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EFFECTS OF pH ON β-HYDROXYBUTYRATE TRANSPORT IN RAT ERYTHROCYTES AND THYMOCYTES

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Summary

Entry of β-hydroxybutyrate into erythrocytes and thymocytes is facilitated by a carrier (C), as judged from temperature dependence, saturation kinetics, stereospecificity, competition with lactate and pyruvate, and inhibition by moderate concentrations of methylisobutylxanthine, phloretin, or α-cyanocinnamate. We studied the dependence of influx and efflux on internal and external pH and [β-hydroxybutyrate]. Lowering external pH from 8.0 to 7.3 to 6.6 enhanced influx into erythrocytes by lowering entry K_m from 29 to 16 to 10 mM, entry V being independent of external pH. Lowering external pH inhibited efflux. At low external pH, external β-hydroxybutyrate enhanced efflux slightly. At high external pH, external β-hydroxybutyrate inhibited efflux. Internal acidification inhibited influx and internal alkalization enhanced influx. Internal β-hydroxybutyrate (βHB) enhanced influx more in acidified than alkalized cells. These data are compatible with coupled $\beta HB^-/OH^-$ exchange, βHB^- and OH^- competing for influx, $C : OH^-$ moving faster than $C : \beta HB^-$, empty C being immobile. They are also compatible with coupled $\beta HB^-/H^+$ copermeation, empty C moving inward faster than $H^+ : C : \beta HB^-$, $H^+ : C$ being immobile, and $C : \beta HB^-$ (without H^+) being so unstable as not to be formed in significant amounts (relative to C, $H^+ : C$, and $H^+ : C : \beta HB^-$).

Introduction

The transport of β-hydroxybutyrate across plasma membranes is facilitated by a carrier which may be the same one that transports lactate, pyruvate, and several other monocarboxylates [1—3]. In thymocytes and erythrocytes [1] β-hydroxybutyrate entry is highly temperature dependent, saturable, stereo-

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

specific, and inhibited by phloretin, methylisobutylxanthine, pyruvate and α-cyanocinnamate at modest concentrations. The equilibrium cell : medium β-hydroxybutyrate ratio suggests that entry is electrically silent and coupled to H^+ entry of OH^- exit. Studies of lactate transport in Ehrlich ascites tumor cells [3] and pyruvate transport in mitochondria [4] have led to the same conclusions, though studies of pyruvate transport in human erythrocytes [2] suggest coupling of monocarboxylate entry with Cl^- exit. The net result of H^+ or OH^- coupling is transfer of the permeant as an acid, which provides for a minimum disturbance of cellular pH, osmolarity and membrane potential in cells undergoing an enhanced production of lactate, pyruvate, β-hydroxybutyrate and/or acetoacetate. In order to gain further insight into the mechanism of monocarboxylate transport, we have studied the dependence of influx and efflux on pH and substrate concentration at the two sides of the membrane.

Methods

The procedures used in this work were essentially those described earlier [1]. For most influx experiments, 100-μl suspensions of erythrocytes (approx. 20%) or thymocytes (approx. 10%) were incubated in 7 × 50-mm flat-bottom vials without or with unlabelled DL-β-hydroxybutyrate. The medium was HEPES-buffered balanced salt solution [1]. After 30 min, the incubator was cooled to 10°C and various concentrations of D-β-hydroxy[^{14}C]butyrate added in 300—500 μl of medium. Samples were transferred to centrifuge tubes for separation of cells from medium. The separated cells were then prepared for counting.

For the efflux experiments, 50-μl suspensions were incubated at 20°C with D-β-hydroxy[^{14}C]butyrate or L-β-hydroxy[^{14}C]butyrate in pH 7.3 medium with 10 mM DL-β-hydroxybutyrate for 30 min, the incubator cooled to 10°C and efflux initiated by dilution with 1 ml medium. Samples were taken at appropriate times for analysis of cell radioactivity.

Results were expressed as β-hydroxy[^{14}C]butyrate in cells (per ml suspension) divided by total β-hydroxy[^{14}C]butyrate (in plus out, per ml suspension) and divided by packed cell volume (approximately the fraction of suspension occupied by cells). These were plotted against time (Figs. 3, 4 and 5). In uptake experiments, the slope of this plot was considered an approximation of the influx coefficient (Table I). The slope could be multiplied by the total D-β-hydroxybutyrate added (per ml suspension) to obtain influx rate as μmol fluxing into 1 ml of cells per min (Fig. 2).

Experiments to estimate the effects of external pH on β-hydroxybutyrate equilibrium were similar to the influx experiments except that labelled β-hydroxybutyrate was added in a small volume on the 100 μl cell suspension 20 min prior to addition of the same label concentration in 500 μl medium with altered pH.

