Biochimica et Biophysica Acta, 508 (1978) 525—538

BBA 77987

CHARACTERIZATION OF β-HYDROXYBUTYRATE TRANSPORT IN RAT ERYTHROCYTES AND THYMOCYTES

BRIAN L. ANDERSEN, HAROLD L. TARPLEY and DAVID M. REGEN

Department of Physiology, Vanderbilt University, School of Medicine, Nashville, Tenn. 37232 (U.S.A.)

(Received August 8th, 1977)
(Revised manuscript received December 1st, 1977)

Summary

A method was developed for study of β-hydroxybutyrate transport in erythrocytes and thymocytes. Critical to the method was a centrifugal separation of cells from medium which took advantage of β-hydroxybutyrate transport's temperature dependence and inhibition by phloretin and methylisobutylxanthine, all of which are demonstrated in this work. These properties suggested mediated transport, as did saturation kinetics and inhibition by several agents including pyruvate and α-cyanocinnamate. Most conclusive in this regard was a 2-fold preference for D- over L-β-hydroxybutyrate. Entry was not Na^+ dependent. It was stimulated by substitution of SO_4^{2-} for most of the Cl^-. The equilibrium β-hydroxybutyrate space was much higher than the Cl^- space of thymocytes, suggesting that β-hydroxybutyrate entry is not associated with net inward negative current and is not coupled to outward Cl^- or inward K^+ movement (assuming that K^+ is at electrochemical equilibrium). Coupling to H^+ entry or OH^- exit is compatible with the result. These findings are consistent with β-hydroxybutyrate entry by the carboxylate transport site which has been studied extensively with pyruvate and lactate as permeants. The Cl^-/HCO_3^- exchange carrier did not appear to contribute significantly to β-hydroxybutyrate transport.

Introduction

Evidence has accumulated over the past several years to indicate that carboxylic acids [1—8] and inorganic anions [9—11] cross biological membranes by mechanisms displaying selectivity (stereospecificity in some

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazolyl-2)-benzene.

instances), saturability, competitive interactions, coupled-exchange interactions, susceptibility to inhibition, and/or other properties indicative of facilitation by specialized membrane sites. The entry of β-hydroxybutyrate (βHB) into the rat brain, across the blood-brain barrier, is the limiting step in cerebral β-hydroxybutyrate utilization [4]. Entry appears to be stereospecific, saturable, highly temperature dependent, and subject to modulation [3,4], the permeability increasing 7-fold during suckling, decreasing subsequently to 1/7 the peak values observed at weaning, increasing 4-fold in adults on a high-fat diet and increasing 2-fold in adults fasting for 5 days. Other permeants such as glucose, 5,5-dimethyloxazolidine-2,4-dione, urea, leucine and valine failed to show these modulations.

These observations stimulated our curiosity about fundamental characteristics of β-hydroxybutyrate transport which could not be examined in the rat brain in vivo. Virtually all studies of monocarboxylate transport across plasma membranes have involved pyruvate and lactate as permeants [6—8]. While these studies suggested that β-hydroxybutyrate (among several monocarboxylates) interacts with the lactate-pyruvate carrier [7,8], it seemed worthwhile to examine β-hydroxybutyrate transport itself. In fact, mechanistic conclusions concerning lactate transport in human erythrocytes [7] were quite different from those concerning lactate transport in Ehrlich ascites tumor cells [8], and one could not be certain which mechanism would apply to β-hydroxybutyrate in a given cell or species. We, therefore, turned our attention to β-hydroxybutyrate transport in suspensions of rat erythrocytes and thymocytes and report here on temperature dependence, substrate dependence, stereospecificity, inhibition by several agents, influence of inorganic ions, and equilibrium cell: medium ratio. Results are consistent with the view that β-hydroxybutyrate entry is mediated by a carrier which couples anion entry with proton entry or with hydroxyl exit resulting in electrically-silent net transport of β-hydroxybutyrate as an acid.

Methods

Incubation medium

Medium similar to that described earlier [12] was prepared from isotonic salt solutions (0.31 osM) in the proportions: 112 NaCl, 5 KCl; 2 $CaCl_2$, 1 $MgSO_4$, 2 sodium phosphate (pH 7.3), 6 Na-HEPES (pH 7.3). Bovine serum albumin (0.2%) and 2 mM glucose were added, and the pH of the final solution checked at 35°C (pH being 7.2—7.3). Henceforth, this solution will be referred to as 'medium'.

Preparation of cells

Thymocytes were prepared as described earlier [12], the cells being labelled with [^{3}H]isoleucine (26 Ci/mmol, 1 μCi in 10 ml cell suspension at room temperature for 20 min) between the two Ficoll density-step centrifugations.

For preparation of erythrocytes, blood was collected at the neck stump of decapitated rats (thymus donors) and stirred with a small wooden stick which took up the fibrin. The defibrinated blood was filtered through a nylon net, diluted with 1.5 volume medium and then treated almost exactly like the thymocyte suspension after its filtration. This further treatment included

centrifugation through a Ficoll layer, resuspension in 10 ml medium, a 10 min incubation (without labelled isoleucine in the case of erythrocytes), a second centrifugation through Ficoll, and resuspension in a volume of medium appropriate for the anticipated experiment.

