Wash-In Methodology and Modeling to Determine Hepatocellular D-Glucose Transport in the Perfused Rat Liver

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Abstract: Wash-in experiments, which may be useful for the study of the disposition of substrates in the liver, have not been well described. To investigate physiological models for wash-in curves, we performed experiments on the perfused livers of male Wistar rats anesthetized with pentobarbital. Test perfusate contained ^{14}C -sucrose as the extracellular marker and ^3H -glucose. Liver perfusions were performed with background glucose concentrations of 5.7, 10.7, 51.2, or 108.5 mM. Outflow time-activity curves were analyzed with the use of four models. The $V_{\max}$ and K_m for the influx of glucose were 1.1 ± 0.03

$\mu\text{mol/s/g}$ and 41 ± 3 mM with the Crone-Renkin early extraction model; 1.4 ± 0.04 $\mu\text{mol/s/g}$ and 36 ± 3 mM with dispersion model analysis; 1.8 ± 0.1 $\mu\text{mol/s/g}$ and 25 ± 4 mM with the Goresky distributed model to fit differentiated wash-in curves; and 2.7 ± 0.6 $\mu\text{mol/s/g}$ and 28 ± 21 mM with compartmental analysis. There was reasonable agreement between the four models, and they yielded results similar to those reported for glucose uptake in other preparations. [The Japanese Journal of Physiology 54: 421–429, 2004]

Key words: D-glucose, wash-in experiment, compartmental, rat liver.

The disposition of substrates in the liver has been studied extensively with the multiple indicator-dilution technique. This involves the injection of a bolus of indicators into the inflow of an organ and an analysis of outflow time-activity curves. The physiological modeling of this technique is well described and include the distributed models developed by Goresky and co-workers [1–3], the dispersion model [4, 5], and the Crone-Renkin early extraction model [6, 7].

By contrast, wash-in experiments have been studied infrequently, and this technique is less well characterized. Recently, we reported the use of wash-in experiments to determine the behavior of carbon monoxide in the liver [8], hind limb [9], and placenta [10]. Wash-in experiments were chosen to simplify the technical difficulties inherent in collecting and

analyzing gaseous substrates. A compartmental model was found to describe the wash-in data adequately. This type of modeling assumes that the vascular and cellular compartments in the liver are well stirred and is a much simpler interpretation of liver physiology than the distributed and dispersion models that have been used to analyze multiple indicator-dilution experiments. Carbon monoxide disposition in the liver has not previously been reported; therefore we wished to explore the modeling of wash-in experiments by using a substrate whose disposition has been elucidated in the liver by means of other techniques.

In this study, wash-in experiments were used to examine the disposition of D-glucose in the perfused rat liver. Several types of physiological models were used to analyze the data, and results were compared with those reported for other methods.

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METHODS AND MATERIALS

Materials. Ninety-five percent O₂/5% CO₂ gas was from Linde Gas (Canberra, Australia). Sucrose, [U-¹⁴C] (sp. act. 10.1 Ci/mmol), and D-glucose, [6-³H] (sp. act. 25–50 Ci/mmol), were purchased from ICN Pharmaceuticals (CA). Sodium heparin was obtained from David Bull Laboratory Victoria, Australia) and pentobarbital sodium from Rhone Merieux Australia Pty. Ltd. (Queensland, Australia). All the other compounds were obtained from Sigma (USA). Wistar rats were obtained from the John Curtin School of Medical Research (Canberra, Australia).

Liver perfusion. Male Wistar rats (7–8 weeks, 216–312 g) were maintained on standard food pellets and water ad libitum. The study was approved by the Australian National University Animal Experimentation Ethics Committee. The rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.). The abdomen was opened through a midline incision, and sodium heparin (200 IU) was injected into the inferior vena cava. The portal vein was cannulated with an 18 G intravenous catheter (Johnson and Johnson, Pomezia, Italy), and an outflow catheter PE-240 tubing (Crichtley Electrical Products, NSW, Australia) was inserted via the right atrium into the inferior vena cava. The liver was perfused *in situ* and in a single-pass mode with Krebs Henseleit bicarbonate buffer equilibrated with 95% O₂/5% CO₂ to pH 7.4 at a flow rate of 25–30 ml/min. The experiment was housed in a thermostatically controlled cabinet at 37°C. Viability was assessed by macroscopic appearance, portal venous pressure measured with a vertical manometer attached to the portal venous cannula, oxygen consumption and outflow bilirubin, alanine transaminase, and γ -glutamyl transpeptidase.

