Biochem. J. (1978) **172**, 407–416
Printed in Great Britain

The Reliability of Rates of Glucose Appearance *in vivo* Calculated from Constant Tracer Infusions

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(*Received* 30 *August* 1977)

The rate of appearance of unlabelled glucose was calculated from tracer data and compared with the actual rate of infusion of unlabelled glucose into an anaesthetized dog with all sources of endogenous glucose production surgically removed. The mean steady-state rate of appearance of unlabelled glucose calculated from the equilibrium specific radioactivity was insignificantly higher (0.3%) than the actual rate of infusion of unlabelled glucose ($n = 6$). During non-steady states, a time-variable volume of distribution of glucose (V) was necessary to predict the rate of appearance of unlabelled glucose correctly from the pool-dependent equation described by Steele [(1959) *Ann. N.Y. Acad. Sci.* **82**, 420–430]. Rapid fluctuations in the rate of appearance of glucose could be predicted reasonably well by using a fixed value of V for 40ml/kg, but by using larger fixed values for V (100–160ml/kg) the rates were inaccurate. The pool-dependent two-radioactive-isotope technique described by Issekutz, Issekutz & Elahi [(1974) *Can. J. Physiol. Pharmacol.* **52**, 215–224] predicted single-step increases in the rate of infusion of glucose reasonably accurately, but the Steele (1959) equation was better at predicting sequential changes in the rate of infusion of unlabelled glucose.

Stetten *et al.* (1951) measured the steady-state turnover of glucose *in vivo* by infusing labelled glucose at a uniform rate until a constant plasma specific radioactivity (isotopic equilibrium) was achieved. Later investigators shortened the time required to reach isotopic equilibrium by giving a priming dose of tracer at the start of the constant infusion (Searle *et al.*, 1954; Steele *et al.*, 1956). Constant tracer infusions were subsequently extended to the measurements of rates of appearance of glucose in non-steady states. The necessary equation for the latter step was described by Steele (1959) and was derived on the assumption of uniform instantaneous mixing of injected labelled glucose with the unlabelled glucose pool. Steele *et al.* (1956) realised that the entire body glucose pool did not mix instantaneously with injected labelled glucose, and the concept of a rapidly mixing pool that was a fraction of the total extracellular glucose pool was advanced. Thus the size of the glucose pool used in the Steele (1959) equation came to equal the product of three terms, the plasma glucose concentration multiplied by the extracellular fluid volume multiplied by the pool fraction. The influence of the pool fraction on the calculated rate of appearance of unlabelled glucose can be profound during rapid changes in specific radioactivity, yet suggested values have ranged from 0.5 to 0.8 (Steele *et al.*, 1956, 1974; Steele, 1964; Cowan & Hetenyi, 1971; Norwich *et al.*, 1974; Radziuk *et al.*, 1974). Concern with not knowing the correct pool fraction in advance has led to other tracer approaches in measuring rates of glucose appearance during non-steady states (Hetenyi *et al.*, 1961; Cowan & Hetenyi, 1971; Norwich, 1973; Issekutz *et al.*, 1974; Norwich *et al.*, 1974). Of these, the two radioactive-isotope method described by Issekutz *et al.* (1974) is particularly appealing because it is pool-independent.

The present study was undertaken to evaluate the influence of the chosen volume of distribution on the rate of appearance of glucose calculated by the Steele (1959) equation and to investigate the technique suggested by Issekutz *et al.* (1974). To obtain a preparation in which the rate of appearance of glucose was limited to a known infusion of unlabelled glucose, we removed the liver and both kidneys from a dog starved for 36h before the operation. With such a model, the rate of appearance of glucose calculated with different pool fractions could be compared with the actual rate of infusion of glucose during both steady and non-steady states. The same dog preparation allowed an evaluation of the ability of the Issekutz *et al.* (1974) equation to predict rapidly changing rates of appearance of glucose.

Methods

Six mongrel dogs weighing 18–21kg were anaesthetized with the short-acting barbiturate thiamyl

sodium (Parke, Davis and Co., Detroit, MI 48232, U.S.A.) after starvation for 36h. Mechanical ventilation was maintained for the duration of each experiment. Two polyethylene (PE 90) cannulae were introduced into the superior vena cava from the external jugular vein for the infusion of labelled and unlabelled glucose. A polyethylene (PE 160) cannula was inserted into the right carotid artery and advanced into the arch of the aorta for the purpose of drawing serial blood samples. The abdomen was opened through a long midline incision and the left kidney removed. A side-to-side porta-caval shunt was then constructed, during which time the portal-venous return was partially occluded for a period of 10min. The brief partial occlusion did not cause venous congestion of the intestine. We then performed a total hepatectomy by the technique described by Markowitz *et al.* (1933). The final stage was the removal of the right kidney and closure of the abdomen.

After completion of the right nephrectomy, a continuous steady infusion of unlabelled glucose was commenced and continued for the next 2.5h (period 1). The constant rate of infusion during period 1 varied between dogs from 1.34 to 2.76mg/min per kg and was given by means of a Harvard syringe pump, except in dog no. 6, when a pump with greater choice of infusion rates was used (Abbott/Shaw Life Care pump; Abbott Laboratories, King of Prussia, PE 19406, U.S.A.). In dogs 1, 3, 5 and 6, the rate of infusion of unlabelled glucose was altered at the completion of period 1. This subsequent phase will be called period 2. The pattern of alteration in the rate of infusion of unlabelled glucose during period 2 varied between dogs and the precise changes in a given case are presented in the Results section. In dogs 2 and 4, an intravenous glucose-tolerance test was performed during period 2, and these data are reported elsewhere (Wolfe *et al.*, 1978).