Assay of cellular ^{14}C

Thymocyte label content was measured essentially as described earlier [12]. Briefly, a sample (200 or 500 μl) of the cell suspension containing β-hydroxy-[^{14}C]butyrate (for a specified period) was transferred from its incubation vial into 5 ml of ice-cold medium layered over 10 ml ice-cold 6% sucrose/1% albumin in 60% medium/40% H_2O in a 15 ml conical centrifuge tube (embedded in ice almost to the lip). Phloretin (0.3 mM) was included in the upper layer except where indicated otherwise. These tubes were then centrifuged 5 min at 5000 $\times g$ in an International PR 2 centrifuge refrigerated at 1°C. The upper 7 ml was aspirated and the upper walls rinsed (from the lip down to the aspiration level); then the next 1.5 cm of supernatant fluid was aspirated and the upper walls again rinsed; then another 1.5 cm supernatant fluid was removed and the walls above this level rinsed. The rinse solution was saline containing 0.005% Triton X100. The remaining supernatant fluid was then aspirated, and the pellet was transferred in four 0.75-ml portions of water through a tube to a counting vial (a vacuum being applied to the counting vial). The aspiration, rinsing and transfer were automated [12] such that eight samples could be processed in 4 min. 10 ml of liquid scintillation counting fluid was added to the counting vial. This was counted in a Beckman LS-230 liquid scintillation spectrometer set for ^{3}H and ^{14}C (and sometimes ^{36}Cl) determinations. The counting fluid was a 2 : 1 (v : v) mixture of toluene and Triton X-100 containing 0.5% PPO and 0.01% dimethyl-POPOP.

After removal from the incubation vials of the samples for determination of cell radioactivity, 20-μl samples were taken from incubation vials and transferred directly to counting vials for determination of the total ^{14}C (cellular plus extracellular) per ml of incubating cell suspension. Cellular ^{14}C (per ml suspension) was divided by total ^{14}C (per ml suspension) to yield the fraction of ^{14}C which was inside cells, ($\beta HB_{in}/\beta HB_{tot}$), an expression which is normalized with respect to day-to-day variations in added β-hydroxy[^{14}C]butyrate. This expression was divided by packed cell volume (or ml of cell pellet packed from 1 ml suspension), thereby normalizing the data with respect to day-to-day variations in cell density. The resulting expression ($\beta HB_{in}/\beta HB_{tot}$)/packed cell volume, is approximately the same as distribution space (milliliters of medium containing the amount in a milliliter of cells) and its initial rate of rise is approximately the influx rate (μmol/min per ml cells) divided by the external concentration (μmol/ml), i.e. the influx coefficient. The expression can be converted to μmol per ml packed cells simply by multiplying by the μmol added per ml incubation volume (see Fig. 4).

Erythrocyte incubations were handled identically except that acid extracts of the cell pellets and of 20-μl suspension samples were prepared before counting. This involved lysis in 1 ml water, acidification with 2 ml of 0.6 M $HClO_4$, centrifugation, and neutralization of 2.5 ml of the clear supernatant with 3 M K_2CO_3. 2 ml of the neutralized extract was counted.

Materials

L-[4,5-$^3H_2$]Isoleucine (26 Ci/mmol) and $Na^{36}Cl$ (14 μCi/mg) were obtained from Amersham; D-β-hydroxy[3-^{14}C]butyrate (0.25 mCi/mg), DL-β-hydroxy-[3-^{14}C]butyrate (0.05 mCi/mg), [G-^{3}H]inulin (100 mCi/mg), 5,5-[2-^{14}C]-dimethyloxazolidine-2,4-dione (9 Ci/mol) were from New England Nuclear. L-β-Hydroxy[3-^{14}C]butyrate was prepared as described earlier [4] except that gradient-elution anion-exchange column chromatography (footnote 1, ref. 4) was used to purify the product. Phloretin was obtained from Nutritional Biochemicals Corp., methylisobutylxanthine, phenylsuccinate and α-cyano-cinnamate from Aldrich Chemical Co., (aminooxy)acetate from Eastman Organic Chemicals, *p*-iodobenzylmalonate from ICN Pharmaceuticals, Inc., and 4-acetamido-4-isothiocyanostilbene-2,2′-disulfonic acid from Polysciences, Inc.

Results

Temperature dependence

In our earliest studies of β-hydroxybutyrate entry at 37°C, we were unable to assess initial entry rates, because equilibration in erythrocytes was nearly complete by 0.1 min and because the uptake course in thymocytes was complicated by rapid metabolism of the substrate after entry. Seeing the rapid rates at 37°C we feared that much of the cellular β-hydroxybutyrate might be lost in the centrifugation step (at 0°C) of the analysis. The experiment of Fig. 1 was, therefore, carried out to determine whether entry would be slow enough at lower temperatures to allow initial rate measurements and whether transport at 0°C would be slow enough for good recovery in the centrifugation step. As

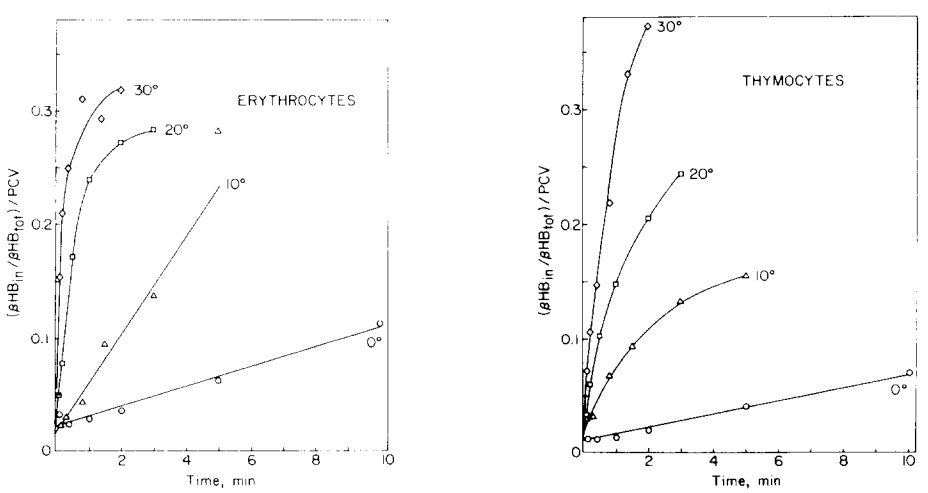

Fig. 1. Temperature dependence of β-hydroxybutyrate entry in a, erythrocytes and b, thymocytes. Cell suspensions (1.5 ml, 13% erythrocytes or 8% thymocytes) in 50-ml round-bottom centrifuge tubes were incubated in a shaking incubator at the indicated temperatures. Uptake was initiated by addition of 10 μl D-β-hydroxy[^{14}C]butyrate (0.23 μCi with 3 μmol unlabeled DL-β-hydroxybutyrate). Samples of 200 μl were transferred to ice-cold density-step gradients in conical centrifuge tubes described in Methods. Phloretin was not present in these tubes, but centrifugation was carried out with dispatch. PCV, packed cell volume.

seen in Fig. 1a, the rate in erythrocytes was still too fast for convenient measurement at 30°C but not at 20°C and lower. At 0°C about 1% of the cell volume equilibrated per min. We would, therefore, expect to lose about 1—2% during centrifugation, since the cells take 1—2 min to pack. We would also expect some gain or loss while the cells awaited centrifugation and, therefore, adopted the practice of centrifuging within 3 min of sampling.

