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EFFECTS OF pH ON β-HYDROXYBUTYRATE EXCHANGE KINETICS OF RAT ERYTHROCYTES

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Summary

The influx of β-hydroxybutyrate into rat erythrocytes at 0°C was measured in the presence of various substrate concentrations and pH values at equilibrium. Under these circumstances, influx measures equilibrium exchange. Acidification (pH 6.3 compared to pH 7.3 and pH 8.3) increased exchange V markedly, increased exchange K_m and increased exchange V/K_m moderately. The data indicate that the base form of the carrier (either lacking H^+ or bearing OH^-) binds β-hydroxybutyrate more tightly than the acid form (either bearing H^+ or lacking OH^-) and that the β-hydroxybutyrate-bearing base form is relatively or completely immobile. If these pH effects reflect titration of the transport-partner site, then the findings favor an A^-/H^+-symport mechanism; for, with this mechanism, a β-hydroxybutyrate-bearing base form of the carrier is expected. The findings do not favor an A^-/OH^--antiport mechanism; for, with this mechanism, a β-hydroxybutyrate-bearing base form is not expected, and OH^- should compete with β-hydroxybutyrate for binding.

Introduction

Studies of pyruvate transport in mitochondria [1], lactate transport in Ehrlich ascites tumor cells [2] and β-hydroxybutyrate transport in erythrocytes and thymocytes [3] have provided evidence for a carrier which catalyzes either monocarboxylate/proton cotransport (A^-/H^+ symport) or monocarboxylate/hydroxyl exchange (A^-/OH^- antiport). It has not been possible to distinguish

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between these two mechanisms [1—3], since: (a) $[H^+]$ and $[OH^-]$ change reciprocally with pH manipulation, and (b) each step in a symport model can be matched by a kinetically equivalent step in an antiport model and vice versa (H^+ association being equivalent to OH^- dissociation). However, some of the ligand combinations which must be postulated for this step-by-step equivalence seem implausible, and we have argued that the two mechanisms should not be kinetically equivalent [3]. Kinetic studies thus far published have indicated that external acidification enhances monocarboxylate entry purely by lowering the monocarboxylate entry K_m, not by increasing the entry V [2,3]. This appeared to be readily accommodated by the antiport hypothesis (where external monocarboxylate and external hydroxyl should be competitive) and to be compatible with symport only if monocarboxylate binding were totally dependent on proton binding [3]. In the present studies of equilibrium exchange at 0° C, acidification enhanced both exchange V and exchange K_m. These effects may provide insights into the transport-coupling mechanism.

Methods

The procedures used in this work were essentially those described earlier [4]. Rat erythrocytes were prepared by defibrination, filtration through nylon net, and centrifugation through 7% Ficoll (in incubation medium at pH 7.3) followed by resuspension in incubation medium at pH 7.3. The cell suspension was then divided into portions each of which was centrifuged through Ficoll in medium buffered at the pH of the eventual experiment, and the cells were suspended in medium buffered at this pH. The above steps were at room temperature.

For each transport test, 150 μl of cell suspension was added to 350 μl of medium containing an amount of unlabelled DL-β-hydroxybutyrate sufficient to give the final concentration stated. This was incubated 20 min at 37°C in a shaking water bath, then transferred to another shaking water bath containing ice slush. The influx measurement was initiated by addition of 5 μl of D-β-hydroxy[3-^{14}C]butyrate (0.5 μCi at 0.13 mCi/mg, New England Nuclear). Four 100-μl samples were taken over the next several minutes.

The samples were squirted into 5 ml ice cold medium (containing 0.3 mM phloretin, 0.7 mM methylisobutylxanthine and 1.0 mM α-cyano-3-hydroxycinnamate) layered over 9 ml medium made dense with sucrose [4]. The separation of cells from medium, extraction and counting were as described previously [4].

