Renal transport of neutral amino acids

Tubular localization of Na\(^+\)-dependent phenylalanine- and glucose-transport systems

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The transport properties for phenylalanine and glucose in luminal-membrane vesicles from outer cortex (pars convoluta) and outer medulla (pars recta) of rabbit kidney were studied by a spectrophotometric method. Uptake of phenylalanine as well as of glucose by the two types of membrane vesicles was found to be Na\(^+\)-dependent, electrogenic and stereospecific. Na\(^+\)-dependent transport of L-phenylalanine by outer-cortical membrane vesicles could be accounted for by one transport system (\(K_A \approx 1.5mM\)). By contrast, in the outer-medullary preparation, L-phenylalanine transport occurred via two transport systems, namely a high-affinity system with \(K_A \approx 0.33mM\) and a low-affinity system with \(K_A \approx 7mM\) respectively. Na\(^+\)-dependent uptake of D-glucose by pars convoluta and pars recta membrane vesicles could be described by single, but different, transport systems, namely a low-affinity system with \(K_A \approx 3.5mM\) and a high-affinity system with \(K_A \approx 0.30mM\) respectively. Attempts to calculate the stoichiometry of the different Na\(^+\)/D-glucose transport systems by using Hill-type plots revealed that the ratio of the Na\(^+\)/hexose co-transport probably is 1:1 in the case of pars convoluta and 2:1 in membrane vesicles from pars recta. The Na\(^+\)/L-phenylalanine stoichiometry of the pars convoluta transporter probably is 1:1. Both the high-affinity and the low-affinity Na\(^+\)-dependent L-phenylalanine transport system of pars recta membrane vesicles seem to operate with a 1:1 stoichiometry. The physiological importance of the arrangement of low-affinity and high-affinity transport systems along the kidney proximal tubule is discussed.

The characteristics of L-phenylalanine and D-glucose transport across basolateral and luminal membrane of proximal tubule have previously been examined by using highly purified vesicles from whole renal-cortical tissue (Aronson & Sacktor, 1974, 1975; Kinne et al., 1975; Evers et al., 1976; Ling et al., 1981; Kragh-Hansen et al., 1982a). The results of these studies established the presence of Na\(^+\)-dependent, electrogenic transport processes for L-phenylalanine and D-glucose in the luminal-membrane vesicles and Na\(^+\)-independent but carrier-mediated transport processes for these metabolites localized at the basolateral membrane of the proximal tubule.

More recently, Turner & Moran (1982a,b,c) studied the characteristics of Na\(^+\)-dependent D-glucose transport by rabbit luminal-membrane vesicles prepared from two regions of the proximal tubule, namely pars convoluta (S\(_1\)-segment) and pars recta consisting of S\(_2\)- and S\(_3\)-segments (for a review, see Maunsbach, 1973). The results of these experiments indicated the existence of two distinct D-glucose-transport systems, with different Na\(^+\)/hexose coupling ratios in these vesicle fractions: a low-affinity system localized in pars convoluta vesicle preparations and a high-affinity system characteristic of the luminal-membrane vesicles obtained from pars recta. In the present investigation we studied the localization of Na\(^+\)-dependent L-phenylalanine-transport systems along the rabbit kidney proximal tubule by using luminal-membrane vesicles prepared from pars convoluta and pars recta by the method described by Turner & Moran (1982a). By contrast with the previous findings of Evers et al. (1976), our experimental data indicate the presence of more than one Na\(^+\)-dependent transport system for L-phenylalanine in luminal-membrane vesicles. Furthermore, an attempt has been made to calculate Na\(^+\)/L-phenylalanine coupling ratios for these systems.
Finally, the heterogeneity of Na\(^+\)-dependent L-phenylalanine transport is compared with the localization of the Na\(^+\)-dependent D-glucose-transport systems.

**Experimental**

**Materials**

Trizma base, Trizma hydrochloride, L-phenylalanine, D-phenylalanine, choline chloride and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium d-glucuronate and L-glucose were purchased from Fluka A.G., Buchs, Switzerland. D-Glucose monohydrate was bought from J. T. Baker Chemicals B.V., Deventer, The Netherlands. 3,3'-Diethyloxadicarbocyanine iodide was supplied by Eastman Kodak Co., Rochester, NY, U.S.A. These and all other reagents were of A.R. grade. All solutions were sterilized before use.