In either cell, the temperature dependence was pronounced, the Q_{10} being about 3—6. This degree of dependence is not expected of a diffusion process (a priori) but is typical of carrier-mediated transport.

In these and other studies intended to characterize β-hydroxybutyrate transport, we used both erythrocytes and thymocytes. In the erythrocytes (lacking mitochondria and β-hydroxybutyrate dehydrogenase) the uptake characteristics are unambiguously referable to transport. The thymocyte was used to determine whether uptake characteristics in a β-hydroxybutyrate-utilizing cell would resemble those in erythrocytes. In most experiments assessing uptake rates, samples were taken before significant bending of the time course and reflected initial uptake rates, which should also be referable to transport and should not be complicated by efflux or by metabolic events subsequent to entry.

Inhibition by phloretin and methylisobutylxanthine

Since β-hydroxybutyrate transport was not entirely stopped at 0°C, we sought an inhibitor which could be added to the upper layer of the centrifuge tube to help slow exchange. Phloretin was tested first because of its reputation for membrane interaction and associated inhibition of glucose transport. It was found to inhibit entry in both cell types without delay. It was more inhibitory at 0°C than at 20°C. Fig. 2 illustrates the inhibitory effect of 0.3 mM phloretin on entry and exit at 0°C.

The cyclic nucleotide phosphodiesterase inhibitor, methylisobutylxanthine, increases cyclic AMP in thymocytes [12] and was tested with hopes of disclosing an influence of cyclic nucleotides on β-hydroxybutyrate entry. Initial studies in which thymocytes were exposed to methylisobutylxanthine for 30 min at 37°C and transport tested at 20°C, showed a strong inhibition by 0.1 mM and higher doses; and we were inclined to suspect a cyclic nucleotide involvement. However, subsequent studies (such as illustrated in Fig. 2) showed (a) erythrocytes to be almost as sensitive as thymocytes to methylisobutylxanthine inhibition, (b) the inhibition in either cell to be as strong at 0°C as at 20°C, and (c) the inhibition to be exerted without delay, all of which argue against cyclic nucleotide mediation and in favor of direct interaction of methylisobutylxanthine with the membrane. A cyclic nucleotide involvement has not been systematically excluded.

As shown in Fig. 2a, phloretin inhibited (solid vs. open symbols) in the presence of methylisobutylxanthine almost as well as it did in its absence and methylisobutylxanthine inhibited (triangles or squares vs. circles) rather well in the presence and absence of phloretin. The two cell types differed in their susceptibilities to the two agents, phloretin sensitivity being greater in erythrocytes and methylisobutylxanthine sensitivity being greater in thymocytes (confirmed in other experiments not shown). This pattern was also

