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Propionate transport in rat liver cells

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Propionate extraction by liver is generally in the range of 95%, which could depend on a transport process across the cell membrane. The study reports conditions in which [^{14}C]propionate uptake can be measured with minimal interferences from metabolism. Propionate uptake by isolated hepatocytes was mediated by two components: a low-affinity component of limited physiological interest and a high-affinity (apparent K_m about 0.15 mM) component. This last component displayed a high capacity but was not Na^+-dependent nor concentrative. Propionate transport was not markedly affected by acetate, butyrate or other C_3 glucogenic compounds; it was inhibited by halogenated monocarboxylates, monochloroacetate and 2-chloropropionate being the most potent. Classical inhibitors of anion transport and of functional-SH groups were ineffective. Propionate uptake was responsive to external pH: stimulated by acidic and depressed by alkaline pH. Hepatic uptake of propionate in vivo was practically quantitative up to 0.8–1.0 mM in afferent plasma, in keeping with the measured capacity of the high-affinity component. It is suggested that propionate uptake is essentially carrier mediated but this process should not be rate limiting for hepatic utilization in physiological conditions.

Introduction

Certain substrates reaching the liver are almost quantitatively removed by the liver under normal conditions, such as ammonia or volatile fatty acids, particularly propionate and butyrate. Volatile fatty acids essentially arise from the bacterial degradation of carbohydrates, in the large bowel for monogastric animals, and in forestomachs for ruminants. Propionate removed by liver cells is activated to propionyl-CoA in mitochondria, then converted into C_4-dicarboxylates which constitute intermediates of the Krebs cycle and may eventually enter into the gluconeogenesis pathway. In ruminants and in herbivorous monogastrics, propionate constitutes the main glucogenic substrate arising from the digestive tract. Even if the contribution of propionate to hepatic metabolism is less in omnivorous animals, its role should not be disregarded since propionate has been reported to affect ureogenesis [1], gluconeogenesis [2] and cholesterol synthesis [3].

The mechanisms which could account for the highly effective uptake of propionate by liver cells are still unclear, except the fact that propionate is readily metabolized by the liver [4]. This feature is shared by butyrate but, for this anion, a cytosolic butyrate-binding protein has been identified [5]. Such a process might improve the hepatic removal of butyrate in the same way as for organic anions and long-chain fatty acids which bind to specific cytosolic proteins [6]. In fact, the question arises as

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SITS, 4-acetamido-4′-isothiocyanatestilbene-2,2′-disulfonic acid.

to whether hepatic uptake of propionate is a carrier-mediated process. At pH observed in portal vein, in the range of 7.3, propionate is almost entirely (99.6%) in the ionized form which should slowly move across the hydrophobic matrix of the cell membrane. Furthermore, recent investigations support the view that the transfer of some organic anions across the liver cell membrane is, to a large extent, mediated by specific carriers as reported for lactate [7,8] and branched-chain ketoacids [9,10].

Since the uptake of propionate is more effective than that of the above anions [11,12], it appeared interesting to examine whether propionate uptake could be mediated by a carrier system. We have therefore attempted to evaluate the kinetic characteristics of this system and to identify physiological factors (metabolites, acid-base equilibrium) or pharmacological ligands which could affect the rate of propionate transport. The aim of this study was also to assess whether plasma membrane transport could constitute a rate-limiting step in the overall hepatic utilization of propionate in some conditions.

Materials and Methods

Chemicals

[1-^{14}C]Propionate (60 mCi/mmol) was purchased from the CEA (Gif-sur-Yvette, France) and [^{3}H]inulin from the Radiochemical Center (Amersham International, Amersham, Bucks, U.K.). Collagenase and albumin were from Boehringer (Meylan, France). 4-Acetamido-4′-isothiocyanatestilbene-2,2′-disulfonic acid (SITS) was from BDH (Poole, Dorset, U.K.). Other inhibitors were obtained from Sigma (St. Louis, MO, U.S.A.) except dichloroacetic and 2-chloropropionic acids (Merck, Darmstadt, F.R.G.). Silicon oils (AR20 and AR200) were from Wacker Chemie (Munich, F.R.G.). Other reagents were commercially available products of the best analytical grade.