**Preparation and enzymic characterization of renal luminal-membrane vesicles**

‘Outer cortical’ tissue (cortex corticis) was obtained by taking slices \(\leq 0.4\) mm thick from the surface of rabbit kidneys. Strips of ‘outer medulla’ tissue (\(\sim 1\) mm thick) were dissected from outer stripe of outer medulla. We always took both outer cortical and outer medullary tissue from the same animals, and two preparations were performed in parallel. Luminal-membrane vesicles were prepared as previously described (Kragh-Hansen et al., 1982b). The vesicle suspensions were stored on ice and used within 6 h. The protein concentration was determined by the method of Lowry et al. (1951), as modified by Peterson (1977), with human serum albumin (Sigma Chemical Co.) as a standard.

The purity of membrane vesicle preparations was examined by measuring various enzymic activities. The activity of alkaline phosphatase, determined by the method of Bessey et al. (1946), in the luminal-membrane vesicle fractions from pars convoluta (‘outer cortex’) and pars recta (‘outer medulla’) was enriched as compared with homogenate by a factor of 8.4 \(\pm\) 1.8 and 12.2 \(\pm\) 2.8 (\(n = 6\)) respectively. (Na\(^+\) + K\(^+\))-stimulated ATPase activity was assayed as described by Ottolenghi (1975) and was decreased to 0.11 \(\pm\) 0.10 and to 0.39 \(\pm\) 0.20 of that of homogenate in the case of pars convoluta and pars recta membrane vesicles respectively. The vesicle preparations were slightly contaminated by mitochondrial proteins [i.e. 4.4\% \(\pm\) 1.9\% of the activities of succinate dehydrogenase (Sheikh, 1972) remained in both types of preparations]. All the above-mentioned data are comparable with those reported by Turner & Moran (1982a). Typical yields were 1.0–1.5 mg and 0.5–1.0 mg of vesicle protein per g of tissue from ‘outer cortex’ and ‘outer medulla’ respectively.

**Microscopic examinations**

The composition of the tissues from ‘outer cortex’ and ‘outer medulla’, and the nature and purity of the membrane vesicle preparations were examined by electron microscopy. For that purpose an adult rabbit was anaesthetized and kidney tissue was fixed in situ essentially as described by Rostgaard & Møller (1980). Small blocks of ‘outer cortex’ and of ‘outer medulla’ tissue were embedded in Epon. Sections of 50–70 nm thickness were double-stained with uranyl acetate and lead citrate and examined in a Zeiss 10B electron microscope at 60 kV. Fig. 1(a) shows an electron micrograph of renal tissue taken from ‘outer cortex’. The Figure shows that the tissue contains numerous proximal convoluted tubules with open lumina. A morphometric study revealed that, of the tubular structures, about 90\% was proximal convoluted tubules and the remaining 10\% were cortical collecting ducts and distal convoluted tubules. No proximal straight tubules were observed. Only the cells in the proximal convoluted tubules possess a brush-border membrane. These findings are in accordance with those of Kaislilng & Kriz (1979). Fig. 1(b) shows a longitudinal section through tissue from ‘outer medulla’. It is seen (as described by Kaislilng & Kriz, 1979) that the tissue, in addition to collecting ducts and blood capillaries, contains numerous (about 50\% of the tubular structures) proximal straight tubules and no proximal convoluted tubules. Of the structures found in the tissue from ‘outer medulla’, only cells of the proximal straight tubules are furnished with brush borders. Electron-microscopical examinations of negatively stained membrane vesicle preparations derived from ‘outer cortex’ or ‘outer medulla’ revealed highly purified, right-side-out brush-border-membrane vesicles (results not shown).

**Transport studies**

The renal uptake of phenylalanine and glucose was studied by the spectrophotometric method previously described (Kragh-Hansen et al., 1982a). A 1.5 ml portion of a buffered aqueous solution of the potential-sensitive dye 3,3'-diethyloxadicarbocyanine iodide, 1.5 ml of a buffered salt solution and 100 u1 of membrane vesicle suspension was mixed in a 1 cm-path-length cuvette. The cuvette was placed in an Amino DW-2a u.v./visible spectrophotometer with a constant temperature in the sample compartment of 20°C. The salt anions permeate into the vesicles faster than the salt cations, resulting in a slight, reversible hyperpolarization of the membranes. The hyper-
polarization was recorded on the spectrophotometer, and at its maximum a small volume of a stock solution of one of the above-mentioned organic solutes or buffer was added, under magnetic stirring, through a small opening in the top of the sample compartment.