Results were expressed as ^{14}C in cells (per ml suspension) divided by ^{14}C in the suspension (per ml suspension) and divided by packed cell volume (approximately the fraction of the suspension occupied by cells). The resulting expression is the volume of suspension containing the amount of β-hydroxy[^{14}C]-butyrate in a milliliter of packed cells, and its dimension is ml/cel, where 'cel' symbolizes ml of packed cells. These were plotted against time (Fig. 1). The initial slope of such a time course (in ml/min per cel) is essentially an influx coefficient, influx rate divided by concentration. The reciprocal of influx coefficient is influx resistance, which is the S/v plotted in Hanes plots.

Results

β-Hydroxybutyrate influx time courses

Fig. 1 shows β-hydroxybutyrate influx time courses at two pH values and four substrate concentrations. Under all conditions, labelled-substrate influx was essentially constant (rectilinear) for the periods of the first three samples. At each concentration, influx was faster at pH 6.3 than at pH 8.4. Saturation of the transport system by unlabelled substrate is expressed as an inhibition of labelled-substrate influx. The system is not readily saturated at pH 6.3.

Effects of pH on β-hydroxybutyrate exchange kinetics

Fig. 2 is a composite of eleven experiments comparing equilibrium exchange kinetics at various pH values. Panel a displays averages from two experiments in which tracer β-hydroxy[^{14}C]butyrate was added to suspensions pre-equilibrated with sodium β-hydroxybutyrate concentrations up to 20 mM. Exchange K_m and exchange V were much higher under acidic conditions than under alkaline conditions.

It was our intention in these experiments to have intracellular pH and substrate concentrations as near as possible to extracellular pH and substrate concentrations. Presumably the Cl^-/HCO_3^- exchange carrier achieves approximate pH equilibrium in a short period of time with a concomitant Cl^- shift. To encourage pH equilibration and reduce the Cl^- shift, the exchange experiment was also carried out in the presence of 20 mM and 40 mM ammonium acetate. As seen in panels b and c, this salt did not affect the result significantly. Acidification enhanced exchange V, exchange K_m and exchange V/K_m as it did in the absence of this salt. Apparently neither ammonium nor acetate interacts strongly with the monocarboxylate carrier.

In another series of experiments, ammonium β-hydroxybutyrate was used in place of sodium β-hydroxybutyrate to minimize the effect of substrate equilib-

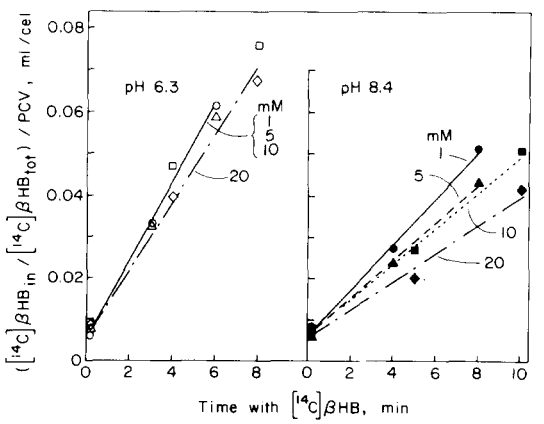

Fig. 1. β-Hydroxybutyrate (βHB) influx time courses. Erythrocytes were pre-equilibrated with 1 mM (○, ●), 5 mM (△, ▲), 10 mM (□, ■) and 20 mM (◇, ◆) unlabelled DL-β-hydroxybutyrate at pH 6.3 and pH 8.4 prior to addition of D-β-hydroxy[3-^{14}C]butyrate at zero min. PCV is packed cell volume, approximately the fraction of the system occupied by cells. The ordinate is proportional to the cell/medium concentration ratio or distribution space.