Preparation of isolated hepatocytes

Male Wistar rats weighing 250 g were housed in air-conditioned quarters with controlled 12 h light and dark cycle (dark cycle from 0.800 to 20.00 h) and received Sanders Laboratory Chow and drinking water ad libitum. Hepatocytes were isolated from fed rats. Collagenase dissociation was essentially performed as outlined by Krebs et al. [13], cells were then purified by centrifugation. Cells viability, estimated by cell membrane refractoriness in phase contrast microscopy ranged from 97 to 99%. The incubations were performed in a Krebs-Henseleit bicarbonate buffer containing 10 mM Hepes and 2% bovine serum albumin (fraction V) previously dialyzed against the same medium.

Transport experiments

Propionate transport was initiated in Eppendorf microfuge tubes containing [1-^{14}C]propionate and [^{3}H]inulin in 100 μl Krebs-Ringer bicarbonate buffer, by addition of 300 μl hepatocyte suspension. The resulting suspension was immediately mixed and incubated at 37°C for 10 s. For measurements of the time-course of propionate uptake, 1.5 ml hepatocyte suspension was incubated at 37°C with labelled propionate and inulin, with appropriate agitation; samples (300 μl) of the suspension were taken at the required time. For both procedures, cells were separated from the medium (within about 2 s) by rapid centrifugation (8000 $\times$ *g*, 10 s) through 250 μl of silicon oil (AR 200/AR 20, 1 : 3, v/v) layered on the top of 50 μl 1.2 M $HClO_4$. After the spin, 100 μl of the supernatant was transferred to counting minivials and mixed with 2 ml of scintillation liquid. The remaining supernatant and about half of the oil layer were then discarded by vacuum aspiration, the microtube was then plunged into liquid N_2 (about 5 s) and the bottom (containing the cell pellets in frozen perchloric acid) immediately cut about 3 mm above the lower meniscus of the oil layer and transferred into a counting minivial. 250 μl of distilled water was added and, after vigorous agitation, 3 ml of scintillation liquid. The correction for extracellular fluid was in the range of 35% of centrifuged water.

For the chemical determination of propionate in the cell pellet fraction, the above procedure was modified: (a) 1000 μl of cell suspension were layered above the oil layer, (b) the 1.2 M perchloric acid under the oil layer contained isobutyric acid, as internal standard for GLC analysis and (c) after the spin, the perchloric extract was brought

to pH 8 with K_2CO_3. The neutralized extract was treated with 20 vol. absolute ethanol, allowed to stand at 0°C for 30 min (precipitation of glycogen and some additional compounds) then evaporated at room temperature.

Sampling procedures and measurement of propionate utilization in vivo

The rats were anaesthetized with sodium pentobarbital (40 mg/kg) and maintained at 37°C on a histological warmer. After laparotomy, 1 ml of blood was withdrawn in heparinized syringes either from the portal vein and then the aorta, or from the hepatic vein (in the left lobe) then the portal vein. Hepatic blood flow was measured by an indicator-dilution method, using *p*-aminohippurate [11]. Briefly, the indicator (0.2 mg/ml in 0.9% NaCl) was infused into a mesenteric vein, with a 0.4 mm needle, at a rate of 0.10 ml/min during 10 min, after administration of priming dose of 0.1 mg. Blood was slowly sampled as described above; if x (%) is the contribution of the portal vein to liver blood supply, the afferent concentration is (x)[portal vein propionate] + $(1-x)$[arterial propionate]; the hepatic balance is the difference [hepatic vein propionate] − [afferent propionate], and the fractional extraction is the ratio hepatic balance/afferent concentration.

Blood was centrifuged ($8000 \times g$, 1 min) and 200 µl plasma was treated by 1 ml absolute ethanol after addition of 50 µl 4 mM sodium isobutyrate. The ethanolic extract was then evaporated to dryness under mild alkaline conditions for subsequent analysis by GLC.

Chromatographic determination of propionate

This was carried out by GLC on a Girdel 30 chromatograph (Delsi, Suresnes, France) fitted with flame ionization detectors. The separation was performed on 2 m × 2 mm i.d. column filled with 20 M poly(ethylene glycol) on chromosorb P. The initial poly(ethylene glycol) coating (5% w/w) was lowered to about 1% with a special thermal treatment [14]. Such columns afford separation of volatile fatty acids at relatively low temperature (100°C) with excellent stability at high sensitivity. Before injection, the samples were redissolved in a minimal volume of 2% H_3PO_4 in order to obtain a final concentration factor of 10.