Results

Characteristics of uptake of phenylalanine and glucose by luminal-membrane vesicles from the ‘outer cortex’

Figs. 2(a) and 2(b) compare the uptake of phenylalanine and glucose by luminal-membrane vesicles from pars convoluta under various incubation conditions. It is seen that addition of either L-phenylalanine or D-glucose to the membrane-vesicle/dye suspension resulted in ‘overshoots’ (reflecting depolarization events) in the presence of extravascular > intravascular sodium salt gradients. However, the magnitude of the ‘overshoots’ is dependent on the sodium salt anion in the following order: Cl⁻ > SO₄²⁻ > D-glucanate. Furthermore, it appears from Fig. 2 that replacing the sodium salts by KCl or choline chloride abolishes the ‘overshoot’ completely. Finally, addition of the stereoisomers D-phenylalanine and L-glucose to membrane-vesicle/dye suspensions in the presence of NaCl gradients do not result in ‘overshoots’, but in only very small absorbance changes.

Fig. 3 shows the absorbance changes measured at the peak of the ‘overshoots’ (30 s uptake values) induced by addition of increasing concentrations of L-phenylalanine or D-glucose to suspensions of membrane vesicles and dye. The results are given as the absorbance changes measured in NaCl minus the absorbance changes in choline chloride. The inset of Fig. 3 shows Eadie–Hofstee analysis of the experimental data. Since the data follow regression lines in this plot, the renal uptake of L-phenylalanine and D-glucose occur via single, but probably different (Kragh-Hansen et al., 1982a), transport systems. The results shown in Fig. 3 can be described by an equation, which in form is identical with the Michaelis–Menten equation, reported in recent papers (Kragh-Hansen et al., 1982b; Jørgensen et al., 1983). The saturation curves were fitted to the experimental data by using a computer–analysed statistical iteration procedure (see Jacobsen et al., 1982). The apparent $K_A$ values (i.e. substrate concentration that gives half-maximal uptake) for Na⁺-dependent renal uptake of L-phenylalanine and D-glucose were calculated to be 1.5 mM and 3.5 mM respectively. We have also determined the $K_A$ values for these substrates by using the initial rate of uptake (4 s uptake value), instead of measuring the peak value of the ‘overshoot’. This approach resulted in more scattered data, but led to very similar $K_A$ values.

An attempt to determine the stoichiometry of the Na⁺/L-phenylalanine and the Na⁺/D-glucose co-transports was performed by using the ‘activation method’ of Turner & Moran (1982b,c), i.e. by measuring the uptake of organic solute in the presence of increasing concentrations of Na⁺. The results of such an experiment, corrected for uptake in choline chloride, are illustrated in Fig. 4, and it is seen that the data follow hyperbolic curves. In Fig. 5(a) the data have been analysed by using the following Hill-type equation (Turner & Moran, 1982c):

$$'Flux' = \frac{\Delta A_{max} [Na^+]^{n}}{K_A^n + [Na^+]^n}$$

The equation assumes the existence of $n$ essential co-operative Na⁺-binding sites per glucose site. As a first assumption, $n$ was put equal to 1. If this assumption can describe the experimental findings adequately, then the results should follow a regression line when plotting ‘flux’/[Na⁺] as a function of ‘flux’ (Turner & Moran, 1982b). In Fig. 5(a) it is seen that this is indeed the case for both L-phenylalanine and D-glucose transport. Our data have also been analysed according to the third and last graphical procedure of the ‘activation method’ (Turner & Moran, 1982b), namely the double logarithmic plot illustrated in Fig. 5(b). Plotted in this way, the data also follow regression lines. The numerical values of the slopes of these regression lines are 1, indicating 1:1 stoichiometries. Thus the results of all three graphical procedures propose a 1:1 ratio for both the Na⁺/L-phenylalanine and the Na⁺/D-glucose co-transport by luminal-membrane vesicles isolated from pars convoluta of the proximal tubules.

