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Evaluation of methods used *in vivo* to estimate the transfer of amino acids and other substrates into the cells of mammary gland

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ABSTRACT

In recent years some approaches were developed to estimate the parameters of transport of amino acids and other substrates into the cell *in vivo* using arteriovenous concentration difference and volume blood flow data with their recalculation according to kinetic model of the system: capillary - interstitium - cell. With the use of literature data and the own results the authors analysed the present feasibility of this method in the studies of mammary metabolism in productive ruminants The model estimates were compared with independent measurements (with ^{13}C-amino acids), the verifying experiments were conducted and some unresolved problems and errors of interpretation were investigated. The method is recommended to study the adaptive shifts in the activity of amino acid transport into the mammary secretory cells during milk formation in various nutritional states.

KEY WORDS: mammary gland, secretory cells, amino acids, transport activity, milk protein yield, modeling

INTRODUCTION

The supply of the cellular biosynthetic machines by the substrates is determined mainly by their concentration in arterial blood, the rate of level of volume blood flow and the state of transport system localized in basolateral membranes of the cell. The quantitative parameters of transport system for many substrates (amino

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acids, etc.) have been studied up to now almost exclusively *in vitro*, but estimation of transport activities in physiological experiments needs specific methodological approaches, that must be noninvasive and take into account the system control of total pool of transporters.

During investigation of metabolic processes in intact organs and tissues with the use of traditional physiological techniques, including measurement of arteriovenous concentration difference and volume blood flow rate, in recent years the relatively simple methods have been developed to estimate parameters of transport into the mammary cells, based on kinetic consideration of mass transfer in the system: capillary - interstitium - cell (Hanigan et al., 1998; Bequette et al., 2000; Cherepanov et al., 2003). The aim of this paper was the further development of this new approach using results of own investigations and literature data, with special attention given to transport of amino acids into mammary secretory cells.

MODEL

The transcapillary exchange of water soluble low molecular mass substrates (e.g., amino acids, glucose, acetate, etc.) takes place, by definition, through water-filled pores: filtration on the arterial side and reabsorption on the venous side of capillaries and in small venules. Designations: c_a, c_v, c_e - concentration of metabolite in plasma of arterial and venous blood, and extracellular fluid respectively, mM. V_{max} - the maximal rate of transfer of metabolite into the cell at high level of this metabolite in the extracellular fluid (mol/time unit per whole organ); Q - volume plasma flow rate (l/time unit). Let us consider a simple system with unidirectional flux of metabolite through basolateral membrane. The total rate of metabolite uptake by the organ is equal to total rate of transport (v) which depends on extracellular concentration with saturation effect:

$$Q\,(c_a - c_v) = \frac{V_{max} c_e}{K_m + c_e} \qquad (1)$$

The value of K_m for many transporters is fairly large compared to mean physiological concentration of the relevant substrate in the extracellular fluid. In these cases the rate of transport approximately linearly depends upon concentration: $v \approx (V_{max}/K_m)\, c_e = T\, c_e$, where T is a rate constant or transport activity expressed in units of clearance per organ (volume unit/time unit).

In equation (1) the changes in venous concentration due to fluid outflow with milk and lymph are not taken into account since these values are negligible (the ratio of daily blood flow to milk yield is in average 500:1). The existence of intensive transcapillary exchange through pores of capillary endothelium permits us to suggest, as a first approximation, that venous concentration of

metabolite as equilibrated with its concentration in extracellular fluid, i.e. $c_v = c_e$. The experimental evidence for this suggestion will be discussed in following section.

Taking these approximations, from equation (1) we have the following relationship:

$$Q\,(c_a - c_v) = c_v T \tag{2}$$

Transforming equation (2) we have the dependence of venous concentration on arterial concentration, which is identical to that described by Hanigan et al. (1988):

$$c_v = \frac{Qc_a}{T+Q} = \frac{C_a}{1+\frac{T}{Q}} \tag{3}$$

Hence, the arteriovenous concentration difference:

$$c_a - c_v = \frac{Tc_a}{T+Q} = \frac{c_v T}{Q} \tag{4}$$

Efficiency of extraction from the blood, $E = (c_a - c_v)/c_a$:

$$E = \frac{T}{T+Q} \tag{5}$$

So, from the relationship (2) we obtain formulas for estimation *T in vivo:*

$$T = \frac{Q(c_a - c_v)}{c_v} \tag{6}$$

or, transforming equation (6):

$$T = Q\frac{E}{1-E} \tag{7}$$

Equation (7) may be used with the aim to recalculate the experimental data described in the literature when the authors do not present the concentration data but use only *Q* and *E*.