Wash-in experiments. The livers were perfused with Krebs Henseleit buffer containing either 5.7, 10.7, 51.2, or 108.5 mM glucose. Fifteen experiments were performed in 10 livers. After equilibration at each glucose concentration, the buffer was changed via a four-way valve to a test buffer maintained at an identical flow rate and containing sucrose [U-¹⁴C] and D-glucose [6-³H]. Outflow samples were collected at 1.4 sec intervals for 5 min with a modified fraction collector (Extech Equipment, Australia). ¹⁴C and ³H activity was measured with a scintillation counter (Model 1900, Packard Instrument Co., CT) after the addition of scintillation fluid (Starscent, Packard Instrument Co., CT).

Data analysis. The outflow activity for each indicator was expressed as a fraction of the measured

inflow activity, and outflow time-activity curves were constructed.

The outflow curves were analyzed by the use of four physiological models to determine the permeability-surface area product (*PS*) for the cellular transfer of D-glucose: the Crone-Renkin model [7, 9, 11], a two compartmental model we developed previously [8], the distributed model of Goresky and colleagues [3, 12, 13], and the dispersion model [4, 5, 14, 15]. The reference substrate was sucrose, which is a non-metabolized substrate that passes unimpeded through the vascular and extravascular spaces (volume of distribution of sucrose, V_s), but does not cross the hepatocellular membrane. For each model, a deviation of the glucose outflow curve from that of sucrose reflects the volume of distribution of glucose in the liver (V_g) and its influx (k_1), and in some models, efflux (k_2) across the hepatocellular membrane (Fig. 1). The value returned for k_1 by each model was used to calculate the *PS* product for glucose. The major differences in the assumptions underlying the models are as follows: In the Crone-Renkin model, a deviation of the glucose curve from the sucrose curve at the earliest time points is considered to reflect only glucose influx [7, 9, 11], but in the other three models, influx and efflux are considered to occur at all time points. In the Goresky model, the shape of the sucrose outflow curve is considered to indicate heterogeneity of the hepatic microvasculature (transit time distribution) [3]. In the dispersion model, the shape of the sucrose outflow curve is considered to be a modified inverse Gaussian curve reflecting dispersion through a labyrinth of interconnected sinusoids [4, 5]. In the compartmental model, perfect mixing is considered to occur within both vascular and the extravascular compartments [8, 9].

Fig. 1. Each model used to analyze the data assumes that the reference substrate, sucrose, travels unimpeded through the vascular and extracellular space (V_s) with a sinusoidal flow rate of Q . There is an influx (k_1) and efflux (k_2) of glucose across the hepatocellular membrane into the hepatocytes.

For the Crone-Renkin model [7, 11], the early extraction, E , was calculated from the relationship:

$$E = \frac{C_{\text{suc}} - C_{\text{glu}}}{C_{\text{suc}}}$$

where C_{suc} and C_{glu} are the outflow dose-normalized concentrations of ^{14}C -sucrose and ^3H -glucose at each time point, respectively. The relationship between time and E was plotted, and the maximum value of E during the plateau phase was used to determine the PS product for glucose influx according to the equation:

$$PS = -Q \ln(1 - E)$$

where PS is the permeability-surface area product for the cellular transfer of glucose and Q the flow rate.

Next, compartmental modeling was used as described previously for the study of the disposition of carbon monoxide in the liver [8]. The sucrose curves were fitted to the equation:

$$C_t = C_{\text{max}} (1 - e^{-Q(t-t_0)/V})$$

where C_t is the outflow concentration at time t and Q the flow rate. The fitted variables were C_{max} , equivalent to the fractional recovery of sucrose; t_0 , the common transit time through catheter and non-exchanging vessels; and V , the apparent volume of distribution of sucrose.