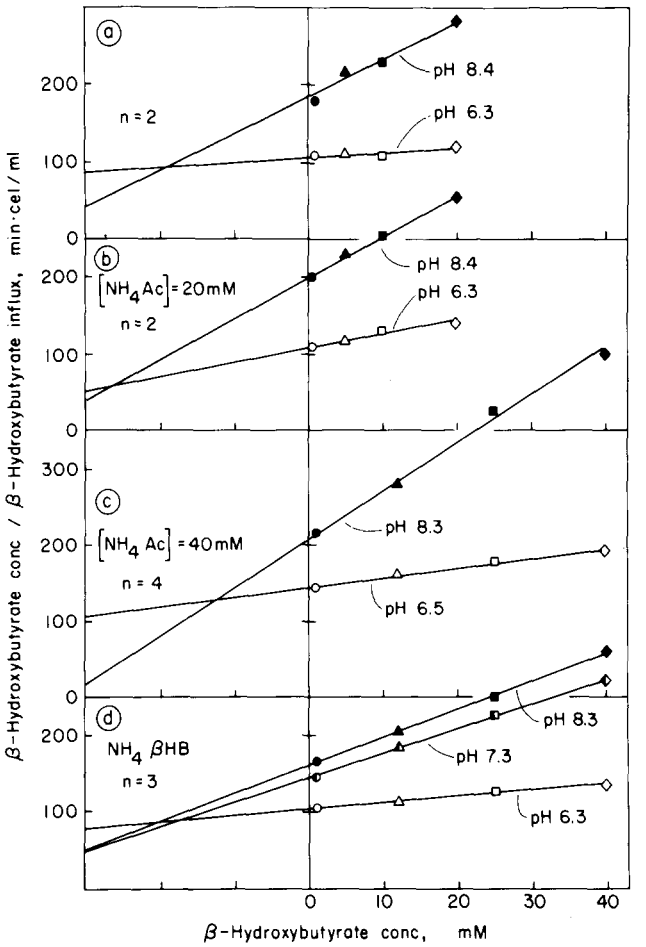

Fig. 2. Effects of pH on β-hydroxybutyrate exchange kinetics (S/v vs. S plots). The data points (S/v) are the reciprocals of time-course slopes such as shown in Fig. 1. The experiment was carried out with 20 mM and 40 mM ammonium acetate in the medium (panels b and c) and with ammonium DL-β-hydroxybutyrate as the independent variable (panel d). These salts were added over and above the inorganic salts of the medium. In these S/v vs. S plots, K_m is the x intercept or the concentration where S/v is twice the y intercept, and V is the reciprocal of slope.

ration on cell pH and Cl^- distribution. This series is shown in panel d, where each point is the average from three experiments. Again it is seen that acidification increased exchange V, exchange K_m and exchange V/K_m, as seen in the other panels. β-Hydroxybutyrate exchange was much more sensitive to a pH change in the acid range than in the alkaline range.

Effects of pH on inhibition by pyruvate

Fig. 3 shows two experiments wherein various sodium pyruvate concentrations were equilibrated at two pH values prior to addition of D-β-hydroxy[^{14}C]-butyrate with 0.1 mM DL-β-hydroxybutyrate. In one experiment, 30 mM ammonium acetate was present in all incubations and in the other experiment ammonium sulfate and sodium pyruvate were present at the same concentrations. With either of these designs, pyruvate inhibited with a K_i of about 15