Characteristics of uptake of phenylalanine and glucose by luminal-membrane vesicles from the ‘outer medulla’

Uptake of phenylalanine and glucose by luminal-membrane vesicles prepared from pars recta and suspended in different salts is shown in Fig. 6. ‘Overshoots’ are only observed when the physiological isomers of the metabolites are added to membrane-vesicle/dye suspensions in the presence of sodium salts, establishing that the two organic solutes are transported by Na⁺-dependent, electrogenic and stereospecific transport systems.

Fig. 7 shows the absorbance changes produced by Na⁺-dependent uptake of increasing concentrations of L-phenylalanine or D-glucose. The results were analysed by using the same approach as outlined in the case of transport by pars convoluta membrane vesicles. A curvilinear plot was obtained when the L-phenylalanine data were shown in an Eadie–Hofstee-type plot (Fig. 7, inset). This is in contrast with L-phenylalanine uptake by pars...
convoluta membrane vesicles (Fig. 3, inset) and suggests the presence of multiple transport pathways (Nord et al., 1982). Computer analysis indicated that the amino acid uptake can be accounted for by assuming two transport systems and gave $K'_A$ and $K'_2$ values of 0.33mM and 7mM respectively. In contrast with L-phenylalanine, D-glucose apparently is taken up via a single transport system with $K_A$ of 0.30mM.

Finally, an attempt has also been made to determine the stoichiometry of the Na$^+$/L-phenylalanine and the Na$^+$/D-glucose co-transport systems localized in pars recta of proximal tubule. Fig. 8 shows uptake of the amino acid and the sugar in the presence of increasing concentrations of Na$^+$. The L-phenylalanine data can be described by a hyperbolic curve, suggesting that the Na$^+$/L-phenylalanine ratio probably is 1:1. By contrast, the D-glucose results show a marked sigmodial dependence on sodium concentration, indicating that more than one Na$^+$ ion is co-transported with one sugar molecule. In Fig. 9 the optical responses induced by a low concentration (Fig. 9a, mainly via high-affinity transport system) and a high concentration of L-phenylalanine (Fig. 9b, mainly via low-affinity transport system) are shown in Hill-type plots (cf. eqn. 1). It is seen from the Figure that $n = 1$ (closed symbols) in contrast with $n = 2$ (open symbols), results in data that can be described by regression lines. An $n$ value of 0.5 (two L-phenylalanine molecules transported together with one Na$^+$ ion) leads to curvilinear plots (results not shown). Fig. 9(c) shows Hill-type analysis of an analogue experiment performed with D-glucose. In this example, $n = 2$ (open symbols), but not $n = 1$ (closed symbols), results in a linear plot. Putting $n = 3$ gives a curvilinear plot (results not shown). Thus the stoichiometries for Na$^+$/L-phenylalanine and Na$^+$/D-glucose co-transports seem to be 1:1 and 2:1 respectively.
Comparison between phenylalanine- and glucose-transport systems

Discussion

Electron-microscopic examinations and measurements of marker-enzyme activities demonstrated that we were able to isolate pars convoluta and pars recta luminal-membrane vesicles of a purity comparable with that of Turner & Moran (1982a) and with that of membrane vesicles prepared from whole renal cortex (Sheikh et al., 1982). These individual segmental preparations of luminal-membrane vesicles provided us with an opportunity to extend our earlier studies on renal uptake of L-phenylalanine (Kragh-Hansen et al., 1982a). For the sake of comparison the results obtained on the uptake of D-glucose under the same experimental conditions are included. Both types of preparations were capable of transporting L-phenylalanine and D-glucose by a Na+-dependent, stereospecific and potential-sensitive process. A similar conclusion was drawn by Turner & Moran (1982a,b,c), who extensively studied the mechanisms of tubular transport of D-glucose in luminal-membrane vesicles from 'outer cortex' and 'outer medulla' by the Millipore-filtration technique. Frömenter and co-workers (Frömenter, 1982; Samarzija & Frömenter, 1982; Samarzija et al., 1982) in electrophysiological studies by microperfusion in vivo found that addition of D-glucose or L-phenylalanine in the presence of an Na+ gradient depolarizes the renal cells, when introduced from the luminal side, confirming the existence of an electrogenic transport mechanism for the re-absorption of these compounds.

