Lactate transport in canine red blood cells

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Objective—To detect monocarboxylate transporters (MCTs) in canine RBC membranes and to determine the distribution of lactate between plasma and RBCs.

Sample population—Blood samples obtained from 6 purpose-bred Beagles.

Procedures—Monocarboxylate transporter isoforms 1, 2, 4, 6, 7, and 8 and CD147 were evaluated in canine RBCs by use of western blot analysis. Lactate influx into RBCs was measured as incorporation of radioactive lactate.

Results—2 MCT isoforms, MCT1 and MCT7, were detected in canine RBC membranes on western blot analysis, whereas anti-MCT2, anti-MCT4, anti-MCT6, and anti-MCT8 antibodies resulted in no signal. No correlation was found between the amount of MCT1 or MCT7 and lactate transport activity, but the ancillary protein CD147 that is needed for the activity of MCT1 had a positive linear correlation with the rate of lactate influx. The apparent Michaelis constant for the lactate influx in canine RBCs was 8.8 ± 0.9mM. Results of in vitro incubation studies revealed that at lactate concentrations of 5 to 15mM, equilibrium of lactate was rapidly obtained between plasma and RBCs.

Conclusions and Clinical Relevance—These results indicated that at least half of the lactate transport in canine RBCs occurs via MCT1, whereas MCT7 may be responsible for the rest, although an additional transporter was not ruled out. For practical purposes, the rapid equilibration of lactate between plasma and RBCs indicated that blood lactate concentrations may be estimated from plasma lactate concentrations. (*Am J Vet Res* 2008;69:1091–1096)

In RBCs, ATP is produced by anaerobic glycolysis, which ends in lactate production. The increase in lactate concentration leads to an increase in the concentration of protons, which have to be exported out of the RBC to prevent excessive acidification. The main mechanisms for proton efflux in RBCs are sodium-proton exchange and proton-lactate cotransport by MCT.[1–5] The MCT family consists of at least 14 members, among which the isoforms 1 to 4 are the most extensively characterized and reported to transport lactate.[6] In addition to lactate, MCTs 1 to 4 have been reported to transport pyruvate, ketone bodies, and short-chain fatty acids.[3,6] Monocarboxylate transporter 8 has been shown to transport thyroid hormones,[7] and MTC10 is a carrier of aromatic amino acids,[8] but knowledge of the physiologic function of the other MCTs is limited.

Lactate transport activity of RBCs has wide interspecies variation.[2,4] In general, the transport activity in ruminant RBCs is low and MCTs are absent or have extremely low activity, which suggests that the efflux or influx of lactate occurs mainly by nonionic diffusion.[2,4] In human RBCs, lactate transport activity is high in comparison to ruminants[4] and the main lactate carrier is MCT1, which is also the only MCT isoform detected in human RBCs.[3] Horses are an exception among the species studied because their lactate transport activity is bimodally distributed with 75% having activities equal to those in humans and the rest having activities similar to those measured in ruminants.[4,9] In contrast to other species studied, horses have 2 MCT isoforms, MCT1 and MCT2, on their RBC membranes. However, irrespective of the large variations in the lactate transport activity, the interindividual variation in the amounts of MCT1 and MCT2 proteins is small,[10] and the reason for the inactivity of MCT1 is apparently the lack of an ancillary protein, CD147, indispensable for the function of MCT1.[10–12] In canine RBCs, lactate transport activity is high in comparison to that in other species and 70% to 90% of the transport appears to be mediated by MCTs.[2,4] The isoform or isoforms of MCT found in canine RBCs are not known.

Abbreviations

CHC	α-Cyano-4-hydroxycinnamate
K_m	Michaelis constant
MCT	Monocarboxylate transporter
pCMBS	p-Chloromercuribenzenesulfonic acid
TBST	Tris-buffered saline solution with 0.1% Tween
V_{max}	Maximal velocity of transport

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At resting conditions, efflux of lactate and protons from RBCs dominates, but during intense exercise, when the efflux of lactate from muscles is high, RBCs may take up lactate from plasma.[13] The accumulation of lactate into RBCs, which has been demonstrated at least in humans and horses,[5,13] may help to maintain the concentration gradient between plasma and the exercising muscle that is the driving force for lactate efflux.

Accumulation of lactic acid into RBCs may influence the comparison of blood and plasma lactate concentrations.[14] If the rate of lactate influx into RBCs is high, the difference between blood and plasma concentrations is smaller than when only a small amount of lactate is transported into RBCs. This is especially important in horses, in which interindividual variations in the lactate transport activity are large.[9,14] The purposes of the study reported here were to detect MCTs in canine RBC membranes and to determine the distribution of lactate between plasma and RBCs.