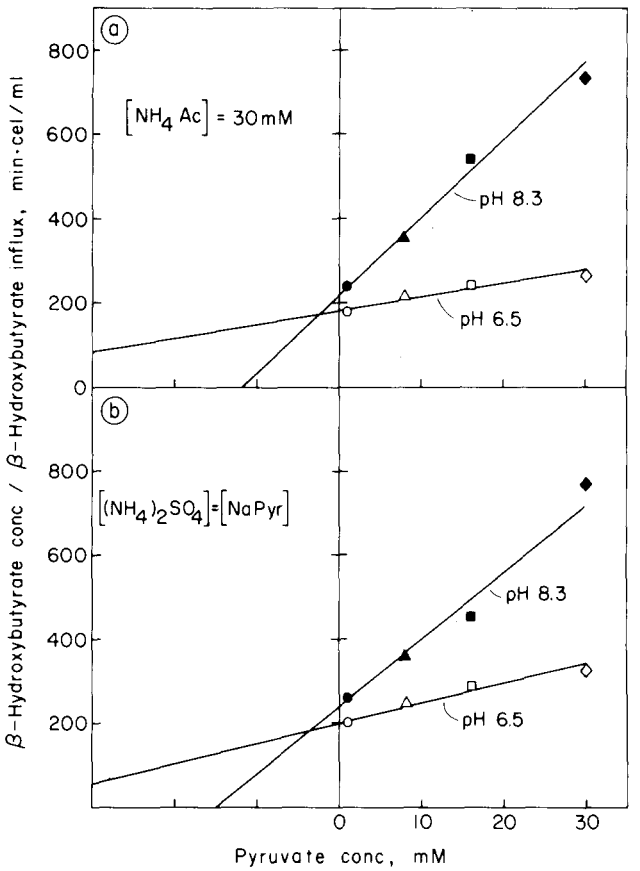

Fig. 3. Effects of pH on inhibition by pyruvate. These experiments were carried out like those of Fig. 2, except that sodium pyruvate concentration was the independent variable. In one series, ammonium acetate was present at 30 mM (panel a), and in the other series, ammonium sulfate was added in equimolar amounts with sodium pyruvate (panel b). In all cases, D-β-hydroxy[3-^{14}C]butyrate influx was measured.

mM under alkaline conditions and with K_i of about 50 mM under acidic conditions.

Discussion

Evidence from several laboratories [1—3] indicates that the monocarboxylate carrier catalyzes net acid transfer, either by A^-/H^+ symport of A^-/OH^- antiport. However, attempts to distinguish between these possibilities are frustrated by several experimental difficulties. The major difficulty is that $[H^+]$ and $[OH^-]$ cannot be altered independently. Moreover, it is difficult or impossible to manipulate extracellular pH without producing an intracellular pH change which is difficult to evaluate. Manipulations of external or internal pH may alter membrane potential and ion distribution, either of which might conveivably mediate an effect of the pH manipulation. One must be concerned with possible effects of pH, unrelated to the role of H^+ or OH^- as a transport partner. One must also be concerned with the effects of pH on substrate protona-

tion. Observations including the present ones, purporting to favor the symport over the antiport mechanism [5,6], are subject to objections and alternative interpretations. Despite this, we felt that the actual effects of pH on monocarboxylate exchange should be examined and considered in relation to this question.

Although it is impossible completely to rule out non-specific effects of pH on the monocarboxylate carrier, it is also true that all pH effects on monocarboxylate transport which have been observed can be accounted for in terms of a role of H^+ or OH^- as a transport partner (as depicted in Figs. 4 and 5). Monocarboxylate entry into cells alkalizes the medium stoichiometrically [2]. External acidification enhances monocarboxylate entry (net entry) and raises the equilibrium cell/medium monocarboxylate ratio [2,3]. External alkalization does the opposite [2,3]. Internal acidification slows monocarboxylate influx whereas internal alkalization speeds monocarboxylate influx [3]. External acidification slows monocarboxylate efflux whereas external alkalization speeds monocarboxylate efflux [3]. External β-hydroxybutyrate largely obscures the effects of external pH on efflux, and internal β-hydroxybutyrate reduces somewhat the effects of internal pH on influx [3]. Since the carrier protein is presumably asymmetrically oriented in the membrane, the symmetry of these effects indicates that the specific pH interactions (of the kinds depicted in Figs. 4 and 5) outweigh any suspected non-specific ones; for, one would not expect the same non-specific pH susceptibility at two different parts of the protein. Moreover, the *trans* effects, which are just as pronounced as the *cis* effects, argue that the pH interactions are with the carrier (as depicted in these models) rather than with the substrate. That the *trans* effects of pH are opposite to the *cis* effects (as expected from the models) also constitutes evidence that the detectable pH interactions are at the transport-partner site rather than elsewhere on the protein; for, a pH interaction elsewhere on the protein could not be vectorial and would affect influx catalysis and efflux catalysis equally.

In view of our failure to find evidence of non-specific pH interactions in experiments where such action might have prevailed, it is reasonable to consider what the effects of pH on equilibrium-exchange kinetics might mean if the interactions were at the transport-partner site rather than at nonspecific areas of the transport protein. In equilibrium exchange studies, the tendency of internal pH to mimic external pH simplifies rather than confounds the interpretation. The possible roles of altered ion distribution and membrane potentials as mediators of pH effects can be tested by use of salts which reduce the impact of pH equilibration on Cl^- distribution and membrane potential. Equilibrium-exchange studies have the further advantage that the conditions surrounding the carrier do not change during a flux measurement, as they do in net-transport studies. There is also a theoretical simplicity to equilibrium-exchange experiments, since there are no chemical potential differences among the various carrier forms and mobility of the monocarboxylate-free carrier base is not involved in equilibrium-exchange rates.