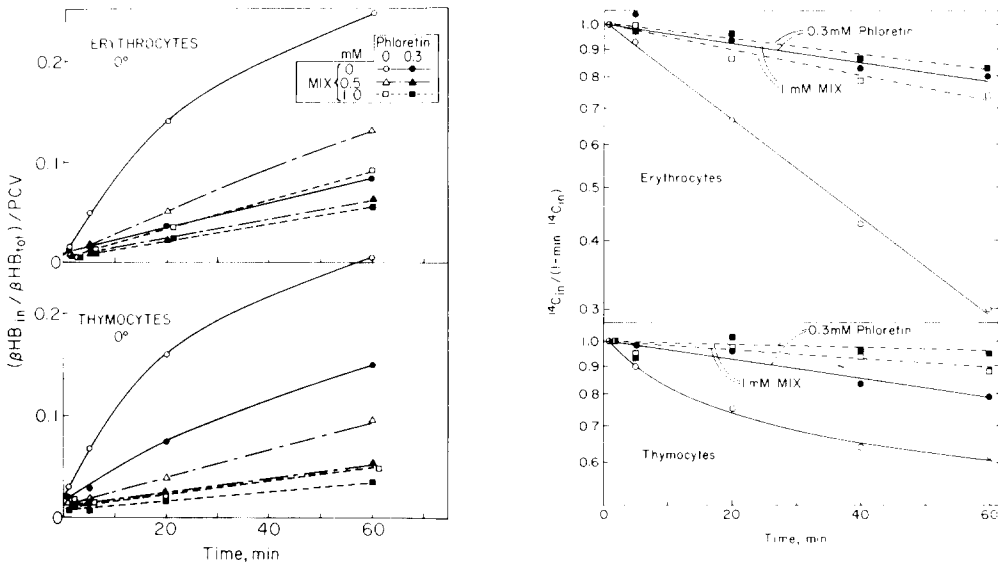

Fig. 2. (a) Effects of phloretin and methylisobutylxanthine on D-β-hydroxy[^{14}C]butyrate uptake. The experiments were carried out at 0°C on the cell batches in b. Cell suspension (1 ml of 13% erythrocytes or 9% thymocytes) was added to 300 μl medium containing methylisobutylxanthine and/or phloretin at 13/3 of the indicated concentrations and 10 μl D-β-hydroxy[^{14}C]butyrate (0.27 μCi with 2.5 μmol DL-β-hydroxybutyrate), and 200-μl samples taken at 1.2, 5, 20 and 60 min. Both phloretin (0.3 mM) and methylisobutylxanthine (0.7 mM) were in the upper layer of the density-step centrifuge tubes. (b) Effects of phloretin and methylisobutylxanthine on ^{14}C exit from cells briefly loaded with β-hydroxy[^{14}C]-butyrate. To 2.5 ml of 21% erythrocytes or 11% thymocyte suspension, 20 μl D-β-hydroxy[^{14}C]butyrate was added (0.5 μCi with 5 μmol DL-β-hydroxybutyrate) and the mixture incubated 10 min at room temperature. These were cooled in ice slush and four equal portions of each were placed on 10 ml ice-cold 7% Ficoll in medium in conical 15-ml centrifuge tubes. After 5 min centrifugation at 1°C, the centrifuge tubes were placed on ice, aspirated half way, filled with saline to the brim and aspirated to the cell pellets. Efflux was initiated when the pellets were resuspended by ejection of 3 ml ice-cold medium from an adjustable 5-ml capacity Pipetman pipet, the suspensions being transferred by this pipet to 50-ml round-bottom plastic centrifuge tubes in the shaking incubator containing ice slush. Samples (500 μl) were transferred to centrifuge tubes containing both 0.3 mM phloretin and 0.7 mM methylisobutylxanthine in the upper layer. Each value of cell radioactivity was divided by that of the first (1-min) samples to show the fractional decline after this time. PCV, packed cell volume.

seen in the efflux data of Fig. 2b, erythrocytes being the more sensitive to phloretin and thymocytes being the more sensitive to methylisobutylxanthine. The combination of inhibitors (solid square) was quite effective, so that the erythrocytes lost only 0.3% per min and thymocytes lost only 0.1% per min. Susceptibility to inhibition by low concentrations of phloretin, methylisobutylxanthine or other agents is consistent with mediated transport. After the inhibitory effects of methylisobutylxanthine were observed, we used this agent at 0.7 mM together with phloretin in the centrifuge tubes. Exit of ^{14}C from erythrocytes was single-exponential while that from control thymocytes was multi-exponential, a substantial fraction being essentially fixed in the cells, presumably in the form of slow-turnover metabolites. This conforms with the expectation that erythrocytes would not metabolize D-β-hydroxybutyrate but thymocytes (containing mitochondria) should.

Stereospecificity

Fig. 3 shows the accumulation of D-β-hydroxy[^{14}C]butyrate, DL-β-hydroxy-[^{14}C]butyrate and L-β-hydroxy[^{14}C]butyrate in the presence of 5 mM unlabeled DL-β-hydroxybutyrate. With either cell the natural isomer (D) was twice as permeable as the unnatural isomer (L). This relation was confirmed by the racemic mixture which showed a permeability half way between the two isomers. Since optical isomers have equivalent diffusion properties, stereospecific entry is very strong evidence for mediated transport. Inasmuch as the mechanism appears to facilitate entry of several monocarboxylic acids [7,8], the modest degree of steric discrimination is understandable.

Substrate dependence

Fig. 4 shows that unlabeled DL-β-hydroxybutyrate interferes with the uptake of D-β-hydroxy[^{14}C]butyrate, a manifestation of saturation kinetics. With

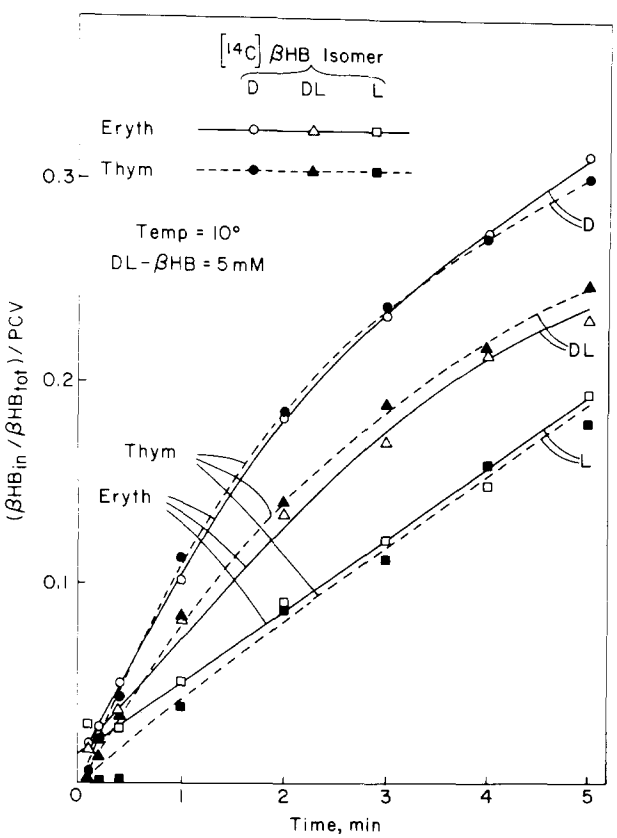

Fig. 3. Stereospecificity of β-hydroxybutyrate entry. To 2-ml cell suspensions (13% erythrocytes or 8% thymocytes) incubating at 10°C, 20 μl of β-hydroxy[^{14}C]butyrate (D, DL or L, 0.4 μCi with 10 μmol DL-β-hydroxybutyrate) was added to initiate uptake. Samples of 200 μl were transferred at the indicated times to the ice-cold centrifuge tubes for analysis of cell label. The upper layer of these tubes contained both 0.3 mM phloretin and 0.7 mM methylisobutylxanthine. PCV, packed cell volume.

either cell, the Hanes plot showed the straight-line relation between resistance and concentration expected of Michaelis-Menten kinetics. The resistance-doubling concentration (K_m) appeared to be somewhat higher in erythrocytes than in thymocytes. Experiments of this kind carried out at 0°C and 20°C also showed saturation kinetics. The apparent K_m values were between 10 and 20 mM and were not obviously dependent on temperature. In view of other evidence suggesting mediated β-hydroxybutyrate entry, it is reasonable to interpret saturation kinetics in terms of a carrier or other specific transport site which is half saturated by 10—20 mM DL-β-hydroxybutyrate.