The glucose profiles were analyzed with a two-compartmental barrier-limited model according to the equation:

$$C_t = C_{\text{max}} \cdot \left\{ 1 + \frac{Q}{V(b-a)} \cdot \left(k_2 \left(\frac{e^{-b(t-t_0)}}{b} - \frac{e^{-a(t-t_0)}}{a} \right) + e^{-a(t-t_0)} - e^{-b(t-t_0)} \right) \right\}$$

where

$$a = \{(k_1 + k_2 + Q/V) - \sqrt{(k_1 + k_2 + Q/V)^2 - 4k_2Q/V}\}/2$$

and

$$b = \{(k_1 + k_2 + Q/V) + \sqrt{(k_1 + k_2 + Q/V)^2 - 4k_2Q/V}\}/2$$

The values for t_0 and Q/V were obtained from the corresponding sucrose outflow profile. k_1 and k_2 are the rate constants for the transmembrane cellular influx and efflux of glucose, respectively. In this model, the volume of distribution of glucose cannot

be estimated directly. Sigmaplot (version 4.0 SPSS, Chicago, IL) was used for the curve fitting procedures. The PS product for glucose influx is equal to the product of k_1 and the extracellular volume, which is equivalent to the apparent sucrose volume.

Third, the distributed model developed by Goresky and co-workers [3] to analyze the behavior of barrier-limited substrates in multiple indicator dilution experiments was used [16–18]. A wash-in curve achieved after a step input function is mathematically equivalent to the integral of a curve obtained after a bolus input [19]. It is not feasible to integrate the complex Goresky equation for barrier-limited behavior after bolus input. Therefore we transformed our wash-in curves into the curves expected after a bolus input by differentiating the wash-in curves according to the equation:

$$C_b = \frac{C_{t+1} - C_t}{t_{t+1} - t_t}$$

where C_b is the expected concentration after a bolus input and C_t and C_{t+1} are the concentrations in the wash-in curve at times t_t and t_{t+1} , respectively. These differentiated curves are effectively equivalent to multiple indicator dilution curves. The recovery and mean transit time for sucrose and glucose were determined from the area under the curve and the ratio of the AUC to the area under the moment of the curve, respectively. The volumes of distribution were determined from the product of the mean transit time and flow rate. The rate constants for the influx (k_1) and efflux (k_2) of glucose were estimated by a regression of the $[^3\text{H}]$ -glucose outflow curve according to the equation for the Goresky model of barrier-limited exchange for a non-eliminated solute:

$$C_{\text{glu}}(t') = e^{-k_1 t'} C_{\text{suc}}(t') + e^{-k_1 t'} \int_0^{t'} e^{[-(k_1 - k_2)\tau]} \times C_{\text{suc}}(\tau) \sqrt{\frac{k_1 k_2 \tau}{t' - \tau}} I_1 [2\sqrt{k_1 k_2 \tau(t' - \tau)}] d\tau$$

where t' is the time following injection corrected for t_0 (common transit time through catheter and non-exchanging vessels), τ is a dummy variable of integration, $C_{\text{suc}}(t')$ and $C_{\text{glu}}(t')$ are the dose-normalized concentrations of sucrose and glucose at time t' , and $I_1[m]$ is a first-order modified Bessel function of argument m . The PS product for the influx of glucose was determined from the product of k_1 and the extracellular volume, which is equivalent to the sucrose volume of distribution. The regression programs were written in QBASIC.

The dispersion model was then utilized. Because of the step input, however, analytical solutions were not directly available. Instead, the non-linear regression was carried in the Laplace domain according to Rowland and colleagues [15]:

$$\bar{C}(s) = \exp \left(\frac{1 - \sqrt{(1 + 4D_N * T * \frac{s(s + k_1 + k_2)}{(s + k_2)})}}{2D_N} \right)$$

with input function:

$$\frac{C_{IN}}{s}$$

The compartmental and dispersion models were also reanalyzed, accounting for catheter effects that represent a distortion of the outflow curves caused by the passage of indicators through the inflow and outflow catheters and non-exchanging large blood vessels. A catheter experiment was performed, and these data were deconvoluted from the outflow curves by fitting the catheter-only profile to a simple mono-exponential function. This was done in the Laplace domain where the outflow curve was represented as the product of the catheter and liver effects.

The rate for glucose influx was determined from the product of the PS products derived from each of these three models and the concentration of the glucose in the perfusate. The rates for glucose influx were fitted to the Michaelis Menten equation with Sigmaplot and the values for K_m and V_{max} for glucose influx determined.