Another extensive study reported in the literature along these lines is that of Barfuss & Schafer (1979, 1981). They studied the uptake of D-glucose, glycine and ß-aminoisobutyric acid in proximal convoluted and proximal straight tubules isolated...
Fig. 3. Kinetics of L-phenylalanine and D-glucose uptake by outer-cortical luminal-membrane vesicles

Uptake of increasing concentrations of L-phenylalanine (●) and D-glucose (○) by luminal-membrane vesicles. The inset shows the same data in an Eadie–Hofstee-type plot. In the inset, y represents ΔA580 - 610, and x represents the concentration (mM) of L-phenylalanine or D-glucose. The intravesicular medium was 310 mM-mannitol/15 mM-Hepes/Tris buffer. The results shown in the Figure are the absorbance changes obtained with an external medium of 155 mM-NaCl/15 mM-Hepes/Tris buffer minus the absorbance changes induced by adding the same metabolite concentration to membrane vesicles suspended in 155 mM-choline chloride/15 mM-Hepes/Tris buffer. The data are from a representative experiment. For further experimental details, see the legend to Fig. 2.

Fig. 4. Effect of Na+ concentration on uptake of L-phenylalanine and D-glucose by outer-cortical luminal-membrane vesicles

Uptake of 1.5 mM-L-phenylalanine (●) and 3.5 mM-D-glucose (○) by luminal-membrane vesicles is shown. The intravesicular medium was 310 mM-mannitol/15 mM-Hepes/Tris buffer. The results shown in the Figure are the optical responses obtained with an external medium of 155 mM-NaCl/15 mM-Hepes/Tris buffer minus the optical absorbance changes measured in the first 4 s after adding L-phenylalanine or D-glucose. The data are from a representative experiment. For further experimental details, see the legend to Fig. 2.

From rabbit kidney by the method of Burg et al. (1966). They found that, in both segments, unidirectional lumen-to-bath fluxes of these solutes exceeded corresponding bath-to-lumen fluxes, and this active uptake of organic compounds was essentially Na+-dependent in accordance with the findings reported in the present paper.

Although qualitatively the properties of L-phenylalanine and D-glucose uptake by the two different types of vesicle preparations are similar, there are quantitative differences, especially in the magnitude of $K_A$ values. Thus, in accordance with the findings of Turner & Moran (1982a) and Barfuss & Schafer (1981), we observed the existence of responses induced by adding the same metabolite concentrations to membrane vesicles suspended in 155 mM-choline chloride/15 mM-Hepes/Tris buffer. The 'fluxes' are calculated on the basis of the absorbance changes measured in the first 4 s after adding L-phenylalanine or D-glucose. The data are from a representative experiment. For further experimental details, see the legend to Fig. 2.
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Fig. 5. Effect of Na⁺ concentration on the uptake of L-phenylalanine and D-glucose by outer-cortical luminal-membrane vesicles

(a) The L-phenylalanine (●) and D-glucose (○) uptake data of Fig. 4 are shown in a Hill-type plot. The intercepts of the regression lines with the x-axis give flux, $F_{co}$ (cf. b). (b) The uptake data of L-phenylalanine (●) and D-glucose (○) shown in a double-logarithmic plot analogous to that of Turner & Moran (1982b). The regression lines have the following equations:

L-phenylalanine: $y = -0.95x + 1.24 \ (r = 0.986)$

D-glucose: $y = -0.98x + 1.55 \ (r = 0.991)$

For further experimental details, see the legend to Fig. 4.

...a low-affinity transport system for D-glucose in the luminal-membrane vesicles from 'outer cortex', and a high-affinity transport system in the outer-medullary-membrane preparations. However, the situation is more complicated in the case of L-phenylalanine. Saturation kinetics experiments clearly showed that the luminal-membrane vesicles from pars convoluta of proximal tubule possess a Na⁺-dependent low-affinity transport system with $K_A \approx 1.5 \text{mM}$. On the other hand, Eadie–Hofstee analysis of the data obtained on vesicles from pars recta revealed the presence of both high-affinity ($K_A \approx 0.33 \text{mM}$) and low-affinity ($K_A \approx 7 \text{mM}$) transport systems for the re-absorption of L-phenylalanine in this region of the nephron.