Results

Effects of external pH on influx rates

If β-hydroxybutyrate entry is coupled to H^+ entry or OH^- exit, then influx

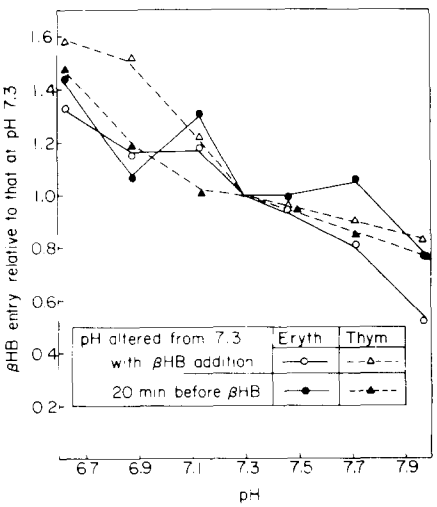

Fig. 1. pH dependence of β-hydroxybutyrate entry. In small counting vials incubating at 20°C, reaction mxitures consisted of 100 μl of cell suspension (17% erythrocytes or 8% thymocytes) at pH 7.3, 500 μl of medium (pH 6.5, 6.8, 7.1, 7.3, 7.5, 7.8, or 8.1) and 5 μl D-β-hydroxy[^{14}C]butyrate (0.1 μCi with 1 μmol unlabelled DL-β-hydroxybutyrate). In some cases, (,) uptake was initiated by addition of cell suspension to the other two components. In other cases (●,▲) the cell suspension and medium incubated 20 min prior to uptake initiation by addition β-hydroxy[^{14}C]butyrate. Erythrocyte samples (○,●) were taken 0.8 min after initiation, and thymocyte samples (△,▲) were taken 1.5 min after initiation. Since the titration curve of medium is rectilinear in this range, it was assumed (for purposes of plotting) that the pH would change from 7.3 five-sixths of the way towards that of the added medium. The resulting 1.3% thymocytes would not affect this relation, and the resulting 3% erythrocytes would not affect it seriously.

rates should be inversely related to pH (see Figs. 6 and 7). As seen in Fig. 1, this expectation was realized, influx being a decreasing function of pH throughout the range tested. The dependence was similar in the two cell types. In one series the pH was altered from 7.3 at the moment of β-hydroxybutyrate addition, and in another series the pH was altered 20 min before β-hydroxybutyrate addition. The dependence may have been slightly less pronounced with the latter procedure, though no convincing difference was seen.

Effects of external pH on influx kinetics

Fig. 2 shows Hanes plots of β-hydroxybutyrate entry into erythrocytes at three separate pH values. In a plot of S/v (i.e., influx resistance) vs. S (external concentration), the slope is $1/V$ and resistance-doubling concentration is K_m. A higher S/v at a given S is a slower rate. Thus, influx was inversely related to external pH as expected from Fig. 1. The pH dependence was not attributable to altered V, the latter being as great at pH 8.0 as at pH 6.6. The influx K_m values, on the other hand, clearly increased with increasing pH, accounting entirely for the reduced influx. This is analogous to the observations of Spencer and Lehninger [3] that increasing pH increases the K_m but does not affect the V of lactate entry into Ehrlich ascites tumor cells.

We have studied β-hydroxybutyrate entry kinetics in thymocytes at these external pHs. The results were similar to those of Fig. 2, entry K_m increased with increasing pH, but entry V was not affected by pH. The influx-kinetics

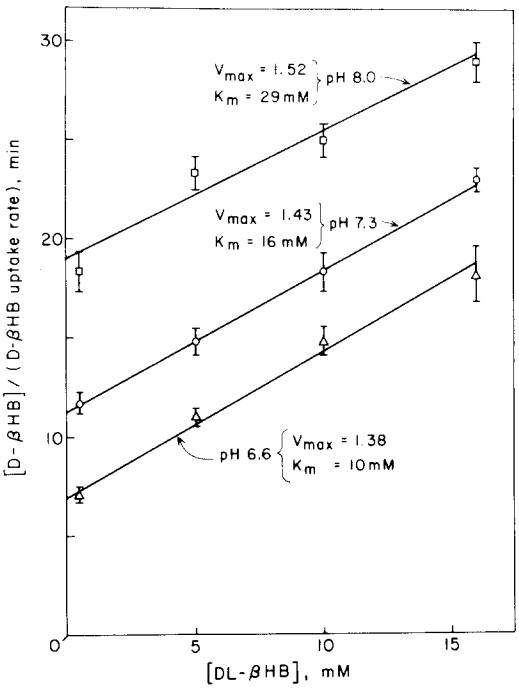

Fig. 2. Effect of external pH on β-hydroxybutyrate entry kinetics. After a preliminary incubation at 20°C, 100-μl erythrocyte suspensions were cooled to 10°C. Uptake was initiated by addition of 300 μl buffered balanced salt solution containing 0.03 μCi D-β-hydroxy[^{14}C]butyrate and DL-β-hydroxybutyrate sufficient to achieve the final concentrations shown. Where indicated the 300 μl was at pH 8.3, 7.3 or 6.3 to achieve final pH values of 8.0, 7.3 and 6.6. Incubations lasted for 0.2, 1, 2 and 3 min, at which times 300-μl samples were taken. Rates were calculated as described in Methods. The ordinate values were calculated as D-β-hydroxybutyrate concentration divided by corresponding rates. The experiment was carried out three times, and in no case did the V show a pH dependence. Average data with standard errors are shown.