Radioactively labelled glucose (New England Nuclear Corp., Boston, MA, U.S.A.) was infused in tracer amounts (25–90nCi/min per kg) with a Harvard syringe pump as follows. (*a*) In all dogs, 15min after starting period 1, a priming dose of [6-^{3}H]glucose was given and immediately followed by a constant infusion of [6-^{3}H]glucose which continued uninterrupted for the remaining 2h of period 1 and during all of period 2. The priming dose/infusion rate ratio was chosen in accordance with the theoretical considerations described by Searle *et al.* (1954), i.e. the ratio was inversely proportional to the rate of infusion of unlabelled glucose during period 1. (*b*) In dogs 1, 3 and 5, a non-primed constant infusion of [U-^{14}C]glucose was commenced at the start of period 2 and continued for the remainder of the experiment. The total rate of fluid administration varied with changes in the rate of infusion of unlabelled glucose. The mean rate of infusion, however, was 0.05ml/min per kg and thus did not represent a significant fluid load.

Arterial blood samples (6ml) were drawn into heparinized syringes at intervals ranging from 2.5 to 15min. When withdrawing samples, an initial 5ml was collected in a separate syringe to clear the catheter completely of 0.9% NaCl. This blood was returned to the animal after the actual collection, and the catheter was then flushed with 6ml of heparinized (20 units/ml) 0.9% NaCl. Expired $^{14}CO_2$ was removed from the laboratory through an exhaust connected to the expiratory side of the Harvard mechanical ventilator.

Analytical procedures

Blood samples were kept on ice until centrifuged (4000*g*/min) at 4°C to separate the plasma. Determination of the plasma glucose specific radioactivity involved the removal of labelled products of glucose metabolism, and ^3H_2O before counting of radioactivity in a liquid-scintillation counter. The extraction was performed by first precipitating the plasma proteins by adding 3ml of 4.73% $Ba(OH)_2$ and 3ml of 5.50% $ZnSO_4$ to 3ml of plasma. The stated concentrations of the $Ba(OH)_2$ and $ZnSO_4$ solutions are adjusted slightly before use so that the pH of the supernatant, resulting from addition of equal volumes of each of these solutions, is 9 (titration against phenolphthalein). The resultant supernatant was passed through a glass column (24cm long × 5mm internal diameter) filled with IRA anion-exchange resin (Amberlite, IRA-400 c.p.; Mallinckrodt, St. Louis, MO, U.S.A.). The resin bed volume was 4.7ml, and the rate of flow across the column was adjusted to 0.5ml/min. The first 2ml of the eluate was discarded to ensure that no dilution occurred, and the remainder was collected. This procedure almost completely eliminates contamination of the glucose radioactivity by labelled organic acids such as lactate and pyruvate (Wolfe & Burke, 1977). Although the eluate contains glycerol, there is relatively little labelled glycerol since glucose specific radioactivity determined by formation of the potassium gluconate derivative is less than 1% different from the specific radioactivity determined by direct scintillation counting and glucose analysis (Kreisberg *et al.*, 1972). Samples (2ml) of the eluate were pipetted in triplicate into counting vials and evaporated to dryness in a vacuum oven at 70°C to eliminate ^3H_2O. The residue was dissolved in 1.0ml of water and counted for radioactivity in 10ml of Aquasol 2 (New England Nuclear Corp.) in a Beckman LS-250 liquid-scintillation counter. The background radioactivity always constituted less than 2% of the total. ^3H_2O and [^{14}C]toluene internal standards were used to correct the observed count rate for quenching and

spillover of ^{14}C counts into the ^{3}H channel. The glucose concentration in the plasma, the eluate and the unlabelled glucose infusion were measured in triplicate by either the glucose oxidase method with colorimetry of *NN*-diethylaniline-4-amino-antipyrine (Kabasakalian *et al.*, 1974) or with a glucose autoanalyser (Beckman).

Calculations

The following equations were used to obtain the data.

(1) The pool-dependent equation derived by Steele (1959):

$$R_a = \frac{F - V[(gl_t + gl_{t+\Delta t})/2][(SA_{t+\Delta t} - SA_t)/\Delta t]}{(SA_t + SA_{t+\Delta t})/2}$$

where: R_a, rate of appearance of unlabelled glucose (mg/min per kg); F, infusion rate of radioactive isotope (nCi/min per kg); V, volume of distribution of glucose (ml/kg); gl, plasma glucose concentration (mg/ml); t, time (min); Δt, increment in time (min); SA, glucose specific radioactivity (nCi/mg).

(2) The rate of appearance at isotopic equilibrium ($SA_t = SA_{t+\Delta t}$):

$$R_a = F/SA$$

(3) The numerical representation of the pool-independent equation derived by Issekutz *et al.* (1974):

$$R_a = [F_C(SAH_{t+\Delta t} - SAH_t) - F_H(SAC_{t+\Delta t} - SAC_t)]/[(SAH_{t+\Delta t} \cdot SAC_t) - (SAC_{t+\Delta t} \cdot SAH_t)]$$

where the symbols are the same as for eqn. (1) and the letter H or C appended to SA and F denotes [6-^{3}H]glucose or [U-^{14}C]glucose respectively.