From equation (5) it follows that extraction efficiency depends non linear on the transport activity with saturation at high values of *T*. The transport activity *T* represents the parameter that in the general case is a function of total pool of

membrane transporters.

The values of activity (rate constants) is usually expressed in units $(time)^{-1}$ but in the case considered the uptake of substrate by the organ (mol/min) is measured and divided by concentration (mol/l), so we obtain l/min. This is an by concentration (mol/l), so we obtain l/min. This is an activity of membrane transporter system, expressed on whole organ. If the animals of compared groups have similar liveweight, age, etc., they have similar mammary volume, in the average, and we may use *T* by the volume of substrate distribution (blood plasma + interstitium).

In the experiment we measure *Ca*, *Cv* and *Q* and transform these measurements to estimate *T*. The question is how much credence we may give to *T* in our attempts to interpret the shifts in *Ca*, *Cv*, uptake and blood flow observed in physiological trials. The task of this paper is to draw attention of experimenters on the need to use this sample alternative parameter instead of A-V difference and *E*.

Analysis of model postulates.

Physiological concentration of many free amino acids in blood and extracellular fluid is smaller than K_m for systems transporting these amino acids into the cells of various tissues (Baumrucker, 1985). In physiological experiments with infusion of radioactive tracers the linear trends of the rate of transport into muscle were established in brood range of concentration for arginine, lysine, treonine and tryptophan in rats (Banos et al., 1973). The value of K_m for histidine transport in the heart was equal in pig to 0.7 mmol/M which exceeds the physiological concentration in blood approximately by a factor of 10 (Hinde and Ahmed, 1998). The value of K_m for methionine transport in rat liver cells was equal to 1.56 mM and physiological concentration was 0.04 mM (Crawhall and Purkiss, 1973). In the experiments with *in situ* perfusion of lactating mammary gland the linear trend of rate of glutamine uptake was observed up to 2 mmol/l, whereas glutamine concentration in blood plasma and in milk was equal to 0.7 and 0.03 mmol/l, respectively (Calvert et al., 1998).

Similar situations are described also for other water soluble metabolites. Experimental data suggest that glucose transporters are not saturated in mammary secretory cells at a mean physiological concentration (Faulkner, 1985; Neville et al., 1990). The uptake of acetate and β-hydroxybutyrate by mammary gland is linear dependent on its concentration in arterial blood (King et al., 1985; Makar et al., 2003).

The existence of intensive transcapillary transfer of low molecular water soluble substrates has been shown to occur in the mammary gland (Prosser et al., 1996; Farr et al., 2000). Using ^{14}C-sucrose with non diffusible tracer Farr

et al. (2000) established that the total rate of filtration expressed as a product of a capillary permeability by the surface area of capillary was equal to 45 ml/100 g of tissue at a volume blood flow 50 ml/100 g of tissue (Prosser et al., 1996). The logic consequence of an intensive transcapillary transfer and mixing of water in the interstitium may be that venous concentration in the mammary gland is representative of extracellular concentration.

Comparison with independent method

The measurements described in the literature (Bequette et al., 2000) were used as a material for analysis. Calculated according to equation (7) with the use of published values of extraction efficiency and mammary blood flow, values of T were compared with the values of rate constant of unidirectional transfer of individual amino acids from interstitium into mammary secretory cells (K_{in}), obtained on the same animals (goats) with the use of mixture of U- ^{13}C- amino acids. During first experimental period the balanced mixture of amino acids was infused, compensating 23% deficit of the requirement for available protein, and during second period the same mixture without histidine was infused; the mammary plasma flow during first and second periods was 0.46 and 0.61 l/min, respectively. By correlating the values of K_{in} with respective values of T the reasonable consistency was revealed for 9 individual amino acids (Figure 1), although the values of T