Results and Discussion

Methodological conditions

A major difficulty encountered in studies dealing with carriers lies in the interferences from metabolism of transported substrates. Endogenous production of propionate in isolated hepatocytes is practically excluded but exogenous propionate is readily metabolized into mitochondria towards Krebs cycle steps, thereafter to glucose or glycogen production. Propionate metabolism is difficult to inhibit: an interesting possibility would be the chemical oxidation of the Co(I) form of vitamin B-12 (involved in the activity of methylmalonyl-CoA mutase) by nitrous oxide. However, assays by gassing 30 min the hepatocytes suspension with nitrous oxide, with parallel gassing with O_2/Co_2 (19/1, v/v), showed that the inhibition of [^{14}C]propionate metabolism was effective but far from complete (about 30%). Since propionate metabolism could hardly be inhibited, the question arises as to whether uptake of label in cell pellets after short term incubations (in the range of 10 s) may be essentially ascribed to propionate alone or is distributed among various intermediates of its metabolism. We have therefore measured propionate uptake by two procedures, at 0.5 mM external propionate: ^{14}C accumulation into cell pellets and determination of effective intracellular concentrations of propionate by GLC. Fig. 1 shows that the evolution of intracellular propionate and of label accumulation followed similar kinetics up to about 20 s. Thereafter, intracellular propionate rapidly levelled off, whereas the accumulation of ^{14}C-labelled products of propionate metabolism accounted for an increasing part of cell pellets label after 20 s incubation. Uptake kinetics of a readily metabolized substrates theoretically provide informations as to transport process only to the extent that transport is rate limiting. There is little doubt that this view is relevant for steady-state conditions. However, there are other conditions in which uptake of label essentially reflects membrane transport: when metabolism is inhibited (but adequate inhibitors are not available) or when metabolism is less active than transport, for initial conditions of substrate uptake for instance. In fact, the metabolism of a small part of the tracer is a problem insofar as it yields products which may

diffuse (or be expelled) out of the cell, such as CO_2 and glucose. Intermediates of propionate metabolism such as CoA derivatives, Krebs-cycle carboxylates and phosphorylated intermediates of gluconeogenesis do not readily diffuse out of hepatocytes. In view of this, we have compared the rate of uptake of label with hepatocytes incubated with [1-^{14}C] or [2-^{14}C]propionate. Owing to randomization in the Krebs cycle at the succinate and fumarate steps, [1-^{14}C]propionate should readily yield $^{14}CO_2$ at the phospho*enol*pyruvate carboxykinase step or at the first revolution in Krebs cycle. In contrast, [2-^{14}C]propionate should not yield $^{14}CO_2$ if directly channeled towards gluconeogenesis ([^{14}C]glucose being then the first diffusible metabolite), and $^{14}CO_2$ would be released from Krebs cycle after two or three revolutions (depending on randomization). In fact, it turned out that no significant differences was observed up to 20 s uptake. Thereafter, the apparent uptake became progressively lower with [1-^{14}C]propionate. This experiment shows that potential differences in the rate of production of diffusible metabolites do not result in noticeable differences in the rate of label uptake, provided that short-term incubations are effected. Consequently, in all subsequents experiments, propionate uptake was studied over 10 s kinetics which affords minimal interference from metabolism.

Time-course of propionate influx in hepatocytes

Fig. 1 shows the time-course of [^{14}C]propionate uptake in a Na^+/Krebs-Ringer bicarbonate medium or in a choline (Na^+-free)/Krebs-Ringer bicarbonate medium, at 0.5 mM external propionate. The rate of propionate uptake was very high, even at this practically physiological concentration since it was 0.15 nmol/10 s per mg cell. Replacement of Na^+ by choline did not affect the rate of propionate entry, regardless the initial external concentration. The uptake of label was linear for about 20 s and a steady-state level was not completely reached up to 2 min. Analysis of propionate uptake were carried out over 10 s. The interpolation of the regression line of propionate uptake practically intercepted the *Y* axis at the origin, suggesting that unspecific fixation of label on hepatocytes was negligible in contrast to branched-chain ketoacids [10]. Time-course studies