The glucose specific radioactivity entered into eqn. (2) was the mean value for the last 30min of period 1. The glucose specific radioactivity measurements entered into eqns. (1) and (3) were derived from a curve fitted visually to the experimentally determined specific radioactivities. The smoothing of the glucose specific radioactivity data by curve fitting was necessary to avoid random experimental error causing implausible values for the time rate of change of specific radioactivity (Cowan & Hetenyi, 1971). The mean and standard deviation of the differences between curve fit and measured values had a normalized value of 0.00018 ± 0.014. A normalized value of the standard deviation of the determination of specific radioactivity has previously been described to be 0.02 (2%) (Cowan & Hetenyi, 1971). Therefore, the fitted curve has not departed from the measured value by more than would be expected on the basis of the random error in the determination of glucose specific radioactivity.

Results

Steady-state conditions

Table 1 presents the comparison between the actual rate of infusion of unlabelled glucose and the rate of appearance of unlabelled glucose calculated from the mean ^{3}H specific radioactivity during the last 30min of period 1. The mean±S.E.M. for the normalized values of the six determinations is 1.003 ± 0.014. The mean calculated rate of appearance is not significantly different from the normalized infusion rate of 1.

Non-steady-state conditions

Period-2 data from dogs 3, 5 and 6 are presented as representative of changes in plasma specific radioactivity and glucose concentration during alterations in the rate of infusion of unlabelled glucose.

In dog 3 (Fig. 1), the rate of infusion of unlabelled glucose was doubled from a constant rate of 1.54 during period 1 to a new constant value of 3.08mg/min per kg for the duration of period 2. The increased infusion of unlabelled glucose was accompanied by an immediate fall in ^{3}H specific radioactivity from the equilibrium value at the end of period 1. The ^{14}C specific radioactivity, however, rose during period 2 in response to the non-primed infusion of [U-^{14}C]glucose which commenced at the start of period 2. Toward the end of period 2, both ^{3}H and ^{14}C specific radioactivities approached a plateau value.

The influence of repeated step changes in the rate of infusion of unlabelled glucose during period 2 in dog 5 is depicted in Fig. 2. As in Fig. 1, the equilibrium ^{3}H specific radioactivity at the end of period 1 is shown at zero time for period 2. The first decrease in the rate of infusion of unlabelled glucose from 2.75 to 2.06mg/min per kg, was accompanied by an immediate rise in ^{3}H specific radioactivity from the equilibrium value. Subsequent decreases and increases

Table 1. *Constant rate of appearance of unlabelled glucose calculated by eqn.* (2)

$$\%\ \text{error} = \frac{\text{Calculated rate} - \text{infusion rate}}{\text{infusion rate}} \times \frac{100}{1}$$

Dog	Rate (mg/min per kg): Unlabelled glucose infused	Appearance calculated by eqn. (2)	% error
1	2.76	2.82	+2.2
2	1.34	1.41	+5.2
3	1.54	1.54	0
4	2.05	2.06	+0.5
5	2.75	2.60	−5.5
6	1.87	1.86	−0.5

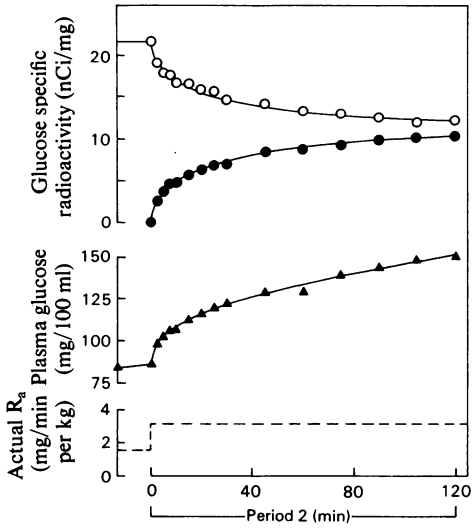

Fig. 1. *Period-2 changes in plasma glucose concentration and specific radioactivity in dog 3*
At 2h before the start of period 2, a priming dose of [6-^{3}H]glucose was injected, and this was followed by a constant infusion of [6-^{3}H]glucose (33.2nCi/min per kg), which continued uninterrupted throughout period 2. A non-primed constant infusion of [U-^{14}C]glucose (38.3nCi/min per kg) was commenced at the start of period 2. The changes in plasma glucose concentration (▲), ^{14}C specific radioactivity (●) and ^{3}H specific radioactivity (○), after a doubling of the rate of infusion of unlabelled glucose [actual R_a (----)] at the start of period 2, are recorded.

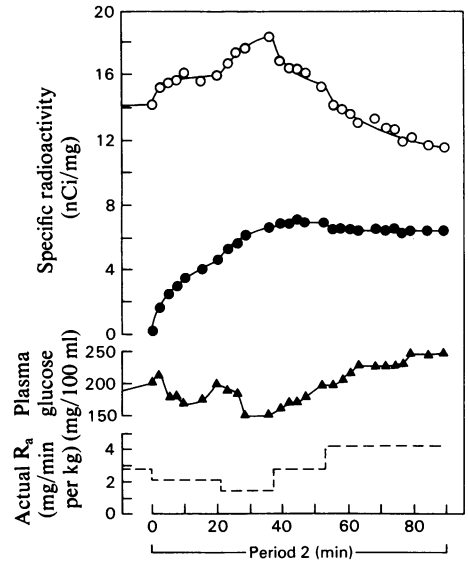

Fig. 2. *Period-2 changes in plasma glucose concentration and specific radioactivity in dog 5*
At 2h before the start of period 2, a priming dose of [6-^{3}H]glucose was injected, and this was followed by a constant infusion of [6-^{3}H]glucose (36.6nCi/min per kg), which continued uninterrupted throughout period 2. A non-primed constant infusion of [U-^{14}C]glucose (27.9nCi/min per kg) was commenced at the start of period 2. The changes in plasma glucose concentration (▲), ^{14}C specific radioactivity (●) and ^{3}H specific radioactivity (○), after sequential step changes in the rate of infusion of unlabelled glucose [actual R_a (----)], are recorded.