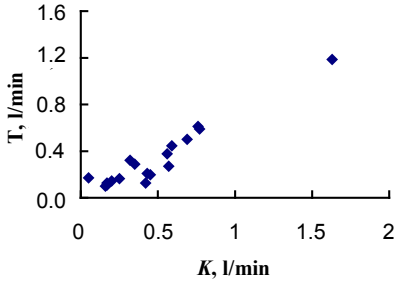

Figure 1. The correlation of the rate constant of unidirectional transfer of individual amino acids from interstitium into mammary secretory cells (*K,* l/min), estimated with the use of U - ^{13}C- amino acids (Bequette et al., 2000), with the activity of net transport (*T,* l/min) estimated on the base of arteriovenous concentration difference and blood flow measured on the same animals. The values are the combined data for 9 amino acids and two experimental periods

were slightly smaller as compared with K_{in}, as should be predicted, since T, as will be discussed in following section, represents the net flux and unidirectional flux is partly compensated by the counter flux from the cell.

Verification of the model in the experiment

The model predicts (see equations (3) and (4) that variations in blood flow will cause the variations in arteriovenous difference and extraction efficiency that are reverse in relation to changes in blood flow. This theoretical prediction was substantiated in the experiment carried out on lactating cows which included 3 periods each of 24 h (Makar et al., 2003). During two last periods the concentrates were removed from the ration with following realimentation to level of that in the first period. During all three periods the level of glucose, free amino acids (α-amine N), β-hydroxybutyrate and acetate did not change significantly, but mammary blood flow decreased and at the end of 24-h experimental period it diminished up to 50% compared to control period; the uptake of substrates decreased synchronous to blood flow dynamics, whereas arteriovenous difference and extraction efficiency during this period increased. The activity of transport during concentrate deprivation was characterized by clearly manifested tendency to decreasing, which was statistically significant for α-amine N.

The second experiment was performed on lactating goats using hyperinsulinaemic euglycaemic clamp (Makar et al., 2007). During insulin infusion the blood level of α-amine N and glucose did not change significantly, but mammary blood flow, uptake of α-amine N and glucose, the activity of transport of α-amine N and glucose, the rate of milk formation and protein yield

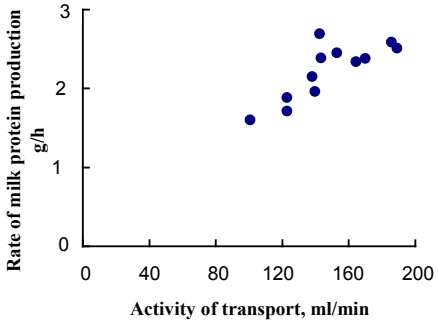

Figure 2. Relationship between the rate of milk protein production and the activity of net amino acid transport (α-amine N) into goat's mammary cells (r =0.8; P<0.001) defined in the experiment with intravenous infusion of insulin and glucose (Makar et al., 2007). The values are the combined data for 3 periods, n=4

were increased, whereas arteriovenous difference for α-amine N was statistically significant decreased and that for glucose did not change compared to control period. Using combined data, the highly significant correlation of the activity of transport of amino acids (α-amine N) with the rate of milk protein production was revealed (Figure 2), whereas extraction efficiency for amino acids did not not be contrary to predictions of the model.

SOME INRESOLVED PROBLEMS AND GUIDELINES TO APPLICATION

From theoretical viewpoint, the validity of the model might be verified by examining the empirical relationships between arteriovenous difference and arterial concentration (see equation 4). Unfortunately, the own experimental and literature data show that linear dependencies on the empirical figures usually are displaced relative to the origin of the coordinates, so the Y-intercept is not equal to 0, as predicted by the model, but is a large negative number (Baldwin and Smith, 1983; Hanigan et al., 1992). The most probable reason for this phenomenon, in our opinion, seems to be the large random error of the experiment, although there may be equally valid alternative explanations of the negative intercept.