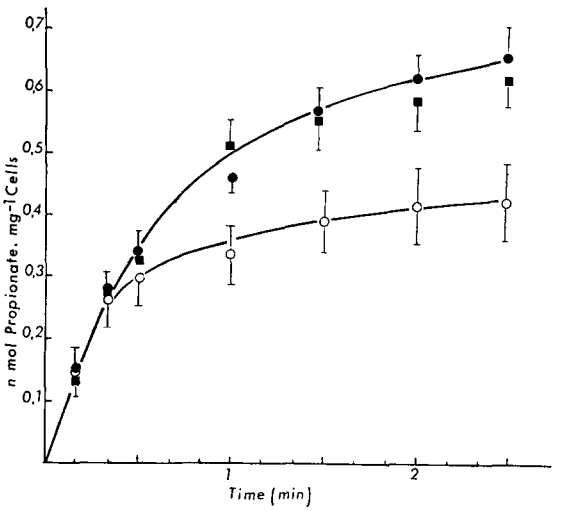

Fig. 1. Time-course of propionate uptake. Hepatocytes were suspended in Krebs-Ringer bicarbonate medium containing 0.5 mM [^{14}C]propionate and [^{3}H]inulin at 37°C. At the requires times, aliquots were transferred into separate microfuge tubes for separation across a silicon oil layer. The supernatant and cell pellets were treated as described in Materials and Methods for propionate determination by GLC (○); in addition an aliquot was withdrawn for parallel determination of radioactivity (●, ■). In a separate batch Na^+ was replaced by choline (■).

of [^{14}C]propionate uptake were also carried out after 5 min preincubation at 0.5 mM propionate and compared with above conditions. It appeared (results not shown) that the kinetics of propionate uptake were not modified by preincubation: this supports the view that unspecific fixation of label on hepatocytes is quite limited and confirms, since propionate metabolism had practically reached steady state, that metabolism minimally interfere with our measurements for short-term (10 s) incubations.

Kinetic analysis of propionate influx

The dependence of the initial rate of propionate uptake on extracellular concentration of substrate was investigated: the entry rate was enhanced with increasing concentrations in the medium, but reached a plateau at very high values. The plot of the initial velocity estimated at 10 s (v) against v/[propionate] was markedly curvilinear (Fig. 2). These results are compatible with a model in which two independent carriers with different affinities contribute to propionate transport. The plots de-

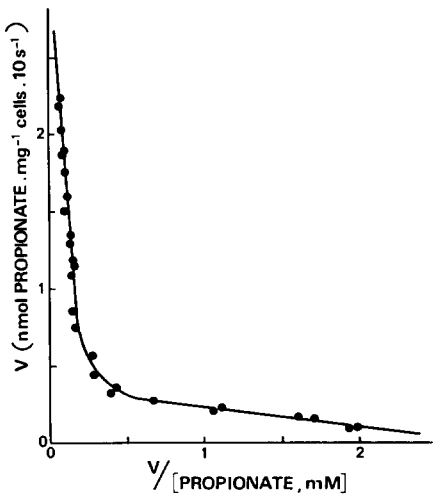

Fig. 2. Kinetics of propionate transport by rat hepatocytes (Woolfe-Augustinson-Hofsee representation). The velocity of propionate transport was measured at various extracellular concentrations. The propionate uptake was measured for 10 s at 37°C.

rived from the experimental points were resolved into two linear components, which affords to determine the kinetic parameters. One of the components represents a relatively high-affinity process with an apparent K_m of about 0.15 mM and a V_{max} of 0.35 nmol/10 s per mg cell. The evaluation of the parameters of the low-affinity component is more difficult, owing to its very high apparent capacity and its low affinity (K_m in the range of 15 mM); since the concentrations of propionate in portal vein are generally in the range of 0.1 to 0.3 mM, the relevance of this component in physiological conditions is questionable. In fact, the Woolfe-Augustinson plot yielded an almost vertical asymptote at high concentrations of propionate, as a result, the low-affinity component is hardly distinguished from passive diffusion itself. In contrast, the properties of the high-affinity component are quite in keeping with a physiological role for this carrier. The capacity of propionate transport (about 2 nmol/min per g cell) appears comparable to that of the monocarboxylates sofar investigated: L-lactate (3–4 nmol/min per g cell) [8] and branched-chain ketoacids (2 nmol/min per g cell) [9]. Propionate transport shares some common features (capacity, high affinity) with branched-chain ketoacids transport described by Kilberg and Gwynn (1983), but markedly differ in its Na^+ requirements [9] since propionate transport is not a Na^+-dependent process and could not therefore be concentrative by such a mechanism.