in the rate of infusion of unlabelled glucose were associated with prompt and reciprocal changes in ^{3}H specific radioactivity. The commencement of the non-primed constant infusion of [U-^{14}C]glucose at the start of period 2 was associated with an initial rise in ^{14}C specific radioactivity, but subsequent changes in the rate of infusion of unlabelled glucose were not all associated with reciprocal rises and falls in ^{14}C specific radioactivity. In particular, ^{14}C specific radioactivity continued to rise after the increase in the rate of infusion of unlabelled glucose at 37min.

Dog 6 did not receive a non-primed [U-^{14}C]glucose infusion during period 2, and so only changes in ^{3}H specific radioactivity are recorded in Fig. 3. The progressive increase and subsequent decline in infusion of unlabelled glucose is associated with a reciprocal pattern of change in ^{3}H specific radioactivity.

Rate of appearance of glucose calculated from pool-dependent eqn. (1)

The period-2 ^{3}H specific radioactivity curves depicted in Figs. 1, 2 and 3 were used to calculate the rate of appearance of unlabelled glucose for each sampling interval. In each case, the rate of appearance was calculated by using a range of volumes of distribution from 25 to 300 ml/kg. To illustrate the influence of the volume of distribution on the calculated rate of appearance of unlabelled glucose, results are presented for volumes of 40, 100 and 200ml/kg. In dog 3 (Fig. 4), the step change in the rate of infusion of unlabelled glucose is predicted best during the first 5min by a volume of distribution of 40ml/kg. As time progressed, larger volumes of distribution were required for the calculated rate of appearance of unlabelled glucose to be within 10% of the actual infusion rate of unlabelled glucose. With dog 5 (Fig. 5), calculations based on a volume of distribution of 40ml/kg predicted the rate of appearance within 25% of the actual value at all times except the interval 0–2.5min. Increasing the volume of distribution to 100ml/kg in dog 5 resulted in larger errors in the calculated rate of appearance at nearly all times and especially immediately after a change in the rate of infusion of unlabelled glucose.

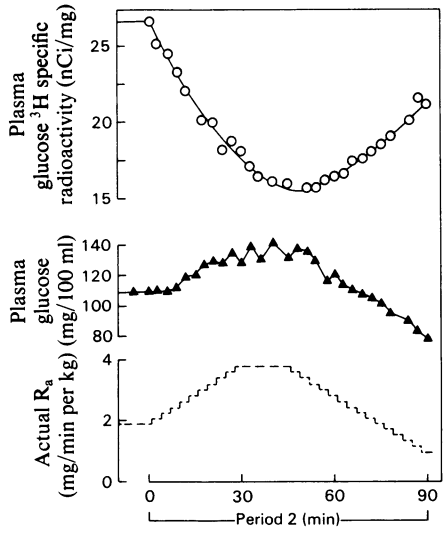

Fig. 3. *Period-2 changes in plasma glucose concentration and specific radioactivity in dog* 6

At 2h before the start of period 2, a priming dose of [6-^{3}H]glucose was injected, and this was followed by a constant infusion of [6-^{3}H]glucose (49.4nCi/min per kg), which continued uninterrupted throughout period 2. The changes in plasma glucose concentration (▲) and ^{3}H specific radioactivity (○), after a progressive increase and subsequent decline in the rate of infusion of unlabelled glucose [actual R_a (----)], are recorded.

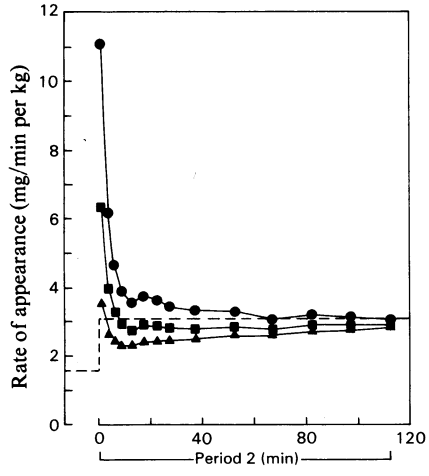

Fig. 4. *Rate of appearance of unlabelled glucose calculated from eqn.* (1) *in dog* 3

The rate of infusion of unlabelled glucose (----) and the rate of appearance calculated from eqn. (1) (——), from the ^{3}H specific-radioactivity data from the curve in Fig. 1, are plotted. The calculated rates of appearance are based on a volume of distribution of 40ml/kg (▲), 100ml/kg (■), or 200ml/kg (●).