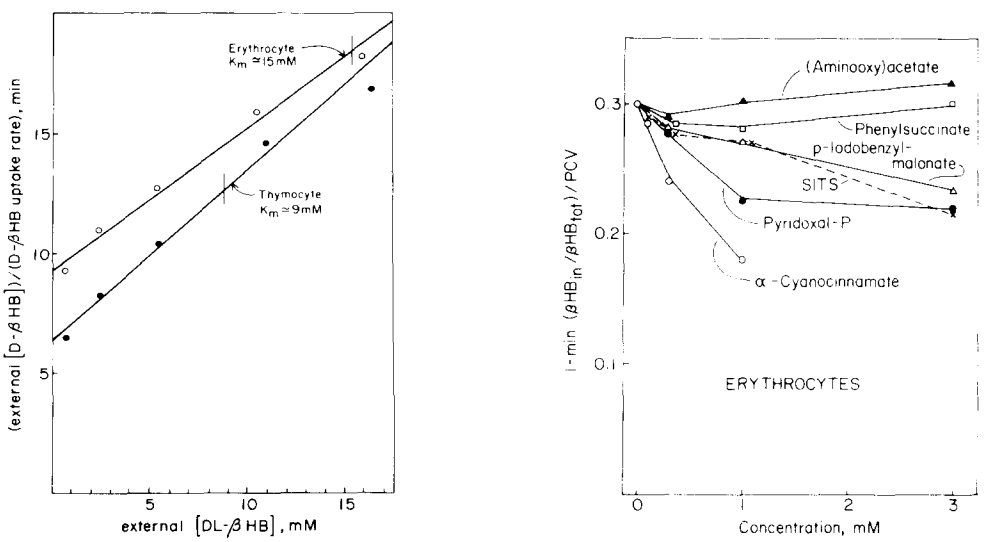

Fig. 4. Concentration dependence of β-hydroxybutyrate entry. To 1-ml cell suspensions (12% erythrocytes or 18% thymocyte) incubating at 10°C, 1 ml of D-β-hydroxy[^{14}C]butyrate (0.2 μCi with 1.2, 4.5, 10, 20 or 30 μmol DL-β-hydroxybutyrate) was added to initiate uptake. At 0.2, 0.5, 1, 2 and 3 min samples (200 μl) were transferred to ice-cold centrifuge tubes containing both 0.3 mM phloretin and 0.7 mM methylisobutylxanthine in the upper layer. Straight lines were drawn through the early points of the ($\beta HB_{in}/\beta HB_{tot}$)/packed cell volume time courses. As expected of a saturable process, the slopes were less steep with higher concentrations. These initial slopes were multiplied by μmol of D-β-hydroxybutyrate added per ml incubation volume to obtain uptake rates (μmol/min per ml packed cells). External D-β-hydroxybutyrate concentration (μmol/ml) was calculated as D-β-hydroxybutyrate added divided by external volume. These were used in the calculation of the ordinate values, concentration/rate, which may be considered transport resistances. External DL-β-hydroxybutyrate concentration was calculated as DL-β-hydroxybutyrate added divided by external volume. This is a Hanes plot of S/V vs. S. (S/V is a resistance expression.) The K_m would be the resistance-doubling concentration (vertical lines) if D and L-β-hydroxybutyrate contributed equally to saturation. Since D-β-hydroxybutyrate is the preferred substrate (Fig. 3) it presumably binds more strongly than L-β-hydroxybutyrate and has a K_m lower than the apparent K_m of the DL mixture.

Fig. 5. Effects of unphysiological anions on β-hydroxybutyrate entry. To small counting vials (50 mm × 15 mm internal diameter) containing 50 μl medium with possible inhibitory agents at 5× the indicated concentrations, 200 μl of cell suspension (15% erythrocytes or 9% thymocytes) was added. The mixture was incubated for 20 min at room temperature and then cooled to 20°C in the shaking incubator. Uptake was initiated by addition of 10 μl D-β-hydroxy[^{14}C]butyrate (0.05 μCi with 0.5 μmol DL-β-hydroxybutyrate). Erythrocyte samples (200 μl) were taken 1 min later (and thymocyte samples 2 min after initiation, data not shown). PCV, packed cell volume.

Effects of physiological ions

It was supposed from other work [6—8] that several monocarboxylates would share a common entry mechanism with β-hydroxybutyrate and that some might have extraordinary affinities for the presumed binding site. We therefore tested whether a modest concentration of such anions would inhibit β-hydroxybutyrate entry markedly. Cells were exposed to the ions both together with and 30 min before uptake initiation, since we felt that transient effects might reflect some kind of disturbance of cell pH or electrical potential rather than competitive inhibition. A competitive effect should persist. As seen in Table I pyruvate was among the more consistent inhibitors, acting on either cell type regardless of whether it was added with or before β-hydroxy[^{14}C]-butyrate. In the thymocytes, acetoacetate and propanoate appeared inhibitory also, and several others appeared to inhibit more when added with the substrate than when added before. The results are consistent with a common mechanism facilitating transport of β-hydroxybutyrate, pyruvate and some other monocarboxylates.

Replacement of most of the Na^+ by K^+ did not reduce β-hydroxybutyrate entry and may have enhanced it temporarily. Obligatory coupling of β-hydroxybutyrate entry with Na^+ entry is, therefore, most unlikely. Replacement of most of the Cl^- by SO_4^{2-} enhanced β-hydroxybutyrate entry dramatically.