RESULTS

Outflow curves. An outflow curve from a wash-in experiment is shown in Fig. 2. There is an overlap of the fitted curves using compartmental and dispersion models, with or without catheter effects. The transformed curve generated by differentiating this wash-in curve is shown in Fig. 3. These differentiated curves resembled multiple indicator-dilution curves seen after a bolus input.

Recovery and volume of distribution. The recoveries and volumes of distribution obtained with the different methods are shown in Table 1. The recoveries of glucose and sucrose were lower with the Goresky model, probably because the tails of the differentiated curves were below the limits of detection and thus prevented extrapolation to infinity. The volume of distribution of sucrose was highest with the dispersion model, and this remained higher even when additional analysis was performed to allow for a correction for catheter effects (0.20 ± 0.05 ml/g).

Transport parameters for glucose transport. The relationship between time and early extraction according to the Crone-Renkin approach is shown in Fig. 4. There is a clear peak of values between 8 and 10 sec when the extraction is above 0.55.

The rate constants and PS products derived by each method are shown in Table 1. There is a three- to four-fold difference in the rate of glucose transport, with Crone-Renkin analysis generating the low-

Fig. 2. Wash-in curves fitted with a compartmental model and dispersion model, with and without catheter effects.

Fig. 3. Curves generated by differentiating the wash-in curves shown in Fig. 1. (The estimated values of k_1 , k_2 , and extracellular volume were 0.217 s^{-1} , 0.246 s^{-1} , and 0.11 ml/g , respectively, for this experiment by use of the Goresky model.)

Table 1. Results for experiments performed with glucose concentration of 5.7 mmol/l ($n = 4$). The results were obtained by (1) compartmental analysis of the wash-in curves; (2) differentiated curves, measurements of AUC and AUMC, and the Goresky model; (3) a dispersion model; or (4) a Crone-Renkin early extraction analysis of wash-in curves.

	Compartmental	Goresky	Dispersion	Crone-Renkin
Recovery sucrose	0.98 ± 0.04	0.89 ± 0.04		
Recovery glucose	0.97 ± 0.04	0.82 ± 0.03^1		
Volume sucrose (ml/g)	0.14 ± 0.06	0.16 ± 0.03	0.24 ± 0.06	
$k_1 (\text{s}^{-1})$	0.6 ± 0.4	0.4 ± 0.05	0.1 ± 0.004	
$k_2 (\text{s}^{-1})$	0.3 ± 0.1	0.2 ± 0.05	0.1 ± 0.004	
$PS (\text{ml/s/g})$	0.07 ± 0.01	0.06 ± 0.01	0.03 ± 0.01	0.02 ± 0.004
Influx rate ($\mu\text{mol/s/g}$)	0.42 ± 0.05	0.32 ± 0.08	0.18 ± 0.05	0.12 ± 0.02

Fig. 4. Relationship between early extraction and time. There is a peak in the values between 8 and 10 s when the extraction is above 0.55. This peak, or plateau, in the extraction is consistent with the assumption that influx exceeds efflux at early time points.

est value, followed by dispersion analysis, Goresky analysis, and finally the compartmental analysis, which gave the highest value.

Michaelis Menten parameters. The effect of glucose concentration in the perfusate on the rate of glucose influx determined by each of the models is shown in Fig. 5. The values for the K_m and $V_{\max}$ obtained with each of the three models are shown in Table 2 and compared to values reported with the use of other techniques.

DISCUSSION

The liver has critical roles in detoxification and in maintaining systemic metabolic homeostasis. Therefore the hepatic disposition of many substrates, including xenobiotics and endogenous chemicals, has been extensively studied. The multiple indicator-dilu-

Fig. 5. The relationship between glucose concentration and the rate of glucose uptake into the liver. We calculated the rates by using a compartmental analysis of wash-in curves, the Goresky analysis of differentiated curves, dispersion modeling, and a Crone early extraction analysis of wash-in curves. The values for K_m and V_{max} are shown in Table 2.