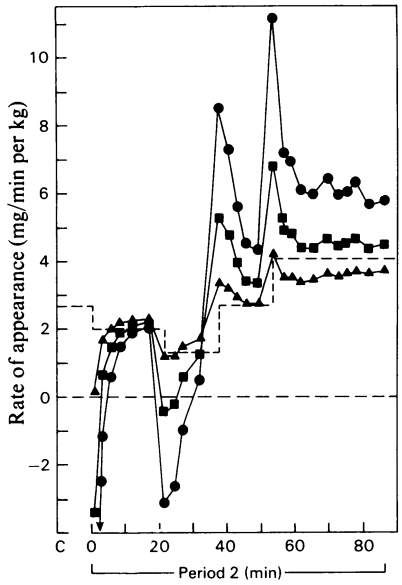

Fig. 5. *Rate of appearance of unlabelled glucose calculated from eqn.* (1) *in dog* 5

The rate of infusion of unlabelled glucose (----) and the rate of appearance calculated with eqn. (1) (——), from the ^{3}H specific-radioactivity data from the curve in Fig. 2, are plotted. The calculated rates of appearance are based on a volume of distribution of 40ml/kg (▲), 100ml/kg (■) or 200ml/kg (●).

A further increase in the volume of distribution to 200ml/kg resulted in errors in the calculated rate of appearance of unlabelled glucose exceeding 200% and the prediction of untenable negative rates of appearance. The influence of the volume of distribution on the calculated rate of appearance of unlabelled glucose in dog 6 is recorded in Fig. 6. When the rate of appearance was calculated with a volume of distribution of 40ml/kg, the actual changes in the rate of infusion of unlabelled glucose were predicted within 18% at each of the 22 determinations between 3 and 75min. The difference between the actual infusion rate and the calculated rate of appearance was increased at most times by basing calculations on a volume of distribution of 100 or 200ml/kg instead of 40ml/kg.

Effective volume of distribution

The volume of distribution that results in the correct calculation of the rate of appearance of unlabelled glucose for each sampling interval will be called the effective volume. The values for ^{3}H specific radioactivity during period 2 for dogs 3, 5 and 6 are depicted in Figs. 7, 8 and 9 respectively. Figs. 7 and 8 also record the effective volume of distribution for the

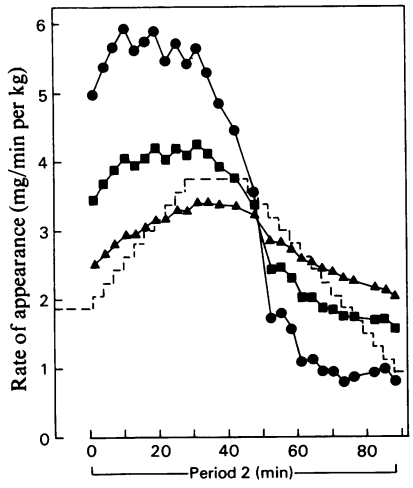

Fig. 6. *Rate of appearance of unlabelled glucose calculated from eqn.* (1) *in dog* 6
The rate of infusion of unlabelled glucose (----) and the rate of appearance calculated from eqn. (1) (——), from the ^3H specific-radioactivity data from the curve in Fig. 3, are plotted. The calculated rates of appearance are based on a volume of distribution of 40ml/kg (▲), 100ml/kg (■) or 200ml/kg (●).

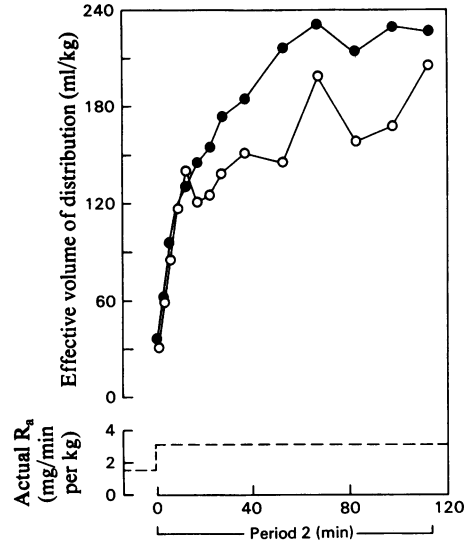

Fig. 7. *Effective volume of distribution in dog* 3
The rates of infusion of unlabelled glucose (----) are recorded along with the corresponding volume of distribution from which these rates could be correctly calculated from eqn. (1) and the data in Fig. 1. The effective volumes for the ^3H specific-radioactivity curve in Fig. 1 (○) and the effective volumes for the ^{14}C specific-radioactivity curve in Fig. 1 (●) are recorded.

^{14}C specific-radioactivity data in dogs 3 and 5 who received a non-primed constant infusion of [U-^{14}C]-glucose during period 2.

In dog 3, who was subjected to a single-step increase in the rate of infusion of unlabelled glucose at the start of period 2, the effective volume of distribution for ^3H increases with time, and there is considerable fluctuation from 15min onwards (Fig. 7). The effective volume for ^{14}C increases rapidly at first and then approaches a plateau value of approx. 230ml/kg towards the end of period 2.

The sequential changes in the rate of infusion of unlabelled glucose in dog 5 resulted in a more complex pattern of change in the effective volume of distribution (Fig. 8). For the ^3H data, the effective volume increased progressively during each period of constant infusion of unlabelled glucose but dropped abruptly at each change in the rate of infusion. The effective volume for the ^{14}C data is different from the effective volume for ^3H, although there are sudden decreases in effective volume coinciding with changes in the rate of infusion of unlabelled glucose. During the final phase of period 2, the effective volume for ^{14}C is consistently negative, whereas for ^3H it is positive.

The smoother alteration of the rate of infusion of unlabelled glucose in dog 6 was associated with yet a different pattern of change in effective volume for ^3H data (Fig. 9). With the progressive increase in the rate of infusion, there was an almost linear increase in effective volume, which continued to rise as the rate of infusion reached a plateau. With the start of the progressive decline in the infusion of nulabelled glucose, there was a dramatic decrease in effective volume, from +183 to −17ml/kg, despite the magnitude of the decrease in glucose infusion rate being only 5%. This decrease in the effective volume was followed by a steady increase as the rate of infusion of glucose continued to decline.

