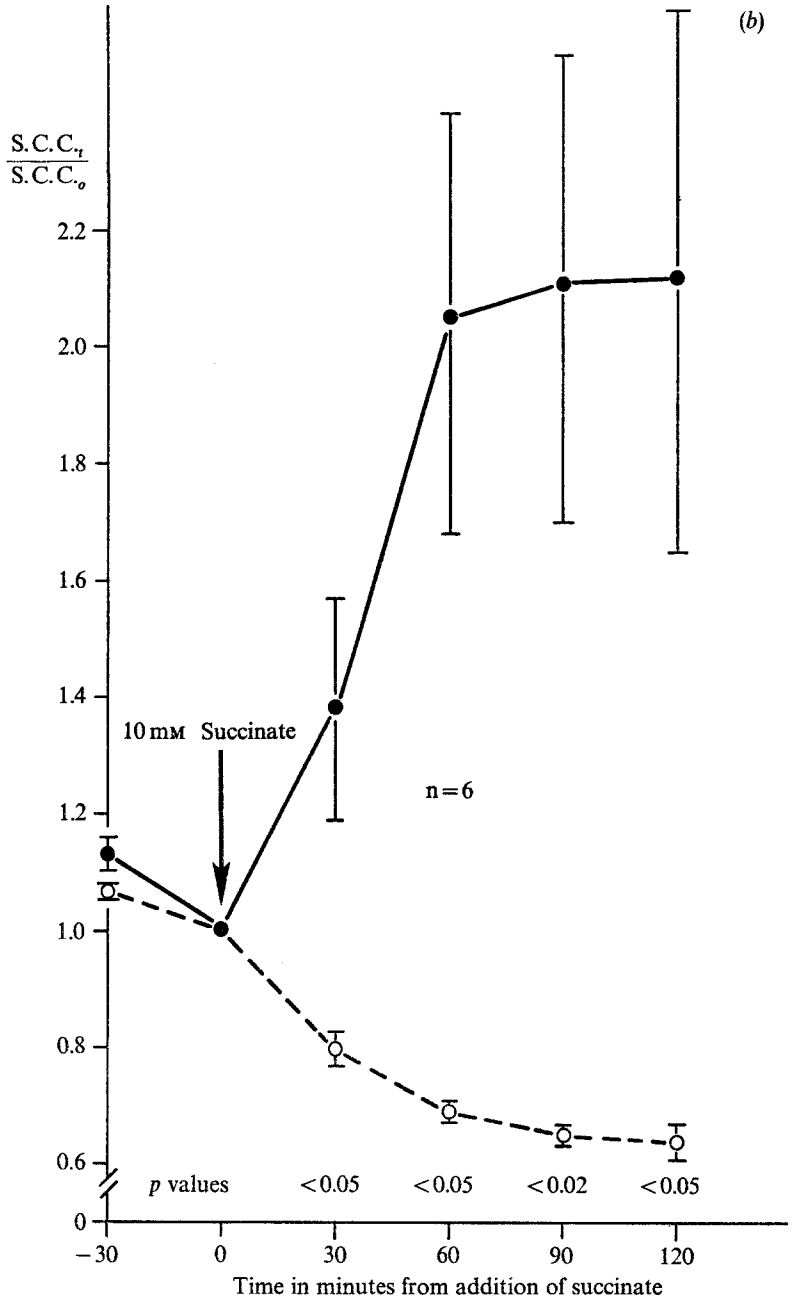

Fig. 3*b*

test substrates observed in this and in previous studies [7, 8, 11]. Cell membranes [1], including those of the toad bladder [10], are ordinarily more permeable to uncharged than to charged molecules, and in our experiments at

pH 6.4 the concentrations in the bathing medium of the uncharged (protonated) carboxylic acids were substantially higher than those employed in previous studies. It therefore seems likely that the stimulation of sodium transport observed on substrate addition at pH 6.4 is simply attributable to a higher rate of penetration of substrate into the transporting epithelial cells at this pH. However, it is also possible that pH affects other variables which influence the rate of substrate utilization (e.g. the activity of enzymes or co-factors, or the coupling of substrate oxidation to sodium transport); indeed the lower rate of sodium transport in hemibladders incubated overnight at lower pH may itself reflect such an influence.

In these studies all substrates were added to the serosal medium only. We have previously found that addition of 20 mM succinate simultaneously to the serosal and mucosal bathing media of freshly-isolated bladders at pH 6.4 can, in some instances, result in inhibition rather than stimulation of the S.C.C. [16]. While this inhibitory effect of succinate may be a function of its intracellular concentration [16], it may in part be ascribable to mucosal addition since inhibition of the S.C.C. in response to mucosal addition of succinate, and also of alpha-ketoglutarate and propionate, has been observed [14] (*also*, J. H. Hess, A. Taylor & R. H. Maffly, *in preparation*). These observations serve to emphasize that in studies with the toad bladder the response to a specific substrate may also vary according to the surface(s) of the bladder to which the substrate is added.

The finding that alpha-ketoglutarate, succinate and fumarate can stimulate the S.C.C. under appropriate experimental conditions supports the conclusion that active sodium transport in the toad bladder is not linked to any specific step of the TCA cycle. Furthermore, the finding that succinate can act synergistically with exogenous aldosterone in the stimulation of sodium transport indicates that, contrary to the postulate of Edelman and co-workers [3, 4, 6, 7], the steps in the TCA cycle prior to the formation of succinate do not play a unique role in the regulatory action of aldosterone. Falchuk and Sharp [5] have drawn similar conclusions on the basis of studies with propionate and malonate. It remains possible that sodium transport is *preferentially* linked to NADH oxidation as proposed by Edelman and co-workers [3, 4, 6], or to formation of ATP at Site I in the electron transport chain [16], and evidence consistent with these possibilities has been presented.

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