Effects of unphysiological organic anions

The experiment of Fig. 5 was carried out as a test for analogies between β-hydroxybutyrate transport and other transport systems of known inhibitor susceptibilities. Of the organic salts tested, α-cyanocinnamate was the most potent inhibitor. This agent is well known as an inhibitor of pyruvate transport across mitochondrial [5] and plasma [6—8] membranes, and its effects strengthen the view [6—8] that β-hydroxybutyrate enters by the monocarboxylate carrier. We have also examined the agent's effects on efflux. At 10°C, 3 mM α-cyanocinnamate inhibited D-β-hydroxybutyrate efflux from erythrocytes by 80% and inhibited L-β-hydroxybutyrate efflux from thymocytes by 60%. Pyridoxal phosphate and 4-acetamido-4-isothiocyanostilbene-2,2'-disulfonic acid (inhibitors of inorganic anion exchange [9—11]) were less potent inhibitors of β-hydroxybutyrate entry. This is consistent with involvement of the inorganic anion-exchange carrier as proposed by Halestrap [7]. As will be seen below, other evidence seems to rule out significant participation of this carrier under physiological conditions. Iodobenzylmalonate was another weak inhibitor. It is known to inhibit three separate mitochondrial carriers exchanging di- and tricarboxylic acids [13].

β-Hydroxybutyrate, Cl^- and 5,5-dimethyloxazolidine-2,4-dione equilibria

Table II shows equilibrium spaces of β-hydroxybutyrate, Cl^- and 5,5-dimethyloxazolidine-2,4-dione in erythrocytes and thymocytes analyzed by two methods. With one method (packed in medium) there was no opportunity for equilibrated permeants to escape during analysis. The other method (density-step centrifugation) involves dilution at cold temperature and physical separation of cells from medium, and those labelled permeants which exchange rapidly at 0°C are lost. The two methods agreed very well with regard to β-

TABLE I

EFFECTS OF PHYSIOLOGICAL ANIONS AND MONOVALENT CATIONS ON β-HYDROXY[^{14}C]BUTYRATE ENTRY

Final incubation volumes were, 610 μl of which 100 μl was cell suspension (17% erythrocytes or 9% thymocytes), 500 μl was medium containing the indicated organic salts at 6 mM or with NaCl component replaced by KCl or Na_2SO_4 (isotonic) as indicated, and 10 μl was D-β-hydroxy[^{14}C]butyrate to give a final concentration of 1.6 mM and 0.2 μCi/ml. In erythrocyte experiments 500-μl samples were taken 1 min after completion of the system, and in thymocyte experiments 500-μl samples were taken two min after completion. To obtain the data in columns headed "With β-hydroxy[^{14}C]butyrate", conditions were imposed at the instant of uptake initiation, since the systems were completed by addition of the 100-μl cell suspensions. To obtain the data in columns headed "Before β-hydroxy[^{14}C]butyrate", the conditions were imposed 30 min prior to uptake inition (the 100 μl cell suspension and the 500 μl altered medium being together at room temperature) and the systems were completed by addition of the 10 μl substrate. All components were at 20°C at the time of uptake initiation. The experiment was repeated twice and average data shown. The effects of pyruvate and of Cl^- replacement by SO_4^{2-} were seen in both experiments with both-uptake inition techniques and in both cell types.

Conditions imposed:	($\beta HB_{in}/\beta HB_{tot}$)/packed cell volume			
	Erythrocyte (1 min)		Thymocytes (2 min)	
	With β-hydroxy[^{14}C]butyrate	Before β-hydroxy[^{14}C]butyrate	With β-hydroxy[^{14}C]butyrate	Before β-hydroxy[^{14}C]butyrate
Control	0.285		0.250	
β-Hydroxybutyrate, 5 mM	0.273	0.250	0.197	0.235
Acetoacetate, 5 mM	0.273	0.260	0.175	0.220
Lactate, 5 mM	0.255	0.290	0.195	0.290
Pyruvate, 5 mM	0.225	0.229	0.208	0.209
Acetate, 5 mM	0.290	0.295	0.197	0.263
Propanoate, 5 mM	0.264	0.260	0.203	0.225
Low Na^+, high K^+	0.317	0.281	0.287	0.237
Low Cl^-, high SO_4	0.590	0.445	0.300	0.270

TABLE II

EQUILIBRIUM DISTRIBUTION SPACES

Suspensions of erythrocytes and thymocytes (without isoleucine label) were incubated at 20°C with [^{3}H]inulin (1 μCi/ml), β-hydroxy[^{14}C]butyrate (0.1 μCi/ml), Na^{36}Cl (0.05 μCi/ml) and 10 mM DL-β-hydroxybutyrate. With erythrocytes D-β-hydroxy[^{14}C]butyrate was used, and with thymocytes L-β-hydroxy[^{14}C]butyrate was used. At various times, samples were centrifuged ($\approx$5000 $\times$ g) in Hopkins Vaccine Tubes for 10 min at 1°C. Supernatant samples (20 μl) were taken for counting, residual supernatants aspirated, and the tubes filled to the brim with saline which was then aspirated. 1 ml of 0.5% sucrose in medium was added and the tubes centrifuged ($\approx$7000 $\times$ g) 20 min at room temperature. Supernatant was aspirated, pellet volume recorded and the cells analyzed for isotope content essentially as described in Methods (thymocytes transferred directly to counting vials, erythrocytes extracted with acid etc.). Cell : medium ratio (of S) was calculated as: (S_{pellet}/[S_{medium}] $-$ $^3H_{pellet}$/[$^3H_{medium}$])/(Volume of pellet) and tabulated under "Packed in medium". For the data under 'Density-step centrifugation', samples were analyzed as in Figs. 2—4, and spaces were calculated: (S_{cell}/[S_{medium}])/(Volume of pellet). Tracer distributions were followed for 300 min, means and standard errors being shown for values on the apparent plateaus.