Effect of inhibitors

In order to further characterize propionate transport, the effects of various monocarboxylates and related substrates have been tested. Table I shows the effect of various substrates at a final concentration of 20 mM in the medium, in the presence of 0.5 mM labelled propionate. Cells were exposed to the tested inhibitors and to propionate at the same time. The volatile fatty acids acetate and butyrate (which are generally available

TABLE I

EFFECTS OF INHIBITORS ON PROPIONATE UPTAKE BY ISOLATED HEPATOCYTES

Incubation conditions were described in Materials and Methods. The various substrates were assayed at 20 mM, in the presence of 0.5 mM labelled propionate over 10 s. The results are the means for triplicate determinations for two experiments.

Inhibitors	Propionate uptake (% of control)
None	100
Aliphatic monocarboxylates and halogenated derivatives	
Propionate	5±3
Acetate	79±5
Butyrate	75±6
Isobutyrate	36±4
Monochloroacetate	14±3
2-Chloropropionate	8±2
Dichloroacetate	53±4
2-Keto monocarboxylates	
Pyruvate	78±8
2-Ketobutyrate	45±3
2-Ketovalerate	51±4
2-Ketoisovalerate	40±3
2-Ketoisocaproate	47±3
2-Ketomethylvalerate	54±4
Others	
L-Lactate	82±9
L-Alanine	105±8
Acetoacetate	70±9
Sulfate	90±8

for hepatic uptake in parallel to propionate) did not noticeably inhibit propionate uptake. The other glucogenic 3-carbon units (pyruvate and lactate) were also ineffective. Some halogenated derivatives of volatile fatty acids displayed a substantial inhibitory effect: the most closely related compound (monochloroacetate) and 2-chloropropionate being the most potent. The branched-chain isomer of butyrate was more efficient than *n*-butyrate and it is noteworthy that the compounds with the same stereoconfiguration (dichloroacetate and 2-chloropropionate) were also inhibitory. Analysis of the inhibition of propionate uptake by varying concentrations of monochloroacetate or 2-chloropropionate was carried out by Dixon plot (Fig. 3). This plot shows that inhibition of propionate uptake was competitive with K_i in the millimolar range: 4 mM for monochloroacetate against 9 mM for 2-chloropropionate. This apparent discrepancy with results in Table I, where 2-chloropropionate appeared more potent, could stem from the fact that high concentrations of 2-chloropropionate (> 10 mM) affect the overall permeability of the cell membrane, as reflected by the higher amounts of [^{3}H]inulin recovered in cell pellets with this compound. In this view, it was examined whether halogenated derivatives of fatty acids affected other unrelated transport systems: Na^+-dependent 2-aminoisobutyrate transport in conditions previously described [15] was unaffected by 20 mM monochloroacetate. It appears that propionate transport is highly specific for carboxylic acids with the formula: CH_3-CH(X)-COOH (or the isosteric equivalent Cl-CH(X)-COOH) since acetate and butyrate were practically uneffective whereas isobutyrate and dichloroacetate were inhibitory. Propionate transport seems less specific as to carbon 2 since it is possible to substitute -H by $-CH_3$ or -Cl but not by polar groups such as -OH or $-NH_2$ (lactate or alanine being ineffective). The effects of 2-ketoacids were also examined: except pyruvate (see above), the other 2-ketoacids investigated exerted a noticeable inhibitory effect, particularly 2-ketobutyrate and 2-ketoisovalerate. It seems thus that an aliphatic chain 'bulkier' than $-CH_3$ is required for inhibition of propionate uptake by 2-ketoacids, but the specificity appears less than for aliphatic carboxylic acids.