Rate of appearance of glucose calculated from pool-independent eqn. (3)

The comparison between rate of infusion of unlabelled glucose and the rate of appearance calculated by the two-isotope technique is presented for dogs 1, 3 and 5 in Fig. 10. In dog 3, the infusion rate of unlabelled glucose during period 2 was double the rate during period 1, and this increase was predicted, within 10%, by eqn. (3). With dog 1, the single-step increase in the rate of infusion of unlabelled glucose was more substantial, 2.76 to 13.8mg/min per kg, and the rate of appearance calculated from eqn. (3) was consistently approx. 27% too high. The more-complex changes in the rate of

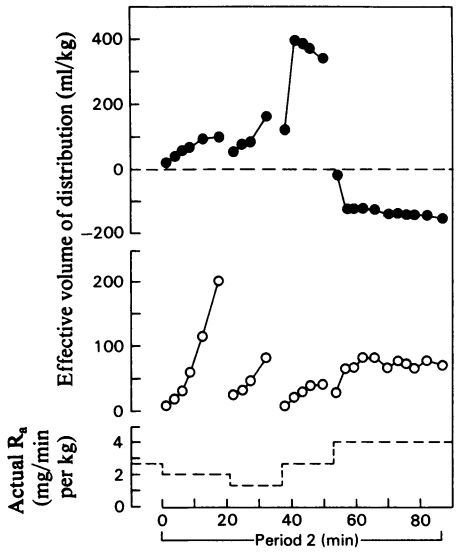

Fig. 8. *Effective volume of distribution in dog* 5
The rates of infusion of unlabelled glucose (----) are recorded along with the corresponding volumes of distribution from which these rates could be correctly calculated from eqn. (1) and the data in Fig. 2. The effective volumes for the ^3H specific-radioactivity curve in Fig. 2 (○) and the effective volumes for the ^{14}C specific-radioactivity curve in Fig. 2 (●) are recorded.

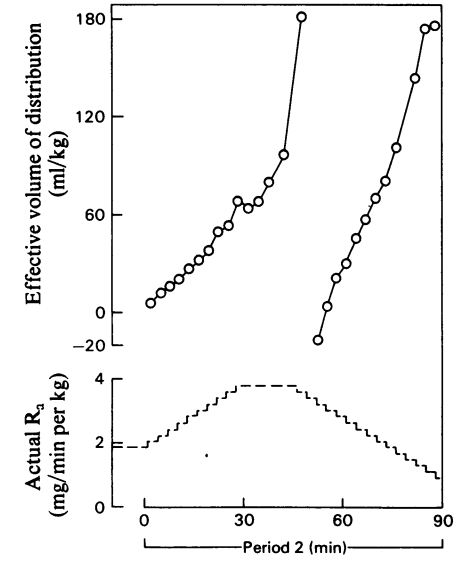

Fig. 9. *Effective volume of distribution in dog* 6
The rates of infusion of unlabelled glucose (----) are recorded along with the corresponding volumes of distribution (○) from which these rates could be correctly calculated from eqn. (1) and the data in Fig. 3.

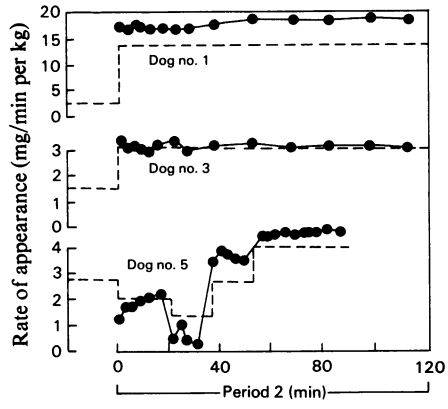

Fig. 10. *Rate of appearance of unlabelled glucose calculated from eqn.* (3) *in dogs* 1, 3 *and* 5
Both the rate of infusion of unlabelled glucose (----) and the rate of appearance (●) calculated from eqn. (3) are recorded for each dog. The data on which these calculations were based are presented for dogs 3 and 5 in Figs. 1 and 2 respectively.

infusion of unlabelled glucose in dog 5 were associated with larger errors in the rate of appearance calculated from eqn. (3). The range of errors was from a 77% underestimate at 31 min to a 41% overestimate at 41 min. Averaging the calculated rate of appearanceduring each known plateau in the rate of glucose infusion resulted in errors ranging from an underestimate of 59% for the period 21–37 min to an overestimate of 34% for the interval 37–53 min.

Discussion

In an overview of the Tracer Methodology Group Conference in 1973, Vranic (1974) emphasized the need for validation *in vivo* of tracer techniques. The infusion into dogs of unlabelled inulin at known rates was subsequently used to study the validity of tracer methods (Norwich *et al.*, 1974; Radziuk *et al.*, 1974). In that model, the Steele (1959) equation predicted the input best when the pool fraction was 0.5–0.8 (assuming a total volume of distribution of 200ml/kg). However, the agreement between known and calculated values was sometimes poor, with errors as high as 50% of the true value when time-coincident values were compared. The agreement could be improved by accepting a 'phase' difference between known and calculated rates (Norwich *et al.*, 1974). Inspection of their data reveals, however, that the phase difference is not constant for the duration of the experiment, a significant obstacle to the investigator interested in the time response to a stimulus. In our opinion, Norwich *et al.* (1974) and Radziuk *et al.* (1974) were unjustified in assessing the percentage error in the

calculated rate of appearance of inulin with a method that avoided direct comparison of time-coincident values. Also these authors acknowledged the potential difficulties of extrapolating their findings in the simple insulin system to the complex glucose system.