Materials and Methods

Animals—The experimental design was approved by the Ethics Committee for Animal Experiments at the University of Helsinki. In these studies, 6 clinically normal purpose-bred Beagles (3 spayed females and 3 castrated males) were used. Dogs were 5 to 6 years old and weighed 14 to 18 kg. They were housed in a group and allowed to exercise outdoors daily in a pen. Dogs were fed commercial dog food twice a day, and water was freely available. Tissue specimens (liver and skeletal muscle) were also taken from a euthanatized dog that was donated to the faculty for teaching and research. This dog was euthanatized by IV injection of thiopental after medetomidine-butorphanol premedication.

Lactate transport activity—To measure the lactate transport activity of RBCs, blood from a jugular vein was collected into tubes containing EDTA. Lactate transport activity was measured, as described previously.[2,9] Red blood cells were separated from plasma by centrifugation (for 10 minutes at 2,000 × *g*), incubated in a large volume of tricine buffer (150mM NaCl; 10mM tricine; pH, 7.6) for 30 minutes at 37°C to remove endogenous lactate, and washed 3 times with the same buffer. The PCV was adjusted to 30% with HEPES buffer (90mM NaCl; 50mM HEPES; pH, 7.4). Groups of RBCs were then incubated in the HEPES buffer containing 0.1, 0.5, 1.0, 2.5, 5.0, 10, or 20mM radioactive lactate (L-[U-^{14}C] sodium lactate; specific activity, 5.62 GBq/mmol)[a] for 15 or 20 seconds at 37°C. Incubation was terminated with ice-cold buffer (150mM NaCl; 10mM 2-[N-morpholino] ethanesulphonic acid; pH, 6.4), and RBCs were washed with the same buffer to remove external radioactivity. Washed RBCs were lysed with 4.2% perchloric acid, the precipitate was removed by centrifugation, and the radioactivity of the supernatant was measured with liquid scintillation counter.[b] All measurements were made in triplicate. The rate of lactate influx is given as µmol of lactate/(mL of RBCs × min). To study the role of MCTs in lactate transport, inhibitors CHC (5mM) and pCMBS (1mM) were used. In the inhibition studies, the lactate concentration was 5mM.

Immunoblotting—Cell membranes for western blot analysis were prepared from washed RBCs, liver tissue, and skeletal muscle tissue, as previously decribed.[10,15] Washed RBCs were hemolyzed with 5mM sodium phosphate buffer at pH 8.0 and washed 3 to 4 times with the same buffer to remove hemoglobin. After determination of protein concentration with the bicinchoninic acid method,[c] the membranes were stored at –75°C until analyzed. Similarly, purified membranes from human RBCs were used as controls. Membrane proteins (total protein, 10 µg [except 20 µg for MCT1]), solubilized in Laemmli buffer,[16] were separated on a 10% (wt/vol) SDS-PAGE gel (200 V; 50 minutes) and electrotransferred (100 V; 1.5 hours) onto a nitrocellulose filter.[d] The nonspecific binding sites were blocked with 10% dry milk in TBST for 1 hour at room temperature (approx 21°C). The filter was incubated with a primary antibody in TBST supplemented with 10% dry milk overnight at 4°C. Thereafter the filter was washed for 15 minutes and 3 times for 5 minutes in TBST, followed by 1 hour of incubation with the secondary antibody in 2.5% dry milk. Filters were washed with TBST for 15 minutes followed by 2 washes for 5 minutes each. Immunodetection was performed with an enhanced chemiluminescence system[e] according to the instructions of the manufacturer. The densities of protein bands were quantified with densitometer[f] connected to appropriate software.[g]

The primary antibodies used were anti-human MCT1 and MCT4 raised in rabbits against C-terminal peptides,[10,h] chicken anti-rat MCT2[i] (peptide NTHNPPSDRDKESSI), rabbit anti-human MCT6[j] (former name, MCT5; 18-amino-acid–peptide sequence within the cytoplasmic terminus of human MCT6), rabbit anti-human MCT7[k] (former name, MCT6; 20-amino-acid–peptide sequence within the cytoplasmic C-terminus of human MCT7), rabbit anti-mouse MCT8[l] (19-amino-acid–peptide sequence within the cytoplasmic C-terminus of mouse MCT8), and unpurified antiserum against CD147 made in rabbits.[m] The presence of MCT7 was tested also with another anti-human MCT7 antibody raised in rabbits (MCT75-A),[n] and the specificity was verified with peptide blocking (MCT75-P).[n] Secondary antibodies were horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin[o] and horseradish peroxidase–conjugated rabbit anti-chicken immunoglobulin.[p]