experiments were also carried out in erythrocytes and thymocytes previously equilibrated with unlabelled β-hydroxybutyrate at the concentrations of labelled β-hydroxybutyrate to be added for the influx measurements. Again, influx K_m increased with increasing external pH, but influx V was not affected by pH. As will be seen in the discussion, an inverse relation between influx V and external pH would have been an interesting finding. We have not observed it.

Effects of external pH and external β-hydroxybutyrate on efflux

In Fig. 3, we plotted the fraction of ^{14}C in cells divided by packed cell volume against time. In those suspensions with rapid efflux, the bending is partly due to the fact that the data are approaching equilibrium values that are around 0.3—0.7. The lines would be straighter had we plotted the difference between the data and their eventual equilibria, and the nearly mono-exponential character of efflux would have been more apparent. We chose not to do this for the following reasons: (a) We did not know the equilibrium being approached under all of these conditions. (b) We did not know that the equilibrium being approached (influx coefficient/efflux coefficient) would be

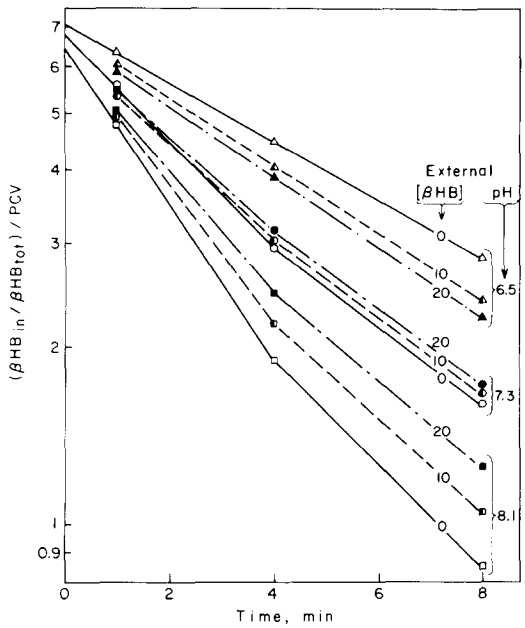

Fig. 3. Effect of external pH and external β-hydroxybutyrate on β-hydroxybutyrate efflux. After equilibration with 10 mM D-β-hydroxy[^{14}C]butyrate in medium at pH 7.3, 50-μl erythrocyte suspensions were cooled to 10°C. Efflux was initiated by addition of 1 ml buffered balanced salt solution at the pH values shown and containing the DL-β-hydroxybutyrate concentrations shown. At the indicated times, 300-μl samples were taken. Average data from three experiments are shown. All of the trends (including the very small inhibition by 10 mM β-hydroxybutyrate at pH 7.3) were so consistent as to be statistically significant with $P < 0.05$. PCV, packed cell volume.

constant with time, in view of the acid movements taking place which might alter cell pH with time. (c) Our conclusions are related to efflux rates or efflux coefficients, which are best appreciated from the early slopes of the plots as presented. Efflux was very sensitive to external pH. In the absence of external β-hydroxybutyrate, an increase in external pH from 6.5 to 8.1 enhanced efflux 3-fold.

It should be emphasized that the effect of external pH on efflux was opposite to its effect on influx, proving that H^+ or OH^- interacts with β-hydroxybutyrate stoichiometrically in a coupled movement, rather than catalytically (e.g. optimizing active site configuration or mobility but not being delivered across membrane). A catalytic action would affect influx and efflux in parallel.

The effects of external β-hydroxybutyrate on efflux depended on the external pH. At low external pH, external β-hydroxbutyrate enhanced efflux modestly but consistently ($P = 0.003$); however, at high external pH, external β-hydroxybutyrate slowed efflux ($P = 0.037$).