It is well known from micropuncture and micro-perfusion studies that a large bulk of filtered D-glucose and L-phenylalanine (approx. 80% of the filtered load) is re-absorbed in the early part of the proximal tubule, corresponding mainly to pars convoluta (for a review, see Silbernagl, 1981). Therefore, the low-affinity transport systems for D-glucose and L-phenylalanine in this segment of the nephron seem capable of efficiently re-absorbing the bulk of these organic compounds in vivo. Consequently, the luminal concentrations of D-glucose and L-phenylalanine in the late proximal tubule probably are low, which in turn obviously requires high-affinity transport systems for efficient reabsorption of the last traces of these important metabolites from the urine. The existence of high-affinity transport systems for D-glucose and L-phenylalanine in outer-medullary-vesicle preparations observed in the present study is in full agreement with expectations and partly describes the important physiological function of...
Fig. 6. Uptake of phenylalanine and glucose by outer-medullary luminal-membrane vesicles

Uptake of 5 mM-phenylalanine (a) and 5 mM-glucose (b) by pars recta luminal-membrane vesicles. In (a), Curves 1–5 depict uptake of L-phenylalanine and Curve 6 describes D-phenylalanine uptake. (b) Curves 1–5 show D-glucose uptake and Curve 6 represents L-glucose uptake. The results shown are from a representative experiment. For further experimental details, see the legend to Fig. 2.

Fig. 7. Kinetics of L-phenylalanine and D-glucose uptake by outer-medullary luminal-membrane vesicles

Na⁺-dependent uptake of increasing concentrations of L-phenylalanine (■) and D-glucose (□) by luminal-membrane vesicles is shown. The inset shows the same data in an Eadie–Hofstee-type plot. The data are from a representative experiment. For further experimental details, see the legend to Fig. 3.
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Comparison between L-phenylalanine and alanine localized in vesicle preparations from pars recta is difficult to explain at present. However, it should be noted that both electron-microscopical examinations and D-glucose-uptake studies ruled out the possibility of appreciable contamination of these vesicle preparations with the membrane fragments from pars convoluta.

Another interesting feature of the present study is the demonstration of a different Na+/solute coupling ratio in vesicles from pars convoluta and pars recta of proximal tubule. By using the same experimental protocol and the procedures of data analysis described by Turner & Moran (1982b,c) we found the Na+/L-phenylalanine and Na+/D-glucose stoichiometry of the outer-cortex transporters to be 1:1. Furthermore, in accordance with the results of Turner & Moran (1982c), the Na+/D-glucose stoichiometry of the outer-medullary transporter was found to be 2:1. By contrast, the Na+/L-phenylalanine-uptake data for both the high-affinity and low-affinity transport systems of pars recta are consistent with a 1:1 stoichiometry. However, a more extended study concerning the coupling ratio of Na+/L-phenylalanine co-transport, including direct radioactive Na+-flux measurements by Millipore-filtration techniques, is desirable before making a final conclusion.

Fig. 8. Effect of Na⁺ concentration on uptake of L-phenylalanine and D-glucose by outer-medullary luminal-membrane vesicles

Na⁺-dependent uptake of 0.33 mM-L-phenylalanine (■) and 0.33 mM-D-glucose (□) by luminal-membrane vesicles. The data are from a representative experiment. For further experimental details, see the legend to Fig. 4.

Fig. 9. Effect of Na⁺ concentration on uptake of L-phenylalanine and D-glucose by outer-medullary luminal-membrane vesicles

Na⁺-dependent uptake of 0.33 mM-L-phenylalanine (a), 9.6 mM-L-phenylalanine (b) and 0.33 mM-D-glucose (c) by luminal-membrane vesicles shown in Hill-type plots using n = 1 (▲, ●, ■, left-side-ordinate axes) and n = 2 (▲, ○, □, right-side-ordinate axes) respectively; cf. eqn. (1) in the text. The data given are average values for three experiments. The regression lines have the following equations:

0.33 mM-L-phenylalanine (▲):  
\[ y = -0.030 x + 2.27 \quad (r = 0.964) \]

9.6 mM-L-phenylalanine (●):  
\[ y = -0.060 x + 25.5 \quad (r = 0.989) \]

0.33 mM-D-glucose (□):  
\[ y = -2.85 x + 1860 \quad (r = 0.936) \]

For further experimental details, see the legend to Fig. 4.
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