Cell and permeant	Cell: medium ratio	
	Packed in medium	Density-step centrifugation
Erythrocyte		
D-β-Hydroxy[^{14}C]butyrate	0.389 ± 0.013 (13)	0.369 ± 0.006 (13)
$^{36}Cl^-$	0.459 ± 0.007 (27)	0.003 ± 0.001 (28)
5,5-Di[^{14}C]methyloxazolidine-2,4-dione	0.524 ± 0.012 (14)	0.005 ± 0.001 (14)
Thymocyte		
L-β-Hydroxy[^{14}C]butyrate	0.624 ± 0.010 (13)	0.618 ± 0.015 (13)
$^{36}Cl^-$	0.145 ± 0.008 (27)	0.150 ± 0.003 (28)
5,5-Di[^{14}C]methyloxazolidine-2,4-dione	0.492 ± 0.033 (14)	0.260 ± 0.034 (14)

hydroxybutyrate spaces, proving the adequacy of the density-step method as it evolved in the course of this work. The method is also adequate for $^{36}Cl^-$ in thymocytes, but worthless for $^{36}Cl^-$ and dimethyloxazolidinedione in erythrocytes (99% being lost) and for dimethyloxazolidinedione in thymocytes (50% being lost). These losses which occurred in about 3 min time, between sampling and pelleting, indicate extremely rapid exchange of Cl^- and dimethyloxazolidinedione in erythrocytes and moderately rapid exchange of dimethyloxazolidinedione in thymocytes at 0°C. The distinctions in exchange rate were confirmed by short time data (not shown) taken in connection with these equilibration studies. In erythrocytes, $^{36}Cl^-$ and dimethyloxazolidinedione were completely equilibrated when the earliest samples were taken at 1 min. In thymocytes, $^{36}Cl^-$ equilibrated with a 30 min half time and dimethyloxazolidinedione had equilibrated by around 20 min (insufficient data to show half time). From the similarity of β-hydroxybutyrate equilibration rates in erythrocytes and thymocytes (Figs. 1—4), which differ at least 100-fold in Cl^- exchange rates, we think it unlikely that the Cl^-/HCO_3^- exchange carrier contributes significantly to β-hydroxybutyrate transport.

There was no large distinction in erythrocytes between the Cl^- space (governed by membrane potential) and the dimethyloxazolidinedione space (governed by cell and medium pH [14]). Both spaces were rather large indicating very little membrane potential and very little pH difference across the membrane. Hence, it would be difficult to say whether β-hydroxybutyrate

space resembled dimethyloxazolidinedione space more or less than it resembled Cl^- space. In thymocytes, on the other hand, the Cl^- space was only 0.15 while the dimethyloxazolidinedione space was 0.49 and the β-hydroxybutyrate space was higher yet. It is reasonable to attribute the low Cl^- space to the membrane potential favoring the outward disposition of anions (according to the Nernst equation). The much higher equilibrium β-hydroxybutyrate space (approximating the water space of 0.6—0.7 ml/ml packed cells [15]) suggested that β-hydroxybutyrate entry is not stoichiometrically accompanied by inward negative current. This most likely results from coupling of β-hydroxybutyrate anion entry with cation entry or with anion exit such that the transport is electrically silent and insensitive to membrane potential *. the major ions are poor candidates for participation in this coupled transport. Coupling with Na^+ would result in much higher cell : medium β-hydroxybutyrate ratios than observed, and β-hydroxybutyrate entry is not Na^+ dependent. Coupling with K^+ or Cl^- would result in much lower ratios such as shown by Cl^-. However, coupling of β-hydroxbutyrate entry with H^+ entry would result in β-hydroxybutyrate distribution similar to the dimethyloxazolidinedione distribution, for the latter weak acid is known to cross the membrane in the electroneutral, protonated form, its equilibrium ratio reflecting the proton concentration difference across the membrane. Coupled exchange of β-hydroxybutyrate with OH^- is equally plausible, since the cell : medium OH^- ratio is the same as the medium : cell H^+ ratio.

Discussion

The pronounced temperature dependence and saturation kinetics of β-hydroxybutyrate entry suggest mediated transport, inasmuch as these properties are typical of well documented carrier-mediated transport processes such as that for glucose. Inhibition by several agents at low concentrations (0.3 mM phloretin, 0.1 mM methylisobutylxanthine, 0.3 mM α-cyanocinnamate, 1 mM pyridoxal phosphate) also argues for mediated β-hydroxybutyrate entry, in that these agents are known to inhibit carrier transport of other permeants. A strong effect of inhibitor on permeation is most simply interpreted in terms of binding of inhibitor to a trasnport catalyst (e.g. a carrier) and/or to adjacent structures. While mediated transport provides a unitary explanation for high temperature dependence, saturation entry kinetics and high sensitivity to several inhibitors, one can construct ad hoc hypotheses by which these properties might be explained in terms of pores. However, the stereospecificity of β-hydroxybutyrate entry would be difficult to explain except in terms of a carrier. Both D- and L-β-hydroxybutyrate have identical diffusion and solubility properties and would pass through a pore or a lipid phase with equal ease. Only a binding step can confer a preference for the D

* The high cell : medium L-β-hydroxybutyrate ratio in thymocytes at 10°C is not due to trapping of label in the cells as slow-turnover metabolites. This isomer is very slowly metabolized in the whole rat [4], which is of course at 37°C. More directly, though, the lack of such metabolites can be appreciated in Fig. 5b of the accompanying paper where about 80% of cellular L-β-hydroxybutyrate escapes the thymocyte in 20 min at 10°C with no sign of a trapped pool. This is to be contrasted with Fig. 2b of this paper where 60% of D-β-hydroxybutyrate appears trapped in thymocytes.

over the L isomer. To explain the preference in terms of a carrier, one postulates that the carrier's binding site provides a better fit for D-β-hydroxybutyrate than for L-β-hydroxybutyrate. Preferential binding of one optical isomer in a fixed pore would not result in preferential movement of either isomer from a racemic mixture. The binding site would equilibrate with adjacent fluid in the pore and would not affect steady-state conductance of one isomer more than the other.