tion technique has been a valuable tool in these studies. In these experiments a bolus of tracers is injected into the inflow of the perfused liver, and outflow time-activity profiles are analyzed. The lagged shape of the outflow curves of vascular markers such as red blood cells is thought to reflect transit time heterogeneity [1, 19]. Goresky and colleagues pioneered the use of the multiple indicator-dilution technique to examine substrate disposition in the liver [1, 3]. This physiological modeling assumes that the difference in the shape of the outflow curves of vascular markers and flow-limited markers such as water is gen-

erated by a distribution of flow-limited markers into nonvascular compartments. Later, more complex models were developed to examine the disposition of substrates that encounter a barrier at the hepatocyte cell membrane, that bind to plasma proteins and red blood cells, or that undergo sequestration in the liver [3, 12, 13, 20]. Other models have been used to analyze hepatic multiple indicator-dilution curves. For example, the model developed by Crone and Renkin to examine the transfer of substrates across capillary membranes [6, 7, 11] has been applied to the liver [21]. This simple model assumes that a deviation of the outflow curve of diffusible marker from that of the vascular marker at early time points is caused by the unidirectional transfer of the substrate into the tissues. More recently, a dispersion model has been described [4, 5, 14]. This and other stochastic models attempt to describe the residence time distribution as known functions, such as lagged Gaussian or log-normal curves.

In contrast to multiple indicator-dilution studies, very few reports have used the wash-in technique to study substrate disposition in the liver. The input function in wash-in experiments is a step function achieved experimentally by switching suddenly from one perfusate to another containing the labeled substrates while maintaining constant flow rate. Outflow samples are collected until steady state equilibrium is achieved. It has been accepted that the integral of the outflow curve after bolus injection is the same as the outflow profile following a wash-in experiment. This was proven theoretically by Meier and Zierler for substrates that undergo flow-limited distribution

Table 2. K_m and V_{max} (mean $\pm$ SEM) for glucose influx in the liver. (Some values of V_{max} are estimated from PS products or rates per mass of protein, dry weight, or ml of intracellular water. In these cases an approximate value has been calculated per gram of wet liver by assuming that intracellular water, dry weight, and protein are all about 50% of wet weight.)

Method	K_m (mmol/l)	V_{max} (μ mol/s/g)
<i>This study</i>		
Crone-Renkin	41 $\pm$ 3	1.1 $\pm$ 0.03
Dispersion	36 $\pm$ 3	1.4 $\pm$ 0.04
Goresky	25 $\pm$ 4	1.8 $\pm$ 0.1
Compartmental	28 $\pm$ 21	2.7 $\pm$ 0.6
<i>Other studies</i>		
Perfused rat liver [16]	48 $\pm$ 10	9.4 $\pm$ 0.9
Perfused rat liver [22]	64 $\pm$ 20	8.8 $\pm$ 1.3
Perfused rat liver [25]	17	~3 (345 μ mol/min/ml intracellular water)
Perfused rat liver [23]	39	6.5
Perfused rat liver [26]	100	7
<i>In vivo</i> dog liver [3]	121	~15 (5.13 mg/ml intracellular fluid/s)
Isolated rat hepatocytes [27]	18	~1 (86 mmol/liter intracellular water/min)
Isolated rat hepatocytes [28]	39	~1 (161 nmol/mg dry wt/min)
Isolated rat hepatocytes [29]	30	~1 (110 nmol/mg protein/min)

behavior [19], but the concept has not been extended to the situation where the indicator undergoes barrier-limited behavior. Nevertheless, we found that differentiating our wash-in curves generated new curves (Fig. 2) almost identical to those we have reported previously for glucose and sucrose by use of the multiple indicator-dilution method [16, 22]. These differentiated curves were suitable for analysis with the use of standard techniques based on the models developed by Goresky.

Previously we reported using wash-in experiments to determine the disposition of carbon monoxide in the perfused rat liver [8], rat hind limb [9], and human term placenta [10]. Wash-in experiments were used because of difficulties related to the collection and analysis of gaseous samples in bolus experiments. In that study we found that a simple compartmental model appeared to fit the outflow curves adequately and returned physiologically plausible values. To make the experiments technically simple, the outflow samples were collected onto blood, and the carbon monoxide concentrations were measured by an assay of carboxyhemoglobin. Although this was a critical technical factor, the data were noisy and too scattered to allow a meaningful assessment of the validity of the compartmental model. Furthermore, there have been no previous reports of the disposition of carbon monoxide for the purpose of validating the compartmental model by comparing the results with those achieved by the use of other techniques.