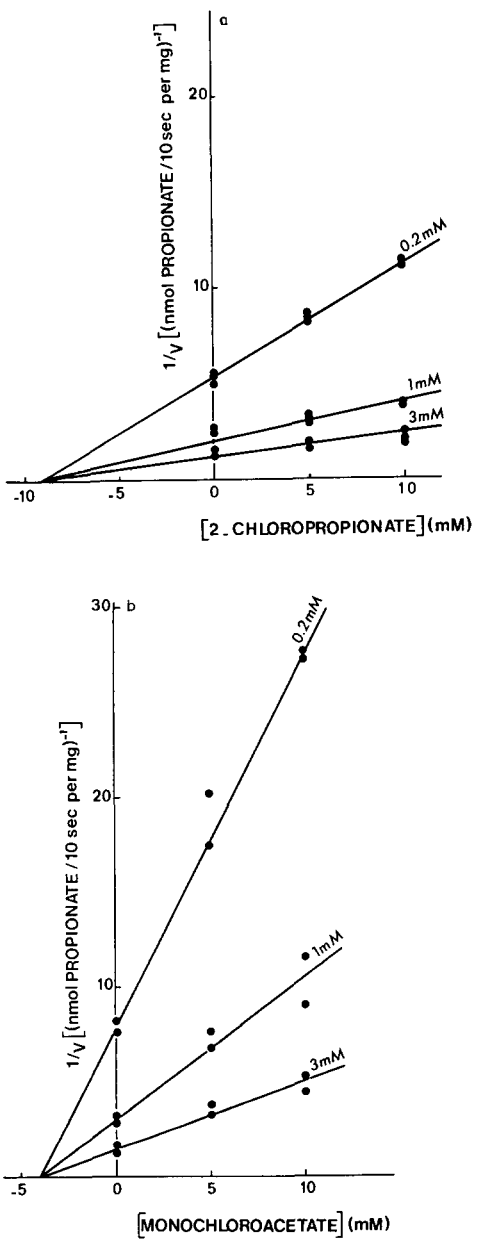

Fig. 3. Dixon plot of the inhibition of propionate transport by 2-chloropropionate (a) and monochloroacetate (b). The initial rate of transport was measured after 10 s of incubation for different concentrations of substrate (propionate) and inhibitors.

It was also interesting to examine the effects of recognized inhibitors of monocarboxylate inhibitors. Owing to the limited solubility of some of them at pH 7.4, they were essayed at 500 μM with

a 10 min preincubation at 37°C; accordingly, the concentration of [^{14}C]propionate was lowered to 0.2 mM. SITS, quercetin and *p*-chloromercuriphenylsulfonate failed to affect propionate transport. More specific inhibitors of monocarboxylate transport (α-cyano 3- and α-cyano 4-hydroxycinnamate) [16] were slightly inhibitory (−16%). However, there is some doubt that propionate could share some common agency with pyruvate, and possibly lactate, since these anions failed to competitively affect propionate uptake. In addition, inhibition by fixation of the cinnamate derivatives on functional -SH groups seems unlikely since *p*-chloromercuriphenylsulfonate was completely ineffective. In the same way, SO_4^{2-} (20 mM) was also ineffective, which support the view that the general anion carrier is not involved in propionate transport.

Effect of pH and possible mechanisms

Propionate uptake was also affected by changes in external pH (Fig. 4a): the rate of uptake was enhanced by decreasing the extracellular pH, these changes being significant over the range of physiological values since uptake was 20–25% enhanced when pH dropped to 7. Conversely, propionate uptake was very responsive to alkaline conditions since it was depressed down to 50% at pH 8. The kinetic characteristics of propionate uptake were assessed for various pH (Fig. 4b), in order to determine if affinity for propionate was affected by changes in H^+. The plot of $1/v$ against $1/s$ demonstrates that the apparent K_m for propionate was not responsive to pH changes. In fact, the influence of external pH could be ascribed to changes in the percentage of the protonated form which is assumed to readily diffuse across the membranes, or to changes in the availability of H^+ if a H^+-propionate anion cotransport was to be operative [17], as suggested for lactate [8]. Furthermore, the existence of an alternative model in which the carrier catalyses the antiport of propionate against some intracellular anion (OH^- for instance) cannot be ruled out [17]. The changes in external pH might also affect the activity of the carrier itself, without apparent modification of the affinity for propionate.