In vitro distribution of lactate—To study lactate distribution between plasma and RBCs, sodium lactate (pH, 7.0) was added to fresh venous blood samples to bring the lactate concentration to 5mM, 10mM, or 15mM. Thereafter, the blood was incubated for 5 minutes at 37°C. The influx of lactate into RBCs was stopped by rapid cooling of the tubes in ice water. Incubations were performed in tubes that contained NaF to prevent endogenous formation of lactate in RBCs. Part of each sample was rapidly centrifuged at 4°C and plasma separated. Lactate concentrations in blood and plasma were measured with a lactate analyzer.[q] The PCV was determined and used for the calculation of RBC lactate concentration according to the following equation:

$$LC = (LB - [1 - PCV] \times LP)/PCV$$

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in which LC was the lactate concentration in RBCs, LB was the lactate concentration in blood, and LP was the lactate concentration in plasma. This formula does not give the exact lactate concentration in RBCs, which would require a correction for water content in plasma and RBCs.[17]

To study the effect of sample storage on lactate distribution, sodium lactate was added to the venous blood samples to bring the concentration to 10mM and incubated for 5, 15, 30, or 60 minutes at room temperature. Lactate concentrations in the blood and plasma were measured as described.

Statistical analysis—Results are reported as mean ± SEM. A Student *t* test (paired) was used to compare sets of incubations, and when > 2 sets were compared, an ANOVA with the Tukey posttest was used. Relationships between measured variables were tested with Pearson correlation analysis. Values of $P < 0.05$ were considered significant.

Results

Lactate transport activity—When washed RBCs were incubated at lactate concentrations of 0.5mM to 20mM, the apparent K_m and V_{max} values for lactate influx were 8.8 ± 0.9mM and 8.3 ± 0.6 µmol/(mL of RBCs × min), respectively, (**Figure 1**). It should be kept in mind that both values represent the sum of all transport mechanisms including nonionic diffusion. At a lactate concentration of 5mM, pCMBS inhibited the influx by 50% and CHC by 70% (**Table 1**).

Immunoblotting—Antibodies used were not specific for canine RBC membranes. Therefore, the reactivity was tested also with cell membranes purified from canine liver and skeletal muscle specimens (**Table 2**). All the antibodies tested in our study cross-reacted with cell membranes of canine liver, canine skeletal muscle, or both.

On RBC membranes, anti-MCT1 antibody recognized a double band at 47 and 49 kDa. A double band was also detected for cell membranes derived from canine liver but not for canine skeletal muscle (results not shown). The amount of MCT1 detected indicated that a large interindividual variation existed (**Figure 2**), with a range from 0.027 to 0.486 arbitrary units. Results of western blot analysis revealed a signal of approximately 97 kDa for MCT7 in canine RBC membranes. When canine RBCs were analyzed after freezing and thawing, the 97-kDa band was attenuated and a band at 48 kDa was observed. The interindividual variation in the amounts of MCT7 (ie, range from 0.153 to 0.182 arbitrary units) was much less than that of MCT1. Human RBC membranes were tested as controls, but no bands were detected with anti-MCT7 antibody. Other MCTs tested (MCT2, MCT4, MCT6, and MCT8) did not result in any signal in immunoblots of canine RBC membranes (results not shown), although they recognized MCTs in cell membranes of canine liver and skeletal muscle tissues (Table 2). The ancillary protein for MCT1, CD147, was detected on western blot analysis at approximately 52 kd in canine RBC membranes.

Amounts of MCT1 or MCT7 were not correlated with the measured rate of lactate influx, whereas the amount of CD147 detected had a positive correlation at all lactate concentrations tested from 0.1mM to 20mM, with the exception of 1mM (**Figure 3**). The amount of CD147 was not correlated with the amount of MCT1 or MCT7.