Therefore we repeated the methodology to examine the substrate, D-glucose, which has been thoroughly characterized in the liver as a result of various methods. We [16, 22] and others [3, 23] have previously reported the use of the multiple indicator-dilution method to examine D-glucose disposition in the liver. The results we obtained by using wash-in experiments are similar to those obtained by using multiple indicator-dilution experiments, except that the values for $V_{\max}$ are lower for wash-in experiments. This is primarily because the values for the extracellular volume obtained with multiple indicator-dilution experiments are greater than those we obtained with wash-in experiments. When the background concentration of glucose was approximately 11 mM, the values for k_1 were 0.47 s^{-1} [22] and $0.52 \pm 0.13 \text{ s}^{-1}$ [16] with multiple indicator-dilution experiments, compared with $0.47 \pm 0.04 \text{ s}^{-1}$ obtained here as a result of wash-in experiments. The extracellular volumes were $0.27 \pm 0.04 \text{ ml/g}$ [22] and $0.37 \pm 0.09 \text{ ml/g}$ [16] for multiple indicator-dilution experiments, compared with $0.15 \pm 0.02 \text{ ml/g}$ for wash-in experiments. Because the PS product is the product of the

extracellular volume and rate constant, the PS and therefore $V_{\max}$ values are greater when the multiple indicator-dilution approach is used. This type of anomaly has been reported previously in the perfused rat hind limb. Heatherington and Rowland [24] noted a consistent overestimation of the vascular space in the perfused hind limb by using bolus methods compared with values obtained by using whole body experiments. They suggested that transient pressure changes caused by injecting the bolus could cause additional vessels to be recruited. When perfusion pressure returns to the baseline level, stagnant pools containing the indicators might develop that will gradually leech out. This will lengthen the outflow tail, thus increasing the mean transit time and estimates of the volume of distribution. In contrast, during wash-in experiments there are no changes in flow rate or perfusion pressure that could introduce this possible artifact. It is of note that the curves we derived by differentiating the wash-in data generated volumes of distribution for sucrose equivalent to those generated by the wash-in curves. This suggests that wash-in curves may not be simple integrals of bolus curves, because of technical differences between the two techniques.

The compartmental modeling appeared to effectively fit the experimental data, as well as the dispersion model (Fig. 2). Catheter effects appeared to be minimal. The values we obtained for the K_m and $V_{\max}$ for glucose influx in the rat liver are similar to those values obtained by using other techniques including the multiple indicator-dilution and steady state techniques in the intact liver or isolated hepatocyte studies. These observations suggest that a simple compartmental model is suitable for the analysis of wash-in curves. The question then arises as to why a simple compartmental model can effectively describe wash-in curves when much more complicated models are required for bolus experiments. It is possible that the intravascular dispersion of markers is different after a bolus-input compared with a step function input. Intravascular dispersion will occur down a concentration gradient; therefore it can occur in two directions after bolus input. After a step input, intravascular dispersion may occur mainly in a single upstream direction, thus simplifying the apparent behavior of markers.

The value for the $V_{\max}$ obtained with the Crone early extraction method was much lower than the value obtained from the compartmental and Goresky models. The Crone method assumes that the transfer is unidirectional at early time points [7, 11]. The early extractions from our wash-in data had obvious

plateaus or peaks (Fig. 4), and the values varied from 0.14 to 0.66 depending on glucose concentration. Values in this range are usually considered to be appropriate for this type of analysis. Even so, this indicates that the transfer of glucose into the cells was rapid, and it is quite likely that even early time points were contaminated by the efflux of glucose from cells. This will lead to an underestimate of the *PS* product and thus the $V_{\max}$. By contrast, the compartmental and Goresky models take into account both the influx and efflux of the glucose and therefore should lead to better estimates of the influx rate constant.

In summary, wash-in curves in the perfused liver can be analyzed by the use of several physiological models, including a simple compartmental model. These analyses returned values for the cellular influx of glucose that are similar to those determined by other techniques. Compartmental modeling may be suitable for the analysis of wash-in curves in the perfused rat liver.

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