Effects of external pH on equilibrium β-hydroxybutyrate space

In Fig. 4, external pH was altered after β-hydroxybutyrate had largely equilibrated in erythrocytes and thymocytes. The abrupt changes in the time courses show that external acidification increased equilibrium β-hydroxy-

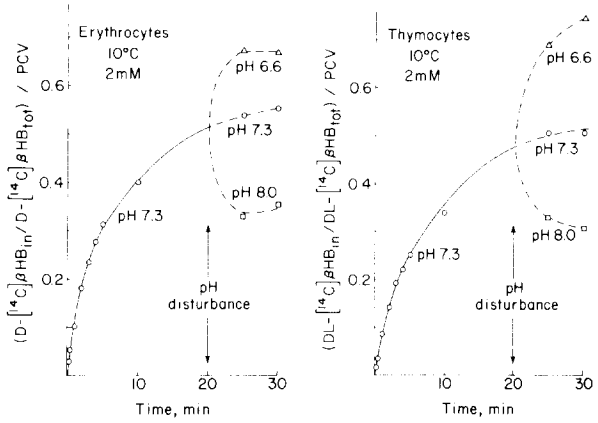

Fig. 4. Effect of external pH on β-hydroxybutyrate equilibrium space. Uptake was initiated by addition of 3 μl D-β-hydroxy[^{14}C]butyrate (0.02 μCi with 0.2 μmol DL-β-hydroxybutyrate) to 100-μl erythrocyte suspensions or by addition of 3 μl DL-β-hydroxy[^{14}C]butyrate (0.02 μCi with 0.2 μmol DL-β-hydroxybutyrate) to 100-μl thymocyte suspensions at 10°C. Individual suspensions were sampled at intervals up to 10 min. At 20 min remaining suspensions were diluted with 500 μl of medium containing 15 μl of the corresponding substrate solution. The diluting medium was at pH 6.5, 7.3 or 8.1, resulting in the approximate external pH values indicated. Samples were taken at 25 and 30 min. PCV, packed cell volume.

butyrate space and alkalization lowered it. These effects appeared more pronounced in thymocytes than erythrocytes, despite the fact that a portion of the D component of the racemic substrate in thymocytes may have been incorporated into slow-turnover metabolities [1]. Although this figure shows nothing which could not be deduced from the influx measurements of Figs. 1 and 2 together with the efflux measurements of Figs. 3 and 5, it does provide a simple demonstration of work being done on the β-hydroxybutyrate diffusion potential as a result of external pH changes. It is reasonable to suppose that the energy source for this work was the altered OH^- diffusion potential or the altered H^+ diffusion potential. The transfer of energy between two diffusion potentials implies coupled movements of the chemical species. The energy source must be dissipated stoichiometrically in doing work on the energy recipient. These thermodynamic considerations are compatible with a variety of coupling mechanisms including those proposed in Figs. 6 and 7.

Effects of internal pH and internal β-hydroxybutyrate on influx

Addition of neutral NH_4Cl to the medium should alkalize cells owing to rapid entry of NH_3. Likewise, addition of neutral $NaHCO_3$ should acidify cells owing to rapid entry of CO_2. As seen in Table I, NH_4Cl enhanced and $NaHCO_3$ reduced influx into thymocytes dramatically, such that the rate with 30 mM NH_4Cl was five times that with 30 mM $NaHCO_3$. These effects were hardly discernible in erythrocytes, owing perhaps to their superior buffering power but more likely to their rapid Cl^-/HCO_3^- exchange. The latter would largely prevent cellular acidification by $NaHCO_3$, since it would allow HCO_3^- to equilibrate almost as quickly as CO_2. It would largely prevent alkalization by NH_4Cl, since it would allow HCO_3^- formed from the excess OH^- to escape,

TABLE I

EFFECT OF INTERNAL pH AND INTERNAL β-HYDROXYBUTYRATE ON INFLUX

In small vials, 100-μl suspensions of erythrocytes (20%) or thymocytes (10%) were incubated at 30°C without and with 16 mM DL-β-hydroxybutyrate (entry or exchange, respectively). The ssuspensions were cooled to 10°C and influx initiated by addition of 300 μl buffered balanced salt solution containing 0.04 μCi D-β-hydroxy[^{14}C]butyrate (βHB*) and sufficient DL-β-hydroxybutyrate to achieve a final concentration of 16 mM. Where indicated, a portion of the NaCl of this 300 μl was replaced by NH_4Cl or $NaCHO_3$ (neutralized with NH_4OH or CO_2, respectively) to achieve the final concentrations shown. The $NaHCO_3$ solution was prepared immediately before use and external pH drifted up only 0.07 unit during the experiment. Incubations lasted for 0.2, 1, 2, and 3 min at which times 300-μl samples were taken. The initial slopes of the time courses which lasted 2 min are shown (see Figs. 1 and 3 of ref. 1 and Fig. 4 of this paper). The experiment was carried out twice with entirely concordant results, the averages of which are shown.