With the aim to give proof to our suggestion we carried out the numerical experiment using method of statistical trials (Monte-Carlo method). The stationary case was analysed when arterial and venous concentrations are constants and the scattering of the experimental points is due to random error. In this case the values of arterial (*ca*) and venous (*cv*) concentrations are normally distributed. Aimed for modeling consecutive measurements of constant concentration, 30 numbers (*zi*) were taken from the table of random numbers with normal distribution, average value M = 0 and variance $\sigma = 1$, and then the values of "measured" arterial and venous concentration were calculated as $Ci = M + zi\ \sigma$ on the assumption that "real" arterial and venous concentration (*Ma* and *Mv*) are measured with the same relative error. So, for each variant of calculation 30 pares of ca and cv values and regression ellipses $cv = f(ca)$ and $(ca - cv) = f(ca)$ were obtained. As might be expected, on these figures the regression cv on ca is absent, but regression lines $(ca - cv) = f(ca)$ are displaced relative to the origin of the coordinates with large negative Y-intercept (Cherepanov et al., 2003). The value of negative intercept changes has been shown to depend on *E*. Thus, extraction efficiency for acetate is larger than for amino acids, therefore, the intercept for acetate (and β-hydroxybutyrate) is not so much.

The results of this analysis suggest that the use of individual measured values of *ca* and *cv* for combined regressions $(ca - cv) = f(ca)$ or $(ca - cv)/ca = f(ca)$ is not correct. The existence of large statistical scattering may be diagnosed if the

large axis of correlation ellipse $cv = f(ca)$ is parallel to X-axis. The dependence predicted by the model may be obtained with larger probability by minimization of statistical scattering, one reason of which seems to be the variability of blood flow among the animals. So, when concentration of each individual amino acids measured simultaneously in one blood probe are used for such combined regression, a postulated linear non displaced dependence is manifested more clearly. This is demonstrated by using the data of a for mentioned experiment of Bequette et al. (2000) (Figure 3). The figure seems to substantiate the model discussed. Indeed, let us consider equation (3):

$$c_a - c_v = \frac{Tc_a}{T+Q}$$

Taking into the account that the values of T do not correlate with values of C_a (for 10 amino acids in the experiment r=-0.29, P>0.05, i.e. correlation ellipse is approximately horizontally oriented) and Q is constant, the points $(C_a\text{-}C_v)$, C_a in the figure, according to this equation, would be distributed with some scattering along regression line with the slope $T/(T+Q)$ and this line will pass the origin of coordinates. This plot does show just this situation.

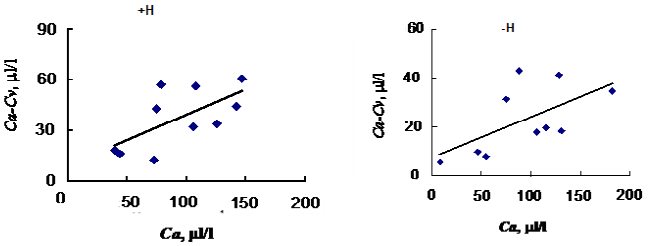

Figure 3. The dependence of arteriovenous concentration difference (*ca - cv*) of ten individual free amino acids in goat's mammary gland on their arterial concentration *(ca)* for control (+H, infusion of balanced mixture) and experimental (-H, infusion of mixture without histidine) periods (calculated from the data of Bequette et al., 2000)

A similar dependence was revealed for mammary glands of sows by defining one regression line of arteriovenous difference for 10 individual amino acids on their arterial concentration using mean values from several measurements (Trottier et al., 1997). The authors pointed out also the absence of any relationship between efficiency of extraction and arterial concentration on combined regression for these amino acids, which is predicted by equation (5).

The model with unidirectional flux of metabolite into the cell is a reduced version in relation to system with bidirectional fluxes (with additional counter flux from the cell). When experimental data obtained in this more a complex system are interpreted using model described on Figure 1, the value of T would be a function of three unknown rate constants. On the other hand, the task is simplified when in series of stationary states studied in chronic experiment the values of intracellular concentration changes proportional to extracellular concentration. It has been shown that in this situation the scheme with counter flux, from formally viewpoint, is equivalent to reduced model in which the value of T represents the activity of net transport from interstitium into the cell (Hanigan et al., 2001; Cherepanov et al., 2003). When the intracellular concentration is disproportionally decreased, this would cause an increase in the observed value of T, so in these cases T is a function of not only of pool of transporters but on the intracellular concentration, too. The data obtained suggest that the stationary values of intracellular concentration of, at least, some metabolites including amino acids and glucose, are characterized usually by linear proportional dependence on extracellular concentration (Clark et al., 1980; Neville et al., 1990). Nevertheless, this aspect needs further investigation.