The effects of acidification to increase exchange V, exchange K_m and exchange V/K_m are in striking contrast to published effects of pH on monocarboxylate entry kinetics. All published studies including our own have shown external acidification to lower monocarboxylate entry K_m and not to increase

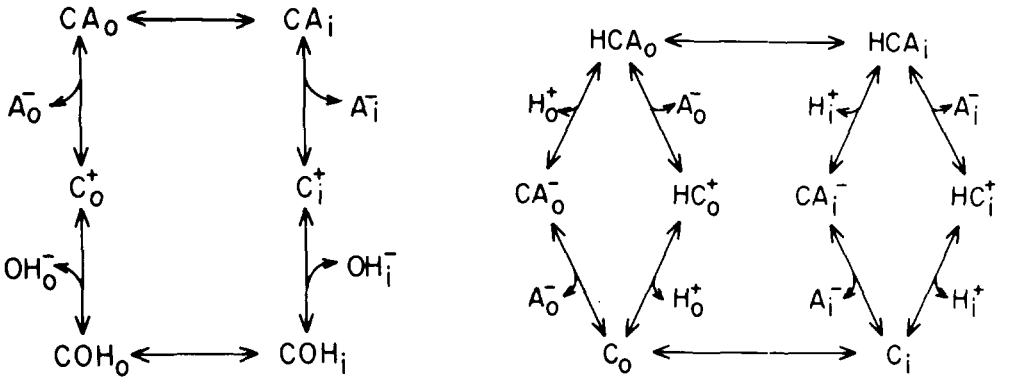

Fig. 4. Expected interactions of an A^-/OH^- antiporter. Monocarboxylate and OH^- are viewed as competing for the anion-binding site of the acid carrier. The COH form would have to be more mobile than the CA form to explain the effect of external β-hydroxybutyrate (βHB) to interfere with the efflux-enhancing action of external alkalization. Interactions of pH with the anion-binding site would not affect βHB entry V or exchange V.

Fig. 5. Expected interaction of an A^-/H^+ symporter. Both the monocarboxylate-loaded base carrier and the monocarboxylate-free acid carrier are viewed as immobile complexes. Stability of the CA^- complex will account for the effect of acidification to increase β-hydroxybutyrate (βHB) exchange V and exchange K_m, and it will account for the effect of external βHB to interfere with the efflux-enhancing action of external alkalization.

entry V. At least two features of an entry experiment may contribute to this result. With low substrate concentrations, external acidification sequesters the carrier in the out-facing orientation (according to either model, Fig. 4 or Fig. 5), so that virtually all the carrier is exposed to the substrate and is in a form which can translocate inward when a monocarboxylate binds. This tends to lower entry K_m. With high substrate concentrations, external sequestration due to external acidification no longer obtains, since the monocarboxylate-loaded acid carrier is mobile. In fact, with high substrate concentrations at low external pH, the carrier tends to be shifted to the in-facing orientation, owing to internal acidification incident to the rapid entry of the monocarboxylate with H^+ or in exchange for OH^-. This tends to lower entry V and obscure any effect of external acidification to enhance entry V. These complexities do not afflict an equilibrium exchange experiment, where one is observing the effects of pH on both sides on saturation of the carrier by monocarboxylate from both sides and on motion of monocarboxylate-loaded carrier in both directions.