Cell type conditions	Influx coefficient (min^{-1}) = $\Delta((\beta HB^*_{in}/\beta HB^*_{tot})/\text{packed cell volume})/\Delta t$	
	β-Hydroxybutyrate entry	β-Hydroxybutyrate exchange
Erythrocytes		
NH_4Cl, 30 mM	0.053	0.068
NH_4Cl, 15 mM	0.048	0.070
Control	0.046	0.065
$NaHCO_3$, 15 mM	0.045	0.063
$NaHCO_3$, 30 mM	0.043	0.056
Thymocytes		
NH_4Cl, 30 mM	0.065	0.070
NH_4Cl, 15 mM	0.045	0.055
Control	0.023	0.036
$NaHCO_3$, 15 mM	0.018	0.031
$NaHCO_3$, 30 mM	0.013	0.028

effectively removing the excess OH^- *. In any case, the effects of internal pH on influx were directionally like the effects of external pH on efflux, indicating a degree of symmetry in the mechanism. Based on the rationale of the experiment, the above interpretation accounts for the effects of NH_4Cl and $NaHCO_3$ in terms of the expected changes in intracellular pH, which were confirmed by dimethyloxazolidinedione distributions (see first-mentioned footnote). We have not specifically ruled out direct stimulation of the carrier by NH_4^+ and direct inhibition by HCO_3^-. However, the use of two agents to alter cellular pH does provide some redundancy, and it would seem to be a perverse coincidence for both agents to exert direct effects in the same directions as their presumed pH-mediated effects and to do this best in the cell type most susceptible to the internal pH changes.

Internal β-hydroxybutyrate typically enhanced influx. The effect was slight in strongly alkalized thymocytes and appeared to be exaggerated in strongly

* Favoring this view is the fact that the cell : medium 5,5-dimethyloxazolidine-2,4-dione ratio (which approaches the medium : cell H^+ ratio and cell : medium OH^- ratio [5]) of thymocytes rose 73% 3 min after addition of 15 mM neutral NH_4Cl and fell 70% 3 min after addition of 15 mM neutral $NaHCO_3$. In erythrocytes, the same procedures increased the ratio 0% and decreased it 40%, respectively. In erythrocytes with Cl^-/HCO_3^- exchange inhibited by 3 mM 4-acetamido-4-isothiocyanostilbene-2,2'-disulfonic acid [6], these procedures increased the ratio 10% and decreased it 74%, respectively.

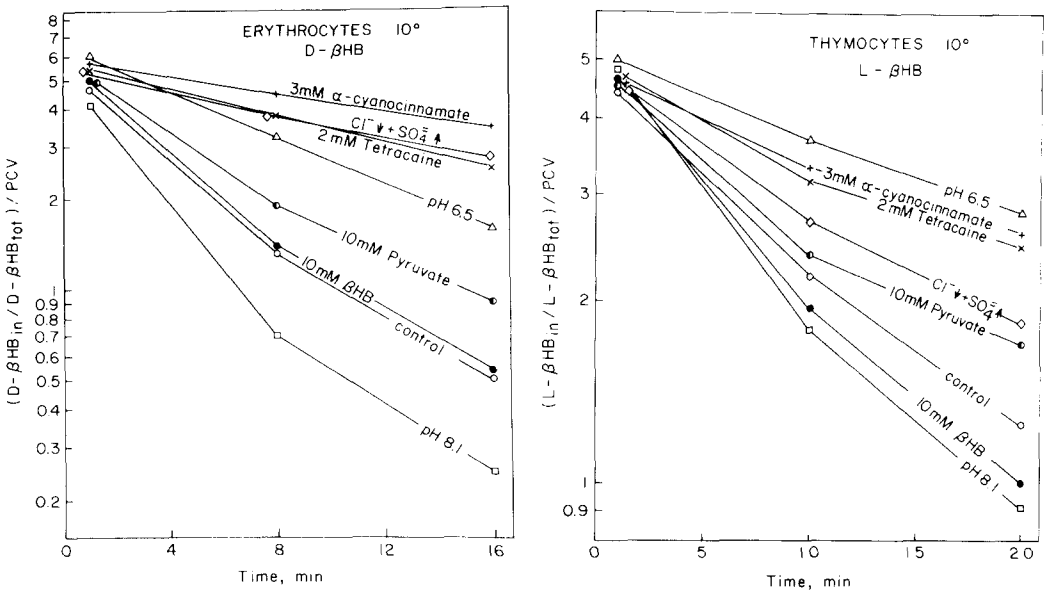

Fig. 5. Effects of external conditions on β-hydroxybutyrate efflux from (a) erythrocytes and (b) thymocytes. After equilibration of erythrocytes with D-β-hydroxy[^{14}C]butyrate and thymocytes with L-β-hydroxy[^{14}C]butyrate, 50-μl suspensions were cooled to 10°C. Efflux was initiated by addition of 1 ml buffered balanced salt solution altered as shown. For the set labelled Cl^- ↓ + SO_4^{2-} ↑, all the NaCl was replaced by isotonic Na_2SO_4. At the indicated times, 300-μl samples were taken. Average data from three experiments are shown. All effects were seen in each experiment. PCV, packed cell volume.

acidified thymocytes. While this corresponds generally to the efflux data of Fig. 3, it is not clear from Table I that there is a degree of cell alkalinity where internal β-hydroxybutyrate would become inhibitory.