Once the theoretical and experimental arguments in support of above described scheme of model calculations has been substantiated, we can ask, in what cases the use of arteriovenous difference and extraction efficiency is correct. For this purpose, we have analysed several situations with various values of plasma flow Q and activity of transport T, which, according to equations (4) and (5) determine arteriovenous difference and extraction efficiency. In the former base state $E = 0.33$, in the second base state $E = 0.75$. For each state the values of Q and T were varied within definite ranges, respective values of E were calculated using equation (5) and all values were expressed as a percentage. In the situation, when the variations in blood flow are larger compared to parallel shifts in transport activity, the shifts of arteriovenous difference and E are the opposite of that in transport activity; i.e. in such situations the use of E as a parameter of transport activity ('tissue avidity') is not correct. When percent increment of blood flow is equal to that of transport activity, the value of E does not change. When blood flow does not change, the value of E adequately characterizes the shifts in transport activity, although it should be pointed out that as value of E increases, it becomes progressively lesser sensible to real shifts in the activity of transport (see equation 7).

Example of using parameter of transport activity compared to arteriovenous concentration difference for amino acids

As an example of differences in interpretation, we discuss some results

obtained in the study of the effect of amino acid availability on the utilization of individual amino acids within the mammary gland. In the study of Guan et al. (2004) arteriovenous balance of individual amino acids was measured in lactating sows fed rations with various level of crude protein (7.8, 13, 18.2, 23.5%). Mammary blood flow in the experimental groups was significantly different (7.14, 7.84, 5.24, 8.41, $l \times 10^{-3}$/d, respectively). The authors indicated that arteriovenous difference for many amino acids was seen to rise as the dietary crude protein level increased, but at high level of crude protein it decreased. This trend might be expected, since mammary blood flow manifested the reverse dynamics. From this viewpoint, the more informative approach would be to correlate venous concentration with uptake of amino acids at various levels of crude protein. The venous concentration of many amino acids, calculated on the base of published data, was increased proportionally to the level of crude protein. After recalculation using formula $T = Q \cdot E/(1-E)$ the clearly tendency of decreasing activity of net individual amino acid transport was revealed as the venous concentration was increased; this decrease was the most evident for leucine (Figure 4).

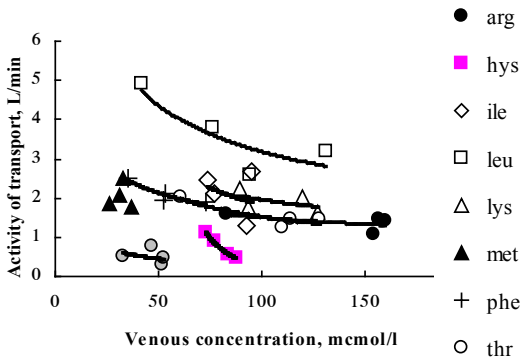

Figure 4. Relationship between the activity of net transport of individual amino acids into the secretory cells of mammary glands and their venous concentration in sows fed diets with various level of crude protein (recalculated from the data of Guan et al., 2004)

In the experiments with infusions of individual amino acids it has been shown previously that transmembrane transfer of amino acids into the mammary secretory cells can be regulated by the cell, in particular, under the deficit of amino acid its activity of transport increased and at excess it decreased (Bequette et al., 2000). The data shown in Figure 4 indicate that this tendency is also holds in monogastric animals (pigs), being manifested not only by infusions but in normal physiological conditions (nutritional states), too.

The discovery of adaptive alterations in the transport activity of basolateral membranes for individual amino acids and other substrates into the mammary secretory cells should attach particular significance to the quantitative estimates of the activity of transport of the main substrates into the cell in vivo. The theoretical and experimental data obtained suggest that the value of extraction efficiency, traditionally used as a measure of "tissue avidity" to substrate, is non-informative in situations with shifts in blood flow and for water soluble substrates it needs to be recalculated to obtain the estimate of transport activity taking into account the kinetic and statistical considerations outlined above. Although the experimental arguments in support of above described simple model calculations have been substantiated in this article, nevertheless, some aspects need further investigations. Therefore, a further study of kinetic and statistical aspects of proper experiment design and model calculations is critically required. These experiments will help to understand how the cell perceives the deficit or excess of substrates and what is the mechanism of integration of metabolic processes at the level of tissue and organ.

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