We thought that there were two experimental models in which we could investigate the ability of tracer techniques to measure rates of appearance of glucose *in vivo*. One possibility was to infuse intact animals with glucose at rates in excess of normal glucose production in the hope that the contribution of endogenous glucose production would be small in relation to the known infusion. This approach does not allow an absolute validation of the measurement of the rate of appearance of glucose, by constant tracer infusion, in steady-state conditions. This is because absolute validation requires a knowledge of the degree of suppression of endogenous glucose production, a value that can only be measured by the tracer technique that one is trying to validate. Net-hepatic and net-renal glucose production can be determined by measuring arteriovenous differences and blood flow across each organ. However, a decrease in net hepatic glucose output during exogenous glucose infusion may be due to increased hepatic glucose uptake and/or a decrease in hepatic glucose production. If an absolute validation is not desired, and one assumes that steady-state rate of appearance of unlabelled glucose can be accurately measured by constant tracer infusion it is possible to determine endogenous glucose production at various constant rates of glucose infusion and hence whether the total rate of appearance during a fluctuating rate of infusion of unlabelled glucose is likely to closely approximate the infused rate. Steele *et al.* (1965) showed that a constant unlabelled glucose infusion caused progressive but usually incomplete suppression of endogenous glucose production as measured with [U-^{14}C]glucose. For example, six of the nine dogs studied by Steele *et al.* (1965) received continuous infusions of glucose at rates 3–5 times their basal endogenous rate of production. In these six dogs, the endogenous glucose production during infusion averaged 9% of the infused rate, 20 or more min after the glucose infusion increased from a value that had already initiated a lesser degree of suppression of endogenous glucose production. It should be pointed out that when turnover is measured with a tracer that does not recycle, e.g. [6-^{3}H]glucose, the degree of suppression of endogenous glucose production is actually less than that recorded by Steele *et al.* (1965) (Wolfe & Burke, 1977). Thus the concept of using a high infusion rate to investigate tracer methodology is restricted by a degree of uncertainty about the actual total rate of appearance, even when the infused rate exceeds the basal physiological rate by a factor of 5. In the light of these considerations, we chose the alternative approach of surgically removing the organs responsible for endogenous glucose production. The latter technique ensures exact knowledge of the input with which the tracer-calculated rate will be compared. An absolute validation of the measurement of a steady-state rate of appearance of glucose *in vivo* is therefore possible, and it is also possible to increase and decrease the rate of appearance of glucose in a precise manner and within a range likely to be encountered physiologically. These obvious advantages need to be tempered by a consideration of the relevance of the findings in surgically prepared dogs to intact animals.

Several pieces of information lead us to believe that the findings in the operated dog have wider relevance. First, the dog, if allowed to awake from anaesthesia, is a viable preparation for 17–20h provided that hypoglycaemia is avoided (Markowitz *et al.*, 1933). For convenience and dog comfort, we chose to use an anaesthetic agent that is cleared by the liver, so that after hepatectomy, the dog remained in the immediately preceding state of anaesthesia without administration of any additional agent. During the course of each experiment, our dogs retained normal eye reflexes, and backbleeding through the carotid cannula was always exceedingly brisk. All dogs required an overdose of anaesthetic agent at the end of the experiment to extinguish life. Perhaps the strongest evidence supporting the wider application of the non-steady-state findings in these dogs is contained within the data as such. That is, the accurate measurement of the rate of appearance of glucose during period 1 implies that the priming dose of tracer and subsequent infusions of tracer and unlabelled glucose are being adequately distributed. Also the time-dependent effective volume of distribution for a non-primed infusion of tracer during a period of constant appearance of unlabelled glucose (period 2 in dog 3; Fig. 7) is a routine finding in the intact dog (Issekutz *et al.*, 1974). This further strengthens the argument that our dogs reflect the same processes of labelled and unlabelled glucose mixing which are present in the intact animal.

One additional methodological point requiring consideration is the choice of procedure for curve fitting. The need to avoid implausible gradients in the specific radioactivity/time curve, owing to random experimental error, is readily appreciated; however, the best method of smoothing the data is not agreed. Cowan & Hetenyi (1971), Norwich *et al.* (1974) and Radziuk *et al.* (1974) have favoured the fitting of polynomial functions to the data. Issekutz *et al.* (1974) argue against polynomial functions and favour the fitting of exponentials. The problems inherent in fitting of exponentials to data have been addressed by Van Liew (1962) and Katz *et al.* (1974). Because it is impossible to prove that any function truly represents events *in vivo*, we have chosen the

simplest method of data smoothing, i.e. visual curve fitting. With this simple method, the differences between our measured and curve-fitted values have been in keeping with the random experimental error of determining specific radioactivity.