Effects of other conditions on efflux

Fig. 5 shows the effects of external pH, substrates, anions and inhibitors on β-hydroxybutyrate efflux from erythrocytes and thymocytes. The L isomer was used in thymocytes because this cell would metabolize some of its D-β-hydroxybutyrate before and during the efflux studies [1]. The effects of external pH on β-hydroxybutyrate efflux from thymocytes were similar to those seen with erythrocytes. The inhibitory actions of α-cyanocinnamate and tetracaine were also similar in the two cells. Pyruvate was inhibitory in both cell types. This was likely a competitive effect exerted by pyruvate after its entry into cells. External β-hydroxybutyrate did not affect efflux from erythrocytes (as already seen at pH 7.3); but it consistently enhanced efflux from thymocytes, much as it did in erythrocytes in a pH 6.5 medium. Substitution of SO_4^{2-} for most of the Cl^- inhibited efflux from erythrocytes more than that from thymocytes *.

* Substitution of SO_4^{2-} for most of the medium Cl^- increased the cell : medium 5,5-dimethyloxazolidine-2,4-dione ratio of erythrocytes by 60%. Substitution of citrate for Cl^- increased it 170%. Thus, the OH^- ratio tended to follow the Cl^- ratio in this cell as expected. These manipulations may have alkalized thymocytes also, but effects were neither large nor consistent.

Discussion

As mentioned in the introduction, several properties of β-hydroxybutyrate entry into erythrocytes and thymocytes indicate carrier-facilitated transport [1]. The equilibrium β-hydroxybutyrate space of thymocytes resembles more the dimethyloxazolidinedione space than the Cl^- space. From 18 measurements, the thiocyanate space of erythrocytes at 10°C in normal medium was 0.47 ± 0.02 and that of thymocytes was 0.14 ± 0.01. This anion equilibrated by 3 min and can be assumed to reflect membrane potential according to the Nernst equation. The similarity between thiocyanate space and Cl^- space [1] indicates that Cl^- also achieves electrochemical equilibrium, even in thymocytes where its movements are slow. The similarity between β-hydroxybutyrate and dimethyloxazolidinedione spaces, both of which are much higher than Cl^- and thiocyanate spaces in thymocytes, remains our best direct evidence for coupling of β-hydroxybutyrate entry with OH^- exit or with H^+ entry and against electrogenic β-hydroxybutyrate entry or β-hydroxybutyrate/Cl^- exchange. Other evidence for carrier coupled β-hydroxybutyrate/OH^- exchange or β-hydroxybutyrate/H^+ copermeation comes from the fact that the dependence of influx and efflux on *cis* and *trans* pH and β-hydroxybutyrate concentrations are all explicable in terms of the models of Figs. 6 and 7 (as will be seen below).

Inconsistencies would arise in attempting to explain the data in terms of electrogenic β-hydroxybutyrate anion transport, pH effects being mediated by their effects on membrane electrical potential. While we have found external pH to affect thiocyanate space of erythrocytes much as it affects equilibrium β-hydroxybutyrate space, external alkalization did not lower thiocyanate space of thymocytes and may have increased it. Hyperpolarization would, therefore, be an unlikely explanation for the effects of external alkalization to lower equilibrium β-hydroxybutyrate space, slow influx and enhance efflux. Likewise, inconsistencies would arise in attempting to explain the data in terms of a β-hydroxybutyrate/Cl^- exchange mechanism, pH effects being mediated by changes in cellular Cl^-. External acidification quickly increases erythrocyte Cl^- owing to the Cl^-/HCO_3^- carrier, and the increased cellular Cl^- would enhance β-hydroxybutyrate influx (as an exchange partner) and reduce β-hydroxybutyrate efflux (by competition). However, the influx enhancement would be a V enhancement, not the K_m reduction observed. Thus, with a large concentration of external substrate but a low concentration of internal exchange partner, entry V would be low owing to sequestration of carrier on the inside. Increasing internal exchange partner would reduce this sequestration, i.e., would allow faster delivery of carrier back to the outside, thereby increasing influx V. Moreover, cellular Cl^- levels could not mediate the pH effects on β-hydroxybutyrate fluxes in thymocytes. In this cell, Cl^- movements are slow (30 min half-time), and at 10°C cellular Cl^- levels are little affected by external and internal pH for at least 30 min (data not shown). For all of the above reasons, we favor either the β-hydroxybutyrate/OH^- exchange mechanism of Fig. 6 or the β-hydroxybutyrate/H^+ copermeation mechanism of Fig. 7. Either provides a unitary explanation of the data.