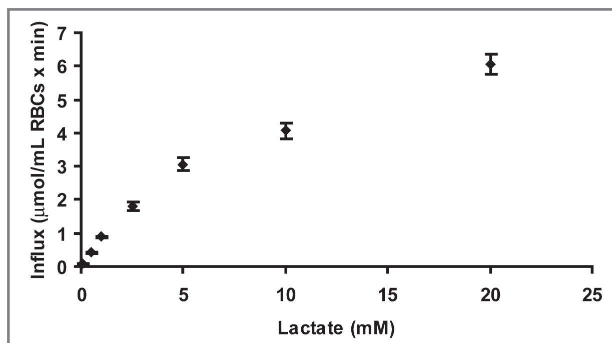

Figure 1—Mean ± SEM total lactate influx into canine RBCs incubated in various concentrations of lactate (n = 6).

Table 1—Effect of inhibitors of MCTs on mean ± SEM lactate influx into canine RBCs. Washed RBCs from 6 dogs were incubated at a lactate concentration of 1mM or 5mM in the presence and absence of CHC (5mM) or pCMBS (1mM).

Inhibitor	Lactate concentration	Lactate transport (nmol/mL of RBCs × min)	Inhibition (%)
None	1mM	918 ± 78	NA
None	5mM	4,029 ± 251	NA
CHC	5mM	1,263 ± 140	69 ± 2
pCMBS	5mM	1,890 ± 165	53 ± 3

NA = Not applicable.

Table 2—Detection of MCT proteins in canine liver, skeletal muscle, and RBCs.

MCT isoform	Antibodies*	Tissue type			Apparent molecular weight (kDa)
		Liver	Skeletal muscle	RBCs	
MCT1	Rabbit anti-human[h]	+	+	+	50
MCT2	Chicken anti-rat[i]	–	+	–	39
MCT4	Rabbit anti-human[h]	–	+	–	47
MCT6	Rabbit anti-human[j]	+	+	–	50
MCT7	Rabbit anti-human[k]	+	+	+	52
MCT8	Rabbit anti-mouse[l]	+	+	–	50

*Superscript letters within column are footnote citations.
+ = Protein detected. – = Protein not detected.

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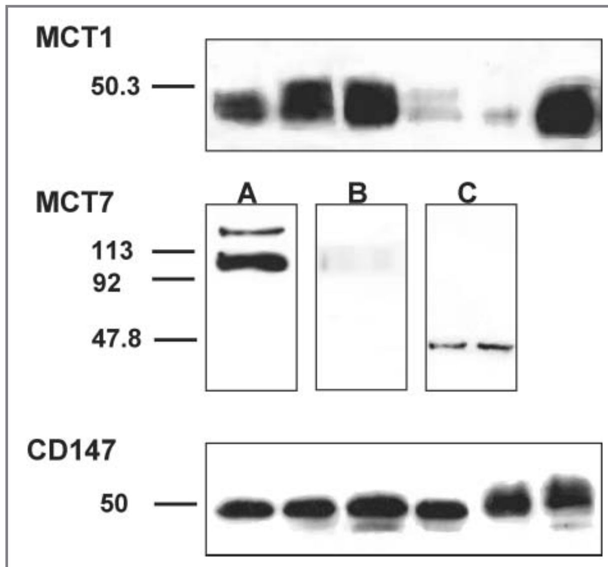

Figure 2—Immunoblots of MCT1, MCT7, and CD147. The 3 panels for MCT7 represent canine RBC membranes (panel A), human RBC membranes (panel B), and freeze-thawed canine RBC membranes (panel C). Values are in kilodaltons.

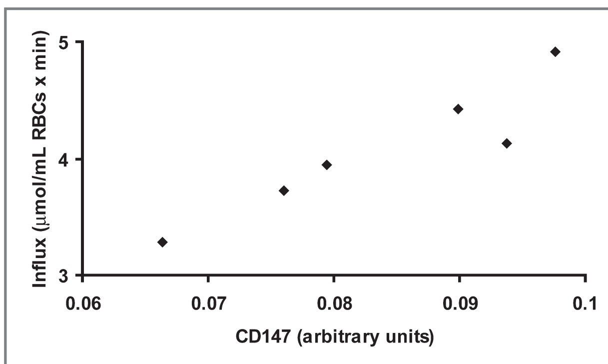

Figure 3—Positive correlation ($r = 0.920$; $P < 0.01$) between the amount of CD147 in arbitrary units, an ancillary protein needed for full activity of some MCT isoforms, and lactate influx into canine RBCs incubated in a lactate concentration of 10mM.