Hepatic uptake of propionate in vivo

This was studied in fed rats in which varying

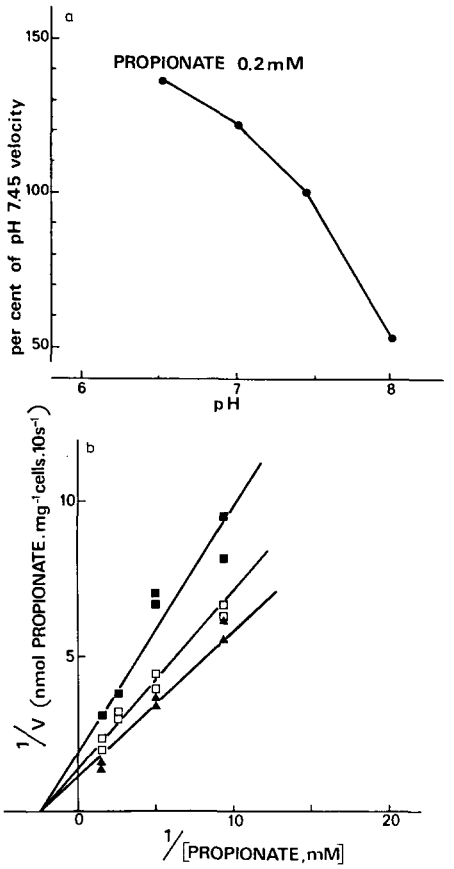

Fig. 4. Influence of pH on propionate uptake by rat hepatocytes. (a) The uptake of 0.5 mM of [^{14}C] propionate was measured at different pH. The results are given as percentage of pH 7.45 velocity. (b) The velocity of propionate transport was plotted against propionate concentrations according to Lineweaver-Burk representation at pH 7.45 (□), 8.02 (■) and 6.40 (▲).

concentrations of portal propionate were obtained by infusion of sodium propionate in saline (0 to 50 mM) into a mesenteric vein at a rate of 0.15 ml/min (see Materials and Methods). As a result, portal propionate varied from physiological values (about 0.2 mM) to about 1.2 mM (Fig. 5). Hepatic uptake of propionate was a linear function of afferent concentrations up to about 0.8 mM, hence a somewhat extraphysiological value. The fractional extraction was 92% in saline-infused controls and remained in this range up to about 0.8 mM in afferent plasma, whereas the efficiency of extraction progressively declined for higher con-

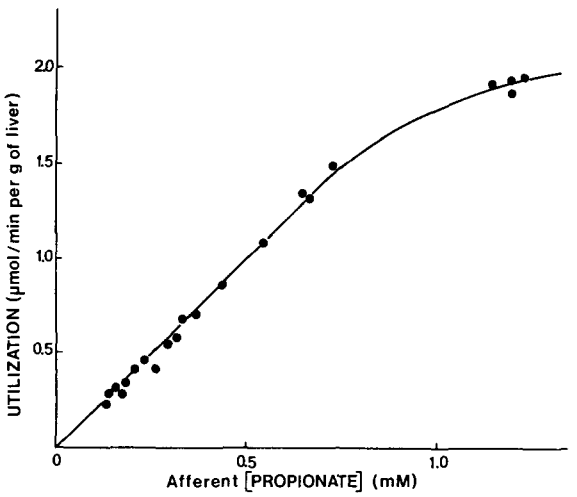

Fig. 5. Rate of propionate utilization by rat liver. Propionate utilization by rat liver ($n = 1$) was plotted against afferent concentrations of propionate which were modified by infusion of propionate into a mesenteric vein. For details, see Materials and Methods.

centrations. Thus, it appears that the liver keeps pace with enhanced supplies of propionate and maintain very low concentrations of propionate in extrasplanchnic blood up to a rate of utilization of about 1.2 μmol/min per g liver. Furthermore, it must be pointed out that V_{max} of propionate transport, at which the high-affinity component should begin to be saturated, is about 2 μmol/min per g cell which corresponds to the value at which the slope of propionate uptake against afferent propionate in vivo deviated from linearity (0.8 mM). This parallel between transport capacities and the hepatic utilization of propionate in vivo suggests that plasma membrane transport is generally not rate limiting. However, this statement has to be qualified since transport processes and cellular metabolism frequently undergo parallel changes, as shown for alanine [18]. In fact, in such a system, transport could be limiting for extreme values of portal propionate: either very high (1 mM, but saturation of metabolic processes cannot be ruled out) or very low, markedly under the K_m value.

The present data suggest that propionate uptake in physiological conditions is almost completely carrier-mediated. Owing to its low K_m and relatively high capacity, this system probably contributes to the highly efficient removal of propionate by the liver. However, this should also be ascribed to the capacity of mitochondrial metabolism since uptake of other glucogenic substrates such as lactate (which carrier as a comparable capacity) [8], or alanine (which carrier is concentrative and adaptative) [18], seldom exceeds 60% of afferent supply. The finding that halogenated monocarboxylates inhibit propionate transport might have some pharmacological relevance since dichloroacetate and 2-chloropropionate have been administrated in large amounts for treatment of hyperlactatemia and lactic acidosis [19,20].

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