The OH^- exchange model is the simpler of the two. Tight coupling is

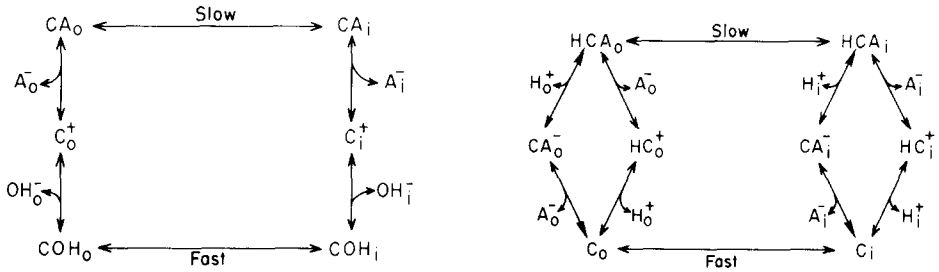

Fig. 6. Essential events of β-hydroxybutyrate/OH^- exchange. Tight coupling [3] requires that the empty carrier, C^+, be immobile. External monocarboxylate, A_o^-, competes with external hydroxyl, OH_o^-, for influx.

Fig. 7. Essential events of β-hydroxybutyrate/H^+ copermeation. Tight coupling requires that singly loaded carrier (HC^+ and CA^-) be immobile or so unstable as not to be formed in significant amounts.

achieved by immobility of free carrier, C^+. According to this model external OH^- and β-hydroxybutyrate compete with each other for external free carrier, hence for influx. The model, therefore, predicts that a rise in external pH will increase the influx K_m but not affect the influx V, as observed. The effects of external pH on efflux (in the absence of external substrate) are also predicted by this model. Without A_o^- the carrier needs OH_o^- in order to return to the inside. Therefore, efflux should be an increasing function of external pH, as observed. At low external pH (low OH_o^-) the carrier would tend to be trapped at the external surface (as the immobile C_o^+), and addition of external substrate would allow the carrier's return to the inside and enhance efflux, as observed. The enhancement by 20 mM external β-hydroxybutyrate was modest compared with that by external alkalinity, despite the fact that 20 mM β-hydroxybutyrate is twice the K_m (external carrier 2/3 occupied). Thus, CA_o would seem to move in slower than COH_o. The enhancement of efflux by external substrate would fade at higher pH where OH^- would allow the carrier's return to the inside. At high external pH, external substrate actually inhibited efflux, again showing that CA_o moves in slower than COH_o. We would, therefore, conclude that the cis and trans interactions of pH and β-hydroxybutyrate are compatible with the OH^- exchange model in which CA movements are slower than COH movements.

The monocarboxylate/H^+ copermeation model is also viable with certain restrictions. In this case, tight coupling is achieved by immobility of single-loaded carrier. According to this model, external acidity would promote influx, since H_o^+ is needed for the mobile HCA_o complex. If the CA_o^- dissociation constant were not too high relative to the A_o^- concentrations studied, then substantial quantity of the immobile CA_o^- complex would be formed under conditions where influx was reduced by alkalinity. Acidification, by increasing the ratio of mobile HCA_o to immobile CA_o^-, would enhance the influx V. Such an effect would strongly favor this model over the OH^- exchange model. Failing to see this V dependence we cannot argue for H^+ copermeation as against OH^- exchange. The lack of V dependence does not rule out the H^+ copermeation model but does argue against formation of significant amounts of

CA_0^- (relative to HCA_0) even in alkaline medium. This is the basis for the large arrow heads leading from the CA^- complex. With this in mind, the large reduction of influx K_m with external acidification can be viewed in terms of the need for H_0^+ in the formation of relatively stable (and mobile) HCA_0, by the HC_0^+ pathway or the CA_0^- pathway or both pathways. According to the H^+ copermeation mechanism, the inhibition of efflux by external acidification (in the absence of A_0^-) is due to the formation of HC_0^+ which is immobile, hence 'sequestered' on the outside at the expense of other forms including internal carrier. The sequestration can be reversed by external substrate, which converts immobile HC_0^+ to mobile HCA_0. This reversal can occur to an extent dependent on the mobility of HCA_0. Since 2/3 saturation of external carrier enhanced efflux modestly compared to reduced acidity (pH 8.1), we conclude that HCA_0 is considerably less mobile than C_0. This is confirmed by the fact that efflux into alkaline medium was slowed by addition of external substrate. We would, therefore, conclude that the *cis* and *trans* interactions of pH and β-hydroxybutyrate are compatible with the H^+ copermeation model in which the CA^- complex is highly unstable and HCA moves slower than C.