The results in Table 1 indicate that the constant infusion of [6-^{3}H]glucose after a priming dose of the same tracer is a very reliable method of determining a steady-state rate of appearance of glucose, the mean value being 0.3% too high with a coefficient of variation of 3.5%. It is often assumed that constant tracer infusions determine steady rates of appearance accurately, but to our knowledge this has not been previously established experimentally for the glucose system. The measurement of a steady-state rate of appearance by constant tracer infusion has, however, been validated *in vivo* in the cortisol system (Paterson & Harrison, 1967). Attempts to validate the measurement of rates of appearance of glucose from the rate of decline of specific radioactivity after a single tracer injection have been unsuccessful, in both the glucose (Hetenyi *et al.*, 1961; Wrenshall *et al.*, 1961) and cortisol (Harrison *et al.*, 1964) systems. However, there is no significant difference between the rate of appearance of glucose calculated from a constant tracer infusion and the rate of appearance calculated from the area under the specific radioactivity/time curve obtained after a single infusion of tracer (White *et al.*, 1969).

In the intact animal, determining the rate of appearance of glucose during non-steady-state conditions by the pool-independent equation of Issekutz *et al.* (1974) requires a correction for difference in the recycling of [6-^{3}H]glucose and [U-^{14}C]glucose. Issekutz *et al.* (1974) determined this correction factor in a preliminary steady-state experiment. Corrections based on such an initial experiment may be inappropriate for subsequent use because of alterations in the percentage of glucose turnover recycled after a perturbation (Wolfe & Burke, 1977). No recycling correction factor was applied to the data in the present paper because in the absence of gluconeogenesis neither tracer can recycle to glucose. Because tracer recycling in our model does not explain the difference between the rate of infusion and the rate of appearance calculated from eqn. (3) the assumptions underlying eqn. (3) and its parent eqn. (1) must be questioned. Our data demonstrate that one of the assumptions involved in the derivation of eqn. (3), that the volume of distribution for both tracers is the same at all times, is incorrect (Figs. 7 and 8). The inability to predict the rate of appearance of glucose, at all times, with a fixed pool fraction also seriously questions the validity of the assumptions involved in the derivation of eqn. (1).

The derivation of the Steele (1959) equation involves a simple application of dilution principles, i.e. eqn. (1) relates the factors that can influence specific radioactivity within a pool in which there is uniform instantaneous mixing of existing, and newly arriving, labelled and unlabelled molecules. To develop the equation, it was necessary to make the assumption that the specific radioactivity of the compartment was not influenced by the net loss of labelled and unlabelled glucose. This assumption will hold if there is no isotope discrimination. It is also necessary to assume that the specific radioactivity of the compartment is not influenced by other unknown factors such as mixing with other compartments that are at a different specific radioactivity. We will call a pool which complies with all of these assumptions an 'ideal pool'. Steele (1959) acknowledged that the entire body glucose pool was unlikely to behave, as a unit, in an ideal manner. He proposed, however, that a fraction of the total glucose pool would meet the assumptions sufficiently well. In our opinion, the consequences of biological pools not behaving in an ideal manner have not been fully appreciated or defined.

The changes in the effective volume of distribution (Figs. 7, 8 and 9) demonstrate that there is no single volume of distribution which will allow the correct calculation of rates of appearance of glucose at all times. Similar time-dependent changes in the effective volume of distribution of glucose have been observed in intact dogs by Issekutz *et al.* (1974). Reinspection of Fig. 5 in the paper of Norwich *et al.* (1974) reveals that the effective volume of distribution for inulin, during a change in inulin infusion rate, is also time dependent. Issekutz *et al.* (1974) suggested that the appropriate pool size should be predicted on the basis of time elapsed from perturbation by using their 'standard curve' for a time-dependent pool. The findings in the present paper (Figs. 8 and 9) indicate that this is an oversimplification and that the effective volume is influenced by sequential perturbations. Unfortunately, it is not possible to know in advance when a perturbation starts (e.g. drug administration and drug action may be separated in time), nor whether the perturbation will cause a single-step change or a sequence of changes in rate of appearance of glucose.

The prediction of a time-dependent pool in our experiments and in intact animals during steady-state conditions (Issekutz *et al.*, 1974) is a consequence of using an equation derived from assumptions that do not hold for biological pools. The incorrect assumption is that the net exit of labelled and unlabelled glucose from the sampled compartment is only dependent on the specific radioactivity within the sampled compartment. The assumption is incorrect because it ignores the effect of mixing between the sampled compartment and the remainder of the glucose space. The inaccurate assumption can,

however, be offset by a correction factor that has become known as a pool fraction. We do not believe that the pool fraction indicates the physical presence of an ideal pool of that size. Rather the pool fraction should be viewed as a factor to compensate for non-ideal pool behaviour. The paradox of predicting a negative volume of distribution is solved by the realization that the effective volume, or pool fraction, is only a correction factor for non-ideal behaviour of biological pools. Not surprisingly, the size and sign of the correction factor are influenced by changes in the rate of infusion of unlabelled glucose which create specific radioactivity gradients between the plasma glucose compartment and the other regions of the total glucose pool. At isotopic equilibrium, the specific radioactivity gradients between compartments no longer exist, and the rate of appearance of glucose can be determined accurately.

Our data indicate that, except at isotopic equilibrium, plasma is not part of an ideal glucose pool. The results also indicate that it is not possible to determine the optimum correction factor (pool fraction) in advance. Pragmatically, 40ml/kg was the best fixed volume of distribution to use in calculating acute changes in the rate of appearance of glucose. This volume approximates the plasma volume of the dog (Albert *et al.*, 1968) and is equivalent to a pool fraction of 0.2 assuming that the total volume of distribution of glucose equals the extracellular fluid volume of 200ml/kg (Walser *et al.*, 1953; Albert *et al.*, 1968).

This work was supported by National Institutes of Health grants nos. GM 070351 and GM 021700. The skilled technical assistance of Miss D. Sahagian is gratefully acknowledged.

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