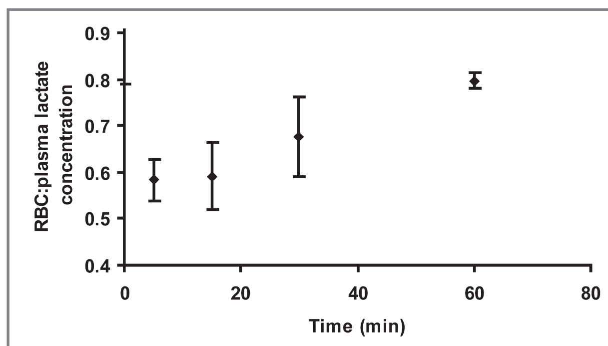

Figure 4—Mean ± SEM RBC-to-plasma lactate concentration ratio after incubation of canine RBCs for 5 to 60 minutes at room temperature in a lactate concentration of 10mM. The increase in the ratio was linear with time ($r = 0.992$; $P < 0.01$).

In vitro incubations—When fresh blood samples were incubated for 5 minutes at lactate concentrations of 5mM, 10mM, and 15mM, the RBC-to-plasma lactate concentration ratio remained constant (mean of all values, 0.69 ± 0.04). When RBCs were incubated at a lactate concentration of 10mM for 5 to 60 minutes, a linear increase ($r = 0.992$; $P < 0.01$) was found in the amount of lactate in RBCs over time (**Figure 4**). At the end of the 1-hour incubation period, 43% of the added lactate on average was in the RBCs.

Discussion

The results of this study confirm the earlier findings that the rate of lactate transport in canine RBCs was similar to that reported previously but substantially higher than in other species, such as humans, ruminants, and horses.[2,4] For example, in comparison to RBCs of humans[4] and horses,[2,4] the rate of transport in canine RBCs at a lactate concentration of 10mM was approximately 1.5 times as high and the difference with ruminants was more than 50-fold.[4] Like several other species,[3,10,18] canine RBCs have MCT1, but we also found MCT7, which, as far as we know, has not previously been found in RBCs. Our results on the use of known inhibitors of MCTs, pCMBS and CHC, indicate that lactate transport in canine RBCs is facilitated by MCTs by at least 70% (Figure 1). Measurement of transport activity and the results of inhibition studies suggest that the activity of MCTs, and not the nonionic diffusion, in canine RBC is higher than that in other species. The high rate of lactate transport in canine RBCs could be the result of the amount of MCT1, the activity of MCT1 through its ancillary protein CD147, or lactate transport via MCT 7.

The MCT1 antibody used in the present study was produced against a 15-amino-acid peptide that corresponds to the C-terminus of human MCT1. Comparison of the peptide with the corresponding canine sequence reveals that 14 out of 15 amino acids are identical, including 2 prolines that affect the angle conformation of the peptide. The only difference was found in the middle of the peptide, where 1 glycine in the human sequence is serine in the canine sequence. We do not know whether this affects the results, but the resulting bands, 47 and 49 kd, were within the range of the reported molecular weight of MCT1 in dogs and other species.[6,15,19] A double band at 47 and 49 kd was also detected for cell membranes derived from canine liver but not for canine skeletal muscle (results not shown) in our study. The reason for the double band is unclear, but similar double bands have been reported.[20] The amount of MCT1 detected in canine RBCs indicated that a large interindividual variation existed, which cannot be attributed to the species specificity of the antibody. The amount of MCT1 detected had no correlation to lactate transport activity, which suggests that the high rate of lactate transport in canine RBCs is not the result of the amount of MCT1.

On plasma membranes, MCT1 forms a complex with CD147[21] and lactate transport occurs only when both proteins are present.[10] In dogs, CD147 was found at an apparent molecular weight of 52 kd, which is close to that found previously in a canine kidney cell line.[22] As in equine RBCs, the amount of CD147 was found to correlate with the lactate transport activity.[10]

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To further study the role of MCT1-CD147 complex in lactate transport, we used pCMBS, which covalently modifies thiol groups in the extracellular part of the CD147 molecule.[12] In canine RBCs, the inhibition of lactate transport was 50%. Our results of experiments with pCMBS also revealed that the correlation between CD147 and the rate of lactate influx disappeared when pCMBS was present, suggesting that the transporter responsible for the MCT1-independent transport was not dependent on CD147.