From the above discussion, it is seen that our views agree with those of Spencer and Lehninger [3] based on studies of lactate transport in Ehrlich ascites tumor cells. They differ from those of Halestrap [2] based on studies of lactate and pyruvate transport in human erythrocytes. He proposed two mechanisms: (1) monocarboxylate/monocarboxylate exchange on a carrier with relatively high affinity for monocarboxylate but incapable of catalyzing net monocarboxylate transport, and (2) low-affinity, high-capacity exchange of monocarboxylate for Cl^- or HCO_3^- on the Cl^-/HCO_3^- exchange carrier. With the substrate concentrations we used, the latter mechanism did not contribute significantly to β-hydroxybutyrate transport in rat erythrocytes; a second pathway of low affinity would lead to a biphasic or bent Hanes plot, which we did not see (Fig. 2). Were the high-affinity mechanism (which is responsible for β-hydroxybutyrate transfer at these concentrations) a monocarboxylate/monocarboxylate exchange carrier, then efflux would depend on external substrate exactly as influx depends on external substrate. In the present studies, efflux was not highly dependent on external β-hydroxybutyrate under any circumstance; and efflux from erythrocytes was actually inhibited by external substrate in normal and alkaline medium. We would, therefore, rule out a monocarboxylate/monocarboxylate exchanger in rat erythrocytes. We did confirm one of the observations which led Halestrap to suspect a monocarboxylate/monocarboxylate exchanger, that monocarboxylate is virtually trapped in erythrocytes suspended in a medium containing a poorly permeable anion in place of Cl^- (Fig. 5a). This, however, can be explained in terms of the net acid transfer mechanisms proposed here and elsewhere [3]. The high cell : medium Cl^- ratio should induce a high cell : medium OH^- ratio, owing to the Cl^-/HCO_3^- exchange carrier (see second-mentioned footnote). The cell alkalinity would then interfere with exit just as external alkalinity interferes with entry (Figs. 1 and 2). The cell alkalinity would also promote influx (Table I and analogy with Fig. 3), accounting for the ability of low-Cl^- medium to enhance monocarboxylate influx [1,2]. The cell alkalinity, hence the inhibited efflux, would be relieved by addition of any permeant which enters as an acid (including

monocarboxylates), providing an explanation for the second observation which led Halestrap [2] to postulate a monocarboxylate/monocarboxylate exchange carrier. In view of these explanations, it seems that the high-affinity mechanism in human erythrocytes may also be a net acid transfer carrier.

It is interesting to consider the homeostatic consequences of the mechanisms proposed here and elsewhere [1,3]. Since *trans*-to-*cis* movement of OH^- is equivalent to *cis*-to-*trans* movement of H^+, either mechanisms automatically moves the positive and negative ionic moieties of acidic fuels (such as lactate and β-hydroxybutyrate) together. Moreover, when a cell suddenly doubles its lactic acid production, the efflux is enhanced owing both to the rise in cell lactate and to the fall in cell pH. This synergism can result in a doubling of lactate efflux with a less-than-doubling of cell lactic acid, i.e., with a modest degree of cell acidification and modest increase in cell osmolarity. The transport mechanism, then, provides at least a partial explanation for the fact that lactic acid output of the perfused heart can increase 13-fold with anoxia and 10-fold with mild ischemia while cell lactate rises less than 3-fold [7]. Finally, the enhancement of production and exit do not themselves alter membrane potential. If the proton and lactate anion produced in glycolysis were to leave by separate paths, each being linearly or hyperbolically dependent on concentration, then cell lactic acid would at least have to double in order for efflux to double. Moreover, the separate paths would disturb membrane potentials or ion distributions or both, since they would either be electrogenic (but not coordinated) or be coupled to electroneutralizing movements of ions. Thus, a coupled monocarboxylate/Cl^- exchange (leaving H^+ movements electrogenic or coupled to ion movements) would seem less satisfactory for excitable cells than transfer of monocarboxylic acid.

The inhibition of efflux by extracellular acidity may be disadvantageous under certain circumstances. This property likely accounts for the reduced lactate efflux coefficients in the severely ischemic as compared to mildly ischemic heart [7], which contribute to the extremely high cell lactic acid concentrations. Perhaps capillary acidosis during and following cerebral anoxia is partly responsible for the reduced lactate efflux coefficients observed under these conditions, resulting in continual lactate accumulation without increased efflux during the anoxic period and very slow reduction of cerebral lactic acid during the recovery period [8].

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References

1 Andersen, B., Tarpley, H. and Regen, D.M. (1977) Biochim. Biophys. Acta 508, 525–538
2 Halestrap, A.P. (1976) Biochem. J. 156, 193–207
3 Spencer, T.L. and Lehninger, A.L. (1976) Biochem. J. 154, 405–414
4 Halestrap, A.P. (1975) Biochem. J. 148, 85–96
5 Roos, A. (1975) J. Physiol. 249, 1–25
6 Rothstein, A., Cabantchik, Z.I. and Knauf, P. (1976) Fed. Proc. 35, 3–10
7 Rovetto, M.J., Whitmer, J.T. and Neely, J.R. (1973) Circ. Res. 32, 699–711
8 Drewes, L.R. and Gilboe, D.D. (1973) J. Biol. Chem. 218, 2489–2496