The postulated facilitating mechanism does not appear to be devoted exclusively to β-hydroxybutyrate. The inhibitory effects of pyruvate and α-cyanocinnamate point to involvement of the monocarboxylate-transport site for which pyruvate appears to be particularly good substrate [5—8]. Preliminary studies in this laboratory have shown β-hydroxybutyrate, phloretin, and methylisobutylxanthine to inhibit lactate and pyruvate transport in erythrocytes. Spencer and Lehninger [8] observed properties of lactate transport in Ehrlich ascites tumor cells essentially similar to those described here for β-hydroxybutyrate transport. Halestrap [7] observed that external β-hydroxybutyrate, like pyruvate and lactate, induced Cl^- efflux from human erythrocytes by an α-cyano-4-hydroxycinnamate-sensitive process. Other evidence for a common monocarboxylate-transport site comes from the age- and diet-dependent modulations of β-hydroxybutyrate permeability at the blood-brain barrier. We found acetoacetate and lactate permeabilities to increase and decrease in parallel with that of β-hydroxybutyrate (unpublished observations of M.C. Sugden, this lab). Oldendorf [2] concluded that lactate and pyruvate cross the blood-brain barrier by a common saturable, stereospecific mechanism. If several monocarboxylic acids, including β-hydroxybutyrate, share the same entry mechanism, then the modest degree of stereospecificity is not surprising. It is interesting that the site has evolved with preference for the naturally abundant isomers of both β-hydroxybutyrate [4] and lactate [2].

The strong inhibitory effect of methylisobutylxanthine was not expected. Like phloretin, methylisobutylxanthine inhibited β-hydroxybutyrate entry in both erythrocytes and thymocytes, was effective immediately at 0°C, and was approximately as effective in the erythrocyte against glucose transport (data not shown) as against β-hydroxybutyrate transport. Other methylxanthines have been reported to inhibit the transport of 2-deoxyglucose, nucleosides, hypoxanthine and choline in cultured hepatoma cells [16] and to inhibit glucose transport in islet cells [17]. The number of affected permeants favors a rather general non-specific interaction of methylxanthine with the membrane, though structural analogy among several transport catalysts is possible. Cyclic nucleotides might be involved in some of these effects.

The equilibrium cell : medium β-hydroxybutyrate ratio favors coupling of β-hydroxybutyrate entry with H^+ entry of OH^- exit. The pH dependences of β-hydroxybutyrate influx, efflux and equilibrium space to be presented in the accompanying paper provide further support for these possibilities. This is consistent with conclusions of Spencer and Lehninger [8] in regard to lactate, pyruvate and β-hydroxybutyrate transport in Ehrlich ascites tumor cells. Either mechanism (H^+ copermeation or OH^- exchange) results in electrically silent transfer of β-hydroxybutyrate as an acid.

Two observations suggested an interaction of inorganic anions with the monocarboxylate carrier, inhibition by 4-acetamido-4-isothiocyanostilbene-2,2'-disulfonic acid and pyridoxal phosphate and stimulation by replacement of Cl^- with SO_4^{2-}. Moreover, Halestrap [7] concluded that some influx of pyruvate, lactate and β-hydroxybutyrate in human erythrocytes occurs on the Cl^-/HCO_3^- exchange carrier. However, the K_m for this pathway was very high, and with physiological monocarboxylate concentrations, its contribution was minor, despite his use of Cl^--free medium. Had he used a normal Cl^- medium, the Cl^-/HCO_3^- carrier would have been largely occupied by Cl^- and would have contributed negligibly to monocarboxylate entry. The Cl^- carrier appears to make little if any contribution to β-hydroxybutyrate entry either in rat erythrocytes or rat thymocytes; for all β-hydroxybutyrate transport properties were similar in the two cells, despite the vast difference in their Cl^- exchange activities. That the Cl^-/HCO_3^- exchange carry is not important to β-hydroxybutyrate transport in thymocytes is further witnessed by the disparity between equilibrium β-hydroxybutyrate space and Cl^- space.

Acknowledgement

This work was supported by U.S. Public Health Service Grants 5 P01 AM07462 and 5 P30 AM17026.

References

1 Drews, L.R. and Gilboe, D.D. (1973) J. Biol. Chem. 218, 2489—2496
2 Oldendorf, W.H. (1973) Am. J. Physiol. 224, 1450—1453
3 Gjedde, A. and Crone, C. (1975) Am. J. Physiol. 229, 1165—1169
4 Moore, T.J., Lione, A.P., Sugden, M.C. and Regen, D.M. (1976) Am. J. Physiol. 230, 619—630
5 Halestrap, A.P. (1975) Biochem. J. 148, 85—96
6 Halestrap, A.P. and Denton, R.M. (1975) Biochem. J. 148, 97—106
7 Halestrap, A.P. (1976) Biochem. J. 156, 193—207
8 Spencer, T.L. and Lehninger, A.L. (1976) Biochem. J. 154, 405—414
9 Cabantchik, Z.I. and Rothstein, A. (1972) J. Membrane Biol. 10, 311—330
10 Rothstein, A., Cabantchik, Z.I. and Knauf, P. (1976) Fed. Proc. 35, 3—10
11 Lepke, S. and Passow, H. (1973) Biochim. Biophys. Acta 298, 529—533
12 Whitesell, R.R., Johnson, R.A., Tarpley, H.L. and Regen, D.M. (1977) J. Cell Biol. 72, 456—469.
13 Robinson, B.H., Williams, G.R., Halperin, M.L. and Leznoff, C.C. (1971) Eur. J. Biochem. 20, 65—71
14 Roos, A. (1975) J. Physiol. Lond. 249, 1—25
15 Whitesell, R.R., Hoffman, L.H. and Regen, D.M. (1977) J. Biol. Chem. 252, 3533—3537
16 Plagemann, P.G.W. and Sheppard, J.R. (1974) Biochem. Biophys. Res. Commun. 56, 869—875
17 McDaniel, M.L., Weaver, D.C. and Lacy, P.E. (1976) Fed. Proc. 35, 1760