The other MCT that was detected in canine RBC membranes in our study was MCT7. According to the manufacturer[k] of the antibody against MCT7, rabbits were immunized with a 20-amino-acid peptide that is within the cytoplasmic C-terminus of MCT7. The exact location of this 20-amino-acid peptide is not known, but comparison of the first 31 amino acids from the C-terminus of human MCT7 to the corresponding sequence in dogs reveals 26 identities. The specificity of the MCT7 band was tested with another MCT7 antibody[n] and the blocking peptide,[n] and the results support the view that the band detected is MCT7. In freshly purified canine RBCs of our study, the apparent molecular weight of MCT7 was 97 kDa, which suggests that MCT7 was present as a dimer or a heterodimer with an ancillary protein. According to Wilson et al,[12] such a dimer of membrane proteins may be stable enough to withstand the denaturating conditions of SDS-PAGE. Also, the finding that after freezing and thawing of canine RBCs, MCT7 was detected at 48 kDa, which is within the range found in rat tissues,[23] supports the view that the 97-kDa band is a dimer. The amount of MCT7 did not correlate with the lactate transport activity, suggesting that either lactate is not a substrate for MCT7 or its activity is regulated by an ancillary protein. So far, MCT7 is an orphan transporter with no known physiologic function, but if it transports lactate, it could explain the results we found.

In accordance with results of earlier studies,[3] we found that human RBCs have only MCT1. The antibody against MCT7 that was used was an anti-human MCT7 antibody and has previously been reported to react with MCT7 in human skeletal muscle.[23]

We calculated the apparent maximal velocity of lactate influx in canine RBCs and found that it was high in comparison to V_{max} in equine RBCs,[9,10] which have MCT1 and MCT2 on their RBC membranes.[10] The apparent K_m value measured in our study is of the same order of magnitude as reported by Skelton et al[2] and lower than the measured K_m in equine RBC membranes under identical conditions.[2,9] Although interpretations of the K_m and V_{max} data are difficult because they include the nonionic diffusion and the activities of anion exchange protein and MCTs, they, together with the inhibition by pCMBS, leave open a possibility that MCT7 is a lactate transporter.

In horses, the bimodal distribution of lactate transport activity causes great interindividual differences in plasma and RBC lactate concentrations.[14] This makes comparison of plasma and blood lactate concentrations impossible and has to be considered, for example, in studies where lactate concentrations are measured after exercise. To study the distribution of lactate in plasma versus RBCs in our study, canine RBCs were incubated with increasing concentrations of lactate. Our results indicate that the relative distribution of lactate between plasma and canine RBCs remained fairly constant throughout the concentration range tested. This indicates that in dogs, as in humans,[24] plasma lactate concentrations can be used to estimate blood lactate concentrations when the PCV is known. Lactate influx into canine RBCs is rapid, and our results indicate that a near equilibrium state is reached in less than the 5-minute incubation time used in this study. However, if one wishes to study plasma lactate concentrations, storage of blood samples at room temperature for more than 15 minutes before plasma is separated leads to increased accumulation of lactate into RBCs, and thus, the concentrations in plasma will be lower than at the time of sample collection.

In summary, the results of this study indicate that in canine RBCs, MCT1 appears to require CD147 for its activity. The MCT1-CD147 complex is responsible for at least 50% of lactate transport into canine RBCs. Another MCT isoform, MCT7, was also detected. Although MCT7 is an orphan receptor, it could explain why the rate of lactate influx in canine RBCs is higher than in other species.

a. L-[U-^{14}C]lactic acid, sodium salt, Amersham Pharmacia Biotech, Buckinghamshire, England.
b. WinSpectral 1414, Wallac Ltd, Turku, Finland.
c. Uptima BCAssay, Interchim, Montlucon, France.
d. Protran, Schleicher and Schuell, Dassel, Germany.
e. SuperSignal WestDura, Pierce, Rockford, Ill.
f. Bio-Rad Fluor-S MultiImager, Bio-Rad Laboratories Inc, Hercules, Calif.
g. Multianalyst, version 1.1, Bio-Rad Laboratories Inc, Hercules, Calif.
h. Sigma Genosys, Pampisford, Cambridge, England.
i. Catalogue No. AB1287, Chemicon, Temecula, Calif.
j. Catalogue No. AB3552P, Chemicon, Temecula, Calif.
k. Catalogue No. AB3554P/AB3322P, Chemicon, Temecula, Calif.
l. Catalogue No. AB3556P, Chemicon, Temecula, Calif.
m. Courtesy of Dr. William Mawby, University of Bristol, Bristol, England.
n. Alpha Diagnostic International, San Antonio, Tex.
o. DakoCytomation, Glostrup, Denmark.
p. Catalogue No. AP19P, Chemicon, Temecula, Calif.
q. YSI 2300 STAT, Yellow Springs Instrument Inc, Yellow Springs, Ohio.

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