The equilibrium-exchange results indicate that acidification increases the mobility of monocarboxylate-loaded carrier but reduces its stability, the dominant effect being the mobility increase such that exchange V/K_m is increased. Since stability effects are mutual, we can assume that monocarboxylate binding favors H^+ dissociation or OH^- binding. Antagonism between carrier acidification and monocarboxylate binding was not expected from the models of Figs. 4 and 5, where the charge in the mobile site of the acid carrier (capable of forming a mobile monocarboxylate complex) should favor monocarboxylate binding. The antagonistic binding interaction must, then, be conformationally mediated. We cannot say from the exchange data whether the pH sensitive site involved in this conformational interaction is the transport-partner site or a site

elsewhere on the protein. To assume that it is the transport-partner site is tantamount to assuming that the A^-/H^+-symport mechanism operates, for the A^-/OH^--antiport model conceives of a single transport site for which A^- and OH^- compete. One does not expect a monocarboxylate-loaded OH^--bearing form. It is also tantamount to assuming that the H^+-free symporter binds monocarboxylate more tightly than does the H^+-bearing symporter. To assume that the conformational pH site is not the transport-partner site involves no commitment as to the coupling mechanism or as to the effect of transport-partner-site titration on monocarboxylate binding.

The pH-interacting site responsible for the V increase with acidification may or may not be the same as that responsible for the increase of monocarboxylate K_m. If it is the transport-partner site, then the V enhancement indicates the presence of an immobile monocarboxylate-bearing base carrier, which is postulated to occur in the A^-/H^+-symport mechanism (as CA^-) but would not be expected of the A^-/OH^--antiport mechanism, in which A^- and OH^- presumably compete for the same site. Acidification cannot increase V unless monocarboxylate can bind to the base carrier to form a relatively immobile complex. If monocarboxylate binding were entirely dependent on carrier acidification (as in the antiport model), then infinite monocarboxylate concentration would convert all of the carrier to the monocarboxylate-bearing acid form (as CA or HCA) regardless of pH, and exchange V would be independent of pH. If the V dependence involves a site other than the transport-partner site, then the same rules would apply to that other site, and the V dependence would not distinguish between the two coupling mechanisms (symport and antiport).

While the above reasoning is rather tortuous, it can be summarized rather simply as follows. If either of the effects of acidification (increased exchange K_m or increased exchange V) is due to titration of the transport-partner site, the effect is evidence for the A^-/H^+-symport mechanism and against the A^-/OH^--antiport mechanism. If neither of the effects of acidification is due to titration of the transport-partner site, then the data are compatible with either mechanism but imply conformational properties of those other pH-interacting sites. Since we know the transport-partner site to be a pH interacting site, since pH interactions at the transport-partner site seem to dominate over non-specific interactions in all published studies, and since the A^-/H^+-symport model can account for all experimental results (including the present ones) in terms of pH actions at this site, it is simplest to believe that monocarboxylate is transported by the A^-/H^+-symport mechanism.

That a more definitive conclusion cannot be drawn seems to be inherent to the problem. Other approaches to this question are subject to the same doubts as to whether the pH interactions are at the transport-partner site. They are also subject to alternate interpretations on other grounds. For example the fact that external monocarboxylates stimulated monocarboxylate efflux from mitochondria more effectively than did alkalization of the medium [5], was taken as evidence for the symport mechanism, apparently with the view that OH^- should be as good an exchange partner as monocarboxylate in an antiport mechanism. However, matrix alkalization in an alkaline medium and matrix acidification by monocarboxylate influx could account for the different effects on efflux, regardless of coupling mechanism. Klingenberg et al. [6] also

expressed a preference for P_i^-/H^+ symport over P_i^-/OH^- antiport in the mitochondrial membrane, based on the effects of pH and phosphate on susceptibility of the phosphate carrier to sulfhydryl reagents. They did not elaborate on the logic connecting this observation with this conclusion. In particular they did not discuss the antiport models which were ruled out. The symport model which they presented would have behaved in every way like the antiport model of Fig. 4.

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References

1 Halestrap, A.P. (1975) Biochem. J. 148, 85—96
2 Spencer, T.L. and Lehninger, A.L. (1976) Biochem. J. 154, 405—414
3 Regen, D.M. and Tarpley, H.L. (1978) Biochim. Biophys. Acta 508, 539—550
4 Andersen, B.L., Tarpley, H.L. and Regen, D.M. (1978) Biochim. Biophys. Acta 508, 525—538
5 Halestrap, A.P. (1978) Biochem. J. 172, 377—387
6 Klingenberg, M., Durand, R. and Guerin, B. (1974) Eur. J. Biochem. 42, 135—150